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EFFECT OF CD40 SILENCING IN MAV-1 INFECTED MH-S CELLS

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Abstract

The murine alveolar macrophage cell line, MH-S, constitutively expresses the immunoregulatory antigen CD40. Previous experiments have demonstrated that CD40 expression is upregulated following infection with the DNA virus, mouse adenovirus 1 (MAV-1), suggesting a role for CD40 in MAV-1 infection. The purpose of this experiment is to investigate the effect of CD40 silencing on MAV-1 replication in MH-S cells. To elucidate the importance of CD40 signaling in MAV-1 replication, CD40 expression was reduced using a short hairpin RNA (shRNA) RNAi system. Five CD40-silenced lines were generated using shRNA and showed between 26-79 percent reduction in cell surface CD40 expression from wild type. To assess the effect of MAV-1 infection on costimulatory markers, CD40 expression was compared between the silenced cell line that showed the greatest level of CD40 suppression (designated NPB5) and wild type cells at 1-day post infection. Flow cytometry revealed CD40 expression was increased in both cell lines following infection with MAV-1. Infected CD40-silenced cultures expressed approximately 50% the level of CD40 as infected wild type cultures. Further characterization of CD40-deficient MH-S cells may reveal novel mechanisms through which MAV-1 infection alters host cell metabolism.

Introduction

Adenoviruses

Adenoviruses are icosahedral, double-stranded DNA viruses that infect a wide range of vertebrate hosts (Zhao et al., 2014). Adenovirus infection of humans predominantly affects adolescents and causes a wide range of upper and lower respiratory diseases (Carbal et al., 2002). Most cases of adenovirus infection are acute (10-14 days), however persistent infections are commonly observed in immunocompromised patients (Chakrabarti et al., 2002; Ghebremedhin, 2014). Adenovirus infection of immunocompromised patients tends to cause more severe infections than observed in immunocompetent patients. Studies have shown that 5-21% of patients are infected with adenovirus following stem cell transplantation and the mortality rate in this patient population is as high as 50% (Chakrabarti et al., 2002). A better understanding of how adenoviruses cause disease may allow for the development of new treatments and decrease the mortality rate in immunocompromised patients.

Mouse Adenovirus - 1

Human adenoviruses (hAd) are species specific and are therefore difficult to experimentally study outside their host (Wadell, 1984). Due to similarities between mouse adenovirus 1 (MAV-1) and human adenoviruses, and the availability of susceptible inbred mice, MAV-1 is an ideal model to study adenoviral infection. MAV-1 and human adenoviruses share a substantial amount of genomic and structural homology (Wigand et al., 1997). For example, MAV-1 and human adenoviruses share approximately 40% sequence similarity between the adenovirus early region 1A (E1A) genes, with increased sequence similarity in the conserved region 2 (CR2) (Ball et al., 1988; Elliot et al., 1995). MAV-1

E1A shares similar functions with hAd E1A, such as binding to host retinoblastoma (Rb) proteins (Fang et al., 2003).

MAV-1 primarily targets endothelial cells throughout the mouse and produces the highest level of virus in the brain, spinal cord and spleen (Lenarts et al., 2009). Most studies on adenovirus infection have been conducted on endothelial cells, however cells of the monocyte lineage also are targeted during infection during MAV-1 infection (Ashley et al., 2009). MAV-1 infection causes pneumonia, hepatitis, encephalitis, gastroenteritis, and disseminated disease involving multiple organs in immunocompromised mice (Ashley et al., 2009). Different strains of inbred mice show varying levels of susceptibility to MAV-1. C57BL/6 mice succumb to fatal hemorrhagic encephalomyelitis (inflammation of the brain) when infected with MAV-1, while BALB/c mice do not (Guida et al., 1995). 40% of the susceptibility trait for MAV-1 infection is controlled by a locus on chromosome 15 containing over 250 identified genes (Welton et al., 2005). It is unclear which genes are responsible for MAV-1 susceptibility. These experiments suggest that there is strong correlation between host allelic variation and susceptibility to MAV-1.

Expression of adenoviral genes is modulated throughout the course of infection. Depending on the stage of infection, adenoviruses produce either predominantly early or late gene products (Figure 1). Early gene products generally promote viral replication and stimulate the transcription of late gene products, while most late gene products are structural proteins, such as the hexamer capsid component (Waye and Sing, 2010).

Early adenovirus gene products include E1A, E1B, E2, E3 and E4 (Fessler and Young, 1998). Early region 1A (E1A) and early region 1B (E1B) gene products alter the cell cycle of infected hosts (McCormick, 2001). The E1A and E1B genes each encode proteins

that promote the transcription of other early gene products. These early gene products interfere with host cell cycle mechanisms by reducing the functionality of the tumor suppressors retinoblastoma protein (Rb) and tumor protein 53 (p53) (Figure 2) (McCormick, 2001). This experiment examines how MAV-1 replication alters host cell metabolism. It is possible that early genes also alter other physiological aspects of their hosts. These alterations may promote viral replication, especially if they can alter immune effectors, such as macrophages.

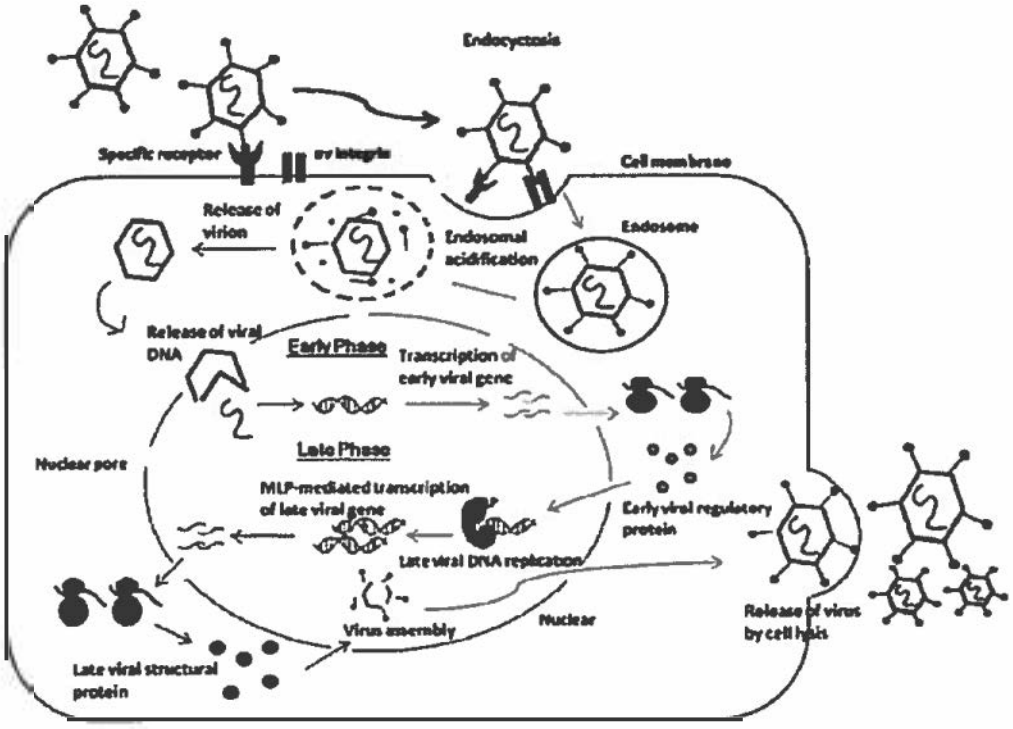

Figure 1. Summary of Adenovirus Infection. (Image from Waye and Sing, 2010)

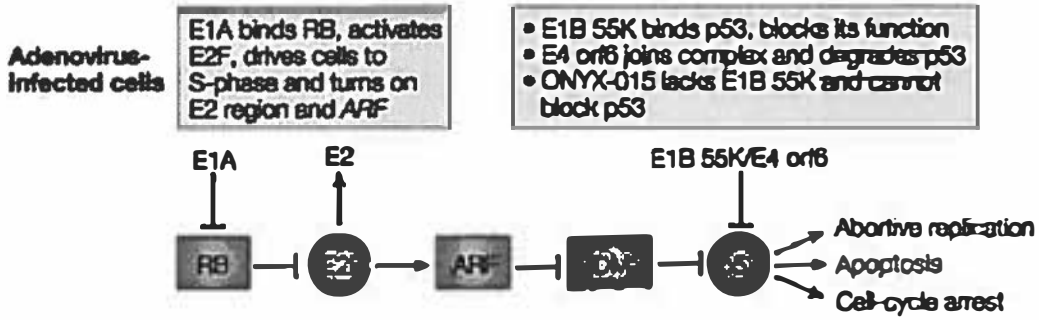

Figure 2. Model of Cell Cycle Manipulation by E1A and E1B. (Image from McCormick, 2001)

Immunological Significance of Macrophages

Macrophages can be found in all tissues as resident populations and can be recruited to other tissues following pathogen invasion or tissue injury (Kennedy and Abkowitz, 1998). Mature macrophages contribute to tissue homeostasis and aid in pathogen clearance through their functions in the innate and adaptive immune system. The main effector functions of macrophages are antigen presentation, phagocytosis of debris and pathogens, and immunomodulation (Stout & Suttles, 2004). Monocytes are derived from myeloid precursors in the bone marrow, spleen and liver (Stout & Suttles, 2004). Monocytes circulate through the blood and can be induced to differentiate into macrophages by exposure to numerous biological factors such as cytokines, chemokines, hormones and immunoglobulins (Stout & Suttles, 2004; Dale et al., 2008).

Macrophages that reside within the alveolar sacs of the lungs are known as alveolar macrophages. Alveolar macrophages serve as the first line of defense against respiratory pathogens through various immunological mechanisms such as phagocytosis, and the

secretion of lysozyme, antimicrobial peptides and proteases (Sibelle et al., 2003). When macrophages are infected by pathogens, these innate and adaptive immune functions can become impaired, leading to the progression of opportunistic infections (Sibelle et al., 2003). MAV-1 infection of macrophages has not been as well studied as other cell types.

