A. SPECIFIC AIMS

A.R21: Correlates of protective cellular immunity in MHC-identical, SIV+ Mauritian macaques

After more than a quarter century of study, we do not have an effective human immunodeficiency virus (HIV) vaccine. The failed STEP trial in 2007 revealed how little is known about the quality and quantity of cellular immune responses necessary to protect against HIV or its nonhuman primate equivalent, simian immunodeficiency virus (SIV). The nature of protective cellular immune responses may be elucidated by studying individuals who spontaneously control HIV/SIV(1). Major histocompatibility complex (MHC) genetics, and presumably MHC-restricted cellular immune responses, are important in establishing and maintaining viral control(1-4).

We have preliminary evidence that Mauritian cynomolgus macaques (MCM) possessing the M3 major histocompatibility complex (MHC) class IB haplotype encoding the alleles *Macaca fascicularis* (Mafa)-B*45, -B*51, and I*100101 (I*10) effectively control SHIV(5) and SIV. These macaques may represent an extremely powerful model for dissecting CD8+ T cell responses responsible for spontaneous control. In this R21 we will test the hypothesis that these alleles restrict SIV-specific CD8+ T cell specificities that reduce in vitro SIV replication by more than 50%. We will explore this hypothesis in two Specific Aims:

Specific Aim 1: Identify immunodominant and subdominant epitope-specific CD8+ T cell responses restricted by Mafa-B*45, -B*51, and -I*10.

Specific Aim 2: Evaluate the capacity of SIV-specific CD8+ T cells restricted by Mafa-B*45, -B*51, and -I*10 to suppress SIVmac239 replication in vitro.

If one or more suppressive CD8+ T cell response(s) restricted by alleles encoded on the MHC M3 haplotype are identified, we will evaluate whether adoptive transfer of these cells can blunt acute SIV replication in an R33. There is thus one milestone governing advancement into the R33:

Milestone 1) Definition of at least one SIV-specific CD8+ T cell response restricted by Mafa-B*45, -B*51, or I*10 that reduces in vitro SIVmac239 replication by 50%.

A.R33: Assess suppression of viral replication by SIV-specific CD8+ T cells in vivo

Disheartening vaccine results are not exclusively the purview of HIV research. Despite declaration of ‘war on cancer’ in 1972 and three subsequent decades of research, cancer vaccines are ineffective(6). Astounding tumor regression has been achieved, however, following adoptive transfers of in vitro expanded tumor-specific CD8+ T cells(7). We hypothesize that expanded CD8+ T cells that suppress viral replication in vitro will reduce the severity of acute SIVmac239 infection in vivo. We will test this hypothesis by completing two specific aims:

Specific Aim 3: Define conditions that enable persistence of adoptively transferred in vitro expanded CD8+ T cells over a 14 day period

Specific Aim 4: Assess whether adoptive transfer of in vitro expanded, SIV-specific CD8+ T cells reduces the severity of acute SIVmac239 infection.

Adoptive transfer experiments could illuminate the types of T cell responses that should be elicited by future vaccines. In 2008 we were the first to describe successful allogeneic adoptive lymphocyte transfer in nonhuman primates(8). This model offers the tantalizing possibility of being the first and only way to unequivocally define the importance of individual immune responses in acute SIV control. Our experience working with Mauritian macaques and performing ex vivo adoptive transfers uniquely positions us to lead these innovative studies.
B. BACKGROUND AND SIGNIFICANCE

B.1: CD8+ T cell responses and HIV vaccines

Individuals living with HIV mount vigorous, virus-specific CD8+ cytotoxic T-lymphocyte (CTL) responses. The discovery of these responses(9, 10) galvanized scientists to explore vaccines that aim to elicit protective CTL responses prophylactically. Conventional immunizations with viral subunits or killed viruses do not efficiently prime HIV-specific CTL(11), prompting exploration of innovative vaccine modalities such as immunization with recombinant adenovirus serotype 5 (Ad5) viral vectors.

The impressive ability of Ad5 vectors to elicit T cell responses in macaques and humans(12) led to the initiation of a Phase 2 test-of-concept trial sponsored by Merck & Co. and the Human Vaccine Trials Network in 2005. Though more than 60% of vaccinees mounted HIV-specific CD8+ T cell responses, the trial was aborted in September 2007 after an interim analysis by the Data Safety and Monitoring Board concluded that continuation of the study would not meet its endpoints of reducing HIV acquisition or early plasma viral load(12).

B.2: Defining protective CD8+ and CD4+ T cell responses in HIV/SIV controllers

The early termination of the Merck Ad5 clinical trial leaves T cell-based vaccines at a crossroads. There is clearly an urgent need for assessing which, if any, prophylactic CD8+ T cells can protect against HIV.

Approximately 1% of all HIV+ people spontaneously control viral replication without therapy(1, 13, 14). This group of controllers is further stratified into ‘elite controllers’ who durably suppress viral replication below 50 copies viral RNA equivalent/mL blood plasma and ‘viremic controllers’ who maintain viral loads lower than 2000 copies / mL(1). The T cell responses mounted by these individuals may provide clues to the responses that should be engendered by vaccines(13).

Viral control is easier to study in macaque cohorts infected with a single strain of SIV; however, T cell responses are still highly variable as a consequence of their diverse major histocompatibility complex (MHC) genetics(15-17). Macaques with uniform MHC genetics would have the same potential to recognize the same T cell epitopes(18, 19) whose contribution to MHC would be directly assessed.

B.3: In vitro measurements of T cell effectiveness

Simple measurements of cytokine production by CD8+ T cells have not successfully defined the correlates of protective immunity in HIV/SIV controllers(13, 20). Production of cytokine by T cells may be analogous to binding to the viral surface by antibodies; necessary, but not sufficient to mediate a biologically relevant effect. Suppression of viral replication in vitro may more closely approximate what happens in vivo and be a more germane measure of T cell quality. There is, therefore, considerable enthusiasm for in vitro T cell suppression assays (IVSAs), functional analogs to neutralizing antibody assays that measure virus production(21, 22).

IVSA assays are straightforward. Cultures of SIV-susceptible CD4+ target cells are infected with SIV immediately before the addition of virus-specific CD8+ T cells. SIV replication, measured as either viral copies in the culture supernatant or the percentage of infected cells, is compared against control samples to which non-specific CD8+ T cells (e.g., MHC mismatched SIV-specific CD8+ T cells or MHC-matched T cells specific for a pathogen other than SIV) are added. IVSA assays more accurately model the complex antigen processing and presentation machinery and provide a clearer picture of which T cells have the greatest capacity to impact virus production, in essence, revealing the T cell equivalent to neutralizing antibodies. Because these assays can use T cell effectors expanded in vitro, head-to-head comparisons of immunodominant and subdominant CD8+(21, 23, 24)T cell specificities are possible.
B.4: MHC-identical Mauritian cynomolgus macaques

We may have discovered an exciting new model for studying spontaneous control of SIV. An exotic invasive population of approximately 50,000 cynomolgus macaques lives on the Indian Ocean island of Mauritius(25), approximately 2000 km from the southeast coast of Africa. This geographically isolated population of monkeys descends from an extremely small founder population introduced to the island within the last 500 years(26). Mauritian cynomolgus macaques (MCM), like their Asian counterparts, are susceptible to SIV and SHIV strains including SIVmac239, SIVmac251, and SHIV89.6P(27, 28), but have dramatically reduced genetic diversity. Six MHC haplotypes account for more than 99% of the MHC diversity in these animals((29) and Section C.2). This provides a model where individuals with the exact same MHC genetics can be infected with the same strain of SIV, facilitating identification and comparison of cellular immune responses and complementing existing systems for studying spontaneous HIV/SIV control.

