SPECIFIC AIMS

Human immunodeficiency virus 1 (HIV-1), the causative agent of AIDS, has its origins in the zoonotic transmission of simian immunodeficiency virus (SIV) from chimpanzees via an older transmission from Old World monkeys. Significant barriers to cross-species transmission of lentiviruses are host-encoded antiviral proteins, called restriction factors, that can inhibit viral infection, replication, and spread. One potent barrier to cross-species transmission of lentiviruses in primates are the APOBEC3 family of restriction factors, of which APOBEC3G is the most effective. Importantly, human APOBEC3 proteins are largely ineffective against HIV-1 due to adaptation of the lentiviral accessory protein Vif (Viral infectivity factor) to bind and degrade these restriction factors. Thus, the adaptation of Vif to bind and antagonize human APOBEC3 proteins was a critical step towards successful zoonotic transmission of lentiviruses; understanding how this adaptation occurs is fundamental to the rational design of evolution resistant Vif inhibitors that can restore potent host restriction factor activity, as well as to prevent future lentiviral zoonotic transmissions. In this proposal, I seek to understand how lentiviral Vif adapts to new species by characterizing the critical changes needed for adaptation to a new host APOBEC3 protein. **Aim 1** will prospectively analyze the evolutionary space within Vif-APOBEC3 interaction interfaces to understand how Vif may evolve to overcome natural APOBEC3-mediated barriers during cross-species transmission. **Aim 2** will investigate how cross-species adaptation of Vif affects viral replication and fitness in non-human primate cells.

Aim 1: Explore the evolutionary space of protein-binding interfaces of HIV-1 Vif. I will determine the ability of HIV-1 Vif to adapt to APOBEC3 proteins of a non-human primate model of HIV-1 infection, the rhesus macaque, by incorporating a library of HIV-1 Vif Loop 5 mutants into an HIV-1 viral vector for selection in cells expressing one of two rhesus macaque APOBEC3G haplotypes.

Aim 2: Investigate the ability of HIV-1 Vif to adapt to polymorphisms in host APOBEC3G. I will examine how macaque-adapted HIV-1 Vif mutants can accommodate natural escape mutations in rhesus macaque APOBEC3G. HIV-1 Vif mutants with broadened specificity towards one or both rhesus APOBEC3G haplotypes will be incorporated into a Simian-tropic HIV-1 (SHIV) to assess replication efficiency in rhesus macaque $CD4^+$ T cells.

This proposal aims to further our foundational knowledge in how lentiviruses cross species among primates to understand how HIV-1 became a human pathogen and to predict future zoonotic transmission events, with the practical benefit of improving SHIVs as challenge viruses for the non-human primate models of HIV-1 infection. The work proposed here will generate tools and data forming the basis of my independent research and will be used towards a Career Transition Award (K22) or independent R01.

Significance

The HIV-1 pandemic originated from zoonotic transmission of SIV infecting chimpanzee (SIVcpz) to humans [1]. Chimpanzees themselves acquired SIVcpz from a recombinant strain between multiple Old World monkey SIVs [1–3]. Cross-species transmission of lentiviruses is dependent on the virus establishing a chronic and productive infection despite innate immune barriers in the new host. In the case of HIV-2, the precursor SIV infecting sooty mangabey monkeys (SIVsmm) is pre-adapted to bypass or antagonize key human restriction factors, allowing direct zoonotic transmission [1]. However, substantial molecular adaptation occurred to facilitate the cross-species transmissions underlying the HIV-1 lineage [1,4,5]. With over 40 species of non-human primates carrying an SIV [6], it is important to understand how lentiviruses adapt to new hosts to proactively assess novel zoonotic threats.

A potent barrier to cross-species transmission of retroviruses in primates are the APOBEC3 family of restriction factors. APOBEC3 proteins package into nascent virions and potently inhibit viral replication by hypermutating the single-stranded viral DNA directly following reverse transcription of the retroviral genome. Lentiviruses encode the accessory protein Vif (Virion Infectivity Factor) to bind and degrade APOBEC3 proteins and help establish productive infection [7]. Human APOBEC3G is the most potent of the APOBEC3 restriction factors, and despite strong antagonism by HIV-1 Vif, its residual activity is correlated with positive prognostic indicators in people living with HIV-1 [8–10]. Therefore, the design and implementation of Vif-specific inhibitors to restore full APOBEC3G activity is a promising avenue to explore for the next generation of anti-HIV-1

therapeutics or as part of an HIV-1 cure strategy to fatally mutate newly activated viruses from a latent reservoir. However, the development of therapies targeting the APOBEC3G-Vif interaction has been largely hindered by the difficulty in co-crystallization of the APOBEC3G-Vif complex.

