Regulation of Neuroinflammatory Factors by Neuroprotective MicroRNAs in HIV-1 Infected Microglia

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Abstract

Despite successful antiretroviral therapy (ART), more than half of individuals living with HIV-1 exhibit HIV-1 Associated Neurocognitive Disorder (HAND). Individuals with HAND experience a spectrum of cognitive, motor, and/or mood dysfunctions. Currently, there are no treatment or prevention methods for HAND. HIV-1 virus enters through the blood-brain barrier (BBB) and establishes infection in CD4+ cells such as macrophages and microglia present in the central nervous system (CNS). Macrophages and microglia are the key targets for HIV-1 in CNS. Infected target cells release inflammatory cytokines and viral proteins that are exposed to neurons. The combination of viral and host factors triggers neuronal damage that results in neurodegeneration, the hallmark of HAND pathology. Identifying new therapeutics is important to reduce the negative effects of HAND, which has great public health significance.

To combat this problem, we propose to target neuroinflammatory factors that have been identified to have a role in the development of HAND using microRNAs (miRNAs). These miRNAs target biological pathways including homeostasis of cellular interaction, regulation of neurotoxic and neuroprotective factors, cell proliferation, and differentiation. In this study, we explore the effects of these candidate miRNAs and their role in regulating the production of neuroinflammatory chemokines and cytokines. By modulating the microglia response to HIV-1

infection, I will be able to understand how normal functioning of microglia in the CNS is interfered by virus infection. Through this knowledge, there will be an increased likelihood of developing novel therapeutic strategies and treatment for neurocognitive disorders.

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Preface

The most important acknowledgment I have is to Ms. Roberta Reis for her patience and mentorship throughout my involvement in Dr. Ayyavoo's laboratory. You trained me to understand the basic fundamentals of laboratory practices, which ultimately became the foundation to my growth and scientific competency. Thank you for going beyond the protocols and teaching me how to critically understand the story behind the science. From troubleshooting shortcomings and failed experiments, to spending 8-hour(s) on multiple days to preform ELISAs together, you truly shaped my graduate school experience. What was once a challenging nuance to me, has become second nature.

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1.0 Introduction

The World Health Organization reported 37.9 million people worldwide living with Human Immunodeficiency Virus Type-1 (HIV-1) in 2018. It has been recognized that this virus progresses into an Acquired Immune Deficiency Syndrome (AIDS) (R. Ellis, Langford, & Masliah, 2007; Jeang, 2007; Price, Perry, & Association for Research in Nervous and Mental Diseases. Meeting, 1994). Although there is not a cure or vaccine available, rising practices in prevention and treatment such as antiretroviral therapy result in viral suppression and reduced transmission (ART) (Walsh et al., 2014). These practices have reduced mortality rates among those infected with HIV/AIDS. Effectively, this life-threatening virus has been reduced to a chronic disease in the past 20 years.

Unfortunately, these therapies do not reduce HIV-1 associated comorbidities including neurodegenerative diseases such as HAND (HIV-1 associated neurocognitive disorders). Recent findings suggest that the central nervous system (CNS) can potentially act as a reservoir for HIV-1 (Gray et al., 2014)**.** Since cART reduced penetration into the CNS, the frequency of HAND is on the rise. Some studies suggest that HIV-1 RNA is harbored exclusively in brain macrophages/microglia, but not astrocytes (Ko et al., 2019). These findings suggest that immune cells, like microglia, are optimal targets for investigation. The public health significance in this case is to have a fluent understanding of how HIV-1 infection and viral protein exposure in the CNS interfere with the normal functioning of microglia. Through this knowledge, there will be an increased likelihood of novel therapeutic strategies and treatment of neurocognitive disorders.

2.0 Human Immunodeficiency Virus-1

2.1 HIV/AIDS Global Epidemic

Since the beginning of the epidemic in 1981, Kaiser Family Foundation reported that approximately 77 million people have become infected with the Human Immunodeficiency Virus Type-1 (HIV-1) (Foundation, 2019). Although HIV/AIDS is a significant health concern in the United States, the hardest hit region due to the infection is in Sub-Saharan Africa. More than two thirds of its population is infected, with Asia and the Pacific following close behind. Regions like the Caribbean as well as Eastern Europe and Central Asia are also affected by the severity of this epidemic (Foundation, 2019). Developing countries still have rather high rates of mortality and morbidity due to the HIV infection (Bhatti, Usman, & Kandi, 2016). Globally, the World Health Organization(WHO) found that this virus continues to have a steady rate of mortality among children, adults, and women (Organization, 2018).

The virus is known to be transmitted sexually but could also be transmitted from mother to child during pregnancy if the mother is not on antiviral medication. Transfusion of contaminated blood or needle exchange with infected individuals are also possible modes of transmission. The use of contaminated needles and injection drug equipment makes individuals with substance use disorders susceptible to infection and has become the primary transmission route for both HIV and hepatitis C (Control, 2019). Various public health interventions, research, and educational programs have been put into place throughout the years in response to this epidemic. Needle exchange programs have helped to reduce the spread by providing clean needles and educating the IV drug abuse population. The greatest frontiers remain to be the reservoirs and sanctuary site and

the effects they play on comorbidities such as HAND. New research through the years has brought us closer to stabilizing the epidemic. It has brought the HIV/AIDS epidemic into the spotlight as a topic of interest in the field of public health.

HIV-1 targets an individuals' CD4+ cells which are white blood cells that play an important role in the immune system. Once this cell count reach below 200 cells/mm3, the infection then progresses into an Acquired Immune Deficiency Syndrome (AIDS). It is the advanced stage of HIV and interferes with the body's immune system; making it difficult to fight off infections. Throughout the past 38 years, there has been advancements in prevention and suppression of the viral replication. This life-threatening condition has been reduced to a chronic disease that's managed through proper antiretroviral therapy. Antiretroviral therapy — also known as highly active antiretroviral therapy (HAART) or combined antiretroviral therapy (cART) — is designed to slow the progression of HIV-1 in infected individuals. UNAIDS reported that AIDS-related deaths have been reduced by more than 51% since the peak in 2004 due to ART. There has been a consistent reduction in mortality from 1.9 million deaths in 2004 and 1.4 million in 2010 (UNAIDS, 2018). In 2017, UNAIDS reported that 940,000 people died from AIDS-related illnesses worldwide. Although there has been great progress, there are still thousands of people dying from this virus.

Recent evidence shows that the central nervous system (CNS) is an important target for HIV-1 (Angstwurm & Neumann, 2019; Weis, Hippius, & European Neuroscience Association. Meeting, 1992; Zayyad & Spudich, 2015). The neurotropic nature of this virus causes neurocognitive dysfunctions, known as HIV-associated neurocognitive disorder (HAND)(Angstwurm & Neumann, 2019). There is a spectrum of severity in HAND ranging from asymptomatic neurocognitive impairment (ANI), mild neurocognitive disorder (MND), and HIV-

associated dementia (HAD) being the most severe (Eggers et al., 2017; Saylor et al., 2016). HAND has become a pressing issue among the older population of infected individuals (Milanini & Valcour, 2017; Price & Sidtis, 1996). Although cART is successful in reducing the viremia in HIV/AIDS, HAND continues to be an underlying occurrence within 50% of those infected with HIV (Cohen, Seider, & Navia, 2015). Individuals with HAND were associated with lower survival rates(R. J. Ellis et al., 1997). These infected individuals continue to have neuronal loss, cognitive decline, and motor dysfunction (Gray et al., 2014; Guduru et al., 2013; McArthur et al., 2003; Price & Sidtis, 1996; Sevenich, 2018). While HAND has shown to be common among HIV+ individuals (Hong & Banks, 2015; Sacktor et al., 2016), HAD was also on the rise in 1996 (Sacktor et al., 2016).

From 1994 to 2001, the incidence and prevalence of HAND was rising simultaneously with the survival rates for AIDS (McArthur et al., 2003). The rise of cART allowed individuals with HIV to live longer. In 2015, an estimated 47% (454,684) of Americans with diagnosed HIV were aged 50 and older (Cohen et al., 2015). Survival rate increased while the frequency in HAD decreased. Individuals that are currently on cART therapy are growing older and are subject to developing age-associated conditions. This presents an opportunity to better understand how HAND and the neuropathogenesis of HIV, including the genome, viral entry and replication, play a role in the CNS. These discoveries could ultimately lead to primary and secondary intervention strategies for HIV-1 infected individuals with HAND.

2.2 The HIV-1 Genome

In order to improve care and prognosis of HIV-1 infections, we need to understand the structure of the virus and how these components relate to HIV-Associated Neurocognitive Disorder (HAND). HIV-1 is grouped in the genus Lentivirus within the family of *Retroviridae* family of viruses and its genome is composed of a single stranded RNA. HIV-1 is a neurotropic virus that infects macrophages and microglia in CNS causing inflammatory and/or neurotoxic host responses within the CNS (Atluri et al., 2015). The genome encompasses nine genes encoding for proteins that will play significant roles in the lifecycle of HIV-1 (Seelamgari et al., 2004). These genes are separated into three different categories such as structural genes (Gag, Pol and Env), regulatory genes (Rev and Tat) and essential accessory genes (Vif, Vpr, Nef, and Vpu) (German Advisory Committee Blood, 2016).

2.2.1 Structural Genes

Structural genes code for the fibrous proteins that maintain the shape of the virus. There are three essential structural genes in HIV-1 referred to as Gag, Pol, and Env(Price et al., 1994). Gag (Group specific antigens) associates with the plasma membrane, where virus assembly takes place (Lu, Heng, & Summers, 2011). It contains precursor proteins like p55 that provide structure to the virus and the protects viral RNA. It is processed to p17 (Matrix), p24 (Capsid), p7 (Nucleocapsid), and p6 proteins. The Pol (Polymerase) gene encodes the viral enzymes responsible for replication such as protease (PR), reverse transcriptase (RT), and integrase (IN) (German Advisory Committee Blood, 2016). These have become key therapeutic targets in ART. By inhibiting these three enzymes, cART has become successful in suppressing viral load in infected

individuals(Leibrand et al., 2019). The Pol gene is responsible for gag processing, reverse transcription and integration of pro-viral DNA (Jeang, 2007). An envelope glycoprotein (Env) is synthesized as a precursor glycoprotein (gp160) and cleaved into the external glycoprotein gp120 and transmembrane glycoprotein gp41 (Fenner & White, 1976). Env-gp41 mediates virus-cell membrane fusion, whereas Env-gp120 binds to receptor CD4 and co-receptor CCR5 or CXCR4. The coreceptor binding initiates conformational changes in the HIV-1 envelope glycoproteins that facilitate the fusion of viral and cell membranes CD4 receptors as well as the initiation of viral entry (Wilen, Tilton, & Doms, 2012).

