MHC-E-RESTRICTED CD8+ T CELL RESPONSES ELICITED BY RHESUS CYTOMEGALOVIRUS VACCINE VECTORS

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CERTIFICATE OF APPROVAL

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Abstract

Vaccination of rhesus macaques with rhesus cytomegalovirus strain 68-1 expressing SIV proteins (RhCMV/SIV) results in stringent control of SIV replication. Approximately fifty percent of vaccinated rhesus macaques exhibit robust control of SIV replication following challenge and eventually clear the virus entirely. However, the mechanism of control and clearance of SIV in strain 68-1 RhCMV/SIV-vaccinated rhesus macaques is unknown. Previous work demonstrated that strain 68-1 RhCMV/SIV vaccination elicits a unique SIV-specific CD8+ T cell response characterized by novel epitope targeting, universal responses elicited regardless of MHC genotype, and wide breadth. We hypothesized this distinct SIV-specific CD8+ T cell response results from unique patterns of MHC restriction. Here, we demonstrate that a large subset of strain 68-1 RhCMV-induced CD8+ T cell responses are restricted by MHC-E molecules, a class of highly conserved, non-classical MHC-I molecules canonically involved in NK cell regulation. SIVgag-specific, MHC-E-restricted CD8+ T cells are able to recognize naturally processed antigen on the surface of both autologous and heterologous SIV-infected CD4+ T cells. In addition, MHC-E is up-regulated on the surface of CD4+ T cells after SIV infection, in contrast to decreased levels of classical MHC-Ia molecules. Finally, we show that MHC-E-restricted CD8+ T cells from rhesus macaques recognize antigen presented across MHC-E molecules from rhesus macaques, humans, and Mauritian cynomolgus macaques, suggesting conservation of MHC-E function across primate species. The findings presented here are the first description of retrovirus-specific, MHC-E-restricted CD8+ T cells, and first identification of MHC-E-restricted CD8+ T cells in non-human primates. Restriction by MHC-E, along with the previously

described MHC-II-restricted class of CD8+ T cells, explains the unique characteristics of the strain 68-1 RhCMV-induced, SIV-specific, MHC-I-dependent CD8+ T cell response, and suggests that virus-specific MHC-E-restricted CD8+ T cell responses may contribute to control and clearance of SIV. Further, induction of a novel MHC-E-restricted CD8+ T cell response of such unprecedented breadth suggests a unique mechanism of CD8+ T cell priming by strain 68-1 RhCMV vectors. Finally, as MHC-E surface expression is often preserved despite pathogen-mediated down-modulation of MHC-Ia, MHC-E-restricted CD8+ T cell responses may remain effective in the face of immune evasion mechanisms. MHC-E appears functionally conserved across primates and exhibits very limited diversity in the human population, and thus mobilizing pathogen-specific MHC-E-restricted CD8+ T cells may serve as a promising approach to T cell-based vaccine development for HIV and other pathogens.

CHAPTER 1: Introduction

I. Human and simian immunodeficiency viruses

Human immunodeficiency virus (HIV) is the causative agent of acquired immune deficiency syndrome (AIDS), a deficiency of cellular immunity characterized by low CD4+ T cell counts and the development of opportunistic infections and malignancies. HIV remains an enormous global health concern. In 2014, there were 36.9 million people globally living with HIV, 2 million new HIV infections, and 1.2 million deaths due to AIDS-related illnesses (*1*). Although treatment with antiretroviral therapy (ART) can control viral replication and reduce the risk of transmission (*2*) (*3*), access and life-long adherence to ART is challenging for some patients and treatment is not curative (*4*) (*5*). In contrast to drug therapy, vaccination against infectious pathogens is among the most cost-effective medical interventions (*6*) (*7*). While the implementation of an effective vaccine would significantly slow the HIV/AIDS epidemic, efforts to develop such a vaccine for HIV have been unsuccessful, largely due to the sequence diversity, adaptability, and immune-evasive capability of the virus. Thus, vaccine strategies that overcome these issues are needed. Infection of Asian macaques with various simian immunodeficiency virus (SIV) strains, primate lentiviruses related to HIV, serves as a powerful experimental animal model of HIV infection that can be employed to test new vaccine strategies (*8*) (*9*). This section discusses the virological and immunological characteristics of HIV/SIV infection, particularly key features that pose challenges for vaccine development.

A. Transmission and disease

The first cases of HIV disease were reported in 1981, describing a rare type of pneumonia caused by *Pneumocystis carinii* (PCP) in five young homosexual men in Los Angeles (*10*). *Pneumocystis carinii*, now known as *Pneumocystic jiroveci*, is a ubiquitous fungus that rarely causes disease in healthy individuals but causes life-threatening pneumonia in immunocompromised patients (*11*). Similar reports of PCP and other opportunistic diseases among homosexual men, hemophiliacs, intravenous drug users, recipients of blood transfusions, female sexual partners of men with symptoms, infants, and Africans accumulated (*12*) (*13*) (*14*) (*15*) (*16*) (*17*) (*18*) (*19*) (*20*). The syndrome became known as Acquired Immune Deficiency Syndrome, or AIDS (*21*), and was discovered to be caused by a retrovirus, eventually named Human Immunodeficiency Virus type 1, or HIV-1 (*22*) (*23*) (*24*) (*25*) (*26*), a virus that infects and destroys CD4+ T cells (*27*) (*28*) (*29*).

HIV is transmitted through contact with bodily fluids of an infected individual, including blood, semen, vaginal fluids, and breast milk (*30*) (*31*). Of the three main modes of HIV transmission, sexual, percutaneous, and perinatal, sexual exposure is the predominant route of infection, with 80% of new infections due to mucosal exposure (*30*) (*31*). Upon primary HIV infection, many patients experience symptoms resembling those of mononucleosis, such as fever, fatigue, lymph node swelling, headache, and rash, that appear within days to weeks following exposure (*32*) (*33*) (*34*). Seroconversion typically occurs weeks to months after infection (*35*). Peak viremia is accompanied by a decline of CD4+ T cell counts in blood that is occasionally accompanied by the development of opportunistic infections (*36*) (*37*) (*38*). However, viremia subsequently declines to a set-point, blood CD4+ T cell counts rebound, and patients often enter clinical latency,

exhibiting little or no clinical manifestations of HIV infection. Plasma viral set point is strongly predictive of the rate of disease progression (*39*). Despite lack of apparent symptoms, viral replication persists, and CD4+ T cells turnover rapidly during this period (*40*) (*41*). For most infected memory T cells, the half-life is less than a day (*42*). This chronic HIV infection results in gradual CD4+ T cell loss, typically occurring over years, and ultimately in the onset of AIDS. AIDS is clinically defined by (1) CD4+ T cell count of less than 200 cells per microliter of blood, or (2) diagnosis with an AIDS-defining illness, including opportunistic infectious diseases such as Candidiasis, Cryptococcosis, Cytomegalovirus disease, and *Pneumocystis jiroveci* pneumonia, and opportunistic malignancies such as Kaposi's sarcoma, Burkitt's lymphoma, and invasive cervical cancer (*43*). Most untreated individuals succumb to AIDS approximately 10 years after infection. Prior to the availability of antiretroviral treatment, the median survival time after AIDS diagnosis was 20 months (*44*). When adhered to, combination antiretroviral therapy controls viral replication and prolongs the life expectancy of HIV-infected individuals (*2*) (*45*).

While most untreated HIV-infected individuals progress as described above, a small subset of untreated individuals (~5-15%), termed long-term non-progressors (LTNPs), progress to AIDS more slowly (*46*). While no official standard definition exists, LTNP cases are typically defined by a CD4+ T cell count of over 500 cells per microliter of blood with no signs of disease sustained for more than 7 years of infection (*47*) (*46*). Long-term non-progressors also tend to have lower viral loads (*47*), and thus often overlap with a group termed elite controllers (ECs), typically defined as individuals with less than 50 copies HIV RNA per milliliter of plasma (*48*) (*49*) (*50*). However, elite

controllers are exceeding rare, making up less than 1% of infected individuals (*51*). Remarkably, some elite controllers have maintained CD4+ T cell counts and undetectable viral loads for over 25 years of HIV infection (*48*), while others have progressed (*52*). Investigating the mechanisms of control in these individuals, as well as in SIV elite controller macaques, can inform vaccine design.

B. HIV/SIV classification

HIV and SIV are lentiviruses belonging to the *Retroviridae* family that exhibit highly error-prone replication in primate hosts (*40*) (*53*). With reverse transciptase's estimated error rate of 10^{-4} to 10^{-5} mutations per base per cycle (*778*) (*54*) (*55*) (*56*), HIV-1 evolves around one million times faster than mammalian DNA (*57*) (*58*) (*56*), and an enormous diversity of HIV/SIV viruses exists among and within infected individuals (*59*) (*60*) (*61*). As described in detail below, this immense genetic diversity greatly diminishes the efficacy of both natural and vaccine-elicited immune responses.

The HIV strains circulating today arose from SIVs that were transmitted to humans from naturally infected African primates, including multiple zoonotic transmissions of SIV from chimpanzees, gorillas, and sooty mangabeys (*62*). There are two major types of HIV: HIV type 1 (HIV-1) and HIV type 2 (HIV-2). HIV-1 is responsible for the vast majority of infections, while HIV-2 is largely contained within West Africa. Circulating HIV-2 strains are descendants of sooty mangabey SIVs (SIVsmm) that crossed into humans (*63*) (*64*) (*65*), including 8 distinct lineages denoted A-H, each resulting from a separate cross-species transmission (*62*). HIV-2 infection is characterized by a longer asymptomatic stage, lower plasma viral load, and lower mortality compared to HIV-1 (*62*). There are four main lineages of HIV-1, called groups

M, N, O, P, each resulting from an independent zoonotic transmission of SIV from chimpanzees or gorillas (*62*). Viruses belonging to group M viruses, or main group, originated from chimpanzee SIV (SIVcpz) and are responsible for the vast majority of HIV-1 infections (*62*), and thus represent the pandemic strain of HIV. During the global spread of HIV-1 group M viruses, a number of population bottlenecks occurred, resulting in nine group M subtypes, A-D, F-H, J-K (*62*). Circulating recombinant forms of these HIV-1 group M subtypes also exist (*66*). Compared to group M infections, HIV-1 group N, O, and P infections are far less prevalent and largely restricted to West Africa (*62*). Molecular clock analysis dates the cross-species transmission event that led to the group M and O epidemics to the early 20th century, while Group N and P infections probably occurred more recently (*67*) (*68*) (*69*) (*70*). Thus, the pandemic group of HIV (HIV-1 group M) has spent over 100 years replicating in human hosts, generating an enormous amount of sequence diversity that poses a significant challenge in developing a globally effective vaccine.

C. Non-human primate models of AIDS

For the study of any infectious disease, a physiologically relevant animal model of infection that recapitulates key features of human pathogenic and immunologic processes is extremely powerful. Among other advantages, animal models allow for direct testing of scientific hypotheses, extensive sampling that would be impossible or unethical in humans, and initial efficacy testing of vaccines and treatments. In particular, non-human primate models provide the most physiologically relevant species to study human disease and disease intervention, and, for reasons described below, serve as standard animal models in HIV/AIDS research.

HIV is species-specific due to inter-species disparities in host co-factors and restriction factors that dictate the ability of the virus to replicate in that host's cells. Thus, HIV-1 is unable to replicate or cause disease in most species other than humans, including commonly used small animal models and most non-human primate species (*71*) (*8*). While chimpanzees can be infected with HIV-1, infection rarely results in the development of disease (*72*) (*73*), and their endangered species status and high maintenance costs preclude the use of chimpanzees as an animal model for HIV/AIDS. Further, the only non-primate virus to cause an AIDS-like syndrome is the feline lentivirus, feline immunodeficiency virus (FIV). However, FIV lacks key proteins present in HIV and SIV, utilizes a different receptor, exhibits broader cellular tropism, and induces a distinct immune response upon infection compared to HIV (*74*) (*8*).

Over 40 African primate species harbor species-specific strains of SIV that are endemic to these natural hosts (*75*) (*76*) (*77*), with SIV infection prevalence ranging from 1% to over 50% depending on the species (*78*). Despite chronic infection with high levels of viral replication, these natural hosts of SIV, such as African green monkeys and sooty mangabeys, do not typically develop AIDS (*79*) (*8*), and thus are unsuitable models for studying pathogenesis. However, SIV infection of non-natural hosts, such as Asian macaques, closely models the course of HIV-1 infection in humans, including gradual CD4+ T cell depletion and progression to AIDS (*8*). The history of simian AIDS in Asian macaques dates back to the 1970's, when an outbreak of B cell lymphomas and opportunistic infections occurred among Asian rhesus macaques housed at the California National Primate Research Center (CNPRC) (*81*). The outbreak was later discovered to be the result of simian AIDS caused by SIVmac (SIV of macaques) (*81*). Prior to the

outbreak, rhesus macaques had been co-housed with healthy, SIV-seropositive sooty mangabeys (*80*) (*81*). While underlying immune deficiency was also noted, the outbreak was not known to be infectious at that time, and few of the surviving healthy SIV-carrying rhesus macaques were sent to other primate centers, including the New England Primate Research Center (NEPRC) (*81*). Thus, SIVmac, which was first isolated at NEPRC in 1985 (*82*), most likely originated from one of the asymptomatic carriers originally co-housed with SIV-infected sooty mangabeys at the CNPRC (*80*) (*81*). The commonly used strains of the SIVmac lineage are the cloned SIVmac239 and uncloned SIVmac251 (*8*). Another lineage of SIV commonly used to infect rhesus macaques, called SIVsm, is derived from SIV of sooty mangabeys that was minimally passaged in rhesus macaques (*83*) (*84*). The commonly used strains of this lineage are the cloned SIVsmE543-3 and uncloned SIVsmE660 (*8*). Both the SIVmac and SIVsm lineages are neutralization-sensitive, utilize co-receptor CCR5, and preferentially replicate in memory CD4+ T cells. As with natural HIV-1 infection, experimental infection of Asian macaques with SIV results in high levels of viremia in the acute phase followed by a reduction in viremia concomitant with the appearance of virus-specific CD8+ T cells, a subsequent prolonged asymptomatic phase, and finally onset of AIDS characterized by the depletion of CD4+ T cells, an increase in viral load, and onset of opportunistic infections (*9*) (*85*) (*86*) (*87*) (*88*). Further, a small percentage of SIV-infected Asian macaques stringently control viral replication, with a phenotype similar to HIV-infected elite controllers (*89*). Thus, SIV-infected Asian macaques, including rhesus, pig-tail, and cynomolgus macaques, serve as relevant experimental models to study HIV/AIDS, including pre-clinical testing of vaccines and therapeutics.

Among non-human primate models of AIDS, SIV infection of Indian-origin rhesus macaques (*Macaca mulatta*) is the most well characterized and widely used. SIVmac251 and SIVmac239 infection of rhesus macaques results in high viral load infection and progressive loss of CD4+ T cells (*90*) (*8*). However, as with any experimental animal model, there are caveats of the rhesus macaque model that must be considered in designing studies and interpreting results. Progression to AIDS occurs faster in SIV-infected rhesus macaques than in HIV-1-infected humans, occurring 0.5-3 years after SIV infection, while untreated HIV-1-infected individuals progress to AIDS ~8-10 years after infection (*8*) (*91*). Further, the genetic diversity among and within SIV strains is lower than the genetic diversity of HIV in humans, particularly with virus grown from molecular clones, such as SIVmac239 (*8*). Thus, vaccines and therapeutics that are broadly effective against SIV infection may not work equally well among diverse HIV strains. In addition, outbred rhesus macaques possess complex MHC genetics compared to humans, with individual rhesus macaques expressing a larger number of MHC-I and MHC-II alleles that present pathogen epitopes to T cells. However, the relative expression of MHC alleles in macaques vary, and there is evidence that the majority of SIV-specific CD8+ T cells are restricted by transcriptionally abundant alleles (*92*). Finally, rhesus macaques possess a larger diversity of tripartite-motif-containing protein 5 (TRIM5) alleles compared to humans, for which only a few minor variants exist (*93*). TRIM5 is a restriction factor and determinant of host-range for retroviruses; in fact TRIM5α was initially identified as a post-entry block to HIV-1 infection in rhesus macaque and other Old World monkey cells (*94*). Rhesus macaque TRIM5 variants confer varying degrees of susceptibility to SIV infection. For example, rhesus macaques

expressing restrictive TRIM5α variants exhibit SIVsmE543-3 viral loads 100- to 1,000-fold lower than animals expressing only non-restrictive variants (*95*).

A more recently characterized Asian macaque model for AIDS is the Mauritian cynomolgus macaque model. SIV infection of cynomolgus macaques is similar to that of rhesus macaques, with the caveat that cynomolgus macaques exhibit slightly lower peak and set-point viral loads (*779*). Mauritian cynomolgus macaques (MCMs) are an insular population of cynomolgus macaques descended from a small founder population that has expanded over the last 500 years (*96*) (*97*). Thus, MCMs exhibit extremely low genetic diversity and possess simplified TRIM5 and MHC genetics relative to other non-human primate models (*98*) (*99*). The majority of MCMs express MHC alleles from seven MHC haplotypes, termed M1 through M7 (*99*) (*100*). Indeed the most common haplotype, M1, is carried by 19% of MCMs and over 4% of MCMs are homozygous for this haplotype (*101*) (*100*). The seven MCM MHC haplotypes are fully defined for MHC-Ia and MHC-II alleles (*100*) (*102*), and thus cohorts of MHC-defined, including MHC-identical, animals can be easily obtained. Thus, MCMs serve as a powerful model to define the relationship between host genetics and control of lentiviral infection (*101*) (*103*) (*104*) (*105*) (*106*) (*107*). Further, access to cohorts of MHC-identical MCMs enable adoptive transfer experiments used to study the protective capacity of various T cell populations (*108*) (*109*) (*110*).

D. HIV/SIV virology

HIV and SIV belong to the *Lentivirus* genus of the *Retroviridae* virus family. Retroviruses are enveloped, single-stranded, positive-sense RNA viruses that replicate through the process of reverse transcription, whereby the viral reverse transcriptase

converts the viral RNA genome into cDNA that is subsequently integrated into the host cell genome, allowing for the transcription and translation of viral genes and packaging of new virions (*111*). Due to the error-prone nature of reverse transcription and high level of viral replication, retroviruses exhibit immense sequence variability and evolutionary potential. Among retroviruses, lentiviruses like HIV/SIV are named for their long incubation periods ("*lente-"* meaning slow in Latin), infecting for months or years prior to the onset of disease. Lentiviruses cause chronic, persistent infections in various mammalian species, including primates, ungulates, and felids (*62*), and can result in a variety of pathologies, including immunodeficiency and neurological disease (*112*) (*86*). This section describes HIV/SIV cellular tropism, life cycle, proteins, and evolution as well as early events of *in vivo* infection, all of which have implications for vaccine design and efficacy.

i. Cellular tropism

HIV/SIV primarily infect CD4+ T cells and macrophages *in vivo*. The restricted cellular tropism of HIV/SIV is largely determined by cellular receptors that allow for viral entry, however other cell-specific factors also impact viral tropism. In order to infect a cell, envelope proteins on the HIV/SIV virion must bind to the receptor, CD4, and co-receptor, either CCR5 or CXCR4, on the surface of the target cell (*113*) (*114*) (*115*) (*116*) (*117*) (*118*). The primary receptor, CD4, is a glycoprotein expressed on the surface of CD4+ T cells, monocytes, macrophages, and dendritic cells (*119*) (*120*) (*121*) (*122*). CCR5 (CD195), or C-C chemokine receptor type 5, binds β-chemokines MIP-1α, MIP-1β, and RANTES, and is expressed on macrophages, dendritic cells, eosinophils, microglia, and some subsets of T cells (*123*) (*124*) (*125*) (*126*). CCR5 is expressed on 5-

25% of peripheral T cells, mostly CD45RA- memory/effector cells (*127*) (*128*) (*117*) (*126*). CXCR4 (CD184), or C-X-C chemokine receptor type 4, binds α-chemokine SDF-1α and is expressed on most peripheral T cells, including both naïve and memory subsets (*127*) (*129*). Individual viral isolates are typically classified by co-receptor usage, either CCR5 or CXCR4; however dual-tropic strains also exist (*780*). CCR5-utilizing viruses typically predominate in the early stages of HIV-1 infection (*130*) (*131*) (*132*) (*133*) (*134*). In about half of HIV-infected individuals, a switch in the viral population to CXCR4-utilizing viruses occurs later in infection; this switch correlates with subsequent accelerated CD4+ T cell decline and disease progression (*135*) (*136*) (*137*) (*138*). In addition to cellular receptors involved in virus entry, additional cellular factors determine HIV/SIV cellular tropism. For example, resting CD4+ T cells are highly resistant to productive HIV-1 infection due to a barrier of cortical actin and abundant expression of SAMHD1, an HIV-1 restriction factor that blocks HIV-1 infection early in reverse transcription (*139*).

ii. Viral life cycle and proteins

Due to the nature of the retroviral life cycle, which includes incorporation into the host genome, HIV/SIV establish irreversible infection in cellular targets. The viral envelope proteins on the surface HIV/SIV virions bind to the receptor, CD4, and co-receptor, CCR5 or CXCR4, on the surface of the target cell, which facilitates fusion of the viral envelope with the target cell plasma membrane and the subsequent release of the viral capsid into the cytosol (*111*) (*140*). Uncoating, reverse transcription of the viral RNA genome into DNA, and assembly of the pre-integration complex occur in the cytosol (*111*) (*140*). The pre-integration complex is then imported into the nucleus, where

the viral integrase protein facilitates integration of the viral DNA into the host cell genome, creating a provirus (*111*) (*140*). Host cell machinery transcribes the proviral cDNA into new viral RNA genomes as well as mRNAs encoding viral proteins, which are exported from the nucleus to the cytosol for translation (mRNAs) and incorporation into virions (genomes) (*140*). New virions assemble and bud from the plasma membrane, after which the viral protease inside each virion cleaves the viral polyproteins into active proteins, creating a mature virion capable of infecting a new target cell (*111*) (*140*).

Integration of HIV/SIV into the host cell genome renders the cell irreversibly infected and also allows for the establishment of viral latency, whereby production of viral proteins and progeny ceases but can later reactivate to produce replication-competent virus (*141*) (*142*) (*143*). The establishment of latency prevents immune recognition of infected cells, and thus latently infected cells avoid immune-mediated destruction (*144*). Further, the latent reservoir persists during antiretroviral treatment (*145*) (*146*) (*147*) (*148*) (*143*) (*149*). The major cellular latent reservoir is resting memory CD4+ T cells (*145*) (*150*) (*151*) (*148*), thought to be previously activated T cells that became infected and survived long enough to revert back to a resting memory state non-permissive for viral gene expression.

HIV/SIV proteins participate in the viral life cycle, but also serve other critical functions, including counteracting cellular restriction factors and facilitating immune evasion. These viral proteins also serve as the antigenic targets of adaptive immune responses. Lentiviruses encode three major viral genes, *gag*, *pol*, and *env*, as well as two regulatory genes, *tat* and *rev*. The major viral genes *gag*, *pol*, and *env* encode polyproteins that are later cleaved into the individual, active proteins. The *gag* gene

encodes a polyprotein that is cleaved by the viral protease during virion maturation into the individual structural proteins of the virus: matrix, capsid, and nucleocapsid (*152*) (*153*). Matrix proteins are myristoylated at the N-terminus, and thus associate with the lipid bilayer, forming a protein layer under the viral envelope (*154*). In HIV, a subset of matrix proteins associate with cellular nuclear import machinery, and thus serve to escort the viral RNA genome to the nucleus for import, allowing the virus to access the nucleus and integrate into the host genome without cell division (*155*) (*156*) (*157*). *Gag*-encoded capsid proteins form the conical core of the virion, surrounding two copies of the viral RNA genome as well as other viral proteins, including *gag*-encoded nucleocapsid proteins and *pol*-encoded enzymes (*158*). Nucleocapsid proteins recognize the packaging signal of the viral RNA genome, mediating incorporation of the viral RNA into virions (*159*). The Gag polyprotein also contains the p2, p1, and p6 regions (*160*) (*161*). Spacers p2 and p1 as well as C-terminal p6 are critical for virion structure and infectivity (*160*) (*162*) (*163*). In addition, the p6 region facilitates incorporation of accessory proteins Vpr and Vpx into virions (*164*) (*165*).

Pol encodes the enzymatic proteins of the virus, including reverse transcriptase, RNase H, integrase, and protease. Reverse transcriptase reverse transcribes the viral RNA genome into cDNA with the assistance of RNase H, which degrades the template RNA in the RNA:DNA intermediate of reverse transcription (*166*). Reverse transcriptase does not possess proofreading activity, and thus reverse transcription is error-prone and produces cDNA copies with point mutations, leading to the continuous production of mutant viruses (*57*) (*58*) (*56*). In addition, reverse transcriptase can "jump" between templates during DNA synthesis, resulting in high rates of recombination and thus further genetic

diversity of progeny viruses (*167*) (*168*) (*169*) (*170*). These characteristics of the viral reverse transcriptase, coupled with the exponential replication of the virus, is a defining feature of HIV/SIV infection that has enormous implications in every aspect of the pandemic, as discussed below. Integrase possesses exonuclease, endonuclease, and ligase activity that facilitate insertion of the viral cDNA into the host genomic DNA in the nucleus (*171*). The *pol* gene is transcribed only as a single *gal-pol* mRNA, which is translated into a single polyprotein and incorporated into virions (*172*) (*158*). Synthesis of the Gal-Pol polyprotein occurs via a slipping mechanism whereby an RNA stem-loop causes stalling of the ribosome and a frameshift (*162*). Similar to Gag-only polyproteins, Gag-Pol polyproteins are cleaved into individual, active proteins by the viral protease during virion maturation (*152*) (*153*). Virions contain a strict 20:1 ratio of Gag to Gag-Pol protein, which is critical for viral assembly and replication (*162*).

The *env* gene encodes the Env polyprotein, containing the gp120 and gp41 subunits of the viral envelope protein. As Env proteins are expressed on the surface of virions as well as the surface of infected cells, these proteins serve as the targets of both neutralizing antibodies that prevent infection of host cells and binding antibodies that mediate other antiviral immune functions, such as antibody-dependent cellular cytotoxicity (ADCC), whereby antibody coats infected cells and facilitates natural killer cell-mediated lysis (*173*). The Env polyprotein is synthesized in the ER, migrates through the Golgi where it is glycosylated, and is then cleaved into the two subunits of the polyprotein by furin, a cellular protease (*174*). The surface subunit, gp120, non-covalently interacts with gp41, which contains a transmembrane domain and localizes the viral envelope protein to the plasma membrane of infected cells and the envelope of

virions, which form Env trimers of the gp120-gp41 heterodimer (*175*) (*176*). Gp120 mediates interaction of Env with the viral surface receptor, CD4, and co-receptor on target cells (*177*), and gp41 subsequently facilitates fusion of the viral envelope and plasma membrane, allowing the virus to enter the target cell (*178*) (*179*).

Retroviruses like HIV/SIV also encode two essential regulatory factors that play critical roles in the replication cycle of the virus: Tat and Rev. Tat serves as a transcriptional transactivator, primarily promoting the elongation phase of provirus transcription (*180*) (*181*) (*182*). Rev facilitates the nuclear export of viral genomic RNA as well as unspliced and incompletely spliced viral transcripts. Most host cellular pre-mRNAs must undergo complete splicing to remove intronic sequences prior to export from the nucleus and subsequent translation (*183*). Thus, Rev export is required for translation of viral proteins translated from unspliced or incompletely spliced viral mRNAs (*183*). Completely spliced viral transcripts encoding Tat, Rev, and Nef can use the normal cellular mRNA nuclear export machinery, and thus Tat, Rev, and Nef are produced early in the Rev-independent phase of translation (*183*). In contrast, the partially spliced and unspliced transcripts encoding Env, Vif, Vpu, Vpr, and Vpx proteins and the Gag and Pol proteins, respectively, rely on Rev for nuclear export, and thus these proteins are not produced until the later, Rev-dependent phase of translation (*183*) (*184*).

In addition to the viral proteins directly involved in the replication cycle, HIV/SIV also encode accessory proteins Nef, Vif, Vpr, and either Vpu (HIV-1) or Vpx (SIV), virulence factors that together serve to enhance viral replication, counteract host restriction factors, and facilitate viral immune evasion mechanisms. With the exception of Vpu, all accessory proteins are detected in virions, and thus can act immediately upon

infection of a new cell (*185*) (*158*). Likely the most relevant HIV/SIV accessory protein to the CD8+ T cell-based vaccine strategies described here is Nef. Nef is critical for high viral loads and viral pathogenesis *in vivo* (*186*) (*187*) (*188*). Nef facilitates down-regulation of MHC class I from the surface of HIV/SIV-infected cells by interfering with cell surface trafficking of MHC-I molecules in the trans-Golgi network (TGN) as well as by promoting endocytosis of MHC-I molecules from the cell surface (*781*) (*189*) (*782*). This function of Nef impedes the ability of virus-specific CD8+ T cells to recognize infected cells (*330*) (*190*) (*738*) (*191*) (*192*) (*193*). Nef also downregulates CD4, MHC-II, and other cell surface receptors (*194*).

iii. Viral evolution

Retroviruses like HIV/SIV exhibit a high degree of sequence variability and evolutionary potential. HIV-1 replicates vigorously, with an estimated 10^{10} virions produced each day cells in a single patient (*195*) (*813*). Coupled with the high rates of mutation and recombination during revere transcription, this exponential replication result in immense genetic diversity among viruses, both at a population-level and within a single infected individual (*70*). Within HIV-1 group M subtypes, variation on the amino acid level is typically 8-17%, but can be as high as 30%, and continues to increase over time (*70*) (*196*). Amino acid variation among group M subtypes is typically 17-35%, but can be as high as 42% (*61*) (*70*). This phenomenon of viral genetic diversity holds true within a single HIV-1 infected individual, where replication gives rise to multiple viral variants, or quasispecies, that change over the course of infection. In some individuals, viral sequences differ by up to 10% (*61*).

This enormous genetic diversity of HIV/SIV has implications for disease progression, transmission, diagnosis, treatment, and vaccine development. For example, subtype D infection is associated with faster disease progression but reduced rates of heterosexual transmission compared to subtype A infection in the same population (*70*). In addition, HIV-1 genetic diversity makes diagnosis and viral load measurements challenging, particularly in areas with limited resources, and existing assays must be checked for the ability to detect emerging variants in order to avoid false negatives (*197*). Treatments and vaccines are also profoundly impacted by HIV/SIV genetic diversity. The effectiveness of antiretroviral therapy varies among strains due to differences in drug resistance mutations (*70*). Similarly, developing a vaccine that is universally effective against all or even most viral variants is an enormous challenge, as vaccine-elicited antibody and T cell responses rely on the recognition of specific viral sequences.

Perhaps the most significant consequence of the exponential, error-prone replication of HIV/SIV, however, is the extreme adaptability of the virus. This conferred evolutionary advantage allows HIV/SIV to adapt to new hosts; in fact, it likely allowed zoonotic SIV to overcome human restriction factors and establish productive infection in the human host (*62*). Furthermore, adaptability allows HIV/SIV to persist in the face of antiviral mechanisms, including antiretroviral drugs and adaptive immune responses, through the development of drug resistance and immune-escape mutations (*198*) (*90*). Thus, current drug and vaccine development programs focus on targeting multiple areas of the virus, through multi-drug treatment or immune response breadth, in an attempt to overwhelm the virus' ability to resist/escape. Immune-driven escape is particularly relevant to the data presented here, and thus is discussed in more detail in section E.

iv. Early events *in vivo*

The most common route of HIV-1 infection is sexual exposure through the genital tract or rectal mucosa; ~80% of adult cases occur via mucosal exposure (*30*) (*31*). In these cases, transmission is thought to be an inefficient process as the mucosa imposes a genetic bottleneck on the infecting virus; ~80% of mucosally transmitted HIV-1 clade B and C infections are initiated by a single virus, often referred to as the transmitted founder virus (*130*) (*199*) (*200*) (*201*) (*202*). A similar genetic bottleneck occurs in SIV infection of rhesus macaques (*203*). Transmission is followed by an eclipse phase of ~10 days before viral RNA is detectable in the plasma. SIV models of vaginal mucosal exposure demonstrate that the virus can cross the mucosal epithelial barrier within hours and establish a small population of infected cells, but local expansion is necessary before dissemination and establishment of systemic infection throughout the secondary lymphoid organs (*204*). At the end of the eclipse period, virus arrives in the draining lymph node, which contains activated CD4+CCR5+ T cells that serve as targets for further infection, allowing for rapid replication and spread to other lymphoid tissues. In particular, the gut-associated lymphoid tissue (GALT) is a major site of HIV/SIV replication due to the presence of high numbers of activated CD4+CCR5+ memory T cells (*205*) (*206*) (*207*). During this period of replication in the GALT, plasma viremia increases exponentially to a peak while CD4+ T cell numbers drop (*208*). This is followed by a decline in viremia over 12-20 weeks, eventually reaching a set point (*40*) (*209*) (*210*). A rebound of CD4+ T cells in blood is observed over this time period; however, GALT CD4+ T cells do not return to normal levels (*205*) (*211*) (*212*).

E. The immune response to HIV/SIV

i. Innate immune response

At mucosal portals of entry, HIV/SIV likely encounter an array of innate inhibitory defenses, including chemokines that block co-receptor-mediated viral entry, recruitment of type I interferon-producing plasmacytoid dendritic cells, and large increases in IFN-γ (*204*). While these innate immune defenses may account for the limited genetic diversity of transmitted viruses and may often protect against acquisition, they are insufficient to control viral replication and systemic spread. The first detectable innate immune response to HIV-1 infection is an increase in the levels of acute-phase proteins, such as serum amyloid A, production of which can be triggered by pro-inflammatory cytokines (*213*). This first wave of acute-phase proteins occurs just before viral RNA is first detected in plasma at greater than 100 copies per milliliter (*213*). A subsequent wave of acute-phase protein production is accompanied by a large cytokine and chemokine response and rapid increase in plasma viremia (*214*) (*215*). The cytokine storm observed during early HIV-1 infection is greater than that observed in acute hepatitis B virus and hepatitis C virus infections, and includes transient increases in IL-15, type I interferons, CXCL10, and IL-10 as well as sustained increases in IL-18, TNF, IFN-γ, and IL-22 (*215*) (*213*). The cellular sources of these cytokines and chemokines are likely infected CD4+CCR5+ T cells, activated DCs, monocytes, macrophages, NK cells, NKT cells, and, later, HIV-specific T cells (*216*) (*207*). Some of the induced cytokines mediate anti-viral activities, such as type I interferons, which inhibit HIV replication and enhance innate and adaptive immune responses (*217*). However, the intense cytokine response may also promote viral replication and mediate immunopathology. For example, the pro-apoptotic effects of type I interferons and TNF can contribute to a loss in

activated DCs and bystander destruction of CD4+ T cells and B cells (*213*). In addition, chemokines produced from plasmacytoid DCs can recruit virus-susceptible CD4+ T cells to the foci of infection (*216*) (*218*).

ii. Adaptive immune response: interplay between virus and host

The adaptive immune response against HIV/SIV includes induction of virus-specific CD4+ and CD8+ T cell responses as well as virus-specific antibody responses. However, this natural adaptive immune response is typically unable to exert complete control over viral replication and, in every case, is unable to eliminate the virus. Indeed, there are no reports of natural clearance of HIV. Decades of research have highlighted a number of concepts that explain this lack of adaptive immune control over HIV/SIV. First, virus-specific adaptive immune responses require time to develop, allowing for an initial period of massive viral replication and for the establishment of systemic infection, including the seeding of viral reservoirs (*219*). This is corroborated by studies demonstrating homogeneity of the virus at peak viremia (*130*) (*199*) (*200*), suggesting a lack of immune-driven selection, both T cell- and antibody-mediated, before and during this time. Second, HIV/SIV infection creates a dysfunctional immune environment by depleting CD4+ T cells that orchestrate immune responses, and causing chronic immune activation (*29*) (*220*) (*221*). Finally, HIV/SIV execute numerous immune evasion strategies, including interference with T cell antigen presentation machinery and viral sequence escape. In particular, keeping up with the rapidly changing viral quasispecies that develops within an infected individual is an immense challenge for the adaptive immune response, particularly as effective adaptive immune responses drive the emergence of viral escape mutants.

a. Viral escape from adaptive immunity

Adaptive immune responses possess exquisite specificity; each clonally expanded response, either antibody or T cell, recognizes a particular part of an antigen, known as an epitope. As adaptive immune responses only carry out their functions upon recognition of specific antigen, the effectiveness of a HIV/SIV-specific immune response is first determined by its ability to effectively recognize the virus. Thus, a key consideration in understanding the adaptive immune response to HIV/SIV infection is the adaptability of the virus. High rates of mutation and recombination during reverse transcription generate enormous viral diversity within a single infected individual (*61*). Measured error rates for reverse transcriptase predict the occurrence of at least one error per viral genome per replication cycle (*90*). HIV/SIV can tolerate sequence variation quite well (*223*), and thus, HIV/SIV possess impressive plasticity, including the ability to escape mounted adaptive immune responses. In immune-driven viral selection, an anti-viral T cell or antibody response drives the selection of viral mutants that are no longer susceptible to that immune response, termed "escape mutants". Conceptually, effective adaptive immune responses will drive the selection of escape mutants with sequence changes that abrogate epitope recognition, as there is a selective advantage for viruses that are no longer targeted by that initial antiviral adaptive immune response. This is in contrast to ineffective adaptive immune responses, which should not drive the selection of escape mutants, as unrecognized viral mutants should not have a selective advantage over viruses that are recognized. Indeed, observations of immune-driven escape provide strong evidence for the effectiveness of particular adaptive immune responses. In both HIV and SIV infection, viral escape from numerous CD8+ T cell responses as well as from Env-

specific neutralizing antibody responses has been observed (*224*) (*225*) (*226*) (*227*) (*228*) (*229*) (*230*) (*231*) (*232*) (*233*) (*783*) (*234*) (*235*) (*236*) (*237*). This can occur quickly; for example, the first CD8+ T cell selected mutations can replace the original sequence of the founder virus within 10 days (*238*) (*239*). Some adaptive immune responses target epitopes in areas critical for protein function, and thus, a mutation in that epitope might exert a fitness cost to the viral escape mutant. Escape from a particular adaptive immune response often involves multiple changes in the viral sequence, suggesting that various mutants emerge until the fittest are selected (*238*). There are demonstrated compensatory mutations that occur in some of these cases, where changes in another part of the viral sequence, often outside of the recognized epitope, can compensate for the fitness cost of the epitope mutation (*231*) (*240*) (*241*) (*242*) (*243*) (*244*) (*245*). Thus, viral evolution creates a continual interplay between virus and host, whereby an effective antibody or T cell response is followed by a wave of viral escape mutants, which may in turn be followed by a new immune response targeting the escape variant (*238*) (*246*) (*247*). Ultimately, the virus outpaces the virus-specific immune response and persistently replicates in the infected host.

b. Antibody and CD4+ T cell responses

Antibodies recognize epitopes on native antigens, and thus directly recognize surface proteins such as the HIV/SIV viral envelope proteins that exist as trimers on the surface of virions and infected cells (*248*). Antibodies can prevent virus from infecting cells, termed neutralization, or can bind and facilitate other immune functions, such as opsonization-mediated phagocytosis, complement activation, and antibody-dependent cellular cytotoxicity (ADCC) (*250*) (*251*) (*252*) (*253*) (*254*) (*255*). In order to neutralize

virus, antibodies must recognize and block crucial structure features of Env and prevent Env from facilitating entry. Unfortunately, Env is the most variable protein of HIV/SIV (*256*), and thus most neutralizing antibodies are only effective against a narrow range of strains (*257*). Antibodies to HIV/SIV develop within weeks to months after infection (*35*) (*258*). The earliest binding antibodies are specific for gp41 and are detected ~2 weeks after first detection of plasma viremia, while gp120-specific antibodies only appear after an additional 2 weeks (*214*) (*259*) (*260*) (*261*) (*262*). However, this initial Env-specific antibody response is non-neutralizing, and does not appear to control viral replication or substantially contribute to the initial decline in plasma viral load (*263*) (*214*). Antibodies that neutralize autologous virus are not detected until at least 12 weeks after transmission (*233*) (*264*) (*259*) (*265*). This initial wave of autologous virus-neutralizing antibodies is typically narrow in epitope-targeting and neutralization activity is only effective against the individual's contemporary and prior viral isolates (*266*) (*255*), and the virus rapidly escapes (*233*) (*264*) (*265*). Broadly neutralizing antibodies, those that are effective against a wide range of strains, develop in ~20% of HIV-infected patients within two years of infection, but usually the virus escapes these antibody responses as well (*267*) (*268*). In addition to viral sequence escape, numerous other antibody evasion strategies are employed by HIV/SIV, including heavy glycosylation of Env that can mask neutralization epitopes and release of "decoy" soluble Env molecules that elicit antibodies poorly reactive with the native Env structure (*90*) (*269*) (*270*) (*271*).

During acute infection, virus-specific CD4+ T cells expand but then decline rapidly and are present at low levels during chronic infection (*272*) (*273*) (*274*) (*275*) (*276*) (*277*) (*278*). HIV/SIV infect and significantly deplete memory CD4+ T cells (*212*)

(*218*) (*279*) (*280*). Further, virus-specific CD4+ T cells are particularly susceptible to infection (*281*). Defining the role of virus-specific CD4+ T cells in viral control and disease progression is complicated by the fact that these cells serve as targets of viral replication. For example, proliferative capacity and polyfunctionality of virus-specific CD4+ T cell responses is preserved in elite controllers compared to progressors, however this is at least partially the consequence of low viral load (*276*). On the other hand, HIV-specific CD4+ T cells from elite controllers secrete higher levels of IL-21 compared to progressors (*285*), and IL-21 has been demonstrated to increase virus-specific CD8+ T cell cytolytic capacity and ability to suppress viral replication *in vitro* (*285*). In addition, particular MHC-II alleles, which present viral epitopes to CD4+ T cells, are associated with control of both HIV-1 and SIV (*810*) (*811*), and there is evidence for CD4+ T cell-driven viral escape (*812*).

c. CD8+ T cell responses

CD8+ T cells recognize non-self peptides presented in the context of major histocompatibility complex class I (MHC-I) molecules on the surface of target cells. For example, during viral infections such as HIV/SIV, infected cells presenting viral peptides can activate virus-specific CD8+ T cells. This recognition occurs in a specific manner whereby each CD8+ T cell response recognizes a specific peptide epitope in the context of a particular MHC-I molecule (*286*). Upon recognition of specific peptide, CD8+ T cells exhibit a variety of antiviral functions, including killing of the target cell and secretion of antiviral cytokines (*287*). In both HIV-1 and SIV infection, the first virus-specific CD8+ T cell responses appear as viremia approaches its peak (*288*) (*289*) (*290*) (*291*) (*87*) (*87*). The virus-specific CD8+ T cell response peaks 1-2 weeks later as

viremia declines to set-point, dropping from a peak of ~10^7 HIV RNA copies per ml of plasma to a set point median of 30,000 within a few weeks (*292*) (*293*). This peak of virus-specific CD8+ T cell responses concomitant with the rapid decline in plasma viremia suggests these cells might be important in viral control.

Indeed, ample evidence supports a role of CD8+ T cell-mediated immunity in controlling viral replication. Early virus-specific CD8+ T cell responses are implicated in the establishment and maintenance of set point viremia (*293*) (*51*) (*294*) (*295*). High frequencies of circulating HIV-specific CD8+ T cells are associated with a reduction in HIV replication and improved clinical outcome (*288*) (*289*) (*296*) (*291*). In particular, long-term non-progressors mount vigorous, highly functional virus-specific CD8+ T cell responses (*297*) (*298*) (*413*). In SIV-infected rhesus macaques, experimental depletion of CD8+ T cells in either acute or chronic infection results in a significant increase in viral replication and accelerated disease progression (*299*) (*300*). Furthermore, in situations of viral suppression, either through antiretroviral treatment or elite control, transient CD8+ T cell depletion of infected rhesus macaques leads to an increase in viral load, which returns to baseline levels with the reconstitution of the CD8+ T cell compartment (*301*) (*302*) (*303*) (*304*). Anti-viral properties of HIV/SIV-specific CD8+ T cells can also be observed *in vitro.* The ability of virus-specific CD8+ T cells to suppress viral replication *in vitro* inversely correlates with the rate of CD4+ T cell decline and viral set point in HIV-1-infected patients (*305*), and cytoxicity of SIV-specific CD8+ T cells correlates with control of SIV replication in SIV-infected rhesus macaques (*306*).

