Characterization of Interferon Regulatory Factor-7 in Defined Subsets of Human Peripheral Blood Mononuclear Cells and Analysis of the Effect of Knockdown on HIV-1 Infection

By

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A thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

In partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

Department of Medical Microbiology and Infectious Diseases

University of Manitoba

Winnipeg, Manitoba

Abstract

Introduction: Interferon regulatory factor-7 (IRF-7), the "master regulator" of type 1 interferon, has shown to orchestrate anti-viral immune responses via fine-tuning expression of interferons and interferon-stimulated genes. IRF-7 expression is upregulated during HIV-1 infection; however, the role of IRF-7 in HIV-1 infection remains controversial, perhaps due to different cell-types used in the studies. In this thesis, IRF-7 expression levels were examined in defined peripheral blood mononuclear cell (PBMC) subsets from both Manitoban and Kenyan donors, and the hypothesis that reducing cellular IRF-7 will render *ex-vivo* CD4+ T cells more susceptible to HIV-1 infection was assessed.

Methods: IRF-7 levels were examined using multi-parametric flow-cytometry in HIV-uninfected Manitoban donors and in HIV-infected and HIV-uninfected volunteers from a well-characterized Kenyan sex worker cohort. IRF-7 expression level was reduced by IRF-7 specific siRNA or shRNA encoded in lentivirus and administered into *ex-vivo* CD4+ T cells by transfection or transduction, respectively. The effects of IRF-7 knockdown on transactivating HIV-1 replication was assessed using p24 ELISA, flow cytometry, absolute qPCR, and Milliplex multiplex assays.

Results: In unstimulated PBMC, IRF-7 was constitutively expressed at low levels in every defined subset of PBMC we examined (CD4, CD8, B cells, NK cells, monocytes, and dendritic cells (DCs)), with the highest expression found in monocytes and DCs compared to lymphocytes (Manitoban donors n=18, 3.3-fold increase, $p \leq 0.0001$) (Kenyan donors n=27, 4.2-fold increase, $p \leq 0.0001$). Endogenous IRF-7 expression level could be up regulated in all defined PBMC subtypes following *ex-vivo* treatment with interferon-αA, and in HIV-1 infected cells (~2 fold, $p \leq 0.05$). There was no significant correlation between the extent of IRF-7 knockdown and the amount of inhibition of HIV transactivation, reflected in p24 production. However, contrary to our hypothesis, we observed less HIV-infected cells (~10%) with IRF-7 knockdown, suggesting that IRF-7 may play a role in HIV infection.

Conclusions: This is the first study that characterized the expression of IRF-7 in defined subsets of

human PBMC from two human populations. Although IRF-7 is constitutively expressed in all cell subsets examined, significantly higher IRF-7 expression in monocytes and DCs suggests these cell types may be critical in early IRF-7 mediated interferon responses. Unexpectedly, it was found that even though IRF-7 had been implicated in orchestrating antiviral events, reducing IRF-7 expression in ex vivo CD4+ T cells, a major target, did not increase the cellular susceptibility to productive HIV infection.

Acknowledgements

I would like to take this opportunity to thank everyone who contributed to my graduate studies.

I would like to thank my supervisors, Dr. Ruey Su and Dr. Blake Ball for giving me the opportunity to be a part of the laboratory and for their guidance and support throughout the years. I would like to thank Ruey for always pushing me to do my best and for helping me find new ways to approach my project. As well I would like to thank Ruey for her help on editing and correcting my thesis. I would like to thank Blake for his continued support and advice throughout my years as a Master's student, as well as for correcting and editing my thesis. I have learnt so many new techniques in this laboratory that will help me be a better scientist.

I would like to thank my advisory committee, Drs. Xi Yang, Abdel Soussi Gounni, and Emmanuel Ho, for providing me with guidance, comments, and criticism. I would like to thank them for supporting my work throughout the years.

I would like to thank my fellow laboratory members and friends: Bernard Abrenica, Andrew Plesniarski, Hillary McCoubrey, Michelle Perner, Jen Juno, Christine Mesa, Jill Waruk, Sandy Kiazyk, Catherine Card, Paul Lopez, Arvelene Camama, and Daniel Underwobele. Thank you for your support and assistance. A special thanks as well to Bernard, Andrew, Hillary, and Michelle for their comments while reviewing my thesis.

I would like to thank the Medical Microbiology staff for their help in navigating the graduate program.

Finally, thank you to my friends and family. Thank you to my parents for supporting me throughout my undergraduate and graduate programs. I would not have able to finish this degree without their continued encouragement and support.

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Introduction:

Since the beginning of the Human Immunodeficiency Virus (HIV) pandemic, almost 78 million individuals have been infected and over 34 million people have died (*1*). By the end of 2015, there was approximately 37 million people living with HIV/acquired immune deficiency syndrome (AIDS) and 2.6 million of these were children (*1, 2*). There was also an estimated 2.1 million people newly infected with HIV in 2015, which averages out to close to 5,500 every day (*1*). As of June 2016, only 17 million people were receiving medicines to treat HIV with the worst affected region being Sub-Saharan African (*1, 3*). The availability of antiretroviral therapy (ART) reduced mortality and morbidity associated with HIV/AIDS significantly. The success of ART is best demonstrated by marked reduction in mother to child transmission (*4*). ARTs however, are expensive and not available to everyone infected with HIV. While ART controls viral load and reduces the risk of transmission, it does not cure HIV-infection and is a life-long treatment. Due to the inability to cure HIV, a preventative vaccine may be the best option in controlling the HIV/AIDS pandemic. To create an effective vaccine, first we must understand the relationship of HIV with the human immune response. This project specifically examined the role of interferon regulatory factor 7 (IRF-7) and its effect on susceptibility to HIV-1 infection and the effects of HIV-1 infection on IRF-7 regulation. The IRF family members are transcriptional regulators, involved in many different biological processes, such as regulating the immune response, cytokine signalling, and apoptosis (*5*). Both IRF-1 and IRF-7 have been shown to regulate anti-viral immune responses via regulating the expression of type 1 and 2 interferons and interferon-stimulated-genes (*5*). IRF-1, a master regulator of anti-viral responses, and IRF-7, a critical regulator of type 1 interferon against pathogenic infection, have both been implicated in HIV-infection (*6-10*). IRF-1 expression is up regulated during HIV-1 infection and IRF-1 binding to the HIV-1 long terminal repeat (LTR) is essential for early trans-activation of the HIV-1 genome (*11*). IRF-7, the “master regulator” of type 1 interferon, can be upregulated during HIV-1 infection and has been implicated in anti-HIV-1 interferon responses. Nevertheless, the role of IRF-

7 in HIV-1 infection remains undefined, perhaps due to different cell-types used in all of the studies.

Classification of HIV-1:

HIV is a Lentivirus that belongs to the family Retroviridae and is responsible for causing AIDS by destroying CD4+ T cells. There are two major HIV subtypes: HIV-1, and HIV-2. HIV-1 is the most virulent and infectious, and has a worldwide distribution (*12*). HIV-1 has an extreme genetic heterogeneity due to several factors, such as the lack of the proof reading ability of the reverse transcriptase (RT), host immune pressure, and recombination events (*13*). HIV-1 is classified into three major phylogenetic groups which include group M, N, and O (*13*). Group M is responsible for the major epidemic worldwide and can be further subdivided into at least 9 clades (*14*). HIV-1 phylogenetic classifications are currently based on either nucleotide sequences from *gag*, *pol*, and *env* of the same isolates, or from full-length genome sequence analysis (*13*). The most prevalent HIV-1 genetic forms are subtypes A, B, and C. Subtype A viruses are generally found in Central and Eastern Africa and in Eastern Europe. Subtype B is found in Central Europe, the Americans, Australia, Southeast Asia, Northern Africa, and the Middle East. While subtype C is predominant in Africa and India. It is believed that HIV-1 originated from the consumption of a Simian Immunodeficiency Virus (SIV) infected chimpanzee in West Africa (*12*). There are four known independent zoonotic transmissions of HIV-1 that occurred from primates (*15*).

HIV-1 Life Cycle:

There are seven stages of the HIV life cycle including binding, fusion, reverse transcription, integration, replication, assembly, and budding. In the first step of the HIV life cycle, **binding**, the virus targets and binds to the CD4 receptor, and to either a CCR5 or CXCR4 co-receptor using its viral envelope proteins (gp120 and gp41) (*16*). HIV can also actively infect macrophages, DC, and Langerhans cells using receptors such as mannose binding protein and DC-Specific Intercellular adhesion molecule-3-Grabbing

Non-integrin (DC-SIGN) (*17-20*). In the second step, **fusion**, the virus attaches to the host CD4+ T cell and the viral envelope fuses with the CD4+ T cell membrane. This allows the virus to enter the cell where it releases HIV RNA and HIV enzymes into the cytoplasm (*21*). In the third step, **reverse transcription**, the virus will use reverse transcriptase to convert RNA into double stranded DNA provirus, which will be translocated to the nucleus (*21*). The fourth step, virus will **integrate** its DNA into the DNA of the host cell by the use of integrase, whereby the host will **transcribe and translate** viral proteins using its own machinery as the fifth step (*16*). The new HIV RNA and proteins created will translocate to the cell surface and **assemble** into immature virions in the sixth step. In the seventh and final step, the new immature, non-infectious, particles will **bud** off from the host cell, followed by the cleavage of the viral Gag and GagPol polyprotein precursors by the viral enzyme protease to generate the mature Gag and Pol proteins and initiate a series of structural rearrangements that ultimately leads to virion maturation (*22*). This creates smaller HIV proteins which results in the development of the Gag core that forms mature, infectious virus (*16, 23*).

HIV Genome Organization and Structure:

The HIV-1 virion is approximately 120 nm in diameter, roughly spherical, and is composed of two copies of a single stranded positive sense RNA enclosed by a capsid (*24*). The HIV-1 genome is less than 10 kb and encodes for more than nine different gene products. It encodes for 3 major structural protein genes: *gag* (group-specific antigen), *pol* (DNA polymerase), and *env* (Envelope), which code for major structural proteins and essential enzymes. *Gag* generates the mature Gag protein matrix (MA or p17), capsid (CA or p24), nucleocapsid (NC or p7), and p6, which encompass proteins for the basic infrastructure of the virus such as the inner core of the viral particle (*25*). *Pol* encodes for reverse transcriptase (RT), which enables the virus to reproduce, integrase (IN), which is necessary to integrate the viral double stranded DNA into the host genome, RNAse H, and HIV protease, which are all encapsulated in the core of the

inner particle formed by the viral capsid protein p24 (*25*). *Env* encodes for glycoproteins of the outer membrane such as outer gp120 (which enables the virus to attach and fuse to cells of the host), and transmembrane gp41 that anchors the glycoprotein complex to the surface of the virion (*25*). Between the core and the envelope is the HIV matrix proteins which are composed of the viral protein p17 (*23*). HIV-1 also encodes for proteins with important regulatory elements (*tat* (Trans-Activator of Transcription) and *rev* (regulator of expression of virion proteins)) and accessary proteins (*vpr* (viral protein r)*, vif* (virion infectivity factor)*, nef* (negative replication factor)*,* and *vpu (*viral protein u)) (*26*). *Tat* is involved in the regulation of the reverse transcription of the viral genome, and *Rev* is important for the synthesis of major HIV-1 proteins (*27*). *Vpr* has an important role in replication of the virus, and also causes host cells to arrest their cell cycle in the G2 phase resulting in the activation of host DNA repair machinery, which may enable integration of the viral DNA (*28*). *Vif* is important for the infectivity of the HIV-1 virions in certain cell types and inhibits apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G (APOBEC3G) (*29*). *Nef* is involved in the replication cycle of the virus, and plays a role in cell apoptosis and increases virus infectivity (*30*). *Vpu* is involved in CD4 degradation, virus particle assembly, and the budding of virions from infected cells. *Vpu* also prevents tetherin which is involved in inhibiting viral release from the cell by “tethering” the viral particles to the cell surface (*31, 32*).

HIV Transmission and Prevention:

HIV transmission occurs through contact with infected bodily fluids by several routes including sexual transmission, vertical transmission (mother to child), and parenteral transmission (injection drug use and contaminated blood products) (*33*). Following mucosal transmission, there is undetectable viral load for up to 10 days which is known as the eclipse period (*34*). There are three stages in HIV infection: acute HIV infection, chronic HIV infection, and AIDS (*35*). Acute infection usually develops within 2 to 4 weeks after an individual is infected with HIV, where virus levels increase exponentially and usually includes flu-

like symptoms such as headache, rash, and fever due to the expression of inflammatory cytokines such as IL-15, IFN-α, and IFN-γ (*35, 36*). In the acute stage, HIV rapidly multiplies and spreads throughout the body destroying CD4+ T cells which includes destruction of mucosal CD4+ T cells. The risk of transmitting HIV is the greatest during the acute infection (*37*). The second stage is chronic HIV infection in which there are low levels of HIV multiplying in the body. People with chronic HIV infection may not have any symptoms but can still spread HIV to others. The final stage of HIV infection, AIDS, occurs when the virus has depleted the immune system of CD4+ T cells, and the body struggles to fight off opportunistic infections. An AIDS diagnosis is given when a person has a CD4+ T cell count of less than 200 cells/mm^3 of blood, and/or one or more opportunistic infections (*35*).

Treatment:

ART is a combination of antiretroviral drugs that individuals infected with HIV can take for life to control viral load (*38*). ART is recommended for everyone infected with HIV, and can lead to a better prognostic outcome (*39*). They also play a direct role in reducing the risk of HIV transmission (*39*). The drugs are grouped into six different drug classes which include: nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), fusion and entry inhibitors, pharmacokinetic enhancers, and integrase strand transfer inhibitors (INSTIs) (*39*). NRTIs and NNRTI's bind to and block HIV reverse transcriptase to prevent HIV from replicating. PIs block HIV protease, which prevents the cleavage of immature HIV and its conversion to a mature virus (*39*). Fusion inhibitors prevent HIV from merging with the host CD4+ T cell membrane, preventing HIV from entering the host cell (*39*). Pharmacokinetic enhancers are used to boost the effectiveness of another drug by interfering with the breakdown of the drug, allowing for it to remain in the body longer (*39*). INSTIs block integrase, which prevents the virus from integrating and replicating. ARTs have also been shown to limit the transmission of HIV-1 from mother to child, and can be effective as pre-exposure prophylaxis (PrEP) (*40, 41*). However, non-adherence can lead to drug resistance and treatment failure and there is still the

risk of possible drug interactions with other medications (*39*). With the high costs and adverse effects associated with a lifetime of ART, there has been an increase in research on finding a cure, or other HIV interventions.

HIV Cure:

Currently there is no cure for HIV-1, mostly due to the establishment of a reservoir of HIV-1 in long-lived pools of latently infected cells that harbor replication-competent virus within patients (*42*). The major form of viral latency is post-integration latency, where the infected activated CD4+ T cells return back to a resting state (*42*). ARTs are unable to eliminate this latent viral reservoir which means that the patient must remain on antiretrovirals for their lifetime (*42*). ARTs are also quite expensive, and can have toxic effects which demonstrate the importance of developing new treatment methods. There are two categories for developing a cure of HIV-1: a sterilizing cure, and a functional cure (*42*). A sterilizing cure would indicate that there is no trace of the virus in the body, such as in the case of the "Berlin patient". This individual had acute myelogenous leukemia, and received a transplantation of hematopoietic stem cells from a donor who was CCR5 delta32 (Δ32) homozygous following whole-body irradiation. CCR5 is used as a co-receptor by certain HIV-1 strains to enter CD4+ T cells and an individual who is homozygous for the delta 32 mutation would therefore be resistant to these strains (*43*). After discontinuation of ARTs, there is still no virus found years later in this patient, which suggests the possibility of a sterilizing cure for HIV (*42*). One of the main challenges for successful therapy is the latent virus reservoir, and much research has been focused on trying to activate viral replication in these cells (reviewed by Shang *et al.* (*44*). Early ART treatment immediately after infection has the potential to reduce the size of the latent reservoir, as demonstrated with the Mississippi baby (*45*). The Mississippi baby received cART at 30 hours of age, but stopped returning for appointments at 18 months. When the child was re-tested at 30 months there was surprisingly undetectable levels of proviral DNA in PBMCs, plasma viral RNA, and HIV-1 antibodies (*45*). However, sadly at the age of 4 years, plasma viral RNA and HIV-1 antibodies were

detected indicating viral rebound (*46*). A functional cure for HIV-1 can be defined as long-term control of virus replication in the absence of antiretroviral therapy (*42*). This is found in a group termed “elite controllers”, who are able to maintain their plasma viremia levels below detectable levels. These individuals have several traits which may explain how they maintain their undetectable viral loads, such as class 1 HLA-B*57 and HLA-B*27 alleles, and potent CD8+ T cell responses (*47*). Due to the difficulty in obtaining either a sterilizing or functional cure HIV, a preventative vaccine may be the best option in controlling the HIV/AIDS pandemic.

Vaccine:

There is currently no successful vaccine for HIV. A vaccine for HIV-1 would need to be affordable, safe, and effective, and would need to produce a strong humoral and cellular immune response against all of the various HIV-1 forms (*13*). There are many challenges for HIV vaccine researchers, including the fact that HIV primarily attacks CD4+ T cells, which are critical regulators of adaptive immune response. A vaccine would need to activate these cells, which could make them susceptible to HIV infection (*48*). A second challenge is that HIV continuously mutates in an infected person resulting in new quasispecies. An effective vaccine would ideally produce a cellular (T cell), and humoral (antibody) immune response to prevent establishment of infection and spread of the virus (*48*). The most promising vaccine trial to date was the RV144 trail in Thailand, which was well tolerated and had a modest 31% reduction in HIV infections in those who received the vaccine (*49*). The vaccine was a prime-boost regiment of two vaccines. First was ALVAC-HIV vaccine (the primer dose), which was a modified canarypox virus. The booster dose was an AIDSVAX B/E vaccine comprised of the glycoprotein 120 subunit. The vaccines were based on the B and E subtypes (*49*). It was found that individuals who produced high levels of a specific antibody against the V2 loop of gp120 were less likely to be infected (*50, 51*). It has been suggested that high antibody-dependent cell-mediated cytotoxicity responses seem to play a major role in protecting

against HIV acquisition (*52, 53*). The results from the trail show the importance in understanding HIV's relationship with host immunity.

Innate Immune Response Against HIV:

The innate immune response includes the first responders to HIV infection, and can restrict viral replication and activate the adaptive immune response. The innate response is less specific than the adaptive response. The activation of several innate immune pathways early after infection is critical for inducing a stronger adaptive immune response, and can also have an important role in HIV-1 disease outcome (*54*). It is still unclear whether early innate immune responses are beneficial or detrimental to the host, as DCs and NK cells are recruited, but so are CD4+ T cells and macrophages, all HIV target cells. Chronic innate immune activation plays a major role in HIV immunopathogenesis and progression to AIDS by leading to immune exhaustion and deregulation that can lead to impaired adaptive responses (*55*). The majority of HIV transmission occurs during sexual contact at the mucosal surfaces, when the virus comes into contact with the vaginal, penile, or rectal epithelial layer and mucosal secretions. Epithelial cells respond to viruses by Toll-Like Receptors (TLRs) which recognize pathogen-associated molecular patterns (PAMPs) that lead to the secretion of cytokines, and recruitment of immune cells (*55*). After crossing the submucosa, the virus comes into contact with target cells and must infect enough target cells in order to survive, the eclipse period (*34, 55*). The innate immune response to HIV-1 triggers TLR2, TLR4, TLR9, TLR7, and TLR8 which results in the activation of DCs and type 1 IFNs (*56*). This inhibits viral replication and activates the immune response. This early triggering of TLRs and DCs results in a cytokine storm which potentially contributes to the immunopathology seen in acute HIV infection (*56*). DCs have a wide distribution, and are in close proximity with the mucosal epithelia making them one of the first cells that HIV will encounter during sexual transmission (*18*). DCs can also trans-infect CD4+ T cells, and may aid in the initial infection by chemokine secretion. Continual DC stimulation may

contribute to T cell exhaustion and immune dysregulation (*18*). DCs also lead to the activation and recruitment of NK cells. NK cells are elevated in acute HIV infection, and recognize and destroy virally infected cells (*55, 56*). NK cells respond to IFN-α and IL-15 created in the cytokine storm during acute infection, and expand rapidly (*56*). HIV-1 uses Nef to evade both T cell and NK cell recognition by downregulating MHC class I expression in infected cells, such as HLA-A and –B molecules, while also reducing NK-activating ligands (*56*). Pattern recognition receptor signalling triggered by TLRs also leads to activation of downstream transcription factors such as IRF-3, IRF-7, and NF-κB, which results in the activation of the antiviral immune response, and activation of interferons and interferon stimulated genes (ISGs). These include restriction factors such as APOBEC3G, Tripartite motif-containing Motif 5α (TRIM5α), SAM domain and HD domain-containing protein 1 (SAMHD1), and tetherin, which have been shown to limit HIV replication and spread (*54*). APOBEC3G induces mutations in the viral genome, TRIM5α binds to retroviruses and targets them for degradation, SAMHD1 inhibits HIV replication in myeloid cells, and tetherin prevents the virus from being released by retaining virions on the cell surface (*55*). There are also a number of mucosal factors that demonstrate anti-HIV activity, such as α-defensins, SLPI, Trappin2, and serpin anti-proteases (*56*). The innate immune system responds and guides the adaptive immune system (cell-mediated and humoral).

Adaptive Immune Response against HIV:

The adaptive immune system provides a more specific immune response to HIV infection. The earliest CD8+ T cell responses to HIV-1 are strong, and usually directed at Env and Nef of the virus as viremia approaches its peak during acute infection (*57-59*). Rapid selection of mutations arises in the virus due to the CD8+ T cell response as the viremia declines to the viral set point (*58*). The T cell response declines rapidly when escape mutations are selected, or through exhaustion, which questions the effectiveness of the early T cell response (*57, 60*). Later the CD8+ T cell response will be to Gag and Pol,

but there will still be escape mutations leading to a decrease in MHC receptor binding, TCR recognition, and epitope processing resulting in insufficient CD8+ immune response (*61*). CD8+ T cells are also important to maintain the viral set point, but only make a small contribution to infected cell death during chronic infection (*57*). HIV-1 infects CD4+ T cells, therefore there is little CD4+ T cell response to infection. The declining viral load may also be in response to massive CD4+ T cell loss, as there is a decreasing pool of target cells for the virus to infect (*62*). The decrease in CD4+ T cells results in immunodeficiency, chronic infection, and subsequently T helper cell function is decreased due to repeated T cell activation. Eventually there is T cell exhaustion due to loss of function and response of the cells (*61*). Another part of the adaptive immune response is the humoral response, of which there are HIV-1 specific responses. B cells are activated to create HIV specific antibodies. However, partly due to inadequate CD4+ T cell response, there are multiple B cell defects leading to poor antibody response and few high affinity neutralizing antibodies created (*63*). In chronic infection there is also decreased specificity to antigens, leading to decreased numbers of memory and activated B cells, and accumulation of exhausted B cells (*63*). Immune activation and inflammation leads to an environment that is more conducive for HIV infection leading to the concept that reduced immune activation may be beneficial for the host.

Immune Quiescence :

HIV preferentially infects activated T cells, conversely infection of quiescent T cells is inefficient (*64*). This is likely due to the fact that there are a number of host factors required for efficient HIV replication that are only present in activated cells (*64*). Immune activation has also been shown to be a factor in immune dysregulation and rapid HIV disease progression (*65*). Due to immune activation being a risk factor for acquiring HIV, a model of immune quiescence has been theorized as being protective from infection. This is based on a phenotype of low baseline immune activation (*64*). Immune quiescence is defined as a

reduction of T cell activation markers (such as CD69+), low levels of pro-inflammatory cytokine and chemokine production in the female genital tract and peripheral blood, and reduced general overall transcription (*64*). Immune quiescence has been observed in a group of HIV Exposed Seronegative (HESN) female sex workers from our well characterized cohort of commercial sex workers (CSW) from the Pumwani district in Nairobi, Kenya that have been repeatedly exposed to HIV for over 7 years and have remained seronegative (*66*). These HESN women demonstrate an epidemiological resistance to HIV, and are considered to be relatively resistant to infection (*67*). It has been shown that HESN women have a higher level of regulatory T (Treg) cells, which leads to suppressed T cell activation in these women. This may protect them by limiting activation of CD4+ T cells (*68*). HESN women have also been shown to highly express several anti-proteases that have anti-inflammatory activity that may limit mucosal immune activation (*69*). In HIV-susceptible individuals, HIV exposure in the context of inflammation can lead to infiltration of the mucosal barrier by CD4+ T cells, and their subsequent infection. Inflammation can drive infection of activated target cells, resulting in dissemination of infection. In HESN individuals, the low levels of T cell activation, increased Tregs, and increased levels of anti-proteases can reduce the availability of activated CD4+ target cells. This limits infection to resting target cells which can possibly be cleared by mucosal HIV-specific T cells or innate mechanisms (*64*). IRF-1 has also been implicated in the immune quiescence phenotype, as it has been shown that there are specific polymorphisms found in IRF-1 (at nucleotide 619 and 6516 and the microsatellite region) that result in reduced IFN-γ stimulated IRF-1 protein expression, reduced likelihood of seroconversion, and resistance to HIV-1 infection but not altered disease progression (*64, 70, 71*). IRF-1 is a transcriptional activator and repressor involved in the regulation of interferons, and can activate transcription of the HIV genome during the early stages of HIV infection (*11, 72*). It has been shown that HESN women have a transient upregulation of IRF-1 expression in response to exogenous IFN-γ, as opposed to HIV-susceptible women who have a sustained IRF-1 response (*73*). These HESN women are not immunosuppressed, as they are able to mount a robust

immune response; however, their immune activation is quickly down regulated, potentially resulting in a return to more quiescent state (*73*). It also has been shown that knockdown of IRF-1 expression by using IRF-1 specific siRNA resulted in a significant decrease in the transactivation of the HIV-1 LTR measured by Gag RNA transcripts and p24 secretion, and that reduced IRF-1 has little impact on IRF-1 regulated immune responses (*74*). Therefore, it is theorized that reduced IRF-1 may be beneficial to the host in the context of HIV infection, as it limits immune activation and initial HIV-1 replication (*74*).

The IRF Family:

IRFs are a large family of transcription factors that play many different roles in important biological processes, such as regulation of the host immune response, cytokine signalling, cell growth, apoptosis, and carcinogenesis (*75-77*). IRFs play a key role in the regulation of interferons (IFNs), which play a vital role in the innate and adaptive antiviral response (*78*). All members of the IRF family share a conserved region in their first 115 amino acids, a series of tryptophan-rich repeats, which contains the N-terminal DNA-binding domain (*77-79*). This DNA-binding domain binds to the interferon-stimulated response element (ISRE), and similar regulatory elements in the promoter of interferons and ISGs (*75, 77, 78*). The C-terminal domain of IRFs is less conserved, and acts as a regulatory domain that is responsible for the different properties of each IRF. This variable domain also determines the functionality of IRFs into transcriptional activators (IRF-1, IRF-3, IRF-7, and IRF-9), repressors (IRF-2 and IRF-8), or both (IRF-1, IRF-2, IRF-4, IRF-5, and IRF-8) (*11, 75, 80*). There have been 10 IRFs discovered in vertebrates with IRF-1-9 found in humans and mice, and IRF-10 found only in avian species (*75, 79, 81*). It has also been suggested by phylogenetic analysis that IRF genes originated around the same time as animal multicellularity, pointing towards their importance in evolutionary history (*75, 79, 81*).

IRF-1/7 Structure:

The human IRF-1 gene is located on chromosome 5, where it encodes for ten exons of which 9 are translated (*82*). IRF-1 is 325 amino acids in length and has a molecular weight of 35 kDa (*83*). Splice variants that skip exon 2 and/or 3 have been identified, however these variants lack the DNA-binding domain and are therefore non-functional (*82*). Variants that skip some combination of exon 7, 8, or 9 have also been discovered, but had weakened transcriptional activity of IRF-1 and altered transcriptional functions (*84*). The human IRF-7 gene is located on chromosome 11, where it encodes for four different isoforms (-A, -B, -C, and -D (also known as -H) (*79*). Isoform A is the standard isoform encoding for a protein of 503 amino acids (*79*). Isoform B contains an internal deletion in the transactivation domain, and lacks 29 amino acids (*60*). IRF-7C encodes a 164 amino acid protein, and contains a premature stop codon (*85*). Isoform D (or H) has an alternative N terminus, which encodes for a protein of 514 amino acids, and has the highest homology to IRF-3 (*86*). The overexpression of IRF-7H has also been shown to activate the IFN-α promoter (*86*). Western blot analysis has shown the molecular weights of isoforms A, B, C, and D to 54, 51, 18, and 56 kDa, respectively (*87*).

IRF-1/7 Expression:

IRF-1 has been shown to be constitutively expressed at low levels in almost all cell types, and has a short half-life of about 30 minutes (*88*). IRF-1 is induced in response to various cytokines (IFN-γ and IL-2), viral infections, and certain hormones (*89, 90*). Constitutive IRF-7 is restricted to certain cell types, such as peripheral blood lymphocytes and DCs, but IRF-7 can be induced in other cell types by IFNs and viral infection (*91, 92*). It has been shown that IRF-7 is constitutively expressed in monocytes, B cells, and plasmacytoid DC (pDCs) in the spleen, thymus, and peripheral blood lymphocytes (*79*). IRF-7 has a half-life of 8 hours in Newcastle disease (NDV) infected cells, and about 1.2 hours in uninfected resting quiescent splenocyte and thymocyte cultures from mice (*93*). pDCs have been shown to be "professional" producers of type 1 IFNs, due to the high levels of IRF-7 and TLR7 in these cells (*79*).

IRF-1/7 Function:

IRF-1 has antiviral, immunomodulatory, apoptotic, and immune cell differentiation effects. Experiments in IRF-1 knockout mice have demonstrated that IRF-1 is a key regulator of macrophage function, NK cell response, Th1/Th2 differentiation, and DC differentiation (*94-97*). IRF-1 is involved in the regulation of many genes during inflammation, immune responses, and cell proliferation. IRF-1 interacts with both IRF-2 and IRF-8 as an antagonist. IRF-2 is an antagonist that represses IRF-1 function by competing for IRF-1 binding sites, and IRF-8 inhibits IRF-1 function by directly binding to it (*98, 99*). IRF-7 is considered the "master" regulator of type I IFN signalling due to its role in providing the second stronger wave of type 1 IFNs. Honda et al. showed that in mice deficient in the IRF-7 gene that IRF-7 is required for the initiation of IFN-α/β genes by the MyD88-dependent pathway and the MyD88-independent pathway (*10*). It has been reported that IRF-7 is expressed at low levels in most cell types (*78*). The low levels of IRF-7 expression suggest that IRF-3 is mainly responsible for the initial induction of IFN, where IRF-7 is responsible for the later up regulation (*78*). This suggests a positive feedback loop where IRF-7 induced IFN expression is involved in the second and larger IFN induction step, which allows for efficient upregulation of type I IFNs during viral infection (*78, 100, 101*). IRF-7 expression is tightly regulated to prevent excessive tissue damage, inflammation, and autoimmunity (*100*).

IRF-1/7 Role in Immune Response:

IRF-1 has been shown to be involved in T cell selection and maturation, promotion of a Th1 response over a Th2 response, and maturation of NK cells, neutrophils, and macrophages (*94*). IRF-1 expression is also essential for the IFN-γ induced expression of low molecular mass polypeptide-2 (LMP-2). LMP-2 is a catalytic subunit of the immunoproteasome that increases the efficiency of endogenous antigen processing (*76, 102*). IRF-1 has an effect on T cell differentiation as IRF-1 knockout mice have

demonstrated that there is a profound reduction in CD8+ T cell numbers and reduced cytotoxic T cell responses. IRF-1 also promotes a Th1 response over a Th2 response (a preferential cell-mediated response versus humoral) (*97*). IRF-7 is primarily activated via two pathways: The endosomal TLR7/9 and TLR3/4 pathway, and the "intrinsic" or RIG-1 related pathway. IRF-7 is important in the first defense against viral infections and has a key role in triggering the adaptive immune response (*76*). As pDCs are major producers of IFNs, producing high levels of IRF-7, leading to the notion that IRF-7 could have a key role in linking the innate and adaptive immune response (*103*). It has also been shown that induction of antigen-specific CD8+ T cell responses is severely impaired in IRF-7 knockout mice, again demonstrating the role of IRF-7 in the adaptive immune response (*104*). Sgarbanti et al. reported that IRF-7 can bind and positively regulate the transcriptional activity of IRF-1 and LMP-2, and that overexpression of a constitutively active form of IRF-7 can positively regulate their promoters. Both of these proteins play an important role in adaptive immunity (*76*). The capability of IRF-7 mediated induction of both IRF-1 and LMP-2 demonstrates its key role in linking the innate and adaptive immune responses.