2.2.2 Regulatory Genes

Regulatory proteins are responsible for controlling the synthesis of proteins and influencing gene transcription. The Regulator of expression of virion proteins (Rev) and Transactivator of HIV gene expression (Tat) are two significant regulatory genes in HIV-1. Rev acts by binding to the Rev responsive element (RRE) on the nuclear membrane. Rev is known for promoting the nuclear export, stabilization, and utilization of the viral mRNAs containing the RRE nuclear protein. Tat mediates the transcriptional initiation and elongation of the virus. Tat also increases the processivity of RNA polymerase to elongate RNA transcripts.

Tat, a regulatory gene in HIV-1, has shown to be present in the CNS receiving cART (Mediouni et al., 2012). ART does not block the secretion of Tat (Mediouni et al., 2012). It has been found that Tat uses a non-saturable mechanism to cross the BBB, and regions such as the hypothalamus, occipital cortex, and hippocampus were highly permeable to the protein (Banks, Robinson, & Nath, 2005; Clark, Nava, & Caputi, 2017). Neurons take up Tat protein and

consequently promote neuroinflammation (Clark et al., 2017). The more time and proliferation the virus has in macrophages and microglial cells, the more neuroinflammation develops.

2.2.3 Accessory Genes

Accessory genes are important for efficient viral replication, virus budding, and pathogenesis (German Advisory Committee Blood, 2016). Negative factor (Nef) is essential for efficient viral spread and disease progression *in vivo*. Nef has been shown to downregulate CD4, and MHC class I molecules. This downregulation helps the gene increase viral infectivity within the host. Viral infectivity factor (Vif) is another accessory gene that induce ubiquitination of host targets (Collins & Collins, 2014). Vif has shown to cause G2 arrest of HIV-infected cells (J. Wang et al., 2007; J. Wang et al., 2008). It promotes infectivity of the virus by proteasomal degradation. It degrades the host antiviral factor, A3G, by marking it for destruction through the ubiquitin/proteosome system (Mehle, Goncalves, Santa-Marta, McPike, & Gabuzda, 2004; J. Wang et al., 2011; Yu, Xiao, Ehrlich, Yu, & Yu, 2004).

Viral protein R (Vpr) targets the nuclear import of pre-integration complexes, arrest of G2 cell growth, transactivation of cellular genes, and induction of cellular differentiation and apoptosis. Viral protein U (Vpu) enhances virion release and degrades CD4 in endoplasmic reticulum (German Advisory Committee Blood, 2016). During oxidative stress in microglia cells, Nef and Vpr produce pro-inflammatory cytokines (Vilhardt et al., 2002).

2.3 Virus Entry and Replication

HIV-1 replication is a multistep process that begins with viral entry in a permissive host cell such as CD4+ T lymphocytes or macrophages (Kurth & Bannert, 2010). CD4+ T cells express CD4 and coreceptor CXCR4 while macrophages on the other hand express CD4 and coreceptor CCR5. Entry of HIV-1 into the cell requires the presence of CD4+ receptors and coreceptors on the cell surface (Fig 1). These receptors interact with protein complexes which are embedded in the viral envelope. These complexes are composed of two glycoproteins, an extracellular gp120 and a transmembrane gp41 (Wilen et al., 2012). When HIV approaches a target cell, gp120 bind to the CD4 receptors and viral attachment to promote further binding to the coreceptor. Coreceptor binding results in a conformational change in gp120(Wilen et al., 2012). This allow gp41 to unfold and insert its hydrophobic terminus into the host membrane. Gp41 then folds back on itself, this draws the virus towards the cell and facilitates the fusion of their membranes(Nisole & Saib, 2004). The viral nucleocapsid enters the cell and breaks open and releases two viral RNA strands and three replication enzymes, protease (PR), reverse transcriptase (RT), and integrase (IN)(German Advisory Committee Blood, 2016).

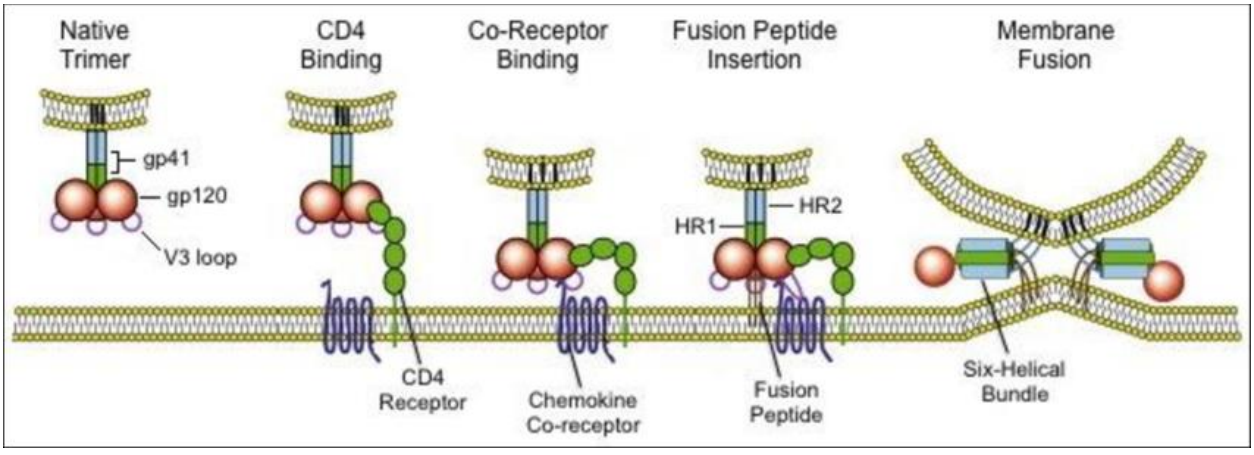

Figure 1: Schematic representation of HIV-1 cell binding and membrane fusion
(Tilton & Doms, 2010)

Once inside the cell, RT begins the reverse transcription of viral RNA. It has two catalytic domains, the ribonuclease H active site and the polymerase active site. Here single stranded viral RNA is transcribed in an RNA/DNA double helix. Ribonuclease H breaks down the RNA, the polymerase then completes the remaining DNA strand to form a DNA double helix. Integrase cleaves a dinucleotide from each 3' end of the DNA creating two sticky ends that gets transferred into the cell nuclease and facilitates integration into host cell genome. Activation of the cell induces transcription of integrated viral DNA into mRNA.

The final step in HIV-1 life cycle is assembly/budding. The viral mRNA migrates into the cytoplasm where building blocks for new virus are synthesized (Nisole & Saib, 2004). Some of them have to be processed by the viral protease. Protease cleaves longer proteins into smaller core proteins. This step is crucial to create an infectious virus. Two viral RNA strands and the replication enzymes then come together, and core proteins assemble around them to form the capsid. This immature viral particle leaves the cell and acquires a new envelope of host and viral proteins. The virus matures and becomes ready to infect other cells. HIV has a high replication speed of about $1x10^{10}$ virions per day, destroying the host immune cells and causing disease progression. HIV replicates rather efficiently and produce new copies of virus or release viruses from infected cells. The virus will be released and proliferate more virus particles that could infect by standing CD4+ T lymphocytes or macrophages.

2.4 HIV-1 Infection in the CNS

The blood brain barrier (BBB) is able to control and restrict cell movement from the peripheral blood in the PNS to the CNS (Ginhoux, Lim, Hoeffel, Low, & Huber, 2013; Joseph,

Arrildt, Sturdevant, & Swanstrom, 2015). A major advantage of the Human Immunodeficiency Virus Type-1 (HIV-1) is its ability to enter the central nervous system (CNS) and cause neurodegeneration. HIV has a neurological effect due to its ability to bypass the BBB, a diffusion barrier that protects the brain from damage(Atluri et al., 2015; McArthur et al., 2003). This advantage emphasizes the importance as to why the genome and viral entry of this virus are carefully studied. The coreceptor binding of CD4+ T lymphocytes, tissue macrophages, and blood monocytes allows the virus to hijack immune cells. Once it integrates it viral genome into the host, HIV-1 is able to establish latency and travel around several anatomical sites. In other words, these immune cells become viral reservoirs for the virus. Macrophages are large phagocytes that are found in tissues around the body including lungs, liver, and bone marrow (Ovchinnikov, 2008). They are also referred to as antigen presenting cells (APCs). Monocytes are simply the precursor of macrophages. Once these immune cells are infected, they can travel across the BBB and enter the brain parenchyma (Fig.2). The brain parenchyma is composed of neurons and glial cells. As the infected monocytes enter the CNS, they differentiate into mature infected macrophages which release infectious virus that are able to infect bystander cells such as macrophages, microglia, and a small percent of astrocytes (Williams et al., 2014). Additionally, infected cells can release viral proteins, Tat and gp120 (Mallard & Williams, 2018). These viral proteins activate the surrounding resident CNS cells which produce proinflammatory cytokines, including TNF-α, IL-1β, and IL-6. Chemokines such as CCL2 and CXCL12 are also produced in this process and this increase promotes additional influx of uninfected and HIV infected monocytes into the brain. The cycle of chronic inflammation within the CNS quickly follows.

In the presence of HIV, neurons are the most affected cells in CNS but they are not infected by the virus (R. J. Ellis, Calero, & Stockin, 2009; Monteiro, Almeida, & Tavares, 2001) Thus, the

model describes that the combination of viral and host factors trigger neuronal damage. This evolves to neurodegeneration, the hallmark of HAND pathology (Gray et al., 2014; Williams et al., 2014). Since the BBB does not allow easy transfer of foreign molecules, treatments for these infections become challenging (Ding et al., 2014; Guduru et al., 2013; Pilakka-Kanthikeel, Atluri, Sagar, Saxena, & Nair, 2013).

