We welcome the opportunity to resubmit our proposal, “Adoptive transfer of immunity elicited by attenuated vaccines.” We are delighted that our initial submission received a priority score of 185 despite major concerns that Specific Aim 2 is premature and should be eliminated. We have taken the two major recommendations of the SRG and eliminated Specific Aim 2 and reduced the project period from five years to three. In addition, we have addressed every other reviewer comment by increasing animal group sizes, providing statistical justification for our group sizes, detailing our immunological analyses in greater detail, and adding a comprehensive assessment of T cell activation in lymphocyte transfer recipients. We also expand the Preliminary Data to show that Mauritian cynomolgus macaques (MCM) are susceptible to SIVmac239Δnef. Throughout the text, substantial changes in the revised application are delineated with a solid line in the left margin. We feel that these improvements suggested by the reviewers have resulted in a greatly improved application.

The major points raised by each reviewer (shown in italics) and our responses are shown below:

Reviewer 1
“the goals of this application and the potential gain in knowledge derived from some of these experiments are highly significant...there is great innovation in the experimental model to be tested...The PI has done a great amount of work that suggests that these experiments are certainly feasible”

We thank the reviewer for this kind assessment of our application’s significance.

“[transfer of T cells] resulted in a transient increase of SIV loads in the recipient animal which he argued as “virus-specific effect of the transferred cells”. This statement is difficult to defend considering the lack of a control experiment...”

[section C.6] We agree with the reviewer that this evidence is anecdotal without control data. We soften the language in the revised application to emphasize that this assertion is speculative.

“...technically speaking only monozygotic twins have identical MHCs”

The term “MHC-identical” has been replaced with the term “MHC-matched” throughout the proposal.

“...successful transfers should also be evaluated by comparison with the viral loads achieved by the group E, immunized with SIVΔnef and challenged with SIVmac239”

[section D.1.3.1] We concur that the group E animals are the “gold standard” representing the maximal level of protection that can be reasonably expected. We modified the text to state that viral loads in Group A-C animals will be compared to both recipients of lymphocytes from naive donors (Group D) and vaccinated animals challenged with SIVmac239 (Group E).

“no previous infections with SIVmac239Δnef have been reported [in MCM]”

[section C.1] We vaccinated two MCM with SIVmac239Δnef on 30 July 2007 to demonstrate susceptibility to this virus. 1 week post-immunization, viral loads in both animals have viral loads greater than 1 x 10^3, consistent with previous immunizations of Indian rhesus macaques. Preliminary results from this ongoing study have been added to the proposal.
“...will presence or absence of detectable viral loads [in SIVmac239Δnef vaccinees] be a requirement for adoptive transfer?”

[section D.1.3.1] In 16 rhesus macaques vaccinated with SIVmac239Δnef, viral loads were consistently low (<1000 vRNA eq/mL) and frequently below the limit of detection (<50 vRNA eq/mL) 6 months post-vaccination (Dr. David Watkins, personal communication). We expect setpoint viral loads in vaccinated MCM to be similarly low and thus will not use viral loads as an inclusion or exclusion criteria for adoptive transfer.

“reports of cytotoxicity and altered cell activity by PKH67”

[section D.1.3.3] We acknowledge this concern and have performed IFN⁠-⁠ELISPOT assays comparing unlabeled to PKH67 labeled effector lymphocytes. INSERT BURWITZ ELISPOT DATA HERE

“very little discussion in terms of general analysis for the immunological assays is provided”

[section D.1.1.1., D.1.3.3-D1.3.6] We include more detailed descriptions of all immunological assays and their analyses in the revised application.

“Additional analysis of soluble factors released in the recipients after cell transfer will also be informative”

[newly added section D.1.3.6] We agree with the reviewer and have added soluble cytokine analyses to the Research Plan.

“A shorter, more focused proposal would certainly be more convincing”

We agree and have eliminated Specific Aim 2 and compacted the study timeline to 3 years.

Reviewer 2

“...The model identified by this group provides a highly original opportunity to address the potential correlates of immune protection from SIV infection...The studies however are well within the capability of this group and collaborators, and the approach is innovative.”

We very much appreciate the reviewer’s supportive comments.

“...viral load set-points appear relatively low diminishing the potential ‘therapeutic index’ in terms of viral loads to be investigated...This finding compounded with the low number of monkeys per group may invalidate potential borderline results”

[section C1 and D.1.3.1] While setpoint viral loads in cynomolgus macaques are lower than in Indian rhesus macaques, acute phase peak viral burdens are similar. Peak viral loads average $5 \times 10^2$ copies / mL plasma in SIVmac239-infected rhesus macaques vaccinated with SIVmac239Δnef. Since we predict that transferred lymphocytes will persist through the first two weeks of infection when explosive SIVmac239 replication typically occurs, we plan to use attenuation of acute phase peak viremia, not reduction of setpoint viral load, as our primary virological endpoint. This is now clarified in the text. Nonetheless, we increase the number of animals in each group as per the reviewer’s suggestion. A previously published power analysis demonstrated that using 5 animals per group is sufficient to detect a peak viral load reduction of $1.50 \log_{10}$ with 90% power in Indian rhesus macaques infected with either SIVmac251 or SHIV89.6P[62]. We will now use 6 animals per group to ensure, even if viral load dynamics of MCM infected with SIVmac239 differ subtly from those used to initialize the power analysis, that we will be able
to detect differences in peak viral load levels with high confidence.

“Missing at this stage are tetramer reagents for this species”

[section D.1.3.3] We have identified a minimal epitope that is likely restricted by Mafa-B*51, an allele on the H3 haplotype and are in the process of producing a tetramer refolded with this peptide. We are aggressively mapping epitopes restricted by alleles on the H1 haplotype and have identified more than 5 CTL reactive regions containing epitopes that we are fine-mapping. We expect that tetramers to at least three responses restricted by alleles on the H1 haplotype by the start of this grant’s project period.

“[could] the activating effect caused by the adoptive transfer [lead] to spikes in viral replication...”

[newly added section D.1.3.6] The reviewer identifies an important oversight in our original submission. We have added a comprehensive assessment of CD4+ and CD8+ T cell activation in adoptive transfer recipients to our experimental plan. It should be noted that any effects adoptive transfer has on viral replication vis-à-vis cellular activation should be similar between the animals that receive lymphocytes from immunized (Groups A-C) or vaccine naive (Group D) donors.

“transfer of [antibody-containing] plasma is not expected to contribute to any significant part in the protection afforded by live attenuated SIV”

[section D.1.5] While we agree that lymphocyte-mediated protection likely mediate protection, we submit that it is premature to exclude a role for antibody-containing plasma in immunity. Though passive transfer of sera alone does not protect monkeys from pathogenic SIV, plasma antibodies and lymphocytes may act in concert to resist superinfection in attenuated vaccinees. We continue to believe that attempting to recapitulate protective immunity by combining lymphocyte transfer and passive immunization is a reasonable alternative approach if the highest dose of lymphocytes alone are not protective.

“At this stage, it might be preferable to eliminate aim 2 altogether.”

As indicated above, Specific Aim 2 has been deleted from the amended proposal.

Reviewer 3

“The proposed study could provide important information concerning the correlates of immune protection in an important SIV macaque animal model system. Since most other populations of macaques are outbred it would not be possible to perform these experiments without bone marrow ablation and subsequent adoptive transfers. The MHC-matched population of macaques presents an important and unique opportunity to gain critical insights into immune protection correlates.”

We thank the reviewer for the favorable overall evaluation of our initial proposal.

“have the investigators adequately addressed the issue of infection and pathogenicity of SIV in this population of macaques?”

[Section C.1] We have infected 7/7 Mauritian cynomolgus macaques after a single exposure with SIVmac239 intrarectally. All developed peak viral loads in excess of $2 \times 10^6$ vRNA eq/mL. As described in the response to Reviewer #1, we now show that SIVmac239Δnef establishes infection in MCM.
A. SPECIFIC AIMS

The best hope for curbing the global HIV/AIDS pandemic lies in the development of an effective, prophylactic vaccine. Despite more than twenty years of study on an enormous number and variety of vaccine candidates, the correlates of protective immunity against HIV are unknown. Determining which immune responses (or combination of immune responses) are most likely to control HIV would focus vaccine research and accelerate the development of an effective vaccine.