In addition, viral escape from CD8+ T cell responses is well documented, suggesting virus-specific CD8+ T cells exert immune pressure on the virus. During the

peak of CD8+ T cell responses and rapid decline of viremia to set point, the viral sequences change dramatically (*200*) (*307*) (*213*). Most of the observed amino acid changes in the virus at this time occur in epitopes recognized by CD8+ T cells (*238*); numerous studies have described virus escape mutations from near the time of viral set point in both HIV-1 and SIV infection (*288*) (*226*) (*308*) (*309*) (*310*) (*225*) (*240*) (*242*) (*311*) (*312*) (*313*) (*314*) (*315*). This rapid replacement of the founder virus with escape mutants suggests the early founder virus-specific CD8+ T cell responses are quite effective at controlling viral replication. However, following viral escape, these initial CD8+ T cell responses decline rapidly (*238*) (*316*), presumably due to the paucity of the original epitope sequence in the viral population. Viral escape from mounted virus-specific CD8+ T cells continues throughout the course of infection (*238*) (*224*) (*225*) (*226*) (*227*) (*228*) (*229*) (*230*) (*231*) (*232*). In some cases, viral escape from an effective CD8+ T cell response late in infection leads to breakthrough of virus and disease progression (*317*). Various types of CD8+ T cell epitope escape have been observed, including changes that abrograte or weaken the ability of viral peptide to bind to the presenting MHC-I molecule, prevent viral peptide recognition by the corresponding T cell receptor, interfere with processing of the viral peptide, or completely delete the epitope (*318*) (*319*) (*317*).

In further support of the role of CD8+ T cells in viral control, particular MHC-Ia alleles are associated with viral control and slower rate of disease progression in both HIV-1-infected patients and SIV-infected rhesus macaques (*320*) (*49*) (*321*) (*322*) (*323*) (*324*). The MHC class Ia genes encode the highly polymorphic antigen presentation molecules that present peptides to CD8+ T cells (*325*) (*326*), and thus the set of MHC-Ia

molecules expressed in an individual governs the epitope targeting of the mounted CD8+ T cell response. Elite controllers have an overrepresentation of "protective" MHC-Ia alleles HLA-B*27, HLA-B*57:01, HLA-B*57:03, and HLA-B*58:01 in humans, and Mamu-B*08 and Mamu-B*17 in rhesus macaques (*49*) (*321*) (*322*) (*323*). While some aspects of the association between MHC and viral control are not fully defined, individuals expressing protective MHC-Ia alleles are more likely to mount dominant and effective CD8+ T cell responses targeting conserved regions of the virus (*227*) (*327*). Conserved regions likely represent areas of critical protein function, where viral mutational escape would exert a fitness cost to the virus. Indeed, associations of MHC-Ia alleles and superior viral control likely result, at least in part, from the presentation of conserved epitopes by particular MHC-Ia molecules, allowing for CD8+ T cell targeting of conserved regions (*328*). HIV-1 infected individuals heterozygous at these loci exhibit slower disease progression (*329*). Thus, as greater diversity of MHC-I genes theoretically means a greater diversity of viral epitopes could be presented, heterozygote advantage suggests that control of HIV replication is associated with the ability to mount a diverse CD8+ T cell response. Qualities of the virus-specific CD8+ T cell response associated with virus control are discussed further in section II.

While virus-specific CD8+ T cells have a clear role in the control of viral replication, they ultimately fail to completely control the virus. The delayed timing of adaptive immune responses and the potential of HIV/SIV for mutational escape, gives the virus a kinetic advantage over host immune responses. In addition, other mechanisms specifically impede effective CD8+ T cell responses. The viral accessory protein Nef interferes with antigen presentation on MHC-I, protecting HIV/SIV-infected cells from

CD8+ T cell-mediated killing (*330*) (*782*) (*189*) (*331*) (*332*) (*781*) (*738*) (*191*) (*192*) (*193*). In addition, recent evidence demonstrates that not all SIV-infected cells are readily accessed by virus-specific CD8+ T cells, and instead viral sanctuaries exist in sites like B cell follicles (*784*). Finally, the continuous high-level antigenic stimulation that occurs during chronic infection, coupled with the lack of CD4+ T cell help, leads to progressive exhaustion of virus-specific CD8+ T cells, rendering these cells unable to exert antiviral functions (*333*) (*334*).

II. HIV vaccine development

Key features of HIV/SIV infection, described above in section I, make developing an effective AIDS vaccine exceedingly challenging. First, the genetic variation and adaptability of the virus pose challenges for adaptive immune responses, as HIV/SIV-specific adaptive immune responses are only effective against a subset of viruses and the virus can evade mounted immune responses through mutational escape. Thus, a globally effective HIV vaccine is an enormous challenge. Second, HIV/SIV possess various immune evasion mechanisms, including viral protein-mediated down-regulation of MHC-I that facilitates CD8+ T cell evasion (*194*). Third, the integration of the viral genome into the host cell chromosome allows for the establishment viral latency, whereby the cell is infected but limited viral replication and virion production allow the virally infected cell to avoid recognition and clearance by the host immune response (*144*). Unlike other infectious diseases, natural immune responses to HIV are ineffective, and thus there is no model immune response on which to base vaccine-mediated immunity. This section discusses the need for an HIV vaccine, vaccine design, and the

successes and failures of tested vaccine strategies, with a focus on CD8+ T cell-based vaccination strategies.

A. The need for an HIV vaccine

An HIV vaccine is needed to slow the AIDS epidemic. Although treatment with combined antiretroviral therapy (cART) limits viral replication in patients, this treatment does not eliminate the virus (*335*) and requires life-long adherence as cART cessation ultimately results in restoration of viral replication and progression to AIDS (*4*) (*142*) (*336*) (*337*). In addition, access and adherence to cART poses a challenge for many individuals. Prevention measures such as vaginal microbicides and pre-exposure prophylaxis have shown some efficacy in reducing HIV-1 transmission (*338*) (*339*) (*340*) (*341*) (*342*), however these measures also require strict adherence for efficacy. In contrast, vaccines require only a handful of administrations to elicit long-lasting protective immune responses (*6*) (*7*). Despite improved access to ART over the past several years, the rate of new infections is still enormous; two million new infections were reported for 2014. Thus, even a moderately effective HIV-1 vaccine would slow the AIDS epidemic.

HIV vaccine strategies must elicit virus-specific immune responses effective against a range of viral strains. While potent broadly neutralizing antibodies have been identified and are effective in preventing infection in a dose-dependent manner upon passive transfer (*343*) (*344*) (*785*) (*345*) (*346*), vaccinologists have been unable to elicit high levels of broadly neutralizing antibody responses in a vaccine. The recent RV144 vaccine trial and other studies suggest that other antibody-mediated functions, such as ADCC, may offer efficacy (*347*) (*348*) (*253*). Vaccinologists have also failed to induce

effective virus-specific CD8+ T cell responses in humans; three major T cell-focused vaccine trials using HIV protein-expressing DNA and Ad5 vectors showed no efficacy. Thus, novel approaches to vaccine development are required.

B. T cell vaccines

i. CD8+ T cell immunology

Activation of T cells occurs through the T-cell receptor, and thus the specificity of a given T cell is dictated by the particular T-cell receptor expressed on its surface (*349*). T-cell receptor genes are rearranged during T cell maturation in the thymus, which results in each T cell expressing a unique T-cell receptor that recognizes a specific epitope presented in the context of a particular MHC molecule (*349*). Indeed, an estimated 2.5 x 10^7 unique T-cell receptors exist in the peripheral naïve T cell pool of a single individual (*350*) (*351*). Naïve T cells, those that have not yet encountered antigen, circulate primarily in secondary lymphoid tissues surveying host cells for their specific antigen (*352*). The development of a T cell response involves priming upon first exposure to the antigen, whereby a naïve T cell is activated by specific antigen, proliferates, and differentiates (*353*). The priming of a T cell response occurs in the T cell areas of secondary lymphoid organs and is thought to require three signals, termed the three-signal model of naïve T cell activation (*354*). The first signal, ligation of the T-cell receptor (TCR), is delivered through the T-cell receptor (TCR) upon interaction with specific peptide complexed with an MHC molecule present on the surface of the antigen-presenting cell. T-cell receptor ligation typically also involves the co-receptor, either CD4 or CD8, contacting the antigen-presentation molecule (*355*). The second signal is the co-stimulatory signal, which must be delivered simultaneously with signal 1; naïve T

cells that receive signal 1 in the absence of signal 2 become anergic. The co-stimulatory signal is often delivered to the T cell through the CD28 receptor upon ligation of B7 co-stimulatory molecules CD80 and CD86 on the antigen-presenting cell. These co-stimulatory molecules are induced on the surface of antigen-presenting cells by innate immune pathways that sense pathogen-associated molecular patterns. In addition, effector CD4+ T cells recognizing the same antigen-presenting cell can induce expression of co-stimulatory molecules on the antigen-presenting cell. The third signal is mediated by cytokines that control the survival, differentiation state, and polarization fate of the activated T cell. These signals ultimately result in the clonal expansion of the activated T cell into a population of primed T cells. As the T cells within this clonal population express identical T-cell receptors, they possess identical specificities, and the population is referred to as a T cell response. Mature, or "licensed", dendritic cells are thought to be primary antigen-presenting cells responsible for priming T cell responses due to their ability to present antigens on both MHC class I and MHC class II and simultaneously provide adequate co-stimulatory signal (*354*). However, other cell types, such as macrophages, also appear able to prime T cell responses (*356*).

The pathway of memory T cell development is not fully delineated. The initial clonal expansion of an activated naïve T cell produces a large pool of effector T cells that carry out effector functions such as cytokine secretion and cytotoxicity (*357*) (*358*) (*359*). This expansion is followed by a period of contraction whereby a large percentage of the effector T cells die, and surviving T cells are maintained for long periods of time as memory T cells (*358*). Memory T cells can be further divided into three broadly-categorized subsets, defined based on homing capacity and effector function: central

memory (T_{CM}), effector memory (T_{EM}), and the recently discovered resident memory (T_{RM}) (*360*). Central memory T cells (T_{CM}) home to T cell areas of secondary lymphoid organs and possess limited effector function, but readily proliferate and differentiate into effectors T cells in response to antigen exposure (*361*) (*360*) (*362*). In contrast, effector memory T cells (T_{EM}) circulate through extralymphoid tissues and exhibit immediate effector function upon antigenic stimulation, but are limited in proliferative capacity (*361*) (*360*) (*362*). Resident memory T cells (T_{RM}) are a recently discovered subset of T cells that do not circulate, but reside permanently in the tissues; the markers of T_{RM} cells are still under investigation (*359*). However, the markers of the T_{CM} and T_{EM} subsets are consistent with their homing and effector capacities, and allow classification of memory T cell responses into one of these subsets (*361*) (*360*) (*362*). For example, CCR7, the lymph node-homing chemokine receptor, is expressed at high levels on T_{CM} but not T_{EM} (*361*). The distribution of memory T cells varies among infections and vaccine strategies, and is likely the result of differences in the levels and persistence of antigen (*363*) (*362*). The decline in antigen levels that occurs with pathogen clearance is accompanied by a shift in the memory T cell response to a T_{CM}-biased population (*362*). For example, agents that provide antigen for a limited amount of time, such as non-persistent pathogens or replication-defective vaccine vectors, predominately elicit central memory T cells (*362*). In contrast, agents that provide persistent levels of antigen maintain effector memory T cells (T_{EM}) in extralympoid tissues (*362*) (*364*) (*365*). As described below, the differentiation state of vaccine-elicited memory T cells likely has implications for vaccine efficacy.

T-cell receptor ligation of an effector or effector memory CD8+ T cell activates a variety of functions. Canonically, activated CD8+ T cells secrete cytokines and kill recognized target cells. The repertoire of cytokines produced and secreted by the T cells varies among CD8+ T cell subsets, but includes TNF-α, IFN-γ, IL-2, IL-4, IL-5, IL-10, and IL-13 (*366*) (*367*). The most commonly detected CD8+ T cell cytokines are TNF-α, IFN-γ , and IL-2 (*368*). TNF-α is a pro-inflammatory cytokine that activates the vascular endothelium and increases vascular permeability, facilitating the entry of immune cells and molecules into tissues and increased fluid drainage to lymph nodes (*369*). TNF-α also induces an antiviral state by increasing MHC-I expression as well as inducing IL-12 and IL-18 production, which can in turn up-regulate CD8+ T cell production of IFN-γ (*370*) (*371*) (*372*). IFN-γ promotes an antiviral state through a variety of mechanisms, including induction of immunoproteasome function and up-regulation of transporter associated with antigen processing (TAP) and MHC-I expression (*373*) (*374*) (*375*) (*376*) (*370*). IFN-γ can also increase expression of TNF family receptors on target cells, effectively increasing susceptibility to apoptosis (*377*) (*378*). IL-2, which is expressed by some subsets of CD8+ T cells, induces T cell proliferation. In addition, CD8+ T cells can kill target cells via release of perforin and granzymes contained in secretory granules (*379*) (*380*) (*381*), or via ligation of surface receptors linked to apoptosis (*382*). In the granule-dependent mechanism of killing, cytotoxic CD8+ T cells release the contents of secretory granules, perforin and granzymes, into the immunological synaptic cleft between the CD8+ T cell and target cell. Perforin oligomerizes to form pores in the target cell membrane and facilitates entry of granzymes into the target cell (*381*) (*383*) (*379*). Granzymes are a family of serine proteases that induce apoptosis primarily via direct

cleavage and activation of caspases (*381*). CD8+ T cell release of secretory granules, or degranulation, can be detected by staining for externalization of CD107, a membrane glycoprotein of secretory granules (*384*) (*385*) (*714*). In the granule-independent pathway of cytotoxicity, CD8+ T cells engage TNF family "death" receptors on the target cell that induce apoptosis, for example via the interaction of Fas ligand on the CD8+ T cell with Fas receptor on the target cell (*382*) (*386*). CD8+ T cells differ in cytokine production and cytotoxic capacity, and thus differ in their antiviral capacities.

ii. CD8+ T cell-based vaccine design

A successful HIV vaccine will likely need to elicit both antibodies and T cells. The enormous genetic diversity of Env, the target of neutralizing antibodies, in circulating strains of HIV-1 (*256*), suggests vaccine-elicited antibody responses will likely only prevent infection in a subset of HIV-exposed individuals. Vaccine-elicited CD8+ T cells can target internal viral antigens that are more highly conserved across HIV-1 strains, however the antigen processing requirements for T cell recognition limit the ability of T cells to provide sterilizing protection. Thus, an ideal vaccine would induce virus-specific antibodies to provide some level of protection against HIV-1 acquisition, and induce virus-specific CD8+ T cells to control viral replication in individuals who become infected, reducing the risk of transmission to uninfected individuals. Targeting HIV/SIV at the earliest stages of infection would offer the best opportunity for prevention and viral control as the early viral population is small, limited in genetic diversity, and more localized to the portal of entry (*204*), and thus more susceptible to immune control. In addition, immune responses acting early in infection could exert antiviral functions prior to the onset of immune dysfunction that rapidly

ensues once systemic infection is established (*204*) (*280*). Finally immune responses able to act early against viral replication could limit the seeding of viral reservoirs, which are established very early after infection (*219*).

Vaccination involves delivering viral antigens or expressing viral antigens in cells via a variety of strategies. Vaccine strategies eliciting CD8+ T cell responses must produce antigen in cells to allow for presentation on MHC-I molecules. The predominate strategies used to elicit strong CD8+ T cell responses against HIV/SIV include immunization with DNA, live attenuated viruses, and recombinant vectored vaccines (*362*). Recombinant vector vaccines can be replication-competent or -incompetent, and include Modified Vaccinia Ankara (MVA) vectors, fowlpox vectors, and adenoviral vectors (*387*). In particular, heterologous DNA prime-recombinant vector boost regimens, such as DNA/Ad5 and DNA/MVA, are particularly effective at eliciting CD8+ T cell responses (*362*) (*387*). Of note, numerous HIV vaccines using replication-competent recombinant vectors are currently in clinical trials, including vesicular stomatitis virus-, vaccinia virus-, measles virus-, and Sendai virus-based vaccines (*388*). For some recombinant vaccine vectors, such as Ad5, pre-existing immunity to the vector itself can diminish vaccine efficacy (*389*) (*390*).

The vaccine delivery system, as well as route and dose, influences not only the overall immunogenicity of the inserted antigens, but also the qualitative characteristics of the elicited immune response. Different vaccines modalities encoding the same viral antigen elicit CD8+ T cell responses that vary in kinetics and differentiation state (*362*). For example, in Mamu-A1*001:01-positive rhesus macaques, the peak of SIV $Gag_{181-189}$(CM9)-specific CD8+ T cells elicited by live attenuated SIV, MVA, and DNA/MVA

vaccine strategies varies in timing and magnitude (*391*) (*392*) (*362*). In addition, the timing and magnitude of CD8+ T cell memory contraction also differ among vaccine strategies (*362*). Memory CD8+ T cell responses elicited by different vaccine strategies also exhibit variation in functional capacity and localization, which is likely the result of differences in differentiation state determined by level and persistence of vaccine-expressed antigen, as described above (*362*).

Despite these differences in vaccine-elicited CD8+ T cell phenotype, the repertoire of epitopes targeted by vaccine-elicited CD8+ T cells is overlapping among various vaccine strategies and infection itself (*391*) (*392*) (*362*) (*393*) (*394*) (*395*) (*786*). While variations do exist in the patterns of CD8+ T cell epitope targeting, this is largely attributed to differences in antigen presentation, particularly MHC-Ia expression, among vaccinated individuals (*786*). However, the targeting of immunodominant epitopes is consistent among vaccine regimens, with the exception of the rhesus cytomegalovirus vaccine vectors (*393*). For example, both the natural and vaccine-elicited CD8+ T cell response to SIVgag in Mamu-A1*001:01-positive rhesus macaques typically includes a GagCM9-targeted response (*396*) (*397*) (*787*) (*391*) (*392*) (*362*) (*393*). Indeed, CD8+ T cell epitope overlap among vaccine strategies is exemplified by the ability of heterologous prime-boost regimens, such as DNA/MVA and DNA/Ad5, to prime and then boost a GagCM9-specific CD8+ T cell response (*391*) (*392*) (*362*) (*393*).

Another aspect of vaccine development is the selection of viral antigens. Factors taken into account include conservation of the antigen and the timing of antigen presentation in infected cells. Theoretically, conserved proteins would offer a greater chance of eliciting a CD8+ T cell response targeting diverse viral strains. Pol and Gag are

the most conserved antigens of HIV/SIV, while Env is the most variable (*256*). However, within antigens, some regions are variable and some conserved. Thus, recent efforts have focused on developing conserved element vaccines, incorporating the most highly conserved regions or epitopes of the viral genome. Another way to overcome virus variability is to include multiple coding sequences for the same antigen, region, or epitope; such multivalent vaccines often include sequences from multiple HIV-1 group M clades. In addition, the timing of antigen presentation in infected cells dictates the onset of CD8+ T cell recognition. HIV-1 viral progeny are produced approximately 24 hours after infection (*398*). Thus, viral antigens presented early in the infectious cycle may serve as advantageous CD8+ T cell targets as early CD8+ T cell recognition could eliminate infected cells prior to virion production. Viral antigens present in incoming virions can be rapidly processed and presented following infection. For example, Gag- and Pol-specific CD8+ T cells recognize virion-derived antigen presented on infected CD4+ T cells as early as 2 hours post-infection (*399*) (*400*), prior to viral integration and gene expression (*401*) (*402*). In contrast, processing and presentation of Env epitopes requires *de novo* Env synthesis (*403*), and Env-specific CD8+ T cells do not recognize infected CD4+ T cells until 18 hours post-infection (*399*). An additional factor in the timing of antigen presentation is Nef-mediated MHC-I down-regulation. Nef down-regulates MHC-I beginning at 12 hours post-infection (*400*), and thus CD8+ T cells specific for viral epitopes presented prior to Nef-mediated MHC-I down-regulation could more effectively recognize infected cells later in infection. Thus, at least some Gag- and Pol-specific CD8+ T cells recognize infected cells prior to Nef-mediated MHC-I down-regulation, while Env-specific CD8+ T cells do not. In agreement with both relative

sequence conservation and early presentation of Gag, the breadth of the HIV Gag-specific CD8+ T cell response is associated with lower viremia (*403*) (*404*). However, conflicting reports exist regarding the benefit of Env-specific CD8+ T cells in HIV control and disease progression (*403*) (*405*) (*406*) (*407*). The associations of CD8+ T cell targeting of particular whole viral antigens with viral control are likely complicated by differences in conservation and timing of presentation among individual epitopes of the same viral antigen. Indeed, differential timing of presentation among epitopes of the same viral protein has been previously demonstrated for epitopes of both Gag and Rev (*408*) (*409*).

Several characteristics of HIV-specific CD8+ T cells are correlated with HIV control, including broad targeting of conserved regions (particularly of Gag) (*404*) (*410*) (*411*) (*412*), polyfunctionality (*413*) (*414*) (*415*), proliferative capacity (*416*) (*417*), cytotoxic capacity (*418*) (*419*) (*788*), and high avidity peptide recognition (*414*) (*420*). Tailoring vaccines to elicit CD8+ T cells with these qualities, either through selection of vaccine antigens or selection of vaccine platform, might improve vaccine efficacy.

C. Previous vaccine trials in humans and vaccine studies in non-human primates

Vaccines can be improved by studying the outcomes and immune correlates of previous vaccine efficacy trials in humans and non-human primates. In the decades since the discovery of the virus, six major HIV-1 vaccine efficacy clinical trials have been conducted. The VaxGen trials, Vax003 and Vax004, utilized bivalent vaccines containing monomeric gp120 subunit in alum adjuvant: AIDSVAX B/E and AIDSVAX B/B, respectively. Administration of the Vax004 AIDSVAX B/B vaccine protected chimpanzees from intravenous challenge (*421*) (*422*). However, these vaccines generated

only limited levels of neutralizing antibodies in phase I and phase II clinical trials and showed no efficacy in reducing HIV-1 acquisition or slowing disease progression among MSM and high risk women in the United States and Europe, nor among injecting drug users in Thailand (*423*) (*424*) (*425*) (*426*) (*427*) (*428*) (*429*) (*430*).

Two subsequent trials focused on stimulating cell-mediated immunity with Merck's replication-deficient recombinant adenovirus serotype 5 (Ad5) trivalent vaccine expressing HIV-1 *gag*, *pol*, and *nef* (*429*) (*430*). The STEP trial, also known as HVTN 502, was conducted among MSM and heterosexual men and women in North and South America, the Caribbean, and Australia. While robust T cell responses to the vaccine antigens were detected by ELISPOT and flow cytometry assays, no reduction in acquisition or long-term control of post-infection viremia was observed and the trial was stopped for futility (*389*). Subsequent analyses revealed evidence of vaccine-elicited immune pressure on the virus in individuals with specific HLA types as well as individuals with responses to particular Gag and Nef epitopes (*431*). However, these analyses also revealed an unexpected transient increased rate of HIV infection in recipients that were both uncircumcised and immune to Ad5 prior to vaccination (*389*) (*390*); the mechanisms behind these findings remain unknown. Excess infections in the vaccine arm were also observed in the Phambili trial (*432*), also known as HVTN 503, which utilized the same vaccine among sexually active heterosexuals in South Africa. HVTN 503 was halted shortly after the STEP trial, during study enrollment (*430*) (*433*). A similar Ad5 vaccine regimen administered to rhesus macaques also failed to protect against infection via either intra-rectal SIVsmE660 or penile SIVmac251 challenges (*434*) (*435*).

The RV144 Thai trial, conducted among heterosexual men and women in Thailand, is the only HIV-1 vaccine trial to show efficacy against HIV-1 acquisition, with a 31% reduction in the frequency of HIV-1 acquisition among vaccinated individuals (*436*). The RV144 vaccine regimen consisted of a canarypox vaccine vector prime followed by two boosts with AIDSVAX B/E, the same bivalent gp120+alum vaccine utilized in the Vax003 trial (*436*) (*429*) (*430*). The recombinant canarypox vaccine vector, called ALVAC-HIV, expresses Gag, Pro, and gp120 linked to the transmembrane anchoring portion of gp41 (*436*). The RV144 vaccine regimen induced binding antibodies against HIV-1 Env and Gag immunogens, CD4+ T cell proliferative responses, neutralizing antibodies, and antibody-dependent cell-mediated cytotoxicity (ADCC) (*437*) (*438*). No significant HIV-specific CD8+ T cell responses were detected (*436*). However, antibodies binding to the V1V2 region of gp120, particularly the IgG1 and IgG3 subclasses that mediate ADCC, correlated with protection from HIV-1 acquisition (*347*) (*348*). The V1V2 region serves critical functions, including the binding of receptor, co-receptor, and $\alpha 4\beta 7$ integrin (*439*), and is the target of neutralizing antibodies (*440*) (*441*) (*442*) (*443*). However, neither neutralization activity nor antibody-dependent cellular cytotoxicity activity correlated with protection (*347*) (*444*) (*348*). In the same study, plasma Env-binding IgA antibodies were directly correlated with infection (*347*). Vaccination did not modify disease course in individuals that became infected; viral set-point, post-infection CD4+ T cell counts, and time to AIDS-defining illness/death were not significantly different among infected individuals in the vaccine and placebo groups (*445*).

Most recently, the HVTN 505 trial utilized a heterologous prime-boost approach with a *gag/pol/env* DNA prime and matching replication-defective recombinant Ad5 boost. Due to the unexpected results in the HVTN 502 and 503 trials using Ad5 vectors, HVTN 505 was conducted in the U.S. among circumcised MSM with negative Ad5-specific antibody titers prior to vaccination (*446*) (*430*). HVTN 505 was halted for lack of efficacy in mid-2013 when primary analysis showed no difference in acquisition of infection between the vaccine and placebo groups, with no significant differences in mean viral load set-point among infected individuals in the two groups (*446*). A similar vaccine regimen encoding SIVmac239 versions of the same antigens protected about 50% of rhesus macaques from acquisition of intra-rectal SIVsmE660, but did not protect against acquisition of SIVmac251 (*447*). However, vaccinated rhesus macaques that acquired SIVmac251 exhibited lower peak plasma viremia than unvaccinated animals. Vaccine protection against acquisition of SIVsmE660 was associated with low levels of neutralizing antibodies in PBMC and Env-specific CD4+ T cell responses (*447*). Additional DNA prime/Ad5 boost regimen studies using only internal antigens found that vaccination fails to protect against SIV acquisition, thus further demonstrating that Env-specific immune responses are required for protection. Administration of this regimen did result in reduced plasma viral loads and increased preservation of memory CD4+ T cell populations in vaccinated animals (*448*) (*449*) (*396*) (*450*), which was correlated with the magnitude of virus-specific CD8+ T cells (*449*) (*396*). However, DNA/Ad5-mediated viral control was transient (*449*) (*396*) (*451*) (*450*), and in some cases only manifested in rhesus macaques expressing Mamu-A1*001:01 (*396*), an MHC-Ia allele associated with lower viral loads (*452*).

Many other T cell-eliciting vaccine modalities have been tested in non-human primates, with variable challenge outcomes that likely result, at least in part, from differences in challenge strains and routes of challenge. The majority of T-cell-eliciting vaccine regimens tested to-date utilize DNA and/or replication-incompetent or –defective recombinant vectors, particularly vectors based on poxviruses and adenoviruses. Modified Vaccinia Ankara (MVA), an attenuated form of vaccinia (*453*), has been extensively tested alone and in combination with DNA vaccination and other vaccine vectors (*454*). MVA vaccination encoding internal antigens results in reduced set point viral load upon SIV challenge (*455*) (*456*). In challenge with a simian-human immunodeficiency virus hybrid SHIV 89.6P, a DNA prime/MVA boost vaccine regimen resulted in reduced peak and set point viremia, increased preservation of CD4+ T cells, and prolonged survival (*392*) (*457*), which was associated with both antibody- and CD8+ T cell-mediated control (*458*) (*459*). Most animals maintained long-term viral control, while loss of control was associated with CD8+ T cell-driven mutational escape and virus-specific CD4+ and CD8+ T cell exhaustion (*460*) (*454*). However, in studies using neutralization-resistant SIVmac239 as a challenge virus, a similar DNA/MVA vaccine regimen only transiently reduced viral loads, and most animals progressed to disease (*461*). However, some of the most effective vaccines include live attenuated vaccines, such as SIVΔNef. Vaccination SIVΔNef elicits robust antibody and T cell responses and can protect against virus acquisition and reduce viral load in animals that become productively infected (*462*) (*463*) (*464*). SIV-specific CD8+ T cell responses contribute to the control of viremia observed with live attenuated vaccines (*303*) (*464*) (*465*) (*466*) (*303*, *467*) (*468*) (*469*). However, the ability of SIVΔNef viruses and other live attenuated

SIV viruses to cause immune dysfunction, T cell depletion, and AIDS as well as their ability to recombine with challenge strains precludes the use of SIVΔNef viruses as vaccines (*464*) (*303*) (*470*). More recently, replication-competent viral vectors have shown efficacy against SIV and/or SHIV challenge (*388*), including vectors based on rhadinovirus, vaccinia virus, vesicular stomatitis virus, rabies virus, Sendai virus, Semliki Forest virus, and rhesus cytomegalovirus (*471*) (*789*) (*472*) (*473*) (*474*) (*475*) (*476*) (*477*) (*478*) (*479*) (*480*) (*450*) (*481*). While correlates of protection and/or virus control have not been defined for most of these vectors, virus-specific CD8+ T cell responses were implicated in vaccine-mediated control in some cases (*789*) (*479*) (*480*).

III. The major histocompatibility complex

Pathogen-specific CD8+ T cells play a critical role in the adaptive immune response against infection. Upon activation, CD8+ T cells can secrete antiviral cytokines and kill target cells, and thus serve to control or clear infections by limiting viral replication and directly eliminating infected cells (*287*). Activation of CD8+ T cells occurs upon recognition of foreign peptides presented by major histocompatibility class I (MHC-I) molecules on the surface of target cells (*349*). The presentation of peptides on MHC-I molecules is an intricate process that involves the breakdown of foreign proteins into antigenic peptides, loading onto MHC-I molecules, and transport to the cell surface for surveillance by CD8+ T cells (*482*) (*394*). The repertoire of peptides presented, and consequently the repertoire of epitope-specific CD8+ T cell responses engendered, is in large-part determined by the set of MHC-I molecules expressed in the individual (*394*). The MHC-I molecules that typically present peptides to CD8+ T cells are encoded by the

highly polymorphic MHC-Ia genes (*325*) (*326*). However, while most CD8+ T cells recognize peptides presented by MHC-Ia molecules, some CD8+ T cells recognize peptides presented by non-classical, more highly conserved MHC-I molecules (*483*). This section focuses on the role of MHC molecules in presenting peptide antigens to T cells. Particularly relevant to the data presented here, presentation of peptides by the non-classical MHC-Ib molecule, MHC-E, is discussed.

A. Classification of MHC-I molecules

Recognition of specific antigen is central to T cell activation both in priming of a naïve T cell upon first antigen encounter and in triggering of antigen-experienced T cells. In contrast to B cells, which recognize native antigens, T cells recognize processed antigens presented by antigen presentation molecules on the surface of target cells (*484*) (*485*). Indeed, the T-cell receptor contacts both the antigenic peptide and the antigen presentation molecule during recognition (*486*) (*487*) (*488*). Thus, the activation of each T cell is "restricted" by the particular antigen presentation molecule presenting the antigenic peptide (*484*). Most T cell antigen presentation molecules are encoded by a cluster of genes called the Major Histocompatibility Complex (MHC), located on human chromosome 6 (*489*). Some MHC molecules carry out alternative, immunoregulatory functions distinct from antigen presentation to T cells.

Major Histocompatibility Complex (MHC) genes are broadly classified into two categories: MHC class I (MHC-I) and MHC class II (MHC-II). MHC-II molecules typically present exogenous peptides to CD4+ T cells, and are expressed by only a subset of cell types, primarily professional antigen-presenting cells such as dendritic cells, macrophages, and B cells (*490*) (*355*). The MHC-I genes are further divided into classical

(MHC-Ia) and non-classical (MHC-Ib) subcategories (*491*). MHC-Ia molecules typically present cytosolic peptides to CD8+ T cells and are expressed by all nucleated cells. The classical MHC-Ia genes are highly polymorphic in the human population, with thousands of alleles identified thus far (*492*). This diversity in MHC-Ia genes reflects the primary role of these molecules in the presentation of diverse peptide antigens to CD8+ T cells (*493*). As each MHC-Ia molecule binds a distinct repertoire of peptides (*494*), diversity of MHC-Ia endows the population with the ability to present many different peptide antigens and, as a result, mount immune responses to many different pathogen epitopes (*495*). In addition, the collection of MHC-I molecules expressed by the individual governs the repertoire of peptides presented and the range of specificities of the CD8+ T cell response mounted in that individual. In contrast to the MHC-Ia genes, non-classical MHC-Ib genes exhibit limited polymorphism, which is thought to reflect the conserved immunoregulatory functions of these molecules (*491*) (*790*). While some MHC-Ib molecules appear to present peptides to T-cell receptors, some also ligate to innate immune receptors, as described below for MHC-E (*483*). The precise functional roles of MHC-Ib molecules are not fully elucidated in most cases (*790*).

In humans, the MHC genes are called human leukocyte antigen (*HLA*) genes; the *HLA-A*, *-B*, and *–C* genes encode classical MHC-Ia molecules, and the *HLA-E*, *-F*, and *–G* genes encode non-classical MHC-Ib molecules. The rhesus macaque homologues of these MHC genes are designated *Mamu*; the *Mamu-A and –B* genes encode classical MHC-Ia molecules, and the *Mamu-E, -F, and –AG* genes encode the non-classical MHC-Ib homologues of *HLA-E, -F,* and *–G*, respectively. In humans, there are three MHC-Ia loci per chromosome, *HLA-A, -B,* and *–C*, and thus individuals express a maximum of 6

MHC-Ia molecules (*496*). In contrast, rhesus macaques possess complex MHC genetics, including a number of duplications and deletions in the MHC-Ia genes (*497*) (*498*). Thus, rhesus macaque MHC chromosomes carry a heterogenous assortment of up to 20 MHC-Ia loci per haplotype (*497*) (*498*).

The structural features of MHC molecules govern the repertoire of peptides presented (*493*). MHC-I molecules are composed of the MHC-I-encoded membrane-spanning alpha (α) chain, or heavy chain, non-covalently complexed with beta2-microglobulin (β2M), a molecule not encoded by the MHC (*499*). The MHC-I α chain dictates most of the function of the MHC-I molecule as the α1 and α2 domains form the peptide-binding groove and contact the T-cell receptor, and the α3 domain anchors the MHC-I molecule in the membrane (*500*). CD8 expressed on the surface of the T cell binds to an invariant site in the α3 domain of MHC-I, and also interacts with the base of the α2 domain (*501*). Due to the closed confirmation of the MHC-I peptide-binding groove, these molecules typically present short peptides of 8 to 10 amino acids in length (*494*). The major sites of polymorphism in variable MHC-Ia molecules are in the α1 and α2 domains forming the peptide-binding region, particularly in the amino acids lining the peptide-binding groove (*325*). Thus, these polymorphic residues govern the diversity of epitopes presented by the particular MHC-I molecule, and consequently the targeting of the mounted CD8+ T cell response.

B. Antigen presentation on MHC-I

In order to successfully reach the cell surface for recognition by antigen-specific CD8+ T cells, antigens must be proteolytically processed into short peptides, loaded onto MHC-I molecules, and transported to the cell surface. The generation of MHC-I-loaded

peptides typically occurs in the cytosol where the proteasome breaks down larger proteins into short peptides (*502*) (*503*) (*504*). The transporter associated with antigen processing (TAP) transports processed cytosolic peptides into the endoplasmic reticulum (ER) (*506*), where assembly of MHC-I molecules occurs (*507*). Newly synthesized MHC-I heavy chains initially complex with chaperone calnexin until β2M binds and facilitates release of MHC-I from calnexin and association with a chaperone complex called the peptide-loading complex (PLC) (*508*). The PLC is composed of two chaperone proteins called calreticulin and ERp57, a TAP-associated protein called tapasin, and TAP itself (*507*) (*509*) (*510*) (*511*). The PLC facilitates peptide loading onto MHC-I by maintaining the MHC-I molecule in a peptide-receptive state and bringing the MHC-I molecule in close association with TAP, through which potential binding peptides are being transported (*507*) (*512*) (*513*). Peptide binding to the MHC-I molecule releases it from the PLC, and the peptide:MHC-I complex is transported through the Golgi apparatus to the cell surface (*507*) (*514*). Thus, the range of peptides presented to CD8+ T cells is also governed by the ability of the antigen processing and presentation machinery to generate the peptide and load it onto MHC-I.

Of note, many pathogens interfere with MHC-I antigen presentation in order to evade CD8+ T cell immunity (*515*). For example, herpes simplex virus 1 (HSV-1), human cytomegalovirus (HCMV), bovine herpesvirus 1 (BHV 1), and Epstein barr virus (EBV) encode viral inhibitors of TAP (*516*). Adenovirus protein E19 retains MHC-I in the ER and prevents tapasin-mediated association of MHC-I with TAP (*517*). As described above, the Nef proteins of HIV and SIV remove MHC-I from the cell surface and retain it in the trans-Golgi network (*782*) (*189*) (*331*) (*332*) (*330*) (*781*) (*738*) (*191*).

However, as described below in section C below, this interference can render infected cells susceptible to natural killer (NK) cells.

C. MHC-E

i. General characteristics of MHC-E

Major histocompatibility complex E (MHC-E) molecules are a class of non-classical MHC-Ib molecules encoded by the MHC-E locus. Homologues of human MHC-E, or HLA-E, have been found in mice (Qa-1b), rats (RT-BM1), and non-human primates (*518*) (*519*) (*520*) (*521*) (*522*) (*523*) (*524*). HLA-E is ubiquitously found on virtually all nucleated cells, with particularly high expression on immune cells (*520*) (*525*) (*526*). HLA-E retains the key structural features of MHC-I molecules, and associates with β2M as well as TAP, tapasin, and calreticulin (*501*) (*527*) (*520*) (*528*).

The MHC-E locus is the most well conserved of all known primate MHC class I genes (*791*) (*524*). In humans and most non-human primates, classical MHC-Ia genes exhibit a large degree of polymorphism in the exons encoding the α1 and α2 domains that form the peptide-binding region (*325*); this positive selection pressure allows for diversity in ligand binding, and thus for presentation of diverse peptide antigens to T cells. In contrast, MHC-E genes in primates exhibit higher rates of synonymous nucleotide substitutions than non-synonymous substitutions in these exons (*521*) (*522*) (*524*), suggesting that the peptide-binding region of MHC-E is under purifying selection to maintain a critical immunological function. This immune function was later revealed when two groups discovered the role of HLA-E in NK cell regulation.

ii. The role of MHC-E in NK cell regulation

HLA-E binds leader sequence peptides derived from positions 3-11 of certain HLA class I molecules, including those of particular HLA-A, B, C, and G molecules (*529*) (*530*) (*528*). In fact, surface expression of HLA-E, which had been controversial until this discovery (*520*) (*525*), is dependent upon the presence of HLA-E-binding leader peptides (*528*) (*530*). HLA-E cannot bind its own leader peptide (*529*) (*528*), and thus HLA-E surface expression correlates with the expression of other MHC-I molecules possessing a leader peptide capable of binding to HLA-E (*528*) (*530*). Studies performed with leader peptides containing various amino acid substitutions demonstrated that positions 2, 7, and 9 were critical for binding to HLA-E (*529*). Surface expression of HLA-E is considered TAP-dependent as HLA-E is not detected on the surface of TAP-deficient cells despite adequate expression of MHC-I molecules with appropriate HLA-E-binding leader peptides (*528*, *530*). However, a separate study detected HLA-E expression on the surface of PBMC from a TAP1-deficient, MHC-Ia-deficient patient, suggesting that TAP-independent surface expression of HLA-E can occur (*531*).

HLA-E/leader peptide complexes bind to CD94/NKG2 receptors present on NK cells and a small subset of T cells (*532*). CD94 belongs to the C-type lectin superfamily and is expressed at the cell surface as a heterodimer with NKG2A, NKG2B, NKG2C, or NKG2E (*533*). The particular CD94/NKG2 heterodimer dictates whether the receptor will activate or inhibit the NK cell's activity (*533*). HLA-E/leader peptide complexes bind to inhibitory receptors CD94/NKG2A and CD94/NKG2B as well as to the activating receptor CD94/NKG2C (*532*). In order to investigate the biological significance of this interaction, several groups employed .221 cells, a mutagenized B-lymphoblastoid cell line, which express low-levels of intracellular HLA-E but are devoid of HLA-A, B, C,

and G molecules, and thus do not express surface HLA-E under normal conditions. Expression of HLA class I molecules or chimeric molecules containing an HLA-E-binding leader sequence capable of stabilizing HLA-E surface expression protects .221 cells from NK cell lysis by NKG2A+ NK cell lines or clones (*532*) (*526*) (*534*) (*535*). This finding was repeated using other NK-susceptible cell types, such as RMA-S cells (*534*) (*536*). This inhibition of NKG2A+ NK cell-mediated lysis occurred in a CD94-dependent and HLA-E-dependent manner (*532*) (*526*). As HLA-E surface expression is dependent on an adequate supply of MHC-I leader peptides and perhaps upon TAP function, these findings implicate MHC-E as a host mechanism to detect defects in MHC-I antigen presentation that often occur upon viral infection or neoplastic transformation. Thus, MHC-E could counteract CD8+ T cell evasion mechanisms by targeting virally infected cells or tumor cells for NK cell lysis. In contrast to HLA-E/leader peptide-mediated inhibition of CD94/NKG2A+ NK cell clones, the activity NK cell clones expressing the activating receptor CD94/NKG2C+ respond poorly to most HLA-E/leader peptide complexes, with the exception of strong activation by HLA-E loaded with leader peptide derived from HLA-G, a non-classical MHC-Ib molecule with restricted placenta-associated tissue expression (*537*). The role of MHC-E in binding MHC-I leader peptides to regulate NK cells is preserved in mice (*538*).

iii. Primate MHC-E

The diversity of the MHC-E locus differs among primate species. There are only two HLA-E alleles present in the human population, which differ by a single amino acid change outside of the peptide-binding groove. Thus, each individual expresses 1 or 2 HLA-E molecules of very limited diversity. In contrast, 28 Mamu-E genes differing by

up to 45 amino acids have been identified in rhesus macaques (Figure 1.1), and individual rhesus macaques express 1-4 different MHC-E molecules (Figure 2.1), suggesting the Mamu-E locus may have undergone a duplication event. Genetically simplified Mauritian cynomolgus macaques carrying MHC haplotypes M1-M4, which represent the vast majority of the MCM population (*100*), express only 4 Mafa-E alleles as a population (Figure 1.1). However, 2 Mafa-E molecules can be expressed from a single haplotype (M4), suggesting a potential duplication event of the Mafa-E locus in MCMs as well (Figure 1.1). Cotton-top tamarins, a non-human primate species with limited sequence variation in MHC-Ia genes (*539*), appear to encode only one MHC-E molecule (*524*). Of note, nearly all primate MHC-E molecules identified thus far share an identical peptide-binding groove (Figures 1.1 and 1.2) (*522*) (*523*) (*524*).

Thus far, a few differences between the two HLA-E alleles have been observed. HLA-E*01:03 is expressed at higher levels on the cell surface compared to HLA-E*01:01, despite equal overall cellular expression, and exhibits higher affinity for leader peptides (*540*) (*541*) (*542*). In addition, different peptide repertoires are eluted from the two HLA-E molecules in transfected cell lines devoid of MHC-I molecules (*543*) (*544*). The two HLA-E alleles are carried at nearly equal frequencies in the human population (*545*), which may reflect balancing selection to maintain these two alleles and implicate functional differences between the two molecules. Indeed, correlative studies implicate HLA-E*01:03 in HIV protection and HLA-E*01:01 in hepatitis C virus protection (*792*) (*793*) (*561*), however the mechanisms behind these associations is unknown. There are no studies evaluating the peptide ligands or immune functions of non-human primate MHC-

E molecules, and thus it is unclear whether individual MHC-E molecules within and across primate species function differently.

iv. Other peptide ligands of MHC-E

Apart from binding peptides derived from MHC-I leader sequences, HLA-E and mouse Qa-1b also bind other peptides, including peptides derived from self, bacterial, and viral proteins (bacterial and viral ligands are summarized in Table 1.1). Similar to the canonical leader sequence-derived peptides, some virally derived HLA-E-binding peptides confer resistance to NK cell-mediated killing (*546*) (*547*) (*548*) (*549*) (*550*). In addition, numerous groups have identified CD8+ T cells recognizing peptide/MHC-E complexes through their T-cell receptor (*551*) (*552*) (*553*) (*554*) (*555*) (*556*) (*557*) (*558*) (*559*) (*560*) (*561*). NK cell and CD8+ T cell recognition of these alternative peptide/MHC-E complexes are described below in sections v and vi, respectively.

