THESIS

MODELING THE EVOLUTION OF SIV INTO HIV USING HUMANIZED MICE

Submitted by

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Graduate Degree Program in Cell and Molecular Biology

In partial fulfillment of the requirements

For the Degree of Master of Science

Colorado State University

Fort Collins, Colorado

Fall 2016

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ABSTRACT

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Acquired Immunodeficiency syndrome (AIDS) is caused by two lentiviruses belonging to the family *Retroviridae*, namely HIV-1 and HIV-2, which originated from SIVcpz and SIVsmm respectively. Multiple independent cross-species transmission events of SIV from chimpanzees (SIVcpz*Ptt*) and gorillas (SIVgor) have given rise to four groups of HIV-1 (M, N, O, P), while transmission from sooty mangabeys (SIVsmm) is responsible for at least 8 groups of HIV-2 (A-H) (Ayouba et al., 2013; Gao et al., 1999; Hirsch et al., 1989). Some of these groups are extremely rare. However, four have established themselves in human populations as pandemic (HIV-1 group M) or epidemic (HIV-1 group O, HIV-2 groups A and B) outbreaks (Faria et al., 2014). While these data suggest that SIV transmission to a human host is by itself not sufficient to establish a new epidemic outbreak, it also implies that viral adaption is necessary for efficient spread of the virus within humans (Marx et al., 2004). However, exact role of factors such as immune selective pressure and genomic changes, contributed to successful SIV adaptation in humans remains unclear.

Study of SIV transmission to humans has been limited by lack of an appropriate model. Ethical constraints prevent experimental challenge of human subjects with SIV and transmission studies have thus far been limited to non-human primates (NHPs) (Chahroudi et al., 2012). While NHP studies have been instrumental to identify key differences in innate and adaptive immune responses that influence transmissibility and virulence of SIV between species, until now experimental *in vivo* challenge of a functional human immune system with SIV has not been

possible. Recently however, a new generation of humanized Rag2$^{-/-}\gamma c^{-/-}$ mice has been established that supports systemic engraftment of a functional human immune system (Akkina, 2013; Denton et al., 2012). These animals are susceptible to HIV-1 infection by mucosal routes and display key features of pathogenic HIV-1 infection in humans, including sustained plasma viremia, CD4+ T cell depletion and increased levels of immune exhaustion markers (Berges et al., 2006; Palmer et al., 2013). Additionally, humanized mice are able to mount adaptive immune responses to HIV-1 infection, producing antibodies and HIV-1 specific T cell responses. In the current study, we used humanized Rag2$^{-/-}\gamma c^{-/-}$ mice to study SIV transmission to humans. We hypothesized that humanized mice could support SIV infection, and that the presence of *in vivo* selective pressures, either innate or adaptive, would drive viral evolution. That is, we expected the majority consensus virus emerging in the plasma of productively infected humanized mice different from the stock virus used to infect the animals. Viral adaptation within infected humanized mice could produce a variant virus more fit for growth and transmission in humans. We infected humanized mice with both SIVsmmE041 and SIVcpzLB715 isolates and measured plasma viral loads and determined complete viral genome sequences. Both SIVsmm and SIVcpz showed stable viral loads over time for up to 7 months. SIVsmm virus from the infected mice was successfully passaged to three successive generations of humanized Rag2$^{-/-}\gamma c^{-/-}$ mice via intraperitoneal route. We were also able to show that human cell-adapted SIVsmm can infect humanized mice through the mucosal route.

Sequence analyses of SIVsmm and SIVcpz output virus from these mice showed many functional mutations in various genes such as Gag, Pol, Env, Vif, Vpx, Vpr and Nef regions. In both viruses, Env regions showed the highest number of mutations suggesting that the envelope region of this virus might be under selective pressure for this virus to be able to replicate in

human cells. We observed that the number of mutations in all genes increased over time, suggesting that the SIVsmm virus is continuously evolving and adapting for successful replication and transmission in these humanized mice. These studies will provide a flexible *in vivo* model for elucidating the mechanisms underlying SIV transmission and gain of function to replicate in humans.

ACKNOWLEDGEMENTS

Thanks are due to my family, friends and committee members for their support during the entire span of my studies. Thanks to Ramesh Akkina, for his financial support to finish my studies here in Colorado State University, Fort Collins.

I can't complete my studies at without big support from my father and mother Satyanrayan Boddeda and Satyavathi, I am really great full to my wife Pavani Beesetty to support me in hard times and also great help in correcting my thesis.

Last but not least special thanks to Dipu Mohan spared lot of his time to correct my thesis and gave me moral support.

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Introduction: Review of Literature

1.1 HIV epidemiology

Acquired immunodeficiency syndrome (AIDS) was recognized as a new disease in 1981, caused by a retrovirus now known as HIV-1, and was one of the most devastating infectious diseases in 1983 (Gallo at al., 1984). According to 2014 global HIV statistics from the Center for Disease Control (CDC), HIV is still a major public health threat. An estimated 36.9 million people are living with HIV, which include 2.6 million children and most of these children were infected from HIV positive mothers during pregnancy and breast feeding. This accounts for a total of 0.8% prevalence globally (UNAIDS). Out of the total 36.9 million people, 25.8 million people are in Sub-Saharan Africa, accounting for 70 percent of the total infected population. Only 54 percent of the people living with HIV know that they indeed harbor the virus. According to the World Health organization's data, a total of 34 million people have died due to HIV infection by the end of 2014. Approximately 1.2 million people die from AIDS every year and about 5,600 people get infected with HIV per day, which translates to 230 newly infected people per hour. Although anti-retroviral therapies are available for controlling HIV infection and slow down the progression to AIDS, only 41 percent of people have access to this therapy. A lot of time and effort has been spent on the development of HIV vaccines, but the feasibility of formulating an effective vaccine still largely remains uncertain (Barouch, 2008; Richman et al., 2009). Thus HIV continues to be an on-going threat and poses a potential threat well into the future. There is a high significance to study the origin of this virus and how it jumped the cross-species barrier, as such information could be used to prevent the spread of this virus and transmission of HIV-like diseases in years to come.

1.2 Origin of HIV-1 and HIV-2

Ever since HIV-1 was discovered in 1981, the reasons for the sudden emergence of this virus were a topic of much scientific curiosity. In 1986 we got the first clue towards this end when a different virus with similar morphological features that causes AIDS was found in West Africa (Clavel et al., 1986). This virus was named HIV-2, was distantly related to HIV-1, but was closely related to simian virus from macaques (Chakrabarti et al., 1987).

Soon after the discovery of HIV-2, many simian immunodeficiency viruses were found in various non-human primates from Sub-Saharan Africa. These viruses mostly clustered closely with HIV-1 and HIV-2 in phylogenetic analysis. Old world monkeys are naturally infected with a group of lentiviruses termed SIVs. Based on the specific primate species of its origin, SIV is represented with a suffix that denotes the primate of origin. For example SIV from chimpanzee is termed SIVcpz, whereas SIV from sooty mangabey monkey is termed SIVsmm (Figure 1). Most of these viruses are non-pathogenic to their natural hosts but cause AIDS like diseases when the virus undergoes cross-species transmission to other hosts.

By comparing genetic organization of these viruses, it was found that there is strong evidence to believe that HIV-1 has originated from SIVcpz from chimpanzees (Huet et al., 1990) and HIV-2 from SIVsmm from sooty mangabey monkeys (Hirsch et al., 1989). This is the first evidence that AIDS had emerged as a result of cross-species transmission of SIVs from apes/monkeys to humans. There are also evidences that HIV-1 and HIV-2 were the result of zoonotic transmission from these primates to humans (Hahn et al., 2000).

1.2.1 Origin of HIV-1

As mentioned previously, HIV-1 originated from SIVcpz by zoonotic cross-species transmission to humans. SIVcpz falls into two lineages known as SIVcpz*Ptt* and SIVcpz*Pts*.

Although there is not much difference between these primate subspecies, viruses from them are at least 30 percent different based on their genetic sequence homology (Haesevelde et al., 1996). These two subspecies of chimpanzees might have separated between Eastern and Central Africa for a long time before the acquisition of SIVcpz, resulting in the divergent sequence of SIVcpz viruses seen in them.

HIV-1 has four subtypes known as M, N, O and P, which we believe originated by independent cross-species transmission events. Out of 4 subtypes, HIV-1 M represents the pandemic strain and has spread to almost every country in the world. HIV-1 group O was discovered in 1990, but this is less prevalent than group M. The group O infection only accounts 1 percent of total HIV-1 infections and is restricted to Cameroon, Gabon and other neighboring countries (Mauclere et al., 1997). HIV-1 group "N" was only identified in 13 patients and is restricted only to Cameroon (Vallari et al., 2010) whereas HIV-1 group "P" was identified only in 1 patient (Plantier et al., 2009).

Phylogenetic analysis revealed that HIV-1 M and N group cluster with and are more related to SIVcpz*Ptt*, HIV-1 P group originated from SIVgor whereas the origin of HIV-1 O group is still unknown (Sharp & Hahn, 2011). Although all phylogenetic studies support the origin of all four strains of HIV-1 from chimpanzee subtype SIVcpz*Ptt* from Central Africa, there is not much evidence for the origin of HIV from SIVcpz*Pts* from Eastern Africa. Although *in vitro* replication and CD4+ kinetics of these both strains are similar (Takehisa et al., 2007), there are a few explanations that support inefficient transmission of SIVcpz*Pts* to humans. One such explanation is that there is not much human interaction with monkeys in Eastern Africa that harbor SIVcpz*Pts* and the other explanation could be that SIVcpz*Ptt* adapted successfully to

overcome human restriction factors such as tetherin to support viral replication in humans whereas SIVcpz*Pts* failed to overcome human restriction factors.

1.2.2 Origin of HIV-2

As mentioned earlier, HIV-2 originated from SIVsmm. HIV-2 is less prevalent than HIV-1 and is mostly restricted to West Africa, with highest prevalence recorded in Guinea and Senegal (De Silva et al., 2008). In 1996 more reports pointed out the fact that SIVsmm circulating in West Africa very closely resembled HIV-2, supporting the hypothesis that SIVsmm transmitted to humans (Chen et al., 1996). HIV-2 replicates at a slower rate in humans compared to HIV-1, and there is no evidence for mother to child transmission of HIV-2; these might be some reasons for the low prevalence and lesser spread of HIV-2 in contrast to HIV-1 (Popper et al., 2000). SIVsmm is found in sooty mangabeys both in the wild and in captivity but is non-pathogenic to its natural host. Although sooty mangabeys are not of much interest for meat production, they are often hunted as agriculture pests and this might be the causal route of transmission to humans.

HIV-2 comprises total 8 distinct subtypes termed as HIV-2 A to HIV-2 H. The Research community believes that these 8 strains resulted from independent transmission of SIVsmm to different human hosts. Only group A and B have spread within human population at higher rates compared to the other subtypes. Group A has been found throughout West Africa whereas group B show high prevalence in Cote d'Ivoire (Peeters et al., 2003; Ishikawa et al., 2001). The remaining subtypes such as HIV-2 C to HIV-2 H were identified in single individuals, suggesting that these subtypes may not have the potential for spread within the human population and hence could be dead-end transmissions. Group C, G, H of HIV-2 have been linked to SIVsmm strain from Cote d'Ivoire, group D closely is related to SIVsmm strain from Liberia and

groups E and F show similarity with SIVsmm strains from Sierra Leone (Gao et al., 1992; Chen et al., 1996).

