Effects of ARVs and HIV on mitochondrial function and immune activation in monocyte derived macrophages in vitro

by

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Master of Science

in

Health Sciences

Carleton University
Ottawa, Ontario

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David Roy
Declaration

I, David L.F. Roy, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.
Dedication

I would like to dedicate this work to my parents, for everything they have done and continue to do for me. For teaching me work ethic, responsibility, accountability, and for giving me every opportunity to live a life of happiness and success. And also to all HIV patients around the world, many of whom live every day in pain and fear, you inspire me.
Abstract

It is estimated that 36.7 million people are living with HIV, 20.9 million of whom are accessing antiretroviral therapy (ART). While ART reduces HIV associated morbidity and mortality, low levels of persistent inflammation in virally suppressed individuals have been shown to drive a form of accelerated aging and contribute to the development of cardiovascular disease, metabolic disorders, dyslipidemia, and neurological impairment. Monocyte and macrophage activation is a central driver of this persistent inflammation but the molecular mechanisms underlying their chronic activation remain poorly understood. My thesis examined if antiretroviral (ARV) drugs contribute to this chronic activation via modulation of mitochondrial function in macrophages. For these studies, I treated uninfected and HIV-infected human monocyte derived macrophages (MDMs) with ARVs (Tenofovir (TFV), Lamivudine (3TC), and Efavirenz (EFV); alone or in combination) and characterized alterations in cell viability and mitochondrial function. I then examined how these alterations affected inflammatory cytokine production. I found individual ARV drugs had distinct effects on mitochondrial function and metabolism. TFV increased cellular reactive oxygen species, gradually shut down cellular respiration, increased cell death and decreased inflammatory cytokine production. 3TC had limited effects on mitochondrial function and cell death. EFV immediately decreased cellular respiration, reprogrammed mitochondrial function, decreased ATP production, decreased expression of complex I of the ETC, increased cell death, increased superoxide and total cellular reactive oxygen species (ROS) production, and increased TNF-α and IFNβ production. ROS scavenger, N-acetyl cysteine (NAC), decreased cell death in TFV and EFV, whereas superoxide scavenger, MitoTEMPO, decreased cytokine production and cell death in EFV. In the context of HIV infection, TFV, when treated at the time of infection, increased cellular ROS production and decreased complex I expression of the ETC. Collectively, results show the newest generation of ARV medications reprogram mitochondrial function and that this reprogramming affects cell viability and cytokine production. These findings may have important implications for HIV-infected patients using ART every day but also at-risk uninfected individuals using ART as pre-exposure prophylaxis (PrEP). Further experimentation must be performed to confirm the link.
between the ARV associated mitochondrial alterations and their role in immune activation in vivo.
Acknowledgements

I would sincerely like to send my gratitude out to my supervisor, Dr. Edana Cassol, for her belief in me, her hard work, her understanding, and her constant motivation to make me a better Master’s student. I have learned an incredible amount over the last two years, more than I had ever expected, and a lot of that is thanks to you.

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<td>Lamuvudine</td>
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<td>ART</td>
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<td>ECAR</td>
<td>Extracellular acidification rate</td>
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<td>EFV</td>
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<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<td>ETC</td>
<td>Electron transport chain</td>
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<td>Group-specific antigen</td>
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<td>Glycoprotein 120</td>
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<td>Glutathione</td>
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<td>Heat stable antigen</td>
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<td>Highly-soluble C-reactive protein</td>
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<td>Integrase Inhibitor</td>
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<td>IP-10</td>
<td>Interferon gamma inducible protein-10</td>
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<td>Interferon regulatory factor</td>
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<td>Macrophage colony stimulating factor</td>
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<td>MA</td>
<td>Matrix</td>
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<td>MAVS</td>
<td>Mitochondrial antiviral signaling protein</td>
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<td>MCP-1</td>
<td>Monocyte chemoattractant protein-1</td>
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<td>MDM</td>
<td>Monocyte derived macrophages</td>
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<td>MFI</td>
<td>Mean fluorescence intensity</td>
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<td>Mfn</td>
<td>Mitofusin</td>
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<td>MMP</td>
<td>Mitochondrial membrane potential</td>
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<td>Multidrug resistance-associated protein 4</td>
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<td>Mitochondrial DNA</td>
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<tr>
<td>mtROS</td>
<td>Mitochondrial reactive oxygen species</td>
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<td>MVEC</td>
<td>Microvascular endothelial cell</td>
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<td>NAC</td>
<td>N-acetyl cysteine</td>
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<td>NAD</td>
<td>Nicotinamide adenine dinucleotide</td>
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<td>NCI</td>
<td>Neurocognitive impairment</td>
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<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
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<tr>
<td>Acronym</td>
<td>Full Form</td>
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<td>NFV</td>
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<td>NOD-like receptor protein-3</td>
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<td>NNRTI</td>
<td>Non-nucleoside reverse transcriptase inhibitor</td>
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<tr>
<td>NP</td>
<td>Nucleoprotein</td>
</tr>
<tr>
<td>NRTI</td>
<td>Nucleoside reverse transcriptase inhibitor</td>
</tr>
<tr>
<td>OCR</td>
<td>Oxygen consumption rate</td>
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<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
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<td>PAPAC27</td>
<td>Pituitary adenylate cyclase-activating peptide 27</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
</tr>
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<td>PBS</td>
<td>Phosphate buffer saline</td>
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<td>PGC-1α</td>
<td>Proliferator-activated receptor-gamma coactivator 1 alpha</td>
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<td>Protease inhibitors</td>
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<td>Polymerase</td>
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<td>Protease</td>
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<td>PrEP</td>
<td>Pre-exposure prophylaxis</td>
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<td>PRR</td>
<td>Pattern recognition receptor</td>
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<tr>
<td>Rev</td>
<td>Regulator of expression of virions proteins</td>
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<td>RLR</td>
<td>RIG-1 like receptor</td>
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<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<td>Reverse transcriptase</td>
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<td>Ritonavir</td>
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<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
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<td>Single nucleotide polymorphism</td>
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<td>SOD</td>
<td>Superoxide dismutase</td>
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<td>Saquinavir</td>
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<td>Tat</td>
<td>Trans-activator of transcription</td>
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<td>T-helper</td>
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<td>Toll-like receptor</td>
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<td>Tumor necrosis factor</td>
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<td>TPV</td>
<td>Tipranavir</td>
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<tr>
<td>TRAIL</td>
<td>TNF-related apoptosis-inducing ligand</td>
</tr>
<tr>
<td>UTT</td>
<td>Universal test and treat</td>
</tr>
<tr>
<td>Vif</td>
<td>Viral infectivity factor</td>
</tr>
<tr>
<td>Vpr</td>
<td>Viral Protein R</td>
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</table>
Vpu: Viral protein U
Vpx: Viral protein X
WHO: World Health Organization
Chapter 1: Introduction

1.1 HIV: History and Pathogenesis

1.1.1 HIV/AIDS Epidemic

June 2019 marked the 38-year anniversary since HIV/AIDS was first described by the CDC in 5 patients in Los Angeles, California, USA\(^1\). Although these patients were among the first identified with disease, the transmission from animals to humans likely occurred between 1920 to 1940; with the probable zoonotic events occurring from chimpanzees (HIV-1) and sooty mangabeys (HIV-2) in Central and West Africa respectively, before making its way to North America via Haiti around the year 1968\(^2\)–\(^4\). Since then, science and medicine have had to put forth a valiant effort in order to dampen the burden of HIV/AIDS on the world community. In 1984 the US Secretary of Health and Human Services predicted a vaccine to be in place within two years\(^5\) but 34 years have come and gone without a vaccine being approved for regular administration\(^6\). Life-saving treatments, however, were approved in 1987 with the discovery and initiation of the antiretroviral (ARV) drug zidovudine (AZT)\(^5\). Although a great advancement in HIV research, AZT has substantial toxic effects on patients, and has since been excluded from common antiretroviral therapy (ART) regimens\(^5\). Nevertheless, this marked a building block for other ARVs to be developed, and widespread access to affected countries around the world began in the late 20\(^{th}\) century and continues into the early 21\(^{st}\) century\(^5\). This vast and rapid administration was in large part to philanthropic and government aid; notably the Bill and Melinda Gates Foundation, the William J. Clinton Foundation, the Global Fund for AIDS, Tuberculosis and Malaria, and the President’s Emergency Program for AIDS Relief which was put in place by president George W. Bush in 2003\(^5\)–\(^7\). These programs, along with others, spread access of ART across low and middle-income countries, saving millions of lives of those infected as well as those exposed to HIV/AIDS\(^5\). Still, it is estimated that over 30 million people have died to due HIV/AIDS since 1981, and over 37 million people are living with the disease today\(^2\)–\(^7\). The most heavily affected area continues to be sub-Saharan Africa, with approximately 1 in 4 of the world HIV-population residing in this area of the world and prevalence rates as high as 57% in some populations of women\(^2\)–\(^7\). The reasons behind higher rates in this area of the world are still up for debate, yet factors such as stigma, poverty, traditional beliefs, polygamous
relationships, governmental policy, and migration appear to be commonly agreed upon theories\textsuperscript{5,8}. Nevertheless, progress continues to be made, with worldwide death rates decreasing by 37.5\% from 2005 to 2013, and another 33\% from 2010 to 2018\textsuperscript{9}. The number of new infections decreased 35\% from 2000 to 2014\textsuperscript{7}, and another 16\% decrease from 2010 to 2018\textsuperscript{9}. However, certain populations continue to be at high risk of infection, including men who have sex with men, people who inject drugs, and sex workers\textsuperscript{10}. These populations are 20 times more likely to acquire the disease compared to the rest of the world population, representing 53\% of new HIV infections in those who reside outside of eastern and southern Africa\textsuperscript{9}. By 2020, it is expected that 81\% of the global HIV population will be on antiretroviral therapy (ART)\textsuperscript{10}, just shy of UNAIDS’s goal of 90\%\textsuperscript{9}. Also by 2020, 21\% of the global HIV population will be over 50 years of age; an increase of 13\% since the year 2000\textsuperscript{10}. As a result, HIV has evolved from a dangerously acute and fatal disease to a more chronic disease. In Canada, approximately 63,110 people were living with HIV at the end of 2016\textsuperscript{11}. Over 50\% of new infections in 2016 were in the male homosexual or bisexual community, although this community only accounts for 2-3\% of the total Canadian male population\textsuperscript{11}. People who inject drugs accounted for 11.3\% of new HIV infections, and indigenous people also accounted for 11.3\% of new infections in 2016 even though they only account for 4.9\% of the total Canadian population\textsuperscript{11}. 9.6\% of Canadians living with the disease in 2016 were indigenous, with the prevalence of HIV rate of in this population being twice as high as the rest of Canada\textsuperscript{11}. This is primarily due to HIV infected aboriginal individuals living in significantly worse social and economic circumstances compared to Caucasians Canadians\textsuperscript{12}. Compared to Canadian Caucasians, HIV-infected aboriginals are more likely to be younger, female, homeless, less educated, and have lower incomes, putting them at higher risk for comorbidities\textsuperscript{12}.

1.1.2 HIV pathogenesis

HIV can be transmitted via sexual intercourse, intravenous drug use, blood transfusion, bone or organ transplantation\textsuperscript{7}. Depending on the mode of transmission, the virus spends a few days at the site of infection (i.e., mucosal tissue) before making its way to the local lymph nodes and then more generally into the bloodstream around day 10, where it begins to spread
exponentially for approximately a month\textsuperscript{7,13}. This timeline, however, can vary between individuals, along with the progression to AIDS, which normally takes approximately 6-10 years in 70-80\% of individuals without treatment\textsuperscript{7,14}. The rate of progression is directly associated with the loss of CD4+ T cells. HIV has been shown to induce cell-death in infected and uninfected CD4+ T cells, through varying mechanisms\textsuperscript{15}. In infected cells, cell death has been shown to occur in three different ways\textsuperscript{15}. Firstly, during abortive infection (i.e. when the viral life cycle ends before integration), where cell death is thought to be mediated by caspase-1 and caspase-3 activation\textsuperscript{16}. Secondly, during HIV integration, cell death appears to be induced via p53 phosphorylation and hence p53-dependant cell death\textsuperscript{17}. Thirdly, following HIV integration, HIV protease has been shown to cleave caspase-8, which then travels to the mitochondria causing depolarization of the mitochondrial membrane and the release of cytochrome c leading to downstream initiation of apoptosis\textsuperscript{18}. This HIV driven apoptosis leads to the gradual depletion of the CD4+ T cell population\textsuperscript{16-18}. In uninfected CD4+ T cells, cell death can be induced through circulating viral proteins such as Vpr, gp120, Tat, and Nef, as well as overexpression of death ligands such as Fas, TRAIL, and TNF\textsuperscript{19}.

Early during infection, HIV targets the gastrointestinal tract, which contains >80\% of memory CD4+ T cells in the body\textsuperscript{20-22}. This targeting selectively depletes Th17 and Th22 subsets, which are vital to gut integrity, and this has been linked to inflammation\textsuperscript{23,24}. This depletion can lead to dysregulation within the gut microbiome which can cause the release of unwanted bacterial products into the bloodstream, also known as microbial translocation\textsuperscript{23-25}. The release of these bacterial products leads to the activation of immunomodulatory signalling pathways, the release of inflammatory cytokines such as tumor necrosis factor-alpha (TNF\textalpha{}), interleukin-6 (IL-6), IL-10, and IL-4, and the subsequent activation of immune cells\textsuperscript{26}. The release of these cytokines due to the virus itself or microbial translocation further induces cell death in CD4+ T cells as well as other immune cells leading to further immunodeficiency\textsuperscript{19}. Markers of microbial translocation have been shown to predict mortality in HIV-infected individuals, as well as comorbidities such as cardiovascular disease (CVD), type 1 diabetes, inflammatory bowel disease, periodontal disease, obesity, and other metabolic disorders\textsuperscript{17}. In HIV patients, positive correlations have been found between the gram-negative bacterial component,
lipopolysaccharide (LPS), and inflammatory markers such as IL-6, TNF-α, and highly-soluble C-reactive protein (hsCRP)\textsuperscript{27}. Similar correlations have also been found between monocyte activation marker, sCD14, and pro-inflammatory cytokine, IL-1β\textsuperscript{27}. In HIV patients, less microbial diversity has been observed, and this is further decreased following the administration of ART\textsuperscript{25,28}. This suggests that ART alone may have a detrimental effect on gut integrity\textsuperscript{19}. Lower bacterial diversity in stool has been shown to be associated with elevated LPS, LPS binding protein (LBP), and monocyte activation (sCD14, sCD163), which are all also markers of inflammation\textsuperscript{25,28}. These events lead to systemic and chronic inflammation seen in HIV-infected patients with or without ART\textsuperscript{29}. This systemic inflammation and drastic depletion of immune cells, predominantly uninfected and infected CD4+ T cells, causes HIV-infected individuals to lose immune function and progress towards acquired immunodeficiency syndrome (AIDS), making them vulnerable to other opportunistic diseases and eventual death due to these comorbidities\textsuperscript{14,15}.

1.1.3 HIV Life Cycle

HIV enters into a host cell and induces viral production through a series of steps known as the HIV life cycle (Figure 1). The cycle begins with the initial binding of the envelope glycoproteins gp120 and gp41, also known as Env, to the host cell’s cell membrane by binding to the CD4 receptor\textsuperscript{30}. This initial interaction leads to a conformational change in Env, allowing for binding to a secondary chemokine co-receptor\textsuperscript{30}. Most isolates of HIV use C-C chemokine receptor type 5 (CCR5) as the co-receptor and are known as R5 HIV, but a subset of viruses can evolve to use C-X-C chemokine receptor type 4 (CXCR-4) and are known as X4 HIV\textsuperscript{30}. X4 HIV is seen more predominantly in later stages of HIV infection\textsuperscript{31}. Based on this tropism, HIV predominantly infects CD4 T cells as well as monocytes, macrophages, and dendritic cells, since they also express these receptors\textsuperscript{2,7}. Following co-receptor binding, complete fusion of the viral and host cell membranes occurs leading to opening and stabilization of the membrane fusion pore, allowing for viral reverse transcriptase (RT), RNAse HCl, Integrase (IN), and viral RNA to enter the host cell\textsuperscript{30}. HIV genetic material consists of two identical copies of single-stranded RNA encoding for 17 viral proteins: capsid (CA), matrix (MA), nucleoprotein (NP), protease (PR),
reverse transcriptase (RT), RNase HCl, integrase (INT), surface glycoprotein (gp120), transmembrane protein (gp41), Tat, Rev, Nef, vif, vpr, vpu, vpx, and tev\(^2\). These proteins all have slightly different roles in viral replication and host cell evasion\(^2\). Upon entry into the cell, viral single-stranded RNA is transcribed into viral double-stranded DNA using viral RT as machinery\(^2,7\). Once viral DNA is produced, RNase HCl degrades the viral RNA, and IN allows for the viral DNA to travel to the nucleus where it is integrated into the host cell’s genome\(^2\). HIV mRNA is transcribed in the nucleus, which then leaves the nucleus for viral proteins to be translated in the cytoplasm\(^7\). New virions are assembled and PR then cleaves the viral Gag-Pol precursor in 9 places in order to allow for the maturation of newly formed viral proteins. This allows for the virus to spread to other cells and restart the viral life cycle\(^3,2\). Antiretroviral therapy was created with the goal of inhibiting this viral life cycle at different critical points in order to decrease the replication and spread of HIV.

Figure 1 The HIV life cycle and role of different antiretroviral therapies (ART).
1.2 Antiretroviral Therapy (ART)

1.2.1 Development of ART

As shown in Figure 1, ART has the ability to inhibit the HIV life cycle at different steps. When HIV was first shown to cause AIDS in 1983, a mad scramble ensued in order to find a cure and prolong the lives of infected individuals. Originally, antiviral medication acyclovir, used for the treatment of herpes simplex virus, was one of the only antivirals on the market. Nucleoside and nucleotide analogues (NRTIs) began being explored such as AZT, a drug first created in 1964 as a cancer drug with no success. AZT hit the market quickly, in 1987, after one small study showed that mortality was dramatically decreased with treatment. Desperate for a cure, AZT began decreasing acute mortality in populations, but toxic side effects and viral resistance became apparent rather quickly, and another solution was needed. Other nucleoside and nucleotide analogs came onto the market in the early 1990s, but were still only used as monotherapies, and viral resistance developed quickly. Soon after, dual-therapy was used with more success than monotherapy, but still effectiveness only lasted for a limited duration. It wasn’t until 1996 when triple therapy, also known as highly active antiretroviral therapy (HAART), was administered and viral resistance really began to decline. As a result, HAART is used to this day. Other drug classes such protease inhibitors (PIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs) also became FDA approved in 1995 and 1996 respectively, which further decreased HIV’s ability to build up resistance. ART is capable of halting the viral life cycle at multiple places in the aforementioned cascade of events. Entry inhibitors inhibit the viral attachment to the CD4 receptor, CCR5 or CXCR4 inhibitors inhibit co-receptor attachment, fusion inhibitors inhibit fusion between the viral and host membranes, nucleoside or non-nucleoside reverse transcriptase inhibitors (NRTIs or NNRTIs) halt the activity of reverse transcriptase, integrase inhibitors (INSTIs) inhibit integration of the virus into the host’s genome, and protease inhibitors (PIs) inhibit protease cleavage necessary for the maturation of infectious viral proteins.

In typical first-line ART regimens in low and middle income countries, two NRTI’s are used in combination with an NNRTI, a PI, or an INSTI. Universal test and treat (UTT) was implemented by the World Health Organization (WHO) in 2015 recommending that ART be initiated in all HIV-infected individuals, regardless of their CD4
count, as supposed to previous guidelines which only recommended ART to only patients with low CD4 counts (below 500 cells/mm$^3$)\textsuperscript{36}. This has been shown to decrease HIV transmission in populations with high rates of HIV\textsuperscript{37}, and is thought to improve patient outcomes even when initiated before symptoms present themselves\textsuperscript{38}. These guidelines have been altered over the evolution of ART, when the drugs were first used alone in treatment. AZT was first used in 1987, it was shown to prevent mother to child transmission in 1994, HAART was first used in 1996, public health systems began providing ART in 1997, a plan for global ART coverage began in 2004, and ART was shown to prevent transmission between couples in 2011\textsuperscript{7,39}.