Two MCM MHC haplotypes, termed M3 and M6, encode MHC class IB alleles significantly associated with in vivo control of SHIV89.6P(5). More than 20% of feral MCM are M3+, the M6 haplotype is much rarer. Interestingly, the M1 haplotype is also very common. 5% of MCM have one M3 haplotype and one M1 haplotype. Thus, 5% of feral MCM share the same MHC alleles, including the M3-encoded alleles Mafa-B*45, -B*51, and -I*10 that are associated with SIV control. These M1/M3 MHC-identical animals have the potential to recognize the same MHC:peptide complexes when infected with SIV, providing an opportunity for studying SIV control in which both the infecting virus and MHC genetics can be held constant.

Another unique opportunity afforded by the identification of MHC-identical MCM is the capability to perform adoptive cell transfer (ACT) experiments. For the remainder of this proposal, ACT refers to adoptive cell transfer with in vitro expanded CD8+ T cells unless otherwise noted. ACT experiments require MHC matching between donor and recipient. We have shown that ex vivo ACT is well-tolerated between MHC-identical MCM without immunosuppression(8), a first in nonhuman primates.

B.5: Antigen-specific T cell Adoptive Cell Transfers

There are significant parallels between T cell responses to solid tumors and HIV/SIV. T cells specific for tumor antigens are often detected in the blood of cancer patients, but as with the T cells directed against HIV/SIV, the clinical relevance of these responses is controversial(30). The importance of T cells is underscored by the observation that they exert selective pressure on both tumor-specific antigens(31) and HIV/SIV(32), resulting in the emergence of T cell-resistant escape mutants. As with HIV/SIV(33), individual susceptibility to certain cancers is markedly increased or decreased by specific major histocompatibility complex class I alleles(34). Given these similarities, promising strategies in clinical oncology may have direct applicability to the study of T cell responses to HIV/SIV.

ACT with CD8+ T cell lines or clones is a clinically validated method for treating certain cancers(35-38). There are astounding examples of cancer regression in ACT recipients (Figure 1); 50% of treatment-refractory metastatic melanomas respond to ACT, with anecdotal reports of complete clinical remission(7).

ACT is conceptually simple. Tumor-specific or virus-specific T cells are expanded in vitro, using either the patient’s own blood or blood from an MHC-matched donor (e.g., an SIV-infected MCM). The expanded lymphocytes are infused intravenously, typically at concentrations exceeding 1 x 10^9 cells per dose(7). Objective tumor regression can occur when donor cells comprise an average of 1.47% of a recipient’s peripheral blood CD8+ T cells(37). Some have reported improved results when patients are conditioned for ACT by short-term, non-myeloablative lymphodepletion and regular infusions of high-dose IL-2(7, 39). Under these conditions, transferred cells can comprise up to 75% of total peripheral CTL for 6-12 months after infusion(7, 38).
B.6: Adoptive T cell therapy in HIV

ACT has been attempted as an HIV immunotherapy with mixed results. Koenig and colleagues derived HLA-A3.1-restricted Nef QK10 CD8+ T cell clones from an HIV+ donor. After *in vitro* expansion, approximately $1 \times 10^{10}$ of these cells were reinfused into the donor. The T cells exerted dramatic selective pressure; nearly 20% of the viruses sequenced after transfer lacked the QK10 epitope entirely (40). Though this ACT regimen did not confer obvious therapeutic benefit, the selection of viral variants by the QK10 CD8+ T cells provides remarkable evidence of function in the context of an established, chronic, systemic infection. A subsequent study demonstrated that adoptively transfered CD8+ T cells traffic to lymph nodes and co-localize with HIV-infected cells (41).

No one has used ACT to induce prophylactic immunity to HIV or SIV, but there is precedent for using ACT in viral prophylaxis. Multiple studies (reviewed in (42)) have used ACT prophylaxis for CMV and EBV in bone marrow transplant recipients. In the largest study, 0 of 39 patients receiving CD8+ ACT developed EBV lymphoma (43).

**ACT studies could determine whether CD8+ T cells alone protect against SIV.** Until now, it has been impossible to perform these experiments. ACT is dependent on MHC-matching of donor and recipient. When ACT is used prophylactically to prevent EBV and CMV, the infused lymphocytes are usually derived from the MHC-matched bone marrow donors. **MHC-identical MCM are the only feasible nonhuman primate model for exploring SIV-specific ACT.**

B.7: Summary

This phased Innovation Grant proposal has two basic objectives. First, we will determine whether any of the CD8+ T cell responses restricted by Mafa-B*45, -B*51, or -I*10 suppress viral replication *in vitro*. If successful, we will investigate whether CD8+ T cell ACT can reduce the explosive viral replication that occurs in the first two weeks of infection. The results from these experiments will inform the development of future vaccines and **provide a robust system for predicting the impact of CD8+ T cells on viral control.**
C. PRELIMINARY DATA

C.R21: Overview

In 2005 our group recognized that the restricted major histocompatibility complex (MHC) diversity of Mauritian-origin cynomolgus macaques (MCM) could enable studies of cellular immunity previously thought impossible in nonhuman primates. Over the ensuing three years we developed the genetic, immunologic, and virologic tools necessary to study SIV-specific CD8+ T cell responses in MCM.

C.R21.1: The MHC genetics of MCM are solved

The MHC is exceptionally polymorphic in Asian rhesus and cynomolgus macaques. Gene duplication, evolution, and recombination make the macaque MHC even more complex than the human HLA(44, 45). Completely identical haplotypes spanning the 5 million base pairs encompassing the MHC class I and II loci are almost never shared in unrelated Asian macaques(46); well-studied alleles such as the Indian rhesus macaque Mamu-A*01 are inherited on mosaic haplotypes that differ from animal to animal. In remarkable contrast, there are only 6 major MHC haplotypes, termed M1-M6 in MCM(29). M7 and M8 haplotypes are detected in less than 1% of MCM.

The eight haplotypes vary in their gene content and population frequency. In a feral population of more than 425 animals, we discovered that the M1, M2, and M3 haplotypes are by far the most abundant(29). MCM homozygous and heterozygous for these three haplotypes are extremely common. More than 5% of MCM are completely MHC-identical, M1/M3 heterozygotes (Figure 2A). All experiments in this proposal will use these MHC-identical, M1/M3 MCM.

We identified all of the MHC class I A and B(29) and MHC class II -DRA, -DRB, -DPA, -DPB, -DQA, and -DQB alleles(47) encoded on the M1 and M3 haplotypes (Figure 2B). In addition, there is an MHC class I 'I allele (Mafa-I*100101) encoded on the M3 haplotype. The I locus likely arose from a recent duplication of an MHC class I B locus and is tightly linked with MHC class IB genes. Unlike conventional MHC class I B alleles, there is very little variability among known MHC class I alleles(48). It is not known whether MHC class I I alleles can present peptides to CD8+ T cells. Since Mafa-I alleles arose from Mafa-B alleles, we consider Mafa-I*10 to be part of the M3 MHC class IB haplotype.

Because the genetics of the M1 and M3 haplotype are solved, we know all the alleles that could potentially restrict CD8+ T cell responses against SIV.

C.R21.2: The linked alleles Mafa-B*45, -B*51, and -I*10 are associated with SHIV/SIV control

The six major MHC haplotypes are associated with variable levels of AIDS virus control. 22 MCM in a vaccine trial were challenged with SHIV89.6P. Viral loads did not differ between vaccine and control groups. When the animals were stratified based on MHC class IB haplotype, clear patterns emerged. Macaques possessing either the M2 or M5 haplotypes had the highest viral loads, while those possessing M3 and/or M6 haplotypes had, on average, 100-fold lower viral loads (Figure 3A)(5). Six of seven animals with the M3 class IB haplotype controlled viremia below the limit of qRT-PCR sensitivity beyond one year post-challenge.

There are several important caveats to this data. Though there does not appear to be a vaccine effect in the M3/M6 MCM, the possibility cannot be excluded. The challenge virus, SHIV89.6P, was less pathogenic than SIVmac239, the highly pathogenic SIV reference strain used widely for vaccine and pathogenesis work. In addition, several of the animals in the study had recombinant MHC haplotypes, which complicated analysis.