1.3 Theories on HIV origin / unresolved issues on origin of HIV

AIDS has grown to pandemic proportions as a disease, however, there are some critical questions that still need to be answered such as where, when, how and why HIV became a deadly pathogen to humans. Although HIV became epidemic in late twentieth century, we are still trying to answer some of the key points such as: i) Why the HIV epidemic began only in the late twentieth century while monkeys have harbored SIV for a very long time? ii) Why only HIV-1 M & O and HIV-2 A & B showed high prevalence in humans and became epidemic, i.e., why are HIV-1 N & P and HIV-2 C to H not circulating in humans or not as prevalent? iii) How long did it take for SIV to mutate in humans and became HIV-1 or HIV-2, iv) Why HIV-2 is geographically specific and contained to only specific regions of West Africa and v) Is HIV transmitted by zoonosis? Stored samples from humans in West Africa had been found to be infected with HIV-1 M and HIV-1 O viruses by 1959 and 1963, respectively (Zhu et al., 1998; Jonassen et al., 1997). But we do not know how much earlier these viruses became entrenched in the human population in West Africa.

There are few theories explaining the above stated unresolved issues of the origin of HIV. Based on available data, we are confident that SIV was first introduced to humans as early as 1930 with a confidence interval of 20 years from 1950 (Korber et al., 2000). There are two major hypotheses to explain the outbreak of HIV and reasons why HIV became an epidemic in the late twentieth century. Hahn et al., 2000, suggested that SIVcpz and SIVsmm were transmitted to humans by exposing human mucosal membranes to infected animals (Hooper, 2000). Transmission of lentivirus from primates to humans by direct exposure to infected animal blood

in the process of hunting, butchering and consumption of uncooked food as shown in figure 2 and 3 is a major factor.

However, this hypothesis by itself might not support the whole zoonosis aspect of HIV because if the direct exposure of these infected monkeys to humans is the primary reason for SIV transmission to humans, then it should have happened much earlier than in the late twentieth century. Some other groups of researchers added additional information to support zoonotic transmission. They believe that there are many other factors involved in the AIDS epidemic of the late twentieth century. The factors proposed are social disruptions with the majority of urbanization happening in the twentieth century, increased prostitution, socio-behavioral changes, and some still unknown factors would have contributed to HIV epidemic in twentieth century (Hahn et al., 2000). In addition, the use of non-sterilized needles for injections and vaccination campaigns might have contributed to serial viral passage from one human to other human and facilitated viral adaptations to new host (Verdrager, 1995). In summary the first hypothesis opines that SIVcpz and SIVsmm entered into the human population by practices such as bush meat hunting, trading and consumption but HIV became epidemic in twentieth century due to urbanization, increase in prostitution, travel and social life changes.

The other hypothesis suggested that the attenuated oral polio vaccine (OPV) trials that began in Congo might be responsible for origin of HIV-1 M group whereas OPV trails from Central and West Africa were responsible for HIV-N and O and HIV-2 respectively (Hooper, 2000). This hypothesis doesn't support the origin of HIV due to following reasons: i) This hypothesis is only based on assumption that OPV trails used both chimpanzee and sooty mangabey kidney cells and there is no evidence that these specific kidney cells were used and ii)

HIV-1 group M is estimated to have originated 10-30 years earlier than the OPV trails were conducted.

In addition to the above stated theories for origin of HIV, another key factor that needs to be mentioned is genetic recombination. There is evidence to show that recombination took place between SIVs and HIV-1 M group. The recombination process of SIVs and HIV are explained in detail in discussion.

Preston Marx and his group made several arguments that AIDS is not a zoonosis and explained the reasoning behind their arguments for why they will not regard AIDS as a true zoonosis (Marx et al., 2004). These arguments are as follows: i) Based on exact definition of zoonosis there must be evidence of SIV directly transmitting to humans, such as in the case with rabies virus transmission through bite of an infected rabid animal, ii) We know there are frequent human exposures to monkeys in Africa and have been for centuries, but only 11 subtypes of HIV-1 and HIV-2 are known, and of that only four HIV-1 M & N and HIV-2 A & B are predominant; if AIDS was a zoonosis there should have been innumerable subtypes of HIV circulating in humans, iii) If AIDS was a zoonosis, it should have risen to epidemic proportions during the slave trade wherein 12.5 million Africans were shipped to various parts of Central and North America and iv) Experimental transmission of SIV from one host to a different host, such as a different monkey species, doesn't cause AIDS like disease. If switching of hosts resulted in AIDS epidemic, then the experimentally infected animal should have developed AIDS. To summarize Preston Marx's theory regarding the evolution of HIV-1 and HIV-2, SIVcpz and SIVsmm did jump from monkeys to humans but it took several passages between different humans before it became a fully adapted and infectious HIV-1 or HIV-2. In this process virus

mutated and acquired host specific adaptations to overcome the host restriction factors and this adapted virus is what we now see as HIV-1 and HIV-2.

1.4 Host-specific adaptations

Humans encode a number of host restriction factors as part of their innate immune system to protect the body from viral pathogens. For successful replication of any virus in a human host, the virus needs to overcome the restriction factors for its successful replication (Malim & Emerman, 2008). Both HIV and SIV also need to interact with these host restriction factors in both humans and monkeys, respectively (Ortiz et al., 2009). The counteractive measure to overcome human restriction factors for any virus is species-specific, so in order for a primate-specific virus to replicate in human hosts it had to specifically adapt to counteract human restriction factors.

The first evidence of host specific adaptations of HIV-1 was found when the analysis of a specific mutation change in every virus which passed from non-human primate to humans was observed. The analysis revealed that the thirtieth position of Gag encoded for Met in all strains of SIVcpz*Ptt* and SIVgor but this amino acid was switched to a basic Arg or Lys in HIV-1 subtypes M, N and P, in most strains (Wain et al., 2007). There is also evidence that the virus with Met 30 replicated more efficiently in chimpanzee CD4+ T cells than the virus with Lys or Arg at Gag 30, whereas the virus with Gag 30 Lys/Arg showed more efficient replication in human CD4+ T cells (Wain et al., 2007). These host specific mutations or adaptations were further supported by infecting chimpanzee with HIV-1 which led to the reversal of the Gag 30 Arg/Lys mutation back to Met. This provided further support for host specific adaptations (Mwaengo & Novembre, 1998) and the importance of a single Gag 30 position for adaptation.

Successful replication in primates, like HIV in humans, is also based on counteracting primate-specific host restriction factors. There are four known host restriction factor families that are involved in SIV cross-species transmission. These include: i) APOBEC3G- Apolipoprotein B editing enzyme catalytic polypeptide like 3G, ii) TRIM5alpha- Tripartite motif 5 alpha protein, iii) Tetherin- also called as CD317 or bone marrow stromal antigen 2 (BST-2) and iv) SAMDH1-SAM domain and HD domain containing protein.

APOBEC3G (A3G) is a family of cytidine deaminases which interfere with the reverse transcriptase of retrovirus. The antiviral activity of A3G is antagonized by the Vif domain of HIV-1 and this antagonism is brought about by forming A3G-Vif ligase complex. This complex includes multiple interactions between Vif domain and A3G. These interactions that form the Vif-A3G ligase complex are also species-specific. For example, A3G of African green monkey (AGM) contains Lys at position 128 whereas human A3G protein has Asp at this position. This single mutation allowed the recognition and regulation of SIVagm and HIV-1 Vif to recognize their specific host (Malim, 2009). APOBEC3G-Vif ligase complex is shown in figure 4.

Tetherin, also called BST-2, shows major anti-viral activity against HIV and SIV. The anti-viral activity of tetherin is accomplished by inhibiting the budding and releasing of virus from infected cells (Tortorec & Neil, 2009). Tetherin consists of three domains- an amino terminal region, extracellular *trans*-membrane domain and carboxy terminal with a glycosylphosphatidylinositol (GPI) anchor. Most SIV use their Nef region to antagonize the anti-viral activity of tetherin by targeting the carboxy terminal cytoplasmic domain (Jia et al., 2009). In contrast in HIV-1, SIV from greater spot-nosed, mona, and mustached monkeys use their Vpu region to degrade the anti-viral activity of tetherin (Iwabu et al., 2009; Rong et al., 2009). Vpu

targets tetherin by attacking *trans*-membrane domain whereas Nef targets the tetherin at its cytoplasmic domain.

The human tetherin gene differs from the non-human primate gene by a five amino acid deletion in the cytoplasmic domain (Sauter et al., 2009). Since Nef binds to the cytoplasmic domain, this 5 amino acid deletion prevents SIVcpz from interacting during transmission to humans, so this virus had to find an alternative to overcome the tetherin anti-viral activity. Gorilla tetherin does not have this deletion, so SIVgor continue to use Nef to overcome tetherin activity. For SIVcpz, when cross-species transmission to humans occurred, this virus could no longer overcome the tetherin activity by their Nef as the Nef could not interact with tetherin, so this virus had to adapt and use Vpu as an alternative to counteract tetherin.

Surprisingly only Vpu of HIV-1 M group showed potential anti-tetherin activity (Sauter et al., 2009), HIV-N group showed very low anti-tetherin activity whereas HIV-O and P groups of Vpu proteins are completely inactive (Kirchhoff, 2010). So when SIVcpz transmission to humans happened, HIV-1 group M ancestral SIVcpz was the only successful strain in the establishment of complete anti-human tetherin activity. This might be one of the reasons why HIV-M is the only subgroup to become a global pandemic (Gupta & Towers, 2009).

Like all SIVs, SIVsmm also uses Nef to counteract tetherin anti-viral activity in its primate host. Since SIVsmm does not have Vpu, when this virus was transmitted to humans, it recruited gp41 of envelope for anti-tetherin activity (Tortorec & Neil, 2009). So far HIV-2 group A is the only HIV-2 that shows anti-tetherin activity.

In summary this evidence clearly support the hypothesis that AIDS is not just a direct zoonosis due to the fact that it was simply not a direct transmission of the ape virus to humans,

but several host specific adaptations played key roles in the origin of HIV-1 and HIV-2 from its primate-specific predecessors SIVcpz and SIVsmm, respectively.

1.5 Animal model for HIV/SIV studies

The fact that HIV does not infect and cause AIDS in any species other than humans poses a significant challenge when it comes to investigating replication kinetics, novel drug compounds and vaccine development (Haigwood, 2004). During the initial days of HIV research there were no animal models available to test the efficacy of anti-retroviral drugs or to evaluate potential vaccine candidates; non-human primates (NHP) models were relied upon heavily to study pathogenesis, mode of infection, test efficiency of drugs, viral clearance, etc., by challenging them with the lentivirus from NHP, SIV.

NHP models for HIV have made crucial contributions in the study of HIV, such as viral diversity, innate and adaptive immune responses, vaccine development, viral reservoirs, etc. Although there are some limitations, the NHP model remains an invaluable tool to further studies in the field of HIV to reach our goals in better understanding of AIDS and devise ways to combat it. Although the NHP model is a great tool to study SIV related disease, many aspects of this model are limited. Although the NHP immune system is comparable to that of a human immune system, due to species-specific receptor tropism there will not be any sustained infection from HIV in NHP model (Polacino et al., 2008). Challenging with SIV instead of HIV in a NHP model and extrapolating the results based on such studies will result in lack of species-specific complexity and will not address all the intricacies of HIV infection but only of SIV. The macaque model was used for the first vaccine development for HIV-1. However, after the third phase scientists were unable to validate any vaccine candidates and the trial failed. In addition to

these limitations, the size and cost of these animal models limit the use of the NHP model (Barouch, 2008).

The smallest available natural model for the study of lentivirus is the cat; lentivirus in cats is called feline immune deficiency virus (FIV). FIV shows a number of similarities with HIV-1 in its structure (Pedersen et al., 1987). FIV shows the same viral replication kinetics like HIV-1 and also shows similar CD4+ T cell decline but FIV also shows CD8+ T cell decline (Brown et al., 1991; Willett et al., 1993). FIV lacks a few accessory proteins compared with HIV. These differences may limit the use of cats as an animal model for HIV and SIV studies. For above stated reasons the development of a humanized model that can mimic human immune system is required. The development of humanized mice model showed promising results with HIV infection.

1.6 Humanized mice

Human hematopoietic stem cells (HSC) can be used to reconstitute the hematopoietic system that can be sustained long term in an *in vivo* system. Humanized mouse models which harbor human HSC in their system will be appropriate to study HIV. Since mouse is small, easy to use and cost-effective in comparison with NHP model, using small animal models like humanized mice have great advantages.