Though vaccines should endeavor to mimic successful immune responses against HIV, examples of experimental or spontaneous control of HIV and its monkey counterpart simian immunodeficiency virus (SIV) are rare:

1. Among vaccines, only live, attenuated strains of simian immunodeficiency virus (SIV) offer consistent protection from pathogenic SIV challenge.
2. A small subset of HIV-infected people and SIV-infected macaques durably control viral replication without therapy.
3. Monkeys treated with antiretrovirals immediately after SIV challenge maintain low levels of virus replication even after therapy is discontinued.

There is an urgent search underway to define the immunological mechanism(s) that unify these three remarkable subgroups. These investigations have been complicated by the inability to isolate and evaluate lymphocyte-mediated protection in vivo. Adoptive transfer of lymphocytes, which have been used successfully to define the minimal requirements for successful immune responses in other systems, have limited application in macaques because of their genetic heterogeneity within the major histocompatibility complex (MHC). Our laboratory has developed an approach to perform lymphocyte transfers using Mauritian cynomolgus macaques (MCM), a geographically isolated population with low genetic diversity. Six MHC haplotypes account for essentially all MHC diversity in MCM, resulting in large groups of MHC-matched animals. In preliminary experiments, transferred lymphocytes persist for at least two weeks.

In this proposal we will test the hypothesis that attenuated SIV elicits protective cellular immune responses that can be functionally transferred between animals. We will test this hypothesis in a single specific aim:

**Specific Aim 1:** Define the number of lymphocytes from SIVmac239Δnef vaccinated macaques necessary to transfer protective immunity to MHC-matched, SIV naive macaques. To accomplish this aim, we will transfer variable numbers of total lymphocytes between SIVmac239Δnef vaccinated macaques and MHC-matched recipients. The recipient animals will subsequently be challenged with SIVmac239.

Until now it has been impossible to use adoptive transfer to assess protective cellular immunity against AIDS viruses in vivo. This proposal builds on a previous NIAID R21 in which we demonstrated that genetically homogenous MCM are susceptible to SIVmac239Δnef and SIVmac239 infection and characterized SIV-specific cellular immunity in these animals. Our research group is uniquely positioned to conduct these experiments as it links together our experience with SIV pathogenesis, MCM genetics, and in vivo lymphocyte transfers.
B. BACKGROUND AND SIGNIFICANCE

B.1: HIV vaccines and the global pandemic

In 2006, more than 4,000,000 people became infected with HIV, bringing the global total to nearly 40,000,000[80]. The number of new infections continues to grow in spite of improved access to treatment and education, underscoring the urgent need for a prophylactic HIV vaccine.

Vaccine research has been galvanized by the creation of the HIV vaccine Enterprise[78], increasing both the number and variety of vaccine approaches that can be pursued simultaneously. Three vaccines are in advanced (Phase IIB or III) clinical trials, while more than thirty additional candidates are progressing through Phase I or IIA clinical trials[31]. None of the vaccines, however, have enjoyed unqualified success in preclinical trials and there is considerable pessimism that they will be only marginally effective in people[37]. Thus, there is a premium on research that will identify next-generation vaccine strategies that improve on those currently in the clinical pipeline.

B.2: HIV vaccines and correlates of protective immunity

A significant obstacle to HIV vaccine development is the lack of universally accepted correlates of protective immunity. There is considerable disagreement about the definition of ‘protective’, at least in part because there are no immune responses that are unequivocally linked with positive outcome following natural infection. Nonetheless, most vaccines currently in testing seek to elicit T cell immunity, often in conjunction with antibody responses. The extraordinary sequence heterogeneity of the viral envelope[7], the paucity of broadly neutralizing antibody epitopes[6], and the failure of the Vaxgen gp120 vaccine in phase III clinical trials[84] has intensified the focus on eliciting cellular immunity by vaccination.

Attempting to compare the quality of immunity elicited by different vaccines is almost impossible. Because ethical concerns preclude the intentional exposure of human vaccine recipients to HIV, vaccinated macaques challenged with SIV are almost always used to evaluate vaccine efficacy and protective immunity. The level of vaccine protection, however, is strongly dependent on the pathogenicity of the challenge virus and host immunogenetics. For example, a vaccine regimen consisting of three inoculations of SIV gag DNA followed by a single inoculation with a recombinant adenovirus vector expressing SIV gag effectively controlled a challenge with the SIV-HIV hybrid virus SHIV89.6P [69] while only animals possessing the MHC class I allele Mamu-A*01 were modestly protected against the highly pathogenic strain SIVmac239 when given the same vaccine[11].

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<tr>
<th>Vaccine</th>
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<td>Live attenuated: (\Delta_{\text{nef}})</td>
<td>59 of 63</td>
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<td>Live attenuated: (\Delta_{3})</td>
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<td>Live attenuated: (\Delta_{5G})</td>
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<tr>
<td>Live attenuated summary</td>
<td>74 of 78 (95%)</td>
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<td>All other vaccine strategies</td>
<td>18 of 256 (7%)</td>
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Figure 1. Attenuated vaccines offer the best protection from pathogenic SIV in Indian rhesus macaques. Protection is defined as consistent 3-log reduction in plasma virus relative to non-vaccinated controls (or low PBMC viral loads / virus titers in experiments predating plasma viral load determination). Figure is modified from Koff et al., 2006.

B.3: Monkeys exposed to attenuated SIV generate protective immune responses

No recombinant vaccine has achieved the level of protective immunity generated by vaccinating macaques with live, attenuated strains of SIV (Figure 1). Animals exposed to an SIVmac239 derivative with a 182 bp deletion in nef (SIVmac239,\(\Delta_{\text{nef}}\)) impressively resist intravenous SIVmac239 challenge[18]. After more than a decade of research, two primary variables that influence the extent of control have been identified. The strength of protection is inversely proportional to the degree of SIV attenuation. Unfortunately, animals exposed to more attenuated viruses
with desirable safety profiles do not resist pathogenic challenge (superinfection) as well as animals inoculated with less attenuated strains[32]. The second key variable is sequence divergence, with the best protection occurring when the attenuated virus closely matches the sequence of the pathogenic challenge strain([87] and David Watkins, personal communication). Both of these variables implicate adaptive immunity in protection. Highly attenuated viruses are unlikely to elicit robust immune responses, while even strong immunity may not be sufficient to protect from rechallenge with a virus that differs within key residues recognized by immune responses.

B.4: Deciphering protective immunity: insights from Friend Virus

Research with Friend virus in mice provides a workable framework for understanding protective immunity elicited by attenuated vaccines[28]. Friend virus (FV) is a retroviral complex composed of the replication-competent but nonpathogenic helper virus (F-MuLV) and a replication-defective, pathogenic spleen focus-forming virus (SFFV). Mice infected with FV develop extensive splenomegaly as infected erythroblasts proliferate. A small number of the infected erythroblasts become malignant, leading to a fatal erythroleukemia in susceptible inbred strains of mice.

Live-attenuated vaccines can elicit protective immunity against FV. By transferring distinct lymphocyte subsets between vaccinees and FV-naive recipients, the correlates of protective immunity against FV have been established. Transfer of 5 x 10^7 vaccine-primed splenocytes completely prevented splenomegaly and reduced plasma viremia to undetectable levels(Figure 2A and[19]). Transferring purified CD8+ T-lymphocytes alone reduced plasma viremia more than 10-fold, while transfers of CD4+ T cells or B cells had modest or minimal effects on viremia (Figure 2B). Follow-up experiments demonstrated that the level of protection afforded by CD8+ lymphocytes during acute infection is directly proportional to the number of transferred cells, but is insufficient to prevent the establishment of persistent infection[21]. Thus, adoptive transfer experiments provide a way of quantifying and evaluating the contribution of cellular immunity to vaccine mediated protection in vivo.

Figure 2. Adoptive transfer studies with attenuated Friend virus define the correlates of protective immunity. (A) Variable numbers of spleen cells from vaccinated donors were transferred into naive syngeneic recipients one day before pathogenic FV challenge. As few as 5 x 10^7 spleen cells completely prevent splenomegaly. (B) Adoptive transfer of individual lymphocyte subsets from vaccinated animals define a critical role for CD8+ T cells in immune protection. This proposal seeks to undertake a similar analysis in macaques vaccinated with attenuated SIV. Figures modified from Dittmer et al., 1999 (A), and Dittmer and Hasenkrug, 2000 (B).
B.5: Adoptive transfer in HIV/SIV

Autologous lymphocyte transfer has been pursued as an HIV immunotherapy for nearly a decade. Purified CD8+ T cell clones[5, 36, 54, 66] and activated CD4+ T cells[41] appear to retain functional activity in vivo. Moreover, autologous PBMC and activated CD4+ T cells derived prior to infection conferred long-term nonprogressor status to SIV-infected macaques[82].

