

THE RELATIONSHIP BETWEEN RESIDUAL VIREMIA AND CHRONIC  
INFLAMMATION IN PATIENTS ON LONG-TERM ANTIRETROVIRAL THERAPY

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# THE RELATIONSHIP BETWEEN RESIDUAL VIREMIA AND CHRONIC INFLAMMATION IN PATIENTS ON LONG-TERM ANTIRETROVIRAL THERAPY

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## ABSTRACT:

Since the development of combination antiretroviral therapy, morbidity and mortality due to AIDS-related malignancies has declined significantly. In most patients, daily administration of a multidrug antiretroviral regimen is sufficient to suppress viral replication and reduce immune activation and inflammation. In spite of this, residual viremia persists for many years in the vast majority of patients, and immune activation remains higher than pre-infection baseline. Chronic immune activation and inflammation are associated with a variety of adverse health consequences including higher risks of cancer, heart disease, liver, and kidney problems. Moreover, these risks are not normalized to those of healthy individuals when patients are on long-term antiretroviral therapy.

Due to the deleterious effects of chronic inflammation, it is desirable to develop treatments aimed at reducing immune activation in patients on suppressive antiretroviral therapy. Unfortunately, the variables that drive chronic inflammation are unclear, but could result from multiple factors including antigenic stimulation via residual viremia, microbial translocation from the gut into the blood due to damage of the gastrointestinal tract, and a reduction in the ability of the immune system to mount rapid immune responses against commonly encountered pathogens. In order to better control chronic immune activation, the variables that influence it must first be determined.

In this thesis, I report on two studies of residual viremia in the context of immune activation. The first is a report of virologic data derived from a clinical trial of patients with sub-optimal reconstitution of the CD4+ T cell compartment. The second is a study regarding the relationship between immune activation, residual viremia, and CD4+ T cell count in the context of regulatory T cells. Neither study found a correlation between immune activation and residual viremia, indicating that immune activation may not be driven by continuous antigenic stimulation provided by persistent virion production.

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## CHAPTER 1: HISTORY OF HIV-1 INFECTION

### 1.1 – HISTORY AND IDENTIFICATION OF HIV-1

In the summer of 1981, a clinical study published in the *New England Journal of Medicine* reported an alarmingly high incidence of *Pneumocystis jirovecii* in a population of gay men in San Francisco<sup>1</sup>. These men presented with acute lymphadenopathy, suggesting some form of systemic infection. Although immunocompromised individuals were known to be susceptible to this pathogen, these men had no obvious reasons for which they should be immunocompromised. It was hypothesized in this report that some known pathogen, such as cytomegalovirus, could be the etiological agent driving *P. jirovecii* infections.

As the number of cases of immunocompromised gay men grew, a search began for the causative agent of this syndrome. The first instance in which this “acquired immunodeficiency syndrome” (AIDS) could be caused by a retrovirus came in 1983 from the work of Françoise Barré-Sinoussi at the Pasteur Institute in France, who discovered a virus containing reverse transcriptase activity from the lymph node from a man with persistent lymphadenopathy<sup>2</sup>. However, since lymphadenopathy is common in a variety of viral infections, there was still debate in the medical community regarding whether AIDS was caused by a known human pathogen. This debate was further bolstered by two publications in *Science* describing the detection and isolation of human T cell leukemia virus (HTLV) from patients with AIDS<sup>3,4</sup>. However, HTLV was known to have a close association with lymphocytes<sup>5</sup>, which the virus could transform but did not kill these cells<sup>5</sup>. These factors were at odds with reports describing transmission of AIDS to hemophiliacs receiving cell-free plasma products<sup>6</sup>, as well as the marked loss

of CD4+ T lymphocytes that became a defining characteristic of AIDS pathogenesis<sup>1</sup>. Clearly, more work was required to resolve these discrepancies.

As Barré-Sinoussi continued her work, she discovered that the virus she isolated, which she named lymphadenopathy-associated virus (LAV) infected and killed CD4+ T lymphocytes in cell culture<sup>7</sup>. In the United States, work by Robert Gallo identified a virus in AIDS patients that demonstrated cross-reactivity with HTLV<sup>8</sup>. Gallo therefore included his virus in the HTLV family, naming it HTLV-III<sup>9</sup>. Shortly thereafter, LAV and HTLV-III were recognized as the primary isolates of the same human retrovirus<sup>10,11</sup>, which differed considerably from other known human retrovirus<sup>12</sup>. Due to these revelations, the AIDS virus was given a unique name: the human immunodeficiency virus, or HIV<sup>13</sup>.

## 1.2 – DEVELOPMENT AND CURRENT STATE OF COMBINATION ANTIRETROVIRAL THERAPY

Following the identification of HIV, it became apparent that most infected individuals remained asymptomatic for many years before showing symptoms. Indeed, the median asymptomatic period between infection and AIDS defining illnesses is approximately 10 years<sup>14</sup>. Despite this, the range of the asymptomatic period varies considerably among infected individuals. In rare circumstances, individuals were unable to mount an effective immune response against the virus and died during the primary infection. In other rare cases, infected individuals remained asymptomatic indefinitely and were labeled “long-term non-progressors.”<sup>15-17</sup> As the virus spread through the homosexual community and later, heterosexual individuals, the need for drugs to treat or cure the infection became a priority. This led to the development of numerous drugs aimed at suppressing viral replication, thereby reducing viral load and CD4+ T lymphocyte destruction.

The first drug developed to treat HIV infection was zidovudine (AZT), a nucleoside-reverse transcriptase inhibitor (NRTI). Initially developed as a cancer drug, it

was discovered that AZT blocked the activity of reverse transcriptase, effectively shutting down the conversion of HIV single stranded RNA to double stranded DNA<sup>18</sup>. Although effective, AZT also blocked cellular & mitochondrial polymerases at high clinical doses and caused severe side effects<sup>19</sup>. Furthermore, AZT could only suppress viral replication transiently before mutations in reverse transcriptase rendered the drug ineffective<sup>20-22</sup>. Throughout the early- and mid-1990's, additional NRTIs were developed<sup>23-26</sup>, as were several drugs that blocked reverse transcriptase at an allosteric site, now known as the non-nucleoside reverse transcriptase inhibitors (NNRTIs) nevirapine<sup>27</sup>, delavirdine<sup>28</sup>, and efavirenz<sup>29</sup>. When used in combination, these drugs could be used at lower doses to reduce side effects, and were effective at maintaining viral suppression, especially in the context of drug resistant mutations<sup>30</sup>. Unfortunately, virus suppression was incomplete and patients still progressed to AIDS due to drug resistant mutants that emerged after weeks to months of dual therapy<sup>31-33</sup>.

