

UNDERSTANDING THE MYELOID HIV RESERVOIR IN HIV-ASSOCIATED  
NEUROLOGICAL DISORDER DURING ANTIRETROVIRAL-TREATED HIV INFECTION

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## ABSTRACT

**Background:** Despite effective antiretroviral therapy (ART), the persistence of latent HIV that produce replication-competent virus remains a major barrier to a cure. Since the development of the quantitative viral outgrowth assay (QVOA) over 20 years ago, there has been significant advancements in the detection and quantification of the replication-competent reservoir. However, the original development and ongoing modifications of the QVOA remains mostly T cell-centric. Recent studies have shown compelling evidence that the myeloid compartment harbor HIV in the setting of ART in tissues, yet only a few studies were tailored to measure myeloid-derived replication-competent HIV in circulation in humans. In addition, the myeloid compartment is reported to play an important mechanistic role in the neuropathogenesis of HIV-associated neurological dysfunction, though it remains unclear whether HIV reservoirs that may be persistent in myeloid cells in the setting of suppressive ART are related to neurocognitive impairment.

**Methods:** We obtained cryopreserved peripheral blood mononuclear cells from chronic HIV-infected individuals on long term ART (>5 years) (n=16) and those who initiated ART early during acute infection and remained aviremic for 2 years (n=8), all of whom had available age- and education-adjusted neuropsychological testing scores. Cells were sorted to ultra-high purity by flow-cytometry for total monocytes and CD4 T cells and validated. Using a modified novel Monocyte TZM-bl Assay (TZA), (MoCo-TZA), the frequencies of cells producing replication-competent HIV (IUPM) were calculated in both total monocytes and CD4 T cells. Total p24 associated with replication-competent HIV produced by these cell types were quantified using a relative light unit to replication-competent HIV-associated p24 standard curve in the MoCo-

TZA. Ratios of total replication-competent HIV-associated p24 and IUPMs, termed infectious potential, were calculated for monocytes and CD4 T cells.

**Results:** Among the 16 ART-suppressed chronic HIV-infected individuals evaluated, 11 had detectable peripheral blood monocytes with inducible replication-competent HIV. Although monocyte IUPMs were significantly lower as compared to CD4 T cells, the infectious potential in the monocyte compartment was slightly higher as compared to the CD4 T cell compartment. Higher surface expression of CD16 on intermediate monocytes correlated with elevated monocyte reservoir measurements. In individuals who were treated early during acute infection, all 8 had detectable circulating monocytes with inducible replication-competent HIV after 2 years of ART. Individuals with cognitive impairment had higher reservoir measurements in total monocytes as compared to those with normal cognition. In addition, higher expression of CD16 on intermediate monocytes correlated with lower executive function and global NP z-scores. No differences in CD4 T cell reservoir measurements were observed between cognitive status groups.

**Conclusion:** Utilizing the MoCo-TZA, we show that inducible replication-competent HIV in circulating monocytes is detectable during long-term chronic infection, as well as in individuals who initiated ART early during acute infection and were on treatment for 2 years. Our results highlight the need to further understand the monocyte-derived replication-competent HIV reservoir during ART-suppression and to further investigate this reservoir compartment in HIV-associated neurocognitive disease treatments and in HIV cure efforts.

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# **CHAPTER 1**

## **BACKGROUND**

## **Beginnings of the HIV Pandemic**

The human immunodeficiency virus (HIV) pandemic clearly remains one of the most important medical and public health issues our society has encountered (1, 2). Although HIV has been suspected of being spread from non-human primates (NHP) to humans throughout sub-Saharan Africa during the early 1900s (3, 4), it was not until the summer of 1981, when the first cases of acquired immunodeficiency syndrome (AIDS) were observed in the US (5, 6), that the world first became aware of this devastating infectious disease. In these early cases, men who have sex with men (MSM) who were previously described as healthy, were afflicted with rare cancers such as Kaposi sarcoma, inexplicable lymphadenopathy, and opportunistic infections that included pneumocystis jirovecii (formally carinii) pneumonia (PCP) and mucosal candidiasis. In the same year, it was discovered that the progressive loss of cluster of differentiation (CD)4+ T cells, as measured by a complete blood cell count (CBC), was the root immunological abnormality that led to the increased risk of infectious and oncological complications (7, 8). Due to the initial cases of the HIV epidemic being comprised of MSM, it was suggested that the reason for the AIDS onset was MSM behavior. This early suggestion was soon dismissed when AIDS was observed in other non-MSM populations. With the clustering of AIDS cases and the dissemination of the disease across different populations, investigators concluded that the etiology of AIDS was an infectious agent that was transmitted by intimate contact, such as through blood or sexual activity (9).

It was hypothesized that the infectious etiology was a retrovirus due to the growing understanding of human and animal retroviruses at that time (10, 11). Human T-lymphotrophic

virus (HTLV)-1 and HTLV-2, two retroviruses isolated and characterized from patients with T cell related malignancies, were the only known type of human viruses that preferentially infected CD4+ T cells (12, 13). Moreover, the risk factors for HTLV-1 infection were similar to the observed risk factors for AIDS; and HTLV-1, along with other related retroviruses, were documented to cause immune deficiency in their respective hosts (10, 14, 15). Thus, with the tools and techniques utilized in the discovery of HTLV-1 and 2, the search for the retrovirus linked to AIDS pushed forward. In 1983, two years after the initial AIDS cases were documented, scientists from France and the US isolated and characterized HIV (16, 17) and, in the following year, established it as the cause of AIDS (18-22). Since these early events of the pandemic, HIV has spread in successive waves in various regions around the globe and, as of 2021, has infected approximately 79.3 million people and, through AIDS-related illnesses, is attributed to the deaths of 36.3 million people worldwide (23).

## **HIV Virology**

### ***Biological origins of HIV-1 and HIV-2***

*Lentivirus*, a genus of the family *Retroviridae*, is composed of enveloped, positive-sense single-stranded RNA viruses that are characterized to cause chronic and fatal disease that have prolonged incubation periods in humans and other mammal hosts. Viruses that fall under the *Lentivirus* genus are present in ungulates (horses, cattle, sheep, and goats), felids (cats), and primates (monkeys, apes, and humans) (24, 25). Primates are the natural hosts for multiple lineages of closely related simian immunodeficiency viruses (SIV) and HIV. In NHPs, SIV naturally infects at least 45 different Old World monkey and ape species from sub-Saharan

Africa, which include chimpanzees (SIV<sub>CPZ</sub>), sooty mangabeys (SIV<sub>SM</sub>), African green monkeys (SIV<sub>AGM</sub>), mandrills (SIV<sub>MND</sub>), and sykes (SIV<sub>SYK</sub>) (26). In contrast, New World (Asian and South American) NHPs are not reported to be natural hosts for SIV, however no large surveys have yet been conducted on wild NHPs in these continents. While SIV is non-pathogenic within their respective natural hosts (27-32), SIV is documented to be pathogenic when transmitted into a different species, as reported in experimental infections of NHPs (33-37), or as been the case in humans.

Zoonotic transmission of SIVs gave rise to two genetically distinct viruses: HIV-1, closely related to SIV<sub>CPZ</sub> and HIV-2, closely related to SIV<sub>SM</sub> (3, 4, 38, 39). Both HIV-1 and HIV-2 cause AIDS in humans, although the disease kinetics are observed to be different. Individuals infected with HIV-2 develop AIDS at a substantially slower progression as compared to individuals infected with HIV-1 (16, 40, 41). These differences in kinetics may be the reason, in part, to HIV-2 infection being restricted within West Africa, while HIV-1 is responsible for the worldwide HIV pandemic.

### ***Genomic elements and proteins involved in the HIV-1 lifecycle***

#### *Regulatory regions and genomic elements of HIV-1*

HIV-1 genome (~9.8 kbp) contains archetypal *gag*, *env*, and *pol* genes that encode viral structural proteins and enzymes, along with a series of regulatory (*tat* and *rev*) and accessory (*vif*, *vpr*, *vpu*, and *nef*) genes that encode for proteins important for efficient viral replication and survival. The HIV-1 genome accommodates these by containing multiple overlapping open reading frames (ORFs) throughout the genome sequence. As a result of reverse transcription by

reverse transcriptase (RT), there are two identical regions at the ends of the HIV-1 integrated proviral DNA sequence known as long terminal repeats (LTRs). LTRs main function is regulating viral RNA synthesis and contains important regulatory regions for the initiation of transcription and polyadenylation. Serving as a promoter region, the LTR contains binding sites for several host cell elements that facilitate RNA Polymerase II activity on the HIV-1 provirus. These elements include transcription factors, such as transcription factor II D (TFIID), that binds to the TATA region upstream of the transcription initiation site. Other host cell transcription factors that are reported to associate with the LTRs are nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B), specificity protein 1 (Sp1), negative regulatory element (NRE), adaptor-like complex 3 (Ap-3-like), and downstream binding factor 1 (DBF-1) (42). The binding of these host cell elements set the stage for assembly of the transcription complex. In addition, the HIV-1 genome encodes three factors that regulate LTR transcription: transactivator of transcription (Tat), viral protein R (Vpr), and negative factor (Nef). Overall, the LTR plays a significant role in the pathogenesis of HIV-1, as it is a central component to its replication.

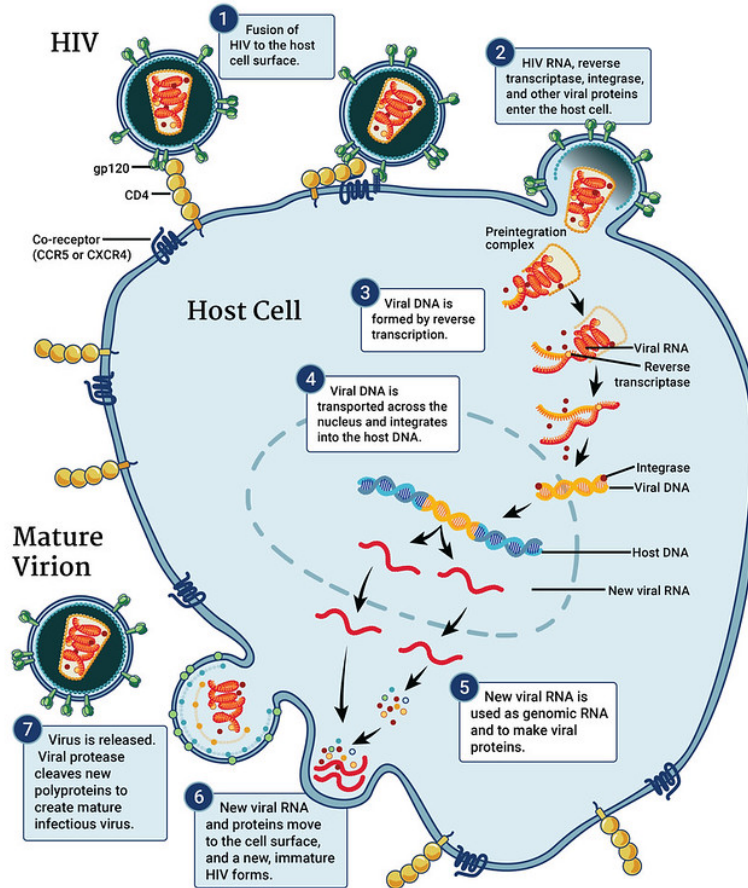
Other important viral genomic elements include Tat-responsive element (TAR) and Regulator for expression of viral proteins (Rev)-responsive element (RRE), which are viral RNA elements that associate with regulatory proteins Tat and Rev, respectively. TAR forms a hairpin stem-loop structure with a side bulge, which is necessary for Tat binding and function. The main function of TAR is the recruitment of cellular RNA polymerase components to the LTR, resulting in the stimulation/upregulation of viral transcription. RRE, on the other hand, is an RNA element present in all unspliced and partially spliced HIV-1 mRNAs (i.e. Gag, Env, and genomic RNAs) and plays a role in the regulation of nuclear export of viral RNA transcripts.

### *Structural proteins and enzymes of HIV-1*

Gag proteins are group-specific antigens and important components to the structure of the virion, in which the expression of these proteins (in the absence of infectious HIV-1 RNA or Env proteins) have been found to be sufficient in the formation of non-infectious, HIV-like particles (43, 44). These proteins are synthesized initially as a polyprotein Gag-Pol precursor (Pr160) that is enzymatically cleaved by the HIV-1 protease (PR) to produce the p55 myristoylated (lipid modification that mediates membrane targeting, protein-protein interactions, and signal transduction) protein (Pr55). Pr55 is further cleaved to form matrix (p17), capsid (p24), nucleocapsid (p7), and proline-rich (p6) proteins. The fate of Pr55 is not to only act as a substrate for PR, but also is an important component to virus assembly at the host cell plasma membrane (45).

Enzymes encoded by the HIV-1 *pol* gene comprise of RT, integrase (IN), and PR. The defining characteristic of retroviruses lies in the properties of RT and the replication cycle of all retroviruses includes a RT-mediated conversion of viral RNA into DNA (Figure 1). Subsequent to the nucleic acid conversion, IN is responsible for the insertion of full-length viral DNA into the host cell genome via the pre-integration complex (PIC) (46-49). When the PIC gains access to the nuclear compartment, the vDNA ends are inserted into a cellular chromosome. This is initiated by the enzymatic action of the IN and is completed by the host cell DNA repair machinery. This integrated vDNA in the host cell genome is referred to as a provirus. PR plays an important role in the later stages of the HIV-1 lifecycle, in that it mediates the formation of mature, infectious virions by the cleavage of the Pr160 and subsequent Pr55. Proteolytic

digestion by the PR on viral proteins is mandatory for the infectivity of HIV-1, hence PR, along with RT and IN, being effective targets of antiretrovirals.



**Figure 1. HIV-1 Replication Cycle.** The first event during the course of infection is attachment of the virus particle to the cell surface, followed by fusion of the viral and cellular membranes delivering the viral core into the cytoplasm of the cell. The process of attachment and fusion is mediated by the interaction of viral glycoprotein spikes (gp120:gp41) with cell surface receptors (CD4 and CCR5 or CXCR4). The interaction of CD4 and co-receptors with gp120 causes a rearrangement in gp41 exposing a hydrophobic fusion domain (50). The fusion domain comes into contact with the target cell membrane and becomes embedded, leading to viral entry. Once inside the cell, the viral envelope dissociates and the viral capsid enters into the cytosol. An important early event in the replication cycle of HIV-1 is the reverse transcription of viral RNA into viral DNA. This is catalyzed by the virus-encoded reverse transcriptase (RT). Several viral and cellular factors, including the reverse transcriptase, form the replication complex, also known as the pre-integration complex (PIC) (51). The PIC is transported to the nucleus and its transport is mediated by nuclear localization signals (NLS) present on viral proteins, particularly on Vpr (52). The viral integrase (IN) catalyzes a series of reactions whereby the intact viral DNA is inserted into the cellular chromosomal DNA forming the provirus. The integration of the

provirus marks the end of the early phase of viral replication and enters the late phase of its life cycle. The provirus goes on to produce multiple copies of the progeny viral RNA and mRNAs that are later translated into viral proteins. HIV-1 proviral transcription is greatly influenced by the activation state of the host cell and is regulated by sequences in 5' long terminal repeat (LTR) of the provirus. Viral assembly involves the transport of viral precursor polyproteins and the envelope glycoproteins to the plasma membrane, followed by the assembly of the viral capsid proteins and finally the packaging of HIV-1 genomic RNA. Budding, the final stage of virus production, is by the process of exocytosis. Viral budding occurs at the plasma membrane of infected CD4<sup>+</sup> T cells, whereas in infected macrophages, budding can occur at intracellular membrane sites, forming vacuoles containing the viral progeny (53). After budding, maturation of the virion occurs when packaged viral precursor proteins are further cleaved by viral-encoded protease (PR) to yield mature HIV-1 virions. [Figure credit: NIAID]

Viral envelope glycoproteins, encoded by the HIV-1 *env* gene, are initially produced as a heavily glycosylated precursor (gp160). Going through the Golgi network of the host cell, gp160 is cleaved by furin or other related proteases, forming the surface (gp120) and transmembrane (gp41) subunits (54). The gp120 and gp41 proteins then remain non-covalently associated, forming the functional, native trimeric gp120-gp41 complex and is transported to the cell membrane, where incorporation into budding virions occurs (55, 56). The Env glycoproteins play a crucial role in viral entry into target cells. The gp120 serves as the binding sites for the primary receptor CD4 and a co-receptor [C-X-C chemokine receptor type 4 (CXCR4) or C-C chemokine receptor 5 (CCR5)], which then induces refolding events in gp41, ultimately fusing the viral envelope to the target cell membrane (57-59).

### *Regulatory proteins of HIV-1*

There are two essential regulatory proteins, Tat and Rev, that are encoded by HIV-1. Tat is a viral, nuclear-acting protein that associates with TAR, which results in the increase of HIV-1 provirus transcription by several hundred fold (60). The Tat-TAR complex recruits positive transcription elongation factor b (P-TEFb), which phosphorylates the C-terminal domain of RNA

polymerase II, increasing transcriptional elongation (61-66). In the absence of Tat, the transcription of HIV-1 is arrested at the TAR element stem loop RNA, which is present at the 5' ends of viral transcripts (64). Rev is also a viral, nuclear acting protein that associates with the RRE element. The Rev-RRE complex is responsible for the nuclear export of unspliced and partially spliced RNA transcripts, which cellular nuclear export machinery is unable to perform alone. Rev binds to the RRE element of the viral transcript and mediates the recruitment of chromosomal maintenance 1 (CRM1), also known as exportin 1 (XPO1), which results then in the export of viral RNAs out of the nucleus (67).

