FACTORS ASSOCIATED WITH THE CONTROL OF SIVsab INFECTION IN BABOONS (*PAPIO PAPIO*)

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B.S., Xavier University, 2011

Submitted to the Graduate Faculty of

the Department of Infectious Diseases and Microbiology of

the Graduate School of Public Health in partial fulfillment

of the requirements for the degree of

Master of Science

University of Pittsburgh

2014

UNIVERSITY OF PITTSBURGH

GRADUATE SCHOOL OF PUBLIC HEALTH

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July 14, 2014

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ABSTRACT

Background. Understanding the mechanisms of simian immunodeficiency virus (SIV) emergence in a new host is a major public health priority, as both HIV-1 and HIV-2 emerged through cross-species transmissions from their respective chimpanzee and sooty mangabey (SM) hosts. We therefore studied the factors associated with the fate of cross-species transmitted SIV infection to a new host by comparing and contrasting controlled and progressive cross-species transmitted SIV infections in an African non-human primate (NHP), the Guinea baboon (*Papio papio*). Baboons, a non-natural host of SIV, were previously reported to carry SIVagm from African green monkeys (AGMs) in the wild and to progress to AIDS when experimentally infected with SIVsmm from SMs.

Methods. Ten baboons were intravenously infected with either SIVsab, the virus naturally infecting the sympatric AGMs (*Chlorocebus sabaeus*) or SIVsmm that naturally infects SMs (*Cercocebus atys*). The impact of intrinsic immunity on SIV infection was assessed by (i) monitoring temporal changes in the host restriction factor (HRF) (APOBEC3G, TRIM5α, SAMHD-1, tetherin and MX2) expression in lymph nodes (LNs) by immunohistochemistry (IHC) and (ii) virus evolution through single genome amplification and sequencing.

Results. Upon exposure to SIV, the viral loads (VLs) peaked at 10-14 days post infection (dpi). Peak VLs were 2-3 logs lower in SIVsab infected baboons, who controlled the virus to undetectable levels at 28-42 dpi and then through follow-up. Conversely, SIVsmm infection was persistent throughout the follow-up and two out of four SIVsmm infected baboons showed signs of disease progression. IHC quantification revealed an increase in the expression of individual HRFs in the LNs from SIVsab infected controller baboons. Conversely, HRF expression was virtually unchanged in SIVsmm infected baboons and in AGM natural hosts. SIV quasispecies characterization identified a mutation rate similar to that observed in the natural host in baboons infected with SIVsmm and baboons infected with SIVsab.

Conclusions. Our results demonstrate that combined rather than individual action of HRFs is the major determinant of the outcome of SIV infection upon cross-species transmission. Therefore, approaches aimed at developing new animal models for HIV research should overcome the overall intrinsic immunity instead of focusing on individual factors.

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PREFACE

I'd like to first acknowledge my advisors Dr. Pandrea and Dr. Apetrei. Thank you for opening your lab to me, sharing your knowledge, your patience, motivational speeches, and pushing me farther than the limits of my own expectations. I never thought I'd be a tertiary author on an original research paper in a major journal or an oral presenter at an international conference at this point in my career. Thank you for allowing me to work on projects outside my own to broaden my scientific horizons. It's been a crazy year and a half in your lab, and I look forward to the next few.

Thank you to my committee members Dr.Ambrose, Dr. Martinson, and Dr. Reinhart for all of your helpful discussion, insight, and flexibility. This project has been elevated to a higher level of scientific standard because of you.

To my labmates and SURP student Adam "Runt" Mobley, thank you for putting up with my stupid jokes, vulgarities, and general obnoxiousness. Your encouragement, empathy, and senses of humor are the things that make the "Pandapet" lab feel like home. I look forward to many more laughs over the coming years.

I'd especially like to thank Dr. Dongzhu Ma for running the PCR experiments featured in this paper and Rich Anand for doing some of the flow analysis used in this paper.

Tom Henderson, "Zeiss guy", I wouldn't know a thing about microscopy without you. Thank you for teaching me what all the buttons do and how to take a good picture.

Thank you to my collaborators in the Estes lab, especially Claire Deleague. Thank you for your helpful discussion particularly with the tricky TRIM5α stain. Thank you the Robertson lab, especially Felix for running the SGAs for this project.

Thank you to my parents. I wouldn't be here without you, literally. The English language does not have a word to adequately express my gratitude. Thanks to my siblings Renee, Chris, and Jill for your encouraging phone calls, texts, dinosaur cards, and coloring book pages.

Thank you to Cincinnati friends for making it still feel like home when I visit and to my new friends in Pittsburgh for making Pittsburgh feel like home. Thank you to my fellow IDM students for valued emotional and academic support. We are lucky to have each other.

Also for obvious reasons, I thank Diet Coke.

1.0 INTRODUCTION

The AIDS pandemic caused by human immunodeficiency virus (HIV) is a large health, economic, and societal burden. With 36 million lives already claimed and 36 million patients currently infected by HIV, it is necessary to devote resources in an effort to curb the destruction caused by HIV (57). Major progress has been achieved in understanding how the virus is spread, allowing for many public health education programs to prevent new infections. Although expensive and not accessible to all who need them, more antiretroviral drugs were developed for HIV and are currently available than for all other viral infections combined. Altogether, antiretroviral therapy (ART) dramatically improved the prognosis of HIV infection, current life expectancy of a newly infected subject being 59 years (14). Still, ART cannot cure infection and no effective vaccine to prevent HIV infection is available thus far.

HIV emerged following cross-species transmissions of the lentiviruses naturally infecting nonhuman primate (NHP) species in Africa, which are generically called simian immunodeficiency viruses (SIVs). A successful infection via cross-species transmission is dictated by many host and viral factors. Understanding the role these factors have on the success or failure of a cross-species transmission event is an essential part of understanding how HIV establishes infection to cause disease, how to prevent more SIVs from jumping the species barrier to humans, and emergence of new epidemic/pandemic strains.

1.1 CROSS-SPECIES TRANSMISSION

Viruses are usually limited to a specific host range as determined by a combination of many host defense and virus specific factors. Generally, most cross-species transmission events are dead-ends, but occasionally a virus can successfully cross the species barrier and successfully spread within a new population. The mechanisms by which cross-species transmission occurs are specific to each virus-host combination and are not well understood.

Many viruses have crossed the human species barrier to humans and some have spread in the human population. Ebola virus' natural host is believed to be Fruit bats, but highly lethal outbreaks spill over and spread into the human population through contact with bat urine or feces. Ebola is also spread from human to human by contact with an infected person's bodily fluids (81). Like Ebola, the Rabies virus natural host is believed to be bats and is spread to wild animal through bat urine or feces. Rabies virus transmission to humans is very rare and usually occurs via saliva from the bite of an infected wild animal Even more rare is human to human transmission, unlike Ebola virus (79). Influenza virus infects humans, birds, and pigs, among other mammals. Influenza can cross the species barrier by using genetic reassortment and genetic drift to evolve past species barriers (67).

As previously mentioned, HIV-1 and HIV-2 are products of successful cross-species transmission from SIVchz and SIVsm respectively. HIV-1 is global and is considered more pathogenic while the less pathogenic HIV-2 is limited to West Africa. In a study that tested 5 subtypes of HIV-2, only 2 were pathogenic while 3 were dead ends (19). However, not all cross-species transmission events are successful, as one human tested positive for antibodies specific to SIVmnd, but did not develop disease from the virus (74). Furthermore, there are reports of African hunters exposed to SIVs through hunting and butchery who show no signs of clinical

disease (29). The mechanisms that determine the success or failure of an SIV cross-species transmission event are largely undetermined.

1.2 HOST RESTRICTION FACTORS

Host restriction factors (HRFs) are proteins that exhibit antiviral properties. HRFs can completely or partially inhibit viral replication and may play a role in the success or failure of cross-species transmission. In general, HRFs are products of IFN-stimulated genes and thus can block virus transmission as part of the broadly-acting interferon (IFN) responses. Note, however, that the transmitted/founder strains were reported to be more resistant to IFNs, which explain why cross-species transmissions of SIVs between NHP species and between NHPs and humans can occur (17). In response to this virus adaptation, multiple HRFs act uniquely in different hosts, with different viruses, and at different stages of the viral life cycle.

Currently, six SIV/HIV host restriction factors have been identified: APOBEC3g (47), MX2 (24, 30), SAMHD1(37, 38), Tetherin (36, 55), TRIM5α (76), and, most recently, nuclear export inhibitor HERC5 (82). While there are most likely many more HRFs yet to be discovered, this study focuses on five (APOBEC3g, SAMHD1, Tetherin, TRIM5α, MX2) HRFs potentially working together, in concert, to have an overall effect on viral replication.

1.2.1 APOBEC3g

In 2003, APOBEC3g, formerly known as CEM 15, was the first HIV specific host restriction factor discovered (84). APOBEC3g is an acronym for apolipoprotein B mRNA-

editing, enzyme-catalytic, polypeptide-like 3G (73), and is the most potent host restriction factor of the several siblings in the APOBEC family of proteins (84). APOBEC3g has two forms: an inactive high-molecular-mass (HMM) and an enzymatically active Low-molecular-mass (LMM) form (11). While APOBEC3g is primarily expressed in $CD4^+$ T cells, dendritic cells and macrophages (the primary targets of the HIV virus) it has also been found in other cell types upon IFN stimulation (75). An important caveat is that APOBEC3g must be packaged into HIV virions as they are exiting an infected cell and acts against HIV in newly infected cells (31).

APOBEC3g acts as a cytosine deaminase thereby removing the amine functional group from cytosine converting it to uracil on the negative strand of DNA during reverse transcription (84). This base pair modification affects the complementary positive strand DNA (G to A mutations) and subsequent mRNA products to induce potentially fatal mutations or drive HIV evolution. *In vitro* studies have also suggested that APOBEC3g works to inhibit reverse transcription altogether by: competitively binding tRNA, inhibiting strand transfer, and inhibiting elongation of reverse transcription products (50). However, these functions have yet to be verified *in vivo* (50).

APOBEC3g is antagonized by the auxiliary protein *Vif* in HIV viruses. *Vif* marks APOBEC3g for ubiquitin degeneration by host proteasomes, thus preventing it from being packaged into the progeny HIV virions (31). Additionally, some evidence suggests that *Vif* directly inhibits encapsidation and impairs translation of APOBEC3g (50).

1.2.2 TRIM5α

In 2004, the second HIV host restriction factor, TRIM5α, was discovered in Owl monkeys, which are resistant to HIV-1 infection (70). TRIM5α is a member of the tripartite

motif (TRIM) family of proteins. Structurally, it contains: RING domains, B-boxes, coiled coils, and a unique carboxy-terminal B30.2 (SPRY) domain not found in the other TRIM5 proteins (76). The RING and SPRY domains are required for antiviral activity, while the B-Box domains assist with but are not required for antiviral function (76). Typically, TRIM5α is located in the cytoplasm of cells, but it also localizes into cytoplasmic bodies (76). Both forms exhibit antiviral capabilities (76).

TRIM5α can restrict a wide range of retroviruses and seems to better restrict retroviral variations outside of host species. For example, rhesus macaque TRIM5α restricts HIV-1 better than human TRIM5α (76, 78). Usually the host TRIM5α does little to restrict the virus when it is in the natural host. Human TRIM5α does little to restrict HIV-1 (76, 78). Thus, TRIM5α can be an excellent mediator of cross-species transmission.

TRIM5α's exact mechanism of restriction is still unclear, but there have been some important clues. First, TRIM5α binds to capsid proteins and appears to accelerate capsid uncoating (77). Premature uncoating itself may disrupt the formation of the reverse transcription complex, thereby inhibiting viral replication. It is also believed that the SPRY domain is important in restriction. One group made chimera TRIM5α proteins by adding an rhTRIM5α SPRY domain to a human TRIM5α, and found that particular chimera is sufficient for HIV-1 inhibition (78). Another mechanistic clue is cyclophilin A. TRIM5α is associated with the cyclophilin A protein. Furthermore to support the relationship between TRIM5α and cyclophilin A, there are reports of a TRIMcyp fusion protein with restrictive properties (56). cyclophilin A's association with TRIM5α can help or hinder viral restriction depending on the host and virus combination as some viruses have mutations that prevent cypA binding (77).

1.2.3 Tetherin

Interestingly, tetherin's existence was predicted before it was discovered. An IFN-induced tethering mechanism acted against HIV virion release that was absent in the presence of *Vpu* supplied the rationale for a microarray search for the protein responsible (45). Tetherin (CD317 or BST-2) was identified in 2008 by Niel *et al.* as a broadly acting protein against many viruses including Lassa virus, Ebola virus, HIV, and SIV (55). Tetherin is an IFN induced protein (55). Tetherin is present on many cell types, including dendritic cells (13), macrophages (12), and T cells (28). It can be located in the cellular membrane or in the trans-Golgi region of the cell in endosomes (54).

Tetherin prevents cell-to-cell spread of HIV infection by binding the virus particles to plasma membrane therefore inhibiting the necessary budding of viral progeny (36, 55). Structurally, tetherin has one transmembrane region that remains in the viral envelope and an anchor region that remains in the host cell membrane. These regions are separated by a long α-helix that has a dimeric, coiled structure (45).

Tetherin is antagonized by three retroviral proteins: *Vpu*, *Nef*, and *Env*. *Vpu* antagonizes tetherin by marking it for degradation (46), or by binding directly to tetherin preventing it from reaching the cell surface (45). In viruses without *Vpu*, there is evidence suggesting that either *Nef* or *Env* proteins can deter tetherin function; however detailed mechanisms have yet to be deciphered (45).

1.2.4 SAMHD1

SAMHD1 stands for SAM domain and HD domain-containing protein 1 and is expressed in dendritic cells, macrophages, and monocytes (37). SAMHD1 usually resides in the nucleus of cells except for those SAMHD1 proteins without a nuclear location sequence (8). Both nuclear and cytoplasmic forms have antiviral capabilities (25). Crystallography reveals that inactive SAMHD1 has a dimer structure with a major and a minor groove (23). dGTP binds to convert SAMHD1 into its enzymatically active form SAMHD1c (23). Together these clues suggest that SAMHD1 interferes with HIV replication.

IFN-induced SAMHD1 has an enzymatic function that converts nucleotides to nucleosides, thereby depleting the pool of available nucleotides for replication (38). Interestingly, SAMHD1 will only hydrolyze GTP when each nucleotide is tested individually, but will hydrolyze all four nucleotides when all are present (23). SAMHD1's depletion of available nucleotides for replication limits reverse transcription and, therefore, HIV infection.

Depending on cellular location, *Vpx* can antagonize SAMHD1, or *vice versa*. In the nucleus, Vpx antagonizes SAMHD1 by recruiting ubiquitin ligases that mark it for degradation in proteasomes (25). In the cytoplasm, SAMHD1 binds *Vpx*, preventing it from reaching the nucleus. Even in the presence of *Vpx*, cytoplasmic SAMHD1 retains antiviral ability (25).

1.2.5 MX2

Myxovirus resistance 2 (MX2) was identified as an HIV host restriction factor in the fall of 2013 by Malim *et al.* despite having been well studied for many years (24). MX1, MX2's cousin, has marked antiviral activity against influenza virus, but not HIV/SIV. Conversely, MX2

does not exhibit any antiviral activity against influenza virus, but has antiviral properties against HIV (24). This protein shows some inhibitory effects on various types of SIV, but most notably with HIV-1 in various cell lines (24, 30).

While the mechanism of action has yet to be determined, MX2 associates with cyclophilin A and loses potency when HIV-1 mutates its capsid protein at residue 88 (30). This suggests that MX2 acts on HIV's capsid in a cyclophilin A-dependent manner (30). MX2 is thought to interfere with HIV DNA's entry into the nucleus or its integration into host DNA, as nuclear aggregation of virus and viral cDNA is suppressed in the presence of MX2 (24). MX2 is located either in the nucleus itself or in the cytoplasm around nuclear pores, thus supporting the hypothesis of nuclear entry/integration interference (24). IFN-α stimulation is required for MX2's antiviral properties (24, 30). Considering MX2's recent identification, not much is known about other functions MX2 may have or what mechanisms of evasion HIV may employ against it.

2.0 HYPOTHESIS AND SPECIFIC AIMS

Most studies investigating the role of HRFs in preventing/controlling HIV/SIV infection in a new host upon cross-species transmissions were performed *in vitro*, on cell lines and generally focused on single host restriction factors. However, their effects might be less clear-cut *in vivo*, but the overall impact might be critical on HIV/SIV pathogenesis and transmission. The case in point is the impact of TRIM5α genotypes on the outcome of chronic SIVsmm infection in RMs. While *in vitro* studies did not find such impact, *in vivo*, different TRIM5α pedigrees of RMs might result in variations of the set point viral loads of 10-1000 fold. We, therefore, developed an animal model system to monitor the impact of HRFs alone or in combination with the outcome of cross-species transmitted SIV infection.

Our overall hypothesis is that **the combined action of multiple host restriction factors will have an overall impact on the control of SIVsab infection in baboons**. The use of this cross-species transmission animal model will provide an excellent environment to assess the role of HRFs as major contributors to the overall success or failure of cross-species transmitted SIV infection.

2.1 AIM 1: TO COMPARE AND CONTRAST THE NATURAL HISTORY AND THE OUTCOME OF SIVSAB OR SIVSMM INFECTION IN GUINEA BABOONS (*PAPIO PAPIO*).

Rationale: Our overall goal is to establish the appropriate animal models to assess impact of host restriction factors on the outcome of the cross-species transmitted viral infection. While both SIVsab and SIVsmm are cross-species transmitted, our hypothesis is that SIVsab and SIVsmm infections will have opposite outcomes in the same baboon species. Therefore, we can use these systems to assess the role of HRFs in these different infection outcomes. As controls, we employed SIVsab in its natural host, AGMs, in which the impact of restriction factors should be minimal. **Hypothesis:** Our hypothesis is that baboons will control the SIVsab infection, but will progress to AIDS when infected with SIVsmm. Due to these major differences in the natural history of infection, these two models of SIV infection represent perfect settings for studying host restriction. **Approach:** Compare and contrast the natural history of SIV infection in the two models by 1) quantifying viral loads in baboons infected with SIVsab or SIVsmm, 2) examining the impact of infection on relevant immune cell populations, and 3) quantifying the levels of systemic immune activation in the two models of progression. **Significance:** The study of two models with completely different SIV infection outcomes offers us an ideal experimental environment to assess the role played by the host restriction factors in SIV cross-species transmission.

