The Dissertation Committee for Edrous Alamer Certifies that this is the Approved Version of the Following Dissertation:

Modulation of Host BRD4 to Repress HIV Replication in Myeloid Cells: Major HIV Reservoirs in Central Nervous System

Committee:

Dr. Haitao Hu, MD, PhD; Mentor

Dr. Lynn Soong, MD, PhD; Co-mentor

Dr. Janice Endsley, PhD

Dr. Shao Jun Tang, PhD

Andrew P. Rice

Dr. Andrew Patrick Rice, PhD

Dean, Graduate School

Modulation of Host BRD4 to Repress HIV Replication in Myeloid Cells: Major HIV Reservoirs in Central Nervous System

by

Edrous Sadig I. Alamer, BS, MS.

Dissertation

Presented to the Faculty of the Graduate School of

The University of Texas Medical Branch

in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

The University of Texas Medical Branch

May 2020

Dedication

Dedicated to my beloved mother **(*Fatma*)** and father **(*Sadig*)**, for teaching me perseverance and for their love, endless support, and encouragement. My brothers and sisters for their countless assistance through this important time of my life. Deeply devoted to my wonderful and lovely wife, ***Omaymah***, for the love, and friendship during all these years of graduate school, whose unending support gave me the strength to persevere. A profound dedication to my son, ***Battal***, for being a close friend and an absolute inspiration. This would have been a much tougher 5 years without you. Thank you for everything!

Acknowledgements

This dissertation won't be possible without the support, guidance, and training of my mentors, Dr**. Haitao Hu** and Dr. **Lynn Soong**. I would like to thank them deeply for their mentorship, invaluable motivation, and availability for listening to my presentations, reviewing manuscripts and giving scientific input and answers to my questions, and giving me the opportunity to grow as a scientist. I would like to extend special thanks to all the former laboratory members: Dr. Qingli Niu, Dr. Fengliang Liu, Xiuzhen Fan, Sarah Auclair and the current members: Chaojie Zhong, Fuquan Long, Lulu Guo for their help and support in the past few years. I would like to thank my dissertation committee members: Drs, Janice Endsley, Shao Jun Tang, and Andrew Patrick Rice, for their agreement to be members of my PhD committee and for their enthusiasm and for providing excellent scientific insight and suggestions that made my project much stringent. I would also like to thank the Microbiology and Immunology graduate program director, Dr. Lynn Soong for her help, guidance and support throughout my years at UTMB. Finally, I would like to thank my wife, my parents and all my family members. The journey of a graduate student is a long journey that could not have been possible without their continuous love, understanding, and support. Special thanks also to Jazan University and to the Ministry of Education in Saudi Arabia for the generous scholarship that supported my graduate studies in the USA.

Modulation of Host BRD4 to Repress HIV Replication in Myeloid Cells: Major HIV Reservoirs in Central Nervous System

Publication No._____

Edrous Sadig I. Alamer, PhD

The University of Texas Medical Branch, 2019

Supervisors: **Haitao Hu** and **Lynn Soong**

Brain-resident microglia and myeloid cells (perivascular macrophages) are important HIV reservoirs in vivo, especially in central nerve system (CNS). Despite anti-retroviral therapy (ART), low-level persistent HIV replication in these reservoirs remains detectable, which contributes to neuroinflammation and neurological disorders in HIV-infected patients. New approaches complimentary to ART to repressing residual HIV replication in CNS reservoirs are needed. Our group has recently identified a BRD4-selective small molecule modulator (ZL0580) that induces epigenetic suppression of HIV. Here, we examined the effects of this compound on HIV in human myeloid cells. We found that ZL0580 induces potent and durable suppression of both induced and basal HIV transcription in microglial cells (HC69) and monocytic cell lines (U1 and OM10.1). Pre-treatment of microglia with ZL0580 renders them more refractory to latent HIV reactivation, indicating epigenetic reprogramming effect of ZL0580 on HIV LTR in microglia. We also demonstrated that ZL0580 induces repressive effect on HIV in human primary monocyte-derived macrophages (MDMs) by promoting HIV suppression during ART treatment. Mechanistically, ZL0580 inhibits Tat transactivation in microglia by disrupting binding of Tat to CDK9, a process key to HIV transcription elongation. High-resolution MNase mapping identified that ZL0580 induces repressive chromatin structure at the HIV LTR. Taken together, our data suggest that ZL0580 represents a potential approach that could be used in combination with ART to suppress residual HIV replication and/or latent HIV reactivation in CNS reservoirs, thereby reducing HIV-associated neuroinflammation.

TABLE OF CONTENTS

List of Tables

List of Figures

List of Abbreviations

HIV	Human immunodeficiency virus
SIV	Simian immunodeficiency virus
CDC	Centers for Disease Control and Prevention
AIDS	Acquired Immune Deficiency Syndrome
ART	Antiretroviral therap
Tat	Transcriptional transactivator
Vif	Viral infectivity factor
Vpr	Viral protein r
Vpu	Viral protein u
DCs	Dendritic cells
RNAP-II	RNA-Polymerase II
LEDGF	Lens epithelium-derived growth factor
nAbs	Neutralizing antibodies
NRTIs	Nucleoside reverse transcriptase inhibitors
NNR-TIs	Non-nucleoside reverse transcriptase inhibitors
NF- ϰB	Nuclear factor kappa B
HAND	HIV-associated neurocognitive disorders
CNS	Central nerve system
BBB	Blood Brain Barrier
CSF	Cerebrospinal fluid
HAD	HIV- associated dementia
NFAT	Nuclear factor of activated T cells
SP1	Specificity protein 1
TFIID	Transcription factor II D
Nuc	Nucleosome
TSS	Transcription start site
HATs	Histone acetyl transferases
CTD	Carboxyl terminal domain
NELF	Negative elongation factor
DSIF	DRB sensitivity-inducing factor
p-TEFb	Transcription elongation factor complex
CDK9	Cyclin-dependent kinase 9
FACT	Facilitates chromatin transcription
HEXIM-1	Hexamethylene bisacetamide-induced protein
7SK snRNA	7SK small nuclear RNA
BRD4	Bromodomain-containing protein 4
H3K9me3	Histone trimethylation of histone H3 lysine 9 trimethylation
H3K27me3	Lysine 27 trimethylation
HMT	Histone methyltransferase
HDAC	Histone deacetylases
YY1	Yin yang 1
LRAs	Latency-reversing agents
PKC	Protein kinase C
TLRs	Toll-like receptors

BET	Bromodomain and extraterminal
DNMTi	DNA methyltransferase inhibitors
LPAs	Latency promoting agents
HSP90	Heat shock protein 90
dCA	didehydro-cortistatin A
BDs	Bromodomain
TFs	Transcription factors
BRDT	Bromodomain testis specific protein
JMJD6	JmjC domain-containing protein 6
PBMCs	Peripheral blood mononuclear cells
MDMs	Monocyte Derived Macrophages
TEER	Transendothelial electrical resistance
GFP	Green fluorescent protein
HPLC	High performance liquid chromatography
MNase	Micrococcal nuclease
Co-IP	Co-immunoprecipitation
CHIP	Chromatin immunoprecipitation

CHAPTER I: General Introduction

1. History, Epidemiology and Classification

Human immunodeficiency virus (*HIV*) is thought to be transmitted in the early 20th century as a result of cross-species transmission of simian immunodeficiency virus (*SIV*) from chimpanzees or gorillas into human beings [1]. In 1981, the Centers for Disease Control and Prevention (*CDC*) reported the observations of rare opportunistic infections in several gay men [2, 3], which was then called "gay-related immune deficiency" [4]. Concurrently, more cases were also reported in several heterosexual partners of infected patients, IV drug users, and some recipients of blood transfusion. Based on these observations, the *CDC* speculated that the syndrome is probably caused by an infectious agent which can be transmitted by blood and other body fluids and this syndrome was then termed *Acquired Immune Deficiency Syndrome (AIDS)* [5]. Later, the agent causing *AIDS* was discovered in 1983 at the Pasteur Institute in France [6], and a year later the virus was also independently discovered by Dr. Robert Gallo research group [7]. It is estimated that by the end of 2018, almost 74.9 million have been infected with HIV, and 32 million have died from *AIDS*-related illnesses. Of those who are currently infected with HIV, almost 79% know that they are infected, and 62% are receiving antiretroviral therapy (ART); 43-63% are virally suppressed [8].

HIV is a member of *lentivirus* which belongs to *Retroviridae* family [9]. The mature forms of HIV virions have envelopes, with 100–120 nm in diameter. There are two strains of HIV: HIV-1 (which was previously described) and HIV-2 which was identified in 1986 [10] and has low viral replication in humans and hence less pathogenesis [11]. There are 4 groups of HIV-1: the main (M), outlier (O), non-M, non-O (N) and P [12]. Group M accounts for almost 98% of global isolates which is subtyped into 9 clades A, B, C, D, F, G, H, J, and K, respectively [12].

2. HIV Biology

2.1. Genome and Virion Structure

HIV is a positive-sense, single-stranded RNA genome. The genomic RNA is 10 kb in length which consists of nine genes: structural (*gag, pol, env),* non-structural *(vif, vpr, vpu, tat, rev,* and *nef)*. *Gag* polyprotein p55 is encoded in the gag gene which is further processed by viral protease to p17 (matrix), p24 (capsid), p7 (nucleocapsid), and p6 proteins. The *pol* gene encodes the *pol* polyprotein p160 which is further processed into three viral enzymes: protease p11, reverse transcriptase p65/51 and integrase p32. The *env* gene encodes for the precursor gp160 which is cleaved by cellular protease into two envelope proteins surface gp120 and transmembrane gp41. Cleavage by viral and cellular proteases are essential for virion maturation and the production of infectious virions. *Tat* (transcriptional transactivator), *rev* and *Nef* encode for proteins which are essential for HIV replication. The other gene-encoding proteins, *Vif* (viral infectivity factor), *Vpr* (viral protein r), and *Vpu* (viral protein u) have roles in evading the innate restriction factors *APOBEC3G*, *SAMHD1*, and *tetherin*, respectively (**Fig. 1A and B**). The mature viral particle consists of two copies of identical single stranded RNA genomes which are surrounded by nucleocapsid proteins. The viral genome is capped at 5'-end and polyadenylated at 3' end and are found associated with viral reverse transcriptase and integrase enzymes. The genomes and viral enzymes are shelled with a capsid consisting of p24 viral protein. The capsid is surrounded by a matrix, P17 which is enveloped in lipid membrane which carries spike protein consisting of trimeric gp120-gp41 complexes (**Fig. 1C**) [13, 14].

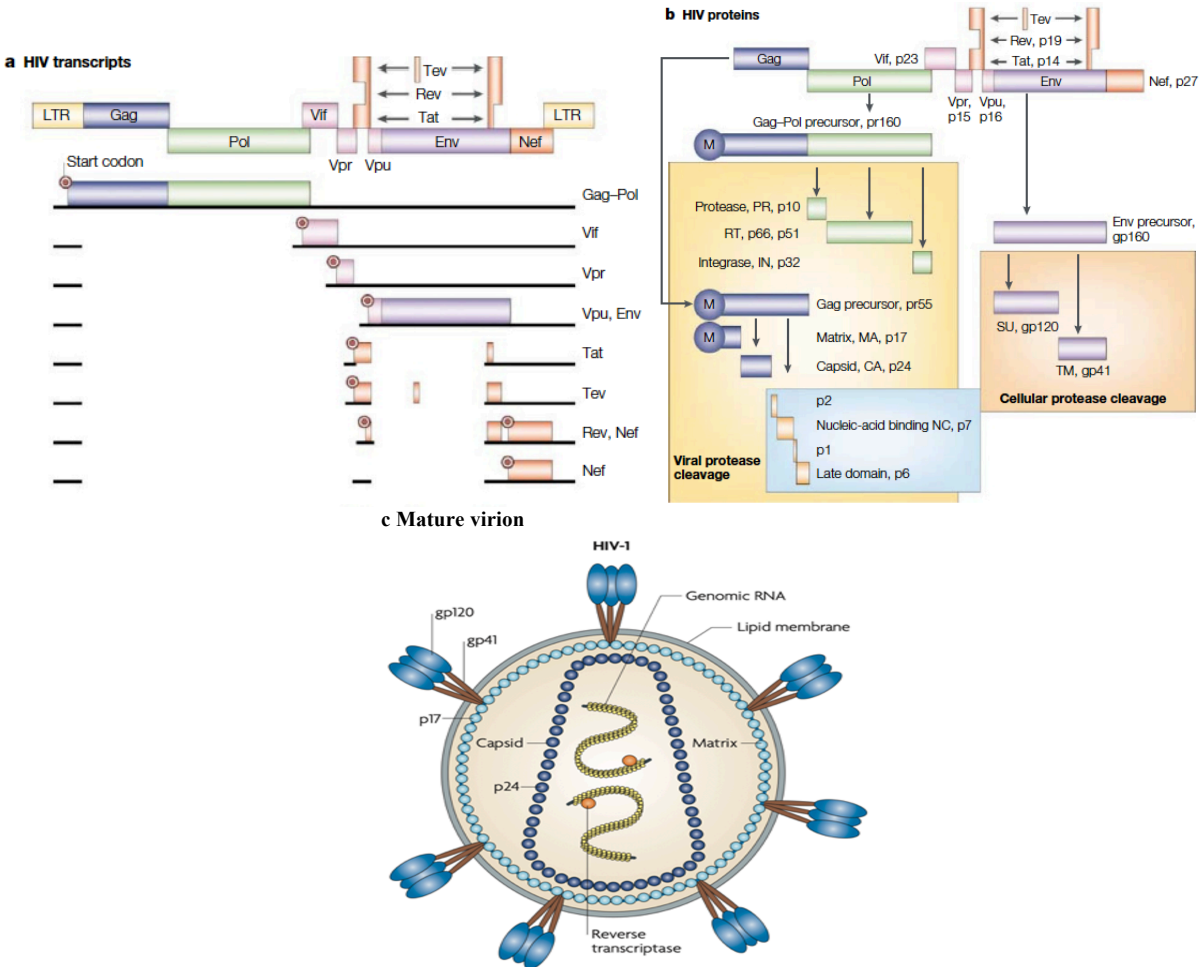

Fig 1. A schematic depiction of HIV genome and mature virion. (A-B) HIV genomic RNA consists of nine genes: structural (*gag, pol, env),* non-structural *(vif, vpr, vpu, tat, rev,* and *nef)* which can be transcribed and translated to different viral proteins. **(C)** The mature viral particle carries two copies of single stranded RNA genomes, associated with viral reverse transcriptase, and integrase enzymes and surrounded by nucleocapsid proteins. The genomes and the viral enzymes are shelled with a capsid consisting and a matrix, which carries spike protein composed of trimeric gp120-gp41 complexes (Adapted from Karlsson Hedestam, Nat Rev Microbiol, 2008; Peterlin, B.M, Nat Rev Immunol, 2003).

3. Replication Cycle

HIV can infect a wide range of cells, including CD4-expressing cells [T helper cells, monocytes, macrophages, and dendritic cells (DCs) and microglia [15, 16], as well as astrocytes through a CD4-independent mechanism [17]. The infection starts when the envelope spike protein (gp120) binds to CD4 receptors. This interaction induces conformational changes and the formation of a bridging sheet between the inner and the outer domains of the gp120

monomer, exposing the secondary binding site to either CCR5 or CXCR4 cellular coreceptors. Based on the coreceptor used, HIV strains are classified into CCR5-tropic (R5 HIV), CXCR4-tropic (X4 HIV), or dual tropisms (R5X4 HIV) [18]. X4 HIV infects CD4 T cells more efficiently than R5 HIV, and this strain dominates in the late stages of infection [18]. The binding of gp120 to cellular receptor and coreceptor leads to viral fusion to the cell membrane and subsequent uncoating and release of viral particle contents into host cell cytosol. The reverse transcriptase then transcribes the single-stranded RNA genome into double-stranded DNA, yielding pre-integration complex (PIC). PIC is then imported into the nucleus, where it can integrate into the host genome by PIC-associated integrase and aided by host chromatin binding protein lens epithelium-derived growth factor (LEDGF). The integrated provirus can be transcribed by host transcription machinery (RNA-Polymerase (RNAP-II) and p-TEFb, which will be discussed in detail later) or remains latent for the life of the cell. The transcribed DNA produces RNAs which are alternatively spliced to give mRNAs for multiple viral proteins as well as genome-length RNAs which are incorporated into the newly formed virions. The viral proteins and the genomic RNA are assembled into immature virions which can bud out of the host cells, resulting in the formation of enveloped membrane. The viral protease then cleaves the Gag polyprotein to produce mature virions (**Fig. 2**) [19].

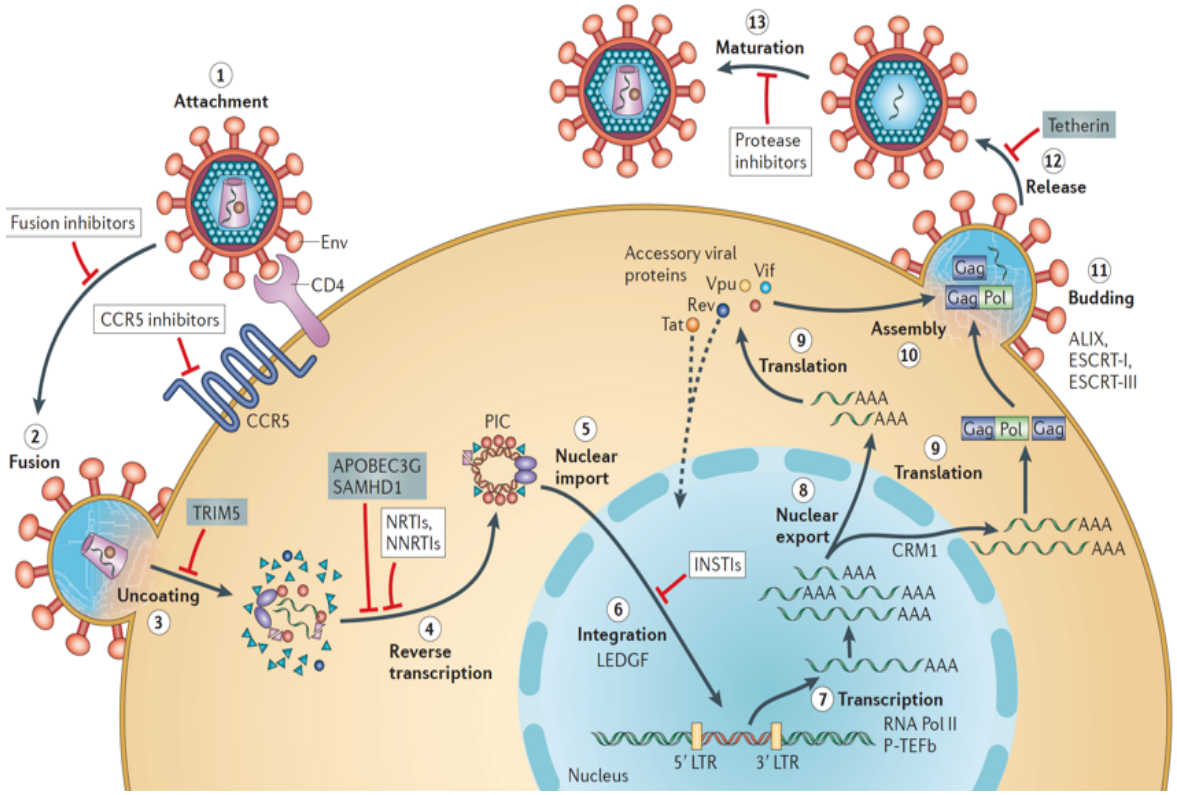

Fig 2. Schematic overview of HIV-1 replication cycle. Binding of infectious virion to the cellular receptor CD4 allows viral fusion, entry to the cell and uncoating of viral contents. Viral genomic RNA reverse-transcribes, forming pre-integration complex (PIC) which is imported into the cell nucleus. The viral DNA integrates into the host genome to form provirus with the aid of viral integrase and host chromatin binding protein lens epithelium-derived growth factor (LEDGF). The provirus is subjected to host transcription machinery and serves as a template for viral replication. Proviral transcription yields mRNAs, which serve as templates for several proteins as well as genome-length RNA which is incorporated into viral particles to produce new virion. The newly formed virion then buds out of cell and is subjected to maturation with the encoded viral protease to produce fully mature and infectious viral particles. Different steps in the HIV life are potential targets in HIV therapy (Adapted from reference 19).

4. HIV Pathogenesis

HIV is a bloodborne virus which can be transmitted by blood and other body fluids including seminal fluid, rectal fluids, vaginal fluids, and breast milk [11]. People with high risk of acquiring HIV infections include men who have sex with men, IV drug users, and sex workers. The virion characteristics such as higher envelope contents, and resistance to IFNα

increase HIV infectivity. The susceptibility to infection increases in HIV negative individuals who have larger number of activated $CD4^+$ T cells at the sites of exposure due to trauma or ongoing infection with other sexually transmitted agents [11]. HIV enters the body mostly through mucosal surfaces of the genital or gastrointestinal tract which harbors high populations of lymphocytes and macrophages [20]. The existence of large populations of 'resting' host $CD4^+$ T cells in the mucosal tissues allows initial viral replication and subsequent spread through bloodstream to the lymphoid tissues [21]. Intense HIV replication then follows in gastrointestinal tract, spleen, and bone marrow which ultimately results in massive infection of susceptible cells [22]. The primary infection is characterized by peak of viremia (10^6 to 10^7 copies per ml of plasma) around 21-28 days post-infection [23], and pronounced depletion of activated as well as memory $CD4^+$ T cell subsets (**Fig. 3**) [22]. Several mechanisms can mediate the depletion of $CD4^+$ T cells, including direct cytopathic effects to the infected cells [11], and pyroptosis and apoptosis of uninfected cells [24, 25]. The cytotoxic T lymphocytes (CTL) then control viral replication and end the acute phase of infection [26]. It takes several (≥ 12) weeks for the production of neutralizing antibodies (nAbs) which fail to fully combat the already generated escape mutants [27, 28]. Clinical latency phase then ensues where the $CD4^+$ T cell counts rebound and the acute intense viral replication is markedly reduced [29]. If infection is left untreated, ongoing viral replication results in generation of HIV-specific CD8 T cells, activation of lymphocytes and/or macrophages by gp120 and Nef, and the release of pro-inflammatory microbial products due to microbial translocation of the gut mucosa [30, 31]. Persistent immune activation can lead to immune system exhaustion [32, 33]. This results in uncontrolled viral replication and significant depletion in $CD4^+$ T cells and finally progressing into end stage of HIV infection, *acquired immunodeficiency syndrome* (*AIDS*). AIDS can be reached within an average of 8 years post infection (except for elite controllers), and this stage is characterized by low levels of $CD4^+$ T cells (≤ 200/μL) and/or the emergence of HIV-

associated opportunistic infections). Additionally, several types of cancers are also reported during AIDS stage [22].

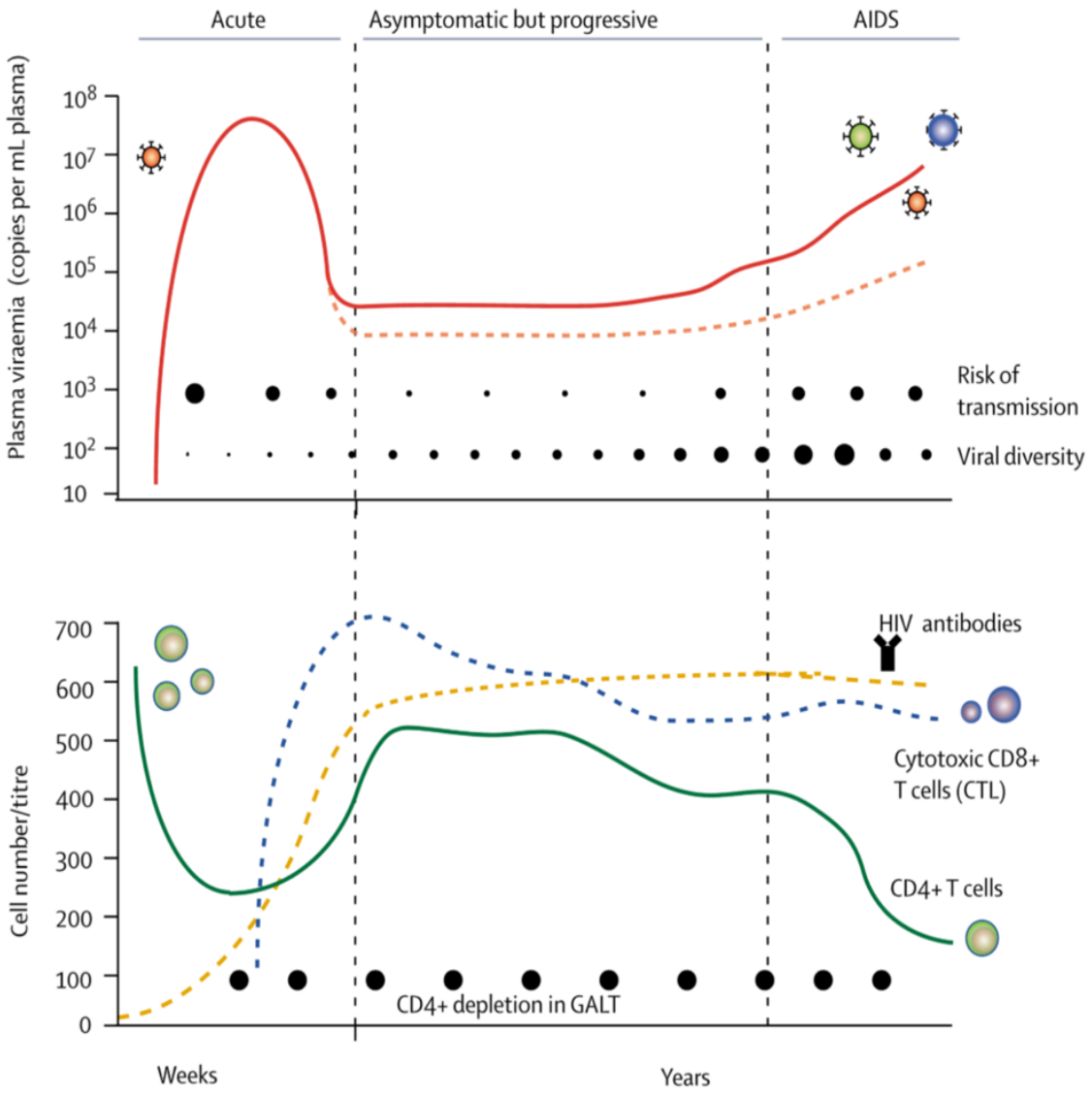

Fig 3. The clinical course of HIV-1 infection. The primary infection is characterized by peak of viremia (red line, top), decrease in $CD4^+$ T cells (green line, bottom), and the lack of HIV-1 specific antibodies (orange line, bottom). $CD8^+$ T-lymphocytes (CTL) then develop (blue line, bottom) resulting in reduction in plasma viral loads (Adapted from reference 22).