The first humanized mice were produced by injection of human stem cells into blood in irradiated mice (Peters et al., 1995; Ganick et al., 1980). Although this model was a good start, the mice showed very low engraftment. To increase the level of engraftment, knockout mice were generated which lacked Hfh11^{nu} and Prkdc^{scid} to develop an immune compromised model (Watanabe et al., 1978; Fried et al., 1996). In 1983 these SCID BALB/c mice displayed human PBMCs but lack of mature host T and B cells (Bosma et al., 1983). After successful production

of SCID mice, SCID-hu mice were generated by injecting human fetal liver and bone marrow cells (McCune et al., 1988). This SCID-hu model was utilized for various studies including gene therapy (Yurasov et al., 1997).

Although SCID-hu mice were a very successful model, the engraftment for human cells reached maximum only up to five percent. To increase the level and stability of human cell engraftment, SCID mice were crossed with non-obese diabetic (NOD) mice to generate NOD-SCID$^{-/-}\gamma c^{-/-}$ (hu-nod) and Rag2$^{-/-}$ $\gamma c^{-/-}$ (Rag-hu) mouse models. Better mouse models were generated by further knock down of interleukin receptor common gamma chain and recombination activating genes (Archer et al., 1997). These further developments promoted stable engraftment of human CD34+ stem cells, by intrahepatic injection that gave rise to multi lineage human hematopoiesis in mouse environment as shown in figure 5. These Rag-hu mice showed higher human immune cell engraftment up to a year (Berges et al., 2010). This mice model is susceptible to HIV-1 and HIV-2 infection.

Further advancements took place in NOD-SCID model by transplantation of human thymic and liver tissue under the kidney capsule followed by hepatic injection of CD34+ cells and this mice model are called BLT mice (blood, liver and thymus) model. This model showed highest level of engraftment but generating this model is hard and expensive.

1.7 Significance of the current project

One of the relevant hypotheses on the origin of HIV suggests that the virus had emerged by crossing the species barrier and by successful adaptation to human host. A suitable model system to evaluate the veracity of this hypothesis has been lacking so far. In the present study, in order to test this hypothesis, we decided to infect humanized mice with SIV and subject the virus through successive passages in humanized mice, in an effort to mimic and recapitulate the events

that would have happened in the wild during the emergence of HIV from SIV. The input virus as well as the passaged viruses will be sequenced by whole genome sequencing. The genomic comparisons would throw light into the crucial mutations and adaptive changes that enabled the successful evolution of SIV to a human pathogen. Sequence data would also give information on viral factors that counteract host restriction factors or novel fixed changes in the genome that are critical for human adaptation of SIV.

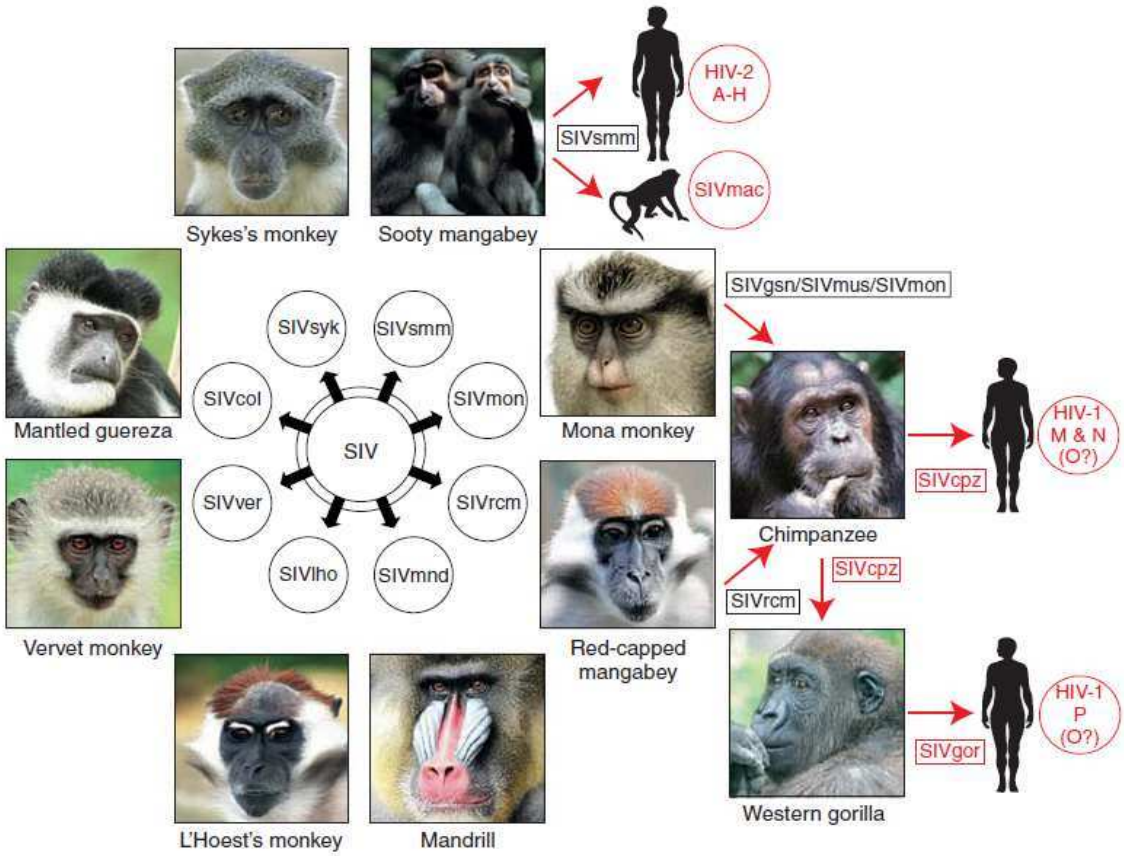

Figure 1: African monkeys are naturally infected with more than 40 different lentiviruses. Here are some examples of SIV strains with primate name as suffix. For example SIV from mandrill and SIV from mona monkey are called SIVmnd and SIVmon, respectively. Here some of cross-species transmission examples shown in red. HIV-2 (A-H) and SIVmac resulted from SIVsmm cross-species transmission to human and Asian monkey (macaque) respectively. HIV-1 M & N originated from cross-species transmission from chimpanzee to humans whereas HIV-P was transmitted from western gorilla to humans (Sharp & Hahn, 2011).

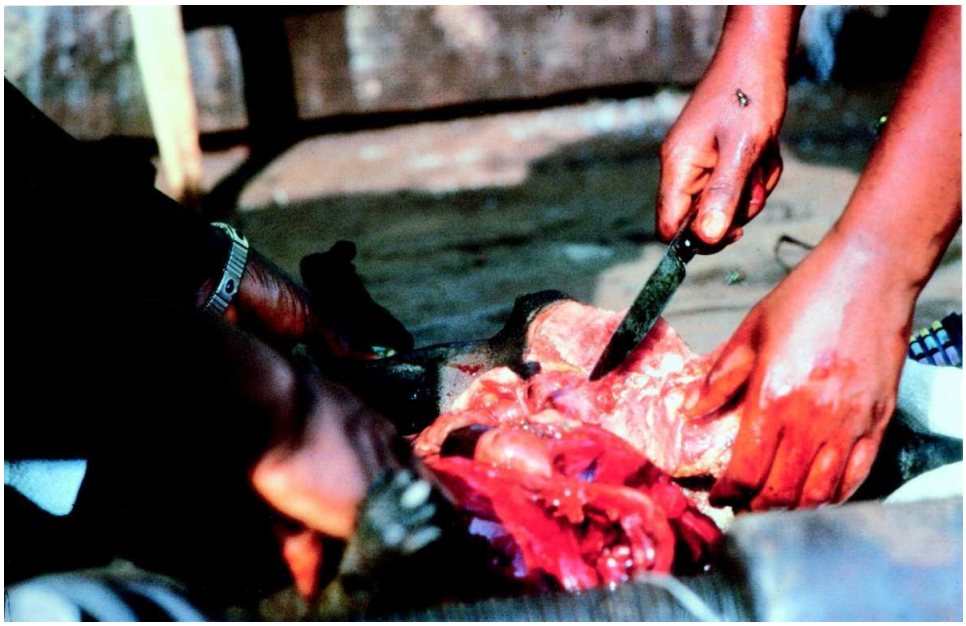

Figure 2: Here is a photograph showing one of the typical meat shops in Africa selling bush meat. A clear example of human contact with infected primates (Photo courtesy of Karal Amman).

Figure 3: Bush meat market in West Africa. Sixty percent of the monkeys from Africa harbor 40 different kinds of SIV. From this photograph, we can clearly appreciate that there is a high risk of human contact with infected animal meat (Photo courtesy of Karal Amman).

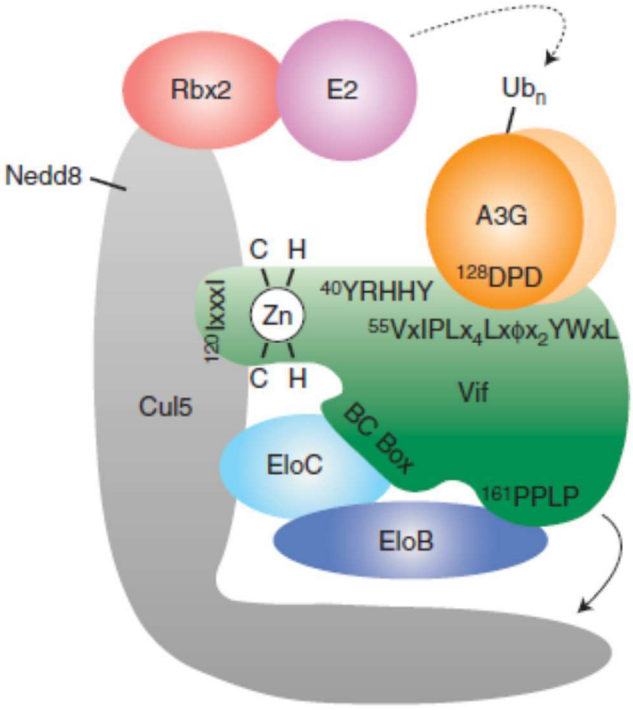

Figure 4: HIV-1 Vif and APOBEC3G ligase complex. HIV-1 Vif shows multiple interactions with elongin B, elongin C and cullin5 through the BC box and proline rich motifs. The formation of HIV-1 Vif-A3G ligase helps the virus antagonize the anti-viral activity of A3G (Sharp & Hahn, 2011).

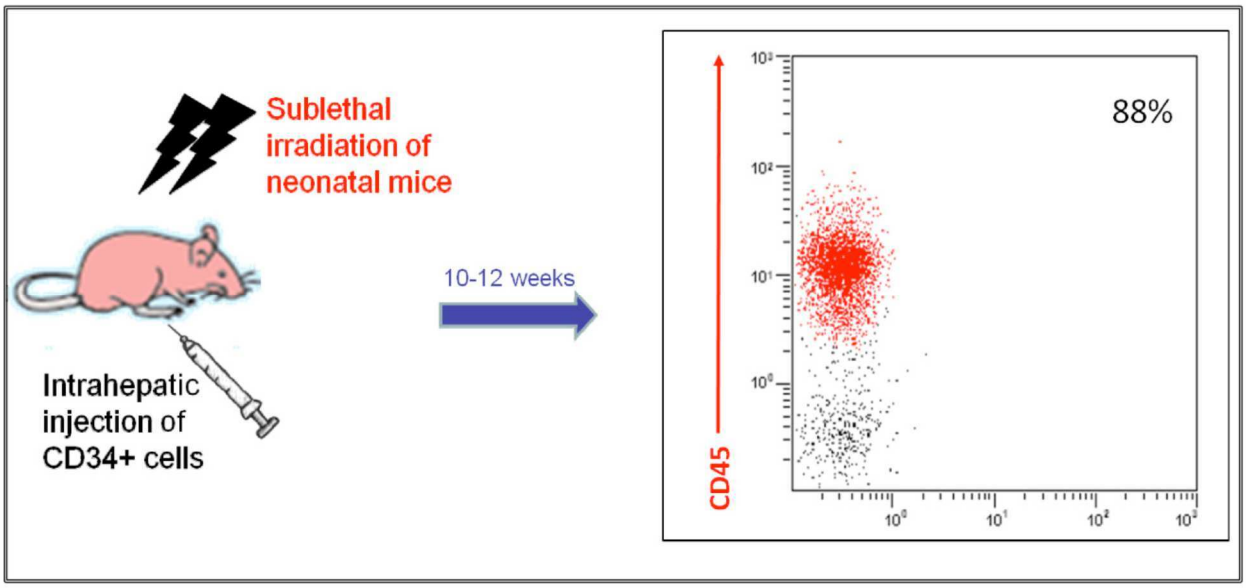

Figure 5: Generation of Rag-humanized mice model. Human fetal liver CD34+ cells were injected intrahepatically into neonatal mice. Peripheral blood was collected after 10-12 weeks and stained with human FITC-conjugated anti-CD45 antibody. Flow cytometry analysis showed 88 percent human T cell engraftment.