MH-S cells (used in this study) are derived from alveolar macrophages from BALB/c mice. Given that BALB/c mice are more resistant to MAV-1 infection than C57BL/6 mice, it is possible that BALB/c mice contain immunological attributes that prevent persistent MAV-1 infections. One possible explanation why BALB/c are more resistant to MAV-1 infection is that they contain alleles that make their macrophages more efficient in recognizing adenoviral infection and promoting a T_{H1} response. Studies have shown that dendritic cells infected with human adenovirus promote the generation of T_{H1} cells (Rea et al., 1999). Dendritic cells and macrophages are both antigen presenting cells, so it is likely that macrophages may be inducing a similar response to MAV-1 infection. T_{H1} responses prepare the immune system to respond to intracellular infections, like viruses, through the secretion of cytokines such as IFN-γ, IL-2, and TNF- β (Romagnani, 1992).

One assumption made during this study is that MH-S cells are representative of alveolar macrophages from BALB/c mice. Due to the finite lifespan of macrophages, MH-S cells were immortalized using simian virus 40 (SV-40), inducing the expression of SV-40 large T antigen (Mbawuike and Herscowitz, 1989). SV-40 large T antigen has many of the properties of E1B, such as binding to p53 and inhibiting its function (Xing et al., 2001). During adenovirus infection E1B protects the virus from p-53 induced antiviral responses, such as apoptosis, which can be induced by E1A (White, 1995). The expression of T antigen

in MH-S cells creates unique limitations in studying adenoviral infection in MH-S cells due to the overlap in functions between E1B and large T antigen.

Nonetheless, MH-S cells are still a valuable model to study pathogenic infection in vitro due to the fact that this cell line maintains many of the functional characteristics of macrophages, such as being esterase positive, peroxidase negative, and expressing the cell surface antigens Ia and Mac-1 (Mbawuike and Herscowitz, 1989). Previous studies have used MH-S cells as an effective model for *Legionella pneumophila* infection of alveolar macrophages (Matsunaga et al., 2001).

MAV-1 infection of monocytes is unique in that monocytes serve as both the target cells for infection and the effector cells that mediate the immune response against the virus (Ashley et al., 2009). Infection of cells of the monocyte lineage could have profound effect on the host immune system through the depletion of effector immune cells. In addition, depletion of monocytes would reduce the number of antigen presenting cells able to stimulate helper T cells and activate the adaptive immune system (Luster, 2002).

CD40

CD40 is a cell surface receptor protein found on B lymphocytes, dendritic cells, follicular dendritic cells, hematopoietic progenitor cells, certain epithelial cells, carcinomas and macrophages (Banchereau et al., 1994). CD40 is a costimulatory antigen belonging to the tumor necrosis family receptor (TNFR) family (Elgueta et al., 2009). Macrophage activation and effector cell functions are, in part, regulated through cellular signaling pathways initiated by CD40 ligation (Stout and Suttles, 2004). CD40 ligand (CD40L) is expressed on activated T-cells and promotes the activation of macrophages (Elgueta et al., 2009). Ligation is the

process through which ligands bind to their respective receptors to initiate cellular signaling pathways.

Ligation between CD40 and CD40 ligands promotes clustering of CD40 in sphingolipid-rich domains of the plasma membrane and recruits TNFR associated factors (TRAF) proteins to the cytoplasmic domain of CD40 (Stout and Suttles, 2004). TRAF proteins initiate a variety of complex cellular signaling pathways leading to the regulation of nuclear factor κB (NFκB), mitogen activated protein kinases (MAPKs) and phospholipase Cγ (PLCγ) signaling pathways (Bishop et al, 2007). Figure 3 summarizes the major functions of CD40 signaling. These pathways alter gene expression to promote a proinflammatory response (Bishop et al, 2007). TRAF proteins are also used by other receptors involved in the immunological activation. For example, TRAFs 2 and 6 are recognized as direct signal transducers for TNF receptors and initiate signaling pathways that promote an antiviral response (Yang et al., 2005).

Ligation of CD40 on macrophages induces the production of numerous cytokines, such as IL-Iβ, TNF-α, IL-6 and IL-8 (Companjen et al., 2002). These cytokines help to recruit and activate other mediators of the immune system. However, sometimes these cytokines can have counterproductive effects when viruses exploit these cytokines to promote their own pathogenesis. For example, expression of TNF-α causes increased transcription of human adenovirus early region 3 (E3) (Korner et al., 1992). Thus, CD40 signaling could potentially increase the expression of transcription factors that promote viral replication.

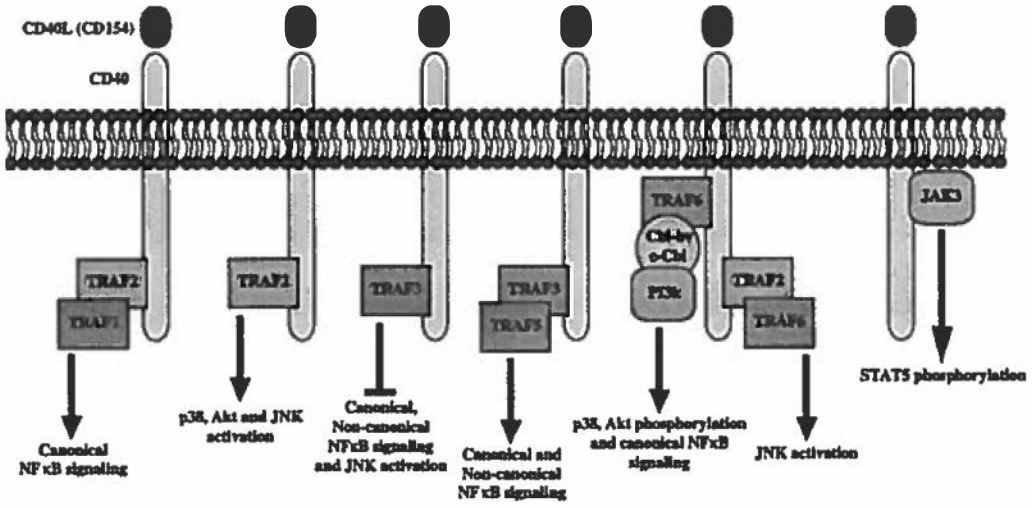

Figure 3. CD40 Signaling Pathways. (Image from Elgueta et al., 2009)

Ongoing research in Michael Angell's laboratory suggests that CD40 surface expression is upregulated in murine alveolar macrophage (MH-S) cells as early as 9 hours after infection with MAV-1. Previous research has shown that CD40 surface expression is not upregulated following infection with UV irradiated virus (data not published). These experiments are consistent with the hypothesis that CD40 upregulation is the result of viral mRNA pathogen associated molecular patterns (PAMPs) recognized by pattern recognition receptors (PRR) in MH-S cells. RIG-I is a PRR that aids in the activation of the innate antiviral immune system through the detection of viral mRNA in the cytoplasm through the initiation of signaling pathways that result in the production of type-I interferons (Xiaomo et al., 2012). A summary of RIG-I detection of viral mRNA is shown below in figure 4.

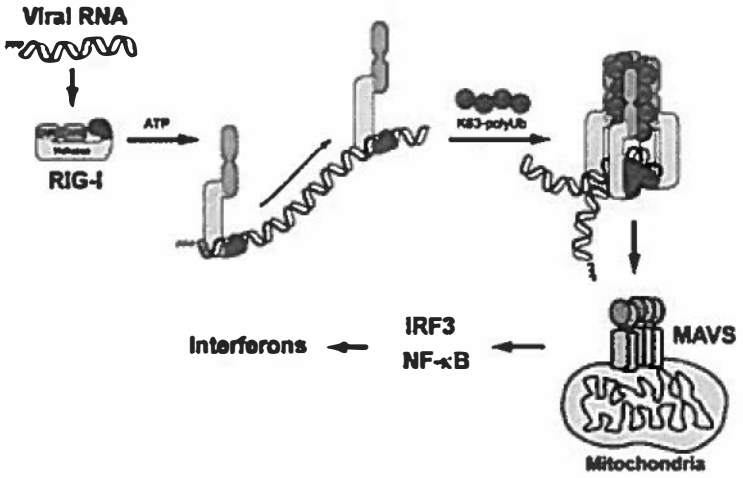

Figure 4. Detection of Viral mRNA by RIG-I. (Image from: Jiang et al., 2012)

This hypothesis is consistent with studies showing that RIG-I deficient mice succumb to fatal MAV-1 infection in vivo within 7 days of infection (Vaysburd et al., 2013). These observations suggest that recognition of viral PAMPS is required for efficient immunological function. UV irradiation of the virus inactivates the virus and blocks successful infection. Thus, it appears that adenovirus needs to be actively replicating to promote CD40 upregulation.

MAV-1 recognition by PRRs may be activating signaling cascades that upregulate the expression of CD40. This study focuses on MH-S cells and the upregulation of CD40 observed following MAV-1 infection is most likely involved in activating macrophages to promote their effector functions. Other targets of MAV-1 infection also possess CD40, such as endothelial cells. Studies have shown that CD40 expression is increased in human endothelial cells infected with human cytomegalovirus between 8 and 72 hours post infection (Maisch et al., 2002).

Endothelial cells are the primary targets of MAV-1 infection, while macrophages are a secondary target (Banchereau et al., 1994; Kotowicz et al., 2000). CD40 expression on endothelial cells is heavily influenced by cytokines, and is suggested to be involved in the development of T-cell inflammatory reactions (Karmann et al., 1995). It is unknown whether MAV-1 infection of endothelial cells induces the same upregulation of CD40 seen in MH-S cells. If CD40 upregulation is the result of PRR signaling, endothelial cells may also upregulate CD40 in response to MAV-1 infection through extracellular and intracellular PRRs (Opitz et al, 2007).