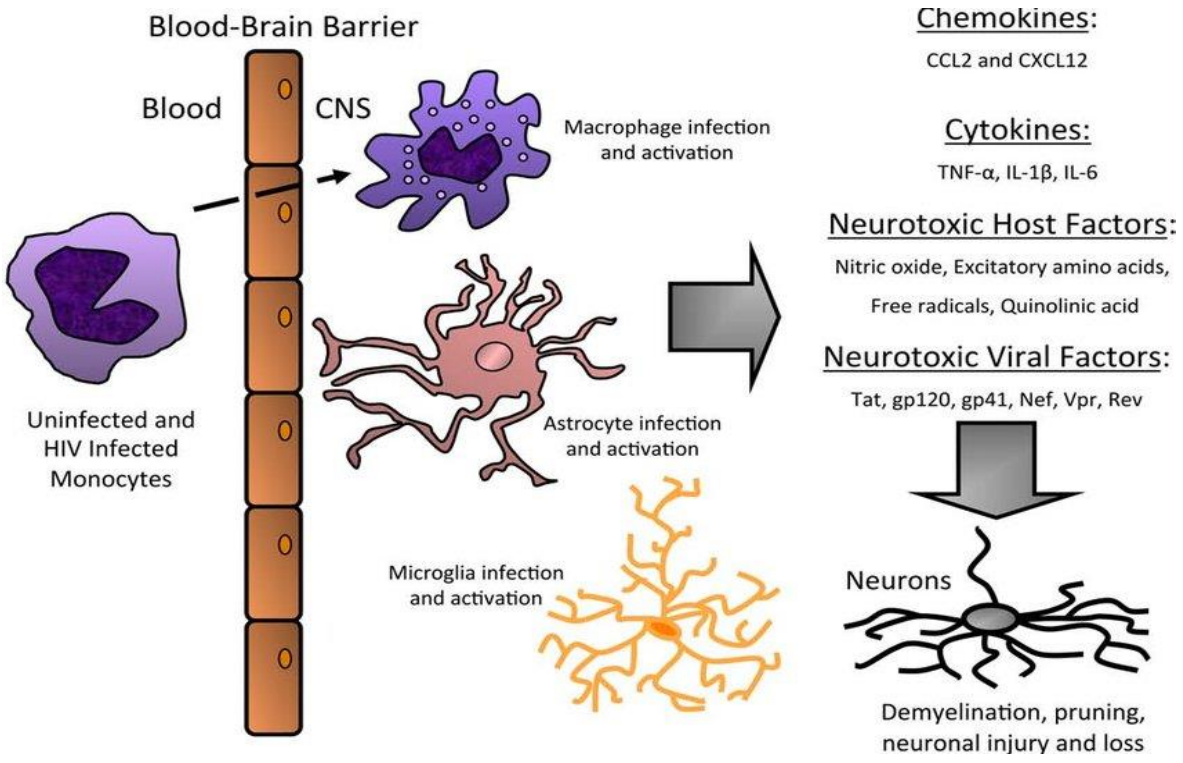

Figure 2: Schematic Representation of Mechanisms of CNS HIV Infection and Damage
(Williams et al., 2014)

2.5 HIV-1-Associated Neurocognitive Disorders

HIV-1 Associated Neurocognitive Disorders (HAND) describes the range of neurocognitive dysfunction associated with HIV-1 infection. It is classified as a spectrum of

neurocognitive impairment ranging from a severity of clinical symptoms(Antinori et al., 2007). It is common for individuals with HAND to have cognitive impairment in motor skills, cognitive speed, verbal fluency, learning memory, and executive function. From least to most severe disorder there is: asymptomatic neurocognitive impairment (ANI), mild neurocognitive disorder (MND), and HIV-associated dementia (HAD) (Saylor et al., 2016). Cohort studies from the U.S. identified 33% had ANI, 12% with MND, and 2% of individuals with HAD (Heaton et al., 2010). ANI, MND, and HAD have criteria that need to be satisfied prior to their clinical diagnosis; patients get their cerebrospinal fluid (CSF) biomarkers analyzed, neuroimaging with MRIs, as well as neuropsychological testing (Ances & Ellis, 2007). Cases of HAD involve a loss in concentration along with slower motor activity among patients (Navia, Jordan, & Price, 1986).

Despite advancements achieved with cART implementation, there is still no specific treatment for HAND. Although the disorders remain stable during cART, they do not resolve overtime (Saylor et al., 2016). Even after instituting cART to individuals that had HAND, the effects from neurocognitive disorders persisted (Heaton et al., 2010; Tozzi et al., 2007). Ultimately, we need alternative antiretroviral agents that can bypass the BBB or improved CNS penetration properties that could serve as effective treatment approaches for HAND.

3.0 Microglia

3.1 Origin and Development of Microglia

Microglia are one of the most important immune cells in the brain (Szalay et al., 2016). These cells are non-neuronal and reside in both the central and peripheral nervous system. The development of microglia begins at an early prenatal stage, with neuronal circuit building, to the postnatal stage, where synapse elimination is present (Ginhoux et al., 2013). It was once believed that microglia stem from neuroectoderm (Piriou, Fedoroff, Jeanjean, & Bercis, 1997), but recent studies have reported that microglia migrates from the periphery with a mesoderm origin (Kettenmann, Hanisch, Noda, & Verkhratsky, 2011). Microglia cells are developed from a yolk sac and enter the brain during early development to gradually occupy defined territory across the CNS (Ginhoux et al., 2013; Ko et al., 2019; Mildner et al., 2007). Such early neonatal development of microglia suggests that they mature with neurons, thus contributing to the wiring of the CNS (Nissen, 2017).

3.2 Role in The Central Nervous System

Microglia are found within both the brain and the spinal cord (Ginhoux et al., 2013). In a healthy and developed brain, resting microglia are mobile so they are able to provide extensive and continuous surveillance for any disturbances in the homeostasis of the brain (Fernandez-Arjona, Grondona, Granados-Duran, Fernandez-Llebrez, & Lopez-Avalos, 2017). This

surveillance monitors synaptic functions and determines the "wiring" of the brain (Wake, Moorhouse, Jinno, Kohsaka, & Nabekura, 2009). If this "wiring" system is disturbed, there will be an excess of excitatory synapses that contribute to neurodegeneration. In addition to their constant surveillance, microglia cells are also responsible for removing dead cells and debris in the CNS.

Resting microglia presents a highly ramified morphology with fine cellular processes(Fernandez-Arjona et al., 2017; Kettenmann et al., 2011). They contain fine branches that produce chemotactic responses— dependent on purine receptors and connexin hemichannels— to the site of lesion or infection (Davalos et al., 2005; Nimmerjahn, Kirchhoff, & Helmchen, 2005). They react to any dysfunction relating to aging, pathology, or neurodegeneration (Fernandez-Arjona et al., 2017; Filiano, Gadani, & Kipnis, 2015; Perry & Teeling, 2013). Any pathologic event in the brain will trigger microglia to activate and cause neuroinflammation in the brain. In 1968, a facial nerve injury model recognized the process of synaptic stripping. Microglia has the ability to physically contact injured neurons, while also being about to remove damaged cells and dysfunctional synapses (Blinzinger & Kreutzberg, 1968). This immune cell represents a critical cellular component in mediating CNS inflammation to ultimately reduce neurodegeneration and shape the synaptic connections.

3.2.1 Microglia Activation and Neuroinflammation

Microglial activation is triggered by Toll-like receptors (TLRs), scavenger receptors, cytokine and chemokine receptors. They become primed by systemic inflammation or neurodegeneration; the priming is caused by changes in the microenvironment. Unfortunately, this priming process makes microglia become susceptible to secondary inflammatory stimulus. Once

activated, microglia have the capability to shift their functional phenotype during the inflammatory response (Graeber, 2010; Stout et al., 2005). These cells gradually alter their morphology into a fully phagocytic form.

In addition to an altered morphology, activated microglia is characterized by high secretion levels of signaling proteins such as chemokines, cytokines, interferons, and excitotoxins (Strunecka, Blaylock, Patocka, & Strunecky, 2018). Activated microglia mediate immune responses in the central nervous system, similar to that of macrophages. Upon exposure to proinflammatory cytokines, IFN- γ and TNF- α, macrophages and microglia that are infiltrating from the circulation become polarized towards a pro-inflammatory (M1) phenotype (Villalta, Nguyen, Deng, Gotoh, & Tidball, 2009). These cells then produce pro-inflammatory cytokines (TNF-α, interleukin (IL)-1β, IL-12). The classically activated, M1 phenotype are activated by pro-inflammatory cytokines, IFN-gamma or TNF-α, while the alternative, M2, phenotype is stimulated by anti-inflammatory cytokines, IL-4 and TGF-ß (Sochocka, Diniz, & Leszek, 2017; Walker & Lue, 2015). M1 have a role in the defense against pathogens through these pro-inflammatory signaling proteins (Colton, 2009). M2 microglia, on the other hand, have anti-inflammatory cytokines that function to control the response of pro-inflammatory proteins (Opal & DePalo, 2000). The challenge with a strong classical microglia activation is the potential for overexpression which will lead to the progression of neurodegeneration (Sochocka et al., 2017). Recent studies proposed that when the brain is absent of microglia, the brain results in more neuronal injury (Szalay et al., 2016). Thus, it is plausible to speculate that skewing the M2 phenotype to M1 might be an interesting therapeutic tool to control inflammation levels and delay or avoid neurodegeneration.

4.0 HIV-1 Pathogenesis and Microglia

4.1 Role of Microglia in HIV-1 Infection

As the predominant resident macrophage in the CNS, microglia cells act as a first line of defense against neural infection and are productively infected by HIV-1 (Garden, 2002). These cells are a target of HIV-1 due to their significant role in the active immune defense for the CNS (Szalay et al., 2016). Microglia express HIV-1 co-receptors CCR5 and CXCR4, and CCR3, these cells are permissive to HIV-1 infection (Albright et al., 1999). Neurons and astrocytes do not have the CD4+ receptor necessary for HIV-1, thus they are not permissive (Gendelman, Lipton, Tardieu, Bukrinsky, & Nottet, 1994). Despite antiretroviral therapies, reservoirs of HIV-1 persist in the body and the CNS has been found to potentially act as a significant reservoir for the virus (Gray et al., 2014).

4.2 Role of Microglia derived Inflammatory Cytokines and Chemokines in HAND

Microglia are thought to play a potential role in HAND related symptoms due to its change in activation state upon HIV-infection. Chronic neurodegenerative diseases were shown to be characterized by high levels of chemokines and cytokines that contribute to disease progression (Smith, Das, Ray, & Banik, 2012; Sochocka et al., 2017). There are also proinflammatory and anti-inflammatory cytokines involved with infection, IL1- ß being one of the many cytokines that get increased in the CNS during lentiviral infection (Chivero et al., 2017). Cytokines such as tumor

necrosis factor, TNF-α, and interleukin-1, IL-1ß, and IL-6, have been shown to be critical pro-inflammatory factors produced by activated microglia in the context of HIV infection (Kany, Vollrath, & Relja, 2019). TNF-α is elevated both in the brain and CSF in HAD patients (Grimaldi et al., 1991).

From previous findings in our laboratory, we concluded that various miRNAs can activate or suppress transduction pathways downstream of cytokines and chemokines(Venkatachari et al., 2017). Candidate microRNAs- miR-let-7a, miR-124, miR-20a and miR-106b were identified by comparing miRNA and mRNA profiles between HIV-1 positive patients with or without HAND by using TimePath and Ingenuity Pathway Analysis (IPA) based canonical pathway. In this study, my goal is to evaluate the role of these specific candidate miRNAs on inflammatory response of microglia during HIV-1 infection. MiR-let-7a and miR-124 were predicted to target chemokine signaling for IL-8, CCL2 and CXCR4. This signaling is done through RHOG, JUN, MAPK3 and RAF1 signal transduction process. MiR-20a and miR-106b, on the other hand, target cytokines such as TNF-α, IL1β and IL-6 through DUSP2, NOTCH1 and FOXO1 signal transduction processes. These findings concluded some neuron-protective role of these miRNAs by reducing cytokine induced neuronal dysfunction and inhibiting the chemotaxis of inflammatory immune cells in CNS.