#### *Accessory proteins of HIV-1*

One of the features of HIV, as well as SIV, that distinguishes them from simpler retroviruses is the expression of accessory proteins. Important in the modulation of the infected intracellular environment, HIV-1 expresses four accessory proteins that ensure optimal conditions for viral persistence, replication and dissemination: viral infectivity factor (Vif), Vpr, viral protein U (Vpu), and Nef. Vif is a viral, cytoplasmic protein that can be in a soluble cytosolic form or membrane-associated form. Vif plays an essential role in the regulation of infectivity of HIV-1 by counteracting the anti-HIV-1 cellular factor apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G (APOBEC3G). APOBEC3G is an editing enzyme that mutates polynucleotides by deaminating cytidine (C) to uridine (U) and, in the absence of Vif, can be packaged into budding HIV-1 virions (68-70). Consequently, when the virus infects a new cell and performs reverse transcription, the packaged APOBEC3G deaminates C to U in the viral negative-sense DNA strand and results in a guanosine (G) to adenosine (A) mutation in the viral positive-sense DNA strand (71, 72). This G to A hypermutation is sufficient to stop viral spread

due to the loss of genetic integrity. Vif counters these antiretroviral effects by binding to APOBEC3G and recruiting a ubiquitin ligase, leading to the polyubiquitylation and degradation of both APOBEC3G and Vif (73, 74).

Vpr is a highly conserved 14 kDa protein that is also incorporated into the budding virion through the interaction of the p6 region of Pr55. Vpr has the ability to localize into the cell nucleus due to the presence of a nuclear localization signal (NLS) (75) and is involved in: 1) nuclear import of the viral PIC, 2) growth arrest of host cell in G2 phase, 3) transactivation of viral transcription in association with the HIV-1 LTR, and 4) apoptosis induction of the host cell (76). Although Vpr has been described to be dispensable for virus replication *in vitro*, it has been shown to play an important role in facilitating the infection of macrophages and dendritic cells (77-80). Thus, Vpr function may be necessary for HIV-1 pathogenesis *in vivo*. In a previous study, six rhesus macaques were infected with pathogenic SIV<sub>mac239</sub> isolates either containing or lacking Vpr and within 16 weeks, wild-type (WT) Vpr sequences were isolated from three of the five animals infected with Vpr null virus (81). Furthermore, the remaining two animals infected with Vpr null virus showed delayed pathogenesis, and reversion to Vpr-expressing virus by 66 weeks (82). These observations paralleled previous work showing reversions of an internal Vpr stop codon to an open reading frame in experimentally infected chimpanzees, as well as in an accidentally infected laboratory worker (83, 84). Altogether, these reports support the importance of Vpr in the pathogenesis of HIV-1.

Vpu is unique to HIV-1, as well as to its close relative SIV<sub>CPZ</sub>, and is a type 1 integral membrane protein that serves two biological functions: 1) degradation of CD4 in the endoplasmic reticulum

(ER) and 2) counteraction of cell-expressed tetherin to enhance virion release from the plasma membrane. CD4, the primary entry receptor of HIV-1, is an important cell surface protein that engages adaptive immune response mechanisms via human leukocyte antigen (HLA)-II. For HIV-1 to evade adaptive immune cell engagement, Vpu recruits a ubiquitin ligase to the cytoplasmic tail of CD4 (85). This Vpu-mediated ubiquitylation occurs when CD4 is retained in the ER while interacting with gp160. How CD4 is ultimately extracted from the ER membrane is not fully understood and it may involve ER-associated protein degradation (ERAD) components, but reports are inconsistent (86, 87).

Nef, similar to Vpu, also plays a role in the modulation of CD4 surface expression on infected cells. Nef, like Pr55, is a myristoylated protein that is associated with the cytoplasmic face of cellular membranes. It is one of the first viral proteins to be expressed following infection, which indicates its important role in helping set the magnitude of infectious virus propagation. The importance of Nef as a critical determinant of pathogenicity has been established in the observed long-term survival of human and NHP, specifically in rhesus macaques, infected with HIV-1 and SIV strains that lack fully intact *nef* genes (88). This observation is further supported by the evidence that Nef may help dictate pathogenic outcome in natural infections of different species in NHP (89). The intracellular trafficking of CD4 within helper T cells and macrophages, which are targets of HIV-1 infection, can be regulated by Nef. As a response to Nef, the endocytosis of CD4 from the surface of infected cells is accelerated. This mechanism proceeds by Nef interacting with the cytoplasmic tail of CD4 and subsequent recruitment of clathrin adaptor protein (AP)-2 complexes, which leads to the internalization of cell surface CD4 via clathrin

coated pits. The internalized surface CD4 is transported to endosomes and then lysosomes for degradation (90).

HLA-I, specifically the HLA-A and -B allotypes, are also downregulated from the surface of infected cells and is mediated by Nef (91). There are two proposed mechanisms of Nef-mediated HLA-I downregulation: first, Nef interacts with the cytoplasmic tail of HLA-I and recruits AP-1 complexes to mis-route HLA-I from the trans-Golgi network to endosomes, rather than to the cell surface, and subsequently to lysosomes for degradation (92-94); the second, Nef assembles a multicomponent Src-family containing intracellular pathway that induces the endocytosis of cell surface HLA-I (95). Despite which mechanism is favored, it can be seen that downregulation of the HLA-I would be advantageous for HIV-1, due to the blunting of cytotoxic CD8 T cell (CTL) recognition of infected cells. Data that supports this immune evasion mechanism is in a rhesus macaque/SIV model that shows mutations in the *nef* gene are associated with heightened functionality of SIV-specific CTL responses (96).

Another important complex that is targeted by Nef is the T cell receptor complex (TCR-CD3). The TCR-CD3 is a critical component of the immunological synapse that forms between antigen-presenting cells (APCs), such as B-cells, monocyte/macrophages, and dendritic cells, with T cells for antigen recognition and T cell activation. In *in vitro* cell co-culture systems where antigen-pulsed APCs contact HIV-1 infected T cells, Nef retarded the formation of immunological synapses by the endocytosis of TCR-CD3 from the infected T-cell surface and its subsequent transport to endosomes (97). It is not fully understood the molecular interactions between Nef and TCR-CD3 that governs its Nef-mediated regulation, though, Nef has been

shown to interact with the  $\zeta$ -chain of CD3, which then leads to the accumulation of lymphocyte-specific protein tyrosine kinase (Lck), an important kinase that is involved in the sustained signaling from the immunological synapse, in the recycling endosome (97). Nef also inhibits the activity of neuronal Wiskott-Aldrich Syndrome protein (N-WASP), a positive regulator of actin polymerization and critical mediator of T cell interaction (98). Enhancement of HIV-1 infectivity via facilitation of the viral core penetrating the cortical actin network during the initial phases of infection is also found to be mediated by Nef. Studies have found that dynamin 2, a GTPase that is required for clathrin-mediated endocytosis, interacts with Nef and is essential for Nef's effect on infectivity (99). Overall, it is evident that Nef manipulates the surface landscape of HIV-1 infected cells in a variety of ways that are advantageous for virus propagation.

### **HIV Acute Infection Immunology and Natural Progression to AIDS**

The natural course of HIV-1 infection, in the absence of effective ART, begins with primary/acute HIV-1 infection and is characterized by the transmission of the virus into a new host organism and establishment of infection in target cells expressing surface CD4 and co-receptors CCR5 or CXCR4. Although HIV transmission includes parenteral and perinatal routes, most infections occur by sexual exposure through the genital tract or rectal mucosa, particularly in areas of microtrauma (100), which allows the virus access to target cells in the submucosa. It has been difficult to fully ascertain in humans the early events during HIV-1 transmission *in vivo*; but studies on mucosal tissue explants infected *in vitro* (101-103), as well as NHPs inoculated intrarectally or intravaginally by SIV (104, 105), have provided important insights in the early dynamics between the virus and the host immune response.

The mucosal epithelium is a highly active front line barrier that can modulate innate defenses against HIV-1 (106-108) and functions as a sentinel and signaling system. It mediates the secretion of important innate factors such as: 1) microbial defensins and other antimicrobial peptides; 2) secretory leukocyte protease inhibitor; 3) microbial enzymes lactoferrin and lysozyme; 4) surfactant protein A; and 5) complement. In addition, the release of chemokines and cytokines recruit plasmacytoid dendritic cells (pDCs) (109, 110), which are potent producers of interferons, as well as other immune cells that further mediate innate defenses and inflammation. Inhibitory chemokines such as stromal cell-derived factor 1 (SDF-1), macrophage inflammatory protein (MIP)-1 $\alpha/\beta$ , and RANTES (for ‘regulated upon activation, normal T cell expressed and secreted’) block viral entry mediated by CCR5 (blocked by MIP-1 $\alpha/\beta$  and RANTES) and CXCR4 (blocked by SDF-1) (111). In addition, MIP-3 $\alpha$  expression has been shown to immediately recruit interferon (IFN)-  $\alpha/\beta$  producing pDCs to the mucosal site and large increases of IFN- $\gamma$  is evident in tissue by 6 days after exposure (112).

During the early stages of infection, the CD4<sup>+</sup> T cell compartment appear to be the major targets of the virus (113), as it has been shown that 1) the number of CD4<sup>+</sup> T cells within mucosal sites of entry outnumber other cellular targets, such as macrophages and dendritic cells (114); and 2) the efficiency of infection by the primary founder virus (transmitted virus) is higher in CD4<sup>+</sup> T cells as compared to what is observed in myeloid cells (115). This may differ later in infection, when virus quasi-species arise and are seen to have equal efficiency of infection in both lymphoid and myeloid cell compartments. This is not to say that the myeloid cellular compartment is not involved in early HIV infection dynamics. There is growing evidence to

suggest that myeloid cells (dendritic and macrophages) do play an important role in early virus dissemination and the formation of virus reservoirs in host lymphoid and non-lymphoid tissues (116-119). Cellular targets, particularly CD4<sup>+</sup> T cells, that are infected during these early events appear to not necessarily have markers of activation, but may be of a resting phenotype with the implication they had some co-receptor expression and a prior state of activation (120). In the gut, preferential infection of T-helper (T<sub>h</sub>) 17 CD4<sup>+</sup> T cells with this minimally activated (resting) phenotype have been reported (121, 122). The primary form of the virus that mediates infection during these early events, either as free or cell-bound virus, is not clear but both has been shown to lead to efficient transmission and productive infection (123).

Within a few weeks of infection, viremia develops. During this point of the infection, the concentrations of virus particles in the blood can reach up to 10<sup>8</sup>/ml. Reports from viral dynamic studies suggest that the virus population doubles every 6 to 10 hours, and that an infected cell, in turn, may have the potential to productively infect 20 new cells (124, 125). This high level of viremia at the primary stage is responsible for the systemic infection of the peripheral lymphoid organs, as well as other susceptible tissue sites. Early understanding of the dynamics of acute infection showed that the variation of HIV-1 observed during primary peak viremia was very limited and majority represent a founder virus. The observed homogeneity of the founder virus indicates that there is no immune-driven selection of escape mutants as viremia increases (115, 126, 127). However, it is important to note that approximately 20-30% of individuals can become infected with multiple founder HIV-1 variants (126-128).

Within a few weeks of initial viremia, the level of the virus in blood circulation decreases to a set-point level, which is known to be an early marker of disease progression (129-131). This coincides with the development of an immune response to HIV-1. Virus-specific CTLs appear early, even before HIV-specific antibodies are detectable (132-135), and play an important role in controlling virus replication (132, 135, 136). Studies in NHP models demonstrate the importance of CTLs in the control of viremia during primary infection. In these models, when CTLs are depleted by CD8-specific monoclonal antibodies and the animals are then challenged with SIV, infected NHPs are unable to control the viremia seen early in primary infection (136). It was observed that an increased number of CD8<sup>+</sup> T cells were present during the acute phase of virus infection, indicating a massive expansion of virus-specific CTLs. Virus-specific CTL responses utilize different methods to inhibit HIV-1 replication, such as the ability to kill infected cells and produce cytokines and chemokines that further modulates the response to the virus. Virus-specific CTLs produce cytokines such as IFN- $\gamma$  that inhibits HIV-1 replication (137, 138), and chemokines MIP-1 $\alpha$ , MIP-1 $\beta$ , and RANTES, which have been shown to suppress HIV-1 replication by competing and downregulating the cellular co-receptor CCR5, resulting in the protection of uninfected cells by limiting the availability of the virus co-receptor (139, 140). Another important component to inhibition of HIV-1 replication by virus-specific CTLs are their cellular lytic mechanisms involving the production of perforins and granzymes (141). Virus-specific CTLs utilize their TCR to recognize viral proteins that have been processed and presented on the surface of infected cells via HLA-I. Upon recognition, the CTLs trigger the lysis of infected cells, which has been shown effective on CD4<sup>+</sup> T cells in-vitro (142). Following the peak in the virus-specific CTL response, the virus sequences are observed to change dramatically due to the immunological pressure. However, despite CTL engagement and the

eventual development of antibodies against HIV-1, it is very rare that the virus is completely cleared from the host. During a long and typically-asymptomatic period, HIV-1 continues to replicate in infected cells and with the decline of CD4<sup>+</sup> T cells, results in the deterioration of the host immune system, and the inevitable onset of clinical immune deficiency or AIDS (143).

### **Treatment of HIV and the Ongoing Battle of Viral Persistence**

Prior to 1996, there were limited options in the treatment of HIV-1 infection. During this time, the management of HIV-1 infection mostly consisted of prophylaxis against opportunistic pathogens and other AIDS-related illnesses that were commonly seen among infected individuals who eventually became severely immunocompromised. It was not until the mid-1990s when inhibitors of the reverse transcriptase and protease, two of the three essential enzymes of the HIV-1 lifecycle, revolutionized the treatment of HIV-1 infection, along with therapy regimens that combined these agents to enhance the overall efficacy and durability. The advent of combination therapy for HIV-1 management was seminal in reducing the morbidity and mortality in PLWH (144). Combination ART (cART) effectively suppresses viral replication and drastically reduces HIV-1 viral load circulating in the plasma to limits below the detection level of most sensitive clinical assays (<50 RNA copies/mL). For the majority of treated PLWH, this results in significant reconstitution of the immune system, as measured by the increase in circulating CD4<sup>+</sup> T cells (145, 146). In addition, the utilization of three antiretroviral agents against at least two distinct molecular targets important in the HIV-1 replication cycle averts viral drug resistance. This is an important feature, particularly because of HIV-1's error-prone reverse transcription process during replication and the diversity of mutants that result. With the

enormous potential for generating genetic diversity, HIV-1 variants with resistance to any one or two drugs may be present in the viral quasi-species before therapy initiation (147). Thus, the success of cART results in part from using drug combinations that decrease the probability of selecting virus clones that have multiple mutations that confer resistance to three antiretroviral drug regimens.

With the proper adherence, cART can suppress viral replication effectively for years, increasing the life expectancy of PLWH. However, cART alone does not eliminate HIV-1 from the host and HIV-1 infection is a chronic infection for which there is currently no cure. The broad implementation of cART uncovered the persistence of HIV-1, both as a latent reservoir and as an expression of low-level viremia (148-150). Studies of PLWH on cART revealed two sources of plasma viremia: 1) short-lived, productively infected CD4+ T cells that produce high levels of virus and then die; and 2) long-lived cells capable of producing viremia that is below the limit of detection of commercial assays (151, 152). The HIV-1 latent reservoir is predominately composed of a subset of CD4+ T cells that harbor latent replication-competent HIV-1 proviruses. With current techniques that assess the viral reservoir, it is estimated that one out of 1000 CD4+ T cells contain an integrated HIV-1 proviral genome. However, only 10% of these measured proviruses are intact and have the capacity to produce replication-competent virions, while the other 90% of proviruses contain defects that are lethal to the virus (153-157). Thus, it can be approximated that one out 100,000 CD4+ T cells harbor a genetically intact provirus. The overabundance of defective proviruses that shroud the low frequency of those intact, along with the lack of a cellular marker to distinguish HIV-1 infected from uninfected cells; these pose significant challenges to understanding the latent cellular reservoir.

While CD4<sup>+</sup> T cells are the primary targets of HIV-1 and are of the majority of cells that make up the latent reservoir, mounting evidence suggest that cells of the myeloid lineage play a significant role in HIV-1 persistence. Recent studies provide compelling evidence that blood monocytes and tissue macrophages in the lung (158, 159), adipose tissue (160), gut-associated lymphoid tissue (GALT) (161, 162), genital tract (163), semen (164), bone marrow (165, 166), and central nervous system (CNS) (167, 168); all have been shown to harbor HIV in the setting of suppressive ART. A variety of attributes make the monocyte and tissue macrophages, including microglia, ideal candidates for contributing to the HIV reservoir, both as carriers and replenishers of the viral reservoir. The macrophage reservoir half-life has historically been underdetermined, yet in the presence of ART, macrophages from SIV-infected rhesus macaques can sustain viremia for several months (119, 169, 170). Myeloid cells are relatively more resistant to apoptosis induced by HIV infection (171), and virus produced by macrophages may be more infectious than virus originating from CD4<sup>+</sup> T cells (172). Moreover, the HIV envelope region undergoes more frequent sequence evolution in blood monocytes as compared to that of resting CD4<sup>+</sup> T cells, suggesting a distinct contribution to plasma viremia. Phylogenetic analyses of HIV-1 sequences indicate that after prolonged cART, viral populations are related to those found in CD14<sup>+</sup> monocytes (173). The non-classical or patrolling monocyte subset (CD14<sup>low</sup>CD<sup>high</sup>) express higher levels of CCR5, a co-receptor for M-tropic HIV strains, and CD4, making them more susceptible to continual viral infection. Circulating monocytes traffic into tissues to later differentiate into tissue macrophages, but have the potential to undergo subsequent differentiation into migratory myeloid dendritic cells, which then traffic to other lymph tissues. Monocytes and macrophages disseminate into most tissues of the body and

mediate HIV spread, particularly into the CNS and lymphoid tissues. Despite the building evidence highlighting the importance of myeloid cells in HIV persistence, the extent of monocyte and tissue macrophages contributing to viral recrudescence is poorly understood and stresses the need to further include more comprehensive evaluation of HIV persistence in the myeloid compartment in both blood and tissue sites. Including the myeloid compartment may provide a more comprehensive evaluation of the dynamics of the HIV-1 reservoir and its' persistence, which may better inform future curative endeavors.

