NEUROPROTECTIVE MICRORNAS COMBAT HIV-1 ASSOCIATED NEUROCOGNITIVE DISORDER (HAND) PATHOGENESIS

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ABSTRACT

HIV-1 associated neurocognitive disorder (HAND) is one of the major HIV-1 co-morbidities prevalent around the world. Early during HIV-1 infection, virus crosses the blood - brain barrier (BBB) and enters central nervous system (CNS) through infiltrating infected monocytes/macrophages. These infiltrating infected monocytes/macrophages serve as the source to initiate secondary infection of resident monocytic cells and glial cells in CNS. Neurons are not directly infected by HIV-1 virus. However, pro-inflammatory cytokines and chemokines as well as viral proteins released by infected/exposed immune and glial cells are neurotoxic and contribute to the neuronal stress and apoptosis. Nearly more than half of individuals living with HIV-1 infection present with some degree of neuronal impairment. Currently, no treatment is available to prevent or treat HAND. Thus, devising a strategy to manage HAND progression and improve the quality of life in HIV-1 positive population will be highly valuable and is of great public health significance.

MicroRNAs are small non-coding RNAs which play an important role in post-transcriptional regulation. Previous studies in our laboratory have identified specific miRNAs that may have a neuro-protective role. In this study, my goal is to evaluate the role of these candidate miRNAs on inflammatory response of monocytes/macrophages and microglia during

HIV-1 infection. I hypothesize that these candidate miRNAs can reduce the proinflammatory response of monocytes/macrophages and microglia, and these inhibitory effects of miRNAs are conserved during HIV-1 infection. In order to test this hypothesis, candidate miRNAs were over expressed in monocytic cell line (THP1) and microglia by using lentiviral expression vectors. Results suggest that miR-20a and miR-106b expression in monocytic cell lines inhibits TNF-α production, while miR-let-7a significantly downregulates IL-6 secretion in response to LPS either in the presence or absence of HIV-1 infection. Similarly, in microglia cells, miR-106b inhibits both IL-6 and IL-8 secretion upon LPS stimulation and miR-20a reduces IL-6 production upon TNF-α stimulation. Evaluation of these candidate miRNAs in J-Lat cells, which contain a single copy of HIV-1 latent HIV-1 virus suggest that, miR-20a, miR-106b, miR-124 and let-7a were able to reduce HIV-1 reactivation. These results indicate that neuroprotective miRNAs may have a direct role in reducing specific neuroinflammatory factor production and may also affect HIV-1 virus production. Thus, these candidate miRNAs could have a potential therapeutic role to minimize neuroinflammatory cytokine induced neuronal dysfunction and thus aid in management of HAND.

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PREFACE

First, I would like to thank my advisor, Dr. Velpandi Ayyavoo for her support and guidance. Her dedication to science was the source of my inspiration and encouraged me every time I encountered failure in experiments. She helped me to remain patient and taught me to think critically throughout the whole project and address the challenges to overcome the experimental failures. Dr.Ayyavoo is always accessible to find help and guidance, be it any experimental troubleshooting or questions related to my project . As I plan to move from her lab soon, I realize that I have been enriched with the knowledge and skills to explore and enjoy science and to apply it for the advancement of health. I would also like to thank Dr. Chen and Dr. Jenkins, for serving as my committee members and providing me invaluable advices and suggestions during my project.

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1.0 INTRODUCTION

The current global estimate for people living with HIV-1 (human immunodeficiency virus type 1) infection is 36.7 million (at the end of 2016) [1]. HIV-1 infection is a global public health concern, and various methods have been developed during the past 20 years to control this pandemic. Combination antiretroviral therapy (cART) has been very successful in controlling HIV-1 transmission and improving the quality of life of HIV-1 patients. This has led to a rapid decline in both incidence and mortality of HIV-1 infection [1]. Consequently, most of HIV-1 positive patients currently are not suffering from acquired immunodeficiency syndrome (AIDS) but many of them are experiencing non-AIDS-related comorbidities including cardiovascular disease, bone loss and neurocognitive disorder [2]. Among those comorbidities, HIV-1 associated neurocognitive disorder (HAND) is observed in more than half of all HIV patients. Definitely, cART has helped to reduce the incidence of most severe form of HAND, HIV-1 associated dementia (HAD), but the milder form of HAND, has become more prevalent. Currently, we do not have any specific therapeutics or treatment options to manage HAND. Thus, devising novel methods to manage HAND is of public health significance, especially, to improve the quality of life in HIV population.

1.1 HIV-1 PATHOLOGY

1.1.1 HIV-1 structure and genome

HIV-1 genome includes nine genes which are responsible for encoding 15 different HIV-1 viral proteins (Figure 1) which are sufficient for HIV-1 virus to successfully establish an infection [3].

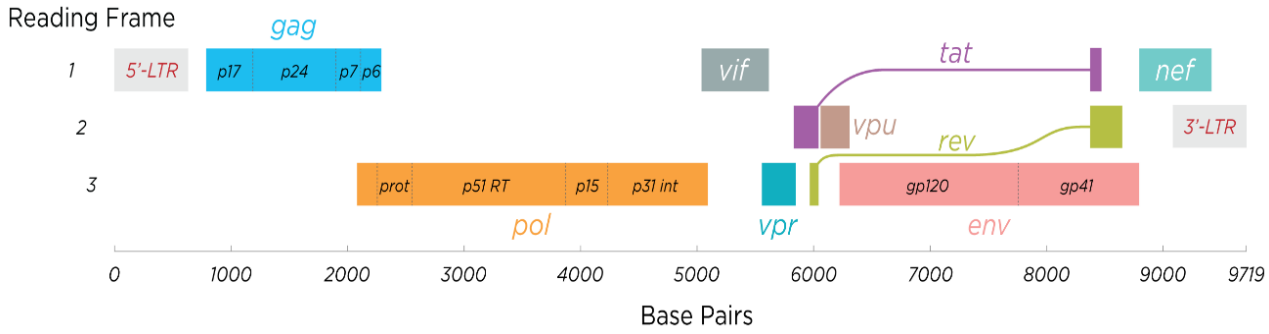

Figure 1. Schematic of HIV-1 virus genomic organization.
Thomas Splettstoesser, www.scistyle.com, 2014.
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HIV-1 genome includes two repeat sequences on either end known as Long Terminal Repeats (LTRs). LTRs serve as the promoter and enhancer of HIV-1 transcription and includes multiple binding sites for both host and viral factors. They regulate the expression of HIV-1 viral proteins encoded by the genome.

Group associated antigen, also known as Gag, is the major structural protein of HIV virion. It has a molecular weight of 55 kilo Dalton (KDa) and is also identified as p55. During virus budding, Gag recruits two copies of the viral RNA and along with other structural proteins., It also facilitates budding of the virion from the cell membrane. Post budding, Gag is processed to form

3 proteins: Matrix (MA), Capsid (CA) and Nucleocapsid (NC). These 3 proteins are critical for protection of the viral RNA genome and essential for establishment of new infection [4].

Polymerase (Pol) is encoded by *Pol* gene. Pol is also a polyprotein which is processed to form 3 important enzymes: reverse transcriptase (RT), integrase (IN) and Protease (PR). RT is responsible for reverse transcription. IN is the viral enzyme that helps proviral cDNA to integrate into host genome, while protease has the ability to proteolytically cleave the viral pro-proteins at specific sites [5].

Envelope (Env) is vital to establish host cell infection. Host protease cleaves the viral pro-protein to form two parts: the surface antigen (SU or gp120) which is able to bind to CD4 receptor and its co-receptor, CCR5 or CXCR4, to initiate membrane fusion by its conformational change. While the trans-membrane protein (TM or gp41) which aids in the process of fusion by sticking its fusion peptide in host membrane to complete the process of fusion [6, 7].

Rev is a viral regulatory protein which is able to bind to Rev Response Element (RRE) to export unspliced viral RNA out of nucleus. This is essential for retrovirus replication since unspliced RNA is unable to leave nucleus in eukaryotic cell [8].

Transactivator of Transcription, also known as Tat, is also a critical regulatory viral protein that enhances the transcriptional activity of HIV-1 LTR. In the absence of Tat, the activity of polymerase will be relatively weak and non-full length viral RNA transcripts will be synthesized [9].

Nef, Negative regulating factor, is also important in HIV-1 infection and viral pathogenesis. HIV-1 Nef is able to downregulate CD4 and MHC class I molecules on the surface of the infected cells, thus helps evading the immune surveillance [10].

Vpr, viral protein R, is another important accessory protein, which is essential for HIV-1 virus to replicate in non-dividing cells like macrophages. Other functions of Vpr include inducing cell cycle arrest and nuclear import of pre-intergration complex [11, 12].

Vpu, viral protein U, is also an accessory protein and has an important role in the process of virion budding as well as CD4 degradation. HIV-1 Vif, Virus infectivity factor, also a viral accessory protein, is essential for degradation of host restriction factor, APOBEC proteins in non-permissive cells including lymphocytes and macrophages [13, 14].

1.1.2 HIV-1 life cycle

HIV-1 infection is a multistep process as pictured in figure 2 [15]. The first step of infection starts with a free viron attaching to the cell surface by the interaction of viral envelope glycoprotein gp120 with host CD4 receptor. Conformational changes occur in both gp120 and gp41 viral proteins following the initial interaction which allows former one to expose binding site to co-receptor CXCR4 or CCR5 (depend on the virus tropism) and the latter one to insert its fusion peptide into the host cellular membrane to complete the fusion of viral membrane with the host cytoplasmic membrane [16].

Following membrane fusion, the contents of the viron are released into the cytoplasm which eventually form a complex called preintegration complex (PIC). In the cytoplasm, the process of reverse transcription of viral RNA to cDNA is initiated by the reverse transcriptase. The newly formed proviral DNA together with the PIC move close to the nuclear pore complex then enter the nucleus via an active transport mechanism. Once the PIC enters nucleus, the proviral DNA is able to integrate into the host genome using integrase [17, 18].

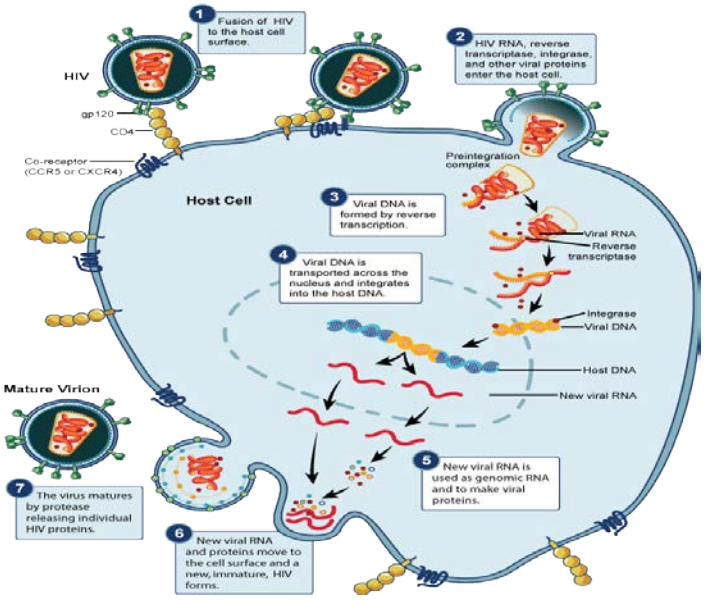


Figure 2. HIV-1 Replication Cycle.
Schematic diagram representing the entry, replication and release of HIV-1 virion from the target host cell.
National institute of Allery and Infectious Diseases
https://www.niaid.nih.gov/diseases-conditions/ *hiv-replication-cycle*

1.2 HIV-1 LATENCY IN CNS

1.2.1 HIV-1 Persistence and therapeutic approaches

cART has been highly successful in controlling virus replication and suppress the viral load in patients to undetectable levels, though complete elimination of HIV-1 infected cells is not currently possible. HIV-1 virus persists to remains transcriptionally silent for long duration only to re-emerge with the discontinuation of cART [19]. This latent reservoir is usually composed of resting CD4+ memory T cells residing in multiple places of human body [20]. In latent cells, the provirus remains transcriptionally silent, however, upon reception of signal from host or viral

factor, for example, TNF-α or Tat, the LTRs in the provirus will be activated and start producing new virus particles resulting in productive infection. In addition, latency cells are typically long-lived cells, making the latent reservoir extremely hard to be eradicated. According to a recent report, current cART drugs require 73.4 years to eradicate latent reservoir with an average half-life of 44 months for the latent cells [21].

