

# **ONCOLYTIC VIRUSES AS A POTENTIAL APPROACH TO ELIMINATE THE HIV RESERVOIR**

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*This thesis is dedicated to all the individuals in the world living with HIV,  
and to their supporters.*

## ABSTRACT

Similar to cancer cells, HIV-infected cells differ from HIV-uninfected cells in that they have altered interferon signaling pathways, the apparent reason for the selectivity of certain oncolytic viruses (OVs). Therefore, it was hypothesized that use of an OV, such as recombinant Maraba virus (MG1), may be a potential approach to eliminate latently-infected cells constituting the HIV reservoir while sparing HIV-uninfected cells. This was studied in U1, ACH-2, OM-10 and J1.1 cells and their respective HIV-uninfected parent cell lines in addition to CD4<sup>+</sup>CD25<sup>-</sup>HLADR<sup>-</sup> cells from HIV-infected individuals on effective antiretroviral therapy. Although MG1 infected and killed latently HIV-infected U1 cells to a greater degree than the HIV-uninfected parent U937 cells, this was not observed in the other HIV-infected cell lines and their respective parent cell lines. Furthermore, results from primary cells suggest that MG1 alone does not appear to eliminate cells which comprise the major HIV reservoir. Challenges of studying the HIV reservoir and priorities for future studies examining the use of OVs as a potential strategy to eliminate the HIV reservoir are discussed.

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## LIST OF ABBREVIATIONS

Ag	Antigen
AIDS	Acquired Immune Deficiency Syndrome
ANOVA	Analysis of variance
BLT	Bone marrow, liver and thymus
CMC	Carboxymethyl cellulose
CDC	Centre for Disease Control
CNS	Central nervous system
Cgp	cytosine-phosphate-guanine
Cdk	Cyclin-dependent kinase
DMEM	Dubecco's Modified Eagle Medium
EC	Elite controller
EGFR	Epidermal growth factor receptor
eIF-2 $\alpha$	Eukaryotic translation initiation factor 2 $\alpha$
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
Gp	Glycoprotein
G	Glycoprotein
GALT	Gut associated lymphoid tissue
GFP	Green fluorescent protein
HAART	Highly active antiretroviral therapy

HDAC	Histone deacetylase
HLA	Human leukocyte antigen
HIV	Human Immunodeficiency Virus
HNSCC	Head and neck squamous cell carcinoma
HCl	Hydrochloric acid
HPV	Human Papilloma Virus
HRP	Horse radish peroxidase
IFN	Interferon
IFNR	Interferon receptor
IFNAR	Interferon- $\alpha/\beta$ receppr
IRF	Interferon regulatory factor
ISG	Interferon stimulated gene
ISRE	Interferon-sensitive response element
IL	Interleukin
JAK	Janus kinase
L	Large protein of the RNA-dependent RNA
Lck	Lymphocyte-specific protein tyrosine kinase polymerase
LTR	Long terminal repeat
M	Matrix
MACS	Magnetic-activated cell sorting
MG1	Recombinant Maraba virus
MOI	Multiplicity of infection
mRNA	messenger RNA
MTT	3-(4,5-dimethylthiazolyl-2)-2,5-

	diphenyltetrazolium bromide
NK	Natural killer
NEJM	New England Journal of Medicine
NFAT	Nuclear factor of activated T cells
NF- $\kappa$ B	Nuclear factor kappa B
N	Nucleocapsid
NNRTI	Non-nucleoside reverse transcriptase inhibitor
OKT3	Anti-CD3 monoclonal antibody
OV	Oncolytic virus
P	Phosphoprotein
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
Pfu	Plaque forming units
PKR	Protein kinase
PMA	Phytohemagglutinin
PSA	Prostate specific antigen
RIG	Retinoic acid inducible gene
SAHA	Suberoxylanilide hydroxamic acid
SIV	Simian Immunodeficiency Virus
STAT	Signal transducers and activators of transcription
TAR	Tat-Tat responsive element axis
Tat	<i>Trans</i> -activator of transcription

TMB	Tetramethyl benzine
TNF	Tumor necrosis factor
Tyk	Tyrosine kinase
UNAIDS	United Nations Program on HIV/AIDS
VL	Viral load
VOR	Vorinostat
VPA	Valproic acid
VSV	Vesicular Stomatitis Virus

## **1. INTRODUCTION**

### **1.1 HIV: From its initial discovery to the state of HIV infection today**

In 1981, the Lancet and the New England Journal of Medicine (NEJM) published a series of articles discussing outbreaks of *Pneumocystis carinii* pneumonia and Kaposi's sarcoma in New York City and San Francisco (Durack, 1981; Gottlieb et al., 1981; Masur et al., 1981). These cases drew considerable attention as these conditions typically affect individuals with profound immune compromise and not the young, otherwise healthy men in whom these illnesses were in fact being observed. Interest and apprehension progressively grew as the death rate was "fearfully high" (Durack, 1981). In fact, by December 1981, two-thirds of patients had already died. As the list of pathogens causing disease in these individuals expanded, it became evident that a cellular abnormality in immune function was at the root of this immune deficiency (Durack, 1981). Investigations continued and, in 1983, Human Immunodeficiency Virus (HIV) was discovered as the causative agent underlying this immune deficiency which culminated in what was termed "Acquired Immune Deficiency Syndrome" (AIDS) (Barré-Sinoussi et al., 1983).

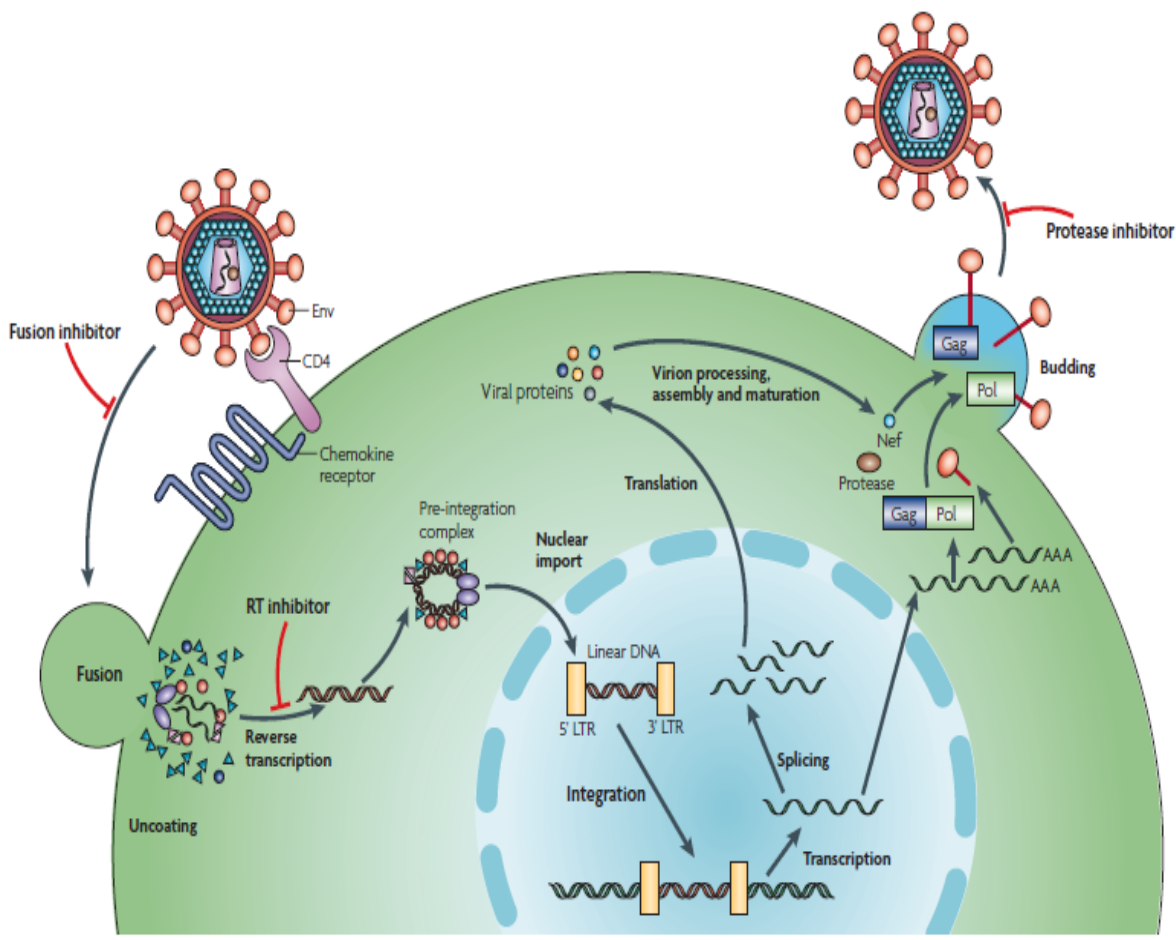
Since the initial recognition of HIV, the landscape of HIV infection has changed tremendously. HIV is now found worldwide and currently infects over 33 million individuals (Centre for Disease Control (CDC), 2012) and greater than 35 million people have died since the beginning of the epidemic (United Nations Program on HIV/AIDS (UNAIDS), 2010). A pivotal turning point in the management of HIV infection was the discovery of highly active antiretroviral therapy (HAART) in 1995 which

suppresses HIV replication to such low levels that individuals today may experience near-normal lifespans (van Sighem, 2010). However, even with the widespread use of HAART, it is still apparent that HIV-infected individuals on HAART do not have immunological profiles which match those of their non-HIV-infected counterparts (Mavigner et al., 2012; Yukl et al., 2010; Wallet et al., 2010). HAART also requires a very high level of adherence in order to yield maximal immune response (de Pee et al. 2012) and may be inconvenient for some patients. It is also very costly, especially in regions of the world such as Sub-Saharan Africa where the prevalence of HIV in some countries may be greater than 35% (UNAIDS, 2010; UNAIDS 2009). Furthermore, efforts to develop a preventative vaccine to date have not yielded encouraging results (Rerks-Ngarm et al., 2009). Therefore, a cure for HIV is greatly needed. Although several strategies have been attempted to date, all of these strategies have encountered obstacles and novel approaches for the eradication of HIV are needed (Lewin et al., 2011).

## **1.2 The HIV lifecycle and pathogenesis**

Mucous membranes, such as the anogenital mucosa, are a frequent portal of entry for HIV but the virus may also be transmitted through blood and transplacentally. HIV infects cells expressing the CD4 molecule in addition to a co-receptor, such as CCR5 on macrophages or CXCR4 on T cells (Khan et al. 1998). After HIV attaches to the CD4 receptor, envelope glycoprotein (gp) 120 is dislodged, exposing domains on the envelope gp41 required for cellular fusion (Moore JP et al. 1990). Following the uncoating of HIV RNA, it complexes with reverse transcriptase, which allows the production of viral DNA from RNA.

Complimentary DNA is transported into the nucleus where integration into host DNA occurs. Viral mRNA and viral genomic RNA are produced from integrated proviral DNA and viral proteins are made from full-length and spliced mRNA. Viral genomic RNA is then incorporated into the capsid and Gag and Gag-Pol polyproteins are processed at the cell surface or within budding virions. Finally, the viral capsid buds through the cell membrane and there is incorporation of viral envelope glycoproteins and other proteins on the cell surface (Han et al., 2007) (FIGURE 1).



**Figure 1: A schematic representation of the HIV lifecycle.** The HIV envelope protein (Env) binds CD4 and, following a conformational change, either the CCR5 (R5 strains) or CXCR4 (X4 strains) chemokine receptor. Entry into host cells occurs by fusion of the virus and host cell membranes. Uncoating of the virus capsid releases the pre-integration complex and reverse transcription begins, generating both linear and circular double-stranded viral complementary DNA. The linear form integrates into the genome of the host cell. Transcription generates both spliced and unspliced viral genomic transcripts, which are transported from the nucleus to the cytoplasm. Viral proteins are then translated and transported to the plasma membrane, where progeny virions are released from the infected cell by budding. Highly active antiretroviral therapy comprises a cocktail of fusion inhibitors, reverse transcriptase (RT) inhibitors and protease inhibitors, and the points in the lifecycle in which each of these inhibitor classes function are shown. LTR, long terminal repeat. Reprinted with permission from Macmillan Publishers Ltd: Nat. Rev. Microbiol. Experimental approaches to the study of HIV-1 latency. Han Y et al. 5, 95-106 (Figure 2). Copyright 2007.

Following HIV entry into the bloodstream, there is widespread dissemination of the virus to the brain, spleen, lymph nodes and other organs (Khan et al., 1998). The acute phase of HIV infection is usually accompanied by a dramatic depletion of CD4+ T cells in the peripheral blood and the massive depletion of memory CCR5+ memory CD4+ T cells in gut associated lymphoid tissue (GALT). This is a consequence of direct killing associated with the abundance of susceptible target cells in GALT (Mattapallil et al., 2005) and bystander killing by apoptosis (Li et al., 2005). Following this there is dissemination of virus to peripheral lymphoid tissue viral reservoirs. Due to the lack of pre-existing immunity, viral replication is initially rapid. Approximately 2-4 weeks following initial exposure, individuals often experience fever, lymphadenopathy, sore throat, rash, myalgias, arthralgias, headache, painful mucocutaneous ulcers, nausea, vomiting and diarrhea (Taiwo et al., 2002). Plasma RNA levels then fall by 2-3 logs and symptoms of the acute retroviral syndrome attenuate as HIV-specific immunity, and especially virus-specific CD8+ cytotoxic T lymphocyte responses, ensue. If untreated, the plasma viral load (VL) will stabilize at an individual's given "set-point" within 6 months of infection (Quinn, 1997). This set-point is an important determinant of the rate of disease progression (Mellors et al., 1996).

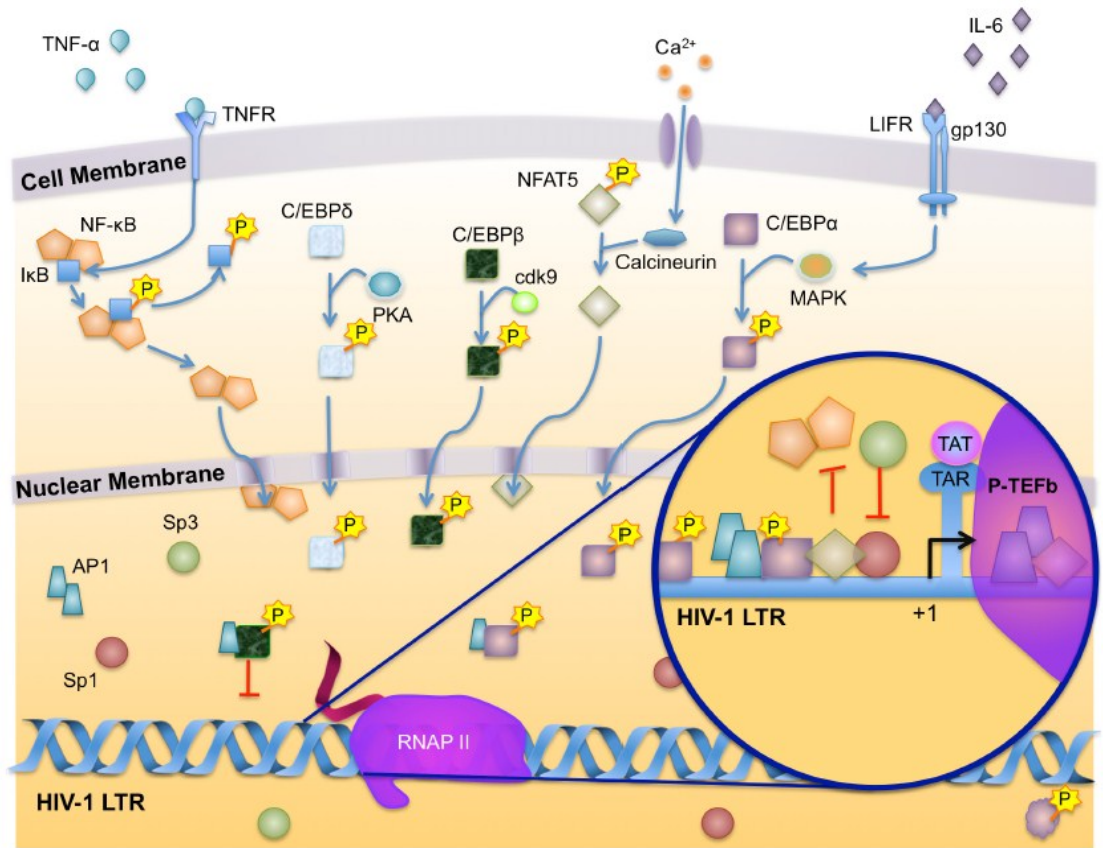
### **1.3 HIV reservoirs and the establishment of latency**

An HIV reservoir refers to "a cell type or body compartment which harbours infectious virus which has more stable kinetics than virus found in peripheral circulation" (Blankson et al, 2002). Within reservoirs, HIV is typically integrated into the host genome in the form

of proviral DNA and is maintained in this state of latency by several different mechanisms (Colin et al., 2009). Although infection of activated CD4<sup>+</sup> T cells with HIV often leads to cell death (Ho et al., 1995; Wei et al., 1995), some activated T cells will transition to a resting memory state prior to being killed by HIV or cleared by the host immune system.

Various mechanisms are involved in maintaining HIV in a latent state. The transition of cells to this resting memory state is accompanied by downregulation of HIV expression (Hermankova et al., 2003; Lassen et al., 2004) through loss of transcription factors, such as nuclear factor-kappa B (NF- $\kappa$ B) and activating protein-1. Alternatively, some transcription factors, such as NF- $\kappa$ B and nuclear factor of activated T cells (NFAT) cannot access the nucleus in resting T cells (Kinoshita et al., 1997; Nabel et al., 1987). Furthermore, through transcriptional interference, transcriptional machinery at the HIV promoter leads to disrupted HIV expression (Duverger et al., 2009; Lenasi et al., 2008). Methylation of cytosine-phosphate-guanine (Cpg) can silence retroviral long-terminal repeat (LTR) promoters and hypoacetylation of histone tails is associated with reduced HIV gene expression (De Ruitjer et al., 2003). Cellular histone proteins may be bound to integrated provirus and hinder access of transcription factors to the LTR (Ylisastigui et al., 2004). Structural changes in chromatin result in reduced mRNA production (Brooks et al, 2003). As viral proteins are not expressed, the immune system does not recognize these cells as being HIV-infected and does not attempt to eliminate these cells. It should be noted that there is little evidence to support the notion that HIV has evolved specific mechanisms that enable it to establish latent infection. Rather, latency appears to be an “incidental by-product” of HIV’s propensity to infected CD4<sup>+</sup> T cells which transition to long-lived memory cells (Marsden et al., 2011). However, some of this HIV is replication-competent

and upon stimulation with cytokines or antigens this state of latency can be reversed by various interactions between HIV regulatory proteins and cellular transcription factors (Colin et al., 2009; Blankson et al., 2002) (FIGURE 2).



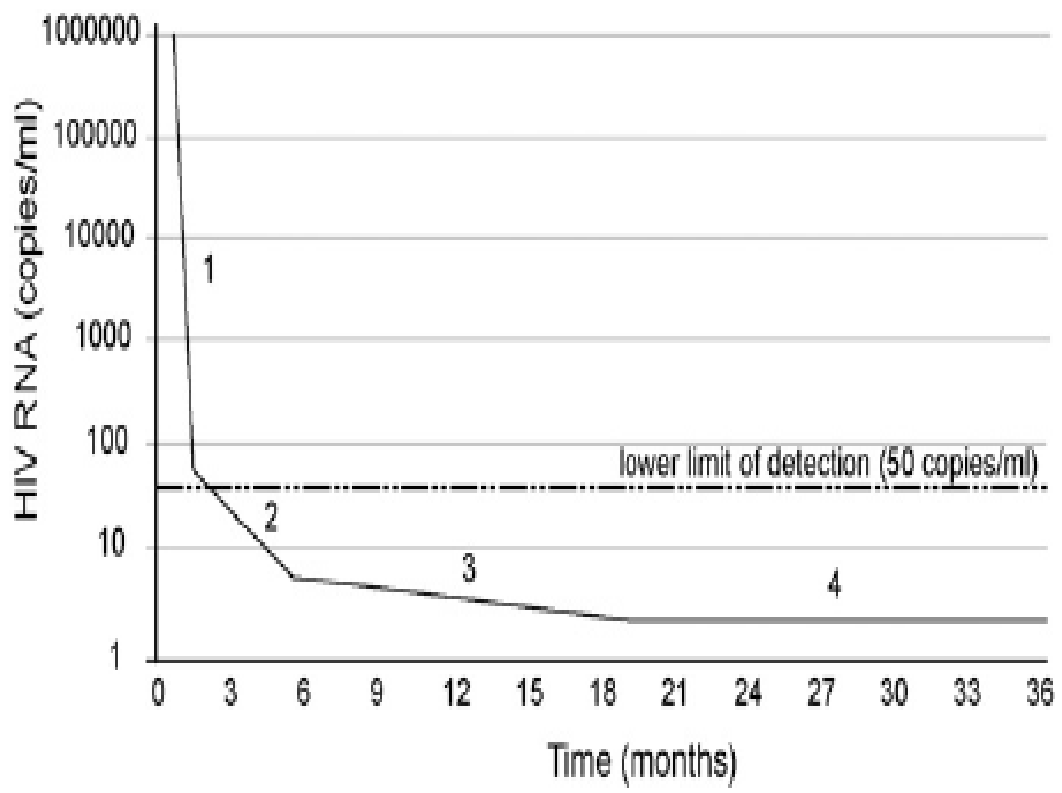
**Figure 2: Regulation of HIV-1 transcription in circulating monocytes.** Transcription of HIV-1 in circulating monocytes is dependent on the ratio of activator to repressor isoforms of transcription factors, the phosphorylation state of transcription factors, and the inducible translocation of NF- $\kappa$ B and NFAT factors from the cytoplasm. NF- $\kappa$ B can be induced to translocate to the nucleus by TNF $\alpha$ -mediated phosphorylation of I $\kappa$ B. NFAT is dephosphorylated in the cytoplasm by calcineurin, which acts in response to calcium levels within the cell. Once it is dephosphorylated, it translocates to the nucleus where it activates transcription by constitutively binding the NF- $\kappa$ B site in the enhancer. Phosphorylation plays a critical role in regulating the activity of C/EBP factors in monocytes. Phosphorylation of C/EBP $\alpha$  by ras-dependent mitogen-activated protein (MAP) kinase, signaled by IL-6 or by cAMP-dependent protein kinase A, results in its nuclear translocation and subsequent transactivation of the LTR. Cyclin-dependent kinase (cdk) 9 specifically phosphorylates C/EBP $\beta$ , which then translocates into the nucleus, binds to the LTR, and leads to an increase in gene expression. Once in the nucleus, C/EBP factors then regulate the activity of AP-1 factors. Relatively high levels of C/EBP $\alpha$  dimerize with the AP-1 factors to form potent activators of transcription. Lower levels of C/EBP $\beta$  balance this activation by binding AP-1 leading to a loss in DNA binding affinity. Sp1 and Sp3 are constitutively expressed in the nucleus. In the presence of Sp1, which is a strong activator, Sp3 competes for binding to the LTR and inhibits activation by Sp1. Reprinted from Regulation of HIV-1 transcription in cells of the monocyte/macrophage lineage. Kilariski E et al. (2009). *Retrovirology*. 6, 118 (Figure 5) under the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/2.0>).

HIV reservoirs can be categorized into a) lymphoid tissues and b) cellular reservoirs, such as CD4+ T cells, macrophages and dendritic cells. Within lymphoid tissues, there are numerous target cells in close proximity and poor antiretroviral penetration. Cellular reservoirs consist predominantly of CD4+ T cells (Moir et al. 2012). Of the CD4+ subsets, latent HIV provirus has been detected mostly within central memory and transitional memory cell subsets (Chomont et al., 2009; Chun et al., 1997). GALT is the predominant site of early HIV replication (Mehandru et al. 2007) and is an important HIV reservoir even despite many years of HAART (Chun et al., 2008; Mehandru et al., 2006). The central nervous system (CNS) and the genital tract are other HIV reservoirs into which antiretrovirals may not penetrate well (Blankson et al., 2002). While on HAART, reasons for HIV persistence include HIV transcriptional latency, reduced entry and effectiveness of antiretrovirals at certain anatomical sites and within certain types of cells, in addition to cell-to-cell transfer of HIV (Sigal et al., 2012).

The implication of viral reservoirs is that there will be rapid rebound viremia upon HAART cessation (Chun et al., 2000; Zhang et al., 2000). Furthermore, due to the continuous entry of cells into the reservoir, there is an archive of all genetic variants of HIV ever harboured by an individual which can later complicate patient management. Perhaps the most important implication, however, is that HIV reservoirs are the major impediment to our ability to cure HIV infection (Chun et al., 2000; Zhang et al., 2000).