## **CHAPTER 2**

### **DISSERTATION SCOPE**

## **Background and research objective**

HIV curative studies have narrowly focused on evaluating and targeting long-lived memory CD4<sup>+</sup> T cells, as this cellular compartment harbors the majority of cell-associated HIV using current quantification assays. As HIV cure trials include carefully planned interruptions of combination antiretroviral therapy (ART) to determine curative efficacy, few to none evaluate myeloid cells, such as blood monocytes and tissue macrophages, as cellular measurements of HIV persistence and reservoir. This partly stems from the debate in the field on the scope of the myeloid compartment as a viral reservoir or contributor to viral rebound. However, recent studies provide compelling evidence that tissue macrophages, including those found in the central nervous system (CNS), harbor HIV in the setting of suppressive ART. While there is evidence of HIV persistence in forms of proviral DNA in circulating blood monocytes, measurement of these cells producing replication-competent virions during ART-suppression has yet to be understood. But, the implications of this observation may be crucial in understanding the dynamics of the HIV reservoir and, more importantly, how the field approaches curative interventions, as well as interventions to reverse/alleviate HIV-associated co-morbidities. One such co-morbidity that has shifted from severe to more milder forms with the advent of ART, but still remains a problem in HIV-infected individuals is neurocognitive impairment (NCI). NCI is estimated to occur in greater than 30% of virally-suppressed individuals on ART who are without other co-morbid causes of cognitive deficits such as cerebrovascular disease. Although mildly impaired, NCI remains a significant public health issue as even mild impairment negatively affects the ability of an individual to work or live fully independent, productive lives. The pathogenesis of HIV-associated neurocognitive impairment during suppressive ART

remains poorly understood. It is hypothesized that HIV-infected, CD16<sup>+</sup> peripheral monocytes migrate from blood circulation across the blood brain barrier, seed HIV in the CNS and establish an inflammatory milieu, which may contribute to neuronal dysfunction and NCI. However, there is still little evidence showing that circulating monocytes produce replication-competent virus, as well as showing its relationship to NCI. The quantitative viral outgrowth assay (QVOA) remains the gold standard for quantifying cellular reservoirs during ART-treated HIV infection and its development was critical in defining resting CD4 T cells as latent reservoirs. Overtime, modifications of the QVOA have been implemented to improve and streamline different steps of the QVOA assay. However, majority of the modifications done on the QVOA remained to be T-cell centric. Although recently, there has been reported modifications to the QVOA assay that are tailored to quantify tissue macrophages that produce replication-competent virus, modifications tailored to assess blood circulating monocytes have not been reported. Therefore, utilizing high-purity multiparametric flow cytometry cell sorting, cryopreserved peripheral mononuclear cells (PBMCs) from ART-suppressed chronic HIV-infected individuals with available age- and education-adjusted neurocognitive performance scores will be sorted from circulating monocytes and CD4 T cells. Evaluation of the replication-competent HIV reservoir in isolated monocytes and CD4 T cells will be assessed using a modified TZM-bl assay developed (MoCo TZA) to evaluate frequency of cells infected with replication-competent HIV (infectious unit per million cells or IUPM) and estimate the number of infectious virions produced per IUPM (Infectious Potential). Comparisons of the inducible replication-competent HIV reservoirs in the monocyte and CD4 T cells compartments will be assessed in acute and chronic HIV disease. In addition, the relationships among the cellular HIV reservoir

measurements and neuropsychological testing scores in individuals chronically infected with HIV will be evaluated.

### **Specific Aims**

**Specific Aim I: To develop a modified viral outgrowth cell co-culture assay that measures the inducible replication-competent HIV reservoir in isolated human peripheral blood monocytes**

*Hypothesis:* Utilizing a HIV infection reporter cell line (TZM-bl cells), the developed HIV viral outgrowth cell co-culture assay (MoCo-TZA) will be able to: (1) detect and quantify the frequency of HIV-infected cells producing replication competent HIV as measured by infectious units per million cells (IUPM) after stimulation and (2) estimate the number of replication-competent virions produced per IUPM (infectious potential) using a relative light unit (RLU) vs. pg of replication-competent HIV-associated p24 standard curve.

**Specific Aim II: To quantify the inducible replication-competent HIV reservoir in peripheral blood monocytes isolated from acute and chronic ART-suppressed PLWH**

*Hypothesis:* Total blood monocytes isolated from acute and chronic HIV-infected individuals who are ART-suppressed will have: (1) detectable frequencies of circulating blood monocytes that produce replication-competent HIV and (2) have measurable infectious potentials.

**Specific Aim III: To evaluate the relationship between the blood monocyte HIV reservoir and neurocognitive impairment (NCI) in ART-suppressed HIV-infected individuals.**

*Hypothesis:* ART-suppressed HIV-infected individuals who have NCI defined by neuropsychological performance testing, will have: (1) higher frequencies of circulating blood monocytes that produce replication-competent HIV and (2) higher monocyte infectious potentials as compared to infected individuals who are cognitively normal. Higher frequencies of blood monocytes producing replication-competent HIV and higher monocyte infectious potentials will be correlated with lower neuropsychological performance scores.

### **Significance**

The pursuit of an HIV cure continues to be steadfast as we continue through the 5<sup>th</sup> decade since the characterization of HIV and its link to AIDS. Understanding mechanisms of HIV persistence, as well as identifying cellular compartments and tissue sites as viral reservoirs, especially during effective ART, are important components to that pursuit. However, majority of the effort elucidating these components have been T cell centric, and as a result, past and current cure initiatives propose interventions that mostly target the CD4 T cell reservoir. With publications characterizing tissue macrophages as a site of HIV persistence during effective ART, myeloid cells represent a non-T cell compartment of the HIV reservoir that may be important to the ongoing presence of HIV in ART-treated individuals and the growing evidence suggest that myeloid cells may need to be considered more in current and future cure approaches. Thus, it is imperative to tailor reservoir measuring assays to myeloid cells so to better characterize this important but often marginalized cellular compartment.

## **CHAPTER 3**

### **SPECIFIC AIM I**

**Development of a modified viral outgrowth cell co-culture assay that measures the inducible replication-competent HIV reservoir in isolated human peripheral blood monocytes**

## Introduction

The majority of the past and current HIV cure trials have concentrated efforts on evaluating and targeting the memory CD4<sup>+</sup> T cell compartment, since these cells harbor a significant proportion of cell-associated HIV as measured by current HIV reservoir quantification assays. As treatment interruption of ART remains the gold standard in determining the outcome of HIV curative designed clinical trials, the contribution of viral persistence in myeloid cells, such as blood monocytes and tissue macrophages, has not been at the forefront. This partly stems from the debate in the field on the scope of myeloid cells as viral reservoirs or contributors to viral persistence. However, recent studies provide compelling evidence that various tissue compartments, including the central nervous system, have been shown to harbor HIV in the setting of suppressive ART (167, 168). Two prominent cure trial cases may also be informative on the importance of myeloid cells as viral reservoirs. In the case of the “Berlin Patient” (174), prior administration of gemtuzumab, a myeloid cell depleting anti-CD33 monoclonal antibody given to treat his acute myeloid leukemia (AML), contributed to a targeted depletion of myeloid cells, which in turn may have eliminated the HIV-infected myeloid reservoir. Although the exact mechanisms of this successful trial remain undefined and speculative, the presumptive importance of this observation can be further supported in the absence of a myeloid cell targeted depletion intervention seen in other human and non-human trials. These trials attempted to recapitulate the case of the Berlin Patient, but all unfortunately resulted in HIV reemergence.

The extent of monocyte and macrophage tissue reservoir compartments contributing to viral recrudescence is poorly understood. Compared to the vast majority of studies tailored to evaluate

the viral reservoir in the CD4 T cell compartment, few studies focus their effort to elucidate the relationship of monocyte/macrophages to HIV reservoir and its persistence. In humanized myeloid-only mice (MoM) infected with HIV, it has been shown that after ART-interruption, one of three mice experienced a delayed viral rebound (116). Due to the absence of human T cells in MoM mice, this study showed for the first time, in vivo, that persistent HIV infection exists in tissue macrophages during ART and the myeloid compartment can contribute to viral rebound after treatment interruption. Although some investigators reported not being able to detect HIV DNA or viral outgrowth in peripheral monocytes isolated from viremic and ART-suppressed patients (116, 117), others have reported detection of HIV in circulating monocyte populations. CD16<sup>+</sup> monocytes isolated from the blood of ART-treated individuals have been shown to harbor HIV DNA (175, 176). In non-human primate studies, SIV was quantifiable in monocytes and macrophages from blood and tissues using a modified QVOA. The macrophage-QVOA (M-QVOA) is specifically tailored to quantify productively infected macrophages (177) through cellular stimulation with TNF- $\alpha$ , a potent activator of myeloid cells (178, 179). Utilizing the M-QVOA technique, macrophages were not only found to be productively infected in the SIV-infected non-human primate model, but also the number of productively infected macrophages varied throughout different tissue sites. Moreover, despite viral suppression by ART, tissue macrophages isolated from SIV-infected macaques continued to be productively infected (180). The evidence in these reports stresses a need for HIV curative studies to include a more comprehensive evaluation of HIV persistence in the myeloid compartment in both blood and tissue. However, in contrast to the number of these studies done in non-human primates, human studies are limited. In this study, TZM-bl cells, a HIV infection reporter cell line, were utilized to develop a monocyte-centric assay to quantify inducible replication-competent HIV in

blood circulating monocytes from chronic ART-suppressed PLWH. Completing this objective will further the understanding of the potential monocyte HIV reservoir and help ascertain failures in past and current cure studies, which may better inform future curative endeavors.

## **Methods**

### ***Cell preparation, immunophenotype staining, and cell sorting***

Following informed consent, PBMCs from Timothy Brown, the first person to be cured of HIV, were isolated by leukapheresis followed by Ficoll gradient centrifugation. PBMCs were cryopreserved in liquid nitrogen storage until use. Cryopreserved PBMCs were thawed and washed with warmed RPMI supplemented with 10% FBS. PBMCs were then surface-stained with CD3, CD4, CD8, CD11b, CD7, CD19, CD20, HLA-DR, CD14, CD16 fluorochrome-conjugated antibodies, and with Propidium Iodide (Live/Dead Stain). Isolated monocytes and CD4 T cells were acquired using the Aria Cell Sorter (BD biosciences) and 100% purity of isolated populations were verified after sort. Purified monocytes and CD4 T cells were pooled separately in 15 ml vials containing RPMI + 10% FBS supplemented with 200 nM raltegravir (NIH AIDS Reagent Program) and 100 nM efavirenz (NIH AIDS Reagent Program) and incubated in 37°C, 5% CO<sub>2</sub> for 3 hours.

### ***Monocyte co-culture TZM-bl cell-based assay (MoCo-TZA)***

#### ***Infectious unit per million cells (IUPM) assessment***

After completion of sort, cells were wash twice with fresh RPMI + 10% FBS and seeded in a limiting dilution (6 replicates / dilution) as indicated in Figure 1 in 96-well tissue culture plates

pre-cultured with TZM-bl cells (NIH AIDS Reagent Program) seeded at  $1 \times 10^4$  TZM-bl cells/well and incubated for 24 hours to ~50% confluence. Monocytes were stimulated with recombinant human TNF- $\alpha$  (R&D systems) at 500 pg/mL and CD4 T cells were stimulated with anti-human CD3/CD28 mAb-coated microbeads (Gibco) at 1:1 bead to T cell ratio). Co-cultures were incubated in 37°C, 5% CO<sub>2</sub> for 3 days. After co-culture incubation, wells were washed once with dPBS and Beta-Glo reagent (Promega) was added to each co-culture well and incubated for 30 mins at RT. Luminescence signal (relative light units; RLU) was detected using a Victor X3 Multilabel Plate Reader (PerkinElmer). A sample well containing cells from HIV-infected participant was considered positive if the luminescence signal was above the mean + 2 SD of the signal obtained from the HIV-uninfected (negative control) sample wells. IUPM was calculated utilizing online software, available at <http://silicianolab.johnshopkins.edu/>.

#### *Infectious virus quantification (Infectious potential)*

Number of virions produced by latently infected cells (frequency determined by IUPM) was estimated by producing a RLU vs. HIV Gag p24 standard curve. ACH-2 cells (NIH Reagent program) were stimulated with recombinant human TNF- $\alpha$  at 500 pg/ml and incubated in 37°C, 5% CO<sub>2</sub> for 48 hours. Culture supernatants were harvested, pooled and assayed by ELISA for HIV-1 Gag p24 (R&D Systems). Viral aliquots (3000 pg/mL HIV-1 Gag p24) were stored in -80°C until use. For each MoCo-TZA experiment, a viral aliquot was thawed and serially diluted (1:4) to 750 pg/mL, 187.5 pg/mL, 46.9 pg/mL, 11.7 pg/mL, 2.9 pg/mL, and 0.7 pg/mL in RPMI + 10% FBS. Each dilution was seeded (100 ul/well) in triplicate into wells pre-cultured with TZM-bl cells seeded at  $1 \times 10^5$  TZM-bl cells/well and incubated for 24 hours to ~50% confluence. In addition, each dilution well was spiked with recombinant human TNF- $\alpha$

(monocyte) or anti-human CD3/CD28 mAb-coated microbeads (CD4 T cells) to maintain culture conditions similar to stimulation wells. Virus seeded wells were incubated and processed with the same parameters as stimulation wells. A standard curve between RLUs and HIV Gag p24 concentrations for each cell culture plate was produced using online software (myassays.com). Amount of HIV Gag p24 produced in positive stimulation wells were determined using standard curve. Infectious potential was determined by dividing the sum of HIV Gag p24 produced in positive stimulation wells by IUPM calculated for the sampled cell compartment.

### ***Statistical Analyses***

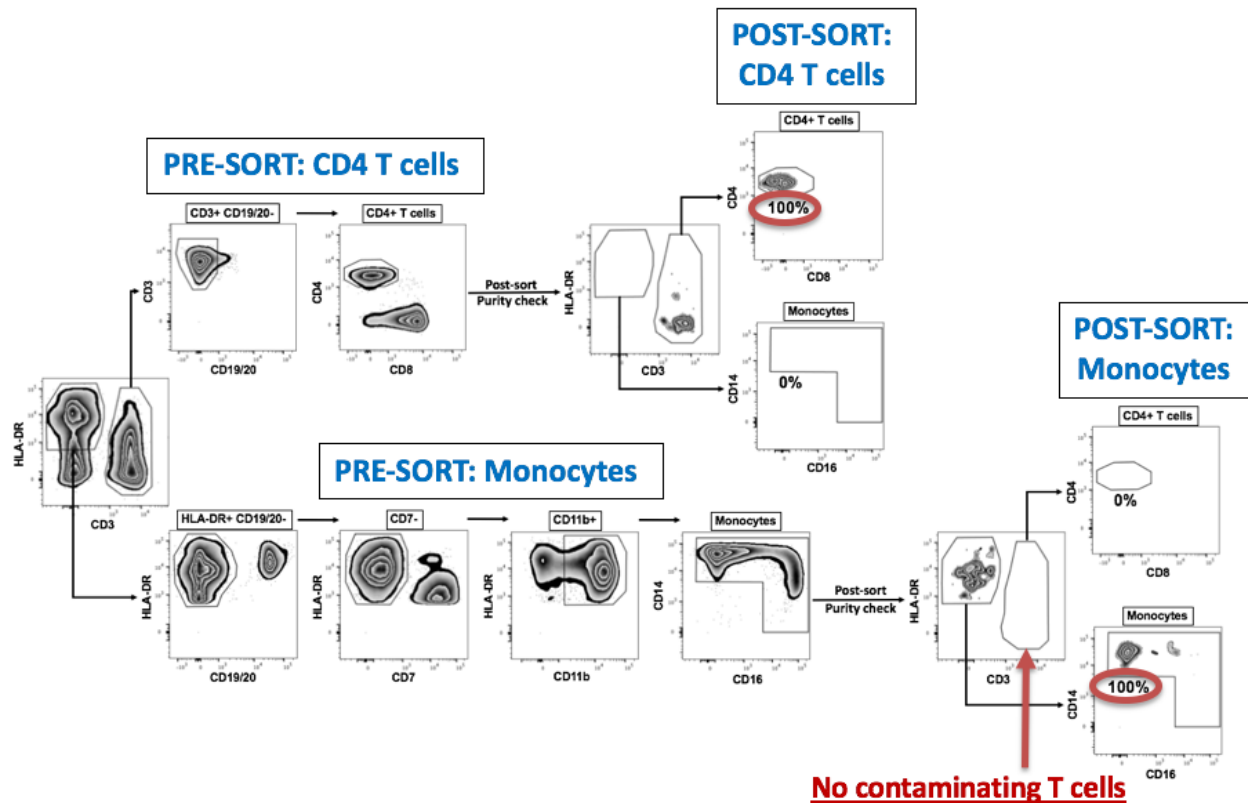
Mann-Whitney U tests and Spearman rho correlations were done in SPSS version 22 software and statistical significance was set at a  $p$ -value  $<0.05$ .