IRFs in HIV Infection:

IRF-1 plays a major role in the early stages of HIV infection. IRF-1 forms a complex with NF-κB at a specific site in the HIV-1 LTR, and also can bind to a region on the LTR shown to be homologous to the ISRE (*11*). IRF-1 was shown to increase HIV-1 LTR gene expression in a dose dependent fashion in Jurkat cells, and has been shown to be upregulated early in HIV-1 infection before the induction of Tat (*72*). As mentioned previously, there are specific polymorphisms found in HESN women that have been shown to be protective by reducing IRF-1 expression and reduced susceptibility (*71*). As well, knockdown of IRF-1 in primary CD4+ T cells results in a significant decrease in the transactivation of the HIV-1 LTR (*74*). IRF-7 has been repeatedly shown to be upregulated during HIV-1 infection, and individuals infected with HIV-1 have higher expression of IRF-7 (*6, 105-107*). IRF-7 levels were found to be positively correlated with

HIV-1 viral load in CD4+ T cells, CD8+ T cells, and CD11c+ cells, and negatively correlated with CD4+ T-cell counts in treatment naïve HIV-1 infected individuals (*105*). This supports the hypothesis that HIV-1 viral load induces IFN-α production perhaps by IRF-7, and consequently higher expression of ISGs (*105, 107*). A similar conclusion was determined by Herbeuval et al. where IFN-α was found to be higher in pDCs in lymphoid tonsillar tissue of individuals with progressive HIV disease compared to non-progressive and uninfected controls and IRF-7 was increased upon infection of pDCs of healthy donors (*107*). It was also found in primary HIV-1 infection that IRF-7 was expressed at a higher level *in ex vivo* pDCs and myeloid DC (mDC) compared to uninfected controls (*106*). IRF-7 expression was also found to be increased in monocyte-derived DCs (MDDCs) after six hours of HIV-1 infection (*6*). These data demonstrate that IRF-7 increases during the course of HIV-1 infection in a variety of different cell types. This may be due to the virus initiating IRF-7 expression for its own use or the host responding to infection and initiating the antiviral response. These data demonstrate the importance of understanding the roles of interferon regulatory factors in HIV infection.

Lentiviral Transduction:

Lentiviral particles can be used as vehicles for the delivery of genes into a wide range of different cell types, including the difficult to transfect cells such as non-dividing mammalian cells (*108, 109*). Lentiviral particles are transduced into cells and integrated into the host cellular genome resulting in long-term expression of the introduced gene both *in vitro* and *in vivo* (*108, 109*). We used lentiviruses as a tool in the laboratory to deliver viral RNA into the DNA of a host cell. Lentiviral vectors are genetically-engineered and contain a viral promoter which can be used to control the expression of a transgene or shRNA (*110*). Lentiviral particles are created, for bio-safety reasons, by the co-transfection of 3 separate plasmids (packaging, expression, and envelope) into a cell line where the proteins encoded by the 3 plasmids will be expressed and assembled into viral particles and then released extracellularly (*110*). To

prevent the generation of replication-competent viral particles the genes that encode for the envelope and for packaging the viral genome are separated into different plasmids and will not be incorporated into the viral particle (*110*). The expression plasmid encodes the gene of interest and the RNA of the expression plasmid will be incorporated into the viral particles. The created lentiviral particles are then transduced into the target cell. The single-stranded viral RNA from the expression vector is reverse transcribed and the double-stranded DNA is imported into the nucleus and stably integrated into the host genome providing long term transcription of the gene or shRNA of interest (*109*). One or two days post the introduction of the single viral RNA, the expression of the protein can be detected (*109*). The envelope of lentiviral particles that are commonly used for research are VSV-G envelope, which has a broad tropism and can transduce almost any cell type. Many lentiviral expression vectors encode a fluorescent gene in addition to the gene of interest, which acts as a reporter and allows for visualization of the transduced cell (*110*).

Electroporation:

Electroporation is a physical transfection method that uses electrical pulses to create temporary pores in cell membranes through which substances such as nucleic acids can pass (*111*). It is a highly efficient, established technique that is thought to be applicable to many cell types (*112*). In electroporation, the host cells and plasmid or siRNA are suspended in a conductive solution. An electrical pulse is discharged through the cell suspension which disturbs the phospholipid membrane (*113*). This results in the temporary breakdown and the formation of pores which allows charged molecules to be driven across the membrane of the cell (*112, 113*). Electroporation can result in both transient and stable transfections. A large number of cells can be transfected in a short time using electroporation (*111*). The disadvantages of electroporation are there is a large amount of cell death caused by the electrical pulse and only partial membrane repair (*111*). It is therefore imperative to ensure good control over

electroporation parameters to ensure efficient DNA transfer with minimal cell damage (*112*). Several laboratories have had successes in using the NucleofectorTM system for electroporating primary cells. Kamata, *et al.* were able to achieve a three-fold decrease in APOBEC3G MFI after nucleofection of primary quiescent CD4+ T cells (*114*). Su, *et al.* were able to knockdown IRF-1 protein expression in unstimulated PBMCs by 25-40% (*74*). Kardava, *et al.* were also able to achieve a 62% knockdown in primary B cells in primary CD72 transcripts measured by quantitative real time RT-PCR (*115*).

Summary:

To summarize, it is very important to understand all aspects of HIV-1 to help aid in the search for a cure. ART an expensive lifelong treatment and therefore a vaccine is critical. To create a vaccine, we must understand the innate and adaptive immune responses to HIV-1. IRF-1 has been implicated in the initial stages of HIV-1 replication and it has been suggested that IRF-7 is involved as well. My project is specifically looking at IRF-1 and IRF-7 expression in Manitoban PBMCs to determine their expression profile as well to see their responses to induction with interferons. As well, IRF-7 is being examined in Kenyan donors from our cohort of well characterized commercial sex workers. The aim is to determine if there are differences in IRF-7 expression between HIV-negative and HIV-positive individuals. The second part of this project is to knockdown IRF-7 expression using both electroporation and lentiviral transduction to determine the role IRF-7 plays in HIV-1 infection.

Section 1 Hypotheses:

The literature has shown that IRF-1 is constitutively expressed in all cell types and IRF-7 is expressed in peripheral blood lymphocytes (*88*), therefore I hypothesized that IRF-1 and IRF-7 were constitutively expressed in all defined subsets of human PBMC examined.

The literature has also shown that IRF-7 expression is increased during HIV infection (*6, 105-107*). I

hypothesized that IRF-7 expression will be higher in the PBMC of HIV-infected individuals compared to HIV-uninfected healthy controls.

IRF-7 expression will be higher in the Kenyan samples compared to the Manitoban due to exposure to HIV-1.

Section 2 Hypotheses:

Reduced IRF-7 expression will result in decreased anti-viral responses rendering CD4+ T cells more susceptible to infection.

There will be little effect on knocking down IRF-7 on its downstream target genes.

Materials and Methods

Ethics

Informed written consent was obtained from all study participants and the studies were approved by The University of Manitoba and Kenya National Hospital Institutional Review Boards.

Study Population (Manitoban and Kenyan Samples)

Manitoban donor samples were collected from staff or students from the University of Manitoba or J.C. Wilt Infectious Diseases Research Centre in Winnipeg, Manitoba. Blood samples from female sex workers, enrolled in the Majengo (ML) cohort, established in 1985 in the Pumwani district, Nairobi, Kenya were also used. Participants in the Majengo cohort fall into one of 4 categories:

1- HIV-infected: These are individuals who tested HIV-1 positive at enrollment.

2- HIV-uninfected/susceptible: These are individuals who tested HIV-1 negative at enrollment however have been in the sex trade for less than 3 years. Epidemiologic observation of this cohort suggests that most of these individuals (85%) would seroconvert.

3 - Seroconverters: These are individuals who were HIV-1 negative at enrollment and seroconverted after follow-up

4 - HIV-exposed seronegative (HESN): Female sex workers who have regular unprotected sex with known HIV-infected clients for more than 7 years.

For my study I will be using samples from HIV-infected and HIV-uninfected/susceptible individuals.

Our Majengo clinic provides sex workers free STI and HIV prevention services such as counseling, condoms, and free primary health care. The individuals in this cohort biannually participated in a resurvey in which they complete a behavioural interview and to donate biological samples such as

blood, cervico-vaginal lavage (CVL) fluid, and cervical mononuclear cells (CMC) for research. The samples used in these experiments are from the resurvey studies.

Collection of Samples

PBMC Isolation from Whole Blood by Density Gradient Centrifugation

Briefly, blood was collected in vacutainers containing heparin and was spun at 524 x g for 7 minutes. Plasma was removed and blood was diluted with phosphate-buffered saline (PBS). Diluted blood was layered over Lymphoprep™ (catalogue number 07861, StemCell Technologies) and then spun at 456 x g for 25 minutes, with no brake. The white cell layer was collected and diluted with complete RPMI 1640 (catalogue number SH3002701, HyClone) (+10% Fetal bovine serum (FBS) (catalogue number F1051, Sigma), + 1% Penicillin-Streptomycin 100X (P/S) (catalogue number MT-30-002-CI, Corning^{TM})) and spun at 335 x g for ten minutes. Cells were washed, counted, and re-suspended in freezing media (FBS +10% Dimethyl Sulfoxide (DMSO) (catalogue number BP231-1, Fisher) in cryovials and frozen in liquid nitrogen (LN_2).

Cell Thawing

Frozen PBMCs samples were thawed in a 37°C water bath until cell pellet was loose and then immediately transferred to pre-warmed complete RPMI using a transfer pipette. Cells were spun at 335 x g for 8 minutes, re-suspended in culture media, and counted. Cells were spun again at 335 x g for 8 minutes, and then re-suspended in complete RPMI for use in the described studies.

Section 1: IRF-1 and IRF-7 Characterization in Manitoban Donor and Kenyan PBMC Subsets

Cell Thawing and Stimulation

Frozen PBMCs were thawed, rested overnight (16-18 hours), and then plated in 12-well plates at a concentration of $1x10^6$ cells/mL in complete RPMI with $2x10^6$ cells/well. To assess the levels of IRF-1 both before and after stimulation, the Manitoban samples were stimulated with Recombinant Human IFN-γ (catalogue number 300-02, Peprotech) at a concentration of 10 ng/mL for 3 hours. To characterize IRF-7, the Manitoban donor and Kenyan samples were stimulated with Recombinant Human IFN-αA (alpha 2a) (catalogue number 11100-1(RD), R&D Systems) at a concentration of 5000 U/mL for 16-18 hours. As a control for the induction of Manitoban donor samples, the expression of Mx-1 was used as positive control, stimulated by Phorbol 12-myristate 13-acetate (PMA) (catalogue number 8139-1MG, Sigma) and Ionomycin (catalogue number 10634-1MG, Sigma) both at a concentration of 50 ng/mL. The expression of IRF-1 stimulated by IFN-γ was used as a positive control for the Kenyan samples.

Cell staining

Cultured cells were transferred into 5 mL polystyrene tubes for staining and flow cytometry. Cells from each well was split into 4 tubes, with approximately $0.5x10^6$ cells/tube. For the Manitoban donor samples, the four tubes were: unstained, surface marker staining + intracellular IRF-1 or IRF-7 staining, surface marker staining + intracellular Mx-1 staining (control), and surface marker staining + secondary antibody, depending on the stimulant. Mx-1 stimulated by PMA and Ionomycin was used as a positive stimulation control for this study. For the Kenyan samples the four tubes were: unstained, surface marker staining + intracellular IRF-7 staining, surface marker staining + IRF-1 intracellular staining (control), and surface marker staining + secondary. After the cells were transferred into the tubes for cell-staining, they were washed with staining buffer (PBS + 2% FBS + 2mM Ethylenediaminetetraacetic acid (EDTA)). Human TruStain FcX (Fc Receptor Blocking Solution) (catalogue number 422302, BioLegend) was added for 10 minutes, followed by a master mix of the surface stain antibody along with a LIVE/DEAD™ VIVID stain, and incubated for 30 minutes at 4°C in the dark. Cells were then washed with

staining buffer and spun for 8 minutes at 524 x g. Cells were fixed using BD (Becton Dickinson) Cytofix/Cytoperm solution (catalogue number 5547414, BD) and incubated for 20 minutes at 4°C and then washed with BD Perm/Wash buffer (1X) (catalogue number 5547414, BD). The primary antibody of IRF-1 (4 µL), IRF-7 (4 µL), or Mx-1 (1 µL), was added and again incubated at 4°C for 30 minutes in the dark. Cells were washed with Perm/Wash buffer, and then the secondary antibody (0.2 µL) was added. After 30 minutes' incubation at 4°C in the dark, cells were washed again with staining buffer and then re-suspended in PBS and stored in the dark at 4°C until flow cytometry analysis. Flow cytometry analysis was performed on a BD LSRII using FacsDiva software.

Table 1. Flow cytometry panel for 11-color staining

Marker	Fluorochrome	Company	Catalogue number
CD3	V500	BD	561416
CD8	FITC	BD	347313
CD4	PE-Cy5	BD	555348
CD14	Pacific Blue	BD	558121
CD16	Alexa Fluor 700	BD	560713
CD56	PE-Cy7	BD	557747
HLA-DR	APC-H7	BD	641393
CD11c	APC	BD	560895
CD19	Brilliant Violet 605	BioLegend	302244
LIVE/DEAD™	Red (Texas-Red)	ThermoFisher Scientific	L-23102
Donkey anti-rabbit IgG	PE	BioLegend	406421
IRF-1 (C-20)		Santa Cruz Biotechnology	sc-497
IRF-7 (H-246)		Santa Cruz Biotechnology	sc-9083
Mx-1 (631-645)		Sigma	SAB1100070

Analysis

The various PBMC subsets were analyzed using FlowJo Software (FlowJo, LLC). I examined CD4+ T cells (CD3+CD4+CD8-), CD8+ T cells (CD3+CD4-CD8+), B cells (CD3-CD19+), NK cells (cytotoxic (CD16+CD56dim) and cytokine producing (CD16-CD56high), monocytes (patrolling (CD14dimCD16+),

inflammatory (CD14+CD16+), and classical (CD14highCD16-), and DC (HLA-DR+CD11c+CD16+).

Table 2. Cell types and markers analyzed for IRF-1 and IRF-7 characterization

Cell Type	Markers
CD4+ T-cells	CD3+CD8-CD4+ (*116*)
CD8+ T-cells	CD3+CD4-CD8+ (*116*)
B Cells	CD3-CD19+ (*117*)
Natural Killer Cells (Cytotoxic Subset)	CD3-CD16+CD56dim (*118*)
Natural Killer Cells (Cytokine-Producing Subset)	CD3-CD16-CD56high (*118*)
Classical Monocytes	HLA-DR+CD14+CD16- (*119*)
Activated Monocytes (Inflammatory)	HLA-DR+CD14+CD16+ (*119*)
Activated Monocytes (Patrolling)	HLA-DR+CD14dimCD16+ (*119*)
DC	HLA-DR+CD3-CD14-CD56-CD19-CD11c+CD16+ (*120*)

For the Kenyan samples, I compared the levels of IRF-7 in HIV-infected individuals compared to HIV-uninfected, susceptible individuals. All statistical analysis was performed using GraphPad Prism, version 5, 6, and 7. I used column statistics (D'Agostino-Perason omnibus normality test) to determine if our sample results followed a normal distribution. I used one-way ANOVA statistical analysis between groups of larger than 2 or the Friedman Test if the results were non-parametric. I used Dunn's multiple comparisons post-test or Tukey's test to compare between the cell populations. Between two paired groups I used t-tests for parametric data and Wilcoxon matched-pair test for non-parametric, for unpaired non-parametric data I used Mann-Whitney test and unpaired parametric data I used un-paired t-test. Mean +/- standard deviation was used for parametric tests and median +/- interquartile range was used for non-parametric tests.

Cell types were analyzed by first gating on singlets, as determined by gating on the diagonal axis of Forward Scatter-Height (FSC-H) by Forward Scatter-Area (FSC-A). Cells that were off the diagonal axis could be clumps or doublets which were excluded from further analysis. Live cells were then selected by

using a LIVE/DEAD™ Fixable Dead Cell Stain. These stains can distinguish live cells from dead as the stain can permeate the dead cell membrane and stain the available amines (*121*). The dead cells are thus stained and can be distinguished from the live cells (*121*). After gating on the live cells, lymphocyte and monocyte sub-populations were gated on the plot of FSC-A by Side Scatter-Area (SSC-A). The lymphocyte population is smaller and less granular than the monocyte population (*122*). Cell types were then determined based on their surface markers.

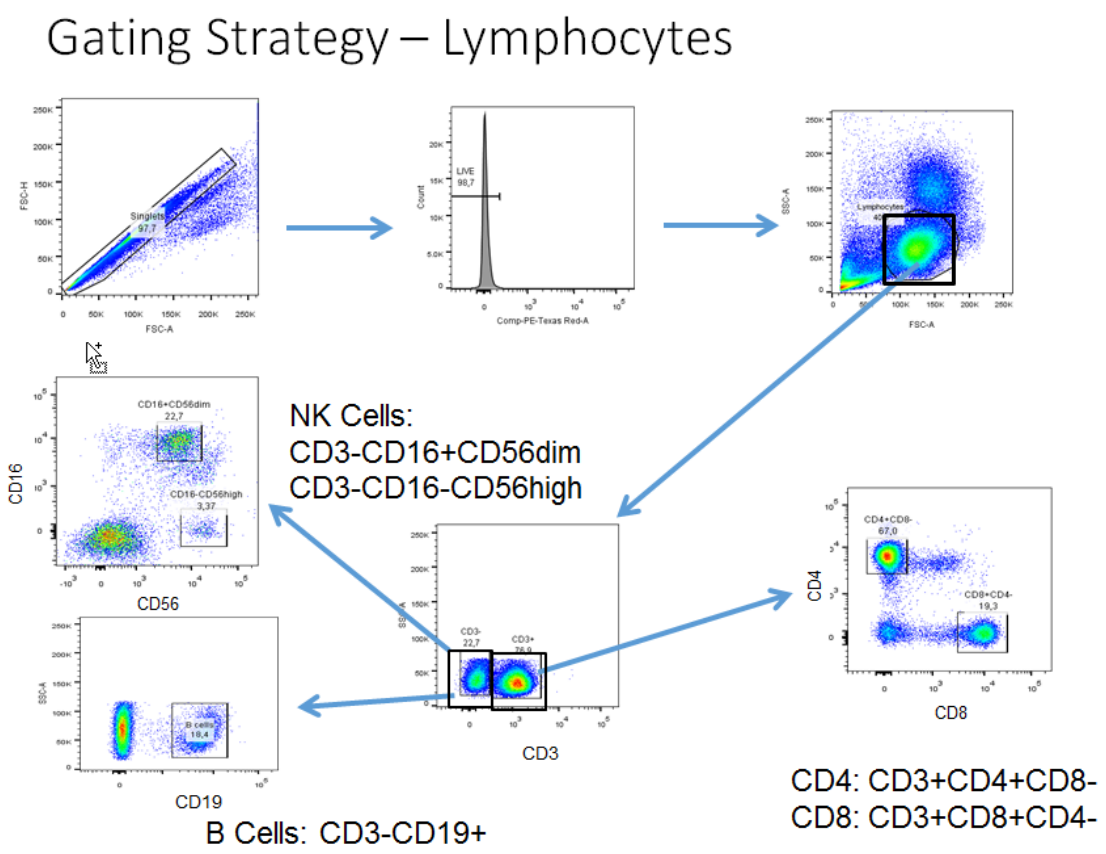


Figure 1. Gating strategy for lymphocytes including CD4+ T cells, CD8+ T cells, B Cells, and NK Cells (cytotoxic and cytokine producing).

Cell types were determined by first gating on singlets. Singlets were determined by gating on the diagonal axis of FSC-H by FSC-A. Live cells were then selected by using a LIVE/DEAD™ Fixable Dead Cell Stain. After gating on the live cells, lymphocyte and monocyte populations were determined by comparing FSC-A by SSC-A. Cell types were then gated on CD3+ or CD3-. The CD3+ subset contained CD4+CD8- T cells and CD8+CD4- T cells. The B cells were determined by gating on the CD3- subset and the CD19+ cells. NK cell populations were determined by gating on the CD16+CD56dim cell subset (cytotoxic NK cells) and the CD16-CD56high cell subset (cytokine producing cells).

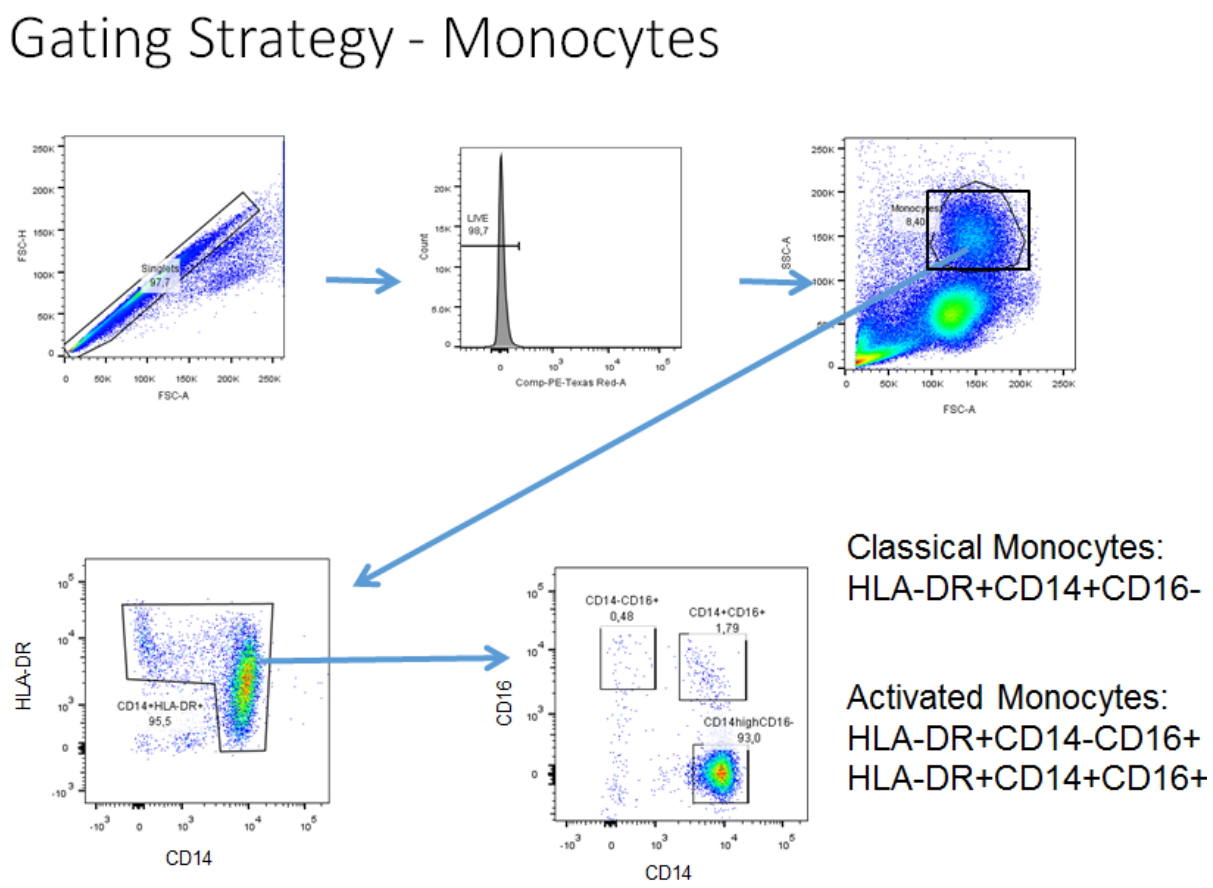


Figure 2. Gating strategy for monocytes including classical monocytes and two different activated monocyte subsets (inflammatory and patrolling).

Cell types were determined by first gating on singlets. Singlets were determined by gating on the diagonal axis of FSC-H by FSC-A. Live cells were then selected by using a LIVE/DEAD™ Fixable Dead Cell Stain. After gating on the live cells, lymphocyte and monocyte populations were determined by comparing FSC-A by SSC-A. The larger and more granular monocyte population was selected. The monocyte populations were determined by gating on HLA-DR+CD14+ subset. The three monocyte populations were determined by gating on CD14 and CD16. The classical monocytes are CD14+CD16-, the inflammatory monocytes are CD14+CD16+, and the patrolling monocytes are CD14+CD16+ (*119*).

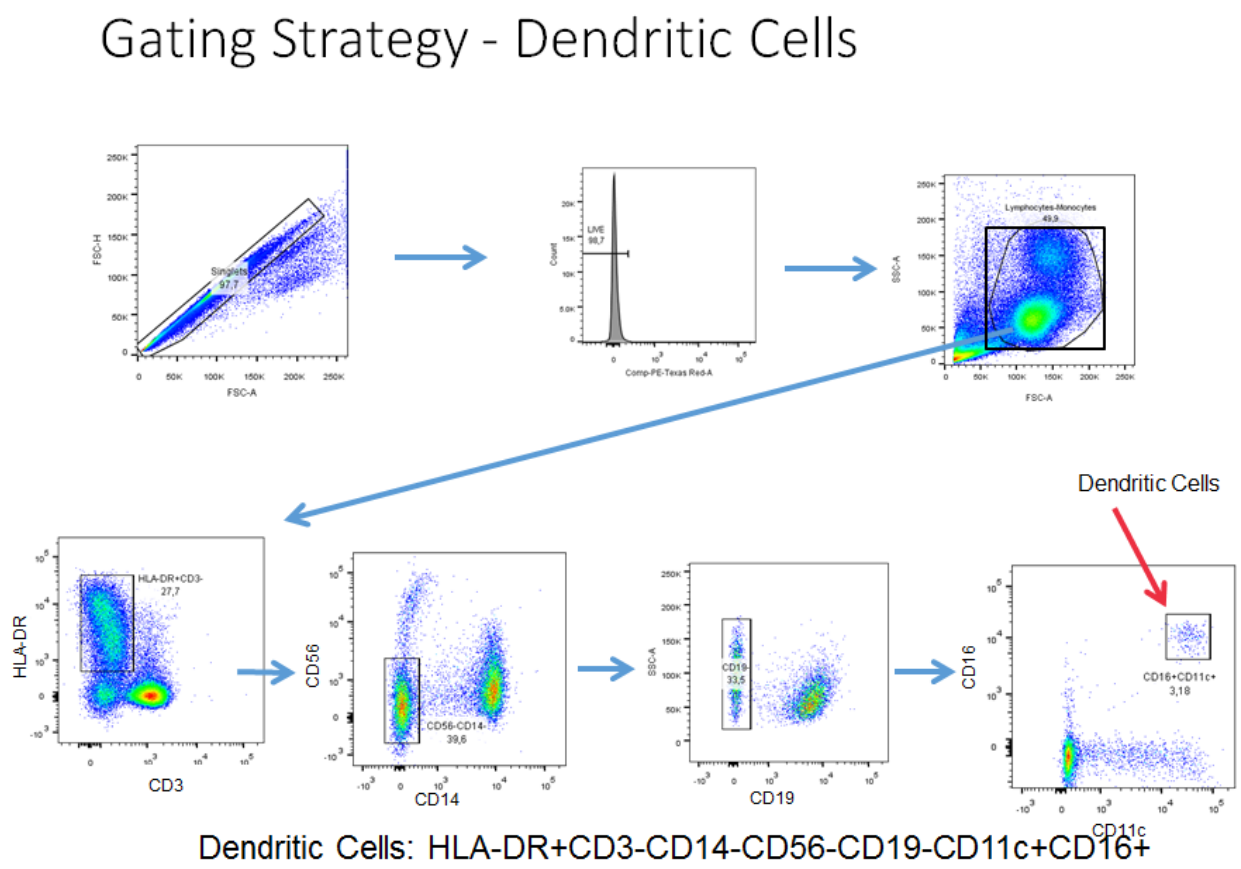


Figure 3. Gating strategy for DCs.

Cell types were determined by first gating on singlets. Singlets were determined by gating on the diagonal axis of FSC-H by FSC-A. Live cells were then selected by using a LIVE/DEAD™ Fixable Dead Cell Stain. After gating on the live cells, lymphocyte and monocyte populations were determined by comparing FSC-A by SSC-A. DCs are an intermediate population between lymphocytes and monocytes and therefore both cell populations were chosen. DCs were determined by gating on the HLA-DR+CD3- population to gate out T cells. Next, cells were gating on CD14-CD56- population to gate out NK cells and monocytes. This was followed by gating on CD19- cells to exclude the B cells. Finally, the DCs were determined by gating on CD11c+CD16+population.

Positive Controls

A positive stimulation control was used to ensure the frozen PBMCs were able to respond to stimulation. Mx-1 was used as a positive control for the IRF-1 studies using Manitoban donors. As it has been well described in the literature that IRF-1 regulates Mx-1 expression, and therefore if an increase in Mx-1 was observed a corresponding in IRF-1 would be as well, since both IRF-7 and Mx-1 are interferon stimulated genes regulated by type I IFNs (*8*). IRF-7 is involved in a positive feedback loop that results in the increase of IFN-αA, which leads to the induction of ISGs such as Mx-1. It is therefore likely that if Mx-1 is increased, IRF-7 should be as well. IRF-1 stimulated by IFN-γ was used as a positive stimulation control for the IRF-7 studies. The controls that we chose to use were determined based on literature and previous data from our laboratory (*71, 73*).

Mx-1 expression in PBMC can be induced by PMA and Ionomycin

To determine if the polyclonal activation (PMA and Ionomycin) was inducing a detectable immune response, Manitoban donors PBMC was stimulated by PMA and Ionomycin as a positive stimulation control.

Mx-1 expression was measured in various PBMC subsets in rested and stimulated cells with PMA and Ionomycin for 16-18 hours (Figure 4). I observed significantly (1.6-fold increase in Mx-1 expression, median unstimulated = 2644 and median stimulated = 4242, p=0.0431) higher expression of Mx-1 in the stimulated cell subset compared to unstimulated. These data demonstrate that our samples, recovered from freezing media were viable and able to respond to stimulation.

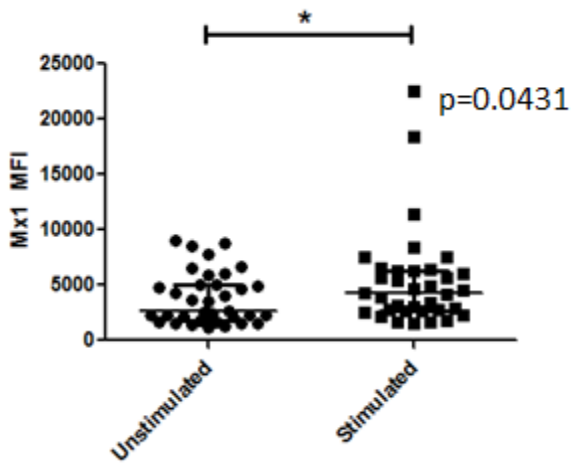


Figure 4. Comparison of the expression of Mx-1 in Manitoban donors in unstimulated and PBMCs stimulated for 16-18 hours.

Flow cytometry was used to measure the expression of Mx-1 in 18 Manitoban donor PBMC samples. This figure shows median fluorescence intensity (MFI) of the PE-secondary antibody for expression of Mx-1 with each point representing the results from a single individual. Paired two-tailed Wilcoxon matched-pairs signed rank statistical tests were performed to assess the expression differences between the groups (95% confidence level, or $p \leq 0.05$). Horizontal bars represent the median +/- the interquartile range.

IRF-1 expression in classical monocytes and CD4+ T cells can be induced by stimulation with IFN-γ.

To determine if frozen PBMCs used in experiments were able to respond to IFN-γ stimulation for our Kenyan samples, IRF-1 expression in classical monocytes and CD4+ T cells that were either unstimulated or stimulated with IFN-γ for 16-18 hours was measured by flow cytometry. Classical monocytes were chosen (CD14highCD16-) as they are the most common monocyte population. CD4+ T cells (CD3+CD4+CD8-) were chosen as a representative of the lymphocyte population as T cells are the most common lymphocyte population I examined and CD4+ T cells are the most common T cells. As well they are both (CD4+ T cells and monocytes) targets of HIV-1.

I saw significantly higher expression of IRF-1 in the stimulated classical monocytes (CD14highCD16-) cell subset compared to unstimulated (unstimulated median = 16555 and stimulated median = 16886, p=0.0354) (Figure 5). A significant increase in IRF-1 was observed in CD4+ T cells (CD3+CD4+CD8-) when comparing fold change after stimulation (fold change= 1.258, p=0.0027).

These results demonstrate that our Kenyan donor monocyte and lymphocyte samples, recovered from freezing media were able to respond to stimulation by exogenous IFN-γ.

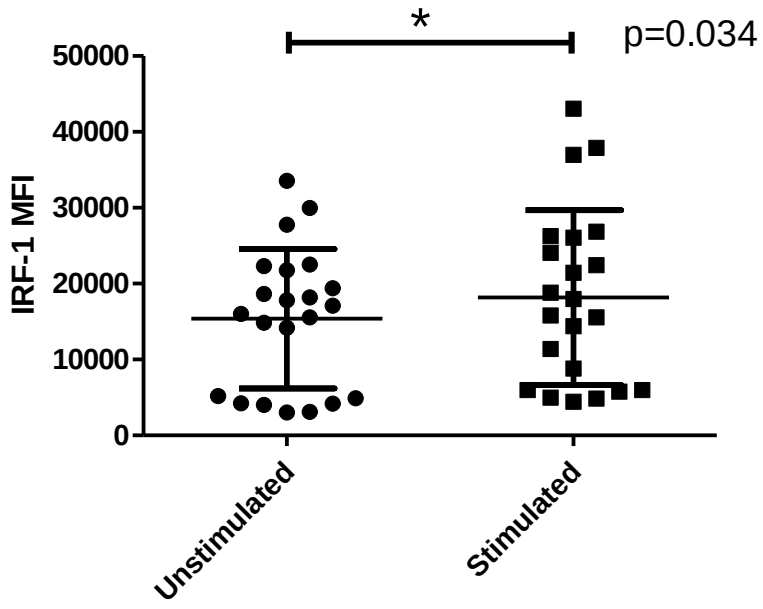


Figure 5. Comparison of the expression of IRF-1 in classical monocytes (CD14highCD16-) from Kenyan donors between unstimulated and classical monocytes stimulated with IFN-γ.