#### **1.4 HIV decay while on HAART**

HIV decline occurs in several phases in response to HAART (Ho et al., 1995; Perelson et al., 1997) (FIGURE 3). The first decline in VL occurs briskly, with a half-life of about 24 hours, and represents the elimination of free virions and HIV-infected CD4+ T cells (Blankson et al., 2002). Subsequently, a slower decline ensues and has a half-life of approximately of 2 weeks (Finzi et al., 1999; Rong et al., 2009). It has been postulated that the second phase of decline results from the elimination of macrophages or virus trapped on the surface of follicular dendritic cells (Blankson et al., 2002).



**Figure 3:** After the initiation of HAART there are four phases of viral decay which have been identified. *Phase 1* corresponds to a virion producing cell population with a half-life of 1-2 days, presumably infected active CD4+ T cells. *Phase 2* corresponds to one or several cell-populations with a half life/lives of 1-4 weeks, such as infected macrophages, dendritic cells or partially activated CD4+ T cells. *Phase 3* corresponds to a population of cells with a half life of 39 weeks. *Phase 4:* a constant phase with no appreciable decline, caused at least partially by the activation of resting memory CD4+ T-cells that start to produce virions. Infected hematopoietic progenitor cells of the monocyte/macrophage cell line could also contribute to this phase. Reprinted from HIV reservoirs, latency, and reactivation: Prospects for eradication. Dahl V et al. (2010). Antivir. Res. 85, 286-294 (Figure 1) with permission from Elsevier Press.

Using this second rate of decay, it was initially predicted that depletion of the HIV reservoir would occur approximately 2-3 years after beginning HAART. However, this was found not to be the case as individuals on HAART for this period of time were still found to harbour latent provirus (Siliciano, 2010). Further investigations revealed a third population of cells undergoing an even slower rate of decay, with a half-life of approximately 39 weeks (Palmer et al., 2008; Siliciano, 2010). A fourth phase has also been described whereby 1-5 copies/ml of HIV RNA have been measured in blood (Maldarelli et al., 2007). There does not appear to be any further significant decay of HIV during this final phase (Maldarelli et al., 2007; Palmer et al., 2008).

The source of the latter two phases of decay have not been fully elucidated. However, the final phase is thought to represent the presence of integrated proviral DNA within resting memory CD4<sup>+</sup> T cells circulating through the lymphoid tissues. Upon antigenic stimulation, these cells enlarge, proliferate and execute their effector immune response. Following this, the majority of these cells die but some will survive and return to a quiescent state (Siliciano, 2010). On effective HAART, the half-life of these cells is approximately 44 months, indicating that greater than 60 years would be required to deplete this reservoir (Siliciano et al., 2003). Furthermore, there is approximately 1 infected cell per million resting CD4<sup>+</sup> T cells and the overall size of this reservoir is estimated to be approximately 1 million cells (Chun et al., 1997).

The difference between the rate of cells leaving and entering the reservoir determines the overall reservoir decay rate. As HAART does not fully suppress all HIV replication (Dornadula et al., 1999), low-level viremia may replenish the reservoir. Cells may be eliminated when they encounter antigens, are reactivated and express viral proteins. This

facilitates recognition of these cells and clearance by the immune system. It appears as though the number of new cells entering the latent reservoir is small relative the overall size of the reservoir (Sedaghat et al., 2007). Sedaghat et al. (2007) have predicted that 0-70 cells enter the reservoir daily, which is a small quantity considering that the overall size of the reservoir is approximately 1 million cells. Thus, the slow rate of cells leaving the reservoir in individuals on HAART appears to be a greater contributor to the maintenance of the reservoir than the small number of cells entering the reservoir on a daily basis (Sedaghat et al., 2007).

### **1.5 Previous attempts to eliminate the HIV reservoir**

Initial attempts to eliminate the latent HIV reservoir have included intensification of antiretroviral therapy by incorporating additional antiretrovirals, an integrase inhibitor or a fusion inhibitor to an individual's existing HAART regimen. Despite numerous attempts, this strategy has been unsuccessful thus far at reducing the HIV reservoir as measured in blood (Dinosa et al., 2009; Ghandi et al., 2010; Gutierrez et al. 2011; McMahon et al., 2010; Yukl et al., 2010).

Immune activation therapy has also been attempted and is based on the rationale that inducing HIV replication will result in cell death, either directly or through mechanisms mediated by the immune system. This method involves administration of a T cell activator in addition to antiretroviral therapy, the latter of which is given to prevent infection of cells not already infected with HIV. However, the challenge is to devise a method to re-activate specifically HIV expression from resting cells without inducing generalized immune

activation and a “cytokine storm.” Generalized immune activation is toxic and also activates target cells, making them vulnerable to infection by newly-produced HIV (Matrayek et al. 2012). Interleukin (IL)-2, anti-CD3 antibodies, IL-7 have been successful at activating latent HIV (Dahl et al. 2010; Johnston et al., 2012). Both IL-2 alone (Chun et al., 1999) and in combination with anti-CD3 monoclonal antibody (OKT3) (Prins et al., 1999) have been studied in HIV-infected individuals. Serious toxicity was observed with high doses of OKT3 (Prins et al., 1999). Furthermore, while IL-2 significantly reduced numbers of latently-infected cells, patients experienced viral rebound upon cessation of HAART (Chun et al., 1999; Davey et al., 1999). IL-7 has garnered interest due to its ability to activate latent HIV without excessive stimulation of host cells (Scripture-Adams et al., 2002) and continues to be studied with interest. While many of these therapies have resulted in varying impacts on HIV DNA or RNA levels, no therapy to date has resulted in HIV eradication or led to a durable decline in HIV burden.

Drugs of particular interest for their potential to induce HIV replication are the histone deacetylase (HDAC) inhibitors. HDACs are enzymes which repress HIV transcription through modulation of chromatin structure. Thus, removing this inhibition of HIV transcription can potentially reverse latency. The anticonvulsant valproic acid (VPA) has been the most extensively studied HDAC inhibitor and has been used in conjunction with HAART in clinical studies in an attempt to reduce the HIV reservoir (Dahl et al., 2010). Many small studies examining the effect of VPA on the HIV reservoir have been conducted but the results were highly variable (Routy et al., 2012; Routy et al., 2005). The first randomized, controlled clinical trial involved 56 HIV-infected individuals with suppressed

blood plasma VL. Treatment allocations consisted either of VPA in addition to HAART for 16 weeks followed by HAART alone for 32 weeks, or HAART alone for 16 weeks then VPA in combination with HAART for 32 weeks. No significant reductions in the frequency of CD4+ T cells containing replication-competent HIV after 16 or 32 weeks of VPA therapy were observed (Routy et al., 2012).

Another HDAC inhibitor which has been examined for its ability to eradicate the HIV reservoir is the cancer therapy suberoylanilide hydroxamic acid (Vorinostat, VOR or SAHA). In a recent clinical trial, a single dose of VOR given to 8 HIV-infected individuals led to increases in biomarkers of cellular acetylation and also resulted in increased expression of HIV RNA expression in resting CD4+ T cells by a mean increase of almost 5-fold (Archin et al., 2012). This landmark study was the first proof-of-concept for the use of HDAC inhibitors as agents to eradicate the HIV reservoir (Archin et al., 2012). Another cancer therapy, prostratin, is also capable of reactivating latent HIV without significantly inducing stimulation and proliferation of resting T cells (Korin et al., 2002). Furthermore, as prostratin induces downregulation of CD4, CXCR4 and CCR5, it has been suggested that it may help prevent HIV spread from newly activated latent provirus to uninfected cells (Kulkosky et al., 2001).

Another drug which has demonstrated promise for reduction of the HIV reservoir is the gold drug auranofin, which is typically used for the treatment of rheumatoid arthritis. In a pilot study involving 6 Simian Immunodeficiency Virus (SIV)mac252-infected macaques with suppressed viral loads on HAART, macaques received intensification therapy with ritonavir-boosted darunavir and auranofin (Lewin et al., 2011). Controls included

macaques with suppressed VLs on HAART who also received intensification therapy but without auranofoin. Upon HAART intensification, a sustained reduction in HIV DNA was observed in those macaques who received auranofoin but not in the control groups. Furthermore, upon discontinuation of therapy, only macaques who had received auranofoin displayed a delayed and reduced rebound in their VL (Lewin et al., 2011).

Although pharmacological approaches examined to date have revealed some promise, a major limitation is that they target only one mechanism of HIV latency. This is a major shortcoming as HIV is kept latent by a multitude of different mechanisms *in vivo* (Blankson et al, 2002). Thus, current pharmacological approaches are unlikely to reactivate and purge all HIV-infected cells from an individual. Therefore, another strategy which has been explored is that of gene therapy. This involves the *ex vivo* genetic modification of host cells followed by reinfusion of the modified cells back into the individual. Attempts have included the excision of CCR5 and CXCR4 from CD4+ T cells through the use of zinc finger nucleases (Johnston et al., 2010; Lalezari et al., 2012; Scherer et al., 2012; Tebas et al., 2012). However, outcomes from studies in the setting of other diseases have raised significant concerns regarding toxicity and even mortality, indicating that gene therapy is not a feasible approach (Edelstein et al., 2007). Other strategies, such as the development of targeted cytotoxic proteins such as immunoconjugates, radioimmunotherapies and immunotoxins are also being explored (Burger et al., 2011). These strategies typically involve the construction of anti-HIV envelope immunotoxins, which consist of target domains such as derivatives of monoclonal antibodies, attached to toxic components (Thrush et al., 1996). In the pre-HAART era, a phase I clinical trial examined the effect of a toxin targeted against HIV. However, it did not demonstrate any antiviral effects *in vivo*

(Ramachandran et al., 1994; Davey et al., 1994). Although diverse methods have been tried to eliminate or reduce HIV reservoirs, it should be emphasized that many of these methods are based on very preliminary data and no strategy has yet been shown to eradicate HIV or result in durable reductions in the reservoir which persist upon on HAART cessation.

### **1. 6 Oncolytic viruses (OVs) as cancer therapeutics**

The idea of using viruses as potential therapeutics originated in the 20<sup>th</sup> century when it was observed, in a few individuals, that cancers would sometimes go into remission following viral infection. As reviewed by Liu et al. (2012), this had been observed following infection with measles virus and after lymphoma and leukemia patients received vaccinia and measles vaccines (Liu et al., 2012). It was thought that the immune compromise of the host may have facilitated virus spread. These observations inspired attempts to use attenuated, wild-type virus strains as therapeutics and it was later observed, for example, that intramuscularly-administered West Nile Virus replicated, spread to tumors and induced remissions in lymphoma patients. Similarly, vaccinia virus was also shown to replicate in and disseminate throughout the bodies and localize to distant tumor sites, resulting in regression of tumors (Liu et al., 2012).

Selectivity of OVs for cancer cells occurs as cancer cells have accumulated genetic and metabolic transformations, such as aberrant interferon (IFN) responses, not observed in normal, healthy cells (Mohr 2005; O'Shea 2005). After infecting cells, these OVs replicate

within cells until lysis occurs, spilling virions which spread throughout the body. The cycle of infection, replication and destruction of more cancer cells continues. Upon cell lysis, release of debris also generates a localized inflammatory response which promotes the recruitment of immune cells (O'Shea 2005; Chiocca 2002). Differences in virus lifecycles may be exploited to design therapies for different medical conditions. For example, poxvirus (vaccinia virus) disseminates systemically and creates pox lesions on skin. Measles and vaccinia travel systemically within lymphocytes and/or monocytes (Liu et al., 2012). Furthermore, OV's are frequently agricultural pathogens to which the vast majority of individuals will not have been previously exposed, hence most individuals will not have pre-existing immunity to these viruses.

The first OV to gain regulatory approval in China for head and neck cancer was H101, an adenovirus-based OV which preferentially targets cells with dysregulated tumor suppressor protein p53 (Garber K, 2006). Initial response rates for H101 in combination with chemotherapy were close to doubled compared to the response rates achieved with chemotherapy alone. However, not all OV's developed since then have been shown to be efficacious (Liu et al., 2012). Currently, there are 6 oncolytic viruses being used in clinical trials. In addition to adenovirus, these include reovirus, measles, herpes simplex, Newcastle disease virus and vaccinia. These viruses have been engineered to be less susceptible to clearance from a host immune response and to target more specifically cancer cells (Donnelly et al., 2012).

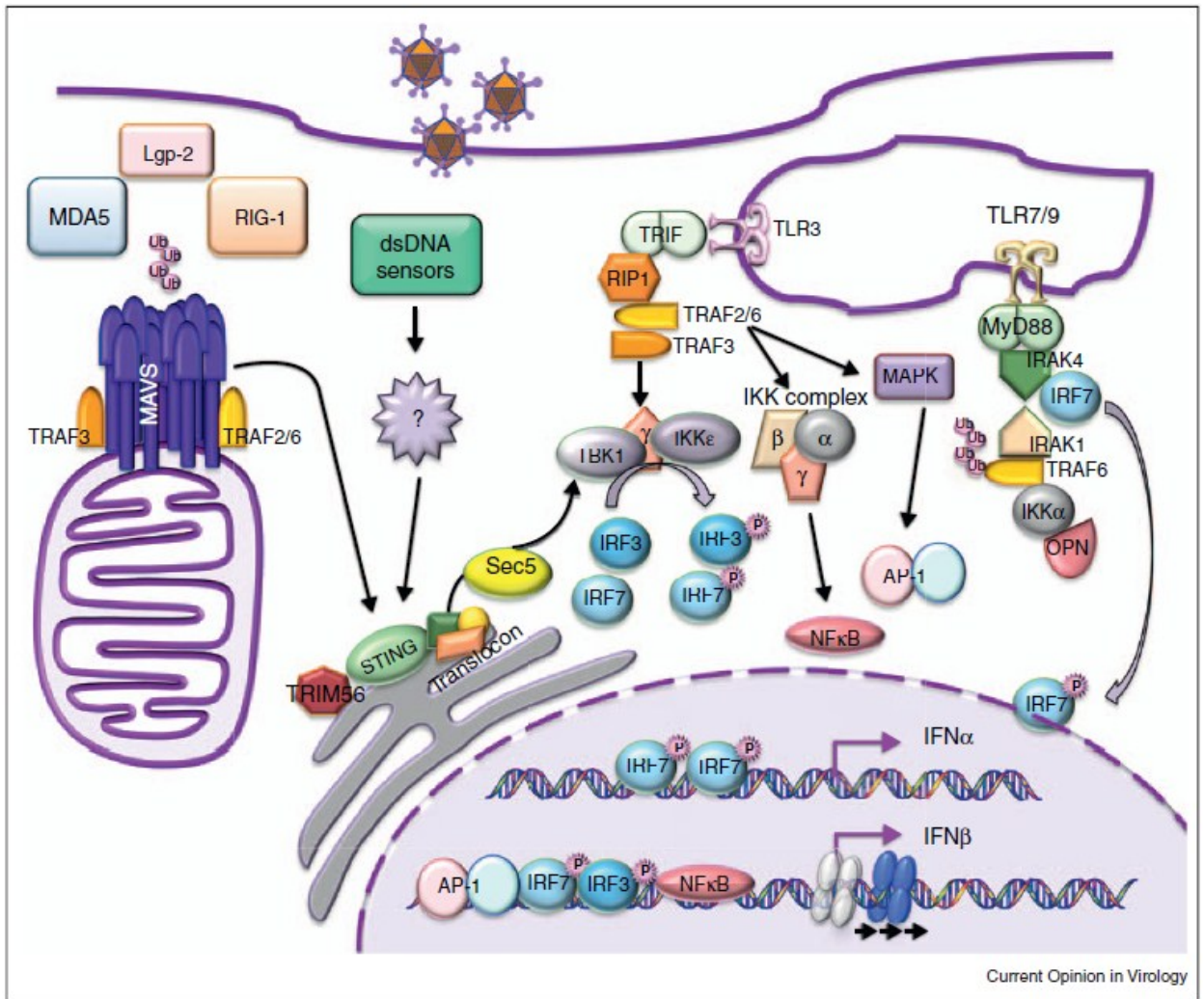
OV's have been given to over 500 individuals and there is accumulating evidence that OV's are safe and generally well-tolerated (Brown et al., 2011; Donnelly et al., 2012; Liu et al., 2007; McCormick et al., 2005). However, OV's still require much study. As reviewed by

Liu et al. (2012), more work is needed to define optimal dosing regimens for each OV, infusion rates, methods of administration (ie, bolus injection or infusion), dosing frequencies and durations of treatment. Likewise, additional work is needed to identify factors predictive of efficacy, such as histologic tumor type, cancer cell viral receptor levels etc (Liu et al., 2012). It has also likely that combination therapy will result in optimal therapeutic potential of OVs and clinical trials have demonstrated synergistic efficacy with virotherapy and chemotherapy for certain tumor types (Adhi et al., 2005).

### **1.7 Rationale for OVs as a potential approach to eliminate the HIV reservoir: Shared defects in interferon signaling between cancer and HIV-infected cells**

IFNs comprise a family of cytokines which are key players in the antiviral innate immune response. They are typically divided into type I and type II IFNs, the former of which include IFN- $\alpha$  and IFN- $\beta$  and the latter of which include IFN- $\gamma$ . IFN- $\alpha$  and IFN- $\beta$  are characteristically secreted by leukocytes and fibroblasts whereas IFN- $\gamma$  is typically secreted by natural killer cells and T cells (Samuel et al., 2001). IFN- $\alpha$  and IFN- $\beta$  induce genes involved with inhibition of translation, induction of apoptosis, release of cytokines and recruitment of immune cells (Levy et al., 2011; Samuel et al. 2001). Upon detection of viral nucleic acids by helicase receptors within the cytoplasm and Toll-like receptors on the endosomal transmembrane, cascades of reactions are initiated which result in the phosphorylation of transcription factors interferon regulatory factor (IRF)-3 and IRF-7. Along with activated NF- $\kappa$ B and c-Jun, these translocate to the cell nucleus where they bind to enhancer and promoter regions of IFN genes. This induces transcription and secretion of IFN- $\beta$  from cells. IFN- $\beta$  then binds to type I interferon receptors (IFN-R),

leading to phosphorylation of IFN-R associated kinases such as Janus Kinase (JAKs). This subsequently results in phosphorylation of signal transducers and activators of transcription (STATs) resulting in dimer assembly. IRF-9 then complexes with activated STATs, translocates to the nucleus, and binds to interferon stimulated response elements (ISREs) in the promoter regions of interferon-stimulated genes (ISGs). IRF-7 is a key ISG whose transcription is promoted by the STAT/IRF-9 complex. Following phosphorylation of IRF-7, it translocates to the nucleus to induce INF- $\alpha$  expression. Furthermore, translational initiation is halted through phosphorylation of eukaryotic translation initiation factor 2 $\alpha$  (eIF-2 $\alpha$ ), which is mediated by eIF-2 $\alpha$  protein kinase (PKR). PKR phosphorylation of eIF-2 $\alpha$  renders it unavailable for translation initiation (Samuel et al., 1993; Levy et al., 2011). A summary of the processes involved in IFN induction by viruses is provided in FIGURE 4 (Levy et al., 2011).



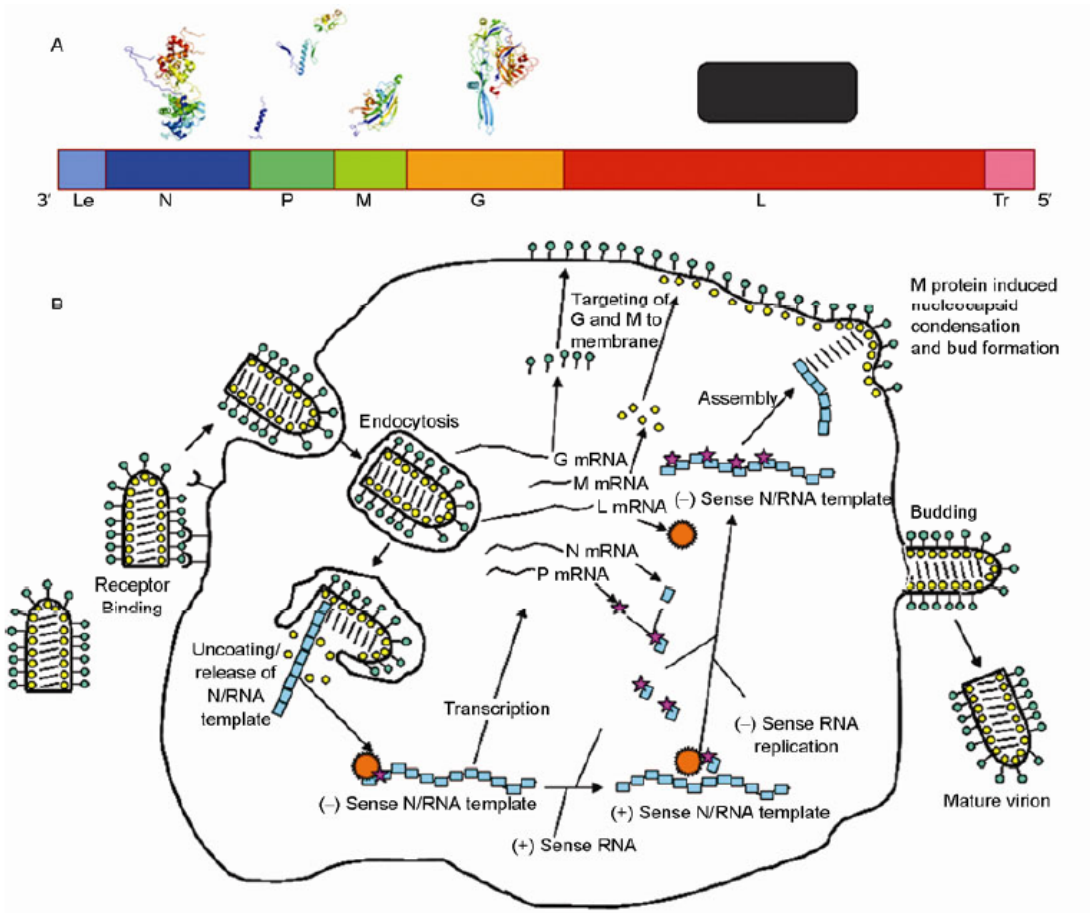
**Figure 4: Induction of IFN by viruses.** Virus infection triggers a complex signaling cascade involving ubiquitin modification and protein aggregation, leading to kinase activation and transcription factor phosphorylation. Signaling is initiated by the detection of the viral nucleic acid by both cytoplasmic helicase receptors and endosomal transmembrane Toll-like receptors, activating distinct signaling pathways that converge on transcription factor phosphorylation. Kinase activation is controlled by signaling protein aggregates that assemble as scaffolds on organellar surfaces, including mitochondria, endoplasmic reticulum, and possibly peroxisomes. Phosphorylated IRF3 and IRF7 and activated NF- $\kappa$ B translocate to the cell nucleus, where they bind to enhancer/promoter regions of IFN genes. IFN- $\beta$  (and probably some IFN- $\lambda$  subtypes) require assembly of a multiprotein enhanceosome that alters chromatin structure, sliding an occluding nucleosome that would otherwise prevent polymerase recruitment. The multiple type I and III IFN genes are largely regulated in analogous fashions, although differential utilization of multiple IRF isoforms, some of which are subject to feedforward and feedback regulation, produces distinct patterns of induction of the individual genes under different circumstances. Reprinted from Induction and function of type I and III interferon in response to viral infection. Levy DE et al. (2011). *Curr. Opin. Virol.* 1, 476-486 (Figure 1) with permission from Elsevier Press.

Aberrant IFN signaling appears to be the reason for selectivity of certain OV's for particular cancer cells (Stojdl et al. 2004; Stojdl et al. 2000). Similar to cancer cells, HIV-infected cells differ from healthy, HIV-uninfected cells (Doehle et al., 2009; Neil et al., 2009). Monocytes derived from individuals with active HIV replication have been shown to have selective defects in IFN- $\alpha$  production (Gendelman et al., 1990). Furthermore, the retinoic acid inducible gene (RIG)-I sensor, which is responsible for the detection of foreign RNA and driving the IFN response, is degraded by HIV protease (Remoli et al., 2011). Accessory protein Vpu is responsible for IFN response factor (IRF)-3 proteolytic degradation (Doehle et al., 2009). Protein kinase (PKR) activity is also targeted by HIV at multiple levels (Clerzius et al., 2011). The HIV Tat protein has been shown to downregulate PKR expression and prevent its induction by IFN-treatment. Tat can also bind directly to PKR and inhibit its activation (Cai et al., 2000). These are only some examples of the mechanisms by which HIV perturbs the innate IFN cellular response. *Therefore, defects in IFN-mediated innate antiviral responses provide a critical link between cancerous and HIV-infected cells and, thus, form the basis for the use of OV's as a potential therapy to eradicate HIV-infected cells.*

### **1.7.1 Recombinant Maraba Virus (MG1) as a promising oncolytic virus candidate**

Both Vesicular Stomatitis virus (VSV) and Maraba virus are from the *Rhabdoviridae* family and are enveloped, non-segmented, single-stranded, negative sense RNA viruses. MG1 is closely related to VSV and its recombinant form, VSV- $\Delta$ 51 (Brun et al, 2010).

