

Anti-CCR5 Antibody for HIV Prevention, Treatment, and Cure

By

Xiao Lan Chang

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CERTIFICATE OF APPROVAL

This is to certify that the PhD dissertation of

Xiao Lan Chang

has been approved

Jonah Sacha, Ph.D., Mentor/Advisor

Nancy Haigwood, Ph.D., Committee Chair

Ashlee Moses, Ph.D., Member

Tim Nice, Ph.D., Member

Fikadu Tafesse, Ph.D., Member

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Abstract

In the absence of a prophylactic vaccine and curative strategies, the daily use of antiretroviral therapy (ART) is essential for preventing and maintaining HIV infection. Although ART plays a vital role in decreasing the numbers of new infections and AIDS-related deaths, transforming this once deadly disease into a manageable condition, we are also observing an increase in global antiretroviral (ARV) drug resistance. Because ART requires daily oral use for a lifetime, suboptimal treatment adherence will accelerate the development of drug resistance. To combat this problem, multiple classes of ARV drugs with different mechanisms of action are being incorporated into first-line drug regimens; however, drug resistance against newer forms of ARV drugs will ultimately rise with increasing usage. To this end, investigating different drug targets with higher genetic barriers to resistance and drug formulations to allow for less frequent dosing will aid in the slowing of the HIV epidemic. This dissertation hypothesizes that an anti-CCR5 antibody Leronlimab (PRO 140; PA14) can phenocopy the natural resistance observed in homozygous CCR5 Δ 32 individuals to prevent viral acquisition and suppress viremia. In Chapter 2, we evaluate the use of Leronlimab in nonhuman primate macaques and human models to find that Leronlimab did not deplete CCR5⁺ target cells, likely due to the lack of immune engagement in the IgG4 subclass. Moreover, we report on a novel method for measuring CCR5 receptor occupancy (RO) by a drug and find that the antiviral potency of Leronlimab correlated with the percentage of CCR5 RO rather than the plasma concentration. In Chapter 3, Leronlimab can suppress rectal challenges of a CCR5-tropic SHIV in a dose-dependent manner, with complete protection observed in the high dose treated group. The protected macaques lack plasma viremia months after complete Leronlimab washout, viral reservoirs in tissues, and CD8⁺ T cell responses against viral peptides. *In vitro* studies show that Leronlimab can protect human

CCR5 wild-type CD4⁺ T cells against viral infections by ten different CCR5-tropic real-world primary HIV isolates similar to the natural protection observed in a homozygous CCR5 Δ 32 individual. Collectively, this chapter demonstrates the effectiveness of targeting a host receptor critical for HIV replication and a treatment method that allows for weekly or biweekly rather than daily dosing. Chapter 4 shows that weekly self-administration of the single-agent Leronlimab effectively maintains virologic suppression of five chronic HIV⁺ individuals for over seven years. As well, weekly Leronlimab dosing potently suppresses high viremia in SHIV-infected rhesus macaques, where two of four macaques reached undetectable levels (< 50 copies/mL) at the end of the study. Viral DNA copies in tissues of Leronlimab-treated macaques are significantly lower than in tissues of untreated macaques, demonstrating a potential effect for Leronlimab to reduce the size of viral reservoirs. Finally, in Chapter 5, we discuss the collective implications of these findings and explore future experimental directions by Leronlimab for HIV prevention, treatment, and curative strategies.

Chapter 1: Introduction

1.1 Human Immunodeficiency virus (HIV)

1.1.1 History

The HIV/AIDS pandemic has been ongoing for the last four decades and remains within the top 10 cause of death in low-income countries (WHO 2020). On June 5th 1981, the CDC published a case report of five healthy homosexual men who presented with unusual pneumocystis pneumonia, Kaposi's sarcoma, and other opportunistic infections that should not be life-threatening in healthy adults, suggesting that these individuals were immune deficient. Since this reporting, HIV has infected approximately 79.3 million people and claimed the lives of 36.3 million people globally (UNAIDS 2020). There are currently around 37.7 million people living with HIV globally. While new infections have dropped by 52% since peak infection in 1997, approximately 1.5 million people were newly infected in 2020, making it a continued global public health problem despite advancements in research, drug development, and outreach programs.

Over 1,200 plasma samples collected from 1959 to 1982 from different regions of Africa showed that approximately 1.7% of the samples were seropositive for HIV. All of the positive samples were from central Africa¹. In particular, one sample (ZR59) collected in 1959 from Kinshasa in the Democratic Republic of Congo showed positive HIV antigens by western blotting and immunoprecipitation assays. Phylogenetic analyses of ZR95 and a second HIV positive lymph node biopsy sample (DRC60) from Kinshasa in 1960 purported that the pandemic started in the first half of the 1920s from a common ancestor infected by an HIV-1 group M strain^{2,3}. The spread of the virus was due to human-to-human transmission²⁻⁵. With rapid

urbanization, over a million people crossed Kinshasa by rail or river in 1940s³, spreading the virus beyond central Africa. The virus eventually spread to North America through Haiti in the early 1970s⁶⁻⁸, where it circulated for over a decade before it was finally reported in 1981.

HIV-related stigma was targeted to the gay community due to first reports of the infection of homosexual men⁹⁻¹¹, resulting in the term Gay-Related Immune Deficiency (GRID) for the syndrome. However, newer cases occurred in heterosexual people¹², intravenous drug users¹¹, blood transfusion recipient¹³, Haitian immigrants¹², infants¹⁴, children¹⁵. In late 1982, the CDC officially named the disease acquired immunodeficiency syndrome (AIDS).

The virus that caused AIDS was first described in 1983 by Luc Montagnier's team at the Pasteur Institute of Paris. The group named the retrovirus was lymphadenopathy-associated retrovirus (LAV) in the family of human T-cell leukemia virus (HTLV). At the time, it was understood that LAV was an RNA virus that formed virions by budding from the host's cell membrane to infect only T cells, not other cell types, including B cells and fibroblasts¹⁶. Luc Montagnier and Francoise Barre-Sinoussi, the corresponding and first authors of that paper, were awarded the Noble Prize in Physiology or Medicine in 2008 for the discovery of HIV. In 1984, Robert Gallo's team at the National Cancer Institute referred to the virus as HTLV-III. They isolated HTLV-III from pre-AIDS or AIDS individuals while detecting no virus in 115 healthy heterosexual individuals, demonstrating that it was the primary cause of AIDS¹⁷. This link was strengthened when Jay Levy's group from the University of California at San Francisco found antibodies against another virus isolate, SF2, which they called AIDS-associated retroviruses (ARV), in all 86 AIDS patients. Sequence comparisons showed that the French and NIH viruses were identical, while SF2 was distinct¹⁸. In 1986, the common name human immunodeficiency virus (HIV) was adopted officially to describe the retrovirus.

The first success in combating the HIV-1 pandemic was in 1987 when the FDA approved the first antiretroviral (ARV) drug, zidovudine (AZT). The drug blocks the viral reverse transcriptase protein used in viral replication. The treatment of HIV+ individuals with AZT led to undetectable viral levels in the plasma, increased T cells, gained weight, and improved fungal infections^{19,20}. In the early 1990s, a combination or a cocktail of ARV drugs with different mechanisms of action was the preferred treatment due to reduced viral replication, morbidity, mortality, and AIDS development, while also increased CD4+ T cell counts^{21,22}. Finally, the first pre-exposure prophylaxis (PrEP) called Truvada received FDA approval in 2012 for the use in high-risk uninfected individuals (for a detailed discussion on ARV drugs, see **Section 1.2 on Antiretroviral Drugs**). The use of ARV drugs, paired with knowledge in managing HIV-related opportunistic infections, drastically decreased the number of deaths and new cases. Now HIV+ individuals on suppressive ART have a comparable life expectancy to HIV- individuals²³.

While ARV drugs successfully control HIV replication, they require daily oral intake for life. In contrast, the development of protective vaccines continues to be a global health priority. The first-generation HIV vaccine candidates aimed to elicit neutralizing antibody production against the HIV Envelope proteins. In contrast, the second generations candidates aimed to induce cell-mediated immunity and more recent vaccine designs use a combination of methods to enhance anti-HIV immune responses. Due to a combination of immense strain variation worldwide and the technical hurdles in developing vaccines that can elicit potent, durable, and broad cellular immunity, there is no vaccine currently to prevent HIV.

1.1.2 Classification

Two species of HIV (HIV-1 and HIV-2) are within the Lentivirus genus and Retroviridae Family. Lentiviruses can infect a range of hosts (human, bird, sheep, horse, reptile, monkeys, and more) to cause chronic diseases in the lungs, joints, nerves, and immune cells. As a single-stranded, positive-sense, enveloped RNA virus, HIV replicates by integrating its genome into the host genome to hijack the host's replication machinery. Because of this mechanism, lentiviruses can persist throughout the cell while maintaining latency to evade the host immune system. Infection can spread within the body to reach many different organ systems and transfer to uninfected individuals through the transfer of virus-containing cells or fluids. In addition to CD4⁺ T cells depletion, an HIV infection can also cause or be associated with cancer (Kaposi's sarcoma), Pneumocytis pneumonia, meningoencephalitis, arthritis, anaemia, encephalopathy, and myelopathy²⁴.

HIV-1 and HIV-2 resulted from the zoonotic transmission of the simian immunodeficiency virus (SIV) in African primates to humans^{25,26}. Transmission likely occurred when a human became infected with animal blood by biting from a primate, during hunting, butchering, or consuming uncooked primate meat. Dozens of different SIV strains occur naturally in many African primates. Genome comparison between SIV and HIV sequences provided strong evidence that SIVcpz in chimpanzees gave rise to HIV-1 while SIVsmm in sooty mangabeys gave rise to HIV-2. While the genomic structures of the two viruses are similar, HIV-1 is much more infectious, transmissible, and virulent than HIV-2²⁷⁻²⁹. Due to lower viral loads in HIV-2 infected people, the sexual and vertical transmission rates are 5-9 fold and 10-20 fold, respectively, lower than rates seen in HIV-1 infected people²⁹. In a large cohort of infected women (n=19,701) from 1988 to 1992, infections caused by HIV-1 increased from 5.0% to 9.2% while they decreased from 2.6% to 1.5% in HIV-2²⁷. Moreover, in over 1.4 million new HIV

infections that occurred in the United States from 1987 to 2009, only 0.01% (n=166) were caused by HIV-2. Of these HIV-2 infected individuals, 81% were from West Africa while 8% were from other parts of Africa³⁰. Collectively, this explains why HIV-1 infection is pandemic, resulting in millions of new infections worldwide every year, while HIV-2 infection is endemic and stable in West African countries.

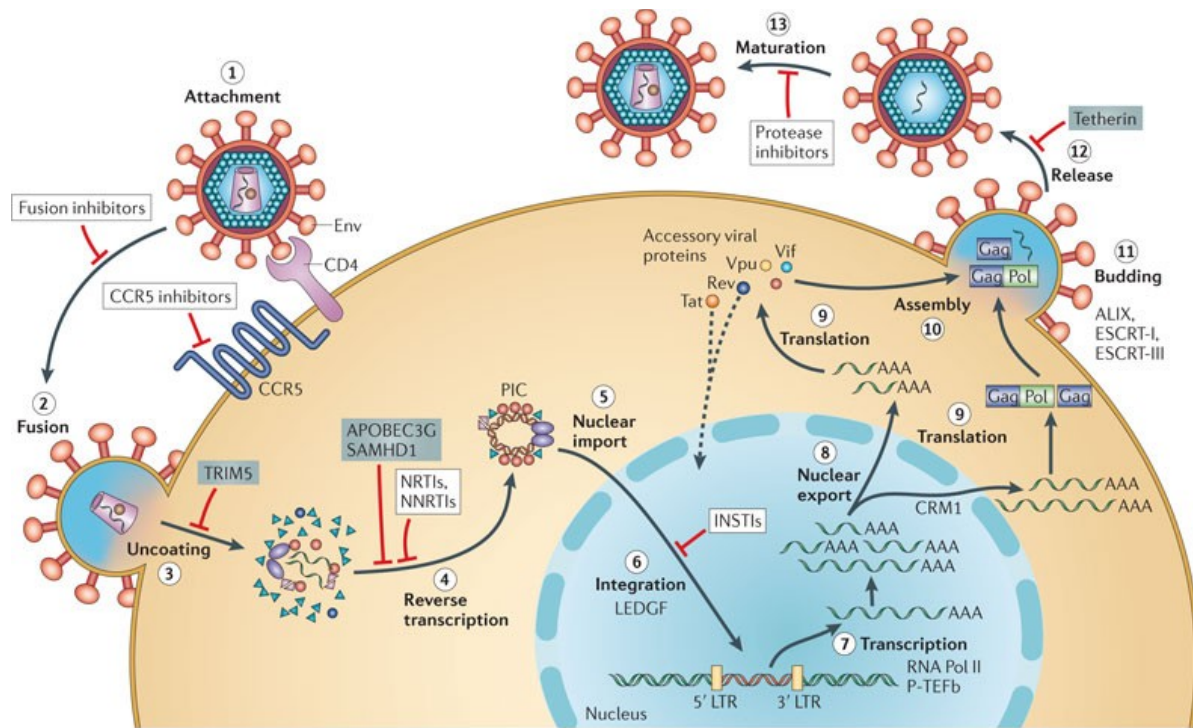
Differences in the envelope sequence classify four groups of HIV-1: M, N, O, and P. HIV-1 group M strains are responsible for the HIV pandemic. In contrast, non-pandemic transmissions of HIV-1 groups O, N, and P localize around the west and central Africa³¹⁻³⁵. While the predicted infection rates for group M and O were similar before 1960³, one reason why group O remained non-pandemic status was due to the poor Tetherin (CD317/BST-2) antagonist ability by Vpu, which allowed host Tetherin to block viral replication. In contrast, the functions of Vpu and Nef in group M evolved to suppress host restriction factor Tetherin, which likely contributed to the rapid and pandemic transmission of group M after the 1960s. Group M is subdivided into different subtypes based on genome sequence and is geographically distinct. The emergence of different subtypes in group M was likely driven by ecological factors, including establishing dominance in a new host population⁸. Circulating recombinant forms (CRFs) can arise from coinfections of different subtypes³⁶. Subtype A is most prevalent in east Africa and Russia; subtype B in North and South America, Europe, and Australia; subtype C in south Africa and India; CRF01_AE in Asia; and CRF02-AG in west Africa^{37,38}. Subtype C is the most prevalent and accounts for approximately 50% of infections worldwide, while subtypes A and B account for 12% and 10%, respectively. It is worth noting that the prevalence of all forms of CRF account for 18% of total global infection³⁷, which is concerning as HIV-1 recombination can increase genetic diversity to exacerbate problems of drug resistance³⁹ and immune escape⁴⁰.

1.1.3 Pathogenesis of HIV

1.1.3.1 Replication cycle

An HIV-1 virion is approximately 145 nm^{41,42} in diameter and consists of two copies of unspliced, positive-sense, single-stranded RNA (ssRNA) that are noncovalently linked together. Identical 5' and 3' long terminal repeats (LTRs) flank the ~10,000 nucleotides genome⁴³. The binding of viral proteins to different regions of the 5' LTR can up- or down-regulate viral genes⁴⁴. For example, certain single nucleotide polymorphisms (SNPs) in the 5' LTR can lowered viral binding affinity to the 5' LTR, resulting in reduced transcription^{44,45}. Three central genes produce viral proteins: 1) gag produces the structural proteins matrix (MA), capsid (CA), nucleocapsid (NC), and p6; 2) pol produces protease (PR), reverse transcriptase (RT), and integrase (IN); and 3) env produces glycoprotein (gp) gp120 and gp41. Additionally, regulatory proteins (Tat and Rev) and accessory proteins (Vif, Vpu, Vpx, Vpr, and Nef) are produced for varying roles.

The HIV-1 life cycle involves six main steps: viral entry, reverse transcription, integration, transcription and translation, assembly and budding, and maturation (Figure 1.1)



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Figure 1.1: Schematic diagram for HIV-1 replication cycle and host protein and ARV drugs inhibition sites.

HIV-1 Env protein attaches to CD4 and CCR5 receptors for cell entry by fusing the host and viral membranes. The capsid is uncoated in the cytosol, where viral RNA is reverse transcribed to viral cDNA. Viral and host proteins engage with the cDNA to generate the preintegration center (PIC), which can travel through the nuclear pores to enter the nucleus. The host protein lens epithelium-derived growth factor/p75 (LEDGF/p75) tethers the viral genome to the host genome to allow for viral integration. The 5' long terminal repeat (LTR) is the promoter site for binding positive transcription elongation factor (p-TEFb) and RNA Polymerase II for the provirus transcription. Synthesized viral mRNA is spliced into varying products to generate different viral proteins and Gag polypeptides required to produce new virions. Fully spliced mRNA use host machinery to exit the nucleus, after which mRNA translation occurs in the cytosol. Accessory viral proteins like Rev reenter the nucleus to assist in the export of unspliced or incompletely spliced mRNA to the cytosol or ER for translation. Gag-Pol polypeptides, Gag polypeptides, accessory proteins, viral RNAs, and more travel to the plasma membrane for viral assembly. The released virion is an immature virus that needs to be processed by the viral protease (PI) that cleaves the Gag and Gag-Pol polypeptides into various enzymatic or structural proteins. Internal arrangement of structural proteins occur to generate a conical capsid core that contains the viral RNA. Host cell restriction factors (TRIM5, APOBEC3G, SAMHD1, and Tetherin) can block HIV-1 replication at varying steps. As well, ARV drugs act by blocking different stages in the viral life cycle, which include fusion inhibitors, nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), integrase inhibitor (INSTIs), and protease inhibitor (PI). From Engelman and Cherepanov 2012. Nature Reviews Microbiology.

On the surface of the viral membrane are three heterodimers consisting of gp120 (facing outwards) and gp41 (attached to the viral membrane) that come together to make a trimer. HIV-1 attaches to a target cell through viral envelope proteins and host cell membrane proteins, including heparan sulfate proteoglycans⁴⁶, $\alpha 4\beta 7$ integrin⁴⁷, and dendritic cell-specific intercellular adhesion molecular 3-grabbing non-integrin (DC-SIGN)⁴⁸. HIV-1 attachment to a

host cell increases the chance for HIV-1 to interact with a CD4 receptor via the highly conserved CD4-binding site (CD4BS) of gp120⁴⁹. Gp120 consists of five conserved domains and five variable loops that determine viral tropism^{50,51} and are highly glycosylated⁵². Binding of gp120 to CD4 receptor leads to conformational changes that expose coreceptor binding sites. Variations of the V3 loop determine whether HIV-1 binds to CCR5 (R5), CXCR4 (X4), or both receptors (R5X4)^{50,53}; however, most new infections are caused by R5 viruses⁵⁴ while X4 viruses typically emerges during late in infection⁵⁵. Binding of the coreceptor will expose a hydrophobic gp41 fusion peptide, which sequentially inserts into the host cell's cell membrane. Tethering between the host and virus membranes brings together the amino- and carboxy-terminal helical regions of gp41 to form a six-helix bundle or fusion pore, allowing viral compounds to enter the cytosol of the host cell⁵⁶. Additionally, HIV-1 can enter the cell's cytosol through endocytosis, followed by membrane fusion in the endosome or dynamin-dependent fusion⁵⁷.

The majority of the reverse transcription occurs in the cytosol of the infected cells; however, studies do show the presence of minus-strand viral DNA within virions, suggesting that a small level of reverse transcription could be occurring before host infection^{58,59}. Reverse transcriptase acts as an RNA-dependent DNA polymerase that synthesizes DNA from RNA and an RNase H endonuclease enzyme that degrades RNA when hybridized with DNA. The 3' end of a host lysine tRNA serves as a primer that binds to the primer binding site (PBS) in the 5' end of the (+) RNA strand. DNA polymerization occurs in a 3' to 5' direction in reverse transcription, while RNase H degrades the complementary RNA. The growing DNA-tRNA complex is transferred to the 3' end of the (+) RNA strand due to a complement region (called R) present at the start and the end of the RNA strand. Because two copies of viral DNA are packaged in each virion, the continuation of the (-) DNA synthesis could switch (+) RNA

template, contributing to viral recombination. RNase H will degrade the majority of the viral RNA; however, a purine-rich region called a polypurine tract (ppt) at the 3' end is resistant to RNase H activity and serves as the primer for the synthesis of (+) DNA from the complementary (-) DNA. Because the Lysine tRNA is still attached to the 3' end of (-) DNA, approximately 18 nucleotides from the lysine tRNA (plus the PBS) will be incorporated into the new (+) DNA. Identical PBS sequences in the (-) and (+) DNA strands complement to form a double-stranded complementary DNA (cDNA). The resulting viral DNA genome is longer than the starting RNA genome due to the addition of an identical LTR sequence to the 5' and 3' ends of the viral genome. The LTR sequence comprises U3, R, and U5 segments. Incomplete RNase H activity against the RNA primers affect the length of U5 and U3: U5 depends on cleavage of the tRNA primer while U3 depends on the cleavage of the ppt primer^{60,61}. Viral genome diversity can arise from the lack of proofreading by reverse transcriptase and the recombination of viral RNA templates during the reverse transcription step.

Next, the preintegration complex (PIC) is formed by viral proteins, host proteins, and viral cDNA interactions. IN consists of an amino-terminal domain or "zinc finger", central catalytic, and carboxy-terminal domains. The first catalytic step of IN is to remove two nucleotides from the 3' end of the cDNA to create blunt ends required for integration into host DNA. The viral DNA ends are bound by four IN dimers to form a tetramer. Next, the PIC travels through the nuclear pore to enter the nucleus, allowing HIV-1 to integrate into resting and undividing cells^{62,63}.

Inside the nucleus, the host protein epithelium-derived growth factor/p75 (LEDGF/p75) tethers the PIC and host DNA together. After which, IN catalyzes the 3'-hydroxyl strand nucleophilic attack of the phosphate backbone of the host genome to form a covalent linkage

between the host and viral DNA. The two ends of the viral DNA attack the host genome in staggered positions that will generate two nucleotide gaps: at the start and end of where the viral DNA is integrated. Host repair machinery will fill and ligate the gaps to generate duplicated 4-5 nucleotides of the host DNA that flank both ends of the integrated viral DNA^{64,65}. HIV-1 is preferentially incorporated into introns, active genes, and highly spliced genes⁶⁶⁻⁶⁸.

While viral DNA can be integrated into the host genome, it can also be degraded in the cytosol or circularized in the cytosol⁶⁹ and nucleus⁷⁰ through interactions with varying host proteins. Resulting circular viral DNA can have one or two LTRs. With a novel quantitative assay, Butler et al. measured viral DNA levels at three stages⁷¹: after reverse transcription, two-LTRs circular DNA, and integrated provirus. The primer sets for the reverse transcription product amplify the left LTR to the 5' end of the *gag* gene; two-LTRs circular DNA use primers that anneal to the junction that joins the two DNA ends; and integrated provirus primers amplified 5'LTR to chromosomal Alu repeats. Because Alu repeats occur at varying distances from a provirus's LTR, it can yield varying PCR products. A standard curve was made using DNA from highly infected cells to generate a heterogeneous provirus population. Using these assays, the group immediately detected reverse transcription products within hours of infection. Peak levels occurred at 12 hours post-infection, which rapidly declined until it was back to baseline by 72 hours. Two-LTRs circular DNA levels shared a similar pattern as reverse transcription products but had a delayed peak level at 24 hours. Lastly, integrated proviruses only became detectable by 24 hours and peaked at 48 hours, after which it plateaued into 72 hours. Peak levels for reverse transcription, two-LTRs circular DNA, and integrated provirus were 20, 0.14, and 1.0 copies per cell, respectively. While the function of circular DNA is unclear,

pathways from reverse transcription products to circularized DNA formation or degradation may provide host protection to limit the number of integrated proviruses.

Once integrated, the provirus can use host machinery to replicate or lie dormant in latency. The control of latency and reactivation is poorly understood. NF- κ B contributes to T cell homeostasis, and thus, NF κ B pathways can be agonists or antagonists to the maintenance of HIV-1 latency^{72,73}. During viral transcription, NF- κ B can induce initiation complex formation at the promoter site by displacing repressor proteins and controlling the transcription of the viral protein called transactivating factor (Tat), which is critical in regulating HIV-1 transcription. The 5' LTR is the promoter region, where Tat binds to a hairpin structured region called the transactivation-responsive region (TAR). The lack of Tat leads to the stalling of RNA polymerase already present at the promoter^{74,75}. Thereby, Tat is involved in the regulation and not the initiation of viral transcription. The formation of the elongation complex requires the binding of Tat and the positive transcription elongation factor (pTEFb), specifically the CDK9 kinase and cyclin T1 components⁷⁶⁻⁷⁹. Binding of Tat and pTEFb at TAR induces conformational changes to pTEFB to activate the CDK9 kinase. Sequential phosphorylation by CDK9 leads to the release of negative elongation factor (NELF) in the promoter region and stalls transcription⁸⁰. Finally, CDK9 hyperphosphorylates critical proteins to begin transcription elongation, including the RNA polymerase II and elongation factor Spt5⁸¹⁻⁸³.

The newly synthesized viral RNA transcript incorporates the start of the R region in the 5' LTR to the end of the R region in the 3' LTR. Both ends of the mRNA are protected with a 5' cap and 3' poly(A) tail. To generate the mRNA templates for various viral proteins, alternative splicing from four donor and eight acceptor sites produces over 40 different spliced mRNA from the original ~10kb mRNA transcript. Splicing occurs in the spliceosome and is regulated by

various splicing enhancers and repressors. Incompletely spliced products of ~4 kb encode for Vif, Vpr, Env, and Vpu while completely spliced products of ~1.8 kb encode for Tat, Rev, and Nef. Tiny completely spliced transcripts, such as Rev, can be exported from the nucleus using the host's transport mechanism. Once translated, Rev returns to the nucleus to facilitate the export of full-length and incompletely spliced mRNA, which generally gets degraded in the nucleus. Rev controls the mRNA export of intron-containing mRNA⁸⁴. Rev binds to the mRNA by the Rev-responsive element (RRE) present in the *env* gene⁸⁵, which is still fully intact in incompletely spliced or unspliced mRNAs. Oligomerization of multiple Rev at RRE leads to the interaction with Crm1 and GTP-bound Ran. The hydrolysis of Ran_{GTP} to Ran_{GDP} provides the energy needed to export the Crm/Rev/mRNA complex through the nuclear pore. In the cytosol, the exchange of Ran_{GTP} to Ran_{GDP} will destabilize the Crm/Rev/mRNA complex, leading to the dissociation of all three components. Rev will then bind to a GDP-bound nuclear import factor to reenter the nucleus for the next round of mRNA export.

In a normal translation process, the ribosome will scan at the 5' cap of the mRNA in a 5' to 3' direction until it recognizes the AUG start codon. However, this mechanism is precluded in HIV-1 mRNA translation due to various reasons: 1) AUG codons are present upstream of the authentic AUG, affecting the translation of the protein of interest; 2) many unspliced mRNAs are bicistronic, including *vpu* and *env*, which means that the two different viral proteins have overlapping open reading frames⁸⁶; and 3) highly-structured regions like the TAR sequence, poly(A) tail, and the primer binding site (PBS) block ribosomal scanning. RNA helicase DDX3 can solve constraints caused by the highly structured regions. Interestingly, DDX3 interacts with Tat proteins to direct binding to the TAR region on the promoter to unwind the hairpin structure of TAR and allow for ribosomal scanning⁸⁷. DDX3 interacts with host proteins at the promoter

site, including eukaryotic initiation factors 1-3 and tRNA, to form the 43S pre-initiation complex (PIC) on the promoter^{88,89}. Next, to bypass the overlapping reading frame between *vpu* and *env*, 43S PIC can “jump” around the mRNA segment in a leaky scanning mechanism to reach the start codon of *env* while bypassing that of *vpu*. One explanation for this mechanism is the weak Kozak consensus sequence at the *vpu* initiation site, which can affect the level of translation^{86,90,91}. While translation can initiate at the 5' cap, translation for the unspliced *gag* and *gag-pol* mRNA can start at Internal Ribosome Entry Sites (IRES) that are present at the 5' LTR (HIV-1 IRES) and within *gag* region (HIV-1 Gag IRES)⁹². Initiation of IRES-dependent translation may be cell-type⁹³ and cell-cycle dependent⁹⁴. Lastly, HIV-1 needs to frameshift by -1 nucleotide to translate Gag and Gag-Pol polypeptides at a ratio of approximately 10:1. The ability to frameshift is regulated by the site that the frameshift occurs at called heptanucleotide slippery sequence (UUUUUUA) and the downstream stem-loop mRNA structure that forms a pseudoknot that pauses translation and leads to a -1 frameshift^{95,96}. The frameshift eliminates the stop codon in *gag* and allows the translation to proceed into *pol*. The ability to produce both peptides is vital because they give rise to different viral proteins during the maturation step: Gag produces MA, CA, SP1, NC, SP2, and p6 while Pol produces PR, RT, and IN.

Each virion will package two copies of HIV-1 (+) RNA, lysine tRNA, Env, Gag peptides, Gag-Pol peptides, and other accessory viral proteins. HIV-1 Gag peptide comprises the MA-CA-SP1-NC-SP2-p6 domains from 5' to 3' direction. MA engages with phosphatidylinositol (4,5) bisphosphate (PI(4,5)P₂)⁹⁷. Moreover, Env proteins interact with MA to incorporate Env proteins into the plasma membrane⁹⁸. CA is responsible for protein-to-protein binding with different Gag peptides, which will come together to form the conical shell in the mature virion. NC binds to the two copies of unspliced viral RNA at a specific packaging sequence (called Ψ) upstream of the

gag start codon. The two copies of viral RNA need to be noncovalently dimerized at the 5' end to form a “kissing” loop hairpin to have effective packaging⁹⁹⁻¹⁰¹. Additionally, each NC contains two zinc finger motifs that interact with the dimerized RNA to improve stability and folding¹⁰². Lastly, host lysine tRNAs are also packaged through PBS interactions at the 5' end, as lysine tRNAs are critical for reverse transcription, as described above. P6 binds to accessory proteins Vpr, Vif, and Nef^{103,104}. SP1 and SP2 are involved in conformational changes during viral maturation. The envelope of an HIV-1 virion is made up of the host's plasma membrane that contains integrated Env proteins that help facilitate cell entry. As described above, Env proteins are heavily glycosylated to shield from neutralizing antibodies. The glycosylation process for Env proteins depends on the endoplasmic reticulum (ER) and Golgi pathway, where Env proteins are translated at the ER, transported to the Golgi for the addition of varying carbohydrate groups, and lastly transported to the plasma membrane for incorporation into viral particles prior to viral budding. Budding from the plasma membrane is controlled by the accumulation of Env proteins at the microdomains and the host's mechanism for endosomal sorting complexes required for transport (ESCRT)¹⁰⁵. The fully budded virion is a non-infectious immature virion. For virion maturation to occur, Gag-Pol polypeptides need to be cleaved by PR to generate viral proteins, including RT, IN, and PR, and sequentially arrangement of structural proteins into a conical capsid core to produce an infectious virion¹⁰⁶.

1.1.3.2 Functions and host interactions of HIV proteins

The Gag peptide contains many structural proteins (MA-CA-SP1-NC-SP2-p6 domains). Matrix MA 5' domain interacts with the transmembrane protein of Env (gp41) during the budding step by promoting the recruitment of Env to the plasma membrane^{107,108}. Particularly

mutations to MA will inhibit Env surface expression and incorporation into the viral lipid membrane¹⁰⁹. The host protein tail-interacting protein of 47 kDa (TIP47) links the interaction of MA and Env, and the lack of TIP47 will inhibit Env incorporation into the membrane of the budding virus¹¹⁰. In a second role, MA is involved in packaging the two RNA copies during viral budding. MA and RNA association is purposed to improve MA binding to membrane lipid PI(4,5)P₂ and protect MA from interactions with cytosolic factors¹¹¹. After PR cleavage, Capsid (CA) will self-assemble into the conical capsid that holds the two RNA copies. Significantly, CA can shield the synthesized viral cDNA from innate sensors¹¹². Nucleocapsid (NC) is another gag domain that binds to viral RNA for assembly via the two zinc finger motifs. Specifically, NC interacts with Vif for the stable binding of lysine tRNA and the two RNA copies in the assembly and maturation steps¹¹³⁻¹¹⁵. In addition to the roles described above, p6 is also involved in encapsulating accessory viral proteins into the assembling virions^{103,104,116,117}. In-between proteins CA and NC there is a spacer peptide called SP1. Mutations to SP1 lead to the inability of Gag to assemble into an immature virus-like particle during the assembly step¹¹⁸. Another spacer peptide, SP2, separates NC and p6 proteins. The function of SP2 is not well known, and the lack of SP2 does not change the infectivity or maturation; however, the lack of cleavage to generate individual NC and p6 is critical to creating infectious mature virions¹¹⁹.

The Gag-Pol peptide contains enzyme proteins (PR, RT, and IN). PR is required for the cleavage of Gag and Gag-Pol polypeptides, allowing for the functional folding of HIV structural (MA, CA, NC, and p6) and enzymatic (RT, IN, and PR) proteins. This process transitions the non-infectious immature virion to an infectious mature virion. Before viral maturation, auto-cleavage occurs in the Gag-Pol peptide by embedded PR to generate fully folded PR enzymes that can go on to cleave other polypeptides^{120,121}. This process is inhibited by neighboring p6,

which is adjacent to PR, to prevent premature protease activities until assembly is complete¹²². RT synthesizes cDNA from the viral RNA copies packaged in the infecting virion. The direct binding of RT to viral integrase is also essential for initiating reverse transcription. Mutations that change IN activity or its interaction with RT can lead to undetectable early reverse transcription products^{123,124}, likely because integrase is involved in the initiation and elongation of reverse transcription¹²⁵. The physical interaction between RT and NC leads to the synthesis of long cDNA transcripts^{126,127}, likely because NC directly binds to lysine tRNA, which is used as the primer for reverse transcription. IN is responsible for integrating cDNA into the host genome to generate the provirus that can hijack the host machinery for viral replication. While RT-IN interaction is vital to enhance reverse transcription, RT acts as a regulator for IN activity. RT can do this by inhibiting the 3' endonuclease and strand transfer activities by IN¹²⁸. Additionally, Rev is another viral protein that can inhibit the activity of IN by promoting the dissociation of LEDGF/p75. This critical protein tethers the viral and host DNA next to each other for strand exchange¹²⁹.

Accessory proteins (Vif, Vpr, Vpu, Tat, Rev and Nef) are also produced using the host machineries to support many steps in the viral life cycle.

Viral infectivity factor (Vif) inhibits the host restriction factor Apolipoprotein B-editing catalytic subunit-like 3 (APOBEC3G). (APOBEC3G) binds to viral RNA and is packaged into the new virions¹³⁰. After the virion infects a cell and is released into the cytosol, APOBEC3G will act as a cytidine deaminase¹³¹ to induce cytosine to uracil mutations in order to 1) trigger DNA degradation by uracil DNA glycosylases that results in decreasing reverse transcription products^{132,133} and 2) causes guanine to adenine mutations in the integrated provirus that can result in amino acid changes and generation of premature stop codons¹³⁴. To combat

APOBEC3G, HIV-1 Vif interacts with cellular proteins (Cul5 and SCF) to poly-ubiquitinate APOBEC3G, which will lead to protein degradation^{131,135,136}. Vif can also bind the mRNA that encodes APOBEC3G to block its translation¹³⁷. Both activities by Vif will reduce the numbers of proteins that can be encapsulated in new virions.

Viral protein R (Vpr) inhibits host lysine tRNA synthetase, which is essential for producing the primer lysine tRNA for reverse transcription; thus, Vpr may have a regulatory role for reverse transcription¹³⁸. It is also involved in the compacted packaging of DNA in the preintegration complex (PIC) involved in the nuclear import of the viral DNA¹³⁹.

Viral protein U (Vpu) downregulates CD4 cell surface expression¹⁴⁰ to promote Env packaging as cell surface CD4 inhibits viral release¹⁴¹. Additionally, host restriction factor Tetherin can tether nascent virions onto the cell surface to prevent viral release from the infected cells¹⁴²⁻¹⁴⁴, which will decrease the number of newly formed virions⁸⁰⁻⁸². Furthermore, the accumulation of tethered virions to the cell surface can promote immune responses, including NK cell-mediated killing, ADCC^{145,146}, dendritic cell activation¹⁴⁷, and downregulation of MHC presentation^{147,148}. To combat Tetherin activity, Vpu can downregulate Tetherin cell surface protein levels by poly-ubiquitination for proteasomal degradation^{142,144}.

Transactivator Tat promotes provirus transcription by binding to the TAR in the 5' LTR promoter. Tat recruits many host proteins to transition from initiation state to elongation stage in viral transcription, including CDK9, cyclin T1, histone acetyltransferase p300, INI1, DSIF (5,6-dichloro-1-β-D-ribofuranosylbenzimidazole sensitivity-inducing factor)^{77,80-82,149}. Furthermore, Tat interacts with viral Vpr to enhance transcription, and the lack of interaction will reduce transcription level¹⁴⁴. While RNA polymerase II is already present in the promoter, the recruitment of the varying proteins and Tat are required to activate the elongation step of the

polymerase¹⁵⁰. As a result, the full-length transcription of the viral genome is dependent on Tat¹⁵¹.

Rev is the primary pathway in exporting mRNA through binding the RRE site located in the *env* gene, which is present in unspliced or incompletely spliced mRNA. Afterwards, host proteins CRM1, RAN_{GTP}, and DDX3 are recruited to the site to help export the mRNA from the nucleus¹⁵²⁻¹⁵⁴. A leucine-rich activation domain is found in the Rev that allows for the continuous shuttling of Rev between the nucleus and cytosol. The lack of this domain will decrease exported viral mRNA due to the inability for Rev to exist from the nucleus¹⁵⁵. Additionally, Rev can downregulate Tat by indirectly reducing quinone oxidoreductase 1 involved in stabilizing stability. Lower Tat levels will decrease viral transcription and promote a stage of latency¹⁵⁶.

Similar to Vpu, negative infectivity factor (Nef) can downregulate CD4¹⁵⁷ and Tetherin¹⁵⁸, which will enhance virion release¹⁴¹ and protect against antibody-mediated immunity¹⁵⁹. Nef can also sequester the major histocompatibility complex (MHC) class I molecules to decrease engagement with CD8+ cytotoxic T cells¹⁶⁰⁻¹⁶².

Finally, envelope proteins gp120 (SU) and gp41 (TM) are transcribed from the *env* gene and incorporated into the viral lipid envelope to assist cell entry and tropism. Gp120 and gp41 bind together as a heterodimer. Three heterodimers interact to form a trimer on the HIV-1 lipid envelope. Gp120 is responsible for binding CD4 receptors, which will induce conformational changes that expose the V3 loop needed to bind to coreceptors⁵⁰⁻⁵³. Binding of Gp120 to the coreceptor will induce additional conformational changes that will expose gp41 to the host membrane layer to initiate membrane⁵⁶. The sequence of the V3 loop in gp120 determines the viral tropism to CCR5 or CXCR4 expressing CD4+ T cells^{50,163}. While CD4+ T cells express

both CCR5 and CXCR4 receptors, CCR5-tropism infections occur in the majority of sexual transmissions⁵⁴. During sexual transmission, viral particles have to go through many barriers, including the mucous layer, and epithelium, before finally reaching the sexual organs. The multiple tissue layers collectively work to positively select for R5-tropic viruses over X4-tropic viruses due to many reasons: 1) the mucosal layer contains anionic proteins that likely binds to the more cationic X4 virus than the less cationic R5 virus¹⁶⁴ and antiviral proteins that will downregulate CXCR4 receptor expressions on surrounding cells¹⁶⁵; 2) higher expression of CCR5 mRNA than CXCR4 mRNA cells in the female genital tract¹⁶⁶; 3) higher preference for R5-tropic viral infections by cell types that are first exposed to HIV-1, including Langerhans cells¹⁶⁷⁻¹⁶⁹, memory T cells^{169,170}, macrophages¹⁶⁷, and dendritic cells¹⁷¹⁻¹⁷³; 4) infected R5-infected dendritic cells can travel to the draining lymph nodes to infect more CD4+ T cells¹⁷²; and 5) intestinal epithelial cells predominately express more CCR5 receptor¹⁷⁴ while constitutive expression of CXCR4 ligand, SDF-1, in the intestinal tract can downregulate CXCR4 receptors¹⁷⁵. Collectively, many barriers contribute to the preferential and positive selection of CCR5-tropism viruses in seeding new infections.

1.1.3.3 Infection timeline

Viral transmission can occur through contact with an infected person's bodily fluid (blood, breast milk, semen, saliva, and vaginal secretion). Approximately 80% of global cases of HIV-1 infections are transmitted through the mucosal surfaces during sexual activity (UNAIDS 2010). The number of founder variants that seed a new infection during sexual transmission is correlated to the recency of the partner's viral infection – partners with acute infection would have a higher risk for multiple-founder variant transmission while infection by a chronically

infected partner could arise from a single viral particle¹⁷⁶. Infections caused by injection needles can have as many as 16 different founder strains¹⁷⁷, likely due to the lack of physical, mucosal, and cellular barriers that restrict sexual transmission.

From the first contact of a viral particle, it can take 2-6 hours for the virus to cross the mucosal barrier, 3-6 days to arrive at the draining lymph nodes, and within a month to establish the viral infection reservoirs¹⁷⁸. Diagnostic tests used to detect an HIV infection commonly use blood to detect viral RNAs and viral antigen CA (p24), which can be detectable at 10-33 and 18-45 days post-exposure, respectively. Additionally, after antibody seroconversion, anti-HIV-1 antibodies are detectable at 23-90 days after viral exposure (<https://www.cdc.gov/hiv/basics/hiv-testing/test-types.html>).

There are three main phases for an HIV infection: acute, chronic, and AIDS. The acute phase is defined by rapid viral replication, high plasma viral titer, and the spontaneous decline in plasma viral titer. Median peak viremia is $6.7 \log_{10}$ copies per mL and occurs approximately 21 days after infection^{179,180}. Due to high plasma viral titers, acutely infected individuals are 26 times more infectious than chronically infected individuals during sexual transmission¹⁸¹. Interestingly, viral titer spontaneously decreases after peak viremia by approximately $2 \log_{10}$ ¹⁸⁰, likely due to the rapid depletion of CD4+ T cells needed to generate new virions¹⁸² and the partial control by virus-specific CD8+ T cell responses¹⁸³⁻¹⁸⁵. After infection, anti-HIV-1 antibodies against the founder viruses can be detected at around 20 days while broadly neutralizing antibodies against escaping variants develop at about three months¹⁸⁶. Individuals in the acute phase commonly experience fever-like symptoms, including fever, headache, rash, and enlarged lymph nodes^{179,180}.

In the chronic phase, individuals are normally asymptomatic and have a long duration of stable viral titers called set-point, which varies considerably. The median set-point in a cohort of 112 volunteers from East Africa and Thailand was 4.4 log₁₀ copies per mL, with a range of 2.5 to 6.0 log₁₀ copies per mL¹⁸⁰. High set-points or the sudden increase in set-points are correlated to AIDS development¹⁸⁷; however, set-points can be decreased with early ART initiation¹⁸⁸. Because viral titers are the lowest during this phase, the risk for sexual transmission is also the lowest compared to acute and AIDS phases. Furthermore, one in 300 infected people are elite controllers who can naturally control infection for many years, with a plasma viral titer of <400 copies per mL^{189,190}. Chronic infection can transition into AIDS in 3-10 years if the individual does not take ART^{191,192}. However, if ART is used, individuals can stay in this stage for decades while living a normal lifespan.

In the last phase, an AIDS diagnosis is defined by having a CD4+ T cell count of fewer than 200 cells/mm³, while a normal CD4+ T cell count is 500-1,600 cells/mm³. This stage is defined by the increase in plasma viral titer and reappearance of symptoms found during acute infection, including swollen lymph nodes, fever, diarrhea, weight loss, rash, etc. However, these individuals can also develop AIDS-defining illnesses: infections (Pneumocystis pneumonia, tuberculosis, candidiasis, cytomegalovirus, meningitis, etc.), cancers (lymphoma and Kaposi's sarcoma), kidney disease, liver disease, neurological complications, and wasting syndrome (weight loss and muscle wasting)¹⁹³. The normal timeframe for AIDS development in an untreated individual is 3-10 years, but it can vary for each individual^{191,192}.

1.1.4 Host immune responses

1.1.4.1 Innate immunity

Sexual transmission of HIV-1 occurs through mucosal tissues that have three barriers of immunity: microbial, physical, and chemical. The microbial barrier contains commensal microbes that can secrete toxins and reduce pathogen adherence that may block HIV-1. Indeed, various peptides secreted by commensal bacteria have been shown to inhibit HIV infectivity^{194,195}. Next, the physical barrier contains epithelium cells that form tight junctions between neighboring cells to prevent pathogen diffusion. Cilia and mucus function to deter pathogens from the epithelium cells, such as secreted mucus from the vaginal epithelium that can interact with commensal bacteria to trap HIV-1 in the mucus layer¹⁹⁶. Lastly, the chemical barrier contains secreted antimicrobial peptides, proteins, and IgA antibodies to block HIV-1 binding. Various secreted mucins have been found to inhibit HIV-1 *in vitro* and enhance antibody binding to HIV-1 virions by mucin engagement^{197,198}.

Even though HIV-1 can penetrate these barriers and attach to a cell, pathogen-associated molecular patterns (PAMPs) of HIV-1 are recognized by the infected cells by pathogen-recognition receptors (PRRs) to activate immune responses. After the HIV-1 CA core enters the host cytosol, CA will interact with host chaperone protein cyclophilin A (CypA) to prevent PRR recognition¹⁹⁹ and block the binding of host restriction factor TRIM5 α to the viral core²⁰⁰. Mutations in the CA proteins will decrease sensitivity to CypA, allowing PRRs to recognize the viral nucleic acid¹¹². Furthermore, the levels of PRR recognition in HIV-1 and HIV-2 infections differ due to variation in their binding affinity to CypA. While CypA has a high affinity for HIV-1 CA and can effectively mask PRR recognition, it has a low affinity to HIV-2 CA, which may contribute to the lower pathogenicity of HIV-2²⁰¹.

Toll-like receptors (TLRs) are a type of PRR that can recognize viral components in the extracellular matrix or within the endosome. TLR2 and TLR4 are membrane-bound receptors

that can recognize gp120, while TLR7 and TLR8 are found within endosomes that bind to ssRNA^{202,203}. Binding of these TLRs will activate a downstream cascade by MyD88 to stimulate NF- κ B, IRF7, and IRF3 induced transcription of proinflammatory cytokines and type 1 IFNs. Furthermore, recognition by TLR8 led to the production of pro-IL-1 β while NOD-like receptor protein 3 (NLRP3) inflammasome led to the activation of caspase 1. This enzyme is required for the processing of pro-IL-1 β to IL-1 β , which mediates pyroptosis in nonpermissive CD4⁺ T cells and promotes inflammatory responses to recruit immune cells to the site of viral infection²⁰⁴.

Cytosolic PRR retinoic acid-inducible gene I (RIG-1) and melanoma differentiation-associated protein 5 (MDA5) can also recognize viral ssRNA²⁰⁵. Both receptors activate the downstream signal for mitochondrial antiviral-signaling protein (MAVS) to stimulate transcription factors IRF3, IRF7, and NF- κ B to promote gene expression for HIV-1 restriction factors, including pro-IL-1 β , IRF target genes, type 1 interferons, and proinflammatory cytokines and chemokines. Additionally, this similar pathway is activated by two other PRRs that recognize HIV-1 cDNA: interferon-inducible protein 16 (IFI16) and cyclic GMP-AMP synthase (cGAS)²⁰⁶⁻²⁰⁸. Here, IFI16 and cGAMP – the second messenger to cGAS – binds to STING adaptor proteins to activate IRF3, IRF7, and NF- κ B induced gene transcription. Newly secreted interferons will bind to the IFN- α/β receptors expressed on self and neighboring cells to activate the Jak-STAT pathway, leading to the upregulation of IFN-stimulated genes (ISGs). ISG gene products can function as HIV-1 PRRs and restriction factors, including IFI16, cGAS, APOBEC3G, SAMHD1, TRIM5 α , and Tetherin²⁰⁹, and are critical to generating antiviral responses within the infected cell. For example, the knockout of IFI16 diminished antiviral response and increased viral replication^{206,210}.

Moreover, APOBEC3G, SAMHD1, TRIM5 α , and Tetherin are critical restriction factors against HIV replication. Apolipoprotein B-editing catalytic subunit-like 3 (APOBEC3G) gets packaged with budding virions to block reverse transcription in the newly infected cells. APOBEC3G can restrict viral infection by deaminating cytidine to uridine during reverse transcription. The uridine-containing cDNA will trigger DNA degradation. HIV-1 Vif can block the function of APOBEC3G by ubiquitination induced protein degradation¹³¹⁻¹³⁷. SAM domain- and HD-domain-containing protein 1 (SAMHD1) is a triphosphohydrolase that converts deoxynucleoside triphosphate (dNTP) to deoxynucleoside (dN) and inorganic triphosphate group²¹¹. The triphosphate group is required to form the phosphodiester bond of a growing DNA chain. A decrease in cytosolic dNTP levels due to SAMHD1 activity will reduce reverse transcription. TRIM5 α self-assembles around the viral CA proteins to prevent downstream HIV infection post cell entry²¹²⁻²¹⁴. TRIM5 α can block HIV replication by many purposed roles: promoting premature uncoating of the viral capsid²¹³, forming a complex with proteasome for degradation²¹⁵⁻²¹⁷, and blocking uncoating while promoting autophagy²¹⁸. Tetherin traffics to the plasma membrane to tether nascent virions on the cell surface, ultimately preventing their release and maturation steps¹⁴²⁻¹⁴⁴. HIV-1 Vpu can combat this step by downregulating Tetherin receptors on the cell^{142,144}.

Viral engagement with varying PRRs pathways can activate macrophages and dendritic cells (DCs), the first cell subsets to be infected during a new infection. These cells can phagocytose HIV-1 to direct it for lysosomal killing or MHC presentation. The sequential activation of natural killer (NK) cells will result in cell killing by releasing perforin and granzymes, ADCC, and cytokine secretion to promote the maturation of DCs, macrophages, and T cells. NK cells express killer-cell immunoglobulin-like receptors (KIRs) that bind with MHC

class I to induce activating or inhibiting signals. In turn, this will allow NK cells to know if they should develop a tolerance or an activation response towards the engaged cell. Because NK cells can also bind to self-MHC class I molecules on healthy cells, inhibitory KIRs are critical for self-tolerance. The downregulation of MHC class I molecules is a potential sign for viral infection. Indeed, HIV-1 Nef downregulates MHC class I surface expression to evade cytotoxic T lymphocyte (CTL) killing¹⁶⁰⁻¹⁶². Normally, NK cells would become activated due to the “missing self” signals²¹⁹; however, evidence shows that HIV-1 can selectively downregulate certain MHC class I allele that allow HIV-1 to maintain NK cell tolerance and enhance NK cell binding to inhibitory KIRs to evade NK cell-mediated killing^{220,221}.

Lastly, the complement system is an important part of the innate immunity that can recognize pathogen cell surface sugars, ficolins, and bound antibodies to promote inflammation, phagocytosis or cell lysis of the pathogen. Indeed, complement-mediated lysis can occur through interactions with virus bound antibodies and mannose-binding proteins²²²⁻²²⁵. However, binding of HIV-1 particles to complement proteins and erythrocytes has been found to enhance HIV-1 infection in PBMC and mucosal Langerhans cell (LCs; one of the early cell types to be infected in the mucosal layer)²²⁶⁻²²⁸.

1.1.4.2 Adaptive immunity

Cells in adaptive immunity use membrane-bound or secreted receptors for recognition. These receptors are produced from genes that underwent somatic recombination of germline DNA, allowing the generation of diverse and highly specific binding against different pathogens. The adaptive immunity is divided into 1) cell-mediated immunity involving T cells and 2)

humoral immunity involving B cells. Once activated, the cells undergo further maturation, differentiation, and hypermutation to increase specificity.

Cell-mediated immunity involves two T cell types – CD4⁺ and CD8⁺ – that recognize a pathogen compound through the T cell receptors (TCRs) expressed on the cell surface. Each TCR comprises an α and β chain with a variable and a constant region. Furthermore, the variable region is generated by somatic V(D)J recombination using many V, D, and J segments. It is estimated that V(D)J recombination could produce approximately 10^{18} different TCRs to combat the unpredictable number of pathogens that could infect the body²²⁹. Activation of naïve T cells occurs when TCR recognizes a viral peptide that is loaded onto an MHC molecule, also termed the cognate antigen. Cognate antigens are generated when an antigen-presenting cell (APC), normally DCs and macrophages, endocytose a pathogen for degradation. A viral peptide is loaded onto an MHC molecule and delivered to the cell surface. CD4⁺ and CD8⁺ T cells engage with MHC class II and class I molecules, respectively. In addition to MHC recognition, naïve T cells also need costimulatory signaling (through binding of CD28 on the T cell and B7 on the APC), and the uptake of APC-secreted cytokines to develop into an effector or memory T cells. Afterwards, effector T cells can migrate to the site of infection, while memory T cells can provide a faster response during reinfection.

Activation of naïve CD4⁺ T cells through response to APC-derived particular STAT transcription factors will lead to the differentiation of five main subsets: 1) Tfh is essential for B cell maturation; 2) Th1 eliminates intracellular pathogens by producing type II IFN that upregulates genes involved in antiviral activities of phagocytes, including macrophages; 3) Th2 produces various effector cytokines for the killing of extracellular pathogens; 4) Th17 controls extracellular bacterial and fungal infections in the mucosal barriers, involves in the development

of autoimmune disease, secrete cytokines (IL-17, etc.) to recruit phagocytes to the site of infection; and 5) Treg helps to maintain immune tolerance. Additionally, CD4⁺ T cells are important for the maturation, proliferation, and survival of CD8⁺ T cells²³⁰⁻²³³. Indeed, HIV-1 specific CD4⁺ T cells produce IL-21 that is important for maintaining memory CD8⁺ T cells²³⁴. As well, IFN γ expressed by CD4⁺ T cells enhances the effector function of CD8⁺ T cells during HIV-1 infection^{235,236}. Collectively, CD4⁺ T cells have dynamic and important roles in adaptive immunity. Unfortunately, they are the target cell population for an HIV-1 infection, which predominately depletes memory CD4⁺ T cells, as well as Th17 cells that protect the intestinal epithelial barriers in gut-associated lymphoid tissues^{182,237}. While the depletion of critical memory CD4⁺ T cells may negatively affect one's health, maintaining memory CD4⁺ T cell homeostasis – where proliferation/surviving cell population is approximately equal to the dying cell population – will allow an HIV+ individual to maintain relatively good health. Furthermore, proper ART use can help an HIV+ individual maintain normal CD4⁺ T cell levels. In a study of HIV+ individuals (n=898), they found that the median CD4⁺ T cell counts following 0 years, at 3 years, and at 7 years of ART use averaged 270, 556, and 532 cells/mm³, respectively, using data from individuals with nonadherence ART use²³⁸. In a hypothetical situation of complete ART adherence, the median CD4⁺ T cell count at 7 years post-ART could increase to 640 cells/mm³. Both cases of incomplete and complete ART adherences resulted in CD4⁺ T cells within the normal range (500-1,600 cells/mm³), highlighting ART's effectiveness but the higher CD4⁺ T cell counts in complete ART adherences. However, failure to maintain memory CD4⁺ T cell homeostasis would result in AIDS, diminished immunity, and a rise in opportunistic infections. A potential cause for this imbalance comes from the reduced thymic output found in HIV+ individuals²³⁹⁻²⁴¹, thereby restricting the regeneration of memory CD4⁺ T cells over time.