Several therapeutic approaches are in development to control or eradicate the HIV-1 latent reservoir. The two major approaches include: sterilizing cure approach, which commits to completely eliminate all persistent HIV-1 virus. The second one is a functional cure, which tries to immunologically control the virus. The “shock and kill” strategy [22] is one of the sterilizing cure methods that currently under great interest. Briefly, this strategy tries to reactivate latent virus with drugs to produce virus leading to cytopathic killing and expose the latent virus containing cells to the immune surveillance and immune-mediated depletion of the latent cells. The normal uninfected cells are protected by cART during this process. However, recent studies have observed a high variation in the response of the latent virus pool to drugs[23].

Thus, in order to get better understanding of HIV-1 latency, in vitro latency models have been developed. Transformed Jurkat cells which is an immortalized human T lymphocyte cell line, have been used to study HIV-1 latency. J-Lat cell line contains latent form of HIV-1 virus that is envelope defective and the nef gene of this virus is replaced by an eGFP gene. Thus, when the provirus is reactivated by agents, such as Tumor Necrosis Factor alpha (TNFα) there will be GFP expression instead of nef expression. However, despite the advantage of genetic and experimental tractability of J-Lat cell, concerns have been raised for the relevance of this model to the actual settings in primary cells and limitations of this model including inherent mutations in proliferation cycles and clonal nature of virus integration sites [24]. Because of these concerns, the first primary model of HIV-1 latency was developed by Sahu in CD4+ lymphocytes [25] and later other primary

latency models have been established with different virus vector in different phenotype of cells for the purpose to study multiple reactivators.

1.2.2 Central Nervous System as Reservoir

The central nervous system (CNS) is a unique anatomical compartment that is isolated from the rest of the human body by blood-brain barrier (BBB). BBB controls and limits the exchange of any substances between blood and brain. However, the BBB is not able to stop HIV-1 virus invading the CNS. HIV-1 virus enters CNS soon after acute infection via infected infiltrating monocytes/macrophages (ref). However, this unique structure is able to impede drugs and other immune cells, which makes CNS the potential reservoir for HIV-1 virus. Evidence that support this hypothesis include the presence of integrated HIV-1 provirus in brain macrophages, microglia and astrocytes samples [26]. The long-lived and reduced immune surveillance nature of those local immune cells makes the CNS a perfect place for HIV-1 virus to hide. Also, 34% of subjects under cART treatment in one study have been found to have detectable level of HIV-1 RNA in Cerebrospinal fluid (CSF) compared to a lower average detectable level of HIV-1 RNA in plasma, suggesting that CNS may have a higher reactivation activity than other parts of body [27]. Finally, the HIV-1 virus found in CNS are phylogenetically distinctive HIV-1 subtypes (YU-2) which may limit the ability of latency reversing agents to facilitate “shock” and “kill” strategy under cART treatment [28]. All these evidences make CNS a possible site as HIV-1 reservoir.

1.3 MACROPHAGE AND HIV-1 ASSOCIATED NEUROCOGNITIVE DISORDER

1.3.1 Role of macrophages in HIV-1 infection

Macrophages are mononuclear leukocytes responsible for phagocytosis and antigen presentation. Macrophages reside in tissues of most part of the body post terminal differentiation [29]. Macrophages express CCR5 co-receptor and thus are capable of supporting HIV-1 infection. However, unlike CD4+ T cell, HIV-1 infection in macrophages does not cause any cytopathic or apoptotic effects, and this is likely due to the immune evasion effects of viral proteins. Viral proteins including Nef, Vpr and gp120 may all play a role in up-regulating NF-κB pathway to avoid TNF-α induced apoptosis in macrophages: soluble Nef can inhibit endosomal V-ATPase to up-regulate NF-κB pathway while Vpr may play a role in reducing TCR-mediated activation of NF-κB activity and last gp-120 may indirectly downregulate TNF-α ligand by increasing macrophage colony-stimulating factor (M-CSF) which is a pro-survival cytokine that modulates the differentiation of monocytes to reduce apoptosis [30-32]. Thus, under the effects of those viral proteins, macrophages are more likely to survive for long time with periodic viral replication and release rather than undergoing apoptosis. Together with the antigen presenting nature, macrophages are able to contribute to the viral susceptibility by continually presenting virus to CD4+ and CD8+ T cells and contribute to the permissiveness of HIV-1 virus by continuous chemokine expression to recruit CD4+ and CD8+ T cells via chemotaxis.

1.3.2 Role of macrophage produced cytokines/chemokines in HIV-1 infection

Cytokines are a broad category of small molecules (5–20 kDa) responsible for changing cell behavior in both the cells which release them and the cells nearby via autocrine and paracrine mechanisms. The cytokines work by binding to receptors on the cell surface, then the signal of cytokines are transduced to nucleus though signal transduction, resultin in modulation of specific gene transcription. While the chemokines are chemotactic cytokines which are able to use a concentration gradient to guide the migration of immune cell to the site of inflammation [33]. They are both important in immune responses and immune cell activation. However, under HIV-1 infection, the virus manipulates cytokine production and immune activation to promote infection and viral replication. For example, TNF-α may facilitate HIV-1 replication by activating host NF-κB pathway to further activate NF-κB binding sites in LTR of HIV-1 genome [34]. Similarly, interleukin 6 (IL-6), as a pro-inflammatory cytokine, has been reported to be a stimulatory cytokine that increases viral replication [35]. Also, interleukin-8 (IL-8), belongs to the C-X-C chemokine family, which is originally responsible for attracting neutrophils to fulfill monocyte adherence, has been shown to stimulate HIV-1 replication [36]. At last, chemokine (C-C motif) ligand 2 (CCL2) which helps to recruit memory T cells, monocytes and dendritic cells, is also believed to function on enhancing the HIV-1 infection.

On the contrary, due to the existence of balance of cytokine network, some cytokines may also play an inhibitory role in viral replication to poise the effect of HIV-1 stimulatory cytokines. For instance, macrophage inflammatory protein 1α and 1β (MIP-1α, MIP-1β), can bind to the CCR5 receptor and thus block the entry of HIV-1 virons. And IL-10 is believed to constrain HIV-1 replication by inhibiting pro-inflammatory cytokine production [37, 38]

1.3.3 Neuropathogenesis of HAND

HIV-1 associated neurocognitive disorder (HAND) is one of the major HIV co-morbidities and the clinical manifestations of HAND are: loss of neuronal density with neuronal damage and loss of dendrities [39]. In the early stage of infection, HIV-1 infected monocytes/macrophages cross the BBB [40] which later enables infection of CNS resident monocytic cells and astrocytes (Figure 3). In the meantime, elevated production of TNF-α reduce the permeability of the BBB leading to further infiltrating of monocytes/macrophages [41]. Together with infected perivascular macrophages, microglia and astrocytes, host factor including pro-inflammatory cytokines and chemokines (e.g. TNF-α, IL-8, IL-1β, IL-6) are secreted, result in increased secretion of neurotoxic substances containing nitric oxide, platelet-activating factor (PAF) and quinolinic acid which stress the neurons. Finally, together with viral factors (Tat, Vpr, gp120), those neurotoxic factors lead to neuronal dysfunction and eventually to neuronal apoptosis [42].

The damage of synapse may also in part responsible for the neuropathogenesis.. Normal synapse maintains physiological level of glutamate in the CNS, which is the major excitatory neurotransmitter to prevent toxic calcium influx in neurons. However, under HIV-1 infection, activated macrophages release excitotoxic glutamate mediated by the N-Methyl-D-aspartate (NMDA) receptor. Glutamate act in a paracrine way entering astrocytes and microglia where they disturb the normal maintenance of glutamate for neurons. Therefore, result in increasing uptake of calcium (Ca2+) in synapse which activate calcium-dependent proteases capable for disrupting the postsynaptic density (PSD). The disrupted PSD then leads to neurodegeneration and synapse disruption [43].

Another interpretation for HAND neuropathogenesis is the possibility of reduced neurogenesis. Chronic neuroinflammation in CNS may result in the interruption of adult

neurogenesis (ANG) by impairing the proliferation and migration of immature neurons as well as expropriating the support from astrocytes for neurons. As a result, patients may suffer from loss of learning ability and anxiety-related behaviors which are the corresponsive clinical manifestations of HAND [44, 45].

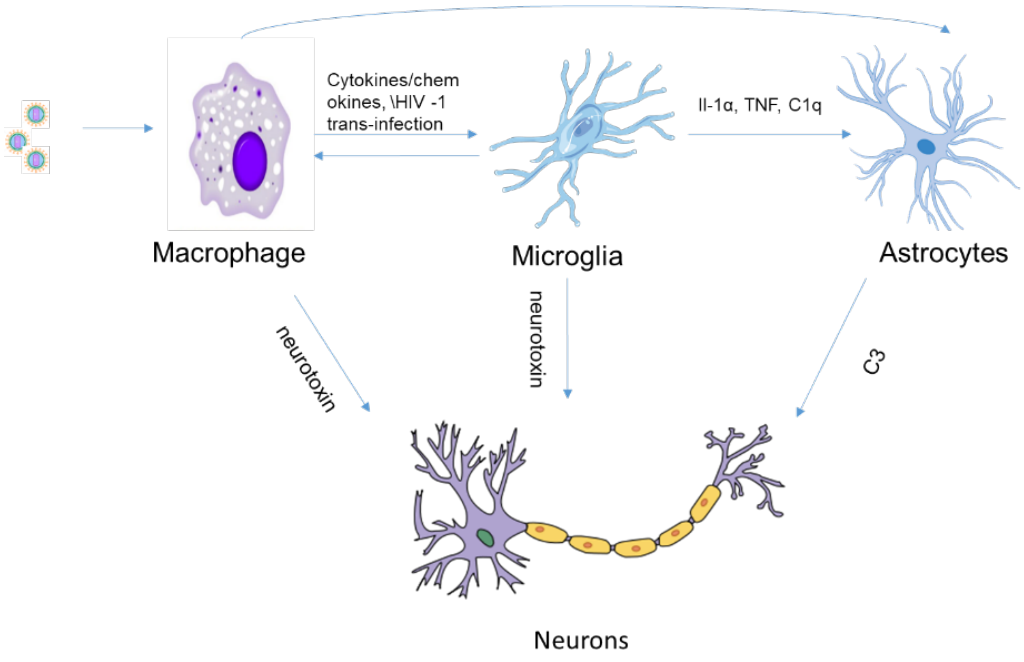


Figure 3. Macrophage-Microglia-Astrocytes-Neurons network: Role in HAND pathogenesis.
HIV-1 virus mainly enters the CNS compartment through infected macrophages which recruit CNS resident microglia and can infect both microglia and astrocytes. Direct HIV-1 infection of neurons is not detected and the neurotoxic effects in HAND are due to viral proteins and the host factors, mainly the proinflammatory factors released by HIV-1 infected/ exposed macrophages, microglia and astrocytes.

