INNATE IMMUNE RESPONSES AND VIRAL CHANGES AFTER ORAL TRANSMISSION OF SIV IN RHESUS MACAQUES

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DEDICATION

This dissertation is dedicated to my parents, Ingrid and Erkan.

First and foremost I would like to thank my mentor Dr. Don Sodora. Don gave me the possibility to work in his laboratory and provided me with several exciting projects. He guided and mentored me throughout my studies and was always helpful with suggestions. His advice was valuable for my life in and outside the lab.

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by

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Recent HIV vaccine trial failures indicated the need to increase our knowledge with regard to mucosal immune responses after exposure to HIV/SIV. The studies presented here utilized oral transmission of simian immunodeficiency virus (SIV) in Rhesus macaques with the goals of (1) determining associations between innate immune responses at different tissue sites and disease progression and (2) assessing the differences in immune and viral changes that occur after oral administrations with low or high SIV doses.

During previous work in the Sodora laboratory, mRNA levels of innate immune responses were assessed at mucosal sites of orally SIV-infected macaques. The studies presented here build upon this work by assessing innate immune modulator transcripts within lymph nodes and peripheral blood. Dividing the SIV+ macaques with regard to their rate of disease progression, rapidly progressing macaques exhibited elevated expression of IFN-α, OAS, CXCL9 and CXCL10 mRNA at lymph nodes. In peripheral blood, only expression of OAS and CXCL10 mRNA was associated with faster disease progression. Assessment of macaques orally infected by high or low doses of SIV revealed that high doses, as expected, resulted in transmission of more virions, and viral replication exhibited slightly faster kinetics early after transmission. Furthermore, high dose macaques exhibited higher levels of the anti-viral factors IFN-α and OAS in tissues. However, other innate and adaptive immune responses were comparable between macaques infected by high or low doses. Also, mRNA expression of immune modulators in peripheral blood was similar between the two groups, with only expression of OAS and CXCL10 transcripts being upregulated. These findings indicate that expression of these two immune modulators is preferentially upregulated during faster disease progression and is independent of viral dose. This suggests that

OAS and CXCL10 transcript levels could potentially be used as diagnostic markers of AIDS progression.

Taken together, these studies indicate that multiple tissue compartments need to be assessed to obtain a complete understanding of immune and viral factors in SIV/HIV disease. A more complete knowledge of the mucosal and systemic immune responses prior to, during and following HIV transmission is likely to lead to new approaches for the development of novel vaccines and therapies to combat HIV infection/disease.

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LIST OF ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
APC	Antigen-Presenting Cell
CCR5	C-C Chemokine Receptor 5
CDC	Centers for Disease Control
cDNA	Complementary DNA
CNPRC	California National Primate Research Center
CTL	Cytotoxic T Lymphocyte
CXCL10	C-X-C Ligand 10
CXCL9	C-X-C Ligand 9
CXCR4	C-X-C Chemokine Receptor 4
DC	Dendritic Cell
DPI	Days Post-Infection
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HAART	Highly active anti-retroviral therapy
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
IFN-α	Interferon alpha
IFN-β	Interferon beta
IFN-γ	Interferon gamma
IL-10	Interleukin-10

IL-12	Interleukin-12
IP-10	Interferon gamma inducible protein 10
IV	Intravenous
LN	Lymph Node
LPS	Lipopolysaccharide
MHC	Major Histocompatibility Complex
Mig	Monokine induced by interferon gamma
NK cell	Natural Killer cell
OAS	2’ – 5’ Oligoadenylate Synthase
OM	Oral Mucosa
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
RB	Rectal Biopsies
RM	Rhesus Macaques
SIV	Simian Immunodeficiency Virus
SM	Sooty Mangabeys
SNPRC	Southwest National Primate Research Center
TNF-α	Tumor Necrosis Factor alpha
YNPRC	Yerkes National Primate Research Center

CHAPTER ONE

General Introduction and Literature Review

HIV introduction

Pneumocystis jirovecii usually does not cause disease in otherwise healthy individuals; however, on June 5th, 1981, the Centers for Disease Control (CDC) published their Morbidity and Mortality Weekly Report (MMWR) describing five homosexual men with *Pneumocystis* pneumonia [43]. A month later, the CDC published another report, describing the equally unusual occurrence of Kaposi's sarcoma in homosexual men [42]. These were the first two reports of what became known a year later as acquired immunodeficiency syndrome (AIDS). It took until 1983 for the causative agent of AIDS to be identified: two independent laboratories reported in the journal *Science* that they isolated a retrovirus from an AIDS patient, namely the groups of Dr. Robert Gallo from the National Institutes of Health in the United States [101] and Dr. Luc Montagnier at the Pasteur Institute in France [25]. Gallo's group later confirmed that the virus they isolated was indeed the cause of AIDS [245] and in 1986 this virus received its name by which it has been known until today, human immunodeficiency virus (HIV) [54]. In Senegal, another human immunodeficiency virus was found soon after the discovery of HIV-1 and

was named HIV-2 [50, 51]. Both viruses share some of their sequence but can differ by more than 55 % on the nucleotide level [116, 181]. While HIV-1 led to a global pandemic over the last 30 years, HIV-2 remained mostly restricted to West Africa. Additionally, the disease course after infection with HIV-2 is much slower and less severe than infection with HIV-1 [140, 181, 196, 197, 319].

Both, HIV-1 and HIV-2 are now recognized at the causative agents of AIDS. However, only HIV-1 has led to a global pandemic. To date, around 30 million people have died from AIDS or AIDS-related syndromes and an estimated 33 million people worldwide are infected with HIV-1 (Figure 1-1). In 2007 alone, there were 2.7 million new infections and 2 million AIDS-related deaths. Sub-Saharan Africa is the hardest hit region by HIV-1 with 22 million people, or two-thirds of all HIV-infected individuals, living there. 1.9 million new infections and 1.5 million deaths occurred in this region of the world. Children carry a heavy burden in this pandemic as well: 2 million are infected worldwide, 1.8 million of them in Sub-Saharan Africa. 370,000 became newly infected in 2007 and 270,000 died. Additionally, almost 12 million children have lost one or both parents to HIV/AIDS in Sub-Saharan Africa alone (all statistics from [324]).

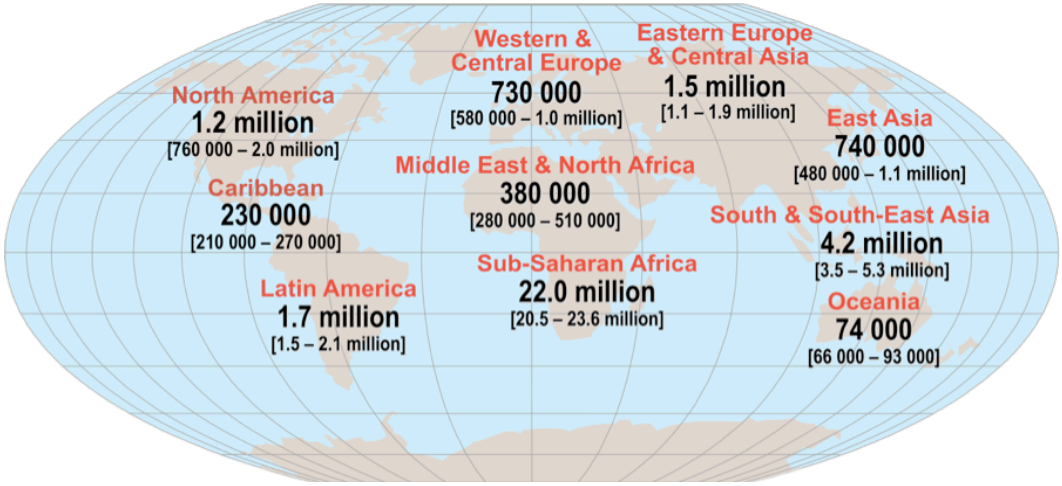

Figure 1-1. Adults and children estimated to be living with HIV, 2007
Worldwide, an estimated 33 million [30 – 36 million] people are infected with HIV. The numbers in brackets represent the ranges of the actual numbers based upon the best available information. Information obtained from the Global Summary of the AIDS epidemic, December 2007, UNAIDS.

HIV biology

HIV-1 and HIV-2 are members of the family of Retroviridae and are in the genus of Lentiviruses. The viral genome of HIV consists of two copies of a single-stranded RNA, which is roughly 10 kb long and codes for 9 proteins [99, 193]: Gag, Pol, Env, Tat, Rev, Vif, Vpr, Vpu and Nef (Figure 1-2, Table 1-1). Gag includes the matrix proteins that form the core, which encapsulates the viral RNA and the viral enzymes polymerase, protease and integrase (encoded by Pol) in addition to the accessory proteins. A

lipid bilayer derived from the host cells during the budding process envelopes the core. The bilayer is studded with host membrane proteins as well as with viral Env gp160 trimers, consisting of gp120 and gp41 [312]. The complete virions are roughly 100 nm in diameter.

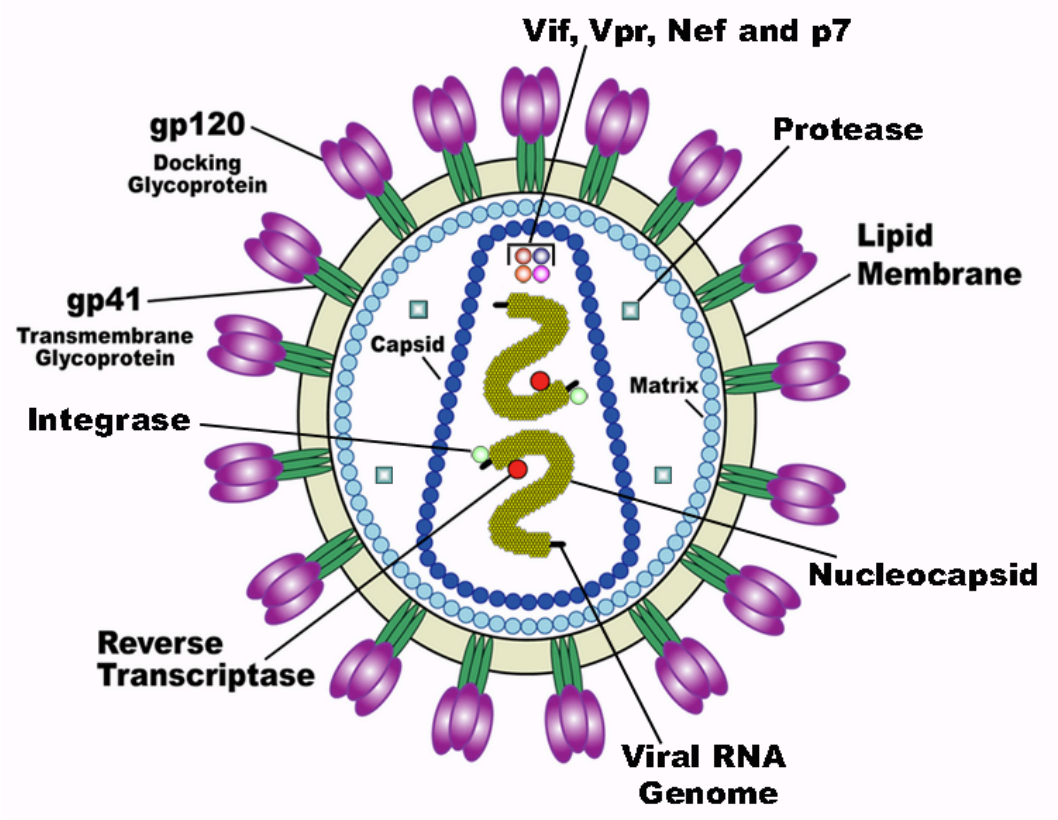

Figure 1-2. Structure of an HIV virion.

To successfully infect a cell, HIV gp120 binds to the CD4 molecule on the surface of appropriate target cells like CD4+ T cells and cells of macrophage lineage. The gp120 molecule undergoes a conformational change so that it then can interact with co-receptors. Several different surface molecules have been identified to serve as co-receptors for HIV, most prominently CCR5 and CXCR4 (reviewed in [15, 288]). CCR5 is typically expressed on effector T cells located at mucosal sites and is usually associated with transmission. In contrast, CXCR4 is usually expressed by naïve and central memory T cells in the circulation or at lymphoid sites and typically becomes a receptor for HIV only later in infection. In fact, HIV develops a tropism for CXCR4 in roughly 50 % of infected individuals and this is usually associated with faster disease progression. After binding CD4 and a co-receptor, another conformational change allows the gp41 molecule to initiate fusion between the lipid bilayer of HIV and the cell membrane. After membrane fusion, the viral core translocates into the cell cytosol where the RNA and additional viral enzymes become uncoated from the surrounding capsid proteins. The reverse transcriptase copies the viral RNA into complementary DNA. This is the process during which mutations in the viral genome occur at an extremely high rate since the viral reverse transcriptase is extremely error-prone and does not possess proofreading abilities. It has been calculated

that during this process every possible mutation at every single base pair occurs once every day [22]. This extreme rate of mutations leads to the development of drug-resistance and immune escape of HIV. Reverse transcriptase also degrades the viral RNA and synthesizes the second DNA strand, leading to double-stranded DNA. The DNA gets transported into the nucleus and the viral enzyme integrase facilitates the integration of the viral DNA into the genomic DNA, called provirus. Certain host transcription factors, like NF-κB, need to be present for the provirus to be transcribed. Transcription factors are upregulated during cell activation, which is why HIV replicates best in activated cells. The proviral DNA is transcribed into mRNA, which is then spliced before exiting the nucleus. In the cytoplasm it is translated into the viral proteins, which are packaged together with viral RNA into new viral particles. These particles bud from the host cell and are ready to infect new cells (Figure 1-3, reviewed in [89, 99, 289]).

Table 1-1. HIV proteins and function.

Gene		Gene Product/Function
Gag	Group-specific antigen	Core and matrix proteins
Pol	Polymerase	Reverse transcriptase, protease, and integrase enzymes
Env	Envelope	Transmembrane glycoprotein. gp120 binds CD4 receptor and CCR5 or CXCR4 coreceptor; gp41 required for viral fusion
Tat	Transactivator	Positive regulator of transcription
Rev	Regulator of viral expression	Important for export of unspliced and singly-spliced viral mRNAs out of the nucleus
Vif	Viral infectivity	Increases viral infectivity by inhibiting cellular APOBEC3G
Vpr	Viral protein R	Transports viral DNA intermediate to nucleus; G_2 cell cycle arrest
Vpu	Viral protein U	Promotes intracellular CD4 degradation and enhances release of virions from membrane
Nef	Negative-regulation factor	Pleiotropic. Enhances virion infectivity. Down-modulates CD4 and MHC class I from cell surface. Affects T cell activation.

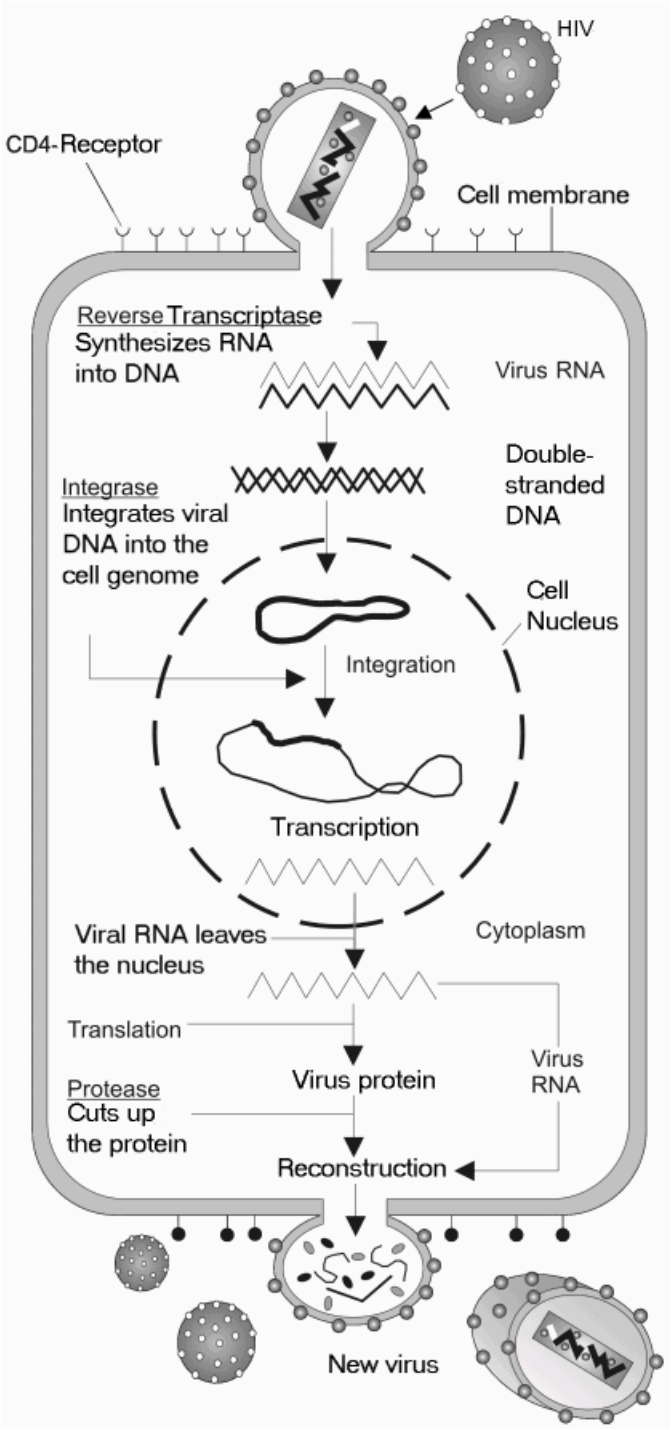

Figure 1-3. HIV replication cycle.

Origin of HIV

Over thirty non-human primate species in Sub-Saharan Africa are naturally infected with simian immunodeficiency virus (SIV) [119, 129, 167] and it is now accepted that SIV represents the origin of HIV-1 and HIV-2 [272]. HIV-2 is genetically most closely related to SIVsm [49, 129], the retroviral species infecting sooty mangabeys (*Cercocebus atys*) in the wild, and thus it is assumed that HIV-2 originated from SIVsm. Additional evidence for this theory comes from the fact that sooty mangabeys are endemic to West Africa, the same region to which HIV-2 is localized. The hypothesis is that this cross-species transmission from mangabeys to humans occurred through hunting, butchering and eating of mangabey bush meat [119, 272]. HIV-1 on the other hand appears to have originated from SIVcpz, the retroviral species infecting chimpanzees (*Pan troglodytes*) in the wild, since sequencing of the HIV-1 and the SIVcpz genome revealed genetic homology between the two [102, 119]. Like HIV-2, it is believed that the cross-species transmission for HIV-1 occurred after butchering and eating of chimpanzee bush meat [119, 123, 272]. Chimpanzees themselves seem to have acquired SIVcpz through a cross-species transmission event after hunting and eating naturally SIV-infected red-capped mangabeys and greater spot-nosed monkeys [119]. It appears that at least three different and distinct cross-species transmissions from

chimpanzees to humans took place, giving rise to the three different HIV-1 groups M, N and O [102, 119, 231]. Group M (main) comprises the largest group of HIV-1 and accounts for the majority of infections worldwide; group O (outlier) contains strains that are highly divergent and are localized to equatorial Africa [63]; and group N (non-M, non O) contains only a small number of strains localized to Cameroon [278].

Natural hosts of SIV infection

As mentioned above, several monkey species in Africa are naturally infected with strains of SIV. These strains vary slightly between the different species and are genetically distinct from HIV-1 and HIV-2. Interestingly, these natural hosts exhibit high viral loads and replication of SIV in blood and tissues, at levels similar to HIV-infected humans [70, 110, 226, 227, 275]. However, most of the African non-human primate species do not get sick and develop signs of simian AIDS [228, 252]. Only a few exceptions have been observed so far in which natural hosts actually exhibited signs of disease [11, 186, 225]. An interesting recent study found that SIV-infected chimpanzees had a 10 to 16-fold higher death rate than their uninfected counterparts [155]. However, this study was only based on 17 infected animals of which 7 died, and more research needs to be done to confirm the pathogenic nature of SIVcpz in

its host. It is interesting to speculate though, that SIV-infection of chimpanzees might represent a “missing link” between the non-pathogenic infections of other SIV-infected natural hosts and the pathogenic infections observed with HIV in humans.

Whatever the reasons for pathogenicity in some of the natural hosts, it is important to note that these findings indicate that SIV is not a harmless virus, but rather that natural hosts co-evolved with the virus and developed a way to control disease in most cases. This is further emphasized by the fact that after cross-species transmission, SIV exhibited a striking increase in pathogenicity in its new hosts in the form of either HIV-1 or HIV-2. The underlying mechanisms of the control of disease and the sustained health in natural hosts of SIV have been subject to intensive research. Although increased or stronger immune responses in natural hosts could have explained the lower pathogenicity of SIV, it was found that neither cellular nor humoral immune responses were enhanced in natural hosts [74, 125, 305, 314]. Furthermore, it is believed that sustained CD4+ T cell levels in the periphery and the gut of natural hosts were a major reason for the non-progression of these animals. Although CD4+ T cell levels are generally preserved in the periphery of natural hosts, a unique cohort of SIV-infected sooty mangabeys was identified that exhibited a profound depletion of blood

CD4+ T cells [207]. The CD4+ T cell numbers have been stable at levels, which would define AIDS in humans, for more than 8 years. Despite this extended time with low levels of CD4+ T cells, these mangabeys have not yet exhibited any signs of simian AIDS [207]. These findings suggest that CD4+ T cells are not the main or at least not the only means by which natural hosts stay healthy; other host defense mechanisms must exist that keep these animals from progressing to simian AIDS. Furthermore, in recent years it has been shown that natural hosts can undergo similar dramatic CD4+ T cell depletions in their intestinal lymphoid tissues during acute infection as is observed in humans [110, 229]. These CD4+ T cell levels are only partially restored during chronic infections. This low number of CD4+ T cells could lead to compromised gastrointestinal mucosal surfaces that are not able to prevent microbial translocation from the intestinal lumen into the systemic circulation. However, it has been shown that despite low levels of CD4+ T cells in the gut of sooty mangabeys, systemic lipopolysaccharides (LPS) levels, as an indicator for microbial translocation, remained low [37], indicating that the mucosal barrier was not compromised in these animals and microbial translocation did not occur. Immune defense mechanisms other than CD4+ T cells must be in place to protect the mucosal surfaces and it has been suggested that Th17 cells might play a role in this protection [35].

Another important factor associated with disease progression that is directly related to the translocation of microbial products is immune activation. It consists of several factors like the expression of activation and proliferation markers on T cells, T cell turnover, levels of activation-induced apoptosis or the levels of proinflammatory cytokines and chemokines in plasma of infected animals or humans. In humans, generalized immune activation during acute and chronic infection is the strongest predictor of disease progression and death in HIV-infected individuals (discussed below) [97, 108, 122, 169, 187, 280, 283, 300]. In contrast, a similar increase in the activation state of the immune system during acute infection in natural hosts of SIV is transient and natural hosts generally resolve this immune activation, leading to low levels during chronic stages of infection [70, 83, 110, 162, 207, 228, 276, 280]. One reason for this limited immune activation could indeed be low levels of LPS (and other microbial products) in the systemic circulation due to decreased microbial translocation [36, 37]. Another reason could be that natural hosts establish a rapid anti-inflammatory and regulatory milieu, which would help in the resolution of the acute immune activation and the maintenance of low levels during chronic infection [83, 162]. Also, natural hosts exhibit low levels of apoptosis during both acute and chronic infection [60, 136, 277]. This is associated with low levels of immune

activation and is another contributing factor to the general maintenance of CD4+ T cell levels. Finally, another marker of immune activation, proinflammatory or anti-viral cytokines in SIV-infected natural hosts tend to be increased during acute infection, but decrease back to baseline and stay low during chronic phases [33, 162, 207, 235]. Taken together, these findings indicate that several mechanisms are synergistically involved to keep natural hosts healthy despite yearlong SIV infection in the presence of high viral loads.

SIV as model for HIV research

To study HIV transmission and pathogenesis, it is necessary to employ animal models for obvious reasons: transmission of HIV entering a new host is impossible to study in humans. Due to the asymptomatic phase right after infection and the flu-like symptoms during acute infection, diagnoses of HIV are harder to obtain early after new infection. This makes it more difficult to study acute infection in humans. Therefore, animal models are needed and natural hosts of SIV infection provide one important model to study HIV. As discussed above, natural hosts of SIV infection exhibit interesting patterns of immune and viral changes throughout their infections, which are for the most part vastly different from what is observed in humans. By studying the differences between humans

and natural hosts and trying to delineate the mechanisms leading to these differences, a better understanding of both diseases can be obtained and findings from these studies might lead to the identification of new approaches for future therapy and vaccine designs for HIV. However, studies in natural hosts have mostly been limited to sooty mangabeys, African green monkeys and mandrills. Other African non-human primates are often not available for research and many of them are also highly endangered, not permitting pathogenesis studies. Also, only a few of the SIV species infecting African monkeys have been sequenced and characterized. In addition, since these species usually do not progress to simian AIDS, it is almost impossible to study disease progression.

However, another non-human primate model to study HIV exists, namely SIV infection of Asian macaques. Asian macaques do not became infected with SIV in the wild; however, accidental cross-species transmission from SIV-infected sooty mangabeys into Rhesus macaques probably in the early 1980s at the California National Primate Research Center led to the emergence of SIVmac [61, 195]. Several other accidental and also experimental cross-species transmissions of different SIV species from natural hosts into non-natural hosts, Asian macaques, resulted in disease progression in these new hosts and led to the establishment of this new animal model of HIV infection [12, 13, 26, 282].

SIV infection of non-natural hosts resembles human HIV infection in many aspects, including the modes of transmission, viral replication, CD4+ T cell depletion in the periphery and the gut, immune activation levels and immune responses towards the virus [69, 127, 128, 178, 182, 200]. Similar to HIV infection in humans, SIV infection of Rhesus macaques leads to different rates of disease progression, from rapid progressors to animals that do not show signs of disease for many years [44, 126, 132, 279, 296]. This animal model is especially important since time, route and dose of infection as well as the number of viral exposures can be manipulated for different studies as needed. In addition, this model can and has been utilized to assess immune and viral parameters early after infection as well as during chronic time points later in infection [20, 21, 105, 144, 205, 263]. Also, several different tissue sites, like lymph nodes, mucosal biopsies, or blood, can be obtained at different times during infection. Since it is difficult to obtain tissues other than blood from humans, SIV infection of Rhesus macaques is an excellent model permitting both virologic and immunologic assessment of critical tissue compartments [1, 5, 106, 208, 331]. SIV infection of both natural hosts like sooty mangabeys and non-natural hosts like Rhesus macaques have been extensively utilized to delineate similarities and differences in immune and viral changes

between the different species as well as to determine the factors leading to disease in humans infected with HIV.

HIV/AIDS clinical symptoms and disease progression

Infection with HIV can be divided into four stages: an incubation period, acute infection, a latency stage and AIDS. The first two to four weeks after transmission in which the virus replicates and establishes infection are usually asymptomatic. This is followed by the acute stage of infection, which usually lasts around 28 days; it is in this stage that the first symptoms usually occur. These can include fever, lymphadenopathy (swollen lymph nodes), pharyngitis (sore throat), rash, myalgia (muscle pain), malaise, and mouth and esophageal sores [57, 149]. The virus replicates to high levels during the acute stage [233] and as a result CD4+ T cell levels drop. At this stage, innate and adaptive immune responses are able to gain control of the viral infection, leading to a decline in viral load and a rebound of CD4+ T cell numbers in the blood; however, CD4 T cell numbers usually do not return to pre-infection levels. This marks the beginning of the clinical latency stage, which can last from weeks to years. During this stage, most patients do not exhibit symptoms of the disease, but are usually still infectious. As the immune system battles the virus, CD4+ T cell numbers decline and the immune system weakens over time,

until a critical threshold is reached. In general, patients begin to develop symptoms of AIDS when their CD4+ T cell levels drop between 200 and 500 cells per micro liter of blood. Clinical AIDS is defined by levels below 200 cells per micro liter of blood.

CD4+ T cell numbers in blood and viral loads have historically been utilized as clinical markers of HIV disease progression, and initiation of therapy usually depends on these levels. However, it has become clear that other factors influence HIV/AIDS disease and should be taken into consideration. It is now known that CD4+ T cells are not only depleted in the blood but almost everywhere in the body. In fact, within a few days after HIV infection, CD4+ T cells in gut-associated lymphoid tissues (GALT) become severely depleted and cell numbers only partially and transiently recover, leaving the intestinal lymphoid tissues with only very few CD4+ T cells for the remainder of the disease [38, 52, 185, 298]. This low number of CD4+ T cells could lead to compromised gastrointestinal mucosal surfaces that are not able to prevent microbial translocation from the intestinal lumen into the systemic circulation. In fact, it has been shown that LPS levels are increased in the circulation of HIV-infected patients [37]. These microbial products, in addition to viral particles, might then lead to a chronic immune activation state by constantly stimulating immune cells through toll-like receptors (TLR) [36, 37]. Indeed, it has

become clear that the activation state of the immune system is an important indicator of disease progression. Early after the discovery of HIV as the causative agent for AIDS, Ascher and Sheppard suggested that the direct effect of HIV on CD4+ T cells was not the only cause of disease progression [16]. They hypothesized that infected macrophages present viral products to T cells, thus leading to generalized immune activation and subsequent T cell anergy and immune dysfunction [16]. Later studies showed that increased T cell activation is a stronger predictor of disease progression and AIDS-related death than CD4+ T cell numbers or viral loads [108, 187], clearly linking immune activation to HIV pathogenesis. These findings have been verified in more recent studies that identify a clear association between the activation state of the immune system and the rate at which HIV-infected patients progress to AIDS [97, 122, 169, 280, 283, 300].

All these factors, including CD4+ T cell decline in the blood and at mucosal sites, viral load and immune activation (Figure 1-4), are hallmarks of HIV/AIDS. They synergize to cause disease and can eventually lead to death if they remain untreated. The first symptoms of AIDS usually include moderate and unexplained weight loss, recurring respiratory tract infections (such as sinusitis, bronchitis), prostatitis, and skin rashes. At this disease stage, HIV-positive individuals are more susceptible to

opportunistic infections, which a healthy immune system could usually control. Oral thrush, tuberculosis, and pneumonia are typical opportunistic infections. Additionally, during this time AIDS patients also develop other protozoal, bacterial, viral and fungal infections as well as neurological disorders and malignancies. Eventually, most untreated HIV-infected individuals will succumb to AIDS (Figure 1-4).

However, it also became clear that a small group of people (5 to 15 %) remained clinically and immunologically stable for years without the need for anti-viral therapy [41, 212, 230, 273]. These long-term non-progressors exhibit low to moderate viral loads and relatively stable CD4+ T cell counts. Although they progress slowly, follow up revealed that most of these patients progress to AIDS eventually [111, 176, 219, 255]. However, a sub-group of the long-term non-progressors is able to suppress viral loads to undetectable levels and remain healthy without therapy. These people have been termed elite controllers and are subject to active research to delineate the causes of their natural ability to control HIV [65, 232].