### **Results**

The TZM-bl cell-based assay (TZA) used to quantify the inducible replication-competent HIV (rcHIV) reservoir in resting CD4 (rCD4) T cells, is a sensitive viral outgrowth technique that utilizes the TZM-bl cell line, which is permissive to CXCR4- and CCR5-tropic HIV infection (181, 182). This cell-line stably expresses CD4, CCR5, and CXCR4 on its surface and contains an integrated copy of the  $\beta$ -galactosidase ( $\beta$ -gal) gene that is under the control of an HIV long terminal repeat (HIV LTR) promoter. TZM-bl cells can report the presence of rcHIV via the expression  $\beta$ -gal when the HIV Tat protein, produced by the integrated HIV-1 provirus, transactivates the HIV LTR promoter of the  $\beta$ -gal gene. The TZA method involves three components: (1) isolation of rCD4 T cells, (2) induction of latent HIV production from rCD4 T

cells using a strong LRA, such as anti-CD3/CD28 antibodies and (3) quantification of induced rHIV in the TZM-bl cells by measuring  $\beta$ -gal expression.

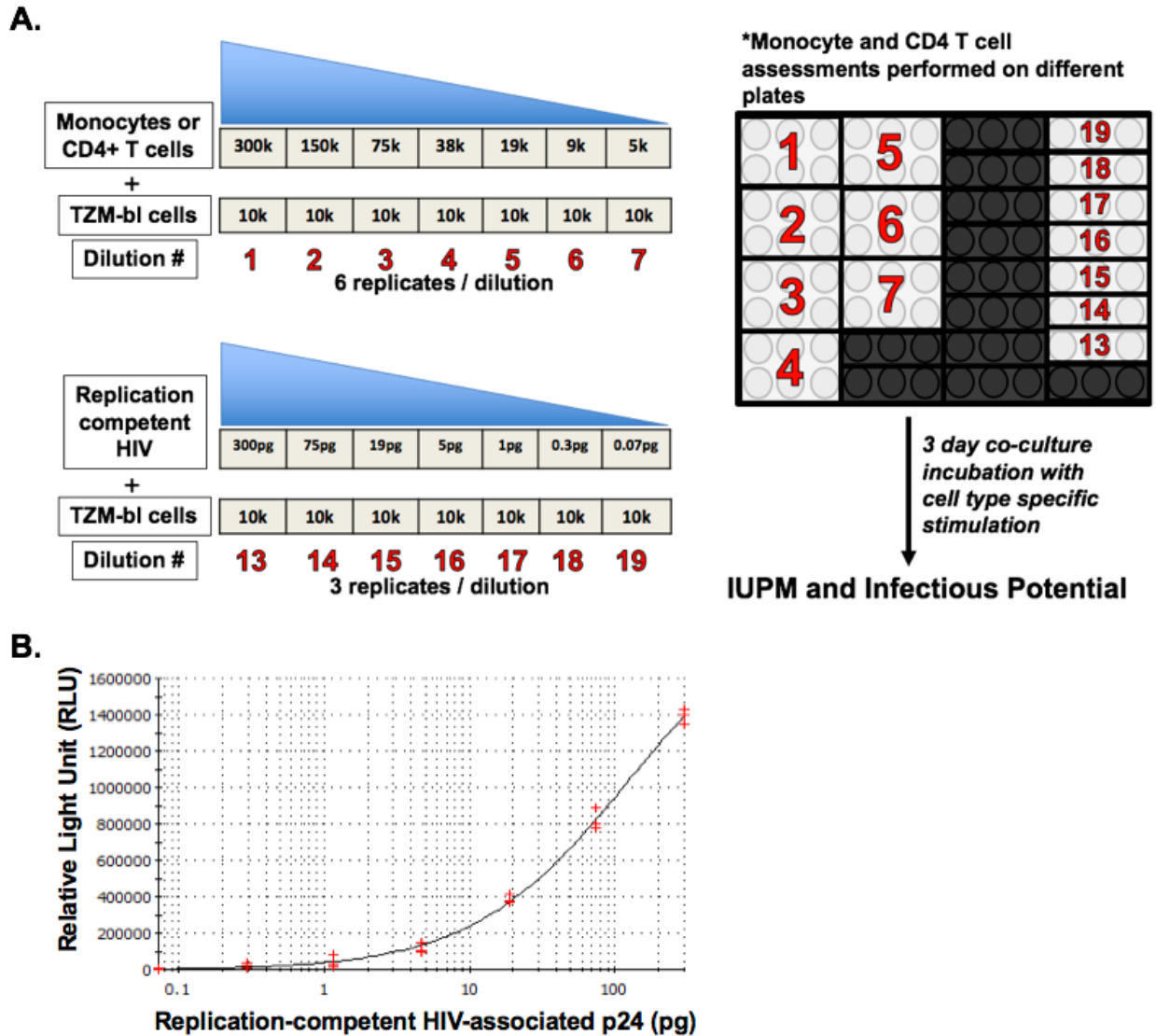
Here we performed a modification of the TZA approach and tailored the assay to specifically evaluate inducible rHIV in blood circulating monocytes referred to as the monocyte co-culture TZA (MoCo-TZA). Unlike the TZA and other viral outgrowth methods that utilize magnetic bead separation to isolate CD4 T cells and monocytes, we utilized multi-parametric flow-cytometry cell sorting to obtain ultra-pure monocyte and CD4 T cell isolations and ensure minimal level of CD4 T cell contamination in the monocyte cell fraction (and vis versa). A comprehensive panel of cell surface markers were used to identify total blood monocytes, which included human leukocyte antigen (HLA) II receptor HLA-DR, integrin CD11b, LPS co-receptor CD14, and Fc $\gamma$ III receptor CD16 (**Figure 1**). CD3, CD19, CD20, and CD7 were utilized as exclusionary markers for T cells, B cells, and NK cells, respectively. Post sort flow-cytometry analysis of isolated monocyte and CD4 T cells documented 100% purity for each isolated cell fraction (**Figure 1**).



**Figure 1.** Plots depict a representative flow gating strategy used to cell sort peripheral blood monocytes and CD4 T cells from cryopreserved PBMCs of ART-suppressed HIV-infected individuals. Sorted monocyte and CD4 T cell populations were verified as 100% pure based on post-sort flow cytometry.

Isolated cells were serially diluted and co-cultured with TZM-bl cells, which were previously seeded 24-hours prior to monocyte and CD4 T cell isolation (**Figure 2**). To induce reactivation of integrated HIV-1 in infected cells, TNF- $\alpha$  and CD3/CD28 mAb-coated microbeads were added to monocyte and CD4 T cell co-culture wells, respectively. After 3 days of co-cultivation with TZM-bl cells, wells were assessed for TZM-bl cell infection of rHIV by measuring relative light units (RLU), which is directly proportional to the amount of  $\beta$ -gal produced by infected TZM-bl cells. A well was considered positive for rHIV when the RLU measurement was 2-standard deviations above the mean of RLU observed in the HIV-negative cell co-culture

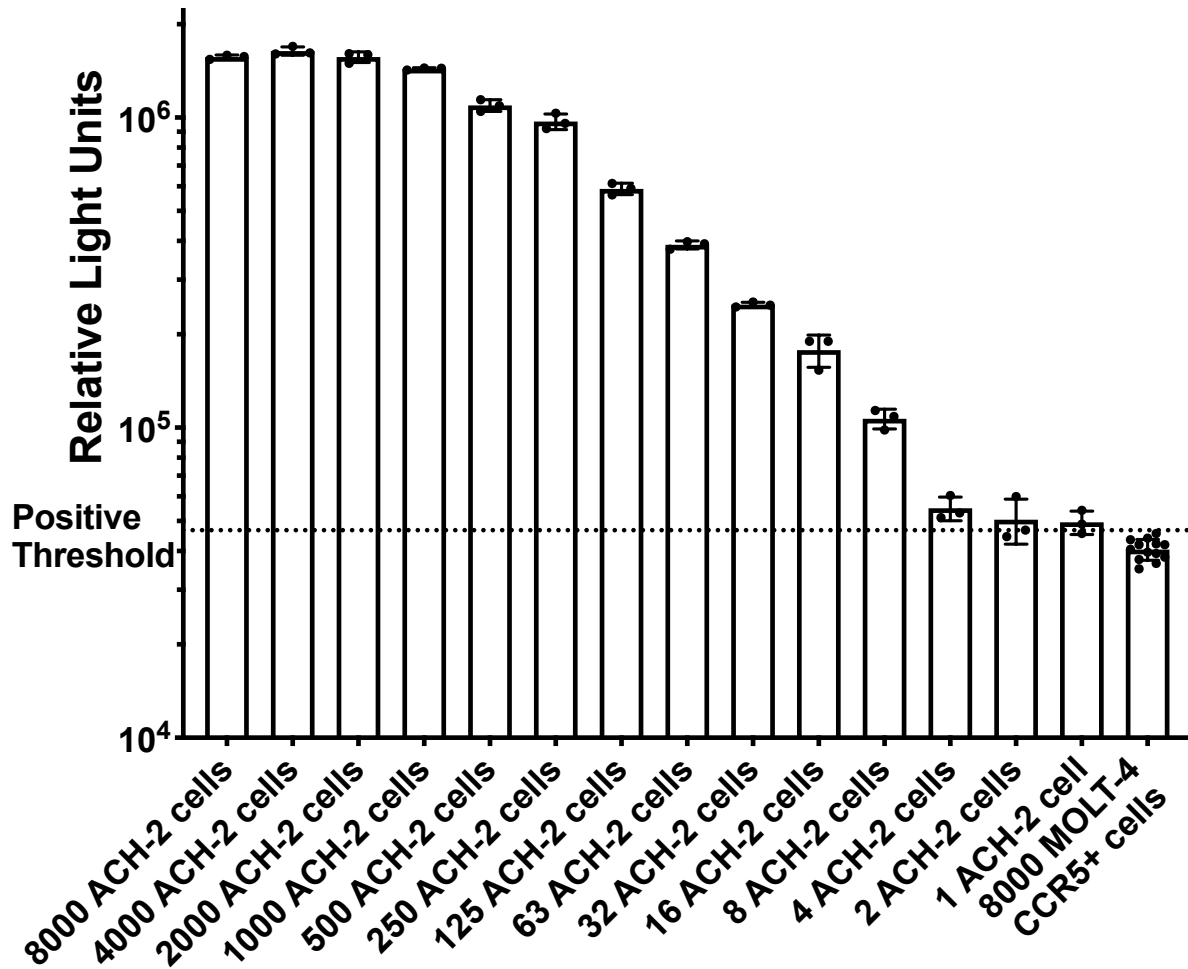
controls. The frequency of cells producing rcHIV according to the number of infectious units per million cells (IUPM) was calculated using limiting dilution statistical analyses.



**Figure 2.** (A) Schematic overview of the MoCo-TZA used to assess IUPM values and quantify inducible replication-competent HIV from sorted monocytes and CD4 T cells. (B) A representative standard curve produced from the positive viral controls used to quantify inducible replication-competent HIV in each cell compartment.

To the determine the sensitivity of the MoCo-TZA to detect HIV-infected cells that produce inducible rcHIV, ACH-2 cells, a latent HIV-infected T cell line that produces rcHIV upon

stimulation with TNF- $\alpha$  were utilized. Using the MoCo-TZA, we detected positive wells diluted to a single ACH-2 cell (**Figure 3**). The variability between culture well replicates for each measured dilution were observed to be low, with a mean coefficient of variability (CV) of 5.96% (**Table 1**).



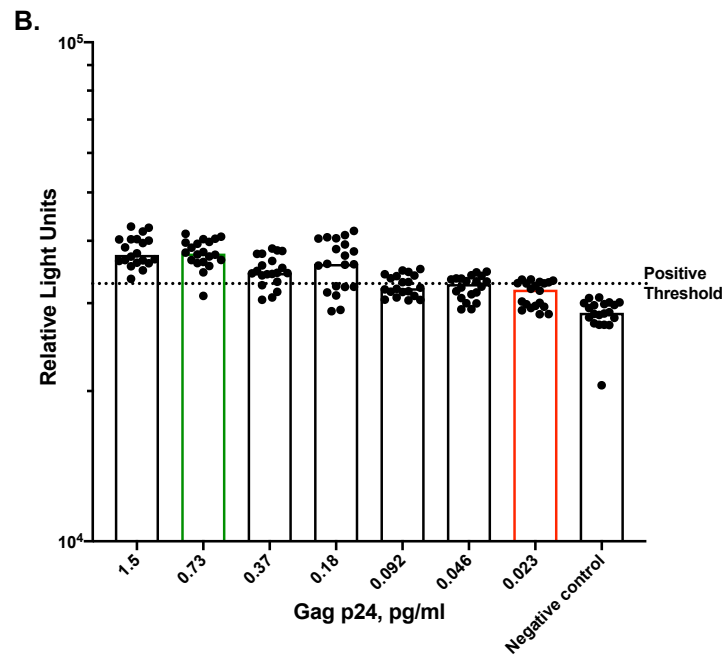
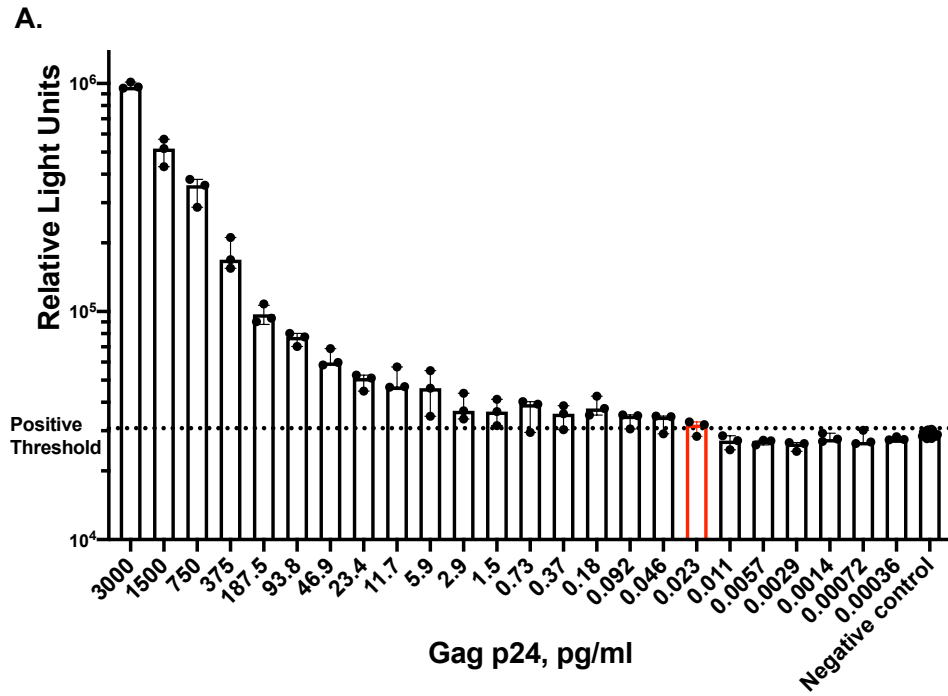
**Figure 3.** Sensitivity testing of MoCo-TZA utilizing serial dilution replicates (n=3) of ACH-2 cells. MOLT-4 (CCR5+) cells served as a negative control. The positive signal threshold was determined by calculating 2 standard deviations above the average RLU signal measured from the MOLT-4 replicates (n=13).

**Table 1. Calculated CV of ACH-2 serial dilution replicates in MoCo-TZA sensitivity testing**

<b># ACH-2 Cells</b>	<b>CV%</b>
8000	1.54
4000	3.10
2000	3.93
1000	0.79
500	4.33
250	5.80
125	4.22
63	2.93
32	1.87
16	11.85
8	7.27
4	8.81
2	16.58
1	8.67
Negative control	7.67
	<b>Mean = 5.96</b>

However, the efficiency of HIV production in ACH-2 cells is quite high and may not reflect the virus production of HIV-infected primary cells. In addition, ACH-2 cells actively divide in cell culture conditions with a doubling time of 24 hours, which is in contrast to primary monocytes that do not divide exponentially when activated in culture. Hence, we serially diluted rHIV

isolated from TNF- $\alpha$  stimulated ACH-2 cell supernatants to evaluate the minimal concentration of rcHIV detected by the MoCo-TZA. Relative to the threshold set by the negative control replicates (n=20), the lowest concentration of virus detected by the MoCo-TZA was 0.023 pg of rcHIV-associated p24/mL (**Figure 4a**). Since 100  $\mu$ l of each dilution concentration was seeded into each well, the lowest absolute amount detected was 0.0023 pg of rcHIV-associated p24 or  $\sim$ 23 rcHIV (**Table 2**). The mean CV across viral dilution replicates was 9.49%. Since only 3 replicates were performed for each dilution in the initial virus dilution experiment, we wanted to further evaluate the sensitivity of the MoCo-TZA by performing more replicates at the lower concentration virus dilutions and determine positive detection rates. Although we continued to observe positive readings at 0.023 pg of rcHIV-associated p24/mL, the MoCo-TZA was only able to yield a positive detection rate of 40% (8 out of 20 replicates) at this concentration (**Figure 4b and Table 3**). A 95% detection rate (19 out of 20) was observed at a concentration of 0.73 pg of rcHIV-associated p24/mL or  $\sim$ 732 rcHIV, with a CV of 6.51%. A lower limit of quantitation (LLOQ) can be estimated as a signal 10 standard deviations above the mean of the negative control signal. LLOQ was estimated to be 1.5 pg of rcHIV-associated p24/mL or 0.1465 pg of rcHIV-associated p24 ( $\sim$ 1,465 rcHIV) (**Figure 4b and Table 2**) and furthermore, we observed a 100% detection rate at this concentration, with a CV of 7.01% (**Figure 4b and Table 3**).