The development of protease inhibitors (PIs) in the mid-1990's was the breakthrough required to achieve durable control of HIV<sup>34</sup>. It was shown that when PIs were used in combination with NRTIs and NNRTIs, viral suppression could be maintained almost indefinitely<sup>35</sup>, given that no previous drug resistant mutations existed and patients adhered to their drug regimens. Since then, viral entry inhibitors<sup>36,37</sup>, integrase inhibitors<sup>38,39</sup>, and second generation NNRTIs and PIs have also been developed. At present, a variety of combination therapies have been validated in clinical trials (discussed in Chapter 3.3), and a number of drugs are available for salvage regimens when first line therapies fail.

## CHAPTER 2 – BIOLOGY OF HIV INFECTION

### 2.1 – TRANSMISSION OF HIV

HIV can be transmitted through three primary routes: (1) unprotected sexual intercourse<sup>1,40-42</sup>, (2) intravenous drug use with contaminated needles<sup>40</sup>, and (3) mother-to-child transmission during birth<sup>43,44</sup>. Although there have been reports of individuals contracting HIV through blood transfusions<sup>6</sup>, modern serum surveillance practices have rendered this method of transmission less common. Although intravenous drug use and mother-to-child transmission are significant methods of transmission, the focus herein will be on unprotected sexual intercourse.

Factors associated with a high risk of HIV infection are well established. Unprotected sex with multiple partners is the most predominant risk factor<sup>45</sup>, as individuals who engage in these activities are at high risk of encountering HIV. The risk of transmission is higher during acute infection<sup>46</sup> or when the infected partner has sexually transmitted diseases other than HIV<sup>47-49</sup>, which results in recruitment of activated CD4+ T lymphocytes to the site of infection. Considering the manner of sex involved, the risk is highest in those who engage in receptive anal intercourse<sup>45</sup>; this activity often damages the rectal mucosa, giving the HIV found in semen access to the immune cells underlying the endothelial layers of the gastrointestinal tract. Unprotected insertive anal intercourse<sup>50</sup> and vaginal intercourse<sup>42</sup> can also transmit HIV, either through the urethral lining of the penis or the vagina mucosa respectively.

Once HIV gains access to the intestinal mucosal lining, free virus or cell-associated virus translocates from the apical surface of the epithelial lining to sub-mucosal sites via resident M cells<sup>51</sup>. Here, the virus infects immune cells in lymph node-

like structures known as Peyer's patches located in the gastrointestinal tract. Replication is slow during the first few days of infection as the virus reproduces in Langerhan's cells, resident macrophages, activated, and resting T cells<sup>52,53</sup>. Within two days, virus disseminates from the initial site of infection to draining lymph node, and viremia develops within 5-7 days<sup>52,53</sup>. At this time, the virus begins to replicate in secondary lymphoid organs and establishes long-lived cellular reservoirs<sup>54</sup>, which become critically important in eradication of HIV during long-term suppressive combination ART.

## 2.2 – ESTABLISHMENT OF LONG-LIVED CELLULAR RESERVOIRS

Due to a number of factors, HIV preferentially infects activated CD4+ T lymphocytes. These include, (1) up-regulation of the HIV co-receptor, CCR5, during T lymphocyte activation<sup>55,56</sup>, (2) an increase in deoxynucleotide triphosphates, which are required for reverse transcription and are rare in resting T lymphocytes, and (3) the availability of transcription factors that are indispensable to HIV transcription, which are synthesized only upon cellular activation. Considering these requirements, latent infection is believed to occur when HIV infects an activated T cell, but the viral lifecycle stalls following integration of the HIV provirus. This is thought to occur when HIV infects an activated cell that is reverting to a resting state. In the context of untreated HIV infection, these events have little clinical meaning; productive infections produce virions and drive HIV pathology, and the rare, transcriptionally silent proviruses are of little pathological consequence. However, upon initiation of cART, productively infected cells are quickly cleared and new rounds of infection prevented<sup>35,57,58</sup>, such that only cells harboring quiescent proviruses remain. The vast majority of infected cells contain replication-deficient proviruses and are clinically irrelevant<sup>59</sup>. However, a small subset consisting of approximately one million infected cells harbor replication-competent provirus that sporadically activate, producing infectious virus capable of reinstate the viral lifecycle in the absence of antiretroviral therapy<sup>59</sup>. These cells were identified by

three groups as circulating, resting CD4+ T lymphocytes<sup>60-62</sup>, and further work has shown they have a memory phenotype<sup>63,64</sup>. It is these cells, which were infected prior to the initiation of cART, that represent long-lived cellular reservoirs of HIV and preclude eradication of HIV by cART alone.

In addition to circulating CD4+ T lymphocytes, a number of anatomical sites have been identified as possible long-lived reservoirs of HIV. The first is the gastrointestinal tract, which harbors up to 70% of the body's immune cells<sup>65</sup>. CD4+ T cells in this location express higher levels of CCR5 than in the periphery, rendering them more susceptible to HIV infection. Indeed, during primary infection, a significant fraction of CD4+ CCR5+ T cells in the gut are eradicated<sup>66</sup>, and this population fails to recover, even during long-term cART<sup>67</sup>. Although a critical site of interest, the infection in the gastrointestinal tract is difficult to study since the number of CD4+ T lymphocytes recoverable by biopsies is extremely limited.

The bone marrow and central nervous system are two other anatomical sites of interest and are similarly limited in the number of recoverable CD4+ T lymphocytes. A number of recent studies have examined the role of bone marrow-derived cells, particularly CD34+ hematopoietic stem cells, as reservoirs of HIV. Initial *in vitro* studies indicated that CD34+ cells were susceptible to HIV infection<sup>68</sup>, and a recent study detected the presence of HIV DNA in CD34+ cells isolated from patients on suppressive cART<sup>69</sup>. Unfortunately, the purity of CD34+ cells in this study was sub-optimal, and a more recent report examining highly pure CD34+ cells in a similar cohort failed to detect HIV DNA<sup>70</sup>. These differences may also be due to viral tropism, as CD34+ T cells are susceptible to CXCR4 tropic viruses (but not CCR5 tropic viruses), which often arise during prolonged chronic infection as patients progress to AIDS.

The central nervous system is particularly difficult to study since tissue from the brain and spinal cord are difficult to obtain in living patients. However, it is known that the viral titer in cerebral spinal fluid decreases dramatically during cART<sup>71</sup>, despite the fact that a number of cART drugs disseminate poorly into the central nervous system<sup>72</sup>.

