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Siv Infected Chimpanzees: Consequences Of Long-Term Infection And Potential Intervention Strategies

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Abstract
Simian immunodeficiency virus of chimpanzees (SIVcpz) is widespread in wild-living chimpanzees and can cause mortality and AIDS-like immunopathology. However, due to limited access to naturally infected chimpanzees, little is known about SIVcpz pathogenesis and potential intervention strategies that might be effective in captivity or in the wild. Given the central role of the intestinal microbiome in mammalian health, I asked whether gut microbial constituents could reveal any insights into SIVcpz-associated pathogenicity. I characterized the gut microbiome and virome of SIVcpz infected and uninfected chimpanzees in Gombe National Park, Tanzania. I found that SIVcpz infected chimpanzees retain a stable gut microbiome throughout much of their natural infection course, with a significant destabilization of bacterial communities observed only in individuals with known immunodeficiency within the last several months before their death. I also explored potential interventions that could be effective in both wild-living and captive chimpanzees. Broadly cross-reactive neutralizing antibodies (bNabs) represent powerful new tools to combat human immunodeficiency virus type 1 (HIV-1) infection. I found that some antibodies and antibody-like inhibitors developed to combat HIV-1 infection are capable of neutralizing genetically diverse SIVcpz and SIVgor strains with considerable breadth and potency, including in primary chimpanzee CD4+ T cells. Identification of these reagents provides an important first step toward translating potential intervention strategies currently developed to treat and prevent AIDS in humans to SIV infected apes. I also report a first case of clinical immunodeficiency in an experimentally SIVcpz infected captive chimpanzee, which improved markedly following antiretroviral treatment. These findings provide new insight into SIVcpz pathogenicity and identify promising new approaches to combat SIVcpz infection.

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SIV INFECTED CHIMPANZEES: CONSEQUENCES OF LONG-TERM INFECTION AND POTENTIAL INTERVENTION STRATEGIES

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Simian immunodeficiency virus of chimpanzees (SIVcpz) is widespread in wild-living chimpanzees and can cause mortality and AIDS-like immunopathology. However, due to limited access to naturally infected chimpanzees, little is known about SIVcpz pathogenesis and potential intervention strategies that might be effective in captivity or in the wild. Given the central role of the intestinal microbiome in mammalian health, I asked whether gut microbial constituents could reveal any insights into SIVcpz-associated pathogenicity. I characterized the gut microbiome and virome of SIVcpz infected and uninfected chimpanzees in Gombe National Park, Tanzania. I found that SIVcpz infected chimpanzees retain a stable gut microbiome throughout much of their natural infection course, with a significant destabilization of bacterial communities observed only in individuals with known immunodeficiency within the last several months before their death. I also explored potential interventions that could be effective in both wild-living and captive chimpanzees. Broadly cross-reactive neutralizing antibodies (bNabs) represent powerful new tools to combat human immunodeficiency virus type 1 (HIV-1) infection. I found that some antibodies and antibody-like inhibitors developed to combat HIV-1 infection are capable of neutralizing genetically diverse SIVcpz and SIVgor strains with considerable breadth and potency, including in primary chimpanzee CD4+ T cells. Identification of these reagents provides an important first step toward translating potential intervention strategies currently developed to treat and prevent AIDS in humans to SIV infected apes. I also report a first case of clinical immunodeficiency in an experimentally SIVcpz infected captive chimpanzee, which improved

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INTRODUCTION

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Section 1.1 - Simian immunodeficiency virus of chimpanzees, the precursor of HIV-1 in humans

HIV-1 and AIDS

Acquired Immune Deficiency Syndrome (AIDS) was first identified in the United States in 1981 (1). It has since caused more than 70 million infections and 35 million deaths worldwide (www.unaids.org). The causative agent is human immunodeficiency virus type 1 (HIV-1), a lentivirus of the *Retroviridae* family. Members of this family are enveloped, single-stranded, positive-sense RNA viruses that utilize a virus-encoded reverse transcriptase to produce DNA from its RNA genome. Viral DNA is then integrated into the host genome, resulting in a persistent infection. HIV-1 primarily infects CD4+ T cells (2), using the CD4 molecule as its entry receptor (3, 4), and CCR5 (5) or CXCR4 (6) as a co-receptor. HIV-1 infection is spread primarily through sexual contact, and is typically associated with a decrease in CD4+ T cell numbers over time and eventual progression to AIDS, characterized by the development of an AIDS-defining illness, such as opportunistic infections, cancer, and/or a decline in CD4 count to lower than 200 cells/ul (7).

The search for the origin of HIV-1 in primate lentiviruses

Following the sudden emergence of HIV-1 infection in the U.S., researchers sought to identify the source of the pandemic. Zoonotic transmission events are responsible for the majority of emerging infectious diseases (9), leading investigators to search for closely related viruses in non-human primates. Identification of a related retrovirus in rehesus macaques, now termed simian immunodeficiency virus (SIV), provided the first evidence that HIV-1 may be of

primate origin. Outbreaks of AIDS-like disease in captive macaques were noted in the early 1980's, which was later associated with a retrovirus featuring similar growth phenotypes, cytopathic effects, and morphology to HIV (10). Others found that this macaque retrovirus (now termed SIVmac) contained proteins of a similar size as HIV, and could be successfully immunoprecipitated with antibodies directed against HIV proteins (11). Another study identified a retrovirus that caused AIDS-like illness and displayed antigenic cross-reactivity to HIV-1 in rhesus macaques that were experimentally inoculated with tissue from sooty mangabeys (12). However, molecular analysis of SIVmac revealed that it was closely related to HIV-2, a virus that is phenotypically and genotypically distinct from HIV-1 (13), leaving the precursor of the pandemic HIV strain elusive (14, 15). SIV was then detected in captive wild-caught sooty mangabeys (16) and African green monkeys (17, 18), broadening the search for SIV strains to African monkeys and apes. Around 40 different primate lentiviruses have since been discovered in African primates, including the genera *Pan* (chimpanzees), *Gorilla*, *Cerocebus* (mangabeys), *Mandrillus* (mandrills), *Colobus*, and *Ceropithecus* (greater spot-nosed and other monkeys) (19). These strains share a similar genomic organization, including the major structural and enzymatic genes *gag* (encoding matrix, capsid, and nucleocapsid proteins), *pol* (encoding protease, reverse transcriptase, and integrase), and *env* (encoding envelope-associated proteins), and accessory genes *tat, rev, vif*, *vpr,* and *nef,* which perform regulatory functions required for viral replication. However, the accessory gene *vpu* is unique to HIV-1, SIVcpz, and SIV strains from the monkeys of the genus *Ceropithecus*, while the *vpx* gene is unique to SIV strains from mangabeys (20). Despite the comparable genomic organization of SIV strains, they exhibit extensive sequence diversity, sharing only 40-50% identity in the more conserved Gag and Pol proteins (21). SIV strains in wild-caught Asian primates have not been detected, suggesting that the ancestor of primate SIV strains likely emerged after the divergence of African and Asian monkeys.

The first evidence of a chimpanzee origin of HIV-1 came from the discovery of a retrovirus infecting two wild-born chimpanzees from Gabon with close antigenic similarity to HIV-1 (22). This virus, termed simian immunodeficiency virus of chimpanzees (SIVcpz), was found to

have the same genomic organization as HIV-1, including *vpu*, which had only been identified in HIV-1 at that time, and a sequence more closely related to HIV-1 than any other primate lentivirus (23, 24). A third case of SIVcpz was then discovered in a captive animal from the Democratic Republic of Congo (25). This strain showed remarkable sequence diversity compared to those previously described, being as distantly related to each other as they were to HIV-1 (25, 26). Extensive screening of captive chimpanzees revealed a scarcity of HIV-1 cross-reactive antibodies in these animals, leading some to doubt that it could serve as a true reservoir at such low frequencies (23, 25-27). Gao and colleagues then characterized a fourth SIVcpz strain, which was closely related to the SIVcpz strains from Gabon (28). This group also found that sizable genetic diversity of the different SIVcpz strains could be attributed to their chimpanzee subspecies specificity, and provided the definitive description of the origin of pandemic HIV-1 from SIVcpz infected chimpanzees (28). HIV-1 is comprised of 4 phylogenetic lineages, group M, the pandemic strain, and groups N (29), O (30), and P (31), which infect far fewer individuals (representing less than 1% of global HIV-1 infections). It was later shown that each HIV-1 group originated from an independent cross-species transmission event of ape SIV strains, with groups M and N originating from SIVcpz and groups O and P originating from SIV infected gorillas (SIVgor) (1). The exact route of transmission from apes to humans is unknown, however, the most likely hypothesis is by exposure to ape blood via bushmeat hunting (32).

The SIVcpz reservoir

The common chimpanzee species, *Pan troglodytes (P.t.*), is composed of four subspecies: *P. t. verus* (located in west Africa), *P. t. ellioti* (located in Nigeria and Cameroon), *P. t. troglodytes* (located in west-central Africa), and *P. t. schweinfurthii* (located in east-central Africa) (33, 34) (Figure 1.1). The HIV-1 pandemic strain arose from transmission of SIVcpz infecting *P. t. troglodytes* chimpanzees (SIVcpzPtt) (28). Studies of the SIVcpz reservoir were difficult, as wild living chimpanzees are endangered, reclusive, and live in very remote habitats.

Anesthetizing wild chimpanzees to collect blood is unethical, because of the health risk associated with an animal losing consciousness and falling from a tree after drug administration. Thus, noninvasive methods to study SIVcpz in wild chimpanzees had to be developed. An initial study of captive and wild-living chimpanzees found that anti-SIVcpz antibodies could be detected in chimpanzee urine and feces using commercially available HIV-1 nitrocellulose strips and Western blot analysis (35). This method showed 100% specificity and 65 to 100% sensitivity in feces and urine, respectively (35). RNA extraction from feces and reverse transcriptase polymerase chain reaction (RT-PCR) could additionally be used for molecular analysis of the infecting strain (35). Expansion of these methods to dozens of field sites and thousands of chimpanzee fecal samples enabled the characterization SIVcpz reservoir (36-40). SIVcpz has been found to infect chimpanzees of the subspecies *P. t. troglodytes* and *P. t. schweinfurthii* and is absent from chimpanzees of the subspecies *P. t. ellioti* and *P. t. verus* (Figure 1.1). *P. t. verus* chimpanzees comprise a vast majority of captive chimpanzees (41), explaining the difficulty in identifying SIVcpz-infected captive chimpanzees in earlier studies. The prevalence rates amongst *P. t. troglodytes* and *P. t. schweinfurthii* chimpanzees varies from 0 – 50% in different communities (35, 36, 39, 40, 42). In contrast, SIV infection in wild monkeys is much more prevalent and uniform between communities (43, 44). These findings suggested a more recent ancestor of SIVcpz than other monkey SIVs, likely following the chimpanzee subspecies divergence. Molecular analysis revealed that the SIVcpz genome is a mosaic, with the *gag*, *pol*, and *nef* genes being more similar to SIVrcm from red-capped mangabeys, and the *vpu*, *tat*, *rev* and *env* genes being more similar to SIVs infecting members of *Ceropithecus* (45). Thus, chimpanzees likely acquired SIVcpz through the cross species transmission and recombination of two monkey SIV strains (Figure 1.1). This scenario is plausible, as chimpanzee habitats overlap with the those of the related SIV hosts, and chimpanzees are known to hunt and eat monkeys (46).

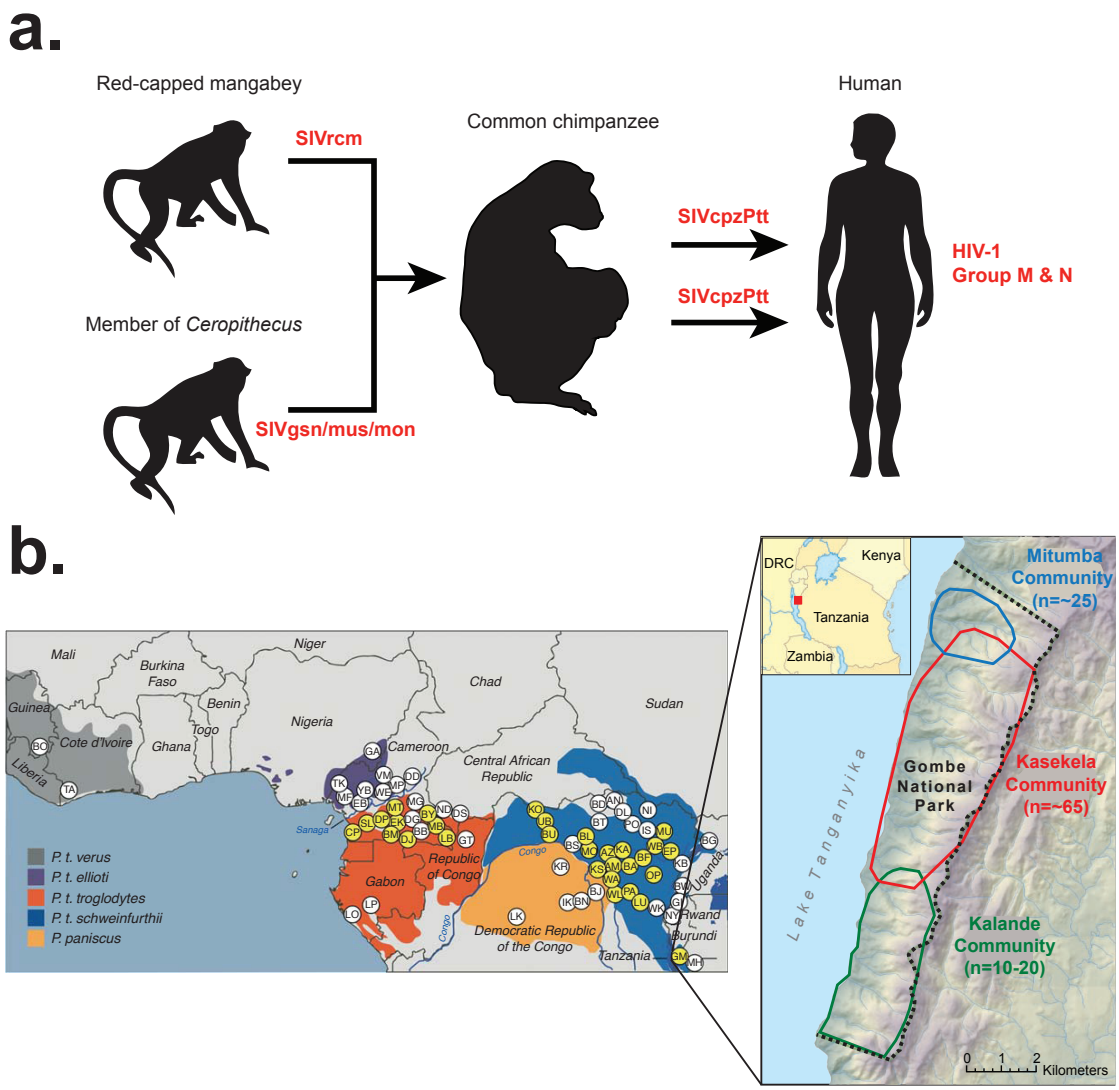


Figure 1.1. Origins and distribution of SIVcpz. a) SIVcpz was acquired from cross-species transmission and recombination of two monkey SIVs. HIV-1 group M and N were a result of two cross-species transmission events from SIVcpz infected chimpanzees to humans. b) A map of the distribution of chimpanzee subspecies and field sites that have been screened for SIVcpz infection (left). Sites where SIVcpz has been detected are represented by yellow circles; sites were SIVcpz has not been detected are in white. A map of Gombe National Park in Tanzania outlines the ranges of the three chimpanzee communities and the approximate population size of each community (right). The maps were modified from Sharp PM and Hahn BH, *Cold Spring Harbor Perspectives in Medicine* 2012 and Keele BF, *Nature* 2009.

SIVcpz natural history studies at Gombe National Park

The most important field site for the study of SIVcpz in wild-living chimpanzees is Gombe National Park in northwestern Tanzania. Gombe covers a narrow area (approx. 35 km^2) between a mountain rift and Lake Tanganyika (Figure 1.1). This biodiverse area is comprised of rift valleys, evergreen and semi-deciduous woodlands, thicket, and grasslands, and is home to *P. t. schweinfurtii* chimpanzees (46). Human deforestation to the north and south of the chimpanzee range resulted in geographic isolation of this population. Jane Goodall began her famous study of these animals in 1960, with important discoveries in the first few years including that chimpanzees hunt and eat meat, and make and use tools (47, 48). Since then, Gombe chimpanzees have been under continuous observation. There are three communities of chimpanzees in Gombe, termed Mitumba, Kasekela, and Kalande (Figure 1.1). Kasekela is the largest community (n≈65) and the first to be habituated to the presence of human observers, Mitumba, smaller community (n≈25) at the northern end of the park, was habituated later, and Kalande, the smallest community (n≈10) to the south, remains only partially habituated to this day (49). The total population of Gombe chimpanzees has decreased since the onset of study in the 1960s from around 120-150 to 96-100 in 2013 (50). The major sources of this population decline are thought to be poaching, intercommunity violence, habitat disturbance, and disease (49), with illness being the most common cause of death of Gombe chimpanzees (51). Major infectious epidemics have been documented in these animals, including sarcoptic mange (52), respiratory disease (51), and polio (53). A comprehensive health monitoring system was established in 2004 that standardized the recording of observational health data and postmortem necropsy evaluations, resulting in frequent and reliable health records of Gombe chimpanzees (54). With over 50 years of observational data, this group of chimpanzees represents a unique and valuable source for infectious disease studies.

SIVcpz was first discovered in Gombe National Park in the early 2000's when analysis of 58 fecal and urine samples from this and two other field sites (located in Cote d'Ivoire and

Uganda) found one male Kasekela resident to be infected (35). Expanded fecal screening of Gombe chimpanzees revealed that SIVcpz was present in all three communities, with prevalences of 5, 17, and 30% for Kasekela, Mitumba, and Kalande, respectively (56). Molecular analysis revealed that full-length SIVcpz genomes could be recovered from fecal samples (55) and phylogenetic analysis showed that Gombe viruses form a monophyletic lineage within all SIVcpzPts strains (57). However, SIVcpz strains from the three communities do not form distinct lineages, suggesting that SIVcpz is transferred between groups, likely through migration of infected females (57). The routes of SIVcpz transmission are similar to HIV-1 infected humans, with both vertical and sexual transmission being observed in Gombe. After biannual screening of Gombe chimpanzees for over seven years, Keele and colleagues discovered that SIVcpz is actually pathogenic in chimpanzees, contrary to previous reports. SIVcpz infected animals showed a 10 to 16 fold increased risk of death compared to uninfected individuals (57). SIVcpz infected females were three times less likely to give birth than uninfected females, and showed high infant mortality, with all four infants born to infected mothers dying in their first year, compared to six of 30 babies born to uninfected mothers. One SIVcpz infected female showed signs of lethargy and weakness only three years after acquiring the virus. At necropsy, Keele and colleagues found that this female showed severe muscle loss, hepatic atrophy, and abdominal abscesses due to parasite infection. Splenic examination revealed severe depletion of CD4+ T cells, signs of lymphatic tissue destruction, and collagen deposition (indicative of overall immune dysfunction (58)). Thus, this animal displayed hallmarks of AIDS that are also observed in humans. Analysis of necropsy samples from two SIVcpz uninfected controls confirmed that CD4+ T cell depletion is abnormal in wild chimpanzees. Two SIVcpz infected animals, who died due to injuries, also showed CD4+ T cell depletion in the spleen, although to a lesser extent than what was seen in the animal with evidence of AIDS. These findings by Keele and colleagues demonstrated that wild-living SIVcpz infected chimpanzees show signs of morbidity and mortality similar that observed in HIV-1 infected humans (59-61), and that infected chimpanzees can develop AIDS-like illness. Further natural history studies in Gombe National Park revealed that

the community with the highest SIVcpz prevalence (Kalande – 46%) was experiencing population decline, while communities with lower prevalences (Mitumba – 13%, Kasekela – 12%) experienced positive growth rates (62). This population decline was not due to habitat quality or access to food, and mathematical modeling indicated that even moderate SIVcpz prevalences could lead to population decline, suggesting that SIVcpz infection could contribute to the mortality burden of already dwindling wild chimpanzee populations.

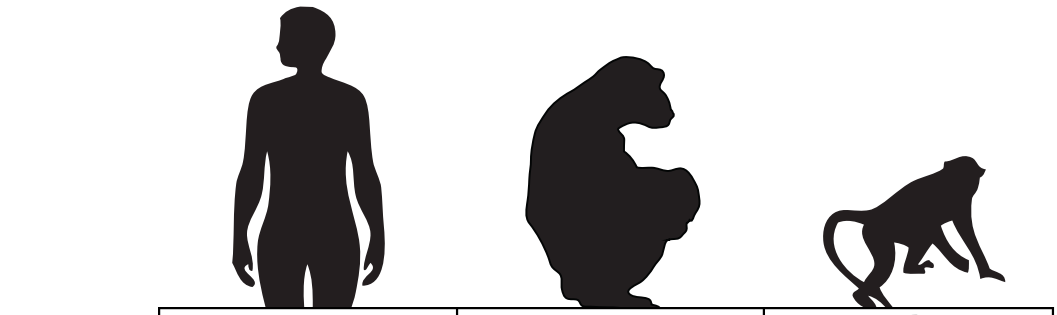
Conclusions

In sum, studies of wild-living chimpanzees have elucidated the origin of HIV-1 and the AIDS pandemic, and the effect of SIVcpz infection on chimpanzee mortality and population dynamics. However, important questions remain unanswered. What factors are involved in SIVcpz pathogenesis in chimpanzees and how do they differ from HIV-1 in humans? To what extent does SIVcpz infection contribute to the catastrophic population decline of wild-living chimpanzees in central Africa, and how might SIVcpz transmission be curbed if medical intervention were to become necessary to rescue endangered chimpanzee populations? Given the phenotypic and genotypic similarities of SIVcpz and HIV-1, as well as chimpanzees and humans, further studies to delineate these topics could not only benefit chimpanzees, but would also inform studies of HIV-1 in humans.

Section 1.2 - Understanding the Consequences of SIVcpz

SIV pathogenesis

SIV infection of most non-human primates is nonpathogenic. However, SIV infection of macaques and chimpanzees, and HIV infection of humans, causes morbidity and mortality. While the pathogenic properties of HIV and SIV infection of humans and monkeys are well described, the mechanisms of pathogenesis in SIVcpz infected chimpanzees are largely unknown. Nonpathogenic hosts of SIV infection, such as sooty mangabeys with SIVsmm or African green monkeys with SIVagm, generally do not develop symptoms of AIDS (19). Their viruses, however, are capable of causing disease in rhesus macaques. This interesting disparity led researchers to investigate the distinguishing features of HIV/SIV in pathogenic versus non-pathogenic hosts (Figure 1.2). Both non-pathogenic and pathogenic hosts show similar kinetics of acute infection, with initial log-phase growth leading to robust levels of peak viremia (63). Viremia persists at a set point throughout the course of chronic infection in both pathogenic and non-pathogenic hosts. HIV/SIV in both pathogenic and non-pathogenic hosts targets activated, short-lived CD4+ T cells, which are depleted in the mucosa during the acute phase of infection (64-67). Peripheral CD4+ T cells, however, are maintained throughout chronic infection in non-pathogenic hosts, but not pathogenic hosts (68). HIV/SIV infection sparks a robust innate immune response regardless of host, including increased levels of proliferating T cells, up-regulation of interferon stimulated genes, and production of type I interferons (69-71). However, unlike pathogenic hosts, this innate immune response is rapidly down-regulated after the acute phase in non-pathogenic hosts (69-71). Chronic systemic immune activation, including increased cell turnover and pro-inflammatory cytokines, is associated with disease progression in pathogenic hosts, but is generally absent from non-pathogenic hosts (72-75). This may be due to a lack of microbial translocation, which triggers immune activation (76), maintenance of Th17 cells, which



Disease course

Pathogenic Hosts	Chimpanzees	Non-Pathogenic Hosts
High levels of mucosal CCR5+ CD4+ T cells	Unknown	Low levels of mucosal CCR5+ CD4+ T cells
High peak viremia	High peak viremia in captive chimpanzees	High peak viremia
Robust acute innate immune response	Unknown	Robust acute innate immune response
Acute depletion of mucosal CD4+ T cells	Unknown	Acute depletion of mucosal CD4+ T cells
High set point viremia	High to moderate set point viremia in captive chimpanzees	High to moderate set point viremia
Chronic immune activation present	Chronic immune activation not observed in captive chimpanzees	Chronic immune activation absent
Loss of mucosal Th17 cells	Unknown	Preservation of mucosal Th17 cells
Microbial translocation	Unknown	Minimal microbial translocation
Presence of gut microbiome dysbiosis	Minimal gut microbiome dysbiosis	Absence of gut microbiome dysbiosis
Increasing viremia over time	Unknown	Stable setpoint
Progressive depletion of peripheral CD4+ T cells	CD4+ T cell depletion observed in wild and captive chimpanzees	Maintenance of peripheral CD4+ T cell levels
Progression to AIDS common	Progression to AIDS documented in wild chimpanzees	Progression to AIDS very rare

Figure 1.2. Host-specific pathogenicity. Comparison of the characteristics of pathogenesis in non-pathogenic hosts of SIV infection, pathogenic hosts of SIV/HIV infection, and chimpanzees with SIVcpz infection.

are key regulators of mucosal immunity (77), or differential regulation of other inflammatory factors (78). Additionally, non-pathogenic hosts have low frequencies of mucosal CCR5+ CD4+ T cells (79), which serve as the main target of SIV infection, indicating that restriction of target cell availability may help reduce SIV pathogenicity in addition to dampened chronic immune responses.

The kinetics of viremia, extent of immune activation, presence of microbial translocation, levels of CCR5+ CD4+ T cells, and other factors key to HIV-1/SIV pathogenicity are largely unknown in naturally SIVcpz infected chimpanzees due to the inaccessibility of blood samples from wild-living apes (Figure 1.2). Limited studies of captive chimpanzees have queried some of these variables. Experimental infection of five captive animals with SIVcpz revealed similar kinetics of viremia to other hosts of SIV/HIV, with an acute peak then chronic set point viral load (80). A follow-up study of seven SIVcpz infected captive animals showed depletion of CD4+ T cells, evidence of lymphoid tissue damage, and increased anti-platelet autoimmune antibodies (81). However, they failed to find significantly increased levels of markers of immune activation and gut integrity. This group thus concluded that SIVcpz infected chimpanzees exhibit less marked pathogenesis than HIV-1 infected humans, but more pathogenesis than other natural hosts of SIV. However, despite these limited studies in experimentally infected chimpanzees, very little is known about the mechanism and extent of pathogenesis in SIVcpz infected chimpanzees.

Immunodeficiency and the microbiome

Pathogenesis studies in wild-living chimpanzees would require a marker of disease that can be detected in fecal material. In recent years, the microbiome, or the community of microbes that are present at a certain site, has become a marker for host health. Recent work suggests that the microbiome has evolved with its host, with evidence for cospeciation of primates with

predominant members of their gut flora, confirming their intimate association (82). The microbiome is inherited at birth, with infants showing compositional similarities to their mother's vaginal tract immediately following delivery (83). Microbiome composition varies greatly by anatomical site, with distinct communities present in the nostrils, oral cavity, skin, stomach, gastrointestinal and genital tracts. Microbiomes vary between individuals (84) and to a lesser extent, within an individual over time (85). External factors can also impact microbiome composition, with diet (86), antibiotic use (87), and even seasonal changes (88) impacting microbial communities. Diseases of multiple anatomic sites can also alter microbiome composition, including psoriasis and the cutaneous microbiome (89), and *Helicobacter pylori* and the gastric microbiome (90). The most well studied microbial communities are those of the gut. Studies of the gut microbiome, often utilizing fecal material, revealed the association of multiple disease states with alterations in bacterial abundances and/or bacterial diversity, termed microbiome dysbiosis. For example, microbes of the *Fusobacterium* species are associated with colorectal cancer (91), Crohn's disease is associated with decreased bacterial diversity (92), and members of *Enterobacteriaceae* are associated with ulcerative colitis (93). The gut microbiome may also influence the outcome of infectious diseases, with the composition of the gut microbiome being associated with the severity of malarial disease in mice (94). While studies of the microbiome are largely observational, understanding these relationships provide important insight into the pathogenesis of multiple diseases.

The gut is a primary focus HIV-1 research, as it serves as the largest reservoir of CD4+ T cells and a primary site of virus replication. Changes in the gut, including disruption of intestinal barriers, depletion of immune cells, translocation of microbial products, and local immune activation, serve as key characteristics of disease progression (95). In addition, late-stage disease is often associated with high incidence of diarrheal disease, which can lead to severe morbidity and/or mortality (96). The profound effect of HIV infection on the gut led many investigators to examine the gut microbiota of HIV infected individuals. Multiple studies of HIV infected and uninfected individuals found that the gut microbiome of HIV-positive patients were

enriched or depleted in certain bacterial strains. Investigators found increased frequencies of *Prevotella* (97-100) and *Proteobacteria* (97, 101, 102), and decreased frequencies of *Bacteroides* (97-100, 102) and *Ruminococcus* (97, 99, 100, 103). However, likely due to variations in sample collection and analysis methods, and the disease state and treatment status of the patients, not all results have been confirmatory. Many groups report significant differences in abundances of bacteria that are not observed by others, with increased abundance of *Prevotella* being the most consistently associated with HIV-1 infection. Inconsistent findings were also reported for alpha diversity, which is a marker of bacterial richness and evenness. Some studies report that HIV-1 infected individuals have decreased alpha diversity (99, 100, 103), while others reported an increase (98), or no difference (97, 101, 102). However, most studies find clustering of HIV-1 infected individuals by beta diversity, a between-individual comparison of bacterial composition; leading investigators to conclude that HIV-1 drives the gut microbiome to be distinct from that of uninfected individuals (98-103). Some investigators have linked gut microbiome dysbiosis with markers of disease progression, including associations with immune cell activation (97, 102), plasma markers of inflammation (101, 102), and microbial translocation (97, 100). Correlations with HIV-1 associated microbiome changes and the gold standard of disease progression, CD4+ T cell counts, were observed in some studies (97, 104, 105), but not others (99, 106). Thus, inconsistent findings leave the role of the gut microbiome in HIV-1 infection unclear. However, these studies have revealed interesting associations that suggest a potential role for gut microbes in HIV-1 pathogenesis.

Using non-invasively collected fecal material, studies of the gut microbiome in wild-living chimpanzees are also possible. Early work showed that chimpanzees and humans have quite similar microbiome compositions, with *Firmicutes, Bacteroidetes, Proteobacteria*, and *Actinobacteria* being the predominant phyla (107). The microbiota of wild-living chimpanzees are also susceptible to environmental factors, with animals of the same community and geographic location showing convergent microbiomes (108, 109). Social behavior also impacts the gut composition of wild chimpanzees, with more diverse and convergent microbiomes observed

during months where chimpanzees exhibit high social activity (110). Age, gender, and genealogy seem to have less pronounced effects on their gut microbiota (108). A preliminary study by Moeller and colleagues investigated the impact of SIVcpz on the chimpanzee gut microbiome. Using longitudinal fecal samples from six SIVcpz-infected individuals and pre-infection samples as controls, Moeller et al. failed to find any bacterial strain that was significantly associated with SIVcpz infection (111). This finding was in contrast to most studies of HIV-1 infected individuals. An increased frequency of some disease-associated genera (*Sarcina, Staphylococcus,* and *Selenomonas*) was observed in some individuals post-infection; however these genera also contain commensal strains and are not indicative of the outgrowth of pathogenic bacteria. No difference in microbiome richness or evenness was observed between pre- and post-infection samples, similar to some studies of HIV-1 infected individuals (97, 101, 102). Investigating the beta diversity of pre- and post- infection samples, Moeller et al. did observe more variation in samples taken after the acquisition of SIVcpz. This implied that while SIVcpz did not have a pronounced effect on the composition of the chimpanzee gut microbiome, it did result in relative instability of microbial communities.

Wild-living gorillas are also infected with SIV, having acquired their strain via cross-species transmission of SIVcpz from chimpanzees (112). The ability of SIVgor to cause disease in these apes is unknown, as wild-living gorillas are very reclusive, making habituation and longitudinal sampling extremely difficult. To look for signs of SIVgor-associated disease in these apes, Moeller and colleagues also investigated the fecal microbiomes of wild gorillas. Utilizing 69 SIV positive samples and 96 SIV negative samples, they found no significant differences in the frequency of bacterial strains, community richness, evenness, or rate of divergence (113). Thus, SIV infection of wild-living apes seems to have a more modest effect on the gut microbiome compared to that of HIV-1 and humans. However, given the study of only six SIV infected chimpanzees to date, a more comprehensive analysis would be required to determine the effect of SIVcpz on the gut microbiome.

Immunodeficiency and the virome

In addition to the bacterial members of the gut, the resident viruses (comprising the virome) may also play a role in HIV/SIV associated pathogenesis. Immunodeficiency has been associated with changes in the virome, including outgrowth of both pathogenic and non-pathogenic viruses (114-117). For example, the blood of immunosuppressed transplant patients has increased frequencies of anelloviruses, a nonpathogenic virus family with small circular DNA genomes (116, 117). An altered virome has also been implicated in HIV and SIV infection. A study of HIV-1 infected patients from the United States and Uganda found that progression to AIDS coincided with the outgrowth of annelloviruses and other commensal viruses (115). TT virus, a member of the annellovirus family, was found to be more prevalent in HIV-1 infected patients by other groups, with TT viremia being inversely associated with CD4+ T cell counts and other predictors of survival (118, 119). Using metagenomic virome analysis, Handley and colleagues investigated fecal samples of 35 SIV infected macaques. They found that SIV infection was associated with a 10-fold increase in the number of viral sequences (114). In contrast, SIV infected African green monkeys, a nonpathogenic host of SIV, did not show expansion of the gut virome. Multiple families of viruses were significantly increased in infected macaques, including picornaviruses and adenoviruses. Adenovirus-associated enteritis was detected by necropsy of SIV infected macaques that had progressed to AIDS, suggesting that the viruses detected by molecular analysis were capable of intestinal damage. Handley et al. also detected parvovirus in the serum of animals with fecal parvovirus expansion, indicating that parvovirus in the gut can invade tissues and enter circulation in the blood. Thus, both nonpathogenic and pathogenic members of the virome may be altered by immunodeficiency. This work suggests that studies of the fecal virome could not only reveal signatures associated with HIV/SIV infection, but could also provide clues to the mechanism of gut pathogenesis.

Conclusions

In sum, the mechanism of HIV- and SIV- associated pathogenesis is complex, and varies depending on the host species. Hosts that have evolved longer with their virus (e.g. African monkey species with SIV) show far less morbidity and mortality than hosts who have acquired their virus more recently (e.g. humans with HIV-1 and Asian macaques with SIV). Where SIVcpz infected chimpanzees lie on this spectrum remains to be determined. Chimpanzees likely acquired their virus relatively recently compared to African monkeys, but thousands of years earlier than the introduction of HIV into human populations (120). Thus, studies of the extent and mechanism of SIVcpz pathogenesis in chimpanzees could reveal interesting insights into how host-specific adaptation affects SIV/HIV-associated disease.

Section 1.4 - Potential Intervention Strategies for SIVcpz Infected Chimpanzees

Introduction

Chimpanzees are an endangered species, with wild populations rapidly decreasing in equatorial Africa (121) in the past several decades. Population numbers are estimated to have decreased from around 1 million chimpanzees in 1990 to around 200,000 in the year 2000 (49). Bushmeat hunting, logging and agriculture-driven deforestation, and infectious disease threaten wild ape populations. Infectious diseases play a significant role in ape population decline, with estimates suggesting that up to one third of wild ape populations may have been killed by the Ebola Zaire strain (121, 122). Due to the critically sparse populations of African apes and the threat of infectious diseases, conservationists and researchers alike have recommended pursuing medical intervention strategies for pathogens that could contribute to extinction of wild apes (123, 124). Due to the perilously low numbers of mountain gorillas (less than 1,000 animals are estimated to remain in the wild), medical treatment of disease has become an integral piece of their conservation plan (125, 126). For example, juvenile mountain gorillas have been treated with anti-parasitics to prevent the spread of mange (127). In addition, vaccination of wild gorillas in the Virungas was performed in response to a measles outbreak (128). Given the endangered status of wild-living chimpanzee populations, and the potential of SIVcpz to contribute to population decline (62), the feasibility of strategies to curb SIVcpz transmission in the wild should explored.

