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The Antiviral Role of CD8+ T cells during Latent Immunodeficiency Virus Infections

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B.S., University of Pittsburgh, 2014

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Abstract

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Human Immunodeficiency Virus (HIV), the infectious agent responsible for Acquired Immunodeficiency Syndrome (AIDS), infects an estimated 37 million people worldwide (UNAIDS, 2018). While antiretroviral therapy (ART) can indefinitely delay the onset of AIDS in HIV-infected individuals and limit the spread of infection, it does not eradicate the virus. The main obstacle in developing a cure for HIV infection is the presence of a population of latently infected cells, termed the viral reservoir, that is established early during infection and persists under ART (Chun et al., 1997b, Finzi et al., 1997, Wong et al., 1997, Siliciano et al., 2003a). A key paradigm in the field of HIV cure, referred to as “shock and kill” (Deeks et al., 2012, Archin et al., 2012), supposes that induction of virus expression (often referred to as “virus reactivation”) in these latently-infected cells (i.e., “shock”) followed by immune-mediated clearing (i.e., “kill”) may substantially reduce the reservoir size and possibly lead to a functional cure for HIV infection. Unfortunately, no latency-reversing agent (LRA) tested to date has successfully perturbed the viral reservoir in human clinical trials (Archin et al., 2014, Spivak et al., 2014, Rasmussen et al., 2014, Søgaard et al., 2015, Elliott et al., 2014, Elliott et al., 2015).

In this dissertation, I seek to address the antiviral role of CD8+ T cells in the maintenance of viral suppression of the latent HIV reservoir and inhibition of latency reversal using the rhesus macaque (RM) model. To this end, we assessed the stability of SIV latency after CD8+ lymphocyte depletion during long-term ART treatment in SIV-infected rhesus macaques. Specifically, we assessed interleukin-15 (IL-15) superagonist complex N-803 as an LRA alone and in combination with CD8 depletion, in addition to CD8 depletion alone. While N-803 alone did not reactivate virus production, its administration after CD8+ lymphocyte depletion induced the most robust and persistent virus reactivation ever observed in humans or macaques. Remarkably, similar results were obtained in a simian-human immunodeficiency virus (SHIV) infection model as well as ART-treated HIV-infected humanized mice, with both the CD8α- and CD8β-targeting depletion antibodies. I also detail the transcriptomic and phenotypic *in vivo* effects of N-803. This dissertation supports the notion that CD8+ T cells are involved in the maintenance of the HIV/SIV latent viral reservoir and encourages the development of CD8+ T cell-based therapeutic vaccine strategies targeting the latent viral reservoir in the search for a cure for HIV/AIDS.

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Important abbreviations

HIV: Human immunodeficiency virus

AIDS: Acquired immunodeficiency syndrome

ART: Antiretroviral therapy

NHP: Non-human primate

RM: Rhesus macaque

SIV: Simian immunodeficiency virus

SHIV: Simian-human chimeric immunodeficiency virus

LRA: Latency-reversing agent

N-803: IL-15 superagonist complex

BLT: Bone marrow-liver-thymus humanized mouse

PBMC: Peripheral blood mononuclear cell

LN: Lymph node

RB: Rectal biopsy

Chapter 1: Introduction

The early HIV epidemic

On June 5, 1981, the CDC's *Morbidity and Mortality Weekly Report* described five cases of a rare Pneumocystis pneumonia in young, previously healthy, gay men living in Los Angeles (CDC, 1981). This led to the report of similar cases of rare opportunistic infections among gay men, primarily in Los Angeles and New York City, and it was recognized that an unidentified, acquired immunodeficiency was emerging (Gottlieb et al., 1981). Initially, the disease was thought to only infect gay men, and was called Gay-Related Immunodeficiency (GRID) leading to a stigma that prevented the allocation of US government funds. It wasn't until the disease was also observed in IV drug user, hemophiliac, and Haitian populations that it was understood that the disease was not specific for homosexuals (Fralick, 1984, CDC, 1982b, CDC, 1983b, CDC, 1983a, CDC, 1982a). The source of the infection was discovered first by Dr. Françoise Barré-Sinoussi and Dr. Luc Montagnier at the Pasteur Institute (Barre-Sinoussi et al., 1983) and quickly thereafter by Dr. Robert Gallo at the US National Cancer Institute (Gallo et al., 1983). The agent was formally named the human immunodefiency virus (HIV) in 1986. We now know that HIV is a lentivirus of the *Retroviridae* family, with a single-stranded, positive sense RNA genome and an encoded reverse transcriptase RNA-DNA polymerase (Levy, 1993). Since 1981, 77.3 million people have been infected with HIV and the epidemic has killed over 37 million people to date through the onset of Acquired Immunodeficiency Syndrome (AIDS) and associated illnesses (UNAIDS, 2018).

The advent of antiretroviral therapy

Fortunately, the battle against HIV/AIDS has come a long way in the nearly four decades since the first few cases of disease. For those with access to care, HIV is no longer a death sentence. Since 1996, combination antiretroviral therapy (ART) has been the standard care for HIV-infected individuals (Collier et al., 1996, D'Aquila et al., 1996, Staszewski et al., 1996), contributing to a decrease in HIV mortality and transmission (CDC, 2006). ART is generally a cocktail of drugs that block specific steps of the HIV replication lifecycle, thus preventing new infection of cells within the host and, when used correctly, viral transmission (Deeks et al., 2015). With the prevention of new infection and the eventual death of previously-infected cells, ART-treated patients see a decrease in the concentration of plasma viremia and achieve viral suppression. With early ART, clinical manifestations are preventable as the disease progression to AIDS is significantly decelerated. Through broad access to effective and less toxic ART regimens, the lifespan of HIV-infected individuals approaches that of the general population (Deeks et al., 2013). Unfortunately, undiagnosed cases, limited/lack of access to care and ART, and poor drug adherence contribute to the roughly 2 million new HIV infections and 1.2 million AIDS-related deaths that occur each year worldwide (UNAIDS, 2015). It is evident that the global handle on the HIV/AIDS epidemic is not sufficient and a cure for the infection remains of utmost importance.

Use of nonhuman primate models for studying HIV

Studying HIV infection in humans has many limitations. For obvious ethical reasons, investigators cannot infect humans with HIV as part of an experimental study. Thus, studies are only able to include previously-infected cohorts, creating much

variation in the sample population in terms of infection and treatment. Access to sites such as the brain and bone marrow are rare, as well as lymph nodes and biopsies from other tissues. Additionally, it is impossible to extensively sample a single subject unless bodies are donated at death.

To overcome these barriers, the simian immunodeficiency virus (SIV) infection model in non-human primates (NHPs) is used to replicate HIV infection in humans without many of the limitations of using human subjects (Micci et al., 2015, Kumar et al., 2016). NHP species utilized to study pathogenic infection include rhesus, pigtail, and cynomolgus macaques. Common viruses include macaque SIV_{mac239} and SIV_{mac251}, or simian-human chimeric immunodeficiency virus (SHIV) strains, which include an SIV backbone and replacement of the SIV envelope with an HIV envelope, providing a more relevant model of HIV transmission and pathogenesis (Pereira et al., 2012). Using animal models allows researchers to control for the infecting virus strain, timing of infection, acquisition of blood and tissue samples, selection of specific MHC class I genotypes, elective animal necropsy, and broad and frequent tissue collections. Of note, SIV infection of rhesus macaques is similar to HIV infection of humans with establishment of peak and set point viremia, onset of AIDS, and resolution of AIDS upon ART initiation, thus providing a convenient and comparable model to study HIV infection of humans (Micci et al., 2015).

HIV transmission and clinical presentation

HIV can be transmitted via blood, breast milk, semen or vaginal secretions from infected individuals (CDC, 2018). Patients with acute HIV infection may experience a flu-like illness characterized by fever, sore throat, lymphadenopathy, and/or rash

(Schacker et al., 1996). Serum antibodies to HIV are not typically high enough during the first few weeks of infection to test positive by an enzyme-linked immunosorbent assay (ELISA), thus a rapid HIV diagnostic test conducted during the early phase of the infection may provide a false negative (Gaines et al., 1988, Tindall and Cooper, 1991). Systemic infection is established with the spread of the virus throughout the body, including to the thymus, spleen, peripheral lymphoid organs, mucosal lymphoid tissues, and the brain (Lackner et al., 2012).

After initial symptoms cease to persist, infected individuals may experience a symptom-free period for upwards of 10 years. During this time, CD4+ T cells, an important cellular arm of the adaptive immune system, are infected with the virus contributing to a decline in their frequency (Klatzmann et al., 1984). The onset of AIDS typically occurs when the CD4+ T cell count falls below 200 cells/μL of blood. During advanced, untreated HIV infection the immune system is unable to protect the body against other sources of infection due to the near absence of CD4+ T cells, chronic immune activation, and dysfunctional CD8+ T cells and B cells (Levy, 1993). The acquired mmunodeficiency leads to susceptibility to opportunistic infections, by organisms such as Pneumocystis jirovecii, mycobacteria, cytomegalovirus, Toxoplasma gondii, and Cryptococcus, as well as the occurrence of malignancies related to viral pathogens such as non-Hodgkins lymphoma and Kaposi's sarcoma (Lackner et al., 2012).

HIV Pathogenesis

The difficulty of HIV eradication, both on an individual and global basis, is owed to the incredible astuteness of HIV as a pathogen. As with most RNA viruses, HIV is

extremely limited by the size of its genome. While the human genome is made of about 3.2 trillion nucleotides, the HIV-1 genome is composed of only 9,500 nucleotides (Luciw, 1996). Incredibly, with only nine genes HIV is able to infect and hijack host cells, producing systemic effects that ultimately lead to AIDS and death if left untreated. Furthermore, the virus has also evolved to infect and deplete CD4+ T cells, targeting the cells of the body that are in place to fight the infection (Klatzmann et al., 1984). Infection recruits more CD4+ T cells to the site, providing a constant supply of target cells for the infection to spread (Silvestri, 2013). The resultant clinical signature of immunodeficiency allows not only HIV, but also other pathogens to spread and eventually kill the host (Lackner et al., 2012). Furthermore, HIV replicates imperfectly due to the error-prone reverse transcriptase RNA-DNA polymerase encoded in the genome (Roberts et al., 1988). These errors introduce mutations that actually help the virus evolve rapidly, allowing for escape mutations against immune recognition or therapy to develop. Next, after reverse transcription into DNA, HIV integrates into the host genome where it can remain latent for years (Vogt, 1997). Thus, an HIV-infected individual remains infectious, regardless of the presence of clinical manifestations, unless viral suppression is achieved. Yet, even after a decade of ART treatment, the sustained presence of a population of latently-infected cells prevents elimination of the viral reservoir (Chun et al., 1997b, Finzi et al., 1997, Wong et al., 1997, Siliciano et al., 2003a). Thus, HIV has evolved to become arguably the smartest pathogen of our time and continues to evade our efforts to fight it.

HIV Lifecycle

HIV virions bind its primary cellular receptor cluster of differentiation 4, simply CD4, via the HIV viral envelope protein (Maddon Pj Fau - Dalgleish et al., 1986, McDougal et al., 1986). CD4 is a member of the immunoglobulin superfamily that typically functions to enhance T cell receptor-mediated signaling and is expressed on helper T lymphocytes, monocytes, macrophages, and dendritic cells. Although HIV can infect an array of cell types, its preferential targets are CD4+ T cells (Klatzmann et al., 1984). Dendritic cells are difficult to infect; instead they are able to capture the virus for trans-presentation to T cells (Wu and KewalRamani, 2006).

The HIV envelope is a glycosylated trimer of gp120 and gp41 heterodimers, with a CD4 binding site within gp120 (Kwong et al., 1998). For fusion and subsequent delivery of the viral core into the cytoplasm of the host cell to occur, HIV must also bind to a coreceptor, the chemokine receptors CCR5 or CXCR4, exposing the hydrophobic gp41 fusion peptide. The membrane of the virion fuses with the lipid membrane of the host cell, establishing entry into the cytoplasm (Wilen et al., 2012). The positive sense single-stranded RNA genome is transcribed into double-stranded DNA via the HIV reverse transcriptase polymerase that is prepackaged in the virion (Hu and Hughes, 2012). The double stranded DNA genome is then imported into the nucleus of the cell, and the pre-packaged HIV integrase enzyme splices open the host DNA and inserts the HIV DNA into the host genome, a process called retroviral integration. Long-translated regions (LTRs) flank each end of the HIV genome and contain the viral promotor region. The virus can then be transcribed via host transcription machinery to yield more genomic HIV RNA or mRNA to be translated into HIV proteins. Two HIV RNA copies,

integrase, and reverse transcriptase are packaged into virions and bud from the infected cell for subsequent infections of neighboring cells. A productively infected cell has a half-life of about 1.6 days (Perelson et al., 1996), during which is produces thousands of progeny HIV virions (De Boer et al., 2010). The HIV provirus, that which is integrated into the DNA of the host, can either direct host transcriptional machinery towards active viral replication or remain latent with the possibility of eventual expression (Vogt, 1997). This makes eradication of HIV from the body extremely difficult, as systemic elimination of the virus requires death of every cell infected with replication competent virus.

Cellular targets of HIV/SIV

Multiple CD4+ T cell subsets exist, varying in frequency, location, activities, and phenotype. After development in the thymus, naïve T cells circulate in the blood and lymphoid tissue in a resting state. Following exposure to cognate antigen, T cells die or persist as memory T cells. Subsets include short-lived effector memory T cells (T_{EM}), which have an activated phenotype and secrete cytokines against the cognate infection, as well as long-lived central memory T cells (T_{CM}), which typically remain petrified in a resting state until reexposure to cognate antigen, leading to rapid activation and response (Mahnke et al., 2013).

CD4+ T cell subtype has been shown to influence HIV infection and replication. Activated CD4+ T cells are superior in their ability to support productive HIV infection (Zack et al., 1990, Stevenson et al., 1990, Chou et al., 1997). Activated CD4+ T cells express the HIV coreceptor CCR5 and have their transcription machinery in the “on-position”, increasing the possibility for provirus transcription and viral replication. Resting naïve CD4+ T cells do not usually express the CCR5 coreceptor and are

consequently more resistant to infection, while resting T_{CM} cells express low levels of CCR5 and have been shown to be significant targets for SIV *in vivo* (Li et al., 2005). Thus, while naïve CD4+ T cells may support integrated HIV DNA, their frequency of infection is usually much lower than memory CD4+ T cells (Palmer et al., 2011). Memory subsets yield different contributions to the HIV reservoir, where central memory cells maintain the highest contribution (Kulpa and Chomont, 2015)

Additionally, target cell availability changes with anatomical location. The population of CD4+ T cells within the mucosal immune system is primarily activated CCR5+ memory CD4+ T cells (Connor et al., 1997, Scarlatti et al., 1997, Long et al., 2002) – the preferential targets of HIV/SIV infection, and are hence heavily depleted during infection (Brenchley et al., 2004b, Mehandru et al., 2004). In contrast to the systemic immune system, the mucosal immune system is massive in size. While productive infection of peripheral CD4+ T cells is quite rare at .01%-1%, infection of mucosal CD4+ T cells is common with estimates of around 60% infected within days of HIV exposure (Brenchley et al., 2004a, Mattapallil et al., 2005). Thus, infection of CD4+ T cells is contingent on coreceptor expression, activation status, and anatomical location.

Loss of CD4+ T lymphocytes

Direct cellular cytotoxicity during HIV replication is likely the cause of death of CD4+ T cells before immunological control during the acute infection (Klatzmann et al., 1984, Okoye and Picker, 2013). Cell death primarily occurs in the mucosal tissues, specifically the gut (Brenchley et al., 2006). Rapid loss of infected cells in the secondary lymphoid tissue results in immunodeficiency that leads to excess pathogen load and

tissue fibrosis (Brenchley et al., 2006). This chronic inflammatory process stimulates immunoregulatory responses that obstruct T cell function (Favre et al., 2010). HIV also hinders the ability of the immune system to regenerate new CD4+ T cells due to damage caused to both stem cells and the thymus during infection (McCune, 2001). Additionally, infection can generate incomplete reverse transcripts that cause an intense inflammatory response and indirect death of local, uninfected bystander cells through a mechanism called pyroptosis (Doitsh et al., 2014). CD4+ T cells that persist show signs of exhaustion and dysfunction that also contribute to the pathogenicity of HIV (Kaufmann et al., 2007, Day et al., 2006, D'Souza et al., 2007).

Chronic immune activation

Though the immune system is compromised during HIV infection, there also appears to be robust immune activation in compartments that include T cells, B cells and antigen presenting cells of the innate immune system. Higher levels of circulating antibodies and proinflammatory cytokines are present in HIV-infected individuals, a result of high activation of interferon-stimulated genes (Woelk et al., 2004, Hyrcza et al., 2007). Additionally, T cells are found to express higher levels of the activation markers CD38 and HLA-DR (Giorgi et al., 1993). Increased expression of markers of immune senescence, such as CD57 (Brenchley et al., 2003), and immune exhaustion, such as PD-1, are observed (Day et al., 2006, Trautmann et al., 2006). T cells expressing these markers were found to be impaired in response to TCR stimulation and these markers are predictors of disease outcome in HIV infection (Giorgi et al., 1993, Deeks et al., 2004, Hazenberg et al., 2003). Furthermore, a hallmark of immune activation is an increase in T cell turnover, as seen in CD4+ and CD8+ T cell populations (Sachsenberg

et al., 1998, Douek et al., 2001), especially within the T_{CM} subset (Picker et al., 2004, Sieg et al., 2005). Lastly, while activated CD4+ T cells are more susceptible to HIV infection, they also appear to be more susceptible to apoptosis (Zack et al., 1990, Sieg et al., 2008).

In theory, there are multiple molecular and cellular mechanisms by which HIV infection can induce immune activation, and these different mechanisms are likely not mutually exclusive and depend on the stage of infection (early, chronic, treated, etc.) (Paiardini and Müller-Trutwin, 2013). The direct innate and adaptive immune responses against the infection are the first, and most predictable, mechanism of immune activation. Activation of HIV-specific T cells and B cells, binding of the mitogenic components of HIV to pattern recognition receptors (PRR) on innate cells, and the activation of infected cells all contribute to immune activation (Beignon et al., 2005, Heil et al., 2004, Meier et al., 2007, Vabret et al., 2012). Antigen load may also correlate with immune activation, as individuals capable of controlling HIV on their own (elite controllers) and ART-treated individuals experience lower viral loads, activation, and inflammation (Barker et al., 1998b, Hileman and Funderburg, 2017). Yet, during controlled infection, when viral loads are beneath the level of detection, the main markers of immune activation and inflammation are still higher than in uninfected individuals (Sauce et al., 2011, Hunt et al., 2003). While ongoing HIV replication most certainly contributes to immune activation, it is not a sufficient explanation. The frequency of activated T cells far exceeds the number of HIV-infected CD4+ T cells, as other cell types including B cells, NK cells, plasmacytoid dendritic cells, and monocytes also show increased levels of activation, turnover, and/or cell death (Doisne et al.,

2004a, Finkel et al., 1995, Hellerstein et al., 2003, Burdo et al., 2011, Brown et al., 2007). In fact, it is the level of immune activation, not viral load, that predicts CD4+ T cell decline (Giorgi et al., 1999, Deeks et al., 2004). Additionally, high viral loads do not always cause pathogenic infection as indicated in studies of natural SIV hosts, where high viral loads persist in the absence of immune activation (Silvestri et al., 2003).

HIV infection when left untreated causes a decline of CD4+ T cells, leading to immune deficiency and host susceptibility to a plethora of other benign infections. These opportunistic and environmental pathogens, as well as reactivated infections (ex. cytomegalovirus and Epstein Barr virus) manifest into disease that activates the immune system, sustaining an inflammatory state (Doisne et al., 2004a). CCR5+ CD4+ T cells are severely depleted from the gastrointestinal tract in the first few weeks of infection and never return to normal, even after treatment initiation (Brenchley et al., 2004b, Mattapallil et al., 2005, Veazey et al., 1998). This depletion is associated with loss of intestinal epithelial cells, disruption of tight junctions, and weakened mucosal intestinal barrier integrity, resulting in the translocation of bacterial and fungal products from the intestinal lumen to the systemic circulation (Brenchley et al., 2008). It is likely that these products contain pathogen-associated molecular patterns (PAMPs) that induce production of pro-inflammatory cytokines such as TNF α, IL-6, IL-1 β, and type I interferons (Emilie et al., 1990, Birx et al., 1990, Molina et al., 1989). Additionally, the Th_{17} lineage of intestinal CD4+ T cells, characterized by IL-17 and IL-22 production, is heavily depleted during HIV-infection (Brenchley et al., 2006, Cecchinato et al., 2008, Raffatellu et al., 2008). IL-17 and IL-22 are thought to be crucial for mucosal immunity and their loss may also contribute to the weaken integrity of the mucosal barrier. SIV

infections of natural hosts and elite controllers, whose infections lack chronic immune activation, show maintenance of the Th_{17} lineage, suggesting that preservation of Th_{17} CD4+ T cells sustains mucosal barrier integrity and helps prevent chronic immune activation (Brenchley et al., 2008, Favre et al., 2009, Salgado et al., 2011, Brandt et al., 2011, Ciccone et al., 2011).

In conclusion, the chronic immune activation observed during pathogenic HIV and SIV infections is maintained by many mechanisms that likely work synergistically. Many of these mechanisms are perpetuated by the immunopathogenic state, further continuing the cycle. Inhibiting viral replication through ART is simply not enough to prevent CD4+ T cell death or fully restore CD4+ T cell frequency due to the residual inflammation that exists even during suppressed infection.

Immune evasion

The hallmark method of immune evasion, as mentioned previously, is the ability of HIV to infect and compromise the host immune system, allowing the virus to hide in plain sight. Additionally, the rapid evolution of HIV within the host is in part responsible for the maintenance of chronicity. An error rate projected at one mutation every 1000 bases coupled with continuing levels of high replication leads to broad variation in HIV (Wu and KewalRamani, 2006, Overbaugh and Bangham, 2001). As opposed to evolution in the eukaryotic kingdom of life, here natural selection takes place with each event of viral replication. Mutant viruses that are capable of replication amid antiretroviral drugs and the immune pressure thrive and propagate, while other variants cease to survive. The HIV envelope is a major site of mutation, consistently escaping recognition by envelope-targeting antibodies. There is much flexibility in the length of

the variable loops and the extent of glycosylation of the envelope protein, which increases the ability to shield epitopes (Wei et al., 2003, West et al., 2014). Several binding sites are only transiently exposed during the entry process, limiting access for antibodies (West et al., 2014). Even within one infected individual, the envelope genomic sequence can vary by .6-1% *per year* (Shankarappa et al., 1999, Piantadosi et al., 2009) and 25-35% between individuals infected with different HIV subtypes (Korber et al., 2001). With such expansive epitope variation, it is challenging to develop a vaccine capable of targeting all HIV variants.

Cellular restriction factors, such as APOBEC3, SAMHD1, and tetherin prevent multiple post-entry activities of the virus. To combat, HIV has accessory proteins that abrogate the activity of the cellular restriction factors. Viral Vif blocks the activity of APOBEC3, which mutates viral genomes (Sheehy et al., 2002). Vpx blocks SAMHD1, which depletes the pool of nucleoside triphosphates required for reverse transcription (Laguette et al., 2011). Vpu blocks the activity of tetherin, which prevents budding and exodus of immature virions (Neil et al., 2008). Viral Nef has an important function, downregulating HLA class I molecule expression in order to reduce the extent of presentation of HIV peptide to cytotoxic T cells and CD4 downregulation to prevent superinfection (Schwartz et al., 1996, Garcia and Miller, 1991). Thus, the accessory proteins encoded within the HIV genome have very efficient and indispensable functions during the pathogenesis of HIV, with immune evasion being a key responsibility.

Natural control of HIV infection

It has long been recognized that a small group of HIV-infected individuals (<1% of the population) are capable of controlling HIV infection independent of ART, in

comparison to chronic progressors. These individuals, termed elite controllers, are able to maintain plasma viremia below the limit of detection of standard PCR assays without treatment (Saez-Cirion, 2013) (Figure 1.1). Elite controllers typically have stable CD4 counts without decline and progression to AIDS. Additionally, post-treatment controllers are HIV-infected individuals who control virus below the limit of detection after interruption of long-term ART (Saez-Cirion et al., 2013).

Just as with HIV infection of humans and SIV infection of rhesus macaques, natural hosts of SIV, sooty mangabeys and African green monkeys, show sustained high levels of plasma viremia, high viral load in the gut and an early, massive intestinal CD4+ T cell depletion. The difference between the two infections is the absence of high, chronic immune activation in natural hosts (Chahroudi et al., 2012, Liovat et al., 2009, Silvestri et al., 2003). We now know that systemic chronic immune activation is the driving force of CD4+ T cell depletion and AIDS (Paiardini and Müller-Trutwin, 2013). Levels of activated CD8+ T cells are more closely associated with shorter survival than viral load or CD4+ T cells (Giorgi et al., 1999). High viremia is not always indicative of progression to AIDS, as a few HIV-infected individuals do not progress to AIDS despite high viral loads (Choudhary et al., 2007, Rotger et al., 2011). Even in the context of ART-treated patients with undetectable viral loads, high levels of T cell activation are observed as compared to healthy controls (Sauce et al., 2011). Elite controllers show levels of T cell activation that is higher than those of both healthy controls and ART-suppressed patients, but T cell activation of chronic progressors is highest (Hunt et al., 2008).

Initially it was thought that defective transmitted viruses may be responsible for the observed control in elite controllers (Deacon et al., 1995, Alexander et al., 2000), however fully replication-competent virus from elite controllers has since been isolated (Blankson et al., 2007). These isolated viruses show viral evolution, evidence that replication within the host has occurred (Bailey et al., 2006), and their replicative capabilities have been shown to be equivalent to that of standard lab strains *in vitro* (Blankson et al., 2007). Sequence analysis of virus isolated from elite controllers does not show any common mutations or significant deletions in the virus (Blankson et al., 2007, Miura et al., 2008). Case studies of an elite controller infected with a second HIV isolate maintained relative control after superinfection (Rachinger et al., 2008) and transmission pairs consisting of a chronic progressor transmitting to a would-be elite controller also suggest that the control observed during elite control is not an artifact of the infecting virus. Host factors therefore likely play the biggest role in the control of HIV replication. Elite controllers typically have an asymptomatic acute infection with higher CD4 counts and lower viral loads than their chronic progressor counterparts, suggesting that control of infection starts early (Goujard et al., 2009, Madec et al., 2005, Okulicz et al., 2009). While differences in innate immune system interactions with HIV between elite controllers and chronic progressors is little understood and neutralizing antibodies against HIV do not appear to be responsible for control, differences in the CD8+ T cells appear to play a promising role during natural HIV control (O'Connell et al., 2009).

Many groups have sought to determine what distinguishes elite controllers from chronic progressors, and specifically seeking to determine whether those differences lie in the activity of CD8+ T cells. Depletion of CD8+ lymphocytes from controller rhesus

macaques results in a transient increase in viremia (Friedrich et al., 2007). Another study found that elite controllers have very high levels of escape mutations, suggesting that CD8+ T cells put great selective pressure on the virus (Bailey et al., 2006). The identification of specific differences in host factors between chronic progressors and elite controllers has defined potential targets for *in vivo* manipulation of HIV/SIV-specific CD8+ T cell-specific responses to achieve better immunological control of the infection.

Peripheral HIV-specific CD8+ T cells from elite controllers are not more frequent, possess no greater specificity or breadth, and show no improved functional avidity when compared to those of chronic progressors (Hersperger et al., 2011c, Migueles et al., 2000, Betts et al., 2001, Migueles et al., 2004). CD8+ T cells of elite controllers were found to be more polyfunctional compared to chronic progressors in response to HIV antigens based on expression of IL-2, IFNγ, TNF α, MIP-1 β and CD107a, but most of these polyfunctional cells are specific to antigens of other viruses such as Epstein Barr virus and cytomegalovirus -- only a relatively small subset is HIV-specific (Betts et al., 2006, Zimmerli et al., 2005, Migueles et al., 2009, Makedonas et al., 2010). Also, many elite controllers do not have any of these polyfunctional cells at all, weakening the probability that polyfunctional CD8+ T cells are solely responsible for the observed control. When considering only HIV-specific CD8+ T cells those of elite controllers, as compared to chronic progressors, were observed to have a dramatically higher proliferative capacity, a greater ability to upregulate granzyme B and perforin production, and a greater cytolytic capacity against autologous HIV-infected CD4+ T cells (Arrode et al., 2005, Horton et al., 2006, Lichterfeld et al., 2004a, Migueles et al., 2002, Migueles et al., 2008). Specifically, during chronic infection, cytolytic potential is

lost rapidly in most HIV-infected individuals, such that only around 15% of HIV-specific CD8+ T cells express perforin, whereas around 40% express perforin in elite controllers (Hersperger et al., 2010). Studies show that HIV-specific CD8+ T cells from elite controllers synthesize greater amounts of cytotoxic granule components, thus increasing their ability to kill infected cells (Saeidi et al., 2015, Hersperger et al., 2010, Migueles et al., 2008, Chen et al., 2012). Elite controllers are found to exceptionally upregulate T-bet expression, which can directly bind to the promoter regions of perforin and granzyme B genes leading to increased expression (Hersperger et al., 2011a, Walker and Yu, 2013). A study found that a large proportion of gag-specific CD8+ T cells from elite controllers coexpress PD-1, CD160, and 2B4, suggestive of a level of exhaustion comparable to chronic progressors (Pombo et al., 2015). However, elite controllers in this study also harbored a large population of gag-specific CD8+ T cells co-expressing CD160 and 2B4 that is associated with cytolytic potential, suggesting that co-expression of inhibitory markers on HIV-specific CD8+ T cells does not always indicate exhaustion. Therefore, the field appears to lack a consensus on the differences in expression and function of inhibitory receptors between chronic progressors and elite controllers, as well as the actual prevalence of exhaustion.

Many elite controllers possess HIV-specific CD8+ T cells restricted by HLA-B*27/*57 that can continue to proliferate throughout chronic infection, whereas the majority of HIV-specific CD8+ T cells restricted by other HLA alleles lose their proliferative capacity (Altfeld et al., 2003, Evans et al., 2000, Kaslow et al., 1996, Klein et al., 1998, Demers et al., 2016, Migueles et al., 2002, Lichterfeld et al., 2004b, Horton et al., 2006). Yet, not all elite controllers have these alleles and even chronic

progressors have been found to have HLA-B*27/*57 genotype. Proliferative capacity of CD8 T+ cells in elite controllers is associated with the up-regulation of perforin and therefore associated with enhanced cytotoxic capabilities (Migueles et al., 2002). In addition, HIV-specific CD8+ T cells from elite controllers synthesize greater amounts of cytotoxic granule components, thus increasing their ability to kill infected cells (Hersperger et al., 2010, Migueles et al., 2008, Chen et al., 2012) and are found to exceptionally up-regulate T-bet expression, which increases the production of perforin and granzyme B (Hersperger et al., 2011a, Walker and Yu, 2013). HLA-B*27/*57-restricted effector CD8+ T cells express high amounts of granzyme B and escape CD4+ T_{REG}-mediated suppression by killing the T_{REG} cells they encounter (Elahi et al., 2011). T_{REG} cells express galectin-P, a ligand for TIM-3 on effector cells. According to this group, HLA-B*27 and HLA-B*57-restricted effector T cells can kill T_{REG} cells but also downregulate Tim-3 avoiding exhaustion through Gal-3/Tim-3 interaction with T_{REG} cells (Elahi et al., 2011).

Host factors, as opposed to viral factors, largely mediate control of HIV infection during elite control and CD8+ T cells play a prominent role in this phenomenon (O'Connell et al., 2009). In conclusion, there appears to be associations between patterns of the anti-HIV activity of CD8+ T cells and status as an elite controller, suggesting that CD8+ T cells are an important factor for the control of HIV infection.

CD8+ T cell responses to HIV/SIV

General CD8+ T cell response to viral infections

CD8+ T cells are a part of the adaptive immune system. During thymic development they mature into cells capable of recognizing foreign antigen presented on

MHC class I molecules on all cells of the body. MHC class I is upregulated during infection, inflammation, and stress, displaying short peptides from intracellular proteins degraded by the immunoproteasome. MHC class I is expressed by all cells of the body. During viral infections, MHC class I molecules present viral peptides and CD8+ T cells with T cell receptors (TCR) specific for that particular foreign peptide-MHC class I complex recognize the infected cell and initiate a response. Traditionally a response to viral infections includes secretion of IFNγ and IL-12; activation of other CD8+ T cells, NK cells, and macrophages; and polarization of CD4+ T cell towards a Th1 phenotype. TCR recognition on CD8+ T cells also causes upregulation of cytotoxic granules, leading to the release of perforin and granzyme into the immunological synapse, resulting in the death of the infected cell. Thus, CD8+ T cells are able to recognize intracellular pathogens, specifically viral infections, and the prototypical response is to kill the infected cells. During chronic viral infections, CD8+ T cells upregulate PD-1 and other inhibitory markers, taking on an exhausted phenotype and downregulate their ability to respond to cognate antigen. This lack of response contributes to the chronicity of some viral infections.

Acute HIV infection

Acute HIV infection of humans is characterized by a transient peak in viremia (2-3 weeks) followed by a post-peak decline to a set-point level of viremia that is a strong predictor of the ensuing rate of progression to AIDS (Mellors et al., 1996). Subsequently, HIV-infected patients experience a slow decrease in CD4+ T cells and gradual deterioration of immune function, including exhaustion of CD8+ T-cells, loss of immune function in the lymph nodes and mucosal tissues, and chronic immune

activation -- leading to increased susceptibility to opportunistic infections and cancer (Fauci et al., 1991, Brenchley et al., 2006, Deeks et al., 2015).

Several lines of evidence suggest that CD8+ T cells play a significant role in the control of virus replication during the acute phase of HIV and SIV infection (Figure 1.1). First, the post-peak decline of viremia only occurs after the emergence of virus-specific CD8+ T cells, suggesting that CD8+ T cells are involved in the initial control of infection (Borrow et al., 1994, Koup et al., 1994). Depletion of CD8+ T cells during acute SIV infection of rhesus macaques results in the abrogation of the post-peak decline of viremia (Matano et al., 1998, Schmitz et al., 1999b), again confirming a critical role in the initial resolution of viral control. In addition, during the first weeks of infection viral mutants capable of escaping the CD8+ T cell response begin to appear and rapidly become fixed in the overall virus population, thus demonstrating a strong evolutionary pressure posed on the virus to escape immunological recognition by CD8+ T cells (Borrow et al., 1997b, Chen et al., 2000, McMichael and Phillips, 1997, Vanderford et al., 2011). Overall, these observations indicate that CD8+ T cells play a significant role in the control of acute HIV infection.

Persistent exposure to HIV antigen during the natural course of HIV infection leads to the progressive dysfunction and “exhaustion” of virus-specific T cells. T cell exhaustion, characterized by altered differentiation, impaired function, and decreased proliferation (Kuchroo et al., 2014), begins soon after peak HIV viremia and persists for the remainder of the infection (Radebe et al., 2015, Cao et al., 2016a). Thus, while HIV-specific CD8+ T cells appear to be necessary for the post-peak decline in viremia during the acute infection, persistent exposure to antigen and chronic inflammation results in

an exhausted state, during which cells are no longer capable of amounting an appropriate response against HIV allowing the infection to persist.

Chronic HIV infection

CD8+ T cells continue to exert some level of control over HIV and SIV replication after the acute phase of infection, as shown by studies in which depletion of CD8+ T cells during chronic SIV infection results in increased viral replication (Chowdhury et al., 2015, Jin et al., 1999, Metzner et al., 2000a). Additionally, viral escape mutants against CD8+ T cell responses continue to appear during the chronic phase of infection (Goulder et al., 1997). However, the combination of virus escape and progressive T cell dysfunction and exhaustion makes HIV- or SIV-specific CD8+ cells increasingly less able to successfully control virus replication (Appay et al., 2002b, Papagno et al., 2004, Pantaleo et al., 1994, Appay et al., 2002a, Champagne et al., 2001a, Betts et al., 2006, Mueller et al., 2001). This loss of CD8+ T cell-mediated control of virus replication is associated with disease progression in chronically HIV-infected individuals (Day et al., 2006, Brenchley et al., 2003). Interestingly, continuous activation of CD8+ T cells in the absence of effective antiviral activity may lead to disease progression (Sodora and Silvestri, 2008), as first suggested by the classical observation that the level of CD8+ T cells expressing the activation markers CD38 and HLA-DR are most closely associated with shorter patient survival than viral load or CD4+ T cell count (Giorgi et al., 1999). In conclusion, CD8+ T cells are involved in control during all stages of infection, responsible for both viral control and persistence.

Expansion of CD8+ T cells and bystander activation

As stated above, during acute HIV infection, there is a large and broad increase in CD8+ T cells which is associated with the post-peak decline of HIV viremia. This expansion of the effector CD8+ T cell compartment is seen in other viral infections (Kolowos et al., 1999, Weiss et al., 1998, Than et al., 1999, Margolick et al., 1993). However, unlike in other viral infections, the expansion is not limited to HIV-specific cells and CD8+ T cell numbers remain elevated throughout the course of the disease (Margolick et al., 1993, Papagno et al., 2004). In fact, HIV-specific CD8+ T cells represent less than 10% of the total CD8+ T cell pool expanded during the acute infection (Betts et al., 2001, Altfeld et al., 2001). CD8+ T cells reactive to latent pathogens, such as cytomegalovirus and Epstein Barr virus reactivate (Rahman et al., 1991), as well as CD8+ T cells specific for non-persistent pathogens, such as influenza and adenovirus, implying that CD8+ T cell expansion occurs through antigen-independent mechanisms as well (Mudd and Lederman, 2014, Bastidas et al., 2014a). This is in contrast to the CD4 compartment, where some expansion of cells reactive to persistent pathogens is demonstrable, but responses to non-persistent antigens are unaffected (Haas et al., 2010). The exact cause of such “bystander activation” remains unclear.

Overtime CD8+ T cell frequency decreases but does not return to pre-infection levels (Cao et al., 2016a). In healthy individuals, the majority of circulating CD8+ T cells are of the naïve and central memory subtypes, but during chronic HIV infection the CD8+ T cell compartment is enriched for effector memory CD8+ T cells (Appay et al., 2002b, Papagno et al., 2004). The expanded CD8+ T cell population in chronically HIV-infected patients shows symptoms of T cell exhaustion and immunosenescence, both of

which are associated with disease progression (Day et al., 2006, Brenchley et al., 2003). Even after the initiation of ART, the bystander contraction of the CD8+ T cell compartment does not return to normal and elevation remains despite virologic control (Serrano-Villar et al., 2014a). As CD4 counts are often lower than pre-infection levels, many ART-treated patients have an inverted CD4:CD8 ratio (Serrano-Villar et al., 2014b, Leung et al., 2013). The initiation of ART during acute infection is associated with a greater CD8+ T cell count reduction than those initiated ART during chronic infection (Cao et al., 2016b). This would suggest that shorter duration of antigen exposure is associated with decreased immune activation and lower CD8+ T cell elevation (Cao et al., 2016b, Jenabian et al., 2015). This elevation of CD8+ T cells occurs not only in the periphery, but is also observed in the lymph nodes of HIV-infected patients (Tedla et al., 1999, Biancotto et al., 2007).

CD8+ T cell escape

CD8+ T cells recognize virally infected cells via viral peptide epitopes presented on MHC class I molecules. The virus with the greatest viral fitness is the variant with a mutated epitope that avoids immune recognition, and these variants predominant until they too are recognized. Immune escape is made highly probably by the error-prone HIV reverse transcriptase enzyme, causing rapid mutation acquisition, an HIV hallmark (Borrow et al., 1997a, Goulder et al., 1997, Koup, 1994, Leslie et al., 2004, McMichael et al., 2010, Phillips et al., 1991). As stated earlier, viral CD8+ T cell escape occurs during both the early and chronic infection, indicative of the necessity for the virus to escape immunological recognition by CD8+ T cells (Borrow et al., 1997b, Chen et al., 2000, McMichael and Phillips, 1997). In treated patients there should theoretically be no

viral replication, and therefore no viral evolution (Von Stockenstrom et al., 2015). Viral reservoirs preserve the pre-ART variants with their escape mutations, but overtime the ability of CD8+ T cells to recognize viral reservoirs diminishes as a function of the time between infection and ART initiation (Jones and Walker, 2016). In fact, more than 98% of proviruses in patients treated during chronic infection harbored escape mutations in dominant epitopes that were unrecognizable to CD8+ T cells, but subdominant CD8+ T cell responses against non-escaped epitopes were still found in each of the patients (Deng et al., 2015). The specific targets recognized by CD8+ T cells is also of importance, as strong T cell responses against HIV gag have been associated with better control of viremia and those targeting HIV env with rapid progression (Kiepiela et al., 2007).

CD8+ T cell exhaustion

Persistent exposure to antigen leads to the progressive dysfunction of virus-specific T cells and “T cell exhaustion”. Exhaustion of chronic virus-specific T cells is characterized by altered differentiation, impaired function, and compromised proliferation and/or survival profile (Kuchroo et al., 2014). In the early stages of exhaustion, HIV-specific T cells have an impaired ability to proliferate in response to antigen, as well as reduced expression of IL-2, IFNγ, TNFα, CCL4/MIP1α, and the degranulation marker CD107a (Betts et al., 2006). Later stages of exhaustion are characterized by apoptosis of virus-specific T cells (Mueller et al., 2001). It is thought that exhaustion of virus-specific adaptive cells contributes to viral persistence during chronic infection, including those of HIV/SIV-specific CD8+ T cells (Kim and Ahmed, 2010). The upregulation of PD-1 on HIV-specific CD8+ T cells from viremic patients is

associated with impaired cytokine production, proliferation, survival, and turnover (Day et al., 2006, Petrovas et al., 2006, Petrovas et al., 2013, Trautmann et al., 2006). Other markers of T cell exhaustion include co-inhibitory receptors LAG-3, CD160, and Tim-3 (Peretz et al., 2012, Brenchley et al., 2003, Desai and Landay, 2010). It was recently shown that while virus-specific CD8+ T cells are initially capable of cytolytic activity, the potential is significantly reduced after acute infection (Roberts et al., 2016, Demers et al., 2016).

The quality of HIV-specific CD8+ T cell responses are impaired during chronic infection (Pantaleo et al., 1994), with decreased cytokine production and survival as well as skewed differentiation (Appay et al., 2002a, Champagne et al., 2001b, Betts et al., 2006, Mueller et al., 2001), all characteristic of T cell exhaustion. Recently it was observed that HIV-specific CD8+ T cells from aviremic individuals display a significant upregulation of PD-1, and this expression is associated with impaired cytokine production, proliferation, and survival (Day et al., 2006, Petrovas et al., 2006, Trautmann et al., 2006). ART initiation does result in modest restoration of polyfunctionality with a partial downregulation of activation and exhaustion markers on T cells (Day et al., 2006, Jones et al., 2008, Kaufmann et al., 2007, Nikolova et al., 2005, Grabmeier-Pfistershammer et al., 2011, Kassu et al., 2011, Streeck et al., 2008, Conrad et al., 2012, Rehr et al., 2008). Thus, persistent exposure of CD8+ T cells to antigen and inflammation brings about an exhausted phenotype, in which they are no longer capable of amounting an appropriate response against HIV/SIV and the infection remains.

ART initiation and barriers to CTL-mediated elimination of the viral reservoir

ART suppresses HIV replication, leading to decreased plasma viremia and a reduction in the antigen required for the mediation of CD8+ T cell responses. This lack of antigen changes the phenotypes and functional profiles of the cell populations that remain after the start of ART (Jones et al., 2016). CD8+ T cell cytotoxic and proliferative capabilities are not restored after ART initiation, although prolonged therapy results in some restoration of polyfunctionality and at least partial downregulation of activation and exhaustion markers (Day et al., 2006, Jones et al., 2008, Kaufmann et al., 2007, Nikolova et al., 2005, Grabmeier-Pfistershammer et al., 2011, Kassu et al., 2011, Streeck et al., 2008, Conrad et al., 2012, Rehr et al., 2008). While protease inhibitors have been observed to impair CD8+ T cell function, other barriers to CD8+ T cell-mediated elimination of persistent viral reservoirs include: sequence diversity/immune escape (Borrow et al., 1997a, Goulder et al., 1997, Koup, 1994, Leslie et al., 2004, McMichael et al., 2010, Phillips et al., 1991), T cell exhaustion/dysfunction (Youngblood et al., 2013), suboptimal epitope targeting (Fellay et al., 2007, Carrington et al., 1999, The International et al., 2010, Kaslow et al., 1996, Pereyra et al., 2014), and compartmentalization/separation of CD8+ T cells from viral reservoirs (Fukazawa et al., 2015, Connick et al., 2007, Folkvord et al., 2005).

It remains unclear whether HIV latency poses as a barrier to CD8+ T cell-mediated eradication, as reviewed in (Jones and Walker, 2016). CD8+ T cells can detect even a single MHC-peptide complex on a cell surface (Irvine et al., 2002) implying that even the slightest bit of translation of HIV gene products is capable of exposing latently infected cells to CD8+ T cell killing. Both unspliced and multiply-

spliced HIV transcripts can be detected in resting CD4+ T cells from HIV-infected individuals (Lassen et al., 2004, Chun et al., 2003, Lassen et al., 2006, Hermankova et al., 2003), yet whether these transcripts are translated may be limited by retention in the nucleus (Lassen et al., 2006), transcriptional interference, and “read-through” transcription (Bullen et al., 2014).