2.2 AIM 2: TO DIRECTLY ASSESS THE CHANGES IN HOST RESTRICTION FACTORS THAT MAY CORRELATE WITH VIRUS CONTROL UPON CROSS-SPECIES TRANSMISSION.

Rationale: Use of two infection models with opposite natural histories of SIV infection based on the same NHP species will permit us to assess the most discrete changes in the HRFs that may play a role in viral control. Our goal is to examine changes in expression of host restriction factors at key time points of SIV infection and control. **Hypothesis:** While each restriction factor acts on different stages of replication and each plays a role in the control of cross-species transmission, it is their aggregated action that will drive the overall outcome of infection. HRF expression will increase in controllers, but will remain constant in progressor baboons and in the natural host. **Approach:** Our approach uses histological techniques to assess dynamics of HRF expression in lymph nodes by 1) an immunohistochemistry (IHC) assessment of HRF protein expression, 2) Quantification of IHC signals, and 3) Immunofluorescence (IF) double stain to attribute expression of HRFs to specific immune cell population(s). **Significance:** This study will examine the expression of five HRFs working in concert to influence the impact of infection, while many studies only focus on one or two of the HRFs.

2.3 AIM 3: TO ASSESS THE HRF IMPACT ON THE VIRUS IN CONTROLLED AND PROGRESSIVE INFECTIONS OF BABOONS.

Rationale: This study will directly assess the effects of APOBEC3g on the virus by monitoring the evolution of the virus quasispecies in controllers and progressors. **Hypothesis:** A

higher APOBEC3g induced G-A hypermutation rate will be observed in controllers and will be directly correlated with the over expression of HRFs. **Approach:** Single genome amplification (SGA) and analysis of SIV quasispecies diversity will be performed to assess hypermutations and sequence changes that might be illustrative of host evolutionary pressures. **Significance:** This approach will directly assess the impact of HRF on the virus itself, an innovation yet to be seen in this field.

3.0 STUDY DESIGN

3.1 THE BABOON IS AN IDEAL ANIMAL MODEL TO STUDY CROSS-SPECIES TRANSMISSION OF SIV/HIV

Due to practical and ethical concerns, the study of SIV cross-species transmission cannot be directly investigated using human subjects. Ethically, one cannot infect humans with potentially dangerous viruses. Practically, a natural cross-species transmission is not usually caught early enough during infection to assess the complex requirements and virus-host interactions necessary for a successful cross-species transmission. Furthermore, if a cross-species event was to occur, the detection tools and methodology may not be readily in place to detect such an event. For example, SIVs have a tremendous genetic diversity while the serology and PCR-based detection kits only cover the diversity of HIV strains, which represent only a minor fraction of the total genetic diversity. Instead, animal models are an acceptable alternative to studying cross-species transmission of SIV/HIV.

With a number of potential animal models available for AIDS research, one should choose the best animal model to study the mechanisms and requirements of a successful cross-species transmission. Ideally, such an animal model must use an African species that is not a natural host of SIV. An African species is more likely to have a similar evolutionary history and ecological features with the natural host. Importantly, a non-natural host of SIV will not have

evolutionary adaptations to SIV. The animal model must compare and contrast the natural history of SIV infection in the new host and in its natural host, and biologically monitor multiple cross-species transmissions with opposite outcomes of infection. Together these considerations should allow us to better understand the requirements for a successful cross-species transmission.

Baboons could serve as an ideal animal model for HIV/SIV investigations, though typically rhesus macaques (RMs) and pigtailed macaques (PTMs) are used to study HIV/SIV. Baboons are not endangered, are larger animals (which is good for large volume blood draws), and breed well in captivity (41). Unlike other NHPs, baboons do not carry the Herpes B virus that is potentially lethal to human researchers (41). Baboons are closely related to both humans and to macaques. In fact, baboons and rhesus monkeys can mate to make a sterile offspring called a rheboon (41). Like humans, baboons have four subclasses of IgG antibody, whereas Rhesus monkeys only have three suggesting this model is closer to humans immunologically (41). Baboons have been used in numerous studies relating to a wide variety of human diseases (41), and have been used to test HIV vaccine components (9, 10, 39, 42). Baboon blood marrow was even used safely in a failed cure transplant attempt for an AIDS patient (49).

Furthermore, baboons are an African NHP species that are not natural hosts of SIV with no baboon-specific SIV being described to date (41). However, multiple species of baboons have been shown to carry SIVs in the wild (in general cross-species transmitted from the sympatric AGM species) (26, 35, 80). The first molecular evidence for an SIV cross-species transmission has been reported in a yellow baboon infected with SIV from AGMs (26). While there are a few cases that suggest SIVs can infect baboons in the wild, it is not known whether baboons progress to an AIDS-like disease status naturally.

Upon experimental exposure, baboons have shown ability to control cross-species transmitted SIVs/HIVs. Thus, baboons infected with SIVmne (6), HIV-1 (52), and SIVmac (15) showed signs of swollen lymph nodes indicative on infection; yet, they demonstrated inconsistent seroconversion, with little–to-no virus isolated from the blood and no signs of clinical disease. Only when infected with the simian-human immunodeficiency virus (SHIV) chimera could the virus be more readily detected in the blood, yet the animals still did not present any signs of clinical disease (1, 34). Upon initial experimental infection with HIV-2, *Hamadryas* baboons showed similar infection patterns as mentioned previously, but upon serial passages, the virus became more pathogenic eventually resulting in an AIDS-like disease (43). Disease progression was more similar to that in humans than other animal models, with high viral loads and $CD4^+$ depletion in the acute phase, a roughly four to seven year healthy phase, and finally a development of an AIDS-like disease (43). Acute infection was initially controlled by $CD8^+$ T cells like human infections (7), and neutralizing antibody response developed at roughly six months and peaked one year post infection (43). It was, therefore, concluded that due to the similar natural history of infection with HIV-1 infection, baboons can be used as an animal model for HIV disease.

For our study, we opted for the use of Guinea baboons (*Papio papio*), which are the baboon species specific to West Africa. The rationale for choosing Guinea baboons is that they are sympatric to the Western species of AGMs, the sabaeus monkey. This is the species extensively used in our lab. We reasoned that since cross species transmission occurred in the wild at different locations (i.e., Kenya and South Africa) between the sympatric AGM and baboon species, the proposed match should be the option of choice for such cross species transmission studies.

3.2 CHOOSING A VIRUS FOR THE STUDY OF CROSS-SPECIES TRANSMISSION

For our studies on cross-species transmission, we employed two SIV strains: SIVSab92018, a virus isolate naturally infecting the West African sabaeus African green monkeys (*Chlorocebus sabaeus*), and SIVsmm, a virus isolated from the sooty mangabeys (*Cercocebus torquatus atys*). While naturally infecting AGMs, SIVagm has been shown to infect baboons in the wild (26). In particular, SIVsab92018 (from here on referenced as SIVsab) strain is ideal for this cross-species transmission study as it has never been passaged *in vitro*, was collected from an acutely infected sabaeus monkey, and has a diverse inocula containing a large proportion of transmitted founder viruses, as shown in previous studies from our lab (3, 5, 21, 22, 62). As Guinea baboons are sympatric and eat AGMs, a plausible route of cross-species transmission can be established in the wild between these two species. To date, it is not known whether baboons progress to AIDS when infected with this virus.

SIVsab has been used in multiple animal model studies. In its natural AGM host, SIVsab causes persistent but nonpathogenic infection (63). Similarly, in the African NHP species, the patas monkeys, SIVsab induces high levels of persistent viral replication with no disease progression (3). SIVsab is pathogenic in pigtailed macaques (PTMs) (61), while RMs control the infection (62).

SIVsmm naturally infects sooty mangabeys (SMs) and is the ancestral virus of HIV-2 (72). After serial passage, HIV-2 was shown to cause AIDS-like disease in baboons experimentally (41, 44), which allowed us to hypothesize that an unadapted SIVsmm transmitted founder strain may cause AIDS-like disease in baboons. This study is the first experimental infection with SIVsmm in baboons.

Experimentally, SIVsmm infection can result in a wide variety of disease outcomes in NHP animal models. SIVsmm is generally nonpathogenic in its natural hosts, the sooty mangabeys, despite high viral loads (33), with only one case of AIDS being described in an old SM that outlived the lifespan of the species (40). Upon cross species transmission, a case of AIDS was also reported in a black mangabey infected with SIVsmm (4). Upon cross-species transmission to Asian NHP species, SIVsmm is pathogenic in rhesus macaques (83) and pigtailed macaques (18).

Both viruses used in this study retain the traditional lentiviral structure of encoding regions 5'-*gag-pol-env*-3' that are flanked by two long terminal repeats (53). The *gag* encoding region produces the capsid proteins P24, P17, and NC (53). The Pol encoding region produces the nonstructural proteins of reverse transcriptase, and protease (to cleave viral polyproteins into individual active proteins) (53). The Env encoding region encodes the envelope proteins of Gp120 and Gp41 used for attachment and entry into the host cell (53).

SIVs may contain a number of accessory proteins that have been associated with virulence in addition to replication functions. In general, all lentiviruses and both viruses used in this study have *vif, vpr, nef, tat,* and *rev* (71). The presence of *vpx* and *vpu* is variable depending on the virus. For example, HIV-1 encodes *Vpu,* but not *Vpx* (71). SIVagm does not have accessory proteins *Vpx* or *Vpu*, while SIVsmm encodes only *Vpx* (71). Of note, these accessory proteins are antagonists to some host restriction factors as mentioned previously. For example, *Vpu* antagonizes tetherin (55); *Vif* antagonizes APOBEC3g (48); and *Vpx* antagonizes SAMHD1 (25). Lack of *Vpx* in SIVsab may render this strain insensitive to this restriction factor.

4.0 MATERIALS AND METHODS

4.1 NHPS AND INFECTIONS

This study involved 10 Guinea baboons and 28 African green monkeys (AGMs). Six baboons were challenged intravenously (IV) with plasma equivalent of 300 Tissue Culture Infectious Doses 50% (TCID50) of SIVagmSab92018 (60) and four were challenged IV with plasma equivalent of 300 TCID50 of SIVsmmD215 (20). All AGMs were infected IV with plasma equivalent of 300 TCID50 of SIVagmSab92018. The animals and infection were monitored for up to 8 months post infection.

The animals were housed at the RIDC Park facility of the University of Pittsburgh in accordance with the recommendations of the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International Standards and with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (2). The Institutional Animal Use and Care Committee of the University of Pittsburgh approved these studies (Protocol # 09039). Efforts were made to minimize animal suffering in agreement with the recommendations of the Weatherall report, "The use of non-human primates in research". The RIDC Park facility is air-conditioned, with an ambient temperature of 21-25°C, a relative humidity of 40%-60% and a 12 h light/dark cycle. Animals were individually housed in suspended stainless steel wire-bottomed cages and provided with a commercial primate diet.

Fresh fruit was provided once daily and water was freely available at all times. A variety of environmental enrichment strategies were employed including housing of animals in pairs, providing toys to manipulate and playing entertainment videos in the animal rooms. In addition, the animals were observed twice daily and any signs of disease or discomfort were reported to the veterinary staff for evaluation. For sample collection, animals were anesthetized with 10 mg/kg ketamine HCl (Park-Davis, Morris Plains, NJ, USA) or 0.7mg/kg tiletamine HCl and zolazepan (Telazol, Fort Dodge Animal Health, Fort Dodge, IA) injected intramuscularly. The animals were sacrificed by intravenous administration of barbiturates prior to the onset of any clinical signs of disease.

4.2 BLOOD AND TISSUE COLLECTION AND PROCESSING

Blood, lymph nodes (LN), and intestinal biopsies (IB) were collected throughout the follow-up as follows: preinfection, at multiple time points of the acute infection, including the viral peak, at the viral set point, during the chronic infection, and at the necropsy. Blood was collected at -14, 0, 7, 10, 15, 22, 29, 43, 73, 101, 134, 166 days post infection (dpi) and at the necropsy. LNs were collected at 0, 10, 29, 101 dpi and at the necropsy. Intestinal fragments of the intestine (jejunum) were obtained by endoscopic guided biopsy at 0, 22, 29, 43, and 101 dpi with additional samples collected at the necropsy.

Within one hour after blood collection, plasma was harvested and peripheral blood mononuclear cells (PBMCs) separated from the blood using Ficoll density gradient centrifugation, as described (66). Lymphocytes from the intestine and LNs were isolated and stained for flow cytometry, as previously described (60, 65, 66). Lymphocytes were isolated

from the axillary or inguinal LNs by gently mincing and pressing tissues through nylon mesh screens (60, 65, 66). Intestinal resections were processed as follows: minced mechanically, washed with EDTA, and subjected to collagenase digestion followed by Percoll density gradient centrifugation, as described (60, 65, 66).

4.3 FLOW CYTOMETRY

Flow cytometry was used to assess the changes in the major immune cell populations and immune activation markers. Whole peripheral blood, LN cell suspensions, and intestinal mononuclear cell suspensions were stained with fluorescently labeled antibodies to markers CD3 (clone SP34-2; all antibodies from BD Bioscience, San Jose, CA, USA unless otherwise noted), CD4 (clone L200), CD8 (clone 3B5, Invitrogen Carlsbad, CA, USA), Ki-67 (clone B56), and HLA-DR (clone L243). For the detection of Ki-67, we employed an intracellular staining technique in which cells were stained with the surface markers, then fixed, permeabilized, and stained for Ki-67. Flow cytometry acquisitions were performed on an LSR II flow cytometer and analyzed with FlowJo software (Treestar, Ashland, OR, USA).

4.4 VIRAL QUANTIFICATION QPCR

SIVsab92108 plasma VLs were quantified with an SIVsab-specific real-time PCR (59). Viral RNA was extracted from 540 µl of plasma using the QIAamp viral RNA extraction kit (QIAGEN, Courtaboeuf, France). Real-time (RT) PCR assays specific for each virus were

developed for SIVagm RNA quantification. Briefly, total RNA was retrotranscribed into cDNA by use of the TaqMan Gold RT PCR kit and random hexamers (PE, Applied Biosystems, Foster City, CA). PCRs were carried out in a spectrofluorometric thermal cycler (ABI PRISM 7700; PE). For SIVsab, quantification was based on the amplification of a 180-bp-located long terminal repeat (LTR) region. The primers and probe for SIVsab were J15S (5′-CTG GGT GTT CTC TGG TAA G-3′), 5′ J15S (5′-CAA GAC TTT ATT GAG GCA AT-3′), and J15P (6-carboxyfluorescein-CGA ACA CCC AGG CTC AAG CTG G-6-carboxytetramethylrhodamine) as previously described (18). SIVsab cDNA was added to the universal master mix (PE, Applied Biosystems), containing 10 μM of each primer and 10 μM of the probe. The PCR mix contained the SIVsab cDNA, 2× universal SybrGreen master mix buffer (Applied Biosystems, Courtaboeuf, France), and primers in a final concentration of 0.3 μM. All PCRs were carried out in duplicate in parallel to a negative non-reverse transcription control reaction. The PCR cycling conditions were identical for all assays: a first cycle of denaturation (95°C, 10 min) was followed by 45 cycles of denaturation (95°C, 10 s), annealing (50°C, 30 s), and extension (72°C, 30 s). Absolute viral RNA copy numbers were deduced by comparing the relative signal strengths to corresponding values obtained for five 10-fold dilutions of standard RNAs that were reverse transcribed and amplified in parallel. In order to construct these RNA standards, larger LTR regions of SIVsab and SIVsmm were PCR amplified. The SIVsab LTR PCR product was obtained by amplifying a SIVsab reference virus (plasmid psab-1) in a PCR with primers LTR2A (5′-AAC TAA GGC AAG ACT TTA TTG AGG-3′) and LTR4S (5′-ACT GGG CGG TAC TGG GAG TGG CTT-3′). The PCR products were cloned into a pCR 2.1 vector (Invitrogen, Groningen, Netherlands). In vitro transcription was then performed using the MEGAscript kit (Ambion, Austin, TX). Known amounts of the SIVsab LTR standard RNAs were used to

determine the target copy numbers. The detection limit of the SIVsab quantification assays was 5 × 10^2 RNA copies/0.5 ml of plasma.

For the quantification of SIVsmmD215, we also employed a specific real-time PCR assay, as described (20). Plasma viral loads were determined by branched DNA assay (bDNA; Bayer Diagnostics, Tarrytown, NY).