Antiretroviral therapy

Given the genotypic and phenotypic similarities of SIVcpz in chimpanzees to HIV-1 in humans, SIVcpz treatment and prevention strategies can be guided by the extensive efforts that

have been performed for humans. The most effective means of treating and preventing HIV-1 to date is antiretroviral therapy (ART). The first antiretrovirals (ARVs) were delivered in the early 1990s, however, due to the error-prone reverse transcriptase and rapid turnover rate of HIV-1, attempts at ARV monotherapy were largely unsuccessful. However, combination therapy of more potent ARVs (cART), led to a significant reduction in HIV-associated morbidity and mortality (129). Dozens of ARVs have since been developed, with the largest fraction targeting the reverse transcription process (130). Two classes of drugs can inhibit this stage of the viral life cycle, nucleoside reverse transcriptase inhibitors (NRTIs), which are analogs of native nucleoside substrates, or non-nucleoside reverse transcriptase inhibitors (NNRTIs), which act non-competitively by binding an allosteric pocket of the reverse transcriptase enzyme. The other enzymatic activities of HIV-1, such as integration (critical for viral latency and protein production) or protease cleavage (involved in virus maturation), are also effective targets of ARVs. Due to the broad activity of these molecules against divergent HIV-1 and even SIVmac strains, and the conservation of these proteins between HIV-1 and SIVcpz, these ARVs would likely be effective against SIVcpz. ART is not only a potent means of reducing viral load and disease progression in HIV-1 infected patients, it can also dramatically reduce the risk of HIV-1 transmission by both sexual and intravenous routes (131-134). While ART would likely prove highly effective against SIVcpz, adherence to daily oral medication is required, making its translation to wild ape populations impossible. Despite promising efforts to generate long-lasting ARVs (135), other strategies to reduce SIVcpz transmission may be required.

Broadly neutralizing antibodies

Monoclonal antibodies have been a recent focus of HIV-1 treatment and prevention strategies, and may provide an alternative to ART (136). Antibody responses to HIV-1 infection develop within a week of detectable viral load; however, this initial response has no effect on HIV-1 viremia (137). Despite gaining some neutralizing activity over time, antibody responses are

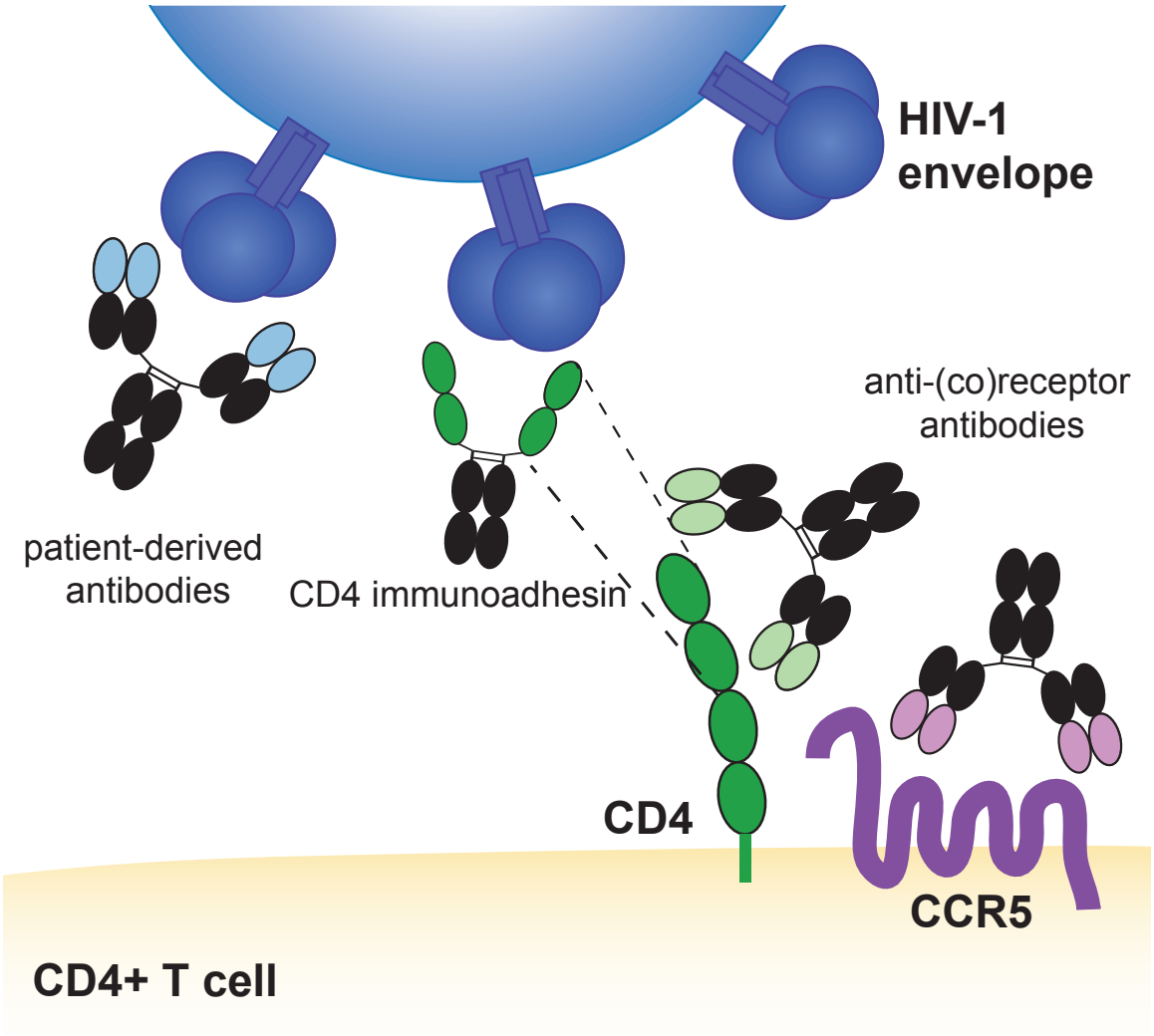


Figure 1.3. Types of HIV neutralizing antibodies. Antibodies targeting the HIV envelope include classical bNAbs that are derived from chronically infected individuals, as well as antibody-like constructs that are engineered to combine domains 1 and 2 of CD4 with antibody constant regions. Monoclonals binding to the HIV receptor CD4, or co-receptor CCR5, can also prevent HIV entry.

incapable of controlling HIV-1 infection throughout the course of infection. Neutralizing responses are thwarted by viral escape through amino acid substitutions and a dynamic glycan shield, which prevents access of antibodies to their conserved epitopes (138). In a subset of HIV-1 infected patients, antibodies capable of neutralizing heterologous variants, termed broadly neutralizing antibodies (bNAbs), develop after several years of infection (139). Development of single cell cloning techniques allowed for high throughput analysis of HIV-1 envelope-reactive antibodies and characterization of bNAbs (140, 141).

Using these methods to screen thousands of patients, a number of broad and potent monoclonal antibodies have been derived. These bNAbs were found to target defined sites of vulnerability on the HIV-1 envelope: the CD4 binding site, variable loops 1 and 2 (V1/V2), also called the V2 apex, variable loop 3 (V3), also called the high-mannose patch, and the membrane proximal external region (MPER). Engineered antibody-like constructs that combine domains of the human receptor with antibody constant regions, termed CD4 immunoadhesins, are also capable of broad and potent neutralizing activity (142-144) (Fig. 1.3). One immunoadhesin, eCD4-Ig, combines an IgG Fc with domains 1 and 2 of CD4 and a CCR5 mimetic peptide (143). This molecule mimics both the HIV-1 receptor and co-receptor and thus binds multiple regions of the gp120 molecule. eCD4-Ig shows the most breadth of any anti-HIV-1 molecule to date, neutralizing every HIV and SIV strain tested (143). Binding of antibodies to the envelope protein not only blocks the virus from entering cells, it has also been shown to accelerate the clearance of bound viruses from plasma (147), stimulate immune responses via Fc-mediated mechanisms (148), and enhance clearance of infected cells (149). Monoclonal antibodies that bind to the HIV receptor or coreceptor (instead of the virus) have also been developed (Fig 1.3). Ibalizumab, a CD4-binding monoclonal that does not inhibit MHC class II binding, and PRO 140, a CCR5-binding monoclonal, are both capable of blocking HIV-1 entry and showed promising results in clinical trials (145, 146).

The long half-lives of monoclonal antibodies provide their potential as long-lasting reagents for both treatment and prevention. Using a chimeric virus that expresses a HIV-1 envelope in the context of a SIV backbone (SHIV), researchers found that passive transfer of monoclonal antibodies prevents virus acquisition after repeated mucosal challenge (150-152). This prophylaxis was extremely effective, providing protection even when using an antibody with relatively low potency at very low concentrations (150). A combination of bNAbs were able to completely suppress viremia in humanized mice with established HIV-1 infection, with antiviral activity lasting for 60 days after the last antibody dose was administered, providing evidence that combination bNAb therapy could provide long-lasting viral suppression (153). Later studies in SHIV infected macaques revealed the ability of combination bNAb therapy to reduce viremia in primates (154, 155). Clinical trials testing the safety and efficacy of bNAb therapy in humans are ongoing. Initials studies report up to a 2.5 log reduction of viremia in HIV-1 infected individuals, with viral suppression lasting up to a month, after administration of a single monoclonal antibody (3BNC117) (156). 3BNC117 was also found to delay the time to viral rebound for up to 19 weeks after cessation of ART in infected individuals, dramatically longer than the average 3-week time to rebound observed without antibody administration (157). Adding to the potential of antibodies for long-term prophylactic or therapeutic activity is the use of antibody gene transfer, pioneered by Lewis and colleagues (158). This method involves the delivery of a gene encoding a monoclonal antibody to muscle tissue via recombinant adeno-associated virus (rAAV) gene therapy vector (158). Skeletal muscle cells are very long-lived, enabling antibody expression for years or even decades. This method proved efficacious as a prophylactic against SIV transmission in macaques and HIV transmission in humanized mice (159-161). Such long lasting activity would be critical for translation to wild-living chimpanzees.

Treating captive chimpanzees

Chimpanzees are not only susceptible to AIDS in the wild. Over 100 chimpanzees were experimentally infected with HIV as part of early vaccine and pathogenesis studies (80, 162-179). Most early transmissions did not result in AIDS-like symptoms, leading researchers to conclude that HIV did not cause disease in chimpanzees (162-164, 168, 170, 180). However, later studies found that HIV-1 was capable of inducing AIDS in captive chimpanzees, with one animal showing rapid reduction in CD4+ T cell numbers to less than 26 cells/ul after only 14 weeks of infection (165). Other chimpanzees showed slower courses of infection, one progressing after 2 years of infection, and another after 3 years of infection (165, 166). Serological analyses found CD4+ T cell loss and increased levels of multiple markers of immune activation in a number of HIV-1 infected chimpanzees, suggesting that chimpanzee "progressors" are more common than previously thought (167, 181). Chimpanzees have also been subjected to experimental infection with SIVcpz. One study performed intravenous, intra-rectal, and intra-vaginal inoculation of eight animals; six of the eight became viremic after a single inoculation (80). Long-term follow-up of these animals revealed that most displayed CD4+ T cell loss and evidence of thrombocytopenia, and those subjected to biopsies all showed evidence of lymphoid pathology (81). One of these chimpanzees had a CD4+ T cell count of 115 cells/ul, and two additional animals showed counts fewer than 300 cell/ul. Thus, a number of captive chimpanzees currently housed in the United States are chronically infected with HIV and SIVcpz, and these animals may be at risk of developing, or have already developed, AIDS. Treatment of a captive chimpanzee with ART has not been reported. However, ethical treatment of these animals may call for monitoring their disease status, and providing appropriate treatment if necessary.

Conclusions

Chimpanzee populations are facing population decline at an alarming rate, are increasingly threatened by infectious diseases, and will go extinct without a comprehensive approach to their conservation. SIVcpz infection contributes to the disease burden of wild

populations, and thus strategies to decrease transmission of SIVcpz should be considered. Extensive work has been invested in small molecules, monoclonal antibodies, and vaccines to improve the outcome of HIV-1 infected individuals and decrease HIV-1 transmission. Many chimpanzees were subjected to HIV-1 infection in order to develop these reagents and remain in captivity without regular monitoring for AIDS progression or efforts to control their viremia. Given the close relation of HIV-1 and SIVcpz, these existing solutions could be easily translated to HIV or SIV infected chimpanzees in the wild or in captivity, providing benefit not only to chimpanzees, but also informing similar studies of humans.

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CHAPTER 2

DESTABILIZATION OF THE GUT MICROBIOME MARKS THE END-STAGE OF SIMIAN IMMUNODEFICIENCY VIRUS INFECTION IN WILD CHIMPANZEES

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The experiments presented in this chapter were designed, executed, and analyzed by myself under the supervision of Beatrice Hahn. Yingying Li, Miguel Ramirez, and Zachary Klase provided technical assistance. Iddi Lipende and Deus Mjungu led the fieldwork that yielded the chimpanzee samples used in this study. Michael Wilson, Anne Pusey, and Elizabeth Lonsdorf provided key insight into the chimpanzee communities at Gombe National Park. Andrew Moeller and Frederick Bushman provided critical reviews of the manuscript. I wrote the manuscript with assistance from Beatrice Hahn.

Section 2.1 – Abstract

Enteric dysbiosis is a characteristic feature of progressive human immunodeficiency virus type 1 (HIV-1) infection, but has not been observed in simian immunodeficiency virus (SIVmac) infected macaques, including in animals with end-stage disease. This has raised questions concerning the mechanisms underlying the HIV-1 associated enteropathy, with factors other than virus infection, such as lifestyle and antibiotic use, implicated as playing possible causal roles. Simian immunodeficiency virus of chimpanzees (SIVcpz) is also associated with increased mortality in wild-living communities, and like HIV-1 and SIVmac, can cause $CD4^+$ T cell depletion and immunodeficiency in infected individuals. Given the central role of the intestinal microbiome in mammalian health, we asked whether gut microbial constituents could be identified that are indicative of SIVcpz status and/or disease progression. Here, we characterized the gut microbiome of SIVcpz infected and uninfected chimpanzees in Gombe National Park, Tanzania. Subjecting a small number of fecal samples (*N*=9) to metagenomic (shotgun) sequencing, we found bacteria of the family *Prevotellaceae* to be enriched in SIVcpz infected chimpanzees. However, 16S rRNA gene sequencing of a larger number of samples (*N*=123) failed to show significant differences in both the composition and diversity (alpha and beta) of gut bacterial communities between infected (*N*=24) and uninfected (*N*=26) chimpanzees. Similarly, chimpanzee stool-associated circular virus (Chi-SCV) and chimpanzee adenovirus (ChAdV) identified by metagenomic sequencing were neither more prevalent nor more abundant in SIVcpz infected individuals. However, fecal samples collected from SIVcpz infected chimpanzees within five months before their AIDS-related death exhibited significant compositional changes in their gut bacteriome. These data indicate that SIVcpz infected chimpanzees retain a stable gut microbiome throughout much of their natural infection course, with a significant destabilization of bacterial (but not viral) communities observed only in individuals with known immunodeficiency within the last several months before their death.

Section 2.2 – Introduction

Simian immunodeficiency virus (SIVcpz) of chimpanzees (*Pan troglodytes*) is the progenitor of human immunodeficiency virus type 1 (HIV-1), the causative agent of the human AIDS pandemic [Sharp & Hahn 2011]. Although only the central (*P. t. troglodytes*) and eastern (*P. t. schweinfurthii*) subspecies are naturally infected, SIVcpz is widespread throughout their habitats in west-central and eastern Africa, with overall prevalence rates of 6% and 14%, respectively [Keele et al., 2006; Li et al., 2012; Rudicell et al., 2011; Santiago et al., 2003; Santiago et al., 2002; Van Heuverswyn et al., 2007]. Conducting non-invasive natural history studies in Gombe National Park, Tanzania, we previously showed that SIVcpz infected chimpanzees have a significantly (10 to 16-fold) increased risk of death compared to uninfected chimpanzees, that infected females are less likely to give birth and have a much higher infant mortality rate than uninfected females, and that SIVcpz can cause $CD4^+$ T lymphocyte depletion and histopathological findings consistent with end-stage AIDS [Keele et al., 2009; Terio et al., 2011]. Moreover, one Gombe community, which exhibited the highest SIVcpz prevalence rate (40%-50%), suffered a catastrophic population decline [Rudicell et al., 2010]. These data indicated that SIVcpz, like HIV-1, is capable of causing substantial morbidity and mortality in infected populations. In HIV-1 infected humans, high plasma viral loads, reduced $CD4^+$ T cell counts, and general systemic immune activation are markers of disease progression [Brenchley et al., 2006; Hazenberg et al., 2003; Mellors et al., 1997]. These parameters cannot be measured in wild-living chimpanzees, since even in habituated communities, such as Gombe, it is neither ethical nor practical to repeatedly tranquilize chimpanzees for blood collection.

Many chronic diseases are characterized by changes in the gut microbiota [Cho & Blaser 2012; Claesson et al., 2012]. In HIV-1 infection, the gastrointestinal tract is a primary site of virus replication, with $CD4^+$ T cell depletion, loss of intestinal immune barriers, translocation of microbial products, and chronic immune activation representing key features of progressive

disease [Brenchley & Douek 2007]. It is thus not surprising that chronic HIV-1 infection has been linked to changes of the gut bacteriome, although results have not always been uniform, most likely because of differences in the types of samples collected, the sequencing and analysis methods used, and the disease and treatment status of the sampled individuals [Dillon et al., 2014; Dinh et al., 2015; Lozupone et al., 2013; McHardy et al., 2013; Mutlu et al., 2014; Vazquez-Castellanos et al., 2015; Vujkovic-Cvijin et al., 2013]. For example, several groups reported an association between HIV-1 infection and decreased alpha diversity, which describes the variety of microbiota (richness) and their relative abundances (evenness) within a sample [McHardy et al., 2013; Mutlu et al., 2014; Vazquez-Castellanos et al., 2015], while others reported an increase [Lozupone et al., 2013], or no effect at all [Dillon et al., 2014; Dinh et al., 2015; Vujkovic-Cvijin et al., 2013]. Similarly, different bacterial taxa have been reported to either be enriched or depleted (i.e., present at a significantly higher or lower frequency relative to the total microbial content) in the gut of HIV-1 infected patients, although several groups found an increase in the relative abundance of *Prevotella* [Dillon et al., 2014; Lozupone et al., 2013; Mutlu et al., 2014; Vazquez-Castellanos et al., 2015] and *Proteobacteria* [Dillon et al., 2014; Dinh et al., 2015; Vujkovic-Cvijin et al., 2013], and a decrease in the relative abundance of *Bacteroides* [Dillon et al., 2014; Lozupone et al., 2013; Mutlu et al., 2014; Vazquez-Castellanos et al., 2015; Vujkovic-Cvijin et al., 2013] and *Ruminococcus* [Dillon et al., 2014; McHardy et al., 2013; Mutlu et al., 2014; Vazquez-Castellanos et al., 2015]. Despite these differences, there is general agreement that compositional changes in gut microbial communities between individuals (beta diversity) are associated with progressive HIV-1 infection [Lozupone et al., 2014; Nwosu et al., 2014].

SIVmac infection of macaques recapitulates many of the clinical sequelae of HIV-1 infection, including the characteristic gut pathology, and represents an important animal model to study HIV-1 pathogenesis [Estes et al., 2010; Li et al., 2005]. It thus came as a surprise when macaques with progressive SIVmac infection were found to have a highly stable gut bacteriome [Handley et al., 2012; Klase et al., 2015; McKenna et al., 2008], although an expansion of their enteric virome was observed [Handley et al., 2012]. These findings raised questions concerning

the mechanisms underlying the changes in intestinal microbiota associated with HIV-1 infection, with factors such as geographic location [Yatsunenko et al., 2012], antibiotic use [Jernberg et al., 2007] and antiretroviral treatment [Klase et al., 2015] implicated as playing potential causal roles. Wild-living chimpanzees are not exposed to drug therapy and maintain relatively stable geographic locations, and thus may represent a more relevant model to examine the impact of a pathogenic primate lentivirus infection on gut microbial diversity.

Previous studies of wild chimpanzees showed that their gut microbiota are very similar to those of humans, both with respect to the types of bacterial constituents present [Moeller et al., 2012] and their modulation by environmental factors such as diet and geographic location [Degnan et al., 2012; Moeller et al., 2013a]. We thus reasoned that analyses of the microbiota in chimpanzee fecal samples might yield insight into the natural history and pathogenesis of SIVcpz infection. Studying a small number (*N*=6) of Gombe chimpanzees before and after they became infected with SIVcpz, we previously found greater fluctuations in bacterial composition as well as a higher abundance of disease-associated bacterial genera in fecal samples collected after SIVcpz acquisition [Moeller et al., 2013b]. However, an analysis of the gut bacteriome of SIVgor infected and uninfected western gorillas (*Gorilla gorilla*) failed to identify such differences [Moeller et al., 2015b], despite the fact that SIVgor originated from the cross-species transfer of SIVcpz and may share some of the same pathogenic properties [Takehisa et al., 2009; Van Heuverswyn et al., 2007]. Here we used both metagenomic and bacterial 16S rRNA gene sequencing to study a much larger number of SIVcpz infected and uninfected chimpanzees in Gombe National Park, Tanzania. Our results indicate a surprising robustness of the chimpanzee gut microbiome throughout much of the duration of the SIVcpz infection course, with significant compositional changes of bacterial, but not viral, constituents observed only in individuals with known (or suspected) immunodeficiency within the last several months before their death.

Section 2.3 – Methods

Chimpanzee fecal samples

Fecal samples were collected from wild-living chimpanzees in Gombe National Park, Tanzania, including members of habituated (Mitumba and Kasekela) and non-habituated (Kalande) communities (for a map of Gombe National Park and the ranges of its three communities, see [Lonsdorf et al., 2015a]). The Kasekela and Mitumba chimpanzees have been under direct observation since the 1960s and 1980s, respectively [Pusey et al., 2007; van Lawick-Goodall 1968], with prospective fecal sampling and SIVcpz diagnostics beginning in 1999 [Santiago et al., 2002]. In Mitumba and Kasekela, stool samples were collected from individually known chimpanzees under direct observation. In Kalande, samples were collected opportunistically. For individual identification, all samples were subjected to mitochondrial, sex, and microsatellite analysis as described elsewhere [Keele et al., 2009; Rudicell et al., 2010]. Fecal material (20-50 g) was placed in conical tubes containing 20 ml of RNA*later* (Ambion), a high salt solution that preserves fecal nucleic acids and allows storage and transport at room temperature. The SIVcpz infection status was determined by screening fecal samples for the presence of virus-specific antibodies by Western blot analysis and/or virion RNA by nested reverse transcriptase polymerase chain reaction (RT-PCR) analysis [Santiago et al., 2003]. AIDS-like immunopathology was detected by necropsy in recovered bodies of a subset of SIVcpz infected individuals as previously described [Terio et al., 2011]. All fieldwork was approved by the Tanzania Commission for Science and Technology, the Tanzania Wildlife Research Institute, and the Tanzania National Parks, and adhered to the American Society of Primatologists' Principles for Ethical Treatment of Nonhuman Primates.

Metagenomic (shotgun) sequencing and analysis

The fecal samples selected for metagenomic sequencing are listed in Table I. Nucleic acids were extracted from fecal samples selected for metagenomic sequencing as described [Minot et al., 2013], with modifications to accommodate RNA*later* preservation and ensure maximal virus nucleic acid recovery. RNA*later* preserved fecal material (1 ml) was vortexed (1 min) and then added to 20 ml phosphate buffered saline (PBS) solution, containing 1% bovine serum albumin (BSA), which increased mammalian virus nucleic acid recovery (not shown). Samples were again vortexed at maximum speed (1 min) and then centrifuged at 1,800 g (5 min) to pellet debris. The supernatant was passed through a 0.45 µm filter (Millipore) to remove bacterial cells and subsequently concentrated by passing through a 100 kDa ultracentrifugal unit (Millipore). Following treatment with 10U RQ1 DNase (Promega), fecal nucleic acids were extracted using the AllPrep DNA/RNA Mini kit (Qiagen), and fecal DNA was quantified using a Qubit 2.0 fluorometer (Invitrogen). Filtering and DNase treatment served to remove bacterial DNA, while at the same time enriching for particle associated (viral) nucleic acids. Both procedures were previously shown not to alter the composition of the fecal bacteriome [Handley et al., 2012]. Fecal DNA was prepared for metagenomic sequencing using the Nextera XT DNA Library Prep Kit (Illumina). Libraries were quality controlled using the Agilent 2200 TapeStation and sequenced on an Illumina MiSeq, using a 500-cycle MiSeq Reagent Kit v2 according to the manufacturer's instructions. Paired-end sequences over 50 base pairs in length were assigned to taxa using a nucleotide BLAST search in combination with a customized nucleotide sequence database that included all RefSeq bacterial and viral reference genomes (http://www.ncbi.nlm.nih.gov/refseq/) as well as all GenBank virus sequence entries. Reads that mapped to mammalian viruses were subsequently blasted against the complete NCBI nonredundant (nr) database (http://blast.ncbi.nlm.nih.gov/) using BLASTx, and those that mapped to non-viral references were removed from the analysis. Tables of bacterial and viral read counts were generated and used for abundance analysis. Bacterial phylogeny was determined using the MEGAN version 4.70.4 software [Huson & Mitra 2012].

Bacterial 16S rRNA gene sequencing and analysis

The fecal samples selected for bacterial 16S rRNA gene sequencing are listed in Table II. DNA was extracted from 0.5 ml of RNA*later* preserved fecal material using the QIAamp DNA Stool Mini Kit (Qiagen) and the QIAcube system. 16S rRNA gene amplification was performed as described [Caporaso et al., 2011; Song et al., 2013], using 100 ng of fecal DNA, the Accuprime Taq DNA Polymerase System (Invitrogen), and V1V2 region directed primers containing Illumina adapters, barcode, and linker regions [Song et al., 2013]. Each fecal sample was amplified on four independent occasions, with the products pooled and purified using AMPure XP beads (Beckman Coulter) before sequencing on the Illumina MiSeq. 16S sequence data were analyzed using QIIME v1.7.0 [Caporaso et al., 2010] and default QIIME parameters unless specified. Sequences were separated by barcode, quality filtered, and operational taxonomic units (OTUs) formed using a cutoff of 97% identity and assigned using the Greengenes database. Samples with less than 50,000 sequences per sample were removed from the analysis. After taxonomic assignment, within-sample (alpha) diversity was calculated using observed species, Chao1 [Chao 1984], Shannon Index [Shannon 2001] and Simpson's Diversity measures [Simpson 1949]. Chao1 is a nonparametric richness calculator, and Shannon and Simpson indices measure diversity both in terms of richness and evenness. Between-sample (beta) diversity was calculated using Bray-Curtis dissimilarity [Bray & Curtis 1957], which measures compositional dissimilarity, as well as Euclidean and weighted UniFrac distances [Lozupone & Knight 2005], which measure the compositional distance between microbial communities. Euclidean and weighted UniFrac distances were also used for principal coordinates analysis.

Detection of chimpanzee stool-associated circular virus and chimpanzee adenovirus in fecal samples

The presence of chimpanzee stool-associated circular virus (Chi-SCV) and chimpanzee (simian) adenovirus (ChAdV) in fecal samples was determined by nested PCR. Primers used to amplify a 214 bp fragment of the Chi-SCV replicase gene included: F1 5'- GGC TTG GTG TTT GTT AGC ACG ATC -3' and R1 5'- GAG ATG GAA CCA AGA AGG GGC -3' for the first round,

and F2 5'- CKA TAG CCG TGT ATA GCT CGG -3' and R2 5'- GGC AAC ATG GGC AAA TCG TGG C -3' for the second round of PCR. First-round PCR amplifications included 35 cycles of denaturation (94°C, 15 sec), annealing (55°C, 30 sec), and elongation (68°C, 1 min) using Platinum *Taq* DNA Polymerase High Fidelity (Invitrogen). One µl of the first-round product was used for the second round PCR, including 45 cycles of denaturation (94°C, 15 sec), annealing (58°C, 30 sec), and elongation (68°C, 1 min). Primers used to amplify a 379 bp fragment of the ChAdV DNA polymerase gene included: F1 5'- TGA TGC GYT TCT TAC CTY KGG TYT CCA TGA G -3' and R1 5'- GAC AGC GAT SCG GAA GAS AGT G -3' for the first round, and F2 5'- GTG ACR AAG AGG CTG TCC GTG TCY CCG TA -3' and R2 5'- TCA CGT GGC MTA CAC YTA CAA GCC AAT CAC -3' for the second round of PCR. Cycling conditions were the same as above. All amplicons were sequenced without interim cloning.

Quantitation of chimpanzee stool-associated circular virus and chimpanzee adenovirus

Chi-SCV and ChAdV viral loads in fecal samples (Table II) were determined by real time PCR, using cloned fragments of the Chi-SCV replicase and the ChAdV polymerase as standards, respectively. Fecal DNA (5 µl) was added to TaqMan Fast Advanced master mix (Applied Biosystems) and amplified using virus specific forward (Chi-SCV: 5'- CGG AAT GTC GAT GAC TAT GAG G -3'; ChAdV: 5'- CKC GGT CCT CCT CGT AGA G -3') and reverse (Chi-SCV: 5'- CTA CAT ACC GCC GTA CAT GAC G -3'; ChAdV: 5'- TGG ACA ACG ACC GCT ACC C -3') primers (750 nM) as well as a virus-specific (Chi-SCV: 5'- FAM-CGC GGT CTT GTA GGA CTA GGC TCG CTA C-BHQ1a -3'; ChAdV: 5'- FAM-CCG GGT CCA GGC CAG CAC GAA GGA AGG-BHQ1a -3') probe (250 nM). Each sample was tested in triplicate. PCR cycling conditions were chosen according to manufacturer's instructions on a 7900HT Fast Real-Time PCR System. Sequence Detection Systems version 2.3 software (Applied Biosystems) was used to quantify viral copy numbers, which were normalized based on the total amount of DNA in the respective fecal sample.

Statistical analysis

The abundances of bacterial and viral taxa in fecal samples from SIVcpz infected and uninfected chimpanzees were compared using the Mann-Whitney test and Prism version 5.0d software (GraphPad). Bonferroni correction was applied when multiple tests were simultaneously performed using Prism or QIIME software. Statistical analysis of bacterial 16S alpha and beta diversity measures was performed using the default parameters of the QIIME v1.7.0 software.

Section 2.4 – Tables

Table 1. Metagenomic sequencing of microbial communities in chimpanzee fecal samples.

Sample	Chimpanzee	Sex	Community[a]	Sample date	SIVcpz status[b]	Minimum number of years infected
GM1586	Ch-64	F	KL	25-Jun-09	pos	13
GM2984	Ch-48	M	MT	31-May-12	pos	13
GM3018	Ch-22	F	KK	28-Jun-12	pos	12
GM2423	Ch-80	F	KK	11-Apr-11	pos	10
GM3149	Ch-52	M	KK	7-Sep-12	pos	12
GM2522	Ch-88	F	KL	11-Jun-11	neg	-
GM2856	Ch-59	M	MT	6-Mar-12	neg	-
GM3028	Ch-09	F	KK	3-Jul-12	neg	-
GM2954	Ch-15	F	KK	13-May-12	neg	-

[a]KK, Kasekela; MT, Mitumba; KL, Kalande.

[b]pos, positive; neg, negative

Table 2. 16S rRNA gene sequencing of bacterial communities in chimpanzee fecal samples

Sample[a]	Chimpanzee[b]	Sex[c]	Community[d]	Sample date	SIVcpz Status[e]	Observable signs of ill health near the time of sampling[f]
GM2541	39	F	MT	18-Jun-11	pos	None
GM2935	39	F	MT	21-Apr-12	pos	None
GM3193	39	F	MT	24-Sep-12	pos	None
GM1425	106	F	KL	5-Nov-07	pos	n/a
GM1542	106	F	KL	19-Jan-09	pos	n/a
GM2321	106	F	KK	11-Jan-11	pos	n/a
GM889	107	F	KL	13-Aug-05	pos	n/a (presumed dead)
GM3256	137	F	KL	9-Oct-12	pos	n/a
GM3483	137	F	KK	15-Apr-13	pos	n/a
GM715	99	F	KK	11-May-05	pos	None
GM783	99	F	KK	12-Jul-05	pos	None
GM958*	99	F	KK	15-Mar-06	pos	None (DOD 30-Nov-06)
GM3401	119	M	KK	12-Jan-13	pos	None
GM3427	119	M	KK	16-Mar-13	pos	n/a
GM3521*	119	M	KK	8-Jun-13	pos	None (DLS 15-Aug-2013)
GM3186	4	M	KK	20-Sep-12	pos	None
GM3429	4	M	KK	21-Mar-13	pos	None
GM3551	4	M	KK	9-Jul-13	pos	n/a
GM329	6	M	KK	20-Aug-03	pos	n/a
GM414	6	M	KK	16-Jul-04	pos	None
GM981	6	M	KK	5-Nov-05	pos	None (DLS 23-Jan-07)
GM944	89	F	KL	26-Apr-06	pos	n/a
GM1277	89	F	KL	6-Aug-07	pos	n/a
GM3496	71	F	KK	25-Apr-13	pos	None
GM2556	133	M	KK	3-Jul-11	pos	None

GM3188	133	M	KK	21-Sep-12	pos	None
GM3492	133	M	KK	25-Apr-13	pos	None
GM1163	21	F	KK	11-Feb-07	pos	Diarrhea
GM1471	21	F	KK	23-Oct-08	pos	None
GM1602*	21	F	KK	12-Aug-09	pos	None (DOD 5-Jan-2010)
GM1598	103	F	KK	25-Aug-09	pos	None
GM2437	103	F	KK	18-Apr-11	pos	None
GM2736*	103	F	KK	7-Oct-11	pos	n/a (DLS 7-Dec-11)
GM3251	22	F	KK	24-Oct-12	pos	n/a
GM3505	22	F	KK	20-Apr-13	pos	None
GM3595	22	F	KK	11-Aug-13	pos	n/a
GM3392	100	M	KL	25-Dec-12	pos	n/a
GM3477	100	M	KL	15-Apr-13	pos	n/a
GM3543	100	M	KL	2-Jul-13	pos	n/a
GM1376	64	F	KL	9-Jun-08	pos	n/a
GM1532	64	F	KL	17-Sep-08	pos	n/a
GM1618	64	F	KL	24-Aug-09	pos	n/a (presumed dead)
GM3066	86	F	KL	19-Jul-12	pos	n/a
GM3328	86	F	KL	29-Nov-12	pos	n/a
GM3590	86	F	KL	29-Jul-13	pos	n/a
GM2678	48	M	MT	5-Sep-11	pos	None
GM3217	48	M	MT	10-Oct-12	pos	None
GM3453*	48	M	MT	7-Jun-13	pos	None (DLS 1-Nov-2013)
GM84	30	F	KK	13-Dec-01	pos	n/a
GM145	30	F	KK	2-Jul-02	pos	n/a
GM337*	30	F	KK	7-Aug-03	pos	n/a (DLS 8-Dec-03)
GM1030	33	F	KK	23-Feb-06	pos	None
GM1320*	33	F	KK	9-Aug-07	pos	None (DLS 2-Mar-08)
GM3075	80	F	KK	30-Jul-12	pos	Weight loss
GM3326	80	F	KK	29-Nov-12	pos	n/a

GM3530	80	F	KL	11-Jun-13	pos	n/a
GM350	45	M	MT	21-Sep-03	pos	n/a
GM704*	45	M	MT	27-Nov-04	pos	Weight loss, diarrhea (DOD 22-Dec-04)
GM1037	36	F	KK	15-Mar-06	pos	None
GM1325*	36	F	KK	28-Jun-07	pos	None (DOD 7-Nov-07)
GM2675	52	M	KK	10-Sep-11	pos	None
GM3167	52	M	KK	14-Sep-12	pos	Diarrhea
GM3333	52	M	KK	5-Dec-12	pos	n/a
GM1718	7	M	KK	7-Sep-09	neg	None
GM2469	7	M	KK	11-May-11	neg	None
GM2673	7	M	KK	7-Sep-11	neg	n/a
GM1389	78	F	KK	22-Jun-08	neg	Diarrhea
GM1624	78	F	KK	5-Aug-09	neg	None
GM1348	109	F	KL	12-Mar-08	neg	n/a
GM1462	109	F	KL	14-Oct-08	neg	n/a
GM1705	109	F	KL	9-Jul-09	neg	n/a
GM3096	97	F	KK	12-Aug-12	neg	None
GM3343	97	F	KK	12-Dec-12	neg	n/a
GM2619	2	F	KK	4-Aug-11	neg	None
GM3508	2	F	KK	14-Apr-13	neg	None
GM295	49	F	MT	18-Oct-03	neg	n/a
GM622	49	F	MT	12-Dec-04	neg	None (DOD 12-Jun-2010)
GM2861	68	M	MT	7-Mar-12	neg	None
GM3027	131	M	KK	3-Jul-12	neg	Respiratory illness
GM3351	131	M	KK	22-Dec-12	neg	None
GM3457	131	M	KK	16-May-13	neg	None
GM195	13	M	KK	14-Jun-02	neg	n/a
GM1238	13	M	KK	28-Jun-07	neg	None
GM3146	51	M	KK	8-Sep-12	neg	n/a

GM3421	51	M	KK	26-Feb-13	neg	n/a
GM3550	51	M	KK	9-Jul-13	neg	n/a
GM719	14	M	KK	4-May-05	neg	None
GM858	14	M	KK	29-Oct-05	neg	n/a
GM975	14	M	KK	12-Mar-06	neg	None
GM3236	15	F	KK	21-Oct-12	neg	None
GM3548	15	F	KK	6-Jul-13	neg	None
GM1104	77	M	KK	1-Jul-06	neg	None
GM1241	77	M	KK	25-Jun-07	neg	None
GM3355	53	F	KK	2-Jan-13	neg	None
GM3475	53	F	KK	7-May-13	neg	None
GM3124	17	F	KK	24-Aug-12	neg	None
GM3402	17	F	KK	15-Jan-13	neg	n/a
GM3511	17	F	KK	7-Apr-13	neg	None
GM87	1	F	KK	31-Dec-01	neg	n/a
GM196	1	F	KK	28-Apr-02	neg	n/a
GM327	1	F	KK	6-Aug-03	neg	n/a
GM1128	93	F	KL	6-Jun-06	neg	n/a
GM1295	93	F	KL	12-Aug-07	neg	n/a
GM3065	110	M	KL	23-Jul-12	neg	n/a
GM3311	110	M	KL	20-Nov-12	neg	n/a
GM3591	110	M	KL	29-Jul-13	neg	n/a
GM881	108	F	KL	25-Jul-05	neg	n/a
GM1000	108	F	KK	16-Mar-06	neg	n/a
GM2632	98	F	MT	14-Aug-11	neg	None
GM3162	98	F	MT	11-Sep-12	neg	None
GM3532	98	F	MT	25-Jun-13	neg	None
GM3386	88	F	KL	20-Dec-12	neg	n/a
GM3572	88	F	KL	16-Jul-13	neg	n/a
GM3620	88	F	KL	15-May-13	neg	n/a

GM1019	54	M	KK	25-Jan-06	neg	Respiratory illness
GM333	25	F	KK	5-Aug-03	neg	n/a
GM445	25	F	KK	30-Jun-04	neg	None
GM1010	25	F	KK	19-Jan-06	neg	None
GM3292	26	F	KK	10-Nov-12	neg	None
GM3503	26	F	KK	11-Apr-13	neg	None
GM1151	27	M	KK	30-Jan-07	neg	None
GM2363	27	M	KK	6-Mar-11	neg	n/a
GM168	36	F	KK	8-Jun-02	neg	n/a

[a]Asterisks highlight samples that were collected from nine SIVcpz infected chimpanzees within 8 months of their death.