To further investigate the importance of M3 class IB haplotypes in SIV control, we examined...
viral loads in a small number of MCM infected with SIVmac239. By 24 weeks post-infection, 4/4 M3+ animals have viral loads less than 1000 copies/mL, in contrast to the average of 14,000 copies/mL in two animals that are M3- (Figure 3B). Though the number of animals in the two groups are unfortunately and unavoidably small, the data is entirely consistent with the SHIV89.6P findings. The MHC class IB alleles encoded on the M3 haplotype, Mafa-B*45, -B*51, and -I*10, may therefore restrict particularly effective CD8+ T cell responses that control SIV replication.

**FIGURE 2. THE MHC GENETICS OF M1/M3 MCM ARE SOLVED.** (A) Frequencies of each MHC haplotype in MCM. M3/X indicates the percentage of MCM that have one intact M3 haplotype, irrespective of what haplotype is on the second chromosome. Note that more than 5% of MCM are MHC-identical, M1/M3 heterozygous. (B) The MHC class I and class II alleles encoded on the M1 and M3 haplotypes are shown. Therefore we know all the MHC alleles that can restrict CD8+ and CD4+ T cell responses in M1/M3 MCM. A hypothetical Indian rhesus macaque haplotype containing Mamu-A*01 is shown to illustrate the difference between a high frequency allele genotype (e.g., Mamu-A*01) and a high frequency haplotype (e.g., M3) containing a set of linked allele genotypes.
We have developed a workflow for rapidly characterizing both immunodominant and subdominant SIV CD8+ T cell responses in MCM. IFN-γ ELISPOT is used to identify immunodominant responses. Bulk PBMC from infected MCM are pulsed with 15-mer peptide pools spanning every SIVmac239 protein. Positive responses are deconvoluted to individual 15-mer peptides, and further fine-mapped to 9-11 amino acid minimal, optimal epitopes by either ELISPOT or intracellular cytokine staining. MHC class I restriction is determined by testing responses against 721.221 B cell lines stably transfected with single MHC class I alleles and pulsed with the minimal, optimal peptide. We have identified one to two immunodominant responses restricted by Mafa-A*25, -A*29, -B*44, and -A*29, -B*44, and -B*51.
-B*51 (Figure 4), verifying the MHC class I restriction of one previously identified response in the process(49). This demonstrates that we can define optimal CD8+ T cell epitopes and determine their MHC class I restriction. Moreover, the detection of a Mafa-B*51-restricted response provides unequivocal evidence that at least one M3 class IB allele is functional.

Whole-proteome scans using peptide pools typically identify only robust, immunodominant responses. Subdominant CD8+ T cell responses are more difficult to map. Testing optimal 8-to-11-mer peptides reveals more subtle responses, but requires thousands of peptides per SIV proteome. To streamline identification of subdominant CD8+ T cell responses, we collaborated with Dr. William Hildebrand to sequence endogenous peptides bound to Mafa-B*51 and four other MCM MHC class I alleles. The sequences revealed the peptide binding motif each allele. Figure 5 shows the motif for Mafa-B*51, along with an immunodominant Mafa-B*51-restricted SIV epitope sequence identified in our laboratory. Motifs for the two other alleles encoded on the M3 class IB haplotype, Mafa-B*45 and Mafa-I*10, are being determined and should be available by this project’s anticipated start date of April 1, 2009.

In this proposal, we will scan the SIV proteome for peptides that fit predicted peptide binding motifs for Mafa-B*45, -B*51, and -I*10. These predicted epitopes will be tested for recognition by CD8+ T cells. Our group has considerable experience with this approach to comprehensive epitope identification; we helped identify subdominant CTL epitopes restricted by the rhesus macaque alleles Mamu-A*01(50) and Mamu-A*02(51).

C.R21.4: In vitro T cell suppression assays in MCM

We are performing in vitro SIV suppression assays (IVSA) with MCM(21, 23, 52). Existing IVSA assays are hampered by significant animal-to-animal variability in SIV growth kinetics. Eliminating this confounding variable would facilitate comparisons of different T cell specificities. We hypothesized that MHC-matched MCM would be histocompatible in vitro, enabling IVSA assays that use target cells from a single animal with multiple allogeneic effector T cell lines. In vitro histocompatibility was tested in a mixed-lymphocyte reaction. As shown in Figure 6, M1/M3 effectors do not proliferate in the presence of irradiated, M1/M3 allogeneic targets. This suggests that we will be able to immortalize a CD4+ T cell line from a single M1/M3 MCM to use as target cells in IVSA with effector CD8+ T cells from allogeneic M1/M3 MCM. Standardizing target cells should facilitate comparisons of the suppressive capacity of T cell
Figure 6. Histocompatibility of M1/M3 MHC-identical MCM in vitro. PBMC from an M1/M3 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 M1/M3 MCM.

Figure 7. Allogeneic CD8+ T Lymphocytes Persist for 2 Weeks in MHC-Matched MCM. 10 million CD8β+ T cells were isolated, fluorescently labeled with PKH-67, and infused into an MHC-matched recipient without immunosuppression. PKH67+ lymphocytes are donor-derived.

While those from animals sharing only one or zero haplotypes are rejected within hours(8), adoptive cell transfers with bulk PBMC and purified subpopulations of CD8+ T lymphocytes were successful. Adoptively transferred lymphocytes persist and traffic in MHC-identical MCM.

C.R33: Overview

We pioneered nonhuman primate lymphocyte adoptive transfers using MHC-matched MCM. Below we describe salient observations from these ex vivo studies and present preliminary data supporting our proposal to adoptively transfer in vitro expanded, immortalized CD8+ T cells.

C.R33.1: Adoptively transferred lymphocytes persist and traffic in MHC-identical MCM

Allogeneic, MHC-matched MCM can be used in adoptive cell transfers without the need for recipient immunosuppression(8). Lymphocytes from MCM matched for both MHC haplotypes persist for more than two weeks (Figure 7), while those from animals sharing only one or zero haplotypes are rejected within hours(8). Adoptive cell transfers with bulk PBMC and purified subpopulations of CD8+ T lymphocytes were successful.
Ex vivo adoptive transfers of CD8β+ T cells in our laboratory have achieved frequencies as high as 1.88% for more than 24 hours without immunosuppression(8), roughly comparable to the average 1.47% associated with improved clinical outcome in tumor immunotherapy(37). Importantly, only a tiny percentage of the total CD8β+ T cells were SIV-specific in our study. The concentration of SIV-specific CD8+ T cells will be much higher when in vitro expanded T cells are infused.

The success of our adoptive transfer experiments using complex lymphocyte populations suggests that MCM in vitro expanded CD8+ T cells should persist at levels consistent with tumor regression and be well tolerated by recipient animals.

**FIGURE 8. ALLOGENEIC LYMPHOCYTES TRAFFIC TO IMMUNE EFFECTOR SITES.** 60 million PBMC were labeled with PKH-67 and infused intravenously into an MHC-matched recipient. The recipient was sacrificed 24 hours later and single-cell suspensions of various effector tissues were examined for PKH-67+ cell trafficking.

**FIGURE 9. CONSISTENT PEAK VIRAL LOADS IN SIVmac239-INFECTED MCM.** Zoomed view of Figure 3B data demonstrating peak viral loads always exceeding $1 \times 10^6$. The yellow box indicates a 1.25log$_{10}$ reduction from $1 \times 10^6$ copies/mL. 80% power to detect a difference of 1.25log$_{10}$ in peak viral load requires five animals per animal group. This level of peak viral load reduction has been associated with vaccine-mediated control of SIV mac239 according to Wilson and colleagues (J. Virol 2006: 5875-5885)
C.R33.2: Peak SIVmac239 Viral Loads in MCM are Predictably High

SIV replication is explosive in the first two weeks of infection, before development of adaptive immune responses. Blunting early viral replication is a goal of Specific Aim 4’s adoptive transfer experiments. We power our experiments to detect a $1.25 \log_{10}$ reduction in peak viral load relative to controls. Fortunately, peak viral loads in MCM always exceed $1 \times 10^6$ copies/mL (Figure 9). We can easily quantify viral loads as low as 50 copies/mL, so a reduction of $1.25 \log_{10}$ (to ~50,000) should be easily measurable. This threshold is in accordance with previous studies demonstrating that 1.0 $\log_{10}$ or greater reduction in peak viral loads are evidence of vaccine-mediated control and correlate with improved outcome. Therefore, the dynamics of untreated SIVmac239 infection in MCM allows us to readily measure statistically significant reductions in peak viral loads.