CD40 upregulation may be the result of macrophage activation in response to MAV-1 infection. Studying the role of CD40 upregulation in vitro is difficult, due to the absence of other immune effectors that may interact with CD40 in vivo. The goals of this study are to develop CD40 silenced cell lines and determine if CD40 silencing impacts MAV-1 infection, in the absence of any stimulation from other immune cell types. However, the true function of CD40 upregulation may be obscured due to the absence of certain costimulatory signals from other cell types that will not be present.

RNA Interference

There are numerous methods that silence the expression of specific genes such as siRNA, RNAi, TALEN and CRISPR (Booettcher & McManus, 2015). This study uses RNA interference (RNAi) to silence the expression of CD40. RNAi can be used to silence the expression of specific genes through the generation of double-stranded RNA (dsRNA), which leads to degradation of corresponding mRNA transcripts (Rao et al., 2009).

Lentiviral vectors provide a fast and efficient means of modulating gene expression in mammalian cells using RNAi (Shearer & Saunders, 2015). Lentiviral mediated RNAi is an attractive option for silencing because the integration of CD40 silencing shRNA into the DNA of the target cell enables constitutive expression of the silencing construct (Rao et al., 2009).

Transcription of shRNA ultimately silences the expression of specific mRNA transcripts based on sequence complementarity. A summary of lentiviral mediated shRNA silencing is shown in figure 5. The lentivirus attaches to the host cell and injects ssRNA containing the shRNA construct. The ssRNA is reverse transcribed to DNA, which can then be integrated into the genome of the target cell. Following the integration of the shRNA sequence, it is expressed by the host cell and cleaved by the enzyme dicer to produce siRNA. siRNA is then able to bind to complementary mRNA transcripts and target them for degradation, thus inhibiting protein expression.

The shRNA transcript used in this study has successfully been used to silence the expression of CD40 in murine bone marrow derived monocytes (Kalantri et al., 2013). The silencing construct contains CD40 shRNA, and a puromycin resistance gene to select for cells expressing the construct. Constitutive expression is provided through the human U6 promoter. U6 is a member of uridine rich small nuclear RNAs (snRNA) (Almada et al., 2013). Other experiments have successfully silenced CD40 expression using similar gene silencing techniques (Hueso et al, 2016; Kawabe et al., 1994).

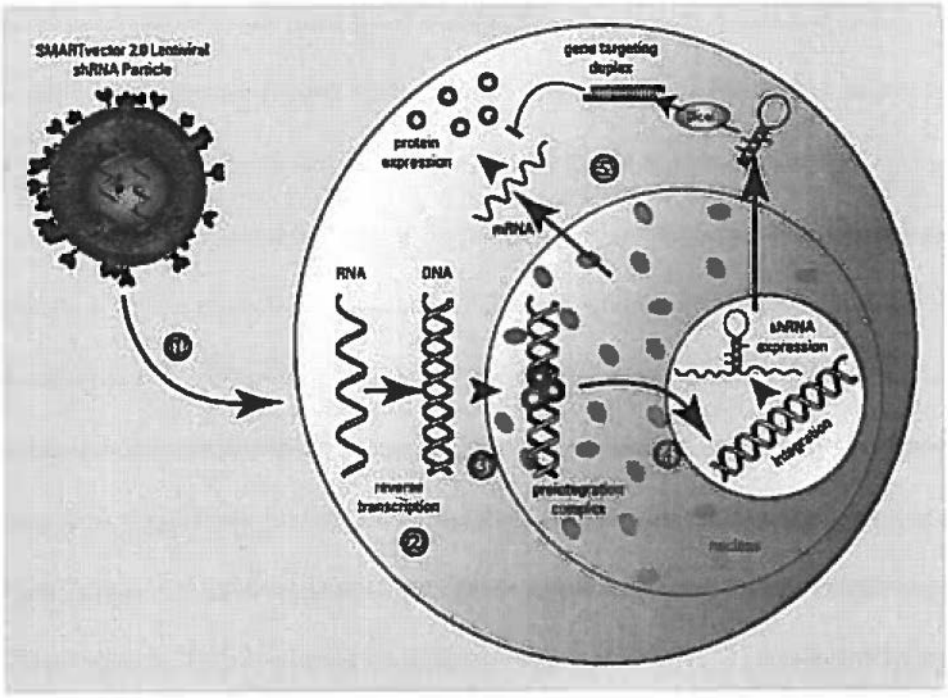

Figure 5. Summary of Lentiviral mediated RNAi. (Image from Liszewski, 2011)

Materials and Methods

Cell lines & culture

Human embryonic kidney (HEK293T) (ATCC® CRL-1573™), murine alveolar macrophage (MH-S) (ATCC® CRL-2019™), and murine fibroblast (3T6) (ATCC® CCL-96™) cells were obtained from the American Type Culture Collection (ATCC). All cells were maintained at 5% CO_2 and 37°C. MH-S cells are Simian Virus 40 (SV-40) transformed alveolar macrophages isolated from the bronchoalveolar lavage of BALB/c mice and are used to study alveolar macrophages and their role in lung pathologies (Mbawuike and Herscowitz, 1989).

HEK293T cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (HIFBS), 4 mM L-glutamine, 1 mM pyruvate and 3.7 g/l sodium bicarbonate. In the initial plating of HEK293T, media was supplemented with 100 IU/mL penicillin and 100 µg/mL streptomycin. MH-S cells were maintained in Roswell Park Memorial Institute (RPMI) media supplemented with 10% HIFBS, 2 mM L-glutamine, 1 mM pyruvate, 1.5 g/l sodium bicarbonate, 10 mM HEPES buffer, 14.3mM 2-mercaptoethanol. 3T6 cells were maintained in DMEM supplemented with 10% HIFBS, 4 mM L-glutamine, 1 mM pyruvate and 3.7 g/l sodium bicarbonate. Media pH was adjusted to 7.2 using 1 N HCl prior to sterilization by filtration using a 0.22 µm filter.

Plasmid Preparation

Plasmids necessary for transfection were prepared by either phenol-chloroform extraction or PureYield® (Promega, A1223) plasmid extraction. The manufacturer's protocol was used to prepare plasmids using PureYield®. Phenol-chloroform extraction was performed according the alkaline lysis method described by Addgene (Addgene, 2016). Glycerol stocks containing competent *E. coli* cells expressing the following plasmids were used to inoculate Luria broth media containing 100µg/ml Ampicillin: CD40 shRNA pLKO.1 plasmid (clone ID: TRCN0000066244, clone name: NM_011611.1-672s1c1; Sigma-Aldrich, St Louis, MO, USA), pMD2.G (Addgene, #12259), and pAdTrack (Addgene, #16404). Cultures were shaken at 300rpm for 18 hours at 37°C. Extracted plasmids were resuspended in Tris EDTA buffer. DNA concentration was determined using a NanoDrop™ spectrophotometer. Plasmid maps for pLKO.1, psPAX2, and pMD2.G are shown in the appendix.

GFP Transfection

HEK293T cells were transfected with 8 µg of pAdTrack using Lipofectamine 2000®. Lipofectamine transfection was conducted per manufacturer's protocol (Thermofisher, 2013). Green Fluorescence Protein (GFP) expression was analyzed by fluorescence microscopy 48 hours post transfection. Cells were counted and the percentage of cells expressing GFP was used as a measure of transfection efficiency. Cells were counted under 200X magnification using fluorescence microscopy on an Olympus IMT-2. The average of the percent GFP expression was averaged for 2 fields chosen at random. Wild type HEK293T cells served as a negative control, and the positive control was AdTrack GFP that was isolated by PureYield® extraction by Dr. Brittany Albaugh (EMU), and has been used successfully in the past.

Lentiviral Production

Lentiviral particles were produced by an adapted protocol provided by the plasmid repository Addgene (Addgene, 2016). HEK293T cells were plated in 60 mm plates to obtain approximately 60% confluency at time of transfection. Media was replaced with DMEM, without serum or antibiotics, 2 hours prior to transfection.

To produce the lentivirus, HEK293T cells were transfected using Lipofectamine 2000®. The transfection inoculum contained 4 µg of CD40 shRNA pLKO.1 plasmid, 3 µg of psPAX2, and 1 µg of pMD2.G. Media for cultures was replaced with DMEM without antibiotics 24 hours post transfection. At 48 and 72 hours post transfection, culture supernatant was harvested and stored at 4°C. The supernatant, containing lentiviral particles,

was centrifuged for 5 minutes at 500 x g / 4°C, filtered through a 0.22 µm filter and stored at -70°C until use. A summary of lentiviral production is shown in figure 6.

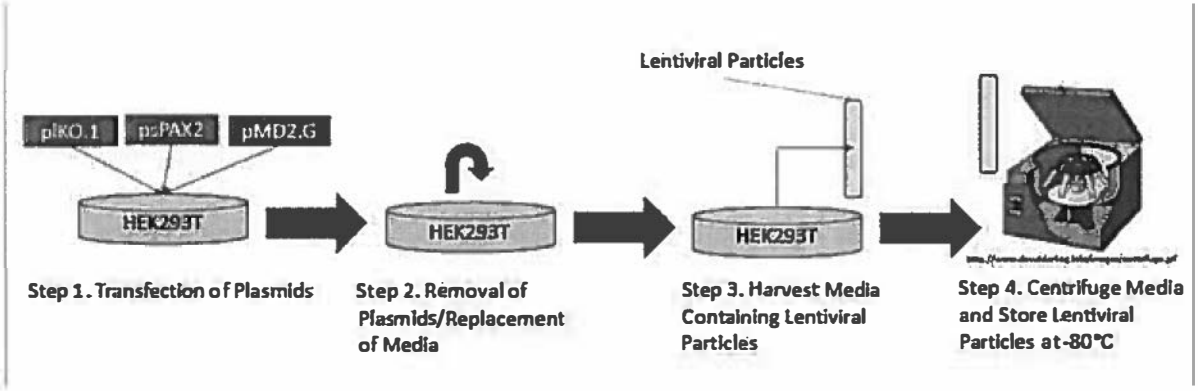

Figure 6. Summary of Lentiviral Production.