Compartmentalization and exclusion

It is thought that perhaps antigen sequestration feeds HIV viral persistence. Most effector CD8+ T cells, including those specific for HIV and SIV, generally lack the proper chemokine receptors to enter the B cell follicle of the lymph node (Connick et al., 2007, Tjernlund, 2010, Vinuesa and Cyster, 2011, Sasikala-Appukuttan et al., 2013, Crotty, 2011). CD4+ T_{FH} cells have been shown to be 30-fold more likely to harbor latently-infected virus than peripheral CD4+ T cells (Fukazawa et al., 2015), perhaps an artifact of the inability of CD8+ T cell localization to the germinal center. The SIV model of elite control is driven by highly effective virus-specific CD8+ T cells that are able to recognize and eliminate at least a subset of infected cells systemically and in the lymph node paracortex without the assistance of latency reversal (Connick et al., 2007). While there is apparent evidence that the lymph node is a site of reservoir sequestration, similar HIV persistence may be facilitated by viral exclusion in immune privileged sites such as the testicles and central nervous system (Galvin and Cohen, 2006, Camelo et al., 2005, Muldoon et al., 2013, Schuppe and Meinhardt, 2005). Other immune privileged sites include the brain, where HIV is thought to replicate in microglial cells independent of CD8+ T cell infiltration and response (Thompson et al., 2011, Gray et al., 2014)

Additionally, the presence of CD4+ and CD8+ tissue–resident memory T cells (T_{RM}) have recently been identified. T_{RM} cells clonally expand in tissue sites and do not readily circulate (Gebhardt et al., 2009, Jiang et al., 2012, Klonowski et al., 2004, Liu et al., 2010, Park and Kupper, 2015, Sathaliyawala et al., 2013, Steinert et al., 2015, Teijaro et al., 2011). It is still unclear whether CD4+ T_{RM} populations contribute to HIV persistence, or whether CD8+ T_{RM} cells are capable of contributing to eradication. It is possible that infected CD4+ T_{RM} that are localized in sites with restricted CD8+ T cell access and may not expose themselves to killing by periodic egress, or that infected CD4+ T_{RM} may harbor escape mutations in CD8+ T cell epitopes that are not well represented in the peripheral blood (Jones and Walker, 2016).

Cytotoxic CD8+ T cell mechanisms

Cytotoxic and noncytotoxic CD8+ T cells have been implicated in being important during the control of HIV infection (Figure 1.3, Table 1). CD8+ T cells have long been characterized by their cytotoxic T lymphocyte (CTL) activity during intracellular infection, most notably during viral infection. CD8+ T cells predominately mediate killing through the secretion of the cytolytic molecules perforin and granzyme contained within lytic granules within the cell (Peters et al., 1991, Shankar et al., 1999) (Figure 1.3, Table 1.1). Degranulation occurs early after CD8+ T cell activation when the lytic granules are released into the immunological synapse formed between the CD8+ T cell and the target cell. Granzymes are serine proteases that induce apoptosis by cleaving caspases (Heusel et al., 1994, Bots and Medema, 2006). Perforin is believed to form pores in the membrane of the cell to allow for delivery of granzyme (Bolitho et al., 2007, Voskoboinik et al., 2006). An essential prerequisite for immunological synapse formation and the

transpiration of degranulation is CD8+ T cell TCR recognition of peptide antigen displayed on MHC class I molecules (Trambas and Griffiths, 2003). Thus, CTL activity is antigen-dependent.

Transcription factors T-bet and Eomes regulate the differentiation and function of effector CD8+ T cells (Cruz-Guilloty et al., 2009, Sullivan et al., 2003, Pearce et al., 2003). While T-bet positively regulates genes associated with perforin, granzyme B, and IFNγ effector function (Jenner et al., 2009, Hersperger et al., 2011b), Eomes positively regulates genes associated with perforin as well as the maintenance of memory CD8+ T cells (Cruz-Guilloty et al., 2009, Pearce et al., 2003, Banerjee et al., 2010, Joshi et al., 2007). The balance between these two transcription factors helps dictate the differentiation and functional pathway of the cell (Joshi et al., 2007, van Aalderen et al., 2015, Buggert et al., 2014, McLane et al., 2013, Paley et al., 2012).

During chronic HIV infection, $Tbet^{hi}$ HIV-specific CD8+ T cells have greater functionality and maintain the ability to express perforin while $T\text{-}bet^{hi}$ $Eomes^{hi}$ HIV-specific CD8+ T cells show reduced differentiation and functionality and increased exhaustion with little to no expression of perforin, and the latter population predominates the HIV-specific CD8+ T cell pool (Hersperger et al., 2011b, Buggert et al., 2014). The loss of HIV-specific CD8+ T cell cytolytic function during chronic infection is a contributing factor to progressive HIV infection (Hersperger et al., 2010, Chen et al., 2009, Migueles et al., 2008, Saez-Cirion, 2007, Appay et al., 2000). Control of viremia is associated with the ability of CD8+ T cells from chronically HIV-infected individuals to upregulate granzyme and perforin *in vitro* (Migueles et al., 2008), and it has also been

shown that the ability of CD8+ T cells to upregulate perforin following stimulation *ex vivo* correlates inversely with viral load (Hersperger et al., 2010).

The peripheral CD8+ T cell response to HIV evolves during the course of the infection. Peripheral blood CD8+ T cell effector responses in subjects with acute HIV infection show a robust and highly activated response with immediate cytotoxic potential within the CD8+ T cell pool (Demers et al., 2016). However, HIV-specific CD8+ T cells lose their ability to upregulate perforin after the resolution of peak viremia, a characteristic that also coincides with reduced T-bet expression, but not Eomes (Demers et al., 2016). While CTL activity appears to be engaged during the acute infection, it seems to be lost during chronic infection resulting in a lack of control and the slow progression to AIDS when left untreated.

Noncytotoxic mechanisms of CD8+ T cell HIV control

Jay Levy, the head of the third laboratory to discover the causative agent of AIDS, observed in 1986 that, in contrast to symptomatic patients, virus could not be isolated from the peripheral blood mononuclear cells (PMBCs) of asymptomatic HIV-infected individuals using standard tissue culture techniques (Walker et al., 1986). Yet, when CD8+ T cells were removed from the peripheral blood cultures of some healthy subjects, virus replication occurred within 7-9 days. Reconstitution of the cultures with autologous CD8+ T cells again suppressed virus replication in a dose-dependent manner, demonstrating that CD8+ T cells play a role in HIV replication *in vitro* and showed that the block in virus production was not due to the killing of virus-infected cells (Walker et al., 1986, Levy et al., 1996). Subsequent studies confirmed that there was no decrease in viability of infected CD4+ T cells cocultured with antiviral CD8+ T cells

(Wiviott et al., 1990). Of note, these original studies used CD8+ T cells from healthy HIV-infected individuals that were preselected to have noncytotoxic anti-HIV responses (CNAR), these original subjects were not necessarily elite controllers, as even some HIV-uninfected individuals were found to have the same activity. Additionally, CD8+ T cells were removed via the Wysocki & Sato panning method (Wysocki and Sato, 1978), which was not effective at removing all CD8+ T cells from PBMCs (Walker et al., 1989). Another study found that the CD8+ T cell anti-HIV activity correlated with the clinical state of the infected individual, where CD8+ T cells from asymptomatic individuals suppressed HIV better than symptomatic and AIDS patients (Mackewicz et al., 1991). Similar findings were found across HIV and SIV strains (Walker et al., 1991b) and the activity was also found to suppress productive infection of macrophages (Barker et al., 1998a).

Levy used two methods to measure the noncytotoxic response of CD8+ T cells during HIV infection *in vitro* (Levy et al., 1996). In the endogenous assay, *ex vivo* HIV-infected CD4+ T cells were cultured in the presence of autologous or heterologous CD8+ T cells at CD8+:CD4+ T cell ratios between 1:20-1:1 (Mackewicz and Levy, 1992, Mackewicz et al., 1991). In the acute infection assay, mitogen-stimulated CD4+ T cells from uninfected donors were acutely infected with HIV ad then cultured with CD8+ T cells from infected individuals at CD8+:CD4+ T cell ratios between 1:4-4:1 (Landay et al., 1993, Mackewicz et al., 1991). The antiviral effect is determined by the extent of reduction of reverse transcriptase activity at various doses of CD8+ T cells relative to CD4+ T cells. Results using these assays demonstrate that the antiviral activity of CD8+ T cells during HIV infection *in vitro* does not require HLA compatibility for efficient

suppression of virus replication (Landay et al., 1993). Many other studies during this era confirmed the presence of an antiviral, noncytolytic CD8+ T cell response that inhibited HIV replication at the level of transcription without killing virus-infected cells (Mackewicz and Levy, 1992, Landay et al., 1993, Mackewicz et al., 1991, Walker et al., 1991a, Tsubota et al., 1989, Brinchmann et al., 1990a, Gomez et al., 1994, Bagasra and Pomerantz, 1993, Hausner et al., 1993). Interestingly, the presence of antiviral CD8+ T cells did not affect activation or proliferation of CD4+ T cells (Landay et al., 1993, Brinchmann et al., 1990b).

Levy and company determined that the noncytolytic response of CD8+ T cells against HIV was related to the secretion of soluble factor(s), which they termed CD8+ Antiviral Factor (CAF). Using both transwell culture systems and also the exposure of CD4+ T cells to CD8+ T cell supernatants, suppression of HIV replication was observed in the absence of physical interaction between CD4+ and CD8+ T cells (Brinchmann et al., 1990b, Mackewicz and Levy, 1992, Mackewicz et al., 1994). Thus, CD8+ T cells may suppress active HIV replication *in vitro* via non-cytolytic mechanisms that are related to the secretion of soluble factors (Mackewicz and Levy, 1992, Levy et al., 1996, Barker et al., 1996, Wiviott et al., 1990, Walker et al., 1991a, Walker and Levy, 1989). Additionally, CAF was shown to be produced by activated HLA-DR+ CD8+ T cells with distinct biochemical and physical properties (Levy et al., 1996, Landay et al., 1993, Barker et al., 1997), including: sensitivity to protease, size range of 10-50kD, and resistance to heat and low pH (Levy et al., 1996, Levy, 2003, Mackewicz et al., 2003a). The maximal secretion of CAF occurs 5-9 days after CD8+ T cell activation (Levy et al., 1996, Mackewicz et al., 1994), unlike secretion of other cytokines which typically occurs

1-3 days after activation (Mackewicz et al., 1994, Landay et al., 1993, Mackewicz et al., 2000a). Additionally, CAF is found in the cytoplasmic fraction of CD8+ T cells, not granules (Mackewicz et al., 2003b). Many soluble factors with anti-HIV activity were tested for CAF activity, including (but not limited to): IL-2, IL-4, IL-10, IL-15, IL-18, TNF α, TNF β, IFN α, IFN β, IFNγ, TGF β, CCL3, CCL4, CCL5, and α-defensins; but none were found to be solely responsible for the CD8+ T cell noncytotoxic anti-HIV response (Levy, 2003, Levy et al., 1996, Brinchmann et al., 1990b, Mackewicz et al., 1994, Mackewicz et al., 2000a) (Rubbert et al., 1997, Walker and Levy, 1989, Mackewicz et al., 1994, Leith et al., 1997). It remains possible that CAF is the activity of multiple factors (Chang et al., 2002).

Despite a significant effort in the laboratory of Dr. Jay Levy, relatively little is known about the exact nature or specific identity of CAF (Mackewicz et al., 1994, Vella and Daniels, 2003, Chang et al., 2003). CAF does not block viral entry, integration, or reverse transcription, nor is it MHC-restricted (Copeland et al., 1997, Mackewicz et al., 2000b, Vella and Daniels, 2003, Chang et al., 2003). Studies have found evidence CD8+ T cells suppress replication by inhibiting viral transcription (Mackewicz et al., 1995) and proviral gene expression (Tomaras et al., 2000, Kiepiela et al., 2007). In addition, CAF is not lentivirus-specific as it was also shown to suppress promotors of other viruses (Le Borgne et al., 2000) and it is not produced exclusively by CD8+ T cells, which led to the hypothesis that CAF is part of the innate immune response (Chang et al., 2002, Le Borgne et al., 2000).

Other immunological factors able to suppress HIV/SIV replication include the β-chemokines CCL3, CCL4, and CCL5 (also known as MIP-1α, MIP-1β, and RANTES,

respectively), which block the entry CCR5-tropic viruses (Alkhatib et al., 1996, Cocchi et al., 1995, Cocchi et al., 2000, Chun et al., 2001). In fact, characterization of CD8+ T cells with a MIP-1 β expression profile has been identified as a correlate of virus control and inhibition (Freel et al., 2012, Ferrari et al., 2011, Freel et al., 2010). HIV/SIV-specific CD8+ T-cells also secret IFNγ, which may play a role in the noncytolytic immune response, however, there is no demonstrable correlation between IFNγ expression and viral load, viral set point, viral clearance, or chronicity, with considerable variation between patients (Lieberman, 2004, Cao et al., 2003, Dalod et al., 1999).

Strong support in favor of the hypothesis that noncytolytic mechanisms of antiviral activity by CD8+ T cells are important in controlling HIV and SIV replication was provided by two independent studies in which the *in vivo* lifespan of productively infected cells was measured in CD8+ lymphocyte-depleted versus non-depleted SIV-infected rhesus macaques (Klatt et al., 2010, Wong et al., 2010). In both studies, SIV-infected macaques were initiated on ART immediately after depletion of CD8+ T cells and the *in vivo* lifespan of productively infected cells was calculated based on the rate of viremia decline under ART using established mathematical models (Perelson et al., 1997, Perelson et al., 1996). Interestingly, both studies showed that the viral decay dynamics at the onset of ART was very similar between CD8+ lymphocyte-depleted macaque and non-depleted animals, thus demonstrating that the relatively short *in vivo* lifespan of productively SIV-infected cells cannot be attributed to cytolytic activity of CD8+ T cells (Figure 1.1 A and B). Instead, the results of both studies are compatible with the hypothesis that noncytolytic mechanisms that do not impact the lifespan of a

productively infected cell are involved in CD8+ T cell-mediated suppression of SIV replication.

The main conclusion of these experiments was independently confirmed by three studies. In the first study, al Basatena et al., sough to determine if the consistent observation of viral escape proves that HIV/SIV-specific CD8+ T cells kill infected cells or could this also be the result of a noncytolytic control (Seich Al Basatena et al., 2013). To this end, these authors developed a 3D cellular automaton model of HIV infection that captures both spatial and temporal dynamics, and reproduces *in vivo* viral dynamics at the cellular and population level. Using this model, al Basatena et al. demonstrated that noncytolytic effector mechanisms can select for viral escape variants. Intriguingly, those viral variants selected by noncytolytic mechanisms of suppression have a slower outgrowth and a lower frequency as compared to those escaping from a cytolytic response, thus suggesting that non-cytolytic responses can provide more durable control of HIV/SIV replication. In the second study, Balamurali et al. investigated the mechanisms of virus-specific CD8+ T cell control during immune escape *in vivo* by using a RT-PCR assay that differentiates wild type virus from escape mutants and studying the dynamics of immune escape in early SHIV infection of pigtail macaques. These authors reasoned that for immune escape mediated by cytolysis, the death rate of wild type-infected cells would be faster than escape mutant-infected cells. However, Balamurali et al. found no significant difference in the rate of decay of wildtype virus compared with escape mutant virus, thus consistent with an epitope-specific, MHC class I-restricted, noncytolytic mechanism of CD8+ T cell control of both wild type and escape mutant variants of SHIV (Balamurali et al., 2010). In the third study, Spits et al.,

tried to identify correlation(s) between markers of CD8+ T cell function that are associated with CTL activity *ex vivo* and the calculated *in vivo* lifespan of productively infected cell by measuring the kinetics of virus decline under ART. The apparently "negative" result that they obtained, i.e., that the lifespan of productively infected cells is similarly short even in patients with the arguably "worse" CTL responses, is consistent with the hypothesis that noncytolytic mechanisms are involved in the anti-HIV effect of CD8+ T cells (Spits et al., 2016).

In conclusion, a number of independent experimental investigations and mathematical analyses suggest that conventional CTL activity does not fully explain the antiviral role of CD8+ T cells in HIV/SIV infection. The possibility that the “CD8 effect” is due to alternative, noncytolytic mechanisms of viral suppression is quite plausible. However, at this time it remains unclear what specific antiviral mechanisms are involved in this phenomenon, and what is the relative contribution of these noncytolytic mechanisms
to the control of HIV or SIV infection *in vivo*.

HIV persistence

The major barrier to eradication of HIV is the presence of latently infected cells that persists in the presence of ART and is maintained for the life time of the patient (Chun et al., 1997b, Finzi et al., 1997, Wong et al., 1997). Termed the “viral reservoir”, this population of cells is long-lived, capable of self-renewable *(in vitro*), and each latently infected cell has a half-life of roughly 44 months (Finzi et al., 1999, Siliciano et al., 2003a). There are several characteristics that cells must meet before they can be considered to have a role in the establishment of latency: cells must, 1) contain a

replication competent integrated provirus, 2) have a mechanism that allows the virus to escape from biochemical decay processes for long periods, 3) have molecular mechanisms in place to suppress viral replication and establish a latent infection, 4) be infected in significant numbers as to contribute to the establishment of a viral reservoir, and 5) have the potential to be activated to produce new viral particles that can reseed the infection (Blankson et al., 2002). Using the SIV infection of rhesus macaque model, it was shown that the viral reservoir is seeded very early following mucosal infection (Whitney et al., 2014a). Early initiation of ART has been shown to improve immune function recovery in HIV infected patients and more importantly reduce the size of the viral reservoir (Saez-Cirion et al., 2013). On the other hand, cessation of ART typically results in viral rebound within two weeks (Davey et al., 1999). Latent infection occurs mostly in resting CD4+ T-cells subsets including central memory, effector memory, T-memory stem cells and naïve T-cells. Overtime there is a progressive reduction of the size of the blood latent reservoir around a core of less differentiated memory subsets (T_{CM} and T_{SCM}) (Jaafoura et al., 2014).

The specific mechanism of reservoir maintenance and persistence is incompletely understood and multiple theories are supported by the literature. Latently-infected cells may have an intrinsic stability that allows them to live for extremely long periods of time (Siliciano et al., 2003a). Evidence also supports the theory that the reservoir is maintained by low levels of homeostatic proliferation (Chomont et al., 2009). Other studies show that the viral reservoir is maintained by residual viral replication occurring under ART (Chun et al., 2005). Perhaps ART is not able to fully penetrate into tissues capable of harboring replicative virus (Fletcher et al., 2014), or viral persistence

in sanctuary sites (testis, brain, eye, etc.) (Cory et al., 2013). These possibilities are not mutually exclusive and the reality is likely a complicated amalgam of mechanisms.

HIV Cure

Two date, two individuals have been successfully cured of HIV. In 2009, an HIV-infected American patient in Germany, “the Berlin Patient” was cured of HIV-1 infection after undergoing treatment for acute myelogenous leukemia with total ablative chemotherapy, radiation therapy, and stem-cell transplantation with donor cells homozygous for CCR5Δ32, with associated graft-versus-host disease (Hütter et al., 2009). Another individual, “the London patient” has since also been cured with a similar strategy (Gupta et al., 2019). In another sadly unsuccessful case, an infant born to a HIV+ mother began receiving ART thirty hours after birth. ART was discontinued when the child was 18 months of age and levels of plasma HIV-1 RNA, proviral DNA in peripheral-blood mononuclear cells, and HIV-1 antibodies, as assessed by means of clinical assays, remained undetectable in the child through 30 months of age (Persaud et al., 2013). However, during a routine clinical care at nearly 4 years of age, the child was found to have detectable HIV levels in the blood, decreased levels of CD4+ T-cells, and the presence of HIV antibodies (NIH News, 2014). This case suggests that very early ART in infants may limit the size of the latent viral HIV reservoir and decrease time to rebound, but ultimately does not cure against long-term persistence of HIV infection.

HIV cure has become an obviously prevalent goal in the field of HIV research. There are two types of achievable cure goals, where cure is defined as the indefinite or permanent absence of plasma viral rebound after ART discontinuation (Chun et al., 2015). A *sterilizing* cure seeks to completely eliminate all traces of HIV in an infected

individual leading to permanent virologic remission, as is the case with the Berlin and London Patients. Alternatively, a *functional* cure attempts to achieve sustained virological remission characterized by replication-competent virus remaining in the body but not causing clinically significant replication or rebound of detectable plasma viremia in the absence of ART (Silvestri, 2013). This state could be maintained by enhancement of the host's endogenous HIV-specific immune response and/or by intermittent administration of immune-mediating agents before or after the discontinuation of ART. This approach is perhaps the most realistic as even in cases of natural control of infection, as with elite controllers, low levels of ongoing viral replication persists, suggesting that complete eradication of HIV reservoirs may not be necessary to achieve sustained virologic remission.

"Shock and Kill" strategy

Strategies additional to ART are necessary to cure HIV, and novel therapies targeting the HIV viral reservoir are of utmost importance. Reversing latency of HIV-infected cells may lead to HIV RNA synthesis, production of viral protein, and release of viral particles, potentially causing direct death of the infected cell and/or indirect killing via recognition by the immune system (Deeks, 2012). Targeted killing of latently-infected cells, particularly during ART, may eliminate the viral reservoir preventing the development of an HIV cure. This therapeutic approach is called the "shock and kill" (Figure 1.4). A latency-reversing agent (LRA) given during a state of viral suppression may be capable of activating CD4+ T cells to "shock" the integrated virus out of latency, exposing the infected cell to cytopathic effects of viral replication and immune recognition. An additional "kill" therapeutic aims to amplify the cytolytic response of the immune system to eliminate infected cells.

The reversal of HIV latency would likely engender a significant increase in plasma

viremia as viral replication is temporarily allowed to resume. Under the assumption that all, or most, latently infected cells synthesize HIV RNA after the administration of an LRA, the “kill” will prove effective if there is a reduction in the size of the reservoir and, most importantly, interruption of ART does not yield a rebound of viremia. Multiple LRAs have already been tested in clinical trials in HIV+ ART-treated patients, but thus far none have shown to elicit the “shock” or “kill” required to eliminate the reservoir, failing to provoke even a minor increase in plasma viremia following LRA administration (Archin et al., 2014, Spivak et al., 2014, Rasmussen et al., 2014, Søgaard et al., 2015, Elliott et al., 2014, Elliott et al., 2015).

CD8+ cells and viral reservoir maintenance

It has recently been shown using the SIV-infected rhesus macaque model that CD8+ T cells are required for maintaining viral suppression during short-term ART treatment (Cartwright et al., 2016). In this study, macaques were infected with SIV_{mac239} and eight weeks later a daily ART regimen was initiated. After 8-32 weeks of therapy, CD8+ lymphocytes were depleted using a monoclonal antibody targeting CD8 α, MT807R1. The depletion of CD8+ lymphocytes resulted in a 72- to 350-fold increase in plasma viremia in 100% of ART-treated macaques. Repopulation of CD8+ T cells was associated with the return of viral control. CD8+ lymphocyte depletion did not significantly change the number of SIV-DNA+ CD4+ T cells in either blood or lymph nodes; thus, it does not appear that CD8+ lymphocyte depletion impacted the size of the viral reservoir. However, there was a significant direct correlation between the amount of cell-associated SIV DNA in CD4+ T cells before depletion and both the peak and the area under the curve of plasma viremia after CD8+ lymphocyte depletion. This suggests that the size of the viral reservoir maintained under ART before CD8+ T cell depletion is a determinant of the ensuing amount of viral production. Longitudinal viral

sequencing by single-genome analysis of SIV_{mac239} envelope at three time points was performed on samples collected from 3 macaques during peak viremia (day 10 post-infection), immediately prior to ART initiation, and after CD8+ lymphocyte depletion. Interestingly, the viral sequences derived from plasma after CD8+ lymphocyte depletion shared more similarities with the virus from peak viremia (day 10) than pre-ART (day 56). This observation supports the hypothesis that a pool of long-lived, latently infected cells infected prior to ART and before the generation of escape mutants rebounds during CD8+ lymphocyte depletion, and that the observed increases in plasma viremia are not caused by *de novo* viral replication. In addition, the study found a significant direct correlation between the level of cell-associated SIV DNA in CD4+ T cells before CD8+ lymphocyte depletion and both the peak and the area-under-the-curve of plasma viremia after depletion. This suggests that the size of the viral reservoir maintained under ART before CD8+ T cell depletion is a determinant of the ensuing amount of virus production.

In this study, a modest increase in CD4+ T cell proliferation was observed likely as a result of homeostatic proliferation of T cells (Barry et al., 2007, Cartwright et al., 2016, Chowdhury et al., 2015). These observations raised the possibility that the observed increases in viral load were a passive consequence of this increased level of CD4+ T cell activation and proliferation, as opposed to the removal of a direct antiviral effect of CD8+ lymphocytes. To address this possibility, our group depleted CD4+ T cells from eight ART-treated SIV-infected macaques and found that while the CD4+ T cells that survived depletion underwent strong homeostatic proliferation (as measured by increased expression of the proliferation marker Ki67) and increased cellular

activation (as measured by increased expression of the markers CD25 and HLA-DR), plasma viremia remained below the limit of detection in all animals and at all time points (Kumar et al., 2018) (Figure 1.1D). Thus, the results of these studies of CD4+ T cell depletion fully support the hypothesis that CD8+ T cells play a previously unappreciated but important direct role in the control of virus production and/or replication in ART-treated SIV-infected rhesus macaques. Further studies with longer follow-up will determine if this effect of CD8+ T lymphocytes is present only in the first several months of ART or persists for longer periods of time under treatment.

It is important to note that in the natural history of HIV and SIV infections both cytotoxic and non-cytotoxic activities result in reduction of virus production and replication, thus acting synergistically in promoting better virus control, with the most obvious example represented by the elite controller phenotype. However, in the setting of ART treatment and in terms of impact on virus persistence and the size of the reservoir, CD8+ T cell mediated cytotoxic activity and CD8+ T cell-mediated suppression of virus transcription may have divergent effects. In particular, while clearance of infected cells via cytotoxic activity will result in a net decrease of the reservoir size, the active suppression of HIV or SIV transcription may paradoxically increase the reservoir size by actively promoting latency. This latter point is of practical importance if we think of ways to manipulate these antiviral roles of CD8+ T cells in ART-treated HIV-infected individuals. In this regard, cytotoxic activity could be enhanced by interventions such as therapeutic vaccinations and/or co-inhibitory blockade. On the other hand, CD8+ lymphocyte depletion could be viewed as a potentially very powerful way to reactivate latent HIV or SIV infection (i.e., latency

reversing agent). Further studies aimed at better elucidating the relative *in vivo* contribution of cytolytic vs. non-cytolytic mechanisms of virus suppression under ART, as well as the molecular pathways that regulate the prevalence of either function of CD8+ T cells, will be crucial to design immune-based interventions that are best suited to reduce the reservoir size in ART-treated HIV-infected individuals.

Chapter 1 summary

In conclusion, HIV is an extremely skilled virus responsible for the worldwide epidemic of our time. It's ability to hide from and dysregulate our immune system eventually converges into the emergence of AIDS and death if left untreated. While antiretroviral therapy has led to a substantial increase in the lifespan of infected individuals, it is not a cure for the infection. Universal eradication of HIV requires a suitable cure. As long as HIV-infected individuals exist there will still be transmission. Curing infected individuals of the disease will decrease the transmissible pool. Nearly forty years after the advent of the epidemic a cure still remains to be found.

CD8+ T cells play a double-edged sword during HIV/SIV infection. While the cells are essential for control of viremia during the acute phase of infection, they proliferate in a non-HIV-specific manner and eventually take on an exhausted phenotype, unable to control the infection. Evidence suggests that CD8+ T cells may actually be contributing to viral persistence, as the presence of CD8+ T cells does not decrease the half-life of productively infected cells. Additionally, CD8+ T cells are required for viral suppression during antiretroviral therapy. These points indicate that CD8+ T cells may be inhibiting

viral replication of HIV/SIV via a non-cytolytic mechanism, specifically via suppression of transcription.

If CD8+ T cells do elicit transcriptional control over HIV, it is possible that reversal of latency requires a CD8+ T cell-free system. In project 1 of my thesis I will explore SIV cure with a latency reversing agent, IL-15 superagonist N-803, in CD8-depleted animals. In projects 2 and 3 I will illustrate the effectiveness of the approach in a translational SHIV-model using sequential depletions with different CD8-targeting antibodies, which opens the door for further studies using HIV-targeting kill approaches.

Chapter 1 Figures

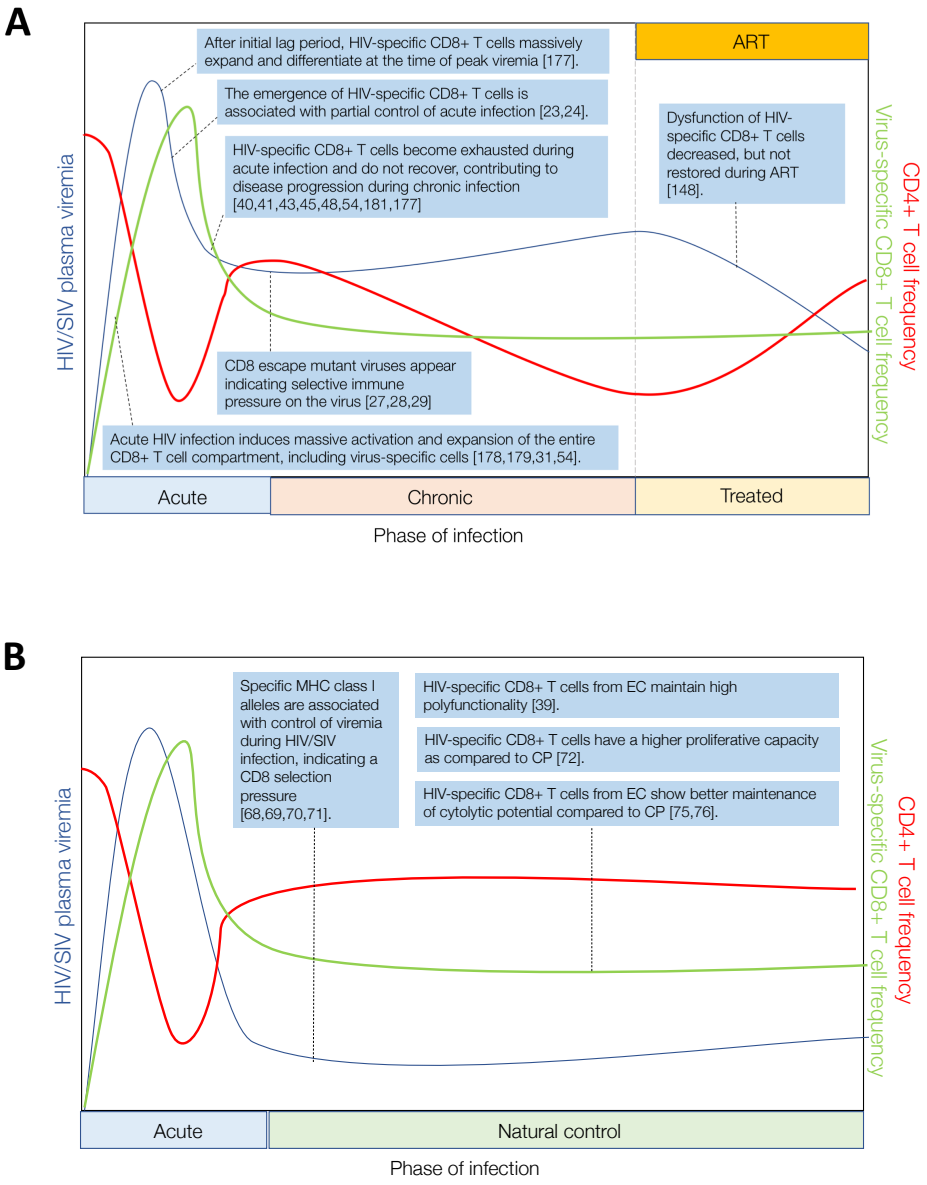

Figure 1: Significant CD8+ T cell events during HIV/SIV infection during A. acute, chronic, and treated infection of chronic progressors, and B. natural control of elite controllers. Abbreviations: SIV: simian immunodeficiency virus; HIV: human immunodeficiency virus; CD: cluster of differentiation; ART: antiretroviral therapy; EC: elite controller; CP: chronic progressor; MHC: major histocompatibility class.

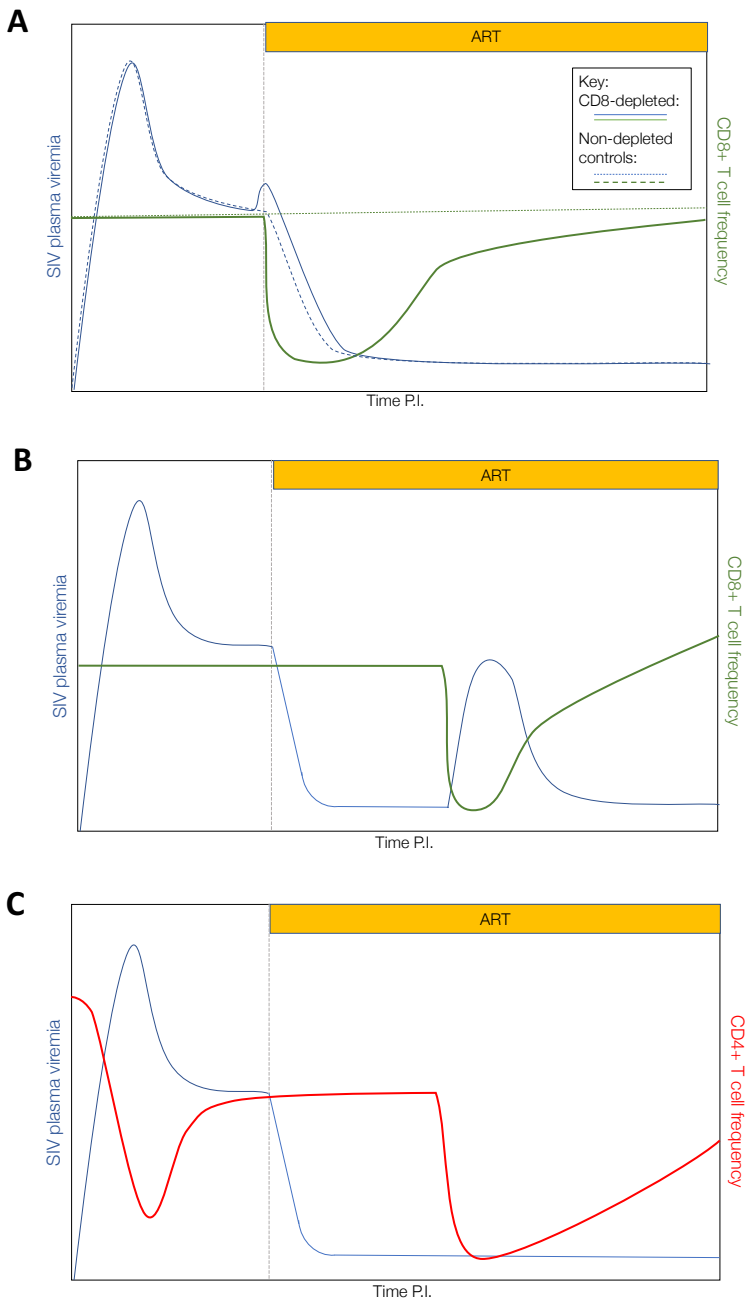

Figure 2: Schematic representations of the association between CD8+ T cell frequency and SIV viral load. A. The initiation of ART in the absence or presence of CD8+ T cells during SIV infection results in similar decay rates of plasma viremia. B. Viral load increases when CD8+ T cells are absent during short-term ART. C. Viral load does not increase when CD4+ T cells are absent during short-term ART. Abbreviations: SIV: Simian immunodeficiency virus; CD: cluster of differentiation; ART: Antiretroviral therapy; P.I.: post-infection.

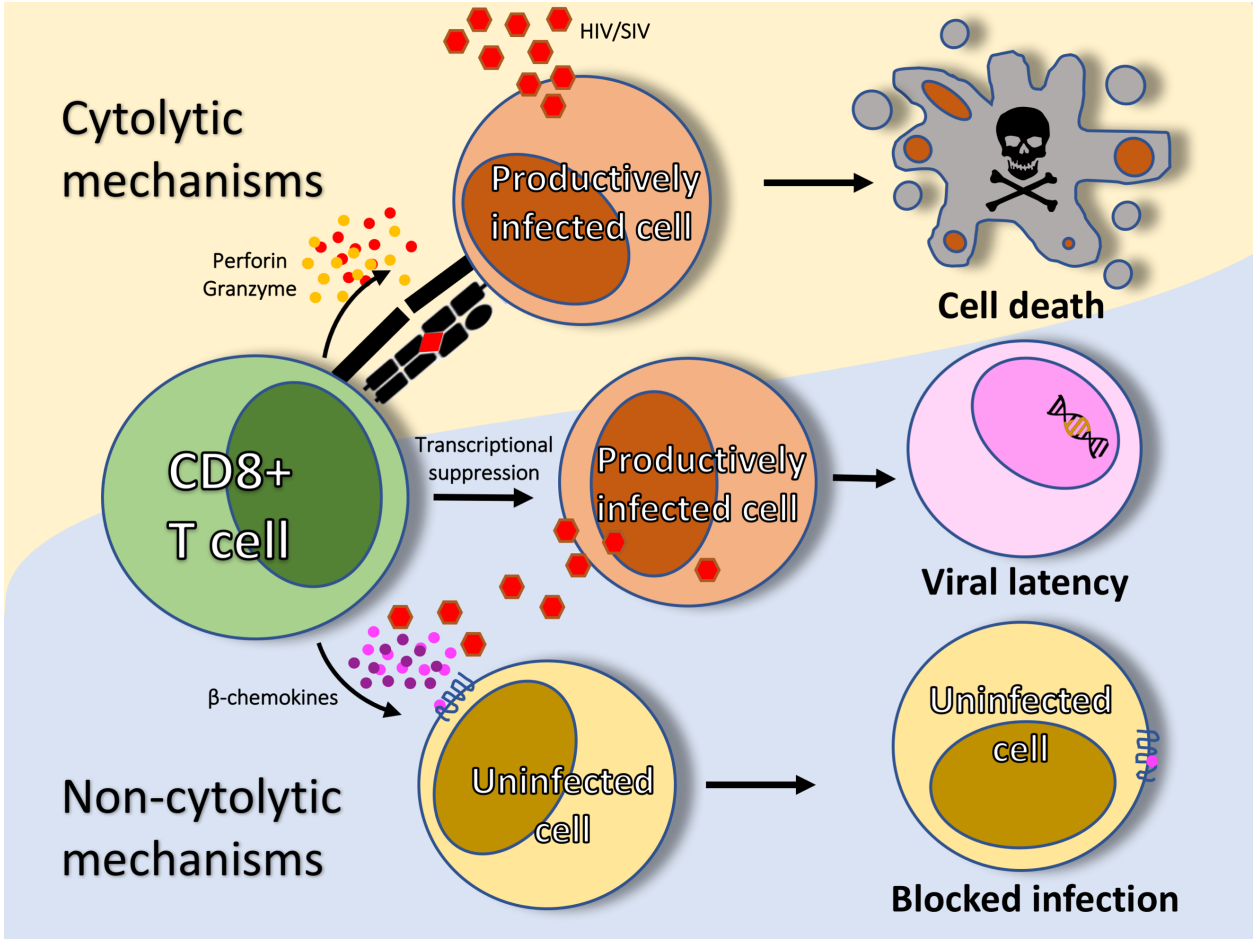

Figure 3: Mechanisms of CD8+ T cell- mediated suppression of HIV/SIV replication. Cytolytic mechanisms of CD8+ T-cells in HIV/SIV infection. Upon CD8 T cell interaction with a productively infected CD4+ T cell, via TCR and co-stimulatory molecules, CD8+ T-cells release granule bound perforin and granzyme which leads to cell death of the infected cell. Non-cytolytic mechanisms of CD8+ T-cells in HIV/SIV infection. CD8+ T-cells also release beta-chemokines in response to HIV/SIV infection, which is hypothesized to protect CD4+ T-cells from becoming infected. Finally, we hypothesize that other non-cytokines mechanisms act on infected CD4+ T cells to block virus expression and promote latent infection.

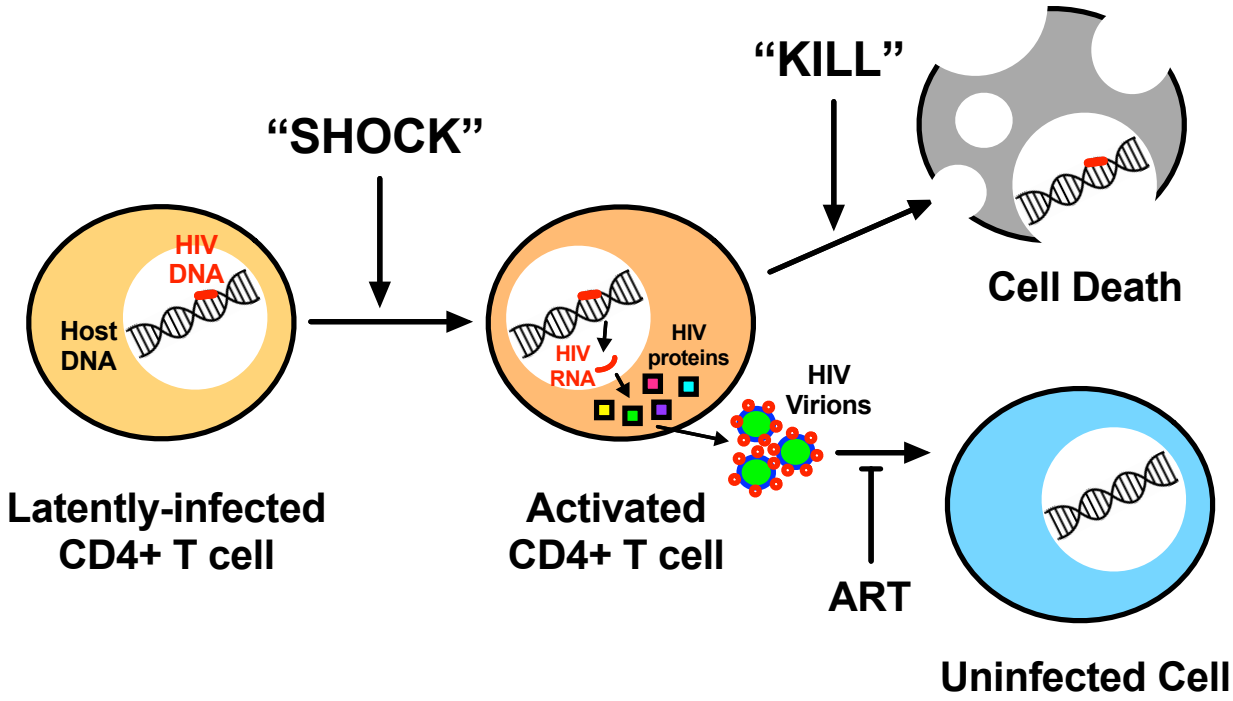

Figure 4: "Shock and Kill" HIV cure strategy schematic. During latent HIV infection, no transcription occurs through the HIV provirus. A latency-reversing agent capable of "shocking" the CD4+ T cell into an active state would induce HIV transcription, resulting in the production of HIV RNA, HIV proteins, and HIV virions. Addition of a "kill" agent would direct the immune response to the infected cell, while ART would prevent de novo infection.