1.2.2 Early Toxic Effects

Early ART came with toxic side effects, as it was shown that frequent bone marrow suppression, nausea, headaches, myalgia, insomnia, and macrocytosis occurred in individuals who were administered AZT\textsuperscript{40}. Compared to the individuals who were administered a placebo, AZT patients had significantly more cases of anemia and neutropenia as well as more AZT patients needed red-cell transfusions compared to the placebo group\textsuperscript{40}. It has also been reported that AZT decreases cell viability \textit{in vitro}\textsuperscript{41} and induces mitochondrial toxicity at physiological concentrations\textsuperscript{42–44}. As years passed, ART improved, with decreases in toxicity shown in NRTIs TFV, emtricitabine (FTC), and lamivudine (3TC) compared to other commonly prescribed ART such as AZT, didanosine (ddI), ritonavir (RTV), lopinavir (LPV), and stavudine (d4T)\textsuperscript{45,46}. As a result, TFV, FTC, and 3TC became commonly used NRTI backbones of first-line regimens seen in most ART, especially in low-middle income countries\textsuperscript{35}. Still, even with the improvement of ART, chronic inflammation persists\textsuperscript{47,48}, along with detrimental effects on metabolism\textsuperscript{49–51}. A common first-line ART regimen consists of TFV/3TC/EFV\textsuperscript{52}, and their chemical structures are shown in Figure 2. Both TFV and 3TC are NRTIs, although TFV is a nucleotide analog and 3TC is a nucleoside analog\textsuperscript{53}. NRTIs inhibit the viral reverse transcriptase by acting as chain-terminators of viral DNA synthesis, impeding the transcription of viral DNA from single stranded viral RNA, and the integration into the host cell genome\textsuperscript{54}. All NRTIs all lack a 3'-hydroxyl yet all also differ in structure\textsuperscript{53}. On top of this, 3TC replaces C3' with a sulfur and has an L-enantiomeric ribose form, whereas has a C-P phosphonate instead of an O5’-P phosphate linkage\textsuperscript{53}. All NRTIs must
be phosphorylated by kinases to gain a triphosphate form before acting in the cell, but TFV requires less phosphorylation due to its already existant phosphonate group\textsuperscript{53}. 3TC and TFV also differ in the kinases used to undergo phosphorylation before activation\textsuperscript{53}. EFV, an NNRTI, also impedes viral DNA synthesis by binding non-competitively to the hydrophobic pocket near the active site of the HIV reverse transcriptase, causing a change in the 3D structure of the enzyme, and leading to its inhibition\textsuperscript{55}.

![Chemical structure of ART drugs tenofovir (TFV), lamivudine (3TC), and efavirenz (EFV).](image)

**Figure 2** Chemical structure of ART drugs tenofovir (TFV), lamivudine (3TC), and efavirenz (EFV).

1.2.3 **Pre-exposure Prophylaxis (PrEP)**

In 2010, ART also began being used to prevent initial infection in uninfected individuals, through pre-exposure prophylaxis (PrEP)\textsuperscript{7}. This marked a great step in the battle against HIV, as now uninfected at-risk individuals could protect themselves. The use of ART in at-risk individuals has also been up for debate, however, with studies showing detrimental effects in treated individuals when inflammation is present\textsuperscript{56}. When an NRTI genital gel, tenofovir (TFV), was used in at-risk individuals, 75\% percent of patients who adhered consistently to the treatment avoided HIV infection in the absence of elevated inflammatory cytokines (less than 3 elevated inflammatory cytokine levels)\textsuperscript{56}. In the presence of elevated inflammatory cytokines (3 or more), however, this number dropped to -10\% effectiveness\textsuperscript{56}. In fact, the results suggested that in the presence of inflammation, TFV did not improve effectiveness compared to the placebo, with less infections occurring in the placebo group\textsuperscript{56}. This suggests that there is more to be understood in the context of ART and immune activation in uninfected patients.
1.3 Non-AIDS Comorbidities

1.3.1 Comorbidities Associated with HIV/AIDS and ART

In HIV-infected individuals on ART, metabolic alterations are thought to be driven, at least in part, by persistent inflammation and immune dysfunction. Inflammatory markers such as CD14, LPS, IL-6, C-reactive protein, CD163, TNF-α, CD27, CD38, neopterin, CXCL10, D-dimer, IL-8, and IL-12 continue to be elevated in HIV patients. These markers have been shown to be associated with morbidity and mortality. Increases in these markers may occur due to continued HIV replication, microbial translocation, and co-infections. HIV-infected patients with optimal CD4+ positive counts (>500 cells/mm<sup>3</sup>) also have increased inflammation, indicating that healthy HIV-infected individuals are still vulnerable to comorbidities.

Furthermore, highly-active antiretroviral treatment (HAART) can decrease T-cell activation to normal levels and suppress viral loads, but monocyte and macrophage activation persists, which can help explain the increased inflammation. Persistent immune activation such as TNF-α and IL-1β secretion can also induce neurocognitive impairment and HIV-associated dementia in HIV-infected individuals. Monocytes are able to penetrate the blood-brain barrier and cause neuronal death leading to disease through the release of inflammatory cytokines. Since monocytes, macrophages, and microglia can be infected at a much more productive level than neurons and astrocytes, this inflammation and subsequent neuronal damage stems from the monocyte transport of HIV across the blood brain barrier causing viral replication and inflammatory cytokine release. While treatment prolongs life and substantially reduces the risk of HIV transmission, it does not completely restore health.

HIV-infected individuals on virally-suppressive ART manifest a phenotype of accelerated aging and experience increased rates of non-AIDS associated comorbidities such as neurocognitive impairment, cardiovascular disease, insulin resistance, hyperglycaemia, dyslipidemia, and diabetes after longer periods of metabolic dysregulation.

1.3.2 Neurocognitive impairment

Neurocognitive impairment (NCI) has been shown in HIV-infected patients, with and without ART. When comparing patients from the pre-cART era (1988-1995) to the cART era (2000-
2007) in HIV asymptomatic patients, patients on cART had more NCI than those not on cART, suggesting that ART does play a role in NCI independent of the HIV infection\textsuperscript{64}. Patients who had documented high levels of early immunosuppression shown by low nadir CD4 or high viremia had higher levels of NCI in both the pre-cART and cART eras, suggesting that there is an irreversible aspect to NCI and this advocates for the need to begin cART at an early stage in viral infection\textsuperscript{64}. Importantly, in HIV patients, a link between inflammation (IL-6), obesity, monocyte/macrophage activation (sCD14 and sCD40L), and neurocognitive impairment has been shown.\textsuperscript{73}

1.3.3 Cardiovascular disease (CVD)

Acute myocardial infarction has been shown to increase by 50\%, after excluding non-HIV-associated risk factors, in HIV-positive patients compared to uninfected individuals\textsuperscript{74}. HIV has also been shown to cause significant increases in hospitalizations due to coronary heart disease and myocardial infarction\textsuperscript{75}. In terms of ART, incidence of heart attack has been shown to increase 10-fold in patients after 4 years of combination ART (cART), with myocardial infarction probability increasing 26\% for the first 4-6 years of cART\textsuperscript{76}. These patients experiencing increased cardiovascular disease also show symptoms resembling metabolic syndrome such as hyperlipidemia, hyperglycemia, and hypertension\textsuperscript{77}, as well as increased artery thickness and hypercholesterolemia\textsuperscript{78}. Due to the increased persistent immune activation, even when viral loads are suppressed and CD4+ T-cell counts are optimal, there is a greater risk for cardiovascular disease in HIV-positive individuals\textsuperscript{74}. Inflammatory cytokine, IL-6, levels have been shown to correlate with cardiovascular disease\textsuperscript{79}. HIV-infected individuals have higher arterial inflammation in the aorta as well as elevated levels of circulating macrophages\textsuperscript{80}. Compared to participants in the Coronary Artery Development in Young Adults (CARDIA) study and the Multi-Ethnic Study of Atherosclerosis (MESA), IL-6 was shown to be 62\% and 152\% higher in HIV-infected patients respectively\textsuperscript{81}. Therefore, it appears that persistent inflammation due to HIV and ART can lead to increased risk of CVD, and monocytes/macrophages have been shown to be direct contributors\textsuperscript{82}. 
1.3.4 Insulin Sensitivity and Diabetes

HIV-infected patients on ART have been shown to have a 4-fold increase in the incidence of diabetes mellitus. Also, more exposure to cART has been shown to correlate with higher incidence of diabetes, and this was also associated with higher levels of cholesterol, HDL, and triglycerides. Dysregulation in lipid metabolism and adipose tissue has been shown to increase immune activation, inflammatory response, oxidative stress, and cell death. It has been shown through metabolic profiling of high-risk HIV-infected patients (CD4 nadir <300 cells/mm³) on protease inhibitors that they express dyslipidemia correlating with microbial translocation, hepatic function, inflammatory cytokine production (IL-6 and TNF-α), and mitochondrial function. It has also been shown in HIV/AIDS patients that plasma triglycerides, inflammatory cytokine levels (TNF-α), and free fatty acids are significantly increased, negatively correlating with CD4+ T cell levels. These findings suggest that there is a benefit in screening for dyslipidemia throughout HIV infection, and that lipid metabolism play a critical role in the infection and immune activation of HIV/AIDS. Increases in immune response and inflammatory cytokine production can also lead to increased insulin, saturated fatty acids, insulin resistance, blood glucose levels and diabetes. Since macrophages, in the classical inflammatory state (M1), and other inflammatory immune cells rely on glycolysis, this increase in blood glucose spurs on immune activation; something that can accentuate the effects of HIV and cell damage. Therefore, it appears that persistent inflammation due to HIV and ART can lead to increased risk of diabetes and other metabolic disorders.

1.4 Monocytes/Macrophages

1.4.1 Role of Macrophages

Macrophages, Greek for “big eaters”, are myeloid cells known for their phagocytic capabilities and are found in all tissues within the body. Known as the most plastic cells of the hematopoietic lineage, they have the ability to alter their activation states based on the environment and alter their function based on cytokine, viral and microbial exposures as well as the tissue microenvironment in which they reside. Macrophages represent the first line of defense against invading microorganisms, play a central role in activating the adaptive immune system, and their dysregulation plays a critical role in the pathogenesis of HIV/AIDS.
system, in regulating inflammatory responses, and in tissue modeling and repair. They differentiate from blood circulating monocytes once recruited into their designated tissue, where they can reside from hours to years, depending on their need and the tissue they are recruited to. Brain resident macrophages for example, known as microglial cells, are thought to live for decades in some cases. Within the tissue, a macrophage’s primary role is to maintain homeostasis of the environment. By ingesting unwanted or potentially harmful material, they are able to test the environment and release cytokines to mount responses when needed.

1.4.2 Macrophage Polarization States

There is a proposed paradigm that macrophages have different polarization states, although it is still not widely accepted and it is still heavily debated. The 3 proposed polarization states are M0 which is activation naïve, M1 which is pro-inflammatory, and M2 which is anti-inflammatory; although these polarization states are thought to be fluid and far more complex than simple groupings. In fact, some believe that there are only two polarization states: classically activated (M1) and alternatively activated (M2). The ability of these states to be achieved and reproduced in vitro is also up for debate, with many not agreeing upon the stimuli used to achieve said polarization states, and the general ability to mimic in vivo polarization states within a petri dish is very difficult. Overall, it is perhaps unrealistic to take macrophages from a human body, and perfectly reproduce the exact environment that they would be succumb to during states of activation in the body. This may be a reason for why it is difficult to translate M1/M2 surface markers from in vitro studies into in vivo situations. Nevertheless, during disease states, these polarization states are achieved through cytokine signaling as well as contact with microbial or viral products, leading to release of pro-inflammatory or anti-inflammatory cytokines in an attempt to regain homeostasis within the tissue environment. During M1 pro-inflammatory responses typically driven by interferon-gamma (IFN-γ), classical pro-inflammatory cytokines such as interleukin-1 (IL-1), tumor necrosis factor alpha (TNFα), IL-6, IL-8, and IL-12 are released which can activate cellular innate immune signaling pathways, as well as IL-12 and IL-23 which lead to T helper cell differentiation (Th1 and Th17) for further
pathogen clearance and systemic inflammation\textsuperscript{91,95,96}. Once the pathogen or damage is under control, however, this inflammation must be dampened or else there runs the risk of damage due to excessive, prolonged, or chronic inflammation\textsuperscript{91,93,96}. Following inflammatory states, macrophages either die off or switch their activation to a more M2-like, anti-inflammatory state, mainly through the production of IL-10\textsuperscript{96}. It should be noted, however, that M2-like states may also be achieved directly following pathogen interaction\textsuperscript{97}. IL-10 inhibits the production of aforementioned pro-inflammatory cytokines as well as inhibits the macrophages' ability to respond to such pro-inflammatory cytokines\textsuperscript{96}. Transforming growth factor beta (TGF-β) is another anti-inflammatory cytokine that dampens macrophage pro-inflammatory cytokine production and responses, as well as T helper cell (Th1 and Th2) proliferation\textsuperscript{96,98,99}. When the production of these cytokines and the responses to them are dysregulated within the immune system, and in macrophages in particular, cytotoxicity and disease has been shown to increase\textsuperscript{96,100}.

1.4.3 HIV Infection in Macrophages
Macrophages have the receptors (CD4) and co-receptors (CCR5 and CXCR4) necessary for HIV infection, and have the ability to be infected in the brain, lungs, and secondary lymphoid tissue\textsuperscript{101}. That being said, macrophages in different tissues have different degrees of infectivity, with vaginal macrophages being more readily infected than gut macrophages, for example\textsuperscript{102}. In general, macrophages are readily infected, but have more of an ability to resist infection in comparison to CD4+ T cells, which infect at higher levels and also experience more HIV-mediated cell death\textsuperscript{103}. This is primarily thought to be due to macrophages' lower levels of CD4 and CCR5 receptor expression compared to CD4+ T cells\textsuperscript{101}. Macrophages have the ability to absorb the virus, protect it, and retain its infectivity before presenting it to T cells, leading to their infection\textsuperscript{104}, and they can also be infected through phagocytosis of infected CD4+ T cells\textsuperscript{105}. Macrophages are known as a principal reservoir for HIV in tissues, as they have the ability to survive longer periods of time as infected cells at different stages of infection, and they can support high levels of viral replication, as seen in late stages of HIV infection when CD4+ T cells are mostly depleted\textsuperscript{106}. Also, during ART, infected macrophage populations have
shown the ability to survive, expressing low and persistent levels of viral replication\textsuperscript{101,103,107}. They have been shown to be more resistant to the effects of ART compared to lymphocytes, showing higher EC\textsubscript{50}\textsuperscript{108}. Microglia, in particular, have shown the ability to resist the effects of ART, as ART has difficulty crossing the blood brain barrier and cells have the ability to efflux ART efficiently in the central nervous system (CNS)\textsuperscript{109}. These latently infected macrophages appear to have the ability to evade the immune system and survive long periods of time, creating long-lasting viral reservoirs\textsuperscript{110}. These viral reservoirs are thought to be key contributors to chronic viral replication leading to chronic immune activation\textsuperscript{25}. Macrophage polarization states also play a role in macrophage infectivity, as it has been shown \textit{in vitro} that classically activated macrophages (M1) and alternatively activated macrophages (M2a) are more resistant to HIV infection\textsuperscript{110,111}. Furthermore, HIV infection causes macrophages to become dysfunctional, even in uninfected macrophages exposed to the HIV environment, also known as bystander cells\textsuperscript{112,113}. HIV-infected and bystander primary human macrophages have also been shown to upregulate TNF-related apoptosis-induced ligand (TRAIL) which can lead to the apoptosis in T cell bystander cells\textsuperscript{114}. As a result, macrophages are an important target in the fight against HIV\textsuperscript{115}.

1.5 Immunometabolism

1.5.1 Overview of Immunometabolism

In the past 5 to 10 years, immunometabolism has come to the forefront of science in order to provide a more detailed and complex understanding of the immune system\textsuperscript{116}. Through this, the science community has come to understand the importance of cell signaling through metabolites, their pathways, and their receptors as well as activation states of different immune cell types\textsuperscript{116}. In recent years, it has been shown that metabolism is not only important for cellular respiration and function, but also for pathogen growth and containment\textsuperscript{117}. The immune system is a highly regulated set of cells and processes that must be able to react and adapt quickly to incoming pathogens or acute trauma\textsuperscript{118}. Cells must have tightly regulated mechanisms of activation and inhibition in order to maintain homeostasis, giving the cell and organism the best chance at survival\textsuperscript{118}. Dysregulation within metabolic pathways can lead to
many detrimental alterations within the immune system such as altered cytokine production leading to undesirable activation states\textsuperscript{116}. There are 6 primary metabolic pathways: glycolytic, TCA cycle, pentose phosphate, fatty acid oxidation, fatty acid synthesis, and amino acid synthesis which are all recruited in different circumstances in order to achieve different metabolic states and reprogramming\textsuperscript{116}. They are all separate, individual pathways, yet they work together and are interconnected\textsuperscript{116}. Mitochondria produce ATP in their inner membrane via oxidative phosphorylation and the electron transport chain (ETC) during aerobic cellular respiration\textsuperscript{119}. The ETC uses electrons provided by the Kreb’s Cycle after oxidizing acetyl coenzyme A (CoA) which can be produced directly by amino acid metabolism or indirectly by glycolysis and beta-oxidation via pyruvate\textsuperscript{119}. This leads to aerobic cellular respiration, which is necessary for cell survival, but it also causes by-products such as reactive oxygen species (ROS) and superoxides (\(O_2^\cdot\)) predominantly through complexes I and III of the ETC\textsuperscript{120}. Superoxides, a type of mitochondrial ROS, are produced from the reduction of oxygen by one electron, and are used mainly in signaling pathways and also to combat invading pathogens, namely viral pathogens\textsuperscript{119,120}. Mitochondrial ROS are known to help the body respond to stress by inducing signaling pathways to allow the body to adapt and reprogram to maintain homeostasis in stressful situations\textsuperscript{160}. It has been shown in knockout mice that macrophages producing more ROS have a better ability to eliminate pathogens\textsuperscript{121}. One mechanism used by ROS linking them to inflammation is the up-regulation of the subset of pattern recognition receptors (PRRs) called toll-like receptor (TLR)\textsuperscript{122}. Toll-like receptors are expressed on innate immune cells with the role of stimulating cells following contact with pathogen-associated molecular patterns (PAMPs) on bacteria, viruses, or fungi\textsuperscript{123}. In macrophages, lipopolysaccharide (LPS) treatment, a toll-like receptor-4 (TLR4) agonist from gram-negative bacteria, can induce proinflammatory and antiviral (Type I Interferon) responses via TLR4 and the NF-KB signaling pathway\textsuperscript{123}. Proinflammatory cytokine production favours the MyD88-dependant pathway, whereas Type I Interferon production favors the My-D88-independent (TRIF-dependent) allowing for proinflammatory and antiviral responses in cells\textsuperscript{123}. In addition, damage to mitochondria can release damage associated molecular patterns (DAMPs) which can bind to TLRs and cause macrophages to activate an immune response leading to persistent
Inflammation also during LPS stimulation, macrophages predominately shift their energy production from oxidative phosphorylation to glycolysis while driving ROS production and an inflammatory phenotype. This inflammatory phenotype was shown to be reversed, however, when ROS production was blocked. Such evidence, along with others, show the importance of the mitochondria in regulating host cell immune responses. In general, oxidative phosphorylation is favored in the presence of oxygen (aerobic respiration), whereas glycolysis is favored in the absence of oxygen (anaerobic respiration). This switching of energy sources and cellular metabolism is dependent on the mitochondria, and critical in the activation of macrophages and other immune cells. Mitochondrial antiviral signaling (MAVS) proteins are another mechanism which link cellular signaling to immune responses. Another type of PPRs called RIG-1 like receptors (RLRs) recognize PAMPs which cause downstream activation of MAVS, which must be localized to the mitochondrial membrane in order to cause downstream effects. This interaction amplifies antiviral responses through the activation of interferon regulatory factor-3 (IRF3) and NF-KB, showing the importance of the mitochondria for host cell antiviral responses. The NLRP3 pathway, a proinflammatory pathway induced by PAMPs and DAMPs, is another pathway used to protect the host via inducing immune responses primarily through the release of interleukin-1-beta (IL-1β). This pathway has also been shown to be activated by ROS, with the main source of this cellular ROS being the mitochondria due to the fact that dysregulation within the mitochondrial inhibited ROS production as well as the NLRP3 pathway. Finally, mechanisms of apoptosis run through the mitochondria, which can also contribute to immune activation or depletion depending on the cell type and circumstance. In macrophages, for example, the clearance of apoptotic or necrotic cells can have different pro or anti-inflammatory effects depending on the mechanism, the effectiveness of clearance, and the timing of cell death. Macrophages can survive long periods of time, or can die off quickly following recruitment due to inflammatory or infectious stimuli. During phagocytosis of apoptotic cells, activated macrophages can also secrete tumour necrosis factor alpha (TNF-α), activate the inflammasome, and release interleukin-1 (IL-1) depending on the mechanism of cell death and caspase production. The intrinsic pathway of cell death is dependent on the mitochondria, with stressors such as...
oxidative stress, viral infection, and DNA damage leading to caspase activation\textsuperscript{139}. Bcl-2, an important regulator of apoptosis, is found on the mitochondrial membrane and is known to inhibit the release of cytochrome c and mitochondrial membrane permeability via Bax and, and Bcl-2 has also been show to regulate ROS production via oxidative phosphorylation\textsuperscript{139,140}. p53, the infamous tumor suppressor involved in over 50% of cancers today, has also been shown to act on the mitochondria\textsuperscript{139}, downregulating inhibitors of apoptosis such as Bcl-2\textsuperscript{141} and survivin\textsuperscript{142}. A loss in p53 has also been shown to switch metabolism from oxidative phosphorylation to glycolysis, and increase ROS production\textsuperscript{143}, showing the importance of the mitochondria and metabolism during apoptosis. Overall, there is clear evidence that the mitochondrion plays a crucial role in immune activation via different mechanisms.