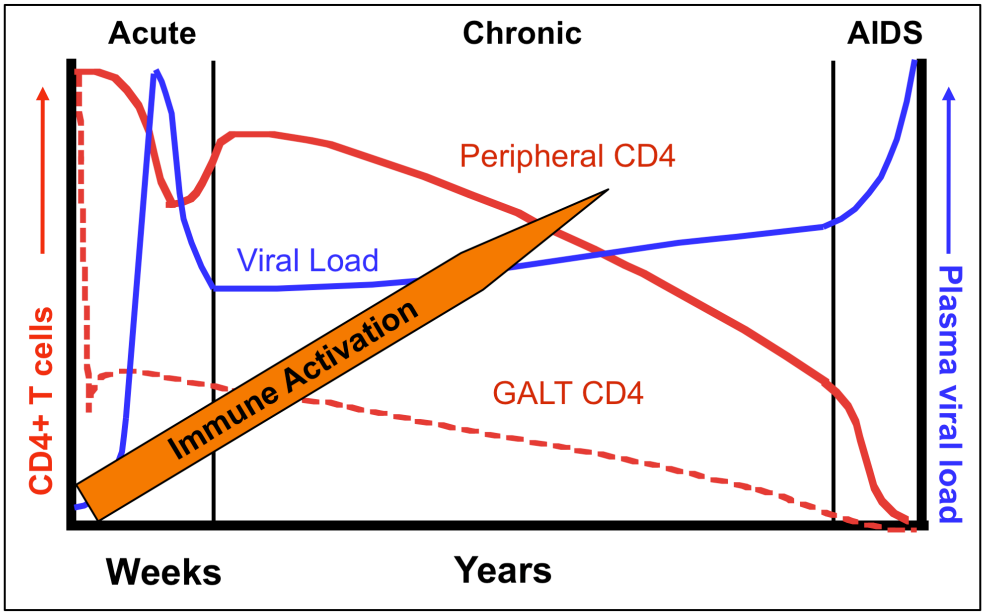

Figure 1-4. Generalized overview of disease progression after HIV infection.

Shown are CD4+ T cell numbers in blood (red solid line) and in gut-associated lymphoid tissues (GALT, red dashed line) as well as HIV RNA viral load (blue solid line) during HIV infection in an untreated average patient. The orange arrow represents the increase in immune activation during HIV disease progression.

HIV treatment

Although there is currently neither a vaccine against nor a cure for HIV, it is no longer a deadly disease but a treatable chronic condition. One of the most important advances in the treatment/therapeutics field was the recognition that several different classes of agents could inhibit viral replication by interfering with different steps within the viral life cycle. AZT

(azidothymidine) was the first drug to be approved for the treatment of HIV/AIDS in 1987. It was shown in placebo-controlled randomized trial to prolong the life of patients with AIDS [92]. However, due to the high mutation rate of HIV, it did not take long until the virus developed resistance to AZT. It became clear that successful therapy would require a combination of drugs, which eventually became known as highly active antiretroviral therapy (HAART). The antiretrovirals used in HAART can be classified by the step of the viral life cycle that the drug inhibits:

- Nucleoside and nucleotide reverse transcriptase inhibitors (nRTI) inhibit reverse transcription by being incorporated into the newly synthesized viral DNA and preventing its further elongation.
- Non-nucleoside reverse transcriptase inhibitors (NNRTI) inhibit reverse transcriptase directly by binding to the enzyme and interfering with its function.
- Protease inhibitors (PIs) target viral assembly by inhibiting the activity of protease, an enzyme used by HIV to cleave nascent proteins for final assembly of new virons.
- Integrase inhibitors inhibit the enzyme integrase, which is responsible for integration of viral DNA into the DNA of the infected cell.
- Entry (or fusion) inhibitors interfere with binding, fusion and entry of HIV-1 to the host cell by blocking one of several targets.

Three or four drugs from different classes are usually taken during HAART. It first became available in 1996 and was based on protease inhibitors and non–nucleoside-analogue reverse-transcriptase inhibitors. It was able to decrease morbidity and mortality in patients with advanced disease [224]. The combination of drugs is supposed to slow the emergence of drug-resistant mutations by inhibiting several different stages of the viral life cycle at the same time. However, HAART also complicates the treatment by the need to take several drugs several times a day, which is not only expensive but can generate unpleasant side effects. Newer one-pill regimens are available, but these are even more expensive and only available in the developed world. Also, long-term HAART is associated with side effects like heart diseases or premature aging. Additionally, viral suppression is lost rapidly when HAART treatment is stopped [151], and discontinuation can increase the risk of opportunistic infections and death [79]. However, HAART has improved the quality of life and the health of HIV-infected individuals; indeed life expectancy has increased from 9 to 11 years without treatment [323] to over 20 years since the introduction of HAART [159]. Current therapy guidelines for HIV-infected individuals are based on the WHO staging systems and the CD4+ T cell count. Depending on the WHO stage,

HAART should be initiated when CD4 T cell levels drop below 350 cells per μL (Stage III) or below 200 cells per μL (Stages I or II).

HIV vaccines

Although treatment that has turned a fatal disease into a chronic illness is now available, HIV/AIDS is still not curable and still contagious. Thus, infected people continue to spread HIV especially as long as they do not know that they are infected. A vaccine able to elicit sterilizing immunity against HIV would be the gold standard to prevent new infections and thus stop the spread of the disease. However, despite more than 25 years of research, scientists have not been able to develop successful vaccine candidates that made it through multi-phase clinical testing. The world's first two phase III HIV vaccine efficacy trials were conducted in 2003 of which one was done in the United States and the Netherlands [95, 121] and the other one in Thailand [234]. Both utilized a recombinant gp120 designed to elicit HIV-specific antibodies. Although individuals who received the vaccine elicited high levels of antibodies, no difference could be observed in either trial between vaccine and placebo groups [107, 234]. The only other phase III clinical trial currently being conducted is taking place in Thailand. Results from this study are expected at the end of 2009 [217].

Most recently, a promising phase IIb efficacy clinical trial was halted prematurely due to ineffectiveness of the vaccine. In 2004, the HIV Vaccine Trials Network (HVTN) started recruiting for STEP study utilizing an attenuated adenovirus vector (Ad5) carrying three HIV subtype genes, gag, pol, nef. This study was supposed to enroll 3000 participants in North and South America, the Caribbean and Australia; however, an interim analysis revealed the ineffectiveness of the vaccine and therefore, the trial was halted in September 2007. In fact, not only did this vaccine prove to be inefficient, it also seemed to increase the risk of certain vaccinated groups to acquire HIV upon exposure. Uncircumcised men and individuals with pre-existing immunity to the adenovirus vector were at higher risk of HIV infection when vaccinated compared to their unvaccinated counterparts [39, 137, 271]. Although several studies have been performed since the end of the study to examine the cause behind this increased infection rate, the mechanisms are still not known. This failure revealed that there is still the need for a better understanding of immune responses following viral exposure, especially at mucosal sites, and led to calls to reexamine the design of future vaccine trials [137, 271]. Based on these results, it is clear that the development of an effective HIV vaccine able to prevent new infections requires more research into the mucosal

immune responses after viral exposure and these efforts will likely take many more years.

Other HIV prevention options

In the absence of an effective HIV vaccine, alternative methods of prevention become even more important. The single most effective measure to prevent the spread of HIV is the use of the male condom. Condoms used properly can prevent the spread of HIV from an infected male individual to his female or male sex partner. Also, a condom can protect the wearer from getting infected by his infected sexual partner. As an additional benefit, condoms prevent infections from other sexually transmitted diseases as well as unwanted pregnancies. A study of sero-discordant couples (in which one partner was HIV-positive and one was HIV-negative) demonstrated that no uninfected partner became infected among couples using condoms correctly and consistently at every act of vaginal or anal sex, whereas 10 percent of those using condoms inconsistently became infected [64]. The female condom has produced some evidence of its efficacy in prevention of HIV [308], however no studies have been conducted to test its effectiveness directly [223]. Antimicrobial agents (microbicides) that are able to stop HIV after intravaginal or intrarectal application would give women another means to

protect themselves from HIV infection. However, most microbicides to date have not proven to be safe or effective. For example, Nanoxynol-9 was recommended for a long time to be used as microbicide to prevent infection since it killed HIV in vitro. However, as a vaginal microbicide it actually increases the risk of HIV infection in female sex workers since it damaged the vaginal mucosa, potentially allowing HIV an easier entry into the body [124, 254, 301]. Today, more microbicides, including newer formulations that contain anti-retroviral compounds, are in clinical trials and being tested for safety and efficacy. These studies include some promising candidates, like for example PRO-2000, a topical gel composed of a synthetic compound non-specifically designed to block attachment of HIV to host cells and thereby prevent infection. Results from a clinical phase IIb trial were presented at the Conference on Retroviruses and Opportunistic Infections (CROI) in Montreal in February 2009, stating that PRO2000 decreases the risk of HIV infection by 30 % in women who received the gel and condoms compared to women who used a placebo and condoms. Although this difference was not statistically significant, it encourages future research on this and other compounds.

Another promising intervention to decrease the spread of HIV is male circumcision. It has been shown in randomized control trials that male circumcision can reduce the risk of HIV infection for men by between

50 and 60 % [17, 23, 113]. The reduction in HIV acquisition rates in these circumcised men is likely due to the removal of a mucosal site (foreskin) that is susceptible to HIV entry. Reducing infection in men should ultimately lead to lower infection rates in women as well; however, a recent study over a 24-month period found that circumcision of HIV-infected men did not lead to a reduction of infection in female partners [317]. This result clearly indicated a need for long-term studies on the benefit for women. Despite this latest finding, the WHO states now that male circumcision is an efficacious intervention for HIV prevention [325]. However, it should be carried out by well-trained medical professional and should be utilized as an additional method or prevention rather than the only one. Taken together, it is important to note that there are many ways to protect uninfected individuals from HIV infection despite the lack of an effective HIV vaccine.

HIV transmission

HIV can enter a new host by one of three major ways: parenteral, through sexual contact or by mother-to-child transmission. In parenteral transmission, the virus gets injected straight into the blood stream, either by intravenous drug-users sharing equipment with HIV-infected individuals, or by blood-transfusion. Today, the latter does usually not

occur since the blood supply is screened for viral and bacterial contaminants. Both transmission via sexual contact and mother-to-child transmission require the virus to cross a mucosal membrane. Sexual transmission can occur during hetero- and homosexual intercourse through the oral, penile, anal or vaginal mucosa with the last two sites being the most common route. Transmission of HIV from an infected mother to her child can occur in utero, during parturition and through breast-milk. Today, parenteral transmission is rare and most worldwide HIV-1 transmission events occur following the translocation of the virus across a mucosal epithelial surface such as the genital, rectal or oral mucosa.

Cells able to be infected by HIV (such as macrophages, dendritic cells and CD4+ T cells) exist at these mucosal sites and likely represent the initial target cells for the virus [47, 147, 206, 328]. Many factors can influence mucosal transmission and need to be considered for future preventative vaccines. In general, the chance of someone becoming infected depends on the infectivity of the infected and the susceptibility of the uninfected partner. It has been shown that associations exist between HIV viral load and the probability to spread the virus: the higher the viral load the more likely transmission will occur. This means that acutely infected individuals or patients in end stages of infection are more likely to

spread HIV than chronically infected individuals on a per act basis since viral loads are much higher during acute infection or during end stages [131]. However, it has also been suggested that the total infection rate during chronic infection is higher due to the fact that chronic infection is much longer and thus more sexual acts can be performed [131]; furthermore, acutely infected individuals usually exhibit flu-like symptoms and feel sick and therefore might not engage in sexual activity as much. As mentioned, another factor influencing HIV transmission is the susceptibility of the uninfected partner, which can be enhanced by for example the presence of sexually transmitted diseases (STD). It has been shown that individuals with recent genital ulcerative, like herpes or syphilis, or non-ulcerative diseases, like gonorrhea, were at significant risk to acquire HIV from an HIV-infected partner [40, 93, 256, 315]. Several mechanisms underlying this increased risk have been suggested, including disruption of the epithelial barrier through genital ulcers, providing a portal of entry to HIV, or the accumulation of activated cells to the mucosal sites, providing more target cells for the virus. Additionally, HIV-infected individuals with concomitant STDs might be more infectious since STDs can lead to increased shedding of HIV into the genital tract, leading to higher viral burden in genital secretions [55, 100, 168, 201, 236]. Finally, several recent randomized control trials have shown that

male circumcision can reduce the risk of HIV infection for men by between 50 and 60 % [17, 23, 113], encouraging the WHO and UNAIDS to now recommend that male circumcision should be performed as a preventative measure against HIV transmission [325]. The propensity of HIV to utilize mucosal transmission to spread to new hosts suggests that developing an effective HIV vaccine will likely require an in-depth understanding of the events at the mucosal sites as well as of the subsequent events that follow at other tissue sites such as lymph nodes (LN) and blood.

Oral transmission

Despite increasing awareness, the misconception still exists that HIV cannot be transmitted through the oral route [257, 266]. In fact, female sex workers in Africa were told to engage in this type of sexual behavior to protect themselves from HIV. However, it has become clear that oral transmission can and does occur either through oral-genital intercourse [34, 257, 258, 266, 309] or via breast-feeding [88, 210, 216, 265]. The latter is especially troublesome in developing countries where the major burden of HIV infection lies and where formula-feeding is not practical due to unsanitary conditions and contaminated water. In fact, up to one third of newborns of HIV-infected mothers become infected through breast-milk [28, 31, 62, 78, 146, 209]. Additionally, other factors exist that can

increase the rate of HIV transmission through the oral route, like the presence of oral infections leading to a disruption of the mucosal barrier [253, 264]. Studies have been conducted to elucidate the mechanisms of viral entry after oral exposure to HIV. For reasons mentioned previously, all these studies have been conducted in the SIV Rhesus macaque model.

To design strategies to prevent viral infection through this route, it is important to know how and where the virus enters. One of the first studies to demonstrate that oral transmission can indeed occur was performed in 1996 [21]: Rhesus macaques were inoculated with cell-free virus orally and all animals became infected. The exact location of viral entry after oral exposure to SIV was not determined. But the authors suggested that the virus likely entered proximal to the stomach, since its extreme acidity would likely destroy the virus [21]. In another study, SIV was applied directly to the tonsils of macaques to assess whether the virus could enter a new host via this lymphoid organ [285]. Virus entered through the tonsils, infected the animals and spread rapidly to be systemic by 7 days post-infection. Virus could not be detected in the intestinal tract before day 23. This indicated that it was unlikely that the virus was swallowed by the animals and infected them through the intestine [285]. In a more recent study, SIV was inoculated into the oral cavity of macaques, allowing it to come in contact with several tissues in the mouth, including the oral

mucosa and the tonsils, before being swallowed [206]. Here, all macaques became infected and virus spread rapidly and could be detected by PCR within 4 days in all tissues assessed. Even more important, the oral and esophageal mucosa as well as the tonsils were the tissues that were SIV-positive within 1 day after viral exposure, indicating that these tissues represented the most likely viral entry sites [206]. These studies and findings emphasize the importance of the oral route for HIV transmission and validate its usefulness as a model to study mucosal transmission. As the oral mucosa is comprised of stratified squamous epithelium, it shows structural similarities to other mucosal sites like the penile and vaginal mucosa. The top layer (or layers) of stratified squamous epithelium can be keratinized and the thickness of this layer determines its protective effect. For example, the skin has a very thick keratinized cell layer. The oral mucosa, especially around the teeth, can be keratinized as well, whereas the vaginal mucosa or the inside of the male foreskin generally do not exhibit thick keratinized cell layers. It is believed that the keratinized cell layer provides some protection against HIV acquisition; the thicker the layer is the less likely transmission with HIV occurs. Due to the structural similarity between the oral mucosa and other mucosal sites, findings from oral transmission studies might be applicable to transmission via these other mucosal sites; however, the amount of keratin in the different

mucosal tissues will likely affect the effectiveness of HIV transmission and this should be considered in future vaccine designs.

Immune responses to HIV/SIV and viral escape

As soon as HIV (or SIV) enters, the immune system of the new host becomes active to fight this infection. Both innate and adaptive immune responses are important in the fight against this virus. However, the virus also starts to immediately employ strategies to circumvent theses immune responses. Unfortunately, in the vast majority of cases, it is the virus that wins this battle if the host remains untreated.

Type I interferons (IFN-α, IFN-β) and interferon-stimulated genes (ISG) are one of the first defenses of the innate immune system against viral infections. Viral components are recognized by pattern recognition receptors, e.g. HIV single-stranded RNA by toll-like receptors (TLR) 7 and 8, and cells are activated to produce these anti-viral response factors. These immune modulators can induce apoptosis of infected cells or render other cells resistant to virus infection [133]. Evidence exists that the interferon system is active during HIV infection, although findings are contradictory: studies have shown that the decline of viral loads in plasma is associated with the presence of type I interferons [98, 310] and that a decrease in type I interferons is associated with the onset of opportunistic

infections in HIV-infected individuals [274]. In fact, treatment of patients with type I interferons is able to reduce viral loads [118, 274]. Furthermore, when plasma viral loads increase and patients develop AIDS, levels of interferon-producing plasmacytoid dendritic cells in the blood decline [71, 222]. In vaccine studies with Rhesus macaques it was shown that IFN-α levels in the blood correlated with SIV-specific IFN-γ producing T cells in macaques that were protected against challenge virus compared to animals that were not protected [2]. On the other hand, the presence of type I interferons does not prevent viral spread and systemic dissemination [1, 98, 106, 310] and in fact, high plasma levels have been associated with poor disease prognosis and high plasma viral loads [1, 106]. It has been argued from studies in the Rhesus macaque model that the interferon response towards SIV comes too little and too late [1, 5]. Several studies were undertaken to delineate the connections and associations between viral infection in humans and monkeys with the presence or absence of other immune mediators like pro-inflammatory cytokines and chemokines. These studies have yielded conflicting results. For example, it has been shown that expression of IFN-γ is high during HIV and SIV infection in several tissue sites including blood, lymph nodes and mucosal sites and does not seem to be associated with slower disease progression or protection [4, 5, 114, 172]. On the other hand,

IFN-γ seems to contribute to protection against challenge after vaccination during acute infection [2] but is associated with a lack of protection later on [2, 3]. Similarly, studies with other cytokines and chemokines have found either protective [103, 106, 158] or pathogenic effects [46, 173, 251].

Cells of the innate immune system also participate in the initial immune response against HIV/SIV. Monocyte-derived cells phagocytose and present antigens to both CD4+ and CD8+ T cells, activate NK cells via IL-12 expression, and help to maintain tissue homeostasis [7]. They upregulate the expression of pro-inflammatory chemokines like MIP-1β, a natural ligand for CCR5, which might be able to prevent HIV-infection of neighboring cells by blocking HIV receptors [175]. However, on the other hand HIV can infect macrophages [80, 194] and this might present a major route of HIV spread within the host, as infected macrophages have been found throughout the body, including brain, mucosal sites and lymph nodes [161, 203, 218, 220, 242]. Additionally, HIV infection of macrophages impairs their immune functions including the ability to phagocytose, intracellular killing and cytokine production [19, 30, 152, 153, 286, 295]. Another important innate cell type, natural killer (NK) cells, play crucial roles during HIV/SIV infection and influence disease outcome, as they are responsible for recognizing and killing of virally infected cells. However, although they are not infected with HIV/SIV themselves,

infection of the host with either of these viruses impairs also their function (reviewed in [9]). Finally, two other innate immune cells, dendritic cells (DC) and γδ T cells, might play beneficial roles during HIV/SIV infection. They seem to impair viral replication, at least in *in vitro* studies, and this might be due to their expression of CCR5-ligands like CCL3 (MIP-1β) or CCL5 (RANTES), which could block the HIV co-receptor [237]. In vaccine studies with Rhesus macaques it has been shown that the an increase in the presence of these two cells at mucosal and lymphoid sites was associated with protection against challenge with pathogenic virus when compared to unprotected animals [177, 294]. However, γδ T cells are also susceptible to immune dysfunction. They can be directly infected with HIV/SIV *in vitro* [138] and it has been shown that their numbers at the intestinal mucosa, blood and spleen decline within seven days after intrarectal inoculation in Rhesus macaques [45]. Additionally, these cells seem to be impaired in their migratory abilities, resulting in potential failure to home to appropriate sites [239] and they seem to lose their proliferative ability in response to antigenic stimulation [238, 329], another sign of immune dysfunction. Taken together, all these findings indicate the important role that is played by the innate immune system against this viral infection. However, they also emphasize the ability of the virus to

elucidate these protective mechanisms and replicate, leading to immune dysfunction.

Similar to the innate immune system, both arms of the adaptive immune system, cellular and humoral immunity, also play major roles during HIV and SIV infections. It has been shown that the drop in viral load at the end of the acute infection stage is associated with the occurrence of virus-specific CD8+ T cells [143, 165, 170, 269] and that the strength and breadth of CD8+ cytotoxic T cells was inversely correlated with viral loads and time to disease [126, 170, 184, 213, 316]. Over time however, HIV and SIV are able to escape this immune response through several mechanisms, including the direct killing of infected CD4+ T cells (needed to initiate cellular and humoral immune responses), killing of CD4+ and CD8+ T cells through bystander apoptosis and by extensive mutations of viral epitopes to prevent immune recognition [73, 112, 141, 145, 160].

The humoral immune response is similarly important for viral control but also similarly vulnerable to viral escape. Viral-specific antibodies are important in controlling viral replication [53, 270] and it has been shown that macaques that progress to simian AIDS at a fast rate fail to develop SIV-specific antibodies [130, 150, 327]. Passive administration of SIV-specific antibodies to Rhesus macaques can block viral infection in uninfected and decrease viremia in infected animals [53, 90, 91, 120,

270]. However, as it has been observed in cellular immunity, HIV and SIV can escape the humoral immune response by similar mechanisms like direct viral killing of CD4+ T cells and mutations of viral epitopes. Additionally, destruction of lymph node architecture, especially germinal centers, occurs during disease pathogenesis as well, inhibiting the initiation of effective immune responses in lymph nodes [68, 73, 189, 293]. Furthermore, in a recent study, it has been shown that similar to the early loss in mucosal CD4+ T cells of the gut, B cells in the gastro-intestinal tract seem to be negatively affected by early HIV infection. During acute stages, B cells are polyclonally and unspecifically activated, lymphoid follicles are damaged and germinal centers are lost [180]. This indicates that, like cellular immunity, humoral immunity is also compromised very early on during HIV infection, further complicating HIV vaccine approaches. As with the innate immune responses, adaptive immunity is critical to controlling viral infection initially. However, the virus can and will escape these immune responses, leading to AIDS if the hosts remain untreated.

Summary

Studies preceding the ones presented here focused on the assessment of innate immune modulators at mucosal sites and their association with

disease progression. Increased expression of innate immune mediators at mucosal sites was associated with slower disease progression. Building upon these results, the studies presented here were designed to obtain a better understanding of what happens at lymphoid tissues and in peripheral blood after SIV (and by analogy also HIV) comes in contact with a mucosal membrane and enters a new host. The experiments presented here focus on the oral transmission route of infection in the SIV macaque model to answer the following questions:

(1) What are immune correlates of disease progression after oral inoculations of SIV? How do these differ between different anatomical locations and can certain immune markers be utilized as predictors of disease progression? (Chapter 3)

(2) Which immune responses and viral changes can be observed after inoculations with low doses of SIV and how do these differ from those in macaques infected with high doses? (Chapter 4)

The results obtained through these studies enhance our knowledge of the immune and viral changes happening after oral inoculations of SIV. These might help in the design of new and improved therapy and vaccine approaches to fight the spread of HIV/AIDS.

CHAPTER TWO

Materials and Methods

Animal Subjects

The macaques used in these studies were colony-bred Rhesus macaques (Macaca mulatta) housed at either the California National Primate Research Center (CNPRC), the Yerkes National Primate Research Center (YNPRC) [208, 214] or the Southwest National Primate Research Center (SNPRC). These animals had the following designations: for CNPRC, RM11 (33291), RM12 (32167), RM13 (32174), RM14 (32296), RM15 (33353), and RM16 (32127); for YNPRC, RM1 (RSm), RM2 (RTq), RM3 (RUh), RM4 (RWp), RM5 (RPc), RM6 (RHj), RCe8, RCo8, RDo8, REi9, RHk8, RIf8, RJj8, RJl9, RKb9, RNr8, ROu8, RUn8, RWi8, RWu8 and RZz8; and for SNPRC, 17742, 18984, 19320, 18412, 18414, 18981, and 19147. The sooty mangabeys utilized in these studies were housed at the YNPRC and had the following designations: SM1 (FFr), SM2 (FBr), SM3 (FCq), SM4 (FCs), SM5 (FRu), and SM6 (FUq). All animals were cared for in accordance with National Institutes of Health guidelines and local Animal Care and Use Committees.

SIV administrations

Macaques housed at YNPRC were previously intravenously inoculated with 1 x 10^4 $TCID_{50}$ of SIVmac239 [81]. Blood samples for analysis were taken on days 7 and 168 post-infection. The CNPRC macaques were orally inoculated with two 1 x 10^5 50% tissue culture infective doses ($TCID_{50}$) of SIVmac251-5/98 [115, 198] 1h apart; this high dose was used to ensure infection. The virus inoculum was slowly dispensed drop-wise from a needleless syringe onto the lower right cheek/gingival area. The virus was allowed to contact the oral gingiva and likely the tonsils prior to being swallowed. Macaques were followed throughout infection and were euthanized following onset of simian AIDS [304] by a pentobarbital overdose in accordance with YNPRC and CNPRC guidelines. The macaques housed at the SNPRC were orally inoculated with low doses of SIVmac251 (Isolate from Ron Desrosiers [61], grown in Rhesus PBMCs at SNPRC), ranging from 1000 to 4000 $TCID_{50}$. The macaques were laid on their left side and the gingiva was dried with gauze. The head was slightly lifted and the virus was dropped on the mandibular molar making sure that the virus made contact with the gingiva. The macaque remained on its left side for at least 5 minutes. After infection, these macaques were followed for two to three months before they were released to other investigators at the SNPRC. The sooty mangabeys used in this study were intravenously

inoculated with plasma from an SIVsmm-infected mangabey [215]. Samples examined here were taken on day 7 for acute time points and between 258 and 287 days for chronic time points.

Tissue collection and processing

During the course of these studies, blood samples were taken from all animals at several time points, and peripheral blood mononuclear cells (PBMCs) were extracted through density centrifugation with Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden). Peripheral blood was assessed for complete blood cell counts (CBC) and absolute T cells, CD4+ T cells, CD8+ T cells, and B cells by flow cytometry. Peripheral blood mononuclear cells were viably frozen at -140 °C and shipped to the Sodora laboratory under nitrogen vapor in a Cryo-Shipper (MVE, Inc., New Prague, Minnesota) or on dry ice. During blood processing to isolate PBMCs, plasma was collected as well and stored at -80 °C. Additionally, numerous lymph node and oral mucosal biopsies were obtained from all Rhesus macaques housed at the CNPRC and the SNPRC. All procedures were performed under ketamine hydrochloride anesthesia (10 mg/kg). Mucosal biopsies consisted of 2 mm punch biopsies of gingival tissue that were placed in either Streck tissue fixative buffer (Streck Laboratories, Inc.) prior to being paraffin embedded or in RNALater (Ambion, Inc.,

Austin, Texas) at -80 °C for RNA isolation. Lymph node biopsies were obtained one each from the left and right axillary and inguinal lymph nodes and preserved as either viable cells, placed into Streck tissue fixative buffer (Streck Laboratories, Inc.) prior to being paraffin embedded or in RNAlater at -80 °C for RNA isolation. To obtain lymph node mononuclear cells (LNMCs), lymph node biopsies were physically disrupted (scalpel microdissection) and crushed through a 70 µm cell strainer (BD Bioscience, San Jose, CA). Cells were washed with PBS prior to use. At necropsy, 15 to 25 tissues were collected, representing both lymphoid and non-lymphoid tissues. All tissues were preserved by several methods: Samples were either snap frozen in liquid nitrogen or submerged in RNAlater and stored at -80 °C for DNA or RNA isolation. Up to one-third of each tissue, including lymph node and mucosal biopsies, were fixed in Streck tissue fixative buffer (Streck Laboratories, Inc., Omaha, Nebraska) or formalin prior to being paraffin embedded.

Magnetic cell sorting

CD14+ monocytes and CD3+ T cells were sorted from PBMCs by MACS magnetic cell separation with supplies from Miltenyi Biotec (Auburn, CA), according to manufacturer's protocol. Briefly, a MACS buffer containing PBS (phosphate buffered saline) pH 7.2, 0.5 % BSA (Bovine Serum

Albumin) and 2 mM EDTA (ethylenediaminetetraacetic acid) was prepared. PBMCs were suspended in this buffer and labeled with CD14 microbeads. The cell solution was applied to a MACS MS column and CD14+ cells were bound to the column under strong magnetic fields. The flow through was collected and used for CD3+ T cell separation. The CD14+ cells were washed on column with the buffer, the magnetic field was removed and the CD14+ cells were eluted with the MACS buffer. The flow through was incubated with biotinylated anti-CD3 antibodies, followed by incubation and binding to anti-biotin microbeads. These were applied to a MS column and CD3+ cells were bound under magnetic fields. Cells were washed on column with the MACS buffer, the magnetic field was removed and CD3+ cells were eluted with the buffer. Aliquots of both the CD14+ and the CD3+ cell populations were labeled with CD3 and CD14 specific antibodies to assess purity by flow cytometry. Purity was >90 % for both populations. Eluted cells were lysed with RLT buffer from the RNeasy Mini Kit (Qiagen, Valencia, CA) and total RNA was extracted as described below.

Nucleic acid extraction

Tissues had been stored in RNAlater and were disrupted by bead-milling using the FastPrep-24 (MP Biomedicals, Solon OH) in the presence of

beads (Lysing Matrix D, MP Biomedicals, Solon OH) and lysis buffer (Buffer RLT, Qiagen, Valencia, CA). PBMCs or LNMCs (lymph node mononuclear cells) were lysed in RLT buffer (Qiagen, Valencia, CA) without the need for prior disruption. Depending on the downstream application, tissue and PBMC lysates were used to either extract genomic DNA and RNA or RNA alone. Genomic DNA and total RNA were extracted simultaneously from the same sample (either tissue or PBMC) using the AllPrep DNA/RNA Mini Kit (Qiagen, Valencia, CA) according to manufacturer's protocol. Briefly, the lysate was homogenized and the DNA bound to a DNA spin column. The flow-through was used for RNA extraction. RNA was bound to the RNA spin column, washed and eluted in RNase-free water. Total RNA was stored at -80 °C until further use. DNA was washed, eluted in Buffer EB (Qiagen, Valencia, CA) and also stored at -80 °C. For purification of RNA only, the RNeasy Mini Kit (Qiagen, Valencia, CA) was used following the manufacturer's protocol. Briefly, tissue or PBMC lysates were homogenized and RNA bound to an RNA spin column. After DNase treatment, the RNA was washed, eluted in RNase-free water and stored at -80 °C.

