CHARACTERIZATION OF INDUCIBLE RESERVOIRS OF HIV-1

by

Anthony Richard Cillo

B.S. in Cell Biology/Biochemistry, Bucknell University, 2010

Submitted to the Graduate Faculty of

the School of Medicine in partial fulfillment

of the requirements for the degree of

Ph.D. in Molecular Virology and Microbiology

University of Pittsburgh

2016

UNIVERSITY OF PITTSBURGH

SCHOOL OF MEDICINE

This dissertation was presented

by

Anthony Richard Cillo

It was defended on

March 31, 2016

and approved by

John M. Coffin, Ph.D., Professor

Paula J. Grabowski, Ph.D., Professor

Ronald C. Montelaro, Ph.D., Professor

Todd A. Reinhart, Sc.D., Professor

Dissertation Advisor: John W. Mellors, M.D., Professor

Characterization of inducible reservoirs of HIV-1

Anthony Richard Cillo, Ph.D.

University of Pittsburgh, 2016

Combination antiretroviral therapy for HIV-1 suppresses viral replication, but is not curative. HIV-1 remains detectable in those on suppressive therapy as cellular proviral DNA and cellular HIV-1 RNA in peripheral blood mononuclear cells (PBMC), and as low-level plasma viremia. A long-lived reservoir of intact proviruses is also retained in the resting CD4+ T cell population. This population of long-lived, latently infected cells is believed to cause life-long persistence of HIV-1. To deplete latently infected cells, a "kick and kill" strategy has been proposed, in which HIV-1 is "kicked" out of proviral latency by small molecule latency reversing agents, leading to the "kill" from viral cytopathic effects or immune mediated clearance. Recent *ex vivo* and *in vivo* studies have concluded that histone deacetylase inhibitors, protein kinase C agonists and Brd4 inhibitors can effect HIV-1 latency reversal. We evaluated this proposed strategy at the level of individual proviruses using a limiting dilution assay, and found that currently used latency reversal agents are not potent activators of HIV-1 latency compared with T cell activation. These latency reversal agents also do not lead to significant depletions of the HIV-1 reservoir in resting CD4+ T cells. We also sought to better understand relationships between molecular biomarkers of HIV-1 persistence and the size of the inducible reservoir. We found that as the frequency of infected cells and their transcriptional activity in PBMC increases, the size of the inducible reservoir also increases. Spontaneous virion production from resting CD4+ T cells and PBMC was also associated with low-level viremia. We also found that the magnitude of the inducible reservoir, and the levels of infectious virus, were higher in total CD4+ T cells compared with

resting CD4+ T cells. Finally, we also found that the size of the inducible reservoir was significantly correlated with the magnitude of viral outgrowth from total and resting CD4+ T cells. Overall, these findings demonstrate that more potent latency reversing agents will be required to reduce the size of the latent reservoir, and suggest that measures of the infectious and inducible reservoirs in those on long-term antiretroviral therapy can be greatly simplified.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PREFACE

First and foremost, I must express my gratitude to my Ph.D. advisor, Dr. John Mellors. Throughout my time as a graduate student, Dr. Mellors has been an inspirational leader, with an unparalleled ability to think and communicate clearly, and possessing a great depth of insight. Dr. Mellors has greatly facilitated not only my understanding of HIV/AIDS, but also has fostered my development as a scientist and a critical thinker. Working with Dr. Mellors, I had the opportunity to present research findings at prestigious national and international conferences, and to be an author or coauthor on 11 publications to date. I am grateful for these opportunities.

I also owe Dr. Todd Reinhart a debt of gratitude for introducing me to research science. Dr. Reinhart is a consummate educator, and provided me with the opportunity to learn how to perform quantitative real-time polymerase chain reactions (an indispensible laboratory technique) in his laboratory the summer before beginning my undergraduate course work at Bucknell University. The opportunity provided by Dr. Reinhart doubtlessly fostered my interest in science at a critical point in my educational career. As a member of my committee, Dr. Reinhart has continued to enhance my development as a professional, and for that I am grateful.

I owe a heartfelt thanks to my committee members for their dedication to helping guide my development as a scientist, and for ensuring that I do not bite off more than I can chew. Their insight at critical junctures was much appreciated, and essential to my timely completion of my doctoral studies.

I am also grateful to Lisa Gundel, who initially recommended me to Dr. Mellors as a potential Research Assistant immediately after I finished my undergraduate coursework. This gesture from Ms. Gundel put me on a path towards graduate school, and I am thankful she connected me to full-time research after my undergraduate studies.

I am immensely thankful for my wonderful coworkers. I can honestly say that I have enjoyed every moment of working in cell culture with Michele Sobolewski, Taylor Simmons and Joshua Cyktor. There have been many long days in the lab, but the great company made the work enjoyable. Elizabeth Fyne also provided critical insight in both cell culture and literary endeavors. It has been a great experience working with all the members of the single copy assay (SCA) team, past (Annie Bedison, Guillaume Besson, Francis Hong) and present (Dianna Koontz, Melissa Tosciano, Mattie Follen), and for philosophical debates with Dr. Hong and Dr. Besson. The hard-working sequencing gurus in the lab (Elias Halvas, fellow student John Bui, Kevin Joseph) have also provided great discussions and insight. Finally, my friend and classmate Jennifer Zerbato has been with me the entire way, and I'm thankful for her ability to make me laugh while simultaneously having a serious discussion about molecular biology.

Finally, I am deeply and profoundly grateful for unwavering encouragement from my fantastic parents, Anthony and Donna Cillo, and my wonderful sisters Amanda and Audrey Cillo. My family and lifelong friends have shaped me into the person I am today, and I would not be here without them.

1.0 INTRODUCTION

The Acquired Immunodeficiency Syndrome (AIDS) epidemic was first brought to the attention of the world by isolated case reports of *Pneumocystis carinii* pneumonia and Kaposi's sarcoma in previously healthy homosexual men without an underlying cause of immunodeficiency[1-3]. Even in these initial reports, there was speculation that a new disease transmitted by sexual contact was responsible for the severe immunodeficiency evident in these index cases. Following the discovery of a severe communicable disease among individuals who had been healthy, the race was on to find the underlying etiologic agent responsible for this disease.

The first report describing a retrovirus associated with AIDS from Barre-Sinousi et al. at the Pasteur Institute was published in 1983 in *Science*[4]. This report was followed in 1984 by four manuscripts from Robert Gallo's laboratory at the National Cancer Institute, also published in *Science*, describing a retrovirus related to human T cell leukemia virus as the causative agent of AIDS, and describing antibodies elicited by this retrovirus in the sera of patients[5-8]. The two groups independently named the virus, with the French group settling on lymphadenopathy associated virus (LAV), and the group from the United States choosing human T cell lymphotropic virus (HTLV-III). These groups and others eventually came to a consensus, calling the etiologic agent of AIDS the Human Immunodeficiency Virus (HIV; this dissertation will focus on HIV type 1, and will therefore refer to HIV as HIV-1 throughout).

1.1 STATE OF ART: A BRIEF HISTORY OF ANTIRETROVIRAL THERAPY

1.1.1 Importance of the development of azidothymidine

After the discovery of a human retrovirus as the causative agent of AIDS, the development of effective therapy to prevent disease progression became a top priority. With retroviruses now garnering the attention of the biomedical research community, groups began to apply extant knowledge of the retroviral lifecycle[9] to investigate viral drug targets. The discovery of an RNA-dependent RNA-polymerase in Rous sarcoma virus by Temin and Mizutani[10] and in murine leukemia virus by Baltimore[11], both in 1970, paved the way for development of antiretroviral therapy against a human retrovirus more than 13 years before HIV-1 was discovered. This example demonstrates the importance of basic research; without an understanding of the retroviral lifecycle, it would not have been possible to detect and identify a retrovirus as the causative agents of AIDS, or to develop antiretroviral drugs. Without the foundation of basic research, many more lives would have been lost to HIV/AIDS.

The development of the first antiretroviral drug, azidothymidine (AZT), a thymidine nucleoside analog was a turning point in the treatment of HIV-1 infection. Like most revolutionary breakthroughs, the discovery that AZT could inhibit viral replication is a rich story, rife with conflict as to who truly made the pivotal discovery. AZT was first synthesized in 1964[12-14] to be tested for the treatment of cancer. However, it was not until years later that AZT was resurrected for investigation as a potential antiretroviral. This resurrection was the result of a number of key players in the pharmaceutical industry and government research positions. Scientists at Burroughs-Wellcome had evidence that one of their drug candidates (AZT)

inhibited replication of MLV *in vitro,* but they lacked an *in vitro* model of HIV-1 infection to test AZT[15]. However, Burroughs-Wellcome was aware that the NCI had recently established an *in vitro* model of HIV-1 replication in the laboratory of Samuel Broder[16,17]. Burroughs-Wellcome then approached scientists at the NCI about testing their potentially promising drug candidate AZT in their *in vitro* system. The results of this collaboration was that in 1985, Mitsuya et al. from Samuel Broder's lab published a seminal manuscript describing the *in vitro* efficacy of AZT in preventing viral replication[18]. These results were followed up with *in vitro* and *ex vivo* confirmatory studies in subsequent years[19,20], demonstrating a surprising efficacy and therapeutic index, considering many at the time did not believe that any therapy would be safe and effective. However, debate would go on for years as to whether the NCI or Burroughs-Wellcome actually discovered the efficacy of AZT and who deserved the patent rights[21].

In parallel with the further investigation of AZT *in vitro,* researchers at the NCI began to take steps into evaluating the clinical efficacy of AZT in patients with advanced AIDS. These pioneering researchers were following the lead of their oncology counterparts at the NCI, and were going from laboratory benches to patient bedsides to investigate whether the findings from the laboratory were clinically relevant. In less than a year after the first publication of AZT as an inhibitor of HIV-1 replication *in vitro,* exploratory clinical results were published, demonstrating that AZT lead to increases in CD4+ T cell counts and restoration of hypersensitivity reactions mediated by CD4+ T cells[22], providing evidence of clinical benefits. Follow-up trials came quickly, and expanded into other potential nucleoside drug candidates, with promising clinical responses at several institutions[23-26].

These first trials laid the framework for phase II placebo controlled trials to evaluate the true efficacy and toxicity of AZT in HIV-infected people. Burroughs-Wellcome sponsored the

multicenter phase II, double-blind placebo controlled trial of AZT in 248 people, which was the first study of its kind. The data safety monitoring board for this trial decided to end the trial early, after clear clinical benefit was observed in the AZT treated arm of the trial[12,27]. This first placebo controlled trial was a major milestone in treatment of HIV-1 infection, as it showed conclusively using clinical endpoints that AZT treatment conferred a significant survival advantage. In one month following the early cessation of the phase II AZT trial for efficacy, a treatment investigational new drug designation was granted for AZT, allowing physicians to prescribed AZT to patients. Finally, in March 1987, the Food and Drug Administration (FDA) granted approval for a New Drug Application (NDA) for AZT. This approval from the FDA led to a federally sanctioned drug to inhibit HIV-1 replication in approximately 2 years from the first publication describing its activity[12].

The placebo-controlled design provided the groundwork for future trials investigating new compounds to suppress viral replication. Other pharmaceutical sponsors of new clinical candidates to suppress replication of HIV-1 were not required to have placebo controls in their early-phase proof of concept trials, due in part to the leadership and forward-thinking of Burroughs-Wellcome. Didanosine (ddI) and dideoxycytidine (ddC) were the next drugs to be approved in October of 1991 and June of 1992, respectively[12]. Thus, the path used to develop AZT as a clinical drug involved collaboration between government agencies and pharmaceutical companies, and the use of a rapid bench to bedside model adopted from oncology trials at the NCI. The FDA also demonstrated leadership by working diligently to translate the clinical findings from Burroughs-Wellcome and the NCI to patients in need of treatment. This eventually led to the new Treatment Investigational New Drug category of regulation in the FDA, wherein drugs can be prescribed by physicians prior to full approval in cases of advanced disease and

where no other treatment options are available[28]. The development of AZT from an effective drug candidate *in vitro* to an FDA-approved drug was a testament to the clear thinking at all stages of scientific development, and a willingness of groups with sometimes divisive interests to work together to achieve something that none could achieve alone. The model for the development of AZT illuminated a pathway forward further drug candidates for the treatment of HIV-1, and it is clear that we would not have the suppressive antiretroviral therapy regimens we have today without the development of AZT.

1.1.2 Development of combination antiretroviral therapy

The development of AZT as the first treatment for HIV-1 infection was pivotal in the HIV/AIDS epidemic, but early enthusiasm from the clinical benefit was quickly dashed. In the first placebo-controlled study of AZT, it was already clear that the clinical benefit was dwindling towards the end of the 24-week trial. The increases in CD4+ T cell counts and recovery of responsiveness to hypersensitivity tests that were associated with the clinical benefit had already begun to reverse in some study participants by the end of the relatively short trial[27]. Indeed, this was an early harbinger of things to come.

Soon after AZT was used broadly to treat HIV-1, it became clear that the responsiveness to therapy waned over time, with AZT only providing approximately three additional years of life after diagnosis of AIDS[29-31]. It was also becoming clear that AZT was associated with significant toxicity[32], and that the virus could evolve resistance to AZT over time[33,34]. Careful analysis demonstrated that the resistance of HIV-1 to AZT therapy was the direct result of specific mutations in the reverse-transcriptase portion of the HIV-1 genome[35,36]. These findings

would have major repercussions, as the study of drug resistance to HIV-1 would come to dominate HIV research for the next two decades.

Appreciation of the limited ability of AZT to suppress viral replication due to the emergence of drug resistance enhanced the race to find new and more potent suppressors of HIV-1 replication. The development of new nucleoside reverse-transcriptase inhibitors (NRTIs) such as ddI[37,38], d4T[39], and ddC[40] were additional tools for the treatment of HIV-1. Combinations of AZT and these newer NRTIs were compared in clinical trials, with combinations performing slightly better than AZT alone[41,42]. A subsequent large trial involving 2,467 participants showed that monotherapy with AZT was inferior to ddI alone, to AZT and ddI, and to AZT and zalcitabine[43]. In the most successful arm of this trial (AZT and ddI), 20% of the participant reached a 50% decline in CD4+ T cell counts, AIDS or death on therapy, with 31 of 613 participants in this arm dying during the 160 weeks of the trial. It was clear that additional classes of compounds would be needed to prolong the suppression of viral replication.

The sobering results of the large-scale clinical trial investigating combinations of other NRTIs with AZT drove the point home that combination therapy with new drugs classes and NRTIs would be required to achieve prolonger viral suppression. Two years after the approval of AZT as the first therapy against HIV-1, the structure of HIV-1 protease[44] was solved, and rational design of inhibitors of HIV-1 protease had begun[45]. Subsequent medicinal chemistry yielded the protease inhibitors Ro-31-8959[46], L-735,524[47] and ABT-358[48], demonstrating sub-micromolar potency for inhibition of HIV-1 protease and favorable therapeutic indices (>1000-fold difference between effective dose and toxic dose[46]) *in vitro,* and minimal toxicity in animals models[48]. In a small scale studies of 49 patients[49], Ro-31-8959 (saquinavir) alone was found to transiently reduce viral load by 80% after eight weeks of therapy, and to transiently increase

CD4+ T cell counts by 60 cells/mm^3 after four weeks. The FDA considered these preliminary results by Kitchen et al.[49] to be sufficient evidence for rapid licensure. During this same time period, both L-735,524/MK-639 (indinavir) and ABT-358 (ritonavir) were undergoing small-scale trials for safety and efficacy, and both were also found to transiently restore CD4+ T cell counts and reduce viral load[50-52]. However, the point that therapies needed to be combined was once again driven home by the emergence of well-defined genetic mutations in the protease region of the HIV-1 genome conferring resistance to protease inhibitors[53-55].

In parallel to the development of protease inhibitors and soon after the development of d4T, came another NRTI known as 3TC with highly potent *in vitro* activity against a range of isolates of HIV-1[56-58]. Although the mutation M184V was shown to be selected by 3TC early on, viruses with this mutation remained susceptible to AZT therapy, and conversely viruses with AZT resistance remained sensitive to 3TC[59-61]. Based on this preliminary *in vitro* data and two early phase clinical studies demonstrating safety and bioavailablity[62,63], it seemed that the combination of AZT and 3TC could potently inhibit HIV-1 replication *in vivo*. Two seminal studies published in 1995 and 1996 showed that this was indeed the case, as participants who received AZT and 3TC did profoundly better than those that received AZT or 3TC alone[64,65]. Importantly, these positive results persisted after 52 weeks of therapy, with CD4+ T cell counts a mean of 60 cell/mm^3 above baseline and mean viral loads a log lower compared with baseline[65]. It seemed that the combination of AZT and 3TC was indicating that researchers and physicians were on the right path to finding a regimen that fully suppressed HIV-1 replication.

The first true test of combination of therapies targeting different aspects of viral replication came from a trial investigating the combination of AZT, ddC, and saquinavir, likely due the fact that these drugs were available prior to the FDA's approval of 3TC and indinavir. It

was unknown at the time that the clinical efficacy of AZT and 3TC would be much superior to AZT and ddC, and *in vitro* studies suggested that the three drug combination of AZT, ddC and saquinavir would be highly synergistic against HIV-1 replication[66-68]. Unfortunately, the encouraging *in vitro* results did not translate to the clinic. Results from the trial of this three drug combination showed that HIV-1 viral loads dropped nearly a $\log_{10}$ after four weeks of therapy, but quickly rebounded to approximately a 0.5 $\log_{10}$ decrease after 16-24 weeks of therapy and remained stably there[69]. CD4+ T cell counts mirrored plasma HIV-1 RNA levels, and by 24 weeks had dropped to only a 10% increase; by week 48, CD4+ T cell count had returned to baseline[69].

About one year following the publication of the results from the combination of AZT, ddC, and saquinavir, results from two trials that combined AZT, 3TC and the newer protease inhibitor indinavir were published[70,71]. The results of these trial were much more positive. In the study by Gulick et al., combination therapy of AZT, 3TC and indinavir dropped viral load by two $\log_{10}$ within eight weeks, and led to a 100 cell/mm^3 increase in CD4+ T cell counts in the same time period[71]. Strikingly, plasma viremia stayed suppressed, with over 80% of study participants on the triple combination having viral loads <500 copies per milliliter of plasma by 24 weeks[71]. Even more amazingly, after 52 weeks of therapy 100% of study participants on the triple drug combination had viral loads <500 copies per milliliter, and 80% had viral loads <50 copies per milliliter. CD4+ T cell counts also continued to rise during this period, reaching a mean increase of 200 cell/mm^3 after 52 weeks of therapy[71]. The trial by Hammer et al. was focused more on clinical outcomes (i.e. AIDS or death), as the cohort studied in this trial were participants with CD4+ T counts <200 cells/mm^3. Focusing on clinical endpoints, this study also had positive results, in that participants were much less likely to have disease progression when

treated with the three drug regimen versus two NRTIs. The results of these two trials clearly demonstrate that combinations of antiretroviral drugs specific to more than one viral target led to the prolonged suppression of plasma viremia, increases in CD4+ T cell counts, and profound clinical benefit.

The development of triple drug combination antiretroviral therapy regimens was a triumph of modern science. Within a little over a decade after the initial discovery of a compound that could inhibit HIV-1 replication *in vitro*, multiple classes of drug compounds targeting reverse transcriptase and protease had gone through inception to testing for efficacy in clinical trials. The ultimate outcome was that HIV-1 went from being an untreatable (and some thought impossible to safely treat) disease, to one in which there was hope for long term viral suppression. It was undoubtedly the combination of a strong foundation of the basic science necessary to understand reverse transcriptase, protease, and viral replication in conjunction with close work between different groups that facilitated the rapid progress in the treatment of HIV-1. The HIV/AIDS epidemic brought together government clinicians working at the NCI with a mind towards bench to bedside approaches to treatment, pharmaceutical companies who had promising drug candidates, the FDA, and patient advocacy groups. The cooperation of these groups at an early stage contributed substantially towards the rapid progress that was achieved. It is prudent to bear this model in mind when working towards breakthrough therapeutics.

1.1.3 Modern ART Regimens and the Status of the War on HIV/AIDS

At the time when the results of the first triple drug combination therapies were being published, much work was concurrently being done on the development of new classes of reverse

transcriptase inhibitors, the non-nucleoside reverse transcriptase inhibitors (NNRTIs). The first NNRTI to be developed was nevirapine[72-75], which was less effective alone or in combination with AZT or AZT and ddI in maintaining suppression of viral replication than three drug regimens with indinavir, 3TC and AZT[70,71]. The next NNRTI developed was efavirenz, which showed improved ability to suppress plasma HIV-1 RNA when paired with AZT and 3TC compared with indinavir, AZT and 3TC, plus was associated with fewer side effects[76]. Efavirenz was also shown to be superior to triple therapy with NRTIs alone[77]. Correspondingly, efavirenz is still a backbone of many regimens used today, and is typically prescribed with the NRTIs tenofovir disoproxil fumarate and 2',3'-dideoxy-5-fluoro-3'-thiacytidine (FTC)[78]. Indeed, efavirenz, tenofovir and FTC are currently available in a one pill, once a day dosing option (known as Atripla®) that was superior to other cornerstone options including boosted protease inhibitors and protease inhibitors plus efavirenz[79].

Other classes of drugs, such as entry inhibitors[80] and integrase inhibitors[81,82] are also constituents of modern ART regimens. Although newer drugs are becoming available, it is worth noting that no combination of drugs has been shown to be virologically superior to efavirenz, tenofovir and emtricitabine. However, the Department of Health and Human Services has moved towards 5 basic regimens, 4 of which include integrase inhibitors plus NRTIs and one of which includes a boosted protease inhibitor plus NRTIs[83]. This movement away from NNRTI based regimens has principally been due to the side effects from efavirenz-based regimens, notably psychological side effects[84]. It is worth noting that efavirenz containing regimens are still recommended as alternative regimens, and that the World Health Organization still recommends efavirenz plus NRTIs as first-line therapy[85].

Despite the overwhelming success that has been achieved and the lives that have been saved by combination antiretroviral therapy, HIV-1 still remains a public health problem in the United States and worldwide. There are still major barriers to overcome to win the battle against HIV-1. Low frequency resistance mutations can lead to subsequent virologic failure on ART[86], demonstrating that resistance still remains a major barrier to long term viral suppression. Modern ART regimens have downsides, including significant long-term toxicity[87-92]. Although many people receiving ART suppress their viral loads, the immune system fails to reconstitute in some individuals[93,94], leading to persistently low CD4+ T cell counts. Though highly effective, ART regimens remain costly in practice; a survey of generic prices reveals that the annual cost of Atripla® is approximately $31,000 based on wholesale prices in the United States, which is obviously cost prohibitive in many contexts[83]. Regardless of these shortcomings, antiretroviral therapy has been responsible for saving over three millon life-years in the United States alone from 1989 to 2006[95].

It is important to remember that as of 2013, nearly 78 million people have acquired HIV-1 since the beginning of the epidemic, and 39 million people have died from AIDS[96]. As of 2014, the World Health Organization estimates that there are nearly 37 million people living with HIV-1 today, with 2 million new HIV-1 infections in 2014 and 1.2 million AIDS deaths[97]. The HIV/AIDS epidemic is far from over, and the most expedient path towards an end to the epidemic has two components: one component in which HIV-1 transmission is prevented by either vaccination, pre-exposure prophylaxis or ART of infected individuals, and another in which those living with HIV-1 infection are cured.

1.2 PERSISTENCE OF HIV-1 AFTER LONG TERM SUPPRESSIVE ART

Combination antiretroviral therapy (ART) has progressed to the point where those who are adherent and do not have resistance mutations, have undetectable levels of HIV-1 RNA in plasma and high CD4+ T cell counts. As long as these individuals continue to take suppressive ART regimens, the virus and the body are in a *status quo*. The virus cannot replicate any further, and the host cannot further reduce the number of latently infected cells in the body. The apparently peaceful *status quo* observed when ART is regularly taken belies the true state of viral persistence.

1.2.1 Viral load: measure of effective therapy becomes measure of persistence

The first assays capable of assessing whether HIV-1 was present in the body came soon after the discovery of HIV-1 as the etiological agent of AIDS. These ELISA-based tested were developed by Robert Gallo's group, and were the first serologic tests capable of identifying those with HIV-1[5,6]. Additional tests were soon available, as well as measures of p24 antigenemia in the plasma as a quantitative marker of virus levels[98,99]. Another early measure of disease progression prior to the development of ART was measures of cellular HIV-1 RNA in peripheral blood mononuclear cells[100], which had moderate predictive power to determine those at risk for early disease progression. One measure that was readily performed, and that was assumed to be the best possible predictor of disease progression, was the quantification of the number and percent of CD4+ T cells in blood[101].

Although the polymerase chain reaction was first described in the mid-1980s[102,103], it had not yet come to the forefront of molecular diagnostics. Manuscripts published back to back in *Nature* in 1995 by Ho et al.[104] and Wei et al.[105] utilized PCR to achieve two distinctly novel results. The first novel finding was that although patients were in a state of clinical latency, it became clear that there was actually a highly dynamic process of viral infection and turnover of infected cells occurring. The second novel result that was achieved by using PCR was the detection of viral variants at the nucleotide level by sequence analysis. Longitudinal sequence analysis showed that nearly complete turnover of viral species occurred in 14 days. Taken together, these two manuscripts shed significant light on the viral dynamics of HIV-1 infection using PCR. These results led to a subsequent manuscript by Perelson et al., where viral dynamics were inferred from the clearance rates observed after administration of ritonavir, and led to the finding that the lifespan of the infected cell is on average 1.2 days, with a generation time of virion infecting one cell, to new virions infecting a second cells of 2.6 days[106].

These results contributed to the time being ripe for the development of a new prognostic marker of progression to AIDS. Recent advances in PCR, coupled with new knowledge about the highly dynamic nature of HIV-1 infection during clinical latency, led to the hypothesis that the number of infected cells in the body would be reflected by the amount of virus in plasma. A small preliminary study had suggested that lower levels of viral RNA in serum was associated with a longer time to disease progression[107]. The hypothesis that HIV-1 RNA levels in plasma could predict the time to disease progression was then tested in two clinical studies. These studies demonstrated that plasma HIV-1 RNA levels were weakly correlated with the CD4+ T cell counts, and that HIV-1 RNA in plasma was a much better predictor of the time to AIDS or death[108,109]. The finding that measures of cell-free viral RNA in plasma were strongly related to

the time to disease progression was striking, with numerous immediate clinical applications. The clearest and most profound clinical application was the *in vivo* evaluation of the efficacy of any given therapy at reducing plasma viral load. Instead of waiting for clinical endpoints such as progression to AIDS or death, investigators could instead rely on plasma viral loads as indicative of the efficacy of a given therapy. The well-documented power and relative ease of viral load testing cemented its status as a predictor of clinical efficacy, and as such it is widely used today to monitor therapy.

After the discovery of fully suppressive ART regimens, plasma viremia was also used to infer the lifespans of infected cell populations. The rationale was that following initiation of fully suppressive ART, all virion production had to be derived from cellular sources that were already infected. Falling levels of virus in the plasma, therefore, would represent the death of the virus producing cells. Using this logic, Perelson et al. found that there were two phases of viral decay, one that was extremely rapid, and a second, longer and slower decay phase[110]. The authors then speculated that extrapolation of this decay would lead to the complete clearance of virus in as few as 3 years. This inference, based on extrapolated data from 1997, is obviously incorrect based on today's knowledge of the latent reservoir, as described below.

One thing that was clear from the initial study of the decay of plasma viremia was that patients on therapy were reaching the limit of detection of clinically available assays, usually within 6 to 12 months. In order to more faithfully assess the decay kinetics of plasma viremia, it would be necessary to develop a new, much more highly sensitive assay. Preliminary attempts were made to lower the limit of detection of viral load assays[111], but a research group at the National Cancer Institute would ultimately develop and validate what would come to be known as the single copy assay (SCA)[112]. The first SCA assay was capable of detecting virus down to

one copy per milliliter of plasma by incorporating ultracentrifugation of up to 7 milliliters of plasma. This assay could indeed readily detect plasma viremia at low levels, and was extensively validated with panels of control samples with known quantities of plasma virus.

SCA was first used to assess whether there were differences in the low-level viremia setpoint after years on different ART regimens. This study revealed that there was no difference between classes of fully suppressive ART regimens and the amount of virus in plasma after long-term suppressive therapy[113]. These findings suggested that, regardless of ART regimen, all people on fully suppressive regimens have approximately the same magnitude of viral persistence in plasma. These findings were followed up shortly thereafter with evidence that low-level viremia in plasma hits a viral set point of approximately 3 copies per milliliter of plasma, and stays there for at least 7 years[114]. This study also found that there were three quantifiable phases of viral decay, with a 4th phase with an infinite half-life. This was largely accepted as evidence that there was no appreciable decay in amount of virus in plasma over time, until a recently published manuscript. Riddler et al. recently found, in a longitudinal cohort of 64 participants, that plasma HIV-1 RNA levels continue to decline with an average rate of loss of 6% and an estimated half-life of 11.5 years[115]. Notably, this study evaluated participants in years 4-12 of suppressive therapy, and therefore had a longer period of time to assess decay than the previous study by Palmer et al.

In summary, studies of plasma viremia in people on long-term suppressive ART have consistently shown several phases of viral decay, followed by a period in which the virus in plasma decays extremely slowly. These studies suggested that the virus produced in plasma during long-term suppressive therapy is likely from cells that were infected prior to the initiation

of therapy, and are either long-lived or are periodically exiting from a latent reservoir and producing virus and dying, or perhaps both.

What is abundantly clear is that once suppressive ART is stopped, plasma HIV-1 RNA levels quickly rebound within days to weeks to levels at or near pre-therapy viral loads, leading to depletion of CD4+ T cells and increased risk of transmission of HIV-1 to others[116-121]. The results from these treatment interruption studies begins to bring to light the fact that, although plasma HIV-1 RNA is suppressed to levels that require large volumes of plasma and highly specialized assays to quantify this low-level viral load, there is still a large reservoir looming under the surface.

Viral load measures for HIV-1 have been useful since PCR technology was brought into the realm of diagnostic molecular biology. At the beginning of the epidemic, viral load was used to assess the rapid turnover of infected cells during clinical latency. Viral load measures guided the assessment of the clinical efficacy of antiretroviral therapy at the turning point of the epidemic, and viral load measures are still being used to assess the amount of virus in patients on long term suppressive ART as well as the time to viral rebound following cessation of ART. Doubtlessly, measures of HIV-1 RNA will remain useful as long as the HIV-1 epidemic continues.

1.2.2 A reservoir of HIV in resting CD4+ T cells

Clinical evidence of persistent low-level viremia in HIV-positive persons on long-term suppressive ART, in conjunction with rapid viral rebound following cessation of ART, is

consistent with the idea of a latent reservoir of HIV-1. While clinical development was correctly focused on identifying markers of viral replication in plasma to develop drugs to extend the lifespans of those living with HIV-1, others were working on elucidating further biological details about the latent nature HIV-1 infection. The outright focus on development of suppressive ART was more immediately important to prolong the lives of those infected with HIV-1, especially given the rapid turnover of infected cells and virions in plasma, compared with elucidating whether there was a latent reservoir of infected cells.

Nevertheless, groups in the mid-1990s were seeking ways to quantify the amount of infectious virus within CD4+ T cells. Investigation of the levels of infectious virus in plasma and cells from people living with HIV-1 has roots in a 1989 manuscript by Ho et al.[99], in which a limiting dilution culture assay with activated cells from HIV-negative donors were cultured with PBMC from HIV-positive people either not receiving therapy or receiving AZT monotherapy. The endpoint of the assay was assessment of p24 by ELISA in cell culture supernatants with twice weekly sampling over a 28-day culture, and inferring the amount of infectious virus present by endpoint assessments. This crude version of a viral outgrowth assay indicated that at least 1 in 400 mononuclear cells were infected with HIV-1 in the periphery, and that the levels of infectious virus in plasma decreased 25-fold following 4 weeks of therapy with AZT, but did not change in mononuclear cells. This assay format was the forerunner of more complicated assays of viral outgrowth to come.

Prior to the use of a viral outgrowth assay, it was first necessary to identify a population of cells that could harbor HIV-1 for the life of the patient. Work by Chun et al. in 1995 isolated resting CD4+ T cells from patients, and quantified integrated HIV-1 DNA in this cell population. While they found integrated HIV-1 DNA in resting CD4+ T cells, they also found that it did not

accumulate over time, drawing the conclusion that the majority of actively infected CD4+ T cells do not convert to infected resting memory CD4+ T cells[122]. This study was the first to identify directly the infection of resting CD4+ T cells.

During the time when Perelson and Ho's modeling suggested that HIV-1 may be eradicated in 3 years with fully suppressive antiretroviral therapy, others were investigating whether there was a latent reservoir that could prevent the eradication of virus from patients with ART alone. The first study to demonstrate replication competence of virus in resting CD4+ T cell was by Chun et al. in 1997, in which they found that overall there was relatively infrequent infection of resting CD4+ T cells during infection[123]. The authors of this study also used samples from both blood and tissues to estimate that the size of the total body reservoir was less than 10^7 cells containing replication competent integrated proviruses in resting CD4+ T cells. This was considered to be a relatively small reservoir, considering the highly dynamic nature of virion production in HIV-1 infection, with greater than an estimated 10^{10} viral particles produced per day[106].

Several months later, two separate laboratories expanded on these preliminary findings by looking for the presence of latently infected cells from patients on suppressive ART regimens[124,125]. The recent development of suppressive ART regimens with indinavir, AZT and 3TC allowed these studies to assess whether there was a latent reservoir in those without levels of detectable viremia in their peripheral blood. Other newer ART regimens, including ritonavir based and d4T based regimens were also available, to name a few. Both Wong et al. and Finzi et al. found that there was replication competent HIV-1 in the peripheral blood of HIV-1 patients despite prolonged suppression of viremia with newer ART regimens. Importantly, the authors also performed sequence analysis to determine if there were drug resistance mutations. Finding

no resistance mutations, the authors concluded that there was a stable reservoir in peripheral blood, and that ART should not be stopped until this reservoir is further evaluated. The modeling data that had been presented earlier in 1997 by Perelson et al. had assumed that there was no third phase of viral decay in plasma, and (critically) that there was no latent reservoir. This study was quickly followed up by Chun et al., who also found that there was an inducible reservoir in CD4+ T cells of those receiving ART[126].

The decision to look at resting CD4+ T cells by Finzi et al. (rather than CD8-depleted PBMC by Wong et al. or total CD4+ T cells by Chun et al.) turned out to have important implications for future research into HIV-1 reservoirs. The study by Finzi et al. was the first to demonstrate that resting CD4+ T cells in patients on suppressive ART harbor replication competent virus. At this point, the stage was set for the elucidation of a life-long reservoir of HIV-1 in patients on fully suppressive therapy. The groundwork had come early, with a rudimentary understanding of the molecular mechanism of HIV-1 proviral latency[127], with work by Chun et al. demonstrating the presence integrated HIV-1 DNA in resting memory CD4+ T cells[122,123], and by Wong et al. and Finzi et al.[124,125] demonstrating the presence of replication competent virus in those on suppressive ART. All that was left to do was to connect the dots, and demonstrate that this supposed reservoir in resting CD4+ T cells was highly stable over time.

The first report of the stability of this reservoir in resting CD4+ T cells came in 1999, with the finding that the half-life of this latent reservoir was nearly 44 months by a mixture of cross-sectional and longitudinal analysis of 34 patients on long term suppressive ART[128]. This preliminary estimate, combined with an estimated total body load of 10^6 latently infected cells as constituting the latent reservoir, led to the inference that it would require an average of 60 years

of uninterrupted therapy for this reservoir to be eradicated, providing evidence of a life-long persistent reservoir in resting CD4+ T cells.

Shortly after the publication of this study, two other manuscripts were published[129,130] suggesting that the decay rate of the latent reservoir in resting CD4+ T cells was substantially less than that found by Finzi et al. Zhang et al. sought to evaluate the decay rate of the reservoir by evaluating the number of sequences present in resting CD4+ T cells over time, and by fitting slopes to sparse longitudinal measures of viral outgrowth data from eight participants[129]. Ramratnam et al., on the other hand, inferred from a negative association between episodes of intermittent viremia and the slope of decay of the size of the infectious reservoir through longitudinal analysis, that replenishment of the reservoir was occurring from ongoing viral replication[130]. Both studies estimated a half-life of the latent reservoir of approximately 6 months, assuming fully suppressive therapy; both studies also shared the idea that there was ongoing viral replication replenishing the reservoir, and therefore not allowing the reservoir in resting CD4+ T cells to decay. These studies also ignored the fact that no HIV-1 DNA bearing resistance mutations was found within the reservoir of those on suppressive ART regimens[124,125].

Ultimately, both of these studies suggesting that the decay rate of the reservoir in resting CD4+ T cell was much less than 44 months were shown to be incorrect. A follow-up study published by Siliciano et al. in 2003 evaluating the longitudinal decay rates of the latent reservoir in 62 participants demonstrated that people on stably suppressive therapy for up to 7 years had similar decay rates, and that the half-life of the latent reservoir was indeed 44 months[131], consistent with the previous result by Finzi et al. in 1999[128].

These studies of the decay rate of the latent reservoir highlight an important theme that is still occurring in HIV/AIDS research today: the argument as to whether ongoing viral replication

is occurring in those on long term suppressive ART. This argument was once again brought to the forefront by recent study asserting that the lower concentration of antiretroviral drugs in the lymphoid compartment causes replenishment of the viral reservoir, with deep sequencing being performed on bulk viral sequence populations cited as evidence of viral evolution[132].

A body of research has demonstrated that intensification of ART regimens does not lead to reductions in low-level viremia[133-135] or increases in 2-LTR circles[136,137], and there is no ongoing replication detectable by highly sensitive and properly performed sequence analysis of individual viral templates in those on long term suppressive therapy[138-140]. Although increases in 2-LTR circles have been reported in those on stable ART, these have primarily been in patients with potentially suboptimal adherence to protease inhibitors[141,142]. Perhaps the strongest evidence for a lack of ongoing replication is that fact that those who are adherent to long term ART regimens do not spontaneously fail these regimens. If any substantial viral replication was occurring, mutations would eventually develop to ART, leading to virologic failure. Continuous replenishment of the reservoir would eventually lead to viral breakthrough. The Centers for Disease Control and Prevention data from 2011 showed that more than 81% of those retained in care and receiving ART for HIV-1 had suppressed viral loads in the United States[143]. The fact that those who are highly adherent to ART can maintain suppressed viral loads for decades is substantial proof that ongoing viral replication does not contribute to replenishment of the viral reservoir.

1.2.3 Clonal expansion as a new mechanism of viral persistence

The dogma of the latent reservoir in resting CD4+ T cells as a key driver of persistence has been the dominant theme regarding HIV-1 persistence since at least 2003, and probably since 1999. While other reservoirs may very well exist in long-lived macrophages and microglia, resting CD4+ T cells are currently the only well-described reservoir that can contribute to life-long persistence of HIV-1 due to the innate immunologic memory of CD4+ T cells. The latent reservoir in resting CD4+ T cells is thought to stochastically reactivate via regular mechanisms of CD4+ T cell activation from memory cells, leading to these cells producing viral proteins, then quickly dying from viral cytopathic effects. The persistence of HIV-1 is therefore mostly attributable to the long lifespan of memory CD4+ T cells[144-146]. Indeed, human CD4+ T cells responses to HCV can be quantified nearly 2 decades after exposure[147], and responses to Vaccinia virus vaccination can be detected nearly 40 years after vaccination[148] in the absence of further antigenic stimulation, demonstrating the long life of memory CD4+ T cells.

There is clearly a persistent long-lived reservoir in resting CD4+ T cells. However, recent evidence has pointed towards another mechanism of viral persistence. Maldarelli et al. and Wagner et al. in 2014 published evidence that the integration site of HIV-1 proviruses can play an important role in the persistence of infected cells over time[149,150]. Maldarelli et al. showed that nearly 40% of the integration sites in one individual were identical, indicating that they were in cell clones and suggesting that extrinsic or intrinsic factors could drive this clonal expansion[149,150]. In one case, the sequence of a drug-sensitive virus in plasma was identical to one massively expanded clone, suggesting that these clonally expanded proviruses can produce virions in the presence of suppressive ART. Some have claimed that these clonally expanded

proviruses are not likely to be replication competent[151], and have been found to be predominantly mutated[152]. However, Simonetti et al. recently reported that the expanded clone that contributed substantially to the plasma viremia observed in one study participant was indeed replication competent[153]. It remains to be seen how frequently clonally expanded populations can be found in plasma, and how often these clonally expanded populations are infectious.

1.3 MOTIVATION FOR A CURE: THE BERLIN PATIENT

1.3.1 Early studies of stem cell transplantation for HIV-1

In those with AIDS, the risk of developing both hematologic and non-hematologic malignancies is much higher due to the compromised state of the immune system and the often-unchecked replication of herpes viruses (i.e., Epstein Barr virus and HHV-8) and human papilloma viruses[154]. However, even following the control of viral replication during the era of fully suppressive ART, the frequency of cancer in those living with HIV-1 is higher[155-162] than those living without HIV-1. As such, oncology and HIV-1 have had an ongoing relationship since the beginning of the epidemic, especially before the invention of suppressive ART regimens. In the time before fully suppressive ART regimens, oncologists attempted to improve the course of HIV-1 infection through donor lymphocyte infusions or transplantations (reviewed in [163]).

The first studies of treatments for HIV-1 in the field of oncology were lymphocyte transfusions in the early 1980s. These first studies showed that they were essentially completely ineffective at treating AIDS, likely due to infection of the transfused donors T cells and the lack

of engraftment of the transfused lymphocytes[164,165]. Another study was undertaken where donor lymphocytes from an HIV-uninfected identical twin were serially infused into an HIV-positive identical twin, based on the principle that this could lead to engraftment in the absence of conditioning chemotherapy[166]. Surprisingly, this showed some benefit, including an increase in CD4+ T cells, but the HIV-positive twin ultimately died 1 year after the serial transfusions began[167]. Together, these initial studies of donor lymphocyte transfusions demonstrated that there was no clinical benefit to simply transfusing in more T cells.

With AZT becoming available in 1987, transplantation oncologists began attempting to investigate the effects of transplantation on HIV-1 persistence in patients for whom transplantation was indicated based on malignancy. Many studies found no effect on HIV-1 persistence following transplantation (reviewed in [163]). However, results from several studies were intriguing. In one study, a man with refractory lymphoma received an allogeneic stem cell transplantation in addition to AZT throughout the transplantation period[168]. Following engraftment, PBMC and bone marrow became negative for HIV-1 by PCR, but unfortunately the patient died shortly thereafter from tumor relapse. Intriguingly, no HIV-1 was detectable by PCR or culture from lymph nodes, bone marrow, tumor or other tissue samples obtained after death. The authors concluded that chemotherapy and transplantation may have eradicated HIV-1, and AZT protected the graft in this context[168]. This basic rationale, of wiping out the host immune system and replacing it with a new one, while protecting the new immune system from being infected with HIV-1, is the same rationale that would lead to the long term remission in the Berlin Patient 20 years later. At the time, it was not known that CCR5 was a major co-receptor for HIV-1 entry[169-173] or that a percentage of the population is homozygous for a deleted version of CCR5[174], and are therefore resistant to HIV-1 infection from CCR5-tropic viruses[175,176].

One year later, a study was conducted in which 16 HIV-positive twins received syngeneic stem cell transplantation from their identical twins, and were randomized to receive AZT or placebo after transplantation[177]. This study found no clinical benefit other than a small increased in CD4+ T cell counts following transplantation, and no differences between the placebo and AZT groups.

Several years later, a woman with AIDS underwent conditioning chemotherapy along with AZT, interferon-α, and anti-HIV-specific cytotoxic T lymphocyte clones as treatment for HIV-1, followed by allogeneic stem cell transplantation from an HLA-identical donor[178]. Engraftment occurred, and the patient was negative for HIV-1 after 30 days, but immunologic reconstitution was poor. The patient ultimately died 10 months after transplantation, and PCR on tissues obtained post-mortem was negative for HIV-1. This study, together with the study by Holland et al. from 1989[168], highlights the potential importance of graft versus host disease in eradicating or greatly reducing the frequency of HIV-1 infected cells. Juxtaposing these studies with the lack of an observed effect in the study following syngeneic transplantation by Lane et al. from 1990[177] further illustrates this point.

One further case of transplantation prior to the advent of suppressive ART is worth reviewing, and it is the case of Jeff Getty, who received a bone marrow transplant from a baboon following non-myeloablative conditioning in 1996 as an experimental treatment for advanced AIDS[179]. This study was approved by the FDA after much convincing from the outspoken and well-known AIDS activist Jeff Getty due to his rapidly deteriorating condition[180,181]. Justifiably, concerns existed about zoonotic transmission of disease from the donor baboon to the recipient, and the possibility of subsequent spread of zoonotic disease from person to person if Mr. Getty was infected with a new pathogen[182]. Fortunately for Mr. Getty, the transplant was relatively

innocuous[179], but was also unsuccessful in treating his HIV-1, although his condition did independently improve. This study raised important concerns about the use of xenogeneic sources of blood stem cells, but also xenogeneic sources of whole organs for transplant, leading some to call for a moratorium on xenotransplantation research[183]. While a moratorium ultimately did not occur[184], more research and an abundance of caution should be used when investigating xenotransplantation.

Overall, stem cell transplantation in the era prior to suppressive ART was largely unsuccessful due mostly to the inability to control the replication of HIV-1, and perhaps partially due to bone marrow toxicity of AZT and the related failure to efficiently reconstitute the immune system following transplantation[185].

1.3.2 Stem cell transplantation in the era of suppressive ART

In the era of suppressive ART, HIV-1 infected persons with hematologic malignancies requiring stem cell transplantation fare remarkably well. The overall quality of health of those living with HIV-1 on suppressive ART regimens has permitted the assessment of preparatory conditioning regimens[186,187], leading to improved outcomes. Reconstitution of the periphery has also been much improved in the context of suppressive ART[188,189].

In particular, autologous stem cell transplantation has improved dramatically in the era of suppressive ART. At this point, there is essentially no difference in terms of outcomes between those with and without HIV-1 when it comes to autologous stem cell transplantation for lymphoma[190-195]. Considering there was a time when having HIV-1 was a contraindication for

receiving a stem cell transplantation, the transplant oncology field has progressed substantially in parallel to the development of ART.

This progress in the treatment of HIV-1 related malignancies with transplantation has led to the ability to inquire as to the effects the process of transplantation itself may have on HIV-1 reservoirs in the context of fully suppressive ART. Studies of autologous stem cell transplantation had generally focused on outcome (as described in the paragraph above), and not directly on whether there was reduced persistence of HIV-1 following autologous stem cell transplantation. To date, three studies have evaluated the impact of autologous stem cell transplantation on HIV-1 persistence. Resino et al. found no change in cellular HIV-1 DNA levels 12 months after transplant in 6 HIV-positive autologous transplant recipients[188]. Simonelli et al. studied 24 HIV-positive patients who underwent autologous stem cell transplantation, finding that levels of HIV-1 DNA in PBMC decreased significant at 24 months post-transplant[189]. Our group studied 10 patients who underwent autologous stem cell transplantation at the City of Hope with minimal interruptions in ART during conditioning and transplantation, and found that plasma viremia was detectable with the single copy assay in 9 of 10 patients, and that HIV-1 DNA was detectable in all 10 patients following transplantation[196]. We also found no relationship between levels of HIV-1 DNA in PBMC and the length of follow-up, with a follow-up of >4000 days. Therefore, it seems clear that autologous stem cell transplantation is not curative nor does it substantially reduce the size of the reservoir, likely due to the infusion of infected cells as a component of the mononuclear cell autograft and incomplete suppression of viral replication in the peri-transplant period. Interestingly, another study by our group also suggested that moderately intensive chemotherapy for HIV-associated malignancies did not lead

to long-term changes in the levels of low-level plasma viremia or cellular HIV-1 DNA in PBMC[197].

Leading up to the success story of the Berlin Patient (published in 2009; see below)[198], many studies of allogeneic stem cell transplantation were attempted in the era of suppressive ART. The vast majority of these cases proved to be entirely unsuccessful, as reviewed by Hütter and Zaia in 2011[163]. In Hütter and Zaia's summary of 20 HIV-positive people who received transplants between 2000 and 2010, many either died shortly after transplant or there was no major impact on the HIV-1 reservoir. In one study by Avettand-Fenoel et al., an HIV-positive 17 year old received an allogeneic stem cell transplantation and concomitant suppressive ART, and subsequently became HIV-1 DNA negative in PBMC[199]. Ultimately, this patient experienced a rapid viral rebound following cessation of ART. In a study of two patients who underwent conditioning and transplantation while receiving ART, one had undetectable levels of HIV-1 DNA in PBMC shortly after transplant, but unfortunately treatment interruption could not be attempted because the patient died soon after[200].

In the era of modern ART regimens, both allogeneic and autologous stem cell transplantation for HIV-associated malignancies are a reality and are commonly performed. What is also clear is that there is almost no role of transplant alone in reducing or eliminating the reservoir of HIV-1 infected cells in patients receiving suppressive ART regimens. Despite having come a long way in learning how to perform hematopoietic stem cell transplantations in those with HIV-1 infection, we were seemingly no closer to a cure for HIV-1.

1.3.3 Timothy Ray Brown and the first cure of HIV

The "Berlin Patient", or Timothy Ray Brown, is an American man who was living with HIV-1 suppressed on ART and working as a translator in Germany, when he was diagnosed with acute myeloid leukemia (AML)[198]. As initial treatment for AML, Mr. Brown received two courses of induction and one course of consolidation chemotherapy, but unfortunately developed severe hepatic and renal toxicity, leading to the discontinuation of ART and subsequent viral rebound. ART was once again started, and viremia was re-suppressed. Despite the initial response of AML to chemotherapy, Mr. Brown experienced a relapsed of AML 7 months later. This relapse was an indication that myeloablative chemotherapy and stem cell transplantation was required.

At this juncture, Dr. Gero Hütter, Mr. Brown's physician at the Chartié Universitätsmedizin Berlin, applied some unique insight. The majority of HIV-1 viral strains enter CD4+ T cells through CCR5, a chemokine receptor expressed on the cell surface[169-173], although some utilize CXCR4 to enter the cell[201-203]. Individuals who are homozygous for the CCR5delta32/delta32 mutation in the CCR5 gene are resistant to HIV-1 infection by CCR5-tropic strains[175,176], and those who are heterozygous are also resistant to infection and exhibit slower disease progression when infected[204,205]. Large-scale studies have determined that the frequency of the delta32 version of the CCR5 allele is approximately 10% in European populations, and at approximately 2-5% across the Middle East and the Indian subcontinent[174]. Importantly, the CCR5delta32 mutation leads to a truncated version of the CCR5 protein that is not expressed on the cell surface[206]. Therefore, those who are homozygous do not express any CCR5, and those who are heterozygous express reduced levels of CCR5 on the cell surface.

Dr. Hütter used the knowledge that homozygosity for the CCR5delta32 allele conferred resistance to HIV-1 infection, and investigated a group of 80 potential stem cell donors from the German Bone Marrow Donor Center who were HLA-identical to Mr. Brown[198]. He found that the frequency of the mutated version of the CCR5 allele was 21% in this cohort of 80, and one donor was homozygous for the delta32 mutation, consistent with population estimates of the frequency of this mutation in European populations[174]. This donor was selected as the stem cell donor for Mr. Brown, and Mr. Brown gave informed consent for the procedure. Mr. Brown was heterozygous at the CCR5 allele prior to the transplantation, though it is unknown if this had any bearing on the clinical outcome.

Mr. Brown underwent conditioning chemotherapy and total body irradiation, as well as prophylaxis against graft versus host disease with antithymocyte globulin[207], all of which substantially depleted T cells. ART was administered throughout conditioning until the day before transplantation, and engraftment subsequently occurred on day 13. Mr. Brown achieved full chimerism, and the wildtype CCR5 allele became undetectable in peripheral blood. Rectal biopsy at day 159-post transplantation showed no expression of CCR5, consistent with 100% chimerism from a delta32 homozygous donor. However, macrophages isolated from the rectal biopsy still expressed wildtype CCR5, likely due to their long lifespan *in vivo*.

Unfortunately, AML relapsed approximately 330 days following transplantation, leading to another round of conditioning chemotherapy and full body irradiation in preparation for another stem cell transplantation from the CCR5delta32/delta32 homozygous donor. Once again, 100% chimerism was achieved, and AML went into remission. Remarkably, ART was never restarted after the first transplant, and Mr. Brown never experienced viral rebound[198].

In a follow up study by Allers et al. 3.5 years after stem cell transplantation, additional evidence was presented for the complete replacement of Mr. Brown's immune system. Tissue samples from the colon, liver, and brain were obtained primarily for the assessment of graft versus host disease, and to attempt to identify the cause of leukoencephalopathy associated with neurological disorders (likely as a result of the extensive toxic chemotherapy and total body irradiation as treatment for AML), and were subsequently assessed for the presence of CCR5 positive cells for research purposes. No cells expressing CCR5 were present in the brain or liver, consistent with replacement of potentially long-lived cells in these compartments with donor-derived cells[208]. Initially, and consistent with the report by Hütter et al.[198], macrophages expressing CCR5 were detectable, but disappeared at later time points. Additionally, antibodies to HIV-1 also faded over time. This wealth of evidence led the authors to conclude that the Berlin patient was likely cured.

Interestingly, Mr. Brown's donor-derived cells expressed CXCR4, and were shown to be susceptible to CXCR4-tropic virus[208]. Initial analyses of the viral species present in Mr. Brown's reservoir suggested that there was ~2% predicted CXCR4-tropic virus[198]. These viruses could theoretically have led to viral rebound by infecting CXCR4 expressing donor cells. Three possibilities exist, with one being that the entire reservoir was eliminated when Mr. Brown's cells were killed by chemotherapy- and graft versus host-mediated toxicity. Another possibility is that HIV-infected cells containing CXCR4-tropic infectious virus were too infrequent to permit viral outgrowth. Finally, it is also possible that there was not any CXCR4-tropic virus in Mr. Brown's reservoir. This last possibility is also supported by the literature, in that the predicted CXCR4-tropic virus actually relied upon CCR5 for entry[209].

These reports documenting the cure of HIV-1 in one patient were received with great enthusiasm and optimism. A commentary published in the journal *Blood* following the report by Allers et al. entitled "The Power of 1 in HIV Therapeutics" captured the sentiment generated by these studies, stating: "The cure of HIV now has a face and a name after a 3-decade fight with HIV. The quest for more practical options for the remaining 33 million people living with HIV worldwide has to continue."[210]

1.3.4 The Boston Patients and subsequent attempts at curative stem cell transplantation

The cure of Mr. Brown following myeloablation and stem cell transplantation with CCR5delta32/delta32 homozygous stem cells as treatment for AML doubtlessly changed the field of HIV-1 research forever. Prior to the cure of Mr. Brown, it was unknown if a cure for HIV-1 was possible, and if it was possible, what it would look like. Mr. Brown and Gero Hütter's team demonstrated that a cure was indeed possible. However, given the drastic measures that had to be taken to cure Mr. Brown (i.e., complete elimination and replacement of his immune system with one that was resistant to HIV-1 infection) and the morbidity that is associated with such treatment, this case of a cure in N=1 is purely inspirational, and is applicable only to a few of the many living with HIV-1 in whom malignancies necessitate stem cell transplantations.

Stem cell transplantation cannot be performed in those living with HIV-1, unless it is done as a last resort for the treatment of a hematologic malignancy, due to the high rates of mortality (1 year survival of 69% for unrelated allogeneic transplantation for AML in the first remission[211], and 59% for chronic myeloid leukemia in the 2nd chronic or accelerated phase [212])

associated with allogeneic stem cell transplantation. Although malignances are more common in HIV-1 infected patients, they are still very rare. This has not stopped physicians from identifying prospective patients with HIV-1 suppressed on therapy and malignancies that necessitate stem cell transplantations. Two such patients whose clinical courses were recently reported have become known as the "Boston Patients". Not unlike the Berlin Patient, these two individuals developed malignancies while on fully suppressive ART regimens[213].

The first of the Boston patients was a male who was diagnosed with Hodgkin's lymphoma in 2006, and whose lymphoma recurred following initial chemotherapy and recurred again following autologous stem cell transplantation, indicating that allogeneic stem cell transplantation was required. This patient underwent reduced intensity conditioning, and received an allogeneic HLA-C-mismatched (7/8 matched alleles) unrelated stem cell transplant. Full donor chimerism was achieved by 216 days after transplantation[213].

The second patient was a male who was initially diagnosed and successfully treated for diffuse large B-cell lymphoma in 2003. However, in 2006 he was diagnosed with mixed-cellularity Hodgkin's disease. His Hodgkin's disease was then treated with chemotherapy, but he relapsed and then underwent an autologous stem cell transplant. Unfortunately, persistent thrombocytopenia and anemia arose, leading to a diagnosis of myelodysplastic syndrome. In 2010, this patient underwent reduced intensity conditioning and received an HLA-matched allogeneic stem cell transplant from a sibling. Full chimerism was achieved by day 220 post-transplant[213].

