The Influence of Mucosal Inflammation on Early Events Following SIV Infection in Rhesus Macaques

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Abstract

Mucosal inflammation is associated with increased HIV infection. However, the virological and immunological mechanisms associated with mucosal inflammation are not well understood. The goal of the studies is to determine the impact of mucosal inflammation on virus acquisition and early events following infection using SIV infection of rhesus macaques. We introduced two forms of mucosal inflammation: induced gingival inflammation (chapter 3 and 4) and *Haemophilus ducreyi*-induced penile inflammation (chapter 5). After successful induction of mucosal inflammation, macaques were non-traumatically exposed to SIV through either the oral or penile route (corresponding to the site of inflammation).

The goal of the study is to understand the ability of mucosal inflammation to influence SIV acquisition, virological and immunological changes associated with SIV infection in rhesus macaques. Following SIV challenges, the macaques were monitored for the frequency of SIV infection, number of founder viruses, plasma viral loads, immune changes at mucosal sites and systemic tissues. Although our results showed that overall SIV acquisition rates (through oral or penile challenge) were not significantly affected in the presence of mucosal inflammation (in the form of induced gingivitis or *Haemophilus ducreyi* related genital inflammation), we observed that mucosal inflammation was associated with an increased number of transmitted founder viruses, indicating that mucosal inflammation can alter host susceptibility to SIV, and by analogy HIV. The influence of mucosal inflammation on SIV pathogenesis in rhesus macaques was assessed. Following oral SIV infection, examination of the level of immune modulators in the oral cavity (gingival crevicular fluid and gingival

biopsy) showed an increased production of IFN-α, OAS and CXCL10 in SIV infected macaques with gingival inflammation, compared to SIV infected control macaques during acute infection. In addition, higher levels of plasma IFN-α and IFN-γ in the peripheral blood were observed in SIV infected macaques with gingival inflammation during acute infection. Furthermore, these macaques were more likely to have OAS upregulation and myeloperoxidase production in the peripheral lymph nodes. These results indicate that mucosal inflammation can modulate early immunological events following SIV infection.

Taken together, these studies demonstrate that mucosal inflammation has influences on not only host susceptibility to SIV but also early immunological changes following SIV infection. The effects between mucosal inflammation and SIV infection have the potential to impact the early stages of the SIV infection with potential implications for treatments or vaccines development.

Chapter 1: General introduction and literature review

HIV epidemics and public health importance

The origins of human immunodeficiency virus-1 and -2 (HIV-1, HIV-2) have been an important subject of research. Recent studies indicate that HIV-1 and HIV-2 infection in humans were the result of zoonotic transmissions of simian immunodeficiency virus (SIV) from African monkeys. Human immunodeficiency virus-1 (HIV-1) originated from SIV in central African chimpanzees (128) and HIV-2 came from SIV in Sooty Mangabeys from West Africa (73). Hunting, butchering, and the trade of monkeys as pets may have contributed to the cross-species transmission of HIV (146). At least three different cross-species transmissions from monkey species to humans occurred, resulting in at least four genetically divergent groups (M, N, O and P groups) of HIV-1 infection in the human population (324, 349, 374). While HIV-2 infection is mostly localized in certain African countries (325), HIV-1 spread to many countries and caused the HIV pandemic, with the M group being responsible for the majority of HIV cases worldwide.

So far, the oldest sample from an HIV infected human (ZR59) can be traced back to 1959 in Congo. The HIV-1 sequences in plasma from ZR59 indicate that their ancestor is group M HIV-1 subtype D (412). Another early case of HIV infection is also identified in Congo (DRC60) (401). HIV-1 sequences from a paraffin-embedded lymph node biopsy back in 1960 indicate that the potential ancestor of DRC60 is group M HIV-1 subtype A. Both viral sequence analyses demonstrate that HIV-1 existed and spread in human populations long before the pandemic started. Whether ZR59 and DRC60 ancestral HIV-1 variants were as pathogenic as current HIV-1 isolates is

unknown. However, it is not until 1981 that the first immunodeficiency patient is officially reported by CDC (1). Later in 1983, HIV-1 is identified as the causative agent of acquired immunodeficiency syndrome (AIDS) (32, 51, 126). Thirty years after identifying the first AIDS patient, HIV-1 infection has become the most important public health issue with an estimated 33 million people being infected by HIV-1 worldwide and more than 2 million new infections every year (2010 UNAIDS report). Epidemics in Sub-Saharan Africa are the largest in the world with 22.5 million people living with HIV, which is around two-thirds of the total number of HIV-1 infected people globally. HIV-1 epidemics are still expanding in Eastern Europe and Central Asia (2012 UNAIDS global report). Obviously, effective ways to stop the HIV-1 epidemic are urgently needed.

Adding to this already massive problem is the fact that HIV-1 infection fuels the incidence and mortality rates of other infectious diseases, such as tuberculosis (TB) and malaria, because HIV-1 severely affects the host immune system and thus decrease the ability of HIV-1 infected patients to control environmental or pathogenic microbes. HIV infection may increase the risk of developing active TB, and TB can accelerate HIV disease progression (265). There are an estimated 1.37 million TB cases in HIV-1-positive individuals worldwide, resulting in 456,000 TB-related deaths in this population, which is the leading cause of death in HIV-1 infected patients (WHO). Similarly, HIV-1-infected patients have a higher risk to develop severe malaria infection (122, 397). Conversely, malaria infection in HIV-1-infected patients increases HIV replication and morbidity (159, 205). The interaction between HIV and other pathogens is complex and efforts need to be made to tackle these problems, such as

how pathogens modulate host immune responses against both pathogens in co-infection patients, how to treat HIV/TB or HIV/malaria co-infected patients and how to alleviate the epidemics of these infectious diseases.

HIV and its replication cycle

HIV-1 and HIV-2 are positive sense, enveloped RNA viruses, classified in the retroviridae family lentivirus genus. HIV virion consists of viral cores, including two copies of the viral genome wrapped with nucleoprotein (NP) along with viral enzymes (reverse transcriptase, protease and integrase) inside capsid, and outer matrix with the envelope protein (gp120 and gp41) integrated in a lipid bilayer which virus acquires when budding out of the cells (Figure 1-1A). The viral genome of HIV-1 is about 9.3 kilobases (kb) in length, similar to other retroviruses, and its genome encodes Gag, Pol, and Env. HIV-1 has six additional reading frames encoding vif, vpr, vpu, tat, rev, and nef regulatory proteins (Figure 1-1B).

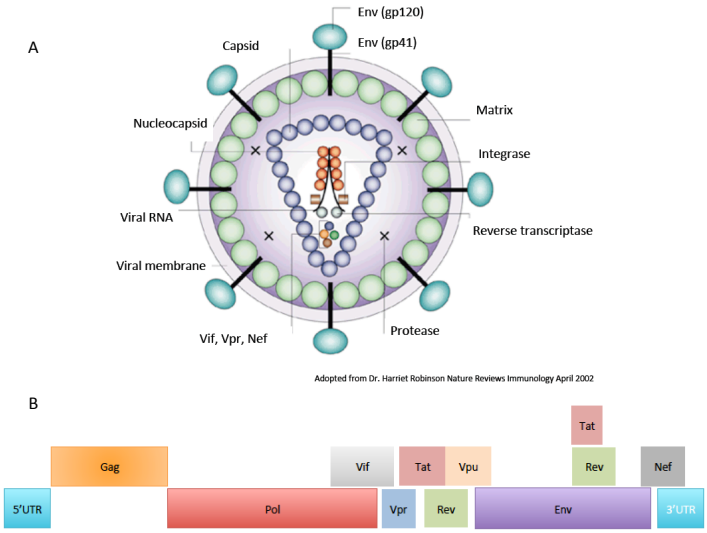


Figure 1-1 The structure of HIV-1 virion (A) and viral genome encoding products (B). The picture was adopted from Dr. Harriet L. Robinson, Nature Reviews Immunology 2, 239-250 (April 2002)

The initial step of HIV infection starts with the HIV envelope protein gp120 interacting with the CD4 molecule, the major receptor present on CD4 T cells, macrophages and dendritic cells. In addition to CD4, HIV also requires co-receptor binding (predominantly CCR5 or CXCR4) to enter the cell, which defines the cell types that HIV can infect (Figure 1-2). Following HIV envelope binding to its receptors, a conformational change of the envelope protein results in the fusion between the virus and the cell membrane, which releases the viral core into the cytoplasm (Figure 1-2).

Once the viral core is released into the cytoplasm, viral reverse transcriptase begins the process of converting viral RNA into a double strand DNA copy (proviral DNA) (Figure 1-2). Reverse transcriptase is an error-prone RNA dependent DNA polymerase without proofreading activity. Therefore, replication errors occur during the reverse transcription process throughout the viral genome and contribute to the generation of mutant viruses (35, 166, 286, 303). As a result, each HIV-1 virion is different within a pool of variants and is known as quasispecies. Up to 35% differences exist between different subtypes of HIV and even within the same subtype, up to 20% of viral diversity can be observed (129, 308). The presence of high HIV-1 diversity indicates that a future HIV-1 vaccine needs to provide protection against a diverse pool of HIV-1. In addition, high error rates during HIV-1 replication also lead to the development of drug resistant strains and immune escape variants, which set the bar even higher to control HIV-1 replication.

Following reverse transcription, viral integrase mediates the transportation of proviral DNA into the nucleus and insertion into the cellular genomic DNA (Figure 1-2).

The site of integration seems to be randomly distributed over the entire host genome. Once integrated, proviral DNA becomes part of the host genomic DNA and replicates with the cells as they divide. These infected cells may be long lived and cannot be distinguished from uninfected cells by the immune system because viral antigens were not expressed in this state. This raises the difficulties of completely eliminating HIV after a systemic infection has been established because all cells that carry proviral DNA need to be destroyed.

After viral RNA synthesis in the nucleus, these viral replication intermediates were transported into the cytoplasm where viral protein synthesis and virion assembly occur. After budding from the cell, these infectious particles were ready to initiate a new round of infection (Figure 1-2).

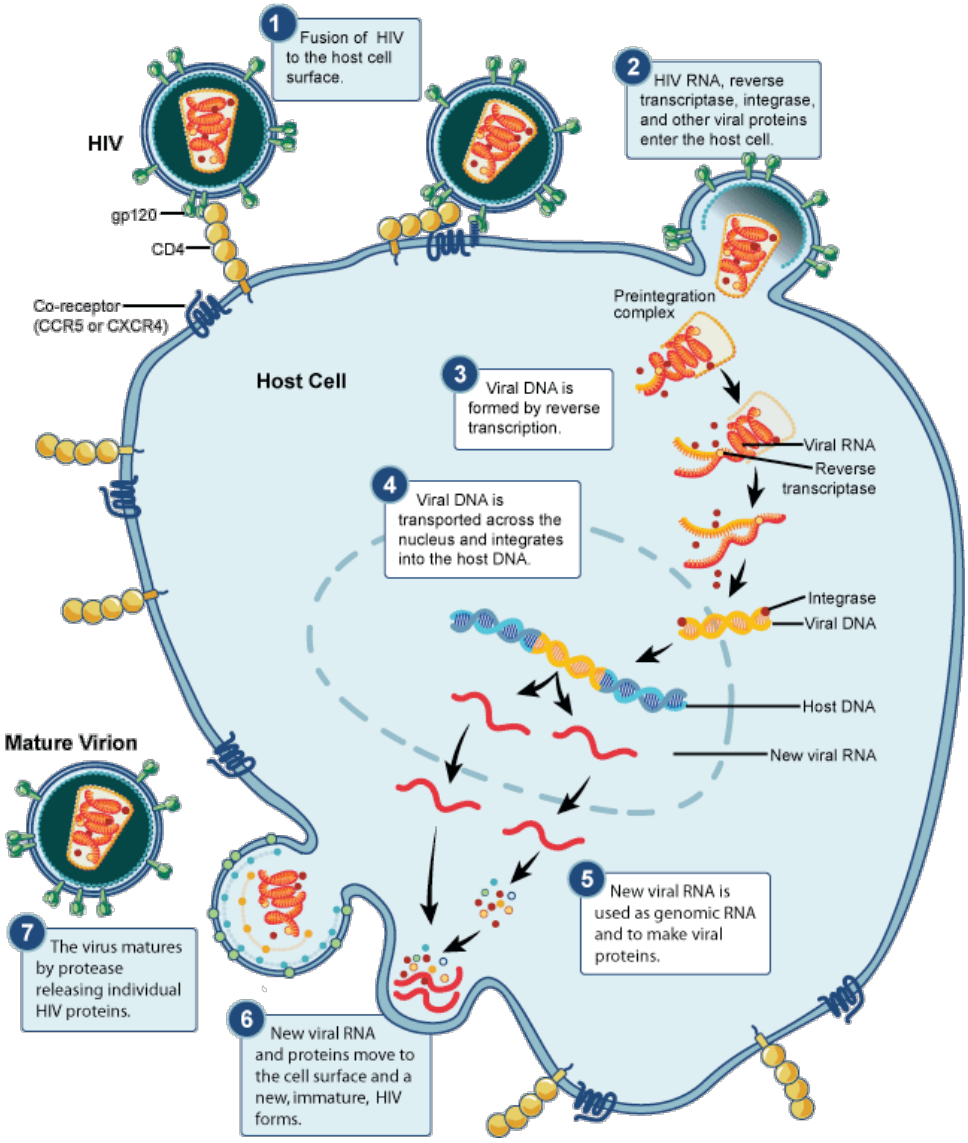


Figure 1-2 HIV-1 replication cycle.

HIV replication involves 1. Virus entry through membrane fusion; 2. uncoating to release viral core into the cytoplasm; 3. Reverse transcription of viral RNA genome to viral DNA; 4. Viral DNA transports and integrates into host DNA; 5. Viral RNA replication and protein synthesis; 6. Virion assembly nearby cell membrane; and 7. Release mature virions.
Figure is provided by National Institute of Allergy and Infectious Diseases Health& research A to Z website (http://www.niaid.nih.gov/topics/HIVAIDS/Understanding/Biology/pages/hivreplicationcycle.aspx)

HIV-1 infection and pathogenesis

Clinically, HIV infection can be divided into three stages: primary/acute infection, latency/chronic infection, and AIDS (acquired immunodeficiency syndrome) (Figure 1-3). During the first few weeks of initial infection, HIV rapidly replicates in CD4 cells and disseminates into systemic tissues with an average of 10^6 to 10^8 viral RNA copies/ml in peripheral blood. In addition to high viral load in peripheral blood, recent studies suggest that the gut-associated lymphoid tissue (GALT) is the major site of HIV viral replication (143, 351). Clinically, individuals may experience a flu-like illness including fever, headache, rash or sore throat, or even no symptoms at all. People infected with HIV tend to be more infectious during the acute stage (50, 161, 403), probably due to the high viral load in the host, as well as relatively infectious/transmission-prone viruses were the major population in newly infected patients. However, most people were not aware of their HIV status until later stages. This imposes difficulties for HIV prevention and the urgent need for early HIV diagnosic tools. The level of HIV circulating in the peripheral blood declines within a few weeks with a partial rebound of CD4 T cell counts because both innate and adaptive arms of the immune system were activated to control some levels of viral replication (279, 290) as well as the balance were reached between CD4 T cell renew and death (13, 200). Around this time, HIV tests might reveal infection by detecting HIV specific antibodies. As most people have a window period that ranges from three to twelve weeks when levels of HIV-specific antibodies were still too low to be detected, retesting is recommended after three months to confirm initial HIV test results. The window period creates another difficulty to study early events of HIV

infection in humans because the timing of HIV infection can only be estimated and clinical specimens were generally not collected for detailed analysis.

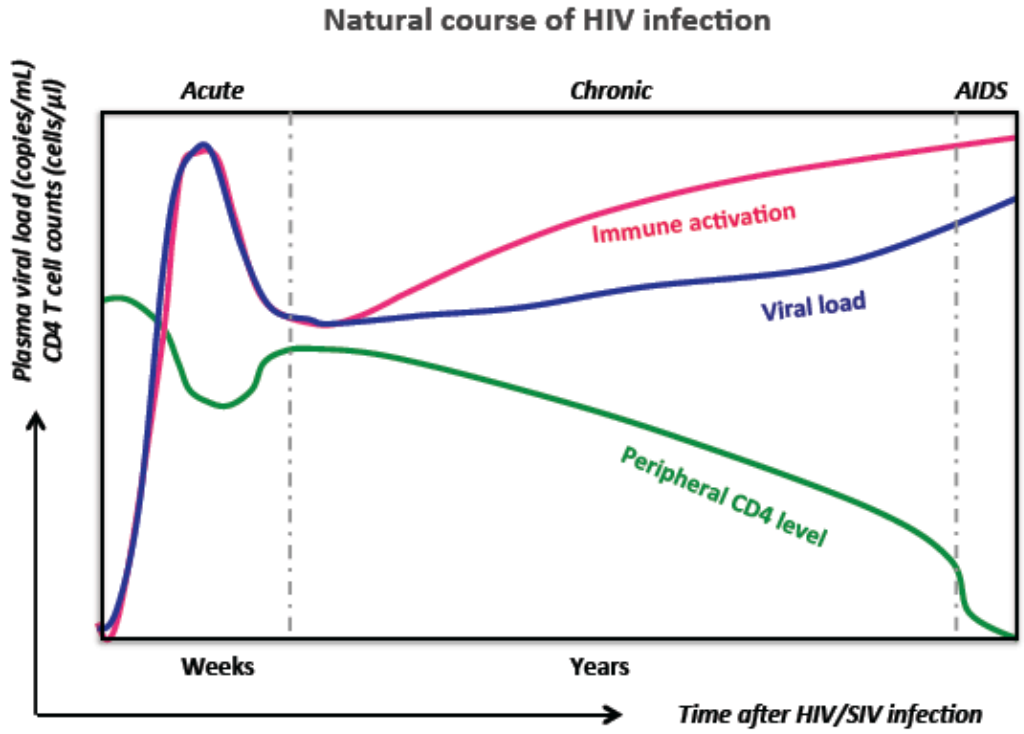


Figure 1-3. Natural history of HIV-1 infection.

Shown are general overview of disease progression after HIV infection without antiviral treatment as virus replicates in high levels (blue line), CD4 T cells numbers in the peripheral blood (green line) decrease, and sustained immune activation (pink line)

The second stage of HIV infection is a result of balancing immune control and productive virus replication to a certain level (known as viral set point) for a period of time (Figure 1-3). The chronic HIV infection stage can range from weeks to years. Within this period, patients generally do not show clinical symptoms but were still infectious enough to spread HIV if prevention strategies were not employed. Clinically, the CD4 T cells decline and plasma viral load were used to monitor the overall health

of HIV infected patients and both parameters serve as guideline for the timing to initiate antiviral therapy in general.

Chronic HIV infection is also characterized by sustained immune activation, including elevated levels of multiple inflammatory cytokines and chemokines (207, 274, 320), rapid immune cell turnover (59, 402), and high expression of proliferation and activation markers on immune cells (227, 300), compared to HIV uninfected healthy individuals. Several studies suggest that sustained chronic immune activation is the driving force of HIV-related immune dysfunction and is a better indicator of disease progression (134, 227) than viral load or CD4 T cell numbers. Immune activation associated with rapid turnover and higher expression of activation or proliferation markers of immune cells is not restricted to CD4 T cells, but occurs also on other immune cells (87, 88, 201, 387). The causes of sustained immune activation in HIV infected patients were multi-factorial and not well understood. One could be the direct effect of HIV replication and its byproducts, such as HIV gp120 binding and signaling through receptors expressed on immune cells, i.e. CD4, CCR5, α4β7 (18, 310, 330), or HIV accessory proteins interfering with cell cycle pathways (344). Second source of immune activation could be the host immune response against HIV. As HIV replicates in the host, the immune system keeps sensing HIV as foreign antigens and initiates both innate and adaptive immune responses to target HIV. This complex interaction has dual effects as the immune system causes selective pressure on HIV, resulting in HIV mutations and in the development of newly evolved HIV populations in the host that initiate additional immune responses. As a result, the host immune system is constantly activated trying to catch up with HIV evolution. The third

source of immune activation may be induced by microbial translocation due to mucosal tissue destruction caused by massive HIV replication in the GALT. As a result, microbial products could translocate from mucosal sites to the systemic circulation. Studies have shown that HIV infected individuals have increased plasma LPS (175, 369), which could trigger Toll-like receptors (TLRs) to induce additional immune activation in response to these microbial products. All these potential driving forces for immune activation were associated with excessive production of inflammatory cytokines and chemokines that deregulate the immune system, impair immune cell function, and induce immune exhaustion (335).

The third stage of HIV infection is characterized by clinical immunodeficiency. As HIV infected patients eventually progress to AIDS (generally defined as CD4 counts below 200 cells per μl blood), opportunistic infections, caused by *pneumocystis carinii*, *Candidas Albicans*, or certain types of cancers (such as Kaposi sarcoma) become life threatening because of the failure of the immune system. In some HIV patients, disease progression is also associated with the emergence of HIV species using CXCR4 co-receptor (295, 359), which further depletes naïve T cells (the major population of CXCR4 expressing cells). Without proper treatment, most HIV infected patients will progress to AIDS.

There is a small group of HIV infected individuals that exhibit slow disease progression, referred to as long-term non-progressors (LTNPs). These people may have been infected with HIV for several years but still maintain CD4 T cell counts (higher than 500 cells per μl) and generally have low plasma viral load (less than 10,000 copies per ml) without antiviral therapy. Within LTNPs, a small group is further

classified as elite controllers (ECs) who have undetectable plasma viral loads for years without antiviral treatment. Despite these features, LTNPs or ECs may eventually still progress to AIDS but at much slower rates, compared to the majority of HIV infected patients. The factors associated with slower disease progression in LTNPs were not fully understood and it is believed that multiple mechanisms were involved including viral, immunological and genetic factors (105, 163, 268, 269). Active research is conducted to understand how these LTNPs maintain their CD4 T cell numbers and how ECs undergo spontaneous controlled virus replication, in order to delineate the protective factors against disease progression for novel therapeutic strategies and vaccine development.

HIV treatment and possible cure?

Several antiviral medications have been specifically developed to inhibit HIV replication. Based on the steps of the HIV life cycle, antiviral drugs can be grouped into 6 classes (U.S. Department of Health and Human Services), including: 1) Non-nucleoside reverse transcriptase inhibitors (NNRTIs) and 2) Nucleoside reverse transcriptase inhibitors (NRTIs) which inhibit or block HIV reverse transcriptase activity; 3) Protease inhibitors (PIs), which block HIV protease; 4) Fusion inhibitors, which block HIV entry into CD4 T cells; 5) CCR5 antagonists, which block CCR5 to prevent HIV entry into CD4 T cells; 6) integrase inhibitors, which inhibit HIV integrase activity. Highly Active Antiretroviral Therapy (HAART) with a combination of three or more anti-HIV medications from at least two different drug classes is prescribed to HIV infected patients. The timing to initiate HAART in depends on several factors, such as CD4 T cell counts in peripheral blood, viral load, overall health, age, co-

infection (i.e. active TB) and pregnancy status. Interestingly, recent studies showed that patients who received antiviral treatment during primary infection have different virological and immunological features, such as reduced HIV reservoir pools (158) and HIV specific CD8 T cell profiles similar to long-term non-progressors (63), when compared to patients who received treatment later during infection. These studies indicate that early inhibition of HIV replication is important and the timing of ART initiation might have beneficial long-term effects on HIV disease progression. However, the availability, the adverse effects of ART regimens as well as the cost-effectiveness for early initiation of HAART in HIV infected patients remain undetermined(348, 357, 400). Current guidelines for HIV treatment do not recommend interruption of therapy once HAART is initiated to reduce the possibility of the development of drug-resistant viruses and the potential for further transmission. In addition, the SMART study further demonstrates that antiviral treatment interruption is associated with higher risk of all cause mortality and opportunistic infections (104) possibly due to inflammation and coagulation dysfunction (81, 208, 334).

HAART can slow down the rate of immune system destruction, thus leading to partial repair of immune function, and delay the timing of AIDS development. However, there is no cure currently available for HIV infection. The integrated HIV genome in non-dividing cells is a huge obstacle to completely eliminate the virus from its host. However, the so-called “Berlin Case” gives some hope: a chronically HIV infected patient also suffered from leukemia and received a bone marrow transplantation from a donor with a mutated CCR5 gene (Δ32/Δ32). This mutation is known to prevent infection with CCR5-tropic HIV and the Berlin patient has remained HIV free for more

than 4 years (11, 170). Although this is a very special case, current researchers in the field are encouraged to explore novel therapies to eradicate HIV in patients. So far the field focuses on compounds that can re-activate latent HIV pools to shorten the lifespan of HIV infected cells with the combination of HAART to prevent further CD4 cells being infected. Another way is to genetically engineer autologous CD4 T cells to become HIV resistant and then transfuse these HIV-resistant cells back into patients to reduce HIV target cells availability. There were several studies that evaluate potential drugs, such as SAHA (suberoylanilide hydroxamic acid) to induce HIV replication in latently infected resting CD4 T cells (17), or the use of zinc finger nucleases (ZFNs) to introduce CCR5 gene mutations to generate HIV-resistant cells from stem cells (162). Although these studies were preliminary, with more interest and efforts in the field to look for new approaches and technologies, a cure for HIV infection may be available in the near future.

HIV transmission and prevention

Transmission through blood and by-product

HIV can be transmitted through contaminated blood, such as blood transfusion, sharing needles, syringes or other sharp instruments. The risk of HIV transmission per exposure through contaminated blood and its byproduct is high, especially through blood transfusion (Table 1-1). Today, HIV transmission through contaminated blood transfusion is rare since

Table 1-1. Risks of HIV acquisition

Exposure route	Risk per 100 exposure	References
Blood transfusion	90	(91)
Needle-sharing Injection drug use	0-7.2	(184)
Anal	0.04-3	(92)
Vaginal	0.05-0.26	(121)
Penile	0.056-0.06	(92)
Oral	0-0.04	(92)

donor blood is now routinely screened for HIV. However, sharing needles or syringes is still a major risk factor of acquiring HIV among injecting drug users (IDU). Public health interventions, including behavior changes and health education, are urgently needed to reduce sharing needles or syringes to specifically target this population to reduce HIV transmission.

Sexual transmission

The major route of HIV transmission is through unprotected sexual intercourse with an HIV infected person. Overall, HIV transmission rates through HIV exposure to vaginal, penile, anal and oral mucosa are low, compared to transmission rates through HIV contaminated blood and its byproducts (Table 1-1). For sexual transmission, the risk of male-to-male transmission is greatest during receptive anal intercourse (302). Among heterosexual transmission, women were more susceptible than men (231). Multiple mechanisms contribute to the differential risk of HIV acquisition between men and women including menstrual cycles, hormone contraception in women, and tissue structure differences in the genital tract of men and women, as well as HIV exposure period. A number of observational studies also suggested that male circumcision is associated with reduced HIV infection in men (27, 139). Indeed, several randomized clinical trials have shown that male circumcision can reduce the risk of HIV acquisition in young men by up to 60% (20, 28, 138) and therefore can be recommended as another HIV prevention strategy for men where safe and affordable circumcision is available. However, circumcision of HIV infected men does not reduce HIV transmission to their female partners (394) and safe sexual

practices are important to prevent HIV transmission. Condom usage is an effective way to prevent HIV transmission as well as other sexual transmitted diseases. However, cultural acceptability and many other physiological factors may reduce people's will to use condoms. Therefore, the development of topical used microbicide before or soon after HIV exposure is also an important research area that could lead to alternative products to effectively prevent HIV transmission, especially to vulnerable populations.

Several studies have demonstrated that the level of HIV in plasma or genital secretions from HIV infected patients is an indicator of HIV transmission, with higher viral loads being associated with a higher risk of transmission (26, 305). With the effectiveness of antiretroviral therapy (ART) to inhibit virus replication, studies were conducted to examine if ART can be incorporated into HIV prevention strategies. In 2011, the HPTN052 study demonstrated that early initiation of HAART in HIV-infected patients reduced sexual transmission to their HIV uninfected partners (76). In addition, the CAPRISA-004, iPrEx, Partner and TDF2 studies demonstrated that either topical use of tenofovir (TDF) gel as a microbicide or taking ART pills as pre-exposure prophylaxis (PrEP) can safely and effectively reduce HIV acquisition in high-risk women, Men who have sex with men (MSM) and IDU (3, 137) (19^{th} CROI at Seattle 2012). The success for these studies is accompanied with high adherence rate to take or use the ART related products and encourage the use of ART as a future prevention strategy before an effective HIV vaccine is developed.

Mother-to-child transmission (MTCT)

Another important route of HIV transmission in children is from an HIV infected mother to her child that can occur in utero, during delivery or through breastfeeding. Without ART intervention, it is estimated that up to 30% of HIV infected mothers transmit HIV to their child (112, 336). The factors associated with MTCT were complex and both mothers and newborns affect the outcome of HIV exposures in infants. On the side of the mothers, recent HIV infection, high plasma viral loads and low CD4 T cell counts were associated with higher rates of MTCT (36, 362). On the side of the child, HIV-specific CD8 T cell response development (113, 177) and higher concentrations of salivary secretory leukocyte protease inhibitor (SLPI) in breast milk (114) were associated with reduced HIV acquisition in HIV-exposed infants. With effective interventions, such as initiating HAART to HIV infected mothers, using ART as prophylaxis for babies or switching to formula feeding if environmental hazards were limited, the rate of MTCT can be reduced to less than 5% (WHO 2010 report). However, active promotion of prevention strategies involving ART for MTCT is controversial. On the side of the mother, debates exist whether all pregnant and breastfeeding HIV infected women should take ART, which regimens to use, the consequences of drug-resistant emergence and its potential influence on the effectiveness of later therapy, the affordability of early initiation of ART, and whether to stop ART in HIV infected women after breastfeeding ends. On the side of the infant, several questions remain largely unknown, such as if there are increased risks of acquiring drug resistant viruses in infants, which may lead to future treatment failure, and if there are side effects of prolonged ART on newborns. Scientific studies at the community level are urgently needed to link these gaps between our knowledge of

HIV and public health interventions. Most importantly, an effective HIV vaccine to prevent HIV infection is highly desirable.

Characteristics of HIV mucosal transmission

Mechanisms of HIV across mucosal barriers

As HIV can be transmitted across different mucosal membranes, it is important to understand that each mucosal tissue is different and HIV may have multiple ways to traverse mucosal barriers. The overall rate of HIV transmission through mucosal exposure is low indicating that mucosal membranes are important and powerful barriers to protect the host from foreign pathogen invasion, including HIV. By using ex vivo tissue explants, it has been shown that HIV can traverse mucosal membranes by a.) migration along with microabration, b.) endocytosis or binding to Langerhans cell and then transfering to CD4 T cells (29, 157), c.) transcytosis by squamous epithelial cells (41). However, the limitations of these studies were that the dynamics of mucosal sites cannot be fully recapitulated in ex vivo model.

Transmission bottleneck

As HIV was presented as diverse viral variants (quasispecies) in chronic infected patients (potential HIV transmitters), interestingly, it has been shown that viral diversity in newly HIV infected patients (recipients) is low (188). Through either cloning followed by sequencing or the heteroduplex mobility assay (HMA), studies have found that the viral diversity is relatively homogenous in newly HIV infected patients (307, 413). These studies also demonstrate the importance of the mucosal barrier and the transmission bottleneck for HIV during mucosal transmission.

With the development of single genome amplification (SGA) and mathematical modeling, the transmission bottleneck during mucosal transmission has been further examined. SGA uses serial dilutions of complementary DNA (cDNA) from viral RNA to a single copy as starting material for PCR amplification, followed by direct sequencing of the PCR products. Compared to cloning/sequencing or HMA, the advantages of SGA include: a) no polymerase-related mutations or recombinations, b) no cloning selection, and c) the proportionality of sequences. Analysis of full length envelope sequences by SGA and mathematical modeling confirm the low viral diversity in recent HIV infected patients (189, 216, 332) and further demonstrate that the majority of HIV infections were initiated by a single or a few viral variants (transmitted or founder viruses) (189, 333). A study from Rolland et al. analyzed nearly full-length HIV genome sequences with SGA and also confirmed the result that most HIV infections (75%) were initiated by a single viral variant (322) (Figure 1-4 left). Infections with multiple viral variants occur at lower rates, but MSM (222), IDU (30), and people with genital inflammation around the time of HIV infection (145, 329) were associated with the acquisition of multiple transmitted viral variants (Figure 1-4 right). The impact of multiple viral variants infection on HIV pathogenesis is still not clear. A study by Sagar et al. found that higher viral diversity during acute HIV infection is associated with a more rapid disease progression (higher viral set point and low CD4 T cells counts) in women (328). However, a study by Gottlieb et al. could not correlate early viral diversity with disease progression indicators in a group of MSM (136). The sample sizes, the studied population and the methods used for determining the number of viral variants in these different studies may contribute to these

discrepancies. Nonetheless, multiple viral variant acquisition sets a higher bar for an HIV vaccine to overcome because an effective vaccine will need to prevent more diverse viral quasispecies to achieve prevention of HIV infection.

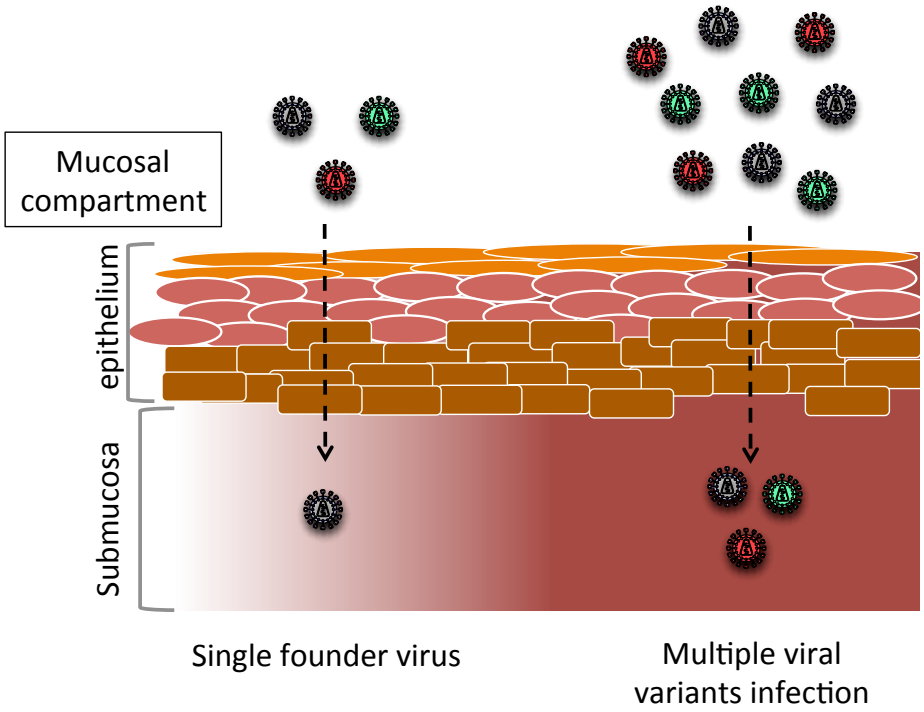


Figure 1-4. HIV transmission bottleneck.

Mucosal membrane consists of epithelial cell layers and submucosa area. In general, single HIV virion (single founder virus) initiates successful infection (left). Multiple viral variants infection occurs at a lower frequency and usually associates with mucosal inflammation (right).

Characteristics of transmitted viral variants/founder virus

The fact that the majority of HIV infections start with single or a few transmitted viral variants brings out the idea that future HIV vaccines may only need to block a few viral variants, despite of huge HIV diversity worldwide. Therefore, many studies were devoted to characterizing the signatures of these transmitted viral variants to understand what makes them preferentially transmitted in a pool of HIV variants. Analysis of the envelope protein of transmitted viral variants shows that these viruses preferentially use the CCR5, rather than the CXCR4, co-receptor (165, 278). In transmitted viral variants of HIV subtype A and C, envelope proteins may have shorter and fewer N-glycosylation sites (71). Reduced glycosylation on envelope proteins of transmitted viruses is associated with higher affinity to the mucosal homing receptor α4β7 (270), which might give transmitted viruses a better fitness to infect activated CD4 T cells at mucosal sites (18, 72). Indeed, detailed analyses of transmitted viruses found that founder viruses were preferentially replicated in activated CD4 T cells, rather than macrophages (278), indicating the importance of future prevention strategies targeting at blocking HIV and CD4 T cells interactions.

Immune response following HIV infection

HIV infection initiates robust innate and adaptive immune responses. HIV can be recognized by triggering pattern recognition receptors (PRR) signaling pathways, including toll like receptor (TLR)7 and TLR8 (218). Following immune sensing, cells were activated to produce many cytokines and chemokines. A study by Stacey et al. demonstrates that multiple cytokines were elevated in association with plasma viremia during acute HIV infection, including rapid and transient increase of IFN-α and IL-15,

rapid and sustained elevation of CXCL10, TNF-α, MCP-1 and additional proinflammatory cytokines including IL-6, IL-8, IL-18, and IFN-γ (354). Interestingly, anti-inflammatory cytokines like IL-10 (354) and IL-1Rα (345) were increased after the waves of inflammatory cytokines, indicating that the immune system tries to balance the activated cytokine network. However, these regulatory cytokines, along with HIV induced indoleamine 2,3-dioxygenase (IDO) expressed on plasmacytoid dendritic cells (pDCs), might also inhibit the development of anti-HIV specific adaptive immune responses (239).

Type I interferons (IFNs), including interferon-alpha and beta (IFN-α and β), were mainly produced by plasmacytoid dendritic cells (pDCs) and mediate antiviral responses as the first line of host immune defense against HIV. IFNs mediate anti-viral response through upregulating interferon-stimulated genes (ISGs) (339), inducing cell death of infected cells and protecting uninfected cells from becoming infected. IFNs can also upregulate expression of host restriction factors, including tripartite motif (TRIM)-5α (331), apolipoprotein B mRNA editing enzyme catalytic polypeptide like 3G (APOBEC3G) (67, 389) and tetherin (225) to specifically interfere with HIV replication and cell to cell spread, as well as HIV recognition by immune cells (277, 293). However, HIV also developed ways to antagonize these antiviral responses, such as the Vpu and Vif proteins targeting tetherin (272) and APOBEC3G (242, 346) for degradation, the Vif and Vpr proteins interfering with interferon signaling pathways by degradation of IRF3 (interferon response factor3) (280) or IRF1 to reduce interferon production (149).

Adaptive immunity includes HIV specific antibody and CD8 T cell responses. Both arms of HIV specific immune responses develop later during HIV infection. The initial antibody response may appear during the first 2 weeks of HIV infection, however, most of these antibodies during early infection may not have neutralizing abilities (366). Autologous neutralizing antibodies generally develop after the first month of infection (315) and studies from Trkola A et al. and Mehandru S et al. find that neutralizing antibodies against HIV can help control virus replication to some extent (255, 368). In addition, HIV specific antibodies can play an important role in HIV prevention (54, 74, 84, 85). Ideally, a future HIV vaccine will be designed to elicit potent and broad neutralizing antibodies that can target diverse HIV variants. However, recent studies found that only 10% – 30% of infected HIV-1 patients develop broad cross-reactive neutralizing antibodies (257). The reasons for this small percentage were not clear, but it is believed that the early HIV infection period plays an important role in developing broad neutralizing antibodies (296). One potential barrier for the development of broadly neutralizing antibodies in HIV infected patients could be that the appropriate epitopes were hidden and therefore, cannot be recognized. Other possibilities include the destruction of germinal centers, where B cells mature and differentiate (220), or early HIV induced B cell dysfunction (364), leading to failure in the generation of cross-reactive broad neutralizing antibodies against HIV.