It has also been established that resident macrophages of the brain, termed microglia, can be infected by HIV<sup>73</sup>. Furthermore, HIV-related neuropathies occur in patients on cART<sup>74</sup>. Whether this is due to the CNS acting as a long-lived reservoir of HIV, or is a side effect from systemic issues such as chronic immune activation, has yet to be determined.

### 2.3 – IMMUNE ACTIVATION OF HIV INFECTION

The immune system responds to HIV infection first through innate immune responses, followed by an adaptive response via HIV-specific CD4+ and CD8+ T lymphocytes. The activation of HIV-specific CD4+ T lymphocytes can be viewed as a double-edged sword. On the one hand, these cells help orchestrate the adaptive immune response against the virus. On the other, activation of these cells provides ideal sites of replication for the virus. In any case, the adaptive immune response is successful in partially controlling viral replication. Indeed, viral load falls considerably ( $<0.5 \log_{10}$ ) from an initial viral load of  $10^6$ - $10^7$  copies of HIV RNA/mL of plasma within 3-5 months of infection<sup>75</sup>. Immune activation remains high during chronic infection due to continuous stimulation of HIV-specific immune responses and can have delirious systemic effects including an increase risk of cancer, cardiovascular damage, and impaired liver and kidney function. Indeed, immune activation during chronic infection was found to correlate strongly with CD4+ T cell decline and progression to AIDS<sup>76-78</sup>.

During long-term cART, viremia drops below the clinical limit of detection, and it could be expected that immune activation would show a similar trend. However, in a subset of patients, immune activation remains high, even when suppression of viral replication has been achieved<sup>79</sup>. It is hypothesized that this could be due to several factors including translocation of microbial products from a gut damaged during acute infection into systemic circulation and chronic immune stimulation by residual viremia. A clinical trial evaluating the effects of rifaximin, an antibiotic that does not diffuse from the

gut, is underway to investigate microbial translocation as a cause of chronic inflammation. The role of residual viremia in chronic immune activation is discussed later in this report.

## 2.4 – HIV VIREMIA IN UNTREATED PATIENTS AND THOSE ON SUPPRESSIVE ART

Viremia is the result of productive cycles of viral replication, resulting in the release of virions into blood. It should be noted that viremia is highly dynamic, in that the half life of free virus in blood is estimated to be approximately 8 hours<sup>80</sup>. During acute infection, viremia can reach over  $1 \times 10^6$  copies of HIV RNA/mL of plasma. Following initiation of cART, viremia undergoes a multiphasic decay consisting of four distinct phases, each with a longer half-life than the previous. The first phase has a half-life of  $t_{1/2} = 1-2$  days and is thought to be due to the rapid decay of free virus and productively infected CD4+ T lymphocytes<sup>80-82</sup>. The second phase has a half-life of  $t_{1/2} = 2-4$  weeks and is due to the slower attrition of infected cells that are partially resistant to the cytopathic effects of HIV, such as macrophages or partially activated CD4+ T lymphocytes<sup>58,83</sup>. During the second phase of decay, viremia falls below the limit of clinical detection, and it was based on the trajectory of viremia, it was estimated that the virus could be cleared from the body in 2-3 years<sup>58</sup>. The discovery of resting CD4+ memory cells with a half-life of 44 months demonstrated that this estimate was far too optimistic<sup>59,61,62</sup>. The third phase, with a half life of  $t_{1/2} = 39$  weeks, continues until viremia plateaus at approximately 1 copy/mL of plasma<sup>84</sup> and remains unchanged for at least seven years ( $t_{1/2} = \infty$ )<sup>85</sup>.

The specific set-point to which viremia decays differs among individuals and is influenced by a number of factors. These include, (1) viral load prior to initiation of cART<sup>84,86</sup>, (2) the duration of time between infection and initiation of cART<sup>86-88</sup>, and (3) the length of time an individual is on suppressive ART<sup>89,90</sup>. Interestingly, the set-point of



residual viremia did not correlate with treatment regimen or CD4+ T lymphocyte count<sup>84</sup>.  
The correlation between residual viremia and immune activation are discussed below.

## CHAPTER 3: SINGLE COPY ASSAYS OF PLASMA HIV-1 RNA

### 3.1 – DEVELOPMENT AND METHODOLOGY OF THE PLASMA HIV-1 RNA SINGLE COPY ASSAY

The first single copy assay was developed at the National Cancer Institute in 2003. By this time, combination antiretroviral therapy achieved durable suppression of plasma viremia such that in most patients, viral load fell below the lower limit of clinical sensitivity (generally 50 copies/mL plasma)<sup>84,91</sup>. Although a number of “home-brew” assays<sup>91</sup> and modifications to Amplicor HIV Monitor test<sup>88,92,93</sup> with varying sensitivities had been developed, they lacked single copy sensitivity and had not been formally validated for use among different research groups. Therefore, the advent of the plasma HIV-1 RNA single copy assay marked the first time residual viremia could be measured reliably with single copy sensitivity<sup>94</sup>.

The single copy assay utilizes a guanidinium isothiocyanate-based extraction method to separate virion-associated HIV-1 RNA from plasma. Whole blood is first subjected to a low-speed centrifugation at 400 x g for 10 minutes at 4°C to pellet cellular components. The plasma is removed subjected to a second, high-speed centrifugation at 1350 x g for 15 minutes at 4°C to remove additional macromolecular complexes. Finally, the plasma is subjected to ultracentrifugation at 150,000 x g for 30 minutes to pellet HIV-1 virions. The supernatant is removed, and the pellet is incubated with 2.0 mg/mL proteinase K at 56°C for 30 minutes to digest the protein components of the virions. 6M guanidinium isothiocyanate supplemented with 0.2 mg of glycogen is then added, and the pellet is incubated for 10 minutes at room temperature to bring all virion components into suspension. Isopropanol is added, and the solution is centrifuged at 21,000 x g for 30 minutes to pellet the nucleic acids. The resulting pellet is then washed

twice with 70% ethanol to remove the guanidinium salts, which would otherwise interfere with downstream PCR steps.

Once the pellet has been washed and dried, the nucleic acids are resuspended in 55 mL of 5 mM Tris-HCl supplemented with 1mM dithiothreitol and 1000 U of RNase inhibitor to stabilize the virion-associated HIV-1 RNA. The RNA is then reverse transcribed into cDNA in a one-to-one ratio by PCR.. Finally, HIV-1 cDNA is quantitatively amplified using primers and probes specific for conserved regions of the HIV-1 gag gene on a Roche Lightcycler platform (Fwd: 5'-CAT GTT TTC AGC ATT ATC AGA AGG A-3'; Rev: 5'-TGC TTG ATG TCC CCC CAC T-3'; Probe: 5'-FAM CCA CCC CAC AAG ATT TAA ACA CCA TGC TAA-TAMRA-3'). A triplicate semi-log dilution of HIV-1 RNA transcripts, ranging from 3-3,000 copies per well, is included with each run to serve as an internal standard curve. The lower limit of quantification, which is dependent on the total volume of plasma assayed, was 0.6 copies/mL for 3.0 mL of plasma.