In this case report, both patients were CCR5delta32 heterozygous, with one wild type CCR5 allele each. Both then received allogeneic stem cell transplantation from CCR5 wild type homozygous donors. Both Boston patients had been heavily pre-treated with chemotherapy, and

had received autologous stem cell transplantations. In this case, both patients engrafted well and achieved full donor chimerism. Some graft versus host disease was reported in each patient, and was controlled with prednisone. HIV-1 DNA in PBMC was detectable pre- and immediately post-transplant in both patients, as was HIV-1 RNA in plasma. However, HIV-1 DNA in PBMC and total CD4+ T cells isolated from both patients were negative for HIV-1 DNA at later time points, corresponding with full chimerism and consistent with replacement of the host immune systems with that of the donor. Levels of HIV-1 antibodies also decreased in both patients, with a five-fold decrease in the first patient 1,266 days post-transplant and a 10-fold decrease in the second patient at 652 days post-transplant[213].

After observing that the levels of HIV-1 DNA in PBMC and total CD4+ T cells became undetectable, and levels of HIV-specific antibodies declined, the investigators sought to more thoroughly evaluate viral persistence in leukapheresis products and rectal biopsies[214]. The authors once again found that no HIV-1 DNA was detectable in PBMC, and that no infectious virus was present in >150 million cultured total CD4+ T cells from these two patients. Rectal biopsies in the second patient revealed no detectable HIV-1 DNA by qPCR. Finally, microchimerism analysis confirmed that only 0.0004% to 0.001% of PBMC were of host origin in these two transplant recipients[214].

These findings prompted the investigators to initiate an analytical treatment interruption study, for which the two patients provided informed consent. Weekly viral load assays were to be performed in the first 10 weeks, followed by viral load testing every one or two weeks after week 10 to carefully assess whether rebound occurred. For the first patient, viral recrudescence occurred 84 days following cessation of ART, with a viral load of 904 copies of HIV-1 RNA per milliliter of plasma 14 days after a negative viral load assay. The second patient remained HIV-1

DNA negative in PBMC at weeks 5 and 18 following treatment interruption, but the patient experienced flu-like symptoms on day 219 post-ART cessation, and was found to have a viral load of 1.9 million copies per milliliter of plasma on day 225 post-ART cessation. This was only 14 days after a negative clinical viral load result in plasma[214]. Virus that was present as HIV-1 DNA in PBMC following resumption of ART was related to virus that was present as HIV-1 DNA in PBMC from before transplantation, suggesting that the source of viral rebound was indeed extremely infrequent latently infected reservoir cells that were still present despite the near-complete replacement of the host's immune system.

These results are ultimately disappointing, both for the patients and for the field of HIV-1 cure research. While disappointing, this study does provide some critical insight into the degree with which the reservoir must be reduced for an eradication cure of HIV-1 to be successful. There are some important differences between these patients and Mr. Brown, most notably that Mr. Brown received a stem cell transplantation from a donor that was CCR5delta32 homozygous[198], so his new immune system was protected from infection, at least from CCR5-tropic virus. Mr. Brown also received a much more intensive conditioning regimen, including total body irradiation and graft versus host disease prophylaxis with rabbit anti-thymocyte globulin, perhaps leading to a more profound initial depletion of Mr. Brown's reservoir. Finally, Mr. Brown also likely experienced a more severe graft versus host disease manifestation, including viscerally and in the central nervous system[198]; this may have also translated into a greater depletion of Mr. Brown's own immune system, and also his HIV-1 reservoir.

Despite these differences, it is important to note that the minimum reduction in HIV-1 DNA from these two patients was approximately 1,000-fold, and yet these patients relapsed at 12 weeks and 31 weeks after ART cessation. Interestingly, Hill et al. have predicted via virologic

modeling that a reduction in the size of the latent reservoir (as measured by viral outgrowth assays) of ~2,000-fold would be required to allow the cessation of ART for 1 year without relapse in most individuals, and that >10,000-fold reductions would be needed to completely prevent viral rebound in the absence of ART[215]. These results highlight the significant difficulties that will be associated with trying to deplete the reservoir of HIV-1; if the reservoir has been depleted to below the limit of detection, as it was in the Boston patients, it is likely that viral relapse will occur in one year following cessation of ART. This modeling work also suggests a disturbing outcome amidst the challenge of evaluating curative strategies, namely that the Berlin patient could one day experience virologic relapse with a CXCR4-tropic virus, if any virus does indeed remain in his body. While this seems unlikely, it is still a possibility.

Ongoing studies are attempting to replicate the success achieved with the Berlin patient, but these studies face several obstacles. The frequency of patients living with HIV-1 that require allogeneic stem cell transplantation is a limiting factor, as is the frequency of CCR5delta32 homozygous individuals who could serve as stem cell donors. Finding an HLA-matched CCR5delta32 homozygous donor for an HIV patient requiring a stem cell transplantation is therefore quite challenging[216]. Follow-up studies in which patients did receive transplantation with CCR5delta32 homozygous stem cells have been attempted in at least 7 instances, but have not been successful. In all cases, the recipient has died within one year following transplant, and CXCR4-tropic virus has rebounded in each case[217,218]. Although repositories of CCR5delta32 homozygous donors have been instituted[219], it seems that the Berlin patient may truly be a unique case, serving primarily as inspiration to achieve a goal that is still quite far off.

1.4 HIV-1 LATENCY REVERSAL AS A CURATIVE STRATEGY

The cure of Mr. Brown's HIV-1 infection by allogeneic stem cell transplantation was important to the field of HIV-1 cure research because it demonstrated that an eradication cure could be achieved, albeit through extraordinary measures. While the allogeneic stem cell transplantation approach cannot be applied for the vast majority of those living with HIV-1, it does serve as a galvanizing force, leading to enthusiasm that a cure can be achieved and renewing interest in investigating curative strategies.

1.4.1 Initial studies investigating HIV-1 latency reversal

A preliminary *ex vivo* study conducted by Chun et al. in 1998 found that combinations of the cytokines IL-6, TNF-α and IL-2 led to the reactivation of HIV-1 expression from cultured resting CD4+ T cells[220]. These findings led to the hypothesis that perhaps IL-2 administration *in vivo* could be used to deplete the latent reservoir through viral cytopathic effects or immune mediated clearance[221]. This hypothesis was put to the test in a group of patients receiving suppressive ART based on one or two NRTI regimens combined with a protease inhibitor, who then received IL-2 either subcutaneously or intravenously. IL-2 therapy had been given to many HIV-1 patients in past studies[222-229], largely as a way to transiently increase CD4+ T cell counts prior to the development of effective ART regimens, and as such there was considerable clinical experience, as well as an appreciation that high dose IL-2 was associated with significant toxicity. A retrospective analysis of those who had received IL-2 therapy in three studies prior to 1995 revealed increases in CD4+ T cell counts and a non-significant trend towards improved clinical

courses in those who had received IL-2 therapy[230]. However, a large scale prospective study published in 2009 that was appropriately powered to evaluate the clinical efficacy of IL-2 therapy found that, despite significant increases in CD4+ T cell counts, there was no clinical benefit of IL-2 therapy in those with suboptimal CD4+ T cell counts on suppressive ART[231].

Bearing this extensive clinical experience with IL-2 therapy in mind, and the appreciation that high-dose IL-2 led to significant toxicity, Chun et al. gave low doses of IL-2 separated by up to 8 weeks. Viral outgrowth assays performed using resting CD4+ T cells isolated from a group of patients that received IL-2 showed statistically significantly lower levels of virus outgrowth compared to a group that did not receive IL-2. Additionally, some in the group that received IL-2 had no detectable viral outgrowth from large numbers of cultured resting CD4+ T cells, or from resting CD4+ T cells isolated from lymph node biopsies, leading to the conclusion that IL-2 may deplete the reservoir in those on suppressive ART[220]. Another study by Prins et al. attempted a similar approach, except that an antibody (OKT-3) specific for CD3 was given in addition to IL-2. This study revealed profound T cell activation and proliferation, and was associated with a dramatic increase in viremia in one of three patients; however, this study also caused transient renal failure and seizures in one of three patients[232]. As such, this intervention was deemed too toxic.

Based on their preliminary results, Chun et al. initiated a treatment interruption study in two patients who had received IL-2 therapy and in whom no replication competent virus could be recovered. Both patients experienced viral rebound within 3 weeks, demonstrating that the virus had not been cleared in these patients[233]. This finding was considered conclusive enough to not pursue additional studies of treatment interruption in patients who had received IL-2 therapy. These findings, in conjunction with the failure of IL-2 therapy to improve outcomes in those with

sub-optimal CD4+ T cell recovery, will likely put an end to the study of IL-2 therapy in HIV-1 infection.

In addition to the investigation of IL-2 to reduce the size of the latent reservoir, a study published in 2005 by Lehrman and Hogue et al.[234] studied the size of the infectious reservoir in four patients who received ART intensification with enfurvirtide, followed by valproic acid (a weak histone deacetylase inhibitor[235,236]) twice daily orally for three months. This study found that there were substantial decreases in the size of the latent infectious reservoir in three of four study participants.

Despite these initially promising findings, follow-up studies in larger cohorts revealed that the reservoir remained stable in those on suppressive therapy[237] and that the reservoir only declined appreciably in a small number of participants following treatment with valproic acid for three months[238]. Another study revealed no changes in the amount of total or integrated HIV-1 DNA in 11 patients receiving valproic aicd compared to 13 controls, and also found no change in the size of the replication competent reservoir[239]. This study also compared treatment interruption in three patients receiving valproic acid as part of a larger French cohort that underwent treatment interruption, and found that there was no difference in the time to viral rebound in the patients who received valproic acid compared to others[239]. Finally, a randomized cross-over style clinical trial studied the effects of valproic acid on the size of the latent reservoir in 56 participants on suppressive ART, and found that it had no effect[240]. These studies led to the conclusion that valproic acid does not have a substantial effect on the size of the latent reservoir in resting CD4+ T cells *in vivo* in those on suppressive ART. Taken together, these early studies using IL-2 or valproic acid to reduce the size of the reservoir indicate that safer, more specific,

and more potent latency reversing agents would be required to reduce the size of the latent reservoir.

1.4.2 Histone deacetylase inhibitors and the kick and kill strategy

Although valproic acid, the first histone deacetylase inhibitor (HDACi) tested, was ultimately found to be ineffective at reducing the size of the latent reservoir in patients on suppressive ART, others hypothesized that the rationale for use of an HDACi was not flawed, and that more potent HDACi were needed. Work elucidating that role of HDAC in suppression of expression from the long terminal repeat (LTR) of HIV-1 is rooted in a publication from 1994, and another from 1997. These two publications together suggested that the transcription factors YY1 and LSF played a role in suppressing viral transcription from the LTR, but the complete mechanism was unclear[241,242]. It was discovered in 2000 that LSF recruits YY1 to the LTR to form a repressor complex, which then recruits the HDAC1 complex to the LTR, leading to molecular repression of the LTR[243]. A follow-up study demonstrated that HDAC1 associates closely with a nucleosome (nuc-1) that is adjacent to the transcription start site in the LTR[244]. Other molecular studies soon followed that ascribed the recruitment of HDAC1 to nuc-1 via different transcription factors than originally proposed, such as NF-KBp50 homodimers[245], c-Myc and Sp1[246], and CBF-1[247]. Regardless of the specific mechanism of recruitment, it is clear that HDAC1 is recruited to the LTR of HIV-1, and leads to the suppression of viral transcription through the deacetylation of histones on nucleosomes associated with the LTR, leading to the close association between viral DNA and the histones and preventing the cellular transcription machinery from accessing the viral LTR.

Ex vivo evaluation of HDACi for their potential role in reversing latency was first performed with valproic acid[248], which was found to be surprisingly effective in reversing latency given the limited responses observed in clinical trials. Two reports in 2009 were published regarding the ability of HDACi to reverse latency *ex vivo*, and once again HDACi were found to be surprisingly effective at reversing latency[249,250]. Vorinostat (also known as suberoylanilide hydroxamic acid or SAHA) was found to induce viral outgrowth from patient-derived resting CD4+ T cells in these two studies, suggesting that SAHA was a potent latency reversing agent and that HDAC1 plays an important role in the maintenance of molecular latency of HIV-1.

1.4.3 Clinical assessments of histone deacetylase inhibitors and disulfiram

A number of clinical studies have been conducted to date to investigate the effects of putative latency reversing agents in terms of their ability to reactivate expression of latent proviruses. Studies have primarily focused on HDACi for the time being, given the literature suggesting the importance of HDAC1 in maintaining proviral latency, and due to the availability of FDA-approved HDACi for use in the clinic, albeit for cancer-related indications.

The first study of SAHA in people came from Archin, et al. and was published in 2012 in *Nature*. This study sought to evaluate the effects of a single dose of oral vorinostat on levels of cellular HIV-1 RNA in resting CD4+ T cell isolated from participants on suppressive ART. The authors studied eight patients, and found that levels of cellular HIV-1 RNA increased in each of these individuals, with a mean increase of 4.8-fold over baseline[251]. Levels of cell-free HIV-1 RNA in plasma were also measured, but did not change. This study was touted as proof of

principle that reversal of proviral latency can be achieved *in vivo* by targeting known mechanisms of proviral latency. However, the study raised more questions than answers, namely whether *de novo* reactivation of transcription was occurring or whether levels of basal transcription were merely being increased, and whether the increases in cellular HIV-1 RNA were associated with depletion of the latent reservoir. These questions served as the impetus for a key portion of my dissertation work.

Two additional studies of SAHA followed the initial report by Archin, et al. One study, from the same group that published the initial report, was a multi-dose evaluation of vorinostat three days in a row, once a week, for eight weeks. The authors found that levels of cellular HIV-1 RNA expression in resting CD4+ T cells increased in only three of the five participants studied after doses 11 and 22, indicating that the responsiveness to SAHA may wane over time[252]. By contrast, a study by Elliot et al. that evaluated the effect of daily 400 mg oral doses of SAHA for 14 consecutive days on the size of the latent reservoir in 20 participants on suppressive ART found that levels of cellular HIV-1 RNA transcription in total CD4+ T cells increased in 18/20 study participants. The median peak fold-increase was found to be 7.4-fold, but plasma viremia, total and integrated HIV-1 DNA, and levels of infectious virus as measured by viral outgrowth were unchanged. Intriguingly, in this study, levels of cellular HIV-1 RNA in total CD4+ T cells remained elevated for 70 days following the last dose of SAHA[253]. The results of these two studies are discordant, likely due to effects from the different numbers of participants studied and the assessment of HIV-1 RNA transcription in total CD4+ T cells in one study versus resting CD4+ T cells in the other study.

Other clinical studies of putative latency reversing agents quickly followed the initial description of SAHA. While mostly HDACi have been studied to date, disulfiram is a notable

exception. Disulfiram is thought to reactivate HIV-1 latency through reduction in levels of phosphatase and tensin homolog (PTEN) protein, the absence of which causes increased phosphorylation of protein kinase B (also known as Akt), leading to activation of NF-κB[254]. A pilot study of 500mg of disulfiram administered daily for 14 days was evaluated for latency reversal effects in a cohort of 16 study participants. Disulfiram was found to not have an effect on the size of the latent reservoir, and did not substantially increase plasma HIV-1 RNA levels, although it may have transiently increased viremia immediately post-dosing in a subset of 6 individuals who were closely monitored[255]. In an expanded study of 34 participants recently published by Elliot et al., administration of disulfiram for three days at doses 500mg, 1000mg and 2000mg was safe and well-tolerated, and increased levels of cellular unspiced HIV-1 RNA significantly, albeit only 1.7-fold above baseline post-dose and 2.1-fold above baseline in the timepoint after dosing[256]. No dose effect was observed, and the overall magnitude of the latency reversal effect was minimal.

Two other HDACi, panobinostat and romidepsin, that are more potent than vorinostat have also been studied clinically. In a phase I/II clinical trial of 20 mg of oral panobinostat given three times per week every other week for eight weeks in 15 study participants, the authors found that levels of cellular HIV-1 RNA in total CD4+ T cells increased significantly, with a median increase of 3.5-fold (ranging from 2.1-fold to 14-fold)[257]. Panobinostat also increased levels of HIV-1 RNA in plasma, although this was assessed with a transcription mediated amplification assay and therefore could not be quantified other than positive or negative. An analytical treatment interruption was also performed, but the median time to viral rebound was 17 days (ranging from 14 to 56 days), suggesting that panobinostat did not have a large impact on the size of the latent reservoir [257].

The study of the potent HDACi romidepsin was performed in a group of 6 study participants with viremia suppressed on ART. These participants received 5 mg/m^2 of romidepsin by infusion over a 4-hour period once weekly for 3 weeks, and levels of cellular HIV-1 RNA were quantified in total CD4+ T cells. Levels of cellular HIV-1 RNA in total CD4+ T cells were found to increase, with fold increases in the range of 2.4- to 5.0-fold. Plasma HIV-1 RNA also increased from <20 copies per milliliter of plasma by Roche COBAS AmpliPrep/TaqMan at multiple timepoints in 5 of 6 study participants, ranging from 46 to 103 copies per milliliter of plasma. From these finding, the authors concluded that romidepsin was a safe and effective latency reversing agent.

Taken together, clinical experience with latency reversing agents to date has focused mostly on HDACi, although disulfiram has also been investigated. The results of trials have generally found that latency reversal agents increase expression of unspliced cellular HIV-1 RNA in either total CD4+ T cells or resting CD4+ T cells. While SAHA and disulfiram have not been found to increases in levels of plasma HIV-1 RNA, panbinostat and romdepsin have reportedly led to increases in plasma viremia. Overall, the effects observed from these latency reversing agents have generally been weak, and have not led to profound reductions in the size of the latent reservoir *in vivo*, as evidenced by the short time to rebound in the panobinostat study and a lack of change in the size of the infectious reservoir by viral outgrowth in the romidepsin study. If we are going to substantially deplete the size of the latent reservoir with the kick and kill approach, it seems that drugs that are better tolerated and that more potently reactivate the latent reservoir will be required.

1.4.4 Pre-clinical assessment of LRAs targeting mechanisms of viral persistence

In addition to the above described HDACi that target chromatin structure to reverse latency, other compounds targeting different known aspects of proviral latency have been proposed and studied in various *in vitro* and *ex vivo* systems. It is worth noting that *in vitro* models of HIV-1 latency have a high degree of variability of responses to different latency reversing agents. Spina et al. recently investigated 5 different *in vitro* models of HIV-1 latency compared with *ex vivo* measures using patient cells, and found that the responsiveness of any one cell model did not necessary translate to the other cell models, or measures of latency reversal from patient cells *ex vivo*[258]. This study acutely highlighted the difficulty associated with using model systems to replicate an extremely complex *in vivo* situation. This problem also extends to the discovery of HIV-1 latency reversing agents at the level of screening systems often used by pharmaceutical companies, namely that a cell line with a predictable response is required for compound screening. Results from any one *in vitro* system should therefore be interpreted with caution, and for this reason *ex vivo* systems using latently infected cells from patients should primarily be used to evaluate promising latency reversal candidates.

Mechanisms of proviral latency are complex and multifactorial, and have been reviewed extensively elsewhere[259-262]. The first step required for the transcription of viral messenger RNA is the ability of the cellular transcriptional machinery to access the LTR. The relevance of chromatin structure, as it relates to suppression of transcription initiation, is described in the sections above about HDACi. Another potentially relevant molecular mechanism of transcriptional suppression is methylation of histones and direct methylation of proviral DNA. Histone methylation has been extensively studied in HIV-infected cell lines, with data showing

that di- (me2) and tri-methylation (me3) of histone H3 at lysine residue 9 (H3K9me2 and H3K9me3), and tri-methylation at histone H3 lysine 27 (H3K27me3) in the histones forming a complex with nuc-1 in the HIV-1 LTR are associated with HIV-1 transcriptional repression[263-268]. The cellular enzymes Suv39H1[264], G9a[266], and EZH2[265] are known to be responsible for the H3K9me3, H3K9me2 and H3K27me3 repressive methylation of histones, respectively, suggesting that inhibition of these enzymes could lead to the reversal of latency.

This hypothesis has been tested in an *ex vivo* system[269] with chaetochin, an inhibitor of Suv39H1-mediated H3K9me3[270], and BIX-01294, an inhibitor of G9a-mediated H3K9me2[271,272]. Bouchat et al. found that chaetochin treatment led to virus production in the culture supernatant from 50% of CD8-depleted PBMC cultures and 86% of resting (in this case, CD3+CD4+HLA-DR-) CD4+ T cell cultures, and that BIX-01294 led to reactivation from 80% of resting (in this case, CD3+CD4+CD25-CD69-HLA-DR-) CD4+ T cells[269]. Although significant proportions of cultures became positive for HIV-1 RNA, it is important to note that levels of HIV-1 RNA produced were higher than maximum reactivation with T cell activation in some donors and were substantially lower than T cell activation in other donors. Additionally, the authors also treated the CD8-depleted PBMC and resting CD4+ T cells with the histone methyltransferase inhibitors (HMTI) for a supra-physiological exposure time of 6 days, and provided no measurements of non-specific T cell activation or cellular viability after culture, raising concerns about the relevance of the reported findings. *In vitro* experiments with EZH2 inhibitors have inconsistently led to viral reactivation[265,273], and inhibition of EZH2 in a primary cell model suggested that it may prime cells for subsequent responsiveness to HDACi treatment[274]. Overall, the lack of a FDA-approved HMTI, limited evidence of the *ex vivo* efficacy of HMTI, and probable off-target

effects from general inhibition of histone methylation contribute to an unclear path forward for use of these compounds for HIV-1 latency reversal.

Methylation of proviral HIV-1 DNA has also been suggested as a mechanism of proviral latency. A cDNA screen in the J-Lat cell line by Kauder et al. identified methyl-CpG binding protein 2 as a major regulator of HIV-1 latency via association with two CpG islands surrounding the transcription start site in the LTR, and that treatment with the DNA methyltransferase inhibitor 5-aza-2'deoxycytidine (5-aza-dC) synergized with NF-κB activators to cause viral gene expression[275]. Blazkova et al. also found that CpG methylation in the LTR was associated with proviral latency *ex vivo*[276]. However, a follow-up study by Blazkova et al. found that methylation of CpG islands within the proviral LTR was relatively infrequent, with only 2.4% of nucleotides methylated in the 5'-LTR of resting CD4+ T cells by sodium bisulfite sequencing. Finally, a study by Bouchat et al. found that treatment with 5-aza-dC did not substantially reactivate virus production from latently infected resting CD4+ T cells, but rather led to high levels of virus production when used to pre-treat cells prior to treatment with HDACi[277]. Taken together, the role of DNA methyltransferase inhibitors such as 5-aza-dC in reactivating HIV-1 transcription may be limited due to infrequent methylation of the CpG islands located within the 5' LTR in latently infected resting cells isolated from patients on long term suppressive therapy.

In addition to the suppressive chromatin structure facilitated by histone acetylation and methylation, many other transcriptional blocks are associated with post-integration HIV-1 latency. After chromatin has been opened sufficiently, key transcription factors can access their cognate binding sites on the HIV-1 genome, and can recruit the cellular RNA polymerase II machinery. The cellular transcription factors Sp1 and NF-κB are both important for the initiation

of HIV-1 transcription[278-280]. While the p50 homodimers of NF-κB present in non-activated cells favor the recruitment of suppressive transcriptional complexes[245], the p50/RelA heterodimers of NF-κB present in activated cells facilitate the recruitment of transcriptional machinery (including p300/CBP), leading to the recruitment of RNA polymerase II to the LTR[281]. As such, the presence of NF-κB heterodimers is thought to be an important step in the reactivation of HIV-1 from latency.

Once NF-κB has been activated, the next step of transcriptional reactivation is the recruitment of the RNA polymerase II machinery, and the interaction between P-TEFb, Tat and RNA polymerase II. The P-TEFb complex, consisting of cyclin T1 and CDK9[282], is utilized via interaction with Tat to drive high-level, full length transcription of HIV-1 RNA[283,284]. However, analogous to the sequestration of the activate version of NF-κB in non-activated cells, P-TEFb is also sequestered in a suppressive 7SK snRNP complex[285]. The bromodomain protein Brd4 can interact with P-TEFb, freeing it from the suppressive 7SK snRNP complex and allowing it to function as part of the super elongation complex[286,287]. However, the interaction of Brd4 with P-TEFb competes with the interaction of Tat and P-TEFb, so the presence of high levels or Brd4 can inhibit reactivation of HIV-1 by preventing high levels of full length transcription[286,287]. An inhibitor of Brd4 named JQ1[288] is being investigated for the potential to reactivate HIV-1 by freeing P-TEFb for interaction with Tat[289]. Different Brd4 inhibitors are currently in early stage development for cancer[290].

The importance of the mechanisms of latency described above has been highlighted by the investigation of compounds targeting specific mechanisms of latency. Chromatin structure can be altered by the inhibition of histone acetyl transferases with the HDACi described above, such as SAHA, panobinostat, and romidepsin. The activation of NF-κB can be achieved by

protein kinase C (PKC) agonists, ideally leading to the reactivation of HIV-1 due to the availability of NF-κB heterodimers. Prostratin was initially used as a PKC agonist[291], but more recently bryostatin has been studied due to its use *in vivo* in cancer trials[292,293]. Ingenol derivatives are also potent *in vitro* PKC agonists[294].

Bullen et al. published a study in 2014 showing that no latency reversing agents induced viral outgrowth from patient-derived resting CD4+ T cells, and that also showed that single agents were ineffective at increasing the transcription of properly poly-adenylated cellular unspliced HIV-1 RNA transcripts, with the notable exception being the PKC agonist bryostatin[295]. This reinforced the appreciation of HIV-1 latency as a multifactorial process and has led recent groups to evaluate combinations of latency reversing agents, rather than single agents. Initial combination studies have focused on the best-described mechanisms of proviral latency, combining HDACi with either PKC agonists or Brd4 inhibitors. Laird and Bullen et al. investigated combinations of latency reversing agents and found that agents targeting PKC and HDAC, and PKC and Brd4 led to substantial latency reversal and exhibited synergy in their reactivation of HIV-1 transcription[296]. Additional studies published recently by Darcis and Kula et al. and Jiang et al. identified combinations of the PKC agonists bryostatin or ingenol-b with JQ1 as potent latency reversing compounds[297], and ingenol-3-angelate and JQ1 as a potent combination of latency reversing compounds[273], respectively. These *ex vivo* studies have demonstrated that combinations of latency reversing agents containing PKC agonists with either HDACi or Brd4 inhibitors are the most potent reactivators of latency across studies. This may be due, in part, to the partial activation of CD4+ T cells as a result of treatment with a PKC agonist[298].

One theme that is universal across these studies is that bulk populations of resting or total CD4+ T cell were treated with latency reversal agents, and the relative activity of the latency reversal agents was inferred from the bulk response. It is important to carefully evaluate these individual and combinations of latency reversal compounds at the level of individual proviruses to ensure that they have significant potency when compared to full T cell activation and unstimulated levels of virion production. Evaluating the effects of these latency reversal agents at the level of individual proviruses, and ascertaining whether these agents have had any effect on the size of the latent reservoir *ex vivo* will be a substantial focus of the ensuing dissertation.

2.0 HYPOTHESIS AND SPECIFIC AIMS

Hypotheses

H1: A latent reservoir of infectious HIV-1 persists in the resting CD4+ T cells of individuals receiving suppressive ART. Latency reversing agents have been proposed to drive proviruses out of latency, leading to depleting of the latent reservoir, but the potency of these agents has yet to be evaluated at the level of individual proviruses. We *hypothesize* that the currently available latency reversing agents will reactivate only a small minority of HIV-1 proviruses to produce virions *ex vivo* compared with T cell activation. Similarly, the available latency reversal agents will not lead to significant depletion of the reservoir from viral cytopathic effects.

H2: Viral persistence can readily be detected in those receiving suppressive ART, but it is unknown how measures of viral persistence are related to the size of the inducible reservoir and to each other. We *hypothesize* that analysis of molecular measures of viral persistence in peripheral blood will reveal that the frequency of HIV-1 infected cells and their transcriptional activity are positively associated with the size of the spontaneous and inducible reservoirs, and can serve as *in vivo* biomarkers of the inducible reservoir.

H3: Finally, we *hypothesize* that there is a greater number of inducible and infectious proviruses within total CD4+ T cells compared with resting CD4+ T cells, and the size of the inducible reservoir in total and resting CD4+ T cells will be positively associated with the magnitude of infectious virus outgrowth in both total and resting CD4+ T cells.

Specific Aims

The hypotheses described above will be evaluated by pursuing the following three specific aims:

1. Quantify the fraction of proviruses in latently infected resting CD4+ T cells isolated from patients on suppressive antiretroviral therapy that can be reactivated to produce virions following *ex vivo* T cell activation or treatment with latency reversal agents, and determine whether latency reversal leads to a reduction in the size of the inducible reservoir.

2. Investigate the underlying clinical and/or virologic factor(s) that are associated with the size of the spontaneous and inducible reservoir in resting CD4+ T cells *ex vivo*.

3. Evaluate the levels of inducible and infectious virus *ex vivo* in total and resting CD4+ T cells to determine if the inducible and infectious reservoirs are larger in total versus resting CD4+ T cells, and to determine if there is a relationship between the size of the inducible reservoir and the magnitude of infectious viral outgrowth in total and resting CD4+ T cells.

3.0 OVERALL MATERIALS AND METHODS

Patient cohorts studied. The work described in this dissertation was performed using peripheral blood samples donated by study participants. Study participants were enrolled either through the University of Pittsburgh Center for AIDS Treatment (PACT) clinic or through Quest Clinical Research (San Francisco, CA). Regardless of where participants were enrolled, local review boards approved the study protocols, and participants provided informed written consent.

Peripheral blood samples. The samples obtained from study participants were either large volume blood phlebotomy (180 milliliters of whole blood, or 360 milliliters of whole blood spread across two 180 milliliter blood draws separated by ~1 hour), or were from leukapheresis products. All blood samples were drawn into either EDTA phlebotomy tubes or leukapheresis bags coated with acid citrate dextrose as anticoagulants. Blood samples were processed as soon as possible, always within 4 hours of phlebotomy.

Processing of peripheral blood samples. When processing whole blood, plasma was first separated from whole blood by centrifuging blood at 500xg for 10 minutes. Plasma was then removed, ensuring that the cellular fraction was left behind. The plasma was then spun a second time at 1350xg for 15 minutes to remove any cellular contamination, and then was divided into

1.5 milliliter aliquots and stored at -80°C for downstream testing. For some leukapheresis donors, plasmapheresis was performed in parallel, and the plasma was processed as described above.

After removing plasma from whole blood, the remaining cellular components were diluted with 37°C phosphate buffered saline containing 10% fetal bovine serum (PBS/FBS). The diluted whole blood was then carefully layered over Ficoll-Paque, and spun at 400xg for 40 minutes at room temperature, with the centrifuge brake turned off to prevent mixing of the separated layers during deceleration of the centrifuge rotator. The peripheral blood mononuclear cells present at the Ficoll boundary where then carefully removed and pooled into one tube. The pooled PBMC were then washed with warm PBS/FBS, counted in a hemocytometer with Trypan blue, and were either cryopreserved or used fresh for experiments.

Isolation of total or resting CD4+ T cells. Isolation of total CD4+ T cells was performed using the total CD4+ T cell isolation kit from Stem Cell Technologies, as per the manufacturer's recommendations. This kit isolates CD4+ T cells through negative selection by depleting cells expressing CD14, CD16, CD19, CD20, CD36, CD56, CD66b, CD123, and glycophorin A using magnetic bead conjugates and a magnet[299]. Briefly, PBMC are resuspended at $50x10^6$ cells per milliliter of RoboSep fluid, and 50 μL of negative selection antibody mixure were added per milliliter of sample. The sample and depletion cocktail were then mixed and incubated for 5 minutes, and then 50 μL of magnetic beads per milliliter of sample were added, and the sample was diluted with RoboSep buffer according to whether the EasySep™ or the BigEasy™ magnet was to be used. Cells were placed on the respective magnet for 5 minutes, allowing the negative selection beads to be drawn towards the outside of the tube due to the magnet field. After the

depletion beads equilibrated to the magnetic field and were firmly adhered to the outer interior portion of the tube, the supernatant containing total CD4+ T cells was removed. This procedure resulted in >95% purity of isolated total CD4+ T cells (see flow cytometry materials and methods below). The isolation of resting CD4+ T cells was performed in two separate ways. For initial studies, total CD4+ T cells were purified by negative selection using the CD4+ T cell Isolation Kit II from Miltenyi Biotec, according to the manufacturer's recommendations, followed by resting CD4+ T cells isolation from total CD4+ T cells by negative selection using anti-CD69, anti-CD25 and anti-HLA-DR antibodies from Miltenyi to deplete activated cells[300]. In later studies, a custom kit from Stem Cell Technologies containing all the antibodies described above for the isolation of total CD4+ T cells plus antibodies to CD25, CD69, and HLA-DR was used to isolate resting CD4+ T cells directly from PBMC following the same procedure described above for isolation of total CD4+ T cells from Stem Cell Technologies. Both of the methods used to isolate resting CD4+ T cells resulted in >95% purity by flow cytometry (see below). The method used in each section of this dissertation is clearly indicated.

Routine cell culture conditions. Routine cell culture is here defined as any cell culture that does not require limiting dilution or viral outgrowth. Isolated cells (e.g., PBMC, total CD4+ T cells, and resting CD4+ T cells) were cultured at $1x10^6$ cells per milliliter in 1 milliliter of culture medium in 48 well plates. Cells were not cultured in the exterior row of the culture plates; these wells were filled with PBS to prevent evaporation of culture medium. Cells were cultured in RPMI-1640 without the phenol red indicator and containing 10% (vol/vol) fetal bovine serum, 1% L-glutamine and 0.6% penicillin/streptomycin. To prevent viral replication, HIV-1 infected cells were cultured in 300 nM efavirenz. For pooled virus recovery assays, up to 6 replicate wells

containing $1x10^6$ total or resting CD4+ T cells were activated with phorbol myristate acetate (PMA; 50ng/mL) and ionomycin (500ng/mL) for 7 days, and then the culture supernatants were removed, pooled, and frozen at -80°C for downstream assessment of virion-associated HIV-1 RNA. Alternatively, we also performed cultures in 6 well plates, with $15x10^6$ PBMC per well in 5 milliliters of culture medium and 24 well plates with $5x10^6$ resting CD4+ T cells per well in 2 milliliters of culture supernatant in the presence of 100 nM efavirenz and 100 nM elvitegravir; these cultures were similarly activated with PMA/ionomycin, but were harvested after either 5 days (PBMC) or 7 days (resting CD4+ T cells). In some instances, cells were reactivated with anti-CD3/CD28 microbeads at a concentration of 3 beads per cell. The culture conditions used for various experiments are clearly stated in the text of this dissertion. Cellular viability was confirmed on the day of harvest using CellTiter-Fluor viability assay, as per the manufacturer. Importantly, cells were counted by hemocytometer prior to CellTiter-Fluor viability assays to ensure that equivalent numbers of cells were assessed for fluorescence viability.

Limiting dilution cultures to quantify fractional provirus expression. To assess the fraction of proviruses that have been reactivated by different treatment conditions, it is necessary to perform a limiting dilution culture setup such that there will be wells that are positive and negative for the viral outcome being assessed (e.g., cellular HIV-1 RNA transcription, virion production, infectious virion production)[301]. This was achieved in two separate formats. The first format was 12 replicate wells (10 wells of treatment condition and 2 wells of medium control) in 1 milliliter of culture medium in 48-well plates beginning at 667,000 resting CD4+ T cells per well, and diluting serially, 3-fold, down to 304 cells per well. In these experiments, resting CD4+ T cells were activation with magnetic beads coated with antibodies to CD3 and CD28 (anti-CD3/CD28

activation beads). Subsequently, we setup experiments with fewer replicates (5 replicates per dilution) and beginning at $1x10^6$ resting CD4+ T cells per well, and diluting serially, 3-fold, down to 457 cells per well. Supernatants were harvested after 7 days of culture for all experimental conditions, and were frozen at -80°C for downstream assessment of virion-associated HIV-1 RNA. The number of proviruses present per well at each dilution was determined by qPCR for total HIV-1 DNA (see Quantification of cellular proviral DNA below), and a maximum likelihood estimate was used to determine the fraction of proviruses that have been reactivated with any given treatment (see statistical analysis section below).

Latency reversal agents. To assess the potency of latency reversing agents for their ability to reactivate proviruses compared with no stimulation and full T cell activation controls, we first pulse treated resting CD4+ T cells with latency reversal agents in a dose- and time-dependent manner reflective of compound exposure that would be achieved with *in vivo* dosing. Resting CD4+ T cells were first pulse-treated with latency reversing agents in 25 cm^2 flasks in 10 milliliters of medium, and were then washed, counted, serially diluted and cultured for 7 days to allow the release of virions into the culture supernatant. The specific concentrations of latency reversal agents, and the timing of the pulse treatments, are described in the results section of Chapter 3. When assessing whether latency reversal agents had any effect on the size of the latent reservoir, a fraction of latency reversing agent-treated resting CD4+ T cells were washed to remove residual compound, and cultured for 5 days in 25 cm^2 flasks. These cells were then counted, serially diluted and maximally activated for 7 days with either anti-CD3//CD28 activation beads or PMA and ionomycin, for a total of 13 days in culture. CellTiter-Fluor cell

viability assays and staining for the assessment of activation marker expression by flow cytometry was performed on days 5 and 13, as described below.

Quantitative viral outgrowth assays. Quantitative viral outgrowth assays were performed on isolated total and resting CD4+ T cells using the methods described in Siliciano and Siliciano, 2005[302]. Briefly, prior to the isolation of patient-derived resting CD4+ T cells or total CD4+ T cells, PBMC from an HIV-negative donor were isolated and CD8-depleted, then activated with PHA to generate target cells for viral outgrowth. On the day of the blood draw from the HIV-positive participant, another blood draw from a negative donor was performed, and whole blood from the HIV-negative donor was gamma-irradiated to generate feeder cells. Once the patient-derived resting or total CD4+ T cells were isolated by negative selection (see above for isolation method), they were then serially diluted in 5-fold serial dilutions beginning with 10^6 cells per well. A total of 12 replicate wells of 10^6 cells per well, and two replicate wells of all subsequent dilutions were utilized for viral outgrowth. The patient-derived cells were mixed with the irradiated feeder cells and allogeneic blasts, and the culture was carried out for up to 21 days, with weekly splitting of the cultures and addition of fresh donor blasts to serve as target cells. Viral outgrowth was scored in each well as positive or negative by p24 ELISA on days 14 and 21. A maximum likelihood estimate for the number of cells responsible for infectious virus production was then evaluated (see statistical analysis section below).

Quantification of virion-associated HIV-1 RNA in culture supernatants. To assess levels of virion-associated HIV-1 RNA in the cell culture supernatants, supernatants were harvested after pre-specified culture times, and were spun at 500xg for 5 minutes to remove cellular debris.

Supernatants were then frozen at -80°C until assessment of virion-associated HIV-1 RNA was performed. Supernatants were then diluted two-fold (for assessment of fractional provirus expression) or were run undiluted (for pooled virus recovery), and HIV-1 RNA was measured by quantitative real-time polymerase chain reaction (qRT-PCR) using the Roche COBAS AmpliPrep/TaqMan version 2.0. The limit of detection was 40 copies of HIV-1 RNA per milliliter of sample for fPVE experiments, and was 20 copies of HIV-1 per milliliter of sample for all pooled virus recovery samples (as these samples were not diluted). Previous control experiments showed that centrifugation of culture supernatants at 24,000xg for 1 hour was sufficient to pellet the virion-associated HIV-1 RNA. Further evidence of the HIV-1 RNA being virion-associated was the fact that it was resistant to DNase I treatment[300].

Quantification of low-level plasma viremia. Plasma viremia (HIV-1 RNA) was quantified in three different ways. First, routine qRT-PCR using the Roche COBAS AmpliPrep/TaqMan version 2.0 platform was performed to assess viral load down to 20 copies per milliliter of plasma. Two distinct single copy HIV-1 RNA assays were also used. The original single copy assay (referred to as "gSCA" due to the fact that the PCR target is gag) was performed as described[112]. A newer single copy assay (refered to as "iSCA" due to the fact that the PCR target is integrase) was also developed during the course of this dissertation[303], and uses improved extraction methods and primers/probe targeting a more highly conserved region of the HIV-1 genome compared to the original gSCA. Large volume adaptations of the iSCA ("mega-iSCA") were implemented in studies in this dissertation to lower the limit of detection and to provide a more accurate assessment of low level viremia, as previously described[303]. The specific assay used, of the three available, is clearly indicated in the text of this dissertion.

Quantification of cellular unspliced HIV-1 RNA and total HIV-1 DNA. Quantification of cellular unspliced HIV-1 RNA and total HIV-1 DNA was performed as previously described[300,304,305]. Briefly, nucleic acid was isolated from $2x10^6$ million by first pelleting the cells, then adding guanidinium hydrochloride and proteinase K solution, followed by sonication and then incubation at 42°C for 1 hour. After this incubation period, guanidinium isothiocyanate was added with glycogen, and an equal volume of 100% isopropanol was added to the extract, followed by centrifugation at 21,000xg for 15 minutes. Next, the supernatant was aspirated, leaving the glycogen/nucleic acid pellet. To wash the pellet, 1 milliliter of 70% ethanol was added, and the sample was once again spun at 21,000xg for 15 minutes. The extract was then resuspended and split into two fractions; one fraction was frozen at -80°C for downstream quantification of DNA, and the other was treated with DNAase I to remove all cellular and HIV-1 DNA. This DNAase treated sample was then re-extracted as described above (without the addition of glycogen). For quantification of HIV-1 DNA, real-time qPCR was performed in 25 μL reactions with 10 μL of input template. If the concentration of the DNA extract was greater than 170 ng/μL by spectroscopy at A260, the sample was diluted to 170 ng/μL. Triplicate HIV-1 DNA reactions using primers/probe targeting integrase[303] were performed, and duplicate reactions targeting CCR5 were performed to normalize for input cell number[306]. For quantification of unspliced HIV-1 RNA, a two-step real-time qPCR was used, with triplicate reactions for HIV-1 RNA targeting integrase[303] and duplicate reactions for the housekeeping gene *IPO8*[300]. If the final concentration of RNA in the extract was greater than 30 ng/μL, the extract was diluted to 30 ng/μL. cDNA synthesis was performed with 10 μL of input RNA in 20 μL of reverse transcription master mix, and real-time qPCR was carried out using the entire

cDNA reaction with 30 μL of LightCycler480® Probes Master real-time PCR cocktail. The iSCA primer probe pair also amplified Vif RNA, but this accounts for approximately 0.21% of the family of 4.0-kb spliced RNA species, and therefore minimally contributes to the measurement of unspliced cellular HIV-1 RNA[307].

Flow cytometric analysis. To assess cell surface phenotypes, we performed flow cytometry using the follow fluorophore conjugated antibodies: CD3-PacBlue, CD4-FITC, CD8-PerCP/Cy5.5, CD25-PE, PD-1-PE/Cy7, HLA-DR-APC. All antibodies were purchased from BD Biosciences. Prior to cell surface staining, cells were stained with a Live/Dead Aqua dye, as per the manufacturer's recommendation. Cells were then washed and stained with fluorochrome conjugated antibodies in stain buffer from BD. Cells were then washed, and fixed with CytoFix™ fixation buffer from BD for 15 minutes on ice prior to running the fixed cells on an LSRII flow cytometer. Acquisition was perfomed using FACS Diva on the LSRII. A minimum of 10,000 events were acquired for all experiments. Cells were first gated on singlet live lymphocytes, followed by populations of interest. Analysis and gating was performed using FlowJo.

Statistical analysis and generation of figures. All figures were generated in GraphPad Prism, with the exception of the correlograms which were generated using R[308], using the reshape[309] and ggplot2[310] packages. All statistical analysis was performed using R[308]. Fractional provirus expression was quantified by using a maximum likelihood estimate in R through a generalized linear model with a binomial distribution and a log link function[300]. Paired and unpaired Wilcoxon signed rank T tests were used to assess differences between groups. Spearman's

correlation was used to assess correlative relationships, and Pearson's correlation was sparingly used to generate 95% confidence intervals for correlation coefficients to compare results between patient groups. Mixed linear effects analysis was performed using the lme4 package[311], with the exception of a preliminary study where the nlme package[312] in R was used; this is noted where appropriate in the body of this dissertation. Type III ANOVA was performed using the Companion to Applied Regression package[313] in R. False discovery rates were corrected using the method of Benjamini and Hochberg[314]. Post-hoc power assessments were performed using the Basic Functions for Power analysis package[315] in R. In all cases, two-sided tests were used, and $\alpha<0.05$ was considered statistically significant. All values that were below the limit of detection for any assay were interpolated as 50% of the limit of detection for the given assay.

4.0 CHAPTER 1. DEVELOPMENT OF A LIMITING DILUTON CULTURE ASSAY TO QUANTIFY HIV-1 LATENCY REVERSAL IN RESTING CD4+ T CELLS FROM PATIENTS ON SUPPRESSIVE ART

Anthony R. Cillo[1], Michele Sobolewski[1], Ronald Bosch[2], Elizabeth Fyne[1], Michael Piatak, Jr.[3], John Coffin[4], John W. Mellors[1]

[1]Division of Infectious Diseases, School of Medicine, University of Pittsburgh; [2]Department of Biostatistics, School of Public Health, Harvard University; [3]AIDS and Cancer Virus Program, SAIC-Frederick, Inc, National Cancer Institute; [4]Department of Molecular Biology and Microbiology, School of Medicine, Tufts University

4.1 PREFACE

A portion of the work described in this chapter has been previously published[300], and is cited where appropriate. As per the *Proceedings of the National Academy of Sciences*, authors retain the copyright of individual articles, while the *Academy* retains an exclusive license to publish these materials. This work has also been presented in part at the Conference on Retroviruses and

Opportunistic infections in 2013 as part of a themed discussion. This work was funded by the Pitt AIDS Research Training Program 5 T32 AI065380-08, the Science Applications International Corporation (SAIC) 25XS119 through the National Cancer Institute, and by the American Cancer Society with support from the F.M. Kirby Foundation.

This work presented in this chapter is in partial fulfillment of dissertation aim 1. John Mellors, John Coffin, and Anthony Cillo designed the experiments; Anthony Cillo, Michele Sobolewski, and Elizabeth Fyne performed the experiments; and Anthony Cillo, John Mellors and Ronald Bosch analyzed the data. Anthony Cillo and Ronald Bosch performed statistical analysis. All other authors contributed technically, conceptually or intellectually to the development of the project described in this chapter.

4.2 INTRODUCTION

As described in the introduction to this dissertation, small molecule latency reversing agents (LRAs) are being rigorously investigated both *ex vivo*[273,295-297,316] and *in vivo*[251-253,255-257,317]. To date, *ex vivo* studies have sought to evaluate whether a given LRA is capable of disrupting known mechanisms of proviral latency by measuring either cellular HIV-1 RNA as a marker of viral gene expression or virus production from cultured peripheral blood mononuclear cells or isolated total or resting CD4+ T cells. Histone deactylase inhibitors (HDACi), most notable suberoylanilide hydroxamic acid (SAHA)[249,250], have been used most extensively *ex vivo* and in several clinical trials[251-253,257,317]. HDACi have been chosen for evaluation clinically based on evidence that they disrupt the nucleosome structure surrounding the HIV-1 LTR, allowing access

of transcriptional machinery and transcription of HIV-1 RNA[318], as described in the Introduction to this dissertation. Clinically, latency reversal has been assessed by measures HIV-1 transcription in PBMC, or as increases in low level plasma viremia.

While changes in transcription of HIV-1 RNA or levels of plasma viremia can be readily measured, it is currently unclear how these results should be interpreted. Several problems exist with currently used methods for the evaluation of latency disruption. First, from bulk measures of cellular HIV-1 RNA or virion-associated HIV-1 RNA from culture, it is not possible to ascertain whether levels of transcription or virion production have been increased *de novo*, or whether LRAs are merely modulating transcription or virion production from cells that are already expressing viral RNA or virions. Second, the potency of latency reversal agents cannot be readily compared at the level of individual proviruses due to the fact that varying numbers of proviruses are likely to produce virions in a bulk culture. Specifically, the same number of total cellular HIV RNA molecules or virions could be produced from a few or many proviruses. Therefore, we sought to develop an *ex vivo* culture system in which LRAs could be compared at the level of individual proviruses[300].

4.3 RESULTS

4.3.1 Establishment of a limiting dilution culture assay to quantify reversal of HIV-1 latency

To evaluate the magnitude of *de novo* latency reversal that is attainable with maximum activation or with latency reversing agents, we have elected to measure the proportion of proviruses detectable by quantitative polymerase chain reaction (qPCR) that can be reactivated with any given treatment. Quantification of HIV-1 latency reversal at the level of individual proviruses requires the segregation of HIV-1 infected cells capable of producing virions into individual replicate wells in a serial dilution culture experiment. The precedent and groundwork for this experimental design is based a relatively crude assay format that was first used in 1989[99] to quantify levels of infectious virus from plasma and peripheral blood mononuclear cells of HIV-1 infected patients. Improvements to the statistical underpinnings of dilution culture assays was described in 1994 by Myers et al. who developed a maximum likelihood framework based on a parametric binomial distribution for assessing the number of events in a given cell population[301]. In the case described by Myers et al., an event is a cell harboring an HIV-1 provirus that is replication competent. The maximum likelihood estimation for a limiting dilution culture assay can be described by a simple equation (Figure 1), where the likelihood is equal to n choose x times one minus e to the negative C times H, times e to the negative C times H. In this equation, C is a parameter value (in our case, the fraction of proviruses that have been reactivated) that is varied to find the maximum likelihood, and H is the number of proviruses per well. Alternative methods to identify the number of events in a cell populations (or any given population) rely on

identifying dilutions in which ~50% of tested reactions are positive, or identifying the endpoints of different assays. However, these methods are much less robust than maximum likelihood estimates, and have notable biases (see the discussion in this chapter for details). The method is described graphically in Figure 2, and can be readily adapted to other assay formats (i.e., the fraction of proviruses that have been reactivated to express unspliced cellular HIV-1 RNA).

$$\text{Likelihood} = \prod_{d=1}^{D} \left(\frac{n_d!}{x_d!(n_d - x_d)!} \right) p^{xd}{}_d \, q^{nd-xd}{}_d$$

$$= \prod_{d=1}^{D} \left(\frac{n_d!}{x_d!(n_d - x_d)!} \right) (1 - e^{-CH}) e^{-CH}$$

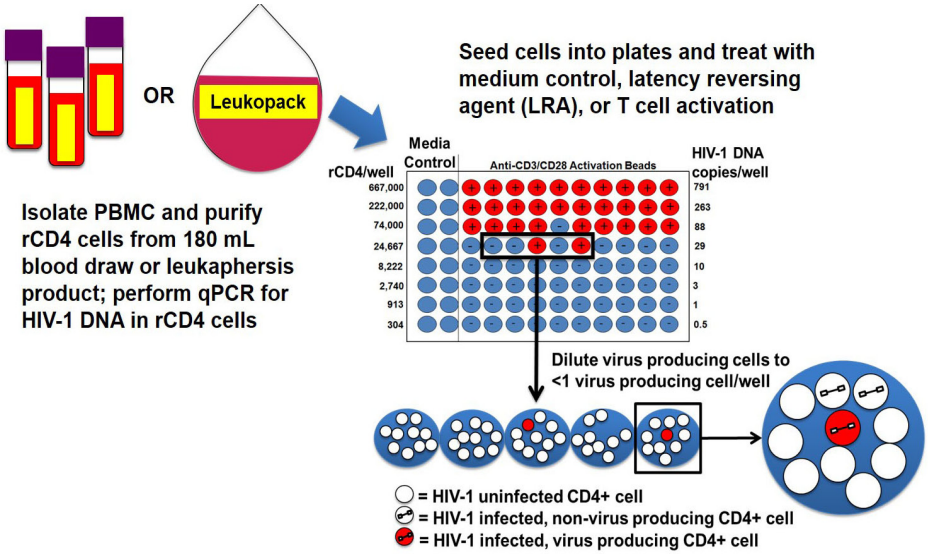
Figure 1. Likelihood equation for a limiting dilution culture assay. The likelihood is equal to the product of dilution one through dilution D of n choose x times the probability of a positive well, times the probability of a negative well at a given dilution. By a first order Taylor approximation, this is equal to e^{-CH} minus one times e^{-CH}, where C is the fraction of proviruses that have been reactivated and H is the number of proviruses per well.

In figure 2, the plot showing the log likelihood demonstrates a varied value of C as described above, where C is the fraction of proviruses that have been reactivated to produce virions. The value of C that maximizes this likelihood plot is the maximum likelihood estimate for fractional provirus expression (fPVE).

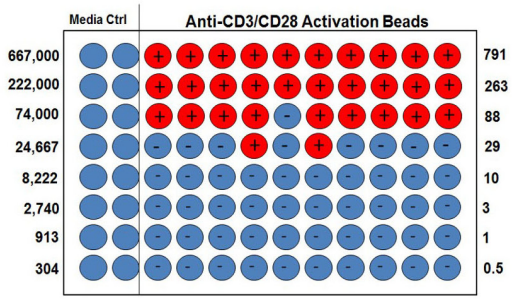
This assay platform can be used to assess the fraction of proviruses that can produce virions in the absence of stimulation, following full T cell activation, or following treatment with

any given latency reversal agent. Using the Roche COBAS AmpliPrep/TaqMan v2.0 platform further facilitates the assay in a higher throughput format, as cell culture supernatants can be removed and up to 42 samples can be run in parallel by automated nucleic acid extraction and qRT-PCR. It is worth noting that the production and release of a virion only requires the expression of gag protein[319], so using qRT-PCR as an endpoint measures virions that contain genomic HIV-1 RNA. This assay is not only applicable to the endpoint of virion production, but could also be used to assess the proportion of proviruses that are transcribing HIV-1 RNA in the absence of stimulation, with full T cell activation or following treatment with any given LRA. For these experiments, we elected to culture the rCD4 cells for 7 days for all conditions, as preliminary experiments demonstrated that virion production peaked at 7 days following T cell activation (data not shown). To assess the potency of SAHA as an LRA, we cultured rCD4 cells with constant exposure to 0.5 μM SAHA for 7 days of culture.

A



B



Proviruses per well	Number of Replicate Wells	Number of Wells with HIV-1 RNA >20 copies
791	10	10
263	10	10
88	10	9
29	10	2
10	10	0
3	10	0
1	10	0
0.5	10	0

C

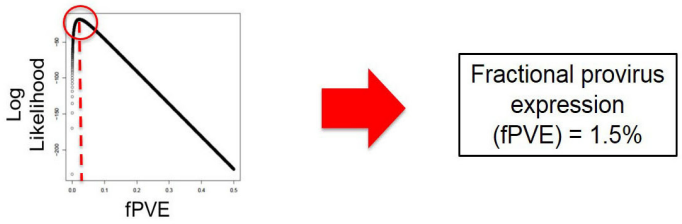


Figure 2. Dilution culture assay to quantify the frequency of proviral latency reversal by a maximum likelihood estimate. **A)** Peripheral blood mononuclear cells are first isolated from large volume blood draws (as in this study) or leukapheresis product. Resting CD4+ T cells are then isolated by negative selection, serially diluted, and seeded into plates. Total HIV-1 DNA in resting CD4+ T cells is assessed by qPCR. Supernatants are harvested from wells after 7 days in culture, and HIV-1 RNA is quantified by Roche COBAS AmpliPrep/TaqMan. As the number of cells per well decreases, the number of inducible proviruses increases per well until an endpoint is reached. **B)** The number of proviruses per well and the number of positive wells are then tabulated. **C)** The tabulated table is then used to perform a maximum likelihood estimate in R using a generalized linear model with a binomial distribution and a log link function. The red circle represents the maximum likelihood, and the fPVE value at this point is the resulting fPVE from the dilution culture data, in this case yielding an fPVE of 1.5% of proviruses have been reactivated to produce virions.

4.3.2 Validation of fractional provirus expression for quantification of latency reversal

Following the theoretical framework described above, we sought to evaluate whether a limiting dilution culture-based assay could be used to ascertain the fraction of proviruses that had been reactivated following any given *ex vivo* treatment of samples obtained from HIV-1 infected patients on long-term suppressive ART. To evaluate fPVE *ex vivo*, we first performed large-volume phlebotomy (180 mL), and isolated peripheral blood mononuclear cells (PBMC) by ficoll-density gradient centrifugation from N=13 study participants on long-term suppressive ART (Table 1). Study participants were male and female, had a mean age of 54 years old, and were suppressed for a mean of 8 years (ranging from 2 to 16 years) on a variety of ART regimens.