4.5 IMMUNOHISTOCHEMICAL (IHC) ASSESSMENT OF HRFS

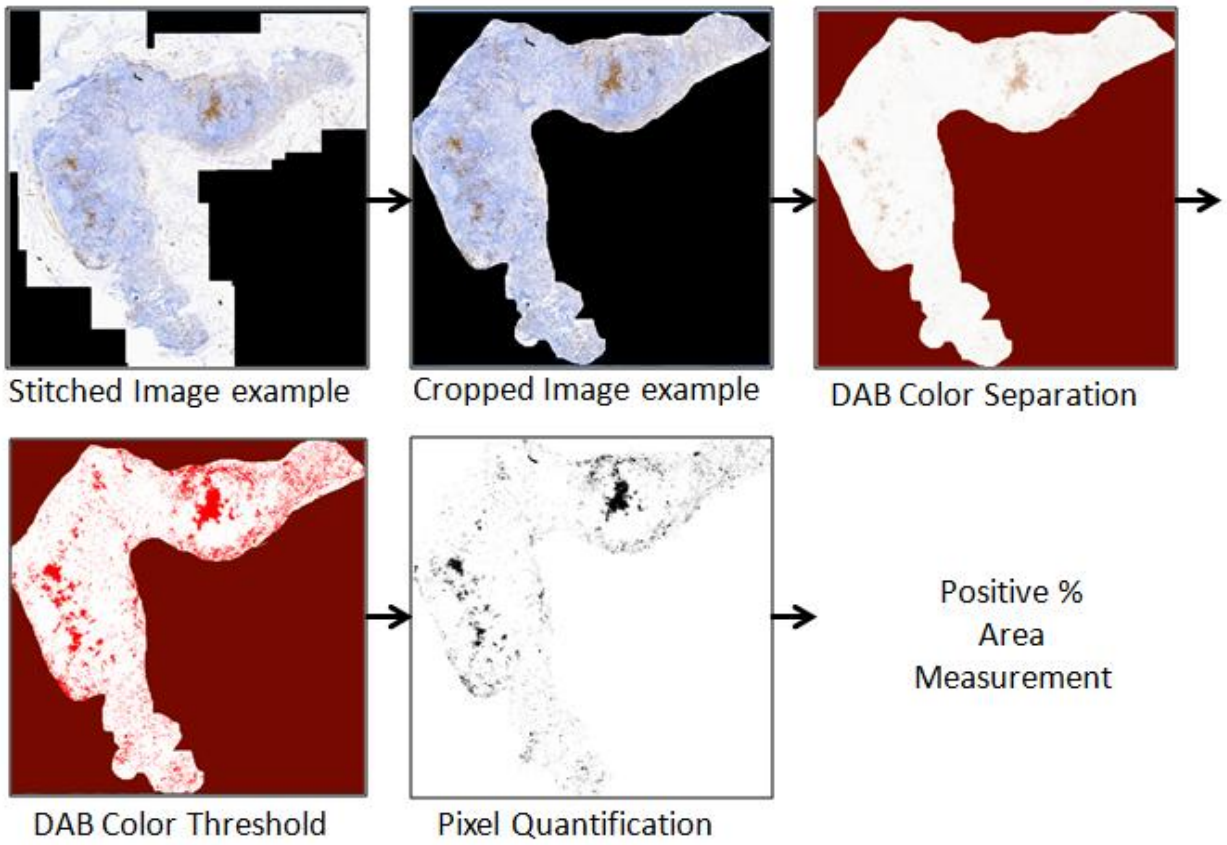
IHC was performed on formalin-fixed or 4% paraformaldehyde-fixed, paraffin-embedded tissue samples. Four µm thick sections were deparaffinized in xylene, rehydrated in ethanol gradient (100, 100, 90, 75), and rinsed in dH_20. For antigen retrieval, the sections were microwaved for 23 minutes in Vector Unmasking Solution (Vector Laboratories, Burlingame, CA). To deactivate endogenous peroxidases, the sections were treated with 3% hydrogen peroxide for 15 minutes. Slides were blocked with Dako blocking serum for 30 minutes (Dako, Carpinteria, CA). Sections were incubated with primary antibody (see **Table 1**) for an hour at room temperature. Vector Vectastain ABC Elite Kit provided animal specific secondary antibody and Avidin-Biotin complex both incubated for 30 minutes (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA). 1X PBS was used for rinses in between steps. For visualization, sections were treated with DAB (Dako, Carpinteria, CA), counterstained with hematoxylin and mounted in xylene based mounting medium with glass coverslips. Either Isotype or appropriate animal universal controls were utilized. Sections were photographed at an overall magnification of 50x.

Table 1. Primary Antibodies and Dilutions Utilized in IHC Staining of HRFs.

Primary Antibody	Type	Dilution	Company	Catalog #
TRIM5α	Polyclonal Goat	1:200	Abcam	ab4389
MX2	Polyclonal Rabbit	1:250	Novus Biologicals	NBP1-81018
APOBEC3G	Polyclonal Rabbit	1:500	Novus Biologicals	NBP1-88592
SAMHD1	Polyclonal Goat	1:100	Santa Cruz Biotechnology	SC-86212
Tetherin	Monoclonal Rabbit	1:100	Abcam	ab134061

4.6 QUANTIFICATION OF IHC

IHC stained slides were photographed at an overall magnification of 50x and unedited for quantification. Individual images of the LNs were stitched together using Image stitching feature of FIJI imaging software (27). DAB color was separated out from tissue samples using Color Deconvolution feature of FIJI imaging software on the hematoxylin and DAB setting (69). The color threshold was set visually per marker and percent area positive was quantified by pixel using color threshold and measure tools in FIJI imaging software (27). See **Figure 1** for a photographical representation of quantification. The averages for each time point were calculated and compared against the preinfection levels to calculate fold change in expression. For the aggregated restriction factor quantification fold changes were added together.



Individual images of partial lymph nodes were stitched together to created composite Lymph node image. Composite images were cropped to include only lymph tissue. Color Deconvulution tool was used to isolate DAB pigment and Color Threshold tool to select for positive DAB signal to quantify percent positive area measurement.

Figure 1. DAB Quantification Schematic.

4.7 IMMUNOFLUORESCENCE (IF) STAINING

Immunofluorescence staining (IF) was performed on formalin-fixed or 4% paraformaldehyde-fixed, paraffin-embedded tissue samples. Four µm thick sections were deparaffinized in xylene, rehydrated in ethanol gradient (100, 100, 90, 75), and rinsed in dH_20. For antigen retrieval, the sections were microwaved for 23 minutes in Vector Unmasking Solution (Vector Laboratories, Burlingame, CA). Slides were blocked with Dako blocking serum for 30 minutes (Dako, Carpinteria, CA). Primary antibodies were incubated for 1hour, followed

by 30 minute incubation with secondary antibody. All secondary antibodies were diluted to match the concentration of primary antibody. Please see Table 2 for primary antibody information and Table 3 for secondary antibody information. Slides were rinsed in 1XPBS or dH20 in between steps and mounted with Fluorescent mounting medium (Dako, Carpinteria, CA). Sections were photographed under oil immersion at 600x.

Table 2. Primary Antibodies and Dilutions Utilized in IF Staining of HRFs.

Primary Antibody	Type	Dilution	Company	Catalog #
TRIM5α	Polyclonal Goat	1:100	Abcam	ab4389
MX2	Polyclonal Rabbit	1:125	Novus Biologicals	NBP1-81018
APOBEC3g	Polyclonal Rabbit	1:250	Novus Biologicals	NBP1-88592
SAMHD1	Polyclonal Goat	1:50	Santa Cruz Biotechnology	SC-86212
Tetherin	Monoclonal Rabbit	1:50	Abcam	ab134061
CD11c	Mouse IgG2a	1:50	Novacastra	CD11c-563
HAM56	Mouse IgM	1:100	Dako	M 0632
CD3	Rabbit polyclonal	1:50	Dako	M 7254
CD3	Mouse monoclonal	1:50	Dako	A 0452
CD 68	Mouse IgG1	1:50	Dako	M 0814

Table 3. Secondary Antibodies and Dilutions Utilized in IF Staining of HRFs.

Secondary Antibody	Type	Company	Catalog #
Alexafluor Donkey α Goat 633	IgG	Molecular Probes	A-11055
Alexafluor Donkey α Rabbit 488	IgG	Molecular Probes	A-21206
Alexafluor Goat α Mouse 488	IgG1	Molecular Probes	A-21121
Alexafluor Goat α Mouse 488	IgG2a	Molecular Probes	A-21131
Alexafluor Goat α Mouse 633	IgG1	Molecular Probes	A-21126
Alexafluor Goat α Mouse 633	IgG2a	Molecular Probes	A-21136
Alexafluor Goat α Mouse 633	IgM	Molecular Probes	A-21046

4.8 SGA OF SIVAGM *ENV* GENES.

To assess the diversity of virus quasispecies at different times post infection and assess the frequency of G-to-A hypermutation, insertions, or deletions that might illustrate the impact of the HRFs on the controlled virus, we used single-genome amplification (SGA) of viral sequences, as described previously (22, 64). Briefly, viral RNA was extracted from plasma samples with an EZ1 virus minikit (version 2.0; Qiagen, Valencia, CA) and reverse transcribed using primer SIVagmENVoutR (5′ GTACCTGGCCCATCAGTGTAATTCTGC-3′) and SuperScript III reverse transcriptase. The first-strand-synthesis reaction mixture contained 1× reverse transcription buffer, 0.5 mM each deoxynucleoside triphosphate, 5 mM dithiothreitol, 2 units/μl of RNaseOUT reagent, 10 units/μl of SuperScript III reverse transcriptase, and 0.25 μM antisense primer. Dilutions of this cDNA were distributed in replicates of 16 PCRs to determine the dilution at which no more than 30% of reactions yielded amplicons, to ensure that most positive reaction mixtures contained a single template molecule. Full-length *env* genes were amplified by nested PCR using 1st-round sense primer SIVagmENVoutF (5′-CAGGTGCTGTAAGCCCAAGACACATC-3′), 1st-round antisense primer SIVagmENVoutR (5′-GTACCTGGCCCATCAGTGTAATTCTGC-3′), 2nd-round sense primer SIVagmENVinF (5′-GCTATCATTGTCCGCTTTGCTTCACTC-3′), and 2nd-round antisense primer SIVagmENVinR (5′-CTCACTGGGAAGCCAACCTCTTCTTC-3′). PCR was performed using Platinum *Taq* High Fidelity polymerase (Invitrogen, Carlsbad, CA) in the presence of 1× PCR buffer, 2 mM $MgSO_4$, 0.2 mM each deoxynucleoside triphosphate, 0.2 μM each primer, and 0.025 units/μl of polymerase in a 20-μl reaction mixture. PCR conditions were 94°C for 2 min, followed by 35 cycles of 94°C for 15 s, 56°C for 30 s, and 68°C for 5 min (first round) or 45 cycles with a 59°C annealing temperature (second round), followed by a final extension of 10

min at 68°C. Amplicons were inspected using 96-well E-gels (Invitrogen) and directly sequenced.

Highlighter plots were created using Highlighter for Nucleotide Sequences v2.2.3 tool from the LANL HIV sequence database (32). APOBEC3g mutation analysis was done with HYPERMUT tool from the LANL HIV sequence database (68).

4.9 MICROSCOPY AND PHOTOGRAPHY

All photographs were taken on Zeiss AX10 Imager M.1 fluorescent microscope with Axiocam MRc5 camera and Axio Vision SE64 Rel 4.8 software package under brightfield or fluorescent settings. Photos were color enhanced and cropped using Adobe Photoshop Elements 9 software package.

4.10 GRAPHING AND STATISTICAL ANALYSIS

All graphs and statistical analyses were generated using GraphPad Prism version 6 software. In each species, post infection time point values for each HRF were compared with preinfection values using the nonparametric Mann-Whitney test. All statistical values of $P < 0.05$ were considered statistically significant.

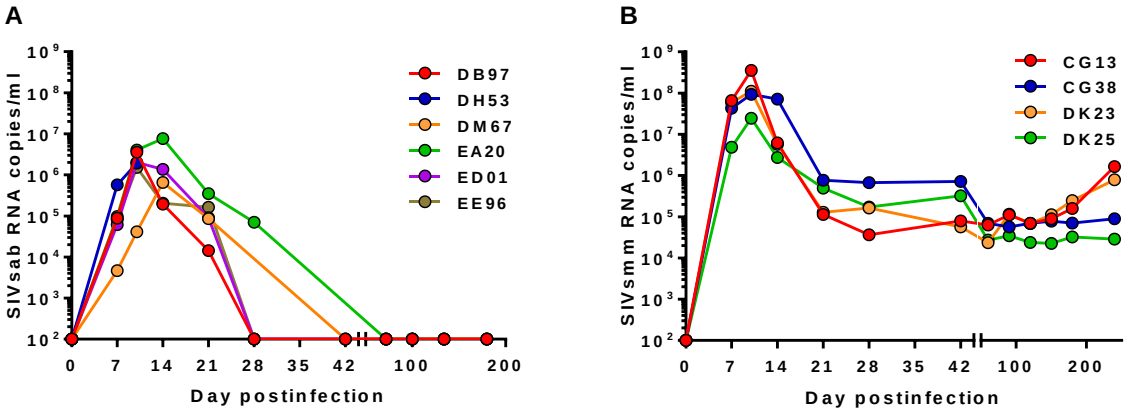
5.0 RESULTS

5.1 AIM 1: COMPARE AND CONTRAST THE NATURAL HISTORY AND THE OUTCOME OF SIVSAB OR SIVSMM INFECTION IN GUINEA BABOONS (*PAPIO PAPIO*).

SIVsab and SIVsmm infection of Guinea baboons. Of the six Guinea baboons IV infected with SIVsab92018, four were followed for more than 6 months post infection (p.i.). The remaining ones died during the follow-up from causes unrelated to SIV infection (complications of the surgery). During the acute infection, two out of six SIVsab infected baboons (DB 97, ED01) showed significant lymphadenopathy. No other clinical sign of acute SIV infection was observed during the follow-up. None of the SIVsab infected baboons had any clinical sign of HIV-associated immune deficiency at the end of the follow-up and all the biological parameters were back at the preinfection levels.

The four Guinea baboons infected with SIVsmm were followed for 8 months pi. During the acute infection they did not show any clinical signs of disease. During the follow-up, two out of four SIVsmm infected baboons (CG13, and DK23) started to lose weight and showed lymphadenopathy, suggestive for disease progression.

5.1.1 Viral Loads



(A) SIVsab controlled infection in baboons. (B) SIVsmm persistent, pathogenic infection in baboons

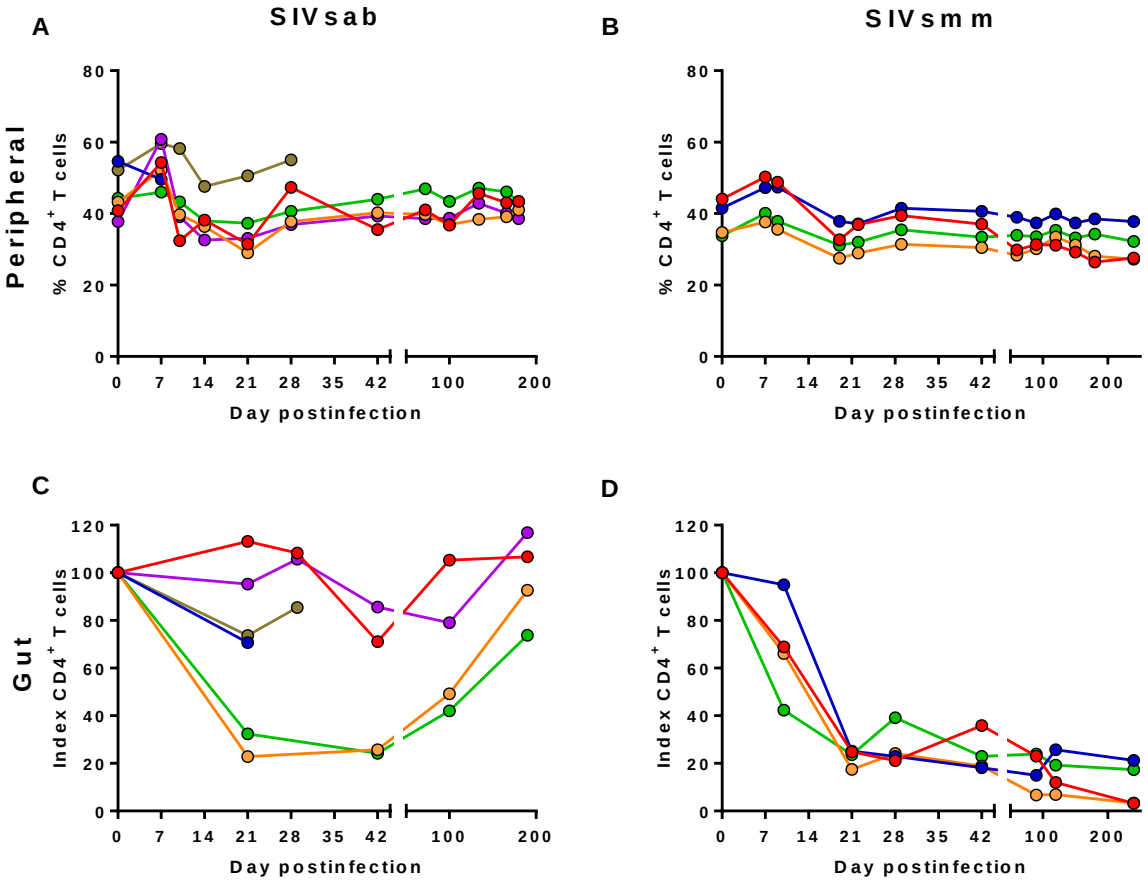
Figure 2. Plasma viral loads in controlled and persistent pathogenic SIV infection.

The viral loads were closely monitored in the two groups of baboons throughout the follow-up. Results are shown in **Figure 2**. In the baboons infected with SIVsab, the viral load peaked at 10^6-10^7 RNA copies/mL **(Figure 2A)**; while in the SIVsmm infected baboons the viral load peaked at 10^9 RNA copies/mL **(Figure 2B)**. The SIVsab infected baboons controlled the virus very rapidly, as early as 28 dpi. Control was maintained throughout the follow-up. In stark contrast, the SIVsmm infected baboons only partially controlled viral replication, reaching a viral set point of 10^5 RNA copies/mL by 42 dpi. The SIVsmm VLs were maintained at these relatively high levels throughout the follow-up, with the exception of the most recent time points when significant increases of the levels of viral replication, suggestive for disease progression, were observed in two (CG13, DK23) of the four SIVsmm infected baboons. These results corroborate the clinical data and are in agreement with the pathogenic, progressive nature of HIV-2 infection in the baboons, as previously reported (44). In the AGMs, the VLs showed the classic pattern of

viral replication in natural hosts, i.e., relatively high peak VLs (10^7-10^8 vRNA copies/ml) followed by partial control at 10^4-10^5 vRNA copies/ml maintained throughout the follow-up (data not shown).

5.1.2 $CD4^+$ T lymphocyte cell counts

$CD4^+$ T cell counts are another important indicator of pathogenicity and disease progression (51). In our study, both peripheral (**Figure 3A,B**) and intestinal (**Figure 3C,D**) $CD4^+$ T cells decreased in SIVsmm infected baboons and remained virtually unchanged in the SIVsab infected baboons. The differences between the two baboon groups were more drastic in the intestine $CD4^+$ T cells, albeit a moderate mucosal $CD4^+$ T cell depletion could be observed in two baboons that presented the highest viral loads. In the remaining baboons, SIVsab infection resulted in a minimal and transient depletion of the mucosal $CD4^+$ T cells , which were rapidly restored during the follow-up (**Figure 3C**). Conversely, in SIVsmm infected baboons, a sharp decline of the mucosal $CD4^+$ T cells occurred by 21 dpi. This $CD4^+$ T cell decline continued during the chronic stage of infection, the levels of $CD4^+$ T cell restoration at the mucosal site being minimal in SIVsmm infected baboons (**Figure 3D**). A severe decline in $CD4^+$ T cells is an indicator of progression to AIDS (16).