5. HIV Therapy and Clinical Outcomes

ART has improved the survival and quality of life of HIV-infected individuals, changing the course of HIV from a life-limiting infection to a chronic disease. ART has significantly reduced the number of new infections [34] and also resulted in 50% decline in the rate of HIV-related deaths since its introduction in the mid-1990s [35] (approximately 1.2 million are averted from deaths annually) (**Fig. 4**). Several classes of ART which target different steps of HIV life cycle are used for the treatment of HIV-1 infections. These include blocking reverse transcription (nucleoside reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNR-TIs), fusion inhibitors, CCR5 co-receptor antagonists, protease inhibitors, and integrase inhibitors (**Fig. 2**) [36]. Combination treatment with at least three different ART regiments is now the standard treatment for newly HIV-infected individuals [37]. ART can effectively suppress active HIV replication and de novo infections. However, ART cannot eradicate or cure the virus since the virus integrates its provirus into the host genome and establishes stable latent infections [38, 39]. Basal/low levels of viremia during proper ART regimen [40, 41], as well as rapid viral rebound during ART treatment interruption [42, 43], were reported in HIV-infected individuals. Several mechanisms are believed to contribute to basal HIV replication during ART including low penetration of ART to tissues, cell-to-cell virion transmission, presence of immune sanctuaries and intermittent activation of latently infected cells due to potential exposure to environmental antigens [29]. Notably, markers of immune activation are observed in ART-suppressed individuals. There are several factors which might contribute to immune activation during ART, including basal/low level of HIV replication in addition to other factors such as CMV and EBV reactivations, and the increased gut bacterial translocation due to HIV infections [31]. Certain ART drugs were also reported to promote immune activation and inflammatory responses [44]. Persistent immune activation during infection increases the intracellular

nuclear factor kappa B (NF-ϰB) levels which further enhance HIV provirus transcription. A repeated cycle in which immune activation induces HIV transcription and the induced HIV transcription promotes immune activation is established [45]. It is postulated that the ongoing low levels of HIV transcription during ART may lead to emergence of ART resistant strains [29]. More recently, it was reported that more than 10% of HIV-infected adults developed resistance to the given ART. This has increased the worrisome levels toward HIV [46], as it is considered unsafe to continue the same medications since resistance could expand further among other groups [46].

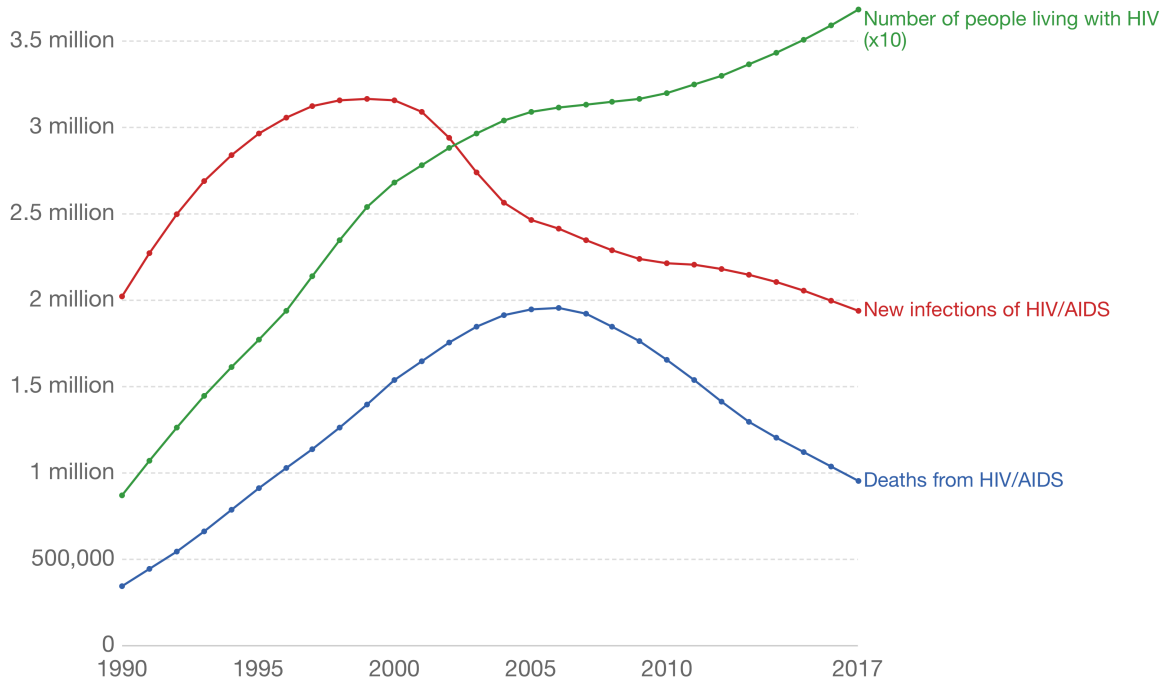

Fig 4. Prevalence, new cases, and death from HIV/AIDS (Adapted from reference 19).

6. HIV Neuropathy and the Role of Myeloid Cells in HIV Neuropathogenesis

HIV has a distinct neurovirulence, resulting in neurocognitive impairment in about 50% of infected individuals, compared to other neurotropic viruses in which only less than 5% of cases show neurological symptoms [47]. Since the introduction of ART, the prevalence of HIV-associated neurocognitive disorders (HAND) remain the same to the pre-ART era [35]. This is presumably to be due to the increased survival of individuals with milder neurological disorders as well as the suboptimal efficacy of ART in brain or related to both reasons [35]. Several symptoms are presented in HAND including memory impairment, disruption in multitasking, attention, impulse control, memory encoding and retrieval, and in some cases bradykinesia, loss of coordination and gait imbalance [35].

Following infection, $HIV^{+}CD14^{+}CD16^{+}$ monocytes [48] transmigrate across the blood–brain barrier (BBB) in response to chemotactic signals where they can establish infection within the central nerve system (CNS). It is generally believed that trafficking monocytes are the main source of HIV in the brain [20], infecting microglia, vascular macrophages and astrocytes (**Fig. 5**). Once HIV provirus becomes integrated into the host-cell genome, the virus becomes latent for years. HIV reservoirs in the brain are established within 3 to 5 days following infection [49]. The brain is considered a sanctuary site for HIV replication due to poor penetration of ART which is imposed by BBB as well as sequestering of HIV in long lived, non-cytopathic cells (brain-resident macrophages and microglia) [50]. Several studies reported the existence of HIV DNA and RNA in brain tissues of ART suppressive, aviremic patients, indicating of persistent HIV replication [51]. Cerebrospinal fluid (CSF) blips are commonly seen in ART-suppressed, aviremic patients who show HAND manifestations [52, 53]. Viral escape (detectable HIV RNA in CSF when undetectable in plasma) are seen in about 10% of infected individuals. Several studies reported the existence of viral mutations (conferring resistance to several classes of antiretroviral drugs) in CSF samples and these

mutations were not detected in their paired blood samples, indicating of ongoing viral replication in CNS [54-56]. HIV infection affects CNS either indirectly resulting in immunodeficiency which increases the susceptibility to opportunistic infections or directly through inducing inflammation which result in neurological syndromes [50]. The pathogenesis of HAND during aviremia is not yet completely understood; however, multiple factors may be involved, including ART neurotoxicity, local CNS inflammation, and co-infections with other viruses such as hepatitis C virus [48].

Brain-resident microglia and perivascular macrophages are the main HIV reservoirs in the CNS which play important roles in HIV neuropathogenesis. Although astrocytes are found to be the most abundantly infected cell types in CNS [48], infection in astrocytes appears to be non-productive [47]. A very recent study showed that in the brains of all virally suppressed cases, viral RNA was detected in macrophages and microglia but not in astrocytes [57]. Thus, astrocytes might not represent a major HIV reservoir [48]. Even in the absence of cellular activation, microglia and macrophages can sustain productive infections [50], which have been evidently correlated with HAND progression [48]. Perivascular macrophages are bone marrow-derived, and terminally differentiated cells. They are long lived, with a half-life of months and are replenished from monocytes crossing the BBB [48]. Microglia are the resident macrophages of the spinal cord and the brain [58], which are originally derived from the primitive myeloid precursors in the yolk sac [59]. Microglia are long-lived, with turn-over rates of years [35]. Unlike perivascular macrophages, microglia undergo cell division, enabling HIV to persist in the brain for long periods [48]. Compared to CD4 T cells, myeloid cells are relatively more resistant to cytopathic effects and apoptosis [60]. A more recent study showed that the efficacies of several ART drugs were reduced in human microglia compared to PBMCs [51]. Activation of microglia is essential to control CNS infections; however, microglial activation following HIV infection could result in chronic inflammation and

neurodegeneration. There are now clear correlations between hyperactivated microglia and HIV-associated dementia (HAD) as well as the less severe conditions known as HIV-associated neurocognitive disorders (HAND) [58].

There are two main mechanisms (viral factors and host factors) which contribute to HAND progression. For the viral factor-related mechanisms, viral protein products such as gp120 and Tat produced by macrophages, microglial or astrocytes have direct neuronal toxic effects. Spike protein (gp120) can interact with cellular receptors, altering the glutamate signaling pathway which can induce cytokine production. Spike protein can also induce further activation of microglia and astrocytes. Tat, in levels less than the concentrations required for normal viral replication, was shown to induces mitochondrial dysfunction and cell death in neurons [47]. The release of other viral proteins such as Rev, and Nef, promote inflammation and neuronal damage (**Fig. 5**) [35]. In terms of host factors, it is assumed that cytokines produced by activated monocytes and macrophages in the periphery can traffic into CNS. These cytokines, in addition to cytokines released by activated astrocytes and microglia of CNS, induce neuroinflammatory states in CNS [47]. Activated perivascular macrophages can also release several factors such as TNF-α, eicosanoids, nitric oxide, platelet activating factor, quinolinic acid, and extracellular matrix degrading proteases which activate astrocytes to release several cytokines and chemokines, altering excitotoxicity [50]. Furthermore, it is also postulated that circulating microbial translocation products derived from gut bacteria resultant from HIV may lead to microglial priming which can contribute in sustaining CNS inflammation (**Fig. 5**) [35].

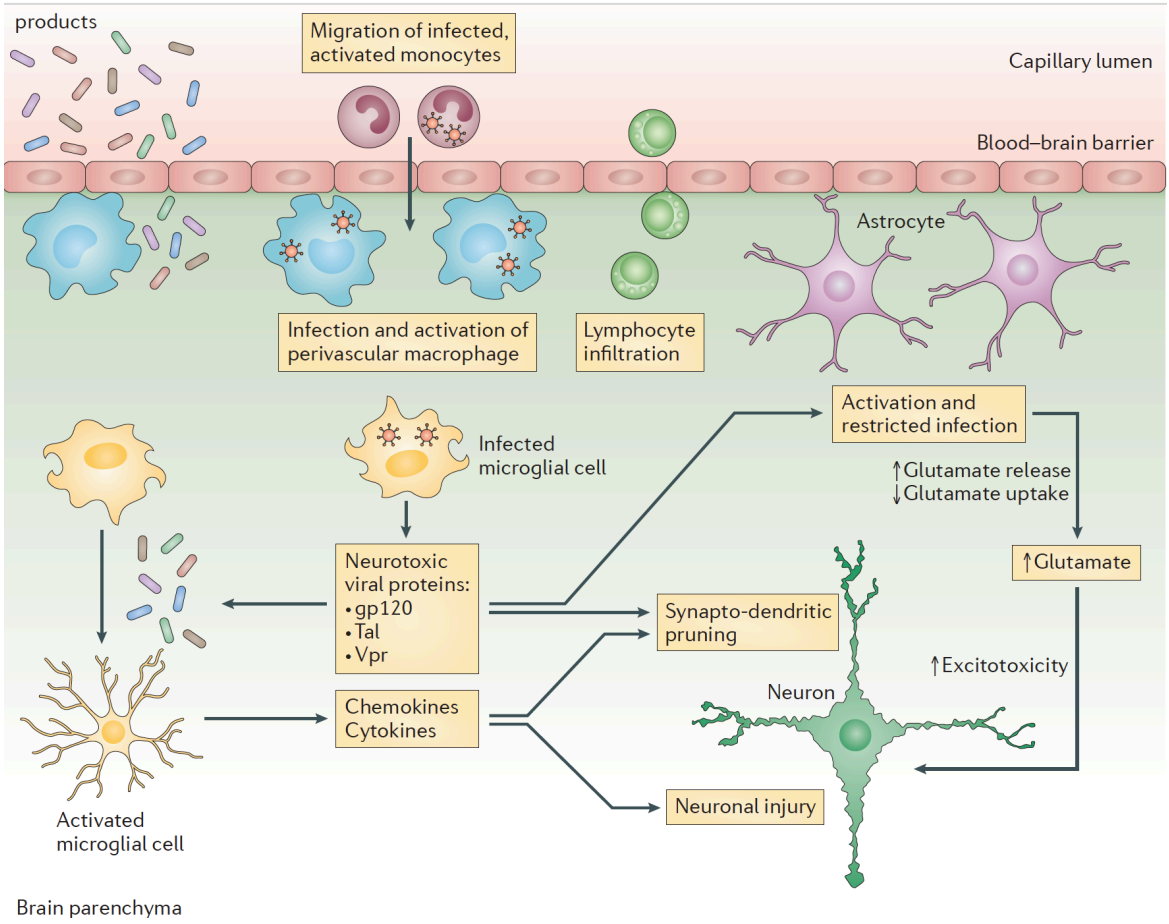

Fig 5. Schematic representation for the proposed mechanisms involved in HIV neuropathogenesis. HIV+ monocytes transmigrate through BBB and establish infection in the CNS. HIV-infected macrophages and microglia release viral proteins which activate astrocytes to release glutamate which accumulate and contribute in neuronal injury. Systemic proinflammatory cytokines and microbial translocation products derived from gut bacteria trigger microglial activation which further induce inflammatory response (adapted from reference 35).

7. Prospects for a Cure for HIV/AIDS

The integration of HIV provirus into the host cell genome and the establishment of latent infection pose major obstacles for HIV eradication or cure [39]. The current antiretroviral therapy can efficiently inhibit active virus replication; however, complete eradication of the virus cannot be achieved even with intensification of ART [38, 39]. Residual viral expression, a driver of inflammation and HIV-associated complications, is reported during ART. The latently infected cells are long lived with half-lives of 44 months for CD4 T cells [61] and

several months to years for macrophages and microglia, respectively [48]. Therefore, it is estimated that it may take more than 73 years of optimal ART regime to fully eradicate HIV reservoirs [61]. Yet, only two cases are reported for complete cure from HIV (Berlin Patient and London Patient). Those patients received hematopoietic stem cells transplantation from human donors with homozygous CCR5-delta 32 mutations [48]. Although this treatment strategy seems effective for HIV cure, regardless of several unsuccessful attempts in-between the two cases, the procedure carries several risks and are not tolerated by most patients [61].

ART targets different steps of HIV life cycle; however, provirus transcription step is not targeted by ART (**Fig. 2**). Due to the limitations of ART, it is logical to identify alternative approaches that are complimentary to ART and target host mechanisms to better regulate HIV transcription (to minimize potential emergence of drug-resistant strains and HIV-associated complications). Accordingly, there are two recently proposed strategies for potential HIV cure: "shock and kill" and "block and lock". These strategies target different factors (host/viral) involved in HIV transcription. To understand the principles of these approaches, firstly, the mechanism of HIV gene transcription needs to be elaborated.

7.1. HIV Transcription Regulation

HIV gene expression is regulated by complex mechanisms which involve multiple viral and cellular factors. Once integrated, HIV provirus is subjected to host cell transcriptional machinery [62]. The 5' LTR serves as a promoter and enhancer for HIV transcription initiation. The 5' LTR has cis-acting DNA elements which serve as binding sites for transcription factors (TFs) including nuclear factor of activated T cells (NFAT), NF-ϰB, specificity protein 1 (SP1) and transcription factor II D (TFIID) [63]. LTR promotor is occupied by at least 2 nucleosomes (Nuc-0 and Nuc-1) which pose barriers for proviral transcription. Nuc-1 is situated downstream of the transcription start site (TSS), blocking transcription elongation in latent cells [64].

Nucleosomes consists of 8 core histones which can be epigenetically modified e.g. acetylation and methylation, altering transcription activity [64]. In productively infected cells, cellular activation mediates the translocation of NF-κB and/or NFAT into nucleus and subsequent binding to their cognate sites [63]. Binding of NFAT, and NF-κB induce the recruitment of histone acetyl transferases (HATs) which induces acetylation of Nucl-1. Acetylation mediates the recruitment of chromatin remodeling protein, PBAF which accumulates at the promoter, allowing re-position of Nucl-1 and subsequent increase in chromatin accessibility [62]. Recruitment of TFIID-associated CDK7 phosphorylates serine 5 in the carboxyl terminal domain (CTD), mediating RNAP-II promoter clearance and transcript elongation initiation. However, escaping RNAP-II complexes are forced into abortive transcription due to negative elongation factor (NELF) and DRB sensitivity-inducing factor (DSIF) [65]. Short completely spliced transcripts, encoding Tat and Rev are produced and translated. Tat binds to TAR, which forms after RNAP-II stalls near the transcription start site (TSS) [63] and this binding recruits several transcription activating proteins. Tat recruits positive transcription elongation factor complex (p-TEFb), which consists of cyclin-dependent kinase 9 (CDK9) and cyclin T1, to the TSS. CDK9 phosphorylates the CTD of RNAP-II as well as the negative factors (DSIF and NELF) [65]. Tat can also recruit active chromatin remodeling PBAF complex which further participates in opening chromatins. Tat also recruits a histone chaperone, FACT (facilitates *chromatin transcription)* complex which interacts with H2A/H2B dimer, enhancing transcription elongation by RNAP-II [66], through destabilizing the nucleosomal structure [64] (**Fig. 6A**). In latently infected cells, p-TEFb is sequestered in inactive form by hexamethylene bisacetamide-induced protein (HEXIM-1) and 7SK small nuclear RNA (7SK snRNA). *Bromodomain*-containing protein *4* (BRD4) (will be discussed in detail later) limits HIV transcription through sequestering p-TEFb, and competitively inhibiting Tat transactivation [64, 66]. Several cellular and viral factors can repress proviral transcription. Low levels of Tat

or phosphorylation of Tat by CDK2 can reduce proviral transcription [67]. In resting cells, NF-κB and NFAT are sequestered in the cytoplasm in inactive forms. NF-κB is found sequestered in the cytoplasm by inhibitors of NF-κB (IκB) and NFAT is found highly phosphorylated in inactive form in cytoplasm [64]. Methylation of histones (Histone trimethylation of histone H3 lysine 9 trimethylation (H3K9me3) and lysine 27 trimethylation (H3K27me3)) is associated with transcriptional repression and is mediated by histone methyltransferase (HMT) which is accumulated in the LTR region of silenced provirus and HMT is relocated after proviral reactivation [68]. Histone deacetylases (HDAC) can deacetylate lysine residues on histone tails, restricting the access of several TFs to the DNA as well as it recruits histone-modifying and chromatin complexes which result in transcriptional repression [69]. HDAC can be recruited by several host factors such as late SV40 factor (LSF), yin yang 1 (YY1), and C-promoter binding (**Fig. 6B**) [64].

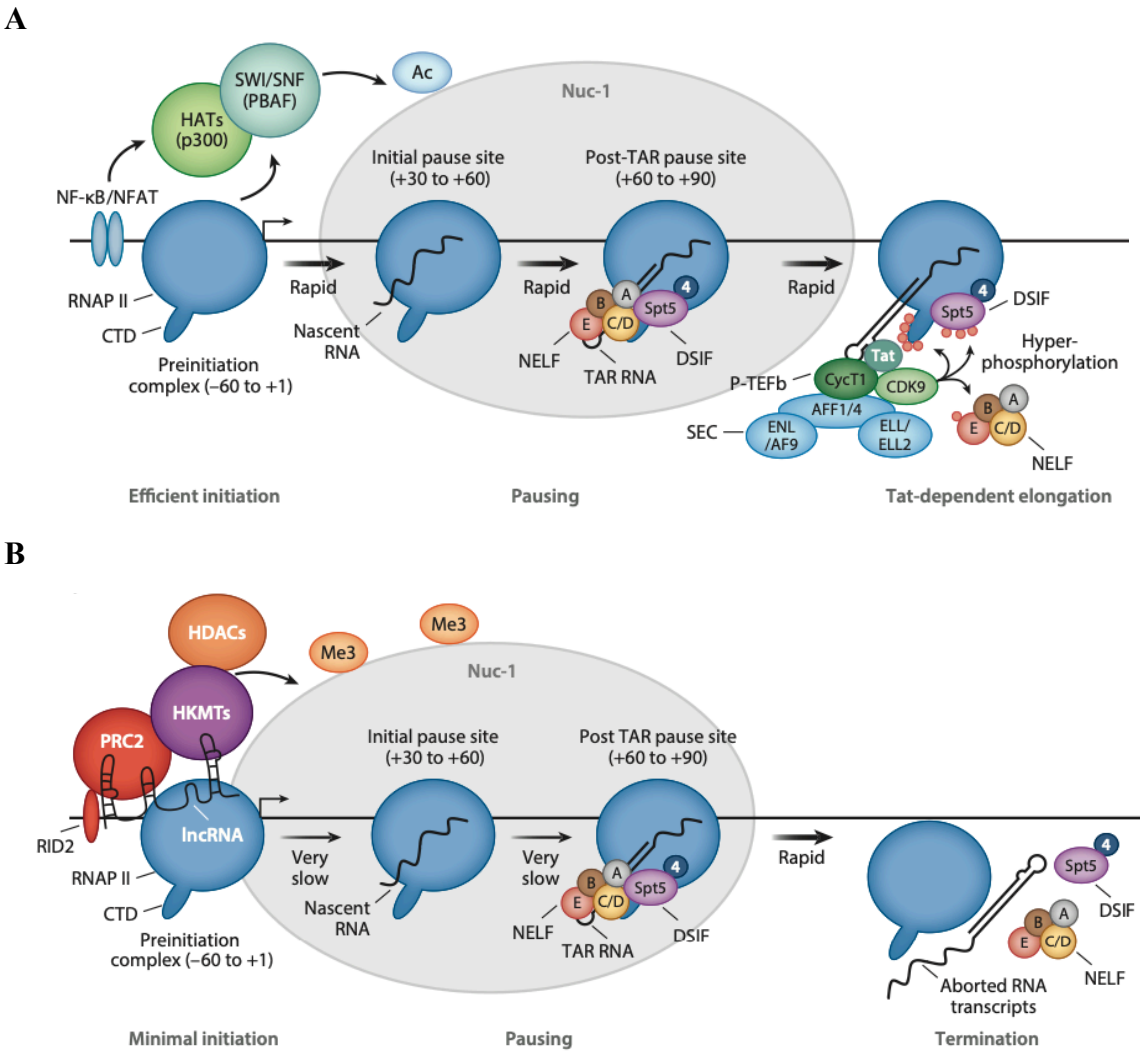

Fig 6. Schematic depiction for HIV proviral transcription regulation. Several factors are involved in inducing productive transcription (**A**) and repressive transcription (**B**) (adapted from reference 65).

7.2. Shock and Kill Strategy

This strategy aims to purge HIV reservoirs through reactivation of latent cells "Shock" with latency-reversing agents (LRAs). The virally productive cells would be then destroyed by immune system or viral cytopathic effects "Kill" [68]. Several agents are used to target different cellular factors, including HDAC inhibitors (HDACi), protein kinase C (PKC) agonists, toll-like receptors (TLRs) agonists, HMT inhibitors, inhibitors of bromodomain and

extraterminal (BET) domain proteins (BETi), and DNA methyltransferase inhibitors (DNMTi) [68]. PKC (such as bryostatin-1, ingenol B and ingenol-3-angelat) and TLR agonists can activate NF-kB and AP-1 leading to p-TEFb release from 7SK snRNP-repressive complex and subsequent transcript elongation [70]. So far, three classes of LRAs have gone through early phases of clinical trials. Data from phase 1 clinical trial showed that PKC induces proinflammatory cytokines short after administration and a follow-up trial with adjusted doses showed no detectable drug in a majority of participants as well as the failure to reverse latency in all participants [70]. In vitro studies showed that TLR7 agonist is efficient in reactivating HIV transcription, and a current clinical trial for TLR7 agonist (GS-9620) is still ongoing [68]. Data from different clinical trial showed that although HDACi induces HIV-1 transcription, no reduction in reservoir size was observed in the trial participants [68]. Several factors are thought to contribute in the ineffectiveness of LRA in clinical trials including diverse mechanisms involved in HIV-1 latency, lack of selectivity of LRAs, variations in patient clinical history and the heterogenous and dynamic of HIV-1 cellular reservoirs success [71].

Regardless of the above-mentioned factors, there are also multiple potential concerns for the application of LRA approach in CNS. Reactivating of latently infected cells in CNS may result in the production of neurotoxic viral proteins, such as Tat and the gp120, and these proteins are implicated in CNS inflammation and neurodegeneration [72]. Additionally, reactivation of latently infected cells may also result in reinfection of new cells in CNS where ART penetration is suboptimal [47]. In vivo study using SIV/macaque model for HIV-related neurocognitive disorders showed that in ART-suppressed animals, LRA induces brain inflammation, and viral RNA in CSF was 10-fold greater than in plasma which also shows unique genotypes that were not present in the periphery [73]. Another important point needs to be carefully considered when using LRA strategy to target CNS reservoirs is the natural resistance of macrophages and microglia to apoptosis and viral cytopathic effect.

7.3. Block and Lock Strategy

This strategy aims to induce deep viral latency through regulating different factors (viral or cellular) that mediate proviral transcription "Block and Lock". Several latency-promoting agents (LPAs) were shown to be effective in repressing HIV transcription including inhibition of FACT, or heat shock protein 90 (HSP90) (involved in NF-kB activation signaling pathway), downregulating of kinases (mTOR and kinase inhibitors) and RNA interference approaches [64]. One of the most advanced and well-studied LPAs is Tat inhibitor, didehydro-cortistatin A (dCA). Tat is the first viral protein expressed upon infection [68]. Inhibition of Tat by dCA results in tight nucleosome/DNA association, increased in deacetylated histone 3 at Nuc-1 and reduction in RNAP-II recruitment to the TSS. In vivo data using BLT mice showed that addition of dCA to ART regime delayed HIV rebound following treatment termination compared to animals treated with ART alone approach [64].

7. 3. 1. Bromodomain Protein: A Promising Target for HIV Silencing

Nucelosomal structure of chromatin consists of 8 core histones, H2A, H2B, H3, and H4 (two copies of each); modifications to these histones play essential role in regulating genome activity [74]. Histone modifications can modulate chromatin compaction through mediating DNA accessibility of several factors including TFs, replication factors, and other repair factors. Proteins that can read modifications in chromatins are called "readers" of epigenetic information. These readers can regulate genome activity through recruiting of several enzymes which regulate gene transcription [75].

Bromodomain (BD) and extra-terminal domain (BET) proteins are a novel family of epigenetic "readers" that play essential roles in genome activity. The BET family consists of 4 members: BRD2, BRD3, BRD4, and bromodomain testis specific protein (BRDT) [75]. BET proteins are normally found in the nucleus, ubiquitously expressed, except BRDT which is exclusively expressed in spermatocytes [76]. BET proteins consist of two conserved N-

terminal bromodomains (BD1 and BD2) and a C-terminal "extra-terminal" domain (ET). BRD4 has an extra domain "carboxyterminal domain" (CTD) that does not exist in other BET members [74] (**Fig. 7A**). BDs bind specifically to the acetylated lysine residues of histone and other proteins [76], **although this binding is shown to be modest** [76]. Each single protein of BET can bind to two acetylated lysine of histones simultaneously. In addition, BET can also interact with transcription factors as well as transcription elongation complex [74] (**Fig. 6B**). Among BET proteins, BRD4 is one of the most well-studied proteins. There are 3 isoforms of BRD4 with different length: long isoform (1362 residues) and two shorter forms (722 and 796 residues, respectively) [75]. BRD4 is involved in regulation of gene transcription through interaction with transcription initiation and elongation complexes, p-TEFb. p-TEFb consists of a regulatory subunit, cyclin T1 and cyclin-dependent kinase, CDK9. Two regions of BRD4 can interact with p-TEFb components. The C-terminal region of BRD4 interacts with the cyclin T1, allowing CDK9 binding to BD2 and interaction of CDK9/BRD2 to acetylated region of cyclin T1 [74]. CDK9 then phosphorylates the CTD of RNAP-II as well as the negative regulators (DSIF and NELF) that inhibit RNAP-II elongation [68]. The ET domain interacts with chromatin remodelers such as the SWI-SNF and CHD2, histone modifiers such as an arginine demethylase, JMJD6 (jmjC domain-containing protein 6), NSD3, a lysine methyltransferase, an arginine demethylase, and NSD3, and a lysine methyltransferase. Interaction of ET domain with those enzymes mediate chromatin remodeling and accessibility to the target DNA [74].