**Figure 4. (A)** Evaluation of the minimal concentration of rcHIV detected by the MoCo-TZA. Three replicates were performed per rcHIV dilution. **(B)** Further evaluation of the minimal concentration of rcHIV detected by the MoCo-TZA. Twenty replicates were performed per rcHIV dilution. Negative control replicates were cell culture media only (no rcHIV). The positive signal threshold was determined by calculating 2 standard deviations above the average RLU signal measured from the negative control replicates.

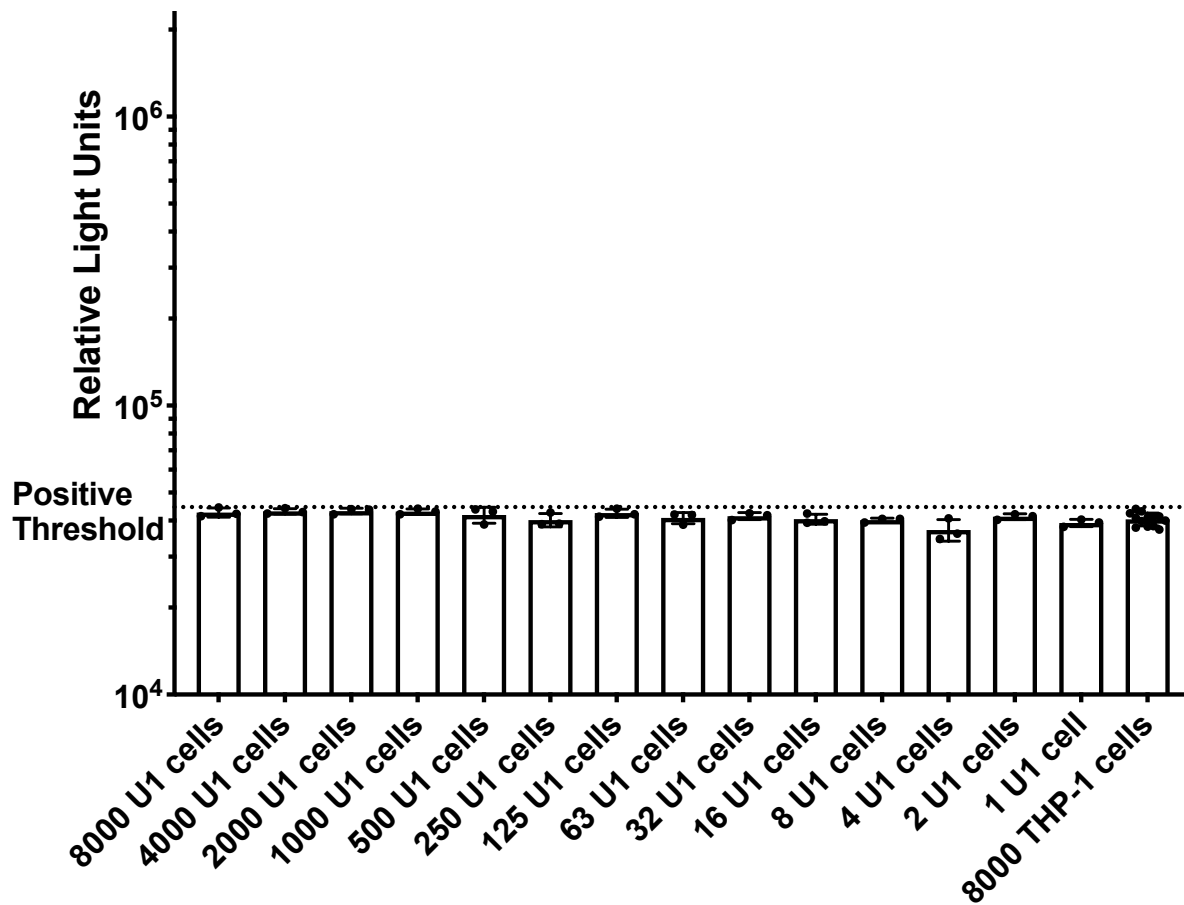
**Table 2. Concentration and CV calculations of rcHIV dilutions performed in minimal rcHIV detection experiment**

Gag p24, pg/ml	Gag p24, pg (100 ul)	# of Virions (~10 <sup>4</sup> HIV/pg)	CV %
3000 pg/ml	300 pg	3,000,000	3.32
1500 pg/ml	150 pg	1,500,000	13.77
750 pg/ml	75 pg	750,000	14.33
375 pg/ml	37.5 pg	375,000	16.55
187.5 pg/ml	18.75 pg	187,500	9.64
93.8 pg/ml	9.375 pg	93,750	6.71
46.9 pg/ml	4.6875 pg	46,875	9.06
23.4 pg/ml	2.3438 pg	23,438	8.45
11.7 pg/ml	1.1719 pg	11,719	12.05
5.9 pg/ml	0.5859 pg	5,859	22.51
2.9 pg/ml	0.2930 pg	2,930	13.54
1.5 pg/ml	0.1465 pg	1,465	13.15
0.73 pg/ml	0.0734 pg	732	16.32
0.37 pg/ml	0.0366 pg	366	12.13
0.18 pg/ml	0.0183 pg	183	9.77
0.092 pg/ml	0.0092 pg	92	7.75
0.046 pg/ml	0.0046 pg	46	9.84
0.023 pg/ml	0.0023 pg	23	7.61
0.011 pg/ml	0.0011 pg	11	7.16
0.0057 pg/ml	0.00057 pg	6	2.49
0.0029 pg/ml	0.00029 pg	3	4.85
0.0014 pg/ml	0.00014 pg	1	4.20
0.00072 pg/ml	0.000072 pg	(0.7)	7.43
0.00036 pg/ml	0.000036 pg	(0.4)	1.68
Negative control	-	-	2.88
			<b>Mean = 9.49</b>

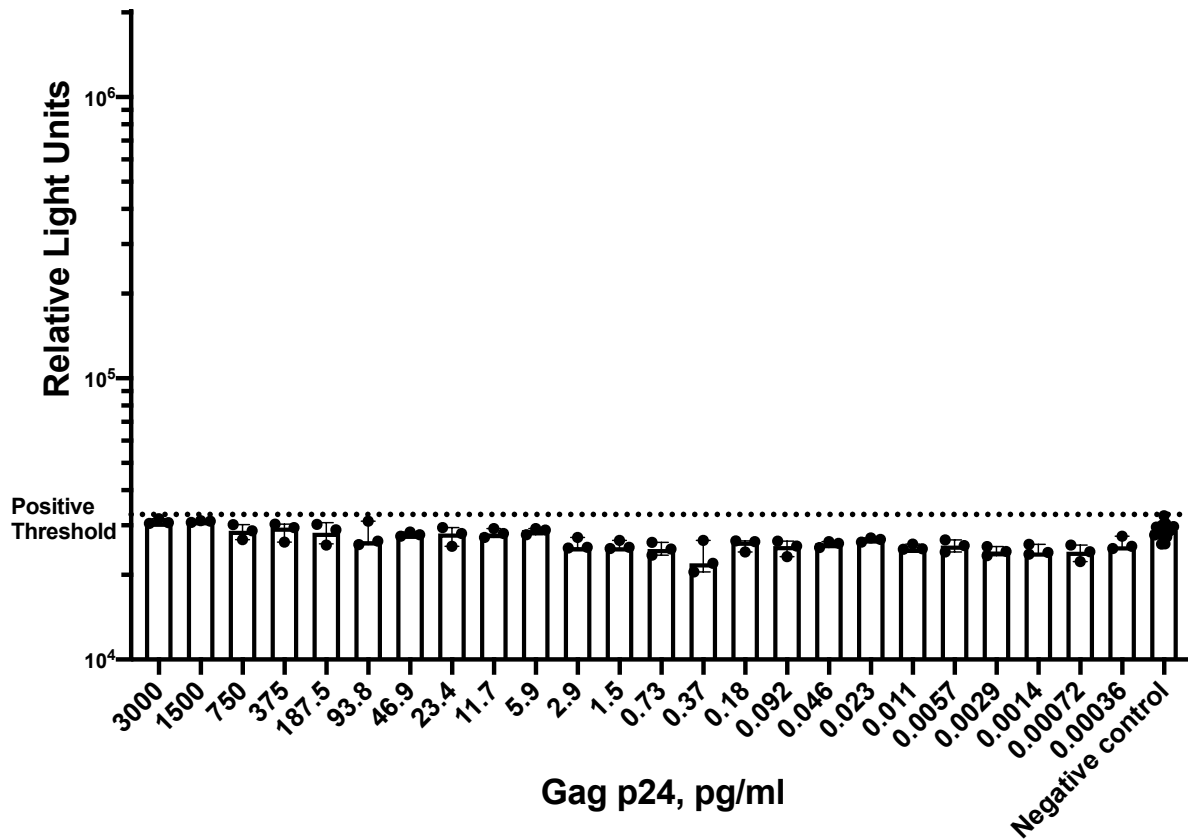
**Table 3. Detection rates and lower limit of quantitation determination of MoCo-TZA**

Gag p24, pg/ml	Gag p24, pg (100 ul)	# of Virions (~10 <sup>4</sup> HIV/pg)	# of Replicates	# of positives	Detection Rate (%)	CV %
1.5 pg/ml	0.1465 pg	1,465	20	20	100%	7.01
0.73 pg/ml	0.0734 pg	732	20	19	95%	6.51
0.37 pg/ml	0.0366 pg	366	20	16	80%	6.97
0.18 pg/ml	0.0183 pg	183	20	13	65%	11.54
0.092 pg/ml	0.0092 pg	92	20	9	45%	4.86
0.046 pg/ml	0.0046 pg	46	20	10	50%	5.48
0.023 pg/ml	0.0023 pg	23	20	8	40%	5.89
Negative control	-	-	20	-	-	7.80
						<b>Mean = 7.00</b>

The capacity of TZM-bl cells to be only sensitive to rcHIV has been previously shown using various latently-infected cell-lines producing replication-defective virions (183). We went on to further assess the specificity of the MoCo TZA to differentiate between rcHIV and replication-defective HIV using U1 cells, a latently-infected monocyte cell-line that produces replication-defective virions upon stimulation with TNF- $\alpha$ . Using the MoCo-TZA, no dilution of U1 cells yielded a positive measurement (**Figure 5**). Similarly, when virions isolated from stimulated U1 cell supernatants were serially diluted and tested with the MoCo-TZA, no dilutions yielded a positive measurement (**Figure 6**). The variability among U1 cell and virus dilution replicates were low at mean CVs of 3.81% and 5.10%, respectively (**Table 4 and 5**).



**Figure 5.** Specificity testing of MoCo-TZA utilizing serial dilution replicates (n=3) of U1 cells. THP-1 cells served as a negative control. The positive signal threshold was determined by calculating 2 standard deviations above the average RLU signal measured from the THP-1 replicates (n=13).



**Figure 6.** Specificity testing of the MoCo-TZA utilizing serial dilution replicates (n=3) of replication-defective HIV isolated from TNF- $\alpha$  stimulated U1 cells. Cell culture media served as a negative control. The positive signal threshold was determined by calculating 2 standard deviations above the average RLU signal measured from the negative control replicates (n=13).

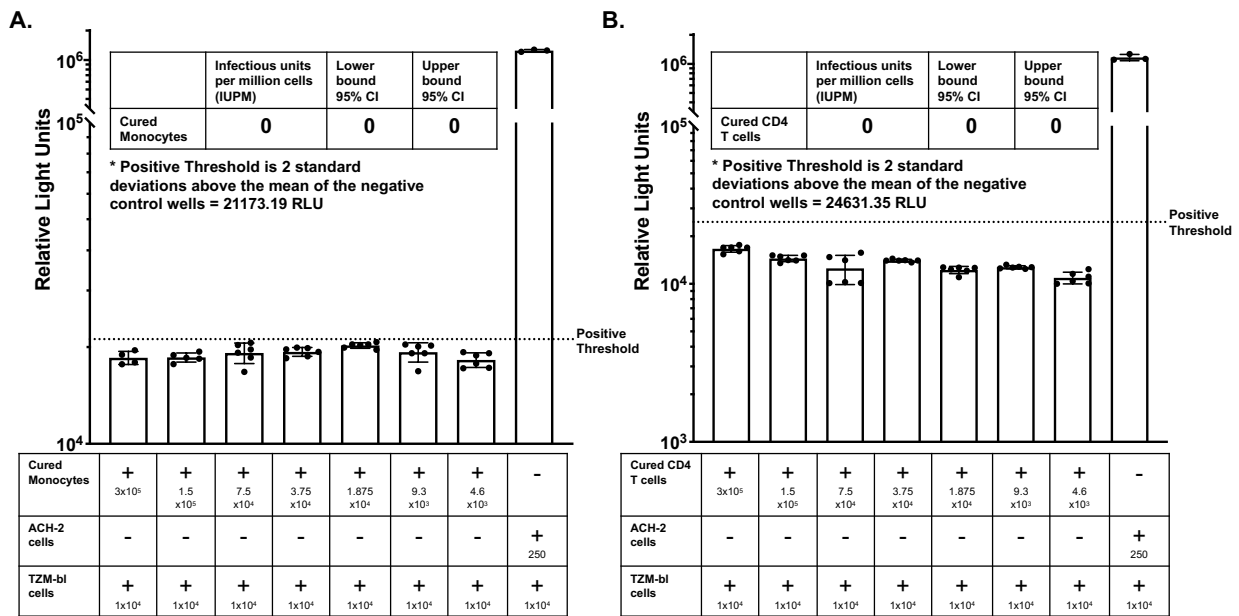
**Table 4. Calculated CV of U1 cell serial dilution replicates in MoCo-TZA specificity testing**

<b># U1 Cells</b>	<b>CV%</b>
8000	3.63
4000	2.20
2000	2.24
1000	2.18
500	6.42
250	5.31
125	3.32
63	4.47
32	2.77
16	3.96
8	1.64
4	8.62
2	2.33
1	2.87
Negative control	5.17
	<b>Mean = 3.81</b>

**Table 5. Concentration and CV calculations of replication-defective HIV dilutions performed in MoCo-TZA specificity testing**

Gag p24, pg/ml	Gag p24, pg (100 ul)	# of Virions (~10 <sup>4</sup> HIV/pg)	CV %
3000 pg/ml	300 pg	3,000,000	2.08
1500 pg/ml	150 pg	1,500,000	0.69
750 pg/ml	75 pg	750,000	6.15
375 pg/ml	37.5 pg	375,000	7.63
187.5 pg/ml	18.75 pg	187,500	8.57
93.8 pg/ml	9.375 pg	93,750	10.65
46.9 pg/ml	4.6875 pg	46,875	1.69
23.4 pg/ml	2.3438 pg	23,438	7.74
11.7 pg/ml	1.1719 pg	11,719	3.67
5.9 pg/ml	0.5859 pg	5,859	2.63
2.9 pg/ml	0.2930 pg	2,930	4.93
1.5 pg/ml	0.1465 pg	1,465	3.65
0.73 pg/ml	0.0734 pg	732	5.33
0.37 pg/ml	0.0366 pg	366	13.62
0.18 pg/ml	0.0183 pg	183	5.02
0.092 pg/ml	0.0092 pg	92	6.44
0.046 pg/ml	0.0046 pg	46	2.40
0.023 pg/ml	0.0023 pg	23	1.74
0.011 pg/ml	0.0011 pg	11	2.37
0.0057 pg/ml	0.00057 pg	6	4.94
0.0029 pg/ml	0.00029 pg	3	3.74
0.0014 pg/ml	0.00014 pg	1	4.29
0.00072 pg/ml	0.000072 pg	(0.7)	6.74
0.00036 pg/ml	0.000036 pg	(0.4)	5.40
Negative control	-	-	5.48
			<b>Mean = 5.10</b>

Although the MoCo-TZA utilizes monocyte and CD4 T cells isolated from HIV-negative individuals as negative controls to establish positive thresholds, we evaluated the monocyte and CD4 T cells isolated from PBMCs of the cured Berlin Patient (174) as an additional negative control. Using the MoCo-TZA, both monocyte and CD4 T cell compartments showed no evidence of rcHIV (**Figure 7a and b**).



**Figure 7.** Utilizing MoCo-TZA to detect production of rcHIV in blood circulating **(A)** monocytes and **(B)** CD4 T cells isolated from the Berlin Patient.

## Summary

The MoCo-TZA utilizes TZM-bl cells, an HIV-infection reporter cell-line, that stably expresses CD4, CCR5, and CXCR4. TZM-bl cells are permissive to HIV and report infection via the production of beta-galactosidase induced by the proviral production of Tat. There are three important components to the MoCo-TZA assay. First, cell populations of interests are isolated by cell sorting using multi-parametric flow-cytometry. Second, isolated cell populations are co-

cultured with TZM-bl cells in stimulation conditions to detect production of replication-competent HIV and calculate IUPMs. Third, the amount of virus produced are estimated using a relative light unit (RLU) vs. pg of replication-competent HIV-associated p24 standard curve. The ratio of total pg of replication-competent HIV-associated p24 measured in positive wells to the IUPM, which we term infectious potential. The lower limit of detection for the MoCo-TZA was observed to be ~0.73 pg/ml at a detection rate of 95% and is specific for replication-competent HIV.