These are single-strand RNA genomes, of approximately 11K nucleotides, encoding 5 viral proteins: N (nucleocapsid), P (phosphoprotein), M (matrix protein), G (glycoprotein), L (large protein of the RNA-dependent RNA polymerase) (Brun et al., 2010; Hastie et al, 2012) (FIGURE 5A). Although VSV can infect virtually all mammalian cells, a specific receptor which it uses to bind to has not been identified. It has been suggested that its host receptor may be either a common molecule among different cell types or that it may be able to use a number of different surface receptors (Luo, 2012). As reviewed by Luo (2012), attachment of the host receptor by VSV initiates endocytosis that leads to VSV uptake in clathrin-coated vesicles (Cureton et al., 2010; Luo, 2012). The acidic intracellular environment induces a conformational change in the G glycoprotein, enabling fusion of the viral and cellular membranes. This results in the release of the nucleocapsid, together with the viral RNA-dependent RNA polymerase. The nucleocapsid is a functional template for viral transcription (Hastie et al., 2012). After transcription of each mRNA, there is targeting of G and M proteins to the cell membrane, where the M protein induces the condensation of the nucleocapsid and budding of the virion (Luo, 2012) (FIGURE 5B). Maraba virus likely undergoes a similar lifecycle to VSV, although the details of the Maraba virus lifecycle have not been previously published.



**Figure 5: A) Genomic structure of vesicular stomatitis virus.** Starting from the 3' end of the viral RNA genome, five viral genes are encoded: nucleocapsid (N), phosphoprotein (P), matrix (M), glycoprotein (G), and the large protein of the viral RNA-dependent RNA polymerase (L). There is a leader sequence at the 3' end and a trailer sequence at the 5' end. The crystal structure of N, M, and G has been solved as shown above in the genome sketch. The crystal structure of P has been solved as fragments and is shown as a possible composition of the fragments. The crystal structure of L is unknown (black box). **B) A schematic illustration of the replication cycle vesicular stomatitis virus.** Reproduced with kind permission from Springer and Sci.China Life Sci. (2012) 55, 291-300. The nucleocapsid of vesicular stomatitis virus. Luo M. (Figure1).

Both VSV and Maraba virus have short life cycles of approximately a few hours within tumor cells and there is virtually no risk of genotoxicity as their lifecycles occur in the cytoplasm and they do not pass through DNA intermediates (Brun et al., 2010). MG1 was chosen for use in this project as it is approaching clinical trials for cancers and it is relatively easy to manipulate genetically. MG1 is a double mutant containing both G protein (Q242R) and M protein (L123W) mutations (Brun et al., 2010). It has been tested both in vitro and in murine models and its mutations in the G and M protein result in attenuated cell toxicity in healthy cells and hypervirulence in cancer cells. MG1 also had a 100-fold greater maximum tolerable dose than wild-type Maraba virus *in vivo* and resulted in durable cures when administered in syngeneic and xenograft models (Brun et al., 2010). Although the features which render cells susceptible to MG1 infection and lysis have not been fully elucidated, it is thought that IFN signaling defects, similar to those observed in VSV- $\Delta$ 51, are implicated to a significant degree (Brun et al., 2010).

The work presented here was performed to evaluate, in a proof-of-principle study, the use of MG1 as a potential therapeutic capable of eliminating HIV-infected cells in vitro. It was also performed in order to describe a system in which one could later study the effects of an MG1 engineered to have increased selectivity for HIV-infected cells while sparing healthy cells.

## **1.8 Hypothesis**

Oncolytic viruses will have a greater propensity to target and kill HIV-infected cells while sparing non-infected cells

## **1.9 Objectives**

### **1.9.1 To determine whether MG1 exerts greater infection and killing of HIV-infected, rather than HIV-uninfected cells, using cell lines**

1.9.1.1 To confirm that HIV-infected cells are able to induce HIV production upon stimulation with appropriate agents

1.9.1.2 To determine the effect of HIV on MG1-infectivity and MG-induced cell death in cell lines

1.9.1.3 To describe the effect of TNF- $\alpha$  on MG1 infectivity and MG1-induced cell death in HIV-infected cell lines

1.9.1.4 To describe the effect of HIV p24 antigen production in HIV-infected cell lines

**1.9.2 To determine whether there is selective replication of MG1, with resultant cell death and decline in HIV production, in cells derived from HIV-infected individuals on HAART**

1.9.2.1 Isolation of CD4+CD25-HLADR- cells from HIV-infected individuals with suppressed VLs on HAART

1.9.2.2 Determining infectivity of MG1 and its ability to induce cell death in cells from patients with HIV infection

1.9.2.3 Determining the effect of MG1 infection on HIV proviral DNA from cells from patients with HIV infection

1.9.2.4 Determining the effect of MG1 infection on p24 antigen and HIV RNA levels in cell-free supernatants from patients with HIV infection

**2. MATERIALS AND METHODS**

**2.1 MG1 viral stock**

**2.1.1 Plaque titer for quantitation of virus**

Green fluorescent protein (GFP)-encoded recombinant Maraba virus, MG1, was a kind gift from Dr David Stojdl and Dr John Bell. U-2 OS cells in Dubecco's Modified Eagle's Medium (DMEM) (GIBCO) were seeded in 6 well plates with  $8.4 \times 10^5$  cells per well and incubated overnight at 37°C in 5% CO<sub>2</sub>. On day 2, 100 µL serial viral dilutions in DMEM

were added to the wells and incubated for 2 hours. Media was then removed and replaced with 2 ml per well of carboxymethyl cellulose (CMC) mixture (3% CMC and 2X DMEM with 20% FBS). The plates were incubated for 3 more days. On day 5, overlays were aspirated from wells and the cell layers were washed once with 1 ml of PBS per well. One ml of 0.1% crystal violet solution was then placed in each well and the plates incubated at room temperature for 15 minutes. Crystal Violet solution was then removed, cells were washed with distilled water and plaques were counted.

## **2.2 Cells**

### **2.2.1 Cell lines**

Cells lines included U1, OM-10, ACH-2 and J1.1 cells, which are chronically-infected cell lines harbouring 1-2 copies of integrated proviral HIV DNA per cell. These four different cell lines were selected as HIV is maintained in a transcriptionally silent or “latent” state within these cells by different mechanisms (Butera et al., 1994; Butera ST et al., 1991; Emiliani et al., 1998; Folks et al., 1989; Folks et al., 1987). Given that HIV is maintained in a latent form within the body by multiple different mechanisms (Blankson et al., 2010), studying the effects of MG1 on cells with different mechanisms of latency was thought to better reflect *in vivo* conditions.

#### U1 and U937 cells

U1 cells are a monocytic cell line chronically infected with 2 copies of integrated HIV proviral DNA (Folks et al., 1987; Folks et al., 1988). They were derived from U937 cells, which had been obtained from a 37-year old male with histiocytic lymphoma (Sundstrom et al., 1976), which were infected *in vitro* with HIV-1 (Folks et al., 1987; Folks et al.,

1988). Each integrated provirus contains 2 distinct forms of *trans*-activator of transcription (Tat) due to mutations in their open reading frame. One form of Tat is mutated at the ATG initiation codon, whereby the first methionine amino acid has become a threonine, resulting in the codon ACG. The second form of Tat contains a histidine to leucine mutation at amino acid 13. These mutations result in HIV being kept in a transcriptionally silent state (Emiliani et al., 1998).

Basally, U1 cells produce small amounts multispliced HIV transcripts (Pomerantz et al., 1990) but expression can be induced through stimulation with tumor necrosis factor (TNF)- $\alpha$ , phorbol esters (Folks et al., 1987; Folks et al., 1988), cytokines such as IL-1 and IL-6 (Poli et al., 1990), cellular transcription factors and other viruses (Poli et al., 1989). It is thought that many of these mechanisms induce transcription through the translocation of NF- $\kappa$ B to the nucleus (Griffin et al., 1989).

U1 cells were obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: U1/HIV-1, from Dr Thomas Folks. U937 cells were obtained from ATCC.

#### ACH-2 and A301 cells

ACH-2 cells (Clouse et al., 1989; Folks et al., 1989) are a lymphocytic T cell line which was developed by infecting the A301 cell line with HIV-1 (Buttke et al., 1992; Folks et al., 1985). These latter cells were derived from a four-year old patient with acute lymphoblastic leukemia (Folks et al., 1985). ACH-2 cells possess a defect in the Tat-Tat responsive element (TAR) axis due to a single point mutation in the TAR region at nucleotide 37 whereby a cysteine is replaced by a threonine. This mutation is located near the loop of the

TAR hairpin (Emiliani et al, 1996). As this region is critical for Tat activity, this mutation renders the integrated provirus unresponsive to Tat (Emiliani et al., 1996).

ACH-2 cells express low levels of reverse transcriptase and p24 antigen in their basal state (Folks et al., 1989). HIV replication can be induced by stimulation with TNF- $\alpha$  and PMA to produce large quantities of HIV (Folks et al., 1989).

ACH-2 and A3.01 cells were obtained from the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: ACH-2, from Dr Thomas Folks.

#### OM-10 and HL-60 cells

OM-10 cells (Butera et al., 1994; Butera et al., 1993; Butera ST et al., 1991) were derived from HL-60 cells infected with HIV-1. HL-60 cells are a promyelocytic cell line which was obtained from a 36-year old woman with acute promyelocytic leukemia (Ghallager et al., 1979). Each OM-10 cell contains a single integrated HIV provirus and protein kinase activity is necessary for maintaining the state of HIV-1 activation. OM-10 cells differ from other cellular models of chronic HIV as they maintain expression of CD4 on their surface until HIV replication is induced. The state of viral activation relates, in part, to intracellular HIV-1 gp160-CD4 complexing. The intracellular association between CD4 and HIV gp160 prevents transport of the complex through the endoplasmic reticulum as a result of inefficient transport of gp160 itself. It has also been suggested that expression of HIV *nef* regulatory gene may mediate a loss of CD4 surface expression (Crise et al., 1990; Garcia et al., 1991; Butera et al., 1991). HIV replication can be induced by TNF- $\alpha$  and PMA (Butera et al., 1993).

OM-10 cells were obtained from the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: OM-10.1, from Dr Salvatore Butera. HL-60 cells were obtained from ATCC.

### J1.1 and Jurkat cells

J1.1 cells (Perez et al., 1991) were derived from Jurkat cells, a mature lymphocytic T cell line, infected with HIV-1. J1.1 cells have defective mobilization of  $\text{Ca}^{2+}$  and IL-1 secretion after cross-linking by anti-CD3 monoclonal antibody. J1.1 cells also have reduced surface density of CD3. It has been suggested that CD3-CD4 interactions are critical during anti-CD3 stimulation to maximize  $\text{Ca}^{2+}$  mobilization and cellular signaling. It has also been suggested that other proteins, such as *tat*, *rev* and *nef*, may contribute to defects observed in  $\text{Ca}^{2+}$  pathways in J1.1 cells (Perez et al., 1991). Like with the other three HIV-infected cell lines discussed, HIV replication in J1.1 cells is induced upon stimulation with  $\text{TNF-}\alpha$  or PMA (Perez et al., 1991).

J1.1 cells were obtained from the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: OM-10.1, from Dr Thomas Folks. Jurkat cells were obtained from ATCC.

#### **2.2.1.1 Cell Maintenance**

Cells were seeded at 0.25 million cells/ml in in RPMI-1640 with L-glutamine (Invitrogen) with 10% FBS (ATCC) and 100  $\mu\text{g/ml}$  of penicillin and 100  $\mu\text{g/ml}$  of streptomycin and passaged every 3-4 days. HL-60 cells were cultured in Iscove's Modified Medium (ATCC) with 20% FBS (ATCC) with the same quantities of antibiotics as the other cell lines and

were also repassaged every 3-4 days.

### **2.2.1.2 p24 antigen ELISA to confirm inducibility of HIV replication**

In order to ensure that it was possible to induce HIV replication from cells chronically-infected with HIV, supernatants from cell lines were tested for p24 antigen production at baseline (prior to stimulation) and daily for 7-14 days after stimulation with TNF- $\alpha$  at 20 ng/mL. This concentration was selected as concentrations any greater than this resulted in too much cell death after 24 hours, as seen by Trypan blue staining, prior to the addition of any MG1. Controls included supernatants from the same cell lines and maintained under the same conditions but without TNF- $\alpha$  stimulation. The U1 line was also tested prior to, and after, stimulation with PMA at 0.5 ng/mL, 1 ng/mL, 2 ng/mL or 3 ng/mL.

As indicated above, TNF- $\alpha$  is known to induce HIV replication in all of these cell lines (Butera et al., 1994; Butera et al., 1991; Emiliani et al., 1998; Folks et al., 1989; Folks et al., 1987). PMA is also known to induce HIV replication in U1 cells and, in fact, is one of the most potent inducers of HIV replication (Folks et al., 1987). Fresh media (either with or without TNF- $\alpha$  or PMA at the appropriate concentrations) was added to cell cultures every 3-4 days. At each time point, 1 mL of suspension was collected and spun down, and the supernatants frozen for p24 antigen ELISA testing at a later date.

Measurement of p24 antigen in supernatants was performed using the HIV-1 p24<sup>CA</sup> Antigen Capture Assay Kit as per the manufacturer's protocol (AIDS & Cancer Virus Program). First, 25  $\mu$ L of Triton X-100 (Sigma) were added to 225  $\mu$ L of thawed supernatants for an hour in order to solubilize HIV p24 from virus particles. A 96 well plate coated with monoclonal antibody to HIV-1 p24<sup>CA</sup> was washed using an automatic plate

washer. One hundred  $\mu\text{L}$  of the lysed supernatants were added to wells in duplicates. The plate was incubated for 2 hours at  $37^\circ\text{C}$  then washed. Rabbit anti-HIV-1 (MN) p24 antibodies were then added to the wells and the plate incubated at  $37^\circ\text{C}$  for an hour. Following this, the plate was washed again and goat anti-rabbit IgG-horse radish peroxidase (HRP) was added. Following another incubation for an hour at  $37^\circ\text{C}$ , the plate was washed and  $100\ \mu\text{l}$  of tetramethyl benzene (TMB) substrate was added, resulting in the formation of a soluble coloured product. The reaction was halted by the addition of  $1\text{N HCl}$  and the product was measured on a plate reader at an absorbance of  $450\ \text{nm}$  with  $650\ \text{nm}$  subtracted. The amount of color developed was proportional to the quantity of capsid protein developed. Hence, the amount of p24 antigen in samples could be determined using a standard curve was prepared using standardized quantities of p24 antigen provided in the kit.

### **2.2.2 Primary cells: CD4+CD25-HLADR- cells**

Whole blood was obtained from 8 healthy, HIV-uninfected donors and 20 HIV-infected individuals on stable HAART regimens for at least 6 months with suppressed VLs.

Written informed consent was obtained from volunteers prior to blood collection with a form approved by the Ottawa Hospital Research Ethics Board.

PBMCs were obtained using Ficoll density centrifugation. CD4+CD25-HLADR- cells were isolated using the Magnetic Activated Cell Sorting (MACS) system through a double-negative selection procedure. First, CD4+ cells were isolated by depleting these cells from PBMCs. This involved using a cocktail of biotin conjugated antibodies against CD8, CD14, CD15, CD16, CD19, CD36, CD56, CD123, TCR $\gamma/\delta$  and CD235a in order to remove

monocytes, neutrophils, eosinophils, B cells, dendritic cells, natural killer cells, granulocytes,  $\gamma\delta$  T cells and erythroid cells. Following this, a CD4<sup>+</sup> T cell microbead cocktail was used to magnetically label and deplete non-target cells.

The negative cell fraction, consisting of CD4<sup>+</sup> T cells, was then treated with anti-CD25 PE and anti-HLA-DR-PE antibodies (Invitrogen), followed by anti-PE conjugated antibodies (Miltenyi Biotec) in order to remove CD4<sup>+</sup> T cells bearing these markers. Isolations were performed according to the manufacturer's protocols (Miltenyi Biotec). Purity of the negative fraction, consisting of CD4<sup>+</sup>CD25<sup>-</sup>HLADR<sup>-</sup> cells, was assessed by flow cytometry after staining cells with PE-conjugated antibodies to CD4 and FITC-conjugated antibodies to CD25 and HLADR (BD Pharmingen). Cells were maintained in RPMI-1640 with glutamine (Invitrogen) with 10% FBS (ATCC), 100  $\mu$ g/mL of penicillin and 100  $\mu$ g/mL of streptomycin.

### **2.3 Effect of HIV on infectivity of MG1 in cell lines**

U1, OM-10, ACH-2 and J1.1 cells, with and without 24 hours of TNF- $\alpha$  pre-stimulation, were infected with GFP-encoded MG1 at multiplicities of infection (MOI) ranging from 0.00001 to 0.01. Controls included the respective HIV-uninfected parent cell lines, U937, HL-60, A3.01 and Jurkat cells. Flow cytometry was performed to assess GFP signal (detected via FL1) as gated on live cells, 18 and 24 hours post MG1 infection. Flow cytometry data was analyzed using FCS Express Version 4.0.

#### **2.4 Effect of HIV on MG1-induced cell death in cell lines**

Cells in the aforementioned conditions were subjected to MTT assay (ATCC) at 18 and 24 hours post MG1 infection to assess viability. This assay uses the reduction of tetrazolium salt MTT (3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) by metabolically active cells to generate compounds such as NADH and NADPH. This, in turn, results in the formation of a purple formazan precipitates which are then solubilized and quantified by spectrophotometry.

Briefly, 100  $\mu$ L of cells at a concentration of 0.5 million/ml were placed in wells 96 well plates and 20  $\mu$ L of MTT reagent (ATCC) was added. This was performed in quadruplets. Cells were incubated for approximately 4-8 hours until a purple precipitate was visualized. One hundred  $\mu$ L of detergent (ATCC) was added to each well and cells were left at room temperature in the dark for approximately 2 hours, following which absorbance was read at 570 nm.

The absorbance for cells without MG1 treatment was used as a baseline and given a viability score of 100%. The absorbance for cells of the same lines which were treated with MG1 were compared to that of untreated cells, and a viability percentage was obtained for cells in each experimental condition. The percentage of viable cells was subtracted from 100% to yield the percentage of cell death.

#### **2.5 Effect of MG1 on p24 antigen production in cell lines**

Twenty-four hours post-MG1 infection, cells from the each experimental condition were spun down and supernatants were collected in order to perform p24 antigen ELISAs, as described above.

In order to confirm that there was no cross-reactivity between the HIV capsid and MG1 capsid proteins, p24 antigen ELISAs were also performed on U937, A3.01, HL60 and Jurkat cells at the same MOIs of MG1 as performed on HIV-infected cell lines.

## **2.6 Effect of MG1 on infectivity and cell death of CD4+CD25-HLADR- cells from patients on effective antiretroviral**

For each individual, CD4+CD25-HLADR- cells were either left untreated as a control, or treated with MG1 at an MOI of 0.01. If sufficient cells were available, cells were also treated with MG1 at an MOI of 0.001. Flow cytometry for GFP expression and MTT assay were performed 24 hours following MG1 infection.

## **2.7 Effect of MG1 on Total DNA and replication-competent HIV on CD4+CD25-HLADR- cells from HIV-uninfected individuals as well as patients on effective antiretroviral therapy**

### 2.7.1 Real-time PCR for Total HIV DNA

Twenty-four hours post MG1 infection, 1 mL of cell suspension was collected from each condition (ie, cells untreated with MG1, cells treated with MG1 at an MOI of 0.01 and cells treated with MG1 at an MOI of 0.001). The suspensions were spun down, supernatants were removed and the cell pellet was frozen at -70C until PCRs were performed.

DNA was extracted using the DNeasy Blood and Tissue Kit DNA (Qiagen), as per the manufacturer's protocol. The quantity of nucleic acid in each condition was quantified using an IMPLEN NanoPhotometer T009 at a wavelength of 260 nm. Ratios of absorbance at 260 nm and 280 nm were used to assess purity of DNA. Quantities of nucleic acid (50-200 ng) were normalized across samples by the addition of the appropriate amount of

RNAase-free water. An iCycler (BioRad) was used for amplification which was done in triplicates using iQ SYBR Green Super Mix (BioRad) and primers for total HIV DNA and RPS18, the latter of which served as the loading control. The iQ SYBR Green Super Mix contains reaction buffer with dNTPs, iTaq DNA polymerase, MgCl<sub>2</sub>, SYBR Green I, fluorescein and stabilizers. Primers for total HIV GAGTCCTGCGTCGCGAGATCT (Yamamoto et al., 2006) at final concentrations of 300 nM. Primers for RPS18 included the forward primer CTGCCATTAAGGGTGTGG and reverse primer TCCATCCTTTACATCCTTCTG. PCR conditions consisted of a step at 95°C for 10 seconds followed by a step at 59°C for 30 seconds and a step at 95°C for 1 minute. This was repeated for 50 cycles.

Negative controls included PBMCs from HIV-uninfected donors while positive controls included ACH-2 cells.

### 2.7.2 Cell stimulation and co-culture to induce HIV replication

In order to quantify replication-competent HIV in cells untreated with MG1 compared to cells treated with MG1 for 24 hours, cells in each condition underwent stimulation and co-culture in 24-well plates based on methods previously described (Chun TW et al., 2010; Chun TW et al., 1999; Chun TW et al., 1997). On day 2, irradiated PBMCs from healthy HIV-uninfected donors were added to cells in each condition (ratio of  $1 \times 10^6$  CD4+CD25-HLADR- cells:  $5 \times 10^6$  irradiated PBMCs). Anti-CD3 (eBioscience) and IL-2 (eBioscience) were added to yield concentrations of 1 µg/mL and 50 Units/mL, respectively. On day 3, CD8-depleted PBMCs from a healthy donor which had been stimulated for 24 hours with anti-CD3 at 1 µg/ml were added to each well (ratio  $1 \times 10^6$  CD4+CD25-HLADR- cells:  $0.8 \times 10^6$  CD8-depleted PBMCs). On day 8, more CD8-depleted PBMCs from a healthy donor

which had been stimulated for 24 hours with anti-CD3 at 1  $\mu\text{g}/\text{mL}$  were added to wells as on day 3. Every 3 days during this period, 33% of the cell suspension was removed from each well and replaced with fresh media. Between days 15-18, suspensions were spun down and supernatants collected and frozen at  $-70^\circ\text{C}$  for future PCR of total HIV RNA and p24 antigen ELISA.

### 2.7.3 Real-time PCR for Total HIV RNA

RNA was extracted from supernatants using the QIAamp Viral RNA Mini Kit (Qiagen). Nucleic acid was quantified using an IMPLEN NanoPhotometer T009 at a wavelength of 260 nm. Ratios of absorbance at 260 nm and 280 nm were used to assess purity of DNA. Quantities of nucleic acid were normalized across samples by the addition of the appropriate amount of RNAase-free water. RNA (50-200 ng) was reverse transcribed to cDNA using the iScript cDNA Kit (BioRad) protocol. This involved the use of a Master Mix solution which included which included buffer and reverse transcriptase enzyme. Reverse transcription was performed on an Eppendorf Thermocycler (Mastercycler) whereby samples were heated to  $25^\circ\text{C}$  for 5 minutes,  $42^\circ\text{C}$  for 30 minutes and  $85^\circ\text{C}$  for 5 minutes and this was followed by a cool-down period. Amplification of cDNA using an iCycler (BioRad) was done in triplicates using the same methods and conditions as described above.

Negative controls included supernatants from PMBCs from HIV-uninfected donors stimulated for 24 hours with TNF- $\alpha$  while positive controls included supernatants from ACH-2 cells stimulated for 24 hours with TNF- $\alpha$ .

#### 2.7.4 p24 antigen ELISA

Supernatants were subjected to p24 antigen ELISA as described above.

### **2.8 Statistical Analysis**

Mean percentages of infectivity and percentages of cell death between HIV-infected and HIV-uninfected cell lines after treatment with various MOIs of MG1 were analyzed using the student's t-test. Mean percentages of infectivity and percentages of cell death between unstimulated cell lines and cell lines pre-stimulated for 24 hours with TNF- $\alpha$  with various MOIs of MG1 were also analyzed using the student's t-test.

Analysis of PCR data was performed using the  $2^{\Delta\Delta Ct}$  principle with the BioRad Gene Expression Analysis Macro Program (BioRad). One-way analysis of variance (ANOVA) with the Kruskal-Wallis test was used to compare DNA and RNA expression ratios across the 3 experimental conditions.