Normally, BET proteins (BRD2/3/4) have regulatory rules in gene transcription, and therefore, their dysfunctions may affect transcriptional homeostasis and lead to human diseases [74]. Therefore, modulation of interactions between BET protein and acetylated lysine represents potential therapeutic targets for human diseases. **As previously mentioned, due to the modest binding of the BDs, several acetyl lysine binding pockets in BDs can bind to**

small molecules with higher affinity and hence, great progress has been gained in targeting and inhibition bromodomain [76]. BRD4 has shown a promising therapeutic target for a variety of human diseases [75]. Stimulus dependent acetylation of RELA/p65 (NF-kB co-activator) at lysine residue induces its binding to BRD4 which leads to the recruitment of p-TEFb and subsequent induction of the transcription of NF-kB-target genes [75]. Furthermore, modulation of BET proteins showed potential applications as antiviral, antifungal and antiparasitic [76]. Inhibition of BET with JQ1 induces HIV transcription through p-TEFb dependent and independent mechanisms. BRD4 and BRD2 have important roles in other viral replication such as HPV, herpesviruses, MCV, and murine leukemia. For instance, inhibition of BET suppresses murine leukemia virus and herpesviruses replications. In *Candida albicans*, Bdf1 (the bromodomain- containing protein 4 (BRD4) homologue) is critical for viability and specific inhibitors of Bdf1 shows potential antifungal activity. In mice model of *Trypanosoma brucei* infection, inhibition of bromodomain containing proteins decreases the parasite virulence [76].

Given the established role of the BRD4 in regulating of HIV transcription, we were interested in identifying novel BRD4 small molecule modulators that can modulate HIV transcription. Through collaboration with a medicinal chemistry group, we have synthesized new class of BRD4 modulators based on structure-guided design. By screening these compounds for their activities in regulating HIV transcription, we have identified and characterized a novel BRD4-selective small molecule, ZL0580, that is distinct from JQ1 and induces epigenetic suppression of HIV in multiple in-vitro and ex-vivo models of HIV infection. Mechanistically, ZL0580 shows more selectivity to BD1 domain of BRD4 as

compared to JQ1 which non-selectively binds to BD1 and BD2 domains of all BET proteins [77]. In addition, ZL0580 has distinct mode binding to BD1 domain of BRD4 as compared to that of JQ1.

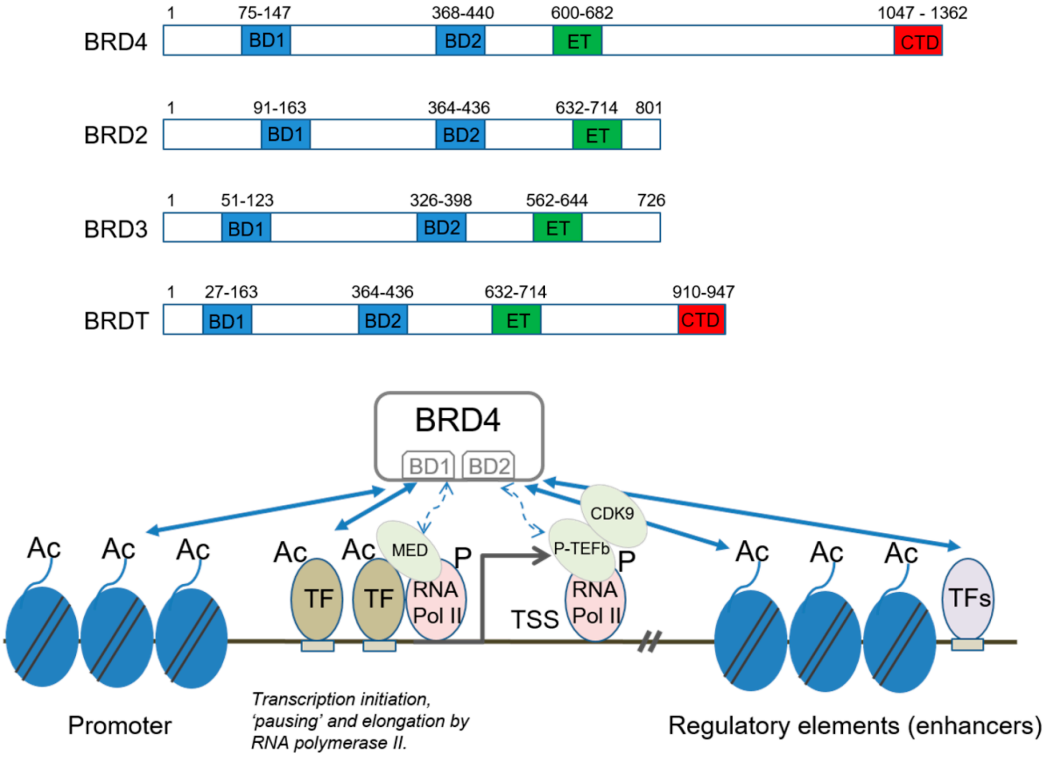

Fig 7. (A) Schematic of domain organizations of BET (bromodomain and extra-terminal domain) family proteins**. (B) f**unctions of BRD4 in regulation of genome activity (Adapted from reference 74)

8. Objectives of this Dissertation

The use of BRD4-selective small molecule modulator (ZL0580) for HIV transcriptional repression in our previous work of CD4 T cell models of HIV infection has demonstrated important findings. ZL0580 represses both induced and basal HIV transcription in multiple in-vitro and ex-vivo cell models. In an ex-vivo human PBMC model, ZL0580 promotes HIV suppression during ART treatment and delays viral rebound after ART termination [77]. Hence, the work presented here aimed to investigate the anti-HIV properties of ZL0580 in cells important in HIV infection of CNS. In the studies conducted in this thesis, we utilized

microglia and multiple myeloid cell models of HIV infection, including U1 and OM10.1 cell lines as well as human primary monocyte-derived macrophages (MDMs), to explore potential activity of this novel class of molecules in suppressing HIV in CNS reservoirs. Additionally, another goal of the current study is to determine if ZL0580 can cross BBB in an in vitro model. This dissertation is intended to provide a proof of concept for the feasibility of repressing HIV transcription in CNS cells through modulation of BRD4. Findings from this work will allow for further studies for the development of this class of molecules as a potential strategy for HIV cure. **Chapter I** aims to provide general background about HIV and the role of BRD4 in HIV transcriptional regulation. **Chapter II** describes materials and methodologies utilized in this work. **Chapter III** presents findings on the effect of ZL0580 on HIV infection in CNS cell reservoirs and discuss these findings in the context of literature. **Chapter IV** summarizes and discuss key findings of this work and the future directions.

CHAPTER II: Materials and Methods

Cell Lines: The microglial cell line (HC69) was a generous gift from Dr. Jonathan Karn (Case Western Reserve University), and was maintained in DMEM-F12 medium (ATCC) supplemented with penicillin G (100U/mL), streptomycin (100 μg/mL), 1% (v/v) FBS, 1 μM dexamethasone (Sigma D4902) and 1x N2 supplement (Gibco-Invitrogen). The U1, and OM10.1 (obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID) were maintained in RPMI 1640 medium (Gibco) supplemented with penicillin G (100 U/mL), streptomycin (100 μg/mL), L-glutamine (2 mM, 0.3 mg/mL) and 10% (v/v) FBS. The Blood-Brain Barrier hCMEC/D3 cell lines were purchased from Millipore Sigma, Burlington, MA. hCMEC/D3 cells were cultured in EBM-2 medium (Lonza, USA) supplemented with fetal bovine serum (5%), basic human fibroblast growth factor (hFGF) (1.0 ng/mL), HEPES (10.0 mM), hydrocortisone (1.4 μM), chemically defined lipid concentrate (1.0%), ascorbic acid (5.0 μg/mL), and penicillin G (100 U/mL), streptomycin (100 μg/mL). Astrocytes (U87MG) (obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID) were maintained in DMEM medium supplemented with penicillin G (100 U/mL), streptomycin (100 μg/mL), L-glutamine (2 mM, 0.3 mg/mL) and 10% (v/v) FBS. Cells were cultured at 37°C in humidified 5% CO_2 incubator.

Small Molecule BRD4 Modulator ZL0580: Design, synthesis, and chemical structure of ZL0580 is described in our recent study [77].

(4-Nitrophenyl) sulfonyl)-*L*-proline (2): To a solution of methyl ((4-nitrophenyl) sulfonyl)-*L*-prolinate (**1**) (500 mg, 1.6 mmol) in 16 mL CH_3OH, $LiOH{\cdot}H_2O$ (334 mg, 8.0 mmol) in 8 mL H_2O was added. The mixture was stirred at room temperature for 4 hours. After concentration, the solution was acidified by 10% HCl to pH = 3 and extracted by dichloromethane (DCM) to obtain **2** (540 mg, quant.) as a white solid. The crude product was used directly in the next step.

(*S*)-1-((4-Nitrophenyl) sulfonyl)-*N*-phenylpyrrolidine-2-carboxamide (3): To a solution of **2** (100 mg, 0.33 mmol) and aniline (31 mg, 0.33 mmol) in 5 mL of DCM, HBTU (321 mg, 1.0 mmol) and DIPEA (295 μL, 1.67 mmol) were added. After stirring at room temperature for overnight, the mixture was extracted with DCM (20 mL × 3). The organic layer was washed with 1 N $NaHSO_4$ (aq.), saturated $NaHCO_3$ (aq.), brine and dried over anhydrous Na_2SO_4. The resulting solution was evaporated, and the residue was purified by PTLC (DCM/MeOH = 70:1) to give the desired product **3** (81 mg, 66%) as a pale-yellow solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.35 (d, J = 8.7 Hz, 2H), 8.05 (d, J = 8.7 Hz, 2H), 7.52 (d, J = 8.0 Hz, 2H), 7.37 – 7.25 (m, 2H), 7.11 (t, J = 7.4 Hz, 1H), 4.32 – 4.18 (m, 1H), 3.68 – 3.56 (m, 1H), 3.29 (dd, J = 16.8, 7.8 Hz, 1H), 2.31 – 2.18 (m, 1H), 1.98 – 1.86 (m, 1H), 1.84 – 1.68 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 168.94, 150.48, 141.93, 137.12, 129.04, 128.97, 124.85, 124.55, 120.20, 120.10, 62.91, 49.85, 30.36, 24.49.

(*S*)-1-((4-Aminophenyl) sulfonyl)-*N*-phenylpyrrolidine-2-carboxamide (4): To a solution of **3** (70 mg, 0.19 mmol) in 10 mL CH_3CH_2OH, NH_4Cl (50 mg, 0.93 mmol) in 3 mL H_2O and Zn dust (123 mg, 1.9 mmol) were added. The solution was allowed to reflux for 0.5 hour. Then it was filtered, and the filtrate was extracted by DCM for three times. The organic layer was dried and concentrated to give **4** as a white foam which was used directly in the next step.

(*R*)-*N*-Phenyl-1-((4-(3-(4-(trifluoromethyl) phenyl) ureido)phenyl)sulfonyl)pyrrolidine-2-carboxamide (ZL0580): To a solution of 4 (0.187 mmol) in 5 mL DCM, 1-isocyanato-4-(trifluoromethyl) benzene (37 mg, 0.2 mmol) was added. After stirring at rt. overnight, the mixture was concentrated and purified by PTLC (DCM/CH_3OH = 50 :1) to give **ZL0580** (65 mg, 66% for two steps) as a white solid. HPLC purity 99.8% (t_R = 19.7 min). ^{1}H NMR (300 MHz, MeOD) δ 7.87 (d, J = 8.8 Hz, 2H), 7.74 (d, J = 8.6 Hz, 2H), 7.68 (d, J = 8.7 Hz, 2H), 7.64 – 7.55 (m, 4H), 7.35 (t, J = 7.7 Hz, 2H), 7.14 (t, J = 7.3 Hz, 1H), 4.24 (dd, J = 7.7, 4.2 Hz, 1H), 3.70 – 3.57 (m, 1H), 3.38 (d, J = 7.6 Hz, 1H), 2.13 – 1.91 (m, 3H), 1.69 (d, J = 5.3 Hz,

1H). ^{13}C NMR (75 MHz, MeOD) δ 171.43, 152.67, 144.19, 142.52, 137.74, 129.56, 128.81, 128.42, 125.75, 125.70, 124.32, 120.44, 118.35, 118.21, 62.61, 49.37, 30.83, 24.31. HR ESI-MS $(M + Na)^+$ m/z = 555.1282 (calcd for $C_{25}H_{24}F_3N_4O_4SNa$: 555.1290).

Cell Treatments and Flow Cytometric Analysis of HIV Expression: Quantification of GFP-expressing cells in microglia (HC69) was carried out by fluorescence-activated cell sorting (FACS) analysis using the BD Accuri™ C6 Flow Cytometer (BD) and LSR-Fortessa. Data were analyzed by using FlowJo software (Tree Star). Unless otherwise stated, HC69 cells were treated with 300 pg/ml TNF-α (R&D 210-TA) +/- 8 μM of ZL0580 or treated with DMSO (NC) for 24 hours. Cells were harvested, washed and resuspended in 300 μl PBS and then analyzed for cell viability stained for viability using trypan blue staining or Aqua Blue LIVE/DEAD (Invitrogen), followed by flow cytometric analyses for GFP expression and cell viability (Aqua Blue). For latent HIV reactivation experiment, cells were treated with DMSO (NC) or 8 μM of ZL0580 for 24 hours, followed by cell wash and continuous culture. Cells were reactivated at the indicated time post treatment with 300 pg/ml TNF-α for overnight. For OM10.1, cells were treated with DMSO (NC) or 20 ng TNF-α with or without 10 μM of ZL0580. For U1, cells were also treated with DMSO (NC) or 0.05 μg/ml PMA with or without 10 μM of ZL0580.

Real-Time PCR Quantification of Cell-Associated HIV RNAs: HIV mRNA quantifications (Gag, early multi-spliced, and GFP) in myeloid cell lines was performed. RNA was extracted using Quick-RNA MicroPrep Kit (Zymo) according to the manufacturer's instructions. Extracted RNA was used to synthesize cDNA using iScriptTM Reverse Transcription Supermix (BioRad). Gene of interest was quantified by qPCR using iTaq Universal SYBR Green Supermix (Bio-Rad) and the CFX Connect Real-Time PCR Detection System (Bio-Rad). PCR reactions and conditions were set as follows: 20 μl of total PCR reaction containing

10 µM primers, 90 ng of cDNA, 10 µl iTaq universal SYBR Green supermix (2X) (Bio-Rad) and molecular grade water were subjected to PCR cycling conditions (95°C for 3 min, 45 cycles of 95°C for 5 sec, 60°C for 30 sec). Primer sequences used in PCR reactions are shown in **Table 1**. Gene expression was normalized to GAPDH and calculated using the $2^{-\Delta\Delta Ct}$ method.

Western Blot Analysis: Briefly, 5×10^6 cells per treatment condition were cultured, and treated as indicated. Cells were harvested and washed twice with PBS. Whole cell extracts were prepared in 1x RIPA Buffer (Sigma 20-188) containing 1x protease inhibitor cocktail (PIC) (Sigma p8340), and 1mM PMSF 1 mM PMSF (Sigma), followed by a brief sonication. Total protein concentration was quantified using Protein Assay Kit (Pierce™, Thermo Fisher Scientific) according to the manufacturer's instructions. Equal amounts of proteins from different treatment conditions (20-30 µg total proteins) were subjected to SDS-PAGE gel separation followed by protein transfer into Immuno-Blot PVDF membranes. Membranes were blocked (5% skim milk powder in TBST) for 1 hr followed by incubation with protein specific antibodies for overnight at 4 °C. The following primary antibodies were used: Tat (catalog 160189, AIDS Reagent Program), p-RNA Pol II-CTD (Ser2) (MA5-23510, Thermo Fisher), BRD4 (730007, Thermo Fisher), NF-κB (33-9900, Thermo Fisher), and GAPDH (2118, Cell Signaling). The membranes were washed 3 times with TBST followed by 1 hr incubation with secondary antibodies (anti-rabbit or anti-mouse IgG-HRP, Thermo Fisher), based on the nature of the primary antibody. Western blot analysis was carried out using Super Signal West Pico Chemiluminescent Substrate (Thermo Fisher) reagent. Markers were used to identify the target protein band. As a loading control, the expression of GAPDH was also measured.

Co-Immunoprecipitation: Briefly, 1.5×10^7 cells per treatment condition were cultured in 6-well plate and treated as indicated. Cells were harvested, washed with PBS and lysed in 500 µl NP-40 lysis buffer that contains 1x PIC (Sigma p8340) and 1 mM PMSF (Cell Signaling 8553),

followed by rotation at 4°C for 1 hr. Cell lysates were centrifuged at 12,000 rpm for 30 mins at 4°C and the supernatants were collected for protein quantification using Protein Assay Kit (Pierce™ , Thermo Fisher Scientific) according to the manufacturer's instructions. 3 µg of anti-Tat (MA1-71509, Thermo Fisher) was incubated with equal amount of protein from each treatment condition rotating overnight at 4°C. The immune complexes were precipitated with 50 µl of streptavidin magnetic beads for 1 hr at 4°C. Beads were washed 6 times using cold NP-40 cell lysis buffer and then subjected to SDS-PAGE electrophoresis. The membranes were blocked and immunoblotted with anti-CDK9 antibody (MA5-14912, Thermo Fisher), followed by wash and incubation with secondary antibody as described above in western blot analysis section.

Chromatin Immunoprecipitation (ChIP) and qPCR: ChIP-IT Express Kit (Active Motif) was used according to manufacturer's instructions. Briefly, 15×10^6 cells were transferred to 6-well plate and treated accordingly as described in the figure legends. At 24 hrs post-treatment, cells were washed and fixed in 1 ml PBS containing 37% formaldehyde (Sigma, F8775) and incubated on shaking platform at RT for 10 min to allow DNA and protein cross-linkage. The reaction was ended with addition of 110 µl 10x Glycine on shaking platform at RT for 5 min. Cell mixtures were then centrifuged at 2,500 rpm for 10 min at 4°C. Supernatants were removed and 1 ml of ice-cold Lysis buffer was added to the pellet for 30 min on ice. Nuclei were pelleted with centrifugation at 5,000 rpm for 10 min at 4°C. Nuclei pellets were re-suspended in 400 µl shearing buffer containing 2 µl PIC and 2 µl PMSF, on ice for 10 min and then subjected to sonication. The mixtures were centrifuged at 15,000 rpm for 10 min at 4°C. The supernatant which is the sheared chromatin was used to set up the ChIP reactions by adding the magnetic beads and 3 µg of anti-Tat (Thermo Fisher) or control mouse IgG (Cell Signaling) for overnight rotation at 4°C. Mixtures were washed multiple times with ChIP buffers followed by chromatin reverse cross-linkage. The DNA was eluted and purified by

phenol-chloroform extraction and then used for qPCR analyses using primers shown in **Table 1**. PCR reactions and conditions were set as described above in PCR quantification section. The results were analyzed by the fold enrichment method with the formula % enrichment=2 - ($C_{T\ IP} - C_{T\ mock}$).

High-Resolution MNase Nucleosomal Mapping: Microglial cells were either treated with ZL0580 (8μM) or left untreated (NC) for overnight then cells in both conditions were re-stimulated with TNF-α (300 pg/ml) for 24 hrs. Cellular cross-linkage was then followed as described in the CHIP assay. Cells were then washed with 1ml buffer B (0.25% Triton-X 100, 1 mM EDTA, 0.5 mM EGTA, 20 mM Herpes, pH 7.6) and then with 1 ml buffer C (150 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 20 mM Herpes, pH 7.6). Cells were washed in cold PBS and ~15× 10^6 cross-linked cells were suspended in 1 ml buffer A (300 mM sucrose, 2 mM Mg acetate, 3 mM CaCl2, 10 mM Tris pH 8.0, 0.1% Triton X-100, 0.5 mM DTT), incubated for 5 min on ice followed by 20 times of dounce-grinding (Tight Pestle, Kontes, 2 ml grinder). Nuclei were pelleted by centrifuging at 4°C, 750 x g for 5 min, followed by washing twice with 1ml of buffer D (25% glycerol, 5 mM Mg acetate, 50 mM Tris pH 8.0, 0.1 mM EDTA, 5 mM DTT) at 15 x 10^6 nuclei/ml. Samples were centrifuged at 4°C, 750xg for 5 min to pellet chromatins and the pellets were suspended in 1ml buffer MN (60 mM KCl, 15 mM NaCl, 15 mM Tris pH 7.4, 0.5 mM DTT, 0.25 mM sucrose, 1.0 mM CaCl2) at 1.5 x 107 nuclei/ml. Then 150 μl of chromatins (2.25 x 10^6 nuclei) were treated with 0, 0.5, 5, 20,50, or 500 U/ml of MNase (USB) for 30 min at 37°C followed by the addition of EDTA (12.5 mM) and SDS (0.5%) to stop the enzymatic reactions. Proteinase K digestion step was followed at 37°C for 4 hrs. The DNA was eluted and purified by phenol-chloroform extraction and then 5 ng/μl of DNA was used for real-time qPCR analysis using primers as shown in **Table 2**. The ΔC_T method was used to calculate fold change and the ratio of the amount of digested DNA to the undigested DNA for each primer was calculated.

In Vitro HIV Infection of Human Primary Monocyte-Derived Macrophages (MDM) and Nested PCR for Quantification of Viral RNA in Cell Supernatants: PBMCs of healthy donors were obtained from the blood bank at the University of Texas Medical Branch (UTMB). Use of de-identified PBMCs of health donors was approved by Institutional Review Board (IRB) of UTMB. Total PBMCs were cultured as adherent monolayers at density of 5×10^6 cells/well in 6-well plates in RPMI 1640 medium (Gibco) (with no FBS) for 2 hrs. Adherent cells were differentiated into macrophages for 6 days in differentiation medium consisting of RPMI 1640 medium (Gibco) supplemented with penicillin G (100 U/mL), streptomycin (100 μg/mL), L-glutamine (2 mM, 0.3 mg/mL), 10% (v/v) FBS and 100 ng/ml GM-CSF (Gibco PHC2015) for 6 days. At day 3 post cell culture, half of the culture medium was changed with fresh differentiation medium. At day 6, cells were infected with HIV (US-1 or JR-FL) using spinoculation (1,200 g for 1 hr) followed by overnight incubation at 37°C in humidified 5% CO2 incubator. Cells were then dissociated non-enzymatically (Sigma C5914) and equal number of cells were distributed into 48-well plates and maintained RPMI 1640 medium (Gibco) supplemented with penicillin G (100 U/mL), streptomycin (100 μg/mL), L-glutamine (2 mM, 0.3 mg/mL) and 10% (v/v) FBS. At day 7 p.i., cells were either untreated (NC), treated with ART alone (400 nM Lamivudine, 400 nM Raltegravir, and 200 nM Efavirenz), or ART plus ZL0580 (2.5 μM). Fresh medium containing the same treatments was changed every 3 days, and HIV production in supernatants was measured by ultra-sensitive nested PCR (as explained later) prior to medium change. Treatments were discontinued only when ART fully suppressed HIV production and HIV production in cell culture were continuously monitored. The establishment of ultrasensitive nested PCR for quantification of HIV production in culture supernatants was performed according to our recent study. Briefly, viral RNA from culture supernatants was extracted using QIAamp® Viral RNA kit (Qiagen) according to the manufacturer's protocol followed by two-step nested PCR approach. Following RNA

extraction, cDNA was synthesized from the extracted viral RNA and were then subjected to the first-round PCR amplification (16 cycles) by using (Gag-Out-F/R) (**Table 1**). The products of the PCR were serially diluted and then subjected to a second-round nested qPCR (40 cycles) using Gag-F/R primer set (**Table 1**). PCR reactions, conditions and analysis were performed as described previously in PCR quantification part. The quantification of HIV copies was performed using the established standard curve.

Establishment of In-Vitro Blood Brain Barrier (BBB) Model

Polycarbonate Transwell inserts (Corning Inc., Corning, NY, USA) (6.5 mm, pore size, 3 μm; 0.33 cm^2 growth area) were coated with Type I rat tail collagen (Fisher Scientific) and then allowed to completely dry. Astrocytes (U87MG) (4 x 10^4) were first seeded into the abluminal side of the inverted transwell and are cultured for 4 hrs. The inserts were then flipped back and hCMEC/D3 cells (50,000 cells/insert) were seeded into the luminal side of transwell filter (**Fig. 8**). For hCMEC/D3 cells, cell passage number less than 10 were used in all experiments. The transwell inserts were then transferred into a 24 well plate and cultured for 5-7 days. The culture medium was DMEM in the lower compartment and EBM-2 in the upper compartment which were replaced every 3-4 days until the cell monolayer became confluent (after 5–7 days). Prior to transport experiments, the formation of tight junction was verified by measuring the transendothelial electrical resistance (TEER) using EndOhm apparatus (World Precision Instruments, Sarasota, FL, USA).

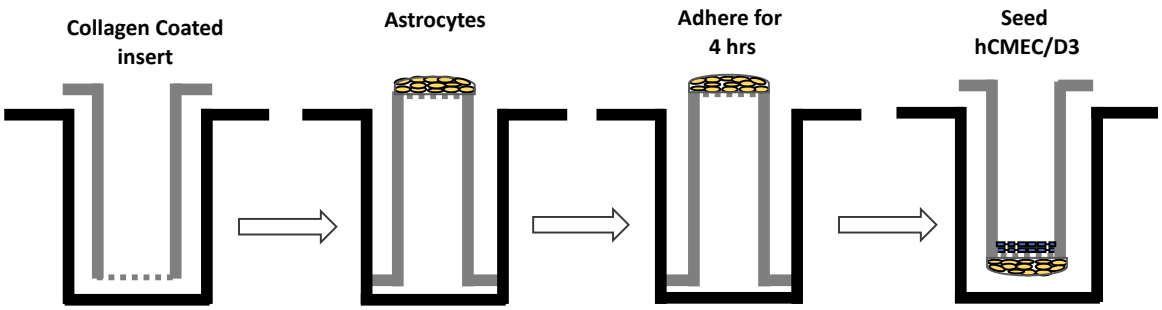

Fig 8. A schematic depiction for the experimental procedure for coculturing astrocytes and hCMEC/D3 for in vitro BBB model.

Functional Determination of ZL0580 Penetration Through BBB: Inserts with cocultured cells were transferred into 24-well plate with previously cultured microglia (HC69). Microglia in the lower chamber were then treated with 300 pg/ml TNF-α or with DMSO (NC). ZL0580 (8 μM based on the total volume in the upper and lower compartments) was introduced into the upper chamber of the transwell insert and cells re-incubated. At the indicated time post treatment, microglia were then harvested, washed and resuspended in 300 μl PBS and analyzed for cell viability by using trypan blue staining, followed by flow cytometric analysis for GFP expression.