HIV specific CD8 T cells can contribute to protection against HIV infection (123, 284, 337) as well as to suppression of HIV replication following infection. Studies from Koup et al. and Borrow et al. showed that the reduction of peak viremia is associated

with the emergence of HIV specific CD8 T cells (43, 202), indicating the role of these cells in controlling HIV replication. In addition, the HIV Nef protein down-regulates MHC-I molecules on HIV-infected cells to evade CD8 T cell mediated cellular immunity (399), indicating the important role of CD8 T cells in controlling HIV replication. The high mutation rates during HIV replication were an important way to escape CD8 T cell responses by introducing mutations on the epitopes that were already recognized by the immune system. With the technology to identify transmitted viral variants, studies further demonstrate that the initial HIV specific CD8 T cells target epitopes on the transmitted viral variants and therefore rapidly select for HIV escape mutants (119, 135). As HIV continuously evolves and escapes, CD8 T cells were constantly trying to keep up, which contributes to chronic systemic immune activation and may drive the CD8 T cell exhaustion which ultimately leads to AIDS development in ART naïve HIV infected patients.

Some HIV-infected patients were long-term non-progressors or elite controllers, partially due to the presence of protective genetic factors, such as the major histocompatibility complex (MHC) class I alleles human leukocyte antigen (HLA)-B57 and B27 (185, 256). One possible mechanism for these MHC alleles to be associated with controlled viral replication is that they might recognize multiple conserved regions of HIV (90) where mutations within these regions would greatly reduce viral fitness or even render HIV not viable. In addition to targeting conserved epitopes, the superior CD8 T cells in long-term non-progressors and elite controllers also exhibit poly-functionality, including secreting multiple cytokines, releasing perforin and granzymes, as well as maintaining their proliferation ability (12, 105). With a better understanding

of how to induce such highly effective CD8 T cell responses, a therapeutic vaccine may be achieved to better control HIV replication and delay AIDS development, as an alternative therapy of life long HAART.

HIV vaccine

Despite the remarkable achievement of HAART for HIV treatment and the potential of extensive use of ART for HIV prevention, a preventive HIV vaccine that elicits sterilizing immunity is still needed and potentially much more cost-effective to control the HIV epidemic. However, after 30 years of HIV research, only a limited number of HIV vaccine candidates have made it to human clinical trials. It is not until 2009, that the RV144 Thai trial showed a partial, 30% efficacy of reducing HIV infection in low risk groups (community based population), indicating that a protective vaccine against HIV acquisition might be possible (311). Before RV144 Thai trial, HIV vaccine trial VAX004 utilizing the vaccine candidate VaxGen, composed of recombinant HIV-1 envelope gp120, did not have the ability to reduce HIV-1 incidence nor control HIV replication (120), despite robust HIV specific antibody responses in the vaccinated participants (133, 299). Another HIV vaccine trial (STEP), utilizing the MRKAd5 vaccine with recombinant adenovirus type 5 vector containing HIV gag, pol and nef genes, was stopped early because the interim analysis showed no vaccine efficacy. In addition, there were more HIV infections in the vaccinated group compared to the placebo group and among newly HIV infected patients, plasma viral load is similar between placebo and vaccinated group (56), despite the induction of robust cell mediated immunity (251, 323). The failure of the VaxGen and MRKAd5 vaccines both showed that either potent antibody responses alone or strong cell

mediated responses alone were not enough to reduce HIV acquisition rates or to decrease HIV replication following infection. These results demonstrate the complexity of HIV infection and a better understanding of correlates of immune protection following HIV exposure is urgently needed for the development of an effective HIV vaccine. More studies were needed to delineate possible factors that alter immune responses in vaccine recipients to help future HIV vaccine development efforts. However, these studies were extremely difficult to conduct in humans; therefore, an adequate animal model is needed to address these unanswered questions.

SIV infection of non-human primates for HIV researches

Simian Immunodeficiency Virus (SIV) refers to a group of retroviruses that infects monkey populations. Species-specific SIV can be found in approximately 40 non-human primate species as natural hosts in Africa. These infections generally were non-pathogenic despite high levels of viral replication, which is considered a result of SIV co-evolution with their natural hosts. Studies of SIV infection in natural hosts, mostly in sooty mangabeys (SM) and African green monkeys (AGM), provide insights into how natural hosts respond to SIV infection without progressing to AIDS and potential future therapies for HIV infection.

Interestingly, accidental transfer of SIVsmm into Asian macaque species, such as Rhesus macaques (RM) can lead to the development of pathogenic SIV infections resulting in AIDS-like diseases that can be used as models for HIV research (219, 241, 384). SIV infection of Rhesus macaques resembles HIV infection of humans in many aspects, including 1) rapid virus replication during acute infection and the establishment of a viral set point during chronic infection; 2) decrease in absolute CD4

T cell counts in peripheral blood and severe loss of CD4 T cells at mucosal sites accompanied by rapid virus replication (49, 223, 383, 411); and 3) progression to AIDS when CD4 T cell counts drop below certain levels. Similar to human HIV infections, the rate of progression to AIDS varies between individual macaques and several parameters were associated with rapid disease progression, including low CD4 T cell counts in the periphery, high plasma viral loads and sustained chronic immune activation(183, 219). Since the earliest events after HIV exposure were difficult to study in humans, SIV infections of Rhesus macaques were frequently used to obtain valuable information about the very early events following viral exposure. The advantages of using SIV infection of Rhesus macaques as a model were that the timing, exposure route, and dose of infection can be controlled, the SIV viral inoculum is well characterized, and tissue samples, such as lymph nodes and mucosal biopsies can be obtained at different times throughout the course of infection. This is important, as studies have shown that analyses of peripheral blood only may not accurately reflect events at tissue sites (82, 144, 408).

SIV infection through mucosal exposures in Rhesus macaques

A recent study from Chenine et al. demonstrated that the minimal dose to achieve systemic SIV infection in the absence of mucosal lesions is lowest through the rectal route, followed by vaginal and finally the oral route (68). This data is consistent with epidemiological observations indicating higher risk of HIV acquisition through rectal exposure than vaginal route and lower risk for oral HIV transmission. The study also indicated that different mucosal membranes and immune

environments exhibit differential susceptibility to SIV, similar to HIV infection. Therefore, SIV infections of Rhesus macaques can be a valuable model to test and evaluate future vaccine or microbicide candidates using different challenge routes.

Early events following mucosal SIV infection

In vivo studies using SIV infection of Rhesus macaques through vaginal exposure demonstrate that only a few clustered cells, predominantly CD4 T cells, have detectable SIV RNA levels at vaginal tissues three to four days after viral exposure indicating that a relatively small number of susceptible CD4 T cells were available at the entry site (130, 409, 410). These small founder populations expand at the mucosal site, trigger a localized innate immune response, recruit more target cells to fuel viral replication, and further disseminate the virus into lymph nodes and distal tissues (224, 258). Note that both HIV and SIV can replicate not only in activated but also resting T cells at the portal of entry following vaginal virus administration (409) and these data point out the important role of CD4 T cells as first target cells, and support the idea that future HIV vaccines will need to induce immune response to provide protection against CD4 T cells being infected at the mucosal sites to prevent heterosexual HIV transmission.

SIVmac strain 251 (SIVmac251) or SIVsm strain E660 (SIVsmE660) each contains diverse quasispecies that can mimic mucosal exposure of HIV. Similar to HIV infection, studies have shown that only a single or a few SIV variants initiate infection in macaques after repeated low dose SIV exposures through the rectal, vaginal or penile route, respectively (190, 235, 358). These studies demonstrated that

repeated low dose exposures of SIV to rhesus macaques recapitulate HIV mucosal transmission in humans and thus validate the use of SIV infections in macaques as a model to study the earliest viral and immune events following mucosal transmission of SIV/HIV. Liu et al. further demonstrated that the dose of SIV exposure can alter the duration of virus dissemination and the number of founder viruses (226), with lower doses of SIV administration being associated with a longer dissemination period and a lower number of founder viruses.

With the ability to sample tissue biopsies of SIV-infected macaques, early events at the mucosal sites following SIV infection start to unravel. The gut has been identified as a major SIV replication site and gut CD4 T cells were severely depleted early and hardly ever recover throughout the entire SIV infection course (248, 350). During early SIV infection, α4β7+ CD4 T cells and Th17 cells were preferentially depleted (47, 181) which results in an unbalanced immune environment at mucosal compartments (62, 115). Disruption of the mucosal immunity balance is associated with mucosal tissue damage (107), microbial translocation (48), and immune cell homeostasis (297) as well as driving force of sustained systemic immune activation during chronic SIV infection. A study from Ansari et al. demonstrated that blocking the gut homing receptor α4β7 on immune cells during acute SIV infection protected macaques from early destruction of mucosal tissue and modulate SIV infection (16). These studies indicate that early events occur at mucosal sites (which may not be reflective in the analysis of peripheral blood) play a central role in SIV, and most likely HIV, pathogenesis.

Immune response against mucosal SIV infection

For the purpose of HIV vaccine development, it is important to understand how the immune system responds to HIV/SIV invasion as well as the interaction between HIV/SIV and immune cells. The SIV infection of macaques model provides precious information regarding early events following SIV exposure to mucosal membranes and mucosal immunity at the site of virus entry. A study from Li et al. demonstrated that within the first day of SIV infection through the vaginal route, MIP-3α (CCL20) is produced by the endocervical epithelium, which further recruited pDCs to the mucosal site. pDCs produce IFN-α/β, MIP-1α (CCL3), and MIP-1β (CCL4) and induce SIV target cells migration to the mucosal site which leads to further virus propagation and dissemination. IL-8 and RANTES were also detected subsequently at the mucosal sites that further enhance mucosal inflammation (224).

Following mucosal SIV infection and dissemination, several plasma cytokines/chemokines, including IFN-γ, IL-1Rα, MCP-1, IL-15, and IL-18 as well as type I interferon, were increased at early times in SIV infected macaques and the dose of the viral inoculum can affect the level of these cytokines in the peripheral blood (226). Transcriptional profile analyses of immune genes comparing SIV infection in natural hosts (AGMs) and rhesus macaques demonstrate that both species up-regulate a variety of innate and adaptive immune regulators during acute SIV infection, indicating that both AGMs and RMs activate their immune systems in response to SIV infection. However, up-regulation of interferon stimulated genes (ISGs) as well as cell proliferation and activation markers, were resolved in AGMs, but

not macaques during chronic SIV infection (44, 150, 174), which emphasizes the role of interferon on SIV pathogenesis and chronic immune activation. During acute SIV infection, pDC levels decline in the peripheral blood, potentially due to virus-mediated cell death (31) and/or pDCs migration into intestinal mucosal tissues (209) and lymph nodes (53, 209) with large amount of type I interferon production at these tissues. Despite the antiviral activity of interferons to inhibit HIV replication, IFN-α treatment in SIV infected macaques has no effect on plasma viral RNA levels (19). These studies indicate that the location of type I interferons may affect the pathogenesis of SIV infection and potentially HIV infection.

Robust cytokine and chemokine production during acute SIV infection initiates the development of adaptive immune responses against SIV. The role of adaptive immune responses is best demonstrated in SIV infections of Rhesus macaques. Studies have shown that passive transfer of SIV specific antibodies can protect macaques from SIV challenge (245, 246), thus providing a rationale for HIV vaccines to induce potent and broad neutralizing antibodies to block HIV entry. However, once macaques were systemically infected with SIV, antibody responses were not sufficient to completely control SIV replication or eliminate the virus during chronic infection (38). Instead, CD8 T cell mediated immunity is required to suppress virus replication (338). In addition, rapid depletion of naïve B cells occurs during early SIV infection (206, 365) that is associated with dysfunctional B cell responses to foreign antigens (195). The dysregulation of B cell functions and destruction of germinal centers during early SIV infection may lead to opportunistic infections and rapid disease progression.

The importance of CD8 T cells in pathogenic SIV infection comes from the experimental depletion of CD8 T cells in SIV infected macaques that result in uncontrolled SIV replication and rapid disease progression (247, 338). Similar to humans, a subset of SIV infected macaques may act as long-term non-progressors or elite controllers and certain types of MHC I alleles, including Mamu-A*01, B*08 and B*17 that recognize conserved epitopes of SIV, were associated with spontaneous control of SIV replication and slower rates of disease progression (229, 405). These studies also provide the rationale to develop CD8 T cell based HIV vaccines to prevent and control HIV infection. Indeed, Hansen et al. demonstrated that vaccine induced robust and sustained SIV specific CD8 T cell responses can reduce SIV infection as well as virus replication following infection (147, 148).

HIV/SIV oral transmission

HIV oral transmission

HIV oral transmission is an important route to spread HIV and can occur via mother to child transmission with virus in breast milk and oral-genital transmission with virus in semen. HIV transmission through breast-feeding remains an important issue in resource-limited areas with up to one third of infants becoming HIV infected through breast milk from their HIV-positive mothers without interventions (192, 271, 290). HIV infected infants tend to have rapid disease progression, with up to 25% mortality within their first two years (275). Currently strategies to prevent HIV infection in newborns include initiation of antiviral treatment for HIV infected moms or using ART as prophylaxis for HIV-exposed infants or switch to formula feeding. However, there are difficulties to use these interventions in the resource-limited area, such as

culture stigma, affordability and accessibility to the ART. HIV transmission through oral-genital intercourse is difficult to clearly define, but case reports have shown that HIV oral transmission in adults can occur (45). In addition, recent studies demonstrated that the HIV uninfected partners in discordant couples can develop HIV specific CD8 T cell responses (292) or neutralizing antibodies at mucosal sites (153) and systemic circulation (152) after oral HIV exposures, indicating that oral sex is not risk-free for HIV transmission.

The upper gastrointestinal (GI) tract includes several histologically distinct tissues, including areas lined with stratified squamous epithelium with (i.e. gingiva) or without keratination (i.e. esophagus), mucosa-associated lymphoid tissue (MALT) (such as tonsils), and glands with columnar epitheliums. Using ex vivo tissue explants, studies have demonstrated that tonsils may play an important role in HIV oral transmission (237, 238). In addition to tonsils, other mucosal areas in the upper GI tract can also be potential virus entry sites after oral exposure to HIV. Indeed, by using a single-layer, polarized epithelial cell model, Tugizov et al. showed that cell-free HIV can efficiently traverse infant and fetal oral epithelia, possibly through transcytosis (371). On the other hand, HIV transverse adult oral epithelia is inefficient and occurs when tight junctions is disrupted (371). Cell-associated HIV during breastfeeding also contributes to HIV acquisition in infants through oral route and HIV infected macrophages may play an important role in carrying HIV through infants epithelium into lamina propria (371).

The reasons for the low HIV infection rates in adults through oral sex is not fully understood and possible explanations include that 1) adult oral epithelium has multi-

layers of stratification that provide mechanical barriers, compare to infants epithelium only has a few layers (372) and 2) adult oral cavity has higher levels of anti-HIV innate proteins (i.e. beta-defensins 2 and 3 (372), secretory leukocyte protease inhibitor (SLPI) (254) and lactoferrin (hLf) (187) as well as other soluble factors, such as CC chemokines in saliva (151)) that can inactivate HIV in the adult oral cavity (373).

Early events following HIV/SIV oral exposures

Studying early events following HIV oral exposures in infants or adults is extremely difficult. By using the macaque SIV infection model, early studies have shown that cell-free SIVmac251 given through the oral route can successfully infect neonates (21, 22, 259) and adult macaques (24, 25, 260). Similar to MTCT of HIV, SIV can be transmitted to infant macaques throughout the period of breast feeding (14). Furthermore, both pathogenic and live attenuated SIV infections of neonatal macaques through the oral route result in rapid disease progression (21, 22, 24). These studies highlight the possibility of oral HIV transmission and further demonstrate that using live attenuated HIV/SIV as vaccines is not feasible.

By using SIV infected rhesus macaques, Stahl-Hennig et al. and Milush et al. demonstrate that SIV can enter through the tonsils or the oral mucosa along the upper GI tract and rapidly disseminate into peripheral blood by four days after oral administration of SIV (259, 355), potentially through the draining lymph nodes around the oral cavity (Figure 1-5). Both studies highlight the rapid dissemination of SIV following oral exposure and raise the issue that future HIV vaccines preventing oral HIV transmission, especially for newborns, might have more difficulties than vaccines

to prevent HIV sexual transmission because of the short time period for immune system to respond.

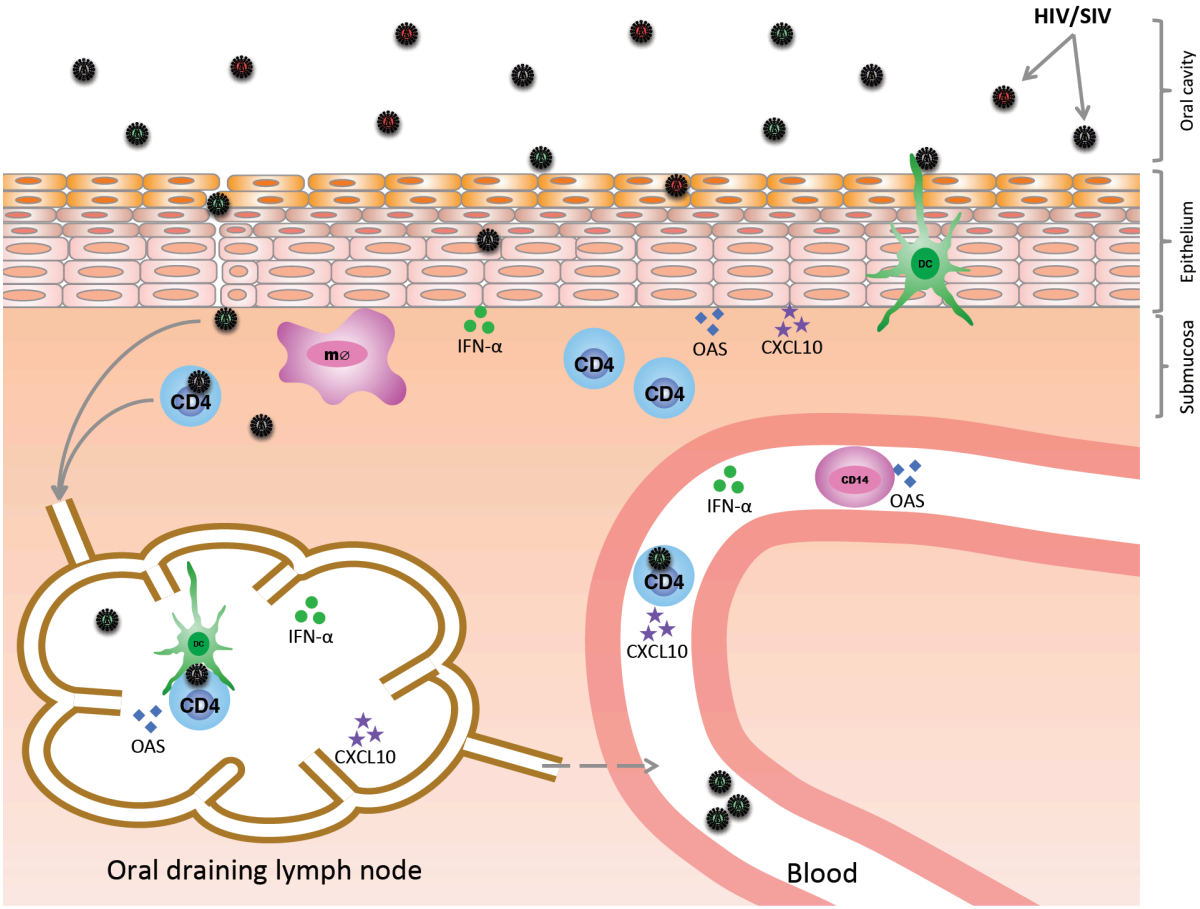


Figure 1-5: Early events following oral HIV/SIV infection.

HIV/SIV can transverse mucosal barrier by moving along microabrations, transcytosis, or captured by dendritic cells (DCs). Following virus come across the mucosal barrier, virus can infect target cells at the mucosal site, disseminate in cell-free or cell-associated format through draining lymph nodes around oral cavity and then peripheral blood.

Target cells in oral mucosa

It has been demonstrated that CD4 T cells, macrophages and Langerhan cells were present in adults and infants oral mucosa (77, 169, 371, 378). By using ex vivo tissue explants, Tugizov et al., showed that HIV transmigration through fetal oral mucosal epithelium can lead to infection of CD4 cells (371). By using SIV infection of

macaques, Milush et al. showed that SIV productively replicated in macrophages and T cells at the upper GI tract four days post infection (259). Both studies point out the important role of both macrophages and CD4 T cells in the oral cavity during early oral SIV infection.

Transmission bottleneck in oral HIV/SIV transmission

Similar to HIV transmission through other mucosal routes, it is believed that transmission bottleneck also exists in oral HIV transmission with a single or only a few viral variants establishing the infection following oral exposure of HIV. Utilizing SIV infected lactating macaques and their infants, it has been shown that most infant macaques were initially infected with a homogeneous SIV population, compared to the diverse population of SIV in the breast milk near the time of transmission (14). Similar results were seen in adult macaques where overall SIV diversity in early infection (week 1-2 post-infection) is low (96). Both studies demonstrate that a transmission bottleneck exists in SIV infection through the oral route, and mostly likely also for oral HIV infection. Furthermore, Durudas et al., showed that oral administration of higher SIV doses results in higher viral diversity during acute infection (96), which is similar to the results of studies by Liu et al. and Varela et al. that the viral dose used for mucosal administration affects the number of transmitted viral variants (226, 382). The dosing effect on the numbers of transmitted viral variants is likely to affect future HIV vaccine efficacy to prevent oral transmission since the viral load in breast milk and semen were variable, ranging from hundreds to millions of viral copies per ml breast milk (95, 341, 361, 398), indicating that newborns

can be exposed to high or low doses of HIV. Furthermore, oral HIV exposure occurs frequently in exclusively breast-feed infants. Prolonged exposure is also possible as breast-feeding can last from months to over a year. Prolonged and highly frequent exposures to potentially high doses of HIV through the oral route may contribute to the acquisition of diverse HIV variants that can result in the failure of future HIV vaccines.

Immune responses following oral HIV/SIV infection

Assessment of immune factors associated with HIV infection or prevention were important for an effective HIV vaccine development to decrease mother-to-child transmission through consuming HIV containing breast milk. However, neonate immunity changes rapidly to adjust for encountering environmental microbes. There were also studies showing the differences between infants and adult immune cells (171, 186, 298) and many questions remain unknown for immune response development in infants. For HIV vaccine development, it is important to dissect the immune factors in newborns that can provide protection against HIV infection and incorporate these immune factors into vaccines to induce protective immune responses against HIV infection. Breast-feeding HIV exposed uninfected infants developed HIV specific T cells response measured by IFN-γ production (176, 204, 230, 285) and HIV specific B cell response with mucosal IgA antibody production (214). Furthermore, early HIV specific IFN-γ responses were associated with decreased HIV-1 acquisition (176, 285). However, HIV-1-infected infants with HIV specific CD8 T cell responses early in their life do not associate with better clinical outcomes (317).

These studies indicate that HIV specific T cell and B cell response were important immune indicators that need to be elicited by vaccines and more information, such as innate immunity, proper adjuvants with cytokines/chemokines milieu, are needed to facilitate the process to develop a preventive HIV vaccine for newborns. By studying SIV infection of rhesus macaques, both adult and neonatal macaques respond quickly to SIV infection and activate the innate immune system following oral SIV administration (4, 5, 22, 25, 96, 98, 99, 260). In oral SIV infection of neonatal macaques, Abel et al. use real-time PCR to examine immune gene expression at different tissue sites and demonstrate that innate immune responses were strongly upregulated in tissues and lymphoid organs close to the oral cavity. The upregulated genes include predominantly inflammation related genes, MIP-1α, TNFα, IL-6, IL-12, and IFN-γ (4, 5). Multiple subtypes of IFN-α and interferon mediated anti-viral genes were also up regulated in different tissues (99, 260), with the highest being in tonsils and draining lymph nodes of the oral cavity, and mild changes in mucosal tissues, such as gingiva and colon. However, the overall antiviral response is insufficient to control virus replication and dissemination. Easlick et al. further demonstrated that IFN-α gene expression levels were positively correlated with levels of virus replication in lymph nodes and the presence of pDCs, indicating that the early interferon response following oral SIV infection is predominantly mediated by pDC migration into lymphoid tissues (99). In addition, several immune modulator genes, including OAS and CXCL10, showed increased expression in gingiva tissues, peripheral lymph nodes and PBMCs in orally SIV infected macaques (98, 260). Interestingly, Milush et al. and Durudas et al. demonstrated that the timing and the location of innate immune

gene up-regulation might affect disease progression in orally SIV infected macaques (98, 260). Consistent with other studies, these results show that early events following oral SIV infection were important for SIV pathogenesis and disease progression.

Potential vaccines to prevent oral HIV/SIV infection

Ideally, an effective HIV vaccine for newborns should be given at birth with extremely high safety and high efficacy to reduce HIV acquisition as well as modulate disease progression if HIV infection occurs. Studies have demonstrated that passive immunization of neonatal macaques can provide protection against oral SIV infection (23, 381), which brings out the potential of vaccines to induce potent antibody responses for prevention of oral HIV infection in newborns (117, 160, 381). However, passive immunization has little or no effect on the control of virus replication or on slowing disease progression (116, 381), which indicates that in addition to potent antibody responses, future HIV vaccines may also need to induce cellular immunity.

DNA vaccines expressing specific antigens to induce immunity is a good way to move forward because of generally lower safety concerns and their ability to induce both humoral and cellular immunity. A recent study from Van Rompay et al. demonstrated that systemic administration of a canarypox virus vector-based SIV vaccine (ALVAC-SIV) or Vaccinia virus Ankara (MVA-SIV) expressing Gag, Pol, and Env did not show protection from infection but could result in reduced viremia following oral SIV challenge in infant macaques (380). Another study tested oral vaccination with a vesicular stomatitis virus based SIV vaccine (VSV-SIV) expressing Gag, Pol and Env followed by a boost with intramuscular immunization of MVA-SIV, but this also failed

to provide protection against oral SIV challenge despite of robust antibody and cell-mediated immune responses at both systemic and mucosal sites (243, 379).

So far, none of the potential vaccine candidates have shown high efficacy in preventing SIV infection of infant macaques with continuous SIV bottle-fed through the oral route. The development of HIV vaccines to prevent oral transmission through breast-feeding in infants may be more difficult, potentially due to continuous exposure to high doses of HIV at high frequency for a long period of time as well as not fully developed organs and immune system. More studies were needed to understand infant immunity, interaction between maternal immune modulators in newborns and the virus, and how to stimulate with optimal antigens in a right way to induce ideal immune responses to prevent HIV infection in infants.

Role of mucosal immunity on HIV/SIV transmission and pathogenesis

For future HIV vaccine and microbicide development, it is important to discover the immune factors that can affect HIV acquisition. Several studies have shown that mucosal inflammation is associated with increased HIV acquisition (179, 180, 313, 314). Several biological mechanisms have been proposed to explain the link between mucosal inflammation and HIV acquisition. One mechanism is that mucosal inflammation induces mucosal membrane disruption, such as genital ulcer, that might facilitate virus entry, which has been reproduced in the SIV Rhesus macaque model (396). Another mechanism could be that mucosal inflammation increases the number of HIV/SIV target cells at mucosal sites that may contribute to increased HIV acquisition. Indeed, Chenine et al. used 10% acetic acid to induce localized buccal inflammation in macaques and demonstrated that an increased number of CD4 T

cells at inflamed tissues is associated with SHIV acquisition (69). Furthermore, inflammatory cytokines can directly upregulate HIV replication (213, 282, 301), indicating that an inflammatory mucosal environment can promote productive HIV infection at the mucosal sites, which may facilitate systemic dissemination following HIV exposure. All these factors related to mucosal inflammation create an environment that promotes HIV replication and establish successful infection. On the other hand, studies of a subset of commercial sex workers who were frequently exposed to HIV but remain seronegative found that low levels of CD4 T cell activation and quiescent CD4 T cell phenotypes were associated with reduced HIV susceptibility (60, 198, 252). Immune quiescence is associated with lower IL-1α, CXCL9 (MIG), CXCL10 levels (212) or higher levels of protease inhibitors, i.e. SLPI (360) and elafin/trappin-2 (173), immune modulators with anti-inflammatory properties, at mucosal sites. In addition, increased antiviral activity, such as higher levels of RANTES (172), IFN-α (156) and increased NK cell activity (340) at mucosal sites, is also associated with protection against HIV infection. Overall, mucosal immune activation favoring virus replication is associated with HIV infection while mucosal immune quiescent inhibiting virus replication is associated with protection against HIV infection (figure 1-5). However, all these studies from humans can only make association conclusions because it is difficult to distinguish whether these responses were results of HIV exposure or these responses were actually determining the outcome of HIV infection. It is important to dissect these immune modulators at mucosal sites to further understand the factors associated with reduced HIV infection

rates so that vaccines can be designed to induce these immune factors to achieve protection against HIV infection.

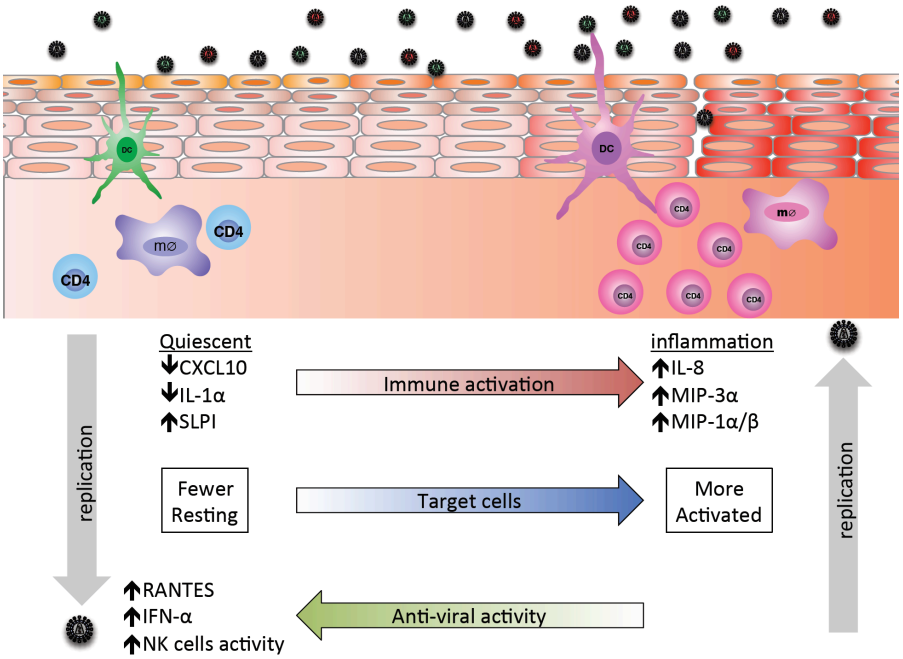


Figure 1-6. The influence of mucosal immunity and HIV acquisition.

Mucosal inflammation is associated with increased HIV infection, potentially due to tissue disruption, increased the numbers of target cells and more activated cells (pink-purple cells) at the mucosal site (right). Mucosal quiescence is associated with protection against HIV infection, potentially due to less target cells availability, cells in resting phenotype (blue-green cell) and elevation of antiviral activity (left).

Innate immunity can play a role in modulating HIV/SIV pathogenesis. Roberts et al. and Bebell et al. demonstrated that the levels of IL-12p40, IL-12p70, IFN-γ, IL-7 and IL-15 in plasma or the levels of IL-1β, IL-6, and IL-8 in cervicovaginal specimens during acute HIV infection could be predictive of disease progression (34, 318). These studies indicate that certain types of cytokines and chemokines, both at mucosal site and in peripheral blood, during early HIV infection can modulate HIV pathogenesis. Similarly, IL-15 treatment during acute SIV infection in macaques results in a 1000-fold increase of the viral set point and in rapid disease progression (264). In contrast, IL-12 administration during acute SIV infection can decrease viral load by 100 fold and slow disease progression in macaques (15). Furthermore, type I interferons accompanied with inflammatory cytokine (i.e.TNF-α, IL-6, CXCL10, and IFN-γ) production at the vaginal mucosa can result in higher viral set points in plasma following vaginal SIV infection in macaques (391). These data demonstrate that early events following HIV/SIV infection were critical and the levels as well as the locations of cytokine/chemokine production during acute infection can have long-term effects on virus replication as well as on disease progression. Clearly, it is important to dissect and understand the complex interactions of cytokine networks during acute HIV/SIV infection and take those into consideration for induction of a proper cytokine milieu to achieve effective immune responses through vaccination against HIV.

Summary

Mucosal immunity may be the key determinant for a host's susceptibility to HIV/SIV, pathogenesis and disease progression. The studies presented here focus on assessing the influence of preexisting mucosal inflammation on oral SIV (and by

analogy HIV) transmission and the early events following oral SIV infection of rhesus macaques. They emphasize the following question:

1. Will oral mucosal inflammation affect the susceptibility of macaques to SIV through the oral route? And what are the mucosal factors associated with successful SIV infection?
2. How does oral mucosal inflammation affect early virological and immunological events following oral SIV infection? Will differences in early events have long-term consequences on disease progression markers, such as chronic immune activation or CD4 T cell counts? Will pre-existing oral inflammation alter the early events following intravenous inoculation of SIV in rhesus macaques?
3. Will induced penile inflammation associate with increased SIV infection through penile challenge? And what are the factors associated with SIV infection through penile challenges? Are these factors similar or different from oral SIV challenge?

These studies will lead to a better understanding of immune correlations of SIV infection and early viral and immune changes following SIV infection through different mucosal routes. The results will help future HIV vaccine development and provide guidelines for optional treatment to reduce mucosal inflammation for public health improvement.

Chapter 2: Material and methods

Part I: oral transmission study

Study animals and gingivitis (mucosal inflammation) induction

The Macaques used in the studies were colony-bred rhesus macaques (Macaca mulatta) housed at the Southwest (SNPRC) National Primate Research Center. Total 16 Macaques were separated into two groups—control group macaques (RM-C1, 26740, 26856, 26968, 26517, 30309, 26744, 26971) with normal food and no ligatures and inflammation-induced group macaques (18978, 19852, 26970, 27270, 19313, 26981, 27238, 30311) with induced gingival inflammation. Gingival inflammation was induced by ligatures tied on the first and second molar and second premolar teeth in four quadrants of the oral cavity using 3–0 silk sutures (Figure 2-1). Soften diets were provided with commercial chow biscuits soaked in warm water for 10 min and drained, and without providing any mechanical oral hygiene throughout the study period(103). All macaques were cared for in accordance with National Institute of Health guidelines and local Animal Care and Use Committee.

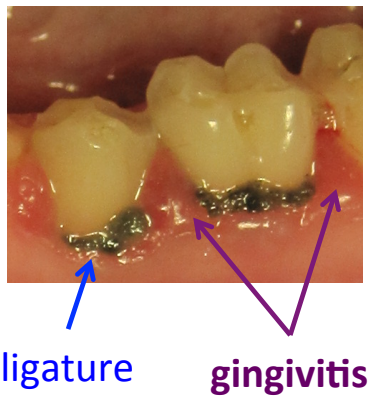


Figure 2-1. Gingival inflammation induction with ligature binding around the teeth

TRIM5α genotyping of macaques

Macaques PBMCs cell pellets were sent to the Johnson lab and TRIM5 genotypes of macaques in this study were determined briefly by isolating genomic DNA from PBMCs and directly sequencing the PCR fragments from the C-terminal domain of TRIM5 as previously described(193, 352). TRIM5 gene polymorphisms were grouped

into TRIM5^{CypA}, TRIM5^{TFP}, and TRIM5^{Q}, representing genotypes as three different classes and six possible genotypes.

MHC genotyping of macaques

9 class I MHC genotypes (Mamu-A*01, A*02, A*08, A*11, B*01, B* 03, B*04, B*08, B*17) of Rhesus Macaques were determined by the Watkins lab. Briefly, pellet PBMCs were lysed to extract genomic DNA with Roche MagnaPure system. Allele specific PCR amplification of genotype Mamu-A*01, Mamu-A*02, Mamu-A*08, Mamu-A*11, Mamu-B*01, Mamu-B*03, Mamu-B*04, and Mamu-B*17 are performed and analyzed with 2% agarose gel to detect allele-specific amplicons (182).

Clinical assessment of gingival inflammation

The ligatures were placed on the teeth from macaques in gingivitis-induced group through out the entire study period. Oral health check was performed every 2 weeks to maintain the status of inflammation while preventing progression to aggressive periodontal disease. Clinical assessment was performed on teeth with ligature binding of 4 quadrants.

A Maryland probe (William's markings) was used to determine plaque index (PI), pocket depth (PD), recession, and bleeding upon probing (BOP) at four sites on each tooth: distobuccal, buccal, mesiobuccal and lingual (premolar, first and second molar) in each quadrant. Clinical attachment level (CAL) values were calculated from the pocket depth and recession measures. A gingival bleeding score, following determination of the pocket depth measure, was obtained.

SIV Virus administration

Quasispecies inoculum SIVmac251 was prepared in Giavedoni lab with virus titer $5.5x10^3$ $TCID_{50}$ per ml in CEM-x-174 cells and previously titrated in vivo to adjust viral inoculation dose for the study(97). In first set study (1^{st} control group: 18993, 26740, 26856, 26968 and gingivitis group: 18978, 19852, 26970, 27270), 1800 $TCID_{50}$ was non-traumatically administered at day 0, 2, 4. Macaques were laid on their side and 1ml SIV was administered through needleless syringe to gingival tissue around the ligature binding teeth to have SIV contact with the gingiva. The macaques remained on the side for additional 5-10 minutes before recovering from sedation. Macaques were examined weekly, up to 4 weeks, for evidence of SIV infection by showing PCR positive with SIV gag gene in PBMC described below. SIV infected macaques were followed for 6-8 months after infection. SIV uninfected macaques were enrolled into next set study but remained in the same group (macaques without gingivitis induction remained in control group and macaques with ligature remained in gingivitis group).

In second and third set studies (2nd control group: 26968, 26517, 30309, 26744, 26971, 2nd gingivitis group: 18978, 19313, 26981, 27238, 30311; 3rd control group: 26968, 30309, 26744, 26971, 3rd gingivitis group: 18978, 27238, 30311), a piece of 3mm whatman paper applied with 1800 $TCID_{50}$ and 2750 $TCID_{50}$ SIVmac251, respectively, were placed on the gingival tissue on the ligature binding teeth at day 0, 2, 4 to increase the contact of virus and gingival tissue to emphasize the influence of mucosal inflammation (Figure 2-2). Similar to 1st set macaques, macaques remained

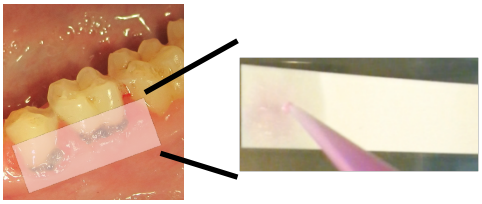


Figure 2-2. SIVmac251 administration with whatman paper.
SIV was put on the whatman paper(right) and then place on top of the inflamed

on the side for additional 5-10 minutes before recovering from sedation and followed up to examine signs of SIV infection. SIV infected macaques were followed up for 6-8 months and SIV uninfected macaques were enrolled into next set study.

For 4th set study, 50 $TCID_{50}$ of SIVmac251 was intravenously injected into control group macaques 26744, 26968, 26971 and gingivitis group macaques 18978, 27238, 30311. After successful SIV infection, macaques were followed with scheduled blood draw and 3 tissues sampling during 6-8 months chronic stage before scheduled necropsy.

Blood and tissue samples collection

Peripheral blood(Figure 2-3)

10-16 ml blood was collected in EDTA anticoagulant tubes at these time points: pre-infection/pre-gingivitis induction, day 0, day 7-9, day 14-16, day 28-30, day 56-58 post-1st virus administration and followed by 6 months monthly blood draw. EDTA blood samples were overnight shipped to the Sodora lab with ice pack. Plasma was collected and stored in -80 °C. Peripheral blood mononuclear cells (PBMC) were purified by standard gradient centrifugation with Ficoll-Paque (GE Healthcare, Uppsala, Sweden) and preserved as cell pellet (average 2 millions cells per tube) in -80°C or viably frozen stored in liquid nitrogen.

Tissues

Average 2 mm punch of gingiva biopsies were obtained and stored in -80 °C with RNAlater (Ambion, TX) and embedded in paraffin (if more than 2 punch biopsies were available) at time points of pre-infection/pregingivitis, day 14-16, and day 28-30 after 1st virus administration (day 0).