[b]Chimpanzees Ch-21 and Ch-36 died of AIDS-related causes as determined by necropsy; Ch-45 died of conspecific aggression and Ch-99 died of conditions resulting from spinal cord injury [Keele et al., 2009]; the cause of death of the other chimpanzees was not determined because their bodies were not recovered.

[c]F, female; M, male.

[d]KK, Kasekela; MT, Mitumba; KL, Kalande.

[e]pos, positive; neg, negative.

[f]Signs of illness observed within one month before or after the date of sample collection [Lonsdorf et al., 2015a]; n/a, data not available. DOD, date of death; DLS, date last seen.

Section 2.5 – Results

Study design

To test for associations between gut microbial communities and SIVcpz infection and/or disease progression, we used both metagenomic (shotgun) and targeted (bacterial) 16 rRNA gene sequencing approaches. Metagenomic sequencing is performed on randomly fragmented DNA and thus provides genetic information for all organisms present in a sample, including bacteria and viruses. However, metagenomic sequencing requires large quantities of fecal material and is computationally intensive. For this reason, we analyzed only a small number of samples (*N*=9, Table I) with the goal of identifying potential microbial indicator species, which could then be studied in a larger number of samples from more individuals using real time PCR. As a second approach, we performed bacterial 16S rRNA gene sequencing, which requires only small amounts of starting material and thus allowed us to use the full range of samples collected from the Gombe chimpanzees over the past 15 years. We also took advantage of the well-validated analysis pipeline that exists for 16S rRNA sequence data [Caporaso et al., 2010], which facilitated a direct comparison with results previously obtained for HIV-1 infected humans [Dillon et al., 2014; Dinh et al., 2015; Lozupone et al., 2013; McHardy et al., 2013; Mutlu et al., 2014; Vazquez-Castellanos et al., 2015; Vujkovic-Cvijin et al., 2013] and SIVcpz infected chimpanzees [Moeller et al., 2013b]. For 16S rRNA gene sequencing, we included up to three fecal samples per individual to control for temporal fluctuations in gut microbial diversity (Table II). The combination of these approaches was intended to ensure unbiased results, while taking full advantage of the contextual knowledge of the sampled chimpanzee hosts.

Metagenomic sequencing fails to identify significant compositional differences between the gut bacteriome of SIVcpz infected and uninfected chimpanzees.

Metagenomic sequencing was performed on fecal samples from four uninfected and five infected Gombe chimpanzees (Table I). To detect both viral (particle-associated) and bacterial nucleic acids, each fecal sample was filtered and DNase treated prior to analysis. Although these procedures resulted in an overall reduction of bacterial DNA, a previous study demonstrated that this decrease was proportional and did not skew the composition of bacterial constituents in the sample [Handley et al., 2012]. The nine fecal samples yielded 15,900,990 high quality paired-end reads, with an average of 1,766,777 reads per sample. Sequences were assigned to bacterial taxa by performing a BLAST search of a database that included all bacterial reference genomes. Consistent with previous results [Degnan et al., 2012], we found that the bacterial phylum *Firmicutes* comprised the largest proportion of the fecal bacteriome in all nine chimpanzees, with *Bacteroidetes*, *Actinobacteria, Proteobacteria*, and *Spirochaetes* constituting the majority of the remaining bacterial phyla (Fig. 1a). Interestingly, fecal samples from the five SIVcpz infected chimpanzees were enriched for members of the family *Prevotellaceae* (P=0.04), although this result was not statistically significant when corrected for multiple tests (Fig. 1b). Nonetheless, this trend was consistent with results from previous studies that had shown an increased abundance of members of the genus *Prevotella* (of the *Prevotellaceae* family) in the intestinal microbiome of HIV-1 infected individuals [Dillon et al., 2014; Lozupone et al., 2013; Mutlu et al., 2014; Vazquez-Castellanos et al., 2015]. None of the other bacterial taxa differed in their relative abundance between SIVcpz infected and uninfected chimpanzees (Fig. 1b).

Chronic SIVcpz infection is not characterized by a distinct gut bacteriome.

Since previous 16S rRNA sequencing was performed on samples from only six Gombe chimpanzees [Moeller et al., 2013b], we designed a follow-up study that included a larger number of samples and individuals. Targeting the V1V2 region of the 16S rRNA gene, we characterized the composition and diversity of gut bacterial communities in 123 fecal samples from 24 SIVcpz infected and 26 uninfected chimpanzees (Table II). Whenever possible, we included two or three

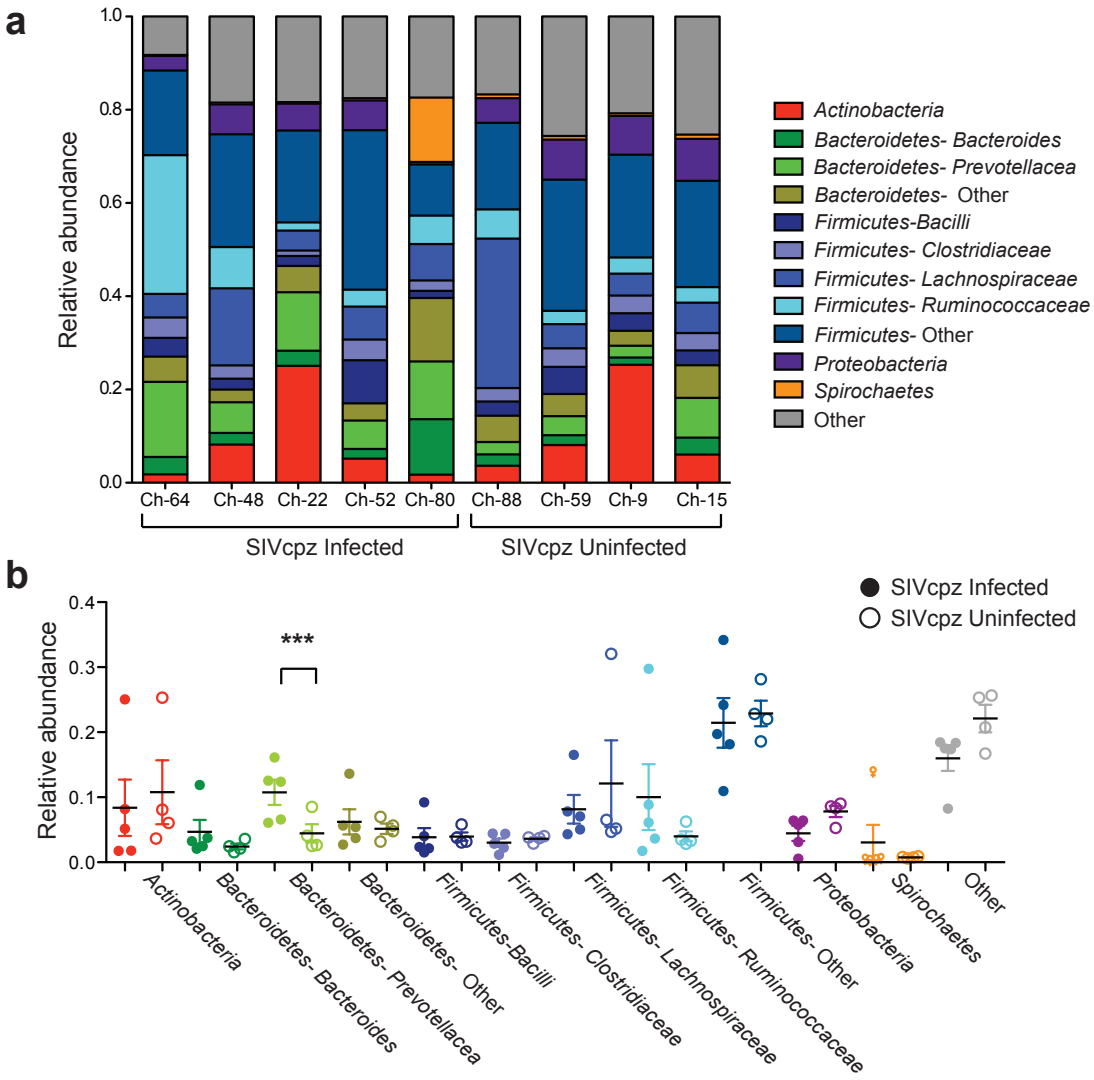

Fig. 1. Composition of the fecal bacteriome in nine Gombe chimpanzees as determined by metagenomic sequencing. (a) Taxonomy bar chart indicating the relative abundance of major bacterial phyla in fecal samples from SIVcpz infected (Ch-64, Ch-48, Ch-22, Ch-52, Ch-80) and uninfected (Ch-88, Ch-59, Ch-9, Ch-15) chimpanzees (see Table I for more information). Only phyla comprising more than 1% of the fecal bacteriome are included and further classified into major families as indicated by different colors (minor *Bacterioidetes* and *Firmicutes* families are combined into "Other" categories). A grey box ("Other") combines bacteria that could not be classified at the phylum level. (b) Relative abundance of major bacterial taxa in fecal samples from SIVcpz infected (closed circles) and uninfected (open circles) chimpanzees. Values are shown as a fraction of the total bacteria detected within each individual. Color-coding is as in (a). Black bars indicate the mean and colored bars the standard error of the mean (SEM). A statistical difference (***) was observed when the relative abundance of the bacterial family *Prevotellaceae* was compared between infected and uninfected chimpanzees using the Mann-Whitney U-test (P=0.04); however, this value did not reach statistical significance (P<0.05) after Bonferroni correction for multiple tests.

samples per individual to control for intra-individual diversity. We also matched each virus positive fecal sample with a virus negative sample from the same community, gender, and collection month to control for environmental, social, and dietary differences [Degnan et al., 2012]. For SIVcpz infected chimpanzees, the last available sample was included to cover as much of the natural disease course as possible. Very few chimpanzees had signs of ill health at or near the time of sampling (Table II) as determined by observational health surveys [Lonsdorf et al., 2015a; Lonsdorf et al., 2015b; Wolf et al., 2015a; Wolf et al., 2015b]. However, 9 SIVcpz infected chimpanzees, including Malaika (Ch-21), Skosha (Ch-30), Titania (Ch-33), Yolanda (Ch-36), Vincent (Ch-45), Rudi (Ch-48), Echo (Ch-99), Mambo (Ch-103), and Eriki (Ch-119) died within eight months of the last sample collection. Previous necropsy studies showed that both Yolanda and Malaika exhibited histopathological findings consistent with full-blown AIDS, while Vincent and Echo died of conspecific aggression and spinal cord injury, respectively [Keele et al., 2009; Terio et al., 2011]. The cause of death for the other five infected chimpanzees as well as one uninfected ape (Ch-49), who died 6 years after the last sample collection, could not be determined because their bodies were not recovered, although Rudi (Ch-48) is suspected to have also died of conspecific aggression (Table II).

Similar to the results obtained from metagenomic sequencing (Fig. 2a), 16S rRNA gene sequencing revealed a predominance of *Bacteroidetes, Firmicutes, Proteobacteria* and *Spirochaetes* in fecal samples from both SIVcpz infected and uninfected chimpanzees (Fig. 2). However, there also was a high degree of compositional variability when longitudinal samples from the same individual were compared. For example, bacteria of the family *Spirochaetacea* comprised 27% of the fecal bacteriome when chimpanzee Ch-80 was sampled in July 2012 (GM3075), but represented only 0.4% and 3% of bacterial communities when the same chimpanzee was sampled again four (GM3326) and 10 (GM3530) months later, respectively. When analyzed in a phylogenetic tree constructed from weighted UniFrac distances, the three samples from chimpanzee Ch-80 did not cluster together (not shown), and this was also true for

a
SIVcpz Infected
SIVcpz Uninfected
Relative abundance
1.0
0.5
0.0
Unclassified Bacteria
Bacteroidetes - Unclassified Bacteroidales 1
Bacteroidetes - Paraprevotellaceae
Bacteroidetes - Bacteroidaceae
Bacteroidetes - Unclassified Bacteroidales 2
Bacteroidetes - Porphyromonadaceae
Bacteroidetes - Prevotellaceae
Bacteroidetes - Unclassified Bacteroidales 3
Bacteroidetes - Unclassified
Firmicutes - Staphylococcaceae
Firmicutes - Streptococcaceae
Firmicutes - Unclassified Clostridiales 1
Firmicutes - Clostridiaceae
Firmicutes - Lachnospiraceae
Firmicutes - Ruminococcaceae
Firmicutes - Veillonellaceae
Firmicutes - Unclassified Clostridiales 2
Firmicutes - Unclassified Coriobacteriales
Firmicutes - Coriobacteriaceae
Firmicutes - Unclassified Clostridia
Firmicutes - Coprobacillaceae
Firmicutes - Erysipelotrichaceae
Firmicutes - Unclassified
Proteobacteria - Alcaligenaceae
Proteobacteria - Unclassified Betaproteobacteria
Proteobacteria - Unclassified Deltaproteobacteria
Spirochaetes - Sphaerochaetaceae
Spirochaetes - Spirochaetaceae
Tenericutes - Anaeroplasmataceae
Other Bacteria

Fig. 2. Composition of the fecal bacteriome in 50 Gombe chimpanzees as determined by 16S rRNA gene sequencing. The relative abundance of major bacterial families (color coded) comprising more than 1% of the fecal bacteriome in at least half of the fecal samples (GM numbers) is shown for SIVcpz infected (top panel) and uninfected (bottom panel) chimpanzees (Ch numbers). Operational taxonomic units (OTUs) are labeled with the highest taxonomic rank as determined by the QIIME software, with those that could not be further classified identified. Brackets denote fecal samples from the same individual (see Table II for more information). Minor bacterial taxa that comprised less than 1% of the microbiome in more than half of the fecal samples are grouped into "Other Bacteria."

longitudinal samples from other chimpanzees. In general, weighted UniFrac distances were not significantly different between longitudinal samples from the same individual and temporally matched samples from different individuals (not shown). Thus, the fecal microbiome of Gombe chimpanzees sampled at different times exhibited as much compositional diversity as did the microbiomes of different chimpanzees sampled at the same time.

To investigate whether *Prevotellaceae* enrichment was a common phenotype of SIVcpz infection, we compared the relative abundance of this family in samples from infected and uninfected individuals. Although the mean *Prevotellaceae* abundance was higher in SIVcpz infected chimpanzees, the difference was not statistically significant (P=0.79). Moreover, a broad search failed to identify bacterial families that were specifically associated with SIVcpz infection status. Thus, there was no significant enrichment or depletion of any bacterial phylum associated with chronic SIVcpz infection.

In addition to changes in bacterial abundances, changes in microbiome alpha and beta diversity have been reported to be associated with HIV-1 infection [Dillon et al., 2014; Dinh et al., 2015; Lozupone et al., 2013; McHardy et al., 2013; Mutlu et al., 2014; Vazquez-Castellanos et al., 2015; Vujkovic-Cvijin et al., 2013]. Alpha diversity is an indicator of both the number (richness) and distribution (evenness) of bacterial taxa within a single sample [Morgan & Huttenhower 2012]. Using Shannon or Simpson indices to summarize alpha diversity, we found no difference between samples of SIVcpz infected and uninfected chimpanzees (not shown). Similarly, there was no difference in richness (observed species, P=0.17) (Fig. 3a) or population size inferred

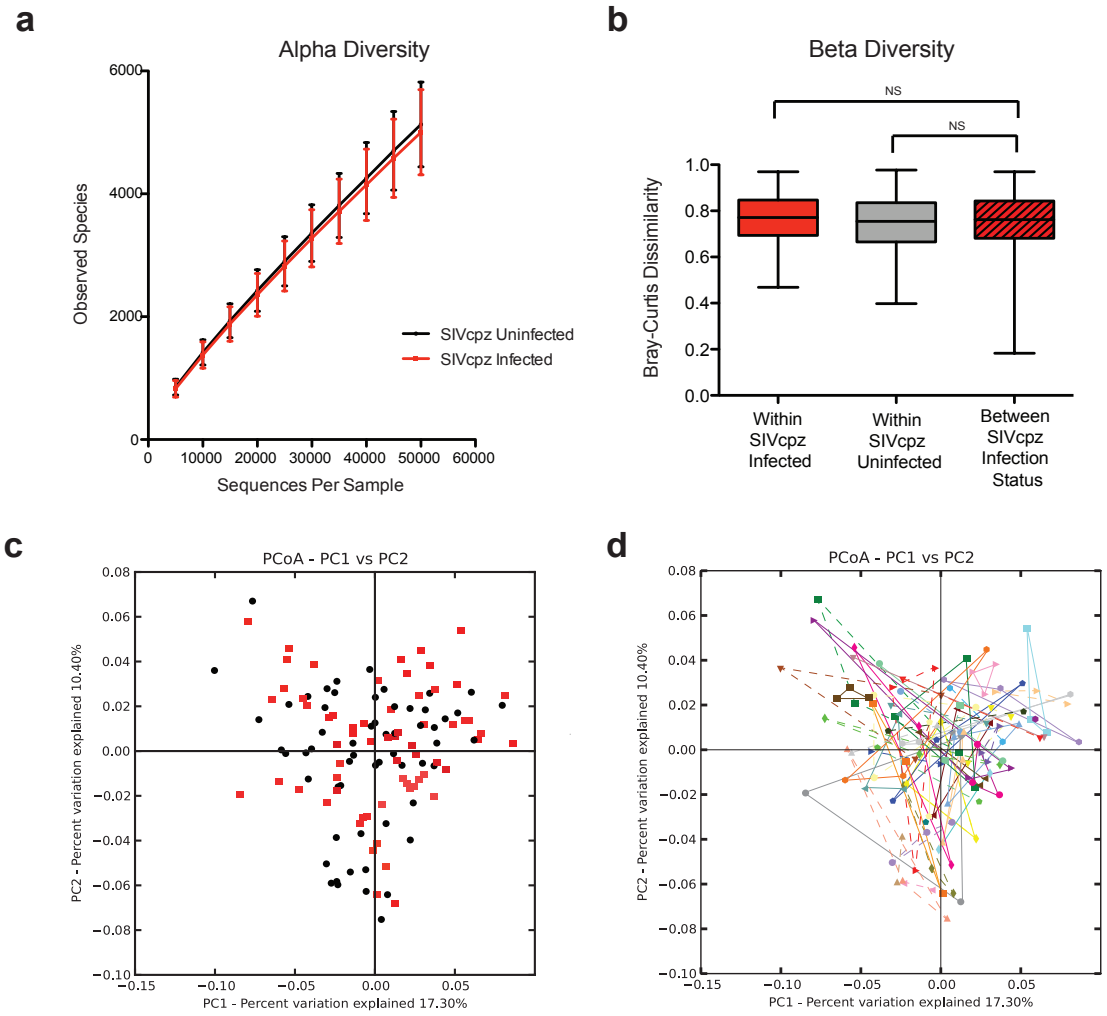

Fig. 3. SIVcpz infected and uninfected chimpanzees are not characterized by a distinct fecal bacteriome. (a) Absence of differences in alpha diversity. The number of observed species of rarefied (sampled at an even depth) OTUs was calculated from 16S rRNA sequencing data for fecal samples from SIVcpz infected (red) and uninfected (black) chimpanzees and plotted using QIIME software. Error bars indicate standard deviation. (b) Absence of differences in beta diversity. Bray-Curtis dissimilarity distances were calculated using the QIIME software for all samples from SIVcpz infected (red) and uninfected chimpanzees (grey), as well as between samples from SIVcpz infected from uninfected individuals (striped). Box plots show the median, upper, and lower quartile ranges for each comparison (indicated by brackets), with whiskers indicating minimum (top) and maximum (bottom) dissimilarities. Statistical analyses were performed using the Mann-Whitney U-test and corrected for multiple comparisons. NS, not significant. (c) Principal coordinates analysis plot (PCoA) constructed from weighted UniFrac distances indicating a lack of primary clustering of bacteriomes from SIVcpz infected (red) and uninfected (black) chimpanzees. (d) Identical PCoA plot as shown in (c), with fecal bacteriomes from the same chimpanzee identified by connecting colored solid (SIVcpz infected) or broken (uninfected) lines.

using the Chao1 estimator (not shown). In addition, there was no significant difference in beta diversity, which measures the extent of similarity between microbiota from different samples [Morgan & Huttenhower 2012]. Comparing Bray-Curtis dissimilarity values (Fig. 3b), we found no significant differences between infected and uninfected groups (P=0.17 and P=0.07 for SIVcpz infected and uninfected groups, respectively). This was also apparent in a principal coordinates analysis (PCoA) of weighted UniFrac distances, which revealed no specific grouping of samples from SIVcpz infected versus uninfected chimpanzees (Fig. 3c). Rather, the intra-individual diversity dominated the beta diversity, with consecutive samples from the same individual spanning the entire PCoA plot (Fig. 3d). Thus, in contrast to HIV-1 infected humans, the fecal microbiota of SIVcpz infected and uninfected chimpanzees were indistinguishable by these measures.

Destabilization of the fecal bacteriome marks the end-stage of SIVcpz infection.

In HIV-1 infected humans, the destabilization of the intestinal microbiome is believed to be caused by a progressive loss of gut immune function [Lozupone et al., 2014], with a normal gut microbiome observed early [Lozupone et al., 2013], but not late [Vujkovic-Cvijin et al., 2013] in infection. Since most of the fecal material subjected to 16S rRNA sequencing was derived from seemingly healthy chimpanzees (Table II), we asked whether samples obtained closer to the time of death might exhibit signs of gut microbiome destabilization. To test this, we calculated all pairwise weighted UniFrac distances between fecal samples from Yolanda (GM1325) and Malaika (GM1602) collected five months before their AIDS-related deaths and fecal samples from all other (infected and uninfected) chimpanzees (N=98) collected more than 8 months before their death (Table II). This analysis revealed a significant compositional change in each of the two immunodeficient chimpanzees relative to the controls, regardless whether samples were analyzed individually (not shown) or in combination (Fig. 4a). However, this same compositional

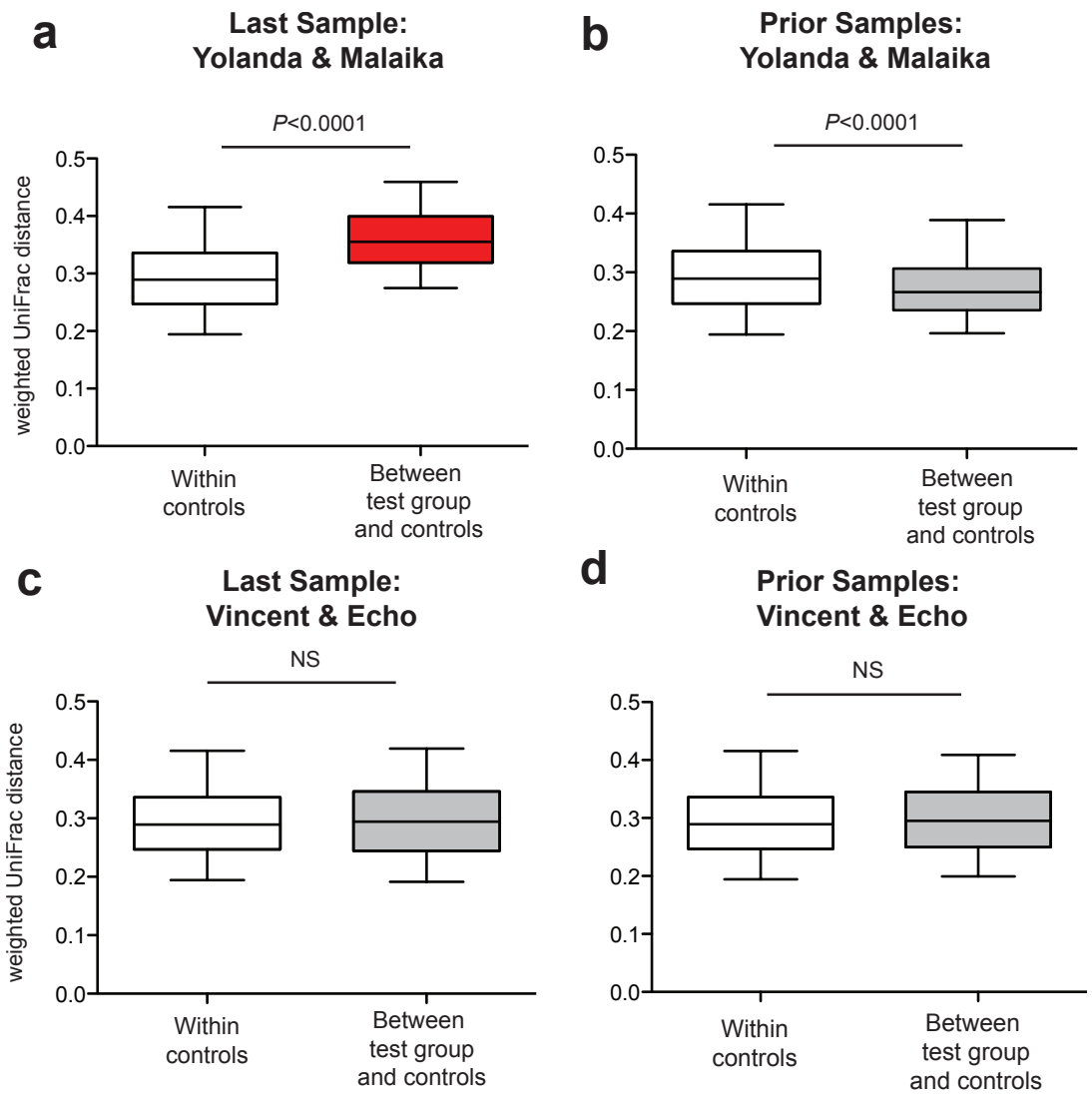

Fig. 4. Pronounced compositional shifts in the gut bacterial communities of SIVcpz infected chimpanzees shortly before their AIDS-related death. (a) Pairwise weighted UniFrac distances of the last fecal samples from Yolanda and Malaika (test group), who died of (necropsy confirmed) AIDS-like disease within five months of sampling, and fecal samples (*N*=98) from all chimpanzees who were sampled more than 8 months before their death (control group). Box plots show the median, upper, and lower quartile ranges with whiskers indicating 95% confidence intervals (CI). Statistical analyses were performed using the Mann-Whitney U-test. Red indicates significantly increased beta diversity. (b) Analysis as in (a), but comparing pairwise weighted UniFrac distances from earlier fecal samples (collected more than 8 months before death) from Yolanda and Malaika (test group) to the same controls. (c) Analysis as in (a), but comparing pairwise weighted UniFrac distances from the last two fecal samples of two SIVcpz infected chimpanzees (Vincent and Echo) who died of trauma-related causes (test group) to the same controls. NS, not significant. (d) Analysis as in (c), but comparing pairwise weighted UniFrac distances from earlier fecal samples (collected more than 8 months before death) from Vincent and Echo (test group) to the same controls. See text and Table II for more details.

shift was not observed when earlier samples from Yolanda (GM1037) and Malaika (GM1163, GM1471) were compared to the same controls (Fig. 4b). Moreover, fecal samples from Vincent and Echo, both of whom died of trauma-related deaths [Terio et al., 2011], did not exhibit destabilization of their gut community composition, regardless of whether they were collected before death (GM704, GM958) (Fig. 4c) (P=0.62), or much earlier during their infection (GM715, GM783, GM350) (Figs. 4d) (P=0.21). Using Euclidean and unweighted UniFrac distances and Bray-Curtis dissimilarity values to measure beta diversity yielded identical results (not shown). Although the number of confirmed AIDS cases in Gombe is small, these data suggest that pronounced compositional changes in the fecal bacteriome portends rapid disease progression and death.

Five additional SIVcpz infected chimpanzees, including Rudi, Skosha, Titania, Mambo and Eriki, disappeared within 7 months of their last fecal sampling (Table II). To examine whether they also experienced a destabilization of their gut bacteriome, we compared the weighted UniFrac distances of their last fecal samples to those of the same controls. Interestingly, fecal samples from Mambo (GM2736) and Eriki (GM3521), both of which were collected two months before their disappearance, also exhibited a significant compositional shift of their bacterial communities (Fig. 5a and 5b), which was not observed when prior samples from these same two individuals (GM3401, GM1598, GM2437) were analyzed (Fig. 5c) (P=0.46). Given these findings, it is possible that Mambo and Eriki also died of AIDS, although this could not be confirmed because their bodies were not recovered. In contrast, no significant compositional changes (P=0.20) were seen for the last fecal samples from Rudi (GM3453), Skosha (GM337) and Titania (GM1320) (Fig. 5d-f). Since these three chimpanzees did not disappear until 4 to 7 months after their last fecal collection (Table II), they may not have been sampled sufficiently close to the time of their death to see compositional changes in their gut bacteriome. Alternatively, they may have died of causes unrelated to their SIVcpz infection. In fact, Rudi was attacked by another chimpanzee shortly before his disappearance and may have died of the inflicted injuries, although

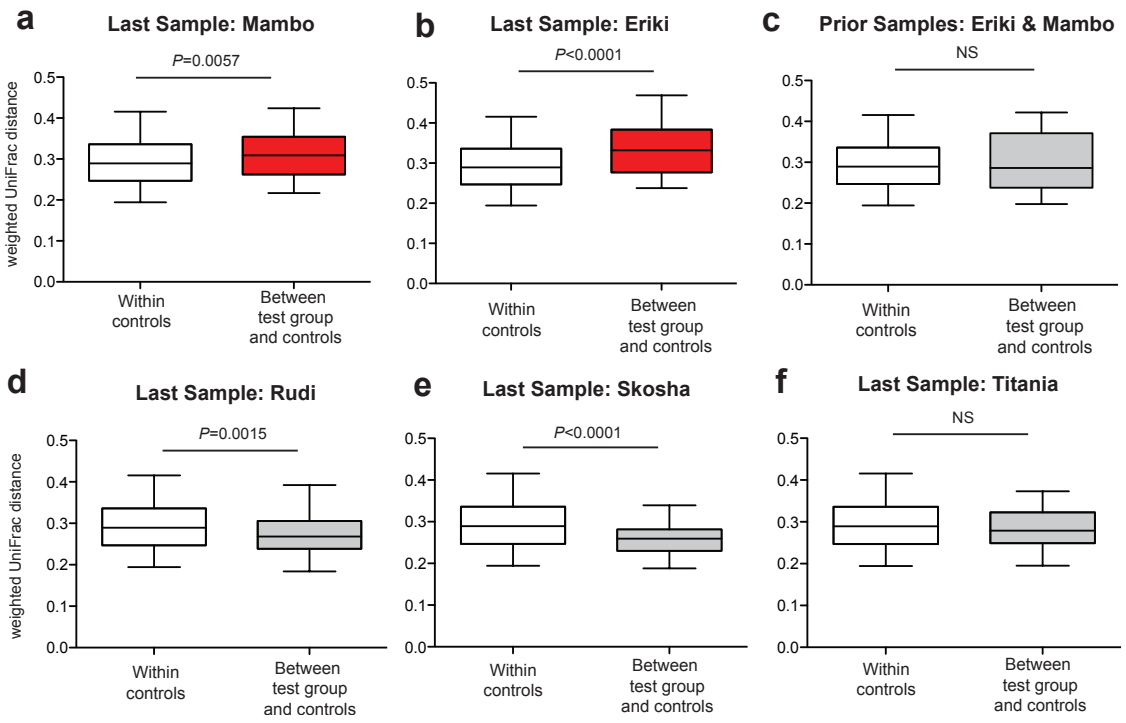

Fig. 5. Pronounced compositional shifts in the gut bacterial communities of two SIVcpz infected chimpanzees shortly before their disappearance. (a) Pairwise weighted UniFrac distances from the last fecal sample of Mambo (test group), an SIVcpz infected chimpanzee who disappeared two months after sample collection, were compared to pairwise weighted UniFrac distances of fecal samples (*N*=98) from all chimpanzees who were sampled more than 8 months before their death (control group). Box plots show the median, upper, and lower quartile ranges with whiskers indicating 95% confidence intervals (CI). Statistical analyses were performed using the Mann-Whitney U-test. Red indicates significantly increased beta diversity. (b) Analysis as in (a), but comparing pairwise weighted UniFrac distances from the last fecal sample of Eriki (test group), an SIVcpz infected chimpanzee who also disappeared two months after sample collection, to the same controls. (c) Analysis as in (a), but comparing weighted UniFrac distances from earlier fecal samples (collected more than 8 months before death) from Mambo and Eriki (test group) to the same controls. (d, e, f) Analysis as in (a), but comparing pairwise weighted UniFrac distances from the last fecal sample of Rudi, Skosha and Titania (test groups), SIVcpz infected chimpanzees who disappeared 5, 4 and 7 months after sample collection, respectively, to the same controls.

this was not confirmed by necropsy. The same results were obtained when Euclidean distances, unweighted UniFrac distances, and Bray-Curtis dissimilarity values were used to calculate beta diversity, except for the last sample from Eriki, which did not show significantly increased unweighted UniFrac distances, and the last sample from Skosha, which did show significantly increased unweighted UniFrac distances. Since unweighted UniFrac distances indicate the presence and absence of bacterial OTUs, without accounting for changes in their relative abundances, these data indicate that the last sample from Eriki likely contained similar microbial taxa as the control chimpanzees, but exhibited significant differences in their relative abundances. In contrast, the last sample from Skosha exhibited differences in some microbial taxa, but the overall composition of her fecal microbiome remained stable.