Compound Release Study: Cocultured inserts were transferred into 24-well plate with media alone in the lower chamber. ZL0580 or Tenofovir (both at 8 μM, based on the total volume in the upper and lower compartments) were introduced into the upper chamber and cocultures were re-incubated at 37°C in humidified 5% CO_2 incubator. Media from the lower compartment was collected at different time points post treatment (0 min, 2 and 24 h) and the compound concentrations in the collected media was quantified by high performance liquid chromatography (HPLC) using the following conditions: Waters μBondapak C18 (300 × 3.9 mm); flow rate 0.5 mL/min; UV detection at 270 and 254 nm; linear gradient from 10% acetonitrile in water to 100% acetonitrile in 20 min followed by 30 min of the last-named solvent.

Statistical Analysis: Where indicated, experiments were analyzed by one-way analysis of variance (ANOVA), followed by Student's t test in Prism 6.0 (GraphPad, San Diego, CA). *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, and ****P ≤ 0.0001.

Acknowledgements: We would like to thank Dr. Jonathan Karn (Case Western Reserve University) for kindly providing the microglial cell (HC69). The study was funded by the NIH grants (R56AI145666; R21AI147903) (H.H.) and UTMB's Institute for Human Infection and Immunity pilot grant (H.H.). Edrous Alamer was supported by the Pre-Doctoral training fellowship from Saudi Arabian Cultural Mission, Jazan University. The following reagents, U1 cell line (Cat. No: 165), OM10.1 cell line (Cat. No: 1319), anti-Tat Ab (Cat. No: 160189), and JR-FL HIV (Cat. No: 395), were obtained from NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH.

TABLE 1 Primer sequences for quantitative PCR quantification of HIV DNA and RNA

Primer	Sequence (5’-3)
Gag-F	5′-GGAAGCTGCAGAATGGGATA-3′
Gag-R	5′-GCTATGTCACTTCCCCTTGG-3′
Gag-out-F	5′-GAGCCACCCCACAAGATTTA-3′
Gag-out-R	5′-AGGGTTCCTTTGGTCCTTGT-3′
GAPDH-F	5'-CAATGACCCCTTCATTGACC-3'
GAPDH-R	5'-GACAAGCTTCCCGTTCTCAG-3'
Early MS-F	5’-CGAAGAGCTCATCAGAACAGTCA-3′
Early MS-R	5’-TTGGGAGGTGGGTCTGCTT-3′
GFP-F	5’-GAGCTGAAGGGCATCGACTT-3′
GFP-R	5’-CTTGTGCCCCAGGATGTTG-3′
5' LTR-F	5′-GTTAGACCAGATCTGAGCCT-3′
5' LTR-R	5′-GTGGGTTCCCTAGTTAGCCA-3′

TABLE 2 Primer sequences for high-resolution MNase mapping

Primer	Sequence (5'-3)
1	**F**-5'GATCTGTGGATCTACCACAC3' **R**-5'GCACCATCCAAAGGTCAGTGG3'
2	**F**-5'CCTGATTGGCAGAACTACACAC3' **R**-5'TCTACTTGCTCTGGTTCAACTGG3'
3	**F**-5'CCTTTGGATGGTGCTTCAAGTTAG3' **R**-5'ATGCTGGCTCATAGGGTGTAAC3'
4	**F**-5'TAAGGAAGAGAAAGAACAGGCTTG3' **R**-5'GAAATGCTAGGAGGCTGTCA3'
5	**F**-5'GAGCCAGCATGGGATGG3' **R**-5'CTCCGGATGCAGCTCTC3'
6	**F**-5'TGACAGCCTCCTAGCATTTC3' **R**-5'CACACCTCCCTGGAAAGTC3'
7	**F**-5'CACATGGCCCGAGAGCTG3' R-5'CCCAGGCCACACCTCCCTGG3'
8	**F**-5'TACTACAAAGACTGCTGACATCG3' **R**-5'TCTGAGGGCTCGCCACTC3'
9	**F**-5'GGGACTTTCCGCTGGGGAC3' **R**-5'CCCAGTACAGGCAAAAAGCAGC3'
10	**F**-5'GGTGTGGCCTGGGCGGGA3' **R**-5'GTTCCCTAGTTAGCCAGAGAGC3'
11	**F**-5'AGTGGCGAGCCCTCAGATG3' **R**-5'AGCAGTGGGTTCCCTAGTTAGC3'
12	**F**-5'TTTGCCTGTACTGGGTCTCTCTGG3' **R**-5'CACAACAGACGGGCACACACT3'
13	**F**-5'GCTCTCTGGCTAACTAGGGAAC3' **R**-5'AGACGGGCACACACTACTTTG3'
14	**F**-5'AGCTCTCTGGCTAACTAGGG3' **R**-5'AAAGGGTCTGAGGGATCTCTAG3'
15	**F**-5'TCTCTGGCTAACTAGGGAACC3' **R**-5'AAAGGGTCTGAGGGATCTCTAG3'
16	**F**-5'AGTGTGTGCCCGTCTGTTGTG3' **R**-5'CTTTCGCTTTCAAGTCCCTGTTCG3'
17	**F**-5'GGTAACTAGAGATCCCTCAGAC3' **R**-5'CTTCAGCAAGCCGAGTCC3'
18	**F**-5'GTGTGGAAAATCTCTAGCAGTG3' **R**-5'CTTCAGCAAGCCGAGTCC3'
19	**F**-5'GCGGAGGCTAGAAGGAGAGAG3' **R**-5'GCTCCCTGCTTGCCCATAC3'
20	**F**-5'AGAGATGGGTGCGAGAGC3' **R**-5'ATTAACTGCGAATCGTTCTAGC3'

CHAPTER III: Results and Discussion

ABSTRACT

Brain-resident microglia and myeloid cells (perivascular macrophages) are important HIV reservoirs in vivo in the central nerve system (CNS). Despite anti-retroviral therapy (ART), low-level persistent HIV replication in these reservoirs remains detectable, which contributes to neuroinflammation and neurological disorders in HIV-infected patients. New approaches complementary to ART to repress residual HIV replication in CNS reservoirs are needed. Our group has recently identified a BRD4-selective small molecule modulator (ZL0580) that induces epigenetic suppression of HIV. Here, we examined the effects of this compound on HIV in human myeloid cells. We found that ZL0580 induces potent and durable suppression of both induced and basal HIV transcription in microglial cells (HC69) and monocytic cell lines (U1 and OM10.1). Pre-treatment of microglia with ZL0580 renders them more refractory to latent HIV reactivation, indicating epigenetic reprogramming effect of ZL0580 on HIV LTR in microglia. We also demonstrated that ZL0580 induces repressive effect on HIV in human primary monocyte-derived macrophages (MDMs) by promoting HIV suppression during ART treatment. Mechanistically, ZL0580 inhibits Tat transactivation in microglia by disrupting binding of Tat to CDK9, a process key to HIV transcription elongation. High-resolution micrococcal nuclease (MNase) nucleosomal mapping identified that ZL0580 induces repressive chromatin structure at the HIV LTR. We also demonstrated in an in vitro BBB model that ZL0580 can partially penetrate BBB and induce HIV suppression in microglia. Taken together, our data suggest that ZL0580 represents a potential approach that could be used in combination with ART to suppress residual HIV replication and/or latent HIV reactivation in CNS reservoirs, thereby reducing HIV-associated neuroinflammation.

1. Introduction

Currently close to 37 million people are infected with HIV worldwide. HIV integrates its provirus into the host cell genome and establishes latent infection, which has posed a major obstacle for HIV eradication or cure [39]. Current standard treatment for HIV infection is anti-retroviral therapy (ART) which consists of a cocktail of antiviral drugs targeting different steps of the HIV replication cycle [78]. ART is highly effective in controlling active viral replication and peripheral viremia, but it does not eradicate HIV infection. Of note, basal low levels of viremia during proper ART regimen [40, 41] as well as rapid viral rebound during ART treatment interruption [42, 43] were reported in HIV-infected individuals. These factors could increase the risks for expansion of ART-resistant strains and persistent inflammation [29]. Therefore, new approaches that are complimentary to ART and target host mechanisms to further suppress HIV transcription are needed.

The CNS is considered a sanctuary site for HIV persistence [72]. It has been indicated that even under ART when HIV is fully suppressed in peripheral blood, low level of persistent HIV replication remains detectable in CNS, which correlates with HAND in infected individuals [79]. In CNS, brain-resident microglia and perivascular macrophages cells are major HIV target cells and represent important HIV reservoirs [80, 81]. Microglia are the resident macrophages of the spinal cord and the brain and are originally derived from the primitive myeloid precursors in the yolk sac [59, 82]. Activation of microglia is essential to control CNS infections; however, microglial activation following HIV infection could result in chronic inflammation and neurodegeneration. There is correlation between hyperactivated microglia and HIV-associated dementia (HAD) as well as the less severe conditions known as HAND [83, 84]. Activated microglia release several proinflammatory cytokines including interleukin (IL)-1β, IL-6, interferon (IFN)-γ, and tumor necrosis factor-α (TNF-α) which in addition to the viral proteins can exacerbate neuronal damage. It is also shown that IL-1β and

TNF-α are potent inducers of HIV transcription in latent HIV reservoirs in brain which could contribute to persistent viral replication [58]. Therefore, efficient control of residual HIV replication in CNS reservoirs is considered critical for reducing neuroinflammation and neurological complications in HIV-infected individuals. It is known that the level of ART penetration into the brain is generally low [79, 85, 86]. In addition, compared to CD4 T cells [87, 88], microglia and macrophages are generally more resistant to cytopathic effect and are less sensitive to some ART drugs for inhibition [51], which could further increase the pool of HIV reservoirs in CNS as well as maximize the potential emergence of drug-resistant strains. Complimentary approaches are needed to further control residual HIV replication and latent HIV reactivation in myeloid cells, especially in CNS.

HIV proviral transcription is controlled by host epigenetic and transcriptional machinery [89]. As an epigenetic reader, the bromodomain (BD) and extra-terminal domain (BET) family protein BRD4 plays an important role in regulating HIV transcription [90-93] and has been explored as a potential therapeutic target [75, 94]. It was reported that BRD4 can compete with HIV Tat for cellular p-TEFb/CDK9, resulting in suppression of HIV transcription elongation [90, 91, 93]. Modulation of BET/BRD4 by a pan-BET inhibitor JQ1 was shown to relieve such competition and therefore reactivate HIV transcription [90, 91]. Accumulating evidence indicates that BRD4 is functionally versatile [95] and its activity on HIV transcription is associated with the partner proteins it interacts with [96]. Based on structure-guided drug design, our recent work characterized a novel BRD4-selective small molecule modulator, ZL0580, that is distinct from JQ1 and induces epigenetic suppression of HIV in human T cells and PBMCs [77].

In this study, we aimed to examine if ZL0580 could suppress transcription and latent reactivation of HIV in CNS reservoirs, including microglia and other myeloid cell populations. By using an immortalized human microglial cell line that harbors integrated HIV provirus [58],

we showed that ZL0580 induced potent and durable suppression of both induced and basal HIV transcription in human microglial cells and in monocytic cell lines (U1 and OM10.1). We also demonstrated that a single treatment of microglial cells with ZL0580 rendered them more resistant to the ensuing latent HIV reactivation. Mechanistically, ZL0580 suppresses HIV transcription in microglial cells by inhibiting Tat transactivation as well as by inducing a more repressive chromatin structures at the HIV LTR. Combination treatment of HIV-infected MDMs with ART and ZL0580 demonstrated that ZL0580 also induces repressive effect on HIV in human macrophages by promoting rapid HIV suppression during ART. Using an in vitro BBB model, we demonstrated that ZL0580 can partially crosses BBB and induce HIV transcriptional suppression in microglia. Consistently, we also showed that ZL0580 treatment mitigates neurotoxic and inflammatory gene expression in HIV-infected microglia.

2. RESULTS

2.1. ZL0580 Modulation of Host Brd4 Suppresses HIV Proviral Transcription and Replication in Myeloid Cells

2.1.1. ZL0580 Suppresses HIV Expression in Microglia and Monocytic Cell Lines

Our recent study has identified ZL0580 as a novel BRD4-selective small molecule modulator that suppresses HIV in human T cells [77]. Here, we examined the effects of ZL0580 on HIV in human myeloid cells which are considered important HIV reservoirs in vivo and play critical roles in the HIV pathogenesis in CNS. To test this, we took advantage of multiple HIV-infected myeloid cell lines (contain integrated HIV provirus), including microglial cells [58], U1 and OM10.1 cells. Microglial cell line (HC69) is the immortalized human microglia which is superinfected with HIV virus carrying a green fluorescence protein reporter, facilitating the study of HIV latency and transcriptional regulation [58]. HIV activation (viral and GFP expression) in HC69 cells can be induced upon treatment with human TNF-α [58, 97]. We first tested the effect of ZL0580 on HIV in the HC69 microglial cells. Cells were treated with DMSO alone (negative control: NC), TNF-α, TNF-α plus ZL0580 (TNF-α/ZL0580) or TNF-α plus JQ1 (TNF-α/JQ1) as a control for 24 hrs. The level of HIV activation was assessed by flow cytometry based on GFP protein expression. We found that ZL0580 significantly suppressed TNF-α-induced HIV activation in microglia (**Fig. 9A** and **B**). To determine whether the suppressive effect of ZL0580 on HIV occurs at the transcriptional level, we quantified expression of mRNAs (early multi-spliced HIV RNA and GFP RNA) at 24 hrs post-treatment and showed that ZL0580 substantially reduced the expression of both RNAs (**Fig. 9C-D**). Next, we examined dose-response effect of ZL0580 on HIV suppression in HC69 cells. Cells were treated with DMSO alone (NC), TNF-α in the absence or presence of different concentrations of ZL0580 (0-16 μM) for 24 hrs. The level of HIV expression was measured by flow cytometry based on GFP expression (percentages of GFP+ cells). We

showed that ZL0580 suppressed TNF-α-induced HIV activation in microglial cells in a dose-dependent manner (**Fig. 9E**). Potential toxic effects of ZL0580 on HC69 cells was assessed by treating them with a wide range of concentrations of ZL0580 (0–128 μM) for various lengths of time (1 and 3 days), followed by LIVE/DEAD aqua blue staining and flow cytometric analysis for cell viability. We observed that ZL0580 did not cause significant cell death at concentrations below 128 μM in resting cells (day 3 p.t.) (**Fig. 9F**) and at concentrations below 64 μM in activated cells (day 3 p.t.) (**Fig. 9G**), indicating that the observed HIV-suppressive effect by ZL0580 (8 μM) in microglia was not simply due to cell toxicity. To validate and expand our findings on HIV-infected microglial cells, we also used U1 and OM10.1 cells which are promonocytic cell lines respectively carrying 2 copies and a single copy of integrated HIV provirus, expressing minimal constitutive HIV-1 production under basal conditions [98] [99]. For U1, cells were treated with DMSO alone (NC), phorbol myristate acetate (PMA), or PMA plus ZL0580 (PMA/ZL0580); for OM 10.1, cells were treated with DMSO alone (NC), TNF-α or TNF-α plus ZL0580 (TNF-α/ZL0580). Consistently, we found that ZL0580 resulted in significant reduction in the expression of HIV mRNA (GAG) in both cell lines at 24 hrs post-treatment as compared to TNF-α or PMA alone treatment (**Fig. 9H-I**) without causing overt cell toxicity (**Fig. 9J**).

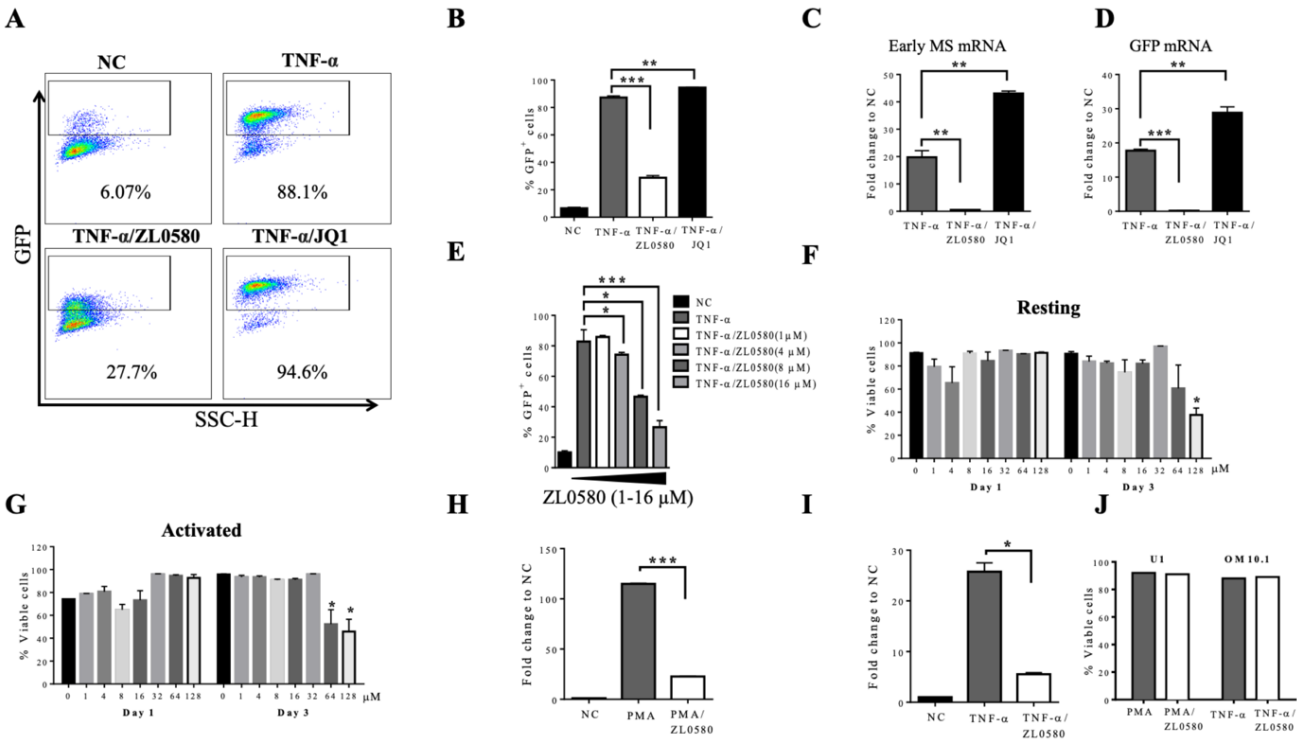

Fig 9. ZL0580 suppresses HIV expression in multiple myeloid cell lines. (A, B) Microglia (HC69) cells were untreated (NC) or stimulated with TNF-α (300 pg/ml) to activate HIV in the absence or presence of ZL0580 (8 μM) or JQ1 (8uM) (as a control) for 24 hrs. (**A**) Representative FACS plots for HIV (GFP+%) in different conditions are shown. (**B**) Quantification of % GFP+ cells from 2 independent experiments. (**C**, **D**) Comparison of HIV transcription in microglia following different treatment conditions as indicated (24 hours). HIV early multi-spliced (MS) RNA (C) and GFP RNA (D) were quantified by q-PCR. Data are shown as fold change to NC. (**E**) ZL0580 suppression of HIV in microglial cells is dose dependent. HC69 cells were untreated (NC) or stimulated with TNF-α (300pg/ml) to activate HIV in the absence or presence of different concentrations of ZL0580 (1, 4, 8, and 16 μM) for 24 hrs. HIV activation was measured by flow cytometry (GFP+%). Quantification of GFP+ cells from 2 independent experiments. (**F, G**) Toxic effect of ZL0580 in microglia during resting (F) and activated (G) conditions. HC69 cells were untreated (NC) or treated with different concentrations of ZL0580 (1, 4, 8, 16, 32, 64 and 128 μM) for 24 hrs. At day 1 and day 3 after treatments, cells viability was measured by flow cytometry based on Aqua blue staining. HC69 cells were stimulated with TNF-α (300pg/ml) in the absence or presence of different concentrations of ZL0580. (1, 4, 8, 16, 32, 64 and 128 μM) for 24 hrs. Day 1 and Day 3 after treatments, cells viability was measured by flow cytometry based on Aqua blue staining. (**H, I**) Effect of ZL0580 treatment on HIV transcription in U1 (H) and OM 10.1 (I) cells. U1 were untreated (NC) or stimulated with PMA (0.05 μg) to activate HIV in the absence or presence of ZL0580 (8 μM) for 24 hrs. OM 10.1 cells were untreated (NC) or stimulated with TNF-α (20 ng) to activate HIV in the absence or presence of ZL0580 (8 μM) for 24 hrs. HIV transcription was quantified by q-PCR. Data are shown as fold change to NC. (**J**) Percentage of viable cells (OM10.1 and U1) in different condition was compared. Statistically significant differences between groups are indicated with *$p \leq 0.05$, **$p \leq 0.01$, and ***$p \leq 0.001$.

2.1.2. The HIV Suppressive Effect of ZL0580 in Microglial Cells is Durable

Next, we asked whether a single dose of ZL0580 has a durable suppressive effect on HIV in microglial cells. We treated the cells with DMSO alone (NC), TNF-α or TNF-α plus ZL0580 (TNF-α/ZL0580), and quantified mRNA (early multi spliced mRNA and GFP mRNA) at different time points post treatment (days 1, 3, 14, 21 and 28), respectively. We found that compared to cells treated with TNF-α alone, ZL0580 treatment (TNF-α/ZL0580) induced significant suppression of HIV transcription through day 21 after single treatment, which waned somewhere between day 21 and day 28 (**Fig. 10A** and **B**). Additionally, we examined if repeated doses of ZL0580 treatments prolong its suppressive effect. Microglia were treated with DMSO alone (NC), TNF-α, or TNF-α plus ZL0580 (TNF-α/ZL0580) at day 0 and then ZL0580 treatment was repeated on day 3 and 7 post initial treatment (three doses of ZL0580). Interestingly, repeated ZL0580 treatments markedly prolonged its suppressive effect on HIV through 41 days post initial treatment (**Fig. 10C** and **D**). Based on the data (fold reduction on day 41) (**Fig. 10C** and **D**), we speculate that the suppressive effect of ZL0580 would remain beyond this time point. To ensure that the suppressive effect was not due to cell toxicity, cell viability prior to experiment termination (day 41) was monitored and found to be comparable between the two treatment conditions (**Fig. 10E**). These data indicate that ZL0580 induces durable HIV suppression in microglial cells.

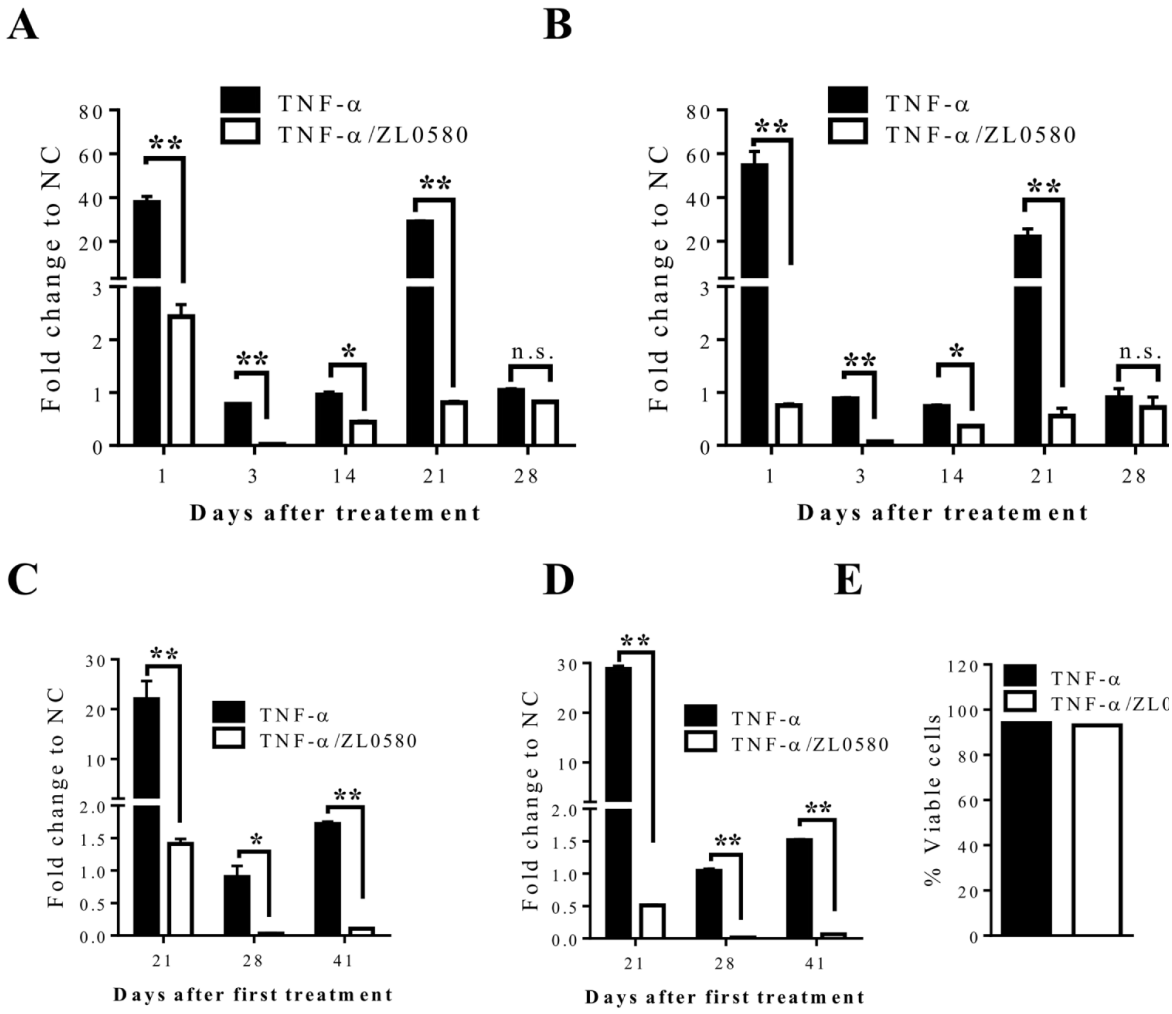

Fig 10. The HIV suppressive effect of ZL0580 is durable in microglia. (**A-B**) HC69 cells were stimulated with TNF-α (300pg/ml) to activate HIV in the absence or presence of ZL0580 (8 μM) for 24 hrs and HIV transcription was quantified by q-PCR at different time points post treatment (p.t.). Expression of HIV mRNA (early multi-spliced (**A**) and GFP mRNA (**B**) in different conditions was quantified by q-PCR. Data was normalized to un-treated (NC) control of each day. (**C-E**) HC69 cells were stimulated with TNF-α (300pg/ml) to activate HIV in the absence or presence of ZL0580 (8 μM) for 24 hrs. ZL0580 treatment was repeated at days 3 and 7 post initial treatment. Expression of HIV mRNA (Early multi-spliced (**C**) and GFP mRNA (**D**) in different conditions was quantified by q-PCR. Data was normalized to un-treated (NC) control of each day. **(E**) Percentage of viable cells at day 41 post treatment was compared between TNF-α and TNF-α/ZL0580 treatment. Statistically significant differences between groups are indicated with *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, "n.s., not significant".