1.6 Mitochondria: Overview and Known Effects of HIV and ART

1.6.1 Role of the mitochondria in the cell

The immune system is a highly regulated set of cells and processes that must be able to react and adapt quickly to incoming pathogens or acute trauma\textsuperscript{118}. Cells must have tightly regulated mechanisms of activation and inhibition in order to maintain homeostasis, giving the organism the best chance at survival\textsuperscript{118}. Much of this adaptation has been shown to be regulated by cellular metabolism; a balance between catabolic and anabolic events\textsuperscript{144}. The mitochondrion is a vital organelle located in the cytoplasm of most mammalian cells, consisting of two membranes compartmentalizing the organelle into an outer membrane, inner membrane, intermembrane space, and a matrix\textsuperscript{145}. The mitochondrion is responsible for many cellular functions such as cellular respiration, energy production, cell signaling, cellular metabolism, cell death and proliferation, hypoxic responses, Ca\textsuperscript{2+} homeostasis, redox balance, reactive oxygen species (ROS) production, tricarboxylic acid (TCA) cycle, as well as others\textsuperscript{145,146}. In the cytosol, glucose is converted into pyruvate via glycolysis and used in the mitochondrion as its main source of energy\textsuperscript{147}. Alternate sources can also be used to produce ATP such as fatty acids via fatty acid β-oxidation (FAO)\textsuperscript{148} and amino acids which are converted to ketone bodies in order to enter the TCA cycle\textsuperscript{149}. The TCA cycle produces reducing agents NADH and FADH\textsubscript{2}\textsuperscript{150}, which create a flow of electrons through the complexes of the electron transport chain (ETC), creating
a proton gradient to produce ATP via ATP synthase (Complex V)\textsuperscript{150}. These electrons, however, can leak out of the complexes (mainly complexes I and III) creating ROS such as superoxides (O\textsubscript{2}\textsuperscript{-}), hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}), and hydroxyl free radicals (· OH)\textsuperscript{150}. The production of ROS under homeostasis is normal as they are cleared by antioxidants such as enzymatic defenses, superoxide dismutase (SOD) and glutathione peroxidase (GSH), or non-enzymatic defenses, vitamin C, vitamin E, or thiols\textsuperscript{151}. SOD has two forms: the manganeses form that scavenges superoxides in the mitochondria, as well as the copper and zink form that scavenges ROS in the cytoplasm\textsuperscript{151}. When mitochondrial function is altered, however, the redox state of the mitochondria can be impacted, creating excessive amounts of ROS leading to cellular oxidative stress and inflammation\textsuperscript{151}.

1.6.2 Mitochondrial Morphology

To take on all these roles within the cell, mitochondria must be plastic organelles, with the ability to reorganize their structure and morphology, via fission and fusion events, in order to adapt to their environment and reprogram the cell to fulfill phenotypic requirements\textsuperscript{145}. Fusion is the act of the mitochondria expanding and fusing together to create networks in metabolically active cells and allowing them to dissipate energy, whereas fission occurs in metabolically dormant cells when the mitochondria fragments and creates smaller mitochondria\textsuperscript{152}. Mitofusins (Mfn1/Mfn2), the protein responsible for fusion, and Dynamin-related protein 1 (Drp1), the protein responsible for fission, are necessary for proper homeostasis development of mitochondria in mammals, as lacking one or the other results in death in mice before birth\textsuperscript{153,154}. Fission and fusion have been shown to alter immune responses and ROS production in brain resident macrophages, microglia, suggesting that mitochondrial morphology plays an important role in regulating inflammation\textsuperscript{155}. Regulation of the number of mitochondria also occurs in a process called ‘mitophagy’, where excess or damaged mitochondria are tagged and transported via isolation vesicles to lysosomes to be recycled\textsuperscript{156}. A lack in controlled mitophagy and an increase in dysfunctional mitochondria can occur due to oxidative stress, apoptotic proteases, and mutations in mitophagic pathways, which can lead to increased cell death signals from the mitochondria\textsuperscript{156,157}. This can disrupt cell
function and cause increased apoptosis leading to toxicity and a dysfunctional immune system\textsuperscript{158}.

1.6.3 Mitochondrial Innate Immune Pathways

In terms or regulating immune responses, mitochondria play a vital role in response to stress, damage, and pathogens\textsuperscript{159}. The cell uses pattern recognition receptors (PRRs) to seek out damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) in order to recognize stress, damage, and pathogens, and activate pathways in order to mount an appropriate response\textsuperscript{147}. Mitochondrial regulation occurs downstream of such receptors\textsuperscript{159}. In antiviral responses, for example, PRRs known as retinoic acid inducible gene-like receptors (RLRs) recognize PAMPs which activate a signaling pathway resulting in mitochondrial release of mitochondrial antiviral signaling (MAVS) protein\textsuperscript{132}, and reactive oxygen species (ROS)\textsuperscript{147}, which lead to antiviral responses and NLRP3 inflammasome activation\textsuperscript{133}. Dysregulation of mitophagy and mitochondrial signaling pathways can lead to dysregulation of mtROS and inflammasome homeostasis, causing increased systemic inflammation and, ultimately, inflammatory disorders\textsuperscript{133}. Furthermore, many damage-associated molecular patterns (DAMPs) have been shown to originate from the mitochondria during dysregulated homeostasis, triggering systemic immune activation and inflammation leading to inflammatory disorders\textsuperscript{160}.

1.6.4 Effects of HIV on Mitochondria

Through the release and regulation of many molecules and pathways, the mitochondrial membrane is of the main cellular mediators of programmed cell death\textsuperscript{161}. Mitochondrial membrane potential is generated by proton pumps in the electron transport chain (complexes I, III, and IV)\textsuperscript{162}. Small fluctuations in the MMP are normal, but larger fluctuations can be detrimental for the cell, and can drive the cell towards death\textsuperscript{162}. A large increase MMP could mean high ATP production, but could also mean high ROS production, whereas a large decrease leads to low levels of ATP production and a reductive state that can be damaging to the cell\textsuperscript{162}.
HIV is known to deplete mtDNA\textsuperscript{150}, reprogram energy production via oxidative phosphorylation\textsuperscript{163}, as well as increase ROS and superoxide production\textsuperscript{164} to allow for spread of the virus\textsuperscript{165}. Inhibition of mtDNA replication can lead to decreases of mtDNA numbers, oxidative stress and, significant decreases (~50\%) in mtDNA have been found in ART-naive patients in peripheral blood mononuclear cells compared to uninfected patients\textsuperscript{166}. HIV is also known to depolarize mitochondrial membrane potentials (MMP) in primary cells and cell lines which increases infection\textsuperscript{167}. The mitochondrion is vital to the initial infection, survival, and spread of HIV in human beings\textsuperscript{168}. In HIV-infected patients, mitochondrial DNA (mtDNA) is significantly decreased (68\%) in patients on ART compared to ART-naive patients, evidence of mitochondrial toxicity that is resolved when the patients discontinue the ART\textsuperscript{169}. There are observed differences, however, between mitochondrial effects in PBMCs, adipose, and muscle tissue in HIV-infected patients\textsuperscript{170}.

1.6.4.1 Trans-activator protein of transcription (Tat)
Tat is a regulatory protein that enhances the efficacy of viral transcription in HIV\textsuperscript{171}, and has been shown to have effects on the mitochondria\textsuperscript{172}. Effects that have been found are: decreases in mitochondrial membrane potential in mouse liver, heart, and brain tissue\textsuperscript{173}, induction of ROS production in neurons\textsuperscript{174}, calcium homeostasis dysregulation in neurons\textsuperscript{175}, and changes in mitochondrial morphology and function in neurons\textsuperscript{172,176}. Exposure of neurons to Tat leads to decreases in mitochondrial diameter and perimeter, and causes fragmentation as well as redistribution of mitochondria within neurons, which leads to loss of mitochondrial transportation leading to dysfunction\textsuperscript{174}. This increase in mitochondrial fragmentation was confirmed through elevated levels of Dynamin-1-like protein (Drp1), a regulator of mitochondrial fission\textsuperscript{174}. A proposed mechanism for these mitochondrial effects is the inflammatory chemokine pathway CCL5 pathway, as activation of this pathway via pituitary adenylate cyclase-activating peptide 27 (PACAP27) lead to the prevention of Tat-induced neuronal cell death in neurons\textsuperscript{174}.
1.6.4.2 HIV-1 viral protein R (Vpr)

Viruses such as HIV have developed ways of eluding immune systems by using viral proteins to inhibit or activate cell death where needed, allowing the virus to infect and propagate before the host dies\(^{177}\). Much of this is done by targeting the mitochondria with viral proteins via dysregulation of MMP, caspase activity, transcription factors, or other signaling pathways such as TNF\(^{177}\). Vpr is an accessory protein that is critical for HIV-1 nuclear import, viral replication of non-dividing cells \textit{in vivo}, and regulation of apoptosis in HIV-infected cells\(^{178}\). Vpr was shown to cause losses in mitochondrial membrane potential (MMP) leading to apoptosis and mitochondrial swelling via the mitochondrial permeability transition pore in jurkat cells\(^{161}\). Furthermore, decreases in ATP production and axonal mitochondrial transport in neurons have been shown causing a lack in intercellular communication that can lead to major neurological diseases and HIV-associated neurocognitive disorder (HAND)\(^{179}\). These effects were shown \textit{in vitro} without the presence of cell death, showing that Vpr can affect the mitochondria without causing cell death\(^{179}\). Vpr was also shown to induce the accelerated aging phenotype of primary neurons \textit{in vitro}, with increases in senescence markers and aging biomarker, β-galactosidase, and decreases in peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC-1α), a key transcriptional factor regulating mitochondrial biogenesis\(^{179}\). Envelope glycoprotein GP120 (gp120) is a glycoprotein located on the surface of the HIV envelope, which is vital to the viral entry into the host cell\(^{180}\).

1.6.4.3 Glycoprotein 120 (gp120)

In addition to cell entry, gp120 is responsible for apoptosis in T cell depletion by binding to the CXCR4 cell surface receptor, resulting in mitochondrial cytochrome c release in HEK-293 and T-cell lines\(^{181}\). The binding of gp120 and CXCR4 initiated apoptosis via cytochrome c release from the mitochondria resulting in the formation of the apoptosome leading to the Caspase-9 and Caspase-3 pathway which results in cell death and the T cell depletion experienced in HIV infection\(^{181}\). These effects were seen independent of the Fas ligand\(^{181}\). Apoptosis was also seen in neurons along with dysregulation of calcium homeostasis and oxidative stress, which can promote mitochondrial dysfunction\(^{175,182}\). In terms of mitochondrial morphology, it was also
shown in neurons that gp120 causes elongation and expansion of mitochondrial size\textsuperscript{183}. Correlating with mitochondrial changes in morphology, it was also shown that gp120, increase mitofusin-1 activity, a mitochondrial fusion protein, and significantly decrease Dynamin-1-like protein (DNM1L) activity, a mitochondrial fission protein, leading to increased neuroinflammation and neurodegeneration as well as elongated mitochondria in primary mouse neurons \textit{in vitro}\textsuperscript{184}. Furthermore, gp120 increases extracellular acidification rate (ECAR)\textsuperscript{184} and decreases mitochondrial respiratory capacity\textsuperscript{183}, suggesting increased glycolysis, decreased oxidative phosphorylation, and decreased energy production as a result.

\textbf{1.6.4.4 Negative regulatory factor (Nef)}

The Nef protein that downregulates cell surface receptors such as CTLA-4, increasing the affinity for the HIV virion to bind at the cell surface and successfully infect CD4\textsuperscript{+} cells\textsuperscript{185}. Nef has also been shown to induce apoptosis is bystander cells; cells which are not infected by HIV but which are exposed to the HIV-infected cell environment\textsuperscript{186}. In primary human brain microvascular endothelial cells (MVECs), apoptosis was induced as a result of Nef stimulation\textsuperscript{186}. Up-regulation of mRNA expression levels of mitochondrial apoptotic pathways were observed during Nef stimulation, suggesting that the mitochondria plays a critical role in the effects of Nef’s apoptotic regulation\textsuperscript{186}. This particular study suggests that Nef and the mitochondria play a role in neuropathogenesis and HAND\textsuperscript{186}.

\textbf{1.6.5 Effects of ART on Mitochondria}

HIV and ART are associated with mitochondrial toxicity and dysfunction\textsuperscript{146}. Children who are exposed to ART and HIV in the perinatal period have higher risk of persistent mitochondrial dysfunction\textsuperscript{187}. ART has been shown to cause mitochondrial toxicity and point mutations in mtDNA in patient skeletal muscle tissue\textsuperscript{188} as well as peripheral blood mononuclear cells (PBMCs)\textsuperscript{150}. HIV-infected patients on ART with and without clinical mitochondrial toxicity have both been shown to have significantly more mtDNA variants compared to HIV-uninfected subjects\textsuperscript{150} as well as HIV-infected patients not on ART\textsuperscript{188}. There were no significant differences, however, between HIV-infected patients experiencing clinical mitochondrial toxicity and those
without such symptoms in PBMCs, suggesting that mtDNA damage occurs independent of mitochondrial toxicity symptoms\textsuperscript{150}. Due to differences in the types of ART found in common HAART regimens, however, it is also important to investigate the effects of ART subclasses individually instead of as a whole.

\textbf{1.6.5.1 Nucleoside reverse transcriptase inhibitors (NRTIs)}

Nucleotide and nucleoside reverse transcriptase inhibitors (NRTI) are the most common ARVs used in first-line ART regimens\textsuperscript{52}. NRTIs have saved the lives of millions of HIV-infected individuals, but have also shown toxic effects on the mitochondria of the cell, potentially leading to inflammation and comorbidities\textsuperscript{52}. A popular hypothesis of NRTI mitochondrial toxicity is the inhibition of DNA pol-\(\gamma\), the sole polymerase responsible for mtDNA\textsuperscript{146}. There are 13 mtDNA genes encoding the ETC in the inner membrane of the mitochondria\textsuperscript{146}. Although NRTIs have a significantly higher affinity for HIV reverse transcriptase than pol-\(\gamma\), they still have a significantly higher affinity and inhibitory effect on pol-\(\gamma\) than any other DNA polymerase\textsuperscript{189}. However, a number of recent studies have also reported dysfunction in the absence of mtDNA depletion suggesting secondary effects of NRTI on mitochondrial function\textsuperscript{190}. These alterations include altered nucleotide phosphorylation and direct mitotoxic effects on mitochondrial respiration and ATP production\textsuperscript{190–192}. In particular, AZT has been shown to inhibit the mitochondrial adenylate kinase and adenosine nucleotide translocator in isolated mitochondria\textsuperscript{193} and promote mitochondrial ROS production by directly inhibiting the electron transport chain\textsuperscript{191,192}. Reactive oxygen species (ROS) are a product of leakage from protons being pumped across the inner membrane of the mitochondria and the electron transport chain (ETC) during oxidative phosphorylation\textsuperscript{194}. ROS production can lead to oxidative stress causing dysregulation in cell-signaling, cell damage, decreased energy (ATP) production, and dysregulation of apoptotic pathways\textsuperscript{194}. The dysregulation of such pathways can cause damage-associated molecular patterns (DAMPs), leading to inflammatory responses. ROS are also important signaling molecules used to mount antiviral and other immune responses\textsuperscript{195}. NRTIs have been shown to affect complex IV activity and inhibit complex I function (a critical producer of ROS)\textsuperscript{196–198}. Another source of ROS production is LPS stimulation via TLR4 signaling pathway,
a vital signaling pathway to inflammatory responses\textsuperscript{195}. ROS production can induce inflammation, and inflammation can also induce ROS production, creating exponential damage, especially if it persists for a long period of time. In recent studies, AIDS patients on cART have shown increased liver oxidative damage and mtDNA loss along with decreased DNA repair function compared to non-cART patients\textsuperscript{199}. This oxidative cellular damage occurs when the production of ROS exceeds the production of protective antioxidants\textsuperscript{199}. HIV infection has been known to decrease cellular glutathione levels, decreasing the ability for HIV-infected cells to inhibit ROS production, and increasing the ability for inflammatory cytokines to stimulate viral replication\textsuperscript{200}. These deficiencies cannot be recalibrated using exogenous antioxidants such as vitamin A and vitamin C\textsuperscript{201}. HIV-infected women on cART have shown evidence of increased mtDNA depletion, oxidative stress, and apoptosis active caspase-3\textsuperscript{202}. In the mouse model, NRTI exposure has shown increases in oxidative damage, DNA repair, and mtDNA loss\textsuperscript{199}. These findings suggest that ROS production as a result of HIV and ART could be a source of inflammation and comorbidities seen in HIV-infected individuals. Telomerase is also known as a vital reverse transcriptase in humans, and telomere length have been shown to be an accurate marker of biological aging, disease, and mortality\textsuperscript{203}. The inhibition or dysfunction of its activity can lead to accelerated decline and aging stemming from mitochondrial dysfunction at the level of oxidative phosphorylation and repression of PGC-regulated oxidative defense genes leading to increased ROS production and oxidative stress\textsuperscript{204}. Importantly, these toxicities can be difficult to reverse and can be life-threatening and careful evaluation of the mechanisms underlying this mitochondrial dysfunction will be critical in reducing this toxicity.

\textbf{1.6.5.2 Non-nucleoside reverse transcriptase inhibitors (NNRTIs)}

NNRTIs are also a widely used class of ARVs that inhibit viral reverse transcription. There are 4 commonly used NNRTIs: Nevirapine (NVP), Efavirenz (EFV), Etravirine (ETR), and Delavirdine (DLV) which are usually used as a 3\textsuperscript{rd} ARV in first-line cART\textsuperscript{52}. NNRTI mitochondrial effects are known to be centered around dysregulation of bioenergetics, ROS production, and cell death\textsuperscript{146}. It has been shown in hepatic cells \textit{in vitro} that EFV significantly increased ROS production, decreased MMP, increased mitochondrial mass, and increased apoptosis via
intrinsic pathway (cytochrome c release and caspase 9 activity)\textsuperscript{146}. These effects were not due to mtDNA numbers as they were not altered in correlation with the mitochondrial alterations\textsuperscript{146}. Also in hepatic cells (Hep3B), EFV has been shown to inhibit complex 1 leading to increased ROS production and decrease ATP production\textsuperscript{205}. In neuronal cell lines and primary neurons, EFV caused mitophagy, decreased ATP production, increased mitochondrial depolarization, and altered mitochondrial morphology\textsuperscript{206}. In mouse neurons, EFV and NVP showed inhibition of complex IV of the ETC\textsuperscript{207}. In umbilical endothelial cells, EFV increased oxidative stress and autophagy while reducing proliferation, and these effects were worsened when combined with the protease inhibitor (PI), nelfinavir (NFV)\textsuperscript{208}. Mrp4 was also explored in the non-nucleoside reverse transcriptase inhibitor (NNRTI) Efavirenz (EFV), where single nucleotide polymorphisms (SNPs) in Mrp4, mainly rs1751034 and rs2274407, accounted for variation in toxicity between individuals using the drug\textsuperscript{209}. EFV has shown similar results for other transporters in human tissue (MRP1 and BCR) leading to CNS pathologies\textsuperscript{209}.