4.3 Role of Non-coding RNAs

Gene expression is regulated at transcriptional, post-transcriptional, and post-translational stages (Hube & Francastel, 2018). A non-coding RNA (ncRNA) is a functional RNA molecule that is transcribed from DNA but not translated into proteins (Hube & Francastel, 2018). These

ncRNAs function to regulate gene expression at the transcriptional and post-transcriptional level (Cannell, Kong, & Bushell, 2008; C. Wang, Ji, Cheng, Chen, & Bai, 2014). Thus far, seven different types of ncRNAs were identified: small interfering RNA (siRNA), micro RNA (miRNA), long non-coding RNA (lncRNA), small nuclear RNA (snRNA), small nucleolar RNA (snoRNA), interference RNA (RNAi), and circular RNA (circRNA). MicroRNAs, and their target genes, have been shown to play a critical role in the innate inflammatory responses mediated by immune cells (Khoshnam, Winlow, & Farzaneh, 2017).

4.3.1 MicroRNAs (miRNAs)

A microRNA (miRNA) is a short non-coding RNA (22 nucleotides) that regulates gene expression pathways post-transcriptionally (Macfarlane & Murphy, 2010). They play an integral role in numerous biological processes, including the immune response, cell-cycle control, metabolism, viral replication, stem cell differentiation and human development. These miRNAs function via base-pairing with complementary sequences within messenger RNA (mRNA) molecules. Various proteins are associated with miRNAs that allow them to function in their environment. Some of these proteins include RNase III-type, dsDNA binding, and argonaute protein. Ultimately, these microRNAs work with various proteins in the nucleus and cytoplasm to maintain the homeostasis of cellular interactions, cell proliferation, cell differentiation, and regulate inflammatory factors.

A microRNA gene is first transcribed as part of a much larger primary transcript and it's transcribed by the Pol II polymerase, the same polymerase that makes the messenger RNA. While still in the nucleus, they are synthesized from pri-miRNAs in two stages by the action of two RNase III-type proteins— Drosha in the nucleus and Dicer in the cytoplasm. Protein such as Drosha and

GRCR8/Pasha are part of the “microprocessor” protein complex. DGCR8 (in human) or Pasha (Drosophila) are dsRNA binding proteins and act as cofactors for Drosha (Luhur, Chawla, Wu, Li, & Sokol, 2014). Drosha cleaves one helical turn from the base of the hair pin. The single stranded RNA then forms a hairpin secondary structure for the pre-miRNA before it matures and exits the nucleus with the help of exportin 5 complex.

Once this pre-miRNA is in the cytoplasm it will come into contact with an enzyme that will cleave the duplex RNA strand, this enzyme is called Dicer. Dicer will then cleave off the loop and form a pre-microRNA duplex. One of the strands of this duplex would be loaded into an argonaute protein to make the silencing complex. Exactly which strand goes into the silencing complex and how that happens is still unknown. One may speculate that the strand that goes into the duplex is the one that has the least stable pairing at its 5’ end. Most of the time, there’s a strong propensity for one of these strands to make it into the silencing complex and from there the miRNAs assemble into RNA-induced silencing complexes (RICS), activating the complex to target messenger RNA (mRNA) specified by the miRNA (Macfarlane & Murphy, 2010). They generally bind to the 3'-UTR (untranslated region) of their target mRNAs and repress protein production by destabilizing the target messenger RNAs (mRNAs) and hybridizing to multiple binding sites (Shirdel, Xie, Mak, & Jurisica, 2011).

Common miRNAs in multiple lineage target and regulate basic cell functions such as growth, differentiation, and apoptosis. There are also cell and tissue specific miRNAs identified. Many miRNAs are regulated by external factors and conditions such as viruses, cancer, or pathogens. The role of miRNA in transcriptional regulation involves in the post-transcriptional regulation of gene expression. They are important in development, growth, immune response

which contributes to their major role in cancer development and other diseases. Recent studies are aiming to use miRNAs as biomarkers and potential therapeutics.

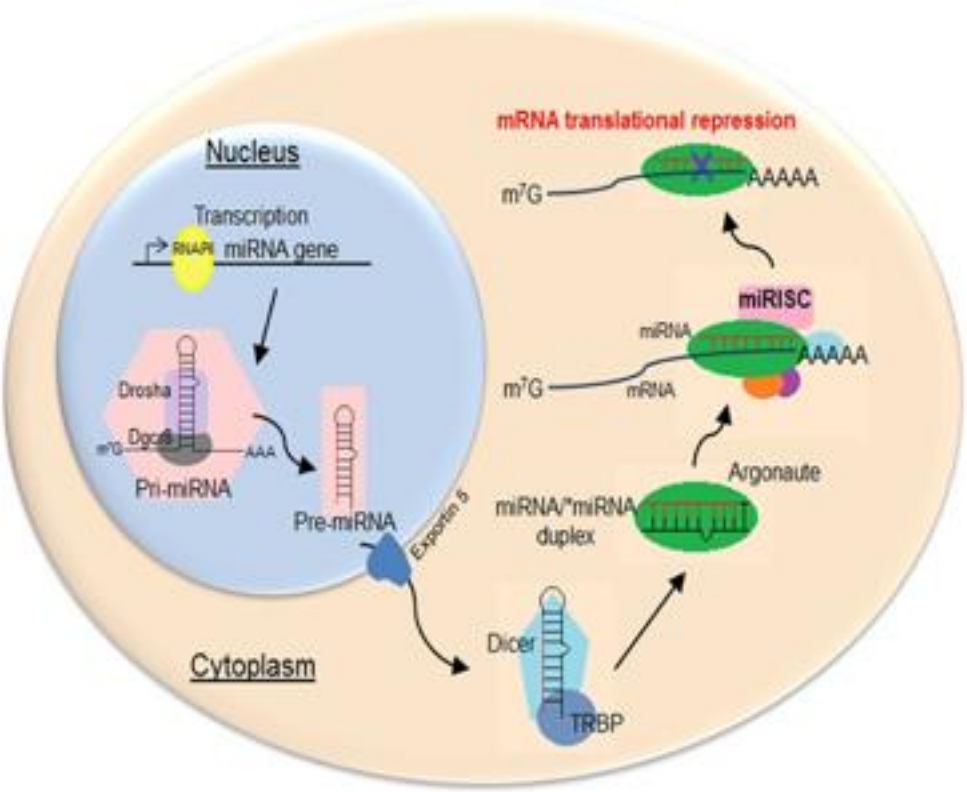

Figure 3: Schematic representation of miRNA biosynthesis
(Hajarnis, Lakhia, & Patel, 2015)

4.3.1.1 miRNA-let-7-a (miRNA-141)

The lethal-7 (let-7) miRNA family, the first miRNA family identified in humans, are abundantly expressed in the brain and exhibit high cross-species sequence conservation (Pasquinelli et al., 2000; Pena et al., 2009). Let-7 miRNAs are the target family because they have shown to regulate cell differentiation, tumor suppression, and suppress LPS-induced apoptosis (Buonfiglioli et al., 2019; Cho, Song, Oh, & Lee, 2015). Ultimately, the miRNA let-7 family controls post-transcriptional gene regulation of innate immune responses. miRNA-Let-7a, specifically, has the potential to act as a regulator for microglia function during inflammation. It

has shown to down regulate of IL-6 and IL-10 cytokines but up-regulation of miR-Let-7a in microglia promotes anti-inflammatory factors and protects microglia against apoptotic damage. (Cho et al., 2015).

4.3.1.2 miRNA-124

miRNA-124 accounts for 25% to 48% of all brain miRNAs (Lagos-Quintana et al., 2002). Over-expression of miR-124 has shown to attenuate the LPS-induced expression of pro-inflammatory cytokines and promote the secretion of neuroprotective factors (Yao et al., 2018). It accomplishes this by regulating the MEKK3/NF-κB signaling pathways. MEKK3 belongs to the protein kinase superfamily and mediates NF-κB transcriptional regulators. NF-κB is a protein complex that controls transcription of DNA, cytokine production and cell survival (Gilmore, 2006). MiR-124 has a potential therapeutic target for regulating the inflammatory response.

4.3.1.3 miRNA-106b

miR-106b is a member of the miR-106b-25 family. Numerous studies have reported that miR-106b acts as an oncogene and regulates tumor viability, cell migration, invasion, and proliferation (Li et al., 2009; Xu et al., 2013; Yin, Chen, Wang, & Zhang, 2019). Phosphatase and tensin homolog (PTEN) is a protein that, in humans, is encoded by the PTEN gene. Mutations of this gene leads to the development of many different cancers, one of them being prostate cancer.

This miRNA downregulates ATP-binding cassette transporter A1 (ABCA1) which is a cholesterol transporter that transfers excess cellular cholesterol onto lipid-poor apolipoproteins. microRNA-106b was identified to significantly decrease ABCA1 levels and impair cellular cholesterol efflux in neuronal cells (Kim et al., 2012). Furthermore, miR-106b dramatically increased levels of secreted amyloid beta (Aβ) by increasing Aβ production and preventing Aβ

clearance. Aβ is the main component of amyloid plaques and is responsible for denoting the peptides involved in Alzheimer's Disease (AD) (Sadigh-Eteghad et al., 2015). Thus, miRNA-106b also downregulates amyloid precursor protein (APP), which is an integral membrane protein that regulates synapse formation and neural plasticity. This explains why miR-106b is downregulated in AD.

4.3.1.4 miRNA-20a

MiRNA-20a is a part of the miRNA-17~92 cluster, which encode for fourteen other miRNAs (Concepcion, Bonetti, & Ventura, 2012). This cluster plays a key role in cell function by inducing enhanced cell proliferation and suppression of apoptosis (Al-Nakhle et al., 2010). MiRNA-20a targets the APP that regulates synapse formation and neural plasticity. When miRNA-20a is overexpressed, it suppresses IL-10 secretion. This would then decreases IL-2, IL-6 and IL8 cytokine production (Reddycherla et al., 2015). These cytokines are known to be regulators of inflammatory responses, which becomes important when we consider their roles in the CNS of HIV-1 infected individuals. This miRNA was also shown to have therapeutic advantages in patients with multiple sclerosis (MS) (Cox et al., 2010), Alzheimer's disease (AD) (Basavaraju & de Lencastre, 2016), and liver carcinoma (Tipanee et al., 2020)

5.0 Hypothesis and Aims

Listed below are the specific aims that were generated to evaluate the regulation of these candidate miRNAs play important role in regulating the production of neuroinflammatory chemokines and cytokines. Our goal is to evaluate the regulation of the specific miRNAs in inflammatory responses using HIV-1 infected microglia cell line, HMC3.