### 3.2 – VALIDATION OF THE PLASMA HIV-1 RNA SINGLE COPY ASSAY

Single copy assays require several forms of validation to ensure single-copy sensitivity is achieved and to establish consistency from run to run. The first is the preparation of HIV-1 RNA standards that serve as an external standard curve and for endpoint dilution to determine assay sensitivity. The second is the use of a low copy number standard, which contains a consistent concentration of HIV-1 RNA.

#### 3.2.1 – Serial Dilution of HIV-1 RNA Transcripts

HIV-1 RNA transcripts are prepared by in vitro synthesis of gag HIV-1 RNA from a plasmid construct. Following synthesis, the RNA of known length is quantified by spectrophotometry, and the transcripts are serially diluted to  $1 \times 10^5$  copies/mL, and

aliquots are stored at -80°C. To validate that the concentration is accurate, the transcripts are further diluted to 1 copy/well and dilutions from 1-100 copies/well are amplified in replicates of 10. According to Poisson statistics, 95% of wells containing 3 copies/well and 63% of wells containing 1 copy/well should be positive for HIV-1 RNA. If transcript amplification deviates from these expected results, the aliquots of HIV-1 RNA are prepared again until the expected results are achieved.

### 3.2.2 – VQA 5 as a Positive Control

A plasma standard known to contain 5 copies of HIV-1 RNA/mL of plasma is included with each run to ensure accurate quantification of low copy number HIV-1 RNA in unknown samples. This standard is prepared off-site by the serial dilution of virion-associated HIV-1 RNA from a viremic donor. The acceptable range for quantitative amplification of this standard is from 2.5-7.5 copies/mL of plasma.

## 3.3 – PRIOR USES OF THE PLASMA HIV-1 RNA SINGLE COPY ASSAY

The plasma single-copy assay has been used extensively to quantify residual viremia for a number of purposes. The three most common applications have been (i) to assess the efficacy of treatment regimens, (ii) to determine whether treatment regimens can be simplified, and (iii) to quantify the impact of treatment intensification on residual viremia. In the second case, treatment simplification is desirable if fewer drugs can be used to maintain suppression of viremia. In the latter case, treatment intensification has been used to assess whether complete suppression of viremia has been achieved.

Several early studies focused on the efficacy of different treatment regimens at controlling viremia. The first report of this kind showed that tenofovir (TDF) was better than stavudine (d4T) when co-administered with efavirenz (EFV) and lamivudine (3TC)<sup>95</sup>. The mean post-treatment residual viremia was lower in the TDF arm (3.8 vs 4.1

copies/mL), and more patients had undetectable viremia (47% vs. 29%). In a later retrospective study, nevirapine (NVP) was found to be better than EFV in achieving undetectable viremia (81% vs. 56%) when used in combination with emtricitabine (FTC) and TDF<sup>96</sup>. However, this study did not utilize randomized groups. A larger, randomized trial of d4T, 3TC, and either nelfinavir or lopinavir/ritonavir showed residual viremia was no different between the two arms<sup>84</sup>. The last of these findings is consistent with complete suppression of residual viremia, regardless of treatment regimen.

Treatment simplification is desirable to reduce medication cost, pill burden, drug toxicities, and drug-drug interactions. To this end, two studies have investigated reducing a standard combination therapy (typically two nucleoside reverse transcriptase inhibitors (NRTIs) and a ritonavir-boosted protease inhibitor) to a simplified regimen. In the first, 81% of patients (17 of 21) experienced no change in residual viremia when their regimen was simplified to ritonavir-boosted lopinavir<sup>97</sup>. Similarly, in the second study, 88% of patients (30 of 34) experienced no change in residual viremia when ritonavir-boosted atazanavir was administered in the absence of NRTIs<sup>98,99</sup>. Therefore, these studies suggest that viral suppression is maintained following treatment simplification in most patients.

In contrast to treatment simplification, treatment intensification has been used to investigate whether complete viral suppression has been achieved by standard combination therapy. Several reports have studied the intensification effects of efavirenz, raltegravir (RAL), and ritonavir-boosted protease inhibitors on residual viremia and found no change in plasma HIV-1 RNA<sup>89,100-104</sup>. Interestingly, two RAL studies yielded results that indicate low-level HIV replication may continue despite no change in residual viremia<sup>100,104</sup>. The first showed a RAL-mediated increase in 2-LTR circles in circulating mononuclear cells following treatment intensification, which is indicative of recent infection events<sup>100</sup>. The second found an increase in cell-associated unspliced HIV-1 RNA in ileal tissue<sup>104</sup>. Neither of these studies has been reproduced, so further investigation is necessary to validate these findings. The most recent intensification studies have been performed using maraviroc (MVC), a viral entry

inhibitor<sup>105,106</sup>. Although one study found an increase in residual viremia<sup>105</sup>, others that examined larger cohorts were unable to reproduce these findings<sup>106,107</sup>. Overall, these studies indicate that current treatments likely achieve full suppression of replication.

### 3.4 – FUTURE SINGLE COPY ASSAYS

Single copy assays capable of quantifying cell-associated HIV-1 nucleic acids are needed to obtain a more complete picture of HIV-1 dynamics during viral suppression. To this end, assays have been developed to quantify cell-associated HIV-1 DNA (spliced + unspliced), 2-LTR circles DNA, and full length HIV-1 RNA. In order to begin with cells, these assays omit the proteinase K digestion, include several sonication steps, and in the case of cell-associated HIV-1 RNA, require DNA digestion. Otherwise, extraction of nucleic acids and quantitative amplification are the same as described in Section 3.1.

### 3.5 – THESIS APPLICATIONS OF THE PLASMA HIV-1 SINGLE COPY ASSAY

The plasma HIV-1 single copy assay is used in two studies described in this report. The first investigates the effects of treatment intensification with MVC on residual viremia. The second examines whether residual viremia correlates with T cell activation in a study of the role of regulatory T cells in patients with virologic suppression. Neither study utilizes cell-associated HIV-1 single copy assays described in Section 3.4.