## **CHAPTER 4**

### **SPECIFIC AIMS II & III**

**Quantify inducible replication-competent HIV reservoir in peripheral blood monocytes isolated from acute and chronic ART-suppressed PLWH and assess the relationship between the myeloid HIV reservoir and neurocognitive impairment**

## Introduction

Despite the widespread use of ART, neurocognitive impairment (NCI) remains a significant morbidity among 15-60% of PLWH. The driving force behind the development of NCI in the context of ART is still highly debatable, in which cognitive deficits experienced by PLWH may be due to direct HIV infection of the CNS, indirect systemic inflammation, or a combination of these along with other mechanisms. HIV invades the brain during the first 2 weeks after primary infection. Once established, viral reservoirs can persist in various different body compartments, such as the gut and central nervous system. Despite majority of latently infected cells are of the CD4 T cell compartment, the cell population likely responsible for the establishment and maintenance of the CNS HIV reservoir are circulating HIV-infected monocytes (184, 185). After HIV-infected monocytes enter the brain, they may differentiate into macrophages and/or release infectious virus that can infect other CNS cells, including microglia and perivascular macrophages. CD14<sup>+</sup> CD16<sup>+</sup> monocytes have been extensively associated with cognitive disorders described in PLWH (184). This monocyte subset is observed to be increased in the peripheral blood in PLWH and has been shown to be permissive to HIV infection and preferentially transigrate across the blood-brain barrier (186). Although the description of HIV-infected monocytes is commonly included in the process of HIV seeding of the CNS, few studies have experimentally characterized the amount of circulating monocytes infected with HIV and the capacity of this cellular compartment to produce replication-competent HIV, particularly in the context of ART suppression. In this study, we utilize a modified QVOA that is tailored to measure replication competent virus in blood circulating monocytes and evaluate the relationship

between the monocyte HIV reservoir and neurocognitive impairment in acute and chronic ART-suppressed PLWH.

## **Methods**

### ***Clinical Characterization***

#### *Chronic HIV Infection (H032) Cohort*

Chronic HIV-infected participants were enrolled in a pilot study (R21NS087951/Ferumoxytol-enhanced Imaging and Quantitative Susceptibility Mapping in NeuroAIDS) to evaluate MRI changes after infusion with Ferumoxytol, a small iron oxide contrast agent. Participants enrolled were 40-65 years old, had a plasma HIV RNA < 20 copies/ml within the last 6 months and at screening, and received ART uninterrupted for > 12 months leading up to the screening period. Neurocognitive assessments of participants enrolled in the pilot study were performed by a trained psychometrist and assessed cognitive domains impacted by HIV. Domains evaluated were attention and concentration [California Computerized Assessment Package (CalCAP), choice RT, Sequential RT, WAIS-IV Digit Span Forward], speed of information processing/psychomotor speed [Trail Making Test-Part A, WAIS-IV Digit-Symbol Coding, Grooved Pegboard, Timed Gait], verbal and visual learning/memory [California Verbal Learning Test (CVLT-II), Brief Visuospatial Memory Test (BVM-T-R)], and executive function [Trailmaking Test-Part B, WAIS-IV Digit Span Backward, WAIS-IV Letter-Number Sequencing, Letter Fluency (FAS), D-KEFS Color-Word Interference Test]. Raw NP scores were transformed to standard z-scores using HIV-uninfected age- and education-matched normative data. Composite z-score (NPZglobal) representing global cognitive performance was

calculated as the arithmetic mean of z-scores of individual cognitive tests. Neurocognitive impairment was defined as global NP z-score (NPZ<sub>global</sub>) < -0.5 or a NP z-score < -0.5 in at least one cognitive domain known to be typically affected by HIV (i.e., executive function, psychomotor speed and attention, learning and memory).

#### *Acute HIV Infection (RV254/SEARCH010) Cohort*

Walk-in clients seeking volunteer counseling and testing at the Thai Red Cross Anonymous Clinic were screened for acute HIV infection by pooled nucleic acid testing and a fourth-generation immunoassay (clinical trials registration NCT00796146). Participants were subsequently categorized as Fiebig stage I–IV. Participants were offered immediate cART via a local protocol (clinical trials registration NCT00796263). Neuropsychological testing has been previously described (187) for the acute cohort. The 4-test NP battery evaluated fine motor function/manual dexterity [Grooved Pegboard test (GP), non-dominant hand], psychomotor speed [Color Trails 1 (CT1), Trail Making A (TM)], and executive function/set shifting [Color Trails 2 (CT2)]. All raw NP scores were transformed to standard z scores using normative data from HIV-uninfected control participants of equivalent age and education. A composite score (NPZ-4), the arithmetic mean of individual sub-domain z-scores, was calculated to provide overall measure on NP performance. Neuropsychological performance data utilized for current study were obtained at 96 weeks (2 years) since ART initiation.

#### ***Cell preparation, immunophenotype staining, and cell sorting***

PBMCs from acute HIV-infected participants after 96 weeks of ART and from chronic HIV-infected participants at baseline of pilot study were isolated using the standard Ficoll gradient

centrifugation technique. PBMCs were cryopreserved in liquid nitrogen storage until use. Cryopreserved PBMCs were thawed and washed with warmed RPMI supplemented with 10% FBS. PBMCs were then surface-stained with CD3, CD4, CD8, CD11b, CD7, CD19, CD20, HLA-DR, CD14, CD16 fluorochrome-conjugated antibodies, and with Propidium Iodide (Live/Dead Stain). Isolated monocytes and CD4 T cells were acquired using the Aria Cell Sorter (BD biosciences) and 100% purity of isolated populations were verified after sort. Purified monocytes and CD4 T cells were pooled separately in 15 ml vials containing RPMI + 10% FBS supplemented with 200 nM raltegravir (NIH AIDS Reagent Program) and 100 nM efavirenz (NIH AIDS Reagent Program) and incubated in 37°C, 5% CO<sub>2</sub> for 3 hours.

### ***Monocyte co-culture TZM-bl cell-based assay (MoCo-TZA)***

#### *Infectious unit per million cells (IUPM) assessment*

After completion of sort, cells were wash twice with fresh RPMI + 10% FBS and seeded in a limiting dilution (6 replicates / dilution) as indicated in Figure 1 in 96-well tissue culture plates pre-cultured with TZM-bl cells (NIH AIDS Reagent Program) seeded at  $1 \times 10^4$  TZM-bl cells/well and incubated for 24 hours to ~50% confluence. Monocytes were stimulated with recombinant human TNF- $\alpha$  (R&D systems) at 500 pg/mL and CD4 T cells were stimulated with anti-human CD3/CD28 mAb-coated microbeads (Gibco) at 1:1 bead to T cell ratio). Co-cultures were incubated in 37°C, 5% CO<sub>2</sub> for 3 days. After co-culture incubation, wells were washed once with dPBS and Beta-Glo reagent (Promega) was added to each co-culture well and incubated for 30 mins at RT. Luminescence signal (relative light units; RLU) was detected using a Victor X3 Multilabel Plate Reader (PerkinElmer). A sample well containing cells from HIV-infected participant was considered positive if the luminescence signal was above the mean + 2

SD of the signal obtained from the HIV-uninfected (negative control) sample wells. IUPM was calculated utilizing online software, available at <http://silicianolab.johnshopkins.edu/>.

*Infectious virus quantification (Infectious potential)*

Number of virions produced by latently infected cells (frequency determined by IUPM) was estimated by producing a RLU vs. HIV Gag p24 standard curve. ACH-2 cells (NIH Reagent program) were stimulated with recombinant human TNF- $\alpha$  at 500 pg/ml and incubated in 37°C, 5% CO<sub>2</sub> for 48 hours. Culture supernatants were harvested, pooled and assayed by ELISA for HIV-1 Gag p24 (R&D Systems). Viral aliquots (3000 pg/mL HIV-1 Gag p24) were stored in -80°C until use. For each MoCo-TZA experiment, a viral aliquot was thawed and serially diluted (1:4) to 750 pg/mL, 187.5 pg/mL, 46.9 pg/mL, 11.7 pg/mL, 2.9 pg/mL, and 0.7 pg/mL in RPMI + 10% FBS. Each dilution was seeded (100 ul/well) in triplicate into wells pre-cultured with TZM-bl cells seeded at  $1 \times 10^5$  TZM-bl cells/well and incubated for 24 hours to ~50% confluence. In addition, each dilution well was spiked with recombinant human TNF- $\alpha$  (monocyte) or anti-human CD3/CD28 mAb-coated microbeads (CD4 T cells) to maintain culture conditions similar to stimulation wells. Virus seeded wells were incubated and processed with the same parameters as stimulation wells. A standard curve between RLUs and HIV Gag p24 concentrations for each cell culture plate was produced using online software (myassays.com). Amount of HIV Gag p24 produced in positive stimulation wells were determined using standard curve. Infectious potential was determined by dividing the sum of HIV Gag p24 produced in positive stimulation wells by IUPM calculated for the sampled cell compartment.

### *Statistical Analyses*

Mann-Whitney U tests and Spearman rho correlations were done in SPSS version 22 software and statistical significance was set at a  $p$ -value  $<0.05$ .

### **Results**

#### *Determination and quantification of replication-competent HIV in monocytes from ART-suppressed chronic HIV-infected individuals.*

Donor samples that were assessed using the MoCo-TZA were collected from participants enrolled in the H032 Chronic HIV Cohort study. All 16 donors were male, on ART at the time of collection, and virally-suppressed (**Table 1**). All individuals were on NRTIs, with the majority (63%) of donors having an integrase inhibitor (INSTI) incorporated into their ART regimen. All donors were chronically-infected with HIV with a median duration of 23 years of infection since HIV diagnosis and were on ART for a median of 18 years since treatment initiation. Although nadir CD4+ T cell counts were low among donors at a median count of 103 cell/uL, CD4+ T cell counts measured during enrollment of the pilot study were relatively normal with a median count of 495 cells/ul (**Table 2**).

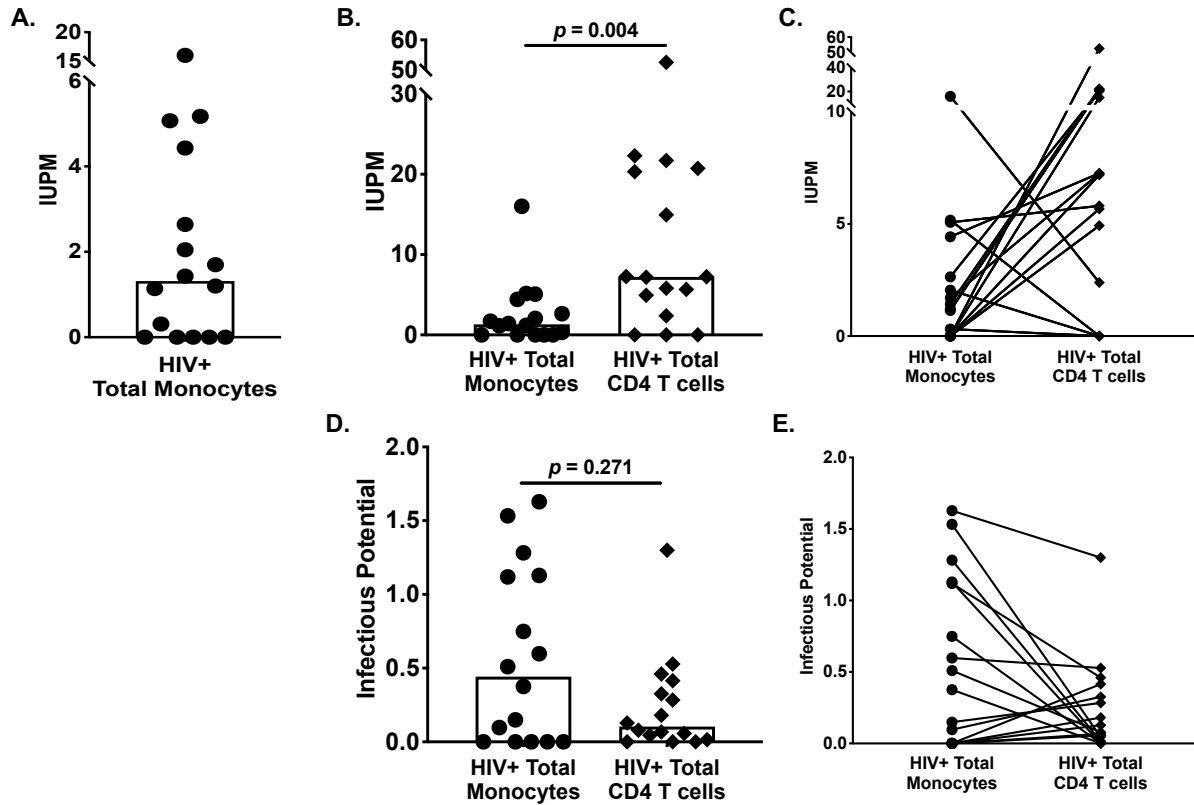
**Table 1.** Demographic and clinical parameters of chronic HIV-infected participants.

Demographic and Clinical Parameters				
Characteristic	Chronic HIV-infected			p-value
	Total (n=16)	Cog. Normal (n=9)	Cog. Impaired (n=7)	
Age, years	58 [53, 61]	55 [51, 59]	61 [58, 64]	0.033
Male, n (%)	16 (100%)	9 (100%)	7 (100%)	1.000
HIV RNA <50 copies/ml, n (%)	16 (100%)	9 (100%)	7 (100%)	1.000
Antiretroviral Medication				
NRTI	16 (100%)	9 (100%)	7 (100%)	1.000
NNRTI	3 (19%)	2 (22.2%)	1 (14%)	1.000
PI	2 (13%)	0 (0%)	2 (29%)	0.175
INSTI	10 (63%)	6 (66.7%)	4 (57%)	1.000
Duration since HIV diagnosis, years	23 [18, 26]	22 [18, 25]	24 [18, 31]	0.408
Duration since ART initiation, years	18 [11, 19]	18 [6, 21]	17 [11, 18]	0.497
Duration of being ART naïve, years	4 [2, 14]	3 [1, 10]	14 [2, 15]	0.497
Duration of education, years	15 [14, 18]	15 [13, 19]	14 [13, 18]	0.756
Neuropsychological performance, z-score				
Global-14	0.12 [-0.46, 0.45]	0.22 [0.10, 0.47]	-0.37 [-1.10, 0.11]	0.055
Executive function	0.64 [-0.22, 1.08]	0.83 [0.18, 1.13]	-0.06 [-1.00, 0.72]	0.108
Learning and memory	0.01 [-0.27, 0.94]	-0.04 [-0.33, 0.88]	0.06 [-0.05, 1.45]	0.606
Working memory	-0.08 [-0.38, 0.08]	-0.06 [-0.33, 0.25]	-0.39 [-0.67, 0.11]	0.261
Psychomotor speed	0.56 [-0.46, 0.73]	0.66 [0.37, 0.82]	-0.51 [-0.99, 0.56]	0.055

**Table 2.** Immunologic parameters of chronic HIV-infected participants

Immunologic Parameters				
Characteristic	Chronic HIV-infected			p-value
	Total (n=16)	Cog. Normal (n=9)	Cog. Impaired (n=7)	
Nadir CD4+ T cells, cells/uL	103 [10, 234]	60 [10, 223]	170 [10, 258]	0.680
CD4+ T cells, cells/uL	495 [387, 771]	492 [372, 798]	668 [415, 783]	0.837
CD4+ T cells, %	32 [19, 43]	31 [19, 42]	32 [20, 45]	0.699
CD8+ T cells, cells/uL	725 [543, 1442]	715 [461, 1690]	734 [536, 1197]	0.758
CD8+ T cells, %	48 [31, 51]	48 [29, 58]	44 [35, 49]	0.700
CD4 CD8 T cell ratio	0.66 [0.40, 1.35]	0.65 [0.35, 1.46]	0.67 [0.45, 1.29]	0.758
Total monocytes, %	8.2 [7.1, 10.2]	8.8 [7.4, 10.7]	7.8 [7.0, 9.9]	0.220
Monocyte subpopulations, %				
Classical (CD14++CD16-)	90.7 [88.7, 93.4]	90.0 [88.9, 93.3]	91.4 [82.4, 95.0]	0.978
Intermediate (CD14++CD16+)	2.5 [1.1, 3.9]	2.4 [0.9, 3.7]	2.6 [1.5, 6.3]	0.681
Non-classical (CD14+/lowCD16++)	2.7 [1.2, 5.4]	2.4 [1.1, 4.2]	3.2 [1.1, 7.9]	0.408