**FIGURE 10. TRANSDUCTION OF T CELL CLONES WITH HTERT MAINTAINS PHENOTYPE AND FUNCTION.** A. A Gag181-189 CM9 CD8+ T cell clone was immortalized with hTERT (CM9-TN). Inhibition of SIV replication in the presence of CM9-TN and the parental CM9 clone is shown. Figure adapted from Minang et al. B. Intracellular IFN-γ production by CM9-TN and CM9 T cell clones is similar following stimulation with synthetic CM9 peptide. C. Concentrations of various cytokines in cell culture supernatants following antigen stimulation are similar between CM9-TN and CM9 clones. Data in panels B and C adapted from Andersen et al.
**C.R33.3: hTERT immortalized T cell lines provide massive numbers of cells for ACT**

ACT requires *in vitro* expansion to generate the massive numbers of lymphocytes needed for infusion. CD8+ T cell lines have limited life-spans *in vitro*(53, 54). As T cells proliferate, decreased telomerase activity leads to telomere shortening(55). One way to overcome T cell senescence is to transduce antigen-specific T cells with human telomerase reverse transcriptase (hTERT)(56). T cell lines expressing hTERT survive indefinitely while retaining the biological properties of primary cells.

Rhesus macaque CD8+ T and CD4+ T cells have been immortalized with hTERT(57, 58). Our collaborator Dr. Ott has continually passaged an hTERT-immortalized CD8+ T cell line for nearly three years with no obvious alternations in its primary properties. Proliferative capacity, cytokine elaboration, cytolytic capacity, surface marker expression, and degranulation are all comparable between a primary CD8+ T cell clone and an hTERT transduced derivative(57, 58). Perhaps most importantly, hTERT-transduced CD8+ T cell clones suppress SIV replication *in vitro* with similar efficiency (Figure 10 and (58)). Therefore, transducing primary cell lines with hTERT provides a virtually limitless supply of CD8+ T cells for ACT. We are currently producing hTERT-transduced Mafa-B*51*-restricted Gag_{146-154}HL9-specific T cell lines.
D. RESEARCH DESIGN AND METHODS

D.R21. Overview: Correlates of protective cellular immunity in MHC-identical, SIV+ Mauritian macaques

The primary goal of the R21 is to define suppressive CD8+ T cells restricted by MHC class IB alleles encoded on the MCM M3 haplotype. We will map these responses in four MHC-identical M1/M3 MCM infected with SIVmac239. The suppressive capacity of the responses we identify will be assessed using in vitro viral suppression assays. We expect that these aims will take two years to complete. The timeline for the R21 experiments is shown in Figure 11.

D.R21. Acquisition of M1/M3 MCM

In our experience, CD8+ T cell epitope mapping requires at least four animals. Responses are not considered authentic unless independently verified in at least two animals at two timepoints. Stochastic influences and selection of immunologic escape variants individualize the strength of individual CD8+ T cell responses, even in MHC-identical MCM that recognize the same possible MHC:peptide complexes. This necessitates using at least three, and ideally four, animals for epitope mapping. Larger groups of M1/M3 MCM would be needed to conclusively determine whether MHC class IB alleles are associated with SIVmac239 control. Analyzing this relationship properly is beyond the scope of an R21 proposal, however, it is a secondary objective that will be evaluated in the four animals purchased for epitope mapping.

We will purchase 4 M1/M3 MCM. 5% of 425 MCM genotyped to date are M1/M3 heterozygotes. Identifying 4 animals with the appropriate genetics requires screening of approximately 80 animals. To account for haplotype frequency fluctuations among shipments of MCM, we will MHC genotype 120 animals. A panel of polymorphic microsatellite markers distributed throughout the MHC will be used to genotype these animals(29). Each MCM MHC haplotype has distinctive microsatellite signatures. Charles River Laboratories (see attached letter of support) allows us to MHC genotype macaques and specifically purchase those with the appropriate genetics.


D.R21.1.1: SIVmac239 infection and viral load monitoring of M1/M3 MCM

The Virology Services Unit of the Wisconsin National Primate Research Center prepares and titers SIV for our research (see attached letter of support). 4 M1/M3 MCM will be infected intrarectally with high dose (50,000 TCID50) SIVmac239, using a challenge stock that previously infected 10/10 MCM((29) and

FIGURE 11. TIMELINE FOR R21 EXPERIMENTS. 4 M1/M3 MCM are necessary. Major events in Specific Aims 1 and 2 are shown in blue and red, respectively.
unpublished data).

Plasma will be collected weekly for the first four weeks of infection and biweekly thereafter for viral load analysis. Virology Services will determine SIV viral loads using a validated qRT-PCR assay that is sensitive to <50 vRNA equivalents/mL plasma. The animals will be monitored for at least 52 weeks or until the development of sAIDS, whichever comes first. None of the M3+ MCM in our preliminary studies developed sAIDS within the first year.

As discussed above, a secondary objective of this Specific Aim is to better define virologic control in MHC-identical, M1/M3 MCM. Controller status will be defined using the algorithm developed for scoring HIV+ humans (1). MCM that maintain viral loads <50 copies/mL at two consecutive sampling timepoints at least two weeks apart will be categorized as ‘elite controllers’, those that maintain viral loads <2000 copies /mL will be categorized as viremic controllers, and those with higher viral loads will be termed non-controllers.

D.R21.1.2: Identification of SIVmac239 CD8+ T cell epitopes restricted by Mafa-B*45, -B*51, and -I*10

Scanning the SIV proteome for sequences that conform with an MHC class I allele’s predicted peptide binding motif is the most robust way to identify new CD8+ T cell epitopes. This approach routinely identifies more than 10 SIV CTL epitopes per MHC class I allele (50, 51, 59), of which generally only 1-2 are strong enough to be detected by stimulation with peptide pools spanning the entire SIV proteome.

The M3 haplotype encodes the MHC class IB alleles Mafa-B*45, -B*51, and -I*10. Our collaborator Dr. William Hildebrand (see attached letter of support) has determined the peptide binding specificity for Mafa-B*51. Peptide binding motifs for the other two alleles should be available by April 1, 2009.

Bioinformatician Dr. Vladimir Brusic (see attached letter of support) has used the binding motif to develop an algorithm for predicting peptide binding to Mafa-B*51. SIVmac239 protein sequences are fragmented into individual peptides and scored for predicted binding. The highest scoring nonamer peptides from Gag are shown in Figure 12. Importantly, the Mafa-B*51-restricted CD8+ T cell Gag 146-154 response, defined initially by stimulation with peptide pools, has the third best fit for the Mafa-B*51 peptide binding motif of all 502 nonamer peptides in Gag. Indeed, its predicted motif fit is in the top 1% of all SIVmac239 nonamers. This inspires confidence that other peptides that fit the Mafa-B*51 motif are targeted by CD8+ T cells.

We will measure CD8+ T cell recognition of the all 8-mer, 9-mer, and 10-mer peptides with predicted binding motif fit in the top 1% of all SIVmac239 peptides. For example, we will examine approximately 40 nonamer peptides (of 3325 total SIVmac239 9-mers) exceeding the Mafa-B*51 binding score threshold of 6.0 (using the scoring system shown in Figure 12, larger positive numbers indicate better motif fit). In total, we expect to test up to 100 individual peptides for each of the three MHC class I alleles for a total of 300 peptides.