Aim 1: Generate lentivirus expressing miRNAs, titer, and stably transduce microglia cell line (HMC3).

- **A.** Transduce normal microglia cell line (HMC3) with candidate miRNAs using a miRNA expression vector.
- **B.** Confirm transduction efficiency via microscopy, and U87-MG assay.

Aim 2: Investigate how lentivirus expressing miRNAs regulate the expression of inflammatory factors in response to LPS and/or HIV-1 infection.

- **A.** Generate HIV-1 virus through transfection of 293T cells with viral plasmids.
- **B.** Transduce normal microglia cell lines with lentivirus overexpressing miRNAs and confirm successful transduction via fluorescent microscope.
- **C.** Infect one set of transduced microglia cells with the HIV-1.
- **D.** Stimulate a separate set of transduced microglia cells with LPS.
- **E.** Quantify levels of cytokine production using ELISA assay.

6.0 Materials and Methods

6.1.1 Cells

HEK293T, U87MG $CD4^+$ $CCR5^+$ and immortalized HMC-3 microglia (ATCC® CRL-3304) were grown in DMEM (Gibco) supplemented with 10% FCS (HyClone), 1% glutamine (Invitrogen) and 1% penicillin-streptomycin (Invitrogen). All cell lines were maintained at 37℃ and 5% CO_2.

6.1.2 HIV-1 Virus production and titration

HEK293T cells were transfected with 5.0 x 10^6 cells in 10ml media in 10 cm^2 tissue culture-grade plates, which were grown for 24 hours prior to transfection to 80% confluence. Thirty minutes prior to transfection, the media was changed to 5 ml per plate. Before transfection, 5μg of purified neurotropic proviral DNA construct pNL43-YU2-Env with enhanced green fluorescent protein (EGFP) as reporter gene was diluted with 250μl of fresh DMEM. Then, 15μl of Polyjet™ transfection reagent (SignaGen) was diluted with 250μl of fresh DMEM. The Polyjet™ dilution was added to the plasmid DNA dilution and left in room temperature for 20 minutes to encapsulate. After 20 minutes, the mixture was added dropwise to the plate. 16 hours post-transfection, the transfected media was removed, and 7 ml of fresh media was added for incubation for the following 24 hours. 24 hours post-transfection, the supernatants were harvested, and 7 ml of fresh media was added for incubation. This process was repeated for the 72-hour harvest of the supernatant post-transfection.

Virus infectivity was quantified by a U87MG assay using the transfected supernatants. Briefly, 3.0 x 10^4 U87MG cells were seeded in triplicate in a 96-well plate for 24 hours. Once the cultures reached 80% confluence, the cells were infected with 1:10, 1:100, 1:1000, and undiluted quantities of virus and incubated for 16 hours. After 16 hours, the media was changed, and the plate was incubated for another 24 hours. The infected cells were then counted in the optimal well (containing 30-150 stained cells) for each replicate and averaged to yield the measure infectivity in infectious particles (IP) per ml. The formula used for calculating the virus titer: lP = Cn × V × DF.

Cn: The total number of green cells

V: The volume of the inoculum.

DF: The virus dilution factor.

6.1.3 miRNA expressing Lentivirus production and Transduction of HMC3

For lentivirus production, 1x 10^6 HEK293T cells were seeded in 10 cm2 tissue culture-grade plates, which were then grown overnight until the cells becoming adherent and reaching 80% of confluence. Two and three quarters (2.75µg) of miRNA expression vector was mixed with 1.0µg of pLP1which stands for packaging plasmids containing gag and pol, 0.5µg of PLP2 (provide Rev gene) and 0.75µg of VSV-G-Env of vesicular stomatitis virus (VSV), respectively, in 250µl of DMEM. Further, 15µl of Polyjet™ transfection reagent was mixed with 250µl of fresh DMEM first and added to the plasmid mixture. After 15 minutes of incubation, the whole mixture was added dropwise to the HEK293T cells. After 24 hours of incubation, 5ml of fresh media was added to the plate to maintain the cells. 48 hours post-transfection, the supernatant was harvested. Lentivirus titer was further quantified by a U87MG assay using 1:10, 1:100, 1:1000 and undiluted

quantities of virus to infect 1.0 x 10^4 U87MG cells with an incubation period of 24 hours. After 24 hours, the media was changed with fresh media and the plate was incubated for another 48 hours. Infectious particles were counted through a green fluorescent microscope 48- and 72-hour post infection.

For transduction, lentivirus overexpressing miRNA supernatants were added to a 12-well plate of HMC3 microglia cells at a multiplicity of infection between 0.1 – 1.0. These cells were then incubated for 24 hours before replacing the wells with fresh media.

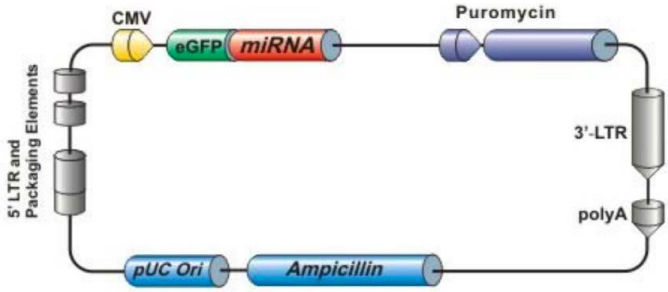

Figure 4: Schematic of miRNA expression vector
The miRNA expression is on a lentivirus backbone with an internal CMV promoter that drives the expression of eGFP and miRNA as a fusion transcript. The lentivirus also includes puromycin resistance gene for selection using puromycin antibiotic.

6.1.4 Cytokine ELISA Quantification

Following infection with HIV-1 or mock infection, cells were treated with LPS (1μg/ml) for 24 hours and the supernatants were collected and analyzed for the presence of TNF-α, IL-1β, and IL-6. Human TNF-α DuoSet ELISA kit (R&D Systems), Human IL-1β DuoSet ELISA kit (R&D Systems) and Human IL-6 DuoSet ELISA kit (R&D Systems) were used for the ELISA analysis. 100μl of 1:60 PBS diluted different capture antibodies were added to a 96-well plate then incubated overnight at 4°C. Next day, the plate was washed three times using wash buffer (0.05% Tween®20 in PBS). Then 100μl of blocking buffer (1% BSA in PBS) was added to each well with

1-hour incubation period at room temperature. After blocking, plates were washed and, dried thoroughly. Harvested supernatants were diluted to an appropriate concentration and then 100µl of each sample was add to each well containing different ELISA capture antibodies. At the same time, an eight-point 2-fold serial dilutions for each cytokine standard was prepared and added to the plate with the highest concentrations ranging from 250pg/ml to 1000pg/ml. Cytokine IL-6 had a maximum of 600pg/ml, TNF-α had 1000pg/ml, and IL-1β had the lowest concentration maximum of 250pg/ml. All the samples and standards were incubated at room temperature for 2 hours. After 2 hours, the plate was washed three times as before, and 100µl detection antibody for each cytokine was diluted to 100 ng/ml in reagent diluent (1% BSA in PBS) then added to each well with an incubation period of 2 hours at room temperature. After 2 hours, the plate was washed three times and Streptavidin conjugated to horseradish-peroxidase (HRP) was then diluted to 1:40 fold in reagent diluent and 100µl was added to each well for 20 minutes.

Tetramethylbenzidine (TMB) substrate SureBlue™ (Seracare Life Sciences Inc.) was used for detection. Equal amounts (50µl) of Substrate Reagent A and Substrate Reagent B were added to each well then incubated for another 20 minutes in dark.

One hundred (100µl) of 2 NH_2SO_4 was then added to stop the reaction and the color intensity was read by ELx800 Absorbance Microplate Reader (Bio-Tek) with KCJunior™ software (Bio-Tek) at 550 nm. Linear trend lines were created from the standard curves with known concentrations and an equation for cytokine calculation were generated by the trend lines with $R2 \geq 0.900$.

6.1.5 Statistical Analysis

Statistics were performed using Graphpad Prism® software (GraphPad Software, Inc.). Comparisons of samples were attempted using a two-sided Student's t-test using a significance level of $p<0.05$.

7.0 Results

7.1 Aim 1: Generate lentivirus expressing miRNAs, titer, and stably transduce microglia cell line (HMC3).

HEK293T cells were seeded and co-transduced with the lentiviral expression vector and a mix of three packaging plasmids, pLP1 (Gag), pLP2 (Rev), and VSV-G-Env. Rev is a transactivating protein that is essential to the regulation of HIV-1 protein expression. Gag, or group specific antigen, is the major structural protein. VSV-G-Env is a vesicular stomatitis virus and it was used to enhance viral entry. Ultimately, within this aim I can conclude that miR-20a and miR106b continue to show the highest level of titer among the other candidate miRNAs. miR-124 also had a high titer but it consistently produced lower titration levels than the other miRNAs. It needed to be concentrated to match the levels of other candidate miRNAs. After this was performed, we observed higher titers in the lentivirus expressing miR-20a, -106b, and -124 (Fig. 5). Scramble served as our negative lentiviral control, while the cells in control were left untransduced thus did not produce any infectious particles. The data shows that all of the candidate miRNAs exhibit higher amounts of infectious particle levels at 48 hours post transduction (hpt) compared to 72 hpt. The representative image visualizes the nuclei (blue) and +eGFP transduced cells (green) from all lentivirus overexpressing candidate miRNAs 72 hpt (Fig. 6). All of these miRNAs displayed traces of +eGFP cells with miR-106b having the highest brightest which shows that the lentivirus was able to infect the HMC3 cells. Puromycin showed to be very toxic to the microglia cell line (HMC3). It proved to be toxic in both levels of puromycin concentration used

(0.25 and 0.50 µg/ml) and was shown to stress out cells as early as 2 days post selection. Thus, this selection process was not continued.