Further hindering development of Vif-targeting therapeutics is the lack of a non-human primate model to study HIV-1 Vif. HIV-1 Vif is unable to antagonize macaque APOBEC3G [11,12], and therefore it is replaced by SIV *vif* genes in the Simian-tropic HIV-1s (SHIVs) that are used as challenge viruses for modeling HIV-1 infection in macaques. Introduction of SIV Vif in SHIVs overcome rhesus macaque APOBEC3 barriers and allow for more productive viral infection [13]. However, the absence of HIV-1 Vif from SHIV/macaque models precludes advanced *in vivo* study of this critical lentiviral accessory protein, hindering the development and evaluation of HIV-1 Vif-specific treatment strategies. This proposal addresses this shortcoming by determining pathways by which an HIV-1 Vif can antagonize macaque APOBEC3 proteins analogous to their human APOBEC3 orthologs, thus providing avenues for engineering SHIVs for *in vivo* studies of HIV-1 Vif biology. An additional goal of this proposal is to understand how Vif can adapt to two different interfaces in the same host species, as APOBEC3G is naturally polymorphic in rhesus macaques [14]. Taken together, this proposal seeks to understand how Vif adapts to new hosts to facilitate cross-species transmission of lentiviruses, while simultaneously improving upon the macaque model of SHIV infection.

Innovation

- Performing combinatorial saturation mutagenesis in a rationally determined region of Vif allows for a complete picture of the biochemical requirements for HIV-1 Vif to bind to human APOBEC3G or adapt to macaque APOBEC3G.
- This project aims to construct novel SHIVs encoding fully macaque-adapted HIV-1 Vif to model the effect of HIV-1 Vif on viral replication in macaque cells, with the ultimate goal of improving upon the SHIV/macaque model of HIV-1 infection.
- The framework of this approach can be expanded to understand how the other HIV-1 accessory proteins, such as Vpr, Vpu, and Nef, adapt to antagonize novel host restriction factors.

Approach

Aim 1: Explore the evolutionary space of protein-binding interfaces of HIV-1 Vif

As a result of longstanding genetic conflict between primate hosts, retroviruses, and endogenous and exogenous pathogens, many of the APOBEC3 proteins are rapidly evolving under positive selection by viral pressures [15–17]. Our group described that two rapidly evolving amino acid positions in hominid APOBEC3G, residues 128 and 130, allow for APOBEC3G to escape Vif antagonism in nearly all SIV strains infecting Old World monkeys, providing a potent barrier against cross-species transmission of these viruses [11,12]. Importantly, an aspartic acid (D) found at site 128 (D128) exclusively in hominid primate APOBEC3G confers protection from most SIV Vif proteins. My recent work used HIV-1 Vif sequence analysis, structural comparisons, and functional virological assays to understand how SIVrcm Vif, the unadapted precursor to SIVcpz and HIV-1 Vif, could adapt to hominid APOBEC3G through mutations introduced to the Loop 5 (L5) region of Vif [5] (**Figure 1**). My work directly linked these adaptations in Vif L5 to the protective K128D mutation present in hominid APOBEC3G that escapes antagonism by other SIV Vif proteins [5]. Importantly, our finding that L5 of Vif accommodates APOBEC3G variants at 128 and 130 is supported by both *in vitro* and *in vivo* evolution experiments in HIV-1 Vif [18] and SIVagm Vif [12], respectively. Identification of Vif L5 as a species-specific determinant for APOBEC3G recognition not only furthers our understanding of how HIV-1 became a human pathogen, but also enables us to explore the evolutionary capacity of HIV-1 Vif to adapt to new hosts, such as those used as models for HIV-1 infection. Importantly, HIV-1 Vif is incapable of restricting APOBEC3G in Old World monkeys due to divergence at residues 128 and 130 [11,12]. **Therefore, we hypothesize that molecular evolution of L5 in HIV-1 Vif is sufficient to facilitate cross-species adaptation towards Old World monkey APOBEC3G**.