\textbf{1.6.5.3 Protease inhibitors (PIs)}

Protease inhibitors (PI) are the third class of most-commonly used drug in first-line HIV cART. There are 9 most commonly used PIs: Saquinivir (SQV), Lopinavir (ABT), Darunivir (DRV), Indinavir (IDV), Tipranavir (TPV), Atazanavir (ATV), Nelfanavir (NFV), Fosamprenavir (FPV), and Ritonavir (RTV)\textsuperscript{52}. PIs inhibit HIV propagation by binding to the HIV-1 protease, inhibiting the cleavage of viral protein particles and subsequent production of viral particles\textsuperscript{210}. Like other ARVs, however, PIs also have their adverse effects on mitochondria\textsuperscript{146}. In human pre-adipocytes, MMP was significantly decreased by the PI, saquinivir (SQV), especially in already-differentiated cells\textsuperscript{211}. In a neuroblastoma cell line (SH-SYSY), ABT and RTV stimulation caused mitochondrial damage, ROS production, and apoptosis\textsuperscript{212}. In human intestinal epithelial cells, SQV, RTV, and NFV induced the formation of massive apoptotic bodies \textit{in vivo} leading to symptoms of diarrhea in treated patients\textsuperscript{213}. In PBMCs, however, there have been contradictory results with increases in MMP and survivability being found in some cases, depending on the cell type and the state of activation of the cell\textsuperscript{214}. These results seem to suggest that in the case of PIs, the mitochondria are very particular in their response and vital to the activation of the
cell, and that PIs have a direct target activity on the mitochondria instead of using intrinsic apoptotic pathways\textsuperscript{214}. In mouse macrophages, RTV, IDV, and ATV were shown to induce endoplasmic reticulum (ER) stress and apoptosis\textsuperscript{215}. Like the other categories or ARVs, PIs also seem to play a vital role in mitochondrial activity that seems to lead towards inflammatory responses and disorders such as cardiovascular disease and HIV-associated Neurological Disorders (HAND). More work must be performed in order to find the link between PI activity, mitochondrial dysfunction, and inflammation.

1.6.5.4\textit{Integrase inhibitors (INIs)}

Integrase inhibitors (INIs) were cleared for use in 2007, and have started being used more recently instead of NNRTI’s and PI’s in cART\textsuperscript{52}. Cases have been shown, however, to have side effects similar to early NRTIs, with symptoms such as jaundice and mitochondrial toxicity\textsuperscript{216}. These symptoms subsided when patients restarted their original cART regimens not including INIs\textsuperscript{216}. INIs have also shown positive effects, however, such as reversing certain symptoms seen in lipodystrophy such as: total body mass, fat mass ratio, limb fat mass, trunk/limb fat mass ratio, fat mass index, and percent leg fat normalized to BMI\textsuperscript{217}. This study also showed increases in mtDNA, PPAR expression, adiponectin, cytochrome b, and monocyte chemoattractant protein-1 (MCP-1) when patients were switched from the NRTI stavudine (d4T) to the INI raltegravir (RAL) for 48 weeks\textsuperscript{217}. This shows that there are still contradicting results in terms of INIs in the context of mitochondrial function, and more evaluation must be performed since it is a newer type or ART. A more recent study found detrimental effects of INIs dolutegravir (DLG) and elvitegravir (EVG) on mitochondrial respiration and cell proliferation in CD4+ T cells\textsuperscript{218}. ART-treated and naïve patient samples were tested \textit{ex vivo} and INIs lead to slower proliferation, lower oxidative phosphorylation, increased ROS production, and increased TNF\textgreek{g} responses compared to PIs and NNRTIs\textsuperscript{218}.
1.7 Rationale

It is estimated that 36.7 million people are living with HIV, 20.9 million of whom are accessing antiretroviral therapy (ART) which is required daily in order to maintain optimal health outcomes\(^9\). Compared to uninfected patients, HIV patients on ART have been shown to have increased inflammation and increased risk of comorbidities such as cardiovascular disease, metabolic disorders, dyslipidemia, and neurological impairment, as well as increased rates of emergency room and hospital visits due to metabolic disorders\(^{47}\). HIV-associated comorbidities carry a substantial financial burden on the healthcare system\(^{219,220}\). HIV-infected patients being treated for metabolic abnormalities were shown to have medical costs that were 3.68 times those of uninfected patients, and pharmacy costs that were 3.13 times higher\(^{221}\). These costs were associated with 3.1 times more hospitalizations and 15.3 times more emergency room visits\(^{221}\). ART treatment has important clinical implications for HIV infected individual patients using ART every day for up to 40 years, as well as at-risk uninfected individuals using ART through pre-exposure prophylaxis (PrEP). Persistent monocyte and macrophage activation have been shown to correlate with increased morbidity and mortality in HIV infected individuals on ART but the mechanisms driving their chronic activation remain poorly understood\(^{47}\). Dysregulation of mitochondrial function and oxidative stress leads to immune activation and inflammation through the circulation of damage-associated molecular patterns (DAMPs)\(^{202}\). Although inflammation is necessary to mount proper immune responses in order to clear harmful pathogens, persistence of this inflammation leads to increased aging and morbidity\(^{222}\). HIV and ART lead to increases in immune activation and systemic inflammation\(^{29}\).

1.8 Hypothesis

As shown in Figure 3, mitochondrial reprogramming driven by HIV and/or ART leads to alterations in mitochondrial function, which affects macrophage activation and the production of inflammatory and antiviral cytokines.
1.9 Project Aims

The aims of this project are as follows: 1. Evaluation of toxic effects of antiretroviral treatment (ART) and effects on mitochondrial morphology and function in uninfected MDMs; 2. Evaluation of the effects of ART with HIV infection on mitochondrial morphology and function in MDMs; 3. Evaluation of the effects of HIV and ART-associated mitochondrial reprogramming on MDM cytokine production and cell viability.
Chapter 2: Effects of antiretrovirals on mitochondrial morphology and function in uninfected monocyte derived macrophages and the link to immune activation

Research question: Does ART associated alterations in mitochondrial function affect immune activation in uninfected MDMs?

2.1 Introduction

2.1.1 HIV as Chronic Disease

By 2020, it is expected that 81% of the global HIV population will be on antiretroviral therapy (ART), and 21% of the global HIV population will be over 50 years of age; an increase of 13% since the year 2000. While treatment prolongs life and substantially reduces the risk of HIV transmission, it does not completely restore health. HIV-infected individuals on virally-suppressive ART manifest a phenotype of accelerated aging and experience increased rates of non-AIDS associated comorbidities such as diabetes, cardiovascular disease, neurocognitive impairment, and cancer. Progress towards improving outcomes for these individuals will depend on the identification of novel strategies for the prevention and treatment of these diseases. Chronic immune activation and inflammation persist in HIV patients on ART. This immune dysfunction is associated with hypercoagulation, tissue fibrosis/damage and organ system dysfunction, which over time contributes to the development of non-AIDS associated comorbidities. The drivers of this activation remain incompletely understood but are thought to include ongoing low levels of HIV replication, secondary coinfections, HIV-mediated breakdown of the intestinal mucosa and chronic exposure to gut microbial products, as well as sustained dysfunction of immunoregulatory factors.

2.1.2 Effects of ART on the Mitochondria

ART has been found to accelerate senescence and aging via its effects on mitochondrial function. Nucleotide and nucleoside reverse transcriptase inhibitors (NRTI) are the most common ARVs used in first-line ART regimens. Typical regimens are comprised of 2 NRTIs as a ‘backbone’ and either one non-nucleoside reverse transcriptase inhibitor (NNRTI), one protease inhibitor (PI), or one intergrase inhibitor as the third component of the combination ART.
RTIs inhibit the transcription of viral RNA into proviral DNA following viral entry into the cell. A popular hypothesis of NRTI mitochondrial toxicity is associated with the inhibition of DNA pol-γ, the sole polymerase responsible for the synthesis of mtDNA. Although NRTIs have a significantly higher affinity for HIV reverse transcriptase than Pol-γ, they also have a significant affinity and inhibitory effect on Pol-γ compared to other DNA polymerases. In HIV-infected patients, mitochondrial DNA (mtDNA) is significantly decreased (68%) in patients on ART compared to those who are treatment naive. Further, evidence suggests mitochondrial toxicity is resolved when the patients discontinue the ART. However, more and more evidence suggests that mitochondrial alterations due to ART go beyond the effects on pol-γ, and are more complex than originally thought. NRTIs have been shown to decrease cell membrane integrity, cell growth, proliferation, and viability as well as an increase in succinate dehydrogenase dysfunction. NNRTI’s mitochondrial effects are known to be centered around dysregulation of bioenergetics, increased ROS production, and increased cell death. Efavirenz (EFV) has been shown to inhibit complex I and IV of the ETC, increase ROS production, decrease ATP production and mitochondrial membrane potential, increase mitochondrial mass, alter mitochondrial morphology, decrease cell proliferation, increase autophagy, and increase apoptosis via intrinsic pathway (cytochrome c release and caspase 9 activity) in different cell types. These findings suggest that ART can be a source of mitochondrial dysregulation. However, it is unclear how this mitochondrial reprogramming effects cell function. Further studies are required in monocyte derived macrophages in order to understand the mechanism of mitochondrial alterations, and their potential link to macrophage activation and inflammation.

2.1.3 The Role of mitochondria in regulating innate immune responses

The immune system is a highly regulated set of cells and processes that must be able to react and adapt quickly to incoming pathogens or acute trauma. Cells must have tightly regulated mechanisms of activation and inhibition in order to maintain homeostasis, giving the organism the best chance at survival. Dysregulation within metabolic pathways can lead to many detrimental alterations within the immune system such as altered cytokine production leading
to undesirable activation states\textsuperscript{116}. The mitochondrion is responsible for many cellular functions such as cellular respiration, energy production, cell signaling, cellular metabolism, cell death and proliferation, hypoxic responses, \(\text{Ca}^{2+}\) homeostasis, redox balance, reactive oxygen species (ROS) production, tricarboxylic acid (TCA) cycle, as well as others\textsuperscript{145,146}. Superoxides, a type of mitochondrial ROS, are produced from the reduction of oxygen, and are used in signaling pathways to combat invading pathogens, namely viral pathogens\textsuperscript{120,228}. It has been shown in knockout mice that macrophages producing more superoxides have a better ability of eliminating pathogens\textsuperscript{121}. One mechanism used by ROS linking them to inflammation is the up-regulation of toll-like receptor (TLR) signaling pathways, the pathway which is essential on macrophages to the innate immune response since it is responsible for recognizing and binding to invading pathogens\textsuperscript{122}. Dysregulation within this system, or long-term activation, even at low levels, can lead to unwanted chronic inflammation\textsuperscript{229}. In addition, damage to mitochondria can release damage associated molecular patterns (DAMPs) which can bind to TLRs and cause macrophages to activate an immune response leading to persistent inflammation\textsuperscript{124}. During LPS treatment, a TLR4 agonist, macrophages predominately shift their energy production from oxidative phosphorylation to glycolysis while driving ROS production and an inflammatory phenotype\textsuperscript{125}. This inflammatory phenotype was shown to be reversed, however, when ROS production was blocked\textsuperscript{125}. Therefore, mitochondria and mitochondrial dysfunction play a vital role in immune activation.

\textbf{2.2 Hypothesis:}

ART in uninfected MDMs leads to alterations in mitochondrial structure and function, which affects macrophage activation and the production of inflammatory and antiviral cytokines.

\textbf{2.3 Methods}

\textit{2.3.1 Macrophage Isolation}

Blood was drawn from healthy male and female donors, aged 20-40 years old, who were selected on a volunteer basis. Using Lymphoprep density gradient medium (Cat #: 07851) from STEMCELL Technologies Inc. (Vancouver, BC), peripheral blood mononuclear cells were separated and isolated from the whole blood and plated at approximately 2 million PBMCs per
mL onto 6, 12, 24, 48, or 96-well Corning cell culture plates in serum free DMEM media from Sigma-Aldrich (St-Louis, Missouri). After a two-hour incubation, non-adherent PBMCs were washed away using Gibco Phosphate Buffer Saline (PBS) (Cat #: 10010023) purchased from ThermoFisher (Waltham, MA) before complete media (10% FBS, 1% PenStrep, 89% DMEM media) was added along with 1µM Recombinant Human M-CSF Protein from R&D Systems (Minneapolis, Minnesota). After 3 days, non-adherent PBMCs were once again washed away using PBS, and fresh complete media containing 1µM M-CSF was added for another 4 days. All treatments, infections, or harvests were performed at day 7 when monocytes were fully differentiated into macrophages.

2.3.2 Cell Harvesting
All cell harvesting was performed using Accutase Cell Detachment Medium from Innovative Cell Technologies, Inc. (San Diego, California) for 30 minutes at room temperature before performing a light scraping. Cells were then collected into different types of tubes depending on the assay, centrifuged at 1,600 rpm for 10 minutes, washed with PBS, before being centrifuged again at 1,600 rpm for 10 minutes.

2.3.3 ARV Treatments
Antiretroviral drugs were purchased from Sigma Aldrich (St-Louis, Missouri) and were catalog numbers: 2020138-50-9 (TFV), 134678-17-4 (3TC), and 154598-52-4 (EFV). The powder form was reconstituted in ddH2O (TFV and 3TC) or DMSO (EFV), aliquoted and stored at -80 degrees Celsius until used. ARVs were diluted to desired concentration (10µM and 50µM) in complete media (10% FBS, 1% PenStrep, 89% DMEM) and added directly to wells.

2.3.4 Assessment of Cell Viability
Changes in cell viability were assessed over time and at defined time points using the Incucyte Zoom Live Cell Analysis System and the Becton Dickinson LSRFortessa flow cytometer, respectively. Live cells analysis was performed using Yoyo-1 Iodide (491/509) staining (cat #: Y3601). Yoyo-1 was acquired from ThermoFisher Scientific (Waltham, Massachusetts), and was
added directly to the wells at the same time as drug treatments at a concentration of 100nM (1:10,000) in complete media (10% FBS, 1% PenStrep, 89% DMEM) before being analyzed using the Incucyte Zoom Live Cell Analysis System on the green channel at 10x magnification. Fluorescent pictures were taken every two hours and analyzed using the Incucyte Zoom software for well confluency and number of green objects were counted. Data was exported, normalized to well confluency and graphed using Graphpad Prism 5. To confirm these results, levels of cell death were evaluated using propidium iodide (PI) staining at 24h and 72h time points. PI stain was acquired from Sigma-Aldrich and was added directly to wells at a concentration of 500ng/mL for 30 minutes at 37 degrees Celsius. Cells were then harvested as shown above and washed with PBS before 10,000 events were acquired using the LSRFortessa flow cytometer in PBS. Analysis was performed using FlowJo version X 10, graphs were made using Graphpad Prism 5, and p-values were calculated using 2-tailed paired t-tests.

2.3.5 Assessment of Mitochondrial Function by Flow Cytometry
Mitochondrial membrane potential was measured using TetremethylRhodamine, Methyl Ester, Perchlorate (TMRM) staining purchased form ThermoFisher Scientific (Cat #: T668). TMRM was added to detached cells in serum free DMEM media at a concentration of 100nM for 30 minutes at 37 degrees Celsius in Falcon round-bottom polystyrene 5mL tubes. Cells were then harvested as shown above and washed with PBS before 10,000 events were acquired using the LSRFortessa flow cytometer in PBS. Analysis was performed using FlowJo version X 10, graphs were made using Graphpad Prism 5, and p-values were calculated using 2-tailed paired t-tests. Total cellular ROS was evaluated using CellROX Deep Red Staining from ThermoFisher Scientific (Cat #: C10422). The stain was added directly to the wells along with drug treatments at a concentration of 5µM in complete media (10% FBS, 1% PenStrep, 89% DMEM), before being analyzed using the Incucyte Zoom Live Cell Analysis System on the red channel at 10x magnification. Fluorescent pictures were taken every two hours and analyzed using the Incucyte Zoom software for mean fluorescence intensity per cell. Data was exported, and graphed using Graphpad Prism 5. Mitochondrial superoxide production was assessed using MitoSOX Red staining, acquired from ThermoFisher.
Scientific (Cat #: M36008). Samples were harvested, as shown above, into Falcon round-bottom polystyrene 5mL tubes, washed with PBS, and centrifuged at 1,600 rpm for 10 minutes. PBS was aspirated and cells were stained with 5µM MitoSOX Red in PBS for 20 minutes at 37 degrees Celsius. Cells were then washed with PBS before and centrifuged at 1,600 rpm for 10 minutes before 10,000 events were acquired using the LSRFortessa flow cytometer in PBS. Analysis was performed using FlowJo version X 10, graphs were made using Graphpad Prism 5, and p-values were calculated using 2-tailed paired t-tests. Results are reported as the percentage of positive cells.

2.3.6 Cellular Bioenergetics – Seahorse XF Analyzer

Macrophages were differentiated for 7 days in 6-well plates before being detached and replated on 8-well Seahorse XFp Cell Culture Miniplates in complete media (10% FBS, 1% PenStrep, 89% DMEM) at a density of 50,000 cells per well. Cell were then left to equilibrate for 48 hours at 37 degrees Celsius prior to testing. The immediate effects of ARVs on mitochondrial function was performed using real-time activation assays and the Mito Stress Test was used to evaluate longer term (24 hours) effects of ARVs on MDM mitochondrial function.

Real Time Activation Assay: On the day of the assay, 180µL of Seahorse media (Seahorse XF DMEM along with 10mM glucose, 1mM pyruvate, and 2mM glutamine; pH 7.4) was added to the wells, and the plates were incubated at 37 degrees Celsius and 0% CO₂ for 1 hour. After one hour, five baseline oxygen consumption rate (OCR) measurements were taken prior to drug treatments, each taking approximately 6 minutes in duration. ARVs were diluted in Seahorse media to 10x concentrations (500µM), and 20µL was added into port A of Seahorse cartridges. Following the 5 baseline OCR measurements, ARVs were injected from the cartridges directly into the wells, and 60 OCR measurements were taken every 6 minutes equaling a total of 360 minutes. OCR measurements were exported and values were graphed using Graphpad Prism 5.

MitoStress Test: MDMs were treated with 50µM ARVs for 24h prior to the assay being performed. Following the 24h treatment, the ARVs were aspirated and the cells were washed
before 180µL of Seahorse media (Seahorse XF DMEM along with 10mM glucose, 1mM pyruvate, and 2mM glutamine; pH 7.4) was added to the wells, and the plates were incubated at 37 degrees Celsius and 0% CO₂ for 1 hour. After one hour, five baseline oxygen consumption rate (OCR) measurements were taken prior to drug treatments, each taking approximately 6 minutes in duration. ETC complex inhibitors were diluted in Seahorse media to 10x concentrations and loaded into ports A, B, and C of the Seahorse cartridges. The inhibitors used, along with their final concentrations, were: Port A, Oligomycin (4µM) – complex V inhibitor; Port B, carbonyl cyanide-p-trifluoromethoxyphenyl hydrazine (FCCP) (1µM) – mitochondrial uncoupling agent; Port C, Rotenone (0.5µM) and Antimycin (0.5µM) – complex I and III inhibitors respectively. Following the 5 baseline OCR measurements, the inhibitors were injected, one at a time, directly into the wells and 3 OCR measurements were taken taking approximately 6 minutes each for a total of 54 minutes. Oligomycin (Port A) was first injected in order to inhibit complex V’s ability to make ATP. This OCR value subtracted by the baseline OCR value provided ATP production. FCCP (Port B) was then injected in order to push all hydrogen ions out of the mitochondria. Rotenone and antimycin (Port C) were then injected in order to inhibit complex I and III, halting electron flow through the ETC. This OCR measurement subtracted by the baseline value provided the basal respiration value. The OCR value following FCCP injection subtracted by the OCR value following the Rotenone and Antimycin injection provided the maximal respiration value. Analysis was performed using Agilent Wave Desktop Software, results were exported and graphs were made using Graphpad Prism 5, and p-values were calculated using 2-tailed paired t-tests.