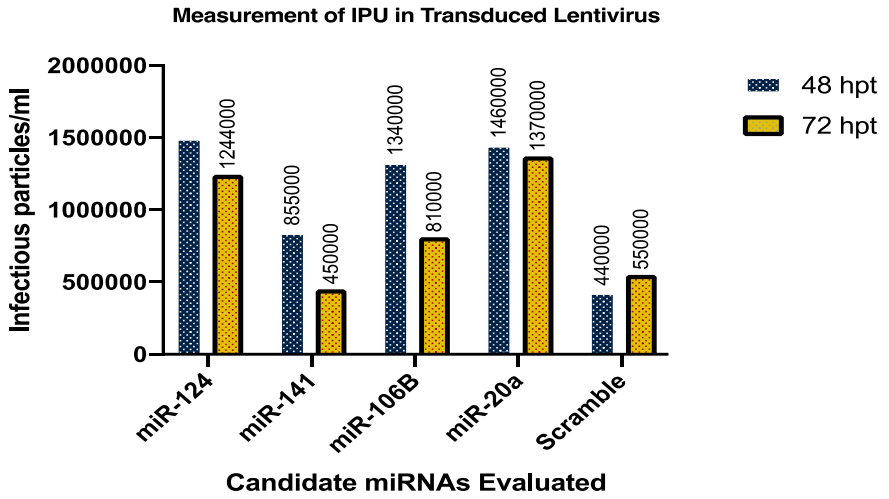

Figure 5: Measurement of Infectious Particle Units (IPU) in Lentivirus Expressing miRNAs
Lentivirus expressing miRNAs were produced by co-transfecting 293T cells with the lentiviral expression vector and a mix of three packaging plasmids. The graph represents the number of infectious particles per mL of the four lentivirus expressing miRNAs and scramble. These titers were measured at 48 hours post-transfection (blue) and 72 hours post transfection(yellow) by counting the number of eGFP+ cells in the lowest lentiviral dilution that were able to infect permissive cells (U87MG, CXCR4+, and CCR5+). MOI of 1.0 for all miRNAs.

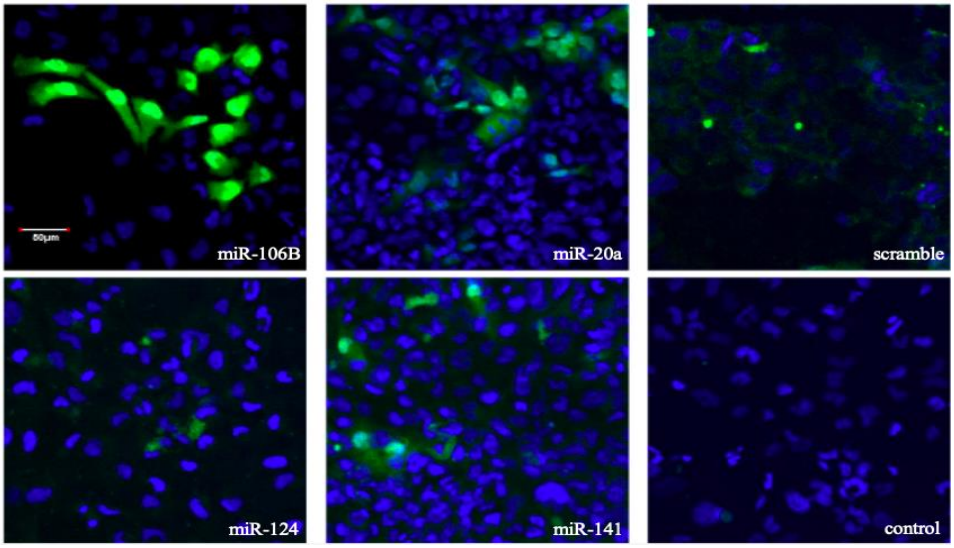

Figure 6: Transduction Efficiency of lentivirus expressing miRNAs in HMC3
The images above were produced by transducing HMC3 cells with the lentiviral expressing miRNAs. The image represents the eGFP+ efficiency of the four lentiviruses expressing miRNAs (green) plus scramble and DAPI stained nuclei (blue). These representative images were taken at 72 hours post-transfection with miR-20a and -106b with a MOI of 1.0 and miR-141, -124, and scramble with a MOI of 3.0.

7.2 Aim 2: Investigate how lentivirus expressing miRNAs regulate the expression of inflammatory factors in response to LPS and/or HIV-1 infection.

The production of an infectious virus stock of YU2-VSV-G was required to stimulate the microglia cell line. The amount of infectious particle units (IPU) the virus stock measured to be 1,500,000 IPU/ml (Fig. 7). This virus stock will be used to infect microglia cells after they are transduced. miR-20a and 106-B have rather higher transduction efficiencies on numerous occasions. Overexpression of miR-106b in microglia cell line HMC3 may have a suppressive effect on IL-1β expression as its levels are decreased after HIV-1 infection. Moreover, the overexpression of miR-106b decreased TNF-α expression, supporting the protective role of this candidate miRNA. There was an increasingly high levels of IL-6 secretion among all candidate miRNAs however from IL-6 within all samples even though they were diluted by 1:50 ratio.

After LPS stimulation, TNF- α and IL-1β levels were measured. miRNA-20a had reduced amounts of TNF produced 24 hours post LPS stimulation (12.14 to 1.43). There was a slight detected of TNF- α 72 hours pre- LPS exposure, but these cytokine levels were not determined in both duplicates 72 hours post LPS (Fig. 8). The 72-hour post stimulation was not determined due to human error, which is depicted as ND*. Overall this miRNA reduced TNF- α levels compared to the lentiviral control (scramble) and the negative control on the right. miRNA-106b had reduced amounts of TNF produced 24 hours post LPS stimulation with a decrease of 16.2-fold change (81.44 to 5.00). There was an increase in TNF- α 72 hours post stimulation but overall it was still less than the lentiviral control, scramble. miRNA-141 had undetectable levels of TNF- α pre- 24 hr, post 24 hr, and pre 72 hours from LPS stimulation (Fig. 8). There was an increase in TNF- α 72-hour post stimulation but these levels were much lower than both controls. TNF- α production

in miRNA-124 showed a small decrease for 24 hours post stimulation (145.71 to 142.14). This miRNA doesn't seem to have a significant reduction in TNF- α levels, which means there are no neuroprotective factors at play with this miRNA. For IL-1β levels, the data shows that miRNA-20a had no increase in IL-1β 24 hours post stimulation with levels being undetectable and/or 0. There was a slight increase 72 hours post stimulation but lower than control and scramble. miRNA-106b had no IL-1β 24 hours post stimulation and no detectable amounts of IL-1β 72 hours post transduction (Fig. 9). miRNA-141 had no detectable levels of IL-1β 24 hours pre and 24 hours post LPS stimulation. There was a huge increase 72 hours pre stimulation, BUT undetectable levels of IL-1β 72 hours post stimulation which is what we care about. miRNA-124 There we no detectable levels of IL-1β pre- LPS stimulation at 24 hours. There was an increase from 0 to 43 pg/ml of IL-1β levels present post stimulation for both 24 hr and 72 hr higher than control and scramble.

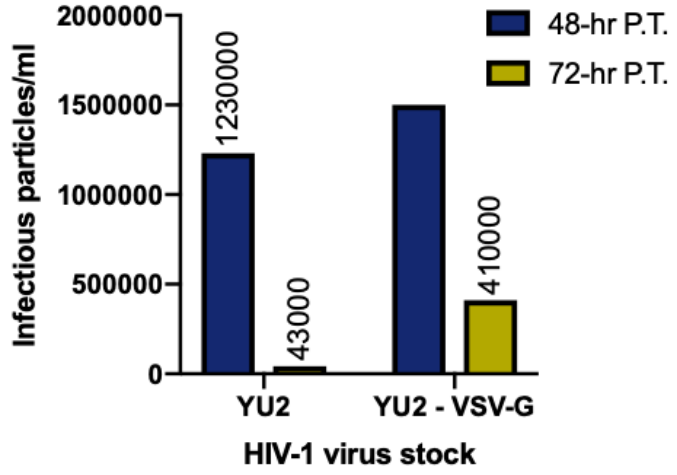

Figure 7: Comparing YU2 and YU-2 VSVG Pseudotyped Viruses
These viruses were produced by transfecting 293T cells with HIV-1 YU2 strain pro-viral construct, with and without VSV-G construct. This graphic represents the titers of YU2 and YU2 -VSVG in permissive cells (U87MG, CXCR4+, and CCR5+). After serial dilutions of virus stocks, titers were measured at 48 hours post-transfection (blue) and 72 hours post-transfection (yellow) by counting the number of GFP+ cells in the lowest dilution.

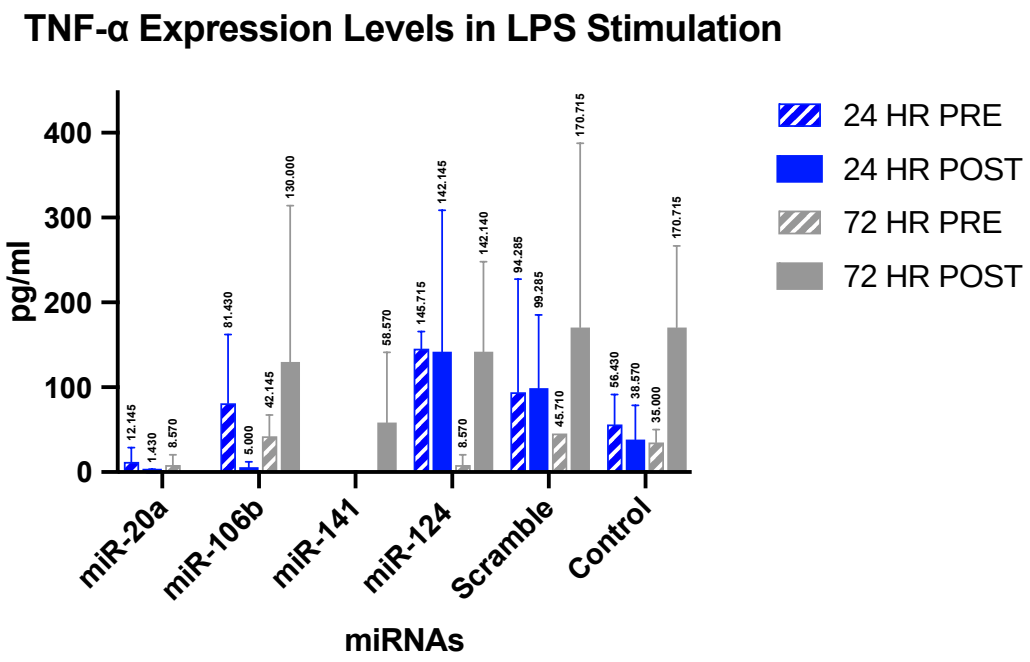

Figure 8: Comparing TNF-Alpha expression in LPS treated cells
HMC3 cells were transduced and overexpressed with candidate lentivirus miRNAs. They were treated with LPS for four hours. Supernatants were collected pre and post treatment at 24- and 72-hours post transduction. Data was analyzed using Two-way ANOVA in PRISM and acquired by performing an ELISA. Data are presented as mean ± SEM (n = 2). *P < 0.05.