2.1.3. ZL0580 Induces Suppression of Basal HIV Expression in Microglial Cells in Resting Condition

In addition to transcriptionally active HIV as described above, we also assessed the effect of the compound on basal HIV transcription in microglial cells under resting conditions. Microglial cells were treated with DMSO alone (NC) or ZL0580 (8 μM), followed by quantification of HIV and GFP RNAs at different time points after treatment (days 1, 3 and 14, respectively). Similarly, treatment of resting microglial cells with single dose ZL0580 resulted in suppression of HIV transcription compared to the untreated cells (**Fig. 11A**). No significance difference in cell viability was observed between NC and ZL0580 treatments on all days examined (**Fig. 11B**). These data altogether demonstrate that ZL0580 can also suppress basal HIV transcription in microglial cells.

Since our data have shown that JQ1 and ZL0580 induce opposing effects on HIV transcription in J-Lat cells [77] and microglia, we next examined whether ZL0580 could antagonize the enhancing effect of JQ1 on HIV transcription in microglia. Cells were respectively treated with DMSO (NC), ZL0580, JQ1, or ZL0580+JQ1 (all at 8 μM) for 24 hrs, followed by assessment of HIV activation by flow cytometry based on GFP expression. Interestingly, ZL0580 could largely antagonize HIV activation induced by JQ1 in microglia (**Fig. 11C** and **D**).

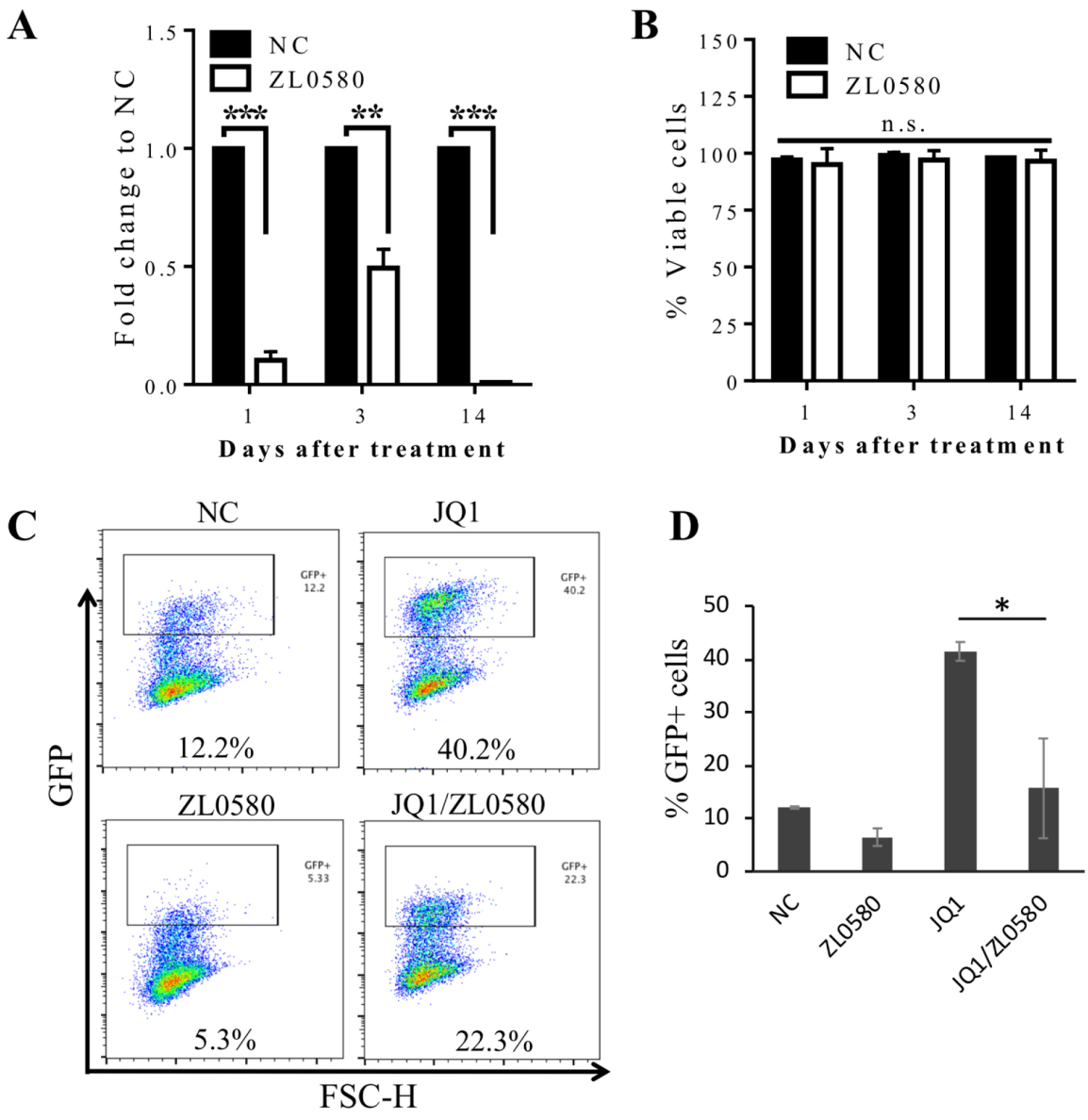

Fig 11. ZL0580 suppresses basal HIV expression in microglial cells (HC69) during resting conditions. HC69 cells were untreated (NC) or treated with ZL0580 (8 μM) for 24 hrs. (**A**) Comparison of HIV early multi-spliced mRNA quantified by q-PCR in different conditions. Data are shown as fold change to NC. (**B**) Percentage of viable cells at different time points post treatment using Trypan Blue assay. (**C**) HC69 cells were untreated (NC) or treated with ZL0580 (8 μM), JQ1 (8 μM) or 8 μM of ZL0580 and JQ1 for 24 hrs. HIV activation was measured by flow cytometry (GFP+%). Quantification of GFP+ cells from 2 independent experiments is shown. Statistically significant differences between groups are indicated with *, $P \leq 0.01$; **, $P \leq 0.001$; ***, $P \leq 0.0001$; n.s., not significant".

2.1.4. Pre-Treatment of Microglial Cells with Single Dose of ZL0580 Induces Durable Resistance to Latent HIV Reactivation

Next, we determined whether ZL0580 could promote the blockage of latent HIV reactivation upon subsequent stimulation of microglia with TNF-α. Microglial cells were either treated with DMSO alone (NC), or exposed to ZL0580 (8 μM) for 24 hrs, followed by cell wash and continuous culture (medium was replaced once every 3 days). Cells of both NC and ZL0580 pre-treated conditions were reactivated with TNF-α at different time points post ZL0580 treatment (days 7, 14 and 21, respectively). HIV activation was assessed by quantifying HIV early multi-spliced mRNA (**Fig. 12A**) and GFP mRNA (**Fig. 12B**). We showed that even under stimulation with this strong reactivator (TNF-α), ZL0580 rendered cells more resistant to latent HIV reactivation in microglial cells (**Fig. 12A** and **12B**). Similarly, this suppressive effect was not due to cell toxicity (**Fig. 12C**). These findings show the durability of the compound in suppression of HIV transcription post a single dose of treatment as well as its potency in rendering cells more resistant to the ensuing latent HIV reactivation.

2.1.5. ZL0580 Inhibits Tat Binding to CDK9

Binding of HIV Tat protein to cellular kinase CDK9 (the catalytic factor component of p-TEFb complex) is a critical step in Tat transactivation and is important for efficient HIV transcription [62]. This process has been shown to be suppressed by cellular BRD4 protein due to the competition with Tat for CDK9 [91, 93]. To understand potential mechanisms underlying ZL0580-induced HIV suppression in myeloid cells (microglial cells), we examined protein-protein interaction between TAT and CDK9 using co-immunoprecipitation (Co-IP) in these cells following ZL0580 treatment or no treatment. Similar to that described above, microglial cells were not treated (NC), or treated with TNF-α alone, TNF-α/ZL0580 for 24 hours. Intriguingly, Tat Co-IP analysis showed that ZL0580 markedly reduced the binding of Tat to CDK9 in microglial cells under TNF-α stimulatory condition (**Fig. 13A**). This was not simply

due to differential input Tat levels, since they were comparable among different treatments (**Fig. 13C**), consistent with our recent report in J-Lat cells [77]. As an important control, total/input CDK9 protein was comparable between the two conditions (**Fig.13A**). A highly consistent pattern was observed in microglial cells under resting conditions where cells were not stimulated, but directly treated with ZL0580 compared to DMSO treatment (**Fig 13B**). Furthermore, CDK9-Tat binding was explored in U1 monocytic cells and we observed similar patterns during activated (**Fig. 13D**) and resting conditions (**Fig. 13E**). These data together indicate that ZL0580 suppresses HIV transcription by inhibiting Tat binding to CDK9 in myeloid cells under both stimulated and resting conditions. We also assessed expression of major cellular factors involved in HIV transcription initiation and elongation (RNAP-II, BRD4, NF-kB). Consistent with the Tat-CDK9 binding result, we observed that ZL0580 treatment reduced RNAP-II activation (phosphorylated RNAP-II) (**Fig 13C**), supporting the idea that ZL0580 induces inhibition of HIV transcription elongation. Of note, unlike RNAP-II, ZL0580 treatment did not appear to affect the expression of other cellular factors BRD4 and NF-kB (**Fig. 13C**).

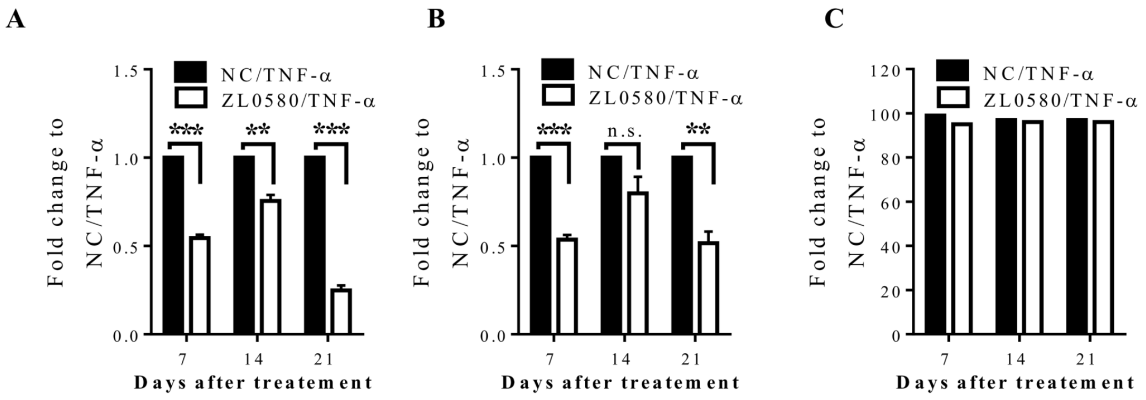

Fig 12. Pretreatment with a single dose of ZL0580 induces durable resistance to latent HIV reactivation in microglia. HC69 cells were untreated (NC) or treated with ZL0580 (8 μM) for 24 hrs, followed by continuous culture. On Day 7, 14, and 21 post initial treatment, cells were stimulated with TNF-α (300 pg/ml) for 24 hours to activate latent HIV. HIV transcription was quantified by q-PCR. Expression of HIV RNA (Early multi-spliced (**A**) and GFP RNA (**B**) was compared between different treatment conditions. (**C**) Cell viability was quantified at different time points and was compared between different treatments. Data are shown as fold change to NC/TNF-α. Statistically significant differences between groups are indicated with **p ≤ 0.01 and ***p ≤ 0.001; n.s., not significant".

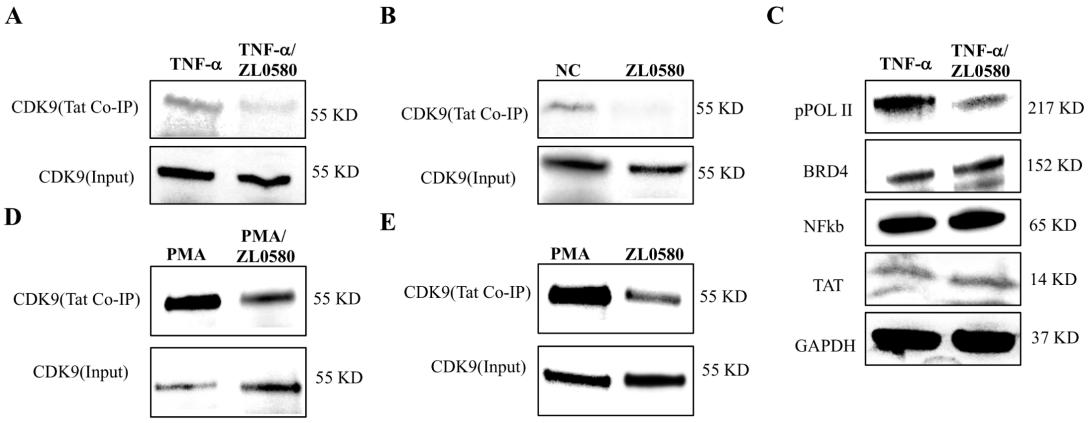

Fig 13. ZL0580 inhibits TAT binding to CDK9 in microglia during activated and resting conditions. (**A**) HC69 cells were stimulated with TNF-α (300 pg/ml) to activate HIV in the absence or presence of ZL0580 (8 μM) for 24 hrs. Co-IP analysis for binding of TAT to CDK9 was shown. Cellular expression of CDK9 protein (input) was shown. (**B**) HC69 cells were treated with ZL0580 (8 μM) or left untreated (NC) for 24 hours. Co-IP analysis for binding of TAT to CDK9 was shown. Cellular expression of CDK9 protein (input) was shown. (**C**) WB measurement of cellular proteins involved in HIV transcription. HC69 cells were treated at indicated in (**A**) and total cellular proteins were extracted for WB analysis at 24 hrs post treatment. (**D**) U1 cells were stimulated with PMA (0.05 μg) to activate HIV expression in the presence of ZL0580 (8 μM) for 24 hrs and cellular proteins were analyzed 24 hrs post treatment. Co-IP was conducted to measure binding of TAT to CDK9. (**E**) U1 cells were treated with ZL0580 (10 μM) or left untreated (NC). Cellular proteins were analyzed 24 hrs post treatment Co-IP was conducted to measure binding of TAT to CDK9.

2.1.6. ZL0580 Inhibits Tat Recruitment to HIV 5' LTR Promoter in Microglial Cells

To directly examine the impact of ZL0580 on Tat transactivation, we next assessed the recruitment of TAT to HIV 5' LTR promoter in microglial cells following ZL0580 treatment under activated condition. First, we treated cells with DMSO alone (NC), TNF-α alone, or TNF-α/ZL0580 for 24 hrs, and then examined binding of TAT to the HIV promoter (a small region overlapping with HIV transcription start site) [91] by using CHIP-qPCR. We found that ZL0580 treatment led to significant reduction in TAT binding to the HIV promoter compared to cells treated with TNF-α alone (**Fig. 14A**). In addition, under resting conditions where cells were only treated with ZL0580 (8 μM) or not treated (NC), we observed a similar reduction pattern induced by ZL0580 compared to NC (data not shown). Furthermore, we quantified the expression of elongated HIV mRNA transcript. We found that ZL0580 treatment resulted in a significant reduction in the expression of elongated mRNA (**Fig. 14B**). Together, these data suggest that ZL0580 inhibits Tat transactivation in microglial cells.

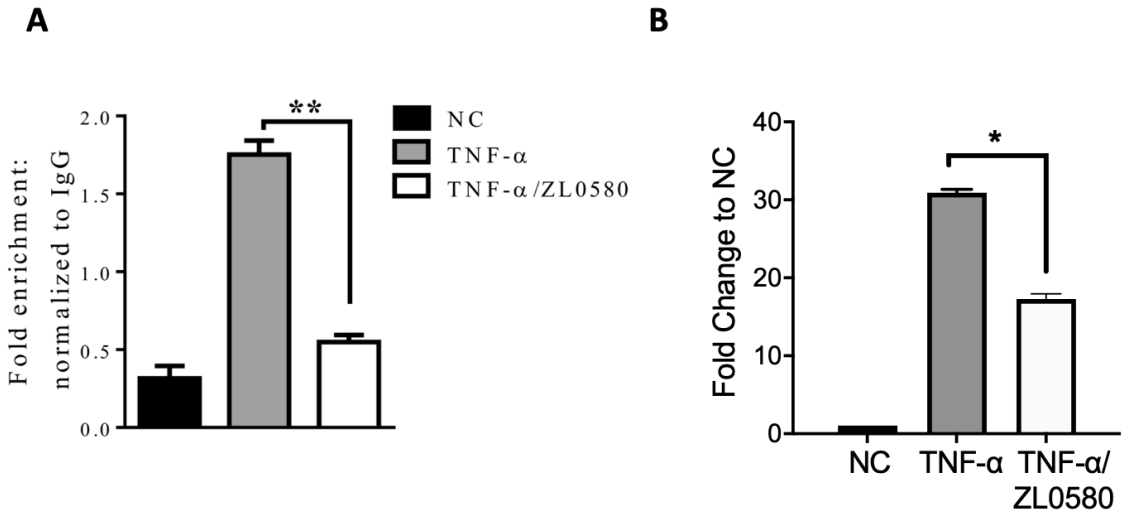

Fig 14. ZL0580 reduces the recruitment of TAT to HIV 5' LTR promoter in microglia and decreases elongated transcript expression. (**A**) CHIP analysis for recruitment of Tat to HIV LTR in HC69 cells under activated condition. Cells were untreated (NC) or treated with TNF-α (300 pg/ml) to activate HIV in the presence or absence of ZL0580 (8 μM) for 24 hrs. CHIP analysis was performed using Tat-specific antibody or control non-specific IgG. Data was normalized to non-specific IgG and was expressed as fold enrichment. (**B**) HC69 cells were untreated (NC) or stimulated with TNF-α (300 pg/ml) to activate HIV in the absence or presence of ZL0580 (8 μM) and the expression of HIV elongated mRNA transcripts was quantified by q-PCR 24 hrs post treatment (p.t.). Statistically significant differences between groups are indicated with **, $p \leq 0.01$.

2.1.7. ZL0580 Induces More Repressive Chromatin Structures in HIV LTR

The organization of nucleosome and chromatin structure (e.g. DNA accessibility) at the HIV LTR correlates with HIV proviral transcription [100]. Our recent study showed that ZL0580 can induce epigenetic reprogramming, leading to a more repressive chromatin structure at the HIV LTR in J-Lat cells [77]. In the present study, we demonstrated that pre-treatment of microglial cells with ZL0580 rendered them more resistant to HIV reactivation (**Fig. 12A** and **12B**), indicating potential epigenetic reprogramming effect of ZL0580 in microglial cells as well. To test this, we employed the high-resolution micrococcal nuclease (MNase) nucleosomal mapping to assess the HIV LTR chromatin profile, as reported previously [77, 100]. Microglial cells were either untreated (NC) or treated with ZL0580 (8 μM) for 24 hrs, followed by activation with TNF-α for 24 hrs. After chromatins were cross-

linked, they were divided into undigested and MNase digested portions. DNA from digested and undigested samples were probed with 20 separate sets of overlapping primers (**Table 2**) to amplify different regions along the HIV LTR (**Fig. 15A**). MNase can cleave nucleosome-free and linker DNA connecting two nucleosomes, while DNA within nucleosomes will be more protected from MNase digestion. This allows the assessment of nucleosomal occupancy and DNA accessibility upon calculating the ratio for PCR products in the digested DNA to that of the undigested control for each primer pair. Intriguingly, we found that treatment of microglia with ZL0580 induced more nucleosomal DNA protection in majority of amplicon regions compared to untreated cells (NC/TNF-a) (**Fig. 15B**). To understand if ZL0580 induces durable effect on HIV LTR nucleosomal structure, the same MNase nucleosome mapping was conducted in microglia at later time point after ZL0580 treatment. Cells were treated with mock (NC) or ZL0580 for 24 hrs, followed by washing to remove residual drugs. On day 6 post initial treatment, cells were activated with TNF-α for 24 hrs. We observed that LTR nucleosomal structure remains repressed, albeit to a lesser extent, in ZL0580-treated cells as compared to mock (NC)-treated cells (**Fig. 15C**). This data is consistent with the observed durable effect of ZL0580 on HIV transcription in microglia. Taken together, these data suggest that ZL0580 remodels HIV LTR chromatins to induce more repressive nucleosomal structure.

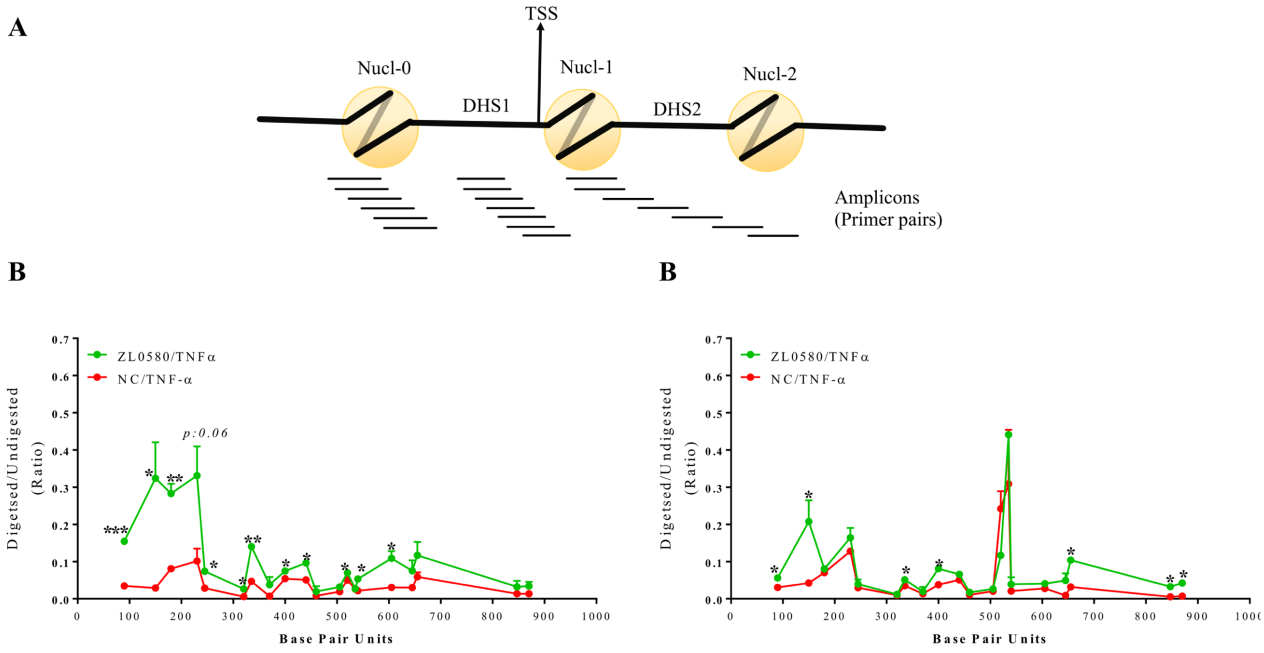

Fig 15. High-resolution MNase Nucleosomal Mapping for HIV LTR in microglia cells. (A) Diagram illustrating PCR amplicons at HIV LTR region covering 40-902 nucleotides corresponding to Nucl-0, DHS1, Nucl-1, DHS2 and Nucl-2. The sizes of PCR products are 100+/-10 bp with approximately 30 bp apart from each other. (**B-C**) Profile of changes in chromatin structure in the HIV LTR in microglia post-treatment. HC69 cells were left untreated (NC) or treated with ZL0580 (8 μM) for overnight. At day 1 (B) and day 6 (C) post-treatment, cells of both conditions were stimulated with TNF-α (300 pg/ml) for 24 hrs. The chromatin profile was determined by calculating the ratio (Y axis) for PCR products in the MNase digested to that of undigested control DNA samples for each primer pair. The X-axis shows the corresponding PCR amplicon and represents base pair units with 0 as the start of LTR Nuc-0. Statistically significant differences between groups are indicated with *p ≤ 0.05, **p ≤ 0.01, and ***p ≤ 0.001.

2.1.8. ZL0580 induces HIV Suppressive Effect on HIV in Human Primary Monocyte-Derived Macrophages (MDMs)

After demonstrating the suppressive activity of ZL0580 on HIV in multiple human myeloid cell lines, we next explored the effect of ZL0580 on HIV in human primary macrophages. In addition, it has been reported that a low level of viral replication remains during ART treatment [40, 41], and importantly, HIV rebound can occur quickly after ART interruption [42, 43]. Hence, we examined the effect of ZL0580 on HIV in macrophages in the

presence of ART treatment. Human MDMs were prepared from monocytes isolated from two donor PBMCs (donor 1 and donor 2). We established MDM HIV infection model by using two different R5 strains (JR-FL and US-1) to understand the breadth of viruses that is potentially affected by ZL0580. Following viral inoculation, productive HIV infection in MDMs was monitored in culture supernatants on day 4 and day 7 by PCR quantification of viral copies. Following establishment of HIV infection in MDMs (typically on day 7 p.i.), cells were either untreated (NC), or treated with ART alone (400 nM Lamivudine, 400 nM Raltegravir, and 200 nM Efavirenz), or ART plus ZL0580 (2.5 μM) (ART/ZL0580). Medium was replaced every three days and maintained the same drugs for continuous treatment. HIV production in supernatants was measured using ultra-sensitive nested PCR [77, 101]. Using this model, our goal was to examine if ZL0580 could promote rapid HIV suppression during ART and prevent or delay HIV rebound in fully suppressed MDMs after treatment interruption. When HIV was fully suppressed by ART or ART/ZL0580, treatments were then stopped to monitor HIV rebound. Interestingly, in US-1 infection, ART alone could effectively suppress HIV, eventually leading to full suppression in both donor MDMs. Of note, ZL0580 (ART/ZL0580) could promote HIV suppression during ART (prior to treatment cessation) (**Fig. 16A**). It appears that ZL0580 could also prevent HIV rebound in the donor 1 MDM after treatment cessation as compared to ART alone (**Fig. 16A**). In JR-FL infection, however, ART failed to effectively suppress HIV in both donor MDMs, indicating that the effects of ART on HIV in macrophages may be dependent of viral strains used. Despite inefficiency of ART in suppressing JR-FL HIV, a consistent observation was that ZL0580 (ART/ZL0580) could also promote HIV suppression during ART in JR-FL infected MDMs (both donors) (**Fig. 16A**). In this case, treatment was not stopped since HIV was not fully suppressed by either ART or ART/ZL0580 treatment. Lastly, cell viability was monitored at the last time point (day 34; prior to experiment termination) and found to be comparable among the three treatment

conditions (**Fig. 16B**). To longitudinally monitor cell viability, a parallel experiment was performed where MDMs were repeatedly treated as described above (NC, ART, ART/ZL0580), followed by collection of cells on day 18 and day 29 (representing middle and late time points after treatments) for cell viability analysis. Similarly, MDM viability was comparable among the treatment conditions at both time points (**Fig. 16C**). Collectively, these data provide evidence that ZL0580 also induces repressive effect on HIV in human macrophages and could promote HIV suppression in combination with ART.