1.4 ROLE OF MIRNA IN HAND

1.4.1 MicroRNA (miRNA) biology

MicroRNAs are 21-23 nt long, non-coding RNAs which have a role in post-transcriptional regulation by altering the stability of target mRNAs [46]. Pri-microRNAs are first transcribed by RNA polymerase II and later cleaved by RNAse III Drosha into pre-microRNA in nucleus [47]. Then the pre-microRNAs are transported out of the nucleus via Exportin 5. In cytoplasm, with the help of RNAse III Dicer, the pre-microRNAs lost their hairpin structure, forming duplex complementary structure. Only one strain of this dsRNA in the structure has the ability to become the mature microRNA and later this strain forms a RNA-induced silencing complex (RISC). This RISC then binds to the 3' untranslated region (UTR) of target mRNAs which leads to degradation, repression or deadenylation of targeted mRNA. It is predicted that nearly more than 60% of protein coding mRNAs in human contain UTR region for miRNA to bind [48]. Thus, miRNAs play an important role in the regulation of all biological pathways including homeostasis of cellular interaction, cell proliferation and cell differentiation. In addition, studies have shown that one miRNA may be able to target multiple mRNAs and one mRNA can also be targeted by multiple miRNAs [49].

1.4.2 Host and viral miRNA

Multiple studies have identified a functional role for miRNAs in cancer, for example, miR-15a and miR-16-1 can induce apoptosis in tumor cells by repressing Bcl-2 which is an anti-

apoptotic protein enriched in malignant cells [50]. Similarly, many researchers also tried to identify the function of miRNAs in infectious disease also. During this process, many miRNAs have been found to correlate with HIV-1 infection. One study found that suppression of miRNA-28, miRNA-150, miRNA-223 and miRNA-382 in monocytes increase HIV-1 infectivity while escalate of those anti–HIV-1 miRNAs in macrophages inhibited HIV-1 replication [51]. MiRNA-221 and miRNA-222 were also found to inhibit HIV-1 entry in macrophages by affecting the CD4 receptor [52] and miR-29a has been shown to target HIV-1 Nef transcription in CD4+ T cells [53]. Thus, these findings implicate that anti–HIV-1 miRNAs may have a key role in protecting immune cells from HIV-1 infection.

On the contrary, miRNAs can also be encoded by many viruses, including HIV-1. HIV-1 encodes microRNAs intending to suppress immune system and thus create a favorable environment for viral invasion and replication. As we mentioned above, HIV-1 transactivation response element (TAR) on LTR has the function on initiating transactivation. Recently studies have also found that TAR region may also act as the source of viral miRNAs [54]. HIV-1 miR-TAR-3p and HIV-1 miR-TAR-5p are two viral miRNAs which were found to target cellular apoptotic factors and proliferation regulators, respectively, to promote viral latency [55]. HIV-1 miR-N367 which derived from Nef gene was reported to control viral transcriptional factors and regulators through negative responsive element in the 5'-LTR to suppress HIV-1 replication [56].

1.4.3 Neuroprotective role of miRNA

The role of miRNAs in the pathogenesis of HAND is an area of interest. Multiple studies have identified a correlation between miRNAs expression level and neuronal function in HAND

[57]. But how these miRNAs protect neuronal function in HAND remains unclear. There are two hypotheses to explain the function of neuroprotective miRNAs: First, miRNAs may have a direct role in protecting neurons. Many studies have shown the function of miRNAs in neuronal recovery after ALS progression and neuron ischemia [58, 59], indicating that a similar mechanism may also exist in the case of HAND. In addition, the miRNAs which have direct role in protecting neurons may also be able to travel from glial cells to neurons by exosomes. For example, miR-26a which derived from astrocytes is reported to potentially increase the neuronal plasticity which is the hallmark of neuronal health [60].

The other hypothesis focuses on the function of miRNAs affecting cytokines received by neurons. Infected neuronal support cells may release large volume of cytokines which stress neurons. In this situation, miRNAs in neuronal support cells which are able to control the cytokine production should also be considered as neuroprotective. As two important neuronal support cells, specific miRNAs in astrocyte and microglia were found to reduce pro-inflammatory cytokine production [61, 62]. However, as we mentioned above, HIV-1 virus initiates CNS infection through infiltrating monocytes/macrophages, which further infects local glial cells and amplify the cytokine storm in the brain. Thus, if we are able to control the scale of the cytokines, the protective role of miRNAs may become more effective. For this reason, we overexpressed candidate miRNAs in monocytes/macrophages to study their effects on cytokine production.

1.4.4 Identification of candidate miRNAs

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 a previously

published paper of our lab [57]. Briefly, total PBMCs from HIV-1 seronegative individuals and HIV-1 seropositive individuals without neurocognitive symptoms, or with MND or HAD were systematic evaluated for of global transcriptome. Candidate miRNAs were upregulated in HAND resistant HIV-1 patients and were predicted to be upstream regulators in HAND. MiR-let-7a and miR-124 were predicted to target chemokine signaling especially for IL-8, CCL2 and CXCR4 through RHOG, JUN, MAPK3 and RAF1 signal transduction process. While miR-20a and miR-106b were predicted to target cytokines including TNF-α, IL1β and IL-6 through DUSP2, NOTCH1 and FOXO1 signal transduction process. Thus, candidate miRNAs could potentially have a neuron-protective role by inhibiting chemotaxis of inflammatory immune cells in CNS and reducing cytokine induced neuronal dysfunction.

2.0 AIMS OF THE PROJECT

The following specific aims focus to evaluate the role of miRNAs to combat HAND

<u>Aim 1</u>: Investigate the role of candidate miRNAs to alter neuroinflammatory chemokines/ cytokines and surface receptor expression in miRNA transduced monocytes and microglia cells.

A. Development of specific miRNA expressing monocytes and microglia using lentiviral expression vectors.

B. Confirm transduction efficiency and candidiate miRNA expression using flow cytometry and q-RT PCR.

C. Evaluate changes in surface receptor expression in transduced monocytes.

D. Verify changes in specific cytokine/chemokines in miRNA transduced monocytes.

E. Analyze the role of exosomes in miRNA mediated effects.

F. Verify changes in specific cytokine/chemokine in miRNAs transduced microglia.

<u>Aim 2</u>: Investigate the ability of these miRNA expressing monocytes/macrophages to reactivate latent HIV-1 using HIV-1 reporter latent cell line.

A. Evaluate the role of miRNA transduced cell lines to reactivate latent HIV-1 virus in J-lat cells.

B. Investigate the host cellular factors responsible for reactivation of latent HIV-1 in J-Lat cells.

3.0 MATERIALS AND METHODS

3.1.1 Cells

HEK-293T and microglia cells were maintained at 37°C, 5% CO_2 in DMEM (Gibco) which contains 10% fetal bovine serum (HyClone), 1% L-glutamine (Invitrogen), and 1% streptomycin (Invitrogen). THP1 cells were similarly maintained in RPMI with 10% fetal bovine serum, 1% L-glutamine, and 1% streptomycin.

3.1.2 Construction of miRNA vector

Lentivirus vectors expressing specific miRNA were obtained from GeneCopoeia™. Figure 4 is an example of hsa-let-7a-1 miRNA vector. The characteristics of this vector include a backbone based on pEZX-MR03 (HIV-based) vector, a 5' LTR with packaging elements and a 3'-LTR with poly A. It also includes a CMV promotor and Puromycin stable selection marker. The reporter gene of this vector is eGFP.

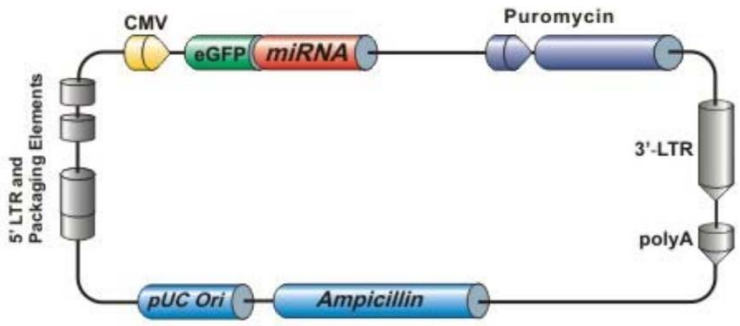


Figure 4. Schematic denoting the structure of miRNA expression vector.
The miRNA expression is on a lentivirus backbone with an internal CMV promoter that drives the expression of eGFP and miRNA as a fusion transcript. The lentivirus also includes puromycin resistance gene for selection against puromycin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

3.1.3 Lentivirus production and Transduction of Target Cells

HEK-293T cells were trypsined by 0.25% of 1X Trypsin-EDTA (gibco) then seeded in 10 cm^2 tissue culture-grade plates, which were then grown overnight until the HEK 293T cells becoming adherent and reaching 80% of confluence.

Two and half (2.5) µg miRNA expression vector was mixed with 0.5µg of PLP1 which stands for packaging plasmids containing gag and pol, and 0.5µg of Vpx expressing plasmid (which functions on degradation of SamHD1 in monocytes) and 0.5µg of PLP2 (provide Rev gene) and pVSV-G-Env of vesicular stomatitis virus (VSV), respectively, in 250µl of DMEM. Then 15µl of Polyjet^{TM} transfection reagent was mixed with 250ul of fresh DMEM first then added to the plasmid mixture. After 15 minutes of incubation, the whole mixture was added drop-wise to the HEK-293T 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 and purified using a 0.22μm filter unit (Steriflip®). Virus titer was then quantified by a TZM-bl assay using 1:10, 1:100, 1:1000 and undiluted quantities of virus to infect 4.0 x 10^4 TZM-bl cells with an incubation period of 6 hours. After 6 hours, the media was changed with fresh media and the plate was incubated for another 48 hours. TZM-bl cells were stained with β- galactosidase substrate for 4 hours and counted in the optimal well to measure infectious particles.

Virus of 0.1 and 1 MOI from the HEK 293T supernatant were added to the washed THP1 cells or microglia cells for transduction and incubated for 24 hours before replacing the cells with fresh media. 72 hours post-transduction, 1μg /ml of puromycin was added to the media to select transduced cells.

3.1.4 Total RNA extraction and cDNA preparation

Total RNA was extracted using *mir*Vana™ miRNA isolation kit as described by the manufacturer. Briefly, cell pellets of miRNA-transduced THP1 cell line were collected by 1000 rpm spinning. 1x10^6 cells were washed with PBS then lysed in 600 μl of Lysis/Binding Solution. 50 μl of miRNA Homogenate Additive was added to the lysed solution with a 30 seconds vortex for mixing. The mixture solution was then left on ice for 10 minutes incubation. 500 μl of AcidPhenol: Chloroform was added to the solution after the incubation with a 30 seconds intense vortexing. The entire solution was then centrifuged at 10,000 rpm for 5 minutes for separation.

After the centrifugation, the upper aqueous layer was carefully transferred to a microcentrifuge tube. Next, one volume of 100% ethanol was added to the microcentrifuge tube then the whole mixture was passed through a RNA binding filter with a short 10,000g centrifugation. Similarly, one time of 700 µl of miRNA Wash Solution 1 and twice of miRNA Wash Solution 2/3 were passed through the filter to clean the RNA on the membrane. After three times of wash, the empty filter cartridge was centrifuged for 30 seconds for removing residual fluid. Then the membrane was transferred to a new collection tube. 100 µl of the heated Elution Solution (95°C) was added to the central of the filter with a 30 seconds centrifugation to get RNA sample. Finally, the concentration of the RNA sample was measured by spectrophotometer (NanoDrop™ 2000).