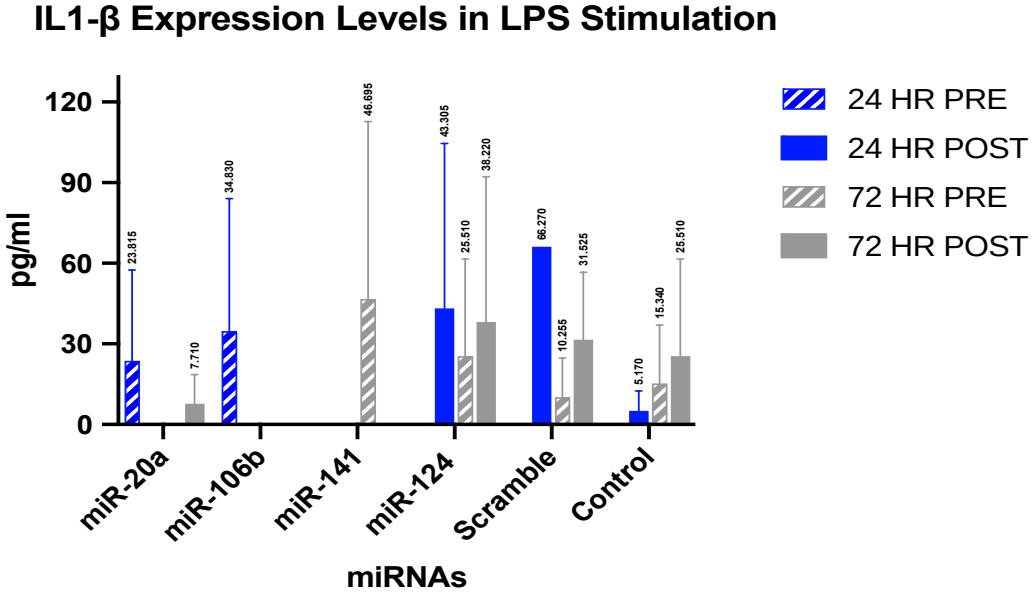

Figure 9: Comparing IL-1Beta expression on LPS treated cells
HMC3 cells were transduced and overexpressed with candidate lentivirus miRNAs. Supernatants were collected pre infection, 24 hpi and 72 hpi. Data was analyzed using Two-way ANOVA in PRISM and acquired by performing an ELISA. Data are presented as mean ± SEM (n = 2). *P < 0.05.

These candidate miRNAs were infected with HIV-1 virus stock and the following cytokines were assessed: TNF- α**,** IL-1β, and IL-6 (Fig. 10-12). miRNa-20a had a slow increase in TNF- α 24 hpi but drops to undetectable levels after 72hpi, supporting a reduction in TNF- α (Fig. 10). miRNA-106b has a slight decrease from pre- to 24 hpi (from 22.86 to 19.285). The 72-hour mark produced undetectable levels of TNF- α. miRNA-141 we see an overall decrease in TNF production in miRNA-141. There were slight levels of TNF- α detected in the pre infection (15.7 pg/ml) but no detectable levels were found post infection for both time points (Fig. 10). miRNA-124 is the only miRNA that had an increase in TNF- α production after infection with HIV-1, but there were undetectable levels of this cytokine at the 72-hour time point. This could be due to the variation of transduction efficiencies among the miRNAs. Over expression of these miRNAs might be distributing the cells and killing them

Notably, IL-6 was diluted 1:50 since the preliminary data observed was outside of the curve (Fig. 11). miR-20a has a high level pre infection, IL-6 levels decreased at the 24 hpi but increased significantly 72 hpi. The 2-way ANOVA analysis identified this specific level as being significant compared to the lentiviral control. miR-106b and miR-141 had similar patterns in IL-6 production. miR-124 exhibited similar patterns but had the lowest levels of IL-6 at the 72 hpi point (Fig. 11).

miRNA-20a had a significant decrease in IL-1β levels, 17-fold decrease specifically, 24 hpi and 0 traces of this cytokine at the 72 hpi (Fig. 12). miRNA-106b and miRNA-141 did not have any detectable trace of IL-1β prior to infection but both had a high spike 24 hpi, still less than scramble levels which reached undetected/zero 72 hpi for both. miRNA-124 had an increase of IL-1β 24 hpi but reached undetectable levels 72 hpi.

From this data presented within this aim, miR-141, miR-106b, and miR-20a showed to downregulate neuroinflammatory cytokines (IL-1β and TNF-α) production in transduced HMC3

cell lines. miR-124 slightly downregulated neuro-inflammatory cytokine IL1β after 72 hours. None of the miRNAs reduced IL-6 production and no therapeutic effects were observed. Puromycin is very toxic to the microglia cell line (HMC3). Therefore, transduction with high MOI (>60-80% EGFP positive) was performed followed by LPS or HIV-1.

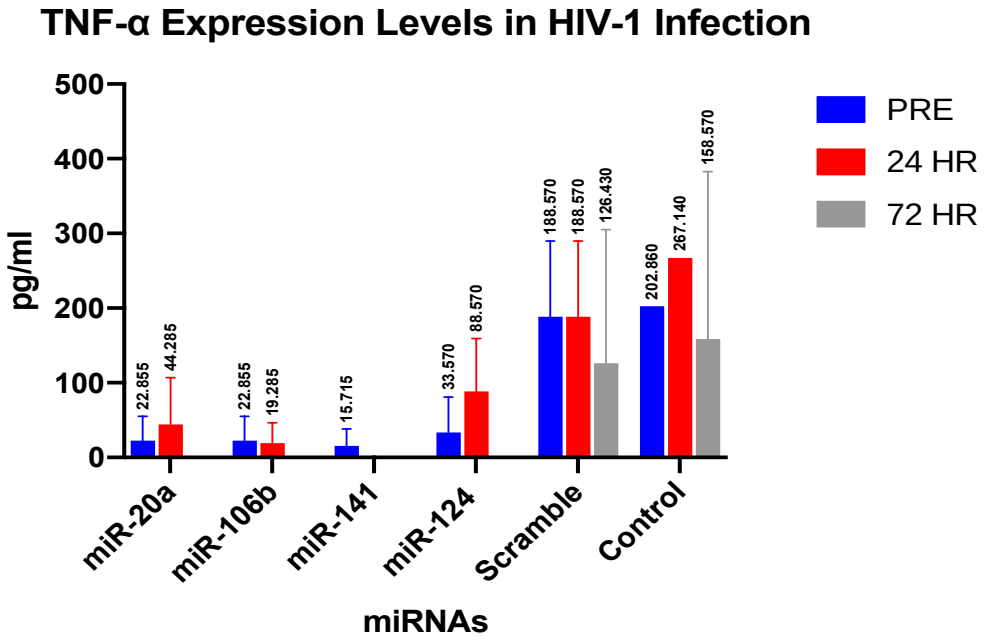

Figure 10: Comparing TNF-Alpha expression on HIV-1 treated cells
These HMC3 cells were transduced and overexpressed with lentivirus miRNAs. They were infected with HIV-1. Supernatants were collected pre infection, 24 hpi and 72 hpi. Data was analyzed using Two-way ANOVA in PRISM and acquired by performing an ELISA. Data are presented as mean ± SEM (n = 2). *P < 0.05.

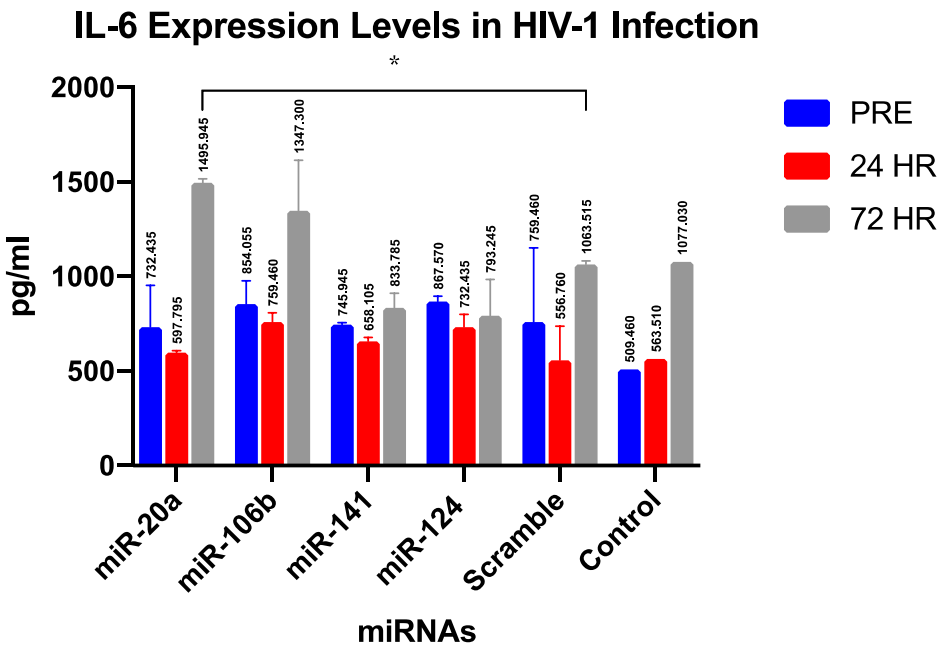

Figure 11: Comparing IL-6 expression on HIV-1 treated cells
These HMC3 cells were transduced and overexpressed with lentivirus miRNAs. Supernatants were collected pre infection, 24 hpi and 72 hpi. These samples were diluted using a 1:50 ratio. Data was analyzed using Two-way ANOVA in PRISM and acquired by performing an ELISA. Data are presented as mean ± SEM (n = 2). *P < 0.05.

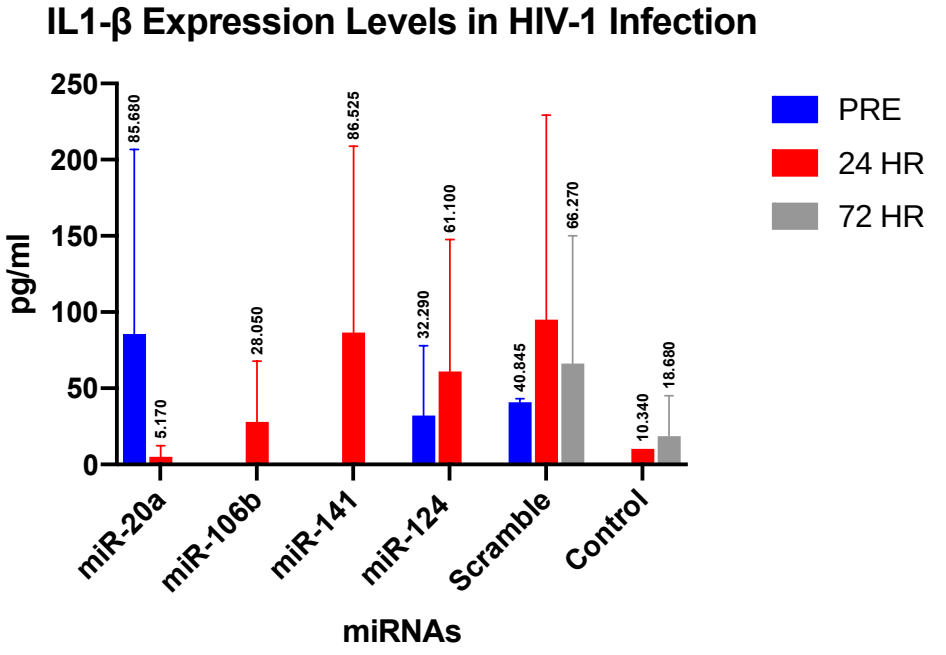

Figure 12: Comparing IL-1Beta expression on HIV-1 treated cells
These HMC3 cells were transduced and overexpressed with lentivirus candidate miRNAs. Supernatants were collected pre infection, 24 hpi and 72 hpi. Data was analyzed using Two-way ANOVA in PRISM and acquired by performing an ELISA. Data are presented as mean ± SEM (n = 2). *P < 0.05.