In order to study how HIV-1 Vif can adapt to a strong barrier of cross-species transmission, I will perform a series of *in vitro* guided evolution experiments by which a library of HIV-1 Vif mutants is challenged against cells expressing human APOBEC3G or one of the two rhesus APOBEC3G haplotypes (methods outlined in **Figure 2**). We have previously found that loop 5 of Vif is responsible for the species-specific recognition of APOBEC3G. I will begin by constructing an HIV-1 Vif library through combinatorial saturation mutagenesis to randomize the four amino acids in Loop 5 through PCR using degenerative NDT codons (N=A/T/G/C, D=A/G/T), resulting in a

total library diversity of both $12^4 = 2.07x10^4$ codon and amino acid combinations. NDT encodes for 12/20 amino acids (RNDCGHILFSYV) representing all of the major biochemical groups with no stop codons [19], and all of the amino acids found in SIV Vif Loop 5 are represented in this library. Importantly, this codon choice also reduces library complexity substantially compared to NNS (N= A/T/G/C, S= C/G), which would give rise to $32^4 = 1.05x10^6$ codon variants and be subject to bottlenecking at the library construction, selection, and sequencing stages.

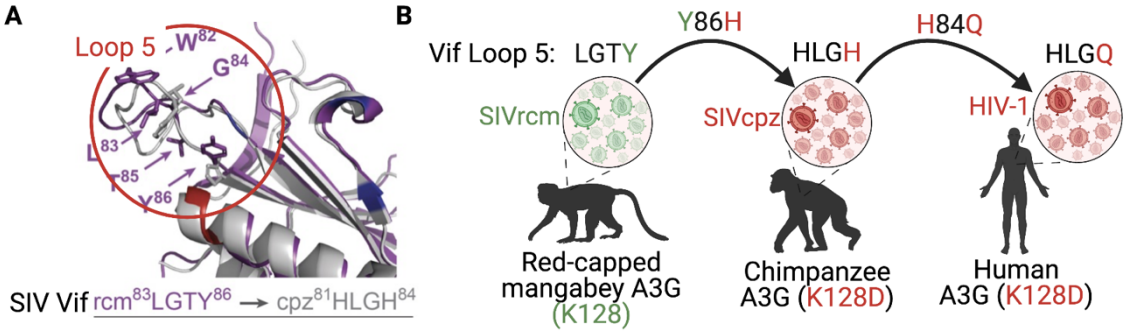

Figure 1. Adaptations in Loop 5 of SIVrcm Vif allowed for hominid APOBEC3G (A3G) antagonism. A) Comparison of the SIVrcm Vif crystal structure with SIVcpz Vif identifies Loop 5 as a variable interaction interface. **B)** Schematic of Vif Loop 5 adaptation to hominid A3G. A single amino acid change in SIVrcm Vif Loop 5 was sufficient to adapt to the protective mutation in hominid A3G, K128D. Figures and additional information in Binning et al., 2019.