(A,B) Peripheral $CD4^+$ T cells in baboons infected with SIVsab or SIVsmm, respectively. (C,D) Mucosal $CD4^+$ T cell in baboons infected with SIVsab or SIVsmm, respectively. Index level is defined as a comparison of percent $CD4^+$ T cell population in comparison to baseline percent $CD4^+$ T cell population.

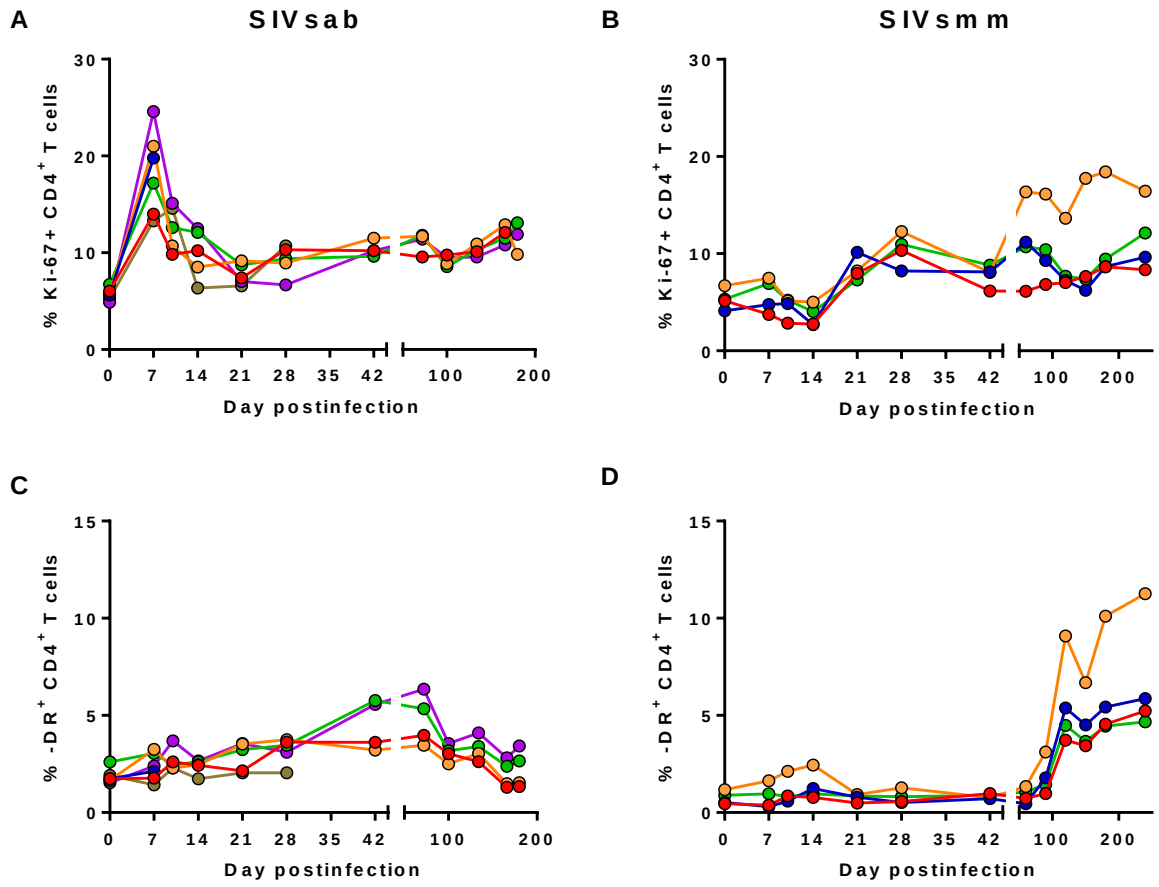
Figure 3. Changes in the $CD4^+$ T cell levels in SIVsab and SIVsmm infected baboons.

5.1.3 $CD4^+$ T Lymphocyte immune activation

Chronic immune activation is the best indicator of HIV/SIV disease progression, being more closely associated with progression than the levels of viral replication or the $CD4^+$ T cell depletion (58). Therefore, we next assessed the levels of immune activation and T cell proliferation between the two groups of baboons. We employed two well-studied markers for

immune activation Ki-67, a cell proliferation marker, and HLA-DR, a MHC-II marker which is used to assess the levels of T cell immune activation. In the controller baboons, Ki-67 transiently increased during acute infection, but rapidly returned to preinfection levels throughout the follow-up **(Figure 4A)**. Unlike the controller baboons, the progressor baboon Ki-67 levels progressively increased throughout chronic infection **(Figure 4B)**.

HLA-DR increased transiently during acute SIVsab infection in the controller baboons, but returned to preinfection levels by late chronic infection **(Figure 4C)**. Conversely, in the progressor baboons, HLA-DR remained close to preinfection levels during the early stages of infection and only showed a sharp increase during late chronic infection **(Figure 4D)**. Overall, immune activation levels returned to preinfection levels in the controller baboons, but progressively increased with disease progression in SIVsmm infected baboons.



(A,B) Expression of KI-67 activation marker by peripheral CD4+ T cells in baboons infected with SIVsab or SIVsmm, respectively. (C,D) Expression of HLA-DR activation marker by peripheral CD4+ T cells in baboons infected with SIVsab or SIVsmm, respectively

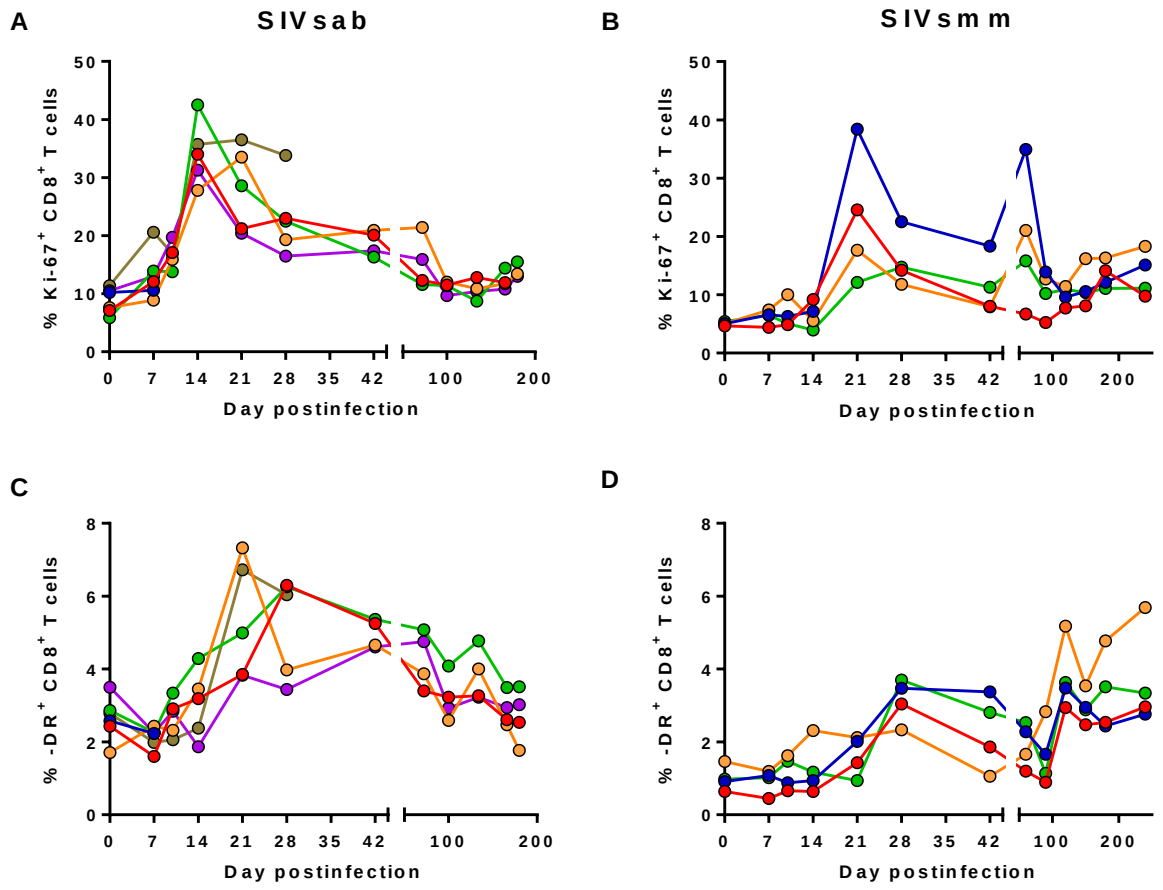
Figure 4. Comparative dynamics of CD4+ T cell immune activation in controller (SIVsab infected) and progressor (SIVsmm infected) baboons.

5.1.4 CD8$^+$ T Lymphocyte immune activation

CD8$^+$ T cell immune activation patterns were similar to those of CD4$^+$ T cells, with a transient increase in the controller baboons and persistent and progressive increases in the progressors. (**Figure 5)**

HLA-DR expression on CD8+ T cells peaks around day 21 post infection in controller baboons, as seen in **Figure 5C**, remains increased through early chronic infection, but returns to

preinfection levels by late chronic infection. In progressor baboons, HLA-DR expression sharply increases by 28, declines to nearly preinfection levels through early chronic infection, then sharply increases during late chronic infection **(Figure 5D)**.



(A,B) Expression of KI-67 activation marker by peripheral CD8+ T cells in baboons infected with SIVsab or SIVsmm, respectively. (C,D) Expression of HLA-DR activation marker by peripheral CD8+ T cells in baboons infected with SIVsab or SIVsmm, respectively.

Figure 5. Comparative dynamics of CD4+ T cell immune activation in controller (SIVsab infected) and progressor (SIVsmm infected) baboons.

5.1.5 SA1 Results Summary

Based on the infection outcome (progressive vs. controlled) and on the major difference in key biological parameters of SIV infection (viral load, CD4+ T cell counts, and immune activation), SIVsab and SIVsmm have completely different natural histories in a single NHP species, the Guinea baboon. Therefore, our study successfully developed an ideal in vivo environment to model the impact of HRFs on cross-species transmitted SIV infections. SIVsab-infected baboons controlled the virus rapidly, preventing/restoring CD4+ cell depletion and successfully limited T cell activation. Consequently, SIVsab infected baboons permit us to monitor the contribution of HRFs to the control of SIV infection upon cross-species transmission. Conversely, SIVsmm induced persistent, progressive infection in baboons, with high chronic set-point levels of viral replication and increase in VLs, persistent $CD4^+$ T cell depletion, and chronic immune activation. SIVsmm infected baboons can be employed to decipher the requirements for a successful cross-species transmission. While many factors may play a role in these differences, we assessed the role of HRFs in driving these different outcomes of SIV infection by focusing on APOBEC3g, MX2, SAMHD1, Tetherin, and TRIM5α in these cross-species transmission models.

5.2 TO DIRECTLY ASSESS THE CHANGES IN HOST RESTRICTION FACTORS THAT MAY CORRELATE WITH VIRUS CONTROL UPON CROSS-SPECIES TRANSMISSION.

To investigate the correlates of virus control/persistence upon cross-species transmission, we next turn our attention to the dynamics of expression of known HRFs in baboons infected with either SIVsab or SIVsmm. At the time when this study was initiated, four such factors were known and we optimized IHC methods for each of them. During the study, MX-2 war further identified as a restriction factor and we optimized a new assay to monitor its expression during controlled and progressive cross-species transmitted infections. HERC5 was reported in May 2014 and we have yet to develop a quantification system for this additional HRF.

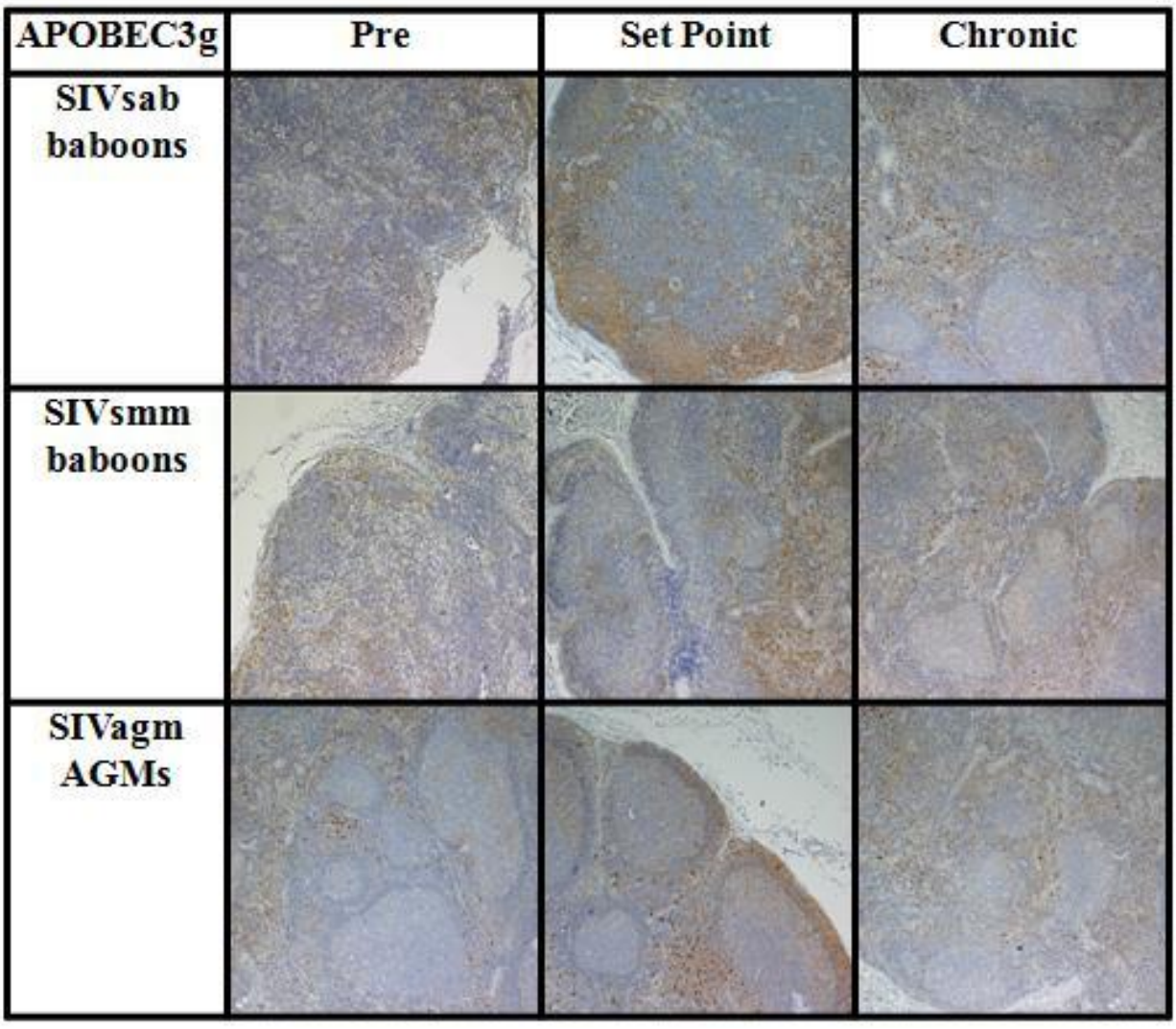
5.2.1 APOBEC3g

The IHC assessment of APOBEC3g expression dynamics over the course of infection indicated an increase in APOBEC3g expression in SIVsab infected baboons that is coicidental with viral control **(Figure 6)**. The progressor baboons infected with SIVsmm and AGM natural hosts had relatively consistent expression of APOBEC3g over the course of infection. Expression of APOBEC3g was generally located in the LN paracortex in the areas directly surrounding the lymphoid follicles, as well as the cortical sinuses.

Quantification of the DAB signal (**Figure 7)**, confirmed that APOBEC3g expression peaked at the point of viral control (29 dpi) at four-fold higher than baseline expression. APOBEC3g expression remained elevated three-fold above the baseline into chronic infection in

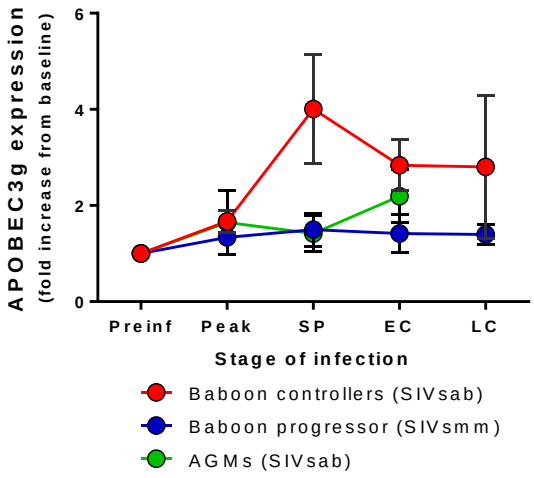
the controller baboons. The progressor baboons and natural hosts had a slight increase from preinfection levels.

Immunofluoresence staining showed that the cell types responsible for the increased APOBEC3g expression are innate and adaptive immune cells (as shown by the colocalization of APOBEC3g marker with expressing CD11c, HAM56, and CD3) **(Figure 8)**.