At the same time of gingival biopsy collection time point, one inguinal lymph node was obtained and preserved in RNAlater or embedded in paraffin block. A small part of lymph node biopsy was physically disrupted and further flowed through 70μm cell strainer (BD Bioscience, San Jose, CA) with 3 times PBS wash and viably frozen as lymph node mononuclear cells (LNMC) in liquid nitrogen.

Gingival crevicular fluid (GCF)

GCF was collected for immune modulators assessment at pre-infection/pregingivitis, 5-7 days before SIV administration, day 14-16 and day 28-30 after 1st SIV administration. Briefly, gingival sites were isolated and dried with cotton gauze.

Absorbent filter strips (Periopaper strips, Oraflow, Inc.) were placed below the gingival margin at mesial sites of a premolar, 1st and 2nd molar teeth in the maxillary and mandibular quadrants on one side of the mouth. The strips were maintained, isolated from saliva, for approximately 15–30 seconds. The filters were removed and the fluid volume determined using a Periotron 8000 (PRO-FLOW, Amityville, NY) that was calibrated, with a standard curve determined for each collection period.

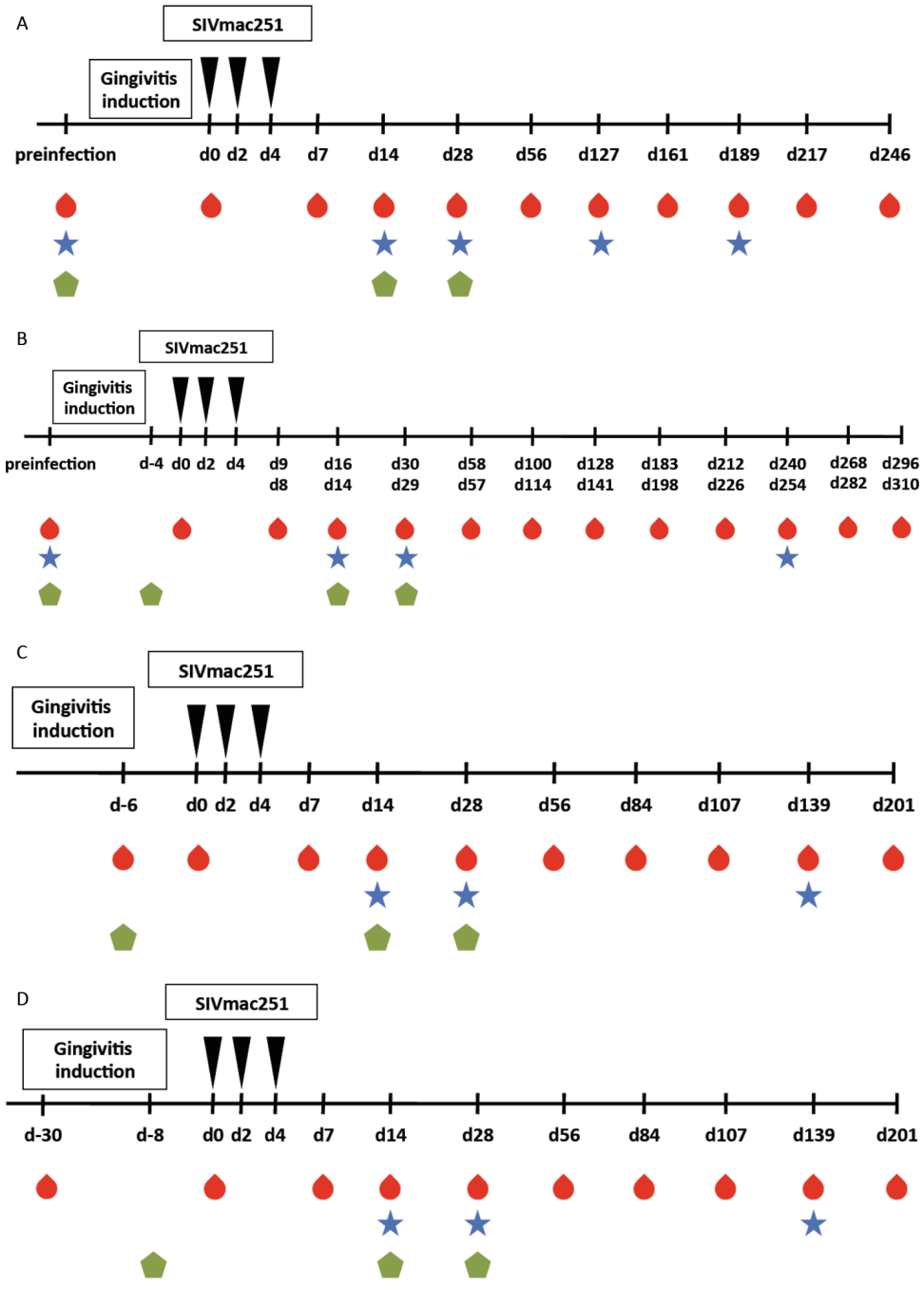


Figure 2-3. Study timeline for oral transmission study.
First set (A), second set (B), third set (C) and fourth set (D). Red drops indicate blood draws; blue stars indicates tissue biopsies (gingival and lymph node biopsies); green pentagons indicate gingival crevicular fluid collection

DNA extraction from PBMC

Cellular DNA from PBMC samples was extracted using Qiagen AllPrep kit (Qiagen, Valencia, CA) or DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA) according manufacturer's protocol. Briefly, 2 millions cell pellets were lysed with 350µl RLT plus buffer, homogenized by vortexing 15-30 seconds and flowed through DNA spin column. Column was washed with RPE buffer twice and eluted in 50µl RNase-free water. PBMC DNA was stored at -20°C before PCR amplification. For using DNeasy Blood & Tissue kit, 10 minutes incubation of PBMC and lysis buffer at 50°C with proteinase K was performed before flowing through DNA spin column.

SIV gag PCR (polymerase chain reaction)

Nested PCR was used to amplify partial SIV Gag region to determine if the macaques were SIV infected. Generally, a mix of PCR reagents was made with 5µl of PBMC DNA or 1st round PCR product, 1µl of SIV gag V1-V2 primer (1^{st} round primers: SIVgagF1- 5'AGA AAG TGA AAC ACA CTG AGG AAG C-3' and SIVgagR1- 5' TCA TCC AAT TCT TTA CTG CTG CA-3'; 2^{nd} round primers: SIVgagF2- 5'ACA GAT AGT GCA GAG ACA CCT AGT GG-3' and SIVgagR2- 5'CTGTCTACATAGCTCTGAAATGGCTC-3') and 5µl of 10x Platinum PCR buffer, 1.2µl of 10mM MgSO4, 1µl of 10mM deoxynucleoside triphosphate, and 0.2µl Platinum Taq in a 50µl reaction (Invitrogen, Carlsbad CA). Same PCR condition were used for both rounds of PCR: 94°C for 5 mins, 35 cycles of 94°C for 30 sec, 55°C for 30sec, 72°C for 45 secs, and final 72°C for 5 mins. PCR products were analyzed by 2% agarose gel with an expectation of a 597 base pair PCR product. SIV infected macaques PBMC DNA during chronic infection stage were included in the PCR as

positive control and water was used for negative controls included in both 1st round and 2nd round of PCR.

Plasma viral load determination

1ml plasma samples were shipped overnight on dry ice to Advanced BioScience Laboratory Inc. (Kensington, MD) and plasma viral load was determined by isothermal nucleic acid sequences-based amplification (NASBA) method using real-time detection of amplified RNA with molecular beacons(215). Plasma viral load was reported as copies RNA per ml with 50 copies/ml as detection limit.

CD4 T cell counts

Absolute CD4 T cell counts were measured as part of complete blood count (CBC) routinely performed with blood draws at Southwest National Primate Research Centers. CD4 T cells are identified by flow cytometry using anti-CD3 and anti-CD4 antibody to have percentage of CD3+CD4+ cells from total cell population. Absolute CD4 T cell counts were calculated as the percentage of CD3+CD4+ cells in lymphocytes from complete blood count.

Assessment of provial DNA SIV envelope V1-V2 sequences by cloning

SIV envelope V1-V2 region nested PCR

Nested PCR was used to amplify SIV envelope V1-V2 region. Generally, a mix of PCR reagents was made with 5µl of PBMC DNA or 1st round PCR product, 1µl of SIV Env V1-V2 primer sets described(352) (1st round PCR primers: SIVenvV1V2-F1: 5'-GGA GGA ATG CGA CAA TTC CCC TCT T(T/C)T GT-3' and SIVenvV1V2-R1: 5'-

CAT TAC ATC TAA GCA AAG CAT AAC CTG G-3'; 2nd round PCR primers: SIVenvV1V2-F1: 5'-CCC AAT AAT GTT TGT CAC AAG ACT C-3' and SIVenvV1V2-R1: 5'-ACC AAG AAT AGG GAT ACT TGG GG-3') and 5µl of 10X Platinum PCR buffer, 1.2µl of 10mM MgSO4, 1µl of 10mM deoxynucleoside triphosphate, and 0.2µl Platinum Taq in a 50µl reaction (Invitrogen, Carlsbad CA). Same PCR condition were used for both rounds PCR: 94°C for 5 mins, 35 cycles of 94°C for 30 sec, 55°C for 30sec, 72°C for 45 sec, and final 72°C for 5 mins. All PCR products were analyzed by 2% agarose gel with an expectation of a 629 base pair PCR product.

TA cloning

Fresh PCR products were cleaned by Qiagen PCR purification kit (Qiagen, Valencia, CA) based on manufacturer's protocol. Briefly, PCR products were mixed with 500 µl PB binding buffer, flowed through QIAquick Spin columns, washed with 500 µl PE washing buffer and eluted with 30 µl autoclaved water. 4 µl purified PCR product was ligated with pCR2.1 TOPO vector at room tempature for 30 minutes and transformed into TOP10 competent cells with overnight growing at 37°C on LB plate selection by Ampicillin (Invitrogen, Carlsbad CA) and X-galactosidase. Individual clone was picked up and grow in 2ml LB medium with Ampicillin for overnight at 37°C. Single round of SIV Env V1-V2 PCR with 5 µl of 10x buffer with $MgSO_4$, 0.5 ul of 10mM SIV Env V1-V2 forward and reverse primer respectively, 0.5µl of 10mM dNTP and EconoTaq (Lucigen, Middleton, WI) was used to confirm the presence of SIV Env V1-V2 region on TOPO vector.

Plasmid DNA extraction and sequence analysis

Plasmid DNA containing SIV Env V1-V2 region were extracted by QIagen mini prep kit (Qiagen, Valencia, CA). Briefly, bacteria were pelleted and lysed with buffer, flow through DNA spin column followed by 2 washes and eluted in 50µl autoclaved water. 3 µl plasmid DNA with 3 µl of M13 forward or M13 reverse primer were used to determine DNA sequences by sequencing core at Seattle BioMed.

Assessment of plasma RNA SIV envelope V1-V2 sequences by single genome amplification (SGA)

Plasma viral RNA extraction

Plasma viral RNA was extracted using Qiagen Ultrasens viral RNA kit (Qiagen, Valencia, CA) according manual. Briefly, 0.8ml buffer AC and 5.6 µl carrier RNA was added to 1ml plasma and incubated at room temperature for 10 minutes so that viral particle was lysed and total RNA was precipitated following spinning at 1200g for 3 minutes. Nucleic acid pellet was re-suspend with 300 µl buffer AR with 20 µl protease K at 40 °C for 10 minutes to digest proteins. 300 µl buffer AB was added to stop protein digestion and entire mixture was transferred to QIAamp spin column following by 5000g spinning for 1 minute. 500 µl of Buffer AW1 and AW2 were used for 2 times of washing. Finally, RNA was eluted in 30 µl buffer AE and stored at -80°C for reverse transcription into cDNA.

Reverse transcription of viral RNA into cDNA

Viral RNA was reversed transcribed into cDNA using superscript III first strand synthesis system for RT-PCR (Invitrogen, Carlsbad CA) according to manufacture's

protocol. Briefly, 8 µl RNA was primed with 1 µl random hexamer (50ng/ µl) and 1 µl dNTP (10mM) at 65°C for 5 minutes and added with cDNA synthesis mix (2 µl 10X RT buffer, 4 µl 25mM MgCl2, 2 µl 0.1M DTT, 1 µl RNaseOUT (40U/ µl) and 1 µl Superscript III RT (200U/ µl)) following incubation at room temperature for 10 minutes, 50°C for 50 minutes allowing cDNA synthesis and 85°C for 5 minutes to inactivate enzymes activity. 1 µl RNase H was added to digest RNA from DNA-RNA hybrid at 37°C for 20 minutes. cDNA were stored at -20 for later PCR reactions.

SIV full length Envelope nested PCR

A master mix of PCR reagents was made with 2µl of cDNA or 1st round PCR product, 0.4µl of 10mM primer sets (1st round PCR primers: 251envF1 (5'-CAG TCT TTT ATG GTG TAC CAG CTT GGA GGA ATG-3' and 251envR1: 5'-GAG GAT CCA TCT TCC ACC TCT CCT AAG AGT C-3'; 2nd round PCR primers: 251envF2: 5'-GGA ACA ACT CAG TGC CTA CCA GAT AAT GGT G-3' and 251envR2: 5'-GTA GGT CAG TTC AGT CCT GAG GAC TTC TCG-3')(358) and 2µl of 10x High Fidelity Platinum PCR buffer, 0.8µl of 10mM MgSO4, 0.4µl of 10mM deoxynucleoside triphosphate, and 0.1µl Platinum Taq High Fidelity polymerase in a 20µl reaction (Invitrogen, Carlsbad CA). PCR conditions are listed as 1st round: 94°C for 5 minutes, 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 68°C for 4 minutes, and final 68°C for 10 minutes. 2nd round PCR condition: 94°C for 5 minutes, 45 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 68°C for 4 minutes, and final 68°C for 10 minutes. All PCR products are analyzed by 0.8% agarose gel with an expectation of a 2316 base pair PCR product.

A 5-fold dilution series was made from cDNA in DEPC water and nested PCR was performed to amplify nearly full length SIV Envelope gene amplicon. The last dilution reaction showing a PCR positive band on the gel and the next dilution point were used to for further replicates. 16 PCR replicates of the last dilution to show a positive band and another 16 replicates of next dilution point were performed to reach less than 30% PCR positive rate, which ensured the amplicons were derived from a single template. Replicates were repeated until average 20 PCR-positive reactions were produced from each SIV infected macaque. PCR products were cleaned by Qiagen PCR purification kit (Qiagen, Valencia, CA) based on manufacturer's protocol. After PCR product clean up, 3 µl of autoclaved water, 3 µl PCR product, 3 µl of 10mM SIVenvV1V2-R1 primer (5'-ACC AAG AAT AGG GAT ACT TGG GG-3') was used to obtain sequence of SIV envelope V1-V2 region by DNA sequencing core at Seattle BioMed.

Sequences analysis

Sequences were proof read and double-checked with single peak at every nucleotide position using the software Bioedit (Ibis Therapeutics, Carlsbad, CA). Verified sequences were aligned and analyzed using neighbor-joining phylogenetic analysis with kimura-2 parameter model, 500 bootstrap value by MEGA version 4.1 (www.megasoftware.net, Center for Evolutionary Functional Genomics, Tempe, AZ). p-distance was calculated by MEGA as dividing the number of nucleotide differences by the total number of nucleotides compared. Aligned sequences were also subject to Highlighter analysis through HIV database online tool to determine the number of viral variants (http://www.hiv.lanl.gov/content/sequence/HIGHLIGHT/highlighter_top.html).

Assessment of immune gene modulators expression in PBMC and tissues biopsy

Cellular RNA extraction from PBMC and tissue biopsy

Gingival biopsies and lymph node biopsies preserved in RNA later were transferred into beads containing tubes, 400 µl RLT lysis buffer provided by Qiagen RNeasy kit (Qiagen, Valencia, CA) was added and subjected to beads beater to disrupt tissue structures. PBMCs were thoroughly vortex to disrupt cell membrane. QIAshredder was also used to homogenize cells and tissue lysate. Cellular RNA was extracted by Qiagen RNeasy kit (Qiagen, Valencia, CA) based on manufacturer's protocol. Basically, homogenized cell lysates went through RNeasy mini spin columns and washed twice with RPE buffer. RNA was eluted with 50 µl RNase-free water and stored at -80°C for later reverse transcribed into cDNA by SuperScript-III first strand synthesis system for RT-PCR (Invitrogen, Carlsbad CA) as described in SGA section.

Quantitative Immune gene expression by real time PCR

Real-time PCR were performed using Applied Biosystem Taqman Master mix with gene specific primers/probes including IFN- α, OAS, CXCL10, IFN-γ, IL-4, IL-6, CXCR3, IL-15, IL-18, TNF-α, TGF-β and IL-10 (table 1). Transcript for Glyceraldehyde-3-phosphate dehydrogenases (GAPDH) gene was used as internal control. Generally, gene specific primers and probes or TaqMan® Gene Expression assays® (Applied Biosystems, Foster City, CA) were mixed with 2X TaqMan® Universal PCR Master Mix (Applied Biosystems, Foster City, CA) and cDNA from transcripts of PBMC or tissues in 96 well plates. Real-time PCR for each gene from each macaque was performed in duplicates with 7500 Real-Time PCR system

(Applied Biosystems, Foster City, CA). Fold change of mRNA level was calculated utilizing delta cycle threshold as relative quantification. Briefly, gene expressions were normalized to housekeeping gene GAPDH at every time point to obtain delta Ct (ΔCt) value (Ct of target gene−Ct of GAPDH). ΔCt of target gene from all macaques in the study at the preinfection/pregingivitis induction time point were averaged to define baseline target gene expression. Gene fold change was calculated as ΔΔCt value where gene expression at different time point post SIV infection compared to baseline gene expression (ΔCt of individual time point−baseline). Fold change was calculated with the formula $2^{-\Delta\Delta Ct}$ (98, 260).

Table 2-1. Primers and probes for quantitative real-time PCR

Gene	**Primer Name**	**Sequence (5' - 3')**
IFN-γ	IFNg Forward	5'-GAA AAG CTG ACC AAT TAT TCG GTA A-3’
	IFNg Reverse	5'-AGC CAT CAC TTG GAT GAG TTC A-3’
	IFNg Probe	5’-FAM-TGA CTC GAA TGT CCA ACG CAA AGC AGT A-TAMRA-3’
IL-10	IL-10 forward	5'-ACC CAG ACA TCA AGG AGC AT-3’
	IL-10 reverse	5'-CCA CGG CCT TGC TCT TGT T-3’
	IL-10 Probe	5’-FAM-TAC GGC GCT GTC ATC GAT TTC TTC-TAMRA-3’
CXCL10	CXCL10 forward	5'-CCT CCA GTC TCA GCA CCA TGA-3’
	CXCL10 reverse	5'-TGC AGG TAC AGC GTA CGG TCC-3’
	CXCL10 Probe	5’-FAM-TTC TGA CTC TAA GTG GCA TTC AAG GAG TAC CTC TCT C-TAMRA-3’
OAS	OAS forward	5'-CTG ACG CTG ACC TGG TTG TCT-3’
	OAS reverse	5'-ACT CTC CCC GGC GAT TTA A-3’
	OAS Probe	5’-FAM-CCT CAG TCC TCT CAC CAC TTT TCA GGA TCA-TAMRA-3’

TNF-α	TNFα forward	5'-GGC TCA GGC AGT CAG ATC AT-3’
	TNFα reverse	5'-GCT TGA GGG TTT GCT ACA ACA-3’
	TNFα Probe	5'-FAM-TCG AAC CCC AAG TGA CAA GCC TGT AGC-TAMRA-3'
CXCR3	CXCR3 forward	5'-CAA CCA CAA GCA CCA AAG CA-3’
	CXCR3 reverse	5'-GCA ACC TCG GCG TCA TTT-3’
	CXCR3 Probe	5’-FAM-CAC TCA CCT CAA GGA CCA TGG CTG G-TAMRA-3’
GAPDH	GAPDH forward	5'-GCA CCA CCA ACT GCT TAG CAC-3’
	GAPDH reverse	5'-TCT TCT GGG TGG CAG TGA TG-3’
	GAPDH Probe	5’-FAM-TCG TGG AAG GAC TCA TGA CCA CAG TCC-TAMRA-3’
IFN-α, TGF-β, IL-4, IL-6, IL-15, IL-18: TaqMan® Gene Expression assays from Applied Biosystems.		

Assessment of cytokines/chemokines in plasma and gingival crevicular fluid by luminex assay

For GCF, cytokines/chemokines were eluted from two filter strips in 50 µl of phosphate-buffered saline(PBS) containing 0.05% Tween 20 and protease inhibitors, and stored at −80°C before examination. Plasma were collected from peripheral blood and store at -80°C before use. An array of cytokines/chemokines in plasma samples including IFNα, IFNγ, CXCL10, IL-17, IL-18, IL-4, IL-6, MCP-1, MIP-1α, MIP-1β and TNF-α were determined by Giavedoni lab. Briefly, Luminex beads were coated with monoclonal antibodies for cytokines/chemokines detection. Plasma samples were thawed and mixed with coated luminex beads in 96-wells microtiter plates. Washes were performed with the help of Multiscreen vacuum manifold. Beads were resuspended with Luminex Sheath fluid and subject to Luminex^{100} system. Raw data as mean fluorescence intensity from beads were analyzed with the MasterPlex QT quantification software (MiraiBio Inc., Alameda, CA) to obtain concentration values(132).

Immunohistochemistry (IHC) staining of lymph node biopsy

Lymph node biopsies preserved in paraffin blocks were sent to SAIC-Frederick, Inc. Frederick National Laboratory (Frederick, MD) for immunohistochemistry staining for Myeloperoxidase and SIV RNA(107).

Assessing development of SIV-specific antibody response

Plasma samples were sent to Pem lab at Louisiana state university to determine the titer of SIV specific antibody. Briefly, concentrations of SIV-specific IgG in plasma were measured by ELISA (240) using high protein-binding microtiter plates (Fisher, St Louis, MO) coated overnight at 4˚C with aldrithiol-2-inactivated SIVCP-MAC (211) viral particles (from Dr. Jeff Lifson, AIDS and Cancer Program, Frederick, MD) that had been lysed with 0.25% v/v Triton X-100 and diluted in PBS to obtain 100ng capsid protein per well. Plates were washed with PBS containing 0.05% Tween-20 (PBST), blocked with 2% goat serum in PBST, then reacted overnight at 4˚C with multiple 3-fold dilutions of standard and each serum sample. The standard was pooled serum from SIV infected macaques; it was calibrated as described (240). Plates were developed the following day using biotinylated anti-human IgG (SouthernBiotech, Birmingham, AL), horseradish peroxidase-labeled neutravidin (Pierce) and TMB (SouthernBiotech). Concentrations of antibody in each test sample were interpolated from standard curves constructed with SoftMax Pro computer software (Molecular Devices, Sunnyvale, CA).

Assessment of C-reactive protein (CRP) in plasma by ELISA

Plasma CRP was measured by commercial ELISA kit from Life Diagnostics, Inc. (West Chester, PA) according to manufacturer protocol. Briefly, defined concentration of CRP was made from 2 fold serial dilution of concentrated CRP (2000ng/mL) to

have 37.5, 18.75, 9.38, 4.69, 2.34, 1.17 ng/mL for standard curve, respectively. Plasma samples were diluted 250 fold with 3 µl plasma and 797µl 1X diluent from the kit. 100 µl diluted samples and standards were added to 8 well strips pre-coated with anti-CRP antibody and incubated at room temperature for 45 minutes on the plate shaker. Wells were washed 5 times with 200 µl wash solution and flicked on the tissue towels to remove residual washing buffer. 100 µl enzyme conjugate reagent was added to each well and incubated at room temperature for 45 minutes on the plate shaker followed by 4 times washing. 100 µl TMB reagent was added and incubated at room temperature for 20 minutes covered in foil. With the reaction between enzyme conguate and TMB reagent, the color of the well turned blue. 100 µl stop solution was then added to each well to stop the reaction and the color of the well turned yellow. OD from each well was measured with wavelengths 450 filter. The concentration of CRP standard and OD were used to determine standard curve (with R^2 higher than 0.99) and calculate the concentration of CRP in plasma sample.

Assessment of cell phenotypes and immune activation markers on immune cells by flow cytometry

Six-color flow cytometry (CD3-Alexa700, CD4-PE, CD8-pacific blue, CD16-APC, HLA-DR-Per-CP-Cy5.5 and Ki-67-FITC) was performed to assess surface and intracellular markers on immune cells from macaques. Whole blood was stained with fluorescent-conjugated antibodies against human which can cross react with macaques molecules. Briefly, 100 µl whole blood was incubated with antibodies staining surface markers for 30 minutes on ice. Red blood cells were lysed by 4ml of erythrocyte lysing solution, washed with PBS. Cell proliferation marker Ki-67 was

stained intracellularly after completing surface markers staining. Data was acquired with CyanTM ADP instrument (Beckman counlter Inc, Fullerton, CA) or BD LSRII (BD Biosciences, San Jose, CA).

Assessment of soluble CD14 (sCD14) and LPS binding protein (LBP) in plasma
Plasma samples were sent to Brenchley lab to examine sCD14 and LBP by ELISA. Each sample was run in duplicates(194).

Part II: penile challenge study

Study animals

The Macaques used in the studies were colony-bred rhesus macaques (Macaca mulatta) housed at the Washington National Primate Research Center (WaNPRC). Total 10 male adult macaques (A09010-A09019) were included in the study. All macaques were cared for in accordance with National Institute of Health guidelines and local Animal Care and Use Committee. Animals were anesthetized with ketamine hydrochloride (10 mg/kg) injected intramuscularly to perform blood and tissue sampling as well as SIV administration.

TRIM5α genotyping of macaques

The TRIM5 genotypes for foreskin study macaques were determined as described above and the results were shown in table 2-2.

Table 2-2 TRIM5 genotypes of macaques

Macaque ID	Genotype
A09010	TRIM Q/TRIM CYPA
A09018	TRIM Q/TRIM CYPA
A09011	TRIM TFP/TRIM CYPA
A09012	TRIM TFP/TRIM Q
A09014	TRIM TFP/TRIM Q
A09013	TRIM TFP/TRIM TFP
A09015	TRIM TFP/TRIM TFP
A09016	TRIM TFP/TRIM TFP
A09017	TRIM TFP/TRIM TFP
A09019	TRIM TFP/TRIM TFP

SIV Virus administration and sampling time line

1st set study included four macaques (A09010, A09011, A09012, A09014). Penile tissues were pulled up to form a cup and held 225µl 8000 $TCID_{50}$ SIVsmE660 to make virus contact with penile/foreskin tissues for 15 minutes. 12ml Peripheral blood was collected at times indicated below in EDTA tube and shuttle from primate center to Seattle Biomed for further processing, including plasma collection and standard Ficoll centrifugation procedure to purify PBMC (Table 2-3).

Table: 2-3 study time line for 1st set study								
Study day	-28	-7	0	4	7	10	14	21
SIVsmE660 inoculation			X					
Blood	X	X	X	X	X	X	X	X

2nd set study included the same four macaques (A09010, A09011, A09012, A09014) as 1st set study. Double volume (450ul) of 8000$TCID_{50}$ SIVsmE660 was given three times every other day with similar virus administration method as described in 1st set study. In addition, surgical type was used to hold the penile tissue cup formation while the macaques recovered. 12ml Peripheral blood was collected indicated below and processed as previous described (Table 2-4).

Table 2-4 study time line for 2nd set study								
Study day	28	30	32	35	42	45	49	56
SIVsmE660 inoculation	X	X	X					
Blood	X			X	X	X	X	X

3rd set study included 5 macaques—macaque A09010 and A09017 were in control group and macaque A09011, A09013, A09015 were in *Haemophilus ducreyi* (HD) group in which macaques were intradermally injected with 5x10^7 pfu *Haemophilus ducreyi* at study day 0. Similar method was used to pull up penile tissue to form a cup for holding 200ul SIVsmE660 (8000$TCID_{50}$) for 10 minutes at study day 2 and 16. Ceftriaxone was intramuscularly injected to treat *Haemophilus ducreyi* infection after second SIV administration (at study day 18 and day 25) in HD group macaques. Foreskin biopsy was taken at study day 18 with one portion of tissue stored in RNAlater and another portion preserved in formalin. 12 ml Peripheral blood was collected at indicated time shown below and processed as previous described (Table 2-5).

Table 2-5 study time line for 3rd set study								
Study day	0	2	9	16	18	23	25	30
infection	X							
ceftriaxone (100 mg/kg)					X		X	
SIVsmE660 inoculation		X		X				
Foreskin biopsy					X			
Blood		X	X	X		X		X

4th set study included 9 macaques—3 macaques (A09011, A09015, A09016) were in control group and 6 macaques (A09010, A09012, A09014, A09017, A09018, A09019) were in HD group in which macaques were intradermally injected with $5x10^7$ pfu at study day -2. Among HD group, macaque A09010, A09012, A09014 received *Haemophilus ducreyi* intradermally and macaque A09017, A09018, A09019 received *Haemophilus ducreyi* subcutaneously. At study day 0, 200 ul 10^5 $TCID_{50}$ SIVmac251 was administered with similar method described with penile tissue pull up to form a cup to hold the SIV for 10 minutes. Macaques infected with *Haemophilus ducreyi* were treated with ceftriaxone at day 16 and 18 in both control and HD group macaques. Foreskin biopsy was taken at study day 16 with one portion of tissue stored in RNAlater and another portion preserved in formalin. 12 ml Peripheral blood was collected at indicated time shown below and processed as previous described (Table 2-6).

Table 2-6 study time line for 4th set study								
Study day	-2	0	7	14	16	21	23	28
***Haemophilus ducreyi* infection**	X							
ceftriaxone (100 mg/kg)					X		X	
SIVmac251 inoculation		X		X				
Foreskin biopsy					X			
Blood		X	X	X		X		X

Determination of SIV infection by SIV gag PCR
SIVgag PCR was used to determine whether macaques were SIV infected as detailed protocols were described in previous part.

Plasma viral load determination
Plasma viral load were determined by Advanced BioScience Laboratory Inc. (Kensington, MD).

CD4 T cell counts
Absolute CD4 T cell counts were measures as part of complete blood count (CBC) routinely performed with blood draws at Washington National Primate Research Center.

Cellular RNA extraction from foreskin biopsy
Foreskin biopsies preserved in RNA later were transferred into Lysing Matrix D beads containing tubes (MP biomedicals LLC. Solon, OH), 400 µl RLT lysis buffer provided by Qiagen RNeasy kit (Qiagen, Valencia, CA) was added and subjected to beads beater to disrupt tissue structures. Cellular RNA was extracted by Qiagen RNeasy kit (Qiagen, Valencia, CA) based on manufacturer's protocol as described in previous chapter.

Examination of immune activation markers by multicolor flow cytometry
8-colors flow cytometry (Table 2-7) was used to assess surface and intracellular markers on macaques PBMC isolated from 15 ml peripheral blood collected and shuttled to Seattle Biomed at the same day of collection.

2 million PBMCs were incubated with a panel of fluorophore-conjugated monoclonal antibodies for extracellular markers in FACS tubes on ice for 30 minutes. Cells were washed with 5ml PBS followed by 1700rpm centrifugation for 10 minutes and supernatant was discarded. After extracellular markers staining, cells were permeabilized with 750ul 1X BD FACS buffer for 10 minutes at room temperature and washed twice. Intracellular marker (Ki-67) antibody was added for 30 minutes staining on ice followed by one wash. Finally, cells were fixed in 1.6% paraformaldehyde and keep on ice until data was acquired using BD LSRII and software FACS DIVA (BD Biosciences, San Jose CA). Generally, 100,000 events were recorded and data was analyzed with FlowJo (Tree Star Inc, Ashland, OR).

Table 2-7 immune activation panel for flow cytometry

Marker	Fluorophore
CD95	APC
Ki-67	PE
HLA-DR	PE-Cy7
CD38	FITC
CD3 (clone SP34-2)	APC-Cy7
CD8	PerCP-Cy5.5
CD4 (clone OKT4)	Pacific blue
Live/dead	Amine Aqua

Hematoxylin and eosin (H&E) staining of foreskin biopsy and imaging

Foreskin biopsies preserved in formalin were sent to Histology Consulting Services (Mohs Histology Consulting Services, Spokane, WA) for tissue embedding, cutting and H&E staining. Slides were sent back to Seattle Biomed and imaged with inverted Microscope.

Chapter 3: The influence of gingival inflammation on oral SIV acquisitions in rhesus macaques

Introduction

The current HIV epidemic is spread primarily during transmission across a mucosal site including the vaginal, penile rectal or oral mucosa. Transmission of HIV via the oral exposure can occur during mother-to-child transmission due to virus in breast milk or oral-genital transmission due to virus in semen. Infants born from HIV infected mothers can acquire the virus prior to birth, during birth or through breastfeeding which accounts for approximately one third of infants that become HIV infected (192, 271, 290). The rate of oral-genital transmission of HIV is difficult to ascertain, although a few studies have documented that transmission can occur during receptive oral intercourse (45). Overall, these epidemiological studies indicate that while oral HIV transmission can occur, it only occurs under certain circumstances and understanding the environmental events or genetic factors that influences oral transmission of HIV would be useful in preventing HIV acquisition via this route.

To investigate the earliest events following oral transmission the simian immunodeficiency virus (SIV) infection of rhesus macaque model have been utilized. Oral SIV administration can initiate a successful infection in both neonates and adults macaques (22, 25, 98, 259, 260). Similar to HIV infection in humans, immune dysfunctions were observed early following oral SIV infection and AIDS like symptoms were developed in the macaques (24, 25). The early virologic events following SIV administration to the oral cavity involve traversing the mucosal surfaces of the upper gastrointestinal tract. Data from our laboratory has provided evidence that orally

applied SIV entered the host through the oral or esophageal mucosa as well as the tonsils (oral mucosal associated lymphoid tissue) (78, 259, 355). SIV infected macrophages and CD4 T cells were detected in oral biopsy within few days following SIV infection, raising the possibility that these cells play key roles in initial virus replication and dissemination during early oral SIV infection (259). Following oral SIV infection, innate immune responses were strongly elicited in oral tissues and draining lymph nodes, predominantly up-regulation of inflammation related genes such as MIP-1α, TNF-α, IL-6, IL-12, and IFN-γ (5, 199, 260). Multiple subtypes of IFN-α and interferon mediated anti-viral genes, including OAS and CXCL10, were also up regulated in oral mucosa (5, 70, 99, 199, 260). These studies indicate that macaques can rapidly respond to SIV invasion following oral exposure, however, early immune activation at the oral tissues is not potent enough to eliminate SIV replication and dissemination.

Studying factors associated with HIV/SIV infection through vaginal, penile, and rectal mucosal sites have provided insights regarding potential immune correlates for mucosal HIV/SIV infection. Epidemiological studies have demonstrated the association between sexual transmitted diseases (STDs) and increased HIV infection (179, 180, 313, 314), indicating that STDs-induced mucosal inflammation can facilitate HIV/SIV infection. Potential mechanisms include that mucosal inflammation can contribute to mucosal membrane tissues damage, which compromises the protecting barrier of mucosal membrane. In addition, mucosal inflammation may accompany with increased numbers of target cells or preferentially increased

activated target cells at the mucosal sites, which can increase the possibility of productive HIV/SIV replication and virus dissemination.

The study described here addresses the question whether pre-existing mucosal inflammation within the mouth impacts oral SIV infection in macaques. In this study, gingival inflammation was induced in macaques and followed by SIV administration into the oral cavity. Our data indicates that induced gingival inflammation had no significant impact on the numbers of rhesus macaques that become SIV infected. However, gingival inflammation was associated with increased numbers of SIV variants being transmitted . Immunologic assessment of the gingival crevicular fluid as well as gingival biopsies determined that certain cytokines/chemokines were up-modulated more robustly in macaques from gingivitis-induced group compared to the controls following oral SIV infection. Overall, this study did not identify the relationship between gingival inflammation and increased SIV infection, but it did identify virologic and immunologic differences that were present following oral SIV infection.

Results

Studied macaques

There were total 16 male macaques enrolled in the study, 8 were assigned into the control group and 8 were assigned into the gingivitis-induced group. The age and the weight of the macaques from control and gingivitis-induced group at the beginning of the study were similar. Three TRIM5 genotypes (Q, TFP, CypA) and nine MHC genotypes (A01, A02, A08, A11, B01, B03, B04, B08, B17) were determined for consideration of potential genetic factors linkage to our results. For MHC genotypes, 5

out of 8 macaques in control group had protective alleles associated with controlled virus replication that need to be considered as a potential factor to explain our data. TRIM5 genotypes of the macaques in the study ($TRIM5^{CypA}$, $TRIM5^{TFP}$, and $TRIM5^{Q}$) were similarly distributed in both groups (Table 3-1).

Table 3-1. Characteristics of studied macaques.

Group	RM ID	Gender	Age	TRIM Genotype#	MHC I Genotype*
Control	**18993 RM-C1**	M	6	Q/CypA	B01,B17
Control	**26740 RM-C2**	M	4	Q/TFP	A02,B17
Control	**26856 RM-C3**	M	4	Q/Q	
Control	**26968 RM-C4**	M	4	TFP/TFP	A08
Control	**26517 RM-C5**	M	5	TFP/TFP	B01,B17
Control	**26744 RM-C6**	M	5	Q/TFP	A02,B01,B17
Control	**26971 RM-C7**	M	5	TFP/TFP	B01
Control	**30309 RM-C8**	M	5	Q/TFP	A01,B01
gingivitis-induced	**18978 RM-G1**	M	6	Q/TFP	
gingivitis-induced	**19852 RM-G2**	M	5	CypA/CypA	
gingivitis-induced	**26970 RM-G3**	M	4	TFP/TFP	A02
gingivitis-induced	**27270 RM-G4**	M	4	Q/Q	B01
gingivitis-induced	**19313 RM-G5**	M	7	Q/Q	
gingivitis-induced	**26981 RM-G6**	M	5	TFP/CypA	
gingivitis-induced	**27238 RM-G7**	M	5	TFP/TFP	B01
gingivitis-induced	**30311 RM-G8**	M	6	TFP/TFP	A08,B01

TRIM5 genotypes includes $TRIM^{Q}$, $TRIM^{TFP}$, $TRIM^{CypA}$ (Johnson lab)
*MHC I genotypes screen includes A01, A02, A08, A11, B01, B03, B04, B08, B17 (Watkins lab)

Gingival inflammation induction

Gingival inflammation was induced in a group of rhesus macaques with a combination of teeth ligatures and a diet of water softened monkey chow (102, 263). Gingival inflammation was evaluated by clinical periodontal measurements and detection of immune modulators in GCF. Around 5-7 days prior to SIV administration, macaques in gingivitis-induced group had higher plaque index (the extent and quantity of tooth-associated bacterial plaque)($p=0.0286$ for 1st set study, $p=0.0079$ for 2nd set study, $p=0.0571$ for 3rd set study, Mann-Whitney U test) and were more prone to bleed when probing ($p=0.0286$ for 1st set study, $p=0.00119$ for 2nd set study, $p=0.0571$ for 3rd set study, Mann-Whitney U test), compared to macaques in control group (Table 3-2), indicating higher levels of gingival inflammation.

Analysis of immune modulators in the gingival crevicular fluid 5 days before SIV administration also showed higher concentration of inflammatory immune modulators, including Gro-α ($p=0.002$), IL-6 ($p=0.002$), IL-8 ($p<0.001$), IL-18 ($p=0.003$), IL-1β ($p=0.0057$), MCP-1 ($p=0.002$), perforin ($p=0.002$), RANTES ($p=0.002$), and sCD14 ($p=0.003$) in our 2nd set study from gingivitis-induced group macaques (Figure 3-1). Based on clinical assessment and immune modulators measurement in GCF, the combination of soft food and silk ligatures successfully induced gingival inflammation in these macaques, compared to control macaques.

Table 3-2: clinical assessment of gingival inflammation 5-7 days before oral SIV administration.