Therapeutic adoptive transfer has also been attempted in HLA-identical, HIV-discordant twins. In one study, the HIV- twin was vaccinated with an Env immunogen derived from his twin’s circulating virus prior to adoptive transfer of peripheral blood lymphocytes. The recipient did not experience any clinical changes except a transient increase in viral load[3]. In another study of HIV discordant twins, unmodified peripheral blood lymphocytes from HIV- donors increased peripheral CD4+ T cell counts during chronic infection[30].

Taken together, these studies illuminate several important features about adoptive transfer studies:

1) **Adoptive transfer studies with both bulk lymphocytes are feasible** in the context of HIV and SIV.
2) **High doses of cells (up to 3 x 10^{10}) are well tolerated clinically**[54].
3) Most adoptive transfer studies in HIV+ individuals use autologous cells; allogeneic adoptive transfer studies are limited to HLA-identical, HIV-discordant twins[3, 30, 68]. The **difficulty of identifying MHC-matched macaques has prevented similar studies in monkeys**.
4) Clinical outcomes have only been assessed during systemic, chronic infection. Prevention or amelioration of incipient viral pathogenicity by adoptive transfer has not been tested.
5) **There is no data on adoptive transfer using lymphocytes harvested from individuals with effective HIV/SIV control**, such as after exposure to attenuated vaccines.

B.6: The Unique Suitability of Mauritian cynomolgus macaques for adoptive transfer studies

Macaque monkeys infected with SIV are, by far, the most widely used nonhuman primate model for HIV research[14]. Rhesus macaques (Macaca mulatta), cynomolgus macaques (Macaca fascicularis), and pig-tailed macaques (Macaca nemestrina) are used in vaccine and pathogenesis testing. Mitochondrial DNA analysis and fossil records suggest that these macaques speciated approximately 2,000,000 years ago[29]. The natural geographic range of these monkeys extends throughout Southeast Asia. The species can be functionally subdivided by geographic origin, as the genetics of macaques from different locations are distinct. The MHC class I alleles of Chinese, Indonesian, Mauritian, and Vietnamese cynomolgus macaques are diverse and largely independent([38] and unpublished data), making identification of MHC-matched donor/recipient pairs almost impossible.

An exotic invasive population of approximately 50,000 cynomolgus macaques exists on the Indian Ocean island of Mauritius[75], approximately 2000 km from the southeast coast of Africa. The contemporary geographically isolated population of monkeys descend from an extremely small founder population introduced to the island within the last 500 years[39]. Mauritian cynomolgus macaques (MCM), like their Asian counterparts, are susceptible to SIV and SHIV strains including SIVmac239, but have dramatically reduced genetic diversity. **Six MHC haplotypes account for more than 99% of the MHC diversity in these animals, with 39% of the animals sharing the most common H1 haplotype([86] and Section C.2)**. This combination of SIV susceptibility and MHC homogeneity **uniquely suits Mauritian macaques for allogeneic adoptive transfer studies** to define distinct correlates of immune protection.
C. PRELIMINARY DATA

C.1: Pathogenic SIV strains including SIVmac239 infect Mauritian cynomolgus macaques

There is ample evidence that many widely used SIVs and SHIVs infect cynomolgus macaques, even if they have not been tested explicitly in Mauritian origin animals. Some of the strains that infect cynomolgus macaques include SIVmac251[17, 51, 57, 64, 77]), SIVmac239[1, 23], SIV SMM-3[12, 77], SHIVsbg[22], SIV316[23], SIV/DeltaB670[27], SIV/17E-Fr[27], SIVmac32H-J5[25, 40], SHIV89.6P[4, 8-10, 35, 47, 64, 77], SHIV-4[48], and RT-SHIV[89]. Additionally, attenuated SIV strains including nef-deleted SIVmac251[13], SIVmac32H-C8[2, 26, 67, 73, 74] have been used successfully in cynomolgus macaques. When compared to Indian rhesus macaques, cynomolgus macaques require a higher dose of virus to consistently establish SIV infection[58] and maintain lower chronic phase viral loads more similar to HIV-infected humans[64, 77].

In an R21-funded pilot study, we demonstrated that MCM are susceptible to SIVmac239. 3 of 3 MCM were infected after a single intrarectal exposure to 50,000 TCID\textsubscript{50} SIVmac239. Peak viremia in all animals exceeded 2 x 10\textsuperscript{6} copies viral RNA per mL blood plasma. The peak viral loads are comparable to Indian rhesus macaques infected with SIVmac239 (Figure 3). In two animals monitored for more than one year, chronic phase setpoints between between 1 x 10\textsuperscript{2} to 1 x 10\textsuperscript{4} copies viral RNA per mL were established.

It is important for our proposed studies that there is a large difference between peak SIVmac239 viral loads in unvaccinated macaques and in macaques vaccinated with SIVmac239\textsubscript{Δnef}. As shown in Figure 3, peak viral loads in SIVmac239 rhesus macaques previously vaccinated with SIVmac239 average 5 x 10\textsuperscript{2} copies viral RNA per mL blood plasma, a reduction of 3.6 log\textsubscript{10} from the lowest MCM SIVmac239 peak viral load. Thus, we believe that a 50-fold (1.5 log\textsubscript{10}) reduction in peak viral loads in adoptive transfer recipients should be resolvable in Mauritian cynomolgus macaques.

We recently infected two MCM with SIVmac239\textsubscript{Δnef}. Peak viremia levels were similar to those previously observed in Indian rhesus macaques and demonstrate that this virus can be used successful in MCM. INSERT PRELIMINARY DATA FROM BURWITZ HERE

C.2: Mauritian cynomolgus macaques have limited MHC diversity

Our laboratory has characterized MHC class I alleles in cynomolgus macaques from different origins. We discovered that there is minimal overlap in the allele sequences of cynomolgus macaques from China, Vietnam, and Mauritius[38]. Surprisingly, we identified a cluster of five MHC class I alleles shared between more than half of the MCM used for allele discovery. We speculated that these alleles segregated on a high-frequency MHC haplotype.

To test this hypothesis, we developed a high-density map of microsatellite markers distributed throughout the 5Mb region containing the MHC class I and MHC class II genes involved in antigen presentation (Figure 4). Microsatellites, also known as simple sequence repeats, are non-coding genetic loci containing variable numbers of simple tandem nucleotide repeats. The short microsatellites (generally 2-5 nucleotide repeat units) are genetically unstable, resulting in size polymorphisms that can be

![Figure 3. Mauritian cynomolgus macaques are susceptible to SIVmac239. Plasma viral loads in MCM during acute infection are very similar to those in Indian rhesus macaques. Rhesus viral loads are the geometric mean of 192 SIVmac239-infected animals and are courtesy of Drs. John Loffredo and David Watkins.](image-url)
Figure 4. Microsatellite map of the macaque MHC. 17 polymorphic microsatellite loci are distributed throughout the 5 Mb MHC region containing MHC class I and MHC class II genes. By determining the allele sizes at the 17 loci in more than 100 Mauritian cynomolgus macaques, we discovered that there are only six MHC haplotypes in this geographically isolated subpopulation.