Synthesis of complementary DNA (cDNA)

The concentration of total RNA was quantified with a NanoDrop ND-1000 spectrophotometer (Fisher Scientific, Wilmington DE) and used for complementary DNA (cDNA) synthesis, utilizing the Super Script First Strand cDNA Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). The synthesis was performed following the company's instructions. Briefly, total RNA was mixed with random hexamers, deoxynucleotides triphosphates (dNTPs), and diethyl-pyrocarbonate (DEPC) treated water and incubated at 60 °C for 5 minutes. Next, magnesium chloride ($MgCl_2$), reverse transcriptase (RT) buffer, dithiothreitol (DTT), RNaseOUT, and SuperScript II RT was added and incubated at room temperature for 10 minutes, followed by 42 °C for 50 minutes (to allow the RT to synthesize the cDNA) and 70 °C for 15 minutes (to inactivate the enzyme). After heating, RNaseH was added and incubated at 37 °C for 20 minutes. Finally, the samples were diluted with DNase/RNase free water so that 1 µL contained the cDNA equivalent to 1 – 2 µg total RNA.

Nested PCR for SIVgag

Nested Polymerase Chain Reaction (PCR) for the SIVgag gene in PBMCs was used to determine whether the orally inoculated macaques indeed became infected. In each nested PCR reaction, 5 µL genomic DNA was

utilized to detect a 597 base pair DNA fragment of the SIVgag gene. The first round primers were 5'-AGAAAGTGAAACACACTGAGGAAGC-3' (forward) and 5'-TCATCCAATTCTTTACTGCTGCA-3' (reverse). In the second round, 2 µL of the first round products were utilized with the following primers: 5'-ACAGATAGTGCAGAGACACCTAGTGG-3' (forward) and 5'-CTGTCTACATAGCTCTGAAATGGCTC-3' (reverse). PCR was performed using a Perkin Elmer GeneAmp® PCR System 2400 with the following conditions: 94 °C for 5 min., followed by 35 cycles of 94 °C for 30 sec., 55 °C for 30 sec., 72 °C for 30 sec., followed by 72 °C for 7 min. Each DNA sample was tested duplicate. Here, the nested PCR approach provided the sensitivity to repeatedly detect 1 - 5 copies of plasmid DNA. SIV-negative macaque PBMCs were utilized as negative controls. Second round PCR products were electrophoresed on a 1 % agarose gel and visualized as a 597 base pair fragment by ethidium bromide staining.

Real-time PCR for SIV gag (Lymph node associated viral loads)

Viral loads in lymph nodes were determined by real-time PCR, based either on genomic DNA (contains the integrated provirus) or on total RNA (contains RNA from released virions). For DNA viral loads, standards were generated from genomic DNA from E11S cells. E11S is a SIVMne-infected Hut78 cell line derived from a single clone, which contains two

copies of SIV integrated per cell [320]. Based on the concentration of the genomic DNA isolated from these cells, the number of integrated SIV can be calculated and used as standard. To determine Ct values, genomic DNA from the unknowns and standards were run in triplicate on a 7500 Real-time PCR system (Applied Biosystems, Foster City, CA) using the following primers and probe specific for SIVgag: GAG5f (5'-ACTTTCGGTCTTAGCTCCATTAGTG-3'), GAG3r (5'-TTTTGCTTCCTCAGTGTGTTTCA-3' and the TaqMan probe GAG1tq (5'-TTCTCTTCTGCGTGAATGCACCAGATGA-3'). Based on the Ct of the standard and the unknown, the SIV copy number of the unknown can be determined and is represented as µg/DNA. For RNA viral loads, real-time PCR with primers and probe specific for SIVgag and GAPDH (Table 2-1) was run with cDNA from lymph node mononuclear cells (LNMC) on a 7500 Real-time PCR system (Applied Biosystems, Foster City CA). Plasmids containing SIVgag and GAPDH sequences were used to generate standard curves from which copy numbers of SIVgag and GAPDH mRNA in LN were derived. LN-associated RNA viral load is expressed as number of SIVgag RNA copies per 10^6 GAPDH RNA copies

Table 2-1. Forward and Reverse Primers and Probes utilized for quantitative real-time PCR

Gene	Oligo	Sequence (5' - 3')
GAPDH	Forward Primer	GCA CCA CCA ACT GCT TAG CAC
	Reverse Primer	TCT TCT GGG TGG CAG TGA TG
	Taqman Probe	5′-FAM-TCG TGG AAG GAC TCA TGA CCA CAG TCC-TAMRA-3′
IFN alpha	N/A	TaqMan® Gene Expression Assay
OAS	Forward Primer	CTG ACG CTG ACC TGG TTG TCT
	Reverse Primer	ACT CTC CCC GGC GAT TTA A
	Taqman Probe	5′-FAM-CCTCAGTCCTCTCACCACTTTTCAGGATCA-TAMRA-3′
IFN gamma	Forward Primer	GAA AAG CTG ACC AAT TAT TCG GTA A
	Reverse Primer	AGC CAT CAC TTG GAT GAG TTC A
	Taqman Probe	5′-FAM-TGACTCGAATGTCCAACGCAAAGCAGTA-TAMRA-3′
TNFalpha	Forward Primer	GGC TCA GGC AGT CAG ATC AT
	Reverse Primer	GCT TGA GGG TTT GCT ACA ACA
	Taqman Probe	5'-FAM-TCG AAC CCC AAG TGA CAA GCC TGT AGC-TAMRA-3'
IL-12	Forward Primer	ACA AAA AGG AAG ATG GAA TTT GGT
	Reverse Primer	GGC CTC ACA TCT TAG AAA GGT CTT A
	Taqman Probe	5′-FAM-CACTGATGTTTTAAAGGACCAGAAAGAACCC-TAMRA-3′
IL-10	Forward Primer	ACC CAG ACA TCA AGG AGC AT
	Reverse Primer	CCA CGG CCT TGC TCT TGT T
	Taqman Probe	5′-FAM-TACGGCGCTGTCATCGATTTCTTC-TAMRA-3′
TGFbeta	N/A	TaqMan® Gene Expression Assay
CXCL8 (IL-8)	Forward Primer	AAG GAA CCA TCT CAC TGT GTG TAA
	Reverse Primer	TTA GCA CTC CTT GGC AAA ACT G
	Taqman Probe	5′-FAM- CTG CCA AGA GAG CCA CGG CCA G -TAMRA-3′
CXCL9 (MIG)	Forward Primer	CAG ATT CAG CAG ATG TGA AGG AA
	Reverse Primer	ACG TTG AGA TTT TCT AAC TTT CAG AAC TT
	Taqman Probe	5′-FAM-CAGCCAAAAGAAAAAGCAAAAGAATGG-TAMRA-3′
CXCL10 (IP-10)	Forward Primer	CCT CCA GTC TCA GCA CCA TGA
	Reverse Primer	TGC AGG TAC AGC GTA CGG TCC
	Taqman Probe	5′-FAM-TTCTGACTCTAAGTGGCATTCAAGGAGTACCTCTCTC-TAMRA-3′
SIVgag	Forward Primer	TCT GCG TCA TCT GGT GCA
	Reverse Primer	TGT CTC TGC ACT ATC TGT TTT G
	Taqman Probe	5'-FAM-CAG AAG AGA AAG TGA AAC ACA CTG AGG AA-TAMRA-3'

Quantification of plasma viral RNA

Viral RNA in plasma was quantified by either of two methods: (1) A Chiron Corporation branch DNA (bDNA) signal amplification assay, version 4.0, specific for SIV [199]. Viral load in the plasma is reported as copies of viral RNA per milliliter of plasma with a limit of detection of 125 copies per milliliter of plasma. (2) By quantitative real-time PCR. RNA was purified from plasma using QIAmp Viral RNA Mini kit (Qiagen, Valencia, CA). The quantitation of SIVmac viral RNA was determined using the RNA Ultrasense One-Step Quantitative RT-PCR System (Invitrogen, Carlsbad, CA) and Taqman methodology. Purified RNA was combined with a RT-PCR reaction mixture consisting of RNA Ultrasense reaction mix, enzyme mix, ROX reference dye, probe, and primers with the following sequences: forward primer 5'-GCCAGGATTTCAGGCACTGT-3', reverse primer 5'-GCTTGATGGTCTCCCACACAA-3' and probe 5'-FAM-AAGGTTGCACCCCCTATGACATTAATCAGATGTTA-TAMRA-3' [179]. The amplification reaction was performed in a 7500 Real Time PCR System (Applied Biosystems, Foster City CA) with the following cycling conditions: 48 °C for 15 minutes, 95 °C for 2 minutes, followed by 42 cycles of 95 °C for 15 seconds and 60 °C for 30 seconds. To generate a standard curve, plasmid DNA containing SIVmac239 Gag sequence was linearized, and in vitro transcribed RNA was produced using the

MEGAscript High Yield Transcription kit (Ambion, Austin TX). RNA concentration was determined spectrophotometrically at OD260. RNA was serially diluted and used in the quantitative Taqman real-time PCR assay to obtain the standard curve. Viral load was determined by comparing Ct values of the standards with known copy number to Ct values of samples. The sensitivity of the assay was 5-10 copies or approximately 500 copies/mL of plasma.

Quantitative real-time PCR for immune modulators

Quantitative real-time PCR was utilized to determine changes in gene expression of several immune modulators including IFN-α, IFN-γ, IL-8, IL-10, IL-12, CXCL9 (Mig), CXCL10 (IP-10), tumor necrosis factor alpha (TNF-α), transforming growth factor beta (TGF-β) and 2'-5' oligoadenylate synthetase (OAS, as described previously [1, 2]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as housekeeping gene. Briefly, gene-specific primers and probes or TaqMan® Gene Expression Assays (Applied Biosystems, Foster City CA) (Table 2-1) were mixed with TaqMan® Universal PCR Master Mix, (Applied Biosystems, Foster City CA) and nuclease-free water and run on a 7300 or 7500 Real-time PCR system (Applied Biosystems, Foster City CA). Fold changes in expression of these genes in SIV-infected macaques compared to uninfected animals

was calculated utilizing delta cycle threshold (ΔCt) values [2, 208]. Briefly, the GAPDH Ct value was subtracted from the Ct value of the target gene generating a ΔCt value. Per gene, an average of ΔCt values was derived for uninfected macaques and subtracted from the ΔCt value of the same gene for an infected macaque to achieve the ΔΔCt value. Fold induction for that gene was then determined by the following formula: $2^{-\Delta\Delta Ct}$ [14]. In the event that the ΔΔCt value was positive, indicating the fold change was less than 1 (a negative fold change), the fold change value was calculated using the following formula: $(-1)*2^{\Delta\Delta Ct}$ (For example, a ΔΔCt value of 3 would result in a fold change of $2^{-3} = 0.125$. To display this fold change graphically as a negative change, the formula would be $(-1)*2^3$ or a -8 fold change). An average fold induction and standard deviation of the target gene was calculated for the uninfected macaques and used to determine whether the target gene in the infected animal was increased or decreased. Changes in mRNA expression of a target gene in an infected macaque was deemed increased or decreased if its fold change was greater than two standard deviations away from the average of the uninfected controls.

Flow cytometric assessment of surface and intracellular markers

6-color flow cytometry was utilized to assess surface and intracellular markers on macaque lymphocytes. Whole blood was stained using directly fluorescent-conjugated human antibodies, cross-reactive to Rhesus macaque cell surface and intracellular proteins. The flow panels utilized are listed in Table 2-2: panels 3-1 and 3-2 were used for chapter 3 and panels 4-1 and 4-2 for chapter 4.

For cell surface staining, 100 µL whole blood was incubated with a panel of fluorochrome-conjugated mAbs in an enclosed compartment under light exclusion for 15 minutes at room temperature. Red blood cells were lysed by a standard whole-blood hemolyzation method using 4 ml of erythrocyte lysing solution (1X BD FACSTM Solution, BD-Biosciences, San Jose, CA), washed twice with PBS, pH 7.4 and fixed with 1.6% methanol free-formaldehyde/PBS (Polysciences inc., Warrington, PA).

Intracellular staining with FITC-labeled Ki67 was performed in separate tubes to analyse cell proliferation in the mayor lymphocyte populations. Ki67 was added after surface staining of 100 µL whole blood, fixation (1X BD FACSTM Solution) and permeabilization with 1X BD PharmLyseTM Solution and incubated at room temperature in an enclosed compartment under light exclusion for 30 minutes. Cells were washed and fixed in 1.6 % formaldehyde as described above. Test tubes

were kept refrigerated until data was acquired using either a CyAnTM ADP instrument (Beckman Coulter Inc, Fullerton, CA) or a BD LSR II (BD Biosciences, San Jose CA). For general phenotypes 10000 events were acquired, and for proliferation analyses 30000-50000 events were recorded. Electronic compensation and analyses was done with either the Summit Software (Version 4.3, Beckman Coulter Inc) or FACS DIVA (BD Biosciences, San Jose CA).

Table 2-2. Panels used for flow cytometry.

Panel 3-1	
mAb specificity	**Fluorescent Dye**
CXCR3	FITC
Ki67	PE
CD8	PerCP
CCR7	PE-Cy7
CD4	APC
CD3	APC-Cy7

Panel 3-2	
mAb specificity	**Fluorescent Dye**
CXCR3	FITC
CD8	PE
HLA-DR	PerCP
CD69	PE-Cy7
CD4	APC
CD3	APC-Cy7

Panel 4-1	
mAb specificity	**Fluorescent Dye**
CD95	Alexa Fluor 488
CD195 (CCR5)	PE
CD4	PerCP-Cy5.5
CD28	APC
CD3	Alexa Fluor 700
CD8	Pacific Blue

Panel 4-2	
mAb specificity	**Fluorescent Dye**
Ki67	FITC
CD4	PerCP-Cy5.5
CD16	APC
CD3	Alexa Fluor 700
HLA-DR	APC-H7
CD8	Pacific Blue

Immunofluorescence assays

Four micron paraffin sections were cut using a Leica 2235 microtome (Leica Microsystems, Bannockburn, IL) and placed on charged slides for immunofluorescence assays. Double fluorescent staining was performed on formalin-fixed, paraffin-embedded tissues by incubating sections with mouse anti-human Ki-67 and rabbit anti-human CD3 antibodies (DakoCytomation, Glostrup Denmark), followed by the appropriate goat anti-mouse or goat anti-rabbit secondary antibodies labeled with Alexa Fluor 488 and Alexa Fluor 568, respectively. Negative controls included normal and infected tissues incubated with anti-rabbit or anti-mouse secondary antibodies only. Stained slides were read on a Leica TCS SP2 laser scanning confocal microscope system (Leica Microsystems, Wetzlar, Germany). Double positive cells were manually counted in lymph nodes, oral mucosa and rectal mucosa (or intestinal lamina propria when rectal mucosa was not available) of normal and SIV-infected Rhesus macaques. At least ten 40x fields were counted per macaque and time point. Results were reported as the mean CD3+Ki67+ double positive cells per 40x power field.

Quantification of immune modulators in plasma

To measure concentrations of immune modulators in plasma, the Luminex assay was utilized. This multiplex assay enables the quantification of multiple analytes in the same sample by utilizing uniquely fluorescent-colored beads, of which each bead is specific for one analyte. Plasma was incubated with a mixture of beads, washed, and incubated with a second set of analyte-specific biotinylated antibodies. After an additional washing step, Streptavidin-Phycoerythrin (PE) was added and the beads were analyzed in a Luminex 100 or 200 system (Invitrogen, Carlsbad CA). Positive signals are measured as events that are double positive for both PE and the unique fluorescence of the analyte-specific bead. Standards of know concentrations were used to quantify the immune modulators in plasma, which are reported as pg/mL.

SIV envelope-specific antibody endpoint titer

Plasma antibody reactivity to detergent disrupted SIVsmB7 envelope proteins [166] was determined in a conconavalin A (ConA) ELISA as previously described [56]. Briefly, SIVsmB7 viral envelope proteins (gp120 and gp41) were captured onto 96 well microtiter plates (Immulon 2HB; Dynex Technologies, Chantilly, VA) coated with ConA. After washing with phosphate-buffered saline (PBS), non-specific binding was blocked by the

addition of dry milk in PBS (blocking solution) to all wells. Heat-inactivated plasma samples were serially diluted in blocking solution and incubated in the SIVsmB7 envelope-coated wells. After an extensive washing, peroxidase-conjugated anti-monkey IgG (Nordic Immunology Laboratories, Tilburg, Netherlands) was diluted in blocking solution, added to each well, incubated and washed. Following the final wash step, all wells were incubated with TM Blue substrate (Seracare, Milford, MA), color was developed by the addition of sulfuric acid, and colorimetric analysis of antibody binding to SIVsmB7 was performed at an optical density of 450 nm (OD450) using a Spectra Max 340 PC (Molecular Devices, Sunnyvale, CA). Endpoint titers were determined to be the last two-fold dilution with an OD450 twice that of normal monkey serum and are reported as the log10 of the reciprocal endpoint titer.

Heteroduplex mobility assay

The heteroduplex mobility assay (HMA) was used to determine the genetic variability of transmitted viral variants in the orally inoculated Rhesus macaques, as previously described [66, 67]. Briefly, genomic DNA isolated from PBMC of acutely infected macaques was used in a nested PCR reaction (described above). The region of the SIV genome analyzed was within the V1-V2 region of the SIVenv gene, which shows a high level

of diversity during SIV infection [8, 221, 290]. The first round amplified a DNA fragment of 629 nucleotides, using the forward primer 5'-GGAGGAATGCGACAATTCCCCTCTT(TC)TGT-3' and the reverse primer 5'-CATTACATCTAAGCAAAGCATAACCTGG-3'. The second round amplified a DNA fragment of 540 bp using the forward primer 5'-CCCAATAATGTTTGTCACAAGACTC-3' and the reverse primer 5'-ACCAAGAATAGGGATACTTGGGG-3'. Each PCR fragment was combined with DNA annealing buffer (Tris [pH 7.5], NaCl, EDTA). The mixture was heated to 95°C for 1-2 min and transferred immediately to ice. This rapid cooling permitted the heteroduplexes to form, and these were separated by electrophoresis through a non-denaturing 5% polyacrylamide gel (250 V for 2 hr 45 min) and observed after staining with ethidium bromide.

Cloning and Sequencing

In addition to HMA, sequencing was used to clarify the genetic variability in the V1-V2 region of the SIVenv gene during acute phases of SIV infection. Genomic DNA was amplified via nested PCR for SIVenv (see above: Heteroduplex Mobility Assay). Fresh PCR product was ligated into pCR2.1 vector and transformed into TOPO competent DH5α cells, using the TOPO TA cloning kit for subcloning (Invitrogen, Carlsbad, CA). A mini

culture was incubated for 1 hour and plated on LB plates containing X-gal (for blue/white screening) and ampicillin. The next day, white colonies (contain PCR product) were picked and incubated with shaking in LB media with ampicillin overnight. Plasmid DNA was extracted the following day, using the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA) according to protocol. Briefly, bacteria were lysed, DNA was bound to a spin column, washed and eluted with nuclease-free water. An aliquot of each sample was restriction-digested and run on an agarose gel to determine presence and size of the PCR insert. Plasmid DNA containing inserts of the expected size were sent to the sequencing facility. Sequencing was performed on a 3730xl DNA Analyzer (Applied Biosystems, Foster City CA) with the following cycling conditions: 1 cycle of 96 °C for 1 min, followed by 25 cycles at 96 °C for 10 sec, 50 °C for 5 sec and 60 °C for 4min. The samples were then kept at 4 °C. Sequences were returned as color-coded electropherogram files and text files and analyzed using MEGA 4.1 (www.megasoftware.net, Center for Evolutionary Functional Genomics, Tempe, AZ). The same program was used to generate phylogenetic trees.

CHAPTER THREE

Immune and viral changes at different tissue sites during progression to simian AIDS.

Introduction

Cytokines, chemokines and other immune modulators play important roles during viral transmission, replication and spread. The model of SIV infection in Rhesus macaques has been particularly useful in assessing the role of immune mediators in different tissue sites at early times following SIV infection [4, 5, 208]. The expression of immune modulators has the potential to impact both SIV transmission as well as SIV induced disease progression. The timing, location, as well as the type of immune modulator elicited have been demonstrated to play crucial roles in this outcome [1-3, 5, 104, 171, 268]. For example, the expression of interferons, pro-inflammatory cytokines and chemokines at the mucosal challenge site can be positively associated with viral replication [4, 5]. Also, increased expression of innate immune modulators in blood and other tissues (e.g. LN, lungs) has been observed during acute and chronic SIV infection [1, 5, 268]. In contrast, assessment of GALT of SIV infected macaques has indicated an inverse association between mRNA

expression of immune modulators and viral replication, as increased gene expression was generally associated with a decrease in viral burden [104]. In addition, vaccine-induced protection is associated with differential innate gene expression at tissue sites that leads to variable outcomes with regard to SIV viral levels [2, 3, 171]. At the oral mucosa, it has been demonstrated that an attenuated SIV vaccine can induce protection following tonsillar inoculation of SIV in Rhesus macaques and was associated with two immune cells, γδ T cells and mature DCs [294].

The activation state of the immune system is likely to be critical for both the frequency of the transmission event as well as the rate that an SIV/HIV infected host progresses to AIDS. During HIV infection an association between immune activation, as defined by the activation state of T cell populations, was first described as potentially having an influence on disease progression by Ascher and Sheppard in 1988 [16] and later by Giorgi and colleagues [108, 187]. These findings have been verified in more recent studies that identify a clear association between the activation state of the immune system and the rate at which HIV-infected patients progress to AIDS [97, 122, 280, 283]. However, most of these studies have assessed T cell activation, only few have examined soluble factors as markers for immune activation and disease progression [18, 142, 169, 284]. Furthermore, low levels of immune activation were associated with a

lack of clinical signs of AIDS in natural hosts of SIV infection, like Sooty mangabeys and African green monkeys [207, 228, 277]. The activation state of the immune system also likely serves a key role for the earliest events of HIV/SIV transmission, viral spread and acute-phase disease progression, although less is known about these earliest events post-infection.

For the first part of this study, Rhesus macaques were infected with SIV via the oral route and followed throughout disease, permitting an assessment of the innate immune responses at different tissue sites to clinical disease outcome. At different time points, expression levels of several immune modulators were assessed at different tissue sites. Earlier studies focused primarily on the oral and rectal mucosal tissues [208], whereas this study focused primarily on LNs and blood, which were assessed for immune modulators during both the acute and chronic phases of the infection. Previous studies by Dr. Kristina Abel assessed expression patterns of 17 different immune modulators in the SIV macaque model [1-3, 5]. Based on their significance for the oral transmission studies in the Sodora laboratory, 13 immune modulators were chosen to assess innate immune responses at the oral and rectal mucosa [208]. Due to their expression profiles, several of these were of particular interest, including IFN-α, an antiviral cytokine, produced mainly

by plasmacytoid dendritic cells upon recognition of viral infection that induces interferon-stimulated genes (ISG) to limit viral replication, and 2'-5' oligoadenylate synthetase (OAS), an ISG that can activate RNase L to degrade viral RNA. Previous studies had indicated that their expression at lymphoid tissues was not sufficient to control viral replication [1], however, expression in peripheral blood was associated with vaccine-induced protection against viral challenge [2]. In contrast, elevated IFN-γ, CXCL9 and CXCL10 expression in lymphoid tissues was associated with viral replication and a lack in vaccine-induced protection against viral challenge [3]. CXCL9 (Monokine induced by IFN-γ, Mig) and CXCL10 (Interferon inducible protein of 10 kD, IP-10) are two pro-inflammatory chemokines that signal through a common receptor, CXCR3, to recruit activated T cells and NK cells to sites of inflammation. Therefore, increased expression in lymphoid tissues of the unprotected macaques might lead to recruitment of more target cells for SIV and thus drive viral replication. In contrast, increased expression of these immune modulators at mucosal sites was associated with slow disease progression [208]. Due to these findings, these immune modulators were chosen for the studies presented here to assess their expression at lymphoid tissues and peripheral blood and their association with disease progression. To obtain a diverse representation of immune responses, the following additional immune modulators were

included in the analysis: IL-12 is produced by dendritic cells and macrophages during innate immune responses and is able to activate NK cells as well as to induce CD4+ T cells differentiation into TH1 cells; IFN-γ, produced by T cells and NK cells, is able to activate macrophages; TNF-α is produced by macrophages and T cells and can activate macrophages and induce inflammation and apoptosis; and IL-10 that is expressed by monocytes and T cells and has anti-inflammatory functions. The studies presented here indicated that four were particularly interesting: IFN-α, OAS and CXCL9 (Mig) and CXCL10 (IP-10). They exhibited distinct patterns of expression at oral mucosa, lymph nodes and peripheral blood that correlated with the rate of disease progression.

Results for OAS and CXCL10 were especially interesting and therefore, in the second part of this study, their expression in peripheral blood of Rhesus macaques and sooty mangabeys was examined to evaluate their usefulness as markers of disease progression. Taken together, assessment of innate cytokines/chemokines in tissues and peripheral blood will be important for unraveling clues to HIV/SIV disease progression that could lead to novel therapy and vaccine approaches.

Results

Oral SIV infection results in varied clinical outcomes.

To assess correlates of disease progression, six Rhesus macaques were non-traumatically orally inoculated with SIVmac251 and followed until they exhibited signs of simian AIDS (sAIDS). The macaques were divided into three groups and follow the same color scheme in the graphs throughout this study: The rapid progressors (RM11, dark red; RM13, pink; and RM14, orange), intermediate progressors (RM12 and RM15, grey), and one slow progressing macaque (RM16, dark green) that did not develop sAIDS for more than 2 years (Figure 3-1A).

Elevated levels of plasma viral loads were associated with more rapid disease progression: the three rapid progressors exhibiting the highest peak and set-point viral loads whereas the slow progressing macaque (RM16) had the lowest peak and set-point viral loads (Figure 3-1B). Similarly, LN-associated viral loads were generally associated with disease progression (Figure 3-1C). The most rapidly progressing macaque (RM11) had persistently increasing LN-associated viral load throughout infection and had the highest LN-associated viral load of all macaques at day 84 post-infection (220,000 RNA copies/106 GAPDH). In contrast, the slow progressor (RM16) maintained low LN-associated viral loads throughout infection and exhibited the lowest viral load at day 84

post-infection (11,000 RNA copies/106 GAPDH). The additional macaques exhibited intermediate LN-associated viral loads and intermediate rates of disease progression

Cytokine/Chemokine expression in oral gingival tissue and lymph nodes.

Expression of innate immune modulatory genes was assessed at both the peripheral LNs (axillary and inguinal) and oral mucosa. The LN biopsies were collected at four time points that were grouped into the early acute phase (7 or 15 days post-infection (dpi)), the later acute phase (21 or 28 dpi), the early chronic phase (45 or 56 dpi) and a final time point that is within the chronic phase of the infection (84 dpi). The range at each phase, for example either taking a sample at 7 or 15 dpi in different macaques, was built into the biopsy recovery plan to optimize the information utilizing a relatively small number of macaques. The oral mucosal biopsies were obtained at three time points (2 or 4, 14 or 21 as well as 70 dpi).

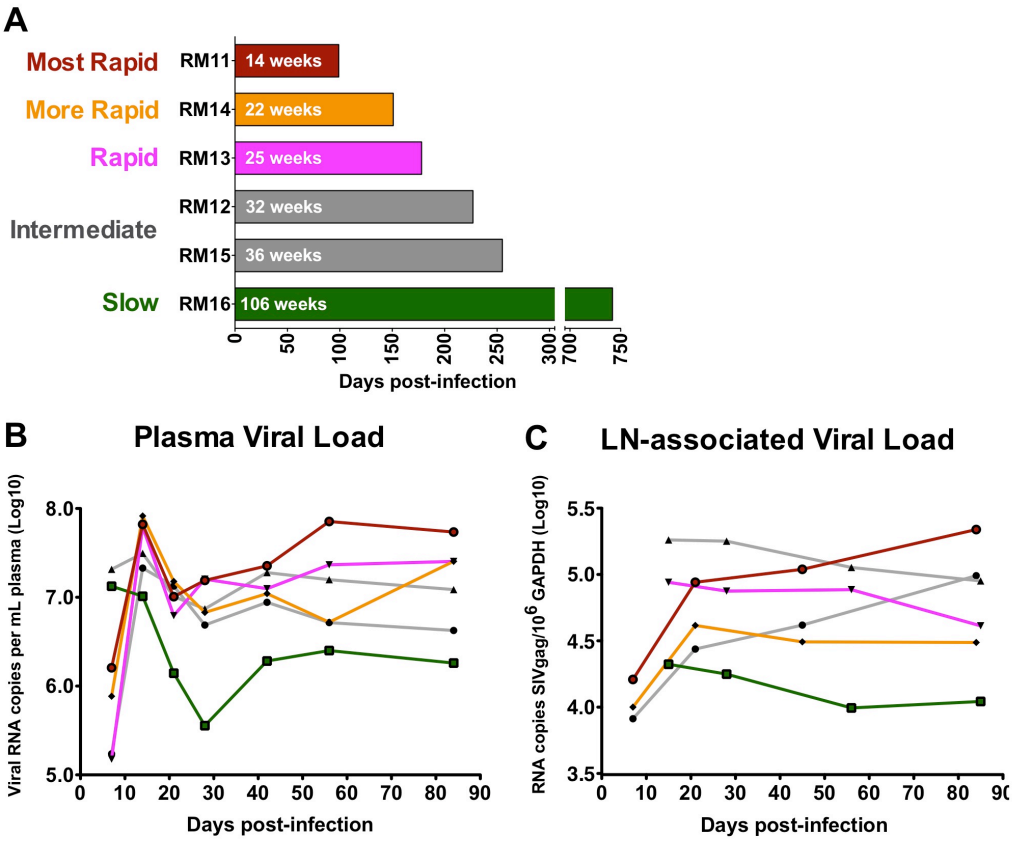

Figure 3-1. Characterization of orally SIV inoculated Rhesus macaques.

(A) Time to progression to simian AIDS. The most rapidly progressing macaque (RM11, dark red) developed simian AIDS after only 13 weeks, followed by two animals progressing to disease within 22 (orange) and 25 (pink) weeks. Two macaques, shown in grey bars (RM12 and RM15), progressed at an intermediate rate (32 and 36 weeks) whereas another macaque did not exhibit any signs of simian AIDS for more than two years (RM16, green). Same color scheme was utilized in other Figures. (B) Plasma viral load, shown as viral RNA copies per mL plasma, measured by bDNA assay. (C) LN associated viral loads, shown as SIVgag RNA copies per 10^6 copies of GAPDH, measured by qRT-PCR.

LN and oral mucosal biopsies were assessed for mRNA levels of eight cytokines and chemokines by real-time PCR: IFN-α and OAS (2'-5' oligoadenylate synthetase) as antiviral modulators, the pro-inflammatory chemokines CXCL9 (Mig) and CXCL10 (IP-10), the pro-inflammatory cytokines IFN-γ, IL-12 and TNF-α as well as the anti-inflammatory cytokine IL-10. Ct values were utilized to determine the average and a two standard deviation range of expression for each gene in uninfected macaques. From this, fold change of gene expression was calculated in the infected compared to uninfected macaques. These fold changes are considered increased or decreased when they are away more than two standard deviations from the average expression in the uninfected macaques. Increases above the two-standard deviation range are indicated by symbols within the blue boxes in Figures 3-2, 3-3 and 3-6.