Mouse MHC-E (Qa-1b) Ligands					
Species source	**Protein source**	**Peptide**	**Recognized by NKG2A+ NK cells**	**Recognized by MHC-E-restricted CD8+ T cells**	**Ref.**
Klebsiella pneumoniae	Urease enzyme	LLLPRGLLL	Unknown	Unknown	(*562*)
Salmonella typhimurium	GroEL	GMQFDRGYL	Unknown	Yes	(*552*) (*553*)
Human MHC-E (HLA-E) Ligands					
Species source	**Protein source**	**Peptide**	**Recognized by NKG2A+ NK cells**	**Recognized by MHC-E-restricted CD8+ T cells**	**Ref.**
Epstein Barr virus	BZLF-1	SQAPLPCVL	No	Yes	(*563*) (*536*) (*555*) (*558*) (*794*)
Influenza virus	Matrix	ILGKVFTLT	Unknown	Unknown	(*563*)
Mycobacterium tuberculosis	Rv0056	FLLPRGLAI	Unknown	Yes	(*557*) (*583*) (*584*) (*585*)
	Rv1518	VMATRRNVL	No	Yes	
	Rv2997	RMPPLGHEL			
	Rv1523	VLRPGGHFL			
	Rv1734c	VMTTVLATL			
	Rv1484	RLPAKAPLL			
Human Cytomegalovirus	UL40	VMAPRTLIL	Yes- inhibits lysis	Yes (NK-CTLs)	(*558*) (*559*)
		VMAPRTLVL			
		VMAPRTLLL			
Salmonella enterica serovar Typhi strain Ty21a	GroEL	AVAKAGKPL	Unknown	Yes	(*560*)
		AMLQDIATL			
		KMLRGVNVL			
		KLQERVAKL			
HIV-1	Gag	AISPRTLNA	Yes- inhibits lysis*	Unknown (HLA-B*57-restricted CD8+ T cells)	(*549*)
Hepatitis C virus	Core	YLLPRRGPRL	Yes- inhibits lysis	Yes, both HLA-A2-restricted and HLA-E-restricted	(*550*) (*561*)
Vaccinia virus	D8L	DGLIIISI	Yes- inhibits lysis**	Unknown (HLA-Cw1-restricted)	(*564*)

*A recent report contradicted this finding, and instead found that this peptide cannot effectively bind CD94/NKG2A or inhibit killing by NKG2A+ NK cells (*565*)
**This peptide is missing the P5 arginine present in other peptides that bind CD94/NKG2A receptors

Table 1.1: Previously identified pathogen-derived MHC-E ligands. Table displays previously reported Qa-1b- and HLA-E-binding ligands and whether the ligand has been demonstrated to be recognized by NKG2A+ NK cells or MHC-E-restricted CD8+ T cells.

v. Regulation of NK cells by virus-derived MHC-E ligands

As discussed above in section ii, MHC-E may serve an important host immune strategy against viruses that interfere with MHC-I antigen presentation, as the failure to deliver the leader peptide/HLA-E inhibitory signal through CD94/NKG2A renders cells susceptible to NK cell-mediated killing. However, viral mechanisms to counteract this MHC-E immune surveillance mechanism have also been discovered. Human cytomegalovirus (HCMV), HIV-1, hepatitis C virus (HCV), and vaccinia virus (VACV) encode peptides that both stabilize HLA-E surface expression and effectively ligate to CD94/NKG2A receptors, inhibiting CD94/NKG2A+ NK cell-mediated lysis. The HCMV UL40 gene encodes an exact mimic of the MHC-I leader sequence-derived VL9 peptide, and thus expression of UL40 prevents NK cell-mediated lysis by CD94/NKG2A+ NK cells (*546*) (*566*) (*547*) (*548*) (*567*). This is particularly relevant for immune evasion by HCMV as HCMV US2-US11 viral proteins interfere with MHC-I antigen presentation through multiple mechanisms in order to evade CD8+ T cell responses (*795*) (*568*) (*569*) (*570*) (*571*) (*572*) (*573*). Indeed, HCMV strains deleted of UL40 cannot inhibit CD94/NKG2A+ NK cell killing (*548*). Of note, the UL40-derived VL9 peptide is presented on MHC-E independently of TAP, and thus retains its NK cell evasion capabilities in the face of viral TAP inhibitor US6 (*548*) (*574*). HIV-1, HCV, and VACV also encode peptides capable of stabilizing HLA-E surface expression and delivering an inhibitory signal to CD94/NKG2A+ NK cells. However, not all peptides capable of stabilizing MHC-E surface expression confer protection from NK cell-mediated lysis (Table 1.1) Multiple groups found that the arginine located at position 5 (P5) of MHC-I leader peptides bound to MHC-E is critical for recognition by

CD94/NKG2A (*575*) (*576*) (*577*) (*549*) (*578*). As shown in Table 1.1, the HLA-E-binding HCMV, HIV-1, and HCV peptides shown to interact with CD94/NKG2A all possess the P5 arginine. However, the VACV HLA-E-binding peptide does not, suggesting that the P5 arginine rule is not absolute. As discussed below, some of these HLA-E-binding viral peptides are targeted by HLA-E-restricted CD8+ T cells.

vi. MHC-E-restricted CD8+ T cells

While MHC-E was first described as a molecule primarily involved in NK cell regulation, evidence that MHC-E serves an additional role as a restricting molecule for CD8+ T cells subsequently emerged. Indeed, the crystal structure of HLA-E revealed that the residues and structural elements required for interaction with both CD8 and TCRα/β are conserved in HLA-E (*501*) (*527*). MHC-E-restricted CD8+ T cells recognizing self, bacterial, and viral peptides exhibit a variety of functions, which are described in this section.

a. Auto-reactive MHC-E-restricted CD8+ T cells specific for TCR-Vβ-derived and tumor-derived peptides

Self peptides presented by MHC-E include peptides derived from certain TCR-Vβ chains (*579*) (*556*). TCR-Vβ-specific, Qa-1b-restricted CD8+ T cells were identified in mice after administration of superantigens, such as Staphylococcus enterotoxin B (SEB), intravenously (*579*). CD8+ T cells from SEB-primed mice lyse autologous CD4+ T cell targets based on expression of particular TCR-Vβ chains, and this recognition is specifically blocked by antisera to Qa-1 (*579*). Similarily, TCR-Vβ-specific, HLA-E-restricted CD8+ T cell lines expanded from human peripheral blood lyse TCR-Vβ-peptide-loaded HLA-E transfectants as well as autologous CD4+ T cell clones expressing

the specific TCR-Vβ in an HLA-E-dependent manner (*556*). The groups that performed these studies proposed that Qa-1b and HLA-E may bind non-leader sequence-derived peptides upon activation, and thus autoreactive Qa-1b-restricted TCR-Vβ-specific CD8+ T cells might serve an immunoregulatory role *in vivo*, controlling the activation of CD4+ T cells. In support of this autoregulatory T cell mechanism, Qa-1-deficient mice develop exaggerated CD4+ T cell responses to self and foreign peptides and increased susceptibility to experimental autoimmune encephalomyelitis (*580*).

Qa-1b-restricted CD8+ T cells also participate in the immune response against tumors with defects in antigen processing (*757*) (*796*). In the context of cellular antigen processing defects, such as TAP inhibition or deficiency, Qa-1b and HLA-E shift from binding predominantly leader sequence-derived peptides to binding a distinct repertoire of self peptides (*581*) (*757*) (*582*) (*797*). TAP-deficient cells activate Qa-1b-restricted CD8+ T cells from mice immunized with TAP-deficient RMA-S cells expressing the co-stimulatory molecule CD80, while TAP restoration decreases the Qa-1b-restricted CD8+ T cell response (*757*). Vice versa, expression of TAP-inhibitor UL49.5 in TAP-positive tumor cells allows for recognition by Qa-1b-restricted CD8+ T cells (*581*). Immunization with TAP-inhibited DCs induces tumor-specific CD8+ T cell responses and protects mice against the outgrowth of TAP-deficient tumors (*758*) (*798*), suggesting that Qa-1b-restricted CD8+ T cells may protect against tumors with defects in antigen processing.

b. MHC-E-restricted CD8+ T cells specific for bacterial peptides

MHC-E-restricted CD8+ T cell responses recognizing bacterial peptides are found in the context of infection with *Listeria monocytogenes, Salmonella typhimurium,* and *Mycobacterium tuberculosis* as well as in the context of typhoid vaccination. In mice, *L.*

monocytogenes and *S. typhimurium* infection elicits pathogen-specific Qa-1b-restricted CD8+ T cells (*551*) (*552*). *L. monocytogenes*-specific Qa-1b-restricted CD8+ T cells lyse infected macrophages as well as infected Qa-1b-transfected cell lines (*551*). In *S. typhimurium*-infected mice, Qa-1b-restricted CD8+ T cells make up the dominant CTL response, comprising 50-60% of *S. typhimurium*-specific CTLs (*552*). One immunodominant *S. typhimurium*-specific CTL response recognized a Qa-1b-presented peptide derived from the bacterial GroEL protein (*553*). This GroEL-specific CTL response was particularly cross-reactive, recognizing cells infected with other Gram-negative bacteria as well as cells loaded with mouse heat shock protein 60 (*553*).

The first pathogen-specific HLA-E-restricted CD8+ T cell responses identified in humans were from individuals latently infected with *M.tuberculosis* (*557*). Clones derived from latently infected individuals secrete IFN-γ in response to HLA-E transfectants treated with supernatant from *M.tb*-infected DCs as well as to HLA-E-transduced mouse cell lines infected with *M.tb* (*557*). A separate group identified HLA-E-restricted CD8+ T cells targeting six *M.tb* H37Rv-derived peptides in individuals responding to *M.tb*-purified protein derivative (PPD) as well as in patients with active TB (*583*) (*584*) (*585*). One Rv2997-derived peptide, RMPPLGHEL, was targeted by HLA-E-restricted CD8+ T cells in over 30% of PPD-responding donors and in 60% of active TB patients (*583*) (*585*). Tetramer staining revealed an increase in HLA-E-restricted CD8+ T cells in active infection compared to latent infection or chemotherapy-treated infection, with the highest frequency of tetramer-positive CD8+ T cells found in patients with HIV co-infection (*584*). Some *M.tb*-specific, HLA-E-restricted CD8+ T cells exhibit cytotoxic activity, lysing peptide-loaded HLA-E transfectants as well as monocytes infected with

M. bovis BCG or *M.tb* (*583*) (*584*) (*585*). Cytolytic *M.tb*-specific, HLA-E-restricted CD8+ T cell clones from PPD-responders inhibit outgrowth of intracellular *M.tb* in primary macrophages (*585*). However, in addition to cytotoxic activity, studies of T cell clones and *ex vivo* tetramer+ T cells revealed that the majority of *M.tb*-specific, HLA-E-restricted CD8+ T cells from both PPD-responders and active TB patients express markers of suppressor function and exhibited an unusual Tc2-like phenotype, predominantly expressing GATA3 and producing IL-4 and IL-13 but not IFN-γ (*585*). In accordance with this phenotype, many clones activated co-cocultured B cells and increased B cell secretion of Ig, particularly IgG and IgM (*584*) (*585*). Clones from PPD-responders also suppressed proliferation and IFN-γ secretion of co-cultured *M.tb*-specific CD4+ T cell Th1 clones (*583*) (*585*), further suggesting a regulatory role for HLA-E-restricted CD8+ T cells in the immune response to *M.tb*. Although all isolated *M.tb*-specific HLA-E-restricted CD8+ T cell clones described in the study expressed GATA3 and predominantly secreted Th2-like cytokines, individual clones usually exhibited either cytolytic or regulatory activity (*583*) (*585*). Thus, the *M.tb*-specific, HLA-E-restricted CD8+ T cell response appears to be multifunctional.

Vaccination with *Salmonella enterica* serovar Typhi strain Ty21a, a live attenuated typhoid vaccine, elicits HLA-E-restricted CD8+ T cells (*560*). *S.* Typhi-specific, HLA-E-restricted CD8+ T cells are detected as early as 4 days after immunization (*586*). These HLA-E-restricted CD8+ T cells effectively lyse *S.* Typhi-infected targets, including allogeneic targets, in a granule-dependent mechanism (*560*). Four *S.* Typhi GroEL-derived peptides stimulated vaccine-elicited CD8+ T cells when loaded onto HLA-E transfectants or HLA-E-expressing BLCL (*560*). The majority of

vaccine-induced *S.* Typhi-specific, HLA-E-restricted CD8+ T cells are multifunctional, secreting both TNF-α and IFN-γ and expressing CD107 (*586*).

c. MHC-E-restricted CD8+ T cells specific for viral peptides

Virus-specific, HLA-E-restricted CD8+ T cells are found in the context of viral infection with human cytomegalovirus (HCMV), Hepatitis C virus (HCV), and Epstein-Barr virus (EBV). Perhaps the most unique case of a virus-specific, HLA-E-restricted CD8+ T cell response is that specific for the HCMV UL40-derived peptide, a virally encoded mimic peptide of MHC-E-binding leader peptides, described above in section v. Three versions of the mimic peptide, each identical to an MHC-Ia-derived leader sequence, are encoded by UL40 in various HCMV strains: VMAPRTLIL, VMAPRTLVL, and VMAPRTLLL (Table 1.1) (*559*). As described above, UL40-derived VL9 mimic peptides loaded on HLA-E inhibit lysis by CD94/NKG2A+ NK cells (*546*) (*566*) (*547*) (*548*) (*567*). However, HCMV UL40-derived mimic peptides can also be targeted by HLA-E-restricted CD8+ T cells (*558*) (*559*) (*587*). This UL40 VL9-specific CD8+ T cell response only occurs in patients infected with a strain of HCMV encoding a UL40 VL9 peptide that does not match any of the endogenous leader-derived VL9 peptides encoded by the individual's MHC-I alleles (*558*) (*559*) (*587*). Presumably, mechanisms of tolerance preclude UL40 VL9-specific, HLA-E-restricted CD8+ T cell responses in cases where UL40 VL9 matches one of the endogenous MHC-I leader-derived VL9 peptides. These UL40 VL9-specific CD8+ T cells were first identified as a unique subset of CD8+ T cells, termed "NK-CTL" due to their expression of inhibitory NK cell receptors, such as CD94/NKG2A, and their ability to lyse NK cell-susceptible targets (*588*). However, NK-CTL recognize HLA-E/peptide complexes through their

TCRα/β rather than through CD94/NKG2A (*588*) (*589*) (*558*), and are only found in CMV-seropositive individuals. Thus, the discovery that NK-CTL are UL40-specific explains these observations as well as their broad cytotoxic activity against some allogeneic, but not autologous, target cells (*588*) (*589*). Upon recognition of "non-self" UL40 peptide, HCMV-elicited HLA-E-restricted CD8+ T cells lyse target cells, proliferate, and secrete IFN-γ, but not IL-2, consistent with their effector memory T cell phenotype (CD45RA+, CD28-, CCR7-) (*559*).

Similar to HCMV UL40-derived peptide, a single HLA-E-binding peptide of HCV also participates in both NK cell and T cell immunity. As described above, peptide YLLPRRGPRL derived from the core protein of HCV binds HLA-E and inhibits lysis by CD94/NKG2A+ NK cells (*550*). The same HCV core peptide is targeted by HLA-E-restricted CD8+ T cells in 40% of HCV-infected patients (*561*). Further, patients possessing this IFN-γ-secreting HLA-E-restricted CD8+ T cell response display significantly lower levels of HCV and higher levels of serum ALT and AST, readouts of liver damage, compared to patients lacking the response (*561*), suggesting these HLA-E-restricted CD8+ T cells might lyse HCV-infected cells. Interestingly, HLA-A2-restricted CD8+ T cells also target this HCV core peptide, but only the HLA-E-restricted CD8+ T cell response correlates with clinical parameters (*561*).

In contrast to HCMV UL40-derived peptide and HCV core-derived peptide, the HLA-E-binding peptide derived from EBV BZLF-1 protein does not appear to interact with CD94/NKG2A receptors (*536*) (*590*). However, this peptide is targeted by HLA-E-restricted CD8+ T cells in EBV-seropositive donors (*590*). In addition, a slight increase in the frequency of HLA-E/BLZF1 SL9 tetramer+ CD8+ T cells is observed in Multiple

Sclerosis (MS) patients compared to healthy controls (*590*), which may reflect an increase in EBV expression in these patients as accumulating evidence suggests EBV is linked to MS (*591*) (*592*) (*593*) (*594*). However, characterization of the functional properties of EBV-specific, HLA-E-restricted CD8+ T cells has not been performed.

vii. Antigen presentation on MHC-E

The pathway of MHC-I-derived leader peptide presentation on HLA-E has been experimentally determined (*595*) (*596*) (*528*) (*530*). As the nascent MHC-I molecule is translocated into the ER, the leader sequence is cleaved off by signal peptidase (SP) located in the ER membrane, and then further processed by signal peptide peptidase (SPP), also located in the ER membrane (*595*) (*597*). SPP cuts the hydrophobic membrane-spanning region of the leader segment, releasing a 14-mer peptide into the cytosol (*595*). The peptide is trimmed into the HLA-E-binding 9-mer by the proteasome (*596*), and then re-imported into the ER by TAP (*528*) (*530*), where it binds to HLA-E and is transported to the cell surface (*528*) (*530*). Tapasin is also required for this process (*528*), and HLA-E associates with TAP, tapasin, and calreticulin (the members of the peptide-loading complex) (*528*). A similar mechanism of leader peptide loading is supported for mouse MHC-E (Qa-1b), with the exception that leader peptide presentation of Qa-1b is proteasome-independent (*598*) (*599*), suggesting other cytosolic or ER proteases in mice can generate the MHC-E-binding 9-mer in the absence of proteasome function.

In contrast to leader peptide presentation, the presentation of pathogen-derived peptides on MHC-E may occur through multiple pathways, particularly as pathogen proteins differ in subcellular localization, and some pathogens interfere with the antigen

processing machinery. Indeed, pathogen-derived peptides exhibit differences in their requirements for the presentation on MHC-E. Presentation of *Listeria*-, *Salmonella*-, and *Mtb*-derived CD8+ T cell epitopes on MHC-E requires intact TAP function (*551*) (*600*) (*552*). In contrast, presentation of HCMV UL40-derived VL9 peptide and vaccinia virus-derived DI8 peptide on MHC-E occurs occurs independently of TAP (*546*) (*566*) (*567*) (*564*), suggesting TAP-independent pathways of MHC-E loading exist.

While the processing and presentation of virally derived peptides on MHC-E remains enigmatic, the process of *Mtb* peptide loading onto MHC-E has been extensively characterized. Bacteria-containing phagosomes can acquire retrotranslocation machinery, which exports proteins from the ER to the cytosol, as well as ER-derived antigen presentation machinery, including MHC-I, TAP, and other members of the PLC (*601*) (*602*) (*603*) (*600*). Presentation of *Mtb*-derived CD8+ T cell epitopes on HLA-E requires retrotranslocation machinery, proteasome, and TAP function, but does not require ER-golgi transport (*557*) (*600*). In addition, HLA-E is enriched in the *Mtb* phagosome after infection, and *Mtb*-specific HLA-E-restricted CD8+ T cells recognize purified phagosome fractions. These data support a model whereby *Mtb* phagosomal antigens exit the phagosome by retrotranslocation, undergo proteasomal processing in the cytosol, and are re-imported into the phagosome by TAP, where they bind to HLA-E. Of note, both *Listeria* and *Salmonella* are localized to phagosomes at some point in their infectious life cycle (*604*) (*605*), and thus may support a similar mechanism of HLA-E peptide loading. In support of this hypothesis, presentation of *Salmonella* peptides to MHC-E-restricted CD8+ T cells is also proteasome-dependent (*557*) (*600*) (*552*) (*553*).

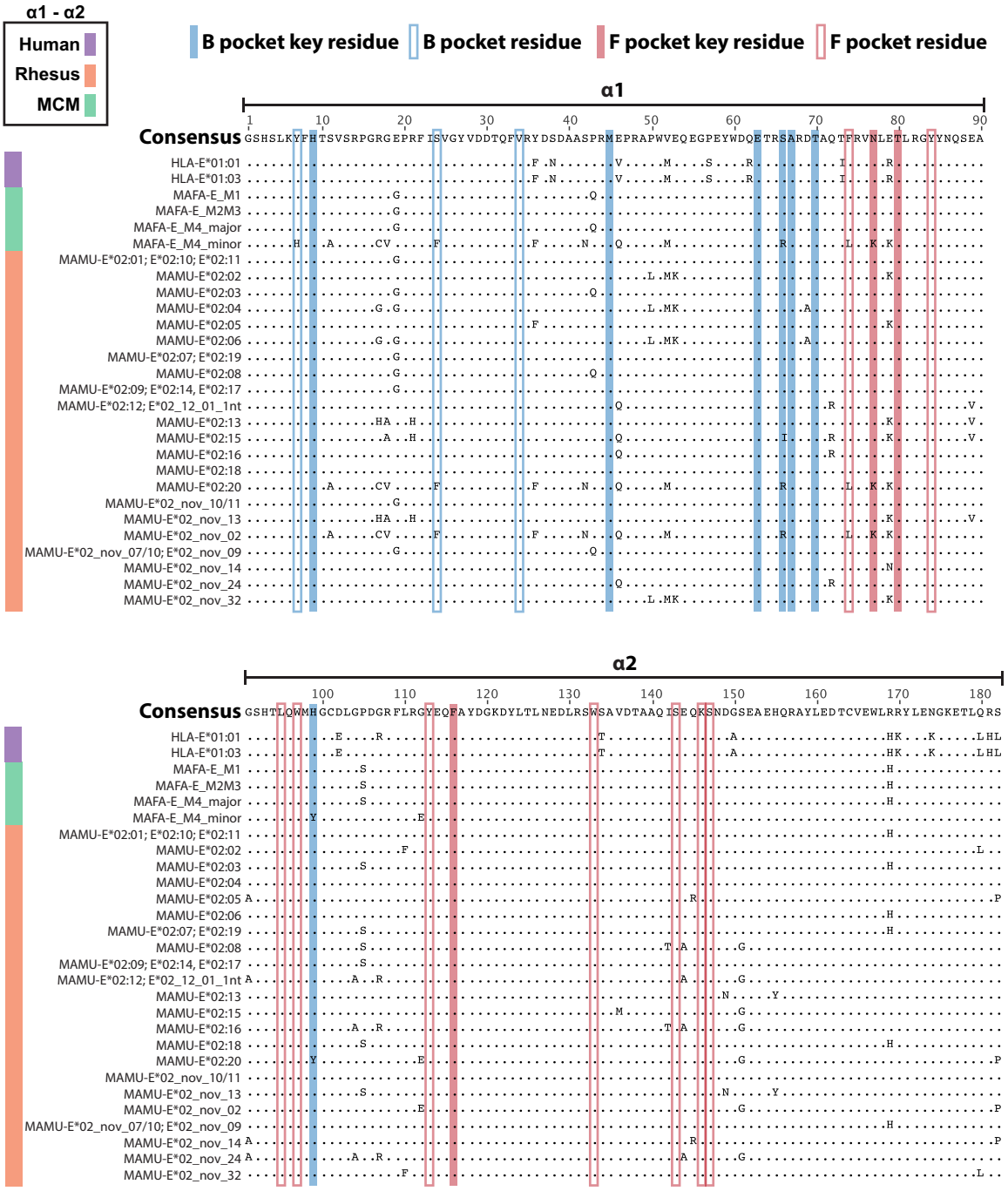


Figure 1.1: MHC-E molecules expressed in humans, rhesus macaques, and Mauritian cynomolgus macaques. Amino acid alignment of the α1 and α2 regions of 2 HLA-E, 28 Mamu-E, and 4 Mafa-E alleles, with the key B and F pocket residues predicted to contact peptide highlighted in blue and red, respectively. B and F pocket residues are conserved among all MHC-E molecules, with the exception of 3 Mamu-E alleles (Mamu-E*02:15, -E*02:20, and -E*02_nov_02) and 1 Mafa-E allele (Mafa-E_M4_minor).

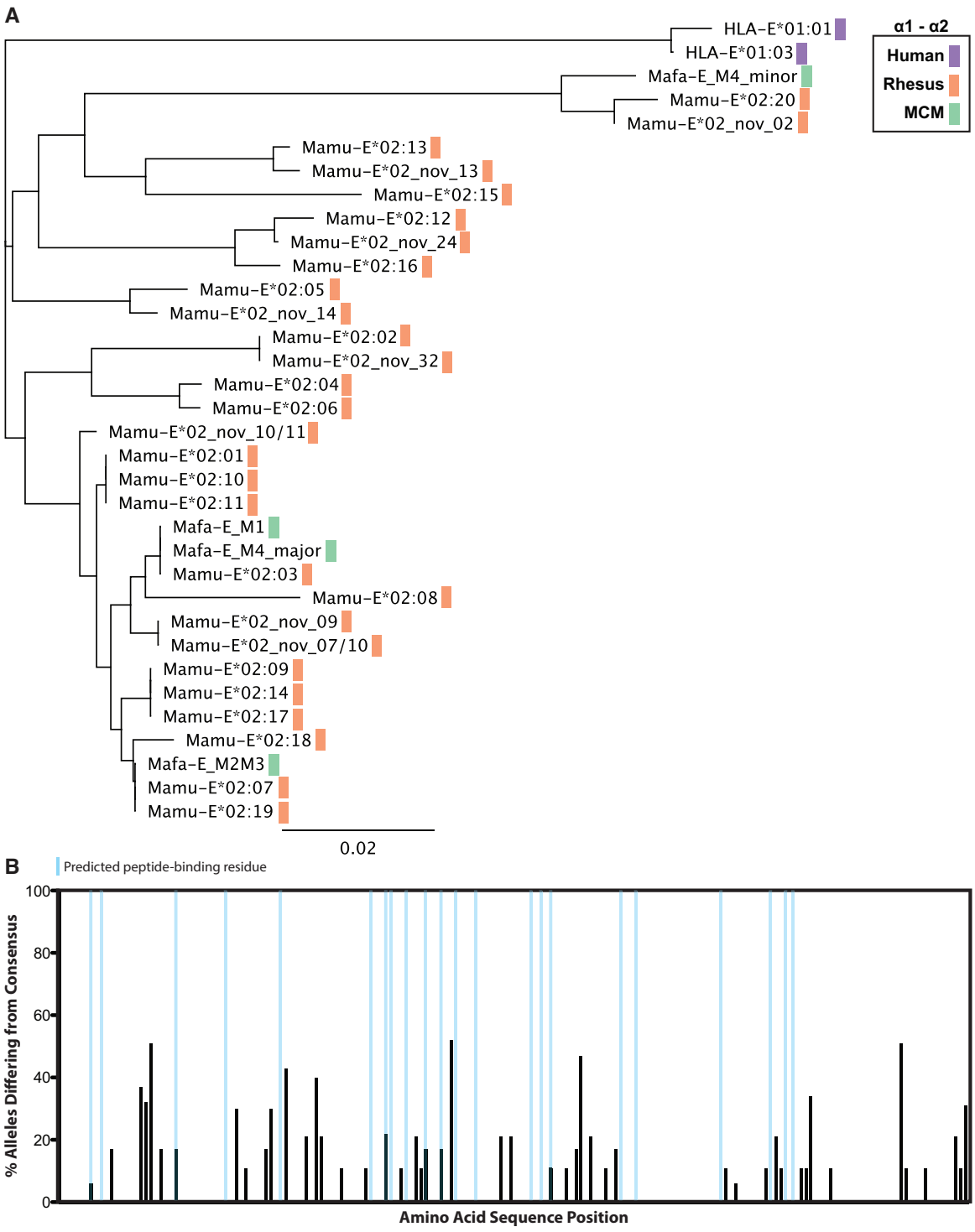


Figure 1.2: Sequence conservation in the α1 and α2 regions of MHC-E molecules expressed in humans, rhesus macaques, and Mauritian cynomolgus macaques. The amino acid sequences of the α1 and α2 regions of 2 HLA-E alleles, 28 Mamu-E alleles, and 4 Mafa-E alleles were aligned (alignment shown in Figure 1.1). **(A)** Neighbor-joining tree of MHC-E molecules is shown. **(B)** Graph displays the

frequency of MHC-E alleles differing from the consensus at each amino acid position of the α1 and α2 region in black. Blue highlighting indicates amino acid residues predicted to line the peptide-binding groove (*606*, *607*).

IV. Cytomegalovirus-based vaccine vectors

Vaccination of rhesus macaques with rhesus cytomegalovirus (RhCMV) expressing SIV antigens is effective against SIV in ~50% of rhesus macaques; efficacy is characterized by stringent control of viral replication and eventual viral clearance. Thus, CMV-based vaccine vectors represent a potentially effective T cell-based vaccine strategy for HIV. This section discusses aspects of CMV virology and immunology relevant to CMV-based vector vaccination, and reviews previous studies utilizing such vectors.

A. CMV virology

Cytomegaloviruses, or CMVs, are ubiquitious betaherpesviruses that infect a range of mammalian species, from mice to humans. CMV persists in infected hosts indefinitely and, like other herpesviruses, is able to go latent with target cells (*799*). Periodically, latent viral genomes reactivate, producing infectious virus that is shed in saliva, urine, genital secretions, and breast milk (*608*). Transmission occurs through exposure to such bodily fluids as well as by intrauterine exposure and transplantation (*609*). An estimated 60-100% of the adult population is infected with human CMV (HCMV), with higher prevalence in developing countries (*610*) (*609*). HCMV infection is generally asymptomatic in immunocompetent individuals, but can cause severe disease in immunocompromised hosts such as AIDS patients, transplant recipients, and congenitally infected infants (*611*) (*800*). A key feature of betaherpesviruses like CMVs is their species specificity; each CMV species is highly adapted to its host species and is unable to infect other species, even those closely related to its host (*610*). In addition, CMVs have the ability to superinfect CMV-positive hosts, despite strong immune

responses to primary infection (*612*) (*613*). CMV infects a wide range of host tissues and multiple cell types, including endothelial cells, epithelial cells, smooth muscle cells, fibroblasts, monocytes, macrophages, dendritic cells, and CD34+ bone marrow progenitors (*614*) (*615*) (*616*). Primary CMV infection involves initial replication in the mucosal epithelium followed by dissemination to myeloid lineage cells such as monocytes and CD34+ bone marrow progenitors, which serve as sites of latent infection (*617*) (*618*) (*619*).

CMVs are enveloped viruses with large double-stranded DNA genomes. The genome of HCMV is ~235 kilobases composed of between 165 and 250 open-reading frames (*620*) (*621*) (*622*) (*623*) (*624*). The HCMV virion consists of nucleocapsid-enclosed double-stranded linear DNA core surrounded by a proteinaceous matrix known as the tegument, all of which is encased in a lipid bilayer (*609*) (*625*). The viral glycoproteins present in the virion envelope interact with cellular surface receptors. Viral glycoproteins gB and gH/gL are essential for virus entry (*626*) (*627*); gB mediates adsorption of HCMV onto heparan sulfate glycosaminoglycans and may assist in entry (*628*) (*629*), while gH/gL mediate entry, either alone or in complex with other envelope glycoproteins (*627*). Entry into fibroblasts requires only the gH/gL complex, while entry into epithelial and endothelial cells requires both gH/gL and the pentameric complex, composed of gH/gL/UL128/UL130/UL131 (*627*). Many cell surface molecules have been described as HCMV receptors, including epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor-α (PDGFRα), integrins, and BST/tetherin (*630*) (*631*) (*632*) (*633*) (*634*) (*635*) (*636*). Entry of HCMV occurs through either direct fusion or through the endocytic pathway, which release the tegument and capsid into the

cytoplasm (*637*). The capsid travels to the nucleus, and the viral DNA is released into nucleus (*637*). During lytic infection, expression of viral proteins occurs in three overlapping phases: immediate-early (IE, 0-2 hours), delayed-early (E, <24 hours), and late (L, >24 hours) (*609*). In some cell types, immediate-early gene expression is silenced, resulting in latent infection characterized by minimal viral gene expression and no production of new virions (*639*) (*640*). The immediate-early proteins activate expression of delayed-early genes, which initiate viral genome replication (*801*) (*641*). After DNA replication, the late genes are expressed, which initiate assembly and egress of new virions (*801*) (*641*). During assembly, the DNA genome is encapsidated in the nucleus, the capsids bud through the nuclear envelope, and assembly occurs in a unique secretory apparatus known as the viral assembly complex (VAC) (*643*) (*644*) (*645*).

B. The immune response to CMV

CMV disease is typically restricted to immunocompromised hosts (*611*) (*800*), suggesting that the immune response to CMV is important in limiting viral pathogenesis. Indeed, numerous studies in humans and animal models provide evidence for the important of immune responses in control of viral replication and protection from CMV disease. However, CMV persists and is often shed in the face of such immune responses (*608*) (*646*) (*647*) (*648*) (*649*) (*650*), demonstrating that host immunity is unable to clear the virus. Thus, CMV infection of immunocompetent hosts represents a balanced interplay between the virus and host, whereby the virus is able to persist but replication is contained to levels that preclude disease development.

Entry of CMV into cells activates several innate immune pathways, resulting in induction of inflammatory cytokines, type I interferons, and costimulatory molecules

such as CD80 and CD86, the latter are crucial in priming effective adaptive immune responses (*651*). For example, HCMV gB/gH envelope glycoproteins activate Toll-like receptor 2 signaling to activate NF-kB signaling (*652*). The innate immune response to CMV also involves natural killer (NK) cells, which survey cells for the presence of activating and inhibitory ligands that alert the NK cell of abnormal cellular processes, such as infection or neoplastic transformation. Upon activation, NK cells secrete cytokines such as IFN-γ and kill target cells. While direct evidence for the role of NK cells in controlling HCMV infection is lacking, patients without NK cells have increased susceptibility to HCMV and can develop severe herpesvirus infections like HCMV disease, and NK cell expansion correlated with control of HCMV infection in a SCID patient lacking T cells and B cells (*653*) (*654*). In support of the importance of NK cells in HCMV infection, HCMV encodes numerous modulators of NK cell activity, including a protein that binds NK cell activating ligands to prevent NKG2D+ NK cell activation and the VL9 mimic peptide that stabilizes HLA-E surface expression and inhibits NKG2A+ NK cell-mediated killing (*802*) (*546*) (*566*) (*547*) (*548*) (*567*).

Adaptive immune responses to primary HCMV infection control HCMV reactivation and prevent uncontrolled viral replication and serious HCMV disease. Binding and neutralizing antibodies against HCMV are readily detected in HCMV-infected individuals, and serve to restrict viral dissemination and limit disease severity (*655*) (*656*) (*657*). Neutralizing antibodies are primarily directed to the envelope glycoprotein gB, but also target envelope glycoprotein gH and complexes gN/gM and gH/gL/UL128/UL130/UL131 (*655*) (*658*) (*659*) (*660*) (*661*) (*662*). However, virus-specific T cell responses are the primary means by which HCMV replication is

controlled. The T cell response to HCMV infection is dominated by effector memory (T_{EM}) and terminally differentiated effector T cells (*363*) (*663*) (*361*). In AIDS patients and transplant recipients, HCMV-specific CD8+ T cell responses limit systemic viral load and/or correlate with protection from CMV disease (*664*) (*665*) (*666*) (*667*) (*668*) (*669*) (*670*) (*671*). Indeed, infusion of HCMV-specific CD8+ T cells into allogeneic bone marrow transplant recipients protects against HCMV-associated clinical complications (*672*) (*673*). The magnitude of the HCMV-specific CD8+ T cell response is very large; a median of 10% of CD8+ T cells in peripheral blood of healthy carriers are HCMV-specific, and this frequency increases in elderly individuals (*674*) (*675*) (*676*) (*677*). In addition, the antigenic targets of the HCMV-specific CD8+ T cell response are extensive, including structural, regulatory, and immunomodulatory proteins spanning immediate-early, early, early-late, and late gene products (*803*) (*678*) (*677*) (*609*). Indeed, comprehensive *ex vivo* T cell assays revealed that more than 70% of HCMV ORFs are targeted by T cells (*803*) (*678*) (*677*). CD8+ T cell responses targeting IE-1, IE-2, and pp65 are immunodominant (*679*) (*803*) (*680*) (*681*) (*682*) (*683*) (*684*) (*678*) (*677*) (*609*). CD4+ T cell responses to HCMV also appear to be critical for HCMV control. Shedding of HCMV in urine and saliva in HCMV-infected healthy children is associated with deficiencies in HCMV-specific CD4+ T cells (*685*). In transplant recipients, low levels of HCMV-specific CD4+ T cells correlate with increased susceptibility to HCMV disease and/or increases in viral load (*686*) (*687*) (*663*), while detection of HCMV-specific CD4+ T cells is associated with protection from HCMV disease (*688*) (*664*) (*689*). Adoptive transfer of HCMV-specific CD4+ T cells reduces HCMV viral loads in allogeneic stem cell transplant recipients (*690*). Similar to the CD8+ T cell response, CD4+ T cell

responses to HCMV are also present at high frequencies and exhibit broad antigen recognition (*677*) (*681*). The major targets of HCMV-specific CD4+ T cell responses are gB, TRL14, and UL16 (*677*) (*681*) (*691*). While many HCMV-specific CD4+ T cells are conventional helper T cells that maintain HCMV-specific antibody and CD8+ T cell responses (*692*) (*673*), some are able to lyse infected cells (*691*) (*804*) (*693*) (*694*).

In the face of such robust immune responses, a key enabler of CMV persistence is viral evasion of host immune responses. CMV possesses an array of immunomodulatory strategies to interfere with host immunity, including dampening induction of interferon-stimulated genes, interfering with antigen presentation to T cells, and evading NK cell responses (*805*) (*695*) (*609*). For example, HCMV encodes a set of related glycoproteins that interfere with antigen presentation on MHC-I to evade CD8+ T cell recognition. US2 and US11 facilitate the degradation of newly synthesized MHC-I molecules (*795*), US3 retains MHC-I in the ER via tapasin inhibition (*568*) (*569*) (*696*), and US6 inhibits TAP-mediated translocation of antigenic peptides into the ER (*570*) (*571*) (*572*) (*573*). Indeed, deletion of the locus homologous to US2-11 from rhesus CMV (Rh182-189) abolishes the ability of the virus to superinfect RhCMV-seropositive rhesus macaques (*697*), demonstrating that these CD8+ T cell evasion genes are required for CMV to establish persistent secondary infection. Of note, these CD8+ T cell evasion genes do not preclude priming of CMV-specific CD8+ T cell responses, but instead interfere with CD8+ T cell recognition of infected cells (*393*), allowing the virus to persist (*697*). This ability to establish persist superinfection is a key advantage of CMV-based vaccine vectors, as described in the next section.

C. CMV as a vaccine vector

i. Rationale for use as a vaccine vector for HIV

Most previous approaches to T cell-based vaccination use non-persistent vectors that produce antigen for a limited time, and thus predominantly elicit central memory T cells (T_{CM}) (*361*), see section II. While central memory T cells are effective against some pathogens (*698*), the massive initial replication of HIV/SIV outpace vaccine-elicited central memory T cells, which require a period of antigen-stimulated anamnestic expansion, differentiation to gain effector functions, and migration to the site of infection in order to act against incoming virus (*362*). Indeed, while some non-persistent vaccine regimens can lower SIV viremia in rhesus macaques, this control is incomplete and usually transient (*449*) (*451*) (*396*) (*450*) (*461*). Further, these non-persistent vaccine regimens have shown no efficacy thus far in humans (*389*) (*430*) (*433*) (*446*).

With these considerations, our collaborators developed a vaccine where SIV antigens are expressed by cytomegalovirus virus (CMV), the prototypical persistent pathogen, in an attempt to elicit SIV-specific effector memory T cells (T_{EM}) capable of acting against the virus at its most vulnerable early stage. A number of characteristics of CMV-elicited immune responses suggest CMV-based vaccine vectors might offer superior control of viral replication and serve as suitable immunization modalities in humans. First, vaccine-elicited T cell responses would be most effective if they act very early after HIV/SIV infection, when the viral population is smaller, less diverse, and more localized to the site of transmission (*204*), see section I. CMV elicits and maintains high-frequency CD4+ and CD8+ T cell responses that are highly T_{EM}-biased (*677*) and, consequently, possess immediate effector function and are present at high frequencies in extralymphoid sites (*362*) 3(*699*) (*700*) (*364*) (*365*), including the vaginal and rectal

mucosa that serve as sites of viral entry and initial replication for the majority of HIV infections (*30*) (*31*). Thus, CMV-elicited T_{EM} cells would be poised to act early against mucosally transmitted HIV. Second, high frequency CMV-elicited T_{EM} responses are maintained throughout the life of the infected individual (*674*) (*675*) (*676*) (*677*) (*681*), and thus are well suited for preventative vaccines. Finally, CMV is able to superinfect CMV-seropositive hosts and prime new T cell responses (*612*) (*613*) (*701*) (*697*) (*450*) (*393*). Thus, while a large proportion of the global population is infected with HCMV, HCMV-based vaccine vectors would be able to elicit antigen-specific T cell responses regardless of CMV status. This is in contrast to Ad5 vector vaccination, where pre-existing immunity to Ad5 can reduce vaccine immunogenicity, and perhaps even increase the risk of HIV acquisition (*702*) (*389*) (*390*). Thus, CMV vectors encoding HIV antigens represent potential candidates for T cell-based HIV vaccines.

Rhesus macaques are an ideal model to test CMV-based vaccine vectors. The relatedness of CMVs mirrors the evolutionary relatedness of their hosts, and thus many genes and gene families present in HCMV are shared by rhesus cytomegalovirus (RhCMV) but not rodent CMVs (*703*) (*610*). The genomes of HCMV and RhCMV are colinear and possess a similar size and structure (*704*) (*705*). Similar to HCMV, infection with RhCMV is persistent, ubiquitous, and typically asymptomatic (*705*). Over 90% of captive rhesus macaques are infected within the first year of life, and the majority RhCMV infections are subclinical (*648*) (*649*) (*650*) (*706*) (*705*). Further, RhCMV infects a similar range of tissues and cell types as HCMV, and is shed in the same bodily fluids (*707*) (*708*) (*709*) (*710*) (*711*) (*647*) (*646*) (*705*). RhCMV is also able superinfect; multiple genetic variants can be isolated from individual immunocompetent RMs (*712*).

Perhaps most significantly for studies of CMV-based vaccine vectors, the host immune response to RhCMV mirrors that observed in HCMV infection. RhCMV-infected RMs develop high frequency, effector memory-biased RhCMV-specific CD4+ and CD8+ T cell responses as well as RhCMV-binding and –neutralizing antibodies (*713*) (*806*) (*714*) (*715*) (*710*) (*706*) (*716*) (*677*). Further, RhCMV encodes homologues of many of the immunomodulatory proteins of HCMV, including the homologues of the US2-11 proteins that interfere with antigen presentation on MHC-I (*704*) (*712*) (*717*) (*718*). Thus, RhCMV infection of rhesus macaques serves as a relevant model of HCMV infection in humans. As SIV-infection of rhesus macaques is a standard model of HIV/AIDS pathogenesis, rhesus macaques can be utilized to study both the immunogenicity of CMV-based vaccine vectors and their efficacy against pathogenic lentiviral infection.

ii. General features of RhCMV vector vaccination

Our collaborators developed RhCMV vaccine vectors expressing SIV antigens based on RhCMV strain 68-1 (*719*) (*704*) (*720*) (*701*) (*697*) (*450*) (*393*) (*481*). Indeed, every SIV challenge study of RhCMV/SIV vector-vaccinated RMs published to-date involved animals administered RhCMV/SIV vaccine vectors engineered from strain 68-1 (*701*) (*450*) (*481*). As described below, this distinction is critical. Most of the open reading frames of RhCMV strain 68-1 encode proteins homologous to known HCMV proteins, including structural proteins, replicative proteins, transcriptional regulators, and immune evasion proteins (*704*). Strain 68-1 is a laboratory-adapted strain of RhCMV passaged in fibroblasts, and thus contains fibroblast adaptions similar to those observed with fibroblast-adapted HCMV, including mutations in homologues of HCMV UL128 and UL130, two components of a pentameric glycoprotein complex that facilitates entry

of CMV into non-fibroblast cells (*704*) (*720*) (*721*). While strain 68-1 RhCMV exhibits attenuated growth *in vivo,* including lower plasma titers and shedding (*712*), the virus is able to establish persistent infection and prime T cell responses in CMV-negative and CMV-positive hosts (*701*) (*697*) (*450*) (*393*) (*481*). The SIV antigens inserted into strain 68-1 RhCMV to make RhCMV/SIV vectors include Gag, Env, a fusion of Rev, Tat, and Nef (Retanef), 5' Pol (encoding protease and reverse transcriptase), and 3' Pol (encoding RNase H and integrase).