8.0 Conclusion

In conclusion, candidate miRNAs play an important role in regulating the production of specific neuroinflammatory chemokines and cytokines. Successful generation of the lentivirus expressing miRNAs were established and stably transduced the microglia cell line, HMC3. Each lentivirus expressing miRNAs had different titer levels after incorporating the appropriate plasmids. This investigation was conducted to ultimately suppress/modulate the expression of pro-inflammatory cytokines that are dysregulated by HIV-infection. To prove this, HIV-1 infection dysregulates miRNA expression, thus altering the expression of host cellular factors that are either neurotoxic or neuroprotective in CNS.

In this study, miRNAs have been introduced and tested to control HAND pathogenesis by reducing specific cytokines and chemokines that are critical in neuropathogenesis of HAND. We were able to modulate the pro-inflammatory response to a more anti-inflammatory response in some candidate miRNAs within select cytokines. We found that candidate miRNAs play an important role in regulating the production of specific neuroinflammatory chemokines and cytokines. The following microRNAs can downregulate IL-1β and TNF-α production in transduced HMC3 cell lines: miR-141, miR-106b, and miR-20a. This supports the idea that the regulation of these candidate miRNAs in inflammatory responses can reduce pro-inflammatory cytokine production during HIV-1 infection. MicroRNA-124 slightly downregulated neuro-inflammatory cytokine IL1β after 72 hours. Candidate miRNAs did not show neuroprotective effects on IL-6 after HIV-1 infection, suggesting that there are no therapeutic effects or regulation with this specific cytokine. We can speculate that this due to the response pattern of each miRNA during LPS stimulation, our positive control.

Ultimately, this evidence can serve to support further efforts in controlling the neurodegenerative comorbidities and cognitive impairment caused by HAND on HIV-1 infected individuals. Within our investigation, certain miRNAs displayed therapeutic potential by downregulating specific proinflammatory cytokines. Understanding the therapeutic effects of these cellular miRNAs dysregulated by HIV-1 infection in microglia cells may provide information for the development of additional antiviral strategies.

9.0 Discussion

Microglia represent a critical cellular component in mediating CNS inflammation to ultimately reduce neurodegeneration and shape the synaptic connections. As the resident macrophage in the CNS, microglial cells activate in the presence of HIV-1 infection and contributes to neuroinflammation. The role of candidate miRNAs was evaluated in transduced microglia cell line. The results suggested that miR-20a and -141 have the ability to reduce levels of TNF-α production as early as 24 hours following a 4-hour LPS stimulation (Fig. 8). Similar results were seen in IL-1β expression levels but through different candidate miRNAs. The results for IL-1β production demonstrated a reduction of cytokine levels by miR-20a and -106b (Fig. 9). After a 4-hour LPS stimulation, miR-20a and -106b can reduce IL-1β production as early as 24 hours post stimulation. This supports the neuroprotective capabilities of specific candidate miRNAs and their effects on various cytokines.

During HIV-1 infection, miR-20a, -106b, and -141 inhibit TNF-α production (Fig. 10). These results support our hypothesis that specific candidate miRNAs have a role in controlling cytokines production. However, lower transduction efficiencies put certain miRNAs at a disadvantage over others. A miRNAs true therapeutic potential should be compared across similar transduction efficiencies. Additionally, other studies in our lab have found similar effects of miRNA on astrocytes, which could also contribute to the neuronal protective role of miRNAs in the Macrophage-Microglia-Astrocytes-Neurons network.

Previous studies have shown that HIV-1 infection increased the production of pro-inflammatory cytokines to promote infection and viral replication (Jacobs et al., 2017; Shebl, Yu, Landgren, Goedert, & Rabkin, 2012). HIV-1 YU2 pseudotype virus was used in the study to

determine whether the transduced candidate miRNAs have similar cytokine inhibition effects during HIV-1 infection. This virus had a titer of 1.5 x10^6 /mL (Fig. 7). Thus, it was not surprising to observe elevated cytokine production during HIV-1 infection. Notably, miR-20a and miR-141 showed consistent inhibition of TNF-α production, in some instances to completely 0pg/ml (Fig. 10). MiRNAs-20a, -106b, and -141 all inhibited cytokine production in the presence of HIV-1 infection, but miRNA-141 is the only one that had 0pg/ml of TNF expression on 24 hpi, the exposure point, and the 72 hpi, viral replication point (Fig 10). These results suggest that some of some candidate miRNAs can inhibit the proinflammatory responses much more than others, miR-20a and miR-106b. The only miRNA that had no therapeutic effect to microglia cells was miR-124. The neuroprotective effects varied among different microRNAs and I speculate that this is due to the variation of transduction efficiencies among the miRNAs. Over expression of these miRNAs might be distributing the cells and killing them. I attempted to have these efficiency levels as high as possible. miR-20a and 106-B had rather higher transduction efficacies on numerous occasions. microRNA-20a usually ranged between 70-80 %, miR-106b averaged out 48.3%, miR-141 averaged out 22%, miR-124 averaged out 23.5%, and scramble averaged out 19.5% (Table 3).

A lentiviral miRNA expression vector with the advantage of stable integration and stable expression of target gene was used to overexpress miRNA in the cell. This study aims to investigate the role of specific miRNAs in cytokine expression within transduced microglia cells, HMC3, during LPS stimulation and HIV-1 infection. Quantitative analysis measured the role of candidate miRNAs in affecting cytokine production in the supernatant by ELISA suggesting that miR-20a, miR-141 were also able to significantly inhibit TNF-α and IL-1β (Fig. 10, 12). However, no differences existed in the IL-6 results between HIV-1 infection (Fig. 11) indicating that the miRNA mediated effect may be time dependent, where the miRNAs can inhibit the cytokine

production early during induction but may not be sufficient at later stages when increasing amount of IL-8 is secreted. Additional experiments are essential to understand this interesting phenomenon.

9.1 Future Directions

These experiments pave the way to apply these specific miRNAs in modulating the inflammatory response in microglia. Future directions can be taken to determining the specific role of miRNA in the neuropathogenesis of HIV-1 within the CNS analyze the mRNA levels of these supernatants through PCR. This study focused on the infection and viral replication of HIV-1 within a microglia cell line. However, since primed microglia have inflammatory properties, the role of miRNA in regulating neuroinflammation can be expanded on by using PBMCs. There have been several hypotheses for other modes of HIV-1 infection within microglia. Future studies can seek to incorporate these approaches with primary cells or periphery blood to study the role of miRNA and ultimately incorporate the important markers available within these cells. In addition, this could also be used to develop miRNA-based therapies. There has been some literature that mentions target gene therapy as a possibility for these noncoding molecules.

Ultimately, this evidence can serve to support further efforts in controlling the effects of HAND on HIV-1 infected individuals. Future directions can be taken to determining the specific role of miRNA in the neuropathogenesis of HIV-1 within the CNS analyze the mRNA levels of these supernatants through PCR.

10.0 Public Health Significance

Globally, 1.7 million children (<15 years) were living with HIV-1 in 2018. This infection does not discriminate against age, sex, race, or SES. It effects a variety of individuals worldwide which means there are millions of people struggling with an autoimmune disease. These people are not only more susceptible to infections, but also to the comorbidities of the infection. HIV-1 is a chronic disease with comorbidities, such as HAND, that will decrease patient productivity through age. Ultimately, this could affect healthcare costs and increase unemployment rates among these individuals.

Throughout the past 38 years, there have been various advancements in public health interventions, research, prevention education and therapies that suppress the viral replication. These efforts have been implemented in response to this epidemic. This life-threatening condition has been reduced to a chronic disease that's managed through proper antiretroviral therapy. Needle exchange programs have helped contain the spread of the virus by providing clean needles and education for drug using populations as well. These sort of efforts and progress have brought us closer to stabilizing the epidemic, but much more efforts are necessary to make this chronic condition easier to live with.

HIV-associated neurocognitive disorders (HAND) is observed in more than half of all HIV-1 patients in a mild form in post-cART era. Even after instituting combined antiretroviral therapy (cART) to HIV-1 seropositive individuals, the effects of neurocognitive disorders persist. The continued presence of HAND, despite aggressive treatment with ART and decrease in viral load, suggests that there is a continued immune response, oxidative stress, and inflammation within the brain. In addition to persistent neurodegeneration, there are no therapeutics available to treat

or prevent the neurodegeneration caused by HAND. This justifies the significance within this investigation.

Table 1: Generation of HIV-1 virus particles with and without VSV-G-ENV pseudotyping

Experiment	Date	Plasmids	eGFP	Titer (per ml)	
				24 hrs	48 hrs
1	10/17/18	Yu2	+	1200	1550
	01/21/19	Yu2/VSVG	++	2400	2200
2	01/26/19	Yu2	-	-	-
	02/04/19	Yu2/VSVG	-	-	-
3	01/31/19	Yu2	++	58500	3050
	02/18/19	Yu2/VSVG	++	67000	3100
4	02/20/19	Yu2	++	UC	UC
	02/25/19	Yu2/VSVG	++	UC	UC
5	02/20/19	Yu2	++	27000	750
	02/26/19	Yu2/VSVG	++	70000	18500

Table 2: Generation of lentivirus overexpressing miRNAs

Experiment	Date	miRNA	eGFP	Titer (per ml)	
				48 hrs	72 hrs
1	4/16/19	miR-20a	+-	X	X
		miR-106b	++	X	X
		miR-141	++	X	X
		miR-124	++	X	X
		scramble	++	X	X
2	4/25/19	miR-20a	++	UC	UC
		miR-106b	++	UC	UC
		miR-124	++	17300	UC
		scramble	++	8750	UC
3	4/25/19	miR-20a	++	1460000	1370000
		miR-106b	++	810000	1340000
		miR-124	++	150000	160000
		scramble	++	440000	55000

Table 3: Transduction efficency of lentivirus overexpressing miRNAss

miRNA	Transduction Efficiency
miR-20a	76.3% (N= 12)
miR-106B	48.3% (N= 14)
miR-141	22% (N= 14)
miR-124	23.5% (N= 12)
Scramble	19.5% (N= 14)

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