Table 1. Participant characteristics for establishment of the fractional provirus expression assay

Participant ID	Sex	Age	Current CD4+ count (cells/mm^3)	Current antiretroviral therapy	Years plasma HIV-1 RNA <50 copies per milliliter
1	Female	56	846	EFV/FTC/TDF	12
2	Male	57	563	ETR/FTC/RAL/TDF/ZDV	4
3	Female	45	477	DRV/FTC/r/TDF	2
4	Female	59	825	EFV/FTC/TDF	7
5	Female	55	1,373	EFV/FTC/TDF	12
6	Male	57	774	EFV/FTC/TDF	3
7	Male	53	1,111	3TC/ABC/NVP/ZDV	10
8	Male	50	380	EFV/FTC/TDF	11
9	Male	65	748	EFV/FTC/TDF	4
10	Male	57	934	EFV/FTC/TDF	16
11	Male	51	534	3TC/ABC/RAL	16
12	Male	43	367	3TC/ABC/RAL	3
13	Female	51	1,131	FTC/RAL/TDF	2
Mean ± SD	ND	54 ± 6	774 ± 309	ND	8 ± 5

SD = standard deviation; ND = not done; 3TC = lamivudine; ABC = abacavir; DRV = darunavir; EFV = efavirenz; ETR = etravirine; FTC = emtricitabine; NVP = nevirapine; r = ritonavir; RAL = raltegravir; TDF = tenofovir disoproxil fumarate; ZDV = zidovudine

From PBMC, we then isolated resting CD4+ T cells (i.e., CD3+CD4+CD25-CD69-HLA-DR-) by sequential bead-based isolation. First, total CD3+CD4+ T cell were isolated by negative selection using the T Cell isolation Kit II from Miltenyi. From these isolated total CD4+ T cells, resting CD4+ T (rCD4) cells were isolated by depleting CD25+/CD69+/HLA-DR+ cells by magnetic separation using Miltenyi columns. This isolation method routinely resulted in >95% purity of isolated rCD4 cells (data not shown). Isolated rCD4 cells were chosen for analysis based on their relative enrichment as reservoir cells for HIV-1[124,126]. These rCD4 cells are quiescent, and are considered to be long-lived cells that facilitate life-long persistent infection of HIV-1 by harboring latent, intact proviruses[124,128,131]. Results for assessment of low-level viremia and total HIV-1 DNA in rCD4 cells are shown in Table 2. We have found previously that 2-LTR circles contribute in a minor way (<5%) to the detection of total HIV-1 DNA in samples isolated from those on suppressive ART (data not shown), and as such total HIV-1 DNA is a close approximation of integrated HIV-1 DNA. At the time of the genesis of this study, a prominent clinical trial had also investigated transcription of cellular HIV-1 RNA in rCD4 cells as an endpoint[251] after treatment with vorinostat, with the hypothesis that increases in cellular HIV-1 RNA in rCD4 cells would represent true disruption of the latent reservoir.

Table 2. Plasma viremia and total HIV-1 DNA in resting CD4+ T cells for establishment of the fractional provirus expression assay

Participant ID	Plasma HIV-1 RNA by Roche COBAS AmpliPrep/TaqMan (HIV-1 RNA copies per milliliter)	Low-level plasma HIV-1 RNA by the single copy assay (HIV-1 RNA copies per milliliter)	Total HIV-1 DNA in resting CD4+ T cells (copies per 10^6 cells)
1	TND	<0.25	136
2	<20	1.5	1,458
3	<20	<0.25	1,110
4	TND	1.8	1,551
5	TND	7.2	2,678
6	<20	6.0	887
7	<20	2.2	350
8	TND	1.3	978
9	TND	2.8	1,957
10	TND	1.8	2,849
11	<20	2.1	2,132
12	TND	3.1	1,503
13	<20	3.3	1,128
Mean ± SD	ND	2.6 ± 2.0	1,440 ± 811

SD = standard deviation; ND = not done

We sought to evaluate fPVE by maximum likelihood estimation in N=13 donors, focusing on medium control and anti-CD3/CD28 treatment in all 13 donors and evaluating the potency of putative LRA vorinostat (suberoylanilide hydroxamic acid; SAHA) compared to medium control and anti-CD3/CD28 activation beads in N=5 donors (Figure 3, Table 3). rCD4 cells were cultured for 7 days with medium, anti-CD3/CD28 activation beads, and continuous exposure to 0.5 μM SAHA.

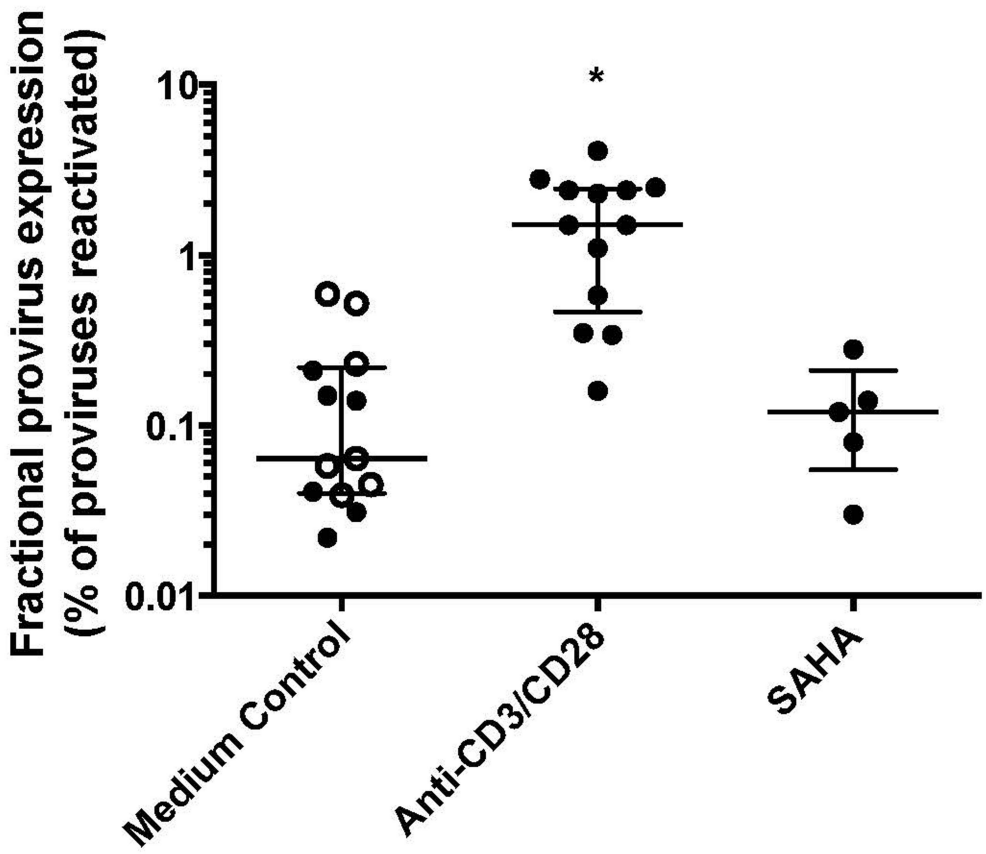


Figure 3. Levels of fractional provirus expression with medium control, anti-CD3/CD28 and SAHA Treatments. From N=13 donors, we found that the median fraction of proviruses that produced virions for medium only treatment, anti-CD3/CD28 treatment and SAHA treatment was 0.041%, 1.5% and 0.12%, respectively. These results are consistent with HIV-1 latency in that few virions were produced in the absence of stimulation, and full T cell activation led to high levels of virion production. However, only a small proportion of proviruses could be maximally reactivated, and a much smaller fraction of proviruses were reactivated with SAHA treatment. A linear mixed effects model was used with the nlme package[312] in R to assess differences between treatment groups, with treatment as a fixed effect and the individual participants as a random effect. This model was significant, and anti-CD3/CD28 was the only significant variable ($p<0.001$, and an asterisk indicates this statistical significance). Some samples did not have detectable virion production at the lowest cell dilutions, and therefore had undetectable fPVE for virion production. The fPVE values for undetectable samples were calculated by assuming that one well at the lowest dilution was positive. Open symbols denote samples that had undetectable fPVE for virion production.

These results reveal the median fraction of proviruses in rCD4 cells that produce virions in the absence of stimulation was 0.041%, the median maximal fraction of proviruses that could be reactivated to produce virions with one round of full T cell activation was 1.5%, and the median fraction of proviruses that were reactivated to produce virions following treatment with SAHA was 0.12%. Individual results from each donor are tabulated in Table 3. In order to ascertain the statistical significance of these findings, a linear mixed effects model, with the treatment condition (i.e., medium control, anti-CD3/CD28, SAHA) as fixed effects and the individual participants as random effects. A mixed effects model was chosen over an analysis of variance (ANOVA) for the analysis of these data due to unbalanced design and the repeated measures; a modified ANOVA controlling for the unbalanced design and repeated measures could be used, but a linear mixed effects model is more flexible and requires less assumptions, and was chosen for these reasons.

This mixed effects analysis revealed that the only significant treatment group in this model was anti-CD3/CD28 treatment ($p<0.001$) while medium control fPVE ($p=0.46$) and SAHA fPVE ($p=0.93$) were not significantly different from each other.

Table 3. Quantification of HIV-1 latency reversal using fractional provirus expression for virion production

Participant ID	Medium Control fPVE	Anti-CD3/CD28 fPVE	SAHA fPVE
1	<0.52%	2.4%	ND
2	<0.058%	1.5%	ND
3	<0.064%	0.58%	ND
4	<0.045%	1.5%	ND
5	<0.039%	0.34%	ND
6	<0.23%	1.1%	ND
7	<0.59%	0.16%	ND
8	0.21%	0.35%	ND
9	0.031%	2.4%	0.12%
10	0.022%	2.5%	0.08%
11	0.14%	2.3%	0.28%
12	0.041%	2.8%	0.03%
13	0.15%	4.1%	0.14%
Median (IQR)	0.041% (0.03% to 0.15%)	1.5% (0.6% to 2.4%)	0.12% (0.08% to 0.14%)
P value	0.46	<0.001	0.93

Next, we sought to quantify latency reversal by assessing the fraction of proviruses that were reactivated to transcribe unspliced cellular HIV-1 RNA in various treatment conditions. A benefit of using the limiting dilution culture is that multiple endpoints for viral reactivation can be evaluated for fPVE, including the fraction of proviruses that are producing virions. To evaluate the fPVE for cellular HIV-1 RNA, nucleic acid was isolated from cultured rCD4 cells (see Overall materials and method section for details) after 7 days of treatment with medium control, anti-CD3/CD28 activation beads or SAHA from the experiments described above. Total RNA was then reverse transcribed into cDNA, and cellular unspliced HIV-1 RNA was quantified by qRT-PCR along with IPO8 mRNA as an internal control. By quantifying cellular HIV-1 RNA from replicate wells at each dilution, the maximum likelihood estimate could be adapted to assess the fraction of proviruses that are transcribing HIV-1 RNA (Table 4).

Table 4. Quantification of HIV-1 latency reversal by fractional provirus expression for cellular HIV-1 RNA

Participant ID	Medium Control fPVE	Anti-CD3/CD28 fPVE	SAHA fPVE	SAHA fPVE / Anti-CD3/CD28 fPVE
12	0.37%	6.8%	0.093%	0.014
13	0.054%	8.2%	0.19%	0.023
Mean ± SD	0.21% ± 0.22%	7.5% ± 0.96%	0.14% ± 0.066%	0.018 ± 0.0067

SD = standard deviation

Evaluating fPVE for cellular HIV-1 RNA revealed that the mean levels of fPVE for medium control treated rCD4 cells was 0.21%, while maximum activation with anti-CD3/CD28 resulted in 7.5% of proviruses produced cellular HIV-1 RNA. SAHA treatment was found to reactivate 0.14% of proviruses, which is a very small fraction of proviruses compared to the fraction of proviruses that could be maximally reactivated to produce cellular HIV-1 RNA. The

median value for cellular HIV-1 RNA fPVE for SAHA was actually less than that for medium control. Of the two donors assessed for cellular HIV-1 RNA fPVE, one showed decreased fPVE following treatment with SAHA.

4.3.3 Assessment of relationships between virion production and cellular HIV-1 RNA transcription

Following the assessment of the fraction of proviruses that could be reactivated to produce virions or transcribe cellular HIV-1 RNA following treatment, we next asked whether levels of cellular HIV-1 RNA transcription were related to levels of virions produced following one round of T cell activation with anti-CD3/CD28 activation beads or following treatment with SAHA (Figure 4).

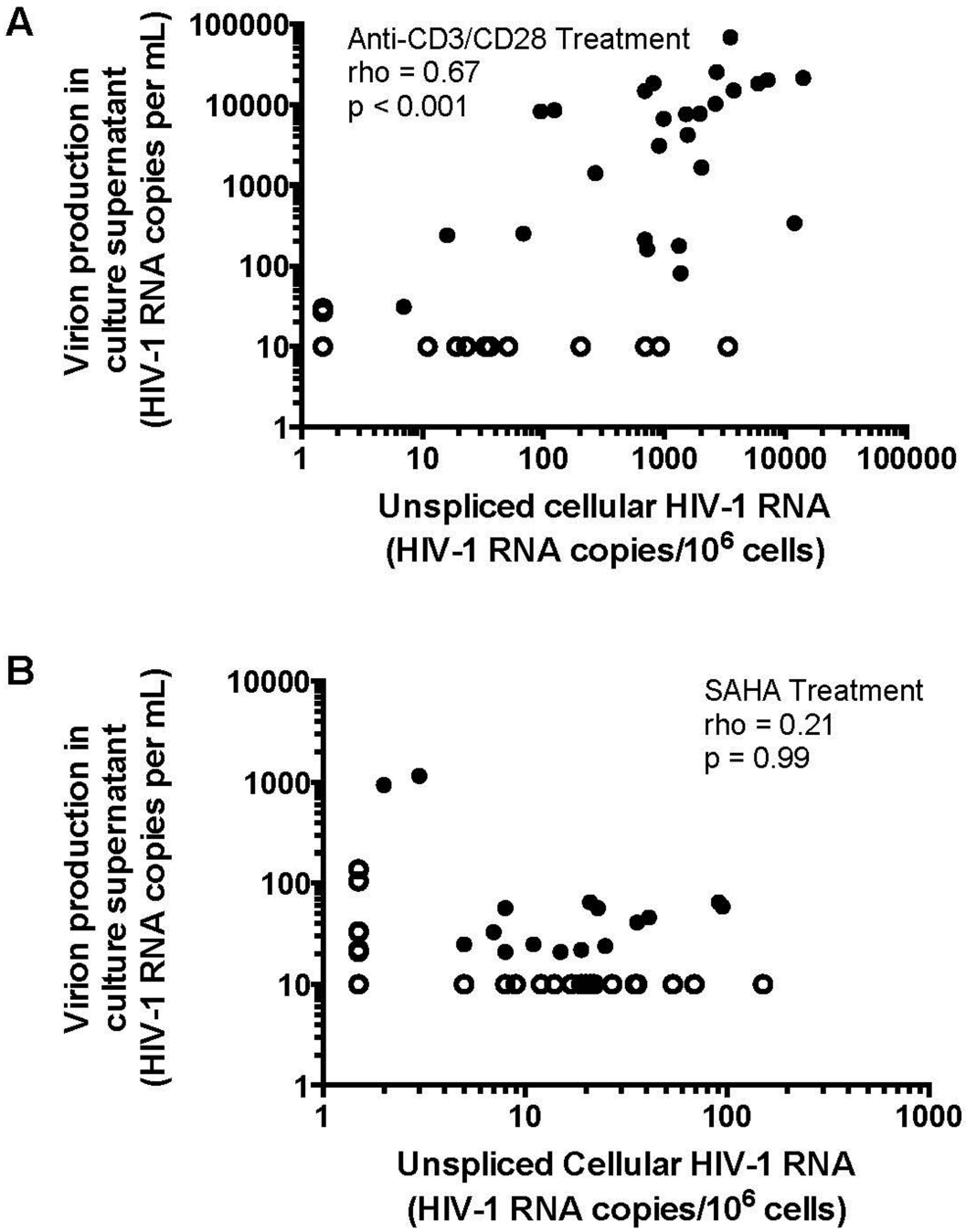


Figure 4. Relationships between virion production and cellular HIV-1 RNA expression. A) Virion production from anti-CD3/CD28 treated rCD4 cells is statistically significantly associated with expression levels of cellular HIV-1 RNA (rho=0.67, p<0.001, analysis corrected for repeated measures[320]). **B)** Virion production from SAHA treated rCD4 cells is unrelated to expression levels of cellular HIV-1 RNA (rho=0.21, p=0.99, analysis corrected for

repeated measures[320]). Open circles denote samples that were undetectable for virion production, unspliced cellular HIV-1 RNA, or both.

This analysis revealed that the levels cellular HIV-1 RNA were strongly correlated with virion production in the context of one round of T cell activation (rho=0.67, $p<0.001$), but that this relationship did not hold up following treatment with SAHA (rho=0.21, p=0.99). This finding is consistent with SAHA non-specifically altering cellular transcription, but not leading to virion production. The failure to produce virions despite the upregulation in expression of cellular HIV-1 RNA in rCD4 cells treated with SAHA may be due to the quiescent nature of rCD4 cells compared with activated CD4+ T cells.

4.4 DISCUSSION

In this series of experiments, we developed an assay to measure the fraction of proviruses that have been reactivated to produce virions or unspliced cellular HIV-1 RNA. The dilution culture assay format, combined with a rigorous maximum likelihood estimate statistical framework, can be readily used to assess the fraction of proviruses that have been reactivated to produce virions or cellular HIV-1 RNA following latency reversal with T cell activation or treatment with putative latency reversal agents. Alternative methods of assessing the frequency of an event based on a dilution culture assay with replicates scored by dichotomous results include methods by Spearman and Karber, Reed and Muench, Dragstedt and Behrens, Litchfield and Wilcoxon, and moving average nonparametric calculation methods[301,321,322]. These methods have generally been applied to the estimate the dilution at which 50% of the replicates would be expected to be positive (ED_{50}). The ED_{50} and frequency of events in a given population as determined by a

maximum likelihood estimate are inherently interchangeable, and can be readily calculated from each through any of the methods described above. However, in terms of performance characteristics and assumptions required by each model, the parametric method described by Myers et al. and adopted for fPVE is the most favorable of these assay formats.

Using this novel assay format, we found that the median fraction of proviruses that could be maximally reactivated was 1.5% in this small group of study participants, and that only 0.12% of proviruses could be reactivated to produce virions following treatment with SAHA. These data indicate that the vast majority of the latent reservoir is not reactivated following SAHA treatment.

We also assessed levels of fPVE for cellular HIV-1 RNA following treatment with anti-CD3/CD28 and SAHA, finding that up a median of 7.5% of proviruses were reactivated to transcribe cellular HIV-1 RNA following T-cell activation, whereas only 0.14% of proviruses were reactivated to transcribe cellular HIV-1 RNA following treatment with SAHA. In participant 12, the fPVE for cellular HIV-1 RNA transcription was 2.4-fold higher compared with virion production, while in participant 13 the fPVE for cellular transcription of HIV-1 RNA was 2-fold greater compared with virion production. However, with SAHA treatment, in participants 12 and 13, the fPVE for cellular transcription fPVE to virion production was 3.1-and 1.3-fold higher. Thus, there seems to be a consistent ratio between the levels of fPVE for viral transcription and virion production in the context of full T cell activation, but the ratio seems to vary following SAHA treatment.

The findings that full T cell activation only reactivates a small proportion of proviruses to produce virions is striking, and implies that 98.5% of proviruses cannot be reactivated to produce virions in one round of T cell activation. While a somewhat higher proportion of proviruses can

be reactivated to transcribe cellular HIV-1 RNA, these results still suggest that only a small number of the proviruses that are detectable by qPCR are capable of undergoing latency reversal following T cell activation. These results are consistent with recent findings by Ho et al., who found that the majority of integrated proviruses that are not replication competent harbor large deletions, are severely mutated by APOBEC, or are missing other key components of the genome[151]. The presence of proviruses that are intact, but that did not replicate in one round of activation in work by Ho et al. suggests that either i.) more proviruses might be able to produce virions following additional rounds of reactivation; or ii.) some of the intact proviruses that did not replicate did indeed produce virions, but these virions did not efficiently infect the allogeneic target cells and lead to subsequent cycles of replication. Which of these two outcomes explain the findings should be pursed in future studies.

The finding that SAHA does not lead to substantial virion production *ex vivo* from rCD4 cells compared with medium control is consistent with findings from Blazkova et al.[316], but differs from earlier findings by Archin et al. in which higher concentrations of cells and a less-sensitive p24 assay was used for detection of virions[249]. Clinical trials of SAHA have been mixed, with initial reports that single-dose SAHA reversed latency by measuring unspliced cellular HIV-1 RNA in rCD4 cells[251], while multiple doses of SAHA did not consistently up-regulate HIV-1 RNA transcription[252]. Elliot et al. found that multiple does of SAHA led to a protracted up-regulation of cellular HIV-1 RNA expression, both generally and for HIV-1 RNA[253], but subsequent modeling work showed minimal depletion of infected cells[323]. These studies have been consistent in that no study showed significant increases in plasma HIV-1 RNA following SAHA administration.

In this study, we found that there was a statistically significant relationship between levels of virion production and cellular HIV-1 RNA following treatment with anti-CD3/CD28 activation beads, but not following SAHA treatment. The relationship between cellular HIV-1 RNA transcription and virion production following T cell activation is likely due to a shift in T cell biology that occurs following activation, where the transcriptional and metabolic profiles of T cells change following activation[324,325]. This process of T cell activation leads to reversal of transcriptional blocks to latency (e.g., chromatin structure[326,327], release of sequestered key transcription factors[245,328]) and post-transcriptional blocks (e.g., transport of multiply-spliced Tat/Rev transcripts[329]) to proviral latency. Alternatively, the majority of unspliced cellular HIV-1 RNA transcripts that are produced following treatment with SAHA might be read-through transcripts[295] that will never be properly translated into HIV-1 proteins. Intriguingly, our results also potentially provide *ex vivo* evidence for an interpretation of the *in vivo* results of clinical trials of SAHA, where levels of cellular HIV-1 RNA are increased following treatment with SAHA, but no substantial virion release (as measured by virion-associated HIV-1 RNA in plasma) was observed following 7 days of culture.

Finally, these data demonstrate that SAHA reactivates a very minor proportion of the total inducible proviruses within the rCD4 cell population. More potent latency reversing agents or combinations of agents targeting multiple factors that contribute to HIV-1 latency will be necessary to achieve latency reversal on a similar magnitude to that achieved by T cell activation. However, care must be taken to find latency reversing agents that do not lead to T cell activation and subsequent cytokine release, as this would represent a significant risk to people who are otherwise healthy on stably suppressive ART. Using the methods described in this

chapter, we can assess the potency of putative LRAs at the level of individual proviruses to determine which (if any) are viable candidates for clinical use.

5.0 CHAPTER 2. INVESTIGATION OF PUTATIVE LATENCY REVERSAL AGENTS IN RESTING CD4+ T CELLS ISOLATED FROM PATIENTS ON SUPPRESSIVE ANTIRETROVIRAL THERAPY

5.1 PREFACE

Anthony R. Cillo[1], Michele Sobolewski[1], Taylor Simmons[1], Elizabeth Fyne[1], Joshua Cyktor[1], John W. Mellors[1]

[1]University of Pittsburgh, Division of Infectious Diseases

The work presented in this chapter is in partial fulfillment of Aim 1 of this dissertation. Anthony Cillo, Michele Sobolewski, Taylor Simmons, Elizabeth Fyne and Joshua Cyktor performed the isolation of resting CD4+ T cells from leukapheresis products, set up extensive limiting dilution cell culture experiments, harvested cell culture supernatants and cells following treatment, and assessed viability of cultured cells. Anthony Cillo, Michele Sobolewski, Elizabeth Fyne and Taylor Simmons performed COBAS AmpliPrep/TaqMan v2.0 for quantification of HIV-1 RNA from culture supernatants. Anthony Cillo performed qRT-PCR for cellular HIV-1 RNA and DNA. Joshua Cyktor performed and analyzed flow cytometry experiments for assessment of activation markers. Anthony Cillo and John Mellors conceived of the project, and Anthony Cillo analyzed the data and performed statistical analysis.

The work in this chapter was funded by the Pitt AIDS Research Training Program 5 T32 AI065380-08, a research grant from Gilead Sciences, and COBAS AmpliPrep/TaqMan kits provided by Roche.

5.2 INTRODUCTION

Following the establishment of an assay that is capable of determining the potency of latency reversal at the level of individual proviruses, we next sought to investigate putative latency reversal agents and to compare their potency to virion production from medium control and T cell activation conditions. We have chosen to evaluate virion production as the major endpoint in our assessment of latency reversal, as the production of virions guarantees that all blocks to proviral latency have been overcome. One could make an argument that infectious virion production should be measured, but assessment of viral outgrowth is technically challenging, particularly at higher cells dilutions, in addition to being labor- and time-intensive; so as such, we chose to evaluate total virion production as an endpoint. As described above, *ex vivo* studies have generally evaluated changes in cellular HIV-1 RNA as an endpoint to determine whether latency reversal has been achieved[273,295,296]. Some groups have also looked at virion production[295-297]. In order to achieve true latency reversal, at a minimum translation of viral proteins must occur. Transcription of cellular viral HIV-1 RNA does not necessarily mean that viral proteins have been translated, and if no viral proteins have been produced, the cell is likely still invisible to immune surveillance and therefore is still in a state of latency. It is also possible to have viral protein translation in the absence of virion production, but it is not currently

possible to evaluate viral protein expression at the sensitivity required for infected cells derived from study participants on suppressive ART. Therefore, we have chosen to focus on virion production, as increases in transcription of cellular HIV-1 RNA does not mean that latency has been reversed or that viral mRNAs have been translated into viral protein, while virion production can serve as a minimum estimate of the amount of HIV-1 protein production.

In this section, we extend our findings from preliminary assessments of fPVE to a more thorough evaluation of putative latency reversing agents targeting known mechanisms of proviral latency. The latency reversing agents we studied can be thought of as tools to evaluate whether known mechanisms of latency that have been described *in vitro* are relevant *ex vivo*, and whether these mechanisms can be targeted and latency reversed without causing global T cell activation. Quantifying the proportion of proviruses that can be reactivated to produce virions with latency reversing agents, compared with full T cell activation, will provide new insights into the potency of these LRAs at the level of individual proviruses.

5.3 RESULTS

5.3.1 Mechanisms of action of putative latency reversal agents studied

To assess mechanisms of latency in rCD4 cells *ex vivo*, we selected a number of latency reversing agents (LRAs) to test in our fPVE assay format. These compounds were selected based on the following criteria:

1. The LRA or combination of LRAs must target known factor(s) associated with proviral latency.

2. The LRA or combination of LRAs must not activate T cells.

3. The LRA or combination of LRAs must have a favorable pharmacokinetic and pharmacodynamic profile for *in vivo* dosing.

4. The LRA or combination of LRAs must not have significant toxicity *in vitro*, *ex vivo*, in animals, or in humans.

With the above criteria above in mind, we choose 6 putative latency-reversing agents, with 4 single agents (2 HDACi, a Brd4 inhibitor (Brd4i) and a PKC agonist) and 2 combinations of agents (HDACi and BRD4i combination, and an HDACi and PKC agonist combination). Table 5 summarizes the LRAs tested, their mechanisms of action, and the concentration and durations of exposure. The concentrations of LRAs used and the duration of treatment were chosen to mimic as closely as possible known or estimated *in vivo* pharmacokinetic and pharmacodynamics properties of the agents. Combination treatments were performed in the presence of both compounds, with the compound requiring a shorter duration of pulse treatment added at the end of the incubation with the first compound. The design of these experiments is more rigorous compared with other *ex vivo* studies that have used non-physiological durations and concentrations.

Table 5. Putative latency reversal agents, proposed mechanism of latency reversal, and *ex vivo* treatment conditions

Compound	General class of compound	Putative mechanism of latency reversal	Concentration of dose and duration of treatment *ex vivo*
Romidepsin	Histone deacetylase inhibitor	Relaxation of chromatin structure surrounding HIV-1 LTR[249,318]	40 nM, 4 hours[330]
Panobinostat			17.5 nM, 30 minutes[331](FARYDAK package insert)
JQ1	BRD4 inhibitor	Free P-TEFb for interaction with Tat[289]	25 μM, 30 minutes (JQ1 Users Guide, Bradner Lab)
Bryostatin	Protein kinase C agonist	Activation of NF-kB and interaction with HIV-1 LTR[291]	10 nM, 1 hour[332,333]
Romidepsin + JQ1	HDACi + BRD4i	Relaxation of chromatin[249,318] and free P-TEFb[289]	40 nM, 4 hours[330] + 25 μM, 30 minutes (JQ1 Users Guide, Bradner Lab)
Bryostatin + Romidepsin	PKC agonist + HDACi	Activation of NF-kB[291] and relaxation of chromatin[249,318]	10 nM, 1 hour[332,333] + 40 nM, 4 hours[330]

5.3.2 Patient characteristics and latency reversal agents studied

After selecting the LRAs listed above, we next assessed the potency of each compound compared with medium control and full T cell activation by anti-CD3/CD28 antibody-coated beads. To do this, we required large numbers of resting CD4+ T cells; accordingly, we enrolled N=9 study participants who were willing to undergo leukapheresis to obtain sufficient PBMC for isolation of adequate numbers of resting CD4+ T cells for the simultaneous assessment of multiple LRAs and controls. Participant characteristics are shown in Table 6. Four males and five females were enrolled, with Caucasian and African American races represented. The mean

age of the study participants was 50 years old, and all study participants had high CD4+ T cell counts at the time of leukapheresis. All study participants were on suppressive ART (plasma viremia <50 copies per milliliter of plasma) for a mean of 8 years, ranging from 3 years to 17 years with. The participants were on a variety of ART regimens (Table 6).

Table 6. Participant characteristics for the evaluation of putative latency reversing agents

Participant ID	Sex	Race	Age	Current CD4+ count (cells/mm^3)	Current antiretroviral therapy	Years plasma HIV-1 RNA <50 copies per milliliter
1	Male	Caucasian	51	ND	3TC/ABC/RAL	17
2	Female	Caucasian	45	ND	EFV/FTC/TDF	3
3	Female	African American	36	809	FTC/RPV/TDF	10
4	Female	African American	57	380	ATV/FTC/r/TDF	5
5	Male	Caucasian	51	603	EVG/c/FTC/TDF	7
6	Male	Caucasian	42	ND	3TC/RAL/ZDV	8
7	Male	Caucasian	46	714	3TC/ABC/EFV	5
8	Female	African American	57	1426	FTC/RPV/TDF	15
9	Female	African American	62	1033	DTG/FTC/TDF	10
Mean ± SD	ND	ND	50 ± 8	828 ± 365	ND	9 ± 5

SD = standard deviation; ND = not done; 3TC = lamivudine; ABC = abacavir; ATV = atazanavir; c = cobicistat; DTG = dolutegravir; EFV = efavirenz; EVG = elvitegravir; FTC = emtricitabine; r = ritonavir; RAL= raltegravir; RPV = rilpivirine; TDF = tenofovir disoproxil fumarate; ZDV = zidovudine

From the leukapheresis product, rCD4 cells were isolated by negative selection in a single step from Ficoll-Hypaque purified PBMC. This single-step isolation was achieved by using magnetic depletion beads from Stem Cell Technologies that targeted all major non-CD4+ T cell lymphocyte populations as well as the antigens CD25/CD69/HLA-DR (see Overall materials and methods for more details). This magnetic bead based separation is efficient, does not require columns, and routinely achieves high purity of the isolated rCD4 cells (>95% CD3+CD4+CD25-CD69-HLA-DR-; data not shown). Levels of total HIV-1 DNA in rCD4 cells were quantified by real-time qPCR using established assays, and the resulting values are shown in Table 7. The mean level of total HIV-1 DNA was 1,770 copies per million rCD4 cells, although there was substantial variation, with the range being 29 to 3,597 copies per million rCD4 cells.

Table 7. Levels of total HIV-1 DNA in resting CD4+ T cells for the assessment of latency reversal agents

Participant identifier	Total HIV-1 DNA per 10^6 resting CD4+ T cells
1	3597
2	428
3	1545
4	3207
5	985
6	29
7	822
8	1725
9	3589
Mean ± SD	1770 ± 1374

The isolated rCD4 cells were then cultured in the presence of medium control, anti-CD3/CD28 antibody-coated beads, or the LRAs of interest in the presence of 300 nM efavirenz to prevent subsequent cycles of viral replication. rCD4 cells were pulse-treated with putative latency reversing agents in 25 cm^2 flasks at the concentrations and durations described in Table 5 for each LRA; cells from the medium only treatment and activation control conditions were immediately serially diluted and placed into wells for culture. Following pulse treatments, treated rCD4 cells were then counted, serially diluted, and placed in serial 3-fold limiting dilution cultures from 10^6 cells per well to 457 cells per well, with 5 replicates at each dilution. These cell were then cultured for 7 days, and individual supernatants were removed and spun at 500xg to pellet cell debris, and were then frozen individually at -80°C for downstream assessment of HIV-1 RNA levels by Roche COBAS AmpliPrep/TaqMan. Each LRA or combination of LRAs was assessed in rCD4 cells isolated from at least 3 separate donors.

5.3.3 Virion production following treatment of resting CD4+ T cells with latency reversing agents

We first assessed virion production from the replicate wells of 10^6 rCD4 cells per well to evaluate the effect of the LRAs on overall virion production from bulk cultured rCD4 cells compared with medium control and anti-CD3/CD28 activation beads (Figure 5). After 7 days of culture, there was substantial virion production from anti-CD3/CD28 treated rCD4 cells (median virion production of 6,464 copies per milliliter of culture supernatant, interquartile range: 3,009 to 51,291 copies per milliliter of culture supernatant), and minimum virion production detection

from medium control rCD4 cells (virion-associated HIV-1 RNA was only detected in 3 of 9 donors in the absence of stimulation or treatment with LRAs). Pulse treatment of rCD4 cells to mimic *in vivo* dosing of LRAs resulted in modest virion production. Most LRAs achieved greater virion production than medium control, but generally produced small amounts of virus compared to T cell activation. Median levels of virion production following romidepsin, JQ1, bryostatin + romidepsin and romidepsin + JQ1 treatment were 61, 60, 29, and 449 and copies per milliliter of culture supernatant, respectively; other LRAs did not have median values above medium control (20 copies per milliliter of supernatant, which was used as the interpolated limited of detection). The average fold-changes in HIV-1 RNA above medium control for romidepsin, JQ1, bryostatin + romidepsin and romidepsin + JQ1 were 3-, 3-, 1.5, and 22-fold, respectively.

To rigorously analyze these results, we applied a linear mixed effects model, using the treatment condition as a fixed effect and the individual participants as a random effect. A Type II Wald chi-squared one-way analysis of variance (ANOVA) found that the linear mixed effects model was statistically significant ($p=0.014$). Unpaired Wilcox tests were then used to assess individual differences in virion production across treatment conditions, as there were uneven numbers of replicate treatments between conditions. Anti-CD3/CD28 was found to be statistically different ($p<0.02$) from all treatments with the exception of the romidepsin + JQ1 treatment ($p=0.063$ for comparison). Romidepsin + JQ1 was also found to be statistically different from medium control ($p=0.03$), suggesting that this condition significantly reverses latency. However, romidepsin + JQ1 was not statistically significant higher than romidepsin ($p=0.13$) or JQ1 alone ($p=0.11$).

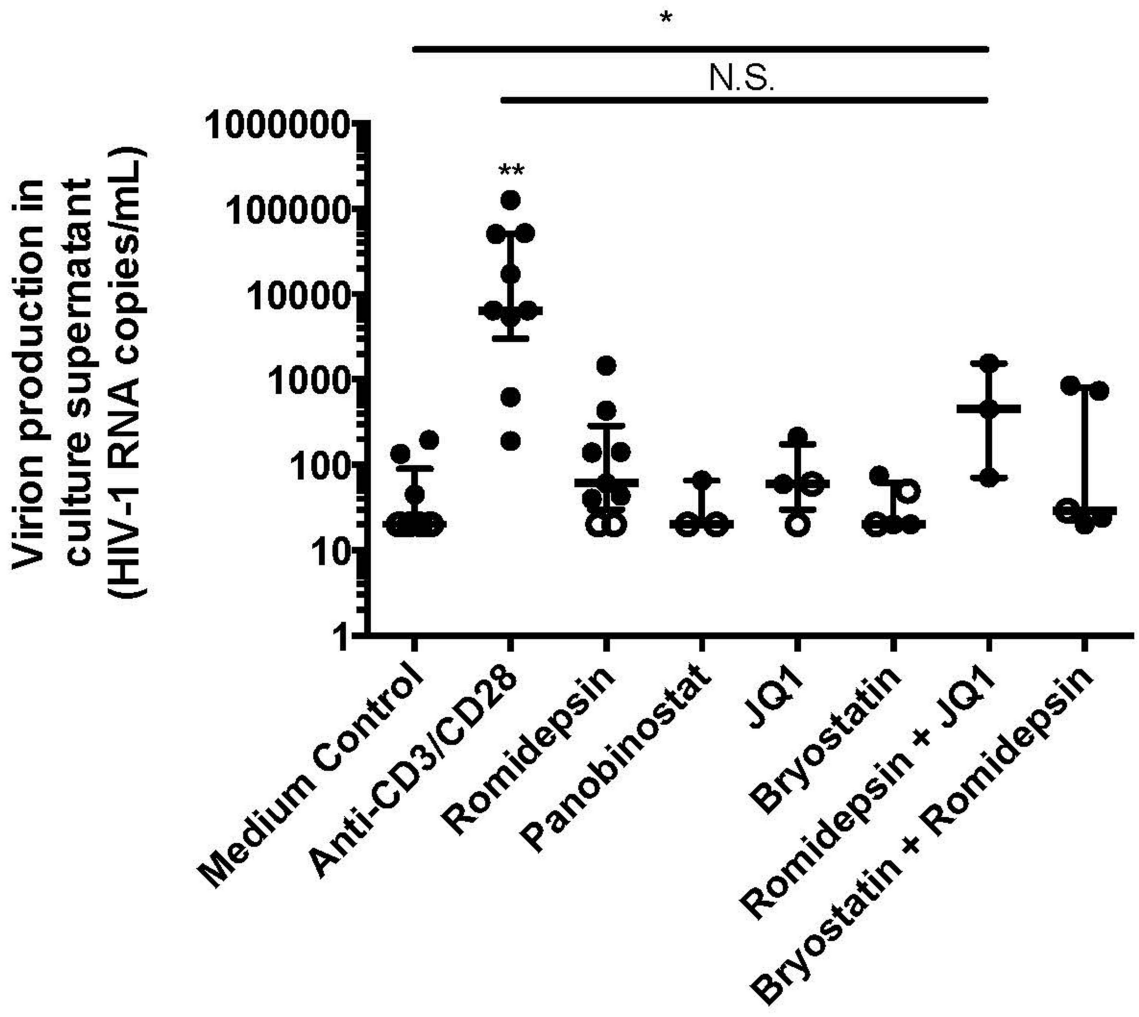


Figure 5. Virion production from bulk cultures of 10^6 resting CD4+ T cells. Median virion production from individual wells containing 10^6 rCD4 cells are shown for the treatment conditions listed. Median and interquartile ranges are shown as summary statistics. A linear mixed effects model, followed by unpaired Wilcox tests were used to assess differences in virion production between treatment conditions. Anti-CD3/CD28 produced significantly more virions than all other treatment conditions (**; $p<0.02$), with the exception of romidepsin + JQ1 ($p=0.063$) (N.S., not significant). Romidepsin + JQ1 produced significantly more virions than medium control ($p=0.03$). All other treatments were not statistically significantly higher than medium control. Open circles denote wells that were negative for virion production. Results were only available from 333,333 rCD4 cells per well for JQ1 and bryostatin from 1 donor, leading to higher levels of undetectable virion production after normalization to 10^6 cells per well.

We next assessed whether romidepsin + JQ1 synergistically led to higher virion production from treated rCD4 cells compared with romidepsin and JQ1 alone. Testing for Bliss independence [273,296,334] leads to a value, Δf, where Δf = 0 is an additive effect of the compounds tested, Δf>0 is synergistic, and Δf<0 is antagonistic. By applying tests for Bliss independence, we found that the mean Δf was -0.003, which is very close to the Δf = 0 for additivity. As such, in the 3 donors studied here with physiologically relevant pulse treatments of LRAs, we did not find evidence of synergy with romidepsin + JQ1.

5.3.4 Quantification of fractional provirus expression following treatment of resting CD4+ T cells with latency reversing agents

When evaluating the levels of virion production following treatment of rCD4 cells with LRAs, we found very modest but quantifiable levels of virion production with the LRAs studied. The levels of virions produced were generally more than were produced with medium only treatment, but were minimal in magnitude compared with latency reversal following maximum T cell activation with anti-CD3/CD28 beads. To better understand the potency of these LRAs at the level of individual proviruses, we utilized the fractional provirus expression assay described in detail in the previous chapter. The fPVE assay format involved limiting dilutions of rCD4 cells from 10^6 rCD4 cells per well to 457 rCD4 cells per well in serial 3-fold dilutions. The results of virion production from 10^6 cells per well (the highest concentration of cells tested with fPVE) were presented in the previous section. The results of the maximum likelihood analysis for fPVE with the LRAs tested, as well as medium control and anti-CD3/CD28 are now shown in Table 8 and Figure 6.

The median fPVE for virion production from medium only treated rCD4 cells was 0.033% of proviruses were reactivated to produce virions, whereas for anti-CD3/CD28 treated cells, a median of 3.8% of proviruses were activated to produce virions. All the LRAs tested reactivated very few proviruses compared with anti-CD3/CD28 treated rCD4 cells, suggesting that their activity is very limited at the level of *de novo* activation of individual proviruses.

Table 8. Percentage of proviruses that are reactivated to produce virions with a given treatment

Participant ID	Fractional provirus expression (% of proviruses reactivated per treatment)							
	Medium Control	Anti-CD3/CD28	Romidepsin	Panobinostat	JQ1	Romidepsin + JQ1	Bryostatin	Bryostatin + Romidepsin
1	0.015	1.9	0.01	0.019	0.0043	ND	ND	ND
2	<0.074	9.4	0.36	0.045	0.08	0.64	ND	ND
3	0.033	3.6	0.09	<0.026	<0.043	0.12	ND	ND
4	0.043	8.2	0.062	ND	0.012	0.088	ND	ND
5	<0.024	3.4	0.043	ND	ND	ND	0.090	0.15
6	<1.4	4.1	<0.77	ND	ND	ND	<0.83	<0.77
7	<0.068	3.8	0.083	ND	ND	ND	<0.023	0.022
8	<0.023	3.5	0.019	ND	ND	ND	<0.017	0.0094
9	<0.008	4.5	0.039	ND	ND	ND	0.0073	0.083
Median	0.033	3.8	0.062	0.019	0.017	0.12	0.012	0.083

ND = not done

The ratio between medium control and anti-CD3/CD28 is 118-fold, demonstrating that full T cell activation leads to profound reversal of latency at the level of individual proviruses. It is clear from Table 8 that LRAs do not achieve anywhere near the degree of latency reversal compared with anti-CD3/CD28. Consistent with levels of virion production, romidepsin + JQ1 treatment reactivated the greatest proportion of proviruses with rCD4 cells. Figure 6 shows a graphical summary of the fraction of proviruses reactivated with given LRAs.

To formally assess differences in fPVE for virion production between groups, we generated a linear mixed effects model, once again with treatment as a fixed effect and individual participants as a random effect. This model was found to be highly statistically significant by Wald's test ($p<0.001$), and unpaired Wilcoxon tests revealed that anti-CD3/CD28 was statistically significant different from all treatment conditions. When analyzing latency reversal by fPVE, romidepsin + JQ1 was not statistically different from medium control ($p=0.064$). This analysis shows that the only treatment to significantly reactivate more proviruses compared with medium control was anti-CD3/CD28.

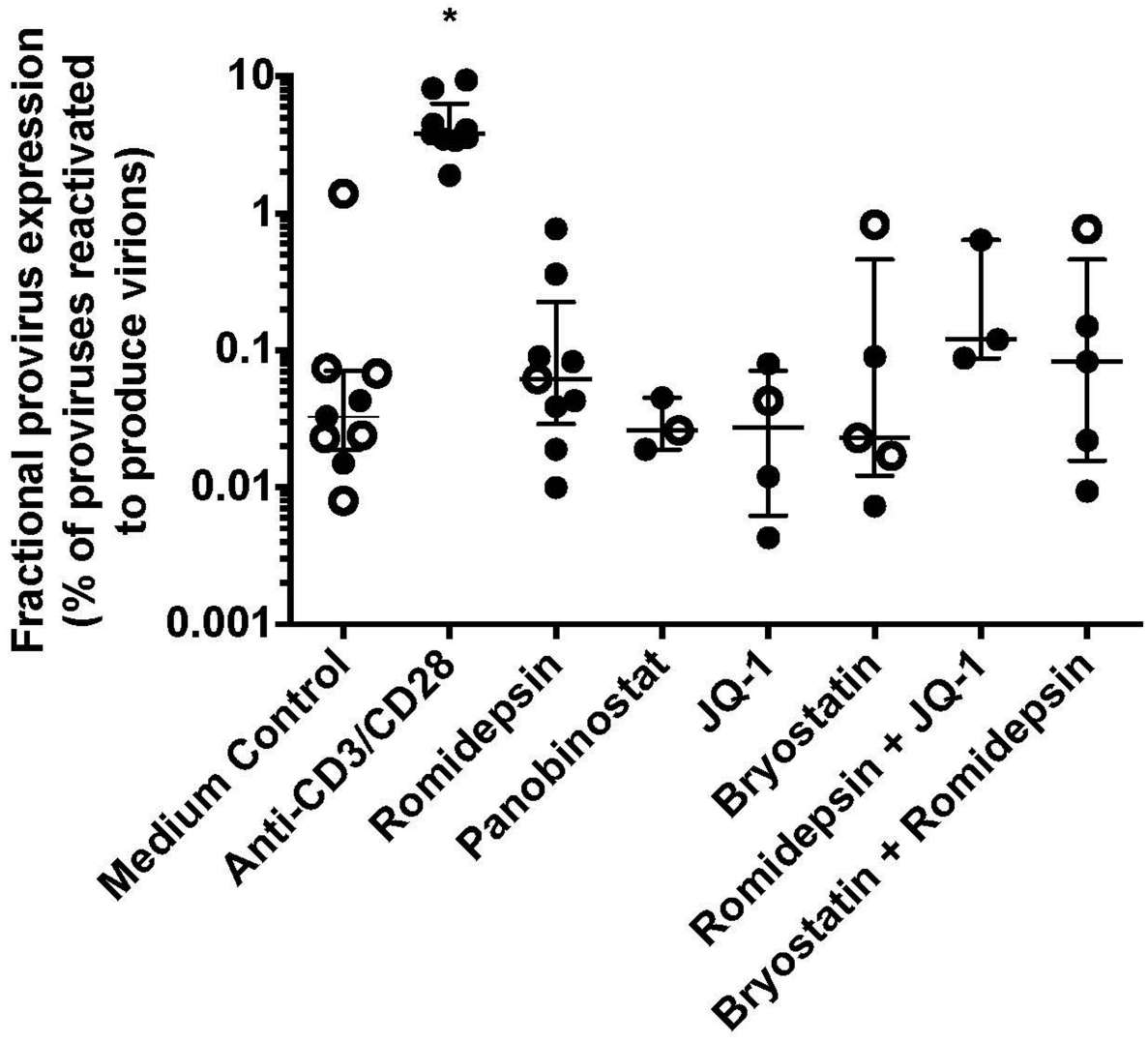


Figure 6. Assessment of fractional provirus expression for medium control, anti-CD3/CD28 and 6 putative LRAs. The fraction of proviruses reactivated in each treatment condition was assessed using a linear mixed effects model, with treatment condition as a fixed effect and individual participants as a random effect, and was found to be statistically significant ($p<0.001$). Subsequent unpaired Wilcoxon tests revealed that only anti-CD3/CD28 led to significantly more virion production compared to all treatment conditions (*). Median and interquartile range is shown for each treatment as summary statistics. Open circles denote samples that were below the limit of detection of fPVE. The limit of detection is based on the number of proviruses detectable by qPCR from each donor.

To assess the relative potency of the LRAs tested, we next used the fPVE value for anti-CD3/CD28 from each individual donor to normalize the magnitude of latency reversal by fPVE (Table 9).

Table 9. Fractional provirus expression normalized for the percent of maximal reactivated proviruses achieved with anti-CD3/CD28 treatment

Participant ID	Percent of proviruses reactivated, normalized for maximum reactivation ([fPVE LRA/fPVE anti-CD3/CD28]*100%)							
	Medium Control	Anti-CD3/CD28	Romidepsin	Panobinostat	JQ1	Romidepsin + JQ1	Bryostatin	Bryostatin + Romidepsin
1	0.79	100	0.53	1.0	0.23	ND	ND	ND
2	<0.39	100	3.8	0.48	0.85	6.8	ND	ND
3	0.92	100	2.5	<0.36	<0.60	3.3	ND	ND
4	0.52	100	0.76	ND	0.15	1.1	ND	ND
5	<0.71	100	1.8	ND	ND	ND	2.7	4.4
6	<34	100	<19	ND	ND	ND	<20	<19
7	<1.8	100	2.18	ND	ND	ND	<0.61	0.58
8	<0.66	100	0.54	ND	ND	ND	<0.49	0.27
9	<0.18	100	0.87	ND	ND	ND	0.16	1.84
Median	0.52	100	1.8	0.48	0.26	3.3	0.31	1.8

The results from this analysis are in agreement with the data presented above, but Table 9 contextualizes the relative potency compared with the maximal size of the latent reservoir that is reactivated in one round of maximum T cell activation. When analyzed as a percentage of maximally inducible proviruses within an individual study participant, we find that romidepsin alone, romidepsin + JQ-1, and bryostatin + romidepsin were the most potent LRAs, but only activated 1.82%, 3.3%, 1.84%, respectively, of the proviruses that were reactivated with anti-CD3/CD28.

5.3.5 Relationships between fractional provirus expression, virion production from bulk culture

To assess relationships between fPVE for virion production and the mean levels of virus produced from bulk-cultured rCD4 cells, we first evaluated whether the fraction of proviruses reactivated to produce virions was correlated by Spearman's correlation to the levels of virus produced into the culture supernatant from replicate wells of 10^6 rCD4 cells (Figure 7). This result revealed that there was no relationship (rho=0.02, p=0.98) between the levels of virus produced in the culture supernatant and fraction of proviruses that had been reactivated.

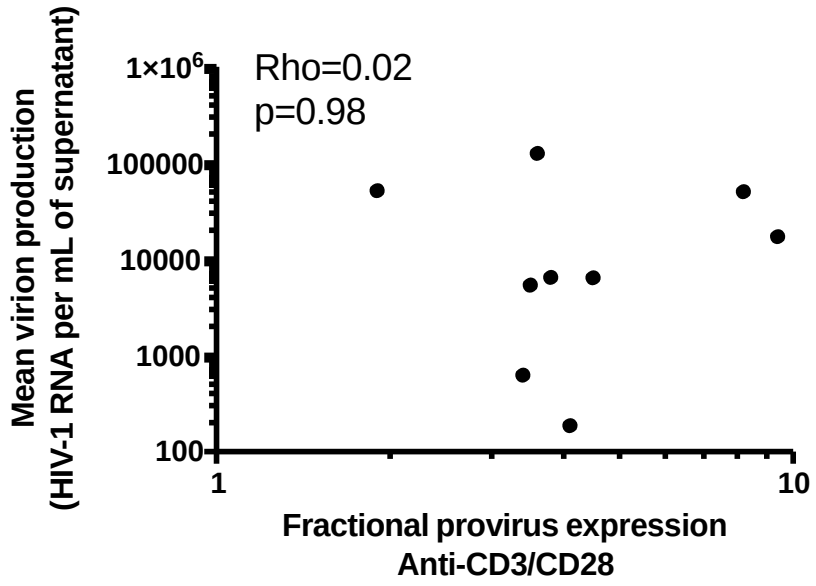


Figure 7. Relationship between mean virion production from 10^6 resting CD4+ T cells and the proportion of proviruses that were reactivated. Mean levels of HIV-1 RNA production in the culture supernatant were compared to the fraction of proviruses reactivated. The percent of proviruses that were maximally inducible following treatment with anti-CD3/CD28 was unrelated to mean virion production, likely due to the different number of proviruses present per 10^6 rCD4 cells, and the variation in the number of proviruses reactivated.

Several factors likely contribute to the lack of an observed relationship between between fPVE for virion production and mean virion production. First, as shown in Table 7, there are different numbers of proviruses per million rCD4 cells. This, combined with variations in the number of proviruses reactivated, likely leads to substantial variation. To correct for these differences, we instead evaluated whether the number of proviruses that were reactivated per million cells (that is, the fraction of proviruses that were reactivated times the number of proviruses per million rCD4 cells) was related to the levels of virion production in bulk culture (Figure 8). Although the relationship is still not statistically significant, a trend is readily apparent compared to Figure 7.

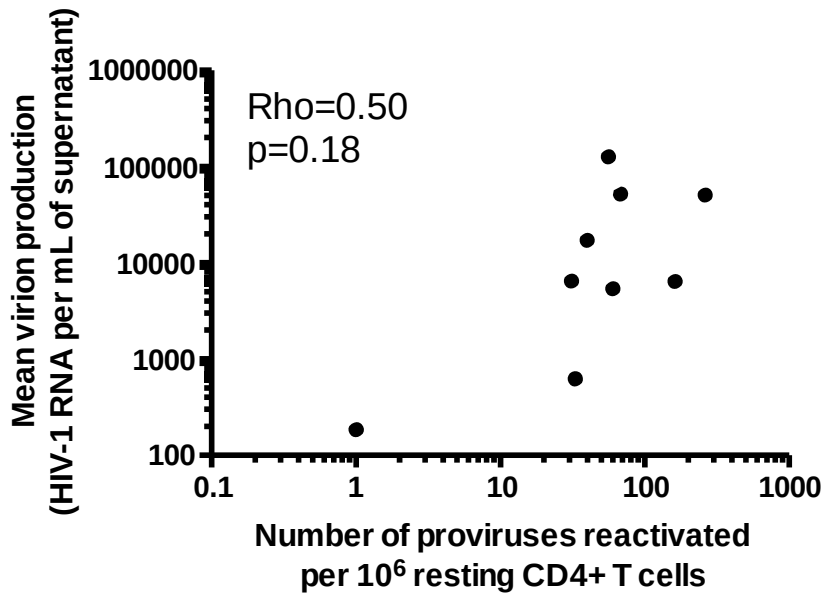


Figure 8. Relationship between the number of proviruses reactivated and mean virion production from resting CD4+ T cells. To determine the number of proviruses reactivated per 10^6 rCD4 cells, the total number of proviruses per 10^6 rCD4 cells was multiplied by the fraction of proviruses that were reactivated to produce virions from a given donor. This analysis revealed an improved trend, though still not statistically significant relationship, between virion production from bulk culture and the number of proviruses that were reactivated.

The relatively weak relationship between the amount of virions produced from bulk culture and the number of proviruses reactivated is likely explained by the fact that individual reactivated proviruses do not produce identical amounts of virions. We have previously described large differences in the number of virions produced from individual reactivated cells[335]. Over a large number of wells, the number of proviruses reactivated and the number of virions produced would positively correlate, but the variation in the amount of virions produced per provirus complicates this correlative assessment. This also highlights the importance of using fPVE, rather than simply assessing levels of virus production from bulk cultured cells to infer the potency of LRAs.

5.3.6 Expression of cell surface activation markers on resting CD4+ T cells treated with LRAs

An important consideration in the assessment of LRAs is whether they lead to changes in expression of cellular activation markers (i.e., CD25, CD69 and HLA-DR) on treated rCD4 cells. Increased expression of activation markers, and associated T cell activation, is likely to be associated with cytokine release and the potential for toxicity *in vivo*. Therefore, LRAs that lead to marked increases in expression of activation markers should probably be pursued with caution.

To assess the levels of activation marker expression following treatment with medium control, romidepsin, bryostatin and bryostatin + romidepsin, we performed flow cytometric analysis of cells was performed with fluorchrome-conjugated antibodies to CD3, CD4, CD25, CD69 and HLA-DR on rCD4 cells on after 5 days of culture (see Overall materials and methods for more information). We found that most treatment conditions did not increase the expression of activation markers, and therefore allowed rCD4 cells to maintain a resting quiescent phenotype (Figure 9). However, levels of CD69 expression were substantially increased in participants 5 and 9 following treatment with bryostatin and bryostatin + romidepsin, and somewhat up-regulated in participant 8 following treatment with bryostatin + romidepsin. Increased expression of CD69 seems to be driven by bryostatin, as cells that were treated with only romidepsin did not exhibit this increase in CD69 expression, but those treated with the combination therapy did.