1st set	Plaque Index (units)	Bleeding on Probing (units)	Probing Depth (mm)	Attachment Loss (mm)
RM-C1	1.54	0.38	1.94	1.88
RM-C2	1.05	0.34	1.27	1.27
RM-C3	1.38	0.54	2.15	1.96
RM-C4	1.40	0.44	1.79	1.79
RM-G1	2.54	1.33	2.25	1.77
RM-G2	2.48	1.79	2.48	2.00
RM-G3	2.13	1.00	2.42	2.19
RM-G4	2.54	1.23	2.21	1.98

2nd set	Plaque Index (units)	Bleeding on Probing (units)	Probing Depth (mm)	Attachment Loss (mm)
RM-C5	1.63	0.42	1.65	1.65
RM-C6	1.52	0.58	1.73	1.63
RM-C4	1.75	0.42	1.63	1.63
RM-C7	1.44	0.48	1.65	1.65
RM-C8	1.65	0.50	1.69	1.69
RM-G1	2.50	1.13	2.31	1.96
RM-G5	2.60	1.60	2.81	2.39
RM-G6	2.48	1.33	2.42	2.11
RM-G7	2.38	1.58	2.23	2.00
RM-G8	2.58	1.88	2.96	2.44

3rd set	Plaque Index (units)	Bleeding on Probing (units)	Probing Depth (mm)	Attachment Loss (mm)
RM-C6	1.63	0.48	1.42	1.38
RM-C4	1.79	0.35	1.46	1.44
RM-C7	1.40	0.10	1.40	1.40
RM-C8	1.67	0.13	1.56	1.54
RM-G1	2.69	0.77	2.10	1.91
RM-G7	2.29	1.21	2.23	1.94
RM-G8	2.58	0.85	2.54	2.25

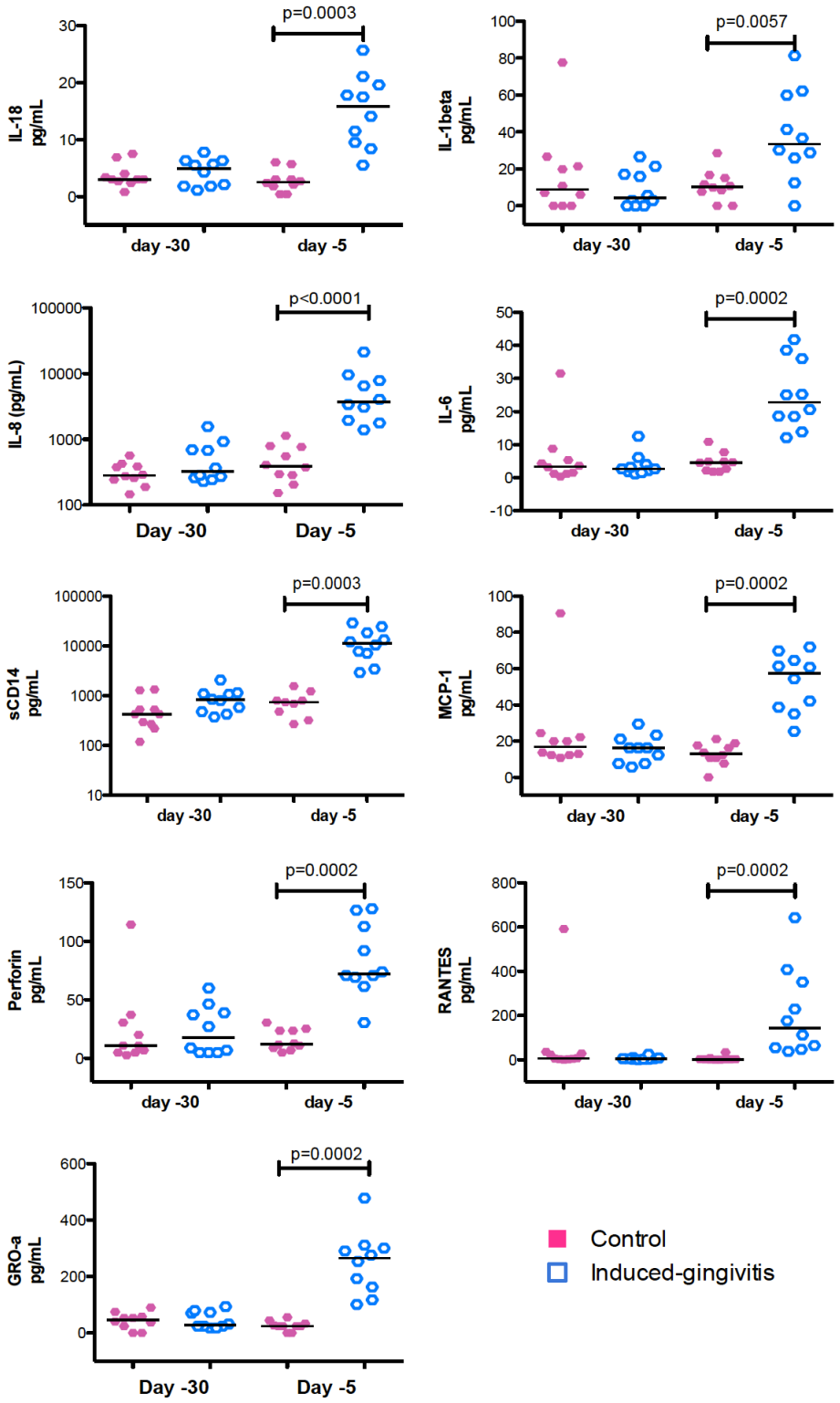


Figure 3-1. The levels of immune modulators in gingival crevicular fluid.
Immune modulators in gingival crevicular fluid from 2nd set study (2 teeth measurements per macaque, total 5 macaques total) were measured by luminex at day -30 (before gingival inflammation induction) and day -5 (after gingival inflammation induction, 5 days prior to SIV administration). p-value was calculated by Mann-Whitney U test.

The impact of gingival inflammation on the rates of oral SIV infection in rhesus macaques

The presence of SIV gag gene in PBMC genomic DNA during first two weeks post 1st SIV administration was used to determine if macaques were SIV infected (Table 3-3). Following three times of oral SIV administration, our 1st set study (using needleless syringe to administer 1800 $TCID_{50}$ SIVmac251 to gingiva) resulted in 3 macaques (RM-C1, RM-C2, RM-C3) in the control group and 3 macaques (RM-G2, RM-G3, RM-G4) in gingivitis-induced group becoming SIV infected. When whatman paper was used to make SIV virus entry more specific and localized to gingival tissues, our 2nd set study (using whatman paper containing 1800 $TCID_{50}$ SIVmac251 to apply on gingiva three times) resulted in 1 macaque (RM-C5) in control group and 2 macaques (RM-G5 and RM-G6) in gingivitis-induced group. Comparing our 1st and 2nd study results, we believe that whatman paper may retain the majority of the virus on the paper, and therefore, lower infection rate was observed in our 2nd set study. In our 3rd set study, we continued to use whatman

Table 3-3: oral SIV infection rate

	Control group		Gingivitis-induced group	
		SIV status		SIV status
1st set	RM-C1	+	RM-G1	-
	RM-C2	+	RM-G2	+
	RM-C3	+	RM-G3	+
	RM-C4	-	RM-G4	+
Infection rate	3 /4 (75%)		3 /4 (75%)	
2nd set	RM-C5	+	RM-G1	-
	RM-C6	-	RM-G5	+
	RM-C4	-	RM-G6	+
	RM-C7	-	RM-G7	-
	RM-C8	-	RM-G8	-
Infection rate	1 /5 (20%)		2 /5 (40%)	
3rd set	RM-C6	-	RM-G1	-
	RM-C4	-	RM-G7	-
	RM-C7	-	RM-G8	-
	RM-C8	+		
Infection rate	1 /4 (25%)		0 /3 (0%)	

paper but with higher dose of SIVmac251 (2750 $TCID_{50}$) application to gingiva for three times, however, only 1 macaque (RM-C8) from control group and no macaque in gingivitis-induced group became SIV infected. Based on these 3 sets studies, the infection rate for 1^{st}, 2^{nd}, 3^{rd} set study respectively is 75%, 20%, 25% in control group and 75%, 40%, 0% in gingivitis-induced group (Table 3-3). Overall, the oral SIV infection rate was similar between control and gingivitis-induced group, indicating that induced-gingivitis may not have dramatic influence on SIV oral acquisition in our study.

The impact of gingival inflammation on SIV plasma viral load and CD4 T cell counts following oral SIV infection

Combining all three sets of studies, there were total 5 macaques in the control group and 5 macaques in the gingivitis-induced group developed systemic SIV infection following oral SIV exposures. Plasma viral load was determined to assess if gingival inflammation has influences on SIV replication following oral SIV administration (Figure 3-2A and 2B). Among SIV infected macaques, RM-C1 and RM-C2 from control group and RM-G3 and RM-G5 from gingivitis-induced group had detectable plasma viral load at day 7 -9 post 1^{st} virus administration. Other macaques had detectable plasma viral load around day 14-16 post 1^{st} virus administration. After acute SIV infection, RM-C5 in control group and RM-G5 in gingivitis-induced group both had controlled virus replication (below detection limit 50 copies/ml) during chronic infection. Macaque RM-C8 in control group also had a trend toward lower viral replication during chronic SIV infection. Overall, viral set points, determined as average plasma viral load obtained between day 60 and day 210, were developed around 10^6 viral RNA copies per ml for SIV infected macaques without controlled virus

replication, and were comparable between control and gingivitis-induced group, indicating that gingival inflammation did not have significant impacts on systemic SIV replication as measured by plasma viral load. Of note that the phenomenon of controlled virus replication during chronic SIV infection in RM-C5 and RM-C8 may be partially due to MHC genotypes, where RM-C5 had well-known protective allele B*17 and RM-C8 had A*01 allele. However, no clear genetic factors were associated with spontaneous controlled virus replication for RM-G5.

CD4 T cell counts in the peripheral blood was determined to assess if gingival inflammation affects disease progression following oral SIV administration, (Figure 3-2C and 2D). Following oral SIV infection, SIV infected macaques exhibited reduced CD4 T cell counts during acute infection. Macaques with low or controlled virus replication (RM-C5, RM-C8 and RM-G5) were more likely to preserve CD4 T cell counts during chronic infection stage. Overall, there were no significant differences with regard to CD4 T cell counts between control and gingivitis-induced groups.

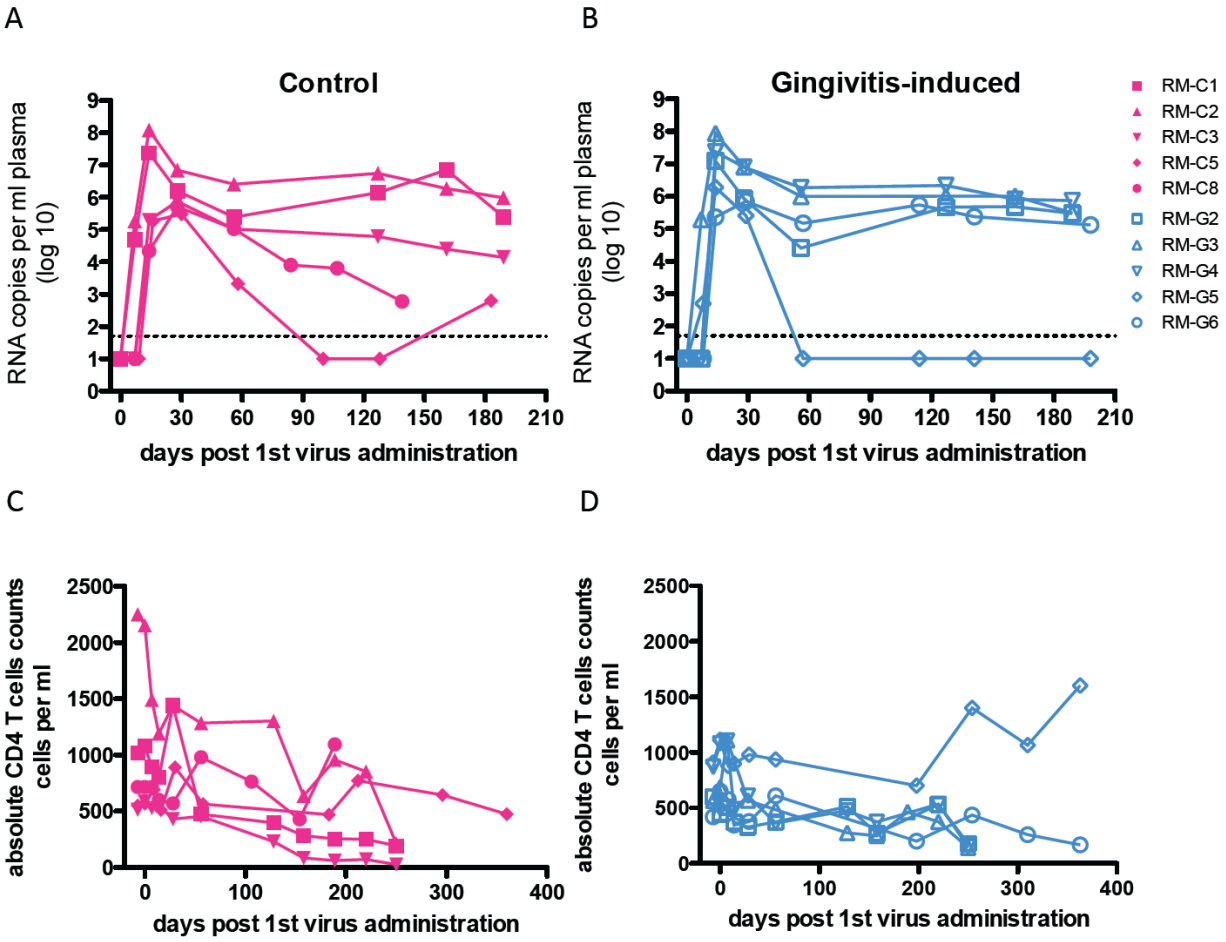


Figure 3-2. Plasma viral load and CD4 T cell counts following oral SIV infection. Plasma viral load shown as Viral RNA copies per milliliter (detection limit as 50 copies per milliliter of plasma shown in dot line) and CD4 T cell counts in orally SIV infected macaques. Plasma viral load and CD4 T cells counts for macaques C1-C8 in control group (A and C); macaques G1-G8 in gingivitis-induced group (B and D)

The impact of gingival inflammation on the number of founder virus following oral SIV administration

To determine if gingival inflammation is associated with increased numbers of founder virus in orally infected macaques, two methods, sequencing of PBMC proviral DNA by cloning and plasma viral RNA by SGA, were used to determine viral sequences from SIV infected macaques. Sequences of highly variable SIV envelope V1-V2 region were obtained from plasma viral RNA and PBMC proviral DNA at the first time point that plasma viral load become detectable (generally between day 7 and 16 post 1st SIV administration) as well as SIVmac251 viral inoculum were analyzed.

Assessment of the SIV envelope PBMC proviral DNA V1-V2 sequences (20 from each macaque on average) resulted in a viral diversity (p-distance) ranging from 0.4%-0.7 % in the control group and 0.3%-1.2% in the gingivitis-induced group. Plasma viral RNA diversity ranges from 0.0%-0.9% in the control group (note that sequences were identical within macaque RM-C3, RM-C5 and RM-C8) and 0.0%-1.4% in the gingivitis-induced group (Table 3-4). Viral diversity was generally higher in PBMC proviral DNA than plasma viral RNA, except RM-C1 in which plasma viral RNA has higher diversity than PBMC proviral DNA. SIV envelope V1-V2 region sampled at day 14 post 1st virus administration from RM-C8 could not be amplified even though re-extraction of PBMC DNA and PCR re-run has attempted with proper positive control.

Table 3-4. SIV Envelope V1-V2 sequences analysis from orally SIV infected macaques

Control	Days post-1st virus administration	No. sequences analyzed		p-distance		Number of founder virus
		PBMC DNA	Plasma RNA	PBMC DNA	Plasma RNA	
RM-C1	7	20	22	0.5%	0.9%	4
RM-C2	7	20	22	0.7%	0.0%	1
RM-C3	14	18	21	0.4%	identical	1
RM-C5	16	13	18	0.5%	identical	1
RM-C8	14	N/A	22	N/A	identical	1
Gingivitis-induced						
RM-G3	7	21	20	1.2%	1.4%	4
RM-G2	14	21	21	0.5%	0.2%	3
RM-G4	14	21	22	0.4%	0.1%	2
RM-G5	14	17	24	0.4%	0.0%	1
RM-G6	14	20	20	0.3%	0.0%	1

One interesting observation was that among 26 PBMC DNA sequences from macaque RM-C5, 13 of 26 sequences had a specific nucleotide deletion within SIV V1-V2 region. Re-amplification of SIV Env V1-V2 region with PCR and cloning yielded the same result, indicating that this mutation was unlikely to be a result of PCR or cloning error. This one nucleotide deletion resulted in a frame-shift and a stop codon in the envelope protein, indicating that these sequences may be replication incompetent variants. It is possible that this variant had this mutation at early time points and accumulated as cells proliferate.

Plasma SIV envelope V1-V2 Sequences from each orally SIV infected macaques were analyzed with highlighter analysis to enumerate the number of founder virus. Viral variants were differentiated based on the assumption that single nucleotide change (exclude predicted APOBEC editing) detected by two or more sequences represent a unique founder virus to rule out random mutation that occurs and accumulates in the sequences. Given the short time period for virus evolution in these macaques (since these sequences were obtained at between day 7 and day 16 after SIV infection) and given the short length of sequences analyzed, more than two nucleotides changes on the sequence is considered as unique founder variant even if only one sequence is presented. Using these criteria, SIV envelope V1-V2 sequences from RM-C1 clearly had 4 different populations of viruses, indicating that RM-C1 was infected by at least 4 founder viruses (Figure 3-3A). For RM-C2, the majority of the sequences were the same except some sequences, which had one nucleotide difference from other variants and were not represented by two or more sequences. These sequences did not fit our criteria to define them as a different founder virus and therefore, the number of founder virus was considered as one (Figure 3-3B). For RM-C3, RM-C5 and RM-C8, SIV envelope V1-V2 sequences from these three macaques were identical and the number of founder virus was considered as one in these macaques (Figure 3-3C to 3E). Phylogenetic tree analysis of PBMC DNA and plasma viral RNA showed that viral sequences from plasma were mingled with sequences from PBMCs and further provide supporting data for our interpretation regarding the numbers of founder virus in each macaque (Figure 3A-3E). In general, 4 out of 5 macaques in the control group were infected with one founder virus.

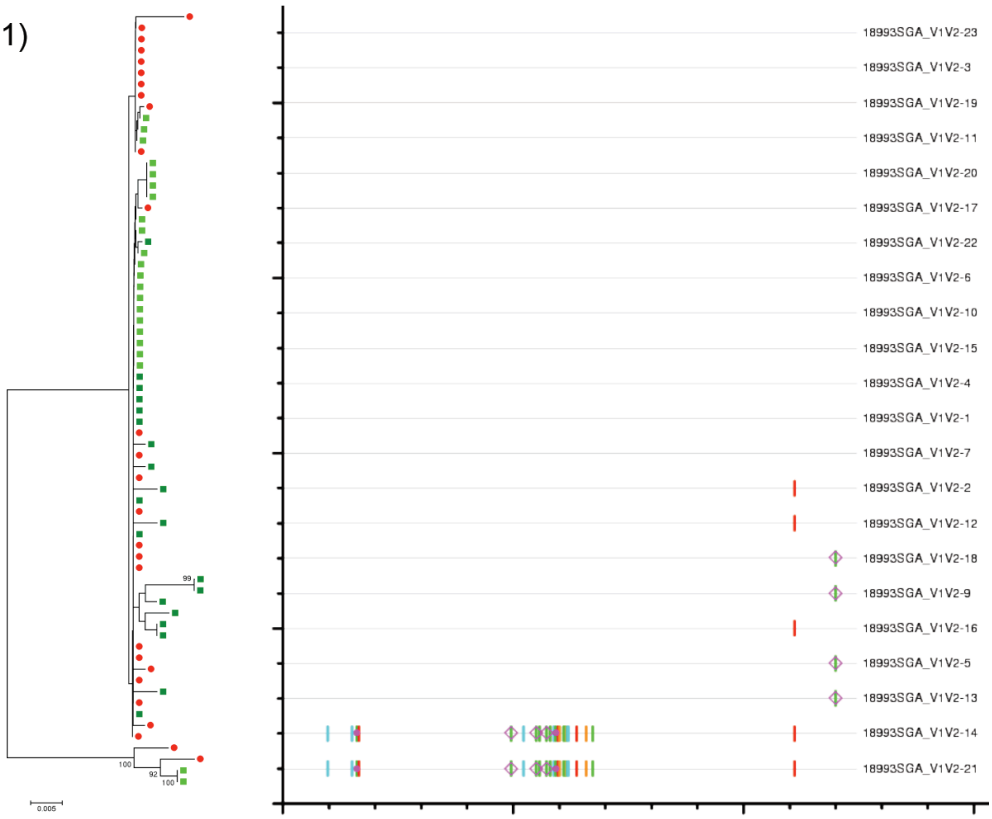
Using the same criteria to determine the numbers of founder virus in macaques from gingivitis-induced group, we found that SIV envelope V1-V2 sequences from RM-G3 had at least 3 populations of viruses (represented by #23, #16 and #6 and #21). However, when SIV envelope V1-V2 sequences from PBMC DNA and plasma viral RNA were combined with SIVmac251 for phylogenetic tree analysis, we found that there was one sequence from plasma and another one sequence from PBMC that were identical and group into another lineage of SIVmac251 inoculum. Although these 2 sequences only differ from majority of the sequences by one nucleotide, we considered these as a 4^{th} variant (Figure 3-4A). For RM-G2, there were 3 different populations of viruses, indicating that macaque RM-G2 was infected by at least 3 founder viruses (Figure 3-4B). For RM-G4, there were a dominant variant population and a minor variant population (represented by 2 sequences #1 and #18). Other than these two major variants, 3 out of 4 changes were considered as APOBEC induced mutations and only represented by one sequence, which did not qualify to be a different variant and therefore we determined RM-G4 had 2 founder viruses (Figure 3-4C). For RM-G5 and RM-G6, the majority of the sequences were the same except some sequences, which had only one nucleotide difference from other variants and were not represented by two or more sequences. These sequences do not fit our criteria to define them as a different founder virus and therefore, the number of founder virus in macaque RM-G5 and RM-G6 was considered as one (Figure 3-4D and 4E). By analysis of phylogenetic analysis of branching patterns, viral sequences from PBMCs also confirmed the numbers of founder virus indicated by SGA analysis

(Figure 4A-4E). In summary, 3 out of 5 macaques from gingivitis-induced group had multiple viral variants to initiate systemic SIV infection.

These reads are combined and summarized in table 5, we observe that 4 out of 5 macaques in control group acquired single viral variant, while multiple variants infected one macaque from control group. In gingivitis-induced group, 3 out of 5 macaques were infected with multiple viral variants. These results indicate that induced gingival inflammation is associated with multiple viral variants infection (OR=6, 95%CI=0.3544-101.5728).

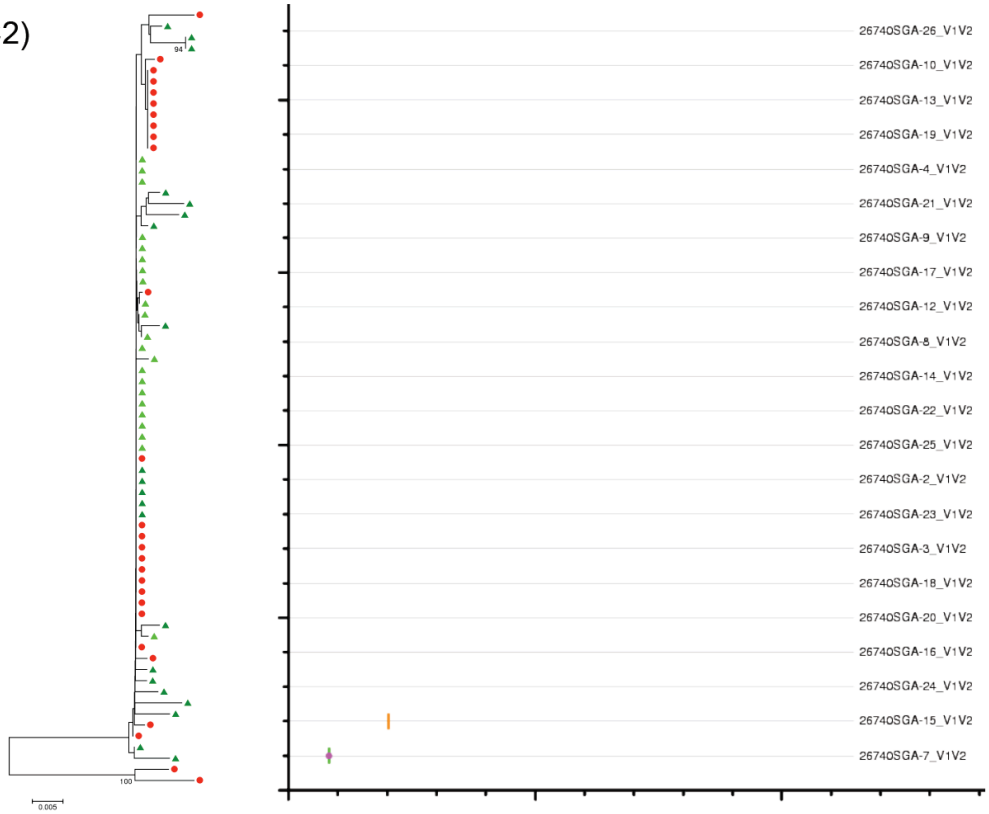
A

18993 (RM-C1)



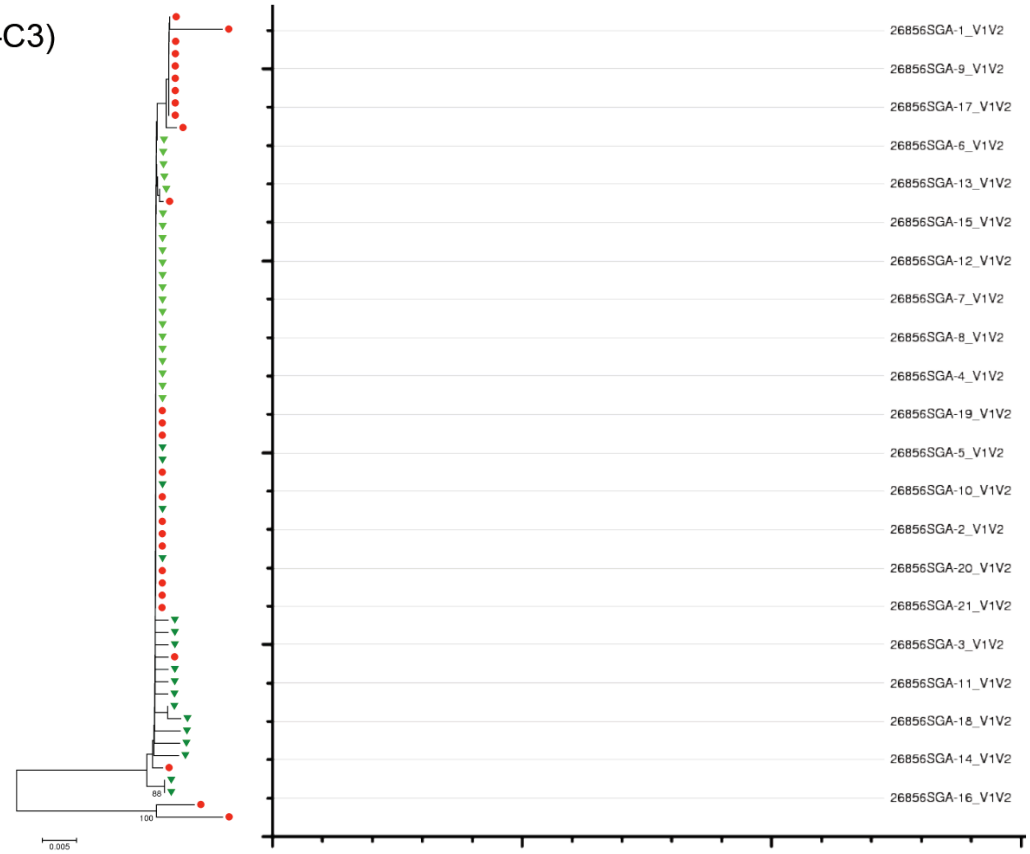
B

26740 (RM-C2)



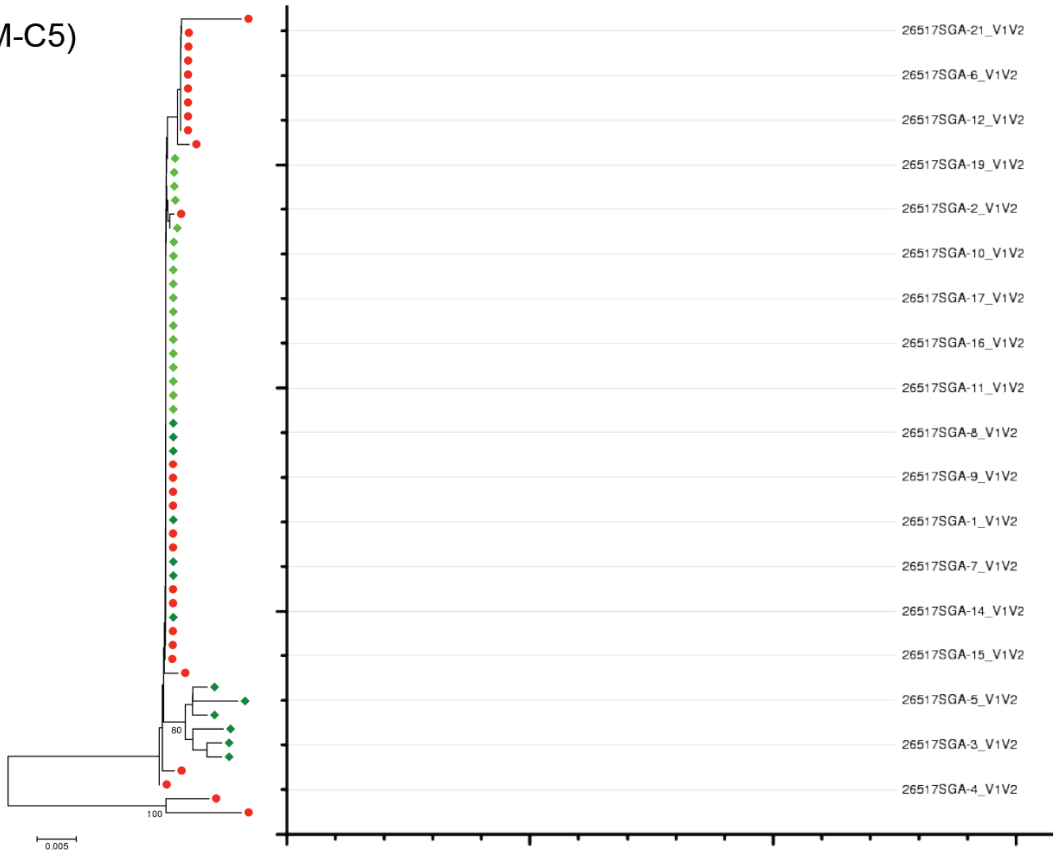
C

26856 (RM-C3)



D

26517 (RM-C5)



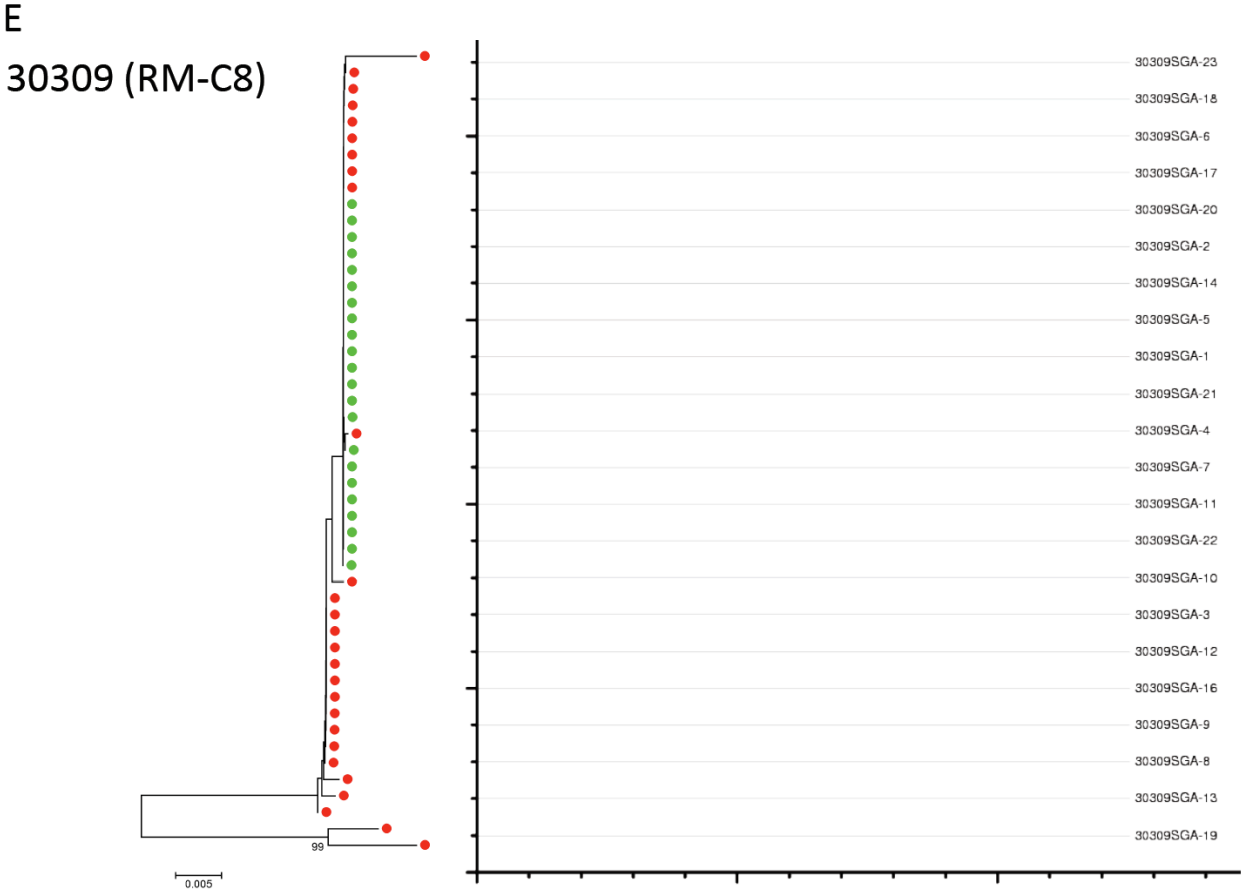
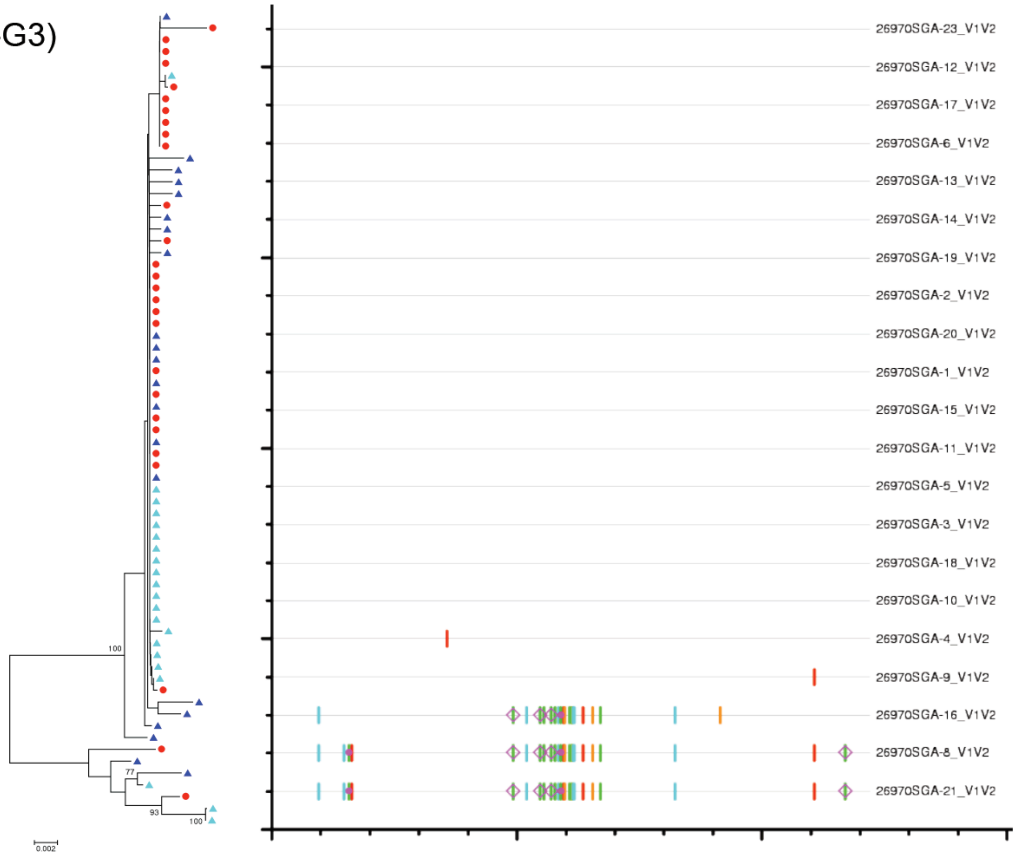
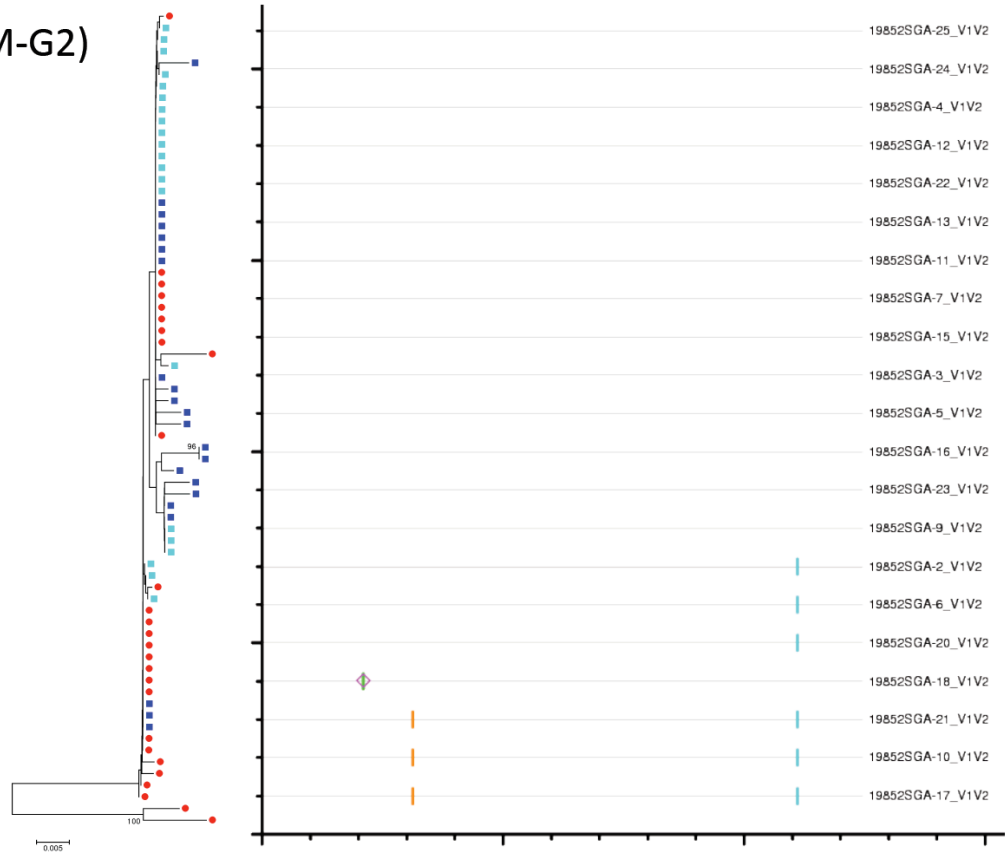