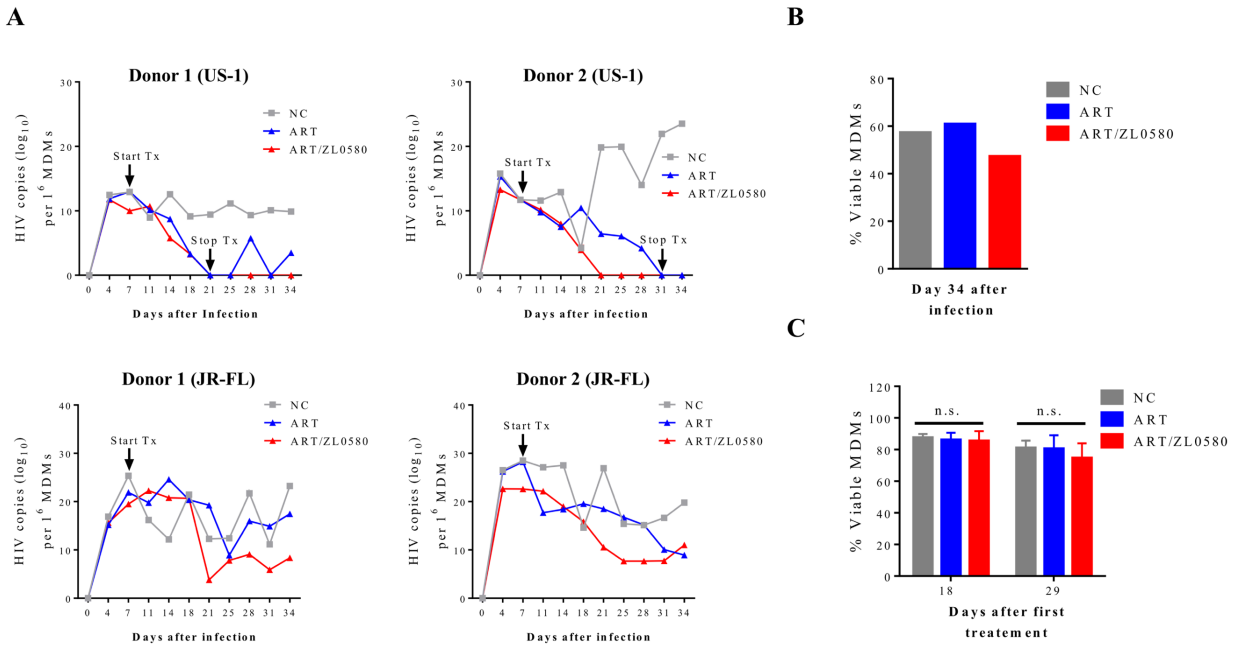

Fig 16. ZL0580 induces suppressive effect on HIV in human MDMs by promoting HIV suppression on ART treatment. **(A)** Monocyte isolated from normal human PBMCs (n = 2; Donor 1 and Donor 2) were differentiated into macrophages (MDM) and then infected with two different R5 HIV strains (US-1 or JR-FL). HIV replication in MDMs was measured on day 4 and day 7 post viral inoculation in culture supernatants to confirm successful infection. On day 7 p.i, cells were untreated (NC) or treated with ART alone or ART plus ZL0580 (2.5 μM). Culture medium was replaced with fresh medium containing same amounts of drugs every 3 days. For US-1 infection (top two panels), treatment was stopped when HIV was fully suppressed in ART alone group. Viral production in supernatants was continuously monitored up to day 34 as the last time point prior to experimental termination. For JR-FL infection (bottom two panels), since our data showed that ART failed to efficiently suppress HIV, treatments were not stopped and maintained to the end of the experiments (day 34). In the experiment, HIV production in culture supernatants was measured every 3 days using the two-step nested RT-qPCR. Viral production kinetics from day 0 to day 34 for each donor is shown. **(B)** Percentage of viable MDM cells at the last time point (day 34) post infection (JR-FL) was measured and compared between different conditions. **(C)** Longitudinal analysis of MDM cell viability following different treatments. MDMs derived from the same two donor PBMCs (not infected) were repeatedly treated with mock (NC), ART alone, or ART plus ZL0580 (2.5 μM) once every 3 days to the end of experiment (day 34). On day 18 and day 29 after first treatments, cells viability was measured by flow cytometry (% viable cells) and compared between different treatments. "n.s., not significant".

2.2 ZL0580 Crosses Blood Brain Barriers in in-Vitro Model and Represses HIV Transcription

2.2.1. ZL0580 Rapidly Penetrates BBB in in Vitro Model

Having demonstrated the suppressive activity of ZL0580 on integrated HIV in multiple human myeloid cells and microglia, we next asked whether ZL0580 can cross BBB and represses HIV transcription. We established a stringent in-vitro model of BBB to mimic the biological barriers that influence drug delivery to the CNS (**Fig. 8**). At day 7 post cell cultures when the cell monolayer became confluent and, the formation of tight junction was verified by measuring the transendothelial electrical resistance (TEER), ZL0580 was introduced into the upper chamber of the BBB and we monitored the drug contents at different time points in the lower chamber using high-performance liquid chromatography (HPLC). As an important control, we also included tenofovir (a widely used component in current antiretroviral regimens) [102] in a separate setup. We observed that ZL0580 demonstrates detectable activity to penetrate BBB in this model compared to the control (**Fig. 17A**). Next, we determined whether ZL0580 is still active after transmigrating across BBB and able to suppress HIV transcription in latently infected microglia. To test this, HC69 cells grown in basal chamber of the BBB model were treated with DMSO alone (negative control: NC), or TNF-α, and then ZL0580 (8 μM) was introduced into the upper chamber of the BBB model. The level of HIV activation was then assessed by flow cytometry based on GFP protein expression at 24 and 48 hrs post-treatment. We found that ZL0580 significantly suppressed TNF-α-induced HIV activation in microglia at both time points (**Fig 17B** and **17C**). To ensure that the transmigration of ZL0580 through the endothelial cells does not induce toxic effect and potential disruption of tight junction integrity, we assessed the cell viability of hCMEC/D3 at 24 hrs after the introduction of ZLL580. We showed that the cell viability was comparable between the treatment groups (**Fig 17D**), suggesting that drug release might not be related to BBB damage.

Taken together, these data demonstrate that ZL0580 can transmigrate through BBB and represses HIV expression in infected microglia.

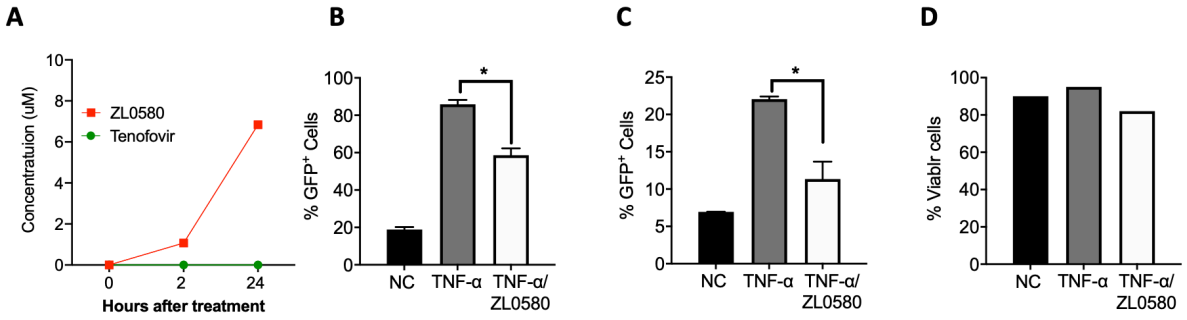

Fig 17. ZL0580 transmigrates through BBB and suppresses HIV expression in microglia. **(A)** ZL0580 (16 μM) or tenofovor (16 μM) were introduced into the upper chamber of the BBB, and the drug content in the lower chamber was quantified by using high-performance liquid chromatography (HPLC) at 2 and 24 hrs after treatment. (**B-D**) HC69 cells grown in basal chamber of the coculture BBB model were treated with DMSO alone (negative control: NC), TNF-α, and then ZL0580 (8 μM) was introduced into the upper chamber of the BBB. HIV activation was measured by flow cytometry (GFP+%) at 24 hrs (**B**) and 48 hrs (**C**) post-treatment. Quantification of GFP+ cells from 2 independent experiments is shown. (**D**) Percentage of viable hCMEC/D3 at 48 hrs post-treatment was compared between NC, TNF-α, and TNF-α/ZL0580 treatment. Statistically significant differences between groups are indicated with **$p \leq 0.01$.

2.2.2. ZL0580 Downregulates Neurotoxic and inflammatory Genes Expression During Acute HIV Infection in Microglia

Next, we assessed whether ZL0580 can mitigate the expression of HIV-mediated neurotoxic and inflammatory genes in HIV-exposed microglia and astrocytes. HIV infection of CNS results in cellular activation and upregulation of several proinflammatory cytokines and chemokines [48]. To directly examine the impact of ZL0580 on genes induced by HIV infection, we infected microglia (C20) with R5 HIV (US-1) in vitro and quantified expression of several genes reported to be involved in HIV neuroinflammation [48] at 48 hrs post-infection. As expected, HIV infection increased RNAs expression of several proinflammatory

cytokines and chemokines (**Fig. 18A**). To test the effect of ZL0580 on these genes during acute infection, we treated cells with DMSO (NC) or 2.5 μM of ZL0580 or a cocktail of ART as a control and then quantified mRNA at 24 hrs post-treatment. We showed that ZL0580 treatment results in substantial reduction the expression of RNAs of these genes (**Fig. 18B**). To ensure that this reduction effect was not due to global changes in the gene expressions, we repeated the experiment in non-HIV-infected microglia. Cells were treated DMSO (NC), 2.5 μM of ZL0580 or cocktail of ART as a control. Then, we quantified mRNA at 24 hrs post-treatment and showed that ZL0580 did not significantly alter the expression of those genes in uninfected cells (**Fig. 18C**). Similarly, we also assessed the effect of ZL0580 on these genes in astrocytes (U87MG). We observed neither HIV-mediated upregulation nor the repressive effect of ZL0580 on expression of these genes (data not shown). Together, these data indicate that ZL0580 mitigates expressions of neuroinflammatory genes in microglia induced by HIV infection.

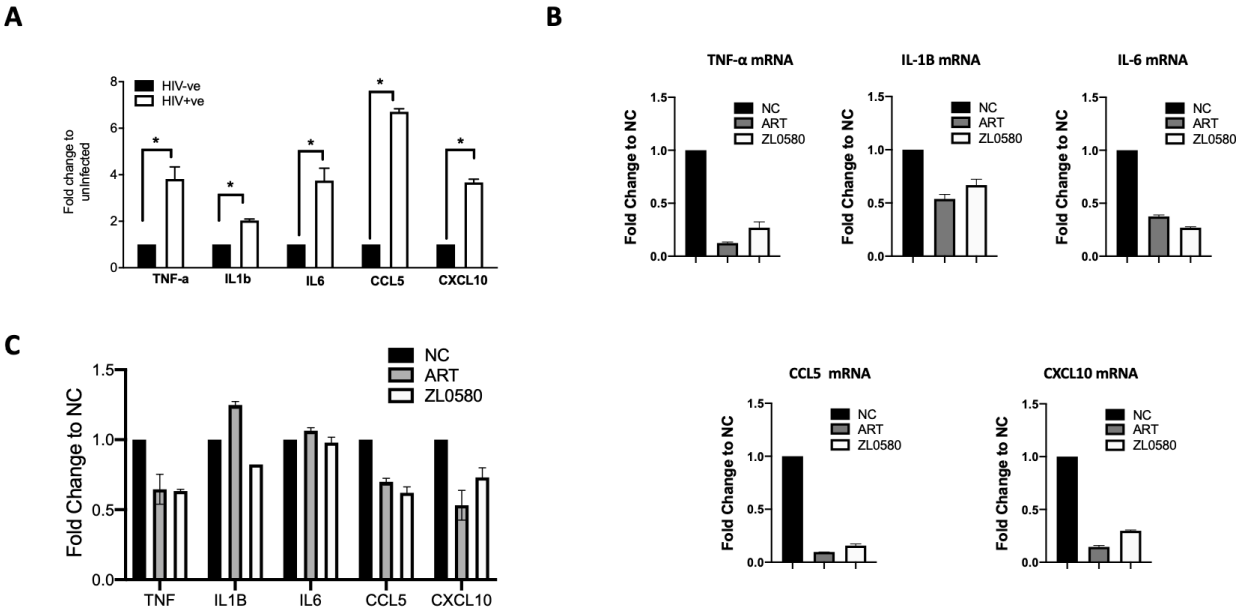

Fig 18. ZL0580 reduces the expression of HIV-mediated neurotoxic genes. (**A**) Microglia (C20) were infected with R5 HIV (US1) and the expression of the indicated genes were quantified by q-PCR at the 48 hrs post infection. Data are shown as fold of change to uninfected cells. (**B**) HIV-infected C20 cells were treated with DMSO (NC), 2.5 μM of ZL0580 or cocktail of ART (400 nM Lamivudine, 400 nM Raltegravir, and 200 nM Efavirenz). The expression of mRNA was quantified at 24 hrs post-treatment. Data are shown as fold of change to infected, DMSO (NC) cells**.** (**C**) Uninfected C20 cells were treated with DMSO (NC), 2.5 μM of ZL0580 or cocktail of ART (400 nM Lamivudine, 400 nM Raltegravir, and 200 nM Efavirenz); the expression of mRNA was quantified at 24 hrs post-treatment. Statistically significant differences between groups are indicated with $^{**}p \leq 0.01$.

3. Discussion

Brain-resident microglia and myeloid cell populations, such as monocytes and perivascular macrophages, play an important role in the establishment, persistence and pathogenesis of HIV, especially in CNS [60]. Our findings demonstrate that modulating host BRD4 with a small-molecule modulator, ZL0580, can potently and durably suppress HIV transcription in several latently infected myeloid cell lines and block events of viral reactivation in microglia. One characterization of this state of suppression is a significant reduction in viral mRNA in an already low level of basal transcription and the durability of the suppression following a single dose of the treatment in microglia. Our data also demonstrate that ZL0580 induces suppressive effect on HIV in human primary macrophages and promotes HIV suppression in combination with ART.

One of the proposed strategies for HIV eradication is the "shock and kill" approach [68]. This strategy aims to reactivate latent viral reservoir during ART regimen using latency-reversing agents (LRA) such as protein kinase C (PKC) agonists and HDAC inhibitors [68]. The virally productive cells can then be lysed due to cytopathic effects or be targeted by HIV specific cytolytic T lymphocytes, whereas infection of new cells will be inhibited by continuous ART treatment [68]. Several in vitro and ex-vivo studies showed that LRA is a potential promising approach to curing HIV; however, data from several clinical trials have not yet shown enough evidence in reducing HIV reservoirs [68]. A potential concern for the application of LRA strategy in CNS is that reactivating of latently infected cells can possibly result in the production of neurotoxic viral proteins, such as Tat and the gp120, and that these proteins have been implicated in CNS inflammation and neurodegeneration [72]. In addition, reactivation of latently infected cells may also result in reinfection of new cells in CNS where ART penetration is suboptimal [47]. A more recent study using simian immunodeficiency virus (SIV) infected macaque model for HIV-related neurocognitive disorders showed that in ART-

suppressed animal, LRA induces brain inflammation and viral RNA in CSF is 10-fold greater than in plasma and presents unique phenotypically distinct genotypes that are not present in the periphery [73]. Recent data showed that low level of ongoing viral replication continues to replenish HIV reservoirs even under ART [41]. Therefore, development of approaches that can repress residual HIV transcription and prevent or delay latent HIV reactivation ("block and lock") are gaining increasing interest. In support, recent several studies showed that inhibition of viral Tat with didehydro-cortistatin A (dCA) [103] or repressing the NF-kB pathway with telomerase-derived peptide (GV1001) [104] or targeting other host factors [105, 106] can repress basal HIV transcription and reactivation. BRD4 plays an important role in HIV transcriptional regulation [91-93]. Our recent study identified a novel small molecule (ZL0580) that can modulate BRD4 to suppress HIV in human T cells [77]. In this study, we further validated and extended this finding via demonstrating that ZL0580 can induce potent and durable HIV suppression in human myeloid cells, including microglia and macrophages. Our findings collectively support the proof-of-concept that targeting host mechanisms to suppress and/or silence HIV by small molecule is feasible in multiple cell types of HIV reservoirs.

Microglia are the major target and reservoir for HIV in CNS with a relatively long-life span [107, 108]. Compared to $CD4^+$ T cells, microglia are more resistant to cytopathic effect and apoptosis [60]. While ART has improved survival and the quality of life of HIV-infected individuals and has reduced the rates of CNS opportunistic infections in HIV^+ people, it is estimated that 15–55% of HIV infected individuals show HAND which is a major cause of morbidity in HIV infected people [35]. In the CNS, ART drugs have low efficiency and this is partially due to their low penetration BBB [85, 86]. Several studies reported existence of HIV DNA and RNA in brain tissues of aviremic patient on suppressive ART, and viral escape mutations were detected in CSF of individuals receiving suppressive ART with undetectable viremia [109], indicating ongoing HIV replication in the CNS. Furthermore, a recent study

showed that the efficacies of several ART drugs were reduced in human microglia compared to PBMCs [51]. Hence, beyond ART, new approaches are needed to further repress HIV activity in microglia. In this study, we showed that ZL0580 induces suppression of HIV transcription in both activated and resting microglial cells (**Fig. 11**), supporting that residual levels of HIV transcription could be controlled by modulating BRD4 in microglia. Mechanistically, BRD4 is an epigenetic reader and interacts with a range of host epigenetic/transcriptional regulators to modulate target gene expression. Our data indicate that ZL0580 manipulates multiple functions of BRD4 to suppress HIV transcription in microglia, including inhibition of Tat transactivation (**Figs. 13-14**) and repression of LTR structure (**Fig. 15**). Based on our recent study [77] and previous literature [90, 91, 93], we speculate that mechanisms by which BRD4 modulates HIV transcription could be associated with cell types and cellular status. In the transformed cell line (e.g. HC69) and activated cells where HIV Tat level is considered relatively high, BRD4 can function to compete with Tat for cellular active CDK9, leading to inhibition of Tat transactivation and transcription elongation (**Figs. 13-14**). In latent conditions when HIV Tat level is considered low, a potential mechanism for BRD4 to inhibit HIV is by engaging chromatin remodeling proteins (e.g. SWI/SNF) (31) to induce repressive HIV LTR nucleosomal structure. Indeed, our data showed that treatment of HC69 cells with ZL0580 induces a more repressive LTR structure which is refractory to subsequent TNF-a reactivation as compared to the mock treatment (**Fig. 15**). This mechanism could help explain why ZL0580 pre-treatment renders microglia more resistant to LRA-stimulated HIV reactivation (possibly due to “repressed” LTR). Nevertheless, additional studies are warranted to further understand mechanisms by which ZL0580 modulates BRD4 to induce repressive LTR structure in myeloid cells, for example, through engaging BRD4-interacting histone modifiers and remodeling proteins [92, 100].

HIV blips may contribute in the replenishment of HIV reservoirs even under optimal ART therapy [110, 111]. Cerebrospinal fluid (CSF) blips are commonly seen in patients with HAND who are on suppressive ART and show undetectable viremia [52, 53]. It was shown that patients who experience no blips revealed faster decay in the viral reservoir compared to patients with blips [112, 113]. Hence, durable repression of HIV activity in CNS reservoirs is important. Our data showed that single ZL0580 treatment induces durable HIV suppression through day 21 in microglia, which waned somewhere between day 21 and day 28 post-treatment (**Fig. 10**). This could be due to gradual loss of activity for ZL0580 or could be related to the fact that newly generated HC69 cells in the culture (cell line continuously proliferate in culture) may be suboptimally exposed to ZL0580. Of importance, the durability of HIV suppression in microglia could be further extended by repeated treatments (**Fig. 10C** and **D**). Based on the data (fold reduction in **Fig. 10C** and **D**), we speculate that the suppressive effect of ZL0580 would remain beyond day 41. Therefore, all these data further support the utility of ZL0580 in durably suppressing HIV in CNS reservoirs. Future studies are warranted to test activity of this compound on HIV reservoirs, including microglia in CNS, in vivo in animal models.

HIV infection of CNS is characterized by dysregulated cytokines and chemokines. Higher CSF levels of proinflammatory cytokines (TNF-a, IFN-a, IL6, IL8, IL1b) and chemokines (CCL5 and CXCL10) were reported in HAND patients who were receiving ART [48]. The severity of HAND is proportionally correlated with the size of activated glial cells. The secretion of these proinflammatory cytokines and chemokines increase the permeability of BBB and also induces the upregulation of adhesion molecules such as E-selectin, adhesion molecule-1 (VCAM-1) and (ICAM-1) on endothelial cells which increase transmigration of immune cells into the CNS. Release of proinflammatory cytokines increases the extra-cellular levels of neurotoxic mediators such as glutamate (from transmigration [48]. Hence, repressing

the expression of HIV-mediated neuroinflammatory genes is important for achieving better clinical outcomes during ART treatment. Indeed, we showed that ZL0580 reduces the expression of several genes that were upregulated during acute HIV infection in microglia (**Fig. 18**). In addition, our data also indicated that ZL0580 could penetrate BBB in the in vitro model and suppresses HIV expression in latently infected, reactivated microglia (**Fig 17**).

Due to limitations in the availability of human primary microglia, we employed MDMs as primary myeloid cell model of HIV infection. Two different HIV strains, US-1 [114-117] and JR-FL [118], were used to infect MDMs to explore potential effects of breath of viruses. In the MDM model, a consistent finding we observed is that ZL0580 could promote HIV suppression during ART treatment in both US-1 and JR-FL infections (**Fig. 16A**), indicating that ZL0580 may have utility in combination with ART to suppress residual HIV replication. A potential limitation of our data is that significant variation was also noted between the two HIV infections in terms of their responsiveness to ART treatment in MDMs. While ART could efficiently suppress US-1 HIV in MDMs, it failed to do so for JR-FL HIV (**Fig. 16A**), suggesting that breath of viruses plays an important role in this model, and that our proposed approach should be further tested with expanded panel of viruses in myeloid cells in the future. Related to this limitation, since ART failed to induce full HIV suppression in JR-FL-infected MDMs (**Fig. 16A**), we were unable to precisely determine in the current study whether ZL0580 could also prevent or delay HIV rebound after treatment cessation in JR-FL-infected MDMs (**Fig. 16A**), which could be further explored in future by using additional donor MDMs and by optimizing JR-FL virus concentration for infection.

In summary, our data demonstrate that the BRD4-selective small molecule ZL0580 induces epigenetic suppression of HIV in human microglial cells and primary macrophages. In the MDM model (ART and ZL0580), we showed that ZL0580 could promote HIV suppression

during ART, indicating its potential utility in combination with ART drugs to suppress residual HIV replication in CNS reservoirs and thereby to minimize HIV-associated neurological disorders. In addition, our study provides new evidence that ZL0580 could penetrate through BBB to repress HIV expression in microglia in an in vitro BBB model. Future studies are warranted to examine the activity of ZL0580 to penetrate BBB as well as the HIV-suppressive efficacy and toxicity of this compound in vivo in HIV or SIV-infected animal models.

CHAPTER IV: Summary, Limitations, and Future Directions

1.1 Summary

Since the start of the epidemic, HIV has resulted in around 32 million deaths due to AIDS-related illnesses. Although ART has resulted in significant decline in HIV-related deaths since its introduction in mid-1990s, ART cannot eradicate or cure the virus [35]. The integration of virus into the human genome poses a major obstacle for HIV eradication or cure. ART targets different steps of HIV life cycle and shows high efficacy in controlling active viral; however, provirus transcription is not targeted by ART (**Fig. 2**). Consequently, frequent basal/low levels of viremia are seen during antiretroviral therapy. These factors could increase the risks for expansion of ART-resistant strains and chronic inflammation [29]. Persistent inflammation is commonly reported in infected individuals receiving ART, presumably harming many tissues [119], and accelerating the progress into non-AIDS co-morbidities such as neurological disorders, metabolic syndrome and cardiovascular diseases [120]. Neurocognitive disease represents one of the important morbidities reported in treated patients with HIV-1 infection [121]. HAND is reported in about 50% of the infected individuals and its prevalence remains the same to the pre-ART. Since ART does not target proviral transcription and due to the poor activity of ART in CNS, in addition to the concerns related to application of "shock and kill" strategy (**discussed in chapter 1**), identifying new approaches that are complementary to ART and target viral or host factors to further repress HIV transcription are needed to minimize basal/low levels of viremia and subsequent inflammation especially in CNS.

Our laboratory has recently identified a novel class of BRD4-selective small molecules with the lead compound as ZL0580 that induce is distinct from the pan-BET inhibitor JQ1 but

induces epigenetic suppression of HIV in multiple in vitro and ex vivo T cell-based cellular models, such as J-Lat cells and human primary CD4 T cells. Our data show that unlike the JQ1 which non-selectively binds to both BD1 and BD2 domains of all four human BET proteins, ZL0580 demonstrates selectivity to BD1 domain of human BRD4. These findings on the suppressive activity of ZL0580 on HIV in T cell systems provided a proof-of-concept for modulation of BRD4 to epigenetically suppress HIV [77].

Utilizing the identified lead BRD4 small molecule modulator (ZL0580), my thesis research focused on our understanding the regulatory activity of this lead compound on HIV in important CNS cell reservoirs. We revealed that ZL0580 induces HIV suppression in microglial cells and other monocytic cell lines (**Fig. 9**). ZL0580 induces potent and durable suppression of both induced and basal HIV transcription in microglial cells. We found that a single dose of ZL0580 repressed HIV transcription up to 21 day after treatment and this suppression can be extended by repeated treatments (**Fig. 10**), supporting that residual levels of HIV transcription could be mitigated by modulating BRD4 in microglia. Importantly, we showed that even under the condition of stimulation with a strong HIV reactivator (TNF-a), one dose of ZL0580 rendered microglia more refractory to subsequent latent HIV reactivation (**Fig. 12**), supporting potential utility of ZL0580 in durably suppressing HIV in CNS reservoirs where blips are commonly seen and believed to contribute to inflammation in ART-suppressed, aviremic HIV-infected individuals. We next explored the activity of ZL0580 in primary human macrophages cells. Due to limitations in the availability of human primary microglia, we employed MDMs as a primary myeloid cell model of HIV infection in CNS. To further explore potential effects of breadth of viruses, we explored the activity of ZL0580 two different HIV strains (US-1 and JR-FL) in MDMs under ART treatment. We revealed that ZL0580 promotes HIV suppression during ART, and that ZL0580

may prevent HIV rebound (observed in one donor infected with US-1), indicating its potential utility in combination with ART drugs to suppress residual HIV replication in CNS reservoirs and thereby could minimize HIV-associated neurological disorders (**Fig. 18**). These intriguing findings led us to investigate the mechanism(s) that might be responsible for such suppression. We found that ZL0580 inhibits Tat transactivation in microglia by disrupting binding of Tat to CDK9 and reduces the recruitment of Tat to HIV 5' LTR promoter, a key process in HIV transcription elongation (**Figs. 13** and **14**). This inhibitory effect was evidenced by suppression of RNAP-II activation and subsequent reduction in mRNA levels of viral elongated transcript (**Fig. 14B**). Next, we investigated the nucleosome structures of HIV LTR, which can be markedly modulated by BRD4 and can affect HIV transcription [100]. Using high-resolution micrococcal nuclease (MNase) nucleosomal mapping, we identified that ZL0580 induces repressive chromatin structure at the HIV LTR (**Fig. 15**), which helps explain why ZL0580 pre-treatment renders microglia more resistant to LRA-stimulated HIV reactivation. We next investigated the ability of ZL0580 to cross BBB and inhibits latent infected microglia in-vitro BBB. Our in vitro model provides evidence that ZL0580 could penetrate BBB, as compared to tenofovir, and significantly suppress TNF-α-induced HIV activation in microglia (**Fig. 17**), supporting its potential utility in combination with ART (which have suboptimal penetration to BBB overall) to suppress residual HIV replication. Lastly, we investigated the effect of ZL0580 on expression of neurotoxic and inflammatory genes induced during acute HIV infection, and we found that ZL0580 substantially reduced the expression of these genes induced by HIV infection in microglia (**Fig. 18**). Therefore, all these data establish a proof-of-concept for the modulation of BRD4 to epigenetically suppress HIV and further support the utility of ZL0580 as a potential approach in combination with ART to suppress residual HIV replication and to reduce HIV-associated neuroinflammation in CNS. Although it

might be challenging to permanently silence all provirus with "block-and-lock" agents, including our molecule, repressing residual HIV and/or delaying viral rebound for few weeks to months or years will still have significant benefits to HIV$^+$ individuals. Finally, inability to achieve functional cure is not considered a failure, as such drugs would still help in reducing basal HIV replication and could be a new potential antiretroviral used in case of drug resistance. A schematic model is presented to summarize major findings of my thesis research (**Fig. 19**).