Generation of CD40 Silenced MH-S Cell Lines

MH-S cells were plated in 60mm plates to obtain approximately 60% confluent flasks at the time of transduction. NPB (1-5) refers to CD40 silenced lines that showed the greatest CD40 suppression. NPB1 and 2 were the earliest transductions using lentivirus produced by plasmids isolated by phenol-chloroform extraction. NPB3, 4 and 5 were transductions using lentivirus produced by plasmids isolated by PureYield® extraction. Twenty-four hours after plating, the media was replaced with fresh media and 1 ml of media containing lentiviral particles was added to each plate. After 24 hours of incubation, the media was replaced with fresh media containing 4 µg/ml puromycin (Sigma Aldrich, P8833) to select for cells containing the CD40 shRNA insert. To assess the effectiveness of puromycin selection, wild type MH-S cells were also grown in media containing 4 µg/ml puromycin and media was replaced every 3 days. Cytotoxicity in non-transduced cells was analyzed 5 days post

transduction to ensure that adequate selection pressure was present due to puromycin. A summary of lentiviral infection is shown in figure 7.

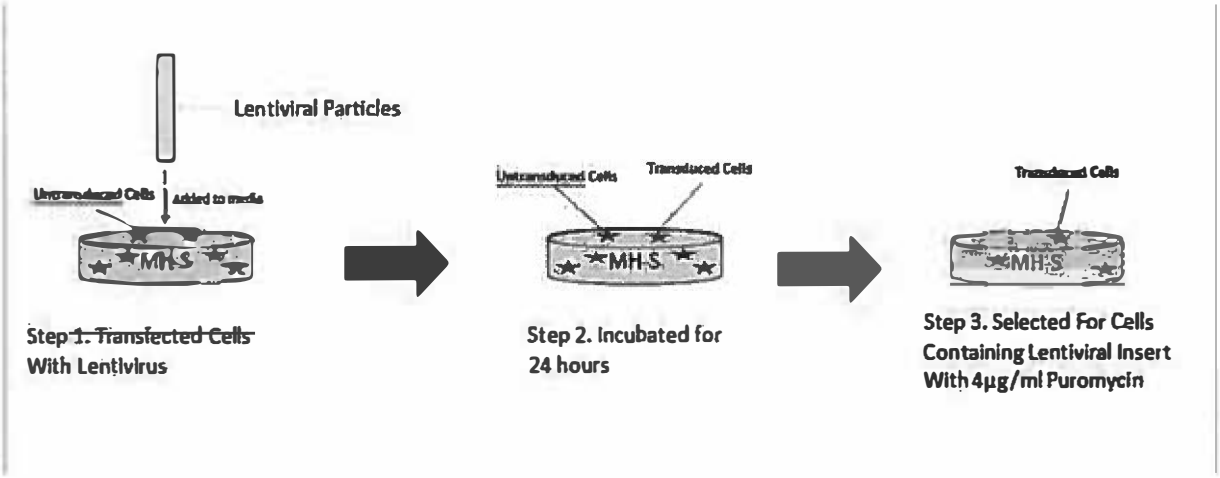

Figure 7. Summary of Lentiviral Mediated CD40 Silencing.

CD40 Stability of Silencing

The stability of CD40 silencing was assessed to determine if the removal of puromycin caused CD40 silenced cells to revert to the wild type phenotype. NPB3 was grown in separate flasks, in media with and without 4 µg/ml puromycin for 11 days. Media was replaced every 2-3 days during incubation period. Cells were analyzed for CD40 surface expression 11 days after separation using the flow cytometric parameters described in the section below.

Flow Cytometric Analysis

Cultured cells were detached from flasks with 5 mL of Accutase (eBioscience, 00-4555-56) and suspended in FACS staining buffer (PBS, 1% bovine serum albumin, 0.1% sodium azide) to obtain a concentration of 10^7cells per mL. One-hundred microliters of cell

solution was used for each reaction, and 1 µl of the following antibodies was added to each reaction to obtain a final antibody concentration of 2µg/ml: anti- mouse CD40 – Allophycocyanin (APC) conjugate (eBioscience, 17-0402-80), and anti-mouse CD86 – (phycoerythrin) PE/Cy7) (eBioscience, 12-5322-81). Rat Ig2a$^{\kappa}$ (eBioscience, 25-4321-81) was used as an isotype control for CD86 staining. Cells were stained on ice for 1 hour in the dark, rinsed with PBS, and fixed with 2% paraformaldehyde in PBS. We used a BD FacsCalibur flow cytometer and analyzed the data we obtained using CellQuest software. The instrument was set up to collect 10,000 of all events for each run. An analysis gate was set to represent 90% of all events based on forward and side scatter. The following instrument settings were used:

Table 1. Flow Cytometry Parameters. (Threshold: FSC Value: 52)

Parameter	Detector	Voltage	Amp Gain	Mode
P1	FSC	E-1	5.41	Lin
P2	SSC	380	1.00	Lin
P3	FL1	500	1.50	Lin
P4	FL2	381	1.00	Log
P5	FL3	650	1.00	Log
P6	FL2-A	N/A	1.00	Lin
P7	FL4	675	1.00	Log

MAV-1 Infection

MH-S wild type cells and a silenced strain, NPB5, were grown to approximately 80% confluence prior to infection. NPB5 cells were used in this experiment because it is the CD40-silenced line that showed the greatest reduction in CD40 expression. Cells were infected with 2 mL of a 1:10 dilution of MAV-1 viral stock and incubated for 1 hr. Based on

the molecular weight of the MAV-1 genome and the DNA concentration of the viral stock, there are approximately 10^{10} viral genomes per mL of viral stock. Based on this concentration, and the assumption that each T75 flask contained approximately 10^7 MH-S cells at the time of the infection, the experimental multiplicity of infection (MOI) is approximately 2000. RT-qPCR analysis of the viral stock demonstrated a detection limit for MAV-1 of approximately 100 viral genomes (data not shown). Thus, the true MOI is more likely between 20-2000. However, because we were unable to plate the virus to determine an actual viral titer, this calculation ofMOI is an estimation. The original MAV-1 stock used to produce the virus was obtained from ATCC (VR-550™) and working stocks were generated from previous infections in 3T6 cells.

Each flask was tilted every 15 minutes to prevent desiccation of cells during infection. After infection, cells were refed with 15mL of RPMI with 2% HIFBS. The media containing virus was not removed prior to refeeding cells. The cultures were incubated at 37°C until their respective analysis times.

At 24 hours pre and post infection, cells were detached with 5 mL of Accutase and washed with 10 mL of PBS per T75 flask. Cells were analyzed for the expression of CD40 and CD86 using flow cytometry.

Results

Lentiviral Plasmid Transfection Verification by GFP

Lipofectamine 2000® transfection efficiency was analyzed to compare the quality of plasmids produced by phenol-chloroform extraction and PureYield® extraction. We isolated GFP plasmid from logarithmically growing *E. coli* cells using phenol-chloroform and PureYield® extraction, and transfected these plasmids into HEK293 cells. Untransfected HEK293 cells served as a negative control to examine the contribution of background fluorescence. We compared these methods of plasmid extraction to optimize the production of lentivirus.

Transfection with GFP-expression plasmid isolated by phenol-chloroform extraction resulted in poor expression and fluorescence (3%) relative to the positive control (44%) and PureYield® GFP (53%) (Figure 7) (Table 2). No fluorescence was observed in the negative control. These results show that GFP plasmids isolated by PureYield® extraction results in more efficient GFP expression than using the same plasmid isolated by phenol-chloroform extraction.

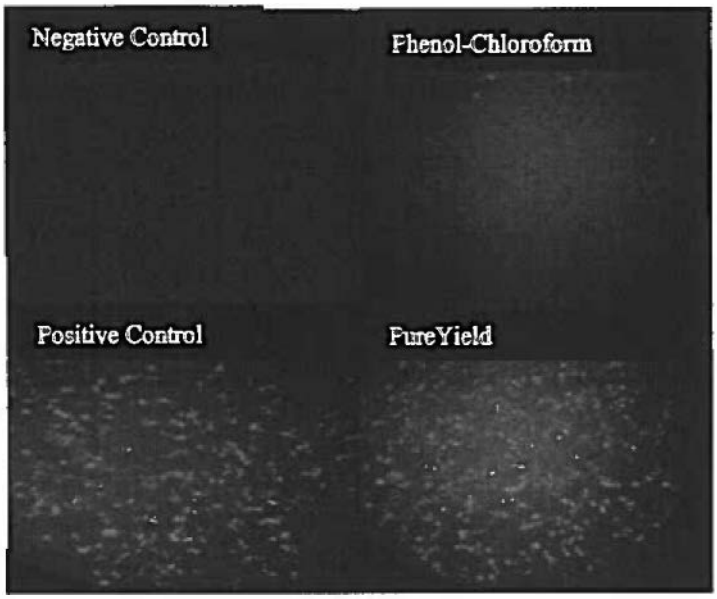

Figure 7. Fluorescence-microscopy analysis of GFP transfection efficiency in HEK293T cells. (Olympus IMT-2, 200X magnification)

Table 2. Percentage HEK293T cells expressing GFP.