Reactivity will be measured by IFN-γ ELISPOT. Though ELISPOT assays may miss some SIV-specific CD8+ T cell responses that fail to elaborate IFN-γ, our experience suggests that this is the most rapid and economical method for scanning a large number of candidate epitopes for CD8+ T cell recognition. A response will be considered positive if it is detected in at least two animals at two different timepoints.

Large numbers of PBMC (>5 x 10^7) will be collected from blood obtained at 4 (acute), 16 (peri-acute), 24 (chronic), and 52 (chronic) weeks post-SIV infection. Each ELISPOT test requires 160,000 PBMC (to test each peptide in duplicate). We can, therefore, test each of the 300 candidate epitopes in each animal at each timepoint, along with positive and negative controls.

Examining patterns of viral escape from CD8+ T cell responses could improve our analysis. Our laboratory routinely sequences complete SIV genomes to examine immunologic escape. We will sequence the dominant plasma virus from each animal at each ELISPOT timepoint, identifying candidate CD8+ T cell epitopes that may be evolving under immunologic pressure. We will couple this information with MHC peptide binding predictions to uncover additional epitopes (Figure 13).
FIGURE 12. PREDICTION OF CD8+ T CELL EPITOPES RESTRICTED BY MAFa-B*51.
A. Screenshot of the web application for predicting peptides that bind to Mafa-B*51. Our collaborator Dr. Brusic will deploy similar applications for Mafa-B*45 and Mafa-I*10 when their peptide binding motifs become available from Dr. Hildebrand. B. Accuracy of Mafa-B*51 CD8+ T cell epitope prediction. The sequence of SIVmac239 Gag was entered into the web application and all 502 nonamer peptides was evaluated for predicted binding to Mafa-B*51. The top 10 predicted binders are shown in the table. Notably, the immunodominant response Gag146-154 is predicted to have the third best binding motif fit for Mafa-B*51 (of all 502 peptides), validating the performance of this algorithm for the identification of additional CD8+ T cell responses.

FIGURE 13. SIV VIRAL SEQUENCE EVOLUTION CAN GUIDE THE IDENTIFICATION OF CD8+ T CELL EPITOPES. SIVmac239 Rev sequence from two M1/M3 animals (CY0111 and CY0113) at four different timepoints (indicated in weeks post-infection). Gaps in sequence coverage are indicated with ‘:‘; the presence of two or more amino acids at a single position is denoted with ‘X‘. The PL9 region, which has a predicted Mafa-B*51 binding score of 8.36 (the third highest in the entire SIVmac239 proteome), accumulates variants consistent with escape from CD8+ T cell pressure. Marrying predicted binding motif fit data with viral variation data will accelerate epitope discovery.
D.R21.3: Anticipated outcomes

We have already identified five SIV-specific CD8+ T cell responses mounted by M1/M3 MCM (see Preliminary Data), including one restricted by Mafa-B*51 (Gag146-154HL9). The concordance between predicted peptide binding, empirically defined CD8+ T cell responses, and viral variation gives us confidence that we will easily identify additional Mafa-B*51-restricted CD8+ T cell responses. Though we cannot know the 'hit rate' of the MHC:peptide binding prediction algorithms a priori, current algorithms are routinely greater than 50% accurate(60). Most CD8+ T cell epitopes recognized by SIV+ rhesus macaques were discovered by systematically testing peptides that bind MHC molecules with high affinity(61), underscoring the merit of this approach to epitope identification.

The peptide binding motifs for Mafa-B*45 and Mafa-I*10 are being defined as part of a separate R24 biodefense grant. We believe these motifs will be available by April 1, 2009. Systematically testing peptides restricted by these alleles will identify additional CD8+ T cell responses. If these motifs are not available by July 1, the anticipated SIV infection date, we will postpone infecting two animals until April 1, 2010. This delay gives Dr. Hildebrand an additional year to complete the Mafa-B*45 and Mafa-I*10 motifs.

A secondary objective of this aim is to better understand SIVmac239 control in M1/M3 MCM. To date we have only infected 3 animals with this combination of MHC haplotypes with SIVmac239, but 2 of the 3 durably controlled viral replication to <500 copies / mL for more than one year. We hypothesize that at least 2 of the 4 newly infected animals will also control SIVmac239 to below 2,000 copies / mL within the first 24 weeks of infection. This outcome could have the important ramification of establishing M1/M3 MCM as the first MHC-identical model for spontaneous control of HIV/SIV-infection.

D.R21.4: Potential problems and alternative approaches

The exact repertoire of T cell responses may vary from animal to animal due to stochastic influences(18, 19). Nonetheless, we expect that immunodominant responses and many subdominant responses will be consistently detected. All Mamu-A*01+ Indian rhesus macaques infected with SIVmac239 mount immunodominant CD8+ T cell responses against the Gag181-189 CM9 CD8+ T cell epitope, despite only sharing the Mamu-A*01 allele.

We elect to characterize responses restricted by Mafa-B*45, -B*51, and -I*10 because of the possible association between CD8+ T cell responses restricted by these alleles and SHIV89.6P(5) and SIVmac239 (Preliminary Data) control. It is important to note that even if none of the four M1/M3 animals durably control SIVmac239, we will still test whether CD8+ T cell responses suppress viral replication in vitro(23), possibly enabling us to meet our milestone for advancement into the R33.

D.R21.2: Specific Aim 2: Evaluate the capacity of SIV-specific CD8+ T cells from M1/M3 MCM to suppress SIVmac239 replication in vitro.

In vitro SIV suppression assays (IVSAs) model in vivo interactions between virally infected target cells and CD8+ T cell effectors. We will use IVSAs to compare the suppressive capacity of CD8+ T cell specificities identified in Specific Aim 1.

D.R21.2.1: Construction of hTERT immortalized CD8+ T cell lines

We will create CD8+ T cell lines specific for each of the epitopes we identify in Specific Aims 1. T cells will be transduced with a recombinant retrovirus expressing human telomerase reverse transcriptase (hTERT) and a C-terminally truncated low affinity human nerve growth factor receptor (LNGFR) as described in Andersen et al.(57). Briefly, recombinant retrovirus is constructed by co-transfection of packaging cell line GP2-293 (Clontech, Mountain View, CA) with a replication-defective, murine leukemia virus based vector expressing hTERT/LNGFR and a commercial vector expressing the envelope protein VSV-G (Invitrogen). The ectopic expression of LNGFR on the T cell surface allows for tracking
of transduced cells both in vitro and in vivo. Transduced CD8+ T cells have been shown to have the same surface marker profiles, IL-2 requirements, and effector cytotoxic functioning as non-transduced T cells(57). Furthermore, hTERT transduced T cells can be grown in culture for extended periods of time without becoming senescent. This advantage will allow our group to comprehensively characterize the in vitro effector quality of our cell lines while scaling to support the large cultures necessary for adoptive cell transfers.

**D.R21.2.2: hTERT immortalizing CD4+ T cells to use as targets for IVSA assays**

SIV replication kinetics in vitro vary from animal to animal, complicating head-to-head comparisons of T cell activity between animals. To minimize this effect, all IVSA assays will utilize the same M1/M3 MCM target cells. We have previously shown that PBMC from MHC-matched animals are histocompatible in vitro, so assaying suppression with standardized M1/M3 target cells is feasible (Figure 7). Before we infect animals in Specific Aim 1, we will collect PBMC and derive individual CD4+ T cell clones. At least eight CD4+ T cell clones will be immortalized with hTERT as described in D.R21.2.1 as Dr. Ott has observed clone-to-clone variation in SIV viral replication (data not shown). Each clone will be infected with SIVmac239. Viral output will be measured by qRT-PCR of the culture supernatant after 96 hours of culture. The clone producing the most virus will be expanded and used as targets for all subsequent IVSA assays. Since all clones were derived from M1/M3 animals, histocompatibility with effector CD8+ T cell lines derived from other M1/M3 MCM should not be an issue.

For each test, 3 wells containing 25,000 target cells each will be activated with the mitogen Concanavalin A (ConA) 96 hours before magnetoinfection with high titer, sucrose gradient-purified SIVmac239 and addition of effector cells.