Chapter 2

Materials and Methods

2.1 Generation of humanized mice

Humanized Rag2$^{-/-}$γc$^{-/-}$ (Rag-hu) mice were prepared by engraftment with human fetal liver derived CD34+ progenitor cells as described in Berges et al., 2006. Mice were maintained at CSU painter animal center. These studies have been reviewed and specifically approved by the CSU institutional animal care and use committee (Protocol 09-1460). Briefly new born mice are conditioned by irradiation with 300 rads and one million human CD34+ cells were injected. Human cell engraftment checked by flow cytometry by staining for human CD45, CD3 and CD4 markers as described in section 2.5.

All the human PBMCs used in the project are isolated from human blood samples received from Garth Englund Blood bank (MTA in file, IBC parf#06-107B). All the mice used during the current project, were maintained at CSU painter animal center. These studies have been reviewed and specifically approved by the CSU institutional animal care and use committee (Protocol 09-1460).

2.2 SIVsmm infection of humanized mice by intraperitoneal injection

Humanized mice were generated by injecting human fetal liver CD34+ hematopoietic progenitor cells into Rag2$^{-/-}$γc$^{-/-}$ mice as previously described (Berges et al., 2006). SIVsmmE041 stock virus (811 TCID 50/ml) was obtained as a gift from Dr. Preston Marx, Tulane National Primate Research Center, Covington, CA. Mice with high human hematopoietic cell engraftment levels (>70% of lymphocytes positive for human CD45) were used for infection study. Virus (100 µl, 811 TCID 50/ml) was injected into these mice by intraperitoneal (IP)

injection at 16 weeks post engraftment. Peripheral blood (150 µl) was collected weekly to assess plasma viral load.

Blood was collected by tail vein puncture, collected in one non-heparinized capillary tube (150 µl) per animal and transferred immediately to EDTA-containing vacutainer tubes (BD Biosciences). PBS (90 µl) was added and centrifuged at 500g for 3 min. Supernatant (200 µl of plasma) was removed for viral RNA extraction with the E.Z.N.A.^{TM} Viral RNA kit (OMEGA Bio-tek, Inc.) as per manufacturer's instructions.

2.3 SIVsmm mucosal infection of humanized mice

Rag-hu mice with high human hematopoietic cell engraftment levels (>65% of lymphocytes positive for human CD45) were used for the infection study. Forty microliters of virus ($1x10^5$ IU/ml) were used to infect these mice by vaginal route at 16 weeks post engraftment. The viral challenge was repeated two more times in the next two days with 40 µl by vaginal route. Peripheral blood (150 µl) was collected weekly to assess plasma viral load. Blood was collected by tail vein puncture and viral loads were determined as described in section 2.2 and 2.4.

2.4 Viral load estimation by qRT-PCR

Viral loads by qRT-PCR were obtained using a C1000 Thermal Cycler (CFX96™ Real-Time System, Bio-Rad) and with iScript™ One-Step RT-PCR kit with SYBR® Green (Bio-Rad). Primers were designed based on the Gag region of SIVsmmE041 published sequence, GenBank: HM059825.1 (Hofmann et al., 2001). Forward: 5'GTCTGCCTCATTTGGTGCATT-3' and Reverse: 5'CACTAGATGTCTCTGCACTAT-3' primers amplify a 98 base pair product. PCR cycling conditions consisted of an initial RT step at 50˚C for 10 min, denaturation at 95˚C for 5 min followed by 40 cycles at 95˚C for 15 sec and 60˚C for 30 sec.

2.5 Flow cytometry for CD4+ engraftment level assessment

FACS analysis was used to check human cell engraftment levels in SIVsmm infected Rag-hu mice and also control non-infected mice. Whole blood was collected in heparinized capillary tubes and centrifuged to separate plasma and blood cells. Fc-block (Jackson Immuno Research Laboratories) was added to blood cell pellet and then stained with hCD-45-FITC (eBioscience), hCD-3-PE (eBioscience) and hCD-4-PE-CY5 (BD Pharmingen) antibodies. Cells were incubated with antibodies at room temperature in the dark for 45 min, and subjected to RBC lysis using an RBC lysing kit (BD Biosciences). After RBC lysis, stained cells were washed twice with wash buffer (BD Biosciences) and analyzed using BD Accuri C6 FACS analyzer. CD4+ levels were measured before infection to get baseline levels. CD4+ cell decline was measured based on comparison between infected and un-infected mice.

2.6 Illumina sequencing and data analysis

Viral RNA was sequenced using Nextera XT DNA Library Prep kit (Illumina) and MiSeq sequencing platform (Illumina). Briefly, viral RNA was amplified using gene specific primers which span the full-length genome. The amplicons from each primer sets were pooled together for each sample and sent for sequencing to Shelby O'Connor core facility at University of Wisconsin, Madison. At the facility, the sequencing was performed using Nextera XT DNA Library preparation kit and the sequence ready library was loaded into the MiSeq Illumina sequencer. The sequence reads were given to us in fasta format, which were analyzed by paired-end reads mapping and variant analysis. The annotations were performed using Geneious software (Biomatters Ltd).

2.7 Cell culture and *in vitro* virus infection

PBMCs and SupT1 cells (human T lymphoblast cell line) were grown in complete RPMI-1640 media with 10% FBS. Antibiotic-antimycotic mix and L-glutamine were added as supplements (Corning Life Sciences). PBMCs were activated with PHA at a final concentration of 2 µg per ml. Both PBMCs and SupT1s were cultured in the medium supplemented with IL-2 at a final concentration of 5 ng per ml. Activated PBMCs and SupT1s were infected with SIVsmm virus at an MOI (multiplicity of infection) of 3. Cell supernatant was harvested every 3 days and viral loads were measured by using qRT-PCR as mentioned in section 2.4. HIV NL4-3 wild-type virus was used as positive control and un-infected cell supernatant was used as negative control.

2.8 SIVsmm propagation by co-culturing with hPBMCs

SIVsmm infected mice were sacrificed at the end of the study (6 months) and different tissues were harvested such as bone marrow, thymus, spleen, mesenteric lymph node and blood (by cardiac puncture). A single cell suspension was made by homogenization and leukocyte fraction isolated by Ficoll-Paque (GE Healthcare). These cells were co-cultured with 10 million human PBMCs in complete RPMI-1640 supplemented with IL-2. Cell supernatant was harvested every 3 days and viral loads measured using qRT-PCR as mentioned in section 2.4 and also by GHOST cell GFP titration, as described in section 2.9.

2.9 Functional titration of SIVsmm using GHOST cells

GHOST R3/X4/R5 cells were cultured in DMEM with 10% FBS and supplemented with 500 µg per ml of G418, 100 µg per ml of hygromycin and 1 ug per ml of puromycin. After 24 hours of plating, GHOST cells were infected with first passage human cell-adapted SIVsmm virus at an MOI of 1 with polybrene (BD Biosciences) at a final concentration of 8 µg per ml.

After 4 hours of infection, media was removed and fresh medium was added. The cells were harvested by trypsinization 48 hours post-infection and GFP expression assessed using FACS analysis.

2.10 SIVcpz strains, infection of Rag-hu mice

The viral copy number of SIVcpzLB and MB in the viral stocks received from Dr. Preston Marx was measured using qRT-PCR and the viral copy numbers were $1x10^4$ per ml for both viruses. Five mice were infected per virus, blood was collected and the viral copy number was determined as described in sections 2.2 and 2.4. No infection was observed, and we assumed the reason might be low viral copy number in inoculums. So we tried to infect the mice using an alternative approach, briefly we cultured PBMCs from un-infected mice and activated them with PHA as described in section 2.7. The activated PBMCs were then infected with SIVcpzLB or MB at an MOI of 3 *in vitro*. After 6 days, the infected PBMCs were directly injected into the mice by IP.

2.11 SIVcpz viral load estimation by qRT-PCR

Viral loads by qRT-PCR were obtained using a C1000 Thermal Cycler (CFX96™ Real-Time System, Bio-Rad) and with iScript™ One-Step RT-PCR kit with SYBR® Green (Bio-Rad). Primers were designed based on the 5' LTR region of SIVcpzMB and Pol region of SIVcpzLB. Forward: 5'GTCTGCCTCATTTGGTGCATT-3' and Reverse: 5'CACTAGATGTCTCTGCACTAT-3' primers amplify a 98 base pair product. PCR cycling conditions consisted of an initial RT step at 50˚C for 10 min, denaturation at 95˚C for 5 min followed by 40 cycles at 95˚C for 15 sec and 60˚C for 30 sec.

2.12 SIVcpz propagation for subsequent passages by co-culturing with hPBMCs

SIVcpzLB infected mice were sacrificed at the end of the study (6 months) and different tissues were harvested such as bone marrow, thymus, spleen, mesenteric lymph node and blood (by cardiac puncture). A single cell suspension was made by homogenization and leukocyte fraction isolated by Ficoll-Paque (GE Healthcare). These cells were co-cultured with 10 million human PBMCs in complete RPMI-1640 supplemented with IL-2. Cell supernatant was harvested every 3 days and viral loads measured using qRT-PCR as mentioned in section 2.4 and also by GHOST cell GFP titration, as described in section 2.9.

Chapter 3

Results

3.1 SIVsmmE041 establishes productive infection and sustained viremia in Rag-hu mice

Five Rag-hu mice engrafted with hematopoietic stem cells from two independent human donors and with engraftment levels higher than 70% were challenged with the stock SIVsmmE041 virus by intraperitoneal injection. The SIVsmmE041 plasma viral loads in these mice were tracked longitudinally over time, using a qRT PCR assay targeting SIVsmmE041 5'LTR region. Our experimental design is shown in figure 6. Plasma viremia was detected in 4 out of the 5 challenged animals by week two with the viral loads increasing further over time and peaking to more than 100 fold the initial viral load at 70 days, before tapering off and eventually falling below the detection limit in all animals after 7 months (Figure 7). One of the challenged mice never became SIV positive and was excluded from further analysis. The three control animals remained negative for virus throughout the experimental time course. The loss of detectable plasma viremia correlated with the progressive loss of engrafted human T cells near the end of the experimental time course (data not shown). In summary we showed SIVsmm can successfully establish infection in Rag-hu mice and can sustain viremia for as long as 7 months post-infection.