Sample	Transfection Efficiency
Negative Control	0%
Positive Control	44%
Phenol-Chloroform GFP	3%
PureYield® GFP	53%

Validation of CD40 Silencing

Flow cytometry was used to visualize CD40 protein surface expression and verify successful silencing of transduced lines. We used the mean fluorescence intensity (MFI) to represent the level of CD40 surface expression of the population. The average MFI of

unstained cells was subtracted to correct for background fluorescence. We observed decreased CD40 expression in 5 transfected lines (hence denoted as NPB1-NPB5). Cells were cultured in media containing 4μg/ml puromycin and analyzed by flow cytometry between 4 and 15 days post lentiviral (shRNA) transfection. Relative expression is based on basal expression of CD40 in wild type MH-S cells. However, the silenced cell lines transduced with lentivirus produced by PureYield® plasmids showed dramatically lower CD40 expression compared to cell lines generated from lentivirus produced with plasmid DNA isolated by phenol-chloroform extraction. Lower silencing efficiency suggests that plasmids isolated by phenol-chloroform extraction result in poor lentiviral yield. A summary of CD40 expression in silenced lines is shown in Figure 8. Among the cell lines generated, NPB5 showed the greatest reduction in CD40 expression and was used for our infection assay.

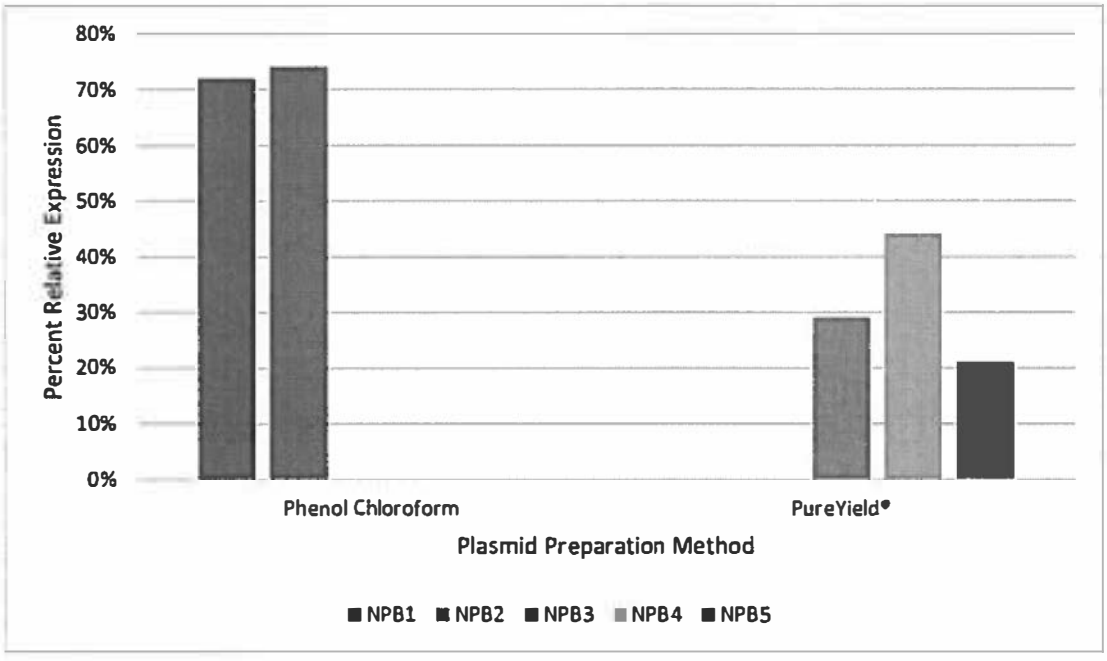

Figure 8. CD40 Expression in Silenced Lines: A Comparison of Plasmid Preparation Methods.

Stability of CD40 Silencing

We examined if the absence of puromycin affects the stability of CD40 silencing. NPB3 cells were grown in media with and without puromycin, and were analyzed by flow cytometry 11 days (4 passages) of cell culture. CD40 expression was slightly upregulated in the cells that had been grown without puromycin (Figure 9). The observed shift represents an approximately 2-fold increase in CD40 expression.

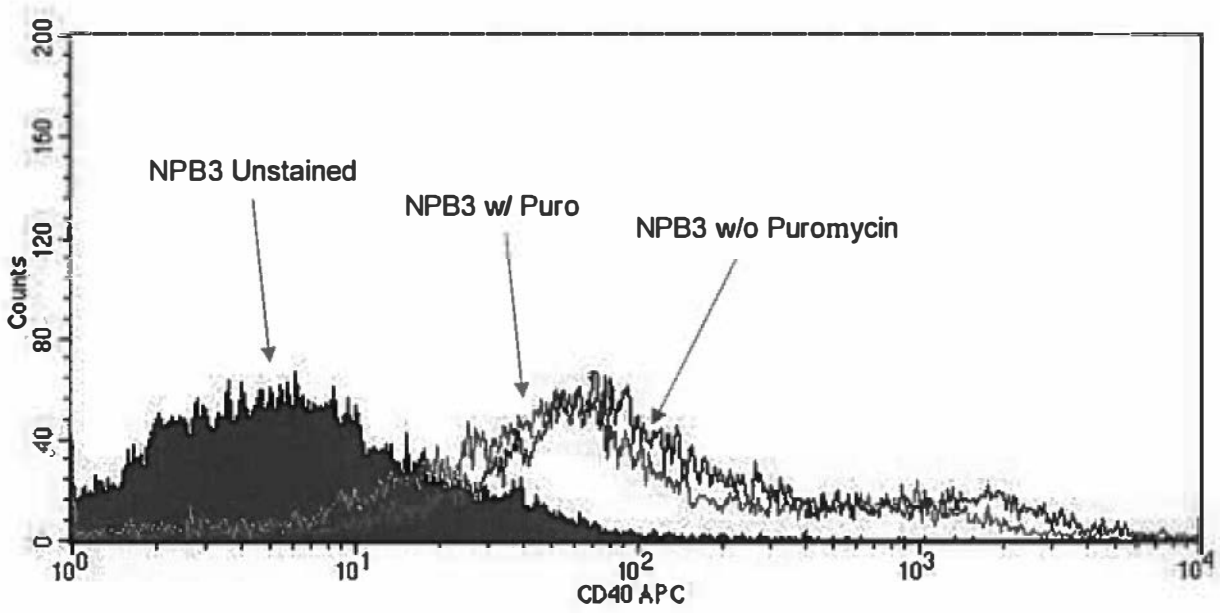

Figure 9. Stability of CD40 silencing. NPB3 cells were grown with (green) and without puromycin (pink) for 11 days and analyzed by flow cytometry. The results show that the removal of puromycin caused an increase in CD40 expression.

CD40/CD86 Expression During MAV-1 Infection

We analyzed surface expression of CD40 and CD86 to determine how MAV-1 infection alters the expression of costimulatory molecules on MH-S cells. Flow cytometry was used to analyze surface expression of CD40 and CD86 24 hours prior to, and 24 hours following, infection with MAV-1. CD86 is another immunological marker found on antigen

presenting cells. CD86 expression was analyzed to verify that silencing of CD40 was specific, and did not alter the expression of other cell surface proteins. The MFI of unstained cells was subtracted to correct for background fluorescence when analyzing CD40, and the MFI of cells stained with a PE/Cy7 isotype control was subtracted to correct for background fluorescence when analyzing CD86 expression. We observed a slight decrease in CD86 expression in NPB5 uninfected and infected cell lines, when compared to wild type MH-S expression under similar conditions.

Flow cytometric analysis revealed CD40 surface expression was upregulated in both the silenced and control cultures following infection with MAV-1. Wild-type MH-S cells showed an approximately 5-fold increase in CD40 surface expression, while NPB5 cells showed a 16-fold increase in CD40 expression. Although elevated relative to basal expression, CD40-levels in infected NPB5 cells were approximately 55% lower relative to CD40 expression in infected MH-S cells. Flow cytograms for CD40 and CD86 are shown in figures 10 and 11. CD40 expression and CD86 expression are also represented in figures 12 and 13. These results confirm that CD40 is upregulated in silenced and wild type MH-S cells by approximately 11-fold and 5-fold, respectively. CD86 is upregulated following infection by MAV-1 by approximately 70% in both cell lines. These results show that CD40 silencing was specific because silencing did not dramatically alter the expression of CD86.