Phase	Finding	Evidence	Reference
Acute infection	CD8 T cells are required for the initial control of HIV viremia.	Depletion of CD8+ lymphocytes from rhesus macaque at the time of SIV infection resulted in abrogation of post peak decline.	(Matano et al., 1998, Schmitz et al., 1999a)
	After initial lag period, HIV-specific CD8+ T cells massively expand and differentiate at the time of peak viremia.	HIV-specific CD8+ T cells exhibit a delay in expansion and differentiation until peak viremia when compartment becomes fully expanded and differentiation in response to systemic proinflammatory cytokine burst, allowing for effective killing of productively-infected cells.	(Takata et al., 2017)
	The emergence of HIV-specific CD8+ T cells is associated with partial control of acute infection.	Increasing frequency of precursor CD8+ T cells specific for HIV-1 gag, pol, and env viral proteins using PBMC from patients experiencing acute HIV infection was correlated with partial resolution of peak viremia.	(Koup et al., 1994, Borrow et al., 1994)
	CD8+ T cells are capable of exerting significant selective pressure on the HIV viral genome.	Identification of the rapid appearance of specific escape mutations in HIV genome.	(Borrow et al., 1997a, Chen et al., 2000)
	Acute HIV infection induces massive activation and expansion of the entire CD8+ T cell compartment	CD8+ T cell frequencies increase during the course of infection in HIV+ individuals and do not return to normal.	(Margolick et al., 1993)
		Activation marker CD38 is up-regulated on Epstein Barr-, Cytomegalovirus- and influenza-specific CD8+ T cells during acute HIV infection, although activation was highest in HIV-specific cells.	(Doisne et al., 2004b)
		HIV-specific CD8+ T cells represent less than 10% of the total CD8+ T cell pool expanded during the acute infection.	(Betts et al., 2001)
		During the acute infection as high as 80%-90% of the entire CD8+ T cell compartment becomes activated.	(Papagno et al., 2004)
	CD8+ T cell expansion can occur through antigen-independent mechanisms.	Microbial products systemically translocated across the gut epithelium contribute to the chronic activation of CD8+ T cells.	(Brenchley et al., 2006)
		Lipopolysaccharide and inactivated HIV activate monocyte-derived dendritic cells, which are capable of activating CD8+ T cells via transpresentation of IL-15. Therefore, proliferation and activation of the CD8+ T cell pool is initiated by cytokines, most notably IL-15.	(Bastidas et al., 2014b)
	HIV-specific CD8+ T cells become exhausted during the acute infection and do not recover.	HIV–specific CD8+ T cells proliferate rapidly upon encounter with cognate antigen in acute infection, but lose this capacity with ongoing viral replication.	(Lichterfeld et al., 2004a)
		HIV-specific CD8+ T cells provide a very early, robust, and highly activated effector response with immediate cytotoxic potential (as measured by perforin expression), but the ability is quickly lost after resolution of peak viremia.	(Demers et al., 2016)
		After full differentiation and expansion, HIV-specific CD8+ T cells reach a hyperproliferation state that is "too strong for too long" and push them to terminally differentiated effector cells that contributes to exhaustion.	(Takata et al., 2017)
Chronic infection	CD8+ T cells contribute to control during chronic HIV infection.	CD8+ T cell depletion during chronic infection results in an increase in viremia that is not controlled until reconstitution of depleted cells.	(Chowdhury et al., 2015, Jin et al., 1999, Metzner et al., 2000b)
	Expanded CD8+ T cell population in chronically HIV-infected patients shows symptoms of immunosenescence.	HIV-specific CD8+ T cells lack of proliferative capability in response to cognate antigen (ex vivo), which could not be overcome by exogenous IL-2 or IL-15. These cells were associated with expression of CD57.	(Brenchley et al., 2003)
		Ex vivo analysis of virus-specific CD8 T cells shows that HIV disease progression correlates with increased proportions of highly differentiated CD8+ T cells, which exhibit characteristics of replicative senescence: CD57 expression, inability to proliferate in response to antigen, and shortened telomeres.	(Papagno et al., 2004)
	The HIV-specific CD8+ T cell compartment has a skewed differentiation pattern towards effector memory during chronic infection.	70% of HIV-specific CD8+ T cells were found to be CD45RA-CCR7-, in contrast to cytomegalovirus-specific CD8+ T cells where only 40% are CD45RA-CCR7-.	(Champagne et al., 2001b)
	Expression of exhaustion markers on HIV-specific CD8+ T cells continues during chronic infection	Persistent antigen during chronic HIV infection contributes to the impairment of HIV-specific CD8+ T cells. HIV-specific CD8+ T cells show significant upregulation of PD-1. Expression correlates positively with impaired function, viral load and inversely with CD4+ T cell count.	(Day et al., 2006, Trautmann et al., 2006,

	and contributes to disease progression.		Petrovas et al., 2006)
		TIM-3 expression on CD8+ T cells correlates positively with viral load and inversely with CD4 counts during chronic HIV infection.	(Jones et al., 2008)
	PD-1 expression on HIV-specific CD8+ T cells is correlated with decreased survival, proliferation, and cytokine expression.	Ex vivo anti-PD-L1 treatment of CD8+ T cells from HIV+ donors led to changes in the ability of the cells to survive, expand, and secrete cytokines.	(Petrovas et al., 2006)
	HIV-specific CD8+ T cells exhibit reduced polyfunctionality during chronic infection.	HIV-specific CD8+ T cells from HIV+ donors exhibit decrease CD107, IFNγ, CCL4, IL-2, and TNFα expression after stimulation.	(Betts et al., 2006)
	HIV-specific CD8+ T cells exhibit impaired cytolytic function during chronic infection	Perforin expression was significantly lower in HIV-specific CD8+ T cells compared to CMV-specific CD8+ T cells of the same donor.	(Appay et al., 2002a)
	CD8+ T cells secrete factors that are capable of suppressing replication of HIV through non-cytolytic mechanisms.	CD8+ T cells were found to release β-chemokines (CCL3, CCL4, and CCL5) with suppressive activities capable of blocking entry of M-tropic viruses.	(Alkhatib et al., 1996, Cocchi et al., 1995, Cocchi et al., 2000)
		Replication of HIV in latently infected, resting CD4+ T cell reservoir is effectively suppressed in ex vivo coculture by autologous CD8+ T cells in EC and ART-treated patients but not ART-naïve patients.	(Chun et al., 2001)
		Identification of the characterization of CD8+ T cells with a MIP-1β expression profile as a correlate of virus control and inhibition.	(Freel et al., 2012, Ferrari et al., 2011)
		CAF suppresses LTR-mediated HIV gene expression in CD4+ T cells.	(Copeland et al., 1997)
		CD8+ T cells suppress replication by inhibiting viral transcription and proviral gene expression].	(Mackewicz et al., 1995, Tomaras et al., 2000, Kiepiela et al., 2007)
		SIV-infected rhesus macaque were initiated on ART in the absence or presence of CD8+ T cells. The rates of viral decay did not differ between the two groups, suggesting that CD8+ T cells do not decrease the lifespan of productively infected cells. Thus, the antiviral mechanism of CD8+ T cells may be non-cytolytic.	(Wong et al., 2010, Klatt et al., 2010)
Natural control	CD8+ T cells are important during the control of SIV viral replication during rhesus macaque controller infection.	Depletion of CD8+ lymphocytes in SIV controller rhesus macaque resulted in a transient and significant increase in viremia and control was reestablished with the reconstitution of CD8+ T cells.	(Friedrich et al., 2007)
	HIV-specific CD8+ T cells from EC maintain high polyfunctionality.	HIV-specific CD8+ showed increase function via expression of 5 functional markers: CD107 (degranulation), IFNγ, CCL4, IL-2, and TNFα.	(Betts et al., 2006)
	HIV-specific CD8+ T cells from EC show better maintenance of cytolytic potential compared to CP.	HIV-specific CD8+ T cells of EC exhibit greater cytolytic capacity compared to CP. The strong ability of EC to kill HIV-infected CD4+ T cells was mediated by the delivery of granzyme B to target cells, an observation not congruent in CP.	(Migueles et al., 2008)
		During chronic infection, cytolytic potential is lost rapidly in most HIV-infected individuals, such that only around 15% of HIV-specific CD8+ T cells express perforin, whereas around 40% express perforin in EC.	(Hersperger et al., 2010)
	HIV-specific CD8+ T cells from EC have a higher proliferative capacity as compared to CP.	High proliferative capacity of HIV-specific CD8+ T cells EC is coupled to increases in perforin expression with relative absence of these functions in CP.	(Migueles et al., 2002)
	Host factors related to CD8+ T cells contribute to the control of HIV infection observed in EC.	CD8+ T cells restricted by certain protective alleles (HLA-B27 and -B57) can resist replicative defects, which permits expansion and antiviral effector activities.	(Horton et al., 2006)

	HIV-specific CD8+ T cells of EC have a higher functional recall memory than CP.	The expansion of CD8+ T cells producing IFNγ alone or in combination with IL-2 in response to gag peptides presented on monocyte-derived dendritic cells is limited in CP compared to EC. This was not observed by CD8+ cells in response to influenza, cytomegalovirus, and Epstein Barr virus.	(Arrode et al., 2005)
	HIV-specific CD8+ T cells put selective pressure on the virus during EC infection.	Sequencing of plasma viremia of EC shows a discordance between the genotypes of the plasma virus and provirus. Specifically, HLA-B*57-restricted Gag epitopes were present in plasma virus but rare in provirus.	(Bailey et al., 2006)
Treated infection	CD8+ T cells are required for the maintenance of viral suppression under ART.	Depletion of CD8+ lymphocytes from SIV+ rhesus macaque during short-term ART results in a rebound of viremia.	(Cartwright et al., 2016)
	HIV-specific CD8+ T cells decline in peripheral blood after the initiation of ART	The longitudinal responses to 95 HLA class I-restricted HIV epitopes were measured using intracellular staining in HIV+ patients beginning ART. A rapid decline in HIV-specific CD8+ T cell response was observed upon initiation of ART. Discontinuation of ART resulted in a rapid increase in HIV-specific CD8+ T cells.	(Casazza et al., 2001)
	Dysfunction of HIV-specific CD8+ T cells is decreased, but not restored during ART.	In a longitudinal study of HIV-infected patients, ART initiation resulted in some restoration of cytokine secretion, increase of IL-7Rα and CD28 expression, and a decline of PD-1 on HIV-specific CD8+ T cells.	(Rehr et al., 2008)
		Defective HIV-specific CD8+ T cell polyfunctionality, proliferation, and cytotoxicity are not restored by ART	(Migueles et al., 2009)
	ART does not resolve CD8+ T cell compartment elevation.	ART does not restore ongoing elevation of CD8 counts despite normalized CD4 count, resulting in a persistently low CD4:CD8 ratio even during virological control. This phenotype is correlated with markers of T cell activation and innate immune active, immunosenescence, and serious non-AIDS events and mortality.	(Serrano-Villar et al., 2014b)
		Early initiation of ART during HIV infection, not prolonged duration of ART, contributes to partial normalization of CD8+ T cell counts.	(Cao et al., 2016b)
	ART is able to partially reverse the exhaustion of virus-specific CD8+ T cells observed during chronic HIV infection.	ART-initiation reverses expression of PD-1 on HIV-specific CD8+ T cells, reversing the functional impairment of these cells that had been caused by the constant presence of HIV antigen.	(Streeck et al., 2008)
		HIV-specific CD8+ T cells from ART-treated patients expressed significantly lower levels of TIM-3 compared with untreated patients and TIM-3 expression was positively correlated with viral load.	(Kassu et al., 2011)

Table 1: Summary of noteworthy studies providing evidence of cytolytic and non-cytolytic antiviral activity of CD8+ T cells during HIV/SIV infection during different phases of infection, including treatment and natural control. Abbreviations: HIV: Human immunodeficiency virus; SIV: Simian immunodeficiency virus; PBMC: peripheral blood mononuclear cell; ART: Antiretroviral therapy; RM: Rhesus macaque; EC: Elite controller; CP: Chronic progressor; CTL: Cytotoxic T lymphocyte; CD: cluster of differentiation; IL: interleukin; IFN: interferon; TNF: tumor necrosis factor; CCL: chemokine (C-C motif) ligand; CCR: chemokine receptor; PD-1: programmed death-1; HLA: human leukocyte antigen; MIP: macrophage inflammatory protein ; CAF: CD8 antiviral factor.

Chapter 2: IL-15 superagonist N-803 administration to SIV-infected long-term ART-treated rhesus macaques induces immunomodulatory effects without SIV reactivation

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Abstract

The main obstacle in the development of an HIV cure is the presence of a population of long-lived, latently-infected cells that persists indefinitely throughout treatment with antiretroviral therapy (ART). Administration of a latency-reversing agent (LRA) during a state of viral suppression could potentially activate CD4+ T cells to "shock" the integrated virus out of latency, exposing infected cells to the cytopathic effects of viral replication and immune recognition. IL-15 superagonist N-803 was recently found to induce HIV antigen expression, reverse HIV latency, and enhance CD8+ T cell killing of cells productively infected with HIV *in vitro* and reduce SIV replication *in vivo* during untreated infection of rhesus macaques. Therefore, N-803 has been proposed as a novel immune-based therapeutic to reduce the virus reservoir under ART. In this study, N-803 was found to transiently modulate peripheral CD4+ T cell, CD8+ T cell, and natural killer (NK) cell populations, specifically through an increase in the expression of genes related to proliferation, metabolism, and antiviral activity via type I and II interferon signaling, as determined by mRNA sequencing. While we did not detect an increase in plasma viremia, cell-associated RNA or DNA, or delay to rebound after ART interruption, we believe that N-803 may contribute to an effective HIV cure when used in conjunction with other HIV/SIV-targeting agents via its ability to modulate the immune system.

Importance

Although antiretroviral therapy effectively suppresses HIV replication, it does not completely eradicate the latent viral reservoir. After decades of viral suppression, interruption of therapy almost invariably results in reactivation of HIV. Reversal of HIV

latency during a state of viral suppression via activation of CD4+ T cells could potentially eliminate the viral reservoir by exposing infected cells to the cytopathic effects of viral replication and immune recognition. To the best of our knowledge, this paper is the first known to report the antiviral effects of high dose N-803 in SIV-infected, ART-treated rhesus macaques. We find that administration of N-803, an IL-15 superagonist complex, to long-term antiretroviral therapy-treated simian immunodeficiency virus-infected rhesus macaques results in transcriptomic and phenotypic modification to CD4+ T cells, CD8+ T cells, and NK cells that reflect immune stimulation and response. Thus, N-803 may function as a latency reversing agents in combination with other anti-HIV/SIV therapeutics.

Introduction

Human immunodeficiency virus (HIV) is the causative agent of acquired immunodeficiency syndrome (AIDS) and infects an estimated 36.7 million people worldwide (Barre-Sinoussi et al., 1983, [internet], 2016). UNAIDS estimates 1 million AIDS-related deaths annually, in addition to 1.8 million new HIV infections (UNAIDS, 2017). While antiretroviral therapy (ART), the standard care for HIV infection, has dramatically reduced the mortality and morbidity of HIV infection, ART fails to cure infection.

The main obstacle in the development of an HIV cure is the presence of a population of long-lived, latently-infected cells that endures even in the presence of ART (Chun et al., 1997c, Finzi et al., 1997, Wong et al., 1997, Siliciano et al., 2003a). This stable virus reservoir occurs primarily within resting memory $CD4^+$ T cells and persists despite decades of therapy (Chun et al., 1997b, Chomont et al., 2009, Chun et al.,

1995, Chun et al., 1997a, Finzi et al., 1997, Wong et al., 1997, Finzi et al., 1999, Siliciano et al., 2003a). Recent data suggests that the HIV viral reservoir is established early during infection and is responsible for viral rebound observed after ART interruption (Whitney et al., 2014b, Ananworanich et al., 2013, Davey et al., 1999, Chun et al., 1999). Therefore, strategies additional to ART are necessary to cure HIV, and novel therapies targeting the HIV viral reservoir are of utmost importance. Targeted killing of latently-infected cells, particularly during ART, may eliminate the viral reservoir preventing the development of an HIV cure. This therapeutic approach is called the "shock and kill". Latency-reversing agents (LRAs) aim to activate CD4+ T cells in order to "shock" the integrated virus out of latency. An additional "kill" therapeutic aims to amplify the immune recognition and cytolytic responses to eliminate infected cells. Multiple compounds have been tested in clinical trials in HIV+ ART-treated patients to reverse latency of HIV-infected cells, but thus far none have been shown to elicit the "shock" or "kill" required to eliminate the reservoir (Archin et al., 2014, Spivak et al., 2014, Rasmussen et al., 2014, Søgaard et al., 2015, Elliott et al., 2014, Elliott et al., 2015).

Interleukin-15, or IL-15, is a cytokine involved in the maintenance of naïve, effector and memory T cells and NK cells. IL-15 is imperative for the proliferative and homing functions essential to mature lymphocytes, as well as the differentiation of effector and memory T cells (Lodolce et al., 1998, Lodolce et al., 2002). N-803 (formally ALT-803), an IL-15 superagonist complex, was found to cause antigen-independent activation of T cell and NK cells (Rhode et al., 2016) and is being tested in clinical trials as a cancer therapeutic (NCT01946789, NCT02099539, NCT02138734, NCT02523469,

NCT02384954, and NCT02559674). Recently, Jones et al. tested multiple LRAs and found N-803 most effectively induced both HIV antigen expression and latency reversal, and enhanced CD8+ T cell killing of productively HIV-infected cells *in vitro* (Jones et al., 2016). Another study showed that when N-803 was given to untreated SIV+ controller macaques, total CD8+ effector and central memory T cell and NK cell populations were transiently elevated in the peripheral blood, while viral loads transiently decreased by ~2 logs, suggesting that N-803 may contribute to control of SIV replication (Ellis-Connell et al., 2017). Moreover, N-803 was recently found to activate and redirect SIV-specific CD8+ T cells from the peripheral blood into B cell follicles, a hot spot of persistent infection (Webb et al., 2018, Fukazawa et al., 2015, Banga et al., 2016). SIV RNA expression and SIV DNA levels were decreased after N-803 treatment in the lymph nodes of controller macaques included in the study. Consequently, N-803 may function *in vivo* as a “shock” in its ability to reverse HIV latency and induce HIV antigen expression. Additionally, N-803 may amplify a “kill” therapeutic in its capacity to activate T cells and NK cells independent of antigen, enhance CD8+ T cell killing of virus-specific cells, and home virus-specific CD8+ T cells to the sites of latent infection.

In this study, we evaluated the ability of N-803 to modulate the immune system to support latency reversal, promote virus reactivation, and decrease the size of the viral reservoir in seven treated SIV+ rhesus macaques. Four weekly doses of 100 μg/kg N-803 were administered to seven SIV-infected RM after one year of ART. Three weeks after the last dose of N-803, animals were interrupted of ART and followed for 6 months before necropsy. Following ART interruption, viral load quickly rebounded without control. There were no significant changes to viral load or cell-associated RNA and DNA

levels in peripheral CD4+ T cells after administration of N-803. On the other hand, phenotypic and transcriptomic analysis of CD4+ T cells, CD8+ T cells, and NK cells following *in vivo* treatment with N-803 revealed a transient increase in markers and gene expression associated with proliferation, activation, metabolism, antiviral responses, and IFNα and IFNγ signaling. The immunomodulatory effects of N-803 indicates its potential to support the reversal of HIV latency and respond to virally-infected cells, thus, we believe that N-803 may contribute to an HIV cure when used in conjunction with another agent(s) capable of reversing latency of the virus reservoir.

Results

N-803 does not induce SIV reactivation in the plasma or cell

Seven Indian-origin rhesus macaques (RM) were infected intravenously with SIV_{mac239} and started on a combination antiretroviral therapy 8 weeks post-infection (Fig. 1A). Animals were kept on ART for 12 months prior to the start of intervention. N-803 was administered as a cycle of 4 weekly doses of 100 μg/kg via subcutaneous route. Animals remained suppressed (viral loads <60 copies/mL) following N-803 treatment (Fig. 1D). The Friedman significance test (non-parametric, matched) was used to evaluate viral load changes as compared to the pre-intervention time point (baseline). Viral loads after intervention were not found to be significantly different than baseline. Additionally, levels of cell-associated RNA were measured before N-803 administration and one week after as a measure of copies of SIV gag RNA per copy of

CD4. N-803 administration did not increase the expression of viral RNA one week after the first dose was administered (Fig 1B).

N-803 does not decrease the size of the total cell-associated SIV DNA reservoir or delay viral rebound following ART interruption

SIV DNA was extracted from pellets pre-intervention, week 1, and week 6 and total cell-associated SIV DNA was measured. There were no significant changes in the frequency of CD4+ T cells harboring cell-associated SIV DNA at weeks 1 or 6 post-intervention (Fig 1C). At 6 weeks post-intervention, rhesus macaques were interrupted of antiretroviral therapy and monitored weekly for roughly 6 months before elective necropsy. Viral loads dramatically increased within three weeks of ART-interruption and remained highly elevated (Fig 1D).

N-803 induces an increase in the frequency of CD8+ T cells in the peripheral blood and lymph node

As IL-15 is important for the homeostasis and differentiation of T cells and NK cells (Lodolce et al., 1998, Lodolce et al., 2002), we assessed changes in the frequencies and proportions of CD4+ T cells, CD8+ T cells, and NK cells in the blood, lymph node, and rectum. Only CD8+ T cells displayed a significant increase in peripheral blood absolute count during intervention as compared to pre-intervention baseline, peaking around weeks 1 and 2 after the first dose of N-803 (Fig. 2A). When calculating the percentage of CD4+ cells and CD8+ cells as part of the parent CD3+ T cell population, the CD8+ T cell subpopulation increased while the CD4+ T cell subpopulation decreased in peripheral blood and the lymph node during the intervention and there were no significant changes to CD4+ and CD8+ proportions of CD3+ T cells

in the rectum (Fig. 2B). NK cell percentages of lymphocytes were also calculated for peripheral blood and interestingly no significant differences were found during multi-dose N-803 treatment (Fig. 2A).

N-803 induces expansion of the effector memory T cell compartments

Memory subsets were calculated as a frequency of the percentage of bulk CD4+ or CD8+ parent T cell. Effector memory CD4+ T cell frequency significantly increased in the peripheral blood during N-803 administration while there was a significant decrease in proportion in the rectum by week 6 compared to baseline (Fig. 2D). A similar trend was observed in CD8+ T cells, where effector memory subpopulation frequency increased in the peripheral blood and additionally the lymph node, yet decreased in the rectum with more durable and significant changes (Fig. 2E). Interestingly, central memory T cells decreased quite significantly in the lymph node by week 6 post-intervention in both CD4+ and CD8+ compartments.

No clear change to percentages of NK cell subpopulations following N-803 treatment

Furthermore, we assessed changes in the frequency of NK cells subtypes. The predominant CD16+CD56- NK cell population is known for its cytolytic activity and limited cytokine production in rhesus macaques (Lanier et al., 1986), while the CD16-CD56+ population is traditionally considered to possess “regulatory” functions (Cooper et al., 2001, Hong et al., 2013). Double negative CD16-CD56- populations are representative of an intermediate between CD16+ and CD56+ NK cells (Hong et al., 2013) and an unclear double positive population. Changes to the frequencies of NK cell subsets during N-803 intervention were minimal, with a modest decrease in the

population of cytolytic CD16+CD56- NK cells by week 6 with a slight increase in the intermediate CD16-CD56- population (Fig. 2C). There was a significant increase of Ki67 expression three days after the first N-803 administration but the effect was not durable (Fig. 2F).

N-803 induces phenotypic changes in bulk CD8+ T cell, CD4+ T cell, and NK cell populations in the peripheral blood, lymph node, and rectum

N-803 treatment produced a transient increase in the expression of proliferation marker Ki67 on bulk CD8+ T cells and NK cells at day 3 (Fig. 2F). Interestingly, there was also a significant increase of Ki67 expression on CD8+ T cells in the lymph node and rectum by week 6 (Fig. 2F). N-803 induced activation of CD4+ T cells in the peripheral blood through week 4 and activation of CD8+ T cells in the lymph node and rectum at week 6 (Fig. 2G). CD8+ T cells in the peripheral blood displayed a late increase in CCR5 expression (Fig. 2H) with no significant changes of expression in the CD4+ T cell compartment.

N-803 induces transcriptomic changes in CD4+ T cells, CD8+ T cells, and NK cells

Sequencing of mRNA reveals upregulation of gene sets associated with cellular proliferation, immune responses, metabolism, and various cell signaling pathways three days after the first administration of N-803 (Fig. 3A). Analysis of enrichment plots reveals transient and cell-specific patterns.

Gene sets related to inflammation and immune response are upregulated following N-803 administration. IFNγ response genes are upregulated at day three in CD4+ T cells, CD8+ T cells, and NK cells, and gene expression significantly falls by

week 2 or 4 (Fig. 3B top), similar results are found for IFNα response genes (Sup. Fig. 1). Additionally, complement genes sets are upregulated in CD4+ and CD8+ T cells.

Not surprisingly, cell cycle- and proliferation-related genes sets are upregulated across cell types. Specifically, G2M checkpoint genes are upregulated at day 3, especially in CD8+ T cells, and expression is lost most rapidly in NK cells by week 2 (Fig. 3B middle). This pattern is consistent in the E2F target and Myc target gene sets (Sup. Fig. 1).

Signaling pathway genes are upregulated differentially across cell types; upregulation of mTORC1 signaling, a gene set associated with T cell activation, was most consistent (Sup. Fig. 1). Other signaling pathways upregulated include TNFα via NFkB, IL-6/JAK/STAT-3, and IL-2/STAT-5 (Fig. 3A). These gene sets appear to be only transiently modulated, with upregulation most prominent on day 3 post N-803 administration in CD4+ T cells (Fig. 4A-C).

Additionally, genes related to metabolism are impacted by N-803 administration. While oxidative phosphorylation genes are down-regulated by CD4+ T cells during weeks 2 and 4 post-N-803 administration, the gene set is upregulated on day 3 in CD8+ T cells and NK cells (Fig. 3B bottom). Glycolysis genes are not significantly different following N-803 administration in CD4+ T cells, but are upregulated in CD8+ T cells and NK cells (Sup. Fig. 1).

From these results, it appears that across CD8+ T cells, CD4+ T cells, and NK cells there appear to be an upregulation of genes involved in the immune response to antigen following N-803 administration. Broadly speaking, this includes proliferation, signaling, inflammation, activation, and metabolism. The level and duration of

enrichment varied across cell types with NK cells appearing to have the least durable response.

Discussion

N-803 is an IL-15 superagonist complex with promising therapeutic potential in the field of HIV and cancer curative strategies. At the time of writing this manuscript, the compound is already being tested in 8 clinical trials, including a study in HIV+ ART-treated patients (NCT02191098). N-803 is relevant to HIV as it was previously shown to reverse HIV latency *in vitro* (Jones et al., 2016), decrease SIV viral replication (Ellis-Connell et al., 2017), and direct SIV-specific CD8+ T cell to the lymph node follicle *in vivo* (Webb et al., 2018).

To the best of our knowledge, this paper is the first known to report the antiviral effects of N-803 in SIV-infected, ART-treated rhesus macaques. Using an ultra-sensitive method of SIV detection in plasma viremia, we were unable to detect any significant changes in plasma viral load following N-803 treatment. Additionally, we were unable to detect reactivation of viral RNA in cells in peripheral blood or lymph node. There were no significant differences between the size of the reservoir weeks 1 and 6 after the start of intervention as compared to pre-intervention values. Interruption of ART is another indicator of the size of the reservoir and upon ART secession all animals quickly rebounded.

Previous studies in macaques with N-803 have shown significant phenotypic alternations to the T cell and NK cell compartments (Rhode et al., 2016, Webb et al., 2018, Ellis-Connell et al., 2017). In the context of long-term ART-treated SIV-infection, N-803 administration induced expansion of the bulk CD8+ T cell compartment and

reduction of the bulk CD4+ T cell compartment in the peripheral blood and lymph node. N-803 did not appear to induce significant changes to the frequency of the bulk NK cell compartment. When comparing subsets, effector memory CD8+ and CD4+ T cells increased in frequency after N-803 administration in the peripheral blood and lymph node. Only modest changes by week 6 were observed in the NK subsets. Additionally, N-803 administration temporarily induced significant Ki67 expression on CD4+ T cells, CD8+ T cells and NK cells in the peripheral blood but the effect was not durable. Ki67 expression appears to be increased 6 weeks after the start of intervention on CD8+ T cells in the lymph node and gut, with a similar pattern observed with PD-1 expression. We also noted a significant and durable upregulation of PD-1 on CD4+ T cells in the peripheral blood after N-803 administration. Lastly, N-803 administration resulted in a delayed upregulation of CCR5 expression on CD8+ T cells in the peripheral blood. These conclusions suggest that N-803 affects CD8+ T cells with initial expansion and increased frequency of effector memory subset in the peripheral blood and lymph node. It was surprising that the effects of N-803 on CD4+ T cell and NK cell populations were less pronounced via flow cytometry.

To this best of our knowledge, this is the first paper detailing the transcriptomic effects of N-803 in T cells and NK cells using the rhesus macaque model. Across cell types we observed an upregulation of gene sets related to cell cycling and proliferation, which supports the notion that IL-15 is involved in T cell and NK cell maintenance and homeostatic proliferation. Interestingly, the level and duration of the effect was cell-type dependent. We observed increases in immune/inflammatory gene sets consistent with the observation that IL-15 induces antiviral activity. Gene sets related to cell signaling

were also upregulated, such as MTORC1 signaling, which supports a role of IL-15 in cellular activation and stimulation. Lastly, metabolic gene sets, important for proliferation, activation, and immune response were upregulated in CD8+ T cells and NK cells. Transcriptome changes following N-803 administration support the notion that the compound is able to activate the immune system, but it is not apparent that N-803 was able to independently reverse SIV latency. An important caveat of this study is the biases induced by sample collection schedules. The limited blood and tissue sampling may have affected our ability to monitor changes in marker expression and subpopulation frequencies. The effects of N-803 on latency reversal may only be evident for a short window after N-803 administration that would have been missed by our weekly blood collections and infrequent tissue collections. Further studies should monitor plasma during the first 24-48 hours following drug administration. In conclusion, while N-803 alone did not appear to independently “shock” the latent SIV reservoir, the immunomodulatory effects observed may heighten the reversal and/or killing potential of other therapeutics when used in combination.

Methods

Animals, SIV-infection, drug administration, and sample collection/processing

This study was conducted using a total of 7 Indian-origin rhesus macaques (RM). All RMs were HLA*B07- and HLA*B17- with the following being HLA*A01+: RVz15, RBn16, and RRn16. Animals were infected intravenously with 3000 TCID50 SIV_{mac239}, and began a three-drug combination ART regimen at eight weeks post-SIV infection. Tenofovir (TDF at 5.1mg/kg/day or PMPA at 20mg/kg/day) and Emtricitabine (FTC at 40mg/kg/day) were both kindly provided by Gilead Pharmaceuticals. Dolutegravir (DTG at 2.5mg/kg/day) was kindly provided by ViiV Pharmaceuticals. Drugs were administered daily by subcutaneous injection. Of note, two animals, REf16 and RVz15 were depleted of CD4+ T cells with CD4R1 (provided by NHP Reagents) four months prior to CD8 depletion for a separate study (Kumar et al., 2018). No rebound of viremia was observed during the depletion of CD4+ T cells and the CD4+ T cell counts at the time of intervention with N-803 were 507 and 954 cells/mm^3, respectfully. After 12+ months of ART, animals were initiated on a dose of N-803, consisting of 4 weekly subcutaneous injections at 100μg/kg. This high dose was deemed safe and effective in previous macaque studies (Webb et al., 2018, Rhode et al., 2016). Longitudinal collection and processing of blood, lymph node biopsies, and rectal biopsies were performed as previously described (Kumar et al., 2018, Cartwright et al., 2016).

All animals were housed at the Yerkes National Primate Research Center, Emory University. These studies were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, and were approved by the Emory University (AWA no.

A3180-01) Institutional Animal Care and Use Committee (IACUC). All animals were anesthetized before the performance of any procedure, and proper steps were taken to ensure the welfare of and to minimize the suffering of all animals in the proposed studies.

Immunophenotyping by flow cytometry

Multiparametric flow cytometry was performed according to a standard protocol on PBMC and LNMC using fluorescently labeled monoclonal antibodies cross-reactive in RM. The following antibodies were used at 37°C for 30 minutes: CCR5 APC (3A9) and CCR7 FITC (150503). Additionally, the following antibodies were at room temperature for 30 minutes: CD3 APC-Cy7 (SP34-2), CD4 BV650 (OKT4), CD8α BV711 (RPA-T8), CD8β PE-Cy5 (SIDI8BEE), CCR5 APC (3A9), CCR7 FITC (150503), CD45RA Pe-Cy7 (5H9), CD62L PE (SK11), CD95 BV605 (DX2), PD-1 BV421 (EH12.2H7), CD16 BV421 (3G8), CD20 PE-Cy5 (2H7), CD14 PE-Cy7 (M5E2), NKG2A (CD159) PE (Z199), CXCR5 PE-eFluor610 (MU5UBEE), CD28 PE-Cy5.5 (CD28.2), and CD56 FITC (NCAM16.2). Cells stained for Ki67 were fixed and permeabilized with Perm II (BD) kit before being stained at room temperature for 30 minutes with Ki67 AF700 (B56). All flow cytometry specimens were acquired with 24 hours on an LSR II (BD Biosciences) equipped with fluorescence-activated cell sorter software (FACS Diva), and analysis of the acquired data was performed using FlowJo software (Tree Star).

Determination of plasma viral load, cell-associated DNA, and cell-associated RNA

For pre-intervention time points, quantitative real-time RT-PCR was performed to determine SIV plasma viral load as previously described with a sensitivity of 60

copies/mL(Taaffe et al., 2010). For the three time points prior to intervention and all post-intervention time points plasma SIV *gag* RNA using quantitative PCR, essentially as described using high sensitivity assay formats(Hansen et al., 2017, Li et al., 2016). Quantification of total cell-associated SIVmac$_{239}$ *gag* DNA was performed as previously described(Chahroudi et al., 2014). The number of *gag* DNA copies per 10^6 CD4+ T cells was calculated by dividing the number of *gag* DNA copies/10^6 PBMC by the percentage of CD3+CD8-CD4+ cells in the PBMC population. CD4+ T cells were isolated from PBMC via the CD4+ T cell isolation kit (Miltenyi) and cell-associated RNA was measured as previously described(Kumar et al., 2018).

Flow cytometry cell sorting

Mononuclear cells isolated from blood were stained with Live/dead, CD3 AF700 (SP34-2), CD4 BV650 (OKT4), CD8 APC-Cy7 (SK1), CD14 PB (M5E2), CD20 PB (2H7), and CD16 PB (3G8), for 30 minutes at room temperature. Alliquots of 50,000 CD4+ T cells (live CD3+CD20-CD14-CD16-CD8-CD4+) and CD8+ T cells (live CD3+CD20-CD14-CD16-CD4-CD8+) were then sorted using a FACS Aria II (BD Biosciences). Mononuclear cells were also separately stained with Live/dead, CD3 AF700 (SP34-2), CD4 BV650 (OKT4), CD8 APC-Cy7 (SK1), CD14 PB (M5E2), CD20 PB (2H7), and NKG2A PE (Z199) to sort aliquots of 50,000 NK cells (Live CD3-,CD20-,CD14-CD4-,CD8+,NKG2A+).

RNAseq

Bulk CD4+ T cells, CD8+ T cells, and NK cells were sorted from fresh PBMCs prior to N-803 administration, day 3, week 2, and week 4 post-administration. Briefly, RNA from sorted cells was collected and extracted and DNA was digested. Libraries

were prepared and normalized, pooled, and clustered on a flow cells for sequencing. RNAseq data were aligned to the MacaM v7.8 assembly of the Indian RM genome. To identify pathways differentially modulated by N-803, Gene Set Enrichment Analysis (GSEA)(Subramanian et al., 2005) was performed on the ranked transcript lists using 1000 phenotype permutations and random seeding. Gene sets used included the MSigDB H (hallmark) gene sets(Liberzon et al., 2011). Heat map colors represent log2 transformed library size normalized read counts scaled to unit variance across transcript vectors and normalized to the baseline median sample value of each transcript.

Statistical analyses

Measurements were performed in Prism Graphpad (version 7.0a) using non-parametric matched Friedman test or non-parametric unmatched Kruskal-Wallis test. Values were compared against baseline pre-intervention. Significance was attributed as *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.000$.

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Chapter 2 Figures

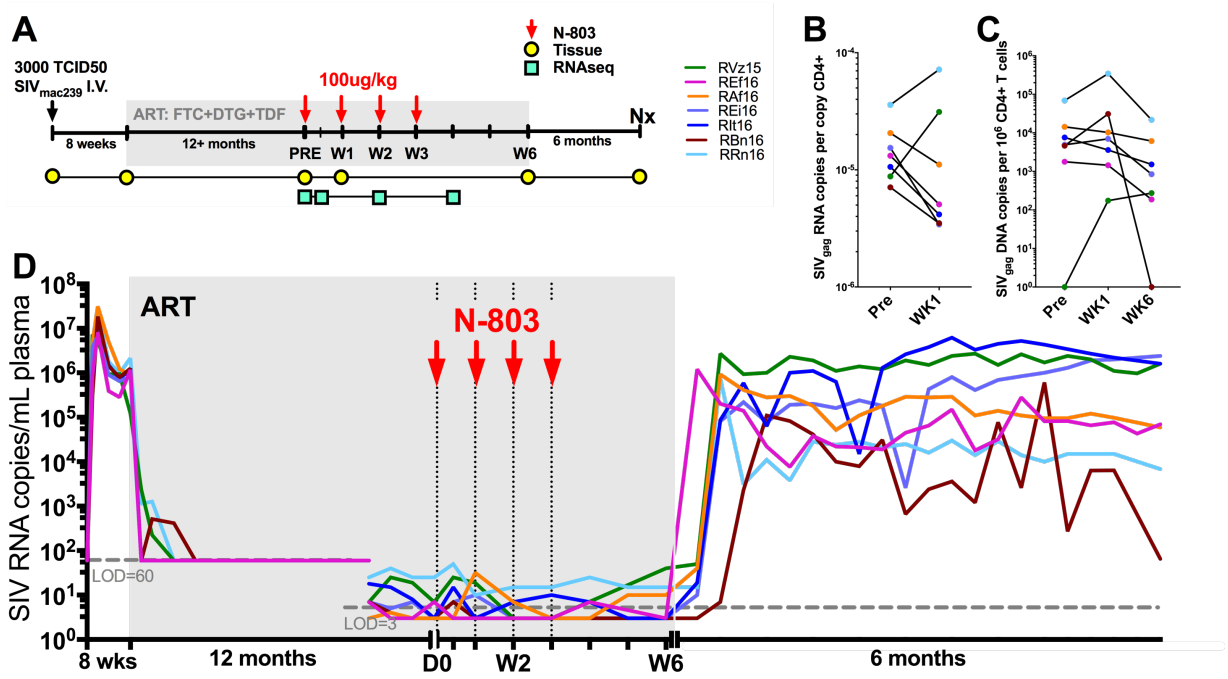

Figure 1: Treatment of SIV+ ART-treated RM with N-803

(A) Study design. 7 RM were infected with 3000 TCID50 SIVmac239 I.V. and initiated antiretroviral therapy (ART) 8 weeks post-infection. 4 weekly doses of 100 µg/kg N-803 were administered after 12 months of ART. 3 weeks after the last N-803 dose animals ART was interrupted. 6 months later animals were necropsied. Blood and plasma were collected routinely throughout study. Tissue (lymph nodes and rectal biopsies) were collected at major time points. PBMCs were sorted for RNAseq analysis at 4 time points. N-803 treatment does not induce any significant changes to the viral load as compared to pre-intervention baseline. Statistics were conducted with a Kruskal-Wallis test (unmatched, non-parametric). P=0.7779. (B) Re-expression of cell-associated viral RNA was assessed by measuring the number of copies of HIV_{gag} in relation to the number of copies of CD4+ and there did not appear to be a significant change after N-803 administration. Statistics were calculated with a Wilcoxon test (paired, non-parametric). P= 0.9375. (C) Cell-associated DNA viral reservoir in CD4+ T cells after intervention. The number of copies of SIV_{gag} DNA in PBMC and the number of copies per 10^6 CD4+ T cells was corrected for using CD4 T cell counts obtained by flow cytometry. DNA was extracted from pellets pre-intervention, week 1 post, and week 6 post. Statistics were calculated with a Friedman test (matched, non-parametric) and compared against the pre-intervention time point. p=0.0854. (D) Change in viral load after infection, ART initiation, N-803 doses, and ART interruption. The limit of detection (LOD) for the first 11 months post-infection is 60 viral copies/mL of plasma. An ultra-sensitive assay was used with a sensitivity of 3 copies/mL of plasma starting at month 11 post-infection. Animals took an average of 4 weeks to suppress viremia below a limit of detection of 60 copies/mL of plasma (standard deviation: 2.9 weeks).

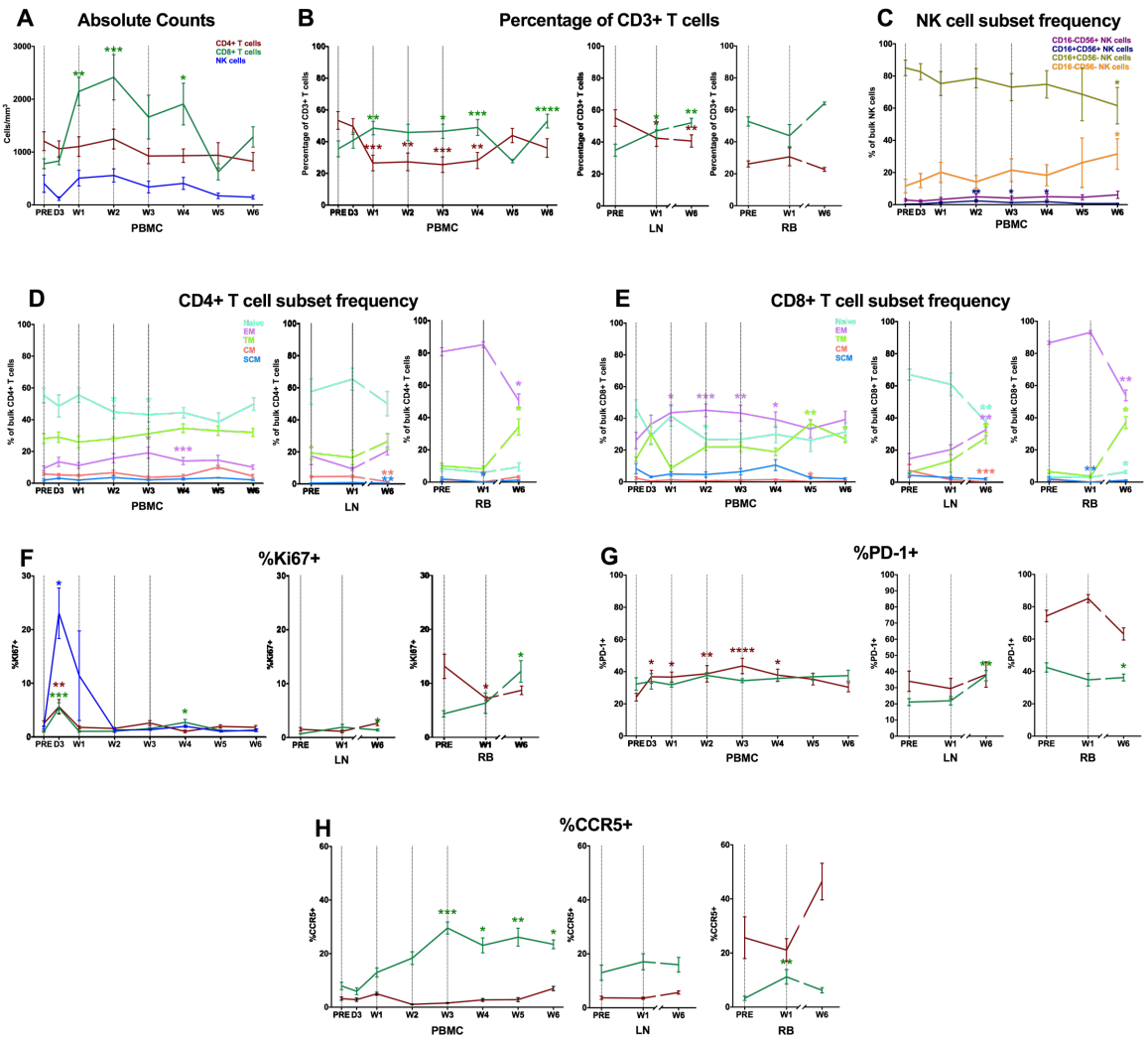

Figure 2: Changes in bulk and subset T cell and NK cell frequencies after N-803 administration

(A) Mean of absolute count of CD4+ T cells (maroon), CD8+ T cells (green), and NK cells (blue) during N-803 treatment. (B) Mean frequency of CD4+ and CD8+ T cell counts as a percentage of parent CD3+ T cell population in peripheral blood (PBMC), lymph node (LN) and rectum (RB). (C) Frequency of NK subsets as a percentage of parent bulk NK population. Frequency of CD4+ T cell subsets (D) and CD8+ T cell subsets (E) as a percentage of parent bulk CD4+ T cell population. *Dashed lines designate N-803 administrations. The sample averages are indicated (±SEM) and a Kruskal-Wallis test was used to compare cell counts between pre-treatment and post-treatment time points.* (F) Changes in Ki67 expression, (G) PD-1 expression, and (H) CCR5 expression. *Dashed lines designate N-803 administrations. The sample averages are indicated (±SEM) and a Kruskal-Wallis test was used to compare cell counts between pre-treatment and post-treatment time points. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.*

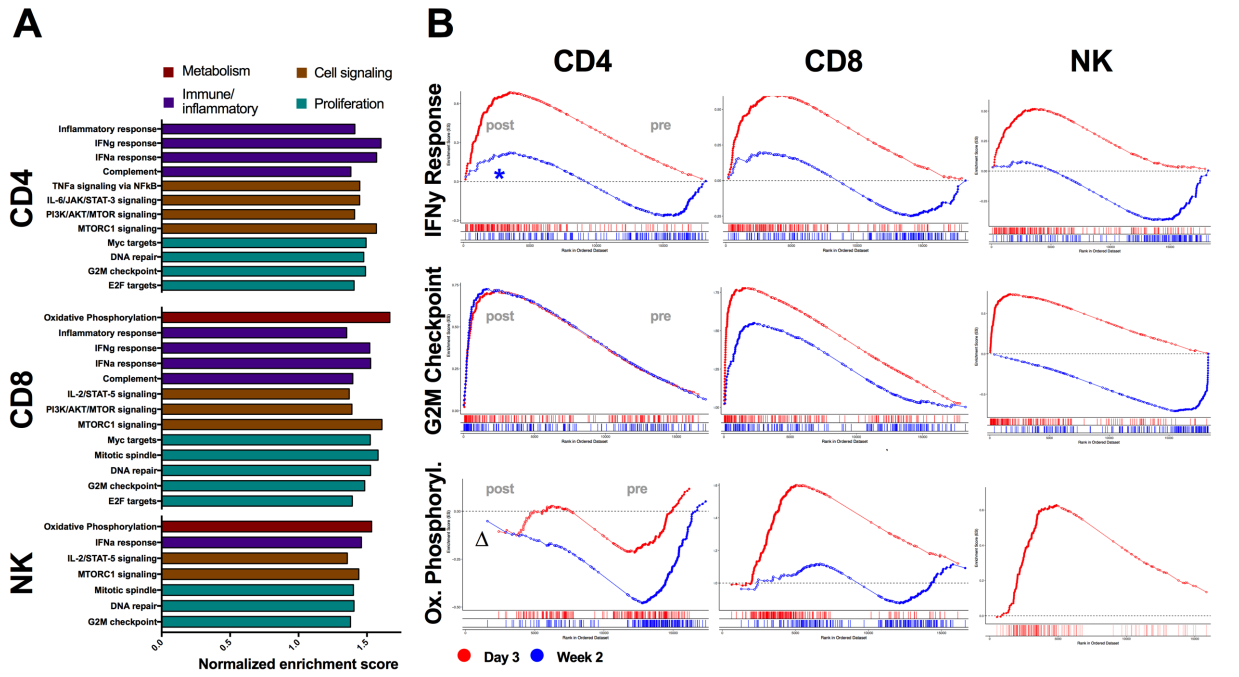

Figure 3: Normalized enrichment scores of genes upregulated after N-803 treatment in CD4+ T cells, CD8+ T cells, and NK cells and enrichment plots of specific gene sets.