Absence of an expanded gut virome in SIVcpz infected chimpanzees

Previous studies of SIVmac infected macaques showed that clinical immunodeficiency can also be manifested by an expansion of the fecal virome [Handley et al., 2012]. To determine whether a similar expansion was occurring in SIVcpz infected chimpanzees, we searched our metagenomic sequences for reads that mapped to mammalian viruses. This analysis identified only a very small number of paired reads (*N*=29), which either mapped to chimpanzee stool associated circular virus (Chi-SCV) (*N*=22) or chimpanzee adenovirus (ChAdV) (*N*=7). Both Chi-SCV [Blinkova et al., 2010] and ChAdV [Cross 2013; Lonsdorf et al., 2014] infections have previously been documented in Gombe chimpanzees. Moreover, most of the virus-specific reads (93%) were recovered from fecal samples of SIVcpz infected chimpanzees (Fig. 6a), which is consistent with data from SIVmac infected macaques, which also had a greater abundance of simian adenoviruses in their shotgun sequenced fecal samples [Handley et al., 2012]. However, the paucity of mammalian virus reads in the metagenomic sequencing data came as a surprise. Although the reasons for this are not entirely clear, partial virus degradation likely played a role,

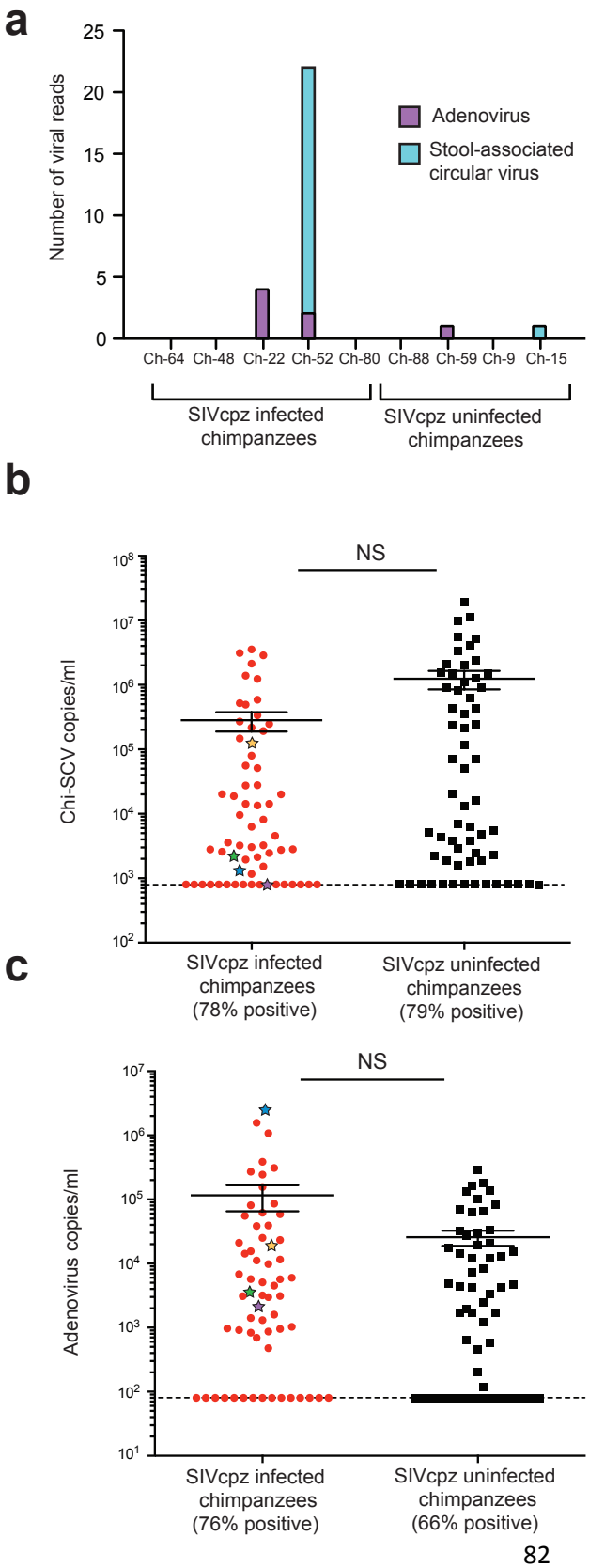

Fig. 6. Absence of an expanded virome in fecal samples from SIVcpz infected chimpanzees. (a) Detection of chimpanzee adenovirus (ChAdV) and chimpanzee stool associated circular virus (Chi-SCV) viral reads in fecal samples by metagenomic sequencing, with the number of paired-end viral reads (color coded) indicated for each individual. Virus infection was independently confirmed by nested PCR amplification and sequencing of ChAdV and Chi-SCV sequences from fecal DNA. (b, c) Chi-SCV (b) and ChAdV (c) viral loads were determined for all fecal samples listed in Table II using a virus-specific real time PCR and expressed as copies per ml of fecal sample (normalized by total sample DNA). The percentage of positive samples in each group is indicated, with the limit of detection denoted by a broken line. For each group, the median and standard error of the mean (SEM) are shown. Fecal samples from four chimpanzees that exhibited a compositional shift in their fecal bacteriome (Figs. 4 and 5) are highlighted (Eriki, blue; Mambo, purple; Malaika, green; Yolanda, orange). Statistical analyses were performed using the Mann-Whitney U test. NS, not significant.

since in contrast to the macaque study Gombe fecal samples were not flash frozen and were shipped (in RNA*later*) at room temperature. Moreover, most of the SIVmac infected macaques were sampled within days of death, while this was clearly not the case for the SIVcpz infected chimpanzees. Reads from plant viruses and bacteriophages were detected, but not further analyzed with respect to SIVcpz infection.

To quantify Chi-SCV and ChAdV infection rates and fecal viral loads in a larger number of SIVcpz infected and uninfected chimpanzees, we developed virus-specific nested and real-time PCR assays. Testing all fecal samples (*N*=123) listed in Table II, we found a high prevalence of Chi-SCV in both SIVcpz infected and uninfected chimpanzees, but no significant difference in fecal viral loads in either group (Fig. 6b). Although both ChAdV infection rates and fecal viral loads were slightly higher in SIVcpz infected than uninfected chimpanzees (Fig. 6c), these differences were not significant (*P*=0.07 and *P*=0.31 for Chi-SCV and ChAdV, respectively), including when values from the known and/or suspected immunodeficient chimpanzees were compared (colored in Fig. 6c). Thus, unlike in SIVmac infected macaques, we did not observe an expansion of the fecal virome in SIVcpz infected chimpanzees, including in the last samples from individuals who died of AIDS-related illness.

Section 2.6 – Discussion

Enteric dysbiosis is recognized as a characteristic feature of progressive HIV-1 infection [Lozupone et al., 2014; Nwosu et al., 2014], but the underlying mechanisms have been the subject of debate [Brenchley 2013; Nwosu et al., 2014]. In particular, the absence of an altered gut bacteriome in macaques with progressive SIVmac infection seemed to argue against lentiviral infection as the main driver of HIV-1 associated enteropathy [Handley et al., 2012; McKenna et al., 2008]. However, SIVmac infection is not an ideal model to assess primate lentivirus induced alterations of host-bacterial interactions since its disease course is greatly accelerated [Staprans et al., 1999]. Moreover, captive macaques differ in lifestyle and diet from naturally infected primates, and are prone to enteritis even in the absence of SIVmac infection [McKenna et al., 2008]. Reasoning that SIVcpz infection constitutes a physiologically more relevant "pathogenic SIV model", we examined the composition of the fecal microbiome in infected (*N*=24) and uninfected (*N*=26) chimpanzees in Gombe National Park. Using both metagenomic and 16S rRNA gene sequencing, we failed to identify significant differences in the abundance, alpha diversity and beta diversity of bacterial communities between SIVcpz infected and uninfected groups (Fig. 3). However, fecal samples from two chimpanzees, who died of an AIDS-like illness, exhibited significant compositional changes five months before their death (Fig. 4). Since earlier samples from these same individuals failed to exhibit the same compositional shifts, it seems likely that effective immune control prevented the destabilization of their gut bacteriome until shortly before their death. These findings are consistent with observational health data that failed to find an association between signs of illness and SIVcpz infection in these same communities [Lonsdorf et al., 2015b; Wolf et al., 2015a]. These findings are also consistent with the absence of gut bacteriome alterations in SIVgor infected western gorillas, the great majority of whom would not have been sampled shortly before their death [Moeller et al., 2015b]. The new data thus provide a plausible explanation for previous discrepant gut microbiome results. It now seems clear that SIVcpz infected chimpanzees are able to maintain a stable gut microbiome for many years, but seem to die relatively rapidly (within only a few months) after the compositional

divergence of their gut microbiome marks the loss of effective immune control. In contrast, HIV-1 infected humans seem to tolerate a more prolonged course of declining immune functions, most likely because of effective medical interventions, which independently contribute to alterations of gut bacterial communities [Jernberg et al., 2007; Klase et al., 2015]. Thus, both HIV-1 and SIVcpz infection are capable of causing a disruption of gut microbial homeostasis, but the timing and circumstances of this disruption and the associated health consequences differ between humans and apes.

Comparing 16S rRNA gene sequences, we noted that the gut bacterial communities from both infected and uninfected chimpanzees exhibited extensive compositional variability when compared over time (Figs. 2 and 3). While this may seem surprising, these results are consistent with a recent study that identified social interactions as the main driver of chimpanzee gut microbiome species richness and compositional fluctuation [Moeller et al., 2015a]. This study showed that the community memberships of individual chimpanzee gut microbiomes were more similar and more species-rich during seasons of high social interaction [Moeller et al., 2015a]. Thus, our failure to identify particular bacterial taxa and/or combinations of taxa to be associated with SIVcpz infection may not be that surprising. Although metagenomic sequencing identified an increased frequency of the bacterial family *Prevotellaceae* in a small number of number of SIVcpz infected chimpanzees, this association did not reach significance when 16S rRNA gene sequences from a larger sample set were compared. Similarly, we previously reported increased bacterial diversity after SIVcpz acquisition in six Gombe chimpanzees, all of whom were also included in the current study [Moeller et al., 2013b]. Again, samples from a small number of individuals allowed detection of microbiome compositional changes, while these same samples (except the very last ones collected before death) failed to show an increased compositional diversity when compared to a much larger number of controls (Figs. 3 and 4). Thus, the extensive compositional variability of the fecal bacteriome in the same individuals over time precludes the identification of subtle differences between infected and uninfected chimpanzees.

In addition to the gut bacteriome, we also investigated the gut virome of SIVcpz infected and uninfected chimpanzees. Metagenomic sequencing identified chimpanzee adenovirus (ChAdV) and chimpanzee stool-associated circular virus **(**Chi-SCV) in fecal samples from four Gombe chimpanzees (Fig. 6a). Suppression of the human immune system is frequently associated with an outgrowth of both pathogenic and non-pathogenic viruses [De Vlaminck et al., 2013; Handley et al., 2012; Li et al., 2013; Young et al., 2015]. For example, human torque teno (TT) virus, a small nonpathogenic DNA virus similar to Chi-SCV, is more prevalent in HIV-1 infected humans and replicates to higher titers in the blood of immunosuppressed individuals [Christensen et al., 2000; Shibayama et al., 2001]. Similarly, anelloviruses (a viral family that includes TT viruses) were more abundant in blood samples from immunosuppressed transplant patients, with an increase in viral titers determined by metagenomic sequencing [De Vlaminck et al., 2013; Young et al., 2015]. Finally, SIVmac infected macaques exhibited higher fecal viral titers of circoviruses, which are related to Chi-SCV, and simian adenoviruses [Handley et al., 2012]. In light of these data, the absence of significant differences in fecal Chi-SCV and ChAdV abundance in SIVcpz infected and uninfected chimpanzees was unexpected (Fig. 6b), especially when this was also the case for the last fecal samples of the four chimpanzees (Yolanda, Malaika, Mambo, Eriki) who exhibited increased bacterial diversity (Figs. 4 and 5). Although there was a trend towards increased titers of adenovirus in fecal samples from SIVcpz infected chimpanzees (Fig. 6c), this finding was nowhere close to the fecal virome expansion observed in SIVmac infected macaques, where mammalian viruses constituted up to 90% of fecal metagenomic reads [Handley et al., 2012]. Unlike the SIVcpz infected chimpanzees studied here, the SIVmac infected macaques were moribund at the time of sampling, with many requiring euthanasia before the completion of the study, which may have influenced the metagenomic results. Nonetheless, adenovirus infection of the intestine is associated with diarrhea, and may therefore contribute to the higher levels of diarrhea observed in SIVcpz infected female chimpanzees [Lonsdorf et al., 2015b].

Noninvasive studies of habituated chimpanzees have revealed much of the natural history of the precursor of the human AIDS virus. While the effects of SIVcpz infection on chimpanzee longevity and population dynamics can be readily monitored, disease progression is difficult to assess without frequent access to blood and tissue samples. The aim of this study was to explore whether characteristic gut microbiome changes could be found that might be suitable to noninvasively monitor disease progression. Although we failed to identify particular bacterial taxa and/or microbiome compositional changes that correlated with SIVcpz infection status, we found an increased beta diversity to mark the end-stage of SIVcpz infection in a small number of apes with necropsy confirmed immunodeficiency. The practical importance of this finding for the management of chimpanzee communities with endemic SIVcpz infection remains to be determined. Fecal microbiome analyses seem of little value in non-habituated communities where longitudinal sampling and observational studies of known individuals are not possible. In contrast, monitoring the fecal microbiome may prove useful in habituated communities, such as in Gombe, where a spike in beta diversity may foreshadow rapid disease progression and death. This could prompt intensified observational studies to more closely monitor signs of ill health, more focused sample collection to identify additional markers of disease progression, and enhanced surveillance to increase the likelihood of recovering tissues for necropsy. Monitoring disease progression in habituated chimpanzees could also be used to identify individuals that might benefit from therapy, provided that the intended intervention is truly necessary, practical in wild settings, and known to be efficacious in chimpanzees [Barbian et al., 2015]. As ape populations continue to dwindle in the wild, such an approach may become increasingly important to ensure their survival [Ryan & Walsh 2011; Walsh et al., 2003; Warfield et al., 2014].

Section 2.7 – Chapter Acknowledgements

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CHAPTER 3

NEUTRALIZATION PROPERTIES OF SIMIAN IMMUNODEFICIENCY VIRUSES INFECTING CHIMPANZEES AND GORILLAS

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The experiments presented in this chapter were designed, executed, and analyzed by myself under the supervision of BHH. JMD, FBR, NFP, SSI, TND provided technical assistance. RPG, APW, CPP, RS, YH, HM, LM, PA, BZ, PDK, RM, GS, MC, DRB, MCN, PJB, DDH, and MF provided monoclonal antibodies and some unpublished data utilizing these reagents. GMS and DRB provided critical reviews of the manuscript. I wrote the manuscript with assistance from BHH.

Section 3.1 – Abstract

Broadly cross-reactive neutralizing antibodies (bNabs) represent powerful new tools to combat human immunodeficiency virus type 1 (HIV-1) infection. Here, we examined whether HIV-1 specific bNabs are capable of cross-neutralizing distantly related simian immunodeficiency viruses infecting chimpanzees (SIVcpz) and gorillas (SIVgor). We found that bNabs directed against the CD4 binding site (n=10), peptidoglycans in variable loops 1 and 2 (V1/V2) (n=3) and at the base of variable loop 3 (V3) (n=5), and epitopes at the interface of surface (gp120) and membrane-bound (gp41) envelope glycoproteins (n=5) failed to neutralize the great majority of SIVcpz (n=11) and SIVgor (n=1) strains. Similarly, llama-derived (heavy chain-only) antibodies (n=6) directed against gp120 and gp41 epitopes were either completely inactive or neutralized only a small fraction (<40%) of SIVcpz strains. In contrast, one antibody targeting the membrane proximal external region (MPER) of gp41 (10E8), antibody-like constructs that function as CD4 and CCR5 receptor mimetics (eCD4-Ig, eCD4-Ig^{mim2}, CD4-218.3-E51, CD4-218.3-E51-mim2), as well as mono- and bi-specific anti-human CD4 (iMab, LM52) and CCR5 (PRO140, PRO140-10E8) receptor antibodies neutralized >90% of SIVcpz and SIVgor strains with low nanomolar (0.13 – 8.4 nM) potency. Importantly, the latter antibodies blocked virus entry not only in TZM-bl cells, but also in Cf2Th cells expressing chimpanzee CD4 and CCR5, and neutralized SIVcpz in chimpanzee CD4+ T-cells with IC_{50} values ranging from 3.6 to 40.5 nM. These findings provide new insight into the protective capacity of anti-HIV-1 bNabs and identify candidates for further development to combat SIVcpz infection.

Section 3.2 – Introduction

Simian immunodeficiency virus (SIVcpz) of chimpanzees (*Pan troglodytes*) is the precursor of human immunodeficiency virus type 1 (HIV-1), the causative agent of AIDS (1). Like HIV-1 in humans, SIVcpz is pathogenic in chimpanzees and can cause substantial morbidity and mortality in its natural host (2, 3). Long-term observational health studies in Gombe National Park, Tanzania, revealed that SIVcpz infected chimpanzees have a 10- to 16-fold increased risk of death compared to uninfected chimpanzees, and that infected females are less likely to give birth and have a much higher infant mortality rate than uninfected females (2). Necropsy studies showed that SIVcpz infected chimpanzees can develop severe $CD4^+$ T lymphocyte depletion and a histopathology consistent with end-stage AIDS (2, 4). Most importantly, the Gombe community with the highest SIVcpz prevalence rate suffered a catastrophic population decline (3). Thus, SIVcpz infection has a substantial negative impact on the health, reproduction and life span of wild-living chimpanzees.

Chimpanzees acquired SIVcpz by cross-species transmission and recombination of SIVs infecting monkeys on which they prey (5). Although only the central (*P. t. troglodytes*) and eastern (*P. t. schweinfurthii*) subspecies are naturally infected, SIVcpz is widespread throughout their ranges in west-central and eastern Africa, with prevalence rates reaching 30% to 50% at certain field sites (6-11). Transmission of SIVcpz occurs by sexual routes and from infected mothers to their infants (2, 3). In addition, virus spreads efficiently between communities through the migration of infected females (3). Thus, SIVcpz has the potential to disperse over long distances and penetrate uninfected populations (7, 10, 12). SIVcpz is also the source of SIVgor, which emerged in western lowland gorillas (*Gorilla gorilla gorilla*) following a single cross-species transmission of a SIVcpz strain from sympatric chimpanzees (13). Although less prevalent than SIVcpz, SIVgor has been identified at several locations throughout southern Cameroon, with some gorilla troops exhibiting high infection rates (14-16). Given its recent emergence from

SIVcpz, SIVgor may share some of the same pathogenic properties. Thus, SIVcpz, and possibly also SIVgor, contribute to the infectious disease burden of wild apes, which are already highly endangered due to extensive habitat destruction and relentless poaching (7, 10, 16-19).

Historically, chimpanzees have been used as an animal model to test new therapies and vaccines for humans, including AIDS vaccines (20-23), a practice that is no longer considered ethical (24). However, the opposite has not been explored, i.e., whether treatment and prevention strategies developed for HIV-1 infected humans could benefit SIVcpz infected chimpanzees. As chimpanzee and gorilla populations are dwindling in the wild, primatologists and conservation groups have become interested in exploring novel avenues to curb the spread of ape pathogens (19, 25). In this context, broad and potent neutralizing antibodies (bNabs) may be of utility (26-29). Although immunogens capable of eliciting such antibodies do not yet exist, several studies have shown that bNabs can prevent the acquisition and/or suppress the replication of HIV-1 and simian-human immunodeficiency viruses (SHIVs) in humanized mice and rhesus macaques, respectively (30-37). For example, antibody-mediated immunotherapy was effective in reducing systemic viral loads and improving immune responses in chronically SHIV infected rhesus macaques (36, 37). Administration of bNabs as purified proteins or transgenes also prevented virus infection in animal models (30-34, 38). Since antibody infusions in wild settings are not feasible, delivery through recombinant vectors, such as adeno-associated virus (AAV), may represent an alternative. AAV has an outstanding safety record in humans as well as other animal species (39-42). Moreover, a single administration by dart or equivalent has the potential to induce long-lasting antibody expression (30, 31, 38, 43). Recombinant (r)AAV vectors could also be used to deliver cocktails of potent neutralizing antibodies, which may be able to reduce systemic viral loads.

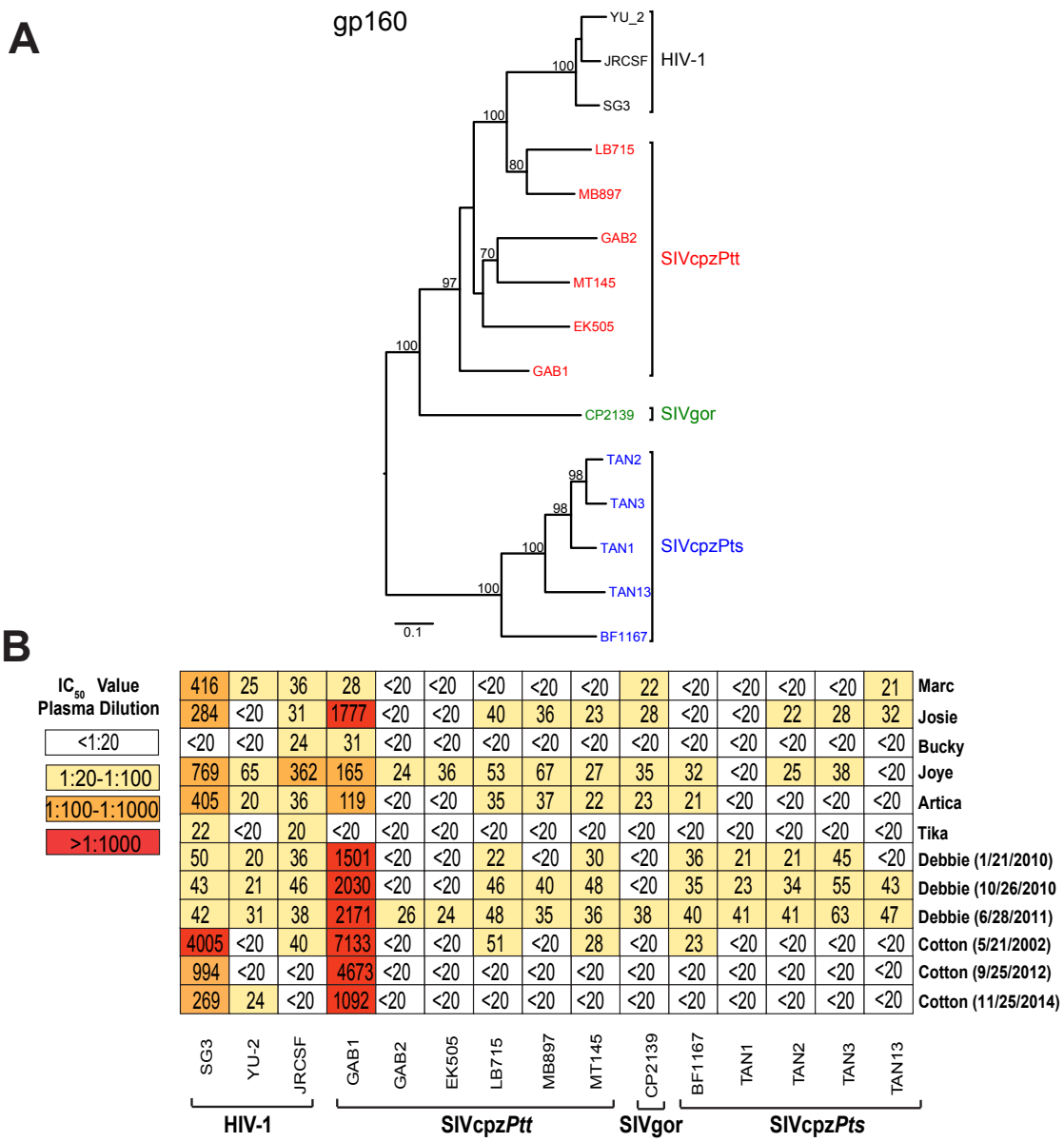
First generation bNabs failed to control HIV-1 replication when administered to infected patients, suggesting that they were of little or no clinical value (44, 45). However, recent advances in HIV-1 specific B cell isolation and antibody cloning techniques have led to the

discovery of a large number of additional bNabs (46), many of which neutralize HIV-1 with much improved breadth and potency (26-29, 47-49). All bNabs target structurally conserved regions on the trimeric HIV-1 envelope (Env) spike, including (i) the CD4 binding site (CD4bs), (ii) peptidoglycans in variable region 1 and 2 (V1V2) and at the base of variable region 3 (V3), (iii) the membrane-proximal external region (MPER), and (iv) glycan associated epitopes at the interface of exterior (gp120) and membrane bound (gp41) Env glycoproteins (50-55). In addition, antibodies directed against the host receptors CD4 and CCR5 (56-59) as well as immunoadhesins containing domain 1 and 2 (D1D2) of CD4 (60-62) have been shown to have substantial anti-HIV-1 activity. While these reagents inhibit a large number of globally circulating HIV-1 strains (63), their capacity to neutralize related lentiviruses from chimpanzees and gorillas has not been examined. Here, we used a panel of SIVcpz and SIVgor infectious molecular clones (IMCs) to show that some, but not all, anti-HIV-1 bNabs and immunoadhesins are capable of neutralizing these viruses. Since the Env proteins of SIVcpz and SIVgor strains are nearly twice as divergent as those of the various HIV-1 group M subtypes, these data yield new insight into the breadth and protective efficacy of anti-HIV-1 antibodies.

Section 3.3 – Results

Plasma samples of long-term HIV-1 and SIVcpz infected chimpanzees lack heterologous neutralization breadth. Almost 50% of HIV-1 positive humans develop some degree of heterologous neutralization breadth within two to four years post infection (64), indicating that the human immune system is capable of targeting conserved epitopes in the HIV-1 envelope (Env) glycoprotein. To determine whether this is also true for chronically infected apes, we tested plasma samples from eight chimpanzees that had been inoculated with HIV-1 and/or SIVcpz 17 to 30 years earlier as part of vaccine and/or pathogenesis studies (Table 1). Although three of these individuals had undetectable plasma virus at the time of sampling, all had high titer antibodies that reacted with HIV-1 Gag, Pol and Env proteins on Western immunoblots, indicating that they were once productively infected (not shown). The remaining five chimpanzees were viremic, with plasma viral loads ranging from 337 to >1,000,000 RNA copies/ml (Table 1). Among the animals with the highest viral loads, Tika (>200,000 RNA copies/ml) was infected with HIV-1/NC, a pathogenic chimpanzee-adapted strain of HIV-1 (21, 23, 65). In contrast, Debbie (>20,000 RNA copies/ml) and Cotton (>1,000,000 RNA copies/ml) were infected with SIVcpzANT (20), an SIVcpz*Pts* strain originally isolated from a wild-caught chimpanzee from the Democratic Republic of the Congo (66). Although Cotton was also exposed to HIV-1/LAV (Table 1), RT-PCR analysis identified SIVcpzANT as the only replicating virus in his plasma. Thus, the latter two animals represent rare examples of captive chimpanzees with chronic SIVcpz infection.

To screen available plasma samples for neutralization breadth, we generated a panel of infectious molecular clones (IMCs) of SIVcpz and SIVgor strains by amplifying viral consensus sequences from fecal samples of wild apes (Fig. 1A). Members of both the SIVcpz*Ptt* and the SIVcpz*Pts* lineage were included, which differed in up to 48% of their Env protein sequence (three previously reported strains of HIV-1 were used for control). All IMCs, except for the T cell line adapted, CXCR4-tropic HIV-1/SG3 strain, used CCR5 as the co-receptor and replicated



	SG3	YU-2	JRCSF	GAB1	GAB2	EK505	LB715	MB897	MT145	CP2139	BF1167	TAN1	TAN2	TAN3	TAN13
Marc	416	25	36	28	<20	<20	<20	<20	<20	22	<20	<20	<20	<20	21
Josie	284	<20	31	1777	<20	<20	40	36	23	28	<20	<20	22	28	32
Bucky	<20	<20	24	31	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20
Joye	769	65	362	165	24	36	53	67	27	35	32	<20	25	38	<20
Artica	405	20	36	119	<20	<20	35	37	22	23	21	<20	<20	<20	<20
Tika	22	<20	20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20
Debbie (1/21/2010)	50	20	36	1501	<20	<20	22	<20	30	<20	36	21	21	45	<20
Debbie (10/26/2010	43	21	46	2030	<20	<20	46	40	48	<20	35	23	34	55	43
Debbie (6/28/2011)	42	31	38	2171	26	24	48	35	36	38	40	41	41	63	47
Cotton (5/21/2002)	4005	<20	40	7133	<20	<20	51	<20	28	<20	23	<20	<20	<20	<20
Cotton (9/25/2012)	994	<20	<20	4673	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20
Cotton (11/25/2014)	269	24	<20	1092	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20

FIG 1. Neutralizing antibody responses in long-term HIV-1 and SIVcpz infected chimpanzees. (A) Phylogenetic relationship of HIV-1, SIVcpz and SIVgor strains used for infectious molecular clone (IMC) construction. A maximum-likelihood phylogenetic tree of Env (gp160) protein sequences is depicted, with sequences color-coded to differentiate HIV-1 (black), SIVgor (green), SIVcpz*Ptt* (red) and SIVcpz*Pts* (blue) strains. Bootstrap values ≥ 70% are shown; the scale bar represents 0.1 amino acid replacements per site. (B) Plasma samples from eight long-term chimpanzees (listed on the right; also see Table 1)) were tested against HIV-1 (n=3), SIVcpz*Ptt* (n=6), SIVgor (n=1), and SIVcpz*Pts* (n=5) strains (bottom) in the TZM-bl neutralization assay (collection date are indicated for samples from the same individual). IC_{50} values (expressed as plasma dilutions) averaged from three replicate experiments are shown, with a heatmap indicating the relative neutralization potency.

TABLE 1 Chimpanzee Clinical History

Ape	Code	Date of Birth (mo/dy/yr)	Virus Strain	Year Infected	Duration of Infection	Plasma (mo/dy/yr)	CD4+ T Cell Count (cells/ul)[a]	Viral Load (RNA copies/ml)	SIVcpz ANT Infection	Virus Specific Antibodies	Refs
Marc	C487	9/06/1981	HIV-1/LAV	1984	30 yrs	11/08/2011	1154	<50	-	pos[c]	21
Artica	C544	2/13/1979	HIV-1/LAV	1986	28 yrs	11/10/2011	1153	4,794	-	pos	23
Joye	C542	2/13/1979	HIV-1/LAV	1986	28 yrs	11/10/2011	2529	337	-	pos	23
Tika	C534	10/03/1978	HIV-1/NC	1997	17 yrs	11/10/2011	4	244,324	-	pos	22
Debbie	X0284	9/23/1985	SIVcpzANT	1996	18 yrs	1/21/2010 10/26/2010 6/28/2011	 nd	nd[d] 26,397 nd	nd pos nd	nd pos nd	20
Cotton	X0115	4/10/1977	HIV-1/IIIB SIVcpzANT	n/a[e] 1996	>18 yrs	5/21/2002 9/25/2012 11/25/2014	 229 220[b]	77,142 1,440,622 861,000	nd pos nd	nd pos nd	20
Bucky	n/a	n/a	n/a	n/a	n/a	11/10/2011	1016	<50	-	pos	n/a
Josie	n/a	5/18/1981	n/a	n/a	n/a	11/10/2011	516	<50	-	pos	n/a

[a]CD4+ T cell counts were performed using blood collected in September 2010 or [b]November 2014
[c]pos, fulfilling criteria for positive Western blot (virus specific antibodies) or SIVcpzANT specific amplification and sequence confirmation (SIVcpzANT infection)
[d]nd, not done
[e]n/a, not available

efficiently in primary human and chimpanzee CD4+ T cells (6, 7, 11, 15, 67-69). Testing available plasma samples in the TZM-bl assay, we found that seven of eight chimpanzees, including the two SIVcpzANT infected individuals, had activity against the easy-to-neutralize (tier 1) HIV-1/SG3 strain (Fig. 1B). All chimpanzee plasmas, except for one (Tika), also neutralized SIVcpzGAB1, with IC_{50} titers exceeding 1:1,000 in three animals. Since SIVcpzGAB1 was cloned from a viral isolate that was extensively propagated in human PBMCs (67), it likely also represents an easy-to-neutralize (tier 1) chimpanzee virus. In contrast, little cross-reactivity was observed against the remaining primary (tier 2) HIV-1 and SIVcpz strains, with most plasma samples containing very low level (<1:50) or no neutralizing activity (Fig. 1B). Longitudinal plasma samples were available for two chimpanzees, one of whom (Cotton) showed no neutralization breadth after over 12 years of infection. The second animal (Debbie) developed antibodies that neutralized all SVcpz strains, but with very low titers (<1:70). Thus, despite the long duration of their infection (Table 1), none of the chronically infected chimpanzees, including the two SIVcpzANT infected animals, developed appreciable neutralization potency against heterologous HIV-1, SIVcpz and SIVgor strains (Table 1).

Anti-HIV-1 CD4 binding site bNabs fail to neutralize SIVcpz and SIVgor strains. Since all primate lentiviruses identified to date use the human CD4 receptor to gain entry into target cells (7, 15, 69, 70) and since the CD4 molecules from humans, chimpanzees and gorillas are closely related (71), we asked whether CD4 binding site (CD4bs) antibodies from HIV-1 infected humans could cross-neutralize SIVcpz and SIVgor strains. Testing VRC01 (28), VCR03 (28), VRC-PG04 (48), VRC-CH30 (48), VRC-CH31 (48), F105 (72), b13 (73), 45-46^{G54W} (74), 45-46m2 (75) and 45-46m7 (75) in the TZM-bl assay, we found that most of these antibodies neutralized the three HIV-1 Env controls potently, with IC_{50} values ranging from of 0.004 to 1.2 μg/ml (a monoclonal antibody directed against herpes simplex virus glycoprotein D served as a negative control). The two exceptions were F105 and b13, which are known to have only limited neutralization breadth and potency (76). In contrast, none of these CD4bs antibodies neutralized

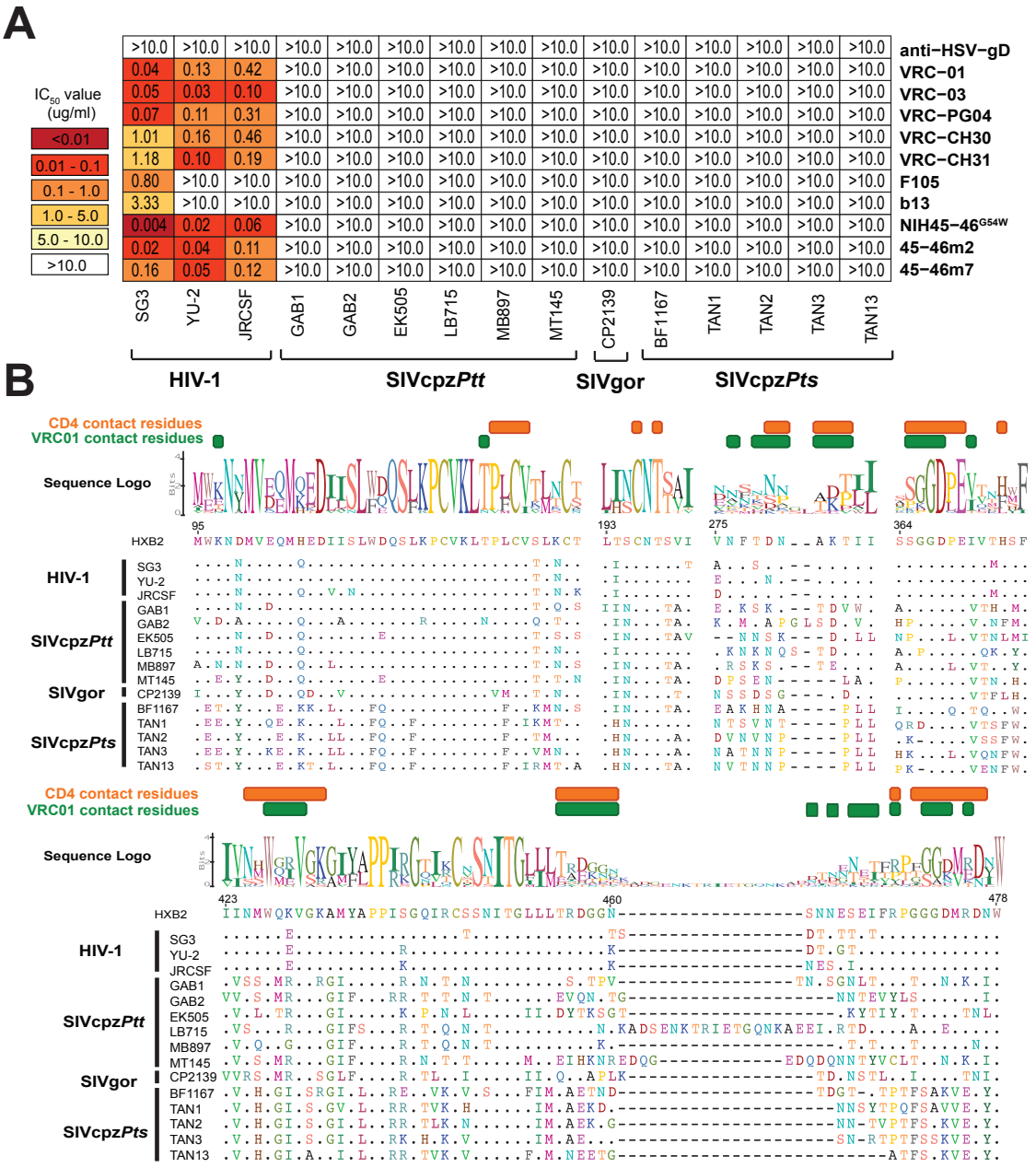


FIG 2. Neutralizing capacity of CD4 binding site antibodies. (A) The ability of CD4 binding site monoclonal antibodies (listed on the right) to neutralize HIV-1, SIVcpz and SIVgor strains (listed on the bottom) is shown. Numbers indicate IC_{50} values (μg/ml) in TZM-bl cells, averaged from three different experiments, with a heatmap indicating the relative neutralization potency. The highest antibody concentration was 10 μg/ml. A herpes virus antibody (anti-HSV-gD) was used as a negative control. (B) Conservation of HIV-1, SIVcpz and SIVgor strains in the CD4 binding region. An alignment of Env protein sequences (left) in regions surrounding the CD4 binding site is shown. CD4 and VRC01 contact resides (indicated in the HXB2 reference strain) are highlighted in orange and green, respectively. A logo plot above the alignment denotes the conservation of each amino acid, with the height of each letter indicating the proportion of the sequences that contain the residue at that site. Dots indicate identity to the HXB2 reference sequence and dashes represent gaps introduced to improve the alignment.

any SIVcpz or SIVgor strain at concentrations of up to 10 μg/ml (Fig. 2A). This was the case despite the fact that the panel included some of the most potent CD4bs bNabs, such as 45-46^{G54W}, which is known to neutralize highly diverse HIV-1 strains with IC_{50} values of <0.05 μg/ml (74).