Figure 3-3. Phylogenetic tree and highlighter analysis of SIV envelope V1-V2 sequences from SIV infected macaques in the control group.
Within the phylogenetic tree, red circles represent viral sequences from SIVmac251 inoculum. Light green represents viral RNA sequences from plasma and dark green represents proviral DNA sequences from PBMC. (A) macaque 18993 (RM-C1); (B)macaque 26740 (RM-C2); (C) macaque 26856 (RM-C3); (D) macaque 26517 (RM-C5); (E) macaque 30309 (RM-C8)

A

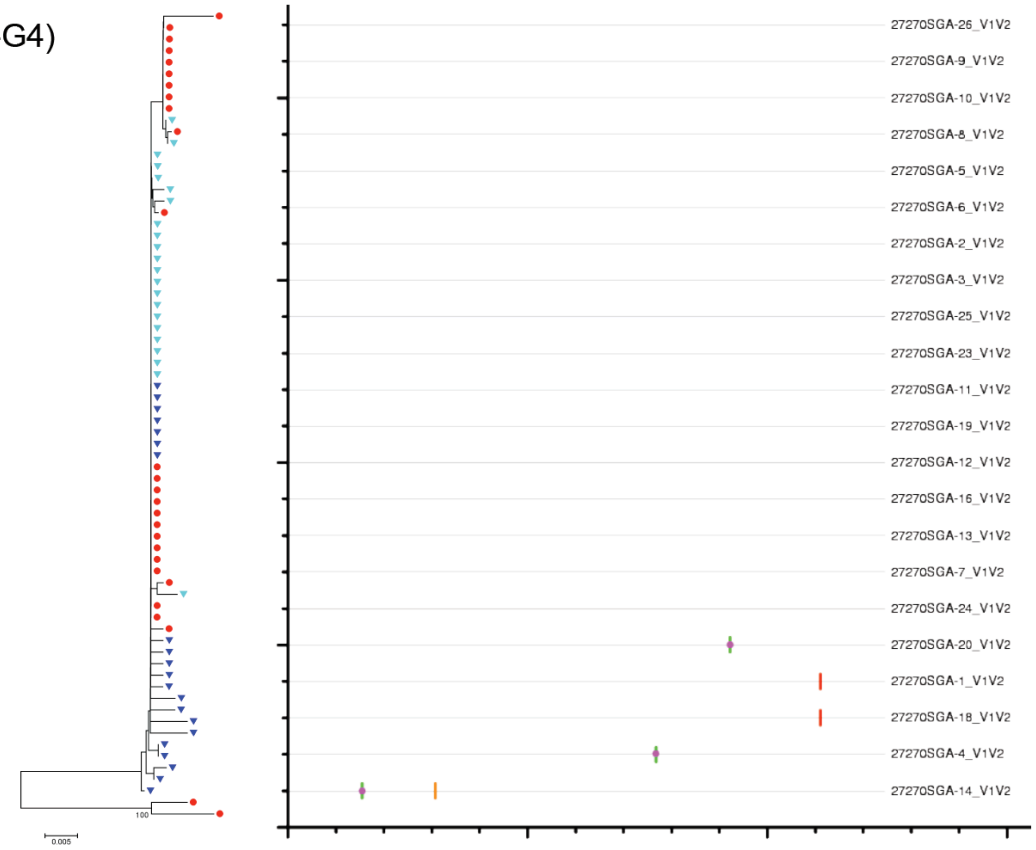

26970 (RM-G3)
26970SGA-23_V1V2
26970SGA-12_V1V2
26970SGA-17_V1V2
26970SGA-6_V1V2
26970SGA-13_V1V2
26970SGA-14_V1V2
26970SGA-19_V1V2
26970SGA-2_V1V2
26970SGA-20_V1V2
26970SGA-1_V1V2
26970SGA-15_V1V2
26970SGA-11_V1V2
26970SGA-5_V1V2
26970SGA-3_V1V2
26970SGA-18_V1V2
26970SGA-10_V1V2
26970SGA-4_V1V2
26970SGA-9_V1V2
26970SGA-16_V1V2
26970SGA-8_V1V2
26970SGA-21_V1V2
100
77
93
100
0.002

B


19852 (RM-G2)
19852SGA-25_V1V2
19852SGA-24_V1V2
19852SGA-4_V1V2
19852SGA-12_V1V2
19852SGA-22_V1V2
19852SGA-13_V1V2
19852SGA-11_V1V2
19852SGA-7_V1V2
19852SGA-15_V1V2
19852SGA-3_V1V2
19852SGA-5_V1V2
19852SGA-16_V1V2
19852SGA-23_V1V2
19852SGA-9_V1V2
19852SGA-2_V1V2
19852SGA-6_V1V2
19852SGA-20_V1V2
19852SGA-18_V1V2
19852SGA-21_V1V2
19852SGA-10_V1V2
19852SGA-17_V1V2
96
100
0.005

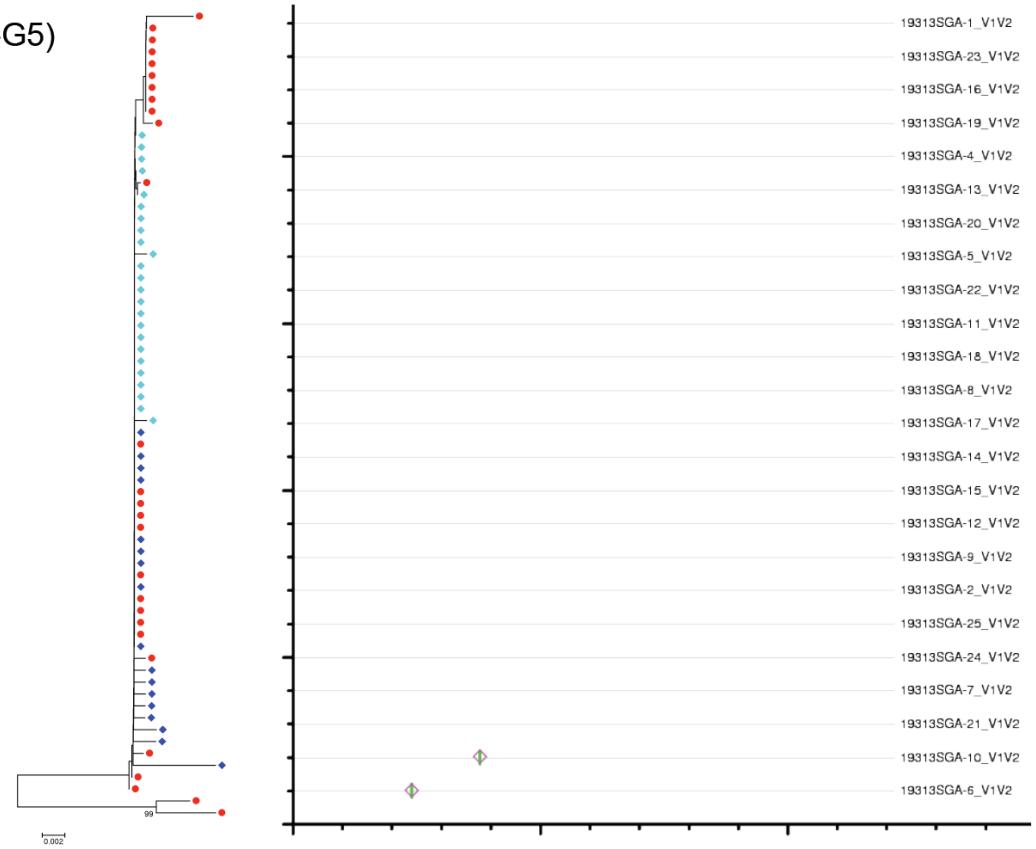
C

27270 (RM-G4)



D

19313 (RM-G5)



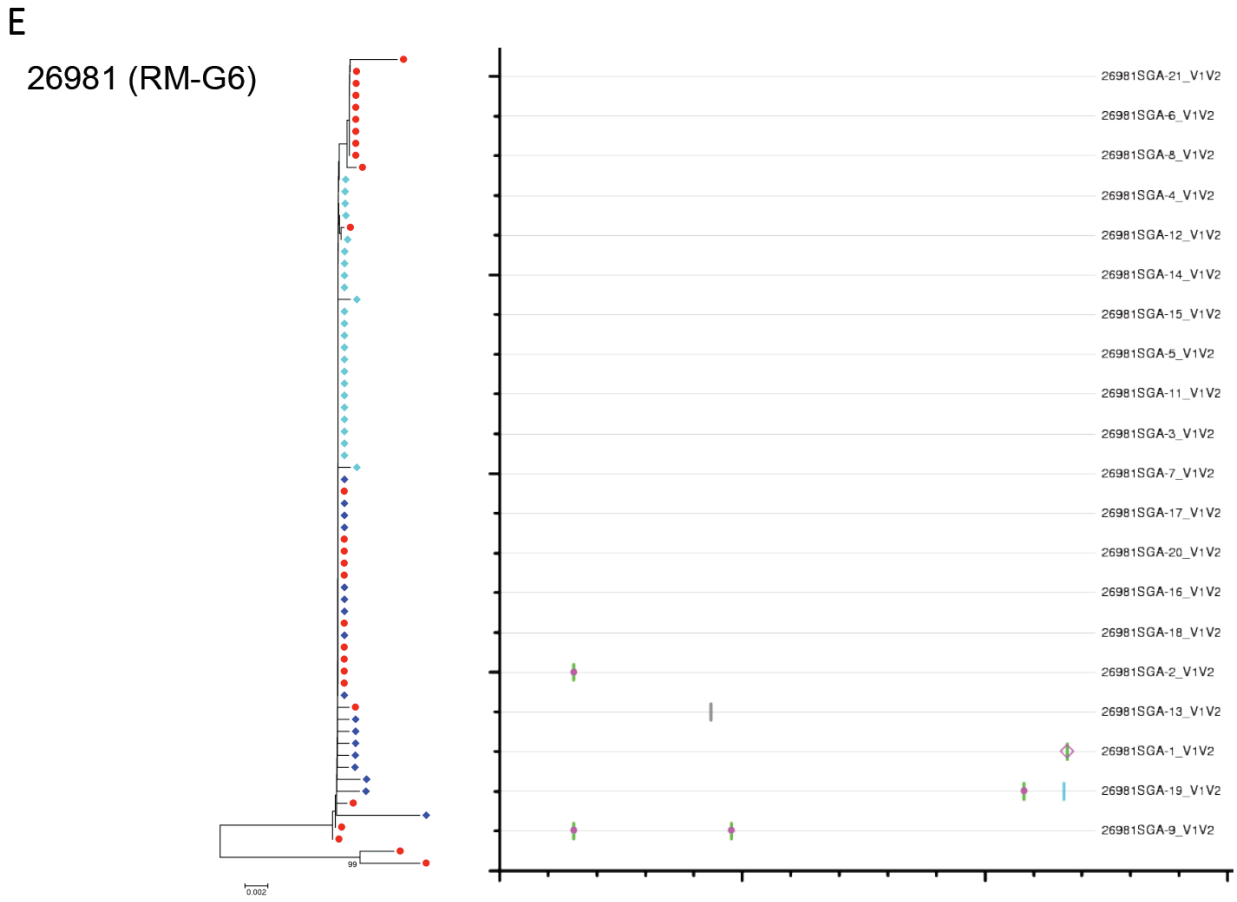


Figure 3-4. Phylogenetic tree and highlighter analysis of SIV envelope V1-V2 sequences from SIV infected macaques in gingivitis-induced group.
Within the phylogenetic tree, red circles represent viral sequences from SIVmac251 inoculum. Light blue represents viral RNA sequences from plasma and dark blue represents proviral DNA sequences from PBMC. (A) macaque 26970 (RM-G3); (B)macaque 19852 (RM-G2); (C) macaque 27270 (RM-G4); (D) macaque 19313 (RM-G5); (E) macaque 26981 (RM-G6)

The influence of gingival inflammation on immune modulators in gingival crevicular fluid (GCF) and gingival biopsy following oral SIV infection.

The levels of immune modulators in gingival crevicular fluid were measured by Luminex assay. SIV infected macaques from control group had increased MIP-1β (p=0.0324), MCP-1(p=0.0035) and IL-18 (p=0.0101), in gingival crevicular fluid at day 14-16 post 1st SIV administration, compared to Pre-SIV infection and Pre-gingivitis induction (Mann-Whitney U test), indicating that SIV induces these immune modulators production. SIV infected macaques in gingivitis-induced group had significantly higher IFN-α (p=0.0002), CXCL10 (p=0.0052), RANTES (p=0.0015), IL-6 (p=0.0008), IL-8 (p=0.0021), IL-18 (p=0.0046), TGF−β (p=0.0003) and sCD14 (p=0.0003) in GCF compared to SIV-infected macaques in control group at day 14-16 post 1st SIV administration. Prior to SIV infection the levels of IFN-α and CXCL10 in gingival crevicular fluid were generally low in the two groups, however after SIV inection the levels of IFN-α and CXCL10 were higher in macaques from gingivitis induced group, indicating synergistic effects of induced gingival inflammation and SIV for IFN-α, CXCL10 production. After day 28-30 post-SIV infections, IL-6 (p=0.0035), IL-8(p=0.0003), IL-18 (p=0.0007), TGF−β (p=0.0011) and sCD14 (p=0.0015) in gingival crevicular fluid remained higher in macaques from gingivitis-induced group than control group (Mann-Whiteney U test) (Figure 3-5a and b). The levels of immune modulators in gingival crevicular fluid were comparable between control and gingivitis-induced group macaques during chronic SIV infection (day 100 to 250 post SIV infection)(data not shown). These results indicate that induced gingival inflammation had transient effects on initial production of immune modulators in gingival crevicular fluid during acute SIV infection.

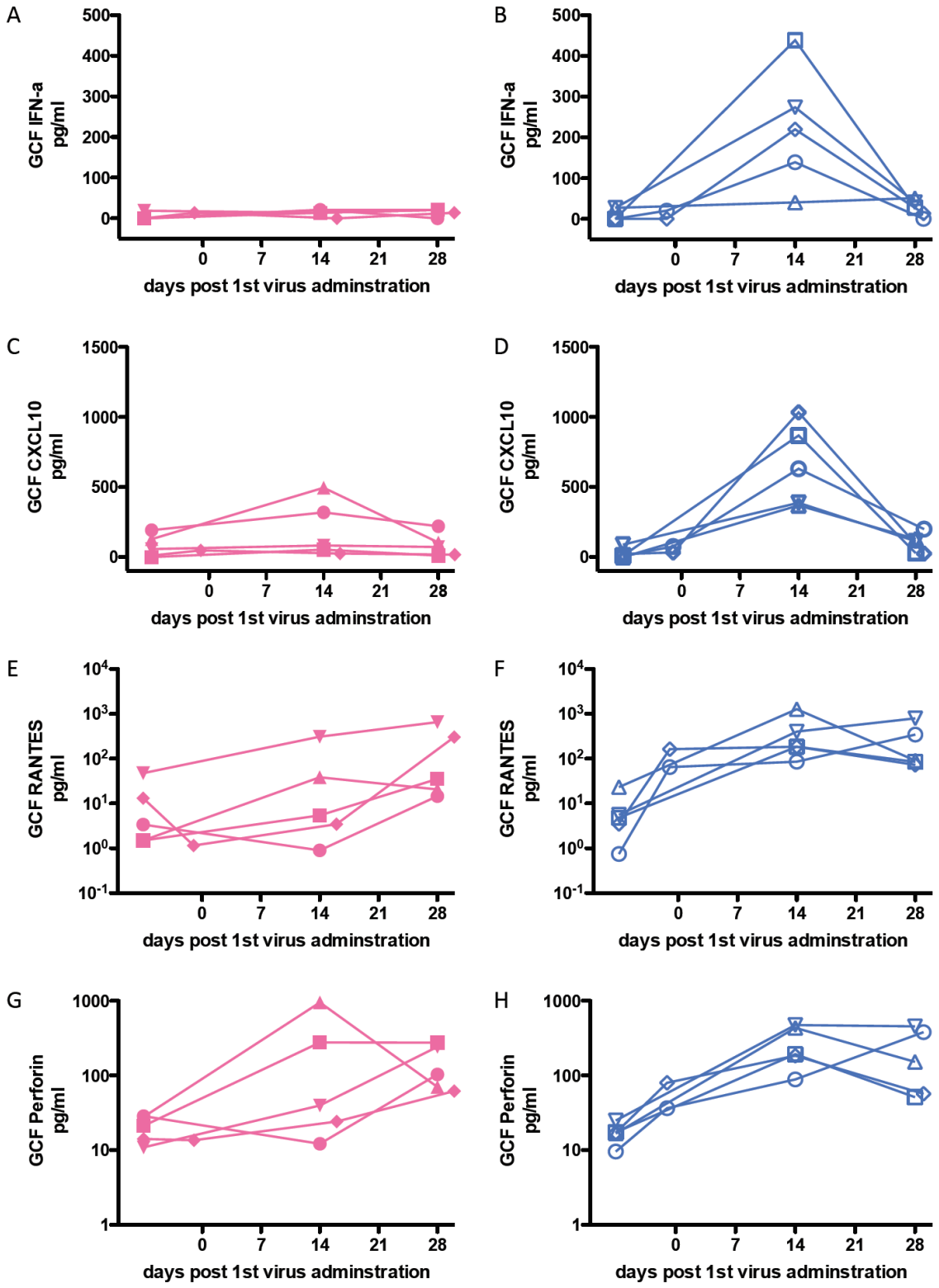


Figure 3-5a. The levels of immune modulators in gingival crevicular fluid from SIV infected macaques before and after oral SIV infection.
Shown are the concentrations of 4 immune modulators from control (A, C, E, G) and gingivitis-induced group (B, D, F, H) macaques (A and B: IFN-a; C and D: CXCL10; E and F: RANTES; G and H: Perforin) measured in crevicular fluid.

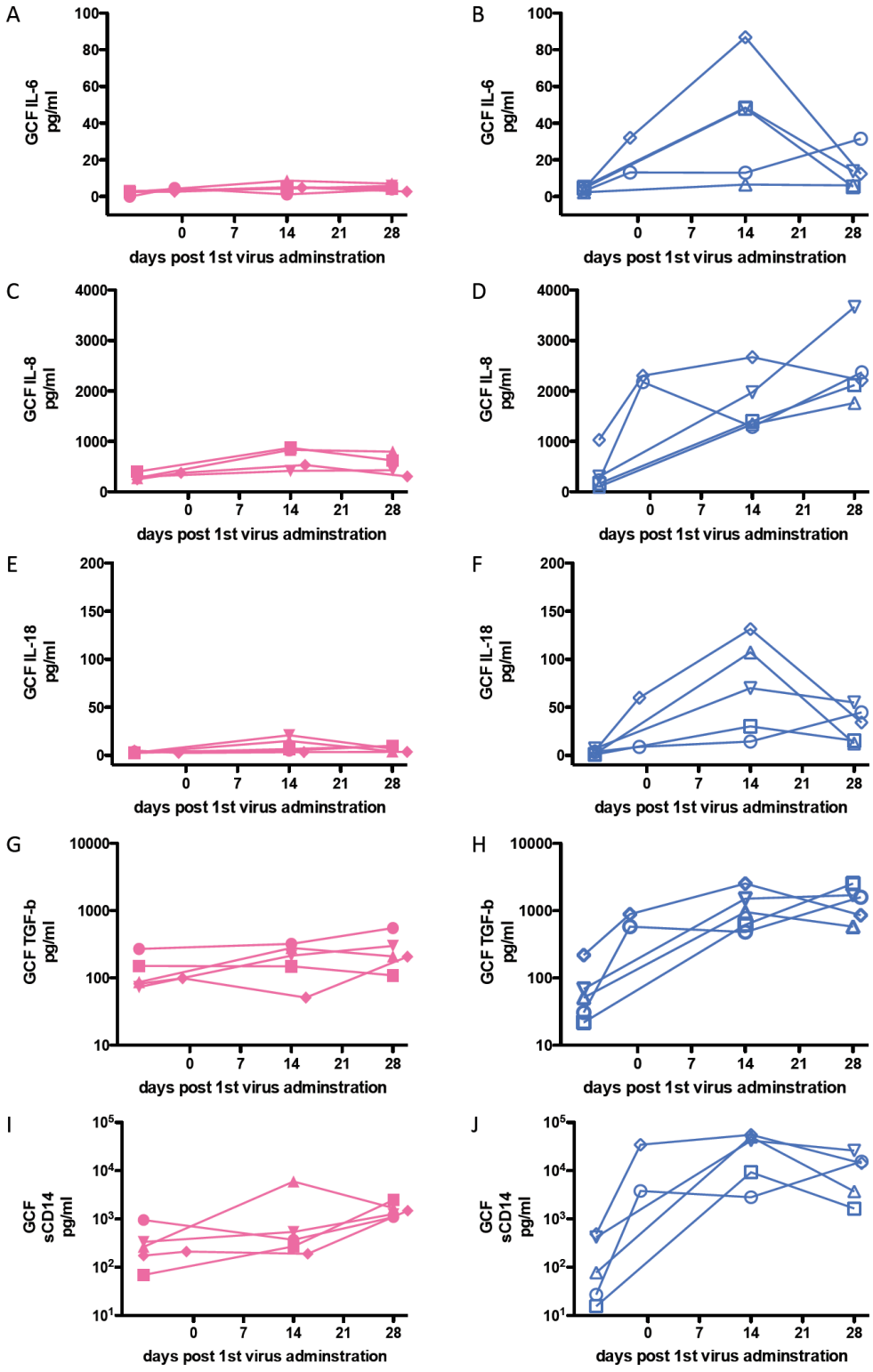


Figure 3-5b. The levels of immune modulators in gingival crevicular fluid from SIV infected macaques before and after oral SIV infection.
Shown are the concentrations of 5 immune modulators from control (A, C, E, G, I) and gingivitis-induced group (B, D, F, H, J) macaques (A and B: IL-6; C and D: IL-8; E and F: IL-18; G and H: TGF-β; I and J: sCD14) measured in crevicular fluid.

Assessment of mRNA transcripts levels for IFN-α, OAS, CXCL10 and CXCR3 by real-time PCR was also performed in punch biopsies obtained from the gingival mucosa of macaques. Compared to baseline, two SIV infected macaques in control group had mild up-regulation of OAS while four SIV infected macaques in gingivitis-induced group had up-regulation of OAS around day 14-16 post 1st SIV administration. The data supports our results from gingival crevicular fluid that SIV infected macaques in gingivitis-induced group had higher levels of IFN-α which initiate antiviral responses in these macaques.

CXCL10 gene expression at gingival biopsy macaques was generally stable during acute SIV infection in control group, except RM-C2 who had increased CXCL10 gene expression around day 14-16 post 1st SIV administration. In contrast, there were four macaques had increased CXCL10 gene expression around day 14-16 post 1st SIV administration in gingivitis-induced group. Around day 28-30 post 1st SIV administration, two macaques from control group and three macaques from gingivitis-induced group had increased CXCR3 gene expression at gingival biopsy, indicating that CXCR3 cell were migrating into the gingival tissues. IL-6 gene expression in most of the control group macaques was below detection by our real-time PCR, indicating low IL-6 gene expression in these macaques. On the other hand, IL-6 gene expression in most of the gingival tissues from gingivitis-induced group macaques was detectable and 2 macaques in gingivitis group had increased IL-6 at week 4 (Figure 3-6).

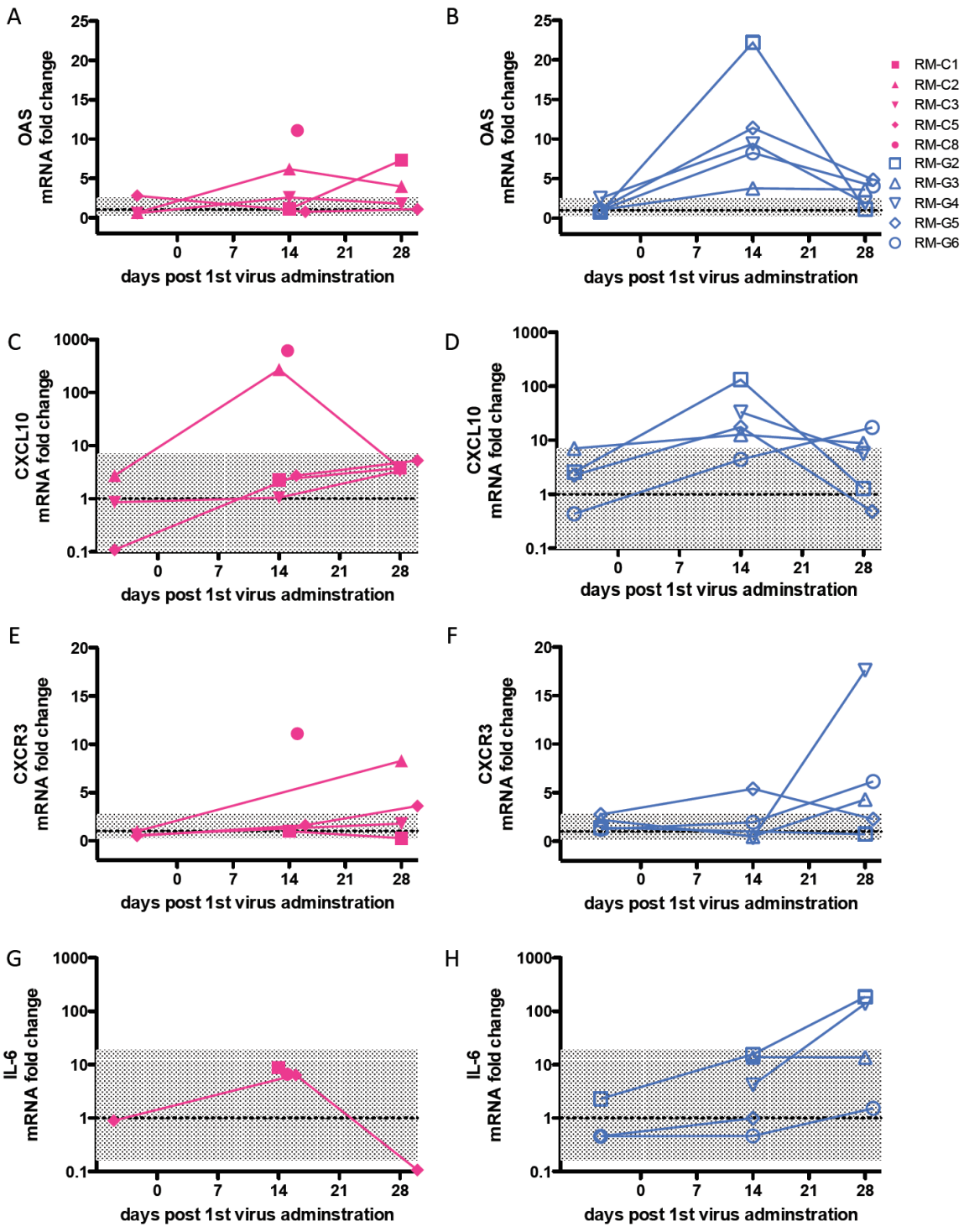


Figure 3-6. Immune modulators transcript fold change in gingival biopsies from SIV infected macaques in control group (A, C, E, G) and gingivitis-induced group (B, D, F, H).

Fold changes of mRNA expression of OAS (A, B), CXCL10 (C, D), CXCR3 (E, F), and IL-6 (G, H) at the time point of pre-SIV infection and pre-gingivitis induction, day 14-16 and day 28-30 post 1st virus administration. The grey area represents the range of each gene expression level, compared to averaged baseline expression.

Discussion

Oral HIV transmission is important in infants receiving HIV containing breast milk and adults with receptive oral intercourses. In order to assess the influence of mucosal inflammation on oral HIV/SIV infection, gingival inflammation was induced in rhesus macaques prior to oral SIV exposures. Gingival inflammation is common in most adults, in the form of gingivitis or periodontal diseases (8-10), which makes our study highly relevant to address oral HIV transmission in adults. Gingival inflammation can also be observed in infants during the period of primary teeth eruption (343). Although the causes of gingival inflammation in infants may not be the same as bacteria-induced inflammation, our study can still provide insights into how gingival inflammation affects HIV oral transmission in newborns.

In our study, we did not observe significant increased numbers of macaques become SIV infection. One of the possible reasons may be due to that only mild gingival inflammation was induced, similar to the levels generally observed in humans with gingivitis. Based on clinical assessment, our macaques in control group also had a mild level of gingival inflammation which may make observing differences between our control and gingivitis groups more difficult. In the future, one potential way to maximize the differences between the control and gingivitis groups would be to first remove all plaque from the teeth prior to initiating the experiment, thereby helping to maximize any differences between the control and mild gingivitis groups.

A second possible reason could be that there are multiple anti-viral proteins and peptides residing in the oral cavity and it is possible that induced gingival inflammation in our study was not strong enough to overcome these inhibitory effects for virus to establish successful infection. Furthermore, compared to cheek and

vaginal mucosa, gingival tissues have extra keratin to prevent virus invasion and it is possible that the inflammation we induced was not severe enough to disrupt the protective barrier of mucosal membranes. Our results contrast previous studies that utilized 10% acetic acid to remove the outer layers of the epithelium and induce a localized buccal inflammation increased macaques' susceptibility to SHIV infection (69). There are differences in these two studies which include the different viruses that were utilized, SIVmac251 in this study and SHIV-1157ipd3N4 in the second study. In addition, the mucosal tissues of virus exposures were different, such as gingival mucosa (our studies) and cheek mucosa. Furthermore, the methods to induce mucosal inflammation are different as Chenine et al. used 10% acetic acid solution to create chemical induced tissue damage at the cheek mucosa at 4 days. Our methods are more biologically relevant through an induction of gingival inflammation (ligatures permitting bacteria and plaque development) that better mimics the types of oral inflammation commonly observed in humans.

Finally, it is possible that differences in the control and gingivitis-induced groups do exist, but they were too small to detect with the number of macaques that were enrolled for the analysis. For example, expectation of 10% difference between control and induced-gingivitis group, sample size calculation suggests that more than 80 macaques per group would be required (http://www.raosoft.com/samplesize.html). With the limitation of the macaque numbers, our study did not have sufficient power to detect small difference between control and gingivitis-induced group.

It is well recognized that there is a transmission bottleneck following mucosal HIV transmission, resulting in low viral diversity during early infection and

characterizing the diversity following transmission can be used as a surrogate marker of HIV acquisition (375). SIV infection of rhesus macaques by mucosal exposures also exhibits the bottleneck phenomenon and previous studies have confirmed that viral dose of exposure can affect the bottleneck (96, 226). In our study, viral diversity (calculated as p-distance) of SIV envelope V1-V2 region from PBMC DNA (determined by cloning and sequencing) was generally greater than plasma RNA (determined by single genome amplification (SGA)). The higher diversity of PBMC pro-viral DNA sequences could come from early APOBEG-induced mutations that accumulated in proviral DNA and PCR errors, editing/recombination during cloning process. These variables confound the ability to use sequences from PBMC DNA to estimate the numbers of founder viruses. Therefore, the numbers of founder viruses were determined by sequences obtained from plasma RNA by single genome amplification (SGA) in our study.

SGA is a recently developed method to examine the numbers of founder virus that initiate systemic SIV infection. With full-length envelope sequences, the mathematical modeling has calculated the maximum number of changes away from a single founder variant is two nucleotides if the samples were examined during the first 2 weeks of HIV/SIV infection (6, 217, 332, 333). Any difference between founder variants should be assumed to be two variants unless the second variant is represented by only one sequence. This is because that with the random mutations that accumulate early, any one given change could have occurred in the host. However, if more than one sequence with the same mutation is observed in an individual at very early following SIV/HIV transmission then it is very likely that the

viral variant was transmitted. In our study, SIV envelope V1-V2 region was analyzed instead of full-length envelope sequence. The shorter length of sequences tends to underestimate the actual number of founder viruses, however, comparisons between the control and inflammation groups is a valid approach as both groups underestimate the number of founder viruses in a same manner. In addition to underestimation the number of founder viruses, what we cannot be sure about is the two sequences with just one nucleotide change away from the majority of the sequences within the SIV V1V2 region. These one nucleotide changed sequences could be mutants from the major lineage or they actually represent a different variant. However, there is no way to distinguish unless more sequences were obtained. If these sequences represent a separate lineage, then a new sequence would show up with the same exact mutation. If these sequences were random mutations, then repeating the SGA would only produce sequences that were identical to the major lineage or different at some other sites. Nevertheless, our study demonstrated that gingival inflammation is associated with multiple viral variants acquisition in macaques. The results are consistent with other studies that mucosal inflammation mitigates transmission bottleneck and allows multiple viral variants infection of the host (145, 329). The data also demonstrate that mucosal inflammation can potentially affect the efficacy of future HIV vaccine and contribute to breakthrough infection if the antigens included in the vaccines do not cover protection against the diverse HIV variants.

SIV infection induces strong immune activation at the entry sites and the periphery (4, 5, 260, 354). In this study, SIV infection induces multiple immune modulators production in the gingival crevicular fluid, including inflammatory cytokines

and antiviral cytokines. Previous studies have indicated that antiviral cytokine production was too little and too late to avoid SIV dissemination from virus entry site

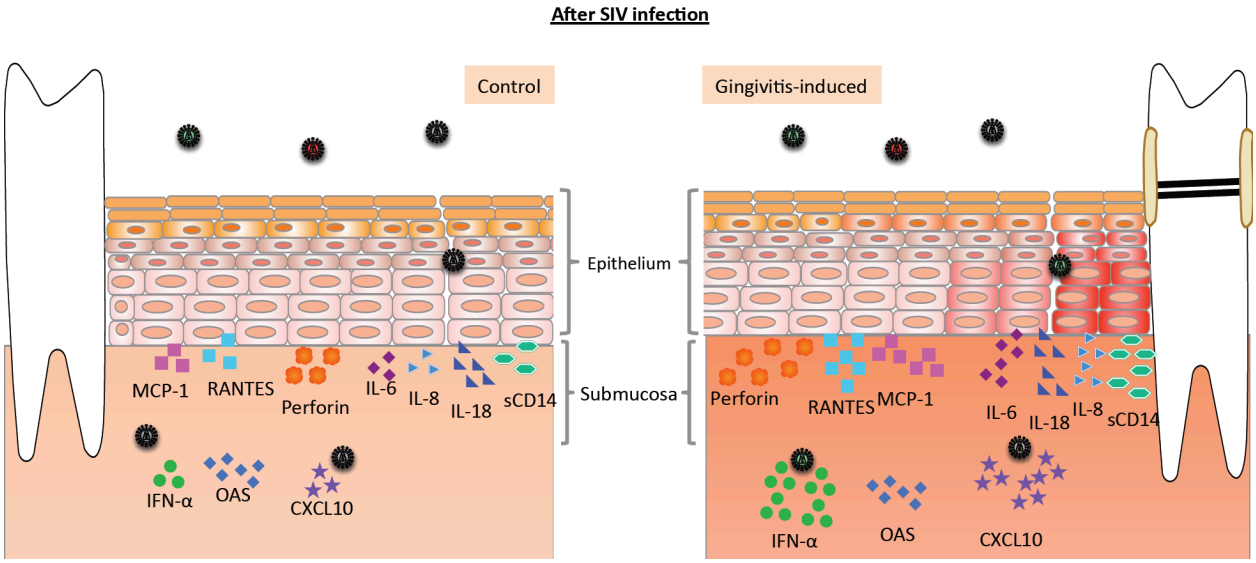


Figure 3-7: Model of immune modulator changes following oral SIV infection. Shown are the levels of immune modulators in gingivial crevicular fluid after oral SIV infection from macaques in the control group (left) and gingivitis-induced group (right). The shapes indicate different immune modulators. The numbers of the symbols does not represent actual concentration, but the trends are indicated.

to distal tissues (4, 5). Indeed, our study showed that the level of IFN-α in gingival crevicular fluid did not have significant increase in SIV infected macaques from control group during acute SIV infection. Interestingly, IFN-α and CXCL10 production was significantly increased at day 14-16 post SIV administration in SIV infected macaques with induced gingivitis, indicating that induced gingival inflammation differentially altered the antiviral response or interferon signaling pathway. Along with other immune modulators associated with induced gingival inflammation, SIV infected macaques from induced-gingivitis group exhibited higher levels of multiple immune modulators during acute SIV infection (Figure 3-8).

In conclusion, induced gingival inflammation did not significantly enhance the numbers of macaques became SIV infected following oral SIV exposures in our study, however, induced gingival inflammation was associated with increased numbers of founder virus infection in the macaque as well as higher levels of immune modulators in the oral cavity of the SIV infected macaques with induced gingivitis. These results indicate that there could be a synergistic effect between mucosal inflammation and SIV infection that can potentially impact the early stages of the SIV infection and interfere with success of treatments or efficacies of vaccines.

Chapter 4: The influence of gingival inflammation on systemic immunological changes following oral SIV infection in rhesus macaques

Introduction

The major route of HIV transmission is through virus across a mucosal site including the vaginal, penile rectal or oral mucosa. Following virus invasion, strong immune activation can be detected at mucosal sites as well as peripheral blood (354). However, human immune system generally fails to clear the viruses and immune activation does not resolve in HIV infected patients during chronic infection. Sustained immune activation is characterized by elevation of multiple inflammatory cytokines/chemokines production (207, 274, 320), rapid immune cells turnover (59, 402), and increased expression of proliferation and activation markers on immune cells (227, 300). Several studies suggest that sustained immune activation is the driving force of HIV-related immune dysfunction and better indicates disease progression(134, 227). All these immune dysfunction are associated with excessive production of cytokines and chemokines(208, 273, 321), highlighting the importance of understanding the roles of cytokines and chemokines in HIV pathogenesis.

HIV oral transmission can occur during mother to child transmission due to virus in breast milk or oral-genital transmission due to virus in semen (192, 271, 290). However, it is difficult to obtain valuable information for vaccine development regarding early events following HIV oral exposure in humans. Instead, SIV infection of rhesus macaques was used to assess immunological changes following mucosal SIV infection (4, 5, 22, 25, 223, 224). By using SIV infection of rhesus macaques model through oral route, our earlier studies have demonstrated that interferon (IFN)-α, interferon stimulated gene 2'-5' oligoadenylate synthetase (OAS) and inflammatory

chemokines CXCL10 were increased at mucosal sites (oral and rectal tissues), peripheral blood and lymph nodes(98, 260). In addition, early elevated expression of OAS, and CXCL10 in lymph nodes are associated with a more rapid disease progression while early increase of these genes at mucosal sites are associated with a slower rate of disease progression in orally SIV infected macaques (98, 260), indicating the influence of the timing and the location of the cytokines/chemokines production on SIV pathogenesis of orally infected macaques.

Mucosal immune activation during acute HIV/SIV infection can have long-term impact on HIV/SIV replication, HIV/SIV-specific adaptive immunity development and disease progression. Study from Wang et al., demonstrated that induction of vaginal inflammation by TLR7 and TLR9 agonists results in higher plasma viral set points after vaginal SIV challenge (390). Study from Sheung et al., showed N. gonorrhoeae induced genital inflammation around the time of HIV infection is associated with higher IFN-γ and MIP-1β secretion by HIV-specific CD8 T cells in the peripheral blood (347). Study from Bebell et al., and Roberts et al., found that during acute HIV infection, elevated inflammatory cytokines IL-1β, IL-6, and IL-8 in cervicovaginal lavage have a significant inverse correlation with systemic CD4 T cell counts (34) while elevated GM-CSF in cervicovaginal lavage is positively correlated with plasma viral set points (318). These results highlight the importance of cytokines/chemokines milieu on HIV/SIV pathogenesis and the potential intervention strategies development to treat mucosal inflammation for slowing down the rate of HIV/SIV disease progression.