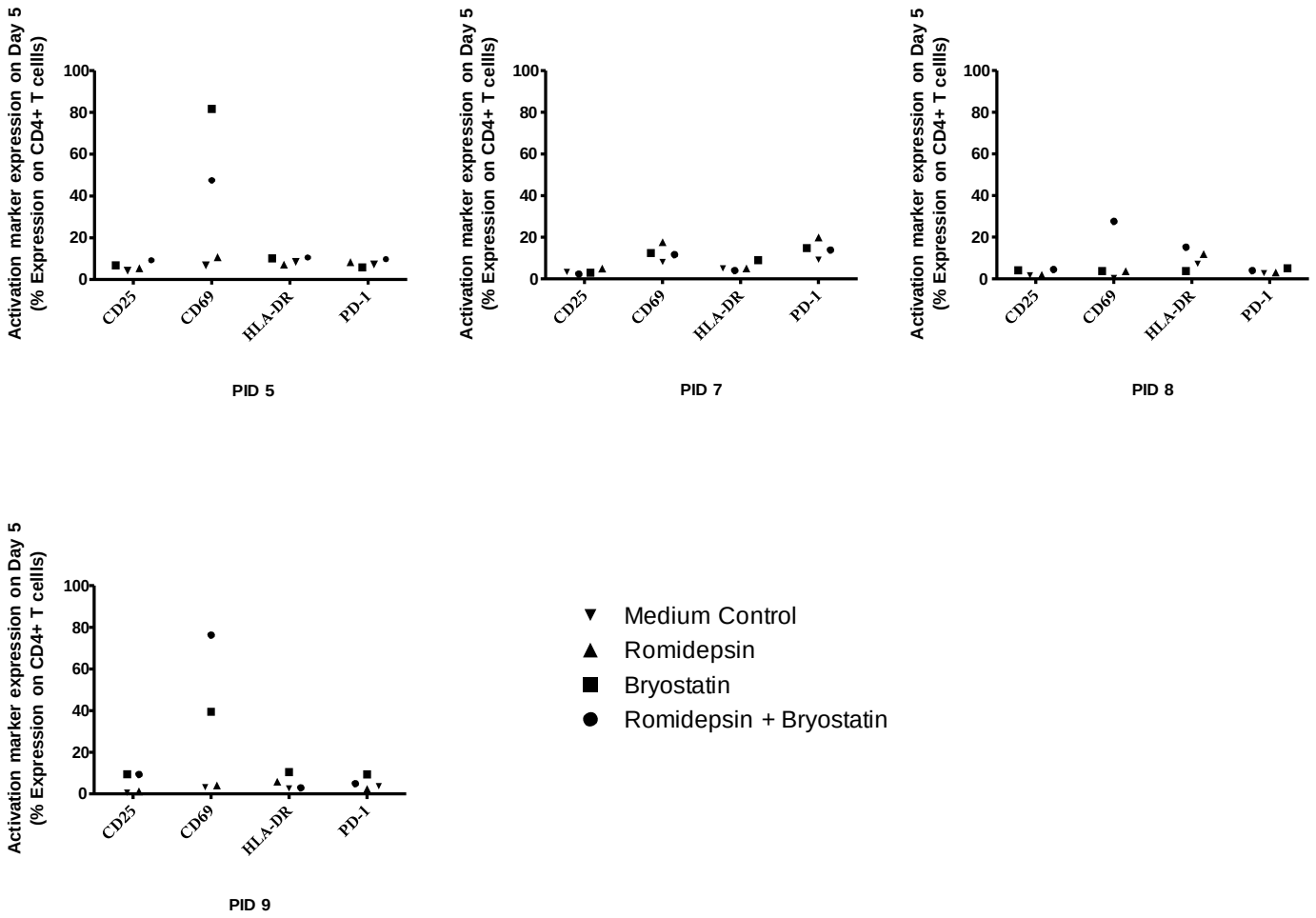


Figure 9. Changes in percent CD4+ T cells expressing activation markers following treatment with LRAs. In 4 donors, levels of activation marker expression following treatment with medium control, romidepsin, bryostatin and romidepsin + bryostatin were assessed. Significant elevation of CD69 expression was observed in participant 5 and 9 following romidepsin and romidepsin + bryostatin treatment, and in participant 8 following bryostatin + romidepsin treatment.

After observing the increased CD69 expression on the cell surface, we next asked whether the level of CD69 expression was associated with the magnitude of latency reversal observed by fPVE for virion production in these donors. The fraction of proviruses that were reactivated with bryostatin + romidepsin treatment (following normalization for the maximal inducible fraction of proviruses from that donor) was strongly positively associated with the levels of CD69 (R=0.86, p=0.14), although this correlation was not statistically significant due to the small number of participants in which bryostatin + romidepsin was studied (Figure 10).

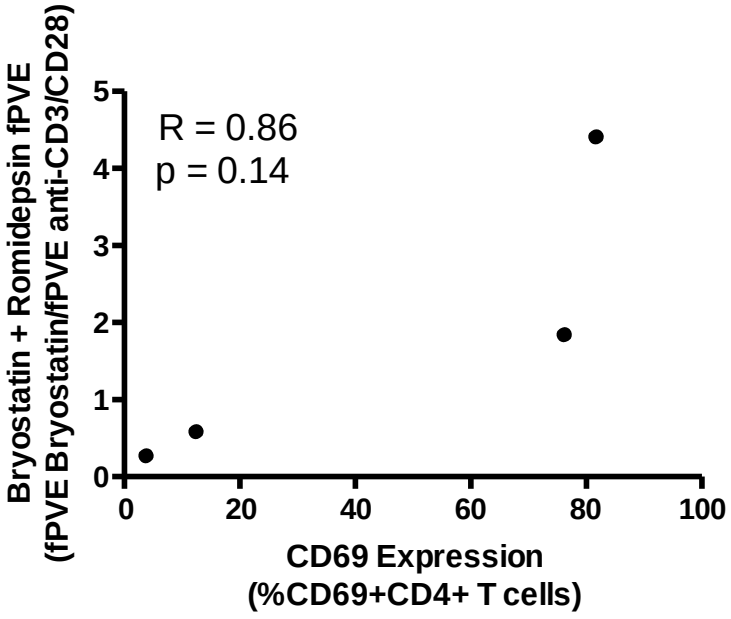


Figure 10. Relationship between CD69 expression and bryostatin + romidepsin fPVE. Levels of CD69 expression on CD4+ T cells on day 5 following pulse treatment with bryostatin + romidepsin was strongly associated (though not statistically significant given the small sample size) with the fPVE for virion production from bryostatin + romidepsin treated rCD4 cells obtained after 7 days of culture. This suggests that the potency of bryostatin + romidepsin is related to cellular activation.

This relationship suggests that the expression of CD69 may be associated with the pharmacokinetic response of rCD4 cells to bryostatin, and indeed activation of NF-κB expression via the PKC pathway leads to increased CD69 expression[336]. Additionally, this result also suggests that the potency of bryostatin + romidepsin is likely related to partial activation of CD4+ T cells following treatment.

5.4 DISCUSSION

In this study, we sought to evaluate the potency of diverse LRAs to activate latent proviruses to produce virions. We elected to focus on virion production as our endpoint, rather than cellular HIV-1 RNA transcription, because virion production indicates full latency reversal, i.e., transcription of cellular HIV-1 RNA, translation of viral proteins, and release of virions. Focusing on cellular transcription of HIV-1 RNA does not indicate whether viral proteins have been produced.

When evaluating latency reversal purely by measuring levels of virion production in culture, we found that fold change in virion production from LRA treated to medium control ranged from 1.5- to 22-fold, but that only romidepsin + JQ1 was statistically significantly higher than medium control in the small numbers of donors studied (N=9). The combination of romidepsin + JQ1 for virion production was found to be additive in this study, despite the independent mechanisms of action of romidepsin and JQ1. Other studies have generally found LRAs to be ineffective in the absence of a PKC agonist[295], with the notable exception being romidepsin[296,330]. Recent studies have also identified combinations of the PKC agonists

bryostatin or ingenol and JQ1 to be synergistic for the reactivation of latency[273,296,297]. While we did observe latency reversal with bryostatin + romidepsin in this study, the magnitude of latency reversal was not as high as has been reported elsewhere. Difference in the patient cohorts studied, including the duration of viral suppression, and differences in treatment duration and timing of assessment of latency reversal by virion production or cellular HIV-1 RNA transcription may have all contributed to the differences observed. Importantly, the pulse treatment conditions chosen for *ex vivo* use to closely reflect durations and concentrations that could be achieved *in vivo* likely contributed to differences between other studies and ours. Others have used overnight, 6 hour, or 48 hour treatments[273,296] or 6 days of continuous treatment[297], none of which represent concentrations or durations of drug exposure that are pharmacologically relevant.

After evaluating levels of virion production from replicate wells of 10^6 rCD4 cells, we next assessed the proportion of proviruses that could be reactivated following treatment with LRAs or full T cell activation with anti-CD3/CD28 activation beads. Similarly to our initial findings, we found that a small fraction of proviruses (3.8% of all proviruses detectable by qPCR) were inducible to produce virions following maximum reversal of latency with full T cell activation, which agrees with previous findings, where 1.5% of all proviruses detectable by qPCR were capable of producing virions[300]. The fraction of proviruses that produced virions from unstimulated rCD4 cells is similar as well in both studies, with 0.044% of proviruses spontaneously producing virions in Cillo et al., and 0.035% of proviruses being responsible for spontaneous virion production in the current study.

The best LRAs in this study achieved a modest 5-6 fold increase in the fraction of proviruses that have are activated to produce virions compared with medium control.

Correspondingly, the best LRAs only achieved reactivation of ~2-3% of the maximum fraction of proviruses that can be induced to produce virions. Our finding that only a small fraction of inducible proviruses in rCD4 cells can be reactivated with current LRAs in a limiting dilution culture format is in contrast with the reported potency of the same LRAs using bulk culture of rCD4 cells[273,296,297].

When comparing our results from fPVE for virion production to levels of virions produced in bulk culture, we found that there was only a trend towards a positive correlative relationship when the number of proviruses reactivated was compared to the level of virion production. This finding is in agreement with our previously published results, where levels of virions produced from individual reactivated cells was highly variable[335]. The presence of individual proviruses that can produce larger amounts of virions compared to other proviruses, and the large degree of variation observed in the levels of virions produced from individual proviruses highlights the importance of assessing the potency of LRAs on the level of individual proviruses.

Finally, we also identified substantial levels of CD69 expression following treatment with bryostatin and romidepsin + bryostatin. These elevated levels have been observed in other studies[273,296,297], but have not been found to be associated with substantial levels of cytokine release from rCD4 cells[273,296]. The magnitude of CD69 expression in these studies is concerning, with levels of CD69 and HLA-DR expression reaching similar levels to that observed with PMA/ionomycin treatment in two studies following treatment with PKC agonists[273,296]; levels of CD69 and HLA-DR expression reached 40% and 20%, respectively in another study with bryostatin[297]. Both these studies also noted the downregulation of CD4 on the surface of T cells treated with PKC agonists, which is a hallmark of T cell activation[337]. Of note, the authors of two

studies conclude that this is beneficial, as it will prevent infection of CD4+ T cells with HIV-1. This conclusion can be questioned because PKC agonists are likely found to be efficient reactivators of latent virus because they activate T cells. Our results, demonstrating a trend towards a greater proportion of proviruses reactivated with bryostatin + romidepsin and higher expression of CD69, is consistent with T cell activation, and implies that partial T cell activation may underlie the mechanism of latency reversal in this context. Great care should be taken to evaluate the potential toxicity associated with PKC agonists, as trends towards general activation of T cells *ex vivo* may be problematic *in vivo*, and could lead to cytokine storms and substantial toxicity.

To achieve substantial reductions in the size of the reservoir via a "kick and kill" strategy, it is clear that more potent latency reversing agents will be required. Reactivation of ~3% of total inducible proviruses will not lead to a functional cure of HIV-1. The potency of the LRAs currently being evaluated are not promising, and the trend towards use of PKC agonists that may causes T cell activation is not a clear path forward. Despite these findings, an important unanswered question is whether these LRAs have achieved a net decrease in the size of the inducible reservoir *ex vivo*. Although we have elected to use the production of virions as an endpoint for latency reversal, it is possible that viral cytopathic effects may occur prior to virion release. As such, evaluating the proportion of proviruses that remain inducible following treatment with LRAs is of interest, and is explored in the next chapter.

6.0 CHAPTER 3. QUANTIFICATION OF THE SIZE OF THE MAXIMALLY INDUCIBLE RESERVOIR FOLLOWING TREATMENT OF RESTING CD4+ T CELLS WITH LATENCY REVERSAL AGENTS *EX VIVO*

6.1 PREFACE

Anthony R. Cillo[1], Michele Sobolewski[1], Taylor Simmons[1], Elizabeth Fyne[1], Joshua Cyktor[1], John W. Mellors[1]

[1]University of Pittsburgh, Division of Infectious Diseases

The work presented in this chapter is in partial fulfillment of Aim 1 of this dissertation, and explicitly is related to assessing whether LRAs can reduce the size of the inducible reservoir. Anthony Cillo, Michele Sobolewski, Taylor Simmons, Elizabeth Fyne and Joshua Cyktor performed the isolation of resting CD4+ T cells from leukapheresis products, treated isolated resting CD4+ T cells with LRAs, and set up many limiting dilution culture experiments in parallel. These same contributors also harvested individual wells of culture supernatants for downstream qRT-PCR. Anthony Cillo, Michele Sobolewski, Elizabeth Fyne and Taylor Simmons performed COBAS AmpliPrep/TaqMan v2.0 for quantification of HIV-1 RNA from culture supernatants. Anthony Cillo performed qPCR for cellular HIV-1 DNA. Joshua Cyktor performed flow cytometry experiments for assessment of activation markers, and analyzed flow

cytometry data. Anthony Cillo and John Mellors conceived of the project, and Anthony Cillo analyzed the data and performed statistical analysis.

The Pitt AIDS Research Training Program 5 T32 AI065380-08, a research grant from Gilead Sciences funded the work described in this chapter. Roche provided COBAS AmpliPrep/TaqMan kits.

6.2 INTRODUCTION

In the previous two chapters, we have discussed the development and implementation of an assay capable of quantifying the magnitude of latency reversal at the level of individual proviruses. The results from these experiments have generally demonstrated that the fraction of proviruses that have been reactivated to produce virions following treatment with LRAs is several orders of magnitude smaller than the fraction of proviruses that can be reactivated to produce virions following full T cell activation with anti-CD3/CD28 beads. However, having chosen to use virion production as our endpoint, we have not evaluated whether LRA treatment leads to significant changes in expression of cellular HIV-1 RNA. Increases in cellular HIV-1 RNA expression may be directly related to viral protein translation, which could cause death of cells harboring reactivated proviruses from viral cytopathic effects prior to virion release.

In this chapter, we seek to extend the findings of previous chapters to evaluate whether viral cytopathic effects from LRA treatment alone can reduce the fraction of proviruses that are maximally inducible. We established a system where we first treat isolated resting CD4+ T cells with LRAs, and then maximally reactivate the T cells in a serial dilution culture format to

evaluate the fraction of proviruses that can be reactivated to produce virions. By comparing the fraction of proviruses that can produce virions between medium only treated cells and LRA treatment, we can assess whether a significant depletion of the inducible reservoir has occurred. *Ex vivo* studies to date have primarily assessed the magnitude of latency reversal and not whether a depletion of the inducible reservoir has occurred[273,295-297,316], with one notable exception being that SAHA treatment of PBMC *ex vivo* did not decrease the size of the infectious reservoir as measured by quantitative viral outgrowth (QVOA) in resting CD4+ T cells[338]. The study by Shan et al.[339] also suggested that infected cells were not killed by viral cytopathic effects alone following latency reversal, and that primed cytotoxic T lymphocytes were required to deplete the reservoir, although the authors utilized an *in vitro* model to demonstrate this. Recent *in vivo* studies of panobinostat and SAHA have also not found reductions in the size of the reservoir following LRA treatment[253,257,323].

The assay format that we have devised is advantageous, as it allows for the overall assessment of changes in the inducible reservoir from viral cytopathic effects. It is possible that increased transcription of cellular HIV-1 RNA leads to increased levels of viral translation, potentially leading to viral cytopathic effects that would be missed by only assessing levels of virion production. As such, the methods established in this chapter are capable of assessing whether the size of the inducible reservoir is indeed reduced by LRA treatment.

6.3 RESULTS

6.3.1 Quantification of the size the inducible reservoir following treatment with latency reversing agents

To assess whether LRAs can reduce the size of the latent reservoir *ex vivo*, we first pulse treated purified rCD4 cells from the donors described in the previous chapter with LRAs, removed the LRA from the medium, and then cultured the cells for 5 days. After 5 days, rCD4 were serially diluted from 10^6 rCD4 cells per well to 457 cells per well and were maximally reactivated with anti-CD3/CD28 (donors 1 through 3) or PMA/ionomycin (donors 5 though 9) for 7 days to calculate fractional proviral expression (an aliquot of cells was also saved for quantification of proviral copy number). The two different activation stimuli (anti-CD3/CD28 beads and PMA/ionomycin) were found to lead to equivalent levels of virion production from rCD4 cells treated in parallel in control experiments (data not shown). After 7 days of culture, supernatants were individually harvested and spun at 500xg for 5 minutes, and were then stored at -80°C for downstream assessment of virion-associated HIV-1 RNA by Roche COBAS AmpliPrep/TaqMan. Individual wells were scored as positive or negative for virion production, and fPVE for virion production was calculated by maximum likelihood estimate as described in the previous chapters.

We quantified changes in the size of the maximally inducible latent reservoir from these eight study participants by comparing fPVE for virion production after maximal T cell activation of LRA treated rCD4 cells to fPVE for virion production from medium only treated rCD4 cells. Total HIV-1 DNA levels were quantified by qPCR from cells that were frozen immediately prior

to maximum reactivation (on day 5) (Table 10). Levels of total HIV-1 DNA were not substantially altered by treatment with LRAs, with the exception of rCD4 cells from participant 6 where a dramatic increase from 62 copies of total HIV-1 DNA per million rCD4 cells to 2,078 copies of total HIV-1 DNA per million rCD4 cells occurred following treatment with romidepsin + bryostatin. It is unclear why this increase in total HIV-1 DNA occurred, but it may be that an HIV-infected rCD4 cell clonally expanded following treatment with romidepsin + bryostatin. As described in the introduction of this dissertation, clonal expansion can occur *in vivo*[149,150]. Overall, the finding of no reduction in HIV-1 DNA is consistent with few proviruses being reactivated following treatment with LRAs, as described in the previous chapter.

Table 10. Levels of total HIV-1 DNA per 10^6 rCD4 cells on day 5 following LRA treatment

Participant ID	Total HIV-1 DNA per 10^6 rCD4 cells on day 5 following treatment			
	Medium Control	Romidepsin	Bryostatin	Romidepsin + Bryostatin
1	2900	967	ND	ND
2	356	415	ND	ND
3	1452	1851	ND	ND
5	825	1086	1188	1133
6	62	123	37	2078
7	1426	1470	1150	974
8	2546	2326	1813	1642
9	1650	1920	2422	1381

ND = not done

To begin assessing whether LRAs can decrease the size of the inducible reservoir, we first assessed levels of virion production from replicate wells of 10^6 treated rCD4 cells (Figure 11). Changes in the size of the maximally inducible reservoir were next quantified following

treatment with romidepsin (N=8), bryostatin (N=5) and bryostatin + romidepsin (N=5). There was a trend towards a reduction in the median amount of virion production following treatment with each LRA, with median virion production of 6,973, 1,548, 595 and 950 copies of HIV-1 RNA per milliliter of culture supernatant for medium control, romidepsin, bryostatin and bryostatin + romidepsin treatments, respectively. Correspondingly, the fold reductions in median virion production between LRA treatment and medium only treatment were 4.5 fold for romidepsin, 11 fold for bryostatin and 7.1 fold for bryostatin + romidepsin. Despite these apparent substantial reductions in the levels of virions produced, a linear mixed effects model with treatment as fixed effects and individual participants as random effects was not statistically significant ($p=0.15$), suggesting that no treatment led to significantly fewer virions produced following maximal activation after treatment with LRAs. The lack of statistical significance observed is likely due to the overlapping interquartile ranges for each of the treatments.

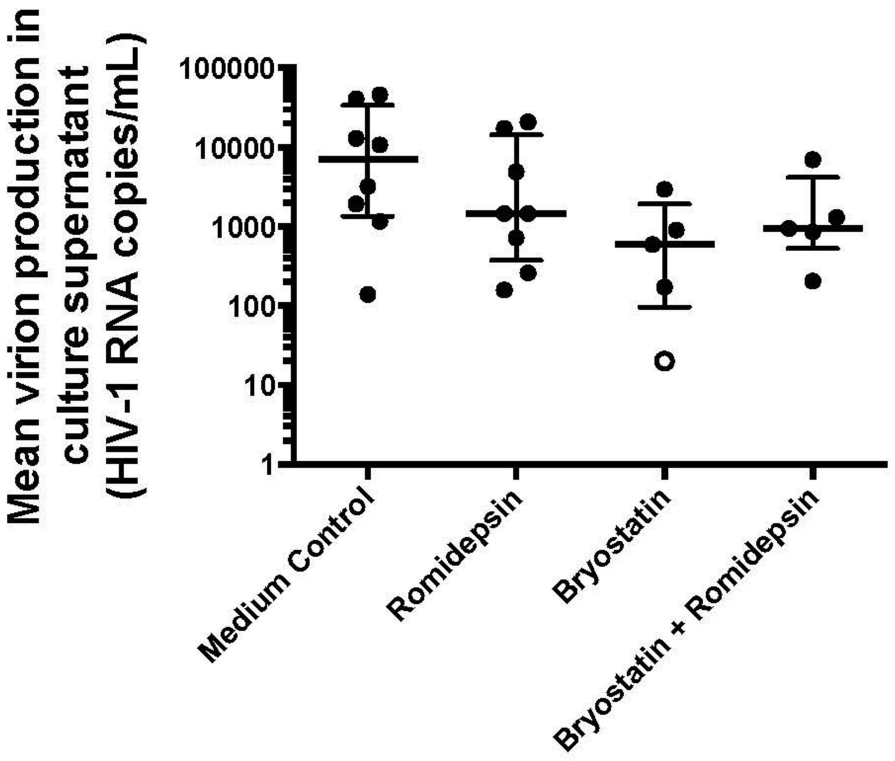


Figure 11. Levels of maximal inducible virion production from rCD4 cells following treatment with LRAs. Levels of virion production were evaluated from replicate wells of 10^6 rCD4 cells activated with full T cell activation. Trends towards reduced levels of virion production were observed following treatment with LRAs, but a linear mixed effects model with treatment as a fixed effect and individual participants as a random effect was not statistically significant (p=0.15). Median and interquartile range are shown as summary statistics, and the open circle denotes a sample with undetectable HIV-1 RNA.

Next, the size of the inducible reservoir following treatment with LRAs was evaluated by assessing fPVE for virion production (Table 11 and Figure 12). Median fPVE from medium control was 1.0%, ranging from 0.12% to 3.4%, while the median maximum fPVE following treatment with LRAs was 0.43%, 0.71% and 0.20% of proviruses inducible for romidepsin, bryostatin, and romidepsin + bryostatin, respectively. However, as is clear from Figure 12, there was substantial inter-patient variability in changes in reservoir size following treatment with LRAs.

Table 11. Assessment of changes in the size of the inducible reservoir following treatment with LRAs

Participant ID	Maximal fraction provirus expression following treatment with LRAs (% of proviruses reactivated to produce virions with maximal activation)			
	Medium Control	Romidepsin	Bryostatin	Bryostatin + Romidepsin
1	0.43	0.31	ND	ND
2	3.4	1.9	ND	ND
3	0.17	0.26	ND	ND
5	2.4	0.54	1.0	0.19
6	1.4	1.6	0.70	0.12
7	1.4	0.43	0.71	1.2
8	0.12	0.31	0.30	0.4
9	0.68	0.42	1.1	0.2
Median	1.0	0.43	0.71	0.20

For example, romidepsin caused a reduction in the size of the maximally inducible reservoir in five of eight participants, while the proportion of proviruses reactivated following romidepsin treatment in the other three participants increased. Similarly for bryostatin and romidepsin + bryostatin, changes in the proportion of proviruses that were reactivated differed between participants; the fraction of inducible proviruses decreased in three of five subjects for bryostatin and four of five subjects for bryostatin + romidepsin treatment.

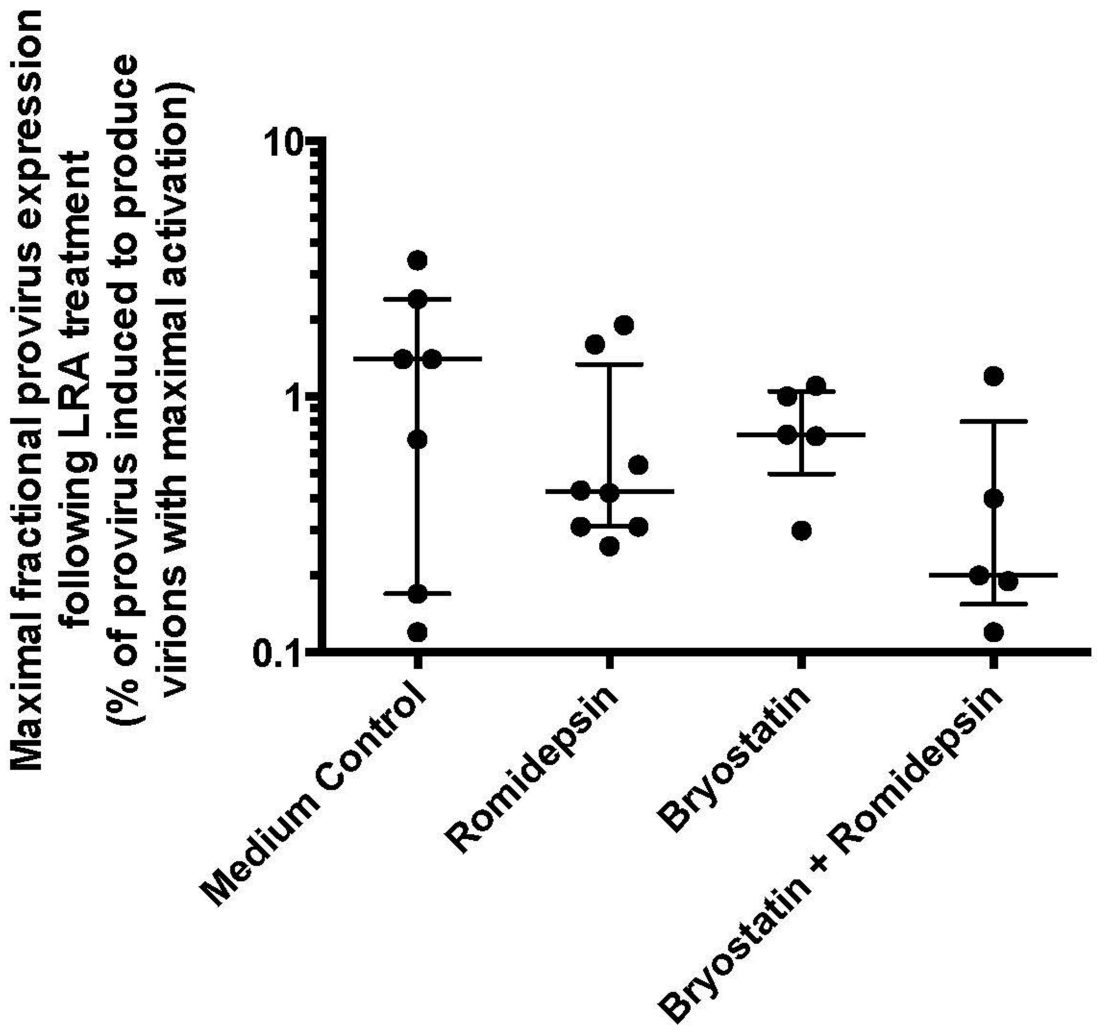


Figure 12. Levels of maximum fPVE following treatment of rCD4 cells with LRAs. rCD4 cells were first treated with LRAs, and were then maximally reactivated with full T cell activation to assess whether LRAs have caused a reduction in the size of the inducible reservoir. While the median is reduced for all LRAs tested compared with medium control, there are substantial inter-participant differences. A linear mixed effects model with treatment as a fixed effect and individual participants as a random effect was not statistically significant (p=0.081), implying that fPVE for virion production was not different between treatment groups. Median and interquartile ranges are shown as summary statistics.

Part of the inter-patient variability observed can be reduced by normalizing the fPVE for virion production following treatment with LRAs by dividing by the fPVE for virion production from the medium only treatment by the fPVE for LRA treatment. Table 12 and Figure 13 show the fold reductions in the fraction of proviruses that are maximally inducible following treatment with LRAs.

Table 12. Fold-change in the size of the inducible reservoir following treatment with LRAs versus medium control

Participant ID	Fold-reduction (Medium Control/LRA) in maximal fPVE following LRA treatment		
	Romidepsin/Medium Control	Bryostatin/Medium Control	Bryostatin + Romidepsin/Medium Control
1	1.4	ND	ND
2	1.8	ND	ND
3	0.67	ND	ND
5	4.3	2.4	13
6	0.91	2.0	11
7	3.2	2.0	1.2
8	0.39	0.40	0.27
9	1.6	0.62	3.5
Median	1.5	2.0	3.5

The median fold reductions in fPVE following treatment with romidepsin, bryostatin, and bryostatin + romidepsin were 1.5, 2.0, and 3.5, respectively, compared with medium control. The most profound reductions in the size of the inducible reservoir occurred with bryostatin + romidepsin treatment, where the fold reductions were as low as 13 and 11 for two donors when

comparing medium only treatment to bryostatin + romidepsin. However, there was also a substantial increase in the size of the latent reservoir in one subject with bryostatin + romidepsin, where the fold reduction was 0.27 for medium to bryostatin + romidepsin fPVE treatment. For romidepsin treatment alone, changes ranged from a 3.2 fold reduction to a 0.39 fold reduction, and results were similar for bryostatin to medium only treatment comparisons, ranging from 2.4 to 0.4 fold reductions. These dichotomous responses are clear in Figure 13, where some donors have substantial reductions in the proportion of proviruses that can be maximally reactivated following treatment with LRAs, demonstrating a heterogeneous response to LRAs across donors.

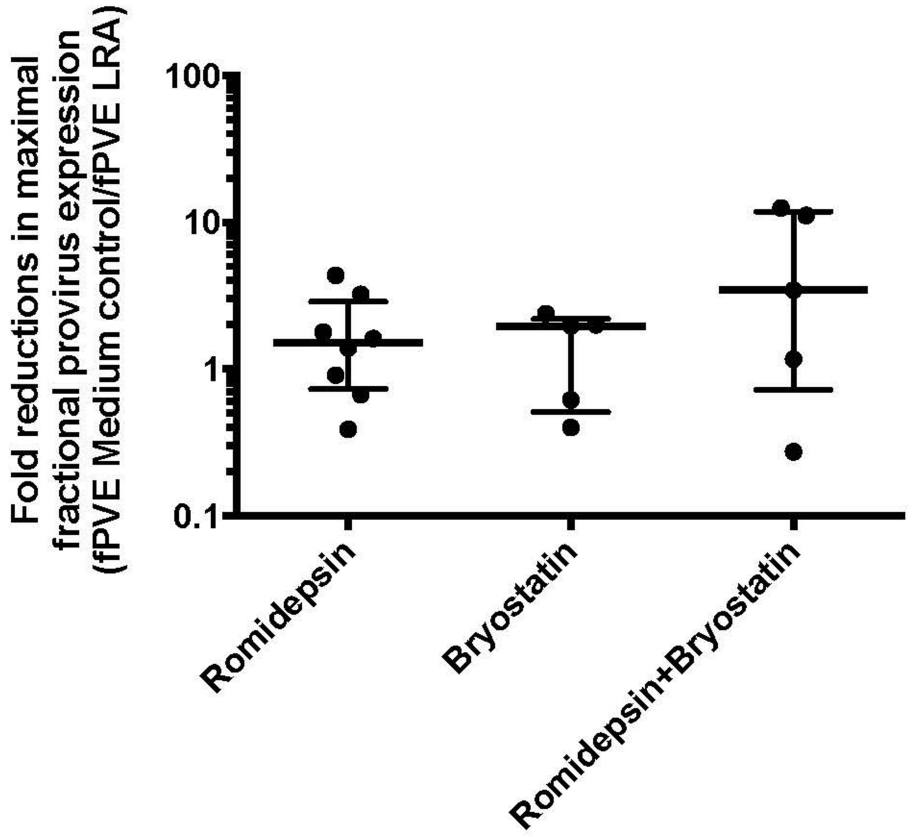


Figure 13. Fold reduction in the size of the inducible reservoir following treatment with LRAs. Changes in the size of the inducible reservoir were assessed by measuring the fold reduction between fPVE for virion production from medium control treated and LRA treated rCD4 cells. Reductions in the size of the inducible reservoir were inconsistent between donors, with some donors have some substantial fold reductions and other having increases

in the size of the inducible reservoir. Some substantial reductions in individual participants for romidepsin + bryostatin were observed.

6.3.2 Analysis of viability and activation marker expression on Day 13 for the assessment of responsiveness to maximal stimulation

One major assumption of the assays we have performed to assess whether the size of the inducible reservoir has changed following treatment with LRAs is that rCD4 cells treated with LRAs can be reactivated to the same magnitude as medium control treated rCD4 cells. If the LRA treated rCD4 cells are refractory to stimulation, the results of these assays will be difficult to interpret, as a decrease in the responsiveness to reactivation would be impossible to separate from a decrease in the size of the inducible reservoir. To assess the responsiveness of rCD4 cells to reactivation following treatment with LRAs, we evaluated the viability of the reactivated cells and the surface immunophenotype on day 13, which is 7 days after stimulation with anti-CD3/CD28 activation beads or PMA/ionomycin.

The assessment of viability was performed on donors 7, 8 and 9 using CellTiter-Fluor (see Overall materials and methods for details). Importantly, the same number of cells was assayed for fluorescence viability from each treatment condition. It is clear from these analyses that the viability of the reactivated cells that were treated with romidepsin and bryostatin + romidepsin was lower compared to the levels of viability from rCD4 cells that served as the medium control (Table 13).

Table 13. Viability of reactivated cells following treatment with LRAs

Participant Identifier	Relative cellular viability following maximal reactivation (fluorescence units LRA/fluorescence units Medium control)		
	Romidepsin/Medium control	Bryostatin/Medium control	Bryostatin+Romidepsin/Medium control
7	0.45	1.0	0.80
8	0.92	1.1	0.85
9	0.53	0.98	0.49
Mean	0.63	1.0	0.71

Romidepsin substantially reduced the viability of cells following 7 days of treatment with full T cell activation in donors 7 and 9, though not substantially in donor 8. Bryostatin treatment did not affect viability, and bryostatin + romidepsin treatment somewhat reduced viability in donors 7 and 8, and substantially reduced viability in donor 9. Notably, donors 7 and 9 had substantial reductions in the size of the inducible reservoir following romidepsin treatment, and this was associated with lower viability of rCD4 cells following maximum reactivation. It is therefore unclear whether these were true reductions in the size of the inducible reservoir, or if virion production was prevented because the cells were less responsive to activation stimuli. For example, the substantial reduction in the size of the latent reservoir following treatment with bryostatin + romidepsin in donor 9 is likely related to reduced cell viability from bryostatin + romidepsin.

To further assess the responsiveness of LRA treated cells to full T cell activation, activation marker expression was assessed in 5 of the 8 donors in whom we evaluated whether

LRAs caused a reduction in the size of the inducible reservoir (Figure 14). It is clear that activation marker expression following treatment with LRAs is heterogeneous, and differs between study subjects. For example, in donors 5 and 6, CD25 expression levels following full T cell activation are the same regardless of LRA treatment, while the levels of CD69 differ based on LRA treatment. However, in donor 6, levels of both CD69 and CD25 are affected by treatment with LRAs. In donors 5, 7, and 9 there were substantial decreases in the size of the inducible reservoir following treatment with romidepsin, but CD69 expression following treatment with RMD in these donors was also substantially lower, suggesting that the responsiveness of the cells may have been affected by treatment with LRAs. Similarly for bryostatin, a reduction in the size of the reservoir was observed for donors 5, 6 and 7, and levels of CD69 expression was lower under these conditions as well. Finally, reductions in the size of the latent reservoir were observed with romidepsin + bryostatin treatment in donors 5, 6, 8 and 9, with levels of CD69 effected in all donors with the exception of donor 9.

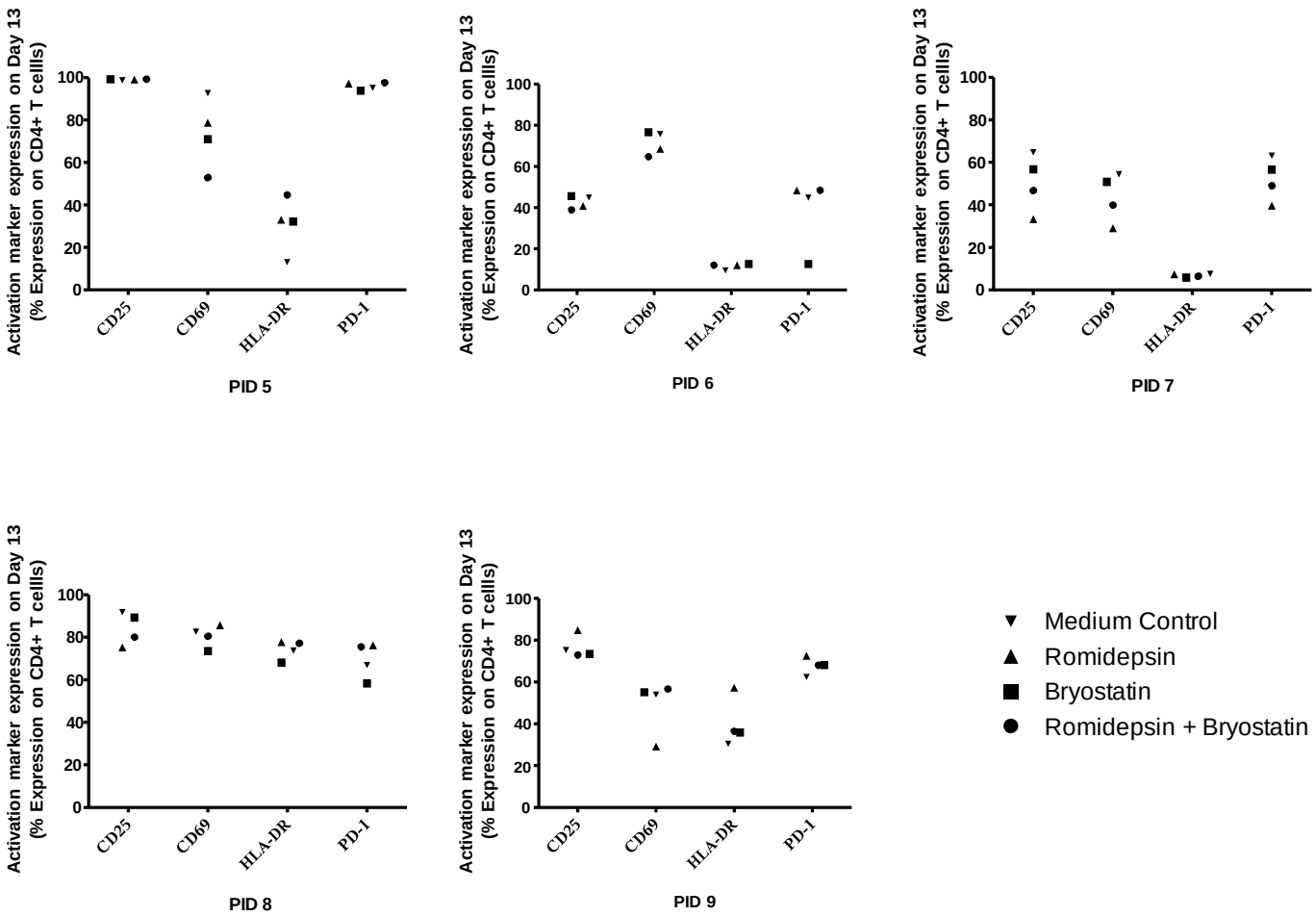


Figure 14. Activation marker expression on maximally reactivated cells following treatment with LRAs. Levels of activation markers on T cells treated with PMA/ionomycin following treatment of rCD4 with LRAs. Inconsistent levels of expression of CD25, CD69, HLA-DR and PD-1 were observed following treatment with LRAs from some donors, complicating analysis of changes in the size of the inducible reservoir following treatment with LRAs.

6.3.3 Relationships between the fraction of proviruses initially reactivated and the reduction in the size of the inducible reservoir

We also sought to evaluate whether there were relationships between the fraction of proviruses that were initially reactivated, and the change in the size of the maximally inducible reservoir following treatment with a given LRA. Due to the small sample sizes and the inability to quantify fPVE due to sensitivity of the assay in some donors (and the relatively small number of proviruses reactivated), relationships between virion production in romidepsin treated rCD4 cells and romidepsin + bryostatin treated rCD4 cells were the only LRAs investigated in this manner (Figure 15).

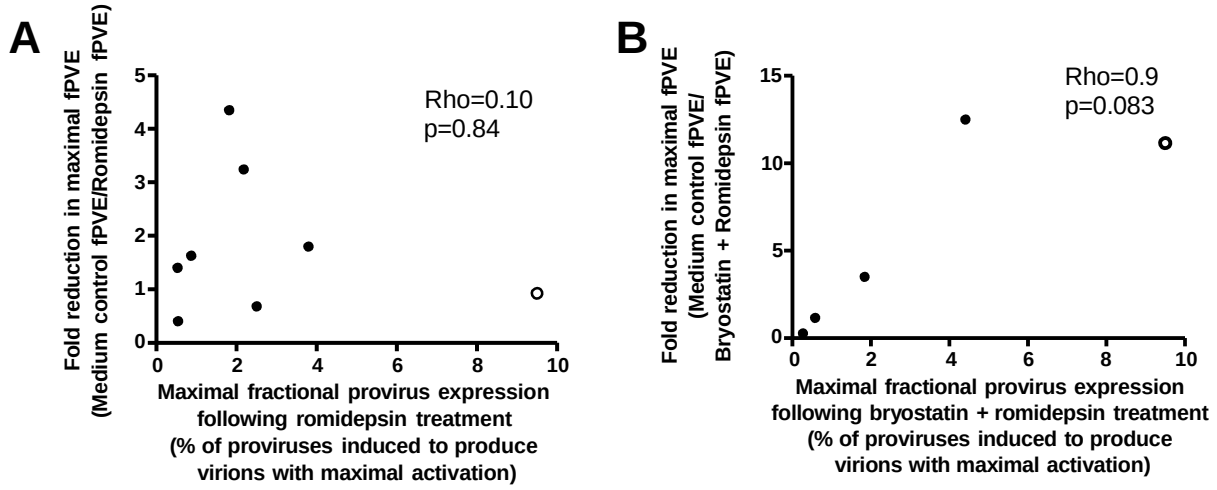


Figure 15. Relationships between fPVE and the change in the size of the maximally inducible reservoir. **A)** The fraction of proviruses that were initially reactivated to produce virions following treatment with romidepsin was not significantly associated with the change in the size of the maximally inducible reservoir following LRA treatment. **B)** The fraction of proviruses that were reactivated to produce virions initially was strongly negatively related to the fraction of proviruses that were reactivated to produce virions (rho=0.9, p=0.083). Although this relationship did not reach statistical significance, there is a clear trend towards fewer proviruses

being maximally reactivated following a larger percentage of proviruses initially reactivated following treatment with LRAs.

In the rCD4 cells that were treated with romidepsin, we found no relationships between the fraction of proviruses that were initially reactivated and the fold reduction in the percentage of maximally inducible proviruses following. However, following treatment with romidepsin + bryostatin, we found a trend towards a strong positive relationship (i.e., the higher the percent of proviruses initially reactivated following treatment with romidepsin + bryostatin, the larger the reduction in the fraction of inducible proviruses that remained). The observed relationship between romidepsin + bryostatin treated fPVE and the maximal fraction of remaining inducible proviruses suggests that there is a proportionality between the fraction of proviruses that were reactivated following treatment with bryostatin + romidepsin, and the fraction of proviruses that undergo viral cytopathic effects following latency reversal. For the cells that were treated with romidepsin alone, the analysis is likely complicated by the fact that viability (and activation marker expression) is substantially reduced following maximal reactivation after romidepsin treatment, and the number of proviruses that are maximally inducible following treatment with romidepsin is artificially reduced due to reduced cellular responsiveness to activation stimuli, and not mechanistically linked to the fraction of proviruses that were initially reactivated.

6.4 DISCUSSION

Studies to date have focused on assessing whether LRAs are capable of reactivating the latent reservoir[273,289,295-297,300,316,330], but have not evaluated whether the size of the inducible reservoir has been reduced following treatment with LRAs. In this study, we have developed a method to

assess whether the size of the inducible reservoir has been depleted following treatment with LRAs. Our method utilizes the previously described fPVE for virion production, and requires treatment of rCD4 cells with medium control or LRAs, followed by subsequent maximal reactivation of rCD4 cells to assess the proportion of proviruses remaining that can be induced to produce virions.

We first assessed whether LRA treatment has any effect on the levels of total HIV-1 DNA in rCD4 cells. With the LRAs tested in this study, we did not find a trend towards significant changes in the amount of total HIV-1 DNA per 10^6 rCD4 cells following treatment compared with medium control. This is consistent with the small fraction of proviruses that are likely to be reactivated following treatment with LRAs, as previously described[300] and discussed in the previous chapter. Intriguingly, we also found a substantial increase in levels of total HIV-1 DNA following treatment with romidepsin + bryostatin in one donor. The mechanism leading to this expansion is currently unknown, but we previously found that levels of HIV-1 DNA can increase upon cellular activation[300]. Given the increases observed in CD69 expression following treatments including bryostatin, it is possible that cellular activation occurred and led to proviral expansion. We can also speculate that treatment with romidepsin + bryostatin may have led to dramatic clonal expansion of a particular integration site in this donor[149,150].

Although levels of total HIV-1 DNA did not change across donors, we did find a trend towards a reduction in the amount of virions produced from LRA treated rCD4 cells compared with medium control treated rCD4 cells. These fold reductions ranged from 4.5 to 11 fold reductions in the amount of virions produced following LRA treatment, but were not statistically significant in a linear mixed effects model due to the overlapping interquartile ranges. However, extrapolating to findings from other studies, reduction in the amount of virions produced that

were observed here are in line with what would be expected if LRAs potently reversed latency and led to viral cytopathic effects to the degree that others have suggested[273,297,330].

Next, we used our fPVE for virion production to evaluate whether the fraction of proviruses that are inducible following treatment with LRAs was substantially reduced compared with medium control. Using fPVE, we found that the median fold-change in the fraction of proviruses that were inducible was 1.5, 2.0 and 3.5, respectively, for romidepsin, bryostatin and romidepsin + bryostatin compared to medium control. At the level of individual donors, romidepsin led to decreases in maximal fPVE for six of eight donors, while bryostatin led to decreases in four of six donors and romidepsin + bryostatin led to decreases in five of six donors.

In order to control for the variation in the fraction of proviruses that were inducible from the medium control condition from each donor, we next evaluated fPVE for virion production as a fold-change for LRAs over medium control. It is clear from this analysis that when normalizing for the maximum fraction of proviruses that can be reactivated in each donor, the fraction of proviruses that can be reactivated has not been substantially reduced following LRA treatment. Although the medians are lower for LRA treated conditions, the interquartile ranges are overlapping. Despite the lack of a consistent decrease across donors, it does appear that some donors have a drop in the fraction of proviruses that are inducible following treatment with LRAs. However, the lack of a consistent decrease across donors is in line with a previous study by Shan et al. that quantified the size of the infectious viral reservoir by quantitative viral outgrowth following treatment of rCD4 cells *ex vivo* with SAHA, and found that there was no change[339]. Lack of an observed change in the size of the reservoir following treatment with LRAs is also consistent with the results from in vivo studies, where treatment with panobinostat did not lead to a change in the length of time to viral rebound following cessation of ART[257], and

treatment with romidepsin[317] did not lead to changes in the size of the reservoir as measured by TILDA[340] or QVOA[302].

An assumption of this experimental design is that the LRA-treated rCD4 cells are equally as responsive to T cell activation stimulus as cells in the medium control condition. To evaluate whether this assumption was true, cellular viability and the expression of activation markers following T cell activation for 7 days was assessed. In the three donors in whom we evaluated cellular viability, we found that romidepsin and bryostatin + romidepsin treatment conditions reduced the cellular viability following T cell activation compared to medium control. This reduction in T cell viability following romidepsin treatment may be related to the impaired cytotoxic T cell response observed following treatment with romidepsin[341]. For romidepsin treatment, the reduction in cellular viability was nearly equivalent to the median fold-change in the fraction of inducible proviruses, suggesting that reductions in viability could explain the apparent reduction in the size of the inducible reservoir. Bryostatin treatment alone did not substantially reduce the viability following maximum reactivation, but viability for bryostatin + romidepsin treatment was somewhat reduced. While viability was reduced for bryostatin + romidepsin, the median fold-change in the fraction of inducible proviruses was greater than the drop in viability, suggesting that decrease responsiveness to activation may not fully explain the drop observed.

Assessment of levels of activation marker expression following full T cell activation revealed that there was heterogeneity in the responsiveness to T cell activation following treatment with LRAs. In donors 5 and 7, there were reductions in the amount of CD69 expression following treatment with LRAs, but levels of CD69 expression were nearly equivalent in the other donors. Donor 7 also had a decrease in levels of CD25 expression

following treatment with LRAs, whereas CD25 expression was consistent in other donors. There was not any clear relationship between viability and activation marker expression, or decreases in the fraction of proviruses that could be reactivated and activation marker expression. From these data, it appears that cellular viability may be a more important and consistent measure to evaluate ability of cells to respond to full T cell activation. However, both measures of cellular viability and activation marker are ultimately informative for the overall state of cells following reactivation. Future studies should also evaluate cytokine production between treatment conditions as a further assessment of equivalent reactivation between conditions.

Finally, we sought to evaluate whether changes in the fraction of proviruses that were inducible following treatment with LRAs was related to the fraction of proviruses that were initially reactivated following treatment with LRAs. The only treatments that had sufficient results to perform this analysis were romidepsin and bryostatin + romidepsin treatment, as bryostatin did not lead to virion production in some donors. We found that the fraction of maximally inducible proviruses was related to the fraction that were initially reactivated for bryostatin + romidepsin treatment, but not for romidepsin treatment. This makes sense in the context of the viability data described above, where it appears that rCD4 cells treated with romidepsin alone were less responsive to T cell activation, while bryostatin + romidepsin treated cells were substantially more responsive. Therefore, the relationship between the fraction of proviruses initially reactivated by romidepsin is likely not related to the fraction of proviruses that can be maximally reactivated following treatment due to the reduced viability of the cells following romidepsin treatment. It is worth noting that this study is preliminary and involves a small number of donors. Future studies should evaluate bryostatin alone and romidepsin + bryostatin treatment in more donors, and should include thorough assessments of whether LRA

treated cells can be activated to the same degree as medium control cells. This could be assessed with cellular viability, activation marker expression, cytokine profiling of cytokines produced from activated cells and RNAseq to evaluate whether transcriptional signatures have changed between treatment conditions. Finally, sequencing of virions produced from activated cells could also be performed and compared to medium control to evaluate whether fewer proviral sequences are produced following treatment with LRAs, consistent with a reduction in the size of the inducible reservoir.

In this chapter, we have extended our findings from assessments of potency of LRAs, to the impact that LRA treatment has on the fraction of proviruses that are inducible by maximum T cell activation. Given the relative impotency of LRAs to reactivate proviral expression, it is not surprising that only marginal reductions in the size of the latent reservoir were observed. Additionally, analysis of the fraction of proviruses that are maximally inducible following treatment with LRAs is complicated by the fact that the viability and expression of activation markers following T cell activation after treatment with LRAs is variable. Differences in viability and ability to be activated following treatment with LRAs is a key point, and prevents us from drawing strong conclusions as to whether the size of the inducible reservoir has truly been reduced.

The analysis of LRAs from the proceeding chapters, in conjunction with this chapter, suggests that the ability of LRAs to disrupt the latent reservoir to the point of virion production are minimal *ex vivo*. Although increased levels of cellular HIV-1 RNA could lead to viral cytopathic effects from increased translation of viral proteins, our results indirectly argue against this possibility, as substantial reductions in the size of the reservoir were not observed, although interpretation is difficult due to the changes in viability and activation marker expression

following treatment with LRAs, as described above. While we did observe a relationship between reactivation and decreases in the fraction of proviruses that were inducible following treatment with bryostatin + romidepsin, the magnitude of the reduction was not impressive. It is possible that a larger portion of cells could produce viral proteins and be cleared by immune effector responses *in vivo*, but we did not seek to address this point in this study. Indeed, Shan et al. demonstrated in a primary cell model that primed cytotoxic T lymphocyte responses led to reductions in the size of the reservoir following treatment with SAHA[339]. Whether immune effector functions can be harnessed to facilitate clearance of the reservoir needs to be evaluated in future studies. Taken together, our results suggest that much more potent LRAs, and increased specificity of LRAs for HIV-1 expression, are needed to achieve a safe and effective kick and kill approach to achieve reservoir reduction.

7.0 CHAPTER 4. BIOMARKERS OF THE INDUCIBLE RESERVOIR OF HIV-1 IN PATIENTS ON LONG TERM SUPPRESSIVE ANTIRETROVIRAL THERAPY

7.1 PREFACE

Anthony R. Cillo[1], Francis Hong[1], Angela Tsai[2], Alivelu Irrinki[2], Jasmine Kaur[2], Jacob Lalezari[3], Derek Sloan[2], Mattie Follen[1], Jeffrey P. Murry[2], John W. Mellors[1]

[1]University of Pittsburgh, Division of Infectious Diseases

[2]Gilead Sciences, Incorporated

[3]Quest Clinical Research

The work presented in this chapter is in partial fulfillment of aim 2 of this dissertation. Angela Tsai, Alivelu Irrinki and Jasmine Kaur isolated PBMC and rCD4 cells from leukapheresis product, set up cultures of PBMC and rCD4 cells, harvested supernatants and assessed levels of virions produced in the supernatant. Anthony Cillo, Francis Hong and Mattie Follen performed qRT-PCR experiments for the quantification of persistence of HIV-1 in plasma, PBMC and rCD4 cells. Jacob Lalezari enrolled study participants for leukapheresis. Anthony Cillo, Derek Sloan, Jeffrey Murry, and John Mellors conceived of the study. Anthony Cillo, Jeffrey Murry and John Mellors analyzed the data, and Anthony Cillo performed statistical analysis.

The work in this chapter was funded by the Pitt AIDS Research Training Program 5 T32 AI065380-08, an R21 from the NIH 1R21AI113102-01, a research grant from Gilead Sciences, and COBAS AmpliPrep/TaqMan kits provided by Roche.

7.2 INTRODUCTION

In the era of modern antiretroviral therapy for HIV-1 infection, regimens are capable of completely suppressing viral replication as long as daily adherence is maintained[79]. As such, those living with HIV-1 now have lifespans approaching those of HIV-1 negative people[342-347], although mortality remains higher from non-AIDS complications in the era of ART[348]. The transformation of HIV-1 infection from a death sentence to a manageable chronic disease is a triumph of modern medicine. This progress was achieved through careful clinical assessments of antiretroviral drugs, which would not have been possible without a biomarker of effective therapy. It was not until the discovery of plasma viremia in uncontrolled HIV-1 infection as a marker of the time to progression to AIDS[108,109], that clinical progress could be evaluated with a molecular biomarker, rather than assessing a time- and cost-intensive clinical result such as time to development of AIDS or death. Therefore, identifying drug candidates that could durably suppress plasma viremia was the key to rapid development of the ART drugs that we have today.

Despite the complete suppression of viral replication as long as therapy is maintained, HIV-1 persists indefinitely, and viral recrudescence occurs within days to weeks following cessation of therapy[116,233,257,349], and seems to rebound from many sources within lymphoid

tissue simultaneously[350]. Persistence of HIV-1 can be detected with specialized assays, such as viral outgrowth assays from rCD4 cells[302], as molecular species (i.e., proviral HIV-1 DNA and cellular HIV-1 RNA) from PBMC and other cellular subsets[300], and as low level viremia in plasma on the order of 1 copy per milliliter of plasma[112-114,303]. Despite the ability of specialized research assays to quantify various forms of viral persistence, the clinical relevance of any given measure of persistence in those on suppressive therapy remains unknown. We are currently in a time period for the assessement of the clinical efficacy of curative interventions that is analogous to the time before the establishment of plasma viremia as a barometer of success. Testing of novel interventions to achieve a cure is occurring in clinical trials, but we do not have a biomarker that unequivocally demonstrates efficacy of any given approach.