## CHAPTER 4 – MEASUREMENT OF IMMUNE ACTIVATION

### 4.1 – MARKERS OF IMMUNE ACTIVATION

Although many distinct markers have been used to characterize T cell activation, only CD38 and HLA-DR were considered in the studies described in this report. CD38, a cyclic ADP ribose hydrolase, is expressed on the surface of a variety of immune cells, but is frequently used as a marker of T cell activation. HLA-DR is a MHC Class II surface receptor that is up-regulated in response to T cell signaling, which results in T cell activation. In contrast to CD38, HLA-DR is a late marker of activation, and is therefore expressed less frequently. In the context of these studies, activated T cells are defined as having a CD38+ HLA-DR+ phenotype.

### 4.2 – FLOW CYTOMETRY APPLICATIONS

Flow cytometric analysis is to determine the frequency of CD4+ & CD8+ activated T cells. Briefly, peripheral blood mononuclear cells are isolated from blood by Ficoll-Paque density gradient separation. The resulting cells are stained with fluorochrome conjugated antibodies specific for CD4, CD8, CD38, and HLA-DR and fixed with 2% paraformaldehyde. The cells are analyzed using an LSR II with FACS Diva software along with positive, negative, and isotype controls within three days of staining.

## CHAPTER 5 – RESIDUAL VIREMIA AND IMMUNE ACTIVATION I

### 5.1 – DESCRIPTION OF A5256: MARAVIROC INTENSIFICATION STUDY

A5256 was designed as a multicenter, open-label, single-arm clinical trial in which 32 participants added MVC (Pfizer Inc.) to their existing suppressive ART regimen, were monitored for 24 weeks, then discontinued MVC and were monitored for an additional 24 weeks. MVC was administered orally twice a day with doses ranging from 150 mg to 600 mg depending on pharmacokinetic interactions with the patients' pre-study medications as specified by the manufacturer. Additionally, patients were asked not to alter their ART regimens during intensification (i.e. week 0 – week 24). Patients who experience virologic failure, defined as two consecutive instances of detectable plasma HIV-1 RNA by the clinical assay used at the patient's local site, discontinued MVC.

Patients underwent two baseline assessments prior to adding MVC to their ART regimens. Patients then provided blood samples at 4, 8, 12, 16, 22, and 24 weeks post-entry (i.e. during intensification) and 36, 46, and 48 weeks post-entry (i.e. after intensification). HIV-1 RNA was quantified at weeks 12, 24, 36, and 48 by a CLIA-certified laboratory using an FDA-approved HIV-1 RNA quantification assay (lower limit of detection of  $\leq 75$  copies/mL).



## 5.2 – PATIENT COHORT

Of the 61 patients screened for this study, 34 met the inclusion criteria and were enrolled (**Table 1**). These included 32 males (94%) including 24 white non-Hispanics (71%), 6 black non-Hispanics (18%), and 4 Hispanics (12%). Patients had a median age of 50 years (range: 41-64 years), a median baseline CD4 T cell count of 153 cells/mL (range: 20-272 cells/mL), a median baseline CD8+ T cell count of 559 cells/mL (range: 20 – 1,365 cells/mL), and a median duration of clinically undetectable viremia of 3.5 years (range: 1.0 – 12.6 years).

Of the 34 patients who enrolled, there were two (6%) who experienced virologic failures upon entry (i.e. at week 0) and stopped treatment intensification. One subject withdrew from the study at week 12, while the other remained in the study follow-up. One additional subject did not continue follow-up after week 22. There were 15 patients (44%) who experienced adverse events, of whom 7 (21%) experienced grade 3 events. The most frequently reported adverse events, aside from “other”, were hepatic, respiratory, and gastrointestinal in manifestation. No grade 4 events were experienced, and no events were severe enough to warrant discontinuation of MVC.

**Table 1: Baseline Characteristics**

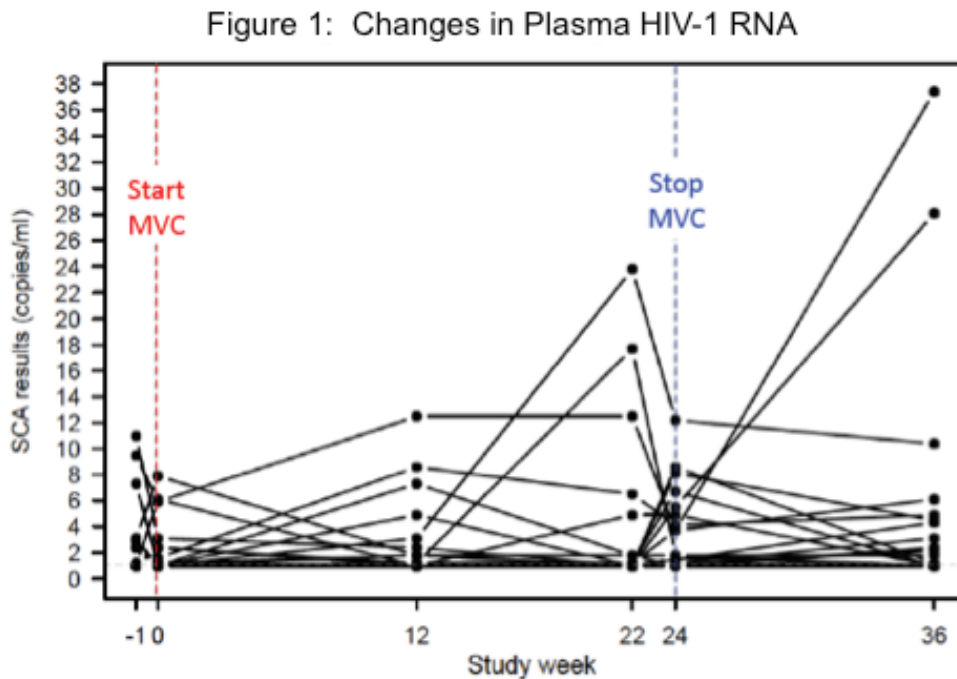
<b>Characteristic</b>	<b>Total Subjects (N=34)</b>
<b>Age (years)</b>	
Median [Q1, Q3]	50 [47, 55]
Mean (standard deviation)	51 (6)
Min, Max	41, 64
<b>Sex</b>	
Male	32 (94%)
Female	2 (6%)
<b>Race/Ethnicity</b>	
White Non-Hispanic	24 (71%)
Black Non-Hispanic	6 (18%)
Hispanic (Regardless of Race)	4 (12%)
<b>Pre-Entry Nadir CD4 Count</b>	
≤50	23 (68%)
51 – 100	9 (26%)
101 – 200	2 (6%)
<b>Pre-Entry CD4 Count Slope (cells/mm<sup>3</sup>/year)</b>	
Median [Q1, Q3]	5.81 [-14.02, 12.13]
Mean (standard deviation)	0.99 (12.75)
Min, Max	-18.17, 18.05
<b>Baseline CD4 Count (cells/mm<sup>3</sup>) †</b>	
Median [Q1, Q3]	153 [119, 203]
Mean (standard deviation)	154 (61)
Min, Max	20, 272
<b>Years with Undetectable Plasma HIV-1 RNA Prior to Entry ‡</b>	
Median [Q1, Q3]	2.95 [1.80, 4.50]
Mean (standard deviation)	3.54 (2.34)
Min, Max	1.00, 12.60

† Baseline CD4 count was calculated as the mean of the last two measurements obtained prior to or at the start of MVC.