(A) Gene set enrichment analysis (GSEA) of RNA-Seq data from blood at day 3 compared with day 0 following N-803. 50,000 bulk CD4+ T cells (live, CD20-, CD16-, CD14-, CD3+, CD8-, CD4+), CD8+ T cells (live, CD20-, CD16-, CD14-, CD3+, CD4-, CD8+), and NK cells (live, CD20-, CD14-, CD3-, NKG2A+) were sorted for the assay. Normalized enrichment scores for select upregulated gene sets depicted, Normalized enrichment score cutoff of greater than 1.35 for upregulated gene sets with a false discovery rate of less than 0.2. (B) Enrichment plots for interferon-gamma response genes (top), G2M target genes (middle), and oxidative phosphorylation genes (ox. phosphoryl; bottom) across cell types. GSEA enrichment plot provides a graphical view of the enrichment score (ES) for a gene set. Most upregulated genes are represented towards the left, including the leading-edge subset. GSEA calculates the ES by walking down the ranked list of genes, and increasing a running-sum statistic when a gene is in the gene set and decreasing when it is not. Red represents day 3, blue represents week 2, as compared to baseline values, with the exceptions of *= day 3 (red) and week 4 (blue), and Δ= week 2 (red) and week 4 (blue).

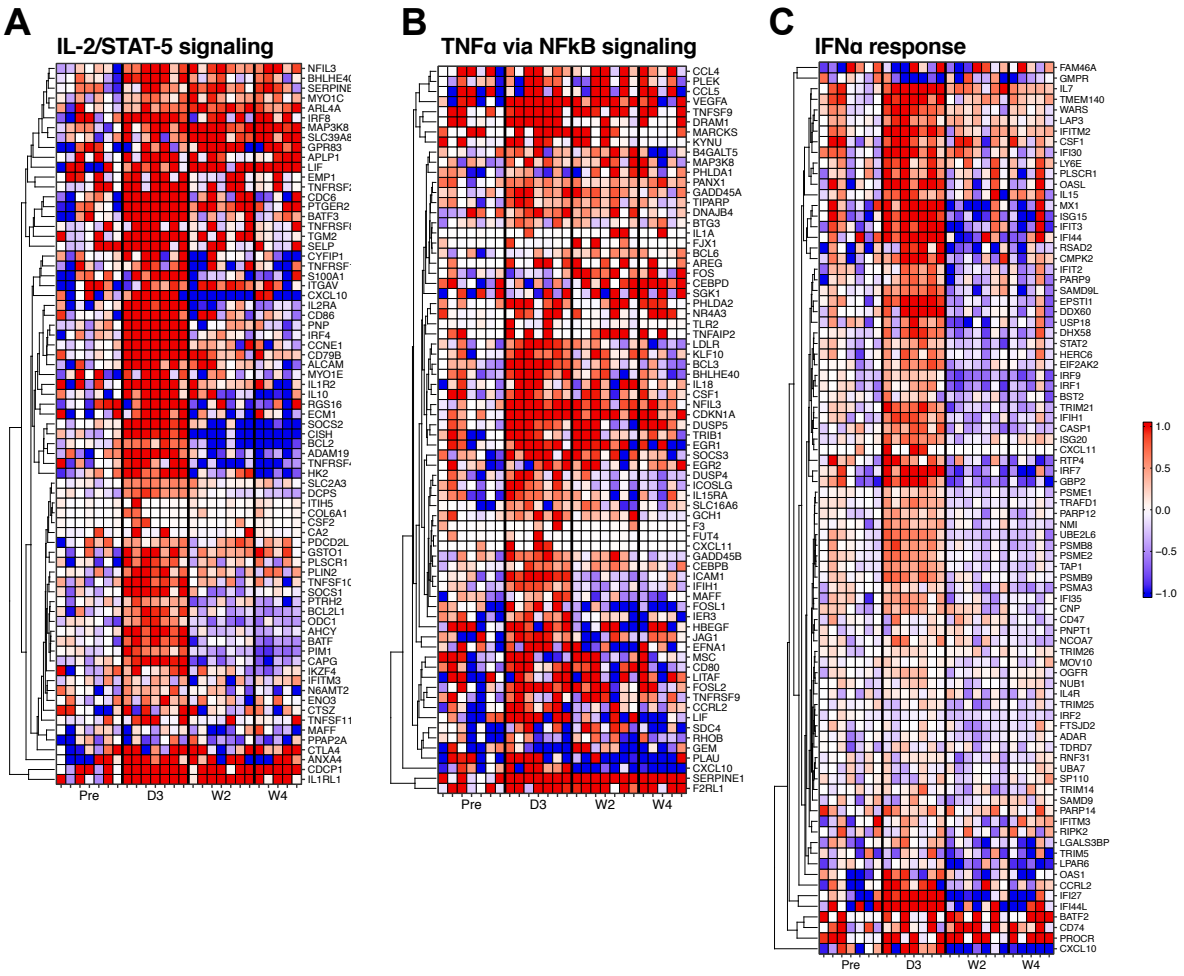

Figure 4: Effect of N-803 on CD4+ T cell signaling pathways.

Heatmap colors represent log2 transformed library size normalized read counts scaled to unit variance across transcript vectors (rows), and normalized to the baseline median sample value of each transcript. Core enriched genes were assessed for the following significantly enriched gene sets in CD4+ T cells, (A) IL-2/STAT-5 signaling, (B) TNFα signaling via NFkB, and (C) IFNα response.

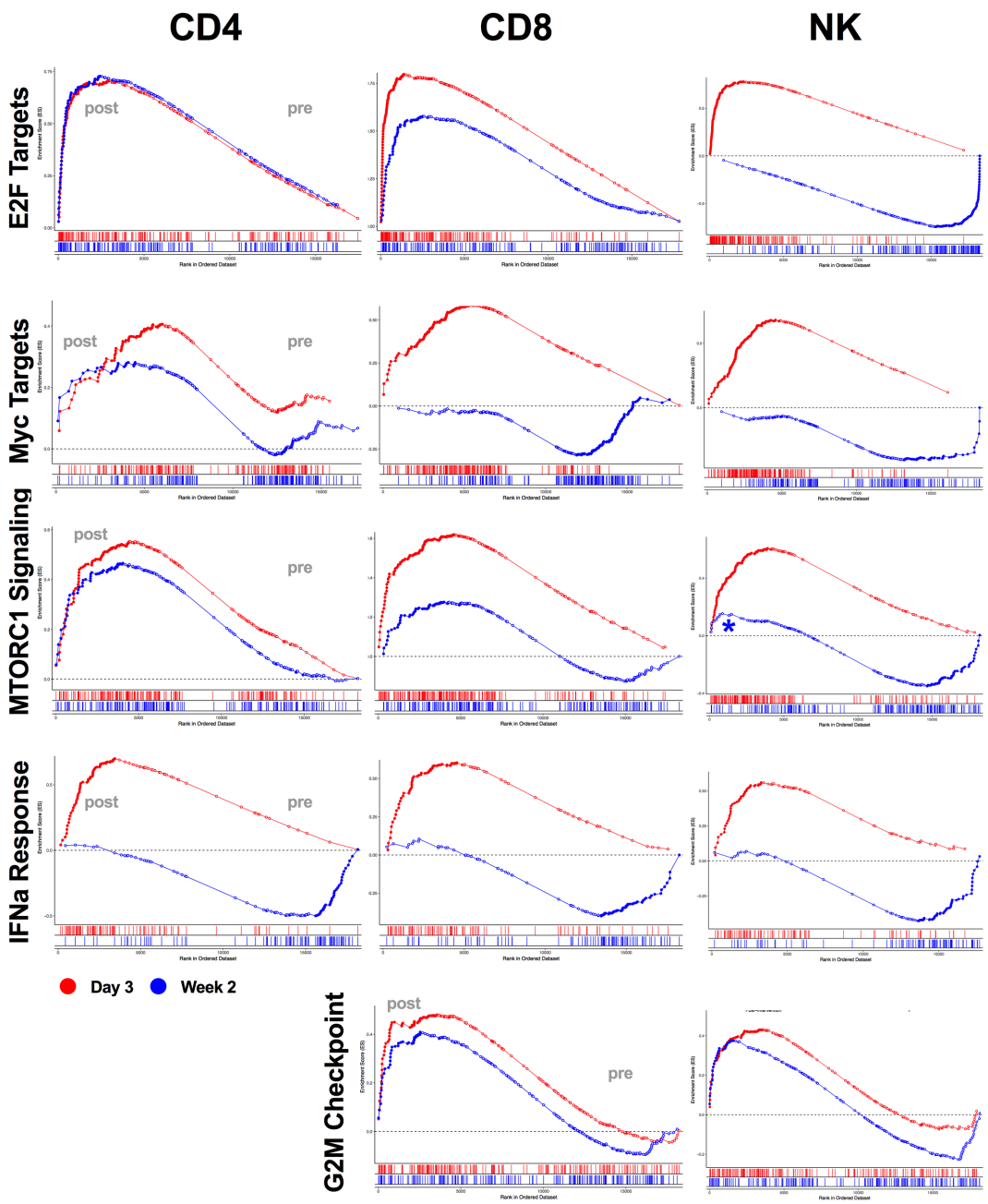

Figure S1: Additional enrichment plots for gene sets upregulated across cell types.

GSEA enrichment plots for E2F targets, Myc targets, MTROC1 signaling, IFNα response, and glycolysis gene sets across CD4+ T cells, CD8+ T cells, and NK cells are depicted. Red represents day 3, blue represents week 2, as compared to baseline values, with the exceptions of *= week 4 (blue). There was no enrichment of the glycolysis gene set in CD4+ T cells during day 3, week 2, or week 4 post-N-803 administration.

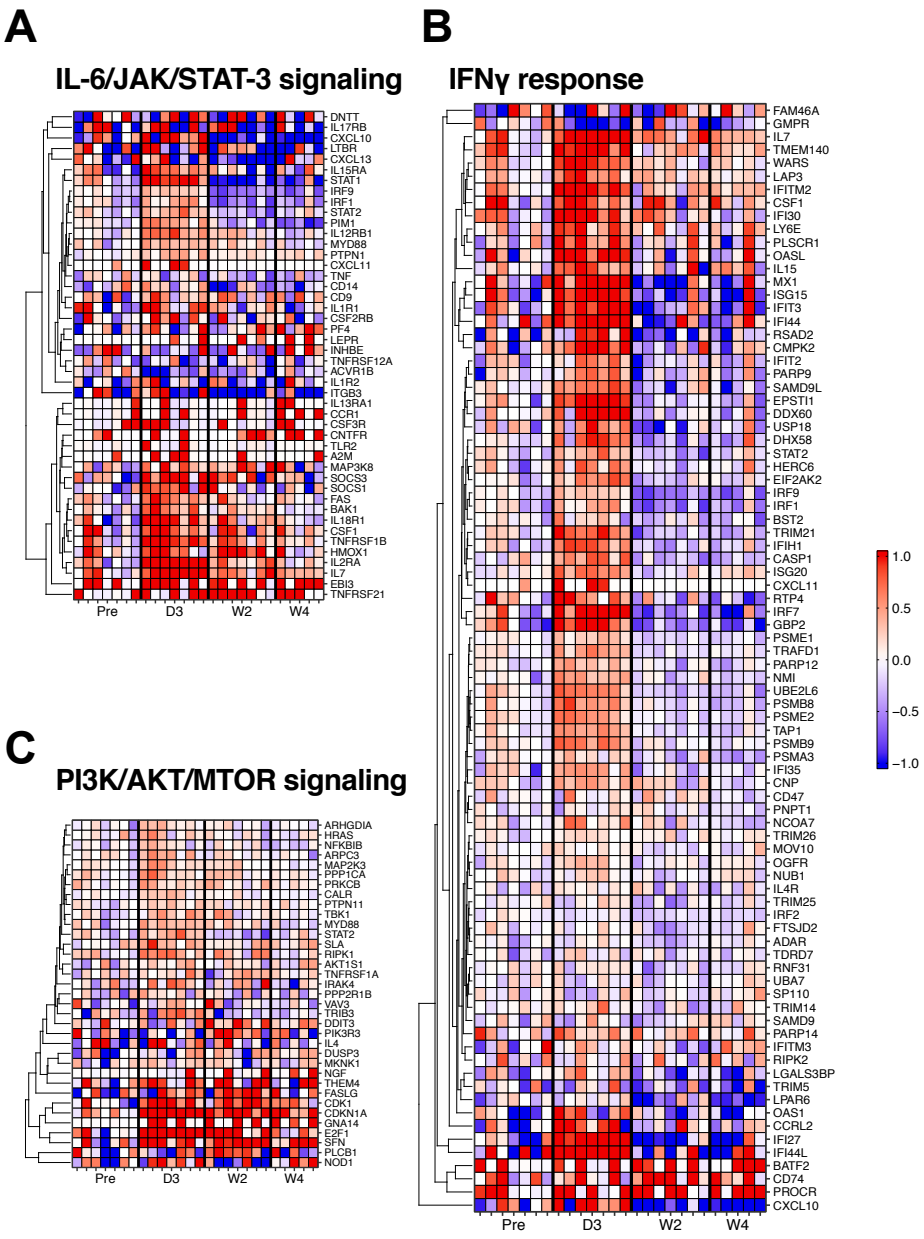

Figure S2: Effect of N-803 on CD4+ T cell signaling pathways (continued).
Heatmap colors represent log2 transformed library size normalized read counts scaled to unit variance across transcript vectors (rows), and normalized to the baseline median sample value of each transcript. Core enriched genes were assessed for the following significantly enriched gene sets in CD4+ T cells, (A) IL-6/JAK/STAT-3 signaling, (B) IFNγ response, and (C) PI3K/AKT/MTOR signaling.

Chapter 3: Robust and persistent SIV and HIV reactivation under ART by N-803 and CD8 depletion

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Abstract

Human Immunodeficiency Virus (HIV) persists indefinitely in antiretroviral therapy (ART)-treated individuals due to a reservoir of latently-infected cells harboring replication-competent virus. To better understand the mechanisms responsible for latency persistence and reversal, we used the interleukin-15 superagonist N-803 in conjunction with CD8+ lymphocyte depletion in ART-treated Simian Immunodeficiency Virus (SIV)-infected macaques. While N-803 alone did not reactivate virus production, its administration after CD8+ lymphocyte depletion induced the most robust and persistent virus reactivation ever observed *in vivo* under ART, with viremia >60 copies/mL in 14/14 animals (100%) and 41/56 samples (73.2%) collected each week after N-803 administration. Strikingly, concordant results were obtained in ART-treated HIV-infected humanized-mice. In addition, we found that co-culture with CD8+ T-cells blocked the *in vitro* LRA effect of N-803 on primary human CD4+ T-cells latently-infected with HIV. These results advance our understanding of the mechanisms responsible for latency reversal and lentivirus reactivation during ART-suppressed infection.

Results

Human Immunodeficiency Virus (HIV) remains a major global health problem with an estimated 1.1 million deaths worldwide annually (UNAIDS, 2017). Despite the major declines in morbidity and mortality associated with the introduction and widespread use of antiretroviral therapy (ART), there is still neither a vaccine nor a cure for HIV infection. The inability to eradicate HIV infection with current therapies is due, at least in substantial part, to the presence of a pool of latently-infected cells harboring integrated replication-competent virus which persists indefinitely in HIV-infected individuals undergoing ART and contributes to rebound viremia when ART is discontinued (often referred to as the "viral reservoir") (Chun et al., 1997b, Finzi et al., 1997, Wong et al., 1997, Siliciano et al., 2003b). A key paradigm in the field of HIV cure, referred to as "shock and kill" (Deeks et al., 2012, Archin et al., 2012), supposes that induction of virus expression (often referred to as "virus reactivation") in these latently-infected cells (i.e., "shock") followed by immune-mediated clearing (i.e., "kill") may substantially reduce the reservoir size and possibly lead to a functional cure for HIV infection. Unfortunately, no latency-reversing agent (LRA) tested to date has successfully perturbed the viral reservoir in human clinical trials. In particular, LRAs belonging to the class of histone deacetylase (HDAC) inhibitors such as vorinostat, panabinostat, and romidepsin failed to induce either robust virus reactivation or reduction of the viral reservoir in ART-treated HIV-infected individuals (Archin et al., 2014, Spivak et al., 2014, Rasmussen et al., 2014, Søgaard et al., 2015, Elliott et al., 2014, Elliott et al., 2015). More encouragingly, in Simian Immunodeficiency Virus (SIV)-infected ART-treated rhesus macaques (*Macaca mulatta*) treatment with Toll-like

receptor 7 (TLR7) agonists GS-9620 and GS-986, was reported to be linked to transient blips of plasma viremia (Lim et al., 2018). However, in additional studies increased plasma viremia has not been observed in response to TLR7 agonist administration to SIV infected rhesus macaques receiving suppressive ART (Del Prete et al., 2019, Bekerman et al., 2019). More recently, persistent remission was observed in a subset of simian/human chimeric immunodeficiency virus (SHIV)-infected macaques that were initiated on ART early after infection before receiving GS-9620 in combination with the broadly neutralizing PGT121 antibody (Borducchi et al., 2018). In all, these published data indicate that novel and more potent approaches for latency reversal and reactivation of the virus reservoir are needed in order to achieve a functional cure for HIV infection.

Infection of rhesus macaques with SIV or SHIV are the most widely used animal models to study the mechanisms by which the viral reservoir is established and maintained under ART, and to test pre-clinically innovative interventions aimed at eliminating, or at least reducing, the viral reservoir *in vivo (Nixon et al., 2017)*. In a previous study, we demonstrated that depletion of CD8+ lymphocytes in SIV-infected ART-treated rhesus macaques was consistently followed by increased plasma viremia, thus indicating that these cells cooperate with ART in suppressing virus production *in vivo* (Cartwright et al., 2016). While the precise mechanisms responsible for this observation remain unclear, phylogenetic analysis of the rebounding virus suggested that silencing of virus transcription may contribute to the antiviral effect of CD8+ lymphocytes in ART-treated macaques. Based on these observations, we hypothesized that CD8+ lymphocyte depletion may combine with LRAs to enhance virus production.

As shown in Figure 1A, the IL-15 superagonist N-803 is a complex of a mutant IL-15 and a dimeric IL-15 receptor αSu/Fc fusion protein (Xu et al., 2013). The engineered structure is at least 25-times more biologically potent than IL-15 as it mimics trans-presentation and the IgG-Fc component confers improved *in vivo* safety and bioavailability (Han et al., 2011, Rhode et al., 2016). In the setting of ART-suppressed lentiviral infection, N-803 has been proposed as a potential agent to target the residual virus pool due to its ability to act *in vitro* as a potent LRA and to strengthen the antiviral cellular immune responses mediated by T and natural killer (NK) cells (Jones et al., 2016).

The current study included a total of 35 SIV-infected macaques that were initiated on ART at day 56 post-infection and treated for at least one year prior to any further intervention. The animals were divided in three groups as follows (Figure 1B): seven macaques were treated with four weekly doses of 100 μg/kg of N-803 (group 1, N-803 alone), fourteen macaques received one dose of the CD8 depleting antibody, MT807R1 (anti-CD8α) at 50 mg/kg iv (group 2, CD8 depletion alone), and fourteen macaques received four weekly doses of N-803 starting at the time of CD8 depletion (group 3, CD8 depletion with N-803). After eventual reconstitution of CD8+ T cells (defined as having a frequency of greater than 100 cells/μL of blood), seven macaques of groups 2 and 3 received an additional cycle of four weekly administrations of N-803. Peripheral blood samples, lymph node biopsies and rectal biopsies were collected at various time points before, during, and after these interventions, and all macaques underwent analytical treatment interruption at week 3 after either CD8+ T cell reconstitution or the last N-803 treatment. As shown in Figure 1C, all animals show

suppression of viremia after one year of ART, with plasma viral loads below the detectable limit of our standard assay (60 copies/mL of plasma)(Taaffe et al., 2010) at the time of the additional interventions in 33/35 animals (94.3%). We also measured residual plasma viremia in our cohort of ART-treated SIV-infected macaques using a more sensitive assay with a limit of detection of 3 copies/mL of plasma(Del Prete et al., 2014) at three monthly sampling points prior to the interventions. Viremia was below 3 copies/mL in 19/35 ART-treated macaques (52.3%), with 26/35 animals (74.3%) showing levels of residual viremia ≤10 copies/mL at the time of intervention (Extended Data Table 1). These results indicate that the level of virus suppression observed in our cohort of macaques was in most cases comparable to that of long-term ART-treated HIV-infected individuals (Dornadula et al.) (Maldarelli et al., Chun et al.).

As shown in Extended Data Figure 1 A-D and consistent with previous studies (Cartwright et al., 2016, Chowdhury et al., 2015), treatment with anti-CD8α MT807R1, with or without N-803, depleted 99.1% of CD3+CD8+ T cells in peripheral blood (standard deviation of 1.8%), 97.9% in lymph nodes (standard deviation of 5.3%), and 99.5% in rectal biopsies (standard deviation of 0.35%). In addition, treatment with MT807R1 alone depleted 93.2% of NK cells in peripheral blood (standard deviation of 7.1%) (Extended Data Figure 1E). As expected based on previous studies, N-803 administration alone resulted in an expansion of CD8+ T cells in the blood and lymph node (Figure 1D-E), as well as increased proliferation of peripheral CD8+ T cells, CD4+ T cells, and NK cells (Figure 1F). Of note, while CD8 depletion alone did not result in a rapid increase in CD4+ T cell proliferation (as measured by Ki67 expression), the combination of CD8 depletion and N-803 resulted in a significant increase of CD4+ T

cell proliferation (Figure 1G; Extended Data Figure 4F-J). The frequency of CD4+ T cells co-expressing Ki67 and the HIV/SIV coreceptor CCR5, i.e., potential target cells of infection, was significantly increased across all groups by the third week after the intervention (Figure 1H). Additionally, CD8 depletion with or without N-803 administration resulted in the expansion of effector memory CD4+ T cells and an increase in PD-1 expression on bulk CD4+ T cells (Extended Data Figure 3F-G).

To better characterize the biological effects of N-803, we conducted a transcriptional analysis using RNA-Seq of sorted CD4+ T cells collected prior to intervention and day 3, week 2, and week 4 after the start of intervention. Regardless of concurrent CD8 depletion, N-803 induced significant upregulation of gene sets associated with cell cycling and proliferation, activation, antiviral responses, and cell signaling (Figure 1I). Specifically, we observed a significant enrichment for genes in the IL-2/STAT-5 signaling gene set, which is also indicative of IL-15 signaling as the receptor for this cytokine shares two out of three subunits with IL-2 and uses STAT-5 as the key adaptor molecule (Figure 1J). In addition, we examined the expression of a set of 25 genes specifically involved in the host-virus interaction during SIV infection and found that N-803 administration induced a consistent and transient upregulation of APOBEC3 in CD4+ T cells, CD8+ T cells, and NK cells (Extended Data Figure 8).

As shown in Figure 2A and Extended Data Table 1, administration of N-803 was not associated with an increase of plasma viremia >60 copies/mL in any of the treated animals, indicating that the IL-15 superagonist is not sufficient to exert a major *in vivo* LRA effect in ART-treated SIV-infected macaques when used alone. As expected based on previous studies(Cartwright et al., 2016), rhesus macaques undergoing CD8+

lymphocyte depletion showed a moderate but significant increase in virus production, with plasma viremia >60 copies/mL detected in 11/14 animals (78.6%) and 18/56 samples (32.1%) collected weekly after CD8 depletion (Figure 2B). Viremia >1,000 copies/mL was observed in 2/14 animals (14.2%) and 2/56 (3.6%) of the same samples (Figure 2B). In all cases, the level of virus production returned to below 60 copies/mL of plasma at the time of CD8+ lymphocyte reconstitution (Figure 2F). Overall, the level of increased viremia observed in this study was consistent with previous studies(Cartwright et al., 2016), even though the magnitude of virus production post-CD8 depletion was somewhat less dramatic, possibly related to the longer period of ART treatment (12 months vs. 2-8 months) (Cartwright et al., 2016). Most remarkably, rhesus macaques treated with N-803 during CD8 depletion showed highly robust and persistent levels of virus production, with viremia >60 copies/mL detected in 14/14 animals (100%) and 41/56 samples (73.2%) and viremia >1,000 copies/mL was observed in 6/14 animals (42.9%) and 13/56 samples (23.2%) (Figure 2C). We would like to emphasize that all seven macaques with full suppression of virus production at the time of intervention with CD8 depletion and N-803 administration (i.e., repeated viral load <3 copies/mL of plasma), demonstrated clear virus reactivation with detectable levels in 26/28 time points one week after each N-803 administration, and above 60 copies/mL in 16/28 of the same samples (Figure 2D). Similar results were obtained in a smaller pilot study in which N-803 administration during CD8 depletion was performed in five fully suppressed $SHIV_{SF162P3}$-infected macaques (Figure 2E; see Chapter 4 for full study). To the best of our knowledge, the level of viremia observed in SIV-infected rhesus macaques treated with combined CD8 depletion and N-803 administration

during long-term ART is the highest and most persistent ever described, as compared to the results of previous “shock and kill” cure strategies tested in humans and nonhuman primates (Archin et al., 2014, Spivak et al., 2014, Rasmussen et al., 2014, Søgaard et al., 2015, Elliott et al., 2014, Elliott et al., 2015, Lim et al., 2018).

After the last treatment with N-803, the level of viremia rapidly declined coincident with the reconstitution of the CD8+ T cell pool, and all macaques returned to <60 copies/mL by week 6 after CD8 depletion with N-803 administration (Figure 2C+G). As expected, CD8+ lymphocyte reconstitution was faster in CD8-depleted rhesus macaques co-treated with N-803 (Extended Data Figure 2E-G), due to the effect of the IL-15 superagonist in enhancing CD8+ T cell proliferation (Figure 1D-F).

We next investigated the correlates of virus reactivation in ART-treated SIV-infected macaques that received CD8 depletion with N-803 administration, and observed that the post-depletion viral load (day 3 through week 6) was negatively correlated to the frequency of CD8+ T cells in the blood (Extended Data Figure 2C). Additionally, the area under the curve (AUC) of virus production was directly correlated with the viral load prior to intervention (Extended Data Figure 2D). Of note, no correlation was found between the level of virus production after CD8 depletion and/or N-803 treatment and either the size of the peripheral blood DNA reservoir (measured as fraction of SIV DNA+ CD4+ T cells) or the level of CD4+ T cell activation (measured as Ki67 or PD-1 expression on CD4+ T cells) (data not shown). To assess whether combined CD8 depletion with N-803 administration induced SIV production in lymphoid tissues, we next analyzed the levels of SIV RNA production in lymph nodes using the RNAscope technology pre-intervention and day 7 post-intervention in five representative

macaques receiving the combination of CD8 depletion with N-803 administration. As shown in Figure 2H, and consistent with the measurements of plasma viremia, we found a statistically significant increase in the percentage of SIV RNA+ cells with high levels of SIV RNA following intervention. No changes were observed in the level of SIV RNA in peripheral CD4+ T cells (Extended Data Figure 5), suggesting that lymphoid tissues are the main source of the observed increased viremia after combined CD8 depletion with N-803 administration.

To confirm the virus reactivation induced by combined CD8+ lymphocyte depletion and N-803 administration in SIV-infected ART-treated macaques in an *in vivo* model utilizing HIV, we next conducted a similarly designed experiment using bone marrow-liver-thymus (BLT) humanized mice (hu-mice) infected with HIV-$1_{JR\text{-}CSF}$ and treated with ART. As shown in Figure 3A-C, HIV-infected hu-mice showed strikingly similar results to those obtained in SIV-infected macaques, with no plasma virus reactivation following administration of N-803 alone, a moderate level of virus reactivation following CD8 depletion alone, and a very robust level of virus reactivation involving 7/8 (87.5%) of hu-mice undergoing CD8 depletion with N-803 administration. Furthermore, we also noted a statistically significant increase in the levels of cell-associated HIV RNA in the spleen and thymic organoid of hu-mice receiving CD8 depletion with N-803 administration (Figure 3D).

The combined data obtained in SIV-infected macaques and HIV-infected BLT humanized indicate that the strong virus reactivation activity induced by N-803 is revealed only in the absence of CD8+ T cells, thus delineating a novel mechanism of latency persistence and/or inhibition of latency reversal that is mediated by CD8+

lymphocytes. To recapitulate this observation in a reductionist *in vitro* model of HIV latency in human cells, we applied the recently developed Latency and Reversal Assay (LARA) (Kulpa DA, 2019) to evaluate how CD8+ T cells affect the virus reactivation activity of N-803 in autologous memory CD4+ T cells that are latently infected with HIV89.6 (Figure 4A). Of note, this assay was conducted using cells derived from HIV-negative donors, thus in the absence of HIV-specific cytotoxic T lymphocytes. As shown in Figure 4B, while N-803 (and its biological counterpart, IL-15) is able to reactivate HIV expression in latently infected CD4+ T cell monocultures, co-culture with activated CD8+ T cells significantly suppresses this ability. These data indicate that CD8+ T cells effectively suppress the latency reversing activity of N-803, and therefore confirm the discovery of a previously unrecognized CD8+ T cell-mediated activity that contributes to the maintenance of *in vivo* primate lentivirus latency.

To next investigate the viral dynamics associated with reactivation following treatment with combined CD8 depletion and N-803 administration in SIV-infected, ART-treated rhesus macaques, we performed a longitudinal sequence analysis of the virus circulating in plasma using single genome PCR amplification (SGA) of the SIV_{mac239}-derived *env* genes. The viral *env* was sequenced at three pivotal time points: (i) day 7 post infection, i.e., peak viral load, (ii) day 56 post infection, i.e., immediately prior to ART initiation (pre-ART), and (iii) during peak virus reactivation, at a time point between 3 days and 4 weeks following treatment with combined CD8 depletion and N-803 administration. We conducted this analysis on a representative subset of six macaques that exhibited robust virus reactivation, resulting in plasma viremia exceeding 800 copies/mL (Figure 2C). Extended Data Figure 9 displays phylogenetic analysis of the

translated Env amino acid sequences of the viruses circulating at the three time points. The peak viral load Env sequences were homogeneous and many were identical to the input SIV_{mac239} sequence; however, the diversity and number of informative sites at the subsequent time points were limited, such that sequences could not be clustered based on time point with significant bootstrap support. The diversity at each time point, while limited, was quantitated by determining the number of amino acid differences from the input SIV_{mac239} Env in each SGA-derived sequence. Extended Data Figure 10A shows that, for all animals, the peak viral load Env sequences have the least differences from the input virus, as expected, while increased divergence was observed at the pre-ART time point and following reactivation in all animals. To gain additional insight into the viral dynamics, the average number of amino acid differences from SIV_{mac239} was calculated for sequences at each time point, and compared with the contemporaneous plasma viral loads for each animal in a correlation matrix. The only significant association that emerged was a direct relationship between Env divergence and plasma viral load during reactivation (Extended Data Figure 10B). Finally, Extended Data Figure 11 shows the location of sequence changes at each time point using highlighter plots of the longitudinal SGA-derived Env amino acid sequences. Overall, this complex virologic analysis supports the hypothesis that treatment with CD8 depletion and N-803 administration induces robust reactivation of a diverse population of viral variants. As no signs of virus evolution emerged from the longitudinal sequence analysis in the macaques experiencing high levels of latency reversal, it is unlikely that the rebounding virus is a product of *de novo* viral replication but rather a reactivated source. In addition, combined CD8 depletion with N-803 administration did not increase the levels of 2-LTR

circles, that are considered a marker of recent lentivirus infection, in peripheral blood mononuclear cells (data not shown).

To next determine whether the treatment regimens induced a decline of the virus reservoir, we first longitudinally measured the level of cell-associated SIV DNA in blood and lymph nodes. As shown in Figure 5A-F, we found that none of the experimental groups showed significant changes in the total fraction of circulating CD4+ T cells harboring viral DNA or in the calculated fraction of lymph node-derived cells harboring viral DNA. To functionally assess the impact of the treatment regimens on the size of the virus reservoir, we performed an analytical treatment interruption (ATI) in all animals three weeks after either CD8 reconstitution and/or the last N-803 treatment. As shown in Figure 5G-I, all animals rebounded within three weeks of interruption and most animals sustained high viral loads until the time of necropsy. It should be noted that in the current study ART was initiated at day 56 post-infection, thus substantially later than in other published non-human primate studies involving ATI, and therefore in the setting of a larger and more disseminated reservoir(Okoye et al., 2018, Borducchi et al., 2018). The rapid rebound after ART interruption was therefore not unexpected as the experimental design was focused on assessing the “shock” effect of CD8 depletion with N-803 administration, with no anticipated impact on the reservoir size in absence of an intervention aimed at clearing the cells that have reactivated SIV production (“kill” phase of the “shock and kill” approach). The absence of a decrease in the level of SIV DNA+ cells after combined CD8 depletion with N-803 administration may be due to the lack of CD8+ T cell-mediated clearance of cells that have reactivated virus expression and/or

the N-803-mediated proliferative expansion of infected CD4+ T cells that have survived the events of virus reactivation.

According to the current paradigm for “shock and kill” interventions in the field of HIV cure, reactivation of virus transcription in latently-infected cells is the first essential step to eliminate the persistent reservoir of replication-competent virus in ART-treated HIV-infected individuals. In this study, we have shown that the administration of the IL-15 superagonist N-803 in both SIV-infected macaques and HIV-infected humanized mice induces a highly robust and persistent reversal of latency only in the setting of CD8+ lymphocyte depletion, thus suggesting a substantial role for CD8+ lymphocytes in suppressing the LRA effect of N-803. Importantly, this novel role of CD8+ lymphocytes in promoting the maintenance of HIV or SIV latency was fully recapitulated in an *in vitro* experimental approach involving co-culture of activated, unprimed CD8+ T cells with autologous, latently HIV-infected human primary CD4+ T cells. To the best of our knowledge, this is the first instance in which a novel approach to manipulate latently infected cells has been independently confirmed in the two best validated and most widely used *in vivo* models for HIV cure interventions and then recapitulated in an *in vitro* experimental system of virus latency. In addition to this conceptual advance, this study defines a very robust “shock” approach which could provide a key experimental system to compare and contrast directly *in vivo* the efficacy of different “kill” interventions (i.e., neutralizing antibodies, CD4 mimetics or immunotoxins). Further studies aimed at identifying the specific molecular pathways used by CD8+ T cells to promote primate lentivirus latency may allow the suppression of this activity, and

therefore will permit a full utilization of the virus reactivating potential of N-803 or other LRAs in the clinical setting without depleting CD8+ lymphocytes.

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providing Raltegravir, and Jim Demarest and ViiV Healthcare for providing Dolutegravir for this study.

Author contributions

J.B.M., A.C., M.P. and G.S. designed the experiments. J.B.M, M.M., E.W. and D.G.C. performed the experiments. S.A.S. performed single-genome PCR and sequencing of SIV RNA and S.A.S. and C.D. performed the sequence-based analyses and wrote relevant portions of the manuscript. J.D.L. performed ultrasensitive viral-load analyses. B.C. performed fluorescence activated cell sorting of live cells. T.H.V. measured viral load, and cell-associated DNA and RNA. J.D.E. and K.B.S. performed RNAscope analysis. S.E.B., G.K.T. and H.W. performed RNA-Seq analysis. M.K., C.R.A.S. and W.O.T. constructed BLT mice. M.K. performed the HIV infection and ART suppression of BLT mice. R.S. performed the viral load measurements, nucleic acid isolations and the analysis of tissue RNA levels for BLT mice. C.R.A.S. and W.O.T. designed and performed the N-803 and CD8 T cell depletion experiments in BLT mice and analyzed the data. A.W. supervised the data collection, analysis, figures, and reporting of all samples from the BLT mice. J.V.G. designed, coordinated and supervised all the BLT experimental work. C.R.A.S., J.V.G., A.W., and W.O.T. wrote and revised the BLT portions of the manuscript. L.F. and D.A.K. performed the *in vitro* studies and wrote the relevant portions of the manuscript. D.M.M, J.T.S., and J.H.L. provided technical support. J.B.M., A.C., and G.S. wrote the manuscript.

Methods

Rhesus Macaque Model

Animals, SIV-infection, antiretroviral therapy, CD8 depletion, and N-803 administration

This study was conducted using a cohort of 35 Indian-origin rhesus macaques housed at Yerkes National Primate Research Center. All macaquess were Mamu*B07- and Mamu*B17- with the following being Mamu*A01+: 77_13, RFr15, 208_13, RPb16, RJt15, RHv15, RAu15, RNa16, RNz15, ROr15, RRb16, RSt15, RAk16, RUs15, Rye16, RVz15, RBn16, and RRn16. All procedures were approved by the Emory University Institutional Animal Care and Use Committee (IACUC) and animal care facilities are accredited by the U.S. Department of Agriculture (USDA) and the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International.

Rhesus macaques were infected intravenously with 10^3 $TCID_{50}$ of SIV_{mac239} (*nef* open). SIV_{mac239} stock was titrated *in vitro* for viral infectivity by standard endpoint titration on CEMx174 cells. The 50% tissue culture infectious dose ($TCID_{50}$) was calculated by the method of Reed and Meunch(REED and MUENCH, 1938). All animals were put on a three drug ART regimen at eight weeks post-SIV infection. Tenofovir (TDF at 5.1mg/kg/day or PMPA at 20mg/kg/day) and emtricitabine (FTC at 40mg/kg/day) were both kindly provided by Gilead Pharmaceuticals. Dolutegravir (DTG at 2.5mg/kg/day) was kindly provided by ViiV Pharmaceuticals. Drugs were administered daily by subcutaneous injection.

After over 12 months of ART, 28 animals were administered one dose of the anti-CD8α-depleting antibody, MT807R1 at 50 mg/kg. The initial 15 animals receiving the

depletion antibody received the administration intravenously. Due to safety concerns, the administration was changed to subcutaneous for the remaining 13 animals. There was no observable effect of the different administration routes on the efficacy of depletion.

At the start of intervention, 21 animals were administered a dose of N-803 either in addition to CD8 depletion (n=14), or without (n=7). N-803 is administered subcutaneously in a cycle of 100 μg/kg once a week for four consecutive weeks.

The study design included a later four dose administration of N-803 in seven animals of groups 1 and 2 that was conducted at the time of CD8+ T cell reconstitution to potentially accelerate the recovery of these cells and improve their antiviral cytotoxic potential. As expected, this second cycle of N-803 induced a faster recovery of CD8+ T cells (data not shown) and was associated with an increase in T cell activation and proliferation that was similar to that observed after the first N-803 cycle (data not shown). The late administration did not result in an increase in plasma viremia.

CD8 depletion with N-803 in SHIV-infected, ART-treated macaques (pilot study)

See chapter 4.

Sample collection and tissue processing

Blood, lymph node (LN), and gut (rectal biopsy, RB) were collected longitudinally including at the time of necropsy and processed for further analyses as previously described(Cartwright et al., 2016).

Immunophenotype by flow cytometry

Multiparametric flow cytometry was performed according to a standard protocol on PBMC and LNMC using fluorescently labeled monoclonal antibodies cross-reactive

in RM. The following antibodies were used at 37°C for 30 minutes: CCR5 APC (3A9) and CCR7 FITC (150503). Then the following antibodies were at room temperature for 30 minutes: CD3 APC-Cy7 (SP34-2), CD4 BV650 (OKT4), CD8α BV711 (RPA-T8), CD8β PE-Cy5 (SIDI8BEE), CCR5 APC (3A9), CCR7 FITC (150503), CD45RA Pe-Cy7 (5H9), CD62L PE (SK11), CD95 BV605 (DX2), PD-1 BV421 (EH12.2H7), CD16 BV421 (3G8), CD20 PE-Cy5 (2H7), CD14 PE-Cy7 (M5E2), NKG2A (CD159) PE (Z199), CD28 PE-Cy5.5 or ECD (CD28.2), CD56 FITC (NCAM16.2). Additional panels included CD69 Pe-Cy5 (FN50), and CD25 APC (M-A251). Cells stained for Ki67 were fixed and permeabilized with Perm II kit (BD) before being stained at room temperature for 30 minutes with Ki67 AF700 (B56).

All flow cytometry specimens were acquired on an LSR II (BD Biosciences) equipped with fluorescence-activated cell sorter software (FACS Diva), and analysis of the acquired data was performed using FlowJo software (Tree Star).

Determination of plasma SIV RNA, and cell-associated RNA and DNA

For pre-intervention time points, quantitative real-time RT-PCR was performed to determine SIV plasma viral load as previously described with a sensitivity of 60 copies/mL(Taaffe et al., 2010). For the three time points prior to intervention and all post-intervention time points plasma SIV *gag* RNA levels were measured using quantitative PCR, essentially as described using high sensitivity assay formats(Hansen et al., 2017, Li et al., 2016). Quantification of total cell-associated $SIVmac_{239}$ *gag* DNA was performed as previously described(Chahroudi et al., 2014). The number of *gag* DNA copies per 10^6 CD4+ T cells was calculated by dividing the number of *gag* DNA copies/10^6 PBMC by the percentage of CD3+CD8-CD4+ cells in the PBMC population.

CD4+ T cells were isolated from PBMC via a CD4+ T cell isolation kit (Miltenyi) and cell-associated RNA was measured as previously described(Kumar et al., 2018).

In Situ RNA analysis and quantification

Viral RNA (vRNA) detection via RNAscope and quantitative image analysis was performed on formaldehyde fixed, paraffin-embedded (FFPE) tissue sections (5μm) according to our previously published protocol (Deleage et al., 2016), with the following minor modifications: heat-induced epitope retrieval was performed by boiling slides in 1x target retrieval (322000; ACD) for 30 min., followed by incubation at 40^{o}C with a 1:10 dilution of protease III (322337; ACD) in 1x PBS for 20 min. Slides were incubated with the target probe SIVmac239 (312811; ACD) for 2 hours at 40^{o}C and amplification was performed with RNAscope 2.5 HD Detection kits (322360; ACD) according to manufacturer's instructions, with 0.5X wash buffer (310091; ACD) used between steps. The resulting signal was detected with Warp Red chromogen (WR806M; Biocare Medical). Slides were counterstained with CAT hematoxylin (CATHE-GL; Biocare Medical), mounted with Clearmount (17885-15; EMS) until dry, coverslipped using Permount (SP15-100; Fisher Scientific), and scanned at 40x magnification on an Aperio AT2 (Leica Biosystems). RNAscope images were analyzed for the total number of vRNA^{+} cells/10^{5} total cells (quantitative) and the relative amount of vRNA present (semi-quantitative) using the ISH module (v2.2) within Halo software (v2.3.2089.27; Indica Labs). The relative amount of vRNA within a single cell was first estimated by quantifying the total area of the vRNA signal spot size (μm^{2}). Since the signal spot size is a function of several steps in the experimental procedures, module settings were established on concomitantly assayed, acutely infected SIV^{+} control slides. To estimate

the signal spot size of a single vRNA molecule, we measured the signal area (min/mean/max) of >10 identifiable individual virions within B cell follicles, which corresponds to two copies of vRNA, and multiplied by 0.5. We set the vRNA minimum signal spot size within the analysis module to exclude detection of a single vRNA molecule and/or integrated viral DNA. Relative vRNA copy numbers present within $vRNA^+$ cells was calculated as [signal spot size within $vRNA^+$ cell (μm^2)/(0.5 x mean signal size for a virion)].