## **3 RESULTS**

### **3.1 Cell lines**

#### **3.1.1 p24 antigen production by HIV-infected cell lines post-stimulation with TNF- $\alpha$ and/or PMA**

Basal levels of p24 antigen prior to TNF- $\alpha$  stimulation were below the level of detection of the assay for all cell lines tested. For HIV-infected cells (untreated with MG1), TNF- $\alpha$  stimulation at a concentration of 20 ng/mL of TNF- $\alpha$  resulted in an increase in p24 antigen production which peaked approximately 2-3 days later. For U1 cells, PMA also increased

p24 antigen production above baseline which peaked approximately 2 days post TNF- $\alpha$  stimulation (FIGURES 6-10). These overall trends were consistent with those previously reported in the literature (Butera et al., 1994; Folks et al., 1987) and suggested that the cell lines were behaving as previously described. Controls included the same cell lines, without TNF- $\alpha$  stimulation, from which p24 antigen levels in supernatants were measured at various time points. Levels of p24 antigen in supernatants were below levels of assay detection for controls.

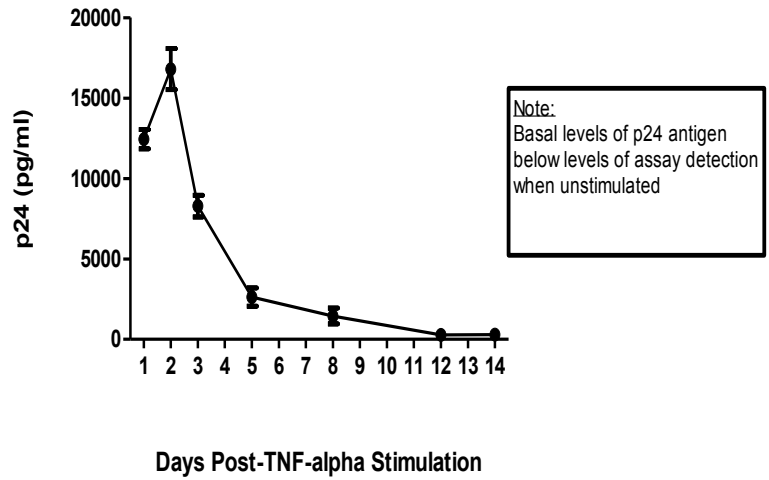


Figure 6

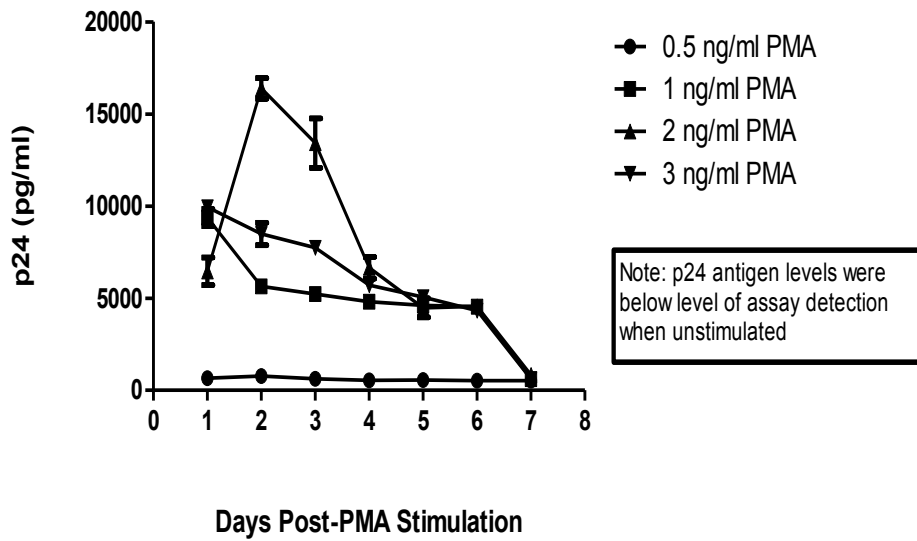


Figure 7

**Figure 6: p24 antigen production by U1 cells with TNF- $\alpha$  stimulation.** U1 cells were stimulated with TNF- $\alpha$  at 20 ng/mL in order to confirm inducibility of HIV replication. Supernatants were collected at baseline (prior to stimulation) and daily for 14 days after stimulation with TNF- $\alpha$ . Controls included supernatants from U1 cells maintained under the same conditions but without TNF- $\alpha$  stimulation. HIV replication was inducible with peak replication observed after 2 days of stimulation.

**Figure 7: p24 antigen production by U1 cells with PMA stimulation.** U1 cells were stimulated with PMA at concentrations of 0.5 ng/mL, 1 ng/mL, 2 ng/mL or 3 ng/mL in order to confirm inducibility of HIV replication. Supernatants were collected at baseline (prior to stimulation) and daily for 7 days. Controls included supernatants from U1 cells maintained under the same conditions but without PMA stimulation. HIV replication was inducible with peak replication observed after 2 days when stimulated with 2 ng/mL of PMA.

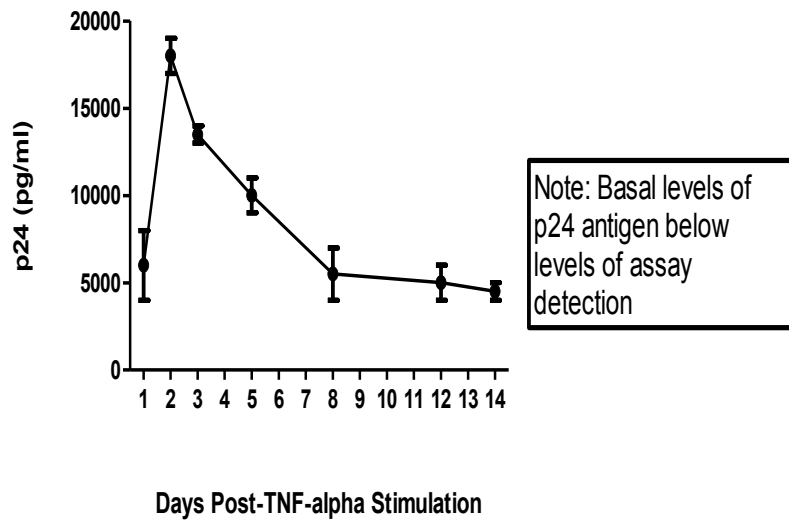


Figure 8

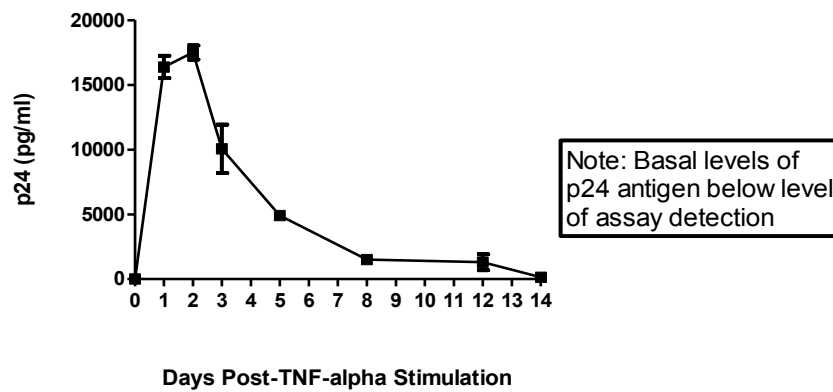
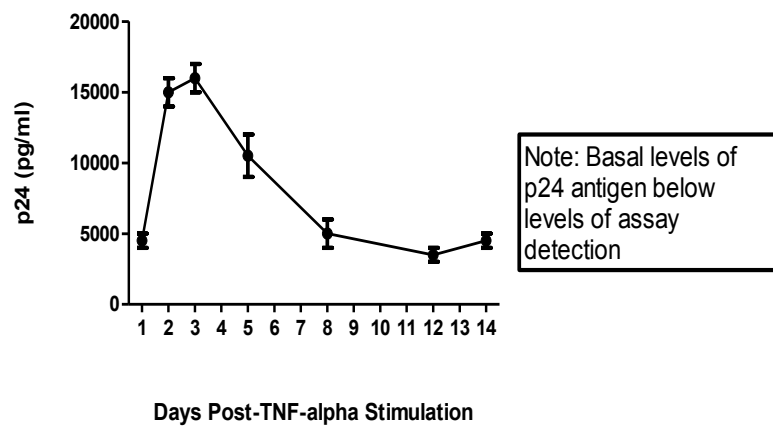


Figure 9

**Figure 8: p24 antigen production by ACH-2 cells with 20 ng/mL of TNF- $\alpha$  stimulation.** ACH-2 cells were stimulated with TNF- $\alpha$  at 20 ng/mL in order to confirm inducibility of HIV replication. Supernatants were collected at baseline (prior to stimulation) and daily for 14 days after stimulation with TNF- $\alpha$ . Controls included supernatants from ACH-2 cells maintained under the same conditions but without TNF- $\alpha$  stimulation. HIV replication was inducible with peak replication observed after 2 days of stimulation.

**Figure 9: p24 antigen production by OM-10 cells with 20 ng/mL of TNF- $\alpha$  stimulation.** OM-10 cells were stimulated with TNF- $\alpha$  at 20 ng/mL in order to confirm inducibility of HIV replication. Supernatants were collected at baseline (prior to stimulation) and daily for 14 days after stimulation with TNF- $\alpha$ . Controls included supernatants from OM-10 cells maintained under the same conditions but without TNF- $\alpha$  stimulation. HIV replication was inducible with peak replication observed after 2 days of stimulation.

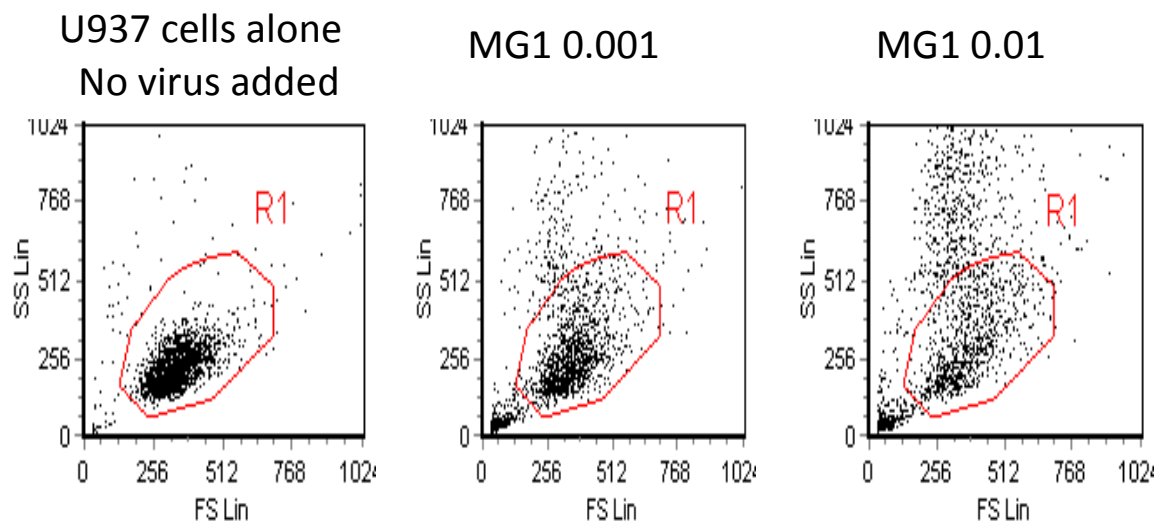


**Figure 10**

**Figure 10: p24 antigen production by J1.1 cells with 20 ng/mL of TNF- $\alpha$  stimulation.** J1.1 cells were stimulated with TNF- $\alpha$  at 20 ng/mL in order to confirm inducibility of HIV replication. Supernatants were collected at baseline (prior to stimulation) and daily for 14 days after stimulation with TNF- $\alpha$ . Controls included supernatants from J1.1 cells maintained under the same conditions but without TNF- $\alpha$  stimulation. HIV replication was inducible with peak replication observed after 3 days of stimulation.

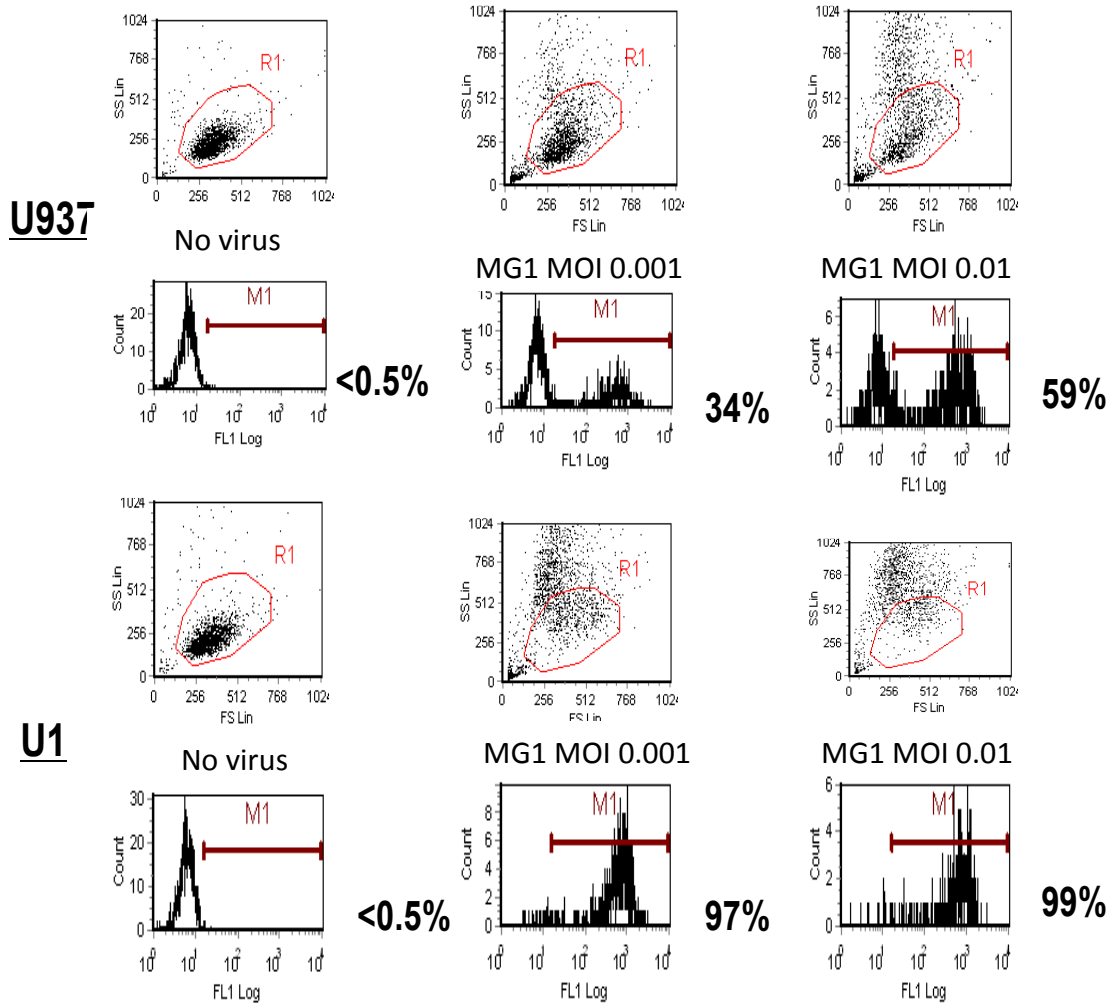
### **3.1.2 Effect of HIV on infectivity of MG1 in cell lines**

Previous preliminary studies had revealed that an MOI of MG1 of 0.1 resulted in an excessive amount of cell death, as determined by Trypan blue staining, in all parent cell lines. Thus, this MOI had been deemed unsuitable for future studies of MG1 with these cell lines. Similarly, at 72 hours post infection with MG1, there had been a very high amount of cell death, making this time point not optimal for the study of MG1 infection and MG-induced cell death in these lines. Therefore, MOIs of 0.0001 through 0.01 were used and infectivity and viability tests were performed at 18 and 24 hours post MG1 infection. Infectivity and viability experiments were performed 3 or more times. An example of the gating strategy using U937 cells is illustrated in FIGURE 11. Examples of scatter plots and histograms comparing infectivity data for U937 and U1 cells are provided in FIGURE 12.



**Figure 11**

**Figure 11: GFP expression as gated on live cells.** Gates were placed around live cell populations. Infectivity was expressed as the percentage of cells within the gate which were GFP-positive. Increased amounts of GFP resulted in higher degrees of infectivity.



**Figure 12**

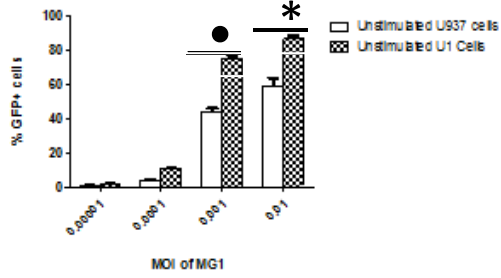
**Figure 12: Examples of scatter plots and histograms for infectivity in U1 versus U937 cells 24 hours post MG1 infection.** U937 and U1 cells were infected in parallel with various MOIs of MG1 and flow cytometry was performed after 18 and 24 hours post-MG1 infection. The scatter plots and histograms on the left-hand side depict the cells without any MG1. The graphs in the middle column depict the cells at 24 hours following MG1 infection at an MOI of 0.001 and the graphs on the right-hand side depict the cells following infection at an MOI of 0.01. HIV-infected U1 cells were infected to a greater degree, as determined by GFP-positivity within the live gate, than HIV-uninfected U937 cells at both MOIs.

All cell lines were easily infected with MG1 at both 18 and 24 hours. Twenty-four hours following MG1 infection, well over 80% of cell lines (except for U937 cells) were infected by MG1 at MOIs of 0.001 and 0.01 when gated on live cells. At lower MOIs of 0.0001 and 0.0001, most cell lines were greater than 40% infected when gated on live cells, except for U1 and U397 cells which displayed much lower levels of infectivity at these two lower MOIs. U1 cells demonstrated a greater propensity to become infected at 18 hours with MG1 than U937 cells at MOIs of 0.001 ( $p=0.0002$ ) and MOI 0.01 ( $p=0.0046$ ). This was also observed at 24 hours post-MG1 infection at MOI 0.001 ( $p<0.0001$ ) and MOI 0.01 ( $p=0.0039$ ). There was no clear difference in infectivity between these two cell lines at MOIs of 0.00001 or 0.0001 at either time point ( $p=NS$ ) (TABLE 1; FIGURE 13). The presence of integrated HIV proviral DNA did not result in increased MG1 infectivity of ACH2, OM-10 or J1.1 cells compared to A3.01, HL-60 and Jurkat cells, respectively, at any MOIs tested (FIGURES 14-16).

**Table 1:** Percentage of GFP positive U1 versus U937 cells post MG1 infection as gated on live cells (means and standard error of the means)

MOI of MG1	U1 cells		U937 cells	
	18 hrs	24 hrs	18 hrs	24 hrs
0.00001	2 (1)	2 (2)	1 (1)	2 (1)
0.0001	11 (1)	13 (1)	4 (1)	15 (2)
0.001	75 (2)	83 (1)	44 (2)	49 (1)
0.01	87 (2)	89(1)	59 (4)	63 (4)

18 hours post MG1 Infection



24 hours post MG1 Infection

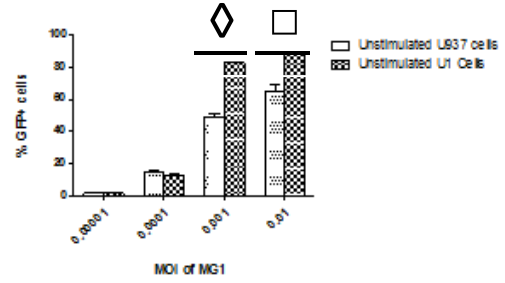
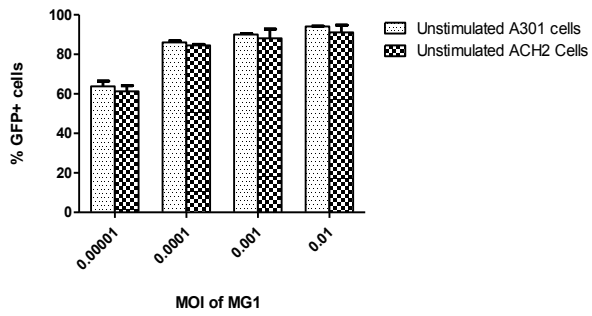


Figure 13

18 hours post MG1 Infection



24 hours post MG1 Infection

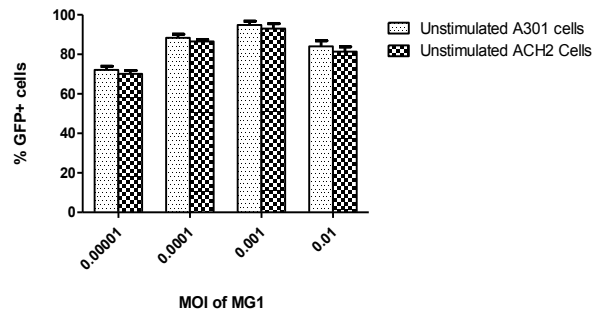
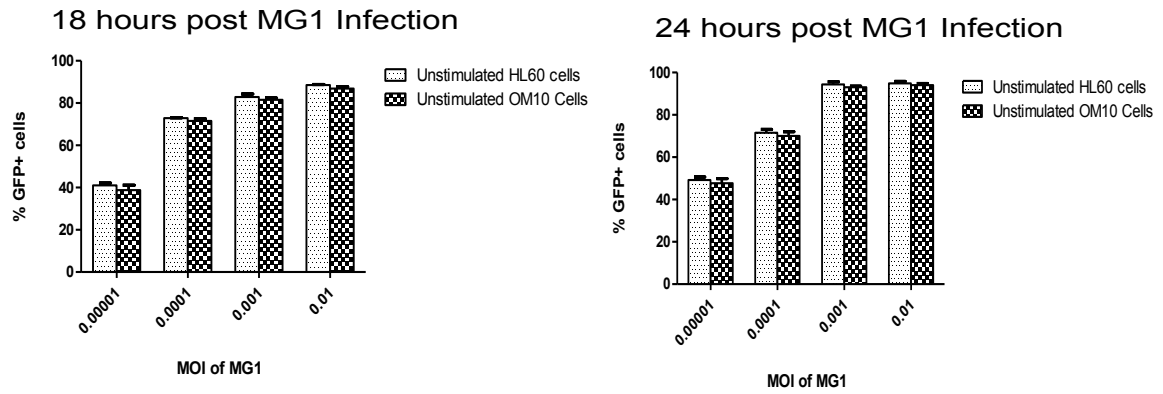


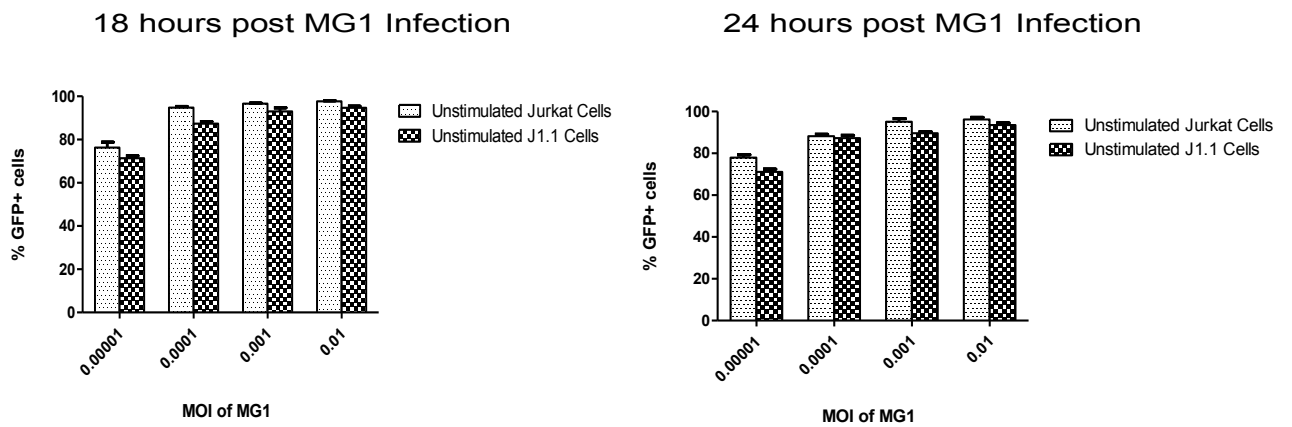
Figure 14

**Figure 13: Greater infectivity of HIV-infected U1 cells compared to HIV-uninfected U937 cells at MOIs of 0.001 and 0.01.** HIV-infected U1 cells demonstrated a greater propensity to become infected at 18 hours with MG1 than HIV-uninfected U937 cells at MOIs of 0.001 (● $p=0.0002$ ) and MOI 0.01 (\* $p=0.0046$ ). This was also observed at 24 hours post-MG1 infection at MOI 0.001 (◇ $p<0.0001$ ) and MOI 0.01 (□ $p=0.0039$ ). There was no clear difference in infectivity between these two cell lines at MOIs of 0.00001 or 0.0001 at either time point ( $p=NS$ ).