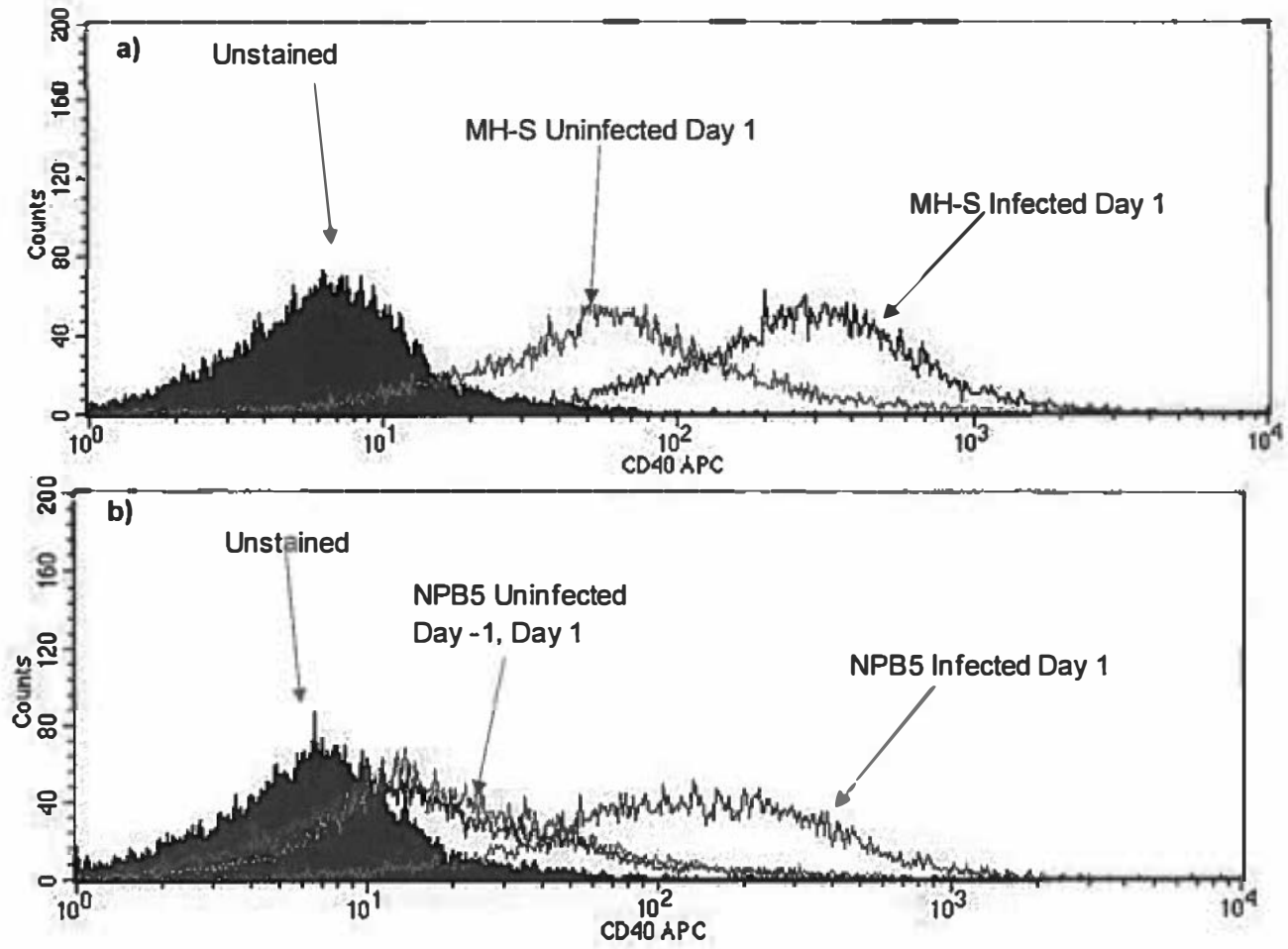

Figure 10. Representative flow cytograms of CD40 expression in MH-S (a) and NPB5(b) cells in response to MAV-1 infection. CD40 expression is reduced in NPB5 cells when compared to MH-S control cells. CD40 surface expression is upregulated in MH-S and NPB5 lines following infection with MAV-1.

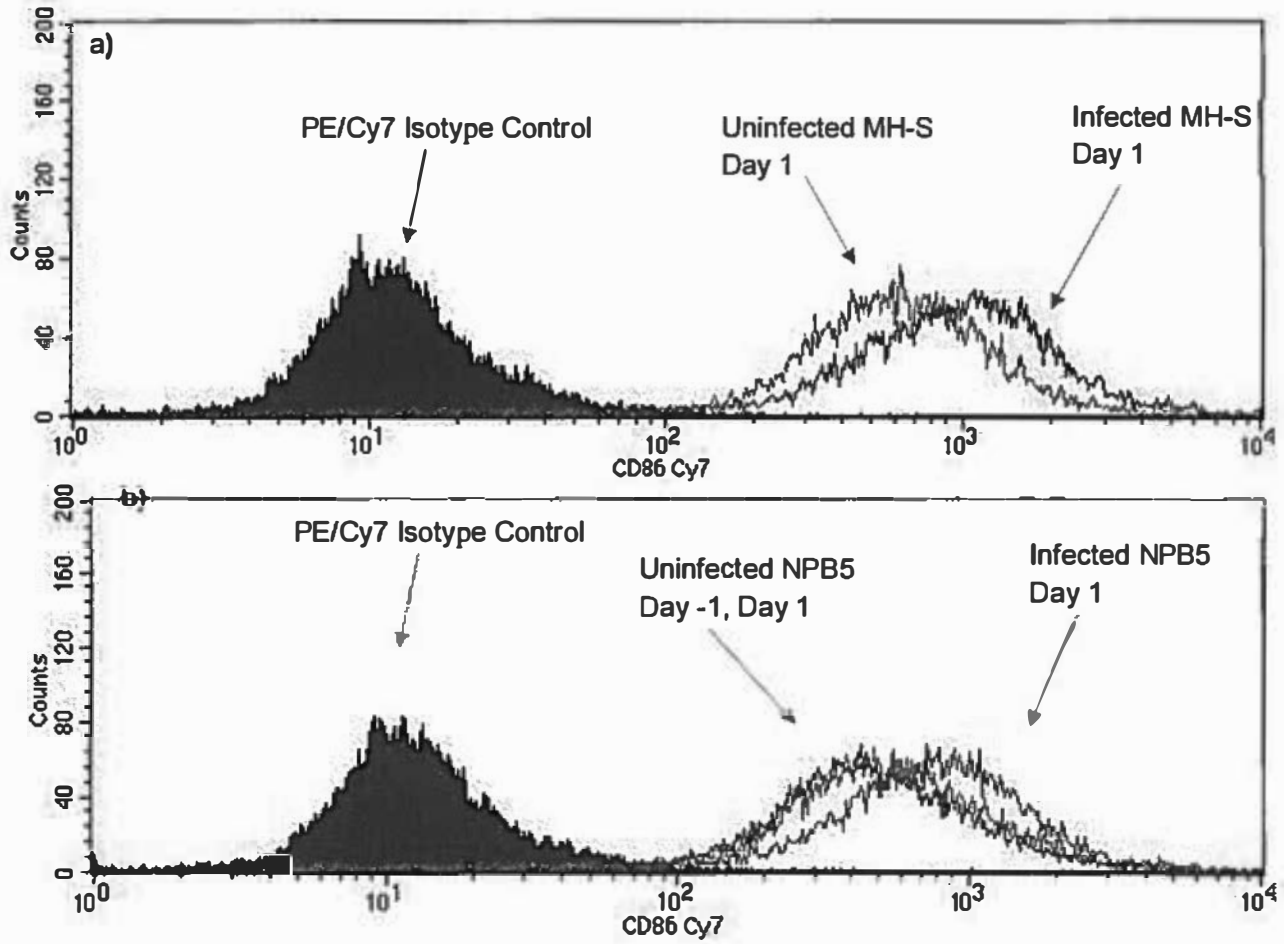

Figure 11. Representative flow cytograms of CD86 expression in MH-S (a) and NPB5(b) cells in response to MAV-1 infection. CD86 is slightly upregulated in MH-S and NPB5 cells following infection. CD86 expression did not change between day -1 and 1 in uninfected NPB5 cells

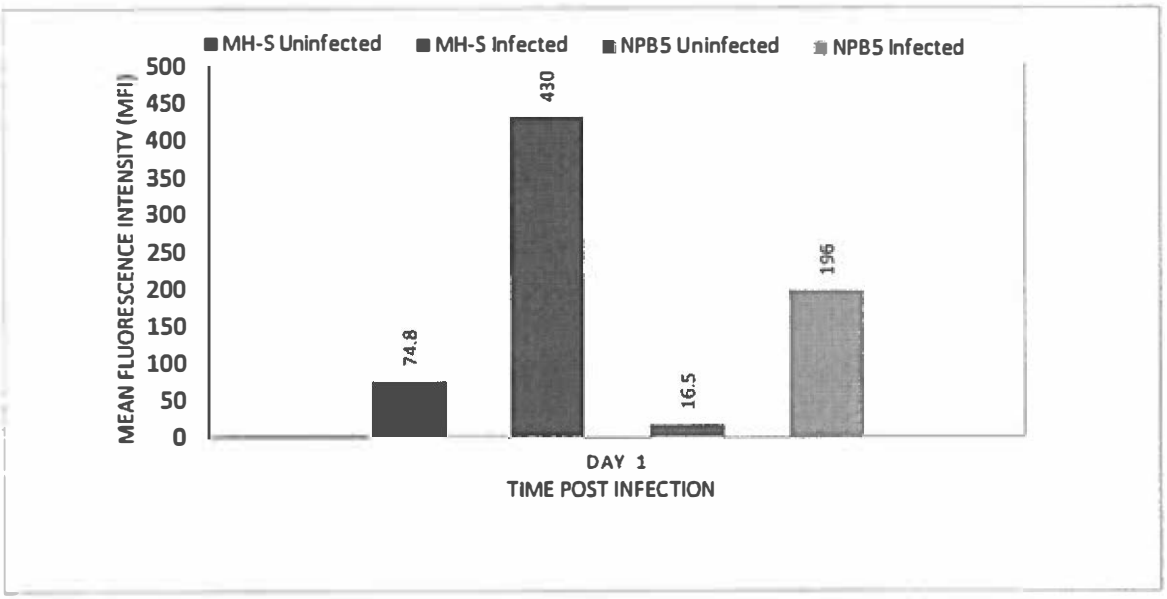

Figure 12. Effect of MAV-1 infection on CD40 Expression. CD40 expression is increased in MH-S and NPB5 cultures at 1 day post infection. CD40 expression is unaltered in uninfected cells between 1 day prior to infection and 1 day post infection (data not shown).