The study described here continues our work with oral SIV infection of rhesus macaques and uses gingivitis induction to further examine the impact of mucosal inflammation on oral SIV infection. Using Luminex to assess plasma cytokines/chemokines and real-time PCR to assess their gene expression changes in PBMCs and peripheral lymph nodes, we demonstrate that induced gingival inflammation is associated with altered immune activation profiles in peripheral blood with higher IFNs production in plasma around day 14-16 post SIV administration. In addition, more macaques with gingivitis have increased OAS gene expression and myeloperoxidase production in peripheral lymph nodes during acute SIV infection, indicating strong interferon mediated antiviral response and polymononuclear cells mediated inflammation at the peripheral lymph nodes. These altered immunological changes between control and gingivitis-induced group do not result in significant differences in SIV specific antibody response, chronic systemic immune activation development, plasma viral load or CD4 T cell counts, therefore these data provide evidence that the impact of gingival inflammation on SIV infection is limited to the acute phase of the infection in the orally inoculated SIV infected macaques.

Results

The influence of gingival inflammation on SIV plasma viral load and CD4 T cell counts following oral SIV infection

Shown in the previous chapter, there were total 5 macaques in control group and 5 macaques in gingivitis-induced group developed systemic SIV infection following oral SIV exposures. Plasma viral load was determined to assess if gingival inflammation had impacts on SIV replication or CD4 T cell declines following oral SIV administration (Figure 4-1). Among SIV infected macaques, RM-C1 and RM-C2 from control group and RM-G3 and RM-G5 from gingivitis-induced group had detectable plasma viral load at day 7 -9 post 1^{st} virus administration. Other macaques had detectable plasma viral load around day 14-16 post 1^{st} virus administration. After acute SIV infection, RM-C5 in control group and RM-G5 in gingivitis-induced group both had controlled virus replication (below detection limit 50 copies/ml) during chronic infection. Macaque RM-C8 in control group also had a trend toward lower viral replication during chronic SIV infection. Overall, viral set points (determined as average plasma viral load obtained between day 60 and day 210) were developed around 10^6 viral RNA copies per ml in SIV infected macaques without controlled virus replication, and were comparable between control and gingivitis-induced group, indicating that gingival inflammation did not have significant impacts on systemic SIV replication.

CD4 T cell counts in the peripheral blood was determined to assess if gingival inflammation affects disease progression following oral SIV administration, (Figure 4-1C-D). Following oral SIV infection, SIV infected macaques exhibited reduced CD4 T cell counts during acute infection. Macaques with low or controlled virus replication (RM-C5, RM-C8 and RM-G5) were more likely to preserve CD4 T cell counts during chronic infection stage. Overall, there were no significant differences with regard to CD4 T cell counts between control and gingivitis-induced groups.

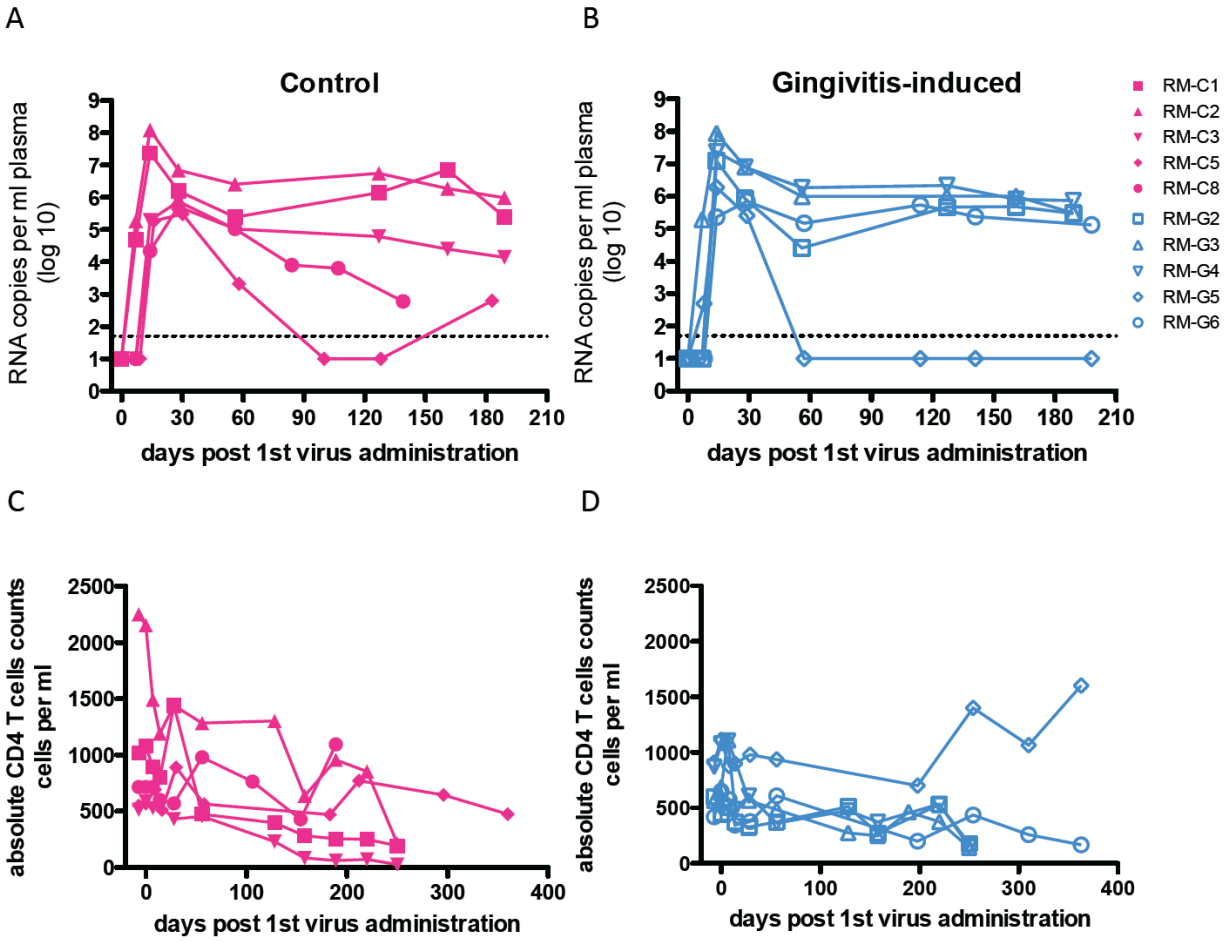


Figure 4-1. Plasma viral load and CD4 T cell counts from orally SIV infected macaques.
Shown are viral RNA copies per milliliter (detection limit as 50 copies per milliliter of plasma shown in dot line) and CD4 T cell counts in orally SIV infected macaques. Plasma viral load and CD4 T cells counts for macaques C1-C8 in control group (A and C); macaques G1-G8 in gingivitis-induced group (B and D)

The impact of gingival inflammation on interferon-α mediated antiviral response in peripheral blood and lymph node.

HIV/SIV infection results in increased production of multiple cytokines and chemokines during acute infection. Interferon-alpha (IFN-α), an antiviral cytokine, is one of the first cytokines shown to be elevated in the peripheral blood following HIV/SIV infection (354). To assess the impact of gingival inflammation on the IFN-α mediated antiviral response, plasma IFN-α was measured by Luminex and its gene expression was measured by real-time PCR (Figure 4-2). The level of plasma IFN-α was elevated during acute SIV infection with peak production around day 7 or day 14 and remained increased at day 28-30 after SIV infection in all macaques, compared to plasma IFN-α level before SIV infection. RM-C1, RM-C2, RM-G3 and RM-G5 with detectable plasma viral load at day 7-9 post 1st SIV administration also had increased IFN-α in plasma at the same time point, indicating plasma IFN-α production was induced in an SIV dependent manner. At day 14-16 after 1st SIV administration, there was a trend toward higher plasma IFN-α from macaques in gingivitis-induced group than control group ($p=0.056$, Mann-Whitney U test).

Interestingly, the mRNA transcripts of IFN-α in PBMC were decreased in SIV infected macaques from both groups, especially in macaques receiving SIV with a needless syringe (1st set study) (Figure 4-2C and 2D). It is possible that the IFN-α expressing cells are being lost from peripheral blood following SIV infection through a migration into tissue compartments. There was no evidence that these cells migrated to the inguinal lymph node as the IFN-α mRNA levels did not exhibit significant elevations during these time points (Figure 4-2E and 2F).

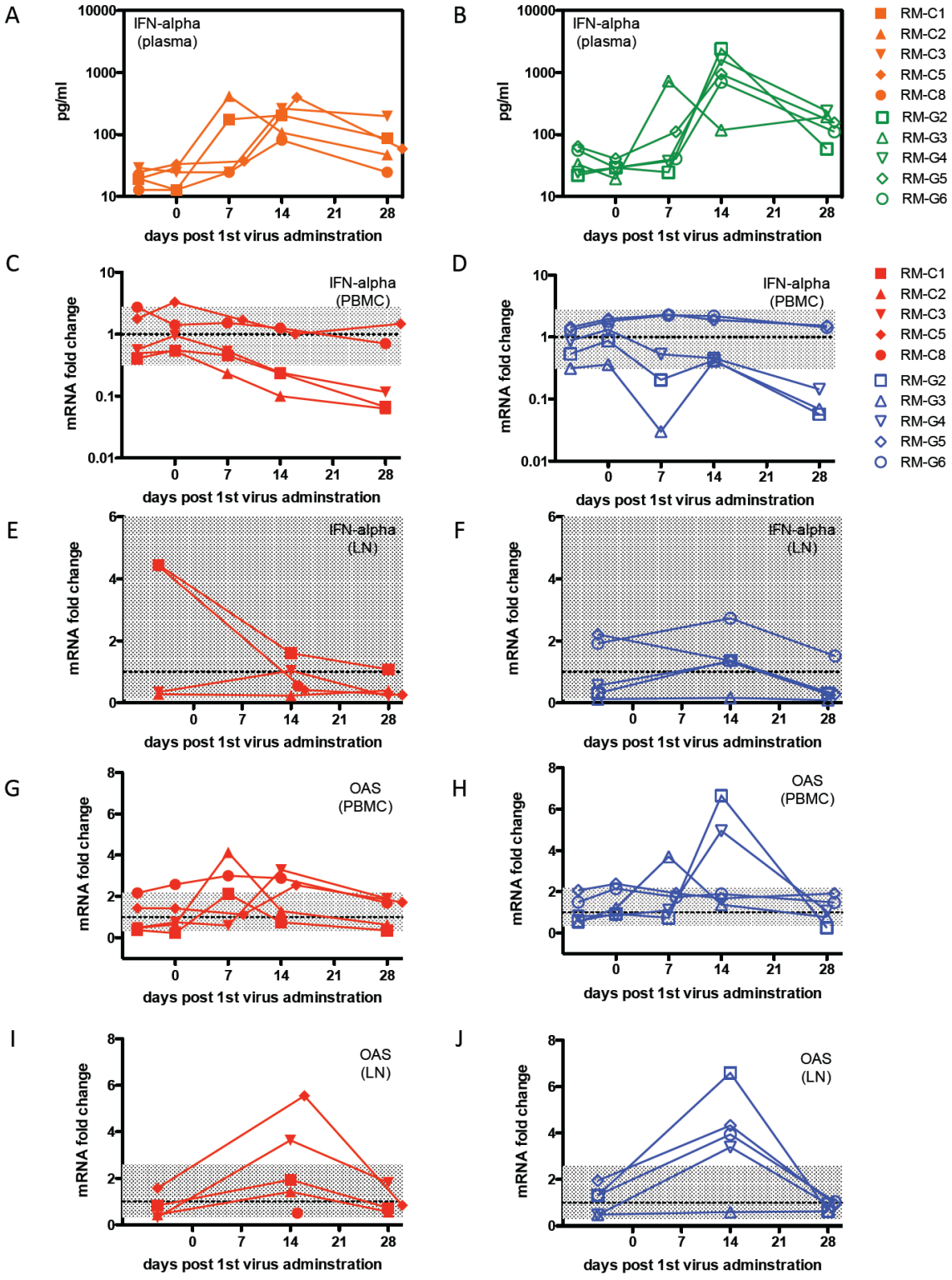


Figure 4-2. IFN-α mediated antiviral response in oral SIV infected macaques from control group (A, C, E, G, I) and gingivitis-induced group (B, D, F, H, J).
Shown are plasma levels of IFN-α (A, B) and fold changes of mRNA expression of IFN-α in PBMC (C, D) and peripheral lymph node (E, F) as well as OAS in PBMC(G,H) and peripheral lymph node (I, J) during acute SIV infection. The grey area represents the range of each gene expression level, compared to averaged baseline expression.

IFN-α mediates an innate antiviral response through induction of several interferon-stimulated genes (ISGs) (77, 378), activation of macrophages (169) and NK cells(371, 373) and modulation of adaptive immune response development. 2'-5'-oligoadenylate synthetase (OAS) is one of the ISGs that respond to IFN and activates RNase L, which results in viral RNA degradation to achieve antiviral activity through inhibition of viral replication. We further examine if increased plasma IFN-α induced robust ISGs expression in SIV infected macaques. In our study, OAS mRNA transcripts were increased in PBMC and peripheral lymph nodes. The level of OAS gene expression fold change was similar between control and gingivitis-induced group macaques (Figure 2G-2J). These results indicate the plasma IFN-α was bioactive and was able to upregulate ISGs gene expression in SIV infected macaques. Consistent with the kinetics of IFN-α production in plasma, macaques RM-C1 and RM-C2 from control group and macaque RM-G3 from gingivitis-induced group with early elevation of IFN-α at day 7-9 also exhibited early OAS gene transcript upregulation in PBMCs at the same time point (Figure 2G-2H). Furthermore, OAS gene expression peaked around day 14-16 post-SIV administration, which is also consistent with timing of peak plasma IFN-a production in most of the SIV infected macaques. The level of plasma IFN-α was associated with the level of OAS gene up-regulation in lymph node day 14-16 after SIV administration. Furthermore, we observed that 4 macaques in gingivitis induced group have increased OAS gene expression around day 14, compared to control group, only 2 macaques have increased OAS gene expression at this time point (Figure 2I and 2J), indicating interferon mediated antiviral response may persist longer at peripheral lymph node following oral SIV infection. Note that mRNA

transcripts of IFN-α and OAS in PBMC may be affected by SIV administration method since SIV infection with whatman paper did not show significant changes of IFN-α or OAS gene expression in PBMC, compared to macaques receiving SIV with needless syringes.

The impact of gingival inflammation on the level of interferon-gamma (IFN-γ) and gene expression in peripheral blood and lymph node.

IFN-gamma (IFN-γ) is another type of interferon that is important for immune response against virus and intracellular bacterial infection. IFN-γ can inhibit virus replication (79), promote NK cell activity (40, 266), increase antigen presentation (131, 249) and enhance lysosome activity in macrophages(221, 370). In HIV/SIV infection, plasma IFN-γ was elevated(110) and IFN-γ gene expression was increased, especially in CD8 T cells (46). In our study, orally SIV infected macaques from control group, generally had stable level of plasma IFN-γ during acute infection (Figure 4-3A). Compared to control group, SIV infected macaques in gingivitis-induced group had a trend toward increased plasma IFN-γ at day 14-16 following 1st SIV administration ($p=0.0556$, Mann-Whitney U test) and statistically higher level of plasma IFN-γ at day 28-30 after SIV administration ($p=0.0362$, Mann-Whitney U test)(Figure 4-3B). Examination of IFN-γ gene expression in PBMC and lymph node did not show significant changes of IFN-γ transcripts between control and gingivitis-induced group (Figure 4-3C-3F). Interestingly, 3 out of 5 macaques (RM-G3, RM-G5, RM-G6) in gingivitis-induced group had gradual increased IFN-γ mRNA levels from day 14-16 to day 28-30 in lymph node, which may contribute to the higher level of IFN-γ in plasma at day 28-30 after SIV infection (Figure 4-3F).

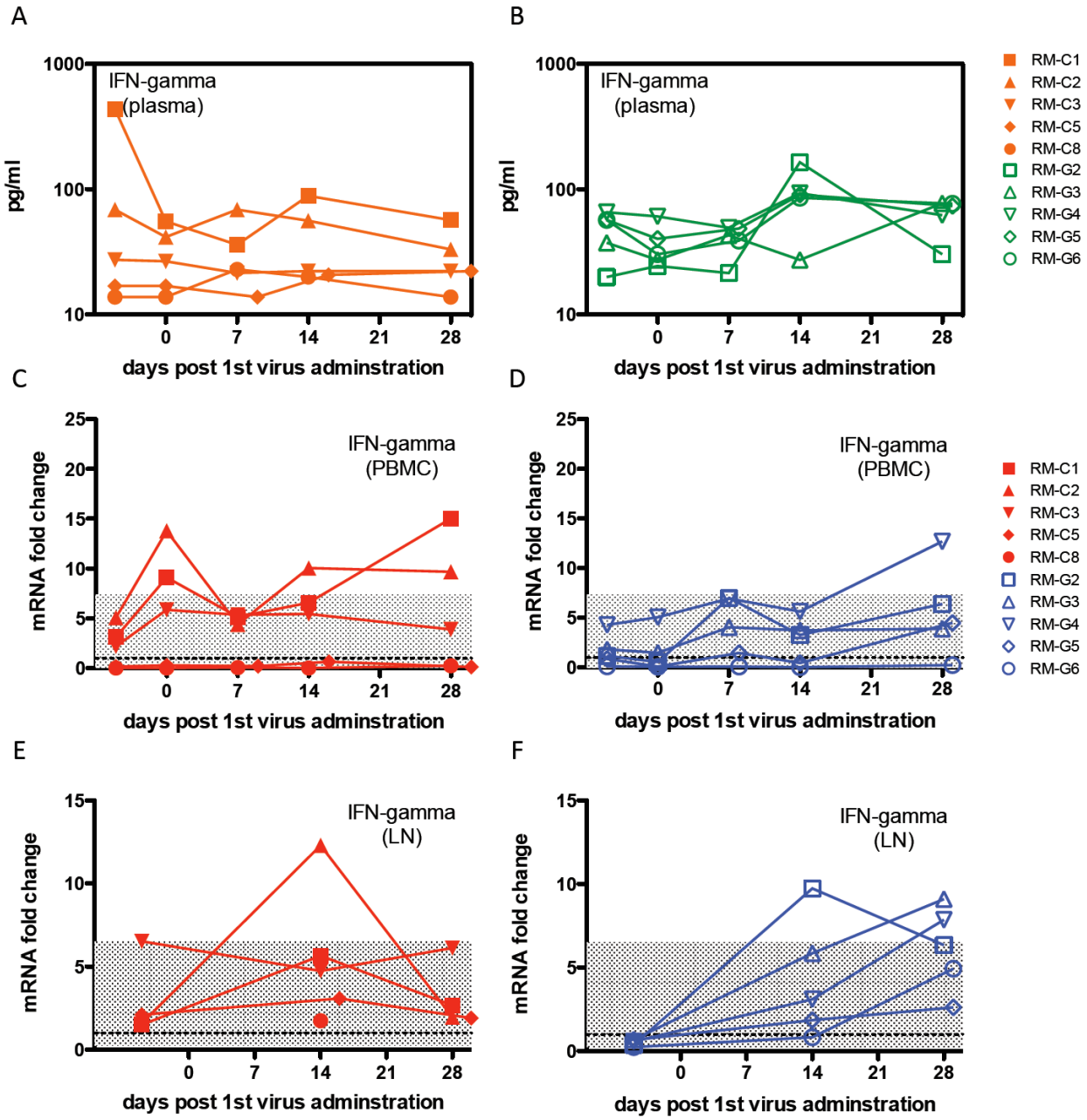


Figure 4-3. IFN-γ protein and gene expression changes in oral SIV infected macaques from control group (A, C, E) and gingivitis-induced group (B, D, F). Shown are plasma levels of IFN-γ (A, B) and fold changes of mRNA expression of IFN-γ in PBMC (C, D) and peripheral lymph node (E, F) during acute SIV infection. The grey area represents the range of each gene expression level, compared to averaged baseline expression.

The impact of gingival inflammation on the level of CXCL10 production and its receptor CXCR3 gene expression in peripheral blood and lymph node.

Inflammatory chemokine CXCL10 is also known as interferon-γ-induced protein 10 kDa (IP-10). As the name suggested, CXCL10 is strongly induced by IFN-γ as well as IFN-α (78, 111, 234). CXCL10 binds CXCR3 receptor, which is expressed mostly on NK cells, type-1 helper (Th1) CD4 T cells and CD8 positive cytotoxic lymphocytes (CTLs), to mediate the migration of CXCR3+ cells into Th1-driven inflammation sites (142, 228, 304, 363). Studies have demonstrated that CXCL10 is produced early and the mRNA levels remain elevated during chronic HIV/SIV infection (98, 260, 354).

Compared to SIV pre-infection, plasma CXCL10 was increased around day 14-16 and remained increased at day 28-30 following oral SIV infection with no significant difference between control and gingivitis-induced group (Figure 4-4A and 4B). CXCL10 gene expression in PBMC was also increased in orally SIV infected macaques from both control and gingivitis-induced group. However, the level of CXCL10 gene expression in PBMC can be up regulated to a higher level at day 7 or day 14 post-SIV infection in macaques with gingival inflammation compared to control group (Figure 4-4C and 4D). The kinetics of CXCL10 gene up-regulation in PBMC was similar to the kinetics of plasma CXCL10 levels. For example, macaques RM-C2 and RM-G3 with increased plasma CXCL10 at day 7 (Figure 4-4A and 4B) both had gene upregulation in PBMC at day 7 (Figure 4-4C and 4D). Macaque RM-C2 and RM-G2 with highest level of plasma CXCL10 among the two groups at day 14 (Figure 4-4A and 4B) also had highest CXCL10 gene upregulation in PBMC at day 14 (Figure 4-4C and 4D).

The level of CXCL10 gene expression changes in lymph node was generally comparable between control and gingivitis-induced group (Figure 4-4E and 4F). In addition, CXCL10 gene expression in lymph nodes may also affect plasma CXCL10 production as increased CXCL10 gene expression at lymph node in macaque RM-C5 16 days post-SIV infection (Figure 4-4E) occurred at the same time as the increase in plasma protein CXCL10 levels (Figure 4-4A). Macaque RM-G2 with highest plasma CXCL10 production at day 14 (Figure 4-4B) also had highest CXCL10 gene up-regulation in the peripheral lymph node (Figure 4-4D and 4F). These results indicate that plasma CXCL10 production was associated with its gene expression in PBMCs and lymph node.

We further examined if CXCR3 expressing cells were recruited into tissue sites in response to increased level of plasma CXCL10 and its gene upregulation in PBMC. CXCR3 gene transcripts were analyzed by real-time PCR. Despite increased CXCL10 gene expression in PBMCs and lymph nodes during acute SIV infection, CXCR3 transcripts did not show significant changes in PBMCs or lymph node from orally SIV infected macaques (Figure 4-4G-4J). However, 4 macaques in gingivitis-induced group show increased CXCR3 gene expression from day 14-16 to day 28-30 (Figure 4-4J), while only 2 macaques in control group had this phenomenon (Figure 4-4I), indicating gingivitis may facilitate CXCR3 cell migration into peripheral lymph nodes at later time points during SIV infection.

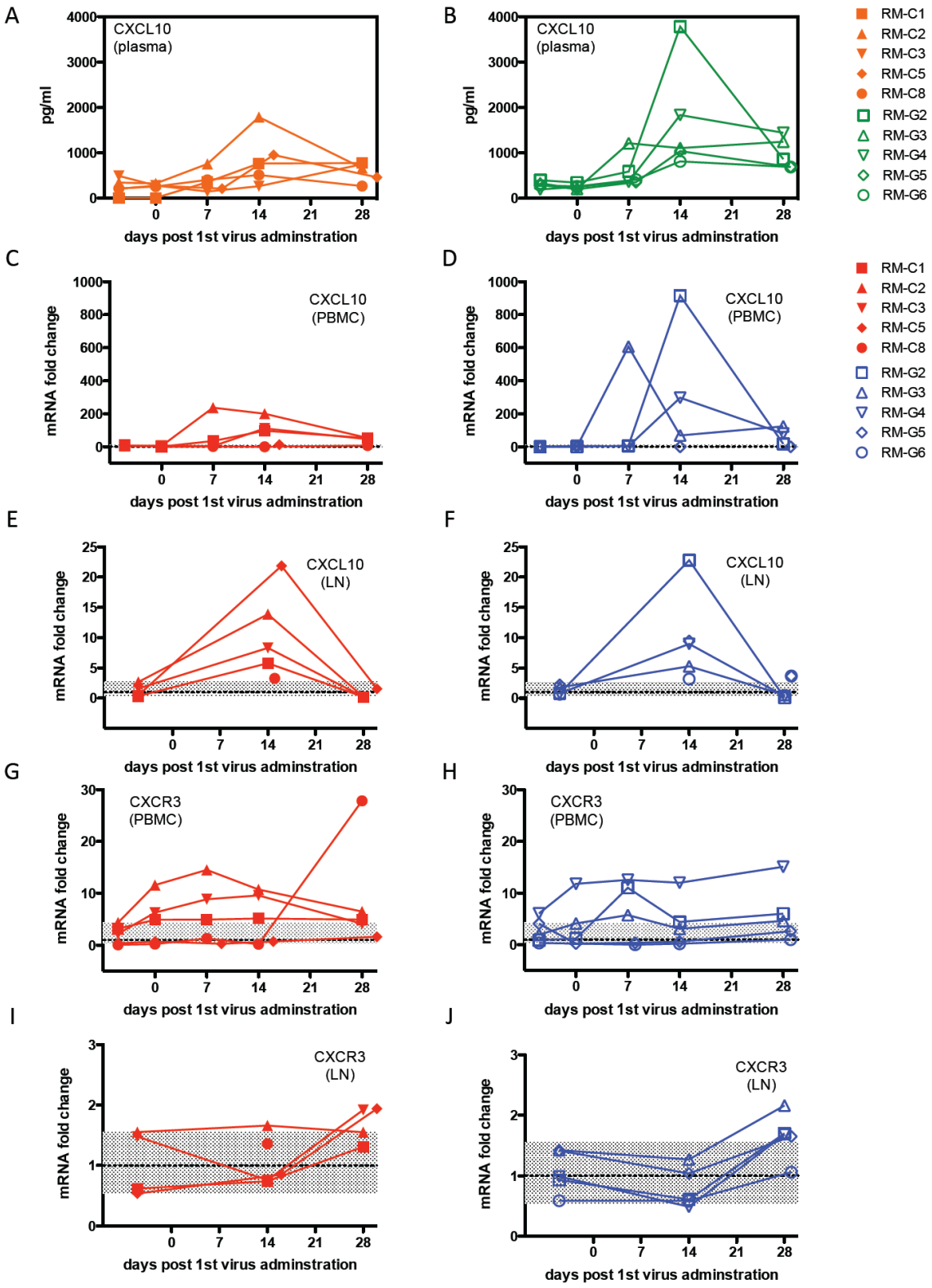


Figure 4-4. CXCL10 and CXCR3 in oral SIV infected macaques from control group (A, C, E, G, I) and gingivitis-induced group (B, D, F, H, J).
Shown are plasma levels of CXCL10 (A, B) and fold changes of mRNA expression of CXCL10 in PBMC (C, D) and peripheral lymph node (E, F) as well as CXCR3 in PBMC (G, H) and peripheral lymph node (I, J) during acute SIV infection. The grey area represents the range of each gene expression level, compared to averaged baseline expression.

The impact of gingival inflammation on IL-6 production and gene expression in peripheral blood and lymph node.

IL-6 is a proinflammatory cytokine produced by monocytes, fibroblasts and endothelial cells (2, 70, 233). IL-6 can drive immune cells differentiation, such as Th17 cells and macrophage development, activate neutrophils, stimulate platelet production and induce acute phase proteins (42, 199, 253, 281, 404). In the context of HIV infection, IL-6 is elevated in HIV-infected patients compared with uninfected persons(273) and high levels of IL-6 is associated with mortality rate and opportunistic infection in HIV infected patients (208, 321).

In our study, orally SIV infected macaques from both groups had no significant elevation of plasma IL-6 except macaque RM-G4 during acute SIV infection (Figure 4-5A and 5B). Interestingly, in SIV infected macaques from control group, IL-6 gene expression in PBMC was increased at day 7 with needleless syringe SIV administration and with a delay increased at day 16 (for macaque RM-C5) or day 28 (for macaque RM-C8) in whatman paper SIV administration (Figure 4-5C). In SIV infected macaques from gingivitis-induced group, IL-6 gene expression in PBMC can be up regulated to a higher level compared to control group (Figure 4-5D). 3 out of 5 macaques from control group and 2 out of 5 macaques from gingivitis-induced group had increased IL-6 gene expression in lymph node with similar level of up-regulation (Figure 4-5E and 5F).

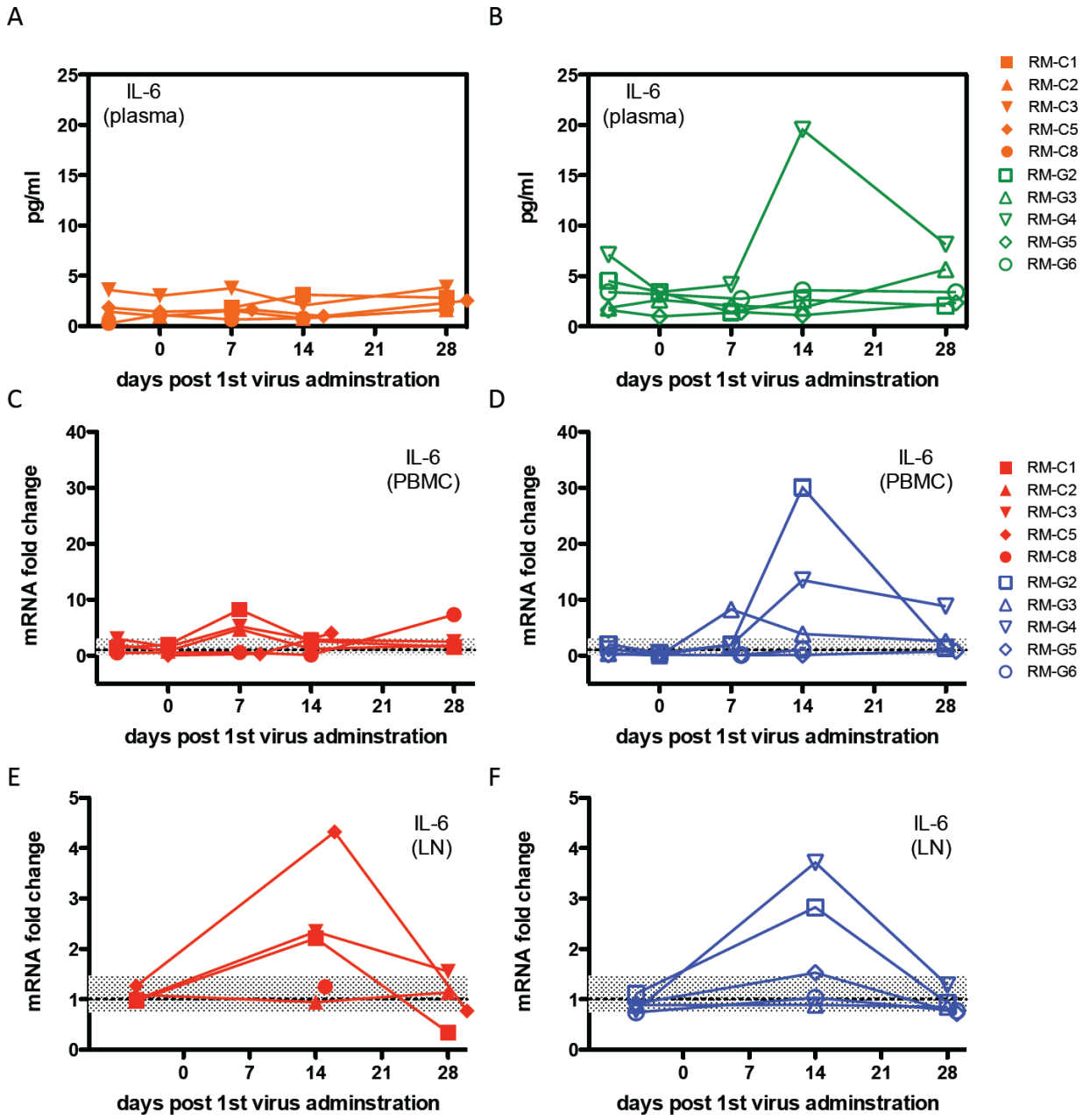


Figure 4-5. IL-6 protein and gene expression changes in oral SIV infected macaques from control group (A, C, E) and gingivitis group (B, D, F).
Shown are plasma levels of IL-6 (A, B) and fold changes of mRNA expression of IL-6 in PBMC (C, D) and peripheral lymph node (E, F) during acute SIV infection. The grey area represents the range of each gene expression level, compared to averaged baseline expression.

The impact of gingival inflammation on IL-15 gene expression in peripheral blood and lymph node.

Interleukin 15 (IL-15) is an inflammatory cytokine that plays a key role in activation, differentiation and proliferation of T cells and NK cells (386), as well as regulates the survival and the function of other immune cells (57). Many cell types constitutively express IL-15 mRNA, however, IL-15 protein secretion is generally low because of tightly controlled translational regulation (57, 291). IL-15 may play dual roles in HIV infection as IL-15-mediated immunity is associated with protection against HIV transmission during breast-feeding (388) while IL-15 is one of the earliest cytokines to be rapidly and transiently produced during acute HIV infection (354) and the level of IL-15 production is likely to impact HIV viremia and viral set point (318).

In our study, the level of plasma IL-15 was not examined. However, IL-15 gene expression in PBMC and lymph node was assessed (Figure 4-6). Overall, there was no significant difference with regard to the level of IL-15 gene expression fold change in PBMC and peripheral lymph nodes between control and gingivitis-induced group macaques. However, we observed a trend toward increased IL-15 gene expression in PBMC from macaques in our 1st set study (needless syringe SIV administration). Similar to IFN-α, RM-C1, RM-C2 and RM-G3 with early detectable plasma viral load at day 7 also had IL-15 gene up-regulation at day 7. The rest of the macaques in our 1st set study with detectable plasma viral load at day 14 had increased IL-15 gene expression at day 14, indicating that IL-15 gene expression may be dependent on the level of SIV replication.

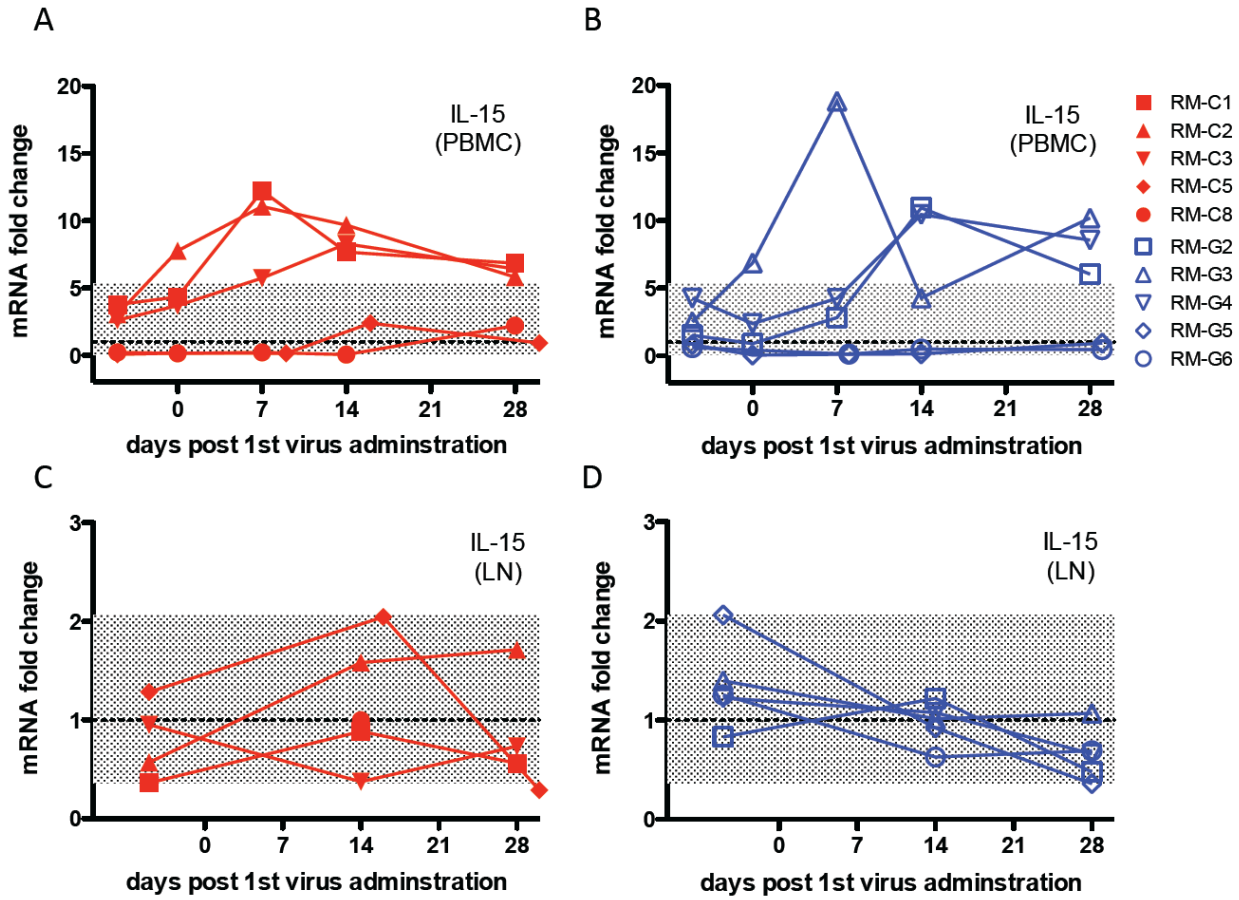


Figure 4-6. IL-15 gene expression changes in oral SIV infected macaques from control group (A, C) and gingivitis group (B, D).
Shown are IL-15 gene expression fold changes in PBMC (A, B) and peripheral lymph node (C, D) during acute SIV infection. The grey area represents the range of each gene expression level, compared to averaged baseline expression.

The impact of gingival inflammation on regulatory cytokine TGF-β and anti-inflammatory cytokine IL-10 production and their gene expression in peripheral blood and lymph node.

TGF-β and IL-10 are the hallmark of regulatory T cells (Tregs). Secretion of TGF-β and IL-10 by Tregs can suppress cytokine production and proliferation of CD4 and CD8 T cells to attenuate an inflammatory response (39). The role of Tregs in HIV infection is still not clear. HIV/SIV infection results in increased Treg (106, 124). However, elevation of Treg frequency, accompanied with TGF-β and IL-10 production

is associated with increased immune activation and tissue fibrosis (108) instead of controlling immune activation.

In our orally SIV infected macaques, the level of TGF-β in plasma fluctuates without obvious patterns associated with SIV replication or gingival inflammation induction (Figure 4-7A and B). The level of plasma IL-10 remained below detection limit of Luminex (data not shown). The gene expression of TGF-β and IL-10 in PBMCs and lymph nodes did not show significant changes following oral SIV infection and no significant difference was observed between the control and gingivitis-induced groups (Figure 4-7 C-J).