3.2 SIVsmmE041 infection leads to CD4+ T cell depletion

A central hallmark characteristic of HIV infections in humans is the depletion of CD4+ T lymphocytes. We have previously showed that HIV infection in Rag-hu mice leads to CD4+ T cell depletion (Berges et al., 2006). To investigate if this loss of CD4+ T lymphocytes also occurs following SIV infection, peripheral blood from SIV infected Rag-hu mice was collected periodically and stained for hCD4 and hCD3. Baseline CD4+ levels were established before infection and were around 68% for all 10 mice used in the study (5 SIVsmm infected and 5 un-

infected mice). All 5 infected mice showed gradual decline of CD4+ T cell levels whereas none of the un-infected mice showed a significant CD4+ T cell depletion over 73 days (Figure 8). In SIVsmm infected mice, CD4+ T cell decline started at 10 days post-infection, which corresponds to the initial time point of detectable plasma viremia, with the mean CD4+ T cell levels declining to 50 percent from the initial 68 percent. We thus showed evidence that SIVsmm can establish viremia in Rag-hu mice, with a resultant CD4+ T cell depletion as its hallmark character.

3.3 Second passage SIVsmm establishes productive infection and sustained viremia in Rag-hu mice

Human cell-adapted SIVsmm establishes productive infection in Rag-hu mice within a shorter time span on subsequent passage (SIVsmm second generation). Once we had established and maintained viremia in Rag-hu mice with SIVsmmE041 stock virus viremia for 7 months, we wanted to passage this first generation virus to a second set of mice in order to obtain second generation viral strains and also to observe any difference in the infectivity/fitness of the first generation virus in establishing infection in Rag-hu mice as compared to the wild-type stock virus. One of the Rag-hu mice with a high SIVsmm viral load, which corresponds to high levels of virus circulating in the peripheral blood, was sacrificed. We isolated PBMCs from the blood, spleen and cells from the bone marrow and lymph nodes were co-cultured with human PBMCs up to 15 days. The supernatants were collected and the viral levels were measured using both qRT-PCR and GFP titration in GHOST cells and titer was determined as 4.05E+05 (Figure 9). We opined that since this SIVsmm were able to infect and actively maintain the infection in the Rag-hu mice for 7 months, these viral strains were human cell-adapted.

Five Rag-hu mice were injected intraperitoneally with this human cell-adapted first passage virus, which was amplified by a 6 day *in vitro* co-culture with human PBMCs. Plasma was collected on a weekly basis and the viral copy numbers were determined by qRT-PCR. We

observed higher viral loads and quicker establishment of infection with the second passage compared to the stock SIVsmm, which was not adapted to human cells, at 10 days post-infection. This difference might be due to the fact that the first passage virus was already human cell-adapted and was thus able to infect the Rag-hu mice more efficiently. Human cell-adapted SIVsmm infections in Rag-hu mice and second passage viral loads are shown in figure 10.

3.4 Second passage SIVsmm showed rapid CD4+ T cell decline

A hallmark characteristic of HIV infections in humans is the depletion of CD4+ T lymphocytes. To investigate if this loss of CD4+ T lymphocytes following infection is same with SIV, peripheral blood from SIV infected Rag-hu mice was collected periodically and stained for hCD4 and hCD3. Baseline CD4+ levels were established before infection and were around 55 or above percent for all the 8 mice used in this study (5 SIVsmm infected and 3 un-infected mice). All 5 infected mice showed gradual decline of CD4+ T cell levels whereas none of the un-infected mice showed a significant CD4+ T cell depletion over a time period of 75 days (Figure 11). In SIVsmm infected mice CD4+ T cell decline started at 15 days post-infection, which corresponds to the initial time point of detectable plasma viremia.

3.5 SIVsmm can successfully establish infection in Rag-hu mice by mucosal route

Humanized $Rag2^{-/-}\gamma c^{-/-}$ mice were generated as described in (Berges et al., 2006). Five Rag-hu mice engrafted with hematopoietic stem cells from two independent human donors and with engraftment levels higher than 65% were challenged with the first passage SIVsmm virus by vaginal route. The SIVsmm plasma viral loads in these mice were tracked longitudinally over time, using a qRT PCR assay based on targeting SIVsmmE041 5'LTR region. Plasma viremia was detected in 4 out of the 5 challenged animals by week 2 with the viral loads increasing further over time and peaking to more than 100 fold the initial viral load at 61 days, before tapering off and eventually falling below the detection limit in all animals after 6 months (Figure

12). The loss of detectable plasma viremia correlated with the progressive loss of engrafted human T cells near the end of the experimental time course (data not shown). In summary we showed SIVsmm can successfully establish infection in Rag-hu mice via vaginal challenge and can sustain viremia for as late as 6 months post-infection.

3.6 SIVsmmE041 mucosal infection leads to CD4+ T cell depletion in Rag-hu mice

A central hallmark character of HIV infections in humans is the depletion of CD4+ T lymphocytes. We have previously showed that HIV infection in Rag-hu mice and SIVsmm infection by intraperitoneal injection leads to CD4+ T cell depletion (Berges et al., 2006). To investigate if this loss of CD4+ T lymphocytes following mucosal SIV infection is same, peripheral blood from SIV infected Rag-hu mice was collected periodically and stained for hCD4 and hCD3.We observed CD4+ T cell decline till 80 days post-infection (data not shown).

3.7 Whole genome sequencing of SIVsmmE041 stock input virus and first passage output virus

To determine if viral adaptation was occurring, we performed next generation Illumina sequencing on viral RNA from the SIVsmmE041 stock virus used to infect our Rag-hu animals, as well as viral RNA isolated from infected animals collected at different time points (days post-infection to as late as 7 months post-infection). Sequence reads were mapped to a SIVsmmE041 reference sequence obtained by de novo assembly by using Geneious software to produce a stock SIVsmmE041 whole genome majority consensus sequence. Open reading frames for all SIVsmm genes were identified and mapped onto the newly generated stock SIVsmmE041 consensus, which was utilized as the reference for mapping sequencing reads from the plasma of infected Rag-hu animals. The consensus majority sequences from Rag-hu animals were analyzed with stock consensus to identify nonsynonymous mutations.

An annotated map of the complete stock SIVsmmE041 genome sequence is shown in figure 13. Genome organization of SIVsmmE041 was found similar to that of HIV-2, containing a Vpx gene, but lacking Vpu. SIVsmmE041 showed 71 percentage nucleotide similarities with HIV-2 strains. Dominant amino acid mutations affecting the Gag, Pol, Env, Nef, Vif and Vpr were detected. No changes in the consensus majority protein coding sequences of Vpx and Tat were observed. While the majority of sites remained relatively neutral with respect to their conversion frequency, several positions appeared strongly selected for substitution mutations, suggesting purifying selection was occurring at these positions.

We compared the amino acid percentage change from the viral population at 28 weeks post-infection, which was the latest time point for the first generation virus in our study, as compared to stock virus sequence created by de novo assembly. We calculated percent amino acid change in each of the genomic regions of the virus obtained from 28 weeks post-infection, compared to the stock virus. The highest percentage change was observed in Env with 16.4, followed by 4 percent for Nef region and 2 percent change in Vif region, whereas other regions such as Gag, Pol and Vpr showed less than two percent change (Figure 14). We believe these changes could potentially be responsible for the successful replication of SIVsmm in human cells from Rag-hu mice.

3.8 SIVsmmE041 sequence shows drift toward HIV-2 with successive Rag-hu passages

It is well established that SIVsmm are the ancestors for and adapted in humans to give rise to HIV-2. To test this hypothesis, we chose to compare the sequences of Env region, which was found to be the region with the highest rate of nonsynonymous mutations, to see if any of these changes represent amino acid sequence as in HIV-2. We compared the sequences from SIVsmmE041 stock, first generation output virus from two mice at 28 weeks post-infection and

4 different sequences of HIV-2 from PubMed. We used Geneious software to align these six sequences using pairwise alignment. We observed amino acid changes at two positions, 120 and 145, which changed from Arg to Lys and Phe to Leu, respectively. HIV-2 Env region contains Lys and Leu at its 120th and 145th position, respectively, as was observed with the first generation SIVsmm terminal virus Env sequence, suggesting a potential drifting and selection of the SIVsmm towards HIV-2 with human cell-adaptation (Figure 15).

3.9 Sequential passaging elicits potential human host cell-adaptation in SIVsmm virus: important Nef R17Y mutation

NefR17Y mutation in the Nef region in SIVmac293 was responsible for its increased virulence in pigtailed macaque and conferred on the virus the ability to infect non-stimulated or resting lymphocytes (Kirchhoff et al., 1999). Interestingly, we observed the same mutation Nef R17Y in the first generation output SIVsmm virus, once we passaged SIVsmm virus in Rag-hu mice (Figure 16). We believe this mutation might be one of the reasons for successful replication of SIVsmm in our mice model.

3.10 Whole genome sequencing of second passage virus SIVsmmE041: adaptation in second generation humanized mice

Second generation sequencing analysis showed new mutations in all the coding regions including Gag, Pol, Env, Nef and Vpu. These new mutations suggest that virus is still evolving and trying to adapt to human cells. The new mutations originated in the Gag region during the second passage are shown in figure 17. When we compared first passage and second passage mutations, we noticed a higher number of mutations in first passage compared to the second passage. This suggests that the stock virus of SIVsmm had to adapt more during the first passage

as this virus has never encountered human cells before. The comparison between percentage amino acid change during first and second passages are shown in figure 18.

3.11 Mutations occurred in first passage are carried over to second passage

We have done pairwise alignment of Gag sequence from the stock, first passage and second passage viruses using Geneious software. Analysis of these alignments illustrated that mutations that occurred in first passage are carried over to the second passage as shown in figure 19.

These results suggests that once the mutations occurred they are carried over and do not mutate back to the original sequence in stock and that all these mutations are adapted to encounter the immune pressure from host. Most of these mutations are observed in multiple animals rather than in a single animal, supporting the statement of viral adaptation to the new host.

3.12 SIVcpzLB715 can establish infection in Rag-hu mice

Rag-hu mice can be infected with SIVcpzLB. Out of five challenged, two mice got infected. One mouse showed stable infection for up to 6 months and the other one showed only sporadic infection. Viral loads are shown in figure 20.

3.13 SIVsmmE041 and HIV-1 establishes productive infection and sustained viremia in Rag-hu mice for 5 months.

The SIVsmmE041 plasma viral loads in these mice were tracked longitudinally over time, using a qRT-PCR assay targeting SIVsmmE041 5'LTR region. Plasma viremia was detected in all challenged animals by week 2 with the viral loads increasing further over time. From the tenth week after HIV-1 infection, viral RNA extracted and viral copies of both SIVsmm and HIV-1 were determined. For the HIV-1 viral load qRT-PCR assay was designed as

described in Berges et al., 2006, targeting HIV-1 5'LTR region. Figure 21 shows SIVsmm and HIV-1 viral loads.

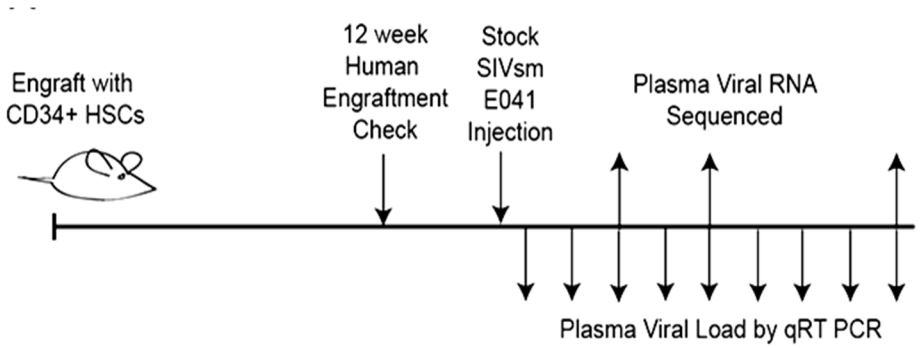

Figure 6: Schematic representation of SIVsmm infection regimen in Rag-hu mice. After 12 week engraftment check, mouse was infected with SIVsmm and plasma virus collected every week and nucleotide sequencing performed at 3 time points.