Flow cytometry was used to measure the expression of IRF-1 in Kenyan donor classical monocyte samples. This figure shows median fluorescence intensity (MFI) of the PE-secondary antibody for expression of IRF-1 with each point representing a single donor. Paired two-tailed T-tests were performed to assess the expression differences between the groups (95% confidence level, or $p \leq 0.05$). Horizontal bars represent the mean +/- standard deviation.

Table 3. Flow cytometry antibody list for electroporation transfection p24 staining

Marker	Fluorochrome	Company	Catalogue number
LIVE/DEAD™	Green (FITC)	ThermoFisher Scientific	L-23101
Donkey anti-rabbit IgG	Brilliant Violet 510	BioLegend	406419
p24	PE	Beckman Coulter	KC57-RD1
IRF-1 (C-20)		Santa Cruz Biotechnology	sc-497
IRF-7 (H-246)		Santa Cruz Biotechnology	sc-9083

Table 4. Flow cytometry antibody list for lentivirus transduction p24 staining

Marker	Fluorochrome	Company	Catalogue number
GFP (Cells)	FITC		
LIVE/DEAD™	Aqua	ThermoFisher Scientific	L34957
Goat anti-rabbit IgG	APC	Jackson	111-136-144
p24	PE	Beckman Coulter	KC57-RD1
IRF-1 (C-20)		Santa Cruz Biotechnology	sc-497
IRF-7 (H-246)		Santa Cruz Biotechnology	sc-9083

Confocal Imaging

PBMC samples from a Manitoban donor were stained with antibodies specific for IRF-1 (clone C-20) (catalogue number sc-497, Santa Cruz Biotechnology) or IRF-7 (clone H-246) (catalogue number sc09083, Santa Cruz Biotechnology) using the protocol for intracellular staining. Since these antibodies are unlabelled, Allophycocyanin (APC) AffiniPure F(ab')$_2$ Fragment Goat Anti-Rabbit IgG (H+L) (catalogue number 111-136-144, Jackson) was used as the secondary for visualization of the primary antibody binding. Samples were re-suspended in staining buffer and allowed to air dry on microscope slides. 1.5H coverslips (catalogue number 474030-9000-000, Zeiss) were then mounted onto the slides using ProLong® Diamond Antifade Mountant with (DAPI 4',6-diamidino-2-phenylindole) (stains nuclei) (catalogue number P36962, ThermoFisher). Mountant was allowed to harden overnight and then

imaged on a Zeiss LSM 700 confocal microscope by a confocal laboratory technician following procedures provided by Zeiss technical experts.

The Effect of IRF-7 Knockdown in CD4+ T Cell Subsets on HIV Infection by Electroporation Transfection

T-Cell Enrichment

PBMCs enrichment was done by using the EasySep™ Human CD4+ T Cell Enrichment Kit (catalogue number 19052, StemCell) and the EasySep™ Human T Cell Enrichment Kit (catalogue number 19051, StemCell) according to manufactures protocol. This is a negative selection process that depletes non CD4+ T Cells or T Cells, depending on the kit. Briefly, cells were re-suspended at 10^7 cells/mL in staining buffer in a 5 mL polystyrene tube. The EasySep Human T Cell Enrichment Cocktail was added at 50 µL/mL and incubated at room temperature for ten minutes. The EasySep D Magnetic Particles were vortexed and then added at 50 µL/mL cells followed by 5 minutes incubation at room temperature. The cell suspension is brought up to 2.5 mL by adding staining buffer and the cells were mixed gently, then placed in the EasySep™ Magnet (catalogue number 18000, StemCell Technologies) for 5 minutes. The tube and magnet were inverted, pouring off the desired fraction, the unlabelled cells, into a new 5 mL polystyrene tube. Cells were centrifuged at 524 x g for 8 minutes, and then re-suspended in complete RPMI and rested for 3 hours.

Electroporation Transfection

Electroporation transfection of CD4+ T cells was done using the P3 Primary Cell 4D-Nucleofector® X Kit S (catalogue number V4XP-3032, Lonza Cologne AG) according to manufacturer's protocol. This kit is specific for primary T cells. In brief, cells were counted and aliquoted into Eppendorf tubes with $1x10^6$/tube. Cells were spun at 845 x g for 3 minutes, supernatant was removed, followed by a second centrifugation for 30 seconds, with the supernatant again removed carefully by pipette. The cell pellets

were re-suspended in 16.4 µL Nucleofector™ Solution along with 3.6 µL supplement/tube. Accell Human IRF1 siRNA, SMARTpool: Accell IRF1 siRNA (catalogue number E-011704-00-0005, Dharmacon), Accell Human IRF7 siRNA, SMARTpool: Accell IRF7 siRNA (catalogue number E-011810-00-0005, Dharmacon), or HS IRF1 1 HP siRNA 20 nMol, 3' Alexa Fluor 647 (catalogue number SI00034083, Qiagen) were added to the appropriate tube. 20 µL of the cell/transfection mixture was added to each 20 µL Nucleocuvette™ Strip well and then placed in the 4D-Nucleofector™ System (catalogue number AAF-1002B, Lonza). The program for high functionality unstimulated primary T cells was chosen which then delivers electric pulses to each well. After transfection, the cells were then pipetted from the wells and added to 230 µL pre-warmed complete RPMI and cultured for 18-20 hours at 37°C. For larger cell volumes, P3 Primary Cell 4D-Nucleofector® X Kit L (catalogue number V4XP-3024, Lonza) according to manufacturer's protocol was used. This kit uses the larger 100 µL Single Nucleocuvette™ and cells were added at concentrations of 1-10x10^6. 82 µL of Nucleofector™ Solution and 18 µL supplement was added to each cuvette. Post-transfection cells were pipetted into 2 mL pre-warmed complete RPMI. Cells were stained for IRF-1 or IRF-7 modulation after transfection.

HIV Infection

CD4+ T cells were counted and washed in serum-free RPMI, plated at 1x10^5 cells/well with 4 µg/mL polybrene in a 96-well plate and then taken to the enhanced laboratory (CL2+) for HIV-infection. The virus was added at an MOI of 3, and the cells were centrifuged for one hour at 37°C at 1000 x g. Cells were then incubated at 37°C for one hour and the plate was agitated every 15 minutes. This was followed by another spin at 524 x g for 8 minutes and a media change which involved the removal of 100 µL from each well and the addition of 100 µL 2x complete RPMI media + 30 U IL-2, and then incubation overnight at 37°C. At 24, 72, and 96 hours post-infection the plates were centrifuged and 135 µL cell culture was removed and added to 1% Triton X-100 (catalogue number BP151-100, Fisher) to

inactivate any residual virus and saved for enzyme-linked immunosorbent assay (ELISA). 135 µL of fresh complete RPMI culture media was added back to the plate and further incubated.

p24 Cell Staining

At 96 hours' post-infection, cells were stained for p24. GolgiPlug™ (catalogue number 554723, BD) was added at a concentration of 1 µL/1 mL of cell culture and the cells were incubated for 6 hours. $1x10^5$ cells were transferred to a 5 mL polystyrene tube and then centrifuged at 524 x g for 8 minutes and then washed with PBS. Cells were stained with LIVE/DEAD™ VIVID stain for 30 minutes at 4°C in the dark and then washed with staining buffer (PBS + 2% FBS + 2 mM EDTA). Cell pellets were re-suspended in 100 µL of 1% paraformaldehyde (PFA) (catalogue number 18814, Polysciences) and then incubated at room temperature for 10 minutes followed by centrifugation at 456 x g for 8 minutes. 100 µL Foxp3 Fixation/Permeabilization (catalogue number 00-5523, eBioscience) buffer was added to the re-suspended cell pellets (in approximately 50 µL) and incubated at room temperature for 15 minutes and then spun at 524 x g for 8 minutes. Cell pellets were re-suspended in 100 µL 1X Perm/Wash Buffer and p24-PE antibody and IRF-1 or IRF-7 antibody was added and incubated for 45 minutes at 4°C in the dark. Cells were spun at 524 x g for 8 minutes, re-suspended and then 0.2 µL secondary antibody was added followed by 30-minute incubation at 4°C. Cells were washed with staining buffer and then re-suspended in 100 µL 1% PFA and 100 µL PBS and then read on a LSRII.

RNA Isolation using Qiagen RNeasy (Plus) Mini Kit

RNA isolation was performed by using the Qiagen RNeasy (Plus) Mini Kit (catalogue number 74136, Qiagen) according to manufacturer's protocol using the buffers provided in the kit; RLT, RW1, and RPE. In brief, RLT buffer with β-Mercaptoethanol (10 µL per 1 mL buffer) was added to lyse the cells and vortexed (350 µL for $< 5x10^6$ cells or 600 µL for $\leq 1x10^7$). The lysate was pipetted directly onto a

QIAshredder column to homogenize the tissue sample and centrifuged at max speed. The sample was then transferred to a gDNA Eliminator column and centrifuged for 30 seconds at ≥8000 x g. 1 volume of 70% ethanol was added to the flow-through to precipitate the RNA, vortexed, and then transferred to a RNeasy (MinElute) spin column and centrifuged at ≥8000 x g for 15 seconds. The spin column was placed in a new collection tube and 700 µL of Buffer RW1 was added, followed by centrifugation at ≥8000 x g. This was followed by two washes with RPE and centrifugation. The column was then spun at maximum speed for 1 minute to dry the spin column membrane. Finally, the spin column was placed in a new 1.5 mL collection tube and 30 µL RNAse-free water was added followed by a final centrifugation at full speed.

Reverse Transcription to cDNA

SuperScript III First Strand Synthesis system (catalogue number 18080-051, ThermoFisher) was performed according to manufacturer's protocol. In brief, 500 ng of RNA (isolated from HIV infections of IRF-7 knockdown), along with 1 µL of 50 ng/µL random hexamers, 1 µL of 10 mM dNTP mix, and up to 10 µL of DEPC-treated water were combined into a 0.2 mL tube and incubated at 65°C for 5 minutes and then chilled on ice for 1 minute. 2 µL of 10X RT buffer, 4 µL 25 mM $MgCl_2$, 2 µL of 0.1 M DTT, 1 µL of RNaseOUT™ (40 U/µL), and 1 µL of SuperScript® III RT (200 U/µL) was added to the RNA/primer mixture and incubated for 10 minutes at 25°C, followed by 50 minutes at 50°C. The reaction was then terminated at 85°C for 5 minutes and chilled on ice. 1 µL of RNase H was added to the tube followed by incubation at 37°C for 20 minutes.

Absolute Quantitative PCR (qPCR)

QuantiTect SYBR Green PCR Kit (catalogue number 204145, Qiagen) was used for all experiments. Primers were created for GFP and IRF-7 by comparing to a reference gene from NBCI. Oligos were

synthesized at the National Microbiology (NML) DNA Core Services. Sequences for IRF-7 primers were: TACACCTTGCACTT GCCCAT for the forward primer and CTACACGGAGGAAC TGCTGC for the reverse. Sequences for GFP primers were: TCACCGACAAGATC ATCCGC for the forward primer and AAGCTGCCATCCAG ATCGTT for the reverse. 18S primers (Hs_RRN_1_SG QuantiTect Primer Assay (200): catalogue number QT00199367) were purchased from Qiagen. Standard curves were created using reference cDNA (qPCR Human Reference cDNA, Oligo(dT)-primed: catalogue number 636693, ClonTech) for 18S. The expression plasmid encoding for GFP and IRF-7 for creating lentiviral particles was used for creating the GFP and IRF-7 standard curves. 1 µL of sample was added to each well for the unknowns for GFP and IRF-7 quantification. 1 µL of 1/10 diluted sample was added to each well for the unknowns for 18S quantification. 96-well plates were read on the LightCycler ® 96 Real-Time PCR System, Roche.

Significantly higher copies of GFP normalized to copies of 18S in CD4+ T cells transduced with lentiviral particles encoding for IRF-7 shRNA.

I have noticed previously the loss of GFP signal after culturing the lentivirally transduced cells for an extended period of time. Even after sorting out the GFP positive cells I can no longer observe the GFP expression by fluorescent microscope or by flow cytometry. I decided to create primers for GFP to ensure that the lentiviral particles were transduced into the cell. To ensure that our cells with lentiviral particles added were positively transduced, I measured levels of GFP in these cells by qPCR. CD4+ T cells transduced with lentivirus particles encoding for IRF-7 shRNA or non-transduced cells were infected with HIV-1 IIIB subtype or laboratory isolate ML 1956 for 96 hours. Following infection, RNA was isolated from the cells and reverse transcribed to cDNA. cDNA was analyzed by absolute qPCR using primers specific for GFP and were able to determine the copies of GFP by comparing to a standard curve of known GFP copy number. The number of 18S copies in a sample were determined by comparing to a standard curve. Values were normalized by dividing the copy number of GFP to the copy number of 18S.

I was able to see statistically significantly more GFP expression in the cells transduced with the lentiviral particles compared to non-transduced cells (701176-fold increase in GFP expression, median control = 8.5e-006 and median transduced cells = 5.96, p=0.0079) (Figure 6).

In conclusion, this confirms that the lentiviral transduction worked but GFP protein expression was down regulated by the cell by yet-to-be-sought mechanisms.

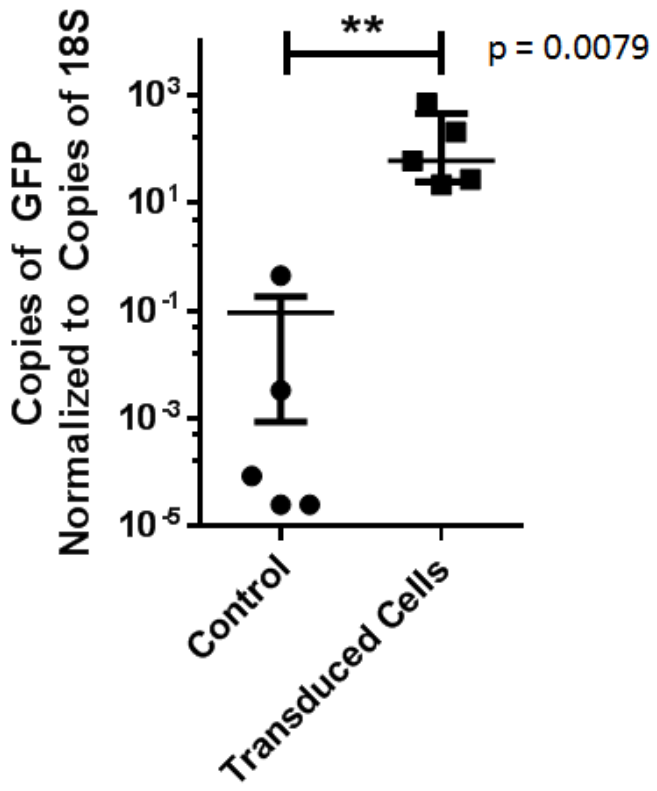


Figure 6. Copies of GFP normalized to copies of 18S in cells transduced with lentiviral particles encoding for IRF-7 shRNA, compared to non-transduced cells measured by qPCR.

CD4+ T cells transduced with lentivirus particles encoding for IRF-7 shRNA or non-transduced cells were infected with HIV-1 IIIB strain or ML 1956 HIV-1 strain for 96 hours. Following infection, RNA was isolated from the cells and reverse transcribed to cDNA. cDNA was analyzed by absolute qPCR using primers specific for GFP. Non-parametric Mann-Whitney statistical tests were performed to assess the expression differences between the groups (95% confidence level, or $p<0.05$).

Significant correlation between copies of IRF-7 normalized to copies of 18S determined by qPCR and levels of IRF-7 (MFI) by flow cytometry.

To determine if there was a correlation between the copies of IRF-7 and the MFI of IRF-7, I compared the copies of IRF-7 generated by qPCR to the levels of IRF-7 determined by flow cytometry. To do this, CD4+ T cells transduced with lentivirus particles encoding for IRF-7 shRNA or non-transduced cells were infected with HIV-1 IIIB strain or ML 1956 HIV-1 strain for 96 hours. Following infection, RNA was isolated from the cells and reverse transcribed to cDNA. cDNA was analyzed by absolute qPCR using primers specific for IRF-7. Cells were also stained at 96 hours post infection for IRF-7. I determined the copy number of IRF-7 by comparing to a standard curve. I then normalized this value to 18S copies determined by comparing to a standard curve. I was able to correlate the amount of IRF-7 copies normalized to 18S copies to the levels of IRF-7 expression in the cells measured by flow cytometry (MFI). There was significant correlation (Pearson $r=0.9297$) between IRF-7 copies by qPCR and IRF-7 expression (MFI) by flow cytometry ($p \leq 0.0001$) (Figure 7). In conclusion, there is a positive correlation between the copies of IRF-7 determined by qPCR and the levels of IRF-7 measured by flow cytometry.

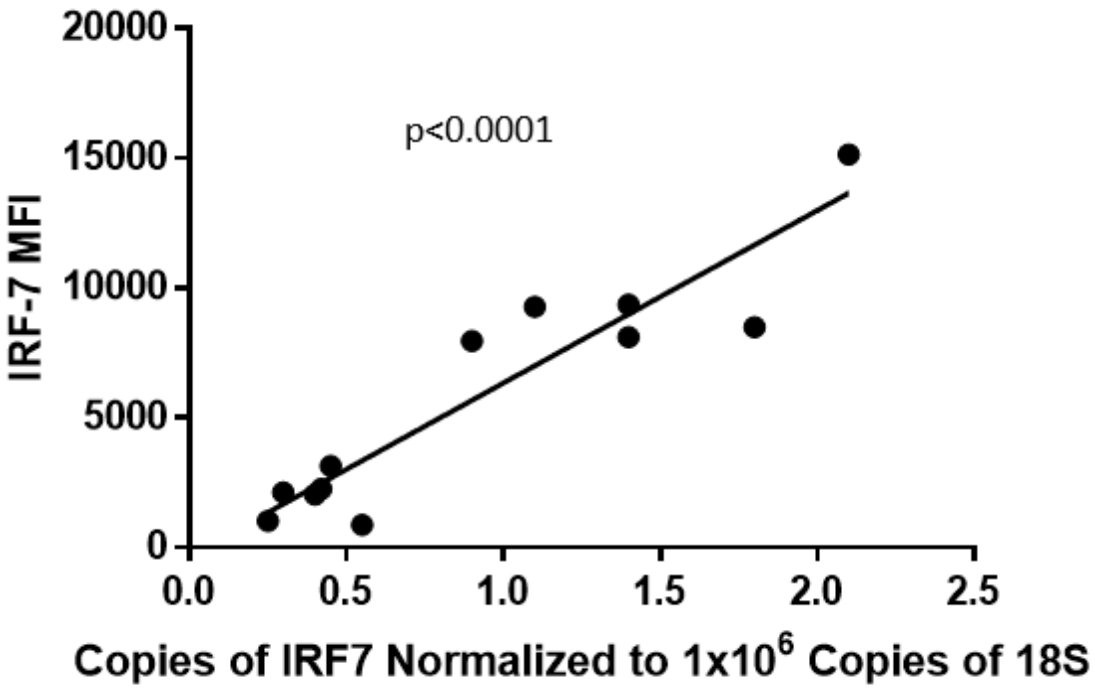


Figure 7. Comparing levels of IRF-7 measured by flow cytometry (MFI) to copies of IRF-7 normalized to copies of 18S measured by qPCR.

CD4+ T cells transduced with lentivirus particles encoding for IRF-7 shRNA or non-transduced cells were infected with HIV-1 IIIB strain or ML 1956 HIV-1 strain for 96 hours. Following infection, RNA was isolated from the cells and reverse transcribed to cDNA. cDNA was analyzed by absolute qPCR using primers specific for IRF-7. Cells were also stained 96 post infection for IRF-7. Parametric Pearson correlation tests were performed to assess the correlation between the groups (95% confidence level, or $p<0.05$).

P24 Enzyme-Linked Immunosorbent Assay (ELISA)

96-well NUNC Maxisorb plates were coated with Primary (Coating) HIV-1 p24 Hybridoma antibody (catalogue number 183-H12-5C, NIH) diluted in coating buffer ((pH 9.6: Na2CO3: catalogue number S7795-500g, Sigma) and NaHCO3: catalogue number S233, Fisher Scientific) and incubated overnight at 4°C in a high-humidity container. All subsequent incubations were performed using a high-humidity maintained container.

The following day, plates were washed 6 times with wash buffer (1x PBS pH 7.4 + 0.05% Tween™ 20 (catalogue BP337500, Fisher) and then blocked with 150 µL of blocking buffer (1X PBS + 2% Goat Serum (catalogue number G6767-100mL, Sigma) + 0.01% Tween 20) in a high-humidity container for 2 hours at 37°C. Plates were washed again 6 times, and then 100 µL of standard (recombinant protein) (catalogue number ab43037, abcam) and samples (tissue culture supernatants) were added to the appropriate wells. The dilutions for the standards are as follows: 50, 25, 12.5, 6.25, 3.13, 1.56, 0.78, 0 ng/mL. The plates were then incubated overnight at 4°C in a high-humidity container.

The following day, plates were washed 6 times with wash buffer, followed by the addition of 100 µL/well secondary (detection antibody) (biotinylated) (catalogue number Ab20774, abcam) and then incubated for 2 hours at 37°C in a high-humidity container. Plates were washed 6 times with washing buffer and then 100 µL/well Streptavidin-Alkaline Phosphatase (SAAP) (catalogue number 016-050-084, Jackson ImmunoResearch) was added and incubated at 37°C for 1 hour in a high-humidity container. Plates were washed again 6 times with washing buffer, and then 100 µL/well substrate buffer (Magnesium Chloride Hexahydrate (catalogue number M35-500, Fisher) + Diethanolamine (catalogue number D45-500, Fisher) + pH with HCL to 9.8 + Phosphatase Tablets ((add when ready to use) (catalogue number S0942-200Tab, Sigma)) was added. The plates were incubated at room temperature and then read at 5 and 15 minutes at 405 nm.

Positive correlation between levels of secreted and intracellular p24.

To measure if there was a correlation between intracellular and extracellular p24, cell supernatants were collected following 96 hours post infection of CD4+ T cells electroporated or lentivirally transduced, and analyzed by ELISA for levels of secreted p24 (ng/µL). Cells were also collected at 96 hours and stained for p24 and analyzed by cell staining and flow cytometry. There was significant correlation (Spearman r=0.4052) between levels of secreted p24 measured by ELISA and the percentage of intracellular p24+ cells (%) measured by flow cytometry (p=0.0142) (Figure 8).

Flow cytometry was used to analyse cells for intracellular p24 (MFI) and compared to secreted p24 measured by ELISA. There was significant correlation (Spearman r=0.5019) between levels of secreted p24 (ng/µL) and levels of intracellular p24 (MFI) measured by flow cytometry (p=0.0016) (Figure 9).

In conclusion, there is a correlation between the levels of secreted p24 (measured by ELISA) to intracellular p24, measured by flow cytometry both using p24% and p24 MFI.

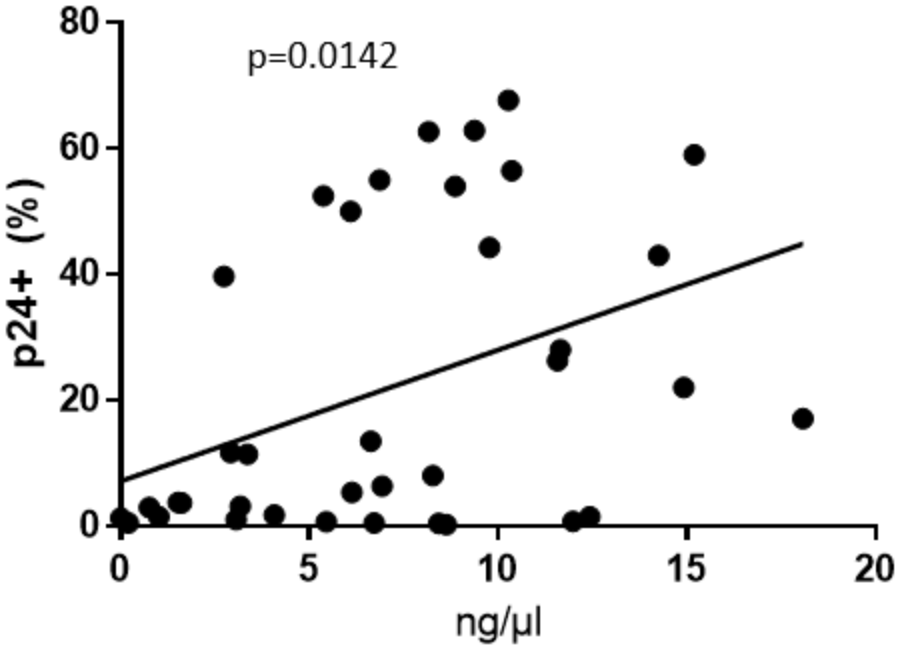


Figure 8. Comparing levels of p24 secreted into supernatants by ELISA (ng/µL) to intracellular p24+ cells by flow cytometry (%).

Cell supernatants were collected at 96 hours post infection and analyzed by ELISA for levels of p24 (ng/µL). Cells were also collected at 96 hours and stained for p24. By comparing HIV-uninfected cell subsets to infected cell subsets I determined the percentage of p24+ cells. Non-parametric Spearman correlation tests were performed to assess the correlation between the groups (95% confidence level, or $p<0.05$).

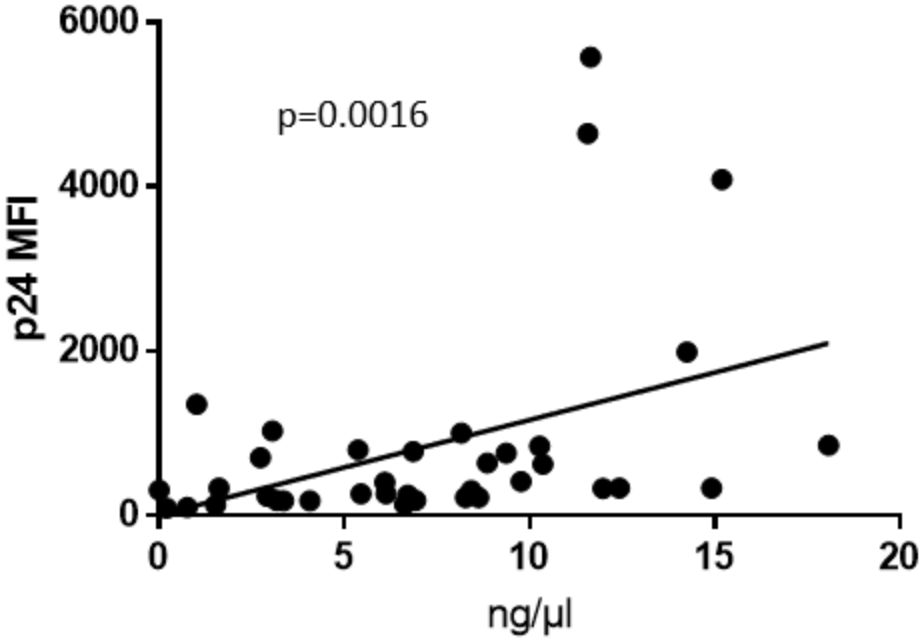


Figure 9. Comparing levels of p24 secreted into supernatants by ELISA (ng/µL) to levels of p24 measured by flow cytometry (MFI).

Cell supernatants were collected at 96 hours post infection and analyzed by ELISA for levels of p24 (ng/µL). Cells were also collected at 96 hours and stained for p24. Flow cytometry was used to measure the expression of p24. This figure shows median fluorescence intensity (MFI) of the PE-secondary antibody for expression of p24. Non-parametric Spearman correlation tests were performed to assess the correlation between the groups (95% confidence level, or $p<0.05$).

Viral Stock Production

PBMCs (50-75x10^6 cells) were stimulated with 5 ug/mL Phytohemagglutinin-L (PHA-L) (catalogue number L2769-10MG, Sigma) in complete RPMI media supplemented with 20 U/mL Human rIL-2 (catalogue number 136, NIH AIDS Reagent Program) for 72-hours at 37°C, 5% CO_2. Cells were then washed with complete RPMI and spun at 524 x g for 10 minutes, counted, and 20x10^6 cells were then infected with 3-4 vials of HIV-1 virus stocks in the level 2+ laboratory. Previously cultured PBMCs were kept in culture to be used later as feeder cells. The cell/virus suspension was incubated for 4 hours at 37°C, 5% CO_2, and then brought up to 10 mL with RPMI supplemented with 20 U rIL-2. The following day (Day 1), the cells were spun for 10 minutes at 524 x g, and then re-suspended in complete RPMI media. On day 4 the cells were washed and counted, an aliquot was removed for p24 ELISA analysis and 20x10^6/10mL feeder cells were added to the infection. If the ELISA was positive, the supernatant was harvested, and the remaining cells were fed with complete RPMI media and monitored by p24 ELISA. The three HIV-1 viruses used were ML 1956 (a clinical isolate, an HIV-1 subtype A1 from Nairobi, Kenya), IIIB and BaL (both HIV-1 subtype B, Laboratory strains).

$TCID_{50}$

PBMCs (50-100x10^6) were stimulated with 5 ug/mL PHA in complete RPMI media supplemented with 20 U/mL human rIL-2, and incubated for 72 hours at 37°C, 5% CO_2. Cells were then harvested, spun at 524 x g for 10 minutes, re-suspended, counted, and then taken to CL2+. In a 96 well plate, 150 µL complete RPMI media was added to A12 and B12 (virus control wells). To columns 1 and 3 to 12, 150 µL complete RPMI media was added except for the rows A and B. 133 µL was added to column 2, except for rows A and B. Virus was thawed and 67 µL was added to column 2, and 50 µL was added to A12 and B12. 50 µL was serially diluted from column 2 to column 12 (C to H only) and the last 50 µL was discarded. 50 µL of the well-mixed PBMCs were added to rows C to H and then incubated at 37°C, 5% CO_2 overnight.

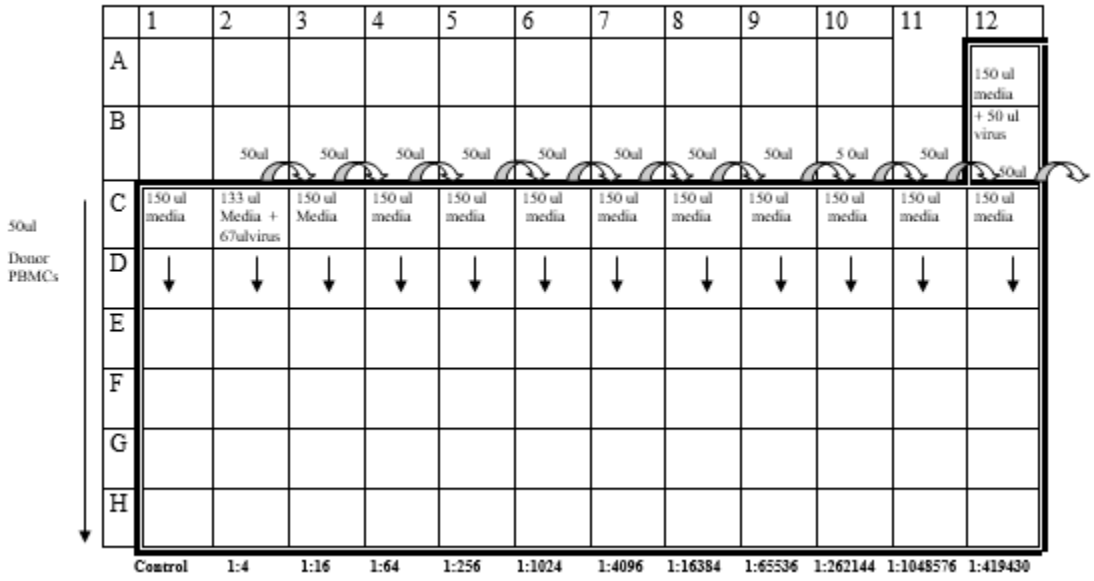


Figure 10. Outline of 96-well plate used in $TCID_{50}$ calculations

The next day (Day 1), 150 µL supernatant was removed and 150 µL complete RPMI media was added back. Plates were spun at 524 x g for 5 minutes and then another 150 µL was removed and 160 µL complete RPMI media was added back. On day 3, 100 µL supernatant was removed and 110 µL complete RPMI media was added back. On day 6, 135 µL of supernatant was removed and added to a final dilution of 1% Triton X-100 for p24 ELISA analysis. 130 µL of complete RPMI media was then added to the plate. Supernatant was again harvested at day 12.

The Effect of IRF-7 Knockdown in CD4+ T Cell Subsets on HIV Infection by IRF-7 Specific Lentivirus

Lentivirus Production

Amplification of Plasmid

IRF1 (V2LHS_133394 172_0387, V2LHS_133391 172_0467, V2LHS_133392 172_0208) and IRF7 (V2LHS_191909 172_0257) GIPZ shRNA lentiviral human clones were purchased from the Lentiviral Core Services at the University of Manitoba. These plasmids contain GFP for visual confirmation of transfection or transduction by fluorescent microscope or by flow cytometry. IRF-1 and IRF-7 cDNA lentiviral vectors were purchased from Applied Biological Materials (cat # LV191802 and LV798857, respectively). These plasmids contain GFP or RFP for visual confirmation of transfection or transduction by fluorescent microscope or by flow cytometry. Plasmid amplification was performed by using the Subcloning Efficiency™ DH5α™ Competent Cells (cat # 18265017, Invitrogen) according to manufactures protocol. In brief, one tube of DH5α™ cells was thawed on ice and then placed in a 1.5 mL microcentrifuge tube. The DH5α™ cells were gently mixed with a pipette tip and then aliquots of 50 µL of the DH5α™ cells were made. 1-10 ng of DNA was added to the DH5α™ cells and gently mixed followed by incubation on ice for 30 minutes. DH5α™ cells were then heat shocked for 20 seconds in a 42°C water bath, followed by a 2 minute incubation on ice. 950 µL of pre-warmed LB broth was added to the tube and then incubated at 37°C for 1 hour on a plate shaker at 225 RPM.