‡ Four (4) subjects had no documentation or recall of the date when their plasma HIV-1 RNA became undetectable

### 5.3 – CHANGES IN RESIDUAL VIREMIA BY STUDY WEEK

Despite clinically undetectable viremia, 11 patients (35%) had detectable plasma HIV-1 RNA by SCA with a median of 2.9 copies/mL (range: 1.2 – 11.0 copies/mL) at pre-entry (week -1). At entry (week 0) 10 patients (32%) had detectable plasma HIV-1 RNA with a median of 2.4 copies/mL (range: 1.2 – 7.9 copies/mL). When MVC was discontinued at week 24, 14 patients (48%) had detectable plasma viremia with a median of 3.9 copies/mL (range: 1.2 – 12.2 copies/mL). Although two patients experienced transient increases in plasma HIV-1 RNA during the intensification period, their viral loads returned to baseline before discontinuation of MVC at week 24. Overall, there was no consistent change in residual viremia during the intensification period or after discontinuation of MVC as shown in **Figure 1**.



*Figure 1: Change in low-level residual viremia among subjects (N=32) before, during and after MVC intensification (N=7 patients never had detectable residual viremia). The y-axis represents plasma HIV-1 RNA as determined by single copy assay, and the x-axis reflects the study week. Intensification occurred between weeks 0 and 24.*

## 5.4 – CORRELATION BETWEEN BASELINE T CELL ACTIVATION AND BASELINE HIV-1 DNA

In addition to examining residual viremia during MVC intensification, we also investigated the relationship between T cell activation and total HIV-1 DNA in the CD4+ compartment at baseline. Regarding CD4+ T cells, the frequency of activation (i.e. HLA-DR+ CD38+) was negatively correlated with baseline total HIV-1 DNA as shown in **Figure 2**. (Spearman correlation = -0.52; p = 0.004). However, a similar correlation was not observed between CD8+ T cell activation and total HIV-1 DNA at baseline as shown in **Figure 3** (Spearman correlation = -0.14; p = 0.48).

Figure 2: Negative Correlation between Baseline CD4+, CD38+, HLA-DR+ T Cells and Baseline CD4+ HIV-1 DNA

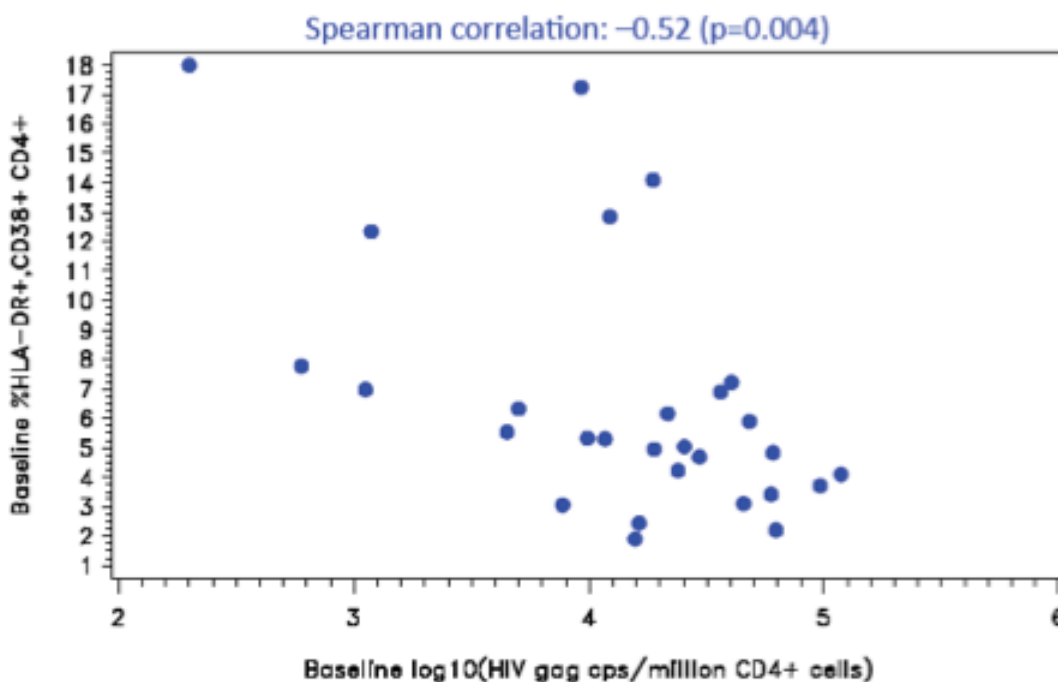


Figure 2: Negative correlation between CD4+ T cell activation and CD4+ cell HIV-1 DNA among subjects (N=28) at baseline. The y-axis represents the percentage of CD4+ T cells with an activated phenotype (i.e. HLA-DR+, CD38+) as determined by flow

cytometry. The x-axis reflects the number of copies of HIV-1 DNA detected per million CD4+ T cells as determined by quantitative amplification of HIV-1 DNA. Of note, no distinction was made between integrated and unintegrated HIV-1 DNA molecules.

Figure 3: Lack of Correlation between Baseline CD8+, CD38+, HLA-DR+ T Cells and Baseline CD4+ HIV-1 DNA