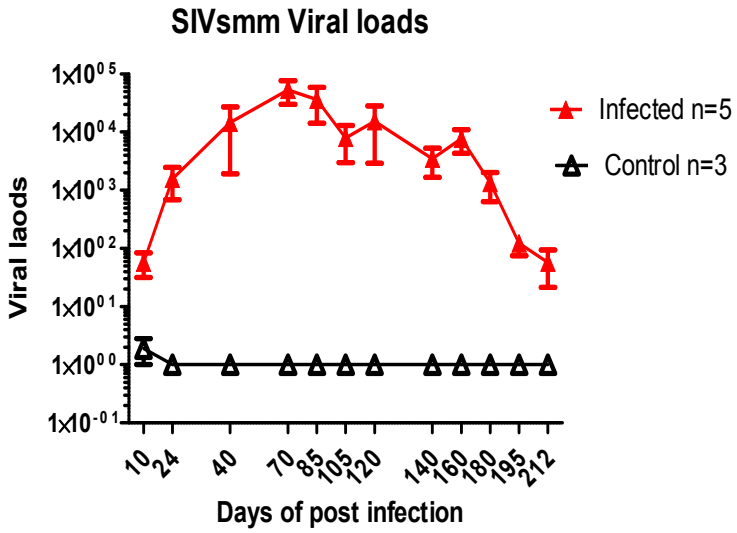

Figure 7: SIVsmmE041 can establish viremia in Rag-hu mice. Plasma viral loads of SIVsmmE041 infected (n=5) and un-infected (n=3) as detected by qRT-PCR.

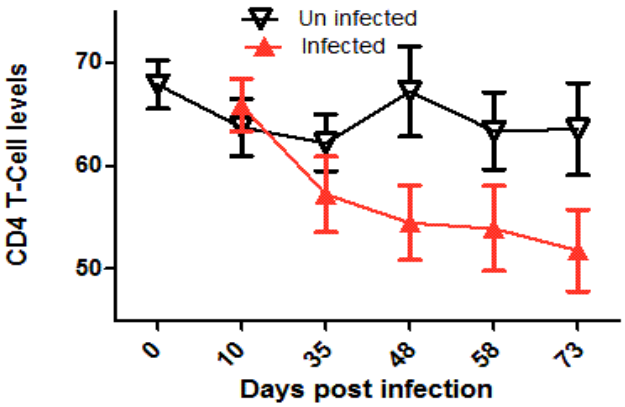

Figure 8: SIVsmmE041 infection leads to CD4+ T cell depletion in Rag-hu mice. Peripheral blood from both infected and un-infected mice was collected every week and stained for hCD3 and hCD4. CD4+ levels shown here are the percentages of CD4+ cells from the entire CD3+ population. Data at each time point represents the mean and standard deviation of n=5.

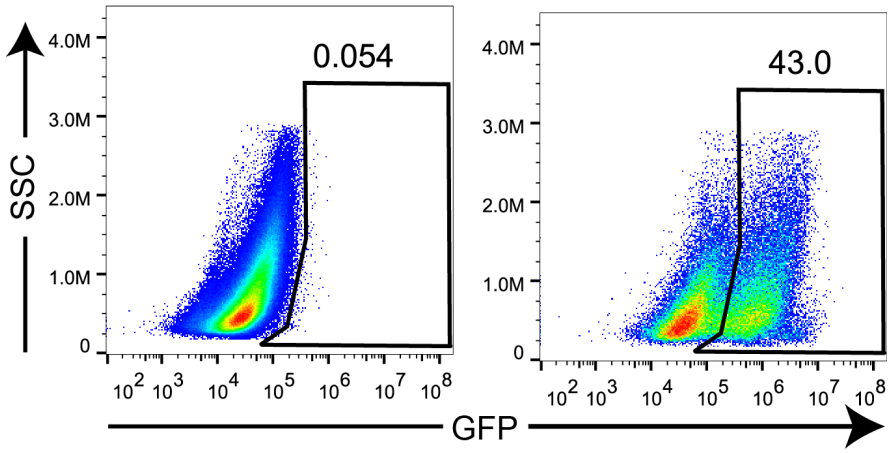

Figure 9: GHOST cells expressing GFP with SIVsmm first passage virus. PMBCs from first generation mice were co-cultured with human PBMCs and the virus was collected every 3 days after infection. Virus from sixth day was titered on GHOST cells. GFP expression was measured by FACS and the calculated virus titer was 4.05E+05.

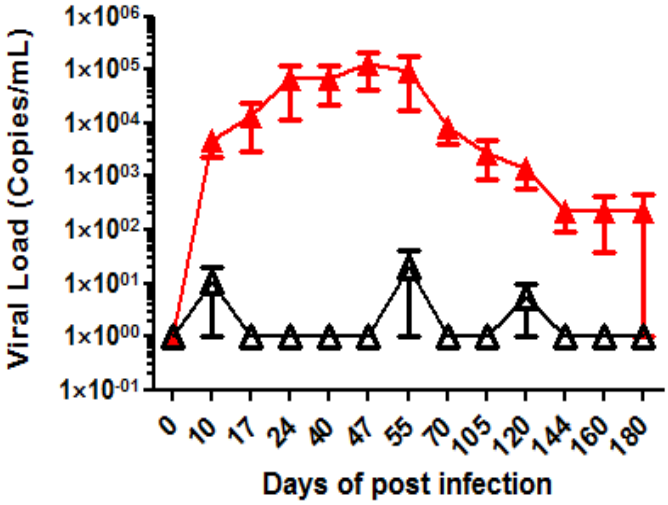

Figure 10: Human cell-adapted SIVsmm infections in Rag-hu mice. Plasma viral loads at different time points in Rag-hu mice infected with first passage human cell-adapted SIVsmm, detected overtime by qRT-PCR.

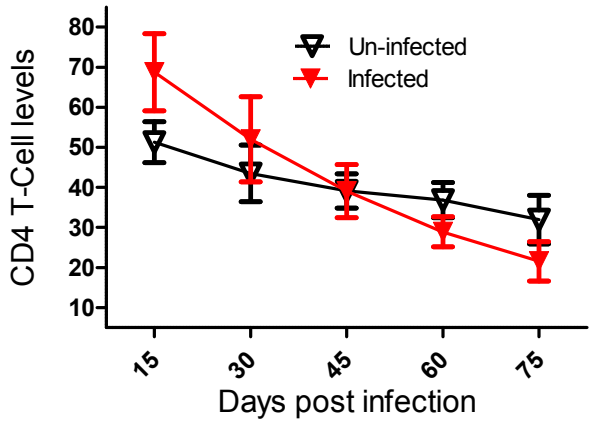

Figure 11: SIVsmmE041 infection leads to CD4+ T cell depletion in second generation Rag-hu mice. Peripheral blood from both infected and un-infected mice was collected every week and stained for hCD3 and hCD4. CD4+ levels shown here are the percentages of CD4+ cells from the entire CD3+ population.

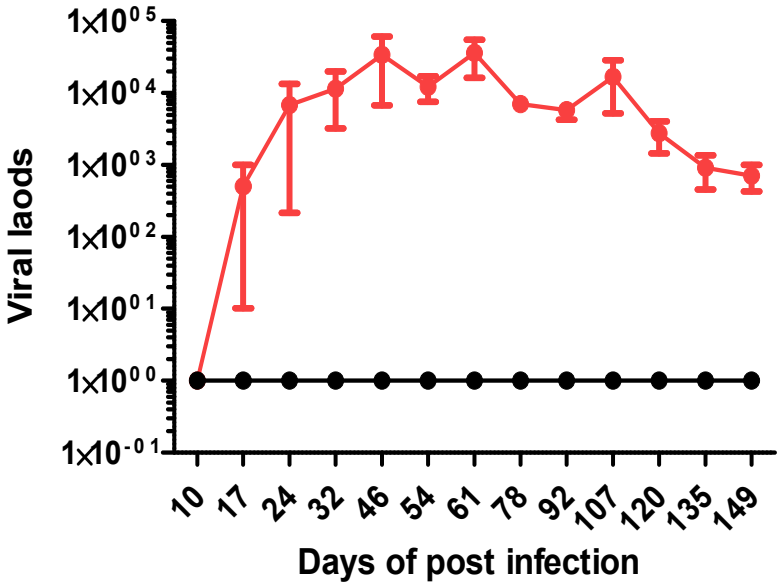

Figure 12: Vaginal challenge of human cell-adapted SIVsmm can establish viremia in Rag-hu mice. Plasma viral loads of SIVsmmE041 infected (n=4) and un-infected (n=5) as detected by qRT-PCR.

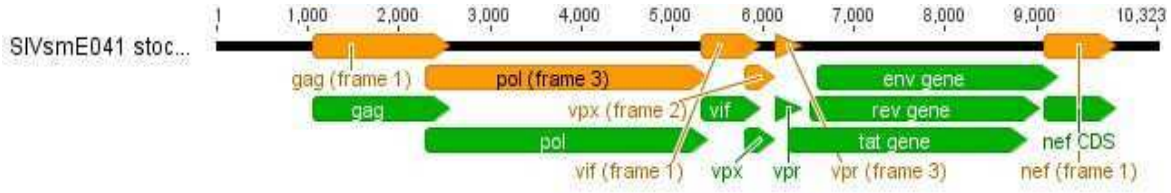

Figure 13: SIVsmmE041 complete genome sequence. The annotated genome sequence of SIVsmmE041 resembles the HIV-2 genome sequence. In addition to sequence similarities both genomes contain the Vpx gene and lack Vpu.

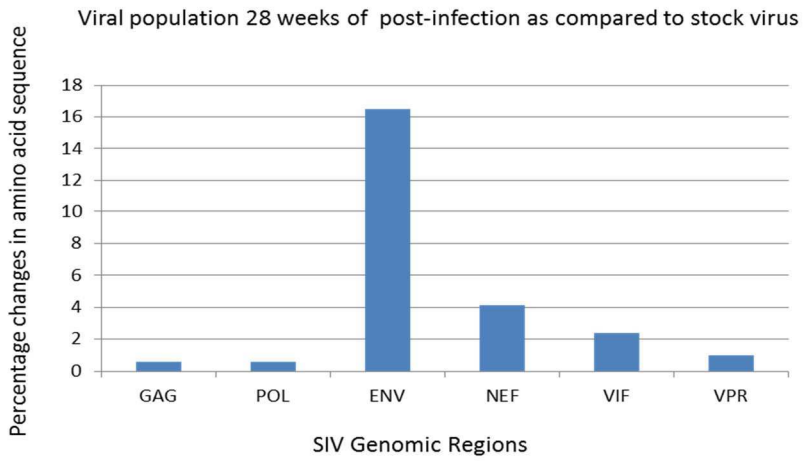

Figure 14: Percentage amino acid change in specific SIV genomic regions of virus collected 28 weeks post-infection compared to parental stock SIVsmm. Env gene showed the highest percentage change (16 percent) followed by Nef (4 percent change).