20-200 µL of this DH5α™ culture was then streaked onto pre-warmed antibiotic selective plates for single colonies that bear the plasmid and cultured at 37°C overnight.

Single colonies on the LB and Ampicillin plates were selected and transferred to 50 mL conical tubes containing 2-5 mL LB + Ampicillin broth for 8 hours at 37°C with vigorous shaking at 225 RPM. 250 µL of the starter culture was then diluted in 250 mL LB + Ampicillin and incubated at 37°C for 12-16 hours with vigorous shaking at 225 RPM. Mini and Maxi preps were performed by using the QIAprep Spin Miniprep Kit (250) (catalogue number 27106, Qiagen) and EndoFree Plasmid Maxi Kit (10) (catalogue number 12362, Qiagen), respectively, according to manufacturer's protocol using the buffers provided in the kit; P1, P2, P3, ER, QBT, QN, and QC. The following protocol described is the EndoFree Plasmid Maxi Kit. The bacterial cells were harvested by centrifugation at 6000 x g at 4°C for 15 minutes. Culture supernatant was discarded and decontaminated in 10% bleach and then the bacterial pellet was re-suspended in 10 mL of Buffer P1. 10 mL of Buffer P2 was added; the tube was then inverted 4-6 times and incubated at room temperature for 5 minutes, followed by the addition of 10 mL of Buffer P3 and inversion of the tube 4-6 times. The lysate was then poured into the barrel of the QiaFilter cartridge and incubated at room temperature for 10 minutes. The plunger was then inserted and the cell lysate was filtered into a new 50 mL tube. 10 mL Buffer ER was added, the tube was inverted 10 times, and then incubated on ice for 30 minutes. 10 mL buffer QBT was then added to a Qiagen-tip 500 followed by the addition of cell lysate. The Qiagen-tip was then washed twice with 30 mL buffer QC, and then eluted with 15 mL buffer QN. DNA was precipitated by adding 10.5 mL isopropanol followed by a centrifugation at 15,000 x g for 30 minutes at 4°C. The pellet was then washed with 5 mL of 70% ethanol and centrifuged at 15,000 x g for 10 minutes at 4°C, and the supernatant decanted. The pellet was allowed to air dry for 5 to 10 minutes and then dissolved in 250 µL buffer TE.

Sequencing

Sequencing was performed on each isolate at the National Microbiology (NML) DNA Core Services. Plasmids

were submitted at a concentration of 150 ng/µL in 5 µL, and primers were submitted at a concentration of 1 µM in a minimum 5 µL reaction volume. Sequencing results were confirmed for homology by using Unipro UGENE and compared to a reference gene from NBCI.

Calcium-Phosphate Transfection

The day before transfection, $15x10^6$ cells of Lenti-X™ 293T Cell Line (catalogue number 632180, ClonTech) growing at linear phase were plated at $15x10^6$ cells in a T175 flask in 40 mL complete DMEM (catalogue number 12430-054, Life Technologies) (DMEM supplemented with 10% FBS and 1% Pen-strep) and incubated overnight at 37°C, 5% CO_2. The following day the media was removed from the flask, and 25 mL complete DMEM with 100 µL 10 mM chloroquine (catalogue number C6628, Sigma) was added to the flask without disturbing the cells and then placed back into the incubator. DNA was prepared in a separate 50 mL tube containing: 12.5 µg of expression vector, 12.5 µg of packaging vector, and 5 µg of envelope vector. H_2O was also added to a final volume of 977 µL. The suspension was mixed well and incubated on ice for 10 minutes. This was followed by the addition of 133 µL $CaCl_2$ drop by drop, mixed vigorously by vortexing, and then incubated on ice for 5 minutes. 1110 µL 2X HEPES-buffered saline (HBS) was then added drop by drop, mixed vigorously by vortexing, and incubated on ice for 20 minutes. The mixture was then added and incubated for an additional 6-8 hours. The mixture was then removed from the flask and 40 mL DMEM supplemented with 10% FBS and 1% Pen-strep was added. The cells were incubated for 3 days at 37°C, 5% CO_2. Supernatant from the calcium-phosphate transfection was then collected and centrifuged at 500 x g for 10 minutes. The supernatant was transferred to a new tube and 1 volume of Lenti-X™ Concentrator (catalogue number 631231, Clontech) was added to 3 volumes of supernatant, mixed by gentle inversion, and then incubated overnight at 4°C. The following day, the cells were spun at 1500 x g for 45 minutes and the supernatant was removed. The pellet was re-suspended in 150 µL PBS and single-used aliquots were created and stored at -80°C.

Stimulation (CD3/CD28 Activation Beads)

CD4+ T cells were stimulated using Dynabeads® Human T-Activator CD3/CD28 (catalogue number 11131D, Life Technologies) according to manufactures protocol. In brief, the Dynabeads® were washed before use by transferring the desired amount of cells and 1 mL staining buffer to a 5 mL polystyrene tube. The tube was vortexed and then placed on an EasySep™ Magnet for one minute and the supernatant was discarded. CD4+ T cells were stimulated by seeding $1x10^6$ cells in 2 mL complete RPMI per well in a 24-well plate and adding 25 µL/well Dynabeads and 30 U/mL rIL-2. The cell/bead suspension was incubated for 3 days at 37°C, 5% CO_2.

Lentivirus Titration

Following the CD4+ T cell activation, cells were pooled into a 15 mL tube and spun down at 524 x g for 8 minutes. The cells were then re-suspended in only RPMI in a 5 mL polystyrene tube and left on an EasySep Magnet for 1 minute at room temperature. The Dynabeads® adhered to the magnet and by inverting the tube the activated CD4+ T cells were collected. Cells were counted and re-suspended at a concentration of $2x10^6$ cells/mL. 50 µL of virus, RPMI, and 4 µg/mL polybrene was added to the wells of a 96-well plate in the varying appropriate dilutions. 50 µL of the cell suspension was added for a final volume of 100 µL per well. The plate was centrifuged at 37°C for one hour at 1000 x g, followed by incubation at 37°C, 5% CO_2 for one hour with tapping the plate every 15 minutes. Following the two-hour incubation, 100 µL of 2X culture media (RPMI + 20% FBS + 2% Pen-strep) with 20 U/mL r-IL-2 final volume was added and the plate was incubated overnight. The following morning the plate was spun at 335 x g for 5 minutes and then 135 µL supernatant was removed and replaced with 135 µL fresh RPMI + 10% FBS + 1% Pen-strep. The plate was incubated at 37°C, 5% CO_2 for three days and periodically checked under a fluorescent microscope for the presence of RFP/GFP. Once RFP/GFP was visible, the cells were harvested. Depending on the level of RFP or GFP visible I used that MOI in further experiments with that specific lentiviral particle. Cells were stained for IRF-1 or IRF-7

expression and analyzed by flow cytometry.

Cell Sorting

The GFP positive cells were sorted by the Flow Cytometry Core at the University of Manitoba. The cells were suspended at a concentration of $10x10^6$ cells/mL in PBS + FBS and filtered to remove clumps. After cell sorting, the cells are collected in RPMI + 30% FBS and rested overnight before infection with HIV at an MOI of 1. Supernatants were collected at 24, 72, and 96 hours post infection.

Cytokine Measurement

Cytokine levels were determined using the Human Cytokine/Chemokine Magnetic Bead Panel from Milliplex MAP multiplex kit (catalogue number HCYTOMAG-60K, Millipore, Billerica, MA). Supernatants collected from HIV infections of cells with IRF-7 knockdown were assessed for the presence of IFN-α2, IFN-γ, interleukin 10 (IL-10), IL-12p70, IL-15, IL-2, IL-6, IL-4, and tumor necrosis factor alpha (TNF-α) according to the manufacturer's overnight protocol. In brief, 200 µL of wash buffer was added to each well of a 96-well plate, followed by incubation at room temperature (20-25°C) for 10 minutes on a plate shaker at 300 RPM. Wash buffer was then removed by decanting. 25 µL of standards or controls was added to the appropriate wells and 25 µL of assay buffer was added to the sample wells. 25 µL of cell culture media (RPMI) was added to the standard and control wells and 25 µL of sample (supernatant) was added to the sample wells. Then, 25 µL of well-mixed detection beads were added to all wells. The plate was then sealed and incubated overnight at 4°C on a plate shaker at 300 RPM. The next day (16-18 hours), the plate was washed twice with wash buffer (200 µL/well). 25 µL of detection antibodies were then added followed by 1-hour incubation at room temperature on a plate shaker. 25 µL of Streptavidin-Phycoerythrin was then added to each well followed by 30 minutes incubation at room temperature on a plate shaker. The plate was washed twice with wash buffer (200 µL/well) followed by the addition of 150 µL of sheath fluid to each well. The plate was then read on the

BioPlex-200 (Bio-Rad, Mississauga, ON, Canada). All statistical analysis was performed using GraphPad Prism, version 7. I used column statistics (D'Agostino-Perason omnibus normality test) to determine if our sample results followed a normal distribution. Between two paired groups I used t-tests for parametric data and Wilcoxon matched-pair test for non-parametric, for unpaired non-parametric data I used Mann-Whitney test and unpaired parametric data I used an un-paired t-test. Minimal detection assay sensitivities were: 2.9 pg/mL for IFN-α2, 0.8 pg/mL for IFN-γ, 1.1 pg/mL for interleukin 10 (IL-10), 0.6 pg/mL for IL-12p70, 1.2 pg/mL for IL-15, 1.0 pg/mL for IL-2, 0.9 pg/mL for IL-6, 4.5 pg/mL for IL-4, 0.7 pg/mL for tumor necrosis factor alpha (TNF-α). For levels of cytokines that were below the detection threshold a value of ½ of the minimum detectable concentration (MinDC) was assigned in the analysis.

Results

Section 1: Phenotyping IRF-1 and IRF-7 Expression in Manitoban and Kenyan Donors

Rationale: IRF-1 has been shown to be constitutively expressed in most cell types, however there are limited studies on cell-type specific IRF-1 expression in PBMCs. There is conflicting data on the expression of IRF-7 in various cell populations, as most studies suggest that IRF-7 is only expressed in certain cell types such as peripheral blood lymphocytes and DC (*92*). Further, they claim that its expression can only be induced in other immune cell types by IFNs and viral infections(*91, 92*).
To better understand IRF-1 and IRF-7 in *ex-vivo* human immune cells the expression of both IRF-1 and IRF-7 was examined in defined PBMC subsets by comparing unstimulated and interferon-stimulated PBMCs. A second goal was to establish the baseline expression of IRF-7 in CD4+ T cells (CD3+CD4+CD8-) to later determine the effects of knocking down IRF-7 expression by both electroporation and lentiviral transduction on HIV replication in these cells. I also aimed to determine if there was an association between HIV infection and IRF-7 expression in HIV-uninfected individuals compared to HIV-infected from Nairobi, Kenya, as the literature suggests that IRF-7 plays a role in HIV infection. It has been repeatedly shown that IRF-7 is upregulated during HIV-1 infection, and that individuals infected with HIV-1 have increased levels of IRF-7 (*6, 105-107*). These studies should provide information on the levels of expression of IRF-1 and IRF-7 in various immune cell populations, which will aid in future studies on these specific cell types. As well, these studies will determine differences in IRF-7 expression in HIV-1 infected individuals compared to uninfected, a factor which may affect their susceptibility to HIV-infection. Increased levels of IRF-7 in HIV-positive populations could suggest that individuals who express higher levels of IRF-7 may be more susceptible to HIV infection, or that HIV infection increases IRF-7 levels in infected individuals.

Hypothesis:

The literature has shown that IRF-1 is constitutively expressed in all cell types and IRF-7 is expressed in peripheral blood lymphocytes (*88*), therefore I hypothesized that IRF-1 and IRF-7 were constitutively expressed in all defined subsets of human PBMC examined.

The literature has also shown that IRF-7 expression is increased during HIV infection (*6, 105-107*). I hypothesized that IRF-7 expression will be higher in the PBMC of HIV-infected individuals compared to HIV-uninfected healthy controls.

IRF-7 expression will be higher in the Kenyan samples compared to the Manitoban due to exposure to HIV-1.

Objectives:

1. Characterize the expression of IRF-1 and IRF-7 in all defined cell subsets CD4+ T cells (CD3+CD4+CD8-), CD8 T cells (CD3+CD8+CD4)-, B cells (CD3-CD19+), cytotoxic NK cells (CD16+CD56dim), cytokine producing NK cells (CD16-CD56high), inflammatory monocytes (CD14+CD16+), patrolling monocytes (CD14dimCD16+), classical monocytes (CD14highCD16-), and DC (HLA-DR+CD11c+CD16+) in blood from healthy local Manitobans.

2. Characterize the responsiveness of IRF-1 and IRF-7 after stimulation with IFN-γ and IFN-αA, respectively, in healthy local Manitoban PBMCs.

3. Compare the expression of IRF-7 in PBMCs between HIV-infected and HIV-uninfected subjects from Nairobi, Kenya and characterize the responsiveness of IRF-7 after stimulation with IFN-αA.

4. Compare the expression of IRF-7 between HIV-uninfected donors from Manitoba and Nairobi, Kenya to HIV-infected donors from Nairobi, Kenya

Experimental Approach:

Expression of IRF-1 and IRF-7 was analyzed in 20 and 18 different Manitoban donor samples, respectively, from the University of Manitoba or J.C. Wilt Infectious Diseases Research Centre. To do this, Manitoban donor blood was collected, followed by the isolation and cryopreservation of PBMCs. At the time of experimentation, the cells were thawed, rested, and stained with antibodies for analysis by flow cytometry to determine cell type and IRF-1 or IRF-7 expression levels. Manitoban donor samples were stimulated with IFN-γ or IFN-αA for IRF-1 and IRF-7 expression, respectively, to measure induction of IRF-1 and IRF-7 responses to immune stimulation. Staining for Mx-1 was used as a positive control in cells stimulated by PMA and Ionomycin.

For studies involving Kenyan samples, PBMCs were isolated in Nairobi, Kenya, and then shipped to Winnipeg. 12 HIV-uninfected and 15 HIV-infected samples were analyzed. At the time of experimentation, the cells were thawed, rested, and stained with antibodies for analysis by flow cytometry to determine cell type and IRF-7 expression. Samples were stimulated with IFN-αA for measuring IRF-7 responses, while IFN-γ was used as a positive stimulation control for IRF-1 (*73, 123*). DCs are not included in all of the analysis, as they were difficult to gate on due to low cell numbers.

Objective 1- Characterization of the expression of IRF-1 and IRF-7 in T cells (CD3+), B cells (CD3-CD19+), NK cells (CD16+CD56dim)/(CD16-CD56high), monocytes (CD14+), and DC (HLA-DR+CD11c+CD16+) in blood from Manitoban donors.

IRF-1 is constitutively expressed in all subtypes of PBMC, and can be further induced by IFN-γ. Not all PBMCs express IRF-7 constitutively, but IRF-7 expression can be induced in all cell types by IFN-αA.

The expression of IRF-1 and IRF-7 was examined in CD4+ T cells (CD3+CD4+CD8-) and classical monocytes (CD14highCD16-) Classical monocytes were chosen (CD14highCD16-) as a representative of the monocyte population as they are the most common monocyte population. CD4+ T cells (CD3+CD4+CD8-) were chosen as a representative of the lymphocyte population as T cells are the most

common lymphocytes I analyzed and CD4+ T cells are the most common T cells. As well they are both (CD4+ T cells and monocytes) targets of HIV-1. I utilized flow cytometry to determine the percentage of cells expressing IRF-1 and IRF-7 in CD4+ T cells (CD3+CD4+CD8-) and classical monocytes (CD14highCD16-) both before and after stimulation with IFN-γ and IFN-αA. I measured IRF-1 expression in 20 donors and IRF-7 expression in 18 donors.

IRF-1 and IRF-7 were expressed at differing levels in CD4+ T cells (CD3+CD4+CD8-) and in classical monocytes (CD14highCD16-). IRF-1 was expressed at a higher percentage than IRF-7 in both CD4+ T cells (CD3+CD4+CD8-) and classical monocytes (CD14highCD16-). There were a higher percentage of classical monocytes (CD14highCD16-) expressing both IRF-1 and IRF-7 compared to the CD4+ T cells (CD3+CD4+CD8-).

The percentage of CD4+ T cells (CD3+CD4+CD8-) expressing IRF-1 before stimulation was between 75.6-99.8%, and after stimulation with IFN-γ increased to 94.2-99.2% (Figure 11 A). There was a significant increase in the percentage of CD4+ T cells (CD3+CD4+CD8-) expressing IRF-1 after stimulation with IFN-γ compared to resting cells from (75.6-99.8%) to (94.2-99.9%) ($p \leq 0.05$). When IRF-7 expression was examined, the percentage of CD4+ T cells (CD3+CD4+CD8-) expressing IRF-7 before stimulation was more variable (~30-100%) among donor samples (Figure 11 B). However, after stimulation with IFN-αA, the percentage of CD4+ T cells (CD3+CD4+CD8-) expressing IRF-7 increased significantly (28.8-99.2%) ($p \leq 0.01$). When the expression of IRF-1 was examined in the monocyte population there was a very high percentage (87.1-100%) of classical monocytes (CD14highCD16-) expressing IRF-1 in rested cells prior to stimulation, and after stimulation with IFN-γ this increased to 96.1-100% (Figure 11 C); this was not statistically significant. There were a higher percentage of classical monocytes (CD14highCD16-) expressing IRF-7 before stimulation (~65-100%) in relation to CD4+ T cells (Figure 11 D). When IRF-7 was examined after stimulation with IFN-αA, the percentage of classical monocytes (CD14highCD16-) expressing IRF-7 increased to 79.0-100% ($p \leq 0.05$). In conclusion, IRF-1 is expressed almost all cells and

can be increased after stimulation with IFN-γ, while the expression of IRF-7 is more variable prior to stimulation but can increase in response to IFN-αA stimulation.

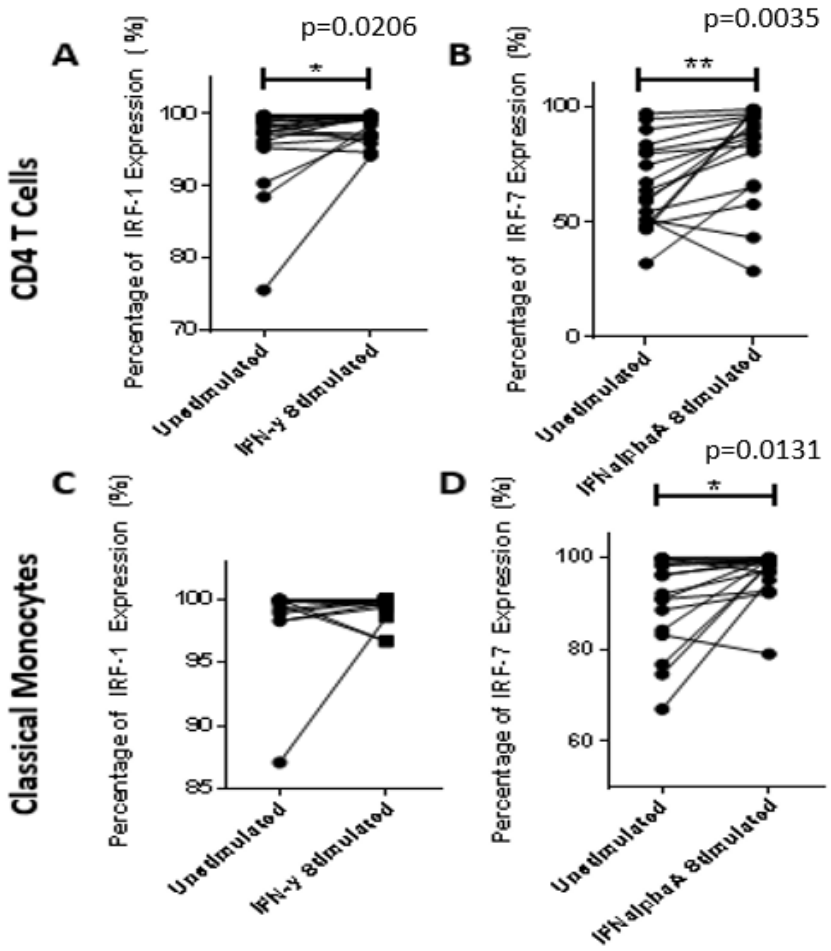


Figure 11. Comparison of the percentage of CD4+ T cells (CD3+CD4+CD8-) (A and B) and classical monocytes (CD14highCD16-) (C and D) expressing IRF-1 (A and C) and IRF-7 (B and D) before and after stimulation in Manitoban donors.

Flow cytometry was used to measure the percentage of cells expressing IRF-1 and IRF-7 in CD4+ T cells (CD3+CD4+CD8-) and classical monocytes (CD14highCD16-) from 20 and 18 Manitoban donors, respectively. These figures show the percentage of cells expressing IRF-1 (A and C) and IRF-7 (B and D) both before and after stimulation. Paired two-tailed Wilcoxon matched-pairs signed rank statistical tests were performed to assess the expression differences between the groups (95% confidence level, or $p \leq 0.05$).

IRF-1 is constitutively expressed in all subtypes of PBMC with the highest expression in inflammatory monocytes (CD14+CD16+), and IRF-7 is expressed in all PBMC subsets with the highest expression in patrolling (CD14dimCD16+) and inflammatory (CD14+CD16+) monocytes.

To determine IRF-1 and IRF-7 expression in defined PBMC immune subsets I used flow cytometry to measure the levels of IRF-1 and IRF-7 expression in unstimulated PBMCs (Figure 12). Levels of IRF-1 and IRF-7 were measured in 20 and 18 donors, respectively. Cell types were first determined based on cell size to differentiate monocytes and lymphocytes. Cell types were then resolved based on cell surface markers (*116-120, 124*).

IRF-1 and IRF-7 are expressed in all cell types examined at differing levels. There appears to be much higher expression of IRF-7 in monocytes compared to lymphocytes, therefore I will assess these groups separately for all analysis from this point forward. IRF-1 and IRF-7 were expressed in every cell subset that was measured. The highest expression of IRF-1 was in the inflammatory monocytes (CD14+CD16+) (median = 11059) compared to the classical (CD14highCD16-) (median = 9018 $p \leq 0.01$) and patrolling (CD14dimCD16+) monocytes (median = 9270 $p \leq 0.001$) (Figure 12 A). Between the lymphocyte populations, there was significantly higher IRF-1 expression in CD4+ T cells (CD3+CD4+CD8-) (mean = 4302) compared to B cells (CD3-CD19+) (mean = 3867 $p \leq 0.01$). There was significantly higher IRF-1 expression in the CD8+ T cell (CD3+CD8+CD4-) (mean = 4426) populations compared to the B cells (CD3-CD19+) (mean = 3867 $p \leq 0.001$), and cytotoxic (CD16+CD56dim) (mean = 4008 $p \leq 0.01$) and chemokine producing (CD16-CD56high) (mean = 4117 $p \leq 0.05$) NK cells.

The highest expression of IRF-7 was in the non-classical cell subsets: patrolling (CD14dimCD16+) (median = 6343 $p \leq 0.001$) and inflammatory (CD14+CD16+) (median =6424 $p \leq 0.001$) compared to classical monocytes (CD14highCD16-) (median = 5430) (Figure 12 B). The highest expression of IRF-7 between the lymphocyte populations was in the cytotoxic NK cell (CD16+CD56dim) (median = 2118) populations. In the unstimulated lymphocytes, I observed significantly higher expression of IRF-7 in the CD4+ T cell

subset (CD3+CD4+CD8-) (median = 1896) compared to B cells (CD3-CD19+) (median = 1588 $p \leq 0.05$). There was also significantly higher expression of IRF-7 in the cytotoxic NK cell subset (CD16-CD56high) (median = 2118) compared to the CD8+ T cell subset (CD3+CD4-CD8+) (median = 1816 $p \leq 0.001$), the B cells (CD3-CD19+) (median = 1588 $p \leq 0.001$), and the cytokine producing NK cells (CD16+CD56dim) (median = 1762 $p \leq 0.001$).

These results show that IRF-1 and IRF-7 are expressed in every cell subset within PBMCs analysed in this project, with the highest levels of expression of IRF-1 in inflammatory monocytes (CD14+CD16+) and the highest levels of IRF-7 in inflammatory (CD14+CD16+) and patrolling (CD14dimCD16+) monocytes.

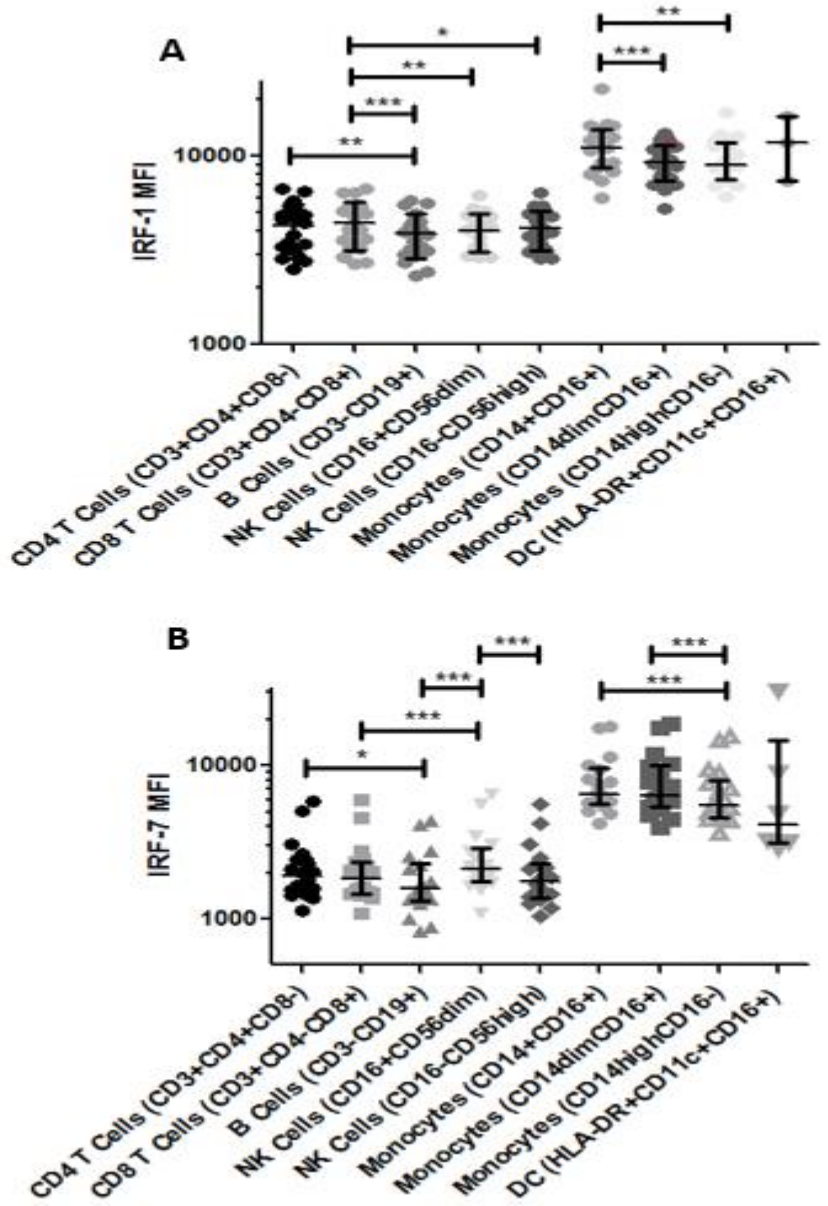


Figure 12. The expression of IRF-1 (A) and IRF-7 (B) in rested Manitoban donor PBMC populations.

Flow cytometry was used to measure the expression of IRF-1 and IRF-7 in 20 and 18 PBMC samples, respectively, from local Manitoban donors. This figure shows median fluorescence intensity (MFI) of the PE-secondary antibody for expression of IRF-1 and IRF-7 with each point representing the results from a single donor. One-way ANOVA and Dunn's and Tukey's multiple comparisons post-test were performed between the lymphocytes and between the monocyte populations to assess the expression differences

between the groups (95% confidence level, or $p \leq 0.05$). Horizontal bars represent the mean +/- standard deviation or median +/- interquartile range.

Highest expression of IRF-1 and IRF-7 in the monocyte and DC sub-populations compared to the lymphocyte (T-, B-, and NK cell populations).

To better demonstrate that IRF-1 and IRF-7 were expressed at a higher level in monocytes (CD14+) and DC (HLA-DR+CD11c+CD16+) compared to lymphocytes, flow cytometry was used to measure IRF-1 and IRF-7 expression in 20 and 18 donors, respectively.

Significantly higher expression of IRF-1 and IRF-7 was observed in the monocyte (CD14+) and DC (HLA-DR+CD11c+CD16+) sub-populations compared to lymphocytes (2.4-fold increase in IRF-1 expression, median lymphocytes = 4045 and median monocytes and DC = 9548, $p \leq 0.0001$) (3.3-fold increase in IRF-7 expression, median lymphocytes = 1854 and median monocytes and DC = 6047 (Figures 13 A and B).

Both IRF-1 and IRF-7 are expressed at a higher level in monocytes (CD14+) and DC (HLA-DR+CD11c+CD16+) compared to lymphocyte populations, which suggests IRF-1 and IRF-7 may play a more important role in DC and monocyte function.

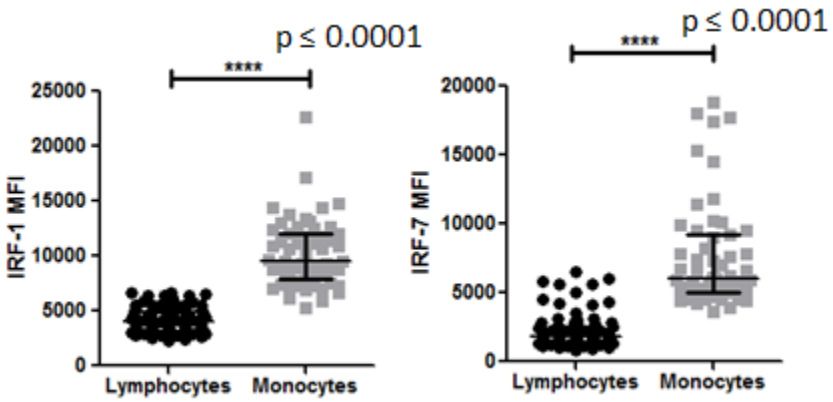


Figure 13. Comparison of the expression levels of IRF-1 (A) and IRF-7 (B) from Manitoban donors in lymphocytes compared to monocytes (CD14+) and DC (HLA-DR+CD11c+CD16+).

Flow cytometry was used to measure the expression of IRF-1 and IRF-7 in 20 and 18 PBMC samples, respectively, from Manitoban donors. This figure shows median fluorescence intensity (MFI) of the PE-secondary antibody for expression of IRF-1 and IRF-7 with each point representing the results from a single individual. Unpaired two-tailed Mann-Whitney statistical tests were performed to assess the expression differences between the groups (95% confidence level, or $p \leq 0.05$). Horizontal bars represent the median +/- the interquartile range.

Objective 2 - Characterizing the responsiveness of IRF-1 and IRF-7 after stimulation with IFN-γ and IFN-αA, respectively in healthy local Manitoban PBMCs

Highest expression of IRF-1 in inflammatory monocytes (CD14+CD16+) after IFN-γ stimulation, and highest expression of IRF-7 in patrolling (CD14dimCD16+) and inflammatory (CD14+CD16+) monocytes in both unstimulated and IFN-αA stimulated PBMCs.

To determine the levels of IRF-1 and IRF-7 in PBMC subsets after stimulation with IFN-γ and IFN-αA, respectively, flow cytometry was used to measure the expression of IRF-1 and IRF-7 after stimulating the cells with IFN-γ for 3 hours or with IFN-αA for 16-18 hours. I measured IRF-1 and IRF-7 expression in 20 and 18 donors, respectively. There appears to be the highest expression of IRF-7 in the monocytes compared to the lymphocytes, therefore these groups were assessed separately. These experiments were performed to determine if expression of IRF-1 and IRF-7 could be upregulated by stimulation with interferons.

All PBMCs studied expressed IRF-1 and IRF-7 in varying levels. The highest expression after stimulation was in the monocytes and DC populations compared to the lymphocyte populations.

In PBMCs stimulated with IFN-γ, the highest expression of IRF-1 was found in the inflammatory monocytes (CD14+CD16+) (median = 15254) compared to the classical (CD14highCD16-) (median = 12338 $p \leq 0.01$) and patrolling (CD14dimCD16+) monocytes (median = 11746 $p \leq 0.001$). Between the lymphocyte populations there was significantly higher expression of IRF-1 in the CD8+ T cell (CD3+CD4-CD8+) (median = 5046) populations compared to the cytotoxic NK cell subsets (CD16+CD56dim) (median = 4919 $p \leq 0.05$) and the cytokine-producing NK cell subset (CD16-CD56high) (median = 4846 $p \leq 0.05$) (Figure 14 A).

In the IFN-αA stimulated PBMCs, the highest expression of IRF-7 was in the non-classical cell subsets (patrolling (CD14+CD16+) (median = 11728) and inflammatory (CD14dimCD16+) (median = 10642))

compared to the classical monocytes (CD14highCD16-) (median = 9560 p≤0.001) (Figure 14 B). The highest expression of IRF-7 in the lymphocyte populations was in the cytotoxic NK cell populations (CD16+CD56dim) (median = 3495). There was significantly higher expression of IRF-7 in the cytotoxic NK cell population (CD16+CD56dim) (median = 3495) subset compared to CD8+ T cells (CD3+CD4-CD8+) (median = 2905 p≤0.001) and B cells (CD3-CD19+) (median = 2708 p≤0.001).