To examine the reasons for this resistance, we compared viral Env sequences for conservation of previously identified CD4 and VRC01 contact residues (77, 78). Not surprisingly, most amino acid residues required for CD4 binding were relatively well conserved, but this was not the case for many VRC01 contact residues (Fig. 2B). For example, residues 461-467 in variable loop 5 (V5), which are known to be critical for VRC01 binding (77), were present in all HIV-1 strains, but exhibited considerable length variations in SIVcpz and SIVgor strains (Fig. 2B). Although the CD4 binding site of SIVcpz and SIVgor strains must be conserved to maintain function, amino acid diversity in neighboring Env regions were likely causing clashes with anti-HIV-1 CD4bs antibodies.

Glycan-dependent variable loop bNabs lack neutralization breadth against SIVcpz and SIVgor. In addition to the CD4 binding site, HIV-1 envelope glycoproteins contain conserved peptidoglycans in variable loops V1 and V2, and at the base of V3, which represent targets for broadly cross-reactive neutralizing antibodies (50, 51). To examine the ability of glycan dependent anti-HIV-1 bNabs to neutralize SIVcpz and SIVgor strains, we tested V1V2-directed antibodies PG9 (26), PG16 (26) and PGT145 (27) as well as the V3 directed antibodies 10-1074 (49), PGT121 (27), PGT128 (27), PGT135 (27) and 2G12 (79) in the TZM-bl assay. Like the CD4bs bNabs, the glycan V3-associated bNabs failed to cross-neutralize SIVcpz and SIVgor strains at concentrations of 10 μg/ml, except for PGT128, which neutralized a single SIVcpz*Ptt* (EK505) virus with an IC_{50} of 0.02 μg/ml (0.13 nM) (Fig. 3A). The V1V2-directed quaternary bNabs PG9 (26), PG16 (26) and PGT145 (27) were slightly more cross-reactive and potently neutralize four of six SIVcpz*Ptt* strains (GAB1, EK505, MB897, MT145) (Fig. 3A). However,

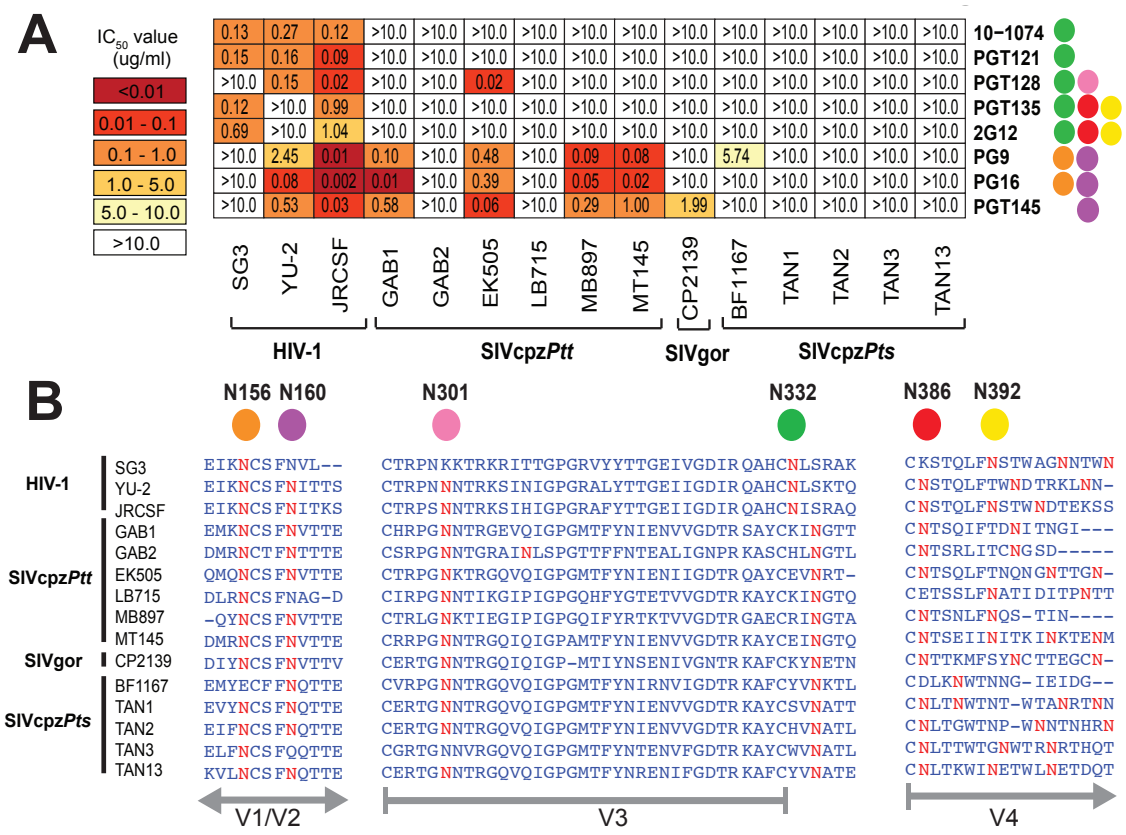


FIG 3. Neutralizing capacity of glycan associated neutralizing antibodies. (A) The ability of peptidoglycan-associated monoclonal antibodies (listed on the right) to neutralize HIV-1, SIVcpz and SIVgor strains (bottom) is shown. Numbers indicate IC_{50} values (μg/ml) in TZM-bl cells, averaged from three different experiments, with a heatmap indicating the relative neutralization potency. Colored circles to the right of each antibody indicate the N-linked glycans that are important for their neutralizing activity (orange - N156, purple - N160, pink - N301, green - N332, red - N386, yellow - N392) (26, 27, 49, 80). The highest antibody concentration used was 10 μg/ml. (B) Conservation of glycans associated with antibody neutralizing activity. An alignment of HIV-1, SIVcpz and SIVgor Env protein sequences is shown, with predicted N-linked glycans (NXS/T) highlighted in red. N-linked glycans known to influence bNab binding are highlighted above the alignment, with HXB2 numbering in black. The position of variable loops (V1/V2, V3 and V4) is shown in grey below the alignment.

these same antibodies lacked activity against the more divergent SIVgor and SIVcpz*Pts* strains. V1/V2 quaternary and glycan V3 bNabs neutralize by engaging the glycan shield and removal of key glycans can abrogate this neutralization (27, 80). For example, 10-1074, PGT121, PGT128, PGT135, and 2G12 all bind a high mannose patch at the base of V3 that is centered around the conserved N332 glycan, while PG9, PG16, and PGT145 contact the N156 and N160 glycans and insert between them to interact with glycan-obscured protein regions (26, 27, 49, 80). Examining SIVcpz and SIVgor Env sequences for NXS/T sequons, we found varying degrees of conservation of these and other relevant N-linked glycosylation sites (Fig. 3B). For example, N332 was conserved in all three HIV-1 Envs, but absent from all SIVcpz and SIVgor Envs, potentially explaining the lack of cross-neutralization of 10-1074, PGT121, PGT128, PGT135 and 2G12. However, SIVcpzEK505 was potently neutralized by PGT128 (Fig. 3A) despite the absence of the N332 glycan (EK505 is also the only strain with a deletion at position 337). Moreover, all SIVcpz and SIVgor strains encode an NXS/T sequon at position 334, which could potentially compensate for the absence of N332 (81). Thus, the lack of N332 is unlikely to be the sole reason for the inability of glycan V3-directed bNabs to cross-neutralize SIVcpz and SIVgor. Similarly, 10 of 12 SIVcpz and SIVgor Envs contained an N-linked glycosylation site at position 160, which is required for neutralization by PG9, PG16 and PGT145 (26, 27). However, these antibodies neutralized only four of the 10 SIVcpz strains, again implicating lack of conservation in other Env regions as a reason for the absence of neutralization breadth.

HIV-1 bNabs directed against the interface of gp120 and gp41 fail to cross-neutralize SIVcpz and SIVgor. The most recently discovered class of anti-HIV-1 bNabs targets glycan associated epitopes at the interface of gp120 and gp41 (52-55). Testing several representatives of this bNab group, including 8ANC195 (53), 35022 (52), PGT151 (55), PGT152 (55) and PGT158 (55), we found that none had cross-neutralization potential (Fig. 4A). With the exception of 35O22, which neutralized a single SIVcpz strain (LB715) at an IC_{50} of 0.5 μg/ml (3.3 nM), all other antibodies failed to block SIVcpz and SIVgor infection at concentrations of up to 10

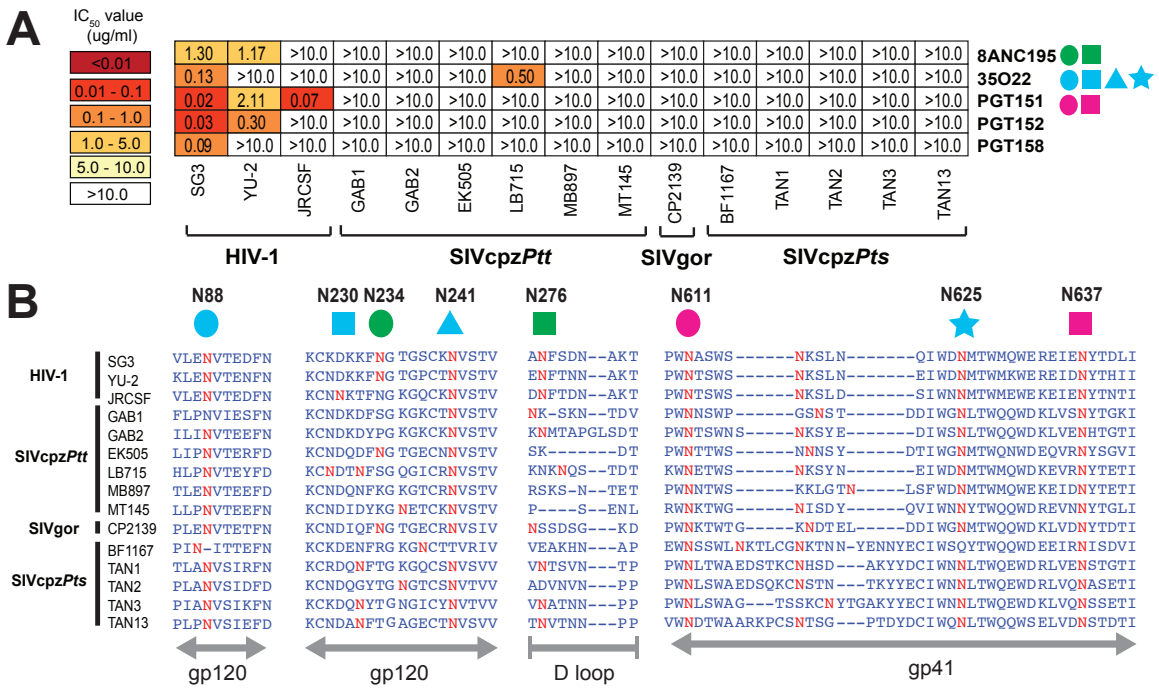


FIG 4. Neutralizing capacity of antibodies targeting the interface of HIV-1 gp120 and gp41 regions. (A) The ability of glycan-associated antibodies (right) to neutralize HIV-1, SIVcpz and SIVgor strains (bottom) is shown. Numbers indicate IC_{50} values (μg/ml) from TZM-bl cells, averaged from three different experiments, with a heatmap indicating the relative neutralization potency. Colored shapes to the right of each antibody indicate the N-linked glycans that are associated with antibody neutralizing activity. Antibody 8ANC195 contacts N234 (green circle) and N276 (green square); 35O22 utilizes N88 (blue circle), N230 (blue square), N241 (blue triangle), and N625 (blue star); and PGT151 requires N611 (pink circle) and N637 (pink square) for optimal neutralization (52-55). The highest antibody concentration used was 10 μg/ml. (B) Conservation of glycans associated with antibody neutralizing activity. An alignment of HIV-1, SIVcpz and SIVgor Env protein sequences is shown, with predicted N-linked glycans (NXS/T) highlighted in red. N-linked glycans known to influence bNab binding are highlighted above the alignment, with HXB2 numbering in black. The position of various Env regions is shown in grey below the alignment.

μg/ml (Fig. 4A). Since these bNabs are also dependent on the presence of certain glycans, we examined the extent of sequence conservation in the corresponding Env regions (Fig. 4B). For example, 8ANC195 requires N-linked glycosylation sites at positions 234 and 276 (53), both of which are highly variable in SIVcpz and SIVgor strains (Fig. 4B). Similarly, 35O22 utilizes N-linked glycosylation sites at positions 88, 230, 241 and 625 (Fig. 4B), one of which (N230) is absent from all SIVcpz and SIVgor strains. Thus, the absence of key N-linked glycosylation sites may be one reason for the lack of cross-neutralizing capacity of this class of bNabs. However, variation in other Env regions must also be involved, since the glycosylation site contacts for PGT151 (N611 and N637) were conserved in all viruses, yet this monoclonal also failed to cross-neutralize.

Camelid antibodies have limited cross-neutralization breadth. Members of the *Camelidae* family produce antibodies that lack light chains and thus comprise much smaller (heavy-chain only) antibodies with long complementarity-determining regions. It has thus been proposed that these single-chain antibodies may be better equipped to bind to small occluded sites on the HIV/SIV Env trimer than full-sized antibodies (82). Indeed, several camelid single-domain antibodies have been shown to potently neutralize genetically divergent strains of HIV-1 (75, 83, 84). To examine whether this cross-reactivity extends to SIVcpz and SIVgor, we tested a panel of llama-derived antibodies, including single domain JM4 (85), J3 (75), 3e3 (86), 2E7 (83) and 11F1F (86) as well as the bivalent Bi-2H10, which contains two molecules of 2H10 joined by a glycine-serine linker (87). Of these, JM4 recognizes a region in gp120 that overlaps both the CD4 and CCR5 binding sites, J3 and 3E3 target the CD4 binding site, 2E7 and 11F1F recognize epitopes in the ectodomain of gp41 (75, 83, 85, 86), and 2H10 targets the membrane proximal external region (MPER) (87). Interestingly, J3 and 3E3 were able to neutralize a limited number of SIVcpz*Ptt* strains at IC_{50} values <5 μg/ml (<13 nM) (Fig. 5A), thus exhibiting greater activity than conventional CD4bs antibodies, which were completely inactive (Fig. 2A). This was also true for the anti-gp41 antibodies 2E7 and 11F1F, which neutralized four SIVcpz*Ptt* and one SIVcpz*Pts*

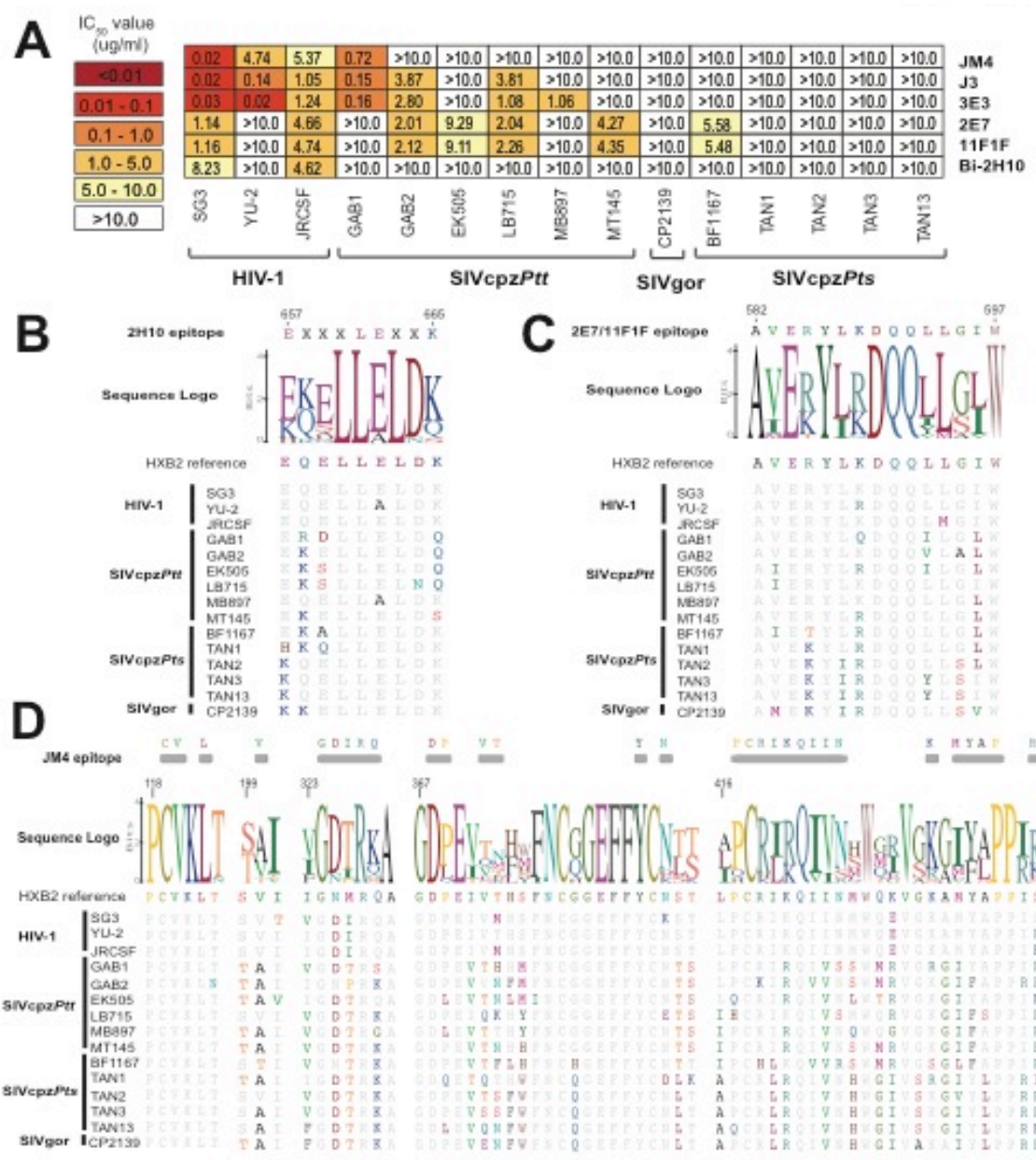


FIG 5. Neutralizing capacity of camelid antibodies. (A) The ability of llama-derived (heavy chain-only) antibodies (listed on the right) to neutralize HIV-1, SIVcpz and SIVgor strains (bottom) is shown. Numbers indicate IC_{50} values (mg/ml) from TZM-bl cells, averaged from three different experiments, with a heatmap indicating the relative neutralization potency. The highest antibody concentration used was 10 mg/ml. (B-D) Conservation of antibody binding epitopes shown in (A). Alignments of HIV-1, SIVcpz and SIVgor Env protein sequences are depicted, with residues identical to the HXB2 reference shown in grey. Logo plots denote the conservation of individual amino acids within each epitope, with the height of each letter indicating the proportion of sequences that contain the residue at that site. An X indicates residues within the 2H10 epitope that do not impact neutralization. JM4 contact residues are indicated on top of the alignment. Sequences are numbered according to the HXB2 reference.

strain at IC_{50} values <10 μg/ml (<40 nM). However, this breadth did not extend to the more divergent SIVcpz*Pts* and SIVgor viruses, and the bivalent MPER antibody Bi-2H10 was unable to neutralize any SIVcpz and SIVgor strains (Fig. 5A).

Since the epitopes of 2H10, 2E7, 11F1F and JM4 have been mapped, we examined the corresponding amino acid sequences in SIVcpz and SIVgor strains (Fig. 5B-D). This analysis showed that none was particularly highly conserved among the ape viruses. All SIVcpz and SIVgor strains, except BF1167, differed by one or more substitutions in the 2H10 epitope (Fig. 5B). This was also true for the partially overlapping 2E7 and 11F1F epitopes, where all SIVcpz and SIVgor sequences differed from the HIV-1 consensus (Fig. 5C). Finally, there was considerable variation in the JM4 epitope (Fig. 5D), with contact residues I326, V372 and M434 conserved in HIV-1, but mutated in most (V372) or all (I326, M434) SIVcpz and SIVgor strains. Thus, epitope variation may explain at least some of the neutralization resistance of SIVcpz and SIVgor strains. However, in contrast to conventional CD4bs and glycan dependent antibodies, llama-derived antibodies neutralized a subset of SIVcpz*Ptt* strains, possibly because of their smaller size.

HIV-1 MPER bNabs neutralize SIVcpz and SIVgor strains. Antibodies targeting the MPER region of gp41 represent still another class of potent anti-HIV-1 bNabs (29, 47). Among those, 4E10 and 10E8 exhibit the greatest breadth, having been shown to neutralize 98% of a large panel (n=181) of HIV-1 pseudoviruses (29, 47). To examine their ability to cross-neutralize ape viruses, we tested 4E10 and 10E8 against our panel of SIVcpz and SIVgor strains. In contrast to all other anti-HIV-1 bNabs, 4E10 neutralized 9 of 12 SIVcpz and SIVgor strains with a mean IC_{50} of 2.7 μg/ml (18.1 nM), while 10E8 neutralized all ape viruses with a mean IC_{50} of 0.7 μg/ml (4.4 nM) (Fig. 6A). An amino acid alignment of the corresponding epitopes revealed considerable sequence conservation (Fig. 6B), likely explaining the remarkable breadth and potency of 10E8 compared to all other anti-HIV-1 bNabs.

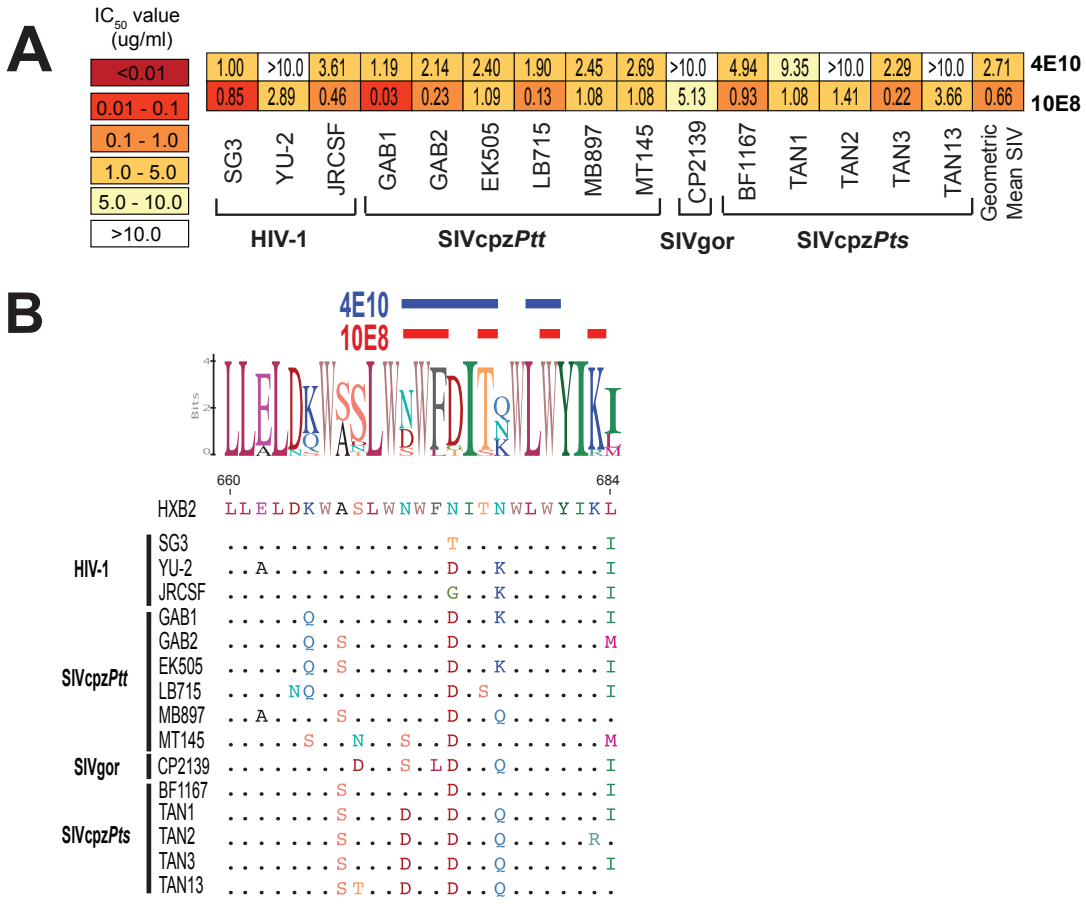


FIG 6. Neutralizing capacity of MPER antibodies (A) The ability of two MPER antibodies (listed on the right) to neutralize a panel of HIV-1, SIVcpz and SIVgor strains (bottom) is shown. Numbers indicate IC_{50} values (μg/ml) from TZM-bl cells, averaged from three different experiments, with a heatmap indicating the relative neutralization potency. The highest antibody concentration used was 10 μg/ml. (B) Conservation of 4E10 and 10E8 epitopes. An alignment of HIV-1, SIVcpz and SIVgor Env protein sequences is depicted, with dots indicating identity to the HXB2 reference sequence. A logo plot denotes the conservation of individual amino acids, with the height of each letter indicating the proportion of sequences that contain the residue at that site. Contact residues of 4E10 (blue) and 10E8 (red) are highlighted above the alignment.

Anti CD4 and CCR5 receptor antibodies potently neutralize SIVcpz and SIVgor strains. Since CD4 and CCR5 protein sequences are highly conserved between humans and apes (71, 88), we asked whether antibodies raised against the human receptors could block SIVcpz and SIVgor infection. Both anti-CD4 and anti-CCR5 antibodies were examined, including ibalizumab (iMab) and PRO140, which have previously been shown to be safe and efficacious in human clinical trials (89-91). Using TZM-bl cells, which express human CD4 and CCR5, we found that both the anti-CD4 antibody iMab (90, 92, 93), and its improved version LM52 (58), neutralized most or all ape viruses with geometric mean IC_{50} values of 0.41 μg/ml (2.8 nM) and 0.12 μg/ml (0.8 nM), respectively (Fig. 7A). PRO140, which targets the CCR5 co-receptor (56), also neutralized all SIVcpz and SIVgor strains with an IC_{50} of 0.34 μg/ml (2.3 nM) (Fig. 7A). Finally, bi-specific versions of iMab, LM52 and PRO140, in which these antibodies were linked to single-chain variable fragments (scFv) of PG9, PG16, PGT128 and 10E8 (59), were potent inhibitors, especially when the fusion partner was also an effective SIVcpz and SIVgor neutralizer. For example, the bispecific iMab-PG9 and iMab-PG16 neutralized the PG9/PG16-sensitive GAB1 and EK505 strains with two orders of magnitude greater potency than iMab alone, but this enhancement was not observed for viruses that were resistant to PG9 and PG16 (Figs. 3A and 7A). By far the most potent anti-receptor antibody was PRO140-10E8, which neutralized SIVcpz and SIVgor strains with a mean IC_{50} value of 0.03 μg/ml (0.13 nM) (Fig. 7A).

To determine whether the anti-human receptor antibodies would block virus entry in cells expressing chimpanzee CD4 and CCR5, we developed a neutralization assay using transiently transfected Cf2Th cells. This canine thymus cell line lacks CD4 and CCR5, but expresses a *tat*-inducible luciferase reporter. Cf2Th cells were co-transfected with either human (Cf2Th-hu) or chimpanzee (Cf2Th-ch) CD4 and CCR5 expression plasmids, and then validated by testing the neutralizing activity of anti-gp120 bNabs (n=3), which should not be affected by entry molecules, against a subset of HIV-1 (YU2) and SIVcpz (GAB1, EK505, MT145, TAN13) strains (Figs. 7B and C). The results showed that IC_{50} values from Cf2Th-hu cells were highly correlated with those

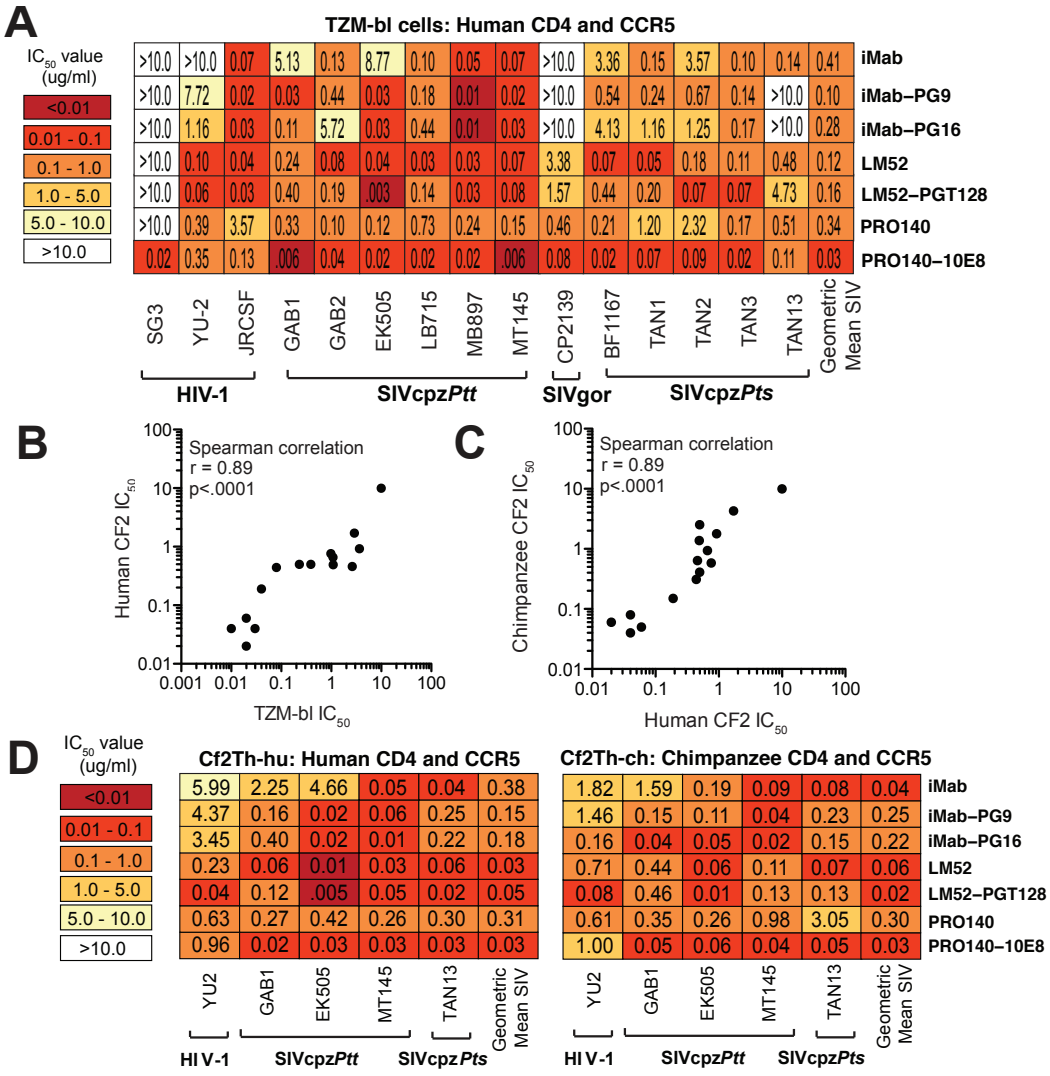


FIG 7. Neutralizing capacity of anti-host receptor antibodies (A) The ability of mono-specific anti-human CD4 (iMab, LM52) and anti-CCR5 (PRO140) antibodies as well as their bi-specific derivatives (iMab-PG9, iMab-PG16, LM52-PGT128, PRO140-10E8) (listed on the right) to neutralize a panel of HIV-1, SIVcpz and SIVgor strains (bottom) is shown. Numbers indicate IC_{50} values (μg/ml) from TZM-bl cells, averaged from three different experiments, with a heatmap indicating the relative neutralization potency. The geometric mean IC_{50} is shown for each antibody (only values for SIVcpz and SIVgor strains that were below 10 μg/ml were included into the calculation). (B) Correlation of TZM-bl and Cf2Th-hu derived neutralization data. Three bNabs (10E8, PG16, eCD4-Ig^{mim2}) were used to neutralize a subset of HIV-1 and SIVcpz strains (YU-2, GAB1, EK505, MT145, TAN13) in TZM-bl and Cf2Th cells expressing human CD4v and CCR5 receptors Cf2Th-hu). IC_{50} values from TZM-bl (x-axis) and Cf2Th-hu (y-axis) cells were plotted and analyzed using the Spearman correlation test. (C) Correlation of Cf2Th-hu and Cf2Th-ch neutralization data. The same three bNabs shown in (B) were used to neutralize the same subset of HIV-1 and SIVcpz strains in Cf2Th cells expressing human (Cf2Th-hu) and chimpanzee (Cf2Th-ch) CD4 and CCR5 receptors. IC_{50} values from Cf2Th-hu (x-axis) and Cf2Th-ch (y-axis) cells were plotted and analyzed using the Spearman correlation. (D) The ability of anti-receptor antibodies (listed on the right) to neutralize a subset of HIV-1 and SIVcpz strains (bottom) in Cf2Th cells expressing either human (left panel) or chimpanzee (right panel) CD4 and CCR5 receptors is shown. Numbers indicate IC_{50} values (μg/ml) averaged from three different experiments, with heatmaps indicating relative neutralizing potencies. The geometric mean IC_{50} is shown for each antibody (only values from SIVcpz strains were included).

from TZM-bl cultures (Fig. 7B), and this was also true for IC_{50} values from Cf2Th-hu and Cf2Th-ch cells (Fig. 7C). Having validated the Cf2Th-ch cell assay, we next examined the neutralizing capacity of the anti-receptor antibodies using a subset of SIVcpz strains (Fig. 7D). Both mono and bi-specific antibodies blocked these viruses in chimpanzee CD4 and CCR5 expressing cells with mean IC_{50} values ranging from 0.30 μg/ml (PRO140) to 0.02 μg/ml (LM52-PGT128). Thus, several anti-human receptor antibodies that potently neutralized SIVcpz strains in cells expressing human CD4 and CCR5 also neutralized these viruses in cells expressing chimpanzee CD4 and CCR5.

CD4 containing antibodies and immunoadhesins potently neutralize SIVcpz and SIVgor strains. Soluble CD4 (sCD4) as well as immunoadhesins, which contain the first two immunoglobulin-like domains (D1 and D1) of CD4 linked to antibody constant regions, are known to have broad anti-HIV-1 activity *in vitro* and *in vivo* (60, 61, 94). To examine their breadth and potency against ape viruses, we tested several members of this inhibitor class. Soluble (s)CD4 (95) and the more bioavailable CD4-Ig (96), in which the D1D2 domains of CD4 are linked to an IgG Fc region (Fig. 8A), neutralized nearly all ape viruses with mean IC_{50} values of 2.0 μg/ml (47.7 nM) and 2.1 μg/ml (23.1 nM), respectively (Fig. 8B). The tetravalent PRO542, in which D1D2 domains are linked to both the constant heavy (C_H) and light (C_L) chains of IgG1 (Fig. 8A), was slightly more potent and neutralized 10 of 12 ape viruses with a mean IC_{50} of 1.1 μg/ml (5.2 nM) (Fig. 8B). Finally, CD4-218.3-E51, a CD4 induced (CD4i) neutralizing antibody (E51) linked to D1D2 via the V_H chain (Fig. 8A), neutralized all SIVcpz and SIVgor strains with a geometric mean IC_{50} of 1.68 μg/ml (8.4 nM) (Fig. 8B). Interestingly, other E51-based constructs that differed in the length or amino acid composition of their linkers (CD4-GS7-E51 (61), CD4-L1-E51, CD4-L14-E51, CD4-L17-E51) were much less broadly acting and/or potent (Fig. 8B), and this was also true for a construct in which the D1D2 domains were linked to an anti-herpes simplex virus antibody (CD4-anti-HSV-gD).