**D.R21.2.3: Evaluation of CD8+ Effector T Cell Viral Suppression**

One effector population will be added to triplicate wells containing target cells at effector to target ratios of 1:10 and 1:20. After 48 hours and again after 96 hours, SIV copies in the culture supernatant will be measured by quantitative, real-time RT-PCR. Intracellular staining for the SIV p27+ antigen will determine how many target cells are infected at each timepoint. In our experience, qRT-PCR and p27 readouts are concordant but provide complementary information. qRT-PCR has a greater dynamic range to detect subtle differences between T cell specificities, however, p27 intracellular staining offers greater precision between assay replicates. It is therefore preferable to do both assays when possible.

**D.R21.2.4: IVSA Assay Schedule and Analysis**

The amount of work necessary to complete Specific Aim 2 can be estimated. Well-characterized Indian rhesus macaque MHC class I alleles fewer than 20 SIVmac239 CD8+ T cell epitopes(61). If Mafa-B*45, -B*51, and -I*10 each bound this number of epitopes, we would need to test 60 individual T cell lines. Individual T cell lines specific for each will be tested in at least three independent IVSA experiments, for a total of 180 IVSA experiments.

Because CD8+ T cells can have antigen-independent effects on viral replication, it is important to use appropriate controls for non-specific CD8+ T cell activity. MHC-mismatched SIV-specific CD8+ T cells are conventionally used as IVSA negative controls(23). Since all of the CD8+ T cell epitopes discovered in MCM thus far are restricted by alleles on the M1 or M3 haplotypes, using MHC-mismatched control negative controls would require us to define SIV-specific CD8+ T cell responses restricted by alleles on a different MCM MHC haplotype, such as M4. Instead, we will use influenza-specific CD8+ T cell responses restricted by alleles encoded on M1 or M3 as the nonspecific negative control for our IVSA assays. We are collaborating with Dr. Thomas Friedrich to identify influenza-specific CD8+ T cell epitopes recognized by M1/M3 MCM (see attached letter of support). More than 150 influenza T cell epitopes have been mapped in humans(62), so we believe it is reasonable to expect that CD8+ T cell responses will be mounted by M1/M3 MCM and detected with contemporary cellular immunology assays.
Dr. Friedrich expects to characterize at least one influenza-specific CD8+ T cell response by April 1, 2009.

‘ Suppressing’ SIV-specific CD8+ T cell lines will be defined as those that reduce either supernatant viral RNA concentrations or the number of p27+ CD4+ target cells more than 50% (at either E:T ratio) when compared to the negative control. This benchmark for suppression by SIV-specific CD8+ T cells was set by Loffredo and colleagues after studying more than 25 individual CD8+ T cell lines.

**D.R21.2.5: Anticipated Outcomes**

We expect to resolve differences in suppressive capacity of different CD8+ T cell lines, with certain specificities demonstrating effective suppression of >50%.

**D.R21.2.6: Potential Problems and Alternative Approaches**

We have successfully performed IVSA assays in our laboratory (data not shown) and demonstrated that MHC-identical MCM are histocompatible *in vitro*. Adapting these assays to use hTERT immortalized CD8+ T cell effectors and standardized hTERT immortalized CD4+ T cell target cells should thus be feasible. Though hTERT immortalization could theoretically alter CD8+ and/or CD4+ T cell phenotypes, our collaborator Dr. Ott has carried a rhesus CD8+ T cell line in culture for almost three years with no obvious alterations in its primary properties. Therefore, we do not expect any significant technical problems executing the IVSA assays in Specific Aim 2.

Previous IVSA studies have defined 50% suppression as the threshold for effective suppression(23), though this definition is admittedly somewhat arbitrary. A secondary goal of the R33 in this proposal is to determine whether 50% suppression *in vitro* is associated with *in vivo* control of SIV replication.

Though we expect Dr. Friedrich to successfully map influenza-specific CD8+ T cell responses for use as negative controls, we have developed an alternative approach in case of unforeseen delays. We infected an M3/M4 MCM with SIVmac239 in July 2007. Characterizing CD8+ T cell responses recognized by alleles encoded on the M4 haplotype has not been a priority, however, we have banked PBMC from this animal that could be used for this purpose. Mismatched effector T cell lines restricted by alleles encoded on the M4 haplotype are not expected to recognize antigen presented by the M1/M3 target cells.

**D.R21: Milestone**

The primary goal of this R21 is to define suppressive CD8+ T cell responses restricted by alleles encoded on the MCM MHC M3 class IIB haplotype. These experiments provide the foundation for the *in vivo* adoptive transfer experiments proposed in the R33. In order to initiate the R33, we will need to achieve a single milestone:

**Milestone 1) Definition of at least one SIV-specific CD8+ T cell response restricted by Mafa-B*45, -B*51, or I*10 that reduces *in vitro* SIVmac239 replication by 50%.

If we discover more than one CD8+ T cell line that meets this criterion, we will advance the one that is most suppressive.
**D.R33: Assess suppression of viral replication by SIV-specific CD8+ T cells in vivo**

The goal of the R33 is to determine whether adoptive cell therapy (ACT) can be used to define a protective role for CD8+ T cell responses against AIDS viruses. ACT with monospecific CD8+ T cells administered intravenously can have powerful, local effects on metastases and certain viral infections. Analogous experiments to assess the value of prophylactic SIV-specific CD8+ T cells against HIV/SIV were technically impossible before the advent of the MCM model.

If the milestone for the R21 is met, we will examine whether preexisting CD8+ T cells protect macaques from pathogenic SIVmac239. We will engraft T cells immediately before, during, and for two weeks after SIV challenge. The endpoints of this experiment are similar to those of macaque vaccine trials; prevention of SIV acquisition and reduction in the severity of acute infection. Animal group sizes will be powered to detect peak viral load differences of $1.25\log_{10}$ between the adoptive transfer recipients and controls. In addition to viral load, the kinetics and distribution of adoptively transfered lymphocytes and other clinical parameters of acute SIV infection will be measured. The schedule of R33 experiments is shown in Figure 14.

**D.R33.3: Specific Aim 3: Define conditions that enable persistence of adoptively transferred in vitro expanded T cells over a 14 day period**

We will establish the parameters for optimal engraftment of hTERT immortalized CD8+ T cells. In this aim we will evaluate in vivo persistence of hTERT-transformed CD8+ T cells in M1/M3 MCM. Adoptively transferred bulk lymphocytes and CD8β+ T cells persist for more than 24 hours in MHC-identical MCM at frequencies comparable to cancer-specific T cells that mediate tumor regression (~1%). We will attempt to maintain this frequency for 14 days, the window immediately after SIV infection when viral replication is most explosive.

**D.R33.3.1: Acquisition of M1/M3 MHC-Matched MCM**

This aim requires 6 M1/M3 MCM, subdivided into three groups of two animals each. Each group will be used for a single adoptive transfer ‘cycle’ that encompasses a series of lymphocyte transfers conducted over a two weeks period. The animals will be MHC genotyped and purchased from Charles River Laboratories as described in D.R21.Acquisition of M1/M3 MCM.

**D.R33.3.2: Expansion of CD8+ and CD4+ T cell specificities for adoptive transfer**

We expect to use the most suppressive CD8+ T cell line identified in Specific Aim 2 for all R33 experiments.

hTERT-immortalized CD8+ T cells will be expanded in vitro until $1 \times 10^{12}$ lymphocytes can be harvested. hTERT immortalized cell lines co-express the LNGFR marker that enables in vivo tracking of hTERT+ cells by flow cytometry. The purity of the expanded, immortalized lymphocytes will be

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**FIGURE 14. TIMELINE FOR THE R33.** Specific Aim 3 optimization cycles are shown in blue. Specific Aim 4 adoptive transfer staggering is shown in red.
assessed by LNGFR staining. Specificity of the expanded cells will be assessed by performing in vitro viral suppression assays as described in Specific Aim 2.