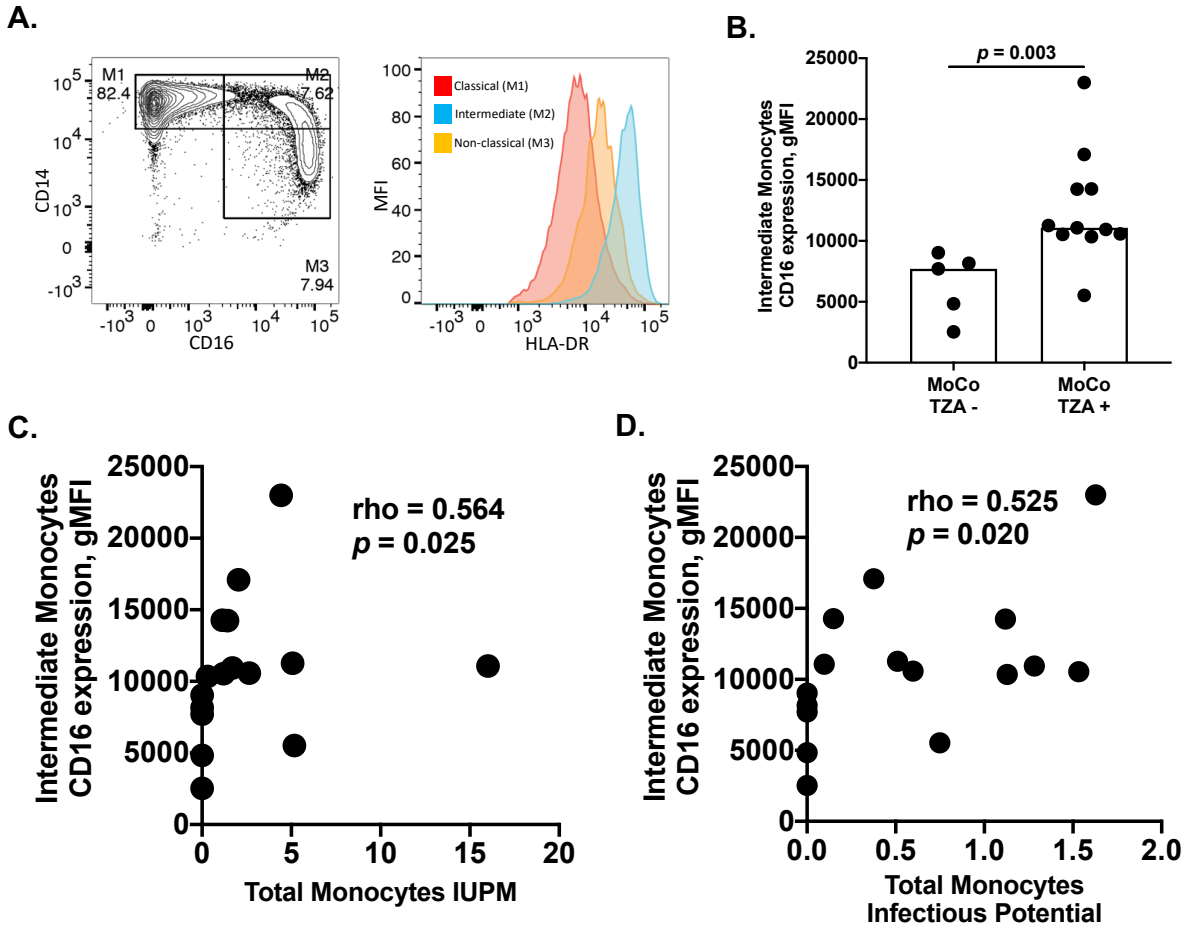
Of the 16 donor PBMCs assessed using the MoCo-TZA, 11 donors (69%) were documented to have blood circulating monocytes with inducible replication-competent HIV at a median IUPM of 1.32 (**Figure 1a**). CD4<sup>+</sup> T cells were also assessed using the MoCo-TZA and 13 out of 16 donors (81%) had circulating CD4<sup>+</sup> T cells with inducible replication-competent HIV at a median IUPM of 7.22, which was found to be significantly higher than what was observed in the monocyte compartment (**Figure 1b**). Furthermore, we observed that the monocyte IUPM:CD4 T cell IUPM ratios appeared to be variable among donors, suggesting the size of the measurable HIV reservoir in the CD4<sup>+</sup> T cell compartment may not necessarily be indicative of the size of the reservoir in the monocyte compartment (**Figure 1c**). Interestingly, we observed that despite the monocyte compartment having a lower frequency of cells producing replication-competent HIV, the amount of virus produced per infectious unit, which here is termed infectious potential, by the monocyte compartment was slightly higher as compared to CD4<sup>+</sup> T cells (**Figure 1d**). Similar to what was seen with the IUPM ratios, the infectious potential ratios of the cellular compartments were variable, which may further suggest independent HIV reservoir dynamics between circulating monocytes and CD4<sup>+</sup> T cells (**Figure 1e**).



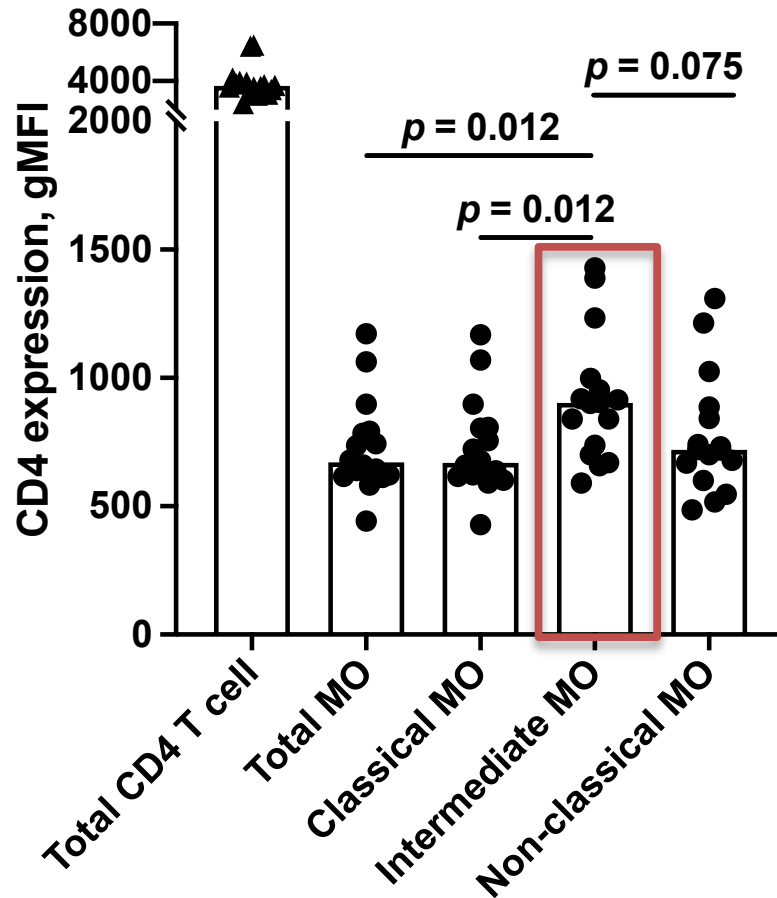
**Figure 1.** (A) Peripheral blood monocytes with inducible replication-competent HIV are detectable using MoCo-TZA in ART-suppressed chronic HIV-infected individuals. (B-C) Comparison of IUPM values calculated by MoCo-TZA between blood circulating monocytes and CD4 T cells. (D-E) Comparison of infectious potentials calculated by MoCo-TZA between blood circulating monocytes and CD4 T cells.

We were then interested to see if particular cellular phenotypes of circulating monocytes and CD4<sup>+</sup> T cells related to the HIV reservoir measurements acquired by the MoCo-TZA. We performed post-hoc analyses on the flow-cytometry sort files to identify 3 circulating monocyte subpopulations based on CD14 and CD16 surface expression: Classical (CD14<sup>+</sup>CD16<sup>-</sup>), Intermediate (CD14<sup>+</sup>CD16<sup>+</sup>), and Non-classical (CD14<sup>+</sup>/-CD16<sup>++</sup>) (**Figure 2a**). In addition, we assessed surface expression of HLA-DR, CD16, and CD4 on the monocyte subpopulations, as well as on CD4<sup>+</sup> T cells. We first assessed if there was a phenotypic difference between donor cells that had a detectable measurement of producing replication-competent HIV by the MoCo-

TZA and donor cells that did not. In the monocyte compartment, we found that donor cells that had a detectable measurement by the MoCo-TZA had a significantly higher expression of CD16 on the intermediate monocyte population as compared to donors that had no detection (**Figure 2b**). Furthermore, we found a significant positive correlation of CD16 surface expression on intermediate monocytes with higher monocyte IUPM and infectious potential measurements (**Figure 2c and d**). We did not observe significant differences or correlations when looking at the other monocyte phenotypes. In the CD4+ T cell compartment, no significant differences or correlations were observed when looking at HLA-DR, CD16, or CD4 surface expression. We show that the higher expression of surface CD16 on the intermediate (CD14+CD16+) subpopulation is correlated with higher frequencies of monocytes with inducible replication-competent HIV (**Figure 2c**), as well as higher infectious potential in the monocyte compartment (**Figure 2d**), which is a measure of the amount of infectious HIV produced per infectious unit. This observation would infer that the intermediate monocyte subpopulation, specifically intermediate monocytes with higher expression of CD16, may be the potential source of inducible replication-competent virus in the circulating monocyte compartment. The potential permissiveness of HIV infection within this particular subpopulation by the higher expression of CD4 as compared to the other monocyte subsets (**Figure 3**), as well as their reported high expression of CCR5 (188, 189) and CXCR4 (190, 191) may further support this inference.



**Figure 2.** (A) Plots depict a representative flow gating used to identify monocyte subpopulations from cryopreserved PBMCs of chronic ART-suppressed HIV-infected individuals. The dot plot represents the gating regions of each monocyte subpopulation based on CD14 and CD16 surface expression: Classical (M1), Intermediate (M2), and Non-classical (M3). HLA-DR surface expression (histogram plot) of each monocyte subpopulation was assessed to facilitate gate placement. (B) Comparison of CD16 surface expression (gMFI) on intermediate monocytes between chronic HIV-infected individuals with undetectable (-) and detectable (+) MoCo-TZA measurement in the total monocyte compartment. (C-D) Scatterplots show correlations (Spearman's rho) with CD16 surface expression on intermediate monocytes to total monocyte IUPM and infectious potential values.



**Figure 3.** CD4 surface expression of blood circulating CD4 T cells and monocyte subpopulations isolated from chronic ART-suppressed HIV-infected participants. Expression levels were determined by multi-parametric flow-cytometry and shown as gMFI.

***Monocytes retain replication-competent HIV in individuals despite 2 years of ART initiated early during acute HIV infection***

We next asked the question if the persistence of virus detected in chronic infection in monocytes could be reduced or suppressed if ART was initiated during acute infection. Using the MoCo-TZA we assessed circulating monocytes and CD4 T cell HIV persistence in blood samples collected from participants enrolled in the SEARCH010/RV254 study. Of the 8 donors assessed, 4 donors were diagnosed and initiated ART during Fiebig 1 (RNA+, p24 antigen-) of acute infection and 4 donors in Fiebig III (IgM+, Western Blot-) or Fiebig V (Western Blot+ without

p31) (**Table 3**). All donors were male, were on ART treatment for a median of 672 days (~2 years), and were virally-suppressed through this period. All donors were on NRTIs, with a majority (75%) having NNRTIs and a smaller proportion (25%) having INSTIs incorporated into their ART regimen. Donors had a median duration of 686 days (~2 years) since HIV infection and a relatively short median duration of 16 days between time of HIV infection and ART initiation (ART naïve). After 2 years of ART, donors had a relatively normal median CD4+ T cell count of 692 cells/ul and high median CD4+ T cell-CD8+ T cell ratio of 1.22 (**Table 4**). Donors who initiated ART during Fiebig I had a significantly higher median CD4+ T cell-CD8+ T cell ratio as compared to individuals who initiated ART during Fiebig III/V.

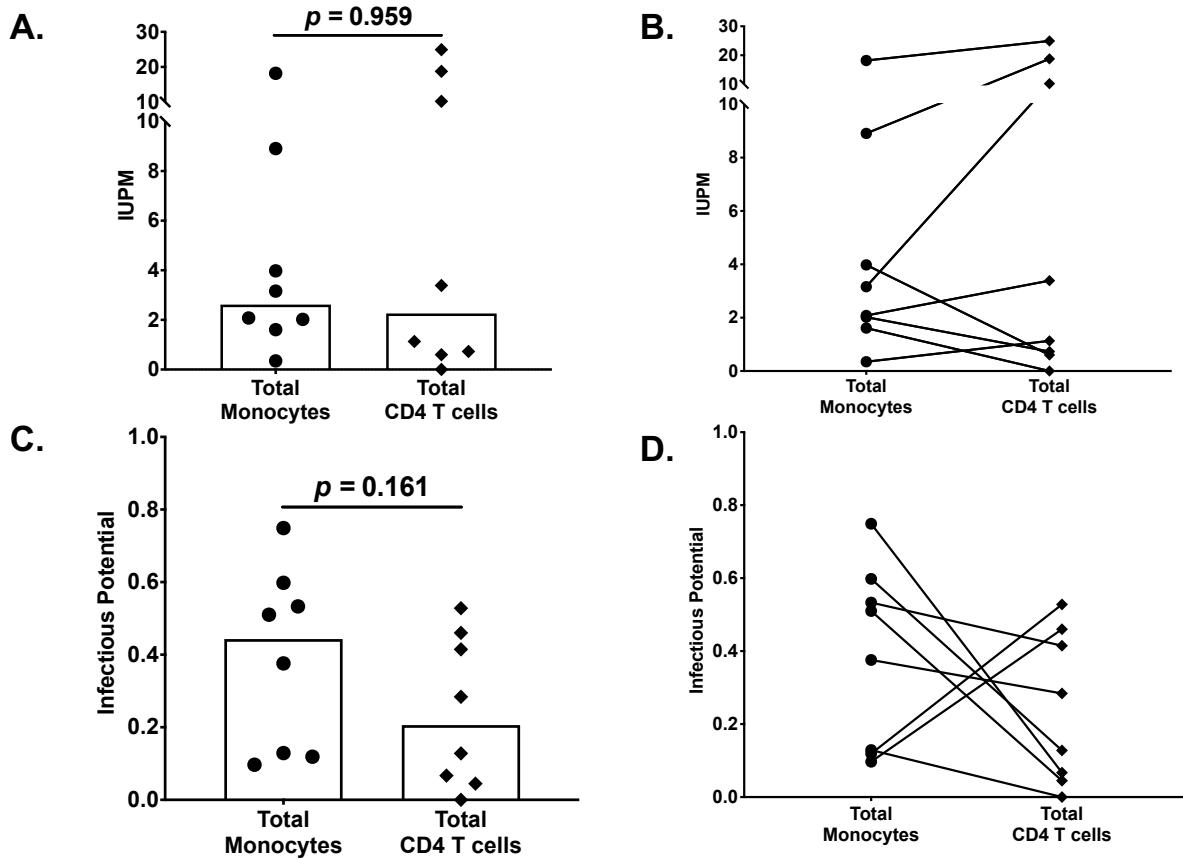
**Table 3.** Demographic and clinical parameters of acute HIV-infected participants

Demographic and Clinical Parameters				
Characteristic	Acute HIV-infected			p-value
	Total (n=8)	Fiebig 1 (n=4)	Fiebig 3,5 (n=4)	
Age, years	32 [26, 43]	36 [25, 47]	31 [26, 40]	0.686
Male, n (%)	8 (100%)	4 (100%)	4 (100%)	1.000
Antiretroviral Medication, n (%)				
NRTI	8 (100%)	4 (100%)	4 (100%)	1.000
NNRTI	6 (75%)	3 (75%)	3 (75%)	1.000
PI	0 (0%)	0 (0%)	0 (0%)	1.000
INSTI	2 (25%)	1 (25%)	1 (25%)	1.000
HIV RNA <50 copies/ml, n (%)	8 (100%)	4 (100%)	4 (100%)	1.000
Duration since HIV infection, days	686 [681, 709]	684 [675, 711]	691 [682, 709]	0.686
Duration since ART initiation, days	672 [670, 674]	673 [659,698]	672 [670, 672]	0.714
Duration of being ART naïve, days	16 [11, 22]	14 [10, 17]	20 [12, 37]	0.343
Duration of education, years	18 [13, 18]	18 [17, 20]	15 [8, 18]	0.314
Neuropsychological performance, z-score				
Global-4	0.46 [0.18, 0.83]	0.68 [0.31, 1.18]	0.33 [0.06, 0.51]	0.343
Grooved Pegboard	0.68 [0.47, 1.18]	0.94 [0.62, 1.51]	0.59 [0.22, 0.88]	0.343
Color Trails 1	0.75 [0.14, 1.26]	1.18 [0.25, 1.68]	0.59 [-0.78, 0.82]	0.200
Color Trails 2	0.37 [0.19, 0.56]	0.36 [-0.38, 0.87]	0.37 [0.22, 0.53]	0.886
Trail Making A	0.30 [-0.03, 0.63]	0.50 [0.04, 1.12]	0.17 [-0.43, 0.40]	0.343