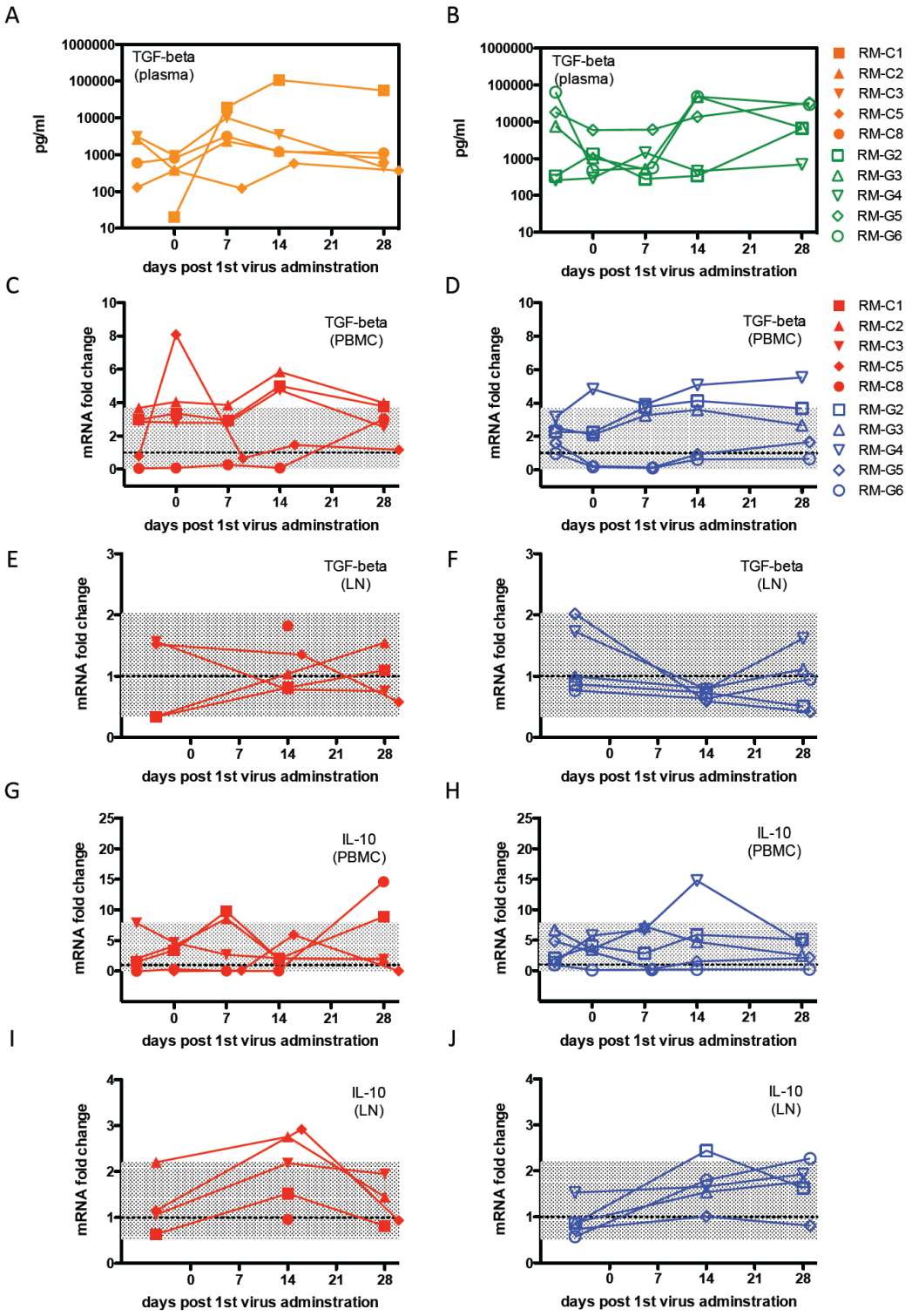


Figure 4-7. TGF-β and IL-10 in oral SIV infected macaques from control group (A, C, E, G, I) and gingivitis-induced group (B, D, F, H, J).
Shown are plasma levels of TGF-β (A, B) and fold changes of mRNA expression of TGF-β in PBMC (C, D) and peripheral lymph node (E, F) as well as IL-10 in PBMC (G,H) and peripheral lymph node (I, J) during acute SIV infection. The grey area represents the range of each gene expression level, compared to averaged baseline expression.

The influence of gingivitis on SIV envelope specific antibody development following oral administration

Cytokines and chemokines direct and shape the adaptive immune response development. Since we observed higher levels of immune modulators in the gingival crevicular fluid and interferons response in the plasma, we determine the influence of differential production of cytokine/chemokine SIV infected macaques between control and gingivitis-induced group on the SIV specific antibody response development following oral SIV administration. As shown in figure 4-9, all orally SIV infected macaques developed SIV envelope specific antibody response. The levels of SIV specific antibody were comparable between control and gingivitis-induced group macaques. RM-C5, RM-C8 and RM-G5 had lower or controlled virus replication during chronic SIV infection tend to have lower level of SIV specific antibody (figure 4-9).

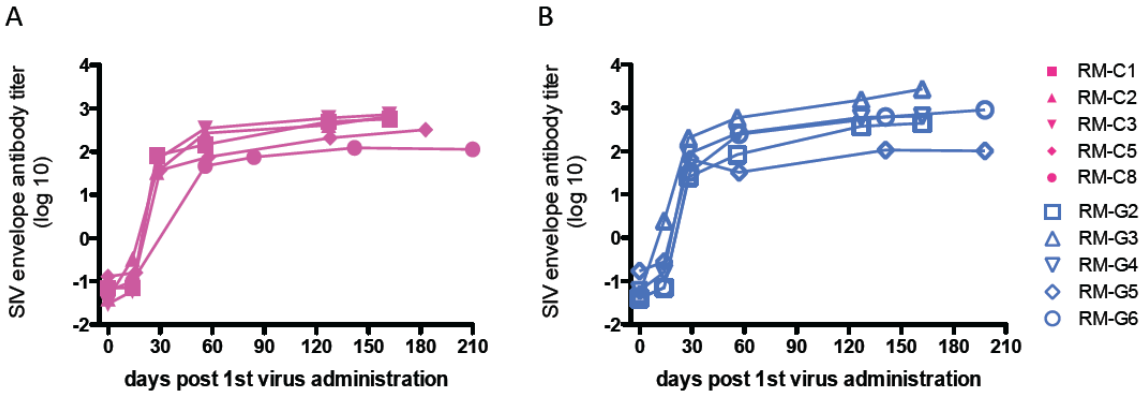


Figure 4-8. SIV envelope specific antibody response orally SIV infected macaques. Shown was SIV envelope specific antibody titer in SIV infected macaques in control group (A) and gingivitis-induced group (B) from acute to chronic infection.

The influence of gingival inflammation on plasma cytokines level during chronic SIV infection

We also examined if the influence of induced gingival inflammation on differential plasma cytokine production persisted into chronic SIV infection. Plasma samples collected between day 150-250 post 1st SIV administration from SIV infected macaques were measured by Luminex. As shown in Figure 4-9, the levels of plasma IFN-α, IFN-γ, CXCL10, IL-6 and other cytokines, such as IL-1Ra, IL-12p40, IL-4, IL-1β, MIP-1β, MCP-1 (data not shown) during chronic SIV infection were generally comparable in SIV infected macaques from control and gingivitis induced groups. Interestingly, RM-C5 and RM-C8 with low or undetectable plasma viral load during chronic infection stage also had low levels of plasma IFN-α, IFN-γ, CXCL10, IL-6. Similarly, RM-G5 in gingivitis-induced group with undetectable plasma viral load had low levels of plasma IFN-α and CXCL10 during chronic SIV infection.

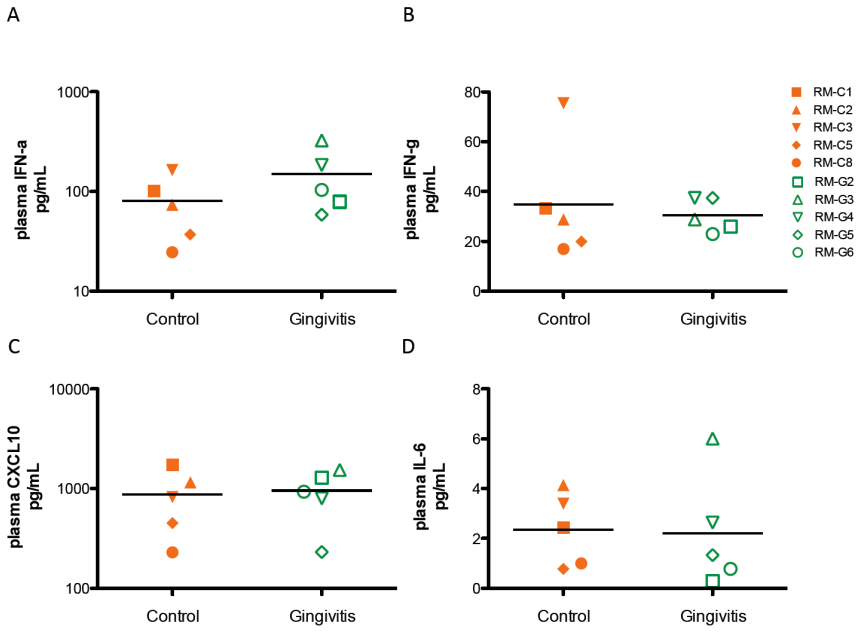


Figure 4-9. The levels of plasma IFN-α, IFN-γ, CXCL10 and IL-6 in orally SIV infected macaques during chronic SIV infection.
Shown are the concentration of plasma IFN-α (A), IFN-γ (B), CXCL10 (C) and IL-6 (D) from SIV infected macaques, measured between day 100-200 post SIV infection.

The impact of gingival inflammation on systemic immune activation following SIV infection

We further examined the impact of induced gingival inflammation on systemic immune activation following SIV infection by measuring: a. expression of proliferation markers on the immune cells, b. plasma C-reactive protein (CRP) and c. plasma soluble CD14 (sCD14).

The percentage of Ki-67 expression on CD8 T cells fluctuated during acute SIV infection and remained stable during chronic SIV infection. The percentage of Ki-67 expression on CD8 T cells was similar between control and gingivitis-induced groups following SIV infection, generally ranging between 10-30%, except macaque RM-G2 who consistently had high levels of Ki-67 expression on CD8 T cells across the study period (Figure 4-10A and 10B). Plasma CRP and sCD14 levels generally remained stable across the study time period for SIV infected macaques in both groups, except RM-C8. The levels of plasma CRP and sCD14 was similar before and after SIV infection and comparable between the control and gingivitis-induced groups (Figure 4-10C to 10F). Interestingly, macaques in the gingivitis-induced group receiving SIV with needleless syringe had higher levels of plasma sCD14 during chronic SIV infection than macaques receiving SIV with whatman paper (Figure 4-10F). Consistent with viral load and CD4 T cell counts data, macaque RM-C5 and RM-G5 with controlled virus replication during chronic SIV infection also exhibited the lowest levels of Ki-67 expression on CD8 T cells, plasma CRP and sCD14 during chronic SIV infection, indicating slower disease progression in these two macaques.

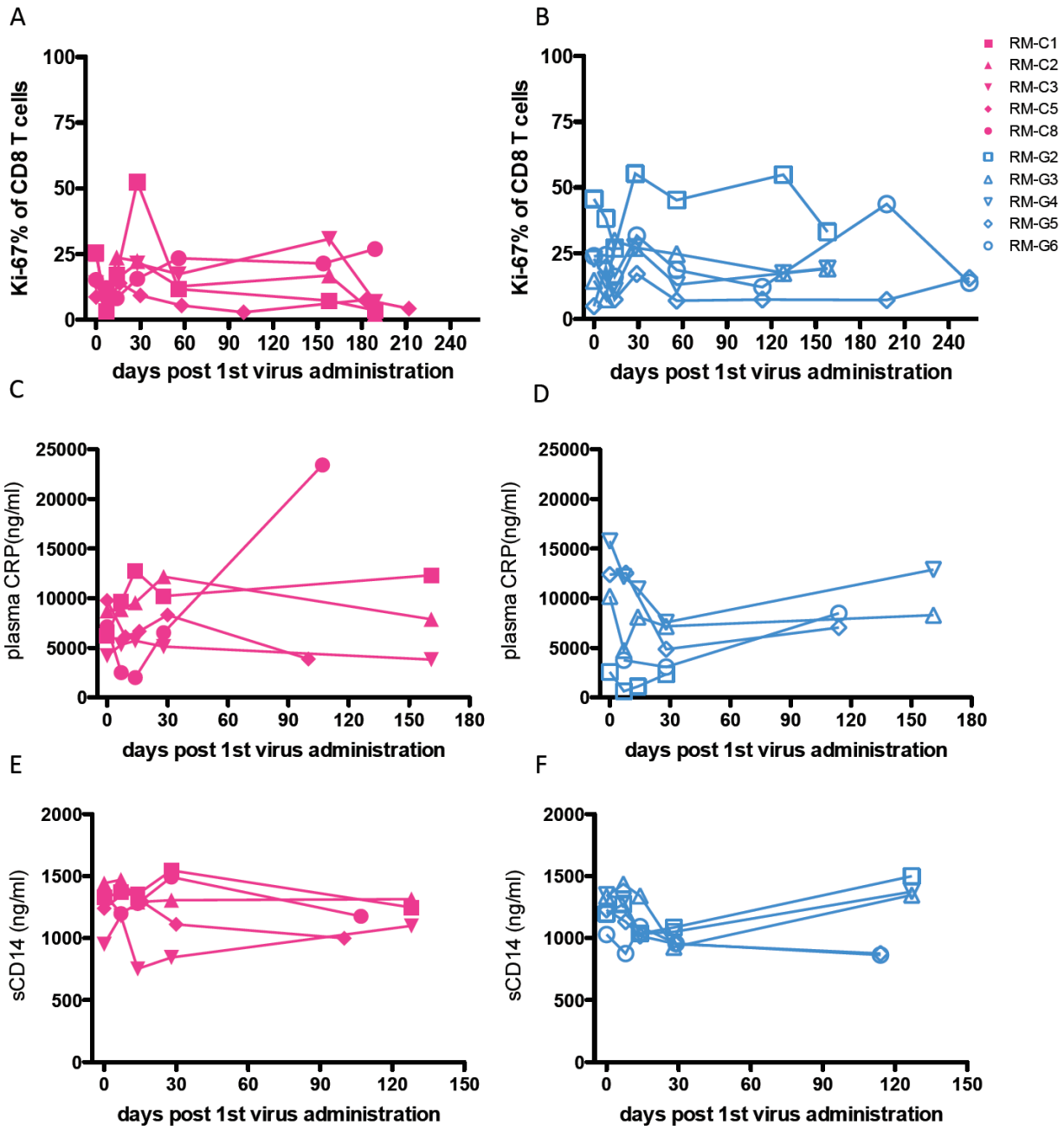


Figure 4-10. The levels of systemic immune activation in orally SIV infected macaques from control (A, C, E) and gingivitis-induced group (B, D, F).
Ki-67 expression on the CD8 T cells (A, B), plasma CRP (C, D) and sCD14 (E, F) were measured in orally SIV infected macaques at pre-SIV infection/pre-gingivitis induction, day 0, day 14-16, day 28-30, and day 100-200 post SIV infection.

The impact of gingival inflammation on virus replication and innate immune response in peripheral blood through intravenous (i.v.) inoculation of SIV

To test if the influence of induced gingivitis on altered innate immune responses following SIV infection were oral route dependent, 6 macaques (n=3 in each group) were intravenously inoculated with 50 $TCID_{50}$ SIVmac251. After a single dose SIV injection, all 6 macaques became SIV infected with detectable plasma viral load at day 7 post-infection, peak virus replication around day 14 and viral set point development during chronic infection with no significant differences between control and gingivitis-induced groups (Figure 4-11A). CD4 T cell counts were decreased following SIV infection with similar rate of CD4 T cell loss between two groups (Figure 4-11B). Changes of IFN-α (Figure 4-11C), OAS (Figure 4-11D), CXCL10 (Figure 4-11E) and IL-6 gene expression (data not shown) in PBMC were comparable between control and gingivitis-induced groups following intravenous SIV inoculation, except 2 macaques in gingivitis-induced group had higher CXCL10 gene expression at day 7 post-infection.

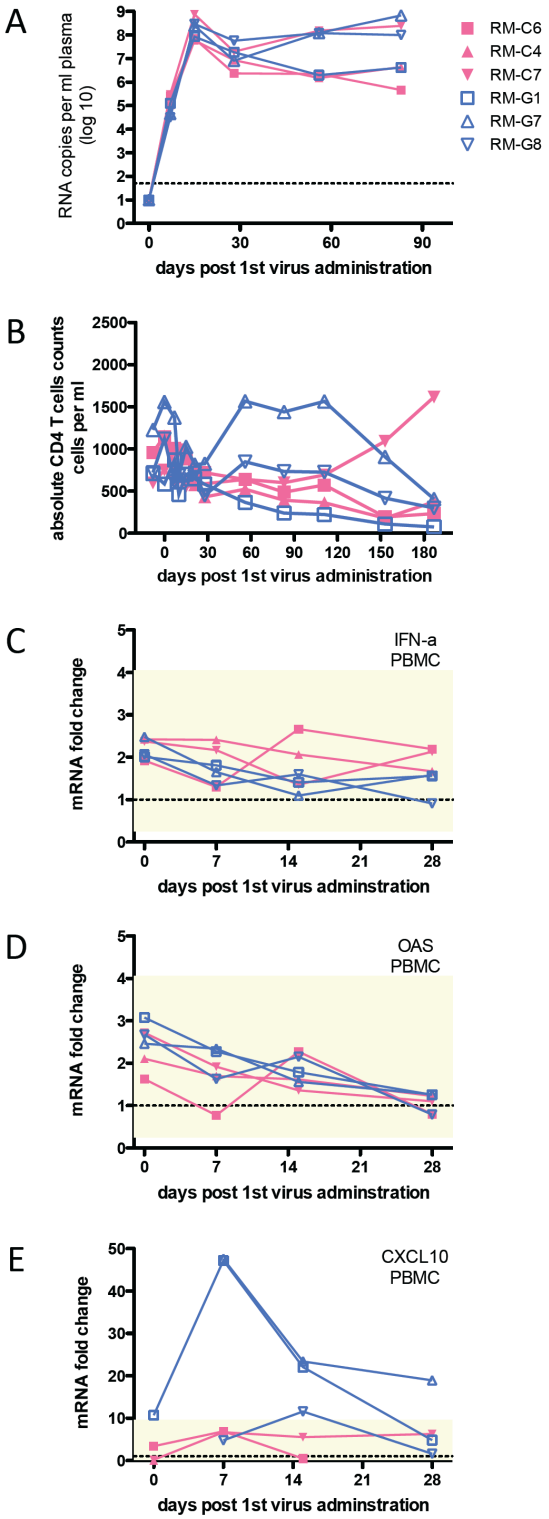


Figure 4-11. Viral and immunological changes following intravenous SIV inoculated macaques with or without induced gingival inflammation.
Shown are Plasma viral load (A); CD4 T cell counts (B); IFN-α (C), OAS (D) and CXCL10 (E) gene expression fold changes in PBMCs during acute SIV infection. The dot line of plasma viral load indicates the detection limit. The yellow area of gene expression fold change indicates the range of gene expression baseline

Discussion

Early events following HIV exposure to mucosal sites are important for identifying immune correlates that can be incorporated into vaccines or therapy development to provide protection of HIV infection or delay the time course to progress to AIDS. Often times this information can only be obtained through studying SIV infection of rhesus macaques. In the study presented here, we examined the influence of pre-existing mucosal inflammation (induced gingival inflammation) on systemic immunological changes following SIV infection through the oral route. Assessment of multiple immune modulator protein levels in plasma and the mRNA level changes in PBMCs as well as peripheral lymph nodes, found that plasma IFN-α and IFN-γ were differentially produced from SIV infected macaques with or without

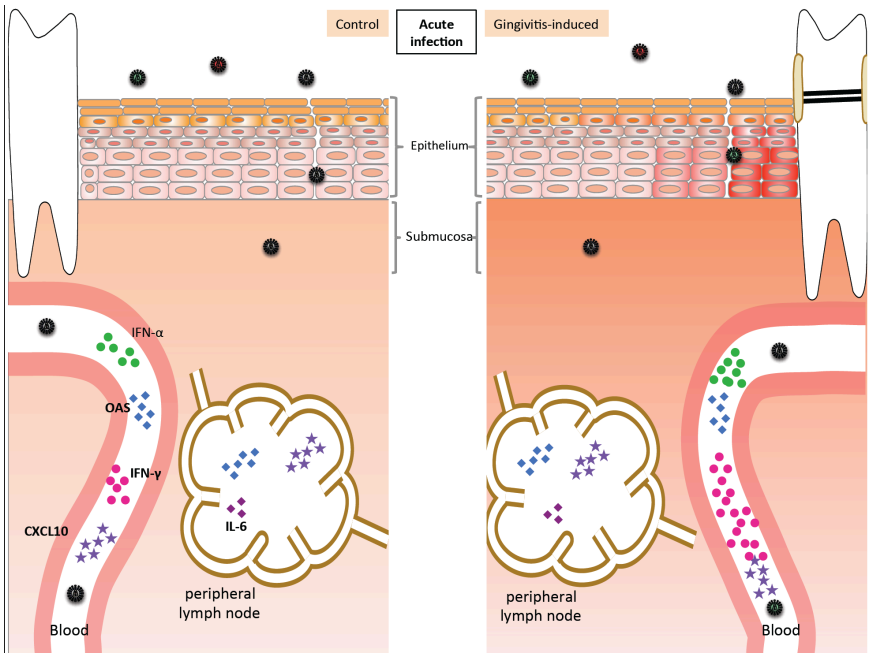


Figure 4-12. Model of systemic immunological changes from orally SIV infected macaques in control (left) and gingivitis-induced groups (right) during acute infection.
Shown are immune modulator (IFN-α, OAS, IFN-γ, CXCL10, and IL-6) changes in peripheral blood and lymph nodes. The numbers of the symbols does not represent actual concentration, but the trends are indicated

induced gingival inflammation during acute SIV infection. OAS and CXCL10 gene expression were also increased during acute SIV infection in PBMC and peripheral lymph nodes. Furthermore, more macaques in gingivitis-induced group had increased OAS gene expression and myeloperoxidase production at the peripheral lymph nodes (Figure 4-12).

During chronic SIV infection stage, the levels of plasma IFN-α, IFN-γ and CXCL10 remained elevated, compared to pre-SIV infection, but were comparable in SIV infected macaques from control and gingivitis-induced groups. Systemic immune activation markers, such as the levels of plasma CRP, sCD14 and the percentage of Ki-67 expressing CD8 T cells, as well as disease progression parameters, including plasma viral load, CD4 T cell counts, were also similar between these two groups,

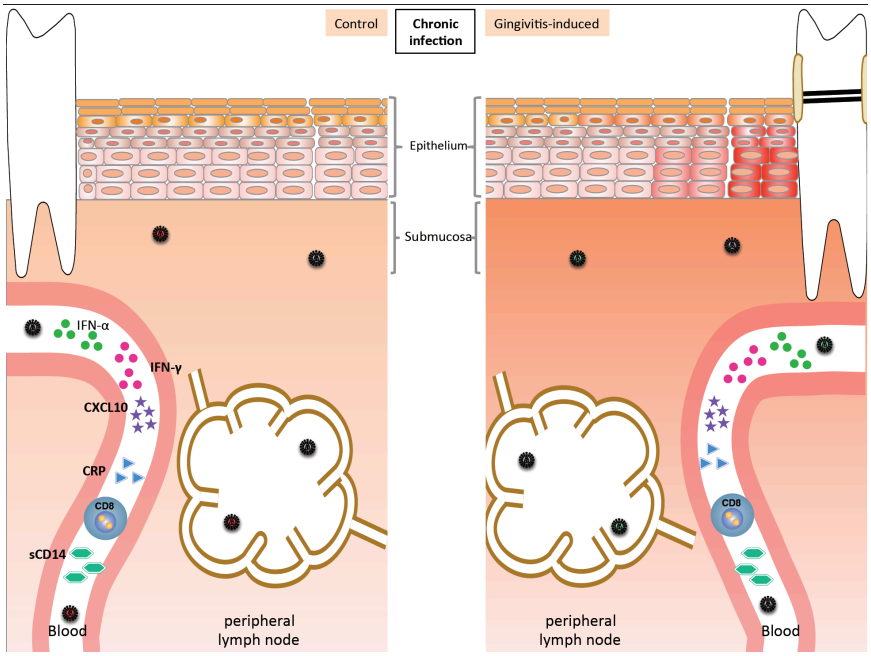


Figure 4-13. Model of systemic immunological changes from orally SIV infected macaques in control (left) and gingivitis-induced groups (right) during chronic infection.
Shown are the levels of immune modulators (IFN-α, IFN-γ, CXCL10, CRP, sCD14) and Ki-67 expressing CD8 T cells in the peripheral blood from orally SIV infected macaques. The numbers of the symbols does not represent actual concentration, but the trends are indicated.

indicating that the presence of induced gingival inflammation does not impact correlates of disease progression in orally SIV infected macaques (Figure 4-13).

The study was originally designed to examine preexisting mucosal inflammation on the rate of oral SIV infection in rhesus macaques. In order to address the contact of SIV with inflamed gingival tissue, whatman paper was used to administer SIV in our 2nd and 3rd set study. Compared to 1st set study using a needleless syringe, we generally observed lower plasma cytokine and chemokine production, mild changes of immune gene expression in PBMCs and controlled virus replication during chronic infection in SIV infected macaques from 2nd and 3rd set study. These observations may be due to the method of SIV administration that a significant amount of SIV were not released by whatman paper and macaques received much lower dose of SIV in 2nd and 3rd set study compared to 1st set study. Indeed, previous studies from our lab and others have demonstrated that lower doses of SIV infection were associated with delayed and less robust cytokine/chemokine production in plasma as well as gene expression in PBMCs (96, 226).

IFN-α is one of the earliest cytokines produced in response to HIV/SIV infection (354). In our study, plasma IFN-α was elevated around day 7 or day 14 and remained increased around day 28 to chronic infection in orally SIV infected macaques. This result is consistent with the result that IFN-α was induced early following HIV/SIV infection. Interestingly, we observed decreased IFN-α mRNA level in PBMCs following oral SIV infection which contradicted the elevation of plasma IFN-α in these macaques. This observation may be explained by the dynamics of immune cells in the circulation. Several studies examined the dynamics and activation of plasmacytoid

dendritic cells (pDCs, major IFN-α producing cells) following SIV infection in rhesus macaques and found that pDCs were decreased in the peripheral blood after SIV infection(31, 52), which may be responsible for the decreased IFN-α mRNA in PBMC. The mechanism(s) that result in reduced pDC in the peripheral blood were not clear. Following SIV infection, pDCs were shown to migrate into mucosal sites, such as gut where pDCs released large amounts of IFN-α (53, 209), as well as increased cell death markers (31). Therefore, both mechanisms are possible and likely occurred simultaneously in this study.

It has been shown in the literature that chronic gingival inflammation can contribute to changes in systemic inflammation markers (100, 101). Several studies have demonstrated that patients with periodontal diseases exhibit higher C-reactive protein (CRP) in peripheral blood (276, 289), TNF-α (33, 93) and IL-6 (267), indicating that bacterial induced gingival inflammation can modulate systemic immune responses. The mechanisms for gingival inflammation associated higher systemic inflammation are complex. As immune cells and secretory proteins can move in and out of the tissues, immune cells may be activated at gingival tissues and then migrate into peripheral blood where cytokines were released. It is also possible that immune modulators were produced at the inflamed tissues and spilled over into the systemic circulation. In addition, severe or aggressive gingival inflammation can induce microbial translocation, such as mild bacteremia or bacterial products, i.e. lipopolysaccharide (LPS), being released into systemic circulation (102, 154) that can further activate the immune system by triggering TLRs of immune cells in the peripheral blood. Examination of LPS-binding protein (LBP) in our study did not show

significant differences between control and gingivitis-induced groups (data not shown), indicating that microbial translocation may not play a major role in driving differential plasma cytokines/chemokines production or immune gene expression in PBMCs. In addition, comparing macaques with intravenous SIV injection in control and gingivitis-induced groups, we did not observe additive or synergistic effects on systemic immune changes between these two groups. This result indicates that SIV infection through oral route, where gingival inflammation was induced, was important to induce differential cytokine/ chemokine production in the peripheral blood.

The major differences in systemic immunological changes of SIV infected macaques between control and inflammation-induced group were related to IFN-α and IFN-γ. The production of IFN-α and IFN-γ depend on sensing of microorganisms, such as viruses and bacteria or their products, by toll-like receptors (TLRs). It is possible that pre-existing gingival inflammation upregulated TLRs expression and therefore induced higher levels of IFN-α and IFN-γ in response to incoming oral SIV administration. Indeed, chronic periodontitis patients express higher TLRs in PBMCs and gingival tissues than healthy controls (37, 58), indicating that pre-existing gingival inflammation can affect TLRs expression, which alters IFN-α and IFN-γ production in response to SIV. Another possibility is that gingival inflammation induces transient higher virus replication in the oral cavity that triggered more robust IFN-α and IFN-γ production in the mucosal sites and then spread into systemic plasma. Indeed, A study from Easlick J et al., demonstrated that IFN-α induction seemed to correlate with the level of virus replication at tissue sites (99). In our study, both mechanisms

may contribute to the higher level of plasma IFN-α and IFN-γ in gingivitis-induced group macaques.

In the last part of the study, we also assessed the influence of mucosal inflammation on immunological changes following intravenous SIV inoculation. We found that IFN-α and OAS gene expression remained stable during the first month of SIV infection, despite high plasma viral load in i.v inoculation macaques. Within two of the gingivitis-induced macaques CXCL10 gene expression in PBMCs in intravenously SIV infected macaques did increase up to 50 fold compare to baseline. The levels of gene expression differences between macaques receiving oral SIV administration or intravenous inoculation could be due to the dose and the route of SIV exposure. Also, one-time intravenous SIV inoculation bypass many immune defense mechanisms that can result in different virus dissemination pattern in the macaques, compared to SIV infection through oral route. These data suggest that the route of SIV infection can affect the induction of immune responses against SIV invasion.

In summary, we demonstrated that SIV infected macaques with induced gingival inflammation exhibited altered interferon-related responses during acute SIV infection following oral SIV exposure. Although the differential productions of IFN-α, OAS, IFN-γ and CXCL10 during acute SIV infection did not show significant impacts on chronic systemic immune activation as well as disease progression indicators (plasma viral load or CD4 T cell count), these observations indicate that pre-existing mucosal inflammation can affect immune induction following HIV/SIV infection and may be a potential factor to alter mucosal delivered vaccines induced immunity.

Chapter 5: Establishment of SIV infection through penile exposure in rhesus macaques

Introduction

The major route of HIV transmission is through unprotected sexual intercourse resulting in HIV exposure to vaginal, penile, anal and oral mucosa. Although men who have sex with men are at high-risk of acquiring HIV, heterosexual men are regularly infected through penile exposure of HIV (262, 326, 327). Despite a large proportion of men acquiring HIV through penile exposure, limited studies have focused on understanding the mechanisms of HIV infection through the male genital tract as well as immune factors that alter HIV susceptibility in males. A recent study from Kigozi et al., found that the risk of male HIV acquisition is increased among men with larger foreskin surface areas, indicating the presence of foreskin tissue is associated with increased HIV acquisition (191). Supporting this, clinical trials have shown that male circumcision can reduce HIV acquisition by 50-60% (20, 28, 138) and is recommended as a part of HIV prevention strategies if possible. These studies point out the important role of the foreskin as a major tissue for HIV infection. Since male circumcision can reduce HIV transmission up to 50%, other tissues of the male genital tract, such as the glans, may also play a role in HIV acquisition and be responsible for the other half of HIV transmission.

The exact mechanisms of how circumcision can reduce HIV acquisition are not clear. However, it is believed that adult foreskin tissue contains high proportions of CD4+ T cells, macrophages, and Langerhan cells (LCs) and circumcision removes potential HIV target cells, therefore, reducing HIV transmission (155, 287). Immune cells, especially LCs in the inner foreskin explants, can rapidly respond to certain

cytokines and induce CD4 T cell infiltration as well as increase their ability to sample environment proteins (109). These results indicate that the inner foreskin may play an important role for initial HIV entry and the contribution from LCs as first target cells for HIV acquisition in male genital tract. Indeed, Ganor et al., demonstrate that efficient HIV transmission can occur in the inner foreskin where LCs pick up HIV and form synapses with T cells thereby transferring HIV to CD4 T cells in which HIV can efficiently replicate (127). Furthermore, following HIV infection of inner foreskin explants, RANTES secretion is increased and CCL20 (MIP-3α) secretion is decreased. Elevated RANTES mediates T cell migration that can contribute to LCs-T cell synapses formation to establish HIV infection (411). These studies suggest that HIV exposure to foreskin mucosa induces immune environment changes in penile tissues and, interestingly, these findings are not consistent with a previous report that CCL20 is increased in vaginal tissues following HIV/SIV infection(224), indicating that the foreskin may have its unique immune mechanisms to interact with foreign pathogens.

Several risk factors are associated with HIV infection in heterosexual men including foreskin inflammation (180), herpes simplex virus type 2 (HSV-2) (342, 385) and genital ulcer diseases, such as chancroid caused by *Haemophilus ducreyi* (236). Possible mechanisms to explain the associations between genital inflammation or sexual transmitted diseases (STDs) with HIV infection are that bacterial or viral infection-induced inflammation recruits immune cells to the genital tract and therefore increases HIV target cell availability at mucosal sites (178) or STDs cause genital ulcers that disrupt mucosal integrity so that HIV can enter the body easily. However, HSV-2 suppression with acyclovir as well as STDs control by mass antibiotic

treatment has no significant effects in reducing HIV-1 transmission (64, 65, 392, 395), indicating that the interaction between HSV, STDs or mucosal inflammation and HIV infection may be more complicated than HIV target cell availability or mucosal integrity. Furthermore, questions with regard to HIV acquisition through penile exposure, such as HIV entry sites, initial target cells along the male genital tract, the immune factors that correlate with HIV infection or protection, the HIV dissemination dynamics, and the immune response following HIV invasion at penile tissues, are largely unknown.

In 2007, HIV vaccine STEP trial interim analysis showed increased HIV infection rates in the vaccination group. Furthermore, uncircumcised vaccinated men were at higher risk of HIV infection (56, 176). The reasons for increased HIV infection in the vaccination group from the STEP trial remain unclear but behavior changes are unlikely to be the major contributor(196). Instead, research into potential biological mechanisms is urgently needed. However, the lack of a well-established animal model, such as SIV infection of Rhesus macaque with penile challenge, has impeded our understanding of mechanisms and factors affecting HIV acquisition through the male genital tract. In addition to vaginal and rectal challenge models of SIV infection in non-human primates, a suitable model to evaluate HIV vaccines or topical use microbicides through penile challenge is also important. Therefore, the goal of the study presented here is to develop a SIV foreskin/penile challenge model in rhesus macaques for future vaccine studies and assess factors associated with successful SIV infection following penile exposure to better understand the mechanisms associated with penile/foreskin HIV infection.

Results and discussions

SIV foreskin/penile challenge model

There were 10 male macaques included in the study, age 4-7 years old and with an average weight of 7.52kg (range from 5.96kg-11.11kg) at the time of study initiation (September 2009) from the Washington Primate Research Center. With the goal of developing a SIV foreskin/penile macaque challenge model and assessing the ability of SIVsmE660 strain to infect Rhesus macaques through the foreskin/penile route, 4 macaques were randomly chosen to receive one dose of 225µl 8000 $TCID_{50}$ SIVsmE660 (1^{st} set study) through penile tissue exposure. When the penile tissues were pulled up to form a cup to hold SIV, both foreskin and glands were exposed to the virus (Figure 5-1). However, examination of the presence of SIV gag in PBMC by nested PCR and plasma viral load were negative, indicating that a single administration of SIVsmE660 was not able to initiate infection in these 4 macaques. This may potentially be due to low pathogenic characteristics of SIVsmE660 or the viral dose of exposure was too low to initiate successful infection. In our 2^{nd} set study, we increased the frequency of SIV administration to 3 times a week (Monday, Wednesday and Friday) and administered higher dose of SIVsmE660 using double the volume of SIV inoculum (450 µl 8000 $TCID_{50}$ SIVsmE660). However, three administrations of higher SIVsmE660 were not successful in initiating systemic infection in these four macaques. The results from these 2 set studies indicate that SIVsmE660 infection through penile exposure in rhesus macaques were not effective.

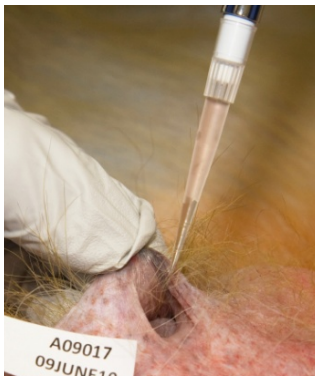


Figure 5-1. SIV administration through penile exposure

It has been reported that TRIM5 genotypes may modulate SIVsmE660 penile acquisition (312, 406). Based on that, we collaborated with Johnson lab and determined TRIM5 genotypes of the macaques in the study and found that three out of four macaques in the 1st and 2nd set study have the permissive allele ($TRIM^{Q}$) that is correlated with successful SIV infection. Therefore, failure to initiate SIVsmE660 infection through penile exposure in our 1st and 2nd studies cannot be attributed to harboring a protective TRIM5 allele in these 4 macaques. Our data from 1st and 2nd set study are consistent with the studies from Ma et al., and Yeh et al., that either SIVsmE660 or SIVmac251 (a more infectious and pathogenic inoculum compared to SIVsmE660) was inefficiently transmitted to macaques through penile exposure (235, 406). In the studies from Ma et al., and Yeh et al., some macaques received 2ml 10^4 $TCID_{50}$ SIVmac251 7 times and remained SIV uninfected. Higher doses of SIV and much more frequent exposures, i.e. two high doses exposure with 4 hours apart, were needed to overcome the penile barrier in rhesus macaques to establish successful SIV infection. These studies demonstrate that the penile tissues from rhesus macaques provide strong protection against SIV, and potentially mimic the difficulties of HIV encountering with male genital tract and provide evidence of overall low transmission rate for HIV through sexual intercourse (140, 141, 393).

Compared to SIV infection via vaginal exposure where repeated low dose challenges were able to initiate successful infection (358) with 30% infection rate with 1000 $TCID_{50}$ SIVmac251, SIV infection through penile route is more difficult to establish systemic infection. This is consistent with epidemiology studies that the odds of male-to-female transmission were greater than female-to-male transmission (283).

The mechanisms to explain differential rates of infection between male and female remain unclear but intrinsic differences between vaginal and penile tissue structures, cell types, or keratinization of the mucosal barrier may play important roles in determining host susceptibility to HIV infection.

The influence of genital inflammation on SIV acquisition through penile exposure

In the literature, the presence of sexually transmitted diseases (STDs), genital inflammation or genital ulcers are associated with increased HIV infection (180, 236, 342, 385). Therefore, we hypothesized that experimentally induced STDs prior to SIV administration can be an alternative method to increase SIV infection of rhesus macaques through penile exposure. STDs and HIV co-infections are also common in HIV endemic areas and establishment of STDs and HIV coinfection model in rhesus macaques will contribute to our understanding of mucosal factors affecting HIV acquisition in populations at higher risk of HIV infection.

In our 3rd set study, we collaborated with Dr. Totten at University of Washington who has extensive experience establishing the primate model for Chancroid pathogenesis studies at Washington Primate Research Center (367). *Haemophilus ducreyi* is the causative pathogen for Chancroid, a sexually transmitted disease characterized by genital ulcers and inflammation, with high prevalence in developing countries (66, 316). In addition, Chancroid is associated with increased HIV transmission. HIV infected patients with chancroid exhibit higher HIV shedding into genital secretions that increases the possibility to transmit HIV to their uninfected partners (125). HIV uninfected people with chancroid are also at higher risk of acquiring HIV (236). To test the hypothesis that pre-existing STDs will enhance SIV

infection through penile exposure, 3 macaques (A09011, A09013, and A09015) were infected with *Haemophilus ducreyi* either through intradermal (A09011 and A09015) or subcutaneous (A09013) injection to foreskin tissue. 2 macaques (A09010 and A09017) were included as control without *Haemophilus ducreyi* infection. As shown in the figure 5-2, *Haemophilus ducreyi* infection of penile tissues showed signs of inflammation at day 4 post *Haemophilus ducreyi* infection and progression to ulceration around day 16 (Figure 5-2). Single dose of 200ul SIVsmE660 (8000$TCID_{50}$) was given 2 days after *Haemophilus ducreyi* infection. In our 3rd set study, none of the macaques were SIV infected (remained negative for SIVgag in PBMC) after receiving first dose of SIVE660. At day 16 post *Haemophilus ducreyi,* all 5 macaques received another dose of SIVsmE660 administration.