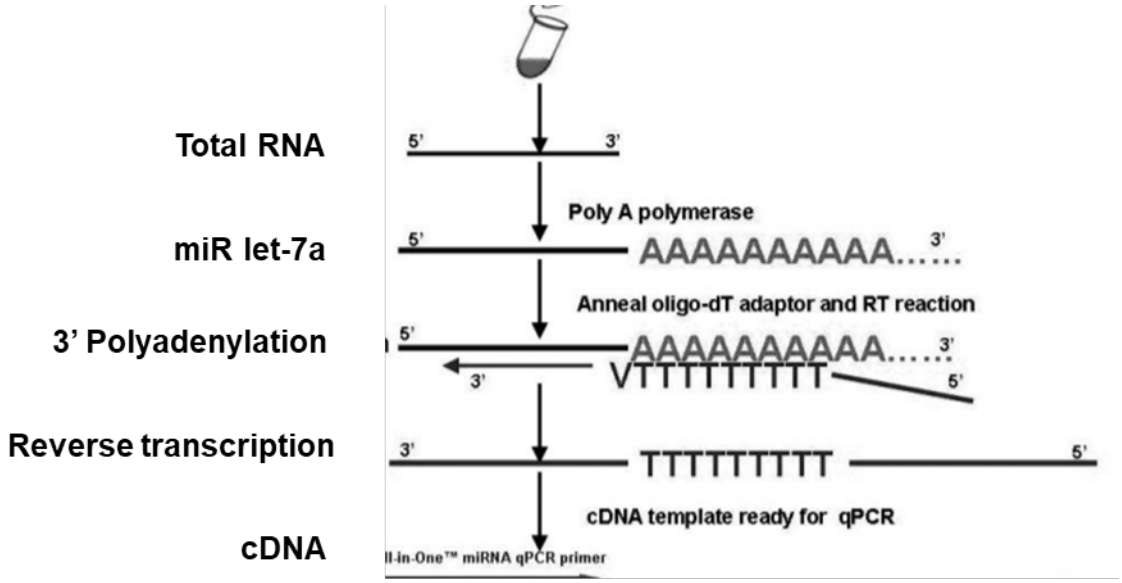


Figure 5. cDNA synthesis for miRNA quantitation.
Adapted from instruction of All-in-One™ miRNA qRT-PCR Detection Kit (GeneCopoeia)

cDNA reverse transcription was performed using All-in-One™ miRNA qRT-PCR Detection Kit (GeneCopoeia) including total RNA obtained from transduced THP1 cell lines. Since miRNA are relatively very short to be detected by regular qRT-pCR, Poly-A tails were added to the 3' end of miRNA before reverse transcription (Figure 5). Following the steps in Table 1, all the reagents were mixed to a final volume of 25 µl. Next, the mixture was incubated at 37°C for 60 minutes for addition of Poly(A) tails by Poly(A) Polymerse following another incubation at 85°C for 5 minutes to inactivate the enzyme. Finally, the cDNA products were ready for next qPCR experiment or it can be stored at –20°C, using specific primers.

Table 1. Reactants for cDNA reverse transcription

Reagent	Volume	Quantity
Total RNA		2 ug
2.5U/µl PolyA Polymerse	1µl	
RTase Mix	1µl	
5xPAP/RT Buffer	5µl	1x
dd H2O (RNase free)	To final 25 µl	

3.1.5 qRT-PCR Quantitation of miRNA expression

All-in-One™ miRNA qRT-PCR Detection Kit (GeneCopoeia) and All-in-One™ miRNA qPCR primer designed for candidate miRNA were used in this process. cDNA generated above

was diluted to 50 times with sterile H_2O before performing qPCR. qPCR mixture was prepared according to table 2 in triplicates, then the samples were run in the ABI Prism 7000 Sequence detection system as per the conditions described in Table 3 In this analysis, the ΔΔCT method was used to generate relative expression and U6 small RNA was included as the endogenous control.

Table 2. Real Time PCR reactants per reaction for miRNA detection

Reagent	Volume	Concentration
2×All-in-One qPCR Mix	10 µl	1×
All-in-One ™ miRNA qPCR Primer (2 µM)	2 µl	0.2 µM
Universal Adaptor PCR Primer (2 µM)	2 µl	0.2 µM
First-strand cDNA (diluted 1:5)	2 µl	
Water (double distilled)	To final 20 µl	

Table 3. Running condition for detection of miRNA

Cycles	Steps	Temperature	Time
1	Initial denaturation	95°C	10 min
40	Denaturation	95°C	10 sec
	2 µl	62°C	20 sec
	2 µl	72°C	At least 10 sec

3.1.6 Intracellular staining and flow cytometry

Intracellular staining of cytokines followed by flow cytometry was used to evaluate the amount of cytokines expressed at single cell level. Intracellular cytokine staining was carried out following the standard staining protocol. Cells (1×10^6) at the mentioned time point were stimulated with LPS (1 µg/ml) in the presence of GolgiStop (2 µl/ml) for a duration of 4 h. Intracellular staining was done with 5 µl PE cy7 anti-human TNF-α antibody (eBioscience), 5 µl APC anti-human IL-6 antibody (BioLegend), and 20 µl Pacific Blue™ anti-human IL-8 antibody (BioLegend). The cells were then washed twice with 1X Perm/Wash buffer and kept in FACS buffer with 1% paraformaldehyde. Flow cytometry analysis was performed on these cells with the LSRFortessa™ cell analyzer (BD Biosciences) and FACSDiva® software (BD Biosciences).

3.1.7 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 IL-8, TNF-α, CCL2, and IL-6. Human IL-8 DuoSet ELISA kit (R&D Systems), Human TNF-α DuoSet ELISA kit (R&D Systems), Human CCL2 DuoSet ELISA kit (R&D Systems) and Human IL-6 DuoSet ELISA kit (R&D Systems) were used for the ELISA analysis. 100 µl 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 100 µl of

blocking buffer (1% BSA in PBS) was added to each well with 1hour 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 same time, an

eight-point 2-fold serial dilutions for each cytokine standard was made and added to the plate with the highest standard concentration, 1000pg/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 (BD OptEIA^{TM}) 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 (100) µl of 2 N H_2SO_4 was then added to stop the reaction and the color intensity was read by ELx800 Absorbance Microplate Reader (Bio-Tek) with KCJunior^{TM} software (Bio-Tek). 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 $R^2 \geq 0.900$.

3.1.8 Total Exosome Isolation

Total exosomes were isolated from the cell supernatant using Total Exosome Isolation Reagent (Invitrogen). Exosomes are small vesicles containing miRNA, cytokines and other signaling molecules which secreted by cells as intercellular messenger. Transduced THP1 cells were cultured in FBS free RPMI media for 24 hours and stimulated with 1µg/ml LPS stimulation. Then 1ml of cell-free culture media was harvested and transferred to a new tube and 500 µl of Total Exosome Isolation reagent was added to the same tube following an overnight incubation at 4℃. Next day, the sample was centrifuged at 10,000 × g for 1 hour at 4℃ to isolate the exosomes. Then the exosome pellet was resuspended in 200µl 1X PBS for further analysis.

3.1.9 Statistical Analysis

The results are expressed as mean ± standard deviation. The data were analyzed using the Student's *t* test for paired samples with a significance cut-off of $p<0.05$.

4.0 RESULTS

4.1. AIM 1: INVESTIGATE THE ROLE OF CANDIDATE MIRNAS TO ALTER NEUROINFLAMMATORY CHEMOKINES/ CYTOKINES AND SURFACE RECEPTOR EXPRESSION IN MIRNA TRANSDUCED MONOCYTIC CELL LINE AND MICROGLIA

4.1.1 Expression of candidate miRNAs in monocytic cell line, THP1

Monocytic cell line, THP1, was transduced with lentivirus vector expressing miRNA. The eGFP coding mRNA transcripts and miRNA transcripts are driven by the same CMV promoter, the eGFP reporter expression reflects the transduction efficiency of the lentivirus and indirectly also reflects the miRNA expression. The percentage of eGFP positive cells for individual candidate miRNA expressing cell line were estimated by flow cytometry. As represented in Figure 6A, after selection with puromycin, more than 90% of the cells express miR-20a, miR-106b, miR-124 and miR-scramble based oneGFP expression, while less than 1% of auto-fluorescence was observed in untransduced THP1 cells and 80% of cells expressing miR-let-7a were eGFP positive. Interestingly, the resistance of these cell lines to puromycin was different. THP1 cells expressing miR-20a, miR-106b and miR-124 were highly resistant to puromycin selection and the eGFP expressing cells can grow in 1μg/ml of puromycin, while miR-scramble and miR-let-7a transduced

THP1 cell lines were only able to tolerate 0.5μg/ml of puromycin which may explain the low transduction efficiency in the miR-let-7a transduced cell lines.

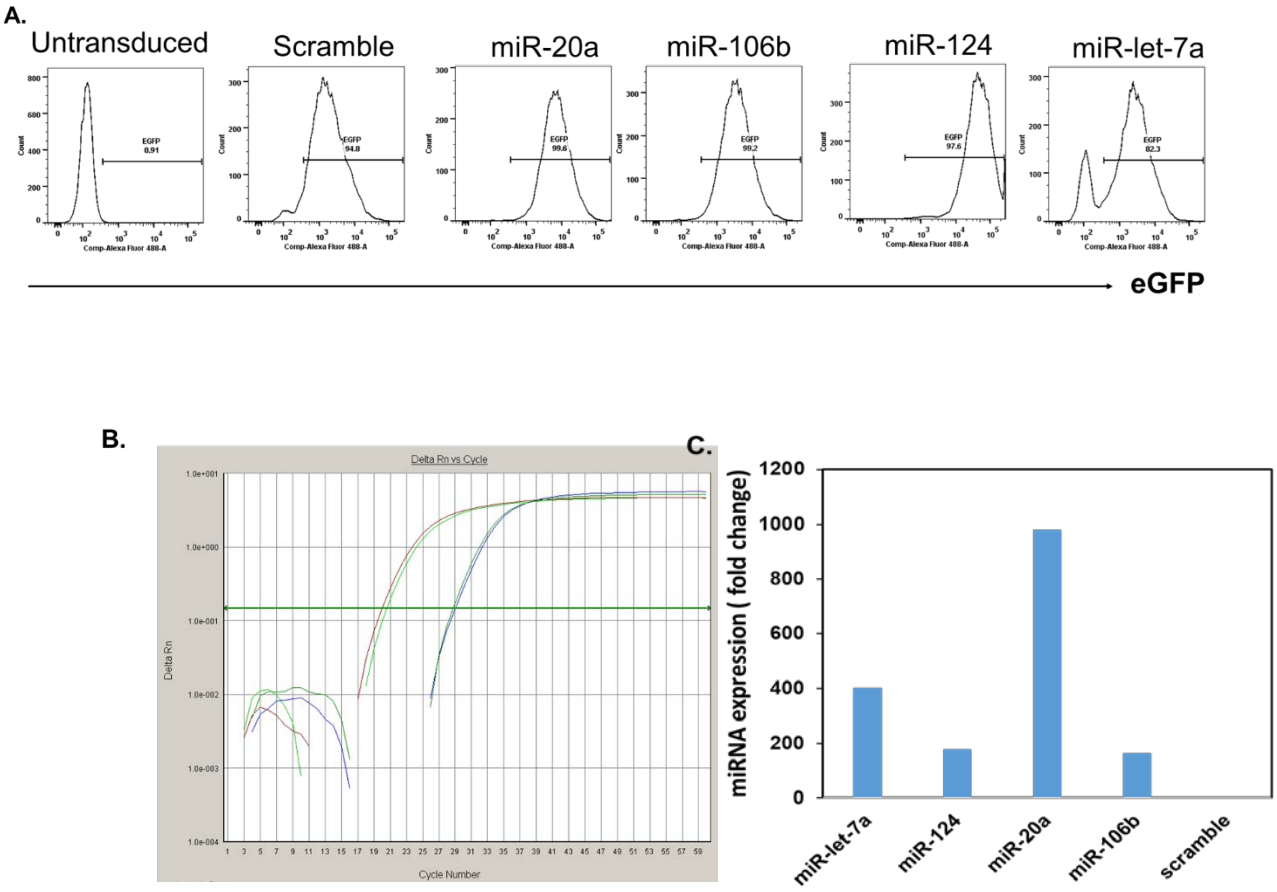


Figure 6. Expression of candidate miRNAs in THP1 cell line.