Expression of these genes at the oral mucosa (Figure 3-2) indicated that in the rapidly progressing macaques, expression of the immune modulators IFN-α, OAS, CXCL9 and CXCL10, was generally similar to uninfected controls, whereas these immune modulators were generally expressed at much higher levels in the slow progressing macaque (Figure 3-2 green circles, up to 730-fold higher than uninfected controls). The slow progressor also exhibited elevated levels of IFN-γ, IL-12 and TNF-α at the oral mucosa for at least one time point, whereas

the levels of IL-10 remained similar in all SIV+ macaques and did not differ from uninfected control macaques (Figure 3-2). The intermediate progressing macaques exhibited mRNA expression levels generally intermediate between the rapid and slow progressing macaques at the oral mucosa (Figure 3-2).

The LNs represent a tissue site important for both initiating immune responses and as a site of viral replication. Compared to uninfected control macaques, the expression of IFN-α, OAS, CXCL9 and CXCL10 at LN was generally upregulated in the rapidly progressing macaques at all time points assessed as demonstrated by symbols from these macaques appearing above the two standard deviation cut-off (Figure 3-3, within blue boxes). In contrast, these same immune modulators were not upregulated compared to uninfected control macaques in the slow progressing macaque. Expression levels of IFN-γ, IL-12, TNF-α and IL-10 in LN were comparable to uninfected control macaques in the SIV+ macaques irrespective of the rate of disease progression (Figure 3-3).

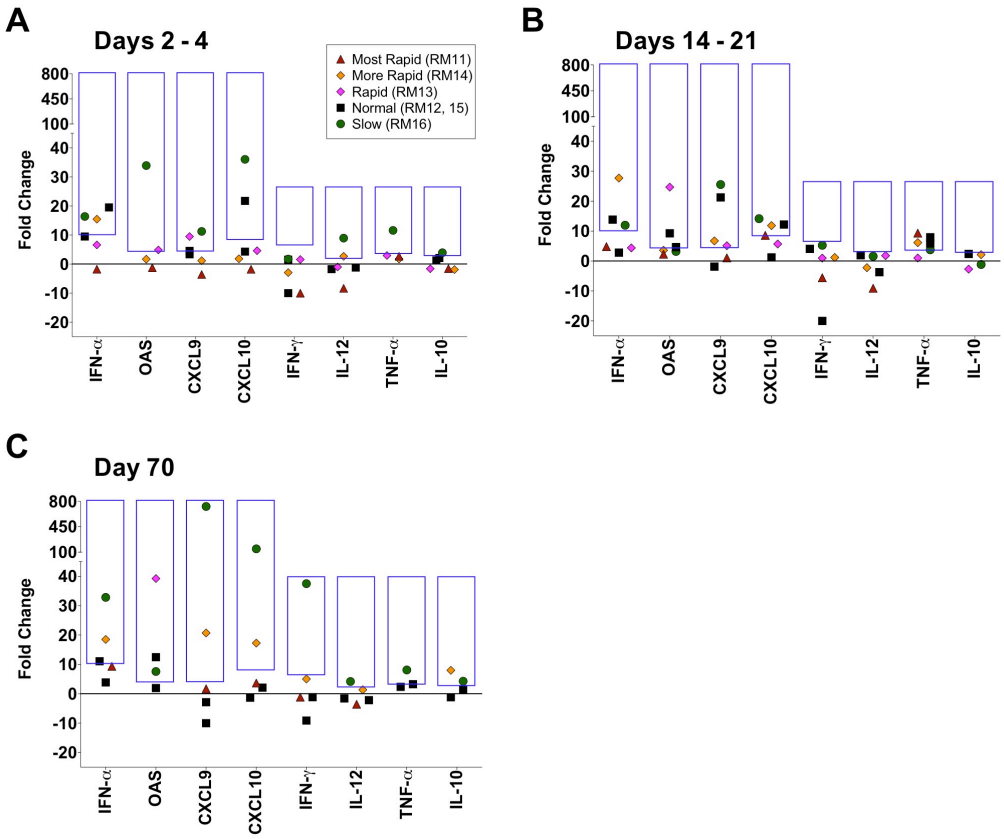

Figure 3-2. Fold changes in immune response gene mRNA expression at the oral mucosa.

Shown are the fold changes of several immune response genes (from left: IFN-α, OAS, CXCL9, CXCL10, IFN-γ, IL-12, TNF-α, IL-10) at the oral mucosa of orally infected macaques at different time points throughout disease progression: 2 – 4 days (A), 14 – 21 days (B) and 70 days post-infection (C). The most rapidly progressing macaque is represented by the red triangles, the second fastest progressor is shown as orange diamonds, and the third with pink diamonds. The two intermediately progressing monkeys are represented by black squares, and green circles show the slow progressor. Symbols within the blue boxes represent mRNA expressions, which are increased more than two standard deviations away from the expression in uninfected animals.

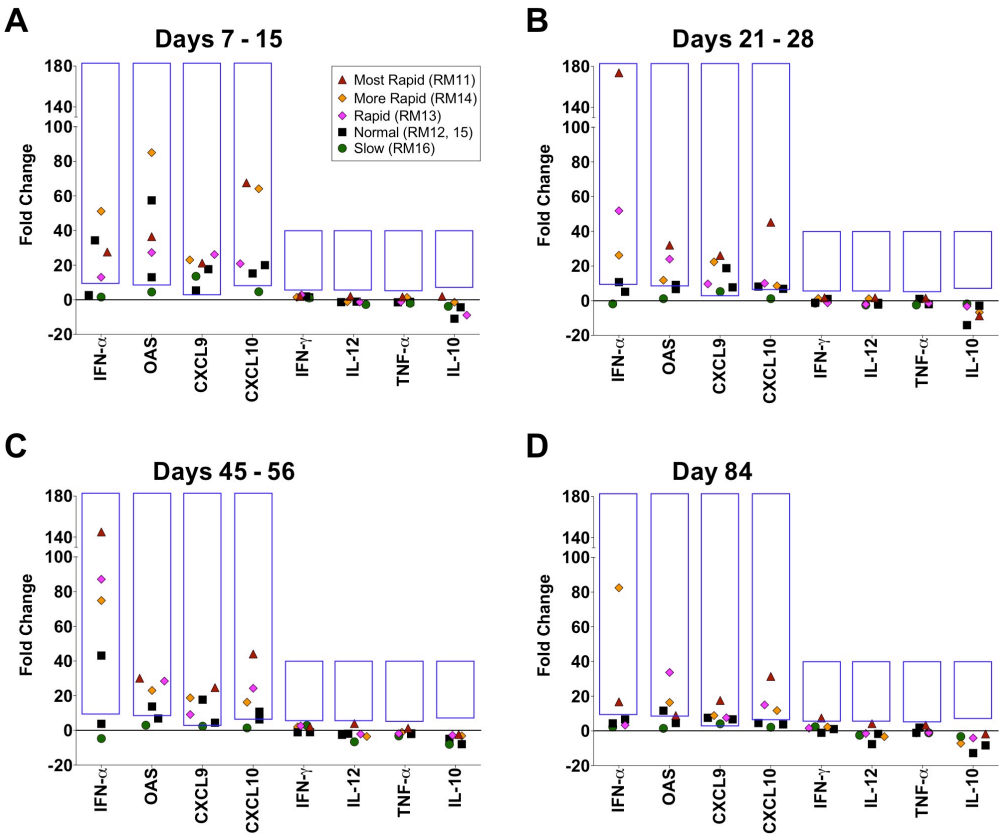

Figure 3-3. Fold changes in immune response gene mRNA expression in LN.

Shown are the fold changes of several immune response genes (from left: IFN-α, OAS, CXCL9, CXCL10, IFN-γ, IL-12, TNF-α, IL-10) in the LN of orally infected macaques at different time points throughout disease progression: 7 – 15 days (A), 21 – 28 days (B), 45 – 56 days (C) and 84 days post-infection (D). See Figure 3-2 for explanation of the color-coding.

Presence of CXCR3+ cells in peripheral lymph nodes.

The chemokines CXCL9 and CXCL10 function by inducing chemotaxis in cells through the binding of their cognate chemokine receptor CXCR3, which is expressed on activated T cells (particularly TH1), natural killer (NK) and plasmacytoid dendritic cells (pDC) [32, 87, 139, 188]. Thus, high levels of CXCL9/10 in LN of rapid progressors suggest that CXCR3+ cells may be recruited to the LN. To determine the levels and phenotypes of CXCR3+ cells in LN, the percentages of CD4+ and CD8+ T cells expressing CXCR3 (Figure 3-4) as well as proliferation (as measured by Ki67) and activation status (as measured by CD69 and HLA-DR) (Figure 3-5) were assessed utilizing flow cytometry. Despite the increased mRNA expression of CXCL9 and CXCL10 in the LN of the rapidly progressing macaques (RM11, RM14, RM13), there was no evidence of increased recruitment of CXCR3+ CD4+ T cells (Figure 3-4A) or CD8+ T cells (Figure 3-4B) compared to the LN of the slow progressor.

Furthermore, the CXCR3+ cells did not generally exhibit any differences with regard to proliferation (Ki67, Figure 3-5A, B) and activation (CD69, Figure 3-5C, D; HLA-DR, Figure 3-5E, F) when compared between the rapid and slow progressing macaques. These findings indicate that there is no correlation between the mRNA

expression of chemokines and the recruitment of their respective target cells at the LNs of SIV+ macaques.

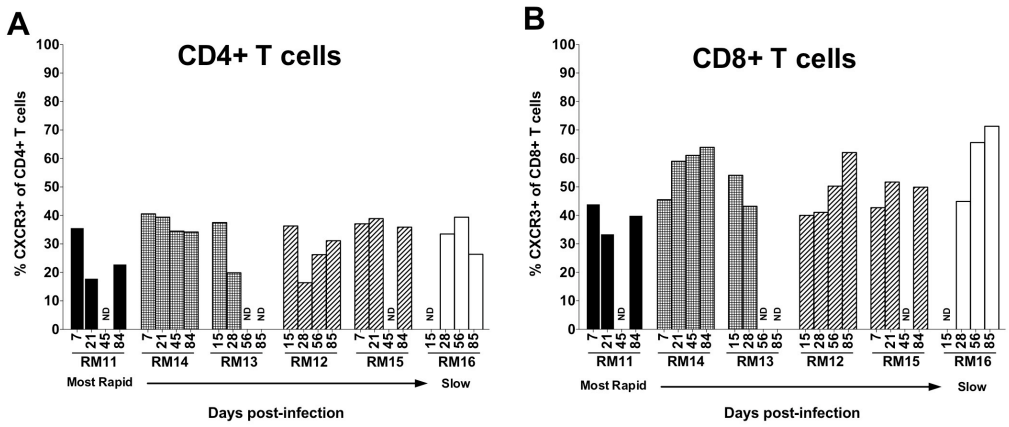

Figure 3-4. CXCR3 expression on LN mononuclear cells.

Expression of the chemokine receptor CXCR3 was assessed by flow cytometry on the CD4+ T cell subset (A) and the CD8+ T cell subset (B) of LN mononuclear cells. Each bar represents one time point of one macaque. ND – Not Determined

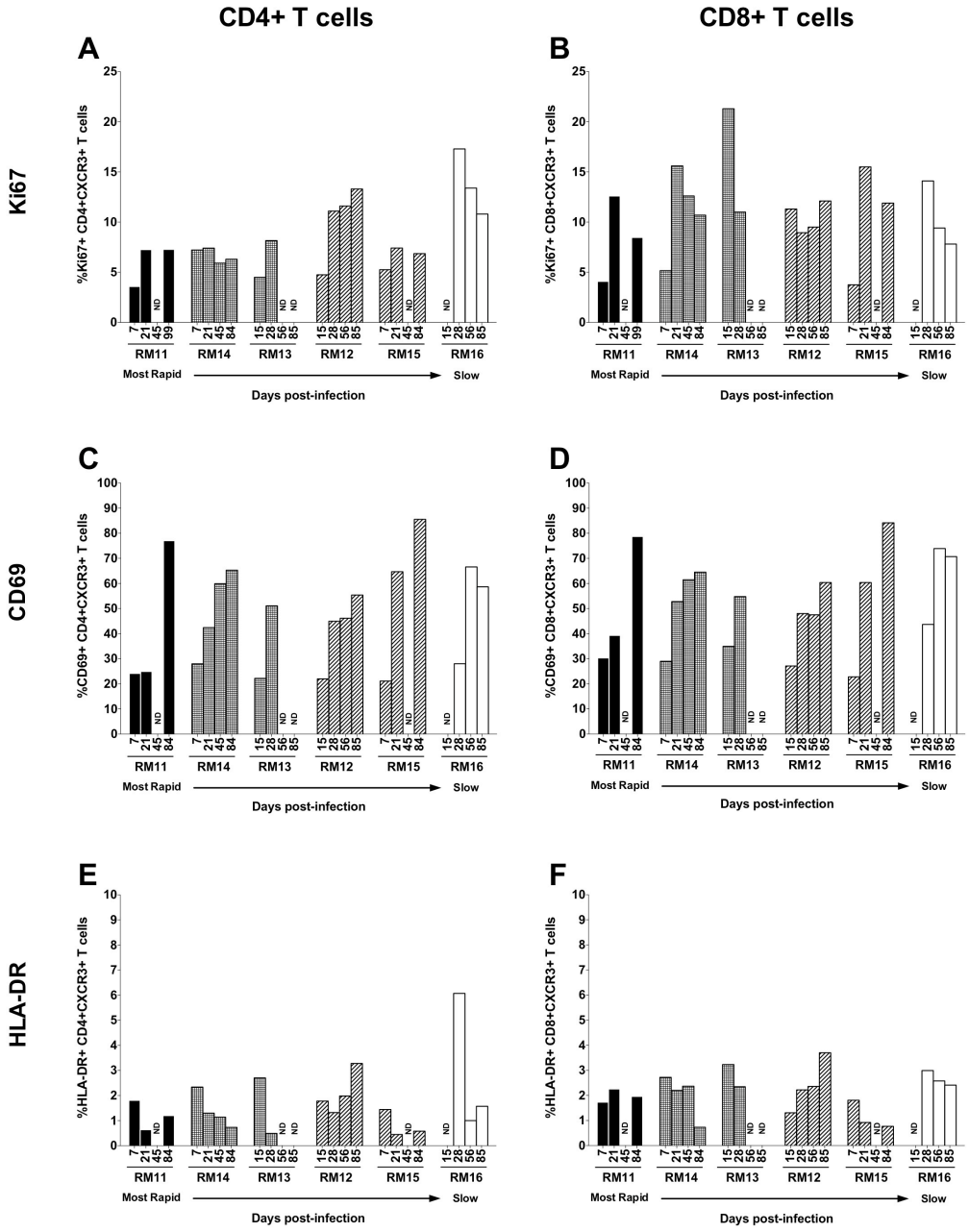

Figure 3-5. Proliferation and activation of CXCR3+ CD4+ and CD8+ T cells.

Expression of the proliferation marker Ki67 and the activation markers CD69 and HLADR were assessed by flow cytometry on CXCR3+CD4+ T cells (A) and CXCR3+CD8+ T cells (B) of LN mononuclear cells. Each bar represents one time point of one macaque. ND – Not Determined

Expression of immune modulators in peripheral blood

Peripheral blood is easy to obtain and often, especially in human studies, the only tissue available. Expression of the same immune modulators as described above was assessed in PBMCs to identify any associations with the rate of disease progression. The results did exhibit some similarities to what was observed in the LNs, however there were some interesting differences as well (Figure 3-6). Whereas the mRNA levels of four of these immune modulators increased in the LNs of macaques with more rapid AIDS progression, only two were elevated within the peripheral blood cells, OAS and CXCL10. This increased expression of OAS and CXCL10 in the peripheral blood of the SIV+ macaques was generally observed within the more rapidly progressing macaques (symbols within blue boxes in Figure 3-6). These immune modulators were not upregulated and similar to uninfected controls in the slowest progressing macaque (Figure 3-6, green circles). The other five immune modulators examined in PBMCs, IFN-α, CXCL9, IFN-γ, TNF-α and IL-10, generally remained similar to levels in uninfected control macaques (Figure 3-6). These findings indicate that out of the genes tested here, mRNA levels of OAS and CXCL10 (IP-10) were associated with disease progression.

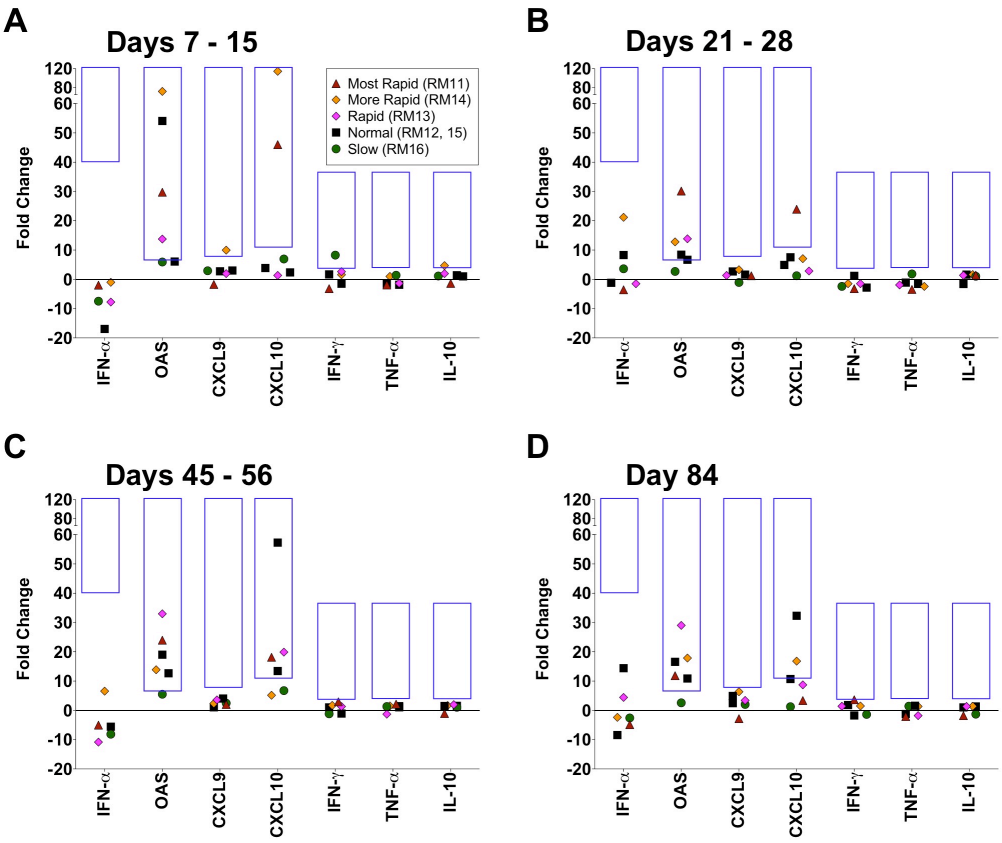

Figure 3-6. Fold changes in immune response gene mRNA expression in PBMCs.

Shown are the fold changes of several immune response genes (from left: IFN-α, OAS, CXCL9, CXCL10, IFN-γ, IL-12, TNF-α, IL-10) in PBMCs of orally infected macaques at different time points throughout disease progression: 7 – 15 days (A), 21 – 28 days (B), 45 – 56 days (C) and 84 days post-infection (D). See Figure 3-2 for explanation of the color-coding.

OAS, CXCL10 and TNF-α mRNA expression at LN, oral mucosa and peripheral blood during acute SIV infection

Since OAS and CXCL10 mRNA expression were upregulated in LNs and PBMCs of those SIV+ macaques that progressed more rapidly to disease, a temporal assessment of these data in each of these tissues was undertaken including the oral mucosa, which has been studied in detail previously [208] (Figure 3-7). The data indicate that CXCL10 and OAS mRNA levels in LNs were generally highest at the earliest time points in the more rapidly progressing macaques (elevated beyond the two standard deviation range) and decline over time (Figure 3-7A, B). In contrast, the CXCL10 mRNA levels at the oral mucosa were highest in the slowest progressing macaque for each of the time points, with elevated levels of CXCL10 most evident at the last time point assessed in this macaque (70 dpi, 150-fold above uninfected levels) (Figure 3-7D). TNF-α mRNA levels are included here as an example of a cytokine/chemokine that did not exhibit any particular pattern with regard to disease progression; levels were similar for all SIV+ macaques (generally at levels comparable to uninfected controls) (Figure 3-7C, F, I).

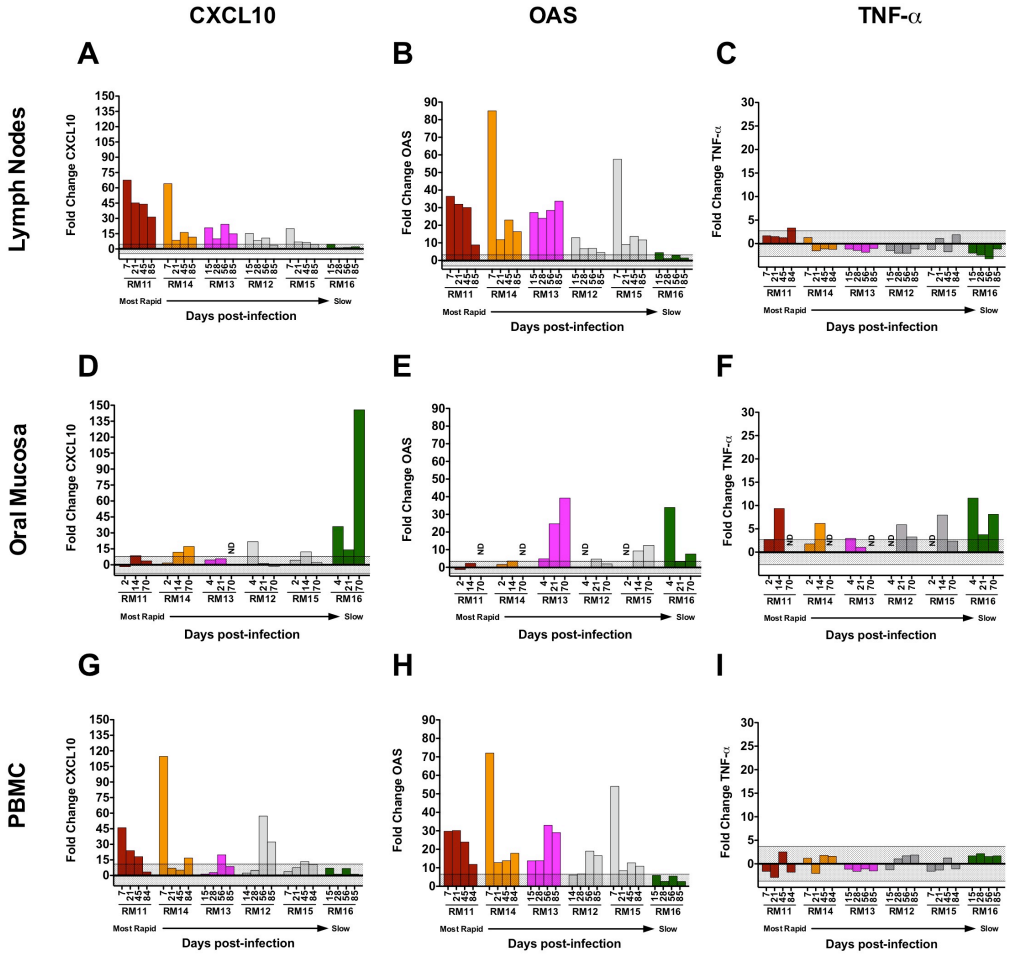

Figure 3-7. Detailed representation of fold changes for CXCL10, OAS and TNF-α.

Fold changes of mRNA expression are shown for CXCL10 (A, D, G), OAS (B, E, H) and TNF-α (C, F, I) at lymph nodes (LN) (A, B, C), oral mucosa (D, E, F) and PBMC (G, H, I). Each bar represents one time point of one macaque. The fold change is considered increased or decreased in infected compared to uninfected macaques when it is greater than two standard deviations (shaded area) away from the average expression in uninfected macaques. ND – Not Determined

One goal of these studies was to ascertain if the cells obtained from the peripheral blood exhibited mRNA levels of these immune modulators that were similar to either the LNs or the oral mucosa. These data indicate that the mRNA levels of CXCL10, OAS and TNF-α are strikingly similar to the data obtained from the LNs, and distinct to the findings from the oral mucosa (Figure 3-7G, H, I). This would indicate that peripheral blood cells have a general up-regulation of CXCL10 and OAS in the more rapidly progressing SIV+ macaques, and lower levels of these immune modulators in the more slowly progressing macaques. TNF-α mRNA levels remained within the two standard deviation range and similar to the uninfected macaques, which is also more similar to the LNs. The other immune modulators were also assessed from the peripheral blood and none exhibited mRNA levels that were positively or negatively associated with the rates of disease progression (Figure 3-6). These data raise the possibility that the assessment of CXCL10 and/or OAS mRNA levels from the peripheral blood cells of SIV+ monkeys or HIV+ patients could provide useful information with regard to the potential for that host to progress rapidly to AIDS.

Comparison of CXCL10 and OAS expression in peripheral blood cells of chronically SIV infected Rhesus macaques and sooty mangabeys.

To assess the association between peripheral blood levels of select immune modulators and rates of progression to AIDS, a retrospective assessment of OAS and CXCL10 expression in six additional chronically SIV-infected Rhesus macaques (Figure 3-8A, C) was undertaken. These macaques had been intravenously inoculated with SIVmac239 resulting in both intermediate progressors to disease (RM1, RM2, RM4, RM5 and RM6) and a non-progressor (RM3) [214]. Of the five intermediate progressors three exhibited increased CXCL10 mRNA levels (Figure 3-8A) and all five had increased OAS mRNA levels in their peripheral blood cells (Figure 3-8C). Interestingly, the sixth macaque that did not exhibit any signs of simian AIDS even at four years post-infection (RM3, star), did not exhibit increased levels of these two immune modulators (Figure 3-8A and C). To further examine these immune modulators, sooty mangabeys were assessed, an African natural host species that replicate SIV to high levels yet do not exhibit any clinical signs of sAIDS [164, 207, 215, 228, 276, 277].

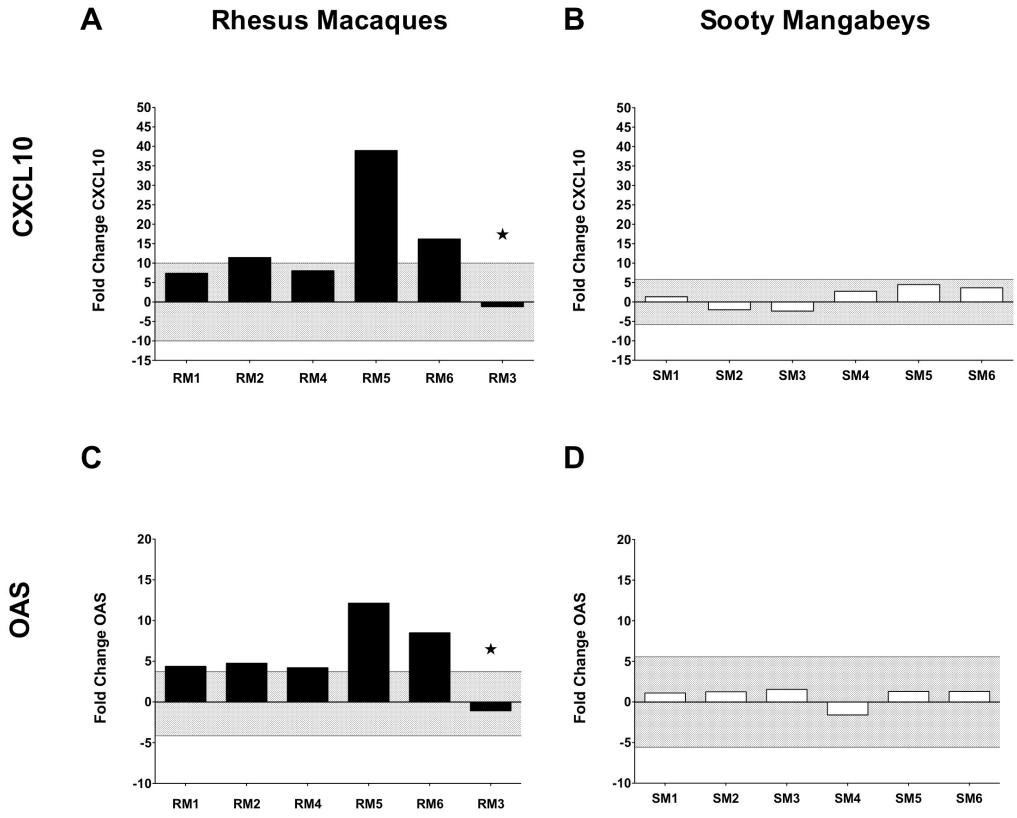

Figure 3-8. Expression of OAS and CXCL10 in PBMC of chronically infected Rhesus macaques and sooty mangabeys.

Fold changes of mRNA expression are shown for CXCL10 in PBMC of chronically (258 – 287 dpi) infected Rhesus macaques (A) and sooty mangabeys (B), as well as fold changes for OAS in macaques (C) and mangabeys (D). Each column represents one chronically infected animal with the star (★) indicating a long-term non-progressor macaque (RM3). The fold change is considered increased or decreased in SIV infected compared to uninfected macaques (A, C) or uninfected mangabeys (B, D) when it is greater than two standard deviations (shaded area) away from the average expression in uninfected animals of the respective species.

Six chronically infected mangabeys were assessed and the real-time PCR data was compared to six uninfected mangabeys to ascertain if the SIV infection altered the mRNA levels of OAS or CXCL10 within their peripheral blood. Similar to the slowly progressing SIV+ Rhesus macaques, the SIV infection of this natural host species did not result in any evidence for increased mRNA levels of OAS or CXCL10 within peripheral blood cells during chronic infection (Figure 3-8B, D).

Comparison of CXCL10, OAS and TNF-α expression in peripheral blood cells of acutely and chronically SIV infected Rhesus macaques.

The results from the chronically infected animals were encouraging to further test the potential of CXCL10 and OAS as diagnostic markers of disease progression by analyzing CXCL10, OAS and TNF-α expression in fifteen additional SIV-infected Rhesus macaques (Figure 3-9). These macaques had been intravenously inoculated with SIVmac239 [81] resulting in both intermediate progressors to disease (REi9, RHk8, RJj8, RJl9, RKb9, RWi8, RWu8, RZz8) and slow progressors (RCe8, RCo8, RDo8, RIf8, RNr8, ROu8, RUn8). PBMC samples were taken during acute (7 dpi) and chronic infection (168 dpi).