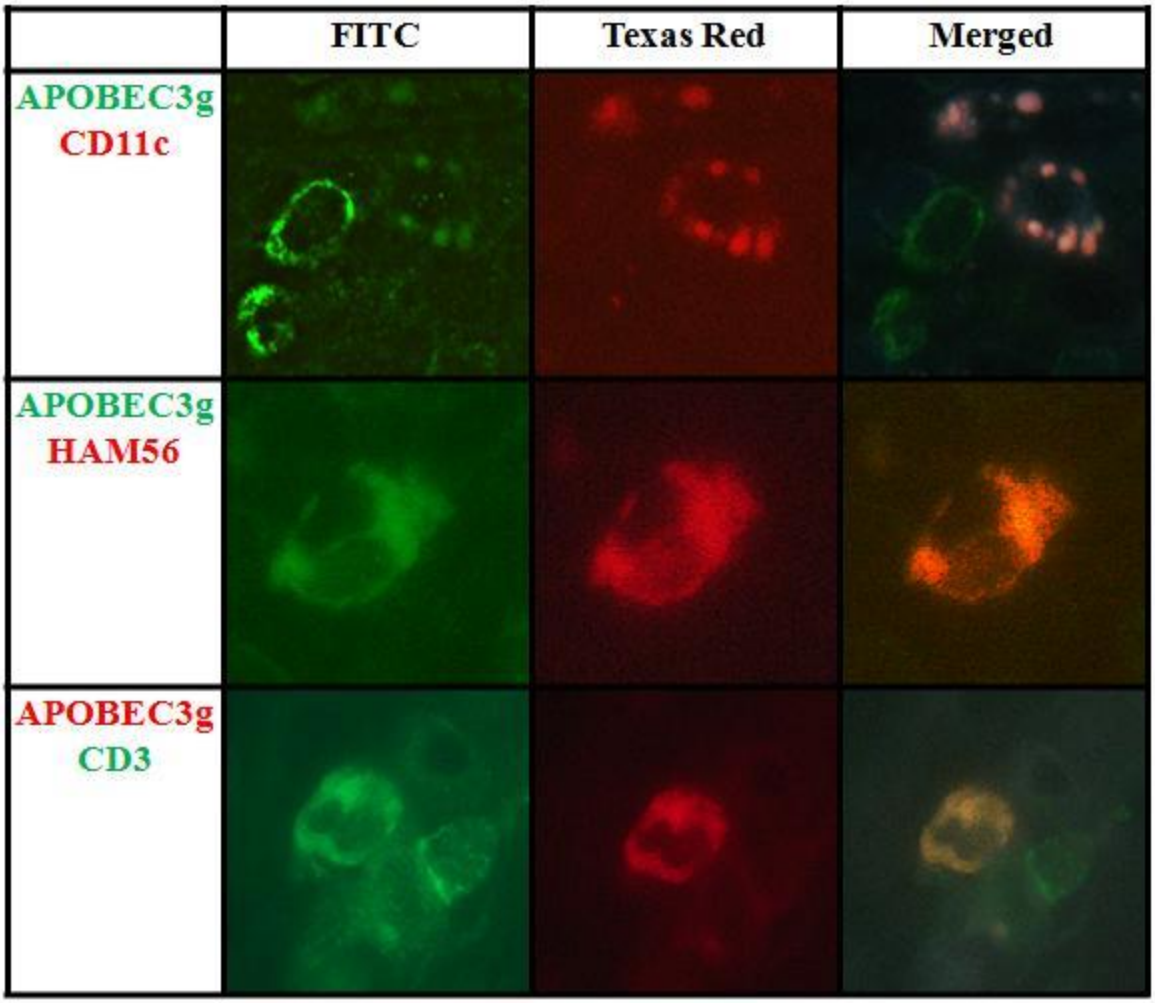
Representative images of immunohistochemistry staining in lymph nodes from preinfection (D0), set point (D29), and chronic infection (D50+). All images at 50x magnification.

Figure 6. Dynamics of APOBEC3g expression in baboons infected with SIVsab, SIVsmm, and in AGMs infected with SIVsab.



Graphical representation of whole lymph node quantification of APOBEC3g expression throughout SIV infection of baboons and AGMs. The average area percent positive were converted to fold change from preinfection levels at peak, set point (SP), early chronic (EC) and late chronic (LC) in controller baboons, progressor baboons, and AGMs. Data points are means from baboons infected with SIVsab (n=6), SIVsmm (n=4), and AGMS infected with SIVsab (n=16)

Figure 7. APOBEC3g levels increase four fold in controller baboons coincidental with viral control.



Combination staining of APOBEC3g (green), CD11c/HAM56 (red), and merged image of double stain. Images photographed at 600x.

Figure 8. Immunofluorescence images of lymph nodes in SIVsab baboon at peak of HRF expression.

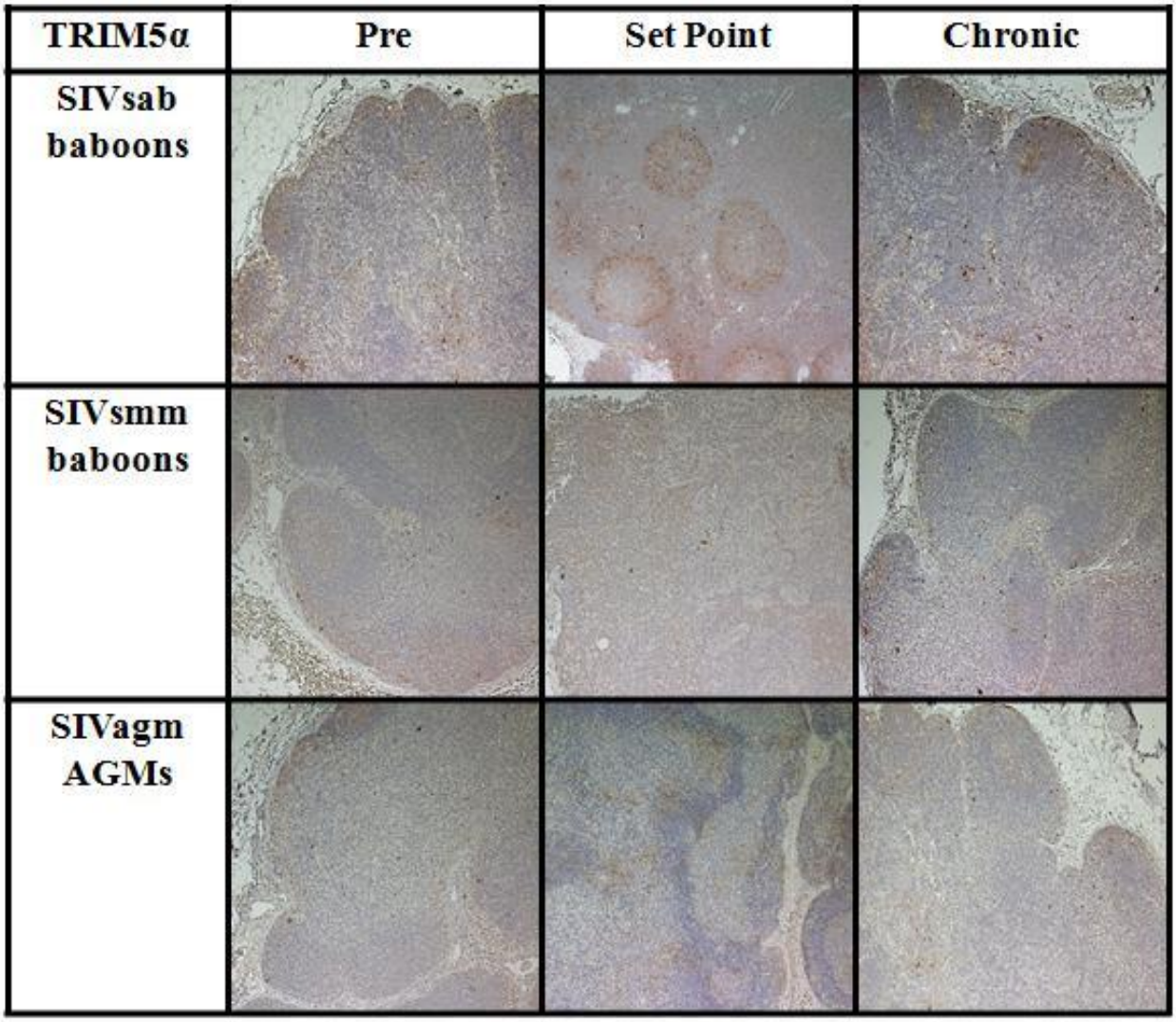
5.2.2 TRIM5α

The IHC assessment indicated an increase in TRIM5α expression which occurred at the timepoint of viral control in SIVsab infected baboons **(Figure 9)**. Conversely, the progressor baboons infected with SIVsmm appeared to express relatively constant levels of TRIM5α throughout infection, similar to the AGM natural host **(Figure 9)**. At the peak of expression,

TRIM5α was generally located in the LN paracortex, but also in the mantle zone of the lymphoid follicles in the controller baboons

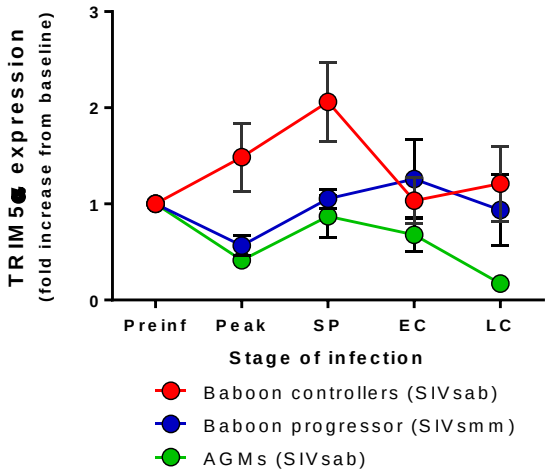
Quantification of the DAB signal (**Figure 10)**, confirmed the TRIM5α expression peak coincidental with viral control (29 dpi) at levels two fold higher than the preinfection expression levels in controller baboons. During chronic infection, TRIM5α expression returned to preinfection levels. Signal quantification also demonstrated that, at the peak of viral replication, TRIM5α expression was lower than the baseline preinfection levels in both the progressor baboons and the AGM natural host and returned to preinfection levels during chronic infection in progressor babons. TRIM5α expression in AGMs remained decreased below preinfection levels during chronic infection

Immunofluoresence staining showed that the cell types responsible for the increased TRIM5α expression are both innate and adaptive immune cells (as shown by the colocalization of TRIM5α marker with expressing CD11c, HAM56 and CD3) **(Figure 11)**.



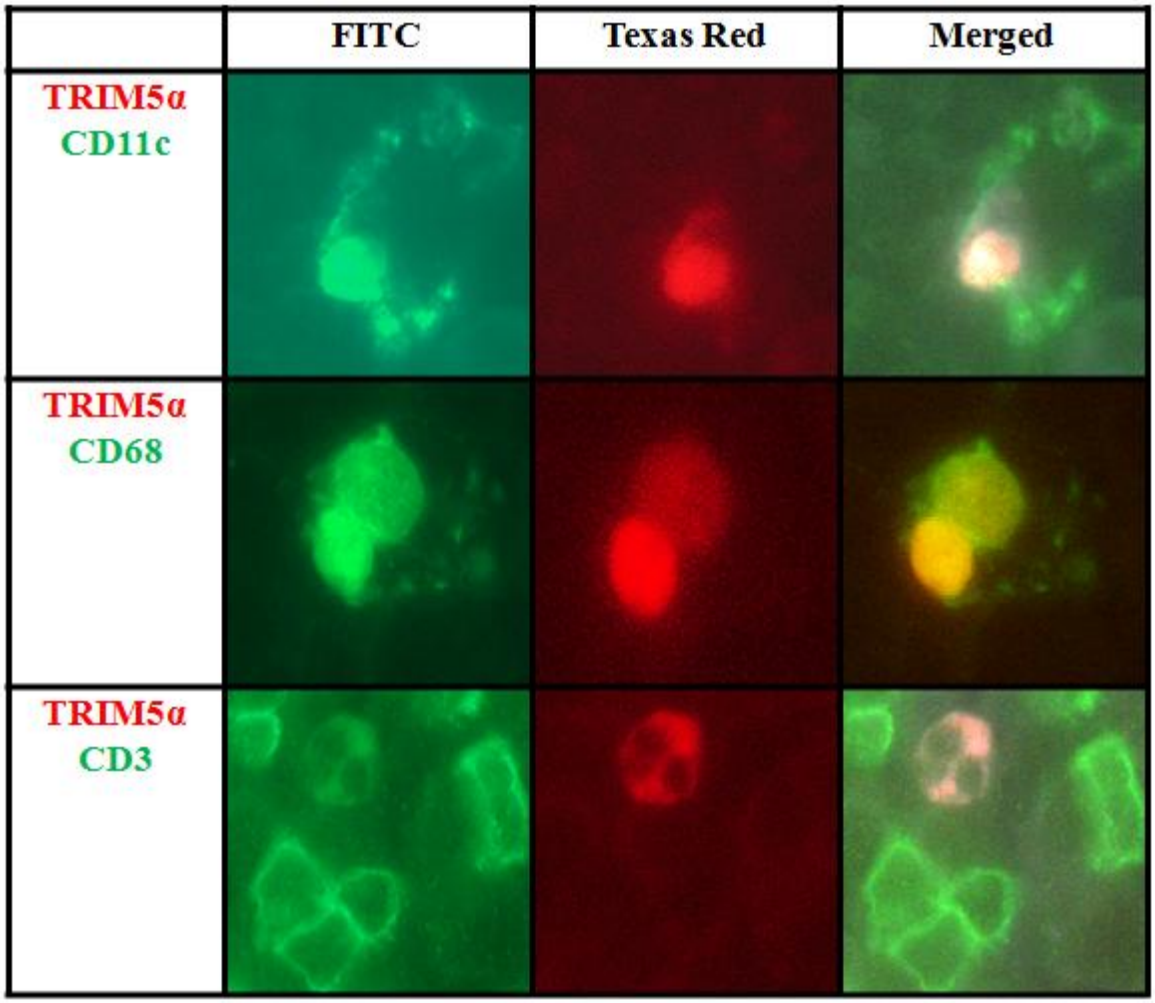
Representative images of immunohistochemistry staining in lymph nodes from preinfection (D0), set point (D29), and chronic infection (D50+). All images at 50x magnification.

Figure 9. Dynamics of TRIM5α expression in baboons infected with SIVsab, SIVsmm, and in AGMs infected with SIVsab.



Graphical representation of whole lymph node quantification of TRIM5α expression throughout SIV infection of baboons and AGMs. The average area percent positive were converted to fold change from preinfection levels at peak, set point (SP), early chronic (EC) and late chronic (LC) in controller baboons, progressor baboons, and AGMs. Data points are means from baboons infected with SIVsab (n=6), SIVsmm (n=4), and AGMS infected with SIVsab (n=16)

Figure 10. TRIM5α levels increase two fold in controller baboons at point of viral control.



Combination staining of CD11c/CD68/CD3 (green), TRIM5α (red), and merged image of double stain. Images photographed at 600x.

Figure 11. Immunofluorescence images of lymph nodes in SIVsab baboon at peak of HRF expression.

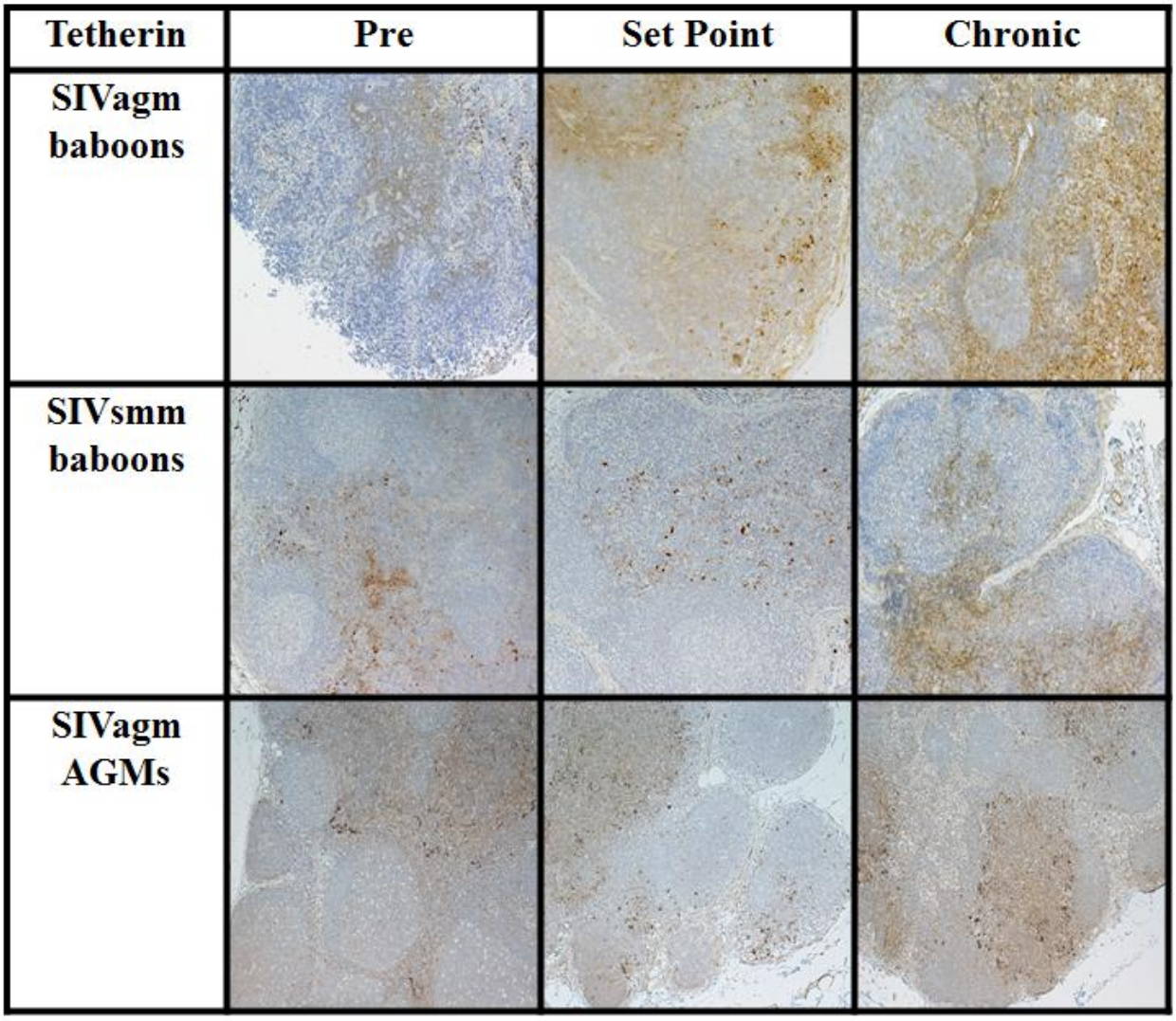
5.2.3 Tetherin

The IHC assessment identified a dramatic increase in tetherin expression coincidental with viral control in SIVsab infected baboons. Tetherin expression then remained increased throughout the follow-up **(Figure 12)**. Conversely, the progressor baboons infected with SIVsmm appeared to express relatively constant levels of tetherin throughout infection, similar

to the AGM natural host **(Figure 12)**. Tetherin was generally expressed in the LN paracortex and the cortical sinuses.