Activated naïve CD8⁺ T cell will become mature cytotoxic T cells (CTL). They can kill infected cells that display the same cognate antigen that led to the activation of the naïve CD8⁺ T cell. The CTL will release perforins and granzymes upon binding to the infected cells. The perforins act to form pores in the cell membrane, allowing granzymes, a serine protease, to enter the cytosol to induce apoptosis. Indeed, multiple nonhuman primate (NHP) studies showed that CD8⁺ T cells are critical in controlling SIV infection^{183,242,243}. However, rapid mutation and downregulating MHC class I molecules allows HIV-1 to escape CD8⁺ T cell immunity. Furthermore, naïve CD8⁺ T cell counts decrease with progressing infection²⁴⁴, likely due to dysfunction of the thymus as in the case with CD4⁺ T cells²³⁹⁻²⁴¹. The upregulation of exhaustion marker PD-1 expressed on HIV-specific CD8⁺ T cells also correlates with dysfunctional HIV-specific CD8⁺ T cells²⁴⁵. Interestingly, approximately 0.18% of HIV-1 infected individuals (n=46,524) are found to have the ability to spontaneously control viral replication and maintain undetectable plasma viral load without the use of ART²⁴⁶. These nonprogressors exhibit strong CTL and weak neutralizing antibody responses²⁴⁷. While the quantity of HIV-specific CD8⁺ T cells does not affect the viral titers, HIV-specific CD8⁺ T cells from nonprogressors show high levels of degranulation, IFN γ , MIP-1 β , TNF α , and IL-2²⁴⁸. Specifically, degranulation is a process where cytotoxic granules (CD107a) containing perforins and granzymes merge into the membrane of activated CD8⁺ T cells in order to release the content into the immunological synapses to reach target cells – HIV⁺ infected cells. Here, perforins will create pores in the cell membrane that will allow granzymes to enter and induce apoptosis in HIV⁺ infected cells. Moreover, genetic analysis of nonprogressors identifies potential protective alleles (*HLA-B*57*, *HLA-B*27*, *HLA-B*14*, *HLA-B*52*, and *HLA-A*25*) and at-risk alleles (*HLA-B*35* and *HLA-B*07*). Especially, SNPs in the peptide-binding grooves suggest that the interaction between the

viral peptide and MHC molecule is a major determinant for HIV-1 control²⁴⁹. Due to the critical role for CD8⁺ T cell-mediated immunity in viral control, a vaccine approach in NHP that targets SIV Gag and uses the CMV vector to induce effector memory CD8⁺ T cell responses; this strategy does not protect from infection after viral challenge but has been shown to result in 50% efficacy in controlling infection²⁵⁰⁻²⁵². SIV was initially detectable in these protected macaques but virus was undetectable after 15-40 months post challenge²⁵². In early 2021, human enrollment began to enable this vaccine to be studied in a phase 1 clinical trial.

In the second arm of adaptive immunity, the humoral immunity comprises B cells that express B cell receptors (BCR). Like TCR, each B cell has a BCR sequence generated from V(D)J recombination of germline gene segments. BCRs consist of 1) a membrane-bound IgM or IgD antibody class and 2) a signal transduction moiety that is a heterodimer of Ig- α and Ig- β . B cell activation occurs in the germinal center, where naïve B cells will survey for antigens captured by follicular dendritic cells (FDCs). Once the BCR binds to the correct cognate antigen, receptor-mediated endocytosis will take in and process the antigen to load onto MHC class II molecules for further engagement to the TCR on Tfh cells. Costimulatory signaling between CD40L on the Tfh cell and CD40 on the B cell will activate B cells for clonal expansion, Ig class switching, and somatic hypermutation. Short-lived plasmablasts are produced for immediate responses by the secretion of antibodies that contain the BCR epitopes. To improve binding affinity to the pathogen, the majority of the activated B cells will undergo light zone and dark zone recirculation. Clonal expansion and somatic hypermutation occur in the dark zone. Activation-induced cytidine deaminase (AID) introduces random base changes to the BCR to generate BCR with high-binding affinity to the pathogen. Afterwards, the activated B cells enter the light zone for another round of antigen exposure. Low-affinity B cells will undergo

apoptosis, while high-affinity B cells undergo Ig class switching. Naïve B cells have IgM and IgD constant regions. During class switching, AID facilitates the removal of the DNA segments for IgM and IgD to allow for the expression of downstream IgG, IgA, and IgE. The expression of different transcription factors will determine if the activated B cell will recirculate to the dark zone for another round of hypermutation, develop into a long-lived plasma cell, or become a memory B cell. Plasma cells will migrate to the bone marrow for long-term antibody secretion of the established BCR sequence. Memory B cells will circulate in the peripheral blood in preparation for secondary exposures, upon which the memory B cells undergo a secondary germinal center response but at a faster rate. As a result, IgG levels peak much earlier during the secondary antigen exposure compared to the primary exposure at ~5 days and ~21 days, respectively²⁵³. Collectively, the combination of V(D)J recombination and somatic hypermutation would generate a B-cell repertoire of over 10^{18} potential BCRs²⁵⁴.

Antibody has effector functions – neutralization, complement activation, opsonization, and ADCC- that can act against pathogens and toxins. The monomeric structure of an antibody is a Y-like structure protein made up of two identical light chains and two identical heavy chains that are linked together by disulfide bonds. The top antigen-binding fragment (Fab) region of the antibody contains the complementarity-determining regions that provide antigen specificity and the bottom constant fragment (Fc) engages with immune cells. There are five classes of human antibody: IgM, IgA, IgD, IgE, and IgG.

IgM is the first antibody class to be secreted by early plasmablasts. IgM consists of five monomers that are joined at the Fc regions by a J chain. As the first antibody class to be secreted, IgM undergoes limited somatic hypermutation and no class switch, resulting in lower affinity and specificity than other antibody classes. However, the pentamer structure connects multiple

Fab regions for enhanced agglutination of pathogens and clustering of antigens to activate the complement system.

IgA provides protection in the mucous membranes. Secreted IgA can be found in both monomeric and dimeric forms. Monomeric IgA can diffuse out from the circulation while dimeric IgA, joined in the Fc region by a J chain, can bind to polymeric immunoglobulin receptor (pIgR) on mucosal epithelial cells for the transport of IgA into the gut lumen. In the gut lumen, IgA can neutralize pathogens to block the pathogens from bypassing the mucosal barrier to infect the host cells.

IgD, IgE, and IgG are secreted as monomers. The function of secreted IgD is not well-known and IgD lack many of the effector functions found in other antibody classes. However, Chen et al. showed that secreted IgD can bind to respiratory bacteria and then sequentially bind to basophils to activate antimicrobial and proinflammatory responses²⁵⁵, potentially providing antigenic specificity to cells in the innate system.

IgE protects against parasites and generates allergic reactions. Secreted IgE are commonly found prebound to resting mast cells. Upon crosslinking with an antibody, the mast cell-bound IgE antibody will activate the mast cells to release granules that contain mediators, including histamine and tumor necrosis factor (TNF). Beyond the sensitization of mast cells, IgE does not have other antibody effector functions like neutralization or complement activation.

IgG is the most abundant antibody class in the blood and is secreted by plasma B cells. Functions of IgG include agglutination and opsonization of pathogens for antibody-dependent cell phagocytosis (ADCP), activation of the complement system, neutralization, and ADCC. Due to the small size, IgG can diffuse into extravascular sites. IgG is the only antibody class that can bind to neonatal Fc receptors (FcRn) to allow for the transport of IgG across the placenta. There

are four subclasses – IgG1, IgG2, IgG3, and IgG4 – that differ in the structure and function. The hinge region is the linkage between the Fab and Fc regions and affect the flexibility of the Fc region. IgG3 has the longest hinge and the most flexible Fab region, followed by IgG1, IgG4, and IgG2²⁵⁶. IgG3 display the highest potency for the activation of the complement system followed by IgG1 and IgG2. As well, all IgG subclasses can neutralize pathogens by engaging with different Fc receptors at varying binding affinity. Contrary to IgG2 and IgG4, IgG1 and IgG3 are robust initiators of ADCC and ADCC; however, the average half-life for IgG1 is 21 days while it is 7 days for IgG3²⁵⁷. Because of the longer half-life and ability to generate cytotoxic effects, IgG1 is the most commonly subclass in antibody-based cancer treatments. As well, therapeutic antibodies that aim to minimize effector functions while maintaining antigen specificity can be developed as a IgG2, IgG4, full Fab region(s) only, or a partial portion of the Fab region called single-chain variable fragment (scFv).

Further discussion on antibody responses against HIV infection can be found in **Section 1.3 - Antibodies for HIV**.

Despite robust adaptive immune responses, no one has fully cleared HIV infection by host immunity alone, and thus it is not possible to study convalescents to determine correlations to protective immunity.

1.1.5 HIV vaccine trials

The first HIV vaccine trial in the United States aimed to generate neutralizing antibody production by vaccinating healthy adults with glycoprotein 160 (gp160) subunits²⁵⁸. While the vaccine was well-tolerated and safe, it elicited low and homologous neutralizing antibody responses. In the latter half of the 1990s, VaxGen generated gp120 subunit vaccines to produce

neutralizing antibody levels in healthy individuals. A phase 3 clinical trial took place in Thailand with 2546 at-risk group of injectable drug users (VAX003)²⁵⁹. Participants received a vaccine with recombinant glycoprotein 120 (gp120) from HIV-1 CXCR4-tropic subtype B and CCR5-tropic subtype B/E. At the 36-month checkup, the vaccination showed no protection against infection, with a tiny nonsignificant efficacy of 0.1%. Approximately 77% of the infection was from subtype E, showing the geographic differences in HIV-1 group M subtypes. Overall, the vaccine did not prevent infection or protect against disease progression. At the same time, VaxGen conducted a second phase 3 trial in the United States and the Netherlands with 5403 participants who were at risk for heterosexual transmission of HIV-1 (VAX004)²⁶⁰. In this trial, the vaccine contained recombinant gp120 proteins from two different CCR5-tropic subtype B strains. Similarly, the vaccine did not protect against infection as the infection rates for the placebo and vaccinated groups were 7.0% and 6.7%, respectively. Furthermore, viral loads did not differ in the placebo and vaccine arms.

The STEP study was a large-scale trial to test the prevention rate using a cell-mediated immunity vaccine²⁶¹. Three thousand participants from North and South America, the Caribbean, and Australia participated in this trial. Participants received an adenovirus vector with encoded HIV-1 *gag*, *pol*, and *nef* genes. Both vaccine and placebo groups showed a 3% infection rate despite ELISPOT analyses showing that 75% and 62% of the vaccinated participants displayed interferon- γ responses to one and two HIV-1 antigens, respectively. The trial concluded that vaccines that induced CTL responses against viral proteins did not prevent viral infection.

In a much earlier attempt to elicit cytotoxic T lymphocyte (CTL) responses, a group from France conducted a phase 1 clinical trial in Zaire in 1986. The group immunized healthy individuals, including the first author himself, with recombinant vaccinia virus-encoded viral

gp160 protein²⁶². The vaccine induced robust immune responses at 63 days post-immunization, which they were able to amplify with repeated boosting with gp160 subunits²⁶³. This trial was unsuccessful and led to many concerns, including vaccine-related complications²⁶⁴ and the ethics of using developing countries for clinical trials; however, it used a “prime and boost” vaccine design that was used in many later clinical trials. In the HVTN 505 trial (ClinicalTrials.gov number, NCT00865566), heterosexual mixed gender participants (n=2504) in the United States received a DNA/rAd5 vaccine²⁶⁵ that consisted of 1) viral DNA of HIV-1 Gag, Pol, and Nef from subtype B and Env from subtypes A, B, and C (the primer) and 2) an adenovirus expressing Gag and Pol from subtype B and Env from subtypes A, B, and C (the booster). Unfortunately, the vaccine was ineffective in preventing HIV-1 infection nor did it reduce viral load setpoints between the vaccine and placebo groups.

In the Thai RV144 trial that took place in the 2000s, 16402 healthy adults who were at risk for heterosexual transmission of HIV-1 received the canarypox vector vaccine that expressed HIV-1 Gag and Pol of subtype B and CRF01_AE HIV-1 gp120, and then later boosted with recombinant gp120 subunits from subtype B/E (ClinicalTrials.gov number, NCT00223080). Compared to the earlier described vaccine trials, this vaccine showed modest protection of 31.2%. However, the vaccine did not decrease viremia nor increase CD4+ T cell counts in the infected vaccine group compared to the placebo group²⁶⁶. At 12 months, they found that 19.7% of the vaccine group had ELSIPOT responses to either Gag or Env peptides, while only 7.3% of the placebo group were responders. They previously found that 96% and 84% of participants in the vaccine group were more responsive to antigens from subtypes B and CRF01_AE by antibody-dependent cell-mediated cytotoxicity (ADCC) compared to the placebo group at 11% and 7%²⁶⁷. Due to the modest protection observed in RV144, another study, HVTN 702²⁶⁸

(ClinicalTrials.gov number, NCT02968849), tried to repeat the same regimen in South Africa by 1) priming with Canarypox vector that expressed subtype C gp120 and subtype B gag160 and protease and 2) boosting with subtype C gp120. Participants (n=5404) from 14 sites in South Africa, where subtype C is the predominant circulating HIV-1. Despite eliciting higher antibody and T cell responses in this study over the RV144 study²⁶⁹, there was no observed protection from viral acquisition, where 5.12% and 4.95% of the participants were infected in the vaccine and placebo groups, respectively. Many reasons may be at play: 1) the two vaccines had different gene sequences and adjuvants than the boosters; 2) higher incidence of infection among women in the placebo group of HVTN 702 (4.2%) over RV144 (0.3%), suggesting that placebo individuals in South Africa had a higher risk of infection than those in Thailand. Furthermore, in the RV144 group, vaccine efficacy decreased from low- to high-risk participants²⁷⁰, which may explain the lack of efficacy in the high-risk South Africa participants; 3) greater genetic diversity in the subtype C in Africa over subtype A/E in Thailand because the RV144 trial took place 15 years earlier; and 4) differences in host genetic factors between the South African and Thai populations as they found that carriers of FCGR2C and HLA-A*02 alleles changed vaccine efficacy in the RV144 study. In summary, the lack of a protective vaccine demonstrates the immense challenge remaining to develop a vaccine that can elicit strong neutralization activity that is broad enough to overcome the hyper-variability of HIV-1 strains²⁷¹⁻²⁷³.

In 2007, Hutter et al. reported on the first known cure of an HIV-1 infected person, Timothy Ray Brown²⁷⁴. Mr Brown lived with HIV-1 for more than ten years before being diagnosed with acute myeloid leukemia. Standard cancer treatment with chemotherapy did not work, and he relapsed, which prompted undergoing an allogeneic stem cell transplantation for his cancer treatment. Because HIV-1 predominately uses CCR5 coreceptor to infect new cells, Hutter et al.

identified an HLA-identical donor that naturally lacked cell surface expression of CCR5 due to the homozygous *ccr5*Δ32 alleles. Mr Brown underwent two rounds of transplantation due to relapse at 332 days after his first transplant. Notably, he discontinued his ART the day before his first transplantation and had been HIV-1 free for 14 years until he passed away in September 2020 from leukemia relapse. Unfortunately, attempts to replicate this cure using donor cells of CCR5 wild type or heterozygous CCR5 Δ32 alleles were unsuccessful²⁷⁵⁻²⁷⁷. In 2019, the second case of HIV-1 cure was in a leukemia patient who underwent stem cell transplantation with homozygous CCR5 Δ32 donor cells^{278,279}, the same method that led to the first HIV-1 cure. The two cases highlight the ability to clear established latent viral reservoirs through stem cell transportation and prevent the establishment of a new viral pool by targeting the CCR5 entry receptor, underscoring the critical role of CCR5 for HIV-1 infection.

1.1.6 The challenge for an HIV cure

A successful HIV infection leads to the integration of proviruses into the genome of the infected CD4⁺ T cells. Once integrated, the provirus will 1) hijack the host transcription machinery to produce new virions; 2) switch to latency which contributes to immune evasion; and 3) replicate alongside normal cellular division to persist within newly developed daughter cells. In addition to persisting in memory CD4⁺ T cells, viral reservoirs can be found in many other cell types – monocytes, macrophages, NK cells, follicular dendritic cells, astrocytes, and microglia – as well as tissues – GALT, lymph node, brain, lung, sexual organs, and peripheral blood²⁸⁰⁻²⁸⁴. The persistent and widely distributed latent viral reservoirs make it difficult to achieve an HIV cure.

While ARV drugs effectively suppress viremia and improve the overall health of HIV+ individuals, the discontinuation of ART will lead to viral rebound at an average of nine days²⁸⁵. Additionally, small levels of viral replication persist in the body despite suppressive ART²⁸⁶⁻²⁸⁹, likely due to limited tissue penetration by ARV drugs²⁹⁰. In an analysis of HIV+ individuals (n=1,057) on suppressive ART, the investigators found that ART did decrease HIV-1 reservoir size by a small amount. Still, the decay diminished over time until it stabilized from the timepoints of 5.4 to 10 years post-ART initiation. Interestingly, 26.6% of the participants were found to have larger viral reservoir sizes despite long-term ART use²⁹¹, demonstrating the limitation in using ARV drugs to eradicate HIV-1.

The "shock and kill" strategy is a potential method for an HIV cure. Here, latency-reversing agents are used to re-activate ("shock") the latently infected virus to "kill" the infected cells by methods including boosting host immunity, ARV drugs, and HIV-specific T cell-inducing vaccines. The first attempt for a "shock and kill" HIV cure was in the RIVER trial. HIV+ individuals were divided into two groups to receive 1) ART only or 2) ART, histone deacetylase inhibitor as the shock, and a prime and boost vaccination method to induce HIV-specific CTL killing. The mean HIV-1 reservoirs in the peripheral blood after ~two years of treatments were 3.02 and 3.06 log₁₀ HIV-1 DNA copies per million CD4+ T cells in the ART only and "shock and kill" groups, respectively²⁹². While the study showed the lack of significant benefits from this particular "shock and kill" strategy, further studies to induce cell re-activation and killing should still be explored. A second and newer method called "block and lock" aims to permanently silence the proviruses to block re-activation. Unlike the previous strategy that aims to activate latent cells, this method uses latency-promoting agents to prevent the activation of latent

cells by targeting various steps in viral replication and signaling pathways, including Tat and LEDGF/p75^{293,294}.

Currently, the only two cases of an HIV-1 functional cure both came after a stem cell transplantation procedure that replaced the immune cells in the infected individual with healthy donor cells that lacked CCR5 expression. Attempts to replicate these results are slowly progressing but are restricted to only HIV+ individuals with cancer. However, successes and failures learned in attempts towards an HIV-1 cure will continue to advance the field.

1.2 Antiretroviral drugs for HIV

1.2.1 HIV drug classes

Current FDA approved drugs are grouped into seven drug classes: 1) nucleoside reverse transcriptase inhibitors (NRTIs), 2) non-nucleoside reverse transcriptase inhibitors (NNRTIs), 3) protease inhibitors (PIs), 4) integrase strand transfer inhibitors (INSTIs), 5) post-attachment inhibitors, 6) CCR5 antagonists, and 7) fusion inhibitors.

NRTI drugs are nucleotide or nucleoside analogues that lack the critical 3' hydroxyl (-OH) group in the pentose sugar that is critical for adding the next nucleotide. While these analogues contain the correct nucleobase that permits correct base pairing, their incorporation into the synthesizing viral DNA chain will lead to early chain termination. Similar to NRTIs, NNRTIs also block HIV reverse transcription by acting as allosteric modulators, where the binding of NNRTIs to reverse transcriptase leads to structural changes that block DNA polymerization. PIs are small chemical compounds that act as substrate analogues for viral protease required for viral maturation. In contrast to NNRTIs, PIs are competitive inhibitors to viral peptides and directly bind to the active sites of viral proteases to prevent the binding by

viral peptides. INSTIs are small chemical compounds that bind directly to the active sites of HIV integrases to prevent the binding of host DNA to the catalytic sites. Ibalizumab is an anti-CD4 antibody that acts as a post-attachment inhibitor, where HIV is blocked from engaging with critical coreceptors needed for membrane fusion. CCR5 antagonist (maraviroc) is a small chemical molecule that binds to the host CCR5 receptor to prevent the fusion step from proceeding. Fusion inhibitor (enfuvirtide) is a peptide compound that binds to the HR1 region of gp41, which is essential for fusing HIV and host membranes²⁹⁵. The PrEP regimen for uninfected humans consist of two NRTIs while the ART regimens for infected humans consist of two different NRTIs and one NNRTI or PI. Additional classes of ARV drugs are used upon multidrug treatment failures.

Drug adherence is critical for the efficacy of ARV drugs, but it can be challenged by ARV drug-related toxicities²⁹⁶⁻²⁹⁸ and the requirement for daily use^{299,300}. Most common adverse effects (AEs) include headache, nausea, rash, gastrointestinal problems, but severe AEs can include hepatotoxicity, liver failure, pancreatitis, and complications with multiple drug interactions³⁰¹. To facilitate adherence to ARV drug regimen, the FDA recently approved the long-acting Cabenuva (cabotegravir; INSTI and rilpivirine; NNTRI) as a once-a-month injection³⁰².

1.2.2 HIV drug resistance

Viral mutations that lead to ARV drug resistances are generated through the high error rate that occurs naturally during the reverse transcription step in HIV replication. This process provides the high genetic variability and survival advantages that allow HIV to evade antiviral host mechanisms. While the error rate for human DNA replication occurs at 1 mutation per 10^{10}

base pairs³⁰³, the lack of proofreading during HIV reverse transcription can generate 1 mutation per 10^3 - 10^5 base pair³⁰³⁻³⁰⁶, leading to approximately 5-10 errors per HIV-1 genome per replication cycle *in vivo*³⁰⁷. This rapid mutation rate can allow new viral pools to emerge that differ from the transmitted/founder strain after 14-27 days^{54,308}, rapidly leading to the increase in HIV genome diversity and the development of drug-resistant variants. Therefore, the use of ART does not generate drug-resistant variants, but rather it provides a fitness advantage for minority populations of drug-resistant variants to outcompete the general viral population to establish dominating infection³⁰⁹.

Mutations that cause drug resistance have been characterized for all commonly used ARV drugs. As previously mentioned, NRTI acts as a nucleotide or nucleoside analogue that lacks a 3' hydroxyl group to cause early DNA chain termination. Mutations on the RT enzyme that lead to NTRI resistance can 1) prevent the incorporation of analogue into the growing viral DNA chain, including the common M184V mutation and 2) promote the entry of adenosine triphosphate into the active site to disrupt the linkage between the drug analogue and the growing cDNA. Similarly, resistance to NNRTIs, PIs, and INSTIs can occur through mutations that block or decrease the binding affinity of the inhibitors to their target enzymes³¹⁰. In particular, resistance to NRTI and NNRTI are the most common as they are the first-line regimens used for both HIV prevention and treatment³¹¹. As a result, the transmission of drug-resistant variants to ART-naïve individuals has increased through the years. Currently, HIV-naïve individuals with high adherence to PrEP can still be infected if their sexual partners harbor drug-resistant variants^{312,313}, termed pretreatment drug resistance (PDR). To combat the problem of ARV drug resistance, HIV+ individuals are given a cocktail of ARV drugs with different mechanisms of

action. Indeed, the survival rate is higher in individuals that receive an ART cocktail of three ARV drugs over two ARV drugs³¹⁴.

1.2.3 Global trend and future projections

Although ARV drugs are a powerful tool to combat HIV-1 infection, the global expansion of ARV drug use is met with the increased development of ARV drug resistance³¹⁵⁻³¹⁷. Here in the United States, 31% of the sampled MSM population was resistant to at least one drug class, with 12% showing multiclass resistance³¹⁸. In a meta-analysis of 170 studies in China, the prevalence of acquired drug resistance – mutations that occur in HIV+ individuals receiving ARV drugs – was 44.7%, where rates for NRTI, NNRTI, and PI resistances were at 31.4%, 39.5%, and 1%, respectively³¹⁹. A multi-continental analysis from trials in Europe, Latin and North America, sub-Saharan Africa, and Asia showed that the prevalence of tenofovir (NRTI) drug resistance in HIV+ individuals was much higher in Africa (56-60%) than in high-income countries (20-22%)³²⁰. This observation is likely due to limited to low healthcare services, ARV drug supplies, virologic monitoring, and drug adherence found in resource-limited areas³¹⁷. Interestingly, prevalence to NNRTI resistance was similar between low- (42-82%) and high- (48-71%) income countries³²⁰. Furthermore, in the recently published plan by the WHO – Global Action Plan on HIV Drug Resistance 2017-2021 – they found that rates for pretreatment NNRTI resistance had rapidly increased in the recent decade, where 7 of the 11 surveilled low-income countries showed rates above 10% (Figure 1.2). If prevalence for drug resistance continues to stay above 10%, then mathematical modeling has shown an increase in AIDS deaths, new infections, and drug costs, all attributable to drug resistance³²¹ (Table 1.1).

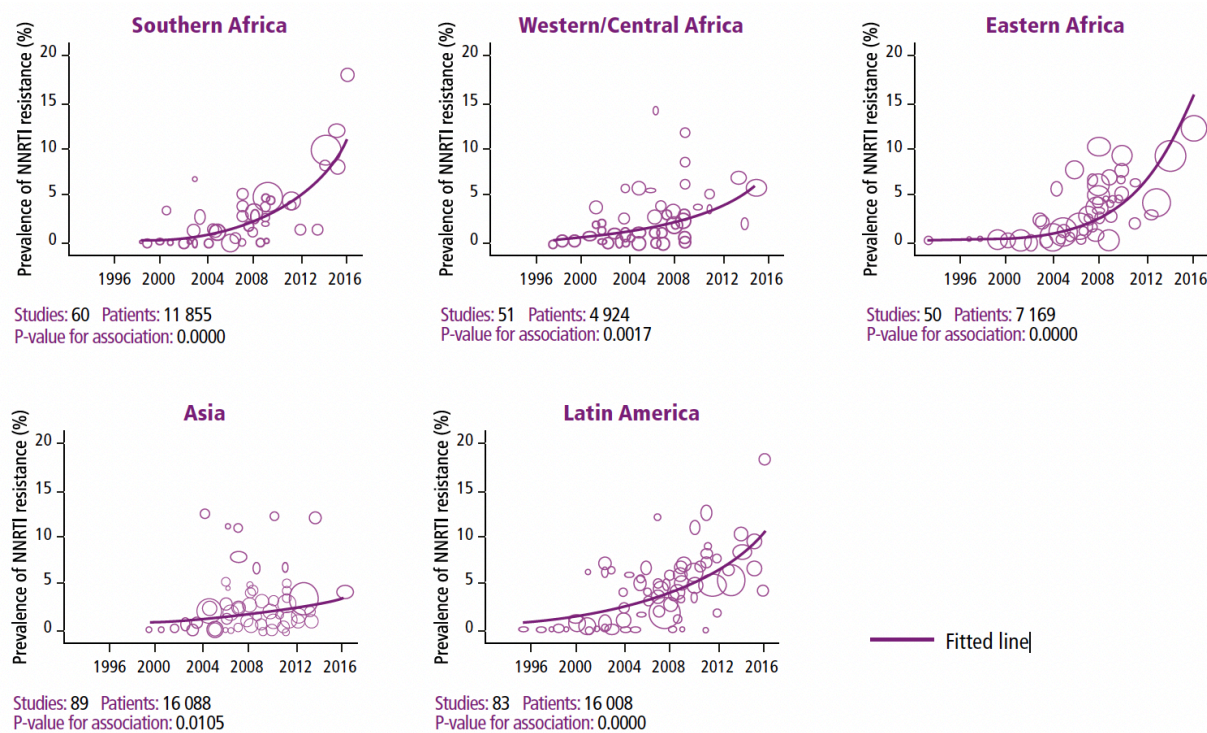


Figure 1.2 Systematic review showing the yearly changes in the prevalence of pretreatment NNRTI resistance by geographical regions. Figure from Report on HIV drug resistance 2017, Geneva, WHO.

	AIDS deaths		New HIV infections		ART costs	
	2016-2020	2016-2030	2016-2020	2016-2030	2016-2020	2016-2030
Amount attributable to HIVDR	135 000	890 000	105 000	450 000	US\$ 0.65 billion	US\$ 6.5 billion
Percentage attributable to HIVDR	5.7%	16%	3.5%	8.7%	2.0%	7.7%

Table 1.1 Mathematical modelling of the predicted impacts of HIV drug resistance. Assuming 10% pretreatment drug resistance to NNRTI. Source: Phillips AN et al. J Infect Dis. 2017;215:1362–5.

1.2.4 The scale of the problem

While NNRTI and NRTI are commonly used as first-line regimens for HIV, the INSTI dolutegravir was recently recommended to be used as first-line treatment in low- and middle-income countries due to treatment failures from traditional first-line regimens^{322,323}. Despite this

suggestion by the WHO, they also acknowledged that resistance to dolutegravir “will invariably emerge” with increasing use. Indeed, the rate of drug-resistant acquisition is found to be directly proportional to the duration of time on ART³²⁴. As INSTIs and PIs increase their utility, drug resistance to these drugs will inevitably occur despite their higher genetic barrier to resistance. Collectively, drug resistance to current FDA-approved drugs will limit treatment options for HIV+ individuals. This exact scenario was reported in a 2020 case study, where a treatment-experienced HIV+ individual was found to be resistant to five classes of ARV drugs (NRTI, NNRTI, PI, INSTIs, and CCR5 antagonist). As a result, this individual's only available treatment options consisted of two FDA-approved drugs - enfuvirtide (fusion inhibitor) and ibalizumab (post-attachment inhibitor) - and experimental drugs³²⁵.

In summary, there is an urgent need to develop and expand ARV drugs to explore 1) drugs with new targets that differ from conventional targets of viral enzymes and 2) drugs with different formulations that can limit drug toxicity and reduce treatment frequency to improve drug adherence.

1.3 Antibodies for HIV

1.3.1 Broadly neutralizing antibodies against HIV capsids

Due to the short half-lives of ARV drugs, once- or twice-daily treatment is a requirement for life³²⁶. Recently, the FDA approved a monthly intramuscular injectable ARV drug; however, the risk for drug toxicities remains³⁰². In contrast, studies for anti-HIV antibody treatments are expanding due to advantages in lowered toxicity, higher specificities, extended half-lives to improve adherence, and engagement with the immune system for enhanced antiviral effects.

Antibody produced after a viral infection is a critical antiviral response by the immune system. Anti-HIV antibodies against capsid proteins gp41 and gp120 start to show up at 13 and 28 days, respectively, after detectable viral loads; however, these antibodies have no effects in controlling viremia despite successful binding to virions^{327,328}. In contrast, neutralizing antibodies (NAbs) that develop months after infection^{327,329} are potent against virions because they can 1) directly block viral interaction with target cells; 2) induce ADCC by the interactions between the Fab portion of the NAb-bound infected cell and the Fc receptors on effector cells like natural killer (NK) cells; and 3) form antibody complexes that augment antigen presentation and adaptive immunity³³⁰⁻³³². Although NAbs can neutralize autologous viruses, they are ineffective against heterologous viruses isolated from other individuals^{329,333}, driving viral envelope evolution over time³³⁴. However, with time, approximately 20-25% of HIV+ individuals can develop cross-reactive antibodies called broadly neutralizing antibodies (bNAbs), which can neutralize a wide breadth of circulating HIV variants³³⁵⁻³³⁷.

The first generation of bNAbs was characterized in the early 1990s^{338,339} and found to be potent in protecting against matched, nearly clonal viral challenges in rhesus macaques^{340,341}. From there, hundreds of new bNAbs have been isolated using advanced techniques, including single B cell culture, neutralization and functional assays, and antigen-specific B cell sorting. These antibodies show greater breadth and require lower concentrations for inhibition³⁴². The wide breadth of the bNAbs is positively correlated with CD4+ T cell depletion, high plasma viremia, and time since infection^{336,343}. Thus, larger amounts of viral antigens and longer exposure time to viral antigens result in affinity maturation of HIV-specific B cells that sequentially enhance the breadth and potency of the antibodies.

HIV can evade antibody recognition in many ways, including residue mutations, conformational constraints, and glycan shields. While these are major contributors to the viral escape of NAbs^{333,344,345}, bNAbs show advantages in many aspects. Current bNAbs bind to conserved peptide and glycan epitopes on viral capsids, including V1/V2 glycan, CD4 binding site, membrane-proximal external region (MPER), V3 glycan, and others^{336,346}. Additionally, continuous somatic hypermutation of antibodies helps maintain neutralization ability to the evolving glycan shield³⁴⁷. Next, the average number of residues that make up the heavy chain complementarity determining region 3 (CDRH3) on human antibodies is approximately 10-14 residues³⁴⁸, while it is much longer in bNAbs (~19 residues)^{349,350}, allowing bNAbs to reach across peptide and glycan shields. The crystal structure of bNAbs PGT 126 and PGT 127 showed that the long CDRH3 could penetrate the glycan shield on the V3 loop to contact conserved glycan and peptide epitopes³⁴⁹. Similarly, when testing a handful of CD4 binding site neutralizing antibodies, only the bNAb b12 could bypass the conformational constraints to bind to the epitopes while NAbs could not^{351,352}.

In protective studies using macaques, bNAbs administered at one day^{340,353}, two days³⁵⁴, and 14 days³⁵⁵ before viral challenges protected macaques from viral infection in a dose-dependent manner^{353,356}. When bNAbs were given to infant macaques within 30 hours after oral exposure, infants were protected from the viral challenge and lacked viral integration in tissues^{357,358}, providing evidence that prevention of HIV vertical transmission may be possible with early treatment. Furthermore, bNAb treatments were potent in suppressing viremia in treatment-free acutely³⁵⁹ and chronically³⁶⁰ infected macaques. In both prevention and treatments, cocktails of more than two bNAbs were more successful than single-agent treatments

of the same bNAbs, as combination treatments protected from the selection of drug-resistant viruses^{360,361}.

The half-lives of ARV drugs and bNAbs are within hours and days, respectively, which allows bNAbs to be explored as weekly or monthly treatments. Furthermore, antibodies can be engineered to improve half-lives. One such way is with the M428L/N434S (-LS) mutation that helps to improve antibody half-life by increasing the antibody's binding affinity to neonatal Fc receptors (FcRn)³⁶². This occurs during the FcRn-mediated transport pathway, where antibodies that are not bound to FcRn while in the endosomes will get sorted into late endosomes and then lysosomes for protein degradation. In contrast, antibodies that remain bound to FcRn will get exocytosed. Indeed, in a protective study using VRC01 and VRC01-LS, macaques that received VRC01-LS exhibited a longer half-life and a 2.5-fold higher trough concentration than macaques that received VRC01. On the day of the viral challenge (14 days after the last bNAb dose), plasma concentrations of VRC01-LS treated macaques ranged from 3.5 to 6.4 µg/mL while approximately 0.1 µg/mL in all VRC01 treated macaques. Because antibody efficacy is dose-dependent, it explained the full protection from viral acquisition seen in VRC01-LS treated macaques (n=3) while VRC01 macaques were only partially protected (n=3; 66%)³⁵⁵.

Because bNAbs still require regular passive transfer of antibodies, various studies have explored the use of gene therapy to deliver bNAb transgenes to cells, allowing the host to produce bNAbs systemically. To this end, studies showed that AAV vectored delivery of bNAbs protected against viral challenges in naïve NHP³⁶³ and suppressed active viremia in infected NHP³⁶⁴. In particular, the “Miami Monkey” remained virologically suppressed for more than three years after AAV-1 gene therapy of three bNAbs³⁶⁴, providing a proof-of-concept for the use of gene therapy in HIV.

Successful macaque studies using bNAbs for HIV prevention and treatment paved the work for human clinical trials. Single or repeated infusions (by IV or SC) of bNAbs in HIV- and HIV+ individuals were found to be safe and well tolerated³⁶⁵⁻³⁶⁸. While macaque studies normally use well-characterized laboratory-adapted viral strains, the circulating viral pools in HIV+ individuals are much more diverse. For reference, the diversity of the global influenza sequences at a given year is comparable to the diversity of the HIV sequences found in one infected individual³⁶⁹. To this end, a single treatment of VRC01 was potent and reduced plasma viremia by 1.1 to 1.8 log₁₀ in ART-untreated humans³⁶⁸ and a single treatment of 10-1074 reduced viremia at a mean 1.52 log₁₀ copies/mL³⁶⁷. Unfortunately, both single-agent antibody treatments selected for less sensitive viruses, which can decrease treatment efficacy and increase the development of drug resistance, promoting the need for combinational therapy. Indeed, combination therapy was safe and potent. The level for viral suppression tracked with bNAb plasma levels^{366,370}. Most importantly, resistant viruses were not selected for during combination ART³⁷⁰. Interestingly, the use of combination ART with bNAbs was associated with increased HIV-1 Gag-specific CD8+ T cell responses, which may be attributable to increase antigen presentation through bNAb-virion complexes³⁷¹.

While bNAbs are tested *in vitro* to have broad neutralization effects, it is unclear whether bNAbs can prevent HIV acquisition from the global circulating strains. To answer this question, a multicenter study³⁷² was conducted in 1) cisgender men and transgender persons in North/South America and Europe (HVTN 704/HPTN 085; N=2699) and 2) heterosexual women in sub-Saharan Africa (HVTN 703/HPTN 081; N=1924). Participants were divided equally into three groups to receive saline placebo, 10, and 30 mg/kg IV VRC01 once-every-eight weeks for 20 months. From the pooled low- and high-dose treated participants, they found that the

protective efficacy for HVTN 704/HPTN 085 and HVTN 703/HPTN 081 was 26.6% and 8.8%, respectively. One potential explanation for the lower rate of protection in the sub-Saharan African study may be due to drug adherence. In these studies, conventional oral PrEP was provided to all participants in the three groups, and thus, should not be a confounding variable in their studies. Even though there were no barriers for oral PrEP availability, women from sub-Saharan Africa still showed a very low drug uptake rate, demonstrating the difficulty to achieving drug adherence in ARV drugs and prompting the need for long-acting agents that allow for less frequent dosing. Finally, neither the low-, high-, or combined dose VRC01 results showed prevention when compared to placebo. In this study, the ability for VRC01 to neutralize a strain is measured by the neutralization inhibitory concentration (IC_{80}). A low IC_{80} means that less VRC01 concentration is required to neutralize 80% of the virus, making the virus a VRC01 sensitive virus, while a virus with a higher IC_{80} is a VRC01 resistant virus. In the clinical trial with VRC01, previous *in vitro* assays predicted that more resistant viral strains with IC_{80} of 10 $\mu\text{g/mL}$ would be neutralized in the treated groups; however, the actual clinical prevention was only observed from viral strains with IC_{80} less than 1 $\mu\text{g/mL}$. Thereby, rather than a 65-81% predicted rate of protection against circulating strains, the real rate of protection was only 30%, which explained the lack of significant prevention compared to placebo. Furthermore, viruses isolated from infected VRC01 treated individuals were more resistant to VRC01 than those isolated from placebo-treated, where the strains IC_{80} was at 8.4 $\mu\text{g/mL}$ and 3.5 $\mu\text{g/mL}$, respectively. Collectively, the selection for resistant variants and minimized potency against the circulating community viral strains support the use of combination bNAbs rather than a single agent.

1.3.2 Antibodies against host cells

Antibodies that target the host cell may be an effective method to combat the wide circulating pool of viruses. The primary CD4 receptor required for HIV infection is a logical target. To this end, the FDA approved an anti-CD4 antibody called ibalizumab (clone 5A8) in March 2018. Ibalizumab is a humanized IgG4 antibody that binds to the extracellular domain 2 of CD4. While ibalizumab does not competitively inhibit the binding of virions to CD4 receptors, occupancy of ibalizumab on CD4 receptor prevents the conformation changes required by gp120 for cell membrane fusion³⁷³. In a phase 3 clinical trial, 40 individuals with multi-drug resistant (MDR) HIV-1 infections and viral loads above 1,000 copies/mL were enrolled to receive 1) ibalizumab with current ART regimen and then 2) ibalizumab with an optimized background regimen. Compared to before ibalizumab, a single IV dose of 2,000 mg ibalizumab reduced mean plasma viral load by 1.1 log₁₀ at six days post-treatment. Next, participants were given a new optimized background regimen that contained at least one ARV drug that they did not have resistance against, and in combination with IV dose (800 mg) ibalizumab every 2 weeks for the next 23 weeks. At the end of the study, the treatment containing optimized background ARV drugs and 800 mg ibalizumab reduced mean plasma viral by 1.6 log₁₀ compared to baseline. Most participants had plasma viral loads under 200 copies/mL, while 25% experienced virologic failure (n=7) and viral rebound (n=3). Nine participants showed higher levels of resistance to ibalizumab than baseline, whereas eight participants lost V5 glycans associated with ibalizumab resistance³⁷⁴. While most AEs were mild to moderate, with diarrhea being the most common symptom, 28% and 10% of the participants experienced grade 3/4 severe AEs and AIDS-defining AEs, respectively. While four participants died due to reasons unrelated to ibalizumab (Kaposi's sarcoma, hepatic failure, lymphoma, and AIDS), one death was found to

be directly due to ibalizumab therapy (immune reconstitution inflammatory syndrome), highlighting a potential risk for ibalizumab therapy³⁷⁵.

UB-421 is another anti-CD4 IgG1 antibody that binds to extracellular domain 1 of CD4 and competitively blocks HIV binding to CD4 receptors. In a phase 2 study³⁷⁶, weekly 10 mg/kg or biweekly 25 mg/kg UB-421 by IV injection helped maintain virologic suppression during ART interruption. Transient episodes of plasma viremia (viral blips) occurred in 28% (n=8) of the participants, which was higher than the 10.3% viral blip frequency found in ART users³⁷⁷. The use of UB-421 was safe and well-tolerated. 28% (n=8) of the participants reported grade 2 or higher AEs, and rash was the most common symptom. Finally, despite being an IgG1 antibody that has effector functions, it did not change CD4 T cell counts during the treatment period.

CCR5 is a critical coreceptor used by HIV infection and targets the FDA approved CCR5 antagonist maraviroc. On the antibody side, HGS101 and Leronlimab are two anti-CCR5 humanized IgG4 antibodies being studied for their anti-HIV potency. HGS101 binds to the extracellular domain 2 of CCR5, directly competing with HIV for CCR5. In PBMC culture work, HGS101 was able to inhibit viral infection by maraviroc-resistant HIV-1, demonstrating the lack of overlapping patterns for resistance between HGS101 and maraviroc despite sharing the same protein target³⁷⁸. However, despite the lack of Fc-mediated effector functions and complement activation in IgG4 antibodies^{257,379}, HGS101 treatment in rhesus macaques led to decreases in the frequency and absolute count of CD4+CCR5+ and CD8+CCR5+ T cells found in the peripheral blood and lymph nodes³⁸⁰, raising concerns over the long-term use of HGS101. Leronlimab will be discussed in **Section 1.5 Anti-CCR5 antibody Leronlimab for HIV**.

1.4 CCR5

1.4.1 CCR5 functions

C-C chemokine receptor type 5 (CCR5) is part of a larger family of seven-transmembrane G protein-coupled receptors involved in cell signaling and migration. There are four superfamilies of human chemokines (C, CC, CXC, and CX3C) and they are grouped by the spacing of two to four cysteine residues found in the N-terminus, center, and C-terminus. There are at least 46 different chemokine ligands in humans that bind to over 18 different chemokine receptors³⁸¹.

CCR5 is found to be expressed primarily on lymphocytes, macrophages, dendritic cells, and natural killer cells, while also found on the epithelium, endothelium, vascular smooth muscle cells, fibroblasts, neurons, astrocytes, and microglia^{382,383}. Pro-inflammatory chemokines CCL3 (MIP-1 α), CCL4 (MIP-1 β), and CCL5 (RANTES) are major CCR5 ligands, as well as ligands MCP-1, MCP-2, MCP-3, and MCP-4. Upon ligand binding to extracellular domains of CCR5, G protein-coupled receptor kinases (GRKs) will phosphorylate CCR5, leading to the binding of beta-arrestin that initiates desensitization of the protein. Internalization of CCR5 will occur through clathrin-mediated endocytosis into clathrin-coated pits. Activated CCR5 will recruit and activate cytosolic proteins, including MAP kinase ERK1, leading to the formation of CCR5 endosomes that either traffick into lysosomes for degradation or recycle back to the cell surface³⁸⁴. CCR5 recycling is independent of protein synthesis, but is dependent on CCR5 dephosphorylation and desensitization within endosomes³⁸⁵.

The main role of CCR5 is to recruit immune cells to sites of inflammation through cell chemotaxis by ligand gradients. Indeed, the number of CCR5+ cells correlated to the severity of inflammation^{382,386}. This critical role in trafficking leukocytes to the sites of infection for viral

clearance is observed in West Nile virus³⁸⁷⁻³⁸⁹, Japanese encephalitis virus³⁹⁰, and influenza^{391,392}. In addition to serving as a chemokine receptor, the interactions of CCR5 and ligands CCL3, CCL4, and CCL5 have various immune activation roles. While CCR5 is not commonly expressed on naïve CD8+ T cells, it is transiently expressed after antigen exposure, resulting in the recruitment of naïve CD8+ T cells into inflammation sites where secreted CCL3 and CCL4 by antigen-specific DCs and CD4+ T cell complexes contribute to CD8+ T cell activation. Inhibition of CCR5 ligands can drastically reduce differentiation of memory CD8+ T cells^{393,394}. IL-12 produced by DCs in response to the parasitic infection of *Toxoplasma gondii* is CCR5 dependent, and the lack of CCR5 is correlated with diminished IL-12, IFN-gamma, and recruitment of CD8+ T cells, all of which are critically required for the control of parasitic infection³⁹⁵⁻³⁹⁷. The interaction of CCR5 and CCL5 inhibits virus-induced macrophage apoptosis, helping to maintain macrophage viability after influenza infection³⁹⁸. Finally, homo- or heterodimerization of CCR5 with CCR2 or CXCR4 results in the recruitment to sites of T cell and antigen-presenting cell (APC) interaction to assist in T cell costimulation³⁹⁹⁻⁴⁰¹.

While CCR5 is involved in many protective roles, there are also negative aspects. To start, the CCR5 receptor is an essential receptor for the infection of HIV^{402,403}, poxvirus⁴⁰⁴, dengue virus⁴⁰⁵, and *Staphylococcus aureus*⁴⁰⁶. Furthermore, CCR5 suppresses cortical plasticity, learning, memory, and recovery after traumatic brain injury⁴⁰⁷⁻⁴⁰⁹. This most likely occurs because CCR5 recruits pro-inflammatory leukocytes into the brain, exacerbating neuroinflammation. In fact, the use of the anti-CCR5 antagonist maraviroc in cognitively impaired HIV+ participants was associated with the decreased influx of monocytes, declined CD8+ T cell activation, and improved neuropsychological performance⁴¹⁰. Also, stroke and traumatic brain injury (TBI) mouse models showed greater improvement with maraviroc

treatment; comparison in a large cohort of stroke patients showed improved outcomes in patients lacking CCR5 expression through a loss-of-function deletion in the *ccr5* alleles⁴⁰⁸. Similarly, CCR5-mediated cellular recruitment is also involved in insulin resistance⁴¹¹ and breast cancer recurrence⁴¹². Moreover, high expression of CCR5 can contribute to cancer progression, tumorigenesis⁴¹³⁻⁴¹⁵, and reduce anti-cancer effects by Toll-like receptor 3 during chemotherapy⁴¹⁶. As expected, the use of anti-CCR5 agents reduced cancer metastasis and enhanced anti-cancer effects in DNA-damaging chemotherapy^{414,417,418}. In summary, CCR5 and its binding ligands are involved in many biological and pathophysiological processes; thereby, knowledge learned by targeting CCR5 for HIV treatment will have a diverse range of applications.

1.4.2 Role of CCR5 in HIV

CCR5 was reported in 1996 to be a critical coreceptor for HIV infection^{402,403}. Viral infection of CD4⁺ T cells was found to be inhibited by CCR5 ligands: macrophage inflammatory protein-1 α (MIP-1 α), macrophage inflammatory protein-1 β (MIP-1 β), and regulated-upon-activation normal T expressed and secreted (RANTES). This could be explained by ligand-mediated internalization of CCR5 receptors that decreased the numbers of cell surface CCR5 receptors for virions to dock and infect cells⁴¹⁹. Furthermore, cells co-transfected with cDNA for CD4 and CCR5 produced the highest infection level versus cDNA from other C-C chemokine receptors, demonstrating the critical role of CCR5 for HIV infection.

In vitro analysis of cells isolated from 25 individuals that remained HIV-free despite experiencing multiple exposures from HIV⁺ sexual partners showed higher resistance to infection than control cells. Protection was not correlated with the density of cell surface CD4⁺

receptors but with the concentration of CCR5 ligands (RANTES, MIP-1 α , and MIP-1 β), suggesting that CCR5 may be a reason for the observed protection. In particular, two individuals (EU2 and EU3) were completely protected from HIV infection during high dose viral exposure⁴²⁰. Liu et al. performed genomic analysis on these two individuals and found a common inherited homozygous defect in the *ccr5* allele that involved a 32 base pair deletion (CCR5 $\Delta 32/\Delta 32$). This deletion led to a frame shift that generated a premature stop codon that reduced the total protein length from 352 amino acids in wild type CCR5 to 215 amino acids in CCR5 $\Delta 32/\Delta 32$. The truncated CCR5 abolished all cell surface expression of the receptors. While activation of other chemokine receptors was unchanged, MIP-1 β mediated activation of CCR5 was completely absent. Furthermore, cells from individuals heterozygous for the allele could still be infected *in vitro* but showed lower HIV replication⁴²¹.

Samson et al. and Huang et al. independently reported on the complete absence of cell surface CCR5 receptors in individuals with CCR5 $\Delta 32/\Delta 32$ alleles in cohorts of 723 and 446 HIV+ Caucasian individuals, respectively. They found that the frequencies of CCR5^{WT/WT} and CCR5^{WT/ $\Delta 32$} alleles were 79.8-89.2% and 10.8-20.2%, respectively^{422,423}. Supporting previously described findings by Liu et al., the mean plasma viremia was higher in individuals with alleles CCR5^{WT/WT} (4.34 log RNA copies/mL) than CCR5^{WT/ $\Delta 32$} (3.95 log RNA copies/mL)⁴²³. Although heterozygous CCR5 $\Delta 32$ cannot fully protect from HIV infection, the deletion of one functional CCR5 allele significantly lowers the incidence of AIDS^{424,425}. Indeed, the density of CCR5 receptors on CD4+ T cells correlated to susceptibility in HIV infection and viral titers in HIV-infected individuals⁴²⁶⁻⁴²⁸.

The frequency of homozygous $\Delta 32$ carriers is approximately 10% for European descent Caucasian populations and essentially nonexistent in non-European populations, including

Asians, and Blacks^{422,423,429}. Within European countries, $\Delta 32$ frequency declines from Northern to Southern countries such that Norway and Greece show allele frequencies of 16.4% and 5.2%, respectively⁴³⁰. The mechanism behind the geographical gradient is explained through two plausible hypotheses. 1) The Viking hypothesis proposed that the variants originated from the Vikings many years ago and spread to Southern countries during the Vikings expansion in the eighth-tenth century CE⁴³¹. This was supported by the high $\Delta 32$ frequencies (~14%) found in known Viking territories in Northern Europe and the estimated age of 700 to 3,500 years for homozygous $\Delta 32$ allele^{432,433}. 2) A second plausible explanation was the biological advantage for selecting $\Delta 32$ carriers over noncarriers. Because the HIV pandemic only occurred in recent history, selection pressure to produce ~10% homozygous $\Delta 32$ frequency likely arose from other pathogen(s). While the waves of bubonic plague (*Yersinia pestis*, carried by rats) were likely bottleneck events, evidence suggests that smallpox was the most likely selecting pathogen. First, smallpox disproportionately affected children under 10 year old while the plague affected all ages, thereby removing the reproductive potential of smallpox-infected people over plague-infected people. Furthermore, the plague only affected Europe for approximately 400 years and was virtually gone after 1750, while smallpox infections continuously occurred in Europe (and in the Americas) for over 2,000 years until the virus was eradicated in the late 1980s. The longer duration of infection in and reduced reproductive potential of smallpox-infected individuals was modeled to produce the ~10% homozygous $\Delta 32$ frequency we observe today. In contrast, the same modeling produced an estimated homozygous $\Delta 32$ frequency of ~0.8% if caused by the plague. Next, geographically, cases of smallpox were more common in Northern Europe than the plague that mainly affected Central Europe. This was in line with the higher homozygous $\Delta 32$ frequency in Northern European countries. Lastly, poxviruses, grouped in the same family as

smallpox, use CCR5 for viral infection of leukocytes. Furthermore, the infection is blocked by using CCL5 and anti-CCR5 polyclonal antibodies, showing a similar mechanism of infection to HIV^{404,434}. In summary, while common pathogens like the plague, measles, chickenpox, and influenza could have produced some selection pressure during short periods in history, the smallpox epidemic was the most likely selection pressure to produce the homozygous $\Delta 32$ frequency that we observe in the current time.

Using the knowledge of natural resistance conferred by homozygous $\Delta 32$, Dr Geo Hutter decided to give a patient who had acute myeloid leukemia and was HIV+, an allogeneic stem cell transplantation from a homozygous $\Delta 32$ donor. That patient was first identified as the "Berlin patient" and later went public as Timothy Ray Brown. After initial treatment with chemotherapy failed and the leukemia relapsed months later, Mr Brown received hematopoietic stem cell transplantation (HSCT) using allogeneic stem cells from an HLA matched donor. Luckily at the time, Mr Brown was living in Germany, which had a large registry for stem cell donors and high homozygous $\Delta 32$ frequency individuals in Northern Europe, and thus, was able to find over 200 potential donors. Of them, Dr Hutter picked a homozygous $\Delta 32$ donor due to the natural resistance to HIV to treat both cancer and HIV. ART cessation occurred one day before HSCT, and to their hypothesis, ARV drugs were not required afterwards, and Mr Brown remained HIV free for over a decade. T cells isolated from Mr Brown lacked anti-HIV responses after HSCT, suggesting that HIV antigens were not present after HSCT to establish new responses in the grafted donor cells. Anti-HIV antibodies were still detectable, likely due to existing plasma cells that commonly persist after HSCT. Most importantly, the use of RNA and proviral DNA PCR assays could not detect HIV genomes in the peripheral blood, bone marrow, or rectal mucosa²⁷⁴, demonstrating that Mr Brown had achieved a sterilizing cure for HIV.

Sequencing of the viral pool before HSCT showed a small pool of CXCR4-tropic viruses (2.9%); however, an outgrowth of CXCR4-tropic viruses was not observed despite him showing normal levels of CXCR4⁺ T cells after HSCT²⁷⁴. To investigate this observation, another group generated the predicted CXCR4-tropic variants and a predicted dominant CCR5-tropic strain from Mr Brown. Using CCR5⁺ only and CXCR4⁺ only cell lines, they found that both predicted CXCR4- and CCR5-tropic strains could only infect the CCR5⁺ cell line and not the CXCR4⁺ cell line. Next, they used the predicted strains to infect PBMCs in the presence of the CCR5 blocker maraviroc or the CXCR4 blocker AMD-3100. They found that maraviroc could block viral replication of the CCR5-tropic strain while AMD-3100 was unable to block viral replications of the CXCR4-tropic strains. Next, they isolated post-HSCT PBMCs from Mr Brown and showed that both patient-derived CCR5- and CXCR4-tropic strains and a control CCR5 strain could not infect these CCR5^{Δ32/Δ32} cells, while only a control CXCR4-tropic strain could do so. Finally, they incubated the patient-derived CXCR4- and CCR5-tropic strains in increasing concentrations of CXCR4⁺ cells to apply selection pressure for CXCR4-tropic infection; however, it did not occur even after 10 weeks in culture, demonstrating the high biological barrier for using CXCR4 in viral infection. Collectively, it showed the dependence of the CCR5 receptor for viral replication despite displaying mutations common for CXCR4-tropism and explained the lack of viral rebound after HSCT with homozygous Δ32 donor cells⁴³⁵.