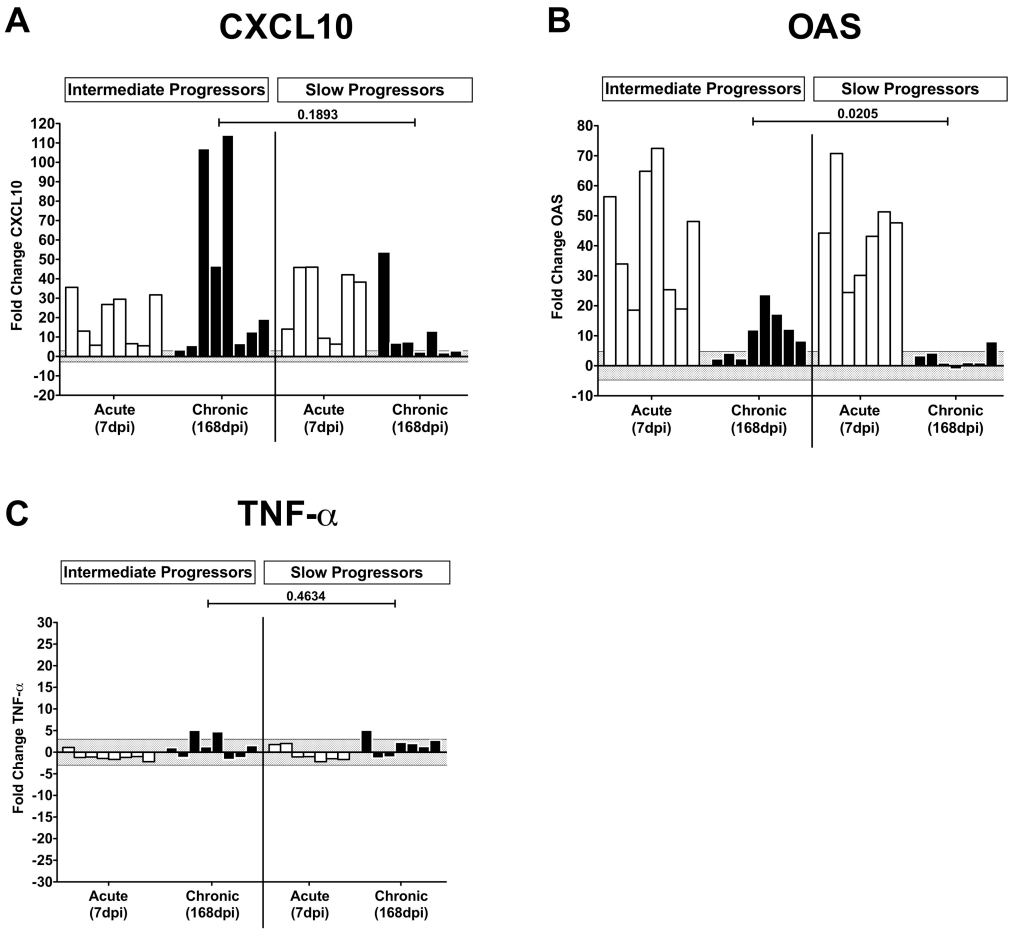

Figure 3-9. Expression of CXCL10, OAS and TNF-α in PBMC of acutely and chronically infected Rhesus macaques.

Fold changes of mRNA expression are shown for CXCL10 (A), OAS (B) and TNF-α (C) in PBMC of acutely and chronically infected Rhesus macaques, divided into intermediate and slow progressing macaques, with the acute time point on the left (open columns, 7 dpi) and the chronic time point on the right (shaded columns, 168 dpi) of each graph. Macaques representing intermediate progressors are (from left to right): REi9, RHk8, RJj8, RJl9, RKb9, RWi8, RWu8, RZz8. Macaques representing slow progressors are (from left to right): RCe8, RCo8, RDo8, RIf8, RNr8, ROu8, RUn8. Each column represents one time point of one infected macaque. The fold change is considered increased or decreased in SIV infected compared to uninfected macaques when it is greater than two standard deviations (shaded area) away from the average expression in uninfected macaques. OAS was statistically different between the chronically infected intermediate and slow progressors. Acutely infected animals were not statistically different for any of the three genes between the two groups.

Interestingly, expression levels of both CXCL10 (Figure 3-9A) and OAS (Figure 3-9B) did not differ between acutely infected Rhesus macaques progressing to disease at slow or intermediate rates. However, differences were observed during the chronic stage of the infection at day 168 post-infection, with the slower progressing macaques tending to exhibit lower levels of these two immune modulators. Indeed, a significant decrease was observed for OAS mRNA levels in the slow compared to the intermediate progressing macaques ($p = 0.0205$). TNF-α levels tended to remain within the range observed in uninfected controls, demonstrating that not all mRNA levels of inflammatory markers were elevated in these macaques (Figure 3-9C). These results identify the further potential for utilizing mRNA levels of CXCL10 and OAS as diagnostic indicators for hosts which would be likely to proceed rapidly to AIDS.

Expression of OAS and CXCL10 in different cell types of macaque PBMCs

To determine cell sources responsible for the mRNA upregulation of OAS and CXCL10 in the peripheral blood of the SIV-infected macaques a magnetic bead separation was utilized. Peripheral blood cells from SIV-uninfected and SIV-infected Rhesus macaques were divided into three categories: CD3+ T cells, CD14+ monocytes and the remaining CD3-

CD14- cell population, representing other PBMCs including B cells, NK cells and dendritic cells. The different cell subsets were sorted to greater than 90 % purity, RNA isolated and quantitative real-time PCR for OAS and CXCL10 mRNA expression was undertaken. Fold changes were calculated based on the expression of these two immune mediators in the same cell subsets from uninfected macaques. In the two SIV-positive animals assessed interesting findings were obtained: OAS expression in CD3+ (Figure 3-10A) and in CD3-CD14- cells (Figure 3-10C) of infected macaques was within the range of uninfected macaques. However, expression of OAS was increased by around 35-fold in CD14+ monocytes of both infected macaques (Figure 3-10B), indicating that this was the cell population responsible for the increase in OAS mRNA in PBMCs of SIV-infected macaques. Similarly, expression of CXCL10 was increased 25- and 15-fold, respectively, in CD3+ cells of the two infected macaques (Figure 3-10D), whereas expression of this gene in CD14+ (Figure 3-10E) and CD3-CD14- cells (Figure 3-10F) of infected macaques was within the expression range of uninfected macaques. These data indicate that the increase in CXCL10 mRNA levels in PBMCs of infected Rhesus macaques resulted from CXCL10 mRNA increases within CD3+ T cells. These findings provide evidence that distinct cell populations are responsible for the production of certain immune mediators, a finding that

will be followed up in future experiments being undertaken in the Sodora laboratory.

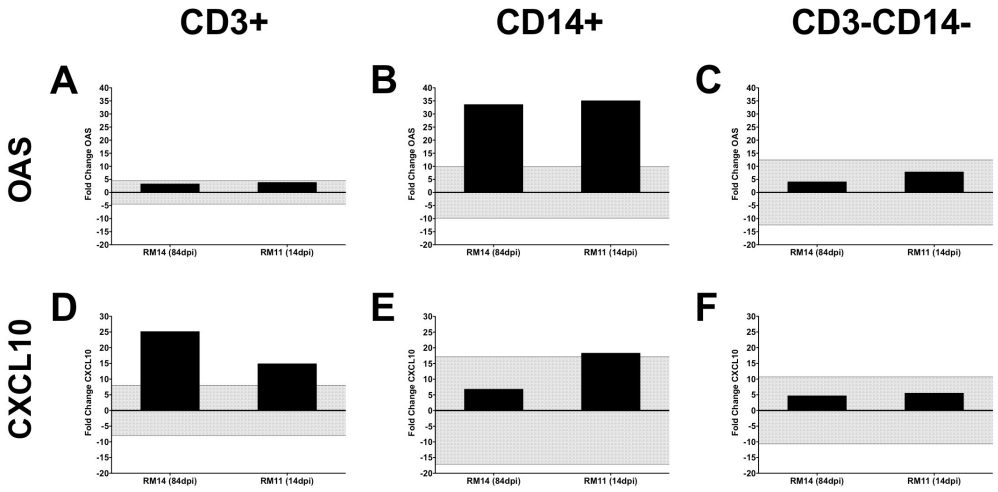

Figure 3-10. Expression of OAS and CXCL10 in different PBMC cell subsets.

Fold changes of expression of OAS (A, B, C) and CXCL10 (D, E, F) are shown in CD3+ cells (A, D), CD14+ cells (B, E) and CD3-CD14- cells (C, F). Expression of a gene in each cell type is compared to expression of that gene in the same cell type of uninfected Rhesus macaques. The fold change is considered increased or decreased in SIV infected compared to uninfected macaques when it is greater than two standard deviations (shaded area) away from the average expression in uninfected animals.

Discussion

Knowledge of the earliest events following SIV administration to mucosal sites will be critical for identifying new therapeutic and vaccine strategies. Often this information is acquired through the assessment of SIV infected macaques that are necropsied, enabling a careful analysis of just this one time point [1, 4, 5, 104, 206, 268]. The approach in this study enabled a characterization of the innate immune response at different tissue sites and different times throughout the acute phase of the infection, while still permitting an assessment with regard to the clinical outcome in these SIV+ macaques. After assessing mRNA levels of eight immune modulators, compelling findings were observed in four genes with roles in innate immunity: IFN-α, OAS and two inflammatory chemokines (CXCL9 and CXCL10). Interestingly, macaques that progressed the fastest to sAIDS exhibited the highest levels of these effector molecules in LNs, with generally lower levels at the oral mucosa. In contrast, the macaques that progressed the slowest to clinical signs of SIV infection exhibited the highest levels of these four effector genes at the oral mucosa, and generally lower levels in the LNs. In peripheral blood, expression of only OAS and CXCL10 was associated with disease progression whereas IFN-α and CXCL9 were expressed at similar levels across macaques.

It is possible that the increase in mRNA levels at mucosal sites, LNs or PBMCs is due to a differential increase of one or more cell types migrating to these sites and expressing these four immune modulators. Alternatively, the cells present at the different sites could express higher levels of certain immune modulators on a per-cell basis due to stimulation by viral products, cytokines or chemokines. In this context, it is important to note that the tissue types assessed here contain differing cell types. The gingival samples consist of epithelial cells and some immune cells, whereas in LN, the majority of cells are comprised of T and B cells as well as antigen presenting cells, such as macrophages and dendritic cells. The cell types assessed in peripheral blood are mononuclear cells comprised mainly of T cells, B cells, NK cells, and monocytes. These differences in cell composition likely play a key role in the differential expression of immune modulators observed at these different tissue sites as well as providing insights into the cell types expressing mRNA of the immune modulators being studied. In fact, results from this study indicate that at least in peripheral blood the main producer cell type for OAS are CD14+ monocytes whereas CD3+ T cells produce CXCL10. These findings are consistent with the fact that both cell types are susceptible to SIV (and HIV) infection. As an interferon response gene, OAS is produced in response to IFN-α/β in autocrine or paracrine signaling after viral infection.

In fact, it has been shown that IFN-β can induce monocytes to produce OAS to much higher levels than other lymphocytes [322].

Regardless of the cell types present at the different tissue sites, the higher levels of immune modulators at the mucosal site very early in infection might aid in an earlier initiation of adaptive immune responses. In fact, the slow progressing macaque exhibited an earlier and stronger induction of SIV-specific antibodies than the other macaques [208]. Additionally, the high levels of cytokines/chemokines at mucosal sites might contribute to an immune environment capable of preventing opportunistic infections, since levels stay high throughout infection. Together, this might lead to slower disease progression. It is interesting to note that other studies also have found benefits of immune responses at mucosal sites. Racz and colleagues showed that the presence of dendritic cells as well as γδ T cells in the tonsils are associated with vaccine-mediated protection against pathogenic viral infection [294]. These cell types might indeed contribute to the heightened cytokine and chemokine expression during slow disease progression. On the other hand, high expression of cytokines and chemokines in LN might contribute to general immune activation rather than to viral control, thus favoring rapid disease progression [84, 86, 247, 280]. In addition, the paucity of CXCR3+ cells despite increased levels of its ligands CXCL9 and CXCL10 at LN of rapid

progressors may represent immunological dysfunction of LN, which is a characteristic of pathogenic SIV and HIV infection [29, 85, 267].

It has been previously observed that the induction of innate immune responses at the vaginal mucosa through TLR ligands prior to SIV inoculation does not protect macaques from infection and elicited a broad range of immune modulators [313]. In contrast, Li et al recently demonstrated that the specific inhibition of certain proinflammatory cytokines and chemokines like MIP-3α at the vaginal mucosa prior to challenge could prevent vaginal transmission of SIV possibly by preventing recruitment of CCR5 bearing cells to the site of virus exposure [183]. These findings, together with the observations here, indicate that immune modulators at different tissue sites might enhance whereas others might inhibit or decrease SIV transmission and/or disease progression.

Although it has been shown that immune activation is a strong predictor of HIV disease progression [16, 108, 122, 187, 283], most of the studies have examined T cell activation. In contrast, the studies here focus on innate immune modulators and their association with disease progression. In LNs and peripheral blood, expression of the IFN-response gene OAS and the chemokine CXCL10 (IP-10) were associated with disease progression. Because these two immune modulators were still upregulated during chronic infection in macaques that showed signs of

simian AIDS, but not in long-term non-progressors, the expression levels of these two chemokines may be useful as a diagnostic marker of hosts that will progress relatively rapid to AIDS. Natural hosts of SIV, like sooty mangabeys, exhibit immune activation only during acute phases of infection, which is transient, and then maintain low levels of immune activation during chronic phases of infection. They generally do not exhibit any clinical signs of sAIDS [70, 83, 110, 162, 207, 228, 276, 277, 280]. Although most of these studies focus on T cell activation, it is interesting to note that innate immune modulators follow a similar trend, as interferon-stimulated gene expression in PBMCs, like OAS or CXCL10, is upregulated during acute infection in sooty mangabeys but declines back to baseline levels after 30 days post-infection [33]. Similar findings could be observed in another natural host species, African green monkeys (AGM) as gene profiling of peripheral blood revealed that genes involved in interferon signaling, like OAS and CXCL10, were upregulated by day 10 but then decreased by day 45 [174]. In addition, Sarkar et al. found that SIV+ macaques with high viral loads had higher numbers of CXCL10-producing cells than animals with low viral loads, and CXCL10 production was inversely correlated with peripheral CD4+ T cell numbers [262]. Chimpanzees infected with HIV that progressed to sAIDS, consistently demonstrated increased levels of CXCL10 in plasma while levels were

undetectable in non-progressing chimps [148]. In addition, there is evidence that CXCL10 (IP-10) might be increased in a specific manner in HIV+ patients as the CXCL10 levels in plasma rapidly increase in HIV-infected individuals and stay elevated in all patients assessed [284]. In contrast, in HBV or HCV infected individuals, plasma CXCL10 (IP-10) levels rise later in infection and are not elevated in every patient [284]. In addition, others have suggested using high levels of CXCL10 in cerebrospinal fluid for the diagnosis of AIDS dementia complex, a common neurological disorder associated with HIV infection and AIDS [48]. In a study published in the mid-1980s, HIV infected patients with AIDS were observed to exhibit elevated levels of OAS prompting the authors to suggest that this immune modulator might be used as a prognostic indicator for progression to AIDS [249]. Findings in natural host species indicate that it might be beneficial to have these immune modulators up early, but that it might be detrimental to keep them elevated as it is observed in non-natural hosts of SIV. These results in combination with the findings from this study warrant further investigation of the use of CXCL10 (IP-10) and OAS as diagnostic markers of immune activation and disease progression in HIV-infected individuals. These analyses could in theory provide a means to identify those hosts, which are intermediate or rapid progressors exhibiting increased levels of CXCL10 and OAS, and

slow or non-progressors that do not have increased CXCL10 and OAS mRNA expression during chronic phases of infection.

In summary, this study has expanded upon previous work that focused on mucosal sites [208] by characterizing the response of innate immune modulators in LN and peripheral blood during both pathogenic and nonpathogenic natural SIV infection. The finding that elevated mRNA levels of certain innate immune modulators at LNs and blood are associated with rapid disease progression, whereas elevations of these same immune modulators at mucosal sites are associated with slower disease progression highlights the complexity that is inherent in primate species, including humans. These diverse outcomes indicate that assessment of multiple tissue compartments is necessary to provide a complete overview of the innate immune response post-SIV (and HIV) infection. However, findings in peripheral blood are still informative as they may provide markers that are useful as predictors of hosts that are likely to progress rapidly to AIDS. The data presented here suggest that it may be beneficial for future therapies or vaccines to induce increased expression of specific immune modulators, including IFN-α, OAS, CXCL9 and CXCL10, at mucosal sites but not the lymphoid tissues or peripheral blood. In addition, the timing and magnitude of this response may be

critical to achieve the desired outcome of inhibiting HIV transmission or disease progression.

CHAPTER FOUR

Immune and viral changes after oral administrations with low or high doses of SIV

Introduction

The theoretically best HIV vaccine would be one that prevents HIV transmission and infection at a mucosal site where most new infections occur. To achieve this lofty goal, there is a need to increase our knowledge with regard to mucosal immune responses in general and after exposure to HIV/SIV in particular. The need for these studies was made evident by the multiple failures of HIV vaccine trials to date [107, 234] and most prominently in September 2007 when it was announced that the Merck sponsored STEP HIV vaccine trial failed; in fact the administration of the Merck HIV vaccine was associated with an increase in the number of HIV infections [39, 137, 271]. A better understanding of the mucosal immune responses prior to, during and following HIV transmission is likely to lead to new approaches in the development of preventative vaccines for HIV.

Sexual transmission of HIV from one person to another depends on multiple factors including the infective potential of HIV in the infected individual and the susceptibility of the uninfected partner. Assessing these

earliest events in humans is difficult, if not impossible, due to the timing of when first samples can be obtained, usually no sooner than 2-4 weeks post-infection when flu-like symptoms become apparent. The SIV infection of macaques is an excellent model system for addressing issues of pathogenesis and transmission of a virus that has many genetic and pathogenic similarities to HIV [69, 127, 128, 178, 182, 200]. Some key advantages of the SIV/macaque model are that the viral doses, time of infection and mode of infection are known, permitting an assessment of the earliest events post-infection. It will be important to examine how many virions are actually transmitted per infection event. A potential future vaccine successful at preventing infection, will need to block these virions at the point of transmission, i.e. the mucosal surface, before they can spread and establish systemic infection. In studies done with human and Rhesus macaque samples from acute infection stages, it has been suggested that the number of virions that are transmitted into a new host is relatively low [117, 156, 204]. To prevent infection, a vaccine would only need to block these few virions, however, it is also true that if only a few virions are required it might be easy for them to escape the initial immune response and initiate the infection.

Most vaccine and pathogenesis studies to date utilized high doses of SIV [115, 134, 163, 164, 206, 208, 285, 299, 303, 328] to ensure that

macaques became infected. These high doses (e.g. 10^5 $TCID_{50}$ or 1 x 10^9 copies of viral RNA) far exceed doses occurring during natural infection, as for example the viral load in breast milk or semen can range from undetectable to 1 x 10^6 copies of viral RNA per milliliter of fluid [75, 307, 321]. In addition, these high doses might overwhelm any anti-viral immune responses. Therefore, transmission studies with low doses of virus are important since these are more likely to mimic naturally occurring doses of HIV and SIV in semen, breast milk or other bodily fluids. HIV infection in humans probably occurs after repeated exposures to low doses of HIV. Studying these immune responses after administration with physiological more relevant low doses of SIV, will enable a comparison of these responses to those of high dose challenges. These studies will be particularly valuable to research studies that utilize low dose SIV administrations to assess vaccines, microbiocides or pathogenesis in the macaque model [4, 202, 302].

The studies described in this chapter were designed to assess immune and viral changes after oral administration with lower doses of SIV and compare these findings to administrations with high viral doses. The goal was to determine how the viral dose impacts the earliest events after viral exposure to a mucosal membrane. In addition, these studies allowed for the identification of an ideal low SIV challenge dose for future

transmission studies to be undertaken in the Sodora laboratory. The strengths of this study are the use of the same route (oral transmission) for low and high viral dose administrations and the in-depth assessment of several immune and viral changes performed with the same experimental techniques for both low and high dose challenged macaques. This allowed for a detailed comparison of macaques inoculated with different doses of SIV via the same route and identified the earliest innate immune events following an SIV exposure.

Results

Macaques and viral administrations

To assess immune responses and viral changes after administrations with low doses of SIV, six Rhesus macaques were challenged with different doses of SIVmac251 through the oral route. These macaques were housed at the Southwest National Primate Research Center (SNPRC) and had the following designations: 17742, 18412, 18414, 18891, 18984, and 19147. To identify the appropriate low dose SIV challenge for oral infection, each macaque underwent a series of three viral administrations with each administration occurring two days after the previous one (days 0, 2 and 4). The macaques that did not get infected after the first series of three doses were given an additional series of three oral SIV

administrations of a slightly higher viral dose after an appropriate resting time. Macaques 17742 and 18984 were first administered a viral dose of 1000 $TCID_{50}$, upon which neither macaque became infected. Approximately two months later, these macaques, that were still SIV-negative, underwent an additional three oral SIV administrations with 4000 $TCID_{50}$, upon which both macaques became infected. A second group of macaques, 18412, 18414, 18981 and 19147, were orally administered SIV with 1000 $TCID_{50}$ with which one macaque, 18981, became infected. The other three macaques became infected after an additional series of oral SIV administrations with 2000 $TCID_{50}$ one month later. These SIV infection results can be summarized as follows: Two macaques (17742, 18984) became infected with 4000 TCID50; three macaques (18412, 18414, 19147) were infected with 2000 TCID50; and one macaque (18981) became infected with 1000 TCID50 (summarized in Table 4-1).

Infection status of each macaque was verified through analysis of peripheral blood cells obtained at 7 and 14 days after SIV administration followed by nested PCR for the SIVgag gene in genomic DNA. Each sample was run in duplicate. Figure 4-1 represents an example of four macaques: PCR products of the second round were run on a 1.5 % agarose gel and visualized.

Table 4-1. SIV Viral doses utilized for oral infection.

Animal ID	Viral Dose SIVmac251 (TCID50)	
	1. Inoculation-Series	2. Inoculation-Series
17742	1000	4000
18412	1000	2000
18414	1000	2000
18981	1000	N/A
18984	1000	4000
19147	1000	2000

uninfected
infected

Macaques 18412, 18414 and 19147 were SIV-positive after 14 days post-infection (dpi) (lanes 1 – 6), whereas macaque #17742 was negative for SIVgag by 7 dpi (lanes 7 – 8). To confirm the presence of genomic DNA in all samples, PCR for β-actin was run simultaneously (Figure 4-1, bottom row). After infection, these macaques were followed for three to four months, at which time none of the macaques had

developed signs of simian AIDS. Blood as well as oral mucosal and lymph node tissue biopsy samples were obtained as outlined in Figure 4-2.

For the high dose experiments, Rhesus macaques housed at the California National Primate Research Center (CNPRC) were utilized and had the following designations: RM11, RM12, RM13, RM14, and RM15. These macaques were non-traumatically orally inoculated with 10^5 $TCID_{50}$ of SIVmac251. Blood, oral mucosal and lymph node biopsies were taken on a regular basis throughout the first 90 days of infection: blood and LN biopsies were collected at four time points (7 or 15, 21 or 28, 45 or 56, as well as 84 days post-infection) and oral mucosal biopsies at three time points (2 or 4, 14 or 21 as well as 70 days post-infection). To distinguish between the macaques given the different doses of SIV, the low dose macaques have a 5-digit ID number whereas the high dose macaques carry an RM identifier.

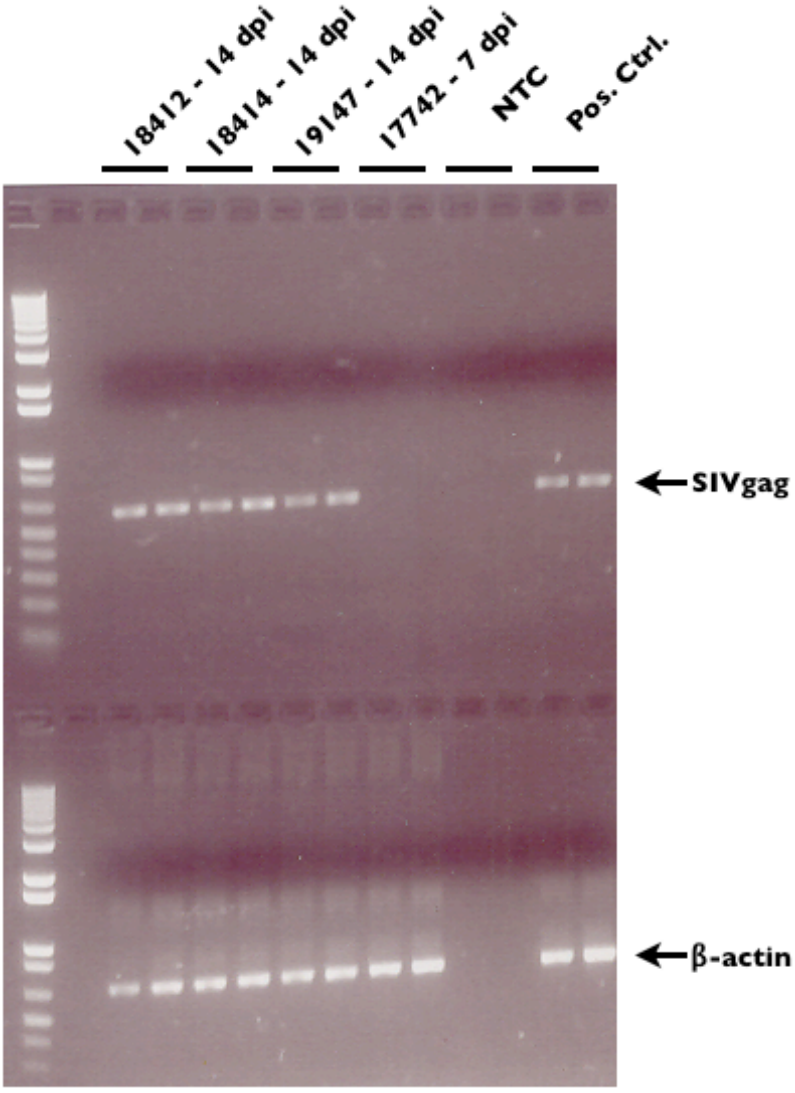

Figure 4-1. Determination of SIV infection status in orally inoculated Rhesus macaques.

Following nested PCR amplification and agarose gel electrophoresis, a 597 base pair DNA fragment of the gag gene could be observed in SIV positive PBMCs. This band was absent in SIV negative macaques (top row). The bottom row represents β-actin, a control for the presence of genomic DNA. NTC – non-template control.

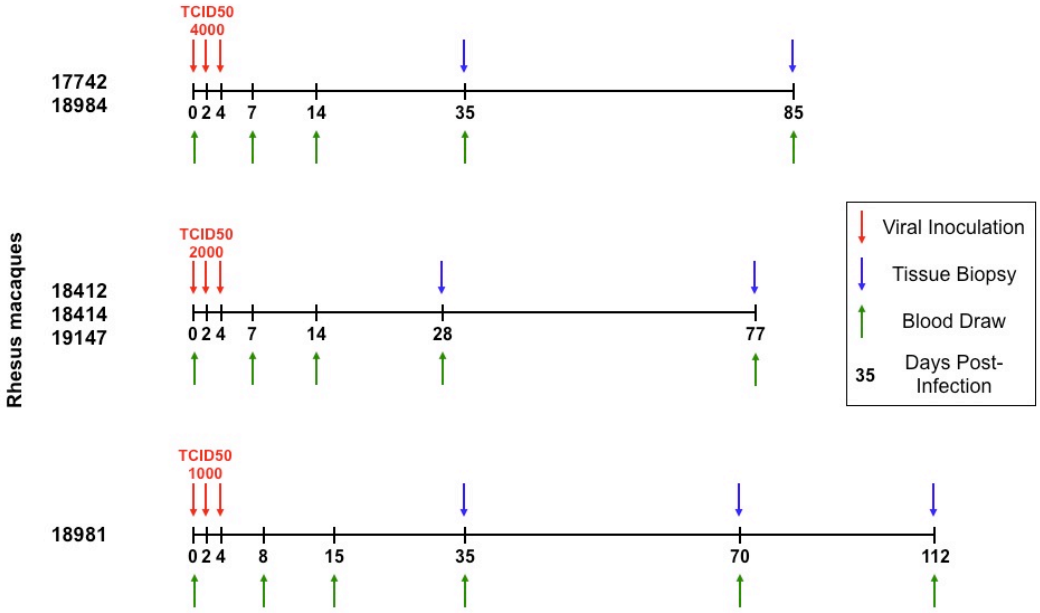

Figure 4-2. Timeline of sampling in orally infected Rhesus macaques

Every Rhesus macaque was inoculated with three doses of SIVmac251. After infection, two or three tissue biopsies (oral and rectal mucosa, lymph nodes) as well as blood samples were taken as indicated.

Plasma viral load in orally inoculated Rhesus macaques

Previous studies from the Sodora laboratory and others have determined that the most likely entry sites of SIV after exposure to the oral mucosa are tissues proximal to the stomach [20, 21], including the tonsils [285] as well as the oral and esophageal mucosa [206]. These studies of SIV infected macaques have also shown that SIV spreads rapidly within the

new host after oral administration and is systemic by 4 to 7 days post-infection [4, 206]. Assessment of viral load is an important measure of viral replication and spread, and were assessed here throughout the first three to four months of infection in the orally inoculated Rhesus macaques (RNA copies per mL of plasma, Figure 4-3). The macaques inoculated with 4000 $TCID_{50}$, 17742 (red line) and 18984 (green line), exhibited peak plasma viral loads between 10^8 and 10^9 viral RNA copies per mL of plasma within 14 days of infection. After that viral load dropped one log and settled at the viral set point of around 10^7 viral RNA copies per mL of plasma (Figure 4-3A). Macaque 18981 (yellow line), which was incubated with 1000 $TCID_{50}$, exhibited a viral peak between 10^6 and 10^7 viral RNA copies per mL of plasma by 14 dpi, and a viral setpoint of 10^3 viral copies at day 112 post-infection. Macaques 18412, 18414, and 19147 were inoculated with 2000 $TCID_{50}$ and plasma levels of virus increased throughout the first 10 days by the time they reached set point levels of around 10^6 viral RNA copies per mL of plasma (Figure 4-3A). Due to the sampling schedule, we cannot be sure that the peak viremia is clearly observed for these three macaques. All animals inoculated with high doses of SIV (10^5 $TCID_{50}$) exhibited peak viral loads around 10^8 viral RNA copies per mL of plasma and viral set points of around 10^7 viral copies (Figure 4-3B).