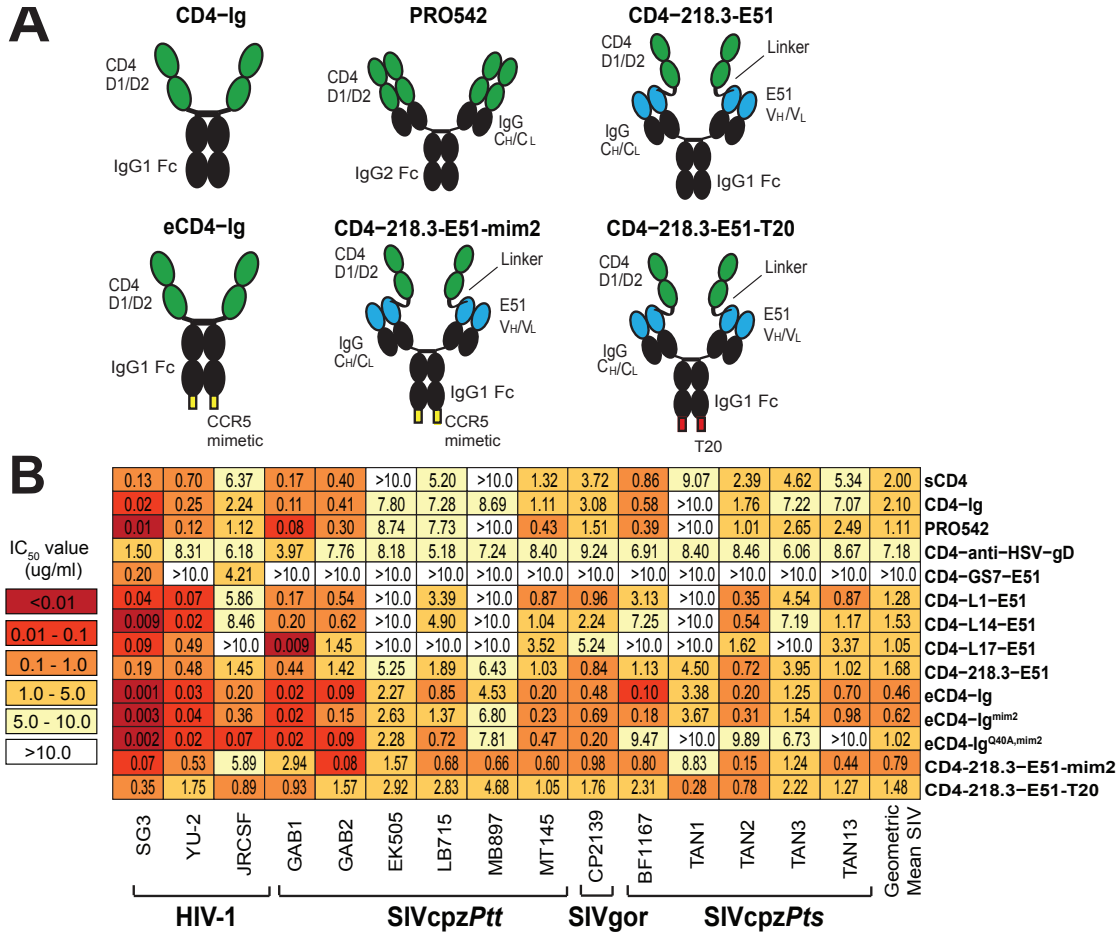


FIG 8. Neutralizing capacity of human CD4 containing immunoadhesins. (A) Schematic representation of six constructs. Human CD4 D1 and D2 domains are shown in green; immunoglobulin (IgG) Fc as well as constant heavy and light (C_H/C_L) regions are shown in black; E51 variable heavy and light (V_H/V_L) regions are shown in blue, CCR5 mimetic peptides are shown in yellow, and the T20 fusion inhibitor is shown in red. (B) The ability of human CD4 containing antibody-like constructs (listed on the right) to neutralize a panel of HIV-1, SIVcpz and SIVgor strains (bottom) is shown. Numbers indicate IC_{50} values (μg/ml) from TZM-bl cells, averaged from three different experiments, with a heatmap indicating the relative neutralization potency. The highest antibody concentration used was 10 μg/ml. The geometric mean IC_{50} is shown for each construct (only values for SIVcpz and SIVgor strains that were below 10 μg/ml were included into the calculation).

Some HIV-1 bNabs use tyrosine sulfation of their complementarity determining regions (CDRs) to mimic CCR5 sulfation and thereby inhibit virus entry (97). Sulfopeptides derived from such antibodies, including a 15 amino acid tyrosine sulfated peptide derived from the CD4i antibody E51, have been shown to reproduce this effect (98). When linked to an antibody Fc domain, this peptide (CCR5mim) was able to block infection of diverse HIV-1 strains (99). However, its neutralization capacity was most improved when fused to the carboxy terminus of CD4-Ig (Fig. 8A). In fact, this enhanced CD4-Ig, or eCD4-Ig, did not only neutralize a diverse panel of HIV-1 strains, but also SIVmac and HIV-2 isolates, with IC_{50} values of <0.05 μg/ml (62). Using TZM-bl cells, we found that eCD4-Ig neutralized SIVcpz and SIVgor strains also more potently than CD4-Ig with a mean IC_{50} of 0.46 μg/ml (4.8 nM). However, this 5-fold enhancement was modest compared to the 20- to 200-fold enhancement observed for HIV-1 strains (62). Moreover, the use of CCR5mim peptide variants previously shown to bind HIV-1 Envs with greater affinity did not improve neutralization: eCD4-Ig^{mim2} was slightly less potent than eCD4-Ig (IC_{50} of 0.62 μg/ml) and eCD4-$Ig^{Q40A,mim2}$ failed to neutralize two SIVcpz*Pts* strains (Fig. 8B). Addition of CCR5mim2 to the carboxy terminus of CD4-218.3-E51 (CD4-218.3-E51-mim2) resulted in a two-fold increased neutralization potency, while addition of the fusion-inhibiting peptide T-20 (100) (CD4-218.3-E51-T20) had very little effect (Fig. 8B). Thus, as previously shown for HIV-1, HIV-2 and SIVmac (62), addition of entry-inhibiting peptides to the carboxy terminus of D1D2 containing antibody constructs enhanced their ability to neutralize SIVcpz and SIVgor strains, although the magnitude of this enhancement was much less pronounced.

Neutralization of SIVcpz in primary chimpanzee CD4+ T cells. TZM-bl and CF2Th-ch cells identified a number of anti-viral and anti-receptor bNabs that neutralized ape viruses with both breadth and potency. To confirm this phenotype in a physiologically more relevant culture system, we tested these antibodies in primary chimpanzee lymphocytes. Chimpanzee CD4+ T cells were isolated from the blood of three donors, activated using autologous macrophages, and used to test the neutralizing capacity of immunoadhesins (CD4-Ig, eCD4-Ig and eCD4-Ig^{mim2}), the

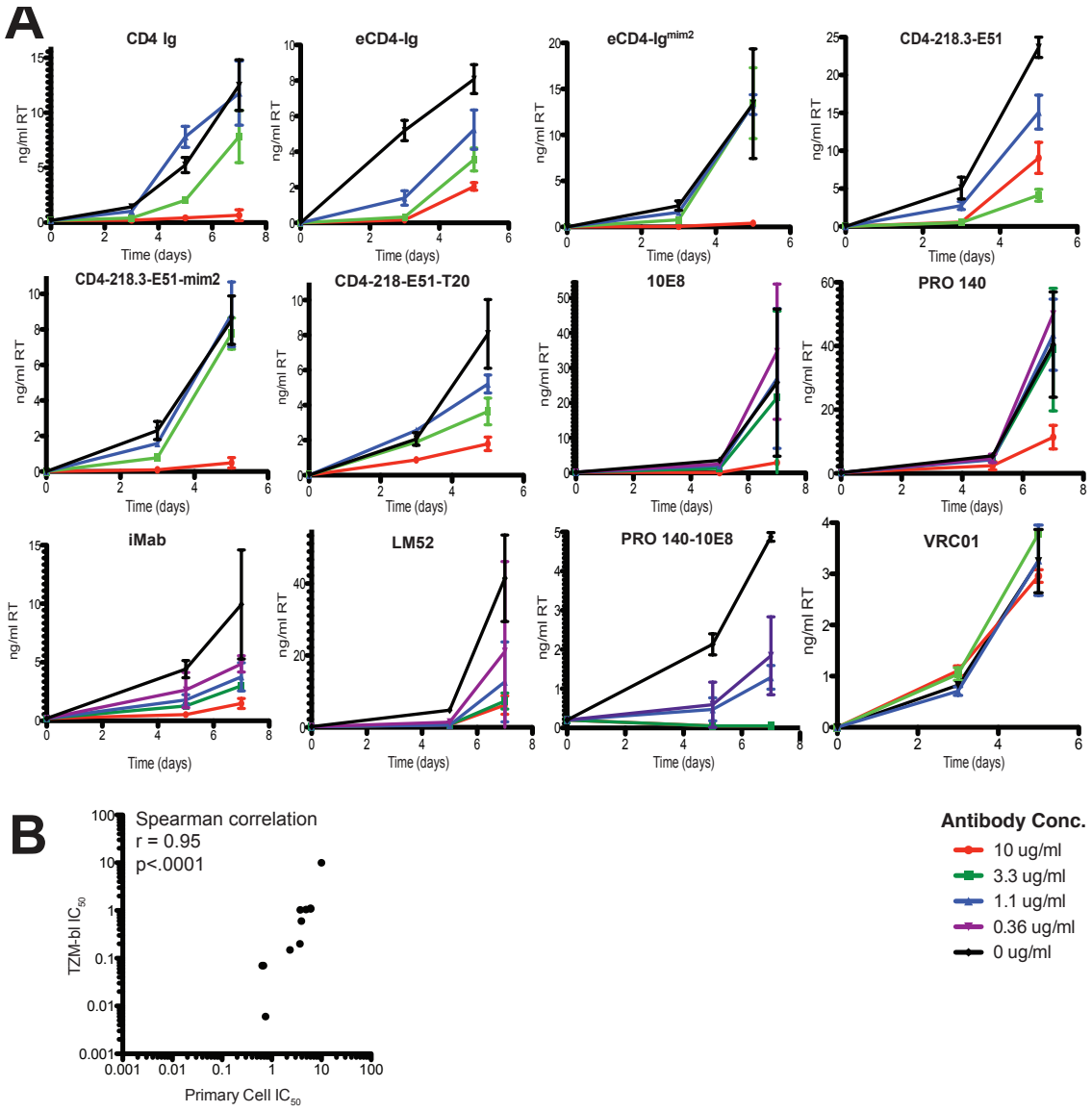


FIG 9. Neutralization of SIVcpz in primary CD4+ T cells. (A) Serial dilutions (red, 10 μg/ml; green, 3.3 μg/ml; blue 1.1 μg/ml; purple 0.36 μg/ml) of the most potent anti-SIVcpz antibodies and immunoadhesins (as determined in TZM-bl cells) were incubated with the SIVcpz strain MT145 before addition to activated primary chimpanzee CD4+ T cells (anti-receptor antibodies were incubated with cells before virus was added). Plots depict MT145 replication after antibody removal, with time (days) plotted on the x-axis and RT activity on the y-axis. Antibody VRC01, which does not neutralize SIVcpz and SIVgor strains, served as a negative control. CD4+ T cells from three different chimpanzee donors were used (one representative experiment is shown). (B) Correlation of IC_{50} values from TZM-bl and primary chimpanzee CD4+ T cells. IC_{50} values averaged from three independent experiments were plotted for chimpanzee CD4+ T cells (x-axis) and TZM-bl (y-axis), and analyzed using the Spearman correlation test.

TABLE 2 Neutralization Potency in Primay CD4+ T Cells

	HIV-1 SG3		SIVcpz MT145	
	IC_{50} (ug/ml)	**IC_{50} (nM)**	**IC_{50} (ug/ml)**	**IC_{50} (nM)**
CD4-Ig	0.02 (±0.02)[a]	0.22 (±0.22)	6.02 (±3.5)	65.4 (±38.0)
CD4-218.3-E51	0.04 (±0.04)	0.20 (±0.20)	3.74 (±1.4)	18.7 (±7.0)
eCD4-Ig	<0.01 (±0.0)	<0.10 (±0.0)	3.68 (±1.1)	38.3 (±11.5)
eCD4-Ig^{mim2}	<0.01 (n/a[b])	<0.10 (±0.0)	5.86 (n/a)	61.0 (n/a)
CD4-218.3-E51-mim2	<0.01 (±0.0)	<0.05 (±0.0)	3.93 (±1.5)	19.7 (±7.5)
CD4-218.3-E51-T20	<0.01 (±0.0)	<0.05 (±0.0)	4.90 (±4.4)	24.5 (±22.0)
VRC01	0.03 (±0.03)	0.20 (±0.20)	>10 (±0.0)	> 66.7 (±0)
10E8	ND[c]	ND	6.07 (±1.82)	40.5 (±12.1)
iMab	ND	ND	0.64 (±0.41)	4.3 (±2.7)
LM52	ND	ND	0.68 (±0.18)	4.5 (±1.2)
PRO140	ND	ND	2.33 (±1.99)	15.5 (±13.3)
PRO140-10E8	ND	ND	0.75 (±0.68)	3.6 (±3.2)

[a]Average of 3 donors shown (±standard deviation)
[b]n/a, not applicable, only performed in 1 donor
[c]ND, not done

MPER antibody 10E8, CD4-CD4i constructs (CD4-218.3-E51, CD4-218.3-E51-mim2, CD4-218.3-E51-T20), and anti-receptor antibodies (iMab, LM52, PRO140, PRO140-10E8) (Fig. 9A). Since chimpanzee blood samples were limited, we were able to test only a single SIVcpz strain (MT145), which was selected based on its ability to replicate efficiently in CD4+ T cells from multiple chimpanzee donors (70). Using serial antibody dilutions (Fig. 9A), we found that MT145 was most potently neutralized by iMab, LM52 and PRO140-10E8 with mean IC_{50} values of 0.64 (4.3 nM), 0.68 (4.5 nM) and 0.75 μg/ml (3.6 nM), respectively (Tables 2). Immunoadhesins were roughly 10-fold less potent, with mean IC_{50} values ranging from 3.7 μg/ml (38.3 nM) for eCD4-Ig to 6 μg/ml (65.4 nM) for CD4-Ig. As expected, the CD4bs antibody VRC01 had no inhibitory activity (Fig. 9A, Table 2). Thus, all antibody constructs that had broad and potent anti-SIVcpz activity in the TZM-bl assay also inhibited SIVcpz infection in chimpanzee CD4+ T cells (Fig. 9B), although the observed IC_{50} values were 10- to 100-fold higher in the primary T cell cultures that permitted multiple rounds of replication (Fig. 9B).

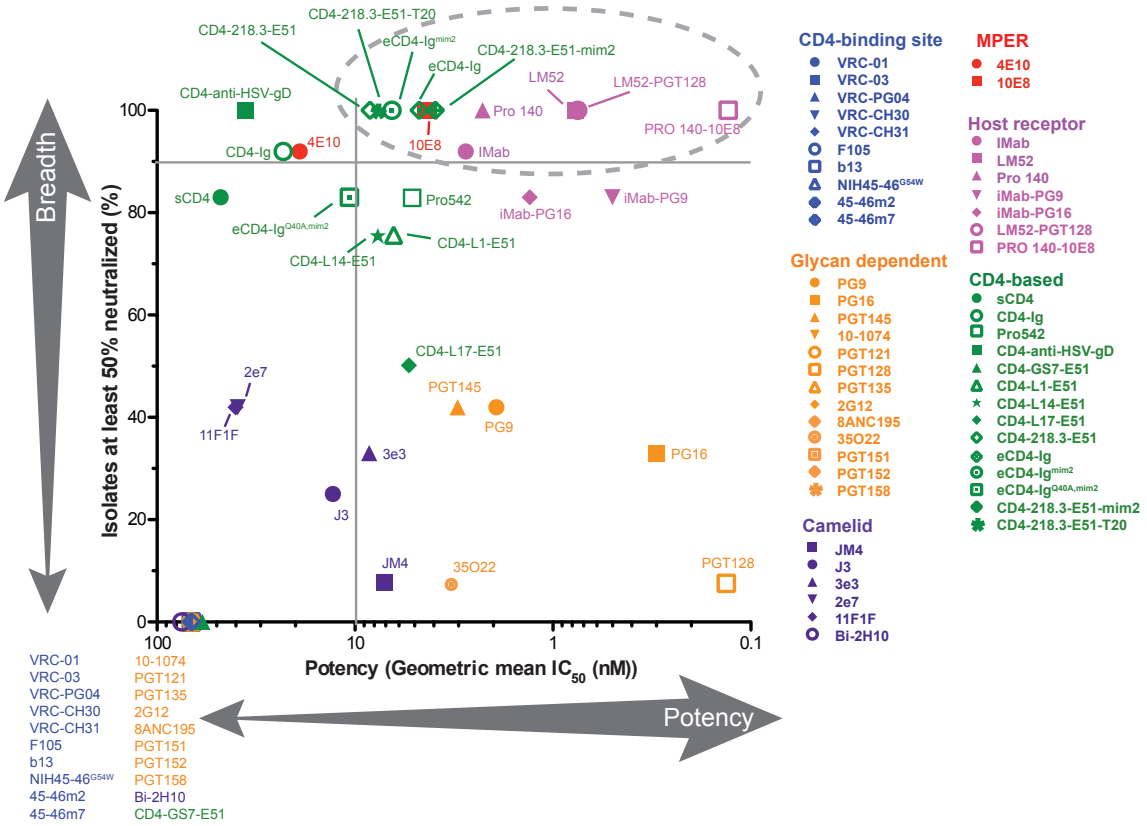


FIG 10. Breadth and potency for antibody-like constructs with anti-SIVcpz and anti-SIVgor neutralizing activity. For each bNab, the percentage of SIVcpz and SIVgor strains neutralized with IC_{50} values of < 10 μg/ml (y-axis) is plotted against the corresponding geometric mean IC_{50} in nM (x-axis). Antibodies and antibody-like constructs are color coded, with those that exhibited no anti-SIVcpz and SIVgor activity listed in the left lower corner. Horizontal and vertical bars denote 90% breadth and 10nM potency, respectively, and the most potent and cross-reactive reagents are circled

TABLE 3 Neutralization Potency (nM) in TZM-bl Cells

	HIV-1			SIVcpzPtt						SIVgor	SIVcpzPts					Geometric	Percentage
	SG3	YU-2	JRCSF	GAB-1	GAB-2	EK505	LB715	MB897	MT145	CP2139	BF1167	TAN-1	TAN-2	TAN-3	TAN-13	Mean[a]	Neutralized[b]
CD4bs																	
VRC-01	0.29	0.84	2.81	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	0%
VRC-03	0.3	0.22	0.63	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	0%
VRC-PG04	0.45	0.76	2.07	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	0%
VRC-CH30	6.73	1.09	3.07	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	0%
VRC-CH31	7.89	0.65	1.28	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	0%
F105	5.3	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	0%
b13	22.2	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	0%
NIH45-46G54W	0.02	0.16	0.41	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	0%
45-46m2	0.14	0.28	0.71	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	0%
45-46m7	1.06	0.34	0.78	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	0%
MPER																	
4E10	6.68	>67	24.06	7.94	14.25	15.97	12.63	16.30	17.93	>67	32.93	62.30	>67	15.26	>67	18.10	75%
10E8	5.67	19.27	3.07	0.20	1.53	7.27	0.87	7.20	7.20	34.20	6.20	7.20	9.40	1.47	24.40	4.39	100%
Glycan-dependent																	
PG9	>67	16.33	0.06	0.69	>67	3.19	>67	0.63	0.51	>67	>67	38.30	>67	>67	>67	1.93	42%
PG16	>67	0.55	0.01	0.07	>67	2.58	>67	0.32	0.15	>67	>67	>67	>67	>67	>67	0.30	33%
PGT145	>67	3.50	0.22	3.85	>67	0.39	>67	1.93	6.64	13.28	>67	>67	>67	>67	>67	3.03	42%
10-1074	0.86	1.81	0.83	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	0%
PGT121	0.97	1.05	0.63	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	0%
PGT128	>67	1.01	0.11	>67	>67	0.13	>67	>67	>67	>67	>67	>67	>67	>67	>67	0.13	8%
PGT135	0.77	>67	6.61	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	0%
2G12	4.63	>67	6.92	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	0%
8ANC195	8.67	7.80	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	0%
35O22	0.87	>67	>67	>67	>67	>67	3.33	>67	>67	>67	>67	>67	>67	>67	>67	3.33	8%
PGT151	0.13	14.07	0.47	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	0%
PGT152	0.20	2.00	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	0%
PGT158	0.60	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	>67	0%
Llama																	
JM4	36.03	67.17	6.82	7.20	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	7.20	8%
J3	0.21	1.35	10.52	1.47	38.65	>100	38.07	>100	>100	>100	>100	>100	>100	>100	>100	12.92	25%
3E3	0.27	0.22	12.42	1.61	27.96	>100	10.83	10.60	>100	>100	>100	>100	>100	>100	>100	8.48	33%
2E7	11.44	>100	46.63	>100	20.14	92.89	20.42	>100	42.66	>100	55.84	>100	>100	>100	>100	39.07	42%
11F1F	11.62	>100	47.42	>100	21.24	91.12	22.56	>100	43.52	>100	54.79	>100	>100	>100	>100	40.13	42%
Bi-2H10	61.42	>75	34.44	>75	>75	>75	>75	>75	>75	>75	>75	>75	>75	>75	>75	>75	0%
Anti-receptor																	
iMab	>67	>67	0.46	34.21	0.86	58.49	0.69	0.36	0.48	>67	22.40	0.98	23.83	0.68	0.96	2.76	92%
LM52	>67	0.64	0.23	1.59	0.50	0.25	0.23	0.22	0.43	22.53	0.46	0.35	1.17	0.76	3.21	0.77	100%
PRO140	>67	2.61	23.81	2.16	0.68	0.80	4.87	1.57	1.02	3.07	1.41	7.97	15.46	1.12	3.43	2.27	100%
iMab-PG9	>48	35.32	0.10	2.56	0.15	0.15	2.08	0.85	0.04	>48	0.09	1.13	3.21	0.67	>48	0.50	83%
iMab-PG16	>48	5.54	0.13	19.68	0.16	0.51	27.24	2.12	0.04	>48	0.15	5.51	5.94	0.83	>48	1.31	83%
LM52-PGT128	>48	0.28	0.14	2.09	0.02	1.94	0.91	0.67	0.12	7.49	0.37	0.95	0.32	0.33	22.52	0.75	100%
PRO140-10E8	0.14	1.65	0.61	0.12	0.08	0.03	0.17	0.10	0.10	0.37	0.03	0.34	0.44	0.10	0.52	0.13	100%
CD4-based																	
sCD4	3.05	16.97	155.3	4.23	9.70	>244	126.9	>244	32.12	90.61	21.07	221.2	58.27	112.79	130.21	47.66	83%
CD4-Ig	0.21	2.70	24.29	1.19	4.40	84.74	79.09	94.40	12.11	33.46	6.31	>109	19.14	78.49	76.79	23.05	92%
PRO542	0.03	0.55	5.33	0.36	1.41	41.60	36.81	>47	2.06	7.18	1.85	>47	4.82	12.62	11.85	5.24	83%
CD4-anti-HSV-gD	7.50	41.57	30.89	19.84	38.81	40.90	25.87	36.19	42.02	46.19	34.57	42.00	42.30	30.28	43.37	35.92	100%
CD4-GS7-E51	1.01	>50	21.03	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	0%
CD4-L1-E51	0.20	0.35	29.31	0.85	2.69	>50	16.93	>50	4.36	4.80	15.63	>50	1.75	22.69	4.33	5.05	75%
CD4-L14-E51	0.04	1.01	42.31	0.98	3.10	>50	24.49	>50	5.22	11.21	36.27	>50	2.68	35.92	5.84	7.64	75%
CD4-L17-E51	0.47	2.46	>50	0.05	7.26	>50	>50	>50	17.61	26.21	>50	>50	8.08	>50	16.87	5.23	50%
CD4-218.3-E51	0.95	2.39	7.24	2.21	7.08	26.25	9.42	32.14	5.16	4.18	5.66	22.52	3.60	19.73	5.09	8.40	100%
eCD4-Ig	0.01	0.26	2.05	0.19	0.97	23.67	8.84	47.19	2.08	4.95	1.03	35.20	2.07	12.98	7.30	4.75	100%
eCD4-Ig^{mim2}	0.03	0.39	3.78	0.22	1.54	27.36	14.22	70.87	2.34	7.19	1.83	38.18	3.18	16.05	10.18	6.49	100%
eCD4-Ig^{G40Amim2}	0.02	0.25	0.69	0.20	0.89	23.70	7.45	81.38	4.89	2.07	98.69	>104	103.0	70.15	>104	10.65	83%
CD4-218.3-E51-mim2	0.34	2.64	29.46	14.70	0.38	7.83	3.38	3.30	2.98	4.88	2.22	4.00	44.17	0.76	6.22	3.92	100%
CD4-218.3-E51-T20	1.75	8.75	4.45	4.64	7.86	14.62	14.15	23.38	5.27	8.78	6.33	11.55	1.40	3.91	11.12	7.58	100%

[a]Geometric mean calculations include only values from SIVcpz and SIVgor strains that reached an IC_{50} at the highest concentration tested

[b]Includes only SIV strains

Section 3.4 – Discussion

In this study, we examined whether antibodies and immunoadhesins known to potently neutralize diverse strains of HIV-1 can cross-neutralize related lentiviruses naturally infecting chimpanzees and gorillas to assess their utility for antibody based strategies to combat ape SIV infections. We found that the great majority of HIV-1 specific bNabs, including those directed against highly conserved Env epitopes such as the CD4 binding site, failed to neutralize SIVcpz and SIVgor strains. However, one antibody directed against the MPER region (10E8) as well as bi-specific CD4 and CCR5 receptor mimetics (eCD4-Ig, eCD4-Ig^{mim2}, CD4-218.3-E51, CD4-218.3-E51-mim2) neutralized 100% of ape viruses with low nanomolar potency (Table 3, Fig. 10). Anti-host receptor antibodies (iMab, PRO140) were also effective neutralizers, especially when fused to the antigen binding regions of potent bNabs such as 10E8 (Table 3, Fig. 10). These data indicate that pandemic HIV-1 shares an extremely limited number of cross-reactive epitopes with its immediate ape precursors. However, the sites of vulnerability that were identified represent attractive targets across the entire HIV-1/SIVcpz/SIVgor clade, and may be of utility to combat other HIV-1 lineages, including group O viruses, which are estimated to have infected ~100,000 people in west central Africa (16).

Neutralizing antibody responses in long-term HIV-1 and SIVcpz infected chimpanzees. Since bNabs isolated from apes may be more appropriate for certain interventions than antibodies derived from humans, we tested plasma samples from eight long-term HIV-1 and SIVcpz infected chimpanzees for evidence of neutralization breadth (Table 1). Although all animals had high titer Env binding antibodies, their plasma samples neutralized only the easy-to-neutralize (tier 1) HIV-1/SG3 and SIVcpzGAB1 strains (Fig. 1B). Four animals had very low (Joye) or undetectable (Marc, Bucky, Josie) plasma viral loads, suggesting insufficient antigenic stimulation as a possible reason for the lack of bNab development (Table 1). In addition, one

animal (Tika) had severe CD4+ T cell depletion and thus may have suffered from immune exhaustion (Table 1). However, two chimpanzees (Debbie and Cotton) had plasma viral titers exceeding 20,000 RNA copies/ml, suggesting sustained productive infection for almost two decades (Table 1). Both were experimentally infected with SIVcpzANT, a naturally occurring SIVcpz*Pts* strain that was administered by mucosal routes using plasma and/or peripheral blood mononuclear cells from a third chimpanzee (20). Despite this "near-natural" infection history, only one of these animals (Debbie) exhibited cross-reactive neutralizing activity, albeit with exceedingly low titers (<1:70) (Fig. 1B). Since the kinetics of bNab development may vary in chimpanzees and humans, it will be important to follow this individual to examine whether neutralizing titers will increase and to map the corresponding antibody specificities. It may also be informative to study autologous neutralization responses and associated viral escape in this and other long-term infected chimpanzees to elucidate why antibody lineages with broad neutralizing capacity develop only rarely. Since very few apes and humans can be studied decades after HIV/SIV infection in the absence of anti-retroviral therapy, this may provide an opportunity to uncover common pathways, as well as roadblocks, to bNab development (101-103).

HIV-1 shares a very limited number of cross-reactive epitopes with SIVcpz and SIVgor strains. The envelope glycoprotein of HIV-1 group M viruses contains five regions of vulnerability that are targeted by broadly cross-reactive neutralizing antibodies. These include the CD4 binding site (28, 48), the MPER region (29, 47), the N332 supersite of vulnerability (27, 49), the N160 antigenic site (26), and glycan-associated regions at the interface of gp120 and gp41 (52, 53, 55). All except the MPER region represent conformational epitopes that span heavily glycosylated and/or structurally flexible protein domains. Since even the most cross-reactive CD4bs bNabs bind an area that extends well beyond the CD4 binding pocket (104), variation in adjacent Env regions can obstruct antibody binding and neutralization (76). SIVcpz and SIVgor Envs vary extensively in regions surrounding the CD4 binding site (Fig. 2B), thus providing an

explanation for their resistance to CD4bs bNabs, including the smaller camelid antibodies (Figs. 2 and 5). Ape viruses also vary in Env regions that are targeted by carbohydrate-associated bNabs, all of which require conservation of key N-linked glycosylation sites (Figs. 3 and 4). Since these are also variable in ape viruses, it is not surprising that almost all glycan dependent bNabs failed to cross-neutralize SIVcpz and SIVgor strains. Nonetheless, antibodies directed against the MPER region neutralized even the most divergent ape viruses (Fig. 6). 4E10 neutralized all SIVcpz*Ptt* strains with a mean IC_{50} of 2.7 μg/ml (18.1 nM), but lacked activity against SIVgor and some SIVcpz*Pts* strains. In contrast, 10E8 neutralized all 12 SIVcpz and SIVgor strains with an IC_{50} of 0.7 μg/ml (4.4 nM). The latter finding may be of interest for "neutralization finger printing" studies (105), since inclusion of SIVgor and select SIVcpz*Pts* Envs into existing pseudovirus panels should permit the differentiation of 4E10-like and 10E8-like bNab specificities in polyclonal patient plasmas.

Anti-receptor antibodies circumvent SIVcpz and SIVgor Env diversity. Antibodies raised against human CD4 and CCR5 receptors are known to inhibit HIV-1 *in vitro* (106) as well as reduce viral loads in infected patients *in vivo* (90). Here, we show that these antibodies also neutralize SIVcpz and SIVgor strains, both in cells expressing human as well as chimpanzee CD4 and CCR5 receptors (Fig. 7). For example, iMab, which blocks HIV-1 infection by binding to the second domain (D2) of human CD4 (107), neutralized nearly all ape viruses with IC_{50} values ranging from 0.41 μg/ml (2.8 nM) in TZM-bl cells to 0.64 μg/ml (4.3 nM) in primary chimpanzee CD4+ T cells (Figs. 7 and 9, Tables 2 and 3). Similarly, LM52 a derivative of iMab with increased neutralization breadth (58), inhibited all 12 SIVcpz and SIVgor strains with IC_{50} values ranging from 0.12 μg/ml (0.77 nM) in TZM-bl cells to 0.68 μg/ml (4.5 nM) in primary chimpanzee CD4+ T cells. Finally, PRO140, which binds a complex epitope spanning multiple extracellular CCR5 domains (108), neutralized all ape viruses with IC_{50} values ranging from 0.34 μg/ml (2.3 nM) in TZM-bl cells to 2.33 μg/ml (15.5 nM) in primary chimpanzee CD4+ T cells. Thus, anti-receptor

antibodies neutralized even the most divergent ape viruses in physiologically relevant target cells (Figs. 9 and 10; Tables 2 and 3).

Although iMab achieved a 1 log reduction in virus titers in chronically HIV-1 infected humans (90, 106), a second anti-CD4 antibody (2D5) afforded only partial protection to rhesus macaques challenged with a simian/human immunodeficiency virus (SHIV) (109). Because anti-receptor antibodies have to block all susceptible target cells, they may require antibody concentrations at the site of virus entry that are difficult to achieve *in vivo* (109). We thus tested bi-specific constructs, in which iMab, LM52 and PRO140 were linked to the antigen binding domains of potent anti-HIV-1 bNabs. Indeed, iMab-PG9, iMab-PG16 and LM52-PGT128 were able to outperform iMab and LM52 (Figs. 7, Table 3), but only when used to neutralize the few ape viruses that were sensitive to PG9, PG16 and PGT128 (Fig. 3). However, the bi-specific PRO140-10E8 neutralized all SIVcpz and SIVgor strains with up to 20-fold increased potency, regardless whether TZM-bl, Cf2Th-ch or primary chimpanzee CD4+ T cells were used as target cells (Figs. 7, 9, 10; Tables 2 and 3). Of all anti-receptor antibodies tested, PRO140-10E8 was by far the most potent, neutralizing 100% of ape viruses with IC_{50} values ranging from 0.03 μg/ml (0.13 nM) in TZM-bl to 0.75 μg/ml (3.6 nM) in chimpanzee CD4+ T cells (Fig. 10). These findings are consistent with the idea that bi-specific anti-receptor antibodies exhibit enhanced neutralizing capacity because they concentrate anti-Env bNabs at the site of virus entry (59). It will be important to test PRO140-10E8 in the SHIV/macaque model to determine whether its *in vitro* potency will translate into *in vivo* protection.

Bi-specific receptor mimetics confer broad anti-SIVcpz and anti-SIVgor activity. Several studies have shown that CD4 containing immunoadhesins exhibit considerable anti-HIV-1 activity and even protected a subset of macaques from SIVmac infection (31, 110). Among these, constructs that use receptor mimicry to simultaneously engage both the CD4 and CCR5 binding sites are the most potent (61, 62). These include CD4-CD4i reagents, in which the D1D2

of CD4 is linked to the heavy chain variable region of a CD4 induced (CD4i) antibody, as well as CD4-Ig based constructs that contain short CCR5-mimetic sulfopeptides on their C-terminus (Fig. 8A). Although both CD4-CD4i and eCD4-Ig constructs neutralized human and ape viruses with nanomolar potency (Table 3), we were particularly interested in eCD4-Ig, because of its smaller size as well as extensive *in vitro* and *in vivo* characterization. Recent studies demonstrated that eCD4-Ig not only neutralizes difficult-to-neutralize (tier 2 and 3) strains of HIV-1, but also inhibits HIV-2 and SIVmac viruses with IC_{50} values of less than 0.01 μg/ml (0.1 nM). Moreover, a rhesus macaque adapted form of eCD4-Ig (rh-eCD4-Ig) was able to protect monkeys from multiple low dose intravenous SHIV challenges when expressed from a recombinant AAV-vector (62). Given these findings, we expected eCD4-Ig to inhibit ape viruses with similar breadth and potency. However, this was only partially the case. Although eCD4-Ig neutralized 100% of SIVcpz and SIVgor strains, IC_{50} values were ~50-fold higher than those previously reported for HIV-1, HIV-2 and SIVmac strains (62). Moreover, this potency was not improved when modified versions of eCD4-Ig (eCD4-Ig^{mim2}, eCD4-Ig^{Q40A}) were used that exhibit enhanced anti-HIV-1 activity (Fig. 8B, Table 3). Overall, eCD4-Ig neutralized SIVcpz and SIVgor strains only five-fold more potently than CD4-Ig, compared to a 20- to 200-fold improvement for HIV-1 strains (62). Thus, eCD4-Ig neutralized ape viruses with the desired breadth, but not with the desired potency.

Although a crystal structure of eCD4-Ig in complex with the HIV-1 Env is not available, modeling suggests that eCD4-Ig binds the HIV-1 Env trimer in a “claw-like” fashion, with two sulfopeptides and one CD4 moiety engaging two protomers within the same Env spike (62). Cross-linking of spike protomers has recently been shown to increase neutralization potency of antibody-like molecules by more than 100-fold (111). It is thus tempting to speculate that the architecture of the SIVcpz/SIVgor envelope spike differs from that of HIV-1, HIV-2 and SIVmac strains in such a way that one or more of the eCD4-Ig interaction sites are altered or occluded. It is also possible that the human CD4i antibody derived E51 peptide does not properly mimic the binding of the chimpanzee and gorilla CCR5 co-receptors, which differ from human and macaque CCR5 molecules in one or two amino acids near the N-terminus (88). It should be noted that

replacing the human CD4 D1D2 domains with the corresponding chimpanzee CD4 D1D2 regions in eCD4-Ig did not increase its potency against ape viruses (unpublished). Since there are no SIVcpz/SIVgor specific bNabs whose Fab fragments could be used for intra-spike crosslinking, it will be important to determine whether the ape virus neutralizing capacity of eCD4-Ig can be improved by altering the length and/or flexibility of the IgG Fc hinge region and/or by improving the binding of the CCR5 mimetic peptide.