2.3.7 Western Blots

Cells were lysed directly on the cell culture plate using Pierce RIPA Buffer and Halt Protease and Phosphatase inhibitor (1:100 dilution) acquired from ThermoFisher with approximately 70µL per 150,000 cells. Cells were collected and centrifuged at 14,000 rpm for 15 minutes at 4 degrees Celsius before being placed at -80 degrees Celsius until use. Bio-Rad Laboratories (Hercules, California) DC assay was used to quantify total protein in each sample, samples were diluted in Bio-Rad Laemmli protein sample buffer for SDS-PAGE (Cat #: 1610747), and ran on
12% TGX Stain-Free FastCast Acrylamide gels from Bio-Rad. The stain-free gels were imaged using the Stain-Free setting on the ChemiDoc MP Imaging System from Bio-Rad, and the gel was transferred onto a PVDF membrane using the Bio-Rad Trans-Blot Turbo System. Membrane was blocked overnight at 4 degrees Celsius in 5% non-fat dry milk diluted in tris-buffered saline-Tween (TBST) buffer (20mM Tris-HCl, 500mM NaCl, and 0.1% v/v Tween 20; pH 7.5), and washed 6x 5 minutes in TBST buffer. Primary antibodies were incubated overnight at 4 degrees Celsius in 5% non-fat dry milk diluted in TBST buffer and washed 6x 5 minutes in TBST buffer. Secondary antibodies were incubated for 1h at room temperature in 5% non-fat dry milk diluted in TBST buffer and washed 6x 5 minutes in TBST buffer. Membranes were incubated in Bio-Rad Clarity ECL Substrate (Cat #: 1705060) for at least 3 minutes out of direct light before membranes were imaged for protein band luminescence. Densitometry was performed using Bio-Rad Image Lab Software. Protein band luminescence was normalized based on the total amount of protein within each lane of the stain-free gel. Values were exported, graphs were made using Graphpad Prism 5, and p-values were calculated using 2-tailed paired t-tests. All antibodies used for Western Blots are outlined below in Table 1 along with dilutions that were used. All protein gels were run at a concentration of 25 µg/mL, at a voltage of 100V.

### Table 1. Antibodies used for Western Blots

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<th>Antibody</th>
<th>Supplier</th>
<th>Catalog Number</th>
<th>Isotype</th>
<th>Host</th>
<th>Dilution</th>
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<tbody>
<tr>
<td>SOD2 (D3X8F) XP Monoclonal Antibody</td>
<td>Cell Signaling Technology</td>
<td>13141</td>
<td>IgG</td>
<td>Rabbit</td>
<td>1:1000</td>
</tr>
<tr>
<td>NDUFB8 Monoclonal Antibody (Complex I)</td>
<td>ThermoFisher</td>
<td>459210</td>
<td>IgG1, kappa</td>
<td>Mouse</td>
<td>1:5000</td>
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<tr>
<td>SDHB Monoclonal Antibody</td>
<td>Abcam</td>
<td>ab14714</td>
<td>IgG2a</td>
<td>Mouse</td>
<td>1:1000</td>
</tr>
<tr>
<td>UQRC2 Polyclonal Antibody (Complex III)</td>
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<td>PAS-30204</td>
<td>IgG</td>
<td>Rabbit</td>
<td>1:5000</td>
</tr>
<tr>
<td>COX4/Complex IV Monoclonal Antibody</td>
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<td>A21348</td>
<td>IgG2a, kappa</td>
<td>Mouse</td>
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<tr>
<td>Anti-Mouse IgG HRP-linked Secondary Antibody</td>
<td>Abcam</td>
<td>ab6789</td>
<td>IgG</td>
<td>Goat</td>
<td>1:5000</td>
</tr>
<tr>
<td>Anti-Rabbit IgG, HRP-linked Secondary Antibody</td>
<td>Cell Signaling Technology</td>
<td>7074</td>
<td>IgG</td>
<td>Goat</td>
<td>1:2000</td>
</tr>
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2.3.8 Human Cytokine ELISAs (CXCL10, TNFα, and IFNβ)

Human CXCL10/IP-10 Quantikine ELISA Kit (Cat #: DIP100), Human TNFα Quantikine ELISA Kit (Cat #: DTA00D), and Human IFNβ Quantikine ELISA Kit (Cat #: DIFNB0) were acquired from R&D Systems (Minneapolis, Minnesota). All antibodies and standards were used from the above-mentioned kits. Antibodies were diluted according to the pamphlets’ recommendations inside the kits. Samples and standards were diluted in Reagent Diluent (1% BSA), and 96-well plates were washed with Wash Buffer (0.05% Tween in PBS). 100µL of diluted capture antibody was added to each well the day before the samples were added and incubated overnight at room temperature. Capture antibody was aspirated, and plates were washed 3 times with 300µL of Wash Buffer. Plates were blocked with 150µL of Reagent Diluent for and plates were incubated 2 hours at room temperature. Blocking solution was aspirated and plates were washed 3 times with Wash Buffer. Samples and standards were prepared at desired dilutions in Reagent Diluent and 100µL was added to each well and plates were incubated for 2 hours at room temperature. Samples were aspirated and plates were washed 3 times with Wash Buffer. 100µL of detection antibody diluted in Reagent Diluent was added to each well and plates were incubated at room temperature for 2 hours. Detection antibody was aspirated and plates were washed 3 times with Wash Buffer. 100µL of Streptavidin-HRP diluted in Reagent Diluent was added to each well and plates were incubated out of direct light at room temperature for 20 minutes. Streptavidin-HRP was aspirated and plates were washed 3 times with Wash Buffer. 100µL of Substrate Solution was added to each well and plates were incubated out of direct light at room temperature for 20 minutes. Without washing, 50µL of stop solution was added to each well, and the plate was read at 450nm using the Bio-Rad iMark Microplate Reader. Values were quantified based on standard curve using Bio-Rad Image Lab Software. Values were exported, graphs were made using Graphpad Prism 5, and p-values were calculated using 2-tailed paired t-tests.

2.3.9 Antioxidant Treatment

MitoTEMPO (Cat #: 1334850-99-5) and N-Acetyl Cysteine (NAC; Cat #: 616-91-1) were acquired from Sigma Aldrich. Solid form stored at -20 degrees Celsius (MitoTEMPO) and 4 degrees
Celsius (NAC) before being weighed and reconstituted in complete media (10% FBS, 1% PenStrep, 89% DMEM) on the day of treatment. Cells were washed with PBS before treatment. MitoTEMPO was added directly to well 1 to 2 hours before ARV/LPS treatment and washed away with PBS before treatment, whereas NAC was added directly to well at the time of ARV/LPS treatment. MitoTEMPO was used at concentrations ranging 125µM and 1mM, whereas NAC was used at concentrations ranging from 1.25mM to 5mM.

2.4 Results

2.4.1 TFV and EFV induce cell death at physiological concentrations:
The toxicology profile of ARV drugs in macrophages remains a greatly understudied area of research\textsuperscript{108}. Using the Incucyte Zoom Live Cell Analysis System, the kinetics of cell death were established for MDMs following treatment of Tenofovir (TFV), Lamivudine (3TC)) and Efavirenz (EFV)), used alone and in combination. ARVs were used at physiological concentrations (10µM and 50µM). These concentrations were chosen because they represent the lower and upper range of levels detected in HIV patient plasma samples\textsuperscript{230–232}. Cells were labelled with Yoyo-1 dye, a DNA stain used to evaluate total cell death. Green cell counts were evaluated every two hours for a total of 72 hours (Figure 4A). Fluorescence was normalized relative to the total confluence of the well. As shown in Figure 4, neither 3TC nor the combination treatment (10µM) was associated with cell death. However, both TFV and EFV at physiological concentrations were associated with some level of cell death. High concentrations of TFV (50µM) was associated with an increase in the number green objects at 18-24 hours post-treatment before plateauing at 36-42 hours. Alternatively, 50µM EFV resulted in a more gradual increase in the number of green objects from 24 to 72 hours. Consistent with these findings, 50µM TFV was associated with a visible loss in well confluency at 72h treatment (Figure 4B). High concentrations of EFV had a lesser effect on cell confluency. These toxicity results were further confirmed using propidium iodide (PI) staining and flow cytometry at 24 and 72 hours post-treatment. PI is DNA stain that is excluded from cells with intact plasma membranes. As observed in the Incucyte analysis, the highest concentrations of TFV (50µM) were associated with significant cell death at 72 hours post-treatment (TFV vs control: p=0.007). Similar trends
were observed following 50µM EFV but the differences were not statistically significant (EFV vs control: p=0.07). Collectively, these results suggest that at the higher end of the physiological range of ARV treatment can lead to cell death in MDMs in vitro. Since cell death has a direct link to mitochondrial function, assessment of mitochondrial function was assessed at early time points (0-24 hours) using 10 and 50 µM concentrations of ARVs, alone and in combination.

Figure 4 Evaluation of cell viability in MDMs treated with ARVs. MDMs were treated in complete media with 50µM ARVs alone and in combination. (A) Cells were treated with Tenofovir (TFV), Lamivudine (3TC), and/or Efavirenz (EFV) and stained with YOYO-1 Iodide
green fluorescent dye. Cell viability was evaluated every 2 hours over the course of 72 hours using the Incucyte Zoom Live Cell Analysis System by Essen Bioscience. Green cell counts were normalized relative to confluency per well; n=5. (B) Representative microscope images captured at 10x magnification at the 72h time point using the Incucyte Zoom Live Cell Analysis System following 50µM ARV treatment. Staurisporine and Sodium Arsenite were used as positive controls. (C) Representative histograms as well as gating strategy from one donor following a 72h treatment of ARVs before cell death was evaluated using Propidium Iodide (PI) as an indicator via the BD LSRFortessa flow cytometer, followed by (D) the percentage of cell death following 24h and 72h ARV treatments given by the percentage of cells expressing positive PI signal; n=5. *p-value<0.05

2.4.2 ARV drugs have differential effects on mitochondrial function in vitro:

NRTIs and NNRTIs have been shown to cause mitochondrial dysfunction in different cell types\textsuperscript{146}. However, emerging evidence suggests the features of mitochondrial reprogramming may vary between cell and tissues\textsuperscript{146}. To better understand the effects on ARV drugs on mitochondrial function in MDM, cells were stimulated with TFV, 3TC and EFV alone and combination at 10 and 50µM. Tetramethylrhodamine, methyl ester (TMRM) was used to evaluate alterations in mitochondrial membrane potential (MMP). TMRM is a stain that is readily sequestered by active mitochondria and accumulates on the negatively charged outer mitochondrial membrane. Using flow cytometry, the level of TMRM staining per cell (mean fluorescent intensity [MFI]) and the percentage of cells with high vs. low TMRM staining were evaluated (Figure 5A). Following short term treatment (6 hours), only 50µM of EFV was found to alter MMP (Figure 5B and C). Specifically, the overall level of TMRM staining per cell was decreased and the % of cells expressing low levels of TMRM was increased. This decrease in MMP was maintained 24 hours post-treatment suggesting an early and sustained reprogramming of mitochondrial function. Alternatively, 50µM of TFV only showed signs of altering MMP at later time points (24 hours; Figure 5B) and these alterations were less pronounced than those observed for EFV. Next, we evaluated if ARV treatment at physiological concentrations affected cellular respiration using the Seahorse XFp Analyzer (Figure 5E and F). Oxygen consumption and pH in cell culture media were used to calculate oxygen consumption rates (OCR) and extracellular acidification rate.
(ECAR), respectively. OCR is an indirect marker of mitochondrial respiration where as ECAR is a marker of the cell’s glycolytic rate. Short and longer-term effects of ARVs were assessed by the real-time activation assay and the MitoStress Test. The real-time assay evaluated changes in OCR and ECAR immediately after drugs were injected onto the cells. The Mito Stress Test was performed on cells after 24 hours of ARV treatment. Consistent with the early effects on MMP, EFV treatment was associated with an immediate drop in MDM OCR levels. Further, despite a very small recovery in OCR between 30-60 minutes, this inhibition of mitochondrial respiration was sustained for up to 6 hours. High levels of TFV (50 µM), on the other hand, had a delayed effect on mitochondrial respiration. A slight drop in OCR was only observed between 240-270 minutes post-treatment (Figure 5D). Interestingly, combination treatment resulted in a near identical profile to EFV alone suggesting this drug may be driving the overall alterations in mitochondrial respiration. Examining the longer-term effects of ARVs on mitochondrial respiration, we found that both 50µM TFV and EFV as well as the combination treatment significantly decreased the basal respiration at 24 hours post-treatment (n=4; Figure 5E). These treatments also significantly reduced the ability of MDM to ramp up mitochondrial respiration in response to stress (FCCP treatment; oxidative phosphorylation uncoupling agent). While we observed a trend towards decreased ATP production following TFV, EFV and combination treatment, these differences were not statistically significant (Figure 5E). Of note, 3TC treatment was associated with a slight increase in cellular OCR levels in some donors. However, these differences were not statistically significant (P<0.05). Further studies are required to understand if these 3TC associated changes are biologically relevant.
Figure 5 Evaluation of alterations in mitochondrial membrane potential and cellular respiration in MDM treated with ARVs alone or in combination. MDMs were treated in complete media with 10 and 50µM ARVs for 6h, 24h, and in real-time. (A) A sample histogram of TMRM staining in MDMs following 24h 50µM ARV treatments. The MFI of TMRM staining for mitochondrial membrane potential (MMP) and percentage of TMRM negative MDMs were quantified via the BD LSРFortessa Flow Cytometer following (B) Levels of TMRM sequestration were evaluated at 6h (n=6) and 24h (n=5) post treatment with 10µM and 50µM ARVs. Results presented as fold change in treated compared to untreated cells. (C) Quantification of TMRM negative cells following 6h (n=6) and 24h (n=5) post treatment with 10µM and 50µM ARVs. (D) Plot showing the oxygen consumption rate (OCR) of MDMs (n=4) pre-injection and post-injection of 50µM ARVs using the real-time activation assay in the Seahorse XF Analyzer by Agilent. (E) An example provided by Agilent of the treatments and measurements taken by the Seahorse XF Analyzer.
during the Seahorse XF Mito Stress Test; followed by the results (F) of basal respiration (left; n=4), maximal respiration (middle; n=4), and ATP production (right; n=3) of MDMs following 24h treatment of 50µM ARVs. Results presented as fold change in treated compared to untreated cells (treatment value/untreated value). *p-value<0.05, **p-value<0.005

2.4.3 ARVs differentially effect electron transport chain (ETC):

Given the differential effects of TFV, EFV and 3TC on MMP and oxygen consumption rates, we next wanted to evaluate if ARV treatment altered protein levels of the various complexes of ETC using Western blot. Antibodies against core components of ETC complexes I, II, III, and IV were used as an overall indicator of complex expression as previously described\textsuperscript{233}. As shown in Figure 6, while TFV did not have a significant effect on ETC complex expression, EFV resulted in an almost complete loss of complex I protein following 24 hours of treatment. In addition to its role as a proton pump, complex I is a major generator of mtROS\textsuperscript{120}. Similar results were observed after treating cells with the combination treatment, again suggesting EFV is the driver of this phenotype. However, the Western blot for one donor did not work for complex I, leaving only two complete donors for the combination analysis. Of note, 3TC treatment was associated with a trend towards decreased complex III expression following 24 hours of treatment. Again, these differences did not reach statistical significance and further studies are required to better understand these findings are biologically relevant.
Figure 6 Effects of ARVs on ETC complex expression. MDMs were treated in complete media for 24h along with 50µM ARVs alone and in combination and ETC complex expression was assessed in cell lysates using Western blot. (A) Representative donor showing alterations in ETC complex expression following 24h ARV treatment and subsequent complex I (NDUFB8 monoclonal antibody; n=3), complex II (SDHB monoclonal antibody; n=4), complex III (UQCRC2 polyclonal antibody; n=4), and complex IV (COX4 monoclonal antibody; n=4) probing. (B-E) Summary of densitometry analysis for complex I (B), complex II (C), complex III (D) and complex IV (E). Analyses were performed using Image Lab Software. Results presented as fold change in treated compared to untreated cells (treatment value/untreated value). *p-value<0.05, **p-value<0.005
2.4.4 EFV, TFV and combination treatment were associated increased mitochondrial superoxide production at 24 hours post-treatment.

Next, we wanted to see if ARV drugs were associated with alterations in redox status of MDM. To do this, we evaluated mitochondrial superoxide production using MitoSOX Red (Figure 7A), levels of the mitochondrial superoxide dismutase-2 (SOD-2) using Western blot and total cellular ROS using CellROX Deep Red, which stains total ROS within the cell. Unexpectedly, we only detected significant alterations in mitochondrial superoxide production at late time points (24 hours). Consistent with the pronounced downregulation of Complex I, EFV treatment and combination treatment had the most pronounced effects (~2 fold increased) on mitochondrial superoxide production at 24 hours (Figure 7B). While there was a trend towards increased mitochondrial superoxide following 6 hours of EFV, these differences were not significant. The variability in the response at 50µM may be related to the increased cell death seen under these conditions. Next, to determine if the accumulation of mitochondrial superoxide production was associated with altered antioxidant responses, we evaluated total SOD2 levels in MDM following ARV treatment. SOD2 is a mitochondrial antioxidant that converts superoxide to the less toxic hydrogen peroxide. Hydrogen peroxide readily diffuses across cell membranes, allowing mitochondrial ROS transfer into the cytosol. As shown in Figure 7C, SOD2 expression appears to be unchanged following ARV treatment, suggesting that the late accumulation of superoxide in mitochondria is not associated with decreased SOD2 protein levels. Finally, we evaluated the kinetics of total cellular ROS production using the Incucyte Zoom Live Cell Analysis System. At this time, there are no validated stains to evaluate similar kinetics of mitochondrial superoxide production using the Incucyte. Consistent with the previous findings, this kinetic analysis found that TFV and EFV but not 3TC was associated with increased cellular ROS. Consistent with the immediate effects of EFV on mitochondrial function, cellular ROS started to accumulate 6-12 hours post-treatment and increased over time. TFV treatment was associated with a significant increase in ROS at 20-24 hours post-treatment (Figure 7D). Further studies are required to determine how much of the total cellular ROS is associated mitochondrial vs. cytosolic production.
Assessment of MDM redox status following ARV treatment. Mitochondrial ROS production of MDM following ARV treatment. MDMs were treated in complete media with 10 µM and 50 µM ARVs alone and in combination. (A) Representative histograms as well as gating strategy from one donor following a 24h treatment of ARVs before mitochondrial superoxide production was evaluated using MitoSox Red indicator via the BD LSRFortessa flow cytometer. (B) Fold change of MitoSOX Red mean fluorescent intensity relative to the untreated control (treatment value/untreated value) following 6h ARV treatment (left, n=10) and 24h ARV treatment (right; n=4). (C) Representative blot of one donor following 24h ARV treatment and subsequent Sod2 (D3X8F) XP monoclonal antibody probing which recognizes endogenous levels of total Sod2 protein, followed by the summary of densitometry analysis of Sod2 expression from all evaluated donors; n=4. Analyses were performed using Image Lab Software, fold change was calculated relative to the untreated control (treatment value/untreated value). (D) Cellular ROS expression over 72-hour stimulation of MDMs with...
ARVs. Mean Fluorescence Intensity (MFI) of CellROX Deep Red was measured every 2h using the Incucyte Zoom Live Cell Analysis System; n=5. *p-value < 0.05

2.4.5 TFV and EFV treatment alter in cytokine production following LPS stimulation:

Next, to determine if ARV treatment is associated with altered cytokine production and responses to inflammatory stimuli, we treated MDM with 50µM of TFV, EFV and 3TC alone and in combination and evaluated cytokine production in the presence and absence of 100ng/mL of LPS. We were specifically interested in the effects of ARVs on TNF-alpha, CXCL10 and IFN-beta production because their expression is regulated by the transcription factors NF-κB and IRF3, which are activated by mitochondrial ROS production\(^{234-237}\). As shown in Figure 8, ARVs alone had no significant effects on cytokine production in MDMs following 6 hours of treatment. However, when MDM were co-treated with ARVs and the inflammatory stimuli LPS for 6 hours, cytokine production was altered. Specifically, whereas 50µM EFV increased TNFα and IFNβ production, 50µM TFV treatment decreased TNFα production (Figure 9). Treatment with high concentrations of TFV was also associated with decreased CXCL10 production in some donors but in combined analyses, these differences were not significant. Interestingly, the combined ARV treatment was associated with an intermediate phenotype with regards to cytokine production. While TNFα and IFNβ production was increased, these differences were not statistically significant. Next, to determine ARVs could prime MDMs to increase responsiveness to inflammatory stimuli, cells were pretreated with ARVs for 6h, washed and then stimulated LPS for an additional 18 hours. Again, 50µM EFV was found to significantly increase TNFα and IFNβ production whereas TFV decreased TNFα production (Figure 10). Combination treatment also increased IFNβ production. Collectively, these results suggest that 50µM of EFV plays a role in activating MDMs and inducing antiviral and inflammatory cytokine production, whereas 50µM of TFV appears to be decreasing inflammatory activation.
Figure 8 Evaluation of the effects of ARVs on inflammatory and antiviral cytokine production. MDMs were treated in complete media with 50µM ARVs for 6h. Cytokine production was quantified in culture supernatant using ELISA. Summary of (A) CXCL10 (n=5), (B) TNF-alpha (n=3), and (C) IFN-beta production from 4 independent donors. Results presented as fold change in treated compared to untreated cells (treatment value/untreated value).