Fluorescence activated sorting (FACS) of live cells

Mononuclear cells isolated from blood were stained with Live/dead, CD3 AF700 (SP34-2), CD4 BV650 (OKT4), CD8 APC-Cy7 (SK1), CD14 PB (M5E2), CD20 PB (2H7), and CD16 PB (3G8), for 30 minutes at room temperature. Aliquots of 50,000 CD4+ T cells (live CD3+CD20-CD14-CD16-CD8-CD4+) and CD8+ T cells (live CD3+CD20-CD14-CD16-CD4-CD8+) were then sorted using a FACS Aria II (BD Biosciences). Mononuclear cells were also separately stained with Live/dead, CD3 AF700 (SP34-2), CD4 BV650 (OKT4), CD8 APC-Cy7 (SK1), CD14 PB (M5E2), CD20 PB (2H7), and NKG2A PE (Z199) to sort aliquots of 50,000 NK cells (Live CD3-,CD20-,CD14-CD4-,CD8+,NKG2A+).

RNA-Seq and data analysis

Bulk CD4+ T cells were sorted from fresh PBMCs prior to intervention, day 3, week 2, and week 4. Briefly, RNA from sorted cells was collected and extracted and DNA was digested. Libraries were prepared and normalized, pooled, and clustered on a flow cells for sequencing. RNA-Seq data were aligned to the MacaM v7.8 assembly of the Indian rhesus macaque genome. To identify pathways differentially modulated,

Gene Set Enrichment Analysis (GSEA)(Subramanian et al., 2005) was performed on the ranked transcript lists using 1000 phenotype permutations and random seeding. Gene sets used included the MSigDB H (hallmark) gene sets(Liberzon et al., 2011).

Single genome PCR amplification of SIVmac239 env sequences

cDNA synthesis and 384-well single genome PCR amplification (SGA) were performed using an approach similar to those previously described(Smith et al., 2016a, Smith et al., 2016b, Burton et al., 2015). Briefly, RNA was extracted from cryopreserved plasma samples using the QIAmp viral RNA kit (Qiagen, # 52906), and reverse transcription was performed using the SuperScript III kit (Invitrogen, #18080-044) with reverse primer SM-ER1 (5'- CTA TCA CTG TAA TAA ATC CCT TCC AGT CCC-3'). cDNA was diluted to result in <30% positive wells for SGA. First round PCR was performed in a 15 μL volume using the Phusion Hotstart II High Fidelity DNA Polymerase (Thermo Scientific, #F537S) with forward primer H2SM-EF1 (5'-CCC TTG AAG GMG CMR GAG AGC TCA TTA-3') and SM-ER1. Cycling conditions were 98°C for 2 min; 10 cycles of 95°C for 15 s, 54°C for 60 s, and 68°C for 4 min; 25 cycles of 95°C for 15 s, 54°C for 60 s, and 68°C for 4 min, adding 5 s to the extension per cycle; 72°C for 30 min; and 4°C hold. Second round PCR was performed with the same enzyme in a 10 μL volume with 1 μL of the first round PCR reaction as template and primers H2SM-EF2 (5'-CAC CTA AAA ART GYT GCT AYC ATT GCC AG-3') and SM-ER2 (5'- ATA AAATGA GAC ATG TCT ATT GCC AAT TTG-3'). Cycling conditions were 95°C for 2 min; 30 cycles of 95°C for 15 s, 54°C for 60 s, and 72°C for 2.5 min; 72°C for

10 min; and 4°C hold. PCR amplicons were purified using Qiaquick PCR Purification Kit (Qiagen #28106).

Sequencing of env amplicons

On average, 26 SGA PCR amplicons per time-point (range 20 to 30) were sequenced by Eurofins Genomic DNA Sanger sequencing using the following primers: SIVmac251seqF1 5'- GGGATATGTTATGAGCAGTCACG-3'; SIVmac251seqF2 5'-ATCCAAGAGTCTTGTGACAAGC-3'; SIVmac251seqF3 5'-AAGAGAGGGAGACCTCACG-3'; SIVmac251seqF4 5'-AGGCCAGTGTTCTCTTCC-3'; SIVmac251seqR1 5'-CTTGTTCCAAGCCTGTGC-3'; SIVmac251seqR2 5'-CCTCTGCAATTTGTCCACATG-3'; SIVmac251seqR3 5'-TCCAAGAAGTCAACCTTTCGC-3'; SIVmac251seqR4 5'-AGCTGGGTTTCTCCATGG-3'(Cartwright et al., 2016). Sequencher v5.1 was used to generate nucleotide sequence contigs, and sequences with mixed peaks in the chromatogram were excluded from further analysis.

Sequence analysis

Geneious v9.1.7 was used to translate nucleotide sequences into amino acids and generate alignments. Amino acid alignments were exported from Geneious in FASTA format and used to generate Highlighter plots to visualize amino acid mismatches (http://www.hiv.lanl.gov/content/sequence/HIGHLIGHT/highlighter_top.html). Phylogenetic Neighbor-Joining consensus trees (Jukes-Cantor, resampling with 100 bootstrap replicates) were created in Geneious using amino acid alignments, and were exported in NEXUS format into Figtree v1.4.4 for further modification (Andrew Rambaut,

Institute of Evolutionary Biology, University of Edinburgh. http://tree.bio.ed.ac.uk/). Phylogenetic trees were presented as unrooted or were rooted on the midpoint. Bootstrap values of greater than 80% are considered significant. Pairwise differences between the infecting SIVmac239 clone and each SGA-derived Env amino acid sequence were determined in Geneious.

Humanized mouse model

Experimental design

The bone marrow-liver-thymus (BLT) humanized mouse model of HIV infection was used to determine the efficacy of CD8 depletion alone, CD8 depletion in combination with N-803, or N-803 alone as a LRA. BLT mice (15-19 weeks post humanization surgery) were exposed to HIV-$1_{JR\text{-}CSF}$ intravenously. ART was initiated 4-5 weeks later. Viremia was durably suppressed by ART for 4 weeks. A single dose of N-803, CD8 depleting antibody or the combination of N-803 and CD8 depleting antibody was administered to HIV-infected and suppressed animals as indicated below. HIV RNA induction was measured on days 4 and 7.

Construction of BLT humanized mice

BLT humanized mice were prepared as previously reported [39-42]. Briefly, a 1-2 mm piece of human liver tissue was sandwiched between two pieces of autologous thymus tissue (Advanced Bioscience Resources) under the kidney capsule of sub-lethally irradiated (200 cGy) 12–15 week-old NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG; The Jackson Laboratory) mice. Following implantation, mice were transplanted intravenously with hematopoietic $CD34^+$ stem cells isolated from autologous human liver tissue. Human immune cell reconstitution was monitored in the peripheral blood of BLT mice by

flow cytometry every 3–4 week as previously described [39-42]. Mice were maintained under specific pathogen-free conditions by the Division of Comparative Medicine at the University of North Carolina, Chapel Hill. Animal experiments were conducted in accordance with NIH guidelines for the housing and care of laboratory animals and in accordance with protocols reviewed and approved by the IACUC at the University of North Carolina, Chapel Hill.

Production of HIV and infection of BLT mice

Stocks of HIV-1_{JR-CSF} were prepared as previously reported [39-41]. The proviral clone was transfected into human embryonic kidney $(HEK)_{293T}$ cells using Lipofectamine™ 2000 (Invitrogen #11668030) following manufacturer's protocol. Viral supernatant was collected 48 hours after transfection and tittered on TZM-bl indicator cells in triplicate to determine the tissue culture infectious units (TCIU) per ml. At least two different titer determinations were performed for each virus stock. BLT mice were exposed to $3x10^4$ TCIU HIV-1_{JR-CSF} via tail vein injection.

Analysis of HIV infection in BLT mice

The peripheral blood plasma viral load was monitored longitudinally by quantitative real-time PCR using a TaqMan® RNA to-C_T™ 1-step kit (Applied Biosystems #4392656). The sequences of the forward and reverse primers and the TaqMan™ probe for PCR amplification and detection of HIV gag RNA were: 5′-CATGTTTTCAGCATTATCAGAAGGA-3′, 5′-TGCTTGATGTCCCCCCACT-3′, and 5′-FAM CCACCCCACAAGATTTAAACACCATGCTAA Q -3′, respectively. Known quantities of HIV gag RNA were run in parallel, creating a standard curve for HIV gag and sample RNA was quantified by extrapolation from the standard curve. All samples

were run and analyzed on an ABI 7500 Fast Real-time PCR System (Applied Biosystems).

HIV DNA levels were measured in tissue cells collected at harvest and cryopreserved in cryopreservation media (10% DMSO: 90% fetal bovine serum). Cells were thawed slowly, counted by trypan exclusion, aliquoted, and pelleted. DNA was extracted from cell pellets using the QIAamp DNA Blood Mini Kit (Qiagen) according to the manufacturer's instructions. RT-PCR was performed with a TaqMan Fast Universal PCR Master Mix (Applied Biosystems). The sequences of the forward primer, reverse primer, and the TaqMan™ probe for amplification and detection of HIV gag DNA were: 5′-CATGTTTTCAGCATTATCAGAAGGA-3′, 5′-TGCTTGATGTCCCCCCACT-3′, and 5′-FAM CCACCCCACAAGATTTAAACACCATGCTAA Q -3′ respectively. As a control, Homo sapiens hemoglobin subunit gamma-2 was ran to quantify the presence of human DNA in each sample. The sequences of the forward primer, reverse primer, and the TaqMan™ probe for amplification and detection of hemoglobin subunit gamma-2 were 5’-CGCTTCTGGAACGTCTGAGATT-3’, 5’-CCTTGTCCTCCTCTGTGAAATGA-3’,

and 5'- FAM TCAATAAGCTCCTAGTCCAGAC-3' respectively. All samples were run and analyzed on an ABI 7500 Fast Real-time PCR System (Applied Biosystems).

ART administration

ART was administered to BLT mice as previously described [43-45] via 1/2″ pellets of irradiated Teklad chow consisting of emtricitabine (1500 mg/kg), tenofovir disoproxil fumarate (1560 mg/kg), and raltegravir (600 mg/kg) (Research Diets).

N-803 and MT807R1 administration

N-803 (0.2 mg/kg in PBS) and control vehicle (PBS) were administered to mice intravenously in a total volume of 200 µL. MT807R1 (3 mg/kg in PBS) and the control vehicle (PBS) were also administered intravenously in a total volume of 200 µL.

Immunophenotypic analysis of BLT mice

Immunophenotyping was performed on peripheral blood samples longitudinally and at harvest on blood and mononuclear cells isolated from the tissues of BLT mice. All flow cytometry data were collected on a BD FACSCanto instrument using BD FACSDiva software (version 6.1.3) and data were analyzed with FlowJo Software (version 10.5.0). Antibodies for the analysis of human immune cell levels include: CD45 APC (clone HIT3a; BD Biosciences #555485), CD3 FITC (clone HIT3a; BD Biosciences #555339), CD4 APC-Cy7 (clone RPA-T4; BD Biosciences #560158), CD33 PE (clone P67.6; BD Biosciences #340679); CD19 PE-Cy7 (clone SJ25C1; BD Biosciences #557835) and CD8 PerCP (clone SK1; BD Biosciences #347314). Flow cytometric gating for expression of lineage specific antigens on human leukocytes was performed as follows: (step 1) forward and side scatter were utilized to set a live cell gate; (step 2) live cells were then analyzed for expression of the human pan-leukocyte

marker $CD45^+$; (step 3) human leukocytes were then analyzed for human $CD3^+$ T cells and $CD19^+$ B-cells and (step 4) T cells were analyzed for human $CD4^+$ and $CD8^+$ expression. The following flow cytometry antibody panel was also used to analyze HLA-DR, CD38 and CD25 expression: CD3 BV421 (clone UCTH1; BD Biosciences #562426), CD4 BV605 (clone RPA-T4; BD Biosciences #562658), CD45 FITC (clone 2D1; BD Biosciences #347463), HLA-DR PerCP (clone L243; BD Biosciences #347364), CD69 PE (clone FN50; BD Biosciences #555531), anti-CD38 PE-Cy7 (clone HB7; BD Biosciences #335790), CD25 APC (clone 2A3; BD Biosciences #340938), CD8 APC-Cy7 (clone SK1; BD Biosciences #557834), and AQUA (ThermoFisher #L35957). Flow cytometric gating was performed as follows: (step 1) forward scatter height and forward scatter area were used to eliminate doublets; (step 2) side scatter area and forward scatter area were used to distinguish leukocytes based on morphology; (step 3) the viability dye AQUA was use to discriminate live cells from dead cells; (step 4) live cells were analyzed for the expression of the human pan-leukocyte marker CD45; (step 5) human leukocytes were then assessed for human CD3 expression to identify T cells; (step 6) T cells were evaluated for expression of human CD4 and CD8; (step 7) human $CD4^+$ or $CD8^+$ T cells were examined for expression of HLA-DR and/or CD38, or CD25. Gates were set with fluorescence minus one controls. Non-specific binding was assessed with isotype controls.

CD8 in vitro suppression assay

Experimental Design

In vitro latently-infected memory CD4+ T cells were generated using the LARA method as previously described(Kulpa DA, 2019) with the following modifications. On day 0, after peripheral blood mononuclear cells (PBMC) were isolated from HIV-naïve buffy coats (New York Blood Center) using SepMate density gradient centrifugation (StemCell, 85460), a portion of PBMCs from each HIV naïve donor were cryopreserved in fetal bovine serum (VWR Life Science Seradigm, 97068-085) + 10% DMSO and stored in liquid nitrogen. On day 8, PBMCs were thawed and rested overnight in RPMI 1640 medium (Fisher Scientific, SH3002701.01) supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin (Corning, 45000-650), and 1% HEPES (Gibco, 15630080); cRPMI) before total CD8+ T cell positive enrichment on day 9 (Miltenyi, 130-045-201). CD8+ T cells were stimulated with anti-CD3/CD28 beads (Dynabeads, 11141D) at a 1:1 ratio plus 30 U/mL IL-2 (R&D system, 202IL050CF) for three days. On day 12 of LARA, CD4+ and CD8+ were prepared for co-culture. HIV latently infected memory CD4+ T cells were washed, counted, and plated for latency reversal in cRPMI in the presence of 100 nM efavirenz, 200 nM raltegravir and 5 μM saquinavir (antiretroviral therapeutics, ART). Activated total CD8+ T cells were removed from the anti-CD3/CD28 beads, washed and resuspended in cRPMI plus ART. CD4+ and CD8+ T cells were co-cultured at a 1:1 or 1:5 ratio at a final density of 1×10^6 cells/mL. CD4+ monocultures were also maintained in parallel. Mono- and co-cultures were then left unstimulated, TCR-activated with 1 μg/mL plate-bound OKT3 and 1 μg/mL soluble CD28 (Biolegend, 302933), or treated with 14 nM N-803 (provided by NantKwest) or

500 ng/mL IL-15 (R&D Systems, 247-ILB). Cells were harvested after 72 hours and analyzed by flow cytometry and qPCR.

Flow cytometry

Multicolor flow analysis of cell surface and intracellular marker expression was performed with a BD FACSymphony flow cytometer. Between 200,000 and 600,000 events were acquired for each sample using the live cell gate. The data was analyzed with FlowJo (v.10).

Antibodies used in this study: CD3 Alexa Fluor® 700 (UCHT1, BD Biosciences, #557943), CD8 BUV737 (SK1, BD Horizon™, #564629), HIV-1 core antigen-FITC (KC57, Coulter Clone, #6604665), CD4 BV421 (SK3, BD Horizon™, #565997), CD45RA APC- eFluor™780 (HI100, Invitrogen, #47045842), CD27 BV650 (O323, Biolegend, #302828), CCR7 Pe-Cy7 (3D12, BD Pharmingen™, #557648), Fixable Viability Dye eFluor™ 506 (Invitrogen eBioscience #65-0866-18).

Integrated/Total/2-LTR HIV-DNA

Cell samples from LARA were used to assess the frequency of integrated HIV DNA as previously described(Vandergeeten et al., 2014).

Statistical analysis

Statistical analyses, including Kruskal-wallis test, Friedman test, one-way ANOVA, and Spearman r, were performed using Prism v7.0 or v8.0. $P < 0.05$ was considered significant. *$P < 0.05$; **$P < 0.01$; ***$P<0.001$, ****$P<0.0001$, and non-

significant values >0.05 were not labeled on figures. Data are represented as mean ±SEM as indicated.

Data availability

Illumina sequencing reads for RNA-Seq experiments were submitted to the NCBI SRA repository and are available at Accession #SRP188630. RNA-Seq datasets were submitted to the NCBI GEO repository and are available at accession number GSE128415. Env nucleotide sequences have been deposited into Genbank under the accession number MK922999-MK923550.

Chapter 3 Figures

Figure 1

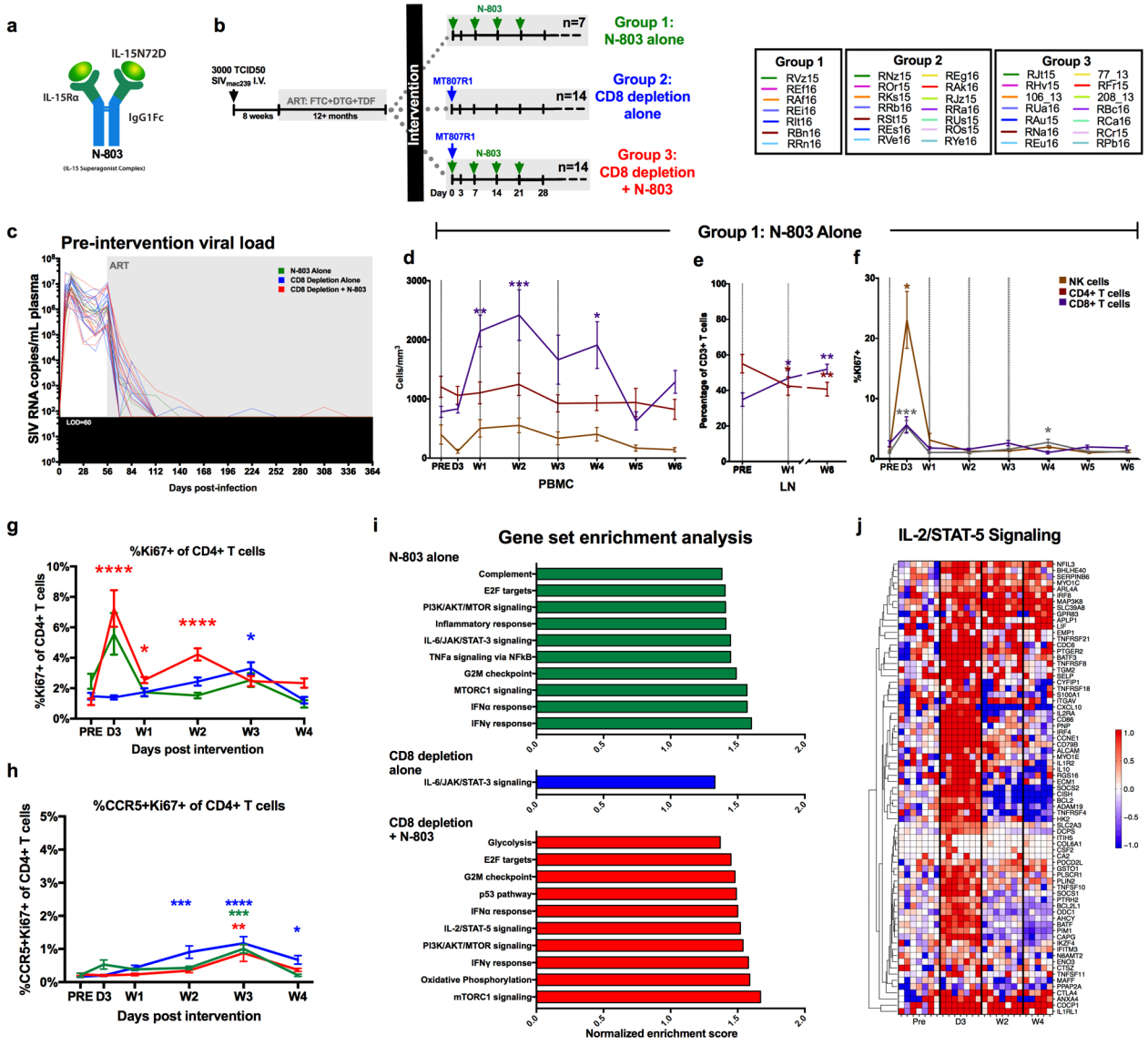


Figure 1 | Study design and phenotypic and transcriptomic effects of N-803. a, IL-15 Superagonist N-803 structure. **b,** Study design with animal key. At intervention phase, green arrows designate 100 μg/kg N-803 administration and blue arrow designates 50mg/kg MT807R1 administration. **c,** Plasma viral load pre-intervention, including infection and initiation of antiretroviral therapy. Limit of detection is at 60 copies of SIV RNA/mL of plasma. Black bar represents viral suppression (<60 copies/mL). Gray represents antiretroviral therapy (ART), starting at day 56 post-infection. Changes to CD4+ T cells (maroon), CD8+ T cells (green), and NK cells (blue) following N-803 administration alone (n=7): **d,** mean cell count frequency, **e,** percentage of CD3+ T cell population, and **f,** Ki67+. **g,** Ki67 expression and **h,** Ki67 and CCR5 coexpression on bulk CD4+ T cells following N-803 alone (green, n=7), CD8 depletion alone (blue, n=14), and CD8 depletion with N-803 administration (red, n=14). **i,** Gene set enrichment analysis (GSEA) of RNA sequencing data from bulk CD4+ T cells comparing gene sets enriched after three days of intervention. Normalized enrichment scores for select upregulated gene sets are depicted, where normalization is group specific. A normalized enrichment score cutoff of greater than 1.35 for upregulated gene sets with a false discovery rate of less than 0.2 was used, in accordance with GSEA guidelines. **j,** Heat map detailing enriched genes in bulk CD4+ T cells in the IL-2/STAT-5 signaling gene set after administration of N-803 alone. Heat map colors represent log2 transformed library size normalized read counts scaled to unit variance across transcript vectors and normalized to the baseline median sample value of each transcript. Sample averages are indicated (±SEM), and Kruskal-Wallis test was used to compare each group to their own baseline. The level of significance for each comparison is indicated above the brackets (**** $p < 0.0001$, ** $p < 0.01$, * $p < 0.05$, none $p > 0.05$).

Figure 2

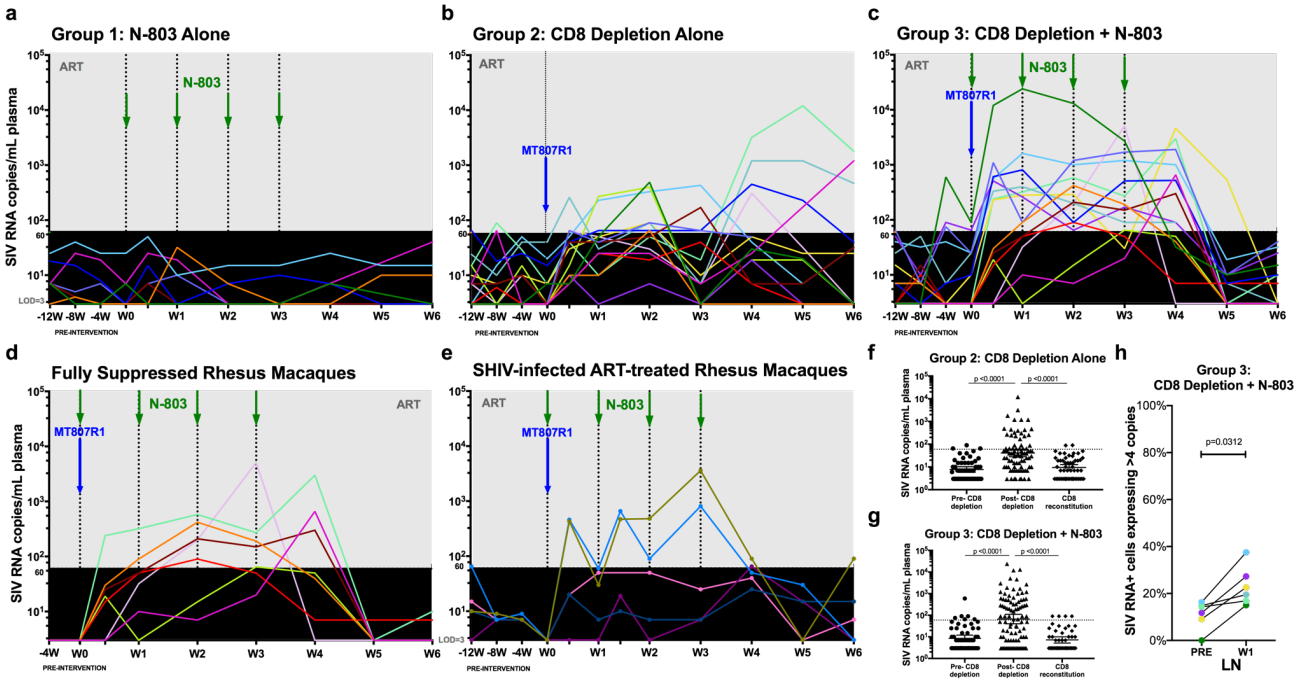


Figure 2 | SIV RNA reactivation occurs in the blood and lymph nodes after the combination of CD8 depletion with N-803 only. a-c, Plasma viral loads following intervention with a limit of detection of 3 copies of SIV RNA/mL plasma. Black bars represent viral suppression <60 copies/mL, where **d,** represents longitudinal plasma viral loads from 7/14 macques with fully suppressed viral load (<3 copies/mL of plasma) prior to CD8 depletion with N-803 administration. **e,** Plasma viral loads following CD8 depletion with N-803 administration in five $SHIV_{162P3}$-infected macaques after six months of ART. Comparison of viral load before CD8 depletion, following CD8 depletion (count <100 cells/μL blood), and after CD8+ T cell reconstitution (>100 cells/μL blood) in macaques treated with **f,** CD8 depletion alone (n=190), and **g,** CD8 depletion with N-803 (n=198). Dotted line at 60 copies/mL. Statistical significance calculated using Kruskal-Wallis multiple comparisons test. **h,** RNAscope determination of the percentage of SIV RNA+ lymph node cells expressing high levels (>4 copies) of viral RNA/cell one week after CD8 depletion with concurrent N-803 administration. Statistics were calculated with a Wilcoxon test.

Figure 3

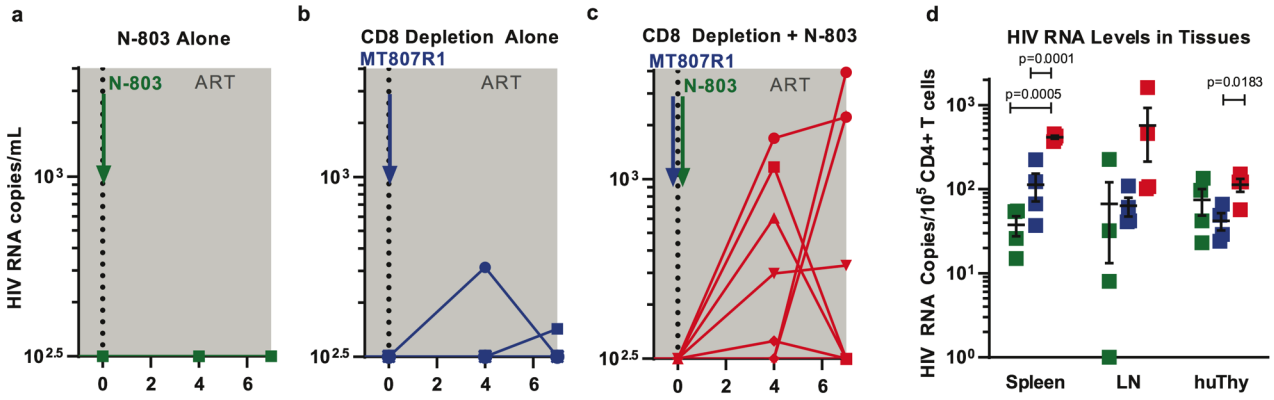


Figure 3 | CD8 depletion with N-803 administration reactivates virus in ART-suppressed humanized bone marrow-liver-thymus mice. Plasma viral load levels of mice treated with a, N-803 alone (orange, n=7), b, CD8 T cell depletion alone (blue, n=8), and c, CD8 depletion with N-803 administration (purple, n=8). Dashed line indicates time when treatment was administered. d, Cell-associated HIV RNA levels in the spleen, lymph node (LN), and human thymus from mice treated with N-803 alone (orange, n=4), CD8 depletion alone (blue, n=4), or in combination (purple, n=4). Sample averages (±SEM) are indicated. Statistical significance was tested using a two-tailed Student's t-test. A P value less than 0.05 was considered significant.

Figure 4

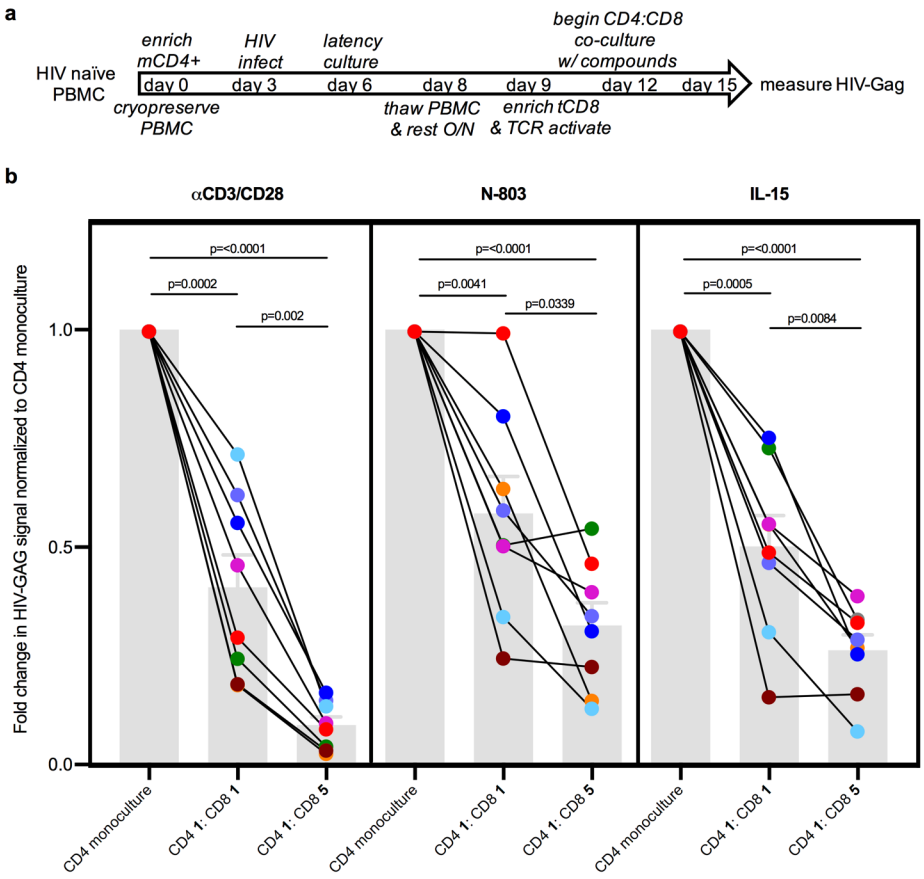


Figure 4 | *In vitro* co-culture of latently-infected human CD4+ T cells with autologus CD8+ T cells results in decreased expression of HIV-GAG during LRA administration. a, Schematic of HIV latency model used in these experiments. Memory CD4+ T cells (mCD4+) are enriched on day 0, and infected *in vitro* on day 3. After infection, mCD4+ are maintained in the antiretroviral saquinavir to prevent viral spreading. On day 6, HIV-infected mCD4+ are cultured in the presence of TGF-beta, IL-7, conditioned medium from H-80 feeder cell line, and saquinavir, efavirenz and raltegravir (additional antiretrovirals). Cryopreserved autologous PBMC were thawed on day 8 and rested overnight (O/N) before enriching for total CD8+ (tCD8+) cells and then TCR activated for three days. On day 12, HIV-infected mCD4+ and TCR-activated tCD8+ are co-cultured in a 1:1 or 1:5 ratio in the presence of an LRA for three days (day 15). **b,** The frequency of HIV-GAG+ CD4+ T cells was quantified by flow cytometry and the fold change compared to frequency in CD4 monocultures was calculated after exposure to anti-CD3/CD28, IL-15 superagonist N-803, or recombinant IL-15. Each color represents a unique donor (n=8) and sample averages (±SEM) are indicated by the grey bar. Statistical significance was tested using a matched one-way ANOVA.

Figure 5

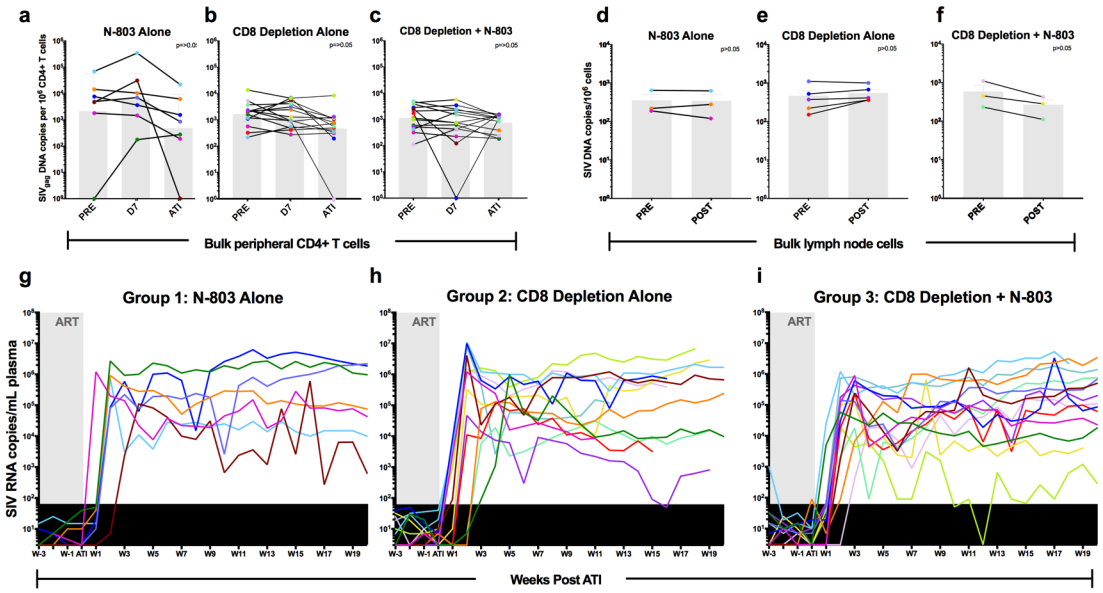
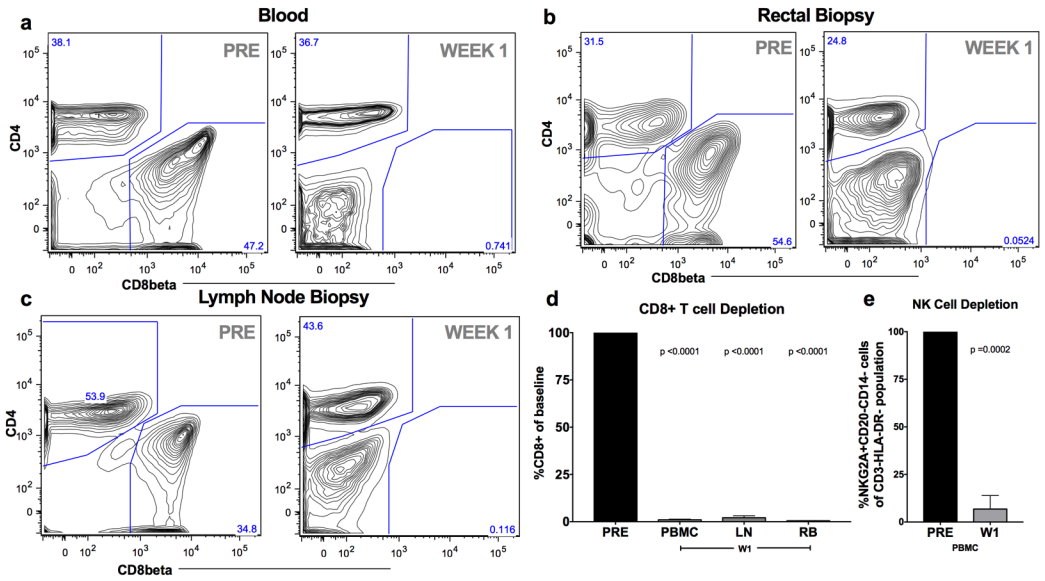


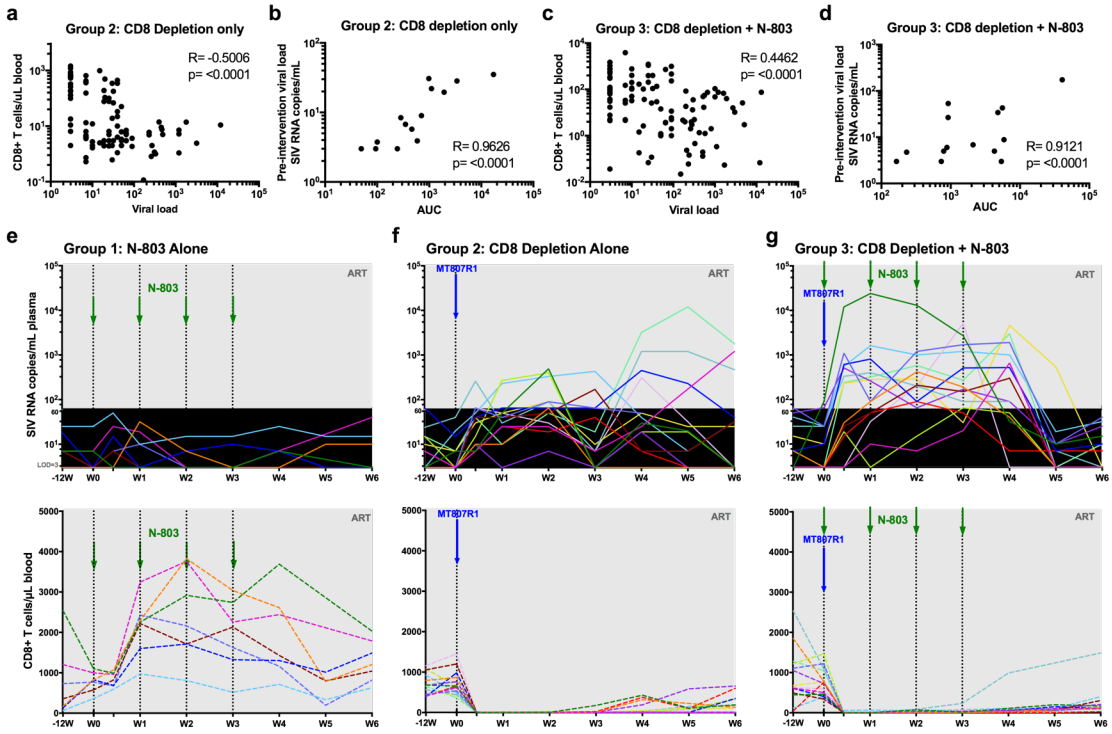
Figure 5 | CD8 depletion with N-803 administration does not decrease the size of the latent SIV viral reservoir. Copies of total cell-associated SIV_{gag} DNA was measured in PBMC and the number of copies per 10^6 CD4+ was corrected using calculated CD4 T cell frequencies for macaques receiving **a,** N-803 alone (n=7), **b,** CD8 depletion alone (n=14), and **c,** CD8 depletion with N-803 administration (n=14). DNA was extracted from pellets pre-intervention (PRE), day 7 post-intervention (D7), and pre-ART interruption (ATI). Friedman tests were used to compare cell counts between pre-treatment and post-treatment time points. SIV DNA was extracted from frozen lymph node pellets and cell-associated SIV DNA levels were measured in animals receiving **d,** N-803 alone (n=3), **e,** CD8 depletion alone (n=5), and **f,** CD8 depletion with N-803 administration (n=3). Statistics were calculated with a Wilcoxon test. The sample averages are indicated by the gray bar on each graph (±SEM). **g-i,** Viral rebound data following ART cessation, three weeks following the last N-803 administration or the reconstitution of CD8+ T cells above 100 cells/μL blood. Animals were followed weekly for around 5-6 months following ART interruption. Limit of detection of 3 copies of SIV RNA/mL plasma. Gray box indicates the time before ART interruption. The black bar represents viral loads <60 copies/mL.

Supplemental Figure 1



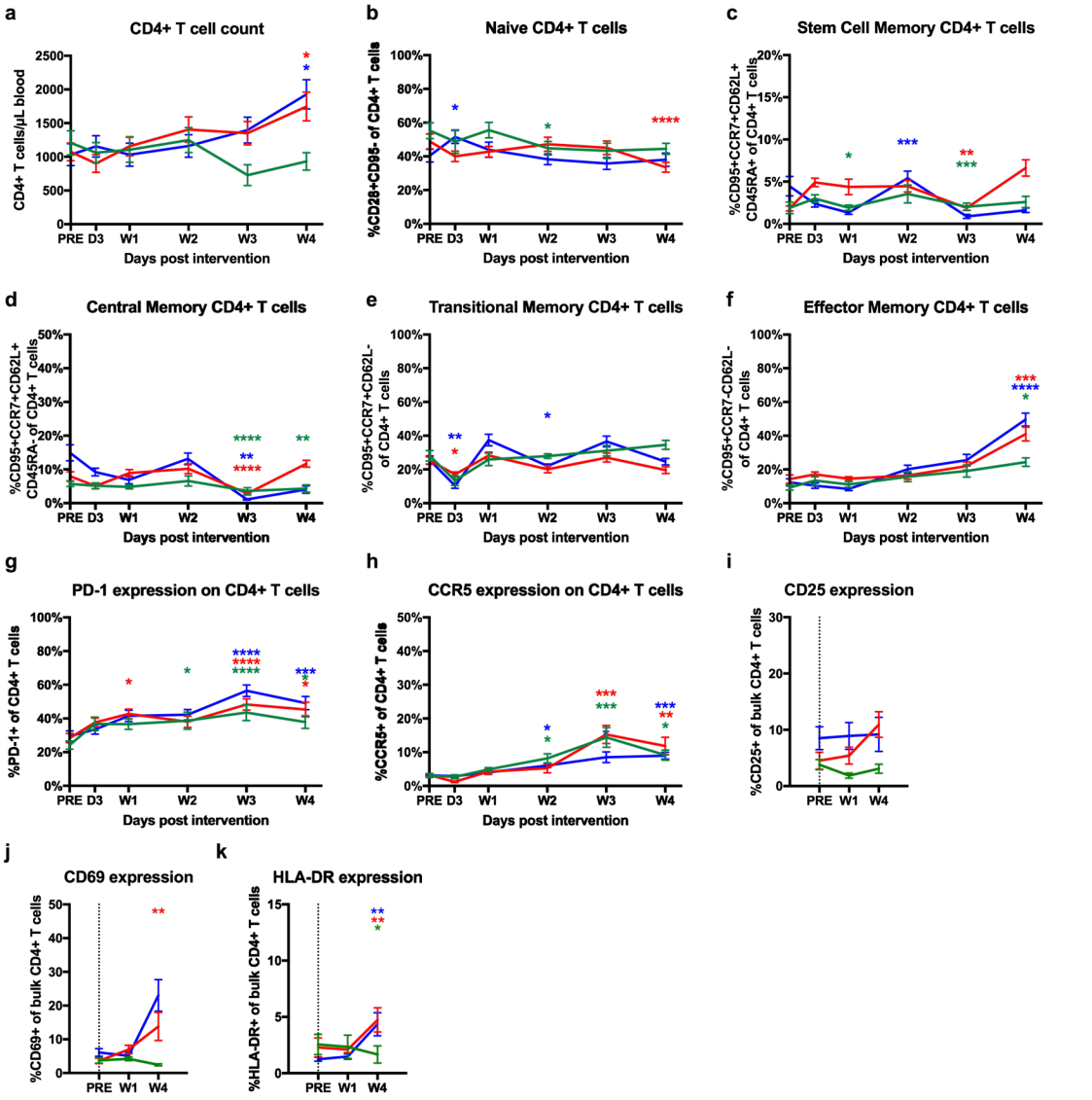
Extended Data Figure 1 | MT807R1 effectively depletes CD8+ T cells in peripheral blood, lymph node, and rectum in addition to NK cells from the blood at day 7. The percentage of CD8+ cells in the CD3+ population seven days post-depletion was compared to pre-depletion levels. Sample flow cytometry shows the absence of a CD8β+ cells as part of the CD3+ T cell population after depletion in **a,** the peripheral blood, **b,** rectum, and **c,** lymph node. **d,** The percentage of CD8β+ cells as compared to pre-depletion baseline was calculated in all CD8-depleted animals (+/- N-803) across blood and tissue. Of note, no major differences in CD8+ T cell depletion were observed between groups receiving N-803 at the time of depletion and those not at day 7. Friedman test was used to calculate the statistical significance of CD8+ cells following depletion in different tissues. (E) Depletion of NK cells in the peripheral blood was assessed in the group receiving CD8 depletion alone using the percentage of NKG2A+CD20-CD14- cells of the CD3-HLA-DR- population as compared to baseline. Statistical significance was calculated using Wilcoxon test. Mean ± SEM (n=14) are shown.