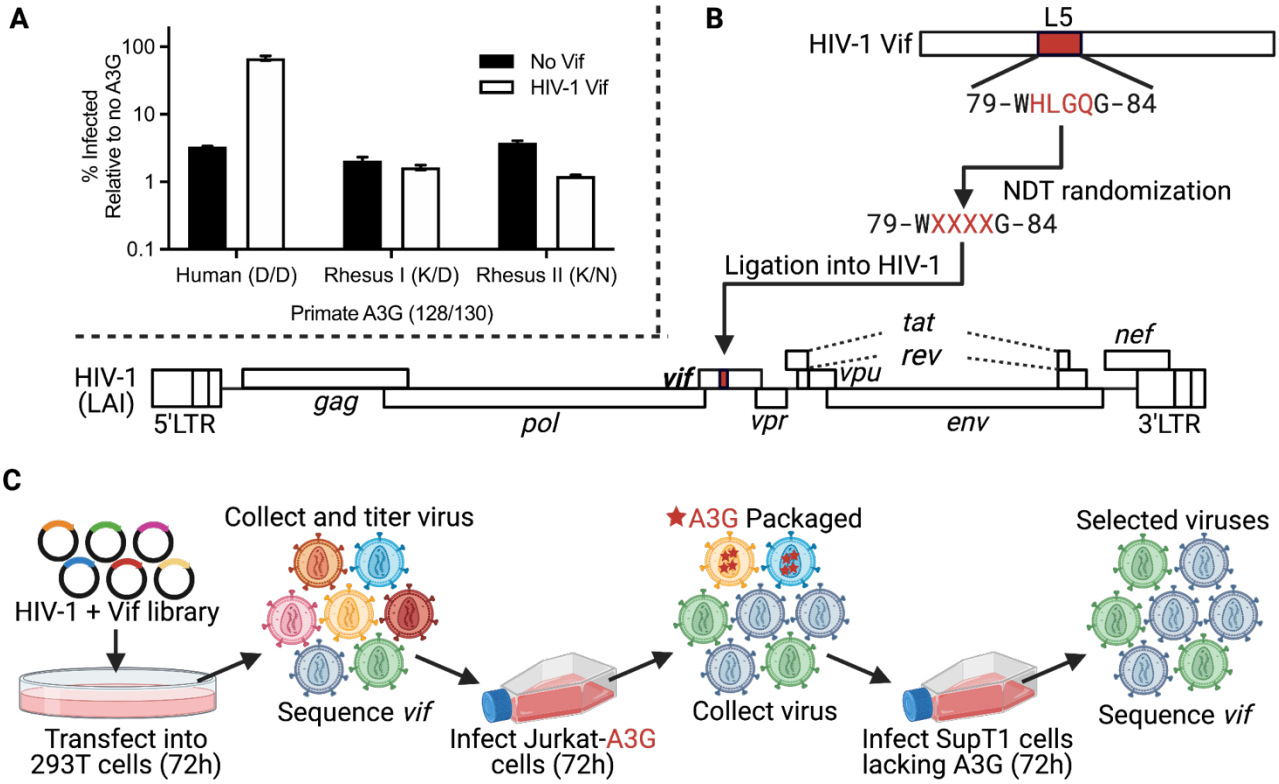

Figure 2: An HIV-1 Vif L5 mutagenesis screen to study cross-species adaptation. A) Single-cycle viral infectivity assays confirm HIV-1 Vif restores infection in the presence of human APOBEC3G (A3G) but not with either rhesus A3G haplotype. **B)** Library generation strategy. Randomization will be performed using PCR with NDT (N=A/C/T/G, D=A/T/G) primers. **C).** Vif mutant selection strategy. Plasmid library from B will generate viral library of Vif mutants, which will be selected in Jurkat T cells expressing human or rhesus A3G. Viruses that emerge from selection will be passaged through SupT1 cells, which lack A3G, to purge viruses that carry A3G from the Jurkat selection.

The Vif mutant library will then be inserted into a replication-competent HIV-1 molecular clone to produce progeny virions in 293T cells lacking APOBEC3 proteins. Viruses containing the Vif mutant library will be titered, deep sequenced, and used to infect Jurkat T cells stably expressing human or rhesus APOBEC3G for 72 hours. This infection timeframe will allow for three viral replication cycles in order to select for viruses expressing Vif

mutants that adequately antagonize APOBEC3G while depleting viruses that are hypermutated by A3G. Viruses will undergo a final 72-hour infection in SupT1 cells, a highly-infectible T cell line lacking APOBEC3 proteins, to enrich viruses that survived APOBEC3G selection. During this step, any viruses that packaged APOBEC3G will be purged from the final selection pool due to hypermutation upon entry into SupT1 cells. Viruses that emerge from the SupT1 infection will again be deep sequenced. Vif sequences from the final infection in SupT1 cells will be compared to viral sequences propagated from 293T cells pre-selection to determine enriched and depleted Vif sequences. I expect the human APOBEC3G control cells to deplete Vif sequences that are detrimental for binding, giving me a comprehensive picture of how human APOBEC3G and HIV-1 Vif bind. I anticipate that only viruses carrying Vif mutants that antagonize rhesus APOBEC3G will survive selection and be enriched in our final sequence pool. Thus, I will be left with HIV-1 Vif sequences and/or biochemical motifs favored for rhesus APOBEC3G antagonism.