In agreement, a second patient was recently reported to achieve a similar sterilizing cure after HSCT with homozygous Δ32 donor cells^{278,279}, further emphasizing the protective role of homozygous Δ32 against predominately CCR5-tropic viral pools. In contrast, studies that attempted HSCT using wild type CCR5 donor cells resulted in viral rebound^{276,277,436}. In particular, two HIV⁺ cancer patients underwent HSCT with wild-type CCR5 donor cells. They

experienced viral rebound at 12 and 32 weeks after ART interruption despite showing a mean 3- $\log_{10}$ reduction in reservoir size after HSCT²⁷⁷.

In summary, the primary use of the CCR5 receptor for HIV infection, natural resistance from a lack of cell surface expression of CCR5 conferred by homozygous $\Delta 32$ alleles, and the involvement of CCR5 in the sterilizing cure in two individuals all support the targeting of CCR5 receptors for anti-HIV strategies.

1.4.3 Anti-CCR5 strategies

Maraviroc is the only anti-CCR5 drug approved by the FDA for HIV treatment. The small-molecule acts as an allosteric modulator by binding to hydrophobic regions on CCR5 to cause structural changes that ultimately blocks HIV binding. In rhesus macaque models, gel-formulated maraviroc protected against vaginal challenges in a dose-dependent and time-dependent manner: the higher concentration (6 nM) and the shorter period between drug application and SHIV viral challenge (30 minutes) protected approximately 20% of the macaques⁴³⁷. However, in a second macaque study, a once-daily oral regimen did not protect macaques against SHIV rectal challenges despite showing high drug concentration in the rectal secretion and tissue. The half-life of CCR5 receptor occupancy by maraviroc was 2.6 days, which supported the daily regimen. Yet, 83% of the maraviroc treated (5/6) and 75% of the control untreated macaques (3/4) were infected after the fifth rectal challenge⁴³⁸. Similarly, in an infant macaque model for mother-to-offspring transmission, repeated oral SIV challenges successfully infected 100% of untreated infant macaques and 60% of maraviroc-treated infant macaques ($p=0.15$), showing a lack of significant protection by maraviroc despite full saturation of CD4⁺ T cells⁴³⁹.

One potential explanation for the lack of drug efficacy is the development of viral mutations that result in resistance against maraviroc. Indeed, maraviroc-resistant variants can utilize maraviroc-bound CCR5 receptors for successful cell entry⁴⁴⁰⁻⁴⁴². Thus, similar to other ARV drugs, maraviroc use should be combined with other ARV drug classes to minimize drug resistance. Maraviroc used as a combinational therapy was conducted in two phase-3 double-blind, placebo-controlled clinical studies. Eligible participants had used or developed resistance against three classes of ARV drugs and had plasma viral loads of more than 5,000 copies/mL. Participants were given a combination treatment of one optimized ARV drug and maraviroc (once-daily or twice-daily) or placebo. After 48 weeks of treatment, participants in the two studies showed a reduced average plasma viral of 1.69, 1.85, and 0.78 log₁₀ copies/mL for once-daily, twice-daily, and placebo-treated groups, respectively. More participants in the maraviroc-treated group had viral titers less than 50 copies/mL than in the placebo group (45% vs 17%). Also, CD4⁺ T cell counts were higher in the maraviroc group than in the placebo group. Viral sensitivity to maraviroc during long-term use was maintained when paired with another ARV drug class with different action mechanisms.

As described earlier, natural chemokines to CCR5 can block HIV infection and this phenomenon was vital for discovering CCR5 as an HIV coreceptor. However, given the short ~10 minutes half-life of chemokines and their pro-inflammatory role, modifications would need to be made for chemokines to be viable therapeutics. One such engineered chemokine is PSC-RANTES, which is modified in the N-terminus that allows for potent inhibition and long-term internalization of CCR5 receptors⁴⁴³. In rhesus macaque models, a single injection of PSC-RANTES provided full protection against SHIV viral challenge in a dose-dependent manner⁴⁴⁴. As well, topical intravaginal treatments with RANTES analogs (PSC, 5P12, and 6P4) protected

rhesus macaques against a single SHIV viral challenge and maintained aviremic for ten weeks⁴⁴⁵. However, CCR5 activation by analog binding can induce inflammation⁴⁴⁴, leading to a potential risk for using chemokine analogs for HIV.

Individuals who are HIV infected or exposed seronegative (ESN) can develop natural anti-CCR5 antibodies. Auto- and monoclonal antibodies (a detailed discussion on monoclonal antibodies can be found in Sections: **1.3 Antibodies for HIV** and **1.5 Anti-CCR5 antibody Leronlimab for HIV**) can recognize different regions of CCR5 to block HIV entry or lead to receptor internalization. Antibodies against CCR5 mainly bind to the N-terminus and extracellular loop 2 (ECL2) of CCR5. HIV binds directly to the N-terminus of CCR5 while ECL2 is important for conformational changes of the HIV envelope for membrane fusion⁴⁴⁶. Hence, anti-CCR5 antibodies can block HIV entry by direct competition to binding epitopes or by introducing steric hindrance to prevent membrane fusion.

Approximately 66-83% of HIV+ mothers have breast milk containing anti-CCR5 antibodies. These antibodies are predominately IgA and IgG, and bind to the ECL2 of CCR5. Indeed, antibody treatment blocked *in vitro* infections of macrophages and dendritic cells in a dose-dependent manner⁴⁴⁷, potentially reducing the risk for mother-to-offspring transmission.

In ESN individuals, anti-CCR5 antibodies recognize ECL2 from CCR5+ cells isolated from the serum^{448,449}, saliva⁴⁴⁹, and genital fluids⁴⁴⁹. Especially, antibodies found in saliva and genital fluids were IgA class, providing a mechanism of protection against the most common route of HIV transmission in the mucosal layers. Furthermore, these antibodies were found to downregulate the expression of cell surface CCR5 on T cells and protect against *in vitro* CCR5-tropic HIV infection⁴⁴⁸. Interestingly, anti-CCR5 antibodies found in 23.5% of long-term non-progressing HIV+ individuals (LTNP) recognize ECL1 of CCR5. While binding to CCR5 N-

terminus or ECL2 could directly block HIV cell entry, the binding of CCR5 ECL1 resulted in long-lasting CCR5 internalization, sequentially decreasing the numbers of CCR5 molecules on CD4⁺ T cells. As expected, these antibodies protected against CCR5-tropic HIV infection and not CXCR4-tropic infections. Furthermore, follow-up timepoints from LTNP participants showed that a loss of anti-CCR5 antibodies was correlated with increased plasma viremia, loss of LTNP status, and requirement for ARV drug use. In contrast, LTNP participants that retained anti-CCR5 antibodies maintained their non-progressing status⁴⁵⁰. In this study, anti-CCR5 antibodies were detected in only ESN (n=128) and LTNP (n=87) individuals while absent in people who were healthy and unexposed (n=500), HIV⁺ on ART (n=135), and HIV⁺ progressors (n=75), showing the role of natural anti-CCR5 antibodies in preventing and controlling HIV.

To provide long-lasting anti-CCR5 strategies, two groups attempted to ablate CCR5 receptors by genetic excision from CD4⁺ T cells in HIV⁺ individuals. Xu et al. reported a case study of an HIV⁺ individual with acute lymphoblastic leukemia. The *ccr5* gene in donor cells was genetically modified by CRISPR-based editing. Approximately 28.8% of the donor CD34⁺ stem cells infused into patients were CCR5-modified to lack CCR5 expression. Infusion of CRISPR-edited cells was safe, and patients achieved full donor chimerism four weeks after transplantation. In the bone marrow, peripheral blood, and circulating CD4⁺ T cells, the percentages of CCR5-modified cells were maintained at approximately 6%, 5%, and 2%, respectively, for at least 19 months after transplantation. Viral load increased during ART interruption at days 221-249 after transplantation. Importantly, the percentage of CCR5 modified CD4⁺ T cells peaked during this period despite increasing plasma viremia and declining cell count, suggesting that CCR5-modified cells were protected from viral infection⁴⁵¹. In another study, Tebas et al. used zinc-finger nucleases (ZFN) to genetically edit the *ccr5* gene in

autologous CD4⁺ T cells of aviremic HIV⁺ individuals (n=12). CD4⁺ enriched T cells that were CCR5-modified at a rate of 11-28% were infused into the individuals without any chemotherapeutic regimens. Infusion of ZNF-modified autologous CD4⁺ T cells was relatively safe, with only one serious adverse event report. At one-week post-infusion, CCR5-modified cells were found at a frequency of 8.8% and 13.9% in the peripheral blood and circulating CD4⁺ T cells, respectively. Because chemotherapeutic regimens were not used to clear unedited cells, the median count of circulating CCR5-modified CD4⁺ T cells declined with time. Similar to the CRISPR-edited case study, viremia rebounded in all patients after treatment interruption. As well, decline to circulating CCR5-modified cells (-1.81 cells per day) occurred less than unmodified cells (-7.25 cells per day), demonstrating the possible protection of CCR5-modified cells from viral replication⁴⁵². CCR5-modified cells could not confer full HIV protection seen in homozygous $\Delta 32$ carriers most plausibly due to incomplete editing of the CD34⁺ and CD4⁺ cell populations; thereby, unmodified cells were still susceptible to viral infection. However, the observed protection of CCR5-modified cells during treatment interruption provides proof-of-concept for the genetic editing of the *ccr5* gene.

The majority of the anti-CCR5 strategies were safe and well-tolerated, even in humans who received genetically edited cells. Furthermore, in a study of 409,693 individuals from the UK Biobank, they found no statistical association between homozygous $\Delta 32$ allele and lifespan. Interestingly, the group did find statistical significance in categories under “certain infectious and parasitic infection,” with p-values of 0.00447 and 0.0057⁴⁵³. This is highly likely due to the previously described roles by CCR5 to control and prevent viral and parasitic infections. Given the overall safety of targeting CCR5 and the critical role of CCR5 for HIV infection, CCR5 is a viable target for HIV therapeutics.

1.5 Anti-CCR5 antibody Leronlimab for HIV

1.5.1 Discovery and characterization

Leronlimab (formerly PRO 140 and PA14) is a humanized IgG4 monoclonal antibody that targets CCR5. It was produced and characterized by Olson et al. in 1999. The clone was produced by immunizing BALB/c mice with murine L1.2-CCR5⁺ cells before splenectomy to produce hybridomas when fused with Sp2/0 cell lines. Supernatants were screened for their ability to inhibit HIV ENV JR-FL fusion and binding to L1.2-CCR5⁺ cells rather than the parental L1.2 cell. Six clones were found to be of interest: PA8, PA9, PA10, PA11, PA12, and PA14 (Leronlimab). While all clones were found by flow cytometry to bind to L1.2-CCR5⁺ T cells, no detection was found when using reduced and denatured CCR5 protein, indicating that binding to CCR5 required conformational epitopes rather than linear epitopes. Next, the group developed 70 alanine point mutations spanning *ccr5* to scan for binding epitopes. HeLa cells expressing these mutated CCR5 proteins were incubated with the six anti-CCR5 clones and the investigators found that binding of Leronlimab required residues in the N-terminus and extracellular loop 2 (ECL2): D2, R168 and Y176. Interestingly, a Leronlimab concentration above approximately 10 µg/mL inhibited calcium mobilization that would normally be induced by natural CCR5 ligands RANTES, MIP-1 α , and MIP-1 β , while the IC₅₀ was 45 µg/mL, serving as a CCR5 antagonist during high Leronlimab concentrations. However, Leronlimab was the most potent of all tested antibodies to inhibit HIV-1 envelope membrane fusion and entry of CD4⁺ T cells. At the same time, it was less efficient in blocking the interaction between gp120 and CCR5⁴⁵⁴. In a time-course inhibition experiment involving SCH-C (anti-CCR5 small molecule), Leronlimab, and T20 (inhibitor of gp41-mediated fusion), Safarian et al. found that the time to achieve half-maximal inhibition by Leronlimab (32 minutes) was much closer to T20

(46 minutes) than SCH-C (9 minutes), indicating that Leronlimab acted towards the end of the HIV-1 entry process⁴⁵⁵.

Using strains with different residues in the V3 loop, Safarian et al. found that the half-maximal inhibition concentrations varied with small molecules but was unchanged for Leronlimab, suggesting that Leronlimab acted independently of the V3 loop sequence. This was in agreement with the findings from Olson et al., where Leronlimab was found to be least efficient in inhibiting the interactions of gp120 and CCR5. As the V3 loop is the most diverse region in gp120, leading to positive selection for drug-resistant strains, the use of Leronlimab could protect against resistance as long as the virus maintains CCR5-utilization⁴⁵⁵. Next, Trkola et al.⁴⁵⁶ used PBMC cultures to show that Leronlimab inhibition was subtype-independent when tested against 17 different R5 viruses from subtypes A, B, C, E, and F. The median IC₉₀ from different subtypes was 2.2 µg/mL. Interestingly, HIV-1_{SF162} from subtype B required the highest Leronlimab concentration (14.6 µg/mL) compared to all tested viral strains. Most importantly, a range of Leronlimab concentrations (0.5 – 25 µg/mL) tested against CXCR4 subtype B viruses neither decreased nor increased viral replication in PBMC cultures. Moreover, they found that Leronlimab could inhibit viral replication in macrophages but required a higher concentration of 16 µg/mL than PBMCs.

1.5.2 Current findings for HIV treatment

Leronlimab has been used in over 1,000 participants and involved in over 20 different clinical trials for HIV, COVID-19, breast cancer, solid tumor, nonalcoholic steatohepatitis (NASH), and graft vs host disease.

In a phase 1b (ISRCTN45537485)⁴⁵⁷ randomized and double-blinded trial to measure the potency of Leronlimab in HIV+ participants, nine participants received placebo treatment and 30 participants received a single IV infusion of 0.5, 2, or 5 mg/kg Leronlimab. Eligible participants harbored only CCR5-tropic viruses, with plasma HIV-1 RNA levels above 5,000 copies/mL, CD4+ T cell counts above 250 cells/ μ L, and received no ART for at least three months prior to treatment.

After a single IV infusion, the average plasma Leronlimab concentrations peaked at 13, 61, and 173 μ g/mL for the 0.5, 2, and 5 mg/kg treated participants, respectively. Afterwards, plasma concentration gradually declined until it was last detected at mean study timepoints of 6.1, 18.2, and 18.9 days for the 0.5, 2, and 5 mg/kg treated participants, respectively. Next, they found that CD4+ T cell counts increased only in the 5 mg/kg treated group ($P=0.55$) compared to baseline, with no changes in the 0.5 and 2 mg/kg treated groups. As well, they observed no depletion of CCR5+ cells in all groups.

The antiviral effects of Leronlimab were dose-dependent and statistical significance was achieved at multiple study timepoints for 2 and 5 mg/kg Leronlimab infused participants, where the mean $\pm$ SD nadir HIV-1 RNA copies in the plasma was reduced by 1.20 ± 0.63 and 1.83 ± 0.41 log₁₀, respectively, when compared to placebo controls (Figure 1.3). On study day 29 post-Leronlimab infusions, the plasma viral loads for all participants returned to baseline values.

All participants in the placebo and Leronlimab-treated groups, excluding one participant in the 0.5 mg/kg treated group, reported at least one adverse event during the course of the study, including headache (31%), lymphadenopathy (28%), and diarrhea and fatigue (21%). One participant in the 5 mg/kg treated group reported symptoms that were consistent with those who received immunoglobulin treatments by IV infusions. Aside from that, no drug-related adverse

events (AEs) or dose-limiting toxicities were reported. Common laboratory analysis to monitor liver and renal functions, serum chemistry, and hematology did not show abnormalities. Therefore, Leronlimab given as IV infusions was well tolerated by participants.

Participants were screened and confirmed to harbor R5-only tropic viruses during all study timepoints except for two individuals. Of the 30 participants who received Leronlimab, only one participant from the 0.5 mg/kg treated group (3.3%) showed dual-tropism at a single timepoint on day 8, which converted back to R5-only tropism for the rest of the study. In contrast, one participant from the placebo group (11%) switched from R5-only to dual tropism for the rest of the study. Next, *in vitro* analyses to measure viral susceptibility to Leronlimab showed no noticeable change in EC₅₀ between the start and end timepoints. Collectively, it suggested that the monotherapy use of an anti-CCR5 therapeutic did not apply selection pressure for non-CCR5 tropism viruses to arise nor changed viral susceptibility to Leronlimab compared to no treatment. Leronlimab was clinically tolerated and did not deplete CCR5⁺ cells in the participants. Overall, this study provided proof of concept for the use of Leronlimab for HIV treatment.

As a follow up to this study, Jacobson et al. conducted a phase 2a clinical trial to test a higher dose of Leronlimab. A total of 31 participants received either a single placebo (n=11), 5, or 10 mg/kg IV infusion of Leronlimab (n=10 per dose). Interestingly, 5 and 10 mg/kg Leronlimab doses achieved the same mean maximum reduction in viral loads at 1.83 log₁₀ from baseline. As well, both doses were statistically significant ($P < 0.0001$) when compared to placebo controls until study day 29, suggesting that monthly Leronlimab treatments may be a good therapeutic regimen. Similar to the previous study, no serious AEs or dose-limiting toxicities were reported. Minor AEs, including headache and nasal congestion, were reported in

all 11 placebo participants (100%) and 15 participants receiving Leronlimab-treated participants (75%). All participants also maintained R5-tropic viruses post Leronlimab treatment, demonstrating the high barrier to resistance⁴⁵⁸.

Although IV infusions provide immediate drug effects, subcutaneous (SC) injections are preferred in most cases due to reduced needs for health care staff, the ability to self-administer at home, and decreased discomfort at the injection site, which collectively may increase patient drug adherence⁴⁵⁹. To this end, Jacobson et al. conducted a phase 2a study⁴⁶⁰ to measure the potency of Leronlimab given by SC injections. Forty-four participants were injected with weekly placebo, weekly 162 mg, biweekly 324 mg, or weekly 324 mg Leronlimab for three consecutive weeks (n=11 per group). Nadir viral load was reduced by mean log₁₀ of 0.23, 0.99, 1.37, and 1.65 by ascending Leronlimab doses compared to baseline. Compared to placebo controls, viremia reduction in all three Leronlimab treated groups were statistically significant. A 324 mg Leronlimab dose is equivalent to approximately 4 mg/kg when assuming an average participant weight of 80 kg, which would explain the lower mean viremia reduction in the 324 mg treated group than 5 mg/kg treated group from prior trials^{457,458}. Importantly, no serious AEs were reported, and the frequencies of injection site-related AEs were similar between placebo and Leronlimab treated groups, indicating the safe use of Leronlimab by SC injections.

Finally, Dhody et al. conducted a phase 2b study to evaluate the long-term efficacy, safety, and tolerability of Leronlimab as a single-agent for virologic maintenance for HIV+ participants after ART cessation⁴⁶¹. In this single-group, open-label study, 41 participants switched from their daily ART regimens to receive weekly Leronlimab SC injections of 350 mg or 4.375 mg/kg when assuming an average participant weight of 80 kg. At the end of the 12-week study, Leronlimab contributed to the virologic maintenance for 23 participants (56.1%),

where their plasma viremia was below 40 copies/mL. At the time of publication, nine participants elected to continue self-administered single-agent Leronlimab for over two years, where tropism screening continued to detect only R5-tropism and viral susceptibility to Leronlimab measured by IC₅₀ and IC₉₀ did not significantly change between pre- and post-Leronlimab treatment. While the tropism maintained R5 in the rebound individuals, the IC₉₀ by rebound and non-rebound participants were 10.8 ± 9.28 and 6.7 ± 6.8 , respectively, suggesting viral resistance to Leronlimab was higher in the rebound group despite maintaining only R5-tropic. The mechanism that led to viral rebound is unknown, but the multi-year use of Leronlimab supports its efficacy, safety, and tolerability and prompted additional pre-clinical and clinical studies.

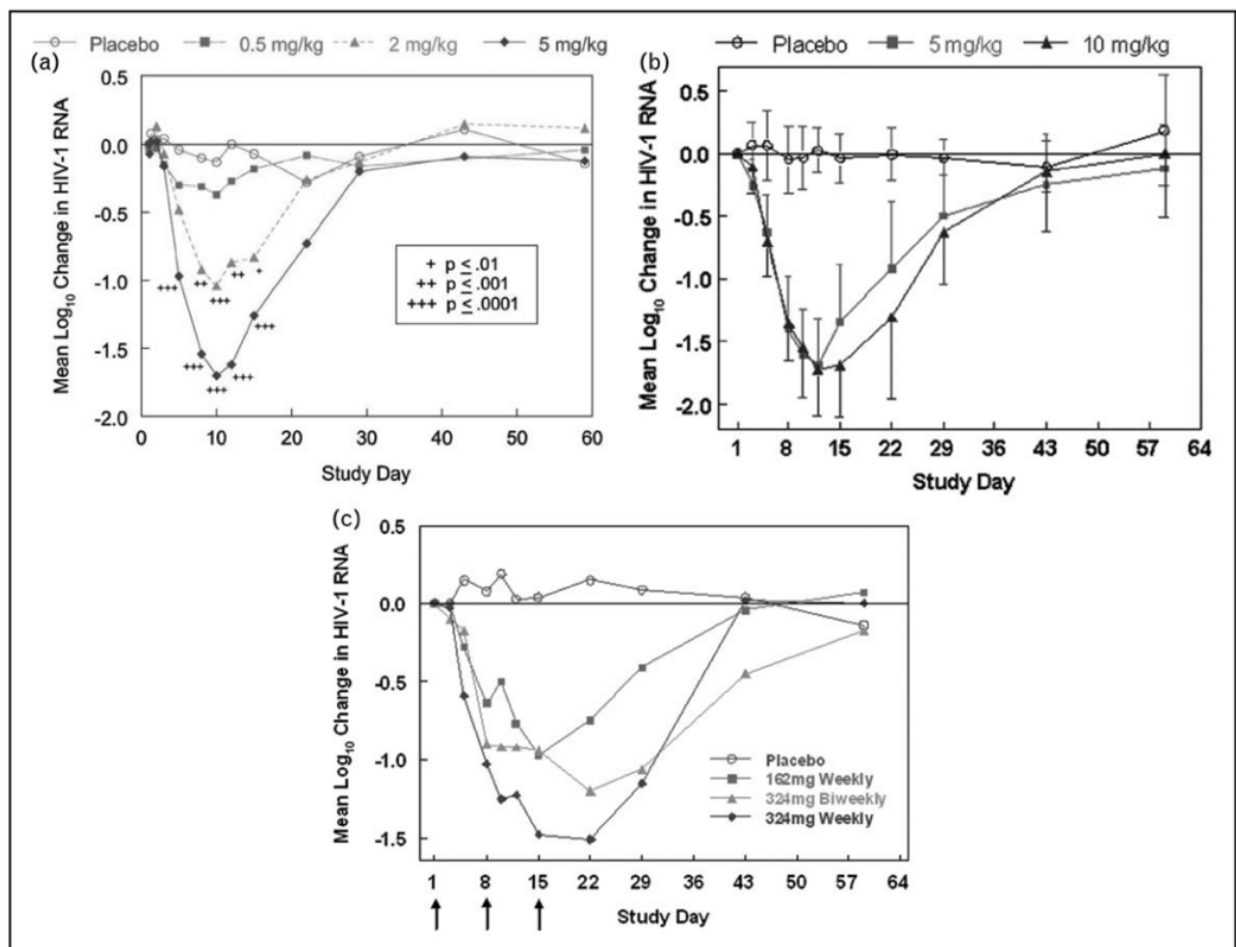


Figure 1.3 Mean log₁₀ change in HIV-1 RNA levels in the plasma compared to baseline. Human participants received placebo or Leronlimab by (a-b)^{457,458} IV infusions or (c)⁴⁶⁰ SC infusions. P values in panel a generated from two-sided t-tests compared to placebo controls. Arrows in panel c indicated Leronlimab dosing. Figure from Thompson, 2018, Curr Opin HIV AIDS.

1.6 Nonhuman primate models for HIV infection

1.6.1 Natural and non-natural SIV hosts

Animal models are important for developing therapies and vaccine candidates; however, finding animal models that can recapitulate the varying aspects of HIV in humans is challenging. While HIV-1 originated from SIVcpz in chimpanzees and HIV-1 infection can proceed in chimpanzee⁴⁶², the model was not suitable to vaccine studies due to severe limitations of numbers, leading to the use of *Macaca species* as alternatives. Ethical, cost, and regulatory reasons led the National Institutes of Health to restrict the use of chimpanzees for biomedical research in 2015, following a study by the Institute of Medicine of the National Academies⁴⁶³.

Previously, we described four key host restriction factors that restrict HIV-1 infection in humans. While HIV-1 viral proteins evolve to combat these restriction factors to allow for HIV-1 replication in humans, this is not the case in other species due to the species-specific activities of these restriction factors. For example, the activity of HIV-1 Vif against APOBEC3 is species-specific. APOBEC3 proteins from mice and NHPs (rhesus macaques and African green monkey) are resistant to HIV-1 Vif and can block HIV-1 replication in NHPs. One exception is chimpanzees, where the APOBEC3 sequences in humans and chimpanzees only differ by 5%⁴⁶⁴. Similarly, NHP TRIM5 α restricts HIV-1 replication in NHPs⁴⁶⁵. SIV Vpx can tag SAMHD1 for proteasomal degradation and allow SIV replication to proceed⁴⁶⁶. However, HIV-1 does not express Vpx and cannot overcome the NHP SAMHD1 restriction on HIV-1 replication. Lastly, HIV-1 viral proteins Vpu, Nef, and Env that normally inhibit human Tetherin will not be

effective against Tetherin from NHPs. Collectively, it is difficult for HIV-1 to replicate and cause normal disease pathogenesis when infecting nonhuman and non-chimpanzee hosts.

Many African nonhuman primate species are natural hosts to SIV due to years of co-evolution between the host and the virus. Of those, the sooty mangabeys (SMs) contain the SIV_{smm} strain that gave rise to HIV-2 and SIV_{mac}, while chimpanzees contain SIV_{cpz} that gave rise to the predominant HIV-1 group M. Studies in natural hosts of SIV found many similarities between pathogenic HIV and SIV infection, including high viremia⁴⁶⁷⁻⁴⁶⁹, rapid turnover of infected cells⁴⁷⁰, depletion of mucosal CD4⁺ T cells⁴⁷¹ and activation of the immune system during acute infection^{467,468}, and weak cellular immunity for AIDS resistance⁴⁷². In contrast to a pathogenic infection, non-pathogenic infection in a natural host results in attenuated chronic immune activation that protects against AIDS development^{467,468}. The low levels of chronic immune activation are associated with healthy natural hosts despite chronic periods of high viremia⁴⁶⁹. Additionally, natural hosts maintain healthy T cell homeostasis⁴⁷³ and restore intestinal CD4⁺ T cells that were depleted during early infection⁴⁷⁴. More importantly, SIV infections do not increase lymphocyte death but, interestingly, increase CD4⁺ T cell proliferation^{9,30} and preserve lymphatic tissue structures^{467,468}. CCR5 expression on all subsets of T cells is much lower in natural hosts than non-natural hosts, with lower CD4⁺CCR5⁺ central memory T cells (T_{cm}) than effector memory T cells (T_{em})⁴⁷⁵. As a result, T_{cm} are partially resistant to SIV infection compared to T_{em}, allowing for the preservation of the T_{cm} pool and stabilization of CD4⁺ T cell levels that are not observed in a pathogenic infection by non-natural hosts. Importantly, the preservation of T_{cm} pool is associated with overall survival and lack of AIDS progression^{476,477}, which may explain the lack of AIDS development in natural hosts.

In contrast to African monkeys, Asian macaques are non-natural hosts to SIV that can provide useful animal models to study HIV infection and therapeutics. Asian macaques that are commonly used in studying SIV infection include rhesus macaques (*Macaca mulatta*; RM), pig-tailed macaques (*Macaca nemestrina*; PTM), cynomolgus macaques (*Macaca fasciculari*; CM), and Mauritian cynomolgus macaques (MCM). Differences in their genetic composition, geographic origin, and fitness towards SIV strains can affect their disease pathogenesis and make them suitable for addressing various scientific questions. RM and PTM are commonly used as models for AIDS because of rapid AIDS development in 1-2 years and 42 weeks, respectively^{478,479}. Furthermore, MHC alleles in RM are highly variable, unlike in humans. While humans have three single polymorphic loci for MHC class I (HLA-A, HLA-B, and HLA-C), RM have two highly variable loci (Mamu-A and Mamu-B) with over 1,200 alleles^{480,481}. Of interest, MHC class I alleles Mamu-A1*001, Mamu-B*008, and Mamu-B*017 are protective alleles that reduce and control viremia in RM⁴⁸²⁻⁴⁸⁴. The high complexity in MHC alleles in RM and the downstream effects on T cell recognition may be a confounding factor in designing experimental groups, especially with the inability to characterize these alleles fully. In contrast, the diversity of MHC alleles in MCM is limited due to geographic isolation and evolutionarily descent from a single female⁴⁸⁵. The numbers of MHC I and II genes in MCM are similar to that in humans⁴⁸⁶. As a result, it is possible to have full MHC-matched experimental groups in studies where T cell responses are important readouts. However, MCMs show attenuated viremia and a higher frequency of natural control than RM and PTM when infected with the same SIV strain⁴⁷⁹, further highlighting the important considerations for choosing an animal model based on the scientific question.

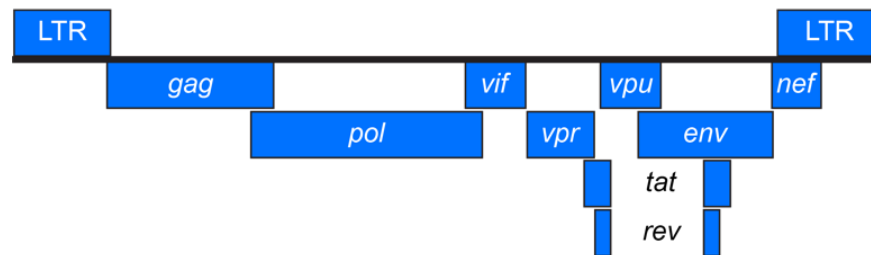
Because RMs are non-natural hosts of SIV, While RMs are non-natural hosts for HIV, a study at the California National Primate Research Center to generate a prion disease model in both RMs and SMs unexpectedly emerged an infectious SIV strain that could infect RMs⁴⁸⁷⁻⁴⁸⁹. The SMs used in the study were natural hosts of SIV, and many were imported directly from Africa, thereby, harboring SIV infections. As SIV was not a pathogen of interest at that time, detection of SIV infection was excluded in the screening process for imported animals. Later genomic sequencing found that many imported SMs were infected with the same strain of SIV_{smm}, and cross-species transmission likely occurred when RMs were inoculated with blood from SIV-infected SM during attempts to generate prion disease in over 1500 monkeys. Many rounds of serial passaging in non-natural hosts likely contributed to the high pathogenic and fitness of the SIV_{mac239} strain commonly used to infect non-natural host animal models.

1.6.2 SIV and SHIV

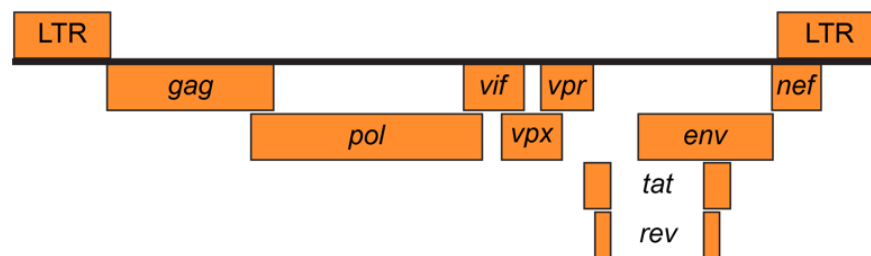
SIV_{mac239} and HIV-1 use CCR5 coreceptors to infect CD4⁺ Tcm cells and generate pathogenesis, but they differ in many critical ways. SIV_{mac239} does not have the *vpu* gene that is present in HIV-1 while it has a *vpx* gene that is not present in HIV-1, which can suppress the activity of macaque SAMHD1. HIV-1 drugs that target varying viral proteins, including reverse transcriptase, integrase, and protease, cannot be studied for anti-SIV activity due to differences in HIV and SIV genes. Furthermore, while HIV-1 and SIV_{mac239} predominately use CCR5 for cell entry, SIV_{mac239} can also use alternate receptors, including CCR2b and STRL33^{490,491}, making it difficult to study HIV-1 entry inhibitors in macaques. Importantly, HIV-1 vaccines that aim to elicit antibody responses or antibody-based agents that target the HIV-1 capsid envelope cannot be studied when using SIV_{mac239} due to differences in the *env* genes. To combat this

problem, chimeric simian/human immunodeficiency virus (SHIV) was created by replacing backbone SIVmac239 *env*, *tat*, and *rev* genes with genes (*vpu*, *env*, *tat*, and *rev*) from a particular HIV-1 isolate of interest (Figure 1.4). *In vivo* serial passages of SHIVs in non-natural hosts help to improve infectability and disease pathogenesis. For example, viremia in a SHIV_{SF16P3} infected RM increased by two-logs in the third and fourth *in vivo* passages through SHIV_{SF16P3}-infected blood and bone marrows⁴⁹².

HIV-1



SIVsmm and SIVmac



Env-SHIV

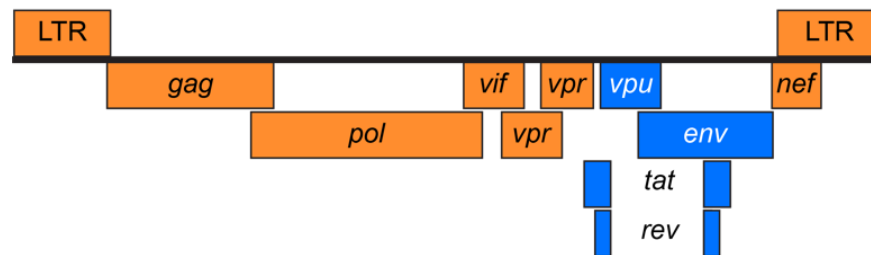


Figure 1.4 Schematic presentation of the genes in HIV-1, SIVsmm, SIVmac, Env-SHIV, and RT-SHIV. Viral genes are shown for HIV-1 (top), SIV_{smm} and SIV_{mac} (middle) and Env-SHIV (bottom). Red colored genes are specific for HIV-1 while blue colored genes are specific for SIV. Figure adapted from Hatzioannou and Evans, 2015 *Nat Rev Microbiol*.

Many SHIV strains have been created using varying HIV-1 *env* genes, but they differ in the disease course and receptor use from their original SIV strains. While SHIV_{HXBc2} replicated in macaque PBMCs *in vitro*, it showed low viremia following rhesus macaque inoculation. In contrast, SHIV_{89.6} replicated poorly in macaque PBMCs *in vitro* but generated high viremia when used to challenge rhesus macaques⁴⁹³. Furthermore, SHIV can evolve to use other receptors, including CXCR4 used by SHIV_{HH12R} and SHIV_{89.6}. Viral challenges by both strains

led to rapid depletion of CXCR4⁺ naïve CD4⁺ T cells rather than the CCR5⁺ memory CD4⁺ T cells infected by the parental SIV strains⁴⁹⁴. The change in cell type targets and shortened timeframe for AIDS-like pathogenesis in CXCR4-utilizing SHIV versus the original CCR5-utilizing SIV may confound experimental designs.

CCR5-utilizing SHIV_{SF16P3} and other CCR5-tropic SHIVs have similar disease courses and infect the same T cell subsets as HIV-1. However, CCR5-tropic SHIV infections in macaques can lead to coreceptor switch to CXCR4-tropic, where the evolved viruses can cause higher viral load setpoint and faster progression to AIDS than in CCR5-tropic HIV infected humans^{495,496}. Using SHIV_{SF16P3}, we have gained a deeper understanding of mucosal transmission⁴⁹⁷ and successfully tested many therapeutics to block against viral transmission^{341,358,437,498}. One such therapeutic class is bNAbs that target varying epitopes on an HIV envelope. Hence, the development and characterization of SHIVs have made it possible to test many different bNAbs, and the successful use of bNAbs in NHP studies laid the groundwork for multiple human clinical trials.

Because ART drugs target various HIV viral proteins, their effectiveness against SIV viral proteins may be limited. To this end, the SIV *pol* gene from a SIVmac backbone was exchanged with an HIV-1 *pol* gene, termed SIV reverse transcriptase or RT-SHIV. RT-SHIV has contributed to our understanding of the development of drug-resistant mutation⁴⁹⁹, the persistence of viral reservoirs⁵⁰⁰, and viral diversity⁵⁰¹ during ART suppression.

Overall, the use of NHP species and SIV strains in HIV research has immensely advanced our understanding of HIV-1 infection, antiviral immunity, viral reservoirs, pathology during full ART drug suppression, and the development of therapeutics and vaccines.

CHAPTER 2: CCR5 receptor occupancy analysis reveals increased peripheral blood CCR5+CD4+ T cells following treatment with the anti-CCR5 antibody Leronlimab

2.1. Abstract

CCR5 plays a central role in infectious disease, host defense, and cancer progression, thereby making it an ideal target for therapeutic development. Notably, CCR5 is the major HIV entry co-receptor, where its surface density correlates with HIV plasma viremia. The level of CCR5 receptor occupancy (RO) achieved by a CCR5-targeting therapeutic is therefore a critical predictor of its efficacy. However, current methods to measure CCR5 RO lack sensitivity, resulting in high background and overcalculation. Here, we report on two independent, flow cytometric methods of calculating CCR5 RO using the anti-CCR5 antibody, Leronlimab. We show that both methods led to comparable CCR5 RO values, with low background on untreated CCR5+CD4+ T cells and sensitive measurements of occupancy on both blood and tissue-resident CD4+ T cells that correlated longitudinally with plasma concentrations in Leronlimab-treated macaques. Using these assays, we found that Leronlimab stabilized cell surface CCR5, leading to an increase in the levels of circulating and tissue-resident CCR5+CD4+ T cells *in vivo* in Leronlimab-treated macaques. Weekly Leronlimab treatment in a chronically SIV-infected macaque led to increased CCR5+CD4+ T cells levels and fully suppressed plasma viremia, both concomitant with full CCR5 RO on peripheral blood CD4+ T cells, demonstrating that CCR5+CD4+ T cells were protected from viral replication by Leronlimab binding. Finally, we extended these results to Leronlimab-treated humans and found that weekly 700 mg Leronlimab led to complete CCR5 RO on peripheral blood CD4+ T cells and a statistically significant increase in CCR5+CD4+ T cells in peripheral blood. Collectively, these results establish two RO

calculation methods for longitudinal monitoring of anti-CCR5 therapeutic antibody blockade efficacy in both macaques and humans, demonstrate that CCR5+CD4+ T cell levels temporarily increase with Leronlimab treatment, and facilitate future detailed investigations into the immunological impacts of CCR5 inhibition in multiple pathophysiological processes.

2.2. Authors, Affiliations, Contributions, and Acknowledgements

Authors: Xiao L. Chang¹, Helen L. Wu¹, Gabriela M. Webb¹, Meenakshi Tiwary¹, Colette Hughes¹, Jason S. Reed¹, Joseph Hwang¹, Courtney Waytashek¹, Carla Boyle¹, Cleiton Pessoa¹, Andrew W. Sylwester¹, David Morrow¹, Karina Taylor¹, Miranda Fischer², Scott Kelly³, Nader Pourhassan³, Rachele M. Bochart², Jeremy Smedley², Christopher P. Recknor³, Scott G. Hansen^{1*}, Jonah B. Sacha^{1,2*}

*Correspondence: Jonah B. Sacha (sacha@ohsu.edu) and Scott G. Hansen (hansensc@ohsu.edu)

Affiliations: ¹Vaccine & Gene Therapy Institute, Oregon Health & Science University, Portland, OR, USA

²Oregon National Primate Research Center, Oregon Health & Science University, Portland, OR, USA.

³CytoDyn Inc., Vancouver, WA, USA

Contributions: JBS conceptualized the studies and experiments. XLC and HLW managed macaque studies, assisted by JSR, JH. CPR ran the clinical trial, while CH, AWS, DW, KT, and SGH performed analysis. MF, RMB, and JS managed the animal care and procedures. XLC, HLW, GMW, MT, CH, JSR, JH, CW, CB, CP, AWS, DM, KT, SK, NP, CPR, SGH, and JBS

investigated. XLC and JBS designed and validated CCR5 RO assays in macaques, while JBS and SGH adapted assay for human clinical use. XLC and JBS drafted the manuscript, and all authors contributed to editing. JBS, SGH, SK, and NP acquired funding.

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2.3. Introduction

C-C chemokine receptor type 5 (CCR5) is a G protein-coupled receptor involved in cell signaling and migration. CCR5 is primarily expressed in lymphocytes, macrophages, dendritic cells, and natural killer cells, but can also be found on the epithelium, endothelium, vascular smooth muscle, and fibroblasts from multiple organs, as well as neurons, astrocytes, and microglia in the central nervous system (CNS)^{382,383}. Chemokines CCL3 (MIP-1 α), CCL4 (MIP-1 β), and CCL5 (RANTES) are the primary CCR5 ligands and are expressed in sites of inflammation to recruit CCR5⁺ immune cells; thus, the number of CCR5⁺ cells often correlate with the severity of inflammation^{382,386,502}.

While CCR5 is known for its role in facilitating human immunodeficiency virus (HIV) infection of CD4⁺ T cells, it has a wide range of roles in normal and pathophysiological processes. In addition to HIV, CCR5 is a critical host receptor for Dengue virus⁴⁰⁵ and *Staphylococcus aureus*⁴⁰⁶ infection. Furthermore, high expression of CCR5 is associated with cancer progression and tumorigenesis⁴¹³⁻⁴¹⁵, development of insulin resistance via adipose tissue

macrophage recruitment ⁴¹¹, and suppression of cortical plasticity, learning, and memory ⁴⁰⁷⁻⁴⁰⁹. Moreover, individuals homozygous for the naturally occurring CCR5 mutation, CCR5Δ32, lack cell surface expression of CCR5 receptors, which has protective effects against HIV infection ^{421,422}, asthma ^{503,504}, severe SARS-CoV-2 symptoms ⁵⁰⁵, and development of rheumatoid arthritis ⁵⁰⁶, and is associated with improved hepatitis B virus infection recovery rates ⁵⁰⁷ and lower incidence of cardiovascular disease ^{508,509}. However, CCR5 is critical for viral clearance after infection by West Nile ³⁸⁹, Japanese encephalitis ³⁹⁰, and influenza A viruses ^{391,392} because of its role in trafficking immune cells to sites of infection. Exemplifying the complexity of CCR5, the lack of CCR5 receptors protected against parasitic *Toxoplasma gondii* infection ⁴²⁵ while the presence of CCR5 was essential for disease control after infection ^{395,396}. Because of the myriad roles played by CCR5, the ability to target CCR5 with therapeutic agents will have a diverse range of applications.

Widely used HIV-specific therapeutics, such as antiretroviral (ARV) drugs and HIV-specific broadly neutralizing antibodies (bNAbs), exert their antiviral effects by directly targeting viral proteins, and thus their efficacy is correlated to plasma concentration ^{366,510,511}. However, for immunomodulatory and antiviral agents that target host receptors, their efficacy relies on the level of receptor occupancy (RO) achieved. Indeed, in HIV infection the density of CCR5 on CD4+ T cells correlated closely to both susceptibility to HIV infection *in vitro* and plasma viral loads in HIV-infected individuals ⁴²⁶⁻⁴²⁸. Additionally, the paucity of CCR5+ CD4+ T cells present in natural hosts of simian immunodeficiency virus (SIV) during infancy protects against mother-to-offspring transmission during breastfeeding by viremic mothers ⁵¹². Thus, the level of CCR5 occupied by a CCR5-targeting drug is a critical predictor of its therapeutic efficacy.

Currently, Maraviroc is the only FDA-approved CCR5 antagonist. Maraviroc inhibits CCR5 internalization following ligand binding, and thus Maraviroc CCR5 RO is indirectly measured by a MIP-1 β internalization assay where CCR5 RO is defined by the percentage of cell surface CCR5 that is not down-regulated following treatment with MIP-1 β ⁵¹³. This indirect method of measuring CCR5 RO results in background RO of approximately 25% ⁵¹⁴, with reports of 120% CCR5 RO in peripheral blood CD4⁺ T cells from both Maraviroc-treated and -untreated rhesus macaques ⁴³⁹. These issues of extremely high background and overcalculation of CCR5 RO are major limitations of the MIP-1 β internalization assay, especially when CCR5 RO is a critical parameter in studying CCR5-blocking agents.

Directly measuring CCR5 RO with monoclonal antibodies also presents challenges as CCR5 expression is a dynamic process that must be controlled for. Indeed, the frequency of CCR5⁺ cells change longitudinally in response to inflammatory and homeostatic stimuli and can be impacted by the CCR5-targeting reagent itself ^{380,438,439,498,515}, leading to inaccuracies in methods that use baseline CCR5 values to calculate CCR5 RO ⁵¹⁶. Not accounting for the ability of CCR5 expression to change over time in the CCR5 RO calculation for the anti-CCR5 antibody HGS004 resulted in baseline pre-treatment CCR5 RO values of 20% in HIV-1 infected participants ⁵¹⁷. Thus, no robust and highly sensitive method for the calculation of CCR5 RO currently exists.

Here, we report on two sensitive methods to measure CCR5 RO by the anti-CCR5 antibody Leronlimab (PRO-140; Vyrologix). We demonstrate the sensitivity of this method to longitudinally quantify CCR5 RO on blood and tissue CD4⁺ T cells from Leronlimab-treated macaques and describe increased levels of CCR5⁺CD4⁺ T cells in the blood of both Leronlimab-treated macaques and humans. Finally, we translate the macaque CCR5 RO method

to Leronlimab-treated, HIV-naïve human participants, demonstrating the direct use for monitoring CCR5 RO by Leronlimab in human clinical trials.

2.4. Materials and Methods

Study approval and design (Nonhuman primates). All animal work occurred at the Oregon National Primate Research Center (ONPRC), a Category I facility that is fully credited by the American Association for Accreditation of Laboratory Animal Care (AAALAC), with approved Assurance (#A3304-01) for the use and care of animals on file with the NIH Office of Laboratory Animal Welfare. Animal experimental care plans, protocols, procedures, and administered reagents were approved by ONPRC Institutional Animal Care and Use Committee (IACUC). The ONPRC IACUC adheres to the national guidelines established in the Animal Welfare Act (7 U.S.C. Sections 2131–2159) and the Guide for the Care and Use of Laboratory Animals (8th Edition) as mandated by the U.S. Public Health Service Policy.

Macaques (*Macaca mulatta* and *Macaca fascicularis*) used in this study were housed at the Oregon National Primate Research Center (ONPRC) in Animal Biosafety level (ABSL)-2+ rooms with autonomously controlled temperature, humidity, and lighting. At assignment, macaques were free of Cercopithecine herpesvirus 1, D-type simian retrovirus, simian T-lymphotropic virus type 1, and Mycobacterium tuberculosis. Macaque specialists designed and oversaw daily wellness and dietary enrichment plans. Ketamine HCl (Ketathesia™, Henry Schein Animal Health) with or without Dexmedetomidine (Dexmedesed™, Dechra, Overland Park, KS) was used to sedate macaques for procedures, including subcutaneous (SC) injections of Leronlimab, venipuncture, tissue biopsy, and viral challenge, and they were performed by certified veterinarians or trained animal technicians with veterinarian oversight.

Adult rhesus macaques (n=12) were used in this study, with six macaques in the single 10 mg/kg SC Leronlimab group and six macaques in the single 50 mg/kg SC Leronlimab group. All but one animal in the 10 mg/kg group were female. Baseline biopsies and whole blood were collected before SC Leronlimab injections. Blood was collected at eight, 24, 48, and 72 hours post Leronlimab injection, and then weekly afterwards. Biopsies were collected at one and four weeks after Leronlimab injection and processed as described below. This was a non-terminal study and macaques were returned to the ONPRC colony after study conclusion.

One adult female chronically SIVmac239-infected Mauritian cynomolgus macaque received weekly doses of 50 mg/kg Leronlimab subcutaneously for 11 weeks.

Study approval and design (Human). De-identified peripheral blood samples were obtained from participants in a phase 2, randomized, double blind, placebo-controlled study to evaluate for the efficacy and safety of Leronlimab treatment in human participants experiencing prolonged SARS-CoV-2 symptoms (termed Long-Haulers). Participants were randomized to receive weekly 700 mg SC Leronlimab or placebo. Participants of all sexes, over 18 years of age, and with prior confirmed positive SARS-CoV-2 RT-PCR test were eligible. Participants experienced at least two clinical symptoms consistent with a SARS-CoV-2 infection for more than 12 weeks. The trial took place at the Arthritis & Rheumatic Disease Specialties in Aventura, Florida and Center for Advanced Research & Education (CARE) in Gainesville, Georgia under the ClinicalTrials.gov identifier (NCT number): NCT04678830. All participants provided written informed consent prior to inclusion in study.

Tissue processing. Whole blood, lymph node, and bone marrow samples were processed as previously described ⁴⁹⁸. Briefly, peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by density gradient centrifugation using Ficoll-Paque PLUS (Cytiva) and centrifuged at 1,860 x g for 30 minutes. Plasma was saved for viral nucleic acid and Leronlimab concentration detection. Buffy coat containing PBMCs was collected and washed with R10 (RPMI 1640 containing 10% fetal bovine serum (FBS)) before use. Lymph nodes were collected in R10, diced with a scalpel, and forced through a 70 µm cell strainer to a single cell suspension in R10. Bone marrow aspirates were collected in R10 and pelleted by centrifugation at 830 x g for 4 minutes. Cell pellets were resuspended by shaking in PBS containing 2 mM EDTA, and then centrifuged at 830 x g for 4 minutes. Cell pellets were then resuspended in 70% isotonic Percoll (GE Healthcare, Buckinghamshire, UK) and layered under 37% isotonic Percoll. Layers were centrifuged at 500 x g for 20 minutes. Mononuclear cells in the interface were collected into R10.

CCR5 RO Assay. *Equation 1 flow staining:* Equation 1 measured for Leronlimab-occupied CCR5 by using anti-IgG4 FITC in comparison to total CCR5 measured by *ex vivo* incubation with a saturating concentration of Leronlimab (5 µg/mL). Three staining tubes were used, with one tube serving as the fluorescence minus one (FMO) control (Supplemental Table 2.1). A minimum of 50 µL of whole blood or 3 x 10⁵ mononuclear cells were used per staining tube. Cells were washed twice with 1mL of PBS by centrifuging at 830 x g for 4 minutes, then supernatants were aspirated to leave ~100 µL, and finally cell pellets were resuspended by vortex. Next, 5 µg/mL of parental, unconjugated Leronlimab was added to tube 3 according to Supplemental Table 2.1 and incubated for 30 minutes at room temperature in the dark. Cells

were washed once with PBS, and then anti-IgG4 FITC was added to tubes 2 and 3 and incubated for 30 minutes at room temperature in the dark. Afterwards, cells were washed once with PBS + 10% FBS and once with PBS. Anti-CCR5 APC was added to tubes 2 and 3, while surface antibodies (CD3, CD4, CD8, CD45, CD95, and CD28) and amine-reactive dye (cell viability) were added to all tubes. A description of antibodies used can be found in Supplemental Table 2.2. Cells were incubated for 30 minutes at room temperature in the dark. For whole blood samples, 1 mL of 1X FACS Lysing solution (BD Biosciences) was added to each tube to lyse red blood cells for 8 minutes, and then immediately centrifuged and washed three times with PBS + 10% FBS. Cells were stored at 4°C prior to running on BD™ LSR II flow cytometer (BD Biosciences). For mononuclear cells, cells were washed twice with PBS and then fixed by adding 100 µL of 2% paraformaldehyde (PFA) and incubating for at least 10 minutes before running on BD™ LSR II flow cytometer (BD Biosciences). Flow analysis was done using FlowJo 10.4, where cells were progressively gated on singlets (FSC-H vs. FSC-A), live, CD45+, CD3+, CD4+/CD8-, and CCR5+ cell populations, as described in Supplemental Figure 2.1. Staining tube 1 served as the FMO control to assist with gating on desired cell populations, tube 2 stained for the frequency of Leronlimab-occupied CCR5+CD4+ T cells, and tube 3 was saturated with Leronlimab *ex vivo* to measure for the total frequency of CCR5+CD4+ T cells. The equation to calculate CCR5 RO using equation 1 is as follows:

$$\%RO = \frac{\%IgG4 (tube\ 2)}{\%IgG4 (tube\ 3)} \times 100\%$$

Equation 2 flow staining: Equation 2 measured for unoccupied CCR5 by using Leronlimab that was conjugated to Pacific Blue (termed Leronlimab-PB). Three staining tubes

were required, with two tubes serving as the FMO controls (Supplemental Table 2.1). Similar to equation 1, a minimum of 50 μ L whole blood or 3×10^5 mononuclear cells were used per staining tube. Cells were washed two times with PBS and anti-IgG4 FITC was added to tube C following Supplemental Table 2.1 and incubated for 30 minutes at room temperature in the dark. Because anti-IgG4 FITC could interact with downstream Leronlimab-PB, leading to false positive staining of anti-IgG4 FITC, cells were washed once with PBS + 10% FBS and at least three times with PBS. Afterward, anti-CCR5 APC was added to tubes B and C, Leronlimab-PB was added to tube C, surface antibodies (CD3, CD4, CD8, CD45, CD16, and CD14) and amine-reactive dye were added to all tubes (Supplemental Table 2.2). Cells were incubated for 30 minutes at room temperature in the dark. Afterward, whole blood or mononuclear cells were lysed with 1X FACS Lysing solution or fixed with 2% PFA, respectively, as described in the staining for equation 1. Samples were collected on a BDTM LSR II flow cytometer (BD Biosciences) and analyzed with FlowJo 10.4, where cells were progressively gated on CD45+, singlets (FSC-H vs. FSC-A), live, CD3+, CD4+/CD8-, and CCR5+ cell populations (Supplemental Figure 2.1). Here, staining tube A served as a FMO control to assist with gating on desired cell populations, and tube B served as a FMO control to assist with gating on IgG4+ and Leronlimab-PB+ cell populations. Tube C stained for cells with unoccupied CCR5 (Leronlimab-PB) and occupied CCR5 (anti-IgG4 FITC), with the total frequency of CCR5+CD4+ T cells represented by the sum of IgG4+ and Leronlimab-PB+ cell frequencies. The equation to calculate CCR5 RO using equation 2 is as follows:

$$\%RO = \frac{\%IgG4 (tube C)}{\%IgG4 (tube C) + \%Leronlimab-PB (tube C)} \times 100\%$$

Lastly, the percentage of CCR5+CD4+ T cells was determined with tube B because it was free from *ex vivo* incubation of unconjugated Leronlimab or conjugated Leronlimab-PB, which, described later in the Results session, was found to stabilize and increase CCR5 expression on CD4+ T cells.