MiRNA transduced THP1 cell lines were selected using puromycin. Then miRNA expression in each cell lines were confirmed by evaluating eGFP expression using flow cytometry and qRT-PCR. **A.** The untransduced THP1 cells are used as control for determining the cut-off. **B.** Amplification plot data of a representative miR-124 quantitation. **C**. Fold change were estimated using the ΔΔCt method relative to untransduced control. U6 snRNA was used as a endogenous control for normalization.

Next, the expression of candidate miRNA in the THP1 cell line were confirmed by qRT-PCR including specific primers. Total RNA was extracted from the transduced THP1 cell lines was included in the cDNA reaction. Before converting to cDNA, Poly-A tails were added to the 3' end of miRNA using Poly-A Polymerse. U6 small nuclear RNA was included for normalization and the expression of the candidate miRNAs in the transduced cells lines relative to untransduced THP1 cells were estimated by $\Delta\Delta$CT method. In Figure 6B, a representative amplification plot of miR-124 is included to demonstrate the increased level of miRNA expression in miRNA transduced monocytes compared to untransduced THP1 cells. Results suggest that the candidate miRNA expressing cell lines have 100-400 fold increase in expression of specific miRNAs (Figure 6C). However, we were unable to detect the expression of scramble miRNA. There is no endogenous expression of scramble miRNA in untransduced THP1 cells and the inability to detect in the lentivirus transduced cell line suggests the possibility of mismatch in primer or probe binding region. These results from flow cytometry and qRT-PCR, suggest that lentivirus transduced THP1 cell lines have a stable expression of candidate miRNAs and can be used in downstream functional analysis.

4.1.2 Effects of candidate miRNAs on cytokine production in monocytic cell line

Previous published results from our lab and others suggest that the candidate miRNAs might potentially target inflammatory cytokines [57]. Here, we evaluated the ability of THP1 expressing candidate miRNAs to respond to Lipopolysaccharide (LPS). LPS binds to TLR-4

receptor, and the downstream signaling induces a strong proinflammtory response in human monocytes and macrophages with production of TNF-α, IL-1β, IL-6 and IL-8. THP1 cells were stimulated with LPS (1μg/ml) for 4 hours in the presence of GolgiPlug, which is a protein transport inhibitor that retains newly synthesized cytokines in the ER. Intracellular staining of TNF-α and IL-8 were performed followed by flow cytometry analysis. The percentage of cytokine positive cells in the eGFP positive cells were determined. Results suggest that 87.5% of the cells were positive for TNF-α in the THP1 cells expressing scramble miRNAs while, miR-20a and miR-106b reduced TNF-α cytokine-positive cells to 0.94% and 5.27%, respectively (Figure 7A). There was no significant decrease in the percentage of cells expressing TNF-α, in miR-124 or let-7a expressing cells. Similarly, in Figure 7B, miR-106b expressing cell line showed a significant decrease in the number of IL-8 positive cells (1.1%) in comparison to miR-Scramble (11.1%). However, more than 35% of eGFP negative cells were observed in the miR-Scramble, miR-20a, miR-124 and miR-let-7a expressing cell lines in the cytokine flow cytometry analysis of IL-8 but not seen in the TNF-α group. However, this was not corresponding to the results in Figure 6 where more than 95% of the cells were miRNA expressing cells. This is likely due to over-permeabilization on those cells resulting in leaking of eGFP proteins and cytokines.

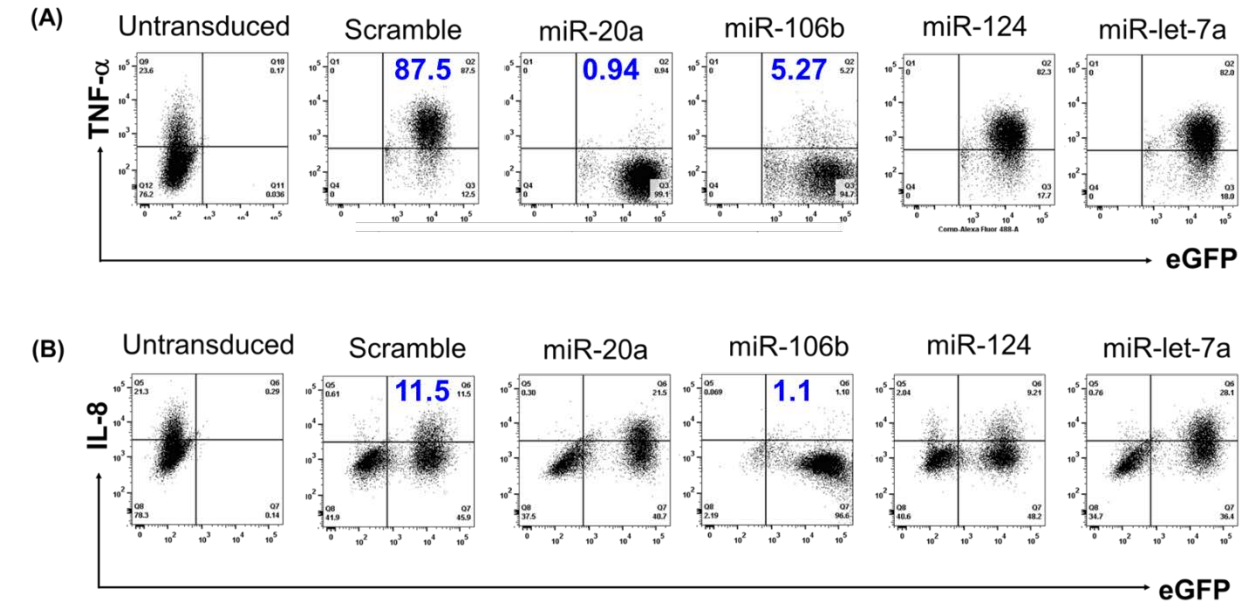


Figure 7. Effects of candidate miRNAs on intracellular cytokine production.

MiRNA transduced THP1 cell lines were stimulated with 1μg/ml LPS for 4 hours in the presence of GolgiPlug. Intracellular cytokine staining was performed for **A.** TNF-α and **B.** IL8 and estimated by flow cytometry.

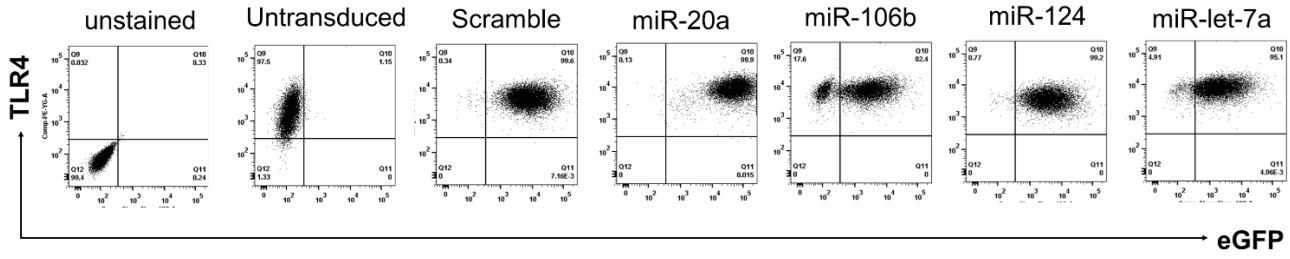


Figure 8. Effects of candidate miRNAs on surface receptor TLR-4 expression.

Cells were stained with specific antibody to detect surface expression of TLR4 and the expression was estimated by flow cytometry.

Considering the multi-target function of miRNAs and the complexity of signal transduction, the TLR-4 receptor may also be targeted by our candidate miRNAs, predictive analysis identifies that miR-124 can directly target TLR-4 expression. Thus, the inhibition of

cytokines by miRNAs may actually result from the loss of TLR4 receptors and their inability to bind to LPS. To evaluate this, the surface expression of TLR4 was evaluated by staining with specific antibody and flow cytometry. Results (Figure 8), suggest that none of candidate miRNAs directly target TLR4 receptor. These results suggest that the ability of miRNA to target cytokine secretion is independent of TLR4 expression.

4.1.3 Expression of cytokines in miRNAs expressing monocytes during HIV-1 infection

Results from intracellular cytokine evaluation suggests that miR-20a and miR-106b reduce TNF-α production in THP1 cells and miR-106b was also able to inhibit IL-8 production in these cells. Next, to confirm the effect of candidate miRNAs on cytokines secreted in the supernatant, especially at later time points, the miRNA expressing THP1 cells were similarly stimulated with 1μg/ml LPS and the cytokines released in the supernatant were estimated by ELISA. As shown in Figure 9A, we observed that miR-20a and miR-106b can inhibit TNF-α when stimulated with LPS. The TNF-α released in the supernatant by THP1 cells expressing miR-20a and miR-106b in response to LPS was ~ 4.4 ng/ml, while THP1 cells expressing scramble control had TNF-α of ~ 32.5 ng/ml. miR-124 also demonstrated a relatively small but significant inhibition of TNF-α production. Interestingly, unlike the results in intracellular staining for IL-8, none of our candidate miRNAs were able to significantly affect the IL-8 production in the supernatant as shown in Figure 9C. This is likely due to difference in the time kinetics of cytokines produced in 4 hours versus 24 hours. Evaluation of IL-6 and CCL2 production in these cells, (Figure 9B and Figure 9D) suggests

that miR-20a reduced IL-6 and CCL2 that was minimal and was not significant. THP1 cells expressing miR-106b had IL-6 and CCL2 levels similar to scramble control. However, THP1 cells expressing miR-let-7a significantly inhibited IL-6 and CCL2 production in the supernatant, as predicted by the miRNA-cytokine regulation network. The IL-6 and CCL2 produced by miR-let-7a expressing THP1 in response to LPS were 8.7 ng/ml and 1874 ng/ml, in comparison to 7,214 ng/ml and 10,862 ng/ml respectively in the scramble control

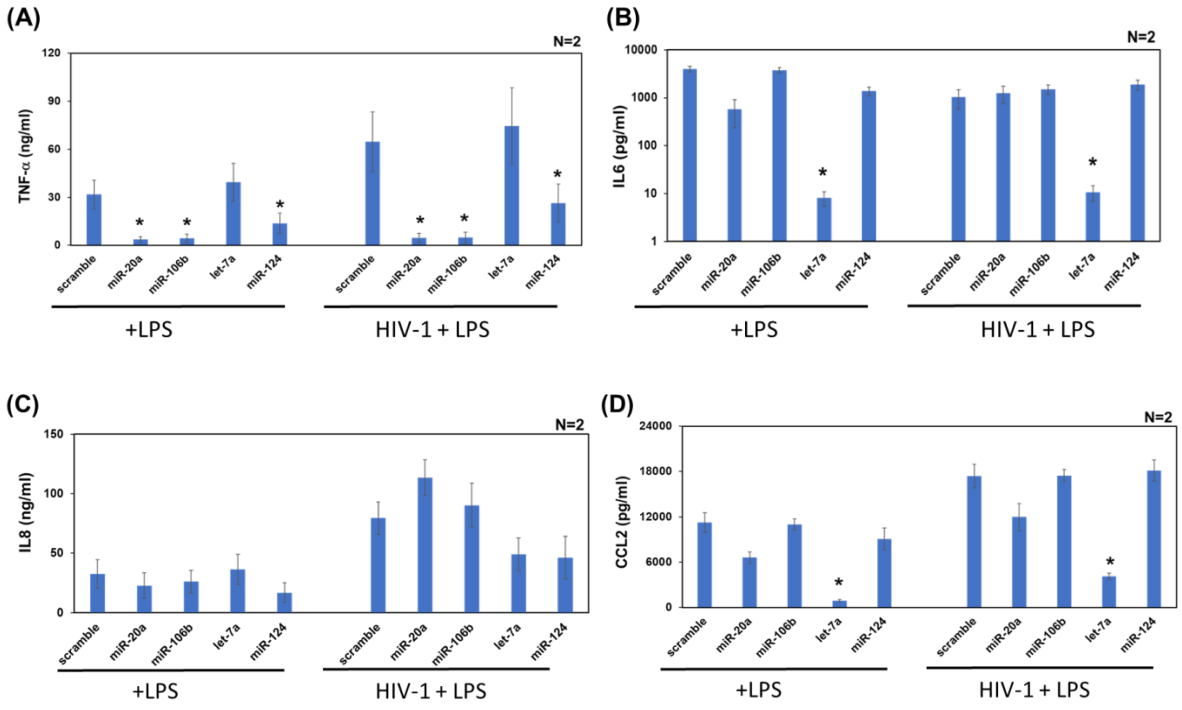


Figure 9. Cytokines production of miRNA expressing monocytes by LPS stimulation in the presence of NL-YU2 virus.