Figure 5. All transcribed MHC class I and class II alleles found on each of the six MCM MHC haplotypes are defined. Individual alleles were cloned and sequenced from MHC homozygous MCM. Resolving the alleles found on each MHC haplotype allows us to identify completely MHC identical MCM for adoptive transfer studies.
Figure 6. Histocompatibility of MHC-identical MCM in vitro. PBMC from an MCM were fluorescently labeled with PKH67 and incubated for one week with irradiated PBMC from four stimulator animals. As responding PBMC proliferate, PKH67 signal is distributed equally between progeny cells. Therefore, decreasing PKH67 signal is associated with increased proliferation. Minimal proliferation was observed when PBMC were incubated with autologous stimulators or with stimulators taken from an outbred, MHC-identical MCM.
C.5: Successful autologous transfer of CD8β T cells in MCM

Since MHC matching is the most significant genetic predictor of transplantation success, we reasoned that MHC-matched Mauritian cynomolgus macaques could be used for lymphocyte adoptive transfers. There is considerable data on the autologous transfer of in vitro expanded T cell clones in HIV infection[5, 36, 61, 76]. We therefore decided to focus initially on transferring CD8β+ T cells. We purified approximately 20 million CD8β+ T cells from 90 mL of blood, labeled the T cells with the green fluorescent dye marker PKH67 for in vivo tracking, and reinfused the T cells (Figure 7A). Immediately following transfer, more than 2% of the total CD3+ CD8+ cells stained PKH67+ (Figure 7B), showing that our transferred cells could be detected in vivo by flow cytometry. Within the first 24 hours, the number of PKH67+ cells in the peripheral blood declined 10-fold, presumably as cells localized to lymph nodes[5]. PKH67+ cells in the blood remained detectable for at least five weeks, attesting to the stability of our fluorescent marker. This experiment demonstrated the feasibility of transferring and tracking viable, purified lymphocyte subsets in Mauritian cynomolgus macaques.

C.6: Successful allogeneic transfer of CD8β T cells in MCM

We next transferred CD8β+ T cells from an SIVmac239-infected MCM into an MHC-matched SIVmac239-infected recipient. Once again, we successfully purified (Figure 8A) and labeled (Figure 8B) CD8β+ T cells from the donor animal. Five minutes after the transfer, we detected circulating PKH67+ lymphocytes at the same frequency as was observed in the autologous transfer (Figure 8C). Within 24 hours, the number of PKH67+ cells in the blood declined 10-fold, again paralleling our autologous transfer. Remarkably, we continued to detect PKH67+ cells in the blood for the next 14 days in the absence of immunosuppressive treatment. We confirmed that the PKH67+ cells were derived from the donor by sorting the PKH67+ cells at day 10 and fingerprinting with a microsatellite marker that
Figure 8. Successful adoptive transfer of CD8β+ T cells between MHC-identical Mauritian cynomolgus macaques. (A) Purity of CD8β+ T cell selection. CD8β+ T cells were positively selected from approximately 90 mL of blood and the purity of the resulting population was assessed by flow cytometry. (B) Essentially all CD8β+ T cells were successfully labeled with the green fluorescent cell linker PKH67. (C) Adoptively transferred cells persist for at least 14 days. The percentage of CD8β+ cells that stain with the cell linker PKH67 at sequential timepoints are shown. PKH67+, CD8β+ T cells can still be easily resolved by flow cytometry as late as 14 days after transfer. (D) Detection of PKH67+ donor cells in the lymph node of the left inguinal lymph node 3 days post-transfer.
differentiates the donor and the recipient (Figure 9). We also collected a lymph node biopsy at day 3 and detected PKH67+ cells, unequivocally demonstrating that the transfused cells retain the capacity to localize to lymph nodes (Figure 8D).

We initially hypothesized that functionally competent T cells would confer clinical benefit and reduce plasma viral loads in the recipient animal. In actuality, we witnessed a dramatic rise in plasma viremia in the recipient animal in the days immediately following the transfer, from 20,000 copies / mL at baseline to 160,000 copies 72 hours post-transfer. The viral loads declined and stabilized at a new setpoint of approximately 50,000 copies / mL in subsequent weeks as PKH67+ cells in the periphery became undetectable (Figure 10A). The nucleotide sequence of the predominant virus in plasma did not change throughout the transfer period, suggesting that the viral load perturbations were not simply due to superinfection with virus from the donor animal.

Though we do not know why the viral loads transiently increased, we found that this was a common theme in adoptive transfers performed during chronic HIV infection[3, 5, 30]. Indeed, Brodie and colleagues suggested that this might be taken as evidence of successful transfer, arguing, “Virus may have been released from target cells into the extracellular space as the transferred CTLs destroyed HIV-infected T cells, and viral production by neighboring infected cells may have transiently increased because of the cytokines (such as TNF) released by the CTLs.” Whether this interpretation explains the observation accurately or not, it is reassuring that our observations in chronically infected MCM mirror those in HIV infected humans. The clinical benefit of adoptively transferred virus-specific lymphocytes may be much more apparent when evaluated in the context of incipient HIV/SIV infection.
C.7: SIV naïve CD8β+ T cells do not affect acute phase SIVmac239 replication

We were initially concerned that lymphocyte transfers alone could have a non-specific suppressive or enhancing effect on SIV replication, modulating the severity of acute infection. To address this issue, $1.1 \times 10^7$ SIV-naïve CD8β+ T cells from an uninfected H1/H1 MCM were infused into an H1/H1 recipient 36 hours prior to intrarectal SIVmac239 exposure. Despite persistence of the transferred cells for more than two weeks, viral loads reached $9 \times 10^6$ copies per milliliter of blood plasma and were indistinguishable from other MCM infected with SIVmac239.

C.8: Billions of lymphocytes can be harvested and transferred between MCM

The highest adoptive transfer dose we propose is $1 \times 10^{10}$ lymphocytes. We demonstrated that this transfer dose is feasible by infecting an H1/H1 MCM with SIVmac239 and sacrificing 3 weeks later at the peak of the cellular immune response. Lymphocytes were purified from mesenteric lymph nodes, peripheral blood, and the spleen. More than $6 \times 10^9$ lymphocytes were collected. Harvesting cells from additional lymph nodes would have undoubtedly resulted in a total yield in excess of $1 \times 10^{10}$ lymphocytes. When $2.2 \times 10^8$ CD8β+ T cells were infused into an H1/H1 homozygous MCM (2 MHC haplotype match), no adverse clinical effects were observed despite the high cell dosage.

C.9: Summary

Our research plan focuses on adoptive transfer of lymphocytes between MHC-matched MCM. We have established a novel set of tools for identifying MHC-matched animals and have demonstrated that these animals can be infected with SIVmac239Δnef and SIVmac239. CD8β T cells persist when transferred between MHC-matched MCM during chronic and incipient SIV infection. Thus our laboratory is uniquely positioned to characterize the correlates of protective cellular immunity in the context of attenuated SIV vaccination.
D. RESEARCH DESIGN AND METHODS

In this proposal, we use adoptive transfers to define the cellular immune responses elicited by live, attenuated SIV strains that are associated with control of pathogenic SIV. A timeline for the proposed experiments is shown in Figure 11. The experimental design is informed by murine studies that delineated the correlates of protective immunity against Friend retrovirus (FV). A landmark achievement in the study of FV immunity was the demonstration that FV-naive recipients transfused with splenocytes from mice vaccinated with attenuated FV resisted pathogenic challenge. We will examine whether protective immunity from SIVmac239Δnef macaques can be similarly be transferred to MHC-matched macaques.

Specific Aim 1: We will define the number of lymphocytes from SIVmac239Δnef vaccinated macaques necessary to transfer protective immunity to MHC-matched, SIV naive macaques.

SIVmac239Δnef-vaccinated macaques resist superinfection with SIVmac239. Plasma viremia following superinfection is generally several orders of magnitude lower than in vaccine naive animals. Attempts to quantify the magnitude of the immune response necessary for control have relied on indirect measurements such as the number of CD8+ T cells staining with a single SIV-specific MHC:peptide tetramer[16] or CTL precursor frequencies[33]. MHC-matched Mauritian cynomolgus macaques allow us to address this question directly by transferring variable number of lymphocytes from vaccinees into SIV naive recipients.

D.1.1.1: Selection of animals

This project requires 48 MHC-defined Mauritian cynomolgus macaques. Identifying these animals and testing their in vitro histocompatibility will take one year (Figure 11). Once identified, the animals will be purchased and transported to UW-Madison in the beginning of year 2. Identifying the animals in one large screen will give us maximal flexibility when selecting donor:recipient groups and will ensure that the animals needed for these experiments will be available.

Every animal will be either homozygous or heterozygous for the MHC H1 haplotype, providing a common set of MHC-restricted responses that can be evaluated. As part of an ongoing NIH R21, we are currently mapping minimal CD8+ T cell epitopes that are recognized in H1 MCM. We expect to complete
mapping and MHC restriction analysis on at least three epitopes restricted by alleles encoded on H1 by the end of 2007. MHC:peptide tetramers to detect T cells specific for these epitopes will be generated in collaboration with Drs. Nancy Wilson and David Watkins at UW-Madison[45]. Other funds are available to support the production of these tetramers; no additional funding for tetramer production is requested in this application.