The total number of lymphocytes in macaques is estimated to be between $1.2 \times 10^{10}$ and $2.5 \times 10^{10}$ (63), compared to $4.6 \times 10^{11}$ in humans (64). Successful T cell immunotherapy trials have used $5 \times 10^{10}$ lymphocytes per infusion (7), or approximately 33% of the recipient’s total lymphocyte pool. A comparable dose in macaques is therefore $8 \times 10^9$ lymphocytes.

**D.R33.3.3: Schedule of CD8+ T cell infusions**

We expect to perform six T cell infusions of $8 \times 10^9$ lymphocytes per animal (Figure 15). Previous studies show that six infusions are well-tolerated (37). Repeated infusions extend the longevity of infused T cells, which is likely to be critical for sustaining activity against SIV during the first weeks of infection. We will infuse the lymphocytes intravenously in sterile 1x PBS as previously described (8). Survival of lymphocytes expanded in vitro is critically dependent on IL-2. In accordance with protocols established for cancer immunotherapy (65), we will administer 720,000 IU/kg IL-2 every 8 hours for starting 24 hours before the first infusion and terminating 72 hours after the last infusion.

**D.R33.3.4: Measuring lymphocyte persistence in vivo**

After each cell transfer, we will assess the frequency and trafficking of transferred lymphocytes. Every hTERT transduced cell also expresses a C-terminally truncated low affinity human nerve growth factor receptor (LNGFR) that is anchored to the cell surface and can be detected by flow cytometry using fluorescent anti-LNGFR antibody.

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**FIGURE 15. EXPERIMENTAL STRATEGY FOR SPECIFIC AIM 3 ANIMALS. A.** Timeline of events during each adoptive transfer cycle. Persistence of cells in blood will be measured daily. Tissue homing to peripheral lymph nodes will be assessed at days 3 and 10. **B.** We expect to improve adoptive transfer results through three iterative refinement cycles. The exact changes from cycle to cycle will depend on the success of the previous cycle; we aim for 1% persistence for 14 days with minimal disruption of the recipients’ normal physiology.
Beginning one day before the first cell infusion, PBMC will be extracted from blood collected daily. Additionally, PBMC will be collected 5 minutes before and 5 minutes after each adoptive transfer. If the cells persist in the periphery for more than two weeks, blood will be sampled once weekly until adoptively transferred cells become undetectable.

Our previous results with ex vivo adoptive transfers show adoptively transferred cells traffic to effector sites including peripheral lymph nodes, spleen, and liver. However, we do not know whether T cell lines will exhibit the same homing profile. We will biopsy inguinal or axillary lymph nodes from each adoptive transfer recipient three days and ten days into each adoptive transfer cycle. LNGFR-expression by purified lymphocytes from these tissues will be measured by flow cytometry to detect transferred cells.

D.R33.3.5: Anticipated outcomes

To our knowledge, no one has adoptively transferred lymphocytes from CD8+ T cell lines in macaques. The T cell lines we use will be allogeneic, but MHC-matched, to the recipients. We readily acknowledge that ACT will require optimization, however, our encouraging results with allogeneic, ex vivo adoptive transfers uniquely positions our group to undertake these studies.

We expect to perform up to three cycles of adoptive transfers to determine the optimal conditions for persistence. Based on our ex vivo data showing demonstrating that 1.8% of adoptively transferred cells persist for more than 24 hours after a single infusion, we believe that serial infusions will result in an average persistence of >1% for more than 14 days. If we achieve this goal in the first cycle of adoptive transfers, the second and third cycles will be dedicated to achieving comparable levels of persistence with lower dosages of IL-2.

The second possible outcome is if the cell transfers show promise (0.5-1.0% 14 day persistence) but the stability or frequency of donor derived cells is lower than we expect. If this occurs, we will adjust cell transfer parameters. The number of lymphocytes transferred may be increased or the dose of IL-2 adjusted.

D.R21.3.6: Potential problems and alternative approaches

If the initial cycle of cell transfers fails (<0.5% 14 day persistence), we will reformulate the protocol for cycles 2 and 3. In cancer studies, non-myeloablative lymphodepletion can improve adoptive transfer success. This conditioning may work by eliminating regulatory T cells, by creating immunologic ‘space’, or by establishing a cytokine profile conducive to cell persistence(7). We prefer to avoid lymphodepletion, if possible, because it may have unforeseen and unpredictable effects on SIV pathogenesis (e.g., depletion of CD4+ cells may dysregulate the typical dynamics of SIV replication). However, if lymphodepletion is necessary to achieve adoptive cell persistence, we will collaborate with Dr. David Sachs (see attached letter of support), an expert in macaque lymphodepletion, to define optimal conditioning parameters. Cyclosporin A(66) treatment or low-dose whole body irradiation(67), for example, have been successfully used in macaques and may extend the persistence of transferred cells. Our partnership with Dr. Sachs provides the expertise to deal with this potentially serious issue, but it does not completely eliminate this concern. Lymphodepletion in macaques is routine, however, and we will compare SIV-specific to influenza-specific T cell lines in Specific Aim 4. This ensures that any effects of preconditioning on SIV pathogenesis or host immunity will be reflected in the animals treated with influenza-specific T cells.

A related concern is that though the T cell lines are MHC-identical to the adoptive transfer recipients, there are differences at minor histocompatibility antigens that could mediate delayed rejection of the transferred cells. If this is occurs, persistence of cells should be greatest during the first infusion and decline with subsequent transfers. Lymphodepleting the recipient may be necessary to sustain durable engraftment. If we see this during the first cycle (with no immunosupression), we will consider optimizing lymphodepletions in cycles 2 and 3 as described above.
D.R33.4: Specific Aim 4: Assess whether adoptive transfer with *in vitro* expanded, SIV-specific CD8+ T cells can reduce the severity of acute SIVmac239 infection.

D.R33.4.1: Selection of M1/M3 MCM

Specific Aim 3 requires 10 MCM, divided into two groups (Groups A,B) of five animals each. All animals will be heterozygous for the M1/M3 MHC haplotypes as described above in R.21. Acquisition of M1/M3 MCM. The 10 animals will be purchased from Charles River Laboratories.

D.R33.4.2: Group assignments

Calculating appropriate animal group sizes for nonhuman primate studies requires care. Purchasing macaques and paying for their care costs thousands of dollars annually per animal. The tendency to use small animal groups because of their high cost must be balanced against the need to get statistically significant results. Parker and colleagues examined viral load variability during acute and chronic SIVmac251 infection to guide animal group size calculations(68). Using five animals per group should provide 80% power to detect differences greater than 1.25 log_{10} in peak viral load. Therefore, we will subdivide our animals as shown in Figure 16. Even though the effect on peak viral load will occur by week 2, we will monitor each set of animals intensively for the first sixteen weeks of infection.

To the maximum extent possible, groups will be balanced with respect to animal weight and age.

The effect of SIV-specific T cell transfers against SIV will be benchmarked against the effect of influenza-specific T cell transfers, which are not expected to have any effect on SIV. We believe this is a more relevant control than comparing animals receiving SIV-specific CD8+ T cell ACT to MCM that receive no ACT.

D.R33.4.3: Adoptive transfer schedule

We will use the optimal schedule for cell infusions determined in Specific Aim 3. 24 hours after the first cell infusion, each animal will be challenged with 50,000 TCID50 SIVmac239 intrarectally using the challenge stock described in D.R21.1.1.

![Adoptive Transfer Schedule Diagram](image)

**FIGURE 16. SPECIFIC AIM 4 EXPERIMENTAL STRATEGY.** A. Animals will receive the optimal ACT dosing determined in Specific Aim 3, beginning one day before SIVmac239 challenge. B. Breakdown of animal groups for Specific Aim 4. 5 animals per group should be sufficient to resolve 1.25log_{10} differences in peak viral loads between animals receiving SIV-specific and influenza-specific T cells.
D.R33.4.4: Assessment of SIV viremia

When animals are anesthetized for cell transfers, we will collect 6mL of blood. Even though MCM are generally smaller than rhesus macaques, even small MCM weighing 4 kg provide 40 mL of blood per month for analysis. This blood will be split between assays to assess SIV status and assays measuring lymphocyte persistence.