Supplemental Figure 2



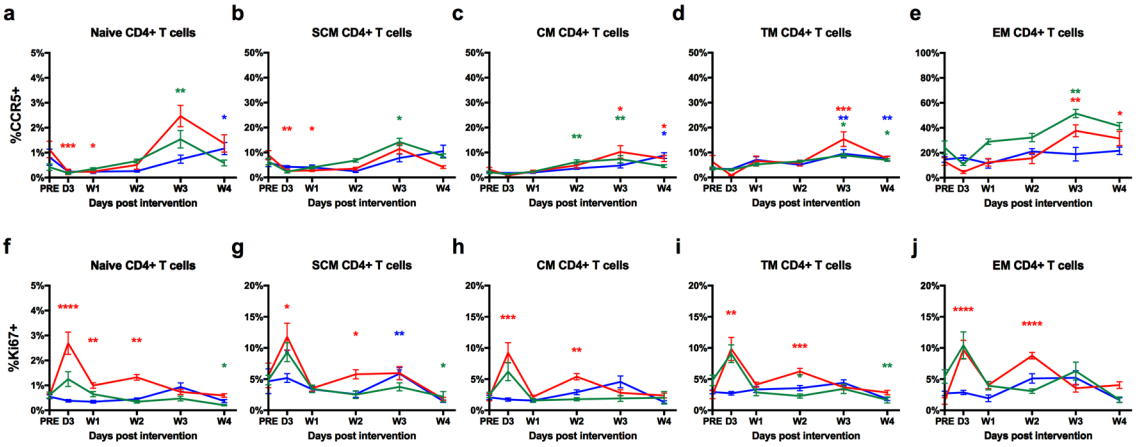
Extended Data Figure 2 | Correlations between virologic and cellular parameters following intervention with CD8 depletion with or without N-803, or N-803 alone. a, Correlation between viral load and CD8+ T cell counts day 0, day 3, and weekly through week 6 (n=103), and **b,** the area under the curve (AUC) and the average pre-intervention viral load following CD8 depletion alone (n=14). **c,** Correlation between viral load and CD8+ T cell counts day 0, day 3, and weekly through week 6 (n=112), and **d,** the area under the curve (AUC) and the average pre-intervention viral load following CD8 depletion with N-803 administration (n=14). Correlation coefficients are calculated using the Spearman's rank-order correlation. **e-g,** Top figure depicts copies of SIV RNA/mL of plasma following each intervention and the bottom figure represents longitudinal CD8+ T cell counts.

Supplemental Figure 3



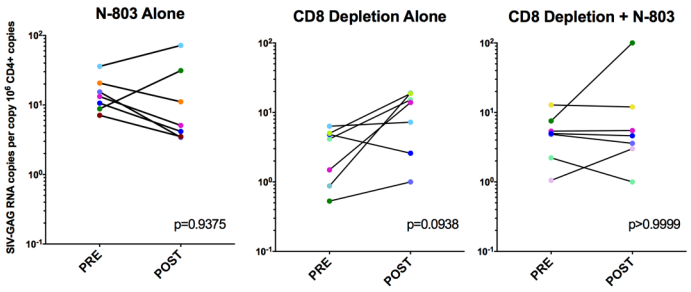
Extended Data Figure 3 | CD4+ T cell subset frequency and activation status following intervention with CD8 depletion with or without N-803, or N-803 alone. Effect of CD8 depletion alone (blue), N-803 alone (green), and CD8 depletion with N-803 administration (green) on frequency of **a,** CD4+ T cells, **b,** naïve CD4+ T cells (CD28+CD95-), **c,** stem cell memory CD4+ T cells (CD95+CCR7+CD62L+CD45RA+), **d,** central memory CD4+ T cells (CD95+CCR7+CD62L+CD45RA-), **e,** transitional memory CD4+ T cells (CD95+CCR7+CD62L-), **f,** effector memory CD4+ T cells (CD95+CCR7-CD62L-), **g,** PD-1+ CD4+ T cells, **h,** CCR5+ CD4+ T cells, **i,** CD25+ CD4+ T cells, **j,** CD69+ CD4+ T cells, **k,** HLA-DR+ CD4+ T cells. Mean ±SEM (n=7 (green), n=14 (blue and red). Kruskal-Wallis test was used to compare cell counts between pre-treatment and post-treatment time points. The level of significance for each comparison is indicated above the brackets (**** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, none $p > 0.05$).

Supplemental Figure 4



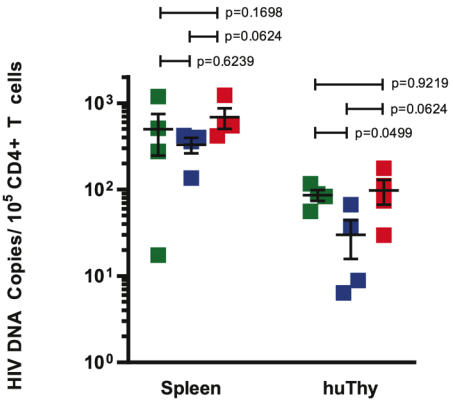
Extended Data Figure 4 | CCR5 and Ki67 expression across CD4+ T cell subsets following intervention with CD8 depletion with or without N-803, or N-803 alone. Effect of CD8 depletion alone (blue, n=14), N-803 alone (green, n=7), and CD8 depletion with N-803 administration (red, n=14) on the expression of CCR5 on **a,** naïve CD4+ T cells (CD28+CD95-), **b,** stem cell memory CD4+ T cells (SCM, CD95+CCR7+CD62L+CD45RA+), **c,** central memory CD4+ T cells (CM; CD95+CCR7+CD62L+CD45RA-), **d,** transitional memory CD4+ T cells (TM; CD95+CCR7+CD62L-), **e,** effector memory CD4+ T cells (EM; CD95+CCR7-CD62L-). **f-j,** Effect of interventions on the expression of Ki67 across CD4+ T cell subsets. Statistical significance was calculated using Friedman test. Mean ± SEM are shown. The level of significance for each comparison is indicated above the brackets (**** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, none $p > 0.05$).

Supplemental Figure 5



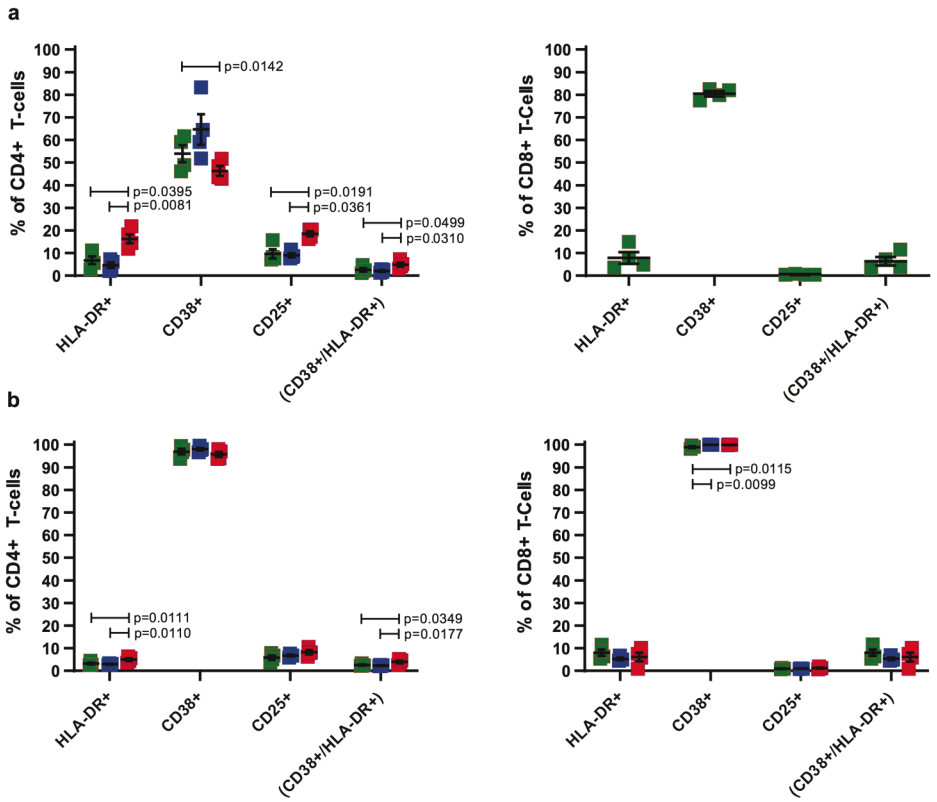
Extended Data Figure 5 | Quantification of levels of cell-associated SIV RNA in peripheral CD4+ T cells prior to and following interventions. a, N-803 alone (n=7), **b,** CD8 depletion alone (n=7), **c,** CD8 depletion with N-803 (n=7). Expression of cell-associated viral RNA was assessed by measuring the number of copies of HIV *gag* in relation to the number of copies of CD4+. Post-intervention time point was chosen one week after the first N-803 administration (N-803 alone group) or at the time of the recorded post-depletion peak viremia (CD8 depletion with or without N-803 groups). Statistical significance was calculated using Wilcoxon test. Mean ± SEM are shown.

Supplemental Figure 6



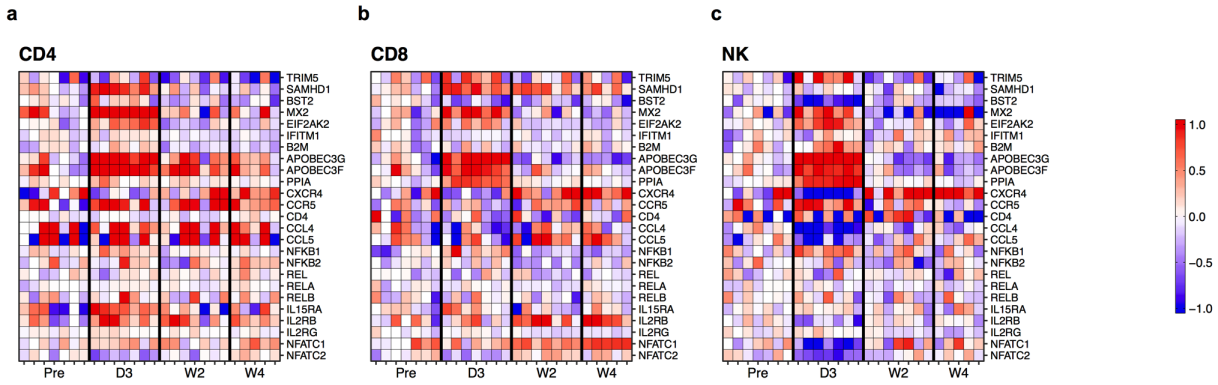
Extended Data Figure 6 | HIV DNA copies in tissues of HIV-infected, ART-Suppressed BLT mice administered N-803, CD8-depleting antibody, or N-803 and CD8-depleting antibody.
Seven days post administration of N-803 (green, n=4), CD8 T cell depleting antibody (blue, n=4), or N-803 and CD8 T cell depleting antibody(red, n=4) to HIV-infected, ART-suppressed BLT mice, total DNA was extracted from mononuclear cells isolated from the spleen and human thymus (huThy). Treatment groups were compared using a Kruskal-Wallis test with a false discovery rate correction. Sample means are indicated by a horizontal bar (±SEM).

Supplemental Figure 7



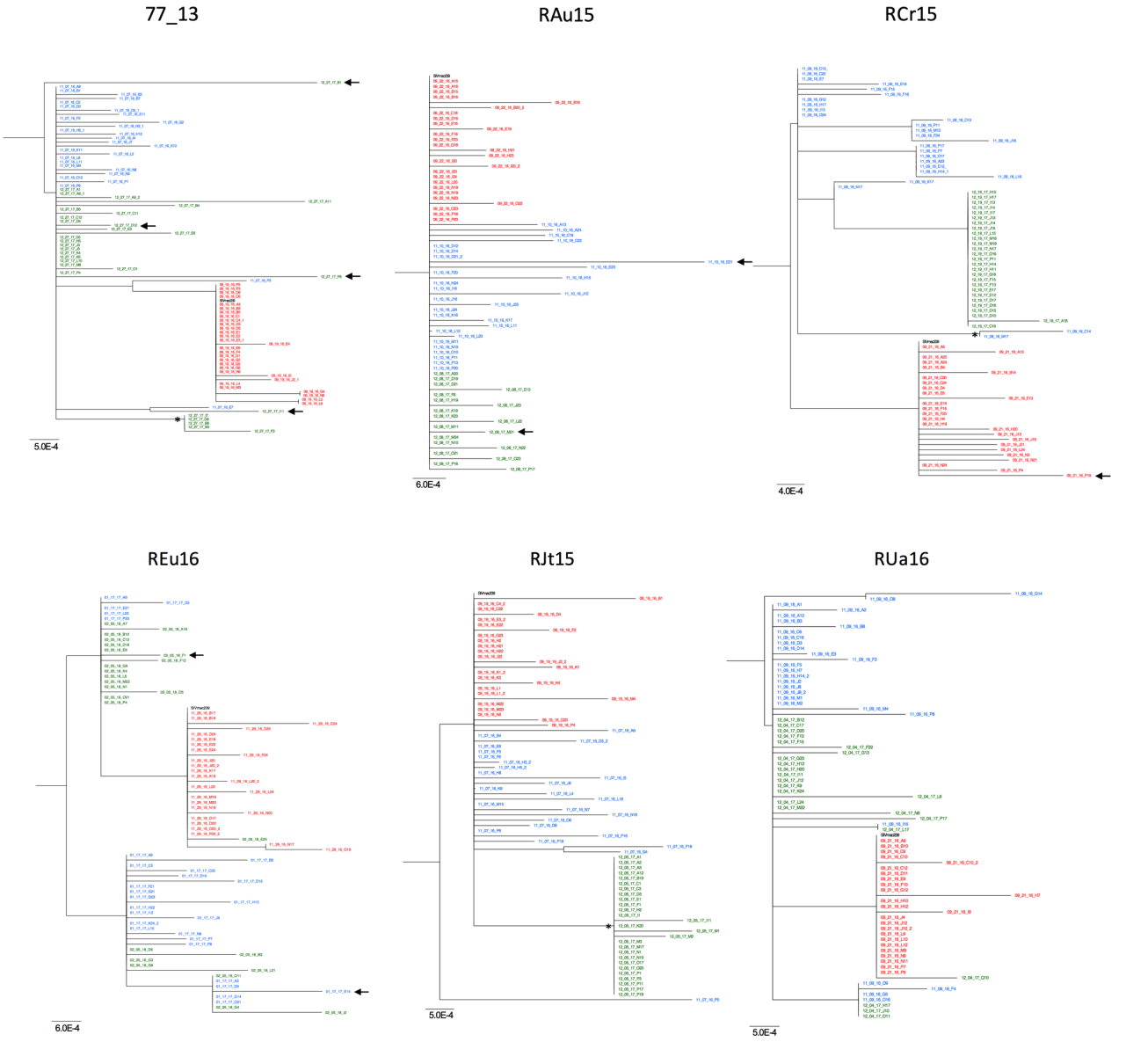
Extended Data Figure 7 | Human T cell activation levels in HIV-infected, ART-suppressed BLT mice administered N-803, CD8-depleting antibody, or N-803 and CD8-depleting antibody.
Percentage of HLA-DR+, CD38+, CD25+, or HLA-DR+/CD38+ was measured in human CD4+ (left) or CD8+ (right) T cells isolated from the spleen (**a**) or human thymus (**b**) of HIV-infected, ART-suppressed BLT mice seven days following administration of N-803 (green, n=4), CD8 depleting antibody (blue, n=4), or N-803 and CD8 depleting antibody (red, n=4). Treatment groups were compared using a Kruskal-Wallis test with a false discovery rate correction. Sample means are indicated by a horizontal bar (±SEM).

Supplemental Figure 8



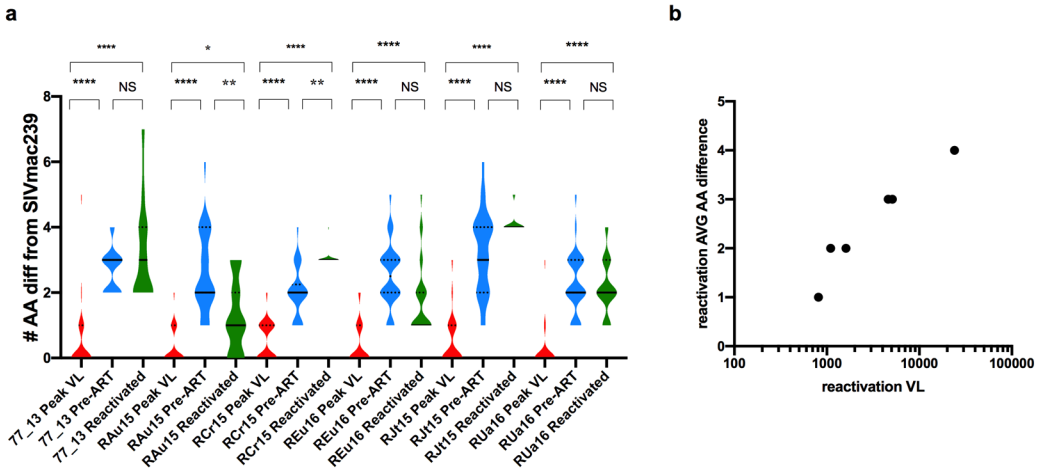
Extended Data Figure 8 | SIV-associated genes and IL-15 subunit genes show a transient change in expression following treatment with N-803 alone. **a,** Bulk CD4+ T cells (CD3+, CD4+ CD8-,CD20-,CD14-), **b,** bulk CD8+ T cells (CD3+,CD4-CD8+,CD20-,CD14), and **c,** NK cells (CD3-CD20-CD14-NKG2A+) were sorted from the peripheral blood pre-intervention, day 3, week 2, and week 4 after the first N-803 administration. RNA was extracted and libraries were prepared, normalized, pooled, and clustered on flow cells for sequencing. RNAseq data was aligned to the MacaM v7.8 assembly of the Indian rhesus macaque genome. Transcripts were analyzed for alignment against a custom gene set with SIV host restriction factors, PPIA (capsid folding protein), SIV receptors, SIV receptor agonists, NFkB subunits (involved in mediating LTR transcription), IL-15 receptor subunits, and NFAT subunits.

Supplemental Figure 9



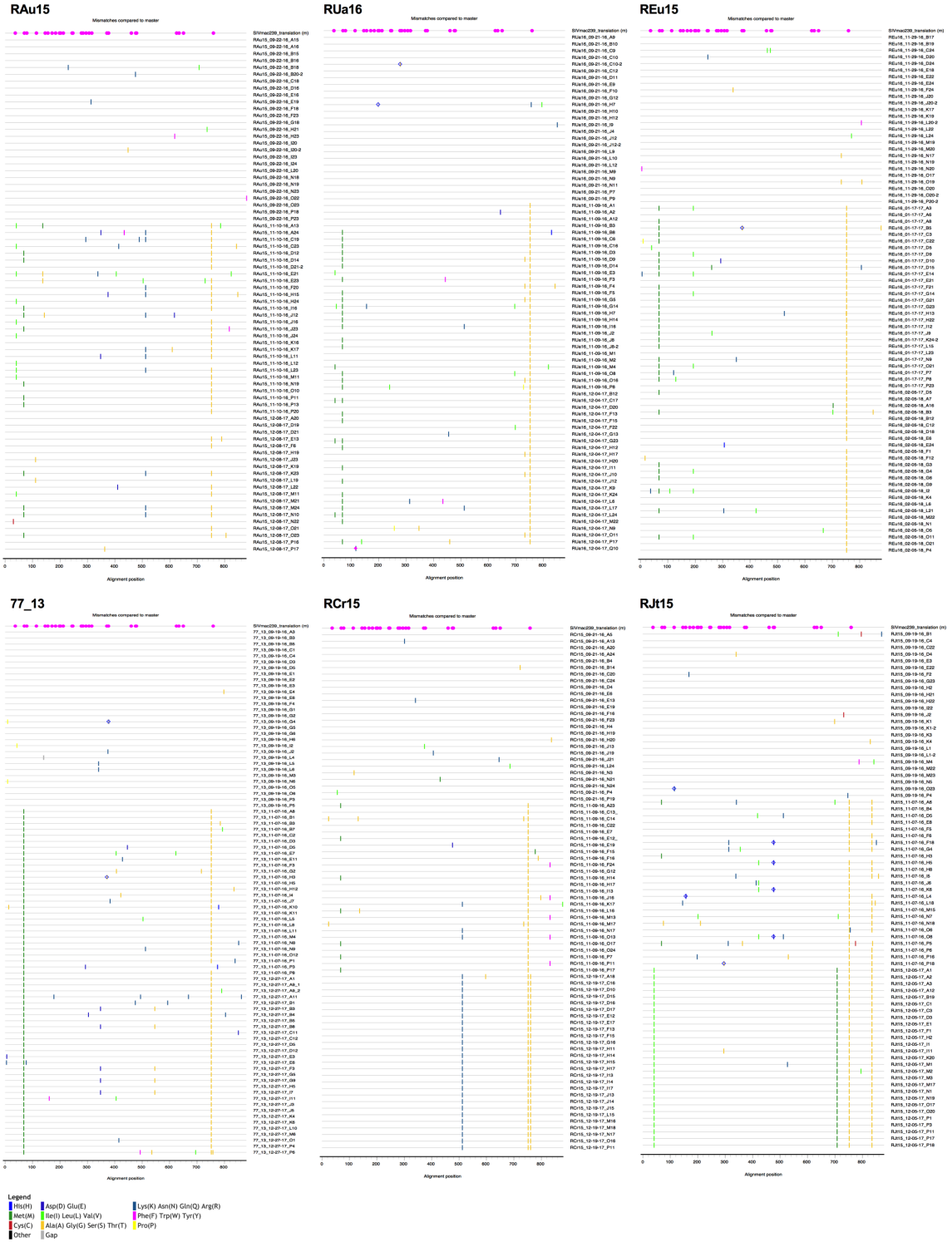
Extended Data Figure 9 | Phylogenetic trees of longitudinal SGA-derived Env amino acid sequences. Phylogenetic trees were generated for six macaques receiving CD8 depletion with N-803 administration using Env sequences from the peak VL (red), pre-ART (blue), and reactivation time points (green). The Env sequence of the SIVmac239 clone used for infection is included in each tree (black). The horizontal bar below each tree indicates the genetic distance. Sequence clusters that are supported with bootstraps greater than 80% are indicated by an asterisk. Env sequences that contain a stop codon are indicated by an arrow.

Supplemental Figure 10



Extended Data Figure 10 | Longitudinal Env amino acid divergence from the input virus and relationship with plasma viral load. The number of amino acid differences between the infecting viral clone SIV_{mac239} and each SGA amplicon was determined using Geneious. **a,** The number of differences for each individual Env sequence is plotted on the y-axis for each animal and time point, as a violin plot, which shows the frequency distribution for each data set, with the median (solid line), and quartiles (dotted lines) indicated. Peak VL (red), pre-ART (blue), and reactivation (green) time points are shown. The animal ID and the 3 time points are indicated below the graph. Statistical differences between time points for each animal were determined by performing multiple comparisons using a Kruskal-wallis test with Dunn's correction. The level of significance for each comparison is indicated above the brackets (**** $p < 0.0001$, ** $p < 0.01$, * $p < 0.05$, non-significant $p > 0.05$). **b,** The average number of sequence differences for each animal at the reactivation time point is plotted on the y-axis, and the corresponding plasma viral loads are plotted on the x-axis on a log10 scale (Spearman $r = 0.97$, $p = 0.01$).

Supplemental Figure 11



Extended Data Figure 11 | Highlighter plots of longitudinal SGA-derived Env amino acid sequences. Highlighter plots were generated for six representative macaques receiving CD8 depletion with N-803 administration using Env sequences from peak VL (red box), pre-ART (blue box), and reactivation (green box) time points. The Env sequence of the SIVmac239 clone used for infection is included as the master (reference) sequence in each plot. The position of N-linked glycosylation sites on the master sequence are indicated by pink circles. Each tick represents an amino acid difference from the master sequence, as is indicated by the legend. Blue diamonds indicate the loss of an N-linked glycosylation site.

Supplemental Table 1

Extended Date Table 1 | Viral loads of SIV-infected, ART-treated Rhesus macaques

		Pre-intervention					Post-intervention					
Group	Animal	Month -3	Month -2	Month -1	Day 0	Day 3	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
N-803 alone	REf16 R	7	< 3	< 3	7	< 3	< 3	< 3	< 3	7		< 3
	RVz15 R	7	25	19	7	25	19	< 3	< 3	1600		40
	RAf16 R	< 3	4	< 3	< 3	< 3	32	7	< 3	< 3	10	10
	REi16 R	7	5	7	< 3	7	10	< 3	< 3	< 3	< 3	< 3
	RIt16 R	18	15	8	< 3	15	< 3	7	10	7	< 3	< 3
	RBn16 R	7	4	< 3	< 3	7	< 3	< 3	< 3	< 3	< 3	< 3
	RRn16 R	25	40	25	25	50	10	15	15	25	15	15
CD8 depletion alone	RNz15 R	< 3	< 3	< 3	7	7	50	490	< 3	30	20	< 3
	ROr15 R	7	65	< 3	< 3	7	25	25	7	25		1200
	RKs15 R	< 3	< 3	< 3	< 3	10	10	65	< 3	< 3		< 3
	RRb16 R				< 3	65	40	90	65	50	Nx	
	REs16 R	65	18	25	15	40	65	65	65	450	230	40
	RSt15 R	7	7	10	< 3	40	40	50	170	7	7	32
	RVe16 R	3	15	50	20	50	230	330	430	40	7	32
	REg16 R	10	7	10	7	30	50	90	10	50	25	25
	RAk16R	< 3	6	< 3	< 3	7	25	19	40	7	< 3	< 3
	RJz15 R	15	< 3	15	< 3	15	270	400	< 3	19	19	< 3
	RRa16 R	< 3	< 3	< 3	< 3	10	< 3	7	< 3	19	< 3	< 3
	ROs15 R	7	< 3	10	< 3	65	50	30	7	310	32	< 3
	RUs15 R	15	90	29	7	40	10	50	19	3200	12000	1800
	RYe16 R	24	10	40	40	260	30	90	7	1200	1200	470
CD8 depletion with N-803	RJt15 R	< 3	< 3	600	90	12000	24000	13000	2700	32	10	15
	RHv15 R	< 3	< 3	< 3	< 3	< 3	10	7	20	660	< 3	< 3
	106_13 R	7	7	< 3	< 3	30	90	420	190	40	< 3	< 3
	RUa16 R	65	7	220	25	1100	90	1200	1700	1900	10	40
	RAu15 R	7	< 3	7	10	610	810	90	510	520	7	10
	RNa16 R	< 3	15	< 3	< 3	25	50	210	150	300	< 3	< 3
	REu16 R	40	130	40	25	557	1610	1000	1200	1000	19	32
	77_13 R	15	7	< 3	10	230	280	290	30	4600	540	< 3
	RFr15 R	< 3	10	< 3	< 3	15	50	90	50	7	7	7
	208_13 R	< 3	< 3	< 3	< 3	19	< 3	15	65	50	< 3	< 3
	RBc16 R	50	10	90	65	510	260	65	170	90	10	25
	RCa16 R	7	7	< 3	< 3	240	320	580	270	3000	< 3	10
	RCr15 R	< 3	< 3	< 3	< 3	< 3	32	200	5100	< 3	< 3	10
	RPb16 R	25	50	7	25	330	400	200	90	90	7	< 3

Ultra-sensitive viral load data with a limit of detection of 3 copies SIV RNA/mL plasma. Time points include months -3 to -1 prior to treatment with N-803 alone, CD8 depletion alone, or CD8 depletion with N-803 administration (pre-intervention) and weekly until week 6 post-intervention with day 3 (post-intervention).

Supplemental Table 2

Extended Date Table 2 | Viral loads of SHIV-infected, ART-treated Rhesus macaques

		Pre-intervention					Post-intervention					
Group	Animal	Month -3	Month -2	Month -1	Day 0	Day 3	Week 1	Day 10	Week 2	Week 3	Week 4	Week 5	Week 6
CD8 depletion with N-803	RKm16	10	9	7	3	430	30	470	480	3600	90	3	90
	CB91	65	7	9	3	460	60	660	90	810	50	30	3
	RPp16	10	7	7	3	20	7	10	7	7	25	15	15
	RRl16	3	7	7	3	3	3	19	3	7	65	15	3
	RYr16	15	7	7	3	20	50		50	25	40	3	7

Ultra-sensitive viral load data with a limit of detection of 3 copies SIV RNA/mL plasma. Time points include weeks -12 to -4 prior to intervention and weekly until week 6 post-intervention with days 3 and 10.

Supplemental Table 3

Extended Date Table 3 | Viral loads of HIV-infected, ART-treated humanized mice

Group	hu-mouse	Pre-intervention	Day 4	Day 7
N-803 alone	1	<346	<346	<346
	2	<346	<346	<346
	3	<346	<346	<346
	4	<346	<346	<346
	5	<346	<346	<346
	6	<346	<346	<346
	7	<346	<346	<346
CD8 depletion alone	1	<346	560	<346
	2	<346	<346	378
	3	<346	<346	378
	4	<346	<346	<346
	5	<346	<346	<346
	6	<346	<346	<346
	7	<346	<346	<346
	8	<346	<346	<346
CD8 depletion with N-803	1	<346	1300	1488
	2	<346	1079	<346
	3	<346	779	<346
	4	<346	546	574
	5	<346	354	<346
	6	<346	<346	1504
	7	<346	<346	1981
	8	<346	<346	<346

Limit of detection of 346 copies HIV RNA/mL plasma at day 0 (pre-intervention), day 4, and day 7 (day of sacrifice).

Chapter 4: Sustained SHIV Reactivation by N-803 in ART-Treated CD8-Depleted Macaques

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Abstract

The “shock and kill” human immunodeficiency virus (HIV) cure strategy attempts to reverse and eliminate the latent viral infection preventing eradication of the virus. Multiple latency reversing agents (LRA) have already been tested in clinical trials and all have failed to expose or decrease the HIV viral reservoir. A recent study by our group was the first to show a robust and persistent induction of plasma viremia during antiretroviral therapy (ART) *in vivo*, following administration of the IL-15 superagonist N-803 in CD8+ T cell-depleted simian immunodeficiency virus (SIV)-infected rhesus macaques. In this proof-of-concept study, we determined whether N-803 will also “shock” the viral reservoir in CD8-depleted, ART-treated rhesus macaques infected with Simian-Human immunodeficiency virus (SHIV), a chimeric and translational virus that allows for testing of HIV-targeting therapeutics with a nonhuman primate model. We observed an increase in plasma viremia following intervention and a decrease in the size of the SHIV viral reservoir in the peripheral CD4+ T cell pool. Thus, this model can be used in future studies in combination with clearance components such as HIV-based vaccine immunogens and HIV-targeting broadly neutralizing antibodies in an effort to develop an HIV cure.

Importance

The persistence of a latent viral reservoir is a major obstacle in the development of an HIV cure. Approaches have attempted to “shock” the infection out of latency to expose the infected cells to the cytopathic effects of viral replication and immune recognition. In this study, we show that administration of the anti-CD8 depleting antibody MT807R1 with concurrent IL-15 superagonist N-803 treatment in ART-suppressed, SHIV-infected rhesus macaques induces a transient increase in viremia and a decrease in the size of the viral reservoir in peripheral CD4+ T cells. As CD8 depletion with N-803 was able to “shock” the latent SHIV viral reservoir, this cure strategy can be combined with HIV-targeting clearance components during future HIV cure studies.

Introduction

Human immunodeficiency virus (HIV) is the causative agent of the acquired immunodeficiency syndrome (AIDS) and infects approximately 36.7 million people worldwide (Barre-Sinoussi et al., 1983, [internet], 2016). UNAIDS estimates there are 1.8 million new HIV infections annually and 1 million AIDS-related deaths (UNAIDS, 2017). While antiretroviral therapy (ART), the standard care for HIV infection, has dramatically reduced the mortality and morbidity of HIV infection, ART fails to provide a cure.

The main obstacle in the development of an HIV cure is the presence of a population of long-lived, latently-infected cells that persists even during ART (Chun et al., 1997c, Finzi et al., 1997, Wong et al., 1997, Siliciano et al., 2003a). This stable viral reservoir presides primarily within resting memory $CD4^+$ T cells and withstands despite decades of therapy (Chun et al., 1997b, Chomont et al., 2009, Chun et al., 1995, Chun et al., 1997a, Finzi et al., 1997, Wong et al., 1997, Finzi et al., 1999, Siliciano et al., 2003a). Recent data suggests that the HIV viral reservoir is established early during infection (Whitney et al., 2014b, Ananworanich et al., 2013) and is responsible for the viral rebound observed after ART interruption (Davey et al., 1999, Chun et al., 1999). Therefore, strategies additional to ART are necessary to cure HIV, and novel therapies targeting the HIV viral reservoir are of utmost importance.

Recent therapeutic approaches have attempted to the reverse HIV latency in order to expose the viral reservoir to elimination, a strategy termed the “shock and kill”. Latency reversal of HIV-infected cells may lead to HIV RNA synthesis, production of

viral protein, and/or release of viral particles, potentially causing direct death of the infected cell and/or indirect killing via recognition by the immune system (Deeks, 2012). A latency-reversing agent (LRA) given during a state of viral suppression may be capable of activating CD4+ T cells to “shock” the integrated virus out of latency, exposing the infected cell to cytopathic effects of viral replication and immune recognition. An additional “kill” therapeutic aims to amplify the cytolytic response of the immune system to eliminate infected cells (Deeks et al., 2015). Targeted killing of infected cells shocked out of a previously latent state, particularly during ART, may eliminate the viral reservoir preventing the development of an HIV cure.

Multiple LRAs have already been tested in clinical trials in HIV+ ART-treated patients. Thus far, none have been shown to elicit the “shock” or “kill” required to eliminate the reservoir, failing to provoke even a minor increase in plasma viremia following LRA administration (Archin et al., 2014, Spivak et al., 2014, Rasmussen et al., 2014, Søgaard et al., 2015, Elliott et al., 2014, Elliott et al., 2015). A recent study by our lab has shown that N-803, an IL-15 superagonist complex, is able to reactivate viral production in CD8-depleted ART-treated SIV-infected rhesus macaques and CD8-depleted ART-treated HIV-infected humanized mice (McBrien et al.). This was the first study to show a robust and persistent induction of plasma viremia during ART *in vivo*. Unfortunately, because no “kill” agent was included in the study design, animals did not show clearance of the viral reservoir.

In this proof-of-concept study, we determine whether N-803 will also “shock” the viral reservoir in CD8-depleted, ART-treated macaques infected with a Simian-Human immunodeficiency virus (SHIV). While SIV infection models are practical for questions

related to pathogenesis, HIV-based vaccine immunogens and broadly neutralizing antibodies cannot be tested directly by challenging with SIV. Typically, SHIV viruses contain a SIV_{mac239} backbone with SIV *env, tat, and rev* genes replaced with corresponding HIV-1 genes. Thus, SHIV models of infection allow the use of therapeutics aimed at HIV to be tested in macaques. In this study, we show that N-803 is able to “shock” the SHIV viral reservoir in CD8-depleted, ART-treated macaques. Additionally, there was a significant decrease in the size of the peripheral CD4+ T cell viral reservoir following intervention. In conclusion, HIV-targeting “kill” agents, such as broadly neutralizing antibodies, CD4 mimetics, vaccinations, or immunotoxins, may be combined with CD8 depletion and N-803 to elicit clearance of SHIV infection in future cure studies.

Results

Study design

Animals were infected intrarectally with a high dose of $SHIV_{SF162P3}$ and viral loads were monitored for twelve weeks following infection. On week twelve post-infection animals initiated a daily three-drug regimen of ART, which was maintained for the remainder of the study (Figure 1). In order to stabilize the latent viral reservoir, animals were treated with ART for six months prior to intervention, with monthly blood draws to record plasma viral load and T cell counts. After six months of ART, animals were administered 50 mg/kg of the anti-CD8α-depleting antibody, MT-807R1, with a cycle of N-803, administered at 100 µg/kg weekly for four weeks. The first N-803 administration was at the time of CD8 depletion. Plasma viral loads were measured 1-2 times per week following intervention. Lymph node and rectal biopsies were collected on the day of intervention and one week after.

N-803 induces an increase in plasma viremia in CD8-depleted, ART-treated macaques

In order to validate the ability of N-803 to reactivate virus in CD8-depleted, ART-treated, SHIV-infected animals, plasma viral load was monitored throughout the study (Figure 2). Following infection, all animals reached an observable peak viral load comparable to HIV infection of humans. ART did result in suppression of viremia in all animals, in fact all animals had plasma viral loads less than 3 copies/mL before intervention. Following administration of anti-CD8 depleting antibody MT807R1 and N-803, 5/5 (100%) animals experienced an increase in plasma viremia within 10 days. 4/5 (80%) animals had at least one time-point with a plasma viral load ≥50 copies/mL and

1/5 (20%) animals had a time-point with a plasma viral load ≥1000 copies/mL. The highest plasma viral load observed under the limitation of blood draw frequency was 3,600 copies/mL. Viral suppression returned after the reemergence of CD8+ T cells, but following the reemergence of NK cells (Supplemental Figures 1 and 2). Of note, within a week of intervention CD8+ T cells and NK cells were significantly depleted in the peripheral blood, lymph node, and rectum as compared to pre-intervention frequencies (Supplemental Figure 3A+B).

Intervention with anti-CD8 depleting antibody MT807R1 with N-803 changes the frequency of immune cell subsets in the periphery, lymph node, and rectum

Peripheral immune cell counts and percentages were measured using flow cytometry and values obtained from complete blood count analysis (Figure 3). Of note, while there was not a statistically significant change in the white blood cell (WBC) count following intervention (Figure 3A), there was a temporary increase in the percentage of B cells and decrease in the percentage and count of peripheral CD3+ T cells (Figure 3E+H). Not surprisingly, the percentage of CD4+ T cells increased following intervention (Figure 3F), as the percentage of CD8+ T cells dropped (Figure 3G). While the CD8+ T cell count remained significantly depleted during the seven weeks of follow-up (Figure 3J), the percentage of NK cells quickly returned to pre-depletion levels by the third week (Figure 3D).

Lymph node and rectal immune cell percentages were also measured pre-ART, pre-intervention, and one week after intervention (Supplemental Figure 4+5). In the lymph node, intervention resulted in a statistically significant increase in monocytes

(Supplemental Figure 4E) but did not have a significant impact on the percentage of CD3+ T cells, CD4+ T cells, or B cells (Supplemental Figure 4A, B, D). The percentage of CD8+ T cells and NK cells in the lymph node was significantly lower following intervention (Supplemental Figure 4C+F). Similarly, while there was no significant change in the percentage of CD3+ T cells or B cells in the rectum following intervention (Supplemental Figure 5B+E), there was a modest increase in monocytes (Supplemental Figure 5E) and a significant depletion of CD8+ T cells and NK cells (Supplemental Figure 5C+F).

Immunologic characterization of CD4+ T cells reveals upregulation of PD-1 on peripheral cells and increased proliferation of lymph node and rectal cells

Flow cytometry was additionally used to assess changes in CD4+ T cell phenotypes and subsets in the peripheral blood, lymph node, and rectum following intervention (Figure 4, Supplemental Figures 6+7). Intervention resulted in a persistent upregulation of the activation and exhaustion marker PD-1 in peripheral CD4+ T cells (Figure 4C). Furthermore, a decrease in the frequency of naïve CD4+ T cells, and expansion of the effector and central memory CD4+ T cell populations was observed (Figure 4D,E,G). In the lymph node, CD4+ T cells showed a significant upregulation of the proliferation marker Ki67 (Supplemental Figure 6B) and a decrease in the frequency of effector memory CD4+ T cells (Supplemental Figure 6E). In addition, Ki67 was upregulated on CD4+ T cells in the rectum (Supplemental Figure 7B).

N-803 administration in CD8-depleted macaques results in a decrease in the size of the peripheral CD4+ T cell reservoir

Peripheral and lymph node CD4+ T cells cell-associated SHIV RNA and DNA were also measured before and after intervention (Figure 5). There was a significant decrease in SHIV DNA levels in peripheral CD4+ T cells one and four weeks following intervention (Figure 5A), indicating a decrease in the size of the reservoir. Cell-associated SHIV RNA levels were not significantly different during these time points (Figure 5B). In the lymph node, neither SHIV DNA or RNA levels appeared to be significantly different one week after intervention.

Discussion

The persistence of a population of latently-infected cells prevents the development of an HIV cure. Reversal of latency aims to expose infected cells to the cytopathic effects of viral replication and immune recognition. Studies from our group suggest that CD8+ T cells suppress HIV/SIV transcription *in vitro* and *in vivo*, thus preventing the reversal of latency during “shock and kill” cure strategies (Cartwright et al., 2016) Franchitti et al., manuscript in preparation, Zanoni et al., manuscript in preparation). Using the SIV model of infection in macaques, we recently showed that IL-15 superagonist N-803 is able to reverse latency but only in the absence of CD8+ T cells (McBrien et. al). These data support the hypothesis that CD8 depletion “unlocks” the viral reservoir, thus allowing for the “shock” induced by a latency reversing agent. Of note, this study did not include a “kill” component poised to target infected cells but serves as a platform to test the ability of such components to clear the reservoir. As only SIV-targeting therapeutics can be tested in the SIV model, an SHIV model of infection

would offer a more translational platform as “kill” therapeutics targeting HIV specifically can be tested.

In this proof-of-concept study, we tested whether administration of IL-15 superagonist N-803 to SHIV-infected, ART-treated, CD8-depleted macaques would induce virus reactivation similar to our previous study (McBrien et. al). Albeit to a lesser degree, the combination of N-803 and CD8 depletion induced an increase in viremia in SHIV-infected macaques. We also observed a decrease in the size of the viral reservoir in peripheral CD4+ T cells following intervention. A caveat of this study is that only five animals were available for use and we did not have N-803 alone or CD8-depletion alone control arms. As we were merely testing the concept that we could apply our “unlock and shock” strategy to a SHIV model in preparation for future and larger studies, we did not find control arms necessary. An additional caveat of this study is that SHIV infections are less pathogenic, often controlling viremia in the absence of ART and producing a smaller viral reservoir. The smaller viral reservoir explains the smaller magnitude of virus reactivation following intervention. Lastly, the anti-CD8 depleting antibody MT807R1 depleted cells expressing CD8α, which includes not only CD8+ T cells but also NK cells. Thus, the “unlock” effect of CD8 depletion could be a result of NK cell depletion. Yet, as the NK cells returned to the peripheral blood before the return of viral suppression (and CD8+ T cells), it is unlikely that NK cells are responsible for the increase in virus expression.

In conclusion, this study confirms that CD8 depletion of ART-treated, SHIV-infected macaques followed by administration of IL-15 superagonist N-803 is able to “shock” the viral reservoir. This model can be applied to future “shock and kill” studies in

macaques with the inclusion of HIV-targeting clearance components, such as vaccines, immunotoxins, CD4 mimetics, broadly neutralizing antibodies, etc. As the strategy involves elimination of cell types with clearance capabilities, future work to elicit the specific mechanism of CD8+ T cell mediated HIV/SIV suppression must be done. Therapeutics must be developed that silence the specific pathway without elimination of the CD8+ T cell. Combining a therapeutic capable of unlocking the suppressive effect of CD8+ T cells on HIV transcription with a latency reversing agent and kill component holds promise towards an HIV cure.

Materials and Methods:

Study approval

All animal experiments were conducted following the guidelines established by the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals(Council, 2011) and performed in accordance with institutional regulations after review and approval by the Institutional Animal Care and Usage Committee (IACUC, 3000065, 2003297, 2003470, and PROTO201700665) at the Yerkes National Primate Research Center (YNPRC; Atlanta, GA). Anesthesia was administered prior to performing any procedure, and the proper steps were taken to minimize any suffering the animals may have experienced.

Animals, SIV-infection, and Antiretroviral therapy

This study was conducted using a total of 5 Indian-origin rhesus macaques (RM) housed at Yerkes National Primate Research Center. All RMs were HLA*B07- and HLA*B17-. All procedures are approved by the Emory University Institutional Animal Care and Use Committee (IACUC) and animal care facilities are accredited by the U.S. Department of Agriculture (USDA) and the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International.

Animals were infected intrarectally with high-dose $SHIV_{162P3}$, administered as a 1:50 dilution of a 2032 TCID50/mL, 10^9 RNA copies/mL, 182 P27 ng/ml stock. 3/5 animals were administered broadly neutralizing antibody targeting V3-glycan PGT-121 (RPp16 1mg/kg, RRl16 and RYr16 0.2 mg/kg) at the time of infection for another study. The presence of the antibody did not prevent infection.