In this study, we sought markers of persistent HIV-1 reservoirs in persons on on long-term suppressive ART and relationships between such markers. The makers we evaluated included molecular measures of HIV-1 persistence and culture-based measures of spontaneous and inducible virion production from both PBMC and rCD4 cells. Inducible virion production, namely levels of HIV-1 RNA in cell culture supernatants following treatment with PMA/iono, was chosen as a culture-based viral endpoint rather than the number of cells harboring infectious proviruses. This choice was made in part because of the tractability of measuring virion production over infectious virion production, but also because it eliminates one level of stochastics, as both virion production and infection of subsequent cells both are likely stochastic events[351-355]. Finally, virion production should logically correlate with the time to viral rebound following cessation of ART, as virions must be produced to infect new cell targets and lead to viral rebound. No studies to date have investigated relationships among makers, or evaluated whether any makers were correlated with inducible and spontaneous virion production from

cultured PBMC and rCD4 cells. We hypothesized that as the frequency of HIV-1 infected cells and their transcriptional activity increases, levels of spontaneous and inducible virion production will concomitantly increase.

7.3 RESULTS

7.3.1 Baseline characteristics of the participants studied

To assess whether biomarkers of virologic persistence are related to levels of spontaneously released and inducible HIV-1 virions production by PBMC and rCD4 cells, we enrolled 21 consecutive HIV-positive study participants who were on suppressive ART for ≥1 year to undergo leukapheresis (Table 14). Leukapheresis was preferred over large volume blood draws to allow for the setup of culture experiments with large number of rCD4 cells and PBMC in parallel. All leukaphereses were performed at Quest Clinical Research (San Francisco, CA), and all participants gave informed written consent. The median age of the study participants was 54 years old, all patients had high CD4+ T cell percentages (median 34% of lymphocytes) and counts (median 725 cells/mm^3) at the time of leukapheresis, and all study participants were male. Low-level viremia was assessed with a large-volume adaptation of the single-copy assay to allow for improved precision and a lower limit of detection[303]. Low-level viremia was quantifiable in 13 of 19 participants tested, and the median level of viremia was 1.1 copies per milliliter of plasma. Total cellular HIV-1 proviral DNA and unspliced cellular HIV-1 RNA were quantified

in PBMC, with cellular HIV-1 DNA (CA HIV-DNA) quantifiable in all 21 study participants and cellular HIV-1 RNA (CA HIV-RNA) quantifiable in 20 of 21 participants.

Table 14. Participant characteristics for the assessment of biomarkers of the inducible reservoir

Participant ID	Age	CD4+ T cell count (cells/mm3)	Percent CD4+ T cells in lymphocytes	Mega-iSCA (copies of HIV-1 RNA per milliliter of plasma)	Cellular HIV-1 DNA (copies per 10^6 PBMC)	Cellular HIV-1 RNA (copies per 10^6 PBMC)	ART Regimen
1	69	742	41.2	1.1	563	74	EFV/FTC/TDF/ RAL
2	54	653	36.3	<0.05	172	82	ND
3	57	587	36.7	1.2	245	40	3TC/ABC/DTG
4	55	762	23.8	1.7	192	3	3TC/ABC/DRV/r
5	51	692	17.3	6	2972	355	DRV/FTC/MVC/ r/RAL/TDF
6	54	607	26.4	2.3	283	37	EFV/FTC/TDF
7	61	1022	29.2	0.81	1061	92	ND
8	58	632	23.4	11	636	177	ETR/MVC
9	50	1345	26.9	6.3	80	35	3TC/ABC/DTG/ DRV/LPV/r/ZDV
10	54	590	26.8	<0.074	611	53	ND
11	52	422	28.1	<0.046	78	4	ATV/DTG/FTC/ r/TDF

12	53	958	38.3	4.0	504	90	ETR/FTC/MVC/ RAL/TDF
13	52	832	39.6	2.8	211	21	3TC/ABC/ATV/ LPV/r
14	64	725	48.3	0.67	453	70	ND
15	64	538	33.6	<0.087	31	15	3TC/ABC/ LPV/r/TDF
16	39	556	39.7	<0.074	160	28	EFV/FTC/TDF
17	56	882	42	ND	7	<1	BMS663068/FTC/ RAL/TDF
18	54	699	36.8	ND	366	13	3TC/ATV/TDF
19	57	780	39	3.0	381	39	ND
20	47	796	36.2	<0.10	289	11	DRV/DTG/r
21	62	783	43.5	0.42	91	2	FTC/RAL/TDF
Median (Interquartile range)	54 (52-58)	725 (607-796)	34 (27-40)	1.1 (0.047-2.9)	283 (160-504)	37 (13-74)	ND

ND = not determined; 3TC = lamivudine; ABC = abacavir; ATV = atazanavir; BMS663068 = fostemsavir; DRV = darunavir; DTG = dolutegravir; EFV = efavirenz; ETR = etravirine; FTC = emtricitabine; LPV = lopinavir; MVC = maraviroc; r = ritonavir; RAL = raltegravir; TDF = tenofovir disoproxil fumarate; ZDV = zidovudine

The median level of CA HIV-DNA was 283 copies per 10^6 PBMC, while the median level of CA HIV-RNA was 37 copies per 10^6 PBMC. Participants had plasma viremia stably suppressed to <50 copies per milliliter of plasma on a variety of ART regimens, including regimens with protease inhibitors, nucleoside and non-nucleoside reverse transcriptase inhibitors, integrase inhibitors, and entry inhibitors.

7.3.2 Correlograms of the relationships between variables

To begin assessing relationships between molecular biomarkers of HIV-1 persistence and culture-based measures of virion production *ex vivo* from participants on suppressive ART, we constructed a correlogram based on Spearman's correlation between the 12 continuous variables assessed in this study (Figure 16). All variables studied are listed on both the x- and y-axis, with the top right hand side of the correlogram showing a heat map derived from the correlation coefficients from Spearman's correlation (rho), with red indicating a strong positive correlation, blue indicating a strong negative correlation, and white representing no correlation. Correlation coefficients that were statistically significant based on uncorrected p values obtained from the pairwise Spearman's correlations are marked with an asterisk (*). The bottom left side of the correlogram shows individual scatterplots of the data from each variable that was used to test for a correlation. From the 66 unique pairwise comparisons tested, we found that 26 were statistically significant when no effort was made to correct for the family-wise type-I error rate or the false discovery rate. It is evident from the correlogram that CA HIV-DNA and CA HIV-RNA in PBMC are strongly correlated with numerous other variables, including measures of spontaneous and inducible virion production from PBMC and rCD4 cells. Levels of total HIV-1

DNA from rCD4 cells are also strongly correlated with some measures of spontaneous and inducible virion production. For culture-based measures of spontaneous and inducible virion production, $15x10^6$ PBMC were plated in 5 milliliters of culture medium in 6 well plates, and $5x10^6$ resting CD4+ T cells were cultured 2 millilter of culture medium in 24 well plates (more details are provided in the Overall materials and methods section).

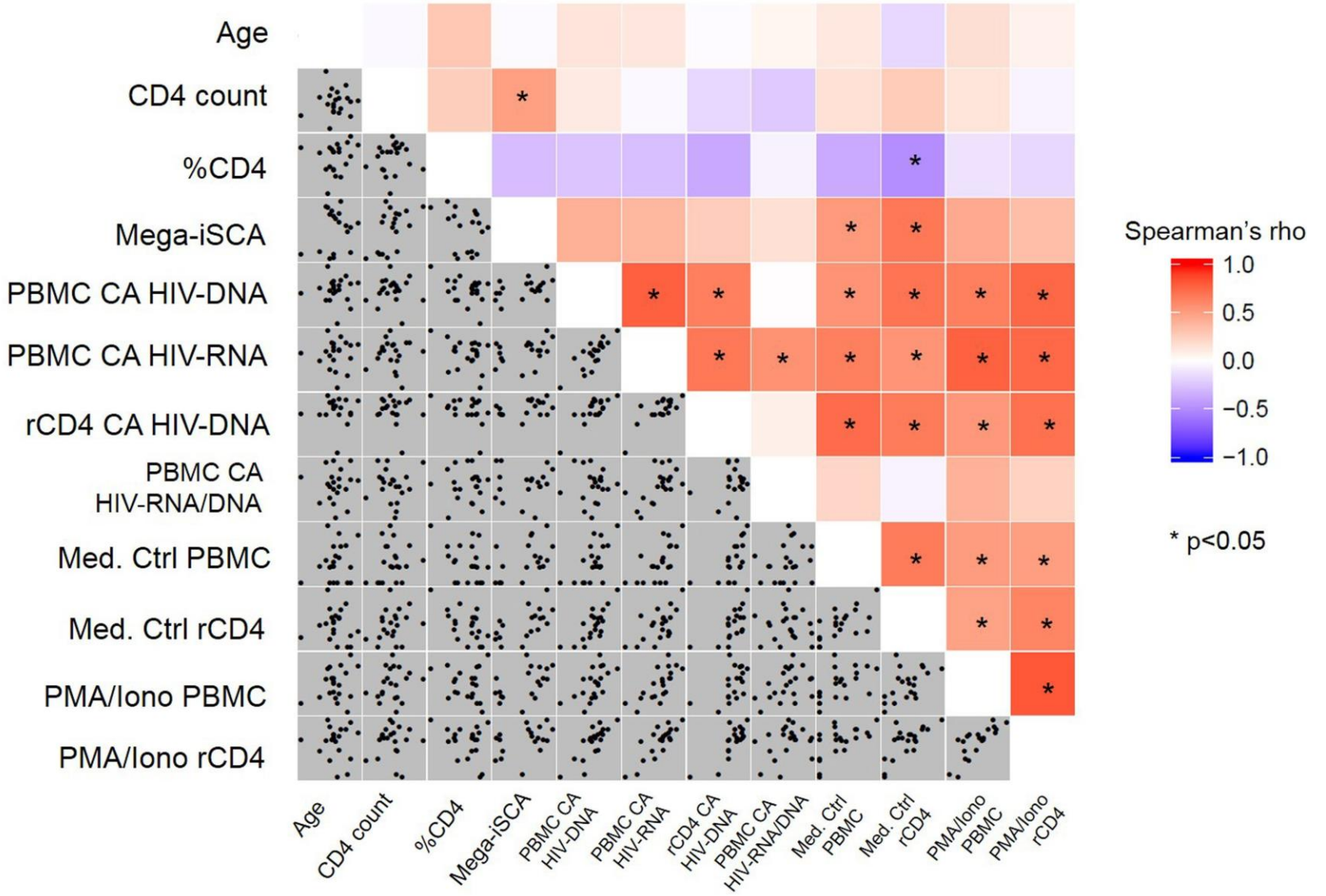


Figure 16. Correlogram of relationships between continuous variables with uncorrected p values. The top right half of the correlogram is a heat map showing the strength of the correlation between variables listed on the x- and y-axis (with strong positive correlations appear red, strong negative correlations appearing blue, and no correlations appearing white). Statistically significant correlations based on uncorrected p values from Spearman's correlations are marked with an asterisk (*). The bottom left half of the graph shows scatterplots of the data that were used for the correlations tested.

Since 66 pairwise comparisons were tested from the same data set in this study, it is important to control either the family-wise type-I error rate (FWER), or the false discovery rate (FDR). While keeping the FWER at α=0.05 (i.e., by using Holm's correction[356]) prevents the inclusion of even one false positive discovery (i.e., rejecting the null hypothesis of no correlation when there is in fact not a statically significant correlation), it is overly conservative and will exclude correlations that actually reject the null hypothesis. As such, an attractive alternative is to control the FDR (i.e., by using the Benjamini and Hochberg correction[314]), and to allow for a proportion of the rejected null hypothesis to be erroneously kept. This method allows for the retention of more power, albeit by a slight tradeoff at the expense of the FWER. Another benefit of FDR correction over FWER correction is that it FDR is both adaptable (allows for a number of falsely rejected null hypothesis based on the total number of rejected null hypotheses) and scalable (the proportion of falsely rejected null hypotheses remains the same regardless of the number of hypotheses rejected)[314]. We elected to use the Benjamini and Hochberg (BH) method to control the FDR since we had a total of 26 null hypotheses rejected out of 66 tested hypotheses; controlling the FWER in this context would be overly conservative, and would likely eliminate many true alternative hypotheses.

A correlogram showing the same relationships described in Figure 16, but with asterisks marking statistically significant correlations following correction of the p values from the Spearman's correlations using the BH method. Overall, 19 of the previously identified 26 correlations were retained after controlling the FDR. Importantly, all of the relationships between CA HIV-DNA and CA HIV-RNA and other variables were retained after controlling the FDR.

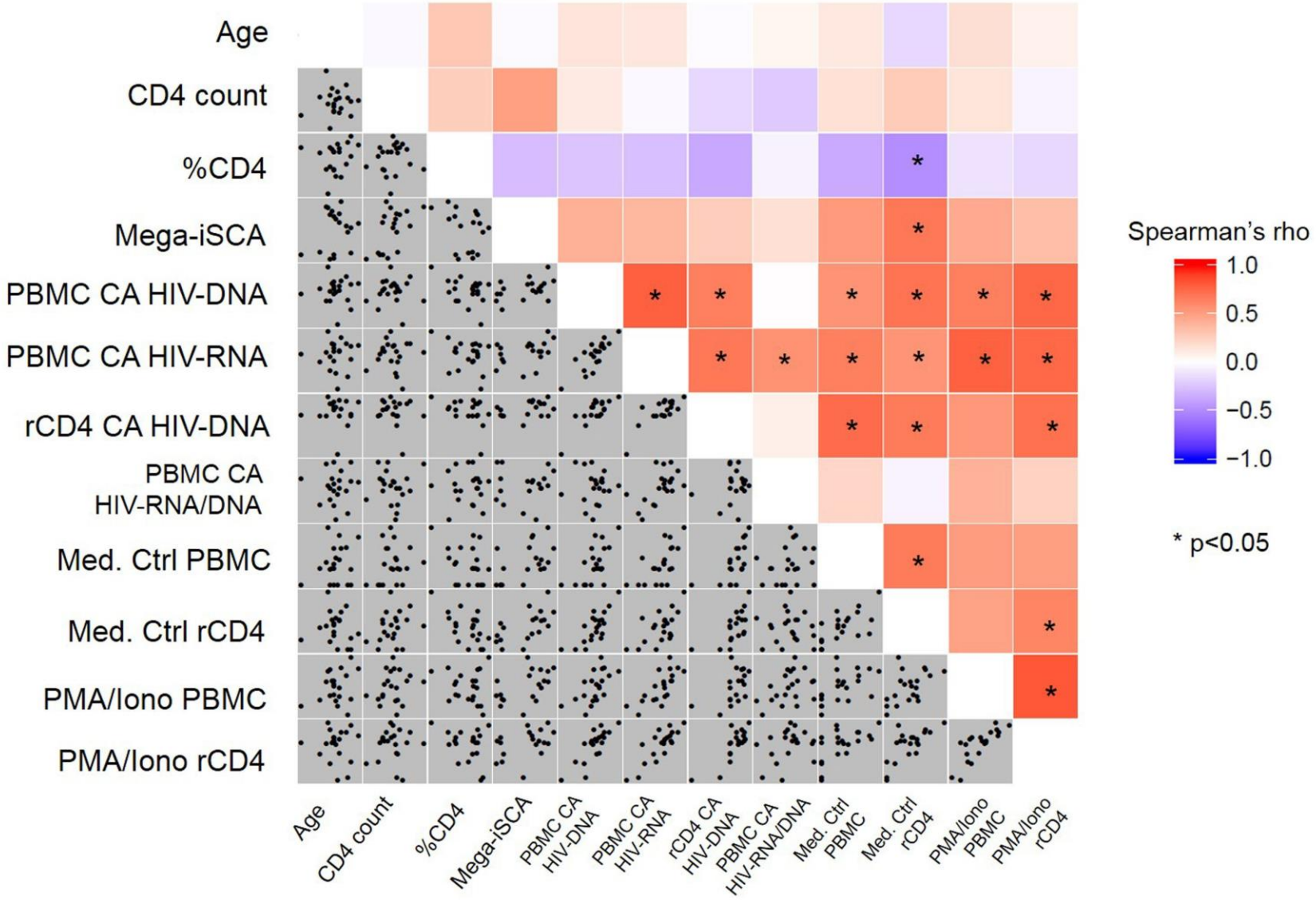


Figure 17. Correlogram showing significant correlations after controlling the false discovery rate. The layout of the correlogram is identical that that in Figure 16, with the exception being that the p values for the Spearman's correlations have been corrected to control the FDR. Of the 26 previously identified statistically significant relationships identified, 19 were retained after controlling the FDR, including all of the previously identified relationship involving CA HIV-DNA and CA HIV- RNA.

7.3.3 Assessment of relationships between individual variables

After using the correlogram to evaluate general relationship between the variables studied, we next sought to specifically evaluate individual relationships in more detail. We first evaluated the relationship between CA HIV-DNA and CA HIV-RNA in PBMC, and found that the frequency of infected cells (CA HIV-DNA) was related to levels of transcription of unspliced HIV-1 RNA (CA HIV-RNA). This finding demonstrates that the great the frequency of infected cells, the higher the HIV-1 transcriptional activity per million PBMC (rho=0.78, FDR-corrected p=0.001).

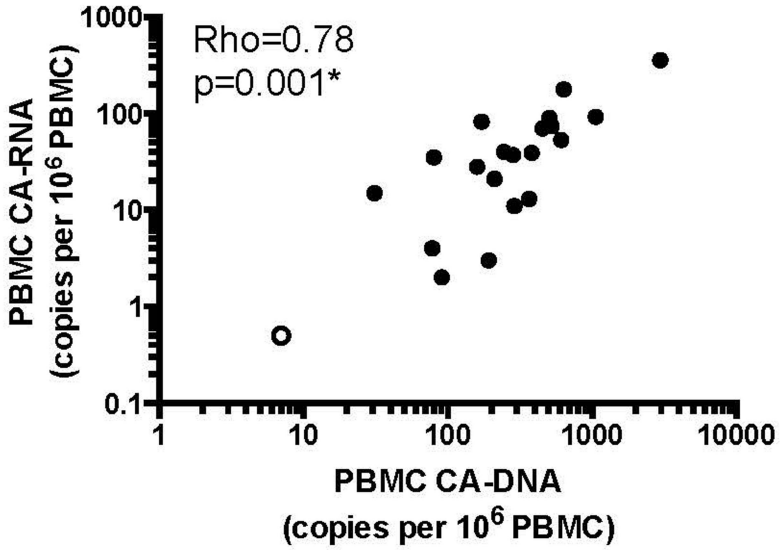


Figure 18. Relationship between levels of cellular HIV-1 DNA and cellular HIV-1 RNA. The frequency of infected cells in PBMC, as measured by qPCR for cellular HIV-1 DNA, is related to levels of HIV-1 transcription, as measured by qRT-PCR for unspliced HIV-1 RNA transcripts. The open symbol denotes a sample with undetectable CA-RNA. The CA-RNA value was interpolated to be 0.5 copies per million PBMC. Asterisk indicates that the p-value has been corrected by controlling the FDR.

After evaluating relationships between the frequency of infected cells and their transcriptional activity, we next assessed whether there was any relationship between levels of low-level viremia measured in plasma and measures of spontaneous virion production from cultured PBMC and rCD4 cells (Figure 19).

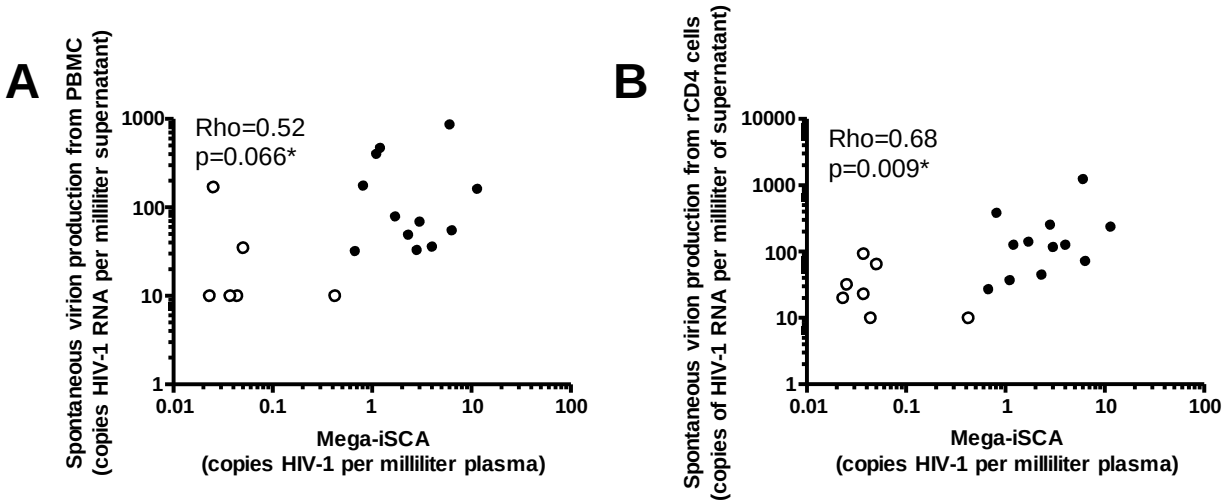


Figure 19. Mega-iSCA is associated with spontaneous virion production. Spontaneous virion release from *ex vivo* cultured PBMC and rCD4 cells was compared to low- level viremia as measured by mega-iSCA. **A)** There was a trend towards a positive correlation after controlling for the FDR between mega-iSCA and spontaneous virion release from PBMC (rho=0.52, p=0.066 controlling for FDR), while **B)** the relationship was statistically significant for virion release from rCD4 cells and mega-iSCA (rho=0.68, p=0.009 controlling for FDR). Open symbols denote samples that were negative by either Mega-iSCA, spontaneous virion production, or both. Undetectable values were interpolated as 50% of the limit of detection. Asterisks indicate that the p-values have been corrected by controlling the FDR.

Low-level viremia, as measured by mega-iSCA, was statistically significantly correlated with spontaneous virion release from both unstimulated PBMC and rCD4 cells cultured *ex vivo* prior to correcting for the false discovery rate. However, after controlling for the FDR, there was still a trend towards a relationship between spontaneous virion release from PBMC and mega-iSCA although it was not significant (rho=0.52, p=0.066 controlling for FDR), while the correlation

remained significant for spontaneous virion release from rCD4 and mega-iSCA (rho=0.68, p=0.009 after controlling for FDR).

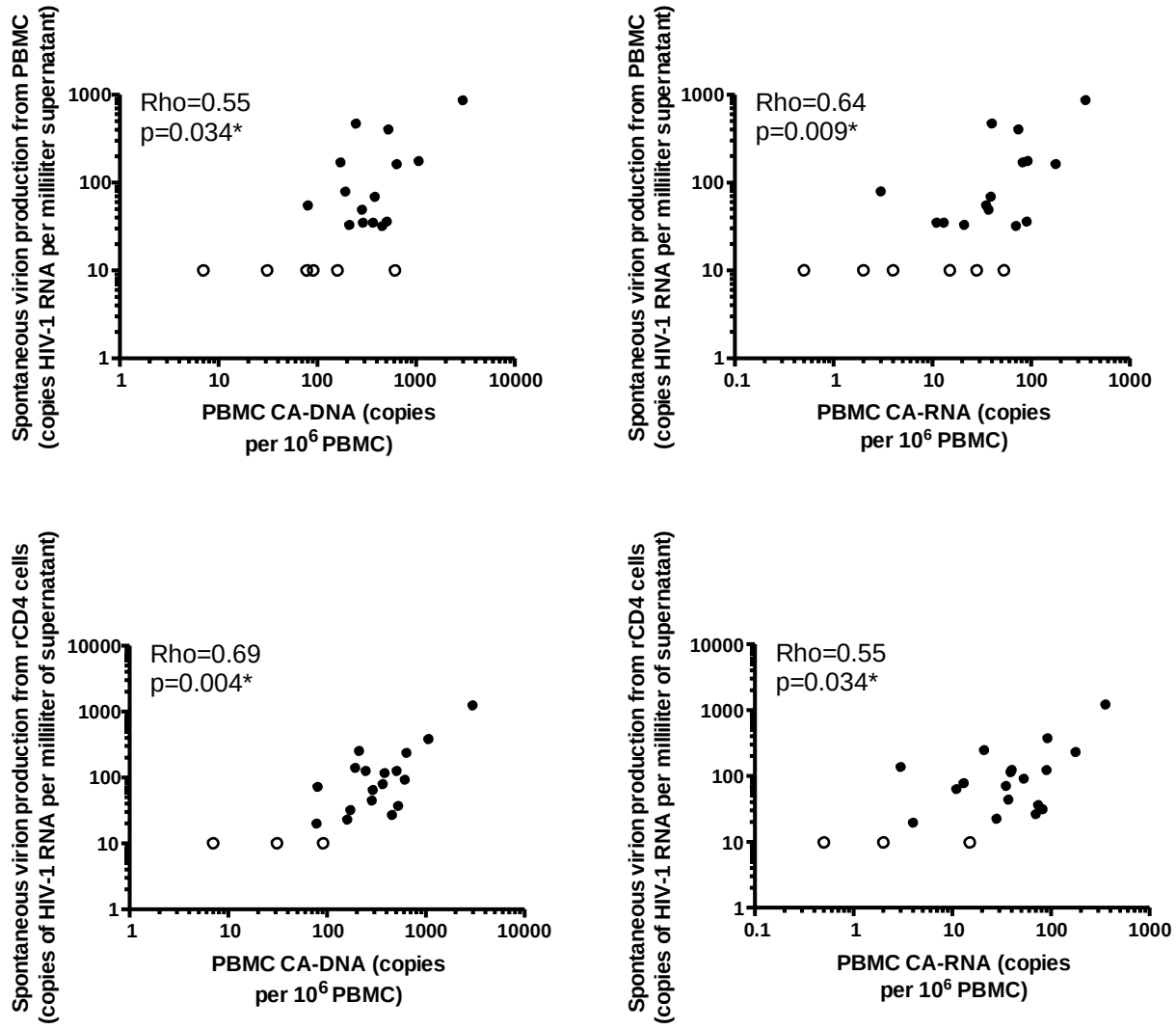


Figure 20. Cellular HIV-1 DNA and RNA from PBMC is associated with spontaneous virion production. Levels of CA HIV-DNA and CA HIV-RNA in uncultured PBMC are significantly associated with levels of spontaneous virion production from both PBMC and rCD4 cells in culture. The frequency of infected cells, and their transcriptional activity, are related to the amount of spontaneous virion release *ex vivo*. All values remained statistically significant after correcting the FDR. Undetectable samples are represented by open symbols, and were interpolated as 50% of the limit of detection. Asterisks indicate that the p-value has been corrected by controlling the FDR.

Next, we assessed whether CA HIV-DNA and CA HIV-RNA were associated with levels of spontaneous virion release from cultured PBMC and rCD4 cells (Figure 20). After controlling the FDR, we found that the frequency of infected cells in PBMC was associated with levels of spontaneous virion production from cultured PBMC (rho=0.55,p=0.034 controlling for FDR) and rCD4 cells (rho=0.69,p=0.004 controlling for FDR). Additionally, we found that levels of HIV-1 RNA transcription in PBMC were significantly related to levels of spontaneous virion production from PBMC (rho=0.64,p=0.009 controlling for FDR) and rCD4 cells (rho=0.55,p=0.034 controlling for FDR). The frequency of infected cells, and their transcriptional activity, in uncultured PBMC are significantly related to the levels of spontaneous virion production from cultured PBMC and rCD4 cells.

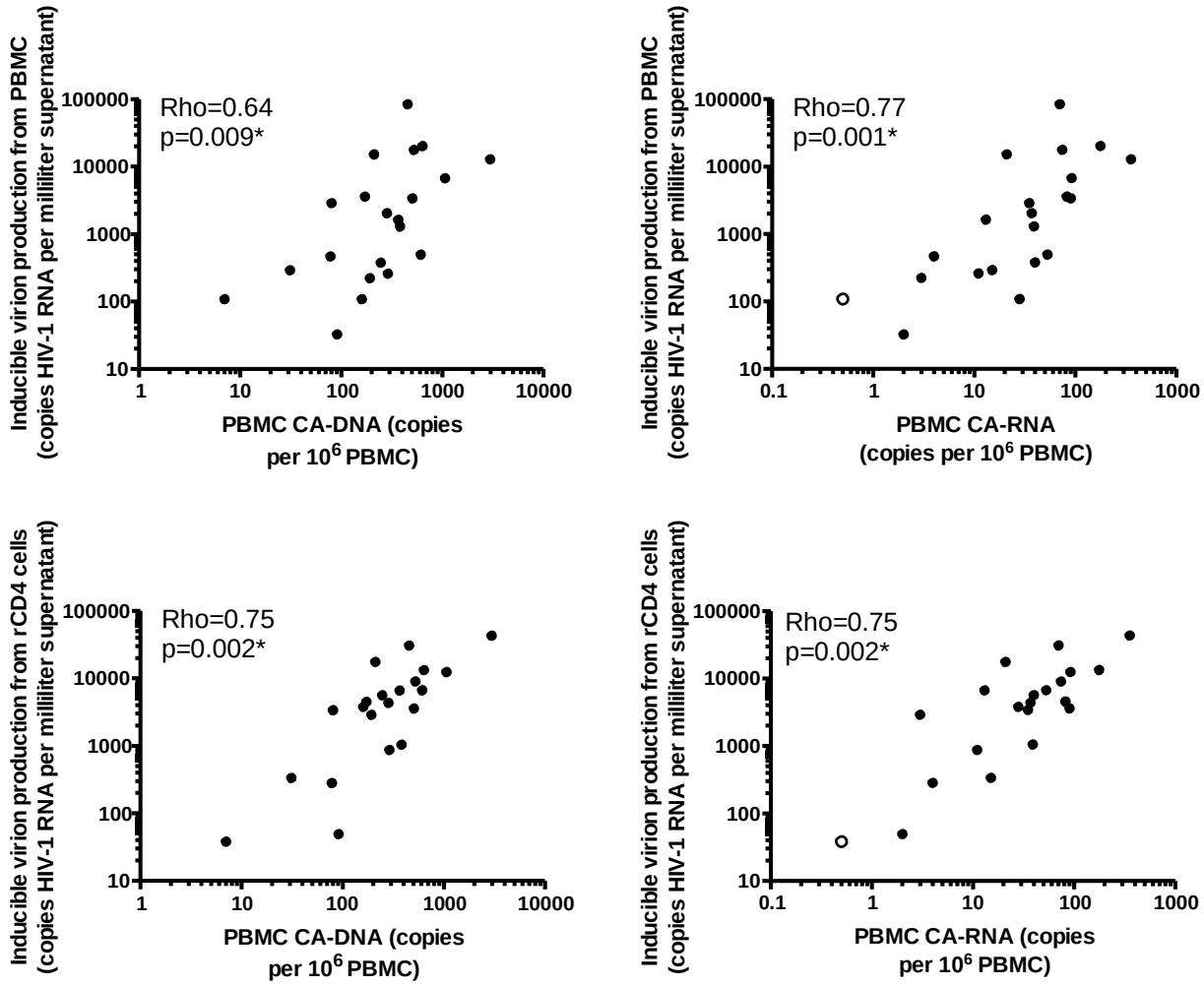


Figure 21. Cellular HIV-1 DNA and RNA from PBMC is associated with inducible virion production. Levels of inducible virion production were quantified and compared with the frequency of infected cells, and the number of cellular HIV-1 RNA transcripts per million uncultured PBMC. This analysis revealed that the frequency of infected cells in PBMC were related to inducible virion production from PBMC and rCD4 cells, and that cellular HIV-1 transcription was also related to the amount of virions that could be produced following T cell activation. The open symbol represents the sample with undetectable CA-RNA, which was interpolated as 50% of the limit of detection. Asterisks indicate correction of p-values by controlling FDR.

We next evaluated whether there was a relationship between the frequency of infected cells and their transcriptional activity in PBMC, and levels of virus that were produced following treatment of PBMC and rCD4 cells with PMA/ionomycin (Figure 21). We found that there were

strong positive relationships between CA HIV-DNA and CA HIV-RNA in PBMC, and the levels of inducible virion production from PBMC and rCD4 cells. Specifically, CA HIV-DNA was strongly associated with levels of virion production from PBMC (rho=0.64,p=0.009 controlling for FDR) and rCD4 cells (rho=0.72,p=0.002 controlling for FDR), demonstrating that the frequency of infected cells is related to the levels of virions that are produced following maximum T cell activation in culture. Levels of CA-RNA in PBMC were also strongly associated with inducible virion production from PBMC (rho=0.77,p=0.001 controlling for FDR) and rCD4 cells (rho=0.75,p=0.002 controlling for FDR), implying that the transcriptional activity of the infected cells in bulk uncultured PBMC is related to the levels of virions that are produced following full T cell activation.

Finally, we evaluated whether the amount of inducible virion production from rCD4 cells was related to the amount of virion production following activation of PBMC in culture (Figure 22). We reasoned that since rCD4 cells are contained within PBMC, levels of virions produced from rCD4 cells should be proportion to levels of virions produced from PBMC. We found that this was indeed the case, and there was a strong relationships between levels of virion production from PMA/ionomycin treated PBMC and PMA/ionomycin treated rCD4 cells in culture (rho=0.81,p=0.0005 controlling for FDR).

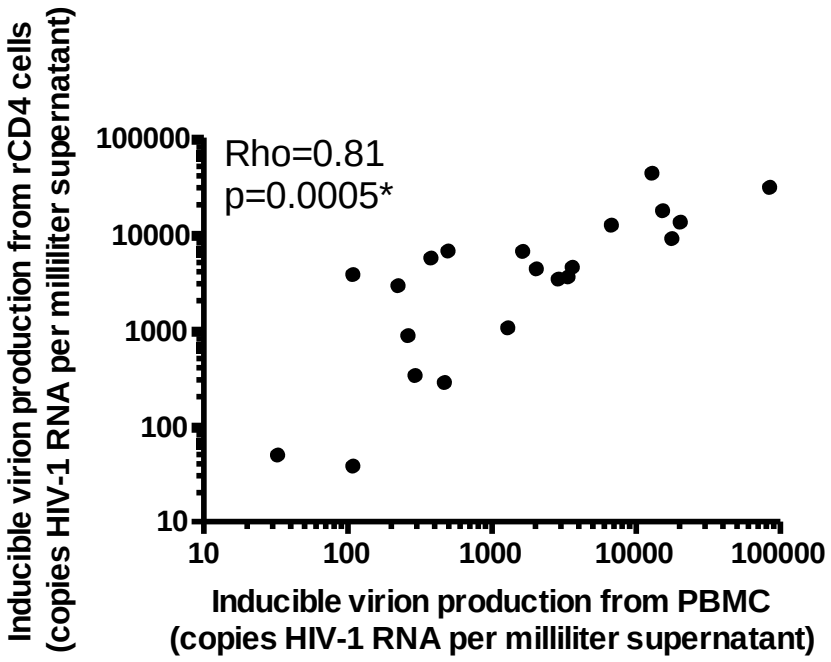


Figure 22. Inducible virion production from PBMC and rCD4 cells are strongly correlated. In parallel cultures of PBMC and rCD4 cells isolated from the same study participants at the same timepoint, we found that there was a strong positive relationship between the virions that were produced following treatment with PMA/ionomycin from both cellular populations. Asterisk indicates a corrected p-value by controlling for FDR.

7.3.4 Interaction networks demonstrating interrelationships between variables

From the data presented thus far, it is clear that there are many interrelationships between the 12 variables that were studied, and particularly many relationships between measures of CA HIV-DNA and CA HIV-RNA and the levels of spontaneous and inducible virion production from PBMC and rCD4 cells. To better display these interrelationships, we constructed an interaction network based on the statistically significant Spearman's correlations between the individual variables studied (Figure 23). In the interaction networks displayed, each node represents an

individual variable and each connection between nodes represents a statistically significant correlation, after correcting for the FDR.

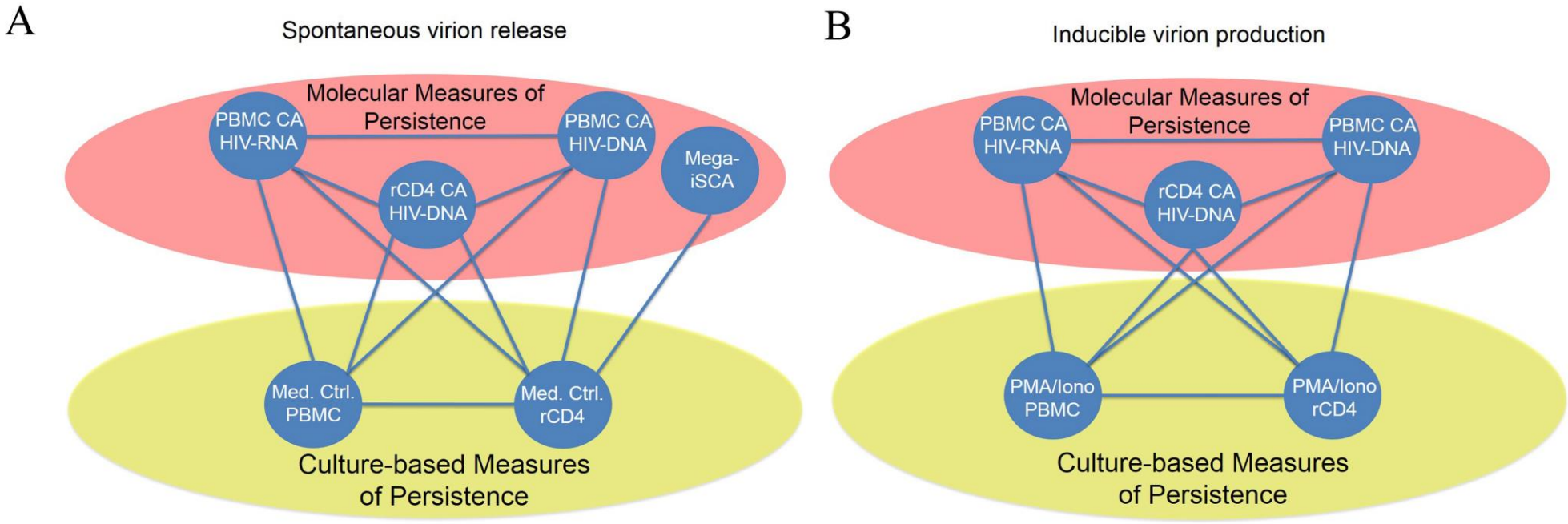


Figure 23. Interaction networks of relationships between virologic variables. Interaction networks are shown, demonstrating the interrelationships between virologic variables, and highlighting interactions between molecular measures of HIV-1 persistence and culture-based measures of virion production. In the interaction networks, each node represents a variable, and the connections between nodes represent statistically significant Spearman's correlations after correcting for FDR. **A)** Relationships between molecular measures of viral persistence from PBMC (CA HIV-DNA and CA HIV- RNA from PBMC, the ratio of CA HIV-RNA/DNA in PBMC, and CA HIV-DNA in rCD4 cells) and plasma (mega-iSCA), and spontaneous virion production are shown. Notably, rCD4 CA HIV-DNA is related to spontaneous virion production from PBMC and rCD4 cells, and levels of persistent viremia *in vivo* (mega-iSCA) was related to spontaneous virion production from rCD4 cells. **B)** Relationships between molecular measures of persistence and culture-based measures of inducible virion production are shown. In this case, PBMC CA HIV-DNA and CA HIV-RNA were related to rCD4 CA HIV-DNA, but unlike CA HIV-DNA/RNA from PBMC, CA HIV-DNA from rCD4 cells was only significantly related to inducible virion production from rCD4 cells and not from PBMC.

7.4 DISCUSSION

Taken together, these results have demonstrated that spontaneous and inducible virion production from sufficient numbers of cultured PBMC and rCD4 cells are related to the frequency of infected cellsand their transcriptional activity following careful assessment by qRT-PCR assays. From a total of 66 variables studied, we initially found that 26 correlations assessed by Spearman's correlation were statistically significant. However, after controlling the false discovery rate, we found that 19 of the pairwise comparisons remained statistically significant, including all of the relationships between measures of total cellular HIV-1 DNA (CA HIV-DNA) and total cellular HIV-1 RNA transcription (CA HIV-RNA) in PBMC.

The relationship observed between the frequency of infected cells and levels of HIV-1 RNA transcription suggests a proportionality between the number of infected cells, and the amount of HIV-1 RNA that is transcribed per million cells. In agreement with these results, this relationship has been recently observed in another study by Hong et al.[304], demonstrating the reproducibility of these results across participant cohorts. While we can make no inference about the level of cellular HIV-1 RNA produced per infected cell or the number of cells that are responsible for virion production, we can deduce that there is proportionality between infected cell frequency and transcriptional activity across study participants.

The findings that CA HIV-DNA and CA HIV-RNA from PBMC are associated with spontaneous virion production from PBMC and rCD4 is novel, and indicates that there is a proportionality between the frequency of infected cells, their basal transcriptional activity, and

their ability to spontaneously release virions without further stimulation. This finding raises two interesting possibilities: i.) there is a reservoir of cells that are producing virions spontaneous and consistently in the absence of any further T cell activation; and ii.) the frequency of infected cells and their transcriptional activity is associated with the proportion of cells in the latent reservoir that are stochastically reactivated at any given time (i.e., more infected cells, the larger proportion that exits that latent reservoir as an activated T cell at a given time). These two possibilities are not mutually exclusive, and both could be driving the size of the spontaneously produced reservoir. The second possibility seems more likely to be a substantial driver of spontaneous virion production, as the prolonged production of HIV-1 antigens from long-lived cell sources would likely render them visible to immune-mediated clearance. From this data, it is not possible to conclude which of these mechanisms is the predominant factor driving spontaneous virion production.

Intriguingly, we also found a relationship between spontaneous virion production from rCD4 cells *ex vivo* and levels of persistent viremia *in vivo* by mega-iSCA. There was also a trend towards a relationship between mega-iSCA and the amount of virions spontaneously released from PBMC, though this relationship was not significant following control of the FDR. These findings highlight the value of this system for evaluating relationships between *ex vivo* variables and *in vivo* measurements of viral persistence. The relationship between levels of viremia *in vivo* and spontaneous virion production from rCD4 cells isolated from the periphery suggests that there is a biological relationship between spontaneous virion production and levels of virions in the periphery, and that measures of persistence in the periphery are associated with body-wide assessments of viral persistence. We did not find any relationships between viral persistence in plasma by mega-iSCA, and levels of CA HIV-DNA or CA HIV-RNA in the periphery, which is

in contrast to an earlier study by Chun et al. that found cellular HIV-1 DNA was associated with persistent plasma viremia[357], though our study was not adequately powered to find the relatively weak relationship they identified.

In terms of assessing the size of the inducible reservoir, to date *ex vivo* studies aimed at quantifying reservoir size have focused predominantly on quantifying the size of the infectious viral reservoir by quantitative viral outgrowth assays (QVOA) from rCD4 cells. Accordingly, studies have sought to correlate other measures of viral persistence with infectious units per million (IUPM) rCD4 cells from QVOA. Previously, relationships have been observed between plasma viremia and QVOA[358], and integrated HIV-1 DNA in PBMC and QVOA[359], and the CA HIV-RNA to CA HIV-DNA ratio in rectal CD4+ T cells and QVOA[359].

Despite the widespread use of QVOA, it has numerous shortcomings, including a requirement for large number of rCD4 cells, irradiated feeder cells, allogeneic blasts as targets for viral infection, and requirements for specialized medium and up to 21-days of coculture. It also has a limited dynamic range due to the infrequency of infectious proviruses in participants on long-term suppressive ART. As such, we have utilized a new assay format, wherein replicate wells of rCD4 cells are plated and activated with PMA/ionomycin, and levels of HIV-1 RNA are assessed in the supernatant by Roche COBAS AmpliPrep/TaqMan, which greatly simplifies measurement of the inducible reservoir, at the cost of having no measurement of infectiousness of the induced viruses.

When comparing the frequency of infected cells and their HIV-1 transcriptional activity in PBMC, we found that both measures were associated with inducible virion production from both rCD4 cells and unfractionated PBMC. These findings were surprising, given recent work by Ho et al., showing that a large proportion of proviruses that were not found to be replication

competent after one round of T cell activation harbor substantial mutations and deletions, rendering them highly defective[151]. Regardless, approximately 12% of these non-induced proviruses were intact in the study by Ho et al., and were therefore potentially inducible. This finding likely complicates measures of the inducible reservoir with simple molecular measures, but the possibility also exists that the non-induced proviruses actually did produce virions and merely were not infectious. Overall, our results suggest that there is proportionality between levels of inducible virus, and total HIV-1 DNA detected by qPCR, which was not found in the study by Ho et al.

Our results demonstrate that simple molecular measures of persistence provide insight into the size of the inducible reservoir in rCD4 cells and PBMC. When broadly considering the factors that are required to permit virion production, it is logical that a greater frequency of infected cells leads to a great number of cells capable of producing virus. Levels of HIV-1 transcription are also related to the size of the inducible reservoir, demonstrating that the frequency of infected cells and the level of transcription both seem to be factors that are associated with the frequency of cells that are capable of producing virions. Once again, this finding implies that there is proportionality between levels of proviruses that can transcribe HIV-1 RNA in their basal state, and the total number of proviruses that are capable of undergoing virion production.

Regardless of the findings of *ex vivo* studies, the standard for the assessment of reservoir size in HIV-1 study participants on long-term suppressive therapy should be the time to viral rebound following cessation of ART. The time to viral rebound following cessation of ART is the most relevant measures of reservoir size, as it is a direct measure of the rapidity with which viral replication resumes, subsequently leading to immune activation, turnover of CD4+ T cells,

and increased risk of transmission and progression to AIDS over time. *In vivo* studies have thus far lent credence to the idea levels of cellular HIV-1 DNA and cellular HIV-1 RNA could serve as biomarkers of the reservoir. The SPARTAC group found a relationship between levels of cellular HIV-1 DNA and the time to viral rebound, using 400 copies per milliliter of plasma as the virologic endpoint[360]. Recently, Li et al. in the AIDS Clinical Trials Group found that time to virologic rebound was associated with levels of cellular HIV-1 RNA on suppressive ART[361]. These two studies contextualize the findings presented here, and demonstrate the feasibility of using molecular measures of cellular HIV-1 RNA and DNA as potential surrogates of the size of the reservoir in those on ART. Furthermore, the relationship between measures of cellular HIV-1 RNA and DNA in PBMC and the size of the inducible reservoir from PBMC and rCD4 cells suggests that simply measuring virions produced in the culture supernatant following activation may be predictive of the time to viral rebound. This possibility is logical, but will need to be rigorously evaluated in future clinical studies of time to viral rebound.

The interrelationships that were uncovered in this study have yet to be elucidated elsewhere, and provide novel insight into the persistence of HIV-1 in patients on suppressive ART. Importantly, these findings assert that molecular measures of persistence are related to each other, and are significantly associated with measures of spontaneously virion release and inducible virion production. Future studies should seek to incorporate measures of infectious proviruses into similar data sets to evaluate quantitative relationships between other measures of persistence and the frequency of infectious proviruses. Ultimately, connecting these findings to the *in vivo* time to rebound following cessation of ART will be important for validating these biomarkers. Robust measures of the time to viral rebound have been elusive, but if these findings hold up in clinical studies, the timeline and complexity of studies to evaluate the effects of novel

therapeutics *in vivo* will both be greatly reduced. Expediency of clinical trials to evaluate interventions will likely be crucial to finding a means to reduce the size of the reservoir in those on suppressive ART, and as such the development of simple and robust biomarkers of the reservoir should be a high priority for curative HIV-1 research.

8.0 CHAPTER 5. ASSESSMENT OF THE SIZE OF THE INDUCIBLE AND INFECTIOUS RESERVOIR IN TOTAL AND RESTING CD4+ T CELLS

8.1 PREFACE

Anthony R. Cillo[1], Michele Sobolewski[1], Taylor Simmons[1], Joshua Cyktor[1], John W. Mellors[1]

[1]University of Pittsburgh, Division of Infectious Diseases

The work presented in this chapter is presented as partial fulfillment of aim 3 of this dissertation. Michele Sobolewski and Taylor Simmons isolated PBMC, total CD4+ T cells and resting CD4+ T cells from peripheral blood, set up cultures for pooled virus recovery and quantitative viral outgrowth, and performed Roche COBAS AmpliPrep/TaqMan v.2.0 and p24 to evaluate virus production and infectious virion production in culture, respectively. Joshua Cyktor performed flow cytometry for activation marker expression, and analyzed the results. Anthony Cillo performed qRT-PCR for the quantification of cellular HIV-1 RNA and DNA from PBMC, total CD4+ T cells and resting CD4+ T cells. John Mellors and Anthony Cillo conceived of the project and analyzed data. Anthony Cillo performed statistical analysis on the resulting datasets.

The work in this chapter was funded by the Pitt AIDS Research Training Program 5 T32 AI065380-08, an R21 from the NIH 1R21AI113102-01, and COBAS AmpliPrep/TaqMan kits provided by Roche.

8.2 INTRODUCTION

HIV-1 is a lifelong persistent viral infection, and has been extensively characterized as persisting within resting CD4+ T cells[123,124], and decaying with an extremely long half-life[128,131]. This reservoir persists throughout the lifetime of the patient due to immunologic memory of the resting CD4+ T cell population that have been exposed to antigens, and due to homeostatic proliferation as a normal biological function to maintain the pool of antigen-experienced memory CD4+ T cells[362]. As such, it is well established that the resting CD4+ T cell population serves as a reservoir of HIV-1 infection that is capable of reseeding viral infection following cessation of ART.

The total CD4+ T cell population can be divided in many ways, including by naïve versus antigen experienced[362,363], memory versus effector[362-364], subsets of central memory CD4+ T cells[362,364], stem cell-like lineages[365], and resting versus activated CD4+ T cells[366,367]. While the reservoir of HIV-1 infection in resting CD4+ T cells has been thoroughly investigated, it is currently unknown whether the activated portion of the total CD4+ T (tCD4) cell population harbors a portion of the total HIV-1 reservoir that contributes to viral rebound following cessation of ART. HIV-1 DNA has been found in activated CD4+ T cells (i.e., CD3+CD4+CD25+/CD69+/HLA-DR+) cells previously[366,367], but it remains unknown if the proviruses in activated cells are producing cellular HIV-1 RNA or virions, or are latent and inducible. Additionally, whether there is any relationship between the magnitude of virion production following activation for tCD4 cells and rCD4 cells, and that amount of infectious virus outgrowth remains unknown.

When evaluating total CD4+ T cells as a potential reservoir, it is important to bear in mind the criteria for latency and for a reservoir in HIV-1 infection[368]. To be truly latently infected, a population of cells must not produce virions in the absence of stimulation, but upon stimulation must produce replication competent virions. To be a reservoir, this cellular population must contribute to life-long persistence of HIV-1 and be capable of leading to viral recrudescence following cessation of ART. Although latent proviruses have been well-described in rCD4 cells, we hypothesized that the size of the latent and infectious reservoir would be larger from tCD4 cells compared with rCD4 cell, suggesting that cell expressing CD25/CD69/HLA-DR can contribute to the latently infected reservoir.

8.3 RESULTS

8.3.1 Study participant characteristics

In this study, we enrolled 11 consecutive participants from the Pitt AIDS Center for Treatment in an IRB-approved study. All study participants gave informed, written consent at the time of enrollment. The participant characteristics are shown in Table 15.

The participants enrolled in this study were Caucasian males and females and African American females. The median age at the time of blood draw was 49 years old. Study participants had a high percentage of CD4+ T cells in PBMC, and were on suppressive ART for a median of 9 years, ranging from 3 to 13 years. Prior to initiation of ART, study participants had a median viral load of 71,000 copies per milliliter of plasma, and a median CD4+ T cell count of

249 cells per mm^3, consistent with the chronic stage of infection. Mega-iSCA confirmed that all participants had suppressed viremia, and median low-level plasma viremia was 0.37 copies per milliliter, with 2 of the 11 participants having undetectable viremia and the other 9 having quantifiable viral loads using the large volume adaptation of the single-copy assay[303].

Table 15. Participant characteristics for the assessment of inducible and infectious viral reservoirs in total and resting CD4+ T cells

Participant ID	Sex	Race	Age	Pre-ART viral load (copies HIV-1 RNA per milliliter plasma)	Nadir CD4 (cell per mm^3 blood)	Mega-iSCA (copies HIV-1 RNA per milliliter plasma)	Percent CD4+ T cells in PBMC	Percent CD8+ T cells in PBMC	Duration of suppression (years)
1	Female	African American	51	59,500	268	0.275	50	42	9
2	Female	African American	36	802,000	20	0.37	40	53	10
3	Male	Caucasian	49	455,000	306	2.2	53	42	ND
4	Female	Caucasian	46	9,740	272	<0.092	24	11	4
5	Male	Caucasian	32	Unknown	259	1.49	48	47	7
6	Male	Caucasian	55	100,000	108	<0.092	69	15	13
7	Female	African American	41	698,000	21	1.6	79	16	3
8	Male	Caucasian	51	3,369	536	0.092	45	49	9
9	ND	ND	55	80,300	157	0.18	56	35	8
10	ND	ND	57	61,600	263	1.7	44	51	4
11	Male	Caucasian	42	24,000	127	1.2	35	58	9
Median (IQR)	ND	ND	49 (42-53)	71,000 (33,000-370,000)	259 (118-270)	0.37 (0.14-1.5)	48 (42-56)	42 (26-50)	9 (5-9)

ND = not done

8.3.2 Magnitude of virion production is larger in total CD4+ T cells versus resting CD4+ T cells

To isolate sufficient numbers of rCD4 and tCD4 cells in this study, we obtained large volume blood draws up to 360 mL by performing phlebotomy for 180 mL of whole blood twice, separated by ~1 hour. To begin to assess differences in the sizes of the inducible reservoir in total CD4+ T cells compared with resting CD4+ T cells, we first assessed the magnitude of spontaneous and inducible virion production from replicate wells of 10^6 tCD4 and rCD4 cells using an assay we refer to as pooled virus recovery (PVR). In PVR, replicate wells of 10^6 cells per well are cultured, and after 7 days of activation, the supernatants are removed, pooled together, and frozen in aliquots for downstream assessment of HIV-1 RNA levels by Roche COBAS AmpliPrep/TaqMan. Using PVR permits the assessment of virion production from larger numbers of rCD4 and tCD4 cells, and the pooled nature of the assay is useful for obtaining a population average without having to perform a large culture; as such, this assay format is amenable to scaling up or down with greater or fewer tCD4 or rCD4 cells following isolation from PBMC (see Overall materials and methods for more information).

To establish the presence of a reservoir within CD4+ T cells that contributes to inducible virion production, it is first important to establish that there is a latent population of proviruses in tCD4 cells. To assess whether this is the case, we cultured tCD4 cells and rCD4 cells in the absence of any stimulation for 7 days in culture with 300 nM efavirenz and 100 nM raltegravir, and quantified virion-associated HIV-1 RNA in culture supernatants by Roche COBAS AmpliPrep/TaqMan v.2.0. We found that the median levels of virion production from both tCD4

cells and rCD4 cells was <20 copies per milliliter of culture supernatant (interpolated value of 10 copies per milliliter of supernatant, which is 50% of the limit of detection of the assay), demonstrating that spontaneous virus production was below the limit of detection for the assay in most individuals (Figure 24). Additionally, spontaneous virion release from rCD4 cells was higher than tCD4 virion release in 4 of the 5 donors that had detectable levels of spontaneous virion production, indicating that despite the presence of activated CD4+ T cells within tCD4 cells, levels of spontaneous virion production were not different (p=0.44, paired Wilcox test).