The high frequency of the H1, H2, and H3 haplotypes leads to a large number of eligible animals. In a previous study of more than 100 MCM, 7.5% were H1/H1 homozygous; 6.8% were H1/H2 heterozygous; and 6.8% were H1/H3 heterozygous (Figure 12 and [86]). Thus, more than 20% of all MCM have one of the three haplotype combinations that will be used in this study. We expect that MHC screening 350 MCM will identify at least 26 H1/H1 homozygote, 23 H1/H2 heterozygote, and 23 H1/H3 heterozygote animals. Therefore, we will purchase blood from 350 MCM owned by Charles River Laboratories (CRL) for MHC screening (see attached letter of support). We will perform microsatellite based typing as previously described to identify animals with the appropriate MHC genetics. We can rapidly screen more than 350 animals per month with this technology, so we can reasonably expect to complete this genetic screen in the first months of the award.

After selecting candidate animals with the appropriate MHC genetics, we will test for in vitro histocompatibility using flow cytometric 1-way mixed lymphocyte reactions[46, 49] (see Preliminary Data section C.4). There will be genetic differences between the MHC-matched candidate animals on non-MHC loci that may influence MLR reactivity. Pairwise experiments using all of the candidate animals will allow us to identify specific MHC-matched donor:recipient groups with the best MLR profiles. PKH67 responder lymphocytes fluorescence will be measured prior to co-culturing with irradiated target lymphocytes. Fluorescence intensity will decline by 50% with each cell division as the PKH67 dye is equally distributed between daughter cells. Measurement of PKH67 fluorescence after 10 days in culture enables calculation of the percentage of input cells that divided and the average number of cell divisions for each dividing cell. Donor:recipient pairs where the mean number of cell divisions is greater than two times the mean number of cell divisions in autologous MLR reactions will be not be used for adoptive transfers. Allogeneic donor:recipient pairs with the lowest MLR proliferation will be prioritized to give the highest probability of adoptive transfer success.

D.1.1.2: Animal group sizes

The 48 animals will be divided into five groups (A-E) as shown in Figure 13. When Indian rhesus macaques are vaccinated with SIVmac239Δnef and challenged with pathogenic SIVmac239, peak plasma viral loads are reduced by an average of 5.0 log₁₀ copies / mL. The peak viral load difference between MCM and Indian rhesus infected with the closely related SIVmac251 is only 0.63 log₁₀ copies / mL[64]. It is therefore reasonable to expect a 4.0-5.0 log₁₀ copies /mL in peak viral load difference between SIVmac239Δnef-vaccinated and sham vaccinated MCM challenged with SIVmac239. Therefore, peak plasma viral load reduction is an appropriate readout for the functional effects of adoptive transfer.

It would be naive, however, to assume that adoptively transferred lymphocytes will completely recapitulate the effects of attenuated vaccination and result in a 4.0-5.0 log₁₀ reduction in peak plasma viral load. Therefore, we will design our study to detect a minimum 50-fold (1.5 log₁₀) reduction in peak plasma viral load with 90% power. This threshold is in accordance with previous studies demonstrating that 1.5 log₁₀ or greater reduction in peak viral loads are evidence of vaccine-mediated control and correlate with improved outcome[34, 55, 70, 85].

Using large sets of viral load data from animals infected with SHIV89.6P and SIVmac251, Parker and colleagues determined group size requirements for detecting significant differences in plasma vira
<table>
<thead>
<tr>
<th>Group</th>
<th>MHC</th>
<th>Donor Animals</th>
<th>Recipient Animals</th>
<th>Donor Vaccine</th>
<th>Adoptive Transfer Lymphocytes</th>
<th>Recipient Challenge</th>
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<tr>
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<td>H1/H1, H1/H1, H1/H2, H1/H2, H1/H3, H1/H3</td>
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<tr>
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<td>7, 8, 9, 10, 11, 12</td>
<td>37, 38, 39, 40, 41, 42</td>
<td>SIVmac239Δnef</td>
<td>5 x 10^{8} (immune sera only)</td>
<td>SIVmac239</td>
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<tr>
<td>D</td>
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<td>---</td>
<td>1 x 10^{10} (3 x naive sera 3 x naive lymphocytes)</td>
<td>SIVmac239</td>
</tr>
<tr>
<td>E</td>
<td>H1/H1, H1/H1</td>
<td>19, 20, 21, 22, 23, 24</td>
<td>--, --, --, --, --, --</td>
<td>SIVmac239Δnef</td>
<td>--</td>
<td>SIVmac239</td>
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</table>

Figure 13. Experimental plan for Specific Aim 1. The effect of lymphocyte dose on SIVmac239 protection will be assessed in Groups A-C. Group D will control for adoptive transfer effects independent of vaccination, while Group E will indicate the maximal level of control expected in SIVmac239Δnef-vaccinated MCM. Parenthetical passive transfers in Groups B and C will be performed only if the Group A adoptive transfers do not protect from SIVmac239 challenge, see D.1.5 for discussion.

Based on these calculations, 5 adoptive transfer recipients per group should be sufficient to detect a 1.5 log_{10} or greater reduction in peak plasma viral load relative to control animals with 90% power (Figure 15). Though peak viral load variability, a key parameter when performing power calculations, may be lower in SIVmac239-infected MCM than in the animals studied by Parker et al. because of the genetic homogeneity, we will be conservative in our study design and use 6 adoptive transfer recipients per group. Six animals per group will also facilitate analyses of adoptive transfer persistence and SIV-specific immunity.

D.1.2: Schedule
Donor animals from Groups A-C and E (Figure 13) will be vaccinated intravenously with SIVmac239Δnef (10 ng Gag p27 per dose) that will be provided by Dr. Ronald Desrosiers. The Group D animals will be sham vaccinated as a control to measure the non-specific effects of adoptive transfer on recipient immunity. Protection from intravenous challenge with SIVmac239 is consistently achieved six months following SIVmac239Δnef vaccination[15]. Each of the Group A-D donor macaques will be sacrificed and exsanguinated six months post-vaccination. Peripheral blood lymphocytes will be purified from the blood. Additionally, lymphocytes will be harvested from lymph nodes (inguinal, axillary, submandibular, mediastinal, mesenteric, and mesocolic) and spleen as described previously[72]. SIV-infected, asymptomatic juvenile rhesus macaques infected with SIV have approximately $2 \times 10^{10}$ total lymphocytes in these tissues([72] and Figure 14). In a preliminary experiment we collected $6 \times 10^9$ lymphocytes from the blood, spleen, and mesenteric lymph nodes of an SIV-infected Mauritian cynomolgus macaque at time of sacrifice. Therefore it is reasonable to expect that we can collect more than $1 \times 10^{10}$ total lymphocytes from each SIVmac239Δnef-vaccinated donor by harvesting cells from blood, spleen, and multiple lymph nodes.

Additionally, we will save large volumes of sera from every exsanguinated macaque in case passive immunizations are indicated.

Because these experiments require intensive sampling in the period immediately following adoptive transfer (Figure 15), we will stagger the vaccinations by group. Among the non-control groups, group A

Figure 14. Absolute numbers of lymphocytes in different macaque organs. White bars indicate uninfected macaques, light grey bars indicate SIV infected macaques without AIDS defining illness, and dark grey bars indicate SIV infected macaques with AIDS defining illnesses. LN=pooled cells from the lymph nodes used in this proposal. Juvenile rhesus macaques with an average weight of about 4 kg were used in this study; this is about the same size as adult Mauritian cynomolgus macaques. Figure modified from Sopper et al., 2003.

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Additionally, we will save large volumes of sera from every exsanguinated macaque in case passive immunizations are indicated.