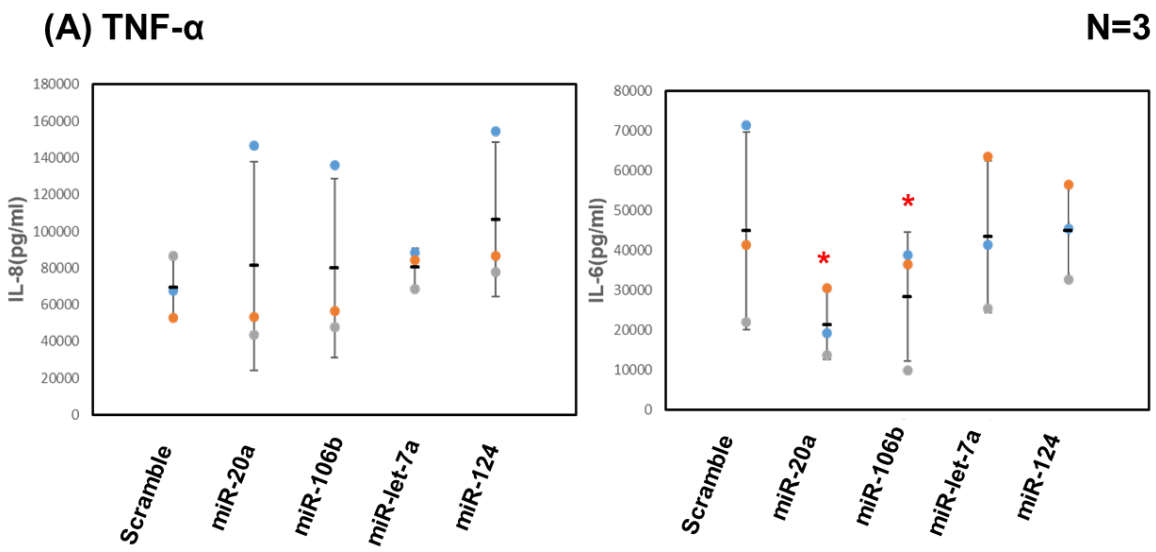
Amount of cytokine secreted were quantified by ELISA for **A.** TNF-α, **B.** IL-6, **C.** IL8 and **D.** CCL2. in supernatants obtained from THP1 cells stimulated with 1μg/ml LPS only (left) for 48 hours plus infection with NL-YU2 virus (right). All the supernatants were harvested after 24 hours post LPS simulation. Figures represent the mean of two experiments, error bars represent the stand deviation between two experiments (*$p < 0.05$).

Next, I evaluated the effects of miRNA in the context of HIV-1 infection, miRNA transduced THP1 cells were infected with 1 MOI of HIV-1 NL-YU2 virus. NL-YU2 virus is a neurotrophic HIV-1 strain isolated from CNS compartment. Forty-eight hours post-infection, the cells were treated with 1μg/ml LPS and the supernatant were collected at 24 hours post stimulation for ELISA analysis. Results suggest that during HIV-1 infection, cytokines were upregulated when compared to uninfected group, especially IL-8 (Figure 9C). As expected, in Figure 9A, we observed a similar patterns of inhibition of specific cytokines by candidate miRNAs, where miR-20a, miR-106b and miR-124 were able to significantly inhibit TNF-α production, and miR-let-7a was able to inhibit IL-6 and CCL2 production even during HIV-1 infection (Figure 9B and 9D). These results suggest that the candidate miRNAs also inhibit specific cytokine production even in the presence of HIV-1infection in monocytic cell line, THP1. Thus, as previously discussed in section 1.3.1, HIV-1 virus can regulate cytokine production to promote infection and viral replication, which may contribute to the elevated level of cytokines during HIV-1 infection. Together, the results suggest that candidate miRNAs have a role in regulating cytokines and may contribute to neuroprotection during HAND pathogenesis.

4.1.4 Expression of cytokines in miRNAs expressing microglia during HIV-1 infection

As previously discussed in section 1.3.1, microglia have a critical role in HAND pathogenesis, and amplify the inflammation of HIV-1 infected macrophages. Microglia also serves as an important intermediate between infected macrophage and neurons in the Macrophage-

Microglia-Astrocyte-Neurons Network. Next, I investigated the role of miRNAs in microglia. Experimental design and methodology to evaluate the role of candidate miRNAs in microglia were similar to the experiments performed with THP1 cells. Briefly, miRNA transduced microglia were stimulated with LPS or TNF-α and the supernatant were collected at 24 hours post-stimulation. Also the changes in cytokine production in response to HIV-1 infection was evaluated. TNF-α was included as an activator in the experiments, since TNF-α is a major cytokine released by infiltrating infected macrophages which can directly activate local CNS resident microglia during HAND neuropathogenesis. Also 10 μg/ml of LPS was used instead of 1 μg/ml LPS since microglia were not responsive to 1 μg/ml LPS (data not shown).



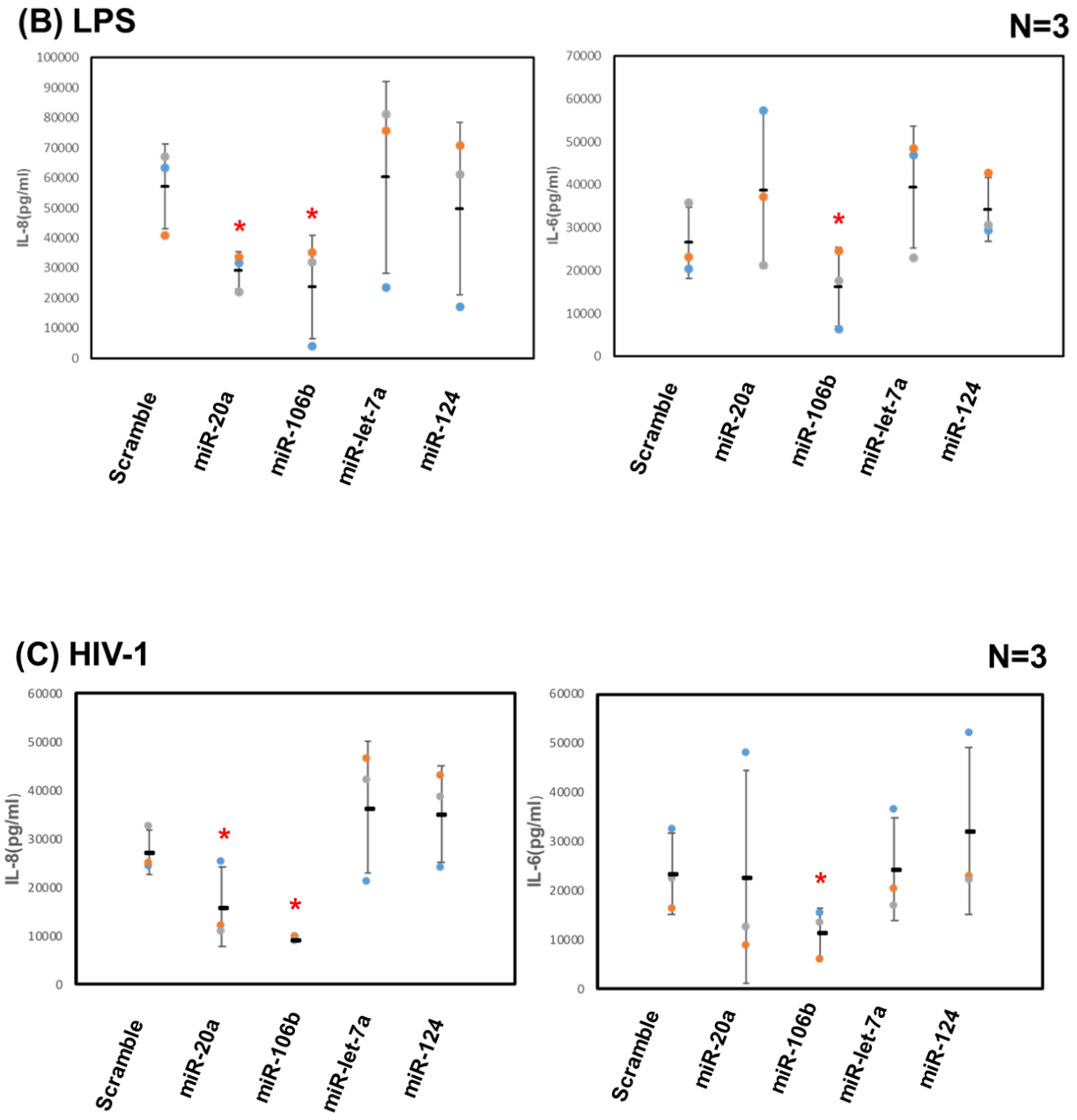


Figure 10. Cytokines production in miRNA expressing microglia stimulated with (A) TNF-a (B) LPS (C) HIV-1 (NL-YU2) infection.

Amount of cytokine secretion in the supernatant were estimated by ELISA (in pg/ml) following stimulation with different stimulators: **A.** TNF-α, **B.** LPS, **C.** NL-YU2 virus. The level of IL-8 secretion was shown on left and IL-6 was shown on right. All the supernatants were harvested after 24 hours of simulation. Error bars represent the stand deviation calculated from three experiments (*$p < 0.05$).

As shown in Figure 10A, we observed that miR-20a and miR-106b significantly inhibited IL-6 production in response to 1 μg/ml of TNF-α. IL-6 released in the supernatant by microglia expressing miR-20a and miR-106b wase 2,040 pg/ml and 2,654 pg/ml, in comparison to an average of 4,380 pg/ml in microglia expressing scramble miRNA. However, no significant inhibition was observed in the production of IL-8 with TNF-α stimulation in the candidate miRNA expressing microglia.

As shown in Figure 10B, IL-8 produced by microglia that expressing miR-20a and miR-106b was half in comparison to the average of 58,000 pg/ml in Scramble miRNA expressing cells. Evaluation of IL-6 in these microglia cells suggests that miR-20a reduced IL-6 from 36,420 pg/ml to 18,320 pg/ml. Interestingly, in monocytes, miR-let-7a was able to reduce IL-6 to a low level compare to scramble but none of miR-20a or miR-106b have shown the ability of IL-8 inhibition. This is likely due to difference in expression of miRNA target transcripts/ protein and the presence of alternate pathways of signal transduction. Results also suggest that certain miRNAs may have a variable response with different stimulators. miR-20a and 106b inhibited IL-8 production, when stimulated by LPS but not with TNF-α; similarly, miR-20a and miR-106b inhibited IL-6 only when stimulated with TNF-α but not with LPS.