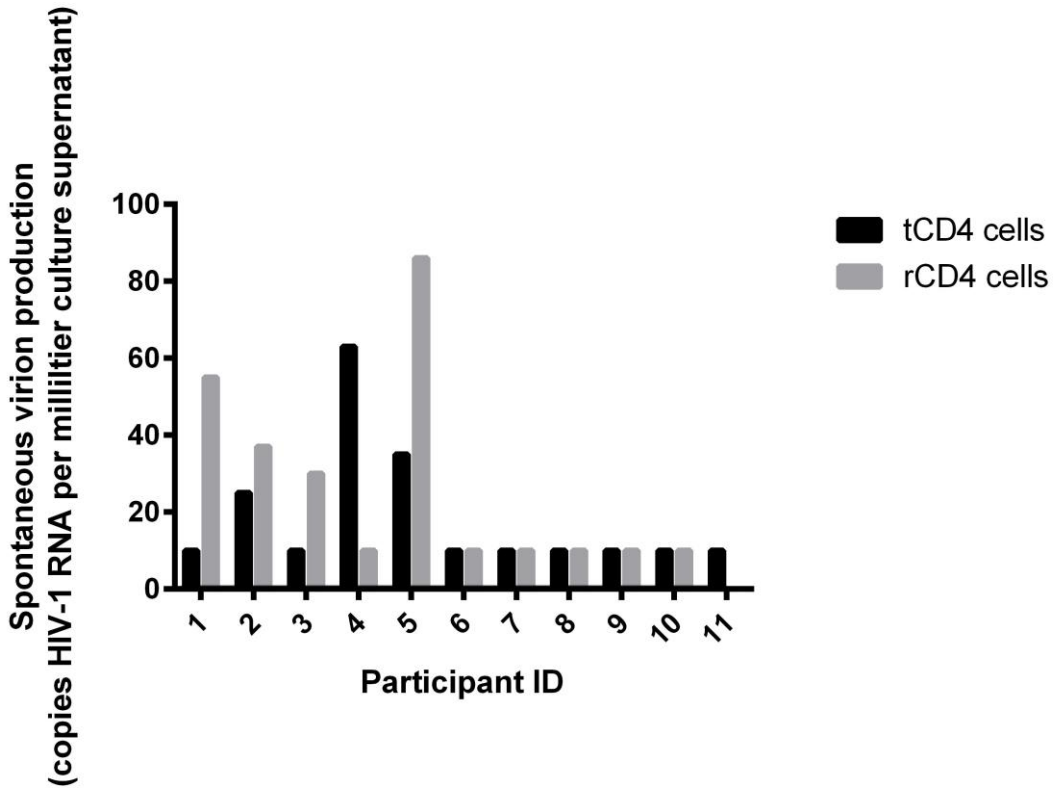


Figure 24. Levels of spontaneous virion production from cultured tCD4 and rCD4 cells. We found that of the 11 participants in this study, 5 had detectable spontaneous virion release from unstimulated cultured tCD4 or rCD4 cells; the other 6 had undetectable levels of virion production from tCD4 and rCD4 cells. In cells from the 5 participants that had detectable virion release, rCD4 cells spontaneously produced more virions in 4 of the 5 donors assessed. These results indicate that there are not appreciable increases in spontaneous virion release from the cultured activated CD4+ T cells within the total CD4+ T cell population.

Using PVR, we next assessed the levels of virion produced from rCD4 cells and tCD4 cells isolated from study participants on long term suppressive ART following 7 days of activation in culture with PMA/ionomycin in the presence of efavirenz (300 nM) and raltegravir (100 nM). We previously determined that 7 days of culture led to peak virion production with full T cell stimulation (data not shown). Median levels of virions produced following T cell activation of tCD4 cells was 4,299 copies per milliliter of culture supernatant, while median virion production from rCD4 cells was 1,728 copies per milliliter of culture supernatant (Figure 25). Overall, the levels of inducible virion production from tCD4 cells were a median of 348-fold higher than spontaneous virion release from these cells. In rCD4 cells, the median fold-increase in inducible virion production over spontaneous virion production was 118-fold, demonstrating the presence of a latent and inducible reservoir within tCD4 cells that is larger than the reservoir within only rCD4 cells.

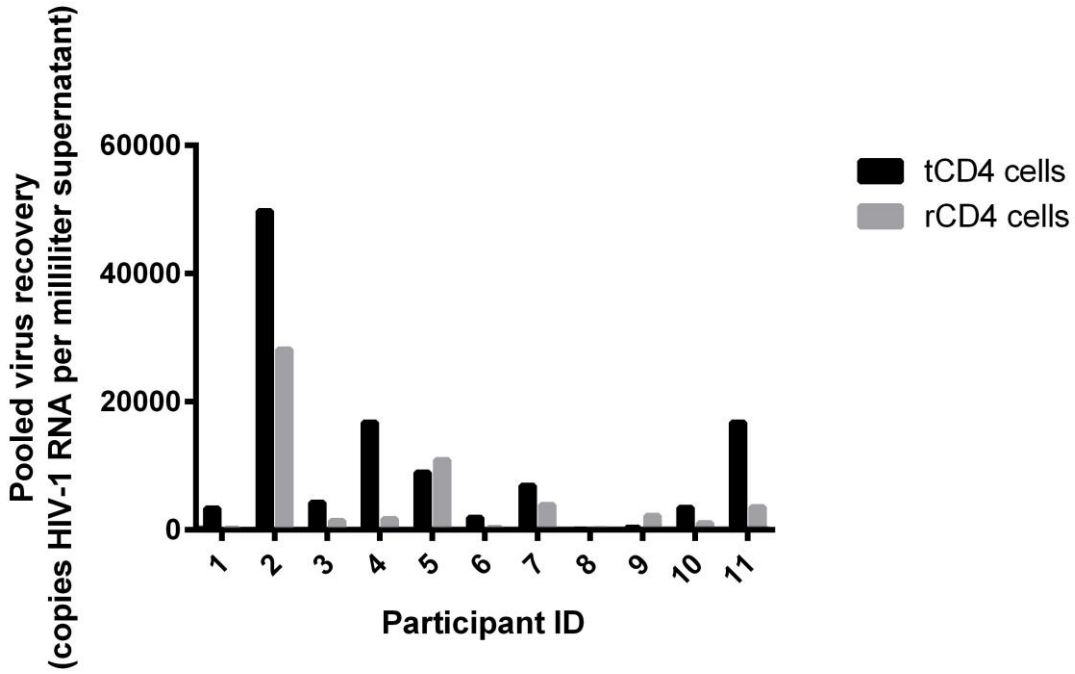


Figure 25. Virion production from pooled virus recovery (PVR) assays on tCD4 and rCD4 cells. Levels of virions produced following activation of tCD4 and rCD4 from the same study participants was assessed in parallel cultures. Median levels of virion production from tCD4 cells were 4,299 copies per milliliter of culture supernatant, while rCD4 cells produced a median of 1,728 copies. Total CD4+ T cells produced more virions compared to rCD4 cells in 8 of 11 participants, while rCD4 cells produced more virions in 3 of 11, although donor 8 only produced 86 copies of HIV-1 RNA from tCD4 cells and 110 copies of HIV-1 RNA from rCD4 cells. Total CD4+ T cells produced statistically significant more virions compared with rCD4 cells (p=0.024, paired Wilcox test).

The observed difference in virion production was statistically significant (p=0.024, paired Wilcox test) in this set of 11 study participants, suggesting that there are more cells capable of producing virions present in tCD4 cells versus rCD4 cells. Since rCD4 cells are present as a subpopulation within tCD4 cells, this result implies that there are inducible proviruses present in the activated population of tCD4 cells (i.e., CD3+CD4+CD25+/CD69+/HLA-DR+ cells).

8.3.3 Overall transcription of HIV-1 RNA is associated with inducible virion production

To begin to assess variables that are associated with the size of the inducible reservoir in tCD4 and rCD4 cells, we first quantified total cellular HIV-1 DNA and unspliced cellular HIV-1 RNA in PBMC (Figure 26). The mean (±standard deviation) for total HIV-1 DNA was 274 (±208) copies per million PBMC, while the mean (±standard deviation) level of cellular unspliced HIV-1 RNA transcription was 127 (±114) copies per million PBMC.

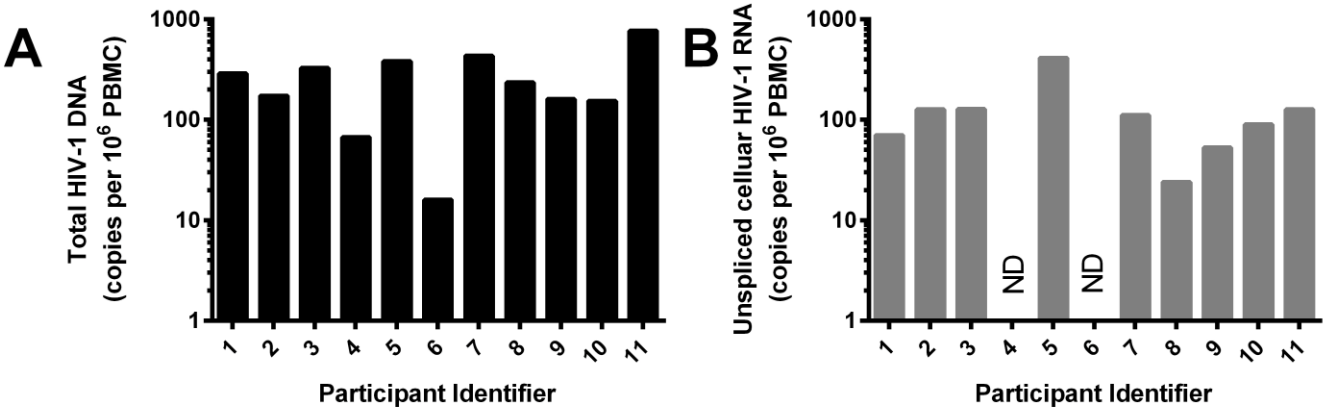


Figure 26. Cellular unspliced HIV-1 RNA and total DNA from PBMC. Levels of cellular unspliced HIV-1 RNA and total HIV-1 DNA from PBMC were assessed using previously described qRT-PCR assays[300,304]. **A)** Levels of total HIV-1 DNA per million PBMC ranged from 16 to 768 copies per million PBMC, with a mean value of 274 (±208) copies per million PBMC. **B)** Levels of unspliced cellular HIV-1 RNA ranged from 24 to 414 copies per million PBMC, with a mean of 127 (±114) copies per million PBMC. Cellular HIV-1 RNA was detectable in all donors, but sufficient cells were not available for assessment of HIV-1 RNA in donors 4 and 6. ND = not determined, due to inadequate numbers of cells available for qRT-PCR analysis.

After assessing levels of total cellular HIV-1 DNA and unspliced HIV-1 RNA in PBMC, we next sought to evaluate factors that were associated with levels of virion production from tCD4 and rCD4 cells. This analysis revealed that the levels of virions that were produced

following activation of tCD4 and rCD4 cells were significantly related to the levels of overall cellular transcription of unspliced HIV-1 RNA per million PBMC (Figure 27).

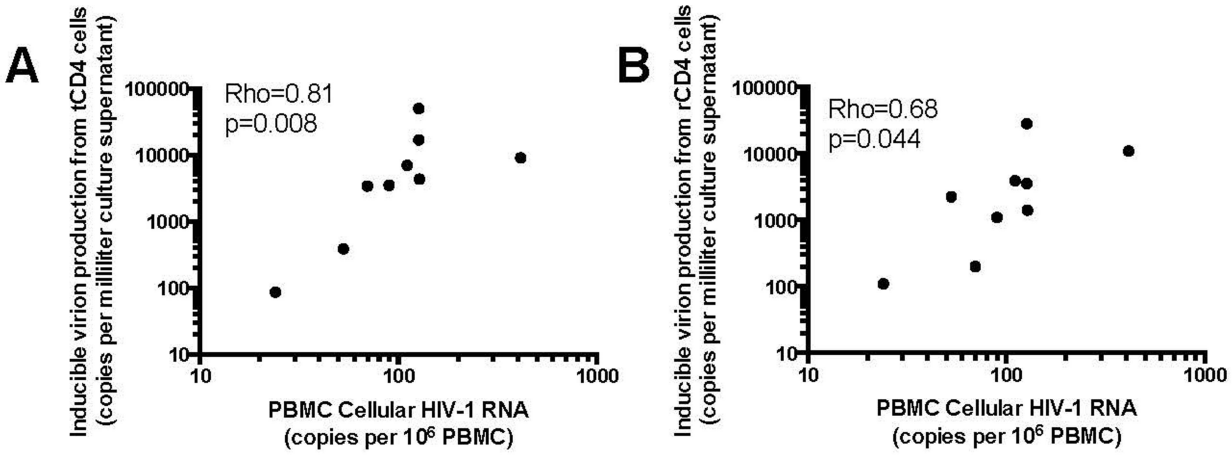


Figure 27. Overall levels of transcription of unspliced cellular HIV-1 RNA is related to inducible virion production. Inducible virion production from tCD4 cells and rCD4 cells is related to the levels of cellular HIV-1 RNA transcription in PBMC. **A.)** Levels of inducible virions from tCD4 cells are significantly related to HIV-1 RNA transcription in PBMC (rho=0.81, p=0.008). **B.)** PBMC cellular HIV-1 RNA transcription is related to inducible virion production from culture rCD4 cells (rho=0.68, p=0.044), although not as strongly as the relationship in tCD4 cells.

Compared with the findings present in the previous chapter, it was surprising that cellular HIV-1 DNA in PBMC was not associated with levels of inducible virion production from rCD4 cells. In this study, the correlation coefficient between cellular HIV-1 DNA in PBMC and inducible virion production from rCD4 cells was rho=0.38, p=0.25 using the nonparametric Spearman's correlation (data not shown). Although the results for indicuble virion production from rCD4 cells were found to be non-Gaussian ($p<0.001$ by Shapiro-Wilk test for normality), if Pearson's correlation is used to generate 95% confidence intervals, the resulting confidence

interval for r is from -0.60 to 0.60, which overlaps with the 95% confidence interval using Pearson's correlation for the data relating PBMC cellular HIV-1 DNA levels to inducible virion production from rCD4 cells (r=0.57 to r=0.92) from the last chapter. Accordingly, a post-hoc power assessment from this study demonstrated that this study only had ~53% power to detect a correlation of r=0.60. Additionally, a correlation between cellular HIV-1 DNA and cellular HIV-1 RNA was not observed in this study (rho=0.52, p=0.15; data not shown), but inspection of the 95% confidence interval using Pearson's correlation (r= -0.49 to r=0.79) demonstrated near overlap with the previously described data (r=0.81 to r=0.97).

8.3.4 Relationships between molecular measures of persistence and inducible virion production

After evaluating differences between the size of the inducible reservoir in tCD4 and rCD4 cells, we next assessed whether differences in molecular measures of persistence (i.e. total cellular HIV-1 DNA and unspliced cellular HIV-1 RNA) in tCD4 or rCD4 cells were related to inducible virion production. Shown in Figure 28 is the frequency of infection and cellular HIV-1 RNA expression per million tCD4 or rCD4 cells. The mean (±standard deviation) level of infection in tCD4 cells was 727 (±578) copies per million tCD4 cells, and the mean (±standard deviation) level of cellular HIV-1 RNA in tCD4 cells was 257 (±211) copies per million tCD4 cells. The mean levels of total HIV-1 DNA in rCD4 cells was 695 (±492) copies per million tCD4 cells, and mean levels of cellular HIV-1 RNA in rCD4 cells was 342 (±309) copies per million rCD4 cells. The frequency of infection was similar in both populations, suggesting that the overall frequency of infection of activated CD4+ T cells is likely similar to that of resting CD4+ T cells,

given that 38% of the tCD4 cells express CD25/CD69/HLA-DR. The trend towards greater levels of cellular HIV-1 RNA present in the resting CD4+ T cell population also implies that overall transcription is lower in the activated CD4+ T cell population within the tCD4 cell population.

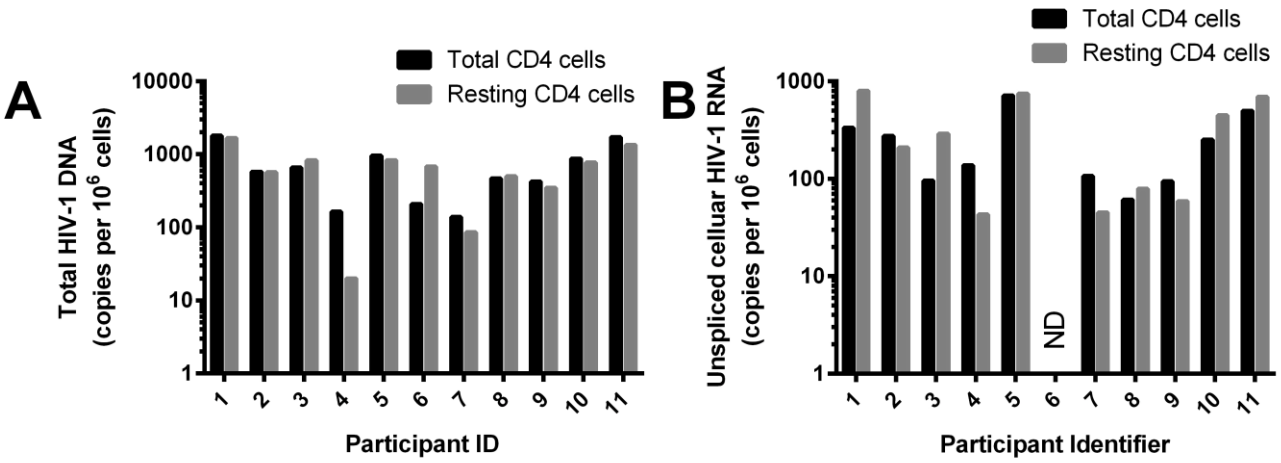


Figure 28. Levels of total HIV-1 DNA and unspliced cellular HIV-1 RNA in total and resting CD4+ T cells. **A)** Levels of total cellular HIV-1 DNA ranged from 139 to 1802 copies per million cells in tCD4 cells, and 20 to 1671 copies per million cells in rCD4 cells. Total HIV-1 DNA was higher in tCD4 cells than rCD4 cells in 8 of 11 donors, was essentially equivalent in 2 donors, and was greater in rCD4 cells versus tCD4 cells in 1 donor. **B)** Levels of unspliced cellular HIV-1 RNA ranged from 61 to 500 copies per million cells in tCD4 cells, and 43 to 802 copies per million cells in rCD4 cells. Cellular unspliced HIV-1 RNA was greater in rCD4 versus tCD4 cells in 6 of 10 evaluable participants, and was greater in tCD4 versus rCD4 cells in 4 of 10 participants.

When comparing relations between tCD4 and rCD4 cells, we found that they were highly interrelated (Figure 29 and Table 16). Given the interrelated nature of measures of molecular persistence in tCD4 and rCD4 cells, these data are presented as an interaction network, with a corresponding table providing the correlation coefficients and p values. The frequency of infection in both tCD4 cells and rCD4 cells was significantly associated with levels of cellular transcription in both tCD4 and rCD4 cells. We also found that the frequency of infection and

cellular transcription in tCD4 cells was related to the frequency of infection and levels of HIV-1 RNA transcription in rCD4 cells. The frequency of infection in tCD4 or rCD4 cells was not related to the frequency of infection per million PBMC (data not shown), nor were the levels of transcription in tCD4 or rCD4 cells related to the level of cellular HIV-1 RNA transcription per million PBMC (data not shown), suggesting that there is variability in the overall percentage of CD4+ T cells as a constituent of PBMC.

In addition to the interrelationships between the viral measures of persistence in the tCD4 cells and rCD4 cells, there were also two noteworthy positive associations between levels of total HIV-1 DNA in tCD4 cells and cell frequencies in PBMC as measured by flow cytometry. Specifically, higher levels of HIV-1 DNA in tCD4 cells were positively associated with both a higher frequency of activated CD4+ T cells in PBMC (i.e., CD3+CD4+CD25+/CD69+/HLA-DR+) and the frequency of CD3+CD8+ T cells in PBMC.

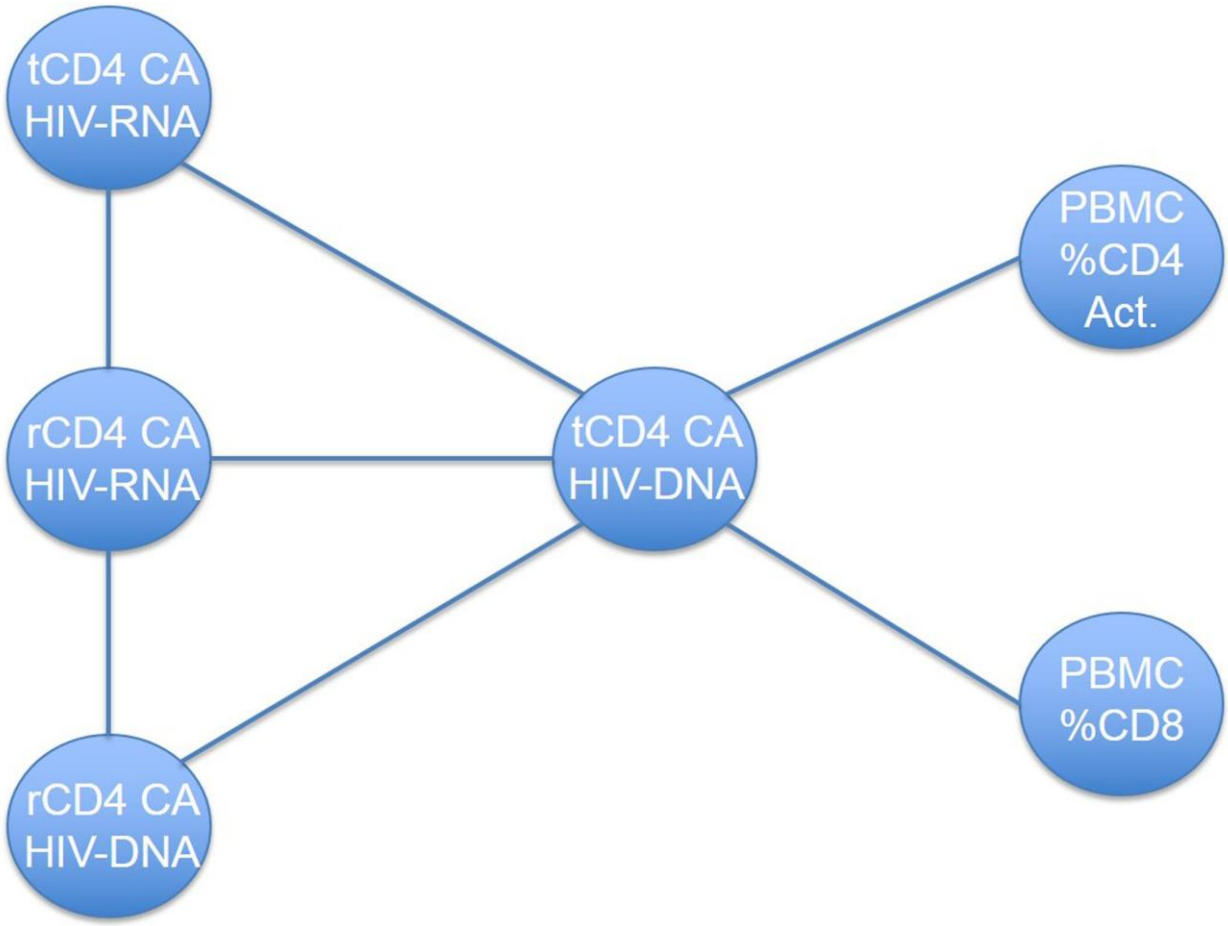


Figure 29. Interrelations between frequency of infection and transcription in tCD4 and rCD4 cells. The relationships between frequency of infection and transcription in tCD4 and rCD4 cells shown as an interaction network. Consistent with previously presented interaction networks, the nodes in this figure are variables, and the lines connecting the nodes represent statistically significant Spearman's correlation coefficients. Frequency of infection and levels of viral transcription are highly interrelated. Total HIV-1 DNA within tCD4 cells was also significant related to the percent of activated CD4+ T cells within the PBMC population (i.e., CD3+CD4+CD25+/CD69+/HLA-DR+) and the percent of CD8+ T cells within the PBMC population (i.e., CD3+CD8+).

Although the interconnected nature of the relationships between measures of viral persistence in tCD4 and rCD4 are not surprising due to fact that rCD4 cells are a cellular population nested within tCD4 cells, it is striking that the correlative relationships are so strong in some cases. Specifically, total cellular HIV-1 DNA in rCD4 and tCD4 are strongly correlated (rho=0.91, $p<0.001$), and cellular unspliced HIV-1 RNA are strongly correlated in rCD4 and

tCD4 cells (rho=0.70, p=0.031) (Table 16). The strength of the relationship between the frequency of infection between tCD4 and rCD4 cells suggests that there is a proportionality between the levels of infection that occurred prior to ART-initiation in both tCD4 cells and rCD4 cells, where higher infection in tCD4 cells is related to higher infection in rCD4 cells. It is intriguing that higher levels of transcription in rCD4 cells are related to higher levels of HIV-1 DNA in tCD4 cells; this suggests that the higher the frequency of infection in tCD4 cells, the higher the frequency of infection and transcriptional activity in rCD4 cells.

Table 16. Relationships between frequency of infection and viral transcription on tCD4 and rCD4 cells

Variable 1	Variable 2	Spearman's rho	P value
tCD4 CA HIV-DNA	tCD4 CA HIV-RNA	0.69	0.031
	rCD4 CA HIV-DNA	0.91	<0.001
	rCD4 CA HIV-RNA	0.98	<0.001
	PBMC %CD4 Act.	0.67	0.028
	PBMC %CD8	0.70	0.021
tCD4 CA HIV-RNA	rCD4 CA HIV-RNA	0.70	0.031
rCD4 CA HIV-DNA		0.94	<0.001

Next, we sought to assess whether there were factors that were related to the fold-difference in the size of the inducible reservoir between tCD4 cells and rCD4 cells. We found that the differences in the size of the inducible reservoir in between these two cell populations was not directly related to any one measure of viral persistence within any cell population (i.e. PBMC, tCD4 or rCD4 cells). The only factor found to be weakly associated with the ratio of virion production from tCD4 cells compared to rCD4 cells was the percent of activated cells within the tCD4 cell population (Figure 30; rho=0.74, p=0.046).

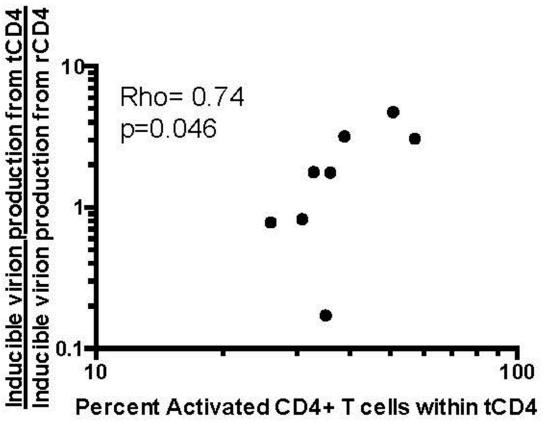


Figure 30. Percent of activated CD4+ T cells is related to the ratio of inducible virion production from tCD4 versus rCD4 cells. The ratio of inducible virion production in tCD4 versus rCD4 cells was positively related to the percentage of activated cells within the tCD4 cell population. This correlation is shows that as the frequency of activated cells within tCD4 cells (that is, non-resting CD4 cells) becomes greater, the level of virion production in the tCD4 cell population becomes greater relative to that in the rCD4 cell population.

While it is unclear exactly why the ratio of inducible virion production from tCD4 cells compared to rCD4 cells increases as the percent of activated CD4+ T cells in the tCD4 cell population increases, we do know that levels of HIV-1 DNA in tCD4 cells increases as the percent of activated tCD4 cells increases, as shown in Figure 29. We showed in the previous chapter that as levels of HIV-1 DNA rise in PBMC and rCD4 cells, levels of inducible virion production from rCD4 cells also rises. Applying the same logic here, as levels of HIV-1 DNA in tCD4 cells rise, there are likely more cells that can produce virions within the tCD4 cell population. Importantly, there is no statistically significant relationship between levels of activation in tCD4 cells and levels of cellular HIV-1 DNA in rCD4 cells (rho=0.62, p=0.12; data not shown). This means that as the frequency of activated CD4+ T cells increases, the levels of rCD4 HIV-1 DNA do not necessarily direct increase (at least not at the same rate that levels of

HIV-1 DNA in tCD4 cells increase), and correspondingly, the number of cells that can produce virions therefore does not increase as quickly as in tCD4 cells. This could potentially lead to a lower ratio of virion production from rCD4 compared to tCD4 as the percent of activated cells within tCD4 cells increases.

8.3.5 Clinical factors associated with the size of the inducible reservoir

One interesting factor that was inversely associated with levels of inducible virion production from both tCD4 cells and rCD4 cells was found to be the age of the study participant. Specifically, the older the study participant, the smaller the amount of virion production that was observed from both tCD4 cells and rCD4 cells (Figure 31). These inverse correlations were of a similar magnitude in both the tCD4 and rCD4 cell populations (rho= -0.77, p=0.006 for tCD4 cells; rho= -0.76, p=0.006 for rCD4 cells). Duration of suppression was not directly associated with the size of the inducible reservoir in tCD4 cells (rho= -0.098, p=0.79) or rCD4 cells (rho= -0.20, p=0.59). Importantly, the age of the study participant was not related to the duration of suppression of the study participant (rho=0.08, p=0.825; data not shown), demonstrating that this observed inverse correlation between age and inducible virion production was not based on duration of suppression.

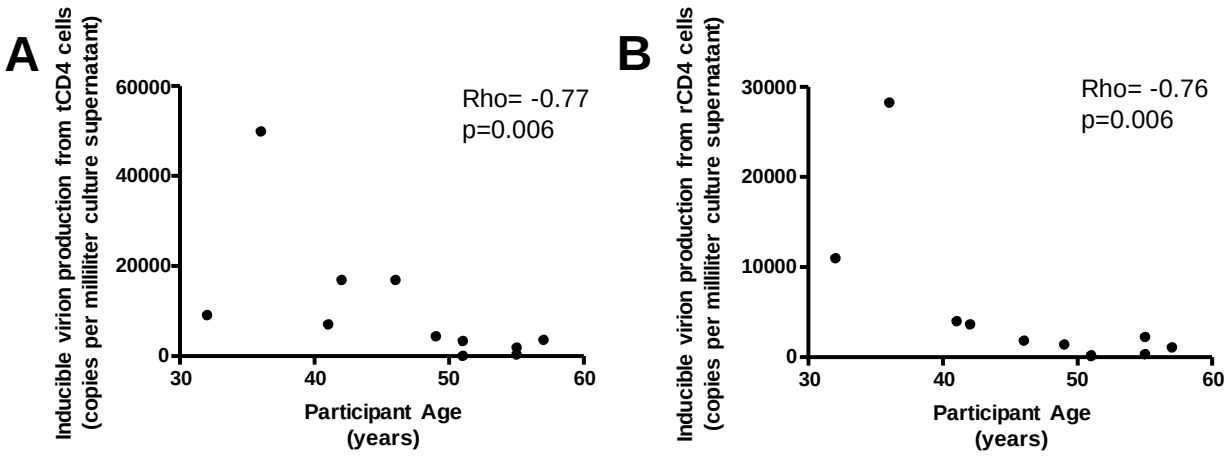


Figure 31. Inverse relationships between age of study participant and inducible virion production. Across the 11 participants in this study, age was found to be significantly inversely associated with age. **A.)** Inverse correlation between age and inducible virion production from tCD4 cells by PVR (rho= -0.77, p=0.006). **B.)** Inverse correlation between age and inducible virion production from rCD4 cells (rho= -0.76. p=0.006), of a very similar magnitude to the relationship between age and inducible virion production from tCD4 cells.

After finding that the age of the participant was inversely related to levels of virions produced, we next evaluated whether other clinical factors were associated with the size of the inducible reservoir. We found, in this cohort of study participants, that the nadir CD4+ T cell count was negatively correlated with the amount of inducible virion production from rCD4 cells, but not tCD4 cells (Figure 32). It is particularly interesting that the nadir CD4+ T cell count from prior to the initiation of ART is related to the size of the inducible reservoir in rCD4 cells following years of suppressive ART.

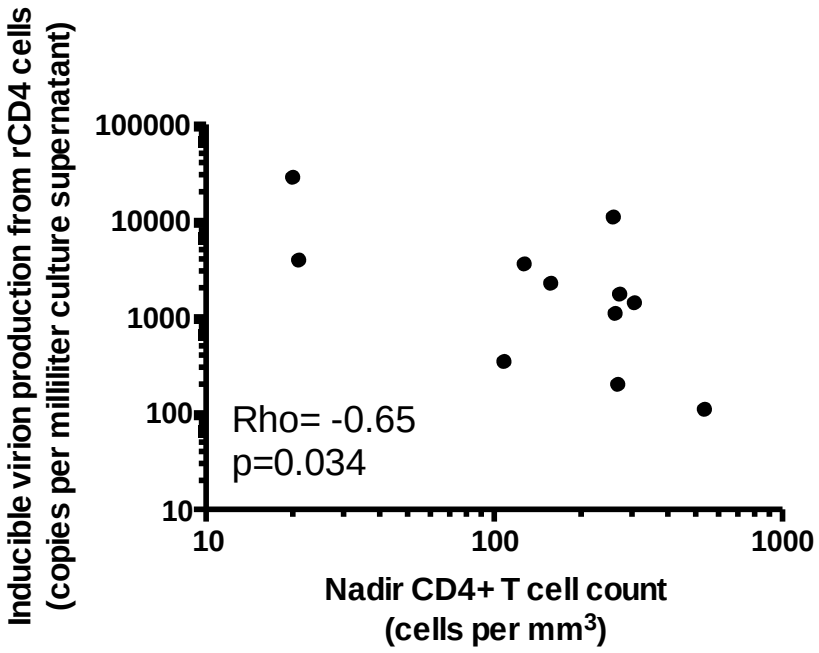


Figure 32. Negative correlation between nadir CD4+ T cell counts and inducible virion production from rCD4 cells. Levels of inducible virion production after years of suppressive ART are significantly inversely correlated with the nadir CD4+ T cell count prior to initiation of ART (rho= -0.65, p=0.034). This finding suggests that the extent of disease progression prior to the initiation of ART is related to the size of the inducible reservoir (i.e., lower nadir CD4+ T cell counts, larger inducible reservoir).

This finding is intriguing, and suggests that the more extensive the disease progression of HIV-1 prior to the initiation of ART, the larger the size of the inducible reservoir in rCD4 cells, but not necessarily tCD4 cells. This also suggests that there are two distinct reservoirs, one in tCD4 cells and another in rCD4 cells, and that the seeding of each reservoir may be through distinct mechanisms. Analysis of single genome sequences from the virions produced from the tCD4 cells and rCD4 cells could provide insight into whether these are indeed distinct populations; if sequences are found in tCD4 cells that are not present in rCD4 cells, it would suggest that cells expressing CD25/CD69/HLA-DR constitute a distinct reservoir.

8.3.6 Size of the infectious virus reservoir in tCD4 cells and rCD4 cells

In parallel to our evaluation of the size of the inducible reservoir in both tCD4 and rCD4 cells, we also assessed the size of the infectious reservoir in both tCD4 cells and rCD4 cells in parallel from the same donors using the quantitative viral outgrowth assay (QVOA; see Overall materials and methods for more information). When assessing levels of infectious viruses in tCD4 and rCD4 cells, we found that tCD4 cells produced infectious virions at a quantifiable level in 7 of the 11 donors studied, while rCD4 cells produced infectious virions in 6 of the 11 donors studied (Figure 33). Of the donors that were negative for infectious virus outgrowth, virus failed to grow out in both tCD4 cells and rCD4 cells in 4 donors. If infectious virus was quantifiable in rCD4 cells, it was also quantifiable in tCD4 cells. In one case, tCD4 cells produced infectious virions, but rCD4 cells were negative for viral outgrowth.

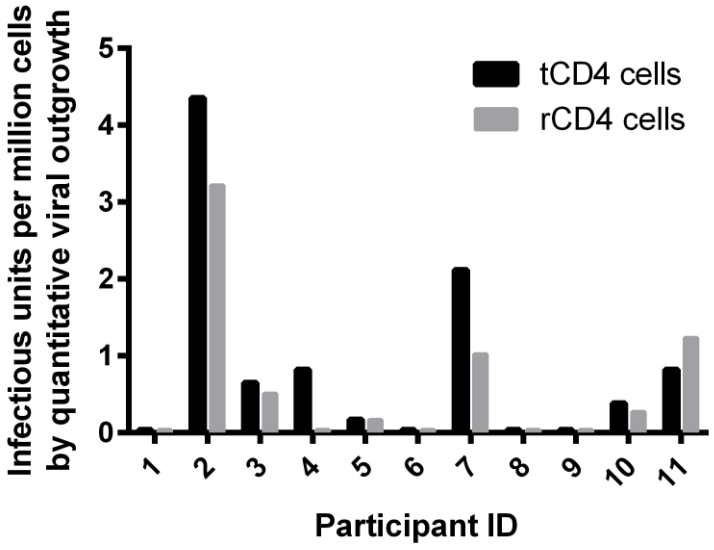


Figure 33. Infectious units per million cells by QVOA from tCD4 and rCD4 cells. In parallel cultures, levels of infectious viral outgrowth were quantified by QVOA from both tCD4 cells and rCD4 cells. In the 7 donors in which infectious virus was produced from tCD4 or rCD4 cells, 6 donors had higher levels of virion production from tCD4 cells (mean fold- difference from tCD4 versus rCD4 cells was 2.9-fold; p=0.11 by paired Wilcox test).

In the 7 donors in which infectious virus was produced from either tCD4 or rCD4 cells, 6 donors had higher levels of infectious virions produced from tCD4 cells. The mean infectious units per million (IUPM) tCD4 cells was 0.86, while for rCD4 cells, the mean IUPM was 0.60; the mean fold-difference between tCD4 IUPM versus rCD4 IUPM was 2.9-fold. Although these results are not statistically significant, it is suggestive of a trend towards higher levels of infectious virus in tCD4 cells versus rCD4 cells. These data, combined with sparse spontaneous release of virions and high levels of inducible virion production from tCD4 cells, suggest that a latent, inducible and infectious reservoir is present in tCD4 cells.

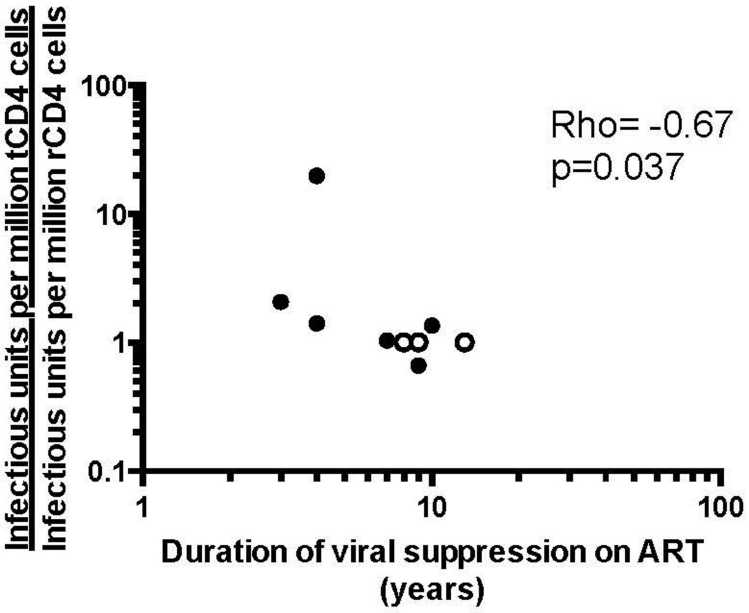


Figure 34. Correlation between duration of suppression and the ratio of the magnitude of viral outgrowth between tCD4 and rCD4 cells. The duration of viral suppression in the 10 study participants with a known duration of suppression was negatively associated with the ratio of infectious virus outgrowth in tCD4 cells versus rCD4 cells (rho= -0.67, p=0.037). Those with shorter durations of viral suppression tend to have a higher ratio of infectious virus in tCD4 cells versus rCD4 cells, and this ratio decreases to approximately 1 (i.e., equal amounts of infectious virus in tCD4 and rCD4 cells) with a longer duration of suppression. Open circles denote participants that had undetectable levels of viral outgrowth in both tCD4 and rCD4 cells; two participants had 9 years of viral suppression and a ratio of infectious virus outgrowth of 1, and were therefore overlapping on the graph.

In the study participants with a known duration of suppression, we found that there was a negative correlation between the duration of suppression and viral outgrowth. The ratio of infectious virus outgrowth was higher in those with short durations of suppression, and approached a ratio of 1 over almost a decade of viral suppression. It is worth nothing that 4 of these 10 study participants had undetectable viral outgrowth in both tCD4 cells and rCD4 cells, resulting in a ratio of 1 that may partially drive this correlation.

8.3.7 Virologic factors associated with the size of the infectious reservoir in tCD4 and rCD4 cells

We next evaluated whether there were virologic factors that were related to the size of the infectious virus reservoir in tCD4 cells and rCD4 cells. The first measure of persistence that we evaluated for relationships to the infectious viral reservoir was low-level viremia by mega-iSCA. From this analysis, we found that the levels of viremia in plasma following long-term suppression of viral replication by suppressive ART were related to the size of the infectious viral reservoir in rCD4 cells, but not tCD4 cells (Figure 35).

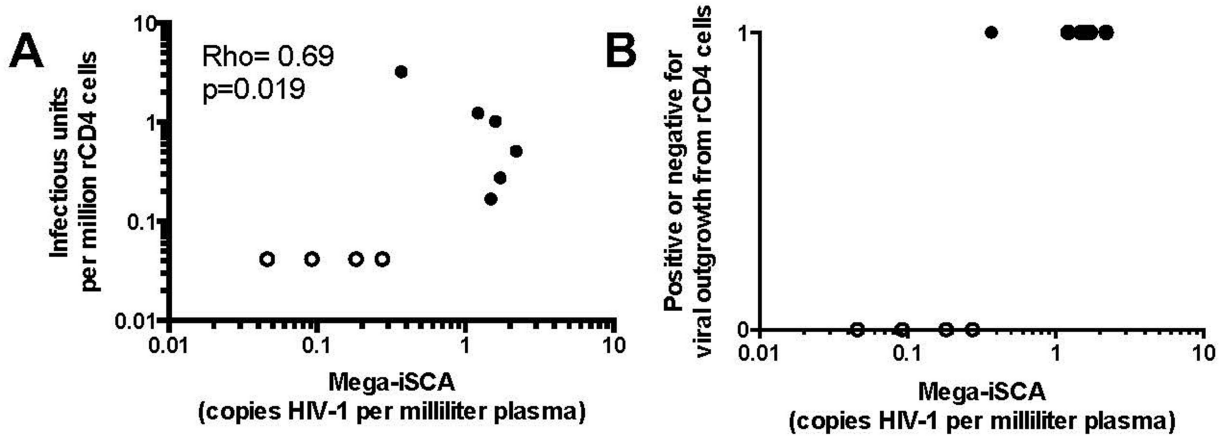


Figure 35. Mega-iSCA is associated with the size of the infectious viral reservoir in rCD4 cells. Relationships between low-level virema as measured by mega-iSCA and the infectious units per million rCD4 cells as measured by QVOA. **A)** Correlation between mega-iSCA and the infectious units per million rCD4 cells (rho=0.69, p=0.019), demonstrating that the levels of infectious virus in rCD4 cells is related to the level of virus present in plasma at any given time. Open circles represent samples that were undetectable by mega-iSCA or QVOA. **B)** Exact logistic regression with dichotomous outcome (i.e. positive or negative by QVOA) as the dependent variable, and levels of plasma viremia by mega-iSCA as a continuous independent variable. In this model, mega-iSCA was found to be a statistically significant predictor (p=0.047), and for each log-increase in mega-iSCA, the log odds of a positive IUPM goes up by 8.6.

The positive correlation between IUPM from rCD4 cells and mega-iSCA suggests that as the viral load increases, the level of IUPM from rCD4 cells increases. This can also be assessed by evaluating the data using an exact logistic regression model. This model indicates that mega-iSCA is a significant predictor of positive or negative viral outgrowth from rCD4 cells, and that a 10-fold increase in mega-iSCA corresponds to a log-odds increase of 8.6 in the odds of positive IUPM result. This analysis also reveals that when plasma viremia is over 0.28 copies per milliliter, infectious viral outgrowth from rCD4 cells is always positive.

Next, we assessed whether there was a relationship between the levels of inducible virus produced in PVR from activated tCD4 cells versus levels of infectious viral outgrowth from

tCD4 cells. This analysis revealed that the levels of virus production from activated tCD4 cells from PVR was statistically significantly correlated with infectious virus outgrowth from tCD4 cells (Figure 36).

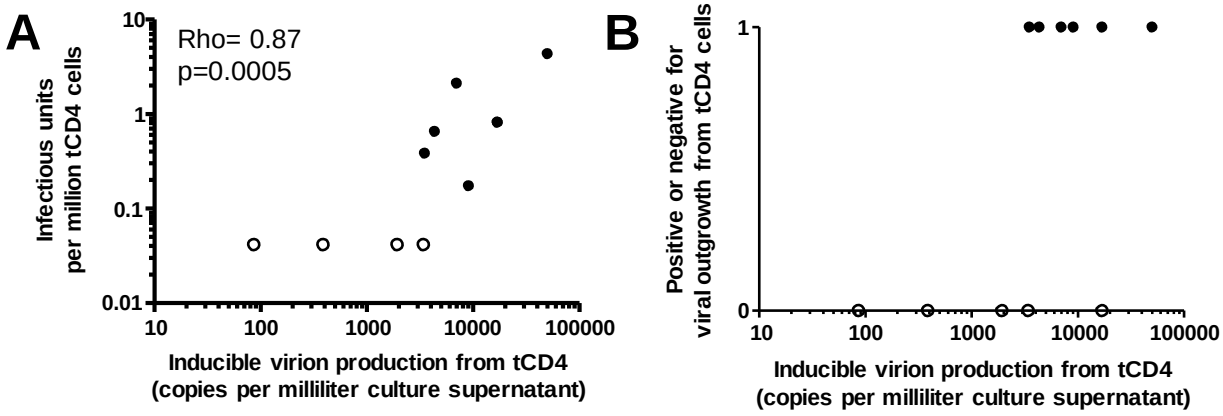


Figure 36. Inducible virion production from tCD4 cells is correlated with viral outgrowth from tCD4 cells. Statistically significant relationships between levels of inducible virion production from tCD4 cells and infectious virus outgrowth from tCD4 cells. **A)** The levels of inducible virus production are statistically significant correlated with levels of IUPM from tCD4 cells (rho=0.87, p=0.0005). Open circles are negative for IUPM from tCD4 cells. **B)** Exact logistic regression for the relationship between positive or negative viral outgrowth from tCD4 cells, and levels of inducible virion production from tCD4 cells. Inducible virion production from tCD4 cells was a significant predictor of negative or positive viral outgrowth (p=0.048), and a log increase in inducible virion production is associated with a log-odds increase of 3.5 for the odds of positive viral outgrowth.

These data demonstrate that there is a quantitative relationship between the levels of virions that are produced following T cell activation, and the levels of virions that are infectious. While there is a strong correlation between the levels of virions produced and the infectious units per million tCD4 cells, an exact logistic regression analysis reveals that although there is a trend towards infectious virion production following increasing levels of inducible virus production, there is no clear point where levels of inducible virion production are associated with infectious

viral outgrowth. Instead, increasing levels of inducible virion production increase the odds of a positive viral outgrowth culture.

Finally, we assessed whether there was a relationship between levels of inducible virion production from rCD4 cells and infectious virion production from rCD4 cells. We once again found that there was a statistically significant relationship between levels of virion production and infectious virion production, consistent with results from tCD4 cells (Figure 37).

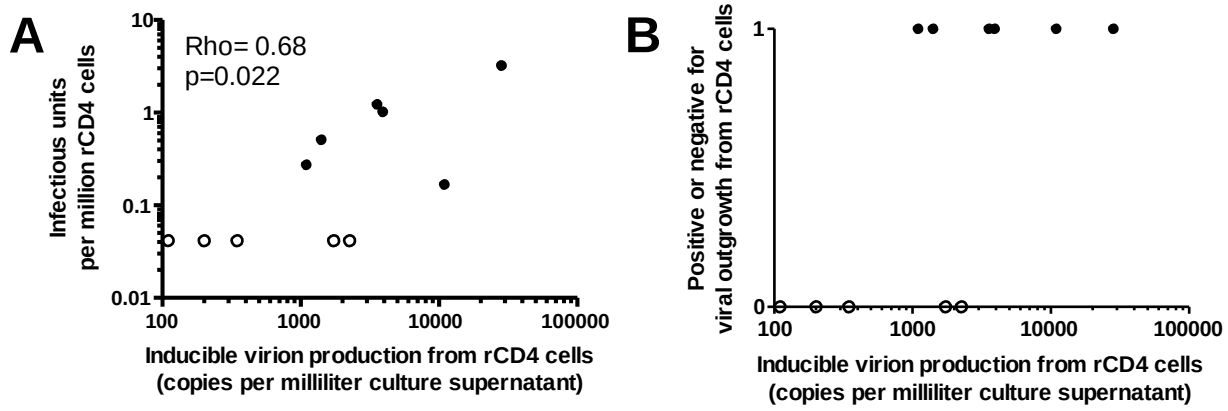


Figure 37. Inducible virion production is correlated with infectious virus production in rCD4 cells. Inducible virion production from rCD4 cells is statistically significant correlated with the number of infectious units per million rCD4 cells. **A)** Levels of virion production as measured by PVR were statistically significantly correlated with IUPM from rCD4 cells as evaluated by QVOA (rho=0.68, p=0.022). **B)** Exact logistic regression with negative or positive QVOA from rCD4 cells as the dependent variable, and levels of inducible virion production from rCD4 cells as the independent variable. Inducible virion production was found to be statistically significant in the exact logistic regression model (p=0.009), and for each log increase in the level of inducible virion production, the log odds of a positive QVOA increase by 9.0.

The correlation between virion production and infectious viral outgrowth by QVOA in rCD4 cells was not as strong (rho=0.68, p=0.022) as the correlation that was found in tCD4 cells. However, in rCD4 cells, the exact logistic regression was very statistically strong, in that the p value for the levels of virion production as a predictor for positive viral outgrowth was very low (p=0.009). Once again, the level of virion production from rCD4 cells did not guarantee viral

outgrowth at any given point, but rather increased the odds of positive QVOA. It is worth noting that the odds of viral outgrowth are high when the levels of virions produced from activated rCD4 cells in culture as measures by PVR was greater than 1000 copies per milliliter of culture supernatant.

8.4 DISCUSSION

In this study, we primarily sought to evaluate whether there was a distinct latent and inducible reservoir in tCD4 cells that was distinct from the well-described reservoir in rCD4 cells[131]. By measuring virion production from unstimulated and PMA/ionomycin treated tCD4 and rCD4 cells, we found that there is a latent and inducible reservoir present in tCD4 cells of a larger magnitude that rCD4 cells. While rCD4 cells are a component of the tCD4 cell population, it is logical that any HIV-1 infected and activated cells within tCD4 cells would be producing viral mRNA, proteins and virions, and should be short-lived due to viral cytopathic effects and the general apoptotic nature of the majority of activated T cells. However, instead we find that there is approximately 3-fold higher virion production when tCD4 cells are activated compared with rCD4 cells, and we can therefore infer that activated CD4+ T cells within the CD4+ T cell population harbor latent and inducible proviruses. These data also suggest that only evaluating rCD4 cells when investigating strategies aimed at reducing the size of the inducible reservoir likely misses a portion of the inducible reservoir in some patients.

We next sought to assess whether any variables were directly related to the level of inducible virion production from tCD4 and rCD4 cells. When looking at virologic measures of viral persistence in PBMC, tCD4 cells, and rCD4 cells, we found that the only factor that was statistically significantly related to the level of inducible virion production in this small group of study participants was the overall level of transcription per million PBMC. This finding is consistent when comparing it to other studies, including the previous chapter of this dissertation and the finding by Li et al. that the time to viral rebound is associated with the level of transcription in PBMC[361]. However, these results also differ from those of the previous chapter and other studies[359] in that the levels of cellular HIV-1 DNA per million PBMC did not correlate with the size of the inducible reservoir. Upon inspection of putative 95% confidence intervals using Pearson's correlation, we find that the value for the correlation coefficient observed in this study overlaps with the confidence interval for the correlation coefficient observed in the previous chapter. Therefore, it is likely that cellular HIV-1 DNA is related to the size of the inducible reservoir, and was just not observed in this small cohort of study participants.

Although a relationship between levels of cellular HIV-1 DNA and cellular HIV-1 RNA was not observed in PBMC, there were significant relationships between levels of cellular HIV-1 DNA and cellular HIV-1 RNA in both tCD4 cells and rCD4 cells, and between tCD4 cells and rCD4 cells. As rCD4 cells are a subset of tCD4 cells, it is not surprising that there was a strong correlation between levels of cellular HIV-1 DNA and cellular HIV-1 RNA in in tCD4 and rCD4 cells. When looking at the relationships between levels of cellular HIV-1 DNA in tCD4 versus rCD4 cells, we found that the frequency of infection in tCD4 versus rCD4 cells was similar. When looking at cellular HIV-1 RNA in tCD4 versus rCD4 cells, we found that HIV-1 RNA was more prevalent per infected cell in rCD4 cells versus tCD4 cells, suggesting non-resting cells in

the tCD4 cell population may be subjected to an environment in which transcription of cellular HIV-1 RNA is selected against.

Other studies have preliminarily sought to evaluate levels of HIV-1 DNA in activated versus resting CD4+ T cell populations. Chun et al. evaluated levels of HIV-1 DNA in activated CD4+ T cells as a means of comparison to levels of HIV-1 DNA in gut-associated CD4+ T cells (which are typically of a more activated phenotype[369]), and found that levels of HIV-1 DNA were somewhat higher in activated versus resting CD4+ T cells[366]. Murray et al. performed a longitudinal analysis of the decay rates of total and integrated HIV-1 DNA in resting and activated CD4+ T cell populations in those initiating ART, and found that by week 52 of ART, levels of total HIV-1 DNA and integrated HIV-1 DNA were similar between activated (defined as CD38+) and resting (defined as CD38-) CD4+ T cells[367], which is consistent with our results between total and resting CD4+ T cells (although we defined resting CD4+ T cells differently).

When looking for factors that explained the differences observed in virion production between tCD4 cells and rCD4 cells, we found that the percent of CD4+ T cells in the tCD4 cell population that express activation markers was positively correlated with the ratio of virion production from tCD4 cells versus rCD4 cells, meaning as there are more non-resting CD4+ T cells in the overall tCD4 population, the virion production from tCD4 relative to rCD4 increases.

We also found that the frequency of infection per million tCD4 cells increases with an increase in the frequency of activated CD4+ T cells in tCD4 cells. Others have found a relationship between levels of immune activation/exhaustion and the size of the reservoir[370,371], although results to date do not not allow for any inference about whether activation is driving reservoir size or reservoir size is driving activation.

Many studies to date have evaluated relationships between inflammation and HIV-1 infection (reviewed by Klatt et al.[372]). A general consensus from these studies is that levels of activation are high in this with uncontrolled viral replication[373-379], and that ART reduces measures of inflammation concomitantly with reductions in plasma viremia[377,380-383], although measures of inflammation remain slightly elevated in those on ART. Many factors, including infection with herpes viruses[384], contribute to these heightened levels of inflammation on ART[385,386], but damage done to the gut mucosa during uncontrolled viral replication likely contributes substantially to chronic inflammation on ART[387-390]. Interestingly, although the frequency of CD4+ T cells in the gut is less than that in peripheral blood[366,369], frequencies of infection are much higher[366,369], but the ratio of transcription to infection seems to be much lower[391]. Finally, recent evidence has indicated that CD4+ T cells may not traffic efficiently to the gut in those with HIV-1 infection[392], and that cells in the periphery expressing homing markers directing them to the gut are enriched for HIV-1 DNA[393]. Together, these lines of evidence may potentially link chronic inflammation derived from the gut to the persistence of infected cells within the tCD4 cell population in the periphery. This hypothesis is highly speculative, and will need to be evaluated in future studies.

In addition to evaluating the size of inducible reservoir in tCD4 and rCD4 cells, we also investigated the size of the infectious reservoir in tCD4 versus rCD4 cells, and found that there was a trend towards a larger infectious reservoir in tCD4 cells compared with rCD4 cells. In the 7 donors from whom infectious virus was recoverable from either tCD4 or rCD4 cells, we found that 5 of these donors produced more infectious virus from tCD4 cells compared with rCD4 cells. Importantly, we also found that low level plasma viremia in participants on long-term suppressive ART was related to the size of the infectious reservoir in rCD4 cells, consistent with

previous findings[358]. Interestingly, all study participants that had a low-level viremia by mega-iSCA that was greater than 0.3 copies per milliliter of plasma were found to have positive viral outgrowth from rCD4 cells. There was also an inverse relationship between the duration of suppression and the ratio of infectious viral outgrowth from tCD4 cells versus rCD4 cells, with a shorter duration of suppression being associated with a higher degree of viral outgrowth from tCD4 versus rCD4 cells. This result suggests that cells containing infectious proviruses in tCD4 cells either transition into the rCD4 population over time, or decay more rapidly than cells containing infectious proviruses in rCD4 cells.

Of critical importance, we found that the levels of virions produced following activation of tCD4 or rCD4 cells were correlated with levels of infectious units per million cells from tCD4 or rCD4 cells, correspondingly. It also appears that there are two distinct points in the infectious viral lifecycle that depend on stochastic events for viral spread. The first stochastic barrier to overcome is sufficient production of virions, and the second stochastic barrier is the infection and spread of virus in susceptable cells. As such, the odds of viral outgrowth increase following with higher levels of virion production, but there does not seem to be a point where production of a certain number of virions guarantees viral outgrowth. Instead, increasing levels of virion production correspond to higher odds of viral outgrowth. This result is inconsistent with the results of Laird et al., who found that any well that contained properly poly-adenylated virion associated HIV-1 RNA was subsequently found to undergo viral outgrowth[394], although we did not measure poly-adenylated transcripts and used activated cells from HIV-negative blood donors for viral outgrowth, while Laird et al. utilized Molt-4/CCR5 cells to propagate virus.