Because these experiments require intensive sampling in the period immediately following adoptive transfer (Figure 15), we will stagger the vaccinations by group. Among the non-control groups, group A

Figure 15. Schematic study plan for Specific Aim 1 adoptive transfers. The intensive sampling required immediately after adoptive transfer and the tight timeline for the proposal requires us to offset the SIVmac239Δnef vaccinations between the groups and manage a large number of concurrent animal groups, necessitating a large personnel commitment. VL = plasma viral load; Per = persistence, homing, and trafficking of PKH67+ cells; T = ex vivo evaluation of SIV-specific CD4+ and CD8+ T cell responses; B = SIVmac239 neutralization assessment; IVSA = in vitro viral suppression assay; Act = T cell activation, memory, and homing phenotype assessment; Cyt = ELISA for soluble cytokine production. Boxed assays will be run on both the donor at time of sacrifice and the recipient immediately after adoptive transfer.
animals receiving $1 \times 10^{10}$ lymphocytes will be processed first. If this lymphocyte dose protects against subsequent SIVmac239 challenge, decreased lymphocyte doses of $1 \times 10^7$ and $5 \times 10^8$ cells will be tested in groups B and C, respectively. If $1 \times 10^{10}$ lymphocytes are not protective, we will reassign the group B and C animals. Group B animals will receive $1 \times 10^{10}$ lymphocytes plus an infusion of antibodies purified from sera from the same immunized donor used for lymphocyte transfer. Group C animals would receive immune sera only as a control. If the passive transfers are deemed necessary, we will also need a series of control animals in which vaccine naive lymphocytes and vaccine naive sera are transferred prior to challenge. Should this be required, the Group D animals will be redistributed for this purpose as shown in Figure 13. Strategically staggering the experiments in this way will allow us to examine synergistic effects of lymphocyte transfer and passive immunization with vaccine-elicited antibodies if high-dose lymphocyte transfer alone is not sufficient for protection. Dr. Dennis Burton (see attached letter of support) will provide assistance and guidance with antibody purification if passive antibody transfers are necessary.

The lymphocytes will be labeled with the fluorescent cell linker PKH67. Specific numbers of lymphocytes, ranging from $1 \times 10^7$ to $1 \times 10^{10}$, will be infused into each donor’s MHC-matched recipient as shown in Figure 13. We limit the number of animals needed for this experiment by titrating lymphocytes from donor animals 7-12 into multiple recipients.

48 hours after lymphocyte transfer, all recipient animals in Groups A-E will be challenged intravenously with 100 TCID50 SIVmac239 (grown in macaque PBMC). Rhesus macaques immunized with SIVmac239Δnef resist IV superinfection with this dose of SIVmac239 six months after vaccination (Dr. David Watkins, personal communication). We will monitor recipient MCM for at least nine months after SIV challenge.

D.1.3: Methods

D.1.3.1: Protection from superinfection. The primary endpoint for this experiment is reduction of SIVmac239 peak plasma viremia relative to the Group D animals mock vaccinated and challenged with SIVmac239. All viral loads will be measured using SIV quantitative RT-PCR (qRT-PCR) performed by the Virology Core at the Wisconsin National Primate Research Center. This service generates thousands of SIV viral loads yearly and is currently adapting qRT-PCR methodologies to discriminate between SIVmac239 and SIVmac239Δnef[52].

In the donors: SIVmac239Δnef viremia will be measured weekly for the first four weeks following vaccination and biweekly thereafter. In 16 rhesus macaques vaccinated with SIVmac239Δnef, viral loads were consistently low (<1000 vRNA eq/mL) and frequently below the limit of detection (<50 vRNA eq/mL) 6 months post-vaccination (Dr. David Watkins unpublished data). We expect setpoint viral loads vaccinated MCM to be similarly low and thus will not use viral loads as an inclusion or exclusion criteria for the adoptive transfers.

In the recipients: After adoptive transfer, viral loads will be measured 5 minutes before SIVmac239 challenge, 5 minutes after SIVmac239 challenge, and twice weekly throughout the first four weeks of infection. Viral loads will be monitored biweekly after the first month of infection. An average peak plasma viral load reduction of 50-fold ($1.5 \log_{10}$) relative to sham vaccinated Group D control animals will be taken as evidence of adoptive transfer-mediated immune control in Group A animals. If this level of protection is not achieved, we will restructure the remaining animal groups to test combination adoptive transfer and passive immunization as described in D.1.2. Viral loads in the adoptive transfer recipients will also be compared to the Group E animals immunized with SIVmac239Δnef and challenged with SIVmac239 to compare adoptive transfer-mediated immune control with the protection engendered by conventional immunization with attenuated SIV.

Because explosive viral replication occurs in the first two weeks of SIV infection, we expect that the most prominent effect of adoptively transferred cells will occur in this period. Nonetheless, it is possible that adoptively transferred lymphocytes will have a beneficial effect on plasma viral loads during chronic
infection. Designing a study to measure differences in chronic phase viral load setpoint requires larger animal groups because of higher interanimal variability. A prohibitively large 24 animals per group would be required to detect a 1.5 log_{10} reduction in setpoint viral load with 90% power at chronic phase setpoint. However, it is important to remember that we may still be able to resolve chronic phase setpoint differences in addition to differences in peak viral loads because the transfers are not statistically independent. In the FV model system, transfer of 1 x 10^7 or more splenocytes prevented FV-induced splenomegaly. While we do not know what minimum dose of lymphocytes will be protective, we expect that all doses higher than this minimum will also be protective.

**D.1.3.2: Persistence of adoptively transferred lymphocytes.** We expect transferred cells to persist in recipients for at least 14 days as previously observed (see Preliminary Data). We will collect blood from the recipient macaques at the same timepoints used for viral load determination and measure the persistence of PKH67+ cells by flow cytometry. PKH67 staining will be combined with cell surface markers for cell lineage (e.g., CD8, CD4, CD20), activation state (e.g., CD69, CD28, CD95), and tissue homing (CCR7, CCR9) to examine the fate of donor-derived lymphocytes in the blood. We will also obtain lymph node biopsies 14 days post-transfer to examine donor lymphocyte persistence in lymphoid tissue.

**D.1.3.3: Functional T cell immunity.** We will measure T cell responses to assess whether the transferred cells retain SIV-specificity and functional activity during immunization and the months following SIVmac239 challenge, before and after the emergence of de novo immune responses in the recipient. At three timepoints after SIVmac239Δnef immunization we will measure the development of vaccine-elicited T cell responses in the donor macaques. The same responses will be evaluated in the recipients after adoptive transfer and SIVmac239 challenge. The exact timepoints when T cell responses will be evaluated are shown in Figure 15.

We are in the process of defining CD8+ and CD4+ T cell epitopes restricted by MCM MHC class I alleles[86] and are currently fine-mapping these epitopes in order to design MHC:peptide tetramers. We are currently working with Dr. Nancy Wilson at the Wisconsin National Primate Research Center to develop these reagents in another project and we expect that epitope sequences and MHC:peptide tetramers specific for at least three CD8+ T cell responses restricted by alleles encoded on the H1 haplotype will be available by the anticipated start date for this project.

The tetramers will be conjugated to the fluorophore PE so that CD8, PKH67, and tetramer staining can be assessed concurrently. The percentage of CD8+, PKH67+, and tetramer+ lymphocytes will be measured at each timepoint following transfer. Individual SIV-specific tetramer+ T cells often comprise more than 1% (and as high as 8%) of total CD8+ lymphocytes during acute infection[56]. We will therefore base our tetramer assays on the assumption that 1% of CD8+ T cells in the recipient are PKH67+ and that 1% of these are SIV-specific for a given tetramer, necessitating approximately 1 x 10^6 total lymphocytes per assay in order to detect 10 tetramer+ events. Though the tetramer+ population is rare, this level of sensitivity is routine at UW-Madison and in other experienced flow cytometry laboratories[59]. Moreover, the frequency of tetramer+ lymphocytes will likely be higher if the initial frequencies of tetramer+ cells or PKH67+ cells is greater than 1% or if the tetramer+ cells proliferate after exposure to SIVmac239.

In addition to measuring the maintenance of SIV-specific CD8+ T cells within the adoptive transfer mixture using MHC:peptide tetramers, we also plan to verify that adoptively transferred cells retain functional capacity. We will use IFN-γ secretion as measured by ELISPOT[86] as a surrogate for functional capacity. While there may be T cells in our transfer mixture that do not elaborate IFN-γ, the epitopes that we expect to have mapped by the start of this project were all identified on the basis of IFN-γ production. To maximize the likelihood of detecting donor-derived, SIV-specific T cell responses by ELISPOT, we will plate 1 x 10^6 cells per well and incubate with individual peptides corresponding to minimal, optimal T cell epitopes[83]. All peptides will be tested in duplicate. Cells stimulated with the lymphocyte mitogen concanavalin A will be included in each assay as a positive control. Assay background will be assessed by incubating cells with an irrelevant nonamer peptide. A response will be scored positive when:
These calculations are performed automatically using a custom ELISPOT analysis system developed by the PI.