Next, to evaluate the effect of HIV-1 infection, miRNA transduced microglia were infected with 1 MOI HIV-1 NL-YU-2 virus. MiR-20a and miR-106b expressing microglia were able to inhibit IL-8 production from the average of 27,300 pg/ml to an average of 14,324 pg/ml and 10,320 pg/ml, respectively, in comparison to Scramble miRNA (Figure 10C). While the evaluation of IL-6 showed that miR-106b inhibited IL-6 by 50%, suggesting that candidate miRNAs are able to

reduce production of cytokines during HIV-1 infection. LPS stimulation was not included post HIV-1 (NL-YU-2) infection for microglia since the infection of virus alone stimulated the microglia to produce up to 46,200 pg/ml of IL-8 and 57,800 pg/ml of IL-6 which is comparable to stimulators.

Together, the results in miRNA-transduced microglia further support the hypothesis that the candidate miRNAs have a role in regulating cytokine production in microglia and monocytes during HIV-1 infection and can play a neuroprotective role by dampening the production of cytokines.

4.1.5 Role of Exosomes derived from miRNAs Expressing Monocytes

Exosomes are small vesicles that act as intercellular messengers for transmission of signaling molecules including cytokines and miRNAs. Thus, exosome may serve as an importance factor in HAND neuropathogenesis. Hence we evaluated the amount of cytokines present in the exosomes and the presence of miRNAs in the exosomes. The amount of cytokine presents in the supernatant harvested from monocytes (FBS-free culture media) and exosome isolated from the same supernatant was evaluated by ELISA. As shown in figure 11A, exosomes derived from miR-20a, miR-106b and miR-let-7a expressing monocytes showed a reduction of IL-6 to 23 pg/ml, 35 pg/ml and 125 pg/ml, respectively, in comparison to 528 pg/ml in Scramble. While similar patterns were observed with the level of IL-8 in exosomes, where we observed that miR-20a, miR-106b and miR-let-7a inhibited IL-8 production in exosomes. However, evaluation of cytokine level in

corresponding supernatant suggest that (Figure 11B), only miR-let-7a and miR-106b inhibited IL-6 and IL-8 production, respectively. This divergence between cytokine in exosomes and supernatant can be due to differences in the time kinetics of cytokine secretion, where the cytokine presents in the exosome represent recent production of cytokine versus the presence of cytokine in the supernatant is the result of accumulation of cytokine released over the entire experimental period (24 hours).

Exosome may also contain miRNAs that can be directly delivered to bystander cells and can be functional. During HAND, miRNA over expressing monocytes or microglia could also have a neuroprotective role by deliveringprotective miRNAs to neurons and other neuronal support cells, such as astrocytes. Thus, a real-time PCR based evaluation was performed to detect the presence of miRNAs in exosomes derived from miRNA overexpressing monocyticcell lines. However, none of the candidate miRNAs were detected in exosomes. This can be due to limit of detection of our PCR assay and its inability to detect low copies of miRNAs present in the exosomes.

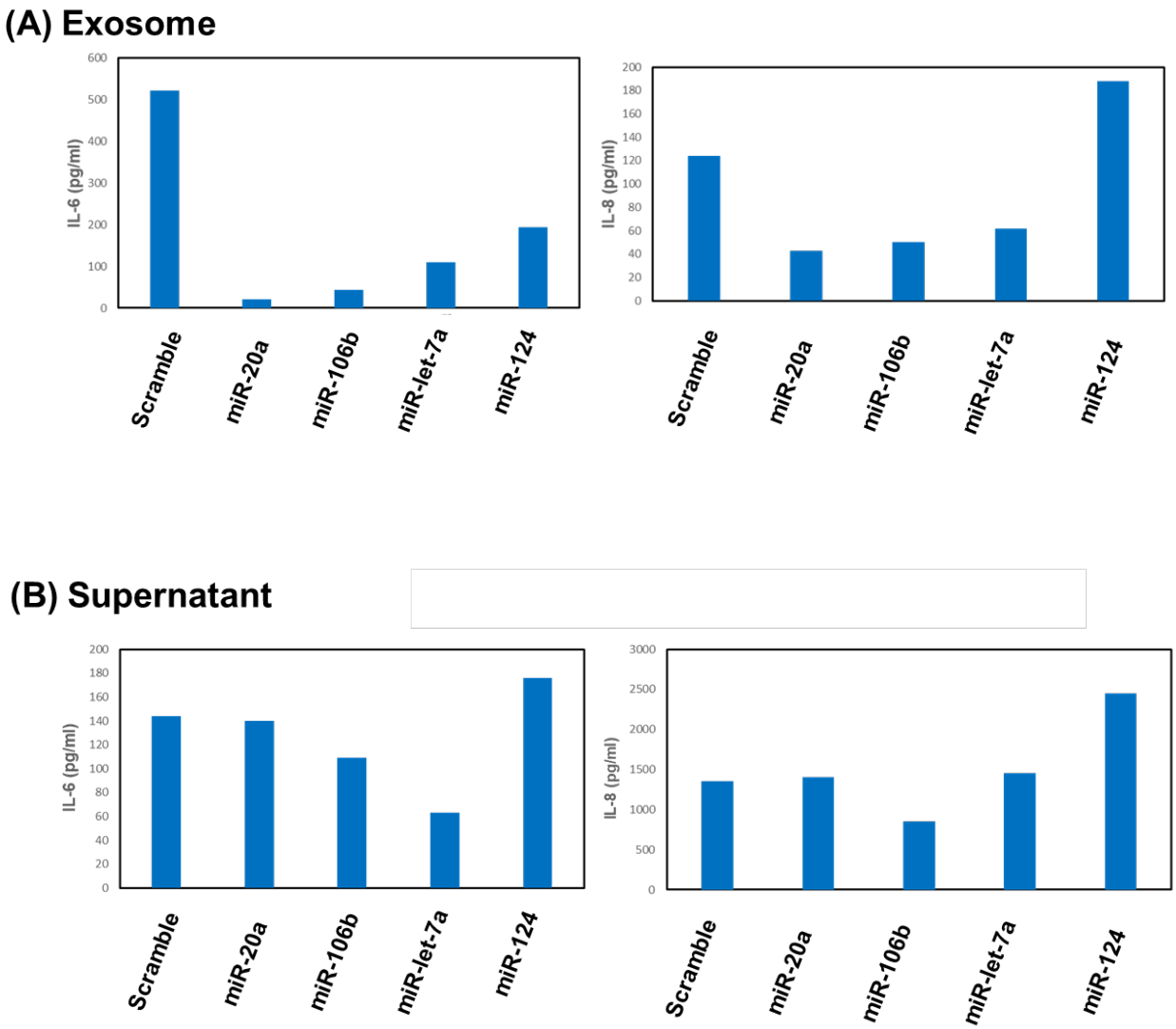


Figure 11. Amount of cytokines in exosomes versus supernatant.

Cytokine production level were estimated by ELISA (in pg/ml) in **A.** Exosome and **B.** supernatant. The level of IL-6 secretion is shown on left and IL-8 is shown on right. The exosomes were extracted from the supernatant and the isolated exosomes were lysed by repeated freeze-thaw cycles.

4.2 AIM 2: INVESTIGATE THE ABILITY OF THESE MIRNA EXPRESSING MONOCYTES TO ACTIVATE LATENT HIV-1 VIRUS USING LATENT HIV-1 REPORTER CELL LINE

In the previous aim, I evaluated the role of candidate miRNAs in regulating neuroinflammatory chemokines/cytokines production in monocytes and microglia. Next, I evaluated if these miRNAs can affect HIV-1 infection. CNS is a potential reservoir for latent HIV-1 virus and CNS viral reactivation can potentially contribute to neuronal toxicity due to the production of viral toxic factors. Thus, investigating the ability of candidate miRNAs to affect virus reactivation and replication is also important.

4.2.1 Characterization of HIV-1 latent reporter cell line by cytokine stimulation

J-Lat cells are derived from Jurkat-based T cell line that harbor an integrated HIV-1 replication defective eGFP reporter virus. The virus does not express HIV-1 envelope and the open reading frame of Nef is interrupted by eGFP gene. The virus in this cell line is latent under normal condition and upon activation results in expression of eGFP reporter. To characterize the ability of cytokines to reactivate the latent virus, $1x10^6$ J-Lat cells were treated with 2-fold serial dilutions of recombinant human TNF-α, IL-6, IL8 and CCL2 proteins, respectively and evaluated for the presence of eGFP by flow cytometry. As shown in the Figure 12A, the reactivation of latent HIV-1 virus in J-Lat cell follows a dose dependent response with increasing concentration of TNF-α. While Figure 12B is a representative figure, other cytokines which do not reactivate latent HIV-1

virus in these cells and are similar to background expression of eGFP. Thus, these results suggest that TNF-α reactivates latent reporter HIV-1 virus in J-Lat cells but not other cytokines included in this study.

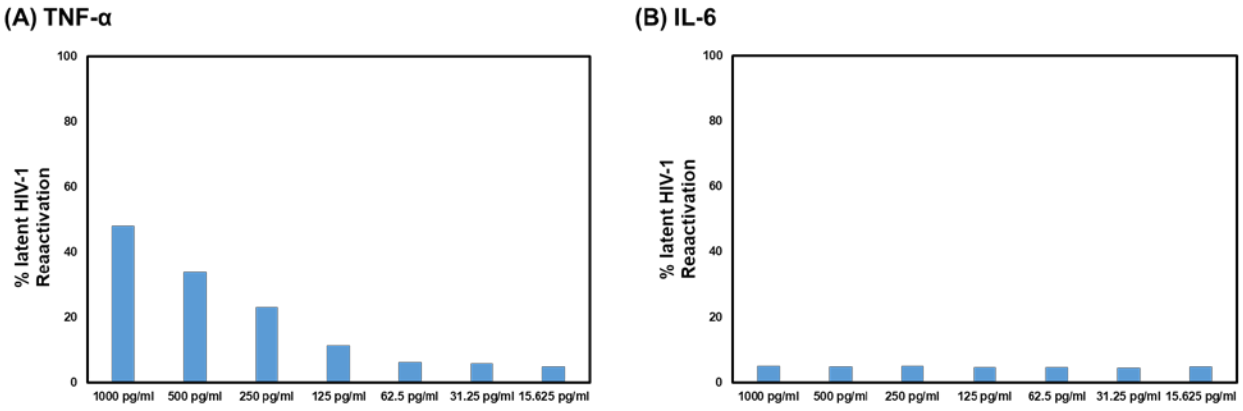


Figure 12. Reactivation of latent HIV-1 in J-lat cells using human cytokines and chemokines.
J-Lat cells were incubated with different concentration (2 fold serial dilution) of human cytokines and chemokines – TNF-α, IL-6, IL8 and CCL2 proteins start from 1000ng/ml. The reactivation of latent HIV-1 virus in J-lat cells were estimated by evaluation of eGFP expression by flow cytometry. Representative data for **A.** TNF-α and **B.** IL-6 are included.