**Table 4.** Immunologic parameters of acute HIV-infected participants

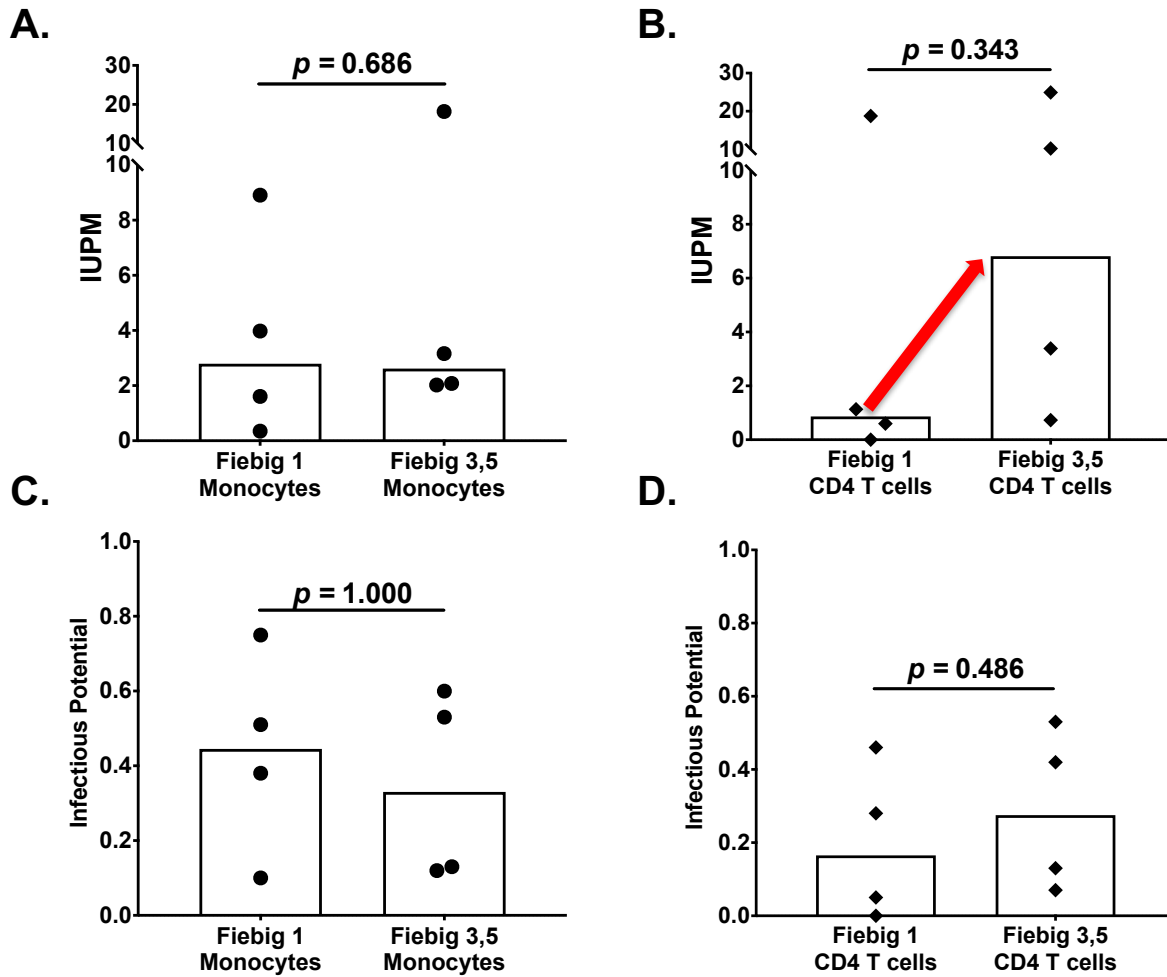
Immunologic Parameters				
Characteristic	Acute HIV-infected			p-value
	Total (n=8)	Fiebig 1 (n=4)	Fiebig 3,5 (n=4)	
CD4+ T cells, cells/uL	692 [556, 841]	692 [563, 757]	749 [508, 1050]	0.886
CD4+ T cells, %	32 [29, 38]	32 [28, 40]	31 [29, 38]	1.000
CD8+ T cells, cells/uL	512 [408, 710]	441 [387, 539]	677 [454, 1009]	0.200
CD8+ T cells, %	25 [22, 31]	23 [20, 25]	30 [26, 34]	0.086
CD4 CD8 T cell ratio	1.22 [1.07, 1.52]	1.49 [1.31, 1.65]	1.10 [1.02, 1.16]	0.029
Total monocytes, %	7.2 [5.8, 9.1]	9.1 [7.5, 9.4]	6.0 [5.2, 7.2]	0.057
Monocyte subpopulations, %				
Classical (CD14++CD16-)	88.3 [87.1, 88.9]	88.9 [87.4, 91.0]	87.5 [87.1, 88.5]	0.371
Intermediate (CD14++CD16+)	4.6 [3.9, 6.1]	5.4 [4.0, 6.7]	4.4 [3.7, 5.1]	0.486
Non-classical (CD14+/lowCD16++)	4.9 [3.6, 5.2]	3.8 [1.9, 4.9]	5.1 [4.8, 6.3]	0.200

Utilizing the MoCo-TZA, all 8 donors had detectable frequencies of monocytes with inducible replication-competent HIV, while 7 of 8 donors (86%) had detectable frequencies of CD4+ T cells with inducible replication-competent HIV (**Figure 4a**). In contrast to what we observed in the chronic HIV cohort (**Figure 1b**), there were no significant differences between the monocyte and CD4+ T cell IUPM measurements. Furthermore, when assessing the relationship between the monocyte and CD4+ T cell IUPM measurements within individuals, there were 2 individuals that had high IUPM measurements for both cell compartments (**Figure 4b**), which was not observed in the chronic cohort (**Figure 1c**). Infectious potentials of circulating monocytes were slightly higher as compared to the infectious potentials determined in the CD4+ T cell compartment (**Figure 4c**), however the infectious potential ratio of monocyte and CD4+ T cell compartments within individuals appeared variable (**Figure 4d**).



**Figure 4.** Inducible replication-competent HIV are detectable using MoCo-TZA in individuals despite 2 years of ART initiated early during acute HIV infection. (A-B) Comparison of IUPM values calculated by MoCo-TZA between blood circulating monocytes and CD4 T cells isolated from . (C-D) Comparison of infectious potentials calculated by MoCo-TZA between blood circulating monocytes and CD4 T cells.

In assessing the reservoir measurements between donors who initiated ART during Fiebig I and Fiebig III/V of acute infection, we found no significant differences of IUPM and infectious potential measures between Fiebig stages in either cell compartment (Figure 5a-d). We want to note that although not significant, early ART initiation during Fiebig I seems to have more of an effect on the CD4+ T cell compartment and lowering the IUPM measurement (Figure 5b). However, in the monocyte compartment, there was no discernable difference of IUPM measurements between Fiebig stages (Figure 5a).

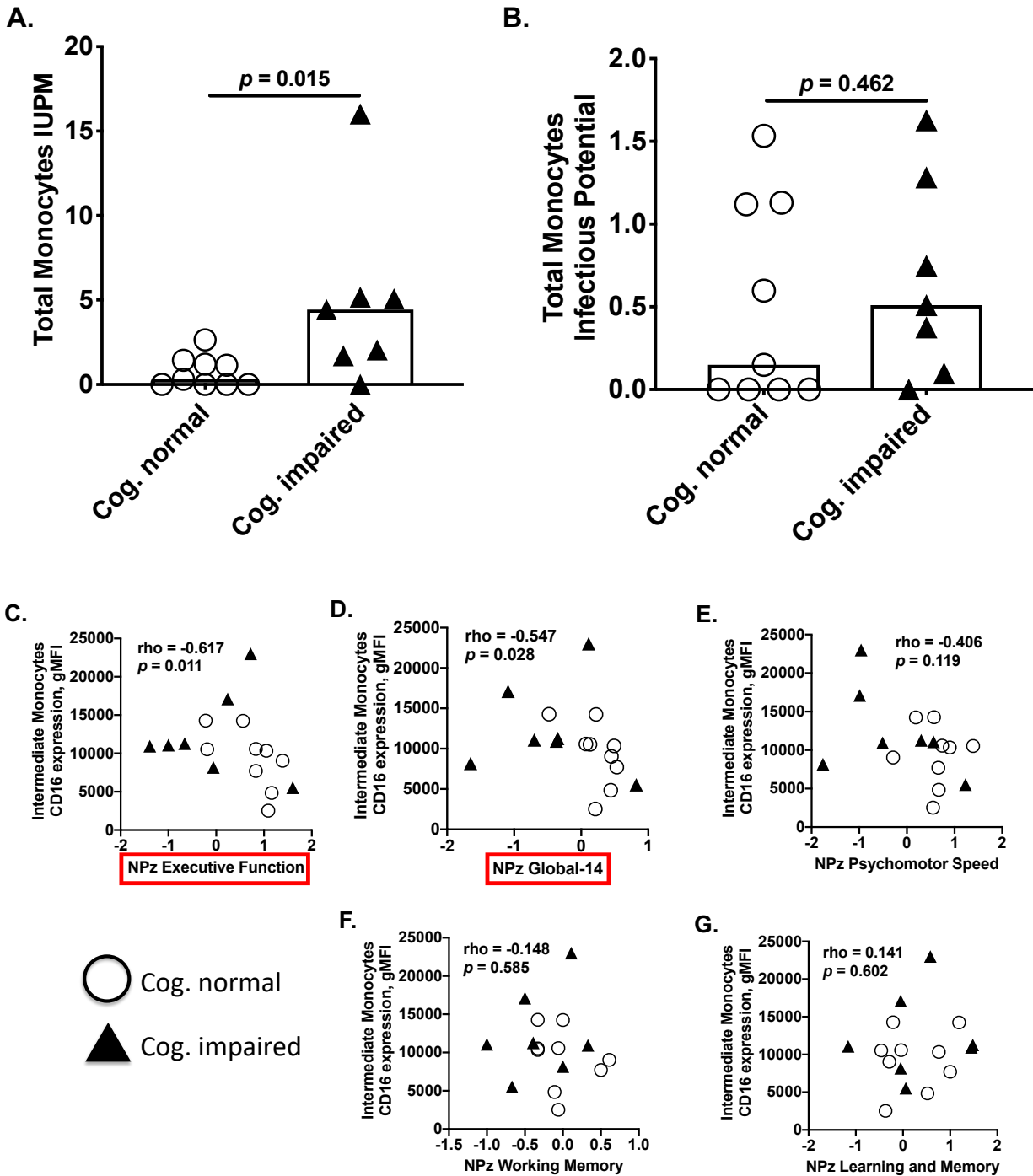


**Figure 5.** (A-B) IUPM and (C-D) infectious potential comparison between Fiebig stages of blood circulating monocytes and CD4 T cells isolated from early ART-treated HIV-infected individuals.

***Relationship between replication competent HIV in monocytes and cognition in HIV infection***

Participants in the H032 Chronic HIV cohort study had available comprehensive neuropsychological performance test scores for various cognitive domains, as well as overall (global) composite performance scores (**Table 1**). Based on the performance scores, 9 of 12 donors were categorized as cognitively normal, while 7 were categorized as cognitively impaired. It's important to note, that although 7 donors met the criteria of impairment set by the study (global neuropsychological score < -0.5 OR neuropsychological score < -0.5 in at least 1

cognitive domain), the level of impairment was observed to be very mild (**Table 1**). We compared monocyte and CD4+ T cell HIV reservoir measurements conducted using the MoCo-TZA between the cognitively normal and cognitively impaired donors. In the monocyte compartment, we observed a significantly higher frequency of circulating monocytes with inducible replication-competent HIV in cognitively-impaired donors, as compared to those who were cognitively-normal (**Figure 6a**). There was no difference in monocyte HIV infectious potentials between the two cognitive groups (**Figure 6b**). Moreover, no significant correlations between total monocyte IUPMs or infectious potentials and global and cognitive domain performance scores were seen in this population. Since higher CD16-expressing intermediate monocytes correlated with higher IUPM and infectious potential measurements in the circulating monocyte compartment, we wanted to assess if this particular subset correlated with neuropsychological performance scores. We observed in the chronic HIV cohort, that a higher proportion of CD16-expressing intermediate monocytes significantly correlate with lower performance scores in executive function (**Figure 6c**), as well as with lower global composite scores (**Figure 6d**). Correlations with psychomotor speed, working memory, and learning and memory domains were not observed to be significant (**Fig. 6e-g**).



**Figure 6.** Relationship between replication-competent HIV in blood circulating monocytes and neurocognitive impairment during chronic HIV disease.

Participants in the SEARCH010/RV254 acute HIV study, similarly all had neuropsychological performance test scores for various cognitive tests, as well as overall (global) composite scores

after 2 years of ART though the test differed from the chronic cohort (reference). Based on the normative data for this population, all 8 donors were observed to have normal median cognitive performance scores after 2 years of ART initiated early during acute infection (**Table 3**).

Moreover, initiation of ART during Fiebig 1 or Fiebig III/V of acute infection had no differential effect in the cognitive performance among all donors assessed. In contrast to what we saw in the H032 HIV Chronic HIV group, we did not observe any relationships between HIV persistence measures of either cell compartment and cognitive performance.

#### **4.4 Summary**

Utilizing the MoCo-TZA, we show that inducible replication-competent HIV in circulating monocytes is detectable during long-term chronic infection, as well as in individuals who initiated ART early during acute infection and were on treatment for 2 years.

The frequency of monocytes producing replication-competent HIV (IUPM) relative to CD4 T cells was observed to be different between cohorts. However, in both cohorts, the amount of replication-competent virus produced per monocyte IUPM (infectious potential) was observed to be slightly higher than CD4 T cells. This may suggest that the monocyte compartment may have a higher efficiency to produce replication-competent HIV per infected cell. Similar observations have been reported in SIV in vitro models, however differences we observed from the CD4 T cells weren't statistically significant, thus this observation needs further follow-up. Our results suggest that the replication-competent HIV reservoir in circulating monocytes may be enriched in the intermediate monocyte subpopulation expressing higher surface CD16. This observation is supported by higher CD4, CCR5, and CXCR4 expression on the intermediate subset relative to

other monocyte subpopulations and the circulation pattern of intermediates migrating in and out of tissues may potentiate these cells to be infected (192). However, how infection of circulating monocytes is mediated in tissues during suppressive ART is unknown. The intermediate subset was also found to be related to lower cognitive performance in the long-term chronic cohort but not the early ART-treated cohort. This may be due to the early ART cohort having normal cognitive performance after 2 years of ART. However, it is unknown what the long-term trajectory of cognition will be beyond 2-years, and questions to consider are: will these individuals have lower cognitive status independent of ageing? If so, when does this happen? and does these processes have any relationship to the monocyte and/or CD4 T cell reservoir? Regional brain volume reductions, particularly in the putamen and caudate, have been reported in the RV254 cohort despite 2 years of ART initiated early during acute infection (193), therefore circulating monocyte and/or CD4 T cell reservoirs, as measured by the MoCo TZA may be related with structural changes rather than performance scores at the 2 year time point, however this needs to be further assessed. Overall, our results highlight the need to further understand the monocyte-derived replication-competent HIV reservoir during ART-suppression and further investigation in this reservoir compartment in HIV-associated neurocognitive disease treatment and HIV cure efforts may be warranted.

## **CHAPTER 5**

### **CONCLUSION AND POTENTIAL FUTURE DIRECTIONS**

The pursuit of an HIV cure continues to be steadfast as we continue through the 5<sup>th</sup> decade since the characterization of HIV and its link to AIDS. Understanding mechanisms of HIV persistence, as well as identifying cellular compartments and tissue sites as viral reservoirs, especially during effective ART, are important components to that pursuit. However, majority of the effort elucidating these components have been T cell centric, and as a result, past and current cure initiatives propose interventions that mostly target the CD4 T cell reservoir. With publications characterizing tissue macrophages as a site of HIV persistence during effective ART, myeloid cells represent a non-T cell compartment of the HIV reservoir that may be important to the ongoing presence of HIV in ART-treated individuals and the growing evidence suggest that myeloid cells may need to be considered more in current and future cure approaches. Thus, it is imperative to tailor reservoir measuring assays to myeloid cells so to better characterize this important but often marginalized cellular compartment

The MoCo-TZA utilizes TZM-bl cells, an HIV-infection reporter cell-line, that stably expresses CD4, CCR5, and CXCR4. TZM-bl cells are permissive to HIV and report infection via the production of beta-galactosidase induced by the proviral production of Tat. There are three important components to the MoCo-TZA assay. First, cell populations of interests are isolated by cell sorting using multi-parametric flow-cytometry. Second, isolated cell populations are co-cultured with TZM-bl cells in stimulation conditions to detect production of replication-competent HIV and calculate IUPMs. Third, the amount of virus produced are estimated using a relative light unit (RLU) vs. pg of replication-competent HIV-associated p24 standard curve. The ratio of total pg of replication-competent HIV-associated p24 measured in positive wells to the IUPM, which we term infectious potential. The lower limit of detection for the MoCo-TZA

was observed to be ~0.73 pg/ml at a detection rate of 95% and is specific for replication-competent HIV.

Utilizing the MoCo-TZA, we show that inducible replication-competent HIV in circulating monocytes is detectable during long-term chronic infection, as well as in individuals who initiated ART early during acute infection and were on treatment for 2 years.

The frequency of monocytes producing replication-competent HIV (IUPM) relative to CD4 T cells was observed to be different between cohorts. However, in both cohorts, the amount of replication-competent virus produced per monocyte IUPM (infectious potential) was observed to be slightly higher than CD4 T cells. This may suggest that the monocyte compartment may have a higher efficiency to produce replication-competent HIV per infected cell. Similar observations have been reported in SIV in vitro models, however differences we observed from the CD4 T cells weren't statistically significant, thus this observation needs further follow-up. Our results suggest that the replication-competent HIV reservoir in circulating monocytes may be enriched in the intermediate monocyte subpopulation expressing higher surface CD16. This observation is supported by higher CD4, CCR5, and CXCR4 expression on the intermediate subset relative to other monocyte subpopulations and the circulation pattern of intermediates migrating in and out of tissues may potentiate these cells to be infected (192). However, how infection of circulating monocytes is mediated in tissues during suppressive ART is unknown. The intermediate subset was also found to be related to lower cognitive performance in the long-term chronic cohort but not the early ART-treated cohort. This may be due to the early ART cohort having normal cognitive performance after 2 years of ART. However, it is unknown what the long-term trajectory of cognition will be beyond 2-years, and questions to consider are: will these

individuals have lower cognitive status independent of ageing? If so, when does this happen? and does these processes have any relationship to the monocyte and/or CD4 T cell reservoir?

Regional brain volume reductions, particularly in the putamen and caudate, have been reported in the RV254 cohort despite 2 years of ART initiated early during acute infection (193), therefore circulating monocyte and/or CD4 T cell reservoirs, as measured by the MoCo TZA may be related with structural changes rather than performance scores at the 2 year time point, however this needs to be further assessed. Overall, our results highlight the need to further understand the monocyte-derived replication-competent HIV reservoir during ART-suppression and further investigation in this reservoir compartment in HIV-associated neurocognitive disease treatment and HIV cure efforts may be warranted.

With the development of the MoCo-TZA, further characterization of the monocyte subpopulation that may be enriched with replication-competent virus may be warranted and may serve as important information, particularly for studies focused on the development of curative strategies. As was shown through our data, increased IUPM values measured by the MoCo-TZA correlated with the intermediate monocyte subpopulation. Cell sorting the classical, intermediate, and non-classical monocyte subpopulations and directly seeding these subsets into the MoCo-TZA may serve as additional confirmation of the findings we observed. It is important to note that conducting such an experiment may require a significant amount of PBMCs since the total monocyte population accounts for ~8-10% of the PBMCs. Another important follow-up study is that of characterizing the virus infected in monocytes. Sequencing the virus produced by the monocyte may serve as way to understand the origins of infections within the myeloid

compartment, since it is still unclear how blood circulating monocytes get infected in context of suppressive ART.

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