**Figure 14: No difference in MG1 infectivity between HIV-infected ACH-2 cells and HIV-uninfected A3.01 cells.** The presence of integrated HIV proviral DNA did not result in increased MG1 infectivity of ACH-2 cells compared to A3.01 at any MOIs tested.



**Figure 15**



**Figure 16**

**Figure 15: No difference in MG1 infectivity between HIV-infected OM-10 cells and HIV-uninfected HL-60 cells.** The presence of integrated HIV proviral DNA did not result in increased MG1 infectivity of OM-10 cells compared to HL-60 at any MOIs tested.

**Figure 16: No difference in MG1 infectivity between HIV-infected J1.1 cells and HIV-uninfected Jurkat cells.** The presence of integrated HIV proviral DNA did not result in increased MG1 infectivity of J1.1 cells compared to Jurkat cells, respectively, at any MOIs tested.

### 3.1.3 Effect of HIV on MG1-induced cell death in cell lines

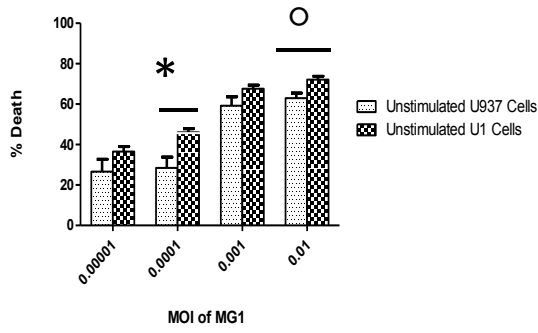
MG1-induced cell death was greater in U1 than U937 cells at several MOIs tested at 18 hours (0.00001, p=NS; 0.0001, p=0.0338; 0.001, p=NS; 0.01, p=0.0421). MG1-induced cell death was also greater in U1 than U937 cells at one MOI 24 hours post MG1 infection (0.00001, p=NS; 0.0001, p=0.0120; 0.001, p=NS; p=NS ) (TABLE 2; FIGURE 17).

**Table 2:** Percentage of dead U1 versus U937 cells post MG1 infection as measured on entire cell population (means and standard errors of the means)

MOI of MG1	U1 cells		U937 cells	
	18 hrs	24 hrs	18 hrs	24 hrs
0.00001	37 (3)	55 (5)	27 (6)	41 (3)
0.0001	46 (2)	74 (2)	28 ( 5)	61 (2 )
0.001	68 (2)	86 (2)	59 (5)	81 (1)
0.01	72 (2)	88 (3)	63 (3)	88 (1)

The presence of integrated proviral DNA also did not appear to affect the degree of MG1-induced cell death in ACH-2 and OM-10 cells compared to their HIV-uninfected controls (FIGURES 18-19). However, J1.1 cells appeared to be more resistant to MG1-induced cell death than the HIV-uninfected Jurkat control cells at several MOIs at 18 hours (0.00001, p=NS; 0.0001, p=NS; 0.001, p=0.0186; 0.01, p=0.0044) in addition to all MOIs at 24 hours (0.00001, p<0.0001; 0.0001, p=0.0002; 0.001, p=0.0001; 0.01, p<0.0001) (FIGURE 20).

18 hours post MG1 Infection



24 hours post MG1 Infection

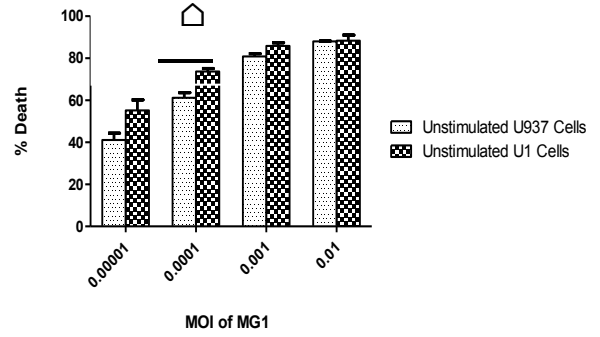
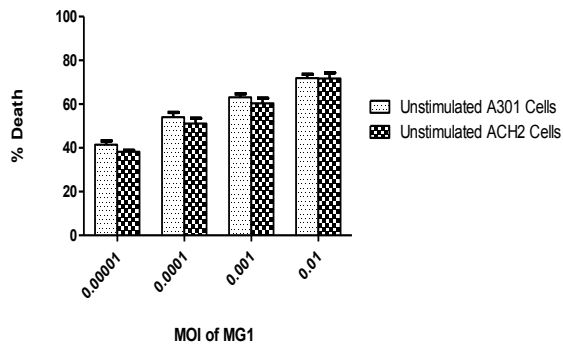


Figure 17

18 hours post MG1 Infection



24 hours post MG1 Infection

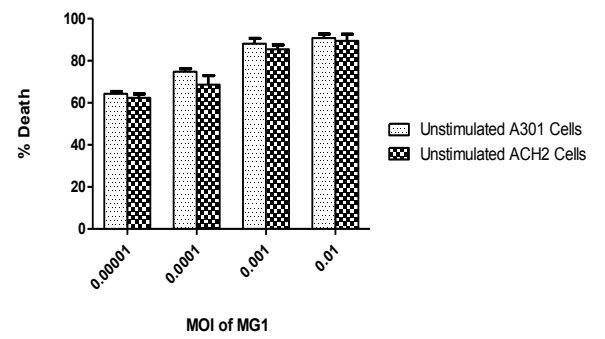
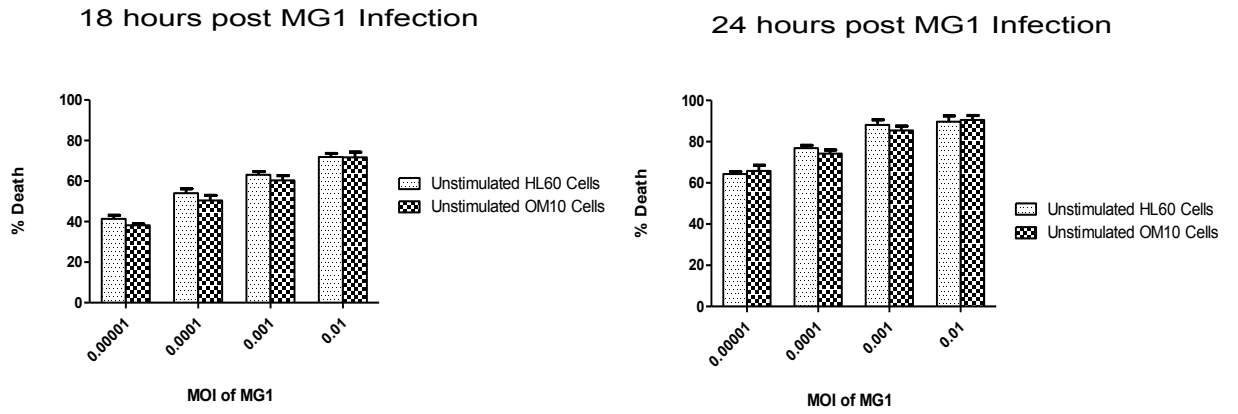


Figure 18

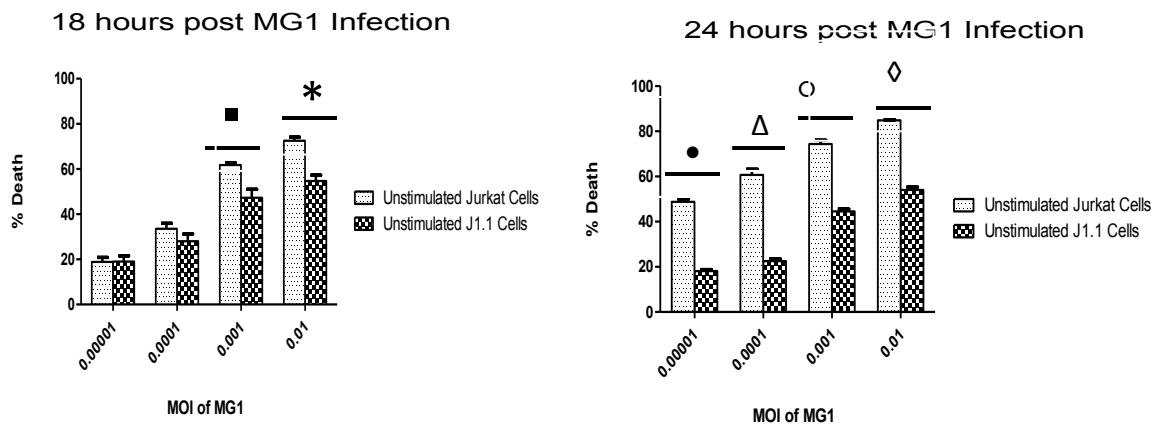
**Figure 17: Greater MG1-induced cell death in HIV-infected U1 cells compared to HIV-uninfected U937 cells at certain MOIs tested.** MG1-induced cell death was greater in U1 than U937 cells at 18 hours for MOI of 0.0001 (\*p=0.0338) and 0.01 (○p=0.0421). MG1-induced cell death was also greater in U1 than U937 cells 24 hours post-MG1 infection at an MOI of 0.0001 (△p=0.120).

.

**Figure 18: No significant difference in MG1-induced cell death between HIV-infected ACH-2 cells and HIV-uninfected A3.01 cells.** The presence of integrated proviral DNA also did not appear to affect the degree of MG1-induced cell death in ACH-2 cells compared to their HIV-uninfected controls.



**Figure 19**



**Figure 20**

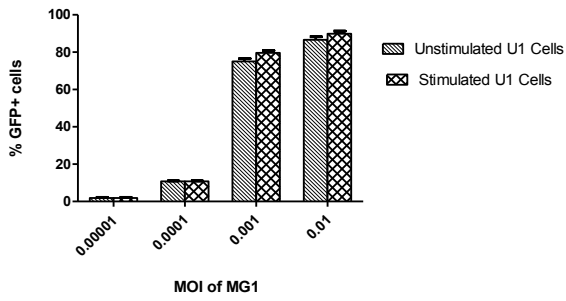
**Figure 19: No significant difference in MG1-induced cell death between HIV-infected OM-10 cells and HIV-uninfected HL-60 cells.** The presence of integrated proviral DNA also did not appear to affect the degree of MG1-induced cell death in OM-10 cells compared to their HIV-uninfected controls.

**Figure 20: Greater MG1-induced cell death in HIV-uninfected Jurkat cells compared to HIV-infected J1.1 cells.** J1.1 cells appeared to be more resistant to MG1-induced cell death than the HIV-uninfected Jurkat control cells at several MOIs 18 hours post-MG1 infection (0.001,  $\blacksquare$ p=0.0186; 0.01,  $*$ p=0.0044) in addition to all MOIs at 24 hours post-MG1 infection (0.00001,  $\bullet$ p<0.0001; 0.0001,  $\Delta$ p=0.0002; 0.001,  $\circ$ p=0.0001; 0.01,  $\diamond$ p<0.0001).

### **3.1.4 Effect of TNF- $\alpha$ pre-stimulation on MG1 infectivity and MG1-induced cell death in HIV-infected cell lines**

TNF- $\alpha$  resulted in a small increase in infectivity of cell lines compared to the same cell lines untreated with TNF- $\alpha$  at certain MOIs, although these differences were often not statistically significant (FIGURES 21-24). TNF- $\alpha$  treatment of cells also resulted in slightly greater overall cell death at certain MOIs compared to the same cells untreated with TNF- $\alpha$ , although once again these differences were often not statistically significant (FIGURES 25-28). Although higher concentrations of TNF- $\alpha$  were tested in an attempt to determine whether one could observe greater infectivity and killing with TNF- $\alpha$  pre-treated cells compared to cells not pre-treated with TNF- $\alpha$  for 24 hours, as previously mentioned the amount of cell death induced by TNF- $\alpha$  prior to MG1 infection was too great (>20%) and thus it was deemed that concentrations greater than 20 ng/mL would be unsuitable for these studies. Concentrations lower than 20 ng/ml (15 ng/ml, 10 ng/ml and 5 ng/ml) also did not result in any major differences in infectivity between cell lines pre-treated and not pre-treated for 24 hours with TNF- $\alpha$ .

18 hours post MG1 Infection



24 hours post MG1 Infection

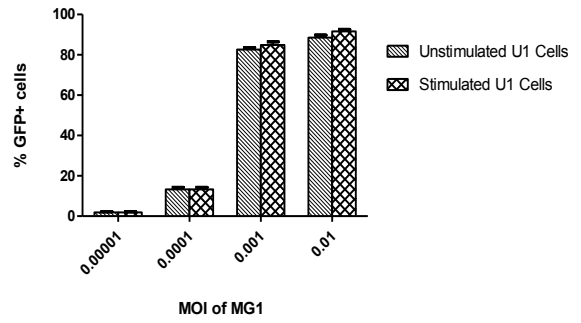
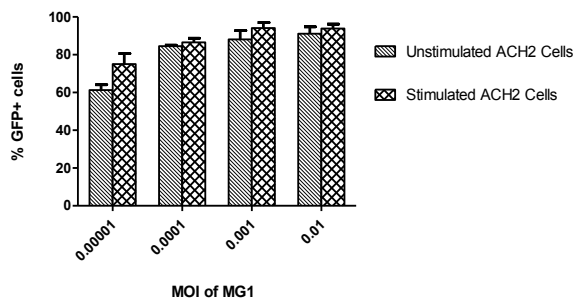


Figure 21

18 hours post MG1 Infection



24 hours post MG1 Infection

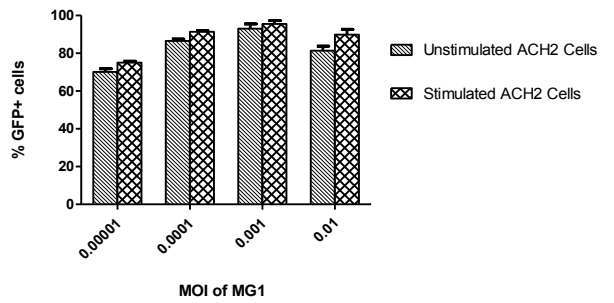
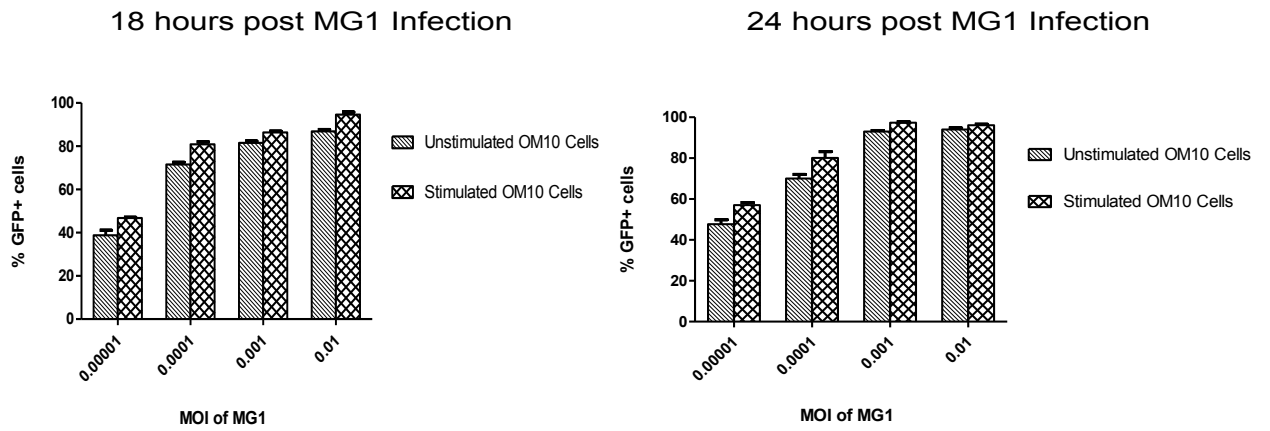


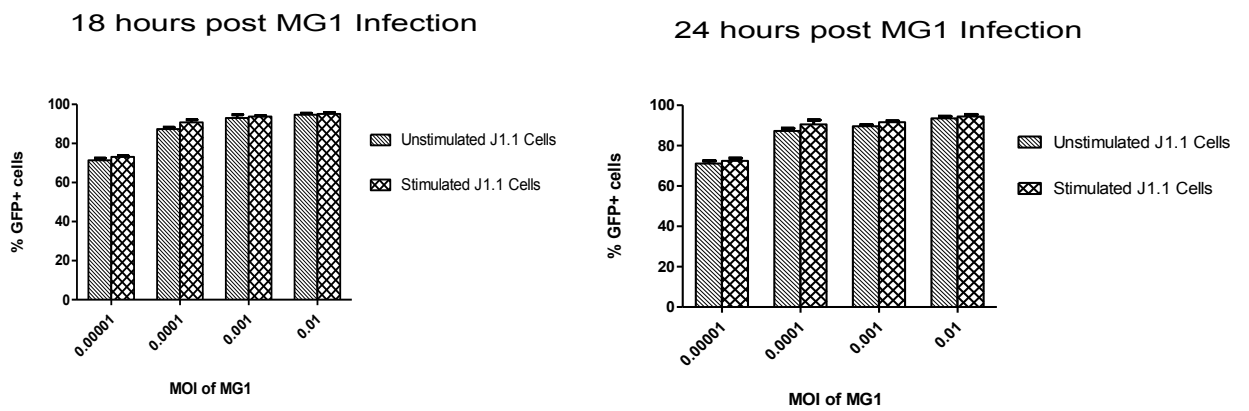
Figure 22

**Figure 21: No significant increase in MG1 infectivity of U1 cells pre-treated compared to U1 cells not pre-treated for 24 hours with TNF- $\alpha$ .** TNF- $\alpha$  pre-treatment at 20 ng/mL resulted in a small increase in infectivity of U1 cells compared to the same cell lines untreated with TNF- $\alpha$  at certain MOIs, although these differences were not statistically significant.

**Figure 22: No significant increase in MG1 infectivity of ACH-2 cells pre-treated compared to ACH-2 cells not pre-treated for 24 hours with TNF- $\alpha$ .** TNF- $\alpha$  pre-treatment at 20 ng/mL did not result in any statistically significant differences in MG1 infectivity compared to ACH-2 cells which were not pre-treated with TNF- $\alpha$ .



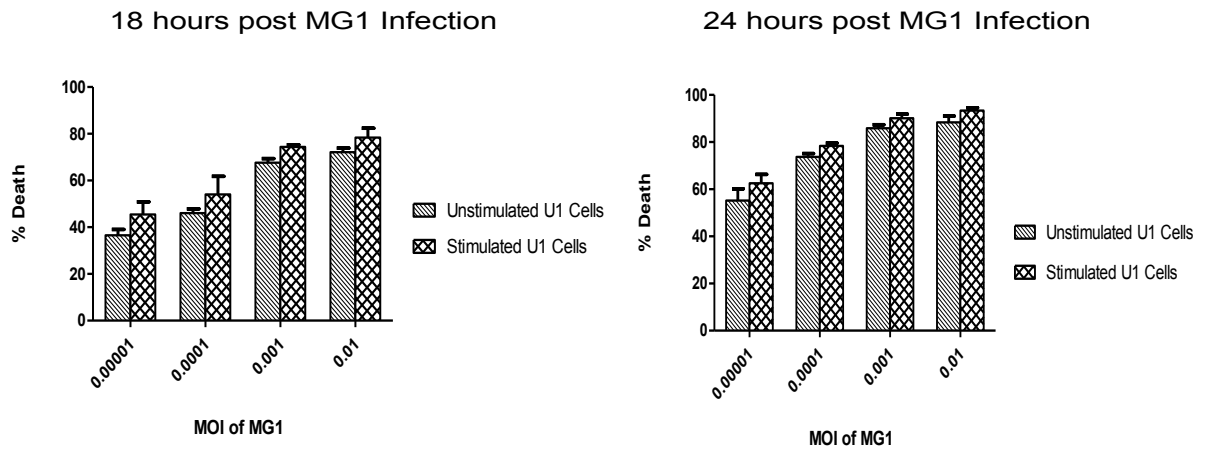
**Figure 23**



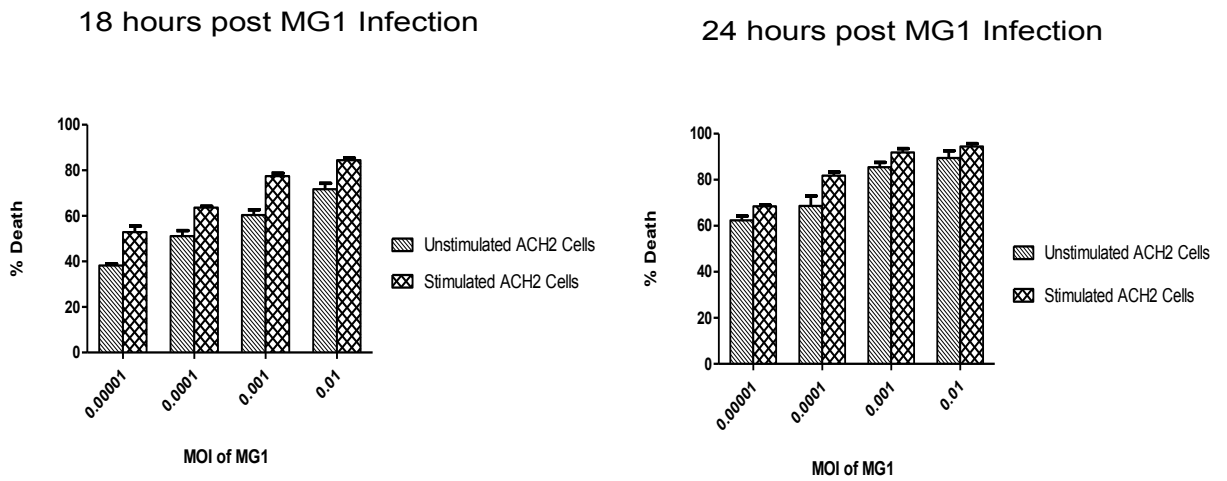
**Figure 24**

**Figure 23: No significant increase in MG1 infectivity of OM-10 cells pre-treated compared to OM-10 cells not pre-treated for 24 hours with TNF- $\alpha$ .** TNF- $\alpha$  pre-treatment at 20 ng/mL resulted in a small increase in infectivity of OM-10 cells compared to the same cells untreated with TNF- $\alpha$ , although these differences were not statistically significant.

**Figure 24: No significant increase in MG1 infectivity of J1.1 cells pre-treated compared to J1.1 cells not pre-treated for 24 hours with TNF- $\alpha$ .** TNF- $\alpha$  pre-treatment at 20 ng/mL did not result in any significant increase in MG1 infectivity of J1.1 cells compared to J1.1 cells which were untreated with TNF- $\alpha$ .