Vectored antibody gene transfer to combat ape pathogens. Wild ape populations will go extinct unless there is a comprehensive approach to their conservation, which -- under certain circumstances -- may need to include medical interventions (19, 25). We have shown in the past that SIVcpz infection can have a substantial negative impact on the health, reproductive success and longevity of chimpanzees in Gombe (2, 3). Because infected chimpanzees in Gombe are individually known, it would be possible to use vectored antibody gene transfer to administer a cocktail of neutralizing antibodies. This may not only benefit the infected individuals, but may also reduce their ability to transmit SIVcpz, which in an isolated population such as Gombe could lead to virus extinction. Vectored antibody gene delivery, alone or in combination with long-acting antiretrovirals (112, 113), could also be used to treat SIVcpz infected chimpanzees in African sanctuaries (114) and/or to vaccinate orphaned chimpanzees prior to releasing them into the wild (115). Finally, vectored antibody gene delivery may be effective against other pathogens, such as anthrax and Ebola (116, 117). Obviously, delivered antibodies must be sufficiently potent and expression levels must be sufficiently high to be effective *in vivo*, but this seems achievable. As shown recently, AAV-delivered rh-eCD4-Ig protected macaques from repeated low-dose intravenous SHIV challenge at serum concentrations as low as 17 μg/ml (62). Moreover, AAV-rh-eCD4-Ig was much less immunogenic than other antibody-based AAV constructs, and functional rh-eCD4-Ig was stably expressed for 40 weeks at concentrations ranging between 17 and 77 μg/ml (62). Obviously, efforts to treat and/or vaccinate wild ape communities would only be

contemplated if their survival was in serious jeopardy and if the safety, efficacy and delivery of the particular intervention were first demonstrated in captivity. However, given the increasing threat of infectious diseases to ape survival, there is an urgent need to explore new ways to curb the spread of pathogens in wild populations. Although further improvements will be necessary, the finding of several antibody-like constructs that are capable of neutralizing SIVcpz and SIVgor strains with nanomolar potency suggests that this goal can be achieved.

Section 3.5 – Materials and Methods

Infectious molecular clones. Full-length infectious molecular clones (IMCs) of HIV-1 strains SG3 (118), YU2 (119) and JRCSF (120), SIVcpz*Ptt* strains EK505 (70), GAB1 (67), GAB2 (68), MB897 (70), and MT145 (70), SIVcpz*Pts* strains BF1167 (7), TAN1 (69), TAN2 (69), and TAN3 (69) and the SIVgor strain CP2139 (15) have previously been reported. To generate additional IMCs, consensus SIVcpz*Ptt* (LB715) and SIVcpz*Pts* (TAN13) sequences were generated from two additional fecal samples (69). Briefly, partially overlapping subgenomic fragments were amplified from fecal RNA, gel purified, and sequenced directly. Chromatograms were examined for positions of base mixtures, and ambiguous sites were resolved as previously reported (69). The resulting proviral consensus sequences were synthesized in 3 non-overlapping fragments (Blue Heron Biotechnology) and ligated using internal restriction enzyme enzymes. In addition, Not1 and Mlu1 restriction enzyme sites were added to the 5' and 3' termini to enable directional cloning into a modified (low copy number) pBR322 vector (15). Since full-length SIVcpz and SIVgor molecular clones are notoriously unstable, plasmids were grown in MAX Efficiency Stbl2 competent cells (Invitrogen) at 30°C, harvested before reaching saturating density, and each IMC was completely sequenced prior to biological analyses to confirm its integrity. The newly derived SIVcpz clones LB715 and TAN13 have been submitted to the National Institutes of Health Research and Reference Program (Rockville, MD) and their nucleotide sequences are available at GenBank.

Phylogenetic analysis. Amino acid sequences inferred from *env* genes of HIV-1 (YU2, GenBank accession number M93258; JRCSF, M38429; and SG3, L02317), SIVgor (CP2139.287, FJ424866), and SIVcpz (LB715, JX178450; MB897, EF535994; GAB2, AF382828; MT145, DQ373066; EK505, DQ373065; GAB1, X52154; TAN2, EF394357; TAN3, EF394358; TAN1, EF394356; TAN13, JQ768416; BF1167, JQ866001) strains were aligned using ClustalW (121), with regions that could not be unambiguously aligned excluded from the analysis. PhyML (version

3) was used to estimate the phylogeny based on a JTT model of amino acid replacement chosen using ProtTest (version 10.2) and a second-order AIC framework (122-124). Ten random-addition-order trees and a neighbor joining tree were likelihood-optimized using subtree pruning-regrafting (SPR) searches. Bayesian posterior probabilities were estimated with MrBayes using a mixed prior model (125).

Chimpanzee plasma. Plasma samples were obtained from eight chimpanzees, who had been experimentally infected with HIV-1 and/or SIVcpzANT decades earlier as part of AIDS pathogenesis and/or vaccine studies (Table 1). Seven of these chimpanzees were sampled at the National Chimpanzee Sanctuary Chimp Haven in Keithville, Louisiana, while the remaining animal (Debbie) was sampled at the Southwest National Primate Research Center (SNPRC) in San Antonio, Texas. For Marc (21), Joye (23), Tika (22), Artica (23), Debbie (20) and Cotton (20), detailed infection histories and clinical follow-up studies have been reported; however, this information was not available for the remaining two animals. Blood samples were collected for veterinary purposes in the context of health examinations. Plasma samples were analyzed for the presence of virus-specific antibodies using an enhanced chemiluminescent Western blot assay as previously described (9). Plasma samples were also tested for viral loads: For HIV-1 infected chimpanzees, this was done using the COBAS AmpliPrep/COBAS TaqMan HIV-1 Test, v2.0 (Roche). For SIVcpzANT infected chimpanzees, a previously reported quantitative (q)PCR designed to detect both HIV-1 and SIVcpz vRNA was used (126). In addition, SIVcpzANT viremia was confirmed by amplifying and sequencing a virus-specific 1.3 kb *gag-pol* fragment from plasma viral RNA (Table 1). Sample collection was approved by the Institutional Animal Care and Use Committee of Chimp Haven and SNPRC, respectively.

Virus stocks. HIV-1, SIVcpz and SIVgor infectious molecular clones (8 μg) were transfected into 293T cells and culture supernatants harvested 72 hours later. Viral stocks were tested for infectivity using TZM-bl cells, a HeLa-derived line that constitutively expresses CD4, CCR5 and CXCR4 receptors, and contains integrated luciferase and b-galactosidase reporter

genes under the control of an HIV-1 LTR (127). TZM-bl cells (8,300 cells per well) were seeded in 96-well plates overnight, incubated with 10-fold serial dilutions of transfection stocks for 48 hours to allow single round infection, and infectious units (IU) were determined by counting the number of β-galactosidase expressing cells.

TZM-bl based neutralization assay. The neutralizing capacity of chimpanzee plasma, anti-HIV-1 monoclonal antibodies, and immunoadhesins was assessed using the TZM-bl assay as previously described (128, 129). Briefly, TZM-bl cells were seeded in 96 well plates (8,300 cells per well) overnight in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS). Serial (5-fold) dilutions of chimpanzee plasma (1:20, 1:100, 1:500, 1:2,500, 1:12,500) or anti-HIV-1 monoclonal antibodies and immunoadhesins (10, 2, 0.4, 0.08, 0.016 μg/ml) were incubated with 4,800 infectious units (IU) of transfection derived virus in a total volume of 100 μl in the presence of DEAE-dextran (40 μg/ml) for one hour at 37°C, and this mixture was then added to TZM-bl cells in 96 well plates. After 48 hours, TZM-bl cells were analyzed for luciferase expression using a Synergy H4 Hybrid microplate reader (Bio-Tek) with Gen5 version 1.11 software. To test the neutralization capacity of anti-CD4 and anti-CCR5 receptor antibodies, 5-fold serial dilutions (10, 2, 0.4, 0.08, 0.016 μg/ml) were incubated with TZM-bl cells in a volume of 50 μl for one hour at 37°C, followed by the addition of virus (4,800 IU in 50 μl) in the presence of DEAE-dextran (40 μg/ml) and further incubation of 48 hours at 37°C. Controls included untreated cells and virus pretreated with normal human plasma or no antibody. Relative infectivity was calculated by dividing the number of luciferase units at each plasma and antibody/immunoadhesin dilution with values obtained for wells that contained normal human plasma or no antibody. Half-maximal inhibitory concentrations (IC_{50}) were determined by linear regression. All monoclonal antibodies were tested in duplicate on three independent occasions. Since available amounts of chimpanzee plasma were limited, samples were tested only once in duplicate.

Cf2Th based neutralization assay. To test the neutralization capacity of anti-host receptor antibodies, we transiently transfected Cf2Th cells with plasmids expressing the chimpanzee CD4 and CCR5 genes. These canine thymus derived cells do not naturally express CD4 or CCR5, but contain a firefly luciferase reporter gene stably integrated under the control of an HIV-1 LTR (2, 3, 130). To generate a chimpanzee CD4 expression plasmid, we extracted RNA from chimpanzee peripheral blood mononuclear cells (PBMCs) and used RT-PCR to generate a cDNA clone of the entire CD4 coding region. Since the chimpanzee CD4 gene is polymorphic (71), we selected one allele that is most predominant in chimpanzees from Gombe National Park. Using site-directed mutagenesis, we then "humanized" this cDNA clone by changing three amino acids to match the (non-polymorphic) human CD4 protein (T34I, V55A and G88E). To generate a chimpanzee CCR5 expression plasmid, we first amplified the human CCR5 gene from PBMC RNA, and then introduced a single (chimpanzee-specific) amino acid substitution (N13D) by site-directed mutagenesis. These expression plasmids were then used to transfect Cf2Th cells in 10 cm dishes at 50% confluency to generate cells that expressed either chimpanzee or human CCR5 and CD4 receptors. 24 hours post-transfection, Cf2Th cells were trypsinized and plated in 96 well plates at a concentration of 6,000 cells per well in DMEM containing 3.5% FBS. Transfected cells were cultured overnight, incubated with serial (5-fold) dilutions (10, 2, 0.4, 0.08, 0.016 μg/ml) of mono- and bi-specific anti-CD4 (iMab, LM52, iMab-PG9, iMab-PG16, LM52-PGT128) and anti-CCR5 (PRO-140, PRO-140-10E8) antibodies in a volume of 50 μl DMEM for one hour, and then infected with 50 μl of virus stock (5,000 IU) in the absence of DEAE dextran, which is toxic to Cf2Th cells. After 48 hours, Cf2Th cells were lysed and analyzed for luciferase expression using a Synergy H4 Hybrid microplate reader (Bio-Tek) with Gen5 version 1.11 software. Relative infectivity was calculated by dividing the luciferase units of wells containing antibodies by the luciferase units of wells lacking antibodies. Half-maximal inhibitory concentrations (IC_{50}) were determined by linear regression. HIV-1/SG3, which requires CXCR4 for entry, was used as a negative control. All monoclonal antibodies were tested in triplicate and the average of 3 replicates is reported.

CD4 T cell based neutralization assay. Left-over blood samples from health examinations of uninfected chimpanzees housed at the Yerkes Regional Primate Center were shipped at room temperature (131) and peripheral blood mononuclear cells (PBMCS) were isolated by gradient centrifugation using Ficoll-Paque Plus (GE Healthcase Life Sciences). Chimpanzee CD4+ T cells were enriched using non-human primate CD4 MicroBeads (MACS Miltenyi Biotec) and magnetic cell sorting (Militenyi Biotec), stimulated with staphylococcal enterotoxin B (Sigma-Aldridge) for 12 to 15 hours (3 μg/ml), and subsequently co-cultivated with autologous monocyte-derived macrophages for optimal activation. After 5 to 6 days, CD4+ T cells were removed from the macrophage feeder layer, placed into DMEM with 10% FBS, and incubated with 30 U/ml interleukin-2 (IL-2). To test the neutralizing capacity of anti-HIV-1 monoclonal antibodies and immunoadhesins, serial dilutions (10, 3.3, 1.1, 0.36 and 0 μg/ml) were incubated with SIVcpzMT145 and HIV-1/SG3 viral stocks (10,000 IU) for one hour at 37°C, and the mixture was then added to $5x10^5$ activated chimpanzee CD4+ T cells in a total of 500μl DMEM containing 10% FBS and 30 U/ml IL-2. The CD4bs antibody VRC01 and uninfected cells were used as negative controls. To test the neutralizing capacity of anti-receptor antibodies, serial dilutions (10, 3.3, 1.1, and 0.36 μg/ml) were first incubated with $5x10^5$ activated chimpanzee CD4+ T cells for 1 hour at 37°C and then exposed to 10,000 IU of virus stocks. After an overnight incubation, cells were washed three times with PBS to remove antibody and non-cell associated virus, suspended in DMEM with 10% FBS and 30 U/ml IL-2, and plated in triplicate in 96 well plates. Supernatant (50 μl) was harvested every 48 hours, starting at day 3 post infection, and replaced with fresh media. To monitor viral replication, supernatants were tested for reverse transcriptase (RT) activity using a colorimetric assay (Roche). Neutralization was calculated by dividing the infectivity (RT activity) of wells with antibody dilutions by the untreated control wells. IC_{50} values were calculated by linear regression. All monoclonal antibodies were tested in CD4+ T cells from three different chimpanzee donors.

Statistical analyses. Statistical analyses were performed using Prism version 5.0d software (GraphPad) and correlations were assessed using Spearman tests. Geometric mean IC_{50} values were calculated using Microsoft Excel version 14.3.9 software.

Nucleotide sequence accession numbers. The nucleotide sequences of SIVcpz strains LB715 and TAN13 as well as the chimpanzee CD4 and CCR5 genes used for transfection are available under Genbank accession numbers JX178450.1, JQ768416, KP235488 and KP235489, respectively.

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CHAPTER 4

ANTIRETROVIRAL THERAPY IMPROVES SIVCPZ-ASSOCIATED IMMUNODEFICIENCY IN A LONG-TERM EXPERIMENTALLY INFECTED WESTERN CHIMPANZEE (*PAN TROGLODYTES VERUS*)

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SECTION 4.1 – ABSTRACT

Background: Simian immunodeficiency virus of chimpanzees (SIVcpz), the precursor of human immunodeficiency virus type 1 (HIV-1), causes increased mortality and AIDS-like immunopathology in infected chimpanzees (*Pan troglodytes*) in the wild. Surprisingly, however, similar findings have not been reported for captive chimpanzees with experimental SIVcpz infection, raising question about the intrinsic pathogenic potential of this chimpanzee lentivirus.

Findings: Here, we report the first case of SIVcpz-associated immunodeficiency in a captive western chimpanzee (*P. t. verus*), who was infected twenty years ago by intrarectal inoculation with an SIVcpz strain (ANT) from a wild-caught eastern chimpanzee (*P. t. schweinfurthii*). Maintaining virus loads of 10^5 to 10^6 viral copies per ml of plasma for the past 15 years, this chimpanzee developed CD4+ T cell depletion (220 cells/μl), thrombocytopenia (90,000 platelets/μl), and persistent soft tissue infections refractory to conventional antibiotic therapy. Combination antiretroviral therapy consisting of emtricitabine (FTC), tenofovir disoproxil fumarate (TDF), and dolutegravir (DGV), resulted in a precipitous decline of plasma viremia (<200 copies per ml) and the rapid resolution of all soft tissue infections. However, difficulties associated with oral administration led to low drug levels in the plasma and the development of FTC resistance mediated by escape mutations identical to those observed in HIV-1 infected humans (M184V/I).

Conclusions: These data indicate that SIVcpz can cause CD4 T cell decline and AIDS-like immunodeficiency in chimpanzees of all major subspecies, including *P. t. verus* apes that are not naturally infected with SIVcpz. Moreover, SIVcpz-associated immunodeficiency can be effectively treated with drugs developed for HIV-1, including infections with strains as divergent as SIVcpzANT. However, just like in HIV-1 infected humans, strict adherence must be ensured to prevent the emergence of drug resistance.

SECTION 4.2 - FINDINGS

SIVcpz naturally infects members of two subspecies of the common chimpanzee (*Pan troglodytes*), the central subspecies (*P. t. troglodytes)* in west central Africa and the eastern subspecies (*P. t. schweinfurthii*) in the Democratic Republic of the Congo (DRC) and countries to the east [1-5]. The other two subspecies in west Africa (*P. t. verus*) and Nigeria/Cameroon (*P. t. ellioti*) are not naturally infected with this virus [3, 6, 7], because SIVcpz represents a complex recombinant of ancestors of SIVs infecting monkeys on which chimpanzees prey [8]. This mosaic genome structure, which is a hallmark of all SIVcpz strains, indicates that chimpanzees acquired SIVcpz by cross-species transmission, but only after *P. t. verus* and *P. t. ellioti* had diverged and were geographically separated from the other two subspecies [6, 8]. Like HIV-1 in humans, SIVcpz has been found to be pathogenic in wild-living chimpanzees. Comprehensive natural history studies in Gombe National Park, Tanzania, revealed that SIVcpz-infected chimpanzees have a 10-16 fold increased risk of death, and can develop CD4+ T cell depletion and an AIDS-like immunopathology [9, 10]. Moreover, SIVcpz infected females exhibited lower birth rates and higher infant mortality compared to non-infected females [9], and one community with high SIVcpz prevalence rates suffered a catastrophic population decline [11]. Thus, SIVcpz differs from non-pathogenic SIVs, such as those infecting sooty mangabeys and African green monkeys, in its ability to cause clinical immunodeficiency and premature death [9-11].

P. t. verus comprises the great majority of captive chimpanzees worldwide, with 95% of founder animals in the United States having originated in West Africa [6, 12]. This explains the paucity of SIVcpz infections in US and European primate centers, although a handful of wild-born SIVcpz infected *P. t. troglodytes* and *P. t. schweinfurthii* apes have been identified in African sanctuaries [12-16]. One *P. t. troglodytes* ape (Goran, CAM155) rescued in Cameroon and studied in captivity for several years developed CD4 T+ cell decline, severe thrombocytopenia, weight loss, and recurring infections, and ultimately died of an AIDS-like illness [13]. A second *P.*

t. schweinfurthii ape (Noah) captured in the DRC and subjected to immunological and virological analyses in a European primate center also exhibited thrombocytopenia and some immunopathology [17], but was lost to follow-up after the transfer to a rescue center for exotic animals in the Netherlands. A third *P. t. troglodytes* ape (Marilyn), who was wild-caught in Africa and utilized for biomedical research in the US, had normal laboratory and clinical findings, but died at age 26 after giving birth to still-born twins [14]. Finally, a number of bushmeat orphans identified in Gabon (GAB1 and GAB2) and Cameroon (CAM3, CAM4, CAM5, CAM13) were shown to be SIVcpz infected, but none of these were studied prospectively since most died shortly after being rescued [15, 16]. Thus, only three naturally SIVcpz infected apes have been studied longitudinally for an extended period of time.

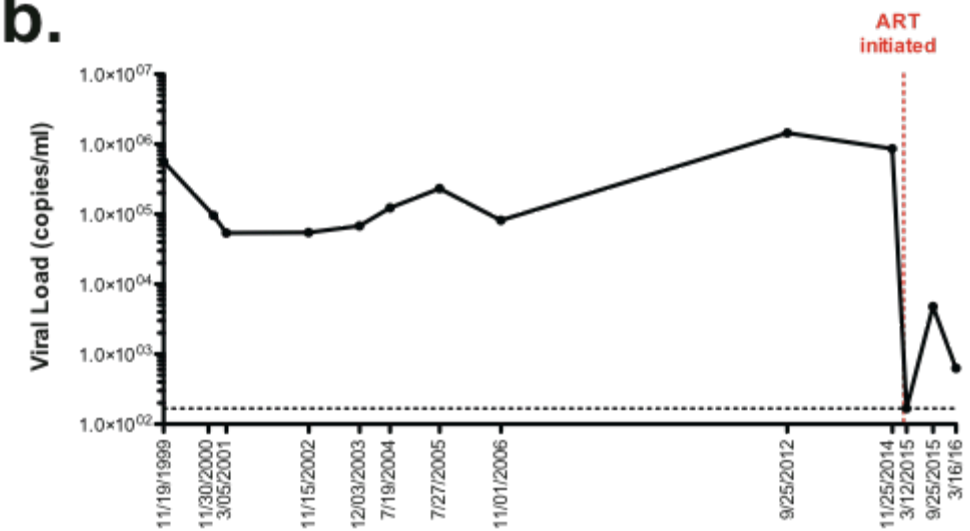
In addition to bushmeat orphans, who acquired SIVcpz prior to their rescue, a handful of chimpanzees have also been experimentally infected with SIVcpz in captivity [17, 18]. Of eight *P. t. verus* apes that were exposed to the same strain of SIVcpz (ANT) by various routes (intra-venous, intra-rectal, and intra-vaginal), six became productively infected [18]. Three of these subsequently died of cardiomyopathy, a frequent cause of death in captive chimpanzees unrelated to SIV infection, while the other three remained clinically healthy after as many as 16 years post-infection. The seeming absence of disease progression in these animals prompted speculation that the increased mortality associated with SIVcpz in the wild was due to factors other than, or in addition to, the lentiviral infection [17]. It was also suggested that members of the *P. t. verus* subspecies, which are not naturally infected, may be relatively more resistant to SIVcpz pathogenicity and/or that the SIVcpzANT strain used for experimental infection was somehow attenuated [17]. Here, we studied one of these chimpanzees after an additional 4-year follow-up, documenting disease progression and clinical immunodeficiency requiring antiretroviral therapy.

Examining the neutralizing antibody responses in long-term HIV-1 and SIVcpz infected chimpanzees [19], we previously discovered that one SIVcpz infected chimpanzee at the Chimp

Fig. 1

a.

b.


ART initiated
Viral Load (copies/ml)
1.0×10^07
1.0×10^06
1.0×10^05
1.0×10^04
1.0×10^03
1.0×10^02
11/19/1999
11/30/2000
3/05/2001
11/15/2002
12/03/2003
7/19/2004
7/27/2005
11/01/2006
9/25/2012
11/25/2014
3/12/2015
9/25/2015
3/16/16

c.

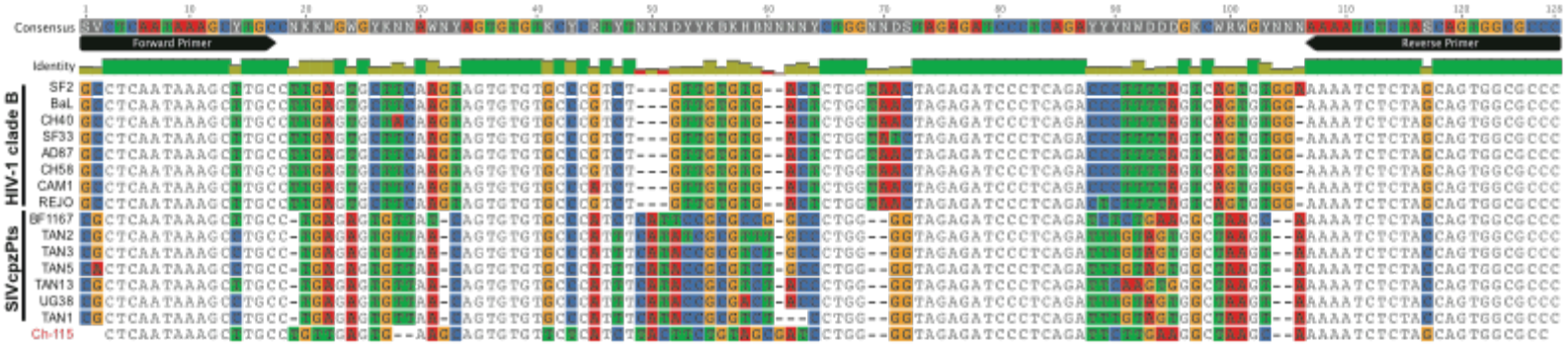

Consensus
Forward Primer
Reverse Primer
Identity
HIV-1 clade B
SIVcpzPts
SF2
BaL
CH40
SF33
AD87
CH58
CAM1
REJO
BF1167
TAN2
TAN3
TAN5
TAN13
UG38
TAN1
Ch-115

Fig. 1. SIVcpz infection in Cotton. (a) Cotton (Ch-115) after initiation of antiviral therapy. (b) Determination of plasma virus load using a validated RT-qPCR method that detects both SIVcpz and HIV-1 [35]. The date when antiretroviral therapy was first initiated (01-19-15) is shown by a red dashed line. (c) Alignment of HIV-1 clade B and SIVcpz*Pts* sequences in the LTR region. Positions where any one of the sequences differs from the others are highlighted in red (A), green (T), orange (G), and blue (C), with dashes denoting gaps introduced into the alignment. The locations of the primers used to amplify this region are shown in black.

Haven sanctuary exhibited particularly high plasma viral loads. This 39-year old male, named Cotton (also termed Ch-115; Fig. 1a), had been experimentally infected with SIVcpz in 1996 after an initial inoculation with HIV-1 [18]. The virus stock was generated by transferring blood from an infected wild-caught eastern chimpanzee (Noah, Ch-No) [20] to his uninfected cage mate (Niko; Ch-Ni), and using plasma collected during the acute phase of the infection for intra-rectal inoculation [18]. Follow up studies showed that Cotton experienced an initial drop in CD4+ T cells counts immediately after SIVcpz infection, followed by the partial restoration and then gradual decline of CD4 T cell counts over time [17]. The most recent CD4+ T lymphocyte counts, performed 18 years post-infection, showed 220 cells/μl (Table 1), compared to 1,500 cells/μl before the infection [17]. Cotton also had a low platelet count of 90,000/ul (normal range 130,500 - 379,930/μl) [21], indicating thrombocytopenia, low albumin levels (2.0 g/dL, normal range 3.3-4.1 g/dL) [21], suggesting malnutrition or chronic inflammatory disease, and persistent soft tissue infections (Table 1). The latter were first noted after his arrival at Chimp Haven and included a necrotizing infection of the hand as well as purulent anal fistulas of the butt pad that persisted despite repeated debridement and administration of multiple rounds of broad-spectrum antibiotics over the course of several months (Table 1). The low CD4+ T cell counts, thrombocytopenia, hypoalbuminemia, and treatment-refractory soft tissue infections strongly suggested that Cotton suffered from SIVcpz induced immunodeficiency.

To confirm that Cotton's clinical symptoms were indeed a consequence of long-term SIVcpz, and not HIV-1, infection, we determined his virus load in the most recent plasma sample as well as in a limited number of archived samples that were still available for the period between

Table 1: Clinical history of Ch-115 since his arrival at Chimp Haven sanctuary in 2006

Date	Clinical symptoms	Treatment Initiated	Platelets (per ul)	CD4+ T cell count (cells/ul)	Viral load (copies/ml)
12/15/09	Wound on hand				
12/19/09	Infection of hand	Oral antibiotics for 7 days			
1/4/10	Necrotizing infection of hand	Debridement			
9/15/10				229	
11/20/11	Draining fistula near anus	Antibiotic wash			
9/25/12					1,440,622
10/25/14	Draining fistula near anus	Oral antibiotics for 7 days			
11/2/14	Draining fistula near anus	Oral antibiotics for 7 days			
11/6/14	Multiple fistulas near anus with thick exudate and sloughing skin	Oral antibiotics for 14 days			
11/10/14	Bloody diarrhea	Ulcer medication			
11/19/14	Draining fistula near anus	Oral antibiotics for 7 days			
11/25/14	Draining fistula near anus	i.v. and topical antibiotics	101,000	220	861,000
12/2/14	Draining fistula near anus	Antibiotics for 30 days			
12/9/14	Exudate near anus still present				
1/19/15	Initiation of combination antiretroviral therapy	Truvada (emtricitabine 200 mg / tenofovir disoproxil fumarate 300 mg) and dolutegravir (50 mg)			
2/10/15	Anal fistulas completely healed				
3/12/15	Blood analysis				167
9/25/15	Blood analysis		90,000		4,760
3/16/16	Blood analysis		91,000		631

1999 to 2014. Since Cotton was exposed to HIV-1 prior to SIVcpzANT inoculation [18], we used a sensitive RT-qPCR method designed to amplify both HIV-1 and SIVcpz strains by targeting highly conserved regions in the viral LTR [22]. This analysis revealed plasma viral titers ranging from 54,000 RNA copies/ml in 2001 to 1,441,000 RNA copies/ml in 2012 (Fig. 1b), significantly higher than previously reported for some of these same time points [17]. Since our RT-qPCR approach was rigorously validated, using both human and chimpanzee plasma samples of known viral content as determined independently by a clinical laboratory, the previous results likely represent an underestimation of Cotton's viral loads. To determine whether SIVcpz, HIV-1, or both comprised Cotton's infection, we sequenced the 121 base pair LTR region used for RT-qPCR analysis (Fig. 1c). Amplicons were prepared for next-generation sequencing (Nextera DNA Library Prep Kit, Illumina, La Jolla, CA, USA), run on a MiSeq (Illumina, La Jolla, CA, USA), and the resulting reads were mapped to available HIV-1 and SIVcpz reference sequences (SIVcpzANT has not been sequenced in the LTR region). This approach yielded 11,679 paired reads, all of which mapped to SIVcpz*Pts* sequences (Fig 1c), indicating that Cotton was solely infected with SIVcpzANT.

Longitudinal samples for SIVcpz infected chimpanzees are extremely rare. To examine the patterns of SIVcpz diversification, we used single genome sequencing (SGS) to characterize his *env* quasispecies over time [23]. Briefly, plasma RNA was extracted, reverse transcribed, and the resulting cDNA end-point diluted to single template levels. 3' half genomes were amplified as described [24], subjected to MiSeq sequencing, and amplicons comprising more than one variant discarded. Phylogenetic analysis of the resulting *env* gene sequences revealed progressive diversification (Fig. 2a), similar to what has been described for HIV-1 infected individuals [25]. For example, the C2-V5 region of the HIV-1 *env* gene has been shown to diverge at a rate of approximately 1% per year, stabilizing after 4 to 6 years at around 4-8% diversity [25]. Using DIVEIN [26] to determine *env* gene diversity in Cotton, we found lower mean diversity values of 1.5 – 3.5% (Fig. 2b), but a similar rate of divergence of 0.77% per year, as measured by linear

Fig. 2

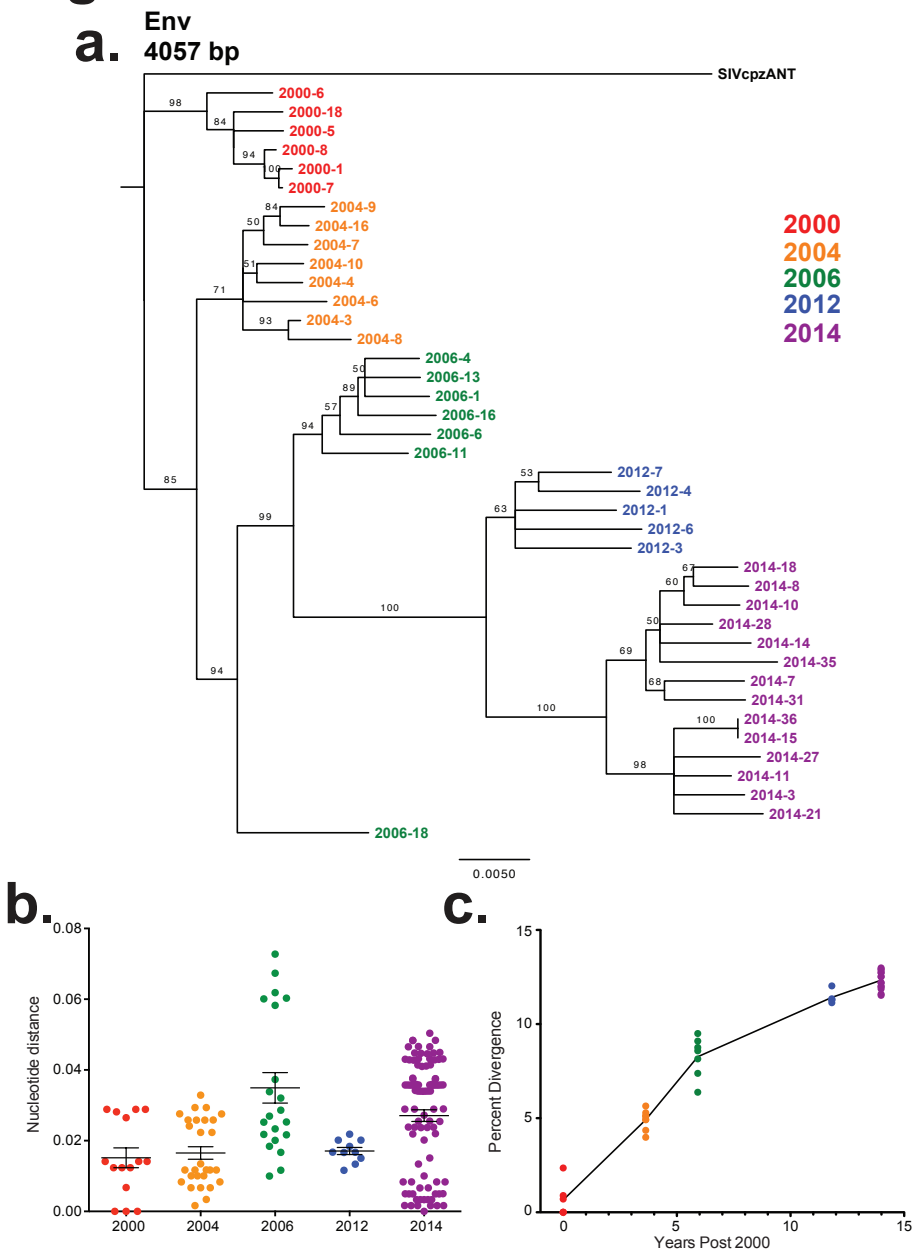


Fig. 2. Evolution of the SIVcpzANT *env* quasispecies over time. (a) Phylogenetic analysis of SGS-derived full-length *env* nucleotide sequences collected at different timepoints following infection. Samples are colored by timepoint and bootstrap values are indicated. The scale bar indicates 0.005 substitutions per site (b) Within sample diversity. The pairwise distance of each sequence shown in panel (a) from other sequences within the same sample is plotted. Mean and SEM are indicated. (c) Pairwise distance of each sequence shown in panel (a) from the year 2000 consensus sequence expressed as % divergence. A black line connects the mean of each timepoint.

regression (Fig. 2c). Of note, because our earliest sample was collected four years after the initial infection, we measured this divergence using the consensus sequence of the year 2000 time point. Excluding the last 2014 time point, the divergence rate was 0.92% per year, nearly identical to the 1% per year estimate for HIV-1 infected humans. Estimates of viral divergence rely on sufficient sequencing depth, and further analyses may be required for more precise estimates. However, these results suggest that the rate of SIVcpz *env* evolution is very similar to that of HIV-1.

Given Cotton's laboratory and clinical findings, combination antiretroviral therapy (cART) was initiated (Table 1), combining the nucleoside reverse transcriptase inhibitors tenofovir disoproxil fumarate (TDF) and emtricitabine (FTC) with the integrase strand transfer inhibitor dolutegravir (DGV), and administering daily doses of 300, 200 and 50 mg, respectively. This regimen was chosen because it potently inhibits HIV-1 and HIV-2 in humans [27-30], as well as SIVmac in experimentally infected rhesus macaques, a virus even more distantly related to HIV-1 than SIVcpzANT [31]. As shown in Fig. 1b, cART reduced SIVcpzANT viremia in Cotton to undetectable levels at the first blood draw after initiation of therapy (Table 1). Moreover, the treatment refractory anal fistulas healed completely within 6 weeks of onset of therapy (Table 1), demonstrating the effectiveness of this regimen in achieving viral control and clinical improvement.

Cotton received cART daily by oral administration in fruit juice, but caretakers noticed that he frequently spit out or did not completely ingest his medication. We thus continued to monitor his virus load in blood samples obtained during his bi-annual medical evaluations. Indeed, six months following initiation of therapy, viremia became again detectable (4,760 RNA copies/ml) (Fig. 1b). To determine whether this rebound was due to insufficient drug levels, one pre-treatment and three post-treatment plasma samples were sent to the Clinical Pharmacology and Analytical Chemistry Core at the UNC Center for AIDS Research to determine plasma

concentrations. This analysis revealed drug levels that were significantly below the 90% inhibitory concentrations (IC_{90}) of DGV (64 ng/ml) and FTC (51 ng/ml) [32, 33] and the 50% inhibitory concentration (IC_{50}) of TDF (60 ng/ml) [34] for all three post treatment samples (Fig 3a). However, higher values were obtained after caretakers diluted the medication in more juice, which also resulted in a concomitant decline of viremia to 631 RNA copies/ml at the last time point. Since insufficient drug levels can lead to drug resistance [35], we used SGS to sequence full-length *pol* genes in pre- and post-treatment plasma samples. An amino acid alignment revealed the emergence of M184V and M184I mutations between the first and second post treatment samples collected at 3/12/15 and 9/25/15, respectively (Fig. 3b). These same mutations are known to confer resistance to FTC in HIV-1 infected humans, and usually are the first drug resistance mutations in a failing ART regimen [35, 36]. Mutations associated with TDF and DVG were not observed, even though drug levels were one log lower than the respective IC_{90} values. Following the discovery of the low drug concentrations and subsequent emergence of FTC resistance, the drug delivery strategy was changed from dissolving tablets in juice to adding crushed tablets to food.