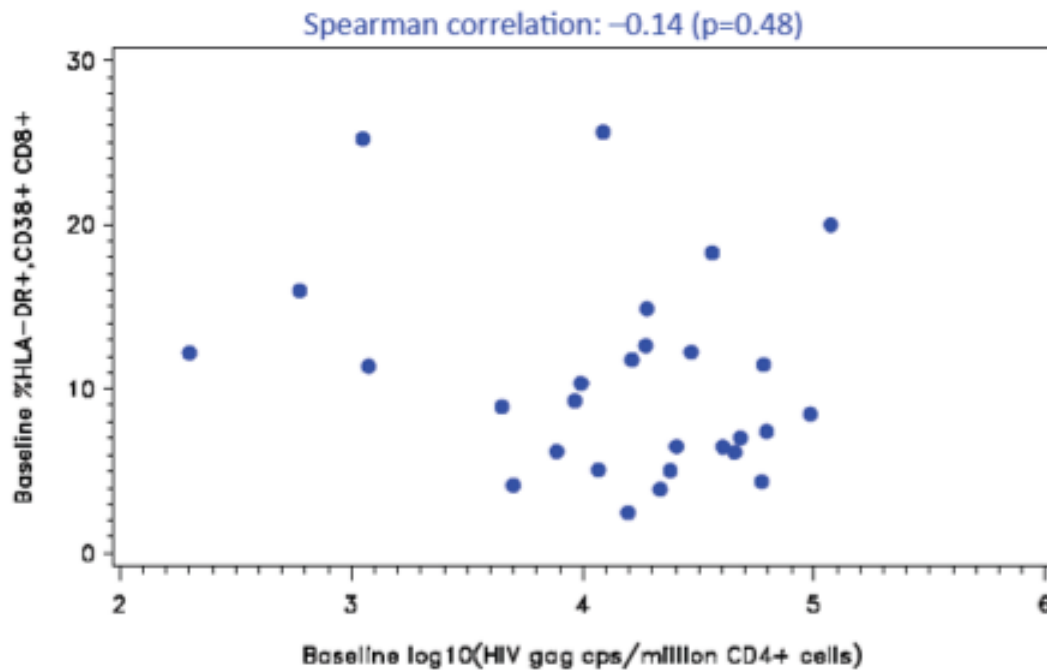


Figure 3: Correlation between CD8+ T cell activation and CD4+ cell HIV-1 DNA among subjects (N=28) at baseline. The y-axis represents the percentage of CD8+ T cells with an activated phenotype (i.e. HLA-DR+, CD38+) as determined by flow cytometry. The x-axis reflects the number of copies of HIV-1 DNA detected per million CD4+ T cells as determined by quantitative amplification of HIV-1 DNA. Of note, no distinction was made between integrated and unintegrated HIV-1 DNA molecules.

## CHAPTER 6 – RESIDUAL VIREMIA AND IMMUNE ACTIVATION II

### 6.1 – STUDY DESCRIPTION

This study was part of the Male AIDS cohort study (MACS) and was approved by the University of Pittsburgh Institution Review board. The cohort consisted of HIV-1 infected subjects (n=36) as well as age matched HIV-1 negative controls (NC; n=10). Specifically, the cohort consisted of treatment naïve patients (n=9) and patients on suppressive ART with CD4+ T cell counts of  $>500$  cells/mm<sup>3</sup> (ART500; n=15) and  $\leq 350$  cells/mm<sup>3</sup> (ART350; n=12). All subjects gave written informed consent and donated 20 mL of blood from which plasma and PBMCs were isolated.

### 6.2 – Correlations between Immune Activation, CD4+ Count, and Residual Viremia

The frequency of activated (i.e. CD38+/HLA-DR+) CD4+ and CD8+ T cells was significantly higher in untreated subjects and those with  $\leq 350$  cells/mm<sup>3</sup> (ART300) than in normal controls or in patients with  $>500$  cells/mm<sup>3</sup> (ART500). This indicates that in the context of antiretroviral therapy, sub-optimal recovery of the CD4+ T cell compartment correlates with high immune activation.

Interestingly, there was no correlation between residual viremia and CD4+ or CD8+ T cell activation. This suggests that residual viremia does not drive chronic immune activation.

Figure 4: Correlations between Immune Activation, CD4+ T Cell Count, and Residual Viremia

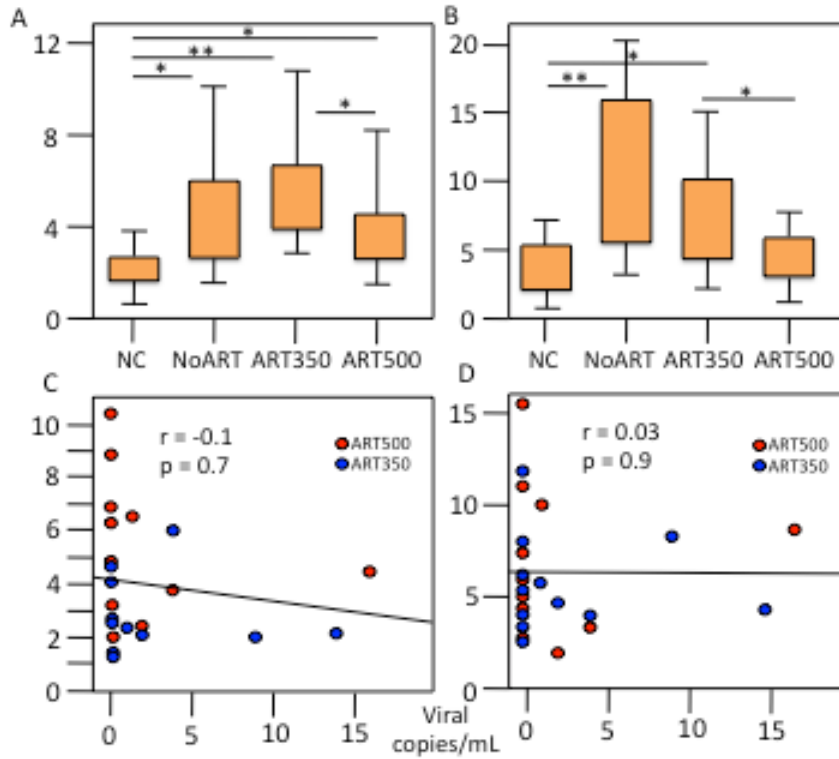


Figure 4: Correlation between CD4+ T cell count and the frequency of activated (A) CD4+ or (B) CD8+ T cells. Correlation between residual viremia and frequency of activated (C) CD4+ or (D) CD8 + T cells. (Courtesy of Bernard Macatangay)

## CHAPTER 7 – SUMMARY AND DISCUSSION

The relationship between chronic immune activation and residual viremia in the context of suppressive antiretroviral therapy is controversial. Specifically, whether chronic immune activation is *caused* by residual viremia or simply correlates with it has been difficult to investigate *in vivo*. In this report, data from two studies show that chronic inflammation is *independent* of residual viremia. In the first study, reduced immune activation mediated by MVC, possibly due to immunosuppressive activity of MVC, was not accompanied by a reduction in residual viremia upon intensification of suppressive regimens with MVC. In the second study, the frequency of immune activation was not dependent on the level of plasma viremia. Together, these data continue to support a model in which chronic immune activation is driven by factors other than residual viremia during long-term antiretroviral therapy.