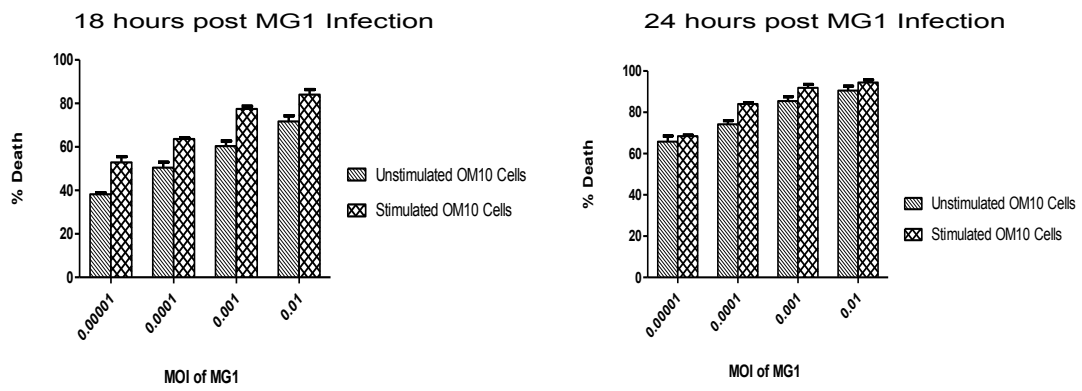
**Figure 25**



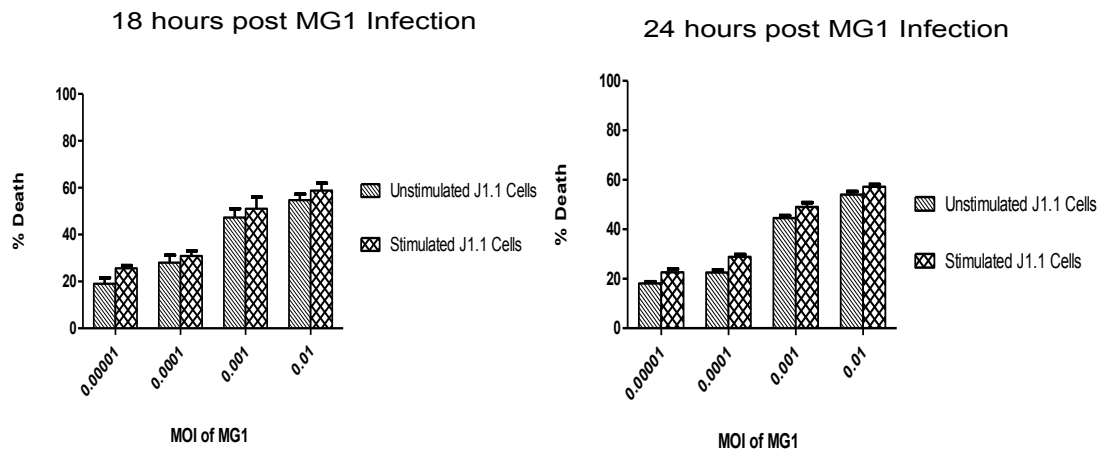
**Figure 26**

**Figure 25: No significant increase in MG1-induced cell death of U1 cells pre-treated compared to U1 cells not pre-treated for 24 hours with TNF- $\alpha$ .** Pre-treatment of cells for 24 hours with TNF- $\alpha$  at 20 ng/mL did not result in a significant increase in cell death compared to cells not pre-treated with TNF- $\alpha$

**Figure 26: No significant increase in MG1-induced cell death of ACH-2 cells pre-treated compared to ACH-2 cells not pre-treated for 24 hours with TNF- $\alpha$ .** Pre-treatment of cells for 24 hours with TNF- $\alpha$  at 20 ng/mL did not result in a significant increase in cell death compared to cells not pre-treated with TNF- $\alpha$



**Figure 27**



**Figure 28**

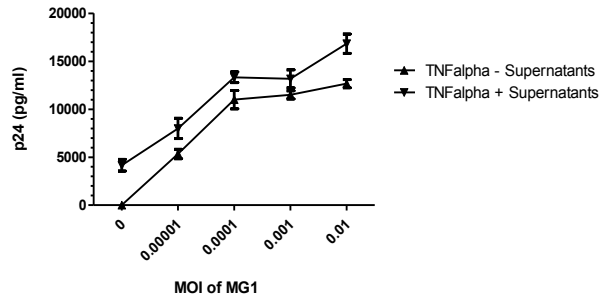
**Figure 27: No significant increase in MG1-induced cell death of OM-10 cells pre-treated compared to OM-10 cells not pre-treated for 24 hours with TNF- $\alpha$ .** Pre-treatment of cells for 24 hours with 20 ng/mL of TNF- $\alpha$  resulted in a small increase in MG1 infectivity but these results were not statistically significant.

**Figure 28: No significant increase in MG1-induced cell death of J1.1 cells pre-treated compared to J1.1 cells not pre-treated for 24 hours with TNF- $\alpha$ .** Pre-treatment of cells for 24 hours with 20 ng/mL of TNF- $\alpha$  resulted in a small increase in MG1 infectivity but these results did not reach statistical significance..

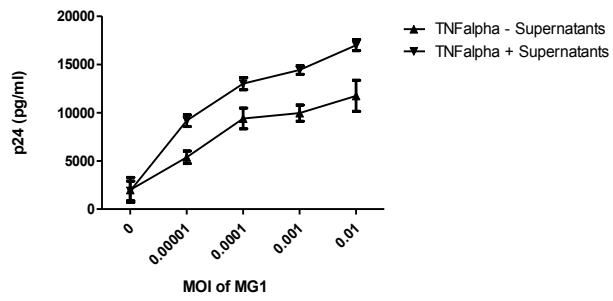
### **3.1.5 Effect of MG1 on p24 antigen production in HIV-infected cell lines**

In all HIV-infected cells lines, p24 antigen levels in supernatants were below the level of ELISA detection when cells were unstimulated. Levels of p24 antigen increased with increasing MOIs of MG1. For all HIV-infected cell lines, p24 antigen measurements in supernatants were higher in cells which were pre-treated with TNF- $\alpha$  for 24 hours (FIGURES 29-32). ELISAs for p24 antigen levels in supernatants were performed in a minimum of 3 separate experiments.

In order to exclude that this increase in p24 antigen production was potentially due to cross-reactivity between the HIV p24 antigen and capsular protein material from MG1, p24 antigen ELISAs were carried out on supernatants from U937, A301, HL-60 and Jurkat cells 24 hours post MG1 infection at an MOI of 0.001 and 0.01 in addition to cell-free media containing MG1. In all of these experiments, no p24 antigen was detected in supernatants.



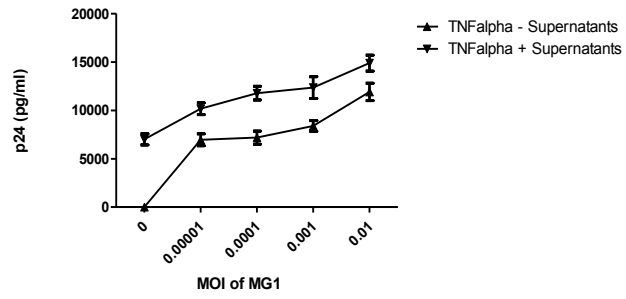
**Figure 29**



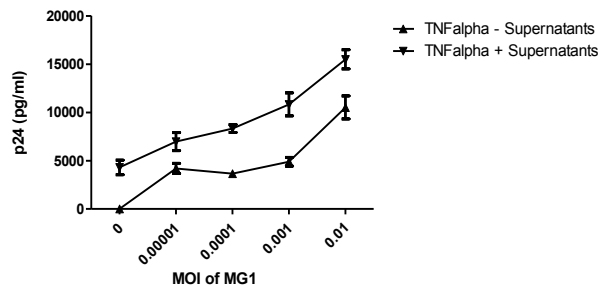
**Figure 30**

**Figure 29: Increasing MOIs of MG1 associated with greater p24 antigen levels in U1 supernatants both with and without TNF- $\alpha$  pre-stimulation.** U1 cells were either pre-treated or not pre-treated for 24 hours with TNF- $\alpha$  at 20 ng/mL. Cells were then infected with MG1 at various MOIs. Twenty-four hours post-MG1 infection, p24 antigen levels in supernatants were quantified by ELISA. p24 antigen levels in supernatants increased with increasing MOIs of MG1, and p24 antigen levels were greater in supernatants from cells which had been pre-stimulated with TNF- $\alpha$  compared to supernatants from cells which had not been pre-stimulated with TNF- $\alpha$ .

**Figure 30: Increasing MOIs of MG1 associated with greater p24 antigen levels in ACH-2 supernatants both with and without TNF- $\alpha$  pre-stimulation.** ACH-2 cells were either pre-treated or not pre-treated for 24 hours with TNF- $\alpha$  at 20 ng/mL. Cells were then infected with MG1 at various MOIs. Twenty-four hours post-MG1 infection, p24 antigen levels in supernatants were quantified by ELISA. p24 antigen levels in supernatants increased with increasing MOIs of MG1, and p24 antigen levels were greater in supernatants from cells which had been pre-stimulated with TNF- $\alpha$  compared to supernatants from cells which had not been pre-stimulated with TNF- $\alpha$ .



**Figure 31**



**Figure 32**

**Figure 31: Increasing MOIs of MG1 associated with greater p24 antigen levels in OM-10 supernatants both with and without TNF- $\alpha$  pre-stimulation.** OM-10 cells were either pre-treated or not pre-treated for 24 hours with TNF- $\alpha$  at 20 ng/mL. Cells were then infected with MG1 at various MOIs. Twenty-four hours post-MG1 infection, p24 antigen levels in supernatants were quantified by ELISA. p24 antigen levels in supernatants increased with increasing MOIs of MG1, and p24 antigen levels were greater in supernatants from cells which had been pre-stimulated with TNF- $\alpha$  compared to supernatants from cells which had not been pre-stimulated with TNF- $\alpha$ .

**Figure 32: Increasing MOIs of MG1 associated with greater p24 antigen levels in J1.1 supernatants both with and without TNF- $\alpha$  pre-stimulation.** J1.1 cells were either pre-treated or not pre-treated for 24 hours with TNF- $\alpha$  at 20 ng/mL. Cells were then infected with MG1 at various MOIs. Twenty-four hours post-MG1 infection, p24 antigen levels in supernatants were quantified by ELISA. p24 antigen levels in supernatants increased with increasing MOIs of MG1, and p24 antigen levels were greater in supernatants from cells which had been pre-stimulated with TNF- $\alpha$  compared to supernatants from cells which had not been pre-stimulated with TNF- $\alpha$ .

### 3.2 Primary cells

#### 3.2.1 Characteristics of patients

Characteristics of the 20 HIV-infected patients are summarized in Table 3. All individuals had well-suppressed peripheral VLs on HAART for much greater than 6 months. Treatment regimens varied, although half of all individuals were on NNRTI (non-nucleoside reverse transcriptase)-based regimens.

**Table 3: Characteristics of Patients with Suppressed Peripheral Blood VLs on HAART (N=20)**

Characteristic	Mean ( $\pm$ SEM)
Age (years)	51(2)
Sex	
% male	80
% female	20
Time from documented seroconversion (years)	10(1)
Duration of suppressed peripheral blood VL (years)	8(1)
CD4 count (cells/ $\mu$ l)	519(44)
% CD4 cells	29(2)
CD4/CD8 ratio	0.78(0.10)
Antiretroviral regimen (% patients)	
NNRTI-based	50
PI-based	25
ISI-based	25

HAART=highly active antiretroviral therapy; ISI=integrase strand inhibitor; protease inhibitor; NNRTI=non-nucleoside reverse transcriptase inhibitor; SEM=standard error of the mean; VL=viral loads

### **3.2.2 CD4+CD25-HLADR- cell yield**

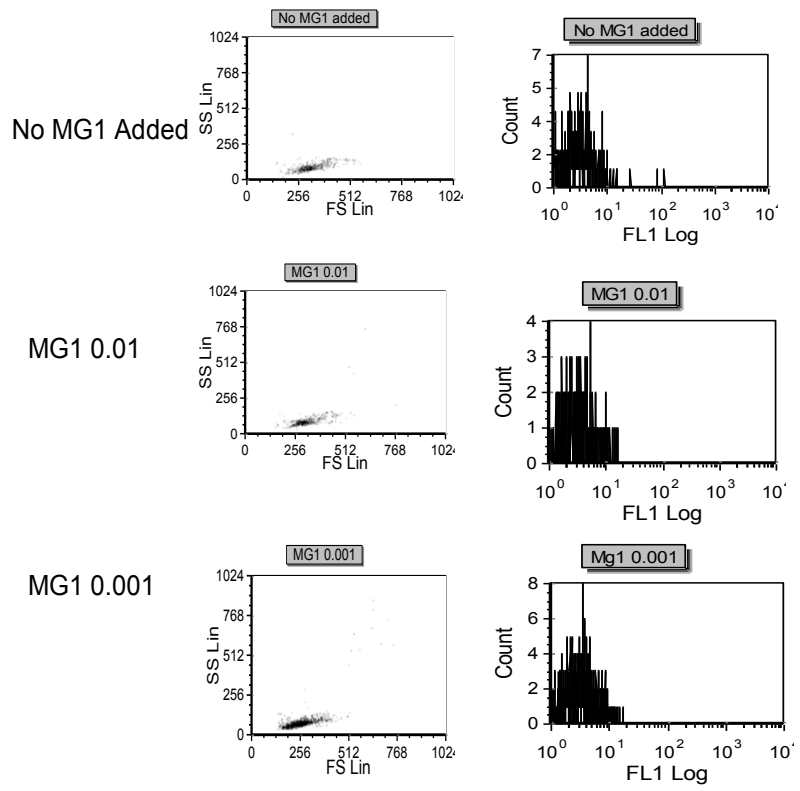
The amounts of CD4+CD25-HLADR- cells isolated from patients varied but were often low in number, ranging from approximately 0.5 million to 6 million cells. Although attempts were made to increase cell yield by adjusting the protocol used on the MACS machine, increase cell yields were often at the expense of decreased purity. Although the purity goal was initially set at 98%, often a purity of 92-95% was achieved in order to ensure that adequate numbers of cells would be available for experiments.

Due to the low number of cells obtained, in addition to the requirement of dividing these cells into smaller portions for performing the various experiments, sufficient cells to test 2 MOIs were only available from 9 HIV-infected patients.

### **3.2.3 Infectivity and viability of CD4+CD25-HLADR- cells from HIV-infected patients on HAART**

CD4+CD25-HLADR- cells untreated with MG1 and cells treated with MG1 from 20 HIV-infected patients did not demonstrate any evidence of productive MG1 infection and viability appeared preserved following 24 hours of MG1 treatment (FIGURE 33).

CD4+CD25-HLADR- cells from 8 healthy, HIV-uninfected donors also did not demonstrate any evidence of productive MG1 infection and viability appeared preserved following 24 hours of MG1 treatment.



**Figure 33**

**Figure 33: No apparent infection of CD4+CD25-HLADR- cells from HIV patients on effective HAART after 24 hours of MG1 treatment.** CD4+CD25-HLADR- cells from 20 HIV-infected individuals with suppressed peripheral blood VLs on HAART were isolated by a double-negative selection procedure. A portion of cells from each individual was untreated with MG1, another portion was treated with MG1 at an MOI of 0.001 and another portion was treated with MG1 at an MOI of 0.01. The cells did not demonstrate any evidence of productive MG1 infection, as assessed by flow cytometry, and viability appeared preserved, as assessed by MTT assay, following 24 hours MG1 treatment.

### **3.2.4 Effect of MG1 on total DNA and replication-competent HIV on CD4+CD25-HLADR- cells from HIV-infected individuals**

Real time PCR for total HIV DNA from cells and total HIV RNA from supernatants were performed for 9 HIV-infected individuals on HAART from whom sufficient quantities of cells were obtained. Relative changes in total DNA and total RNA expression for MG1-treated cells were compared to changes in total HIV DNA and total HIV RNA expression in cells untreated with MG1. Results were variable across patients for both total HIV DNA and total HIV RNA expression in response to MG1 treatment. Median total DNA and median total RNA expression for untreated cells were set to 1.0. Median relative fold expression, relative to untreated cells after MG1 treatment for 24 hours with an MOI of 0.001 was 1.15 and for an MOI of 0.01 was 1.15. Median relative fold expression of total HIV RNA for an MOI of 0.001 was 0.595 and for an MOI of 0.01 was 0.933. Due to the non-Gaussian distribution, one-way ANOVAs using Kruskal-Wallis tests were performed. There were no statistically significant differences in the expression of total HIV DNA ( $p=0.158$ ) or total HIV RNA ( $p=0.891$ ) for MG1-treated versus MG1-untreated cells (FIGURE 34-35). p24 antigen levels in supernatants were below the level of ELISA detection for all 20 patients.

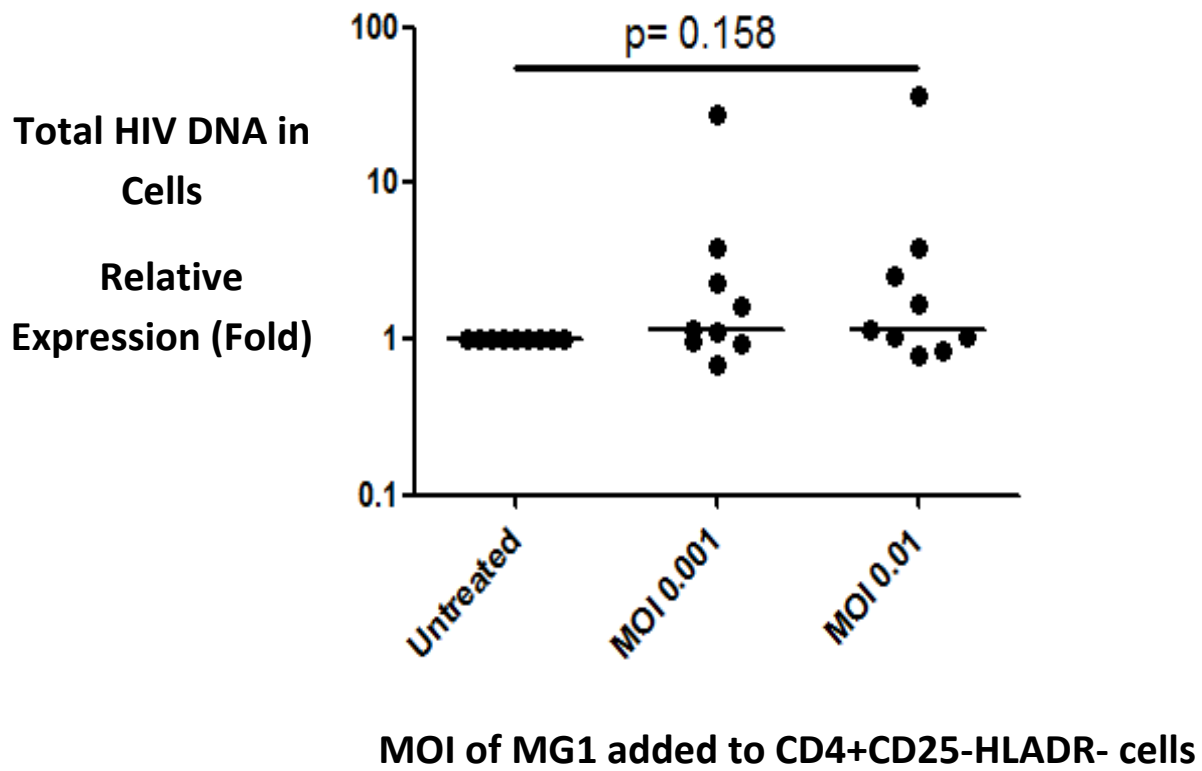


Figure 34

**Figure 34: PCR for total HIV DNA from CD4+CD25-HLADR- cells from HIV-infected individuals on HAART 24 hours after MG1 infection.** Real-time PCR for total HIV DNA from cells was performed for 9 HIV-infected individuals on suppressive HAART. Relative changes in total DNA expression for MG1-treated cells were compared to changes in total HIV DNA expression for cells untreated with MG1 for 24 hours. There were no statistically significant differences in the expression of total HIV DNA for MG1-treated versus MG1-untreated cells.



**Figure 35: PCR for total RNA from supernatants of CD4+CD25-HLADR- cells from HIV-infected individuals on HAART 24 hours after MG1 infection and following a 2-week stimulation and co-culture period.** Cells from 9 HIV-infected individuals were either left untreated or treated for 24 hours with MG1 at MOIs of 0.001 or 0.01. After a 2 week cell co-culture period, supernatants were collected and RT-PCR was performed to quantitate relative changes in total RNA expression from supernatants of cells which had been treated with MG1 and for those which had not been treated with MG1. No statistically significant differences were found in total HIV RNA expression in supernatants between cells which had been treated with MG1 and supernatants from cells which had not been treated with MG1.

## **4. DISCUSSION**

### **4.1 Cell lines**

#### **4.1.1 Greater infectivity and killing of U1 cells compared to U937 cells**

The results of the current study demonstrated that all cell lines were easily infected by MG1 and that there were high levels of cell death after 24 hours of MG1 treatment. Given that MG1 has a propensity to infect cells with aberrant IFN signaling, as frequently observed in cancer cells, this suggests that the cell lines examined in this study likely possess IFN signaling abnormalities to some degree.

IFN plays an important role in regulating the cell cycle through its effects on cycle E protein. After Cycle E forms a complex with cyclin-dependent kinase (CDK), the complex promotes the transition of the cell from the G1 to the S phase of growth. Upregulation of cycle E can hasten the phases of the cell cycle, resulting in uncontrolled cell growth (Zhang et al., 2007). In the absence of IFN- $\alpha$ , U937 cells strongly express Cycle E mRNA but this is down-regulated when cultured in media containing IFN- $\alpha$  (Zhang et al., 2007). Thus, through downregulation of cycle E expression, INF- $\alpha$  may suppress proliferation and facilitate apoptosis of U937 cells (Fernie et al., 1991). HL-60 cells have also been shown to have IFN- $\gamma$  and IFN- $\beta$ -related defects. As discussed by Zhou et al. (2008), the fact that IFN- $\gamma$  can induce apoptosis in HL-60 cells supports the notion that type I IFN signaling pathways may be altered in these cells (Zhou et al., 2008). There is also some evidence to

suggest that IFN signaling pathways may be altered in A3.01 and Jurkat cells (Bednarik et al., 1989; Scheller et al., 2002; Yang et al., 2009).

There is less literature describing IFN signaling defects in HIV-infected cell lines compared to the parent cell lines. Munier et al. (2008) demonstrated that IRF-8, a transcription factor, binds to an IFN-sensitive response element (ISRE) and regulates gene expression by genes stimulated by IFNs. Decreased expression of IRF-8 following reactivation of latency suggests that IRF-8 may contribute in the maintenance of the latent state in U1 cells (Munier et al., 2008). Furthermore, HIV replication in U1 cells is known to affect PKR which, as previously mentioned, is involved in the IFN signaling cascade (Muto et al., 1999). Based on the results of the current study, one may postulate that U1 cells could potentially harbour IFN signalling deficiencies of a greater magnitude than U937 cells. However, it is not possible to say with certainty that IFN signaling abnormalities are the only defects rendering these cells especially susceptible to MG1 infection and killing. Indeed, it is very plausible that additional mechanisms could also be implicated.

TNF- $\alpha$  prestimulation induced HIV replication, as was expected. HIV replication involves production of factors, such as Tat and Vpu, which are known to interfere with IFN production (Cai et al., 2000; Clerzius et al., 2011; Doehle et al., 2009). Therefore, one may have expected to see greater infectivity and killing by MG1 within HIV-infected cell lines which were pre-stimulated with TNF- $\alpha$  than HIV-infected cell lines which were not pre-stimulated with TNF- $\alpha$ . Although this was observed, the differences in infectivity and killing between these conditions were fairly small. It is possible that inducing HIV replication with TNF- $\alpha$  prestimulation for 24 hours was too short a time period to appreciate the ability of HIV to exert any significant influence on IFN responsiveness or

signaling. Hence, this may have obscured the ability to determine whether MG1 may have a greater propensity to infect and kill cells prestimulated with TNF- $\alpha$ . Similarly, the reason for the absence of any significant difference in MG1 infectivity and/or MG1-induced cell death at some higher MOIs is unclear at this time. It is possible that the system was “saturated” with virus at these higher MOIs, obscuring any clear differences inherent between the two cell lines.

There did not appear to be any clear differences between ACH2, OM-10 and J1.1 cell lines and their respective parent cell lines with respect to MG1 infectivity after 18 or 24 hours of MG1 treatment. One could hypothesize that these HIV-infected cells and their respective parent cell lines possess similar degrees of IFN signaling anomalies. Again, prestimulation of cells for 24 hours with TNF- $\alpha$  did not consistently result in any significant differences in the likelihood of these cells becoming infected by MG1. Once again, this may be due to the fact that 24 hours of HIV replication was too short a time period to induce any noticeable differences in IFN signaling and responsiveness.

Although ACH-2 and OM-10 cells were killed to a similar extent as their respective parent cell lines, killing of Jurkats exceeded that of the HIV-infected J1.1 cells. It is possible that the kinetics of infectivity and killing between these cell lines differed. For example, perhaps the overall rates of infectivity and killing were greater in J1.1 cells than in Jurkat cells. Percentage infectivity was examined on live cells only and was measured at particular time points. However, viability data was obtained by examining the entire cell population and was cumulative over time. These differences may explain why infectivity and viability data were not necessarily proportional. Examining infectivity on the total cell

population at more times points may have also revealed differences between cell lines more clearly. Similarly, examining viability over a longer period of time may have demonstrated larger differences between cell lines. Use of dyes, such as Annexin V and Propidium Iodide, to assess cellular viability by flow cytometry may have perhaps provided more robust viability data and may have enabled for the elucidation of the mechanisms involved in MG1-induced cell death. Although mechanisms of cell death by MG1 were not elucidated in the current work, it is likely death is mediated, in large part, by apoptosis as is frequently observed in other cell lines in response to VSV or MG1 treatment (Brun et al., 2010).