Combined equations 1 and 2 flow staining: In the clinically-adapted CCR5 RO assay, changes were made to 1) fluorophore conjugates to adjust for the higher-wattage lasers used in FACSymphony A5 (BD Biosciences), a machine that allowed for easier detection of rare events, 2) the FACSymphony A5's lack of a 651nm (green) laser and expanded 488nm (blue) laser, 3) antibody clones to improve species reactivity to macaque and human samples, and 4) combine both CCR5 RO equations into one staining panel to minimize the number of cells required for staining. Here, four staining tubes were required, with two tubes serving as FMO controls (Supplemental Table 2.1). At least 50 μ L of whole blood or 3×10^5 PBMC samples were placed into each staining tube and samples were washed two times with PBS. Next, 5 μ g/mL of the parental, unconjugated Leronlimab was added to tube H4 (Supplemental Table 2.1) and incubated for 30 minutes at 2-8°C in the dark. Afterward, tubes H1 and H2 were washed once with 3 mL cold PBS while tubes H3 and H4 were washed with 1 mL cold PBS with 10% mouse serum (Equitech) in order to minimize nonspecific binding of anti-human IgG4 FITC. Cells in tubes H3 and H4 were then incubated with 100 μ L of cold mouse serum and incubated for 60 minutes at 2-8°C followed by another wash of 1 mL cold PBS with 10% mouse serum. Anti-IgG4 FITC and 100 μ L of cold mouse serum were then added to tubes H3 and H4 and incubated for 30 minutes at 2-8°C in the dark. Next, cells were washed once with DPBS and 0.1% BSA and then at least three times with PBS. Anti-CCR5 APC was added to tubes H2, H3, and H4, Leronlimab-PB was added to tube H3, surface antibodies (CD3, CD4, CD8a, CD45, CD16,

CD14, CD28, and CD95), and amine-reactive dye (cell viability) were added to all tubes (Supplemental Table 2.3). Cells were incubated for 30 minutes at room temperature in the dark. Finally, whole blood or PBMC samples were lysed with 1X FACS Lysing solution, incubated for 10 minutes in the dark, and then immediately washed once with DPBS and 0.1% BSA. Cells were then permeabilized with 0.5 mL 1.5X FACS Lysing solution and 0.05% Tween-20, incubated for 10 minutes in the dark, and then washed twice with DPBS and 0.1% BSA. Samples were run using the FACSymphony A5 (BD Biosciences) and analyzed with FlowJo 10.4. Cells were progressively gated on CD45⁺, singlets (FSC-H vs. FSC-A), live, CD3⁺, CD4⁺/CD8⁻, and CCR5⁺ cell populations (Supplemental Figure 2.1). Here, tube H1 and H2 served as FMO controls to assist with gating on cell populations, tube H3 stained for the frequency of Leronlimab-occupied CCR5 with anti-IgG4 FITC and Leronlimab-unoccupied CCR5 with Leronlimab-PB, and tube H4 was saturated with Leronlimab *ex vivo* to measure for the total frequency of CCR5⁺CD4⁺ T cells. The equations to calculate CCR5 RO are as follow:

$$\%RO = \frac{\%IgG4 (tube H3)}{\%IgG4 (tube H4)} \times 100\% \quad Equation 1$$

$$\%RO = \frac{\%IgG4 (tube H3)}{\%IgG4 (tube H3) + \%Leronlimb-PB (tube H3)} \times 100\% \quad Equation 2$$

***In vitro* CCR5 ligand binding and staining.** PBMCs were incubated with or without 5 µg/mL of Leronlimab for 30 minutes at 37°C. Next, cells were incubated at 37°C for an additional 30 minutes with no additional ligand or 50 nM of one of the following CCR5 ligands: MIP-1α, MIP-1β, or RANTES. Afterwards, cells were stained with CCR5 (clone 3A9), CD3, CD4, and

CD8 cell surface receptors and amine-reactive dye. Samples were collected using the BDTM LSR II flow cytometer and analyzed with FlowJo 10.4 by gating on live, CD3+, CD4+/CD8-, singlet, and CCR5+ cell populations. Data was presented as normalized %CCR5+ for each treatment condition that was normalized to frequency observed in Leronlimab-untreated, ligand-free cells from each respective donor.

Measurement of Leronlimab concentration in plasma. Enzyme-linked immunosorbent assay (ELISA) was used to detect for Leronlimab levels in plasma samples, as previously described ⁴⁹⁸. Briefly, plates were coated with 1.5 µg/mL PA22 (CytoDyn, Vancouver, WA) in carbonate-bicarbonate buffer (ThermoFisher) overnight at 4°C. Plates were washed three times with PBS-T (PBS with 0.1% Tween-20) and blocked for at least two hours in room temperature with Blocking Buffer (PBS with 0.4% Tween-20 and 10% bovine serum albumin (Fisher Scientific). Standard curve generated with serial dilutions of Leronlimab and samples were plated onto blocked plates and incubated for 30 minutes at room temperature. Plates were washed three times with PBS containing 0.5 M NaCl, and then incubated with 20,000-fold diluted mouse anti-human IgG4 pFc'-horseradish peroxidase (Southern Biotech) in Blocking Buffer for 30 minutes at room temperature. Finally, plates were washed three times with PBS-T and 3,3',5,5'-Tetramethylbenzidine (TMB) substrate (Southern Biotech) was added to develop the plates for two minutes, after which, 1 N H₂SO₄ was added to stop the reaction. Developed plates were read on Synergy HTX Multi-Mode Microplate Reader (BioTek) using the Gen5 v3.10 software to read two absorbance wavelengths: 650 nm for the developing reaction and 450 nm for the developed reaction. Plasma concentration for each sample (µg/mL) was determined using the generated standard curve, with an assay limit of detection of 0.0226 µg/mL.

Viral nucleic acid detection. SIV nucleic acid detection assays were performed by members of the ONPRC Molecular Virology Core. Detection of SIV nucleic acids was performed as previously described^{252,498,518}. Briefly, viral nucleic acids from 300 µL of plasma were extracted using LEV Viral Nucleic Acid Kit and the Maxwell 16 instrument (Promega, Madison, WI) following manufacturer's protocols. RT-qPCR reaction was performed to quantify SIV viral RNA in the plasma. The reaction used the total extracted RNA, 900 nM of SGAG21 forward primer (GTCTGCGTCATPTGGTGCATTC), 900 nM of SGAG22 reverse primer (CACTAGKTGTCTCTGCACTATPTGTTTTG), and 250 nM of pSGAG23 probe (5'-6-carboxyfluorescein [FAM]-CTTCPTCAGTKTGTTTCACTTTCTCTTCTGCG-black hole quencher [BHQ1]-3') for a final reaction volume of 30 µL. Standard curve was created by using *in vitro* transcribed SIVgag RNA that was serially diluted in 5 ng/µL yeast tRNA (Sigma R5636). Applied Biosystems QuantStudio 6 Flex instrument (Life Technologies) was used to run the RT-qPCR reactions at the following setting: 50 °C for 5 min; 95 °C for 20 s; [95 °C for 3 s, 60 °C for 30 s] × 45 cycles. The limit of quantification for this assay is 50 copies/mL.

Statistical Analysis. Wilcoxon signed-rank test was used to analyze %CCR5+CD4+ T cells between baseline and Leronlimab treatment timepoints in macaques. Two-way repeated measures analysis of variance (ANOVA) with Sidak's correction for multiple comparisons was used to compare the %CCR5+CD4+ T cells between Leronlimab-treated and placebo human participants. Analysis was performed using GraphPad Prism Version 8.31 (332).

2.5. Results

Development of the Leronlimab CCR5 Receptor Occupancy Assay

Rhesus macaques are vitally important pre-clinical models used to validate potential prophylactic and therapeutic modalities as they share similar immune systems with humans. Indeed, CCR5 sequence, structure, and function are highly conserved between the two species⁵¹⁹. Previously, we demonstrated that Leronlimab specifically binds the same CCR5 epitope on the surface of human and rhesus macaque leukocytes⁴⁹⁸. Therefore, to facilitate both pre-clinical macaque and clinical human studies, we analyzed the effect of Leronlimab on CCR5 expression on CD4⁺ T cells from both species.

First, we examined the impact of treatment with a saturating concentration of 5 mg/mL Leronlimab on surface CCR5 levels of primary human and macaque CD4⁺ T cells *in vitro*. Similar to Maraviroc, Leronlimab stabilized cell surface CCR5 and directly increased the frequency of CCR5⁺CD4⁺ T cells from humans and rhesus macaques (Figure 2.1A). Next, we explored if Leronlimab-occupied CCR5 was resistant to internalization following treatment with CCR5 ligands, a phenomenon that forms the basis for the MIP-1 β internalization Maraviroc CCR5 RO assay^{513,514}. In samples without Leronlimab, we observed CCR5 internalization in response to MIP-1 α , MIP-1 β , and RANTES (Figure 2.1B). Following treatment with Leronlimab, we found increased frequencies of CCR5⁺CD4⁺ T cells where CCR5 was resistant to internalization following treatment with all three CCR5 ligands, indicating that Leronlimab both stabilized surface CCR5 expression and prevented its internalization. Thus, it is critical to account for this Leronlimab-induced increase in surface CCR5 levels for CCR5 RO measurements.

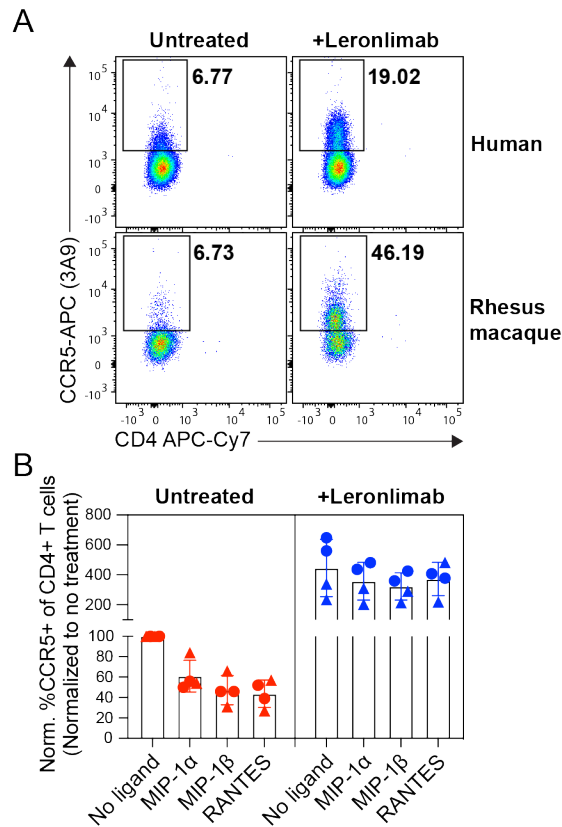


Figure 2.1. Leronlimab increases cell surface CCR5 expression that is resistant to internalization. PBMC from humans (n=2; triangles) and rhesus macaques (n=2; circles) were incubated without (left, red) or with (right, blue) 5 μ g/mL Leronlimab for 30 minutes at 37°C, then with either no treatment or 50nM of one of the following CCR5 ligands: MIP-1 α , MIP-1 β , or RANTES, for an additional 30 minutes. After incubation, cells were stained for CCR5 (clone 3A9) and CD3, CD4, and CD8 surface markers. Cells were gated within live, CD3+, CD4+/CD8-, singlet populations. **(A)** Representative flow plots. **(B)** Graphs show normalized frequencies of CCR5+ of CD4+ T cells to frequency observed with untreated cells (no Leronlimab, no CCR5 ligands) from the respective donor.

To measure CCR5 RO, we designed Leronlimab CCR5 RO assays based on methods previously established for RO measurements of anti-PD-1 antibodies in clinical trials^{520,521}. The Leronlimab CCR5 RO assays consist of the three following critical components: 1) the CCR5-specific antibody clone 3A9 that does not compete with nor sterically hinder Leronlimab binding of CCR5 to track overall CCR5 expression, 2) the anti-human IgG4 antibody clone HP6025, which binds to the humanized IgG4 Fc of Leronlimab and 3) Pacific Blue-labeled Leronlimab (termed Leronlimab-PB), which binds to free CCR5 receptors not occupied by Leronlimab (Figure 2.2A). To measure the percentage of CCR5 RO on the surface of CD4+ T cells, two different methods were used as described in detail in the Materials and Methods section. CCR5 RO equation 1 measures Leronlimab-bound receptor by using the anti-human IgG4 antibody to measure Leronlimab-occupied CCR5 receptor directly (Figure 2.2A). CCR5 RO equation 2 measures unoccupied CCR5 receptors by using Pacific Blue-conjugated Leronlimab. In both

methods, CCR5 staining with the 3A9 antibody is used to account for weekly variations in CCR5 expression prior to calculation of CCR5 RO (Supplemental Figure 2.1). CCR5 RO for Equation 1 is defined by the percentage of cells CCR5+ (measured by clone 3A9) and Leronlimab+ (measured by anti-human IgG4) divided by the percentage of cells CCR5+ and Leronlimab+ following incubation with 5 mg/mL, a saturating concentration of unlabeled Leronlimab (Figure 2.2B-C).

$$\%RO = \frac{\%IgG4}{\%IgG4 \text{ (ex vivo saturating Leronlimab)}} \times 100\% \text{ (Equation 1)}$$

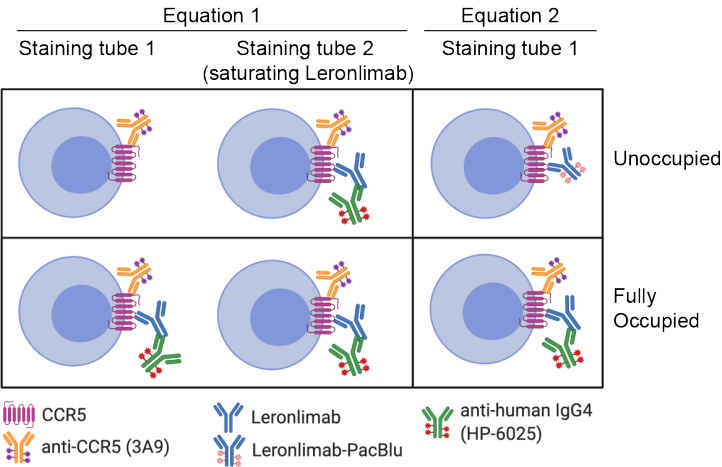
CCR5 RO for Equation 2 is defined as the percentage of cells CCR5+ (measured by clone 3A9) and Leronlimab+ (measured by anti-human IgG4) divided by the percentage of cells CCR5+ and Leronlimab+ (measured by the sum of anti-human IgG4 and Leronlimab-PB) cells following incubation with 5 µg/mL Leronlimab-PB.

$$\%RO = \frac{\%IgG4}{\%IgG4 + \%Leronlimab - PB} \times 100\% \text{ (Equation 2)}$$

Next, we tested the Leronlimab CCR5 RO assay using longitudinal peripheral blood samples from a rhesus macaque that received a single 50 mg/kg subcutaneous (SC) dose of Leronlimab. Representative flow cytometric plots and calculated CCR5 RO values using the two equations at three different study weeks are shown to demonstrate how the RO values are calculated (Figure 2C). We observed no CCR5 RO on peripheral blood CD4+ T cells immediately prior to Leronlimab dosing, followed by 100% CCR5 RO within eight hours

following the SC injection (Figure 2.2D). CCR5 RO was then maintained at approximately 100% until the Leronlimab plasma concentration fell below 5 µg/mL at approximately six weeks post injection. Following this, CCR5 RO continually decreased to baseline level following complete washout of Leronlimab from plasma. Importantly, both RO methods yielded similar CCR5 RO measurements throughout the study and correlated with the Leronlimab plasma concentration. These results demonstrate the sensitivity and reproducibility of the CCR5 RO assay for monitoring Leronlimab RO *ex vivo*.

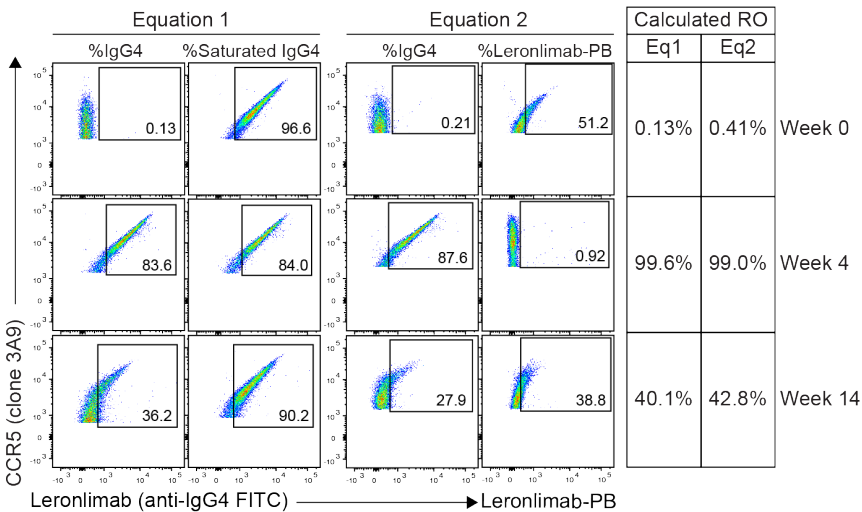
A



B

$$\%RO = \frac{\%IgG4}{\%IgG4 \text{ (ex vivo saturating Leronlimab)}} \times 100\% \quad (\text{Equation 1})$$
$$\%RO = \frac{\%IgG4}{\%IgG4 + \%Leronlimab-PB} \times 100\% \quad (\text{Equation 2})$$

C



D

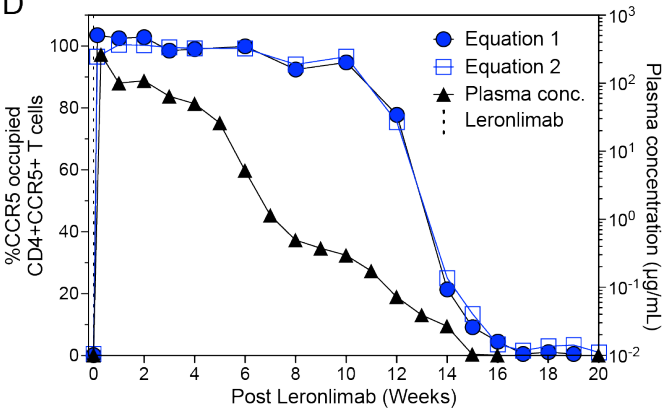


Figure 2.2. CCR5 Receptor Occupancy Assay Overview.

(A) Flow cytometry diagram showing the interactions between anti-CCR5, Leronlimab, Leronlimab-PacBlu (PB), and anti-human IgG4 for the two equations using a CCR5 unoccupied (top) and fully occupied (bottom) scenario. **(B)** Equations for calculating CCR5 RO. **(C)** Representative flow cytometry plots displaying the different components needed to calculate for the two equations using a rhesus macaque that received a single 50 mg/kg SC Leronlimab injection. Equation 1 used %IgG4+ events within CD45+, singlet, live, CD3+, CD4+/CD8-, and CCR5+ events. Equation 2 used %IgG4+ and Leronlimab-PB+ events within CD45+, singlet, live, CD3+, CD4+/CD8-, and CCR5+ events. Table on the right shows the calculated CCR5 RO calculated by the two equations at study weeks 0, 4, and 14 post single Leronlimab injection. **(D)** Left Y-axis is for CCR5 RO by Leronlimab on peripheral blood CD4+CCR5+ T cells calculated by equation 1 (solid blue circle) and equation 2 (open blue square). Right Y-axis is for the longitudinal plasma concentration (solid black triangle) in blood samples from the treated macaque.

Leronlimab treatment increases CCR5+CD4+ T cell counts in healthy rhesus macaques

To further verify that CCR5 RO calculated by equation 1 and equation 2 were robust methods to longitudinally track Leronlimab CCR5 RO over time in blood and tissue, as well as to monitor the impact of CCR5 RO on CCR5+CD4+ T cell levels, we treated 12 rhesus macaques with a single 10 mg/kg or 50 mg/kg SC Leronlimab injection (n=6 per group). As expected, Leronlimab plasma levels peaked at lower levels and washed out more rapidly in macaques who received 10 mg/kg versus 50 mg/kg (Figure 2.3A). In both groups, however, Leronlimab treatment yielded full CCR5 RO on peripheral blood CD4+ T cells by eight hours post injection, and maintained >90% CCR5 RO for an average of 12.8 days and 32.6 days for the 10 mg/kg and 50 mg/kg groups, respectively (Figure 2.3B).

CCR5 antagonists impact expression of cell surface CCR5 and thus modulate the levels CCR5+CD4+ T cells circulating *in vivo*. Maraviroc treatment increased CCR5 expression on peripheral blood T cells in both humans and macaques, but the effect on CCR5 expression on tissue-resident T cells is unclear^{438,515,522}. In contrast, longitudinal treatment of macaques with the anti-CCR5 antibody HGS101 did not increase CCR5 expression, but rather decreased levels of CCR5+CD4+ T cells in both peripheral blood and lymph nodes³⁸⁰. Therefore, we next sought to assess the impact of Leronlimab on CCR5+CD4+ T cells levels in both peripheral blood and within tissues. Following administration of Leronlimab at either dose we observed an increase in the frequencies of CCR5+CD4+ T cells circulating within peripheral blood that tracked with CCR5 RO (Figure 2.3C). In line with the rapid stabilization of CCR5 *in vitro* shown in Figure 2.1, statistically significant increases in peripheral blood CCR5+CD4+ T cell frequencies were found as early as eight hours post injection and maintained at every timepoint examined throughout the first week post injection (Figure 2.3D). Next, we examined the impact of

Leronlimab on CCR5+CD4+ T cells from lymph node and bone marrow biopsies. Similar to peripheral blood results, we found no CCR5 RO on lymph node and bone marrow CD4+ T cells prior to Leronlimab injection, followed by high levels of CCR5 RO on CD4+ T cells from these tissues at one week post injection (Figure 2.3E). Reflective of plasma concentration and peripheral blood CCR5 RO results, levels of CCR5 RO by Leronlimab on tissue CD4+ T cells were very low at four weeks post injection in the 10 mg/kg treated group, while significant, but variable CCR5 RO levels remained on tissue CD4+ T cells from the 50 mg/kg treated group (Figure 2.3E). In line with these CCR5 RO values, we observed a statistically significant increase in frequencies of tissue CCR5+CD4+ T cells at one week post Leronlimab injection, concomitant with high levels of CCR5 RO on CD4+ T cells in those tissues (Figure 2.3F). Upon loss of CCR5 RO at week four post Leronlimab injection, the levels of CCR5+CD4+ T cells decreased and were no longer statistically different from pre-injection levels. These observations demonstrate that Leronlimab treatment increases CCR5+CD4+ T cell frequencies in both the peripheral blood and within lymphoid tissues, and that this phenomenon depends upon the degree of Leronlimab occupancy of CCR5.

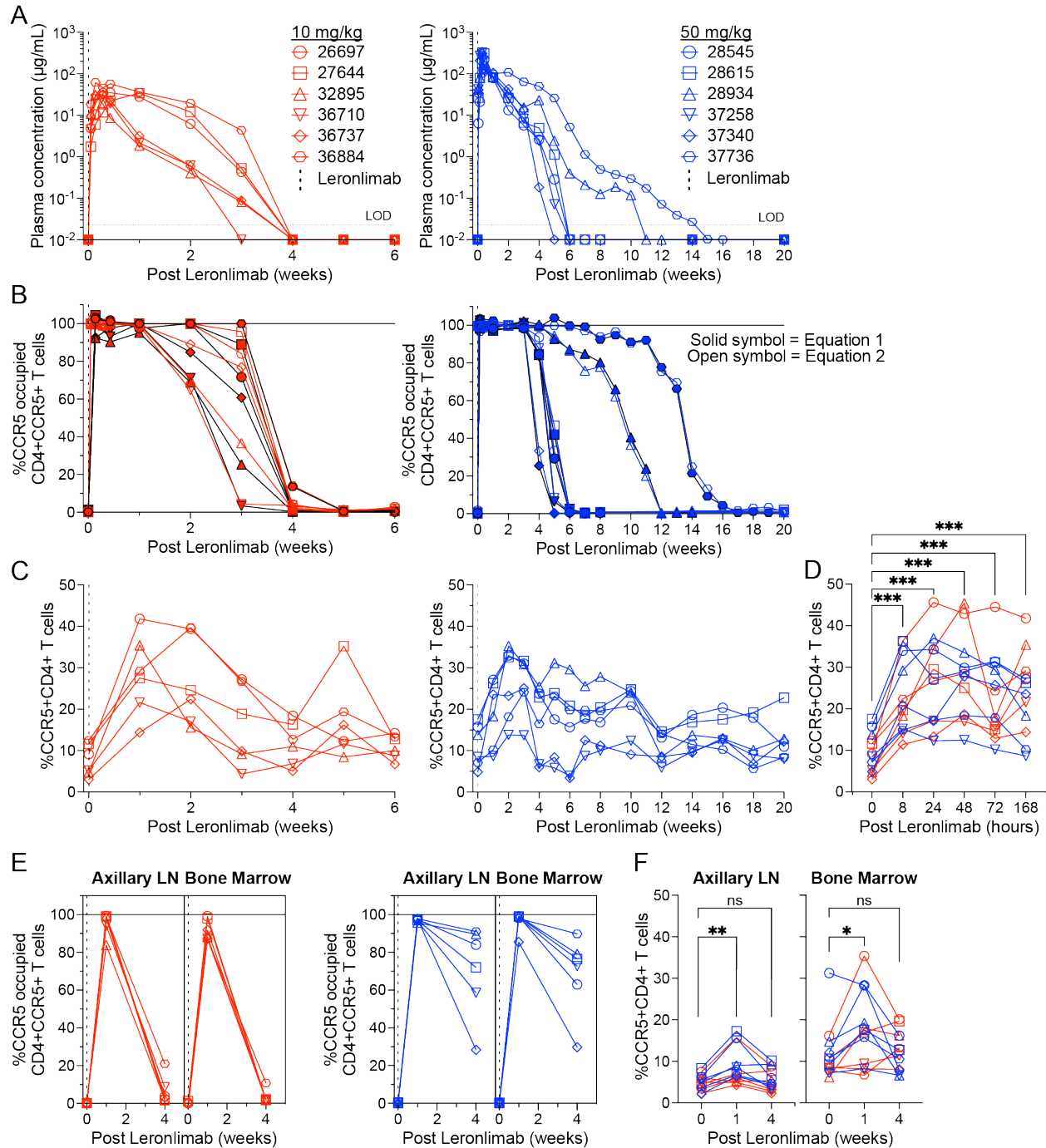


Figure 2.3. CCR5+CD4+ T cell frequencies increase with CCR5 RO. Leronlimab-naïve, SIV-naïve, rhesus macaques received a single 10 mg/kg (n=6; red; left) or 50 mg/kg (n=6; blue; right) SC Leronlimab. **(A)** Longitudinal Leronlimab concentration in plasma. **(B)** Longitudinal CCR5 RO by Leronlimab on peripheral blood CD4+CCR5+ T cells. **(C-D)** Peripheral blood CCR5+CD4+ T cell frequency for (C) longitudinal weekly timepoints, separated by treatment group and (D) hourly timepoints for both treatment groups within the first week post Leronlimab. **(E-F)** Axillary lymph node and bone marrow (E) CCR5 RO by Leronlimab and (F) %CCR5 on tissue-resident CD4+ T cells. (B and E) Solid symbol represents CCR5 RO calculated from equation 1 while open symbol represents CCR5 RO calculated from equation 2. P-values in panels D and F generated by Wilcoxon signed-ranked test; * p < 0.05; ** p < 0.01; *** p = 0.0005.

Leronlimab treatment suppresses SIVmac239 replication despite increasing CCR5+CD4+ T cell levels

The observed increase in CCR5+CD4+ T cells in Leronlimab-treated macaques raised the possibility that Leronlimab could inadvertently exacerbate ongoing SIV infection by providing additional susceptible targets for viral replication. To study the impact of Leronlimab on CD4+ T cell dynamics and viral replication in an ongoing SIV infection, we treated a chronically SIVmac239-infected Mauritian cynomolgus macaque with weekly 50 mg/kg SC Leronlimab injections for 11 weeks.

Similar to a single 50 mg/kg injection, complete CCR5 RO on peripheral blood CD4+ T cells was observed one week after the first dose and subsequently maintained for weeks after the final dose. However, compared to a single 50 mg/kg injection, repeated weekly 50 mg/kg injections for 11 weeks resulted in a longer duration of plasma Leronlimab, where complete Leronlimab plasma wash out and loss of CCR5 RO occurred at study weeks 32 and 36 post first dose, respectively (Figure 2.4A and 2.4B). As expected, based on our results above in SIV-naïve macaques, the frequency of CCR5+CD4+ T cells circulating in peripheral blood tracked with CCR5 RO, where it immediately increased after the first injection, began to decline with Leronlimab plasma washout, and returned to baseline level with loss of CCR5 RO (Figure 2.4C). Importantly, the increased frequencies of CCR5+CD4+ T cell targets did not exacerbate SIV replication. Instead, Leronlimab potently and completely suppressed SIV replication for approximately 20 weeks, during the time period where both full CCR5 RO and increased CCR5+CD4+ T cells were present in the blood (Figure 2.4D). As the Leronlimab plasma concentration declined and CCR5 RO was lost on CD4+ T cells, viral rebound occurred. SIVmac239 plasma viremia ultimately returned to pre-Leronlimab levels after complete loss of

CCR5 RO. Therefore, the Leronlimab-induced increase in CCR5+CD4+ T cell targets did not exacerbate ongoing SIV replication; rather, the binding of Leronlimab to the CCR5 co-receptor used for viral entry protected these cells from infection and greatly diminished ongoing SIV replication, resulting in minimal plasma viremia during the period of complete CCR5 RO.

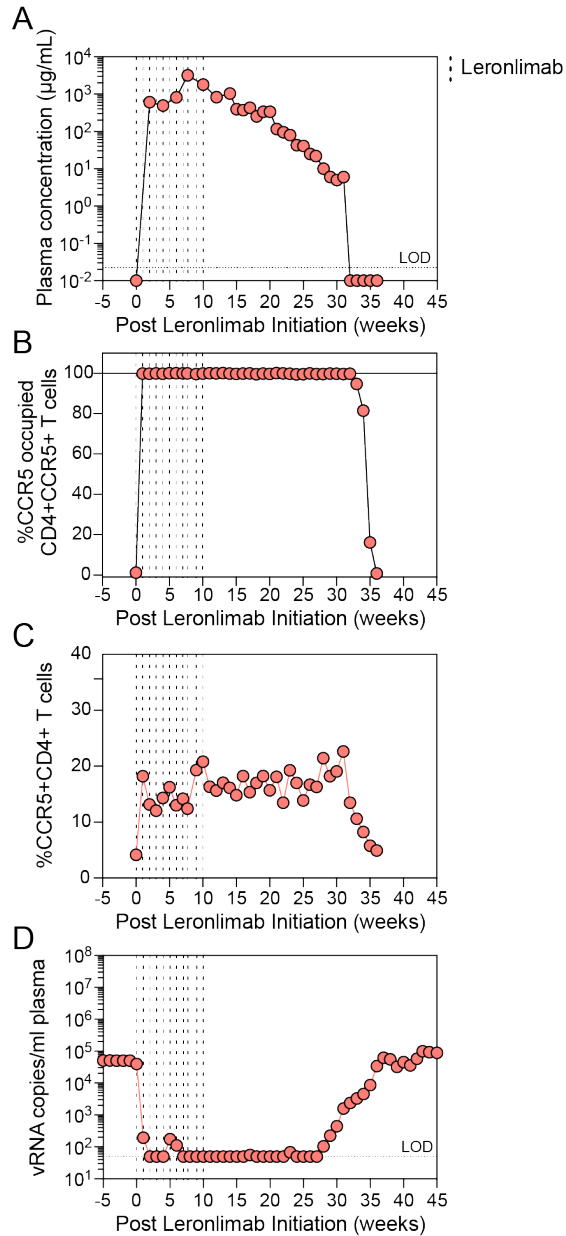


Figure 2.4. Leronlimab treatment of an SIVmac239-infected macaque. A cynomolgus macaque (n=1; 36484) was chronically infected with SIVmac239 prior to receiving weekly 50 mg/kg SC Leronlimab. **(A)** Longitudinal Leronlimab concentration in plasma. **(B)** Longitudinal CCR5 RO by Leronlimab on peripheral CD4+CCR5+ T cells. **(C)** Longitudinal peripheral blood CCR5+CD4+ T cell frequency. **(D)** Longitudinal SIV RNA copies per mL plasma. Vertical dashed lines represent each SC Leronlimab injection.

Measurement of CCR5 RO in Leronlimab-treated, HIV-free human participants.

Currently, Leronlimab is undergoing testing in clinical trials for both HIV and HIV-unrelated indications. With the successful demonstration of the CCR5 RO assay in nonhuman primate models, we next sought to extend the pre-clinical CCR5 RO technique for clinical applications. While the majority of the antibodies utilized in the macaque-specific assay are human-specific antibodies that cross react with the macaque orthologue, we adapted and refined the antibody clones utilized for optimal staining on human cells (Supplemental Table 2.2). To determine the performance of the clinical-grade CCR5 RO assay, we obtained blinded clinical samples from eight participants enrolled in a phase 2, two-arm, randomized, double blind, placebo-controlled study on the effects of Leronlimab treatment in long COVID-19 (ClinicalTrials.gov Identifier: NCT04678830). Enrolled participants were randomized to receive either weekly 700 mg SC Leronlimab injections or placebo throughout an eight-week study period, with a total of three clinical visits at weeks 0, 4, and 8 after the first Leronlimab initiation. As in our pre-clinical study models, the two equations calculated comparable CCR5 RO percentages from all three clinical visits in Leronlimab-treated human participants (Figure 2.5A). Furthermore, the CCR5 RO assay correctly identified individuals treated with Leronlimab versus placebo following unblinding, as treated individuals presented at baseline with no CCR5 RO, but then achieved full CCR5 RO on peripheral blood CD4⁺ T cells at the two follow-up clinical visits, while placebo-treated participants never presented with CCR5 RO (Figure 2.4B). In line with the preclinical macaque results, we observed increased frequencies of circulating CCR5⁺CD4⁺ T cells following Leronlimab dosing. After Leronlimab initiation, frequencies of CCR5⁺CD4⁺ T cells circulating in the peripheral blood in Leronlimab-treated participants were statistically higher than in untreated participants (Figure 2.4C). Thus, the longitudinal CD4⁺ T

cell CCR5 RO in Leronlimab-treated participants and lack of measurable CCR5 RO in Leronlimab-untreated participants demonstrates the robustness and sensitivity of the clinical CCR5 RO assay. Indeed, in both macaques and humans, CCR5 RO in the absence or presence of saturating plasma concentrations of Leronlimab was approximately 1% and 99%, respectively (Table 2.1). Cumulatively, these results establish precise methods to measure CCR5 RO in a pre-clinical nonhuman primate species and in human participants in clinical trials, and demonstrate that Leronlimab CCR5 RO induces increased frequencies of CCR5+CD4+ T cells.

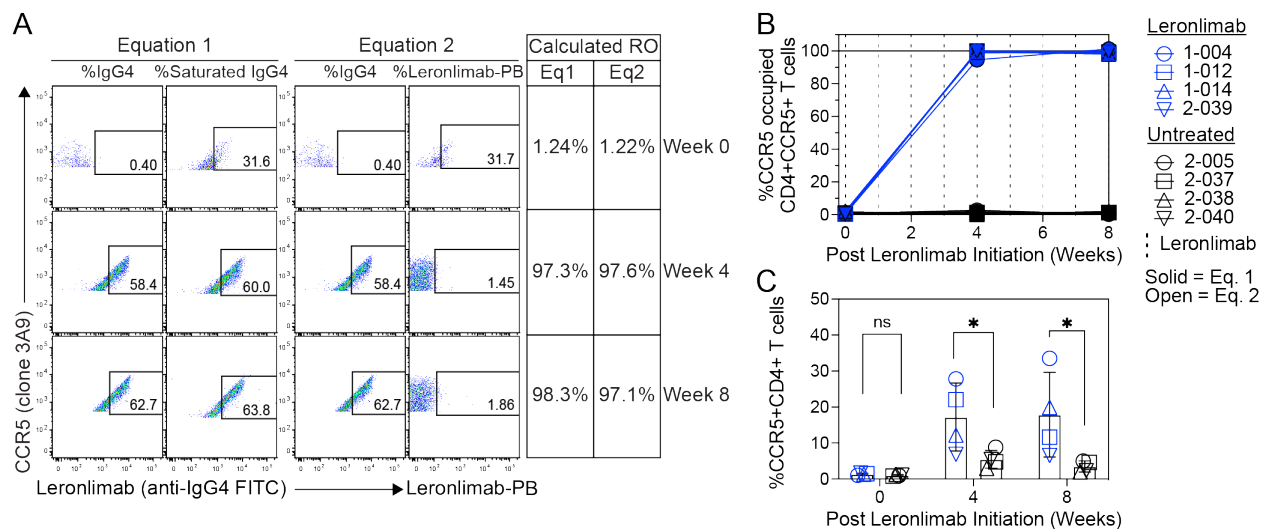


Figure 2.5. CCR5 RO assay on human samples. Blood samples were collected from human participants in ClinicalTrials.gov Identifier: NCT04678830 at study weeks 0, 4, and 8 post Leronlimab initiation. Leronlimab-treated (n=4; blue) participants received weekly 700 mg SC Leronlimab, while controls were untreated (n=4; black). **(A)** Representative flow cytometry plots showing the different components required to calculate CCR5 RO for equation 1 and 2. Table on the right shows the calculated RO at study weeks 0, 4, and 8. **(B)** Longitudinal CCR5 RO by Leronlimab on peripheral blood CD4+ T cells calculated with equation 1 (solid blue) and equation 2 (open blue). **(C)** Longitudinal CCR5+CD4+ frequency in peripheral blood. Vertical dashed line represents each SC Leronlimab injection. P-values in panel C calculated by two-way repeated measures ANOVA with Sidak's correction for multiple comparisons; * p < 0.05; ** p < 0.01.

Leronlimab condition	Species	Mean % CCR5 RO (SD, N)	
		Equation 1	Equation 2
Untreated	Human	1.02% (0.62, 8)	0.65% (0.52, 8)
	Macaque	0.38% (0.41, 12)	0.08% (1.06, 12)
Treated	Human	98.39% (2.47, 4)	99.73% (0.57, 4)
	Macaque	100.83% (4.33, 12)	99.84% (0.59, 12)

Table 2.1. Calculated CCR5 RO by Leronlimab on peripheral blood CD4+ T cells from Leronlimab-treated or untreated humans and macaques.

2.6. Discussion

Here, we created and validated two different methods of calculating CCR5 RO by the anti-CCR5 antibody Leronlimab. The methods generated comparable longitudinally CCR5 RO percentages in rhesus macaques that received a single 10 mg/kg or 50 mg/kg SC Leronlimab injection. Additionally, both methods were highly sensitive, with baseline values of 1% and fully saturated values of 99% when tested in human and nonhuman primates. These results are in contrast to the commonly used MIP-1 β internalization assay utilized for Maraviroc, which is associated with background levels of approximately 25% in human samples ⁵¹⁴ and yields values in excess of 100% in maraviroc-treated and -untreated rhesus macaques ⁴³⁹. Higher RO percentages calculated by the MIP-1 β internalization assay may be due to fluctuating CCR5 frequencies or incomplete CCR5 internalization upon MIP-1 β binding. In contrast, our methods did not depend on receptor internalization and all mathematical components used were gated on CCR5+ cells, compensating for any fluctuation in CCR5 frequency and allowing for precise

calculation of RO. Finally, the pre-clinical Leronlimab CCR5 RO assay was extended into human participants, demonstrating the ability to longitudinally and robustly monitor CCR5 RO.

Similar to maraviroc, we found that Leronlimab stabilized surface CCR5 molecules and prevented its internalization following ligand binding. Indeed, this shared feature of both drugs likely explains their shared ability to increase frequencies of CCR5+CD4+ T cells in both humans and macaques. Because CCR5+CD4+ T cells are target cells for HIV/SIV infection, increasing the frequencies of susceptible cells could raise concerns of increased HIV/SIV replication. However, we found that weekly Leronlimab treatment in a chronically SIVmac239-infected macaque fully suppressed plasma viremia for over 20 weeks despite a rise in the CCR5+CD4+ T cell frequency immediately after the first Leronlimab injection. Both suppression of viral replication and increased CCR5+CD4+ T cell levels were temporally associated with full CCR5 RO on peripheral blood CD4+ T cells, underscoring the need to measure CCR5 RO in studies utilizing CCR5-blocking agents.

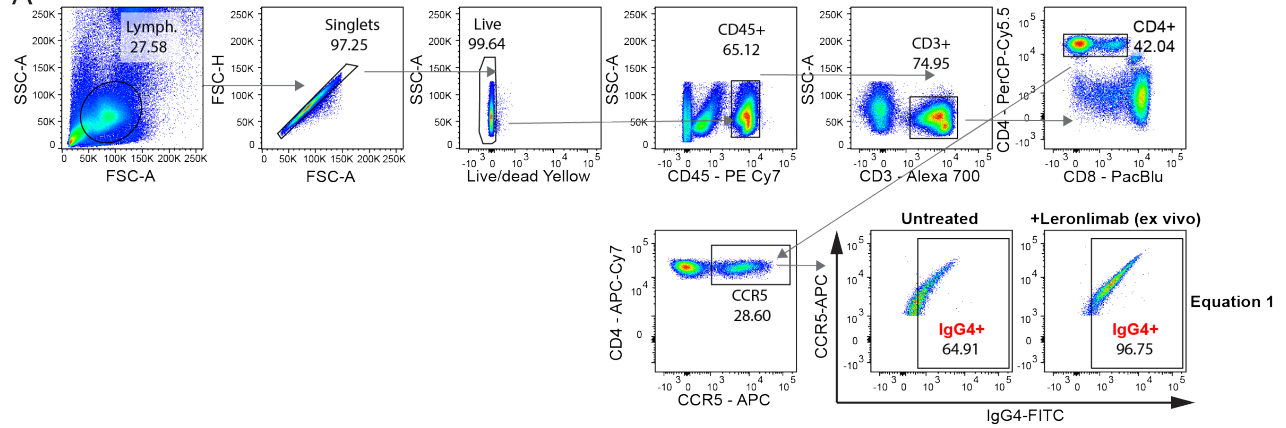
Because CCR5 is involved in multiple pathophysiologic processes, Leronlimab is being explored in clinical trials for both HIV and non-HIV indications. In HIV-positive participants, Leronlimab suppressed plasma viremia after a single 5 or 10 mg/kg SC Leronlimab injection, while Leronlimab monotherapy contributed to the maintenance of viral suppression for over six years^{457,458,460,461}. Moreover, the only two cases of HIV cure occurred after hematopoietic stem cell transplantation (HSCT) with donor cells homozygous for CCR5 Δ 32^{274,278}, while similar studies using donor cells that were wild-type CCR5 or heterozygous CCR5 Δ 32 led to eventual viral rebound²⁷⁷. For this reason, there is intense focus on genetically disrupting *ccr5* to abolish cell surface CCR5 expression in HIV-positive individuals^{452,523}. However, CCR5 has many protective roles, making it difficult to predict the long-term biological effects or consequences of

permanently disrupting *ccr5* expression in humans. A logical approach to reproduce the phenotypic protection seen in homozygous CCR5 Δ 32 individuals is to instead use CCR5-blocking pharmacologic agents such as Leronlimab. Moreover, the therapeutic use of Leronlimab extends beyond HIV treatment due to its diverse roles. CCR5 is expressed in over 95% of triple-negative breast cancers⁴¹⁸ and influences breast cancer progression⁴¹⁵. In a murine model, Leronlimab prevented and reduced breast cancer metastasis suggesting a role for Leronlimab in the treatment of neoplasia⁴¹⁷. As CCR5 is central in inflammatory immune responses, it is currently being studied as a therapeutic for severe and critical SARS-CoV-2 infections^{516,524-526} and graft-versus-host disease (GVHD), where Leronlimab treatment reduced xeno-GVHD after HSCT of human cells to mice⁵²⁷. Finally, Leronlimab is currently in phase 1 and 2 clinical studies to treat metastatic colorectal cancer, nonalcoholic Steatohepatitis, and long COVID after SARS-CoV-2 infection, demonstrating the diverse applicability of this safe and effective CCR5-targeting agent.

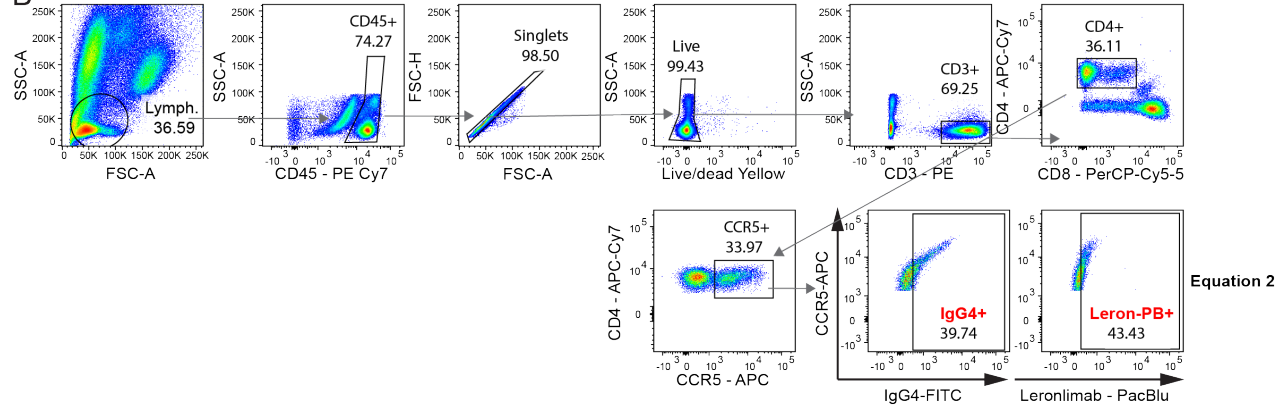
The appeal of monoclonal antibody-based therapeutic agents is growing due to their longer half-life and promising safety profile, and this is reflected in the dramatic rise in FDA approvals and commercial use of antibody treatments in recent years^{528,529}. The CCR5 RO assays described here will be an important study measurement for any CCR5 antibody-based agent, and can be modified for any antibody-based agents that have a clearly defined cell surface protein target. In the case of the anti-CCR5 antibody Leronlimab, the ability to accurately measure CCR5 RO will advance both pre-clinical and clinical studies, furthering our understanding of the immunological impacts of CCR5 for multiple pathophysiologic processes.

2.7. Supplemental Data

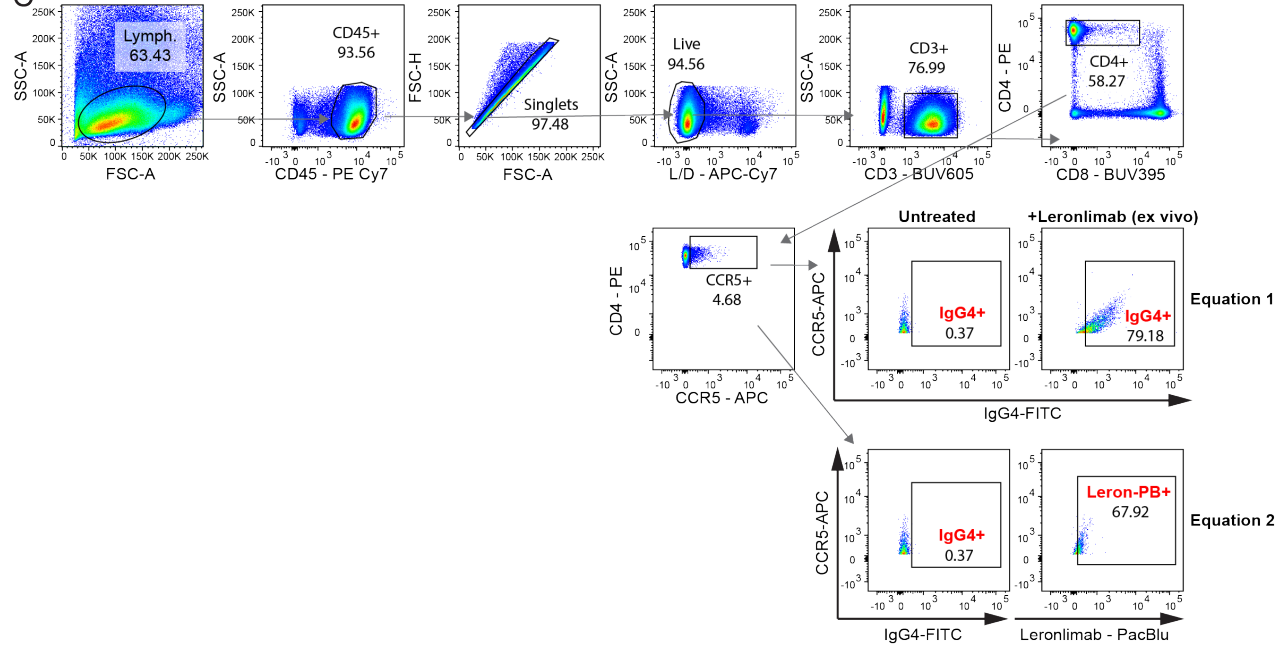
A



B



C



Supplemental Figure 2.1. Representative flow cytometry gating strategies for CCR5 RO using (A) equation 1 and (B) equation 2 in partially-occupied macaques, and (C) equation 1 and 2 in unoccupied human.

CCR5 RO	Species	Tubes	Leronlimab	Leronlimab-PB	Anti-IgG4 FITC	Anti-CCR5 APC
Equation 1	Macaque	1	-	-	-	-
		2	-	-	+	+
		3	5 µg/mL	-	+	+
Equation 2	Macaque	A	-	-	-	-
		B	-	-	-	+
		C	-	5 µg/mL	+	+
Combined equations 1 and 2	Human	H1	-	-	-	-
		H2	-	-	-	+
		H3	-	5 µg/mL	+	+
		H4	5 µg/mL	-	+	+

Supplemental Table 2.1. Staining tubes for CCR5 RO equation 1 and equation 2 used in macaque and human analysis.

<i>BD LSR II</i>			Macaque Equation 1		Macaque Equation 2	
Laser	Filter	Fluorophore Detector	Pre-Surface	Surface	Pre-Surface	Surface
-	-	-	LRM-pure		LRM-pure	
-	-	-				
Violet	450/50	PacBlue		CD8 α (RPA-T8)		LRM-PB
Violet	525/50	BV510				
Violet	560/40	QD655				
Violet	585/42	BV570				
Violet	605/40	BV605		L/D		L/D
Violet	660/40	BV650				
Violet	705/70	BV711				
Violet	780/60	BV786				
Blue	515/20	FITC	IgG4 (HP-6025)		IgG4 (HP-6025)	
Blue	710/50	PerCP Cy5.5		CD4 (L200)		CD8 (SK1)
Green	575/25	PE		CD95 (DX2)		CD3 (SP34-2)
Green	610/20	PE CF594		CD28 (CD28.2)		CD14 (RMO52)
Green	660/40	PE Cy5				
Green	710/50	PE Cy5.5				
Green	780/40	PE Cy7		CD45 (D058-1283)		CD45 (D05-1283)
Red	660/20	APC		CCR5 (3A9)		CCR5 (3A9)
Red	710/50	A700		CD16 (SP34-2)		CD16 (3G8)
Red	780/60	APC-Cy7				CD4 (OKT4)

Supplemental Table 2.2: Antibody table for BD LSR II. Antibodies used in the CCR5 RO staining in macaque and human, with antibody clone names listed in parentheses. LRM-Pure = unconjugated, parental Leronlimab; LRM-PB = Leronlimab-Pacific Blue; L/D = live/dead.

<i>BD FACSymphony</i>			Human RO	
Laser	Filter	Fluorophore Detector	Pre-Surface	Surface
-	-	-	LRM-Pure	
-	-	-		
UV	378/29	BUV395		CD8 α . (RPA-T8)
UV	515/30	BUV496		CD16 (3G8)
UV	740/35	BUV737		CD95 (DX2)
Violet	450/50	PacBlue		LRM-PB
Violet	525/50	BV510		
Violet	586/15	BV570		
Violet	610/20	BV605		CD3 (UCHT1)
Violet	670/30	BV650		
Violet	710/50	BV711		CD14 (M5E2)
Violet	780/60	BV786		
Blue	530/30	FITC	IgG4 (HP-6025)	
Blue	575/25	PE		CD4 (SK2)
Blue	610/20	PE CF594		CD28 (CD28.2)
Blue	710/50	PerCP Cy5.5		
Blue	780/60	PE-Cy7		CD45 (2D1)
Red	670/30	APC		CCR5 (3A9)
Red	710/50	A700		
Red	780/60	APC-Cy7	L/D	

Supplemental Table 2.3: Antibody table for BD FACSymphony. Antibodies used in the CCR5 RO staining in macaques and human, with antibody clone names listed in parentheses. LRM-Pure = unconjugated, parental Leronlimab; LRM-PB = Leronlimab-Pacific Blue; L/D = live/dead.

CHAPTER 3: Antibody-based CCR5 blockade protects macaques from mucosal SHIV

3.1. Abstract

In the absence of a prophylactic vaccine, the use of antiretroviral therapy (ART) as pre-exposure prophylaxis (PrEP) to prevent HIV acquisition by uninfected individuals is a promising approach to slowing the epidemic, but its efficacy is hampered by incomplete patient adherence and ART-resistant variants. Here, we report that competitive inhibition of HIV Env-CCR5 binding via the CCR5-specific antibody Leronlimab protects rhesus macaques against infection following repeated intrarectal challenges of CCR5-tropic SHIV_{SF162P3}. Injection of Leronlimab weekly at 10 mg/kg provides significant but partial protection, while biweekly 50 mg/kg provides complete protection from SHIV acquisition. Tissue biopsies from protected macaques post challenge show complete CCR5 receptor occupancy and an absence of viral nucleic acids. After Leronlimab washout, protected macaques remain aviremic, and adoptive transfer of hematologic cells into naïve macaques does not transmit viral infection. These data identify CCR5 blockade with Leronlimab as a promising approach to HIV prophylaxis and support initiation of clinical trials.

3.2. Authors, Affiliations, Contributions, and Acknowledgements

Authors: Xiao L. Chang^{1,2,†}, Gabriela M. Webb^{1,2,†}, Helen L. Wu^{1,2}, Justin M. Greene¹, Shaheed Abdulhaqq¹, Katherine B. Bateman¹, Jason S. Reed¹, Cleiton Pessoa¹, Whitney C. Weber¹, Nicholas Maier¹, Glen M. Chew³, Roxanne M. Gilbride¹, Lina Gao², Rebecca Agnor², Travis Giobbi², Jeffrey Torgerson², Don Siess², Nicole Burnett², Miranda Fischer², Oriene Shiel², Cassandra Moats², Bruce Patterson⁴, Kush Dhody⁵, Scott Kelly⁶, Nader Pourhassan⁶, Diogo M.

Magnani⁷, Jeremy Smedley², Benjamin N. Bimber^{1,2}, Nancy L. Haigwood¹, Scott G. Hansen¹, Timothy R. Brown⁸, Lishomwa C. Ndhlovu^{9,*}, Jonah B. Sacha^{1,2,*}

[†]These authors contributed equally

* These authors jointly supervised this work

*Correspondence: Jonah B. Sacha (sacha@ohsu.edu)

Lishomwa Ndhlovu (lnhlovu@med.cornell.edu)

Affiliations: ¹Vaccine & Gene Therapy Institute and ²Oregon National Primate Research Center, Oregon Health & Science University, Portland, OR, ³University of Hawaii, Honolulu, HI, USA, ⁴IncellDX, Menlo Park, CA, USA, ⁵Amarex Clinical Research LLC, Germantown, MD, USA, ⁶CytoDyn Inc., Vancouver, WA, USA, ⁷MassBiologics of the University of Massachusetts Medical School, Boston, MA, USA, ⁸Palm Springs, CA, USA, ⁹Department of Medicine, Division of Infectious Disease, Weill Cornell Medicine, New York, NY, USA.

Contributions: XLC, GMW, HLW, JMG, SA, KBB, JSR, CP, WCW, NM, GMC, RMG, TG, JT, BNB, and DS planned and performed experiments, assisted by BP, NP, SK, DMG, NLH, SCH, DMM, TRB, LCN, KD, and JBS. XLC managed the animal study. NB, MF, OS, CM, and JS managed the animal care and procedures. LG and RA performed statistical analyses. JBS conceived of and supervised all experiments, analyzed and interpreted data, and wrote the paper, assisted by XLC, GMW, and HLW.

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of the Hawaii Center for AIDS, Director, Dr. Cecilia Shikuma and the Leukapheresis team at Queens Medical Center for support and commitment in specimen sampling from the study participant. Reagents used in these studies were provided by the NIH Nonhuman Primate Reagent Resource. The following reagent was obtained through the AIDS Reagent Program, Division of AIDS, NIAID, NIH: human IL-2 from La Roche and the HIV-1 60 International Isolate Panel. We acknowledge the macaques that contributed to this study. This manuscript is dedicated to Timothy Ray Brown, who inspired, supported, and materially contributed to this research. Macaque icons used in Figure 3.2a were created on BioRender.com. This work is supported by National Institute of Allergy and Infectious Diseases (NIAID) grants R01 AI129703, R01 AI54559, and R21 AI54559 to JBS, K01 OD026561 to JMG, U24 AI126683 to DMM, and the Oregon National Primate Research Center Core grant P51 OD011092 from the National Institutes of Health, Office of the Director.