Figure 9 Evaluation of the effects of ARV treatment in combination with LPS treatment on inflammatory and antiviral cytokine production. MDMs were treated in complete media with LPS
(100 ng/ml) or with a combination of LPS (100 ng/ml) and ARVs (50µM). Evaluation of (A) CXCL10 (n=5), (B) TNF-alpha (n=3), and (C) IFN-beta (n=3) production were performed using ELISA analysis. Fold change for above graphs are represented in (D) and (E). IFN-beta fold change was not included since two of the donors had no measurable levels of production in the control. Results presented as fold change in treated compared to untreated cells (treatment value/untreated value). *p-value<0.05, **p-value<0.005

**Figure 10** Evaluation of the effects of ARV treatment in combination with 100ng/mL LPS treatment on inflammatory and antiviral cytokine production. MDMs were treated in complete media with 50µM ARVs for 6h followed by 100ng/mL of LPS treatment for 18h. Evaluation of (A) CXCL10 (n=5), (B) TNF-alpha (n=3), and (C) IFN-beta (n=3) production in culture supernatant were performed using ELISA analysis. Fold change for above graphs are represented in (D), (E), and (F). Results presented as fold change in treated compared to untreated cells (treatment value/untreated value). *p-value<0.05, **p-value<0.005
2.4.6 Antioxidants and ROS scavengers reduce ARV associated cell death:

We wanted to determine if the ARV associated increase in mitochondrial and total cellular ROS contributed to its toxicity. To do this, cells were treated with 50µM of TFV or EFV in the presence or absence of MitoTEMPO or N-acetylcysteine. MitoTEMPO (MT) is a mitochondria-specific antioxidant that selectively scavenges mitochondrial superoxide. N-acetylcysteine (NAC) boosts glutathione synthesis reducing total overall cellular ROS production. Cells were pretreated for 1 hour with MT and then washed. NAC was maintained in the co-culture for the entire experiment. For these experiments, we again used Yoyo-1 dye and Incucyte Zoom Live Cell Analysis System to evaluate the kinetics of cell death following treatment. Green cell counts, or numbers of dying cells, were determined and normalized to the confluence of the well. As shown in Figure 11, MT at 125µM, 250µM, and 500µM was unable to reduce cell death following TFV treatment. In fact, pre-treatment with this mitochondrial superoxide scavenger seemed to slightly increase the green object counts suggesting some level of toxicity when combined with TFV. Alternatively, MT had varying effects on EFV treated cells. At low concentrations, MT increased EFV associated cell death. However, at 500 µM it slightly decreased cell death suggesting mitochondrial superoxide may play a role in EFV associated death. NAC, on the other hand, was better able to reduce cell death. At 1.25mM and 2.5mM, it reduced TFV and EFV associated cell death suggesting the accumulation of cytosolic ROS may play a more central role in these processes.
Figure 11 Contribution of ROS production to ARV associated cell death. MDMs were pre-treated with ROS scavengers prior to administration of ARVs. Levels of cell death were assessed every hour for 72 hours using YOYO-1 stain in the IncuCyte Zoom System. Four different concentrations of MitoTEMPO (0µM, 125µM, 250µM, 500µM) were used to scavenge mitochondrial superoxide production in TFV (A) and EFV (B) treated MDM. Four different concentrations of N-acetylcysteine (NAC) (0mM, 1.25mM, 2.5mM, 5mM) were also used to upregulate glutathione production and reduce total cellular ROS in TFV (C) and EFV (D) treated MDM; n=2.

2.4.7 Antioxidants and ROS scavengers reduce cytokine production:
We wanted to determine if the ARV associated increase in mitochondrial and total cellular ROS contributed to cytokine production. To do this, MitoTEMPO and NAC were used at 500uM and 1.25mM respectively. MDM were pre-treated with MitoTEMPO for 2 hours, and NAC was used as a co-treatment with ARVs and LPS. MDM were treated with 50uM of TFV or EFV for 6h, and
100ng/mL of LPS for 18h following ARV treatment. As shown in Figure 12, TNF-α and IFN-β in production was assessed in cell supernatants using ELISA. NAC and MitoTEMPO both decreased TNF-α and IFN-β production, with MitoTEMPO having the more drastic effects. These preliminary results suggest that ROS and superoxide production play critical roles in TNF-α and IFN-β production. However, further work is required to validate these findings in additional donors.

Figure 12 Evaluation of the effects of ARV treatment in combination with 100ng/mL LPS treatment, following a 2h pre-treatment with MitoTEMPO or a co-treatment with NAC, on inflammatory and antiviral cytokine production. MDMs were pre-treated with 500µM MitoTEMPO for 2h in complete media. MDMs were then treated in complete media with or without 1.25mM NAC as well as 50µM ARVs for 6h, followed by 100ng/mL of LPS treatment with or without 1.25mM NAC for 18h. Evaluation of (A) TNF-alpha (n=1), and (B) IFN-beta (n=1) production in culture supernatant were performed using ELISA analysis.

2.5 Discussion

In summary, this chapter characterizes alterations seen in uninfected MDMs such as cell viability, mitochondrial morphology and function, and cytokine production. To our knowledge, this type of characterization has not yet been investigated at these concentrations with links to immune function. Therefore, these findings represent novel findings encapsulating effects of ART on immunometabolism.
It has been previously demonstrated in different cell types, at different concentrations, that TFV and EFV have detrimental effects on cell viability \textit{in vitro}\textsuperscript{208,225,238–240}. However, since macrophages are known to be more resistant to the effects of ART\textsuperscript{108}, it is unclear if these effects will be seen in MDMs. Furthermore, we wanted to understand the effects of these drugs at physiological levels that are similar to the range found in human plasma\textsuperscript{230–232}. Finally, we wanted to investigate the effects of the ARVs individually, but also when treated in combination, in order to simulate cART in patients. In the current study, we found that TFV and EFV at concentrations towards the higher end of the physiological range, 50\(\mu\)M, induced cell death beginning around 24h. These results suggest that TFV and EFV could have detrimental effects on macrophage viability in HIV infected patients as well as uninfected patients using them as PrEP, especially when used every day for long periods of time. HIV patients have been shown to have increased levels of oxidative stress due to HAART\textsuperscript{241}. In fact, those who adhered more to treatment had higher oxidative stress\textsuperscript{241}. Most of the toxicity characterization in the context of patients and ART has been done in kidney and liver disease, since this is where the highest dysfunction is seen due to mitochondrial toxicity\textsuperscript{242}. In our context, macrophage depletion is important due to its potential detrimental effects on innate immunity within patients. Macrophages represent the first line of defense against invading microorganisms, play a central role in activating the adaptive immune system, in regulating inflammatory responses, and in tissue modeling and repair\textsuperscript{91}. Depletion of macrophages has been shown in mice to lead to long-term virus-induced morbidity and mortality\textsuperscript{243}.

These toxicity assays were also conducted to identify time points that should be used moving forward into other assays. We were interested in mitochondrial alterations and differences in cytokine production that were occurring prior to cell death. Therefore, time points were used at 24h and under for mitochondrial and cytokine assays. Decreased mitochondrial membrane potential\textsuperscript{162}, decreased oxidative phosphorylation\textsuperscript{244}, increased superoxide production\textsuperscript{245}, and increased total cell ROS production\textsuperscript{246} are all examples of mitochondrial alterations that have been shown to lead to apoptosis, but have been characterized more extensively in hepatic,
renal, and neuronal tissue. Therefore, we wanted to further evaluate these results by investigating mitochondrial functional mechanisms that could be responsible for the observed cell death following ART in MDMs.

Mitochondrial membrane potential (MMP) is the potential for positively charged hydrogen ions to flow across the mitochondrial membrane, generated by the flow of electrons through complexes I, II, and IV\(^{162}\). This process is necessary under aerobic conditions, as it is used to produce energy (ATP) within the cell\(^{162}\). Although increases in MMP could mean increases in energy production, increased or decreased MMP for extended periods of time could be detrimental to the cell\(^{162}\). TFV and EFV have been shown to impact MMP in different cells types, impacting the cell’s ability to produce energy\(^{146}\). However, since macrophages are known to be more resistant to the effects of ART\(^{108}\), it is unclear if these effects will be seen in MDMs and if these alterations would affect cell function. In the current study, we found that TFV and EFV treatment at 50\(\mu\)M, decreased the overall MMP in MDMs, and lead to a larger population of MDMs that were shut off of MMP. These effects presented themselves in EFV at an earlier time point (6h) and persisted, whereas TFV’s effects showed up later (24h) and were not significant (\(p=0.08\)). These alterations in MMP were not seen in 3TC, as the results were not significantly different than the untreated control. Therefore, these effects do seem to be consistent with the losses in cell viability, with the most pronounced effects occurring at 24h, the same time point in which cell viability began to decrease. These alterations are important in the patient context due to the importance of MMP in antiviral response. It has been shown that cells lacking MMP lose their ability to mount RLR-mediated antiviral reponses\(^{247}\). Therefore, the current study’s results suggest that infected or uninfected cells exposed to HIV in patients, that have decreased MMP due to ART, could have a decreased ability to mount antiviral responses and protect the cell against infection or viral production. Furthermore, due to the MMP’s importance on electron flow, proton flux and ATP production, it can be indicative of alterations in oxidative phosphorylation and ROS production\(^{248}\). Therefore, we wanted to further evaluate these results by investigating oxidative phosphorylation, superoxide and total cellular ROS production.
Oxidative phosphorylation is the most efficient way of producing ATP under aerobic conditions, with the electron transport chain being comprised of five complexes that produce a proton gradient and proton flux across the inner mitochondrial membrane to produce energy (ATP)\textsuperscript{249}. In macrophages, the balance between glycolysis and oxidative phosphorylation is critical to inflammatory and anti-inflammatory phenotypes, with M1 macrophages favoring glycolysis and M2 macrophages favoring oxidative phosphorylation\textsuperscript{250}. Dysregulation within oxidative phosphorylation can therefore alter immune function\textsuperscript{250}. NRTIs and NNRTIs have been shown to cause alteration in cellular respiration in hepatic cells, endothelial cells, kidney cells, adipocytes, and cardiac cells, but more characterization is required in MDMs\textsuperscript{146}. In the current study, EFV had an immediate impact on cellular respiration at 50\textmu M, as the real-time measurements of oxygen consumption rate (OCR) showed a dramatic decrease from the time that it was injected. TFV at 50\textmu M also appeared to have an effect, although more gradual, taking longer to show its effects. These effects persisted, as seen through decreased basal respiration, maximal respiration, and ATP production following 24h treatments of 50\textmu M TFV and EFV. 3TC, however, did not decrease oxidative phosphorylation and, in fact, the mean was slightly higher than the untreated control for basal respiration, maximal respiration, and ATP production, although not significant. Aerobic cellular respiration is necessary for cell survival, and it also causes by-products such as reactive oxygen species (ROS) and superoxides (O$_2^-$) predominantly through complexes I and III of the ETC\textsuperscript{120}. As mentioned, a decrease in oxidative phosphorylation is associated with an inflammatory phenotype\textsuperscript{250} and increased ROS production\textsuperscript{120}, similar to a phenotype that is expressed following LPS stimulation\textsuperscript{123}. Therefore, we wanted to evaluate the effects of ART on ETC complex expression, superoxide production, and total cell ROS production in order to understand if specific complexes within the ETC are leading to this inhibition, and what functional consequences might be associated with them.

As mentioned, complexes within the ETC are vital to energy production within the cell\textsuperscript{249}. Dysregulation within these complexes can lead to increased ROS production\textsuperscript{120}, and dysfunctional immune activation\textsuperscript{251}. For example, in mice with a NDUFS4 deletion (a subunit of complex I), macrophages shifted towards a pro-inflammatory M1 phenotype, suggesting that
complex I plays a vital role in suppressing inflammation. Therefore, we wanted to evaluate complex I, II, III, and IV expression following in vitro ART. In hepatic cells (Hep3B), EFV has been shown to inhibit complex I leading to lipid accumulation in the cytoplasm, and in mouse neurons, EFV showed inhibition of complex IV leading to adverse CNS effects. In terms of NRTIs, they have been shown to inhibit complex I function and affect complex IV activity associated with mitochondrial toxicity and decreased mtDNA. TFV specifically has been shown to decrease complex I, II, IV, and V activity in rat kidney tissue. However, as mentioned for previous assays, macrophages are known to be more resistant to the effects of ART, and it is unclear if these effects will be seen in MDMs. In the current study, EFV, when treated at 50 µM for 24h, decreased complex I expression significantly. These results suggest that significant and rapid alterations shown in oxidative phosphorylation and MMP following EFV treatment may be due to inhibitory effects on complex I. Complex I, as mentioned, also plays a crucial role in superoxide and ROS production, and this observed inhibition could lead to alterations in superoxide and ROS production, which could in turn activate inflammatory signaling. Finally, this significant decrease in complex I expression could lead to a shift of the MDMs towards a pro-inflammatory M1-like phenotype. Therefore, these results further interested us in the investigation of superoxide production, total cellular ROS production, and inflammatory cytokine production.

2.5.5 Superoxide and Total Cellular ROS Production

As mentioned, ROS are generated through cellular respiration and metabolism, and a balance must be maintained in order to avoid oxidative stress on the mitochondria and the cell. ROS production can lead to oxidative stress causing dysregulation in cell-signaling, cell damage, decreased energy (ATP) production, and dysregulation of apoptotic pathways. This oxidative cellular damage occurs when the production of ROS exceeds the production of protective antioxidants. ROS have also been shown to be important signaling molecules, critical to inflammatory and antiviral immune responses, especially in TLR4 signaling and immune activation. Superoxides, the main type of mitochondrial ROS, are produced from the reduction of oxygen by one electron, which leak out of the ETC.
mainly through ETC complexes I and III, and are used mainly in signaling pathways or to combat invading pathogens, but also have toxic abilities if they accumulate\textsuperscript{119,120}. It has been shown in hepatic cells \textit{in vitro} that EFV significantly increases cellular ROS and superoxide production\textsuperscript{146,257}. TFV has been shown to deplete antioxidant levels leading to increased ROS production and nephrotoxicity\textsuperscript{258}. In the current study, EFV and TFV showed increases in total cellular ROS over 72h, with an increase beginning around the 6-12 hours following EFV treatment, and 20-24 hours following TFV treatment. EFV, however, was the only drug to significantly increase superoxide production following a 24h treatment. EFV appears to be having more an impact on the mitochondria due to its effects on mitochondrial specific ROS accumulation, which could lead to differential downstream immune activation. Therefore, although both TFV and EFV lead to increases in total cellular ROS, they appear to be through different mechanisms. This is consistent with decreases seen in cell viability, cellular respiration, oxidative phosphorylation, and MMP; they are all occurring but at different rates and to different degrees. Furthermore, EFV’s increase in superoxide production is consistent with its inhibition of complex I. HIV patients have been shown to have increased levels of oxidative stress due to HAART\textsuperscript{241}. In fact, those who adhered more to treatment had higher oxidative stress\textsuperscript{241}. Therefore, these differences observed in the literature and the current study could be leading to chronic immune activation that has been shown in HIV-infected patients on ART\textsuperscript{48}.

\textbf{2.5.5 Inflammatory and Antiviral Cytokine Production}

Cytokines are signaling secretory proteins which are secreted by immune cells in order to communicate and direct immune responses when needed\textsuperscript{235}. Cytokines are produced and released predominantly by macrophages and T helper cells during different activation states in order to prime and regain homeostasis within the immune system\textsuperscript{235}. Although cytokine production and immune activation are necessary to repair and fight pathogens, potential chronic ART-induced immune activation due to everyday treatment can be detrimental to patients\textsuperscript{47}. In the current study, we investigated CXCL10, TNF\textgreek{a}, and IFN\textgreek{b} in order to evaluate the effects of ARVs on inflammatory and antiviral cytokine production. CXCL10, also known as interferon gamma inducible protein-10 (IP-10), has been shown to have antiviral\textsuperscript{237} and
inflammatory\textsuperscript{236} capabilities. TNF\textsubscript{α} is an inflammatory cytokine produced primarily by macrophages in order to mediate inflammation through TNF receptors on cells, via the NF-KB signaling cascade\textsuperscript{235}. IFN\textsubscript{β} is a widely expressed antiviral type I interferon that is produced in abundance by macrophages, among other cells, during times of pathogen encounter\textsuperscript{234}. In the current study, we decided to investigate these three cytokines due to their link to ROS signaling and mitochondrial pathways\textsuperscript{259–261}. The bacterial ligand found on gram-negative bacteria, LPS, which stimulates through TLR4, was also used during this investigation in order to enhance cytokine production. CXCL10, TNF\textsubscript{α}, and IFN\textsubscript{β} have all been shown to increase production following TLR4 stimulation\textsuperscript{259–261}. LPS stimulation was also appropriate in the context of ART, since it simulates a scenario seen commonly in HIV infection, microbial translocation\textsuperscript{262}. In the current study, 50\textmu M EFV decreased CXCL10 production, but increased TNF\textsubscript{α} and IFN\textsubscript{β} production when treated with 100ng/mL LPS for 6h, or when treated prior to LPS for 6h with a subsequent 18h LPS treatment. 50\textmu M TFV had an opposite effect on TNF\textsubscript{α} production, as it decreased levels of TNF\textsubscript{α}, and no significant effects were shown in terms of CXCL10 or IFN\textsubscript{β} production. This again points to different mechanisms due to mitochondrial alterations, since EFV appears to have a more immediate and drastic effect on mitochondrial function, leading to the differential effects that are seen following TFV and EFV treatment. These results indicate that 50\textmu M of EFV treatment is inducing increases in immune activation. These effects may be associated with chronic immune activation commonly seen in long-term ART users. One of the main differences seen between TFV and EFV treatment was superoxide production. Due to superoxides’ known signaling capabilities on mitochondria-driven immune activation\textsuperscript{254}, we wanted to focus on this aspect moving forward, as well as general ROS production, through superoxide and ROS scavengers. This would help us understand, more in depth, the mechanisms behind the functional effects that were seen following treatments.

As shown in the current study, 50\textmu M TFV and EFV induced cell death in MDMs. It has been shown that cell viability can be decreased due to excess superoxide and ROS production\textsuperscript{255}, and thus scavenging their accumulation may help increase cell viability. MitoTEMPO, a superoxide scavenger, and N-acetyl-L-cysteine (NAC) an inducer of antioxidants, were used in order to
scavenge superoxides and total cellular ROS respectively. Three concentrations for each scavenger were used based on concentrations that have been used in the literature as well as in our lab, in order to evaluate which concentrations could be toxic and which concentrations could decrease toxicity in MDMs following ARV treatment. For MitoTEMPO, all three concentrations slightly increased TFV-mediated cell death, whereas the higher concentration of 500µM decreased cell death following EFV treatment. This was not surprising given EFV treatment showed increased superoxide production and TFV treatment did not, therefore scavenging superoxides should have more of an effect on EFV-mediated cell death. For NAC, the higher concentration of 5mM increased cell death following both TFV and EFV treatment, whereas the two lower concentrations of 1.25mM and 2.5mM decreased cell death following both TFV and EFV treatment. Therefore, it is apparent that our previous results of increased ROS production following TFV and EFV treatment, and increased superoxide production following EFV treatment, have effects on cell death. In order to complete our story, we will also evaluate the effects of these scavengers on ART-mediated cytokine production. Using concentrations of 500µM of MitoTEMPO and 1.25mM of NAC, we will evaluate previously explored cytokines, TNFα and IFNβ, in order to evaluate if these scavengers can reverse cytokine production differences that we saw following ART. This could confirm the effects of superoxides and ROS on immune activation in the context of ART, and link mitochondrial alterations to immune activation as well in the context of ART.
Chapter 3: Effects of antiretrovirals and electron transport chain inhibition on HIV infection and mitochondrial function in monocyte derived macrophages

Research questions: Does ART associated mitochondrial reprogramming in HIV-infected MDMs resemble what was seen in uninfected MDMs? Does the inhibition of different complexes within the electron transport chain impact HIV infection in MDMs?