An interesting phenomenon which has been described is the ability of 2 viruses to induce “cooperative oncolytic activity,” such as the synergistic tumor cell killing seen with vaccinia virus and VSV. As discussed by Le Boeuf et al. (2012), infection of the cell by vaccinia virus inhibits IFN secretion necessary for VSV oncolysis, thus enhancing the efficacy of both viruses. Similarly, Human Papilloma Virus (HPV) can halt IFN production in some tumor cells by binding to Tyk2, inhibiting its interaction with the Type I IFN receptor (IFNAR) and therefore blocking the downstream reactions which typically ensue (Li et al., 1999). Le Boeuf et al. studied the sensitivity of four cervical carcinomas (HPV+) and four head and neck squamous cell carcinomas (HNSCC) (HPV-) to VSV treatment. They found that the cervical carcinoma cell lines, which harboured HPV, were more susceptible to VSV infection and lysis than the HNSCC cell lines. They also showed that HPV-E6 expression attenuated the IFN response induced by VSV (Le Boeuf et al., 2012). Therefore, it is also possible that HIV infection may enhance MG1-mediated oncolysis of cells.

#### **4.1.2 Advantages and disadvantages of cell line models of HIV latency**

Cell line models of latency have both advantages and disadvantages. Advantages include the fact that they are relatively constant over time and yield reproducible experimental results. Furthermore, each cell is known to harbour 1-2 copies of integrated proviral DNA unlike primary cells whereby only 1 in a million cells contain integrated proviral DNA. Cell lines also grow rapidly and thus large quantities of cells can be obtained, making them useful for screening a series of compounds for their ability to reactivate HIV or to eliminate HIV-infected cells.

Despite their advantages, however, cell lines models of latency also have many disadvantages. Notably, these are cancer cells, which harbour abnormal signaling pathways and are continuously proliferating. Thus, these cells are inherently different from resting CD4+ T cells of patients on HAART. Cell lines may also harbour HIV which is kept in a transcriptionally silent or latent state by methods which may not accurately reflect *in vivo* conditions (Yang et al., 2012). Furthermore, over the years it is possible that the cell lines may have mutated. Therefore, it is not possible to say, with absolute certainty, that the cell lines used in the present study are necessarily identical to the ones which were characterized decades ago.

Of relevance to the current work examining the effects of latent HIV infection on OV infectivity and killing is the fact that cell lines are cancer cells which, as previously discussed, are known to harbour aberrant signaling pathways and a multitude of other abnormalities. Thus, it is likely that the very high levels of infectivity and cell death which

were observed related, at least in part, to the fact that these are cancer cells. The fact that no infectivity or killing of PBMCs or CD4<sup>+</sup> cells from healthy donors were observed highlights this difference.

Although not used in the current work, other cell line models of HIV latency exist. For example, Jurkat-derived J-lat cells have GFP encoded in their genome and this is expressed upon HIV activation, facilitating the detection of latency reversal (Weinberger et al., 2005). However, the J-lat cell line was not used in the current study as the GFP encoded within the genomes of these cells may have interfered with the detection and accurate quantitation of GFP expression due to MG1 infection. Cells of another line, also derived from Jurkat cells, have a genome which consists solely of LTRs, Tat-coding sequences and reporter genes. It was previously demonstrated that fluctuations in Tat levels were able to activate HIV within these cells, due to the positive feedback loop (Weinberger et al., 2005).

Despite their limitations, cell line models of latency still have a role in the study of OV<sub>s</sub> as a potential approach to eliminate the HIV reservoir given that an eventual goal of this project is to engineer constructs of MG1 which are selective for HIV-infected cells while sparing non-HIV-infected cells. The study of various MG1 constructs will require a cell system which yields reproducible results in order to detect the effect of systematically altering various components of MG1.

#### **4.1.3 Increasing p24 antigen levels with increasing MOIs of MG1: Increased HIV replication versus increased cell lysis?**

Results of the current study suggest that MG1 may induce HIV replication as increasing levels of p24 antigen were measured from supernatants of cells treated with increasing MOIs of MG1. One may hypothesize that MG1, similar to other viruses, induces HIV replication by stimulation of the HIV LTR or via an effect on the HIV Tat protein. It is also possible that there was not an actual increase in p24 antigen production but that the increasing p24 antigen levels which were measured with increasing MOIs of MG1 were actually reflective of increased cell lysis. p24 antigen does not exist independently within cells but is part of the pr55gag precursor protein, of which p24 antigen is a component. The precursor protein is later cleaved, releasing free p24 antigen outside of the cell. The ELISA used in these studies can detect, qualitatively, p24 antigen which is a component of the pr55gag polyprotein. However, quantitation of the p24 antigen component of the pr55gag polyprotein within the cell is not accurate. One method to determine whether increased MOIs of MG1 result in increased p24 antigen levels due to cell lysis would be to select a protein which is known to exist exclusively within the cell. If this protein is then detected in supernatants, and if the quantity of this protein increases with increasing MOIs, this would suggest that increasing p24 antigen levels measured were in fact due to general cell lysis and release of pr55gag polyprotein rather than *de novo* HIV production. Furthermore, even if MG1 is found to induce HIV replication, this does not necessarily diminish the possible role of OV's as a component of an eradication strategy for HIV as it may indicate that MG1 could serve as a latency-reversing agent. It is also possible that these results would not be

observed with other OVs. Hence, more OVs should be examined for their ability to selectively target, replicate within, and kill HIV-infected cells.

Another possible explanation for the findings of increased p24 antigen levels with increasing MOIs of MG1 is a possible cross-reactivity between the capsid of MG1 with the HIV p24 capsid as detected by the ELISA. The only other virus known to cross-react in this ELISA is SIV, and this is to a very minor degree (The AIDS and Cancer Research Program). Furthermore, as previously outlined, p24 antigen levels in supernatants from HIV-uninfected cell lines after 24 hours of MG1 infection were quantified in order to exclude the possibility of cross-reactivity between the HIV p24 capsid and the MG1 capsid protein. There was no detectable p24 antigen in these samples, eliminating the possibility of cross-reactivity between the p24 antigen capsid protein from HIV and the MG1 capsid protein. Overall, the significance of the p24 antigen studies are not entirely clear at this time as these findings were observed in cell lines which, as previously discussed, do not accurately represent *in vivo* conditions.

## **4.2 Primary Cells**

### **4.2.1 Primary cell results and challenges of studying the HIV reservoir using primary cells**

Treatment of patient cells with MG1 at an MOI of 0.01 or 0.001 for 24 hours did not result in any apparent infection or killing of patient cells. Furthermore, total DNA or total RNA levels were not altered. These MOIs were selected given that they resulted in high levels of

infectivity and cell death in cell lines. It is possible that patient cells did not become infected by MG1 as only 1 in a million cells from individuals on effective HAART are thought to be HIV-infected (Chun et al., 1997). Therefore, the vast majority of the cells obtained were likely HIV-uninfected and may be expected to behave in a manner somewhat similar to cells isolated from HIV-uninfected individuals. Of note, it was decided to test for total DNA rather than integrated proviral DNA, despite the fact that the latter is more representative of the HIV reservoir, due to the low yield of CD4+CD25-HLADR- cells. It must be kept in mind that there were multiple experimental conditions which required the division of these cells into separate, smaller portions. Furthermore, although the decision was made to examine total HIV DNA within cells and total HIV RNA within supernatants, no significant changes were observed in total HIV DNA or total HIV RNA despite treatment of cells with two MOIs of MG1. Therefore, future studies should examine the effects of MG1 at higher MOIs.

Examining the frequency of resting CD4+ T cells from actual patients on HAART which contain latent but replication-competent virus is the “gold standard” method to assess the HIV reservoir in peripheral blood (Lewin et al., 2012). However, in the context of studying MG1 infectivity and killing, latently-infected cells do not have any distinct surface markers or features which allow for their clear identification from cells which do not contain HIV. Furthermore, CD4+CD25-HLADR- cells are present in low numbers, especially in patients who have low total CD4+ counts. The fact that the cells of interest are available in such low numbers makes it difficult to perform experiments where quantitation is accurate. Although leukapheresis can greatly augment the numbers of cells isolated from patients, this poses an inconvenience for patients and is not accessible in all research centers.

Furthermore, the cells must be stimulated and co-cultured for 2-3 weeks with cells from HIV-uninfected donors in order to reactivate latent provirus (Chun et al., 2007), a process which has been described in the literature as “labour-intensive” (Lewin et al., 2012). Similarly, as with any type of *in vitro* work on primary cells, it is unclear how removing these cells from the body may change their phenotypic or functional characteristics.

Another challenge with studying resting CD4<sup>+</sup> T cells from patients on HAART is that less than 1% of the total integrated DNA is replication-competent (Chun et al., 1997). Due to epigenetic changes or silencing, HIV DNA may be positioned within regions of chromosomes where its expression is defective (Eisele et al., 2012). Moreover, as discussed by Marsden et al. (2011), HIV within the cellular genome is that of the original HIV strain with which individuals were initially infected. This eliminates the possibility of genetically altering the virus to investigate the role of specific regions of the viral genome, such as transcription factor binding sites, in latency activation (Marsden et al., 2011). Thus, the low numbers of cells obtained from patients and the variability amongst patients, coupled with the lengthy co-culture process, renders these cells unfeasible for the screening large quantities of compounds for therapeutic potential (Sundberg, 2000).

#### **4.3 Determining the optimal method to quantify the HIV reservoir**

As reviewed by Lewin et al. (2012), during replication the majority of DNA within cells is full-length, linear and unintegrated (Chun et al., 1997). A small portion of total HIV DNA is integrated and an even smaller amount is replication-competent (Chun et al., 1997). As

most of the unintegrated HIV DNA will degrade (Burinsky et al., 1991; Pierson et al., 2002; Zack et al., 1992; Zhou et al., 2005), integrated HIV DNA is thought to be a better surrogate of replication-competent virus than is total HIV DNA (Agosto et al., 2011; Graf et al., 2011). Integrated proviral DNA may be quantified using Alu-LTR PCR, a nested PCR (Sonza et al., 1996). This involves a second amplification cycle using both primers within the HIV LTR (O'Doherty et al., 2002). However, this method also involves multiple replicates and controls, increasing the number of steps required (O'Doherty et al., 2002; Lewin et al., 2008). As noted by Lewin et al. (2012), measuring total HIV DNA may also overestimate the size of the reservoir as much of this provirus does not have the ability to contribute to viremia (Lewin et al., 2012). Others have argued that measures of total and integrated DNA may be interchangeable in HIV-infected patients on HAART given that unintegrated DNA has a short half-life and most of the HIV DNA will be integrated when replication is inhibited by HAART (Koelsch et al., 2008; Chomont et al., 2009; Brenchley et al., 2004).

With regards to HIV RNA, an ultrasensitive PCR assay has been developed which can measure viremia to levels as low as 1 copy/ml (Palmer et al., 2008). However, a large volume of plasma (7 ml) is required. As with HIV DNA, it is also unclear whether this low-level RNA represents replication-competent virus (Brennan et al., 2009; Sedaghat et al., 2007).

#### 4.4 Primary cell models of HIV latency

In order to engineer a recombinant MG1 selective for HIV-infected cells, large numbers of cells which behave in an identical manner will be required. As cell lines are artificial models of HIV latency, a possible solution would be to implement a primary cell model of HIV latency (Yang et al., 2011; Bosques et al., 2009). Although no models of HIV latency entirely simulate *in vivo* conditions, a primary cell model of latency would provide the best compromise between cell volume, reproducibility and *in vivo* conditions. Furthermore, almost all cells in these models contain HIV provirus, thus facilitating the study of the effects of MG1 on HIV-infected primary cells.

Primary resting CD4<sup>+</sup> T cells are characterized by certain features, such as small cell size, G<sub>0</sub> cell cycle status, low levels of activation markers and absence of nuclear forms of certain transcription factors (Yang et al., 2011). Establishing a primary cell model of HIV latency involves taking these cells from healthy HIV-uninfected donors, stimulating them with various cytokines and activating agents to render them susceptible to HIV infection, and infecting them with a laboratory-based strain of HIV. The cells then naturally return to a state of quiescence. An example of a primary cell model is that developed by Bosque et al. (2009) whereby T cells from healthy, HIV-uninfected donors are first stimulated for 7 days *in vitro*. They are then infected with an HIV strain which is *env*-deficient and is, hence, replication incompetent, ensuring there is no production of new HIV virions. The surviving cells become memory cells harbouring latent provirus. Such cell models have been useful in delineating the mechanisms by which HIV is kept latent in primary cells. For example, Bosques et al. (2009) demonstrated that lymphocyte-specific protein tyrosine

kinase (Lck) and NFAT, but not NF- $\kappa$ B, were important for the reactivation of latent HIV in primary cells (Bosques et al., 2009). As reviewed by Yang et al., (2011), several primary cell models have been described, and these vary in terms of agents used to reverse latency and the cell culture conditions. These primary cell models also differ with respect to the yield of cells obtained and their degree of quiescence (Yang et al., 2011).

Despite their advantages, the establishment of primary cell systems for the study of HIV latency poses many challenges. Firstly, it is difficult to create *in vivo* conditions *in vitro* (MacLeod et al., 2010; Murrack et al., 2004). Ideally, physiologic conditions should be reproduced whereby cells can be kept viable but HIV latency is not reversed. T cells in culture often need to be stimulated by growth-promoting cytokines such as IL-2 or IL-7 to remain viable. However, these cytokines may also reactivate latent HIV to a certain extent (Chun TW et al., 1998; Brooks DG et al., 2003; Wang FX et al., 2005). As all of the primary cell models to date have certain drawbacks (Yang et al., 2011), further work is still required in order to optimize many of the primary cell models of latency.

#### **4.5 Other cellular reservoirs of HIV**

The current work only examined cells derived from peripheral blood. However, given the wide diversity of cell types which comprise the HIV reservoir, examination of the mechanisms by which different cells support the persistence of HIV is merited. Of relevance to the study of OVs as a potential mechanism to eliminate the HIV reservoir, it will be necessary to determine the effect of OVs on primary cells other than lymphocytes. For example, in contrast to CD4<sup>+</sup> T cells, macrophages may survive for 6 months

following HIV infection due to their resistance to the cytopathic effects of HIV (Gendelman et al., 1998; Orenstein et al., 1988). Macrophages and microglial cells are also the principal infected cell types in the CNS (Gartner et al., 1986), which can be the anatomical reservoir with limited access to antiretroviral drugs. CD8<sup>+</sup> T cells can also become infected by HIV (Semenzato et al., 1995) and may indeed harbour significant amounts of HIV provirus (Imlach et al., 2001). Some subsets of Natural Killer (NK) cells have also been shown to carry high burdens of HIV proviral DNA (Valentin et al., 2002). Similarly, several subsets of monocytes exist in peripheral circulation (Ziegler-Heitbrock et al., 1993) and are important given that their migration and trafficking are thought to distribute HIV to various regions in the body, including the central nervous system (Persidsky et al., 1999). Dendritic cells have also been recognized as important in the storage and dissemination of HIV throughout the body. HIV can be stored in endosomes without degradation for several days and spread to T cells (Geijtenbeek et al., 2000; Kwon et al., 2002). Large numbers of cells accumulate within lymphoid tissue in the first few weeks of infection (Lore et al., 2002) and are thought to contribute to the dramatic rise in infected CD4<sup>+</sup> cells found in these tissues (Zhang et al., 1999). Similarly, B cells in lymphoid tissue and peripheral blood carry HIV attached to their surface which can infect CD4<sup>+</sup> T cells via cell-to-cell contact. In fact, cell-to-cell spread of HIV infection has been shown to be more efficient at spreading HIV than through the infection of cells by free virions (Jakubik et al., 2000). Cell-to-cell transmission also permits HIV to escape recognition and clearance by the immune system and the effects of antiretrovirals (Sigal et al., 2012). Hence, the mechanisms by which cells interact in order to sustain the HIV

reservoir must also be clearly elucidated in order to develop therapies to inhibit these processes.

#### **4.6 Different anatomical compartments and tissue reservoirs of HIV**

Peripheral blood cells have typically been studied due to the ease with which they can be obtained, both in clinical practice and in the research setting. However, it has become increasingly evident that immunological events in the peripheral blood frequently do not mirror those in other anatomical compartments. It is well-recognized that the largest HIV reservoir within the body is found in gut associated lymphoid tissue (GALT), where levels of HIV DNA of patients on HAART are found to be 10-fold higher than in peripheral blood (Yukl et al., 2010; North et al., 2010; Chun et al., 2008; Yukl et al., 2010). This likely relates, in part, to the fact that the immunological pressures within these compartments differ from those within the peripheral blood. For example, the phenotypic and functional characteristics of macrophages within the subepithelial lamina propria are distinct from monocytes within peripheral blood (Smith et al., 1997; Smith et al., 2001). HIV quasispecies isolated from gastrointestinal tissues have also been found to possess genetic and molecular differences, such as in the *env*, *pro* and reverse transcriptase genes, supporting the notion of the gastrointestinal tract as a distinct anatomical compartment (van der Hoek et al., 1998). Furthermore, the majority of studies which have examined the ability of HAART to restore gut immunity have demonstrated partial, and not complete, immune reconstitution (reviewed by Costiniuk et al., 2012). Peripheral lymphoid tissue is another major HIV reservoir and findings from studies on peripheral lymphoid tissue

underscore the importance of cell-to-cell transmission in maintaining the HIV reservoir (Cheynier et al., 1994). The CNS is another important HIV reservoir with an immunological environment that is distinct from that in peripheral blood, due in part to the blood-brain-barrier and blood-cerebrospinal fluid barrier in the choroid plexus (Saksena et al., 2003). Similarly, the male genital tract possesses a blood-testis barrier. Lack of correlation between plasma and semen VLs have been reported in some studies (Gupta et al., 1997; Liuzzi et al., 1996; Saksena et al., 2003). Furthermore, although there is evidence that the lungs and kidneys may also serve as reservoirs of HIV, their overall importance in maintaining the reservoir is unclear (Saksena et al., 2003). Therefore, different anatomical compartments and tissues represent different immunological environments and it is likely that factors that allow the establishment and maintenance of latency may differ in blood and tissues. Understanding the optimal methods to process various body tissues and fluids in order to quantify and characterize the HIV reservoir accurately within various compartments will be necessary in the future in order to study the therapeutic potential of OVs.

#### **4.7 Animal model of HIV latency**

Currently, the humanized BLT (bone marrow, liver, thymus) mouse is the only animal model of HIV latency (Denton et al., 2011). These are immunodeficient mice which undergo a preconditioning regimen, are implanted with human thymus and liver tissue and are then transplanted with CD34+ hematopoietic progenitor cells from human fetal liver (Denton et al., 2011). Advantages of the BLT mouse model is that they these mice are

susceptible to HIV acquisition by the same routes used in humans, namely vaginally, rectally and intravenously (Brainard et al., 2009). The course of HIV infection in BLT mice closely mimics that observed in humans and currently prescribed antiretrovirals are efficacious at reducing their VLs (Denton et al., 2008; Denton et al., 2010; Denton et al., 2011). Of particular relevance to the study of HIV reservoirs, these mice contain CD4+ T cells which are latently-infected with HIV and these cells can be stimulated to produce HIV. The frequency of CD4+ T cells which contain HIV is similar to that in humans on HAART (Denton et al. 2011; Marsden et al., 2012). However, this latter feature may also pose a challenge to the study of reservoirs, as was discussed with regards to the challenges of studying primary cells from patients on suppressive HAART.

Murine models are advantageous among animal models given their affordability and relative ease of maintenance compared with other animals (Johnston et al., 2012). However, given that mice are not naturally susceptible to infection with HIV, they require major manipulation. Thus, a major concern is whether findings from murine studies are generalizable to humans (Johnston et al., 2012). Several primate models have also been used to study HIV and the advantage of using primates is that they are natural hosts of SIV. However, again results generated from primate studies raise the issue of whether findings from these studies are generalizable to humans (Johnston et al., 2012).

#### **4.8 Sterilizing versus functional cure?**

A sterilizing cure requires eradication of all latently HIV-infected cells from the body of an infected person (Johnson 2012). In contrast, a functional cure implies that there may still be

detectable HIV present within the body, but it is present in such low amounts that the host immune system is able to suppress it in the absence of antiretrovirals. Furthermore, as it is present in such low quantities, it does not lead to any negative sequelae for the host and it is non-transmissible to others.

The “Berlin patient” is the only example of any individual achieving a sterilizing cure. This gentleman had acute myeloid leukemia and underwent a bone marrow transplant with a donor who had a 32-based pair deletion in the CCR5 gene (Hutter et al., 2009). Despite discontinuing HAART after his transplant and remaining off HAART for 4 years, there is no detectable HIV in his body despite extensive investigations of various tissue sources (Hutter et al 2009; Allers et al. 2010). However, as suggested by Lewin et al., it is possible that chemotherapy, total body irradiation and low-grade graft-versus-host disease are factors which may have contributed to his sterilizing cure (Lewin et al., 2012).

As discussed by Lewin et al., (2012), elite controllers (ECs) are often provided as examples of HIV-infected individuals who represent “functional cures” and have, in fact, inspired vaccine design (Lewin et al., 2012). These individuals are able to control HIV blood plasma to levels below 50 copies/ml in the absence of HAART. They have also been found to harbour smaller reservoirs of HIV DNA, as measured by HIV DNA in various CD4+ subsets in peripheral blood (Deeks et al., 2007; Descours et al., 2009) as well as in rectal tissue (Avettand-Fenoel et al., 2008; Dalmaso et al., 2008). The ability of ECs to maintain immunologic and virologic controls relates to certain HLA class I genes (Fellway et al., 2007) and robust HIV-specific CD4+ and CD8+ T cell responses in blood and mucosal lining (Hersperger et al., 2011; Ferre et al., 2010; Ferre et al., 2009). However, despite their immunological ability to control HIV replication, their immune profiles still

differ from HIV-uninfected individuals as they have greater levels of immune activation (Hunt et al., 2008).

#### **4.9 The road ahead for the study of oncolytic viruses as a potential approach to eliminate the HIV reservoir**

The work presented here was an initial attempt to study the effect of a recombinant Maraba virus, MG1, on HIV-infected cell lines and primary cells from HIV-infected individuals on HAART. Outcomes examined included infectivity, killing and effect on HIV burden. These initial studies highlighted some challenges involved in the study of HIV reservoirs which are consistent with the challenges reported in the literature.

Although cell lines were highly infected by MG1, the relative lack of infection of primary cells may reflect the fact that primary cells do not harbour aberrant signaling pathways or the fact that higher MOIs of MG1 should be used in the future. Due to significantly greater infectivity and killing of HIV-infected U1 cells compared to U937 cells, it is likely that these cell lines should be used when examining the effect of engineering an MG1 to be selective for HIV-infected cells while sparing HIV-uninfected cells. However, due to the malignant nature of U937 cells, it is unlikely that it will be possible to achieve complete sparing of U937 cells. Furthermore, whether results obtained from U1 and U937 cells can be generalizable to individuals on HAART is unlikely. However, as demonstrated by the current work, the use of primary cells from HIV patient on HAART is challenged by low yields of cells. A primary cell model of HIV latency will need to be implemented for future studies of MG1 as a potential agent to eliminate HIV-infected cells while sparing HIV-

uninfected cells. Such a primary cell model will provide the best compromise between *in vivo* conditions and the ability to obtain large numbers of cells necessary for these studies.

Although monocytic and lymphocytic cell lines were studied in the present work, future studies should examine the effect of MG1 on other cell types, such as monocyte-derived macrophages, dendritic cells as well as gut-associated lymphoid tissue. The latter may be obtainable through routine colonoscopy screening. Although it would also be meritorious to examine the effect of MG1 on HIV DNA burden in peripheral lymphoid tissue, this latter tissue is not readily obtainable, precluding its study for the time being.

Other OV's also need to be explored for their ability to eradicate HIV. As previously mentioned, some OV's, such as measles and vaccinia viruses, are transported in monocytes and lymphocytes (Liu et al., 2012). Hence, it is logical that such OV's may be able to eliminate these cells. Indeed, it is possible that multiple OV's which target different cell populations and have a tropism for different anatomical compartments may be needed to eradicate HIV from all cells and body sites. For example, given the neurotropism of rhabdoviruses, it is possible that this feature may make them ideal candidates to target CNS reservoirs of HIV infection.

As with other eradication strategies, combination therapy will likely be required and the use of HDAC inhibitors appears promising due to preliminary successes from studies examining reversal of HIV latency with compounds such as SAHA (Archin et al., 2012). Furthermore, viral sensitizing agents, which have shown promise in cancer research (Diallo et al, 2010), are another group of compounds which merit exploration in the context of HIV eradication. Finally, as with virtually all strategies aimed at eliminating the HIV reservoir,

OVs will need to be used in conjunction with HAART in order to prevent viral replication and infection of uninfected cells.