3.3. Introduction

Pre-exposure prophylaxis (PrEP) is effective for HIV prevention, where drug concentrations in the blood are strongly correlated with protection^{296,297}. However, the efficacy of PrEP is hindered by incomplete drug adherence⁵³⁰ and the global rising rate for ART drug-resistance³¹⁵, resulting in incidents of multidrug-resistant HIV infections despite high adherence to PrEP^{312,531}. Potent broadly neutralizing antibodies (bNAbs) represent a potential alternative to ART-based PrEP. Yet, bNAbs also remain susceptible to antibody-resistant HIV strains, and alternate preventative modalities with complementary mechanisms of action are needed^{370,372,532,533}.

HIV can utilize either the CCR5 or CXCR4 co-receptor for entry into CD4⁺ T cells, yet CCR5 is the primary co-receptor used during transmission of HIV^{54,167,313}. Accordingly, individuals with a natural genetic deficiency in CCR5 via a homozygous 32-base pair deletion in *ccr5* (CCR5^{Δ32/Δ32}) are highly resistant to HIV infection^{421,422}. Further underscoring the central role of CCR5 in viral spread *in vivo*, the only two documented cases of HIV cure occurred in the setting of allogenic stem cell transplantation using CCR5^{Δ32/Δ32} donor cells^{534,535}. CCR5 therefore represents an ideal target for HIV prevention, yet small-molecule CCR5 inhibitors like Maraviroc have yielded disappointing results as PrEP agents^{438,536} and alternate CCR5-specific strategies are needed.

Leronlimab is an anti-CCR5 humanized IgG4 antibody currently in clinical trials for HIV therapy as a once-weekly, subcutaneous injection with a favorable safety profile in over 1,000 volunteers across multiple studies⁵³⁷. In contrast to Maraviroc, which interferes with HIV Env attachment to CCR5 by allosteric modulation, Leronlimab binds to the same CCR5 extracellular loop-2 and N-terminus domains used by HIV Env, thereby directly outcompeting HIV for binding to CCR5⁴⁵⁴. A single 10 mg/kg dose of Leronlimab lowered plasma viral loads by approximately 100-fold for two weeks in HIV-positive individuals^{457,458,460}. When utilized as once weekly self-administered subcutaneous monotherapy, Leronlimab maintained undetectable plasma viral loads in HIV-infected individuals for over two years⁴⁶¹, with the longest successful monotherapy patients now reaching over six years of continual use (Chang et al, manuscript in preparation). The ability to self-administer Leronlimab at home as a subcutaneous injection augurs well for its adherence profile as a PrEP agent. Finally, in both single dose and multiyear monotherapy studies, no viral co-receptor switching occurred, underscoring the high genetic barrier to developing Leronlimab resistance. Based on these antiviral, safety, and user-friendly

characteristics, we hypothesized that Leronlimab could be utilized as an effective PrEP strategy with the potential for high patient usage and set out to establish the efficacy of Leronlimab-based PrEP in the macaque model of HIV.

3.4. Materials and Methods

HIV and SHIV stocks. HIV-1 isolates were obtained from the NIH AIDS Reagent Program, with the majority from the HIV-1 60 International Isolate Panel (Cat #11412). The SHIV_{SF162P3} stock (harvest 4, dated 9/16/2016, 173.3 ng/ml p27, 1×10^9 vRNA copies/ml, 2.67×10^5 TCID₅₀/ml in TZM-bl cells, 1.28×10^3 TCID₅₀/ml in rhesus PBMC) used in the PrEP animal study was generated, characterized, and kindly provided by Nancy Miller.

HIV and SHIV *in vitro* infection assays. Human peripheral blood mononuclear cells (PBMC) were first depleted of CD8⁺ T cells with Human CD8 Microbeads (Miltenyi) and then sequentially enriched for CD4⁺ T cells with Human CD4 Microbeads (Miltenyi) following manufacturer's instructions. The resulting CD4⁺CD8⁻ T cells were incubated at 2×10^6 cells/mL in R15-100 media (RPMI1640 with antibiotic/mycotic, 15% fetal bovine serum, and 100 U/mL IL-2) and activated for 24 h with a stimulating cocktail containing CD3, CD49d, CD28 antibodies (BD Biosciences), and Staphylococcal enterotoxin B (Toxin Technologies, Inc.). After 24 h, cells were washed two times and incubated for 2-3 additional days in R15-100 before viral infection. At day 0 of infection, 5×10^5 cells were incubated with or without the desired concentration of Leronlimab for 1 h at 37°C. Next, cells were infected with the desired HIV isolate from Supplemental Table 3.1 by spinoculation for 2 h at 1,200 x g at room temperature (RT). Cells were washed four times with R15-100 to remove free viruses and cultured with R15-

100 media plus the same concentration of Leronlimab used during the pre-treatment step. An additional 5×10^5 cells were left uninfected and kept in culture as the uninfected control. Cultures were maintained by replacing 50% of the culture with new media containing the desired concentration of Leronlimab every other day for 5 days, when cells were harvested for intracellular p24 staining by flow cytometry.

Rhesus macaque (RM) PBMC were depleted of CD8⁺ T cells by staining with NHP CD8-PE (Miltenyi) followed by anti-PE Microbeads (Miltenyi) then subsequently enriched for CD4⁺ T cells with NHP CD4 Microbeads (Miltenyi), following the manufacturer's instructions. Purified RM CD4⁺ cells were activated and maintained in culture similarly to human CD4⁺ T cells, as described above. For the spreading assay with HIV-1 Ba-L, HIV-1 LAI, and SHIV_{SF162P3}, CD4⁺ T cells were isolated, activated, infected, and cultured as described above but with the following changes. HIV-1 Ba-L, HIV-1 LAI, and SHIV_{SF162P3} were used to infect at a multiplicity of infection (MOI) of 1×10^{-5} (FFU/cell) and kept in culture for 6 days before p24 or p27 intracellular staining. For detection of intracellular HIV p24 or SHIV p27 by flow cytometry, cells were stained for CD3, CD4, CD8, and amine-reactive dye for viability for 30 min at RT in the dark. Cells were washed once with PBS, spun down at $830 \times g$ for 4 min, and fixed with 2% paraformaldehyde (PFA) for 30 min in 4°C. Afterwards, cells were washed once with FACS buffer (10% bovine growth serum in PBS) and stained with p24 or p27 antibodies in 100 μ L of Permeabilization Medium B (Thermofisher) for 1 h at RT in the dark. Cells were washed once with FACS buffer and fixed again with 2% PFA for more than 30 min before collecting on LSR-II instrument and FACSDIVA version 6.1 (BD Biosciences, Franklin Lakes, NJ). Data was analyzed using FlowJo v10 (Tree Star) by gating on singlet, live, CD3⁺, CD8⁻, and p24⁺ or p27⁺.

Human blood donors. Healthy human donor whole blood was purchased from Innovative Research and processed in-house to PBMC by density gradient centrifugation using Ficoll-Hypaque. Blood was collected with K2 EDTA anticoagulant and tested negative for the following viral markers: HIV-1 RNA, antibodies to HIV, antibodies to Hepatitis C Virus (HCV), HCV RNA, Hepatitis B Virus (HBV) DNA, Hepatitis B Surface Antigen (HbsAg), and Syphilis. CCR5 expression was confirmed via flow cytometry. Leukapheresis samples were collected from TRB following informed consent under the Research and Institutional Review Committee (RIRC) of the Queens Medical Center approval number RA-2014-307 of the H026 Study protocol. TRB consented to publish identity in this study. Individual consented to identification in this study.

Quantitation of CCR5 expression levels. To measure the frequency of CCR5-expressing cells, PBMCs were incubated with 5 µg/mL of unlabeled Leronlimab for 30 min at RT in the dark, and then washed once with PBS. Anti-human IgG4 was used as a secondary antibody to detect surface-bound Leronlimab for 30 min at RT in the dark. Cells were washed once with FACS buffer and once with PBS, and then stained for CD3, CD4, CD8, CCR5 (via antibody clone 3A9, which is specific to a distinct, non-competitive CCR5 epitope than Leronlimab), and amine-reactive dye for 30 min at RT in the dark. Cells were washed twice with PBS and fixed with 2% PFA before collecting through the LSR-II instrument and FACSDIVA version 6.1. Samples were analyzed by gating on singlet, live, CD3⁺, CD4⁺/CD8⁻, and CCR5⁺ (via clone 3A9) and/or human IgG4⁺ events. The number of CCR5 molecules on the cell surface was measured with quantitative cytometry using the Quantum Molecules of Equivalent Soluble Fluorochrome

(MESF) kit (Bangs Laboratories, Inc). PE-conjugated Leronlimab used to quantify surface CCR5 expression and PE-labelled microspheres for standard curve generation were provided by IncellDX. Phenotypic staining was done using CD3-, CD4-, CD8-, CD14-, CD16-specific antibodies used as described above. T cell memory subset determination was defined as Central Memory (Human: CCR7+ CD45RA-, RM: CD28+ CD95+), Effector Memory (Human: CCR7- CD45RA-, RM: CD28- CD95+), and Naïve (Human: CCR7+ CD45RA+, RM: CD28+ CD95-). Gating was done using FlowJo v10. MFI was used to attain MESF according to the manufacturer's protocol.

Leronlimab CCR5 receptor occupancy (RO). To measure the percentage of CCR5 RO on the surface of CD4+ T cells, we developed the RO equation shown in Supplemental Figure 3.5.

$$\% \text{ RO} = \frac{\% \text{ IgG4}}{\% \text{ IgG4} + \% \text{ Leronlimab-PB}} \times 100\%$$

The equation measures unoccupied CCR5 receptors by using Pacific Blue-conjugated Leronlimab (termed Leronlimab-PB). CCR5 RO is defined as the percentage of cells CCR5+ (measured by clone 3A9) and Leronlimab+ (measured by anti-human IgG4) divided by the percentage of cells CCR5+ and Leronlimab+ (measured by the sum of anti-human IgG4 and Leronlimab-PB) cells following incubation with a saturating concentration of Leronlimab-PB. This method is based on RO assays for anti-PD-1 antibodies in clinical trials⁵²¹.

PBMC or single cells from tissue homogenates ($0.3 - 1 \times 10^6$) were stained with anti-human IgG4 for 30 min at RT in the dark. Next, cells were washed once with FACS buffer and three times with PBS and then stained with CD45, CD3, CD4, CD8, CD16, CD14, amine-

reactive dye, CCR5 (via antibody clone 3A9), and Leronlimab-PB for 30 min at RT in the dark. Finally, cells were washed twice with PBS and fixed with 2% PFA for more than 30 min before collecting on LSR-II instrument and FACSDIVA version 6.1. Using FlowJo v10, cells were gated on CD45⁺, singlet, live, CD3⁺, CD4⁺, and CCR5⁺ (via 3A9 staining) events. The CD4⁺ CCR5⁺ population was further gated on human IgG4⁺ or Leronlimab-PB⁺ events.

Rhesus macaques (RM). All study RM were housed at the Oregon National Primate Research Center (ONPRC) in ABSL-2+ rooms with autonomously controlled temperature, humidity, and lighting. At assignment, all study RM were free of cercopithicine herpesvirus 1, D-type simian retrovirus, simian T-lymphotrophic virus type 1, and *Mycobacterium tuberculosis*. RM were typed for the MHC alleles Mamu-A*01, Mamu-A*02, Mamu-B*17, and Mamu-B*08, with Mamu-B*17 and/or -B*08 positive animals excluded when possible or placed into control groups when not possible to exclude biasing results. All attempts were made to pair housed RM during the study period. When RM were single cage-housed due to infection status, they had visual, auditory and olfactory contact with other animals, and an enhanced enrichment plan was designed and overseen by RM behavior specialists. RM were fed commercially prepared primate chow twice daily and received supplemental fresh fruit or vegetables daily. Fresh, potable water was provided via automatic water systems. RM were sedated with ketamine HCl or Dexmedetomidine for procedures, including subcutaneous Leronlimab administration, venipuncture, bronchoalveolar lavage, tissue biopsy, and SHIV challenge. For spleen and mesenteric lymph node biopsies, animals were sedated with isoflurane. At scheduled endpoints, RM were euthanized with sodium pentobarbital overdose (>50 mg/kg) and exsanguinated via the distal aorta, and tissue collection at necropsy was performed by a certified veterinary pathologist.

RM care and all experimental protocols and procedures were approved by the ONPRC Institutional Animal Care and Use Committee (IACUC). The ONPRC is a Category I facility. The Laboratory Animal Care and Use Program at the ONPRC is fully accredited by the American Association for Accreditation of Laboratory Animal Care (AAALAC) and has an approved Assurance (#A3304-01) for the care and use of animals on file with the NIH Office of Laboratory Animal Welfare. The IACUC adheres to national guidelines established in the Animal Welfare Act (7 U.S.C. Sections 2131–2159) and the Guide for the Care and Use of Laboratory Animals (8th Edition) as mandated by the U.S. Public Health Service Policy.

For the Leronlimab PrEP study, a total of 18 adult RMs were used and divided between three experimental groups: (1) six RMs in the control group, (2) six RMs in the 10 mg/kg weekly Leronlimab-treated group, and (3) six RMs in the 50 mg/kg every two weeks Leronlimab-treated group. The three groups were gender matched, with two females and four male RMs in each group. See Supplemental Table 3.3 for the gender, age, and MHC type of these animals. Animals received their first injection of Leronlimab one week before the initiation of viral challenges, and continued to receive Leronlimab until study week seven, for a total of nine Leronlimab injections for group 1 (10 mg/kg-treated group) and a total of five Leronlimab injections for group 2 (50 mg/kg-treated group). At study week zero, all three groups received their first intrarectal (IR) SHIV_{SF162P3} challenge with a 1:400 dilution (3.2 TCID₅₀ measured from rhesus PBMC, dose based on *in vivo* macaque titration experiments, Nancy Miller personal communication) delivered via atraumatic installation into the rectum with a needleless syringe. Viral challenge then continued every week until confirmed infection or until study week seven for a total of eight consecutive weekly IR challenges. Animals were euthanized as described above, with the timing dictated by the following guidelines: (1) ten weeks after acquisition of SHIV_{SF162P3} infection,

which was confirmed by plasma viremia, or (2) eight weeks after complete washout of plasma Leronlimab and loss of Leronlimab CCR5 RO on CD4⁺ T cells in blood. Three SHIV-naïve RMs served as adoptive transfer recipients for tissue homogenates from PrEP RM as described. All Leronlimab utilized in these studies was clinical-grade material provided by CytoDyn at a concentration of 175 mg/mL.

Processing of blood and tissue. Whole blood was collected into EDTA-treated or non-anticoagulant tubes (BD Biosciences). Blood in EDTA-tubes was assessed for complete blood counts using an ABX Pentra 60 C+ Hematology Analyzer. Blood in non-anticoagulant tubes was spun down at 1,860 x g for 10 min to separate serum from clotted blood, and then assessed for serum chemistry values using an ABX Pentra 400 Chemistry Analyzer. PBMC and plasma were isolated from whole blood in EDTA-treated tubes by density gradient centrifugation using Ficoll-Hypaque. Bronchoalveolar lavage fluid was collected in PBS and filtered through 70-µm cell strainers. Lymph node and spleen were collected in RPMI 1640 containing 10% FBS (R10), diced into tiny pieces with a scalpel, and forced through 70-µm cell strainers to collect single-cell suspensions. Bone marrow was collected in R10, pelleted by spinning at 830 g for 4 min. Cell pellet was resuspended by vigorously shaking in PBS containing 2nM EDTA. Cell suspension was spun down again at 830 g for 4 min and resuspended in 70% isotonic Percoll (GE healthcare, Buckinghamshire, United Kingdom) before underlaying in 37% isotonic Percoll. Cells were spun at 500 g for 20 minutes with brake. Mononuclear cells in the center interface were collected and washed in R10. Duodenum and colon were collected in R10 and diced into tiny pieces with a scalpel. Tissues were placed into a 50 mL conical tube containing R3 (RPMI 1640 and 3% FBS) with 0.5 M EDTA, and then incubated in 37°C with 225 rpm shaking for 30

min to remove mucus coating. After 30 min, cells were poured over a tea strainer and sequentially washed three times with Hank's Buffered Salt Solution (HBSS) to remove EDTA and mucus coating. Tissues were returned into a 50 mL conical tube containing R3, 0.2 mg/mL collagenase (Sigma-Aldrich, St. Louis, MO) and 0.2 mg/mL DNase I (Roche, Indianapolis, IN), and then incubated in 37°C with 225 rpm shaking for 1 h. Digested tissues were filtered through a 70-µm cell strainers. Cell flow throughs were spun at 840 g for 4 min. Pellet was resuspended in 70% isotonic Percoll and underlaid in 37% isotonic Percoll, before spinning at 500 g for 20 min with brake. Mononuclear cells in the center interface were collected and washed in R10.

To quantify Leronlimab concentration and ADA in the blood, plasma was heat inactivated by incubation at 56°C for 30 min, and then spun for 20 min at 12,000 x g to pellet residual debris. Resulting supernatant was transferred to a new tube and stored at -80°C until assayed. To quantify Leronlimab concentration in tissues, tissues were finely diced and 10 – 1,000 mg of tissue of interest were placed into Lysing Matrix tubes (MP Biomedicals) and 300-500 µL of complete EDTA-free Protease Inhibitor Cocktail (Sigma Aldrich) in PBS was added. Tissue disruption was achieved by beating in a Beadbeater (Biospec) device for three cycles of 1 min beating and 1 min on ice. Supernatant from the tissue homogenate was transferred to a new tube and spun for 20 min at 12,000 x g to pellet residual debris. Resulting supernatant was transferred to a new tube and stored at -80°C until assayed.

Viral nucleic acid detection. Nucleic acid from plasma and PBMC cell pellets were extracted using the Maxwell 16 instrument (Promega, Madison, WI) following the manufacturer's protocol, which uses the LEV Viral Nucleic Acid Kit for plasma and the LEV Whole Blood Nucleic Acid kit for cell pellets.

Nucleic acid from tissues were extracted by first placing tissues in Lysing Matrix tubes (MP Biomedicals) with 1 mL Tri-reagent (Molecular Research Center, TR-118). Then, vortexed to soaked tissues in Tri-reagent and placed on wet ice. Tissues were grinded using the MagNA Lyser rotor (Roche Life Science) for 1-2 cycles depending on the size of the tissue, alternating between the MagNA Lyser rotor and wet ice. Tissue homogenates were briefly spun down to pellet the beads and supernatant was pipetted out for nucleic acid extraction. Supernatant was mixed with 1/10 Bromochloropropane (BCP) to Tri-reagent volume. Mixture was vortexed and incubated for 5 min in room temperature, before spinning at 12,000 g for 15 min at 4°C to achieve phase separation. For RNA extraction, the upper aqueous layer was pipetted into a new tube containing 12 µL of glycogen (Thermo Scientific) and 0.5 mL isopropanol. Sample was inverted to mix and spun at 15,000 g for 10 min in room temperature. Isopropanol was carefully discarded without losing pellet, and then washed with 0.7 mL 75% ethanol twice by spinning at 15,000 g for 10 min at room temperature. After discarding the ethanol, RNA pellet was dried in room temperature for 10-15 min. Finally, RNA resuspension buffer (10 mM Tris, pH 8) was added to the RNA-containing tube and incubated in 37°C heat block for 15 min to elute. Resuspended RNA was stored in -80°C until assayed. For DNA extraction, the interphase and organic layers, not used for RNA extraction, were mixed with 0.5 mL of DNA extraction buffer, containing 4 M Guanidine Thiocyanate (Sigma), 50 mM Sodium Citrate (Sigma), and 1 M Tris base. Reaction was vortexed and incubated for 5 min in room temperature, before spinning at 12,000 g for 15 min 4°C to achieve phase separation. DNA-containing upper aqueous layer was pipetted into a new tube containing 12 µL of glycogen (Thermo Scientific) and 0.4 mL isopropanol. The remaining steps follow RNA extraction protocol described above, with the exception of the DNA resuspension buffer (10 mM Tris, pH 9) used to elute the DNA pellet.

Viral copies were measured by RT-qPCR or qPCR that targets a highly conserved sequence of Gag⁵¹⁸. The assays used the SGAG21 forward primer (GTCTGCGTCATPTGGTGCATTC), SGAG22 reverse primer (CACTAGKTGTCTCTGCACTATPTGTTTTG), and pSGAG23 probe (5'-6-carboxyfluorescein [FAM]-CTTCPTCAGTKTGTTCACCTTCTCTTCTGCG-black hole quencher [BHQ1]-3') (Supplemental Table 3.4). All viral detection assays were performed by members of the ONPRC Molecular Virology Core, who were blinded in the treatment conditions of each animal.

To quantitate SHIV viral RNA in plasma, RT-qPCR reactions were performed using the TaqMan Fast Virus 1-Step Master Mix (Applied Biosystems). The reactions used the total RNA extracted from 300 μ L of plasma, with 900 nM SGAG21, 900 nM of SGAG22, and 250 nM pSGAG23 in a final volume of 30 μ L. Viral RNA copies per reaction were calculated with a standard curve created by using in vitro transcribed SIVgag RNA that was serially diluted in 5 ng/ μ L yeast tRNA (Sigma R5636). SIV-positive plasma RNA was used as positive control and nuclease free water was used as negative control. Reactions were run with the Applied Biosystems QuantStudio 6 Flex instrument (Life Technologies) using the following thermal conditions: 50°C for 5 min; 95°C for 20 sec; [95°C for 3 sec, 60°C for 30 sec] x 45 cycles. The limit of quantification for this assay is 50 copies/mL.

To detect SHIV viral RNA copies in cell pellets and tissues, a two-step RT-qPCR reaction was performed⁵³⁸, where 2.5 μ g RNA was synthesized to complementary DNA (cDNA) using 20 U Superscript II RT (ThermoFisher/Invitrogen), 5 mM MgCl₂, 0.5 mM dNTPs, 1 mM dithiothreitol (DTT), 150 ng random hexamers, 1x TaqMan PCR buffer (with 0.05% gelatin and 0.02% Tween-20), and 20 U RNAaseOut in a final volume of 30 μ L. The reaction was performed with an Applied Biosystems ABI 9700 instrument using the following thermal

conditions: 25°C for 15 min, 42°C for 40 min, 90°C for 15 min, 25°C for 30 min, and 5°C hold. Following reverse transcription, qPCR was performed by adjusting the reaction to contain 1.25 U Platinum Taq (Applied Biosystems), 600 nM of SGAG21, 600 nM of SGAG22, 100 nM pSGAG23, 1x TaqMan PCR II buffer, 4.5 mM MgCl₂, and 50 nM ROX passive reference dye, in a final volume of 50 µL. Viral RNA copies per reaction were calculated with a standard curve created by using in vitro transcribed SIVgag RNA that was serially diluted in 100 ng/µL yeast tRNA (Sigma R5636). Reactions were performed in an Applied Biosystems ABI 7500 instrument using the following thermal condition: 95°C for 2 min; [95°C for 15 sec, 60°C for 1 min] x 45 cycles.

To detect SHIV viral DNA copies in cell pellets and tissues, qPCR reactions were done using Taqman Fast Advanced Master Mix (Life Technologies). Extracted DNA was first heated at 95°C for 5 min then placed on ice. The reactions used 2.5 µg of DNA, with 600 nM of SGAG21, 600 nM of SGAG22, and 100 nM pSGAG23. Viral DNA copies per reaction were calculated with a standard curve created with linearized plasmid DNA containing the SIVgag sequence that was serially diluted in TE buffer containing 2.5 ng/mL SIV-negative rhesus genomic DNA as carrier. Reactions were run with the Applied Biosystems QuantStudio 6 Flex instrument (Life Technologies) using the following thermal conditions: 50°C for 2 min; 95°C for 20 sec; [95°C for 1 sec, 60°C for 20 sec] x 45 cycles. The limit of quantification for this assay is 10 copies/million cells for cell pellets and 7 copies/million cells for whole tissue biopsies.

Leronlimab measurement in plasma and tissues. Enzyme-linked immunosorbent assay (ELISA) was used to detect free Leronlimab in plasma. Half-area 96-well Costar Assay Plates (Corning) were coated with the anti-idiotypic antibody PA-22, provided by CytoDyn, at 1.5

µg/mL in carbonate-bicarbonate buffer (ThermoFisher) and incubated overnight. Plates were washed three times with PBS-T (PBS + 0.1% Tween-20) and blocked with blocking buffer (PBS + 0.4% Tween-20 + 10% BSA) for at least 2 h at RT. Leronlimab concentration was calculated with a standard curve created with a serial titration of Leronlimab diluted in blocking buffer with a range of 4.7 ng/mL to 300 ng/mL. Heat-inactivated plasma samples were also diluted with blocking buffer. After incubating for 30 min at RT, plates were washed three times with 0.5 M NaCl in PBS and incubated with 1:20,000 dilution of mouse anti-human IgG₄ pFc'-horseradish peroxidase (HRP) (Southern Biotech) in blocking buffer for 30 min at RT. Plate was washed three times with PBS-T and developed for 2 min using 3,3',5,5'-Tetramethylbenzidine (TMB) substrate (Southern Biotech). Reaction was stopped with 1 N H₂SO₄. Plates were read on the Synergy HTX Multi-Mode Microplate Reader (BioTek) and data was collected using software Gen5 v3.09 at two absorbance wavelengths: 650 nm for the developing reaction and 450 nm for the developed reaction after the reaction was stopped with 1 N H₂SO₄. Final OD was determined by OD_{450 nm} minus OD_{650 nm}. The limit of detection for the assay is 22.5 ng/ml. Leronlimab in tissue was quantified in supernatants prepared from tissue homogenates by ELISA as described above. Further, the mass of the total protein in the collected tissues was determined by the Pierce Coomassie Plus Bradford Assay (ThermoFisher) following manufacturer's instructions. Tissue concentration of Leronlimab is reported as the ng of Leronlimab per mg of total protein.

Measurement of Leronlimab Antidrug Antibodies (ADA). Half-area 96-well Costar Assay Plates (Corning) were coated with 2 µg/mL Leronlimab (Cytodyn, Vancouver, WA). Plates were washed with PBS-T three times and blocked with blocking buffer for 2 h at RT. Plates were then washed three times with PBS-T. Heat-inactivated plasma samples were serially-diluted in blocking buffer, added to the plates in duplicate, and incubated at RT for 30 min. Plates were

then washed three times with 0.5M NaCl in PBS. To determine ADAs from RM, a secondary antibody recognizing rhesus IgG (anti-rhesus IgG1/3[1B3]-HRP, NHP Reagent Resource) and conjugated to HRP was added. Plates were incubated at RT for 30 min, then washed three times with PBS-T. TMB solution (Southern Biotech) was added at RT for 2 min and the reaction was stopped with 1N H₂SO₄. Absorbance was read at 450 nm on a Synergy plate reader (BioTek).

ADA titers are defined as the reciprocal of the highest dilution of the sample that yields a positive result (*e.g.* dilution of 1/2460 = titer of 2460). A positive result was defined as twice that of background values.

Env Sequencing. Viral sequencing and analysis were adapted from previously published genome-wide SIVmac239 sequencing protocols. Viral RNA was isolated from virus stocks and plasma samples using QIAamp MinElute Virus Spin Kit following manufacturer's instructions. Complementary DNA was generated with the SuperScript III One-Step RT-PCR with Platinum Taq (ThermoFisher). SHIV *env* Forward primer (GGCATAGCCTCATAAAATATCTG) and the SHIV *env* Reverse primer (ACAGAGCGAAATGCAGTGATATT) were used to amplify a ~4.5kb amplicon spanning the *env* gene (Supplemental Table 3.4). RT-PCR reactions were performed on Eppendorf Mastercycler Pro S Thermal Cyclers using the following thermal conditions: 50°C for 30 min; 94°C for 2 min; [94°C for 15 sec, 60°C for 1 min, 68°C for 4 min] x 2 cycles; [94°C for 15 sec, 58°C for 1 min, 68°C for 4 min] x 2 cycles; [94°C for 15 sec, 60°C for 1 min, 68°C for 4 min] x 45 cycles; 68°C for 10 min; and hold at 4°C. The resulting 4.9 kb fragments were purified on a 1% agarose gel and purified using NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel). Dual-indexed Illumina MiSeq-compatible libraries were then prepared using the Nextera XT DNA Sample Prep Kit, and purified with AMPure XP magnetic beads

(Beckman Coulter). Libraries were analyzed on an Agilent 2100 Bioanalyzer using the HS DNA kit (Agilent), normalized to 2 nM, pooled at an equimolar ratio, and sequenced in parallel on the Illumina MiSeq. Sequence reads were processed^{539,540}, where raw data were trimmed using Trimmomatic version 0.39 and aligned to the SHIVSF₁₆₂ reference sequence (GenBank Accession No. KF042063.1) using BWA-MEM version 0.7.17-r1188^{541,542}. All bases of the alignment were evaluated and single nucleotide polymorphisms (SNPs) and deletion/insertion polymorphisms were called for bases with a quality score above 17. Importantly, the identity of the associated read was retained for each SNP, which allowed the phase of SNPs to be considered. This information allowed amino acid translations to be calculated based on the sequence of each individual read, as opposed to the consensus sequence. SNP analysis and visualization of mutations was performed using the SequenceAnalysis module, written for LabKey Server 21.3⁵⁴³.

T cell Assays. SHIV-specific CD8⁺ T cell responses in PBMC were measured by flow cytometric intracellular cytokine staining (ICS)⁴. 1×10^6 PBMCs were incubated with overlapping 15-mer peptide pools spanning SIVmac239 Gag or Vif open reading frame and co-stimulated with CD28 and CD49d antibodies (eBiosciences) for 1 h, followed by the incubation with Brefeldin A (Sigma-Aldrich) for an additional 8 h. Stimulation with rhesus cytomegalovirus (RhCMV) lysate served as a positive control while incubation without antigen served as background control. Cells were surface stained with antibodies for CD3, CD4, CD8, and amine-reactive dye, fixed with 2% PFA, permeabilized with BD FACS Lysing Solution (BD Biosciences), and stained intracellularly for IFN- γ , TNF- α , and CD69. Samples were collected on LSR-II instrument and FACSDIVA version 6.1 and analyzed with FlowJo v10 (Tree Star) by

gating on singlet, live, CD3⁺, CD4⁻, and CD8⁺ cells. Responding CD8⁺ T cells were measured by Boolean gating on cells that are CD69⁺/TFN- α ⁺ and/or CD69⁺/IFN- γ ⁺.

Adoptive transfer. To confirm sterilizing protection, cells from the infected control animals (all six animals) or uninfected Leronlimab-treated animals (four animals in the 10 mg/kg group and all six animals in the 50 mg/kg group) were adoptively transferred into one SHIV-naïve RM per animal group, as detailed in Supplemental Table 3.5. Cells were prepared an hour before infusion by resuspending in 1 mL of Hank's Buffered Salt Solution (HBSS) with 15 U/mL heparin. Recipient RM were sedated with ketamine HCl (8-20 mg/kg) or Telazol (2-5 mg/kg) and prophylactically treated with Benadryl (5 mg/kg) prior to infusion of donor cells. Donor cells were slowly infused intravenously with an infusion pump at a maximum rate of 22 ml/kg/hr. Animals were monitored for at least 2 h for post-procedural complications.

Antibodies. The following conjugated antibodies were used in these studies: a) from BD Biosciences, D058-1283 (CD45; PE Cy7; 1:100; cat# 561294), SP34-2 (CD3; Alexa 700; 1:100; cat# 557917), SP34-2 (CD3; PE; 1:20; cat# 552127), LP200 (CD4; PerCP-Cy5.5; 1:40; cat# 552838), RPA-T8 (CD8; PacBlu; 1:40; cat# 558207), SK1 (CD8; TruRed; 1:100; cat# 341051) 3GB (CD16; Alexa 700; 1:100; cat# 560713), 25723.11 (IFN- γ ; APC; 1:100; cat# 502512), 6.7 (TNF- α ; PE; 1:100; cat# 554513), 3A9 (CCR5; APC; 1:100; cat# 560748), SK1 (CD8; BUV737; 1:50; cat# 612754), L200 (CD4; BUV395; 1:200; cat# 564107), FN50 (CD69; PE-Texas Red; 1:100; cat# 562617), SP34-2 (CD3; Pacific Blue; 1:100; cat# 558124), b) from BioLegend, OKT4 (CD4; APC-Cy7; 1:100; cat# 305612), RPA-T4 (CD4; APC; 1:100; cat# 300537), c) from Beckman Coulter, RMO52 (CD14; PE-Texas Red; 1:40; cat# IM2707U), KC57 (HIV Gag p24; FITC; 1:100; cat# 6604665), d) from Sigma, HP-6025 (IgG4; FITC; 3:100; cat# F9890), and e)

from SouthernBiotech, HP6023 (mouse anti-human IgG₄ pFc'; HRP; 1:20,000; cat# 9190-05), f) from NHP Reagent Resource, 1B3 (anti-rhesus IgG1/3; HRP; 1:5000). The following unconjugated antibodies were used: a) 55-2F12 (SIV Gag p27; NIH AIDS Research and Reference Reagent Program), conjugated in-house to FITC using PierceTM FITC Antibody Labeling Kit (ThermoFisher) and used at approximately 1:100 depending on the efficacy of conjugation, b) PA-14 (Leronlimab; CytoDyn), conjugated in-house to PacBlu using Pacific BlueTM Antibody labeling Kit (ThermoFisher) and used at approximately 1:80 depending on the efficacy of conjugation, c) anti-idiotypic antibody, PA-22 (CytoDyn). Live/dead Fixable Yellow Dead Cell Stain Kit and Near-IR Dead Cell Stain Kit (ThermoFisher) were amine-reactive dyes used at 1:1000 dilution to assess cell viability.

Statistical analyses. Time to infection was assessed by log-rank test. Differences in CCR5 expression percentages were measured by nonparametric Kruskal-Wallis test, and differences in the number of CCR5 surface molecules was assessed by nonparametric Mann-Whitney test. Statistical significance was determined at the significant alpha level of 0.05. Statistical analyses were conducted using GraphPad Prism software version 6.0 (GraphPad Software, La Jolla, California).

3.5. Results

Because cell-associated virus plays an important role in mucosal transmission⁵⁴⁴, we first assessed the ability of Leronlimab to inhibit HIV cell-to-cell transmission in an *in vitro* spreading assay. In neutralization assays, Leronlimab inhibits diverse HIV isolates with an IC₅₀ value comparable to HIV broadly neutralizing antibodies (bNAbs) such as 10E8, PGT128, and 3BNC117^{545,546}. However, in line with previous findings of reduced neutralizing activity on cell-to-cell transmission⁵⁴⁷, 100 µg/ml Leronlimab was necessary to fully prevent cell-to-cell spread of CCR5-tropic HIV in the *in vitro* culture system of purified, highly activated CD4⁺ T cells (Supplemental Figure 3.1). When treated with this concentration of Leronlimab in the spreading assay, CD4⁺ T cell targets from CCR5 wild type donors became fully resistant to infection with CCR5-tropic HIV while still supporting replication of CXCR4- and dual-tropic HIV, identical to the infection pattern observed with CD4⁺ T cell targets from a CCR5^{Δ32/Δ32} donor (Figure 3.1a). Using a panel of 25 HIV isolates from multiple clades (Supplemental Table 3.1), we confirmed the ability of Leronlimab to mimic the resistance conferred by the CCR5^{Δ32/Δ32} phenotype onto CD4⁺ T cells from CCR5 wild type donors, thereby protecting these cells from infection with CCR5-tropic isolates from multiple geographical origins (Figure 3.1b).

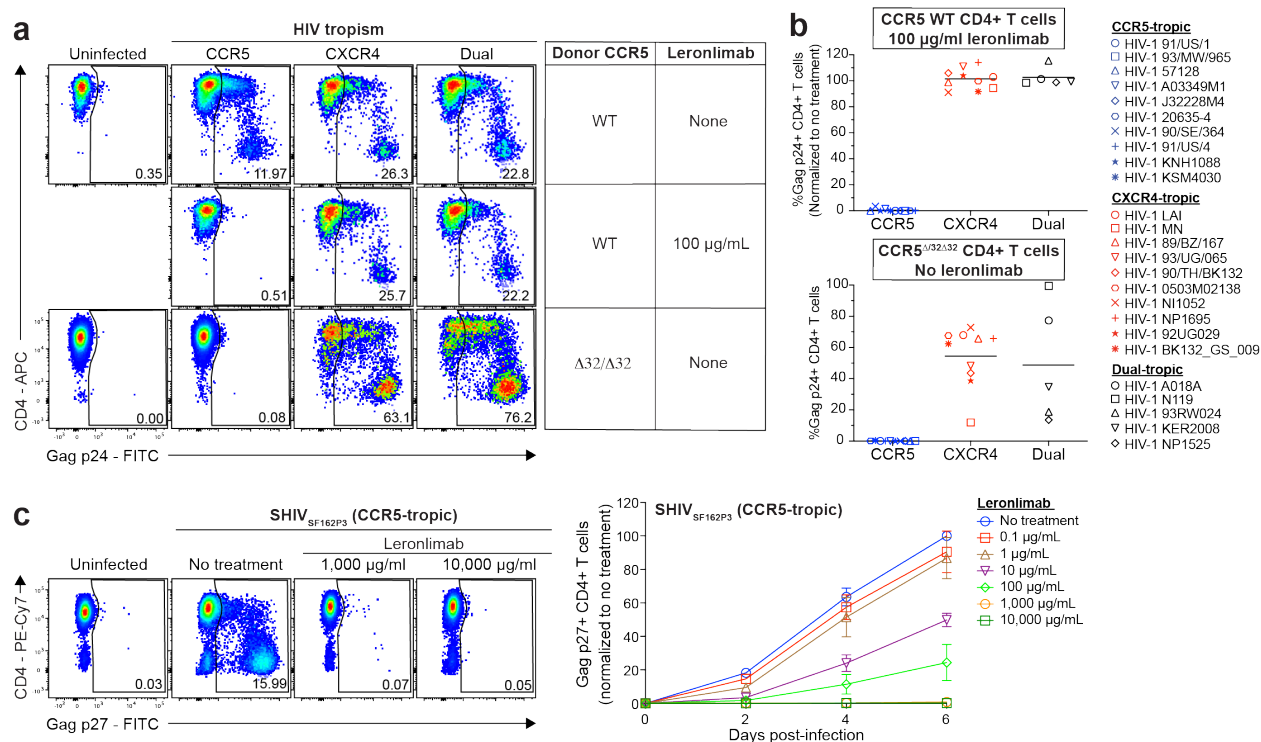


Figure 3.1. Leronlimab blocks the spreading of CCR5-utilizing strains *in vitro*. (a) Representative flow cytometry plots displaying intracellular Gag p24 staining of untreated CCR5 wild-type (WT), Leronlimab-treated CCR5 WT, and untreated CCR5^{Δ32/Δ32} human CD4+ T cells in HIV-1 infection assay with CCR5-tropic (blue), CXCR4-tropic (red), and dual-tropic viruses (black). (b) Summary of the mean Gag p24 levels from HIV-1 spreading assays performed on CCR5 WT human CD4+ T cells treated with 100 µg/ml Leronlimab (top) or untreated CCR5^{Δ32/Δ32} human CD4+ T cells (bottom). Each data point represents the mean Gag p24 levels of CCR5 WT human (n=3) or CCR5^{Δ32/Δ32} human (n=1; two replicates) CD4+ T cells. Each viral tropism group represents the mean Gag p24 levels of ten CCR5-tropic, ten CXCR5-tropic, and five dual-tropic viral infections. Gag p24 levels of CCR5 WT human CD4+ T cells were normalized to “no treatment”. (c) Spreading assay using SHIV_{SF162P3}, a CCR5-utilizing virus. RM CD4+ T cells (n=3) were infected and treated with increasing amounts of Leronlimab. (Right) Summary (mean ±SEM) of the longitudinal infection as measured by Gag p27 levels, with all values normalized to day 6 “no treatment”.

CCR5 is highly conserved between humans and rhesus macaques⁵¹⁹, and accordingly, Leronlimab specifically bound CCR5 on the surface of macaque CD4+ T cells (Supplemental Figure 3.2a and b). However, while the frequency of CCR5+ CD4+ T cells was similar between human and macaques in peripheral blood, macaque CD4+ T cells expressed a higher number of CCR5 molecules on a per-cell basis, with central memory CD4+ T cells expressing the highest levels overall (Supplemental Figure 3.2c). This higher per-cell expression of CCR5 on macaque CD4+ T cells suggested that inhibition of SHIV infection in macaque CD4+ T cells may require

higher concentrations of CCR5-targeted competitive inhibitors such as Leronlimab compared to inhibition of HIV infection in human CD4⁺ T cells. Indeed, a 10-fold higher concentration of Leronlimab was required to achieve full inhibition of SHIV_{SF162P3} infection in macaque cells compared to HIV in human cells *in vitro* (Figure 3.1c and Supplemental Figure 3.1a).

The primary co-receptor used during mucosal transmission is CCR5^{54,167,313}. Thus, we explored whether Leronlimab could act as PrEP to protect macaques from repeated low-dose intrarectal (IR) SHIV_{SF162P3} challenges. The study design, shown in Figure 3.2a, consisted of three groups of macaques (n=6 per group, individual macaque information listed in Supplemental Table 3.2) that served as untreated controls (group 1), weekly 10 mg/kg Leronlimab-treated (group 2), or biweekly 50 mg/kg Leronlimab-treated animals (group 3). The 10 mg/kg dose in macaques is an allometrically scaled human clinical dose of 350 mg Leronlimab. Due to the emergence of ADA, the allometrically scaled human clinical dose of 700 mg Leronlimab was adjusted to 50 mg/kg dose in macaques, a dose that does not elicit ADA likely due to high zone tolerance⁵⁴⁸, with dosing changed to bi-weekly to more closely approximate plasma levels from weekly doses in humans. Thus, these two macaque doses of Leronlimab represent the lowest and highest doses currently used in clinical trials. Animals received their first subcutaneous Leronlimab injection one week prior to the start of IR challenges. At study week zero, all animals received their first IR challenge with 3.2 TCID₅₀ of SHIV_{SF162P3} that continued for eight consecutive weeks. We selected SHIV_{SF162P3} for the following two reasons: (1) the parental Env from HIV-1_{SF162} is CCR5-tropic, yet particularly resistant to CCR5-targeting agents⁵⁴⁵, and (2) SHIV_{SF162P3} can switch to CXCR4 co-receptor use in macaques via defined amino acid substitutions in Env V3 loop⁴⁹⁵. Uninfected animals received their last IR challenge and Leronlimab injection at study week seven. After this point, infected

animals were euthanized at 10 weeks after confirmed infection while aviremic, protected animals were longitudinally monitored and euthanized eight weeks after loss of Leronlimab receptor occupancy (RO) on peripheral blood CD4⁺ T cells.

In all animals, Leronlimab was well-tolerated and showed no adverse clinical effects, similar to the high safety profile reported previously in multiple clinical trials^{457,458,460,461,537}. Serum chemistry and complete blood count parameters did not differ significantly between groups nor change significantly over the study period (Supplemental Figure 3.3). Although no significant change in peripheral blood T cell counts or frequencies were found, we observed a dose-dependent increase in peripheral blood CCR5⁺ T cell frequencies and absolute counts during Leronlimab treatment that subsequently returned to baseline levels concomitant with Leronlimab washout, likely reflecting the previously-described ability of Leronlimab to interfere with CCR5-mediated chemotaxis⁴⁵⁴ (Supplemental Figure 3.4).

All untreated control macaques in group 1 became infected by the seventh intrarectal challenge, while two of six macaques in group 2 (weekly 10 mg/kg) became infected ($p=0.001$). In contrast, no macaques in group 3 (50 mg/kg biweekly) became infected ($p=0.0005$), indicating that Leronlimab protected macaques from SHIV acquisition in a dose-dependent manner (Figure 3.2b). To track CCR5 RO, we established and validated an *ex vivo* Leronlimab RO assay (Supplemental Figure 3.5). All six macaques in group 3 maintained full CCR5 RO on peripheral blood CD4⁺ T cells throughout the challenge phase (Figure 3.2c). As expected with the high dose, these macaques did not develop ADA (Figure 3.2d), and maintained sufficient plasma levels with a half-life of 7.5 weeks after the last Leronlimab injection at study week seven (Figure 3.2e). Following washout of plasma Leronlimab and loss of CCR5 RO on peripheral blood CD4⁺ T cells, we monitored animals for an additional eight weeks for emergence of occult

SHIV infection. All group 3 macaques remained aviremic until necropsy, suggesting sterilizing protection from acquisition (Figure 3.2f). In the four protected group 2 animals (weekly 10 mg/kg), we observed earlier loss of CCR5 RO following the final Leronlimab dose, low level ADA development, and, similar to all group 3 animals, no emergence of occult SHIV infection following Leronlimab washout and loss of CCR5 RO, again indicating sterile protection from acquisition (Figure 3.2c-f). Two group 2 animals, 37032 and 34487, however, did become infected. 37032 developed strong ADA that led to rapid loss of CCR5 RO, clearance of plasma Leronlimab in plasma, and acquisition of SHIV (Figure 3.2c-f). 34487 acquired infection despite maintaining peripheral blood CD4⁺ T cell CCR5 RO and Leronlimab plasma levels similar to group 2 protected animals. Interestingly, following the final challenge this animal had the lowest Leronlimab levels in the colon of any treated animals without ADA (Figure 3.3b), suggestive of incomplete CCR5 RO in the rectum that resulted in suboptimal protection during viral challenges. Following infection, 34487 experienced the lowest peak plasma viremia of all infected animals and was the only animal to control SHIV_{SF162P3} to undetectable levels, suggesting antiviral effects by Leronlimab similarly seen in clinical trials of monotherapy treatment of HIV-positive individuals^{457,458,460,461,537} (Figure 3.2f, Supplemental Figure 3.6). However, we cannot exclude the potential of spontaneous control of SHIV replication. We sequenced the V3 loop of *env* and found that virions in the plasma remained CCR5-tropic for both animals, excluding the possibility of infection by CXCR4-tropic SHIV variants (Supplemental Figure 3.7).

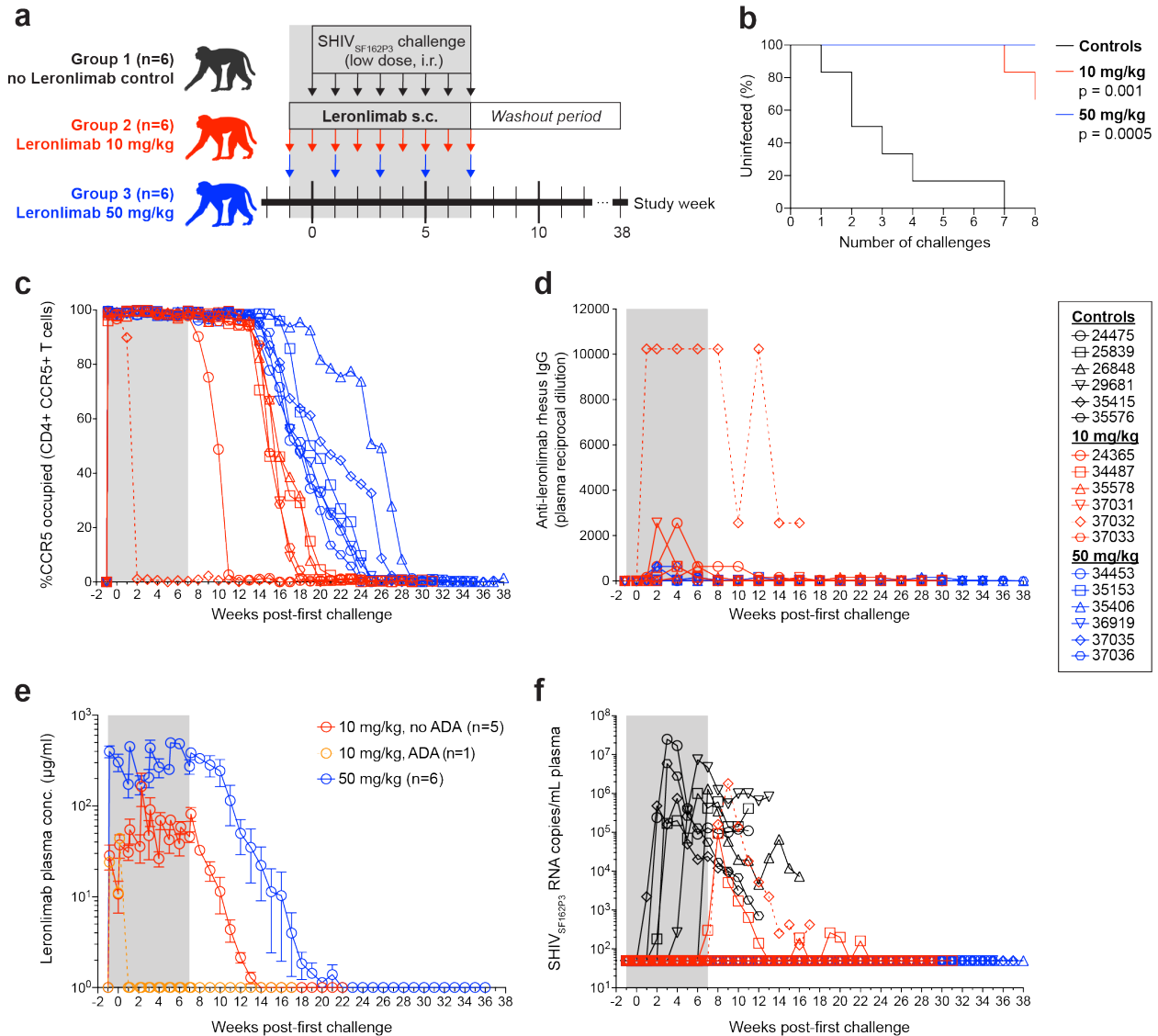


Figure 3.2. Leronlimab pre-exposure prophylaxis protects rhesus macaques from intrarectal SHIV_{SF162P3} acquisition. (a) Study outline. Eighteen rhesus macaques were challenged with 3.2 TCID₅₀ of SHIV_{SF162P3} weekly via intrarectal inoculation for eight consecutive weeks. Group 1 received no Leronlimab (black, n=6) while Group 2 received weekly 10 mg/kg Leronlimab (red, n=6) and Group 3 received biweekly or once-every-two-weeks Leronlimab (blue, n=6). (b) Kaplan-Meier curve comparing percentage of uninfected to the number of SHIV_{SF162P3} challenges, analyzed by log-rank test with Dunn's correction. (c) Longitudinal CCR5 receptor occupancy levels on peripheral blood CCR5+ CD4+ T cells in Leronlimab-treated macaques as determined by flow cytometry (see Methods). (d) Longitudinal anti-Leronlimab rhesus IgG levels in plasma of Leronlimab-treated macaques. (e) Mean ($\pm$ SEM) longitudinal Leronlimab plasma concentrations in Groups 2 and 3, separated by development of persistent anti-Leronlimab rhesus IgG antibody levels (ADA = anti-drug antibodies; orange). (f) Longitudinal SHIV_{SF162P3} plasma viral loads. Horizontal dashed line denotes assay limit of quantification (50 copies/ml); Gray boxes in a, c-f denote the Leronlimab treatment phase for Leronlimab-treated macaques. Legend at right shows symbols used in panels c, d, and f identifying individual rhesus macaques. The macaque that developed persistent anti-Leronlimab rhesus IgG antibody levels (37032) is shown as a dashed line throughout panels c-f.

To determine if protection correlated with CCR5 RO on tissue and mucosal resident CD4⁺ T cells, we collected lymph node and endoscopic duodenum biopsies and bronchoalveolar lavages (BAL) during the first IR challenge and again one week following the final IR SHIV challenge. We observed full CCR5 RO on CD4⁺ T cells from these tissues throughout the IR challenge phase, except for animal 37032 who lost CCR5 RO due to ADA (Figure 3.3a). To assess Leronlimab tissue penetration, we biopsied various anatomical locations following the final SHIV challenge. As expected, higher levels of Leronlimab were present in group 3 versus group 2 animals, with the lowest colon levels found in 34487 (Figure 3.3b). Finally, we examined tissues collected during necropsy and confirmed an absence of Leronlimab and CCR5 RO on CD4⁺ T cells from these tissues, demonstrating that the lack of plasma viremia in protected animals was not due to residual Leronlimab in tissue.

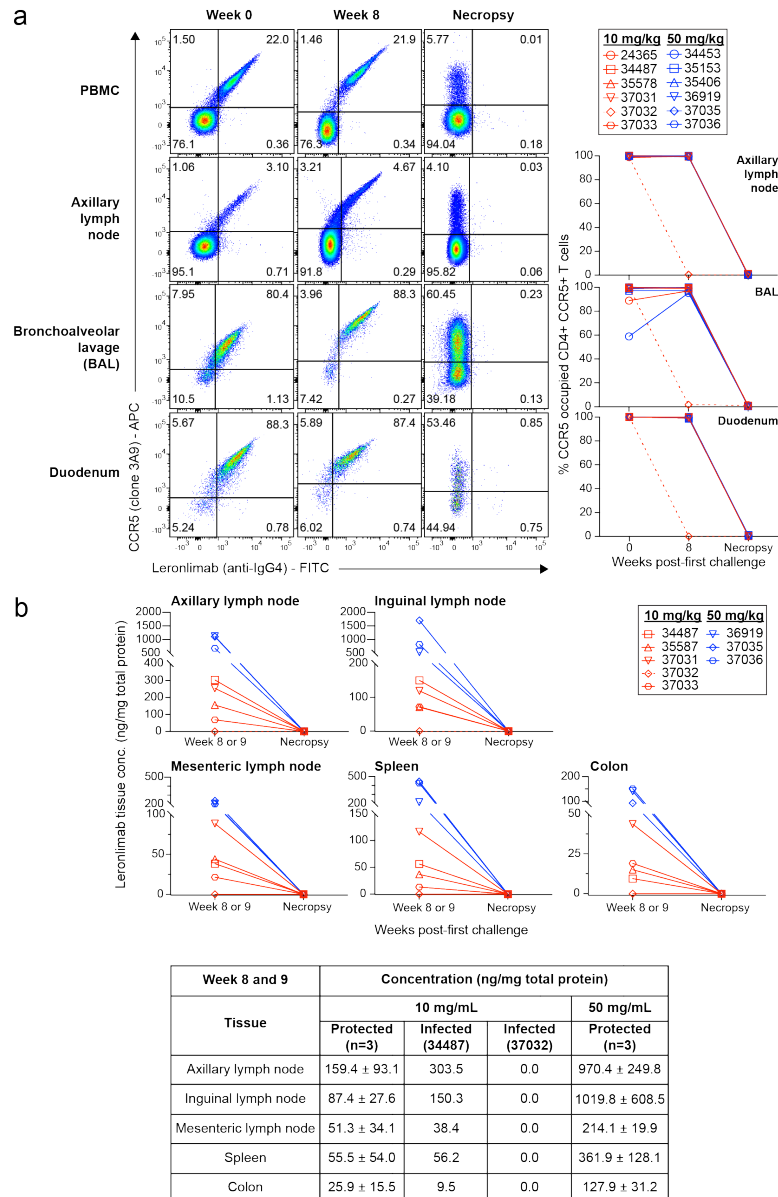


Figure 3.3. Longitudinal Leronlimab tissue levels and CCR5 receptor occupancy. Showing 10 mg/kg treated animals (red) and 50 mg/kg treated animals (blue). **(a)** On the left, representative flow cytometry plots showing the co-staining of anti-CCR5 (clone 3A9) and Leronlimab (by anti-human IgG4, clone HP-6025) on CD4+ T cells from PBMC, axillary lymph node, bronchoalveolar lavage, and duodenum. Tissues were taken from three timepoints: week 0 (one-week post first Leronlimab treatment; week of first viral challenge), week 8 (one-week post final Leronlimab treatment; one-week post final viral challenge), and necropsy (varying for all animals). Flow cytometry plots for all three timepoints and tissues followed the 50 mg/kg treated animal, 35153. On the right, individual CCR5 RO on longitudinal CD4+CCR5+ T cells from axillary lymph node, bronchoalveolar lavage, and duodenum. Dashed line shows the CCR5 RO of 37032, the animal that developed ADA. **(b)** Tissue concentration of Leronlimab for five 10 mg/kg treated animals and three 50 mg/kg treated animals during two timepoints: week 8/9 and necropsy. On the bottom, summary of the tissue concentration in mean values for the three protected 10 mg/kg treated animals; individual values for the infected 10 mg/kg treated animals, 34487, and 37032; and mean values for the three protected 50 mg/kg treated animals.