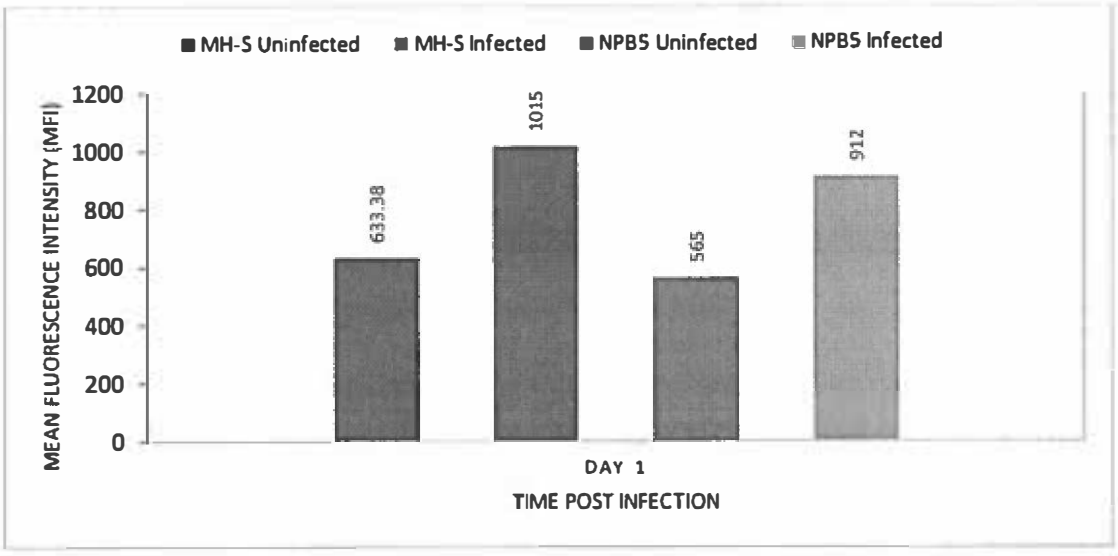

Figure 13. Effect of MAV-1 Infection on CD86 Expression. NPB5 expressed slightly lower levels of CD86 when compared to MH-S cells in both uninfected and infected cultures. MH-S and NPB5 lines showed increased CD86 expression following infection with MAV-1. CD86 expression is unaltered in uninfected cells between 1 day prior to infection and 1 day post infection (data not shown).

Discussion

Lentiviral Plasmid Transfection Verification by GFP

We compared the transfection efficiency of a GFP-expression plasmid to examine how the method of DNA preparation alters DNA uptake and expression in HEK293T cells. The initial lentiviral transductions (NPB1 & NPB2) were conducted using lentivirus generated from plasmids extracted by phenol-chloroform extraction and showed poor CD40 suppression. We hypothesized that plasmids extracted by phenol-chloroform extraction may not be efficiently expressed in HEK293 cells. Poor plasmid transduction would result in poor lentivirus production and lower lentiviral titer, resulting in reduced the probability of successful shRNA integration and CD40 silencing. Optimizing plasmid transfection efficiency improved lentiviral production and the subsequent silencing of CD40 of MH-S cells. We compared AdTrack GFP plasmids that were isolated from phenol-chloroform and PureYield® extractions.

Plasmid extraction by PureYield® led to more successful transfection of the GFP plasmid, as assessed by fluorescence microscopy. No fluorescence was observed in the non-transfected control samples, suggesting that the background fluorescence in HEK293T cells is negligible in our analysis. Cells that were transfected with GFP-plasmid isolated by phenol-chloroform showed very low transfection efficiencies. Poor GFP-plasmid transfection suggests that lentiviral production will also be poor using this method. Flow cytometry data supports that CD40 is suppressed to a greater level in cells that were transduced with lentivirus produced by plasmids isolated by PureYield® extraction. The transfection efficiency was comparable between the positive control (produced in another laboratory) and

our PureYield® extracted GFP plasmid, which is to be expected given that both plasmids were obtained using the same extraction method.

The low level of expression in phenol-chloroform extracted GFP suggests that plasmids isolated by phenol-chloroform extraction are not suitable for expression in HEK293T cells. Phenol-chloroform extraction may have carried some endotoxin from the bacterial culture. This could alter the efficiency of HEK293T cells to take up exogenous DNA. PureYield® extraction kits are specifically designed to neutralize endotoxin contamination, which may explain the differences in expression between the two methods. It also is possible that phenol and/or chloroform may have been carried over during plasmid preparation, which would have been toxic to the cells. These data suggests that lentivirus production by plasmids that were extracted by phenol-chloroform will be less efficient, result in lower viral titers in harvested media, and be less efficient at silencing CD40.

Generation of CD40 Deficient MH-S cells

Five CD40 silenced lines were generated from lentivirus produced by plasmids extracted by phenol chloroform extraction (NPB1-2) and PureYield extraction (NPB3-5). Lines NPB1-2 showed 26-28% reduction in cell surface expression of CD40 compared to wild type controls. Lines NPB3-5 showed a 56-79% reduction in cell surface expression of CD40 compared to wild type controls. Multiple factors can influence the degree of suppression such as plasmid purification method, puromycin concentration and culture conditions. These results support our GFP assay, which suggests that lentiviral production plasmids extracted by PureYield® result in a higher transfection efficiency than plasmids extracted by phenol-chloroform extraction.

Stability of Silencing in the Absence of Puromycin

We compared CD40 expression in cells that had been maintained in media with and without puromycin. Stable expression of the silencing construct is essential because subsequent MAV-1 infection studies would be conducted in the absence of puromycin. In addition, because puromycin is a protein synthesis inhibitor, we wanted to ensure that any effects we observe in the CD40 silenced cells are due the silencing construct and not interactions with puromycin.

Our results showed that the effect of CD40 silencing is slightly reduced in cells that were grown without puromycin for 11 days. This increase in expression suggests that the removal of puromycin as a selection pressure, results in drift towards wild type CD40 expression levels. The MAV-1 infection in this experiment used cells that were grown without puromycin for only 2 days, so it is unlikely that the same level drift would be observed in our cells.

CD40/CD86 Expression Following MAV-1 Infection

Flow cytometric analysis shows that CD40 surface expression is increased in silenced and unsilenced cell lines following infection with MAV-1, supporting previous experiments that have shown an approximately 4-fold increase in CD40 expression following infection with MAV-1. We analyzed CD86 as a marker to determine if CD40 silencing was specific and did not impact the expression of other antigens. MH-S cells showed approximately 5-fold greater expression of CD40 following infection with MAV-1. Uninfected NPB5 cells showed approximately 80% reduction in CD40 relative to wild type MH-S cells. NPB5 showed an approximately 15-fold increase in CD40 compared to uninfected NPB5 cells. This

dramatic increase in expression is largely due to the low level of CD40 expression observed in silenced cells. Infected NPB5 cells expressed almost 3 times as much CD40 as uninfected MH-S cells, and half as much CD40 as infected MH-S cells.

We did not account for the dramatic CD40 upregulation occurring to the extent that it did in the CD40 silenced lines. An ideal silencing construct would maintain effective silencing even after infection with MAV-1. However, infected CD40 silenced lines still showed a 2-fold reduction in CD40 expression compared to wild type infected cells. A 2-fold difference may still be large enough to reveal the role of CD40 up regulation following MAV-1 infection.

It was hypothesized that CD86 would be unaffected by CD40 silencing. The data suggest that CD40 silenced lines showed a slight reduction in CD86. Infection with MAV-1 caused an increase in CD86 expression in silenced and unsilenced cell lines. Both cell lines showed approximately the same relative upregulation (+66%).

These results are consistent with the hypothesis that CD40 and CD86 are upregulated in MAV-1 infected cells to promote macrophage activation and antigen presentation. CD40 has been shown to be upregulated as early as 9 hours post infection (Angell, M.G. personal communication). Previous RT-qPCR data has also shown that MAV-1 early genes are expressed during this timeframe (Angell, M.G, personal communication). Given that CD40 coincides with the period of MAV-1 early gene expression, it is likely, but not proven, that MAV-1 upregulation is the result of pattern recognition receptors responding to MAV-1 infection by upregulating CD40 and other costimulatory markers to promote activation of naïve T-cells. Similarly, HIV-1 Tat protein has been shown to cause an upregulation in CD40, CD80 and CD86 in monocyte derived dendritic cells, and promote macrophage

maturation, antigen presentation, and a T-helper 1 response (Fanales-Belasio et al., 2002). Although Tat has its own effector functions in viral replication, it is also a potential target for PRRs (Mogensen et al., 2010). These observations support the hypothesis that CD40 and CD86 upregulation are the result of macrophage activation. However, it is still unclear as to whether MAV-1 can benefit from this upregulation.

It has been shown that adenoviruses can activate their own transcription in response to some inflammatory cytokines induced by CD40 signaling (Korner et al., 1992). Through this mechanism, MAV-1 would be able to exploit host inflammatory cytokines to promote its own pathogenesis. This prompts inquiry into the true function of CD40 signaling. Classically, it would be assumed that this upregulation of CD40 is the result of macrophage activation and functions to activate other aspects of the immune system. While this may be partially true, it also possible that CD40 upregulation promotes MAV-1 replication.

Future Directions

We did not anticipate that MAV-1 infection would reduce the effects of our silencing construct. Manufacturers commonly market silencing constructs that that boast 80% silencing efficiencies. Thus, the ideal silencing method would maintain silencing near this level, even after infection with MAV-1. Potential methods of increasing the silencing efficiency include using multiple CD40 shRNA transcripts in a silencing cocktail, or using a CRISPR system, that could be used to inactivate the CD40 (Cong et al., 2013). It is likely that the latter method will be more effective due to the CRISPR system modifying the CD40 DNA within the genome. In comparison, shRNA mediated silencing leaves the CD40 gene intact, while degrading the CD40 mRNA transcripts.

However, MAV-1 infected CD40 silenced MH-S cells still maintained a 2-fold reduction in CD40 expression compared to wild type, and may be a valuable tool to study the role of CD40 upregulation in vitro. Studies have shown that a 2-fold difference in CD40 expression on myeloid derived suppressor cells is sufficient to dramatically alter gene expression (Ding et al., 2015). Therefore, the silenced cells generated in this study may still be used to further elucidate the role of CD40 signaling in macrophage cells by conducting a similar experiment with the addition of CD40 ligand to activate CD40 signaling. This would provide a clearer description of the role of CD40 in vivo, where other costimulatory antigens would be present to activate these pathways (Stout and Suttles, 2004).