Quantification of the DAB signal (**Figure 13)**, confirmed the tetherin expression peak coincidental with viral control (29 dpi) at levels six fold higher than the preinfection levels. This increased expression persisted throughout the chronic infection in the controller baboons. In the progressor baboons, tetherin expression was double the baseline preinfection levels at the viral setpoint and remained slightly elevated during chronic infection. Finally, in the SIVagm infected AGMs, tetherin expression was fairly constant throughout infection.

Immunofluoresence staining showed that the cell types responsible for the increased tetherin expression are innate and adpative immune cells (as shown by the colocalization of tetherin marker with expressing CD11c, HAM56, and CD3) **(Figure 14)**.



Representative images of immunohistochemistry staining in lymph nodes from preinfection (D0), set point (D29), and chronic infection (D50+). All images at 50x magnification.

Figure 12. Dynamics of Tetherin expression in baboons infected with SIVsab, SIVsmm, and in AGMs infected with SIVsab.

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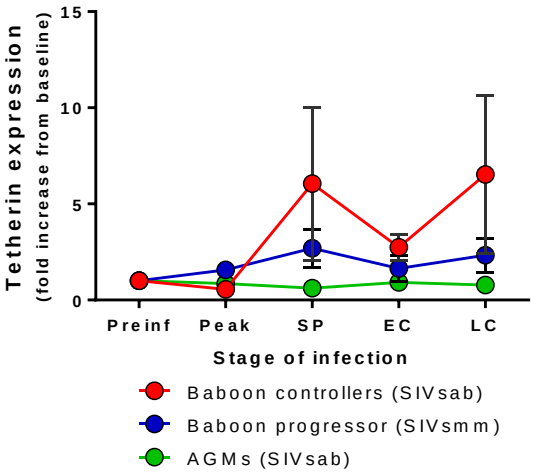
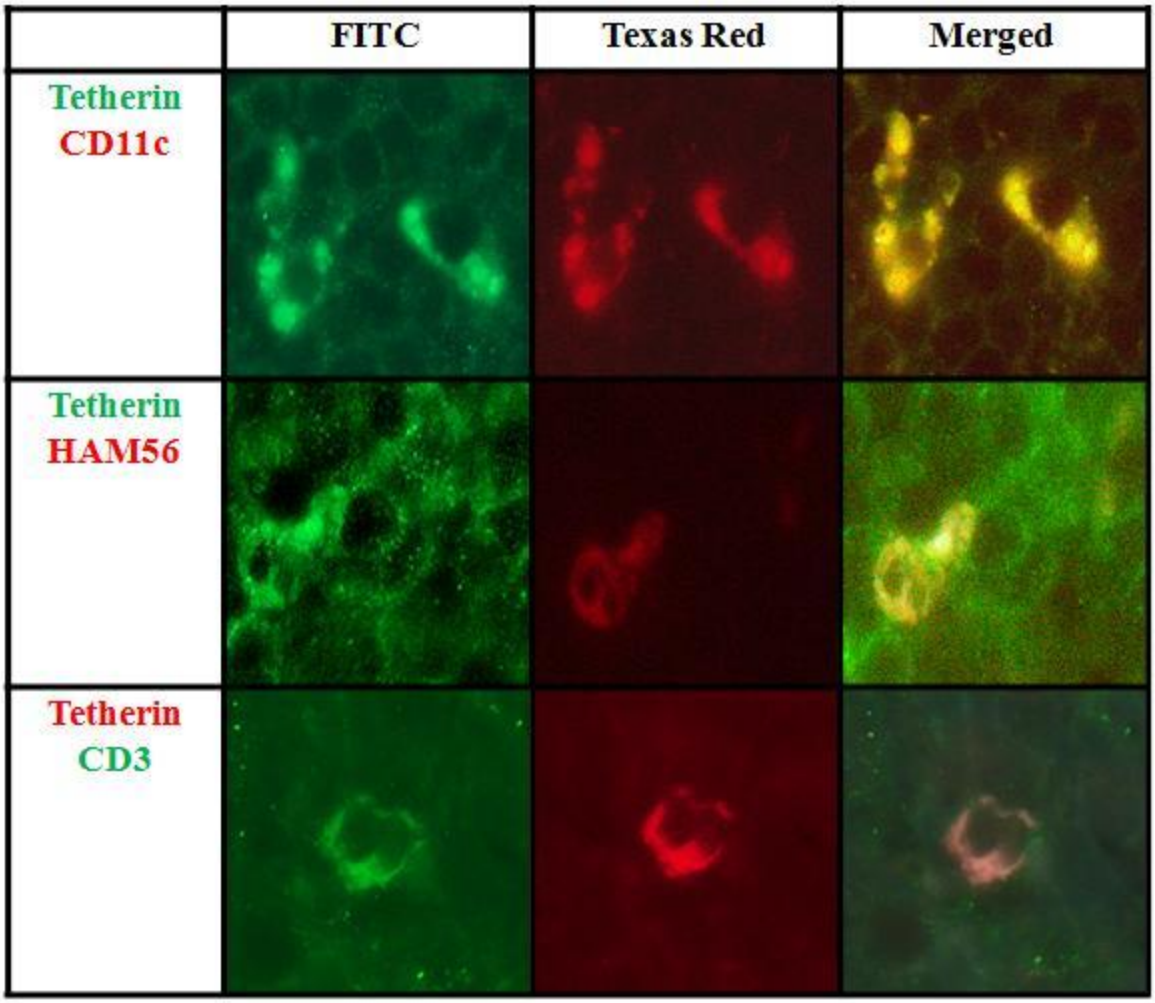


Graphical representation of whole lymph node quantification of tetherin expression throughout SIV infection of baboons and AGMs. The average area percent positive were converted to fold change from preinfection levels at peak, set point (SP), early chronic (EC) and late chronic (LC) in controller baboons, progressor baboons, and AGMs. Data points are means from baboons infected with SIVsab (n=6), SIVsmm (n=4), and AGMS infected with SIVsab (n=16)

Figure 13. Tetherin levels increase seven fold in controller baboons at point of viral control and in late chronic infection.



Combination staining of Tetherin (green), CD11c/HAM56 (red), and merged image of double stain. Images photographed at 600x.

Figure 14. Immunofluorescence images of lymph nodes in SIVsab baboon at peak of HRF expression.

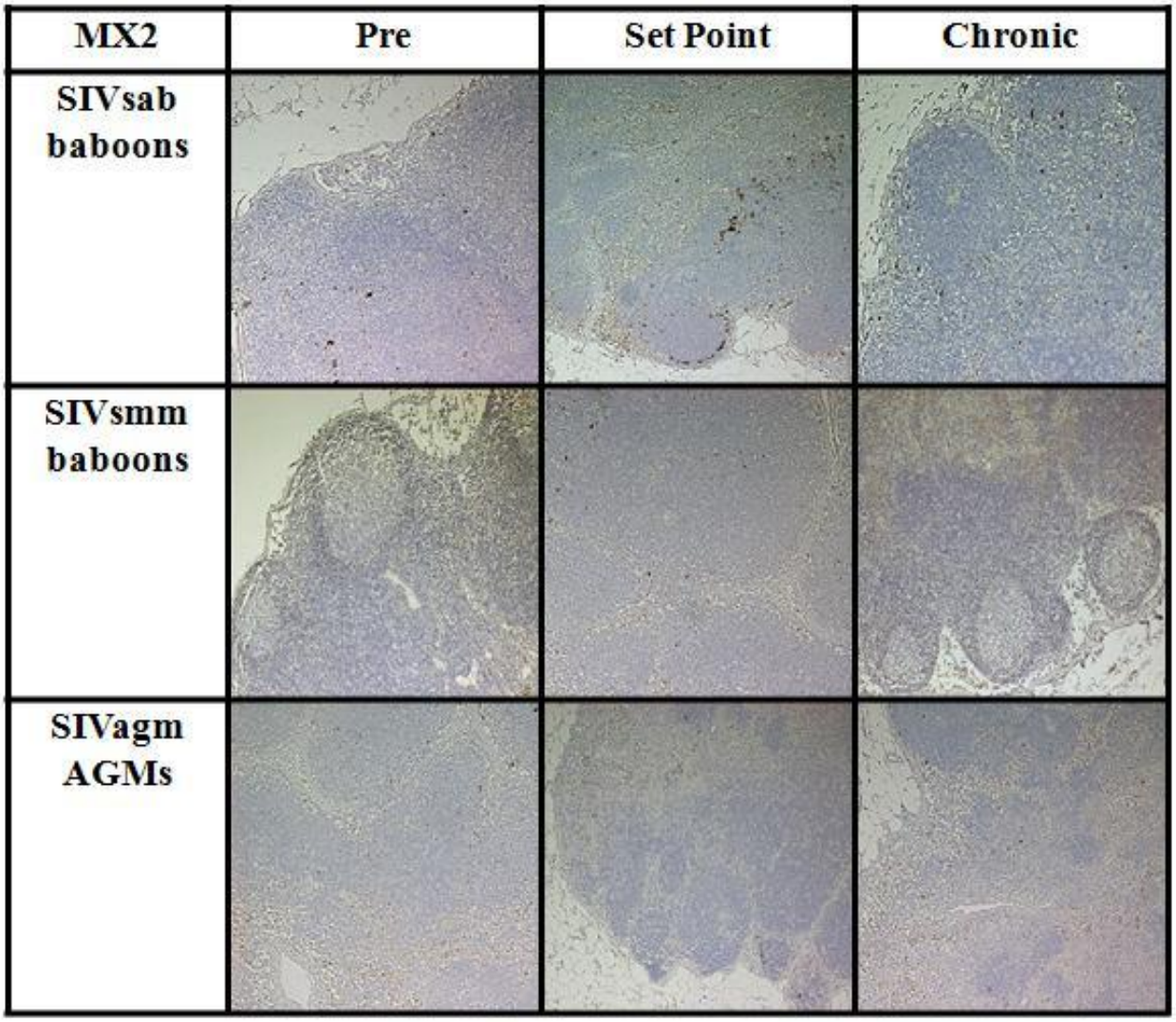
5.2.4 MX2

The IHC assessment of MX2 expression dynamics over the course of infection identified an increased MX2 expression in the controller baboons **(Figure 15)**. Conversely, the progressor baboons infected with SIVsmm appeared to express relatively constant levels of MX2 throughout infection, similar to the AGM natural host **(Figure 15)**. MX2 was generally

expressed in the LN paracortex and cortical sinuses. At the peak of MX2 expression, positive signals were also found in the mantle zone of lymphoid follicles.

Quantification of the DAB signal (**Figure 16)**, showed that MX2 expression peaked at seven fold increase compared to baseline preinfection levels coincidental with the peak of viral replication (10 dpi) and remained increased (3-fold) at the time of viral control (29 dpi). In both progressor baboons and AGMs expression of MX2 remained virtually unchanged from preinfection levels throughout infection.

Immunofluoresence staining showed that the cell types responsible for the increased MX2 expression are innate and adaptive immune cells (as shown by the colocalization of MX2 marker with expressing CD11c, HAM56, and CD3) **(Figure 17)**.



Representative images of immunohistochemistry staining in lymph nodes from preinfection (D0), set point (D29), and chronic infection (D50+). All images at 50x magnification.

Figure 15. Dynamics of MX2 expression in baboons infected with SIVsab, SIVsmm, and in AGMs infected with SIVsab.

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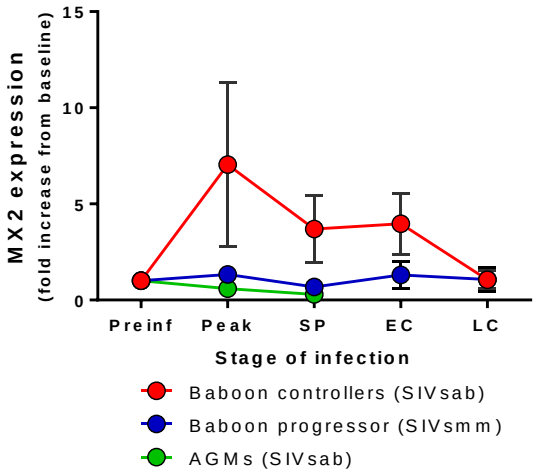
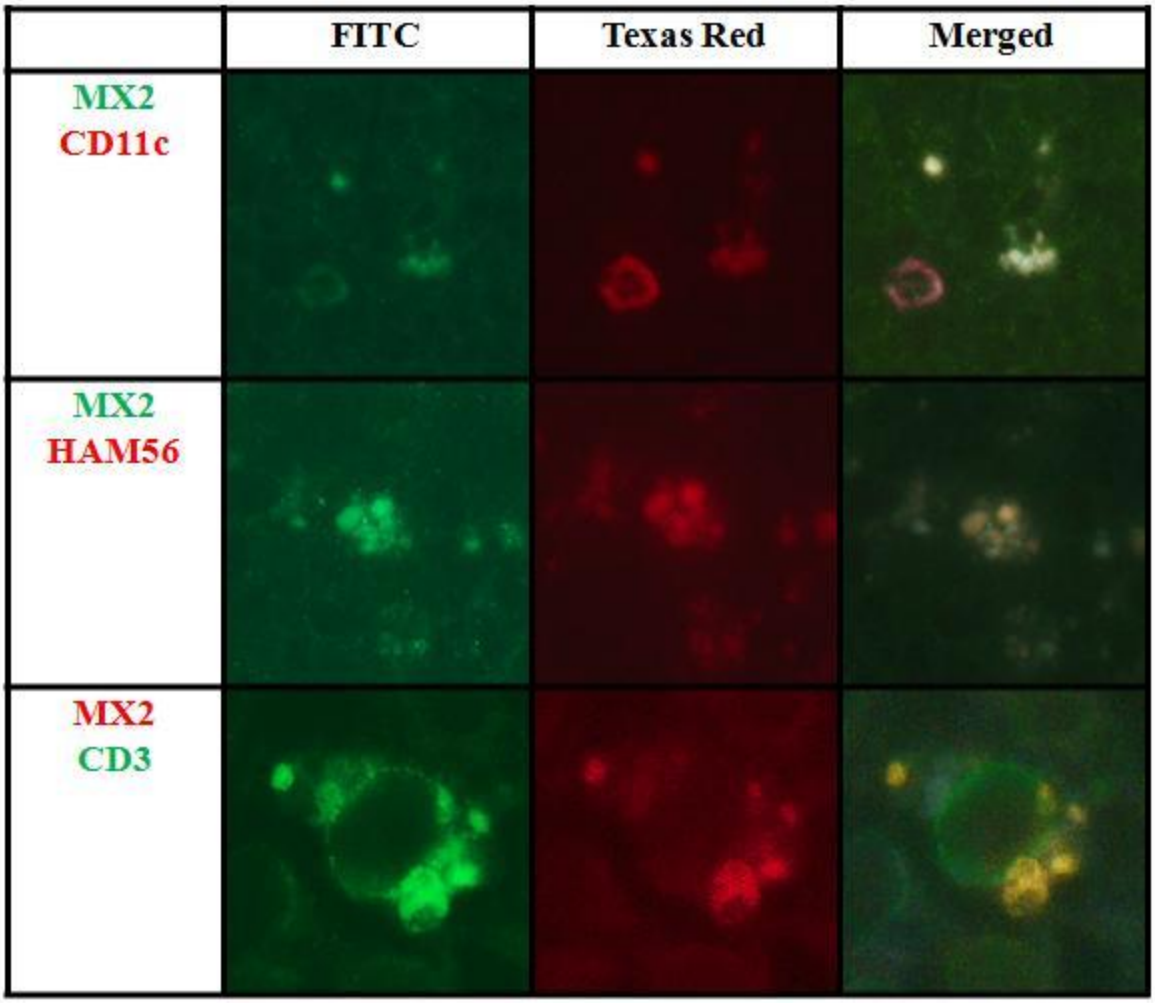


Graphical representation of whole lymph node quantification of MX2 expression throughout SIV infection of baboons and AGMs. The average area percent positive were converted to fold change from preinfection levels at peak, set point (SP), early chronic (EC) and late chronic (LC) in controller baboons, progressor baboons, and AGMs. Data points are means from baboons infected with SIVsab (n=6), SIVsmm (n=4), and AGMS infected with SIVsab (n=16)

Figure 16. MX2 levels increase seven fold in controller baboons at peak of viral replication.



Combination staining of MX2 (green), CD11c/HAM56 (red), and merged image of double stain. Images photographed at 600x.

Figure 17. Immunofluorescence images of lymph nodes in SIVsab baboon at peak of HRF expression.