To assess if Leronlimab mediated sterile protection from challenge, we measured cell-associated SHIV DNA and RNA levels in multiple anatomical locations by tissue biopsy after the final challenge (study weeks eight to nine) and at necropsy (Figure 3.4a-b). As expected, SHIV nucleic acid was readily detected in macaques with plasma viremia, including in all untreated controls and the two infected group 2 animals, 34487 and 37032. However, many tissues from 34487 at necropsy were below the limit of quantification suggesting Leronlimab

may have limited viral spread post acquisition. In contrast, we found no samples positive for SHIV DNA or RNA from the four aviremic group 2 animals and all six group 3 animals. Because Gag- and Vif-specific CD8⁺ T cells are a sensitive readout of occult SIV infection²⁵², we longitudinally monitored for their emergence in all macaques. In line with longitudinal plasma and cell-associated viral load results (Figure 3.2f and 3.4a-b), SHIV Gag- and Vif-specific CD8⁺ T cells responses developed in all viremic animals while it was absent in all aviremic animals, despite the presence of CMV-specific CD8⁺ T cells in all macaques (Figure 3.4c and Supplemental Figure 3.8). Finally, after confirming that Leronlimab and CCR5 RO was not present in tissues collected at necropsy (Figure 3.3), we adoptively transferred pooled hematologic cells into one SHIV-naïve recipient macaque per group. Lymph node (axillary, inguinal, and mesenteric), bone marrow, and spleen cells from all six infected group 1 animals, the four protected group 2 animals, and all six group 3 animals were pooled by group and infused at final cell counts of 7.8×10^8 , 6.9×10^8 , and 9.7×10^8 cells, respectively (Supplemental Table 3.3). Transfer of SHIV_{SF162P3} infection occurred with the infusion of cells from infected control animals, but not isolated cells from protected animals in group 2 or 3 (Figure 3.4d). Together, these results indicate that Leronlimab mediated sterile protection against mucosal acquisition of SHIV_{SF162P3} in the aviremic animals.

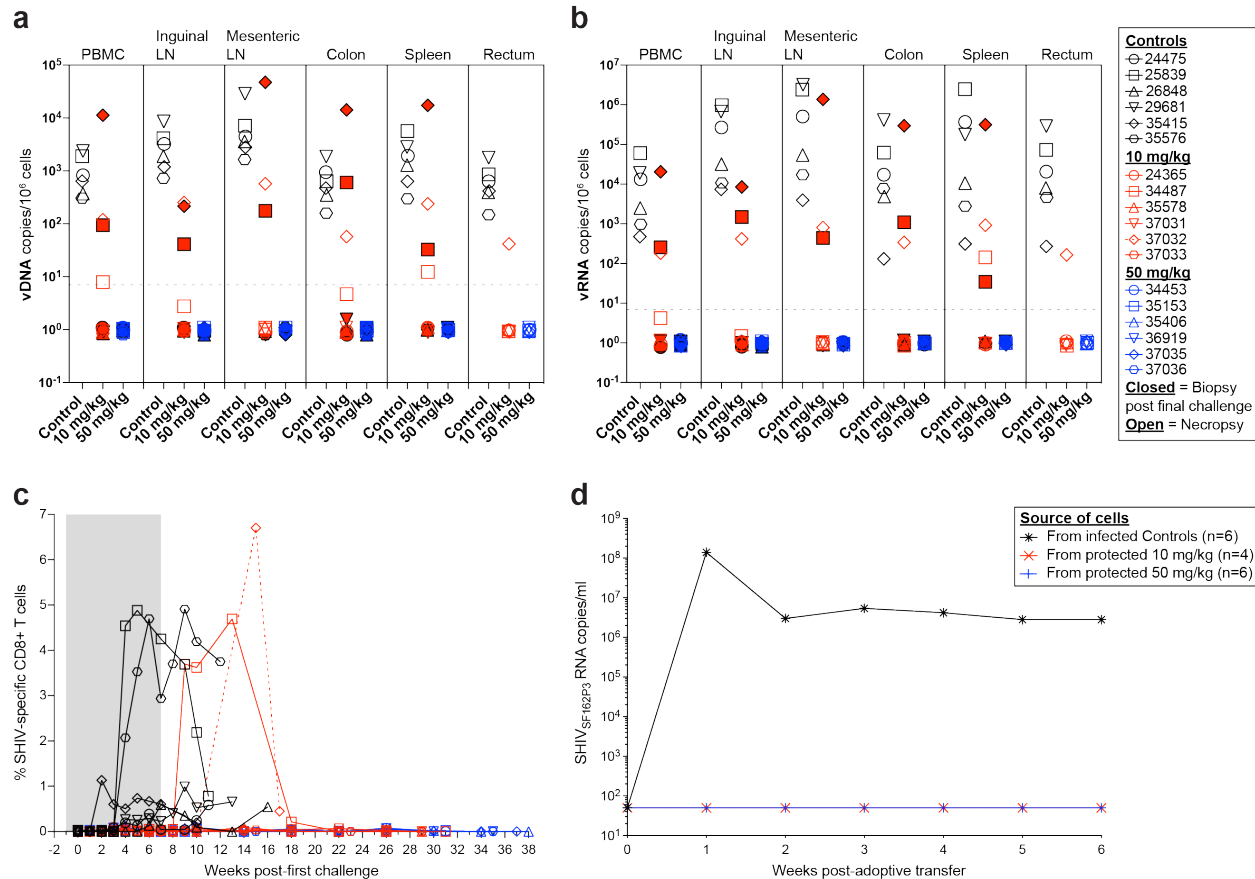


Figure 3.4. Validation of protected Leronlimab-treated rhesus macaques. Showing untreated control animals (black), 10 mg/kg treated animals (red), and 50 mg/kg treated animals (blue). **(a,b)** Cell-associated SHIV_{SF162P3} DNA (a) and RNA (b) viral loads after final challenge (closed symbols, biopsies at study week 8 or 9) and at necropsy (open symbols). Horizontal dashed lines in a-b denote assay limit of quantification of 7 copies/10⁶ cells. **(c)** Longitudinal SHIV-specific CD8⁺ T cell responses as determined by intracellular cytokine staining. Positive percentage of responses were determined by Boolean gating to CD8⁺ T cells that were CD69⁺/TFN- α ⁺ and/or CD69⁺/IFN- γ ⁺. Gray box denotes the Leronlimab treatment phase for Leronlimab-treated macaques. **(d)** Longitudinal SHIV_{SF162P3} plasma viral loads in adoptive transfer recipients infused with cells harvested from macaques of each group upon euthanasia (one recipient per group). Each adoptive transfer recipient received a combined infusion of cells from six infected control macaques, from four aviremic 10 mg/kg treated macaques, or from six aviremic 50 mg/kg treated macaques (see Supplemental Table 3). Horizontal dashed line denotes assay limit of quantification (50 copies/mL).

3.6. Discussion

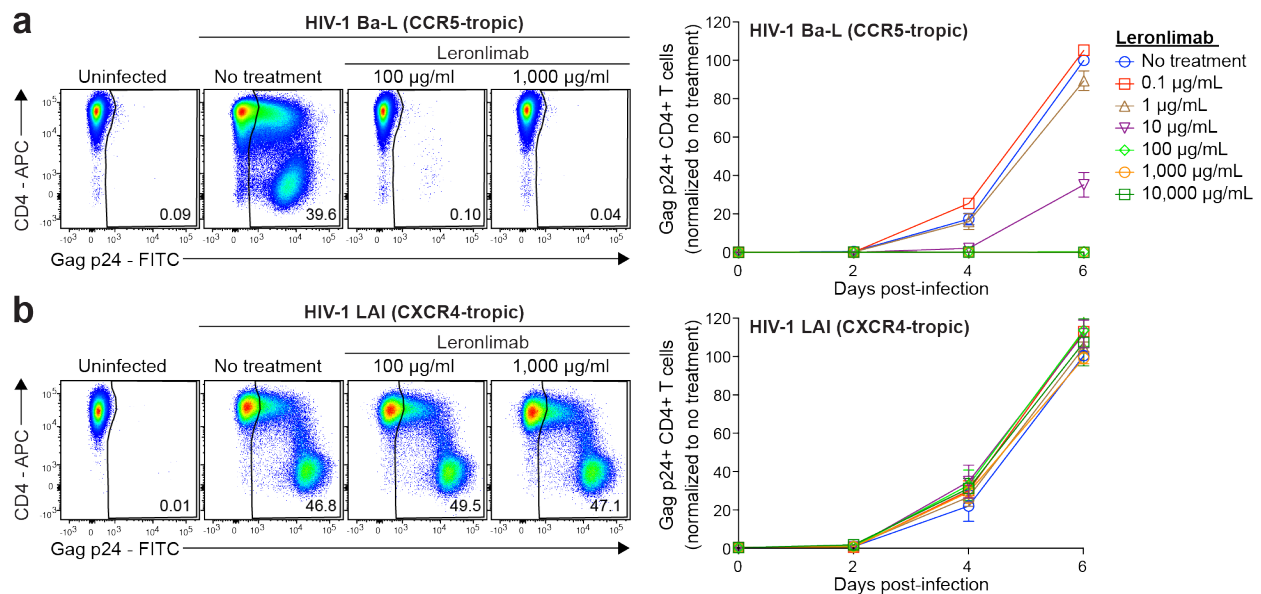
The efficiency of daily ART-based PrEP is heavily dependent on patient adherence⁵³⁰ and absence of drug-resistance variants⁵³¹. Even new preventative approaches like long-acting injectable cabotegravir can lead to the emergence of drug-resistant variants, especially if initiated during undiagnosed acute infection⁵⁴⁹. Thus, alternative and cost-reducing methods are needed,

including the use of antibody-based PrEP. Leronlimab is administered subcutaneously at home, offering advantages over bNAb-based PrEP that requires IV infusion and intra-muscular injectables that are administered in a clinic by a health care provider. Given the significantly longer Leronlimab plasma half-life in humans versus macaques, coupled with the lower per-cell CCR5 expression on CD4⁺ T cells in humans, it is possible that once monthly Leronlimab may be sufficient to prevent HIV acquisition. With advances in antibody Fc engineering to enhance plasma half-life⁵⁵⁰, minor sequence modifications could extend Leronlimab to a once-quarterly injection to further improve adherence by lowering the frequency of administration.

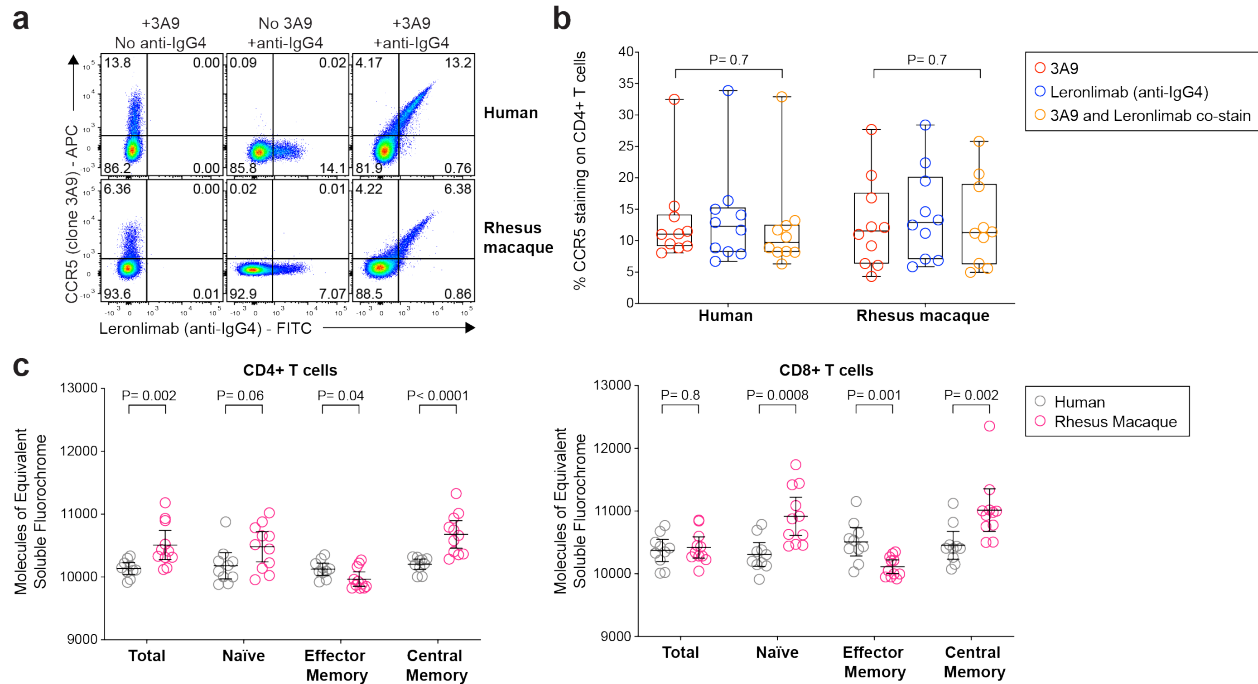
Because CCR5 is the primary HIV co-receptor used by virions during transmission^{54,167,313}, development of viral resistance is difficult, as demonstrated by the previously documented protection from infection in CCR5^{Δ32/Δ32} individuals^{421,422}. Indeed, no escape mutants were observed with long-term Leronlimab monotherapy use in HIV⁺ individuals, in contrast to treatment with bNAbs^{532,533}. This is likely due to targeting of the primary CCR5 co-receptor rather than epitopes on Env by bNAbs that permit selection for bNAb-resistance variants. Infection with CXCR4- or dual-tropic HIV isolates is possible, but such events represent a small minority of transmission due to multiple layers of restriction on CXCR4-tropic viruses in the mucosa^{9,35}. Further, the ability of Leronlimab to fully protect animals from rectal transmission of SHIV is in contrast to a previous study where the small-molecule CCR5-inhibitor Maraviroc failed to protect animals challenged with 10 TCID₅₀ SHIV_{SF162P3} despite sufficiently high drug concentrations in rectal tissues⁴³⁸. Thus, competitive inhibition of the CCR5 Env interaction represents a powerful approach for PrEP. Finally, beyond targeting CCR5 for prophylaxis, significant effort is focused on genetic engineering approaches to knock out CCR5 in HIV cure strategies⁴⁵². The results presented here show that the use of Leronlimab

could mimic the same protection from acquisition seen in CCR5 $\Delta 32/\Delta 32$ individuals via a reversible pharmacological therapy that does not require genetic modifications that risk off-target effects. Given that the CCR5 $\Delta 32/\Delta 32$ phenotype carries no risk for life span⁴⁵³ and that multi-year Leronlimab use has been safely demonstrated, future studies are warranted to explore the utility of Leronlimab in HIV cure and prevention.

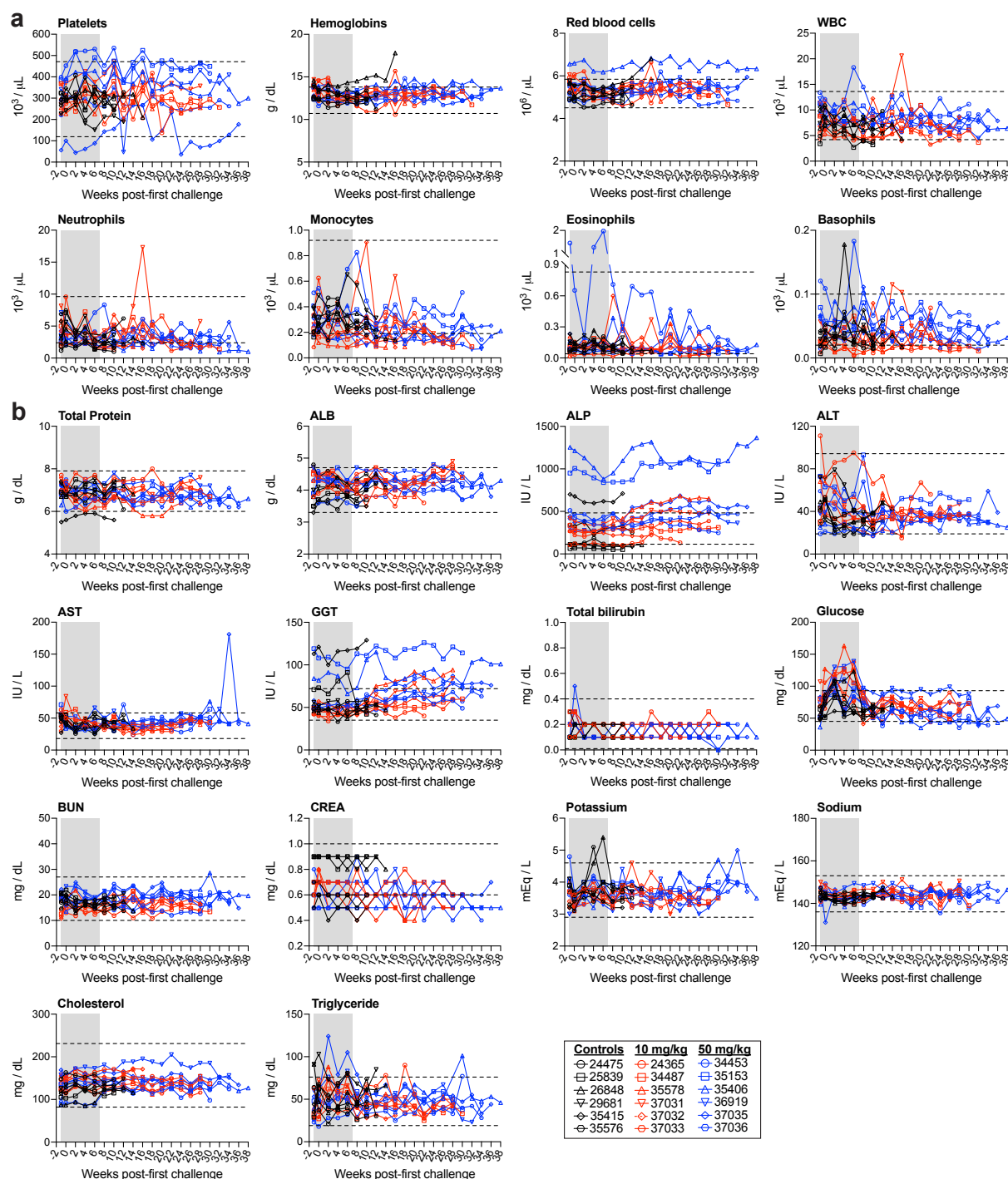
3.7. Supplemental Data



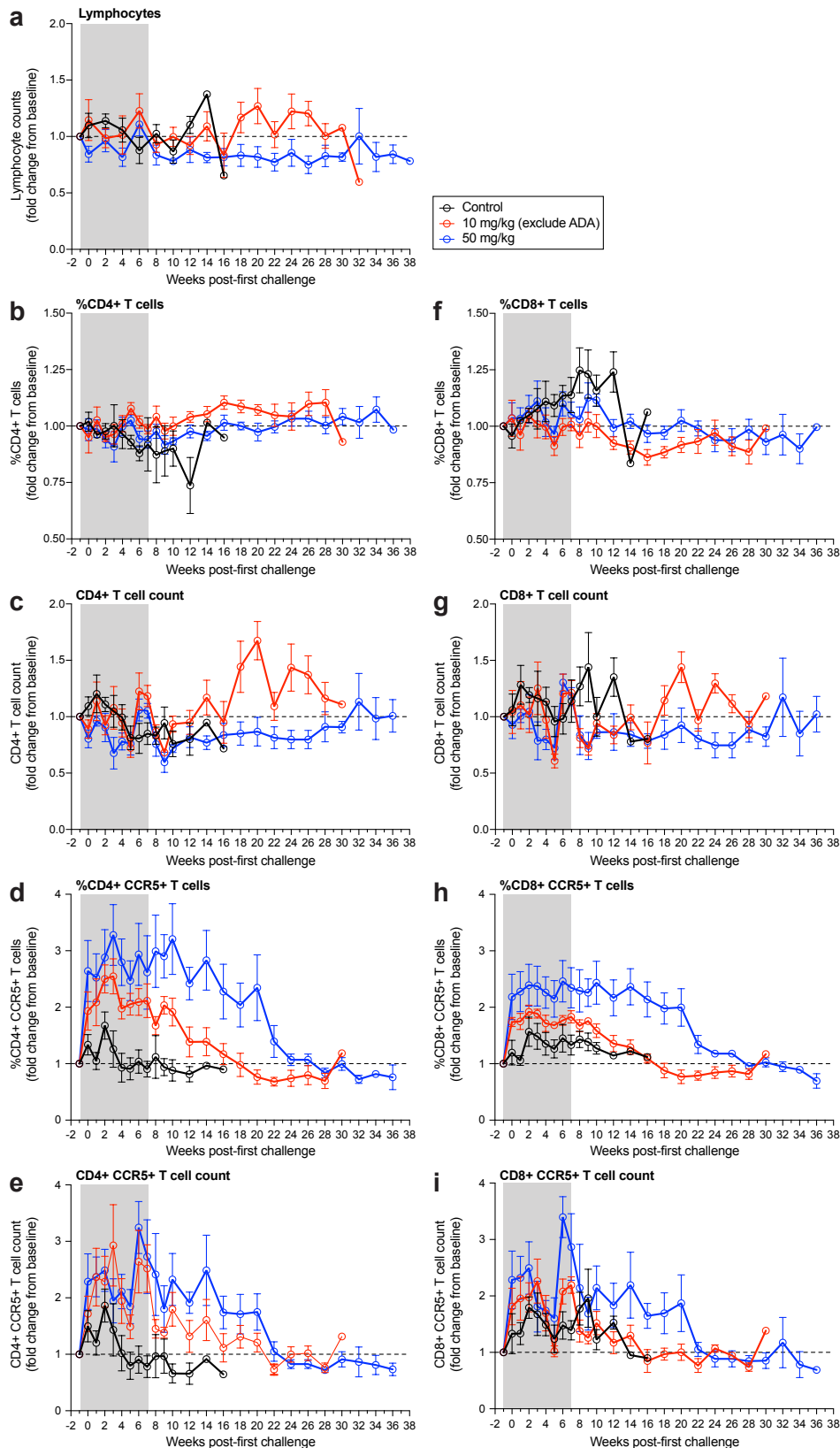
Supplemental Figure 3.1. Leronlimab blocks spreading infection of CCR5-utilizing HIV *in vitro*. CCR5 WT CD4+ T cells (n=3 donors) were infected and treated with or without Leronlimab at different concentrations. Cells were cultured for six days, and media (with Leronlimab at the desired concentration) was replenished on day 2 and day 4. On day 6, cells were stained for intracellular Gag protein. (Left) Representative flow cytometry plots displaying day 6 of the intracellular Gag p24 staining of **(a)** HIV-1 Ba-L, a CCR5-utilizing virus, **(b)** HIV-1 LAI, a CXCR4-utilizing virus, (Right) Summary (mean $\pm$ SEM) of the longitudinal infection, with all values normalized to day 6 “no treatment”.



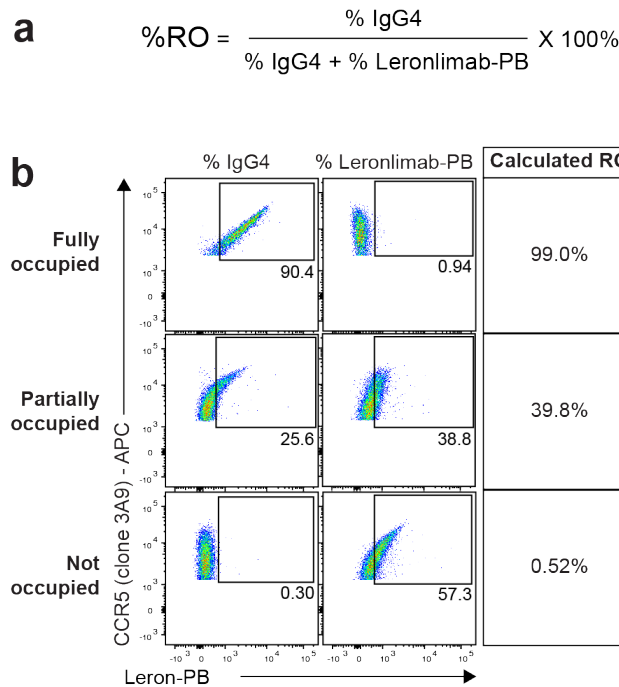
Supplemental Figure 3.2. Leronlimab binds to CCR5 on human and macaque cells. (a) Representative flow cytometry plots of CCR5 receptor staining on CD4+ T cells. Cells were stained with anti-CCR5 antibody (clone 3A9) only, Leronlimab only, or both antibodies. The secondary antibody, anti-human IgG4 (clone HP-6025) was used to stain Leronlimab. (b) Summary of the %CCR5 receptor on CD4+ T cells from human (n=10 donors) and macaque (n=10 donors) PBMC as determined by the three different staining methods. Box plots are presented where the middle line is the median, the lower and upper hinges correspond to 25th and 75th percentiles – interquartile range – and the upper and lower whiskers extend to the maximum and minimum values. Differences between the three staining methods was assessed by one-sided nonparametric Kruskal-Wallis test followed by Dunn’s correction for multiple comparisons. (c) Summary (mean $\pm$ 95% confidence interval) of the number of CCR5 receptors on total and memory subset CD4+ T cells (left) and CD8+ T cells (right) from human (n=10 donors) and macaque (n=10 donors) PBMC. Described in the Methods, the number of CCR5 receptors was equivalent to the number of detectable fluorochromes, using PE-conjugated Leronlimab. P-values calculated from two-sided nonparametric Mann-Whitney test. P-values <0.05 is significant.



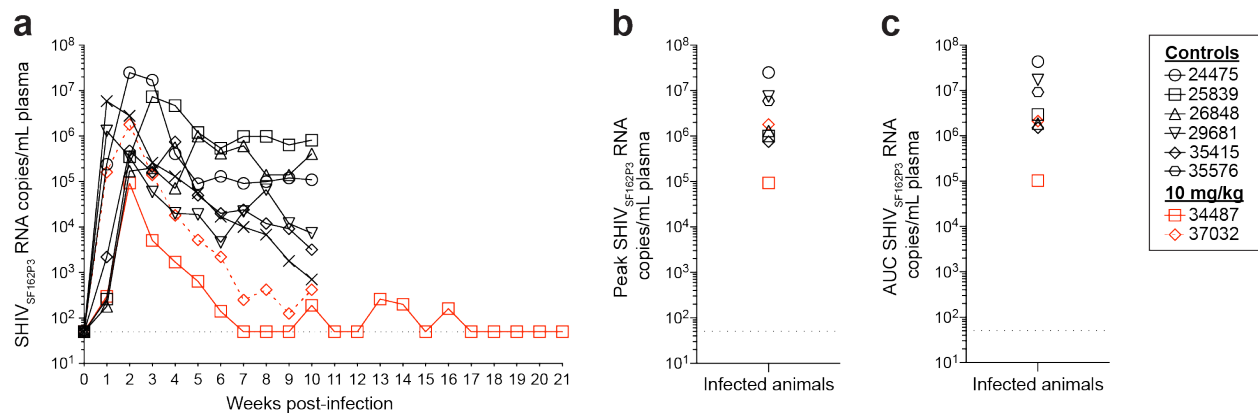
Supplemental Figure 3.3. Longitudinal complete blood count and serum chemistry. Longitudinal values are shown for control group (n=6, in black), 10 mg/kg group (n=6, in red), and 50 mg/kg group (n=6, in blue). The gray box indicates Leronlimab treatment period that started on study week -1 and ended on study week 7. Two dotted lines indicate the standard reference range for each parameter in rhesus macaques housed at ONPRC. **(a)** Peripheral blood levels of platelets, hemoglobin, red blood cells, white blood cells (WBC), neutrophils, monocytes, eosinophils, and basophils. **(b)** Serum levels of total protein, albumin (ALB), alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyltransferase (GGT), total bilirubin, glucose, blood urea nitrogen (BUN), creatinine (CREA), potassium, sodium, cholesterol, and triglyceride.



Supplemental Figure 3.4. Longitudinal lymphocyte counts. Mean ($\pm$ SEM) of control group (n=6, in black), 10 mg/kg group (n=5, excluding 37032, who developed ADA, in red), and 50 mg/kg group (n=6, in blue). The gray box indicates Leronlimab treatment period that started on study week -1 and ended on study week 7. Panels A-I show longitudinal peripheral blood values normalized to baseline value at study week -1. Dotted line at Y=1 denotes fold change from baseline for: **(a)** Lymphocyte counts. **(b)** Percentage of CD4+ T cells. **(c)** CD4+ T cell count. **(d)** Percentage of CD4+ CCR5+ T cells. **(e)** CD4+ CCR5+ T cell count. **(f)** Percentage of CD8+ T cells. **(g)** CD8+ T cell count. **(h)** Percentage of CD8+ CCR5+ T cells. **(i)** CD8+ CCR5+ T cell count. Study week -1 to week 7 was the period of Leronlimab treatment.

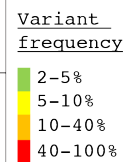


Supplemental Figure 3.5. Calculation of CCR5 receptor occupancy (RO). (a) Equation used to calculate CCR5 RO. (b) Representative flow cytometry plots displaying the different components required to calculate RO and different occupancy levels: fully occupied, partially occupied, and not occupied by Leronlimab. Briefly, cells were gated on CD45+, singlet, live, CD3+, CD4+/CD8-, CCR5+, and either IgG4+ or Leronlimab-PB+ events. Table at right shows the calculated percentage of CCR5 RO.

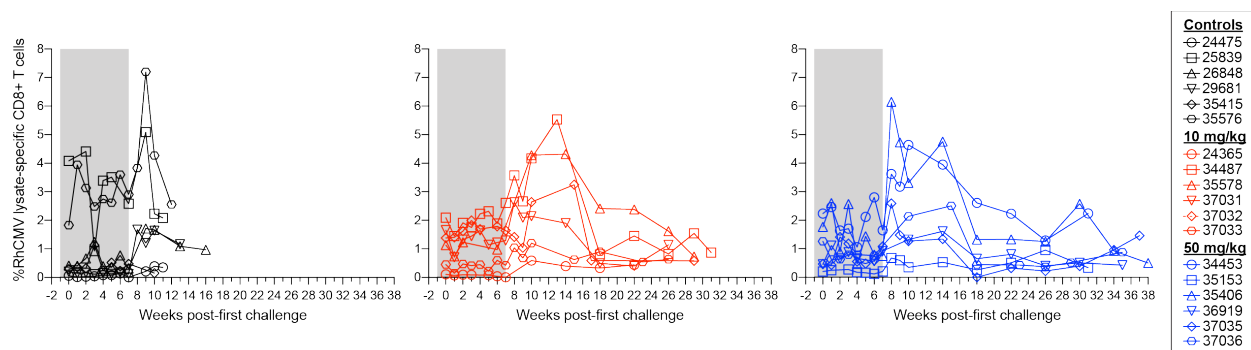


Supplemental Figure 3.6. Plasma viremia comparison of SHIV-infected macaques. Legend at right shows symbols used in panels A-C identifying individual rhesus macaques. Horizontal dashed line denotes assay limit of quantification (50 copies/ml); undetectable plasma viral loads graphed as 50 copies/mL. (a) Longitudinal SHIV_{SF162P3} plasma viral loads shown in weeks post-infection. Six infected control animals and one 10 mg/kg animal (37032) that developed ADA were euthanized at ten weeks after infection while one 10 mg/kg animal (34487) was euthanized after five consecutive weeks of aviremia. (b) Summary of highest SHIV_{SF162P3} plasma viral load for each infected animal. (c) Summary of area under the curve (AUC) for each infected animal.

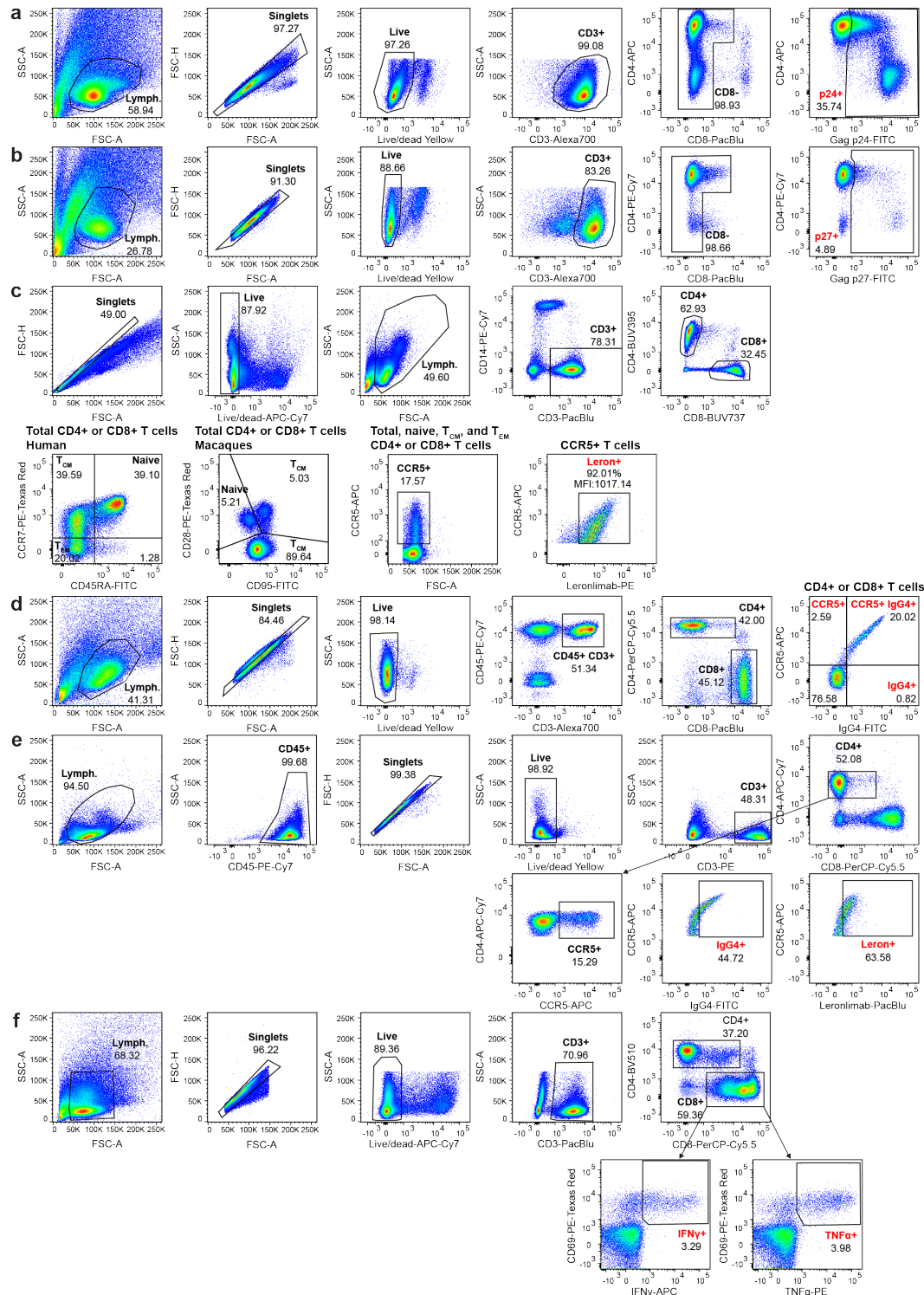
		Virus	Coreceptor	V3 loop	
		SHIV SF 162P3	R5	CTRPNNNTRKSIPI--GPGKAFYATGDIIGDIRQAHC	
		Stock	R5		
		BR24N	X4	Y.I....R.HR.....	
		HXB2	X4	R.R.QR.....V.I.K-. .NM.....	
		Ams-32	X4	S.....R.HR.....T. N.....	
		CA28NL	X4	H.--RRWR--.....	
Control	24475	Week 2			
		Week 6			
		Week10			
	25839	Week 2			
		Week 6			
		Week10			
	26848	Week 2			
		Week 6			
		Week10			
	29681	Week 2			
		Week 6			
		Week10			
	35415	Week 2			
		Week 6			
		Week10			
10 mg/kg	34487	Week 2			
		Week 4			
		Week 6			
		Week13			
	37032	Week 2			
		Week 4			
		Week 6			
		Week 8			



Supplemental Figure 3.7. Assessment of V3 loop sequences from infected macaques in PrEP study. The *env* gene was sequenced from plasma RNA at three timepoints post-infection: week 2, 6, and 10. “SHIV SF 162P3” is the theoretical consensus sequence. “Stock” is the sequence of the stock virus used for viral challenges. “BR24N”, “HXB2”, “Ams.32”, and “CA28NL” are sequences of CXCR4-utilizing isolates related closely to SHIV_{SF162P3}. All sequences are aligned to “SHIV SF 162P3”, where the dots denote similarities and the dashes indicate gaps. Legend at right denotes the variant frequency. Variants <2% were excluded.



Supplemental Figure 3.8. Longitudinal RhCMV lysate-specific CD8+ T cells responses. In the same ICS experiment to determine SHIV-specific CD8+ T cell responses (Figure 3.2c), longitudinal PBMC samples were stimulated with RhCMV lysate as a positive control. Cells were stained and collected on a flow cytometer as described in the Methods. Using FlowJo v10, cells were gated for live, CD3+, CD4-, and CD8+. Percentage of RhCMV lysate-specific responses were determined by Boolean gating to cells that were CD69+/TFN- α + and/or CD69+/IFN- γ +. Summary of longitudinal changes of RhCMV lysate-specific CD8+ T cell responses for the Control animals (left), 10 mg/kg treated animals (middle), and 50 mg/kg treated animals (right). Gray box denotes the Leronlimab treatment phase for Leronlimab-treated macaques.



Supplemental Figure 3.9. Flow cytometry gating strategy. Representative flow cytometry gating strategy to determine the a) percentage of intracellular Gag p24 presented on Fig. 1a-b and Supplemental Figure 3.1a-b; b) percentage of intracellular Gag p27 presented on Figure 3.1c; c) number of CCR5 receptors presented on Supplemental Figure 3.2c; d) percentages for CCR5, Leronlimab (by the secondary antibody, anti-human IgG4-FITC), CD4+ T cells, and CD8+ T cells presented on Figure 3.3a, Supplemental Figure 3.2a-b, and Supplemental Figure 3.4a-i; e) percentage of CCR5 receptor occupancy used in Figure 3.2c, Figure 3.3a, and Supplemental Figure 3.5b; f) percentage of ICS used in Figure 3.4c and Supplemental Figure 3.8.

Co-receptor	HIV-1	Subtype/CRF	GenBank No.	TCID ₅₀ used per infection
CCR5	HIV-1 KHN1088	A	AF457063	2.80E+01
	HIV-1 KSM4030	A	AF457079	3.15E+01
	HIV-1 91/US/1 (GS004)	B	AY173952	6.49E+03
	HIV-1 Ba-L	B	AY713409	8.24E+04
	HIV-1 91/US/4	B	AY173955	4.09E+03
	HIV-1 93/MW/965	C	AY713413	1.42E+01
	HIV-1 20635-4	C	AY713414	7.56E+01
	HIV-1 90/SE/364	C	AY713416	1.30E+04
	HIV-1 57128	D	AF484502	1.01E+01
	HIV-1 A03349M1	D	AF484518	4.56E+02
	HIV-1 J32228M4	D	AF484516	5.56E+01
CXCR4	HIV-1 92UG029	A	AY713407	3.65E+03
	HIV-1 LAI	B	K03455	8.67E+03
	HIV-1 MN	B	M17449	2.39E+01
	HIV-1 89/BZ/167	B	AY173956	4.83E+02
	HIV-1 90/TH/BK132	B	AY173951	1.33E+02
	HIV-1 BK132/GS/009	B	AY173951	1.46E+04
	HIV-1 93/UG/065	D	AY713418	3.20E+00
	HIV-1 0503M02138	CRF01_AE	AY713424	6.67E+02
	HIV-1 NI1052	CRF01_AE	AY713423	7.60E+01
	HIV-1 NP1695	CRF01_AE	AY713419	1.33E+02
Dual	HIV-1 93RW024	A	AY713406	7.25E+03
	HIV-1 KER2008	A	AF457052	4.60E+03
	HIV-1 A018A	B	U12738	1.24E+05
	HIV-1 N119	B	HQ898266	4.50E+05
	HIV-1 NP1525	CRF01_AE	AY713420	9.05E+01

Supplemental Table 3.1. HIV-1 isolates utilized for *in vitro* spreading assay. Table showing the isolates used in the spreading assays with information on co-receptor tropism, subtype, GenBank, and TCID₅₀ used to infect targets in the assay shown in Figure 3.1.

Group	Animal	Gender	Age (Years)	MHC	
				B*08	B*17
Control	24475	F	15.60	-	-
	25839	M	13.52	-	-
	26848	M	12.54	-	-
	29681	M	9.44	-	-
	35415	M	3.26	+	-
	35576	F	3.58	-	-
10 mg/kg	24365	F	15.48	-	-
	34487	M	4.95	-	-
	35578	M	3.83	-	-
	37031	M	3.76	-	-
	37032	F	3.53	-	-
	37033	M	3.79	-	-
50 mg/kg	34453	F	4.65	-	-
	35153	M	3.81	-	-
	35406	M	3.80	-	-
	36919	M	3.08	-	-
	37035	M	2.96	-	-
	37036	F	3.00	-	-

Supplemental Table 3.2. Demography of macaques. Table showing the animal ID, gender, age, and MHC type for animals used in the PrEP study. Note the presence of one Mamu-B*08+ animal in the control group.

Animal group	Recipient animal ID	Donor animal ID	Axillary LN	Inguinal LN	Mesenteric LN	Bone Marrow	Spleen	Tissue distribution per animal	Numbers of adoptively transferred cells
Control	26047	24475	4%	4%	4%	0%	4%	16%	7.8E+08
		25839	4%	4%	4%	0%	4%	16%	
		26848	1%	4%	8%	0%	4%	16%	
		29681	4%	6%	3%	0%	4%	17%	
		35415	4%	4%	4%	0%	4%	16%	
		35576	4%	3%	4%	4%	4%	19%	
10 mg/kg	34279	24365	2%	1%	5%	0%	16%	24%	6.9E+08
		35578	5%	4%	10%	1%	5%	26%	
		37031	5%	3%	10%	1%	5%	25%	
		37033	5%	3%	10%	3%	5%	26%	
50 mg/kg	37037	34453	4%	4%	4%	4%	4%	18%	9.7E+08
		35153	4%	4%	4%	4%	4%	18%	
		35406	4%	4%	4%	1%	4%	16%	
		36919	2%	3%	3%	2%	4%	15%	
		37035	3%	3%	4%	4%	4%	17%	
		37036	1%	1%	4%	4%	7%	17%	

Supplemental Table 3.3. Source of cells used in adoptive transfer. Table showing the source of donor cells infused into each adoptive transfer recipients, with one recipient per experiment group. From the control group, all infected animals were donors. From the 10 mg/kg group, four aviremic animals were donors, excluding 34487 and 37032. From the 50 mg/kg group, all aviremic animals were donors. Mononuclear cells came from axillary lymph node, inguinal lymph node, mesenteric lymph node, bone marrow, or spleen. Shown are the percentage of combined tissues coming from each donor animal and the total cell count adoptively transferred to each recipient animal.

Primer Name	Primer Sequence (5' – 3')	Purpose
SGAG21 Forward	GTCTGCGTCATPTGGTGCATTC	Viral load quantitation in plasma, PBMC, tissues
SGAG22 Reverse	CACTAGKTGTCTCTGCACTATPTGTTTTG	Viral load quantitation in plasma, PBMC, tissues
pSGAG23 probe	5'-6-carboxyfluorescein [FAM]-CTTCPTCAGTKTGTTTCACTTTCTTCTGCG-black hole quencher [BHQ1]-3'	Viral load quantitation in plasma, PBMC, tissues
SHIV <i>env</i> Forward	GGCATAGCCTCATAAAATATCTG	Sequencing SHIV <i>env</i>
SHIV <i>env</i> Reverse	ACAGAGCGAAATGCAGTGATATT	Sequencing SHIV <i>env</i>

Supplemental Table 3.4. Primer names and sequences. List of primers used in this study.

CHAPTER 4: Suppression of human and simian immunodeficiency virus replication with CCR5-specific antibody Leronlimab in two species.

4.1. Abstract

The CCR5-specific antibody Leronlimab is being investigated as a novel immunotherapy that can suppress HIV replication with minimal side effects. Here we studied the virological and immunological consequences of Leronlimab in chronically CCR5-tropic HIV-1 infected humans (n=5) on suppressive antiretroviral therapy (ART) and in ART-naïve acutely CCR5-tropic SHIV infected rhesus macaques (n=4). All five human participants transitioned from daily combination ART to self-administered weekly subcutaneous (SC) injections of 350 mg or 700 mg Leronlimab and to date all participants have sustained virologic suppression for over seven years. In all participants, Leronlimab fully occupied CCR5 receptors on peripheral blood CD4+ T cells and monocytes. In ART-naïve rhesus macaques acutely infected with CCR5-tropic SHIV, weekly SC

injections of 50 mg/kg Leronlimab fully suppressed plasma viremia in half of the macaques. CCR5 receptor occupancy by Leronlimab occurred concomitant with rebound of CD4+ CCR5+ T-cells in peripheral blood, and full CCR5 receptor occupancy was found in multiple anatomical compartments. Our results demonstrate that weekly, self-administered Leronlimab was safe, well-tolerated, and efficacious for long-term virologic suppression and should be included in the arsenal of safe, easily administered, longer-acting antiretroviral treatments for people living with HIV-1.

4.2. Authors, Affiliations, Contributions, and Acknowledgements

Authors: Xiao L. Chang¹, Jason S. Reed¹, Gabriela M. Webb¹, Helen L. Wu¹, Jimmy Le³, Katherine B. Bateman¹, Justin M. Greene¹, Cleiton Pessoa¹, Whitney C. Weber¹, Joseph Hwang¹, Miranda Fischer², Cassandra Moats², Oriene Shiel², Rachele M. Bochart², Hugh Crank², Don Siess², Travis Giobbi², Jeffrey Torgerson², Rebecca Agnor², Lina Gao², Kush Dhody⁴, Jay P. Lalezari³, Ivo Sah Bandar⁵, Alnor M. Carnate⁵, Alina S. Pang⁵, Michael J. Corley⁵, Scott Kelly⁶, Nader Pourhassan⁶, Jeremy Smedley², Benjamin N. Bimber^{1,2}, Scott G. Hansen¹, Lishomwa C. Ndhlovu⁵, Jonah B. Sacha^{1,2*}

*Correspondence: Jonah B. Sacha (sacha@ohsu.edu)

Affiliations: ¹Vaccine & Gene Therapy Institute and ²Oregon National Primate Research Center, Oregon Health & Science University, Portland, OR, USA. ³Quest Clinical Research, San Francisco, CA, USA. ⁴Amarex Clinical Research LLC, Germantown, MD, USA. ⁵Department of Medicine, Division of Infectious Diseases, Weill Cornell Medicine, New York, NY, USA. ⁶CytoDyn Inc., Vancouver, WA, USA.

Contributions: KD, JPL, SK, NP and JL designed and conducted clinical studies, with data analyzed by XLC and JSR. JBS conceptualized the study and experiments. XLC managed the macaque study, assisted by JSR. XLC, JSR, GMW, HLW, KBB, JMG, CP, WCW, JH, DS, TG, JT, ISB, AMC, ASP, MJC, BB, SGH, LCN, and JBS investigated. MF, CM, OS, RMB, HC, and JS managed the animal care and procedures. RA and LG performed statistical analyses. XLC and JBS drafted the manuscript, and all authors contributed to editing. JBS, SK, and NP acquired funding.

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4.3. Introduction

Combination antiretroviral therapy (ART) has drastically improved survival and increased life expectancy for people living with HIV ⁵⁵¹. However, it is not curative and requires daily adherence for life. Furthermore, the prevalence of antiretroviral (ARV) drug resistance has significantly increased in recent years ³¹⁵, with future projections for increasing AIDS-related deaths, new infections, and costs for HIV maintenance all attributable to ART failure due to drug resistance ³²¹. To address this problem, the World Health Organization emphasized the need for new ART strategies in their Global Action Plan on HIV Drug Resistance ³¹⁶.

Both small-molecule ARVs ⁵⁵² and broadly neutralizing antibodies (bNAbs) ³⁶⁷ can lead to the emergence of drug-resistant variants. Alternatively, a promising approach may be to target the conserved major coreceptor CCR5 used by HIV-1 for CD4+ T-cell entry. Indeed, individuals homozygous for a 32-base pair deletion in *ccr5* are highly resistant to infection by HIV-1 strains encoding a variety of CCR5-tropic envelope sequences ⁴²². Furthermore, recent attempts in curative strategies have been directed at CCR5 ⁵⁵³, making it an ideal target for HIV-1 therapies.

Leronlimab (PRO 140, Vyrologix) is an anti-CCR5 humanized monoclonal IgG4 antibody tested in multiple clinical trials for HIV therapy as a weekly SC injection, with favorable safety and tolerability profiles in over 1,000 volunteers ⁵⁵⁴. Similar to HIV, Leronlimab binds to the extracellular loop-2 and N-terminus domains of CCR5, thereby directly outcompeting HIV for CCR5 engagement and blocking entry to CD4+ T-cells ⁴⁵⁴. This mechanism of action is in contrast to FDA-approved CCR5 small-molecule inhibitor maraviroc that binds to CCR5 by allosteric modulation, allowing maraviroc-resistant HIV variants to utilize maraviroc-bound CCR5 for cell entry ^{440,441}. In contrast, viral susceptibility to Leronlimab did not change after Leronlimab treatment ^{457,461}, demonstrating its high genetic barrier to viral resistance. Additionally, a single Leronlimab injection reduced plasma viremia by approximately 100-fold ^{457,458} while, in the CD01 study (ClinicalTrials.gov NCT02175680), weekly SC Leronlimab maintained virologic suppression in 56.1% of CCR5-tropic HIV-infected participants (n=41) for 12 consecutive weeks after ART interruption ⁴⁶¹. Here, we report that uninterrupted weekly Leronlimab treatments resulted in over seven years of virologic suppression in five chronically CCR5-tropic HIV-infected human participants and reduced plasma viremia by 4.29 log₁₀ in four acutely CCR5-tropic SHIV-infected rhesus macaques, demonstrating long-term safety and antiviral efficacy of Leronlimab for HIV-1 therapies.

4.4. Materials and Methods

Ethics Statement: *Human subject research.* Studies were approved by Western Institutional Review Board® (WIRB) (WIRB Protocol Numbers: 20140327 and 20142157) and by the Institutional Review Board. Declaration of Helsinki and the International Conference on Harmonization Good Clinical Practice Guideline were used to conduct studies. Trials took place at the Quest Clinical Research in San Francisco, California (ClinicalTrials.gov Identifiers: NCT02175680 and NCT02355184). All participants provided written informed consent prior to inclusion in studies.

Human studies were an open-label, single arm, phase 2b clinical trials to evaluate the efficacy, safety, and tolerability of Leronlimab (Vyrologix) monotherapy for maintenance of viral suppression in subjects who were stable on combination antiretroviral therapy (Table 4.1). HIV-infected participants of all sexes were eligible if they were older than 18 years of age, were stable on antiretroviral therapy for the last 12 months, had two or more potential alternative ART regimen options, and had nadir CD4 T-cell counts of > 200 cells/mm³. At the Screening Visit, eligible participants had exclusive CCR5-tropic virus as determined by Trofile™ DNA assay and no documented detectable viral loads (< 50 HIV-1 RNA copies/mL) within the last 12 months. Participants with CXCR4-tropic or dual-mixed tropic viruses, hepatitis B infection, AIDS-defining illness, and history of using entry, attachment, CCR5 co-receptor, or fusion inhibitors were excluded from the study.

Leronlimab was supplied by CytoDyn, Inc. Eligible participants received weekly SC injections of 350 mg Leronlimab (NCT02175680) for 12 weeks. Total treatment duration was 14 weeks, where participants had a one week overlap with an existing ART regimen at the start and conclusion of study. After which, consenting participants continued weekly SC injections for at

least 160 weeks (NCT02355184) (Figure 4.1A). Plasma HIV-1 RNA levels were measured using a quantitative assay (*Abbott Real time*), where the lower limit of detection was 40 copies/mL. Participants were monitored weekly during the first 12 weeks, once-every-two weeks from study weeks 12-52, and monthly thereafter. CD4⁺ T-cells counts were monitored using the TruCount Assay (LabCorp). Participants re-initiated their previous ART regimen if plasma HIV-1 RNA level was >400 copies/mL on two consecutive blood draws at least three days apart.

Animal research: Rhesus macaque care plan and all experimental protocols, procedures, and administered reagents were approved by the ONPRC Institutional Animal Care and Use Committee (IACUC). The ONPRC is a Category I facility. The Laboratory Animal Care and Use Program is fully accredited by the American Association for Accreditation of Laboratory Animal Care (AAALAC), with an approved Assurance (#A3304-01) for the use and care of animals on file with the NIH Office of Laboratory Animal Welfare. The ONPRC IACUC adheres to national guidelines established in the Animal Welfare Act (7 U.S.C. Sections 2131–2159) and the Guide for the Care and Use of Laboratory Animals (8th Edition) as mandated by the U.S. Public Health Service Policy. As well as using the standards of the US NIH *Guide for the Care and Use of Laboratory Animals* (National Academies Press, 2011).

Macaques (*Macaca mulatta*) used in this study were housed at ONPRC in Animal Biosafety level (ABSL)-2+ rooms with autonomously controlled temperature, humidity, and lighting. At assignment, macaques were free of *Cercopithecine* herpesvirus 1, D-type simian retrovirus, simian T-lymphotrophic virus type 1, and *Mycobacterium tuberculosis*. Macaques were typed for the MHC alleles Mamu-B*17, and Mamu-B*08. Macaques positive for Mamu-B*17 and/or B*08 were excluded when possible or placed into the control group when it was not possible to exclude biased results. All attempts were made to pair house macaques during the

study period by gender and treatment group. When compatible partners were not available, macaques had auditory, visual, and olfactory contact with neighboring macaques and were provided with an enhanced enrichment plan designed and overseen by a macaque behavioral specialist. Macaques were fed Purina LabDiet 5000 (Purina Mills International, St. Louis, MO) twice daily and received daily food enrichment (e.g., fresh fruit, vegetables). Automatic water systems provided fresh and potable water. Ketamine HCl (Ketathesia™, Henry Schein Animal Health) with or without Dexmedetomidine (Dexmedesed™, Dechra, Overland Park, KS) was used to sedate macaques for procedures, including subcutaneous injections of Leronlimab, venipuncture, tissue biopsy, and SHIV challenge. At the study endpoint, macaques were euthanized with sodium pentobarbital overdose (>50 mg/kg) and exsanguinated via the distal aorta. Aboard certified veterinary pathologist performed and collected tissues at necropsy.