As expected, administration of strain 68-1 RhCMV/SIV vectors to either RhCMV-naïve or naturally RhCMV-infected rhesus macaques results in clinically benign infection. Strain 68-1 RhCMV/SIV vectors persist in vaccinated rhesus macaques, with antigen-expressing RhCMV shed in urine and saliva indefinitely but no measurable viremia (*701*) (*450*). This shedding is consistent with previous findings that healthy RhCMV-infected adult RMs often shed virus in urine, saliva, and genital secretions (*646*) (*647*) (*648*) (*649*) (*650*).

Strain 68-1 RhCMV/SIV vectors do not elicit strong antibody responses to SIV. In some vaccinated rhesus macaques, SIV-binding antibodies are detected at very low titers (≤1:10 plasma dilution), but they are unable to neutralize SIVmac239 or a neutralization-sensitive, tissue culture-adapted variant of SIVmac251 (*701*) (*450*) (*481*). However, all rhesus macaques administered strain 68-1 RhCMV/SIV vectors develop and maintain high frequency effector memory-biased CD4+ and CD8+ T cell responses against the inserted SIV antigens indefinitely, with >90% of peripheral blood SIV-specific CD8+ T cells possessing a T_{EM} phenotype (*701*) (*450*). Consistent with this T_{EM}–biased response, SIV-specific T cell responses are detectable in a variety of tissues,

including extralymphoid sites such as the liver, intestinal mucosa, and bronchoalveolar lavage (*450*) (*393*). In addition, the induced SIV-specific CD4+ and CD8+ T cell responses are polyfunctional, consistent with previous studies of CMV-elicited T cells (*722*) (*723*). RhCMV-induced, SIV-specific CD4+ T cells are equally split between transitional T_{EM1} (CD28+CCR7-) and fully differentiated T_{EM2} (CD28-CCR7-) phenotypes and produce combinations of TNF, IFN-γ, IL-2, and MIP-1β, with the majority of responding cells producing at least three of these effector molecules (*701*). RhCMV-induced, SIV-specific CD8+ T cells are nearly exclusively fully-differentiated T_{EM2} cells and, consistent with this, produce a combination of TNF, IFN-γ, and MIP-1β and externalize CD107, but do not produce significant amounts of IL-2 (*701*).

iii. Efficacy of RhCMV/SIV vector vaccination

Multiple challenge studies performed to-date have demonstrated that vaccination with strain 68-1 RhCMV/SIV vectors is able to stringently control SIV in challenged rhesus macaques (*701*) (*450*) (*481*). Strain 68-1 RhCMV/SIV vector vaccination is effective in 50% of vaccinated rhesus macaques after repeated, limiting dose intrarectal or intravaginal challenge with SIVmac239 and in two of six vaccinated rhesus macaques after low dose intravenous challenge (*481*). The hallmarks of strain 68-1 RhCMV/SIV vector-mediated control are consistent regardless of the route of challenge and, as described below, differ from that of other HIV/SIV vaccines and elite control.

First, the outcome of challenge among strain 68-1 RhCMV/SIV-vaccinated rhesus macaques is binary. RhCMV/SIV-vaccinated rhesus macaques that do not control viral replication (non-controllers) manifest mean peak and plateau phase viral loads similar to that observed in unvaccinated progressors (*701*) (*450*) (*481*). In contrast, RhCMV/SIV-

vaccinated controller rhesus macaques exhibit an acute peak of viremia followed by rapid control of viral replication periodically interrupted by blips of plasma virus (*701*) (*450*) (*481*). This binary efficacy is also reflected in CD4+ T cell levels, whereby RhCMV/SIV controllers do not exhibit any decrease in CD4+ T cell numbers in peripheral blood or frequency in bronchoalveolar lavage, while RhCMV/SIV non-controllers exhibit significant CD4+ T cell depletion similar to that observed in unvaccinated controls (*450*) (*481*). This binary outcome in both control of viral replication and CD4+ T cell preservation suggests that once progressive infection is established, RhCMV/SIV vector-elicited T cells are unable to exert control over viral replication. Indeed, such binary efficacy is reminiscent of successful antibody-based vaccines like the RV144 vaccine regimen, where vaccine-elicited antibodies are able to provide some level of protection against acquisition, but are unable to alter the course of HIV/SIV infection once infection is established (*445*). However, RhCMV/SIV vector-mediated binary efficacy is in contrast to the consistent challenge outcome observed with conventional T cell-based vaccines, such as standard benchmark DNA/Ad5 vaccine regimens, where all vaccinated rhesus macaques manifest progressive infection with reduced early phase viral loads compared to unvaccinated controls (*449*) (*451*) (*396*) (*450*).

Second, despite viral spread, controller strain 68-1 RhCMV/SIV vaccinees maintain control of viral replication and eventually appear to clear the virus entirely. After the acute peak of viremia following SIV challenge, RhCMV/SIV controller vaccinees quickly control virus replication to lower than 30 copies of SIV RNA per milliliter of plasma. This robust control of viral replication is occasionally interrupted by blips of plasma viremia, which decrease in magnitude and frequency over time until they

eventually disappear by ~75 weeks post-SIV challenge (*701*) (*450*) (*481*). The level of viral control offered by RhCMV/SIV vectors is superior to that conferred by T cell-based vaccine regimens like DNA/Ad5, which is incomplete and often transient (*449*) (*451*) (*396*) (*450*). Despite stringent control of viral replication in RhCMV/SIV vector-protected RMs, SIV spreads from the site of inoculation in RhCMV/SIV controller animals. SIV RNA and DNA is detectable in the lymph nodes, bone marrow, spleen, and liver of protected animals necropsied within 24 days of plasma virus control, albeit at much lower levels than those found in unvaccinated SIV-infected progressors (*481*). Further, replication-competent SIV is able to be co-cultured from draining lymph nodes and spleen of protected animals necropsied at such early time points (*481*). However, RhCMV/SIV controller animals necropsied between 69-180 weeks after infection, after the disappearance of plasma virus blips, show no signs of infection (*481*). Viral clearance in RhCMV/SIV controller RMs at these later time-points is supported by lack of tissue-associated SIV RNA and DNA by ultra-sensitive qRT-PCR and qPCR, inability to co-culture replication competent virus from lymphoid tissues, decline in CD8+ T cell responses to an SIV antigen not included in the vaccine (Vif) to undetectable levels, lack of induction of SIV Env-specific antibody responses, and the failure of 6×10^7 hematolymphoid cells from protected animals to initiate infection in SIV-naïve RM upon adoptive transfer (*481*). These data support immune-mediated control and progressive clearance of SIV infection in RhCMV/SIV controller RMs, and constitute the only report of vaccine-mediated clearance of an established lentiviral infection.

In addition, strain 68-1 RhCMV/SIV vector-mediated control is insensitive to depletion of either CD8+ or CD4+ T cells from blood (*450*). Administration of anti-

CD8α or anti-CD4 monoclonal antibody to RhCMV/SIV controller RMs does not result in any rebound in plasma viremia or tissue-associated SIV RNA levels (*450*) (*481*). In contrast, administration of anti-CD8α to unvaccinated, ART-suppressed, DNA/Ad5-vaccinated, or elite controller RMs results in a transient increase in plasma viremia that returns to pre-depletion levels concomitant with the return of CD8+ T cells (*299*) (*300*) (*301*) (*302*) (*303*) (*304*) (*450*). While transient depletion of CD8+ T cells by anti-CD8α is complete in the blood, this depletion is incomplete in the tissues, particularly in extralymphoid sites such as bronchoalveolar lavage where only ~50% depletion is observed (*450*) (*481*). As RhCMV/SIV vector-elicited SIV-specific CD8+ T cells are T_{EM} in phenotype and present at high frequencies in such extralymphoid sites, it is possible that sufficient numbers remain after depletion to maintain control over viral replication. However, lack of viral rebound in RhCMV/SIV controller RMs after CD8+ or CD4+ T cell depletion could also reflect very low levels of virus or the ability of the other T cell subset to compensate for the depleted subset (i.e. CD4+ T_{EM} cells may compensate for the depletion of CD8+ T_{EM} cells). Regardless, this suggests resilient control of SIV replication by RhCMV/SIV vector-elicited immune responses.

Finally, strain 68-1 RhCMV/SIV vector-mediated control is not associated with protective MHC-Ia alleles (*701*) (*450*) (*481*). This is in contrast to other T cell-based vaccine regimens as well as natural SIV infection, where rhesus macaques expressing particular MHC-Ia alleles or TRIM5 alleles manifest superior control over viral replication or slower disease progression (*449*) (*724*) (*321*) (*322*) (*324*). The observed lack of association between protection and MHC-Ia alleles is explained by the findings described in the next section and in Chapter 2.

Control and clearance of SIV replication in strain 68-1 RhCMV/SIV-vaccinated rhesus macaques is likely mediated by SIV-specific T cell responses. Vaccination does not induce significant SIV-specific antibody responses (*701*) (*450*) (*481*). Innate immune responses can also be discounted as all challenged rhesus macaques harbored natural strains of RhCMV prior to vaccination, and all previous studies conducted SIV challenges at least 300 days after the last RhCMV/SIV vaccination (*701*) (*450*) (*481*).

iv. CD8+ T cell responses elicited by RhCMV/SIV vaccine vectors

Surprisingly, strain 68-1 RhCMV/SIV vector vaccination elicits an SIV-specific CD8+ T cell response that is distinct from that induced upon SIV infection or conventional vaccination (*393*). First, the canonical dominant and sub-dominant SIV-specific CD8+ T cell responses are not induced upon strain 68-1 RhCMV/SIV vaccination (*393*). For example, strain 68-1 RhCMV/SIVgag-vaccinated Mamu-A1*001:01-positive RMs do not mount responses to $SIVgag_{181\text{-}189}$(CM9), and Mamu-A1*002:01-positive animals do not mount responses to $SIVgag_{71\text{-}79}$(GY9). Instead, strain 68-1 RhCMV/SIV vectors elicit SIV-specific CD8+ T cell responses targeting novel viral epitopes (*393*). Indeed, the SIVgag epitopes targeted by strain 68-1 RhCMV/SIVgag-elicited CD8+ T cells are non-overlapping with epitopes targeted by MVA/SIVgag-, Ad5/SIVgag-, and DNA/SIVgag+IL-12-elicited CD8+ T cells, as evidenced by the failure of these conventional vaccine regimens to boost SIVgag epitope-specific CD8+ T cell responses primed by strain 68-1 RhCMV/SIVgag, and vice versa (*393*). This lack of epitope overlap was further confirmed by comprehensive deconvolution of epitopes targeted by SIVgag-specific CD8+ T cells in RMs administered strain 68-1 RhCMV/SIVgag vectors, RM administered conventional SIVgag-encoding vaccines, and

RMs with controlled SIV infection (*393*). Thus, strain 68-1 RhCMV/SIV vectors elicit SIV-specific CD8+ T cell responses targeting novel viral epitopes.

Second, strain 68-1 RhCMV/SIV vaccination elicits a breadth of CD8+ T cell responses about 3-fold as wide as conventional vaccination or SIV infection (*393*). For example, rhesus macaques vaccinated with strain 68-1 RhCMV/SIVgag target an average of 46 of 125 SIVgag 15-mer peptides (with 11 amino acid overlap), corresponding to a minimum of ~32 distinct epitopes (*393*). In contrast, conventionally vaccinated and SIV-infected controller RMs mount responses to 15-mers corresponding to a minimum of 9-14 SIVgag epitopes (*393*). Thus, strain 68-1 RhCMV/SIV vector vaccination induces a broad SIV-specific CD8+ T cell response.

Third, particular SIVgag epitopes, termed "supertopes", were universally targeted by the CD8+ T cells of every strain 68-1 RhCMV/SIVgag-vaccinated rhesus macaque, with many additional SIVgag epitopes frequently targeted by the CD8+ T cells of over 40% of animals (*393*). This frequent CD8+ T cell targeting of particular SIVgag epitopes occurs despite the fact that rhesus macaques are outbred, and no MHC-Ia allele is shared among all vaccinated animals (*393*). This frequent, sometimes universal, CD8+ T cell targeting of SIV epitopes among MHC-disparate rhesus macaques is unique to strain 68-1 RhCMV/SIV vaccination.

These unique properties of the strain 68-1 RhCMV-induced SIV-specific CD8+ T cell response were partially explained by two key findings. First, RhCMV gene Rh189, which encodes a homologue of HCMV US11, inhibits canonical SIV-specific CD8+ T cell priming, as deletion of this gene results in induction of canonical SIVgag-specific CD8+ T cell responses in addition to the novel SIVgag-specific CD8+ T cell responses

elicited by strain 68-1 RhCMV/SIVgag (*393*). Second, two-thirds of the strain 68-1 RhCMV-elicited SIVgag-specific CD8+ T cell responses are unconventionally restricted by MHC-II molecules (*393*). Unconventional restriction by MHC-II explains the novel targeting of a large subset of the strain 68-1 RhCMV-induced, SIV-specific CD8+ T cell response. Further, the demonstration that single MHC-II molecules could present multiple SIVgag peptides to MHC-II-restricted CD8+ T cells, and that MHC-II-restricted CD8+ T cells could recognize a single SIVgag peptide in the context of multiple MHC-II molecules explains the wide breadth and universal targeting, respectively, of the induced MHC-II-restricted SIV-specific CD8+ T cell response. However, these two findings did not explain the differential targeting of the remaining one-third of SIVgag-specific CD8+ T cell responses induced upon strain 68-1 RhCMV/SIV vector vaccination, which is the focus of the study presented here.

CHAPTER 2: Broadly targeted CD8+ T cell responses restricted by major histocompatibility complex E

I. The problem and scientific approach

The specific mechanism of viral control conferred by vaccination with strain 68-1 RhCMV/SIV vectors is unknown. As strain 68-1 RhCMV/SIV vector vaccination stringently controls viral replication and eventually clears the virus entirely in ~50% of vaccinees, defining the mechanism of control would be valuable to both HIV vaccine development and cure research. The demonstrated association of the peak vaccine-phase SIV-specific CD8+, not CD4+, T cell response magnitude with vaccine-mediated protection, coupled with the lack of vaccine-elicited SIV-directed antibody responses,

suggests vaccine-elicited CD8+ T cells mediate viral control in strain 68-1 RhCMV/SIV-vaccinated RMs. While the T_{EM}-biased phenotype of the RhCMV-induced SIV-specific CD8+ T cell response supports the ability of these CD8+ T cells to intercept virus early and control viral replication, the distinct SIV epitope targeting exhibited by these cells, particularly their wide breadth, might also be critical to strain 68-1 RhCMV/SIV-mediated control. Further characterization of these strain 68-1 RhCMV-induced SIV-specific CD8+ T cell responses is required to delineate how such responses might influence the course of SIV infection and facilitate vaccine efficacy.

While the majority of SIVgag-specific CD8+ T cell responses induced by strain 68-1 RhCMV/SIV vector vaccination were found to be restricted by MHC-II molecules, the remaining one-third of SIVgag epitope-specific CD8+ T cell responses, including two "supertope" responses, were blocked by pan-MHC-I monoclonal antibody W6/32, and thus were MHC-I-dependent (*393*). An explanation for the differential targeting exhibited by these MHC-I-dependent SIV-specific CD8+ T cell responses required further investigation. In particular, while promiscuous presentation of a single peptide by multiple MHC-II molecules has been previously documented (*725*), such promiscuous presentation by MHC-Ia molecules has not. Thus, the generation of universal MHC-I-restricted CD8+ T cell responses by strain 68-1 RhCMV/SIV vectors was particularly enigmatic. As this subset of non-canonical MHC-I-dependent, SIV-specific CD8+ T cell responses is elicited by protective vaccination with strain 68-1 RhCMV/SIV vectors, we set out to characterize these cells. We hypothesized that the differential targeting of the strain 68-1 RhCMV-induced, MHC-I-dependent SIV-specific CD8+ T cell response results from unique patterns of MHC restriction.

In order to characterize the subset of MHC-I-dependent SIV-specific CD8+ T cell responses elicited by strain 68-1 RhCMV/SIV vector vaccination, we first attempted to identify restricting MHC-I molecules for these CD8+ T cell responses. As described above in section III, the particular MHC molecule capable of presenting the specific CD8+ T cell epitope and activating the CD8+ T cell, is known as the restricting MHC molecule for that particular CD8+ T cell response. We reasoned that priming of MHC-I-dependent universal CD8+ T cell responses in every strain 68-1 RhCMV/SIV-vaccinated RM could potentially result from (1) promiscuous presentation of the same SIVgag peptide by multiple MHC-Ia molecules, or (2) presentation of SIVgag peptides by non-classical MHC-I molecules that exhibit limited diversity among animals. Thus, we MHC-typed four strain 68-1 RhCMV/SIVgag-vaccinated RMs whose SIV gag epitope-specific CD8+ T cell responses had been previously mapped (*393*), and made transfectant cell lines expressing single MHC-I molecules, both classical and non-classical, encoded by these RMs. We utilized this panel of single MHC-I-expressing transfectants to present SIVgag peptides to CD8+ T cells from these vaccinated RMs, in order to identify restricting molecules for these MHC-I-dependent SIVgag-specific CD8+ T cell responses.

The repertoire of epitope-specific CD8+ T cell responses induced upon infection or vaccination is largely determined by the ability of MHC molecules expressed in the animal to bind and present particular peptides. As such, knowledge of the restricting MHC allele for a particular CD8+ T cell response would likely explain the novel patterns of epitope targeting observed in strain 68-1 RhCMV/SIV-vaccinated rhesus macaques. Indeed, this was previously demonstrated with the identification of MHC-II molecules as

the restricting elements for a large subset of strain 68-1 RhCMV-induced, SIV-specific CD8+ T cells (*393*). Further, identification of the restricting MHC allele would facilitate further characterization of the CD8+ T cell response by allowing for creation of MHC tetramer reagents that can be used to isolate a pure population of epitope-specific T cells for functional assays and T-cell receptor sequencing, both of which could shed light on the contribution of such T cell responses to strain 68-1 RhCMV/SIV vector-mediated control. Thus, we believed our approach of identifying restricting MHC molecules would lay the foundation for future studies of these unique MHC-I-dependent SIV-specific CD8+ T cell responses and their role in efficacy against SIV.

II. Author contributions

All the work presented here was performed by the author, with the following exceptions: MHC typing (Figure 2.1A) and epitope mapping (Figure 2.8). In some instances, experiments were performed with technical assistance from Scott G. Hansen and Colette M. Hughes (ICS stimulations), Katherine B. Hammond (MHC-I transfectants, blood processing), and Jason S. Reed (blood processing, Miltenyi sorting).

III. Materials and methods

Vaccines

The construction, characterization, and administration of 1) the strain 68-1 RhCMV vectors expressing SIVgag and SIV5'-pol (amino acids 97-441), 2) the strain 68-1.2 RhCMV vector expressing SIVgag, and 3) the MVA vectors expressing SIVgag have been previously reported (*393*) (*450*) (*481*) (*701*).

Macaques

A total of 74 purpose-bred male or female rhesus macaques (RM; *Macaca mulatta*) of Indian genetic background were used in the experiments reported here. These animals included 42 RM vaccinated with strain 68-1 RhCMV vectors expressing SIVgag, 9 RM vaccinated with strain 68-1.2 RhCMV/gag vectors, 11 vaccinated with SIVgag-expressing MVA/gag vaccines, and 12 unvaccinated RM with controlled SIVmac239 infection (plateau phase plasma viral loads < 10,000 copies/ml). All RM were used with the approval of the Oregon National Primate Research Center Institutional Animal Care and Use Committee, under the standards of the US National Institutes of Health Guide for the Care and Use of Laboratory Animals. RM used in these experiments were free of cercopithicine herpesvirus 1, D-type simian retrovirus, and simian T-lymphotrophic virus type 1. Selected RM were MHC-I- genotyped by deep sequencing, as previously described (*726*). Briefly, amplicons of Mamu class I sequences were generated via amplification of cDNA by PCR using high-fidelity PhusionTM polymerase (New England Biolabs) and a pair of universal MHC-I-specific primers with the following thermocycling conditions: 98°C for 3min, (98°C for 5s, 57°C for 1s, 72°C for 20s) for 23 cycles, and 72°C for 5min. Each PCR primer contained a unique 10 bp Multiplex Identifier (MID) tag along with an adaptor sequence for 454 SequencingTM (5'-GCCTCCCTCGCGCCATCAG-MID-GCTACGTGGACGACACG-3'; 5'-GCCTTGCCAGCCCGCTCAG-MID-TCGCTCTGGTTGTAGTAGC-3'). Resulting amplicons span 190bp of a highly polymorphic region within exon two. The primary cDNA-PCR products were purified using AMPure XP magnetic beads (Beckman Coulter Genomics). Emulsion PCR and pyrosequencing procedures were carried out with Genome Sequencer FLX instruments (Roche/454 Life Sciences) as per the

manufacturer's instructions. Data analysis was performed using a Labkey database in conjunction with Geneious-Pro® bioinformatics software (Biomatters Ltd.) for sequence assembly. Expression of Mamu-E*02:04 and Mamu-E*02:11 in Rh21826, Rh22034, Rh22436, and Rh22607 was confirmed by extracting RNA from PBMC using the AllPrep DNA/RNA Mini Kit (Qiagen) and amplifying full-length MHC-E by RT-PCR using primers that bind to the 5' and 3' untranslated regions (forward: 5'-TCTAAGCTTCACGACTCCCGACTATAAAG-3'; reverse: 5'-AGCGGCCGCTTGCACACAAGGCAGCTGTC-3') with the following thermocycling conditions: 50°C for 30min, 94°C for min, (94°C for 15s, 52°C for 30s, 68°C for 30s) for 40 cycles, 68°C for 5min(SuperScript III One-Step RT-PCR system, Invitrogen). Amplicons were cloned into pCEP4 with HindIII/NotI restriction sites, and Sanger sequenced. Cells from four Mauritian cynomolgus macaques were also utilized; MCMs were MHC-typed as previously described (*110*).

Antigens and Antigen-Presenting Cells

The synthesis of sequential 15-mer peptides (overlapping by 11 amino acids) comprising the SIVgag proteins, as well as specific 9-mer peptides within these proteins, was performed by Intavis AG, based on the SIVmac239 Gag sequence (Genbank Accession #M33262). All peptides are identified by the position of their inclusive amino acids from the n-terminus (e.g., Gagxx-yy). Consecutive 15-mers (as well as 9-mers within these 15mers) are also designated by their position starting from the n-terminal 15-mer [e.g., Gag1-15 (1) is 15-mer #1; Gag5-19 (2) is 15-mer #2, etc.]. Unless otherwise specified, these peptides were used in T cell assays at 2μg/ml. Autologous B-lymphoblastoid cell lines (BLCL) were generated by infecting RM PBMC with Herpesvirus papio, as

previously described (*393*). Mammalian expression vectors for Mamu class I molecules were generated by ligating each allele into pCEP4 KpnI/NotI or HindIII/NotI restriction sites. Plasmids were cloned in DH5α *E. coli* (Life Technologies), sequence confirmed, and electroporated into MHC-I-negative K562, 721.221, or RMA-S cells (*727*) using Nucleofector II/Kit C (Lonza). Transfectants were maintained on drug selection (Hygromycin B). HLA-E*01:03-expressing K562 cells were generously provided by T. van Hall (described in (*582*)). All transfectants were routinely confirmed for surface expression of MHC-I by staining with pan-MHC-I antibody clone W6/32. Throughout use in T cell assays, RNA from MHC-I transfectants was extracted using the AllPrep DNA/RNA Mini Kit (Qiagen), amplified by RT-PCR using primer pairs flanking a highly polymorphic region within exon 2, and sequence confirmed by Sanger sequencing. Antigen-presenting cells were pulsed with peptides of interest at a final concentration of 10μM for 90 minutes then washed three times with warm PBS and once with warm R10 (RPMI 1640 supplemented with 10% fetal bovine serum, L-glutamine, and antibiotic/mycotic) to remove unbound peptide before combining with freshly isolated PBMC at an effector:target ratio of 10:1. In order to stabilize MHC-E surface expression (*728*) (*526*), MHC-E transfectants were incubated at 27°C for >3 hours prior to use in assays and maintained at 27°C throughout peptide incubation until combined with effectors. Autologous SIV-infected target cells were generated by isolation of CD4+ T cells from PBMC with CD4 microbeads and LS columns (Miltenyi Biotec), activation with a combination of IL-2 (NIH AIDS Reagent Program), Staphylococcus enterotoxin B (Toxin Technologies Inc.), and anti-CD3 (NHP Reagent Resource), anti-CD28, and anti-CD49d mAbs (BD Biosciences), and spinoculation with sucrose-purified SIVmac239,

followed by 3-4 days of culture. Prior to use in T cell assays, SIV-infected target cells were purified using CD4 microbeads and LS columns (Miltenyi Biotec), as previously described (*399*). Infected target cell preparations were confirmed to be >95% CD4+ T cells prior to infection and >50% SIV-infected following enrichment and were used at an effector:target ratio of 40:1 (effectors: CD8β+ T cells) or 8:1 (effectors: CD8+ T cell line effectors). In these experiments, uninfected, activated CD4+ T cells served as negative control APCs [uninfected targets from SIV+ RM were cultured with 400 μM tenofovir (NIH AIDS Reagent Program)]. To assess surface bulk MHC-I and MHC-E expression, SIV-infected CD4+ T cells were generated as described above without post-infection purification and stained for surface MHC-I (clone W6/32), MHC-E (clone 4D12; anti-mouse IgG1 clone M1-14D12), CD3, and CD4, followed by intracellular SIV Gag.

T Cell Assays

SIV-specific CD8+ T cell responses in peripheral blood mononuclear cells (PBMC) or CD8+ T cell lines were measured by flow cytometric ICS. Brief, PBMC were isolated from anticoagulant-treated whole blood by Ficoll density gradient centrifugation (GE Healthcare). Where indicated, CD8β+ cells were isolated from PBMC via Miltenyi sorting using CD8β-PE and anti-PE beads. CD8+ T cell lines were generated as previously described. Briefly, PBMC was stimulated with peptide-pulsed, irradiated autologous BLCL weekly and cultured in R15 supplemented with 100 units/ml IL-2 (NIH AIDS Reagent Program). PBMC, isolated CD8β+ T cells, or CD8+ T cell lines were incubated with antigen (peptides, peptide-pulsed antigen-presenting cells, or SIV-infected CD4+ T cells) and the co-stimulatory molecules CD28 and CD49d (BD Biosciences) for 1 hour, followed by addition of Brefeldin A (Sigma-Aldrich) for an additional 8 hour.

Co-stimulation with no antigen, unpulsed antigen-presenting cells, or uninfected CD4+ T cells served as background controls. In restriction assays, peptide-pulsed MHC-I-negative parental cell lines served as additional negative controls. The MHC association (MHC-Ia, MHC-E, MHC-II) of a response was determined by pre-incubating isolated PBMC, antigen-presenting cells, or SIV-infected CD4+ cells for 1 hr at room temperature (prior to adding peptides or combining effector and target cells and incubating per the standard ICS assay) with the following blockers: 1) the pan anti-MHC-I mAb W6/32 (10mg/ml), 2) the MHC-II-blocking CLIP peptide (MHC-II-associated invariant chain, amino acids 89-100; 20μM), and 3) the MHC-E blocking VL9 peptide (VMAPRTLLL; 20μM), alone or in combination (blocking reagents were not washed, but remained throughout the assay). In some experiments, the Mamu-A1*001:01-binding peptide CM9 (CTPYDINQM; 20μM), or the Mamu-A1*002:01-binding peptide GY9 (GSENLKSLY; 20μM) were used as blocking controls. Stimulated cells were stained and analyzed as described below and previously described (*393*). Cells were washed with 1x PBS, surface stained for 30 minutes, washed with FACS (1X PBS supplemented with 10% fetal bovine serum), fixed with 2% paraformaldehyde, permeabilized with saponin buffer, and stained intracellularly for 45 minutes. Sample collection was performed on an LSR-II instrument (BD Biosciences), and analysis was done using FlowJo software (Tree Star). In all analyses, gating on the light scatter signature of small lymphocytes was followed by progressive gating on the CD3+ population and then the CD4-/CD8+ T cell subset. For epitope deconvolution experiments (Figure 2.8), strict response criteria were used to prevent false positives. In these studies, a response to a given 15-mer peptide was considered positive if the frequency of events clustered as CD69+, TNF-α+ and IFN-γ+

was >0.05%, with background <0.01% in at least 2 independent assays. The classification of an individual peptide response as blocked was based on >90% inhibition by blockade relative to the isotype control. To be considered MHC-E-restricted by blocking, the individual peptide response must have been singly blocked by both anti-MHC-I clone W6/32 and MHC-E-binding peptide VL9, and not blocked by the CLIP peptide. To be considered MHC-II-restricted by blocking, the individual peptide response must have been blocked by the CLIP peptide and not blocked by either the anti-MHC-I clone W6/32 or the MHC-E-binding peptide VL9. To be considered MHC-Ia-restricted by blocking, the individual peptide response must have been blocked by the anti-MHC-I clone W6/32 and not blocked by either the MHC-E-binding peptide VL9 or the MHC-II-binding CLIP peptide. Responses that did not meet these criteria were considered indeterminate. Minimal independent epitope numbers were estimated from the positive responses identified by testing of consecutive 15mer peptides by the following criteria: single positive peptide of same restriction type = 1 independent epitope; 2 adjacent positive peptides of same restriction type = 1 independent epitope; 3 adjacent positive peptides of same restriction type = 2 independent epitopes; 4 adjacent positive peptides of same restriction type = 2 independent epitopes; and 5 adjacent positive peptides of same restriction type = 3 independent epitopes.

Antibodies

The following conjugated antibodies were used in these studies: a) from BD Biosciences, L200 (CD4; AmCyan), SP34-2 (CD3; PacBlu), SK1 (CD8a; TruRed, AmCyan), 25723.11 (IFN-γ; APC, FITC), 6.7 (TNF; APC), MAb11 (TNF; Alexa700), b) from Beckman Coulter, L78 (CD69; PE), 2ST8.5H7 (CD8β; PE), Z199 (NKG2A/C or

CD159a/c; PE), c) from Biolegend, W6/32 (pan-MHC-I; PE), OKT-4 (CD4; PE-Cy7), B1 (TCRγ/δ; Alexa647), d) from Miltenyi Biotec, M-T466 (CD4; APC), e) from eBiosciences, M1-14D12 (mouse IgG1; PE-Cy7). The following unconjugated antibodies were used in these studies: a) from Advanced BioScience Laboratories, 4324 (SIV Gag p27), b) from LSBio, 4D12 (HLA-E), c) W6/32 (pan-MHC-I). LIVE/DEAD Fixable Yellow Dead Cell Stain (Life Technologies) was used to assess cell viability.

IV. Results

A. Non-classical MHC-E molecules restrict SIVgag-specific, MHC-I-blocked CD8+ T cell responses in strain 68-1 RhCMV/SIVgag-vaccinated rhesus macaques

In order to identify restricting MHC-I molecules for the subset of MHC-I-dependent CD8+ T cell responses mapped in strain 68-1 RhCMV/SIVgag-vaccinated rhesus macaques, we conducted previously described MHC restriction assays (*393*) using a panel of single MHC-I-expressing cell lines to present SIVgag peptides to CD8+ T cells. Four strain 68-1 RhCMV/SIVgag-vaccinated rhesus macaques were typed for expression of MHC-I transcripts by deep sequencing (Figure 2.1A). We expressed a subset of identified MHC-I molecules in MHC-I-negative 721.221 or K562 cells, which resulted in detectable MHC-I surface expression (Figure 2.1B). To identify the restricting MHC-I molecule of a particular SIVgag-specific CD8+ T cell response, single MHC-I-expressing transfectants were pulsed with SIVgag 15-mer, washed to remove excess peptide, and combined with peripheral blood mononuclear cells (PBMC) from a strain 68-1 RhCMV/SIVgag-vaccinated rhesus macaque (Figure 2.2A). CD8+ T cell reactivity was determined by intracellular cytokine staining (ICS) for cytokines TNF-α and IFN-γ (Figure 2.2B). In these assays, we found that supertope-specific CD8+ T cells did not

respond to SIVgag$_{273-287}$(69) or SIVgag$_{477-491}$(120) pulsed onto classical MHC-Ia molecules expressed in the macaque, but rather recognized peptide presented by non-classical MHC-E molecules, Mamu-E*02:04 and Mamu-E*02:11 (Figure 2.3). We extended this restriction analysis to an additional 10 SIVgag 15-mer-specific CD8+ T cell responses present in the four studied macaques, and found that, in every case, SIVgag-specific CD8+ T cells responded to peptide pulsed onto Mamu-E*02:04 and Mamu-E*02:11 (Figure 2.4). In contrast, most of the tested SIVgag-specific CD8+ T cell responses did not respond to peptide-pulsed transfectants expressing classical MHC-Ia molecules expressed in the macaque (Figure 2.4). Three SIVgag-specific CD8+ T cell responses, directed at Gag$_{69-83}$(18), Gag$_{129-143}$(33), and Gag$_{197-211}$(50), were able to recognize peptide presented by classical MHC-Ia molecules Mamu-A1*001:01 or Mamu-A1*002:01(Figure 2.4). However, the presence of CD8+ T cell responses directed at these SIVgag peptides did not correlate with expression of Mamu-A1*001:01 or Mamu-A1*002:01 in vaccinated rhesus macaques (Figure 2.5), suggesting recognition of Gag$_{69-83}$(18), Gag$_{129-143}$(33), and Gag$_{197-211}$(50) in the context of these classical MHC-Ia molecules likely reflects cross-recognition rather than priming by Mamu-A1*001:01- or Mamu-A1*002:01-presented peptide. Thus, MHC-I restriction analysis identified twelve MHC-E-restricted, SIVgag-specific CD8+ T cell responses in strain 68-1 RhCMV/SIVgag-vaccinated rhesus macaques.

In order to confirm SIVgag-specific CD8+ T cell responses were restricted by MHC-E, we attempted to specifically block MHC-E-restricted CD8+ T cell responses using MHC-E-binding peptide VMAPRTLLL (VL9). Peptide VL9 is derived from positions 3-11 of the leader sequence of a subset of MHC-Ia molecules and canonically

binds HLA-E with high affinity to regulate NK cells (*529*) (*530*) (*528*) (*532*) (*526*) (*534*) (*535*). Antigen-presenting cells were pre-incubated with VL9 or irrelevant Mamu-A1*001:01- or Mamu-A1*002:01-binding peptide prior to addition of the SIVgag 15-mer of interest in the MHC-I restriction assay described above. Pre-incubation of autologous BLCL or Mamu-E transfectants with VL9 blocked the $SIVgag_{273-287}$(69)- and $SIVgag_{477-491}$(120)-specific supertope CD8+ T cell responses, while pre-incubation with irrelevant peptide did not (Figure 2.6A). We confirmed VL9 blocking was specific for MHC-E by titrating the concentration of VL9 blocking peptide pre-incubated on antigen-presenting cells, which resulted in a dose-dependent increase in blocking of the $SIVgag_{477-491}$(120)-specific, MHC-E-restricted CD8+ T cell response to both peptide-pulsed autologous BLCL or Mamu-E*02:11 transfectant with increasing concentrations of VL9 blocking (Figure 2.6B). In contrast, even high concentrations of VL9 peptide blocking did not affect recognition by SIVgag-specific, A1*001:01-restricted CD8+ T cells (Figure 2.6B). Thus, pre-incubation of antigen-presenting cells with VL9 specifically blocks MHC-E-restricted CD8+ T cells.

Extending these blocking studies to the other ten identified MHC-E-restricted, SIVgag-specific CD8+ T cell responses demonstrated that pre-incubation of MHC-E transfectants with VL9 blocked all twelve identified MHC-E-restricted, SIVgag-specific CD8+ T cell responses, while pre-incubation with irrelevant peptide did not affect SIVgag peptide recognition (Figure 2.7). In order to discern the proportion of strain 68-1 RhCMV-induced CD8+ T cell responses restricted by MHC-E, we utilized MHC-E-blocking peptide VL9 as well as pan-MHC-I antibody W6/32 and MHC-II-blocking peptide CLIP to restriction-classify every previously mapped SIVgag-specific CD8+ T

cell response in a total of 42 animals (Figure 2.8 and (*729*)). PBMC were pre-incubated with blocking reagents for one hour prior to adding SIVgag 15-mer peptides in the ICS assay setup, as previously described (*393*). Every strain 68-1 RhCMV-elicited, SIVgag 15-mer-specific CD8+ T cell response previously shown to be blocked by pan-MHC-I antibody W6/32 (*393*) was also blocked by VL9, while CD8+ T cell responses previously classified as MHC-II-restricted were impervious to VL9 blockade (Figure 2.8). This pattern of blocking was distinct from that of MVA/gag-vaccinated, SIV-infected, and strain 68-1.2 RhCMV/SIVgag-vaccinated rhesus macaques, where nearly all of the SIVgag-specific CD8+ T cell responses were blocked by pan-MHC-I antibody W6/32, but not VL9 (Figure 2.8), suggesting these animals mounted a predominantly classically MHC-Ia-restricted CD8+ T cell response against SIV. Thus, every MHC-I-dependent, SIVgag-specific CD8+ T cell response elicited in strain 68-1 RhCMV vaccinees was found to be restricted by MHC-E, with a median of 20 distinct MHC-E-restricted, SIVgag-specific CD8+ T cell responses elicited in each animal (*729*). This strain 68-1 RhCMV-induced pattern of MHC restriction, with two distinct subsets of CD8+ T cells restricted by either MHC-E or MHC-II, was consistent with all tested vaccine inserts, including SIVpol and *Mycobacterium tuberculosis* antigens RpfA, Ag85B, and ESAT-6, with a similar density of ~4 MHC-E-restricted epitopes per 100 amino acids of protein length (*729*). Thus, strain 68-1 RhCMV consistently elicits a large subset of MHC-E-restricted CD8+ T cell responses, regardless of the nature of inserted antigen.

B. SIVgag-specific, MHC-E-restricted CD8+ T cells targeting supertopes Gag69 and Gag120 are conventional CD8α/β T cells

Previous reports HLA-E-restricted CD8+ T cells demonstrated TCRγ/δ usage or expression of inhibitory NK cell receptors (*730*) (*731*) (*732*) (*733*) (*734*) (*588*) (*589*) (*558*) (*559*). Thus, we investigated whether strain 68-1 RhCMV-induced MHC-E-restricted CD8+ T cells belonged to one of these unconventional T cell subsets. Particularly relevant to the studies described here, a previous report found HCMV-elicited, HLA-E-restricted NK-CTLs express inhibitory NK cell receptor CD94/NKG2A, possess a CD8βdim, CD45RA+, CD28-, CD27-, CCR7-, and CD56+ phenotype, and produce IFN-γ but not IL-2 upon stimulation (*559*). MHC-I-dependent SIVgag-specific CD8+ T cells from strain 68-1 RhCMV/SIVgag vaccinees (demonstrated here to be MHC-E-restricted) were previously shown to be predominantly effector memory in phenotype (CD28-, CCR7-) and to produce IFN-γ but not substantial amounts of IL-2 upon stimulation (*701*) (*450*) (*393*). Thus, we conducted additional phenotypic analyses of these cells. Backgating on CD3+ T cells responding to either $SIVgag_{273\text{-}287}$(69)- and $SIVgag_{477\text{-}491}$(120)-pulsed autologous BLCL in bulk PBMC revealed these T cells were uniformly CD4-, CD8α/β+, TCRγ/δ-, and NKG2A/C- (Figure 2.9). Thus, supertope-specific MHC-E-restricted CD8+ T cells in strain 68-1 RhCMV vaccinees do not belong to the NK-CTL subset observed in HCMV infected patients. Furthermore, this analysis suggests that T cell recognition of SIVgag peptide bound to MHC-E does not occur through an unconventional TCRγ/δ receptor nor through HLA-E-binding NK cell receptors NKG2A or C. Thus, strain 68-1 RhCMV-elicited MHC-E-restricted CD8+ T cells likely recognize peptide in the context of a conventional TCRα/β. Unfortunately, we could not find a commercially available rhesus macaque cross-reactive TCRα/β-specific antibody to verify this hypothesis. These data suggest that MHC-E-restricted,

SIVgag-specific CD8+ T cells in strain 68-1 RhCMV/SIVgag-vaccinated RMs are conventional CD8α/β T cells.

C. SIVgag-specific, MHC-E-restricted CD8+ T cells recognize naturally processed antigen on the surface of SIV-infected CD4+ T cells

MHC-E-restricted CD8+ T cells from strain 68-1 RhCMV/SIVgag vaccinees respond to SIV peptides *in vitro*, but these T cells must recognize naturally processed antigen on the surface of SIV-infected cells in order to participate in immune responses against the virus and contribute to *in vivo* control. Previous analysis demonstrated that both MHC-I-dependent and MHC-II-dependent SIVgag-specific CD8+ T cells from strain 68-1 RhCMV vaccinees recognize SIV-infected CD4+ T cells (*393*). As every MHC-I-dependent SIVgag-specific CD8+ T cell response was demonstrated to be restricted by MHC-E based on the restriction and blocking assays shown above (Figures 2.3, 2.4, 2.6, 2.7, 2.8), we assessed the ability of MHC-I-dependent SIVgag-specific CD8+ T cells to recognize SIV-infected CD4+ T cells in an MHC-E-dependent manner. CD4+ cells were isolated from strain 68-1 RhCMV/SIVgag-vaccinated rhesus macaque PBMC, activated, infected with SIVmac239, and enriched for SIV-infected cells by isolating cells with down-regulated surface CD4, a consequence of productive HIV/SIV infection mediated by Nef (*735*) (*736*) (*737*). In order to assess recognition by the distinct restriction-classified subsets of strain 68-1 RhCMV-elicited CD8+ T cells, we incubated SIV-infected CD4+ T cell targets with various blocking reagents, including pan-MHC-I-blocking antibody W6/32, MHC-II-blocking peptide CLIP, and MHC-E-blocking peptide VL9, prior to combining targets with autologous CD8β+ effector cells from PBMC (Figure 2.10A). CD8+ T cell reactivity to SIV infected CD4+ T cell targets was

determined by ICS detection of TNF-α and IFN-γ production. CD8+ T cells from strain 68-1 RhCMV/SIVgag vaccinees responded to autologous SIV-infected CD4+ T cell targets, but not uninfected counterparts. Single blockade with W6/32, CLIP, or VL9 failed to completely block the CD8+ T cell response to SIV-infected CD4+ T cell targets (Figure 2.10B,C) However, SIV-specific CD8+ T cell recognition was completely blocked by a combination of W6/32 and CLIP, effectively inhibiting both the MHC-I-dependent and MHC-II-dependent SIVgag-specific CD8+ T cell subsets (Figure 2.10B,C). Strain 68-1 RhCMV-elicited SIV-specific CD8+ T cell recognition was also completely blocked by a combination of VL9 and CLIP in the absence of W6/32 (Figure 2.10B,C), demonstrating that every MHC-I-dependent CD8+ T cell response displaying reactivity to SIV-infected CD4+ T cell targets recognized antigen in the context of MHC-E molecules. Further, a CD8+ T cell line specific for supertope $SIVgag_{477\text{-}491}$(120) also responded autologous SIV-infected targets, and this recognition was inhibited by single blockade with either pan-MHC-I-blocking antibody W6/32 or MHC-E-blocking peptide VL9 (Figure 2.11A,C). In addition, the $SIVgag_{477\text{-}491}$(120)-specific CD8+ T cell line, which was derived from Rh22607, also recognized heterologous SIV-infected targets from Rh21826 and Rh22034 in a VL9-dependent manner (Figure 2.11D). In contrast, addition of W6/32, either alone or in combination with CLIP, completely blocked recognition of autologous SIV-infected targets by CD8+ T cells from MVA/gag-vaccinated, SIV-infected, or strain 68-1.2 RhCMV/gag-vaccinated rhesus macaques, while dual VL9 and CLIP blockade failed to block recognition (Figure 2.10B,C). This pattern of blockade was consistent with restriction analysis of SIVgag-specific CD8+ T cells from these cohorts (Figure 2.8), confirming a predominantly classical MHC-Ia-

restricted CD8+ T cell response to SIV in these animals. Further, recognition of SIV-infected targets by a SIVgag$_{181\text{-}189}$(CM9)-specific, Mamu-A1*001:01-restricted CD8+ T cell line derived from an SIV-infected rhesus macaque was blocked by W6/32, but not VL9, further confirming VL9 to be a specific inhibitor of MHC-E-restricted CD8+ T cell responses (Figure 2.11B,C). Collectively, these data demonstrate that strain 68-1 RhCMV-induced, SIVgag-specific, MHC-E-restricted CD8+ T cells recognize naturally processed antigen presented on the surface of SIV infected CD4+ T cells, confirming that at least some of the identified MHC-E-binding SIVgag peptides, including supertope SIVgag$_{477\text{-}491}$(120), are processed and presented on MHC-E during SIV infection.