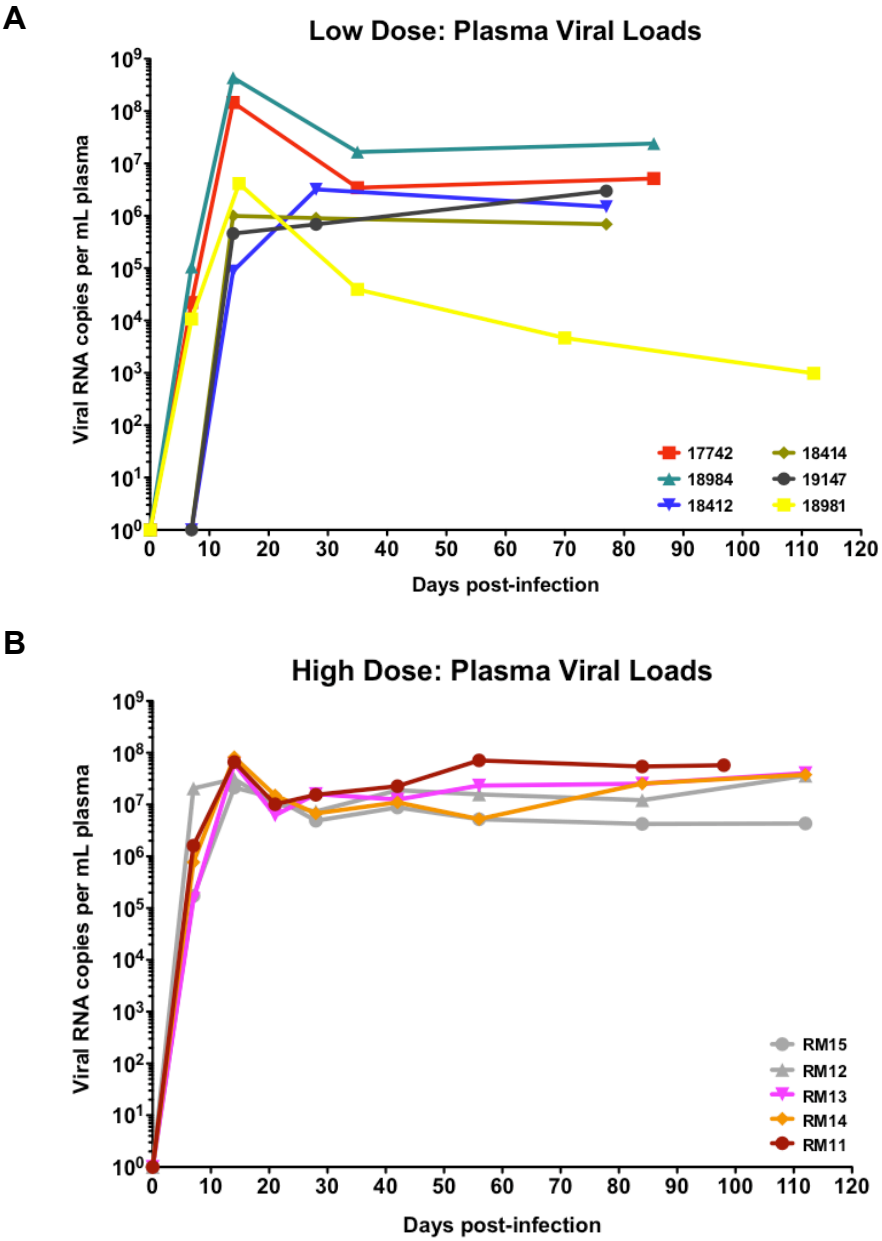

Figure 4-3. Plasma viral loads.

Viral RNA copies per mL of plasma are shown for macaques inoculated with low doses (A) or high doses of SIV (B).

Interestingly, detection of virus in plasma is delayed in some macaques, as viral loads are still undetectable at 7 days post-infection for three of the SIV low dose macaques. However, statistical assessment of the set point viral loads (days 70 to 85 post-infection) revealed no significant difference between the two groups of macaques ($p = 0.121$). These findings indicate that low or high dose administrations to the oral mucosa can lead to infection with similar viral loads during chronic time points.

Assessment of SIV genetic diversity at early time points following oral administration of SIV in low or high dose challenges

To prevent SIV/HIV from infecting a new host a successful vaccine needs to induce an adaptive, along with a possible innate, immune response to inhibit HIV entry. Here, two methods were used to determine the diversity of the early viral determinants responsible for initiating infection, the heteroduplex mobility assay (HMA) and sequencing of SIV DNA. The V1-V2 region of the SIV env gene exhibits a high level of diversity throughout SIV disease course [8, 221, 290] and is the best choice to assess viral diversity. Genomic DNA obtained from peripheral blood cells was subjected to nested PCR to permit the sampling of SIV diversity at the earliest time points assessed (7 or 14 dpi).

To obtain a qualitative assessment of viral diversity, the PCR product of the SIV env V1-V2 region was subjected to a heteroduplex mobility assay (HMA). The PCR products were heated to denature the DNA double-strands, followed by rapid cooling that induces heteroduplex formation. Heteroduplexes are DNA double-strands of which the two single strands are not a perfect match since they arise from variants that are genetically polymorphic. Therefore, they migrate slower than homoduplexes on a polyacrylamide gel and thus can be identified after visualization on the gel. Previous studies have determined that the greater the number of heteroduplexes observed on a gel, the larger was the genetic variety in the starting material [66, 67, 281]. Figure 4-4 shows the HMA results for the SIV stock inoculum (lane 1) and the macaques inoculated with low doses of this viral stock (lanes 2 to 7). Five of the macaques did not exhibit the slowly migrating heteroduplexes that are represented in the inoculum during these early time points (lanes 2, 3, 5 to 7). This provides evidence for a low level of viral diversity in these five macaques early after infection, indicating that only a very limited number of virions were transmitted after viral administration. However, one macaque, 18412, did exhibit these slowly migrating heteroduplexes (lane 4), indicating that multiple SIV variants traversed the oral mucosa to initiate an infection in this macaque. Further sequencing analysis of the

SIV V1-V2 region was undertaken and a phylogenetic tree generated using the neighbor-joining method of the MEGA 4.1 software (www.megasoftware.net, Center for Evolutionary Functional Genomics, Tempe, AZ). The resulting tree enabled an assessment of sequences obtained from each macaque (eight to eleven samples per macaque) as well as from the viral inoculum (five sequences), each of which is presented as a separate color (Figure 4-4). For five of the macaques the sequences group together, indicating low viral diversity at this early time point after infection, confirming the HMA analysis. This is indicative of transmission of only a limited number of viral variants in these macaques. The only exception to this very limited diversity is macaque 18412 (blue dots): the PCR clones of this macaque did not group together but rather are split onto different branches of the phylogenetic tree, indicating a higher viral diversity in this macaque. These results confirm the findings of the HMA analysis in that five of the six macaques that were administered a low viral dose exhibit only limited viral diversity early after infection, indicating that only a limited number of variants crossed the oral mucosa and elicited an infection following a low dose challenge.

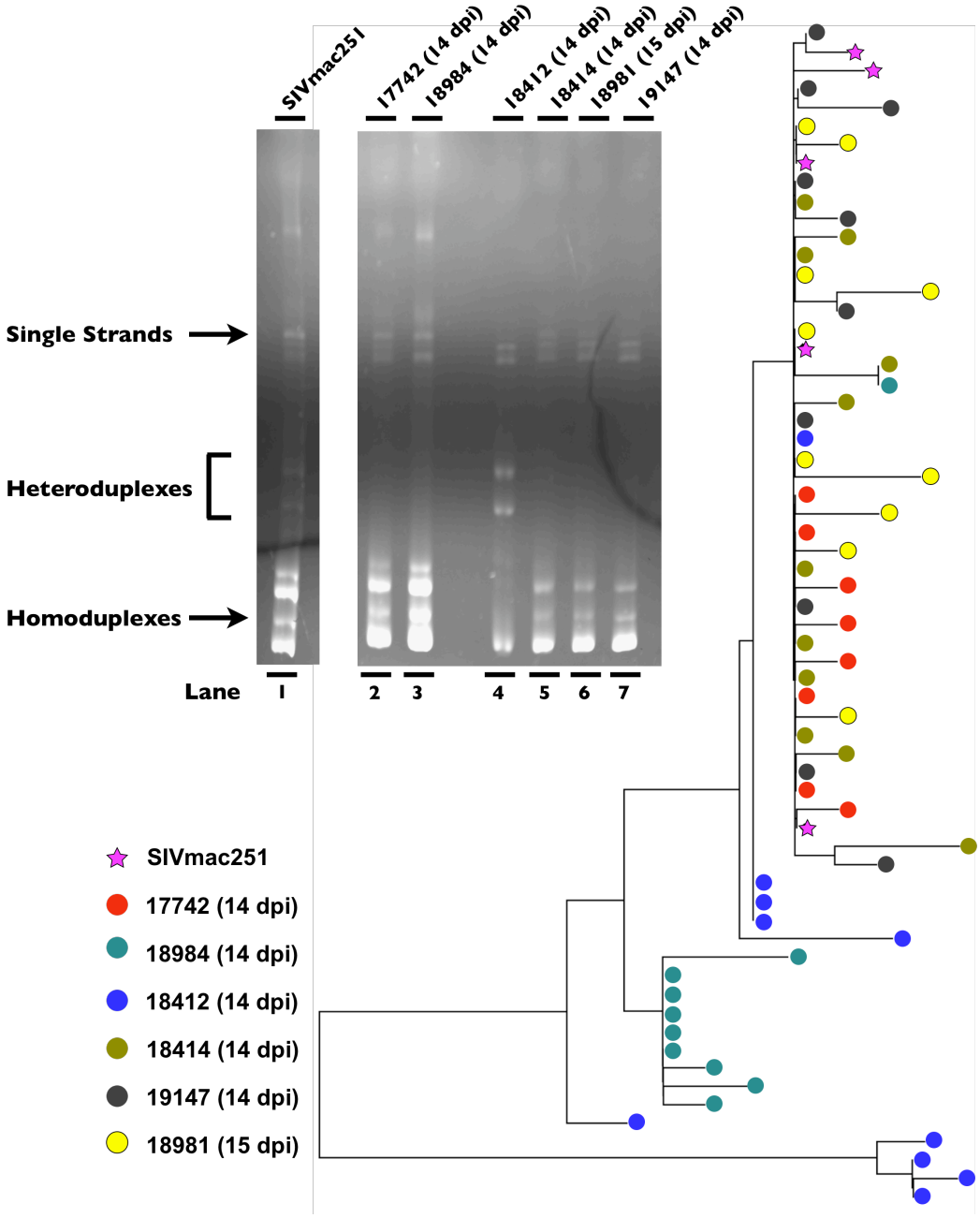

Figure 4-4. Heteroduplex Mobility Assay and Phylogenetic tree of macaques inoculated with low doses of SIVmac251.

All samples were obtained from PBMCs of orally inoculated Rhesus macaques. Lane 1 shows the viral inoculum (SIVmac251) of which low doses were used to infect the macaques shown in lanes 2 to 7. Dots colored the same on the phylogenetic tree indicate different clones of one time point per macaque.

Similar analyses were done for the macaques inoculated with high doses of SIV. In contrast to the findings from the low dose challenge, four of five macaques infected by a high dose of SIV exhibit slowly migrating heteroduplexes (Figure 4-5, lanes 1 to 4). Indeed, the sequencing analysis and resulting phylogenetic tree (Figure 4-5) confirms the findings from the HMA analysis. The env V1-V2 sequences from each macaque are spread across multiple branches of the tree indicating a high level of viral diversity early after infection in these macaques. These findings obtained from the heteroduplex mobility analyses together with the sequencing data indicate that multiple viral variants were transmitted and established infection in macaques that were given a high dose oral administration of SIV.

Comparing the two different dosing strategies reveals that macaques that were administered a high dose of SIV exhibited higher viral diversity early after infection than macaques administered a low dose. This indicates, not unexpectedly, a direct association between the amount of SIV administered to the oral mucosal membrane and the number of viral variants that are able to traverse the mucosal barrier and establish infection. Importantly, previous studies have determined that early viral diversity is relatively limited [66, 154, 260, 330] after natural exposure to HIV. Therefore, the use of low dose SIV administrations would be predicted to result in an early infection viral diversity more representative

of a natural infection, and thus low doses would be more applicable for vaccine or microbiocide studies.

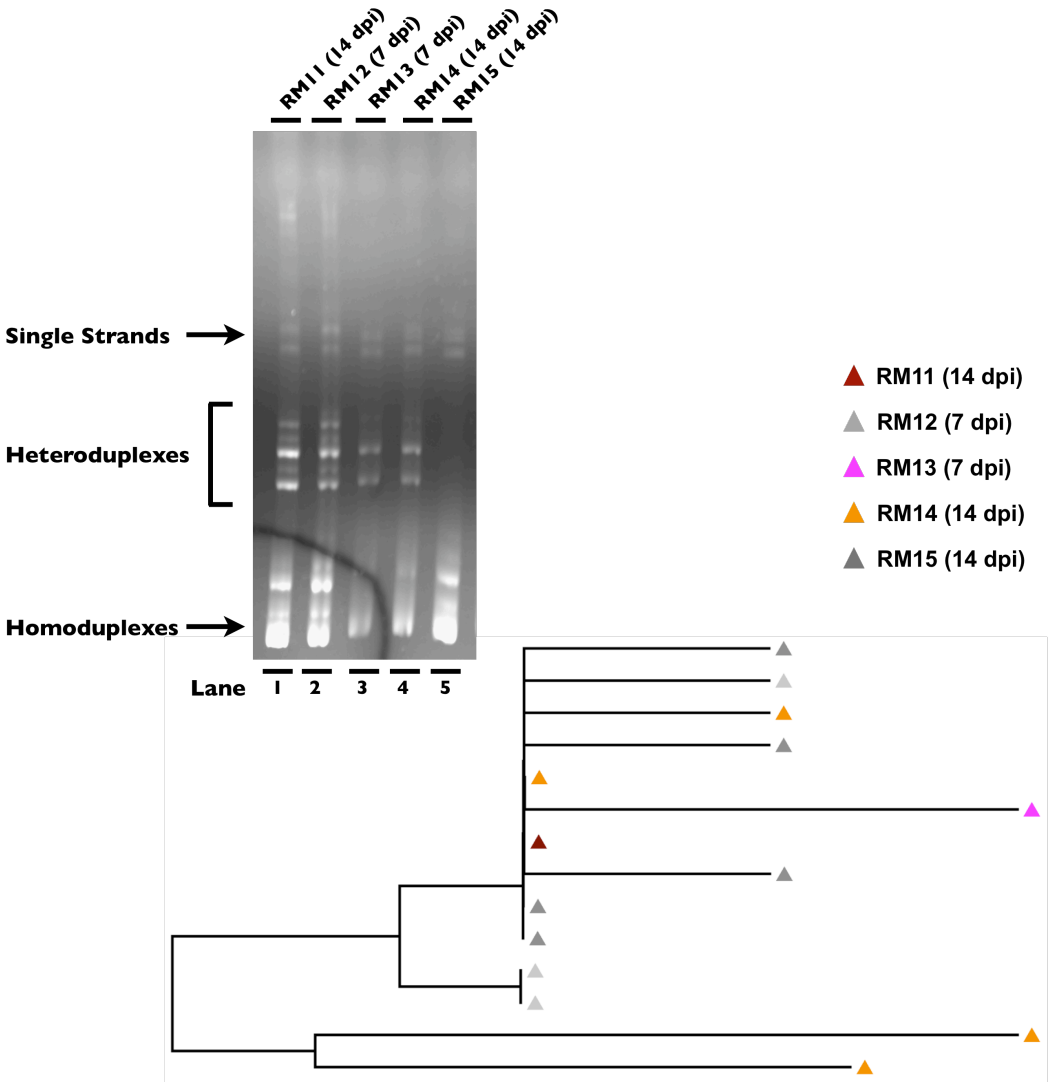

Figure 4-5. Heteroduplex Mobility Assay and Phylogenetic tree of macaques inoculated with high doses of SIVmac251.

All samples were obtained from PBMCs of orally inoculated Rhesus macaques. Lanes 1 to 5 show the 5 macaques inoculated with high doses of SIVmac251. Dots colored the same on the phylogenetic tree indicate different clones of one time point per macaque.

Expression of immune mediators is similar in macaques inoculated with low or high doses of SIV

The expression of immune mediators was assessed at different time points and different tissue sites (including the oral mucosa, peripheral lymph nodes and blood) to identify immunologic differences between the high and lose SIV dosed macaques. Expression levels were determined as mRNA fold changes in the SIV-infected macaques when compared to an average expression of the same mRNA transcript from four uninfected control macaques. For RNA extractions, tissue biopsies were homogenized and PBMCs were obtained from whole blood. PBMCs and tissues were lysed, RNA was extracted (using the Qiagen RNeasy Mimi Kit), cDNA synthesized and real-time PCR performed for eight immune mediators including IFN-α, OAS, IL-12, TNF-α, IFN-γ, CXCL9, CXCL10 and IL-10. All low dose and high dose macaques were assessed at both acute (2 to 28 dpi) as well as chronic (45 to 112 dpi) time points. These are combined in a scatter plot showing all samples for one gene grouped together. Symbols within blue boxes represent samples that are more than two standard deviations higher than the average expression of the same gene in uninfected control macaques (Figures 4-6, 4-7, and 4-8).

At the oral mucosa (Figure 4-6), the anti-viral response with IFN-α and OAS is more pronounced in macaques inoculated with high doses of

SIV, indicated by the higher number of symbols within the blue boxes. In fact, a statistically significant difference between fold changes of IFN-α expression could be observed between macaques inoculated with high doses compared to the low dose macaques (t test, $p = 0.0039$, Figure 4-6B). Expression of proinflammatory chemokines (CXCL9 and CXCL10) is similar between the SIV low and high dose groups, with some evidence for an increased CXCL9 and CXCL10 expression in some of the macaques. Differences were however observed in the TH1/proinflammatory cytokines IFN-γ and TNF-α with the low dose macaques producing an increase of IFN-γ transcripts and the high dose macaques upregulating TNF-α. The anti-inflammatory cytokine IL-10 transcripts are generally at a level comparable to the uninfected macaques, with only a few samples in either group exhibiting an upregulation of this cytokine after SIV infection.

Similar findings could be observed in the lymph nodes of these macaques (Figure 4-7). Fold changes of IFN-α and OAS are significantly higher in macaques SIV-inoculated with high doses compare to low doses (t test, $p = 0.024$ [IFN-α], $p = 0.0006$ [OAS], Figure 4-7B).

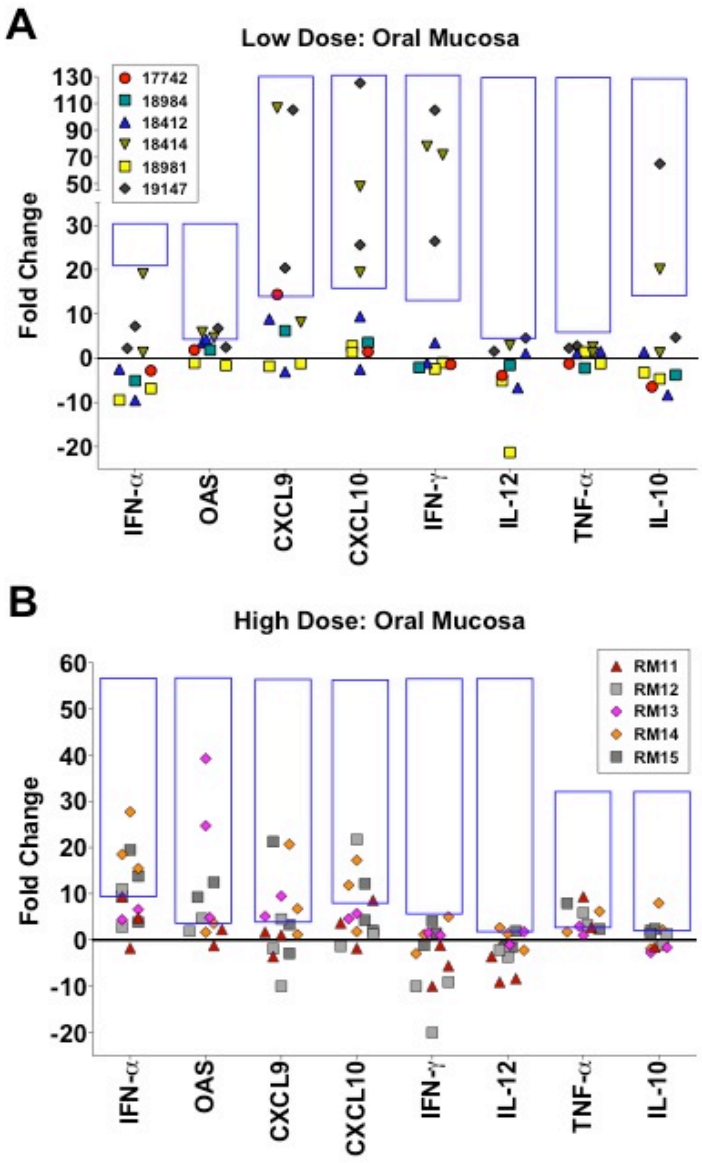

Figure 4-6. mRNA expression of immune modulators at the oral mucosa.

Shown are the fold changes of eight immune response genes (from left: IFN-α, OAS, CXCL9, CXCL10, IFN-γ, IL-12, TNF-α, IL-10) at the oral mucosa of macaques infected orally with low doses (A) or high doses (B) of SIV. Symbols of the same color represent the same macaque and each symbol represents a different time point of sampling. Symbols within the blue boxes represent mRNA expression levels that are increased more than two standard deviations away from the expression in uninfected macaques. Biopsies were obtained between days 28 and 35, 70 and 85 and on day 112 for the low dose macaques and on days 2 or 4, 14 or 21 as well as 70 dpi for the high dose macaques.

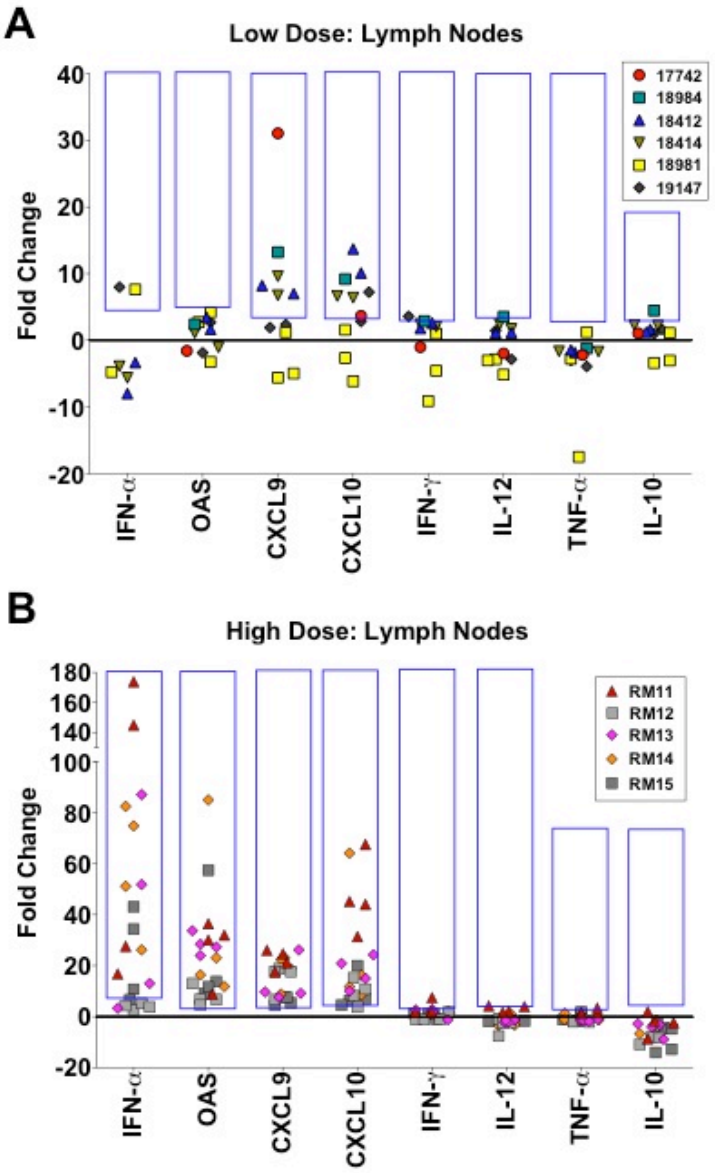

Figure 4-7. mRNA expression of immune modulators at peripheral lymph nodes.

Shown are the fold changes of eight immune response genes (from left: IFN-α, OAS, CXCL9, CXCL10, IFN-γ, IL-12, TNF-α, IL-10) at lymph nodes of macaques infected orally with low doses (A) or high doses (B) of SIV. See Figure 4-6 for color-coding. Biopsies were obtained between days 28 and 35, 70 and 85 and on day 112 for the low dose macaques and on days 7 or 15, 21 or 28, 45 or 56 as well as 85 dpi for the high dose macaques.

Expression of the proinflammatory chemokines CXCL9 and CXCL10 was increased in both the SIV-inoculated low as well as high dose macaques, whereas expression of the other immune modulators (IFN-γ, IL-12, TNF-α and IL-10) was generally not different than expression in uninfected macaques (Figure 4-7, all symbols outside the blue boxes).

The expression of these immune mediators was also assessed in peripheral blood cells (Figure 4-8). Although the same panel of immune modulators was assessed in peripheral blood cells, IFN-α and IL-12 could not be reliably detected in the uninfected control macaques. Thus, fold changes could not be calculated and so these were left out of the Figure. The remaining immune modulators assessed, OAS, CXCL9, CXCL10, IFN-γ, TNF-α and IL-10, exhibited very similar expression patterns between macaques inoculated with low doses (Figure 4-8A) and high doses of SIV (Figure 4-8B). OAS and CXCL10 were upregulated in many of the low and high dose macaques whereas CXCL9, IFN-γ, TNF-α and IL-10 expression levels were generally expressed at levels similar to uninfected control macaques.

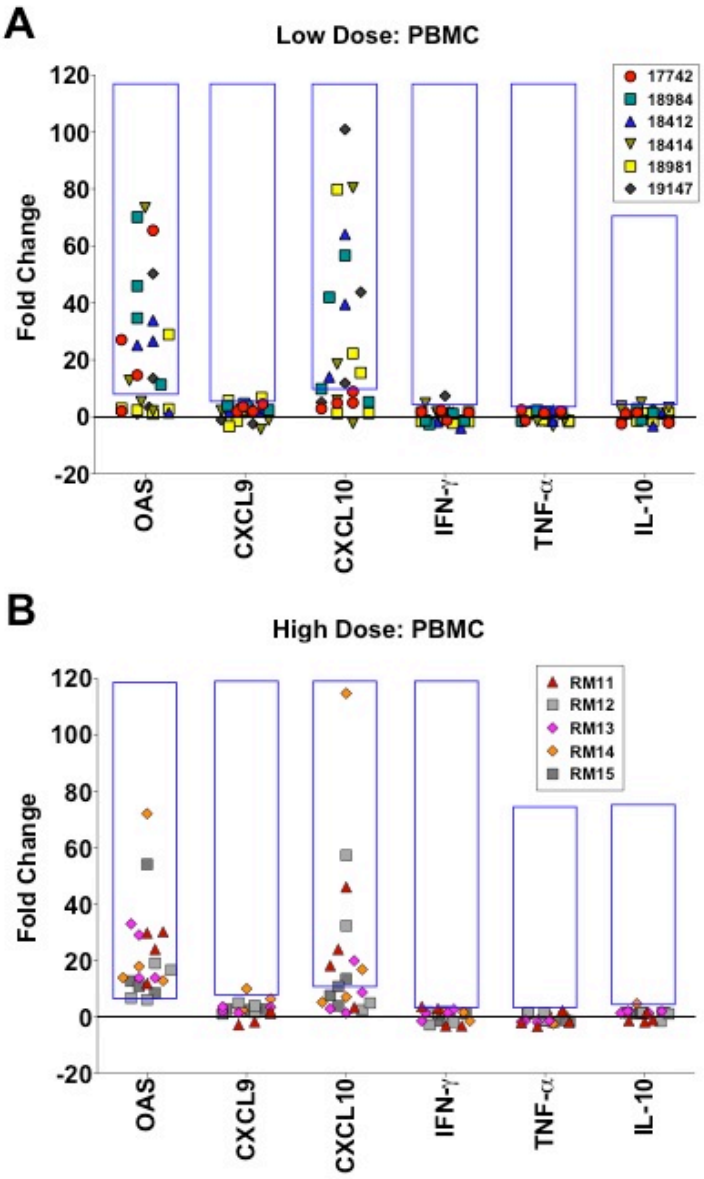

Figure 4-8. mRNA expression of immune modulators in peripheral blood.

Shown are the fold changes of six immune response genes (from left: OAS, CXCL9, CXCL10, IFN-γ, TNF-α, IL-10) in PBMCs of macaques infected orally with low doses (A) or high doses (B) of SIV. See Figure 4-6 for color-coding. PBMCs were obtained between days 7 and 14, 28 and 35, 70 and 85, and on day 112 for the low dose macaques and on days 7 or 15, 21 or 28, 45 or 56 as well as 85 dpi for the high dose macaques.

To further delineate the distinctions at the different tissue sites and different time points, expression patterns of three immune modulators, OAS, CXCL10 and TNF-α, were examined for both the low and high dose SIV infected macaques (Figure 4-9). Expression of OAS (Figure 4-9A, B) and CXCL10 (Figure 4-9C, D) was generally upregulated in PBMCs of Rhesus macaques regardless of whether they were infected by a low (Figure 4-9A, C) or a high dose of SIV (Figure 4-9B, D), and fold changes reached similar levels. Interestingly though, expression of OAS and CXCL10 in PBMCs was delayed in macaques inoculated with low doses of SIV until day 14 post-infection. On the other hand, some genes, including TNF-α (Figure 4-9E, F), were within the range of the uninfected control macaques, not influenced by infection of either a low or high dose SIV challenge. This indicates that heightened gene expression in peripheral blood was specific for OAS and CXCL10 and not observed in other genes like TNF-α.