Taken together, we report here the first case of clinical immunodeficiency in a captive western chimpanzee, who was experimentally infected with a naturally occurring SIVcpz*Pts* strain. While Cotton's laboratory values do not meet the formal definition of AIDS (<200 CD4+ T cells per ul), his low CD4+ T cell count, refractory soft tissue infections, and marked clinical improvement after initiation of cART strongly suggests that SIVcpz caused his immunodeficiency. We also show that SIVcpz infection and associated immunodeficiency can be effectively treated with antiretroviral therapy developed for HIV-1, but that a lack of adherence to the drug regimen can result in the emergence of drug resistance mutations similar to what has been observed in HIV-1 infected humans. Importantly, a considerable number of HIV-1 and SIVcpz infected chimpanzees are still housed in US primate centers. This study, together with previous reports of disease progression in experimentally HIV-1 infected chimpanzees [37-39], indicates that animalswith high viremia and reduced CD4+ T cell counts are at high risk of developing AIDS and

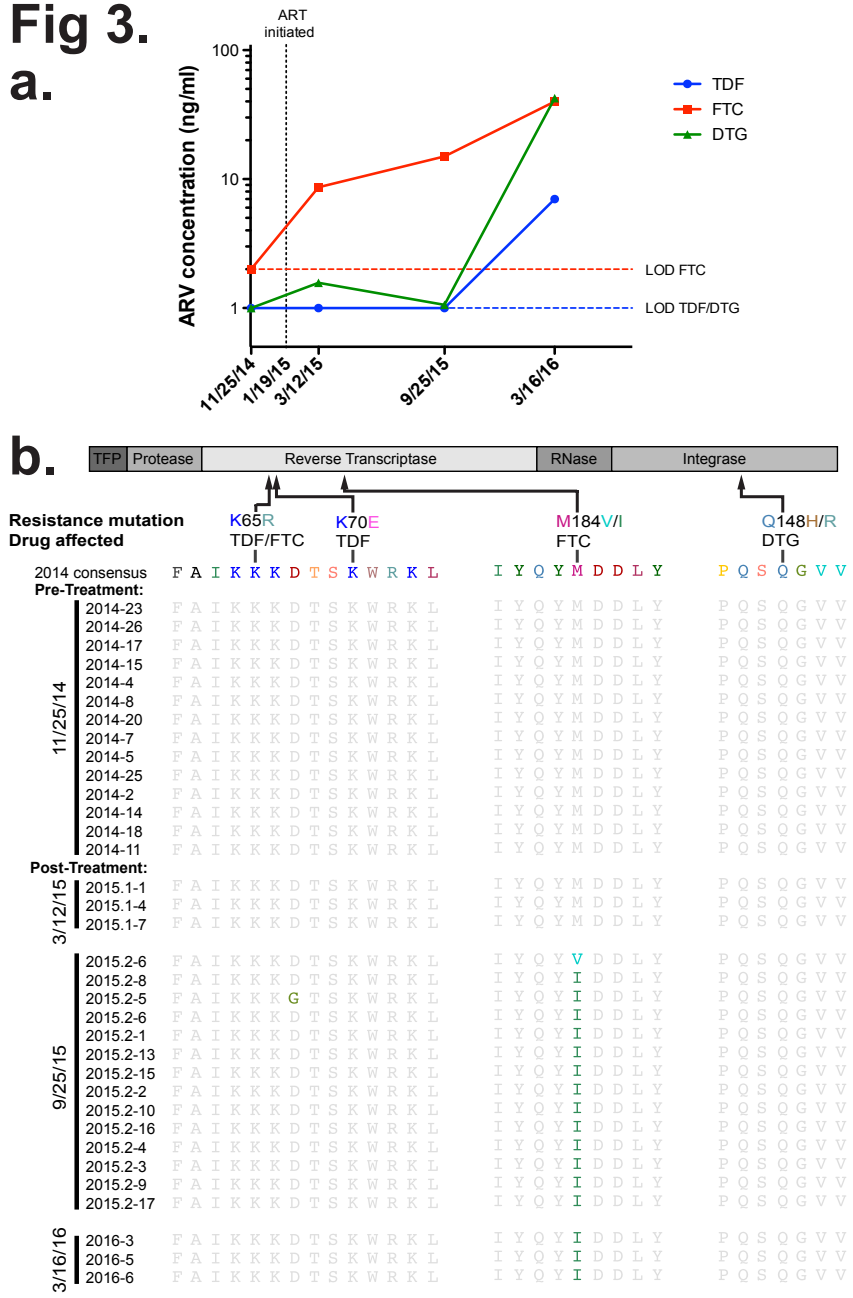


Fig. 3. Emergence of resistance to FTC. (a) Plasma concentrations of tenofovir disoproxil fumarate (TDF, blue), emtricitabine (FTC, red), and dolutegravir (DTG, green) are shown for one pre-treatment and three post-treatment samples as depicted in Fig. 1b. Limits of detection (LOD) for FTC as well as TDF and DTG are shown as red and blue dotted lines, respectively. (b) A map of the *pol* gene products is shown with mutations associated with TDF, FTC, and DTG resistance in HIV-1 infections outlined below. An amino acid alignment of the regions associated with resistance mutations is shown, with residues altered from the pre-treatment consensus in color. Residues identical to the pre-treatment consensus are shown in grey.

should be treated to prevent clinical disease.

This study also questions the hypothesis that SIVcpz may be intrinsically less pathogenic than HIV-1 or that SIVcpzANT is attenuated [17]. Of the six chimpanzees initially infected with this virus, three died of cardiomyopathy, a common cause of death in captive chimpanzees, with one of these animals (Niko) exhibiting a CD4+ T cell count of 276 cells/ul at the time of death [17]. In addition to Cotton, two other experimentally SIVcpz infected chimpanzees are still alive. One was reported to have a CD4+ T cell count of 115 cells per μl, which represents an AIDS-defining criterion [17], while the second animal (Ch-284) has thus far maintained low viral loads and normal CD4 counts 16 years post infection [17]. The average time to AIDS progression in untreated HIV-1 infected humans has been estimated to range between 10-15 years (www.who.int). Given the long survival times of some experimentally SIVcpz infected chimpanzees, it is possible that progression to disease is more protracted in captive settings, although the small number of animals that have been studied precludes definitive conclusions. The clinical and laboratory findings reported here for Cotton demonstrate that SIVcpz is a pathogenic lentivirus that can cause immunodeficiency and clinical disease both in captivity and in the wild.

SECTION 4.3 - DECLARATIONS

Ethics approval and consent to participate

Sample collection was approved by the Institutional Animal Care and Use Committees of ChimpHaven and the Southwest Foundation for Primate Research.

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Authors' contributions

HJB designed and executed most experiments, and analyzed all data. RJJ and CSB provided veterinary care and clinical data for Cotton. EFK and GHL assisted with viral diversity analyses. FBR, TD, and YL provided technical assistance. MSS, KJB, JL, and GMS provided critical medical advice. HJB and BHH wrote the manuscript with input from all authors.

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CHAPTER 5

CONLUSIONS AND FUTURE DIRECTIONS

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Section 5.1

Investigating SIVcpz Pathogenesis in the Gut

HIV-1 infection of humans is associated with significant gut pathology, including depletion of immune cells, disruption of intestinal barriers, immune activation, and diarrhea (4, 5). Consistent with this, several groups have associated dysbiosis of the gut microbiota with HIV-1 infection (6-13). To determine if SIVcpz infection has a similar impact on the gut of infected apes, I investigated the fecal microbiome of wild-living chimpanzees in Chapter 2. Using 16S sequencing of longitudinal samples from 24 SIVcpz infected and 26 uninfected animals, I found no significant difference in bacterial abundances, or microbiome richness and evenness between these groups. Principal coordinates analysis revealed that microbiomes of SIVcpz infected animals do not cluster together by beta diversity, and are instead interspersed with those of SIVcpz uninfected animals. These data suggest that unlike reports of HIV-1 infected humans, SIVcpz infection of chimpanzees is not associated with a distinct gut microbiome. However, samples collected from animals within six months of their eventual AIDS-related death showed pronounced compositional shifts in the gut microbiota. This destabilization was not observed in earlier samples from these animals, nor in samples taken from other animals within six months of non-AIDS-related deaths. Studies of SIV infected rhesus macaques revealed an expansion of viral populations in fecal samples (14), so I also investigated the fecal virome of SIVcpz infected animals using metagenomic sequencing and quantitative PCR. I found a trend towards increased titers of adenovirus, but no significant differences between infected and uninfected animals.

The disparity between reports of the microbiota in HIV-1 and SIVcpz infection could be due to differences in how the virus affects the gut or, alternatively, it could be due to differences in the cohorts tested. The chimpanzees used in my study all share a common home range, diet,

have similar social interactions, and do not take any medications. Studies of humans are much harder to control for these factors. Before the publication of my manuscript, studies of the gut microbiome of HIV-1 infected individuals utilized samples from European and American patients exclusively. With sexual contact of men who have sex with men (MSM) being the most predominant mode of transmission in the US and Europe, samples from MSM likely comprised a majority of those tested. Lifestyle differences (diet, exercise, etc.) are known to have a significant effect on the composition of the gut microbiota (15). Thus, if not properly controlled, disparities between HIV-1 infected and uninfected individuals could be due to the lifestyle of HIV positive patients, rather than the infection itself. In fact, a recent study published after the submission of my manuscript found that MSM had bacterial profiles very similar to those reported for HIV-1 infected individuals, including a higher prevalence of *Prevotella* and increased richness and diversity compared to non-MSM individuals (16). When controlling for sexual orientation, this group found no evidence for HIV-1 associated gut dysbiosis in two large European cohorts. Another group that investigated MSM HIV-negative controls found that these subjects exhibited a *Prevotella*-rich microbiome, previously associated with HIV-1 infection (17). Only minor disturbances to the gut microbiome were observed in HIV positive individuals using this group of MSM controls (17). To my knowledge, no other group controlled for sexual practice in their studies of HIV and the microbiome. Thus, the significant compositional changes observed in earlier reports of HIV-1 infected individuals may well be entirely the result of other factors associated with MSM individuals, instead of HIV-1 infection itself. A recent report by Monaco and colleagues investigated the fecal microbiome of a cohort from Uganda, where heterosexual transmission predominates. They found that changes in microbiome composition and richness were only observed in patients with low CD4+ T cell counts (<200 cell/s ul), and were not associated with HIV status (18). They also found an absence of clustering of HIV infected individuals using beta diversity measures, even in those with CD4+ T cell counts below 200 cells/ul. While these findings require replication, they are consistent with our findings in SIVcpz

infected chimpanzees, with stability of the gut microbiome despite HIV-1/SIVcpz infection until progression to AIDS.

The virome of SIVcpz infected chimpanzees also seemed relatively unaffected compared to previous reports in the literature. A study of rhesus macaques showed dramatic expansion of gut viruses in SIVmac infected animals, including increased frequencies of multiple families of viruses (14). In comparison, I found only insignificantly increased frequencies of simian adenovirus. This may also be due to differences in the types of cohorts studied. The SIVmac infected macaques used in the fecal virome study progressed very rapidly to AIDS, with several animals requiring euthanasia before the completion of the study. In comparison, most of the animals included in my study had been infected for years with no observable signs of illness. Thus, proliferation of gut viruses might only be observed in animals experiencing the late stages of SIV infection. In support of this, a more recent study of SIVmac infected macaques found an expansion of adenoviruses only in animals that rapidly succumbed to AIDS, and not those that survived through the duration of the study (19). Importantly, these researchers also found that several families of viruses were significantly associated with gastrointestinal disease regardless of SIV infection, supporting the probable role of AIDS-related disease in virome expansion and not the infection itself. A recent study of a Ugandan cohort found no differences in the frequencies of gut viruses between HIV-positive and negative subjects (18). However, patients with a CD4+ T cell count less than 200 cells/ul showed increased frequencies of adenoviruses and anelloviruses. Thus, two studies of both humans and macaques found adenoviruses to be associated with low CD4+ T cell counts. Adenovirus is a source of diarrheal disease and has been previously linked to SIV-associated pathology in the gut of infected macaques (14). Long-term longitudinal studies of clinical signs of illness in Gombe chimpanzees revealed that SIVcpz infected females showed significantly increased incidences of diarrhea (20). In addition, an SIVcpz infected chimpanzee that exhibited diarrhea when sampled within a few months of his death showed the highest adenovirus titer of all of the animals tested in my study. Thus, while not being a feature common to all infected animals, the insignificantly increased titers of

adenoviruses observed in SIVcpz infected wild chimpanzees may reflect SIV-associated pathology.

Based on my results and recent well-controlled studies in humans and macaques, dysbiotic microbiota seem to be associated only with late stages of disease and not HIV/SIV infection per se (Fig 5.1). Thus, it seems that many of the gut manifestations associated with HIV-1 infection, including local CD4+ T cell depletion, immune activation, microbial translocation, and impairment of the gut barrier (4), are not sufficient to cause significant microbial dysbiosis. Instead, gut pathology associated with end-stage disease may be causing the observed alterations of gut microbiota. AIDS progression is often characterized by enteric disease, including the outgrowth of opportunistic infections that cause diarrhea (cytomegalovirus, Cryptosporidium, Giardia lamblia) and idiopathic diarrhea (21, 22). In addition, chronic wasting is a hallmark of AIDS, and is caused in part by gut malabsorption, highlighting the abnormal gut of AIDS patients. Thus, AIDS-associated symptoms such as diarrhea, gastrointestinal infections, lethargy, and decreased appetite may be to blame for the observed alteration of gut microbiota, rather than the virus itself, since these factors have been shown to also alter microbial communities in non-HIV infected patients (15, 23-26).

Additional studies are needed to confirm the causes and consequences of SIVcpz and HIV-1-associated microbiome dysbiosis. Confirmation that HIV-1 infection is not sufficient to induce significant enteric microbial dysbiosis would require better-controlled studies of the American and European cohorts who originally showed microbiomes distinct from uninfected individuals. Test groups and controls should have similar sexual practices, with similar diets and activity levels, and should be properly controlled for antibiotic and antiretroviral use. All of these factors impact gut microbial communities (15, 16, 27, 28) and may be significantly different between HIV-1 infected and uninfected individuals.

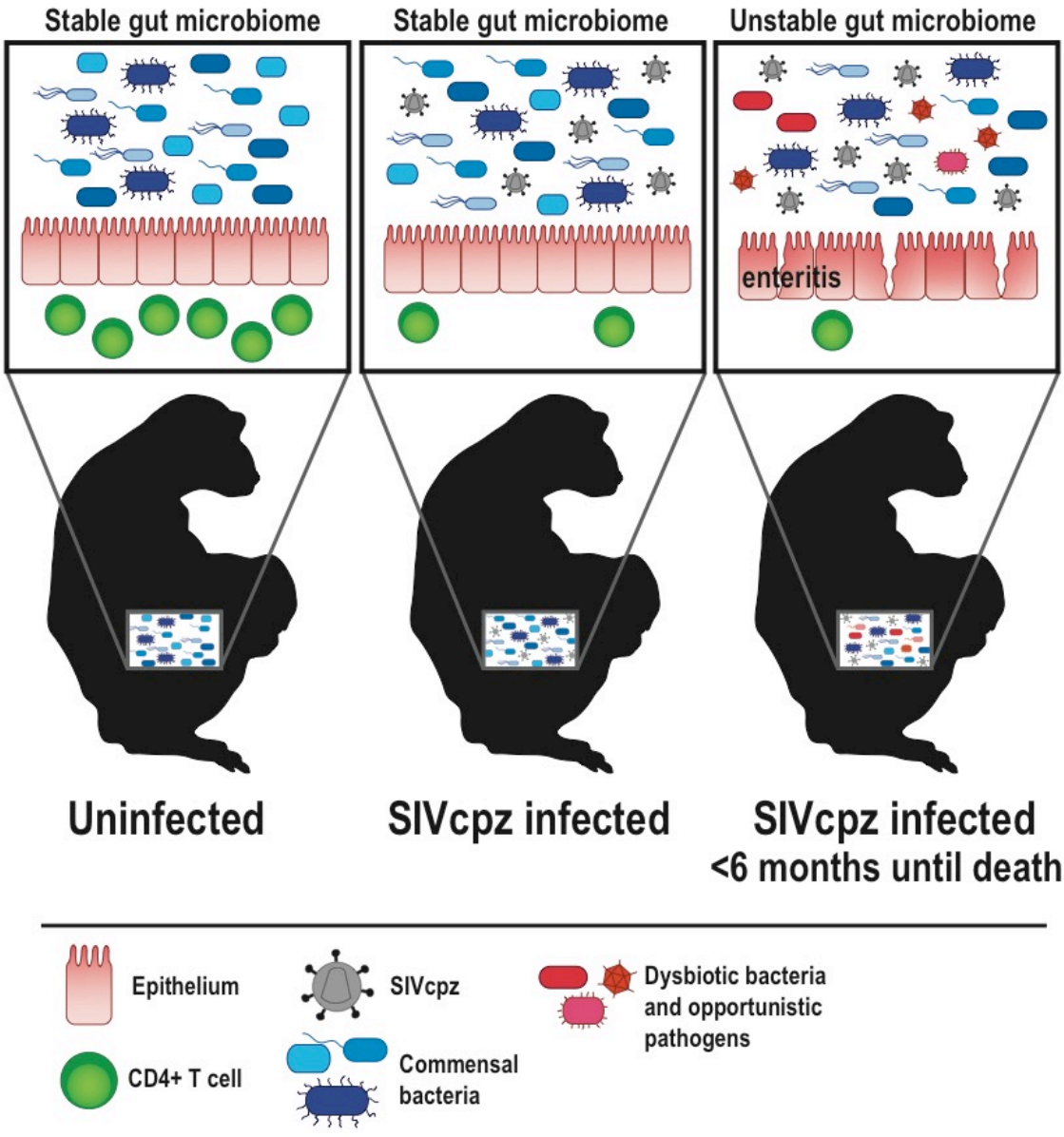

Fig. 5.1. Model of the gut microbiome in SIVcpz infected chimpanzees based on results reported in Chapter 2, as well as studies in HIV-1 infected humans and SIV infected macaques. Destabilization of the gut microbiome occurs only at the end stages of SIVcpz infection. AIDS-associated symptoms and gastrointestinal disease may destabilize gut microbial populations with opportunistic infections likely contributing.

Practical uses of the finding of dysbiotic bacteria in late-stage SIVcpz infection for the study of chimpanzees remains to be determined. Given the lack of bacterial or viral species associated with progressive SIVcpz infection, the gut bacteriome and virome of SIVcpz infected chimpanzees are unlikely to be useful as markers of disease in non-habituated communities. However, detecting a rise of microbial beta diversity in a SIVcpz infected chimpanzee in Gombe could foreshadow rapid disease progression and death, prompting intensified observational studies to record signs of ill health associated with SIVcpz disease progression. It could also help alert field workers to more closely track the animal's whereabouts, in order to increase the likelihood of recovering tissues for necropsy, which is a rare occurrence in wild-living chimpanzees.

A different fecal marker of disease progression would be extremely useful, especially in non-habituated ape communities where SIV-associated morbidity and mortality is near impossible to determine. In HIV-1 infected humans, plasma viral load is monitored as a sign of disease progression. In wild chimpanzees, viral loads cannot be determined with accuracy, as quantitative RT-PCR (qRT-PCR) lacks the sensitivity to detect RNA in fecal samples. A qRT-PCR method was recently developed that targets the strong stop DNA region of viral genome and is broad enough to detect multiple groups of HIV-1 and SIVcpz and SIVgor strains (29). I found that this method is able to detect SIVcpz in Gombe chimpanzee fecal samples, potentially because strong stop DNA is more stable than single stranded RNA. However, I found fecal viral loads to vary substantially over time using this method. The cause of this remains to be determined, but I suspect that sample degradation may strongly influence viral load estimations. HIV-1 infection is known to cause inflammation of the gut (30), resulting in lymphocyte activation, increased virus replication, and accelerated disease progression (31, 32). Since it is impractical to assess cytokine mRNA levels or T cell activation markers in wild-living chimpanzees, we could use proteins associated with inflammation as alternative markers (33, 34). Fecal calprotectin, a by-product of neutrophils, was found to be significantly increased in HIV-1 infected individuals (35,

36), and in preliminary studies, we have found this to also be true for SIVcpz infected chimpanzees. However, again we found fecal calprotectin values to be too variable in longitudinal samples to yield a reliable marker of disease progression. Thus, further studies are needed to find a reliable marker of disease progression, which would also allow the monitoring of disease in non-habituated chimpanzees and SIV infected gorillas.

Section 5.2

Anti-SIVcpz Interventions in Wild and Captive Chimpanzees

HIV-1 infection of humans can be readily controlled by antiretroviral therapy. However, treatment of wild-living chimpanzees with current antiretroviral regimens is not feasible. Nonetheless, chimpanzees are critically endangered by disease and habitat destruction, and SIVcpz infection is a component of their disease burden. SIVcpz intervention strategies capable of translation to wild ape populations deserve further thought. Broadly cross-reactive neutralizing antibodies (bnAbs) are exciting new tools being developed for long-term treatment and/or prevention of HIV-1. In order to be useful for SIV infected chimpanzees, bnAbs would need to be capable of neutralizing diverse SIVcpz strains. To test this, I examined the ability of existing HIV-1 bnAbs for their ability to neutralize 11 SIVcpz strains derived from wild-living chimpanzees in Chapter 3. I found that most bnAbs derived from chronically infected humans, including those targeting the CD4 binding site and glycan-dependent variable loop epitopes, failed to neutralize the majority of SIVcpz strains. However, antibodies that mimic the CD4 or CCR5 (co)receptors were able to neutralize highly divergent SIVcpz strains. In addition, antibodies that bind to CD4 or CCR5 also potently blocked infection of SIVcpz strains. This work provided new insight into the cross-reactivity of anti-HIV-1 reagents with SIVcpz, and also identified candidates that could be further developed for use in wild-living apes.

Given the very broad HIV cross-reactivity of some bnAbs, I was surprised to find only limited cross-neutralization of even the “best” anti-HIV-1 antibodies to SIVcpz. All SIVcpz infectious molecular clones readily utilize human CD4 for entry, suggesting functional conservation of the CD4 binding site. In spite of this, all 10 of the most broad and potent CD4 binding site antibodies failed to neutralize even a single SIVcpz strain, including at a high concentration of 10 ug/ml. Analysis of the amino acid sequence of SIVcpz and HIV-1 strains revealed that the CD4 binding site of HIV-1 and SIVcpz are only conserved at CD4 contact

residues, and not surrounding motifs. Most patient-derived antibodies targeting gp120 recognize complex conformational epitopes that span genetically and structurally flexible regions (37-40). Variation in the structure or sequence of these epitopes mediates resistance of HIV-1 strains to bnAbs (41). The only envelope site of vulnerability that involves a linear epitope is the MPER domain (42, 43), which is uniquely sensitive to cross-neutralization of HIV-1 antibodies to SIVcpz strains. Thus, variation in the structure, sequence, and glycosylation of the regions that are not required for effective entry likely preclude binding of anti-HIV-1 antibodies to SIVcpz. This suggests that the SIVcpz envelope could be conformationally distinct from HIV-1. SIVcpz has not been crystallized, so no structural information exists. Structures of SIVcpz envelopes could reveal which sites are most highly conserved between HIV-1 and SIVcpz. These sites would likely include regions involved in HIV/SIV entry, and would contain the epitopes of antibodies that are most cross-reactive between HIV-1 and SIVcpz strains. Such epitopes would not only be useful for generating reagents capable of neutralizing SIVcpz strains, they would also likely represent a highly conserved region amongst HIV-1 strains and could serve as targets for the development of HIV-1 antibodies through vaccination.

Engineered antibody-like proteins that mimic CD4/CCR5 and monoclonals that target CD4/CCR5 itself avoid the targeting of non-conserved regions. These reagents are thereby capable of neutralizing diverse SIVcpz strains. Considering the possibility of therapeutic or prophylactic use in chimpanzees, these reagents would need to be optimized to avoid clearance by the chimpanzee immune system, and to increase the potency to maximize their activity. To avoid anti-construct host immune responses, we could generate chimpanzee versions of the most potent constructs. Human and chimpanzee CD4 D1 domains differ at amino acids 34 (I34T), 55 (A55V) and 88 (G88E). Of these, I34T creates an additional N-linked glycosylation site in close proximity to the HIV/SIV Env binding site, which may affect the neutralization properties of the immunoadhesins. Based on passive protection studies in macaques (44) and attainable rAAV-mediated antibody expression levels (45-47), clinically suitable antibodies would need to neutralize SIVcpz with IC50 values of 0.01-0.1 μg/ml. Several anti-CD4 and CCR5 receptor

antibodies meet these criteria, since they neutralize 100% of SIVcpz strains with IC50 values <0.06 μg/ml both in human and chimpanzee cells. Immunoadhesins were also highly cross-reactive, but neutralized SIVcpz strains with much reduced (up to 50-fold) potency compared to HIV-1. Of these, eCD4-Ig, which combines CD4 D1D2 with an IgG Fc and CCR5 mimetic sulfopeptides, is of particular interest because of its small size, exceptional potency against HIV-1 and SIVmac (0.1 nM), and extensive in vitro and in vivo characterization (47). Recent data showed that the macaque version of eCD4-Ig was able to protect monkeys from multiple SHIV challenges when expressed from a recombinant AAV-vector (47). Since rh-eCD4-Ig also did not elicit unwanted anti-antibody responses in macaques, this receptor mimetic seems to represent an ideal candidate to combat SIVcpz infection. However, in order to be of clinical utility, its anti-SIVcpz potency has to be increased. Modeling suggests that eCD4-Ig binds the HIV-1 Env trimer in a "claw-like" fashion, with two sulfopeptides and one CD4 moiety engaging two protomers within the same Env spike (47). I thus hypothesize that the architecture of the SIVcpz envelope differs from that of HIV-1 such that one or more of the eCD4-Ig interaction sites are altered or differently spaced. To test this hypothesis, we could utilize the "molecular ruler" approach developed in the Bjorkman laboratory, which utilizes DNA of known sizes to measure inter-epitope distances on gp120 (48). Since successful cross-linking of spike protomers increases the neutralization potency of antibody-like molecules by more than 100-fold (48), we could use this approach to probe the overall architecture of the SIVcpz spike in comparison to that of HIV-1 and SIVmac, which are highly sensitive to eCD4-Ig. This information could then be used to modify the length and flexibility of the eCD4-Ig hinge region, which was previously shown to be critical for the neutralization capacity of eCD4-Ig in macaques. It is also possible that CCR5mim, a human CD4i antibody-derived peptide, does not optimally interact with the chimpanzee CCR5 co-receptor, which differs from human and macaque CCR5 in one and two amino acid residues near the N-terminus, respectively (49). We could mutate this peptide in the chimpanzee version of eCD4-Ig, similar to what was done in human eCD4-Ig to improve its avidity and neutralization potency (50-52). We could also change the Fc portions of our antibodies or immunoadhesins to chimpanzee

IgG1 and/or IgG2 to ensure conservation of ADCC and other effector functions.

Before investigating safety and efficacy in chimpanzees, the candidate bNabs and immunoadhesins would first need to be tested in a smaller animal model. Bone marrow-liver-thymus (BLT) mice recapitulate human hematopoietic lineages in a mouse model (53-55), and may serve as an effective model given the ability of SIVcpz strains to establish infection in human PBMCs. One group recently investigated the ability of BLT mice to support SIVcpz infection, and found that multiple SIVcpz strains replicated efficiently in this model (56). Immunodeficient NSG mice could alternatively be reconstituted with chimpanzee PBMCs, as was already performed using macaque cells (165). I performed a small pilot study in NSG mice reconstituted with primary human CD4+ T cells (n=6) and found that SIVcpz replicates efficiently in these animals and that viral loads can be transiently reduced following the administration of potent antibodies. Two mice were treated with a cocktail (0.5 mg) of two CD4 immunoadhesins (eCD4-Ig and CD4-218.3-E51mim2), as well as MPER antibody 10E8 and anti-CCR5 antibody PRO140, which resulted in a 4-fold reduction of virus load in one mouse (the other mouse died before a second blood sample could be obtained). Although these results are preliminary, they show that humanized mice are susceptible to SIVcpz infection and can be used to examine the efficacy of anti-SIVcpz neutralizing antibodies. If a primate animal model were desired, a chimpanzee equivalent of the simian-human immunodeficiency virus (SHIV) could be generated, using a new and customizable technique developed by Hui Li and colleagues (57). This method results in a virus containing a SIVmac backbone (allowing for effective replication in macaques) that expresses an HIV or SIVcpz envelope of your choosing (in order to test anti-envelope reagents) (57). bNabs and immunoadhesins should be tested as both preventatives and therapeutics, and tested individually and in combination to determine their combined potency.

The use of anti-SIVcpz interventions in the wild would only be considered if safety and efficacy was first proven in captivity, and if a chimpanzee community were in serious jeopardy at least in part due to SIVcpz infection. Given the increasing threat of infectious pathogens to

chimpanzee survival, there is an urgent need to develop methods to curb the spread of disease in wild apes, and groups have already undertaken such interventions for other ape pathogens (58). Habituated chimpanzees like those in Gombe National Park would provide ideal candidates because field workers can easily identify infected individuals. Thus, infected chimpanzees could be targeted individually with anti-SIVcpz therapy, benefiting the infected individual by preventing disease progression, but also benefiting the community by reducing the likelihood of transmission. Using this approach, SIV uninfected chimpanzees would not need to be subjected to the intervention. Apes in sanctuaries could also benefit from treatment in order to prevent AIDS-like illness and transmission to other animals housed in the same facility (59). In addition, orphaned chimpanzees who became SIV infected through vertical transmission could be treated before their release into the wild, preventing the introduction or spread of SIVcpz into their new community (60). In these scenarios, long-lasting treatment would be useful, as daily oral ART may not be feasible. Vectored antibody gene delivery, which results in extremely long-lasting antibody expression, could be combined with long-acting antiretrovirals, which are currently in development (61), in order to effectively reduce and maintain low levels of virus replication without the risk of viral escape (Fig. 5.2). The development of antibody-based therapies would also provide an important proof-of-concept that could be applied to other diseases that affect chimpanzee populations such as anthrax and Ebola (62, 63). Effective monoclonal antibodies could be delivered using the same gene therapy vector, resulting in a fast and long-lasting strategy to curb the spread of pathogens for which a safe and effective vaccine and/or therapy has not yet been developed.

Over 100 chimpanzees have been subjected to HIV and SIV infection in captivity (64). Unlike wild chimpanzees, captive chimpanzees could be treated with daily ART. However, the efficacy of existing ARVs against SIVcpz strains has not been reported. In Chapter 4, I described the SIVcpz-associated immunodeficiency and subsequent ARV treatment of a captive, experimentally infected chimpanzee. This work revealed several important findings that could

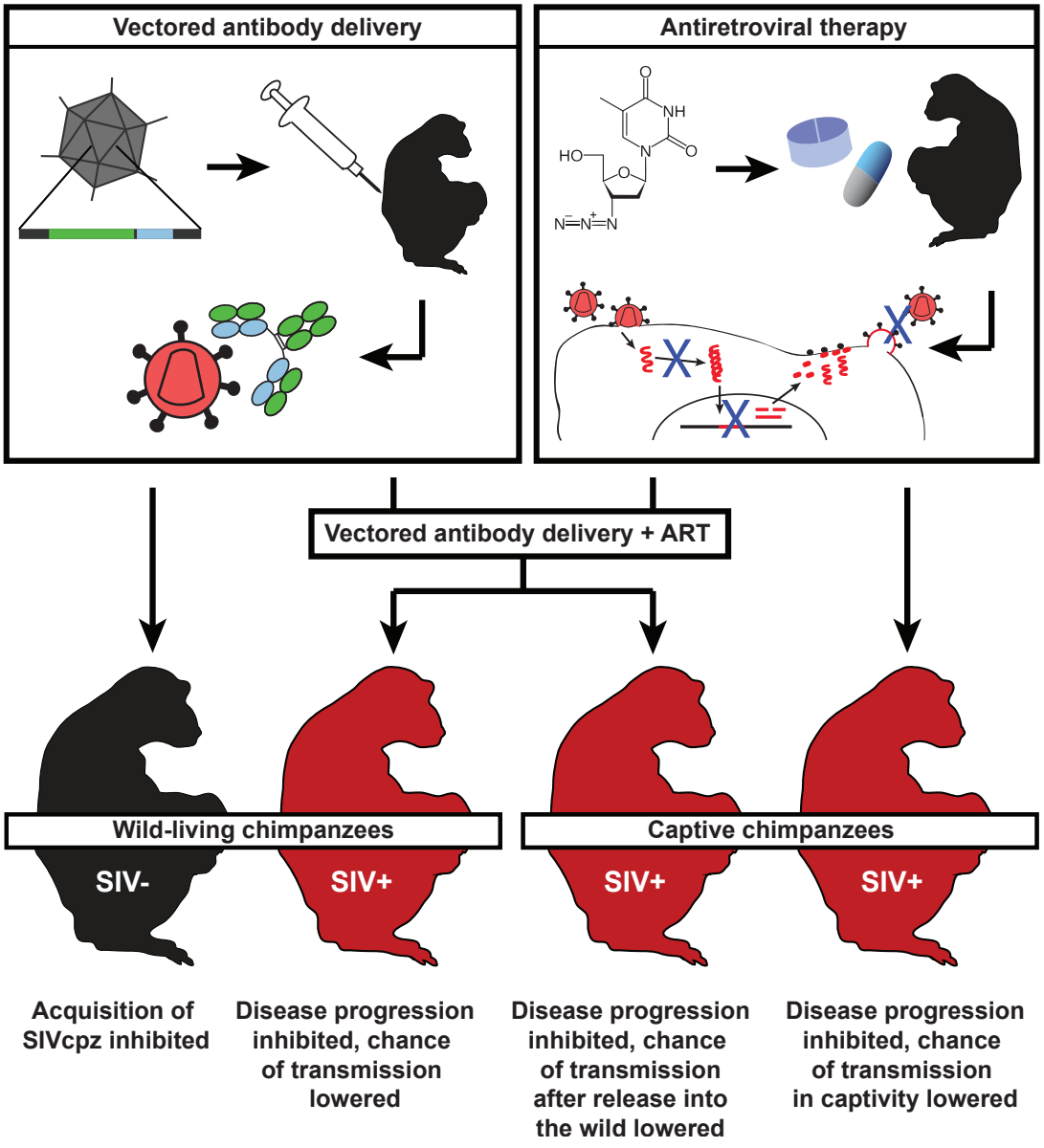

Fig. 5.2. Potential uses of SIVcpz intervention strategies. Vectored antibody delivery involves the packaging of genes encoding a monoclonal antibody in a gene therapy vector. This is delivered to skeletal muscle via injection, which continuously produces the SIVcpz neutralizing antibody protein. Antiretroviral therapy involves ingestion or injection of small molecules that block SIVcpz replication, usually by targeting enzymes essential for HIV/SIV reverse transcription, integration, and maturation. Treatment of chimpanzees in the wild would require the long-lasting products of antibody gene delivery. Chimpanzees in a captive environment could be given daily ART. Animals with active SIV replication would require initial treatment with ART before antibody gene delivery to preclude antibody escape during the initial ramping stages of antibody concentrations.

influence the veterinary care of captive chimpanzees infected with SIVcpz. First, SIVcpz is sufficient to drive immunodeficiency, even in animals with ready access to veterinary care and free of the pathogens and environmental hazards that may burden wild-living communities. Second, *P. t. verus* chimpanzees, a subspecies that is naturally free of SIVcpz infection, are susceptible to SIVcpz-associated immunodeficiency. Third, ARV regimens designed for treatment of HIV-1 infected humans can effectively reduce SIVcpz replication *in vivo*. Fourth, insufficient levels of these drugs can drive viral escape through mechanisms similar to HIV-1 infected humans. This has important implications for the care of captive SIVcpz infected chimpanzees. To our knowledge, no recommendations to monitor the infection and disease state of HIV and SIV infected captive chimpanzees exist in primate centers or sanctuaries. These animals can progress to AIDS and possibly even transmit the infection, while existing ARV regimens designed for humans can prevent this unnecessary disease. Thus, captive HIV/SIV infected chimpanzees would greatly benefit from regular viral load and CD4+ T cell enumerations and appropriate therapies.

In summary, these results contribute to the understanding of the biology of SIVcpz, as well as provide a foundation to combat this infection in chimpanzees. Due to the endangered status of wild chimpanzees and the ethical inability to perform invasive studies in these animals, studies of SIVcpz in chimpanzees are inherently constrained. However, studies of noninvasively collected material have thus far yielded an understanding of the natural history of SIVcpz in its natural host, clues to its mechanism of pathogenesis, and potential methods to alleviate SIVcpz-associated disease in chimpanzees. Further work and innovation could not only benefit chimpanzees, but also inform our understanding of HIV-1.

Section 5.3

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