4.2.2 Verify the ability of miRNA transduced monocytes to reactivate latent HIV-1 virus in J-lat cells

Cells harboring latent HIV-1 virus are potentially present in the CNS compartment and the brain-resident memory T cells (bTRM) could also play a role in latent HIV-1 reactivation related to neuronal injury. Thus, investigating the role of monocytes/macrophages in HIV-1 latency regulation in T cells and evaluation of the role of miRNA in this process is also of great importance in HAND. To

evaluate the role of miRNA in monocyte/macrophage-mediated effect on HIV-1 latency, 0.1 million J-Lat cells were treated with the supernatant derived from 1μg/ml LPS stimulated miRNA expressing monocytes for 24 hours and the eGFP expression was evaluated by flow cytometry. As shown in Figure 13, J-lat cells that were treated with supernatant from LPS-stimulated miR-20a and miR-106b expressing monocytes showed ~ 10% eGFP (latent HIV-1 reactivation), in comparison to Scramble which was considered as 100%. While J-lat cells treated with supernatant from LPS-stimulated miR-124 and miR-let-7a expressing monocytes also exhibited more than 50% of reduction in latent HIV-1 reactivation. These results suggest that miRNAs might have an indirect role in regulation of HIV-1 latency reactivation by inhibiting TNF-α production.

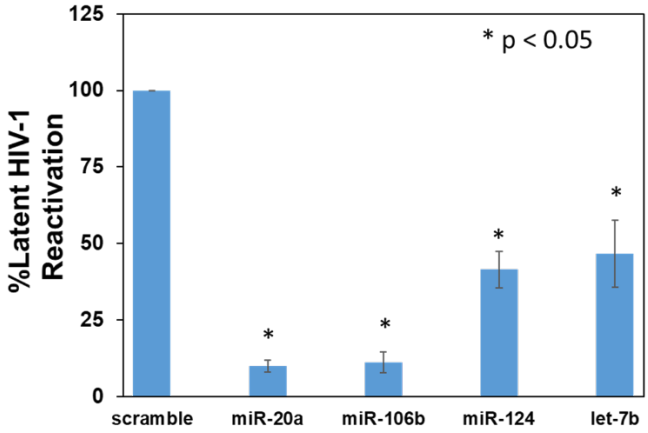


Figure 13. Reactivation of latent HIV-1 in J-lat cells using supernatant from monocytes.
Supernatant from THP1 cells stimulated with 1μg/ml LPS were collected 24 hours post stimulation and incubated with J-lat cells for 24 hours. The reactivation of latent HIV-1 virus in J-lat cells were estimated by evaluation of eGFP expression by flow cytometry. All the values were normalized to scramble, which is considered as 100%.

5.0 DISCUSSION

HIV infection in monocytes/macrophages plays an essential role in the establishment of CNS infection. As we previously discussed, infected monocytes/macrophages cross the blood-brain-barrier and initiate local infection of resident monocytic cells and astrocytes. Through the Macrophage-Microglia-Astrocytes-Neurons network, proinflammatory cytokines and viral proteins secreted by infected immune and neuronal support cells in CNS act as neurotoxins to induce neuronal stress, leading to neuronal dysfunction and subsequently HAND. Multiple studies have observed changes in cytokine profiles during HIV-1 infection of CNS [63, 64] and studies in our lab have demonstrated the differences in miRNA expression during HAND progression [57]. Thus, it will be interesting to verify if miRNA expression is correlated with the changes in cytokine profile and if the identified miRNAs play a role in the regulation of cytokine in CNS.

In our previous studies [57], computational methods predicted that miR-let-7a, miR-124, miR-20a and miR-106b to be the upstream regulators of cellular factors involved in development of HAND. MiR-let-7a and miR-124 were predicted to target chemokine signaling specifically related to IL-8, CCL2 and CXCR4 through RHOG, JUN, MAPK3 and RAF1 and thus could potentially have a neuron-protective role by inhibiting chemotaxis of inflammatory immune cells and reducing associated pro-inflammatory response. While miR-20a and miR-106b, which share seed sequence with miR-17-5p, were predicted to target cytokines including TNF-α, IL1β and IL-6 through DUSP2, NOTCH1 and FOXO1 and thus could potentially protect neurons. Also, other

published studies have identified a role of candidate miRNAs in cytokines regulation. For example, one study demonstrated that miR-124 can prevent monocyte migration and inflammation by targeting CCL2 in pulmonary fibrosis models in mouse [65] and miR-20a is tested to be a negative regulator of inflammation in rheumatoid arthritis [66]. TNF-α, IL1β, IL-6 and CCL2 have been well established to have a role in the onset and progression of HAND [67-69]. Thus, it will be interesting to investigate the effect of candidate miRNAs in targeting cytokines including TNF-α, IL-6 and CCL2 in HAND.

This study aims to investigate the role of specific miRNAs in cytokine expression during HAND using HIV-1 infected monocytes/macrophages and microglia. A lentiviral miRNA expression vector with the advantage of stable integration and stable expression of target gene [70] was used to overexpress miRNA in the cell. The expression of miRNAs were then confirmed by both flow cytometry and qPCR as seen in Figure 6. Although scramble was not detectable at transcriptional level by qPCR, the scramble miRNA expressing lentivirus transduced cell line has more than 95% of eGFP positive cells as detected by flow cytometry. This failure of detection of scramble can be due to inefficient design of scramble miRNA specific primers.

Intracellular analysis of cytokines, TNF-α and IL-8 suggest that miR-20a, miR-106b significantly inhibited TNF-α production and miR-106b also reduce IL-8 production in monocytes (Figure 7). Follow-up studies to confirm the role of miRNAs to affect cytokines production in the supernatant by ELISA suggest that miR-20a, miR-106b were also able to significantly inhibits TNF-α production which further support the results from intracellular staining (Figure 9). However, differences exist in the IL-8 results between intracellular cytokine staining (Figure 7B) and

estimation in supernatant by ELISA, (Figure 9C) indicating that miRNA mediated effect may have a time dependent effect, 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.

To determine whether the tested candidate miRNAs have similar cytokine inhibition effects during HIV-1 infection, HIV-1 NL-YU2 virus was used in the study. Previous studies have shown that HIV-1 infection increased the production of pro-inflammatory cytokines to promote infection and viral replication [71, 72]. Thus, it was not surprising to observe elevated cytokine production during HIV-1 infection. Notably, miR-20a, miR-106b consistently showed the function of inhibition of TNF-α production, while miR-let-7a was also able to inhibit IL-6 and CCL2 production (Figure 9), indicating that miRNAs inhibit cytokines production even in the presence of HIV-1 infection. These results suggest that the candidate miRNAs can inhibit the proinflammatory response amplified by HIV-1 infection.

Microglia have a major role in HAND pathogenesis, as the resident phagocytic cells, microglia support HIV-1 infection and helps in amplification of inflammatory reaction which are detrimental for optimal functioning of neurons. The role of candidate miRNAs was evaluated in microglia. Results (Figure 10) suggest that miR-20a and miR-106b inhibit IL-6 production upon TNF-α stimulation and miR-20a and miR-106b reduce IL-8 production with LPS stimulation, while, during HIV-1 infection, miR-20a and miR-106b inhibit IL-8 production. These results support our hypothesis that specific candidate miRNAs have a role in controlling cytokines production. However, higher concentration of LPS was needed to activate microglia. This may

explain by the fact that microglia are mainly responsible for the maintenance of the neurons, while the macrophages are specialized in phagocytosis of pathogens and antigen presenting role thus, lead to different sensitivity between macrophage and microglia towards endotoxins [73]. 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.

Exosomes were also evaluated in this study (figure 11) to understand the relationship between exosomes and cytokines level. We found that exosomal contents derived from miR-20a, miR-106b and miR-let-7a expressing monocytes cell lines showed a reduction of IL-6 and IL-8 while the corresponding supernatant derived from miR-let-7a and miR-106b cell lines shown the cytokines IL-6 and IL-8 are inhibited, respectively. These differences could be due to the time kinetics of production/secretion of cytokines regulated by miRNAs.

Exosome also serve as the delivery machine for miRNA [60]. Thus, it is possible that the neuronal protection can be the direct consequence of miRNA delivery though the exosomal communication between neuron and support cells like microglia and astrocytes. For this reason, future experiment should also focus on the indirect role of miRNAs.

In addition, the ability of these miRNA expressing monocytes and macrophages to activate virus reactivation were also evaluated. In figure 13, all four miRNAs especially miR-106b and miR-20a were found to reduce latent HIV-1 reactivation in J-Lat cells. As it shown in figure 12, TNF-α was a major host factor responsible for reversal of HIV-1 latency in J-Lat cells. Other studies have shown that TNF-α is capable of reactivating the latent virus through nuclear factor

binding to the NF-kB sites in the LTR [21]. Thus, we believe that miRNAs have a role in inhibition latency reactivation and viral replication by inhibiting cytokine secretion upon stimulation.

6.0 FUTURE DIRECTIONS

Results strongly support that miR-20a and miR-106b have the ability to reduce cytokine production in monocytes and microglia cell lines. However, current study only focused on studying the function of individual miRNA. Thus, evaluating combinations of candidate miRNAs could be utilized to further potentiate the effects of cytokine inhibition. Also, my focus in the current study was to evaluate the role of miRNAs on cytokines and chemokines that have previously identified to have a major role in HAND pathogenesis. Follow up experiments to evaluate the direct and indirect effect of miRNA on neurotoxic and neuroprotective factors will be very useful, similar the effect of miRNA in transduced cells and the effect on bystander cells will help to design better therapeutics for HAND.

As previously discussed, miRNA can also directly or indirectly affect HIV-1 virus replication and production of viral proteins. In this study, only the function of miRNAs on HIV-1 host factors have been investigated while the viral factors could also play an important role in the neuropathogenesis of HAND. It has been reported that number of miRNAs could direct target HIV-1 RNA through base pair complementarity. For example, miR-28 and miR-150 have been shown to target the 3′ UTR of viral mRNAs to inhibit viral translation[74]. Thus, future studies could focus on whether our candidate miRNAs are able to control the viral replication and viral protein production.

In addition, the current study utilized monocytes and microglia cell lines to study the effect of candidate miRNAs. In future, inclusion of monocytes derived macrophage model will be valuable to study the function of candidate miRNAs in primary cells. While in Aim 2, HIV-1 latency reactivation study was based on the J-lat cell, which is a Jurkat-based T cell line. *In vitro* primary models of HIV-1 latency such as Sahu and Tyagi models could be used to study the effect of candidate miRNAs in primary HIV-1 latency models.

Furthermore, a 3D model of macrophage, microglia and neurons should be developed to investigate the role of candidate miRNAs in the Macrophage-Microglia-Astrocytes-Neurons network. Thus, we can evaluate the role of candidate miRNAs in 3D CNS organoid *in vitro* model similar to those present in brain.

7.0 PUBLIC HEALTH SIGNIFICANCE

HIV-1 infection is a global public health concern and various methods have been developed to control the pandemic of HIV-1 infection. Among them, the cART therapy has achieved a great success in improving HIV-1 patient life quality. As a result, majority of HIV-1 patients are not suffering from acquired immunodeficiency syndrome (AIDS) nowadays, but many of them are still experiencing HIV-1 associated comorbidities. Among those comorbidities, HIV associated neurocognitive disorder (HAND) is observed in more than half of all HIV-1 patients in a mild form in post-cART era. Clinically, the severity of HAND can be divided into three stages: asymptomatic neurocognitive impairment (ANI), mild neurocognitive disorder (MND), and HIV associated dementia (HAD). The successful coverage of highly active anti-retroviral therapies (HAART) has significantly lower the incidence of HAD and MND. However, the ANI remains as the most common co-morbidity of HIV-1 infection. Nearly more than half of individuals living with HIV-1 infection present with some degree of neuronal impairment. Currently, no treatment is available to prevent or treat HAND or ANI. Thus, finding novel methods to manage HAND is of public health significance to improve quality of life in HIV-1 population. 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. Thus, these miRNAs could have the potential as therapeutics to minimize neuronal dysfunction resulting from HAND by preventing neuroinflammation.

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