Taken together, the results of this study begin to shed light on the nature of the viral reservoir in those on long-term suppressive ART. Consistent with the plethora of functions that

CD4+ T cells contribute to *in vivo*[395,396], the persistence of HIV-1 within tCD4 cells is indeed complicated. In addition to the well-described viral reservoir in rCD4 cells that is thought to lead to life-long persistence of HIV-1, we find that there is a reservoir present in tCD4 cells, and that this reservoir leads to levels of virion production that are greater than those produced from rCD4 cells alone. We have shown this reservoir to be latent and inducible, and to contain replication competent proviruses. In particular, to better understand the tCD4 cell reservoir, activated CD4+ T cells will need to be evaluated independently of rCD4 cells. Direct quantification of HIV-1 RNA and DNA in activated versus tCD4 versus rCD4 cells will be an important component of future studies. The interplay between the latent reservoir in rCD4 cells and tCD4 cells harboring latent, inducible, infectious virions also needs to be further clarified. While levels of cellular HIV-1 DNA and RNA in tCD4 cells and rCD4 cells are correlated, it is not clear why levels of cellular HIV-1 RNA transcription in tCD4 cells are lower compared to rCD4 cells, and whether rCD4 cells exit from the latent reservoir and enter in the tCD4 cell compartment, or whether the tCD4 cells harbor a distinct reservoir. The existence of this reservoir in tCD4 cells further complicates curative strategies, and the biology of this novel reservoir needs to be thoroughly evaluated in future studies.

9.0 OVERALL CONCLUSIONS AND FUTURE DIRECTIONS

The projects described in this dissertation began in 2012, at which time the only latency reversal agent that had entered clinical trials was vorinostat[251], and the relative potency of vorinostat compared to other HDACi and whether HDACi had an effect on the inducible reservoir were unknown. The results from the trial of vorinostat suggested that it was an active latency reversal agent, and that the proof of principle of "kick and kill" had been demonstrated. This first clinical report of the activity of vorinistat was the impetus for the study of latency reversal agents at the level of individual proviruses, the development of an assay to assess fractional provirus expression, and the investigation of the potency of latency reversal agents targeting other known mechanisms of proviral latency.

At the time when this dissertation began, the concept of total inducible virion production versus viral outgrowth had not yet been investigated, and the only way to measures the size of the reservoir *ex vivo* was to evaluate viral outgrowth[302]. Initial studies conducted in the AIDS Clinical Trials Group by Gandhi et al. suggested that the size of the infectious reservoir was correlated with the degree of low-level viremia in plasma[358], and another study by Eriksson et al. demonstrated that integrated HIV-1 DNA in PBMC was associated with the magnitude of viral outgrowth[359]. These two studies served as a foundation for our investigation of the nature of viral persistence. The small amount of data in these initial studies was sufficient to spur us to answer

questions that were important to the field at the genesis of this dissertation project, and that they remain important at the completion of this dissertation.

At the outset of this dissertation, the goals were : i.) to develop and implement an assay to assess the potency of latency reversal agents targeting known aspects of proviral latency at the single proviral level, and to determine whether LRA treatment reduces the size of the latent reservoir *ex vivo*; ii.) to quantify relationships between spontaneous and inducible virion production from resting CD4+ T cells, and to relate these measures to other molecular biomarkers of HIV-1 persistence *in vivo*; and iii.) to determine whether a reservoir of latent, inducible, and infectious HIV-1 was present in total CD4+ T cells, and to ascertain whether levels of total inducible virus were related to infectious virus outgrowth.

9.1 FRACTIONAL PROVIRUS EXPRESSION REVEALS POOR POTENTCY OF PUTATIVE LATENCY REVERSING AGENTS

9.1.1 Establishment of fractional provirus expression

Our initial goal was to determine the most suitable assay format to assess the fraction of proviruses that spontaneously produced virions, and the fraction that could be induced to produce virions following maximum T cell activation. An assay that could determine the fraction of proviruses reactivated with any given treatment could answer the open question as to the potency of vorinostat at the level of individual proviruses. The best method to achieve this goal was found to be a limiting dilution culture assay, where replicate wells of rCD4 cells were serially

diluted and treated with medium control (negative control), anti-CD3/CD28 activation beads (positive control) or LRAs of interest. Following 7 days of culture, the supernatants were harvested from these wells, and the amount of virion associated HIV-1 RNA present in the supernatant was evaluated. By knowing the number of cells per well (and therefore the number of proviruses present per well), we were able to apply a parametric binomial maximum likelihood estimate to evaluate the proportion of proviruses that were responsible for virion production in any given treatment condition (Chapter 1). We then used this fractional proviral expression (fPVE) assay to evaluate the fraction of proviruses that were responsible for virion production from rCD4 cells following treatment with medium control, anti-CD3/CD28 or vorniostat, and found that only 1.5% of proviruses could be activated with anti-CD3/CD28, 0.041% were spontaneously producing virions, and 0.12% were activated to produce virions following treatment with vorinostat (Chapter 1). We also found a significant relationship between unspliced cellular HIV-1 RNA expression and virion production following anti-CD3/CD28 treatment, but not following vorinostat treatment, likely due to the inability of vorinostat to reverse all blocks associated with proviral latency. This study established the framework for the assessment of other LRAs described in Chapter 2 of this dissertation, and provided the first inkling that putative LRAs are weak activators of the reservoir in rCD4 cells at the level of individual proviruses.

9.1.2 Investigation of the potency of putative LRAs and the relevance of previously described mechanisms of proviral latency

After establishing that fPVE was a robust way to assess the reversal of HIV-1 latency in rCD4 cells at the level of individual proviruses, we next sought to evaluate whether targeting known mechanisms of proviral latency with putative LRAs was effective *ex vivo*. We selected a panel of latency reversal agents based on previously described mechanisms of proviral latency, and disqualified agents that were thought to cause T cell activation. The selected agents tested whether histone acetylation, availability of P-TEFb, activation of NF-κB, or combinations of these mechanisms of proviral latency could be specifically therapeutically targeted to permit virus production (Chapter 2).

Investigating these agents led to the conclusion that, although some LRAs could activate virion production, no LRA or combination of LRAs led to activation of a significant proportion of latent proviruses compared with anti-CD3/CD28 treatment. Treatment of rCD4 cells with anti-CD3/CD28 activated a median of 3.8% of proviruses, while treatment with romidepsin, JQ1, bryostatin, romidepsin + JQ1 and bryostatin + romidepsin each reactivated 0.062%, 0.017%, 0.012%, 0.12% and 0.083% of proviruses (Chapter 2). Additionally, treatment of rCD4 cells with bryostatin, a known PKC agonist, led to activation of rCD4 cells in some donors as evidence by increased expression of CD69. This increase in expression of activation markers could be potentially dangerous *in vivo*, as non-specific immune activation can lead to cytokine related illness. These findings demonstrate that targeting one or a few known mechanisms of proviral latency *ex vivo* is not sufficient to adequately reverse proviral latency, and that targeting cellular pathways can lead to increased expression of activation markers and cellular activation.

This is the only in depth study of LRAs that has evaluated their relative activity compared to T cell activation at the level of individual proviruses, and provides important information about the relative inactivity of LRAs. The ability of LRAs to reactivate only a few proviruses is not currently appreciated. However, we did not assess the potency of latency reversal at the level of cellular HIV-1 mRNA in this study, and it is possible that expression of cellular HIV-1 RNA was increased, possibly leading to translation of viral proteins and death from viral-mediated cellular cytopathic effects or from immune clearance.

9.2 EVALUATION OF CHANGES IN THE SIZE OF THE INDUCIBLE RESERVOIR IN RESTING CD4+ T CELLS FOLLOWING TREATMENT WITH LATENCY REVERSING AGENTS

The experiments described in Chapter 2 indicated that there is no significant increase in virion production following the treatment of rCD4 cells with LRAs. This finding, though important, does not preclude the possibility that cellular transcription of HIV-1 mRNA may have been modulated, leading to the translation of viral proteins and death of reactivated cells through viral cytopathic effects. To evaluate this possibility, we performed a series of experiments in which rCD4 cells were first treated with LRAs and then maximally reactivated to assess whether there was a decrease in the size of the inducible reservoir. In this context, if the inducible reservoir had been depleted by LRAs, this would be clear by a reduction in virion production or fPVE from the treated cells compared to medium control cells. Importantly, we also assessed both viability and

activation marker expression following maximum reactivation to elucidate whether the rCD4 cells had become less responsive to reactivation.

Using the experimental approach described above, we found that the median reductions in the size of the latent reservoir following treatment with romidepsin, bryostatin and bryostatin + romidepsin compared to medium control was 1.5, 2.0, and 3.4 fold respectively (Chapter 3). Although it initially appeared that these LRAs may have an effect on the size of the inducible reservoir following maximum reactivation, this finding is confounded by the fact that the viability is substantially lower for romidepsin and bryostatin + romidepsin treated cells compared with medium control following full latency reversal. Thus, it appears that these cells become somewhat resistant to activation following treatment with romidepsin and bryostatin + romidepsin, complicating the conclusion that fewer proviruses are inducible due to the depletion of the latent reservoir rather than simply resistance to T cell activation stimuli. It is possible that bryostatin may reduce the size of the inducible reservoir, as it was not found to substantially reduce viability upon maximum stimulation. However, there was some variability in activation marker expression following treatment with bryostatin (Chapter 3).

The results from Chapter 3 demonstrate once again the importance of assessing the fraction of proviruses that are inducible following treatment with LRAs. Although most of the treatment conditions led to reduced viability following subsequently maximum reactivation, it is interesting to note that bryostatin appears to cause a reduction in the median size of the inducible reservoir, albeit a modest 2 fold reduction. These results are the first to assess whether latency reversal agents cause reductions in the size of the inducible reservoir.

9.3 FREQUENCY OF INFECTED CELLS AND THEIR TRANSCRIPTIONAL ACTIVITY IN PBMC CORRELATE WITH THE SIZE OF THE INDUCIBLE RESERVOIR IN RESTING CD4+ T CELLS

The goal of this study was to evaluate whether there were relationships between molecular measures of viral persistence and the size of the inducible reservoir in rCD4 cells in study participants on long-term suppressive antiretroviral therapy. In collaboration with Gilead Sciences, we evaluated levels of cellular HIV-1 DNA and RNA in PBMC, low-level viremia in plasma by large-volume single copy assay, and inducible and spontaneous virion production from cultured rCD4 cells in 21 study participants (Chapter 4). To date, this is the first study to examine relationships between measures of viral persistence in conjunction with their relationship to the amount of spontaneous virion production and the size of the inducible reservoir.

Our led to the elucidation of a number of important relationships that were not previously appreciated. Principal among these was the finding that simple measures of cellular HIV-1 RNA and DNA in PBMC were strongly associated with the size of the inducible reservoir in rCD4 cells. There has been much discussion recently regarding whether proviruses detectable by qPCR are intact, harbor large internal deletions, are hypermutated, or are otherwise unable to produce virions, and whether the magnitude of the reservoir is comparable to molecular measures of persistence[151,359]. This is a valid concern, but the reality is that the frequency of infected cells likely correlates with the frequency of cells that are capable of producing virions following reactivation, a proportionality that has likely not been appreciated elsewhere thus far.

In addition to the finding that cellular HIV-1 RNA and DNA in PBMC are related to the size of the inducible reservoir, we also found that the magnitude of low-level viremia present in plasma was correlated with the magnitude of spontaneous virion production from both PBMC and rCD4 cells. This is an important finding, in that it connects the *in vivo* measure of viral persistence to peripheral measures of the basal virion production, and implies that virion release in the periphery is the source of plasma viremia (Chapter 4).

This study identified substantial and previously unknown relationships between molecular measures of HIV-1 persistence and the size of the inducible reservoir in rCD4 cells in study participants on long-term suppressive ART. These findings beg further study of the reservoir in rCD4 cells, specifically whether measures of inducible virion production are associated with the size of the infectious reservoir in rCD4 cells. In addition, this study had assumed that the reservoir of HIV-1 is primarily in rCD4 cells, and did not evaluate the presence of a viral reservoir within the activated CD4+ T cell population.

9.4 PRESENCE OF AN INDUCIBLE INFECTIOUS RESERVOIR OF HIV-1 WITHIN TOTAL CD4+ T CELLS, AND RELATIONSHIP BETWEEN INDUCIBLE AND INFECTIOUS VIRION PRODUCTION

The final objective of this dissertation was to assess whether there is a cellular population within total CD4+ T cells, distinct from rCD4 cells, that harbors an inducible infectious viral reservoir. We also proposed to evaluate whether there is a relationship between the size of the inducible reservoir and the magnitude of viral outgrowth in total and resting CD4+ T cells. To evaluate

this, we quantified levels of inducible virus in tCD4 cells and rCD4 cells, and levels of infectious virus in tCD4 cells and rCD4 cells in parallel. If there is a reservoir in tCD4 cells that is distinct from rCD4 cells, we would expect to observe higher levels of virion production and infectious virion production from tCD4 cells, in the absence of high levels of spontaneous virion release from productively infected cells (Chapter 5).

In a small cohort of 11 study participants on long-term suppressive ART, we found that levels of inducible virion production were 2.5-fold higher from tCD4 cells compared with rCD4 cells, and that this difference was statistically significant. Levels of infectious virion production from tCD4 cells also tended to be higher versus rCD4 cells, although this did not reach statistical significant in this cohort due to the small size, and the censoring of several donors who did not have detectable infectious virus in either tCD4 or rCD4 cells. In the 7 donors with quantifiable infectious virion production from either tCD4 cells or rCD4 cells, we found that 6 of 7 donors had higher levels of infectious virus outgrowth in tCD4 cells versus rCD4 cells, and that the mean fold-difference in viral outgrowth between tCD4 and rCD4 cells (2-9-fold difference) paralleled that observed in inducible virion production between tCD4 and rCD4 cells.

In agreement with the observed fold-change in tCD4 versus rCD4 cells for virion production and infectious viral outgrowth, we also found important, statistically significant correlations between the levels of total inducible virion production and viral outgrowth in tCD4 cells and rCD4 cells. This finding suggests that higher levels of virion production are associated with higher levels of infectious virion production, which is in partial agreement with the finding by Laird et al. that any wells in a modified viral outgrowth assay that were positive on Day 7 for virion production, subsequently became positive by p24 for viral outgrowth[394]. Our results differ from that of Laird et al. in that virion-associated HIV-1 RNA was detected following reactivation

of rCD4 and tCD4 cells from all study participants, and did not guarantee viral outgrowth after subsequent protracted co-culture. Finally, in agreement with a previous study within the AIDS Clinical Trials Group by Gandhi et al.[358], we found that levels of low-level viremia measured by large-volume single copy assays were statistically significantly correlated with levels of infectious virus within rCD4 cells, though not tCD4 cells.

The results presented in Chapters 4 and 5, when considered simultaneously, suggest that assessing the size of the latent reservoir in those on long term suppressive ART can be considerably simplified. The interrelated nature of the molecular markers of viral persistence also suggest that measuring only a few markers of persistence likely provides substantial information about this size and state of the reservoir. These findings should greatly accelerate the study of *in vivo* interventions aimed at reducing the size of the reservoir in those on suppressive ART.

9.5 FUTURE DIRECTIONS

9.5.1 Investigation of latency reversal agents specific for HIV-1

The investigation of latency reversal agents to reactivate latently infected CD4+ T cells has generally been viewed as a promising strategy to perturb the latent reservoir, and to make it amenable to immune clearance. However, these studies suggest that, when measuring the potency of LRAs at the level of individual proviruses, that their activity is quite limited. Additionally, studies that evaluated whether LRAs have reduced the size of the latent reservoir

ex vivo were complicated by the reduced viability of rCD4 cells to reactivation after treatment with LRAs. While bryostatin treatment seemed to cause a reduction in the size of the reservoir, it did so at the cost of partial activation of rCD4 cells, which suggests that *in vivo* dosing may be associated with side effects that are ultimately too toxic for those who are otherwise relatively healthy on suppressive ART.

The potential for current LRAs to reduce the size of the latent reservoir is therefore limited. One factor that is shared by the compounds that are currently being tested as LRAs is that they are all specific for host cell functions, and therefore are relatively non-specific for HIV-1 activation. ART, on the other hand, is highly specific for the virus (rather than the host), and therefore is maximally efficacious at suppressing viral replication at favorable therapeutic indices. The use of LRA compounds targeting host cellular factors that have previously been identified to be associated with latency is therefore never going to be as efficacious as targeting a specific aspect of the virus itself, although at this point it is unclear how this could be achieved.

The functions of CD4+ T cells *in vivo* are highly varied, and the immune system has a large degree of redundancy and plasticity, leading to a high degree of heterogeneity in the physiology of individual cells. While this complexity is appreciated *in vivo*, much of it is lost in cell lines by the vary nature of homogeneity they strive to achieve. This has been observed in comparison studies of different cell lines and primary cell models of HIV-1 infection, where no one model captures all aspects of proviral latency *in vivo* or *ex vivo*, leading to significant departures from physiological behavior and limited relevance of the established cell models[258]. The use of cell lines to identify and characterize factors associated with proviral latency has doubtlessly led to an oversimplification of the mechanisms that contribute to proviral latency *in*

vivo. This highlights an important shortcoming of homogenous cell lines that should be considered at early stages of development of LRAs.

Targeting a specific aspect of the virus itself in a latent cell would be an admirable goal, given the problems discussed above with targeting cellular pathways. This is an apparent paradox though, as the latent provirus does little to favor transition to latency from active infection; the provirus is more passenger than driver in the trip to latency. There are a few options that would be worth considering, such as a mimic of Tat protein that would foster highly processive full-length transcription of HIV-1 RNA. In our initial study of vorinostat, we found that there was not a relationship between levels of unspliced cellular HIV-1 RNA transcription and virion production, suggesting a block between transcription and translation. These findings suggest that another option for a virus-specific latency reversing agents would be a drug that mimics the activity of Rev, bringing HIV-1 transcripts into the cytosol for efficient translation into HIV-1 proteins. Developing small molecules that are bioavailable and that can perform all the functions usually achieved by large proteins with multiple protein interaction sites *in vivo* would certainly be a challenge, but may ultimately be more successful that targeting cellular aspects of proviral latency. At this juncture, the path forward for latency reversal agents is uncertain at best.

9.5.2 Investigation of the non-resting cells within tCD4 cells that harbor proviruses

We have found, for the first time, that there is a latent and inducible population of cells within total CD4+ T cells that harbor latent, inducible and infectious proviruses. The latent infectious reservoir in resting CD4+ T cells has been well described, and is thought to lead to rebound

plasma viremia following cessation of ART. While the reservoir in rCD4 cells is certainly an important reservoir, it is likely only part of the picture. It seems that cells expressing one or more activation markers, representing various degrees of activation, also satisfy the conditions required to be a latent reservoir.

One important question that arises from these data is which cells in the tCD4 pool that express CD25/CD69/HLA-DR are the most relevant reservoir in tCD4 cells. Characterizing the relative contribution of these cells to the total reservoir in tCD4 cells will be important to more fully understanding the biology of this reservoir. Comparing the frequency of infection, and the amount of HIV-1 RNA transcription in activated versus total versus resting CD4+ T cells will be an important next step, followed by characterization of the proviral and HIV-1 mRNA species that are present in each cell population. This will lead to insight as to whether the activated CD4+ T cells are transitioning out of the latent reservoir or represent a novel, stable species in those on long term ART. If the tCD4 cell compartment harbors a biologically distinct reservoir, evaluating whether this reservoir size is associated with viral rebound will be an important task. Finally, if this reservoir is truly distinct and biologically relevant, novel therapeutics should be investigated to eliminate these cells. These cells may be more amenable to therapeutic targeting, since they are likely to be more metabolically active than their rCD4 cell counterparts.

9.5.3 Evaluation of biomarkers of time to viral rebound following cessation of ART

Perhaps the most natural extension of the findings presented in this dissertation will be to prospectively evaluate the biomarkers pertaining to the size of the inducible reservoir as predictors of the time to virologic rebound. From the results presented here, the simplest markers

of the size of the latent reservoir would be molecular measures of plasma viremia, and cellular HIV-1 RNA and DNA in unfractionated PBMC. However, one could imagine scenarios wherein all the replication competent (~1 cell per million rCD4 cells) proviruses capable of producing virions following activation (2-4%) would be eliminated, with a minimal effect on the frequency of infected cells. As such, it seems that the most robust measure of the size of the inducible reservoir would be virion production by PVR from total CD4+ T cells. This measure of reservoir size would be the best for several reasons: i.) tCD4 PVR correlations with the size of the infectious reservoir in tCD4 cells, which is of a larger magnitude than the size of the reservoir in rCD4 cells; ii.) tCD4 PVR also correlations with the level of cellular transcription of HIV-1 in PBMC, demonstrating a connection to a molecular measure; iii.) virion production is a robust measure of the size of the inducible reservoir, as it has a large dynamic range, is a surrogate for the number of proviruses that have been reactivated, and is relatively easy to measure.

Although prospective trials are expensive and time consuming, the type of interruption trial where participants are closely monitored for viral rebound should be relatively safe for study participants, as long as rebound is identified without delay and ART is reinitiated in a timely manner. It will also be an important step forward for the prospective assessment of biomarkers of the inducible reservoir. Simplification of measures of the inducible reservoir will greatly expedite progress towards therapies that reduce the size of the reservoir by eliminating the need for expensive and time-consuming treatment interruptions studies in small scale proof of principle trials, the type of which have been advocated for in HIV-1 curative research[397]. Large-scale treatment interruption trials could then be used as the final validation tool for very promising strategies that have been advanced to later-stage clinical development.

BIBLIOGRAPHY

1 Centers for Disease Control and Prevention. A cluster of Kaposi's sarcoma and Pneumocystis carinii pneumonia among homosexual male residents of Los Angeles and Orange Counties, California. *MMWR Morb Mortal Wkly Rep* **31**, 305-307 (1982).

2 Gottlieb, M. S. *et al.* Pneumocystis carinii pneumonia and mucosal candidiasis in previously healthy homosexual men: evidence of a new acquired cellular immunodeficiency. *N Engl J Med* **305**, 1425-1431, doi:10.1056/NEJM198112103052401 (1981).

3 Centers for Disease Control and Prevention. Pneumocystis pneumonia--Los Angeles. *MMWR Morb Mortal Wkly Rep* **30**, 250-252 (1981).

4 Barre-Sinoussi, F. *et al.* Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). *Science* **220**, 868-871 (1983).

5 Schupbach, J. *et al.* Serological analysis of a subgroup of human T-lymphotropic retroviruses (HTLV-III) associated with AIDS. *Science* **224**, 503-505 (1984).

6 Sarngadharan, M. G., Popovic, M., Bruch, L., Schupbach, J. & Gallo, R. C. Antibodies reactive with human T-lymphotropic retroviruses (HTLV-III) in the serum of patients with AIDS. *Science* **224**, 506-508 (1984).

7 Popovic, M., Sarngadharan, M. G., Read, E. & Gallo, R. C. Detection, isolation, and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS. *Science* **224**, 497-500 (1984).

8 Gallo, R. C. *et al.* Frequent detection and isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk for AIDS. *Science* **224**, 500-503 (1984).

9 Weiss, R., Teich, N., Varmus, H. & Coffin, J. *RNA tumor viruses*. (1985).

10 Temin, H. M. & Mizutani, S. Viral RNA-dependent DNA Polymerase: RNA-dependent DNA Polymerase in Virions of Rous Sarcoma Virus. *Nature* **226**, 1211-1213 (1970).

11 Baltimore, D. Viral RNA-dependent DNA Polymerase: RNA-dependent DNA Polymerase in Virions of RNA Tumour Viruses. *Nature* **226**, 1209-1211 (1970).

12 Broder, S. The development of antiretroviral therapy and its impact on the HIV-1/AIDS pandemic. *Antiviral research* **85**, 1, doi:10.1016/j.antiviral.2009.10.002 (2010).

13 Yarchoan, R. & Broder, S. Development of Antiretroviral Therapy for the Acquired Immunodeficiency Syndrome and Related Disorders. *New England Journal of Medicine* **316**, 557-564, doi:10.1056/NEJM198702263160925 (1987).

14 Horwitz, J. P., Chua, J. & Noel, M. Nucleosides. V. The Monomesylates of 1-(2'-Deoxy-β-D-lyxofuranosyl)thymine1,2. *The Journal of Organic Chemistry* **29**, 2076-2078, doi:10.1021/jo01030a546 (1964).

15 Schedler, D. J. A. Drug Discovery: A History (Sneader, Walter). *Journal of Chemical Education* **83**, 215, doi:10.1021/ed083p215.1 (2006).

16 Mitsuya, H. *et al.* Suramin protection of T cells in vitro against infectivity and cytopathic effect of HTLV-III. *Science* **226**, 172-174 (1984).

17 Mitsuya, H., Matsukura, M. & Broder, S. Rapid in vitro systems for assessing activity of agents against HTLV-III/LAV. In: Broder S (ed) AIDS: modern concepts and therapeutic challenges. Marcel Dekker, New York (1987).

18 Mitsuya, H. *et al.* 3'-Azido-3'-deoxythymidine (BW A509U): an antiviral agent that inhibits the infectivity and cytopathic effect of human T-lymphotropic virus type III/lymphadenopathy-associated virus in vitro. *Proceedings of the National Academy of Sciences of the United States of America* **82**, 7096-7100 (1985).

19 Perno, C. F. *et al.* Inhibition of human immunodeficiency virus (HIV-1/HTLV-IIIBa-L) replication in fresh and cultured human peripheral blood monocytes/macrophages by azidothymidine and related 2',3'-dideoxynucleosides. *J Exp Med* **168**, 1111-1125 (1988).

20 Mitsuya, H. & Broder, S. Inhibition of the in vitro infectivity and cytopathic effect of human T-lymphotrophic virus type III/lymphadenopathy-associated virus (HTLV-III/LAV) by 2',3'-dideoxynucleosides. *Proceedings of the National Academy of Sciences of the United States of America* **83**, 1911-1915 (1986).

21 Harvard Law Review. Patent Law. Pharmaceuticals. Federal Circuit Upholds Patents for AIDS Treatment Drug. Burroughs Wellcome Co. v. Barr Laboratories, Inc., 40 F.3d 1223 (Fed. Cir. 1994). *Harvard Law Review* **108**, 2053-2058, doi:10.2307/1341954 (1995).

22 Yarchoan, R. *et al.* Administration of 3'-azido-3'-deoxythymidine, an inhibitor of HTLV-III/LAV replication, to patients with AIDS or AIDS-related complex. *Lancet* **1**, 575-580 (1986).

23 Yarchoan, R. *et al.* Response of human-immunodeficiency-virus-associated neurological disease to 3'-azido-3'-deoxythymidine. *Lancet* **1**, 132-135 (1987).

24 Yarchoan, R. *et al.* Phase I studies of 2',3'-dideoxycytidine in severe human immunodeficiency virus infection as a single agent and alternating with zidovudine (AZT). *Lancet* **1**, 76-81 (1988).

25 Yarchoan, R. *et al.* In vivo activity against HIV and favorable toxicity profile of 2',3'-dideoxyinosine. *Science* **245**, 412-415 (1989).

26 Yarchoan, R. *et al.* Long-term toxicity/activity profile of 2',3'-dideoxyinosine in AIDS or AIDS-related complex. *Lancet* **336**, 526-529 (1990).

27 Fischl, M. A. *et al.* The efficacy of azidothymidine (AZT) in the treatment of patients with AIDS and AIDS-related complex. A double-blind, placebo-controlled trial. *N Engl J Med* **317**, 185-191, doi:10.1056/nejm198707233170401 (1987).

28 Food and Drug Administration. New Drug, Antibiotic, and Biologic Drug Product Regulations. Document number: 82N-0394 (1987).

29 Hirsch , M. S. & D'Aquila , R. T. Therapy for Human Immunodeficiency Virus Infection. *New England Journal of Medicine* **328**, 1686-1695, doi:doi:10.1056/NEJM199306103282307 (1993).

30 Vella, S., Giuliano, M., Pezzotti, P. & et al. SUrvival of zidovudine-treated patients with aids compared with that of contemporary untreated patients. *JAMA* **267**, 1232-1236, doi:10.1001/jama.1992.03480090080031 (1992).

31 Fischl, M. A., Richman, D. D., Causey, D. M. & et al. PRolonged zidovudine therapy in patients with aids and advanced aids-related complex. *JAMA* **262**, 2405-2410, doi:10.1001/jama.1989.03430170067030 (1989).

32 Richman, D. D. *et al.* The Toxicity of Azidothymidine (AZT) in the Treatment of Patients with AIDS and AIDS-Related Complex. *New England Journal of Medicine* **317**, 192-197, doi:doi:10.1056/NEJM198707233170402 (1987).

33 Rooke, R. *et al.* Isolation of drug-resistant variants of HIV-1 from patients on long-term zidovudine therapy. Canadian Zidovudine Multi-Centre Study Group. *Aids* **3**, 411-415 (1989).

34 Larder, B. A., Darby, G. & Richman, D. D. HIV with reduced sensitivity to zidovudine (AZT) isolated during prolonged therapy. *Science* **243**, 1731-1734 (1989).

35 Larder, B. A., Kellam, P. & Kemp, S. D. Zidovudine resistance predicted by direct detection of mutations in DNA from HIV-infected lymphocytes. *Aids* **5**, 137-144 (1991).

36 Larder, B. A. & Kemp, S. D. Multiple mutations in HIV-1 reverse transcriptase confer high-level resistance to zidovudine (AZT). *Science* **246**, 1155-1158 (1989).

37 Lambert, J. S. *et al.* 2′,3′-Dideoxyinosine (ddI) in Patients with the Acquired Immunodeficiency Syndrome or AIDS-Related Complex. *New England Journal of Medicine* **322**, 1333-1340, doi:10.1056/NEJM199005103221901 (1990).

38 Cooley, T. P. *et al.* Once-Daily Administration of 2′,3′-Dideoxyinosine (ddI) in Patients with the Acquired Immunodeficiency Syndrome or AIDS-Related Complex. *New England Journal of Medicine* **322**, 1340-1345, doi:10.1056/NEJM199005103221902 (1990).

39 Browne, M. J. *et al.* 2′,3′-Didehydro-3′-deoxythymidine (d4T) in Patients with AIDS or AIDS-Related Complex: A Phase I Trial. *Journal of Infectious Diseases* **167**, 21-29, doi:10.1093/infdis/167.1.21 (1993).

40 Abrams, D. I. *et al.* A Comparative Trial of Didanosine or Zalcitabine after Treatment with Zidovudine in Patients with Human Immunodeficiency Virus Infection. *New England Journal of Medicine* **330**, 657-662, doi:10.1056/NEJM199403103301001 (1994).

41 Collier, A. C. *et al.* Combination therapy with zidovudine and didanosine compared with zidovudine alone in HIV-1 infection. *Ann Intern Med* **119**, 786-793 (1993).

42 Meng, T. C. *et al.* Combination therapy with zidovudine and dideoxycytidine in patients with advanced human immunodeficiency virus infection. A phase I/II study. *Ann Intern Med* **116**, 13-20 (1992).

43 Hammer, S. M. *et al.* A Trial Comparing Nucleoside Monotherapy with Combination Therapy in HIV-Infected Adults with CD4 Cell Counts from 200 to 500 per Cubic Millimeter. *New England Journal of Medicine* **335**, 1081-1090, doi:10.1056/NEJM199610103351501 (1996).

44 Navia, M. A. *et al.* Three-dimensional structure of aspartyl protease from human immunodeficiency virus HIV-1. *Nature* **337**, 615-620 (1989).

45 Roberts, N. A. *et al.* Rational design of peptide-based HIV proteinase inhibitors. *Science* **248**, 358-361 (1990).

46 Craig, J. C. *et al.* Antiviral properties of Ro 31-8959, an inhibitor of human immunodeficiency virus (HIV) proteinase. *Antiviral Res* **16**, 295-305 (1991).

47 Vacca, J. P. *et al.* L-735,524: an orally bioavailable human immunodeficiency virus type 1 protease inhibitor. *Proc Natl Acad Sci U S A* **91**, 4096-4100 (1994).

48 Kempf, D. J. *et al.* ABT-538 is a potent inhibitor of human immunodeficiency virus protease and has high oral bioavailability in humans. *Proceedings of the National Academy of Sciences of the United States of America* **92**, 2484-2488 (1995).

49 Kitchen, V. S. *et al.* Safety and activity of saquinavir in HIV infection. *Lancet* **345**, 952-955 (1995).

50 Stein, D. S. *et al.* A 24-week open-label phase I/II evaluation of the HIV protease inhibitor MK-639 (indinavir). *Aids* **10**, 485-492 (1996).

51 Markowitz, M. *et al.* A Preliminary Study of Ritonavir, an Inhibitor of HIV-1 Protease, to Treat HIV-1 Infection. *New England Journal of Medicine* **333**, 1534-1540, doi:10.1056/NEJM199512073332204 (1995).

52 Danner, S. A. *et al.* A Short-Term Study of the Safety, Pharmacokinetics, and Efficacy of Ritonavir, an Inhibitor of HIV-1 Protease. *New England Journal of Medicine* **333**, 1528-1534, doi:10.1056/NEJM199512073332303 (1995).

53 Molla, A. *et al.* Ordered accumulation of mutations in HIV protease confers resistance to ritonavir. *Nat Med* **2**, 760-766 (1996).

54 Condra, J. H. *et al.* Genetic correlates of in vivo viral resistance to indinavir, a human immunodeficiency virus type 1 protease inhibitor. *J Virol* **70**, 8270-8276 (1996).

55 Condra, J. H. *et al.* In vivo emergence of HIV-1 variants resistant to multiple protease inhibitors. *Nature* **374**, 569-571, doi:10.1038/374569a0 (1995).

56 Schinazi, R. F. *et al.* Activities of the four optical isomers of 2',3'-dideoxy-3'-thiacytidine (BCH-189) against human immunodeficiency virus type 1 in human lymphocytes. *Antimicrob Agents Chemother* **36**, 672-676 (1992).

57 Coates, J. A. *et al.* The separated enantiomers of 2'-deoxy-3'-thiacytidine (BCH 189) both inhibit human immunodeficiency virus replication in vitro. *Antimicrob Agents Chemother* **36**, 202-205 (1992).

58 Coates, J. A. *et al.* (-)-2'-deoxy-3'-thiacytidine is a potent, highly selective inhibitor of human immunodeficiency virus type 1 and type 2 replication in vitro. *Antimicrob Agents Chemother* **36**, 733-739 (1992).

59 Tisdale, M., Kemp, S. D., Parry, N. R. & Larder, B. A. Rapid in vitro selection of human immunodeficiency virus type 1 resistant to 3'-thiacytidine inhibitors due to a mutation in the YMDD region of reverse transcriptase. *Proc Natl Acad Sci U S A* **90**, 5653-5656 (1993).

60 Schinazi, R. F. *et al.* Characterization of human immunodeficiency viruses resistant to oxathiolane-cytosine nucleosides. *Antimicrob Agents Chemother* **37**, 875-881 (1993).

61 Boucher, C. A. *et al.* High-level resistance to (-) enantiomeric 2'-deoxy-3'-thiacytidine in vitro is due to one amino acid substitution in the catalytic site of human immunodeficiency virus type 1 reverse transcriptase. *Antimicrob Agents Chemother* **37**, 2231-2234 (1993).

62 van Leeuwen, R. *et al.* Evaluation of safety and efficacy of 3TC (lamivudine) in patients with asymptomatic or mildly symptomatic human immunodeficiency virus infection: a phase I/II study. *J Infect Dis* **171**, 1166-1171 (1995).

63 van Leeuwen, R. *et al.* The safety and pharmacokinetics of a reverse transcriptase inhibitor, 3TC, in patients with HIV infection: a phase I study. *Aids* **6**, 1471-1475 (1992).

64 Bartlett, J. A. *et al.* Lamivudine plus zidovudine compared with zalcitabine plus zidovudine in patients with HIV infection. A randomized, double-blind, placebo-controlled trial. North American HIV Working Party. *Ann Intern Med* **125**, 161-172 (1996).

65 Eron, J. J. *et al.* Treatment with lamivudine, zidovudine, or both in HIV-positive patients with 200 to 500 CD4+ cells per cubic millimeter. North American HIV Working Party. *N Engl J Med* **333**, 1662-1669, doi:10.1056/nejm199512213332502 (1995).

66 Craig, J. C., Whittaker, L., Duncan, I. B. & Roberts, N. A. In vitro anti-HIV and Cytotoxicological Evaluation of the Triple Combination: AZT and ddC with HIV Proteinase Inhibitor Saquinavir (Ro 31-8959). *Antiviral Chemistry and Chemotherapy* **5**, 380-386, doi:10.1177/095632029400500605 (1994).

67 Craig, J. C., Duncan, I. B., Whittaker, L. & Roberts, N. A. Antiviral Synergy between Inhibitors of HIV Proteinase and Reverse Transcriptase. *Antiviral Chemistry and Chemotherapy* **4**, 161-166, doi:10.1177/095632029300400305 (1993).

68 Johnson, V. A., Merrill, D. P., Chou, T. C. & Hirsch, M. S. Human immunodeficiency virus type 1 (HIV-1) inhibitory interactions between protease inhibitor Ro 31-8959 and zidovudine, 2',3'-dideoxycytidine, or recombinant interferon-alpha A against zidovudine-sensitive or -resistant HIV-1 in vitro. *J Infect Dis* **166**, 1143-1146 (1992).

69 Collier, A. C. *et al.* Treatment of Human Immunodeficiency Virus Infection with Saquinavir, Zidovudine, and Zalcitabine. *New England Journal of Medicine* **334**, 1011-1018, doi:doi:10.1056/NEJM199604183341602 (1996).

70 Hammer, S. M. *et al.* A controlled trial of two nucleoside analogues plus indinavir in persons with human immunodeficiency virus infection and CD4 cell counts of 200 per cubic millimeter or less. AIDS Clinical Trials Group 320 Study Team. *N Engl J Med* **337**, 725-733, doi:10.1056/nejm199709113371101 (1997).

71 Gulick , R. M. *et al.* Treatment with Indinavir, Zidovudine, and Lamivudine in Adults with Human Immunodeficiency Virus Infection and Prior Antiretroviral Therapy. *New England Journal of Medicine* **337**, 734-739, doi:doi:10.1056/NEJM199709113371102 (1997).

72 Cheeseman, S. H. *et al.* Phase I/II evaluation of nevirapine alone and in combination with zidovudine for infection with human immunodeficiency virus. *Journal of acquired immune deficiency syndromes and human retrovirology : official publication of the International Retrovirology Association* **8**, 141-151 (1995).

73 Havlir, D. *et al.* High-dose nevirapine: safety, pharmacokinetics, and antiviral effect in patients with human immunodeficiency virus infection. *J Infect Dis* **171**, 537-545 (1995).

74 Havlir, D., McLaughlin, M. M. & Richman, D. D. A pilot study to evaluate the development of resistance to nevirapine in asymptomatic human immunodeficiency virus-infected patients with CD4 cell counts of > 500/mm3: AIDS Clinical Trials Group Protocol 208. *J Infect Dis* **172**, 1379-1383 (1995).

75 D'Aquila, R. T. *et al.* Nevirapine, zidovudine, and didanosine compared with zidovudine and didanosine in patients with HIV-1 infection. A randomized, double-blind, placebo-controlled trial. National Institute of Allergy and Infectious Diseases AIDS Clinical Trials Group Protocol 241 Investigators. *Ann Intern Med* **124**, 1019-1030 (1996).

76 Staszewski , S. *et al.* Efavirenz plus Zidovudine and Lamivudine, Efavirenz plus Indinavir, and Indinavir plus Zidovudine and Lamivudine in the Treatment of HIV-1 Infection in Adults. *New England Journal of Medicine* **341**, 1865-1873, doi:doi:10.1056/NEJM199912163412501 (1999).

77 Gulick , R. M. *et al.* Triple-Nucleoside Regimens versus Efavirenz-Containing Regimens for the Initial Treatment of HIV-1 Infection. *New England Journal of Medicine* **350**, 1850-1861, doi:doi:10.1056/NEJMoa031772 (2004).

78 Gallant, J. E. *et al.* Tenofovir DF, Emtricitabine, and Efavirenz vs. Zidovudine, Lamivudine, and Efavirenz for HIV. *New England Journal of Medicine* **354**, 251-260, doi:doi:10.1056/NEJMoa051871 (2006).

79 Riddler, S. A. *et al.* Class-sparing regimens for initial treatment of HIV-1 infection. *N Engl J Med* **358**, 2095-2106, doi:10.1056/NEJMoa074609 (2008).

80 Gulick , R. M. *et al.* Maraviroc for Previously Treated Patients with R5 HIV-1 Infection. *New England Journal of Medicine* **359**, 1429-1441, doi:doi:10.1056/NEJMoa0803152 (2008).

81 Walmsley, S. L. *et al.* Dolutegravir plus Abacavir–Lamivudine for the Treatment of HIV-1 Infection. *New England Journal of Medicine* **369**, 1807-1818, doi:doi:10.1056/NEJMoa1215541 (2013).

82 Steigbigel, R. T. *et al.* Raltegravir with Optimized Background Therapy for Resistant HIV-1 Infection. *New England Journal of Medicine* **359**, 339-354, doi:doi:10.1056/NEJMoa0708975 (2008).

83 Department of Health and Human Services. Guidelines for the Use of Antiretroviral Agents in HIV-1-infected Adults and Adolesents. (2016). Accessed March 16, 2016. <http://aidsinfo.nih.gov/guidelines>.

84 Mollan, K. R. *et al.* Association Between Efavirenz as Initial Therapy for HIV-1 Infection and Increased Risk for Suicidal Ideation or Attempted or Completed SuicideAn Analysis of Trial DataSuicidal Ideation or Attempted or Completed Suicide With Efavirenz. *Annals of Internal Medicine* **161**, 1-10, doi:10.7326/M14-0293 (2014).

85 World Health Organization. Consolidated guidelines on the use of antiretroviral drugs for treating and preventing HIV infection. (2016). Accessed March 17, 2016.

86 Li, J. Z., Paredes, R., Ribaudo, H. J. & et al. Low-frequency hiv-1 drug resistance mutations and risk of nnrti-based antiretroviral treatment failure: A systematic review and pooled analysis. *JAMA* **305**, 1327-1335, doi:10.1001/jama.2011.375 (2011).

87 Périard, D. *et al.* Atherogenic Dyslipidemia in HIV-Infected Individuals Treated With Protease Inhibitors. *Circulation* **100**, 700-705, doi:10.1161/01.cir.100.7.700 (1999).

88 Friis-Moller, N. *et al.* Combination antiretroviral therapy and the risk of myocardial infarction. *N Engl J Med* **349**, 1993-2003, doi:10.1056/NEJMoa030218 (2003).

89 Gallant, J. E. *et al.* Efficacy and safety of tenofovir DF vs stavudine in combination therapy in antiretroviral-naive patients: a 3-year randomized trial. *Jama* **292**, 191-201, doi:10.1001/jama.292.2.191 (2004).

90 Cooper, R. D. *et al.* Systematic Review and Meta-analysis: Renal Safety of Tenofovir Disoproxil Fumarate in HIV-Infected Patients. *Clinical Infectious Diseases* **51**, 496-505, doi:10.1086/655681 (2010).

91 Grigsby, I. F., Pham, L., Mansky, L. M., Gopalakrishnan, R. & Mansky, K. C. Tenofovir-associated bone density loss. *Therapeutics and Clinical Risk Management* **6**, 41-47 (2010).

92 Worm, S. W. *et al.* Risk of Myocardial Infarction in Patients with HIV Infection Exposed to Specific Individual Antiretroviral Drugs from the 3 Major Drug Classes: The Data Collection on Adverse Events of Anti-HIV Drugs (D:A:D) Study. *Journal of Infectious Diseases* **201**, 318-330, doi:10.1086/649897 (2010).

93 Grabar, S. *et al.* Clinical outcome of patients with HIV-1 infection according to immunologic and virologic response after 6 months of highly active antiretroviral therapy. *Ann Intern Med* **133**, 401-410 (2000).

94 Kaufmann, G. R. *et al.* CD4 T-lymphocyte recovery in individuals with advanced HIV-1 infection receiving potent antiretroviral therapy for 4 years: the Swiss HIV Cohort Study. *Arch Intern Med* **163**, 2187-2195, doi:10.1001/archinte.163.18.2187 (2003).

95 Walensky, R. P. *et al.* The Survival Benefits of AIDS Treatment in the United States. *Journal of Infectious Diseases* **194**, 11-19, doi:10.1086/505147 (2006).

96 World Health Organization. Global Health Observatory data: HIV/AIDS Global situation and trends. (2013) Accessed March 17, 2016.

97 World Health Organization. HIV/AIDS Data and statistics: Global summary of the HIV/AIDS epidemic, December 2014. (2014). Accessed March 17, 2016.

98 Goudsmit, J. *et al.* Expression of human immunodeficiency virus antigen (HIV-Ag) in serum and cerebrospinal fluid during acute and chronic infection. *Lancet* **2**, 177-180 (1986).

99 Ho, D. D., Moudgil, T. & Alam, M. Quantitation of human immunodeficiency virus type 1 in the blood of infected persons. *N Engl J Med* **321**, 1621-1625, doi:10.1056/nejm198912143212401 (1989).

100 Saksela, K., Stevens, C. E., Rubinstein, P., Taylor, P. E. & Baltimore, D. HIV-1 Messenger RNA in Peripheral Blood Mononuclear Cells as an Early Marker of Risk for Progression to AIDS. *Annals of Internal Medicine* **123**, 641-648, doi:10.7326/0003-4819-123-9-199511010-00001 (1995).

101 Tsoukas, C. M. & Bernard, N. F. Markers predicting progression of human immunodeficiency virus-related disease. *Clinical Microbiology Reviews* **7**, 14-28 (1994).

102 Saiki, R. K. *et al.* Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239**, 487-491 (1988).

103 Saiki, R. K. *et al.* Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* **230**, 1350-1354 (1985).

104 Ho, D. D. *et al.* Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. *Nature* **373**, 123-126, doi:10.1038/373123a0 (1995).

105 Wei, X. *et al.* Viral dynamics in human immunodeficiency virus type 1 infection. *Nature* **373**, 117-122, doi:10.1038/373117a0 (1995).

106 Perelson, A. S., Neumann, A. U., Markowitz, M., Leonard, J. M. & Ho, D. D. HIV-1 dynamics in vivo: virion clearance rate, infected cell life-span, and viral generation time. *Science* **271**, 1582-1586 (1996).

107 Hogervorst, E. *et al.* Predictors for Non- and Slow Progression in Human Immunodeficiency Virus (HIV) Type 1 Infection: Low Viral RNA Copy Numbers in Serum and Maintenance of High HIV-1 p24-Specific but Not V3-Specific Antibody Levels. *Journal of Infectious Diseases* **171**, 811-821, doi:10.1093/infdis/171.4.811 (1995).

108 Mellors, J. W. *et al.* Prognosis in HIV-1 Infection Predicted by the Quantity of Virus in Plasma. *Science* **272**, 1167-1170 (1996).

109 Mellors, J. W. *et al.* Plasma Viral Load and CD4+ Lymphocytes as Prognostic Markers of HIV-1 Infection. *Annals of Internal Medicine* **126**, 946-954, doi:10.7326/0003-4819-126-12-199706150-00003 (1997).

110 Perelson, A. S. *et al.* Decay characteristics of HIV-1-infected compartments during combination therapy. *Nature* **387**, 188-191 (1997).

111 Dornadula, G. *et al.* Residual HIV-1 RNA in blood plasma of patients taking suppressive highly active antiretroviral therapy. *Jama* **282**, 1627-1632 (1999).

112 Palmer, S. *et al.* New real-time reverse transcriptase-initiated PCR assay with single-copy sensitivity for human immunodeficiency virus type 1 RNA in plasma. *J Clin Microbiol* **41**, 4531-4536 (2003).

113 Maldarelli, F. *et al.* ART suppresses plasma HIV-1 RNA to a stable set point predicted by pretherapy viremia. *PLoS Pathog* **3**, e46, doi:10.1371/journal.ppat.0030046 (2007).

114 Palmer, S. *et al.* Low-level viremia persists for at least 7 years in patients on suppressive antiretroviral therapy. *Proc Natl Acad Sci U S A* **105**, 3879-3884, doi:10.1073/pnas.0800050105 (2008).

115 Riddler, S. A. *et al.* Continued Slow Decay of the Residual Plasma Viremia Level in HIV-1-Infected Adults Receiving Long-term Antiretroviral Therapy. *J Infect Dis* **213**, 556-560, doi:10.1093/infdis/jiv433 (2016).

116 Davey, R. T., Jr. *et al.* HIV-1 and T cell dynamics after interruption of highly active antiretroviral therapy (HAART) in patients with a history of sustained viral suppression. *Proc Natl Acad Sci U S A* **96**, 15109-15114 (1999).

117 Hamlyn, E. *et al.* Plasma HIV viral rebound following protocol-indicated cessation of ART commenced in primary and chronic HIV infection. *PLoS One* **7**, e43754, doi:10.1371/journal.pone.0043754 (2012).

118 Quinn, T. C. *et al.* Viral Load and Heterosexual Transmission of Human Immunodeficiency Virus Type 1. *New England Journal of Medicine* **342**, 921-929, doi:10.1056/NEJM200003303421303 (2000).

119 Oxenius, A. *et al.* Stimulation of HIV-specific cellular immunity by structured treatment interruption fails to enhance viral control in chronic HIV infection. *Proc Natl Acad Sci U S A* **99**, 13747-13752, doi:10.1073/pnas.202372199 (2002).

120 Wit, F. W. *et al.* Safety of long-term interruption of successful antiretroviral therapy: the ATHENA cohort study. *Aids* **19**, 345-348 (2005).

121 El-Sadr, W. M. S. S. G. CD4+ Count–Guided Interruption of Antiretroviral Treatment. *New England Journal of Medicine* **355**, 2283-2296, doi:10.1056/NEJMoa062360 (2006).

122 Chun, T. W. *et al.* In vivo fate of HIV-1-infected T cells: quantitative analysis of the transition to stable latency. *Nat Med* **1**, 1284-1290 (1995).

123 Chun, T. W. *et al.* Quantification of latent tissue reservoirs and total body viral load in HIV-1 infection. *Nature* **387**, 183-188, doi:10.1038/387183a0 (1997).

124 Finzi, D. *et al.* Identification of a reservoir for HIV-1 in patients on highly active antiretroviral therapy. *Science* **278**, 1295-1300 (1997).

125 Wong, J. K. *et al.* Recovery of replication-competent HIV despite prolonged suppression of plasma viremia. *Science* **278**, 1291-1295 (1997).

126 Chun, T. W. *et al.* Presence of an inducible HIV-1 latent reservoir during highly active antiretroviral therapy. *Proc Natl Acad Sci U S A* **94**, 13193-13197 (1997).

127 Garcia-Blanco, M. A. & Cullen, B. R. Molecular basis of latency in pathogenic human viruses. *Science* **254**, 815-820 (1991).

128 Finzi, D. *et al.* Latent infection of CD4+ T cells provides a mechanism for lifelong persistence of HIV-1, even in patients on effective combination therapy. *Nat Med* **5**, 512-517, doi:10.1038/8394 (1999).

129 Zhang, L. *et al.* Quantifying residual HIV-1 replication in patients receiving combination antiretroviral therapy. *N Engl J Med* **340**, 1605-1613, doi:10.1056/nejm199905273402101 (1999).

130 Ramratnam, B. *et al.* The decay of the latent reservoir of replication-competent HIV-1 is inversely correlated with the extent of residual viral replication during prolonged anti-retroviral therapy. *Nat Med* **6**, 82-85, doi:10.1038/71577 (2000).

131 Siliciano, J. D. *et al.* Long-term follow-up studies confirm the stability of the latent reservoir for HIV-1 in resting CD4+ T cells. *Nat Med* **9**, 727-728, doi:10.1038/nm880 (2003).

132 Lorenzo-Redondo, R. *et al.* Persistent HIV-1 replication maintains the tissue reservoir during therapy. *Nature* **530**, 51-56, doi:10.1038/nature16933 (2016).

133 Dinoso, J. B. *et al.* Treatment intensification does not reduce residual HIV-1 viremia in patients on highly active antiretroviral therapy. *Proceedings of the National Academy of Sciences* **106**, 9403-9408, doi:10.1073/pnas.0903107106 (2009).

134 Gandhi, R. T. *et al.* The effect of raltegravir intensification on low-level residual viremia in HIV-infected patients on antiretroviral therapy: a randomized controlled trial. *PLoS Med* **7**, doi:10.1371/journal.pmed.1000321 (2010).

135 McMahon, D. *et al.* Short-course raltegravir intensification does not reduce persistent low-level viremia in patients with HIV-1 suppression during receipt of combination antiretroviral therapy. *Clin Infect Dis* **50**, 912-919, doi:10.1086/650749 (2010).

136 Besson, G. J., McMahon, D., Maldarelli, F. & Mellors, J. W. Short-course raltegravir intensification does not increase 2 long terminal repeat episomal HIV-1 DNA in patients on effective antiretroviral therapy. *Clin Infect Dis* **54**, 451-453, doi:10.1093/cid/cir721 (2012).

137 Gandhi, R. T. *et al.* No effect of raltegravir intensification on viral replication markers in the blood of HIV-1-infected patients receiving antiretroviral therapy. *J Acquir Immune Defic Syndr* **59**, 229-235, doi:10.1097/QAI.0b013e31823fd1f2 (2012).

138 Nottet, H. S. *et al.* HIV-1 can persist in aged memory CD4+ T lymphocytes with minimal signs of evolution after 8.3 years of effective highly active antiretroviral therapy. *J Acquir Immune Defic Syndr* **50**, 345-353, doi:10.1097/QAI.0b013e318197eb04 (2009).

139 Josefsson, L. *et al.* The HIV-1 reservoir in eight patients on long-term suppressive antiretroviral therapy is stable with few genetic changes over time. *Proc Natl Acad Sci U S A* **110**, E4987-4996, doi:10.1073/pnas.1308313110 (2013).

140 Kearney, M. F. *et al.* Lack of detectable HIV-1 molecular evolution during suppressive antiretroviral therapy. *PLoS Pathog* **10**, e1004010, doi:10.1371/journal.ppat.1004010 (2014).

141 Buzon, M. J. *et al.* HIV-1 replication and immune dynamics are affected by raltegravir intensification of HAART-suppressed subjects. *Nat Med* **16**, 460-465, doi:10.1038/nm.2111 (2010).

142 Hatano, H. *et al.* Increase in 2-long terminal repeat circles and decrease in D-dimer after raltegravir intensification in patients with treated HIV infection: a randomized, placebo-controlled trial. *J Infect Dis* **208**, 1436-1442, doi:10.1093/infdis/jit453 (2013).

143 Bradley, H. *et al.* Vital Signs: HIV diagnosis, care, and treatment among persons living with HIV--United States, 2011. *MMWR Morb Mortal Wkly Rep* **63**, 1113-1117 (2014).

144 Michie, C. A., McLean, A., Alcock, C. & Beverley, P. C. Lifespan of human lymphocyte subsets defined by CD45 isoforms. *Nature* **360**, 264-265, doi:10.1038/360264a0 (1992).

145 Tough, D. F. & Sprent, J. Turnover of naive- and memory-phenotype T cells. *J Exp Med* **179**, 1127-1135 (1994).

146 Mohri, H., Bonhoeffer, S., Monard, S., Perelson, A. S. & Ho, D. D. Rapid turnover of T lymphocytes in SIV-infected rhesus macaques. *Science* **279**, 1223-1227 (1998).

147 Takaki, A. *et al.* Cellular immune responses persist and humoral responses decrease two decades after recovery from a single-source outbreak of hepatitis C. *Nat Med* **6**, 578-582, doi:10.1038/75063 (2000).

148 Jing, L. *et al.* CD4 T-Cell Memory Responses to Viral Infections of Humans Show Pronounced Immunodominance Independent of Duration or Viral Persistence. *Journal of Virology* **87**, 2617-2627, doi:10.1128/JVI.03047-12 (2013).

149 Maldarelli, F. *et al.* HIV latency. Specific HIV integration sites are linked to clonal expansion and persistence of infected cells. *Science* **345**, 179-183, doi:10.1126/science.1254194 (2014).

150 Wagner, T. A. *et al.* HIV latency. Proliferation of cells with HIV integrated into cancer genes contributes to persistent infection. *Science* **345**, 570-573, doi:10.1126/science.1256304 (2014).

151 Ho, Y. C. *et al.* Replication-competent noninduced proviruses in the latent reservoir increase barrier to HIV-1 cure. *Cell* **155**, 540-551, doi:10.1016/j.cell.2013.09.020 (2013).