In conclusion, after stimulation of IRF-1 and IRF-7 with IFN-γ and IFN-αA, respectively, I found the highest induction of IRF-1 in inflammatory monocytes (CD14+CD16+) and the highest induction of IRF-7 in inflammatory (CD14+CD16+) and patrolling (CD14dimCD16+) monocytes.

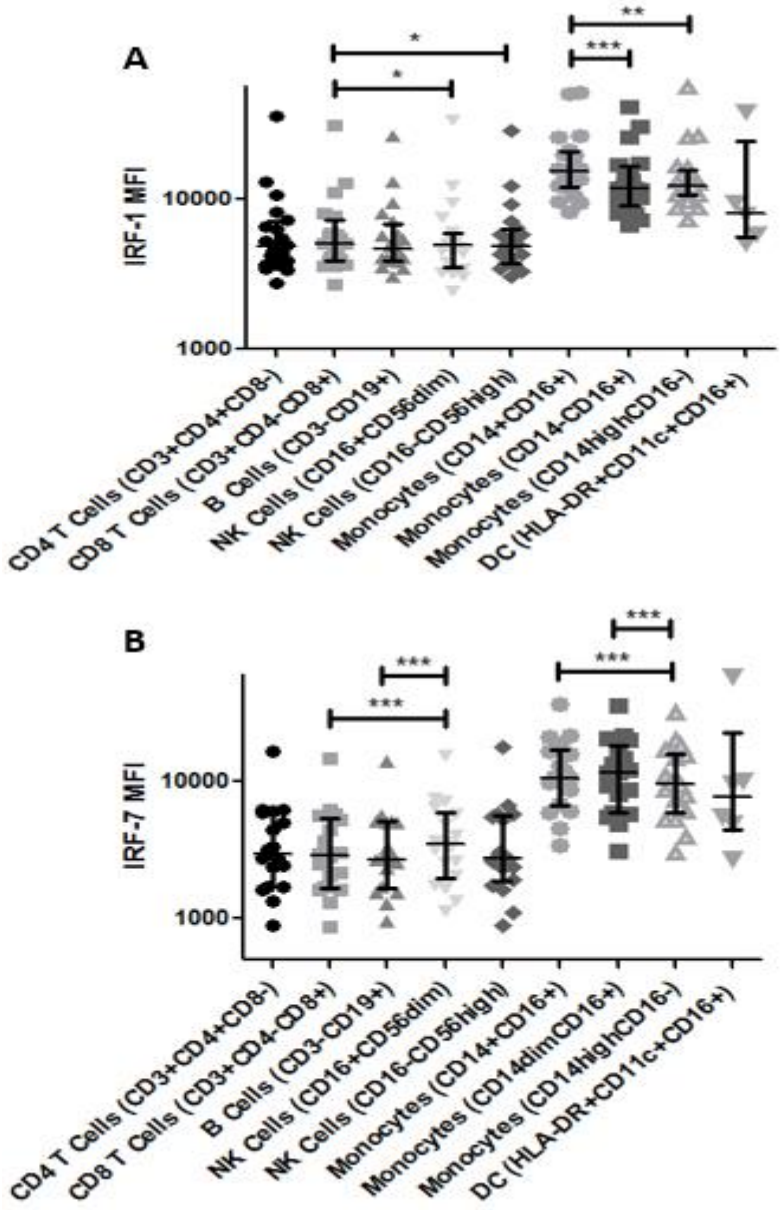


Figure 14. The expression of IRF-1 and IRF-7 in Manitoban donor PBMC populations after stimulation with IFN-γ and IFN-αA, respectively.

Cells were stimulated with IFN-γ for 3 hours to analyze IRF-1 expression and with IFN-αA for 16-18 hours for IRF-7 expression. Flow cytometry was used to measure the expression of IRF-1 and IRF-7 in 20 and 18 PBMC samples, respectively, from Manitoban donors. This figure shows median fluorescence intensity (MFI) of the PE-secondary antibody for expression of IRF-1 and IRF-7 with each point representing a single donor. One-way ANOVA and Dunn's multiple comparisons post-test were performed between the

lymphocytes and between the monocyte populations to assess the expression differences between the groups (95% confidence level, or $p \leq 0.05$). Horizontal bars represent the median +/- the interquartile range.

Responsiveness to stimulation by IFN-γ highest in patrolling monocytes (CD14dimCD16+) when comparing fold change in IRF-1 expression and B cells (CD3-CD19+) have the highest responsiveness to IFN-αA stimulation compared to all other PBMC subsets when comparing fold change in IRF-7 expression.

To determine the fold change of expression after stimulation (a different readout of IRF-1 and IRF-7 responsiveness), fold change differences were calculated by dividing the MFI of the stimulated samples by the MFI of the unstimulated samples (Figure 15). I measured 20 and 18 samples for IRF-1 and IRF-7 expression, respectively. The monocytes and lymphocytes were assessed separately. DCs were not included in the analysis as there were too few samples.

Almost all PBMC subsets demonstrated a moderate increase in IRF-1 post interferon induction with an average increase of 1.68-fold. For IRF-1 the response to stimulation was significantly higher in the patrolling monocytes (CD14dimCD16+) (1.619-fold increase) compared to the inflammatory monocytes (CD14+CD16+) (fold increase= 1.346 $p \leq 0.01$) and the classical monocytes (CD14highCD16-) (fold increase= 1.29 $p \leq 0.001$). Between the lymphocyte populations the fold change increase after stimulation was in the B cell subset (CD3-CD19+) (1.304-fold increase) compared to the cytotoxic (CD16+CD56dim) (1.241-fold increase $p \leq 0.05$), and cytokine producing (CD16-CD56high) (1.233-fold increase $p \leq 0.01$) NK cells (Figure 15 A).

For IRF-7, almost all PBMC subsets demonstrated an increase in IRF-7 post interferon induction with an average fold increase of 1.76. After comparing the fold change after stimulation with IFN- αA between the lymphocyte populations**,** the response to stimulation was significantly higher in the B cell subset (CD3-CD19+) (1.574-fold increase) compared to the CD8+ T cell subset (CD3+CD4-CD8+) (1.441-fold increase $p \leq 0.01$) and the cytotoxic NK cells (CD16+CD56dim) (1.482-fold increase $p \leq 0.01$) (Figure 15 B).

In conclusion, the highest responsiveness to stimulation by IFN-γ when analysing IRF-1 expression was in

the patrolling monocytes (CD14dimCD16+) and for IFN-αA when analysing IRF-7 expression was in B cells CD3-CD19+).

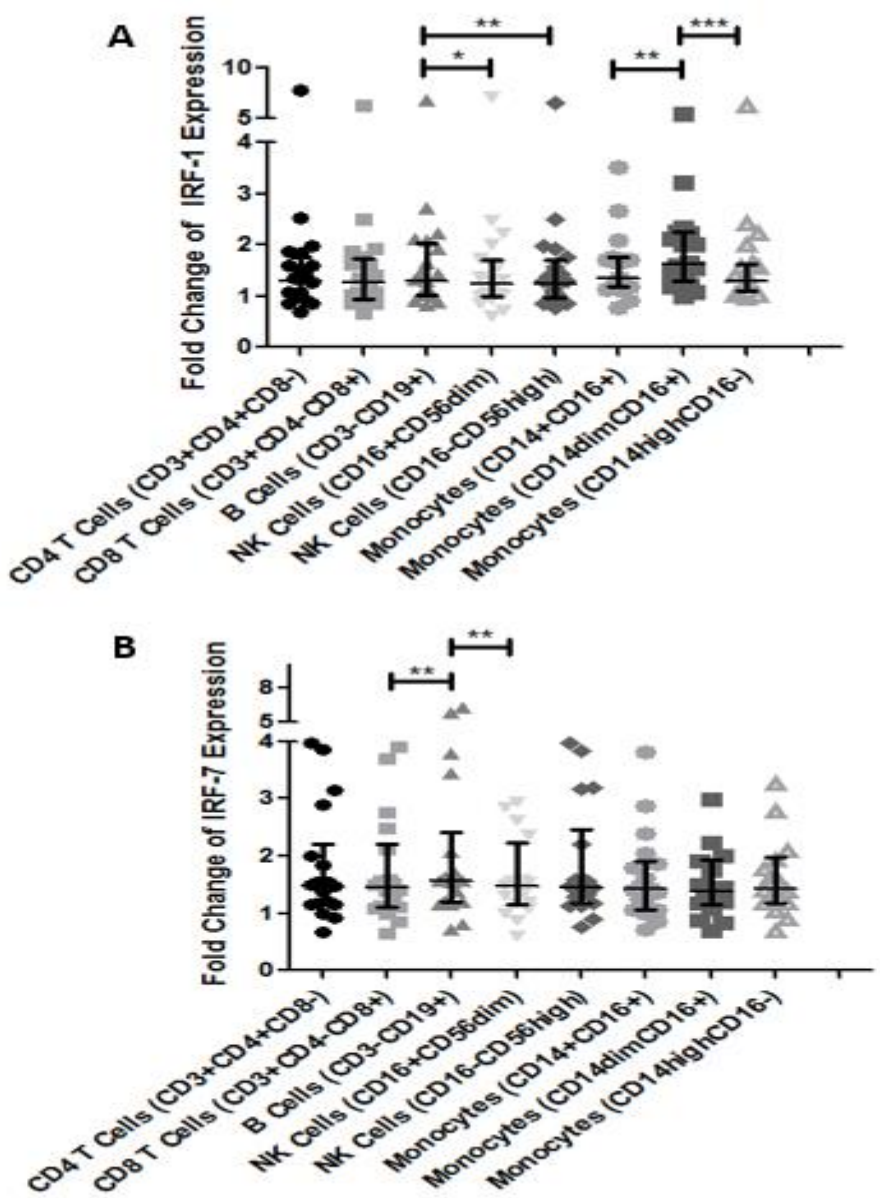


Figure 15. Comparison of the fold change difference of IRF-1 (A) and IRF-7 (B) expression in Manitoban donors of IFN-γ and IFN-αA stimulated cells compared to unstimulated in various cell populations.

Flow cytometry was used to measure the expression of IRF-1 and IRF-7 in 20 and 18 PBMC samples, respectively from Manitoban donors. This figure shows fold change differences by dividing the MFI of the PE-secondary antibody for expression of IRF-1 and IRF-7 of the IFN-γ and IFN-αA stimulated samples,

respectively, by the MFI of the unstimulated samples. One-way ANOVA and Dunn's multiple comparisons post-test were performed between the lymphocytes and between the monocyte populations to assess the expression differences between the groups (95% confidence level, or $p \leq 0.05$). Horizontal bars represent the median +/- the interquartile range.

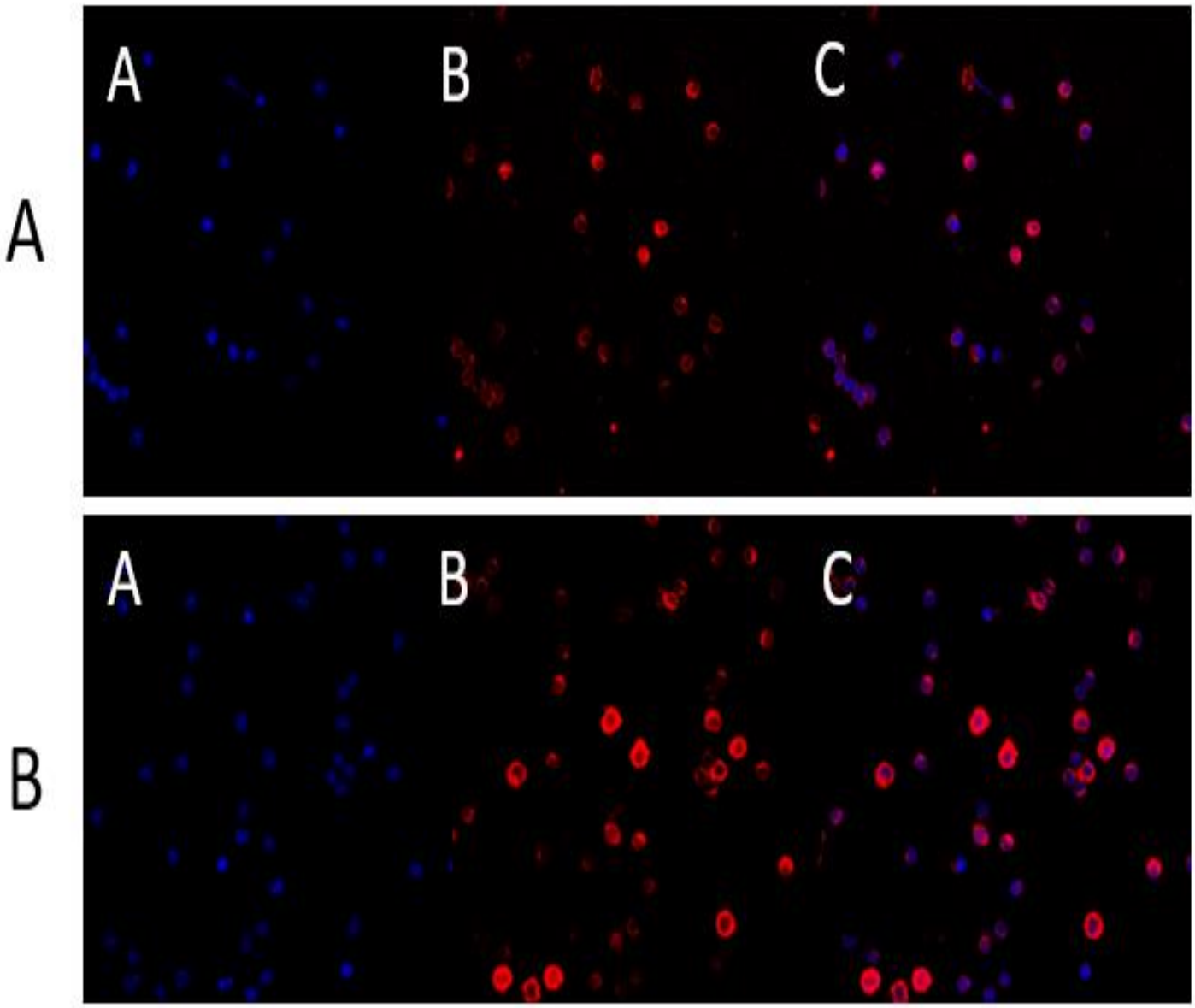
IRF-1 and IRF-7 expressed primarily in the cytoplasm in unstimulated PBMCs.

To determine the location in the cell where IRF-1 and IRF-7 are expressed, antibody protein staining and confocal microscopy were used to measure IRF-1 and IRF-7 expression in both unstimulated and IFN-γ and IFN-αA stimulated PBMCs in 2 donors each. PBMCs were stained with antibodies specific for IRF-1 and IRF-7, and then mounted onto slides using a mountant stain containing DAPI (stains nuclei). We were unable to measure for quantitative amounts of fluorescence due to being unable to standardize mean fluorescence intensity's. The images shown are representatives of what was seen in all images taken.

IRF-1 and IRF-7 were identified in both the cytoplasm and in the nucleus. IRF-1 was found to be mostly cytoplasmic in unstimulated PBMCs. However, the unstimulated imaging of IRF-1 shows that it is possible to also identify IRF-1 in the nucleus (Figure 16 A). After stimulation with IFN-γ, there appears to be a qualitative increase in IRF-1 expression indicated by increased brightness of the red channel fluorescence intensity (Figure 16 B).

Similar to IRF-1, IRF-7 is mostly cytoplasmic in unstimulated PBMCs (Figure 16 C). After stimulation with IFN-αA there appears to be a qualitative increase in IRF-7 indicated increased brightness of the red fluorescence intensity (Figure 16 D).

Both IRF-1 and IRF-7 appear to be mostly cytoplasmic in unstimulated cells, and appear to increase in expression after stimulation.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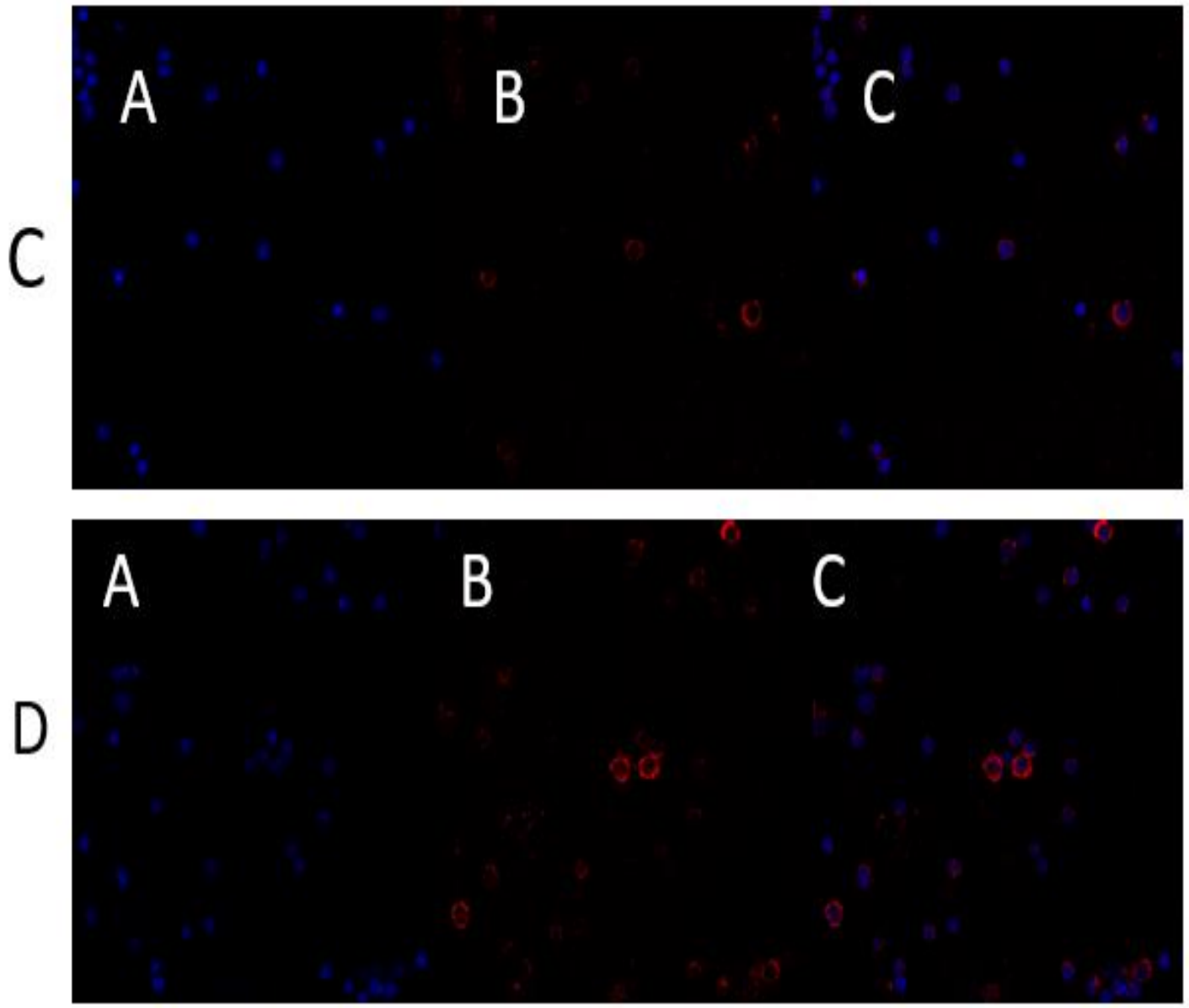


Figure 16. Localization of IRF-1 and IRF-7 expression in unstimulated and IFN-γ and IFN-αA stimulated PBMCs.

Immunofluorescence localization of IRF-1 (A and B) and IRF-7 (C and D) was performed on unstimulated PBMCs (A and C) and PBMCs stimulated with IFN-γ (B) and IFN-αA (D). Blue DAPI staining depicts the nucleus. A: depicts only the nuclear DAPI stain, B: depicts the IRF-1 or IRF-7 stain (goat anti-rabbit APC antibody), and C: depicts the overlay of images A and B.

Objective 3 - Comparing the expression of IRF-7 in PBMC subsets between HIV-infected and HIV-uninfected subjects from Nairobi, Kenya and characterizing the responsiveness of IRF-7 after stimulation with IFN-αA.

IRF-7 expression can be induced in Kenyan PBMCs by stimulation with IFN-αA.

Flow cytometry was used to determine the percentage of cells expressing IRF-7 both before and after stimulation with IFN-αA. Only IRF-7 was analyzed due to reduced availability of PBMCs, as there were minimal samples per donor available. As well, only IRF-7 knockdown was to be analyzed in subsequent studies. Classical monocytes were chosen (CD14highCD16-) as they are the most common monocyte population. CD4+ T cells (CD3+CD4+CD8-) were chosen as a representative of the lymphocyte population as T cells are the most common lymphocytes I analyzed and CD4+ T cells are the most common T cells. As well they are both (CD4+ T cells and monocytes) targets of HIV-1. Samples from 12 uninfected donors and 15 infected donors were analyzed. In Kenyan donors, the percentage of unstimulated CD4+ T cells (CD3+CD4+CD8-) expressing IRF-7 ranged from 41.7-89.4% in HIV-uninfected donors and 16.2-91.4% in HIV-infected donors (Figure 17, A and B). Upon stimulation with IFN-αA the percentage of CD4+ T cells (CD3+CD4+CD8-) expressing IRF-7 increased significantly (63.9-92%) ($p=0.048$) in the HIV-uninfected group. In HIV-infected subjects upon stimulation with IFN-αA the percentage of CD4+ T cells (CD3+CD4+CD8-) expressing IRF-7 increased to 51.6-94.5%. There were also a higher percentage of rested classical monocytes (CD14highCD16-) expressing IRF-7 compared to CD4+ T cells (CD3+CD4+CD8-). The percentage of resting classical monocytes (CD14highCD16-) expressing IRF-7 ranged from 40.6-99.4% in HIV-uninfected individuals and from 2.2-99.2% in HIV-infected (Figure 17, C and D). Upon stimulation with IFN-αA the percentage of classical monocytes (CD14highCD16-) expressing IRF-7 increased in the HIV-uninfected donors' cells to 58.3-100%, and increased statistically significantly in the HIV-infected to 15.4-100% ($p=0.0397$).

The percentage of cells expressing IRF-7 was very similar between the lymphocyte subsets and between the monocyte subsets, with the highest percentages found in the monocyte sub-populations.

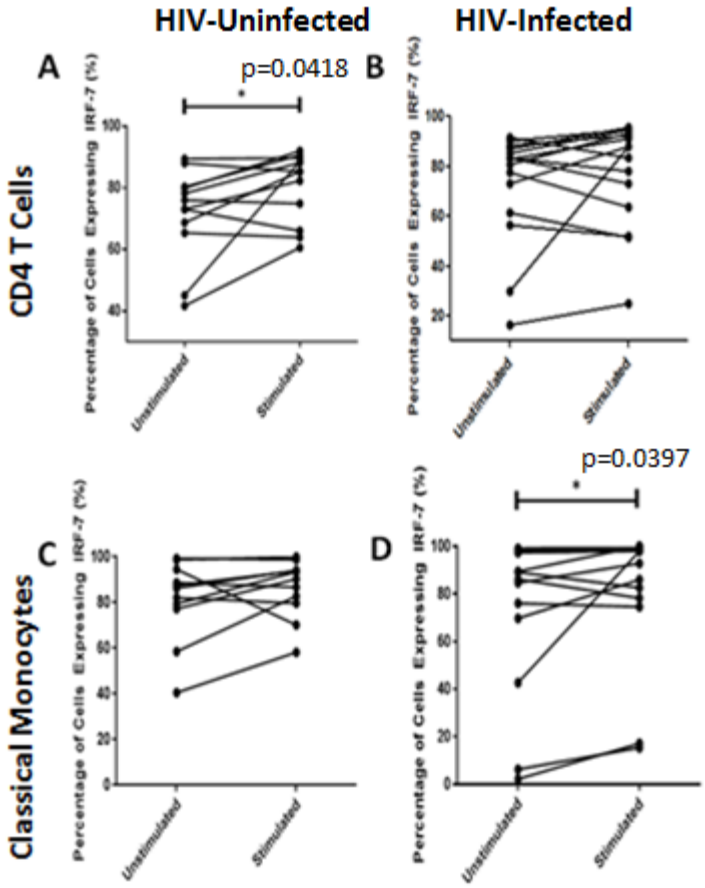


Figure 17. Comparison of the percentage of CD4+ T cells (CD3+CD4+CD8-) (A and B) and classical monocytes (CD14highCD16-) (C and D) expressing IRF-7 before and after stimulation with IFN-αA in HIV-uninfected (A and C) and HIV-infected (B and D) Kenyan Donors.

Flow cytometry was used to measure the percentage of cells expressing IRF-7 in 12 samples from HIV-uninfected and 15 samples from HIV-infected Kenyan donors. This figure shows the percentage of cells expressing IRF-7 both before and after stimulation with each point representing the results from a single individual. Paired two-tailed Wilcoxon matched-pairs signed rank statistical tests were performed to assess the expression differences between the conditions (95% confidence level, or $p \leq 0.05$).

In unstimulated Kenyan donors, higher IRF-7 expression was found in inflammatory monocytes (CD14+CD16+), compared to other subsets of PBMC.

Flow cytometry was used to measure IRF-7 expression in 12 Kenyan HIV-uninfected and 15 HIV-infected individuals (Figure 18). IRF-7 is expressed in all cell types examined at differing levels. There appears to be the highest expression of IRF-7 in the monocytes compared to the lymphocytes, therefore these groups were assessed separately. The highest expression of IRF-7 was observed in the inflammatory monocytes (CD14+CD16+) (HIV-uninfected mean = 8055) (HIV-infected median = 8463) compared to the other PBMC sub-populations. I also found the lowest IRF-7 expression in the cytokine-producing NK cells (CD16-CD56high) (HIV-uninfected mean = 1325) (HIV-infected median = 1395) compared to all other lymphocyte subsets.

In the HIV-uninfected subsets between the monocyte populations, there was higher expression of IRF-7 in the inflammatory monocytes (CD14+CD16+) (mean = 8055) compared to the classical monocytes (CD14highCD16-) (mean = 6298 $p<0.05$) (Figure 18 A). Within the lymphocytes there was significantly higher expression of IRF-7 in the uninfected CD4+ T cells (CD3+CD4+CD8-) (mean = 1677) compared to B cells (CD3-CD19+) (mean = 1486 $p<0.05$) and cytokine producing NK cells (CD16-CD56high) (mean = 1325 $p<0.001$). There was also significantly higher IRF-7 expression in the CD8+ T cells (CD3+CD4-CD8+) (mean = 1767) compared to the B cells (CD3-CD19+) (mean = 1486 $p<0.01$) and cytokine producing NK cells (CD16-CD56high) (mean = 1325 $p<0.001$). There was significantly higher expression of IRF-7 in the cytotoxic NK cells (CD16+CD56dim) (mean = 1639) compared to the cytokine producing NK cells (CD16-CD56high) (mean = 1325 $p<0.01$).

In the HIV-infected cell subsets within the monocyte populations there was significantly higher expression of IRF-7 in the inflammatory monocytes (CD14+CD16+) (median = 8463 compared to the patrolling monocytes (CD14dimCD16+) (median = 6696 $p<0.01$) and the classical monocytes (CD14highCD16-) (median = 6813 $p<0.01$) (Figure 18 B). Within the lymphocyte populations there was

higher expression of IRF-7 in the CD4+ T cells (CD3+CD4+CD8-) (mean = 1898) compared to the cytokine producing NK cells (CD16-CD56high) (mean = 1454 $p<0.001$). There was also higher expression of IRF-7 in the CD8+ T cell (CD3+CD4-CD8+) (mean = 1958) populations compared to B cells (CD3-CD19+) (mean = 1665 $p<0.05$) and the cytokine producing NK cells (CD16-CD56high) (mean = 1454 $p<0.001$). As well as significantly higher expression of IRF-7 in the cytotoxic NK cell populations (CD16+CD56dim) (mean = 1864) compared to the cytokine producing NK cells (CD16-CD56high) (mean = 1454 $p<0.001$).

The highest IRF-7 expression for both HIV-uninfected and HIV-infected donors was found in inflammatory monocytes (CD14+CD16+) and the lowest expression of IRF-7 was in the cytokine producing NK cell subsets (CD16-CD56high).

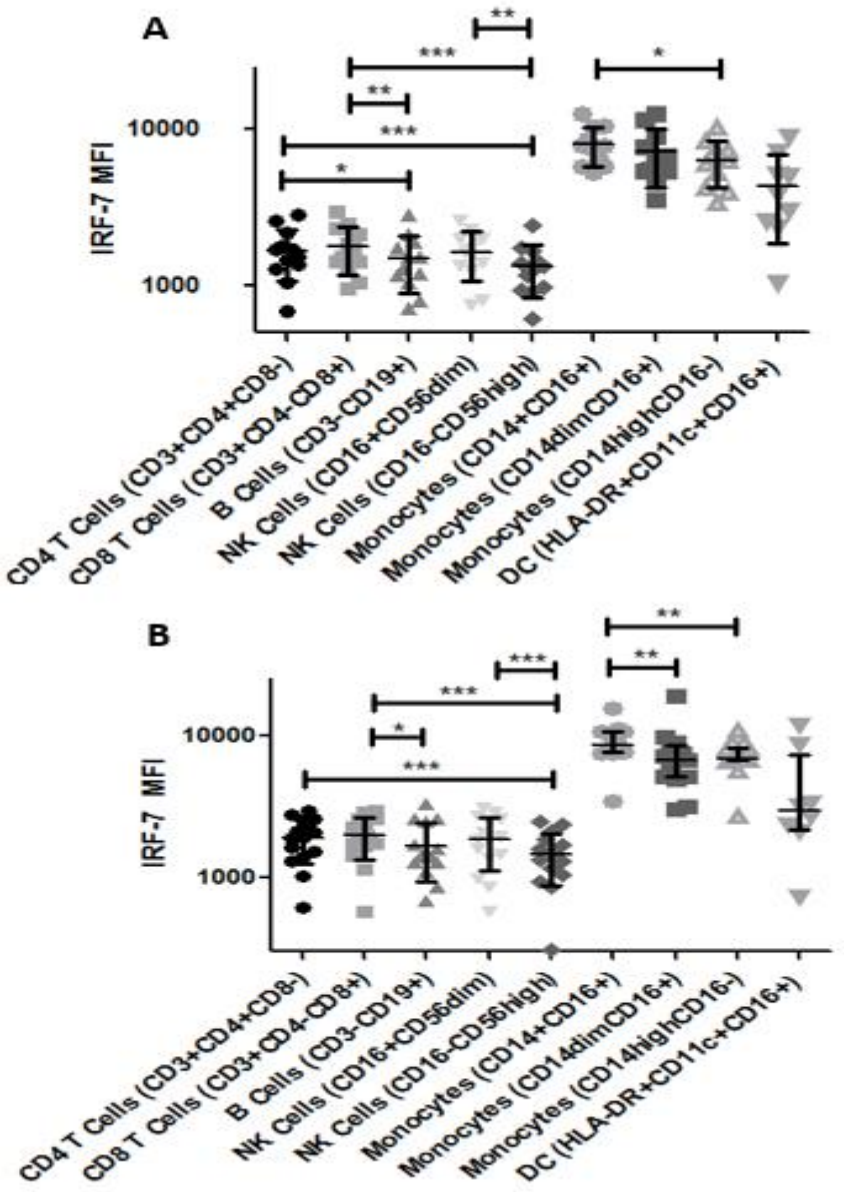


Figure 18. Comparison of the expression levels of IRF-7 from HIV-uninfected Kenyan donors (A) and HIV-infected Kenyan donors (B) in various unstimulated cell types.

Flow cytometry was used to measure the expression of IRF-7 in 12 PBMC samples from HIV-uninfected and 15 HIV-infected Kenyan donors. This figure shows median fluorescence intensity (MFI) of the PE-secondary antibody for expression of IRF-7 with each point representing the results from a single individual. One-way ANOVA and Dunn's and Tukey's multiple comparisons post-test were performed between the lymphocytes and between the monocyte populations to assess the expression differences

between the groups (95% confidence level, or $p \leq 0.05$). Horizontal bars represent the mean +/- the standard deviation or the median +/- the interquartile range.

Higher expression of IRF-7 in the monocyte and DC sub-populations compared to the lymphocyte (T-, B-, and NK cell populations).

To better demonstrate that IRF-7 was expressed at a higher level in monocytes (CD14+) and DC (HLA-DR+CD11c+CD16+) compared to lymphocytes, flow cytometry was used to measure IRF-7 expression in 12 HIV-uninfected and 15 HIV-infected donors. IRF-7 was expressed at a higher level in monocytes and DC compared to lymphocyte populations in both HIV-uninfected and HIV-infected cell subsets. Significantly higher expression of IRF-7 was found in the monocyte (CD14+) and DC (HLA-DR+CD11c+CD16+) sub-populations compared to lymphocytes (HIV-uninfected: 4.2-fold increase in IRF-7 expression, median lymphocytes = 1808 and median monocytes and DC = 7563, $p \leq 0.0001$) (HIV-infected: 4.1-fold increase in IRF-7 expression, median lymphocytes = 1813 and median monocytes and DC = 7517, $p \leq 0.0001$) (Figure 19 A and B).