Pitfalls and alternative approaches

I may find that the HIV-1 Vif L5 region cannot adapt to either of the variants of rhesus APOBEC3G used for this study. If this is the case, I will shift my attention towards the F1-Box region of HIV-1 Vif, constituting amino acids 14-DRMR-17. Importantly, mutating F1-Box in HIV-1 Vif to 14-SEMQ-17 moderately restores antagonism of rhesus APOBEC3G, demonstrating that HIV-1 Vif is capable of degrading rhesus APOBEC3G to some extent [20]. Thus, saturation mutagenesis and *in vitro* selection of the F1-Box region is also a viable approach to identify HIV-1 Vif mutants capable of gaining antagonism towards rhesus APOBEC3G. In the event that I am unable to select for Vif mutants using the high-throughput method described, I will utilize a fluorescence-based system that has previously been developed and optimized to screen for small-molecule inhibitors and cellular factors that affect the Vif-APOBEC3G interaction [21–24]. Briefly, my HIV-1 Vif library will be stably introduced into cell lines expressing Green Fluorescent Protein (GFP)-tagged rhesus APOBEC3G (GFP-rhA3G). Cells that express macaque-adapted HIV-1 Vif will have reduced fluorescence, due to degradation of GFP-rhA3G, and can be sorted by fluorescence-associated cell sorting. Sorted cells can be deep-sequenced to determine Vif sequences capable of degrading GFP-rhA3G.

Statistical considerations

Ideal sampling size was calculated based on the probability of achieving full coverage of a given library size [25], and I determined that a sampling size of 3.35×10^5 is required for a 0.95 probability of full coverage. This sample size is relatively small, as our group routinely prepares libraries containing up to 3.0×10^6 clones. The number of Jurkat-A3G cells used for selection will start at 2×10^6 per condition, roughly 100x the library size to minimize bottlenecking library diversity. Using the Illumina MiSeq platform, sequencing depth will exceed 2 million reads to ensure 100x coverage of each codon variant. Sequence enrichment scores will be calculated as the ratio of selected sequences versus the initial input read counts per million. Selection screens will be performed in two biological replicates, and correlations between replicates will be calculated using Spearman's rank correlation. Comparisons of enrichment between groups will be calculated using Student's unpaired *t*-tests, or one-way ANOVA for comparisons of more than two groups.

Aim 2: Investigate the ability of HIV-1 Vif to adapt to polymorphisms in host APOBEC3G.

The polymorphic nature of APOBEC3G in rhesus macaques provides an intriguing challenge towards adapting an HIV-1 Vif to recognize both relevant rhesus APOBEC3G haplotypes simultaneously. APOBEC3G is polymorphic in several Old World monkeys, and variations within residues 128 and 130 strongly affect Vif antagonism [11,12]. An experimental evolution study in African Green Monkeys suggests that APOBEC3G heterozygosity may confer an adaptive constraint on SIV Vif and prevent the broadened specificity required to accommodate both interfaces of APOBEC3G [12]. However, several examples of Vif accommodating more than one interface within a single host exist in nature [11,12]. Thus, while APOBEC3G polymorphisms in monkeys protect against Vif adaptation initially, broadened specificity against host APOBEC3G haplotypes appears to be a common strategy underlying cross-species adaptation of Vif. However, the molecular adaptations Vif utilizes to accommodate escape mutations in APOBEC3G while still maintaining binding with the ancestral host APOBEC3G interface remain unexplored.

I will first screen macaque-adapted HIV-1 Vif mutants through single-cycle infectivity assays as in **Figure 2A**. I will insert selected HIV-1 Vif mutants into a luciferase reporter proviral HIV-1 vector to produce viruses in 293T cells coexpressing human APOBEC3G, either haplotype of rhesus APOBEC3G, or a combination of both rhesus

haplotypes. Viruses produced in these cells will be used to infect SupT1 cells to determine antiviral efficacy of each of the APOBEC3G proteins in the presence of HIV-1 Vif mutants.

Macaque-adapted HIV-1 Vif mutants that most potently antagonize rhesus APOBEC3G in single-cycle infectivity assays will be tested in rhesus macaque 221 cells, an immortalized $CD4^+$ T cell line [26]. I will introduce selected mutants into the minimal SHIV vector NL-DT5R for infection of these cells (**Figure 3A**). SHIV NL-DT5R contains 93% of the HIV-1 genome and uses SIVmac239 Vif in place of HIV-1 Vif [27]. Importantly, SHIV NL-DT5R is only able to replicate in macaque cells due to SIVmac239 Vif, demonstrating that unadapted HIV-1 Vif abrogates viral replication of this vector [27]. A spreading infection scheme will be utilized where rhesus macaque 221 cells will be challenged with a low multiplicity of infection to assess the replication of SHIV expressing macaque-adapted HIV-1 Vif over a 12-day period (**Figure 3B**). We will also assess the amount of endogenous rhesus APOBEC3G remaining in cells and viruses in the presence of macaque-adapted HIV-1 Vif through immunoblot. Finally, I will perform deep sequencing of integrated SHIV NL-DT5R genomes following the 12-day infection to assess rhesus APOBEC3G activity in the presence of wild-type HIV-1 Vif, macaque-adapted HIV-1 Vif, and SIVmac239 Vif using a hypermutation assay developed by our lab [28]. This work will be performed in collaboration with Dr. Amit Sharma, PhD, an Assistant Professor at The Ohio State University and an expert in SHIV vectors and non-human primate models of HIV-1. I anticipate that adapting HIV-1 Vif towards rhesus macaque APOBEC3G will provide valuable information towards understanding how Vif is able to evolve around barriers of cross-species transmission while generating practical new tools such as engineered SHIVs for *in vivo* studies of HIV-1 Vif in the macaque models of HIV-1 infection.