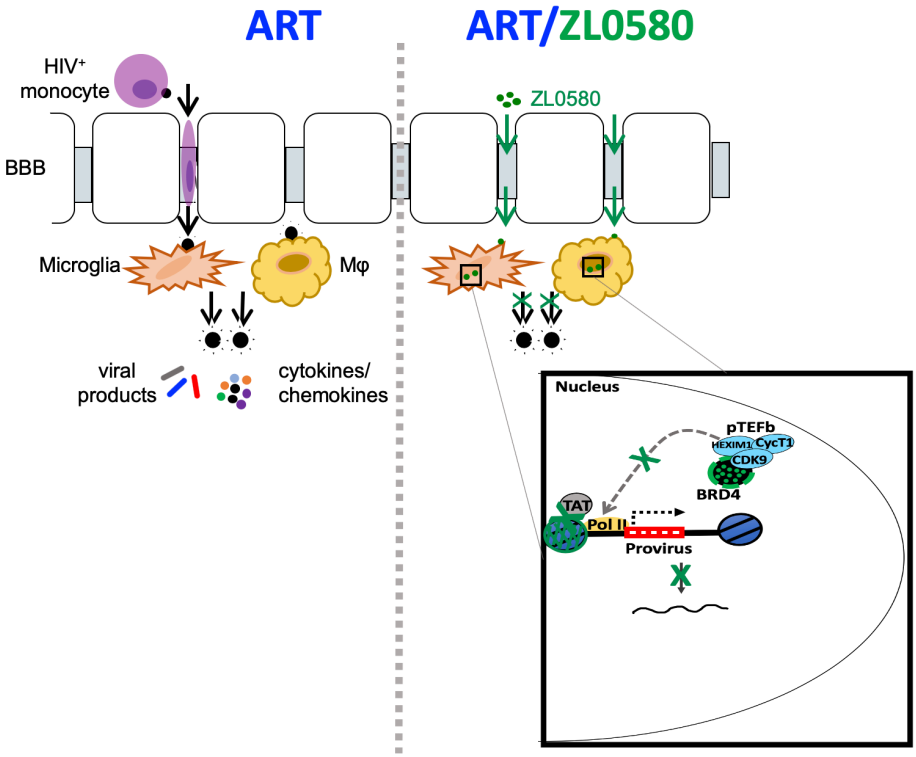

Fig 19. A proposed model illustrating the activity of ZL0580 in CNS. ZL0580 crosses BBB and suppresses HIV expression and subsequently the reduces neurotoxic genes**.**

1.2. Limitations and Future Directions

Despite that many intriguing findings that were obtained from studies conducted in this dissertation, several areas still require further optimization and investigations. The proposed future

directions which I believe could improve and move forward the development of this class of molecules for regulation of HIV transcription in CNS are outlined below:

1.2.1. Further understanding of mechanisms of action: We postulate that ZL0580 induces HIV suppression through Tat dependent and Tat independent mechanisms. In our studies we disclosed key factors underlying Tat-dependent mechanism, such as decreased Tat binding to CDK9 and to HIV 5' LTR promoter (**Figs. 13** and **14**), and enhanced binding of BRD4 to CDK9 and HIV 5' LTR promote [77]. However, Tat-independent mechanism which is evidenced by the induction of more repressive chromatin structure in HIV LTR (**Fig. 15**) requires further exploration. The findings that JQ1 and other BRD-inhibitors can reactivate latent HIV-1 in cells defective in the TAR/Tat axis (ACH-2 and U1) or lacking Tat (J-Lat A72) supported that BRD4 can regulate HIV transcription in Tat-independent manner [66]. Confirming Tat-independent mechanism of ZL0580 can be pursued using J-Lat A72 cells and J-Lat A2 cells (which harbors an LTR-GFP construct, and LTR-Tat-IRES-GFP construct), respectively [122]. The repression of HIV activity by ZL0580 in both cell lines will confirm our postulation that ZL0580 suppresses transcription via both mechanisms. Next, it would be intriguing to further investigate engagement and interaction of BRD4 with important chromatin remodeling proteins (SWI-SNF and CHD2, histone modifiers arginine demethylase, JMJD6, NSD3, a lysine methyltransferase, an arginine demethylase, and NSD3, a lysine methyltransferase) as well as the recruitment of these proteins to HIV LTR nucleosomes, which could reveal additional mechanisms concerning how ZL0580/BRD4 induces repressive chromatin structure at the HIV LTR region. This could be explored by using techniques such as coimmunoprecipitation (Co-IP) and CHIP assays. Findings from this proposed direction will further expand our knowledge of biology of BRD4 and its roles in HIV infection and other human diseases.

1.2.2. Expand the testing for activity of ZL0580 on multiple HIV viruses and CNS cellular reservoirs: Based on our previous work [77], we hypothesized that ZL0580 promotes HIV suppression during ART and delays HIV rebound after ART termination in MDMs. We tested our hypothesis by using two different strains of HIV R5 (US-1 and JR-FL). Although we observed that ZL0580 promotes HIV suppression during ART treatment in both US-1 and JR-FL infections (**Fig. 16A**), significant variation was also noted between the two HIV infections in terms of their responsiveness to ART treatment, suggesting that breath of viruses plays an important role in this model. Therefore, our proposed approach should be further tested with expanded panel of viruses in myeloid cells in the future. In the current studies, we were not able to fully confirm the ZL0580 induces delay in HIV rebound in both strains. Further studies using expanded panel of viruses and additional donor MDMs as well as optimized JR-FL virus concentration for infection in MDMs need to be carried out in the future. In addition, since different ART regimens have been used for HIV treatment, it would also be important to understand if ART regimens influence the effects of ZL0580 on HIV. In this study, we have not yet tested this point but speculate that ART regimen would matter. For example, if protease inhibitor, instead of integrase inhibitor, is included in the ART regimen, we expect that more proviruses would be established in cells, which would require higher concentration or more doses of ZL0580 to achieve sustained HIV suppression. Future studies should also test impact of different ART regimens on the effectiveness of ZL0580 in suppressing HIV in myeloid cells and other HIV reservoirs. Additionally, due to limitations in the availability of human primary microglia, we employed MDMs as primary myeloid cell model of HIV infection. Hence, it is still essential to test the activity of ZL0580 in primary microglia cells. In addition, we showed that ZL0580 shows penetration through BBB and can suppress HIV in

microglia in vitro (**Fig. 17**). Although we did not observe significant toxic effect of ZL0580 in the BBB cells post drug penetration, potential disruption of BBB tight junction cannot be fully excluded from the current studies. Therefore, assessing tight junction integrity using transendothelial electrical resistance (TEER) during and after treatments need to be conducted in future experiments.

1.2.3. Testing off-target effects: Potential off-target effects should be carefully considered for therapeutic approaches that target host mechanism (e.g. BRD4) to suppress HIV, although host-targeting approaches provide some critical advantages (e.g. sustained suppression and limited drug resistance). Thus far, our data support that HIV LTR is a major target by ZL0580/BRD4, although potential effects of ZL0580 on non-HIV gene targets are not fully excluded. In our recent study [77], we examined effects of ZL0580 on phenotypes (e.g. receptor, co-receptor, and activation markers) and gene-expression profile of human T cells (17 selected genes critical for T-cell functions). We noted that ZL0580 does not appear to induce a broad impact on T cells. While these focused analyses are helpful, they are not unbiased approaches. Conducting future studies using global genomic approaches, such as RNA-Seq (to examine transcriptomic profile) and CHIP-Seq (to examine recruitment of BRD4 to gene promoters) on myeloid cells and other cell types are required. This will provide more comprehensive characterization on whether and to what extent modulation of BRD4 by ZL0580 induces global or substantial off-target effects in HIV-infected target cells.

1.2.4. Evaluate pharmacodynamic and toxicological properties: Although the data from my dissertation research and our recent work [77] support a potent activity and safe profile of ZL0580 regarding cellular toxicity and off-target effects, additional studies are still needed to move forward. Further chemical optimization of this class of molecules based on the structure of ZL0580

should be pursued. Such optimization can improve target specificity and selectivity, efficacy, and stability of candidate compounds. Pharmacological and toxicological properties of this class of compounds, such as drug metabolism and in vitro & in vivo DMPK (bioavailability, half-life, clearance and metabolic profile), should be evaluated as well in the future.

References

1. Korber B, Muldoon M, Theiler J, Gao F, Gupta R, Lapedes A, et al. Timing the ancestor of the HIV-1 pandemic strains. Science. 2000;288(5472):1789-96. PubMed PMID: 10846155.

2. (CDC) CDC. Kaposi's sarcoma and Pneumocystis pneumonia among homosexual men--New York City and California. MMWR Morb Mortal Wkly Rep. 1981;30(25):305-8. PubMed PMID: 6789108.

3. (CDC) CDC. Pneumocystis pneumonia--Los Angeles. MMWR Morb Mortal Wkly Rep. 1981;30(21):250-2. PubMed PMID: 6265753.

4. Altman LK. New Homosexual Disorder Worries Health Officials. New York Times. May 11, 1982.

5. (CDC) CDC. Possible transfusion-associated acquired immune deficiency syndrome (AIDS) - California. MMWR Morb Mortal Wkly Rep. 1982;31(48):652-4. PubMed PMID: 6819440.

6. Barré-Sinoussi F, Chermann JC, Rey F, Nugeyre MT, Chamaret S, Gruest J, et al. Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). Science. 1983;220(4599):868-71. PubMed PMID: 6189183.

7. Gallo RC, Salahuddin SZ, Popovic M, Shearer GM, Kaplan M, Haynes BF, et al. Frequent detection and isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk for AIDS. Science. 1984;224(4648):500-3. PubMed PMID: 6200936.

8. UNAIDS. Global HIV & AIDS statistics. 2019.

9. Goff SP. Retroviridae. In: Knipe DM, Howley PM, editors. Fields Virology. 2. 6th ed. Philadelphia, PA: Lippincott Williams and Wilkins; 2013. p. 1425 - 73.

10. Clavel F, Guétard D, Brun-Vézinet F, Chamaret S, Rey MA, Santos-Ferreira MO, et al. Isolation of a new human retrovirus from West African patients with AIDS. Science. 1986;233(4761):343-6. PubMed PMID: 2425430.

11. Deeks SG, Overbaugh J, Phillips A, Buchbinder S. HIV infection. Nat Rev Dis Primers. 2015;1:15035. Epub 2015/01/01. doi: 10.1038/nrdp.2015.35. PubMed PMID: 27188527.

12. Sharp PM, Hahn BH. Origins of HIV and the AIDS pandemic. Cold Spring Harb Perspect Med. 2011;1(1):a006841. doi: 10.1101/cshperspect.a006841. PubMed PMID: 22229120; PubMed Central PMCID: PMCPMC3234451.

13. N BH. HIV Infection «HIV Tat Protein, a Key Factor in Pathogenesis and Immune System Dysregulation: Implication of IL-10. 9789533074429, editor. Croatia: INTECH; 2011.

14. Peterlin BM, Trono D. Hide, shield and strike back: how HIV-infected cells avoid immune eradication. Nat Rev Immunol. 2003;3(2):97-107. Epub 2003/02/04. doi: 10.1038/nri998. PubMed PMID: 12563294.

15. King SR. HIV: virology and mechanisms of disease. Ann Emerg Med. 1994;24(3):443-9. PubMed PMID: 7915889.

16. Freed EO, Martin MA. Human Immunodeficiency Virus: Replication. In: Knipe DM, Howley PM, editors. Fields Virology. 2. 6th ed. Philadelphia, PA: Lippincott Williams and Wilkins; 2013. p. 1502 - 60.

17. Liu Y, Liu H, Kim BO, Gattone VH, Li J, Nath A, et al. CD4-independent infection of astrocytes by human immunodeficiency virus type 1: requirement for the human mannose receptor. J Virol. 2004;78(8):4120-33. Epub 2004/03/30. doi: 10.1128/jvi.78.8.4120-4133.2004. PubMed PMID: 15047828; PubMed Central PMCID: PMCPMC374297.

18. Gorry PR, Ancuta P. Coreceptors and HIV-1 pathogenesis. Curr HIV/AIDS Rep. 2011;8(1):45-53. doi: 10.1007/s11904-010-0069-x. PubMed PMID: 21188555.

19. Engelman A, Cherepanov P. The structural biology of HIV-1: mechanistic and therapeutic insights. Nat Rev Microbiol. 2012;10(4):279-90. Epub 2012/03/17. doi: 10.1038/nrmicro2747. PubMed PMID: 22421880; PubMed Central PMCID: PMCPMC3588166.

20. Kruize Z, Kootstra NA. The Role of Macrophages in HIV-1 Persistence and Pathogenesis. Front Microbiol. 2019;10:2828. Epub 2019/12/24. doi: 10.3389/fmicb.2019.02828. PubMed PMID: 31866988; PubMed Central PMCID: PMCPMC6906147.

21. Haase AT. Perils at mucosal front lines for HIV and SIV and their hosts. Nat Rev Immunol. 2005;5(10):783-92. doi: 10.1038/nri1705. PubMed PMID: 16200081.

22. Simon V, Ho DD, Abdool Karim Q. HIV/AIDS epidemiology, pathogenesis, prevention, and treatment. Lancet. 2006;368(9534):489-504. Epub 2006/08/08. doi: 10.1016/S0140-6736(06)69157-5. PubMed PMID: 16890836; PubMed Central PMCID: PMCPMC2913538.

23. McMichael AJ, Borrow P, Tomaras GD, Goonetilleke N, Haynes BF. The immune response during acute HIV-1 infection: clues for vaccine development. Nat Rev Immunol. 2010;10(1):11-23. Epub 2009/12/11. doi: 10.1038/nri2674. PubMed PMID: 20010788; PubMed Central PMCID: PMCPMC3119211.

24. Finkel TH, Tudor-Williams G, Banda NK, Cotton MF, Curiel T, Monks C, et al. Apoptosis occurs predominantly in bystander cells and not in productively infected cells of HIV- and SIV-infected lymph nodes. Nat Med. 1995;1(2):129-34. PubMed PMID: 7585008.

25. Garg H, Mohl J, Joshi A. HIV-1 induced bystander apoptosis. Viruses. 2012;4(11):3020-43. Epub 2012/11/09. doi: 10.3390/v4113020. PubMed PMID: 23202514; PubMed Central PMCID: PMCPMC3509682.

26. Goonetilleke N, Liu MK, Salazar-Gonzalez JF, Ferrari G, Giorgi E, Ganusov VV, et al. The first T cell response to transmitted/founder virus contributes to the control of acute viremia in HIV-1 infection. J Exp Med. 2009;206(6):1253-72. Epub 2009/06/01. doi: 10.1084/jem.20090365. PubMed PMID: 19487423; PubMed Central PMCID: PMCPMC2715063.

27. Wei X, Decker JM, Wang S, Hui H, Kappes JC, Wu X, et al. Antibody neutralization and escape by HIV-1. Nature. 2003;422(6929):307-12. doi: 10.1038/nature01470. PubMed PMID: 12646921.

28. Richman DD, Wrin T, Little SJ, Petropoulos CJ. Rapid evolution of the neutralizing antibody response to HIV type 1 infection. Proc Natl Acad Sci U S A. 2003;100(7):4144-9. Epub 2003/03/18. doi: 10.1073/pnas.0630530100. PubMed PMID: 12644702; PubMed Central PMCID: PMCPMC153062.

29. Martinez-Picado J, Deeks SG. Persistent HIV-1 replication during antiretroviral therapy. Curr Opin HIV AIDS. 2016;11(4):417-23. Epub 2016/04/15. doi: 10.1097/COH.0000000000000287. PubMed PMID: 27078619; PubMed Central PMCID: PMCPMC4900428.

30. Marin B, Thiébaut R, Bucher HC, Rondeau V, Costagliola D, Dorrucci M, et al. Non-AIDS-defining deaths and immunodeficiency in the era of combination antiretroviral therapy. AIDS. 2009;23(13):1743-53. doi: 10.1097/QAD.0b013e32832e9b78. PubMed PMID: 19571723; PubMed Central PMCID: PMCPMC3305466.

31. Capeau J. Premature Aging and Premature Age-Related Comorbidities in HIV-Infected Patients: Facts and Hypotheses. Clin Infect Dis. 2011;53(11):1127-9. Epub 2011/10/13. doi: 10.1093/cid/cir628. PubMed PMID: 21998279.

32. Dornadula G, Zhang H, VanUitert B, Stern J, Livornese L, Ingerman MJ, et al. Residual HIV-1 RNA in blood plasma of patients taking suppressive highly active antiretroviral therapy. JAMA. 1999;282(17):1627-32. PubMed PMID: 10553788.

33. Palmer S, Maldarelli F, Wiegand A, Bernstein B, Hanna GJ, Brun SC, et al. Low-level viremia persists for at least 7 years in patients on suppressive antiretroviral therapy. Proc Natl Acad Sci U S A. 2008;105(10):3879-84. Epub 2008/03/10. doi: 10.1073/pnas.0800050105. PubMed PMID: 18332425; PubMed Central PMCID: PMCPMC2268833.

34. Ritchie MRaH. HIV / AIDS: OurWorldInData.org; 2020 [updated 2020].

35. Saylor D, Dickens AM, Sacktor N, Haughey N, Slusher B, Pletnikov M, et al. HIV-associated neurocognitive disorder--pathogenesis and prospects for treatment. Nat Rev Neurol. 2016;12(4):234-48. Epub 2016/03/12. doi: 10.1038/nrneurol.2016.27. PubMed PMID: 26965674; PubMed Central PMCID: PMCPMC4937456.

36. Archin NM, Sung JM, Garrido C, Soriano-Sarabia N, Margolis DM. Eradicating HIV-1 infection: seeking to clear a persistent pathogen. Nat Rev Microbiol. 2014;12(11):750-64. Epub 2014/11/18. doi: 10.1038/nrmicro3352. PubMed PMID: 25402363; PubMed Central PMCID: PMCPMC4383747.

37. unaids. HIV treatment: unaids; 2020.

38. Dahabieh MS, Battivelli E, Verdin E. Understanding HIV latency: the road to an HIV cure. Annu Rev Med. 2015;66:407-21. doi: 10.1146/annurev-med-092112-152941. PubMed PMID: 25587657; PubMed Central PMCID: PMCPMC4381961.

39. Siliciano RF, Greene WC. HIV latency. Cold Spring Harb Perspect Med. 2011;1(1):a007096. doi: 10.1101/cshperspect.a007096. PubMed PMID: 22229121; PubMed Central PMCID: PMCPMC3234450.

40. Fletcher CV, Staskus K, Wietgrefe SW, Rothenberger M, Reilly C, Chipman JG, et al. Persistent HIV-1 replication is associated with lower antiretroviral drug concentrations in lymphatic tissues. Proc Natl Acad Sci U S A. 2014;111(6):2307-12. doi: 10.1073/pnas.1318249111. PubMed PMID: 24469825; PubMed Central PMCID: PMCPMC3926074.

41. Lorenzo-Redondo R, Fryer HR, Bedford T, Kim EY, Archer J, Pond SLK, et al. Persistent HIV-1 replication maintains the tissue reservoir during therapy. Nature. 2016;530(7588):51-6. Epub 2016/01/27. doi: 10.1038/nature16933. PubMed PMID: 26814962; PubMed Central PMCID: PMCPMC4865637.

42. Hatzakis AE, Touloumi G, Pantazis N, Anastassopoulou CG, Katsarou O, Karafoulidou A, et al. Cellular HIV-1 DNA load predicts HIV-RNA rebound and the outcome of highly active antiretroviral therapy. AIDS. 2004;18(17):2261-7. doi: 10.1097/00002030-200411190-00006. PubMed PMID: 15577538.

43. Davey RT, Bhat N, Yoder C, Chun TW, Metcalf JA, Dewar R, et al. HIV-1 and T cell dynamics after interruption of highly active antiretroviral therapy (HAART) in patients with a history of sustained viral suppression. Proc Natl Acad Sci U S A. 1999;96(26):15109-14. doi: 10.1073/pnas.96.26.15109. PubMed PMID: 10611346; PubMed Central PMCID: PMCPMC24781.

44. Lagathu C, Eustace B, Prot M, Frantz D, Gu Y, Bastard JP, et al. Some HIV antiretrovirals increase oxidative stress and alter chemokine, cytokine or adiponectin production in human adipocytes and macrophages. Antivir Ther. 2007;12(4):489-500. Epub 2007/08/03. PubMed PMID: 17668557.

45. Appay V, Sauce D. Immune activation and inflammation in HIV-1 infection: causes and consequences. J Pathol. 2008;214(2):231-41. Epub 2007/12/29. doi: 10.1002/path.2276. PubMed PMID: 18161758.

46. Mega ER. Alarming surge in drug-resistant HIV uncovered: Nature; 2019.

47. Ellis R, Langford D, Masliah E. HIV and antiretroviral therapy in the brain: neuronal injury and repair. Nat Rev Neurosci. 2007;8(1):33-44. doi: 10.1038/nrn2040. PubMed PMID: 17180161.

48. Wallet C, De Rovere M, Van Assche J, Daouad F, De Wit S, Gautier V, et al. Microglial Cells: The Main HIV-1 Reservoir in the Brain. Front Cell Infect Microbiol. 2019;9:362. Epub 2019/11/12. doi: 10.3389/fcimb.2019.00362. PubMed PMID: 31709195; PubMed Central PMCID: PMCPMC6821723.

49. Whitney JB, Hill AL, Sanisetty S, Penaloza-MacMaster P, Liu J, Shetty M, et al. Rapid seeding of the viral reservoir prior to SIV viraemia in rhesus monkeys. Nature. 2014;512(7512):74-7. Epub 2014/07/22. doi: 10.1038/nature13594. PubMed PMID: 25042999; PubMed Central PMCID: PMCPMC4126858.

50. McArthur JC, Brew BJ, Nath A. Neurological complications of HIV infection. Lancet Neurol. 2005;4(9):543-55. Epub 2005/08/20. doi: 10.1016/S1474-4422(05)70165-4. PubMed PMID: 16109361.

51. Asahchop EL, Meziane O, Mamik MK, Chan WF, Branton WG, Resch L, et al. Reduced antiretroviral drug efficacy and concentration in HIV-infected microglia contributes to viral persistence in brain. Retrovirology. 2017;14(1):47. Epub 2017/10/16. doi: 10.1186/s12977-017-0370-5. PubMed PMID: 29037245; PubMed Central PMCID: PMCPMC5644262.

52. Grennan JT, Loutfy MR, Su D, Harrigan PR, Cooper C, Klein M, et al. Magnitude of virologic blips is associated with a higher risk for virologic rebound in HIV-infected individuals: a recurrent events analysis. J Infect Dis. 2012;205(8):1230-8. doi: 10.1093/infdis/jis104. PubMed PMID: 22438396; PubMed Central PMCID: PMCPMC3308904.

53. Taiwo B, Bosch RJ. More reasons to reexamine the definition of viral blip during antiretroviral therapy. J Infect Dis. 2012;205(8):1189-91. doi: 10.1093/infdis/jis109. PubMed PMID: 22438394.

54. Strain MC, Letendre S, Pillai SK, Russell T, Ignacio CC, Gunthard HF, et al. Genetic composition of human immunodeficiency virus type 1 in cerebrospinal fluid and blood without treatment and during failing antiretroviral therapy. J Virol. 2005;79(3):1772-88. Epub 2005/01/15. doi: 10.1128/JVI.79.3.1772-1788.2005. PubMed PMID: 15650202; PubMed Central PMCID: PMCPMC544082.

55. Cunningham PH, Smith DG, Satchell C, Cooper DA, Brew B. Evidence for independent development of resistance to HIV-1 reverse transcriptase inhibitors in the cerebrospinal fluid. AIDS. 2000;14(13):1949-54. Epub 2000/09/21. doi: 10.1097/00002030-200009080-00010. PubMed PMID: 10997399.

56. Venturi G, Catucci M, Romano L, Corsi P, Leoncini F, Valensin PE, et al. Antiretroviral resistance mutations in human immunodeficiency virus type 1 reverse transcriptase and protease from paired cerebrospinal fluid and plasma samples. J Infect Dis. 2000;181(2):740-5. Epub 2000/02/11. doi: 10.1086/315249. PubMed PMID: 10669367.

57. Ko A, Kang G, Hattler JB, Galadima HI, Zhang J, Li Q, et al. Macrophages but not Astrocytes Harbor HIV DNA in the Brains of HIV-1-Infected Aviremic Individuals on Suppressive Antiretroviral Therapy. J Neuroimmune Pharmacol. 2019;14(1):110-9. Epub 2018/09/07. doi: 10.1007/s11481-018-9809-2. PubMed PMID: 30194646; PubMed Central PMCID: PMCPMC6391194.

58. Garcia-Mesa Y, Jay TR, Checkley MA, Luttge B, Dobrowolski C, Valadkhan S, et al. Immortalization of primary microglia: a new platform to study HIV regulation in the central nervous system. J Neurovirol. 2017;23(1):47-66. Epub 2016/11/21. doi: 10.1007/s13365-016-0499-3. PubMed PMID: 27873219; PubMed Central PMCID: PMCPMC5329090.

59. Greter M, Lelios I, Croxford AL. Microglia Versus Myeloid Cell Nomenclature during Brain Inflammation. Front Immunol. 2015;6:249. Epub 2015/06/16. doi: 10.3389/fimmu.2015.00249. PubMed PMID: 26074918; PubMed Central PMCID: PMCPMC4443742.

60. Le Douce V, Herbein G, Rohr O, Schwartz C. Molecular mechanisms of HIV-1 persistence in the monocyte-macrophage lineage. Retrovirology. 2010;7:32. Epub 2010/04/09. doi: 10.1186/1742-4690-7-32. PubMed PMID: 20380694; PubMed Central PMCID: PMCPMC2873506.

61. Elsheikh MM, Tang Y, Li D, Jiang G. Deep latency: A new insight into a functional HIV cure. EBioMedicine. 2019;45:624-9. Epub 2019/06/23. doi: 10.1016/j.ebiom.2019.06.020. PubMed PMID: 31227439; PubMed Central PMCID: PMCPMC6642357.

62. Mbonye U, Karn J. Transcriptional control of HIV latency: cellular signaling pathways, epigenetics, happenstance and the hope for a cure. Virology. 2014;454-455:328-39. Epub 2014/02/26. doi: 10.1016/j.virol.2014.02.008. PubMed PMID: 24565118; PubMed Central PMCID: PMCPMC4010583.