D. SIV infection increases expression of MHC-E on the surface of rhesus macaque CD4+ T cells

Both HIV and SIV encode the accessory protein Nef, which selectively down-regulates classical MHC-Ia molecule expression on the cell surface (*782*) (*332*) (*331*), a mechanism employed by the virus to evade CD8+ T cell responses (*330*) (*738*) (*190*) (*191*) (*192*) (*193*). HLA-E and Mamu-E molecules are resistant to Nef-mediated down-regulation as a result of amino acid differences in the cytoplasmic tails of MHC-E molecules compared to Nef-susceptible MHC-Ia molecules (*739*) (*332*). In fact, one report found that HLA-E is up-regulated on the surface of CD4+ T cells infected with HIV *in vitro* (*549*). Thus, we assessed changes in Mamu-E surface expression during *in vitro* SIV infection. CD4+ cells were isolated from 16 rhesus macaques, activated, infected with SIVmac239, and surface-stained for bulk MHC-I or MHC-E at Day4 post-infection (Figure 2.12A). As expected, bulk MHC-I levels were decreased on the surface of productively SIV-infected CD4+ T cells (SIVgag p27+, CD4low) compared to

uninfected cells in the same well (SIVgag27-, CD4high,Figure 2.12A). In contrast, surface MHC-E levels were increased on SIV-infected CD4+ T cells (Figure 2.12A). The HLA-E monoclonal antibody clone 4D12 used in these experiments was confirmed to be specific for MHC-E and cross-reactive with Mamu-E by staining a panel of single MHC-I transfectants (Figure 2.12B). Thus, SIV infection up-regulates MHC-E on the surface of rhesus macaque CD4+ T cells, in contrast to SIV-induced down-regulation of bulk MHC-I molecules, suggesting that SIV-specific CD8+ T cells restricted by MHC-E may be particularly effective against SIV-infected CD4+ T cells.

E. Primate MHC-E molecules exhibit functional conservation in the presentation of peptide to primed CD8+ T cells

Human HLA-E molecules exhibit reduced sequence diversity compared to Mamu-E molecules of rhesus macaques (*521*), as described above in section III. Thus, we sought to investigate whether sequence differences among HLA-E and Mamu-E molecules resulted in functional differences in each molecule's ability to present peptide antigens to CD8+ T cells. Using Mamu-E*02:04 and Mamu-E*02:11 transfectants in MHC-I restriction assays described above, we demonstrated that strain 68-1 RhCMV-elicited CD8+ T cells from rhesus macaques lacking Mamu-E*02:11 (Rh22034 and Rh22436) could nevertheless recognize SIVgag peptide presented on Mamu-E*02:11 (Figure 2.4). To extend these studies, we employed two new transfectants singly expressing Mamu-E*02:20 and HLA-E*01:03 (Figure 2.1B, Figure 2.13A), two MHC-E molecules that differ most significantly from the Mamu-E*02:04 and Mamu-E*02:11 molecules previously demonstrated to present SIVgag peptides to CD8+ T cells in strain 68-1 RhCMV/SIVgag-vaccianted rhesus macaques (Figures 1.1, 1.2, 2.3, 2.4). In

particular, Mamu-E*02:20 differs at 5 amino acid positions predicted to line the peptide-binding groove of MHC-E, while nearly all other MHC-E molecules in humans and rhesus macaques are completely conserved at these positions (Figures 1.1, 1.2). All twelve Mamu-E-restricted CD8+ T cell responses tested reacted to SIVgag 15-mer presented on Mamu-E*02:20 and HLA-E*01:03 in addition to Mamu-E*02:04 and Mamu-E*02:11 (Figure 2.13B), regardless of whether the rhesus macaque expressed the particular allele (Figure 2.1A). Further, when the optimal 9-mer SIVgag peptides (mapping in (*393*) and (*729*)) were pulsed onto single MHC-E transfectants at decreasing concentrations and combined with PBMC from each of four strain 68-1 RhCMV/SIVgag-vaccinated rhesus macaques, CD8+ T cells equivalently recognized peptide presented by HLA-E*01:03, Mamu-E*02:04, and Mamu-E*02:20 transfectants at peptide doses ranging from 10μM to 1 nM (Figure 2.14A, B). In some cases, Mamu-E*02:04 and Mamu-E*02:20 were capable of presenting peptide at a 10-fold lower dose (0.1 nM) compared to HLA-E*01:03 (Figure 2.14A, B). Dose response of CD8+ T cells to peptide-pulsed BLCL reflected the results found with peptide-pulsed MHC-E transfectants (Figure 2.14C).

Mauritian cynomolgus macaques (MCMs) represent an alternative non-human primate model of SIV infection and a potential alternative model to study CMV-elicited CD8+ T cells (*740*). As described above, MCMs possess simplified MHC genetics; the majority of MCM express MHC alleles from seven haplotypes, termed M1 through M7. MHC-Ia and MHC-II alleles expressed from these seven haplotypes have been fully defined (*100*) (*741*). Sixty-four percent of MCMs carry one of the 4 most common MHC haplotypes (M1, M2, M3, and M4), and our collaborators recently identified MHC-E

molecules expressed from these haplotypes by deep sequencing full-length MHC-E cDNAs from haplotype homozygous MCMs (Figure 1.1). In order to test Mafa-E for the ability to present SIVgag peptides to Mamu-E-restricted CD8+ T cells, we made a transfectant expressing the Mafa-E molecule encoded by the M1 haplotype (Figure 2.15A). When pulsed with a pool of the 11 MHC-E-binding optimal 9-mer SIVgag peptides, the Mafa-E transfectant, and not MHC-I-negative parental cell line K562, was able to stimulate CD8+ T cells from four strain 68-1 RhCMV/SIVgag-vaccinated rhesus macaques (Figure 2.15B). Further, strain 68-1 RhCMV/SIVgag-elicited, MHC-E-restricted CD8+ T cells from rhesus macaques were able to recognize SIV-infected CD4+ T cells derived from Mauritian cynomolgus macaques expressing a variety of MHC haplotypes as well as SIV-infected CD4+ T cells derived from heterologous rhesus macaques (Figure 2.15C, D). Collectively, these data suggest that Mamu-E-restricted CD8+ T cells can cross-recognize peptide presented by Mamu-E molecules not expressed in the rhesus macaque as well as peptide presented by MHC-E of other primate species, including HLA-E and Mafa-E. Further, rhesus, human, and Mauritian cynomolgus macaque MHC-E molecules, even those with amino acid differences in predicted peptide-contact positions, effectively present at least some of the same peptides to SIVgag-specific CD8+ T cells.

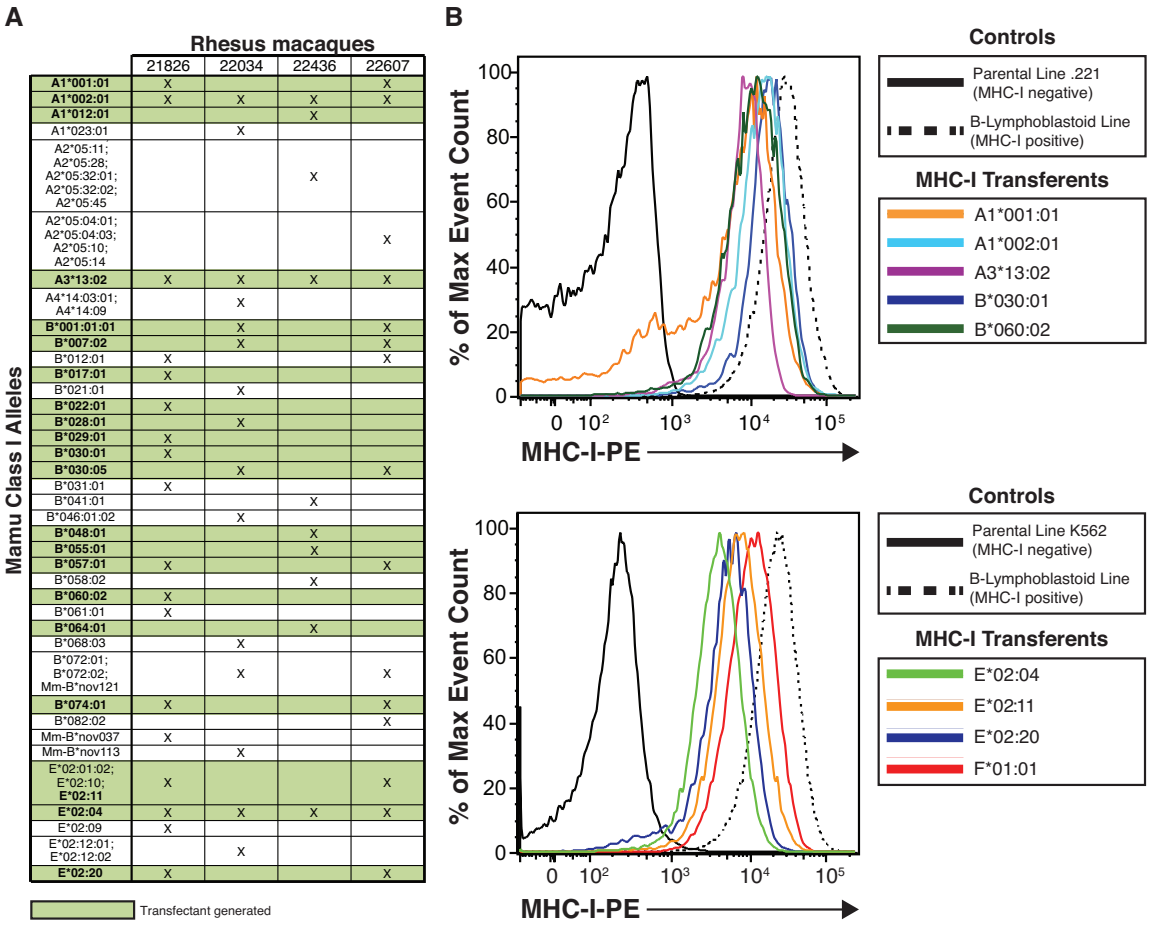


Figure 2.1: Validation of transfected cell lines expressing single MHC-I molecules corresponding to MHC-I molecules expressed by 4 strain 68-1 RhCMV/SIVgag-vaccinated RM. (A) 4 strain 68-1 RhCMV/SIVgag-vaccinated RMs were Mamu-A, -B, and -E typed by Roche/454 pyrosequencing. Green highlight indicates alleles selected for MHC-I transfectant generation. Where multiple alleles are listed, a transfectant expressing the bolded allele was produced. **(B)** One MHC-Ia (top) or MHC-Ib (bottom) allele was transfected into a parental (MHC-I negative) cell line (.221 cells or K562 cells) and stained with pan-MHC-I monoclonal mAb W6/32. MHC-I-expressing B-lymphoblastoid cells (BLCL) served as a positive control, while the MHC-I negative parental cell lines were used as negative controls.

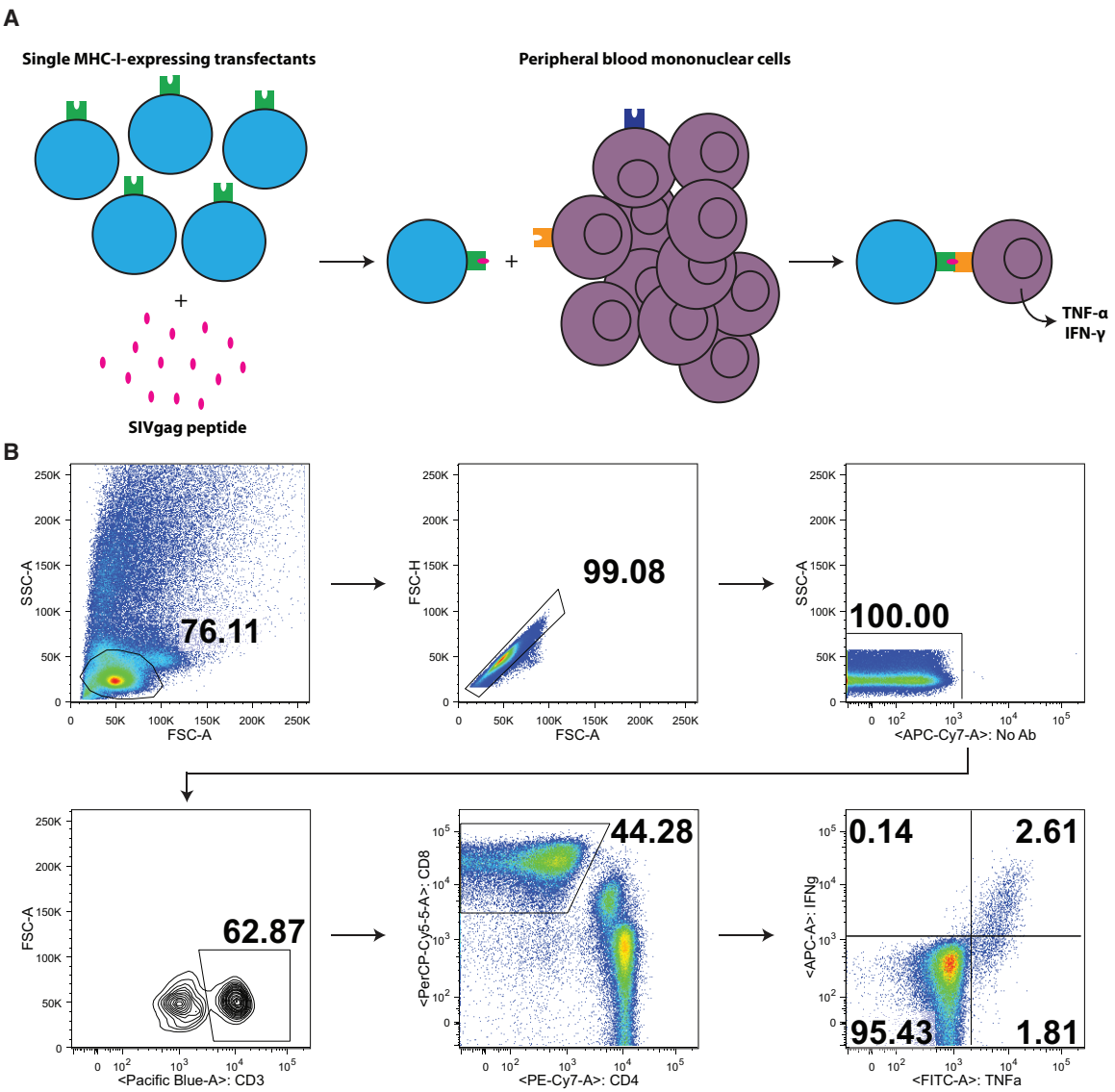


Figure 2.2: Schematic of MHC-I restriction assay. (A) Single MHC-I-expressing transfectants are incubated with SIVgag peptide at a final concentration of 10μM for 90 minutes, washed three times with 1X PBS and once with R10 to remove unbound peptide, and combined with freshly isolated peripheral blood mononuclear cells (PBMC) from a strain 68-1 RhCMV/SIVgag-vaccinated rhesus macaque. Positive CD8+ T cell recognition of SIVgag peptide in the context of the particular MHC-I molecule expressed by the transfectant results in induction of cytokines TNF-α and IFN-γ. **(B)** Brefeldin A (BFA) is added one hour after combining antigen-presenting cells and PBMC, trapping cytokines in the endoplasmic reticulum for detection by intracellular cytokine staining. Eight hours after addition of BFA, cells are stained for surface expression of CD4 and CD8α, permeabilized with saponin, stained for CD3, TNF-α, and IFN-γ,

and collected on a BD LSR-II instrument. Plots displaying TNF-α versus IFN-γ are gated for CD8+ T cells using the following progressive gating strategy: lymphocytes, singlets, APC-Cy7-negative cells (dump channel), CD3+ cells, and CD4-CD8α+ cells.

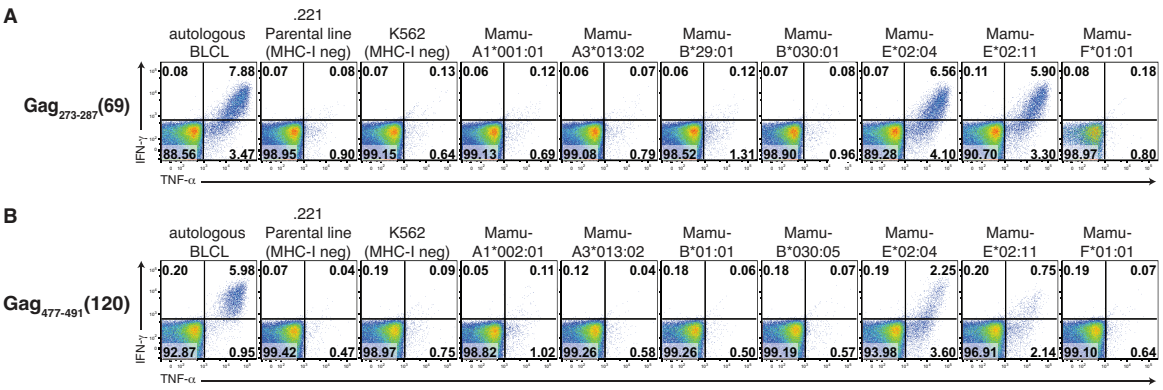
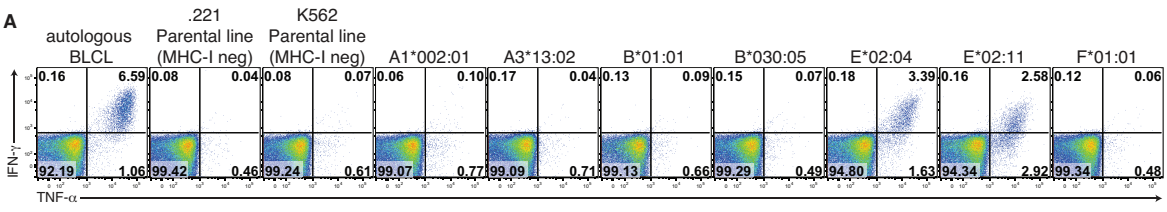


Figure 2.3: MHC-E molecules restrict SIVgag supertope-specific CD8+ T cell responses. PBMC from representative strain 68-1 RhCMV/SIVgag-vaccinated rhesus macaques were combined with SIVgag peptide pulsed onto autologous BLCL (positive control), MHC-I-negative cell lines (.221 and K562; negative controls), or the indicated transfectants expressing single MHC-I molecules encoded by the animal (MHC typing shown in Figure 2.1A; schematic shown in Figure 2.2A). CD8+ T cell recognition was determined by detection of TNF-α and IFN-γ in flow cytometric ICS, using the gating strategy shown in Figure 2.2B. Representative flow plots display MHC-I restriction analysis of **(A)** SIVgag$_{273\text{-}287}$(69)-specific or **(B)** SIVgag$_{477\text{-}491}$(120)-specific CD8+ T cell responses in Rh21826 and Rh22034, respectively. Results shown are representative of those obtained with PBMC from 4 strain 68-1 RhCMV/SIVgag-vaccinated rhesus macaques, see Figure 2.4 for comprehensive restriction analysis.



B

Rh21826	Gag_{61-75} (16)	Gag_{69-83} (18)	Gag_{89-103} (23)	$Gag_{117-131}$ (30)	$Gag_{129-143}$ (33)	$Gag_{197-211}$ (50)	$Gag_{257-271}$ (65)	$Gag_{273-287}$ (69)	$Gag_{385-399}$ (97)	$Gag_{433-447}$ (109)	$Gag_{473-487}$ (119)	$Gag_{477-491}$ (120)
BLCL	-	-	+	-	+	+	+	+	-	-	+	+
221	-	-	-	-	-	-	-	-	-	-	-	-
K562	-	-	-	-	-	-	-	-	-	-	-	-
A1*001:01	-	-	-	-	-	+	-	-	-	-	-	-
A1*002:01	-	-	-	-	+	-	-	-	-	-	-	-
A3*13:02	-	-	-	-	-	-	-	-	-	-	-	-
B*029:01	-	-	-	-	-	-	-	-	-	-	-	-
B*030:01	-	-	-	-	-	-	-	-	-	-	-	-
E*02:04	-	-	+	-	+	+	+	+	-	-	+	+
E*02:11	-	-	+	-	+	+	+	+	-	-	+	+
F*01:01	-	-	-	-	-	-	-	-	-	-	-	-

Rh22034	Gag_{61-75} (16)	Gag_{69-83} (18)	Gag_{89-103} (23)	$Gag_{117-131}$ (30)	$Gag_{129-143}$ (33)	$Gag_{197-211}$ (50)	$Gag_{257-271}$ (65)	$Gag_{273-287}$ (69)	$Gag_{385-399}$ (97)	$Gag_{433-447}$ (109)	$Gag_{473-487}$ (119)	$Gag_{477-491}$ (120)
BLCL	+	+	+	-	-	-	+	+	+	+	-	+
221	-	-	-	-	-	-	-	-	-	-	-	-
K562	-	-	-	-	-	-	-	-	-	-	-	-
A1*002:01	-	-	-	-	-	-	-	-	-	-	-	-
A3*13:02	-	-	-	-	-	-	-	-	-	-	-	-
B*001:01	-	-	-	-	-	-	-	-	-	-	-	-
B*030:05	-	-	-	-	-	-	-	-	-	-	-	-
E*02:04	+	+	+	-	-	-	+	+	+	+	-	+
E*02:11	+	+	+	-	-	-	+	+	+	+	-	+
F*01:01	-	-	-	-	-	-	-	-	-	-	-	-

Rh22436	Gag_{61-75} (16)	Gag_{69-83} (18)	Gag_{89-103} (23)	$Gag_{117-131}$ (30)	$Gag_{129-143}$ (33)	$Gag_{197-211}$ (50)	$Gag_{257-271}$ (65)	$Gag_{273-287}$ (69)	$Gag_{385-399}$ (97)	$Gag_{433-447}$ (109)	$Gag_{473-487}$ (119)	$Gag_{477-491}$ (120)
BLCL	-	-	-	+	-	+	+	+	-	+	+	+
221	-	-	-	-	-	-	-	-	-	-	-	-
K562	-	-	-	-	-	-	-	-	-	-	-	-
A1*002:01	-	-	-	-	-	-	-	-	-	-	-	-
A3*13:02	-	-	-	-	-	-	-	-	-	-	-	-
B*048:01	-	-	-	-	-	-	-	-	-	-	-	-
E*02:04	-	-	-	+	-	+	+	+	-	+	+	+
E*02:11	-	-	-	+	-	+	+	+	-	+	+	+
F*01:01	-	-	-	-	-	-	-	-	-	-	-	-

Rh22607	Gag_{61-75} (16)	Gag_{69-83} (18)	Gag_{89-103} (23)	$Gag_{117-131}$ (30)	$Gag_{129-143}$ (33)	$Gag_{197-211}$ (50)	$Gag_{257-271}$ (65)	$Gag_{273-287}$ (69)	$Gag_{385-399}$ (97)	$Gag_{433-447}$ (109)	$Gag_{473-487}$ (119)	$Gag_{477-491}$ (120)
BLCL	-	+	+	-	+	-	+	+	+	+	+	+
221	-	-	-	-	-	-	-	-	-	-	-	-
K562	-	-	-	-	-	-	-	-	-	-	-	-
A1*001:01	-	+	-	-	-	-	-	-	-	-	-	-
A1*002:01	-	-	-	-	+	-	-	-	-	-	-	-
A3*13:02	-	-	-	-	-	-	-	-	-	-	-	-
B*001:01	-	-	-	-	-	-	-	-	-	-	-	-
B*030:05	-	-	-	-	-	-	-	-	-	-	-	-
E*02:04	-	+	+	-	+	-	+	+	+	+	+	+
E*02:11	-	+	+	-	+	-	+	+	+	+	+	+
F*01:01	-	-	-	-	-	-	-	-	-	-	-	-

Figure 2.4: Comprehensive analysis of the MHC-Ia and MHC-Ib specificity of twelve SIVgag 15-mer peptide-specific CD8+ T cell responses in four strain 68-1 RhCMV/SIVgag-vaccinated rhesus macaques. (A) Flow cytometric ICS plots showing a representative MHC restriction analysis of the $SIVgag_{433-447}$(109)-specific CD8+ T cell response in Rh22034. PBMC were combined with SIVgag 15-mer peptide pulsed onto autologous BLCL (positive control), MHC-I-negative cell lines (.221 and K562; negative controls), or the indicated transfectants expressing single MHC-I molecules encoded by the animal (MHC typing shown in Figure 2.1A; assay schematic shown in Figure 2.2A). CD8+ T cell recognition was determined by detection of TNF-α and IFN-γ in flow cytometric ICS, using the gating strategy shown in Figure 2.2B. **(B)** Table shows comprehensive MHC-I restriction analysis of twelve SIVgag 15-mer peptide-specific CD8+ T cell responses in PBMC of 4 indicated RM (MHC-typing shown in Figure 2.1A), as described in (A). Combinations that resulted in CD8+ T cell responses above background (antigen-

presenting cells with no peptide) are indicated by + signs (blue boxes); combinations that did not result in CD8+ T cell responses above background are indicated by - signs (open boxes). MHC-I alleles expressed in each RM are indicated in red; non-expressed alleles are shown in white (except expression of Mamu-F*01:01 is unknown).

Macaque:	A1*001:01	A1*002:01		Gag_{69-83} (18)	$Gag_{129-143}$ (33)	$Gag_{197-211}$ (50)
Rh22607	+	+		+	+	-
Rh21826	+	+		-	+	+
Rh25545	+	-		-	-	-
Rh25565	+	-		+	-	+
Rh22034	-	+		+	-	-
Rh28819	-	+		+	-	-
Rh28808	-	+		-	+	+
Rh22436	-	+		-	-	+
Rh24194	-	+		-	-	+
Rh27517	-	-		+	+	-
Rh22063	-	-		+	-	-
Rh27473	-	-		+	-	-
Rh27715	-	-		+	-	-
Rh29483	-	-		+	-	-
Rh25222	-	-		-	+	+
Rh29208	-	-		-	+	+
Rh29212	-	-		-	+	+
Rh22624	-	-		-	+	-
Rh29482	-	-		-	-	+
Rh21756	-	-		-	-	-

Figure 2.5: Classical MHC-Ia allomorphs capable of presenting SIVgag peptides to strain 68-1 RhCMV/SIVgag-elicited CD8+ T cells are not the restricting MHC alleles for these T cell responses. Twenty strain 68-1 RhCMV/SIVgag-vaccinated RM were MHC-typed for the presence of Mamu-A1*001:01 and Mamu-A1*002:01 and tested for CD8+ T cell responses specific for $SIVgag_{69-83}$(18), $SIVgag_{129-143}$(33), and $SIVgag_{197-211}$(50). As shown in Figure 2.4, Mamu-A1*001:01 can present $SIVgag_{69-83}$(18) and $SIVgag_{197-211}$(50), and Mamu-A1*002:01 can present $SIVgag_{129-143}$(33). The presence of Mamu-A1*001:01 or Mamu-A1*002:01 in the rhesus macaque does not track with the detection of CD8+ T cells specific for these three peptides.

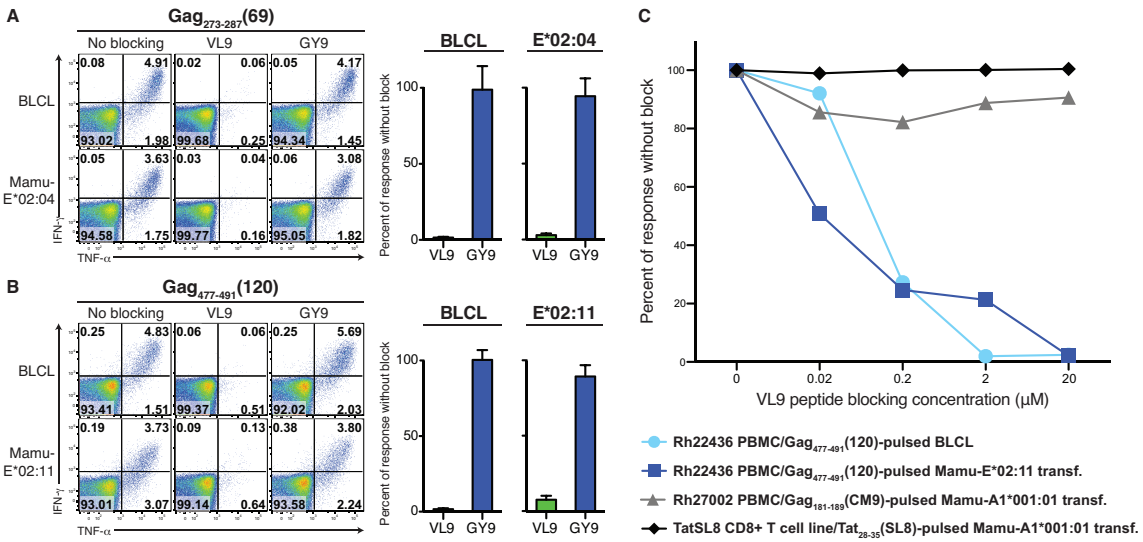


Figure 2.6: MHC-E-binding peptide VL9 specifically blocks MHC-E-restricted CD8+ T cell responses targeting SIVgag supertopes. (A, B) Flow cytometric ICS plots on left show a representative blocking experiment. The indicated antigen-presenting cells, either autologous BLCL or MHC-E transfectants, were pre-incubated with MHC-E-binding peptide VMAPRTLLL (VL9) or irrelevant A1*002:01-binding peptide SIVgag$_{71\text{-}79}$(GY9) prior to addition of SIVgag 15-mer peptide and combination with PBMC from a strain 68-1 RhCMV/SIVgag-vaccinated rhesus macaque, as described in Figure 2.2A. CD8+ T cell recognition was determined by detection of TNF-α and IFN-γ in flow cytometric ICS, using the gating strategy shown in Figure 2.2B. Representative flow plots display blocking of **(A)** SIVgag$_{273\text{-}287}$(69)-specific and **(B)** SIVgag$_{477\text{-}491}$(120)-specific CD8+ T cell responses in Rh21826. Bar graphs on the right show results from four rhesus macaques response (mean±SEM TNF-α+IFN-γ+ CD8+ T cell response frequencies, normalized to the response observed with no peptide blocking). **(C)** Verification of VL9 blocking specificity for MHC-E-restricted CD8+ T cell responses. The indicated antigen-presenting cells were pre-incubated with increasing concentrations of VL9 prior to pulse with the SIVgag$_{477\text{-}491}$(120) 15-mer or optimal Mamu-A1*001:01-presented SIVgag$_{181\text{-}189}$(CM9) or SIVtat$_{25\text{-}35}$(SL8) peptides. These antigen-presenting cells were then incubated with the indicated effectors for flow cytometric ICS analysis, as described in (A, B). Data represent the percentage of TNF-α+IFN-γ+ CD8+ T cells, normalized to the response observed with no peptide blocking. Rh22436 is a strain 68-1 RhCMV/SIVgag-vaccinated RM, while Rh27002 is SIV-infected. Note that increasing concentrations of VL9 peptide progressively block the

ability of MHC-E-expressing antigen-presenting cells to activate SIVgag$_{477\text{-}491}$(120)-specific CD8+ T cells from a strain 68-1 RhCMV/SIVgag-vaccinated RM, but have no effect on conventionally MHC-Ia-restricted CD8+ T cells specific for SIVgag$_{181\text{-}189}$(CM9) or SIVtat$_{28\text{-}35}$(SL8).

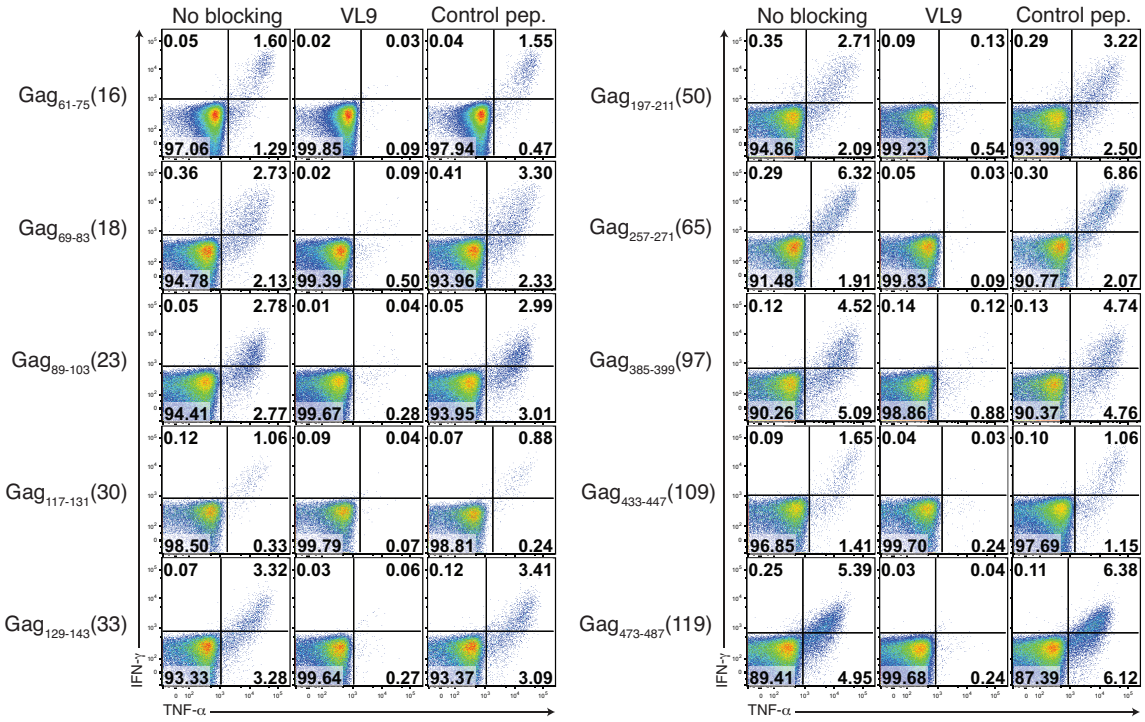


Figure 2.7: MHC-E-binding peptide VL9 specifically blocks ten identified MHC-E-restricted CD8+ T cell responses. Flow cytometric ICS plots show representative blocking experiments. MHC-E transfectants were pre-incubated with MHC-E-binding peptide VL9 or control peptide prior to addition of SIVgag 15-mer peptide and combination with PBMC from a strain 68-1 RhCMV/SIVgag-vaccinated rhesus macaque, as described in Figure 2.2A. CD8+ T cell recognition was determined by detection of TNF-α and IFN-γ in flow cytometric ICS, using the gating strategy shown in Figure 2.2B. The following MHC-E transfectants were utilized: Mamu-E*02:04 for the SIVgag$_{69-83}$(18), SIVgag$_{89-103}$(23), SIVgag$_{385-399}$(97), and SIVgag$_{433-447}$(109) responses and Mamu-E*02:11 for the SIVgag$_{61-75}$(16), SIVgag$_{117-131}$(30), SIVgag$_{129-143}$(33), SIVgag$_{197-211}$(50), SIVgag$_{257-271}$(65), SIVgag$_{473-487}$(119), and SIVgag$_{477-491}$(120) responses. Control peptides consisted of either Mamu-A1*002:01-binding peptide SIVgag$_{71-79}$(GY9) or Mamu-A1*001:01-binding peptide SIVgag$_{181-189}$(CM9). Along with Figure 2.6, these data indicate that the VL9 peptide blocks CD8+ T cell recognition of twelve MHC-E-presented 15-mer peptide epitopes.

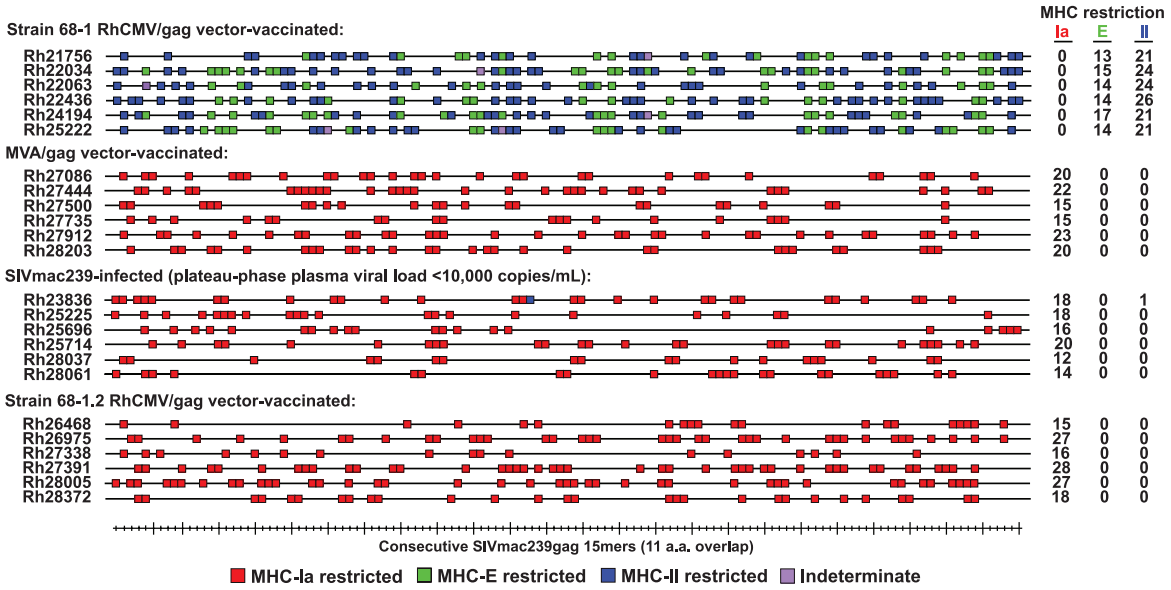


Figure 2.8: Every MHC-I-dependent CD8+ T cell response elicited by strain 68-1 RhCMV/SIVgag vaccination is restricted by MHC-E. CD8+ T cell responses to SIVgag were epitope-mapped using flow cytometric ICS to detect recognition of 125 consecutive 15-mer SIVgag peptides (with 11 amino acid overlap) in rhesus macaques vaccinated with the indicated SIVgag-expressing viral vectors or infected with SIVmac239. Peptides resulting in specific CD8+ T cell responses are indicated by a box, with the color of the box designating MHC restriction as determined by blocking with pan-MHC-I mAb W6/32, MHC-E-binding peptide VL9, or MHC-II-binding peptide CLIP. MHC-Ia-, MHC-E-, and MHC-II-restriction was based on >90% response blocking by W6/32 alone (red), W6/32 alone and VL9 alone (green), and CLIP alone (blue), respectively, with responses not meeting these criteria labeled indeterminate (purple). The minimal number of independent epitopes in each MHC restriction category is shown at right for each rhesus macaque. Analysis of additional RMs (strain 68-1 RhCMV/SIVgag n=36, MVA/gag n=5, SIVmac239-infected n=6, strain 68-1.2 RhCMV/SIVgag n = 3) are shown in the supplementary material of *Hansen & Wu* et al. (*729*).

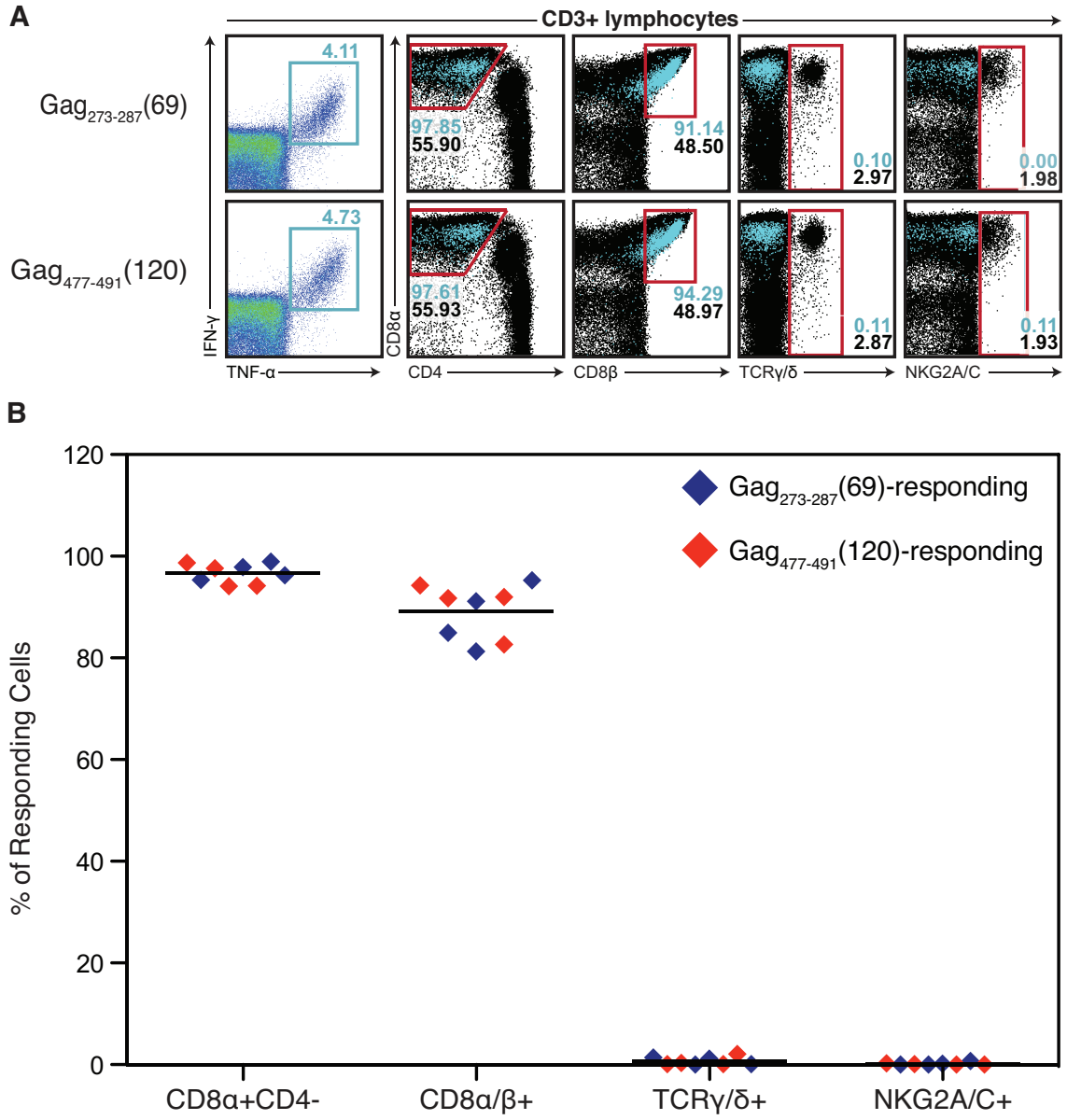


Figure 2.9: Strain 68-1 RhCMV/SIVgag-elicited, MHC-E-restricted, SIVgag supertope-specific CD8+ T cells exhibit a conventional CD8α/β+, TCRγ/δ-, NKG2A/C- phenotype. (A) Representative flow cytometric analysis of the phenotype of SIVgag supertope-specific, MHC-E-restricted CD8+ T cells elicited by strain 68-1 RhCMV/SIVgag vaccination. PBMC from Rh21826 were stimulated with autologous BLCL pulsed with either the SIVgag$_{273-287}$(69) or SIVgag$_{477-491}$(120) MHC-E-presented supertopes, and responding vs. non-responding CD3+ lymphocytes (responding defined as TNF-α+IFN-γ+; gate shown in left plot) were cell surface phenotyped by flow cytometric ICS. Responding cells and non-responding cells within the CD3+ T cell gate colored blue and black, respectively; the percentage of cells

within each population falling within the red gated regions of each plot are indicated in the same colors. Note that supertope-responding cells (blue) are almost entirely CD8α/β+, TCRγ/δ-, and NKG2A/C-negative. **(B)** Summary of the phenotypic analysis of MHC-E-restricted CD8+ T cells responding to autologous BLCL pulsed with $SIVgag_{273-287}$(69) or $SIVgag_{477-491}$(120) in four strain 68-1 RhCMV/SIVgag-vaccinated rhesus macaques. The graph shows the percentages of responding CD3+ T cells (TNF-α+IFN-γ+) that possess the indicated phenotypes.