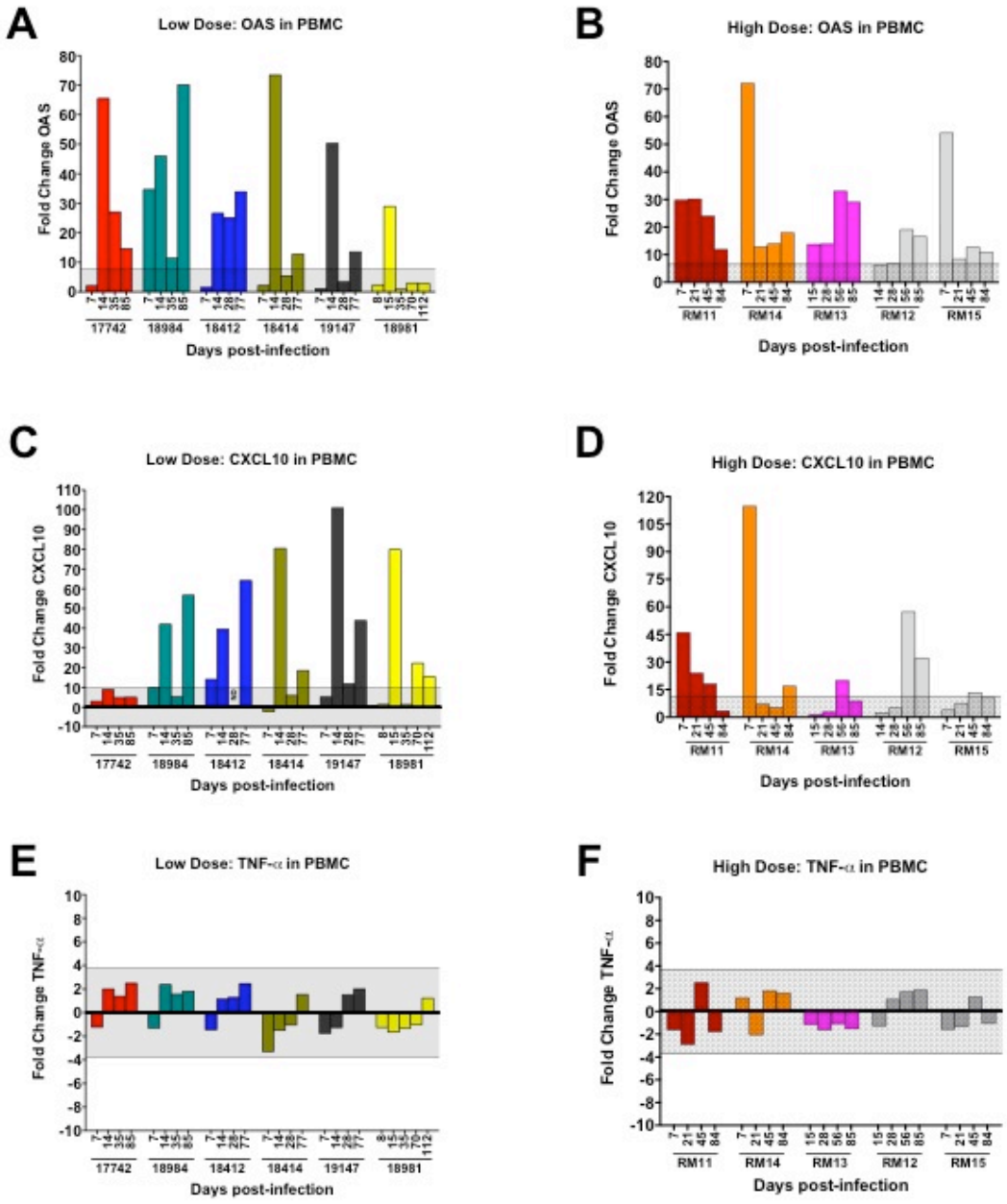

Figure 4-9. mRNA expression in peripheral blood.

OAS (A, B), CXCL10 (C, D) and TNF-α expression (E, F) are shown for PBMCs of Rhesus macaques orally inoculated with low doses (A, C, E) or high doses (B, D, F) of SIVmac251. Each bar represents the fold change of expression at one time point compared to uninfected macaques. The shaded area represents two standard deviations of the average expression of the corresponding gene in uninfected macaques. ND – not determined.

In summary, eight immune modulators were assessed at the oral mucosa and in lymph nodes. Expression of pro- and anti-inflammatory cytokines was generally not different between macaques administered low or high doses of SIV. Interestingly though, the anti-viral response with expression of IFN-α and OAS was more pronounced in tissues of the high dose macaques. In PBMCs, all six genes assessed exhibited similar expression patterns with only OAS and CXCL10 being upregulated in both macaque groups.

Comparison of cytokines and chemokines in plasma of Rhesus macaques inoculated with low and high doses of SIV

To further assess immune modulators in these SIV infected macaques, plasma immune proteins were assessed in both the low and high dose macaques by Luminex. The read-out of Luminex is concentration in pg/mL, however, to take assay and macaque variability into account, these values were normalized by dividing the concentration obtained for every time point by the concentration of the same protein at the 0 time point (0 dpi). Values bigger than 1 indicate increases whereas values smaller than 1 indicate decreases induced by the SIV infection. The fold changes of three representative pro-inflammatory cytokines in macaques inoculated with low or high doses of SIV are shown: IL-12 (Figure 4-10A,

B), IL-18 (Figure 4-10C, D) and IL-1Ra (Figure 4-10E, F). IL-12 levels increased in some of the macaques early after infection and in some macaques late, while IL-18 and IL-1Ra levels increased early on in most macaques before decreasing again. Interestingly though, the cytokine levels were generally similar between the low and high dose macaques. Statistical analysis revealed that there was no significant difference between the levels of IL-12 ($p = 0.2548$), IL-18 ($p = 0.1235$) and IL-1Ra ($p = 0.3422$) during chronic time points (70 – 85 dpi) between macaques administered low doses of SIV compared to macaques administered high doses. Importantly, these data show a general increase in the levels of pro-inflammatory cytokines after infection is established in macaques inoculated with either low or high doses of SIV.

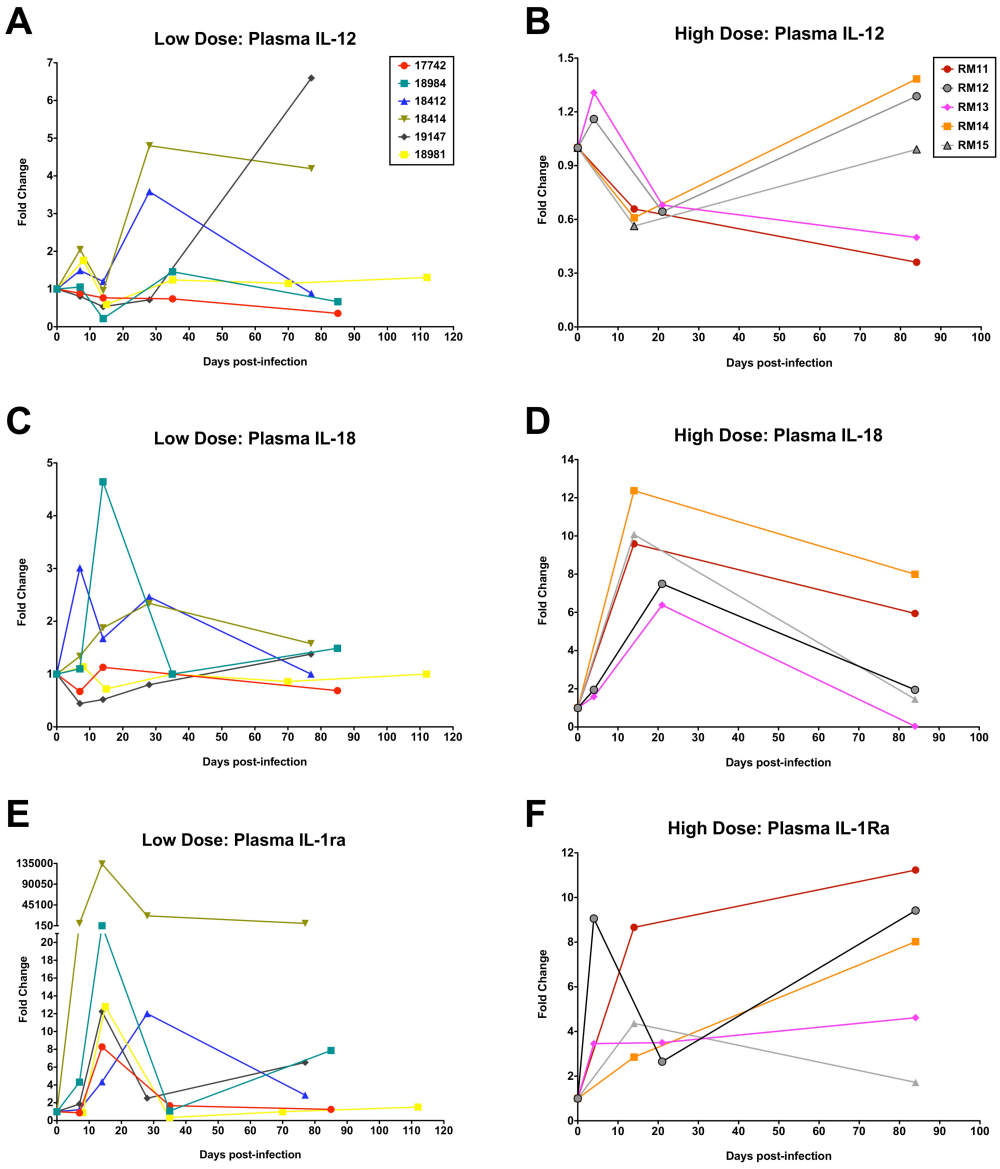

Figure 4-10. Levels of proinflammatory cytokines in plasma.

Plasma levels of the proinflammatory cytokines IL-12 (A, B), IL-18 (C, D) and IL-1Ra (E, F) are shown for Rhesus macaques infected with low doses (A, C, E) or high doses of SIVmac251 (B, D, F). Cytokine levels were determined by Luminex and fold changes were calculated by normalizing every time point to time 0.

T cell activation in Rhesus macaques inoculated with low doses of SIV

Evidence suggests that the level of immune activation during the chronic phase of HIV-infection is a strong predictor of disease progression and mortality [97, 108, 122, 187, 280, 283]. To examine whether immune activation occurs also after oral administrations with low doses of SIV, Ki67, a proliferation marker, was quantified on T cells both in peripheral blood via flow cytometry and in tissues via immunofluorescence staining. As can be seen in Figure 4-11, all Rhesus macaques inoculated with low doses of SIV experienced an initial drop and then a dramatic increase in the percentage of Ki67+ CD8+ T cells between 20 and 30 days post-infection. After this increase, Ki67 expression of CD8+ T cells dropped in most macaques but remained steady at levels statistically higher than during pre-infection (paired T test, $p = 0.0465$) (Figure 4-11).

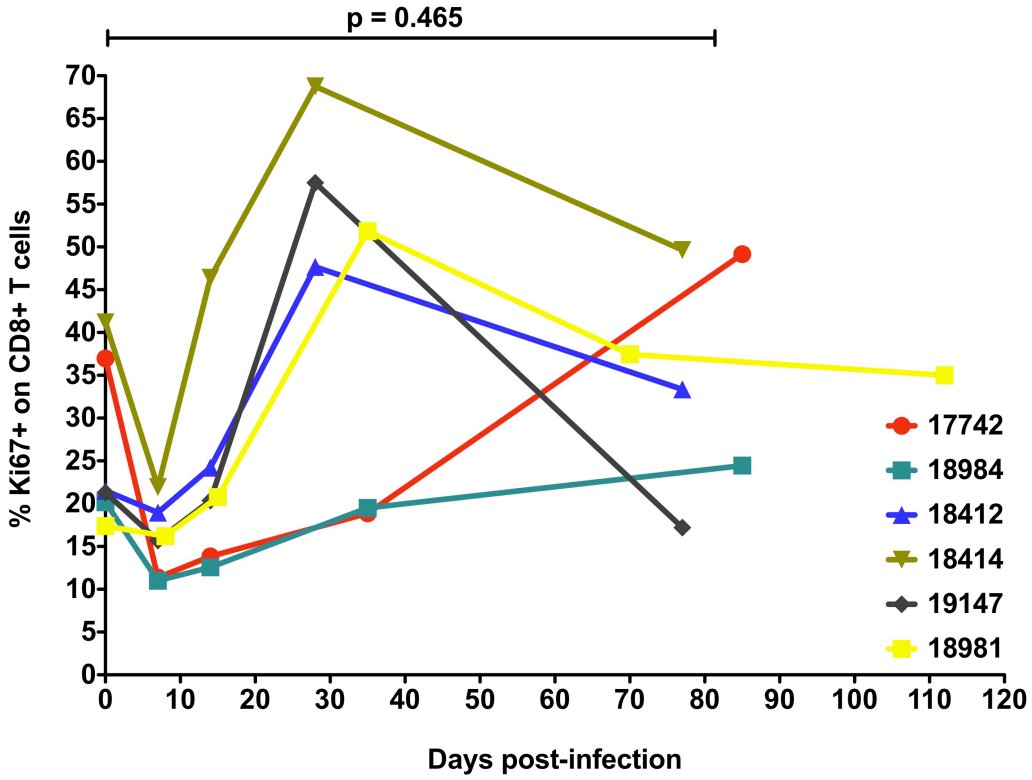

Figure 4-11. T cell activation in blood in orally inoculated Rhesus macaques.

Ki67 was utilized as marker of T cell activation and its expression on CD8+ T cells in peripheral blood was assessed by flow cytometry. A paired T test was conducted to assess whether the levels of Ki67+CD8+ T cells were significantly higher during chronic infection compared to preinfection levels. The p value is indicated.

Immune activation as measured by Ki67 expression on CD3+ T cells could also readily be observed in several tissues, including lymph nodes and mucosal sites. Figure 4-12 shows representative staining of

CD3 (green) and Ki67 (red) in these tissues of one macaque in which CD3+Ki67+ cells are marked by white arrows. Manual counting of CD3+Ki67+ cells in the different tissues yielded the following results (reported are the numbers of CD3+Ki67+ cells as the mean of at least ten 40x fields):

Lymph nodes	0dpi: 3 cells	85dpi: 26 cells
Oral mucosa	0dpi: 0-1 cells	85dpi: 3-4 cells
Rectal mucosa	0dpi: 0-1 cells	85dpi: 3-4 cells

Usually, only very few or no Ki67-CD3 double-positive cells could be observed in lymph nodes (Figure 4-12A), oral mucosa (Figure 4-12C) or rectal mucosa (Figure 4-12E) in uninfected macaques. However, during chronic infection (85 dpi) there was a large increase of CD3-Ki67 double-positive cells present in lymph nodes of low dose orally infected macaques (Figure 4-12B). Similarly, increases in proliferation of CD3+ T cells could be seen at the oral mucosa (Figure 4-12D, white arrows) and the rectal mucosa (Figure 4-12F, white arrows). Taken together, these findings indicate that oral administrations with low doses of SIV result in increased immune activation similar to what has been observed previously in macaques inoculated with high viral doses. Since increased immune activation is a hallmark of SIV/HIV infection, this finding validates the use of the low dose model via the oral route.

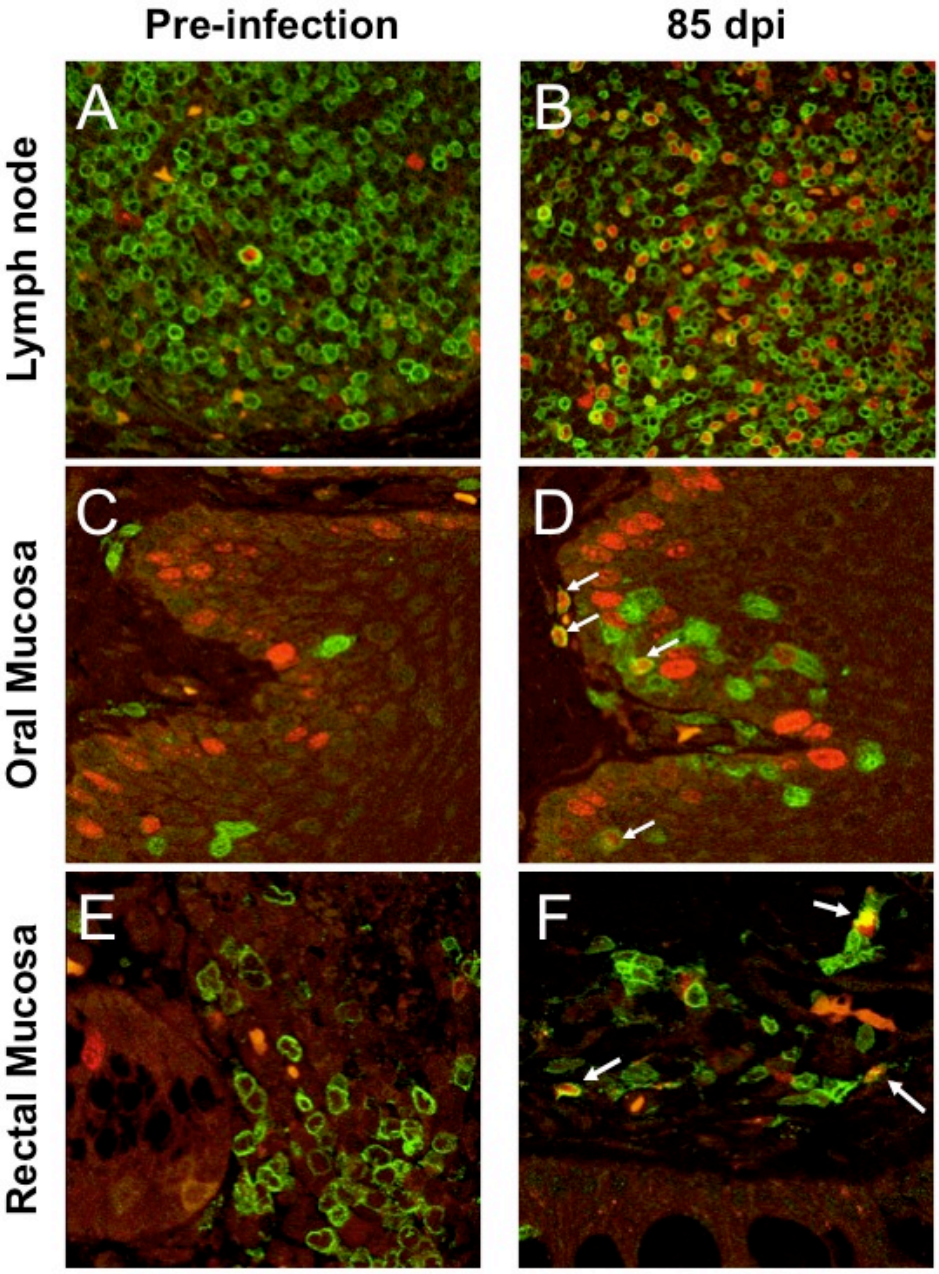

Figure 4-12. T cell activation in tissues of orally inoculated Rhesus macaques.

Ki67 expression in lymph nodes (A, B), at the oral mucosa (C, D) and at the rectal mucosa (E, F) was assessed on T cells by immunofluorescence staining. A pre-infection time point (A, C, E) and 85 dpi (B, D, F) was utilized. CD3 is stained in green, whereas Ki67 is stained in red. Double-positive cells are marked by white arrows in D and F.

Comparison of antibody responses

SIV-specific antibody levels in plasma were assessed throughout the study period in both groups of macaques inoculated with low doses as well as high doses of SIVmac251. All six macaques inoculated with low doses seroconverted between two and three weeks following infection (Figure 4-13A) and exhibited similar kinetics of antibody levels. Antibody levels in three macaques had reached their peak by around four weeks and then remained steady, whereas antibody levels continued to rise in the other three macaques. The five Rhesus macaques inoculated with high doses of SIV exhibited similar kinetics of antibody levels compared to the low dose macaques (seroconvertion between two and four weeks post-infection) (Figure 4-13B). However, one rapid progressor (see Chapter Three) could not maintain antibody levels (RM11, dark red, Figure 4-13B) for more than one week and an additional rapid progressor (RM13, pink, Figure 4-13B) exhibited a lower peak than all other macaques. The remaining three macaques increased their SIV-specific antibodies continuously throughout the study period. Generally, both macaques inoculated with low doses as well as macaques infected with high doses exhibit similar trends of SIV-specific antibody developments, indeed no significant difference between the SIV-specific antibody levels was observed between the two groups.

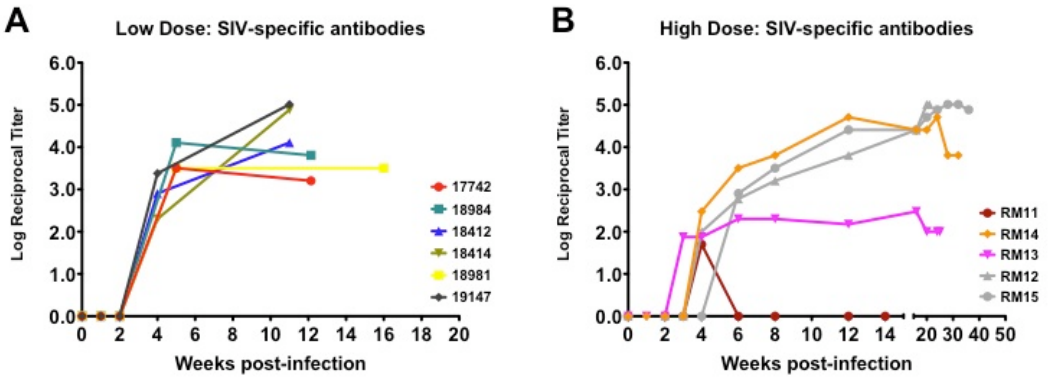

Figure 4-13. SIV-specific binding antibodies in orally inoculated Rhesus macaques.

SIV-specific antibodies in plasma of orally inoculated Rhesus macaques are shown for (A) macaques infected with low doses and (B) macaques infected with high doses of SIV. Antibody endpoint titers were analyzed for reactivity to SIVsmB7 envelope proteins by ELISA. Endpoint titers were determined to be the last two-fold dilution with an OD450 twice that of normal monkey plasma and are reported as the log10 of the reciprocal endpoint titer.

Discussion

Studying the pathogenesis of SIV requires the macaques to become infected when inoculated. To ensure this, high viral doses are usually used, which greatly exceed the amount of virus naturally occurring in bodily fluids. It is possible that the large amount of virus simply overwhelms the immune response, thus initiating infection in an artificial

way and impacting results from vaccine and pathogenesis studies that utilize this approach. Administrations with lower viral doses would mimic naturally occurring amounts more closely, and infections resulting from this type of administration might reproduce immune responses more accurate and comparable to those occurring in naturally infected hosts. Indeed, in recent years researchers have been starting to use low dose administration models in pathogenesis and vaccine studies [4, 202, 302]. The studies presented here represent an effort to characterize the immune and viral changes that occur after administrations with low viral doses. Additionally, these studies enabled a comparison of results obtained from low and high dose administrations via the oral route that might be used to validate the results of previous and future high dose studies.

An advantage of the study presented here was that its design allowed for the testing of several different low viral doses. To assess factors influencing transmission in future studies, it will be important to utilize an ideal viral dose. A dose too high would mask any potential effects of a confounding factor since macaques would become infected regardless of any preexisting condition. The virus would possibly just overwhelm the immune response. Similarly, a dose too low would not lead to infection at all. Statistically, a dose that is able to infect 50 % of inoculated “normal” macaques would be ideal. Experiments with this dose

could then be performed to assess whether a certain condition increases (more than 50 % macaques infected), decreases (less than 50 % macaques infected) or does not change the actual transmission event. This approach would allow deciphering immune and viral changes impacted by the preexisting condition. By testing different low viral doses in the presence of a healthy oral mucosa and their effect on macaques, a dose of 2000 $TCID_{50}$ was determined to be optimal for future transmission and vaccine experiments.

The comparison of macaques inoculated with low viral doses to the ones infected by high doses revealed some interesting similarities and differences. Plasma viral load as an important and obvious indicator of viral infection was not statistically different during chronic infection between the two groups. Interestingly, detection of virus in plasma was delayed in some macaques, as viral loads are still undetectable at 7 days post-infection for three of the SIV low dose macaques. This result calls for a more detailed assessment of the viral replication kinetics early after transmission to delineate true differences in early viral load development. This would potentially require taking more frequent samples, maybe even daily, during the first couple of weeks of infection. Further characterizations of viral changes were undertaken and assessment of viral diversity early after the transmission event revealed that high dose

administrations led to a slightly higher viral diversity than low dose administrations. This indicates that high dose administrations to the oral mucosa result in the transmission of more virions, which might explain the slightly higher replication kinetics observed in the high dose macaques early on.

Although slightly more virions were transmitted after exposure of the oral mucosa to a high dose inoculum, this is not comparable with the large amount of viral diversity observed after an intravenous infection, during which the virus does not need to cross any mucosal barriers [10, 115, 281]. It is interesting to note that although a low viral inoculum leads to only a limited number of transmitted viral variants, it is still sufficient to induce disease and immune responses similar to the ones observed after high dose infections. In this respect, the data presented here are in agreement with previous studies in humans and non-human primates assessing the viral diversity during acute infection with HIV and SIV, respectively. Although Miller et al. did not examine the viral diversity directly, they showed that after vaginal administration in Rhesus macaques only a limited number of virions are responsible for initiating infection [204]. Using single-genome amplification, another group calculated the number of viral variants responsible for establishing persistent infection after intrarectal administration with SIV to be 1 to 5

[156]. Similarly, other studies showed that also during HIV-infection in humans, only a few, maybe down to one, viral variants get transmitted and establish infection. This appears to be independent of the HIV subtype, as it has been shown for subtypes A [117], B [154] and C [6, 117]. This small genetic founder population provides both an opportunity as well as an obstacle for any future preventative vaccine. On one hand, a vaccine would only need to block a few virions; on the other hand though, it is also true that if only a few virions are required it might be easy for them to escape the initial immune response and initiate the infection. Additionally, a vaccine-induced immune response must respond to these few virions within a short time frame to prevent systemic infection. And this time window is relatively small due to the fact that virus spreads rapidly and can be found systemically after just four to seven days [4, 206].

Assessment of gene expression of immune modulators at the oral mucosa and peripheral lymph nodes in macaques infected by low or high viral doses exhibited some interesting trends. Both pro- and anti-inflammatory cytokine and chemokine responses were similar between the low and high dose macaques in both tissues. However, the macaques inoculated with high viral doses exhibited increased levels of the anti-viral response genes IFN-α and OAS at the mucosa and in lymph nodes. In fact, fold changes of IFN-α at both tissues, and OAS at lymph nodes were

significantly increased compared to the macaques inoculated with low viral doses. This might be an indication of a more pronounced anti-viral host response in tissues towards the higher number of virions transmitted after high dose administrations. Interestingly though, expression of both anti-viral and pro-inflammatory immune mediators in PBMCs did not differ between the two macaque groups. Only the expression of the anti-viral effector OAS and the pro-inflammatory chemokine CXCL10 were upregulated in PBMCs. However, it is interesting to note that a detailed analysis of these two immune modulators over time revealed that their expression in PBMCs was delayed early after infection, as expression of both OAS and CXCL10 remained unchanged at 7 days post-infection. Similar to the delay in viral replication, this might be due to the lower number of virions transmitted after lower dose administrations. The studies presented in Chapter 3 suggested the use of OAS and CXCL10 as diagnostic markers for disease progression since they remained elevated during chronic infection in macaques that progressed to simian AIDS more rapidly. In the studies presented here, almost all macaques exhibited increased levels of these two immune modulators in PBMCs early in infection (14dpi). However, some macaques, like 18414 and 18981, then decreased expression levels of both markers and this might indicate that they will progress to simian AIDS more slowly than the other macaques. It

is interesting to note that despite some differences in the innate immune responses between the two groups, adaptive immune responses are similar as measured by the levels of SIV-specific antibodies. In fact, statistical analysis revealed that antibody levels in macaques inoculated with low and high doses of SIV were not different at 6 or 12 weeks post-infection. This finding validates the low dose oral administration model since SIV-specific antibody levels in plasma after low dose oral administrations were comparable to those found in plasma after intravaginal [1, 2], intrarectal [240, 287] or even intravenous infections [241, 259].

It is disconcerting to see that even the administration with low doses of SIV leads to disease, and immune responses mounted are not sufficient to control viral replication and spread. These findings are in agreement with previous observation, as one study, also utilizing low viral doses via the oral route to infect infant Rhesus macaques, found that the virus spread rapidly after transmission and could be found in the circulation by day 7 [4]. The macaques were able to mount innate immune responses but they were dominated by pro-inflammatory cytokines rather than anti-viral effectors at the mucosal entry site [4]. Similar results have been obtained after vaginal administration: the strongest innate immune responses, comprised of pro-inflammatory cytokines, were seen in the

genital tract and were lowest in systemic lymphoid tissues, possibly driving the infection rather than limiting it [5]. It has been suggested that the potentially beneficial production of antiviral immune mediators (like type I interferons and interferon-stimulated genes) at mucosal sites occurs too little and too late to prevent spread of the virus [1, 5]. These studies yielded similar results regarding innate immune effectors to the ones presented here and thus also validate the use of the low dose oral transmission model.

Further characterization of the macaques inoculated with low viral doses revealed several indicators of immune activation, which is a typical sign of SIV and HIV pathogenesis and a strong predictor of AIDS disease progression and mortality [97, 108, 122, 187, 280, 283]. Plasma cytokine levels were increased in these macaques, as were the levels of proliferating CD8+ T cells in peripheral blood. Furthermore, infected macaques exhibited marked increases in T cell proliferation in several tissues, including oral and rectal mucosa as well as lymph nodes. This is similar to what has been observed for high dose inoculum experiments, as plasma cytokine levels were also increased in the high dose macaques presented here. Furthermore, previous studies from the Sodora laboratory demonstrated an early peak in levels of Ki67+ CD8+ T cells in peripheral blood and increased activation levels throughout infection in macaques

intravenously infected with SIV [214]. Additionally, others have reported similar immune activation states in peripheral blood and in tissues of SIV-infected Rhesus macaques [81, 211, 275].

Taken together, the studies presented here are the first to detail an in-depth assessment of immune and viral changes in Rhesus macaques inoculated orally with low and high doses of SIV. The similarity between the two groups in most aspects examined (including viral loads, innate and adaptive immune responses as well as immune activation) indicates that high dose inoculum studies yield accurate findings with regard to disease progression and pathogenicity in macaques. However, the differences observed here during the early phases of infection between the SIV high and low dose macaques indicate that the low dose is a better model when studying the transmission event or events early after viral exposure. Therefore, it will be important for vaccine and transmission studies to employ low doses of virus that mimic natural occurring viral doses more closely. The results presented here have led to a better understanding of early mucosal and systemic events after low dose administration and can be used as baseline for studies utilizing low doses to aid in the development of new therapy and vaccine approaches to fight HIV/AIDS.

CHAPTER FIVE

Final Discussion and Future Directions

Final Discussion

More than 33 million people worldwide are infected with the human immunodeficiency virus with 2.7 million new infections in 2007. To date, almost 30 million people have died of the resulting acquired immunodeficiency syndrome, with 2.7 million deaths in 2007 alone. Almost 300,000 of these were children; in addition, more than 12 million children became orphans or half-orphans due to AIDS-related deaths of their parents [324]. With more than 60 million people affected by HIV/AIDS, this is a major public health threat and the development of new therapies and, even more important, preventative measures is crucial. A vaccine able to prevent new infections upon exposure to the virus would be an ideal tool to help stop the spread of the virus. However, the recent failures of HIV vaccine trials [107, 234, 271] indicate that there is still a long way to go until the development of an efficient human vaccine against HIV. Furthermore, the failures made clear that the immune responses and the viral changes after exposure of the virus to a mucosal membrane are still not sufficiently understood. The work presented here served the purpose to enhance this knowledge by examining immune and viral changes after

the virus infects a new host through a mucosal route. For this purpose, the animal model of HIV/AIDS, SIV infection of Rhesus macaques, was utilized to assess immune and viral factors after oral inoculation of SIV.

A previous study from the Sodora laboratory had focused on mucosal expression of immune mediators in orally inoculated Rhesus macaques [208]. These data demonstrated that slow disease progression was associated with high expression levels of IFN-α, OAS, CXCL9 and CXCL10 at mucosal sites [208]. This early robust innate immune response might aid in an early initiation of an anti-SIV adaptive immune response and might be a sign of a healthy mucosa during a pathogenic SIV/HIV infection, able to suppress onset of opportunistic infections. The studies presented in chapter 3 built upon this previous work and focused on the expression of innate immune modulators in lymph nodes (LN) and peripheral blood and their role during disease progression. Based on the findings presented here, a model is proposed for how immune modulators might influence the earliest events following a successful SIV, and by analogy HIV, infection. Figure 5-1 depicts a representation of events occurring during rapid progression to disease (left) as well as during slow progression to disease (right).