3.1 Introduction

3.1.1 HIV as Chronic Disease
HIV-infected individuals on virally-suppressive ART manifest a phenotype of accelerated aging and experience increased rates of non-AIDS associated comorbidities such as diabetes, cardiovascular disease, neurocognitive impairment, and cancer\textsuperscript{47,48}. Progress towards improving outcomes for these individuals will depend on the identification of novel strategies for the prevention and treatment of these diseases. Chronic immune activation and inflammation persist in HIV patients on ART\textsuperscript{3}. This immune dysfunction is associated with hypercoagulation, tissue fibrosis/damage and organ system dysfunction, which over time contributes to the development of non-AIDS associated comorbidities\textsuperscript{81}. The drivers of this activation remain incompletely understood but are thought to include ongoing low levels of HIV replication, secondary coinfections\textsuperscript{47,48}, HIV-mediated breakdown of the intestinal mucosa and chronic exposure to gut microbial products\textsuperscript{223}, as well as sustained dysfunction of immunoregulatory factors\textsuperscript{57}.

3.1.2 The Role of Macrophages in HIV Infection
Macrophages have the receptors (CD4) and co-receptors (CCR5 and CXCR4) necessary for HIV infection, and have the ability to be infected in the brain, lungs, and secondary lymphoid tissue\textsuperscript{101}. That being said, macrophages in different tissues have different degrees of infectivity, with vaginal macrophages being more readily infected than gut macrophages, for example\textsuperscript{102}. In general, macrophages are readily infected, but have more of an ability to resist infection in comparison to CD4+ T cells, which infect at higher levels and also experience more HIV-
mediated cell death\textsuperscript{103}. This is primarily thought to be due to macrophages' lower levels of CD4 and CCR5 receptor expression compared to CD4+ T cells\textsuperscript{101}. Macrophages have the ability to absorb the virus, protect it, and retain its infectivity before presenting it to T cells, leading to their infection\textsuperscript{104}, and they can also be infected through phagocytosis of infected CD4+ T cells\textsuperscript{105}. Macrophages are known as a principal reservoir for HIV, as they have the ability to survive longer periods of time as infected cells at different stages of infection, and they can support high levels of viral replication, as seen in late stages of HIV infection when CD4+ T cells are mostly depleted\textsuperscript{106}. Also, during ART, infected macrophage populations have shown the ability to survive, expressing low and persistent levels of viral replication\textsuperscript{101,103,107}. They have been shown to be more resistant to the effects of ART compared to lymphocytes, showing higher EC\textsubscript{50}\textsuperscript{108}. Microglia, in particular, have shown the ability to resist the effects of ART, as ART has difficulty crossing the blood brain barrier and cells have the ability to efflux ART efficiently in the central nervous system (CNS)\textsuperscript{109}. These latently infected macrophages appear to have the ability to evade the immune system and survive long periods of time, creating long-lasting viral reservoirs\textsuperscript{110}. These viral reservoirs are thought to be key contributors to chronic viral replication leading to chronic immune activation\textsuperscript{25}.

3.1.3 Effects of HIV and ART on the Mitochondria
HIV and ART together are associated with mitochondrial toxicity and dysfunction\textsuperscript{146}. Children who are exposed to ART and HIV in the perinatal period have higher risk of persistent mitochondrial dysfunction\textsuperscript{187}. HIV alone is known to deplete mtDNA\textsuperscript{150}, reprogram energy production via oxidative phosphorylation\textsuperscript{163}, as well as increase ROS and superoxide production\textsuperscript{164} to allow for spread of the virus\textsuperscript{165}. HIV is also known to depolarize mitochondrial membrane potentials (MMP) in primary cells and cell lines which increases infection\textsuperscript{167}. The mitochondrion is vital to the initial infection, survival, and spread of HIV in human beings\textsuperscript{168}. Most importantly in the context of HIV infection and mitochondria is the reprogramming in order to evade host-cell antiviral responses. During pathogen stress, RIG-1 receptor signaling causes the mitochondria to undergo reprogramming in order to release mitochondrial antiviral-signaling proteins (MAVS) and trigger antiviral immune responses (IFN
responses\textsuperscript{263,264}. Upon infection, HIV has the ability to maintain mitochondrial homeostasis through regulating mitophagy, fusion and fission events, and mitochondrial membrane integrity in order to avoid IFN responses\textsuperscript{263,264}. It has been shown that HIV has direct effects on inhibiting the RIG-1 pathway, leading to decreased IFN responses, and allowing the virus to infect and propagate\textsuperscript{261}.

ART has been found to accelerate senescence and aging\textsuperscript{224}, with a main source of this being due to mitochondrial toxicity. Nucleotide and nucleoside reverse transcriptase inhibitors (NRTI) are the most common ARVs used in first-line ART regimens\textsuperscript{52}. Typical regimens are comprised of 2 NRTIs as a ‘backbone’ and either one non-nucleoside reverse transcriptase inhibitor (NNRTI), one protease inhibitor (PI), or one intergrase inhibitor as the third component of the combination ART (cART)\textsuperscript{52}. RTIs inhibit the transcription of viral RNA into proviral DNA following viral entry into the cell\textsuperscript{52}. A popular hypothesis of NRTI mitochondrial toxicity is the inhibition of DNA pol-\textgamma, the sole polymerase responsible for the synthesis of mtDNA\textsuperscript{146}. Although NRTIs have a significantly higher affinity for HIV reverse transcriptase than Pol-\textgamma, they still have a significantly higher affinity and inhibitory effect on Pol-\textgamma than any other DNA polymerase\textsuperscript{189}. However, more and more evidence suggests that mitochondrial alterations due to ART go beyond pol-\textgamma, and are more complex than originally thought\textsuperscript{146}. NRTIs have also been shown to decrease cell membrane integrity, cell growth, proliferation, and viability as well as an increase in succinate dehydrogenase dysfunction\textsuperscript{225}. NNRTI’s mitochondrial effects are known to be centered around dysregulation of bioenergetics, increased ROS production, and increased cell death\textsuperscript{226}. Efavirenz (EFV) has been shown to inhibit complex I and IV of the ETC, increase ROS production, decrease ATP production, decrease MMP, increase mitochondrial mass, alter mitochondrial morphology, decrease cell proliferation, increase autophagy, and increase apoptosis via the intrinsic pathway (cytochrome c release and caspase 9 activity) in different cell types\textsuperscript{205–208,226,227}. These findings suggest that mitochondrial alterations as a result of ART can be a source of mitochondrial dysregulation, but more investigation must be performed in monocyte derived macrophages in order to understand the mechanism of mitochondrial alterations, and their potential link to inflammation due to macrophage activation and dysfunction.
3.2 Hypothesis

Similar to uninfected MDMs, mitochondrial reprogramming driven by ART in HIV-infected MDMs leads to alterations in mitochondrial function. Also, inhibition of the electron transport chain individual complexes decreases HIV infection.

3.3 Methods

3.3.1 Macrophage Isolation

Blood was drawn from healthy male and female donors, aged 20-40 years old, who were selected on a volunteer basis. Using Lymphoprep density gradient medium (Cat #: 07851) from STEMCELL Technologies Inc. (Vancouver, BC), peripheral blood mononuclear cells were separated and isolated from the whole blood and plated at approximately 2 million PBMCs per mL onto 6, 12, 24, 48, or 96-well Corning cell culture plates in serum free DMEM media from Sigma-Aldrich (St-Louis, Missouri). After a two-hour incubation, non-adherent PBMCs were washed away using Gibco Phosphate Buffer Saline (PBS) (Cat #: 10010023) purchased from ThermoFisher (Waltham, MA) before complete media (10% FBS, 1% PenStrep, 89% DMEM media) was added along with 1µM Recombinant Human M-CSF Protein from R&D Systems (Minneapolis, Minnesota). After 3 days, non-adherent PBMCs were once again washed away using PBS, and fresh complete media containing 1µM M-CSF was added for another 4 days. All treatments, infections, or harvests were performed at day 7 when monocytes were fully differentiated into macrophages.

3.3.2 Cell Harvesting

All cell harvesting was performed using Accutase Cell Detachment Medium from Innovative Cell Technologies, Inc. (San Diego, California) for 30 minutes at room temperature before performing a light scraping. Cells were then collected into different types of tubes depending on the assay, centrifuged at 1,600 rpm for 10 minutes, washed with PBS, before being centrifuged again at 1,600 rpm for 10 minutes.
3.3.3 ARV Treatments

Antiretroviral drugs were purchased from Sigma Aldrich (St-Louis, Missouri) and were catalog numbers: 2020138-50-9 (TFV), 134678-17-4 (3TC), and 154598-52-4 (EFV). The powder form was reconstituted in ddH$_2$O (TFV and 3TC) or DMSO (EFV), aliquoted and stored at -80 degrees Celsius until used. ARVs were diluted to desired concentration (10µM and 50µM) in complete media (10% FBS, 1% PenStrep, 89% DMEM) and added directly to wells.

3.3.4 Western Blots

Cells were lysed directly on the cell culture plate using Pierce RIPA Buffer and Halt Protease and Phosphatase inhibitor (1:100 dilution) acquired from ThermoFisher with approximately 70µL per 150,000 cells. Cells were collected and centrifuged at 14,000 rpm for 15 minutes at 4 degrees Celsius before being placed at -80 degrees Celsius until use. Bio-Rad Laboratories (Hercules, California) DC assay was used to quantify total protein in each sample, samples were diluted in Bio-Rad Laemmli protein sample buffer for SDS-PAGE (Cat #: 1610747), and ran on 12% TGX Stain-Free FastCast Acrylamide gels from Bio-Rad. The stain-free gels were imaged using the Stain-Free setting on the ChemiDoc MP Imaging System from Bio-Rad, and the gel was transferred onto a PVDF membrane using the Bio-Rad Trans-Blot Turbo System. Membrane was blocked overnight at 4 degrees Celsius in 5% non-fat dry milk diluted in tris-buffered saline-Tween (TBST) buffer (20mM Tris-HCl, 500mM NaCl, and 0.1% v/v Tween 20; ph 7.5), and washed 6x 5 minutes in TBST buffer. Primary antibodies were incubated overnight at 4 degrees Celsius in 5% non-fat dry milk diluted in TBST buffer and washed 6x 5 minutes in TBST buffer. Secondary antibodies were incubated for 1h at room temperature in 5% non-fat dry milk diluted in TBST buffer and washed 6x 5 minutes in TBST buffer. Membranes were incubated in Bio-Rad Clarity ECL Substrate (Cat #: 1705060) for at least 3 minutes out of direct light before membranes were imaged for protein band luminescence. Densitometry was performed using Bio-Rad Image Lab Software. Protein band luminescence was normalized based on the total amount of protein within each lane of the stain-free gel. Values were exported, graphs were made using Graphpad Prism 5, and p-values were calculated using 2-tailed paired t-tests.
All antibodies used for Western Blots are outlined below in Table 1 along with dilutions that were used. All protein gels were run at a concentration of 25 µg/mL, at a voltage of 100V.

Table 2. Antibodies used for Western Blots

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Supplier</th>
<th>Catalog Number</th>
<th>Isotype</th>
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3.3.5 HIV NL4.3 BAL-IRES-HSA Transfection/Amplification

The plasmids to produce HIV NL4.3 BAL-IRES-HSA CCR5 tropic virus was acquired from Dr. Michel Tremblay at the Université de Laval. 20ug of the viral plasmid was transfected into HEK293T cells using Lipofectamine 2000 and Opti-MEM I Reduced Serum Media, both acquired from ThermoFisher Scientific. HEK293T cells were approximately 85% confluent at the time of transfection, and were incubated at 37 degrees Celsius for 48 to 72 hours. Supernatants were collected at 48 and 72-hour time points and centrifuged for 10 minutes at 2000 rpm before being aliquoted into 2mL tubes and stored at -80 degrees Celsius until use. To quantify the amount of virus produced, 500µL of supernatant was lysed using 1% Triton X for 1 hour at 37 degrees Celsius and p24 levels were quantified using the Frederick National Laboratory for Cancer Research protocol as shown below.

3.3.6 HIV NL4.3 BAL-IRES-HSA Infection of MDMs and ARV Treatments

MDMs were washed with warm PBS and HIV NL4.3 BAL-IRES-HSA viral stock was thawed. HIV NL4.3 BAL-IRES-HSA was diluted to 100ng/mL and was added directly to wells in complete media (10% FBS, 1% PenStrep, and 89% DMEM), along with 5ug/mL polybrene (Cat #: TR-1003) acquired from Sigma-Aldrich. The virus was left to incubate for 24 hours before cells were washed and media was replaced with complete media not containing virus. For 14-day experiments, as shown in Figure 13, supernatants were collected at days 4, 7, 10, and 14, and
cell harvesting occurred at day 14 along with staining. For 7-day experiments, supernatants were collected at day 4 and 7, and cell harvesting occurred at day 7 along with staining. Following supernatant collections, 300µL of fresh complete media was added to each well. 10µM ARVs were either added at the time of infection, or along with the 300µL of fresh media following supernatant collection. Once ARVs had been added to a designated well, fresh media with 10µM ARVs was replaced every 3 or 4 days.

3.3.7 Quantification of number of HIV infected MDMs and cell death

In order to quantify the percentage of HIV-infected cells in vitro, we used the HIV NL4.3 BAL-IRES-HSA R5 tropic virus which produces a heat stable antigen (HSA) on the cell surface via a glycoprophatidylinositol anchor once MDMs are infected. To quantify the number of infected cells, CD24 FITC Staining (Order #: 130-099-188) was used in order to tag the HSA protein. This gave us the ability to discriminate between HIV-infected and uninfected cells in order to quantify the differences between the two populations. In order to quantify the number of dead cells, eBioscience Fixable Viability Dye (FVD) eFluor 450 Staining (Cat #: 65-0863-14) was co-stained with CD24-FITC. Cells were washed with PBS, PBS was aspirated, cells were detached using protocol seen above, and cells were centrifuged at 1,600 rpm for 10 minutes. FcR Blocking Reagent (Order #: 130-059-901) purchased from MACS Miltenyi Biotech (Cambridge, MA) was diluted 1:10 in FcR buffer (0.5% BSA, 2mM EDTA, 7.2 pH in PBS), 50µL was added to each tube, and samples were incubated at 4 degrees Celsius for 15 minutes. CD24 FITC Antibody (Order #: 130-099-188), purchased from MACS Miltenyi Biotech (Cambridge, MA) was diluted 1:50 in FcR buffer (0.5% BSA, 2mM EDTA, 7.2 pH in PBS) and FVD Antibody, purchased from ThermoFisher Scientific (Waltham, MA) was diluted 1:1000 in FcR buffer (0.5% BSA, 2mM EDTA, 7.2 pH in PBS), and both were added to tubes. Samples were vortexed and incubated at 4 degrees Celsius for 15 minutes. Samples were washed with PBS, centrifuged at 1,600 rpm for 10 minutes, 200µL of 2% paraformaldehyde (PFA) was added to each tube, cells were vortexed well and left to fix for 15 minutes at room temperature 10,000 events were acquired using the LSRFortessa flow cytometer. Analysis was performed using FlowJo version X 10, graphs were
made using Graphpad Prism 5, and p-values were calculated using 2-tailed paired t-tests. Results are reported as the percentage of positive cells.

3.3.8 p24 ELISA (Viral Production Detection)

Supernatants were collected and centrifuged at 1,600 rpm for 5 minutes before being lysed in 1% Triton-X for 1 hour at 37 degrees Celsius. All antibodies were obtained from Frederick National Laboratory for Cancer Research, Frederick, MD; NIH AIDS Reagent Program. Capture Ab was diluted 1:1440 in PBS, 100µL of Capture Ab solution was added to each well of a 96 well plate, plate was sealed and stored overnight at 4 degrees Celsius. Capture Ab was aspirated and patted dry plate on clean paper towel, 300µL of Blocking Solution (1% BSA in PBS) was added to each well, plate was incubated for 30 minutes at 20 degrees. Blocking Solution was then aspirated and plate was patted dry on a clean paper towel. Plates were then used or stored at 4 degrees Celsius, upside down, for up to 1 month if needed. Samples were diluted in Sample Diluent (1% BSA, 0.2% Tween-20 in Hyclone RPMI-1640 Media (GE Healthcare Life Sciences, Chicago, IL (Cat #: SH30027.01)) at desired concentrations (5000pg/mL-78.13pg/mL), 100µL of standard and samples were added to each well. The plates were incubated at 37 degrees Celsius for 2 hours or overnight at 4 degrees Celsius if needed. Plates were washed 5x with 300µL of Wash Buffer (0.5mL Tween-20 + 100mL PBS), Primary Ab was diluted 1:200 in Primary Ab Diluent (10% FBS, 2% NMS, in Hyclone RPMI-1640 Media), 100µL of Primary Ab solution was added to each well except blank/background wells, and plates were incubated at 37 degrees Celsius for 1 hour. Plates were washed 5x with 300µL of Wash Buffer, Secondary Ab was diluted 1:40,000 in Secondary Ab diluent (2% Normal Mouse Serum, 5% Normal Goat Serum, 0.01% Tween-20 in Hyclone RPMI-1640 Media), 100µL of Secondary Ab solution was into each well except blank/background wells, and plates were incubated for 1 hour at 37 degrees Celsius. Plates were washed 8x with 300µL of Wash Buffer, 100µL of TMB Peroxidase Substrate were added into each well, plates were incubated at room temperature until lowest concentration on standard curve began to turn blue, then 100µL of 1N HCl stop solution was added to each well. Plate was read at 450nm with a reference of 655nm using the Bio-Rad iMark Microplate Reader. Values were quantified based on standard curve using Bio-Rad Image Lab Software.
Values were exported, graphs were made using Graphpad Prism 5, and p-values were calculated using 2-tailed paired t-tests.

3.3.9 ETC Inhibitor Treatment

Inhibitors were diluted in complete media (10% FBS, 1% PenStrep, 89% DMEM) at concentrations of: 100nM (Rotenone), 1µM (antimycin), and 3.125mM (Na Azide). Rotenone was used to inhibit complex I of the ETC, antimycin was used to inhibit complex III, and Na Azide was used to inhibit complex IV in order to evaluate their effects on HIV-infection (HSA-positive cells) and HIV production (p24). Cells were treated at the same time as 100 ng/mL infection of HIV NL4.3 BAL-IRES-HSA, and media was changed the next day for fresh complete media containing the same concentration of inhibitors. Supernatants were collected at day 3 of infection, and cells were harvested and stained using the above CD24 protocol before 10,000 events were acquired using LSRFortessa flow cytometer in PBS. Analysis was performed using FlowJo version X 10, graphs were made using Graphpad Prism 5, and p-values were calculated using 2-tailed paired t-tests. Results are reported as the percentage of HSA positive cells.