The H1 haplotype is found in nearly 40% of MCM. Mapping additional T cell responses restricted by alleles encoded on this haplotype, though ancillary to our primary objective of studying adoptive transfer mediated protection, will be valuable to other investigators who are using these MCM for SIV pathogenesis research. Therefore we will also perform a series of IFN-γ ELISPOT assays using peptide pools spanning the entire SIVmac239 and SIVmac239Δnef proteomes. Individual peptides from reactive peptide pools will be tested in a subsequent IFN-γ ELISPOT and optimal epitopes determined. We have a panel of B cell lines that each express a single MHC class I allele encoded on the H1 haplotype, allowing determination of epitope restriction. We expect to identify at least 10 additional SIV-specific T cell responses restricted by the five alleles encoded on the H1 haplotype in this analysis.

D.1.3.4: Antiviral efficacy of donor cells in vitro: While the detection of SIV-specific lymphocytes indicates that an individual has generated an immune response to SIV, it does not measure functional suppression of viral replication. Since our goal is to transfer functional immunity that suppresses viral replication, we will measure whether lymphocytes from the donor animals have the capacity to suppress SIV viral replication in cells from an MHC-matched recipient animal in vitro. Friedrich and colleagues have shown that PBMC isolated from SIV infected animals can suppress viral replication in autologous CD8-depleted cells that have been superinfected with SIVmac239[24]. Because our donor:recipient pairs are MHC-matched, we believe that it will be possible to adapt this assay to study allogeneic target and effector populations.

We will generate CD4+ target cells by isolating PBMC from the recipient animals, depleting CD8+ cells by negative selection, and phytohemagglutinin-stimulating the remaining lymphocytes for 24 hours in order to increase the number of SIV-susceptible targets. The CD8- targets will be incubated for 4 hours with SIVmac239 at a multiplicity of infection of 5 x 10^{-5}.

To generate effector cells, PBMC will be isolated from the MHC-matched donor animals at 2, 4, and 6 months after SIVmac239Δnef immunization. Varying concentrations of effector cells will then be incubated with the SIVmac239-infected target cells. After 7 days, the entire cell population will be stained for intracellular p27 to measure whether the extent to which donor cells are suppressing viral replication.

The target cells will be plated at a density of 5 x 10^5 cells per well in a 24 well plate and incubated with effector cells for 8 days[44]. Every other day, 0.5 mL of supernatant will be removed and replaced with fresh culture media. SIV viral load in the supernatant will be determined as described in D.1.3.1. The target cells will also be stained for intracellular SIV p27. These measurements will be used to calculate the extent to which donor cells are suppressing viral replication. As a control for non-specific lymphocyte suppression and to provide a baseline for viral suppression readings, target cells will be cocultured with SIV naive PBMC in each assay. When high concentrations of individual CD8+ T cell lines are used in suppression assays, greater than 20-fold reductions in viral replication (as measured by either supernatant viral load or intracellular SIV p27 staining)[43, 44]. We will therefore define ‘effective’ in vitro suppression as a 20-fold or greater viral suppression relative to the SIV naive controls.

By examining viral suppression in vitro in parallel with examining protection in vivo, we can determine whether strict suppression of viral replication in vitro is indeed a measure of functional immunity that can be transferred from donor to recipient. We expect that PBMC from all SIVmac239Δnef vaccinated donors, but not sham vaccinated donors, will suppress viral replication in vitro. However, it is possible that viral suppression will occur in vitro, but not in vivo. This result may indicate either that we have not transferred enough lymphocytes to suppress viral replication in the recipient or that the
transferred cells did not reach the sites of viral replication and were thus limited in their antiviral effect. Alternatively, the donor cells may not possess antiviral activity in vitro, but they will in vivo. This result may also suggest that the in vivo environment provides other factors that promote suppression of viral replication that cannot be replicated in vitro.

D.1.3.5: Neutralizing antibody activity: Our collaborator Dr. Ronald Desrosiers will measure SIVmac239 neutralizing antibody activity as described previously[88]. Concerns that SIVmac239 is an inappropriate challenge virus because of its neutralization resistance are unfounded. SIVmac239 is regularly neutralized by plasma from SIVmac239-infected monkeys (Dr. Ronald Desrosiers, unpublished data). How does this compare to the human situation with HIV-1? **SIVmac239 neutralizing titers appear to be at the low end of the range of titers observed by Richman and colleagues against the homologous infecting HIV-1 strain[65].** Measuring neutralizing antibody activity, therefore, is essential given recent data showing that CD20+ B cells are associated with SIVmac239 control[53].

In the donors: Neutralizing antibody activity will be measured 4 weeks, 12 weeks, and 24 weeks after SIVmac239Δnef vaccination.

In the recipients: Neutralizing antibody activity will be measured before adoptive transfer, at the time of adoptive transfer, 1 week post-SIVmac239 challenge, 4 weeks post-SIV challenge, and 12 weeks post-SIV challenge. These timepoints are selected to assess antibodies produced by transferred B cells both before and after the development of de novo antibody responses in the recipients.

To measure neutralization antibody activity, a secreted engineered alkaline phosphatase (SEAP) reporter cell assay will be used[50]. In this assay, SEAP is under transcriptional control of the SIV long terminal repeat, such that SEAP expression is proportional to the amount of input virus. A fixed amount of SIVmac239 is incubated with 1:2 to 1:200 dilutions of heat-inactivated plasma. SEAP activity is measured by incubating cultures with a chemoilluminencescent SEAP substrate. Sera with neutralizing antibody activity decrease the amount of SEAP relative to sera from naive controls. In unpublished data from the Dr. Desrosiers laboratory, 7/7 SIVmac239 infected Indian rhesus macaques developed neutralizing antibodies with an average 50% neutralization titer of 1:48. Negative controls show no reduction in SEAP activity at any dilution, providing confidence that any reduction in SEAP activity is the consequence of neutralizing antibodies.

D.1.3.6: T cell activation in adoptive transfer recipients: Though we hypothesize that transferred lymphocytes will confer clinical benefit, adoptive transfer may disrupt immunological homeostasis, with unforeseen consequences. Unfractionated, autologous PBMC transfers in HIV+ patients increase peripheral CD4+ counts by 25% two weeks post-transfer, an increase that is difficult to reconcile with the number of transferred cells (<1% of systemic lymphocytes)[79]. Activation and proliferation of CD4+ T cells in MCM adoptive transfer recipients could potentially increase the pool of SIV-susceptible target cells[42]. We will use two complementary approaches to measure T cell activation in the adoptive transfer recipients.

We will phenotype both donor and recipient lymphocyte populations using three panels of activation markers and one panel of memory phenotype markers as shown in Figure 16. The activation makers HLA-DR, CD38, CD69, CD25, and CD70 will be measured alongside the memory markers CD95 and CD28. Six color flow cytometry using a BD LSRII will allow us to measure each of these parameters in conjunction with CD3, CD4, CD8, and PKH67. All of these antibodies have been used successfully with cynomolgus macaque samples as confirmed by the NIH Nonhuman Primate Reagent Resource, the manufacturer, or use in our lab.