All animals were put on a three-drug ART regimen at twelve weeks post-SHIV infection, after the washout period of PGT-121 had passed. Tenofovir (TDF at 5.1mg/kg/day or PMPA at 20mg/kg/day) and Emtricitabine (FTC at 40mg/kg/day) were both kindly provided by Gilead Pharmaceuticals. Dolutegravir (DTG at 2.5mg/kg/day) was kindly provided by ViiV Pharmaceuticals. Drugs were administered daily by subcutaneous injection.

CD8 depletion and N-803 administration

After 6 months of ART, animals were administered one dose of the anti-CD8α-depleting antibody, MT-807R1 at 50 mg/kg subcutaneously. N-803 was administered subcutaneously in a cycle of 100 µg/kg once a week for four consecutive weeks starting at the time of CD8 depletion.

Sample collection and tissue processing

Blood, lymph node (LN), and gut (rectal biopsy, RB) were collected prior to ART initiation, prior to intervention, and one week after intervention and processed for further analyses as previously described(Cartwright et al., 2016).

Immunophenotype by flow cytometry

Multiparametric flow cytometry was performed according to a standard protocol on PBMC, LNMC, and rectal cells using fluorescently labeled monoclonal antibodies cross-reactive in RM. The following antibodies were used at 37°C for 30 minutes: CCR5 APC (3A9) and CCR7 FITC (150503). Then the following antibodies were at room temperature for 30 minutes: CD3 APC-Cy7 (SP34-2), CD4 BV650 (OKT4), CD8α

BV711 (RPA-T8), CD8β PE-Cy5 (SIDI8BEE), CCR5 APC (3A9), CCR7 FITC (150503), CD45RA Pe-Cy7 (5H9), CD62L PE (SK11), CD95 BV605 (DX2), PD-1 BV421 (EH12.2H7), CD16 BV421 (3G8), CD20 PE-Cy5 (2H7), CD14 PE-Cy7 (M5E2), NKG2A (CD159) PE (Z199), CD28 ECD (CD28.2), and CD56 FITC (NCAM16.2). For panels with intracellular stain, cells were fixed and permeabilized with Cytofix/cytoperm kit (BD) before being stained at room temperature for 30 minutes with Ki67 AF700 (B56).

All flow cytometry specimens were acquired on an LSR II (BD Biosciences) equipped with fluorescence-activated cell sorter software (FACS Diva), and analysis of the acquired data was performed using FlowJo software (Tree Star).

Determination of plasma SIV RNA, and cell-associated RNA and DNA

Plasma SHIV *gag* RNA was performed using quantitative PCR to determine SHIV plasma viral load essentially as described using high sensitivity assay formats(Hansen et al., 2017, Li et al., 2016). CD4+ T cells were isolated from PBMC and LNMC via the CD4+ microbeads kit (Miltenyi) prior to intervention, one week after intervention, and 4 weeks after (PBMC only). Quantification of total cell-associated $SHIV_{162P3}$ *gag* DNA and RNA were performed as previously described(Chahroudi et al., 2014, Kumar et al., 2018).

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providing N-803, Diogo Magnani and the NHP Reagent Resources for the MT-807R1 antibody, Romas Geleziunas and Gilead Pharmaceuticals for providing Tenofovir and Emtricitabine, and Jim Demerest and ViiV Healthcare for providing Dolutegravir for this study.

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Author contributions

J.B.M., A.C., M.P. and G.S. designed the experiments. J.B.M, E.W. and D.G.C. performed the experiments. J.D.L. performed ultrasensitive viral-load analyses and cell-associated DNA and RNA. J.T.S. and J.H.L. provided technical support. J.B.M., and G.S. wrote the manuscript.

Chapter 4 Figures

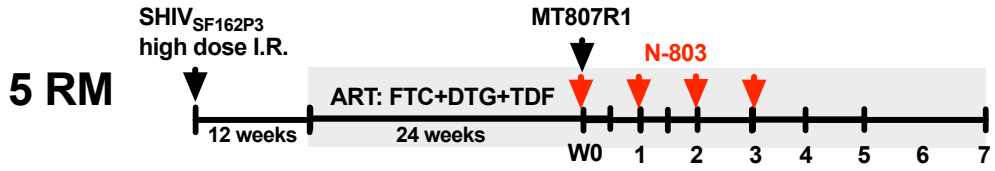


Figure 1: Study Design. Animals initiated antiretroviral therapy 12 months after SHIV infection, and intervention was started 24 weeks after ART initiation. Numbers on axis designate week post-intervention. Red arrows designate 100 μg/kg N-803 administration and black arrow designates 50 mg/kg MT807R1 administration. RM: rhesus macaques, I.R: intrarectal, ART: antiretroviral therapy, TDF: Tenofovir, FTC: Emtricitabine, DTG: Dolutegravir, CD8dep: CD8 depletion with MT807R1, N-803: IL-15 superagonist complex N-803.

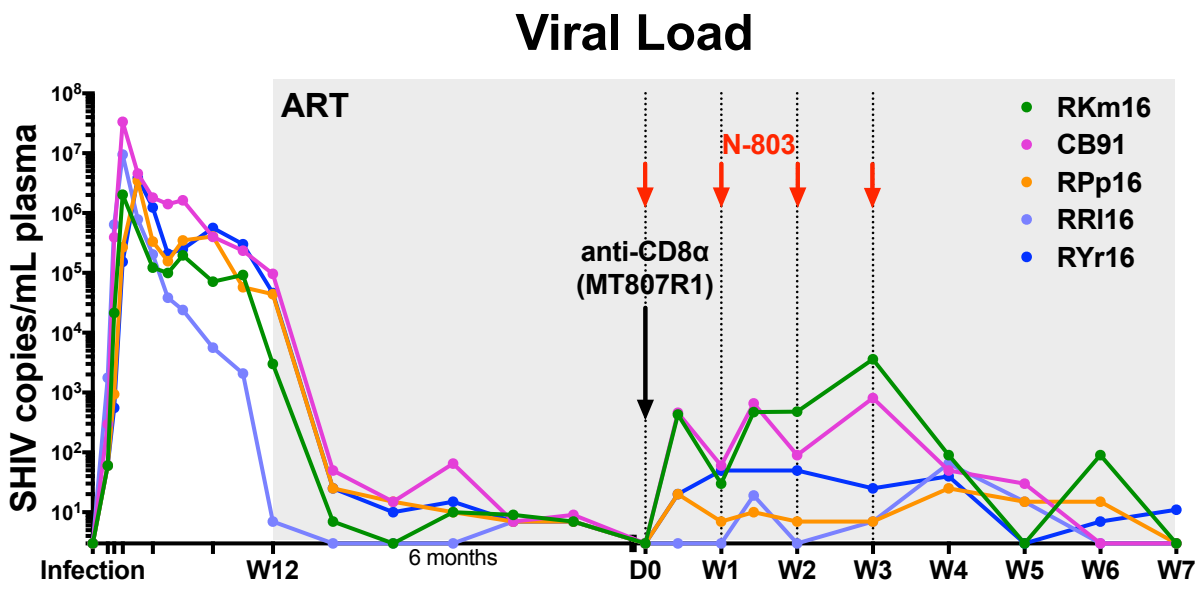


Figure 2: N-803 induces an increase in plasma viremia when administered in the absence of CD8+ T cells. Changes in viral load throughout infection, ART treatment, and intervention. Limit of detection is represented by a grey dashed line at 3 copies/mL. Pre-intervention time points and post-intervention time points are at different time scales.

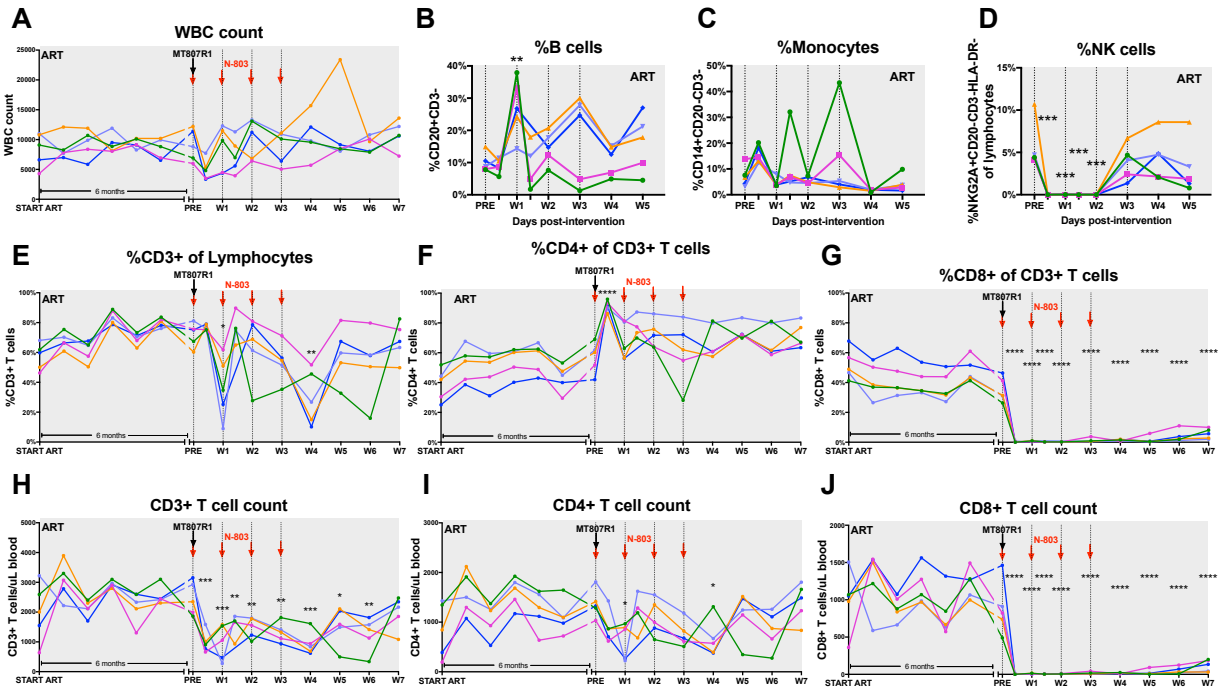


Figure 3: Peripheral blood cell frequencies pre- and post-intervention. A) White blood cell (WBC) count was recorded from the complete blood count. B) The percentage of B cells was calculated as the proportion of CD20+CD3- cells of the viable leukocyte population. C) The percentage of monocytes was calculated as the percentage of CD14+CD20-CD3- cells as a proportion of the viable leukocyte population. D) The percentage of NK cells was calculated as the percentage of NKG2A+CD20-CD3-HLA-DR- cells as a proportion of the viable leukocyte population. E) The percentage of CD3+ T cells was calculated as the proportion of viable leukocyte. F-G) The percentage of CD4+ and CD8+ T cells were calculated as the proportion of CD3+ cells. H-J) T cell counts were calculated using the percentages in (E-F) and the WBC count in (A) for the same time point. One-way ANOVA was used to determine statistically significant differences between pre-intervention and post-intervention values.

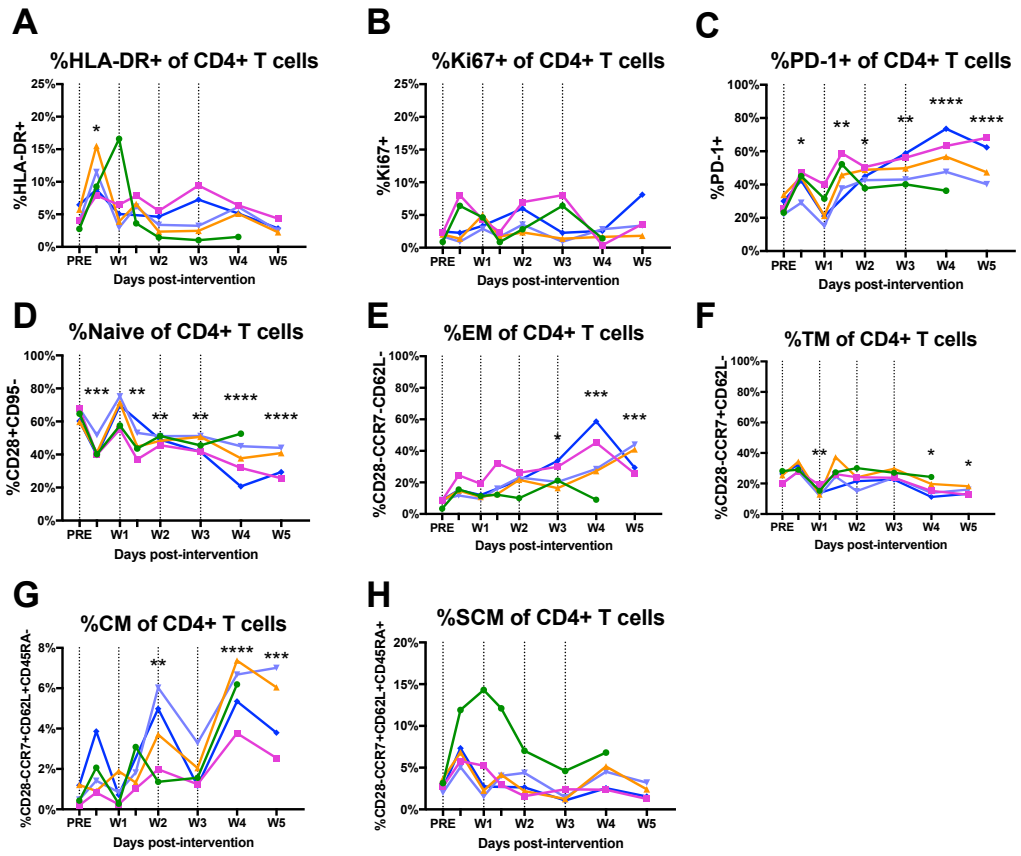


Figure 4: Peripheral blood CD4+ T cell phenotypes and subsets pre-ART, before intervention, and one week after. All percentages were calculated as the proportion of the CD4+ T cell population. A) HLA-DR was used as a marker of cellular activation. B) Ki67 was used as marker of proliferation. C) PD-1 was used as a marker of cellular exhaustion. D) Naïve cells were defined as the CD28+CD95- population. E) Effector memory (EM) were defined as the CD28-CCR7-CD62L- population. F) Transitional memory (TM) were defined as the CD28-CCR7+CD62L- population. G) Central memory (CM) were defined as the CD28-CCR7+CD62L+CD45RA- population. H) Memory stem cell (SCM) were defined as the CD28-CCR7+CD62L+CD45RA+ population. One-way ANOVA was used to determine statistically significant differences between pre-intervention and post-intervention values.

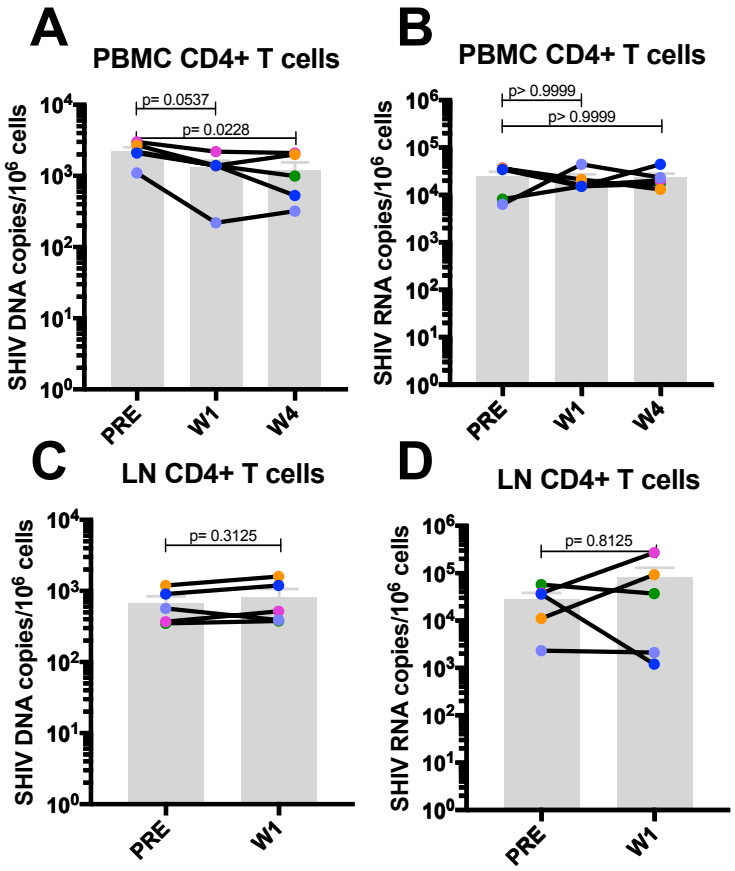
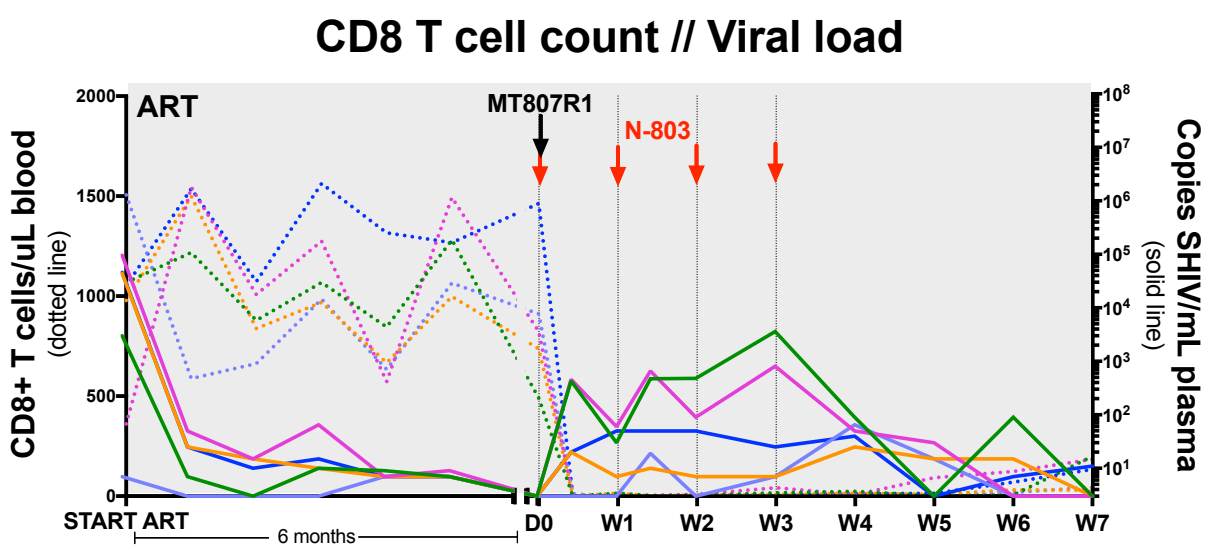
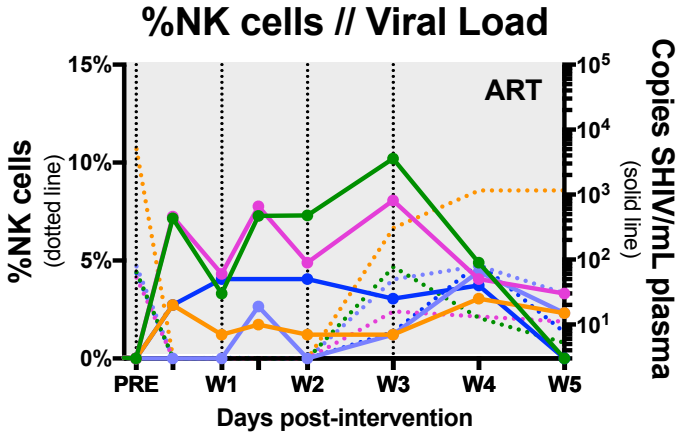


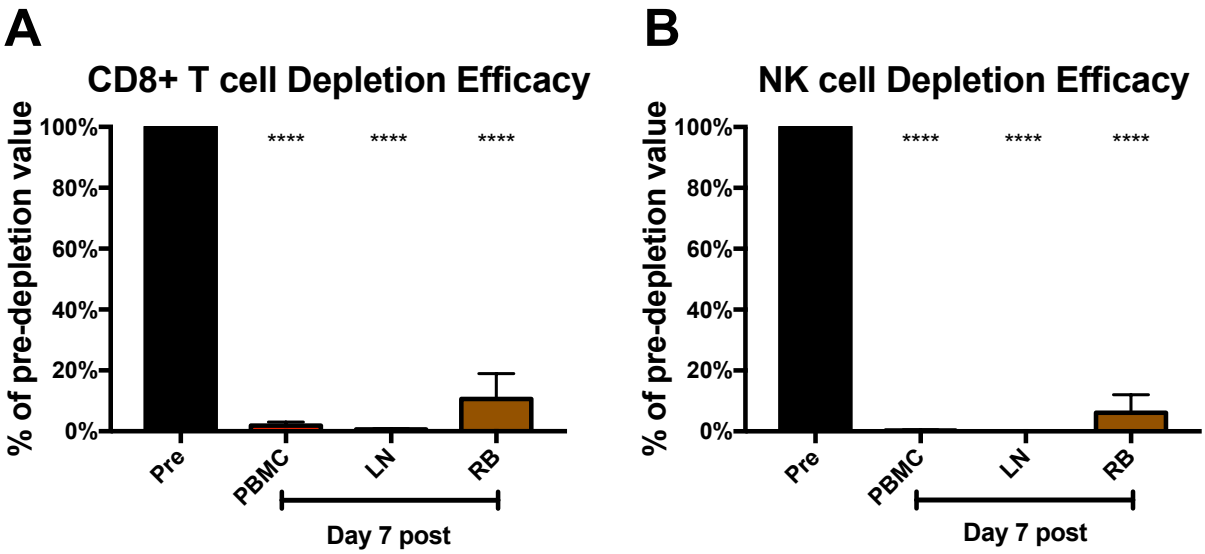
Figure 5: Levels of cell-associated SHIV decrease in peripheral CD4+ T cells following treatment with N-803 in CD8-depleted animals. Bulk CD4+ T cells were isolated from PBMCs and lymph node mononuclear cells (LNMC) pre-intervention, week 1 post-intervention, and week 4 post-intervention (PBMC only). Quantification of cell-associated SHIV DNA (A) and SHIV RNA (B) from peripheral CD4+ T cells. C-D) Cell-associated SHIV DNA and RNA from CD4+ T cells in the lymph node. Wilcoxon test was used to test significance in PBMC samples, and Friedman test was used for lymph node samples.



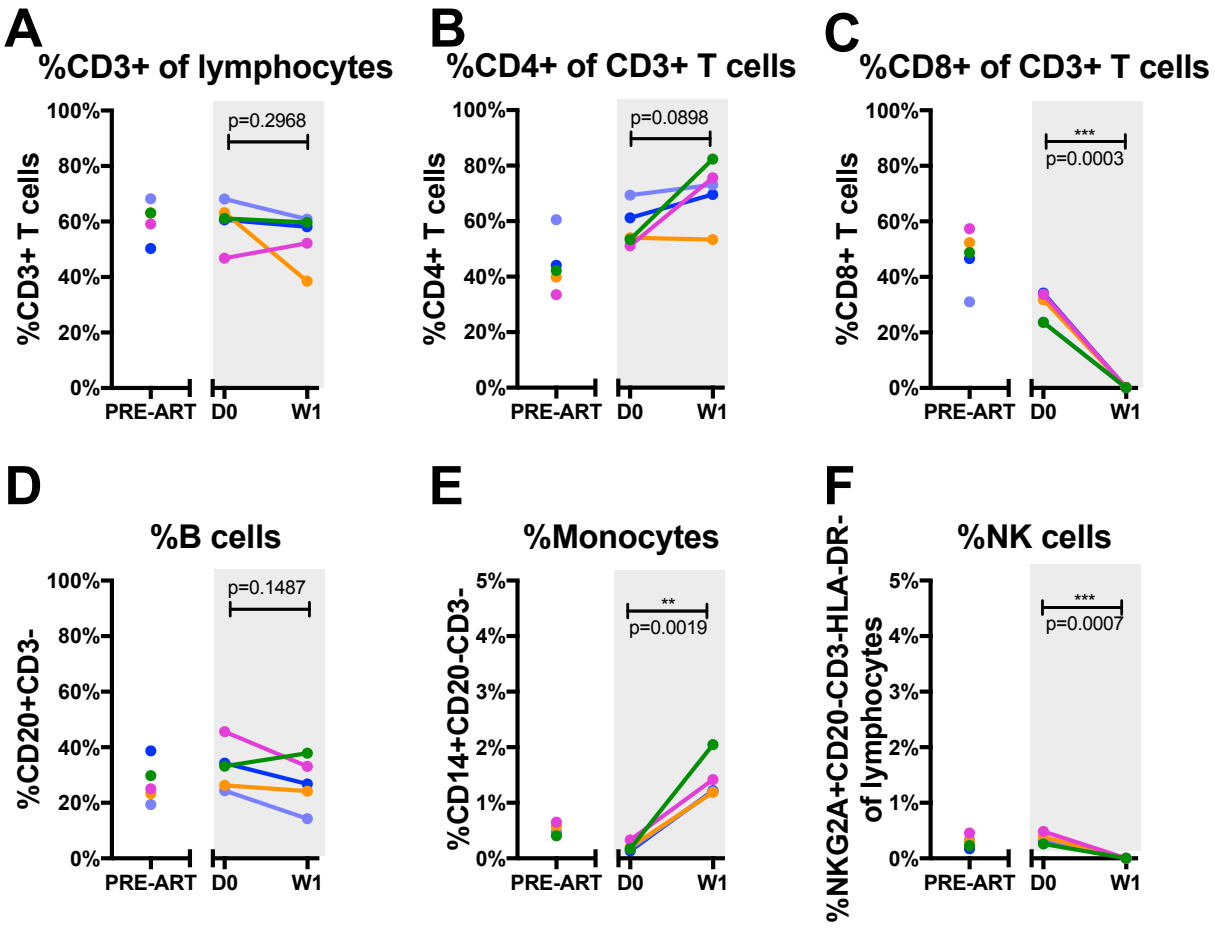
Supplemental Figure 1: Plasma viral loads with CD8+ T cell count during antiretroviral therapy. Plasma viral loads are represented on the right axis (solid line) and peripheral blood CD8+ T cell counts are represented on the left axis (dotted lines). The limit of detection of the number of copies of SHIV is 3 copies/mL of plasma. Animal color key is consistent with Figure 3.2.



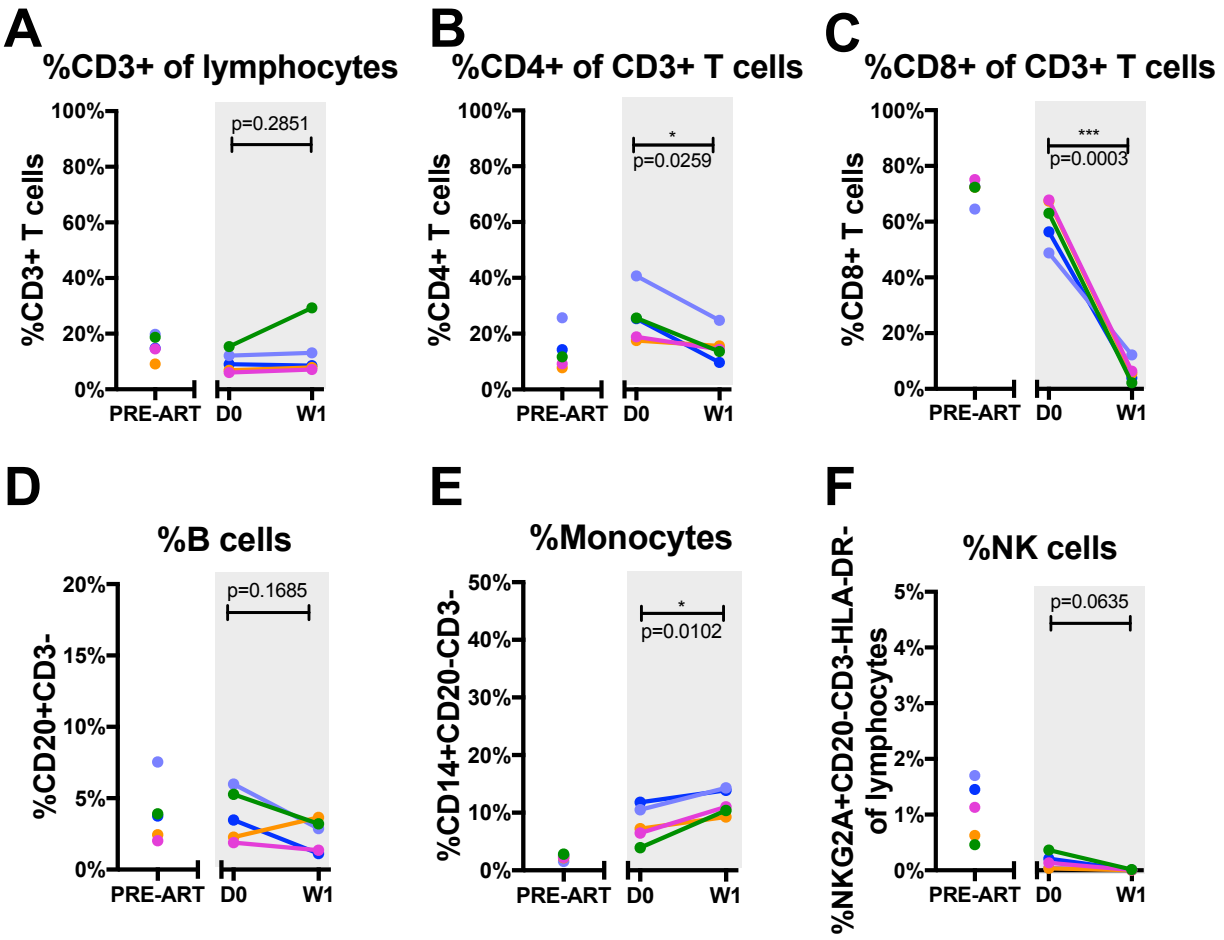
Supplemental Figure 2: Plasma viral loads with the percentage of NK cells in the periphery during antiretroviral therapy. Plasma viral loads are represented on the right axis (solid line) and peripheral blood NK cell percentages (NKG2A+CD20-CD3-HLA-DR-) are represented on the left axis (dotted lines). The limit of detection of the number of copies of SHIV is 3 copies/mL of plasma. Animal color key is consistent with Figure 3.2.



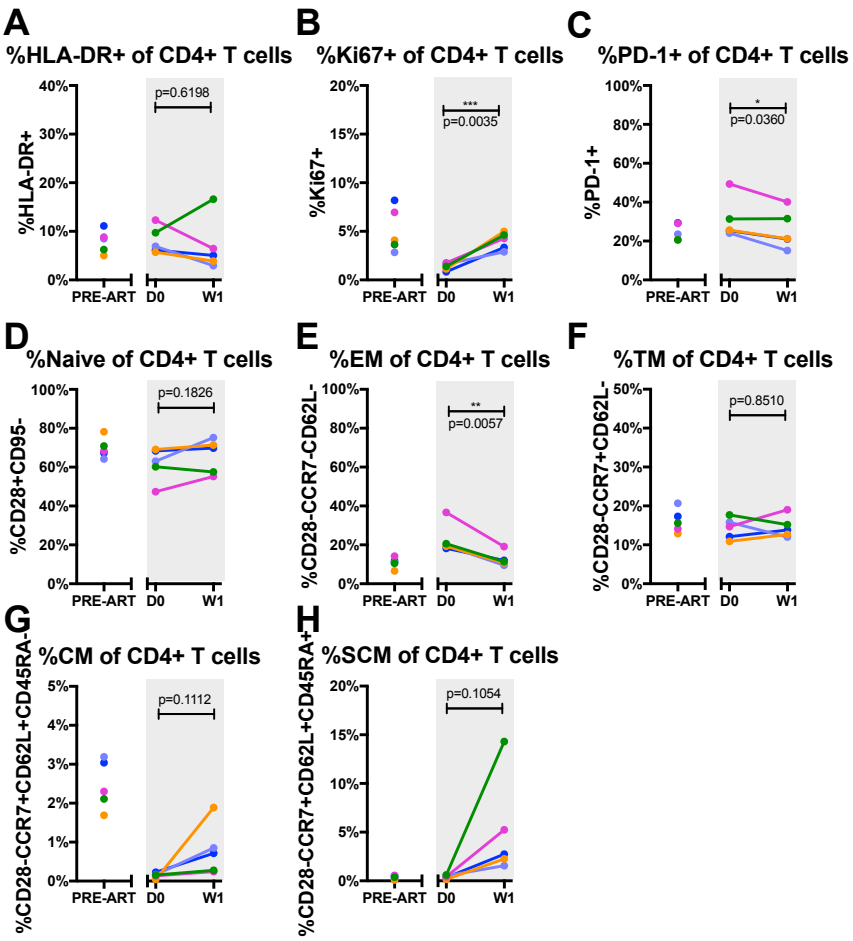
Supplemental Figure 3: Efficacy of CD8+ lymphocyte depletion day 7 post-intervention. Determined by calculating the percentage of the cell type at day 7 as a proportion of the percentage pre-intervention (day 0). All values were statistically significant ($p<0.0001$) at day 7 across tissues, as determined using a paired T test. Pre: pre-depletion; PBMC: peripheral blood mononuclear cell; LN: lymph node; RB: rectal biopsy.



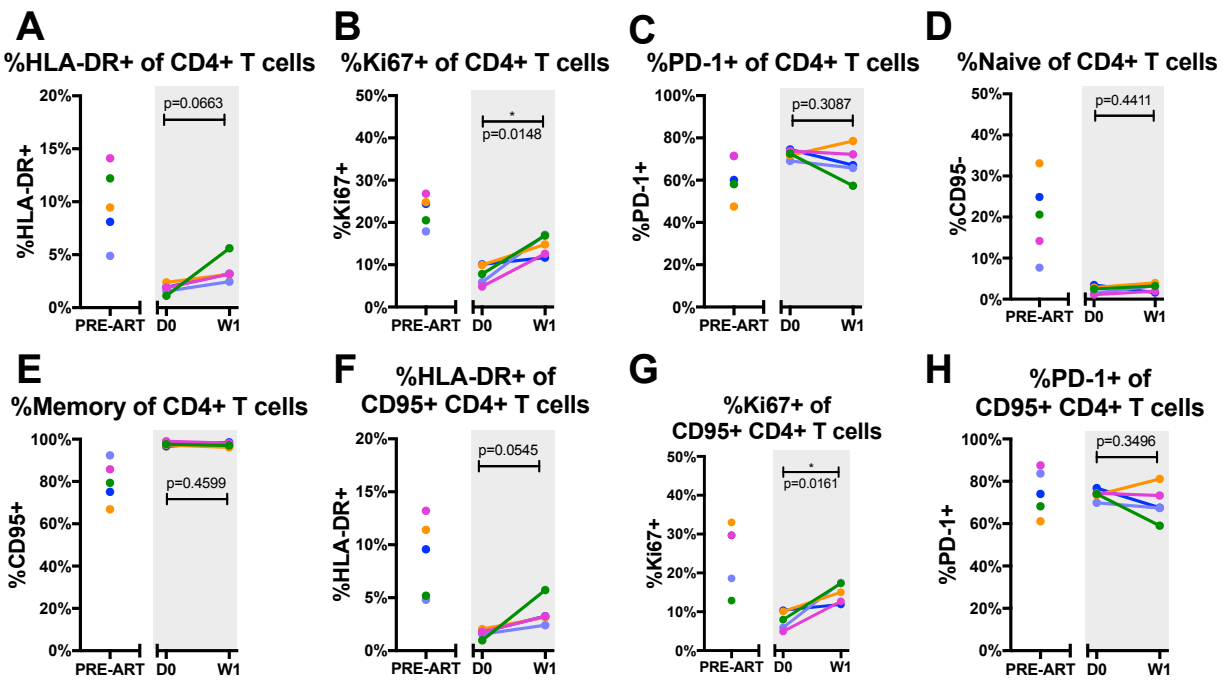
Supplemental Figure 4: Lymph node cell subsets pre-ART, before intervention, and one week after. A) The percentage of CD3+ T cells was calculated as the proportion of viable lymphocytes. B-C) The percentage of CD4+ and CD8+ T cells were calculated as the proportion of CD3+ cells. D) The percentage of B cells was calculated as the proportion of CD20+CD3- cells of the viable lymphocyte population. E) The percentage of monocytes was calculated as the percentage of CD14+CD20-CD3- cells as a proportion of the viable lymphocyte population. F) The percentage of NK cells was calculated as the percentage of NKG2A+CD20-CD3-HLA-DR- cells as a proportion of the viable lymphocyte population. Paired T tests were used to determine statistically significant differences between pre-intervention and week 1 post-intervention values.



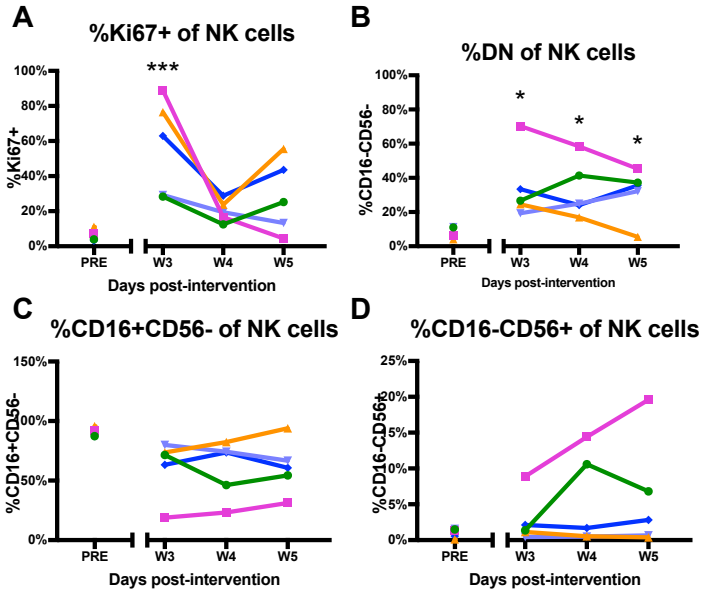
Supplemental Figure 5: Rectal biopsy cell subsets pre-ART, before intervention, and one week after. A) The percentage of CD3+ T cells was calculated as the proportion of viable lymphocytes. B-C) The percentage of CD4+ and CD8+ T cells were calculated as the proportion of CD3+ cells. D) The percentage of B cells was calculated as the proportion of CD20+CD3- cells of the viable lymphocyte population. E) The percentage of monocytes was calculated as the percentage of CD14+CD20-CD3- cells as a proportion of the viable lymphocyte population. The percentage of NK cells was calculated as the percentage of NKG2A+CD20-CD3-HLA-DR- cells as a proportion of the viable lymphocyte population. Paired T tests were used to determine statistically significant differences between pre-intervention and week 1 post-intervention values.



Supplemental Figure 6: Lymph node CD4+ T cell phenotypes and subsets pre-ART, before intervention, and one week after. All percentages were calculated as the proportion of the CD4+ T cell population. A) HLA-DR was used as a marker of cellular activation. B) Ki67 was used as marker of proliferation. C) PD-1 was used as a marker of cellular exhaustion. D) Naïve cells were defined as the CD28+CD95- population. E) Effector memory (EM) were defined as the CD28-CCR7-CD62L- population. F) Transitional memory (TM) were defined as the CD28-CCR7+CD62L- population. G) Central memory (CM) were defined as the CD28-CCR7+CD62L+CD45RA- population. H) Memory stem cell (SCM) were defined as the CD28-CCR7+CD62L+CD45RA+ population. Paired T tests were used to determine statistically significant differences between pre-intervention and week 1 post-intervention values.



Supplemental Figure 7: Rectal biopsy CD4+ T cell phenotypes and subsets pre-ART, before intervention, and one week after. All percentages were calculated as the proportion of the CD4+ T cell population (A-E) or the memory CD4+ T cell population (F-H). A+F) HLA-DR was used as a marker of cellular activation. B+G) Ki67 was used as marker of proliferation. C+H) PD-1 was used as a marker of cellular exhaustion. D) Naïve cells were defined as the CD95- population. E) Memory were defined as the CD95+ population. Paired T tests were used to determine statistically significant differences between pre-intervention and week 1 post-intervention values.



Supplemental Figure 8: Peripheral blood NK subsets and proliferation pre-ART, before intervention, and one week after. All percentages were calculated as the proportion of the NK cell population. A) Ki67 was used as marker of proliferation. B) Double negative cells were defined as CD16-CD56-. C) The CD16+CD56- subset represents NK cells with a cytotoxic phenotype. D) The CD16-CD56+ subset represents NK cells with a cytokine-producing phenotype. One-way ANOVA was used to determine statistically significant differences between pre-intervention and post-intervention values.

Chapter 5: Combination of CD8β depletion and IL-15 superagonist N-803 induces virus reactivation in SHIV-infected, long-term ART-treated rhesus macaques

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Abstract

The “shock and kill” strategy predicates that virus reactivation in latently infected cells is required to eliminate the HIV reservoir. In a recent study, we show robust and persistent induction of plasma viremia in ART-treated SIV-infected rhesus macaques (RMs) undergoing CD8a depletion and treated with the IL-15 superagonist N-803 (McBrien et al.). Of note, in the study we used an antibody targeting CD8α, therefore depleting NK cells, NKT cells, and γδ T cells in addition to CD8+ T-cells. In this proof-of-concept study, we tested whether virus reactivation can be induced by administration of N-803 to SHIV-infected, ART-treated RMs that are selectively depleted of CD8+ T-cells via the CD8β-targeting antibody CD8b255R1. CD8β depletion was performed in five $SHIV_{SF162P3}$-infected RMs treated with ART for 12 months and with plasma viremia consistently below 3 copies/ml. All animals received four weekly doses of N-803 starting at the time of CD8b255R1 administration. Induction of detectable plasma viremia was observed in three out of five RMs, with the level of virus reactivation seemingly correlated to the frequency of CD8+ T-cells following CD8b depletion as well as the level of virus reactivation observed when the same animals underwent CD8a depletion and N-803 administration after 24 weeks of ART. These data indicate that CD8β depletion and N-803 administration can induce virus reactivation in $SHIV_{SF162P3}$-infected RMs despite suboptimal depletion of CD8+ T-cells and profound ART-induced suppression of virus replication, thus confirming a critical role for these cells in suppressing virus production and/or reactivation *in vivo* under ART.

Importance

The “shock and kill” HIV cure strategy attempts to reverse and eliminate the latent viral infection preventing eradication of the virus. Latency-reversing agents tested in clinical trials to date have failed to expose or decrease the HIV viral reservoir. IL-15 superagonist N-803, currently involved in a clinical trial for HIV cure, was recently shown by our lab to induce robust and persistent induction of plasma viremia during ART in three *in vivo* animal models of HIV infection. These results suggest a substantial role for CD8+ lymphocytes in suppressing the latency-reversal effect of N-803 by promoting the maintenance of viral latency. In this study, we tested whether the use of a CD8β-targeting antibody, which would specifically deplete CD8+ T cells, would yield similar levels of virus reactivation. A modest induction of plasma viremia was observed, which heavily correlated with the efficacy of the CD8 depletion strategy.

Introduction

Human immunodeficiency virus (HIV) is the causative agent of acquired immunodeficiency syndrome (AIDS) and infects approximately 36.7 million people worldwide (Barre-Sinoussi et al., 1983, [internet], 2016). UNAIDS estimates there are 1.8 million new HIV infections annually and 1 million AIDS-related deaths (UNAIDS, 2017). While antiretroviral therapy (ART), the standard care for HIV infection, has dramatically reduced the mortality and morbidity of HIV infection, ART fails to provide a cure.

The main obstacle in the development of an HIV cure is the presence of a population of long-lived, latently-infected cells that persists even during ART (Chun et al., 1997c, Finzi et al., 1997, Wong et al., 1997, Siliciano et al., 2003a). This stable viral reservoir presides primarily within resting memory $CD4^+$ T cells and withstands despite decades of therapy (Chun et al., 1997b, Chomont et al., 2009, Chun et al., 1995, Chun et al., 1997a, Finzi et al., 1997, Wong et al., 1997, Finzi et al., 1999, Siliciano et al., 2003a). Recent data suggests that the HIV viral reservoir is established early during infection (Whitney et al., 2014b, Ananworanich et al., 2013) and is responsible for the viral rebound observed after ART interruption (Davey et al., 1999, Chun et al., 1999). Therefore, strategies additional to ART are necessary to cure HIV, and novel therapies targeting the HIV viral reservoir are of utmost importance. Infection of rhesus macaques with simian immunodeficiency virus (SIV) or simian-human chimeric immunodeficiency virus (SHIV) are the most widely used animal models to study the mechanisms by which the viral reservoir is established and maintained under ART, and to test pre-clinical interventions aimed at eliminating, or at least reducing, the viral reservoir *in vivo* (Nixon

et al., 2017).

The “shock and kill” HIV cure strategy endeavors to reverse HIV latency and expose the viral reservoir to elimination via therapeutic approaches. A latency-reversing agent given during a state of viral suppression may be capable of activating CD4+ T cells to “shock” the integrated virus out of latency, leading to HIV RNA synthesis, production of viral protein, and/or release of viral particles, potentially causing direct death of the infected cell and/or indirect killing via recognition by the immune system (Deeks, 2012). An additional “kill” therapeutic aims to amplify the cytolytic response of the immune system to eliminate infected cells (Deeks et al., 2015). Targeted killing of infected cells shocked out of a previously latent state, particularly during ART, may eliminate the viral reservoir preventing the development of an HIV cure.