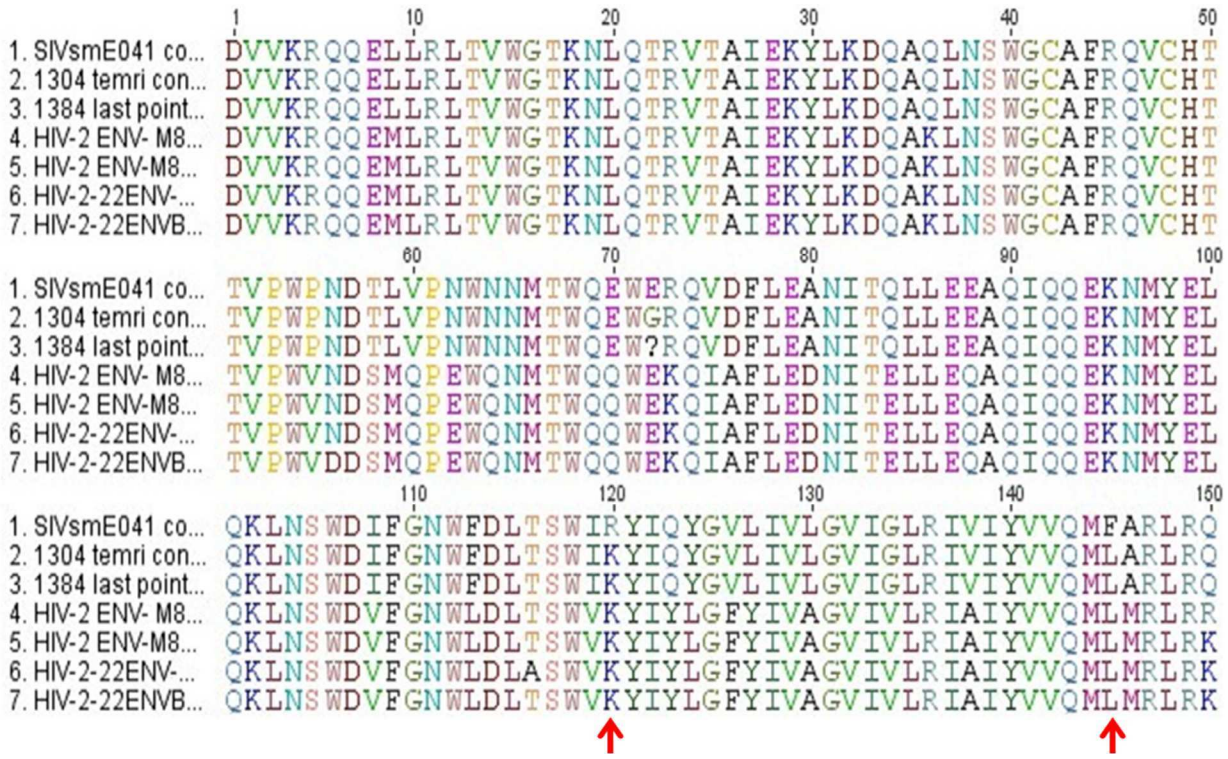

Figure 15: Pairwise alignment of Env region from SIVsmmE041 stock virus with HIV-2. Pairwise alignment of Env region from SIVsmmE041 stock virus (1), first generation SIVsmm terminal output virus from 2 different mice (2, 3) and 4 HIV-2 strains from PubMed (4 to 7). Red arrows show the mutated amino acid positions, 120 and 145. First passage SIVsmm virus shows homology with HIV-2 at these positions, evolving from the parental input virus.

Figure 16: Pairwise alignment of the first 45 amino acids of Nef from SIVsmm stock virus and first passage output virus obtained at 7 months post-infection from two mice. The blue box highlights the position 17, which bears Arg in SIVsmm stock virus, which mutated to Tyr in the first passage virus.

Figure 17: Additional adaptive mutations occurred in the second passage SIVsmm viruses. Shown here is the pairwise alignment of the Gag region of the SIVsmm stock (1), first passage viruses (2 and 3) and second passage viruses (4, 5, 6, 7). The point mutation at position 234 is highlighted, where the origin R (Arg) in stock input virus and the first generation virus has changed to T (Thr) in the second generation.

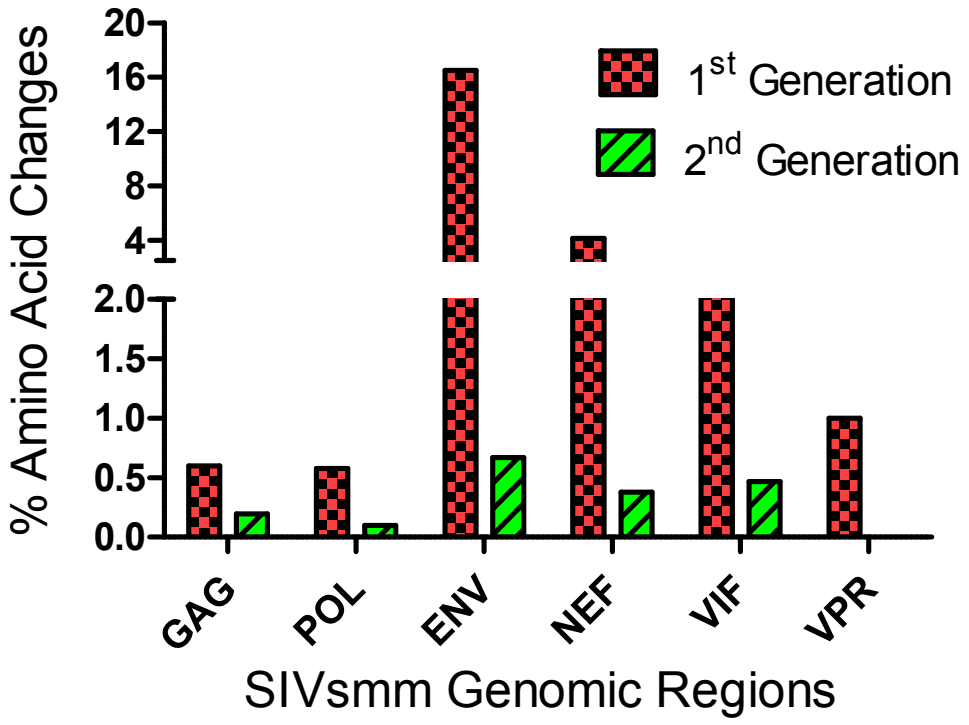

Figure 18: Percentage amino acid change in different SIV genomic regions at the end of first and second passage. The percent change in amino acids is shown on Y-axis and the different genomic regions are shown on X-axis. Virus from both passages showed highest changes in Env region. Overall first passage showed a higher percentage of mutations compared to second passage.

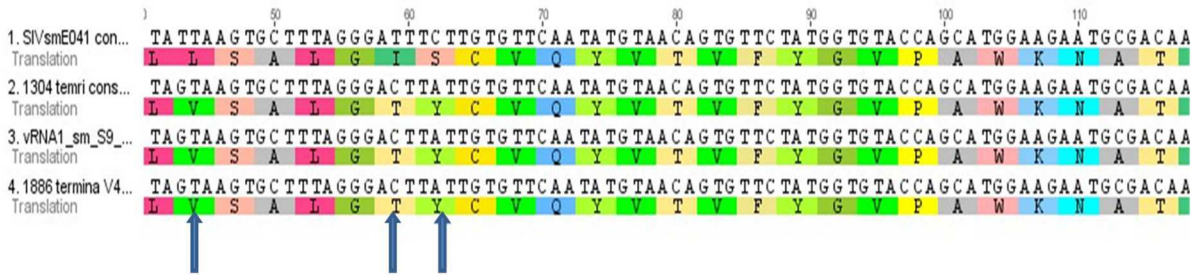

Figure 19: Mutations that occurred in first passage are carried over to the second passage. Shown here is a pairwise alignment of 118 amino acid sequence from Gag region of stock (1), first passage virus (2) and second passage viruses (3 and 4). Blue arrows represent mutations that were observed in first passage and carried over to the second passage.

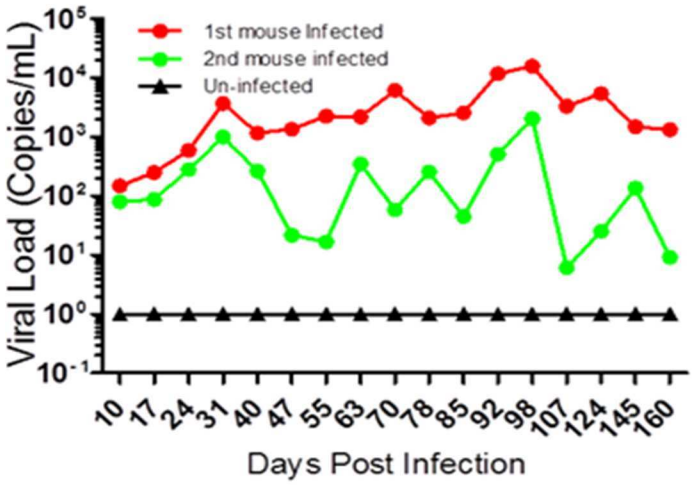

Figure 20: SIVcpzLB715 can establish viremia in Rag-hu mice. Plasma viral loads of SIVcpz LB infected mice were determined by qRT-PCR. Red line shows stable infection of one SIVcpz LB infected mouse and green line shows intermittent viral loads in second SIVcpzLB infected mice. Stable infection showed positive till 160 days post-infection.

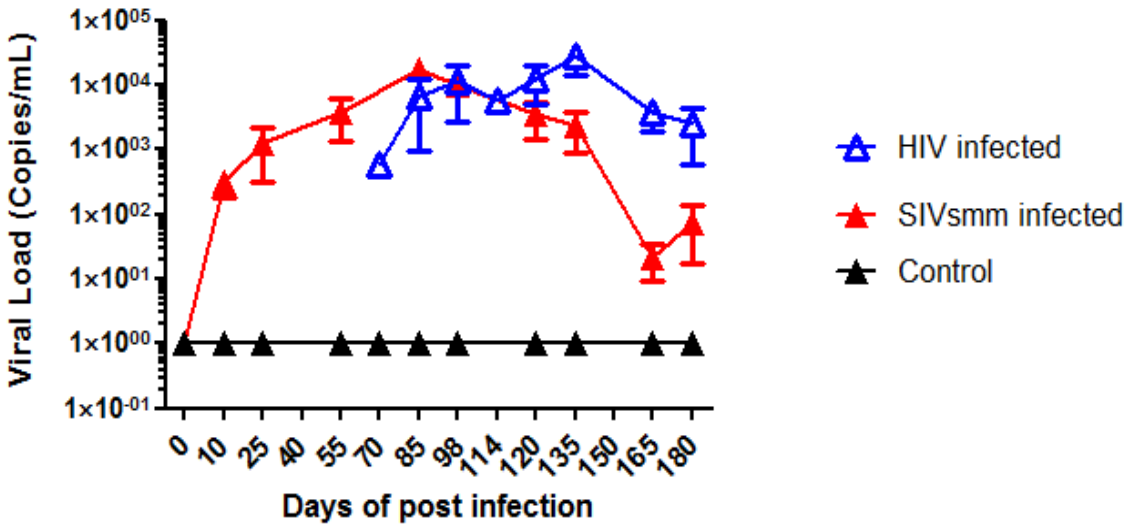

Figure 21: SIVsmm and HIV-1 can establish viremia in Rag-hu mice simultaneously. Plasma viral loads of SIVsmmE041 infected and HIV-1 infected samples determined by qRT-PCR. Red line shows SIVsmm viral loads peaks at 85 days post infection whereas blue line shows HIV-1 viral loads and peaked at 135 days post infection. Control remained negative for both viruses.

Chapter 4

Discussion and Future Directions

Acquired immune deficiency syndrome (AIDS) is caused by human immunodeficiency virus (HIV) which was first discovered in 1983. From the beginning of the HIV pandemic until the end of 2012, more than 40 million people have died from AIDS. Although AIDS related deaths began declining by the mid-2000s, according to the World Health Organization, it still remains among the top ten leading causes of death. AIDS is also considered the most devastating pandemic ever in human history. About 95% of HIV infections throughout the world are caused by HIV-1 (more specifically HIV-M) whereas HIV-2 is mostly confined to West Africa.

HIV arose from the cross-species transmission of simian immunodeficiency viruses whose natural hosts are chimpanzees (SIVcpz) and sooty mangabeys (SIVsmm), resulting in HIV-1 and HIV-2 respectively. Cross-species transmission alone is not sufficient for the evolution of HIV from SIV however, and multiple other factors contributed to its emergence, including selective pressure, immune suppression, genomic mutations, and host restriction factors. Of these, restriction factors play a key role for successful adaptation of SIV in humans. Effective replication of SIV in a new host is dependent on successful counteraction of host restriction factors. Numerous host restriction factors have been identified to date, but only four are hypothesized to exert significant inhibitory effects on viral replication: i) APOBEC3G - Apolipoprotein B editing enzyme catalytic polypeptide like 3G, ii) TRIM5alpha - Tripartite motif 5 alpha protein, iii) Tetherin - also called as bone marrow stromal antigen 2 (BST-2) and CD317, and iv) SAMDH1 - SAM domain and HD domain containing protein.