152 Cohn, L. B. *et al.* HIV-1 integration landscape during latent and active infection. *Cell* **160**, 420-432, doi:10.1016/j.cell.2015.01.020 (2015).

153 Simonetti, F. R. *et al.* Clonally expanded CD4+ T cells can produce infectious HIV-1 in vivo. *Proc Natl Acad Sci U S A* **113**, 1883-1888, doi:10.1073/pnas.1522675113 (2016).

154 Boshoff, C. & Weiss, R. Aids-related malignancies. *Nat Rev Cancer* **2**, 373-382, doi:http://www.nature.com/nrc/journal/v2/n5/suppinfo/nrc797_S1.html (2002).

155 Goedert, J. J. The epidemiology of acquired immunodeficiency syndrome malignancies. *Seminars in oncology* **27**, 390-401 (2000).

156 Besson, C. *et al.* Changes in AIDS-related lymphoma since the era of highly active antiretroviral therapy. *Blood* **98**, 2339-2344 (2001).

157 Engels, E. A. *et al.* Trends in cancer risk among people with AIDS in the United States 1980-2002. *Aids* **20**, 1645-1654, doi:10.1097/01.aids.0000238411.75324.59 (2006).

158 Bonnet, F. *et al.* Changes in cancer mortality among HIV-infected patients: the Mortalite 2005 Survey. *Clin Infect Dis* **48**, 633-639 (2009).

159 Simard, E. P. & Engels, E. A. Cancer as a cause of death among people with AIDS in the United States. *Clin Infect Dis* **51**, 957-962, doi:10.1086/656416 (2010).

160 Shiels, M. S. *et al.* Cancer burden in the HIV-infected population in the United States. *J Natl Cancer Inst* **103**, 753-762, doi:10.1093/jnci/djr076 (2011).

161 Simard, E. P., Pfeiffer, R. M. & Engels, E. A. Cumulative incidence of cancer among individuals with acquired immunodeficiency syndrome in the United States. *Cancer* **117**, 1089-1096, doi:10.1002/cncr.25547 (2011).

162 Gopal, S. *et al.* Meeting the challenge of hematologic malignancies in sub-Saharan Africa. *Blood* **119**, 5078-5087, doi:10.1182/blood-2012-02-387092 (2012).

163 Hutter, G. & Zaia, J. A. Allogeneic haematopoietic stem cell transplantation in patients with human immunodeficiency virus: the experiences of more than 25 years. *Clin Exp Immunol* **163**, 284-295, doi:10.1111/j.1365-2249.2010.04312.x (2011).

164 Davis, K. C., Hayward, A., Ozturk, G. & Kohler, P. F. Lymphocyte transfusion in case of acquired immunodeficiency syndrome. *Lancet* **1**, 599-600 (1983).

165 Hassett, J. M., Zaroulis, C. G., Greenberg, M. L. & Siegal, F. P. Bone marrow transplantation in AIDS. *N Engl J Med* **309**, 665, doi:10.1056/nejm198309153091114 (1983).

166 Thomas, E. *et al.* Bone-marrow transplantation (first of two parts). *N Engl J Med* **292**, 832-843, doi:10.1056/nejm197504172921605 (1975).

167 Lane, H. C. *et al.* Partial immune reconstitution in a patient with the acquired immunodeficiency syndrome. *N Engl J Med* **311**, 1099-1103, doi:10.1056/nejm198410253111706 (1984).

168 Holland, H. K. *et al.* Allogeneic Bone Marrow Transplantation, Zidovudine, and Human Immunodeficiency Virus Type 1 (HIV-I) InfectionStudies in a Patient with Non-Hodgkin Lymphoma. *Annals of Internal Medicine* **111**, 973-981, doi:10.7326/0003-4819-111-12-973 (1989).

169 Alkhatib, G. *et al.* CC CKR5: A RANTES, MIP-1alpha, MIP-1beta Receptor as a Fusion Cofactor for Macrophage-Tropic HIV-1. *Science* **272**, 1955-1958 (1996).

170 Choe, H. *et al.* The beta-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. *Cell* **85**, 1135-1148 (1996).

171 Deng, H. *et al.* Identification of a major co-receptor for primary isolates of HIV-1. *Nature* **381**, 661-666, doi:10.1038/381661a0 (1996).

172 Doranz, B. J. *et al.* A dual-tropic primary HIV-1 isolate that uses fusin and the beta-chemokine receptors CKR-5, CKR-3, and CKR-2b as fusion cofactors. *Cell* **85**, 1149-1158 (1996).

173 Dragic, T. *et al.* HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5. *Nature* **381**, 667-673, doi:10.1038/381667a0 (1996).

174 Martinson, J. J., Chapman, N. H., Rees, D. C., Liu, Y. T. & Clegg, J. B. Global distribution of the CCR5 gene 32-basepair deletion. *Nature genetics* **16**, 100-103, doi:10.1038/ng0597-100 (1997).

175 Liu, R. *et al.* Homozygous Defect in HIV-1 Coreceptor Accounts for Resistance of Some Multiply-Exposed Individuals to HIV-1 Infection. *Cell* **86**, 367-377, doi:10.1016/S0092-8674(00)80110-5 (1996).

176 Samson, M. *et al.* Resistance to HIV-1 infection in caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene. *Nature* **382**, 722-725, doi:10.1038/382722a0 (1996).

177 Lane, H. C. *et al.* Syngeneic Bone Marrow Transplantation and Adoptive Transfer of Peripheral Blood Lymphocytes Combined with Zidovudine in Human Immunodeficiency Virus (HIV) Infection. *Annals of Internal Medicine* **113**, 512-519, doi:10.7326/0003-4819-113-7-512 (1990).

178 Contu, L. *et al.* Allogeneic bone marrow transplantation combined with multiple anti-HIV-1 treatment in a case of AIDS. *Bone marrow transplantation* **12**, 669-671 (1993).

179 Michaels, M. G. *et al.* Baboon bone-marrow xenotransplant in a patient with advanced HIV disease: case report and 8-year follow-up. *Transplantation* **78**, 1582-1589 (2004).

180 Pennisi, E. FDA panel OKs baboon marrow transplant. *Science* **269**, 293-294 (1995).

181 Press, T. A. in *New York Times* (Joshua Tree, California, 2006).

182 Boneva, R. S., Folks, T. M. & Chapman, L. E. Infectious Disease Issues in Xenotransplantation. *Clinical Microbiology Reviews* **14**, 1-14, doi:10.1128/CMR.14.1.1-14.2001 (2001).

183 Bach, F. H. *et al.* Uncertainty in xenotransplantation: individual benefit versus collective risk. *Nat Med* **4**, 141-144 (1998).

184 Wadman, M. FDA turns down moratorium demand on xenotransplants. *Nature* **391**, 423-423 (1998).

185 Kaplan, L. D. *et al.* Low-Dose Compared with Standard-Dose m-BACOD Chemotherapy for Non-Hodgkin's Lymphoma Associated with Human Immunodeficiency Virus Infection. *New England Journal of Medicine* **336**, 1641-1648, doi:10.1056/NEJM199706053362304 (1997).

186 Molina, A. *et al.* High dose therapy and autologous stem cell transplantation for human immunodeficiency virus-associated non-Hodgkin lymphoma in the era of highly active antiretroviral therapy. *Cancer* **89**, 680-689 (2000).

187 Ratner, L. *et al.* Chemotherapy for human immunodeficiency virus-associated non-Hodgkin's lymphoma in combination with highly active antiretroviral therapy. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **19**, 2171-2178 (2001).

188 Resino, S. *et al.* Short communication: Immune reconstitution after autologous peripheral blood stem cell transplantation in HIV-infected patients: might be better than expected? *AIDS Res Hum Retroviruses* **23**, 543-548, doi:10.1089/aid.2006.0071 (2007).

189 Simonelli, C. *et al.* Immune Recovery after Autologous Stem Cell Transplantation Is Not Different for HIV-Infected versus HIV-Uninfected Patients with Relapsed or Refractory Lymphoma. *Clinical Infectious Diseases* **50**, 1672-1679, doi:10.1086/652866 (2010).

190 Ambinder, R. F. The same but different: autologous hematopoietic stem cell transplantation for patients with lymphoma and HIV infection. *Bone marrow transplantation* **44**, 1-5, doi:10.1038/bmt.2009.105 (2009).

191 Krishnan, A. *et al.* Autologous stem cell transplantation for HIV-associated lymphoma. *Blood* **98**, 3857-3859 (2001).

192 Krishnan, A. *et al.* Durable remissions with autologous stem cell transplantation for high-risk HIV-associated lymphomas. *Blood* **105**, 874-878, doi:10.1182/blood-2004-04-1532 (2005).

193 Krishnan, A. *et al.* HIV status does not affect the outcome of autologous stem cell transplantation (ASCT) for non-Hodgkin lymphoma (NHL). *Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation* **16**, 1302-1308, doi:10.1016/j.bbmt.2010.03.019 (2010).

194 Michieli, M., Mazzucato, M., Tirelli, U. & De Paoli, P. Stem cell transplantation for lymphoma patients with HIV infection. *Cell transplantation* **20**, 351-370, doi:10.3727/096368910x528076 (2011).

195 Re, A. *et al.* High-Dose Therapy and Autologous Peripheral-Blood Stem-Cell Transplantation As Salvage Treatment for HIV-Associated Lymphoma in Patients Receiving Highly Active Antiretroviral Therapy. *Journal of Clinical Oncology* **21**, 4423-4427, doi:10.1200/jco.2003.06.039 (2003).

196 Cillo, A. R. *et al.* Plasma viremia and cellular HIV-1 DNA persist despite autologous hematopoietic stem cell transplantation for HIV-related lymphoma. *J Acquir Immune Defic Syndr* **63**, 438-441, doi:10.1097/QAI.0b013e31828e6163 (2013).

197 Cillo, A. R. *et al.* Impact of chemotherapy for HIV-1 related lymphoma on residual viremia and cellular HIV-1 DNA in patients on suppressive antiretroviral therapy. *PLoS One* **9**, e92118, doi:10.1371/journal.pone.0092118 (2014).

198 Hutter, G. *et al.* Long-term control of HIV by CCR5 Delta32/Delta32 stem-cell transplantation. *N Engl J Med* **360**, 692-698, doi:10.1056/NEJMoa0802905 (2009).

199 Avettand-Fenoel, V. *et al.* Failure of bone marrow transplantation to eradicate HIV reservoir despite efficient HAART. *Aids* **21**, 776-777, doi:10.1097/QAD.0b013e3280b01836 (2007).

200 Woolfrey, A. E. *et al.* Generation of HIV-1-specific CD8+ cell responses following allogeneic hematopoietic cell transplantation. *Blood* **112**, 3484-3487, doi:10.1182/blood-2008-05-157511 (2008).

201 Bleul, C. C. *et al.* The lymphocyte chemoattractant SDF-1 is a ligand for LESTR/fusin and blocks HIV-1 entry. *Nature* **382**, 829-833, doi:10.1038/382829a0 (1996).

202 Feng, Y., Broder, C. C., Kennedy, P. E. & Berger, E. A. HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. *Science* **272**, 872-877 (1996).

203 Oberlin, E. *et al.* The CXC chemokine SDF-1 is the ligand for LESTR/fusin and prevents infection by T-cell-line-adapted HIV-1. *Nature* **382**, 833-835, doi:10.1038/382833a0 (1996).

204 Dean, M. *et al.* Genetic restriction of HIV-1 infection and progression to AIDS by a deletion allele of the CKR5 structural gene. Hemophilia Growth and Development Study, Multicenter AIDS Cohort Study, Multicenter Hemophilia Cohort Study, San Francisco City Cohort, ALIVE Study. *Science* **273**, 1856-1862 (1996).

205 Marmor, M. *et al.* Homozygous and heterozygous CCR5-Delta32 genotypes are associated with resistance to HIV infection. *J Acquir Immune Defic Syndr* **27**, 472-481 (2001).

206 Benkirane, M., Jin, D. Y., Chun, R. F., Koup, R. A. & Jeang, K. T. Mechanism of transdominant inhibition of CCR5-mediated HIV-1 infection by ccr5delta32. *J Biol Chem* **272**, 30603-30606 (1997).

207 Bacigalupo, A. *et al.* Thymoglobulin prevents chronic graft-versus-host disease, chronic lung dysfunction, and late transplant-related mortality: long-term follow-up of a randomized trial in patients undergoing unrelated donor transplantation. *Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation* **12**, 560-565, doi:10.1016/j.bbmt.2005.12.034 (2006).

208 Allers, K. *et al.* Evidence for the cure of HIV infection by CCR5Delta32/Delta32 stem cell transplantation. *Blood* **117**, 2791-2799, doi:10.1182/blood-2010-09-309591 (2011).

209 Symons, J. *et al.* Dependence on the CCR5 coreceptor for viral replication explains the lack of rebound of CXCR4-predicted HIV variants in the Berlin patient. *Clin Infect Dis* **59**, 596-600, doi:10.1093/cid/ciu284 (2014).

210 Parker, R. & Sereti, I. The power of 1 in HIV therapeutics. *Blood* **117**, 2746-2747, doi:10.1182/blood-2011-01-324921 (2011).

211 Center for International Blood and Marrow Transplant, a contractor for the C.W. Bill Young Cell Transplantation Program operated through the U.S. Department of Health and Human Services, Health Resources and Services Administration, Healthcare Systems Bureau. U.S. Patient Survival Report, AML- Acute myeloid leukemia- 1st remission, from 2008 through 2012. < http://bloodcell.transplant.hrsa.gov/RESEARCH/Transplant_Data/US_Tx_Data/Survival_Data/survival.aspx>. Accessed April 13, 2016.

212 Center for International Blood and Marrow Transplant, a contractor for the C.W. Bill Young Cell Transplantation Program operated through the U.S. Department of Health and Human Services, Health Resources and Services Administration, Healthcare Systems Bureau. U.S. Patient Survival Report, CML- Chronic myeloid leukemia- 2nd or subsequent chronic or accelerated phase from 2008 through 2012. <http://bloodcell.transplant.hrsa.gov/RESEARCH/Transplant_Data/US_Tx_Data/Survival_Data/survival.aspx>. Accessed April 13, 2016.

213 Henrich, T. J. *et al.* Long-Term Reduction in Peripheral Blood HIV Type 1 Reservoirs Following Reduced-Intensity Conditioning Allogeneic Stem Cell Transplantation. *The Journal of Infectious Diseases* **207**, 1694-1702, doi:10.1093/infdis/jit086 (2013).

214 Henrich, T. J. *et al.* Antiretroviral-free HIV-1 remission and viral rebound after allogeneic stem cell transplantation: report of 2 cases. *Ann Intern Med* **161**, 319-327, doi:10.7326/m14-1027 (2014).

215 Hill, A. L., Rosenbloom, D. I., Fu, F., Nowak, M. A. & Siliciano, R. F. Predicting the outcomes of treatment to eradicate the latent reservoir for HIV-1. *Proc Natl Acad Sci U S A* **111**, 13475-13480, doi:10.1073/pnas.1406663111 (2014).

216 Hutter, G. & Thiel, E. Allogeneic transplantation of CCR5-deficient progenitor cells in a patient with HIV infection: an update after 3 years and the search for patient no. 2. *Aids* **25**, 273-274, doi:10.1097/QAD.0b013e328340fe28 (2011).

217 Hutter, G. More on shift of HIV tropism in stem-cell transplantation with CCR5 delta32/delta32 mutation. *N Engl J Med* **371**, 2437-2438, doi:10.1056/NEJMc1412279#SA1 (2014).

218 Kordelas, L. *et al.* Shift of HIV tropism in stem-cell transplantation with CCR5 Delta32 mutation. *N Engl J Med* **371**, 880-882, doi:10.1056/NEJMc1405805 (2014).

219 Petz, L. *et al.* Hematopoietic Cell Transplantation with Cord Blood for Cure of HIV Infections. *Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation* **19**, 393-397, doi:10.1016/j.bbmt.2012.10.017 (2013).

220 Chun, T. W., Engel, D., Mizell, S. B., Ehler, L. A. & Fauci, A. S. Induction of HIV-1 replication in latently infected CD4+ T cells using a combination of cytokines. *J Exp Med* **188**, 83-91 (1998).

221 Cohen, J. Exploring how to get at--and eradicate--hidden HIV. *Science* **279**, 1854-1855 (1998).

222 Carr, A. *et al.* Outpatient continuous intravenous interleukin-2 or subcutaneous, polyethylene glycol-modified interleukin-2 in human immunodeficiency virus-infected patients: a randomized, controlled, multicenter study. Australian IL-2 Study Group. *J Infect Dis* **178**, 992-999 (1998).

223 Davey, R. T., Jr. *et al.* Subcutaneous administration of interleukin-2 in human immunodeficiency virus type 1-infected persons. *J Infect Dis* **175**, 781-789 (1997).

224 Jacobson, E. L., Pilaro, F. & Smith, K. A. Rational interleukin 2 therapy for HIV positive individuals: daily low doses enhance immune function without toxicity. *Proc Natl Acad Sci U S A* **93**, 10405-10410 (1996).

225 Kelleher, A. D. *et al.* Effects of IL-2 therapy in asymptomatic HIV-infected individuals on proliferative responses to mitogens, recall antigens and HIV-related antigens. *Clin Exp Immunol* **113**, 85-91 (1998).

226 Kovacs, J. A. *et al.* Increases in CD4 T lymphocytes with intermittent courses of interleukin-2 in patients with human immunodeficiency virus infection. A preliminary study. *N Engl J Med* **332**, 567-575, doi:10.1056/nejm199503023320904 (1995).

227 Kovacs, J. A. *et al.* Controlled trial of interleukin-2 infusions in patients infected with the human immunodeficiency virus. *N Engl J Med* **335**, 1350-1356, doi:10.1056/nejm199610313351803 (1996).

228 Simonelli, C. *et al.* Interleukin-2 in combination with zidovudine and didanosine is able to maintain high levels of CD4 cells and undetectable HIV viraemia. *Aids* **12**, 112-113 (1998).

229 Witzke, O. *et al.* Comparison between subcutaneous and intravenous interleukin-2 treatment in HIV disease. *Journal of internal medicine* **244**, 235-240 (1998).

230 Emery, S. *et al.* Pooled analysis of 3 randomized, controlled trials of interleukin-2 therapy in adult human immunodeficiency virus type 1 disease. *J Infect Dis* **182**, 428-434, doi:10.1086/315736 (2000).

231 Committee, I.-E. S. G. a. S. S. Interleukin-2 Therapy in Patients with HIV Infection. *New England Journal of Medicine* **361**, 1548-1559, doi:10.1056/NEJMoa0903175 (2009).

232 Prins, J. M. *et al.* Immuno-activation with anti-CD3 and recombinant human IL-2 in HIV-1-infected patients on potent antiretroviral therapy. *Aids* **13**, 2405-2410 (1999).

233 Chun, T. W., Davey, R. T., Jr., Engel, D., Lane, H. C. & Fauci, A. S. Re-emergence of HIV after stopping therapy. *Nature* **401**, 874-875, doi:10.1038/44755 (1999).

234 Lehrman, G. *et al.* Depletion of latent HIV-1 infection in vivo: a proof-of-concept study. *Lancet* **366**, 549-555, doi:10.1016/S0140-6736(05)67098-5 (2005).

235 Göttlicher, M. *et al.* Valproic acid defines a novel class of HDAC inhibitors inducing differentiation of transformed cells. *The EMBO Journal* **20**, 6969-6978, doi:10.1093/emboj/20.24.6969 (2001).

236 Phiel, C. J. *et al.* Histone deacetylase is a direct target of valproic acid, a potent anticonvulsant, mood stabilizer, and teratogen. *J Biol Chem* **276**, 36734-36741, doi:10.1074/jbc.M101287200 (2001).

237 Siliciano, J. D. *et al.* Stability of the Latent Reservoir for HIV-1 in Patients Receiving Valproic Acid. *Journal of Infectious Diseases* **195**, 833-836, doi:10.1086/511823 (2007).

238 Archin, N. M. *et al.* Valproic acid without intensified antiviral therapy has limited impact on persistent HIV infection of resting CD4+ T cells. *AIDS (London, England)* **22**, 10.1097/QAD.1090b1013e3282fd1096df1094, doi:10.1097/QAD.0b013e3282fd6df4 (2008).

239 Sagot-Lerolle, N. *et al.* Prolonged valproic acid treatment does not reduce the size of latent HIV reservoir. *Aids* **22**, 1125-1129, doi:10.1097/QAD.0b013e3282fd6ddc (2008).

240 Routy, J. P. *et al.* Valproic acid in association with highly active antiretroviral therapy for reducing systemic HIV-1 reservoirs: results from a multicentre randomized clinical study. *HIV Med* **13**, 291-296, doi:10.1111/j.1468-1293.2011.00975.x (2012).

241 Margolis, D. M., Somasundaran, M. & Green, M. R. Human transcription factor YY1 represses human immunodeficiency virus type 1 transcription and virion production. *J Virol* **68**, 905-910 (1994).

242 Romerio, F., Gabriel, M. N. & Margolis, D. M. Repression of human immunodeficiency virus type 1 through the novel cooperation of human factors YY1 and LSF. *J Virol* **71**, 9375-9382 (1997).

243 Coull, J. J. *et al.* The human factors YY1 and LSF repress the human immunodeficiency virus type 1 long terminal repeat via recruitment of histone deacetylase 1. *J Virol* **74**, 6790-6799 (2000).

244 He, G. & Margolis, D. M. Counterregulation of chromatin deacetylation and histone deacetylase occupancy at the integrated promoter of human immunodeficiency virus type 1 (HIV-1) by the HIV-1 repressor YY1 and HIV-1 activator Tat. *Mol Cell Biol* **22**, 2965-2973 (2002).

245 Williams, S. A. *et al.* NF-kappaB p50 promotes HIV latency through HDAC recruitment and repression of transcriptional initiation. *EMBO J* **25**, 139-149, doi:10.1038/sj.emboj.7600900 (2006).

246 Jiang, G., Espeseth, A., Hazuda, D. J. & Margolis, D. M. c-Myc and Sp1 contribute to proviral latency by recruiting histone deacetylase 1 to the human immunodeficiency virus type 1 promoter. *J Virol* **81**, 10914-10923, doi:10.1128/jvi.01208-07 (2007).

247 Tyagi, M. & Karn, J. CBF-1 promotes transcriptional silencing during the establishment of HIV-1 latency. *The EMBO Journal* **26**, 4985-4995, doi:10.1038/sj.emboj.7601928 (2007).

248 Ylisastigui, L., Archin, N. M., Lehrman, G., Bosch, R. J. & Margolis, D. M. Coaxing HIV-1 from resting CD4 T cells: histone deacetylase inhibition allows latent viral expression. *Aids* **18**, 1101-1108 (2004).

249 Archin, N. M. *et al.* Expression of latent HIV induced by the potent HDAC inhibitor suberoylanilide hydroxamic acid. *AIDS Res Hum Retroviruses* **25**, 207-212, doi:10.1089/aid.2008.0191 (2009).

250 Archin, N. M. *et al.* Expression of latent human immunodeficiency type 1 is induced by novel and selective histone deacetylase inhibitors. *Aids* **23**, 1799-1806, doi:10.1097/QAD.0b013e32832ec1dc (2009).

251 Archin, N. M. *et al.* Administration of vorinostat disrupts HIV-1 latency in patients on antiretroviral therapy. *Nature* **487**, 482-485, doi:10.1038/nature11286 (2012).

252 Archin, N. M. *et al.* HIV-1 expression within resting CD4+ T cells after multiple doses of vorinostat. *J Infect Dis* **210**, 728-735, doi:10.1093/infdis/jiu155 (2014).

253 Elliott, J. H. *et al.* Activation of HIV transcription with short-course vorinostat in HIV-infected patients on suppressive antiretroviral therapy. *PLoS Pathog* **10**, e1004473, doi:10.1371/journal.ppat.1004473 (2014).

254 Doyon, G., Zerbato, J., Mellors, J. W. & Sluis-Cremer, N. Disulfiram reactivates latent HIV-1 expression through depletion of the phosphatase and tensin homolog. *Aids* **27**, F7-f11, doi:10.1097/QAD.0b013e3283570620 (2013).

255 Spivak, A. M. *et al.* A pilot study assessing the safety and latency-reversing activity of disulfiram in HIV-1-infected adults on antiretroviral therapy. *Clin Infect Dis* **58**, 883-890, doi:10.1093/cid/cit813 (2014).

256 Elliott, J. H. *et al.* Short-term administration of disulfiram for reversal of latent HIV infection: a phase 2 dose-escalation study. *The Lancet HIV* **2**, e520-e529, doi:10.1016/S2352-3018(15)00226-X (2015).

257 Rasmussen, T. A. *et al.* Panobinostat, a histone deacetylase inhibitor, for latent-virus reactivation in HIV-infected patients on suppressive antiretroviral therapy: a phase 1/2, single group, clinical trial. *Lancet HIV* **1**, e13-21, doi:10.1016/S2352-3018(14)70014-1 (2014).

258 Spina, C. A. *et al.* An in-depth comparison of latent HIV-1 reactivation in multiple cell model systems and resting CD4+ T cells from aviremic patients. *PLoS Pathog* **9**, e1003834, doi:10.1371/journal.ppat.1003834 (2013).

259 Richman, D. D. *et al.* The challenge of finding a cure for HIV infection. *Science* **323**, 1304-1307, doi:10.1126/science.1165706 (2009).

260 Siliciano, R. F. & Greene, W. C. HIV Latency. *Cold Spring Harbor Perspectives in Medicine:* **1**, a007096, doi:10.1101/cshperspect.a007096 (2011).

261 Van Lint, C., Bouchat, S. & Marcello, A. HIV-1 transcription and latency: an update. *Retrovirology* **10**, 67, doi:10.1186/1742-4690-10-67 (2013).

262 Kumar, A., Darcis, G., Van Lint, C. & Herbein, G. Epigenetic control of HIV-1 post integration latency: implications for therapy. *Clinical Epigenetics* **7**, 1-12, doi:10.1186/s13148-015-0137-6 (2015).

263 Bernhard, W. *et al.* The Suv39H1 methyltransferase inhibitor chaetocin causes induction of integrated HIV-1 without producing a T cell response. *FEBS letters* **585**, 3549-3554, doi:10.1016/j.febslet.2011.10.018 (2011).

264 du Chene, I. *et al.* Suv39H1 and HP1gamma are responsible for chromatin-mediated HIV-1 transcriptional silencing and post-integration latency. *Embo j* **26**, 424-435, doi:10.1038/sj.emboj.7601517 (2007).

265 Friedman, J. *et al.* Epigenetic silencing of HIV-1 by the histone H3 lysine 27 methyltransferase enhancer of Zeste 2. *J Virol* **85**, 9078-9089, doi:10.1128/jvi.00836-11 (2011).

266 Imai, K., Togami, H. & Okamoto, T. Involvement of histone H3 lysine 9 (H3K9) methyltransferase G9a in the maintenance of HIV-1 latency and its reactivation by BIX01294. *J Biol Chem* **285**, 16538-16545, doi:10.1074/jbc.M110.103531 (2010).

267 Marban, C. *et al.* Recruitment of chromatin-modifying enzymes by CTIP2 promotes HIV-1 transcriptional silencing. *Embo j* **26**, 412-423, doi:10.1038/sj.emboj.7601516 (2007).

268 Pearson, R. *et al.* Epigenetic silencing of human immunodeficiency virus (HIV) transcription by formation of restrictive chromatin structures at the viral long terminal repeat drives the progressive entry of HIV into latency. *J Virol* **82**, 12291-12303, doi:10.1128/jvi.01383-08 (2008).

269 Bouchat, S. *et al.* Histone methyltransferase inhibitors induce HIV-1 recovery in resting CD4(+) T cells from HIV-1-infected HAART-treated patients. *Aids* **26**, 1473-1482, doi:10.1097/QAD.0b013e32835535f5 (2012).

270 Greiner, D., Bonaldi, T., Eskeland, R., Roemer, E. & Imhof, A. Identification of a specific inhibitor of the histone methyltransferase SU(VAR)3-9. *Nature chemical biology* **1**, 143-145, doi:10.1038/nchembio721 (2005).

271 Chang, Y. *et al.* Structural basis for G9a-like protein lysine methyltransferase inhibition by BIX-01294. *Nat Struct Mol Biol* **16**, 312-317, doi:10.1038/nsmb.1560 (2009).

272 Kubicek, S. *et al.* Reversal of H3K9me2 by a small-molecule inhibitor for the G9a histone methyltransferase. *Molecular cell* **25**, 473-481, doi:10.1016/j.molcel.2007.01.017 (2007).

273 Jiang, G. *et al.* Synergistic Reactivation of Latent HIV Expression by Ingenol-3-Angelate, PEP005, Targeted NF-kB Signaling in Combination with JQ1 Induced p-TEFb Activation. *PLoS Pathog* **11**, e1005066, doi:10.1371/journal.ppat.1005066 (2015).

274 Tripathy, M. K., McManamy, M. E., Burch, B. D., Archin, N. M. & Margolis, D. M. H3K27 Demethylation at the Proviral Promoter Sensitizes Latent HIV to the Effects of Vorinostat in Ex Vivo Cultures of Resting CD4+ T Cells. *J Virol* **89**, 8392-8405, doi:10.1128/jvi.00572-15 (2015).

275 Kauder, S. E., Bosque, A., Lindqvist, A., Planelles, V. & Verdin, E. Epigenetic regulation of HIV-1 latency by cytosine methylation. *PLoS Pathog* **5**, e1000495, doi:10.1371/journal.ppat.1000495 (2009).

276 Blazkova, J. *et al.* CpG Methylation Controls Reactivation of HIV from Latency. *PLoS Pathog* **5**, e1000554, doi:10.1371/journal.ppat.1000554 (2009).

277 Bouchat, S. *et al.* Sequential treatment with 5-aza-2'-deoxycytidine and deacetylase inhibitors reactivates HIV-1. *EMBO molecular medicine* **8**, 117-138, doi:10.15252/emmm.201505557 (2015).

278 Ross, E. K., Buckler-White, A. J., Rabson, A. B., Englund, G. & Martin, M. A. Contribution of NF-kappa B and Sp1 binding motifs to the replicative capacity of human immunodeficiency virus type 1: distinct patterns of viral growth are determined by T-cell types. *J Virol* **65**, 4350-4358 (1991).

279 Suñé, C. & García-Blanco, M. A. Sp1 transcription factor is required for in vitro basal and Tat-activated transcription from the human immunodeficiency virus type 1 long terminal repeat. *Journal of Virology* **69**, 6572-6576 (1995).

280 Perkins, N. D. *et al.* A cooperative interaction between NF-kappa B and Sp1 is required for HIV-1 enhancer activation. *Embo j* **12**, 3551-3558 (1993).

281 Gerritsen, M. E. *et al.* CREB-binding protein/p300 are transcriptional coactivators of p65. *Proc Natl Acad Sci U S A* **94**, 2927-2932 (1997).

282 Price, D. H. P-TEFb, a cyclin-dependent kinase controlling elongation by RNA polymerase II. *Mol Cell Biol* **20**, 2629-2634 (2000).

283 Wei, P., Garber, M. E., Fang, S. M., Fischer, W. H. & Jones, K. A. A novel CDK9-associated C-type cyclin interacts directly with HIV-1 Tat and mediates its high-affinity, loop-specific binding to TAR RNA. *Cell* **92**, 451-462 (1998).

284 Mancebo, H. S. *et al.* P-TEFb kinase is required for HIV Tat transcriptional activation in vivo and in vitro. *Genes Dev* **11**, 2633-2644 (1997).

285 Yang, Z., Zhu, Q., Luo, K. & Zhou, Q. The 7SK small nuclear RNA inhibits the CDK9/cyclin T1 kinase to control transcription. *Nature* **414**, 317-322, doi:10.1038/35104575 (2001).

286 Bisgrove, D. A., Mahmoudi, T., Henklein, P. & Verdin, E. Conserved P-TEFb-interacting domain of BRD4 inhibits HIV transcription. *Proc Natl Acad Sci U S A* **104**, 13690-13695, doi:10.1073/pnas.0705053104 (2007).

287 Yang, Z. *et al.* Recruitment of P-TEFb for stimulation of transcriptional elongation by the bromodomain protein Brd4. *Molecular cell* **19**, 535-545, doi:10.1016/j.molcel.2005.06.029 (2005).

288 Filippakopoulos, P. *et al.* Selective inhibition of BET bromodomains. *Nature* **468**, 1067-1073, doi:10.1038/nature09504 (2010).

289 Zhu, J. *et al.* Reactivation of latent HIV-1 by inhibition of BRD4. *Cell Rep* **2**, 807-816, doi:10.1016/j.celrep.2012.09.008 (2012).

290 Odore, E. *et al.* Phase I Population Pharmacokinetic Assessment of the Oral Bromodomain Inhibitor OTX015 in Patients with Haematologic Malignancies. *Clinical pharmacokinetics* **55**, 397-405, doi:10.1007/s40262-015-0327-6 (2016).

291 Williams, S. A. *et al.* Prostratin antagonizes HIV latency by activating NF-kappaB. *J Biol Chem* **279**, 42008-42017, doi:10.1074/jbc.M402124200 (2004).

292 Prendiville, J. *et al.* A phase I study of intravenous bryostatin 1 in patients with advanced cancer. *British journal of cancer* **68**, 418-424 (1993).

293 Zonder, J. A. *et al.* A phase II trial of bryostatin 1 in the treatment of metastatic colorectal cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research* **7**, 38-42 (2001).

294 Jiang, G. *et al.* Reactivation of HIV latency by a newly modified Ingenol derivative via protein kinase Cdelta-NF-kappaB signaling. *Aids* **28**, 1555-1566, doi:10.1097/qad.0000000000000289 (2014).

295 Bullen, C. K., Laird, G. M., Durand, C. M., Siliciano, J. D. & Siliciano, R. F. New ex vivo approaches distinguish effective and ineffective single agents for reversing HIV-1 latency in vivo. *Nat Med* **20**, 425-429, doi:10.1038/nm.3489 (2014).

296 Laird, G. M. *et al.* Ex vivo analysis identifies effective HIV-1 latency-reversing drug combinations. *J Clin Invest* **125**, 1901-1912, doi:10.1172/JCI80142 (2015).

297 Darcis, G. *et al.* An In-Depth Comparison of Latency-Reversing Agent Combinations in Various In Vitro and Ex Vivo HIV-1 Latency Models Identified Bryostatin-1+JQ1 and Ingenol-B+JQ1 to Potently Reactivate Viral Gene Expression. *PLoS Pathog* **11**, e1005063, doi:10.1371/journal.ppat.1005063 (2015).

298 Valge, V. E., Wong, J. G., Datlof, B. M., Sinskey, A. J. & Rao, A. Protein kinase C is required for responses to T cell receptor ligands but not to interleukin-2 in T cells. *Cell* **55**, 101-112 (1988).

299 Stem Cell Technologies. <http://www.stemcell.com/en/Products/All-Products/EasySep-Human-T-Cell-Enrichment-Kit.aspx>. (2015).

300 Cillo, A. R. *et al.* Quantification of HIV-1 latency reversal in resting CD4+ T cells from patients on suppressive antiretroviral therapy. *Proc Natl Acad Sci U S A* **111**, 7078-7083, doi:10.1073/pnas.1402873111 (2014).

301 Myers, L. E., McQuay, L. J. & Hollinger, F. B. Dilution assay statistics. *J Clin Microbiol* **32**, 732-739 (1994).

302 Siliciano, J. D. & Siliciano, R. F. Enhanced culture assay for detection and quantitation of latently infected, resting CD4+ T-cells carrying replication-competent virus in HIV-1-infected individuals. *Methods Mol Biol* **304**, 3-15, doi:10.1385/1-59259-907-9:003 (2005).

303 Cillo, A. R. *et al.* Improved single-copy assays for quantification of persistent HIV-1 viremia in patients on suppressive antiretroviral therapy. *J Clin Microbiol* **52**, 3944-3951, doi:10.1128/JCM.02060-14 (2014).

304 Hong, F. *et al.* Novel assays to measure total cell-associated HIV-1 DNA and RNA. *J Clin Microbiol*, doi:10.1128/JCM.02904-15 (2016).

305 Venneti, S. *et al.* Longitudinal in vivo positron emission tomography imaging of infected and activated brain macrophages in a macaque model of human immunodeficiency virus encephalitis correlates with central and peripheral markers of encephalitis and areas of synaptic degeneration. *The American journal of pathology* **172**, 1603-1616, doi:10.2353/ajpath.2008.070967 (2008).

306 Malnati, M. S. *et al.* A universal real-time PCR assay for the quantification of group-M HIV-1 proviral load. *Nat Protoc* **3**, 1240-1248, doi:10.1038/nprot.2008.108 (2008).

307 Ocwieja, K. E. *et al.* Dynamic regulation of HIV-1 mRNA populations analyzed by single-molecule enrichment and long-read sequencing. *Nucleic acids research* **40**, 10345-10355, doi:10.1093/nar/gks753 (2012).

308 Team, R. C. R, A Language and Environment for Statistical Computing. (2013).

309 Wickham, H. Reshaping data with the reshape package. *Journal of Statistical Software* **21** (2007).

310 Wickham, H. *ggplot2: Elegant Graphics for Data Analysis*. (Springer-Verlag, 2009).

311 Bates, D., M, M., Bolker, B. & Walker, S. Fitting linear mixed-effects models using lme4. *Journal of Statistical Software* **67**, 1-48, doi:10.18637/jss.v067.i01 (2015).

312 Pinheiro J, B. D., DebRoy S, Sarkar D. nlme: Linear and Nonlinear Mixed Effects Models. *R package version 3.1-127* (2016).

313 Fox, J. & Weisberg, S. *An {R} Companion to Applied Regression*. Second Edition edn, (Sage, 2011).

314 Benjamini, Y. & Hochberg, Y. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society. Series B (Methodological)* **57**, 289-300 (1995).

315 Champely, S. *et al.* Basic Functions for Power Analysis. (2015).

316 Blazkova, J. *et al.* Effect of histone deacetylase inhibitors on HIV production in latently infected, resting CD4(+) T cells from infected individuals receiving effective antiretroviral therapy. *J Infect Dis* **206**, 765-769, doi:10.1093/infdis/jis412 (2012).

317 Sogaard, O. S. *et al.* The Depsipeptide Romidepsin Reverses HIV-1 Latency In Vivo. *PLoS Pathog* **11**, e1005142, doi:10.1371/journal.ppat.1005142 (2015).

318 Keedy, K. S. *et al.* A limited group of class I histone deacetylases acts to repress human immunodeficiency virus type 1 expression. *J Virol* **83**, 4749-4756, doi:10.1128/JVI.02585-08 (2009).

319 Swanstrom, R. & Wills, J. W. in *Retroviruses* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor (NY), 1997).

320 Follmann, D., Proschan, M. & Leifer, E. Multiple outputation: inference for complex clustered data by averaging analyses from independent data. *Biometrics* **59**, 420-429 (2003).

321 Finney, D. J. *Statistical method in biological assay*. Vol. 3rd (Macmillan, 1978).

322 Hamilton, M. A. in *Statistics in Toxicology* (ed D. and Franklin Krewski, C.) 66-81 (Bordon Breach Science Publishers, 1991).

323 Ke, R., Lewin, S. R., Elliott, J. H. & Perelson, A. S. Modeling the Effects of Vorinostat In Vivo Reveals both Transient and Delayed HIV Transcriptional Activation and Minimal Killing of Latently Infected Cells. *PLoS Pathog* **11**, e1005237, doi:10.1371/journal.ppat.1005237 (2015).

324 Chtanova, T. *et al.* Identification of T cell-restricted genes, and signatures for different T cell responses, using a comprehensive collection of microarray datasets. *J Immunol* **175**, 7837-7847 (2005).

325 Stentz, F. B. & Kitabchi, A. E. Transcriptome and proteome expression in activated human CD4 and CD8 T-lymphocytes. *Biochem Biophys Res Commun* **324**, 692-696, doi:10.1016/j.bbrc.2004.09.113 (2004).

326 Rafati, H. *et al.* Repressive LTR nucleosome positioning by the BAF complex is required for HIV latency. *PLoS Biol* **9**, e1001206, doi:10.1371/journal.pbio.1001206 (2011).

327 Verdin, E., Paras, P., Jr. & Van Lint, C. Chromatin disruption in the promoter of human immunodeficiency virus type 1 during transcriptional activation. *EMBO J* **12**, 3249-3259 (1993).

328 Bosque, A. & Planelles, V. Induction of HIV-1 latency and reactivation in primary memory CD4+ T cells. *Blood* **113**, 58-65, doi:10.1182/blood-2008-07-168393 (2009).

329 Lassen, K. G., Ramyar, K. X., Bailey, J. R., Zhou, Y. & Siliciano, R. F. Nuclear retention of multiply spliced HIV-1 RNA in resting CD4+ T cells. *PLoS Pathog* **2**, e68, doi:10.1371/journal.ppat.0020068 (2006).

330 Wei, D. G. *et al.* Histone deacetylase inhibitor romidepsin induces HIV expression in CD4 T cells from patients on suppressive antiretroviral therapy at concentrations achieved by clinical dosing. *PLoS Pathog* **10**, e1004071, doi:10.1371/journal.ppat.1004071 (2014).

331 Clive, S. *et al.* Characterizing the disposition, metabolism, and excretion of an orally active pan-deacetylase inhibitor, panobinostat, via trace radiolabeled 14C material in advanced cancer patients. *Cancer Chemother Pharmacol* **70**, 513-522, doi:10.1007/s00280-012-1940-9 (2012).

332 Sun, M. K. & Alkon, D. L. Bryostatin-1: pharmacology and therapeutic potential as a CNS drug. *CNS Drug Rev* **12**, 1-8, doi:10.1111/j.1527-3458.2006.00001.x (2006).

333 Zhang, X. *et al.* Preclinical pharmacology of the natural product anticancer agent bryostatin 1, an activator of protein kinase C. *Cancer Res* **56**, 802-808 (1996).

334 Bliss, C. I. THE TOXICITY OF POISONS APPLIED JOINTLY1. *Annals of Applied Biology* **26**, 585-615, doi:10.1111/j.1744-7348.1939.tb06990.x (1939).

335 Bui, J. K., Mellors, J. W. & Cillo, A. R. HIV-1 Virion Production from Single Inducible Proviruses following T-Cell Activation Ex Vivo. *J Virol* **90**, 1673-1676, doi:10.1128/JVI.02520-15 (2015).

336 Lopez-Cabrera, M. *et al.* Transcriptional regulation of the gene encoding the human C-type lectin leukocyte receptor AIM/CD69 and functional characterization of its tumor necrosis factor-alpha-responsive elements. *J Biol Chem* **270**, 21545-21551 (1995).

337 Pelchen-Matthews, A., Parsons, I. J. & Marsh, M. Phorbol ester-induced downregulation of CD4 is a multistep process involving dissociation from p56lck, increased association with clathrin-coated pits, and altered endosomal sorting. *J Exp Med* **178**, 1209-1222 (1993).

338 Shan, L. *et al.* Stimulation of HIV-1-Specific Cytolytic T Lymphocytes Facilitates Elimination of Latent Viral Reservoir after Virus Reactivation. *Immunity* **36**, 491-501, doi:http://dx.doi.org/10.1016/j.immuni.2012.01.014 (2012).

339 Shan, L. *et al.* Stimulation of HIV-1-specific cytolytic T lymphocytes facilitates elimination of latent viral reservoir after virus reactivation. *Immunity* **36**, 491-501, doi:10.1016/j.immuni.2012.01.014 (2012).

340 Procopio, F. A. *et al.* A Novel Assay to Measure the Magnitude of the Inducible Viral Reservoir in HIV-infected Individuals. *EBioMedicine* **2**, 872-881, doi:10.1016/j.ebiom.2015.06.019 (2015).

341 Jones, R. B. *et al.* Histone deacetylase inhibitors impair the elimination of HIV-infected cells by cytotoxic T-lymphocytes. *PLoS Pathog* **10**, e1004287, doi:10.1371/journal.ppat.1004287 (2014).

342 Bor, J., Herbst, A. J., Newell, M.-L. & Bärnighausen, T. Increases in Adult Life Expectancy in Rural South Africa: Valuing the Scale-Up of HIV Treatment. *Science* **339**, 961-965 (2013).

343 collaboration, A. t. c. Life expectancy of individuals on combination antiretroviral therapy in high-income countries: a collaborative analysis of 14 cohort studies. *Lancet* **372**, 293-299, doi:10.1016/s0140-6736(08)61113-7 (2008).

344 Lima, V. D. *et al.* The combined effect of modern highly active antiretroviral therapy regimens and adherence on mortality over time. *J Acquir Immune Defic Syndr* **50**, 529-536, doi:10.1097/QAI.0b013e31819675e9 (2009).

345 Mills, E. J. *et al.* Life expectancy of persons receiving combination antiretroviral therapy in low-income countries: a cohort analysis from Uganda. *Ann Intern Med* **155**, 209-216, doi:10.7326/0003-4819-155-4-201108160-00358 (2011).

346 Nakagawa, F., May, M. & Phillips, A. Life expectancy living with HIV: recent estimates and future implications. *Curr Opin Infect Dis* **26**, 17-25, doi:10.1097/QCO.0b013e32835ba6b1 (2013).

347 Samji, H. *et al.* Closing the Gap: Increases in Life Expectancy among Treated HIV-Positive Individuals in the United States and Canada. *PLoS ONE* **8**, e81355, doi:10.1371/journal.pone.0081355 (2013).

348 Palella, F. J., Jr. *et al.* Mortality in the highly active antiretroviral therapy era: changing causes of death and disease in the HIV outpatient study. *J Acquir Immune Defic Syndr* **43**, 27-34, doi:10.1097/01.qai.0000233310.90484.16 (2006).

349 Joos, B. *et al.* HIV rebounds from latently infected cells, rather than from continuing low-level replication. *Proc Natl Acad Sci U S A* **105**, 16725-16730, doi:10.1073/pnas.0804192105 (2008).

350 Rothenberger, M. K. *et al.* Large number of rebounding/founder HIV variants emerge from multifocal infection in lymphatic tissues after treatment interruption. *Proc Natl Acad Sci U S A* **112**, E1126-1134, doi:10.1073/pnas.1414926112 (2015).

351 Merrill, S. J. The stochastic dance of early HIV infection. *Journal of Computational and Applied Mathematics* **184**, 242-257, doi:http://dx.doi.org/10.1016/j.cam.2003.09.057 (2005).

352 Singh, A. in *Decision and Control (CDC), 2012 IEEE 51st Annual Conference on.* 4918-4923.

353 Tan, W. Y. & Wu, H. Stochastic modeling of the dynamics of CD4+ T-cell infection by HIV and some Monte Carlo studies. *Mathematical biosciences* **147**, 173-205 (1998).

354 Weinberger, Ariel D. & Weinberger, Leor S. Stochastic Fate Selection in HIV-Infected Patients. *Cell* **155**, 497-499, doi:10.1016/j.cell.2013.09.039 (2013).

355 Weinberger, L. S., Burnett, J. C., Toettcher, J. E., Arkin, A. P. & Schaffer, D. V. Stochastic gene expression in a lentiviral positive-feedback loop: HIV-1 Tat fluctuations drive phenotypic diversity. *Cell* **122**, 169-182, doi:10.1016/j.cell.2005.06.006 (2005).

356 Holm, S. A Simple Sequentially Rejective Multiple Test Procedure. *Scandinavian Journal of Statistics* **6**, 65-70 (1979).

357 Chun, T. W. *et al.* Relationship between residual plasma viremia and the size of HIV proviral DNA reservoirs in infected individuals receiving effective antiretroviral therapy. *J Infect Dis* **204**, 135-138, doi:10.1093/infdis/jir208 (2011).

358 Gandhi, R. T. *et al.* Residual plasma viraemia and infectious HIV-1 recovery from resting memory CD4 cells in patients on antiretroviral therapy: results from ACTG A5173. *Antivir Ther* **18**, 607-613, doi:10.3851/IMP2543 (2013).

359 Eriksson, S. *et al.* Comparative analysis of measures of viral reservoirs in HIV-1 eradication studies. *PLoS Pathog* **9**, e1003174, doi:10.1371/journal.ppat.1003174 (2013).

360 Williams, J. P. *et al.* HIV-1 DNA predicts disease progression and post-treatment virological control. *Elife* **3**, e03821, doi:10.7554/eLife.03821 (2014).

361 Li, J. Z. *et al.* The size of the expressed HIV reservoir predicts timing of viral rebound after treatment interruption. *AIDS* **30**, 343-353, doi:10.1097/QAD.0000000000000953 (2016).

362 Chomont, N. *et al.* HIV reservoir size and persistence are driven by T cell survival and homeostatic proliferation. *Nat Med* **15**, 893-900, doi:10.1038/nm.1972 (2009).

363 Brenchley, J. M. *et al.* T-Cell Subsets That Harbor Human Immunodeficiency Virus (HIV) In Vivo: Implications for HIV Pathogenesis. *Journal of Virology* **78**, 1160-1168, doi:10.1128/JVI.78.3.1160-1168.2004 (2004).

364 Soriano-Sarabia, N. *et al.* Quantitation of replication-competent HIV-1 in populations of resting CD4+ T cells. *J Virol* **88**, 14070-14077, doi:10.1128/jvi.01900-14 (2014).

365 Buzon, M. J. *et al.* HIV-1 persistence in CD4+ T cells with stem cell-like properties. *Nat Med* **20**, 139-142, doi:10.1038/nm.3445 (2014).

366 Chun, T.-W. *et al.* Persistence of HIV in Gut-Associated Lymphoid Tissue despite Long-Term Antiretroviral Therapy. *Journal of Infectious Diseases* **197**, 714-720, doi:10.1086/527324 (2008).

367 Murray, J. M. *et al.* HIV DNA subspecies persist in both activated and resting memory CD4+ T cells during antiretroviral therapy. *J Virol* **88**, 3516-3526, doi:10.1128/jvi.03331-13 (2014).

368 Eisele, E. & Siliciano, R. F. Redefining the viral reservoirs that prevent HIV-1 eradication. *Immunity* **37**, 377-388, doi:10.1016/j.immuni.2012.08.010 (2012).

369 Yukl, S. A. *et al.* Site-specific differences in T cell frequencies and phenotypes in the blood and gut of HIV-uninfected and ART-treated HIV+ adults. *PLoS One* **10**, e0121290, doi:10.1371/journal.pone.0121290 (2015).

370 Hatano, H. *et al.* Cell-based measures of viral persistence are associated with immune activation and programmed cell death protein 1 (PD-1)-expressing CD4+ T cells. *J Infect Dis* **208**, 50-56, doi:10.1093/infdis/jis630 (2013).

371 Cockerham, L. R. *et al.* CD4+ and CD8+ T cell activation are associated with HIV DNA in resting CD4+ T cells. *PLoS One* **9**, e110731, doi:10.1371/journal.pone.0110731 (2014).

372 Klatt, N. R., Chomont, N., Douek, D. C. & Deeks, S. G. Immune activation and HIV persistence: implications for curative approaches to HIV infection. *Immunol Rev* **254**, 326-342, doi:10.1111/imr.12065 (2013).

373 Catalfamo, M. *et al.* HIV infection-associated immune activation occurs by two distinct pathways that differentially affect CD4 and CD8 T cells. *Proc Natl Acad Sci U S A* **105**, 19851-19856, doi:10.1073/pnas.0810032105 (2008).

374 Catalfamo, M. *et al.* CD4 and CD8 T cell immune activation during chronic HIV infection: roles of homeostasis, HIV, type I IFN, and IL-7. *J Immunol* **186**, 2106-2116, doi:10.4049/jimmunol.1002000 (2011).

375 Day, C. L. *et al.* PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. *Nature* **443**, 350-354, doi:10.1038/nature05115 (2006).

376 Deeks, S. G. *et al.* Immune activation set point during early HIV infection predicts subsequent CD4+ T-cell changes independent of viral load. *Blood* **104**, 942-947, doi:10.1182/blood-2003-09-3333 (2004).

377 Kaplan, R. C. *et al.* T cell activation and senescence predict subclinical carotid artery disease in HIV-infected women. *J Infect Dis* **203**, 452-463, doi:10.1093/infdis/jiq071 (2011).

378 Liu, Z. *et al.* CD8+ T-lymphocyte activation in HIV-1 disease reflects an aspect of pathogenesis distinct from viral burden and immunodeficiency. *Journal of acquired immune deficiency syndromes and human retrovirology : official publication of the International Retrovirology Association* **18**, 332-340 (1998).

379 Srinivasula, S. *et al.* Differential effects of HIV viral load and CD4 count on proliferation of naive and memory CD4 and CD8 T lymphocytes. *Blood* **118**, 262-270, doi:10.1182/blood-2011-02-335174 (2011).

380 Hunt, P. W. *et al.* T cell activation is associated with lower CD4+ T cell gains in human immunodeficiency virus-infected patients with sustained viral suppression during antiretroviral therapy. *J Infect Dis* **187**, 1534-1543, doi:10.1086/374786 (2003).

381 Lederman, M. M. Immune restoration and CD4+ T-cell function with antiretroviral therapies. *Aids* **15 Suppl 2**, S11-15 (2001).

382 Moore, R. D. & Keruly, J. C. CD4+ cell count 6 years after commencement of highly active antiretroviral therapy in persons with sustained virologic suppression. *Clin Infect Dis* **44**, 441-446, doi:10.1086/510746 (2007).

383 Robbins, G. K. *et al.* Incomplete reconstitution of T cell subsets on combination antiretroviral therapy in the AIDS Clinical Trials Group protocol 384. *Clin Infect Dis* **48**, 350-361, doi:10.1086/595888 (2009).

384 Hunt, P. W. *et al.* Valganciclovir reduces T cell activation in HIV-infected individuals with incomplete CD4+ T cell recovery on antiretroviral therapy. *J Infect Dis* **203**, 1474-1483, doi:10.1093/infdis/jir060 (2011).

385 Estes, J. *et al.* Collagen deposition limits immune reconstitution in the gut. *J Infect Dis* **198**, 456-464, doi:10.1086/590112 (2008).

386 Estes, J. D., Haase, A. T. & Schacker, T. W. The role of collagen deposition in depleting CD4+ T cells and limiting reconstitution in HIV-1 and SIV infections through damage to the secondary lymphoid organ niche. *Seminars in immunology* **20**, 181-186, doi:10.1016/j.smim.2008.04.002 (2008).

387 Brenchley, J. M. *et al.* Microbial translocation is a cause of systemic immune activation in chronic HIV infection. *Nat Med* **12**, 1365-1371, doi:10.1038/nm1511 (2006).

388 Favre, D. *et al.* Critical loss of the balance between Th17 and T regulatory cell populations in pathogenic SIV infection. *PLoS Pathog* **5**, e1000295, doi:10.1371/journal.ppat.1000295 (2009).

389 Favre, D. *et al.* Tryptophan catabolism by indoleamine 2,3-dioxygenase 1 alters the balance of TH17 to regulatory T cells in HIV disease. *Sci Transl Med* **2**, 32ra36, doi:10.1126/scitranslmed.3000632 (2010).

390 Klatt, N. R. *et al.* Loss of mucosal CD103+ DCs and IL-17+ and IL-22+ lymphocytes is associated with mucosal damage in SIV infection. *Mucosal immunology* **5**, 646-657, doi:10.1038/mi.2012.38 (2012).

391 Yukl, S. A. *et al.* Differences in HIV burden and immune activation within the gut of HIV-positive patients receiving suppressive antiretroviral therapy. *J Infect Dis* **202**, 1553-1561, doi:10.1086/656722 (2010).

392 Mavigner, M. *et al.* Altered CD4+ T cell homing to the gut impairs mucosal immune reconstitution in treated HIV-infected individuals. *J Clin Invest* **122**, 62-69, doi:10.1172/jci59011 (2012).

393 Gosselin, A. *et al.* Peripheral blood CCR4+CCR6+ and CXCR3+CCR6+CD4+ T cells are highly permissive to HIV-1 infection. *J Immunol* **184**, 1604-1616, doi:10.4049/jimmunol.0903058 (2010).

394 Laird, G. M. *et al.* Rapid quantification of the latent reservoir for HIV-1 using a viral outgrowth assay. *PLoS Pathog* **9**, e1003398, doi:10.1371/journal.ppat.1003398 (2013).

395 Farber, D. L., Yudanin, N. A. & Restifo, N. P. Human memory T cells: generation, compartmentalization and homeostasis. *Nat Rev Immunol* **14**, 24-35, doi:10.1038/nri3567 (2014).

396 Streeck, H., D'Souza, M. P., Littman, D. R. & Crotty, S. Harnessing CD4(+) T cell responses in HIV vaccine development. *Nat Med* **19**, 143-149, doi:10.1038/nm.3054 (2013).

397 Bacchetti, P., Deeks, S. G. & McCune, J. M. Breaking Free of Sample Size Dogma to Perform Innovative Translational Research. *Science Translational Medicine* **3**, 87ps24-87ps24 (2011).