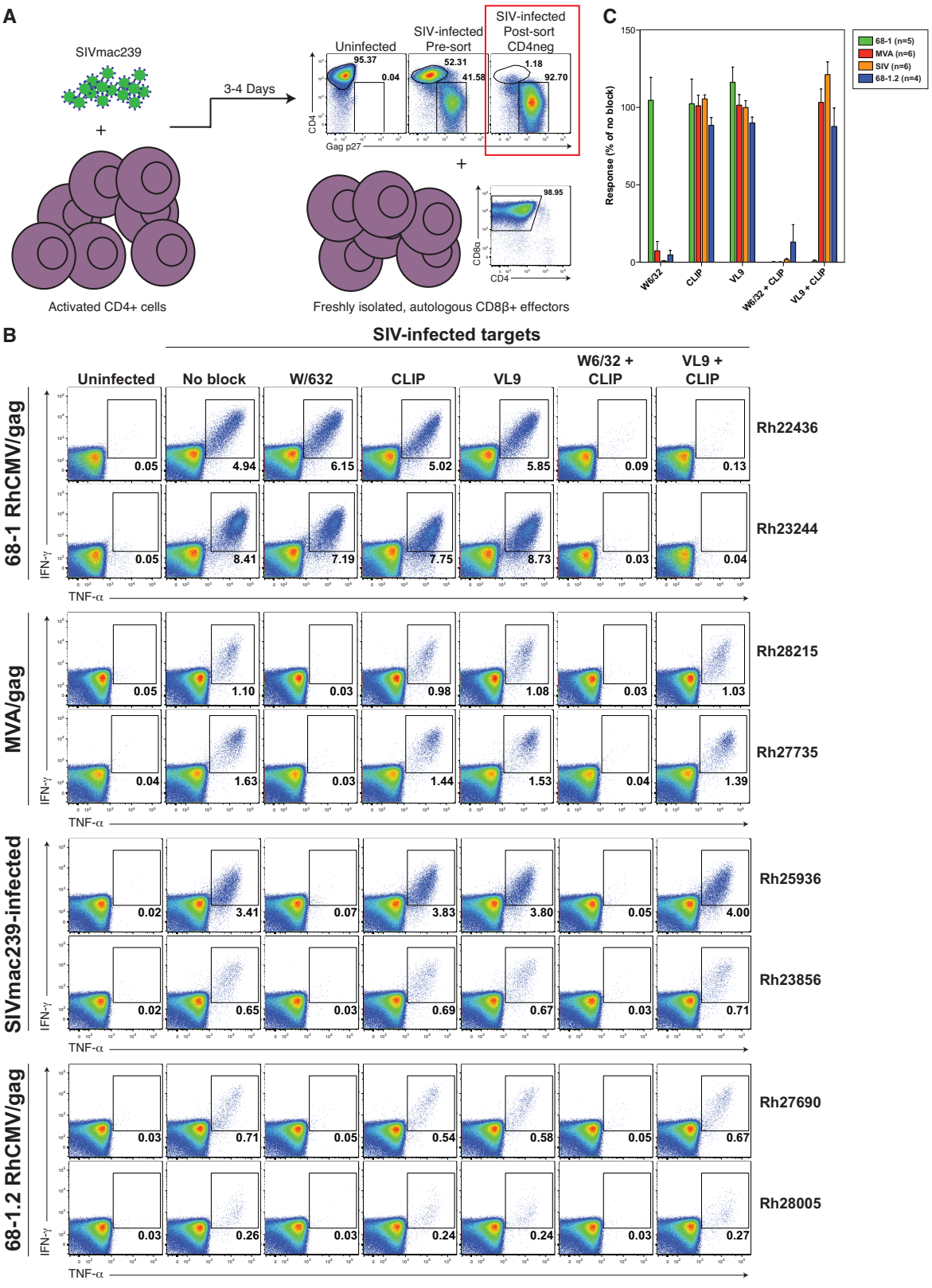

A
SIVmac239
3-4 Days
Uninfected
SIV-infected Pre-sort
SIV-infected Post-sort CD4neg
95.37
0.04
52.31
41.58
1.18
92.70
CD4
Gag p27
98.95
CD8α
CD4
Activated CD4+ cells
Freshly isolated, autologous CD8β+ effectors
C
Response (% of no block)
68-1 (n=5)
MVA (n=6)
SIV (n=6)
68-1.2 (n=4)
W6/32
CLIP
VL9
W6/32 + CLIP
VL9 + CLIP
B
SIV-infected targets
Uninfected
No block
W/632
CLIP
VL9
W6/32 + CLIP
VL9 + CLIP
68-1 RhCMV/gag
MVA/gag
SIVmac239-infected
68-1.2 RhCMV/gag
IFN-γ
TNF-α
Rh22436
Rh23244
Rh28215
Rh27735
Rh25936
Rh23856
Rh27690
Rh28005

Figure 2.10: MHC-E-restricted, strain 68-1 RhCMV/SIVgag-elicited CD8+ T cells recognize SIV-infected CD4+ T cells. (A) Schematic of SIV recognition assay is shown. CD4+ cells are isolated from PBMC, activated, and infected with SIVmac239 for 3-4 days to generate targets. SIV-infected targets are enriched for SIV-infected cells by isolation of CD4-negative cells, a consequence of SIV infection. Flow plots at top show uninfected targets and SIV-infected targets prior to and after enrichment (gated on live, CD3+ cells). Uninfected targets or SIV-infected, post-sort CD4-negative targets are combined with freshly isolated, autologous CD8β+ cells from PBMC. CD8+ T cell recognition is determined by detection of TNF-α and IFN-γ in flow cytometric ICS, using the gating strategy shown in Figure 2.2B with the addition of a live cell gate. **(B)** Flow cytometric ICS plots show representative SIV-infected CD4+ T cell recognition by CD8β+ T cells isolated from PBMC of rhesus macaques vaccinated with the indicated SIVgag-expressing viral vectors or infected with SIV. Plots show CD8β+ T cell recognition of autologous SIV-infected CD4+ T cells alone (no block), in the presence of anti-MHC-I mAb W/632 plus CLIP, or in the presence of VL9 plus CLIP. All plots gated on live, CD3+, CD8+ cells. Bar graph on right shows SIV recognition results from 4-6 rhesus macaques per group. Data represent the percentage of responding TNF-α+ and/or IFN-γ+ CD8+ T cells (gated as shown in B), normalized to the response observed with no peptide blocking (mean±SEM).

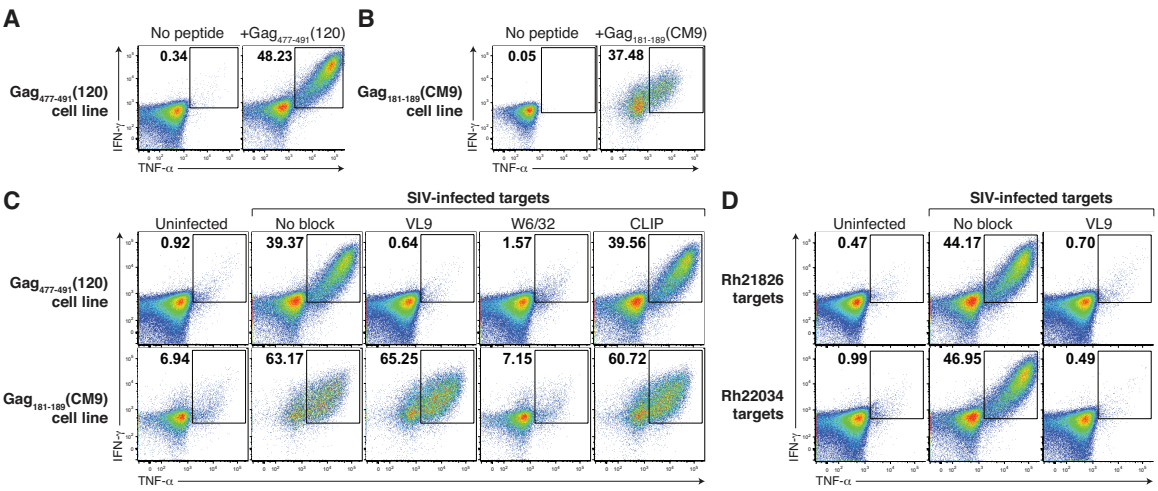


Figure 2.11: An SIVgag supertope-specific CD8+ T cell line recognizes SIV-infected CD4+ T cells in an MHC-E-dependent manner. (A, B) Flow cytometric ICS plots showing peptide recognition by **(A)** an MHC-E-restricted, SIVgag$_{477\text{-}491}$(120)-specific CD8+ T cell line and **(B)** a Mamu-A1*001-restricted, SIVgag$_{181\text{-}189}$(CM9)-specific CD8+ T cell line. All plots gated on live, CD3+, CD8+ cells. **(C)** Flow cytometric ICS plots showing SIV-infected CD4+ T cell recognition by CD8+ T cell lines. Both the MHC-E-restricted, SIVgag$_{477\text{-}491}$(120)-specific and the Mamu-A1*001-restricted, SIVgag$_{181\text{-}189}$(CM9)-specific CD8+ T cell lines were used as effectors in SIV recognition, as described in Figure 2.10A. Plots show CD8+ T cell recognition of Rh22607 (Mamu-A1*001+) SIV-infected CD4+ T cells alone (no block) or in the presence of blocking with anti-MHC-I mAb W/632, MHC-E-binding peptide VL9, or MHC-II-binding peptide CLIP. All plots gated on live, CD3+, CD8+ cells. **(D)** Flow cytometric ICS plots showing MHC-E-restricted, SIVgag$_{477\text{-}491}$(120)-specific CD8+ T cell line recognition of heterologous SIV-infected CD4+ T cells derived from Rh21826 and Rh22034, as described in C.

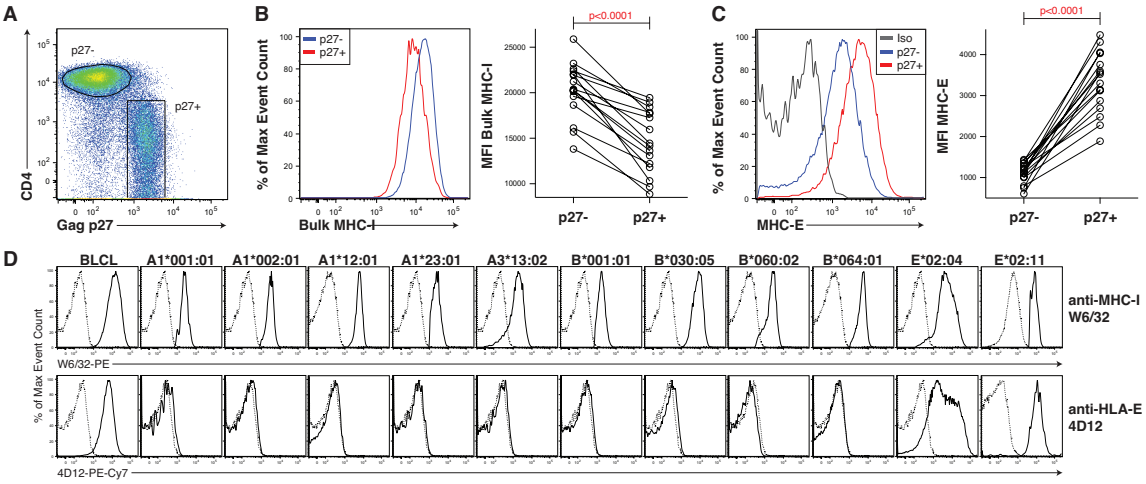


Figure 2.12: MHC-E is up-regulated on the surface of SIV-infected CD4+ T cells. (A, B, C) CD4+ cells were isolated from PBMC, activated, and infected with SIVmac239 for 4 days. **(A)** Flow cytometric plots showing gating scheme for uninfected (SIVgag p27-/CD4high) versus productively SIV-infected cells (SIVgag p27+/CD4low) in the same culture. Plots gated on live, CD3+ cells. **(B, C)** Surface expression of bulk MHC-I **(B)**, as determined by staining with mAb W6/32, or MHC-E **(C)**, as determined by staining with mAb 4D12, is shown on productively SIV-infected and uninfected CD4+ T cells in the same cultures, gating scheme shown in A. Left panels show representative flow cytometric histograms. Right panels depict the mean fluorescence intensity (MFI) of bulk MHC-I or MHC-E-specific staining on the surface of *in vitro* SIV-infected versus uninfected CD4+ T cells derived from a total of 16 unrelated RM. P values were determined by the paired Student's T test. **(D)** Validation of the specificity of MHC-E-specific mAb 4D12. Mamu-Ia and Mamu-E were expressed the murine cell line RMA-S, which expresses human β2-microglubulin. Histograms showing surface staining of single rhesus macaque MHC-Ia or MHC-Ib transfectants by the pan-MHC-I mAb W6/32 (top row) versus the MHC-E-specific mAb 4D12 (bottom row). Rhesus macaque BLCL were used as a positive control, whereas the parental RMA-S cell line was used as a negative control (light gray histogram). Note the restriction of 4D12 reactivity to the Mamu-E transfectants.

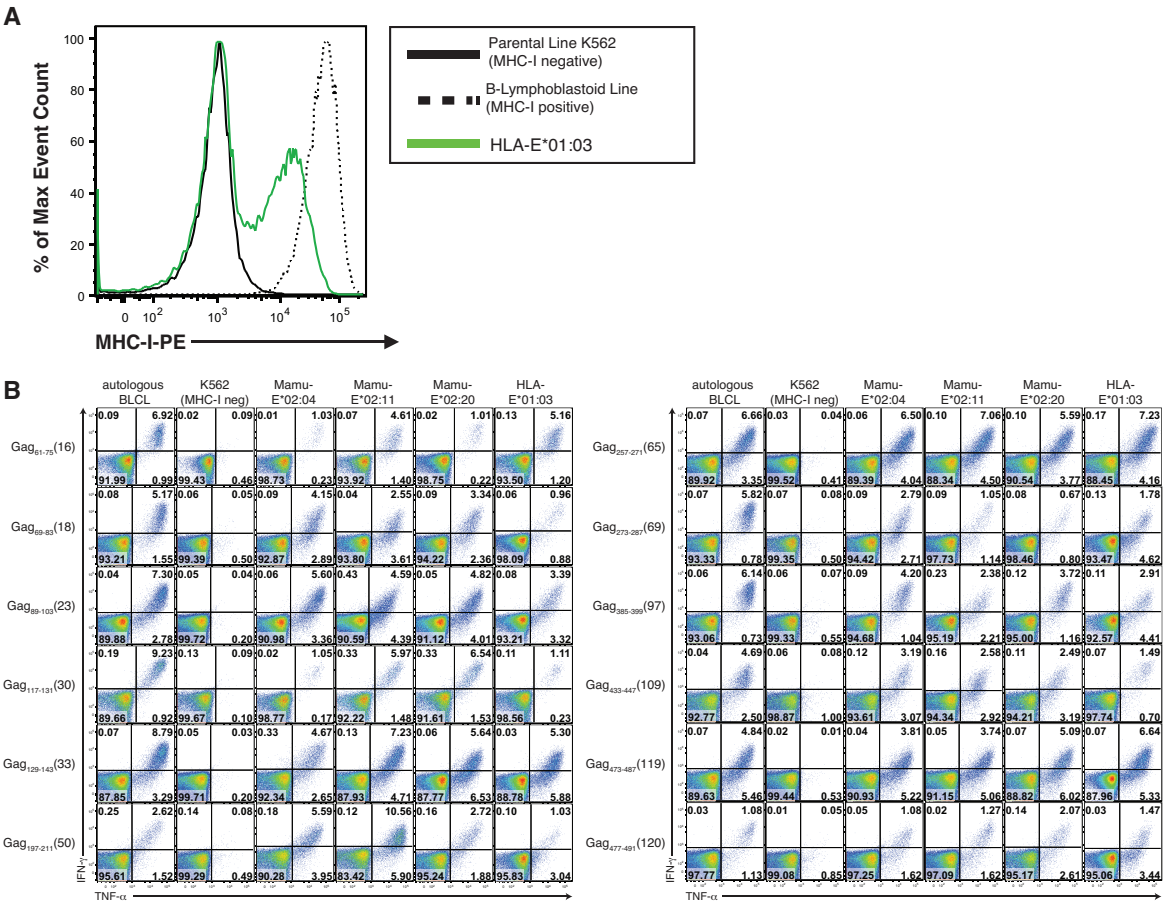


Figure 2.13: Strain 68-1 RhCMV/SIVgag-elicited CD8+ T cells recognize peptide across rhesus macaque and human MHC-E molecules. Flow cytometric ICS plots showing CD8+ T cell recognition of peptide presented by various human and rhesus MHC-E molecules. PBMC from strain 68-1 RhCMV/SIVgag-vaccinated rhesus macaques [Rh21826: $SIVgag_{89\text{-}103}(23)$, $SIVgag_{129\text{-}143}(33)$, $SIVgag_{257\text{-}271}(65)$, $SIVgag_{473\text{-}487}(119)$; Rh22034: $SIVgag_{61\text{-}75}(16)$, $SIVgag_{69\text{-}83}(18)$, $SIVgag_{271\text{-}287}(69)$, $SIVgag_{385\text{-}399}(97)$, $SIVgag_{477\text{-}491}(120)$; Rh22436: $SIVgag_{197\text{-}211}(30)$, $SIVgag_{197\text{-}211}(50)$] were combined with SIVgag 15-mer peptide pulsed onto autologous BLCL (positive control), MHC-I-negative cell lines K562 (negative control), or the indicated MHC-E transfectants. CD8+ T cell recognition was determined by detection of TNF-α and IFN-γ in flow cytometric ICS, using the gating strategy shown in Figure 2.2B. Note that all twelve MHC-E-restricted 15-mer peptide epitopes studied can be effectively presented to strain 68-1 RhCMV/SIVgag-elicited CD8+ T cells on all 3 tested Mamu-E molecules and on HLA-E. Since MHC-E expression levels on the different transfectants vary from each other and with each experiment, the strength

of the MHC-E-mediated presentation of these 15-mer peptides cannot be directly compared by relative response frequencies, but rather should be compared by endpoint dilution analysis of optimal 9-mer peptides for each epitope on the same transfectant (see Figure 2.14).

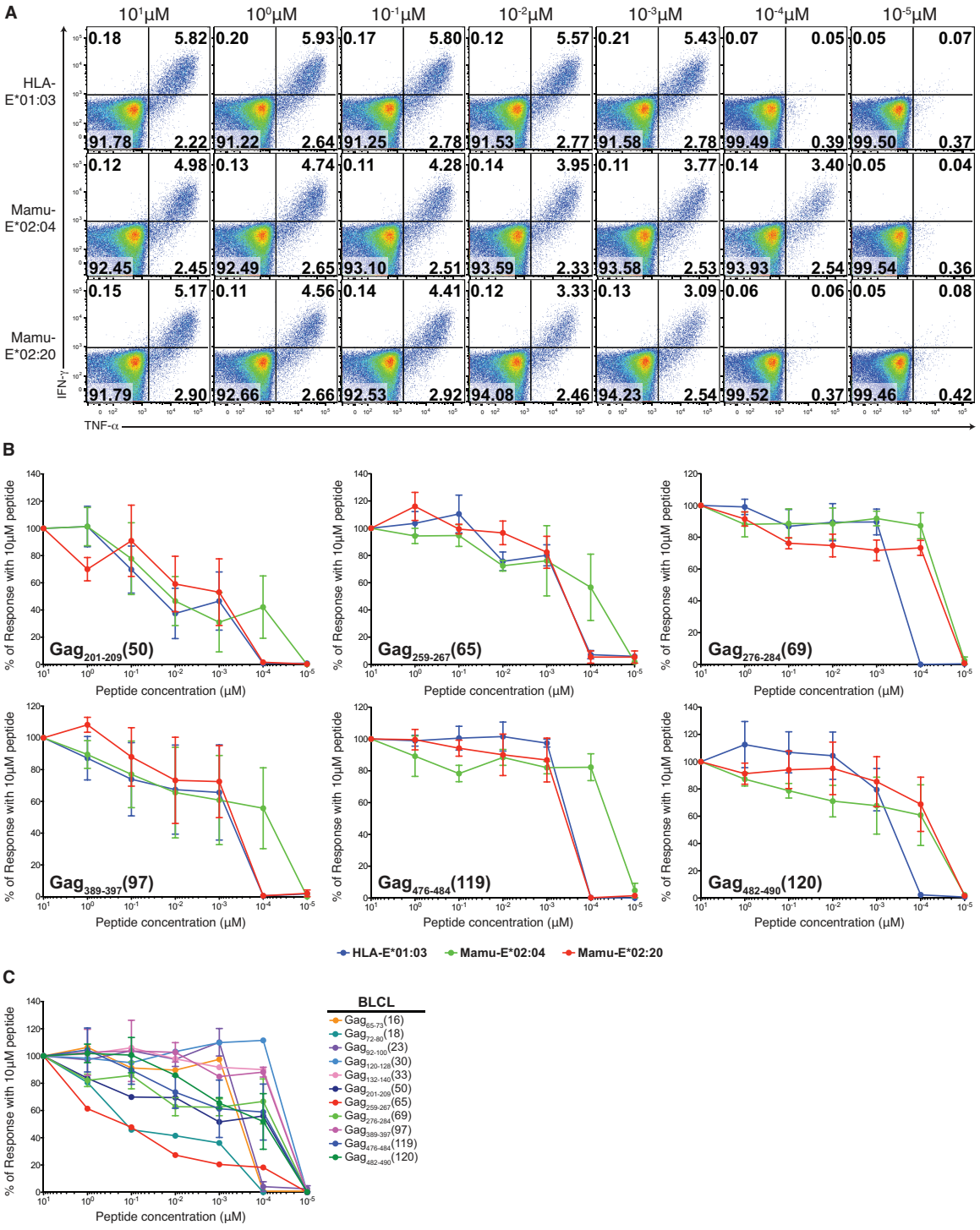


Figure 2.14: Dose response of MHC-E-restricted CD8+ T cells to optimal SIVgag 9-mers pulsed on human and RM MHC-E transfectants and BLCL. Autologous BLCL or MHC-E transfectants were pulsed with the indicated concentration of the optimal SIVgag 9-mer peptide epitopes, washed, and combined with PBMC from strain 68-1 RhCMV/SIVgag-vaccinated rhesus macaques for flow cytometric

ICS detection of responding CD8+ T cells, as described in Figure 2.2A. In these experiments, numbers of MHC-E transfectants combined with PBMC were normalized based on surface MHC-E expression. **(A)** Representative flow cytometric ICS plots showing the dose response to SIVgag$_{476\text{-}484}$(119) in Rh22607. **(B, C)** Graphs show the dose response for CD8+ T cells responding to the indicated pulsed antigen-presenting cells (mean±SEM TNF-α+IFN-γ+ CD8+ T cell response frequencies, normalized to the response observed with APCs pulsed with 10μM peptide dose). Each graph shows data from (B) 3-4 or (C) 1-2 strain 68-1 RhCMV/SIVgag-vaccinated rhesus macaques.

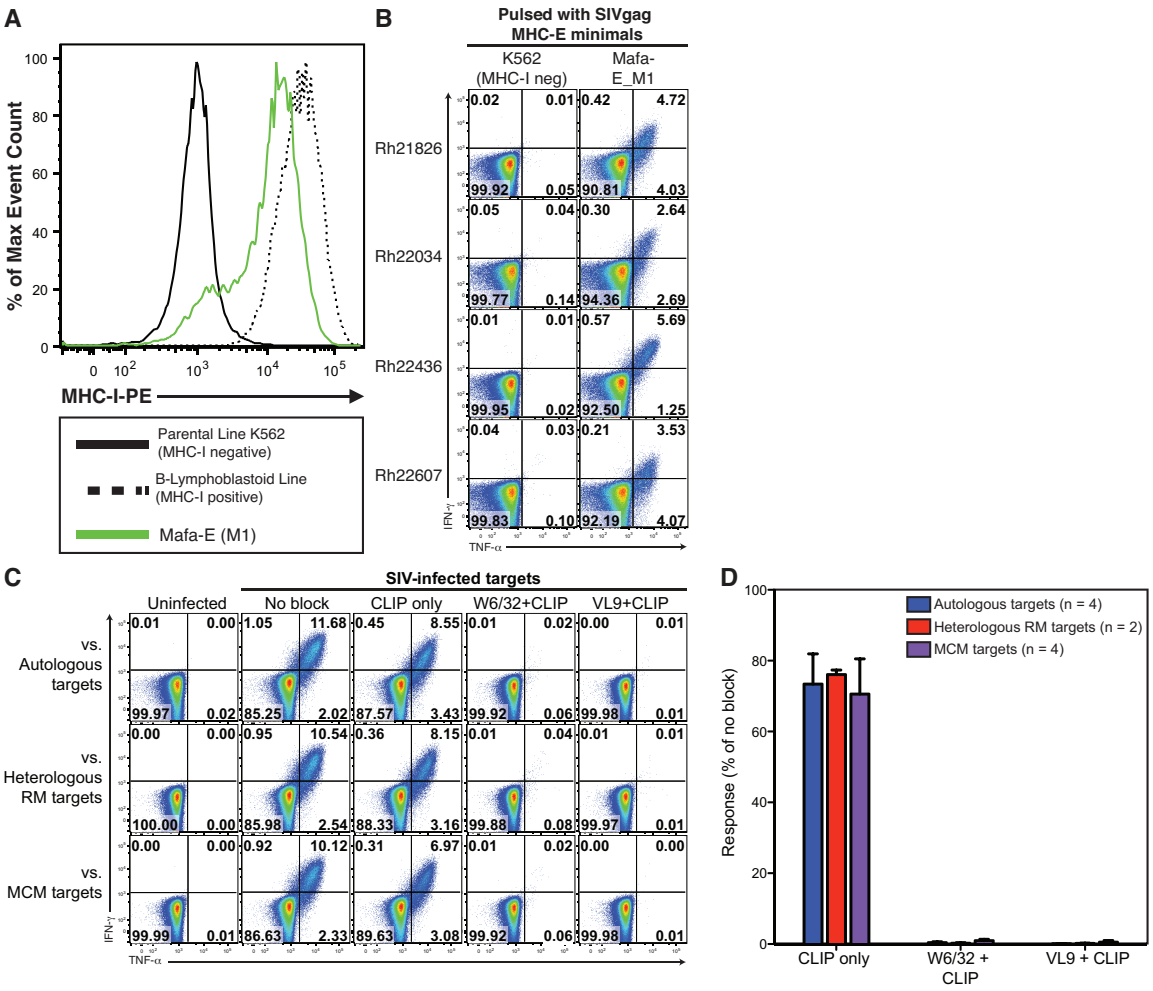


Figure 2.15: Strain 68-1 RhCMV/SIVgag-elicited CD8+ T cells recognize peptide presented by Mauritian cynomolgus macaque MHC-E molecules. (A) The Mafa-E allele expressed from the MCM MHC haplotype M1 was transfected into a parental MHC-I negative K562 cells and stained with pan-MHC-I monoclonal mAb W6/32. MHC-I-expressing B-lymphoblastoid cells (BLCL) served as a positive control, while parental MHC-I negative K562 cells served as a negative control. **(B)** Flow cytometric ICS plots showing CD8+ T cell recognition of peptide presented by Mafa-E. PBMC from 4 strain 68-1 RhCMV/SIVgag-vaccinated rhesus macaques were combined with MHC-I negative parental cell line K562 (negative control) or Mafa-E transfectant from (A) pulsed with a pool of the 11 SIVgag MHC-E-binding minimals (listed in Figure 2.14C), as described in Figure 2.2A. CD8+ T cell recognition was determined by detection of TNF-α and IFN-γ in flow cytometric ICS, using the gating strategy shown in Figure 2.2B. **(C)** Representative flow cytometric ICS plots show SIV-infected CD4+ T cell recognition by CD8β+ T cells isolated from PBMC of a strain 68-1 RhCMV/SIVgag-vaccinated rhesus macaque (Rh21826), performed

as outlined in Figure 2.10A. Plots show CD8β+ T cell recognition of SIV-infected CD4+ T cells derived from the RM (autologous), from a heterologous RM, or from an MCM under the following conditions: alone (no block), in the presence of CLIP alone, in the presence of anti-MHC-I mAb W/632 plus CLIP, or in the presence of VL9 plus CLIP. All plots gated on live, CD3+, CD8+ cells. **(D)** Bar graph shows SIV recognition results from 2-4 strain 68-1/SIVgag-vaccinated rhesus macaques for each type of target. For MCM targets, SIV-infected CD4+ T cells from four different MCM carrying different MHC haplotypes (M1/M1, M2/M3, M1/M5, and M4/M4) were utilized. The following non-autologous combinations were performed: Rh21826 effectors vs. Rh22436 targets, Rh21826 effectors vs. MCM 32843 (M2/M3) targets, Rh22034 effectors vs. MCM 32845 (M1/M5) targets, Rh22436 effectors vs. Rh21826 targets, Rh22436 effectors vs. MCM 32848 (M4/M4) targets, and Rh22607 effectors vs. MCM 32851 (M1/M1). Data represent the percentage of responding TNF-α+IFN-γ+ CD8+ T cells (gated as shown in B), normalized to the response observed with no peptide blocking (mean±SEM).

CHAPTER 3: Discussion and future directions

The restriction and blocking analyses performed in this study demonstrate that vaccination with strain 68-1 RhCMV vectors reliably elicits broadly targeted MHC-E-restricted CD8+ T cells. This is the first description of retrovirus-specific, MHC-E-restricted CD8+ T cells, and the first description of MHC-E-restricted CD8+ T cells in non-human primates. These results explain the unique epitope targeting of the strain 68-1 RhCMV-induced, MHC-I-dependent CD8+ T cell response, and support the existence of a novel mechanism of unconventional CD8+ T cell priming by RhCMV. As MHC-E-restricted CD8+ T cells recognize SIV-infected CD4+ T cells, these cells likely contribute to control of SIV replication observed in strain 68-1 RhCMV/SIV-vaccinated rhesus macaques. In addition, these data provide evidence for functional conservation of MHC-E in primates, including humans, and thus support the use of non-human primates models to study HLA-E-restricted CD8+ T cells. Harnessing pathogen-specific MHC-E-restricted CD8+ T cell responses might be near universally effective against other human infectious diseases, as MHC-E exhibits extremely limited polymorphism in humans and MHC-E surface expression must be preserved to evade NK cells. However, the mechanism of MHC-E-restricted CD8+ T cell priming as well as the protective capacity of MHC-E-restricted CD8+ T cells against HIV/SIV and other pathogens require further investigation.

I. MHC-E restriction explains the unique properties of the strain 68-1 RhCMV-elicited, MHC-I-dependent CD8+ T cell response.

Restriction by MHC-E explains the non-canonical epitope targeting, universal epitope targeting, and wide breadth of the strain 68-1 RhCMV-induced, MHC-I-

dependent CD8+ T cell response. First, while the lack of canonical epitope targeting is attributed to Rh189 (US11), unconventional restriction by MHC-II (previously described) (*393*) and MHC-E (described here) explains the targeting of novel SIV epitopes by strain 68-1 RhCMV-induced CD8+ T cells. As the data presented here constitute the first description of retrovirus-specific, MHC-E-restricted CD8+ T cell responses, no MHC-E-binding CD8+ T cell epitopes of HIV or SIV had been previously described and, consequently, these MHC-E-binding epitopes represent entirely novel retroviral CD8+ T cell epitopes. Further, the nature of the MHC-E-binding epitopes identified here is distinct from that of previously described MHC-E ligands. Under normal cellular conditions, MHC-E binds a limited peptide repertoire composed almost exclusively of peptides derived from the leader sequences of other MHC-I molecules (*599*) (*742*) (*797*), which possess a defined MHC-E-binding motif including hydrophobic anchor residues at positions 2 and 9 (*529*). While MHC-E has been demonstrated to present select bacterial and viral peptides to CD8+ T cells in the context of infection and vaccination, this presentation is typically limited to peptides possessing a similar motif to MHC-E-binding leader peptides (see Table 1.1). However, the data shown here and accompanying data in (*729*) reveal MHC-E can bind a much broader repertoire of peptides that diverge from this canonical motif (Table 3.1). Not only are these novel MHC-E-binding epitopes presented in the context of strain 68-1 RhCMV vaccination, but we also demonstrated that at least some of these MHC-E-binding SIVgag peptides are naturally presented during SIV infection of both rhesus macaque and Mauritian cynomolgus macaque CD4+ T cells (Figures 2.10, 2.11, and 2.15). We, and others, have looked for MHC-E-restricted CD8+ T cells in SIV-infected rhesus macaques and HIV-infected patients, but have thus

far failed to identify such responses. Why CD8+ T cell responses are not normally engendered against these naturally presented MHC-E-binding SIVgag epitopes during SIV infection is unclear, and provides further evidence that strain 68-1 RhCMV vectors alter normal CD8+ T cell priming. This is discussed further in section II below.

Peptide	Sequence	Recognition frequency (%)
$SIVgag_{65\text{-}73}$(16)	APLVPTGSE	44
$SIVgag_{72\text{-}80}$(18)	SENLKSLYN	34
$SIVgag_{92\text{-}100}$(23)	EKVKHTEEA	49
$SIVgag_{120\text{-}128}$(30)	KTSRPTAPS	41
$SIVgag_{132\text{-}140}$(33)	GGNYPVQQI	30
$SIVgag_{201\text{-}209}$(50)	QIIRDIINE	56
$SIVgag_{259\text{-}267}$(65)	VGNIYRRWI	51
$SIVgag_{276\text{-}284}$(69)	RMYNPTNIL	100
$SIVgag_{389\text{-}397}$(97)	KPIKCWNCG	61
$SIVgag_{475\text{-}484}$(119)	LGKQQREKQ	71
$SIVgag_{482\text{-}490}$(120)	EKQRESREK	100

Table 3.1: CD8+ T cell recognition frequency of eleven MHC-E-binding SIVgag optimal 9-mers among strain 68-1 RhCMV/SIVgag-vaccinated rhesus macaques. Table displays the sequence and recognition frequency of eleven SIVgag optimal 9-mers targeted in strain 68-1 RhCMV/SIVgag-vaccinated rhesus macaques (n=42). The mapping of these optimal 9-mers is shown in (*729*).

Second, restriction by a highly conserved MHC molecule such as MHC-E explains the observed universal targeting of the supertopes $SIVgag_{273\text{-}287}$(69) and $SIVgag_{477\text{-}491}$(120), as well as the frequent targeting of other epitopes (Table 3.1) (*729*). This high degree of sequence conservation among MHC-E molecules suggests that every rhesus macaque encodes an MHC-E molecule capable of presenting each of the two supertopes and priming a CD8+ T cell response. Indeed, we demonstrated that three Mamu-E molecules, including one molecule with differences in the predicted peptide

contact residues (Mamu-E*02:20), were each able to present all 12 MHC-E-binding SIVgag peptides to primed strain 68-1 RhCMV-induced CD8+ T cells (Figure 2.13), providing evidence that different Mamu-E molecules are able to present the same SIVgag peptides to CD8+ T cells. However, this prompts the question, why isn't every MHC-E-restricted CD8+ T cell response universal? Similar gaps in priming exist for HLA-E-restricted CD8+ T cell responses in humans, where the diversity of MHC-E is even more limited compared to rhesus macaques (*561*) (*590*) (*583*). The explanation for these observed gaps in priming is unknown, but may reflect differences in antigen presentation or differences in T-cell receptor clonotypes among animals. Another possible explanation is lack of detection of particular responses at the time of epitope identification experiments. Indeed, fluctuations in the magnitude of HCMV epitope-specific CD8+ T cell responses have been previously reported (*674*) (*743*). Finally, computed binding affinities suggest that $SIVgag_{276\text{-}284}$(69) and $SIVgag_{482\text{-}490}$(120) optimal 9-mers possess the highest binding affinities for MHC-E among the eleven MHC-E-binding SIVgag optimal 9-mers tested (*729*), so perhaps the universal priming of these two CD8+ T cell responses reflects their superior binding affinities, which may be significant for priming CD8+ T cell responses, but not for recognition by already-primed CD8+ T cells.

Third, and perhaps most surprisingly, the breadth of the strain 68-1 RhCMV-induced, MHC-I-dependent CD8+ T cell response is explained by the ability of MHC-E to present a highly varied peptide repertoire. A median of 20 SIVgag epitopes are targeted by MHC-E-restricted CD8+ T cells in strain 68-1 RhCMV/SIVgag-vaccinated animals, in comparison to a median of 11 and 14.5 epitopes targeted by MHC-Ia-restricted CD8+ T cells in conventionally vaccinated (MVA, Ad5, and DNA+IL-12) and

SIV-infected controller RMs, respectively (*729*). This breadth of MHC-E-restricted CD8+ T cell responses is impressive considering there is a larger diversity of MHC-Ia molecules expressed in each individual RM compared to MHC-E molecules (Figure 2.1), and appears to result from the surprisingly diverse array of peptides bound by MHC-E molecules. Indeed, 87% of the overlapping 15-mer peptides spanning SIVgag were targeted by MHC-E-restricted CD8+ T cells in at least one of the 42 vaccinated RMs tested here. Of note, the breadth of antigen-specific MHC-E-restricted CD8+ T cells is accompanied by an even wider breadth of MHC-II-restricted CD8+ T cells specific for the same antigen that are also engendered in these vaccinated animals (*393*).

The unique patterns of epitope targeting and MHC-E/II-restriction of strain 68-1 RhCMV-induced CD8+ T cell response are not limited SIVgag, as animals vaccinated with vectors encoding SIV pol and *Mtb* antigens mount antigen-specific CD8+ T cell responses of similar breadth and restriction patterns, including universal targeting of supertopes (*729*). In addition, RhCMV IE1-directed CD8+ T cells in RhCMV seronegative rhesus macaques vaccinated with these vectors also share these characteristics (*729*), suggesting this unique strain 68-1 RhCMV-induced CD8+ T cell targeting also applies to RhCMV antigens in the vector backbone. The significance of these unique properties of strain 68-1 RhCMV-elicited CD8+ T cells is discussed below in section III.

II. Priming of MHC-E-restricted CD8+ T cells by strain 68-1 RhCMV

While there are descriptions of MHC-E-restricted CD8+ T cell responses specific for epitopes of a few bacterial and viral pathogens, there are no descriptions of an MHC-E-restricted CD8+ T cell response with the wide breadth and diverse nature of targeted

epitopes exhibited by the strain 68-1 RhCMV-induced CD8+ T cell response described here. Thus, the mechanism behind the consistent priming of broadly targeted MHC-E-restricted CD8+ T cell responses by strain 68-1 RhCMV vectors likely constitutes a novel mechanism of CD8+ T cell priming.

The confirmed presentation of SIVgag epitopes on MHC-E during SIV infection (Figures 2.10, 2.11 and 2.15) raises the question of why this antigen presentation fails to prime MHC-E-restricted CD8+ T cell responses during SIV infection (Figure 2.8 and 2.10). This may reflect differences in the levels of antigen presented on MHC-E in SIV-infected cells versus RhCMV-infected cells, whereby levels of MHC-E antigen presentation on SIV-infected cells are insufficient to prime CD8+ T cell responses (*744*). Or, perhaps the relatively low level of MHC-E surface expression compared to that of MHC-Ia molecules normally favors priming of MHC-Ia-restricted CD8+ T cell responses in lieu of those restricted by MHC-E. In any case, this phenomenon suggests that priming of MHC-E-restricted CD8+ T cells is precluded under most conditions, but strain 68-1 RhCMV vectors are able to circumvent this preventative mechanism. Importantly, strain 68-1 RhCMV lacks expression of a number viral genes, including the Rh157.5 and Rh157.4 genes that encode RhCMV homologues of HCMV UL128 and UL130. Our collaborators previously demonstrated that natural RhCMV infection as well as vaccination with RhCMV/SIV vectors based on an alternative strain of RhCMV, called strain 68-1.2, which contains intact expression of Rh157.5 and Rh157.4, results in a CD8+ T cell response distinct from that of strain 68-1 (*393*). This response is more narrowly targeted, completely lacks supertope responses, and is entirely MHC-I-dependent (*393*). In conjuction with the data presented here, our collaborators confirmed

that this shift in the CD8+ T cell response is mediated by Rh157.5 and Rh157.4 (*729*). Vaccination with strain 68-1.2 RhCMV/SIVgag in which Rh157.5 (UL128) and Rh157.4 (UL130) have been specifically deleted completely alters the induced CD8+ T cell response to one similar to strain 68-1 vaccination, characterized by wide breadth, supertope targeting, and MHC-E- and MHC-II-restricted responses. Conversely, vaccination with strain 68-1 RhCMV/SIVgag in which expression of Rh157.5 (UL128) and Rh157.4 (UL130) has been repaired, results in a CD8+ T cell response indistinguishable from that of strain 68-1.2, a more narrowly targeted response lacking supertope-specific and MHC-E- and MHC-II-restricted CD8+ T cells. Thus, generation of both MHC-E- and MHC-II-restricted CD8+ T cell responses by RhCMV vectors only occurs in the absence of Rh157.5 (UL128) and Rh157.4 (UL130), suggesting RhCMV naturally primes unconventional CD8+ T cell responses in the absence of interference by these two viral proteins. RhCMV 157.5 and Rh157.4 genes encode homologues of HCMV UL128 and UL130, two components of the pentameric receptor complex involved in CMV infection of non-fibroblast cells (*721*). In addition, HCMV UL128 exhibits chemokine activity (*745*). The specific functions of RhCMV 157.5 (UL128) and Rh157.4 (UL130) that prevent the generation of MHC-E- and MHC-II-restricted CD8+ T cells require further investigation, but could involve either of these previously ascribed functions or an entirely novel function.

The inherent features of RhCMV infection that promote priming of unconventional MHC-E- and MHC-II-restricted CD8+ T cell responses are also incompletely characterized. However, studies of RhCMV vector vaccination and CMV biology to-date implicate particular aspects of CMV infection in MHC-E-restricted CD8+

T cell priming. First, the ability of RhCMV 157.5 (UL128) and Rh157.4 (UL130), members of a receptor complex that facilitates RhCMV entry, to abolish priming of these unconventional CD8+ T cell responses suggests virus cellular tropism may be critical. Of note, pathogens that elicit HLA-E-restricted CD8+ T cells are all able to infect myeloid-lineage cells (*746*) (*557*) (*747*) (*748*) (*749*) (*750*) (*751*). Thus, the ability of strain 68-1 RhCMV to infect myeloid-lineage cells may be critical in priming MHC-E-restricted CD8+ T cells. Second, both HCMV and RhCMV encode viral mimics of canonical MHC-E-binding VL9 peptides within the UL40 and Rh67 genes, respectively, a strategy employed to up-regulate surface MHC-E/VL9 and evade NK cell recognition (*807*) (*567*) (*566*) (*546*) (*548*). The demonstrated interplay between this virally encoded VL9 peptide and MHC-E suggests this peptide may also facilitate MHC-E-restricted CD8+ T cell priming. High levels of Rh67-derived VL9 peptide might stabilize MHC-E molecules and facilitate delivery to a compartment rich in RhCMV vector-encoded peptides where peptide exchange could occur, effectively enhancing antigen presentation on MHC-E and allowing for priming of MHC-E-restricted CD8+ T cells. Indeed, in conjunction with the data presented here, our collaborators provided evidence that HLA-E maintains a relatively rigid peptide binding groove that remains open in the absence of peptide (*729*). Thus, such a peptide exchange mechanism, similar to that observed with CLIP and MHC-II in endosomal compartments (*752*), is plausible for VL9 and MHC-E. In further support of this, in macrophages, the majority of newly synthesized HLA-E molecules traffic to intracellular autophagy-lysosomal vesicles, rather than the cell surface (*753*). A third aspect of CMV infection that might allow for priming of MHC-E-restricted CD8+ T cells is the formation of the virion assembly complex. HCMV-encoded microRNAs facilitate

restructuring of the Golgi apparatus and formation of a virion assembly compartment, or VAC, in the cytoplasm of infected cells (*644*) (*643*) (*754*) (*755*), where host proteins from the Golgi, ER, and endosomes accumulate along with viral glycoproteins and tegument proteins (*644*) (*754*) (*756*). Thus, VAC formation might facilitate interactions between MHC-E present in secretory networks and RhCMV vector-encoded antigenic peptides, and ultimately enable the priming of antigen-specific MHC-E-restricted CD8+ T cell responses.