3.3.10 Quantification of total cellular ROS during HIV infection

For analyses of total cellular ROS at following 7 days of HIV-HSA infection, CellROX Deep Red was used to stain ROS. samples were detached, as described above, into Falcon round-bottom polystyrene 5mL tubes, washed with PBS, and centrifuged at 1,600 rpm for 10 minutes. PBS was aspirated and cells were stained with 5µM CellROX Deep Red in complete media (10% FBS, 1% PenStrep, 89% DMEM) for 20 minutes at 37 degrees Celsius. Cells were then washed with PBS, centrifuged at 1,600 rpm for 10 minutes, and PBS was aspirated before 10,000 events were acquired using the LSRFortessa flow cytometer in PBS. Analysis was performed using FlowJo version X 10, graphs were made using Graphpad Prism 5, and p-values were calculated using 2-tailed paired t-tests. Results are reported as the percentage of positive cells and as mean fluorescence intensity (MFI), the latter being used to describe the level of expression on a population of positive cells.
3.4 Results

3.4.1 Early ARV treatment reduces the number of HIV-infected MDM but does not alter viral production after the establishment of infection:

To evaluate the effects of ARVs on mitochondrial function in the context of HIV infection, we first needed to optimize our infection model using HIV NL4.3 BAL-IRES-HSA. This strain of HIV produces a murine heat stable antigen (HSA), which is expressed on the surface of infected cells. This expression allows us to investigate alterations in infected and uninfected bystander cells from the same well. TFV, 3TC and EFV (alone and in combination; 10µM) were administered to the cells at 0, 4, 7 and 10 days post-infection. The number of infected cells (HSA-positive cells) were quantified using flow cytometry and de novo viral production was evaluated using a p24 ELISA (Figure 13A). As shown in Figure 13B, when ARVs were administered at the time of infection (day 0) and maintained in the culture media for 14 days, they significantly reduced the total number of infected cells (>3.7 fold) suggesting that these drugs alone and in combination can prevent MDM infection. When ARVs were administered at day 4 post-infection and maintained in the culture media for 10 days, the number of HIV infected cells was reduced by approximately half. However, when drugs were provided at 7 or more days post-infection, they seemed to have a limited ability to reduce the number of infected cells. These data may suggest that by day 7, the spreading phase of infection may be mostly complete in our system. Given their mechanism of action, reverse transcriptase inhibitors had a limited capacity to prevent de novo viral production (Figure 13C). Based on these optimization experiments, we focused on the effects of ARVs on mitochondrial function during spreading infection (administration of ARVs at day 3 post-infection). ARVs provided at day 0 post-infection was used as a control. Further, given the significant cell death observed at day 14, endpoint assays were performed at day 7 post-infection (Figure 14).
Examination of the effects of ART on 14-day HIV infection in MDM. MDMs were infected in complete media with 100ng/mL of HIV NL4.3 BAL-IRES-HSA for 14 days. Cells were treated with 10μM ARVs alone or in combination at 0, 3, 7 and 10 days post-infection. (A) Schematic of infection protocol. (B) Levels of infection were assessed in MDM treated with 14, 10, 7 and 3 days of ARVs. The percent of infected MDM (% HSA-positive cells) were performed 14 days post-infection via the BD LSRFortessa Flow Cytometer; n=2. (C) Levels of virus production were assessed in culture supernatant at day 14 post-infection using an HIV p24 ELISA; n=2.
Figure 14 HSA Day 7 Examination of the effects of ART on 7-day HIV infection in MDM. MDMs were infected in complete media with 100ng/mL of HIV NL4.3 BAL-IRES-HSA for 7 days. Cells were treated with 10µM ARVs alone or in combination at 0 and 3 days post-infection. (A) Levels of infection were assessed in MDM treated with 7 and 3 days of ARVs. The percent of infected MDMs (% HSA-positive cells) were performed 7 days post-infection via the BD LSRFortessa Flow Cytometer; n=4. (C) Levels of virus production were assessed in culture supernatant at day 14 post-infection using an HIV p24 ELISA; n=3. *p-value<0.05

3.4.2 HIV is the main driver of ROS production during spreading infection:

Up to this point, we have used live cell stains to evaluate the effects of ARVs on mitochondrial function in uninfected cells. However, these stains cannot be used in the HIV model as the cells need to be fixed prior to analysis. The only stain available to evaluate ROS production in HIV infected cells was the CellROX Deep Red. For these studies, living cells were stained, fixed and total levels of cellular ROS were examined in infected (HSA-positive) and uninfected (HSA-negative) cells using flow cytometry (Figure 15A). When drugs were provided at infection and maintained in the culture media for 7 days, only TFV significantly increased ROS production in MDM (Figure 15B). This increase in ROS was associated with a slight downregulation of complex I of the ETC (Figure 16A and C). However, in the context of spreading HIV infection (ARVs given
3 days post-infection and maintained for 4 days in media), the drugs seemed to have a limited effect on ROS production. In uninfected cells (HSA-negative MDM), ROS levels were less than those observed in HSA-positive MDM and similar between ARV treated and untreated HSA-negative cells (Figure 15C). The HIV associated increase in cellular ROS production at day 7 post-infection was not significantly altered following ARV treatment in HSA-positive cells (Figure 15D). Consistent with these findings, complex I expression was not altered in spreading HIV infection (Figure 16A and B). While these results are preliminary and require further investigation, they may suggest that HIV infection and potentially exposure to the virus may prevent ARV associated mitochondrial reprogramming.
Figure 15 ROS production in HIV infection following ARV treatment. MDMs were infected in complete media with 100ng/mL of HIV NL4.3 BAL-IRE-HSA for 7 days. Total cellular ROS levels were evaluated using CellROX Deep Red via the BD LSRFortessa Flow Cytometer. Levels of cellular ROS in cells not exposed to HIV (Mock), HIV infected (HSA-positive cells) and uninfected bystander cell (HSA-negative cells) from the same culture well were quantified using the mean fluorescent intensity (MFI) of CellROX Deep Red compare to those treated with ARVs. (A) Cellular ROS levels in MDMs treated with ARVs at the time of infection (day 0). Given low levels of infection, results are presented as MFI of the total MDM population; n=3. (B and C) Levels of cellular ROS production were assessed in uninfected bystander (HSA-negative) (B) and infected cells (HSA-positive cells) (C) from the same culture well. Cells were infected with HIV and treated with ARVs at 4 days post-infection; n=6. (D) Representative gating strategy shows how
viable macrophages were selected for from the total event population, doublet cells were excluded, dead cells were excluded using eBioscience Fixable Viability Dye 450 eFluor (FVD 450), and HSA-negative cells were compared to HSA-positive cells for CellROX Deep Red fluorescence using a CD24-FITC antibody. Results presented as fold change in treated compared to untreated cells (treatment value/untreated value). *p-value<0.05

**Figure 16** Effects of ARVs on ETC complex I expression in HSA-exposed MDMs. MDMs were infected in complete media with 100ng/mL of HIV NL4.3 BAL-IRES-HSA for 7 days. Cells were treated with 10µM ARVs alone or in combination at 0 and 4 days post-infection. ETC complex I expression was assessed in cell lysates using Western blot. (A) Representative donor showing alterations in ETC complex I expression following 4-day and 7-day ARV treatment in combination with 7-day HIV NL4.3 BAL-IRES-HSA infection and subsequent complex I (NDUFB8 monoclonal antibody. (B and C) Summary of densitometry analysis for complex I following (B) 3-day ARV treatment and (C) 7-day ARV treatment; n=3. Analyses were performed using Image
Lab Software. Results presented as fold change in treated compared to untreated cells (treatment value/untreated value). *p-value<0.05

3.4.3 Flux through the ETC is required to support HIV infection:
As our preliminary studies suggest that HIV and not ARVs drive cellular ROS production in MDM, we next wanted to see if HIV infection was dependent on mitochondrial function. Specifically, we wanted to see if HIV infection was dependent on flux through the ETC. For these studies, we used the complex I inhibitor rotenone (100nM), complex III inhibitor antimycin (1µM), and complex IV inhibitor sodium azide (3.125µM). Inhibitors were added at the time of infection (day 0) and supernatants and cells were collected 3 days post infection with HIV NL4.3 BAL-IRESHSA. As shown in Figure 17, all 3 complex inhibitors significantly reduced the number of infected MDM (HSA+ cells). Inhibition of complexes III and IV seemed to have the most pronounced effect on reducing the number of infected cells but these differences were not statistically significant. Interestingly, while inhibition of the various complexes of ETC reduced overall p24 production compared to untreated cells, high levels of p24 were produced by few infected MDM (Figure 17C). These findings suggest that the ETC is require for HIV entry into the cell but perhaps not to support de novo viral production. Further studies are required to understand the specific dynamics of mitochondrial reprogramming in HIV infection over time.
The effects of electron transport chain (ETC) complex inhibition on HIV infection in MDM. MDMs were infected in complete media with 100ng/mL of HIV NL4.3 BAL-IRE-HSA for a total of 3 days with ETC complex inhibitors for the duration of infection. (A) Visual representation of which complexes the different drugs inhibited following 3-day HIV NL4.3 BAL-IRE-HSA infection in combination with 3-day treatment of 100nM Rotenone (Complex I), 1µM Antimycin (Complex III), and 3.125µM Na Azide (Complex IV). Following this treatment and sample collection, 3 assays were performed: (B) HSA positive cells via the BD LSRFortessa flow cytometer; n=4, (C) HIV p24 viral production via ELISA analysis; n=4, and (D) CXCL10 production (pg/mL) via ELISA analysis; n=3. *p-value<0.05, **p-value<0.005

3.5 Discussion

In summary, this chapter characterizes alterations in mitochondrial function in HIV-infected and HIV-exposed bystander cells following ARV treatment and ETC complex inhibitor treatments in vitro. To our knowledge, this type of characterization has not yet been investigated at these
concentrations in macrophages. Therefore, these findings represent novel findings encapsulating effects of ART on immunometabolism and HIV infection.

An optimization experiment was first carried out using 10µM ART at different time points in order to understand the effects of ARV treatment on in vitro MDM HIV infection and p24 viral production. ARV treatment decreased the percentage of HIV-infected cells in a time dependant manner, although treatment at 7 or 10 days post-infection had limited effects compared to when treated at the time for infection (day 0) or 3 days post-infection. Interestingly, p24 viral production was not inhibited to the same degree as the percentage of HIV-infected cells, suggesting that, although there are less infected cells in the ARV-treated samples, the overall population is still producing an equivalent amount of virus. These results are preliminary, and more investigation needs to be performed in order to understand if the infected cells are producing more p24 when treated with ARVs. If so, a mechanism would need to be investigated in order to understand why these ARV-treated and HIV-infected cells are producing more virus that ARV-naïve cells, if it could be linked to mitochondrial dysfunction, and if it could be linked to subsequent immune activation via cytokine production. The effects of ART on the mitochondria in infected cells could be leading to more optimal metabolic environments that could be enhancing the virus’ ability to propagate, although this is very hypothetical at this point. Overall, this optimization allowed us to confirm that our ARV treatments were inhibiting infection, and it also allowed us to pick a time point in which there was a sizeable population of HIV-infected cells, but still inhibition of HIV infection to a degree. This was important as we wanted to isolate HIV-infected cells in the context of ARV treatment in order to evaluate mitochondrial effects within this population. Therefore, we moved forward into mitochondrial functional assays using ARV treatments at 0 and 4 days post-infection, with analyses being run at 7 days post-infection.

ROS are generated through cellular respiration and metabolism, and a balance must be maintained in order to avoid oxidative stress on the mitochondria and the cell\textsuperscript{255}. ROS production can lead to oxidative stress causing dysregulation in cell-signaling, cell damage,
decreased energy (ATP) production, and dysregulation of apoptotic pathways\textsuperscript{194}. This oxidative cellular damage occurs when the production of ROS exceeds the production of protective antioxidants\textsuperscript{199}. ROS have also been shown to be important signaling molecules, critical to inflammatory and antiviral immune responses, especially in TLR4 signaling and immune activation\textsuperscript{256}. Superoxides, the main type of mitochondrial ROS, are produced from the reduction of oxygen by one electron, which leak out of the ETC\textsuperscript{43}. Superoxides are produced mainly through ETC complexes I and III, and are used mainly in signaling pathways or to combat invading pathogens, but also have toxic abilities if they accumulate\textsuperscript{119,120}. ROS is also known to play a critical role in controlling infection, making the dynamics of ROS production complex within the context of HIV\textsuperscript{265}. In general, alterations in cellular redox status have been mostly studied in cells that are not infected by HIV that have been implicated in clinical disease. In these cases, ROS appears to be increased during HIV infection, although the results are inconsistent\textsuperscript{265}. In terms of ART, it has been shown in hepatic cells \textit{in vitro} that EFV significantly increases cellular ROS and superoxide production\textsuperscript{146,257}. TFV has been shown to deplete antioxidant levels leading to increased ROS production and nephrotoxicity\textsuperscript{258}. In the novelty of the current study, was that ROS production was evaluated in infected and uninfected cells following ARV treatment at two different time points, in order to evaluate the effects of ARV treatment on ROS production before infection is established, and once HIV infection is established for 4 days. 10\textmu M of TFV showed increases in total cellular ROS when treated for 7 days (at the time of infection), but this increase was not shown when treated for 3 days (at 4 days post-infection). EFV, on the other hand, increased ROS production when treated for 3 days (at 4 days post-infection), but not when treated for 7 days (EFV at the time of infection). Therefore, although both 10\textmu M of TFV and EFV lead to increases in total cellular ROS in the context, they occur at different time points in treatment and infection, and appear to be through different mechanisms. This is consistent with what was seen in uninfected MDMs, as both TFV and EFV increased ROS, but at different time points and by different magnitudes, and through different mechanisms. HIV patients have been shown to have increased levels of oxidative stress due to HAART\textsuperscript{241}. In fact, those who adhered more to treatment had higher oxidative stress\textsuperscript{241}. Therefore, these differences observed in the literature and the current study
could be leading to chronic immune activation that has been shown in HIV-infected patients on ART\(^{48}\). Since ROS and superoxide production is fueled predominantly through complexes I and III, these results interested us in complex expression following 3 and 7-day ARV treatment in parallel with 7-day HIV infections.

Complexes within the ETC are vital to energy production within the cell\(^{249}\). Dysregulation within these complexes can lead to increased ROS production\(^{120}\), and dysfunctional immune activation\(^{251}\). For example, in mice with a NDUFS4 deletion (a subunit of complex I), macrophages shifted towards a pro-inflammatory M1 phenotype, suggesting that complex I plays a vital role in suppressing inflammation\(^{252}\). Therefore, we wanted to evaluate complex I expression following in vitro ARV treatment in the context of HIV infection. In hepatic cells (Hep3B), EFV has been shown to inhibit complex I leading to lipid accumulation in the cytoplasm\(^{205}\). In terms of NRTIs, they have been shown to inhibit complex I function associated with mitochondrial toxicity and decreased mtDNA\(^{196-198}\). TFV specifically has been shown to decrease complex I activity in rat kidney tissue\(^{253}\). HIV infection has been shown to decrease complex I, II, and III, while also increasing complex IV and V activity in T-cells\(^{163,266}\). However, as mentioned for previous assays, macrophages are known to be more resistant to the effects of ART and HIV infection\(^{108}\), which make it unclear if these effects will be seen in MDMs. In the current study, complex I expression was evaluated following ARV treatment and HIV infection at two different time points, in order to evaluate the effects of ARV treatment on complex I expression before infection is established, and once HIV infection is established for 4 days. TFV, when treated at 10µM for 7 days (at the same time as infection), significantly decreased complex I expression. These results were not seen when ARV treatment was administered for 3 days (at 4 days post-infection). Complex I, as mentioned, plays a crucial role in superoxide and ROS production\(^{120}\), and this observed inhibition could lead to alterations in superoxide and ROS production, which could in turn activate inflammatory signaling\(^{254}\). These results suggest that significant increases in ROS production following 7-day TFV treatment, along with HIV infection, may be due to the inhibitory effects seen on complex I. As mentioned, this significant decrease in complex I expression could lead to a shift of the MDMs towards a pro-inflammatory M1-like
phenotype. Therefore, these results further interest us in the investigation of inflammatory cytokine production. Moving forward, complexes II, III, and IV should be investigated in order to have a full picture of the ETC in the context of ARV treatment and HIV at 0 and 4 days post-infection. Further characterization of the effects of ARV treatment and HIV infection on oxidative phosphorylation should be performed through functional assays such as the Mito Stress Test in order to gain an overall understand of the bioenergetic profiles of the MDMs.

HIV is known to deplete mtDNA\textsuperscript{150}, reprogram energy production via oxidative phosphorylation\textsuperscript{163}, as well as increase ROS and superoxide production\textsuperscript{164}, suggesting that it does depend to a certain degree on the mitochondria to allow for spread of the virus\textsuperscript{165}. As mentioned, HIV infection has been shown to decrease complex I, II, and III, while also increasing complex IV and V activity in T-cells\textsuperscript{163,266}, which suggests that HIV infection also depends to some degree on interaction with ETC complexes. Characterization, however, does not appear to have been performed in order to evaluate the direct dependence of HIV infection and viral production on individual complexes within the ETC. Therefore, the current study set out to evaluate the effects of complex I, III, and IV inhibition using rotenone, antimycin, and sodium azide respectively, on HIV infection and production. Results showed that all complex inhibitors decreased HIV-infection and HIV-production significantly 3 days post-infection, suggesting that HIV depends on individual complex activity in order to establish infection. Furthermore, this suggests that regulating cellular metabolism is paramount to controlling HIV infection. In T cells, it has been shown that increased cellular metabolism is associated with susceptibility to HIV infection, and inhibiting metabolism decreases HIV infection\textsuperscript{267}. The current study’s results are preliminary, and more characterization must be performed in order to fully understand the mechanism behind the decreased infection. Moving forward, more mitochondrial and metabolic characterization must be performed. Cell viability, mitochondrial membrane potential (MMP), ROS production, and bioenergetics are all avenues that can be explored in order to fully understand the functionality of the mitochondria and MDMs during this resistance to infection, ultimately linking these effects to immune activation through inflammatory and antiviral cytokine production. The ultimate goal would be to understand the
distinct metabolic profiles of chronically-infected MDMs, in order to identify and eliminate chronically-infected cells and viral reservoirs in HIV patients who experience chronic immune activation due to chronic HIV infection.
Conclusion
In summary, TFV and EFV treatments alone and in combination appear to affect mitochondrial function in uninfected MDM, decreasing cell viability and leading to downstream alterations in cytokine production (Figure 18). Both treatments lead to increased cellular ROS production, as well as decreased MMP, cellular respiration, oxidative phosphorylation, and ATP production. However, unlike TFV, EFV treatment also lead to increased superoxide production and decreased complex I expression. We also found that EFV treatment amplified cytokine production (TNF-α and IFN-β) in response to LPS stimulation, which was reversed by pre-treating the cells with MitoTEMPO suggesting superoxides play a central role in this process. We believe this accumulation of mitochondrial superoxides, causes the formation of mitochondrial DAMPs, which increases inflammatory signaling to LPS stimulation. Further studies are required to confirm this hypothesis. Alternatively, the accumulation of high levels of cytosolic ROS following TFV treatment appear to gradually shut down mitochondrial function resulting in reduced immune responsiveness and eventually cell death. Collectively these data suggest that EFV may be reprogramming the mitochondria from the inside-out, whereas TFV appears to be acting more from the outside-in. However, in both instances the long-term accumulation of total cellular ROS (mitochondrial and cytosolic) results in cell death. Due to the importance of ROS in immune activating signaling pathways, this increase in ROS due to ART in uninfected MDM may be playing an important role in chronic immune activation seen in HIV-infected individuals with viral suppression. Furthermore, individuals using ART as a preventative measure through PrEP may be at risk to increased ROS production and subsequent chronic immune activation, which could also increase their risk of comorbidity. In the future, further research will be required in order to prevent increases in ROS production as well as treatments for the ROS that is being produced in excess.
Interestingly, these alterations appear to differ in the context of HIV infection (Figure 19). When drugs were provided at the same time of infection, TFV but not EFV was associated with decreased complex I expression and increased ROS production. These data suggest that in the absence of productive HIV infection, that ARV drugs are still able to reprogram mitochondrial function. However, if drugs were provided after the establishment of productive infection, ARVs were unable to reprogram mitochondrial function. Instead, any reprogramming seems to be driven by exposure to HIV or by HIV infection itself. Specially, HIV infection (HSA-positive cells) was associated with a significant increased in ROS production, which was not altered by ARV treatment. Similarly, treatment of exposed but uninfected MDMs to ARVs (HAS-negative) did not upregulate ROS production suggesting exposure to HIV and/or the local microenvironment is the main driver of mitochondrial reprogramming. Further, we found that HIV infection and viral production is dependent on flux through the ETC, as inhibiting complexes I, III, and IV
resulted in a decrease in the overall percentage of HIV-positive MDM as well as de novo HIV production. These findings suggest that targeting metabolism, and specifically the mitochondria, may be a means of HIV therapy in the future. Cells with reduced electron flux and oxidative phosphorylation appear to have lower rates of infection. Therefore, targeting MDM with unique metabolic profiles may be a means of targeting HIV-infected populations, and specifically at different stages of infection when these cells may be more metabolically active. Since HIV-infected cells rely heavily on mitochondrial function and electron flux, targeting the mitochondria of these cells may be a means of clearing infection. However, more extensive research must be performed to fully understand these mechanisms.

Figure 19 Summary of effects of ART and HIV on mitochondrial in HIV-infected MDM.
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