Another key area of research for future studies on OVs as potential agents to eliminate the HIV reservoir will be to determine whether primary cells which contain HIV proviral DNA harbour any specific signaling defects. Of relevance to MG1 and VSV would be to explore whether primary cells which contain integrated proviral DNA harbour any defects in IFN signaling or responsiveness. As previously discussed, cells with actively replicating HIV possess multiple defects related to IFN signaling and responsiveness. It has previously been shown that plasma from individuals with advanced HIV supports VSV replication to high titres. As the presence of IFN in the plasma of these individuals was found to be only partially responsible for the inhibition of VSV replication, Piasecki et al. suggest that there could be a specific HIV-VSV interaction (Piasecki et al., 2010). Therefore, given the significant similarities between VSV and MG1, it is possible that there could also be a specific MG1-HIV interaction. Future studies should aim to determine whether primary cells with latent HIV infection possess any abnormalities shared with cancer cells which may render the cells susceptible to oncolysis.

In the event that future studies demonstrate that MG1 is not effective at reducing or eliminating the HIV reservoir independently, MG1 may still serve a role as an agent which can reverse HIV latency. After the reversal of latency, other therapies with the potential to actually eliminate these cells, and therefore reduce the HIV burden, could be administered. Furthermore, OVs may even have a role in the management of HIV-infected individuals even if they are not used therapeutically. For example, it has been shown that a VSV replication assay was useful for monitoring the effect of antiretroviral therapy and Piasecki

et al. (2010) suggest that a VSV replication assay could be used as an alternative to the evaluation of viral load, CD4 count and stage of HIV infection (Piasecki et al., 2010)

The ultimate goal is to construct a recombinant OV dependent on the presence of HIV for its selective replication. Strategies used to design OVs to be selective for cancer cells can be extrapolated to engineer OVs selective for HIV-infected cells, but this process will be facilitated by clarifying which signaling pathways are abnormal in cells with latent HIV infection. For example, knockout of the E1B protein in ONYX-015 renders it incapable of replication within cells with functional p53 pathways (Bischoff et al., 1996). Therefore, if it were established that cells with latent HIV-infected also harbour p53 defects, ONYX-015 may then potentially be effective for the elimination of cells harbouring HIV provirus. Similarly, the oncolytic poxvirus JX-594 has been shown to possess several mechanisms for cancer selectivity, with replication being activated in cells with abnormalities in epidermal growth factor receptor (EGFR)/Ras pathways, altered thymidine kinase levels and resistance to IFN type 1 (Parato et al., 2012). Like ONYX-015, this later virus has also been given to humans in clinical studies, including a recent randomised phase II dose-finding clinical trial (Heo et al., 2013), and thus it may be worthwhile exploring its potential use as a therapeutic agent for cells with latent HIV infection given its favourable safety profile (Heo et al., 2013).

Use of HIV-specific promoters may be another approach to increase selectivity for HIV-infected cells. Incorporation of a prostate-specific antigen (PSA) promoter in the adenovirus genome resulted in its ability to reduce prostate specific antigen levels (Small et al., 2006). In the context of HIV infection, a strategy which has already been attempted included the incorporation of HIV ligands into an OV (Schnell et al., 1997). Schnell et al.

engineered a strain of VSV by replacing the G protein with expression cassettes for CD4 and CXCR4. After infecting T cells with both HIV and the engineered VSV, they noted that infectious HIV levels dropped almost 1000-fold (Schnell et al., 1997).

In addition to increased selectivity, methods to increase the efficacy of OV's have been attempted in the cancer field and thus these strategies may also be extrapolated to the development of an OV to treat HIV-infected cells. Incorporating therapeutic genes with pro-apoptotic effects is a method which has been used to enhance the efficacy of OV's in cancer (Los et al., 2009). Furthermore, understanding the microenvironment of tumor cells has been exploited in cancer therapeutics (Breitbach et al., 2013), and thus it is possible that a better understanding of various microenvironments within anatomical reservoirs may enable the exploitation of these features to create more potent OV's directed against cells with latent HIV-infection. OV's can also be engineered to evade immune recognition and response through NKG2D-ligand surface expression, as was demonstrated with VSV (Jensen et al., 2011) and through the use of carrier cells (Iguchi et al., 2012) and microbubbles (Greco et al., 2006). Finally, safety and tolerability will be especially important in getting a novel therapy for HIV approved, given that the majority of patients on effective HAART are generally doing quite well from an overall health perspective (Johnston et al., 2012).

## 5.0 CONCLUSION

MG1 generally infects and kills latently HIV-infected U1 cells to a greater degree than the HIV-uninfected parent U937 cells and may be a promising model to facilitate further studies of MG1 as a potential therapy for the eradication of latently HIV-infected cells. ACH2, OM-10 and J1.1 cell lines, and their respective parent cell lines, do not appear to be promising models in which to study the effects of MG1 as there were no significant differences consistently observed in infectivity or killing between ACH2 and A301 cell lines nor between OM-10 and HL-60 cell lines. Meanwhile, although Jurkat and J1.1 cells became infected with MG1 to a similar degree, killing of Jurkats exceeded that of the HIV-infected J1.1 cells. Preliminary results from primary cells suggest that MG1 alone does not appear to eliminate cells which comprise the major HIV reservoir. However, such low numbers of CD4<sup>+</sup>CD25<sup>-</sup>HLADR<sup>-</sup> cells were obtained, rendering it challenging to truly elucidate the effect of MG1 on these cellular HIV reservoirs.

Priorities for future studies examining the ability of OV<sub>s</sub> as a potential strategy to eliminate the HIV reservoir should include the implementation of a primary cell model of HIV latency. This will enable the generation of large numbers of primary cells, almost all of which will harbour HIV proviral DNA. Higher MOIs of MG1 should be examined, in addition to the use of OV<sub>s</sub> other than MG1. Similarly, potential roles for adjunctive agents, such as HDAC inhibitors and viral sensitizers, merit exploration. It is also important to determine whether cells from HIV-infected individuals on HAART harbour any defects in IFN signaling or responsiveness, or if they share any important features with cancer cells. Addressing these issues will help guide the construction of a recombinant OV selective for HIV-infected cells while sparing HIV-uninfected cells. Although the work presented here is

very preliminary, OVs represent a novel approach to eliminate the HIV reservoir. Given the tremendous success in cancer research, many lessons from the oncology field can be transferred to the study of HIV reservoirs and eradication. Multidisciplinary approaches will be required in order to optimize the development an effective OV-based therapy that is safe, well-tolerated and effective at eliminating the HIV reservoir.

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**Cecilia Costiniuk, BSc Pharm, MD, FRCPC**  
Curriculum Vitae  
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***Education/Training:***

- Sept 2011-Feb 2013 Master's in Microbiology and Immunology (Clinician Investigator Program), University of Ottawa
- July 2009-June 2011 Infectious Diseases Fellowship, University of Ottawa (Chief Resident July 2010-January 2011)  
-Successfully completed the Royal College of Canada Infectious Diseases Exam
- July 2006-June 2009 Internal Medicine Postgraduate Medical Education Program, University of Ottawa  
-Successfully completed the Royal College of Canada Internal Medicine Exam
- Sept 2003-May 2006 Doctor of Medicine, McMaster University
- Sept 2000-April 2003 Bachelor of Science in Pharmacy, Memorial University of Newfoundland  
-Successfully completed the Canadian Pharmacy Licensing Exam
- Sept 1996-April 1999 Bachelor of Arts, Major in Psychology, University of Western Ontario

***Scholarships and Awards:***

- 2012 Ontario HIV Treatment Network (OTHN) Abstract/Travel Scholarship
- 2012 The Juan A. Embil Award for Excellence in Infectious Diseases Research: Outstanding contribution to Infectious Diseases Research by a trainee (national award)
- 2012 CIHR-Canadian HIV Trials Network (CTN) Fellowship
- 2012 University of Ottawa Research Fellowship Award
- 2011 Association of Medical Microbiology and Infectious Disease of Canada (AMMI)/Astellas
- 2011 Canadian Association of HIV Research (CAHR)/Viiv Healthcare Master's Scholarship in HIV Research (declined)
- 2011 University of Ottawa Research Fellowship Award
- 2003 Glaxo-Harlow Pharmacy Clinical Clerkship Travel Award: Harlow, England
- 2002 Apotex Pharmacy Literary Challenge National Finalist

- 2002 Apotex Pharmacy Patient Counselling Competition Award
- 2002 Alcohol and Drug Dependency Commission Commemorative Pharmacy Academic Award
- 2001 Pharmaceutical Supplies Ltd Pharmacy Academic Award
- 2000 Altimed Pharmacy School Entrance Award: Outstanding Score on the PCAT (Pharmacy College Admission Test)
- 1999 University of Western Ontario Governor General's Medal: Runner-up
- 1999 University of Western Ontario Grad Pact Award
- 1998 University of Western Ontario Faculty of Social Sciences Scholarship
- 1998 University of Western Ontario In-course Scholarship
- 1998 University of Western Ontario Honour W Award

### *Manuscripts Published and In Press*

- 1) **Costiniuk C.** Pills, Chills and Dr Seuss: The Pharmacist's Role in Helping Patients Recover From Drug Dependency. *Canadian Pharmaceutical Journal (Supplement): Apotex Student Literary Challenge National Finalists 2003*; 12:18.
- 2) **Costiniuk C.** The Prescription Conundrum: Factors That Impact Prescribing Practices. *McMaster University Medical Journal* 2004; 2: 38-40.
- 3) Gano D and **Costiniuk C.** Message from the Editors. *McMaster University Medical Journal* 2005; 3: 4.
- 4) Boggild AK, **Costiniuk C**, Kain KC and Pandey P. Environmental Hazards in Nepal: Altitude Illness, Environmental Exposures, Injuries, and Bites in Travelers and Expatriates. *Journal of Travel Medicine* 2007; 14(6): 361-368.
- 5) **Costiniuk C**, Mills E and Cooper C. Evaluation of Oral Cannabinoid-containing Medications for the Management of Interferon and Ribavirin-Induced Anorexia, Nausea and Weight Loss in Patients Treated for Chronic Hepatitis C Virus. *Canadian Journal of Gastroenterology* 2008; 22(4): 376-80.
- 6) **Costiniuk C**, Camacho F and Cooper C. Erythropoietin Use for Interferon-Ribavirin-Induced Anemia in Chronic Hepatitis C is Not Associated with Increased Rates of Cardiovascular Disease, Thrombosis, Malignancy or Death. *Clinical Infectious Diseases* 2008; 47(2): 198-202.
- 7) **Costiniuk CT**, Sharapov AA, Rose GW, Veinot JP, Desjardins MA, Brandys TM and Suh KN. *Mycobacterium bovis* Abdominal Aortic and Femoral Artery Aneurysms Following Intravesical Bacillus Camille-Guerin Therapy for Bladder Cancer. *Cardiovascular Pathology* 2010; 19(2):e29-32.
- 8) Cooper C, **Costiniuk C** and Giguere P. HIV-HCV Co-infection therapeutic outcomes have not improved over time. *HIV Clinical Trials* 2009; 10(3): 203-6.
- 9) Hibbert B, **Costiniuk C**, Hibbert R, Joseph J, Alanazi H, Simard T, Dennie C, Angel J and O'Brien ER. Cardiovascular Complications of *Salmonella enteritidis* Infection. *Canadian Journal of Cardiology* October 2010; 26(8): 323-5.
- 10) **Costiniuk C**, Le Saux N, Doja A, Jacob P, Karnachow T and Hui C. Miller-Fisher Syndrome in a Toddler with pH1N1 Infection. *Journal of Child Neurology* 2011; 26(3): 385-388.

- 11) **Costiniuk CT**, McCarthy AE, Talreja H, Zimmerma D, Liu TT, Owen E and Angel JB. Acute Renal Failure and Disseminated Intravascular Coagulation Associated with Rifampin in the Treatment of TB. *International Journal of TB and Lung Disease* 2011; 15(1):1.
- 12) Akl EA, Oxman AD, Herrin J, Vist G, **Costiniuk C**, Blank D and Schunemann HJ. Using Alternative Statistical Formats for Presenting Risks and Risk Reductions. *The Cochrane Database of Systematic Reviews* 2011; 16(3): CD006776.
- 13) **Costiniuk C**, de Souza C and Voduc N. Pulmonary Actinomycosis in a Male with a Tracheal Bronchus. *Canadian Respiratory Journal* 2011; 18(2): 84-86.
- 14) **Costiniuk C**, Wang B, Johnstone D, Toye B, Veinot JP, and Desjardins M. *Coxiella burnetii* Culture-negative Endocarditis Diagnosed by 16S rRNA Sequencing of Heart Valve Tissue. *Pathology* 2011; 43(5): 513-515.
- 15) **Costiniuk C** and Angel J. ALVAC-HIV as a Prophylactic and Therapeutic Vaccine: Highlights From Over a Decade of Clinical Trials. *Future Virology* 2011; 6(12): 1481-1492.
- 16) Akl EA, Oxman AD, Herrin J, Vist G, **Costiniuk C**, Blank D and Schunemann HJ. Framing of Health Information Messages. *The Cochrane Database of Systematic Reviews* 2011 (12): CD006777.
- 17) **Costiniuk C**, Fergusson DA, Doucette S and Angel JB. Discontinuation of Prophylaxis for *Pneumocystis jirovecii* Pneumonia with CD4+ T cell Count <200 cells/uL when HIV Viral Load is Suppressed on Antiretroviral Therapy: A Systematic Review. *PLoS ONE* 2011; 6(12): e28570.
- 18) **Costiniuk C**, Cooper L, Doucette S and Kovacs. Parasitic Disease Screening Among HIV Patients from Endemic Countries in a Toronto Clinic. *Canadian Journal of Infectious Diseases and Medical Microbiology*; 2012.
- 19) **Costiniuk C**, Hibbert B, Filion L, Kovacs C, Benko E, O'Brien E and Angel J. Endothelial Progenitor Cell Levels are Not Reduced in HIV-infected Men. *The Journal of Infectious Diseases* 2012; 205(5): 713-717.
- 20) **Costiniuk C**, Hibbert B, Filion L, O'Brien E and Angel J. Response to Lopez et al. Circulating Endothelial Progenitor Cells and HIV. *The Journal of Infectious Diseases* 2012; 206(9): 1480-1481.
- 21) **Costiniuk C**. Book Review: Models of Protection Against HIV/SIV: Avoiding AIDS in Humans and Monkeys. Gianfranco Pancino, Guido Silvestri and Keith Fowke (Ed). Elsevier Academic Press. *International Journal of Infectious Diseases* 2012; 16(8): e643.
- 22) **Costiniuk C**, LaPorte C and Angel B. Surreptitious Antiretroviral Use as a Cause of Apparent Elite Control. *AIDS Patient Care and STDs* 2012; 26(11): 645-646.
- 23) **Costiniuk C**, Kovacs K, Routy JP, Singer J, Gurunathan S, Sekaly RP and Angel J. Human Immunodeficiency Virus Rebound in Blood and Seminal Plasma Following Discontinuation of Antiretroviral Therapy. *AIDS Research and Human Retroviruses* 2012; Sept 25 Epub ahead of print.
- 24) **Costiniuk C** and Angel J. Human Immunodeficiency Virus and the Gastrointestinal Immune System: Can Highly Active Antiretroviral Therapy Restore Normal Gut Immunity? *Mucosal Immunology* 2012; 5(6):596-604.

- 25) **Costiniuk C**, Hibbert B, Simard T, Ghazawi F, Angel B, O'Brien E. Circulating Endothelial Progenitor Cells in HIV Infection: A Systematic Review. *Trends in Cardiovascular Medicine* 2013; Feb 5 Epub ahead of print,
- 26) **Costiniuk C** and MacPherson P. Neurocognitive and Psychiatric Changes as the Initial Presentations of Neurosyphilis: 3 Illustrative Case Reports. *Canadian Medical Association Journal* 2013; Feb 25 Epub ahead of print.

### *Abstracts Published*

Costiniuk C, Côté S, Carrasco-Medina L, Angel J. Oncolytic Viruses as a Potential Approach to Eliminate the HIV Reservoir. *Canadian Association of HIV Research (CAHR) Annual Conference Program, April 2012.*

### *Manuscripts Submitted*

- Chun TW, Justement JS, Murray D, Kim CJ, Blazkova J, Hallahan CW, Benko B, **Costiniuk CT**, Kandel G, Ostrowski M, Kaul R, Moir S, Casazza JP, Koup RA, Kovacs C, Fauci AS. Effect of Antiretroviral Therapy on HIV Reservoirs in Elite Controllers: Implications for Eradication of Virus.

### *Presentations*

- 2013: Oncolytic viruses as a potential approach to eliminate the HIV reservoir (Oral). *Association of Medical Microbiology and Infectious Diseases of Canada (AMMI) Annual Conference. Quebec City, April 2013.*
- 2013: Oncolytic viruses as a potential approach to eliminate the HIV reservoir (Oral). *CIHR Canadian HIV Trials Network (CTN) Postdoctoral Fellow's Breakfast. Vancouver, April 2013.*
- 2013: Oncolytic viruses as a potential approach to eliminate the HIV reservoir (Poster). *Canadian Association of HIV Research (CAHR) Annual Conference. Vancouver, April 2013.*
- 2012: Oncolytic viruses as a potential approach to eliminate the HIV reservoir (Poster). *Ottawa Hospital Research Institute Research Day, Ottawa, November 2012.*
- 2012: Oncolytic viruses as a potential approach to eliminate the HIV reservoir (Oral). *Ontario HIV Treatment Network (OHTN) Conference, Toronto, November 2012.*
- 2012: Oncolytic viruses as a potential approach to eliminate the HIV reservoir (Poster). *Biochemistry, Microbiology and Immunology Poster Day. University of Ottawa, Ottawa, May 2012.*
- 2012: Rhinocerebral Mucormycosis Suppressed with Posaconazole in a Bone Marrow Transplant Recipient (2<sup>nd</sup> author; Poster). *Canadian Bone Marrow Transplant Group, Toronto, April 2012.*

- 2011: Posaconazole: A Role for Antifungal Therapeutic Drug Monitoring (Oral). *Haematology/Infectious Diseases Academic Half-Day, University of Ottawa, November, 2011*
- 2011: Circulating Endothelial Progenitor Cells in HIV-infected Men as a Potential Marker of Cardiovascular Risk (Oral). *Department of Medicine Annual Resident Research Day, University of Ottawa, May 2011.*
- 2011: Discontinuation of Prophylaxis for *Pneumocystis jirovecii* Pneumonia with CD4+ T cell Count <200 cells/uL when HIV Viral Load is Suppressed on Antiretroviral Therapy: A Systematic Review. (Poster). *Canadian Association of HIV Research (CAHR) Annual Conference, Toronto, Ontario, April 2011*
- 2010: Parasitic Disease Screening Among HIV Patients From Endemic Countries in a Toronto Clinic. (Poster Presentation). *Canadian Association of HIV Research (CAHR) Annual Conference, Saskatoon, Saskatchewan, May 2010 and Department of Medicine Annual Resident Research Day, University of Ottawa, May 2008.*
- 2008: Erythropoietin Use for Interferon-Ribavirin-induced Anemia in Chronic Hepatitis C is Not Associated with Increased Rates of Cardiovascular Disease, Thrombosis, Malignancy or Death (Poster Presentation). *Department of Medicine Annual Resident Research Day, University of Ottawa, May 2008.*
- 2008: Evaluation of Oral Cannabinoid-containing Medications for the Management of Interferon and Ribavirin-induced Anorexia, Nausea and Weight Loss in Patients Treated for Chronic Hepatitis C (Poster Presentation). *Canadian Digestive Disease Week (CDDW) and Annual Canadian Association for the Study of the Liver (CASL) Winter Meeting, Montreal, Quebec, March 2008.*
- 2008: Bacilli Can Go Anywhere. (Oral presentation). *Association of Medical Microbiology and Infectious Disease (AMMI) of Canada Conference Clinical Vignettes Presentations, March 2008.*
- 2007: Evaluation of Oral Cannabinoid-containing Medications for the Management of Interferon and Ribavirin-induced Anorexia, Nausea and Weight Loss in Patients Treated for Chronic Hepatitis C (Poster Presentation). *Department of Medicine Annual Resident Research Day University of Ottawa, May 2007.*

#### ***Extracurricular, Volunteer and Teaching Experience:***

- 2010-2012: University of Ottawa Medical School: Review lecture on HIV
- 2010-2011: University of Ottawa Family Medicine Academic Day Antimicrobial Workshop facilitator
- 2010-2011: University of Ottawa Medical School: Review lecture on HIV

- 2008-2009: University of Ottawa Internal Medicine Journal Club: Co-organizer
- 2007-2008: University of Ottawa Internal Medicine Curriculum and Evaluations Committee
- 2007-2008: University of Ottawa Internal Medicine Admissions Committee for International Applicants
- 2007-2008: University of Ottawa Professional Skills Development Tutor for Medical Students
- 2004-2006: McMaster International Women's and Children's Health Committee
- 2004-2005: McMaster University Medical Journal (MUMJ): Executive Editor
- 2004-2005: McMaster Internal Medicine Interest Group: Lecture Coordinator
- 2003-2006: McMaster Medical School Curriculum Committee
- 2003-2004: McMaster University Medical Journal (MUMJ): Ethics Section Editor
- 2003-2004: McMaster Medical French Interest Group
- 2003-2004: McMaster Medical School Admissions Committee
- 2001-2003: Memorial University Pharmacy Society: Educational Coordinator
- 2001-2002: St Catharines Hotel Dieu Hospital Oncology IV Manual Committee
- 2000-2001: Memorial University Pharmacy School Academic Council
- 1999-2000: Romania Tutova Orphanage Volunteer
- 1999-2000: Teacher of English as a Second Language (TESL) Certification

### ***Reviewer for Journals:***

- PLoS ONE
- International Journal of Infectious Diseases
- Journal of Infectious Diseases Reports

### ***Contributions to Team Grants:***

- Canadian Foundation for AIDS Research (CANFAR) (Oncolytic viruses as a potential approach to eliminate the HIV reservoir)
  - \$25,000 awarded to Principal Investigator for 2012-2013
- Department of Medicine Research Priority Award (Oncolytic viruses as a potential approach to eliminate the HIV reservoir)
  - \$115,000 awarded to Principal Investigator for 2012-2014
- Canadian Institute of Health Research (CIHR) (Oncolytic viruses as a potential approach to eliminate the HIV reservoir)
- American Association for AIDS Research (AMFAR) (Oncolytic viruses as a potential approach to eliminate the HIV reservoir)
- Bill and Melinda Gates Foundation (Oncolytic viruses as a potential approach to eliminate the HIV reservoir)

- Department of Medicine Research Priority Award (Endothelial Progenitor Cells in HIV Infection)
  - \$50,000 awarded to Principal Investigator for 2009-2011

### ***Professional Development:***

- Canadian HIV Trials Network (CTN) Spring Meeting 2013; Vancouver, BC.
- Canadian Association of HIV Research (CAHR) Annual Conference 2013; Vancouver, BC.
- Association of Medical Microbiology and Infectious Disease (AMMI) of Canada Annual Conference 2013; Quebec City, QC.
- Ontario HIV Treatment Network (OHTN) Scientific Meeting 2012; Toronto, ON.
- Canadian HIV Trials Network (CTN) Fall Meeting 2012; Toronto, ON.
- Clinician Investigator Trainee Association of Canada Annual Meeting 2012; Ottawa, ON
- Canadian HIV Trials Network (CTN) Spring Meeting 2012; Montreal QC.
- George Washington University Infectious Diseases Board Review Course 2011; Washington DC, USA.
- Canadian Association of HIV Research (CAHR) Annual Conference 2011; Toronto, ON.
- New Investigator Workshop (CAHR): 2011; Toronto, ON.
- HIV and Cardiovascular Disease Workshop 2010; Ottawa, ON.
- HIV Reservoirs Workshop: Towards a Cure 2010; Vienna, Austria.
- International AIDS Society (IAS) Conference 2010; Vienna, Austria
- New HIV Investigator Workshop (CAHR) 2010; Saskatoon, Saskatchewan.
- Canadian Association of HIV Research (CAHR) Annual Conference 2010; Saskatoon, SK.
- John Hopkins' Infection Control Review Course 2010; Baltimore, Maryland.
- Ottawa Hospital Research Training Course 2009; Ottawa, ON.
- McGill University Course on Tropical and Parasitic Diseases 2008; Montreal, QC.
- Canadian Digestive Disease Week (CDDW) and Annual Canadian Association for the Study of the Liver (CASL) Winter Meeting 2008; Montreal, QC.
- Association of Medical Microbiology and Infectious Disease (AMMI) of Canada Annual Conference 2008; Vancouver, BC.
- Albert Einstein University: HIV Management: The New York Course 2007; New York, USA.
- International Women's and Children's Health Annual Conferences 2003-2006, Hamilton, ON.

## **CONTRIBUTIONS OF COLLABORATORS**

MG1 was provided by Dr David Stojdl and Dr John Bell.

Feras Al-Ghazawi assisted with data analysis of the PCR results.

Dr. Paul MacPherson and Sandra Côté contributed ideas to the study design.