IRF-7 is expressed at a higher level in monocytes (CD14+) and DC (HLA-DR+CD11c+CD16+) compared to lymphocytes in Kenyan populations. This suggests IRF-7 may be more relevant to monocyte function, which may have importance to these cells function in the antiviral response.

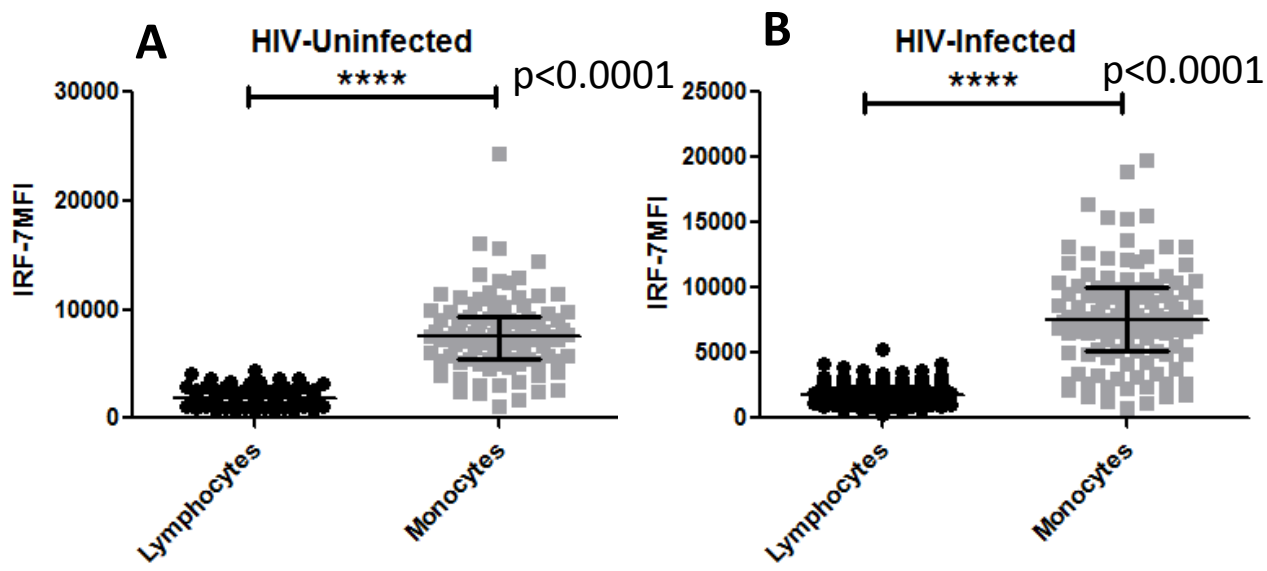


Figure 19. Comparison of the expression levels of IRF-7 from HIV-uninfected (A) and HIV-infected (B) Kenyan donors in lymphocytes compared to monocytes (CD14+) and DC (HLA-DR+CD11c+CD16+). Flow cytometry was used to measure the expression of IRF-7 in 12 HIV-uninfected and 15 HIV-infected PBMC samples from Kenyan donors. This figure shows median fluorescence intensity (MFI) of the PE-secondary antibody for expression of IRF-7 with each point representing the results from a single individual. Unpaired two-tailed Mann-Whitney statistical tests were performed to assess the expression differences between the groups (95% confidence level, or $p \leq 0.05$). Horizontal bars represent the median +/- the interquartile range.

Higher IRF-7 expression was found in inflammatory monocytes (CD14+CD16+) for HIV-uninfected Kenyan donors after stimulation and lower IRF-7 expression was found in the DC (HLA-DR+CD11c+CD16+) subset of the IFN-αA stimulated, HIV-infected samples.

Flow cytometry was used to measure IRF-7 expression after stimulation with IFN-αA in 12 HIV-uninfected and 15 HIV-infected donors from Kenya, after stimulating the cells for 16-18 hours.

IRF-7 is expressed in all cell types examined at differing levels. There appears to be the highest expression of IRF-7 in the monocytes compared to the lymphocytes, therefore these groups were assessed separately.

In the HIV-uninfected donors, I found the highest expression of IRF-7 in the inflammatory monocytes (CD14+CD16+) (median = 9754) compared to the classical monocytes (CD14highCD16) (median = 7726 $p \leq 0.001$) (Figure 20 A).

In the HIV-infected donors between the monocytes the lowest expression of IRF-7 was in the DC (HLA-DR+CD11c+CD16+) (mean = 5172). There was significantly higher expression of IRF-7 in the inflammatory monocytes (CD14+CD16+) (mean = 11947) compared to the patrolling monocytes (CD14dimCD16+) (mean = 8372 $p \leq 0.001$) and classical monocytes (CD14highCD16-) (mean = 8827 $p \leq 0.001$). Between the lymphocyte populations I found higher expression of IRF-7 in the CD4+ T cell subset (CD3+CD4+CD8-) (median = 1962) compared to the cytokine-producing (CD16-CD56high) (median = 1835 $p \leq 0.05$) NK cells (Figure 20 B).

The highest expression of IRF-7 was found in the inflammatory monocyte populations (CD14+CD16+).

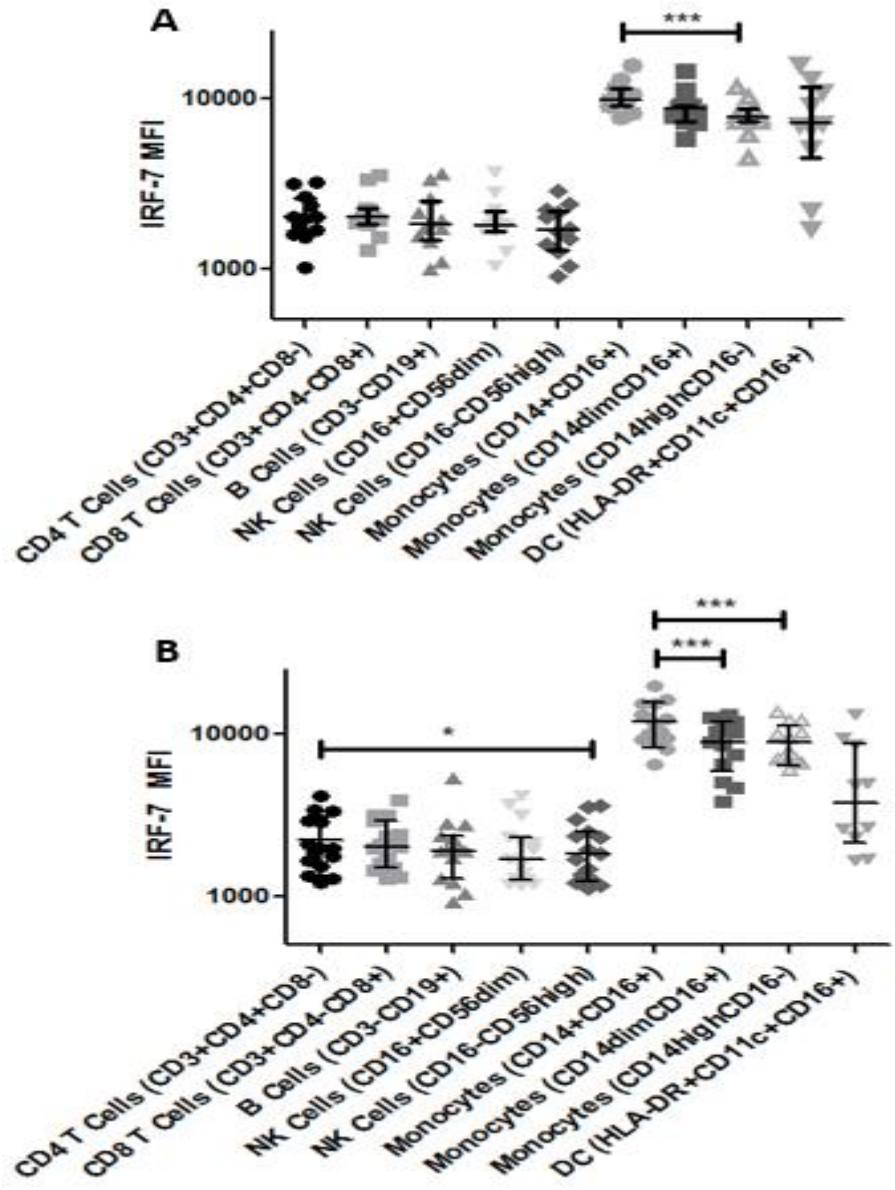


Figure 20. Comparison of the expression levels of IRF-7 from HIV-uninfected Kenyan donors (A) and HIV-infected Kenyan donors (B) in different cell populations stimulated with IFN-αA.

Cells were stimulated with IFN-αA for 16-18 hours to analyze IRF-7 expression. Flow cytometry was used to measure the expression of IRF-7 in 12 PBMC samples from HIV-uninfected and 15 PBMC samples from HIV-infected Kenyan donors. This figure shows median fluorescence intensity (MFI) of the PE-secondary antibody for expression of IRF-7 with each point representing a single donor. One-way ANOVA and Dunn's and Tukey's multiple comparisons post-test were performed between the

lymphocytes and between the monocyte populations to assess the expression differences between the groups (95% confidence level, or $p \leq 0.05$). Horizontal bars represent the mean +/- the standard deviation or the median +/- the interquartile range.

In HIV-uninfected and HIV-infected Kenyan samples, greater IRF-7 responses (fold changes) were found in the B cells (CD3-CD19+) and cytokine-producing NK cells (CD16-CD56high), respectively.

To determine the fold change of expression after stimulation fold change differences were calculated by dividing the MFI of IRF-7 of the IFN-αA stimulated samples by the MFI of the IRF-7 unstimulated samples in 12 HIV-uninfected and 15 HIV-infected donors (Figure 21). The monocyte and lymphocyte groups were assessed separately. Almost all PBMC subsets demonstrated a moderate increase in IRF-7 post interferon induction (fold increase for HIV-uninfected = 1.33 and fold increase for HIV-infected = 1.35). When comparing between the monocyte populations and between the lymphocyte populations for HIV-uninfected individuals there were no significant differences in fold change after stimulation (Figure 21 A).

In the HIV-infected Kenyan samples, after comparing the fold change after stimulation with IFN- αA**,** the response to stimulation was significantly higher in the cytokine-producing NK subset (CD16-CD56high) (1.424-fold increase) compared to the CD8+ T cells (CD3+CD4-CD8+) (1.193-fold increase $p \leq 0.001$) and the cytotoxic NK cells (CD16+CD56dim) (1.172-fold increase $p \leq 0.001$). There was also significantly higher fold change after stimulation in the B cell subset (CD3-CD19+) (1.246-fold increase) compared to the CD8+ T cells (CD3+CD4-CD8+) (1.193-fold increase) ($p \leq 0.05$ Figure 21 B).

In the HIV-infected donors the highest fold change was in the cytokine-producing NK cells (CD16-CD56high).

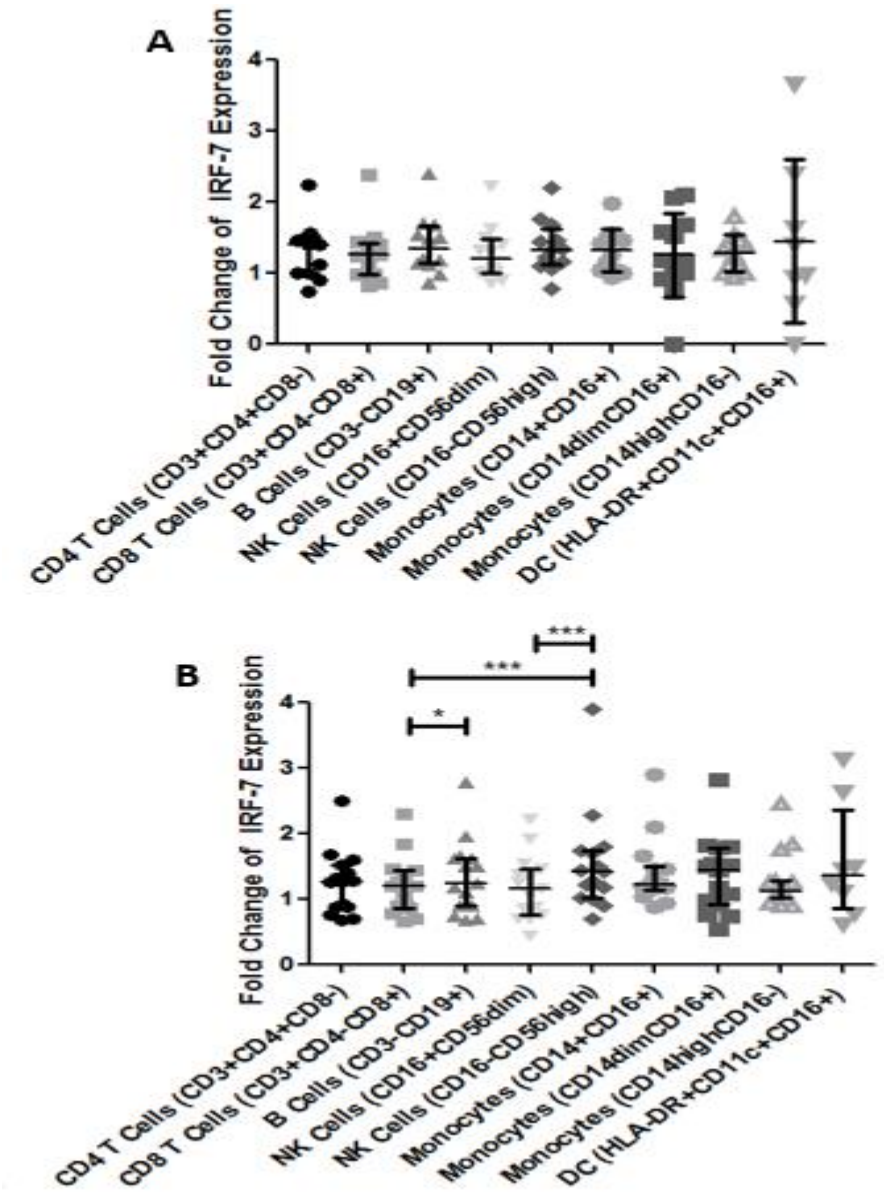


Figure 21. Comparison of the fold change of IRF-7 expression in HIV-uninfected (A) and HIV-infected (B) Kenyan donors of IFN-αA stimulated cells compared to unstimulated in various cell populations. Flow cytometry was used to measure the expression of IRF-7 in 12 PBMC samples from HIV-uninfected and 15 PBMC samples from HIV-infected Kenyan donors. This figure shows fold change differences by dividing the MFI of the PE-secondary antibody for expression of IRF-7 of the IFN-αA stimulated samples by the MFI of the unstimulated samples. One-way ANOVA and Dunn's and Tukey's multiple comparisons post-test were performed between the lymphocytes and between the monocyte populations to assess

the expression differences between the groups (95% confidence level, or $p \leq 0.05$). Horizontal bars represent the mean +/- standard deviation or median +/- the interquartile range.

Objective 4 - Compare the expression of IRF-7 between HIV-uninfected donors from Manitoba and Nairobi, Kenya to HIV-infected donors from Nairobi, Kenya

There were no significant differences in the intensity or the level of IRF-7 expression in the PBMCs from the HIV-uninfected and HIV-infected donors.

To determine if there were any differences between HIV-infected and HIV-uninfected donors, the MFI of the unstimulated and IFN-αA PBMCs for the 18 Manitoban donors and 12 HIV-uninfected Kenyan and were compared to 15 HIV-infected Kenyan blood donors. I observed no significant differences between the two groups. To summarize, it appears as if there are no significant differences in levels of IRF-7 at baseline or upon stimulation between HIV-infected and HIV-uninfected individuals, both from Manitoba and Nairobi, Kenya.

Summary of Section:

IRF-1 and IRF-7 were expressed in all cell subsets analyzed. The highest expression of both IRF-1 and IRF-7 were in the monocyte and DC populations compared to the lymphocytes. IRF-1 is expressed in a higher percentage of CD4+ T cells (CD3+CD4+CD8-) and classical monocytes (CD14highCD16-) than IRF-7 but both can be induced by interferon. Inflammatory monocytes in most cases express the highest levels of IRF-1 and IRF-7, with B cells (CD3-CD19+) and cytokine producing NK cells (CD16-CD56high) expressing the lowest. Samples from Kenya from HIV-uninfected and HIV-infected cells had the same IRF-7 expression profiles as the Manitoban donors, and no significant differences were found between the infected and uninfected samples.

Section 2: IRF-7 Knockdown

Rationale: The effect of repressing IRF-1 before viral infection has been studied in relation to its effect on HIV-1 infection, however, there is little information available in respect to IRF-7 (*74*). Overexpression of IRF-1 and IRF-7 was shown to limit HIV-1 and Hepatitis C Virus (HCV) replication in a fibroblast cell line, but conflicting data were reported when IRF-1 was overexpressed in Jurkat T cell lines (*8, 77*). While knockdown of IRF-1 in primary CD4+ T cells resulted in significant decrease of the transactivation of the HIV-LTR (*74*), there is conflicting data on the effects of modulating IRF-7 expression before HIV infection. Overexpression of IRF-7 by poly I:C stimulation in cervical tissues from HIV-1 seronegative women increased the antiviral response resulting in decreased HIV-1 transcription and viral release (*125*). Another study showed that deleting the IRF-1/7 binding site from the HIV-1 LTR resulted in decreased viral infectivity (*6*). IRF-7 has also been shown to be upregulated during HIV-1 infection, and individuals infected with HIV-1 have higher IRF-7 expression (*6, 105-107*). These findings made primarily in cell lines do not necessarily represent the effects of HIV-1 *in vivo* and require further validation in primary untransformed CD4+ T cells, which are the primary targets of HIV replication. Knocking down IRF-7 prior to HIV infection will help us determine if reduced levels of IRF-7 lead to decreased or increased levels of HIV replication. It is unknown whether IRF-7 preferentially infects cells with increased levels of IRF-7, or if IRF-7 increases in these cells in response to HIV-1 infection. Determining the levels of IRF-7 during the course of HIV-1 infection and analyzing the expression of IRF-7 in infected cell subsets compared to uninfected could help address these questions.

Hypothesis:

Reduced IRF-7 expression would result in decreased anti-viral responses, measured by cytokine release, and rendering CD4+ T cells to be more susceptible to HIV-1 infection.

There will be little effect on knocking down IRF-7 on its downstream target genes.

Objectives:

1. Determine the effects of IRF-7 knock-down using electroporation on cellular susceptibility to infection.

2. Determine the effects of IRF-7 knock-down using lentivirus on cellular susceptibility to infection.

3. Determine the effects of IRF-7 knockdown on cytokine release using Milliplex multiplex assays.

Experimental Approaches:

PBMCs were isolated from Manitoban donors by Ficoll-hypaque density gradient centrifugation and then cryopreserved until use. Cells were thawed, enriched for CD4+ T cells by EasySep™ Human CD4+ T Cell Enrichment Kit, and rested. For electroporation transfection cells were electroporated using the Lonza 4D-Nucleofector™ System with either scrambled control siRNA or siRNA specific for IRF-7, resulting in knockdown. Cells were rested overnight (16-18 hours) prior to HIV infection. For lentivirus transduction CD4+ T cells were stimulated with CD3/CD28 Dynabeads® for 72 hours prior to transduction with lentiviral particles encoding IRF-7 shRNA. The lentiviral particles were created by 3-plasmid calcium transfection into a Lenti-X™ 293T cell line, and then harvested 3 days later. After transduction, the cells were rested for 3-5 days prior to HIV infection. Cells were infected with HIV-1 subtype IIIB or a donor laboratory isolate ML 1956 subtype A1 using a multiplicity of infection (MOI) of 3 for the unstimulated electroporation experiments, and an MOI of 1 for the experiments using stimulated transduced cells. Supernatants were collected at 24, 72, and 96 hours and analyzed by ELISA and by Human Cytokine/Chemokine Magnetic Bead Panel Milliplex assay for IFN-γ, IL-10, IL-6, TNF-α, IFN-α2, IL-12p70, IL-15, IL-2, and IL-4. Cells were stained for intracellular p24 at 96 hours post infection. Since p24 is secreted from infected cells, Golgi-Plug was added to stop p24 from being secreted 6 hours prior to staining. RNA was also isolated from cells at 96 hours post-infection, reverse-transcribed into cDNA, and

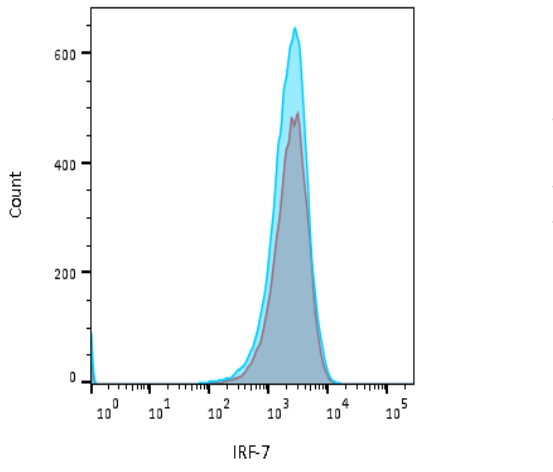
then analyzed by qPCR to validate flow cytometry data and transduction efficiency.

Objective 1 - Comparing the efficiency of IRF-7 knockdown on primary CD4+ T cells using electroporation (Nucleofection)

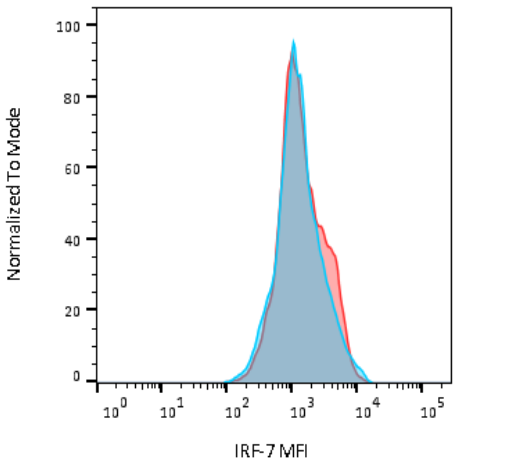
IRF-7 expression was reduced in primary CD4+ T cells after electroporation with IRF-7 specific siRNA compared to a scramble control.

As IRF-7's role in HIV replication in primary CD4+ T cells has not yet been defined, decreasing IRF-7 levels would allow the determination of its role in HIV replication. To measure the efficiency of IRF-7 knockdown by electroporation primary CD4+ T cells were transfected by electroporation (Nucleofection) with IRF-7 specific siRNA compared to a scramble control. After considerable attempts at optimization, only samples from 2 subjects achieved IRF-7 knockdown in this pilot study. There was little knockdown achieved using electroporation transfection. Intracellular IRF-7 levels (MFI) were assessed by flow cytometry, and a knockdown of 5-7% was achieved in cells from 2 different donors (Figure 22 A and B). Electroporation transfection of primary CD4+ T cells did not yield a significant knockdown, and therefore Lentivirus vectors were used in further experimentation. Samples were still infected with HIV-1 to determine if moderate decrease in IRF-7 expression inhibited HIV-1 replication.

A)



	Sample Name	Subset Name	Median : Comp-BV510-A
	D241 IRF7_IRF7_014.fcs	FSC-A, SSC-A subset	2223
	D241 Scramble_IRF7_011.fcs	FSC-A, SSC-A subset	2320



	Sample Name	Subset Name	Median : PE-A
	D178 IRF7_IRF7_019.fcs	FSC-A, SSC-A subset	1165
	D178 Scramble_IRF7_013.fcs	FSC-A, SSC-A subset	1242

B)

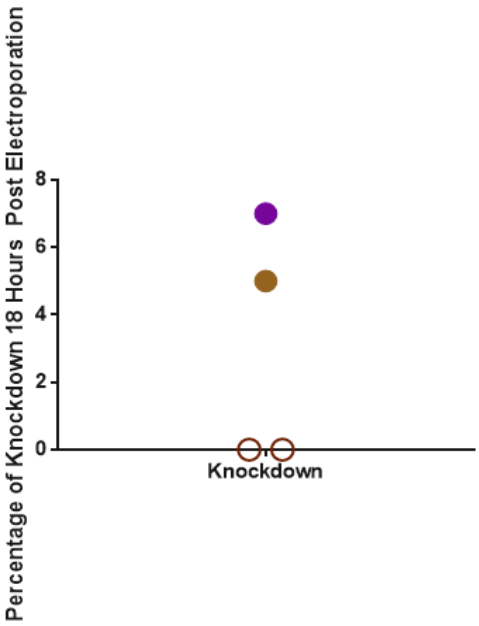


Figure 22. Percentage of IRF-7 knockdown in CD4+ T cells after electroporation transfection prior to HIV-1 infection (A and B).

The percent of IRF-7 knockdown was calculated by comparing the levels of IRF-7 expression (MFI) in CD4+ T cells electroporated with IRF-7 specific siRNA to cells electroporated with scramble control siRNA 18 hours post electroporation. Figure A shows histograms comparing IRF-7 scramble siRNA (pink) to IRF-7 specific siRNA (blue). Figure B shows the percentage of IRF-7 knockdown. Closed circles are cells electroporated with IRF-7 specific siRNA. Open circles are cells electroporated with scramble control siRNA.

IRF-7 knockdown resulted in less p24+ CD4+ T cells 96 hours after infection with HIV-1 in electroporated transfected cells.

To measure the effect of IRF-7 knockdown on HIV infection, CD4+ T cells from 2 subjects were electroplated with scramble control siRNA and IRF-7 specific siRNA were infected with HIV-1 IIIB (n=2) for 96 hours. Following infection, supernatants were collected at 24, 72, and 96 hours post-infection, and then analysed by ELISA for p24 secretion. Cells were also stained for IRF-7 expression (MFI) and p24 (%) at 96 hours-post infection. Samples were still infected with HIV-1 even though there was little knockdown, to determine if moderate decrease in IRF-7 expression inhibited HIV-1 replication. Detectable p24 was found in both conditions when comparing both intracellular p24 (measured by flow cytometry) and secreted p24 (measured by ELISA). The sample size was too small to analyze for differences in p24 MFI in control compared to knockdown.

Even in the presence of 5-7% knockdown, analysis revealed that visually there appeared to be less p24 positive CD4+ T cells after infection in the cells with the knockdown (n = 2, 0.5-20.6%) compared to the control (n = 2, 2.95-21.10%) measured by flow cytometry at 96 hours post infection. However, the data was not statistically significant (p=0.5) (Figure 23).

There were not enough data points to determine the correlation between the percentage of knockdown before infection and the percentage of p24+ cells.

At 24, 72, and 96 hours post infection there were no significant differences between secreted p24 measured by ELISA in the cells with the IRF-7 knockdown compared to control (24 hours p=1.0, 72 hours p=0.5, 96 hours p=0.5). However, when comparing all time points there appears to be less p24 in the cells with the IRF-7 specific knockdown (n = 6, median control = 3.831 and median knockdown = 2.423 p=0.1875) (Figure 24) however this data is not significant.

The sample size was too small to accurately determine differences between the groups.

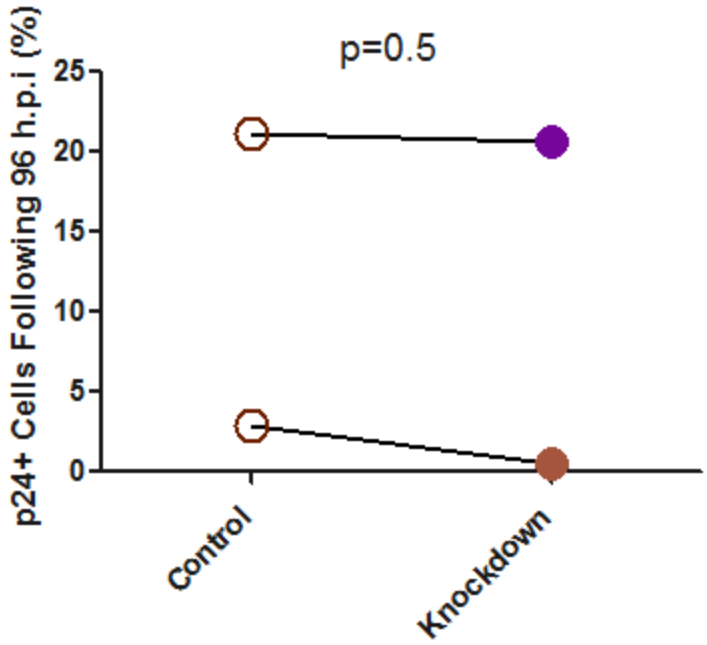


Figure 23. Percentage of p24+ CD4+ T cells after 96 hours HIV-1 infection in electroporated cells.

Comparison of the percentage of p24% CD4+ T cells 96 hours after infection with HIV-1 IIIB. Open circles are cells electroporated with scramble control siRNA. Closed circles are cells electroporated with IRF-7 specific siRNA.

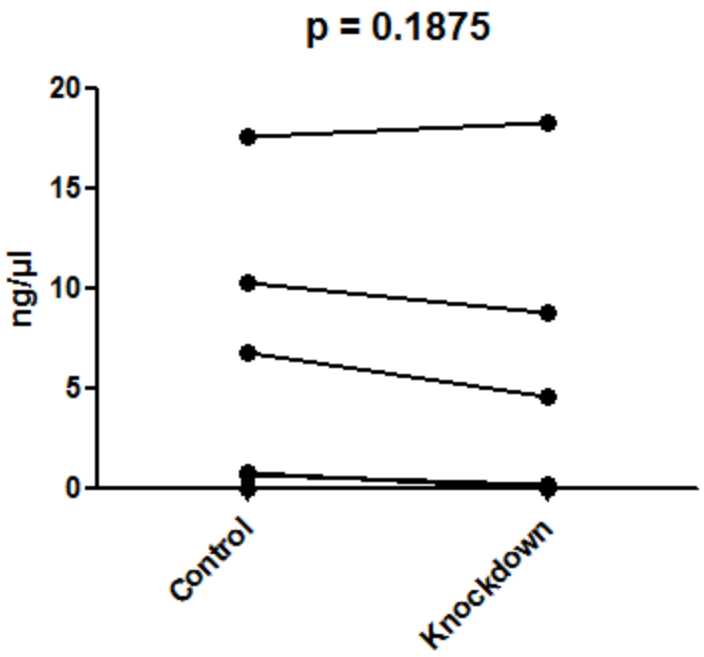


Figure 24. Comparing levels of p24 (ng/µL) in cells electroporated with control siRNA to cells electroporated with IRF-7 specific siRNA.

Cell supernatants were collected at 24, 72, and 96 hours post infection from infection of electroporated cells, and analyzed by ELISA for levels of p24 (ng/µL). Paired Wilcoxon signed rank tests were performed to assess the differences between the groups (95% confidence level, or $p<0.05$).

No differences between levels of IRF-7 (MFI) before and after infection with HIV-1.

To see if HIV infection induces IRF-7 expression, IRF-7 was detected in cells (n=2 control siRNA and n=2 IRF-7 siRNA) before (at time point 0) and after HIV-1 infection (96 hours post infection) by cell staining for IRF-7 and flow cytometry.

Low levels of IRF-7 were detected in cells regardless of HIV infection. There were no significant differences between IRF-7 expression comparing MFI before and after infection with HIV-1 (n=2, control: before infection median = 1781 and after infection median =1639; IRF-7 knockdown: before infection median =1694 and after infection median = 1321; p=1) (Figure 25).

There appears to be no statistically significant changes in IRF-7 expression during the course of HIV-1 infection.

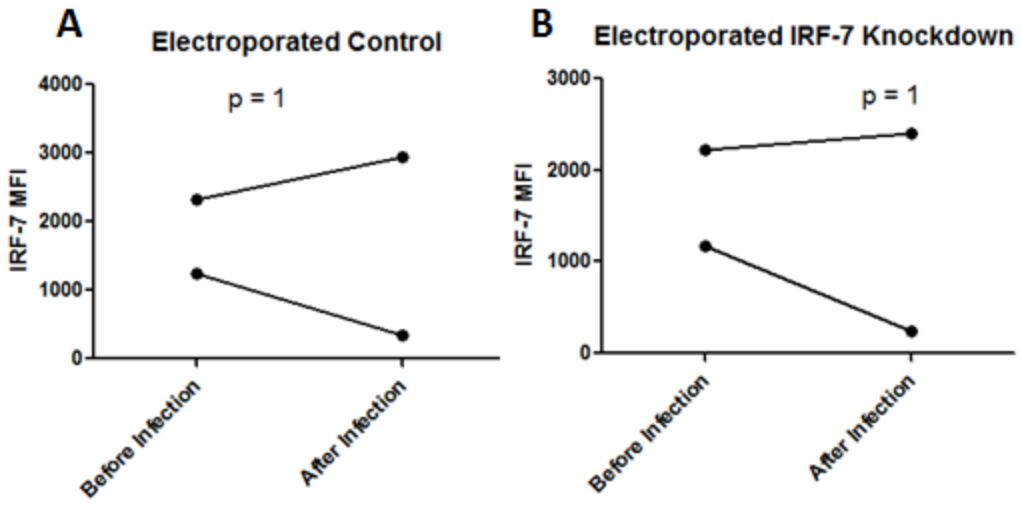


Figure 25. Comparing levels of IRF-7 (MFI) in cells electroporated with control siRNA (A) to cells electroporated (B) with IRF-7 specific siRNA before and after infection with HIV-1.

Flow cytometry was used to measure the expression of IRF-7 both before and after infection with HIV-1 from 2 control samples and 2 knockdown samples. This figure shows median fluorescence intensity (MFI) of the PE-secondary antibody for expression of IRF-7 with each point representing the results from a single individual. Paired two-tailed Wilcoxon signed rank tests were performed to assess the expression differences between the groups (95% confidence level, or $p<0.05$).