63. Cary DC, Fujinaga K, Peterlin BM. Molecular mechanisms of HIV latency. J Clin Invest. 2016;126(2):448-54. Epub 2016/01/06. doi: 10.1172/JCI80565. PubMed PMID: 26731470; PubMed Central PMCID: PMCPMC4731164.

64. Vansant G, Bruggemans A, Janssens J, Debyser Z. Block-And-Lock Strategies to Cure HIV Infection. Viruses. 2020;12(1). Epub 2020/01/16. doi: 10.3390/v12010084. PubMed PMID: 31936859; PubMed Central PMCID: PMCPMC7019976.

65. Rice AP. Unexpected Mutations in HIV-1 That Confer Resistance to the Tat Inhibitor Didehydro-Cortistatin A. MBio. 2019;10(4). Epub 2019/07/09. doi: 10.1128/mBio.01547-19. PubMed PMID: 31289189; PubMed Central PMCID: PMCPMC6747727.

66. Mousseau G, Valente ST. Role of Host Factors on the Regulation of Tat-Mediated HIV-1 Transcription. Curr Pharm Des. 2017;23(28):4079-90. Epub 2017/06/24. doi: 10.2174/1381612823666170622104355. PubMed PMID: 28641539; PubMed Central PMCID: PMCPMC5731639.

67. Mbonye U, Karn J. The Molecular Basis for Human Immunodeficiency Virus Latency. Annu Rev Virol. 2017;4(1):261-85. Epub 2017/07/19. doi: 10.1146/annurev-virology-101416-041646. PubMed PMID: 28715973.

68. Darcis G, Van Driessche B, Van Lint C. HIV Latency: Should We Shock or Lock? Trends Immunol. 2017;38(3):217-28. Epub 2017/01/07. doi: 10.1016/j.it.2016.12.003. PubMed PMID: 28073694.

69. Archin NM, Kirchherr JL, Sung JA, Clutton G, Sholtis K, Xu Y, et al. Interval dosing with the HDAC inhibitor vorinostat effectively reverses HIV latency. J Clin Invest. 2017;127(8):3126-35. Epub 2017/07/18. doi: 10.1172/JCI92684. PubMed PMID: 28714868; PubMed Central PMCID: PMCPMC5531421.

70. Spivak AM, Planelles V. Novel Latency Reversal Agents for HIV-1 Cure. Annu Rev Med. 2018;69:421-36. Epub 2017/11/04. doi: 10.1146/annurev-med-052716-031710. PubMed PMID: 29099677; PubMed Central PMCID: PMCPMC5892446.

71. Lin CMV, editor THE CURRENT STATUS OF LATENCY REVERSING AGENTS. CROI: CONFERENCE ON RETROVIRUSES

AND OPPORTUNISTIC INFECTIONS; 2019: CROI.

72. Marban C, Forouzanfar F, Ait-Ammar A, Fahmi F, El Mekdad H, Daouad F, et al. Targeting the Brain Reservoirs: Toward an HIV Cure. Front Immunol. 2016;7:397. Epub 2016/10/18. doi: 10.3389/fimmu.2016.00397. PubMed PMID: 27746784; PubMed Central PMCID: PMCPMC5044677.

73. Gama L, Abreu CM, Shirk EN, Price SL, Li M, Laird GM, et al. Reactivation of simian immunodeficiency virus reservoirs in the brain of virally suppressed macaques. AIDS. 2017;31(1):5-14. Epub 2016/11/30. doi: 10.1097/QAD.0000000000001267. PubMed PMID: 27898590; PubMed Central PMCID: PMCPMC5131686.

74. Hajmirza A, Emadali A, Gauthier A, Casasnovas O, Gressin R, Callanan MB. BET Family Protein BRD4: An Emerging Actor in NFkappaB Signaling in Inflammation and Cancer. Biomedicines. 2018;6(1). Epub 2018/02/09. doi: 10.3390/biomedicines6010016. PubMed PMID: 29415456; PubMed Central PMCID: PMCPMC5874673.

75. Liu Z, Wang P, Chen H, Wold EA, Tian B, Brasier AR, et al. Drug Discovery Targeting Bromodomain-Containing Protein 4. J Med Chem. 2017;60(11):4533-58. Epub 2017/03/02. doi: 10.1021/acs.jmedchem.6b01761. PubMed PMID: 28195723; PubMed Central PMCID: PMCPMC5464988.

76. Cochran AG, Conery AR, Sims RJ, 3rd. Bromodomains: a new target class for drug development. Nat Rev Drug Discov. 2019;18(8):609-28. Epub 2019/07/06. doi: 10.1038/s41573-019-0030-7. PubMed PMID: 31273347.

77. Niu Q, Liu Z, Alamer E, Fan X, Chen H, Endsley J, et al. Structure-guided drug design identifies a BRD4-selective small molecule that suppresses HIV. J Clin Invest. 2019;130:3361-73. Epub 2019/07/22. doi: 10.1172/JCI120633. PubMed PMID: 31329163; PubMed Central PMCID: PMCPMC6668673.

78. Saag MS, Benson CA, Gandhi RT, Hoy JF, Landovitz RJ, Mugavero MJ, et al. Antiretroviral Drugs for Treatment and Prevention of HIV Infection in Adults: 2018 Recommendations of the International Antiviral Society-USA Panel. JAMA. 2018;320(4):379-96. Epub 2018/07/26. doi: 10.1001/jama.2018.8431. PubMed PMID: 30043070; PubMed Central PMCID: PMCPMC6415748.

79. Heaton RK, Clifford DB, Franklin DR, Woods SP, Ake C, Vaida F, et al. HIV-associated neurocognitive disorders persist in the era of potent antiretroviral therapy: CHARTER Study. Neurology. 2010;75(23):2087-96. doi: 10.1212/WNL.0b013e318200d727. PubMed PMID: 21135382; PubMed Central PMCID: PMCPMC2995535.

80. Thompson KA, Cherry CL, Bell JE, McLean CA. Brain cell reservoirs of latent virus in presymptomatic HIV-infected individuals. Am J Pathol. 2011;179(4):1623-9. Epub 2011/08/30. doi: 10.1016/j.ajpath.2011.06.039. PubMed PMID: 21871429; PubMed Central PMCID: PMCPMC3181362.

81. Churchill MJ, Gorry PR, Cowley D, Lal L, Sonza S, Purcell DF, et al. Use of laser capture microdissection to detect integrated HIV-1 DNA in macrophages and astrocytes from autopsy brain tissues. J Neurovirol. 2006;12(2):146-52. Epub 2006/06/27. doi: 10.1080/13550280600748946. PubMed PMID: 16798676.

82. Ginhoux F, Prinz M. Origin of microglia: current concepts and past controversies. Cold Spring Harb Perspect Biol. 2015;7(8):a020537. Epub 2015/07/03. doi: 10.1101/cshperspect.a020537. PubMed PMID: 26134003; PubMed Central PMCID: PMCPMC4526747.

83. Rock RB, Gekker G, Hu S, Sheng WS, Cheeran M, Lokensgard JR, et al. Role of microglia in central nervous system infections. Clin Microbiol Rev. 2004;17(4):942-64, table of contents. Epub 2004/10/19. doi: 10.1128/CMR.17.4.942-964.2004. PubMed PMID: 15489356; PubMed Central PMCID: PMCPMC523558.

84. Watkins CC, Treisman GJ. Cognitive impairment in patients with AIDS - prevalence and severity. HIV AIDS (Auckl). 2015;7:35-47. Epub 2015/02/14. doi: 10.2147/HIV.S39665. PubMed PMID: 25678819; PubMed Central PMCID: PMCPMC4319681.

85. Kaul M. HIV-1 associated dementia: update on pathological mechanisms and therapeutic approaches. Curr Opin Neurol. 2009;22(3):315-20. doi: 10.1097/WCO.0b013e328329cf3c. PubMed PMID: 19300249; PubMed Central PMCID: PMCPMC2779773.

86. Chan P, Brew BJ. HIV associated neurocognitive disorders in the modern antiviral treatment era: prevalence, characteristics, biomarkers, and effects of treatment. Curr HIV/AIDS Rep. 2014;11(3):317-24. doi: 10.1007/s11904-014-0221-0. PubMed PMID: 24966139.

87. Perelson AS, Essunger P, Cao Y, Vesanen M, Hurley A, Saksela K, et al. Decay characteristics of HIV-1-infected compartments during combination therapy. Nature. 1997;387(6629):188-91. doi: 10.1038/387188a0. PubMed PMID: 9144290.

88. Wong JK, Hezareh M, Gunthard HF, Havlir DV, Ignacio CC, Spina CA, et al. Recovery of replication-competent HIV despite prolonged suppression of plasma viremia. Science. 1997;278(5341):1291-5. PubMed PMID: 9360926.

89. Hakre S, Chavez L, Shirakawa K, Verdin E. Epigenetic regulation of HIV latency. Curr Opin HIV AIDS. 2011;6(1):19-24. doi: 10.1097/COH.0b013e3283412384. PubMed PMID: 21242889.

90. Zhu J, Gaiha GD, John SP, Pertel T, Chin CR, Gao G, et al. Reactivation of latent HIV-1 by inhibition of BRD4. Cell Rep. 2012;2(4):807-16. doi: 10.1016/j.celrep.2012.09.008. PubMed PMID: 23041316; PubMed Central PMCID: PMCPMC3523124.

91. Li Z, Guo J, Wu Y, Zhou Q. The BET bromodomain inhibitor JQ1 activates HIV latency through antagonizing Brd4 inhibition of Tat-transactivation. Nucleic Acids Res. 2013;41(1):277-87. doi: 10.1093/nar/gks976. PubMed PMID: 23087374; PubMed Central PMCID: PMCPMC3592394.

92. Conrad RJ, Fozouni P, Thomas S, Sy H, Zhang Q, Zhou MM, et al. The Short Isoform of BRD4 Promotes HIV-1 Latency by Engaging Repressive SWI/SNF Chromatin-Remodeling Complexes. Mol Cell. 2017;67(6):1001-12 e6. doi: 10.1016/j.molcel.2017.07.025. PubMed PMID: 28844864; PubMed Central PMCID: PMCPMC5610089.

93. Bisgrove DA, Mahmoudi T, Henklein P, Verdin E. Conserved P-TEFb-interacting domain of BRD4 inhibits HIV transcription. Proc Natl Acad Sci U S A. 2007;104(34):13690-5. doi: 10.1073/pnas.0705053104. PubMed PMID: 17690245; PubMed Central PMCID: PMCPMC1959443.

94. Padmanabhan B, Mathur S, Manjula R, Tripathi S. Bromodomain and extra-terminal (BET) family proteins: New therapeutic targets in major diseases. J Biosci. 2016;41(2):295-311. PubMed PMID: 27240990.

95. Donati B, Lorenzini E, Ciarrocchi A. BRD4 and Cancer: going beyond transcriptional regulation. Mol Cancer. 2018;17(1):164. Epub 2018/11/24. doi: 10.1186/s12943-018-0915-9. PubMed PMID: 30466442; PubMed Central PMCID: PMCPMC6251205.

96. Li Z, Mbonye U, Feng Z, Wang X, Gao X, Karn J, et al. The KAT5-Acetyl-Histone4-Brd4 axis silences HIV-1 transcription and promotes viral latency. PLoS Pathog. 2018;14(4):e1007012. Epub 2018/04/24. doi: 10.1371/journal.ppat.1007012. PubMed PMID: 29684085; PubMed Central PMCID: PMCPMC5933813.

97. Alvarez-Carbonell D, Garcia-Mesa Y, Milne S, Das B, Dobrowolski C, Rojas R, et al. Toll-like receptor 3 activation selectively reverses HIV latency in microglial cells. Retrovirology. 2017;14(1):9. Epub 2017/02/06. doi: 10.1186/s12977-017-0335-8. PubMed PMID: 28166799; PubMed Central PMCID: PMCPMC5294768.

98. Folks TM, Justement J, Kinter A, Dinarello CA, Fauci AS. Cytokine-induced expression of HIV-1 in a chronically infected promonocyte cell line. Science. 1987;238(4828):800-2. doi: 10.1126/science.3313729. PubMed PMID: 3313729.

99. Butera ST, Perez VL, Wu BY, Nabel GJ, Folks TM. Oscillation of the human immunodeficiency virus surface receptor is regulated by the state of viral activation in a CD4+ cell model of chronic infection. J Virol. 1991;65(9):4645-53. PubMed PMID: 1678437; PubMed Central PMCID: PMCPMC248919.

100. Rafati H, Parra M, Hakre S, Moshkin Y, Verdin E, Mahmoudi T. Repressive LTR nucleosome positioning by the BAF complex is required for HIV latency. PLoS Biol. 2011;9(11):e1001206. Epub 2011/11/29. doi: 10.1371/journal.pbio.1001206. PubMed PMID: 22140357; PubMed Central PMCID: PMCPMC3226458.

101. Mousseau G, Kessing CF, Fromentin R, Trautmann L, Chomont N, Valente ST. The Tat Inhibitor Didehydro-Cortistatin A Prevents HIV-1 Reactivation from Latency. MBio. 2015;6(4):e00465. doi: 10.1128/mBio.00465-15. PubMed PMID: 26152583; PubMed Central PMCID: PMCPMC4495168.

102. Best BM, Letendre SL, Koopmans P, Rossi SS, Clifford DB, Collier AC, et al. Low cerebrospinal fluid concentrations of the nucleotide HIV reverse transcriptase inhibitor, tenofovir. J Acquir Immune Defic Syndr. 2012;59(4):376-81. Epub 2012/01/06. doi: 10.1097/QAI.0b013e318247ec54. PubMed PMID: 22217676; PubMed Central PMCID: PMCPMC3299895.

103. Kessing CF, Nixon CC, Li C, Tsai P, Takata H, Mousseau G, et al. In Vivo Suppression of HIV Rebound by Didehydro-Cortistatin A, a "Block-and-Lock" Strategy for HIV-1

Treatment. Cell Rep. 2017;21(3):600-11. doi: 10.1016/j.celrep.2017.09.080. PubMed PMID: 29045830; PubMed Central PMCID: PMCPMC5653276.

104. Kim H, Choi MS, Inn KS, Kim BJ. Inhibition of HIV-1 reactivation by a telomerase-derived peptide in a HSP90-dependent manner. Sci Rep. 2016;6:28896. Epub 2016/07/01. doi: 10.1038/srep28896. PubMed PMID: 27363520; PubMed Central PMCID: PMCPMC4929463.

105. Jean MJ, Hayashi T, Huang H, Brennan J, Simpson S, Purmal A, et al. Curaxin CBL0100 Blocks HIV-1 Replication and Reactivation through Inhibition of Viral Transcriptional Elongation. Front Microbiol. 2017;8:2007. Epub 2017/10/17. doi: 10.3389/fmicb.2017.02007. PubMed PMID: 29089933; PubMed Central PMCID: PMCPMC5651003.

106. Zhao R, Li Y, Gorantla S, Poluektova LY, Lin H, Gao F, et al. Small molecule ONC201 inhibits HIV-1 replication in macrophages via FOXO3a and TRAIL. Antiviral Res. 2019;168:134-45. Epub 2019/05/31. doi: 10.1016/j.antiviral.2019.05.015. PubMed PMID: 31158413; PubMed Central PMCID: PMCPMC6620139.

107. González-Scarano F, Martín-García J. The neuropathogenesis of AIDS. Nat Rev Immunol. 2005;5(1):69-81. doi: 10.1038/nri1527. PubMed PMID: 15630430.

108. Ioannidis JP, Reichlin S, Skolnik PR. Long-term productive human immunodeficiency virus-1 infection in human infant microglia. Am J Pathol. 1995;147(5):1200-6. PubMed PMID: 7485383; PubMed Central PMCID: PMCPMC1869535.

109. Clifford DB. Viral escape in cerebrospinal fluid--an achilles heel of HIV therapy? J Infect Dis. 2010;202(12):1768-9. Epub 2010/11/04. doi: 10.1086/657343. PubMed PMID: 21050117.

110. Chun TW, Nickle DC, Justement JS, Large D, Semerjian A, Curlin ME, et al. HIV-infected individuals receiving effective antiviral therapy for extended periods of time continually replenish their viral reservoir. J Clin Invest. 2005;115(11):3250-5. doi: 10.1172/JCI26197. PubMed PMID: 16276421; PubMed Central PMCID: PMCPMC1265878.

111. Jones LE, Perelson AS. Transient viremia, plasma viral load, and reservoir replenishment in HIV-infected patients on antiretroviral therapy. J Acquir Immune Defic Syndr. 2007;45(5):483-93. doi: 10.1097/QAI.0b013e3180654836. PubMed PMID: 17496565; PubMed Central PMCID: PMCPMC2584971.

112. Siliciano JD, Kajdas J, Finzi D, Quinn TC, Chadwick K, Margolick JB, et al. Long-term follow-up studies confirm the stability of the latent reservoir for HIV-1 in resting CD4+ T cells. Nat Med. 2003;9(6):727-8. doi: 10.1038/nm880. PubMed PMID: 12754504.

113. Ramratnam B, Mittler JE, Zhang L, Boden D, Hurley A, Fang F, et al. The decay of the latent reservoir of replication-competent HIV-1 is inversely correlated with the extent of residual viral replication during prolonged anti-retroviral therapy. Nat Med. 2000;6(1):82-5. doi: 10.1038/71577. PubMed PMID: 10613829.

114. Auclair S, Liu F, Niu Q, Hou W, Churchyard G, Morgan C, et al. Distinct susceptibility of HIV vaccine vector-induced CD4 T cells to HIV infection. PLoS Pathog.

2018;14(2):e1006888. doi: 10.1371/journal.ppat.1006888. PubMed PMID: 29474461; PubMed Central PMCID: PMCPMC5841825.

115. Hu H, Eller MA, Zafar S, Zhou Y, Gu M, Wei Z, et al. Preferential infection of human Ad5-specific CD4 T cells by HIV in Ad5 naturally exposed and recombinant Ad5-HIV vaccinated individuals. Proc Natl Acad Sci U S A. 2014;111(37):13439-44. doi: 10.1073/pnas.1400446111. PubMed PMID: 25197078; PubMed Central PMCID: PMC4169982.

116. Hu H, Nau M, Ehrenberg P, Chenine AL, Macedo C, Zhou Y, et al. Distinct gene-expression profiles associated with the susceptibility of pathogen-specific CD4 T cells to HIV-1 infection. Blood. 2013;121(7):1136-44. doi: 10.1182/blood-2012-07-446278. PubMed PMID: 23258923; PubMed Central PMCID: PMC3575757.

117. Liu F, Fan X, Auclair S, Ferguson M, Sun J, Soong L, et al. Sequential Dysfunction and Progressive Depletion of Candida albicans-Specific CD4 T Cell Response in HIV-1 Infection. PLoS Pathog. 2016;12(6):e1005663. doi: 10.1371/journal.ppat.1005663. PubMed PMID: 27280548; PubMed Central PMCID: PMC4900544.

118. O'Brien WA, Koyanagi Y, Namazie A, Zhao JQ, Diagne A, Idler K, et al. HIV-1 tropism for mononuclear phagocytes can be determined by regions of gp120 outside the CD4-binding domain. Nature. 1990;348(6296):69-73. Epub 1990/11/01. doi: 10.1038/348069a0. PubMed PMID: 2172833.

119. Deeks SG, Tracy R, Douek DC. Systemic effects of inflammation on health during chronic HIV infection. Immunity. 2013;39(4):633-45. Epub 2013/10/22. doi: 10.1016/j.immuni.2013.10.001. PubMed PMID: 24138880; PubMed Central PMCID: PMCPMC4012895.

120. Zicari S, Sessa L, Cotugno N, Ruggiero A, Morrocchi E, Concato C, et al. Immune Activation, Inflammation, and Non-AIDS Co-Morbidities in HIV-Infected Patients under Long-Term ART. Viruses. 2019;11(3). Epub 2019/03/02. doi: 10.3390/v11030200. PubMed PMID: 30818749; PubMed Central PMCID: PMCPMC6466530.

121. Sereti I, Altfeld M. Immune activation and HIV: an enduring relationship. Curr Opin HIV AIDS. 2016;11(2):129-30. Epub 2016/02/06. doi: 10.1097/COH.0000000000000244. PubMed PMID: 26845672; PubMed Central PMCID: PMCPMC4748385.

122. Besnard E, Hakre S, Kampmann M, Lim HW, Hosmane NN, Martin A, et al. The mTOR Complex Controls HIV Latency. Cell Host Microbe. 2016;20(6):785-97. Epub 2016/12/16. doi: 10.1016/j.chom.2016.11.001. PubMed PMID: 27978436; PubMed Central PMCID: PMCPMC5354304.

Vita

Edrous Sadig I. Alamer, BS, MS.

PRESENT POSITION

Graduate Research Assistant
Department of Microbiology and Immunology
The University of Texas Medical Branch
301 University Boulevard,
Galveston, Texas, 77555-1061
Phone: 409-772-0926 (office) 312-479-6657 (cell)
Email: edalamer@utmb.edu

EDUCATION

Sept 2015 – Present

Ph.D. Student
Department of Microbiology and Immunology
The University of Texas Medical Branch at Galveston
Mentors: Dr. Haitao Hu and Dr. Lynn Soong

Jan 2013 – May 2015

Master of Health Science in Medical Laboratory Sciences (Thesis Track).
Quinnipiac University, Hamden, CT
Mentor: Dr. Sheldon Campbell,
MD, PhD (Yale University)

Jan 2009 – May 2011

Bachelor of Science (BSc) in Biomedical Science
University of Portsmouth, Portsmouth, United Kingdom
Mentor: Dr. Vitaly Zinkevich, MD, PhD

Jan 2002 – May 2005

Associate Degree of Health Science in Laboratory Sciences.
Jazan University, Jazan, Saudi Arabia.

May 2005 – Nov 2009

Intern, Clinical Laboratory Sciences
King Fahd Central Hospital, Jazan, Saudi Arabia

PROFESSONAL EXPERIENCES

Sep 2015 – present

Graduate Research Assistant,
Department of Microbiology and Immunology
The University of Texas Medical Branch at Galveston,
TX, USA

Jan 2012 – Oct 2012	Teaching Assistant, Immunology, Department of Microbiology and Immunology Jazan University, Jazan, Saudi Arabia
Jul 2006 – Oct 2008	Clinical laboratory technologist King Fahd Central Hospital, Jazan, Saudi Arabia

RESEARCH INTEREST

My overall research interest is to study the host-pathogen interaction of Infectious Diseases and the molecular mechanisms underlying pathogen replication.

CURRENT RESEARCH INTERESTS

My research interest focuses on studying the molecular mechanisms underlying pathogen replication, including Human Immunodeficiency Viruses (HIV), to better understand and to gain more insights into the identification of novel approaches which could serve as potential therapeutics. My goal is to use the knowledge learned from our studies to develop innovative strategies which could counter HIV and other infectious diseases.

DOCTORAL RESEARCH

Department of Microbiology and Immunology, The University of Texas Medical Branch at Galveston.
Mentors: Dr. Haitao Hu and Dr. Lynn Soong
Thesis title: Modulation of Host BRD4 to Repress HIV Replication in Myeloid Cells: Major HIV Reservoirs in Central Nervous System

MASTER'S DEGREE RESEARCH

School of Health Sciences, Quinnipiac University, Hamden, CT
Mentor: Dr. Sheldon Campbell, MD, PhD (Yale University)
Field of Study: Mycobacteria
Thesis title: Comparison of the Vitek MS and Bruker Matrix-assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF) for the Identification of Mycobacterial Species using different extraction protocols and its correlation to routine methods of diagnosis (HPLC, Probes and Nucleic Acid Sequencing) at Veterans Affairs [VA] hospital and Yale hospital, New Haven, CT.

UNDERGRADUATE'S DEGREE RESEARCH

School of Pharmacy and Biomedical Sciences, University of Portsmouth, Portsmouth, UK
Mentor: Dr. Vitaly Zinkevich, MD, PhD

Field of Study: Human colonic bacteria
Thesis title: The Effect of Rifampicin on Mixed Population of Bacteria Present on Human Colon.

FUTURE RESEARCH PLAN:

After graduation with the Ph.D, my goal is to "try" to pursue postdoctoral studies with a long term goal is to return to Saudi Arabia where, where with collaboration, I will establish my laboratory to continue my researches, investigating host-pathogen interaction and the underlying mechanisms of pathogen replication. I have already secured a faculty job at Jazan University where I will run my laboratory

MEMBERSHIP IN SCIENTIFIC SOCIETIES:

Institute of Biomedical Science (IBMS), UK

American Society of Clinical Laboratory Science (ASCLS), USA

American Association of Immunologists (**AAI**), USA

HONORS AND AWARDS:

August 2019	Mardelle Susman Scientific Writing Award The University of Texas Medical Branch at Galveston
August 2012	Awarded a fully funded Scholarship from the Ministry of Higher Education, Saudi Arabia to pursue postgraduate studies abroad in the field of microbiology and immunology
May 2015	Academic Excellence Award, MHS in Medical Laboratory Sciences. Quinnipiac University, USA.
August 2008	Awarded a fully funded Scholarship from the Ministry of Health, Saudi Arabia to pursue undergraduate studies abroad in the field of Biomedical sciences
May 2005	Exemplary Student Acknowledgement and Appreciation Certificate, Jazan University, Saudi Arabia.

PUBLICATIONS

1. **Edrous Alamer**, Chaojie Zhong, Zhiqing Liu, Qingli Niu, Fuquan Long, Lulu Guo, Benjamin B. Gelman, Lynn Soong, Jia Zhou, Haitao Hu. Epigenetic suppression of HIV in myeloid cells by the BRD4-selective small molecule modulator ZL0580 [published online ahead of print, 2020 Mar 18]. **J Virol**. 2020;JVI.01880-19. doi:10.1128/JVI.01880-19

2. Qingli Niu, Zhiqing Liu, **Edrous Alamer**, Xiuzhen Fan, Haiying Chen, Janice Endsley, Benjamin B. Gelman, Bing Tian, Jerome H. Kim, Nelson L. Michael, Merlin L. Robb, Jintanat Ananworanich, Jia Zhou, and Haitao Hu. Structure-guided drug design identifies a BRD4-selective small molecule that suppresses HIV. **J Clin Invest.** 2019;129(8):3361–3373. Published 2019 Jul 22. doi:10.1172/JCI120633

3. **Edrous Alamer**, Victor H. Carpio, Samad A. Ibitokou, Michelle L. Kirtley, Inaia R. Phoenix, Michael M. Opata, Kyle D. Wilson, Yingzi Cong, Sara M. Dann, Ashok K. Chopra, Robin Stephens. Dissemination of non-typhoidal Salmonella during Plasmodium chabaudi infection affects anti-malarial immunity. **Parasitol Res**. 2019;118(7):2277–2285. doi:10.1007/s00436-019-06349-z

4. **Alamer E,** Lynn Soong, Haitao Hu. HIV Transcription Regulation via Host BRD4: How modulation of Host BRD4 can suppress HIV? **In preparation (*Review*)**

PRESENTATIONS

- A Novel Approach to Epigenetically Suppress HIV in Myeloid Cells. **Poster presentation** at Keystone symposium: Functional Cure and the Eradication of HIV (March 24-28, 2019/Whistler, British Columbia, Canada
- Epigenetic suppression of HIV in myeloid cells by a BRD4-modulating small molecule. **Oral presentation**, Baylor College of Medicine, Houston, Texas. April 2019
- Epigenetic Modulation of HIV Transcription in Myeloid Cell, UTMB, Galveston, Texas, USA. **Poster presentation**, McLaughlin Symposium, March 2018, UTMB.