PBMC and plasma fractions will be purified. SIV plasma viremia will be assessed by quantitative real-time RT-PCR (qRT-PCR). Validated SIVmac239 qRT-PCR assays with a dynamic range from 3x10^1 - 1x10^10 copies / mL blood plasma are available from Virology Services at the Wisconsin Primate Research Center (see attached letter of support). Viral loads will be determined at each of the six cell transfers in the first two weeks of infection, and weekly thereafter for the first sixteen weeks of infection.

D.R33.4.5: Adoptive cell transfer persistence and lymphocyte dynamics

The same blood, PBMC, and plasma fractions collected in D.R33.4.4 will be used to evaluate the transferred cells.

Persistence of the transferred cells will be measured by flow cytometry for LNGFR(69), a marker engineered for expression specifically on the transferred lymphocytes. MHC:peptide tetramers, if available at the time of study, will be used to confirm the specificity of adoptively transferred CD8+ lymphocytes.

We will also examine persistence of transferred cells in tissues. Lymph node biopsies will be obtained 3 and 10 days after the first adoptive transfer. Single-cell suspensions will also be prepared from the tissues. Flow cytometry for LNGFR and/or MHC:peptide tetramer staining will quantify the frequency of infused cells.

While it may seem unorthodox, studying viral escape may be the best way to assess the function of transferred cells in the absence of significant viral load reduction. **If viral loads remain high in the presence of SIV-specific CD8+ T cells, we expect the transferred cells to exert strong selective pressure** as described previously in a study of therapeutic adoptive transfer(40). We will sequence the gene containing the adoptively transferred CD8+ T cell epitope twice weekly during the first two weeks of infection, weekly through week 6, and biweekly until week 16. **SIV escape may not be as rapid if viral loads in the recipients are controlled** by the transferred T cells. Nonetheless, we can successfully sequence SIV CD8+ T cell epitopes even when plasma viral loads are lower than 200 copies / mL(28), so we can monitor viral evolution even if transferred cells strongly attenuate viral replication.

Cell infusion may perturb lymphocyte dynamics in the recipient monkeys. Complete blood counts will be obtained from each animal at each timepoint. Absolute CD4+ and CD8+ T cell counts will be assessed by flow cytometry.

The cytokine milieu in the host, as well as recognition of SIV antigens, could modulate the phenotype of the transfused lymphocytes. Intracellular cytokine staining will be coupled with LNGFR surface staining to assess the capacity of infused lymphocytes to elaborate cytokines, an indirect proxy of T cell activity. Before transfer, we will measure expression of IFN-gamma, IL-2, IL-4, IL-10, perforin, granzyme B, and TGF-β production by the activated, in vitro expanded T cells. Production of the same cytokines will be assessed twice weekly after SIVmac239 infection.

D.R33.4.6: Anticipated outcomes

We have designed a series of experiments that will assess whether ACT with in vitro expanded CD8+ T lymphocytes can block or reduce the severity of acute SIV infection. If macaques receiving SIV-specific T cells exhibit lower peak viral loads than controls administered influenza-specific T cells, we will have strong evidence that SIV-specific T cells alone have an ameliorative effect on SIV replication. Alternatively, accelerated selection of viral variants within the epitope targeted by adoptively transferred CD8+ T cells may provide functional evidence of antiviral activity.

The experiments are designed to test whether massive numbers of CD8+ T cells alone can suppress
viral replication. Reductions in viral load following vaccine delivery are not as unambiguous; vaccines designed to elicit specific T cell responses may engender other confounding innate or adaptive immune responses.

D.R33.4.7: Potential problems and alternative approaches

We have experience measuring SIV viral load, determining CD4+ T cell counts, sequencing SIV genes and genomes, detecting labeled lymphocytes ex vivo, and analyzing intracellular cytokine staining. Therefore, we do not anticipate any technical problems executing these experiments.

The adoptive transfer itself, however, may have unpredictable effects on SIV pathogenesis and the host's immune system. For example, therapeutic adoptive transfers of HIV-specific CD8+ CTL resulted in transient increases in plasma viral load(41). The adoptive transfer, if not coupled to a lymphodepletion, may activate CD4+ T cells, creating additional targets for viral replication. Alternately, if lymphodepletive conditioning is required for adoptive transfer persistence, SIV-susceptible target cells will be lost. In both cases, we control for non-specific adoptive transfer and/or lymphodepletion effects by comparing infusions of SIV-specific T cells to infusions of influenza-specific T cells.

Additionally, our preliminary data suggests that M1/M3 MCM may spontaneously control chronic SIV viral replication; indeed, this observation forms our theoretical basis for identifying CD8+ T cell responses restricted by Mafa-B*45, -B*51, and -I*10. Therefore, we expect a fraction of the M1/M3 animals to control SIV after mounting de novo adaptive immune responses to the virus. These responses emerge coincident with the decline in peak viral load(70), suggesting that explosive viral replication in the first two weeks is unchecked by de novo T cell responses. Peak viral loads in every MCM infected with SIVmac239 exceed 1 x 10⁶ copies / mL by two weeks post-infection, providing a window to measure the effect of adoptively transferred cells on viral replication in vivo.

The intensive sampling regimen requires significant mobilization of both laboratory and veterinary resources. We will therefore stagger these experiments. The 10 total animals will be processed in subgroups of 2 animals each. We expect that the planning, execution, and analysis of data from each subgroup will take approximately 5 months, including the 16 weeks required for ACT and SIV infection. It should be feasible to process 4-6 animals per year and complete these experiments in years 4 and 5 of the R33.

This experiment requires an influenza-specific CD8+ T cell response that has not yet been identified. We readily acknowledge that this may look like a vulnerability to this proposal. Dr. Friedrich's interest in influenza pathogenesis and immunity in MCM mitigates this risk. He recently submitted an NIH R21 proposal to characterize CD8+ T cell responses in M1/M3 MCM and has already received pilot funding from UW-Madison's Institute for Clinical and Translational Research to begin this effort. Even if the identification of influenza-specific CD8+ T cell responses is not complete by the time we initiate Specific Aim 2, we expect to identify at least one response by the time Specific Aim 4 adoptive transfer begin in April, 2012. If the availability of an influenza-specific CD8+ T cell line is doubtful as of April, 2011, we will start mapping CD8+ T cell epitopes from other, non-retroviral viruses endemic to macaques (e.g., CMV(71)).

A failure to protect macaques from SIV does not mean that the Specific Aim 4 experiments are unsuccessful, nor would it absolutely disprove a role for CD8+ T cells in SIV protection. The first description of adoptive cell transfer in cancer was published twenty years ago(72) and this therapeutic approach is just now coming to fruition. We designed these experiments to take advantage of the collective experience of the cancer community during the ensuing two decades, but adapting this concept to the study of SIV/HIV will likely be an iterative process. Success for this experiment is broadly defined as the persistence of adoptively transferred cells and some evidence of their antiviral function, either through attenuation of viral replication or acceleration of viral immune escape.
Summary

The role of HIV/SIV-specific CD8+ T cells in prophylaxis has never been tested directly in vivo due to the lack of a relevant adoptive transfer system. MHC-matched MCM provide a model for investigating adoptive transfer of cellular immunity. In this proposal we will define SIVmac239 epitope-specific CD8+ T cell responses restricted by three MHC class I alleles and evaluate their quality. If successful, we will investigate whether massive numbers of CD8+ T cells directed against a single epitope and present immediately before, during, and after SIVmac239 challenge blunt the severity of acute infection. We are uniquely positioned to undertake these studies given our investigative team's unique experience pioneering adoptive transfers in Mauritian cynomolgus macaques. These experiments are bold and innovative, fitting squarely within the rubric of the R21/R33 Innovation Grant Program.