Higher expression of IRF-7 (MFI) in HIV-1 p24+ subsets of cells compared to uninfected.

The efficiency of HIV-1 infection is poor as not all cells in the HIV-1 treated culture became infected. Therefore, the expression of IRF-7 was compared in the p24+ (HIV-infected) cells versus p24- (uninfected) cells within the same culture that was treated with siRNA. To determine if there is an association between IRF-7 and HIV infection the expression of IRF-7 was examined in CD4+ T cells that were electroporated with scramble control or IRF-7 specific siRNA, and then infected with HIV-1 for 96 hours. Flow cytometry was used to determine the level of IRF-7 (MFI) at 96 hours post-infection. Included in this analysis were subjects where 0% knockdown was achieved. HIV-1 infected cells (p24+) cells were compared to HIV-1 uninfected cells (p24-) at 96 hours post infection to determine if there were differences in the levels of IRF-7 expression.

IRF-7 was detected in all groups, regardless of HIV status. There was significantly higher expression of IRF-7 in p24+ cell subset compared to the p24- cell subset, control siRNA (n=10, mean uninfected = 1417 and mean infected = 2620; p=0.0372) or IRF-7 specific siRNA (n=10, mean uninfected = 1415 and mean infected = 2819; p=0.0288) (Figure 26 A and B).

IRF-7 levels are higher in HIV infected cells compared to uninfected cells regardless of IRF-7 knockdown.

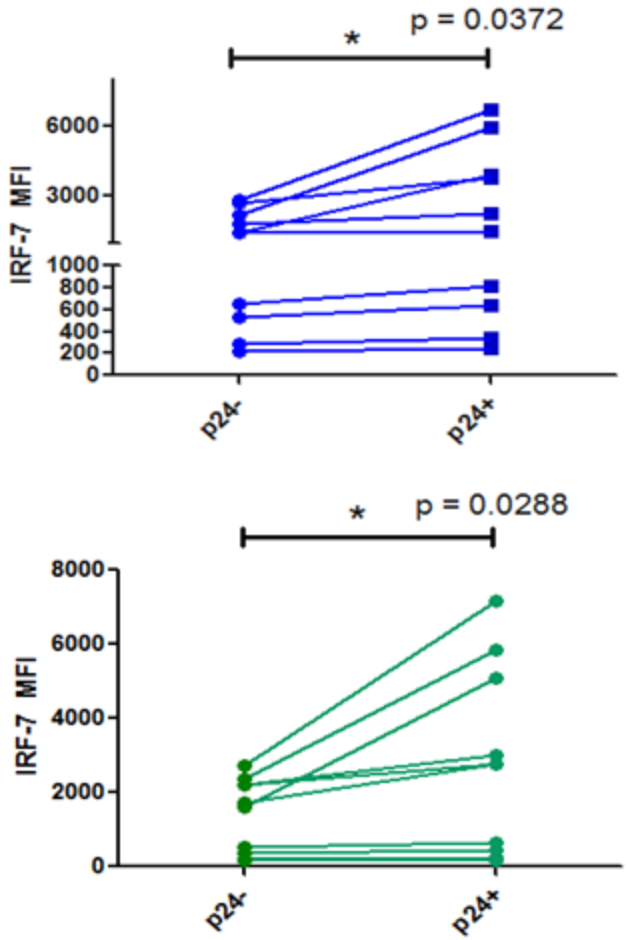


Figure 26. Levels of IRF-7 in uninfected compared to infected cell subsets in CD4+ T cells electroporated with scramble control siRNA (A) and IRF-7 specific siRNA (B).

Flow cytometry was used to measure the expression of IRF-7. This figure shows median fluorescence intensity (MFI) of the PE-secondary antibody for expression of IRF-7 with each point representing the results from a single individual. Paired two-tailed t-tests were performed to assess the expression differences between the groups (95% confidence level, or $p<0.05$).

Objective 2 - Comparing the efficiency of IRF-7 knockdown on primary CD4+ T cells using lentiviral particles (lentivirus)

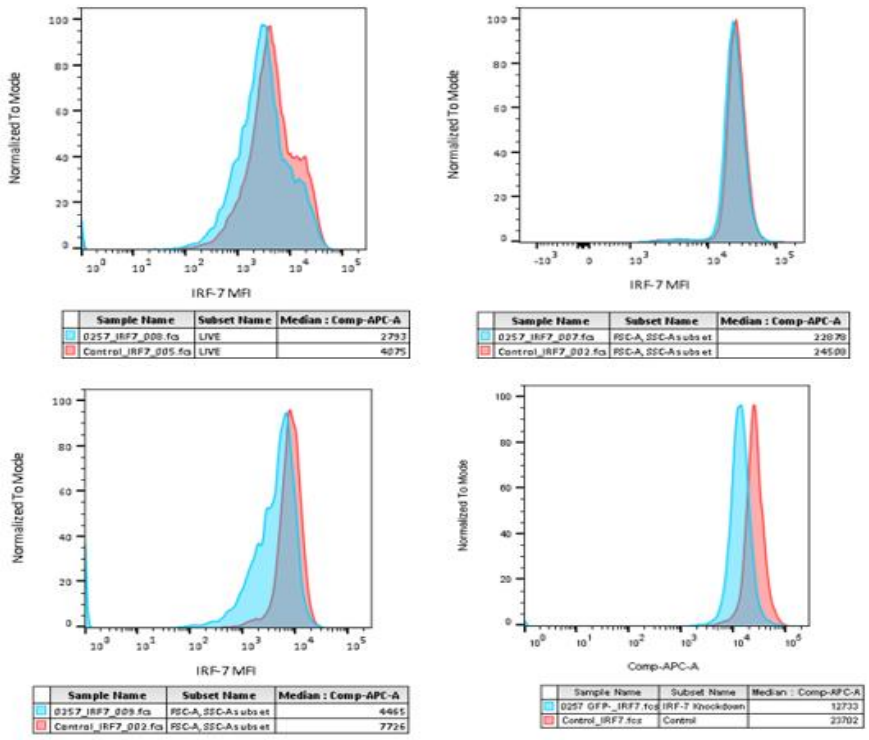
IRF-7 expression was reduced in primary CD4+ T cells after transduction with the lentiviral particles encoding for IRF-7 shRNA, compared to the untransduced controls.

Lentiviral vectors were used to knockdown IRF-7 expression as electroporation did not yield significant knockdown. To measure the efficiency of lentiviral transduction in knocking down IRF-7 expression primary CD4+ T cells were transduced with lentiviral particles encoding for IRF-7 shRNA and compared to non-transduced cells.

Lentivirus appears to be more efficient than electroporation at knocking down IRF-7 cellular expression. Using 4 different donors, a range of 7-83% knockdown could be achieved (Figure 27 A and B).

The knockdown achieved by lentiviral transduction (7-83%) was superior to the knockdown achieved by electroporation transfection.

A)


Normalized To Mode
IRF-7 MFI
Sample Name | Subset Name | Median : Comp-APC-A
0257_IRF7_008.fcs | LIVE | 2793
Control_IRF7_005.fcs | LIVE | 4075
Normalized To Mode
IRF-7 MFI
Sample Name | Subset Name | Median : Comp-APC-A
0257_IRF7_007.fcs | FSC-A, SSC-A subset | 22079
Control_IRF7_002.fcs | FSC-A, SSC-A subset | 24500
Normalized To Mode
IRF-7 MFI
Sample Name | Subset Name | Median : Comp-APC-A
0257_IRF7_009.fcs | FSC-A, SSC-A subset | 4465
Control_IRF7_002.fcs | FSC-A, SSC-A subset | 7726
Normalized To Mode
Comp-APC-A
Sample Name | Subset Name | Median : Comp-APC-A
0257 GFP-_IRF7.fcs | IRF-7 Knockdown | 12733
Control_IRF7.fcs | Control | 23702

B)

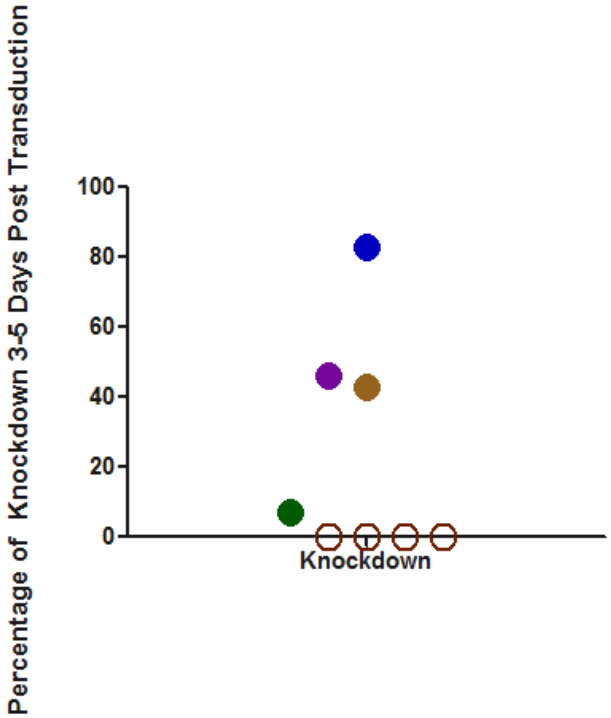

Percentage of Knockdown 3-5 Days Post Transduction
100
80
60
40
20
0
Knockdown

Figure 27. Percentage of IRF-7 knockdown in CD4+ T cells after lentivirus transduction prior to HIV-1 infection (A and B).

The percent of IRF-7 knockdown was calculated by comparing the levels of IRF-7 expression (MFI) in CD4+ T cells transduced with lentiviral particles encoding for IRF-7 shRNA to non-transduced cells. Figure A shows histograms comparing non-transduced cells (pink) to IRF-7 specific lentivirally transduced cells (blue). Figure B shows the percentage of IRF-7 knockdown. Closed circles are cells transduced with lentiviral particles encoding for IRF-7 shRNA. Open circles are non-transduced control cells.

Although the data is not statistically significant, IRF-7 knockdown leads to a trend of reduced % of p24+ CD4+ T cells after HIV-1 infection in the cells transduced with lentiviral particles.

To determine the effect of knocking down IRF-7 by lentiviral transduction on HIV replication, CD4+ T cells from 4 subjects were transduced with lentivirus particles encoding for IRF-7 shRNA or non-transduced cells were infected with HIV-1 IIIB (n=4) or laboratory isolate ML 1956 (n=2) for 96 hours. Following infection, supernatants were collected at 24, 72, and 96 hours post-infection, and then analysed by ELISA for p24 secretion. Cells were also stained for IRF-7 expression (MFI) and p24 (%) at 96 hours-post infection. Detectable p24 was found in both conditions when comparing both intracellular p24 (measured by flow cytometry) and secreted p24 (measured by ELISA).

Analysis revealed that visually there appeared to be less p24 positive CD4+ T cells after infection in the cells with the knockdown (n = 6, 1.28-43.20%) compared to the control (n = 6, 1.74-59.10%) measured by flow cytometry at 96 hours post infection. However, the data was not statistically significant (p=0.1563) (Figure 28).

When compared to control cells there appeared to be reduced p24 expression (MFI) in the knockdown cells compared to the control cells, however this was not statistically significant (n=6, control mean = 3775 and knockdown mean = 3455, p=0.6557) (Figure 29).

The data was analyzed to determine if there was a correlation between the amount of IRF-7 knockdown prior to infection with HIV-1 and the amount of p24 decrease between the control and IRF-7 knockdown. There was no correlation between the percentage of knockdown prior to HIV-infection and the percentage of p24+ cells when comparing percentage of p24+ cells in the knockdown compared to the control cells (% p24+ control - % p24+ knockdown) (n=6, p=0.0573, r=-0.7945) (Figure 30).

At 24, 72, and 96 hours post infection there were no significant differences between p24 (ng/μL) production in the cells with IRF-7 knockdown compared to control (24 hours p=0.6875, 72 hours p=0.1563, 96 hours p=0.3125). When combining all time points, the data is not significant but trending

towards less p24 in cells with IRF-7 knockdown (n=18, mean control = 6.837 and mean knockdown = 4.872; p=0.1057) (Figure 31). It is possible that knocking down IRF-7 prior to HIV infection may result in less CD4+ T cells becoming infected, or that HIV-1 may be preferentially infecting the cells with higher expression of IRF-7. Unfortunately, the data is not significant, rendering the data inconclusive.

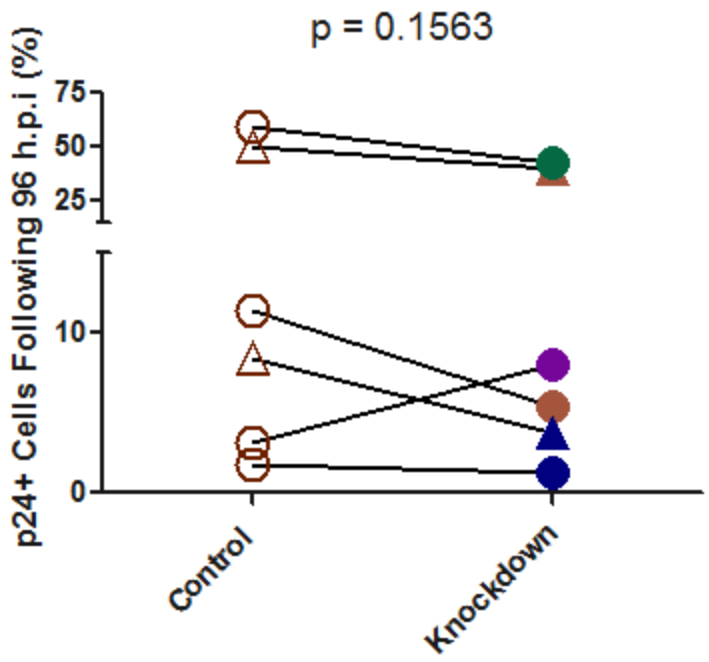


Figure 28. Percentage of p24+ CD4+ T cells after 96 hours HIV-1 infection in lentivirally transduced cells.

Comparison of the percentage of p24% CD4+ T cells 96 hours after infection with HIV-1 IIIB or ML 1956. Open circles/triangles are non-transduced cells. Closed circles/triangles are CD4+ T cells transduced with lentiviral particles encoding for IRF-7 shRNA. Circles are cells infected with HIV-1 IIIB. Triangles are cells infected with a donor laboratory HIV-1 strain ML 1956.

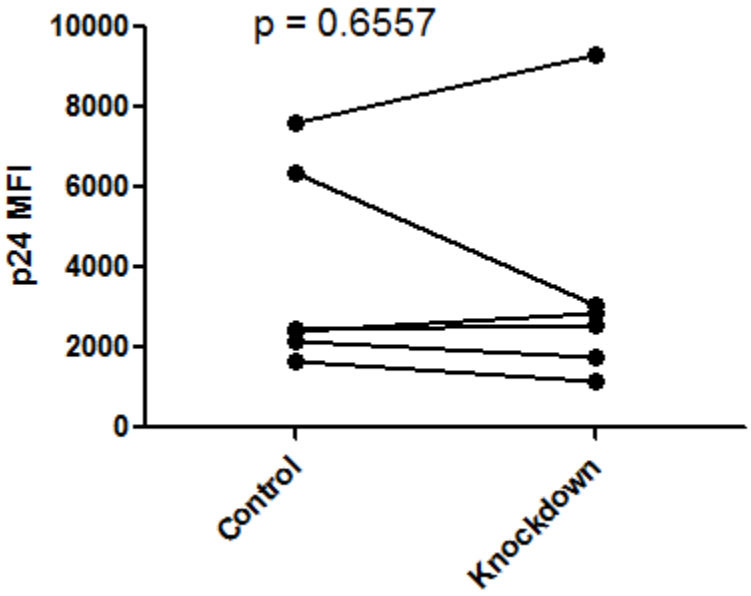


Figure 29. Expression of p24 (MFI) after 96 hours of HIV-1 infection in lentivirally transduced cells. Flow cytometry was used to measure the expression of p24. This figure shows median fluorescence intensity (MFI) of the PE-secondary antibody for expression of IRF-7 with each point representing the results from a single individual. Paired two-tailed t-tests were performed to assess the expression differences between the groups (95% confidence level, or $p<0.05$).

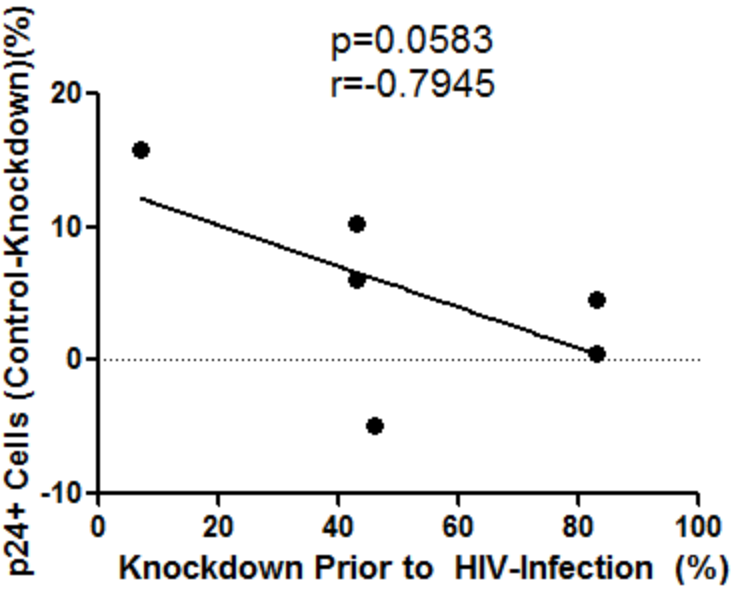


Figure 30. Comparing knockdown of IRF-7 prior to HIV-Infection (%) to the difference of p24+ (%) cells in control to IRF-7 knockdown measured by flow cytometry (MFI).

Flow cytometry was used to measure the expression of IRF-7 prior to HIV infection and determine the percentage of knockdown (%). Flow cytometry was also used to determine the percentage of p24% cells after HIV-1 infection for 96 hours. This figure shows the percentage of IRF-7 knockdown and the percentage of p24+ cells. Non-parametric Spearman correlation tests were performed to assess the correlation between the groups (95% confidence level, or $p<0.05$).

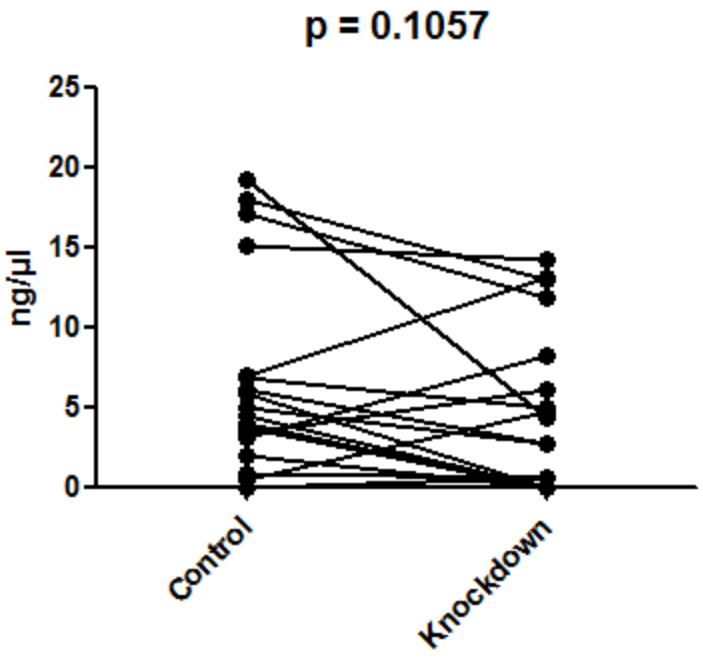


Figure 31. Comparing levels of p24 (ng/µL) in control and transduced CD4+ T cells for IRF-7 specific knockdown.

Cell supernatants were collected at 24, 72, and 96 hours post infection, and analyzed by ELISA for levels of p24 (ng/µL). Paired t-tests were performed to assess the differences between the groups (95% confidence level, or $p<0.05$).

No differences between the levels of IRF-7 (MFI) before and after infection with HIV-1.

To see if HIV infection induces IRF-7 expression, IRF-7 was detected in cells (n=6 control and n=6 transduced) before (at time point 0) and after HIV-1 infection (96 hours post infection) by cell staining for IRF-7 and flow cytometry.

Low levels of IRF-7 were detected in cells regardless of HIV infection. There were no significant differences between IRF-7 expression comparing MFI before and after infection with HIV-1 (for control n=6: before infection median = 7726 and after infection median = 2190, p=0.0625; for IRF-7 knockdown n=6: before infection median = 4465 and after infection median = 2705; p=0.4375) (Figure 32 A and B). There is no change in IRF-7 expression over the course of HIV-1 infection.

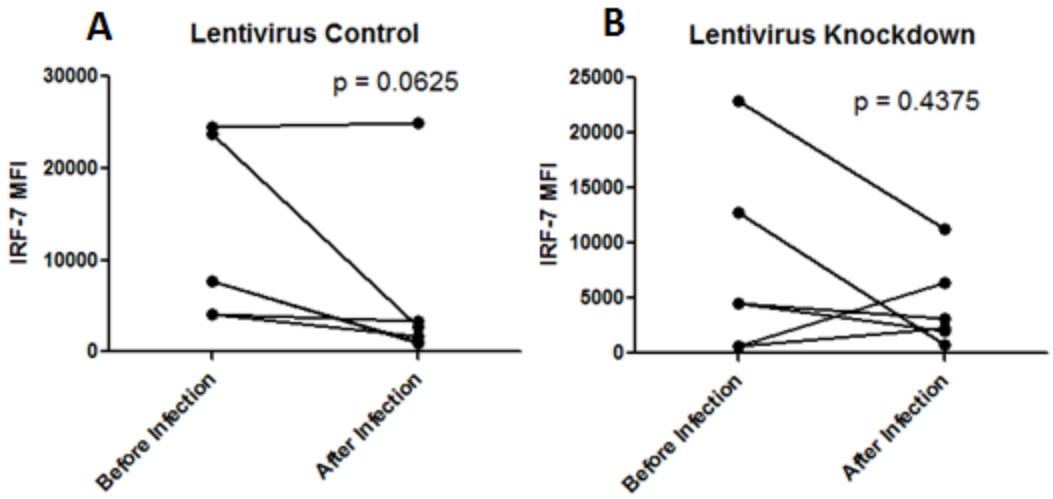


Figure 32. Comparing levels of IRF-7 (MFI) in control cells (A) to cells transduced with IRF-7 encoded lentivirus (B) before and after infection with HIV-1.

Flow cytometry was used to measure the expression of IRF-7 both before and after infection with HIV-1 from 6 control samples and 11 knockdown samples. This figure shows median fluorescence intensity (MFI) of the PE-secondary antibody for expression of IRF-7 with each point representing the results from a single individual. Paired two-tailed Wilcoxon signed rank tests were performed to assess the expression differences between the groups (95% confidence level, or $p<0.05$).

Higher expression of IRF-7 (MFI) in HIV-1 p24+ cell subsets, compared to the uninfected (p24-) subsets.

The efficiency of HIV-1 infection is poor as not all cells in the HIV-1 treated culture became infected. Therefore, the expression of IRF-7 was compared in the p24+ (HIV-infected) cells versus p24- (uninfected) cells within the same culture. To determine if there is an association between levels of IRF-7 and HIV infection, untransduced CD4+ T cells and cells transduced with lentiviral particles were infected with HIV-1 for 96 hours. Flow cytometry was used to determine the level of IRF-7 (MFI) at 96 hours post infection. All samples were kept for analysis, even when there was no detectable knockdown achieved by lentivirus transduction. Levels of IRF-7 in the p24+ cell subset representing HIV infected cells were compared to the p24- cell subset. HIV-1 infected cells (p24+) cells were compared to HIV-1 uninfected cells (p24-) at 96 hours post infection to determine if there were differences in the levels of IRF-7 expression.

Detectable IRF-7 was detected in all groups, regardless of HIV status. There was significantly higher expression of IRF-7 in the p24+ cell subset compared to the p24- cell subset in the control cells (n=9, uninfected mean = 4352 and infected mean = 8540, p=0.0426) and lentivirally transduced cells (n=13, uninfected median = 2223 and infected median = 7563, p=0.0002) (Figure 33 A and B).

It appears that cells infected with HIV-1 produce higher levels of IRF-7, or that HIV infects cells with increased IRF-7 expression more efficiently.

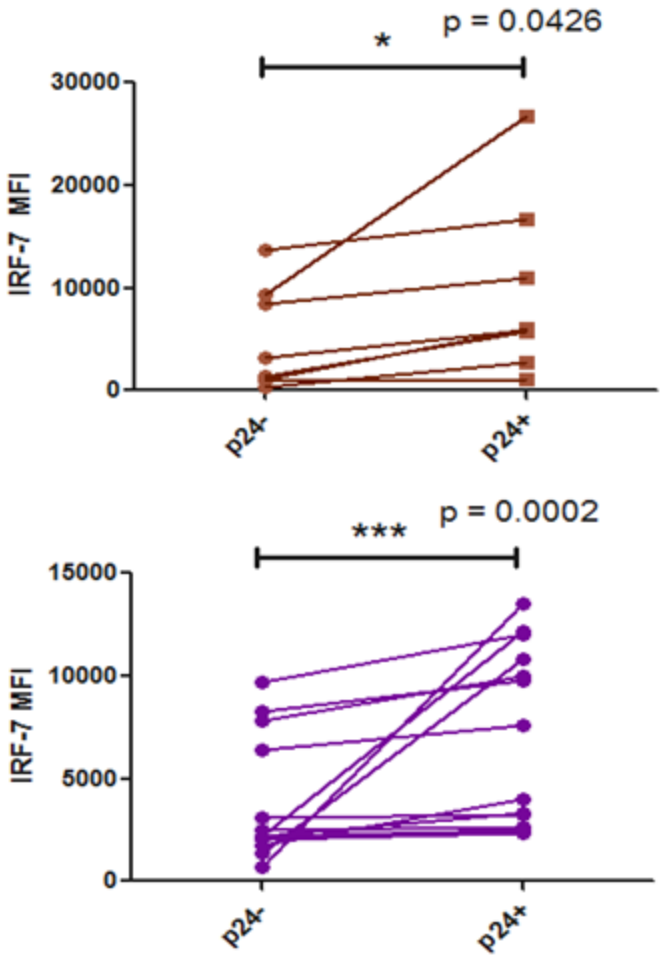


Figure 33. Levels of IRF-7 in uninfected compared to infected cell subsets in non-transduced CD4+ T cells (A) and transduced with lentiviral particles encoding for IRF-7 shRNA (B).

Expression of IRF-7 was measured by antibody staining. This figure shows median fluorescence intensity (MFI) of the PE-secondary antibody for expression of IRF-7 with each point representing the results from a single individual. Paired t-tests were performed to assess the expression differences between the groups (95% confidence level, or $p<0.05$).

Objective 3 - Determine the effects of IRF-7 knockdown on cytokine release using Milliplex multiplex assays

Increase in IL-6 and TNF-α at 24 and 96 hours post HIV-infection.

To determine if HIV-1 infection, length of infection (24 hours compared to 96 hours), or IRF-7 knockdown had an effect on cytokine release I measured IFN-γ, IFN-α2, IL-6, TNF-α, IL-4, IL-10, IL-12p70, IL-2, and IL-15 by Milliplex multiplex assay on cell supernatants collected from CD4+ T cells at 24 and 96 hours post infection. IRF-7 knockdown was achieved in primary CD4+ T cells either by transfection with IRF-7 specific siRNA, or by transduction with lentiviral particles encoding for IRF-7 shRNA. The goal was to determine if there were differences in cytokine expression in HIV uninfected compared to infected CD4+ T cells at 24 and 96 hours post-infection. As well, the effect of IRF-7 knockdown on cytokine secretion was assessed (control blue dots, IRF-7 knockdown black dots).

There was significantly higher expression of IL-6 in the infected cell subset compared to uninfected at both 24 and 96 hours post-infection (n=11, 24 hours: uninfected median = 0.45 and infected median = 7.370, p=0.0156) (n=12, 96 hours: uninfected median = 0.45 and infected median = 2.495 p=0.0313) (Figure 34 A and B). When analyzing the IL-6 data at 24 and 96 hours post-infection, cells with control (blue dots) had no significant differences between uninfected and infected (24 and 96 hours p=0.2500). The cells with IRF-7 knockdown had no significant differences (24 hours p=0.1250 and 96 hours p=0.2500). This may be due to the small sample size.

There was significantly higher expression of TNF-α in the infected cell subset compared to uninfected at 24 hours post-infected (n=11, uninfected median = 14.93 and infected median = 22.82, $p \leq 0.01$) (Figure 34 C). When analyzing the TNF-α data at 24 hours for differences between uninfected and infected for control and IRF-7 knockdown (blue dots) there were no significant differences (control p=0.1250) and knockdown (p=0.0625).

HIV-1 infection results in an increase in IL-6 and TNF-α, however the majority of cytokines showed no differences.

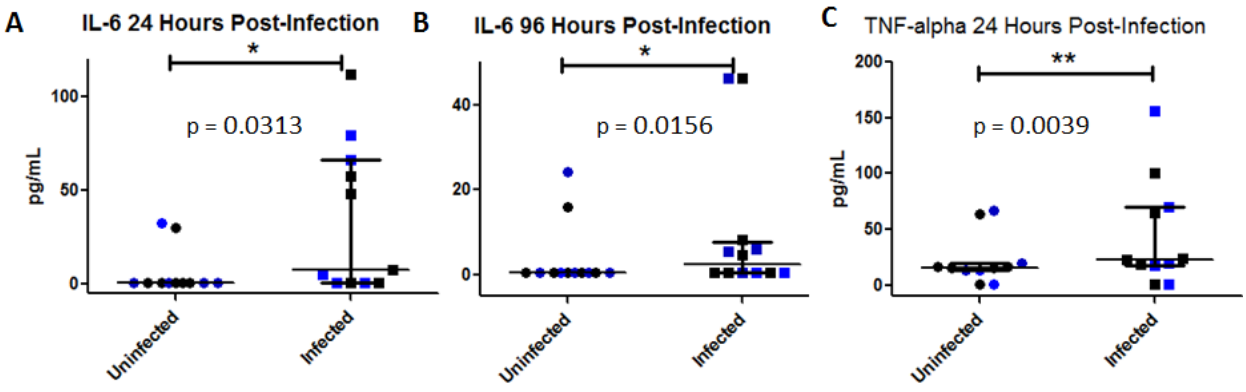


Figure 34. Comparing levels of IL-6 and TNF-α in uninfected and infected cell subsets at 24 and 96 hours post-infection by Milliplex assay.

IRF-7 knockdown was achieved in primary CD4+ T cells either by transfection with IRF-7 specific siRNA or by transduction with lentiviral particles encoding for IRF-7 shRNA. Supernatants were collected at 24 and 96 hours post-infection and analyzed by Human Cytokine/Chemokine Magnetic Bead Panel Milliplex assay for changes in IL-6 and TNF-α expression. Blue dots are from control CD4+ T cells and black dots are from cells with IRF-7 knockdown. Paired t-tests or Wilcoxon matched-pairs signed rank tests were performed to assess the differences between the groups (95% confidence level, or $p<0.05$).

Decrease in IFN-α2, IL-4, and IFN-γ production in the IRF-7 knockdown cells, compared to control.

To determine if knocking down IRF-7 had an effect on cytokine release I measured IFN-γ, IFN-α2, IL-6, TNF-α, IL-4, IL-10, IL-12p70, IL-2, and IL-15 by Milliplex multiplex assay. IRF-7 knockdown was achieved in primary CD4+ T cells either by transfection with IRF-7 specific siRNA or by transduction with lentiviral particles encoding for IRF-7 shRNA. Supernatants were collected at 24 and 96 hours post-infection, and analyzed by Human Cytokine/Chemokine Magnetic Bead Panel Milliplex assay.

There was significantly lower expression of IFN-α2 in the cells with IRF-7 specific knockdown compared to control (n=20, control median = 5.280 and knockdown median = 4.720, p=0.0092) when including HIV-1 uninfected and infected, and all time points (Figure 35 A). When data was analyzed looking only at 24 or 96 hours or only at uninfected or infected supernatants data was found to be not significant.

There was also significantly lower expression of IL-4 in the cells with IRF-7 specific knockdown compared to control (n=20, control mean = 21.10 and knockdown mean = 13.24, p=0.0391) at 96 hours post-infection (Figure 35 B). Data was also analyzed to determine if there were differences in HIV infected (red dots) (p=0.3125) and uninfected (p=0.25) samples.

There was significantly higher expression of IFN-γ in the supernatants collected from the control cells compared to the cells with IRF-7 knockdown at 96 hours post-infection in the supernatants of samples infected with HIV-1 (n=6, control median = 93.32 and knockdown median = 64.89, p=0.0313) (Figure 35 C).

There were no differences in IL-10, IL-12p70, IL-15, and IL-2 expression between 24 and 96 hours post infection, uninfected compared to infected, and in control cells compared to IRF-7 specific knockdown.

Knocking down IRF-7 results in decreased IFN-α2, IL-4, and IFN-γ expression at 96 hours post-infection. IL-10, IL-12p70, IL-15, and IL-2 expression was unchanged between all tested parameters.