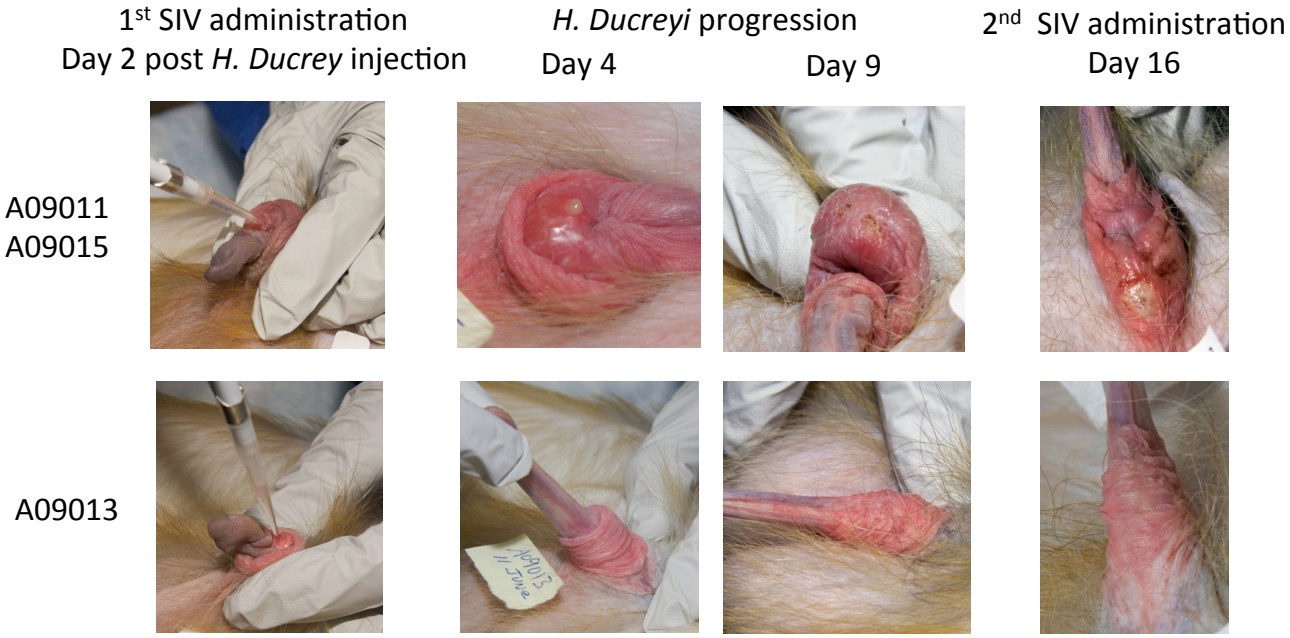


Figure 5-2 *Haemophilus ducreyi* infection lesion progressions and SIV administration through penile exposure.
Shown are *Haemophilus ducreyi* induced genital inflammation developments in macaques A09011 and A09015 (upper) and A09013 (lower) from day 2 (left) to day 16 (right).

Interestingly, macaque A09013 infected with *Haemophilus ducreyi* without signs of genital inflammation became SIV positive after receiving second SIVsmE660 administration. Plasma viral load of macaque A09013 exhibited a transient high viral replication during acute infection and remained low or undetectable (50 copies/ml indicated as dot line) during chronic infection (Figure 5-3). Genetic factors, such as MHC or TRIM5 genotypes, may contribute to spontaneous controlled virus replication in this macaque. MHC genotypes information for the macaques in this study was not available but we knew macaque A09013 had homozygous TRIM$^{TFP/TFP}$ genotype that may provide intermediate ability to control SIVsmE660 replication (312, 406). Furthermore, it is possible that the strains of SIVsmE660 (originated from sooty mangabey) used in our study cannot replicate well in rhesus macaques. Further studies such as obtaining sequence information of our SIVsmE660 may provide insights into if our viral inoculum was sensitive or resistant to TRIM5 restriction. Infection of our SIVsmE660 as well as other sources of SIVsmE660 or SIVmac251 in vitro with macaques PBMCs would be another way to test our SIVsmE660 replication ability compared to other SIV strains.

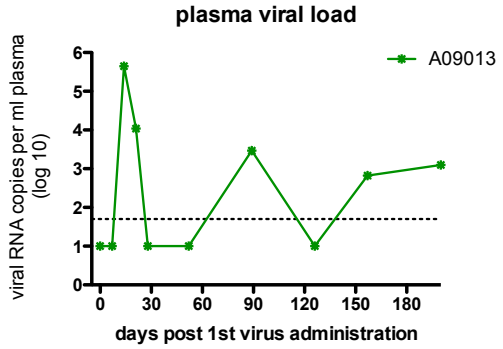


Figure 5-3: plasma viral load of macaque A09013

We further analyzed potential immune factors that may be associated with successful systemic SIVsmE660 infection of this macaque through penile exposure by assessing the level of systemic immune activation, the proportion of activated CD4 T cells in the periphery as well as immune genes expression and target cells at foreskin. We found that successful SIVsmE660 infection in macaque A09013 was not associated with increased systemic immune activation (measured by Ki-67 expression on CD8 T cells and the percentages of CD38+HLA-DR+ CD8 T cells) or the proportion of activated CD4 T cells (Ki-67+ or CD38+HLA-DR+ CD4 T cells) in the peripheral blood around the time of SIV administration (Figure 5-4). Interestingly, an antibiotic treatment for *Haemophilus ducreyi* infection with ceftriaxone was likely to induce increased systemic immune activation as we observed dramatic increased cell activation markers expression on CD4 and CD8 T cells after ceftriaxone treatment in macaque A09011 and A09015 (Figure 5-4C and G).

Analysis of immune genes (RANTES, CCR5, TNF-α, IL-8, IFN-α, IFN-γ, and TGF-β) expression levels with foreskin biopsy sampled at day 18 post *Haemophilus ducreyi* (2 days after 2nd SIV administration) did not find any correlation of increased gene expression and SIV infection (Figure 5-5). Most of the gene expression levels examined in macaque A09013 were comparable to SIV exposed but uninfected control macaques. Note that 2 SIV negative macaques co-infected with *Haemophilus ducreyi* had higher RANTES, TNF-α, IL-8, IFN-α, IFN-γ and CCR5 gene expression at foreskin, indicating these 2 macaques indeed had mucosal inflammation that was consistent with their clinical signs of genital ulcer development.

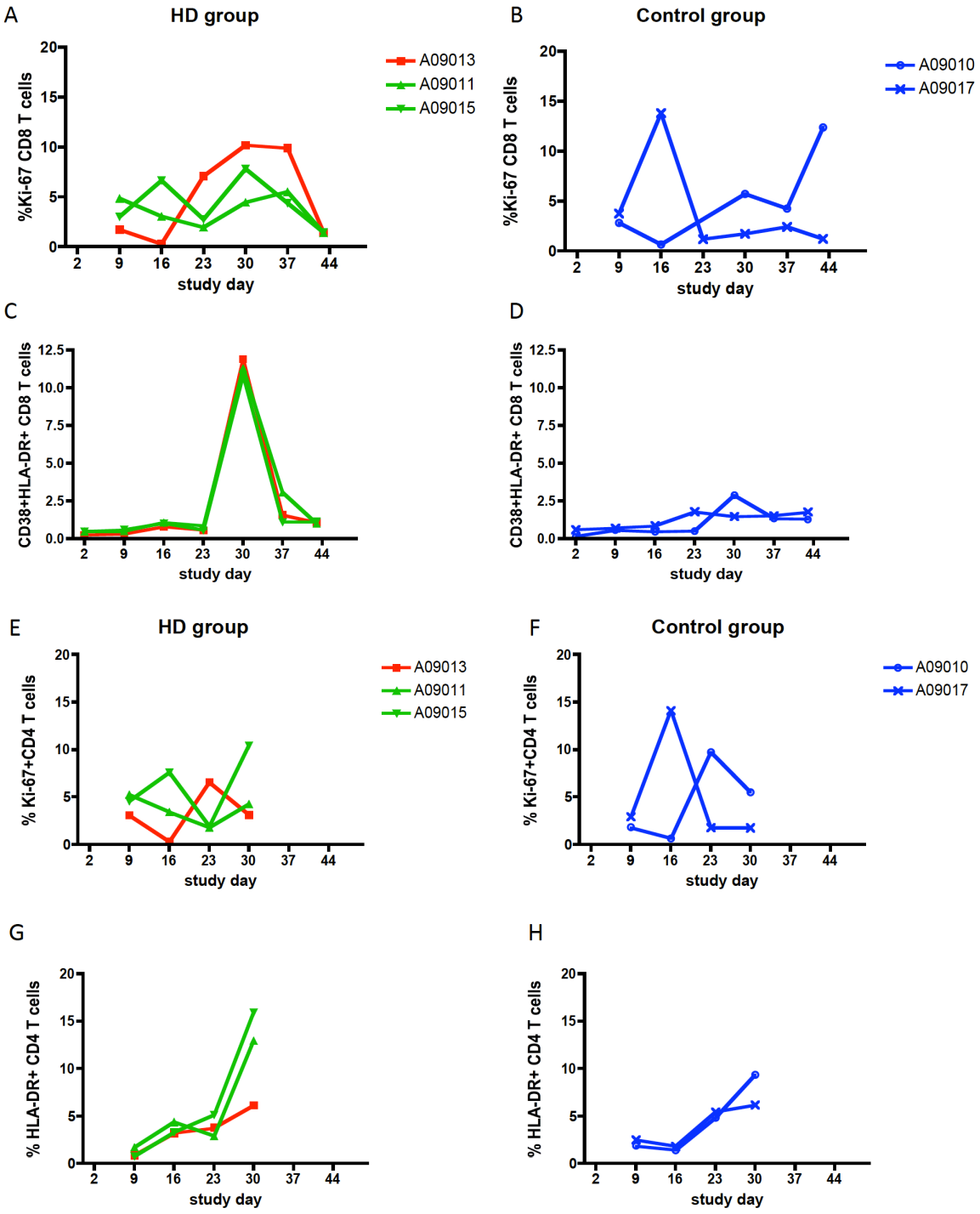


Figure 5-4. Systemic immune activation and activated CD4 T cells in 3rd set study macaques.
Shown are the percentages of Ki-67+ CD8 T cells (A, B), CD38+HLA-DR+ CD8 T cells (C, D), Ki-67+ CD4 T cells (E, F) and CD38+HLA-DR+CD4 T cells (G, H) from macaques infected with (A, C, E, G) or without (B, D, F, H) *Haemophilus ducreyi*. The macaque became SIV infected was shown in red.

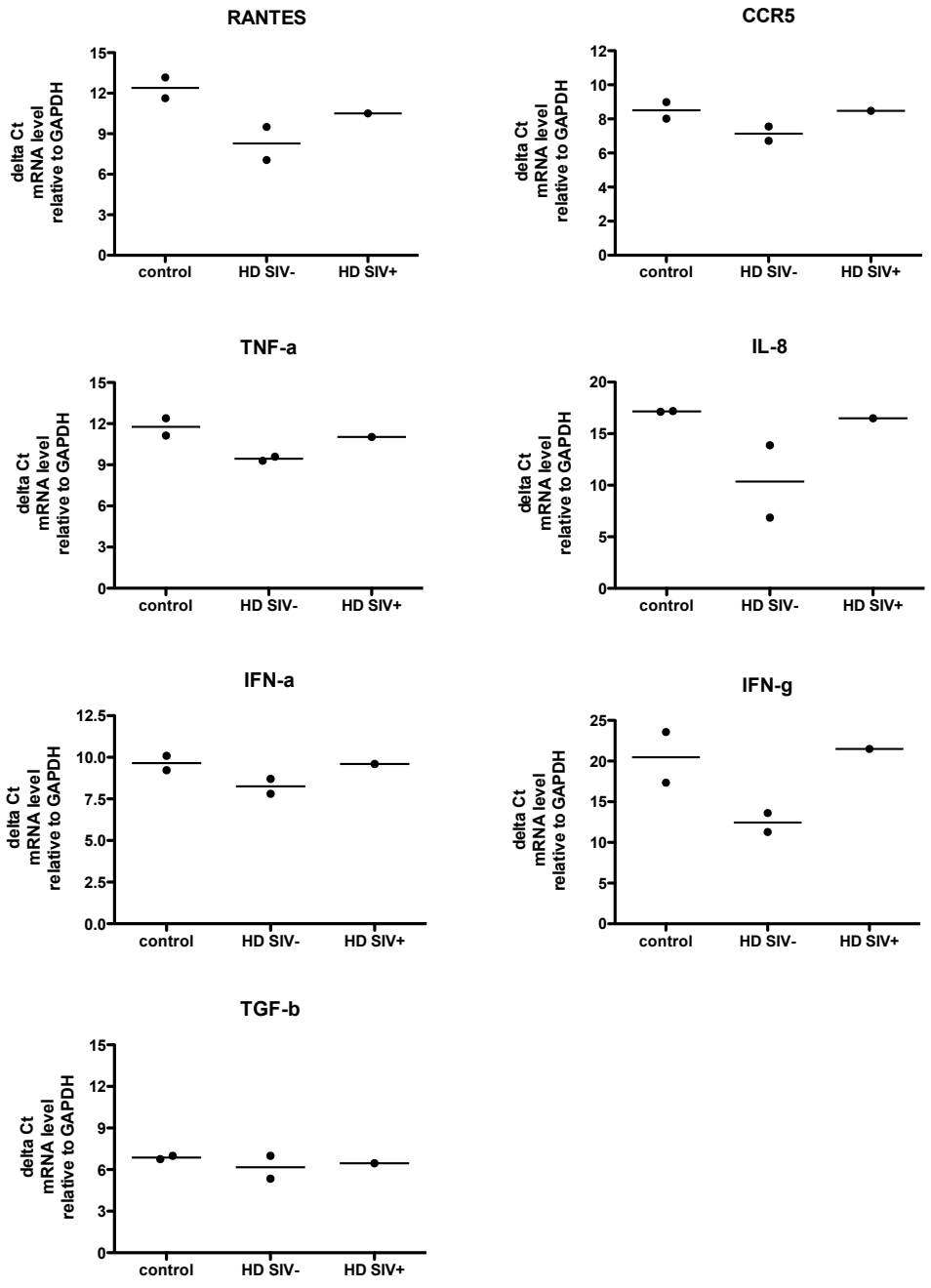


Figure 5-5. Immune gene transcript levels in foreskin biopsy.
Shown are seven (A: RANTES, B: CCR5, C: TNF-α, D: IL-8, E: IFN-α, F: IFN-γ, G: TGF-β) immune genes mRNA level in foreskin biopsy taken at day 18 after *Haemophilus ducreyi* infection (2 days after second SIV exposure). mRNA expression was indicated as delta Ct value relative to GAPDH expression. The lower delta Ct value indicates the higher the mRNA level.

Histological analysis with hematoxylin and eosin staining (H&E staining) of foreskin biopsy taken at day 18 post *Haemophilus ducreyi* (2 days after 2nd SIV administration) demonstrated that macaque A09010, A09017 as well as A09013 had normal foreskin tissue histology (Figure 5-6). On the other hand, macaque A09011 exhibited mild epithelial hyperplasia, moderate inflammation with scattered eosinophils and neutrophils spreading into the epithelium at foreskin biopsy. Similarly, foreskin biopsy from macaque A09015 showed mild epithelial hyperplasia, mild increased vascularity, mild to moderate inflammation with significant eosinophils and scattered neutrophil infiltration.

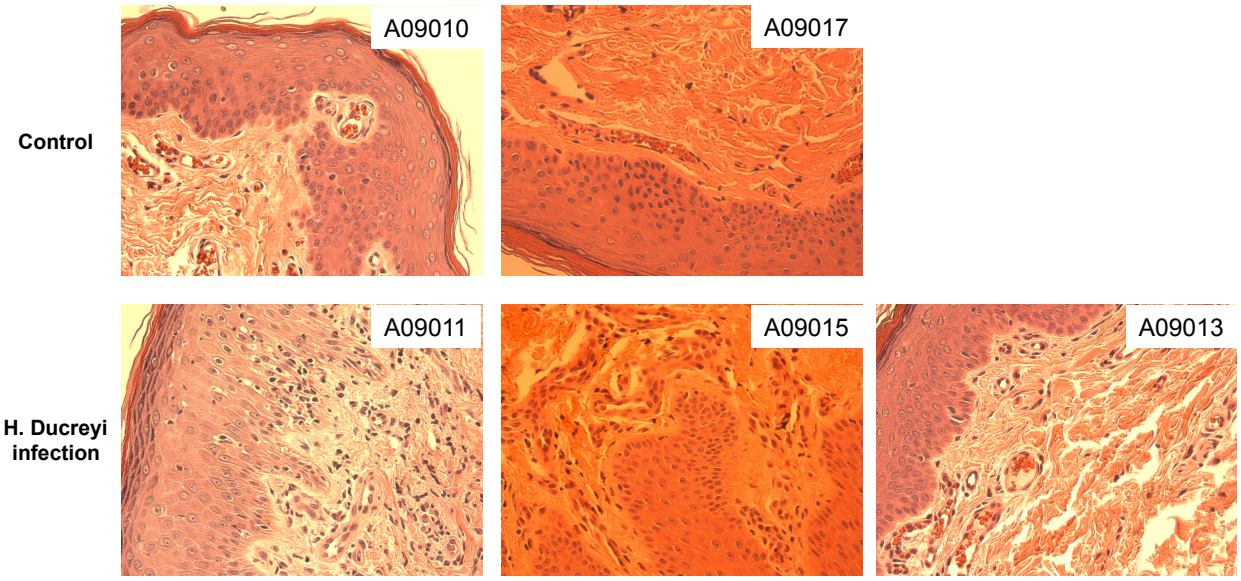


Figure 5-6. Histological examination of foreskin biopsy at day 18 after *Haemophilus ducreyi* infection.
Shown are H & E staining of foreskin biopsy taken at day 18 after *Haemophilus ducreyi* infection (2 days after 2nd SIV penile exposure) in rhesus macaques.

The H&E staining result for macaque A09013 is consistent with immune gene expression levels at the foreskin biopsy. Despite *Haemophilus ducreyi* injection, this macaque had similar immune gene expression levels at the foreskin biopsy as control

macaques (Figure 5-5) and normal foreskin tissue histology (Figure 5-6). On the other hand, *Haemophilus ducreyi* infected macaques A09011 and A09015 with increased immune gene expression at the foreskin biopsies (Figure 5-5) exhibited severe cell infiltration and clinical signs of inflammation (Figure 5-6). These results also fit the observation that macaque A09013 never showed classic lesions of *Haemophilus ducreyi* infection while macaque A09011 and A09015 developed genital ulcers (Figure 5-2) around day 18 post *Haemophilus ducreyi* infection.

From our 3rd set study, we were unable to identify immune factors associated with successful SIV infection through penile route in macaque A09013 but we noticed that macaque A09013 was accidentally injected subcutaneously for *Haemophilus ducreyi* infection. Therefore, we further hypothesized that subcutaneous mucosal inflammation can enhance penile SIV infection. In our 4th set study, 3 macaques were put in control group (no *Haemophilus ducreyi* infection), 6 *Haemophilus ducreyi* naive macaques were put in HD group (with *Haemophilus ducreyi* infection; 3 with intradermal injection and 3 with subcutaneous injection). We also switched our virus inoculum from low pathogenic and low titer (8000$TCID_{50}$) of SIVsmE660 to high pathogenic and more concentrated (10^5 $TCID_{50}$) SIVmac251 to increase the possibility of SIV infection. A similar timeline from 3rd set study was utilized for our 4th set study. Due to the observation that ceftriaxone treatment may increase systemic immune activation, in our 4th set study, all 9 macaques were treated with ceftriaxone with or without *Haemophilus ducreyi* infection. However, none of the macaques in our 4th set study were SIV infected, despite 2 subcutaneously *Haemophilus ducreyi* infected macaques showing moderate swelling (without external lesions) and 1

intradermal *Haemophilus ducreyi* infected macaque developing ulcerations with purulent discharge (data not shown).

Combining our 3rd and 4th set study, we had 1 macaque successfully infected with SIV through penile exposure. However, we were unable to identify immune correlates associated with successful SIV infection. Interestingly, we did not observe significant enhancement of SIV infection through penile route due to pre-existing *Haemophilus ducreyi* infection. In the previous study from Totton et al., genital lesions caused by *Haemophilus ducreyi* generally started with vesicles or pustules within 2 days after *Haemophilus ducreyi* infection and progressed to ulcers around 7-13 days post *Haemophilus ducreyi* infection and the lesions can persist for another 11-15 days. Other clinical symptoms include edema of foreskin tissues or hemorrhagic lesions that can also be exhibited in nonhuman primates (367). The lesion progressions in our study were similar with the previous report. We observed swelling around day 2 after *Haemophilus ducreyi* infection when the first dose of SIV was administered and lesions progressed to ulcers in some macaques around day 16 after *Haemophilus ducreyi* infection when the second dose of SIV was administered (Figure 5-2). Assessing immune gene expression at the foreskin also found higher mRNA levels of immune genes (Figure 5-5), indicating the presence of mucosal inflammation in these macaques, which was consistent with clinical symptoms.

Several mechanisms were proposed to explain how chancroid affects HIV susceptibility, including disruption of mucosal integrity that provides a portal of entry for HIV, increased HIV susceptible cells in the genital tract (167, 353), enhanced CCR5 expression on macrophages which increase HIV invasion (168) and

Haemophilus ducreyi antigen specific T cells promoting HIV replication in vitro (377). In our study, we observed foreskin inflammation accompanied with neutrophil and eosinophil infiltration by H&E staining at day 18 post *Haemophilus ducreyi* infection (2 days following 2nd SIV administration). It is possible that the early phase of chancroid induced inflammation was innate immune cell driven and CD4 cells migrating into inflamed tissues occurred at later time points which exceeded our experimental schedule and therefore we did not observe increased SIV infection despite clinical signs of genital inflammation and ulcers.

Analysis of immune gene expression by real-time PCR at foreskin biopsy in our study showed increased RANTES and CCR5 transcripts in 2 macaques with obvious signs of chancroid induced genital inflammation, which has also been described in the study with human cutaneous experimental *Haemophilus ducreyi* infection (168). It is possible that increased RANTES recruits CCR5 positive immune cells in foreskin tissues. However, high levels of RANTES in foreskin tissues can also compete with virus for co-receptor binding and therefore provide protection against SIV infection in these two macaques. Determining the protein level of RANTES and the relative ratio of RANTES/CD4 positive cells (SIV target cells) in foreskin biopsies may provide insights if high levels of RANTES could be the reasons for no significant increased SIV infection in macaques despite of severe genital inflammation. Furthermore, it may be important to understand if there are preferential CCR5 positive CD4 cells recruiting to foreskin tissue by RANTES and identify which cell population plays more important role in SIV (and potentially HIV) acquisition through penile exposure.

From our analysis of systemic and mucosal immune factors, we were unable to identify the immune factors associated with SIV infection in macaque A09013 who received subcutaneous injection of *Haemophilus ducreyi* without clinical signs of genital inflammation. It is possible that needle injection of *Haemophilus ducreyi* resulted in mucosal integrity disruption and was not fully recovered when the SIV was given, which would highlight the important role of the mucosal barrier of penile tissues in preventing SIV infection through the male genital tract. Overall, we were not able to establish successful SIV infection through penile exposure in rhesus macaques. However, recent studies have published successful infection of SIVsmE660 or SIVmac251 through penile exposure (235, 306, 406). The differences between virus administration methods may be critical. In studies from Yeh et al., and Ma et al., the glans and inner foreskin were emphasized to make contact with SIV and resulted in successful SIV infection while our method pulled up penile tissues to hold SIV, which was not focused and resulted in less virus exposure to these two sites of the male genital tract. Thus, the glans and inner foreskin may be the primary site of virus entry in male genital tract.

Early events associated with penile SIV infection, the influence of penile/foreskin mucosal immunity on SIV/HIV transmission and the biological explanation of reduced HIV infection in circumcised men are not well understand because of the lack of a proper model in non human primates. With the establishment of SIV infection through penile exposure in rhesus macaques, additional questions can be answered and these studies will contribute to HIV vaccine prevention product

development for men as well as provide a valuable model to evaluate preclinical vaccine efficacy or intervention strategies.

Chapter 6: Final discussion and potential future directions

HIV infection is an important public health issue with more than 33 million people living with HIV. In addition to being a life-threatening disease, HIV infection has significant social and economic impacts, especially in developing countries. After 30 years of HIV research several preventive strategies are employed to prevent HIV acquisition, such as using ARV as prophylaxis, circumcision, screening blood donations and encouraging condom usage. However, an effective vaccine against HIV infection is still urgently needed to reduce the HIV epidemic. The difficulties in obtaining mucosal samples from humans and to examine, in detail, early events immediately after HIV exposure to a mucosal membrane have impeded the discoveries of the immune correlates to HIV infection or protection. Therefore, SIV infection of rhesus macaques has become an important model to study these important questions regarding the early events following SIV infection and the discovery of potential protective immune correlates that can be incorporated into future vaccine design. The studies described here evaluated the impact of mucosal inflammation (in the form of induced gingival inflammation and *Haemophilus ducreyi* induced genital inflammation/ ulcers) on virus acquisition using SIV infection of rhesus macaques and advance our knowledge about the factors that affect SIV/HIV mucosal transmission.

Our results showed that overall SIV acquisition rates (through oral or penile challenge) were not significantly affected with the presence of gingival or penile inflammation. However, gingival inflammation was associated with multiple founder viruses infection, indicating that mucosal inflammation can alter host susceptibility to

SIV, and by analogy HIV. In addition, we describe that gingival inflammation during acute SIV infection can alter early immunological events following SIV infection. These results demonstrate that mucosal inflammation can modulate HIV/SIV infection.

Mucosal inflammation can be a double edge sword for HIV/SIV acquisition. Mucosal inflammation associated with increased proinflammatory cytokines and chemokines can potentially result in mucosal membrane disruption (55, 80, 164, 294) and recruit target cells to the mucosal sites (69, 244, 309). In addition, enhancement of immune cell functions due to inflammatory stimulation, such as dendritic cells sampling foreign antigen from the mucosal lumen (109) could also help virus cross the mucosal barrier and gain access to target cells. All these factors create a microenvironment that favors HIV/SIV replication and, therefore, increase the possibility for HIV/SIV to establish systemic infection after mucosal exposures to HIV/SIV. On the other hand, mucosal inflammation could potentially result in increased production of anti-HIV proteins and peptides in the mucosal compartment, such as beta-defensins 2 and 3 (372), secretory leukocyte protease inhibitor (SLPI) (254), lactoferrin (hLf) (187), elafin/trappin-2 (173), or beta-chemokines and anti-viral cytokines, which could partially inhibit HIV/SIV replication. Mucosal sites are dynamic and the influence of mucosal inflammation on HIV/SIV acquisition may depend on a fine balance between these two scenarios.

Based on the results from previous studies showing the association of genital inflammation and increased HIV infection (179, 180, 261, 313, 314), we hypothesized that pre-existing mucosal inflammation would increase SIV infection in rhesus macaques following oral and penile mucosal exposures. However, in our studies,

induced gingival inflammation or *Haemophilus ducreyi* induced penile inflammation, did not significantly enhance SIV infection following oral or penile SIV exposures, respectively. Our results suggest that not all inflammation at mucosal sites will result in increased HIV/SIV acquisition. Since mucosal inflammation is a double edge sword for HIV/SIV acquisition, some threshold of anti-HIV activity might need to be overcome to bias the mucosal environment favoring virus replication. It is possible that one factor alone, such as increased CD4 positive cells at the mucosal site, may not be sufficient to enhance HIV/SIV infection. Instead, multiple factors in combination could change host susceptibility to HIV/SIV, i.e. disruption of mucosal membrane integrity combined with accumulation of activated CD4 positive cells at the mucosal site. Indeed, studies from Chenine et al and Weiler et al., both showed that increased virus infection rate in rhesus macaques is associated with severe mucosal membrane disruption as well as significant CD4 T cell infiltration at the site of virus exposure. Severe mucosal inflammation is more likely to result in changes of membrane integrity and target cells toward a mucosal environment that favors virus replication. A recent study from Mlisana et al., demonstrated that women with STI-related genital inflammation were at higher risk of HIV acquisition than women without STIs and the numbers of STIs were predictive of HIV infection (261), indicating that the severity of mucosal inflammation is more likely to alter host susceptibility to HIV/SIV, potentially through increased target cell availability, reduced protective effect of mucosal barriers or the combination of both.

For potential future studies, it is possible to use SIV infection of rhesus macaques model to conclusively determine the role of mucosal membrane integrity,

and target cell availability on oral SIV acquisition and test the hypothesis that the combination of mucosal membrane disruption and increased target cell availability at the same time would increase oral SIV acquisition. First, to examine if physical disruption of mucosal membrane alone can enhance SIV infection in rhesus macaques, a method to introduce disruption of mucosal barrier alone could be employed to a group of macaques, i.e. using a cytobrush to scrape off some layers of epithelium of the oral mucosa (Figure 6-1, group A). Mucosal membrane biopsies would be sampled to confirm and examine the levels of mucosal membrane disruption prior to SIV exposure. SIV could be given through the dripping method to the site where the cytobrush was applied and examine if more macaques became SIV infected in the cytobrush versus the control group. The results would indicate that physical disruption of the mucosal membrane plays an important role in virus acquisition through oral exposure. Second, to test if increase in target cell availability alone can enhance SIV infection in neonate macaques, a method to induce increasing CD4 T cell numbers and their activation status would be used, i.e. treat with lipopolysaccharide (LPS) or Monophosphoryl Lipid A (MPL, a LPS derivative), to stimulate TLR4 signaling and initiate immune cell recruitment (Figure 6-1, group B) (391). LPS is a common antigen present on outer membrane of gram-negative bacteria and LPS from oral cavity, such as P. gingivalis or E. coli, can signal through TLR4 to drive T cell expansion and differentiation (75, 89, 197, 250, 407). A plastic ring could be placed on the oral mucosal site and LPS/MPL could be applied inside the ring (69) to address the mucosal area affected by the LPS/MPL treatment. Examination of changes in target cell numbers and activation status in the oral

mucosal biopsies following LPS/MPL treatment would be important to gather information about the dynamics of immune cell infiltration, how long will they sustained, and their activation status. These preliminary analyses would also be used to adjust the concentration of LPS/MPL for oral mucosal treatment of the macaques (Figure 6-1 group B top). After detailed analysis of LPS/MPL-induced target cell changes at the oral mucosa, SIV could be administered via the similar method as LPS/MPL administration to restrict the virus exposure to the LPS/MPL treated site. Compared to a control group, if more macaques with LPS/MPL treatment became SIV infected, the result would indicate increased target cells, in quantity and quality, at the mucosal membrane plays a big role in virus acquisition. It is also possible that the combinations of the two factors are required to enhance SIV infection; i.e. mucosal integrity disruption and target cell recruitment. To test this, a group of macaques would be treated with LPS/MPL and the cytobrush and a group of control macaques would receive no treatment prior to challenge with SIV (Figure 6-1 group C). In all groups, saliva and blood samples would also be collected weekly and examined for anti-HIV immune factors (i.e. SLPIs or cytokines/chemokines) and systemic effects of immune changes over the different treatment groups to examine the influence of anti-HIV/SIV immune factors on the rate of oral SIV infection. If the combination of membrane disruption and increased target cells at the oral mucosa further enhances SIV infection in macaques, this would point out the importance of both mucosal membrane and target cell availability on oral SIV acquisition. The potential studies proposed here would provide further details of factors influencing oral SIV/HIV infection and inform design of effective vaccines or microbicides, whether to target

minimizing the number of activated CD4 positive cells at the mucosal sites, and/or preventing mucosal membrane integrity disruption, and/or enhancing the expression of anti-HIV immune factors to reduce successful HIV infection in populations.

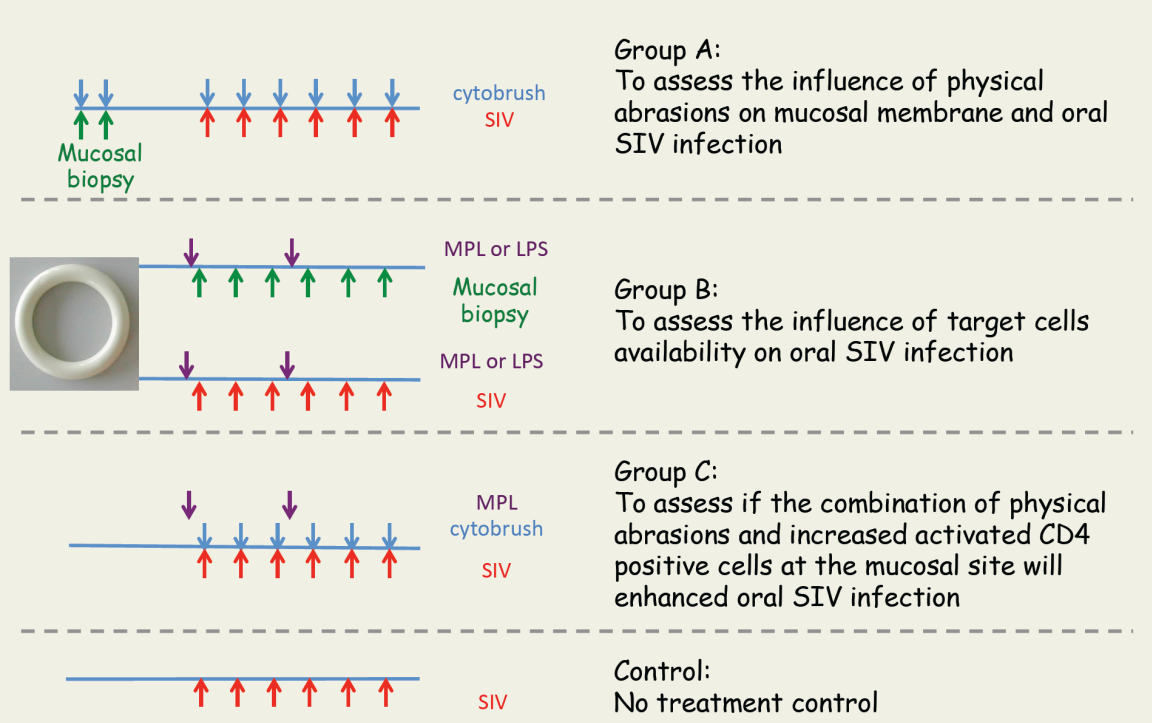


Figure 6-1. Schematic representation for potential future studies to test the factors affecting oral SIV infection in neonate macaques.
Using SIV infection of neonate macaques to examine the role of mucosal membrane integrity and target cell availability in oral acquisition. Group A was designed to test if physical abrasions, induced by solid food diets, enhance oral SIV infection. Group B was designed to test if increased activated CD4 T cells enhance oral SIV infection. Group C was designed to test if the combination of physical abrasion and increased activated CD4 T cells at the mucosal site enhances SIV infection

SIV infection through penile tissue was inefficient in our study, only 1 macaque was SIV infected, despite the presence of penile inflammation or high dose challenge; indicating the threshold set up for SIV penile infection to overcome is relatively high. Another potential study direction for penile SIV infection is to induce epithelial disruption and examine the role of penile epithelium on SIV acquisition in rhesus macaques. Nonoxynol-9 (N-9) could be a good candidate to induce mucosal membrane damage through the penile route. N-9 has been widely used as an active component of spermicides in condoms. However, frequent N-9 usage has adverse effects on the vaginal mucosa and increases the risk of HIV infection in women due to the damage of vaginal surfaces (376). *In vitro* and *in vivo* studies have clearly demonstrated the damage to epithelial surfaces and the subsequent inflammatory response following N-9 treatment (83, 118, 203, 288, 319). It is possible to apply lubricant containing N-9 daily to penile tissues of rhesus macaque, examine the changes of penile/foreskin membrane integrity and challenge with SIV to examine if penile tissue integrity dirsuption would increase SIV infection through penile exposure.

The influence of mucosal inflammation may not be restricted to HIV acquisition but also the immune response following HIV infection. So far, only a few studies have addressed the influence of pre-existing mucosal inflammation on HIV infection pathogenesis and the literature have shown that mucosal inflammation during acute HIV/SIV infection can have long-term effects on HIV/SIV pathogenesis. A study from Sheng et al., showed that genital inflammation (caused by N. gonorrhea) around the time of HIV infection was associated with higher IFN-γ and MIP-1α secretion by HIV-specific CD8 T cells in peripheral blood (347). Bebell et al., and Roberts et al., further

demonstrated that genital inflammation during acute HIV infection (determined by elevation of inflammatory cytokines in cervicovaginal lavage) had a significant inverse correlation with the number of CD4 T cell counts in the periphery and positive correlation with plasma viral load during chronic HIV infection (34, 317). By using SIV infection of rhesus macaques, induction of vaginal inflammation by TLR agonists results in higher virus replication after vaginal SIV challenge (391). These results suggest that mucosal inflammation may play a role in modulating the disease course of HIV/SIV infection.

Results from the study presented here provide better understanding of the influence of oral inflammation on systemic immunological changes following oral SIV infection. In our study (chapter 4), we observe higher IFN-α and IFN-γ production in plasma during acute SIV infection from macaques with induced gingival inflammation, compared to control macaques. This result provides supporting evidence that mucosal inflammation around the time of virus infection can modulate HIV/SIV infection. Interestingly, results from our study and previous studies are not identical with others. The study from Bebell et al., Roberts et al., and Wang et al., correlate the genital inflammation directly with disease progression surrogates (CD4 T cell counts and plasma viral load) while the study from Sheung et al., correlates the genital inflammation with HIV specific adaptive immunity during chronic infection but not disease progression. Our study showed transiently higher interferon production during acute infection but no significant impact on chronic immune activation or disease progression indicators. These differences may be due to the mucosal sites of virus exposure (the route of HIV/SIV infection) as well as the differential levels of mucosal

inflammation between these studies. For example, TLR agonist-induced vaginal inflammation and N. gonorrhea induced genital inflammation may be more severe than induced gingival inflammation. In addition, different types of mucosal inflammation may result in differential TLRs expression on immune cells, which can affect downstream signaling pathways to initiate immune responses against incoming HIV/SIV. Indeed, studies have shown that different TLR ligand activation can have differential effects on immune cell activation, i.e. survival or death (7, 61, 210), and immune gene expression, i.e. cytokine or chemokine production (86, 130, 232). Therefore, not all types of mucosal inflammation would have similar impacts on HIV disease progression and some types of mucosal inflammation may actually help HIV specific adaptive immune response development.

Recently in the field, concerns have been raised about the possibility for future HIV vaccines to induce immune changes that could potentially increase the risk of HIV infection. The STEP trial (56, 94) and a similar study using SIV infection of rhesus macaques model recapitulating the STEP trial (99) both demonstrated that the Adenovirus-5 vector based HIV/SIV could enhance HIV-acquisition. Similarly, Staprans et al. demonstrated enhanced SIV replication and rapid disease progression in macaques vaccinated with an attenuated recombinant varicella-zoster virus expressing SIV envelope vaccine (356). Although a follow-up study found that the effects of vaccine-induced increased risk for HIV infection wane over time (94), these studies indicate that vaccine induced immune changes (adjuvants, HIV/SIV specific antigens, vaccine vectors) have the potential to modulate host susceptibility to HIV/SIV at certain time points following vaccination. The inflammatory properties of

induced-gingivitis in our study are interesting because it modulated the immune changes to incoming SIV without increased SIV oral acquisition. If we were able to identify the key differences between our induced gingival inflammation versus the inflammation that does increase HIV acquisition, this may also inform HIV vaccine design to avoid the increased risk of HIV infection. It is possible to explore the potential for using oral bacteria, i.e. Porphyromonas gingivalis, as vaccine vector to orally deliver HIV antigen to induce gingivitis-like immune activation to facilitate host response to HIV antigen as part of vaccine design.

In summary, our studies presented here assessed the influence of mucosal inflammation on HIV/SIV acquisition and immune changes following SIV infection at mucosal and systemic tissues sites in detail. The results indicate that mucosal inflammation can alter hosts' susceptibility through mucosal exposure and modulate immune changes at mucosal as well as peripheral tissues following SIV infection. With the potential future studies and more understanding of the factors related to HIV/SIV infection, the results will provide insights for future vaccine and microbicide development to effectively reduce HIV acquisition in HIV uninfected individuals.

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