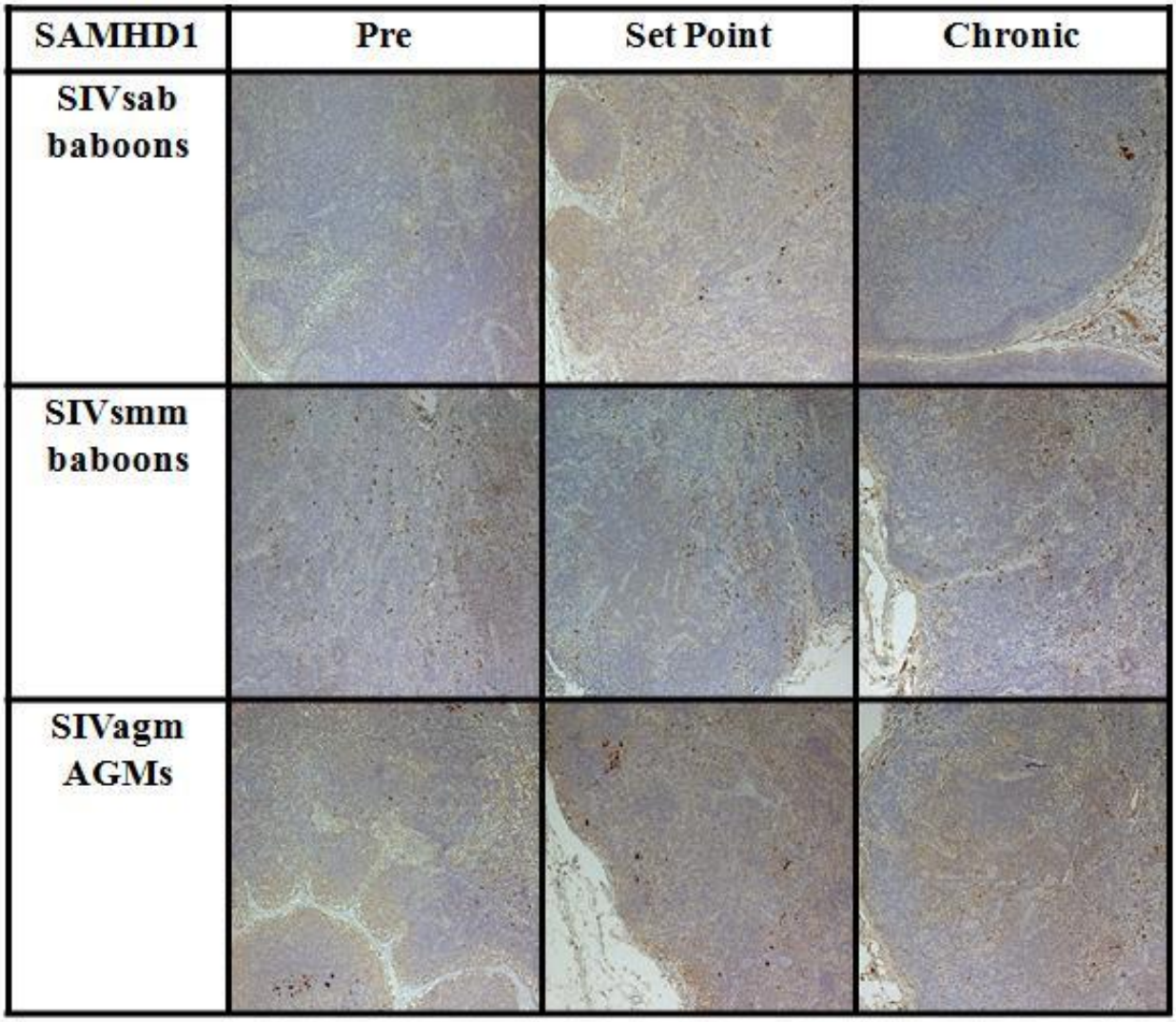
5.2.5 SAMHD1

Differently from all the other HRCs, the IHC SAMHD1 expression remained virtually unchanged during SIVsab infection in the controller baboons **(Figure 18).** Furthermore, SAMHD-1 expression also remained virtually unchanged in the progressor baboons infected with SIVsmm and in the AGM natural host of SIVsab, with only slight increases being observed

in progressor baboons at the late chronic time points. SAMHD1 was generally expressed in the LN paracortex and in the cortical sinuses.

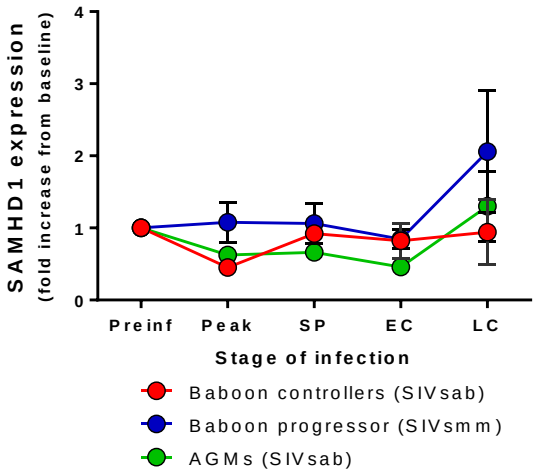
Quantification of the DAB signal (**Figure 19**) confirmed the IHC observations and showed only a 1.5-2-fold increases in SAMHD-1 expression in the late chronic time points in progressor baboons. An explanation for this the lack of increase in SAMHD-1 expression in controller baboons is that SIVsab does not contain a *vpx* gene, which is the known antagonist of SAMHD-1. Interestingly, progressor baboons, which were infected with SIVsmm, the prototype virus containing a vpx gene exhibited a higher expression of SAMHD-1 than controllers.

Immunofluoresence staining showed that the cell types responsible for the increased SAMHD-1 expression are both innate and adaptive immune cells (as shown by the colocalization of SAMHD-1 marker with expressing CD11c, HAM56 and CD3) **(Figure 20)**.



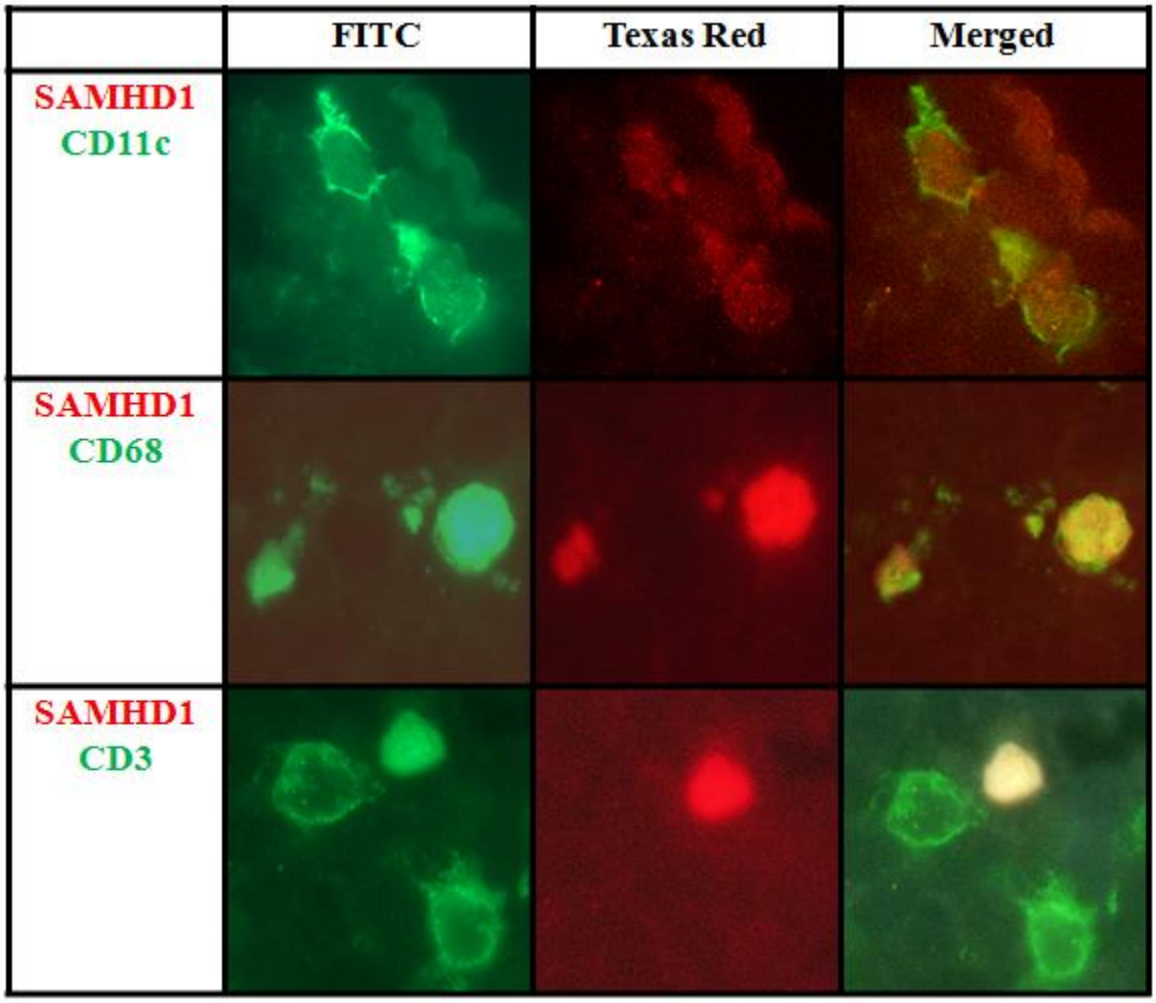
Representative images of immunohistochemistry staining in lymph nodes from preinfection (D0), set point (D29), and chronic infection (D50+). All images at 50x magnification.

Figure 18. Dynamics of SAMHD1 expression in baboons infected with SIVsab, SIVsmm, and in AGMs infected with SIVsab.



Graphical representation of whole lymph node quantification of SAMHD1 expression throughout SIV infection of baboons and AGMs. The average area percent positive were converted to fold change from preinfection levels at peak, set point (SP), early chronic (EC) and late chronic (LC) in controller baboons, progressor baboons, and AGMs. Data points are means from baboons infected with SIVsab (n=6), SIVsmm (n=4), and AGMS infected with SIVsab (n=16)

Figure 19. SAMHD1 levels increase two fold in progressor baboons in late chronic infection.



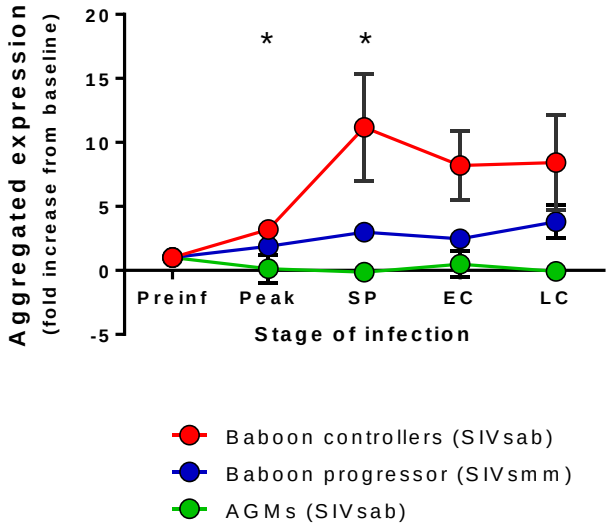
Combination staining of CD11c/HAM56/CD3 (green), SAMHD1 (red), and merged image of double stain. Images photographed at 600x

Figure 20. Immunofluorescence images of lymph nodes in SIVsab baboon at peak of HRF expression.

5.2.6 Aggregation of Five markers

Our results identified a clear trend for increased expression of HRFs in controlled infection and relatively constant expression throughout the progressive infection. However, none of these increases reached statistical significance, due to either a high variability between animals and to the relatively small number of animals included in this study. While each of this

factors could impact the virus at different time points of the virus cycle, we reasoned that the overall expression of the HRFs is illustrative for their combined action of these factors rather than the individual values. Therefore, in order to conclude the importance of these factors on the control of infection, we aggregated their impact by adding the quantification results for each of the individual factors. Results are shown in **Figure 21**, and clearly demonstrate that the increase in HRF expression is significant at the time points preceding and coinciding with viral control ($p<0.005$). Conversely, in either baboons infected with SIVsmm or AGMs infected with SIVsab, aggregation of the results did not identify significant increases in the HRC expression.



Sum aggregation of fold expression changes of five HRFs tested previously. Statistical significance is based on non-parametric Mann-Whitney test with significance of $P<0.005$.

Figure 21. Aggregated fold increase (sum) expression of host restriction factors significantly increases at point of viral control in controller baboons.

5.2.7 SA2 results summary

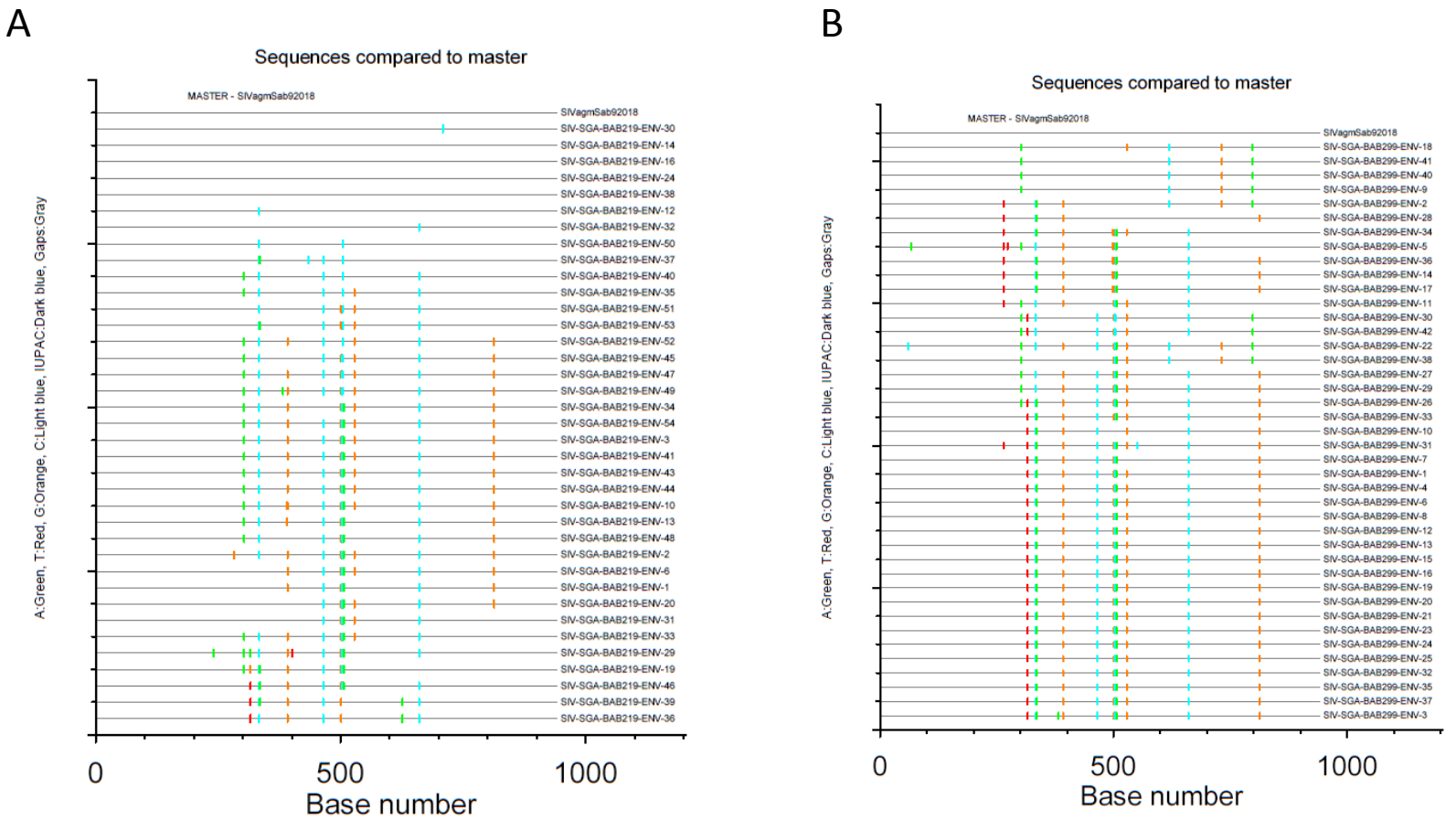
Four of five HRFs tested had increased expression in the LNs of the controller baboons coincidental with viral control as demonstrated visually by IHC and quantitatively by whole LN quantification. No significant increase in HRF expression occurred in the progressor baboons or in the natural AGM host. Additionally, these HRFs were found to be expressed in both innate and adaptive immune cell populations, all target cells of SIV. These differences in expression demonstrate *in vivo* that HRFs may play a critical role in the success or failure of a cross species transmission event. We therefore next focused on measuring the impact of intrinsic immunity on the virus to identify one of the mechanisms through which a cross-species transmitted infection can be controlled.