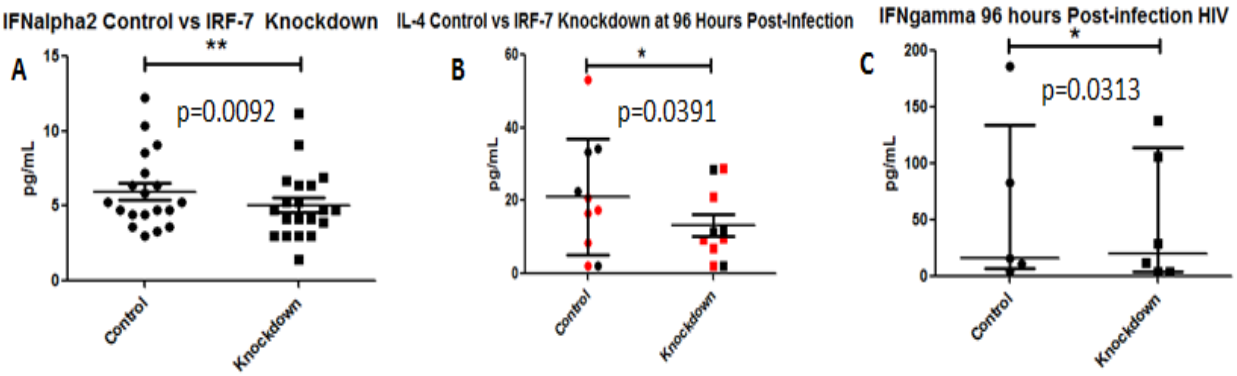


Figure 35. Comparing levels of IFN-α2, IL-4, and IFN-γ in control cells compared to cells with IRF-7 specific knockdown by Milliplex assay.

IRF-7 knockdown was achieved in primary CD4+ T cells either by transfection with IRF-7 specific siRNA or by transduction with lentiviral particles encoding for IRF-7 shRNA. Supernatants were collected at 24 and 96 hours post-infection, and analyzed by Human Cytokine/Chemokine Magnetic Bead Panel Milliplex assay for changes in IFN-α2, IL-4, and IFN-γ expression. For panel B, red dots are from cells infected with HIV-1. Paired t-tests and paired Wilcoxon signed rank tests were performed to assess the differences between the groups (95% confidence level, or $p<0.05$).

Summary of section:

It is possible that knocking down IRF-7 expression prior to HIV-1 infection (whether it be by electroporation or lentivirus) may lead to less HIV-1 replication as measured by percentage of p24+ CD4+ T cells, p24 MFI (intracellular), or secreted p24 (extracellular) however this data however was not significant. There are higher levels of IRF-7 in the HIV-infected cell subsets compared to the uninfected cell subsets (the p24+ cells compared to the p24- cells in the same culture), which suggests that either the virus is inducing IRF-7 to its own benefit or the cell is inducing IRF-7 in the antiviral response to HIV-1. Another possibility is that the virus is infecting cells expressing high levels of IRF-7. This may be the case, as IRF-7 does not increase during the course of infection. A decrease in IFN-α2, IL-4, and IFN-γ was observed in the cells with IRF-7 knockdown, which would lead to less inflammation and may be beneficial for the cell as the environment would be less conducive to HIV-1 infection. Overall, it appears as if less IRF-7 prior to infection may be beneficial to the host.

Discussion

Due to the increases in new infections and deaths due to HIV/AIDS and the continued global epidemic of HIV, it is critical to look for novel strategies to fight new HIV infections such as a preventative vaccine. To create an effective vaccine, we must first better understand the relationship between the virus and the host. IRFs play a key role in the regulation of the immune response, and while some research has been conducted on the effects of IRF-1 in relation to HIV, there is little known about the role of IRF-7. IRF-1 has been shown to play an important role in the transactivation of the HIV-1 LTR and consequently its viral transcription. There has been conflicting results on the role of IRF-7 in HIV-1, with studies suggesting that overexpressing or knocking down IRF-7 leads to decreased HIV-transcription or infectivity (*6, 8, 125*). It is therefore imperative to analyze the expression of these transcription factors in human primary cells and their relationship with HIV infection.

IRF-1 and IRF-7 are expressed in all defined cell subsets of human PBMC.

The percentage of cells expressing IRF-1 and IRF-7 was analyzed using CD4+ T cells (CD3+CD4+CD8-) as a representative of the lymphocyte population as T cells are the most common lymphocyte population analyzed and CD4+ T cells are the most common T cells, and classical monocytes (CD14highCD16-) as a representative of the monocyte population as they are the most common monocyte population. As well they are both (CD4+ T cells and monocytes) targets of HIV-1.

In the Manitoban donors the percentage of CD4+ T cells (CD3+CD4+CD8-) expressing IRF-1 in the absence of stimulation was quite high (>75%). After stimulation almost all (~100%) CD4+ T cells (CD3+CD4+CD8-) express IRF-1. The percentage of classical monocytes (CD14highCD16-) expressing basal levels of IRF-1 before stimulation was also very high (>85%). Following stimulation with IFN-γ, almost all monocytes express IRF-1 which shows that IRF-1 can be induced in almost 100% of cells, suggesting an important role of IRF-1 in IFN-γ stimulated immune response which includes the antiviral

response.

The expression of IRF-7 in the Manitoban donors for both the CD4+ T cells (CD3+CD4+CD8-) and classical monocytes (CD14highCD16-) was more variable than IRF-1 before and after stimulation. In the CD4+ T cell (CD3+CD4+CD8-) subsets there was substantial diversity between the levels of IRF-7 expression (>30%). After stimulation with IFN-αA there was a significant increase in the percentage of CD4+ T cells (CD3+CD4+CD8-) expressing IRF-7 in these cell types (>80%) ($p \leq 0.01$). In the classical monocyte (CD14highCD16-) populations there was also quite a bit of biological diversity between the levels of IRF-7 expression in unstimulated cells (>65%). After IFN-αA stimulation the percentage of classical monocytes (CD14highCD16-) increased significantly to around 100% in most cases ($p \leq 0.05$). Similar results were also observed in the Kenyan samples. In CD4+ T cells (CD3+CD4+CD8-) in HIV-uninfected donors the percentage of cells expressing IRF-7 increased after stimulation from 41.7-89.4% to 63.9-92%, and in HIV-infected donors from 16.2-91.4% to 51.6-94.5%. In the classical monocytes (CD14highCD16-) in HIV-uninfected donors the percentage of cells expressing IRF-7 increased from 40.6-99.4% to 58.3-100%. In the infected cell subset IRF-7 expression increased from 2.2-99.2% to 15.4-100% ($p \leq 0.05$).

The similarities between high IRF-1 and IRF-7 expression in monocytes compared to lymphocytes speak to their similar roles in the innate immune response as they are both involved in type I interferon expression. The differences in IRF-1 and IRF-7, such as higher overall percentage of cells expressing IRF-1, may suggest that higher basal levels of IRF-1 are required. This induction shows that our cells do respond to interferon, as well as that even if not all cells are expressing IRF-1 and IRF-7 their expression can be induced. The higher levels of IRF-1 and IRF-7 in the monocyte populations compared to lymphocytes may point to the importance of these transcription factors in the innate immune response. Thus it may be important in the context of infection for IRF-1 and IRF-7 to be first responders to infection.

Higher IRF-1 and IRF-7 in the inflammatory monocytes (CD14+CD16+) may have a role in maintaining the inflammatory reaction.

IRF-1 and IRF-7 were expressed in all cell subtypes tested. In unstimulated and IFN-γ stimulated PBMCs IRF-1 was expressed at the highest level in inflammatory monocytes (CD14+CD16+), and was expressed at significantly higher levels than the patrolling monocytes (CD14dimCD16+) and the classical monocytes (CD14highCD16-).

IRF-1 is involved in the activation of macrophages by pro-inflammatory signals that include IFN-γ (*123, 126*). IFN-γ is important in linking the innate to the adaptive immune response, and is involved in the antiviral response(*123*). IFN-γ is produced by NK cells (innate immunity) and by CD4+ and CD8+ T cells once antigen immunity develops (adaptive immunity). IFN-γ has been shown to be a potent activator of macrophages, and it has been suggested that IFN-γ may modulate monocyte differentiation (*127, 128*). Thus, the high level of IRF-1 in inflammatory monocytes (CD14+CD16+) may not be surprising, as IFN-γ is a pro-inflammatory signal and is critical for monocyte function.

In both unstimulated and IFN-αA stimulated PBMCs the highest expression of IRF-7 was in the non-classical monocyte subsets (inflammatory (CD14+CD16+) and patrolling (CD14dimCD16+) compared to the classical monocytes (CD14highCD16-) ($p \leq 0.001$). After stimulation IRF-7 forms a complex with IRF-3 and travels to the nucleus where it induces IFN expression by binding to the ISRE. IRF-7 is expressed at lower levels in most cell types compared to IRF-3, suggesting that IRF-3 is involved in the initial induction of IFNs and IRF-7 is responsible for the late up regulation (*78, 101*). IRF-7 is involved in a positive feedback mechanism where IRF-7 is upregulated by IFNs where it in turn upregulates IFN expression (*101*). Stimulation of IRF-7 expression by type I IFNs (IFN-αA) would result in the positive feedback mechanism, which would upregulate IRF-7 expression in these activated cell subsets. Type I IFNs are also involved in the inflammatory response. IRF-1 and IRF-7 may be expressed at higher levels in the

monocyte populations due to their role in innate immunity, and their importance in being first responders to infection. IRF-1 and IRF-7 both play a major role in the antiviral response, so quick responses to infection would be ideal.

Higher induction of IRF-1 in patrolling monocytes and IRF-7 in B cells after stimulation with IFN-γ and IFNα-A respectively.

For IRF-1 the highest fold change after stimulation with IFN-γ was in the patrolling monocyte subset. Patrolling monocytes (CD14dimCD16+) patrol the vasculature, and are recruited/respond to sights of inflammation where they remove debris and dying cells during disease states and reduce inflammation (*129, 130*). It is possible that the anti-inflammatory patrolling monocytes (CD14dimCD16+) increased at such high levels in response to the high levels of IRF-1 in the inflammatory monocytes (CD14+CD16+) to bring down inflammation. The highest fold increase in the lymphocyte populations was in the B cell (CD3-CD19+) subset, which may be to initiate an adaptive immune response. IRF-1 was also expressed at the lowest levels in B cells (CD3-CD19+). In the literature it has been shown that mice deficient in IRF-1 had no effect on B cell development which suggests that IRF-1 is not important in these cells (*131*). It has also been shown that B cells constitutively express IRF-1 at low levels during B cell maturation (*132*). These data are conflicting, however suggests that IRF-1 appears to not be critical, but low levels may be necessary to maintain B cell function.

For IRF-7 the highest fold change after stimulation was in the B cell (CD3-CD19+) subset. The B cell (CD3-CD19+) subset also had the lowest IRF-7 level among the stimulated cell subsets. Low levels of IRF-7 in the lymphocyte populations suggests that IRF-7 may play a bigger role in the innate immune response, rather than in T and B cell responses in the adaptive immune response. Perhaps the large induction of IRF-7 after stimulation may be due to B cell innate immune responses.

Higher IRF-1 and IRF-7 expression in non-lymphocytic cell subsets, perhaps reflect on the innate function of monocytes (CD14+) and DCs (HLA-DR+CD11c+CD16+).

Both IRF-1 and IRF-7 are expressed in all cell types examined, however they are both clearly expressed at a higher level in monocytes and DCs compared to lymphocytes. When comparing IRF-1 and IRF-7 expression between both unstimulated and stimulated PBMC subsets the highest expression (MFI) was found in the monocyte and DC populations compared to the lymphocyte populations (T-, B-, and NK cell populations) ($p \leq 0.0001$). These data were also observed in the Kenyan samples, both in the HIV-uninfected and HIV-infected cell subsets.

This could be explained by the larger size of monocytes and DCs compared to lymphocyte populations (*133*). The larger volume of the cells would associate with an increased volume in transcripts and transcriptional regulators. A more likely explanation, however, has more to do with the role of monocytes and DCs in the innate immune response. Monocytes are precursors to macrophages and DCs, which both have substantial roles in the regulation of the innate immune response (*134*). Macrophages are involved in direct pathogen clearance by phagocytosis, and play an important role in downregulation of the inflammatory response (*135*). DCs are antigen presenting cells that are able to direct an adaptive immune response, and influence the type of immune response (Th1/Th2) (*136*). DCs respond to innate activation, express innate antigen receptors, take up antigen, and present antigen (*136*). Innate immunity requires immediate responses. Having a responding factor ready would allow for a more rapid response.

As IRF-7 is the "master regulator" of type I IFN responses and the various elements of IFN responses, it is reasonable to speculate that IRF-7 has many important roles in the function of these cells whether they be the innate or adaptive immune response (*10*). Thus, IRF-7 may be expressed at higher levels in monocytes and DCs to prime these cells for rapid IFN production. It has been suggested that the

induction of IRF-7 expression is needed for monocyte differentiation to macrophages, and that overexpression of IRF-7 alone is sufficient to trigger differentiation; therefore, increased IRF-7 may be an integral part of monocyte differentiation (*137*). However, IRF-7 shares a DNA binding site with other IRFs (including IRF-1), which may also play a role (*137*). IRF-1's role in the adaptive response takes longer to establish, and the differentiation of T cells take time. Therefore, having higher levels in the innate immune cells would result in a quicker response to infection.

A future research question to address would be to reduce IRF-1 and IRF-7 expression in innate cells to see if that would affect the efficiency and the rapidness of the innate responses. Knocking down IRF-1 and IRF-7 expression in monocytes would be interesting, as these were the cell types with the highest expression, and then these cells could be infected with HIV-1. It would be interesting to see if there are differences in viral replication in the cells with the knockdown or without, to see the role of IRF-1 and IRF-7 in the innate immune response.

PBMCs were also stained for IRF-1 and IRF-7 for visualization by confocal microscopy to determine where in the cell they were expressed. I was able to visually confirm that the majority of IRF-1 and IRF-7 expression in unstimulated cells was in the cytoplasm. I was also able to confirm that I was able to stain for IRF expression in the nucleus, as seen in the confocal images. There are also a number of papers that used the same methodology to stain for IRF-7 expression in the nucleus and cytoplasm (*138, 139*). This helps to validate the antibodies and staining conditions used.

High IRF-7 expression in inflammatory monocyte, and low IRF-7 expression in DC, subsets of HIV-1-infected patients, suggest possible dysregulation of IRF-7 in HIV-1 infected patients.

In the Kenyan HIV-uninfected and infected cell subsets the highest expression of IRF-7 was found in the inflammatory monocytes (CD14+CD16+). These results are similar to the results for the Manitoban donors. In both the HIV-uninfected and infected cell subsets the lowest levels of expression were in the

B cells and cytokine producing NK cells (CD16-CD56high). In the PBMC samples from HIV-infected donors from Nairobi, Kenya, within the monocyte and DC populations, the lowest levels of IRF-7 were in the DC populations. DCs are one of the first cell types that HIV encounters during sexual transmission, due to their close proximity to the mucosal epithelia (*18*). DCs are poorly infected by HIV, however they are involved in trans-infecting the major HIV target CD4+ T cells (*18*). The low levels of IRF-7 found in the DCs from HIV-infected women may suggest possible overall immune dysregulation. DCs are responsible for linking the innate and the adaptive immune response, and it is possible that lower levels of IRF-7 may lead to the impaired immune responses often seen in HIV infection.

There were also low levels of IRF-7 in the NK cell populations. During HIV infection, a decrease in cytotoxic and cytokine producing NK cells during HIV infection leading to an 'anergic' NK cell population that is CD16+CD56-. This population is rare in healthy individuals, and is characterized by impaired cytokine secretion and increased inhibitory receptors on NK cells (*140*). HIV-1 has been known to dysregulate NK-cell responses, resulting in reduced cytotoxicity (*141*). This could explain the low levels of IRF-7 expressed in these cells.

In the IFN-αA stimulated PBMC samples from HIV-infected donors from Nairobi, Kenya there was significantly higher IRF-7 expression in the inflammatory monocytes (CD14+CD16+) compared to the other monocyte populations (patrolling (CD14dimCD16+) ($p \leq 0.01$) and classical monocytes (CD14highCD16-) ($p \leq 0.001$)) and the DC populations (HLA-DR+CD11c+CD16+) ($p \leq 0.01$).

In the HIV-uninfected populations there were no differences in fold change in response to stimulation by interferon between the lymphocyte and monocyte populations. In the HIV-infected populations between the lymphocytes, the highest fold increase after stimulation was in the cytokine-producing NK cells (CD16-CD56high) and the B cells (CD3-CD19+).

There were no significant differences between the fold change after stimulation with IFN-αA between the HIV-uninfected and HIV-infected groups, suggesting that the IRF-7 responsiveness was not affected

by HIV-infection. There were also no significant differences between either the unstimulated HIV-uninfected compared to the HIV-infected individuals, or the IFN-αA stimulated HIV-uninfected compared to the HIV-infected individuals. This suggests that IRF-7 may play a role in initial HIV infection during acute infection, but may not play a role in chronic infection.

Similar observations of the levels of IRF-7 were made in Manitoban blood donors and Kenyan female-sex workers (FSW), suggesting that these observations are independent of the biological effects of the sex trade.

In unstimulated PBMC samples from HIV-uninfected donors from Nairobi, Kenya there is significantly higher expression of IRF-7 in the inflammatory monocytes (CD14+CD16+) compared to the classical monocytes (CD14highCD16-) ($p \leq 0.001$) and the DC populations ($p \leq 0.01$). The expression of IRF-7 was lowest in B cells (CD3-CD19+) and cytokine-producing NK cells (CD16-CD56high), similar as to what was seen in the Manitoban donors.

In the IFN-αA stimulated PBMC samples from HIV-uninfected donors from Nairobi, Kenya between the monocyte populations there was significantly higher expression of IRF-7 in the activated cell subsets inflammatory (CD14+CD16+) ($p \leq 0.001$) and patrolling (CD14dimCD16+) ($p \leq 0.05$) compared to the classical monocytes (CD14highCD16-). Again, these data are very similar to the data found in the activated cell subsets for the Manitoban donors.

To summarize, the highest expression of IRF-7 was found in the inflammatory monocytes (CD14+CD16+) for both the HIV-infected and HIV-uninfected individuals. These data are similar to the levels of IRF-7 from the Manitoban donors, which suggests that levels of IRF-7 are not affected by the different lifestyles of the donors (Kenyan donors are female sex-workers), and that increased STI risk and sexual activity may not affect IRF-7 levels.

IRF-7 knockdown may lead to less p24+ CD4+ T cells.

7% knockdown of IRF-7 was achieved by electroporation with IRF-7 siRNA and 83% knockdown of IRF-7 was achieved by using lentiviral particles encoding for IRF-7 shRNA. The cells were infected with either HIV-1 IIIB or a donor laboratory isolate ML 1956 HIV-1 subtype for 96 hours, followed by the collection of supernatants and cell staining for p24 and IRF-7. I found that the cells that had the IRF-7 specific knockdown prior to infection had a smaller percentage of p24+ cells after infection even though this was not statistically significant. These observations were found in both the electroporation and lentivirus knockdown. I also found statistically significantly higher levels of IRF-7 in the infected cell subset compared to the uninfected (control and knockdown, $p \leq 0.05$ and $p \leq 0.001$). This leads to the question of whether IRF-7 induction during HIV infection plays any role in host defense against HIV infection, or whether IRF-7 expression is induced by HIV-infection to facilitate viral replication and dissemination. Based on the data it appears as if knocking down IRF-7 expression prior to HIV infection resulted in reduced HIV-infection (about a 10% decrease), however these data were not found to be significant. It is possible that the virus may be using IRF-7 for its viral transcription, similar to IRF-1. IRF-1 is involved in the transactivation of the HIV-1 LTR, and can bind to two distinct sites on the HIV-1 LTR: the ISRE and at the NF-κB binding sites (*11*). It has also been shown that knocking down IRF-1 20-40% in primary CD4+ T cells prior to HIV infection resulted in more than 90% decrease in the transactivation of HIV genes (p24 measured by ELISA and gag mRNA measured by qPCR) (*74*). It has also been theorized that IRF-7 may bind to the ISRE similarly as IRF-1 but this has yet to be shown (*6*). It is therefore possible that IRF-7 binds to the ISRE along with IRF-1, and may be involved in the transactivation of the HIV-1 LTR.

It is not to say that IRF-7 does not have a role in anti-viral responses. IRF-7 is part of the innate immune response's 'first line of defence'. This infection study was performed with a 90% enriched CD4+ T cell population, and hence might not have addressed the role of IRF-7 in monocytes and DCs where IRF-7 expression was found to be higher than in CD4+ T cells. Evidence from other groups that augment IRF-7

expression level using various means prior to infection with HIV-1 strongly support the role of IRF-7 in anti-viral response. Such discrepancy in observed data may be explained by the differences in the cell types examined.

Rollenhage, *et al.* showed that IRF-7 expression stimulated by poly I:C in cervical tissues from HIV-1 seronegative women increased the antiviral response, and consequently decreased HIV-1 transcription and viral release. Tissues or PBMCs were treated with poly I:C for six hours prior to HIV-1 infection and then HIV-1 reverse transcription, integration and gene transcription were measured by real-time PCR (*125*). They observed a decrease in infection which was proposed to be due to enhancement of IRF-7 by stimulation of the RIG-1/MDA5 signalling pathway. This paper also reduced IRF-7 expression using IRF-7 specific siRNA in cervical tissues. They observed an upregulation of HIV-1 gene transcription and NF-κB transcription in total cervical tissues (*125*). They also demonstrated lower IRF-7 expression in PBMCs at day 3 post-infection, and then higher expression at day 5 post-infection. This demonstrates that lower IRF-7 at the beginning of HIV-1 infection may be critical in the initial HIV infection for the virus, but increases at day 5 may initiate the anti-viral response. Once the cells are infected (later after HIV-exposure) higher IRF-7 mediated antiviral responses may be critical in controlling the dissemination/spread of the viral infection. Another article looked at IRF-7 over-expression using lentiviral vectors in STAT1-deficient fibroblasts and human hepatoma cells, where it was shown that IRF-7 overexpression inhibited HIV-1 replication (*8*). These data together suggest that overexpressing IRF-7 prior to HIV-1 infection leads to reduced HIV-1 replication.

Another article looked at the effect of deleting the IRF-1/7 binding site from the HIV-1 long terminal repeat (LTR) in primary monocyte-derived DCs (MDDCs) (*6*). An HIV-1 BAL IRF-1/IRF-7 binding site deletion mutant was created by replacing the ISRE with the Zeicher linker sequence (*6*). The deletion of the binding site resulted in decreased virus infectivity, which suggests that the induction of IRF-1 and IRF-7 during the early phase of viral infection of MDDCs may aid in viral replication (*6*). This paper does

not focus on IRF-7 specifically, and this could be due to IRF-1 on its own. Sirois et al. looked at HIV-1 infection in primary macrophages after modulation of IRF-7 expression (*9*). They found that downregulation of IRF-7 by siRNA decreased HIV-1 replication, whereas overexpression of IRF-7 by transfection of pcDNA3-IRF7-myc resulted in an increase in viral replication measured by p24 ELISA (*9*). The data observed in my experiments were not significant, however there was a trend suggesting that knocking down IRF-7 prior to HIV-1 infection resulted in decreased HIV-1 replication similar as to what was seen in these experiments. I did however see an increase in IRF-7 in the HIV-1 infected cells, which may be due to the virus preferentially infecting activated CD4+ T cells which were expressing higher levels of IRF-7 or the virus increased IRF-7 after infection to its own use. It is also possible that the cell increased IRF-7 as a process of the antiviral response in response to infection.

It was suggested by Sirois *et al.* that such as in primate models, that higher levels of type I IFN are associated with a pathogenic state (*9, 142*). These data further imply that the virus undermines the innate immune response, and uses it to its own advantage.

IRF-7 cannot enhance the activity of the HIV-1 LTR on its own in primary human macrophages, and may require interaction with another protein such as IRF-1 or facilitate the translocation and activity of NF-κB (*9, 11*). It is possible that, similar to IRF-1, HIV-1 requires IRF-7 for its viral transcriptional activity, and that IRF-7 expression increases shortly after infection is required for continual viral replication.

Another possibility is that HIV-1 is preferentially infecting cells with increased IRF-7 levels. This would explain the higher levels of IRF-7 in the HIV-infected cell subsets. HIV-1 preferentially infects activated CD4+ T cells, so these data are not surprising. Since I did not see an increase in IRF-7 expression after 96 hours of infection compared to time point 0, this also leads to me to believe that the virus is infecting already activated CD4+ T cells expressing high levels of IRF-7. If the virus was inducing IRF-7 expression or if the cells were responding to infection (initiating the antiviral response) there should be an increase in IRF-7 at 96 hours post infection. These data lead me to believe that lower IRF-7 prior to HIV-1

infection is beneficial, as there is a decrease in activated CD4+ T cells for the virus to infect. This could be another target for induction of immune quiescence, which has been hypothesized as a method to protect against HIV-1 infection (*64*). The cells which express the highest levels of IRF-7 were not tested in the knockdown experiment when in theory, they are best candidate for knockdown. One would expect if these cells were subject to knockdown, the results might be different.

Increase in IL-6 and TNF-α after HIV-1 infection.

Supernatants were collected at 24 and 96 hours post-infection, and analyzed using microbead array assay (Milliplex multiplex assay). Post HIV-1 infection, higher levels of IL-6 were found in the infected cell culture supernatant, compared to the uninfected, at both 24 and 96 hours post-infection. This is in agreement with studies showing IL-6 production was detected in the cell culture supernatant of HIV-infected cells (*143*). IL-6 is a pro-inflammatory cytokine produced by monocytes, macrophages, and T cells in response to HIV, and it is involved in T cell activation and B cell differentiation and maturation (*144*). It is possible that increased IL-6 is solely due to the addition of viral culture, which is supernatant from PHA-stimulated PBMCs infected with HIV-1 that would most likely contain large amounts of IL-6. However, the data shows increased IL-6 at both 24 and 96 hours post-infection, and at 96 hours post-infection there were multiple removals of cell supernatants and replacement with fresh media. Therefore, any residual viral supernatant added at time point 0 would be diluted.

TNF-α is also a pro-inflammatory cytokine produced by a variety of cells, including CD4+ T cells, but is mainly secreted by macrophages. It is involved in biological processes such as cell proliferation, apoptosis, and differentiation (*144-146*). TNF-α has been shown to increase in response to HIV-1 infection, and a positive correlation between TNF-α and increased HIV-1 plasma load has been seen (*147*). TNF-α has also been shown in the literature to stimulate HIV-1 replication in T cells (*143*). During HIV-1 infection there is an increase in dying cells, which would lead to an increase in pro-inflammatory

cytokines such as TNF-α.

Decrease in IFN-α2 and IL-4 after IRF-7 knockdown.

I finally wanted to analyze whether there were differences in cytokine release between cells with IRF-7 knockdown compared to control cells. To do this I compared supernatants from cells with either IRF-7 specific knockdown or control using microbead array assay (Milliplex multiplex assay). Post HIV-1 infection, I found decreased IFN-α2 and IL-4 in the supernatant from cells with IRF-7 specific knockdown compared to the control group. IRF-7 is considered the 'master' regulator of type I IFNs, and therefore it is not surprising that a decrease in IRF-7 would lead to a decrease in IFN-α2 production (*10*). In terms of HIV infection, IFN-α is a double edged sword. IFN-α has been shown to be critical during the acute phases of HIV infection, but damaging to host immune function during chronic infection. Decreasing, but not abolishing, IFN-α expression might not impact the host negatively in the context of HIV infection (*148*).

There is little known about IRF-7 in the context of IL-4 regulation. IL-4 is involved in the differentiation of Th0 cells to Th2, where it is involved in promoting a humoral immune response (*149*). The release of IL-4 has been shown to be induced by HIV-1 gp120 in CD4+ T cells, and Th2 cells have been shown to be more efficient in replicating HIV-1 virus (*150, 151*). It is possible that less IL-4 leads to less infection, and less pathogenesis. It therefore might be advantageous for the IRF-7 knockdown cells to have less IL-4 production in terms of HIV infection.

Reduced IFN-γ in the IRF-7 specific knockdown cells compared to control cells may lead to reduced inflammation, and a reduction of activated CD4+ target cells leading to decreased HIV-1 infection. Increased IFN-γ secretion at 96 hours could also be associated with initiation of the adaptive immune response and disease control (*152*).

There weren't any significant differences between IL-10, IL-12p70, IL-15, and IL-2 between uninfected

and infected, 24 compared to 96 hours post-infection, and in IRF-7 specific knockdown compared to control. There was very minimal IL-10, IL-12p70, and IL-15 detected by the assay which led to no significant differences. As well, I added IL-2 to our media during infections, so it was again not surprising that there were no significant changes.

Increases of IL-6 and TNF-α after HIV-1 infection are not surprising, as these have both been well documented in published literature. A decrease in IFN-α2 due to IRF-7 knockdown is also expected, as IRF-7 is involved in regulating IFN-α expression. A reduction in IFN-γ may be advantageous to the cell, as this may lead to reduced inflammation and reduced activated target CD4+ T cells. Little is known about IL-4 in relation to IRF-7, however it is possible that reduced IL-4 may lead to reduced HIV-1 infection.

Conclusions

I was able to characterize IRF-7 expression in a variety of blood immune cell subsets from Manitoban donors and HIV-infected and HIV-uninfected female sex workers from Nairobi, Kenya. I determined that IRF-7 was expressed at a higher level in monocytes and DCs compared to lymphocytes. I also observed up-regulation of cellular IRF-1 by exogenous IFN-γ in Manitoban donors and IRF-7 by exogenous IFN-αA in both Manitoban and Kenyan donors. I found no differences in the level of IRF-7 expression and the proportion of PBMCs expressing IRF-7 between HIV-uninfected and HIV-infected donors from Nairobi, Kenya.

The second part of my thesis determined the effects of IRF-7 knockdown on cellular susceptibility to HIV-1 infection. I successfully knocked down IRF-7 expression in CD4+ T cells using electroporation transfection and lentiviral particles encoding IRF-7 shRNA. I observed that reducing cellular IRF-7 expression prior to HIV-1 infection with a laboratory strain, IIIB, and a clinical isolate, ML 1956, resulted in less CD4+ T cells being infected measured through decreased percentage of p24+ cells observed at 96 hours post-infection. This data was not significant, however, and as a result I cannot indicate any true

difference yet. I also observed higher IRF-7 expression in the p24+ cells (i.e. infected cells) when compared to p24- cells (i.e. uninfected). These suggest a potential link between IRF-7 and HIV, and I speculate that IRF-7 may play a role in regulating HIV-1 transcription at the early stage/phase of HIV-1 infection similar to IRF-1. The effects of IRF-7 knockdown on cellular cytokine secretion in response to HIV-1 infection exhibited a decrease in IFN-α2, IFN-γ, and IL-4. It is possible that HIV-1 is infecting cells expressing higher levels of IRF-7. Knocking down IRF-7 prior to HIV-1 infection could result in less activated CD4+ T cells, and less HIV-1 infection. These points together prompt an overarching hypothesis that knocking down IRF-7 expression in HIV-1 target cells (i.e. CD4+ T cells) prior to infection may reduce cellular susceptibility to HIV-1 infection and limit the spread of the virus, as well as perhaps limit the pathogenesis of the disease by hampering inflammatory cytokine production during the later stages of infection. This study does not suggest that IRF-7 works on its own in regulating HIV-1 replication and antiviral response, and it may work with other proteins such as IRF-1. The molecular mechanisms underlying IRF-7's involvement in cellular susceptibility to HIV-1 infection require further study, and how IRF-7-regulated IFN-α2 and IL-4 expression affect HIV-replication, and perhaps pathogenesis during late stages of HIV-infection, will require detailed kinetic study.

Study Limitations

Possible limitations of this study are that only CD4+ T cells were examined in the knockdown studies not all PBMCs. The results may be different when the CD4+ T cells were placed back in with the other cell subsets.

Using a scramble or empty control vector for the lentiviral vector studies would have been a more appropriate control than non-transduced cells. Transducing the cells with lentiviral particles may lead to some effects. However, the electroporation transfection results support my data found for the lentiviral vector transduction studies.

Statistical significance does not always mean physiological significance. All results must be analyzed with this in mind.

Future work.

Immediate future work in following up on these findings includes: (1) determining whether or not IRF-7 binds to the HIV-1 LTR directly by chromatin immunoprecipitation (ChIP), which would be useful in determining IRF-7's role in HIV-1 transcription; (2) determining how IRF-7 knockdown affects cellular susceptibility to infection with other viruses, such as influenza or herpes simplex virus, since IRF-7 has been documented to play critical roles in these infections via the interferon signalling pathway; (3) determining the effects of knocking down IRF-7 expression in monocytes on HIV-1 infection, as they expressed the highest levels of IRF-7 (4) determining the effects of overexpressing IRF-7 prior to HIV-1 infection on cellular susceptibility and on antiviral inflammatory responses during late stages of HIV-infection to see if higher levels of IRF-7 would result in an increase in HIV-1 infection.

Impact of these findings.

Since the beginning of the HIV-1 pandemic about 34 million individuals have died due to HIV-related causes (*153*). Even with successful ARTs there is still a need to create alternative treatment strategies, as ARTs do not eradicate the virus, and ART is a life-long treatment with undesirable side-effects and the opportunity for the emergence of drug-resistance. My data suggests that decreasing IRF-7 prior to HIV-1 infection may be beneficial to host cells *in vivo*, however there are still a number of questions that remain. It is clear that susceptibility to HIV-infection is a multi-factorial phenomenon, and the potential effect of IRF-7 knockdown on HIV-1 infection alone is not sufficient to create a strong preventative strategy. For the best effect it would need to be combined with other strategies, and its effects would

have to be better understood. Continued research on IRF-7 holds promise, and may lead to findings that contribute to tools for a successful HIV prevention strategy.

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