After viral exposure to a mucosal membrane, the virus crosses it to establish infection (Figure 5-1, top) and spreads to lymph nodes (middle)

and peripheral blood (bottom). During the earliest time points after infection, the innate immune response is activated to contain the infection. Immune modulators like cytokines and chemokines play crucial roles during these processes. Previous studies by Dr. Kristina Abel assessed expression patterns of 17 different immune modulators in the SIV macaque model [1-3, 5]. Based on their significance for the oral transmission studies in the Sodora laboratory, 13 immune modulators were chosen to assess innate immune responses at the oral and rectal mucosa [208]. Due to their expression profiles, several of these were of particular interest, including IFN-α, OAS, CXCL9, CXCL10, IL-12, TNF-α, IFN-γ and IL-10. IFN-α is an antiviral cytokine, produced mainly by plasmacytoid dendritic cells upon recognition of viral infection that induces interferon-stimulated genes (ISG) to limit viral replication, and 2'-5' oligoadenylate synthetase (OAS) is an ISG that can activate RNase L to degrade viral RNA. Previous studies had indicated that their expression at lymphoid tissues was not sufficient to control viral replication [1], however, expression in peripheral blood was associated with vaccine-induced protection against viral challenge [2]. In contrast, elevated IFN-γ, CXCL9 and CXCL10 expression in lymphoid tissues was associated with viral replication and a lack in vaccine-induced protection against viral challenge [3]. CXCL9 (Monokine induced by IFN-γ, Mig) and CXCL10 (Interferon

inducible protein of 10 kD, IP-10) are two pro-inflammatory chemokines that signal through a common receptor, CXCR3, to recruit activated T cells and NK cells to sites of inflammation. Therefore, increased expression in lymphoid tissues of the unprotected macaques might lead to recruitment of more target cells for SIV and thus drive viral replication. In contrast, increased expression of these immune modulators at mucosal sites was associated with slow disease progression [208]. Due to these findings, these immune modulators were chosen for the studies presented here to assess their expression at lymphoid tissues and peripheral blood and their association with disease progression. Together, these eight immune modulators IFN-α, OAS, CXCL9, CXCL10, IL-12, TNF-α, IFN-γ and IL-10 represent a diverse spectrum of immune responses, including antiviral as well as pro- and anti-inflammatory cytokines/chemokines. The studies presented here indicated that four were particularly interesting: IFN-α, OAS and CXCL9 (Mig) and CXCL10 (IP-10). They exhibited distinct patterns of expression at oral mucosa, lymph nodes and peripheral blood that correlated with the rate of disease progression.

In LN of rapid progressors (Figure 5-1, left, middle), these four immune modulators were upregulated several fold compared to uninfected macaques: IFN-α up to 180-fold (light purple symbols), OAS up to 80-fold (light blue), CXCL9 up to 20-fold (yellow) and CXCL10 up to 60-fold

(green). In contrast, the slow progressor did not exhibit increases in these four immune modulators and expressed them at levels in the range of uninfected macaques (Figure 5-1, right, middle). Elevated expression of CXCL9 and CXCL10 in LN of rapidly progressing macaques suggested that more CXCR3+ cells would be recruited to the LN. However, this was not the case (depicted by the crossed-out cells in the rapidly progressing macaques, Figure 5-1, left middle), and all macaques exhibited similar numbers of CXCR3+ cells in their LN. It is hypothesized that immune dysfunction of LN is a marker of disease progression [29, 85, 267] and the inability to recruit the appropriate target cells despite the presence of the ligands indicates an additional sign of dysfunction in this immunological important compartment.

In peripheral blood of orally inoculated Rhesus macaques, the same immune mediators were examined but in this compartment only the expression of OAS (light blue) and CXCL10 (green) were associated with disease progression (Figure 5-1, bottom). The rapidly progressing macaques exhibited large fold change increases of OAS (up to 80-fold) and CXCL10 (up to 120-fold) compared to uninfected macaques (Figure 5-1, bottom left). In contrast, the slow progressor exhibited expression levels of these two immune modulators similar to uninfected macaques (Figure 5-1, bottom right). These findings indicate that in addition to the

traditionally used viral loads and CD4+ T cell counts, the expression of innate immune modulators has the potential to serve as markers for disease progression.

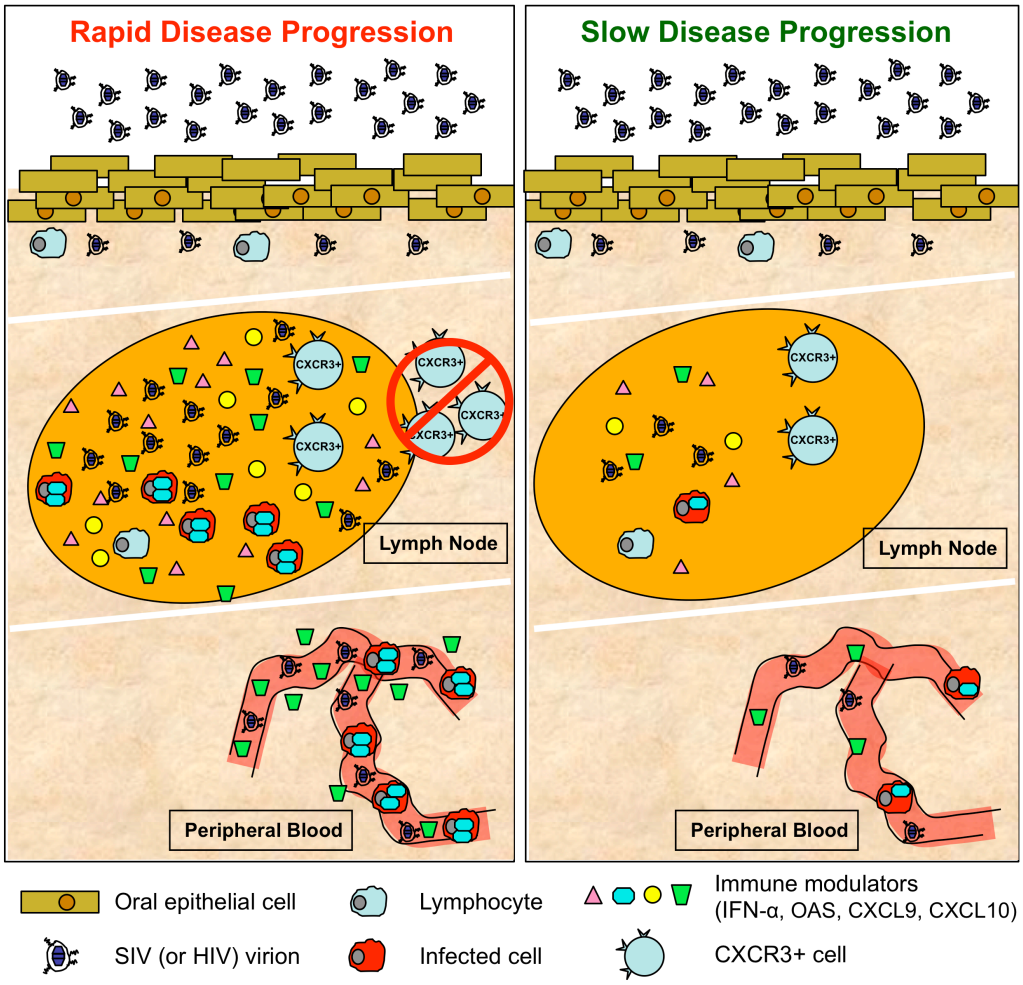

Figure 5-1. Model of differences in disease progression after oral administrations of SIV.

Model for how immune modulators might influence the earliest events following a successful SIV, and by analogy HIV, infection. It depicts a representation of events occurring during rapid progression (left) as well as during slow progression to disease (right).
The number of symbols does not represent actual fold change, but trends are indicated, i.e. higher number of symbols indicates higher fold change.

Many studies to date including the one described above utilized high doses of SIV for vaccine and pathogenesis studies. High doses are necessary to ensure infection of the animals; however, these doses exceed naturally occurring viral amounts in bodily fluids. Due to this large number of virions present at a mucosal site, events happening early might be affected since the early immune response might simply be overwhelmed. This might especially impact transmission and vaccine studies since the earliest viral and immune events happening after mucosal exposure to a virus are crucial for these types of studies. Low doses on the other hand more closely mimic natural occurring viral amounts and might therefore be more appropriate for vaccine and transmission studies. The studies presented in Chapter 4 aimed at examining immune and viral changes after inoculations with low viral doses via the oral route. These results were compared to those obtained after high dose inoculations. Based on this in-depth comparison of immune and viral changes in macaques inoculated with low and high doses of SIV, a model is proposed that explains the similarities and differences between the two groups. Figure 5-2 depicts the events happening after exposure to high (left) and low doses of SIV (right) during acute (A) and chronic infection (B).

After administration of SIV to the mucosal membrane, high doses resulted in a greater number of viral variants transmitted compared to low dose inoculations (Figure 5-2A, top), although both methods led to successful infection regardless of the number of virions transmitted. During acute infection at very early time points, some of the low dose macaques exhibited a slight delay in viral replication kinetics that might be due to the lower number of virions transmitted, resulting in lower viral loads (depicted as less virions in the low dose macaques in lymph nodes (Figure 5-2A, middle) and peripheral blood (Figure 5-2A, bottom)). However, during chronic infection viral loads were similar in macaques of both groups (Figure 5-2B). In lymph nodes of macaques that received high doses of SIV, a more robust antiviral response could be observed both during acute and chronic infection: IFN-α (light purple) and OAS (light blue) both exhibited more elevated fold changes in the high dose macaques in comparison to the low dose macaques (Figure 5-2A and B, middle). This increase in anti-viral gene expression is potentially due to the higher number of virions transmitted since more virions might have been able to spread to peripheral LN. However, inflammatory gene expression during chronic infection was generally similar between macaques that received high and low dose challenges (Figure 5-2B, middle, yellow and green symbols).

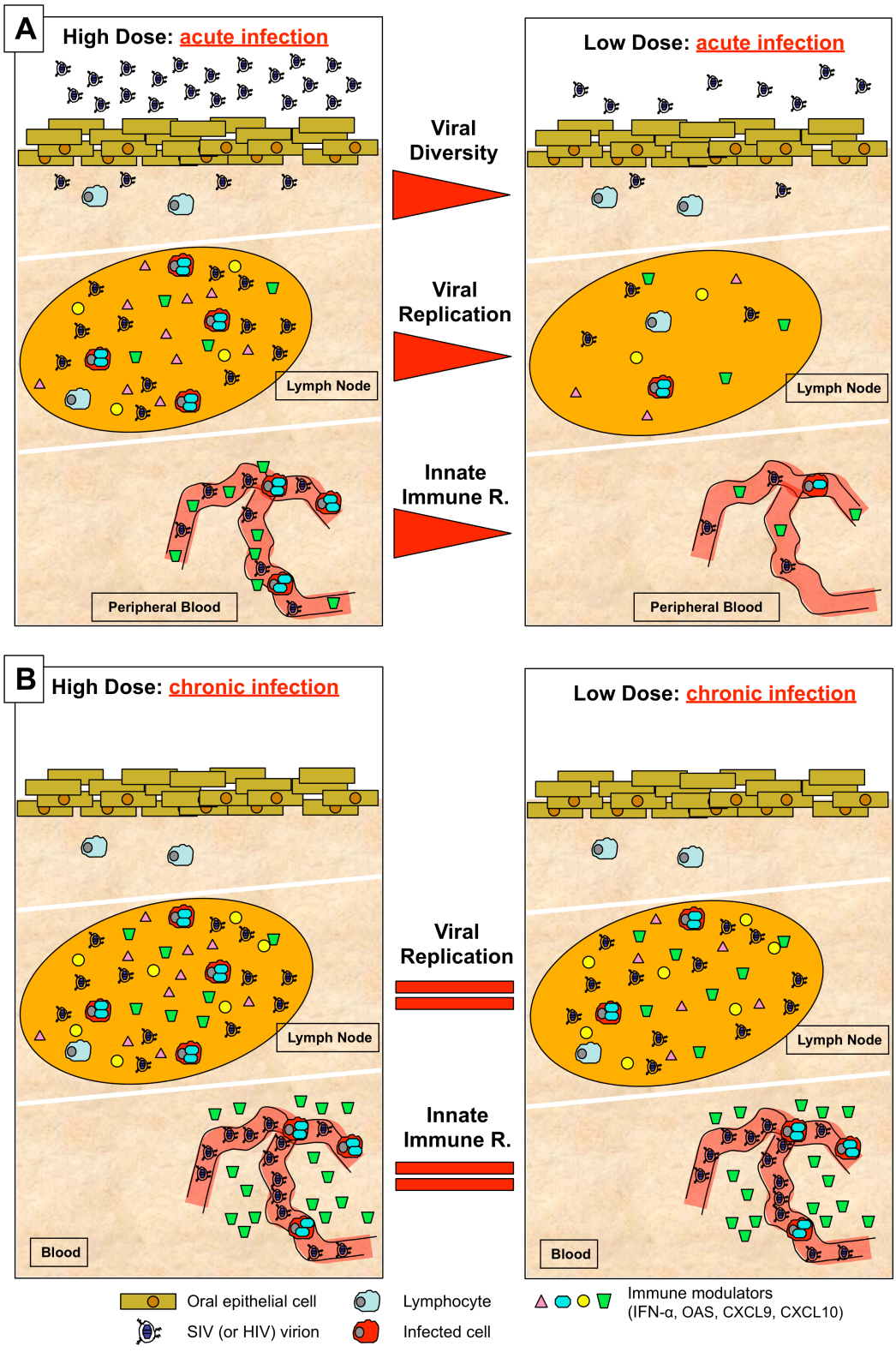
A
High Dose: acute infection
Low Dose: acute infection
Viral Diversity
Viral Replication
Innate Immune R.
Lymph Node
Lymph Node
Peripheral Blood
Peripheral Blood
B
High Dose: chronic infection
Low Dose: chronic infection
Viral Replication
Innate Immune R.
Lymph Node
Lymph Node
Blood
Blood
Oral epithelial cell
SIV (or HIV) virion
Lymphocyte
Infected cell
Immune modulators
(IFN-α, OAS, CXCL9, CXCL10)

Figure 5-2. Model of low and high dose oral administrations of SIV.
Model depicting the events happening after exposure to high (left) and low doses of SIV (right) during acute (A) and chronic infection (B). It indicates the similarities and differences between the two groups.
The number of symbols does not represent actual fold change, but trends are indicated, i.e. higher number of symbols indicates higher fold change.

In peripheral blood, expression changes were only observed for OAS (light blue) and CXCL10 (green). The low dose macaques exhibited a slight delay of expression kinetics of these two immune modulators during acute infection (Figure 5-2A, bottom). During chronic infection, responses in peripheral blood were very similar between the two groups with OAS and CXCL10 transcripts upregulated in PBMCs of macaques administered high or low doses of SIV (Figure 5-2B, bottom).

Not surprisingly, these findings indicate that both high and low doses of SIV can establish infection as soon as the virus crosses a mucosal membrane and is able to spread due to an immune response that is not sufficient to prevent this initial replication. Based on the findings and models presented here it also became clear that immune and viral events occurring during the chronic phases of infection are similar regardless of the infecting viral dose, indicating that high dose inoculations are suitable for studies of disease progression and pathogenicity. However, early

immune and viral changes can be impacted by the viral dose and therefore, a low dose approach mimicking natural viral amounts is a better model when studying the transmission event or events early after viral exposure.

It is noteworthy that in both studies CXCL10 and OAS were upregulated in PBMCs of Rhesus macaques during chronic stages of infection. Interestingly, assessment of PBMCs from sooty mangabeys, natural hosts of SIV infection that do not progress to sAIDS, revealed increased levels of innate immune mediators during acute infection [33, 174] which however then decreased to low levels during chronic infection as observed here. Based on these results, expression of OAS and CXCL10 might be used as diagnostic markers to identify hosts that will progress to AIDS at a faster rate. The use of these two immune modulators as markers of disease progression might be beneficial for HIV-infected individuals to determine start and dosage of anti-viral therapy. A brief discussion of the biology of these two molecules will help in the understanding of their origins and functions in general and in HIV disease.

CXCL10 is also known as IP-10 (Interferon inducible protein of 10 kD) and was first discovered in 1985 by Luster and colleagues as a gene that is inducible by rIFN-γ in cell lines as well as human mononuclear cells, fibroblasts and endothelial cells [191]. The 5'-flanking region of the

CXCL10 gene contains an interferon-stimulated response element (ISRE) and two NF-κB sites [190, 306], and it is now known that CXCL10 can be induced not only by IFN-γ but also but type I interferons as well as LPS [191, 306]. Furthermore, exposure to HIV-1 can induce CXCL10 expression in monocyte-derived macrophages and dendritic cells [96]. It was first described by Taub et al. that CXCL10 can act as chemoattractant for activated CD4+ T cells and NK cells [291, 292]. Soon after, the receptor for CXCL10 was identified [188] and named CXCR3. Consistent with the chemoattractant properties of CXCL10, its expression has been demonstrated on activated T cells, especially TH1 cells, and NK cells [32, 139, 188]. Thus, expression of CXCL10 leads to the recruitment of CXCR3+ cells including activated T cells and NK cells to sites of infection. Since CCR5+ cells are generally found within the CXCR3+ CD4+ T cells [246, 248], CXCL10 might lead to the recruitment of the ideal target cells for HIV/SIV. CXCL10 is a secreted protein of 77 amino acids and can be produced by several different cell types including lymphocytes, monocytes, keratinocytes and endothelial cells. The data presented here indicated that CD3+ T cells were the major source of CXCL10 mRNA within PBMCs of the orally SIV-inoculated Rhesus macaques. Since CD4+ T cells are one of the major target cells for SIV, it is interesting to speculate that the infected cells produce the CXCL10 to recruit more

activated cells that would fight the infection. However, from the finding that elevated CXCL9 and CXCL10 levels in LN did not yield an increase in CXCR3+ cells at that compartment, it is possible that this is true for peripheral blood as well. To overcome the lack of recruited cells, an even higher production of the chemokine might be the result, which is observed here.

The intracellular protein 2',5'-oligoadenylate synthetase or OAS is an interferon-inducible gene and part of the antiviral interferon system. After infection with HIV, several intracellular receptors including TLR7, RIG-I or MDA-5 can recognize viral nucleic acids. This leads to a signaling cascade culminating in the induction of type I interferons. Binding of IFN-α or IFN-β to their cell surface receptors leads to another signaling cascade resulting in the induction of interferon-response genes. Like CXCL10, the 5'-flanking region of the OAS gene contains an ISRE and therefore, OAS can be induced by IFN-γ, IFN-α or IFN-β [27, 250, 311]. After induction, OAS becomes activated and stimulated by double-stranded RNA (dsRNA) produced during viral infections. The exact nature of these dsRNA has not been defined precisely but they could include single-stranded transcripts that have double-stranded character due to secondary structures or RNA duplexes as replicative intermediates [261]. A well-defined activator of OAS is the HIV TAR RNA sequence present at the 5'-end of HIV

transcripts. It forms a stable secondary structure able to bind to OAS and thus activate it [192]. After activation, OAS then catalyzes the synthesis of 2',5'-oligoadenylates [157]. These nucleic acids bind to monomeric RNase L, a latent endoribonuclease, and this binding leads to its activation and induces the formation of active homodimeric RNase L [72]. Activated RNase L is able to cleave both mRNA and rRNA thus leading to inhibition of protein expression [94, 326]. By these means, OAS establishes an antiviral state in the cells in which it is activated. In theory, this could be any cell expressing interferon-receptors; however, the findings presented here indicated that CD14+ monocytes contributed substantially to the increased OAS mRNA expression observed in the orally SIV-inoculated macaques. Only a small percentage of blood monocytes have been shown to be infected with SIV; however, the fact that type I interferons act in paracrine also on neighboring cells, and monocytes are able to produce OAS to much higher levels than other lymphocytes [322] would be a potential explanation for the elevated OAS levels in monocytes.

Based on these findings, it is likely that expression of CXCL10 and OAS is induced, at least in part, by SIV infection itself. This response however, is not sufficient to limit viral replication and spread, as the macaques continue to exhibit high viral loads. It might be possible that these two molecules do not function properly as it has been demonstrated

here, that elevated expression of CXCL10 in lymph nodes does not lead to an increased influx of CXCR3+ cells. Also, despite elevated expression of OAS, SIV replication is not suppressed.

It is also interesting to consider that although the oral route of infection is only one route through which new HIV infections occur, other mucosal sites contain similar epithelial cell structures (stratified squamous epithelium) like the vaginal or penile mucosa. Therefore, it is likely that the findings from the oral mucosa can be applied to other mucosal sites as well, These studies indicate that it is necessary to assess different types of immune responses at multiple tissue compartments to obtain a complete understanding of events happening early after infection with SIV (and also HIV). Generating an immune response that is quick enough and in the right tissues will be a major obstacle for any vaccine to successfully prevent the virus from infecting a new host. It is likely that a more complete knowledge of the earliest innate and adaptive immunity events will lead to approaches for novel vaccines and therapies to combat HIV infection/disease.

Future Directions

Results from both chapters presented here open up new avenues of research to bring forward our knowledge about events happening at

mucosal sites and systemically after viral exposure. Based on these findings, two areas will be of special interest: disease progression and transmission. The identification of diagnostic markers in peripheral blood of humans to predict disease progression would be of enormous interest to physicians to get a better idea when to initiate antiviral therapy. However, the studies presented here have assessed the immune markers OAS and CXCL10 in non-human primates. A few promising studies have been done with these immune mediators in humans. There is evidence that CXCL10 (IP-10) might be increased in a specific manner in HIV+ patients as the CXCL10 levels in plasma rapidly increase in HIV-infected individuals and stay elevated in all patients assessed [284]. In contrast, in HBV or HCV infected individuals, plasma CXCL10 (IP-10) levels rise later in infection and are not elevated in every patient [284]. Additionally, others have suggested the use of CXCL10 in cerebrospinal fluid for the diagnosis of AIDS dementia complex [48] or OAS protein levels in PBMCs as prognostic indicator for progression to AIDS [249]. Based on these earlier studies and the findings presented here, an important future experiment would be to confirm that the expression of OAS and CXCL10 in peripheral blood of HIV-infected individuals is indeed a useful marker for disease progression in humans. To that effect, blood samples should be taken from HIV-infected patients during acute stages, as well as chronic and end

(AIDS) stages of infection. Comparing the expression levels of these two (and possibly other) immune mediators to uninfected individuals would support the hypothesis that expression of OAS and CXCL10 are indeed markers of disease progression. Additionally, it would also be interesting to examine whether heightened expression levels do actually lead to increased protein levels as well; this can be done in the non-human primate model as well as with human samples.

This would then allow one to further test whether the expression and presence of these two molecules would merely be a consequence of infection or whether it could be a driving force behind immune activation and viral replication. Rhesus macaque, sooty mangabey or human cells could be used for *in vitro* infections with the appropriate virus (i.e. SIV or HIV). To examine the effect of OAS on viral replication in this system, type I interferons could be added to the cell cultures prior to or after infection with SIV/HIV. Similarly, the cell cultures could be exposed to differing concentrations of CXCL10. Expression of activation markers on cells (e.g. Ki-67 or HLA-DR on T cells), cytokine/chemokine expression and viral replication would be the read-outs of these experiments to assess the impact of OAS and CXCL10 on infected cells. In fact, one study showed that CXCL10 (IP-10) stimulated HIV replication in macrophages and lymphocytes *in vitro* [173]. Furthermore, it also showed that blocking the

chemokine or its receptor decreased viral replication [173]. If this can indeed be confirmed in additional studies, then this, in addition with the findings presented here that heightened expression of OAS and CXCl10 are associated with disease progression, would open up new therapeutic possibilities by blocking these immune modulators *in vivo* to limit viral replication and possibly disease progression in infected individuals. The knowledge of which cell types produce these immune modulators, would help in this endeavor by providing specific targets for blocking of OAS or CXCL10.

Another important area of research based on findings presented in chapter 3, is to delineate the mechanisms by which increased immune modulators at mucosal sites aid in the slower disease progression observed. It was shown that the two proinflammatory chemokines CXCL9 (Mig) and CXCL10 (IP-10) were upregulated at the oral mucosa in SIV+ macaque with a slow rate of disease progression. It will be interesting to see whether the presence of these two chemoattractants will indeed lead to an increase in the number of CXCR3-expressing cells, namely NK and T cells, to the oral mucosa. This could be accomplished through either a flow-cytometric approach or by immunofluorescence staining for CXCR3 and cell-specific markers. To delineate the influence of the presence or absence of these cell types on disease progression, the macaque model

could be utilized to block the action of the two proinflammatory chemokines by blocking them through CXCL9/10-specific or CXCR3-specific antibodies. Furthermore, in a different set of experiments, CXCL9 or CXCL10 could be applied to the oral mucosa to experimentally recruit their target cells to the oral mucosa of SIV-infected macaques. Both types of experiments could then be analyzed to determine the impact these two immune modulators and their target cells exert on disease progression.

These experiments will be especially interesting in light of the timing of immune activation at mucosal sites. As discussed above, epidemiological and observational studies in humans have shown that the presence of other infections at mucosal sites lead to an increased risk of acquiring HIV after exposure. This could be due to an activated immune state at the infection site, including the presence of more target cells for the virus, or due to micro-breaks in the mucosa caused by the presence of other infections enabling easy entry of viral particles. In fact, it has been previously observed that the induction of innate immune responses at the vaginal mucosa through TLR ligands prior to SIV inoculation elicited a broad range of immune modulators but did not protect macaques from infection [313]. Furthermore, macaques treated with these TLR ligands prior to viral exposure exhibited higher viral loads than macaques not treated [313]. Together with the findings presented here this would mean

that the timing of immune activation at a mucosal site is crucial: increased activation prior to exposure might increase the risk of infection, whereas increased activation after infection might be beneficial by prolonging the time until disease.

In this regard, studies assessing factors influencing transmission are another important research area for future experiments. As mentioned above, the reasons for the increased risk to acquire HIV infection in the presence of other sexually transmitted diseases is not known and can have several causes, including the presence of more target cells or microbreaks. In fact, it has been shown that preexisting inflammation due to infection at the vaginal mucosa can lead to microbreaks, ulcers and thinning of the mucosal barrier, potentially leading to easier viral entry [244]. The studies presented in chapter 4 laid the groundwork for experiments assessing factors influencing transmission, which are ongoing in the Sodora laboratory. To study these factors, it is necessary to utilize an appropriate dose; a dose too high would lead to infection regardless of confounding factors while doses too low would not infect animals at all. By testing different low viral doses in the presence of a healthy oral mucosa and their effect on macaques, a dose of 2000 $TCID_{50}$ was determined to be optimal in these experiments. Furthermore, immune and viral changes after these low dose oral inoculations were analyzed in

detail. These “baseline” results can be used as comparison while studying factors influencing oral transmission. Future experiments will assess the impact of pre-existing inflammation, gingivitis, on the ability of the virus to cross the mucosal layer. Gingivitis is a common human oral infection, occurring in 70 to 90 % of the human adult population [24, 109, 135]. It is an inflammation of the gingiva, the oral epithelial cells around the teeth, caused by bacterial plaque that accumulates in the small spaces between the teeth and the gums. Gingivitis usually occurs without loss of tooth attachment but leads to an influx of lymphocytes and monocytes to the sites of inflammation, thus accumulating potential target cells for HIV/SIV. Rhesus macaques usually do not develop gingivitis due to their diet of crunchy monkey-chow that reduces the amount of gingivitis-inducing plaque on their teeth. However, gingivitis can be experimentally induced by tying silk ligatures around the teeth and feeding soft food. The silk ligatures provide a niche for bacteria and the softened food prevents the scraping of the teeth [76, 77]. By this approach, the impact of preexisting inflammation alone (in the absence of oral lesions caused by more severe periodontitis) can be studied as the confounding factor potentially influencing transmission. Macaques with and without gingivitis will be inoculated orally with a low dose of SIV (determined in chapter 4) and monitored carefully for successful infection. Both groups will be compared

to assess the impact of gingivitis on transmission of SIV and resulting immune responses. Experiments in this study will assess similar parameters as described in chapter 4, including viral loads and viral diversity as well as innate and adaptive immune responses at different tissue sites. The working hypothesis is that increased oral inflammation will lead to increased transmission in macaques with gingivitis. In addition, it will be interesting to follow these macaques longitudinally to determine whether the inflammation at the mucosal site will also have an influence on the rate of disease progression. These studies are important for several reasons: gingivitis is present in a large number of adult people throughout the world and could have a potential impact on HIV transmission for people engaging in oral intercourse. This is especially troublesome since the misconception exists that oral sex is less dangerous to acquire HIV [257, 266] and sex workers in Africa get instructed that engaging in this supposedly lower risk behavior will keep them HIV-free. Together with the fact that gingivitis and periodontal diseases are even more widespread in Africa [82], the risk of acquiring HIV in these settings might be greater than previously thought.

All these studies will enhance the knowledge about mucosal transmission of SIV/HIV. The studies presented here, as well as many of the future experiments suggested, focus on oral transmission of the virus.

It will be important to assess as many mucosal sites as possible to be able to compare differences and similarities of transmission across a variety of mucosal sites. In addition to the oral route of infection, especially vaginal and rectal transmission have been studied in great detail due to their importance in natural settings [5, 58, 59, 134, 154, 177, 199, 204, 243, 281, 297, 299, 318]. An additional mucosal site, the male foreskin, has not been studied as much as other mucosal sites, possibly due to the difficulty assessing this mucosal compartment. However, the male foreskin might be important in HIV transmission: vaccinated individuals in the Merck STEP vaccine trial seemed to be at higher risk to acquire HIV than their unvaccinated counterparts (placebo group) and further analysis revealed that uncircumcised men (i.e. men with a foreskin) in the vaccinated group exhibited increased risk of infection, indicating a potential role of the foreskin in transmission [39]. The Rhesus macaque model could be utilized to assess this route and evaluate its potential as mucosal challenge route for future SIV/HIV vaccine trials. Experiments are currently ongoing in the Sodora laboratory in which macaques are being experimentally inoculated with SIV via the foreskin. It will be interesting to determine the viral diversity after infection through the foreskin/penile route and compare the number of viral variants responsible for establishing infection to other routes of mucosal transmission. This will

serve the purpose to determine whether selection of certain viral variants occurs through this route and how this might differ from other routes.

The studies presented here together with those done previously by the Sodora laboratory and others indicate that systemic immune activation later in infection is detrimental to the host [97, 108, 122, 187, 280, 283]. The association between the presence of other sexually transmitted diseases and the increased risk of HIV acquisition imply that an activated immune state at mucosal sites prior to infection is also harmful. However, there might be a small window of opportunity during which upregulation of certain types of immune modulators at mucosal sites is beneficial by prolonging the time until disease. The future studies suggested here will examine the mechanisms of mucosal (and systemic) immune responses against the virus in more detail, providing insights for future vaccines or therapies that might hopefully be developed to block virus before infection can be established.

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