Nine adult macaques were used in the study, with five macaques in the control group and four macaques in the Leronlimab-treatment group. See Supplemental Table 4.3 for the ID number, gender, age, weight, and MHC alleles of these animals. Animals were balanced between the control and Leronlimab-treatment groups by gender. At the beginning of the study, all macaques were intravenously infected with 1,000 TCID₅₀ of the SHIV_{SF162P3} stock provided by Nancy Haigwood³⁵⁷. Macaques received no treatments until study week-3, after which, Leronlimab-treated macaques (n=4) initiated weekly SC 50 mg/kg Leronlimab for 12 consecutive weeks, while untreated control macaques (n=5) received no treatments. Sites for the Leronlimab injection alternated every three weeks between the dorsal scapular and dorsal thoracic areas. At week-15, animals were euthanized and tissues were collected and processed, as described below.

Mass Cytometry (CyTOF). Mass cytometry was performed as previously described⁵⁵⁵. Briefly, Maxpar Direct Immune Profiling Assay reagents were purchased from Fluidigm Inc. Cryopreserved peripheral blood mononuclear cells from each of the human participants receiving Leronlimab during study week 297-304 were analyzed. Live cells were blocked with Human TruStain FcX for 10 minutes, then stained with a panel of metal-labeled antibodies against 30 cell surface markers (Supplemental Table 4.6) for 30 minutes on ice and then washed twice. Stained cells were then fixed with fresh 1.6% formaldehyde for 10 minutes, aspirated, and stained with intercalator-IR. Samples were acquired on a Helios Mass Cytometer and resulting FCS files normalized using a bead-based normalization algorithm and analyzed with Maxpar Pathsetter software.

T-cell Assays. T-cell responses in PBMC were measured by flow cytometric intracellular cytokine staining (ICS) as previously described²⁵². One million PBMC were incubated with overlapping 15-mer peptide pools spanning HIV-M (subtype B) Gag or Nef, and co-stimulated with CD28 and CD49d antibodies (BD Biosciences) for 1 hour, followed by incubation with Brefeldin A (Sigma-Aldrich) for 8 hours. Similarly, stimulation using human cytomegalovirus (HCMV) 15-mer peptide pools spanning IE1 and pp65 served as positive controls, while no antigen incubation served as background controls. Cells were surface stained with antibodies against CD3, CD4, CD8, CD95, CD28, and amine-reactive dye, and then fixed with 2% paraformaldehyde (PFA). BD FACS Permeabilizing Solution (BD Biosciences) was used to permeabilize the membranes before staining intracellularly for IFN- γ , TNF- α , and CD69. Samples were collected on a BDLSR-II instrument with FACSDIVA version 6.1, and analyzed with FlowJo v10 (Tree Star) by gating on singlet, live, CD3+, CD4+ or CD8+ cells. Positive

responding CD4⁺ and CD8⁺ memory (CD95⁺) T-cells were measured by Boolean gating on CD69⁺/TFN- α ⁺ and/or CD69⁺/IFN- γ ⁺ cells. Presented as HIV positive responses were sums of individual HIV Gag and Nef responses, while positive HCMV responses were sums of individual HCMV IE1 and pp65 responses.

SHIV stock. SHIV_{SF162P3} stock (day 7 post-infection harvest, dated 03-17-17, 170 ng/mL p27 by ELISA, and 6,68x10⁵ TCID₅₀/mL in TZM-bl cells) was generated, characterized, and kindly provided by Nancy Haigwood ³⁵⁷.

Processing blood and tissue. Whole blood for complete blood count (CBC), plasma, and peripheral blood mononuclear cell (PBMC) processing was collected with EDTA-treated tubes (BD Biosciences). CBC was measured using an ABX Pentra 60 C+ Hematology Analyzer. PBMC and plasma were isolated from whole blood by density gradient centrifugation using Ficoll-Hypaque (Sigma Aldrich) by spinning at 1,860 x g for 30 minutes. Plasma was isolated from the top layer while PBMC was isolated from the buffy coat and washed once with R10 (RPMI 1640 containing 10% fetal bovine serum (FBS)). Whole blood for serum chemistry analysis was collected in non-anticoagulant tubes and spun down at 1,860 x g for 10 minutes to separate serum from clotted blood. Serum chemistry levels were assessed using an ABX Pentra 400 Chemistry Analyzer.

Lymph nodes and spleen were collected in R10, diced into fine pieces with a scalpel, and forced through a 70- μ m cell strainer into R10 to collect single cell suspensions. Red blood cells from spleen-derived single cell suspensions were lysed using the ACK lysis buffer (ThermoFisher) following the manufacturer's protocols. Bronchoalveolar lavage fluid was

collected in phosphate-buffered saline (PBS; Fisher Scientific) and filtered through a 70- μ m cell strainer.

Gastrointestinal tissues were collected in R10, diced into fine pieces by a scalpel, placed into a 50 mL conical tube containing R3 (RPMI 1640 containing 3% FBS) with 0.5 M EDTA, and incubated in 37°C with 250 rpm shaking for 30 minutes to remove mucus coating.

Afterward, tissues were poured over a tea strainer to remove EDTA and mucus coating, and then sequentially washed three times with Hank's Buffered Salt Solution (HBSS; Fisher Scientific) by pouring over a tea strainer between each wash. EDTA-free tissues were placed into a new 50 mL conical tube containing Digestion Media (R3, 0.2 mg/mL collagenase (Sigma-Aldrich, St. Louis, MO) and 0.2 mg/mL DNase I (Roche, Indianapolis, IN)), and then incubated in 37°C with 250 rpm shaking for 1 hour. Digested tissues were filtered through a 70- μ m cell strainer and spun down at 830 x g for 4 minutes to pellet the cells. The pellet was resuspended in 70% Percoll (Cytiva) and underlaid in 37% Percoll before spinning at 500 x g for 20 minutes. Single cell lymphocytes were collected from the center interface into R10 and stained.

Rectum, cervix, and vagina were processed with the gastrointestinal tissue protocol described above, with the following change: HBSS was used in replacement to R3 with 0.5 M EDTA during the first 30 minutes incubation step. As well, the liver was processed with the gastrointestinal tissue protocol, with the following change: exclusion of the first 30 minutes incubation step. Finally, brain regions were processed with the gastrointestinal tissue protocol described above, with the following changes: 1) exclusion of the first 30 minutes incubation step and 2) incubation of tissues in Digestion Media at 37°C without shaking.

Leronlimab CCR5 receptor occupancy (RO). Two methods were used and validated to measure the percentage of CCR5 RO by Leronlimab on the cell surface (Supplemental Figure 4.3). Equation 1 measured bound CCR5 receptors by using anti-human IgG4 to directly measure Leronlimab-occupied CCR5 receptors, while Equation 2 measured unoccupied CCR5 receptors by using Pacific Blue-conjugated Leronlimab (termed Leronlimab-PB). These two complementary methods were based on RO assays for anti-PD-1 antibodies in clinical trials ⁵²¹.

$$\% RO = \frac{\% IgG4}{\% Saturated IgG4} \times 100\% \quad Equation 1$$

From CCR5+ (measured by clone 3A9) cells, RO for Equation 1 was defined by the percentage of Leronlimab+ (measured by anti-human IgG4) divided by the percentage of Leronlimab+ following incubation with a saturating concentration of unlabeled Leronlimab. To perform the staining, $0.3 - 1 \times 10^6$ PBMCs or single cells from tissue homogenates were incubated with or without 5 µg/mL of unlabeled Leronlimab to achieve Leronlimab saturation of cell surface CCR5 receptors for 30 minutes at room temperature (RT) in the dark. Cells were washed once with PBS, spun down at 830 x g for 4 minutes, aspirated, and stained with anti-human IgG4 for 30 minutes at RT in the dark. After washing once with FACS buffer (PBS with 10% FBS) and once with PBS, cells were stained with CD45, CD3, CD4, CD8, amine-reactive dye, and CCR5 (via antibody clone 3A9) for 30 minutes at RT in the dark. Finally, cells were washed twice with PBS and fixed with 2% PFA for more than 30 minutes at 4°C before collecting on LSR-II instrument and FACSDIVA version 6.1. Using FlowJo v10 (Tree Star), cells were gated on CD45, singlet, live, CD3+, CD4+, CCR5+, and anti-human IgG4+.

$$\% RO = \frac{\% IgG4}{\% IgG4 + \% Leronlimab - PB} \times 100\% \quad \text{Equation 2}$$

From CCR5+ (measured by clone 3A9) cells, RO for Equation 2 was defined as the percentages of cells positive for Leronlimab (measured by anti-human IgG4) divided by the sum of anti-human IgG4 and saturating concentration of Leronlimab conjugated to Pacific Blue (termed Leronlimab-PB), as previously described⁴⁹⁸. Briefly, 0.3 – 1 x 10⁶ PBMC or single cells from tissue homogenates were washed with 10% mouse serum (Fisher Scientific) in PBS and then incubated with 100 µL of 100% mouse serum for 1 hour at RT in the dark to block nonspecific Fc receptors binding to anti-human IgG4 antibodies by monocytes. Cells were washed with 10% mouse serum, replenished with new 100 µL of 100% mouse serum, and incubated with anti-human IgG4 for 30 minutes at RT in the dark. Next, cells were washed once with FACS buffer and four times with PBS. Cells were stained with CD45, CD3, CD4, CD8, CD16, CD14, amine-reactive dye, CCR5 (via antibody clone 3A9), and Leronlimab-PB for 30 minutes at RT in the dark. Lastly, cells were washed twice with PBS and fixed with 2% PFA for more than 30 minutes before collecting on LSR-II instrument and FACSDIVA version 6.1. Using FlowJo v10 (Tree Star), cells were gated on CD45+, singlet, live, CD3+, CD4+, and CCR5+ events. The CD4+ CCR5+ population was further gated on anti-human IgG4+ or Leronlimab-PB+ events.

Measurement of Leronlimab concentration. Enzyme-linked immunosorbent assay (ELISA) was used to detect Leronlimab in plasma and tissues as previously described⁴⁹⁸. Briefly, anti-idiotypic antibody PA22 (CytoDyn, Vancouver, WA) was used to coat half-area 96-week Costar Assay Plates (Corning) at 1.5 µg/mL in carbonate-bicarbonate buffer (ThermoFisher) and

incubated overnight at 4°C. The next day, plates were washed three times with PBS-T (PBS with 0.1% Tween-20) and blocked with Blocking Buffer (PBS with 0.4% Tween-20 and 10% bovine serum albumin (Sigma)) for at least two hours in RT. A standard curve was generated with serial Leronlimab dilutions that ranged from 4.7-300 ng/mL. Plasma samples were first heat-inactivated for 30 minutes at 56°C and spun for 20 minutes at 12,000 x g to pellet residual debris. Supernatants were used for ELISA by diluting in Blocking Buffer with at most four appropriate dilutions to assay. Blocked plates were washed three times with PBS-T. Diluted plasma and standard samples were added into the plates in duplicates and incubated for 30 minutes in RT. Afterward, plates were washed three times with 0.5 M NaCl in PBS. Mouse anti-human IgG4 pFc'-horseradish peroxidase (Southern Biotech) was diluted 20,000-fold with Blocking Buffer, added to the plates, and incubated for 30 minutes in RT. Plates were finally washed three times with PBS-T and developed for two minutes with 3,3',5,5'-Tetramethylbenzidine (TMB) substrate (Southern Biotech). Reactions were stopped with 1 N H₂SO₄. Plates were read on the Synergy HTX Multi-Mode Microplate Reader (BioTek) and Gen5 v3.10 at two absorbance wavelengths: 650 nm for the developing reaction and 450 nm for the developed reaction. The final OD used for calculation was obtained by subtracting OD_{450 nm} with OD_{650 nm}. Plasma concentration (µg/mL) was determined using the generated standard curve, with an assay limit of detection of 0.0226 µg/mL.

To quantify Leronlimab concentration in tissues, 2-5 mm² pieces were placed into Lysing Matrix tubes (MP Biomedicals), with 200-500 µL (adjust volume based on tissue size) of complete EDTA-free Protease Inhibitor Cocktail (Sigma Aldrich) in PBS. Tissue was disrupted by beating in a Beadbeater (Biospec) device by three cycles of 1 minute beating and 1 minute on ice. Supernatant from the tissue homogenate was transferred into a Sarstedt tube and spun for 20

minutes at 12,000 x g to pellet residual debris. Protein-containing supernatant was transferred into a new Sarstedt tube and stored at -80°C until assayed. Leronlimab concentration in the tissue, presented as ng of Leronlimab to mg of total protein, required two ELISAs to determine 1) Leronlimab concentration as described above and 2) total protein concentration measured with Pierce Coomassie Plus Bradford Assay (ThermoFisher) following manufacturer's instructions.

Measurement of anti-drug antibody (ADA) against Leronlimab. ELISA was used to detect ADA against Leronlimab in plasma as previously described⁴⁹⁸. Briefly, 2 µg/mL Leronlimab (Cytodyn, Vancouver, WA) was diluted in carbonate-bicarbonate buffer (ThermoFisher) and coated into half-area 96-well Costar Assay Plates (Corning) overnight at 4°C. The next day, plates were washed three times with PBS-T and blocked with Blocking Buffer for at least two hours in RT. Afterward, blocked plates were washed three times with PBS-T. Heat-inactivated plasma samples were serially diluted in Blocking Buffer with six serial dilutions and plated onto blocked plates in duplicates for 30 minutes in RT. After this incubation step, plates were washed three times with 0.5 M NaCl in PBS and incubated with 5,000-fold diluted secondary antibody that recognized rhesus macaque IgG and conjugated to HRP (anti-rhesus IgG1/3[1B3]-HRP; NHP Reagent Resource; 0.5 mg/mL) in Blocking Buffer. Plates were incubated at RT for 30 minutes, with the remaining steps following the quantification ELISA described above. ADA titers were defined as the reciprocal of the highest dilution of the sample that yielded a positive result (*e.g.*, dilution of 1/2460 = titer of 2460). A positive result was defined as twice that of background value for each individual macaque.

Viral nucleic acid detection. Detection of integrated HIV-1 DNA was performed as previously described^{555,556}. Briefly, HIV-1 viral DNA was isolated from PBMCs and enriched for CD4+ T-cells to purities of more than 95% with an EasyStep Human CD4+ T-cell enrichment kit (Stemcell Technologies). DNA was extracted from enriched CD4+ T-cells using AllPreP DNA/RNA/miRNA Universal Kit (Qiagen) and measured by a ND-2000 spectrophotometer (NanoDrop Technologies) for purity and concentration. To determine integrated HIV-1 DNA copies in CD4+ T-cells, DNA samples were first pre-amplified using LTR-Alu specific primers ULF1, Alu1 (5'-TCCCAGCTACTGGGGAGGCTGAGG-3') and Alu2 (5'-GCCTCCCAAAGTGCTGGGATTACAG-3'). Pre-amplification was performed in 50 µL reaction volumes using 5 µL Taq Buffer, 3 µL of MgCl₂, 0.6 µL of each dNTP, 1.5 µL of each primer, 0.5 µL of Taq polymerase, and 5 µL of DNA template. Reactions were performed on a GeneAmp PCR System 9700 (Applied Biosystems Inc.) with the following thermal conditions: 95°C for 8 minutes; [95°C for 1 minute, 55°C for 1 minute, 72°C for 15 minutes] x 12 cycles. Integrated HIV-1 DNA samples were quantified with a qPCR TaqMan assay using Lambda T (5'-ATGCCACGTAAGCGAAACT-3') and UR2 (5'-CTGAGGGATCTCTAGTTACC-3'; HXB2 583-602), and coupled with a FAM-BHQ probe (5'-CACTCAAGGCAAGCTTTATTGAGG-3'). The reaction volume was 20 µL, containing 10 µL of 2x TaqMan Universal Master Mix II including UNG (Life technologies), 0.25 µL of 100 µM per primer, 0.4 µL of 10 µM probe, and 5 µL pre-amplified PCR product (1:10 dilution). Reactions were performed on a StepOne Plus Real-time PCR System (Applied Biosystems Inc) with the following thermal conditions: 50°C for 2 minutes; 95°C for 10 minutes; [95°C for 15 seconds and 60°C for 1 minute] x 60 cycles. A standard curve was created with the CD3 gene, which allowed for the calculation of cell-equivalent DNA copy numbers.

Detection of SHIV nucleic acid was conducted previously described^{498,518}. Briefly, all SHIV nucleic acid detection assays were performed by members of the ONPRC Molecular Virology Core, who were blinded in the treatment conditions of each animal for all time points. Maxwell 16 instrument (Promega, Madison, WI) was used to extract nucleic acid from plasma and PBMC cells using LEV Viral Total Nucleic Acid Kit and LEV Whole Blood Nucleic Acid kit, respectively, following manufacturer's protocols. Tissues were disrupted in Tri-reagent, and extraction of DNA and RNA was performed as previously described^{252,498}. Viral copies were measured by RT-qPCR or qPCR that targeted a highly conserved sequence of Gag by using the SGAG21 forward primer (5'-GTCTGCGTCATPTGGTGCATTC-3'), SGAG22 reverse primer (5'-CACTAGKTGTCTCTGCACTATPTGTTTTG-3'), and pSGAG23 probe (5'-6-carboxyfluorescein [FAM]-CTTCPTCAGTKGTTTCACTTTCTCTTCTGCG-black hole quencher [BHQ1]-3').

RT-qPCR reactions were performed to quantitate SHIV viral RNA copies in plasma. The reactions used the TaqMan Fast Virus 1-Step Master Mix (Applied Biosystems), all RNA extracted from 300 µL of plasma, 900 nM SGAG21, 900 nM of SGAG22, and 250 nM pSGAG23. A standard curve was created by using in vitro transcribed SIVgag RNA serially diluted in 5 ng/µL yeast tRNA (Sigma R5636), with known SIV-positive plasma RNA and nuclease-free water serving as the positive and negative controls, respectively. Applied Biosystems QuantStudio 6 Flex instrument (Life Technologies) was used to run the reactions, with the following thermal conditions: 50°C for 5 minutes; 95°C for 20 seconds; [95°C for 3 seconds, 60°C for 30 seconds] x 45 cycles. The limit of detection for this assay is 50 copies/mL.

To detect SHIV viral RNA copies in cell pellet and tissue samples, 2.5 µg of extracted RNA samples were synthesized to complementary DNA (cDNA) using 20 U Superscript II RT

(ThermoFisher/Invitrogen), 5 mM MgCl₂, 0.5 mM dNTPs, 1 mM dithiothreitol (DTT), 150 ng random hexamers, 1x TaqMan PCR buffer (with 0.05% gelatin and 0.02% Tween-20), and 20 U RNAaseOut for a final volume of 30 µL. Reactions were ran using the following thermal conditions: 25°C for 15 minutes, 42°C for 40 minutes, 90°C for 15 minutes, 25°C for 30 minutes, and 4°C hold. Using the entire RT-PCR reactions, qPCR reactions were performed by adding 1.25 U Platinum Taq (Applied Biosystems), 600 nM of SGAG21, 600 nM of SGAG22, 100 nM pSGAG23, 1x TaqMan PCR II buffer, 4.5 mM MgCl₂, and 50 nM ROX passive reference dye for a final volume of 50 µL. A standard curve was created by using in vitro transcribed SIVgag RNA serially diluted in 100 ng/µL yeast tRNA (Sigma R5636). qPCR reactions were performed in an Applied Biosystems ABI 7500 instrument using the following thermal conditions: 95°C for 2 minutes; [95°C for 15 seconds, 60°C for 1 minute] x 45 cycles.

To detect SHIV viral DNA copies in cell pellet and tissue samples, 2.5 µg of extracted DNA samples were first heated at 95°C for 5 minutes, placed on ice to chill, and then added to Taqman Fast Advanced Master Mix (Life Technologies), 600 nM of SGAG21, 600 nM of SGAG22, and 100 nM pSGAG23. A standard curve was created by serial dilutions of a linearized plasmid containing the SIV gag sequencing using TE buffer that contained 2.5 ng/mL SIV-negative rhesus macaque genomic DNA. Applied Biosystems QuantStudio 6 Flex instrument (Life Technologies) was used to run qPCR reactions with the following thermal conditions: 50°C for 2 minutes; 95°C for 20 seconds; [95°C for 1 second, 60°C for 20 seconds] x 45 cycles. The limit of detections for this assay are 10 copies/million cells for cell pellets and 7 copies/million cells for tissue biopsies.

Env sequencing. SHIV_{SF162P3} Env sequencing and analysis were performed as previously described⁵³⁹⁻⁵⁴³. Briefly, QIAamp MinElute Virus Spin Kit (Qiagen) was used to extract viral RNA from viral stock and plasma samples following manufacturer's instructions. Complementary DNA was generated with the SuperScript III One-Step RT-PCR with Platinum Taq (ThermoFisher) with the following primers: SHIV *env* Forward primer (GGCATAGCCTCATAAAATATCTG) and SHIV *env* Reverse primer (ACAGAGCGAAATGCAGTGATATT), giving a ~4.5kb amplicon of *env*. Eppendorf Mastercycler Pro S Thermal Cyclers was used to run the RT-PCR reaction, using the following thermal conditions: 50°C for 30 min; 94°C for 2 min; [94°C for 15 sec, 60°C for 1 min, 68°C for 4 min] x 2 cycles; [94°C for 15 sec, 58°C for 1 min, 68°C for 4 min] x 2 cycles; [94°C for 15 sec, 60°C for 1 min, 68°C for 4 min] x 45 cycles; 68°C for 10 min; and held at 4°C. PCR amplicons were purified using 1% agarose gels and NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel). Following manufacturer's instructions, Nextera XT DNA Sample Prep Kit (Illumina) was used to generate dual-indexed Illumina MiSeq-compatible libraries and then purified with AMPure XP magnetic beads (Beckman Coulter). Dual-indexed libraries were analyzed on an Agilent 2100 Bioanalyzer using the HS DNA kit (Agilent) before normalizing to 2 nM and pooling to an equimolar ratio for Illumina MiSeq run. Sequences were processed as previously described^{539,540}, where Trimmomatic version 0.39 and BWA-MEM version 0.7.17-r1188^{541,542} were used to trim the raw data and align to the consensus SHIV_{SF162} sequence (GenBank Accession No. KF042063.1). Deletion and insertion polymorphisms and single nucleotide polymorphisms (SNPs) were called for bases with a quality score above 17. Identity of the associated read was retained for each SNP, allowing the phase of SNPs to be considered. This allows for amino acid translations to be calculated based on the sequence per individual

read rather than on the consensus SHIV_{SF162} sequence. SequenceAnalysis module, written by LabKey Server 21.3 ⁵⁴³ was used to analyze SNPs and visualize mutations.

Antibodies. The following conjugated antibodies were used in these studies: a) from BD Biosciences, D058-1283 (CD45; PE Cy7; cat# 561294), SP34-2 (CD3; Alexa 700; cat# 557917), SP34-2 (CD3; PE; cat# 552127), LP200 (CD4; PerCP-Cy5.5; cat# 552838), RPA-T8 (CD8; PacBlu; cat# 558207), SK1 (CD8; TruRed; cat# 341051) 3GB (CD16; Alexa 700; cat# 560713), 25723.11 (IFN- γ ; APC; cat# 502512), 6.7 (TNF- α ; PE; cat# 554513), 3A9 (CCR5; APC; cat# 560748), SK1 (CD8; BUV737; cat# 612754), L200 (CD4; BUV395; cat# 564107), FN50 (CD69; PE-Texas Red; cat# 562617), SP34-2 (CD3; Pacific Blue; cat# 558124); b) from BioLegend, OKT4 (CD4; APC-Cy7; cat# 305612), RPA-T4 (CD4; APC; cat# 300537); c) from Beckman Coulter, RMO52 (CD14; PE-Texas Red; cat# IM2707U); d) from Sigma, HP-6025 (IgG4; FITC; cat# F9890); e) from SouthernBiotech, HP6023 (mouse anti-human IgG4 pFc'; HRP; 1:20,000; cat# 9190-05), f) from NHP Reagent Resource, 1B3 (anti-rhesus IgG1/3; HRP; 1:5000).

The following unconjugated antibodies were used: a) PA-14 (Leronlimab; CytoDyn), conjugated in-house to PacBlu using Pacific BlueTM Antibody labeling Kit (ThermoFisher) and used at approximately 1:80 depending on the efficacy of conjugation, b) anti-idiotypic antibody, PA-22 (CytoDyn). Live/dead Fixable Yellow Dead Cell Stain Kit and Near-IR Dead Cell Stain Kit (ThermoFisher) were amine-reactive dyes used at 1:1000 dilution to assess cell viability.

Statistics. Two-way repeated measures analysis of variance (ANOVA) was used for longitudinal outcome measures to compare plasma viral load and normalized change from baseline for

%CCR5, lymphocytes, CD4+, and CD8+ cells between the Leronlimab-treated and control groups over the 15-week study period. Two-way repeated measures ANOVA was also used to compare tissue vDNA and tissue vRNA changes between treatment groups over six tissues: PBMC, lymph nodes (axillary and mesenteric), spleen, colon, and cervix. Since in a typical experiment using repeated measures, two measurements taken at adjacent times are more highly correlated than two measurements taken several timepoints apart, optimal covariance structure chosen by Bayesian Information Criteria (BIC) was used to account for within-subject correlation. One-way ANOVA was used to compare peak and area-under-curve plasma viral load between treatment groups. Due to right-skewed data, some analyses were performed on log-transformed data. For normally distributed data, analyses were conducted on untransformed data, including normalized change from baseline for %CCR5 of CD8+ T-cells, lymphocytes, CD4+ T-cell, and CD8+ T-cell. Multiplicity adjusted p-values following Tukey procedures were presented. Statistical significance was determined at the significant alpha level of 0.05. Analyses were performed using SAS version 9.4, specifically PROC MIXED.

4.5. Results

Leronlimab treatment in HIV-infected human participants for over seven years.

To evaluate the long-term safety, tolerability, and efficacy of weekly SC Leronlimab in humans, five virologically-suppressed participants from the CD01 study (ClinicalTrials.gov NCT02175680) were enrolled in the CD01-Extension study (ClinicalTrials.gov NCT02355184) to continue self-administration of weekly Leronlimab for at least 160 weeks (Figure 4.1A and Table 4.1). In this ongoing study, cohort 1 (n=2) received weekly 350 mg Leronlimab and cohort 2 (n=3) increased their weekly doses from 350 mg to 700 mg. As previously reported,

Leronlimab was well-tolerated ⁴⁶¹ and no sustained changes were observed in longitudinal serum chemistry profiles (Supplemental Figure 4.1).

ID	Sex	Age (Years)	Average weight (kg)	Duration of HIV infection (years)	Prior ART regimen	Class
01-037	Male	39.9	70	10	TDF/FTC/RPV	NRTI/NNRTI
01-038	Male	63.7	80.5	37	RAL/DTG	INSTI
01-057	Male	32.6	83.5	15	TDF/FTC/EVG/c	INSTI/ NRTI
01-061	Female	73.8	59.5	9	TDF/FTC	NRTI
01-064	Male	51.9	97	9	TDF/FTC/EVG/c	INSTI/ NRTI

Table 4.1. Human participant demographics and clinical information. TDF = tenofovir disoproxil fumarate; FTC = emtricitabine; RPV = rilpivirine; RAL = raltegravir; DTG = dolutegravir; EVG/c = elvitegravir/cobicistat; NRTI = nucleoside reverse transcriptase inhibitor; NNRTI = non-nucleoside reverse transcriptase inhibitor; INSTI = integrase strand transfer inhibitor.

Leronlimab treatment maintained virologic suppression in all five participants for over seven years (Figure 4.1B-C). Four of the five participants experienced infrequent, transient episodes of plasma viremia, termed viral blips, while one participant from cohort 2 (01-061) was aviremic in all sampled timepoints (Figure 4.1D and Supplemental Table 4.1). Participant 01-038, who has the longest duration HIV infection of all study participants at 37 years, presented with the highest number of blips. Viral blips occurred in 9.8% of the sampled timepoints (n=205) in cohort 1 and 3.5% of the sampled timepoints (n=256) in cohort 2. Although the viral blip frequency for all participants overall at 6.3% was higher than the 2.0% observed in 4,449 sampled timepoints from 735 HIV-infected individuals on long-term ART ³⁷⁷, the small sample size precludes statistical analysis. Mean ($\pm$ SD) plasma viral loads during these transient viremic episodes were 282.2 ($\pm$ 498.0) and 207.3 ($\pm$ 362.8) HIV-1 copies/mL for cohort 1 and 2, respectively (Figure 4.1E). Lastly, viral blips in most participants spontaneously resolved by the next timepoint, indicating lack of emergence of Leronlimab-resistant variants.

Low CD4⁺ T-cell counts in the blood is a hallmark of untreated HIV. Here, four of the five participants had longitudinal CD4⁺ T-cell counts within the normal reference range (Figure

4.1F-G). Notably, 01-038 was the only participant with CD4⁺ T-cell counts below the normal range, likely attributable to the length of HIV infection in this participant. Interestingly, the dose change from 350 to 700 mg sequentially increased CD4⁺ T-cell counts in all three participants from cohort 2.

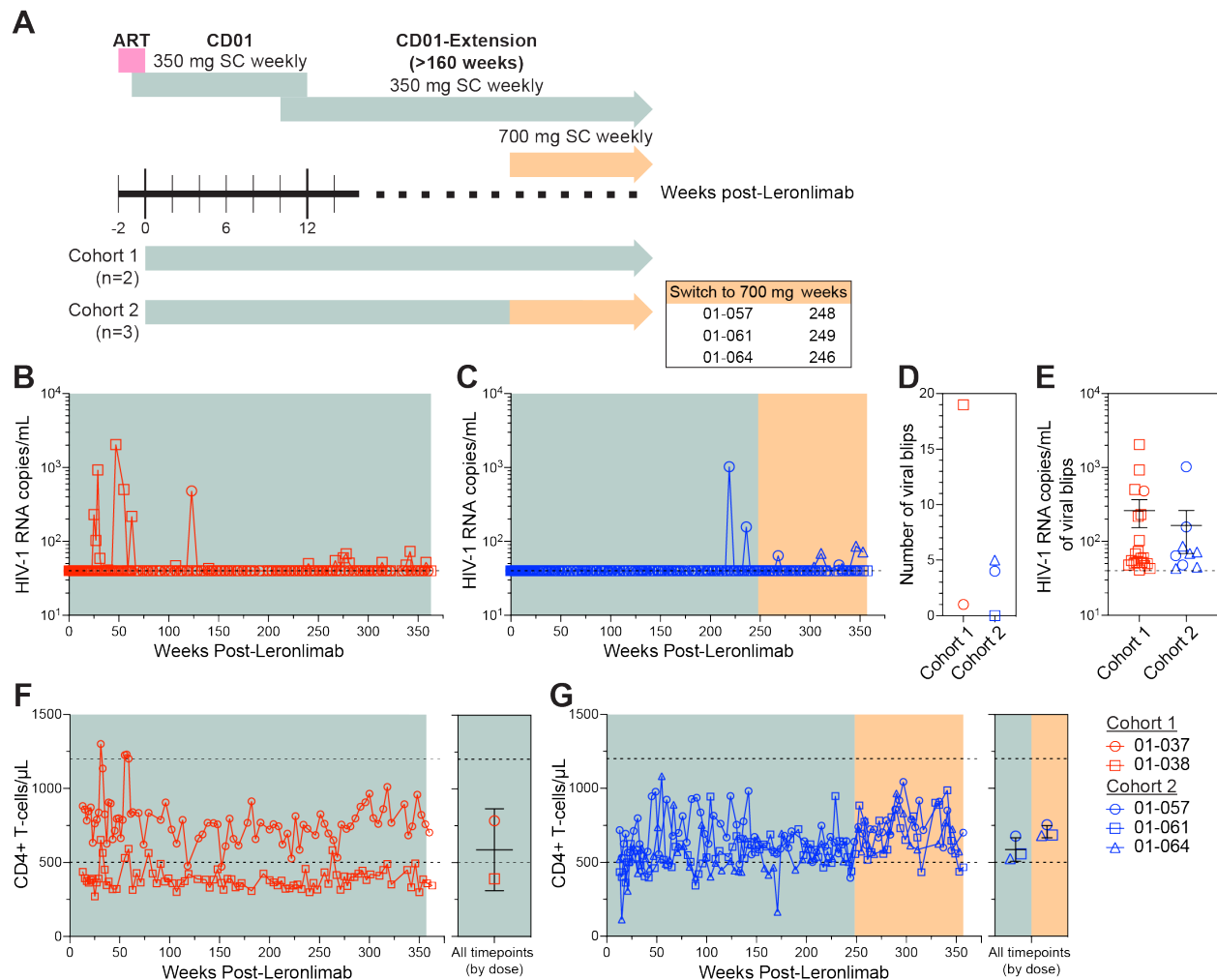


Figure 4.1. Leronlimab maintained virologic suppression in humans for over seven years. (A) Study outline for CD01 and CD01-Extension studies (ClinicalTrials.gov: NCT02175680 and NCT02355184). One-week prior to ART interruption, cohort 1 (n=2; red) and cohort 2 (n=3; blue) received weekly SC 350 mg injections. After weeks 246-249, cohort 2 switched to weekly SC 700 mg. Longitudinal HIV-1 RNA copies/mL in plasma for (B) cohort 1 and (C) cohort 2. (D) Total number and (E) mean HIV-1 RNA copies/mL of viral blips (>40 copies/mL). (B-C, E) Horizontal dashed line denotes assay limit of detection (LOD) of 40 copies/mL; undetected viral loads graphed at LOD. (F-G) Left graph shows longitudinal CD4⁺ T-cell counts in the blood and right graph shows mean ($\pm$ SD) for all CD4⁺ T-cell timepoints based on the treatment dose for cohort 1 (E) and cohort 2 (F). (F-G) Two horizontal dotted lines represent the normal range for CD4⁺ T-cells. Gray and orange boxes denote 350 and 700 mg injections, respectively.

To more closely investigate the long-term effects of Leronlimab, we collected blood samples at one timepoint per participant during study week 297-304. We detected integrated HIV-1 DNA in CD4⁺ T-cells from four of the five participants, with 01-038, the individual with the longest duration of HIV infection and most viral blips, harboring the greatest copy number of integrated HIV-1 DNA in CD4⁺ T-cells (Figure 4.2A). Thus, despite the presence of a latent HIV-1 reservoir in blood CD4⁺ T-cells, the absence of plasma viremia suggested that weekly Leronlimab was responsible for the maintenance of virologic suppression after ART interruption. Next, we performed mass cytometry (CyTOF) to profile 37 immune cell populations from participants' PBMCs, which did not reveal any notable depletion of subsets of CD4⁺ T-cells, CD8⁺ T-cells, B cells, NK cells, monocytes, and dendritic cells (Supplemental Figure 4.2 and Supplemental Table 4.2).

Because Leronlimab exerts its antiviral efficacy by outcompeting HIV for CCR5 binding, full CCR5 receptor occupancy by Leronlimab is required for a maximal antiviral response. To this end, we found that both cohorts had comparable mean ($\pm$ SD) plasma concentrations of 48.6 ($\pm$ 5.4) and 46.1 ($\pm$ 27.0) μ g/mL for cohort 1 and 2, respectively, likely due to the prolonged treatment period (Figure 4.2B). To quantify the saturation of cell surface CCR5 receptors by Leronlimab, we developed two complementary methods to measure free or Leronlimab-bound CCR5 receptors via flow cytometry (Supplemental Figure 4.3), which were used interchangeably to calculate for the percentage of CCR5 receptor occupancy (RO) by Leronlimab⁵⁵⁷. In all five participants, we observed near-complete CCR5 RO by Leronlimab on CD4⁺ T-cells, CD8⁺ T-cells, and CD14⁺ monocytes in the blood (Figure 4.2C). Together with the stringent viral control observed over seven years, these findings demonstrated that both doses of Leronlimab tested

were sufficient to fully occupy CCR5 and protect CCR5+CD4+ T-cells from CCR5-tropic HIV-1 replication.

To evaluate if Leronlimab treatment would interfere with T-cell responses to HIV, we performed intracellular cytokine staining (ICS) on peripheral blood mononuclear cells (PBMC) using HIV and positive control human cytomegalovirus (HCMV) peptide antigens. Participants showed varying CD4+ memory T-cell responses to HIV despite being responsive to HCMV. In contrast, all participants showed distinct CD8+ memory T-cell responses (Figure 4.2D-E), confirming that long-term Leronlimab use did not abrogate cytotoxic T-cell responses to viral antigens.

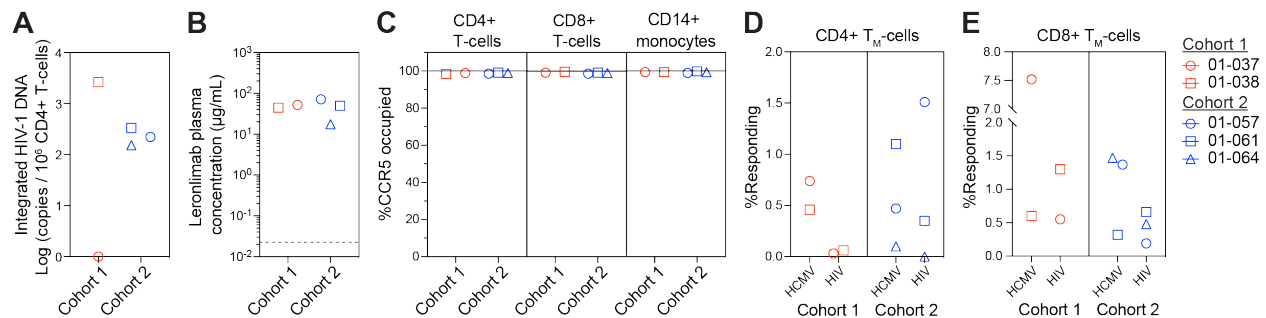


Figure 4.2. Analyses of long-term Leronlimab treatment in humans. Blood was collected from 01-037, 01-038, 01-057, 01-061, and 01-064 at weeks 304, 301, 297, 299, and 297, respectively. **(A)** Integrated HIV-1 DNA copies in CD4+ T-cells. **(B)** Leronlimab concentration in the plasma. Horizontal dashed line denotes LOD ($0.0226 \mu\text{g/mL}$). **(C)** CCR5 RO by Leronlimab on CD4+ T-cells, CD8+ T-cells, and CD14+ monocytes. Intracellular cytokine staining of CD95+ memory **(D)** CD4+ and **(E)** CD8+ T_M -cells stimulated with HCMV (IE1 and PP65) and HIV (Gag and Nef) peptides. Positive responses were determined by Boolean gating with CD69+TFN- α + and/or CD69+IFN- γ +

Collectively, our findings demonstrated that Leronlimab treatment in humans was safe, well-tolerated, and maintained virologic suppression for over seven years. Weekly SC dose of 350 mg or 700 mg Leronlimab resulted in near-complete CCR5 RO on peripheral blood cells and suppression of CCR5-tropic HIV-1 replication, without depletion of immune system subsets or abolishment of antiviral cytotoxic T-cell immunity.

Leronlimab treatment in SHIV-infected rhesus macaques for 12 weeks.

Experimental studies in nonhuman primates provide opportunities not possible in human clinical trials, including the ability to frequently and extensively sample anatomical sites of interest and synchronize infections⁵⁵⁸. We previously reported that Leronlimab, a humanized antibody, cross-reacted with macaque CCR5⁴⁹⁸. To explore the impact of Leronlimab in acute infection, we intravenously infected nine macaques with 1,000 TCID₅₀ of CCR5-tropic SHIV_{SF162P3}. We selected SHIV_{SF162P3} for two reasons: 1) the parental Env, CCR5-tropic HIV-1_{SF162}, is particularly resistant to CCR5-targeting agents⁴⁵⁶ and 2) SHIV_{SF162P3} can switch tropism to use non-CCR5 coreceptors in macaques infection, which can help assess risk of viral escape⁴⁹⁵. SHIV_{SF162P3} infection proceeded without treatment for three weeks, after which four macaques received weekly SC injections of 50 mg/kg Leronlimab for 12 consecutive weeks. The remaining macaques (n=5) served as untreated controls. At week-15, all animals were euthanized and anatomical tissues were collected for analysis (Figure 4.3A and Supplemental Table 4.3).

Consistent with Leronlimab treatment in humans, Leronlimab treatment in macaques was well-tolerated, with no major changes observed in longitudinal complete blood counts and serum chemistry parameters. However, absolute cell counts for lymphocytes, CD4⁺ T-cells, and CD8⁺ T-cells did trend higher in Leronlimab-treated macaques than in control macaques, as previously reported⁴⁵⁷ (Supplemental Figure 4.4, Supplemental Figure 4.5, and Supplemental Table 4.4).

One week following the first Leronlimab dose, we observed near-complete CCR5 RO by Leronlimab on CD4⁺ T-cells and CD14⁺ monocytes from PBMC (Figure 4.3B and Supplemental Figure 4.6A) and axillary lymph node (Supplemental Figure 4.6B-C), which was maintained throughout the study. The mean ($\pm$ SD) Leronlimab plasma concentration was 702.3 ($\pm$ 401.8) μ g/mL (Figure 4.3C). Anti-drug antibody (ADA) responses against the humanized

antibody, Leronlimab, may spontaneously develop in macaques, yet, we observed absent or minimal levels that did not impact plasma concentration or CCR5 RO (Supplemental Figure 4.6D).

Prior to Leronlimab treatment, there was no statistically significant difference between control and Leronlimab-treated macaques for both peak and area-under-the-curve (AUC) plasma viral loads (Supplemental Figure 4.6E-F). After receiving the second dose of Leronlimab, we observed a statistically significant reduction in weekly plasma viral loads in Leronlimab-treated macaques compared to controls, with viral loads that fell below the limit of detection in two of four macaques (Figure 4.3D, Supplemental Figure 4.6G and Supplemental Table 4.4). At the end of the study, the average plasma viral load for Leronlimab-treated macaques was 4.29 log₁₀ lower than control macaques. Furthermore, we found no modifications within the Env V3 loop indicative of escape via CXCR4 usage, an alternative coreceptor for cell entry (Supplemental Figure 4.7).

Concomitant with near-complete CCR5 RO in the peripheral blood, Leronlimab-treated macaques exhibited a statistically significant resurgence in SHIV-susceptible CCR5+CD4+ T-cells, while control macaques experienced sharp and sustained decline in the same cell type (Figure 4.3E and Supplemental Table 4.4). Similarly, CD8+CCR5+ T-cells increased after Leronlimab treatment (Supplemental Figure 4.6H), showing that the anti-CCR5 Leronlimab did not deplete CCR5+ cells, likely due to low effector functions exhibited by IgG4²⁵⁷, but rather, Leronlimab rescued and protected CCR5+ cells during active viremia.

Next, we measured cell-associated SHIV DNA and RNA levels in multiple tissues. In line with plasma viral load results prior to Leronlimab treatment, viral DNA (vDNA) in PBMCs and viral RNA (vRNA) in PBMCs and axillary lymph nodes showed no significant difference

between the two groups; however, vDNA in the axillary lymph nodes of control macaques were statistically higher than Leronlimab-treated macaques (Figure 4.3F-G). After 12 weekly Leronlimab treatments, we detected statistically lower vDNA in PBMCs, axillary and mesenteric lymph nodes, and spleen in the Leronlimab-treated group compared to the controls, with the colon approaching statistical significance ($p=0.053$), while vRNA levels in these tissues were comparable between the two groups.

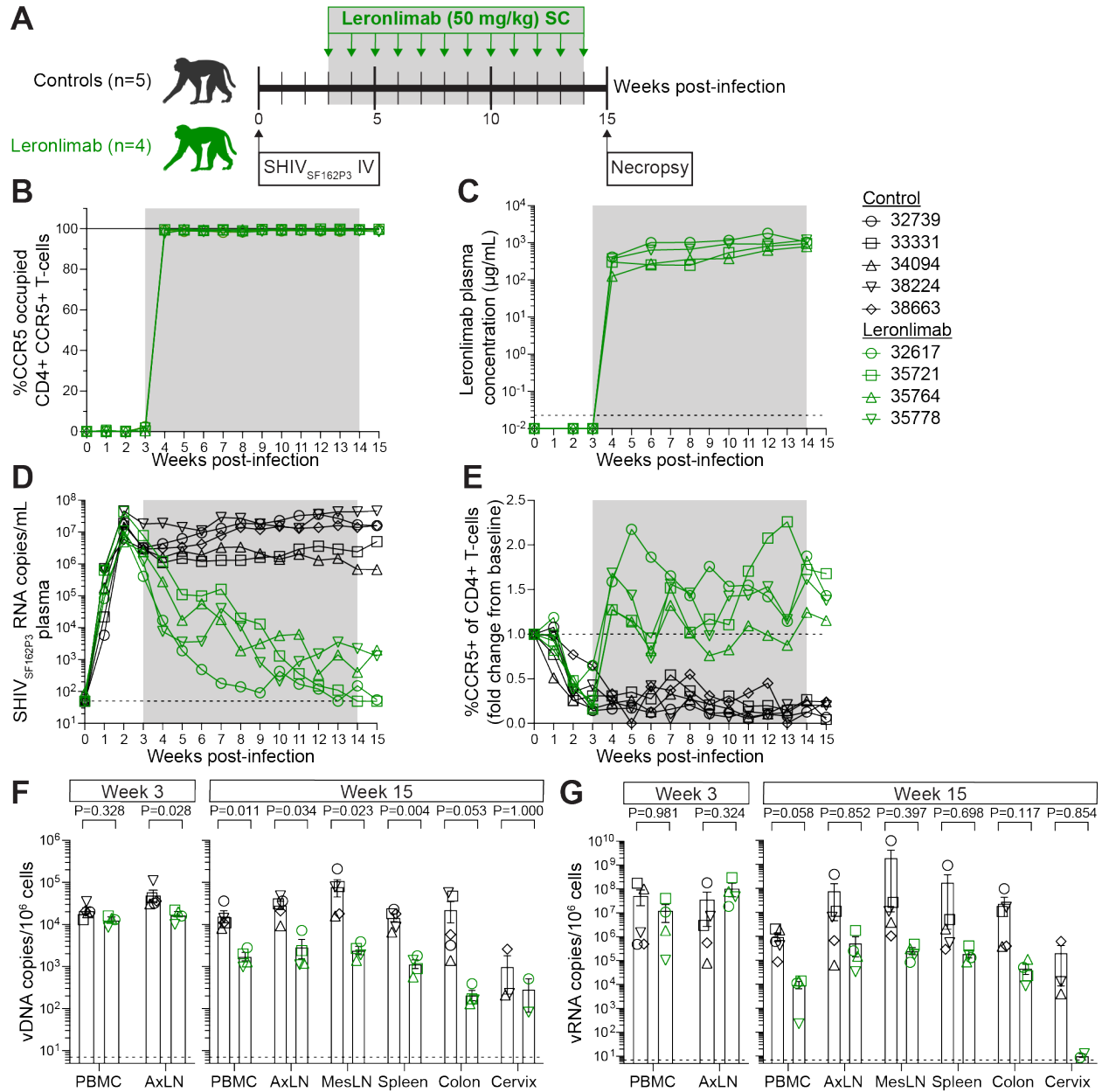


Figure 4.3. Leronlimab suppressed SHIV_{SF162P3} viremia in macaques. Macaques were infected intravenously with 1,000 TCID₅₀ SHIV_{SF162P3}. Leronlimab-treated group (n=4, green) received weekly SC 50 mg/kg starting at week-3 post-infection and untreated controls (n=5, black). **(A)** Study outline. **(B)** Longitudinal CCR5 RO levels by Leronlimab on blood CD4+CCR5+T-cell. **(C)** Longitudinal plasma concentration; horizontal dashed line denotes LOD (0.0226 μg/mL). **(D)** Longitudinal SHIV_{SF162P3} RNA copies/mL in plasma. Horizontal dashed line denotes LOD (50 copies/mL); undetectable viral loads graphed at LOD. **(E)** Longitudinal fold change from week-0 (baseline) for CD4+CCR5+ T-cell percentage in blood. (D-E) Weekly P-values can be found in Supplemental Table 4. **(F-G)** Mean (±SEM) cell-associated SHIV_{SF162P3} (F) vDNA and (G) vRNA at week-3 (pre-treatment) and week-15 (necropsy). Horizontal dashed lines denote LOD (7 copies/10⁶ cells). PBMC = peripheral blood mononuclear cell, AxLN = axillary lymph node, MesLN = mesenteric lymph node. (F-G) P-values calculated by two-way repeated-measures ANOVA with Tukey-Kramer adjustment. Gray box represents period of Leronlimab injections.

Because ARVs tissue distribution is heterogenous^{289,290}, we investigated the degree of tissue penetrance by Leronlimab. During euthanasia at week 15, saline was perfused into Leronlimab-treated macaques to remove peripheral blood from tissues to allow for the assessment of tissue-resident Leronlimab. We detected a wide range of concentrations from all sampled tissues, and concomitantly measured near-complete CCR5 RO by Leronlimab in non-brain tissues. Leronlimab CCR5 RO in brain tissues was ~70%, with the lowest tissue concentration of all sampled tissues found in brain (Figure 4.4A-C and Supplemental Table 4.5). As expected, we found no Leronlimab bound to CCR5 receptors on tissue-resident CD4⁺ T-cells from control macaques (Figure 4.4D and Supplemental Figure 4.8). Noticeably, levels of CCR5⁺CD4⁺ T-cells were significantly lower in tissues from controls than Leronlimab-treated macaques due to SHIV-mediated depletion.

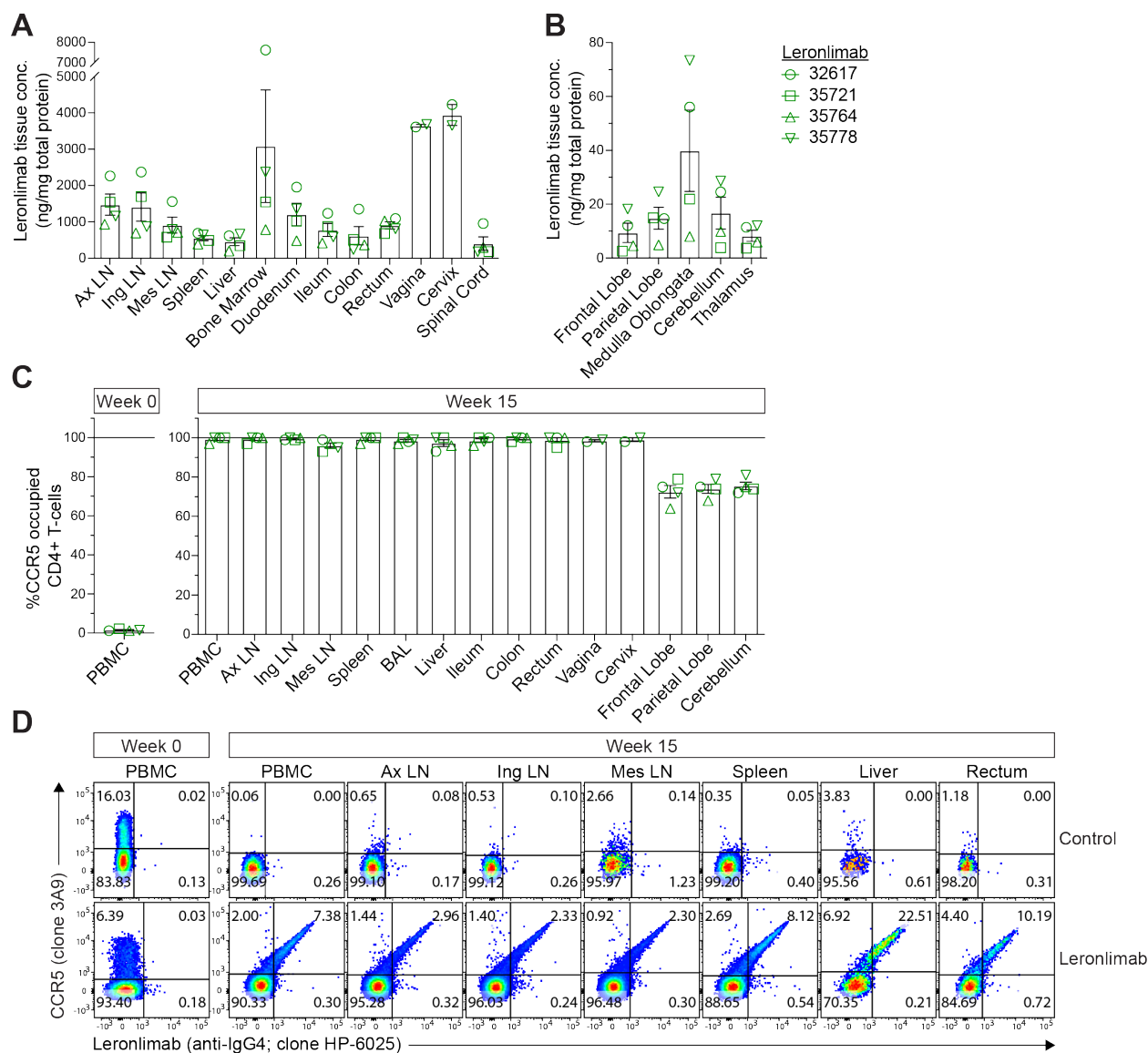


Figure 4.4. Tissue penetration by Leronlimab. Tissues were collected at week-15 (necropsy). Tissue concentration in **(A)** non-brain and **(B)** brain tissues. **(C)** CCR5 RO by Leronlimab on CD4+ T-cells. Panels A-C show mean ($\pm$ SEM). **(D)** Representative flow cytometry plots showing the costaining of anti-CCR5 (clone 3A9) and Leronlimab (by anti-human IgG4, clone HP-6025) on CD4+ T-cells from control (38224) and Leronlimab-treated (35778) macaques. PBMC = peripheral blood mononuclear cell, Ax LN = axillary lymph node, Ing LN = inguinal lymph node, Mes LN = mesenteric lymph node, BAL = bronchoalveolar lavage.