5.3 AIM 3: TO ASSESS THE HRF IMPACT ON THE VIRUS IN CONTROLLED AND PROGRESSIVE INFECTIONS OF BABOONS.

5.3.1 SGA analysis of *env* sequences from controller baboons

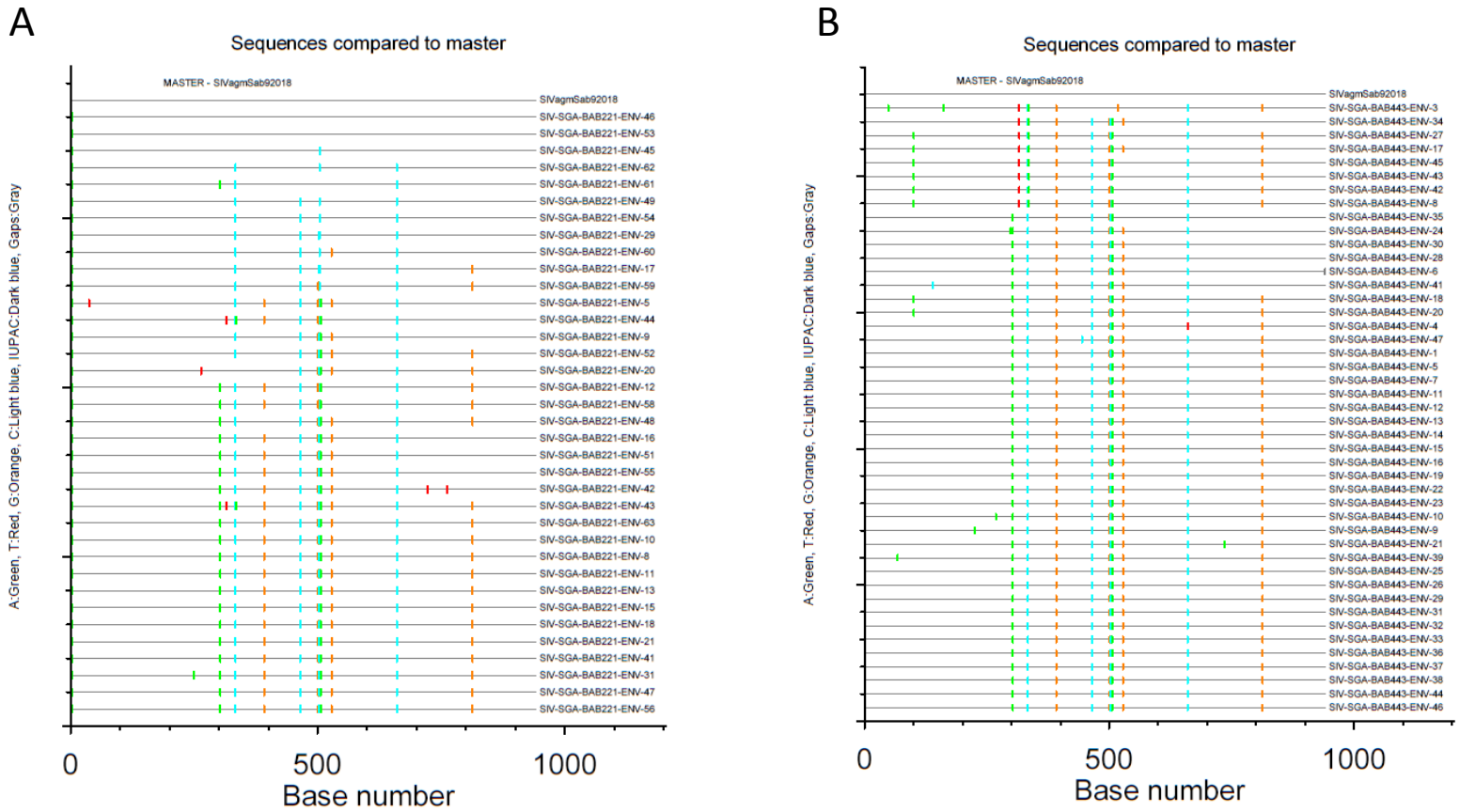
Plasma samples collected at the peak of viral replication and at the last time point where a detectable viral load was observed were subjected to SGA and direct sequencing in an attempt to identify the imprint of intrinsic immunity on virus evolution. This approach had two major shortcomings: first, we could only select very early time points (before 29 dpi) that showed detectable viral loads. Second, the virus that we amplified was the one that was replication competent and, probably the least impacted by the restriction factors. Indeed, comparative

analyses of the viral diversity in three controller baboons (DB97, EA20, ED01), at the peak of viral load (10 dpi) and at the time of viral control (29 dpi) failed to identify a major impact of APOBEC3g on the virus **(Figures 22-24)**, the rates of mutation being similar to those observed in the natural host of SIVsab, the AGM (data not shown). Therefore, in a second attempt to document the impact of intrinsic immunity on virus evolution we performed SGA on the DNA from the PBMCs collected at the peak (10 dpi), set-point (42 dpi) and during the chronic infection (90 dpi) to document accumulation of defective viruses as a result of host restriction. Preliminary evidence points to a direct impact of APOBEC3g on viral control as hypermutation rates increase in the PBMC derived *env* from controller baboons DB97 from peak infection to the time of viral control as seen in **Figure 25.** More sample analysis is in progress.



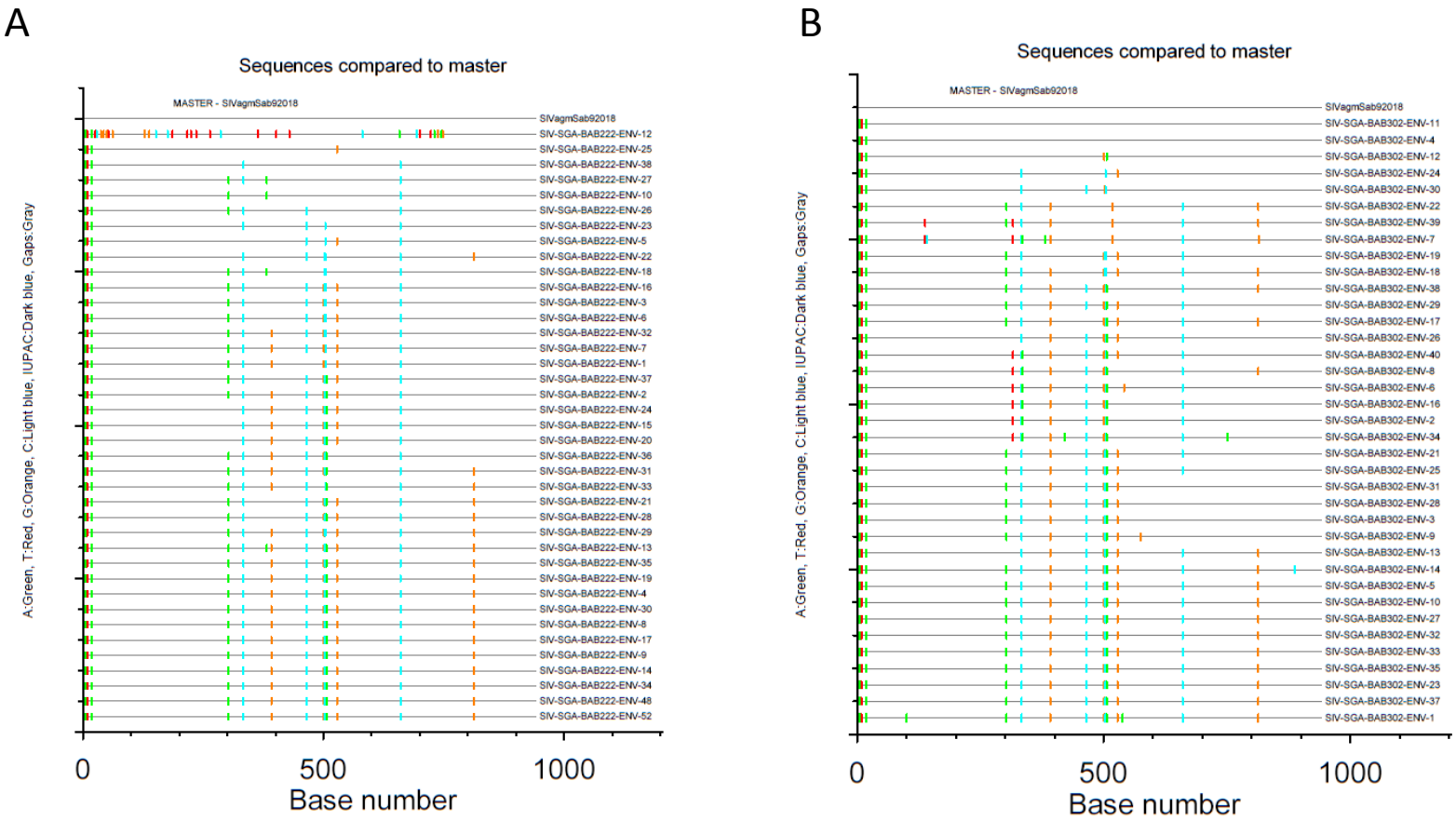
(A) peak of viral replication (D10) and (B) viral control (D22). Highlighter tick colors correspond with the nucleotide as listed A:Green, T:Red, G:Orange, C:Light blue, IUPAC:Dark blue, Gaps:Gray.

Figure 22. Highlighter Plot assessing the sequences of plasma SIV *Env* in controller baboon DB97.



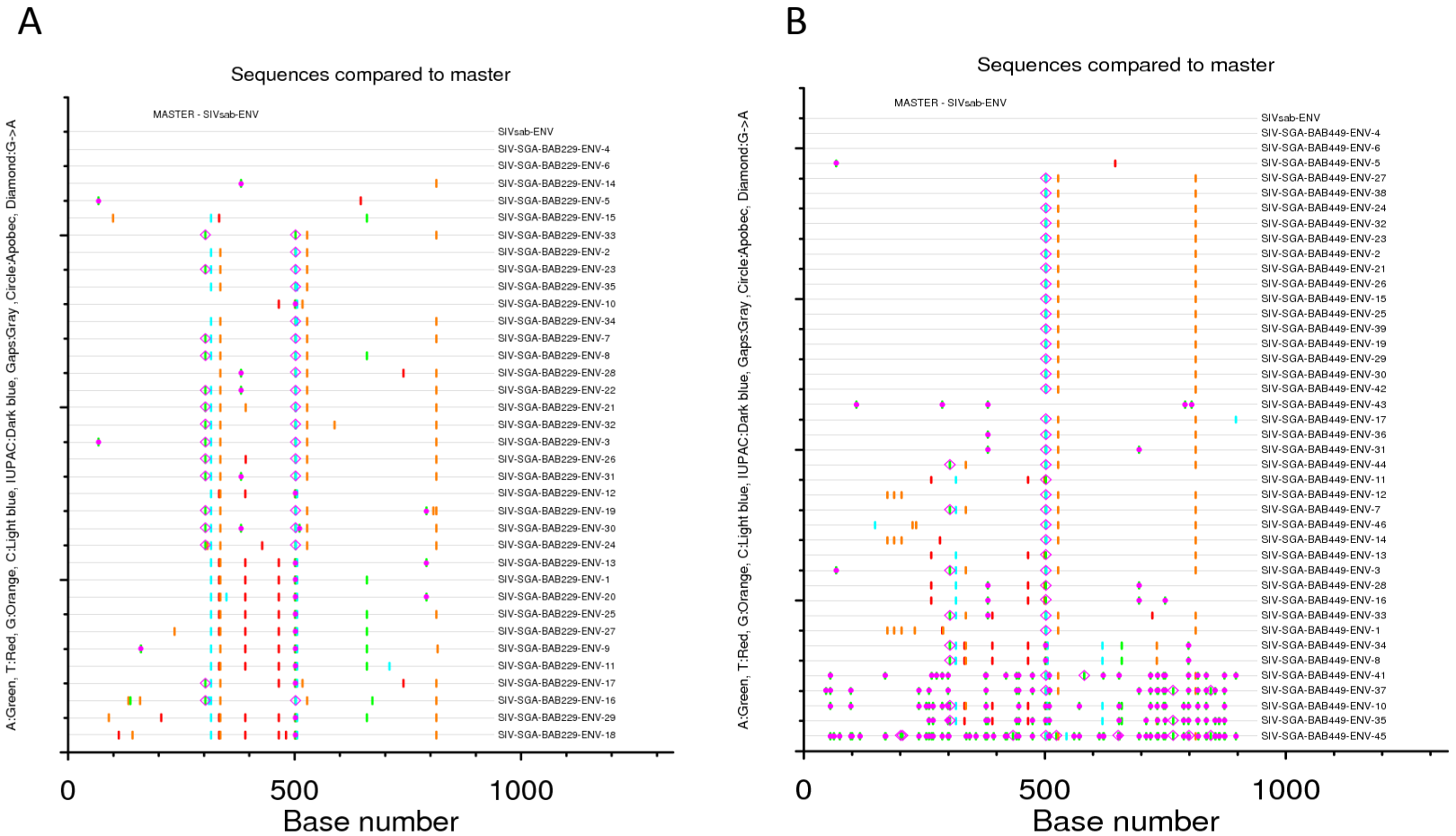
(A) peak of viral replication (D10) and (B) viral control (D43). Highlighter tick colors correspond with the nucleotide as listed A:Green, T:Red, G:Orange, C:Light blue, IUPAC:Dark blue, Gaps:Gray.

Figure 23. Highlighter Plot assessing the sequences of plasma SIV *Env* in controller baboon EA20.



(A) peak of viral replication (D10) (B) during course of viral control (D22). Highlighter tick colors correspond with the nucleotide as listed A:Green, T:Red, G:Orange, C:Light blue, IUPAC:Dark blue, Gaps:Gray

Figure 24. Highlighter Plot assessing the sequences of plasma SIV *Env* in controller baboon ED01.



(A) peak of viral replication (D10) (B) during course of viral control (D44). Highlighter tick colors correspond with the nucleotide as listed A:Green, T:Red, G:Orange, C:Light blue, IUPAC:Dark blue, Gaps:Gray. APOBEC specific: Pink.

Figure 25. Highlighter Plot assessing the sequences of PBMC derived SIV *Env* in controller baboon DB97.

5.3.2 SA3 Results Summary

There was no significant change in the mutation rates in the *env* sequence of SIV found in the plasma of the controller baboons from peak of infection to point of viral control. Preliminary evidence points to a direct impact of APOBEC3g on viral control as hypermutation rates increase in the PBMC derived *env* from controller baboons DB97 from peak infection to the time of viral control.

6.0 DISCUSSION

SIV cross-species transmission among NHPs and between NHPs and humans is an important area of study as both HIV-1 and HIV-2 jumped species to humans. Additionally, humans are exposed in Central/West-central Africa (i.e., the area of HIV emergence) to a plethora of highly divergent viruses related to HIVs. It is not well understood what factors determine the success or failure of a cross-species transmission event. To address these questions and to provide the scientific community with an adequate animal model for the study of the factors governing SIV cross-species transmission, we developed a new baboon animal model. It consists of the use of a single species of monkeys and two different viruses resulting in two completely opposed pathogenic outcomes: completely controlled SIV infection (in baboons infected with SIVsab) and persistent, progressive infection (in baboons infected with SIVsmm). Compared to the Asian RM non-natural host traditionally used, this model uses the African Guinea baboon non-natural host that lives sympatrically with the AGM natural host. Thus, a cross-species transmission event is plausible in the wild, where cross-species transmission events occur naturally. Furthermore, another innovation of our study is that differently from the majority of the in vivo approaches, we used unadapted plasma SIV stocks that predominantly contained transmitted founder strains which therefore have a similar resistance to IFNs as the strains that can be transmitted in natural cross-species transmissions.

In the past decade HRFs have been identified and rapidly changed the field of HIV study. As this is a newly growing field, most studies focus on one or two of the HRFs using mostly *in vitro* models. This study furthers the HRF field first by using an *in vivo* NHP model, and an experimental challenge approach that models natural transmission as closely as possible. Second, this study assessed the combined effect of multiple HRFs as a driving factor in cross-species transmission rather than individual action of the HRFs in artificial in vitro systems. While each factor is important, it is the combined action that will drive the overall response to cross-species transmission.

Our protein expression quantification analysis suggests that, while the individual expressions of each HRF upon cross-species transmitted infections only marginally correlate with the virus control, their aggregated expression is clearly statistically associated with control of infection. While our analysis relied on the quantification of protein expression and not necessarily of protein action, our results clearly support that all together these proteins could be drivers of cross-species transmission. Our quantification was based on an innovative approach in which we quantified HRF expression on the whole lymph node rather than on common "regions of interest".

An indirect validation of our approach is represented by the results of SAMHD1 quantification. This was the only HRF whose expression did not increase at the time of virus control in SIVsab infected baboons. This is not unexpected, as SAMHD1 acts through Vpx and SIVsab does not contain this accessory gene. Interestingly, in the pathogenic counterpart of the model, SAMHD1 increased at the time of disease progression. Note, however, that the increase in SAMHD1 expression was not directly correlated with disease progression. The significance of these changes in SAMHD1 expression with disease progression has to be further investigated.

Our results also support previous studies reporting that HRFs are expressed in relevant cell types that are the major target cells of HIV (12, 13, 28, 37, 75). We found that HRFs were expressed by dendritic cells, macrophages, and T cells, which are all major targets of HIV/SIV. As these are the physiologically relevant target cells, HRF expression in these cells is likely critical to virus restriction.

Unexpectedly, SGA analysis revealed no significant changes in mutation rates in plasma virus. While virus subjected to the HRFs should ideally have a higher mutation rate or marks of intrinsic immunity pressures, our data suggest otherwise. However, we have initially attempted to characterize the impact on the plasma virus. By definition, the virus in plasma is replication competent and should a dramatic impact of the HRFs on the virus occurred, that virus would not have been present in plasma, as hypermutations, frame shifts, deletions and insertions rendering the virus impaired for replication would prevent such a virus to be present in large amounts in plasma. We therefore, recently reasoned that to assess the impact on viral quasispecies we should focus on the intracellular virus and performed SGA on the viral DNA contained in PBMCs and LNs. Upon completion of such assays, virus derived from PBMC's does have higher instance of APOBEC associated mutations, indicating APOBEC3g induces hypermutation and indicates direct impact on viral control.

In summary, our study demonstrates a significant increase in HRF protein expression in controller baboons relative to virtually unchanged HRF expression in progressor baboons and natural hosts. This HRF expression increase occurs in the physiologically relevant HIV target cell populations, suggesting that viral restriction through HRFs is a key mechanism of control in vivo upon cross-species transmissions. While plasma virus *env* sequences showed no hypermutation, PBMC derived *env* showed an increase of APOBEC3g specific hypermutations

pointing to a direct effect of APOBEC3g on virus control. Overall, our study suggests that HRF expression does play a major role in the success or failure of cross-species transmission.

7.0 FUTURE DIRECTIONS

While this study makes advancement in the understanding of what role HRFs play in cross-species transmission, much more progress can be made. Further staining and quantification of HRF activity at mucosal sites would benefit this study. Including the recently discovered HERC5 HRF in all assays would provide a more comprehensive view of host restriction. A combined IHC stain of all HRFs that can be performed simultaneously would also benefit this study. A combined stain could be quantified using the same quantification technique each individual marker was subjected to and produce more reliable results. Such an analysis is in progress.

Additionally, the experiments performed in this study were based on IHC expression. IHC is a notoriously difficult assay to perform and the quantification techniques used have a mild degree of subjectivity. A qRT-PCR assay for each HRF would provide an excellent viral mRNA expression complement to the protein-based assays performed here. qRT-PCR quantification is less subjective than the quantification techniques used in this study. Note, however that, while less subjective, qRT-PCR is the quantification of mRNA, and not necessarily quantifying the expression of or action of each protein.

8.0 PUBLIC HEALTH SIGNIFICANCE

This research has many applications to the field of public health. In particular understanding cross-species transmissions, of which HIV-1 and HIV-2 are products of, is essential to preventing the occurrence and spread of new HIVs and may help control the current HIV pandemic. The use of an animal model is the closest possible way to model cross-species transmission that occurs in the wild. This research establishes a new baboon animal model for cross-species transmission, which is an essential part of HIV/AIDS research.

The role of HRFs and what impact they may have in determining the success of cross-species transmission is also important to public health. This research suggests that the collective action of multiple HRFs is a major determinant to the success of cross-species transmission and further understanding could prevent the establishment of new HIVs. In addition to cross-species transmission mediation, HRFs may provide many therapeutic opportunities to prevent or treat HIV infections in the future.

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