This staining strategy will enable separable measurements of CD4+ and CD8+ T cell activation in both the donor and recipient lymphocytes. Activated cells are expected to be HLA-DR+, CD38+, CD69+ and CD25+. We will look for an increase over baseline staining for these surface markers. Performing this comprehensive series of surface stains will require 3 x 10^6 lymphocytes per timepoint. The frequent
### Activation Panel I

<table>
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<tr>
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<th>Fluorochrome</th>
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<td>CD4 T cell identifier, MHC class II co-receptor</td>
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### Activation Panel II

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### Activation Panel III

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### Memory Phenotype Panel

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<td>CD3</td>
<td>SP34-2</td>
<td>Pacific Blue</td>
<td>Associated with T cell receptor (T cell marker)</td>
</tr>
<tr>
<td>CD95</td>
<td>DX2</td>
<td>APC</td>
<td>Binds FAS ligand and induces apoptosis</td>
</tr>
<tr>
<td>CD28</td>
<td>15E8</td>
<td>Biotin/Strep PerCP Cy5</td>
<td>Receptor for costimulation (activation of naïve T cells)</td>
</tr>
<tr>
<td>CCR5</td>
<td>3A9</td>
<td>PE</td>
<td>Co-receptor for SIVmac239</td>
</tr>
<tr>
<td>PKH67</td>
<td></td>
<td>FITC</td>
<td>Donor derived lymphocyte identifier</td>
</tr>
</tbody>
</table>

### Homing Panel

<table>
<thead>
<tr>
<th>Marker</th>
<th>Clone</th>
<th>Fluorochrome</th>
<th>Biological Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4</td>
<td>L200</td>
<td>PerCP Cy5.5</td>
<td>CD4 T cell identifier, MHC class II co-receptor</td>
</tr>
<tr>
<td>CD8</td>
<td>RPA-T8</td>
<td>Pacific Blue</td>
<td>CD8 T cell identifier</td>
</tr>
<tr>
<td>CD3</td>
<td>SP34-2</td>
<td>Alexa Fluor 700</td>
<td>Associated with T cell receptor (T cell marker)</td>
</tr>
<tr>
<td>CCR7</td>
<td>150503</td>
<td>APC</td>
<td>Homing to lymph nodes</td>
</tr>
<tr>
<td>B7</td>
<td>FIB504</td>
<td>PE</td>
<td>Integrin associates with alpha4 (CD49d) to home to non-pulmonary mucosal sites</td>
</tr>
<tr>
<td>PKH67</td>
<td></td>
<td>FITC</td>
<td>Donor derived lymphocyte identifier</td>
</tr>
</tbody>
</table>

**Figure 16. Flow cytometry staining study plan.** In order to understand the activation, phenotype, and homing of T cells within the adoptive transfer population, we will comprehensively analyze the phenotype of lymphocytes using antibodies specific for cell surface markers. All antibodies except those shaded have been used successfully in cynomolgus macaques. The two shaded antibodies have been successfully used in rhesus macaques. PKH67 is included in each staining panel to differentiate donor-derived from recipient lymphocytes. Additional antibodies may be included as novel macaque flow cytometry reagents become available.
blood sampling required for viral load in the weeks following adoptive transfer limit the number of large blood draws that provide the requisite number of lymphocytes. We will therefore perform this analysis in the recipient animals 4 weeks before adoptive transfer, at the time of adoptive transfer, and once weekly throughout the first four weeks of infection.

Cytokine ELISA assays will also be employed to determine whether proinflammatory cytokine levels increase following adoptive transfer. Commercial ELISA kits that have been validated in cynomolgus macaques are available for proinflammatory cytokines TNF-alpha, IL-1, IL-12, and IFN-gamma and antiinflammatory cytokines IL-4 and IL-10. Plasma concentrations of these cytokines will be assessed 24 hours, 3 days, and 2 weeks following adoptive transfer. As a baseline, cytokine concentrations will be determined in the recipient animals four weeks before adoptive transfer.

D.1.4: Anticipated Outcomes

We do not know of any previous studies examining the transfer of SIV- or HIV-specific cells prior to transmission. There is, however, an anecdotal report that 2/2 cats resisted FIV challenge after receiving cell infusions from MHC-matched donors[63]. Therefore, it is reasonable to anticipate that a similar protective threshold exists in SIV-challenged monkeys. Demonstrating protection with total lymphocytes, including antibody-producing CD20+ B cells, would provide unequivocal evidence for cellular immunity in control mediated by attenuated vaccines and would establish a framework for defining the minimal correlates of protection. In the Friend virus model, whole splenocytes provided a benchmark for assessing the protective value of individual lymphocyte subsets and lymphocyte combinations[20, 21].

Using our preliminary data as a guide, we expect the transferred cells to persist for at least 14 days. Fortuitously, this suggests that donor cells will be present throughout the window of explosive SIV replication that happens immediately after infection.

D.1.5: Projected Difficulties and Alternative Approaches

Allogeneic adoptive transfer studies have been impossible to perform in nonhuman primates before our discovery of MHC-matched MCM, thus there is scant data informing our experimental design.

One concern in our experimental design is that lymphocyte transfers may have non-specific suppressive or enhancing effects on SIV replication. We doubt this will be problematic as we infected an MCM with SIVmac239 48 hours after adoptive transfer with 1 x 10^7 CD8β+ T cells from an uninfected donor. The peak viral load in this animal was 9.0 x 10^6, characteristic of naive MCM infected with the same virus. Nonetheless, we address this concern by including a group of mock-vaccinated controls (Group D) and comprehensively measuring T cell activation and cytokine production. We are also aware that the maximal proposed transfer of 1 x 10^10 lymphocytes may have unforeseen consequences on the recipient monkeys, even though a comparable lymphocyte dose has been used in humans with minimal toxicity[54]. Because we have previous experience isolating, labeling, and adoptively transferring CD8β+ T cells, we do not foresee any significant technical hurdles with these experiments.

It is also possible that clinical benefits afforded by adoptive transfer may be greater than predicted by perturbations in peak viral load alone[81]. Since the cells in our pilot experiment persisted for approximately two weeks, peak viral load (which typically occurs approximately two weeks after infection) is the most logical indicator of clinical effect and our groups are powered to detect significant differences in this parameter. We will, however, continue to monitor all animals challenged with SIVmac239 for at least nine months to assess whether the adoptive transfer has any effect on establishment of setpoint plasma viral load.

Another potential difficulty is that the transferred lymphocytes, collected from various tissues, will home to tissues and be difficult to detect in blood. When lymphocytes from blood, spleen, and mesenteric lymph nodes were transferred, a higher percentage of donor cells was observed in a lymph node biopsy than in blood 9 days post-transfer. Nonetheless, donor-derived PKH67+ cells could still be detected in the blood. To further address this issue, we will examine the trafficking and homing phenotype of PKH67 labeled lymphocytes in the blood and will collect a lymph node biopsy 14 days post-transfer to directly
SIVmac239Δnef-infected CD4+ T cells will establish infection in the recipient animals before exposure to SIVmac239 since as few as 2 SIV-infected cells can establish intravenous infection[71]. The recipient animals will be challenged with SIVmac239 48 hours after cell transfer so carryover SIVmac239Δnef will not be present long enough to elicit protective immunity in the recipient[73]. We are developing qRT-PCR viral load diagnostics that differentiate between SIVmac239Δnef and SIVmac239 in order to assess the contribution of each to total viral burden. While specific transfer of SIV-negative cells would theoretically remove this confounding variable, cell separation purity would need to exceed 99.99999%.

Perhaps the most significant concern is that even at the highest lymphocyte dose, we will not observe protection against SIVmac239 in the recipients. Again, limited data from the FIV model suggests that lymphocytes alone can be protective[63]. However, it is possible that lymphocytes, in the absence of preexisting antibodies or other non-lymphocytic components, are insufficient to reconstitute protective immunity against SIVmac239. This observation could potentially prompt investigations into other immunological consequences of live, attenuated vaccination. Alternately, it is possible that failure to protect is due to methodological aspects of vaccination, adoptive transfer, or challenge. However, it is impossible to control for each of these complicated issues in these early-stage experiments.

Nonetheless, we have established a schedule and contingency plan that allow us to adapt to this possibility. It is unlikely that lymphocyte doses lower than $1 \times 10^{10}$ will be effective if the highest dose is not protective. Therefore we will test this high dose first. If it is protective, we will test lower lymphocyte doses in groups B and C. If the group A animals receiving $1 \times 10^{10}$ are not protected, we will use the group B and C animals to investigate the combined effect of $1 \times 10^{10}$ lymphocytes and antibody-containing immune sera.

Summary and Overview
Adoptive transfer studies are pivotal for understanding immunity to pathogens. Until now, these studies have not been possible in macaque monkeys, the most relevant model for HIV pathogenesis and vaccine research. Here we propose a series of provocative experiments designed to test whether cellular immunity is responsible for protection by live, attenuated SIVmac239Δnef. Successful completion of these aims will identify immune responses that should be elicited by rationally designed vaccines and will generate an entirely novel, innovative, and significant framework for studying HIV/AIDS.

LITERATURE CITED


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