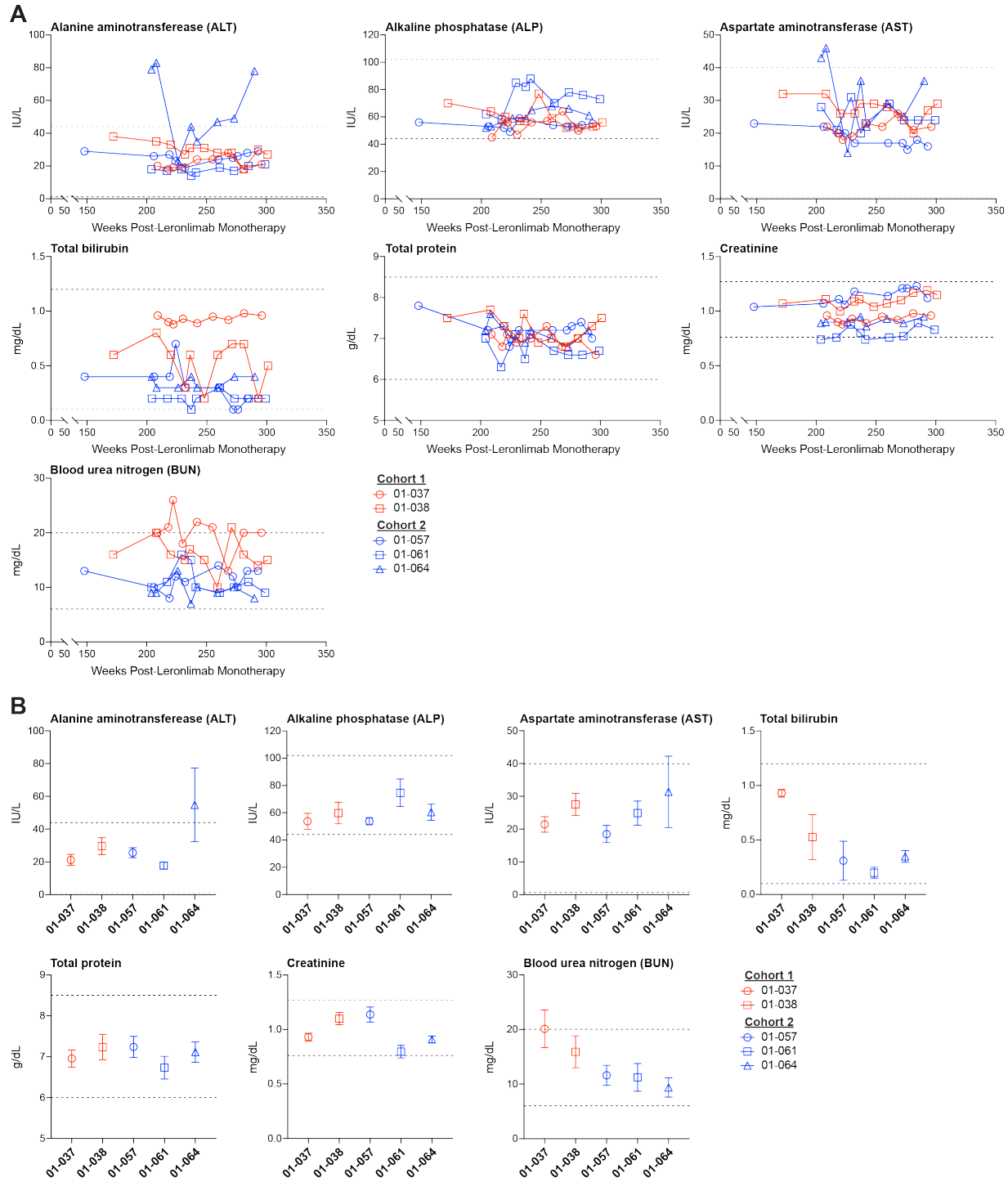
4.6. Discussion

In summary, the studies of Leronlimab treatment in chronically-infected humans and acutely-infected macaques described here support the safety, efficacy, tolerability, and potency of Leronlimab as an anti-HIV therapeutic. Although the clinical study was limited in sample size,

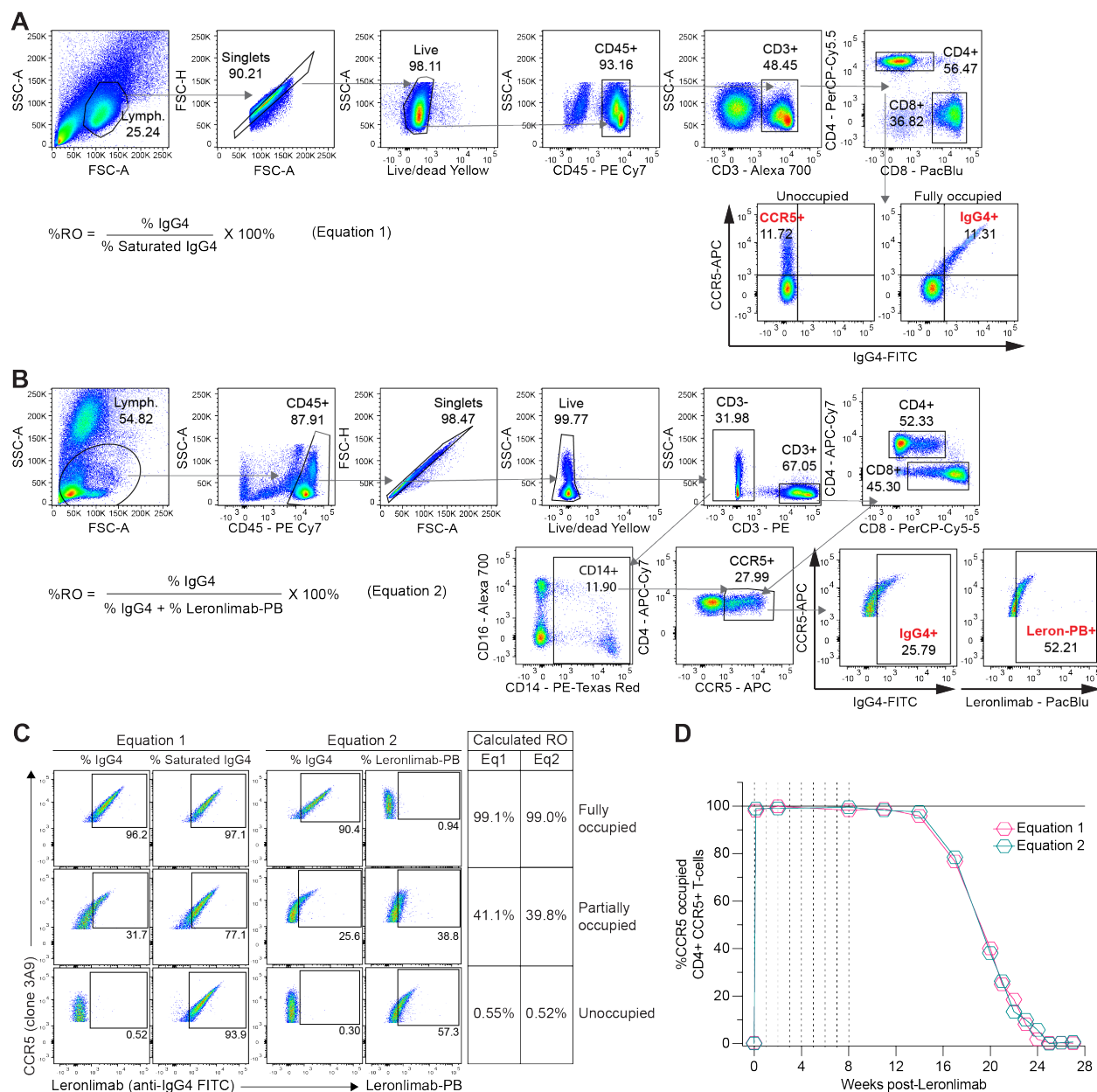
the power of this study came from the >7 years duration of Leronlimab treatment, which, to our knowledge, is the longest antibody treatment for HIV reported to-date. There are many potential benefits for the use of antibody-based drugs, including long-acting formulations to allow for infrequent dosing, higher target specificity to decrease side effects, and enhanced tissue penetrance in gastrointestinal tissues not seen with current ARVs^{290,559}. A switch from daily oral pills to weekly or monthly antibody injections would substantially improve user experience and lower adherence-associated risks of drug-resistance.

Currently, maraviroc is the only FDA-approved CCR5 inhibitor for HIV. In a 10-day monotherapy study, twice-daily 300 mg maraviroc reduced viral load by 1.84 log₁₀⁵¹³. A similar reduction of 1.83 log₁₀ was observed from a single Leronlimab injection of 5 or 10 mg/kg^{457,458}, which is the approximate range (4.34-11.76 mg/kg) given to participants in our study based on their individual average weights. While both drugs are CCR5-targeting and show similar efficacy in short-term use, the difference in their mechanism of action allows maraviroc-resistant HIV to continue infecting maraviroc-occupied CD4+CCR5+ T-cells⁴⁴¹, perpetuating the development of ARV resistance. In contrast, the successful protection from CCR5-tropic HIV-1 replication in humans for over seven years and in acutely-infected, ART-naïve macaques demonstrates the high genetic barrier to resistance by Leronlimab. Collectively, these findings suggest that Leronlimab is a promising entry inhibitor candidate. Future studies are warranted to expand and characterize the use of Leronlimab as an HIV therapeutic.

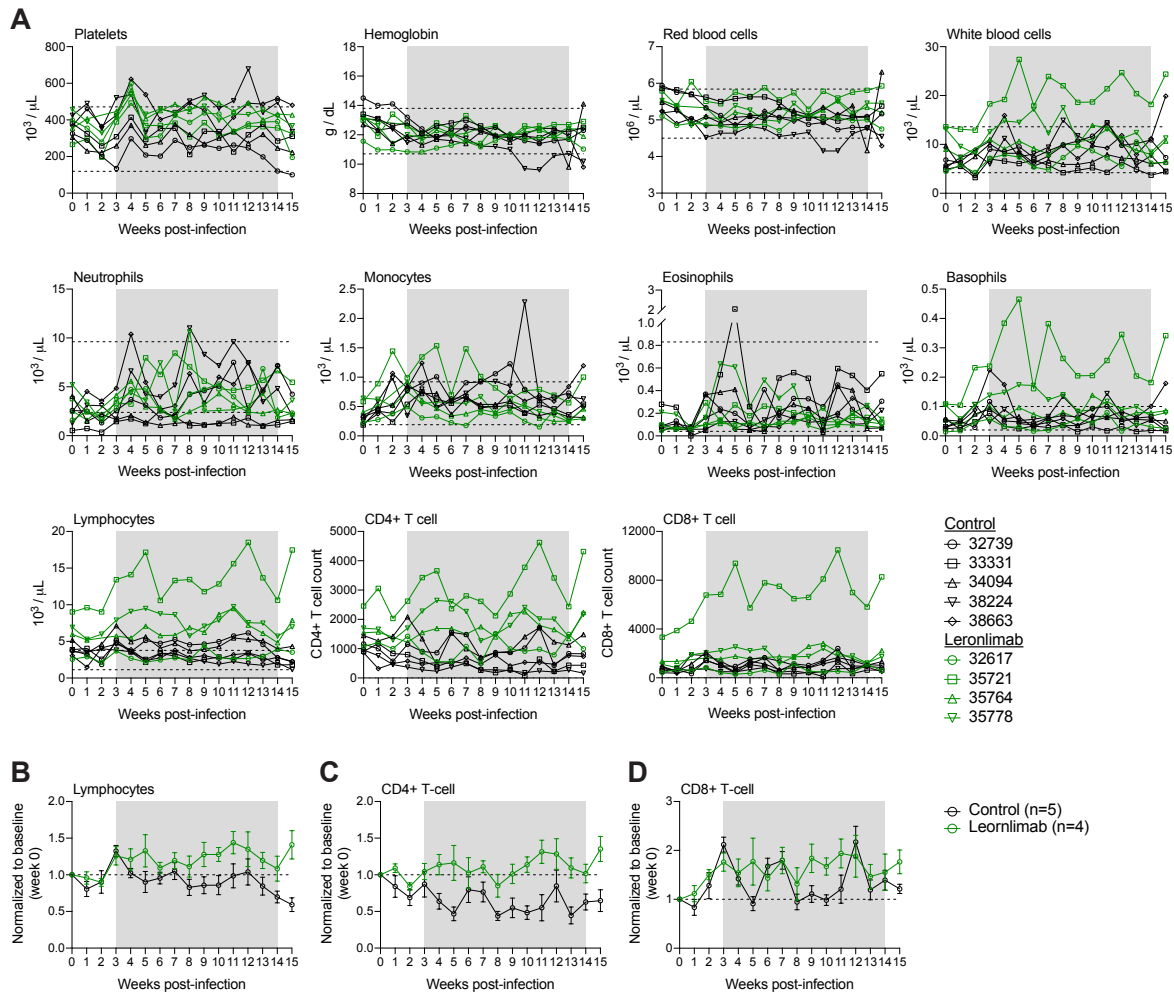
4.7. Supplemental Data



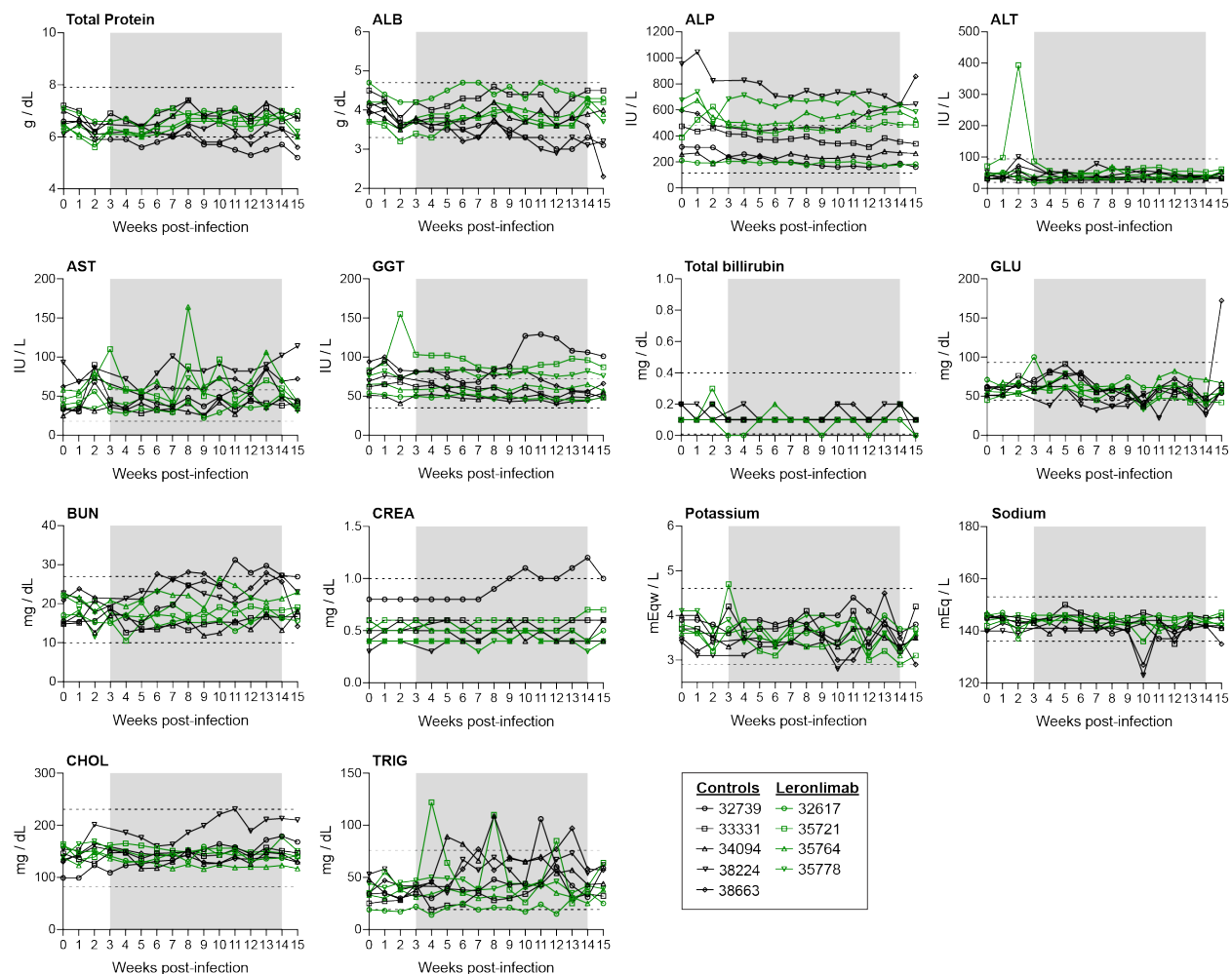
Supplemental Figure 4.1. Serum chemistry of human participant. Serum values are shown for cohort 1 (n=2; red) and cohort 2 (n=3; blue). For each participant, longitudinal (A) individual timepoints and (B) mean ($\pm$ SD) timepoints of alanine aminotransferase (ALT), alkaline phosphatase (ALP), aspartate aminotransferase (AST), total bilirubin, total protein, creatinine, and blood urea nitrogen (BUN). The two horizontal dotted lines in all panels represent the normal ranges in healthy, uninfected humans.



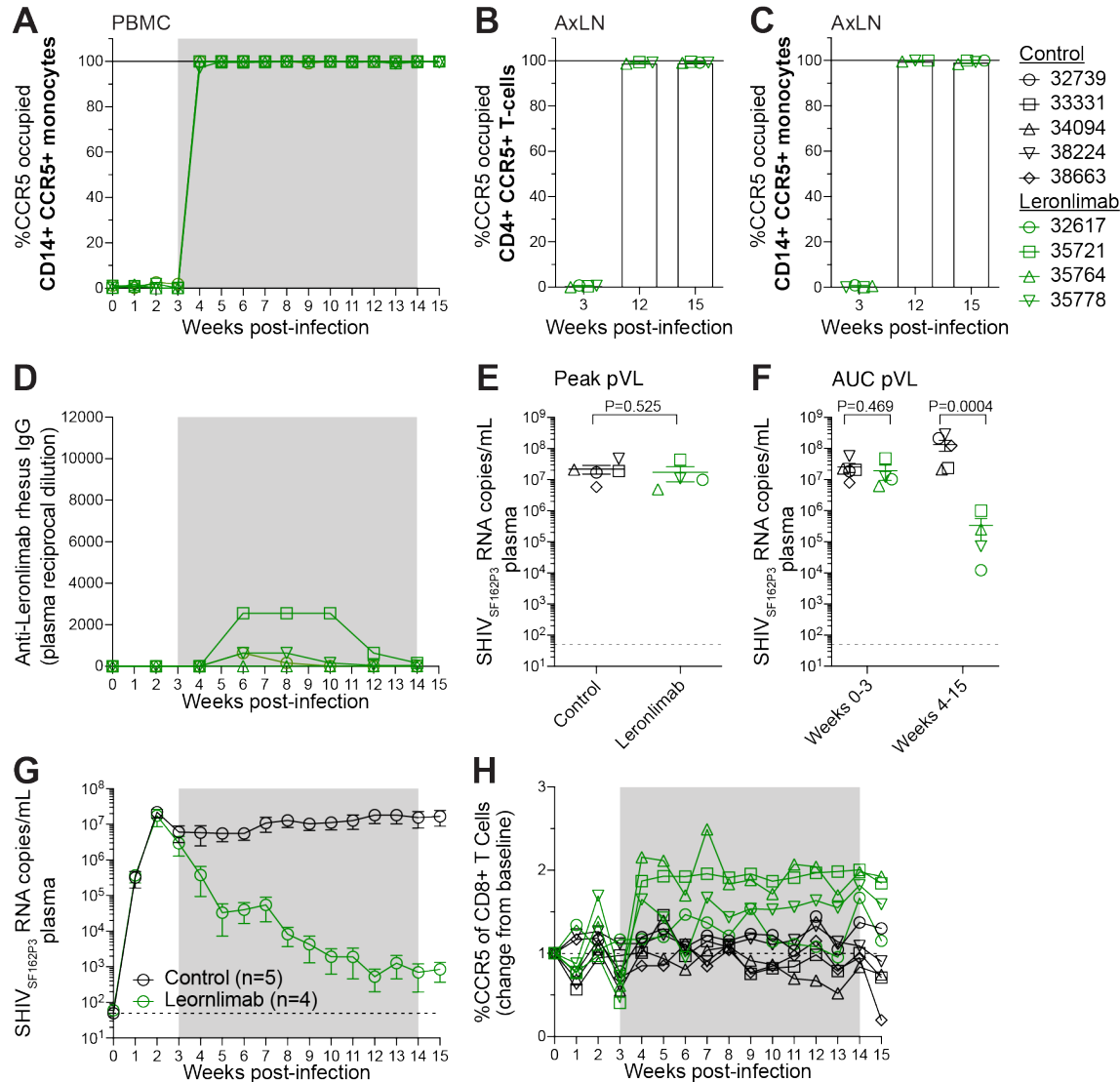
Supplemental Figure 4.3. CCR5 receptor occupancy (RO) assays. (A-B) Representative flow cytometric analysis for (A) equation 1 and (B) equation 2 used to calculate for CCR5 RO by Leronlimab. **(C)** Representative flow cytometry plots displaying the different components required to calculate each equation, and at different levels of CCR5 RO: fully occupied, partially occupied, and unoccupied. Equation 1 used the frequency of IgG4+ events within the CD45+, singlet, live, CD3+, CD4+/CD8-, and CCR5+ population. Equation 2 used the frequency of IgG4+ and Leronlimab-PB+ events within the CD45+, singlet, live, CD3+, CD4+/CD8-, and CCR5+ population. Table at right shows the calculated percentages of CCR5 RO using the two equations. **(D)** Graphic comparison of longitudinal CCR5 RO on CD4+ T-cells in the blood calculated by each equation. Dashed vertical lines represents Leronlimab treatment.



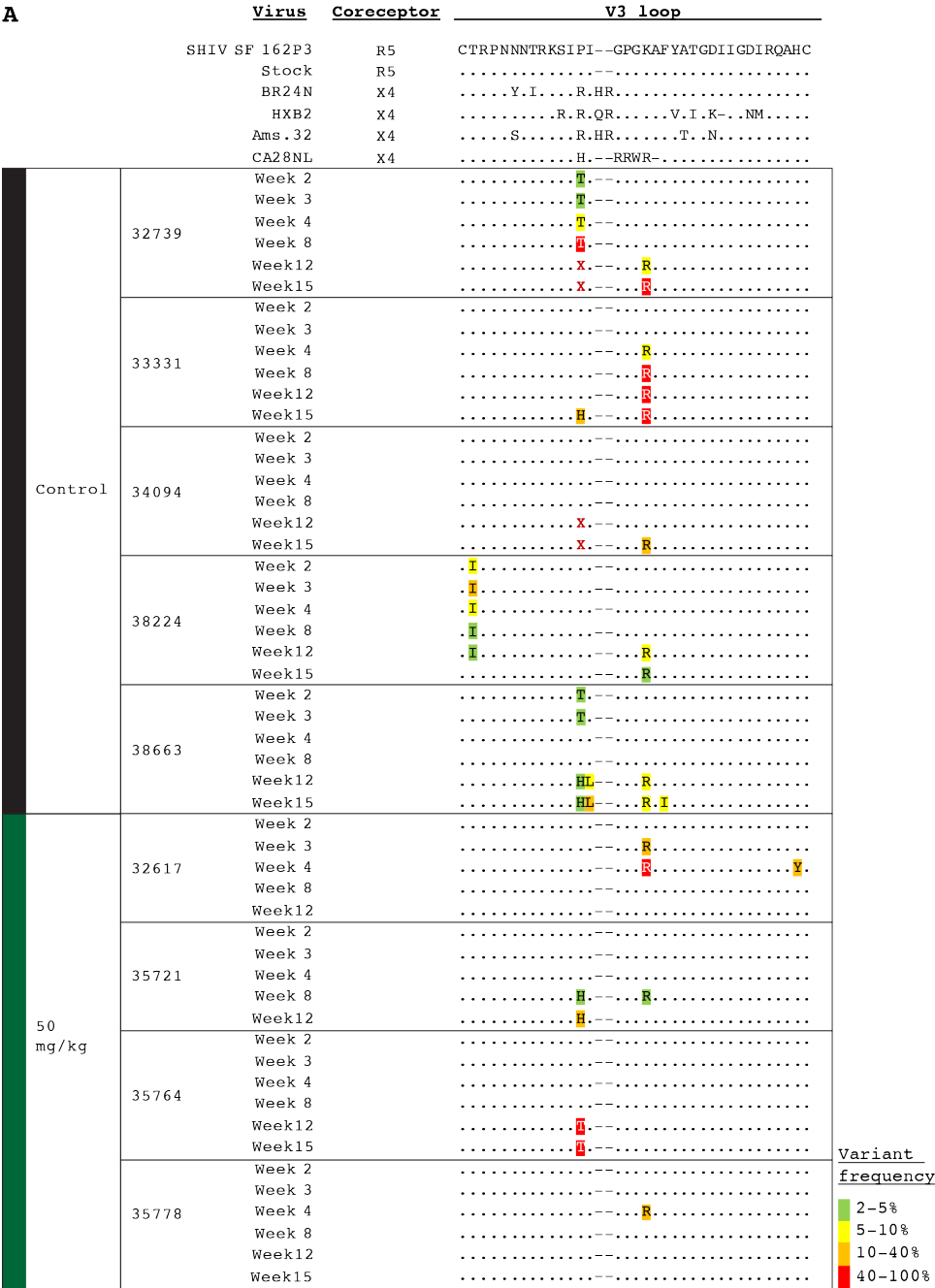
Supplemental Figure 4.4. Longitudinal complete blood counts in macaques. Longitudinal values are shown for the control (n=5; black) and Leronlimab-treated (n=4; green) macaques. **(A)** Absolute counts for peripheral blood counts of platelets, hemoglobin, red blood cells, white blood cells, neutrophils, monocytes, eosinophils, basophils, lymphocytes, CD4+ T-cells, and CD8+ T-cells. The two horizontal dotted lines indicate standard reference range for each parameter in macaque housed at ONPRC. **(B-D)** Longitudinal mean ($\pm$ SEM) for (B) lymphocytes, (C) CD4+ T-cells, and (D) CD8+ T-cells, and weekly P-values can be found in Supplemental Table 4. Gray box represents period of Leronlimab treatment.



Supplemental Figure 4.5. Longitudinal serum chemistry parameters in macaques. Albumin (ALB), alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyltransferase (GGT), glucose (GLU), blood urea nitrogen (BUN), creatinine (CREA), cholesterol (CHOL), and triglyceride (TRIG). The two horizontal dotted lines indicate standard reference ranges for each parameter of rhesus macaques housed at ONPRC. Gray box represents period of Leronlimab treatment.



Supplemental Figure 4.6. Additional data corresponding to Fig. 4.3. (A) Longitudinal CCR5 RO levels by Leronlimab on blood CCR5+ CD14+ monocytes in Leronlimab-treated macaques. **(B-C)** CCR5 RO levels of axillary lymph node (AxLN) of CCR5+ **(B)** CD4+ T-cells and **(C)** CD14+ monocytes. **(D)** Longitudinal anti-Leronlimab rhesus IgG levels in plasma. **(E-F)** Mean ($\pm$ SEM) plasma viral load comparison of the control (n=5) and Leronlimab-treated macaques (n=4) for **(E)** peak plasma viral loads and **(F)** area-under-the curves (AUC); P-values were calculated with one-way ANOVA test. **(G)** Mean ($\pm$ SEM) longitudinal plasma viral load. **(H)** Longitudinal changes in CCR5+ CD8+ T-cell frequency in blood. Graphs show fold change from baseline (week-0). Weekly P-values found in Supplemental Table 4 were calculated by two-way repeated measures ANOVA with Tukey-Kramer adjustment. Gray box represents period of Leronlimab treatment.

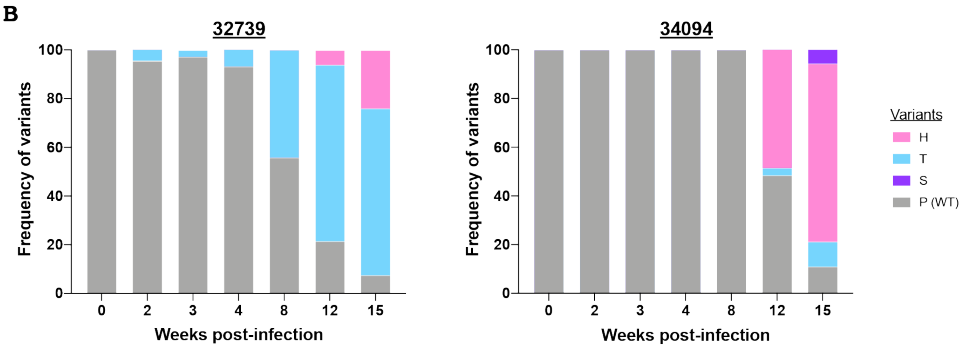


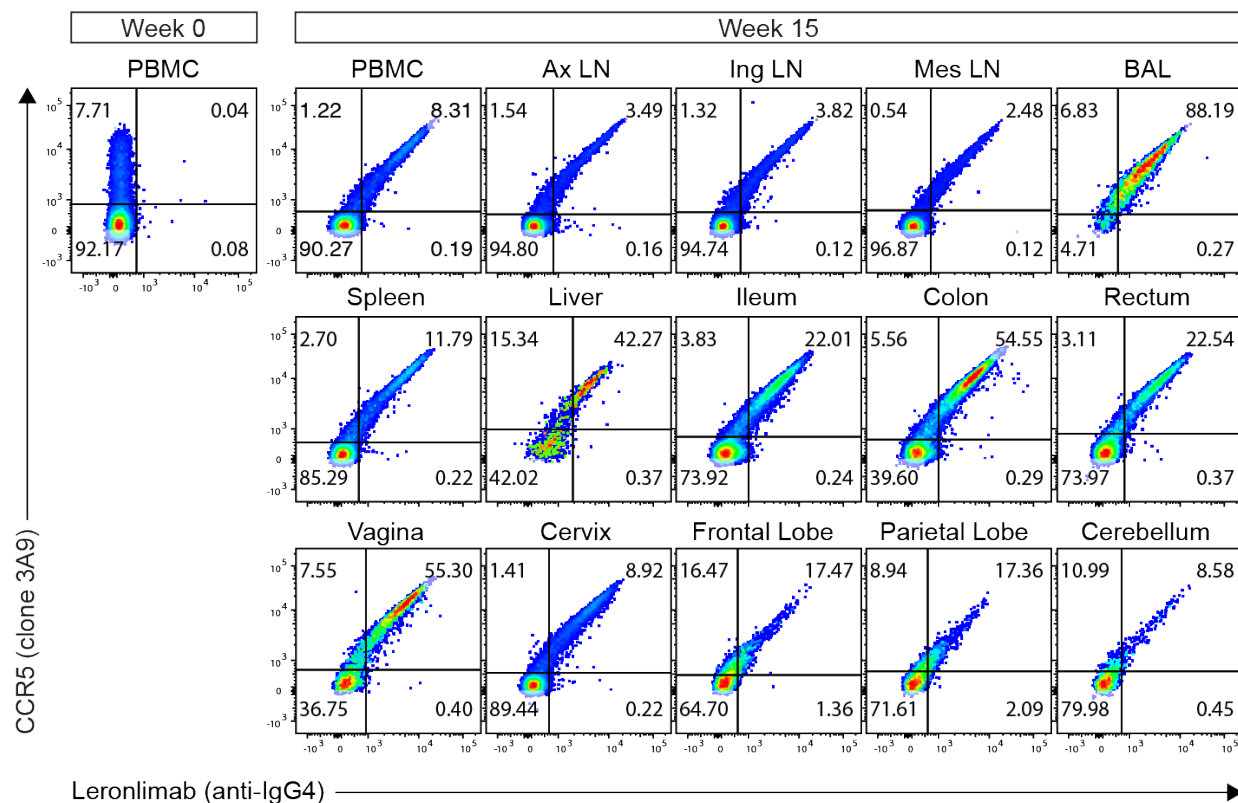
Supplemental Figure 4.7. Assessment of Env V3 loop sequences from SHIV-infected macaques.

Plasma vRNA from six timepoints were isolated and *env* was sequenced. Two

Leronlimab-treated animals, 32617 and 35721, had undetectable viremia at week-15 and thus excluded at that timepoint. (A) Amino acid alignment of Env sequences. Consensus sequence for “SHIV SF 162P3” serves as the reference sequence. “Stock” is the sequence of the challenge stock virus used to infect all the animals at the start of the study. “BR24N”, “HXB2”, “Ams.32”, and “CA28NL” are sequences of CXCR4-utilizing isolates related to SHIVSF162P3. Dots denote identical amino acids and the dashes indicate gaps compared to the reference sequence. Legend at right denotes the variant frequency. Variants <2% were excluded. Red “X” residue indicates more than one variant with a frequency of >2% at that position.

(B) Change in variant frequency for two control animals, 32739 and 34094, at position 13 of the V3 loop (or position 306 in Env protein).





Supplemental Figure 4.8. Additional representative flow cytometry plots corresponding to Figure 4.4D. Representative flow cytometry plots showing the co-staining of anti-CCR5 (clone 3A9) and Leronlimab (by anti-human IgG4, clone HP-6025) on CD4⁺ T-cells from Leronlimab-treated macaque, 32617.

Plasma viral load above LOD (>40 copies/mL)		
ID	Study week	Viral load
01-037	123	481
01-038	25	230
	27	103
	29	929
	31	59
	39	41
	47	2039
	55	505
	63	218
	107	47
	140	43
	240	50
	267	55
	275	60
	278	68
	281	51
	314	52
	338	48
01-057	219	1025
	236	157
	268	64
	329	48
01-064	304	45
	311	69
	332	43

Supplemental Table 4.1. HIV-1 RNA copies/mL above LOD (>40 copies/mL) and the corresponding study week. List of all study week timepoints with plasma HIV-1 RNA copies/mL values higher than the limit of detection (>40 copies/mL).

	01-037	01-038	01-057	01-061	01-064
CD3 T cells	60.23	24.84	43.76	44.91	40.47
CD8 T cells Total	26.36	13.22	24.13	16.03	19.86
CD8 T cells Naive	5.76	1.25	2.86	3.36	2.92
CD8 T cells Central Memory	1.37	1.07	0.58	0.52	1.40
CD8 T cells Effector Memory	2.32	4.61	10.62	2.42	5.62
CD8 T cells Terminal Effector	16.90	6.28	10.08	9.73	9.92
CD4 T cells Total	31.33	11.06	16.45	22.92	12.50
CD4 T cells Naive	5.62	0.36	4.44	7.57	1.38
CD4 T cells Central Memory	14.40	4.83	5.07	5.42	4.94
CD4 T cells Effector Memory	2.22	1.89	2.75	2.54	2.09
CD4 T cells Terminal Effector	9.09	3.98	4.19	7.38	4.09
Treg	0.19	0.09	0.09	0.13	0.12
Th1-like	0.70	0.13	0.16	0.42	0.28
Th2-like	3.80	2.38	1.95	3.60	3.50
Th17-like	1.52	0.64	0.68	1.03	1.64
Gamma Delta T Cells	2.41	0.56	3.18	5.75	7.45
MAIT & NKT CD4- T Cells	0.13	0.00	0.00	0.22	0.65
B Cells	6.00	7.38	13.65	7.33	4.23
B Cells Naive	4.00	6.61	11.11	6.10	3.31
B Cells Memory	1.98	0.77	2.46	1.16	0.91
B Cells Plasmablasts	0.02	0.01	0.07	0.06	0.01
NK Cells	13.03	35.37	6.05	11.64	14.57
Early NK	3.10	7.32	1.34	2.98	5.23
Late NK	9.93	28.04	4.71	8.66	9.34
Monocytes Total	14.23	20.38	26.70	21.75	29.66
Monocytes Classical	11.82	17.57	24.10	18.82	26.25
Monocytes Transitional	1.61	2.14	2.12	1.93	2.56
Monocytes Non-Classical	0.80	0.67	0.48	1.00	0.84
Dendritic Cells	1.26	1.02	1.44	2.22	1.16
pDC	0.22	0.13	0.10	0.08	0.35
mDC	1.04	0.89	1.34	2.14	0.81

Supplemental Table 4.2. Percent of total immune cells in long-term Leronlimab treatment in humans.
Table shows immune cell percentages in the five human participants.

Group	Animal	Gender	Age (Years)	Weight (kg)	MHC alleles	
					B*08	B*17
Control	32739	Male	6.05	8.2	-	-
	33331	Male	5.15	9.5	-	-
	34094	Female	4.50	4.60	-	-
	38224	Female	1.93	3.1	-	-
	38663	Female	1.61	2.4	-	N/A
Leronlimab	32617	Female	5.77	5.3	-	-
	35721	Male	3.19	4.4	-	-
	35764	Male	2.74	4	-	-
	35778	Female	3.21	5	-	-

Supplemental Table 4.3. Rhesus macaque demographics. Table shows animal ID, gender, age, and MHC alleles. N/A = not sequenced.

Weeks post-infection	plasma viral load (Fig. 3D)	%CCR5 of CD4+ T-cells (Fig. 3E)	%CCR5 of CD8+ T-cells (fig. S6H)	Lymphocyte (fig. S4B)	CD4+ T-cells (fig. S4C)	CD8+ T-cells (fig. S4D)
0	1	1	1	1	1	1
1	1	1	1	1	1	1
2	1	1	1	1	1	1
3	1	1	1	1	1	1
4	0.3202	0.0009	0.1342	1	0.8102	1
5	<.0001	0.0122	0.6481	0.9091	0.171	0.7195
6	0.0009	0.024	0.7081	1	1	1
7	0.0087	0.0001	0.0078	1	0.9981	1
8	<.0001	0.0685	0.6766	0.9997	0.9739	1
9	<.0001	0.0003	0.0352	0.9226	0.9043	0.9301
10	<.0001	<.0001	0.3453	0.9241	0.2715	0.9628
11	<.0001	<.0001	0.0629	0.8384	0.0687	0.9296
12	<.0001	<.0001	0.4392	0.9987	0.9477	1
13	<.0001	0.0003	0.1065	0.9919	0.2931	1
14	<.0001	0.0324	0.0125	0.9675	0.9875	1
15	<.0001	<.0001	0.006	0.01	0.1521	0.9983

Supplemental Table 4.4. Individual P-values for longitudinal panels in macaques. Table shows the weekly P-values for longitudinal panels in plasma viral load (Fig. 3D), CCR5+ CD4+ T-cells (Fig. 3E), CCR5+ CD8+ T-cells (fig. S6H), lymphocyte (fig. S4B), CD4+ T-cell (fig. S4C), and CD8+ T-cell (fig. S4D). P-values were calculated by two-way repeated measures ANOVA with Tukey-Kramer adjustment. P-value <0.05 was significant and highlighted in orange.

Leronlimab/total protein (ng/mg)			
Tissue	Mean	SEM	N
Ax LN	1475.7	291.5	4
Ing LN	1408.4	388.4	4
Mes LN	911.5	220.8	4
Spleen	553.4	68.1	4
Liver	456.7	110.3	4
Bone Marrow	3082.5	1547.5	4
Duodenum	1204.2	307.9	4
Ileum	778.7	179.1	4
Colon	618.6	251.1	4
Rectum	903.7	96.1	4
Vagina	3646.4	35.7	2
Cervix	3938.9	285.9	2
Spinal Cord	403.9	185.6	4
Frontal Lobe	9.4	3.6	4
Parietal Lobe	14.8	4.0	4
Medulla Oblongata	39.9	15.1	4
Cerebellum	16.7	5.9	4
Thalamus	8.2	2.1	4

Supplemental Table 4.5. Summary of tissue concentrations corresponding to Fig. 4A-B. Ax LN = axillary lymph node, Ing LN = inguinal lymph node, and Mes LN = mesenteric lymph node. Tissue concentration represented as Leronlimab (ng) over total protein (mg)

Metal Tag	Antibody	Clone
89Y	Anti-human CD45	HI30
141Pr	Anti-human CD196/CCR6	141Pr
143Nd	Anti-human CD123	6H6
144Nd	Anti-human CD19	HIB19
145Nd	Anti-human CD4	RPA-T4
146Nd	Anti-human CD8a	RPA-T8
147Sm	Anti-human CD11c	Bu15
148Nd	Anti-human CD16	3G8
149Sm	Anti-human CD45RO	UCHL1
150Nd	Anti-human CD45RA	HI100
151Eu	Anti-human CD161	HP-3G10
152Sm	Anti-human CD194/CCR4	L291H4
153Eu	Anti-human CD25	BC96
154Sm	Anti-human CD27	O323
155Gd	Anti-human CD57	HCD57
156Gd	Anti-human CD183/CXCR3	G025H7
158Gd	Anti-human CD185/CXCR5	J252D4
160Gd	Anti-human CD28	CD28.2
161Dy	Anti-human CD38	HB-7
163Dy	Anti-human CD56/NCAM	NCAM16.2
164Dy	Anti-human TCRgd	B1
166Er	Anti-human CD294	BM16
167Er	Anti-human CD197/CCR7	G043H7
168Er	Anti-human CD14	63D3
170Er	Anti-human CD3	UCHT1
171Yb	Anti-human CD20	2H7
172Yb	Anti-human CD66b	G10F5
173Yb	Anti-human HLA-DR	LN3
174Yb	Anti-human IgD	IA6-2
176Yb	Anti-human CD127	A019D5
103Rh	Cell-ID Intercalator	

Supplemental Table 4.6. 30-marker antibody human CyTOF Panel. 30 markers used in the human CyTOF panels grouped by antibody clone and metal tag.

CHAPTER 5: Implication and future directions

5.1 Implications

5.1.1 Characterization and effectiveness of CCR5-targeting Leronlimab

In the absence of a prophylactic vaccine and functional cure for HIV infection, ARV drugs are essential for the effective treatment of HIV+ individuals. However, the prevalence of ARV drug resistance is rising globally year-to-year, warranting the investigation of novel drugs with different mechanisms of action and formulation than the commonly used ARV drugs. Antibody-based drugs offer many advantages over chemical compounds, including reduced toxicity (dependent on the antibody target), extended half-lives, high specificity to the target, and Fc-mediated immune functions for certain IgG subclasses. To this end, this dissertation focused on the anti-CCR5 antibody Leronlimab.

Anti-HIV bNAbs can elicit Fc-mediated effector functions to enhance antiviral responses^{331,332}; however, similar immune responses would not be ideal for Leronlimab since Leronlimab binds to host cells. Unlike anti-HIV bNAbs, Leronlimab is an IgG4 subclass that does not display complement activation and has low binding affinities to Fc receptors²⁵⁷. Interesting, the FDA-approved anti-CD4 antibody, ibalizumab, is also an IgG4 subclass and works similarly to Leronlimab in inhibiting HIV binding to host cells, making them ideal blockade antibodies that do not engage with the immune system. Indeed, in three separate NHP studies to investigate Leronlimab for pharmacokinetics, HIV prevention, and HIV treatment, we did not observe depletion of CCR5+ target cell populations. In contrast, the susceptible CCR5+CD4+ T cell population was rescued and protected during active viremia due to Leronlimab treatment. Furthermore, in HIV-naïve macaques, the treatment of Leronlimab

temporarily increased the frequencies and absolute cell counts of CCR5+CD4+ and CCR5+CD8+ T cells, which returned to baseline levels after Leronlimab washout. The observed Leronlimab-mediated increases to CCR5+ T cells may be beneficial in maintaining healthy CD4+ T cell homeostasis that is important to protect against AIDS development in HIV+ individuals. Importantly, the lack of Leronlimab-mediated depletion of host cells is ideal for long-term therapeutic use.

Next, the challenge in an HIV cure is due to the existence of persistent viral reservoirs. While ART can suppress plasma viremia in HIV+ individuals, it can only marginally reduce the size of the viral reservoirs during long-term suppressive ART²⁹¹. A novel attempt to reduce viral reservoir size using a latency-reversing agent and HIV primed CTL responses was ineffective²⁹². The work described here demonstrated the potential for Leronlimab to prevent the seeding of a viral reservoir and to reduce the viral reservoir size in weekly Leronlimab treatments. Chapter 3 described Leronlimab as PrEP in an NHP model for HIV infection. Leronlimab protected all 50 mg/kg treated macaques from repeated intrarectal challenges. In the 10 mg/kg treatment group, Leronlimab fully protected four of the six macaques. We found that one animal became infected due to the rapid clearing of Leronlimab by host ADA responses, while the second animal contained the lowest Leronlimab concentration in the colon that likely led to insufficient protection. Importantly, all aviremic animals in both 10 and 50 mg/kg treated animals lacked detectable viral DNA copies in the tissues examined, demonstrating the effectiveness of targeting the first step in the HIV lifecycle – host cell entry. Moreover, limiting the events of high error-prone reverse transcription can restrict the number of variants to prevent HIV drug escape. In Chapter 4, we found that the use of Leronlimab as a therapeutic agent for acute SHIV-infected macaques significantly reduced viral replication, with two of the four treated animals showing

undetectable viremia. The number of viral DNA copies detected in varying tissues from Leronlimab-treated macaques was also statistically lower than in untreated macaques. However, it is worth noting that early initiation of ART can also reduce the reservoir size⁵⁶⁰. Future comparison studies between Leronlimab and ART would provide a better insight into the extent of viral reservoir clearance by Leronlimab. In particular, the combinational effects of pairing Leronlimab and ART may enhance viral reservoir clearance. The durability of the reduction in the reservoir is not known.

The main concern for the monotherapy treatment of any anti-HIV therapeutics – both ARV drugs and antibodies – is the development of drug-escaping variants. However, the single-agent Leronlimab was effective in suppressing plasma viremia in five HIV+ participants for over seven years. Transient episodes of detectable plasma viremia (> 40 copies/mL) did occur. Still, they were all controlled without any change from the Leronlimab-only regimens, suggesting that the overall viral pools continued to be CCR5-tropic. One plausible reason is the protective role conferred by blocking HIV cell entry. Without the ability for HIV to enter a host cell, downstream steps for reverse transcription that generate viral diversity and cDNA integration that establish viral reservoirs will not occur in the uninfected host cells. Furthermore, most of the transient episodes occurred during periods of immune activation, including vaccination, herpes outbreak, upper respiratory infection, and hospitalization. As described in **Section 1.4 CCR5 for HIV**, antigen exposure can lead to transient upregulation in CCR5 receptors on T cells in order to traffic to sites of inflammation. Thereby, these short periods of breakdown infections may be due to insufficient Leronlimab dosages that could not completely occupy the newly synthesized CCR5 receptors on CD4+ T cells in response to immune activation events.

5.1.2 Recapitulation of homozygous CCR5 Δ 32 phenotype with Leronlimab

The resistance against HIV infection in hosts who are homozygous for CCR5 Δ 32 has been extensively documented; however, there are no reliable tools to replicate this protection in the clinical setting. Procedures to replace an individual's immune cells with allogenic HSCT with homozygous CCR5 Δ 32 donor cells are not scalable for the millions living with HIV, and gene editing tools to disrupt *ccr5* expression show low rates of genome editing. In the work described here, we tested the CCR5-blocking Leronlimab and found a number of positive results that support its ability to mimic the homozygous CCR5 Δ 32 mediated protection.

First and foremost, Leronlimab treatment of CD4⁺ T cells resulted in complete protection against infection by 10 different CCR5-tropic HIV-1 isolates listed in the “Panel of 60 International Human Immunodeficiency Virus Type 1 Isolates” (HIV Reagent Program). The different subtypes tested – A, B, C, D, and CRF01 – did not influence the degree of protection. These results agreed with a previous study that found Leronlimab to be subtype-independent⁴⁵⁶. Importantly, Leronlimab-untreated CCR5 Δ 32/ Δ 32 CD4⁺ T cells and Leronlimab-treated CCR5^{WT} CD4⁺ T cells conferred the same degree of protection during viral infection, demonstrating that the blockade of CCR5 receptors via Leronlimab can produce the same clinical protection from infection observed in homozygous *ccr5* Δ 32 individuals.

Second, the homozygous *ccr5* Δ 32 deletion is a germline variant that affects all cell types expressed throughout the body. Thereby, CD4⁺ T cells found in the peripheral blood or the mucosal layers equally lack CCR5 expression. To phenocopy the protection of homozygous *ccr5* Δ 32, potential therapeutics must have broad tissue penetration to protect cells from HIV

infection, particularly to the epithelial layers where HIV first comes into contact with host cells. Indeed, our investigation showed that Leronlimab effectively trafficked to mucosal sites, including the rectum and vagina, and completely bound CCR5 receptors on CD4+ T cells isolated from these tissues.

Lastly, monocytes are major viral reservoirs and can transfer HIV infection through cell-to-cell spreading^{168,171,284}. Similar to the second point, potential therapeutics must have specificity to a range of cell types. This is important as HIV can infect many cell types beyond CD4+ T cells, including monocytes, macrophages, Langerhans cells, NK cells, follicular dendritic cells, astrocytes, and microglia²⁸⁰⁻²⁸⁴, to establish viral reservoirs. To this end, we found that Leronlimab was capable of binding to monocytes isolated from the peripheral blood and lymph nodes. Due to the high specificity exhibited by antibodies, Leronlimab could likely bind to a diverse number of cell types as long as the cells express cell-surface CCR5 receptors.

In summary, Leronlimab exhibited subtype-independent protection against varying CCR5-tropic HIV-1 isolates, sufficient anatomical distribution to protect cells in sites of first HIV contact, and high binding specificity to CCR5+ cells, collectively demonstrating its ability to mimic the clinical protection observed in homozygous CCR5 Δ 32. However, the main caveat that remains is the need to dose in a manner that can guarantee complete CCR5 binding by Leronlimab.

5.2 Future directions for Leronlimab

5.2.1 Combination therapies with ARV drugs or bNAbs

In Chapter 4, we reported on the clinical trial results of five individuals who were on Leronlimab monotherapy maintenance for over seven years. To our knowledge, this is the

longest case of single-agent anti-HIV drug treatment. However, it is important to acknowledge the other participants in the trial who experienced viral rebound but were all successfully re-suppressed after ART re-initiation. Viral escape could be the reason for the viral rebound but viruses detected from the rebound participants continued to be CCR5-tropic but required higher concentrations of Leronlimab to inhibit HIV replication than viruses from non-rebound participants. A plausible explanation for the virologic failure may be the low dosages used in the study, in contrast to higher dosages used in bNAb clinical trials. However, other unknown factors may be at play. For this reason, Leronlimab would be a more suitable drug candidate for combinational therapy with ARV drugs and bNAbs.

Interestingly, the pairing of Leronlimab and bNAbs may enhance the Fc-mediated functions of bNAbs. While Leronlimab prevents HIV binding to CCR5, it does not stop HIV from binding to the primary receptor CD4. The binding of HIV to CD4 will trigger conformational changes that expose potential bNAb epitopes, including the V3 loop that is recognized by antibodies 447-52D and F425-B4e8^{561,562}. Furthermore, engineered bi- and tri-specific antibodies using Leronlimab and one or more bNAbs demonstrated greater antibody potency and breadth than the parental Leronlimab *in vitro* studies⁵⁶³. To this end, using our NHP models to test various combinations of bNAbs and Leronlimab could optimize the antibody treatment regimens for HIV prevention and treatment.

5.2.3 Antibody engineering

ARV drugs effectively suppress plasma viremia but require daily or twice-a-day regimens for life. In contrast to small molecules, antibodies have a longer half-life to allow for less frequent dosing. Importantly, antibodies can be engineered to improve half-life and potency.

One such mutation is the M428L/N434S (-LS) mutation that increases the half-life of an antibody by increasing the binding affinity to the neonatal Fc receptor (FcRn)⁵⁵⁰. Introducing this mutation to the anti-HIV bNAb VRC01 resulted in a longer half-life, higher trough concentration (the antibody concentration in the blood immediately before the next dosage), and full protection against viral challenges than the parental VRC01³⁵⁵. To this end, we collaborated with Dr Diogo Magnani (MassBiologic) to produce the Leronlimab-LS variants. The Sacha Lab is currently conducting a comparative study on the pharmacokinetics of parental Leronlimab and Leronlimab-LS, with plans to investigate the effect of reducing dose frequency in HIV prevention and treatment NHP models. More importantly, the findings of these studies will pave the experimental design for clinical trials aiming to study Leronlimab as an HIV PrEP agent for high-risk individuals in Thailand and Brazil.

HIV-1 can lose V5 glycans on its envelope during ibalizumab treatment to escape from ibalizumab neutralization³⁷⁴. However, the addition of N-linked glycans to the light chains of ibalizumab can enhance antiviral effects and restore neutralization against ibalizumab-resistant viral strains. In particular, one mutant fully neutralized the 118 HIV-1 isolates tested *in vitro* and showed a 10-fold higher potency than the parental ibalizumab⁵⁶⁴. The addition of glycans onto ibalizumab likely introduced steric hindrance that furthered the physical blockage of HIV-1 to CD4+ T cell receptor. This mechanism of drug escape by HIV may be a plausible scenario for the viral rebound observed in Leronlimab monotherapy treated participants despite maintaining CCR5-tropism. Since ibalizumab and Leronlimab generate steric hindrance to block HIV-1 cell entry, the addition of various glycans to Leronlimab may increase its potency, as observed in ibalizumab.

5.2.3 Curative strategies

One potential barrier to reproducing the HIV cure observed in the Berlin²⁷⁴ and London^{278,279} patients is the difficulty of finding an HLA-matched donor who is also a homozygous CCR5 Δ 32 carrier. Moreover, northern Europeans have the highest frequency of homozygous CCR5 Δ 32 alleles, while it is essentially nonexistent in individuals who are not of European descendants. It is worth noting that both cases of HIV cure occurred in Europe. Dr. Jonah Sacha has developed an MCM model of allogeneic HSCT in his laboratory to study this method for HIV cure. Using this model, they found that a graft-versus-host (GvHD) response that arose after allogeneic HSCT was the major contributor to clearing the viral reservoir in the HIV+ recipient. However, the engrafted donor cells continue to be susceptible to viral infection, especially since a single HIV particle is sufficient to produce a clinical infection^{54,176}. Thus, the absence of CCR5 receptors on the donor immune cells will protect them from HIV infection. For this reason, attempts to reproduce an HSCT-mediated HIV cure with wildtype CCR5 donor cells were unsuccessful^{276,277}. We have already described the ability of Leronlimab to mimic the protection observed from homozygous CCR5 Δ 32 carriers. For this reason, we hypothesize that the use of Leronlimab will protect against infection of engrafted wildtype or heterozygous CCR5 Δ 32 donor cells during HSCT. To test this hypothesis, CytoDyn – the company that manufactures Leronlimab – and amfAR have formed a partnership to study the use of Leronlimab in six HIV+ leukemia patients who will undergo HSCT treatment for leukemia.

A second method for a functional HIV cure comes from the anti-CD19 chimeric antigen receptor (CAR) T therapy for B-cell leukemia⁵⁶⁵. CAR works by displaying an unconventional receptor specific to an antigen to induce T-cell mediated cytotoxic killing of the target. Indeed, an anti-HIV CD4 ζ CAR T therapy – CD4 receptor linked to the CD3 ζ activation chain – was

developed to engage with the HIV gp120 envelope proteins⁵⁶⁶. A phase 2 clinical trial showed stable engraftment and detectable CD4ζ CAR edited cells in 98% of the participants (n=43) after 11 years of infusion, but it found minimal antiviral effects for controlling plasma viral load⁵⁶⁷. One potential explanation is the use of the CD4ζ CARs by HIV to infected the edited cells, thereby, producing a bigger pool of susceptible cells for HIV infection. Another method for anti-HIV CAR T therapy comes from the use of broadly neutralizing antibodies (bNAbs)^{568,569}. However, a study that used bNAbs as CARs showed an accelerated rate in the development of CAR-escaping HIV mutants⁵⁶⁹. Despite the many limitations, we learned that anti-HIV CAR cells were safe and persisted in the body for over a decade⁵⁶⁷; however, novel CARs need to be explored, ideally, one that is independent of the HIV strains. The work described in this dissertation support the investigation of a novel curative strategy for HIV+ infected individuals using Leronlimab as the CAR to target CCR5 receptors. To this end, Dr. Sacha and colleagues are creating a Leronlimab CAR T therapy to study in the NHP models.

Lastly, gene therapy can allow for the systemic expression of Leronlimab by host cells. A previous study showed that a gene therapy that expressed secreted Leronlimab scFvs was potent in reducing viral replication *in vitro*⁵⁷⁰. Yet, *in vivo* NHP studies showed that ADA responses could develop against systemic expressed bNAbs after AAV-based gene therapy. In contrast, minimal ADA responses were observed when the same bNAbs were passively transferred³⁶⁴. However, Martinez-Navio et al. found that the AAV gene therapy led to multiyear expressions of bNAbs in animals that lacked ADA development. In turn, the expressed bNAbs levels were sufficient to prevent IV viral challenges in the naïve macaque⁵⁷¹ and suppress plasma viremia in SHIV-infected macaque³⁶⁴. These successful primate studies provide proofs-of-concept for AAV delivery of anti-HIV antibodies, including Leronlimab. Although there are many barriers to gene

therapy, the ability of an individual to systemically express Leronlimab would be a promising substitute for prophylactic vaccines since 1) Leronlimab blockade is independent of HIV strains⁴⁵⁶ and 2) CCR5-tropic viruses initiate the majority of sexual transmissions of HIV⁵⁴.

5.3 Concluding thoughts

In the absence of a prophylactic vaccine and functional cure for HIV, ARV drugs are essential for effective treatment of HIV+ individuals. However, the prevalence of ARV drugs is rising globally year-to-year and warrants the need to explore compounds with a different mechanisms of action than those in commonly used ARV drugs. A promising drug target is the CCR5 coreceptor used for HIV cell entry. The dissertation work described here demonstrates the effectiveness of the anti-CCR5 antibody Leronlimab in preventing and suppressing HIV infections. Throughout our investigation, we are reminded of the essential role that CCR5 plays in HIV prevention, treatment, and cure. As there is currently no viable precision genetic method to edit out CCR5 expression in adults, the passive transfer of Leronlimab proves to be an effective method to block the engagement of HIV and CCR5, which mimics the protection that occurs naturally in homozygous CCR5 Δ 32 individuals. The future holds promise for future studies exploring Leronlimab and its potential to prevent, treat, and cure HIV.

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