Immune activation and inflammation are widely associated with disease, and the correlation between immune activation and progression to AIDS in untreated HIV infection is now widely recognized<sup>78</sup>. Although immune activation peaks during acute infection, it was observed to decrease to a steady set-point during chronic infection; this set-point also represents an independent indicator of CD4+ T cell decline and progression to AIDS<sup>76,108</sup>. Therefore during chronic, untreated infection, a positive feedback loop between immune activation and viral replication is established. There are numerous consequences to this chronic inflammation including fibrosis of secondary lymphoid organs, impaired T cell function, microbial translocation via damaged gut mucosa, and irreparable attrition of the memory T cell compartment. The most striking *in vivo* evidence supporting the causal relationship between immune activation and disease comes from primate studies of SIV<sup>109</sup>. Although SIV replicates quite well in old world monkeys, immune activation and disease progression remains low.



Upon initiation of antiretroviral therapy, HIV replication is suppressed and immune activation is reduced due to a lack of antigenic stimulation. Despite the paucity of virus in the blood, immune activation fails to normalize in many patients and remains high<sup>79</sup>. These patients suffer from many of the same ailments seen in untreated infection and are at increased risks of non-AIDS defining illnesses such as cardiovascular, liver, kidney, and neurological diseases, as well as cancer. The reasons for chronic immune activation are unclear, but may include (1) antigenic stimulation by residual viremia, (2) microbial translocation through damaged gut mucosa<sup>110</sup>, and (3) incomplete reconstitution of the CD4+ T cell compartment. Therefore, it is critical to determine the causes of chronic immune activation in order to better control inflammation-associated diseases.

In the A5256 study, the role of antigenic stimulation in response to residual viremia was addressed. To date, no study has conclusively demonstrated that intensification of existing suppressive ART regimens further reduces residual viremia. These include studies that have examined NNRTIs<sup>101</sup>, PIs<sup>101</sup>, integrase inhibitors<sup>89,102,103</sup>, and MVC<sup>106,107</sup>. Our data is in agreement with these reports in that we found no durable reduction in residual viremia. Interestingly, immune activation was reduced during intensification and was partially reversed when MVC administration ceased. These results are consistent with several studies that have reported MVC-induced reductions in immune activation. A study by Funderburg et al. (n=57) compared the effects of MVC and efavirenz on markers of immune activation in treatment naïve patients<sup>111</sup>; the levels of CD4+CD38+ cells, D-dimer, and CRP in peripheral blood were all found to decrease during MVC intensification. Similarly, a report by Vitrello et al. (n=20) demonstrated MVC-dependent reduction in soluble CD30 (a marker of T cell activation) in patients on suppressive ART<sup>112</sup>. Lastly, a study by Wilkin et al. (n=34) showed the frequencies of CD4+ T cells expressing markers of immune activation (CD38+ and CD38+/HLA-DR+) and proliferation (Ki67+) decreased during MVC intensification, and increased following cessation of MVC<sup>107</sup>. These studies (and ours) differ from a report by Hunt et al. (n=45), who described an increase in CD38+HLA-DR+

in peripheral CD8+ T cells after 24 weeks of MVC intensification<sup>113</sup>; moreover, this study found the frequencies of CD4+ and CD8+ T cells expressing CD38 and HLA-DR nearly doubled in rectal tissue. Interestingly, this study also reported a decrease in soluble LPS, but an increase in soluble CD14. The reasons for the disparities between these results are unclear, but may be related to unrecognized difference in patient cohorts or specific methods used to quantify fluctuations in markers of immune activation.

Although residual viremia did not correlate with immune activation, analysis of baseline data revealed an inverse correlation between CD4+ T cell activation and total HIV-1 DNA in the CD4+ T cell compartment ( $\rho = -0.52$ ;  $p = 0.004$ ). In contrast, a recent study by Hatano et al. on a large cohort of treated subjects ( $n = 109$ ) found a modest *positive* correlation between CD4+ T cell activation and proviral HIV-1 DNA ( $\rho = 0.16$ ;  $p = 0.057$ )<sup>114</sup>. This is also true of an earlier study investigating correlations between CD8+ T cell activation and viral “blips.” In theory, elevated CD4+ T cell activation may drive HIV expression by fostering an environment conducive to intracellular HIV-1 RNA expression. This could result in cell death, either through cytopathic effects of viral proteins or through cytotoxic responses by innate and adaptive immune cells. Since this process would lead to attrition of proviruses capable of expressing HIV-1 RNA, it would be reflected as relatively low levels of total HIV DNA in the CD4+ T cell compartment.

In addition to the role of residual viremia driving immune activation, the relationship between CD4+ T cell count and immune activation was also examined. Untreated patients and those on ART with sub-optimal T cell recovery (ART350) exhibited high levels of immune activation compared to patients with appreciable T cell recovery (ART500) and healthy controls. Since CD4+ T cells are critical to coordinating the adaptive immune response against HIV, low T cell counts compromise the ability of the adaptive immune system to respond to infections. Although residual viremia does not appear influence immune activation, other antigenic stimulation from microbial translocation, co-infections (hepatitis B and C), and pathogens that are typically well controlled in healthy individuals (e.g. CMV & EBV) are potential causes. Further studies

utilizing techniques to perturb these different parameters while monitoring changes in immune activation are required to fully elucidate the factors that contribute to chronic immune activation.

Although informative, these studies have several limitations that must be acknowledged. First, the MVC study was a single-arm trial without a control group, so small effects on residual viremia as well as variables other than MVC intensification cannot be addressed. Second, only one primer/probe set specific for a conserved region of the HIV-1 genome was used to assay residual viremia. At most, 48% of patients had detectable residual viremia, and it is possible that primer/probe mismatches resulted in false negatives. For both studies, pre-therapy samples were not available, so no plasma remains to test this hypothesis. Finally, these studies did not consider ongoing replication in anatomical sites not reflected in the blood, such as the gastrointestinal tract, genital tract, and central nervous system. It is conceivable that limited, ongoing replication occurs in these sites and helps drive chronic immune activation

Despite the limitations, these data contribute to our knowledge of chronic immune activation and its relationship to residual viremia and CD4+ T cell count. The absence of changes to residual viremia during MVC intensification strongly supports existing evidence that (1) residual viremia does not originate from ongoing cycles of HIV-1 replication, and (2) residual viremia does not drive immune activation. This is further supported by the lack of correlation between immune activation and residual viremia in the second study. Although MVC may have utility in treating individuals with drug resistance mutations against first-line therapies, it may be useful in blunting immune activation in patients on suppressive ART, as well as in other maladies driven by immune activation; indeed, MVC has been shown to have utility in the context of graft versus host disease<sup>115</sup>. Our study attempted to help discern the relationship between residual viremia and immune activation. We found no relationship between these parameters, and the concept that immune activation drives residual viremia was not supported.

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