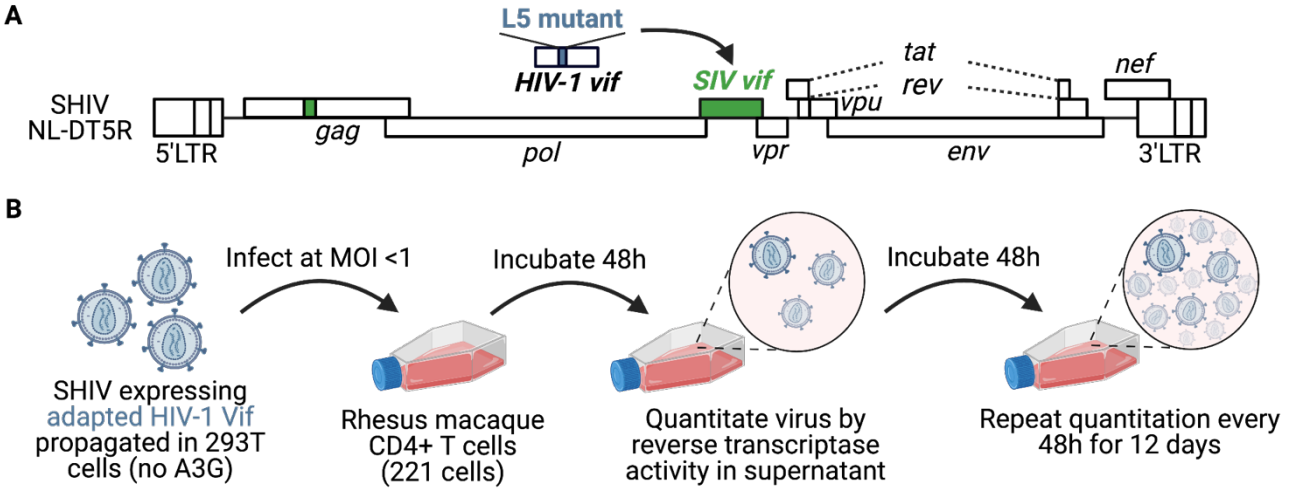

Figure 3: Validation of macaque-adapted HIV-1 Vif. A) Schematic of the SHIV NL-DT5R genome. Green regions represent sequences from SIVmac. Macaque-adapted HIV-1 Vif will be added in place of SIV Vif. **B)** Spreading infection framework to asses adapted HIV-1 Vif activity in rhesus macaque CD4+ T cells.

Pitfalls and alternative approaches

I may find that mutations within Loop 5 of HIV-1 Vif can only accommodate one specific rhesus APOBEC3G interface and is unable to broaden specificity towards both haplotypes. I will genotype the *apobec3g* gene present in rhesus macaque 221 cells to determine which haplotype is present and move forward with the HIV-1 Vif mutants that accommodate that haplotype. If rhesus macaque 221 cells are heterozygous for both haplotypes, I can genotype alternative rhesus macaque $CD4^+$ T cell lines or engineer compatible rhesus APOBEC3G haplotypes through homology directed repair using CRISPR-Cas9 and single-stranded oligodeoxynucleotides [29].

Statistical Considerations

Single-cycle infectivity assays will be performed in biological triplicate and presented as normalized infection compared to a no A3G control. Mean normalized infectivity will be calculated and compared between groups using Student's unpaired *t*-tests or one-way ANOVA for comparisons between two or more groups. Spreading infection experiments will measure the reverse transcriptase (RT) activity in the viral supernatant every 48 hours [30], and mean RT activity will be calculated and compared between groups using Student's unpaired *t*-tests or one-way ANOVA for comparisons of more than two groups. Infection experiments and biochemical validation via immunoblot will be performed in biological triplicates.

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