While multiple latency-reversing agents have been tested in clinical trials in HIV-infected ART-treated patients, none have been shown to elicit the “shock” or “kill” required to eliminate the reservoir, failing to provoke even a minor increase in plasma viremia following latency-reversing agent administration (Archin et al., 2014, Spivak et al., 2014, Rasmussen et al., 2014, Søgaard et al., 2015, Elliott et al., 2014, Elliott et al., 2015). A current clinical trial seeks to disrupt the HIV reservoir using a novel latency-reversing agent, IL-15 superagonist N-803 (ClinicalTrials.gov Identifier: NCT02191098). N-803 is a complex of a mutant IL-15 and a dimeric IL-15 receptor αSu/Fc fusion protein (Xu et al., 2013). The engineered structure is at least 25-times more biologically potent than IL-15 as it mimics trans-presentation and the IgG-Fc component confers improved *in vivo* safety and bioavailability (Han et al., 2011, Rhode et al., 2016). A recent study by our lab has shown that N-803 induces robust and persistent plasma viremia in CD8-

depleted ART-treated SIV-infected rhesus macaques, CD8-depleted ART-treated HIV-infected humanized mice, and CD8-depleted ART-treated SHIV- infected rhesus macaques (McBrien et al.). A caveat of this study was the use of MT807R1, an anti-CD8α antibody, as the mechanism for CD8+ T cell depletion. CD8α is expressed on CD8+ T cells, as well as NK, NKT and γδ T cells, resulting in depletion of multiple cellular subtypes and complicating determination of the specific role of CD8+ T cells alone.

In this proof-of-concept study, we sought to determine whether N-803 is capable of "shocking" the viral reservoir in ART-treated SHIV-infected macaques depleted of CD8+ T cells using an antibody targeting CD8β, which is specifically expressed on CD8+ T cells. In order to directly compare the efficacy of both antibodies, we performed this experiment on a small cohort of rhesus macaques that were previously depleted of CD8+ T cells using the antibody targeting CD8α, the results of which have been recently published (McBrien et al.).

Results

Study design

Animals were infected intrarectally with a high dose of $SHIV_{SF162P3}$ and twelve weeks later initiated on a daily three-drug regimen of ART which was maintained for the remainder of the study. In order to stabilize the latent viral reservoir, animals were treated with ART for six months prior to the first intervention, consisting of administration of 50 mg/kg of the anti-CD8α-depleting antibody, MT807R1, with a cycle of N-803, administered at 100 μg/kg weekly for four weeks. Six months after administration of

MT807R1, following the reconstitution of CD8+ T cells, animals were administered 50 mg/kg of the anti-CD8β-depleting antibody, CD8b255R1, with an additional cycle of N-803 (Figure 1). While sequential interventions allow comparison of the immunological effects of each intervention strategy, longer ART treatment at the time of CD8β depletion with N-803 prevents virological comparisons.

N-803 administration in CD8β-depleted, ART-treated, SHIV-infected macaques results in modest virus reactivation

As previously published, N-803 resulted in robust and persistent virus reactivation in CD8α-depleted, ART-treated, SIV_{mac239}-infected macaques, and these results were recapitulated, albeit to a lesser degree, in a $SHIV_{SF162P3}$-model of infection (McBrien et al.). While $SHIV_{SF162P3}$ is derived from an SIV_{mac239} backbone, replacement of SIV *tat, rev,* and *env* with a corresponding sequence from HIV_{SF162} renders the infection less immunogenic (Luciw et al., 1995). Thus, it was not surprising that the level of virus reactivation following CD8α depletion with N-803 was smaller in the SHIV model as compared to the SIV model. In this study, animals were treated with ART for one year prior to intervention and viral loads were steadily less than 3 copies/mL of plasma. Following CD8β depletion with N-803, viral loads increased above 3 copies/mL in 3/5 animals (Figure 2A). While the observed level of virus reactivation was modest, it was less impressive than levels observed after both the previous CD8α depletion with N-803, and, as expected, CD8α depletion with N-803 in ART-treated, SIV-infected macaques. Additionally, CD8β depletion with N-803 did not result in a decrease in the size of viral reservoir (Figure 2B) or an increase in peripheral CD4+ T cell activation or proliferation (Figure 2C-E).

CD8+ T cell depletion using the anti-CD8β antibody was suboptimal compared to the anti-CD8α antibody

CD8α is expressed on CD8+ T cells at a very high density, either as a homodimer or a heterodimer with CD8β, while CD8β is expressed at a low density, always in a heterodimeric form with CD8α (Baume et al., 1990). Thus, while both MT807R1 and CD8b255R1 antibodies are of the IgG1 isotype, they target epitopes with varying levels of availability which may affect their ability to deplete CD8+ T cells. In fact, previous studies utilizing CD8b255R1 as a CD8+ T cell depletion strategy have observed suboptimal CD8+ T cell depletion in rhesus macaques (Nishimura et al., 2017, Martins et al., 2017). Therefore, we evaluated the ability of CD8b255R1 to deplete CD8+ T cells, in direct comparison with previous depletion with MT807R1. As shown in Figure 3A+B, the percentage and frequency of peripheral CD8+ T cells reached a brief nadir early after CD8β depletion with N-803. In contrast, CD8α depletion with N-803 resulted in a sustained decrease in CD8+ T cells. Additionally, the percentage of CD8+ T cells was not significantly different in the periphery, lymph node, and rectum one week after CD8β depletion with N-803 (Figure 3C-E). While some animals saw an increase in the percentage of CD8+ T cells across tissues following CD8β depletion with N-803, the fold change was not significantly different than following CD8α depletion with N-803 (Figure 3F). We also calculated the frequency of circulating NK cells following both interventions (Figure 3G). While CD8α depletion with N-803 resulted in a transient depletion of NK cells, as predicted, CD8β depletion with N-803 did not deplete the NK cell population.

Level of virus reactivation is correlated with efficacy of CD8+ T cell depletion and post-depletion viral loads are correlated between sequential depletions.

A previous study by our group indicated that the level of virus reactivation following N-803 in CD8-depleted, ART-treated, SIV-infected rhesus macaques is negatively correlated with post-depletion CD8+ T cell frequency (McBrien et al.). Therefore, we hypothesized that the level of virus reaction following N-803 administration would be dependent on the ability of the anti-CD8 antibodies to deplete CD8+ T cells. We found very strong negative correlations between both the percentage of CD8+ T cells and the frequency of CD8+ T cells in the periphery and the plasma viral load (Figure 4A+B), suggesting that the mere modest level of virus reactivation observed following CD8β depletion with N-803 is due in part to suboptimal CD8+ T cell depletion. Additionally, we sought to determine whether there was a correlation between post-depletion viral loads observed following CD8β depletion with N-803 and the previous intervention of CD8α depletion with N-803. We found that the level of virus reactivation was correlated between sequential depletions (Figure 4C).

Discussion

The “shock and kill” HIV cure strategy requires the reactivation of latent virus for eradication of the HIV reservoir. A recent study by our lab was the first to show a robust and persistent induction of plasma viremia during ART via IL-15 superagonist N-803 in CD8+ lymphocyte-depleted macaques and humanized mice (McBrien et al.). These results suggest a substantial role for CD8+ lymphocytes in suppressing the latency-reversal effect of N-803 by promoting the maintenance of viral latency. A limitation of

these models was the use of a depleting antibody targeting CD8α, which is expressed on CD8+ T cells in addition to NK cells, NKT cells, and γδ T cells, complicating the specific role of CD8+ T cells alone. In this proof-of-concept study, we tested whether administration of N-803 to SHIV-infected, ART-treated macaques depleted of CD8+ T cells using a CD8β-targeting antibody would similarly induce virus reactivation. To specifically compare the efficacy of the depletion strategy, we performed the intervention using anti-CD8β antibodies and N-803 in five SHIV-infected, ART-treated macaques that were previously intervened with anti-CD8α antibodies and N-803 (McBrien et al.).

Intervention using CD8β depletion with N-803 resulted in modest virus reactivation in ART-treated, SHIV-infected macaques. As expected, the observed level of virus reactivation was lower than in our previous study using a SIV model of infection (McBrien et al.). While intervention with CD8α and N-803 was effectively able to deplete CD8+ T cells in the periphery and rectal tissue, the depletion observed following intervention with CD8β and N-803 was suboptimal. CD8+ T cells were not significantly depleted across tissues one week after administration of CD8b255R1 with N-803. Thus, multiple administrations of CD8b255R1 may be necessary for effective and sustained CD8+ T cell depletion. Additionally, we found that the level of virus reactivation was highly correlated with the frequency of CD8+ T cells following depletion with either intervention, suggesting that the lower level of virus reactivation following CD8β depletion with N-803 was due to higher residual levels of CD8+ T cells.

In conclusion, the level of virus reactivation following CD8 depletion and N-803 is dependent on the efficacy of the CD8 depletion strategy, where a depletion strategy

specifically targeting the CD8+ T cell population resulted in suboptimal depletion and modest virus reactivation. Future macaque “shock and kill” cure strategies incorporating CD8 depletion should utilize either antibodies targeting CD8α or deliver multiple administrations of CD8b255R1 for persistent and robust virus reactivation.

Materials and Methods

Study approval

All animal experiments were conducted following the guidelines established by the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals (Council, 2011) and performed in accordance with institutional regulations after review and approval by the Institutional Animal Care and Usage Committee (IACUC, 3000065, 2003297, 2003470, and PROTO201700665) at the Yerkes National Primate Research Center (YNPRC; Atlanta, GA). Anesthesia was administered prior to performing any procedure, and the proper steps were taken to minimize any suffering the animals may have experienced.

Animals, SIV-infection, and antiretroviral therapy

This study was conducted using a total of five Indian-origin rhesus macaques housed at Yerkes National Primate Research Center. All RMs were HLA*B07- and HLA*B17-. All procedures are approved by the Emory University Institutional Animal Care and Use Committee (IACUC) and animal care facilities are accredited by the U.S. Department of Agriculture (USDA) and the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International.

Animals were infected intrarectally with high-dose $SHIV_{SF162P3}$, administered as a 1:50 dilution of a 2032 TCID50/mL, 10^9 RNA copies/mL, 182 P27 ng/ml stock. 3/5 animals were administered broadly neutralizing antibody targeting V3-glycan PGT-121 (RPp16 1mg/kg, RRl16 and RYr16 0.2 mg/kg) at the time of infection as part of a previous study. The presence of the antibody did not prevent infection.

All animals were put on a three-drug ART regimen at twelve weeks post-SHIV infection, after the washout period of PGT-121 had passed. Tenofovir (TDF at 5.1mg/kg/day or PMPA at 20mg/kg/day) and Emtricitabine (FTC at 40mg/kg/day) were both kindly provided by Gilead Pharmaceuticals. Dolutegravir (DTG at 2.5mg/kg/day) was kindly provided by ViiV Pharmaceuticals. Drugs were administered daily by subcutaneous injection.

CD8 depletions and N-803 administration

After 6 months of ART, animals were administered one dose of the anti-CD8α-depleting antibody, MT-807R1 at 50 mg/kg subcutaneously. N-803 was administered subcutaneously in a cycle of 100 µg/kg once a week for four consecutive weeks starting at the time of CD8 depletion. Six months after CD8α depletion, animals were administered one dose of the anti-CD8β-depleting antibody, CD8b255R1 at 50 mg/kg subcutaneously. Again, N-803 was administered subcutaneously in a cycle of 100 µg/kg once a week for four consecutive weeks starting at the time of CD8 depletion.

Sample collection and tissue processing

Blood was collected longitudinally throughout the study. Fine needle aspirates of the lymph node (LN) and rectal tissue (rectal biopsy, RB) were collected prior to intervention, and one week after intervention and processed for further analyses as previously described (Cartwright et al., 2016).

Immunophenotype by flow cytometry

Multiparametric flow cytometry was performed according to a standard protocol on PBMC, cells collected from lymph node fine needle aspirates, and rectal cells using fluorescently labeled monoclonal antibodies cross-reactive in RM. In addition to the viability dye LIVE/DEAD Fixable Far Red, the following antibodies were used at room temperature for 30 minutes: CD3 APC-Cy7 (SP34-2), CD4 BV650 (OKT4), CD8α BV711 (RPA-T8), CD8β PE-Cy5 (SIDI8BEE), CD28 ECD (CD28.2), HLA-DR PerCP-Cy5.5 (G46-6), PD-1 BV421 (EH12.2H7), Ki67 AF700 (B56), CD20 PE-Cy5 (2H7), CD14 PE-Cy7 (M5E2), and NKG2A (CD159) PE (Z199). CD8α was used to identify CD8+ T cells following CD8β depletion, and CD8β was used to identify CD8+ T cells following CD8α depletion due to epitope masking by the depleting antibody.

All flow cytometry specimens were acquired on an LSR II (BD Biosciences) equipped with fluorescence-activated cell sorter software (FACS Diva), and analysis of the acquired data was performed using FlowJo software (Tree Star).

Determination of plasma SHIV RNA and cell-associated SHIV *gag* DNA

Plasma SHIV *gag* RNA was performed using quantitative PCR to determine SHIV plasma viral load essentially as described using high sensitivity assay formats (Hansen et al., 2017, Li et al., 2016). CD4+ T cells were sorted from PBMC via CD4 MicroBeads (Miltenyi 130-091-102), purified CD4+ T cells were preserved as a dry pellet, and the quantification of total cell-associated *gag* DNA was performed as previously described (Chahroudi et al., 2014).

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Author contributions

J.B.M., A.C., M.P. and G.S. designed the experiments. J.B.M., E.W., A.W., and D.G.C. performed the experiments. T.H.V. measured cell-associated DNA. J.T.S. and J.H.L. provided technical support. J.B.M. and G.S. wrote the manuscript.

Chapter 5 Figures

Figure 1

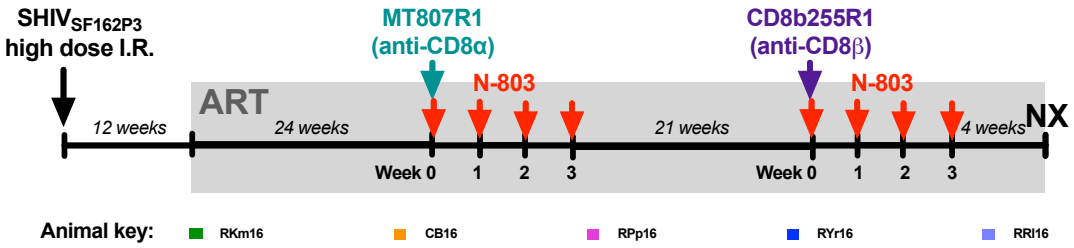

Figure 1: Study Design. Animals were infected intrarectally with high-dose SHIV$_{SF162P3}$, administered as a 1:50 dilution of a 2032 TCID$_{50}$/mL, 109 RNA copies/mL, 182 P27 ng/mL stock. All animals were put on a three drug ART regimen at twelve weeks post-SHIV infection consisting of tenofovir (TDF at 5.1mg/kg/day or PMPA at 20mg/kg/day), emtricitabine (FTC at 40mg/kg/day), and dolutegravir (DTG at 2.5mg/kg/day). ART was administered daily by subcutaneous injection for the remainder of the study. After 24 weeks of ART, animals were administered one dose of MT807R1 (anti-CD8α) at 50 mg/kg subcutaneously. N-803 was administered subcutaneously in a cycle of 100 µg/kg once a week for four consecutive weeks starting at the time of CD8α depletion. 24 weeks after administration of MT807R1 with N-803, animals were administered one dose of CD8b255R1 (anti-CD8β) at 50 mg/kg subcutaneously. N-803 was administered subcutaneously in a cycle of 100 µg/kg once a week for four consecutive weeks starting at the time of CD8β depletion. An elective necropsy was performed seven weeks after administration of CD8b255R1 with N-803. Abbreviations: I.R.: intrarectal, ART: antiretroviral therapy, N-803: IL-15 superagonist complex, NX: necropsy.

Figure 2

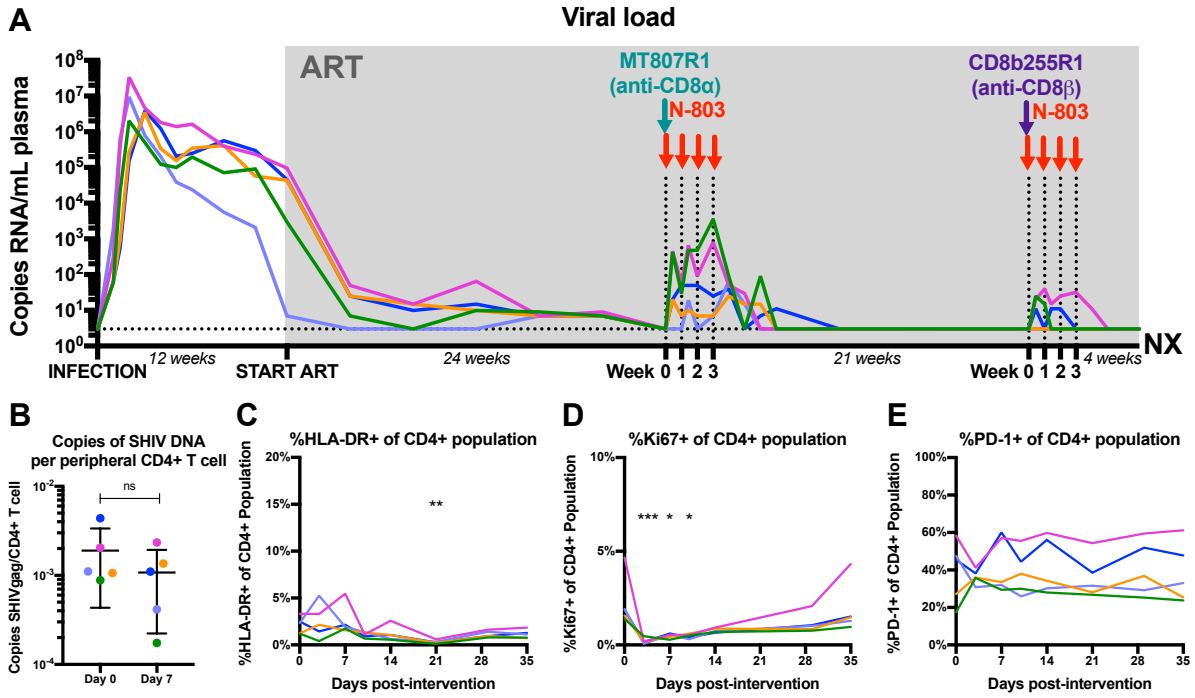

Figure 2: CD8β depletion with N-803 results in a modest virus reactivation correlated with CD8+ T cell frequencies, but does not reduce the size of SHIV viral reservoir. A) Longitudinal plasma viral load starting at the time of infection, through ART initiation, CD8α depletion with N-803, CD8β depletion with N-803, and ending with animal necropsy. The limit of detection of the assay was 3 copies of SHIV RNA/mL of plasma. (B) Change in the level of cell-associated SHIV DNA in sorted, bulk peripheral CD4+ T cells. Statistics were calculated using a Wilcoxin test. Changes in the expression of (C) HLA-DR, (D) Ki67, and (E) PD-1 on peripheral CD4+ T cells following intervention with CD8β depletion and N-803. Statistics were calculated using Friedman tests and the level of significance for each comparison is indicated above the brackets (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, none $p > 0.05$). Each color designates a specific animal. Abbreviations: ART: antiretroviral therapy, NX: necropsy.

Figure 3

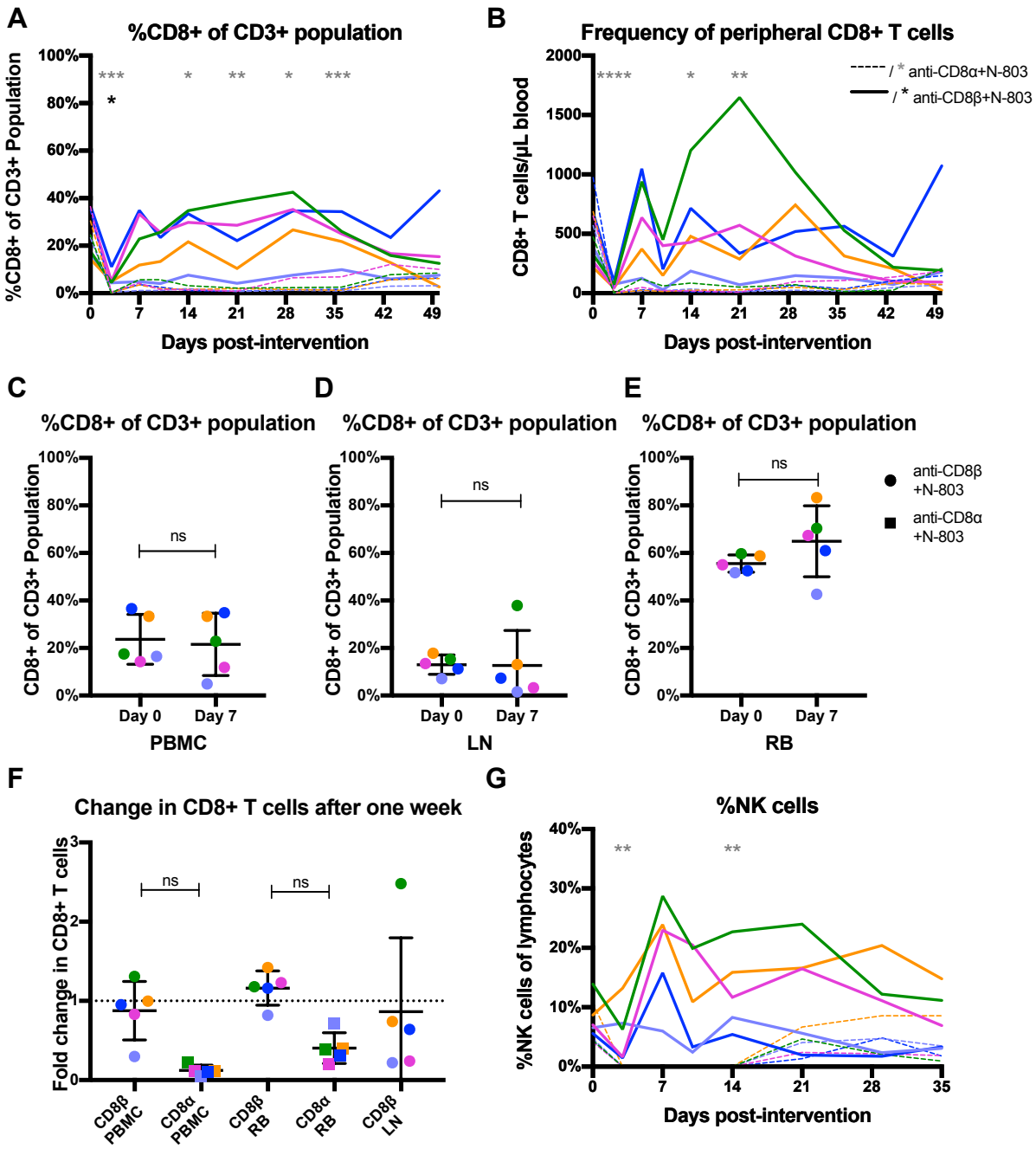

Figure 3: Reduction of CD8+ T cells following anti-CD8α or anti-CD8β. A) Percentage of peripheral CD8+ cells from the CD3+ T cell population at the time of intervention (day 0) through day 49. B) Longitudinal frequency of peripheral CD8+ T cells. Percentage of CD8+ cells from the CD3+ T cell population at the start of intervention and after seven days in the (C) peripheral blood, (D) lymph node (via a fine needle aspirate), and (E) the rectum (via a rectal biopsy). (F) Fold change of CD8+ T cells seven days after the start of intervention in the peripheral blood and rectum with CD8β depletion and CD8α depletion. This was calculated by dividing the percentage of CD8+ T cells at day 0 from day 7. Of note, fine needle aspirates of the lymph node were not collected following CD8α depletion. (G) The percentage of NK cells (CD3-CD20-CD14-HLA-DR-NKG2A+) as a proportion of lymphocytes longitudinally following the start of interventions. Intervention with anti-CD8β with N-803 is shown with solid lines or circles, and astericks denoting statistical significance are black. Intervention with anti-CD8α is shown with dashed lines or squares, and asterics denoting statistical significance are grey. Statistics for A, B, and G were calculated using Friedman tests and C-F were calculated using two-sided Wilcoxon tests. The level of significance for each comparison is indicated above the brackets (**** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, none $p > 0.05$). Each color represents a specific animal. Abbreviations: PBMC: peripheral blood mononuclear cells, LN: lymph node, RB: rectal biopsy, ns: not significant.

Figure 4

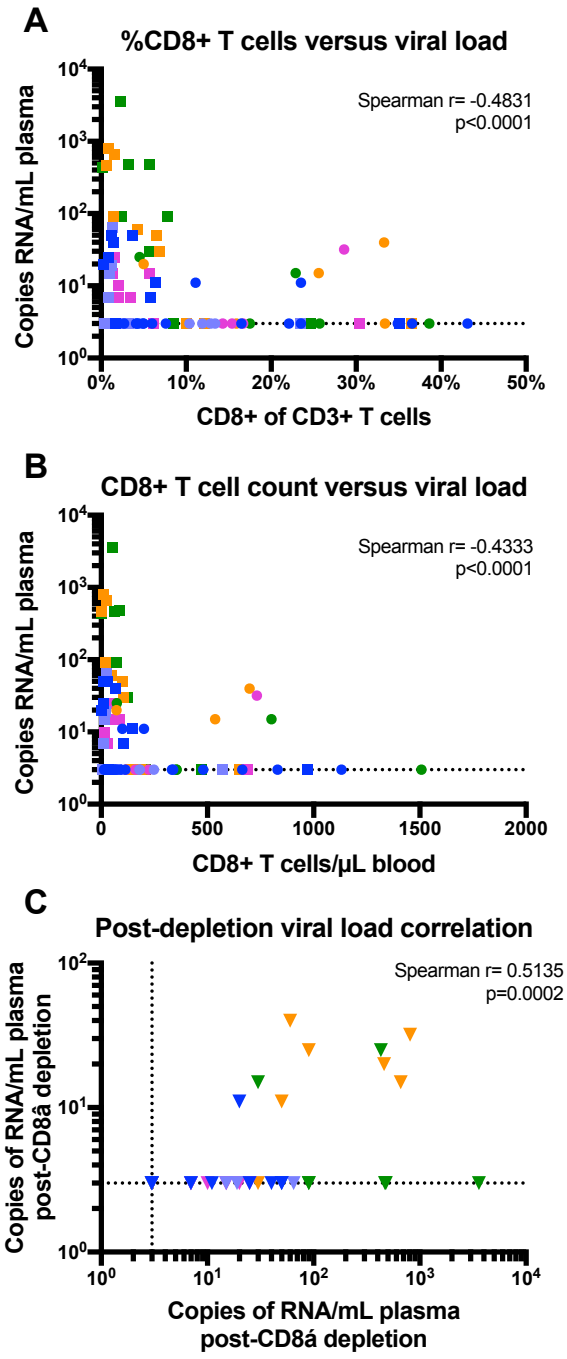

Figure 4: Level of virus reactivation is correlated with the efficacy of CD8+ T cell depletion and previous depletions. Correlation between (A) the percentage of peripheral CD8+ T cells and plasma viral load, and (B) peripheral CD8+ T cell count and viral load. Counts and viral loads from both depletion strategy were combined for the correlations. (C) Correlation between plasma viral loads following CD8α depletion and CD8β depletion. Correlations were calculated via Spearman r. Each color designates a specific animal. Circles represent intervention using CD8β depletion with N-803, squares represent intervention using CD8α depletion with N-803.

Chapter 6: Discussion

The HIV epidemic today

When Health and Human Services Secretary Margaret Heckler announced in 1984 that Dr. Robert Gallo of the National Cancer Institute had found the cause of AIDS, she optimistically expressed their hope that a vaccine against the virus could be produced within two years (Heckler, 1984). Thirty-five years later we have still not seen a vaccine or an end to the epidemic. Fortunately, the battle against HIV/AIDS has come a long way since the era before the causal agent, method of spread, and treatment were known. Since 1996, combination antiretroviral therapy (ART) has been the standard care for HIV-infected individuals, contributing to a decrease in HIV mortality and transmission (Collier et al., 1996, D'Aquila et al., 1996, Staszewski et al., 1996, CDC, 2006). Other victories include heightened awareness and reduced stigma, improved diagnostics of infected individuals, and increased access to antiretrovirals and pre-exposure anaphylaxis (PrEP). Yet, undiagnosed cases, incomplete access to care and ART, and drug adherence concerns contribute to the roughly 2 million new HIV infections and 1.2 million AIDS-related deaths that occur each year worldwide (UNAIDS, 2015). Since 1981, 77.3 million people worldwide have been infected with HIV. It is evident that the global handle on the HIV/AIDS epidemic is not sufficient and a cure for the infection remains of utmost importance.

Earlier this year, United States President Donald Trump proposed a plan to eradicate the spread of HIV in the US by 2030*, Ending the HIV Epidemic: A Plan for America*. The multi-agency initiative will focus on states and counties with the highest burden of disease to reduce new infections nationally by 90 percent in the next ten

years. Their four strategies include **diagnosing** all people with HIV as early as possible after infection, **treating** the infection rapidly to achieve sustained viral suppression, **protecting** people at risk for HIV using potent and proven prevention interventions, and **responding** rapidly to detect and respond to growing HIV clusters and prevent new HIV infections (HIV.gov, 2019). As broad public health initiatives have successfully reduced the number of new transmissions (Frieden et al., 2005), more resources being allocated into epidemic control alongside foci-driven efforts have the potential to improve the lives of HIV-infected Americans and prevent tens of thousands of new infections in the next decade.

Nonetheless, as long as HIV remains endemic within populations, we will never be capable of truly eradicating the virus. Tackling the HIV epidemic in the United States will be a constant battle until the spread of new infections is eliminated globally. Unfortunately, more than 95% of HIV infections occur in developing countries (UNAIDS, 2015), where high-budget HIV eradication campaigns are unrealistic. The development of a preventative vaccine could vastly contribute to a decrease in new infections, but unless the vaccine is administered universally, there will still be spread through the presence of previously-infected individuals. The only hope for a complete eradication of HIV/AIDS is through a therapeutic cure of the infection, where infection can be eliminated at the source.

Hope for a cure

As described in Chapter 1, HIV is an incredibly smart pathogen. The virus attacks the body's very defenses against it, the immune system, leaving it weakened and inept against other infections. Additionally, the virus mutates quickly, inducing changes that

allow variants to escape against immune recognition or therapy. To further increase the virus' ability to pass on new infections, HIV integrates into the host cell where it can remain in a state of latency. Viral latency allows for the prolonged lifespan of the host (and prolonged spread during untreated infection), as well as the persistence of a population of cells that are not affected by antiretroviral therapy. For these reasons, a cure against HIV remains elusive.

Yet, a cure is possible. Timothy Ray Brown, or "the Berlin patient" was cured of HIV in 2009 after receiving total body irradiation and a hematopoietic stem cell transplant from a donor homozygous for CCR5Δ32 to treat a concomitant diagnosis of acute myeloid leukemia (Hütter et al., 2009). The CCR5 mutation found in some Europeans yields cells resistant to infection with HIV variants that interact with the CCR5 coreceptor, thus these individuals are effectively immune to HIV infection (Liu et al., 1996). It is unknown whether the stem cell transplant or irradiation was responsible for the elimination of infected cells in Timothy Ray Brown's body, and some hypothesize that graft-versus-host disease cleared the infection (Pitman et al., 2018). Additionally, just this year another patient, "the London patient," was cured of HIV using a similar strategy for treatment of concomitant Hodgkin's lymphoma (Gupta et al., 2019). While these two cases represent success stories, the approach has been repeated and failed in others (Hutter, 2014). Additionally, because of the high mortality rate associated with hematopoietic stem cell transplant, this HIV cure strategy is unrealistic for HIV-infected individuals without a separate diagnosis requiring the transplantation.

Another HIV cure strategy involves the use of gene therapy. In these strategies, gene editing systems are used to eliminate or disrupt the HIV-coreceptor gene (inducing

HIV resistance similar to those with the CCR5Δ32 mutation) or the HIV provirus. Gene therapy editing via CRISPR/Cas-9 has been tested in animal models and *in vitro* (Liu et al., 2017, Xu et al., 2019, Dash et al., 2019), in addition to phase 1 and 2 clinical trials (NCT03020524, NCT02500849, NCT00295477, NCT01734850, NCT02225665, and NCT01013415). Gene editing of the HIV genome will likely require multiple HIV targets to avoid resistance and enhanced delivery strategies to access the low frequency of infected cells in the blood and tissue (Pitman et al., 2018).

Others have attempted to cure HIV using broadly neutralizing antibodies targeting the HIV envelope protein, which neutralize a broad range of free virus and clear cells expressing viral antigen on their surfaces (Lu et al., 2016, Schoofs et al., 2016). When the broadly neutralizing antibodies 3BNC117 and VRC01 were administered to ART-treated HIV-infected patients, a delay in viral rebound was observed after interruption of ART (Bar et al., 2016, Scheid et al., 2016). While a powerful idea, development of an effective broadly neutralizing antibody is difficult due to the heavy glycosylation of HIV envelope and quick generation of viral resistance. Additionally, antibody-based molecules that incorporate the antigen specificity of multiple broadly neutralizing antibodies to multiple binding sites on the virus hold promise. These bi- and tri-specific antibodies neutralize HIV with greater breadth and potency than their parent broadly neutralizing antibodies (Huang et al., 2016, Xu et al., 2017). Furthermore, dual-affinity retargeting molecules (DARTs) are engineered heterodimers comprising the variable domains of two different monoclonal antibodies specific for the HIV envelope and CD3 (Sloan et al., 2015). Neither bi-specific and tri-specific antibodies, nor DARTs have entered clinical trials.

Chimeric antigen receptors (CARs) are engineered receptors comprised of an extracellular domain (usually a single variable chain from a monoclonal antibody), a transmembrane domain, and an intracellular domain (comprised of the ζ signaling chain of CD3 with or without other intracellular domains of costimulatory receptors). Another HIV cure strategy involves the engineering of T cells with a CAR consisting of an anti-HIV envelope broadly neutralizing antibody variable chain in the extracellular domain, targeting HIV-infected cells for death (Liu et al., 2016, Hale et al., 2017). This strategy is currently being tested in a clinical trial (NCT03240328).

Other HIV cure strategies include immune system modulation. In the case of therapeutic HIV T cell vaccines, boosting human responses against HIV may eliminate virus infected cells. Several studies have assessed the combination of a latency reversing agent with a T cell vaccine (Leth et al., 2016, Hansen et al., 2011, Sneller et al., 2017, Borducchi et al., 2016). Additionally, immune checkpoint blockade may enhance the function of HIV-specific T cells by reversing T cell exhaustion and additionally activate latently-infected cells to induce latency reversal (Wykes and Lewin, 2018, Chew et al., 2016), Harper et al., unpublished). Clinical trials are already underway testing immune checkpoint blockers in HIV-infected individuals with a concomitant malignant disease (NCT02408861, NCT02595866, NCT03304093, NCT03354936, NCT03367754, and NCT03426189). Reprograming the immune system to target the infection remains one of the biggest challenges in the development of a cure.

“Shock and Kill” approach

The HIV cure strategy explored in this thesis attempts to expose virus-infected cells for clearance by the immune system. In this strategy latency reversing agents aim to “shock” the HIV provirus out of latency and “kill” agents target infected cells for death. LRAs and kill components include many of the strategies already discussed, such as T cell vaccines, broadly neutralizing antibodies, immune checkpoint blockers, etc. A successful HIV cure using this strategy assumes that complete “shock” is feasible, and that these “shocked” cells are capable of immune recognition and death. What further complicates this strategy is that no gold standard for testing latency reversing agents or clearance components exists.

Unfortunately, no LRA has proven successful in clinical trials. In particular, LRAs belonging to the class of Histone De-Acetylase (HDAC) inhibitors such as vorinostat, panabinostat, and romidepsin failed to induce either robust virus reactivation or reduction of viral reservoir in ART-treated HIV-infected individuals (Archin et al., 2014, Spivak et al., 2014, Rasmussen et al., 2014, Søgaard et al., 2015, Elliott et al., 2014, Elliott et al., 2015). More recently, the Toll-like receptor 7 (TLR-7) agonists GS-9620 and GS-986 showed some virus reactivation in SIV-infected ART-treated rhesus macaques up to 1,000 copies/mL of plasma (Lim et al., 2018). However, the LRA effect of TLR7 agonists was short-lived, as plasma viremia peaked 24 to 48 hours after treatment and then invariably returned to baseline by day 4 or 7 after administration of each drug. More recently, persistent remission was observed in a subset of simian/human chimeric immunodeficiency virus (SHIV) infected macaques that were

initiated on ART early after infection before receiving GS-9620 in combination with the broadly neutralizing PGT121 antibody (Borducchi et al., 2018).

Multiple *in vitro* models using cell lines or primary cells have been developed to test “shock and kill” cure therapeutics, but the results differ across models and especially when applied *in vivo*, as occurred with the HDAC inhibitors brought to clinical trials. Often times, primary cell *in vitro* models test compounds against latently-infected CD4 T cell monocultures – a hyper-simplified facsimile of the immune system. Relevant to this thesis, these models intentionally do not contain CD8+ T cells.

CD8+ T cells thwart an HIV cure

Decades ago, Dr. Jay Levy showed that CD8+ T cells suppress HIV replication *in vitro* (Walker et al., 1986). Using sorted peripheral CD4+ T cells and CD8+ T cells from seropositive HIV-infected donors, he observed that CD8+ T cells suppress HIV replication greater than ninety percent via an undefined, diffusible molecule (Walker and Levy, 1989) and additionally, that the mechanism was of a non-cytolytic nature (Wiviott et al., 1990, Walker et al., 1991a, Mackewicz and Levy, 1992). This activity of CD8+ T cells contrasts the conventional CD8+ T cell antiviral activity involving cytotoxic T cells (CTLs) that kill virus-infected cells. It is no wonder that LRAs, while functioning *in vitro*, are unable to “shock” the latent *infection in vivo* where infection dynamics are complicated by other immune cells, notably CD8+ T cells.

Recent *in vitro* studies by our own group support the hypothesis that CD8+ T cells inhibit transcription of HIV, thus establishing and maintaining HIV latency. In the first of these models (Zanoni et al., manuscript in preparation), CD4+ T cells are cultured alone or with CD8+ T cells just hours after infection. Three days later HIV

infection (via an EGPF reporter protein) and HIV transcription are measured. While the number of HIV-infected cells is consistent across monocultures and cocultured, there is a decrease in HIV transcription when CD8+ T cells are included in the culture. An additional model cultured CD4+ T cells alone or with CD8+ T cells during the establishment and reversal of latency, and found that when CD8+ T cells were included in the culture there was an increase in latent infection and a decrease in latency reversal (Kulpa et al., manuscript in preparation). These observations support the notion that CD8+ T cells induce HIV latency through a mechanism of transcriptional suppression. This hypothesis is further supported by the *in vivo* studies detailed in this thesis, where the latency reversing agent, IL-15 superagonist N-803, is only able to shock latent SIV, SHIV, and HIV infection in the absence of CD8+ T cells. We hypothesize that CD8+ T cell depletion released the HIV transcriptional block induced in their presence. In fact, we observed the largest "shock" in nonhuman primate and human studies when N-803 was administered to CD8-depleted macaques, even during antiretroviral therapy. This suggests that CD8+ T cells prose as a barrier to an HIV cure by promoting the maintenance of HIV latency, which contradicts the traditional assumption that CD8+ T cells kill HIV-infected cells.

Unlock, Shock, and Kill

If the hypothesis that CD8+ T cells inhibit virus transcription and prevent the reversal of latency during "shock and kill" HIV cure strategies is true, targeting this activity of CD8+ T cells is necessary. In effect, we need to "unlock" access to the HIV reservoir in order to facilitate a "shock." While CD8 depletion was well tolerated in our cohort of macaques, it is unlikely that CD8 depletion would ever be approved for human

use. Therefore, it remains vital that the mechanism of CD8+ T cell-induced HIV suppression is deciphered. Once the mechanism is established, we can develop compounds targeting this activity in order to “unlock” access to the reservoir safely in humans. Further combination with a latency-reversing agent and clearance agent hold promise towards an HIV cure. A limitation of this strategy is that the mechanism of CD8+ T cell suppression may not be HIV-specific, in which case inhibiting the activity may “shock” a gamut of infections out of latency. A current study by our group seeks to sequence the virome of CD8+ T cell-depleted animals from our SIV+ ART-treated cohort (McBrien et al., unpublished). As an HIV cure is an alternative to antiretroviral therapy, the strategy needs to be just as safe, if not safer, than ART.

Additionally, our model of SIV/SHIV-infected, CD8-depleted macaques can be used as a platform to test superior “shock” and “kill” therapeutics. In this thesis, we only discussed the use of CD8 depletion with one latency reversing agent, N-803, and without a clearance component. Latency reversing agents have never had a positive control *in vivo* until now, and clearance components have never before had a testing platform as virus reactivation *in vivo* had yet to be successful. Once latency reversal is improved and a method of clearance of virus-infected cells is established, these reagents could be combined with a compound targeting the HIV suppressive effect of CD8+ T cells.

A caveat of our “unlock, shock, and kill” strategy is that by inhibiting the activity of CD8+ T cells, we may inadvertently be decreasing our ability to kill virally-infected cells. Most therapeutic vaccines and immune checkpoint blockade strategies target the activity of CD8+ T cells for viral clearance. Thus, clearance agents that are mediated by

NK cells, B cells, complement, or other immune components may be key. This may include broadly neutralizing antibodies, CD4 mimetics, or immunotoxins. Another recent hypothesis is to combine CD8 depletion with an LRA and a BCL-2 inhibitor to produce an “unlock, shock, and suicide,” as recent studies show that inhibition of BCL-2 using Venetoclax selectively targets HIV-infected cells for apoptosis *in vitro* and reduces the size of the HIV reservoir (Cummins et al., 2017, Cummins et al., 2016). In these studies, HIV killing of CD4+ T cells that replicate HIV was shown to involve HIV protease-mediated cleavage of procaspase 8 to generate a fragment Casp8p41. Casp8p41 directly bound and activated the proapoptotic protein BAK, resulting in apoptosis of the infected cell. Additionally, the fragment also bound to the antiapoptotic protein BCL-2, which prevented apoptosis. Furthermore, they found that central memory CD4+ T cells from HIV+ individuals had a heightened expression of BCL-2 relative to procaspase 8, which could be a possible explanation for the persistence of HIV in central memory CD4+ T cells despite generation of the Casp8p41 fragment. Treatment of HIV-infected cells *in vitro* with the BCL-2 inhibitor Venetoclax prevented binding of Casp8p41 to BCL-2, allowing the fragment to find to BAK, inducing apoptosis of virus-infected cells and a decrease in the size of the reservoir (Cummins et al., 2017, Cummins et al., 2016). As Venetoclax is FDA-approved for patients with chronic lymphocytic leukemia, the drug has already met safety guidelines.

Another consideration is that CD8+ T cell suppression of HIV transcription may be one of many mechanisms maintaining the HIV viral reservoir. It remains unknown the contribution of CD8+ T cell non-cytolytic activity to preservation of this population, and targeting an activity of CD8+ T cells alone may only release a piece of the block.

Conclusion

To close, an HIV cure is essential to the worldwide elimination of HIV and should be prioritized alongside preventative measures. While preventative measures already have a profound effect on the spread of HIV, efforts towards an HIV cure have been slow. We hypothesize that CD8+ T cells pose a major block to “shock and kill” HIV cure strategies, as their activity includes the suppression of HIV transcription, preventing the reversal of latency. In this thesis, I detail the results of multiple studies that support this hypothesis. In chapter 3 we most notably use the model of ART-treated SIV infection of rhesus macaques to show that IL-15 superagonist N-803 functions as an LRA only in the absence of CD8+ T cells (the transcriptomic and phenotypic changes induced by N-803 are discussed in chapter 2). The results of this study showed the most robust and persistent reactivation of plasma viremia under ART observed to date. We further supported these results by repeating the experiment in an ART-treated HIV infection of humanized mice model and an *in vitro* model of HIV latency. In chapter 4 we show these results can be repeated using an ART-treated SHIV infection model in rhesus macaques, a prerequisite for future studies incorporating an HIV-targeting “kill” therapeutic into the strategy. Last, in chapter 5 we perform a sequential depletion in the small cohort of ART-treated SHIV-infected macaques, this time utilizing the CD8β-targeting CD8 depletion antibody – selectively depleting CD8+ T cells. In all of these studies and *in vivo* models, plasma viremia increased following exposure to the LRA N-803 in the absence of CD8+ T cells. Studies determining this specific mechanism of CD8+ T cell transcriptional control of HIV/SIV latency and development of a drug capable of inhibiting this activity is of utmost importance. Combining drugs targeting the

suppressive activity of CD8+ T cells with the most promising latency reversing agents and clearance components may one day be the key towards an HIV cure.

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