In a broader sense, there are many other perspectives to examine the function of CD40 upregulation following MAV-1 infection. One could observe CD40 expression in MAV-1 BALB/c and C57BL/6 alveolar macrophages. If CD40 upregulation is the result of an immunological process found in BALB/c mice, but not C57BL/6, we would expect to see an increase in CD40 expression in BALB/c macrophages, but not C57BL/6 macrophages. C57BL/6 derived alveolar macrophages are available from the ATCC (ATCC, 2017). However, the use of immortalized cell lines creates special limitations when studying adenoviral replication. MH-S cells are derived from BALB/c derived macrophages, but are altered due to immortalization with SV-40. To reduce the number of confounding variables, an ideal experiment would be conducted using mice, since this would provide the most accurate depiction of MAV-1 infection in mice.

In conclusion, further characterization of the CD40 silenced cell lines generated in this study may reveal the role of CD40 upregulation following MAV-1 infection. However, the cell lines we generated may not provide optimal CD40 silencing during MAV-1

infection. It may be worthwhile to generate a new CD40 silenced cell line using a silencing construct that maintains CD40 suppression following MAV-1 infection. The results of this study support previous experiments that have shown a 5-fold increase in CD40 expression is observed following infection with MAV-1. In addition, we observed a slight increase in CD86 following infection with MAV-1. Future studies could further characterize CD40 silenced MH-S cells to understand the role of CD40 and CD86 upregulation following infection with MAV-1.

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Appendix

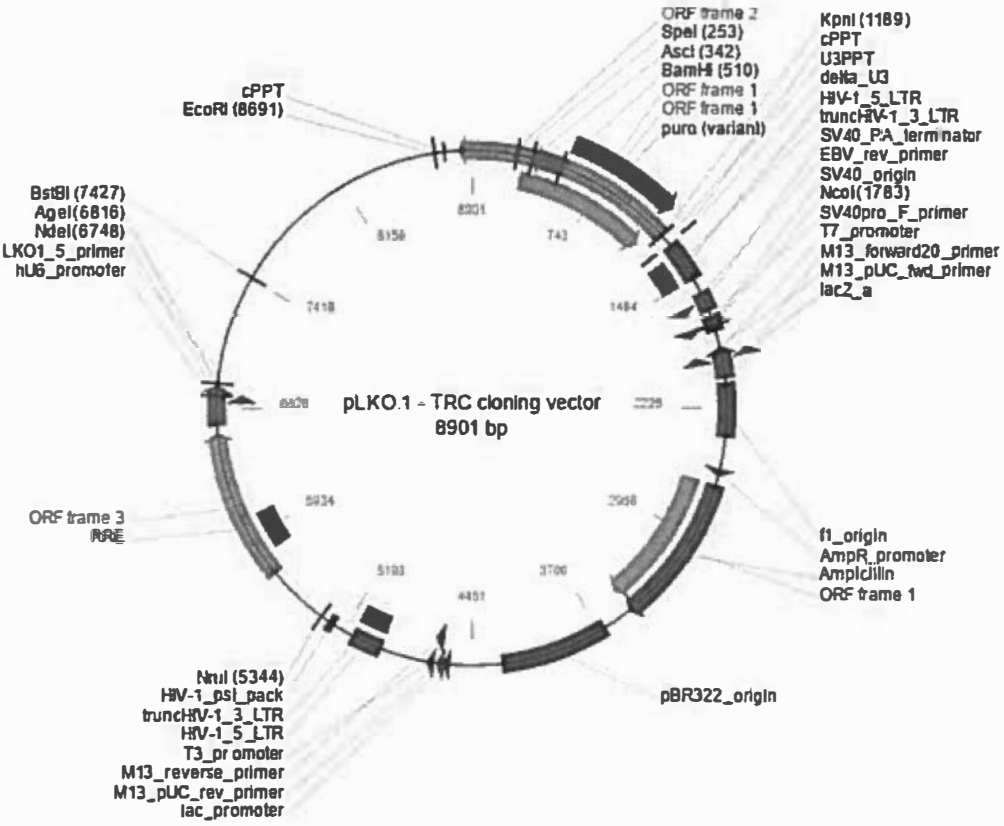

Figure 14. Plasmid map of pLKO.1.

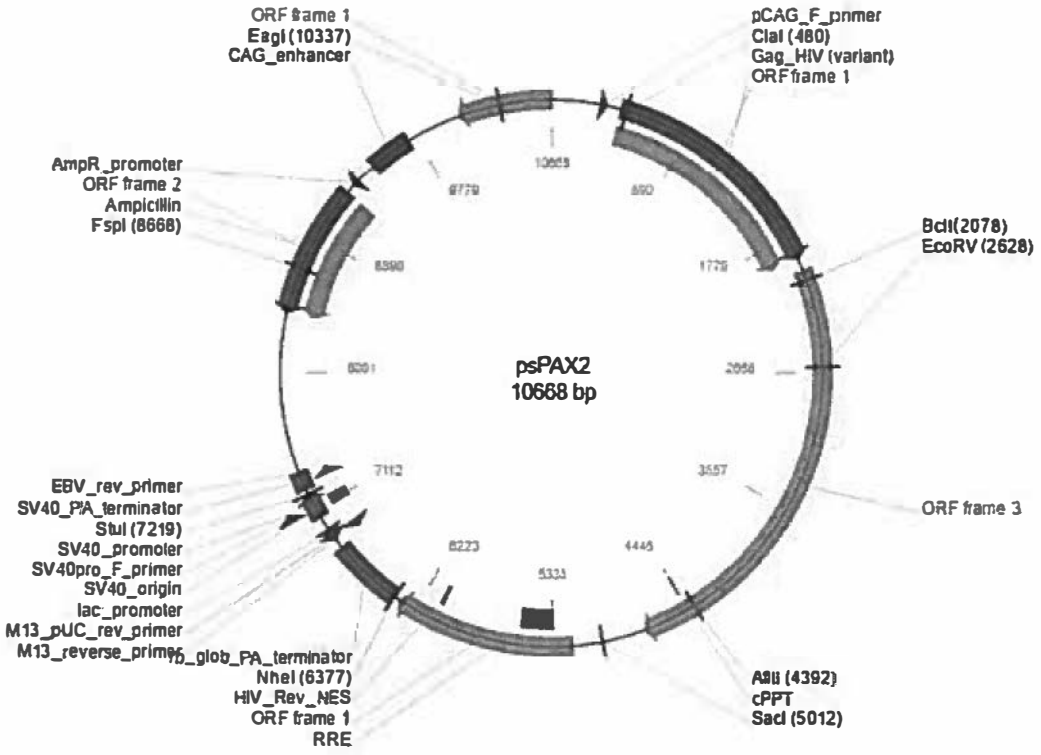

Figure 15. Plasmid map of psPAX2.

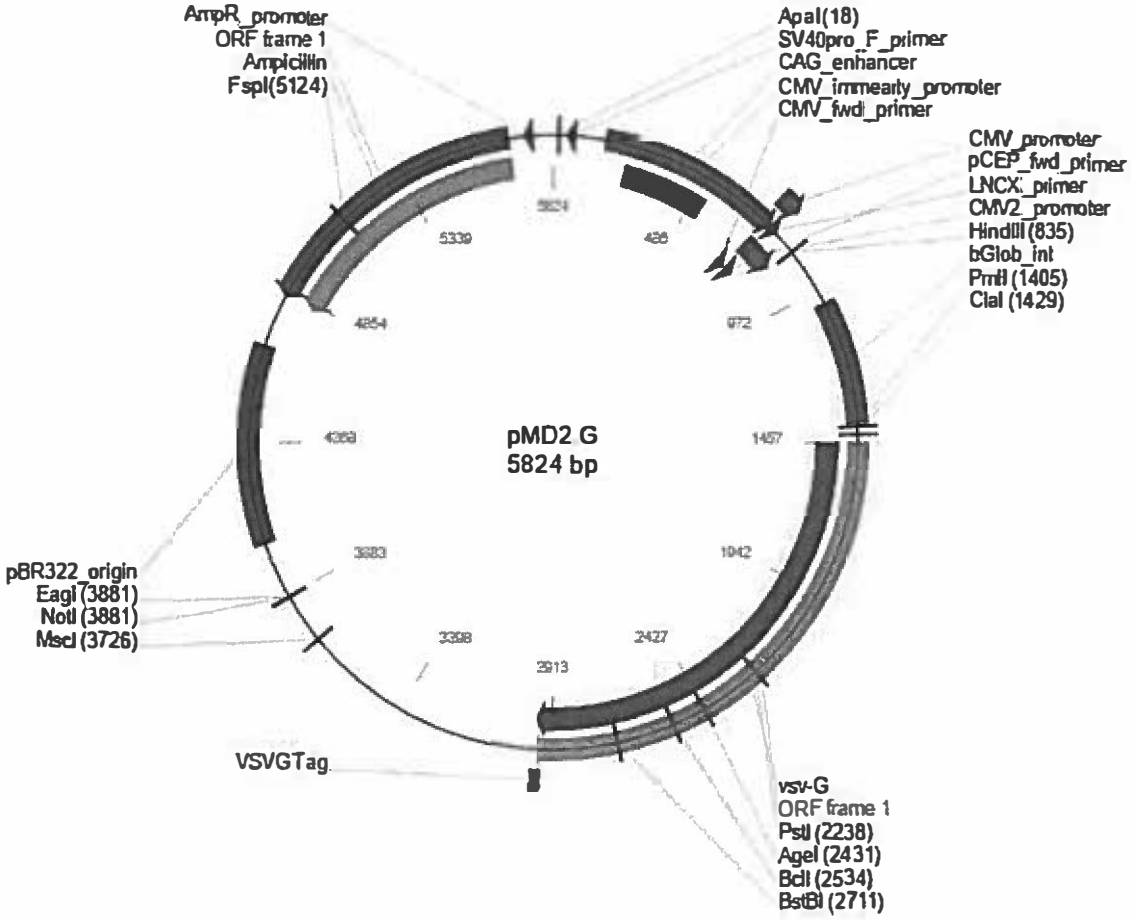

Figure 16. Plasmid map of pMD2.G.