Tetherin is a human restriction factor that impedes viral activity by inhibiting the release of virus from infected cells. In non-human primates, the SIV accessory protein, Nef is able to

counteract tetherin, allowing for successful viral replication. However, SIV Nef cannot counteract human tetherin due to the lack of five amino acid codons in the carboxyl terminus of tetherin. Because SIV Nef is ineffective against human tetherin, SIVcpz developed an alternate approach to overcoming this restriction factor using its viral protein U (Vpu). SIVsmm, on the other hand, does not harbor the Vpu gene, and instead recruited its enveloping protein gp41 to counteract tetherin. This suggests these viruses adapt according to their host and these adaptions are species specific. Although we know that HIV is still adapting, the molecular mechanisms by which this mechanism occurs is largely unknown. Although SIVsmm and SIVcpz are capable of zoonotic transfer to humans, only HIV-1 M and HIV-2 A sub-groups became global pandemics. Thus understanding genomic changes in these viruses as they adapt to human hosts is critical.

Non-human primate (NHP) models are commonly used to study the molecular mechanisms of the SIV/HIV adaptations. This model system has been used to investigate multiple aspects of the disease, such as viral pathogenesis, viral evolution, and drug development against the virus (Haigwood, 2004). The accidental transmission of SIVsmm to Asian macaques at a national primate research center is responsible for the origin of the highly pathogenic strain, SIVmac. The emergence of this virus offers an excellent example of viral adaptation to a novel host (Hirsch et al., 1989). The NHP model has been used to evaluate anti-tetherin adaptions of SIV in pigtailed macaques (Schmitz & Korioth-Schmitz, 2013). The NHP model is a valuable tool to study host specific adaptations but it is not necessarily a physiologically relevant model to assess SIV adaptation to a human system.

SIVsmm is a lentivirus that infects sooty mangabeys (*Cercocebus torquatus atys*). SIVsmm evolved into HIV-2 by cross-species transmission from sooty mangabeys to humans in West Africa, an event that was first discovered in 1992 (Gao et al., 1992). SIVsmm infection in

sooty mangabeys shows high viral loads without any CD4+ depletion. Hence it doesn't cause disease in its natural host, but it causes severe disease and CD4+ depletion in non-natural hosts such as rhesus macaques (Chahroudi et al., 2012). Although HIV-2 is not highly prevalent in humans except for a few cases in West Africa, there is evidence for continuing cross-species transmission to humans as a new HIV-2 strain (HIV-2-07ICTNP03) has been recently identified in humans (Ayouba et al., 2013). So there is a possibility for the emergence of a new HIV strains in the near future and studying these cross-species transmission adds great value to the field of HIV research. In this project, we also established a new model for SIV infection in Rag-hu mice.

Here we used humanized mice model to study the evolution of HIV from SIV and the corresponding adaptations of SIV to a human immune system. To study the *in vivo* evolution of HIV-2 from SIVsmm and HIV-1 from SIVcpz, we used new generation humanized mice harboring a transplanted human immune system (Rag-hu mice), prepared by injecting human hematopoietic CD34+ stem cells into neonatal mice.

In this study, we infected humanized mice model with either SIVsmm or SIVcpz. Virus was collected from the plasma of infected mice at regular intervals, and the viral genome sequenced and evaluated for the genomic mutations during each successive passage (generation). To date, by serial passage of virus through Rag-hu mice, three successive times at six month intervals, three generations of virus were generated. The overall mutation rate was evaluated by whole genome sequencing and the genetic distance determined between the stock virus and the adapted virus after each passage. Our results showed that when Rag-hu mice were challenged with SIVsmm or SIVcpz via intraperitoneal (IP) injection, mice were successfully infected and maintained viral loads for up to 6 months. We also observed the hallmark characteristic of HIV infection in SIVsmm infected mice: decline of CD4+ T cells over time. This suggests that the

infection also leads to pathology. These data demonstrate that SIVsmm and SIVcpz can cross the species barrier and infect human immune cells *in vivo*.

We also showed that SIVsmm can be transmitted from one animal to another by IP injection of lymphocytes from an infected donor. Thus far, the virus has been passaged three times in Rag-hu mice and showed successful viral replication and a decline in CD4+ T cells. The infection rate was 100% during all three passages, and the fourth passage of this virus is ongoing. We also observed early onset of viral replication in the second generation of infected mice. Therefore, despite a more aggressively replicating virus in the second passage, viral set points were equivalent in the first and second passages, and there was no significant difference in the CD4+ T cell decline between the two passages.

As mentioned previously, HIV-1 originated from chimpanzees by zoonotic cross-species transmission to humans. SIVcpz fall into two lineages known as SIVcpz*Ptt* and SIVcpz*Pts*. Although there is not much difference between these two primate subspecies, viruses from them differ by at least 30 percent in their genetic sequence homology (Haesevelde et al., 1996). HIV-1 has four subtypes known as M, N, O and P, which we believe are evolved by an independent cross-species transmission events. Out of 4 subtypes, HIV-1 M represents the pandemic strain and has spread to almost every country in the world.

Since we already showed successful infection and sequence analysis of SIVsmm, we extended our work to SIVcpz strains which was the precursor for HIV-1. We obtained 5 different strains of SIVcpz (SIVcpzMB, SIVcpzLB, SIVcpzEK, SIVcpzMT and SIVcpzGab) as a kind gift from Dr. Preston Marx, Tulane National Primate Research Center, Covington, CA. These strains were named after the field places in Cameroon (Figure 22), where each strain was isolated from the wild chimpanzees.

Sequence analysis showed that in the first viral passage, the envelope region of the virus exhibited the highest mutation rate compared to the rest of the genome. This suggests that mutation of the Env region is essential for the adaption of these viruses to a human host. Nef and Vif also displayed numerous mutations during this initial passage. Gag and Pol genes showed very few mutations, though we anticipate identifying more mutations in these regions in further generations. We observed one key mutation in Nef, R17Y (Arg to Tyr) which was previously shown to result in a more pathogenic strain of SIVsmm in pigtailed macaques. We anticipate a mutation in Gag 30 in further passages. We observed additional mutations in the second generation, suggesting that the virus continues to evolve and adapt to its new host over multiple passages. Upon comparison of the first and second passage sequences, we noted that all the mutations from first passage were carried over to the second passage virus. This suggests that mutations from one passage become fixed and are passed on to subsequent passages.

In previous experiments we showed that introducing SIVsmm via IP injection resulted in an active infection in Rag-hu mice and the infection was sustained for more than six months. However to mimic the natural infection process we introduced SIVsmm vaginally in the mice and noticed that 80 percent of the animals got infected and the infections were sustained for 6 months. The vast majority of HIV-1 infections in nature occur through mucosal route either by vaginal (majority) or rectal infection. Pediatric HIV infection results from oral uptake of maternal fluids (Mofenson, 1997). Studies on macaque virus showed that active infection of the virus can be obtained by any mucosal route such as vaginal, rectal, cervical, and oral in animals (Ruprecht et al., 1998; Miller et al., 1989). When virus was introduced by IP, it directly enters the blood stream and all the immune cells are readily available for infection. Whereas when the virus was introduced through vaginal route, it has to cross the mucosal barrier to get into the

blood stream. The initial events during vaginal infection will also be different compared to IP as the virus will now encounter different cell types that it has to pass or escape through. Hence we are expecting to observe different viral mutations or adaptations based on the method of infection (vaginal route or IP).

Here we demonstrated that SIVsmm can infect Rag-hu mice via mucosal challenge. Mucosal challenge resulted in only 80% infection rate relative to IP challenge. This may be due to the fact that the mucosal barrier presents a bottle-neck for the viral infection which is not present in IP challenge where the virus is injected directly into the mouse. During mucosal challenge, only viruses that breach this barrier and establish infection in the resident cells can successfully colonize the animal. It would be interesting to compare the genetic changes resulting from mucosal challenge to those generated in an IP challenge as well as the parental input stock virus. We have isolated RNA from the virus collected from the mice challenged mucosally, and intend to sequence these viral preps in the near future. The direct comparison of these genetic sequences will shed further light onto essential adaptive changes necessary for SIV to overcome the mucosal bottle-neck and establish infection in a humanized system.

The adaptive mechanisms that enabled cross-species transmission of SIV to humans are complex. There is evidence for a different mechanism of HIV evolution which is co-infection with different lentiviruses and their apparent recombination that occurred in the wild. A few strong arguments for such notable recombination events in the wild are: i) recombination between SIVcpz and its ancestor: SIVcpz appears to have evolved by recombination between SIVrcm from red capped mangabeys and SIVmon from greater spot nosed and mona monkeys (Bailes et al., 2003; Courgnaud et al., 2003), ii) SIVagm from African green monkey in West Africa is a result of recombination between SIVagm and SIVrcm (Jin et al., 1994) and iii)

SIVmnd-2 is the result of recombination between SIVrcm and SIVmng-1(Hu et al., 2003, Beer et al., 2001). Here we are trying to investigate if a recombination event can happen between SIV and HIV. For recombination to occur between SIVsmm and HIV-1, both the viruses should be able to infect and replicate in the same cell.

Here we showed SIVsmm and HIV-1 co-infection can successfully establish viral replication in $Rag2^{-/-}\gamma c^{-/-}$ mice. We initially challenged Rag-hu mice with SIVsmm and established steady viral loads. After 9 weeks post-infection with SIVsmm, the same mice were challenged with HIV-1. We observed both viruses replicating simultaneously in the mice for 3 more months.

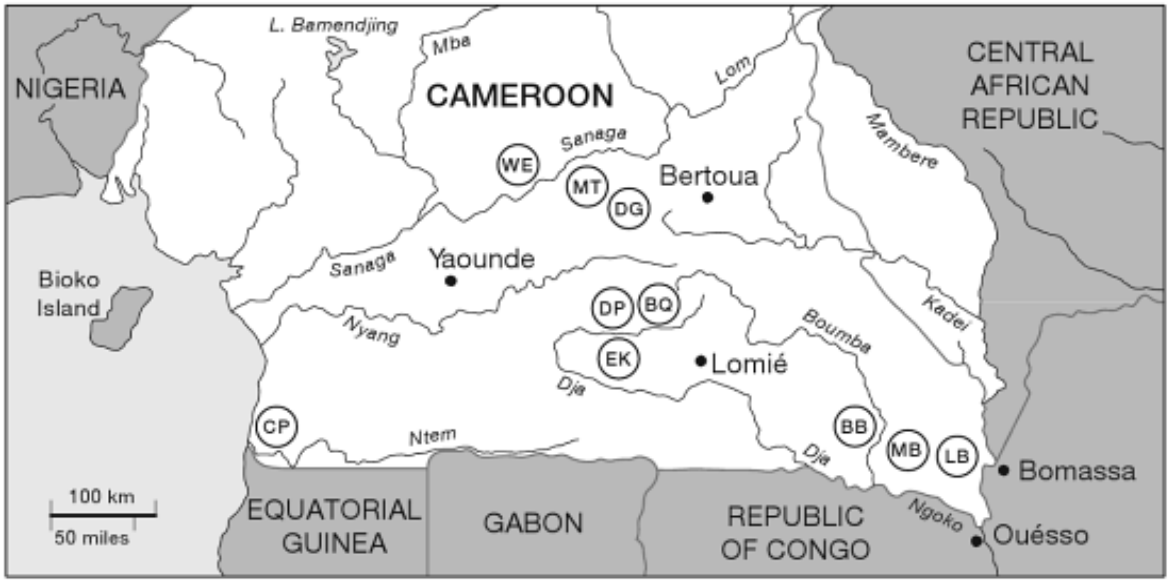

Figure 22: Map of Cameroon showing the field areas where different strains of SIV originated. The map is showing the field areas where different strains of SIV were isolated from wild chimpanzees. SIV isolated from MT region of Cameroon was named as SIVcpzMT. Others are also named accordingly.

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