The idea of peptide-loading on MHC-E in the VAC is similar to the demonstrated loading of *Mtb*-derived peptides on HLA-E in the *Mtb* phagosome, where HLA-E and other members of the antigen presentation machinery are enriched following *Mtb* infection (*600*). In this case, newly synthesized HLA-E is not required from presentation of *Mtb* antigen on HLA-E and thus peptide loading in the phagosome likely involves recycled HLA-E. This type of presentation appears to facilitate priming of MHC-E-restricted CD8+ T cell responses, as a large number of *Mtb*-specific CD8+ T cell responses are restricted by MHC-E (Table 1.1) (*583*) (*583*) (*585*). This supports a hypothesis that the nature of antigen presentation normally precludes the generation of MHC-E-restricted CD8+ T cells, but infection with certain pathogens, such as *Mtb* and CMV, alters the antigen presentation environment in a way that facilitates priming of MHC-E-restricted CD8+ T cells. Additional evidence in support of this hypothesis are reports of defects in antigen presentation machinery shifting the repertoire of peptides presented by Qa-1b and HLA-E from predominantly MHC-I leader sequence-derived peptides to diverse, alternative peptide ligands that do not uniformly exhibit the canonical MHC-E-binding motif (*757*) (*582*) (*543*) (*544*). Indeed, the CD8+ T cell response to

TAP-deficient tumors is dominated by Qa-1b-restricted CD8+ T cells, and vaccination with DCs expressing the potent TAP inhibitor UL49.5 from Bovine herpesvirus-1 protect mice against TAP-deficient tumor challenge (*796*) (*757*) (*758*). Further, in addition to *Mtb* and CMV, many other pathogens demonstrated to elicit MHC-E-restricted CD8+ T cells have been reported to alter MHC-I antigen presentation in some way (*759*) (*760*) (*761*) (*762*) (*763*) (*568*) (*569*) (*570*, *696*) (*571*) (*572*) (*573*) (*764*). For example, Epstein Barr virus encodes a TAP inhibitor, BNFL2a, and a mediator of MHC-I degradation, BILF1 (*765*) (*766*), similar to CMV. Thus, modifications in antigen presentation created upon infection may allow for the generation of MHC-E-restricted CD8+ T cell responses.

Interesting, the CMV-derived VL9 peptide can be targeted by HLA-E-restricted CD8+ T cells in cases where the infecting strain of HCMV encodes a VL9 peptide that does not match any of the VL9 leader-derived peptides encoded by the individual's endogenous MHC-I alleles. The existence of UL40-derived VL9-specific HLA-E-restricted CD8+ T cell responses in some HCMV-infected patients in face of UL128/UL130 expression by wildtype HCMV is contrary to the ability of RhCMV UL128/UL130 homologues to abolish priming of MHC-E-restricted CD8+ T cells in rhesus macaques. One explanation is that priming of this particular VL9-specific MHC-E-restricted CD8+ T cell response occurs by a different mechanism than that of RhCMV-elicited CD8+ T cells, specifically one that is unaffected by expression of UL128/130. Another explanation is UL128/130 of HCMV function differently than Rh157.5 and Rh157.4 of RhCMV, and thus the UL128/UL130 homologue-mediated inhibition of MHC-E-restricted CD8+ T cell priming observed in rhesus macaques does not occur in HCMV infection of humans. However, this seems unlikely as priming of additional

HCMV-specific, HLA-E-restricted CD8+ T cell responses would be expected, but no other HLA-E-restricted CD8+ T cell responses have been identified in HCMV infection. In rhesus macaques, the most common MHC-I leader sequence-derived VL9 peptide is VMAPRTLLL, and this is identical to the peptide encoded by Rh67 of RhCMV strain 68-1. Thus, most, if not all, rhesus macaques encode a self VL9 peptide identical to the Rh67 VL9 mimic, and accordingly none of the rhesus macaques studied here mounted a response to VMAPRTLLL during blocking experiments. Infection of rhesus macaques with RhCMV strains possessing mutated Rh67-derived VL9 sequences, mismatched from the endogenous MHC-I-derived leader sequences in the animal, would assess whether VL9-directed MHC-E-restricted CD8+ T cell responses can be engendered in the presence of UL128/UL130 homologues in rhesus macaques, as occurs in humans. However, this particular VL9-targeted CD8+ T cell response may be a unique case of MHC-E-restricted CD8+ T cell priming distinct from that of broadly targeted MHC-E-restricted CD8+ T cell responses engendered by strain 68-1 RhCMV.

III. The role of MHC-E-restricted CD8+ T cells in strain 68-1 RhCMV/SIV-mediated control of SIV

While there is no direct evidence that MHC-E-restricted CD8+ T cells, specifically, confer control of SIV replication, these responses constitute one of only two CD8+ T cell subsets elicited by protective strain 68-1 RhCMV vector vaccination, the other being MHC-II-restricted CD8+ T cell responses. SIV challenge of rhesus macaques vaccinated with strain 68-1.2 RhCMV vectors, which do not elicit MHC-E- or MHC-II-restricted CD8+ T cell responses, will assess whether unconventional restriction of SIV-specific CD8+ T cells is critical to viral control conferred by strain 68-1 RhCMV vector

vaccination. If unconventionally restricted CD8+ T cells are critical for SIV control after challenge, further experiments will be necessary to assess whether both subsets of unconventionally restricted CD8+ T cells (MHC-E-restricted and MHC-II-restricted), or just one, are critical in this regard. In addition, the mechanism by which this unconventional restriction endows RhCMV vector-elicited CD8+ T cell responses with the ability to control SIV replication would require further investigation.

Key to elucidating the mechanism of protection mediated by unconventionally restricted CD8+ T cells is a comprehensive characterization of the function and antiviral capacity of strain 68-1 RhCMV-elicited, SIV-specific CD8+ T cells. Our collaborators previously demonstrated that the MHC-I-dependent SIVgag supertope-specific CD8+ T cells, shown here to be MHC-E-restricted, are polyfunctional upon activation, producing cytokines TNF-α, IFN-γ, and MIP-1β and externalizing CD107 (*393*). However, additional functions of strain 68-1 RhCMV-induced MHC-E-restricted CD8+ T cells, such as direct cytolytic action against SIV-infected cells, require further investigation. Most previously identified pathogen-specific HLA-E-restricted CD8+ T cells, such as those specific for *Salmonella enterica*, *Mycobacterium tuberculosis*, Epstein Barr virus, HCMV, and HCV, have been demonstrated to lyse target cells, secrete IFN-γ and granzyme B, and proliferate upon antigen recognition (*563*) (*536*) (*555*) (*558*) (*794*) (*557*) (*583*) (*558*) (*559*) (*560*) (*561*). However, recent characterization of *Mtb*-specific HLA-E-restricted CD8+ T cell clones show that some clones lack the ability to produce IFN-γ and instead possess an alternative, regulatory phenotype. While the former cytolytic phenotype is probably more applicable to strain 68-1 RhCMV-induced MHC-E-restricted CD8+ T cells due to their consistent induction of IFN-γ and externalization of CD107

upon antigen recognition, alternative functions of these cells cannot be excluded at this time.

While comprehensive characterization of the function and antiviral capacity of strain 68-1 RhCMV-induced MHC-E-restricted CD8+ T cells has not been performed, an SIV-specific immune response mediated by MHC-E-restricted CD8+ T cells offers potential advantages over that mediated by classical MHC-Ia-restricted CD8+ T cells. First, MHC-E is resistant to Nef-mediated downregulation by both HIV and SIV, in contrast to other MHC-Ia molecules (*767*) (*332*); this selectivity protects HIV- and SIV-infected cells from NK cell-mediated lysis (*739*) (*549*). Thus, while Nef-mediated MHC-I-downregulation prevents some MHC-Ia-restricted CD8+ T cell responses from effectively lysing SIV-infected cells (*330*) (*738*) (*190*) (*191*) (*192*) (*193*), MHC-E-restricted CD8+ T cells would be unaffected. In fact, MHC-E is actually up-regulated on the surface of both HIV- and SIV-infected CD4+ T cells (*549*) (Figure 2.12). Shang *et al.* also observed up-regulation of MHC-E in the female reproductive tract of rhesus macaques within 24 hours of intra-vaginal SIV infection, particularly in Langerhaans cells, myeloid-derived dendritic cells, within the genital epithelium (*768*). While the mechanism behind surface MHC-E up-regulation during HIV/SIV infection is unclear, it may reflect the ability of MHC-E-binding HIV/SIV epitopes to stabilize MHC-E surface expression, a phenomenon observed for VL9 and other MHC-E binding peptides, see section III. However, Shang *et al.* also found up-regulation of MHC-E mRNA transcripts, suggesting a separate, perhaps additional, mechanism of MHC-E up-regulation (*768*). In any case, up-regulation of MHC-E on the surface of SIV-infected cells suggests that

MHC-E-restricted CD8+ T cells may be superior to MHC-Ia-restricted CD8+ T cells in recognizing infected cells.

A second potential advantage lies in the ability of MHC-E to present a broad range of diverse epitopes. Strain 68-1 RhCMV-elicited MHC-E-restricted CD8+ T cell responses exhibit wide breadth, targeting an average of 4 epitopes per 100 amino acids of SIVgag and 4.4 epitopes per 100 amino acids of SIV 5' pol (*729*). Indeed, the breadth of MHC-E-restricted, SIVgag-specific CD8+ T cell responses alone exceeds the breadth of the entire SIV-gag-specific CD8+ T cell response engendered upon SIV infection or Ad5-, MVA-, or DNA+IL-12-based vaccination (*729*). Wider breadth of epitope targeting increases the probability that mounted virus-specific CD8+ T cell responses will target conserved epitopes, where escape would incur at a high fitness cost to the virus, as well as target at least one epitope of diverse viral strains, including quasispecies that develop within the infected individual. Furthermore, breadth hampers the ability of the virus to escape the mounted immune response. Indeed, broadly targeted HIV-specific CD8+ T cell responses are associated with control of viral replication (*403*) (*411*) (*412*). In order to exert antiviral functions and contribute to *in vivo* virus control, antigen-specific CD8+ T cells must recognize naturally processed antigen on the surface of SIV-infected cells. While it is unknown whether the entire breadth of MHC-E-binding epitopes described here are naturally presented during SIV infection, we provide evidence here that at least some are (Figures 2.10, 2.11, 2.15), and thus at least some of the MHC-E-restricted CD8+ T cell responses identified here are capable of participating in immune responses against the virus.

Importantly, while all strain 68-1 RhCMV/SIV-vaccinated rhesus macaques develop broadly targeted MHC-E-restricted CD8+ T cell responses, only ~50% control SIV upon challenge. This disparity suggests that, while MHC-E-restricted CD8+ T cells might be critical to control of SIV, restriction by MHC-E alone is insufficient to confer vaccine efficacy. As viruses like HIV/SIV replicate and establish systemic infection very quickly, immediate control of viral replication by effector memory CD8+ T cell responses is likely critical in RhCMV vector efficacy. Thus, stochastic variations in the magnitude and breadth of effector memory CD8+ T cell responses in the tissues at the time of challenge might plausibly explain the lack of universal efficacy among rhesus macaques. In addition, a unique feature of RhCMV vector-mediated control is eventual viral clearance, constituting the first report of vaccine-mediated clearance of HIV or SIV. This clearance likely partially reflects the ability of RhCMV-elicited CD8+ T cells to quickly control viral replication and reduce seeding of viral reservoirs, as these reservoirs are smaller in RhCMV/SIV controller animals compared to animals on ART (*450*). However, does clearance also reflect a unique ability of RhCMV-elicited CD8+ T cells to eliminate viral reservoirs, and, if so, is unconventional restriction by MHC-E and MHC-II paramount to this ability? One intriguing possibility lies in the immense breadth of the strain 68-1 RhCMV-induced, SIV-specific CD8+ T cell response, mediated by both MHC-E- and MHC-II-restricted CD8+ T cells. In HIV-1 infection, the vast majority of latent viruses carry CTL escape mutations that they acquire early in infection, which renders them resistant to later elimination. Perhaps, the strain 68-1 RhCMV-induced, SIV-specific CD8+ T cell responses are so broad and effective, that the virus is unable to escape or is only able to escape a small subset of responses. In this case, the persistent

RhCMV-induced, SIV-specific CD8+ T cell responses might be sufficiently broad as to recognize and eliminate any reactivated latent virus.

IV. MHC-E peptide presentation to CD8+ T cells is conserved across primate species

In this study, we demonstrate the ability of MHC-E from three primate species, human, rhesus macaque, and Mauritian cynomolgus macaque, to present a shared peptide repertoire to MHC-E-restricted CD8+ T cells (Figures 2.3, 2.4, 2.13, 2.14, 2.15). Recognition by MHC-E-restricted CD8+ T cells reflects two properties of MHC-E: (1) the ability to bind peptide ligand, and (2) the ability of MHC-E/peptide complexes to ligate T-cell receptors on MHC-E-restricted CD8+ T cells. While MHC-E molecules might differ in their affinities for a particular peptide, peptide dose titration experiments demonstrate near identical presentation of 9-mer optimal peptides by Mamu-E*02:04, Mamu-E*02:20, and HLA-E*01:03 from peptide doses of 10uM to 1nM (Figure 2.14). Mamu-E*02:04 and Mamu-E*02:20 appear able to effectively present some SIVgag 9-mers at a 10-fold lower dose (0.1nM) compared to HLA-E*01:03, suggesting a slightly enhanced binding affinity of these MHC-E molecules for particular 9-mers. Among MHC-E molecules, Mamu-E*02:20 and HLA-E*01:03 are most divergent from other MHC-E molecules in the α1 and α2 regions that form the peptide-binding groove (Figure 1.2). In particular, while most human, rhesus, and MCM MHC-E molecules are completely conserved at the predicted peptide-binding residues, Mamu-E*02:20 differs at 5 of these positions (Figure 1.1). However, Mamu-E*02:20 and HLA-E*01:03 are each able to present the same peptides as Mamu-E*02:04 and Mamu-E*02:11.

There may be subtle differences in the ability of various MHC-E molecules to prime CD8+ T cell responses to the same peptide. However, if this were the case, we might expect a broader MHC-E-restricted CD8+ T cell response in rhesus macaques expressing a greater number of MHC-E molecules. This trend is not observed in the subset of strain 68-1 RhCMV/SIVgag-vaccinated rhesus macaques that have been typed for MHC-E molecules (Figure 3.1).

MHC-E-restricted CD8+ T cell recognition of peptide across MHC-E molecules not only indicates functional conservation among primate MHC-E molecules in the presentation of peptides, but also demonstrates the promiscuity of strain 68-1 RhCMV-elicited, Mamu-E-restricted CD8+ T cells. For example, the only MHC-E molecule expressed in Rh22436 is Mamu-E*02:04 (Figure 2.1), and consequently all of its MHC-E-restricted CD8+ T cell responses must be primed by Mamu-E*02:04-presented epitopes. However, these Mamu-E*02:04-restricted CD8+ T cells recognize SIVgag peptides presented by Mamu-E*02:11, Mamu-E*02:20, HLA-E*01:03, Mafa-E expressed from the M1 MCM haplotype, and at least one Mafa-E expressed from the M4 MCM haplotype (Figures 2.4, 2.13, 2.14, and 2.15). This suggests that the T-cell receptors of strain 68-1 RhCMV-induced, MHC-E-restricted CD8+ T cells do not discriminate between different MHC-E molecules, at least at the stage of antigen recognition. Whether particular MHC-E molecules are more likely to prime CD8+ T cells with particular T-cell receptor clonotypes requires further investigation.

The high level of functional conservation in peptide presentation among Mamu-E molecules likely allows for the observed frequent, sometimes universal, CD8+ T cell targeting of MHC-E-binding epitopes among strain 68-1 RhCMV-vaccinated rhesus

macaques, regardless of MHC genotype (*393*) (*729*). This frequency of epitope targeting among individuals far exceeds that observed for MHC-Ia molecules (*393*) (*729*), suggesting that eliciting MHC-E-restricted CD8+ T cells might make T cell-based vaccines more effective in a larger percentage of individuals. Importantly, MHC-E is even more conserved in the human population, with only 2 HLA-E alleles that differ by a single amino acid, and thus MHC-E-binding epitopes might be even more frequently targeted in humans than in rhesus macaques. While few studies have screened patients for particular epitope-specific HLA-E-restricted CD8+ T cell responses, an HLA-E-restricted CD8+ T cell response to the HCV core epitope YLLPRRGPRL is detected in 40% of chronically infected HCV patients (*561*). Similarly, an HLA-E-restricted CD8+ T cell response to the *Mtb* Rv2997 epitope RMPPLGHEL is detected in 30% of PPD-responding donors (*583*).

This report contains the first description of MHC-E-restricted CD8+ T cells in non-human primates. There are very few studies describing MHC-E function in non-human primates, and thus it is unknown whether macaque MHC-E molecules possess functions different from that of HLA-E. However, the data presented here provide evidence that, at least in antigen presentation to primed CD8+ T cells, MHC-E function appears conserved across primate species.

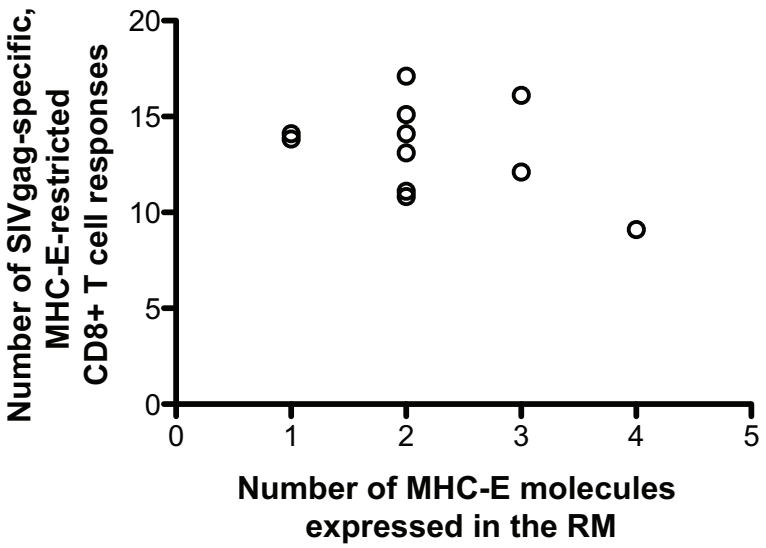


Figure 3.1: The number of MHC-E molecules expressed in an individual RM does not correlate with the number of primed SIVgag-specific, MHC-E-restricted CD8+ T cell responses primed. Graph displays 11 strain 68-1 RhCMV/SIVgag-vaccinated rhesus macaques. MHC-E molecules were identified by deep sequencing. Cases where amplicon sequences could not distinguish between multiple MHC-E molecules (see Figure 2.1) were counted as one MHC-E molecule. The number of primed SIVgag-specific, MHC-E-restricted CD8+ T cell responses is based on the minimal number of independent epitopes (see Figure 2.8 and Methods).

V. Implications for vaccine design

This study demonstrates that MHC-E-restricted CD8+ T cells constitute a large proportion of the CD8+ T cell response engendered upon strain 68-1 RhCMV vector vaccination. Vaccination with strain 68-1 RhCMV/SIV vectors stringently controls viral replication in ~50% of challenged rhesus macaques. Thus, vaccine strategies eliciting unconventional MHC-E- and MHC-II-restricted CD8+ T cells represent an attractive new approach to vaccine development, for both HIV and other pathogens.

Successful mobilization of broadly targeted MHC-E-restricted CD8+ T cells in an HIV vaccine would offer numerous advantages. First, as described above, MHC-E-restricted CD8+ T cell responses would likely remain effective in the face of Nef-mediated MHC-I-downregulation, in contrast to MHC-Ia-restricted CD8+ T cells. Second, the wide breadth of MHC-E-restricted CD8+ T cells increases the probability that vaccine-elicited CD8+ T cells would target diverse viral strains and conserved epitopes, and decreases the likelihood of viral escape. Third, MHC-E-restricted CD8+ T cell response have not been identified in the context of HIV and SIV infection, and SIV-specific MHC-E-restricted CD8+ T cells target an entirely distinct set of epitopes from CD8+ T cell responses elicited during SIV infection. Thus, HIV/SIV have not evolved under immune pressure from MHC-E-restricted CD8+ T cells, and thus vaccine-elicited MHC-E-restricted CD8+ T cells would likely be effective against transmitted escape variants (*769*) (*311*) (*770*).

The finding that broadly targeted MHC-E-restricted CD8+ T cells are engendered against every tested vaccine insert to-date, including SIV, *Mtb*, and RhCMV antigens, suggests that the binding repertoire of MHC-E is much more diverse than previously

thought. Consequently, MHC-E is likely able to present epitopes from diverse pathogen proteins, and under the correct conditions, prime CD8+ T cell responses. Thus, vaccines mobilizing MHC-E-restricted CD8+ T cells may also be effective for other pathogens. In particular, many pathogens other than HIV/SIV interfere with MHC-I antigen processing and presentation to evade pathogen-specific CD8+ T cell responses (*771*). However, if this mechanism interferes with leader peptide-mediated stabilization of surface MHC-E, the infected cell becomes susceptible to NK cell-mediated killing. Thus, similar to HIV/SIV, many of these pathogens have also evolved mechanisms to preserve or increase MHC-E surface expression, see section III. However, the inevitable result of simultaneous evasion of both CD8+ T cell- and NK cell-mediated immune responses by this mechanism is that infected cells are rendered susceptible to MHC-E-restricted CD8+ T cells. Thus, for these pathogens, vaccine-elicited MHC-E-restricted CD8+ T cells might be particularly effective.

VI. Future directions

Future studies of strain 68-1 RhCMV-induced, MHC-E-restricted CD8+ T cells should define their contribution to SIV control, particularly relative to MHC-II-restricted CD8+ T cells. Indeed, vectors that elicit MHC-II-, but not MHC-E-, restricted CD8+ T cells have been identified, and challenge studies of rhesus macaques vaccinated with these vectors will assess whether MHC-E-restricted CD8+ T cells are critical to vaccine efficacy. In addition, comprehensive functional characterization of MHC-E-restricted CD8+ T cells *ex vivo* would be valuable in assessing how these cells influence the course of SIV infection. Such analyses would include assessing MHC-E-restricted CD8+ T cells for the ability to suppress viral replication, kill SIV-infected target cells, and proliferate

in response to antigen, perhaps performed in comparison to MHC-II-restricted CD8+ T cells or control MHC-Ia-restricted CD8+ T elicited by SIV infection or conventional vaccination. This type of characterization requires isolation of MHC-E-restricted CD8+ T cells from bulk PBMC, which can be achieved with tetramer reagents or other capture assays that are currently under development. In addition to functional characterization, sequencing the T-cell receptors expressed by individual epitope-specific MHC-E-restricted CD8+ T cells may provide an explanation to the promiscuity of MHC-E-restricted CD8+ T cells as well as additional clues about antiviral efficacy. In particular, we are interested in whether strain 68-1 RhCMV-elicited MHC-E-restricted CD8+ T cell responses use public TCR clonotypes. A public clonotype is a TCR amino acid sequence specific for a particular epitope/MHC that is found in more than one individual (*772*) (*773*). Usage of these public clonotypes by antigen-specific CD8+ T cells is associated with control of both HIV and SIV infection (*808*) (*809*) (*414*) (*774*). For example, the immunodominant Mamu-A1*001:01-restricted CD8+ T cell response targeting $Gag_{181-189}$(CM9), a conserved epitope, is associated with control of SIVmac239 in rhesus macaques. However, when the CD8+ T cells targeting this epitope used TCRs with a limited public repertoire, they failed to control replication following viral escape (*787*). Thus, knowledge of the TCR sequence of MHC-E-restricted CD8+ T cells among rhesus macaques might provide an explanation for the 50% efficacy rate observed with strain 68-1 RhCMV vector vaccination. Finally, direct sequencing of ligands eluted from MHC-E in SIV-infected cells (*775*) would facilitate identification of the particular SIV epitope-specific MHC-E-restricted CD8+ T cell responses that are capable of participating in immune responses against the virus.

As eliciting pathogen-specific MHC-E-restricted CD8+ T cell responses may benefit vaccine strategies for HIV as well as other pathogens, future studies should also focus on elucidating the mechanism of MHC-E-restricted CD8+ T cell priming by RhCMV. This can be achieved by using virus vectors with specific deletions and by studying the functions of viral genes implicated in this process, such as Rh67 and Rh157.5/Rh157.4. Such studies may lead to new vaccine strategies capable of eliciting MHC-E-restricted CD8+ T cells, for example other vectors expressing the required RhCMV genes along with vaccine antigens.

More broadly, the data presented here lay the foundation for studies of basic MHC-E biology, including antigen presentation on MHC-E and how MHC-E is influenced by infection with various pathogens. Eluting and sequencing ligands from MHC-E molecules may identify more subtle differences in peptide binding repertoire among various primate MHC-E molecules and would assess changes in the peptide repertoire of MHC-E during infection, for example with CMV or HIV/SIV. In addition, the pathway of antigen presentation on MHC-E requires further investigation, and the MHC-E-restricted CD8+ T cells identified here could aid these studies by serving as a functional readout for epitope presentation on MHC-E after blocking various cellular processes and antigen presentation pathways, for example with inhibitors of TAP or the proteasome. Indeed, such studies would likely assist in elucidating the mechanism of MHC-E-restricted CD8+ T cell priming by RhCMV.

Finally, key aspects of MHC-E immunobiology, including NK cell and T cell biology, have not been investigated in non-human primates. For example, the relationship between MHC-E and NKG2A+ NK cells observed in humans has not been demonstrated

in any non-human primate species. However, HLA-E/leader peptide tetramers stain rhesus NK cells (*575*), suggesting the role of MHC-E in NK cell regulation is conserved in rhesus macaques. Of note, we have verified an HLA-E-specific antibody, clone 4D12, that cross-reacts with Mamu-E and Mafa-E (Figure 2.12 and data not shown). With this verified antibody, we can perform a comprehensive assessment of MHC-E expression on different cellular subsets in rhesus macaques and cynomolgus macaques, without complications from non-specific staining observed previously (*777*). In addition, this MHC-E-specific antibody can detect changes in MHC-E surface expression during infection (Figure 2.12), and thus could identify pathogens for which pathogen-specific, MHC-E-restricted CD8+ T cell responses might be effective.

Developing an additional animal model for CMV-based vaccine studies would also be advantageous. In particular, CMV vaccination of humans will involve HCMV, rather than RhCMV, vaccine vectors, as primate CMVs exhibit exquisite species specificity. Differences between HCMV and RhCMV vector backbones, or inherent host differences between humans and rhesus macaques, could translate into differences in vaccine-elicited CD8+ T cells and ultimately vaccine efficacy. Here, we show that the presentation of CD8+ T cell epitopes by MHC-E is conserved in Mauritian cynomolgus macaques. Of note, we have isolated the CMV strains naturally infecting MCMs (CyCMV), and cloned one of these as a bacterial artificial chromosome, which can serve as a backbone for creating CyCMV vaccine vectors analogous to strain 68-1 RhCMV vectors. Thus, a model of CMV vector vaccination in Mauritian cynomolgus macaques is currently being established. This model will assess the ability of CMV vectors to elicit unconventional MHC-E- and MHC-II- restricted CD8+ T cells, and ultimately mediate

control of SIV, in another primate species, particularly one where the diversity of MHC-E more closely mirrors that of humans (Figure 1.1).

VII. Summary and conclusions

In summary, we have shown that strain 68-1 RhCMV/SIVgag vector vaccination elicits MHC-E-restricted CD8+ T cell responses targeting a broad set of diverse SIVgag epitopes. These MHC-E-restricted CD8+ T cell responses constitute the entire MHC-I-dependent CD8+ T cell response in vaccinated rhesus macaques. In addition, some SIVgag epitope-specific, MHC-E-restricted CD8+ T cell responses are frequently, sometimes universally, engendered among vaccinated rhesus macaques. SIVgag-specific, MHC-E-restricted CD8+ T cells possess a conventional CD8α/β+, TCRγ/δ-, NKG2A/C- T cell phenotype and are able to recognize naturally processed antigen presented on the surface of SIV-infected CD4+ T cells. In contrast to bulk MHC-I, MHC-E is up-regulated on the surface of SIV-infected CD4+ T cells. Finally, SIVgag-specific, MHC-E-restricted CD8+ T cells from vaccinated rhesus macaques are able to recognize peptide presented across rhesus macaque, human, and Mauritian cynomolgus macaque MHC-E molecules.

These results demonstrate that broadly targeted pathogen-specific, MHC-E-restricted CD8+ T cell responses can be reliably elicited by strain 68-1 RhCMV vaccine vectors, and suggest strain 68-1 RhCMV possesses a unique mechanism to facilitate priming of MHC-E-restricted CD8+ T cell. SIVgag-specific, MHC-E-restricted CD8+ T cells likely participate in immune responses against SIV in challenged vaccinees, as at least some SIV epitopes are presented on MHC-E during CD4+ T cell infection. In addition, these MHC-E-restricted CD8+ T cell responses will likely retain the ability to recognize infected cells in the face of Nef-mediated MHC-I down-regulation. Finally,

MHC-E peptide presentation to CD8+ T cells is conserved across primate species, supporting the possibility that CMV-based vaccine vectors will elicit MHC-E-restricted CD8+ T cells in humans. As MHC-E surface expression must be preserved to evade NK cell-mediated killing, pathogen-specific MHC-E-restricted CD8+ T cell responses may be effective against HIV and other pathogens well versed in immune evasion.

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Appendix 1: Identification and spontaneous immune targeting of an endogenous retrovirus K envelope protein in the Indian rhesus macaque model of human disease

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Author contributions

The author wrote the manuscript and performed the experiments shown in Figures 4, 5b, S4, and S5 as well as replicates of experiments shown in Figure 2C, 2E, 2F, and 3.

I. The problem and scientific approach

Endogenous retroviruses (ERVs) comprise approximately 8% of the human genome. While the vast majority of ERVs no longer possess coding capacity for the production of viral mRNAs and proteins, some ERV open reading frames (ORFs) remain intact (*1*) (*2*). Human ERV (HERV) activity is normally repressed in healthy tissues, however, activation of HERV ORF transcription and protein production is detected in numerous developmental and pathological contexts, including embryogenesis, pregnancy, neoplasia, autoimmunity, neurodegeneration, and viral infection (*3*) (*4*) (*5*) (*6*) (*7*) (*8*) (*9*) (*10*). In addition, HERV-specific cellular and humoral immunity has been reported in humans, particularly in the context of exogenous retroviral infection with human immunodeficiency virus (HIV) (*10*) (*11*) (*12*) (*13*). However, ERV activity and ERV-specific immune responses remain poorly defined in the physiologically relevant rhesus macaque model frequently used to study human reproduction, development, neurology, and infectious disease. Therefore, we investigated here if rhesus macaques harbor a functional equivalent of HERV-K (HML-2).

II. Materials and methods

Rhesus macaques

A total of 82 purpose-bred male or female rhesus macaques (RM) (*Macaca mulatta*) of Indian genetic background were used in this study including 34 SIV-infected RM, 46 SIV-naïve RM, and 2 RM with time points taken before and after SIV infection. Rhesus macaques were housed at the Wisconsin National Primate Research Center, New England Primate Research Center, or the Oregon National Primate Research Center. All protocols were approved by the respective Institutional Animal Care and Use Committees, under

the standards of the US National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Identification of SERV-K1

We used HERV-K consensus sequences (*14*) to search the rhesus macaque genome for the simian orthologue of ERV-K. Sequence analysis was conducted using MacVector (Cary, NC, USA) and Geneious (Auckland, New Zealand) software.

mRNA capture

Several micrograms of mRNA were purified from BLCL utilizing the Qiagen (Valencia, CA, USA) RNeasy kit and Oligotex RNA kit according to the manufacturer's protocols. cDNA libraries were made from this mRNA with the Invitrogen (Carlsbad, CA, USA) Superscript II cDNA synthesis kit according to the manufacturer's protocol. The cDNA was ligated into the pCMVsport vector and transformed into stbl4 electromax cells from Invitrogen (Carlsbad, CA, USA). Greater than 2×10^5 colonies were plated out from the transformation and were physically scrapped off the plates to make a plasmid prep of the cDNA library. Probes of approximately 50nt in length were designed based on conserved sequences found in an alignment of multiple known ERV. The following probes were ordered from IDT (Coralville, IA, USA) as 5′ biotinylated probes that had been HPLC purified:

5′CAGCTAYGGCTGCTGTAGCAGGAGTTGCATTGCACTCTTCTGTTCAGTC3′ (envelope sequence),

5′GCTGCCAATCCTCCAGTTAACATAGATGCAGATCAACTATTAGGAATAG3′ (gag sequence), and

5′CACTATTATTAACATATACTTCAATAGGATTATCCATCCATGTAACTGCC3′

(envelope sequence). The cDNA library was enriched for the target molecules utilizing the biotinylated probes and the RecA affinity capture method of Zhumabayeva et al. (*15*, *16*). Specifically, the buffer described by Zhumabayeva et al. was mixed with aqueous Adenosine 5′-[γ-thiotriphosphate tetralithium salt (stored at −80 in small aliquots in a 2:1 ratio with ATP) from Sigma-Aldrich (St. Louis, MO, USA) and 50ng of probe and 2µg of RecA protein in a 30µl reaction volume. This was incubated at 37°C for 15 min. Then 5µg of cDNA library was added and a further 37°C incubation of 20 min was done. The RecA was then inactivated by adding 10 % SDS and Proteinase K as described by Zhumabayeva et al. The Proteinase K was incubated for 10 min at 37°C. Finally, the Proteinase K was inactivated by adding 1 µl of 100 mM PMSF. Once this was thoroughly mixed, 20 µl of M-270 Dynal paramagnetic streptavidin beads from Invitrogen (Carlsbad, CA, USA) that were previously washed twice with a binding buffer consisting of 10 mM TrisHCL, 1 M NaCl, and 1 mM EDTA were added to the reaction. This was gently mixed by hand for 30 min at room temperature. The beads were washed three times with 10 mM TrisHCl, 2 M NaCl, and 1 mM EDTA wash buffer to remove unbound plasmid. A last wash was done with pure water at 37°C. The beads were resuspended in 50 µl of TE buffer and extracted with an equal volume of phenol:chloroform:isoamyl alcohol. The aqueous phase was ethanol precipitated and resuspended in 10 µl of TE of which 0.5 µl was used for heat shock transformation of stbl3 cells from Invitrogen (Carlsbad, CA, USA). The resulting colonies were plasmid purified and Sanger sequenced to screen for the target sequences. The resulting clones are typically enriched for target molecule to better than 50 %, although the percentages can vary widely. The non-target molecules are generally random in their nature and probably represent non-specific sticking to the

streptavidin beads. Sequence analysis was conducted using MacVector (Cary, NC, USA) and Geneious (Auckland, New Zealand) software. Accesssion numbers for captured SERV-K1 Env mRNA are KU363810 and KU363811.

T cell assays

ELISPOT screening was carried out as previous described (*17*) using 15-mer peptides (with 11 amino acid overlap) spanning the entire SERV-K1 Env open reading frame or the entire SIVmac239 proteome. Intracellular cytokine staining assays were carried out as previously described (*18*) (*17*). Positive responses shown were confirmed using peptides synthesized by three independent sources.

Antibody detection

Heat-inactivated serum was diluted 1:1000 and used in a peptide-based ELISA assay, as previously described (*19*), against overlapping 15-mer peptides (with 11 amino acid overlap) spanning either the entire SERV-K1 Env open reading frame or the entire SIVmac239 Env open reading frame. ELISA was conducted in duplicate, using absorbance at 450 nm to determine binding antibodies.

Serological tests for antibodies

Intuitive Biosciences performed serology assays. Individual serum samples were screened for presence of antibodies to a panel of viral pathogens using CSA: Simian Expanded Array and CSA: Simian Detection Kit (Intuitive Biosciences, Madison WI), following the standard manufacturer's protocol. Briefly, samples were diluted 1:100 in the supplied Sample Dilution Buffer and incubated on the CSA: Simian Expanded Array for 1 hour at room temperature. Each sample well was washed five times with Wash Buffer, and a 1:5000 dilution of anti-Simian IgG in Sample Dilution Buffer was added to

each well and incubated at room temperature for 1 h. After washing fives times with Wash Buffer, a 1:100 dilution of gold conjugate reagent in gold conjugated diluent was added to each well and incubated at room temperature for 45 min. After five repeat washes with Wash Buffer, each well was rinsed using 1× rinse buffer. SilverQuant reagent A and B were quickly mixed and added to each well, incubating for 3 min while protected from light. Each sample well was quickly rinsed with ultrapure water several times, and the arrays dried with nitrogen gas at approximately 80 psi. Each array was scanned and analyzed using the AthenaQuant System (Intuitive Biosciences). A report was generated with intensity of each spot recorded by antigen as a mean of five replicate spots, in relative intensity units. Cut off values as specified by the manufacturer were used to determine positive and negative designations for each sample. When a sample generates significant intensity of signal on the array, this indicates that the animal is seropositive to the virus represented by the antigen on the array. This does not detect presence of virus, merely presence of specific IgG to the virus, which is indicative of previous exposure or latent and/or subclinical infection.

III. Results

A. Identification of a spliced SERV-K1 Env mRNA

To assess whether a biologically active ERV might exist in the genome of Indian rhesus macaques, we focused on HERV-K (HML-2), the youngest and most active of all ERVs in the human genome (*20*). To this end, we used consensus HERV-K sequences to scan the rhesus macaque genome, where we identified three proviral insertion sites on chromosomes 5, 11, and 12. The proviral insertion on chromosome 12 exhibited the greatest sequence homology to HERV-K, with 89 % nucleotide homology to HERV-K

Gag, Pro, Pol, and Env open reading frames; we termed this provirus simian ERV-K1 (SERV-K1) (Appendix 1 Figure S1). While the Pro and Pol reading frames contained frameshift mutations leading to premature stop codons, the Gag and Env ORFs remained intact with the potential to code for full length proteins (Appendix 1 Figure 1A). To assess whether the SERV-K1 provirus could produce protein-coding mRNA transcripts, we used mRNA capture to generate a cDNA library from a macaque in which we detected a large SERV-K1 Env T cell response (r02120, see below). The cDNA library from r02120 contained a spliced mRNA transcript coding for the SERV-K1 Env protein possessing canonical structural features of a retroviral Env protein, including a signal leader peptide (L), a RX(K/R)R consensus cleavage site separating the surface (SU) and transmembrane (TM) envelope subunits, a fusion domain, an immunosuppressive domain, and a transmembrane anchor domain (Appendix 1 Figure 1B, C; Figure S2). Phylogenetic analysis confirmed that SERV-K1 is part of a simian 'sister' lineage of the HERV-K (HML-2) lineage that expanded in the hominid germline (Appendix 1 Figure 1D; Figure S3). As HERV-K HML2 Env and SERV-K1 Env share >90% sequence homology, it is likely that, as described for HERV-K Env in humans (*20*), SERV-K1 Env retains functional activity in rhesus macaques, and thus may serve as a target for immune responses.

B. SERV-K1 Env-specific T cell responses are rare, but can be high frequency

Because SERV-K1 Env retained the ability to code for functional protein, we next assessed whether this activity in rhesus macaques was sufficient to trigger SERV-K1 Env-specific immune responses. To this end, we generated a set of overlapping 15-mer peptides spanning the SERV-K1 Env protein sequence and performed IFN-γ ELISPOT

using PBMC from both SIV-infected and uninfected rhesus macaques. We selected SIV-infected macaques due to the well-documented association between HIV-1 infection and increased HERV activity (*21*). Although we screened 29 chronically SIV-infected rhesus macaques (16 progressors and 13 elite controllers), 4 SIVΔNef vaccinated rhesus macaques, and 26 SIV-naïve rhesus macaques we identified only one rhesus macaque mounting T cell responses against SERV-K1 Env (Appendix 1 Figure 2A). As mentioned above, this SIV-infected progressor, r02120, was the same macaque from which we had captured the SERV-K1 Env-encoding mRNA, providing further evidence that SERV-K1 was transcriptionally active in this animal.

Although SERV-K1 Env-specific T cells were only rarely detected in our cohort of macaques via IFN-γ ELISPOT, we identified one response in r02120 targeting SERV-K1 $Env_{526–540}$ LL15 with surprisingly high magnitude (Appendix 1 Figure 2B). Given that impurities in commercially prepared peptides can result in false positive responses in IFN-γ ELISPOT (*22*) (*23*), we confirmed this high magnitude T cell response using SERV-K1 $Env_{526–540}$ LL15 peptide synthesized by three independent sources (data not shown). Intracellular cytokine staining (ICS) revealed that this high frequency response was CD4+ T cell mediated (Appendix 1 Figure 2C). Strikingly, the SERV-K1 $Env_{526–540}$ LL15-specific CD4+ T cell response was larger in magnitude than the entire SIVmac239-specific CD4+ T cell response in r02120 (Appendix 1 Figure 2D), and could be detected in ELISPOT at concentrations of 1 μM (Appendix 1 Figure S4). Further analysis of this unusual CD4+ T cell response revealed that SERV-K1 $Env_{526–540}$ LL15-specific CD4+ T cells were uniformly effector memory in character (Appendix 1 Figure 2E), suggesting continual exposure to low levels of antigen. SERV-K1 $Env_{526–540}$ LL15-specific CD4 + T

cells were also highly polyfunctional, as evidenced by secretion of cytokines (TNF-α and IFN-γ) and chemokine (MIP-1β) and degranulation (measured by CD107a) (Appendix 1 Figure 2F). The effector memory phenotype coupled with the high frequency of responding T cells is reminiscent of the phenomenon of "memory inflation", which is seen with chronic pathogens such as herpes viruses (*24*).

In addition to the large SERV-K1 $Env_{526–540}$ LL15- specific CD4+ T cell response, r02120 also mounted a CD8+ T cell response to SERV-K1 $Env_{108–115}$ PA8 (Appendix 1 Figure 3A). Similar to the CD4+ T cell response, SERV-K1 $Env_{108–115}$ PA8-specific CD8+ T cells could be detected in IFN-γ ELISPOT at concentrations of 100 nM (Appendix 1 Figure S4) and were uniformly effector memory in phenotype (Appendix 1 Figure 3B). Thus, while SERV-K1 Env-directed T cell responses are not frequent in SIV-infected rhesus macaques, rare, high frequency, responses can be detected, suggesting that SERV-K1 is active in at least a subset of these animals.

C. SERV-K1 Env-specific antibodies are common in rhesus macaques

As we had captured an mRNA encoding SERV-K1 Env with all the structural elements of a functional envelope protein, and detected both CD8+ and CD4+ T cells targeting this protein in r02120, we asked whether SERV-K1 Env-specific antibody responses would be present in this animal. To assess this, and to identify the immunogenic domains of SERV-K1 Env, we performed a peptide-based ELISA assay to screen for antibodies directed to SIVmac239 Env and SERV-K1 Env in rhesus macaque r02120. As anticipated, we detected robust antibody responses to SIVmac239 Env (Appendix 1 Figure 4A). However, we also observed robust antibody responses against SERV- K1 Env in the serum of this macaque (Appendix 1 Figure 4B). As the sequence

homology between these two envelopes is low (~19 % amino acid homology), the SERV-K1 Env-specific antibodies detected are unlikely the result of cross-reactive SIVmac239 Env-specific antibodies. Furthermore, we found similar antibody targeting of SERV-K1 Env in 3 additional SIV-infected and 2 SIV-naïve rhesus macaques (Appendix 1 Figure 4B). Interestingly, one of the highly targeted regions in this macaque, SERV-K1 $Env_{541–555}$ TM, is fully conserved with HERV-K Env and a major humoral target in HIV-infected humans (*10*). Focusing on six frequently targeted SERV-K1 15-mer peptides, we screened an additional 21 SIV-naïve rhesus macaques, and found consistent antibody targeting of these six SERV-K1 15-mer peptides in all animals tested (Appendix 1 Figure 4C). These data demonstrate that the observed SERV-K1 Env antibody responses represent genuine targeting of SERV-K1 Env and not cross-reactive SIV-specific antibody responses. Thus, SERV-K1 Env protein expression is sufficient to commonly trigger SERV-K1 Env-directed antibody responses in rhesus macaques.

D. Viral infection history does not explain the presence of ERV-specific T cell responses in r02120

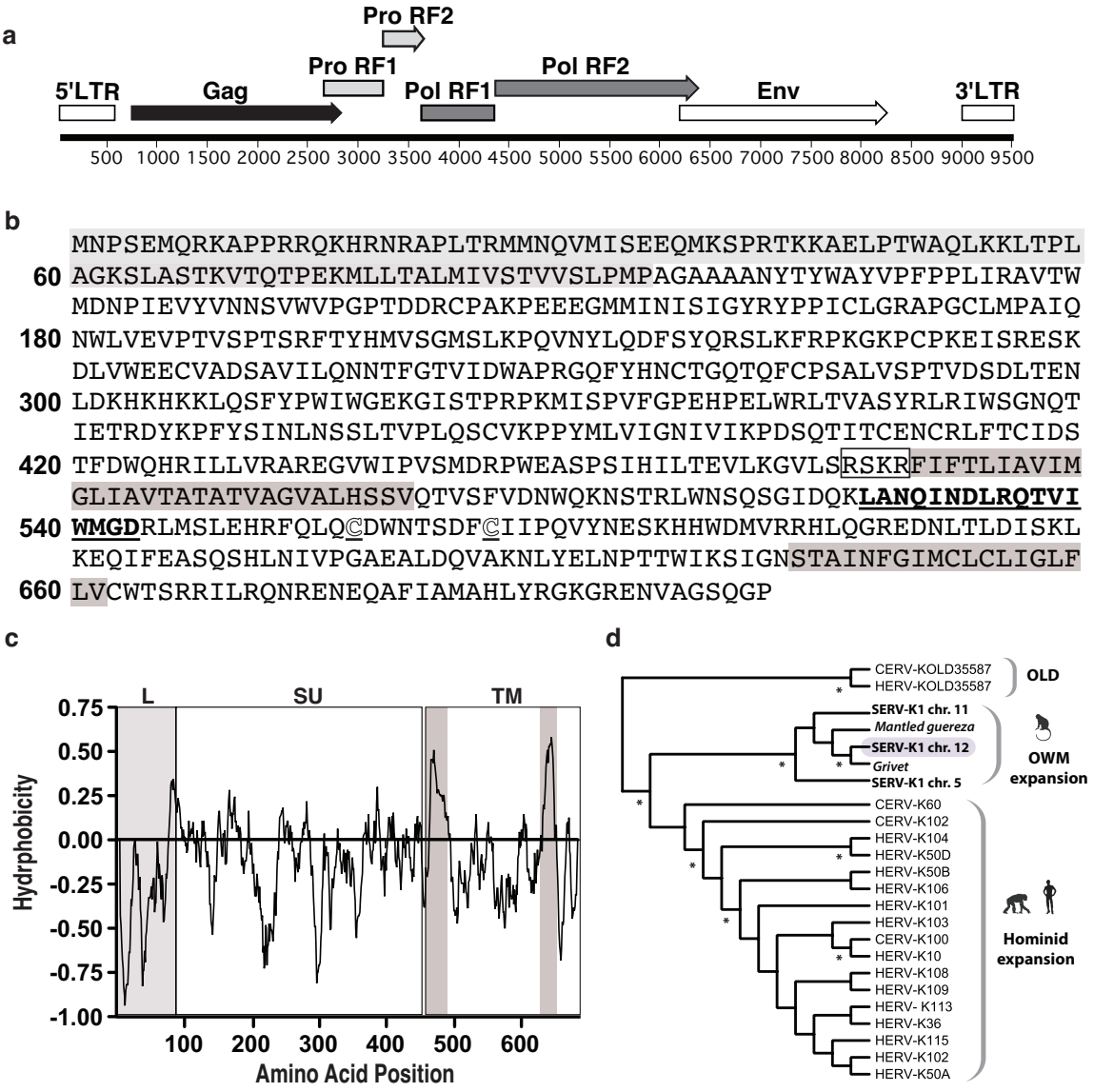
Previous studies reported that infection with viruses such as HSV-1, HIV-1, and cytomegalovirus resulted in an increase in ERV expression in human cells in vitro (*25*) (*26*). To investigate whether the viral infection history of r02120 accounted for its ability to mount SERV-K1 Env-specific T cell responses, we screened a cohort of 24 rhesus macaques, including r02120, for previous viral infections by plasma serology. The pattern of antibody detection against various retroviruses (Simian T lymphotropic virus, Simian retrovirus types one and five, Simian foamy virus), herpes viruses (Herpes B virus, Cytomegalovirus, Lymphocryptovirus, Rhesus rhadinovirus), and paramyxoviruses

(Measles virus) did not reveal any correlation between previous viral infections and the presence of SERV-K1-specific immune responses (Appendix 1 Figure 5A). In order to specifically examine whether the observed T cell recognition of SERV-K1 Env LL15 and PA8 in r02120 was the result of cross-reactive SIV-specific T cell responses, we attempted to align these SERV-K1 Env epitopes to SIVmac239 Env and did not find any SIVmac239 Env peptides with greater than 33 and 38 % identity to SERV-K1 LL15 and PA8, respectively (Appendix 1 Figure S5). Furthermore, longitudinal analysis of both the SERV-K1 $Env_{526–540}$ LL15-specific CD4+ T cell response and $Env_{109–115}$ PA8-specific CD8+ T cell response in r02120 revealed that these responses were present prior to SIV infection, in contrast to a $Vif_{97–104}$ WY8-specific CD8+ T cell response that only arose following SIV infection (Appendix 1 Figure 5B). Thus, the LL15- and PA8-specific T cell responses in r02120 likely represent genuine targeting of SERV-K1 Env and not cross-reactive SIV-specific T cell responses. This is in line with a previous report indicating lack of SIV-induced up-regulation of SERV activity in the cells of rhesus macaques (*18*). Thus, viral infection status alone does not predict SERV-K1-specific T cell immunity.

IV. Discussion

In conclusion, these data suggest that, although ERVs have become endogenous self-antigens, ERV activity can generate specific immune responses. The mechanisms by which this ERV-specific immunity is induced and how tolerance to ERV antigens, if any, is overcome, require further investigation. However, these data contribute to the growing body of evidence that ERVs are not inert genomic fossils, but rather represent dynamic

protein-coding products that impact developmental, pathological, and immunological processes.

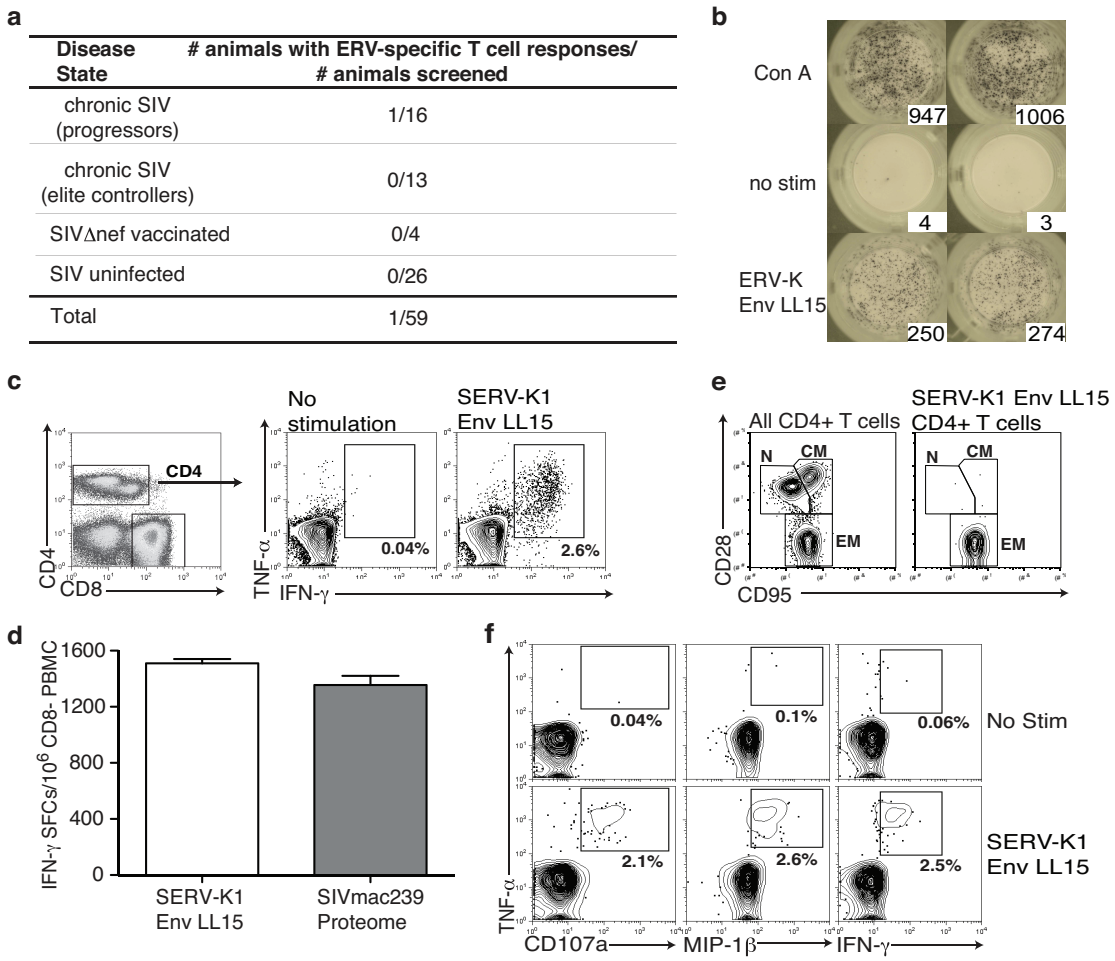


Appendix 1 Figure 1: Identification of macque ERV-K provirus and a fully-spliced SERV-K1 Env mRNA. **A)** Using human ERV-K sequences to search the rhesus macaque genome for ERV proviral insertions, we identified a proviral insertion of SERV-K1 on macaque chromosome 12. Genomic organization of this SERV-K1 provirus is shown. **B)** Using mRNA capture, we identified a fully-spliced mRNA encoding the SERV-K1 Env protein. Predicted protein translation of the captured mRNA is shown with canonical Env structural features: leader sequence (light grey), R-X-K/R-R cleavage site (boxed), fusion domain (grey highlight), immunosuppressive domain (bolded and underlined), conserved cysteine residues (white, underlined Cs), and transmembrane anchor domain (grey highlight). **C)** Hydrophobicity plot of SERV-K1 Env protein with features highlighted as in part B. **D)** Phylogram showing the

relationship of the simian ERVs examined here to previously characterized HERV-K HML-2 insertions in chimpanzees and humans. The phylogram was generated using maximum likelihood as implemented in PHYML (*27*) with 1000 bootstrap replicates. The tree is rooted on HERV-K(OLD) pro- viruses previously determined to be ancestors of the HML-2 lineage in Old World primates (*28*). The macaque locus examined here is indicated by background shading. Asterisks indicate nodes that were recovered in >95 % of bootstrap trees. OWM old world monkey, Chr chromosome.

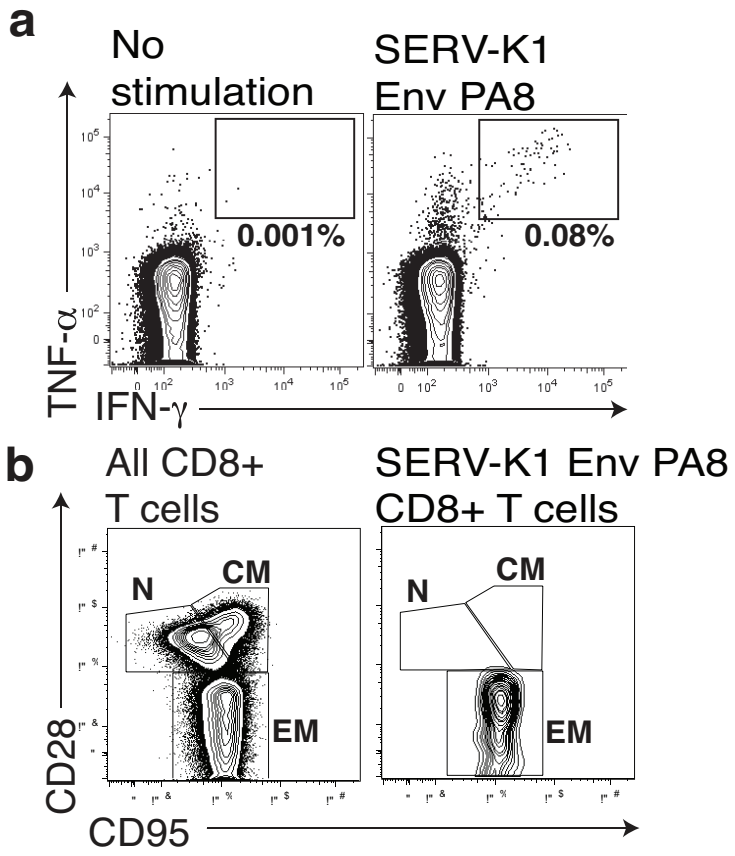
a

Disease State	# animals with ERV-specific T cell responses/ # animals screened
chronic SIV (progressors)	1/16
chronic SIV (elite controllers)	0/13
SIVΔnef vaccinated	0/4
SIV uninfected	0/26
Total	1/59

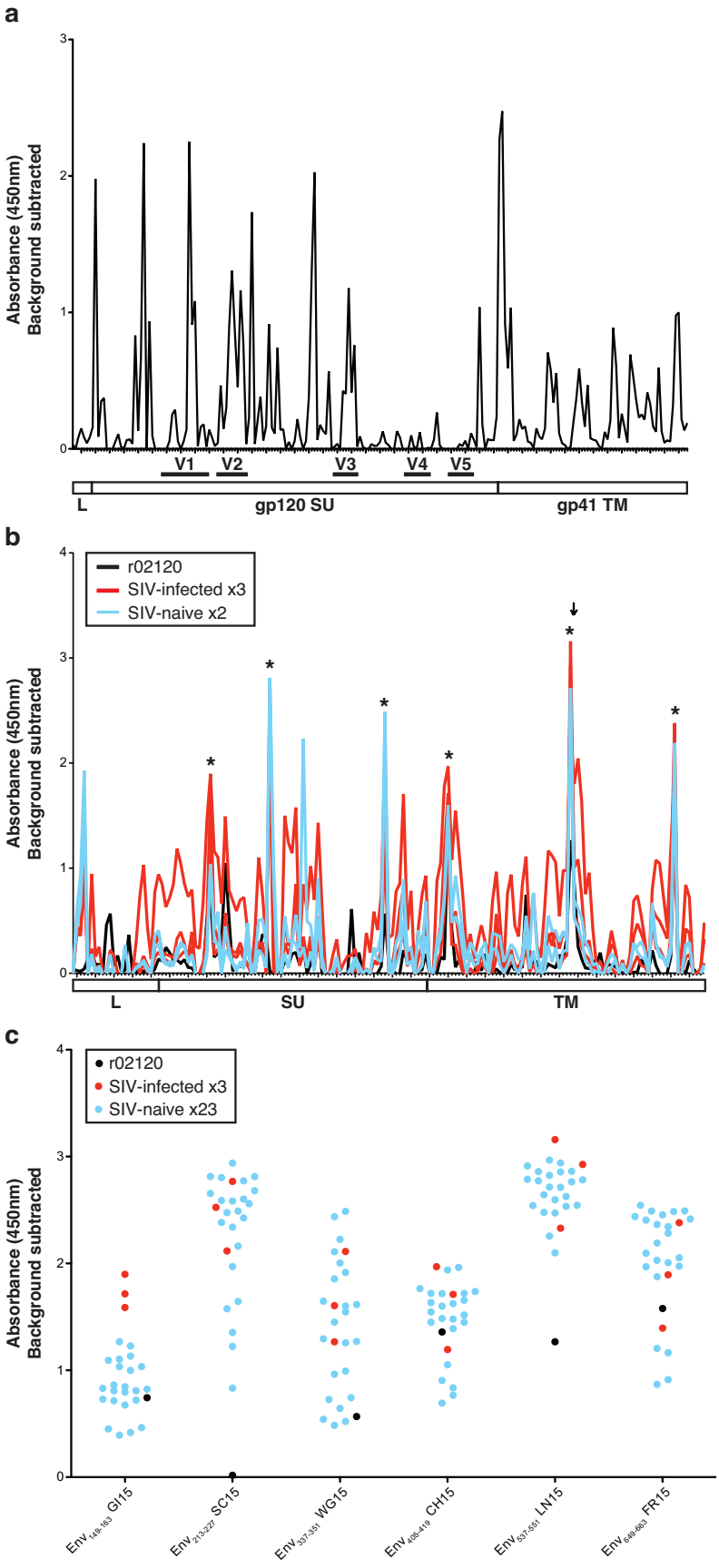


Appendix 1 Figure 2: SERV-K1 Env-specific T cell responses are rare, but can be high frequency. A) Fifty-nine Indian rhesus macaques were screened for SERV-K1 Env T cell responses using overlapping 15-mer peptides spanning SERV-K1 Env in IFN-γ ELISPOT. Table shows summarized results. **B)** Raw IFN-γ ELISPOT images of SERV-K1 Env LL15-specific T cell response identified in r02120 performed in duplicate alongside positive control (Concanavalin A, Con A) and negative control (no stimulation, no stim) wells. **C)** Intracellular cytokine staining (ICS) flow plots showing TNF-α and IFN-γ cytokine induction in SERV-K1 Env LL15-responding T cells. Gating scheme shown on left displays the CD3+ cell population, demonstrating that the SERV-K1 Env LL15-specific T cell response is CD4+ T cell-mediated. **D)** ELISPOT was performed on CD8-depleted PBMC from r02120 stimulating with either SERV-K1 Env LL15 peptide, or a pool of overlapping 15-mer peptides spanning the entire SIVmac239 proteome. Graphs display summed responses performed in duplicate. SFCs = IFN-γ spot forming cells. **E)** Flow plots display memory subset staining of either bulk CD4+ T cells (left) or SERV-K1 Env LL15-responding CD4+ T

cells (right, gated by TNF-α/IFN-γ induction shown in part C. Memory subsets are labeled as follows: N naïve, CM central memory, EM effector memory. Note SERV-K1 Env LL15-specific T cells are uniformly effector memory in phenotype. **F)** ICS flow plots showing functionality of SERV-K1 Env LL15-specific CD4+ T cells based on induction of TNF-α in conjuction with IFN-γ, MIP-1β, and CD107a staining. Plots are gated on CD3+CD4+ as in part C. Note frequency of response of double positive cells is similar among plots, suggesting LL15-specific CD4+ T cells are polyfunctional. Of interest, screening of two half-siblings of r02120's dam and four siblings of r02120's sire revealed no SERV-K1 Env-specific T cell responses.



Appendix 1 Figure 3: SERV-K1 Env-specific CD8+ T cell response. A) ICS flow plots showing TNF-α and IFN-γ cytokine induction in SERV-K1 Env PA8-responding T cells. **B)** Flow plots display memory subset staining of either bulk CD8+ T cells (left) or SERV-K1 Env PA8-responding CD8+ T cells (right, gated by TNF-α/IFN-γ induction shown in part A. Memory subsets are labeled as follows: N naïve, CM central memory, EM effector memory. Note SERV-K1 Env PA8-specific T cells are uniformly effector memory in phenotype.


a
Absorbance (450nm)
Background subtracted
V1
V2
V3
V4
V5
L
gp120 SU
gp41 TM
b
r02120
SIV-infected x3
SIV-naive x2
Absorbance (450nm)
Background subtracted
L
SU
TM
c
r02120
SIV-infected x3
SIV-naive x23
Absorbance (450nm)
Background subtracted
Env148-163 GI15
Env213-227 SC15
Env337-351 WG15
Env405-419 CH15
Env537-551 LN15
Env649-663 FR15

Appendix 1 Figure 4: SERV-K1 Env-specific antibodies are common in rhesus macaques. A) Overlapping 15-mer peptides spanning SIVmac239 Env were used in a peptide-based ELISA for detection of specific antibodies in the serum of r02120. **B)** Overlapping 15-mer peptides spanning SERV-K1 Env were used in a peptide-based ELISA for detection of specific antibodies in the serum of 02120 (black) as well as the plasma of 3 additional SIV-infected (red) and 2 SIV-naïve RMs (blue). Arrow indicates SERV-K1 $Env_{541–555}$ 15-mer, which is an epitope targeted in HIV-infected patients. Peptides indicated with asterisks (*) are shown in part C. Graphs in A, B indicate the major subunits and/or variable loops (V1–V5) of the Env proteins. **C)** ELISA detection of antibody responses to six frequently targeted SERV-K1 15-mer peptides in plasma from 21 SIV-naive rhesus macaques (blue). SIV-infected rhesus macaques from part B are shown for reference. All graphs display the average of two duplicate wells, background subtracted.

a

Animal ID	STLV	SRV5	SRV1	Measles	Herpes B	HSV-1	CMV	LCV	RRV	SFV
r02120							+	+		
r94048					+	+	+	+		+
r99002							+	+	+	+
r03011							+	+	+	+
r99055							+	+		+
r07046							+	+		
r07045				+			+	+	+	
r07039								+		
r07047								+		
r01016							+	+	+	+
r05033								+		
r99080							+	+	+	+
r99047							+	+		+
r01061										+
r06004							+	+		+
r07015							+	+		+
r90132					+	+	+		+	+
r03159							+	+	+	+
r99037							+	+	+	+
rh1999							+		+	+
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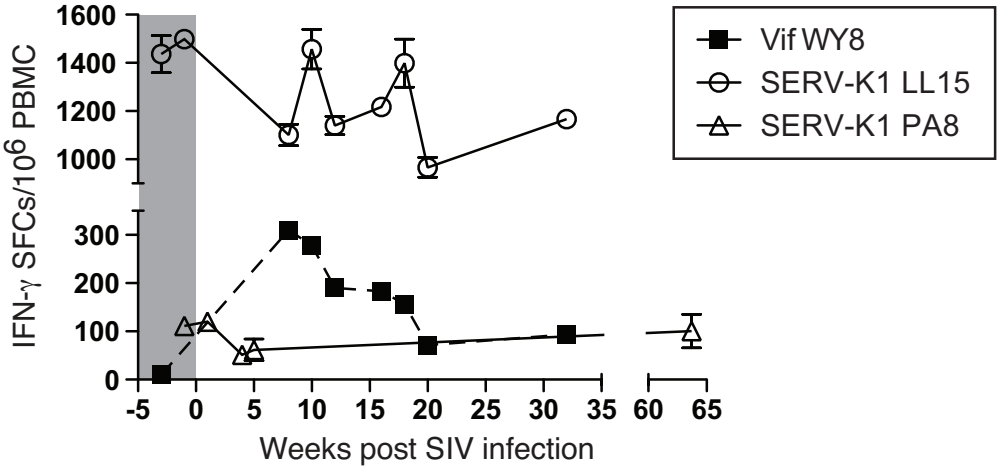
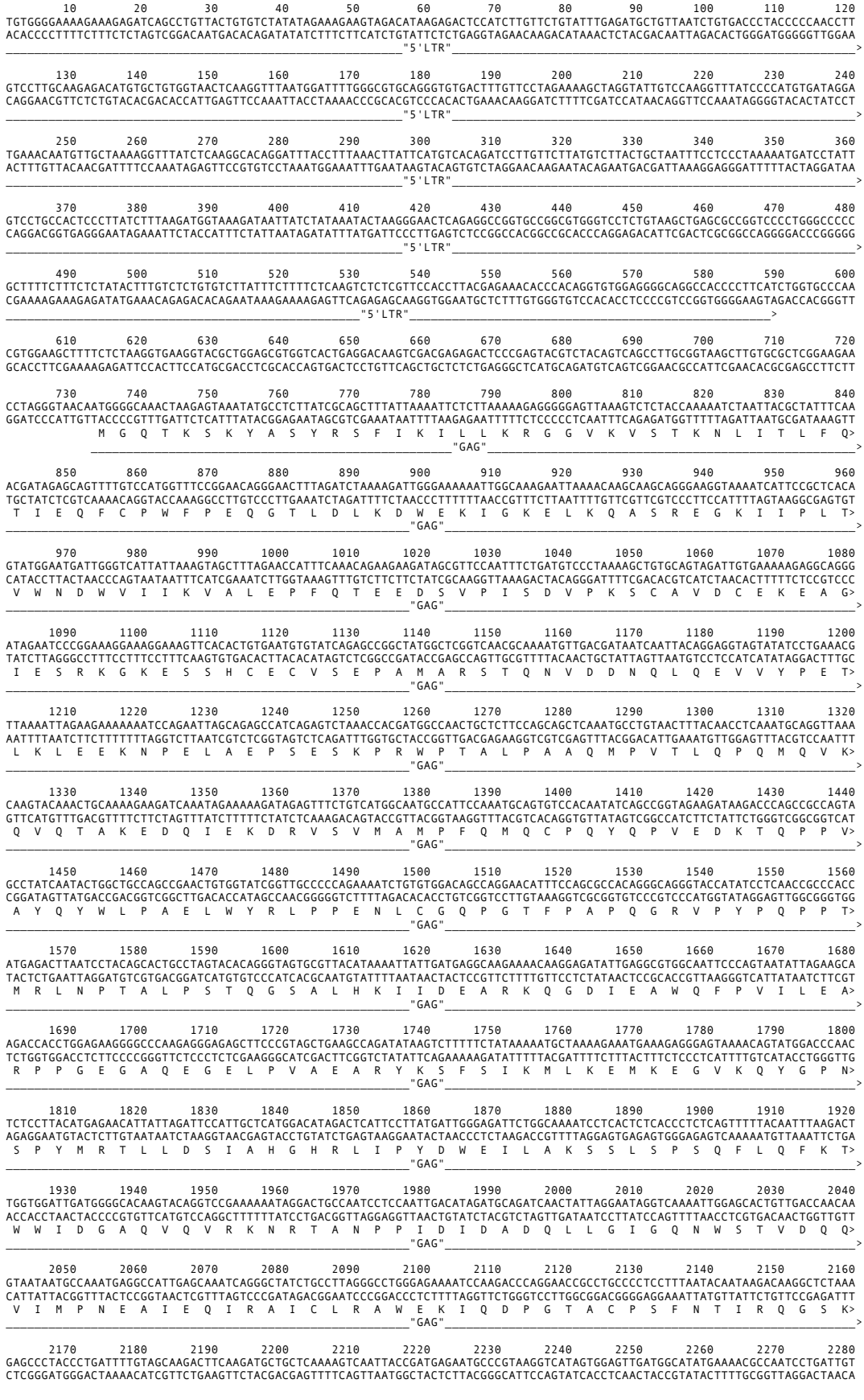


Appendix 1 Figure 5: Viral infection history does not explain the presence of ERV-specific T cell responses in r02120. A) Twenty-four rhesus macaques were screened for previous viral infections by plasma serology. Plasma was tested for antibodies against Simian T Lymphotropic virus (STLV), Simian Retrovirus Groups 1 and 5 (SRV1 and SRV5), Measles virus, Herpes B virus, Herpes Simplex virus type 1 (HSV-1), Cytomegalovirus (CMV), Lymphocryptovirus (LCV), Rhesus rhadinovirus (RRV), and Simian

Foamy Virus (SFV). Rhesus macaque r02120, the only macaque in this group with detectable SERV-K1 Env-specific T cell responses, is boxed in blue. Note previous infection history does not explain r02120's ability to mount SERV-K1 Env-specific immune responses (for example, r02120 and r07046 have identical plasma serology against these tested viruses). **B)** Longitudinal ELISPOT values for IFN-γ spot forming cells (SFCs) measured in r02120 PBMC against either SERV-K1 Env LL15, SERV-K1 Env PA8, or SIV Vif WY8 are shown as mean ± SD of triplicate wells performed at the indicated times. The grey box indicates pre-SIV infection.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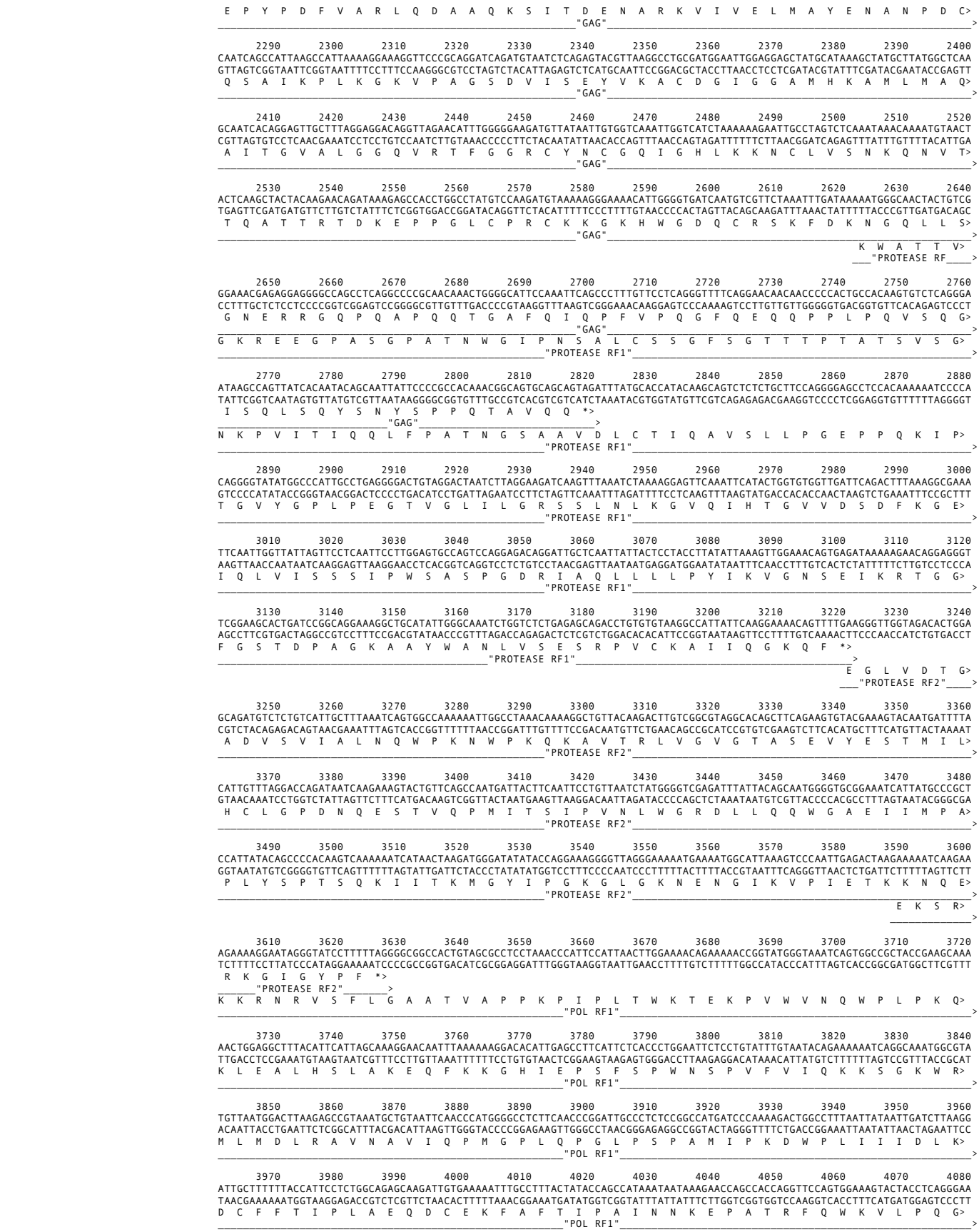
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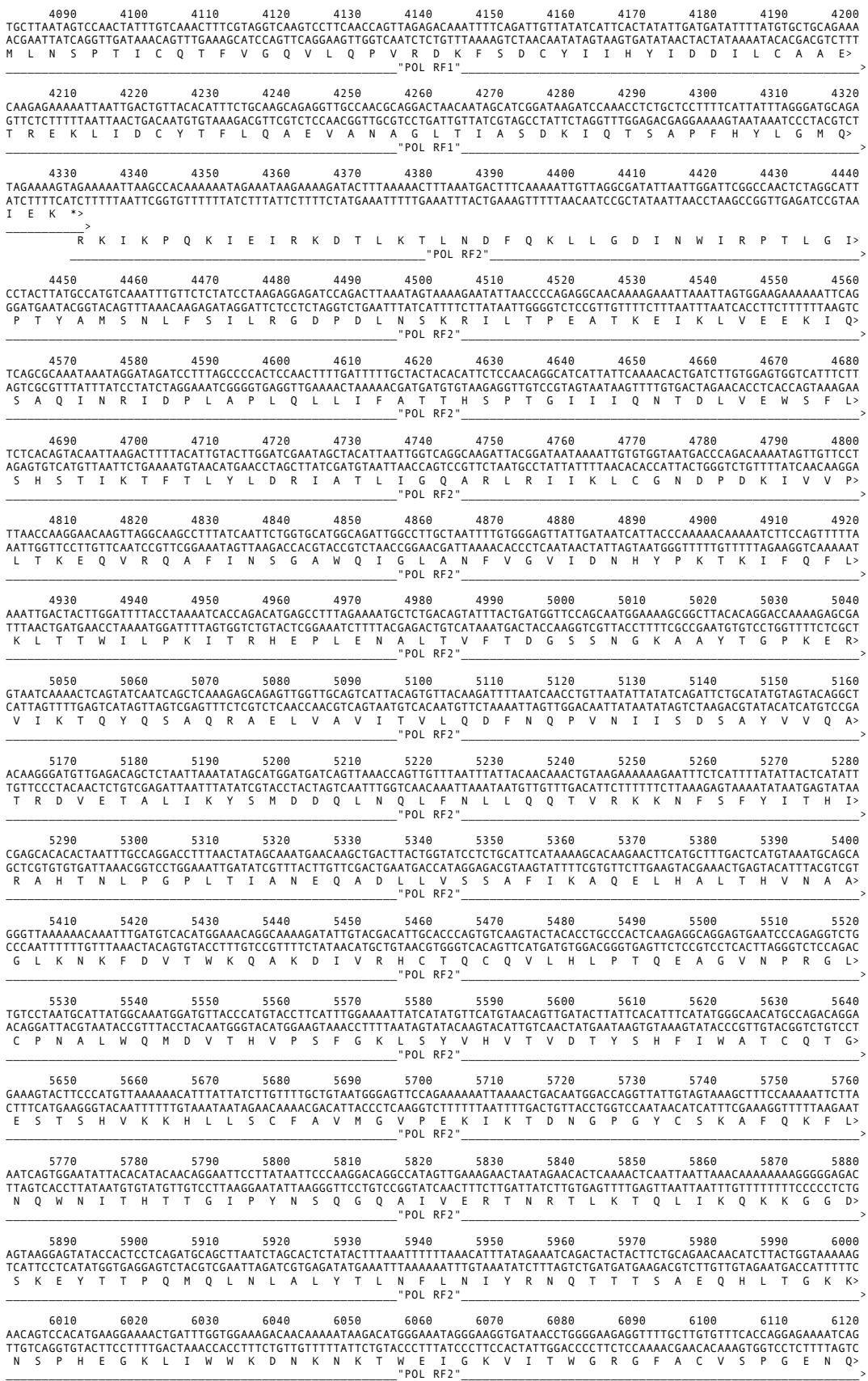
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G L K N K F D V T W K Q A K D I V R H C T Q C Q V L H L P T Q E A G V N P R G L>
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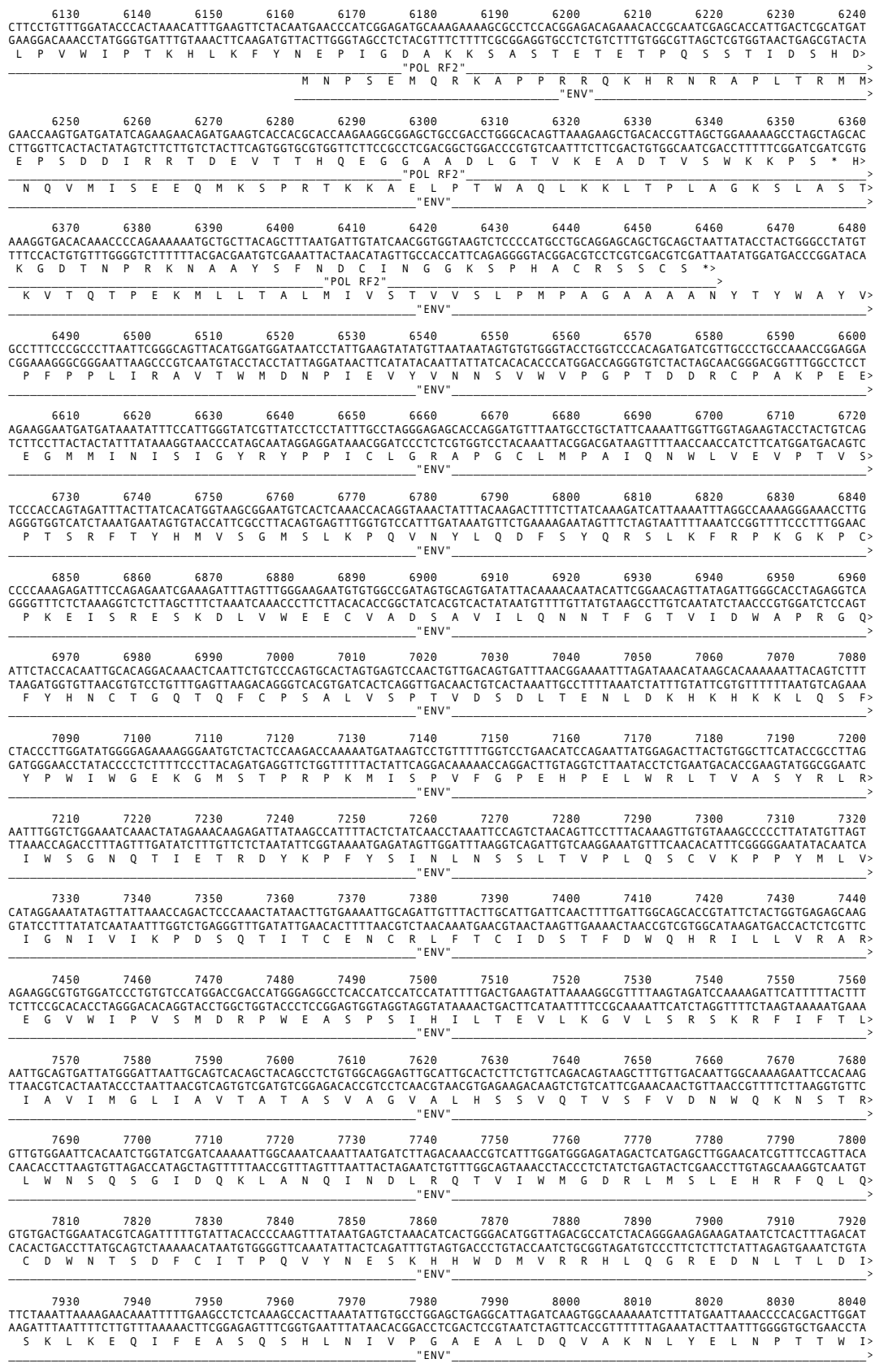
"POL RF2"



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L P V W I P T K H L K F Y N E P I G D A K K S A S T E T E T P Q S S T I D S H D>
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M N P S E M Q R K A P P R R Q K H R N R A P L T R M M>
"ENV"

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"POL RF2"
N Q V M I S E E Q M K S P R T K K A E L P T W A Q L K K L T P L A G K S L A S T>
"ENV"

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K G D T N P R K N A A Y S F N D C I N G G K S P H A C R S S C S *>
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"ENV"

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P F P P L I R A V T W M D N P I E V Y V N N S V W V P G P T D D R C P A K P E E>
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F Y H N C T G Q T Q F C P S A L V S P T V D S D L T E N L D K H K H K K L Q S F>
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"ENV"

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"ENV"

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"ENV"

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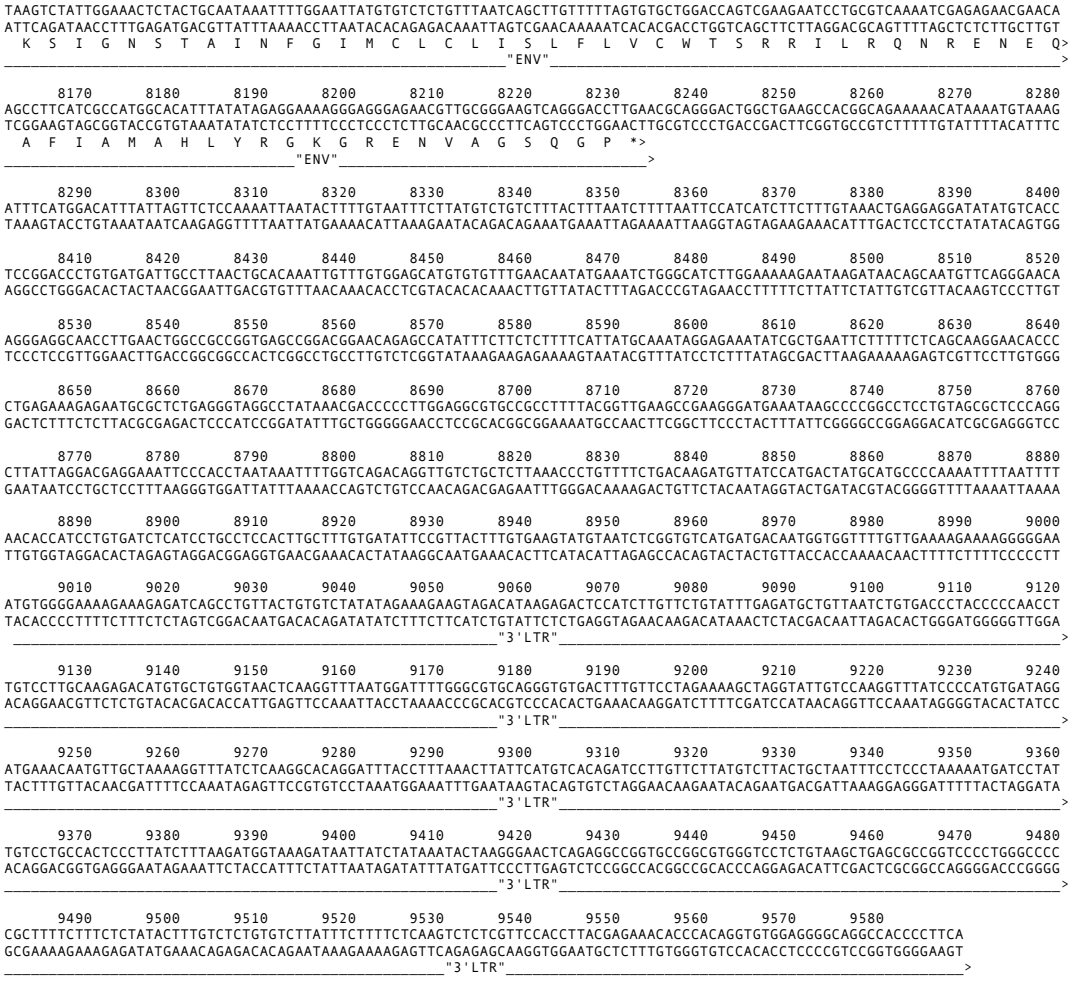

Appendix 1 Figure S1: SERV-K1 proviral sequence. Nucleotide sequence of SERV-K1 provirus from chromosome 12 of rhesus macaque r02120 is shown. Long terminal repeat sequences (LTRs) as well as coding sequences for Gag, Pro, Pol, and Env open reading frames are annotated. Note Pro and Pol ORFs contain frameshift mutations resulting in premature stop codons, while Gag and Env ORFs appear intact. RF= reading frame.

```
r02120 provirus (12)    1 MNPSEMQRKAPPRRQKHRNRAPLTRMMNQVMISEEQMKSPRTKKAELPTWAQLKKLTPLA  60
r02120 Env mRNA v1      1 MNPSEMQRKAPPRRQKHRNRAPLTRMMNQVMISEEQMKSPRTKKAELPTWAQLKKLTPLA  60
r02120 Env mRNA v2      1                                                               0


r02120 provirus (12)   61 GKSLASTKVTQTPEKMLLTALMIVSTVVSLPMPAGAAAANYTYWAYVPFPPLIRAVTWMD 120
r02120 Env mRNA v1     61 GKSLASTKVTQTPEKMLLTALMIVSTVVSLPMPAGAAAANYTYWAYVPFPPLIRAVTWMD 120
r02120 Env mRNA v2      1                                PMPAGAAAANYTYWAYVPFPPLIRAVTWMD  30
                                                         ******************************


r02120 provirus (12)  121 NPIEVYVNNSVWVPGPTDDRCPAKPEEEGMMINISIGYRYPPICLGRAPGCLMPAIQNWL 180
r02120 Env mRNA v1    121 NPIEVYVNNSVWVPGPTDDRCPAKPEEEGMMINISIGYRYPPICLGRAPGCLMPAIQNWL 180
r02120 Env mRNA v2     31 NPIEVYVNNSVWVPGPTDDRCPAKPEEEGMMINISIGYRYPPICLGRAPGCLMPAIQNWL  90
                          ************************************************************


r02120 provirus (12)  181 VEVPTVSPTSRFTYHMVSGMSLKPQVNYLQDFSYQRSLKFRPKGKPCPKEISRESKDLVW 240
r02120 Env mRNA v1    181 VEVPTVSPTSRFTYHMVSGMSLKPQVNYLQDFSYQRSLKFRPKGKPCPKEISRESKDLVW 240
r02120 Env mRNA v2     91 VEVPTVSPTSRFTYHMVSGMSLKPQVNYLQDFSYQRSLKFRPKGKPCPKEISRESKDLVW 150
                          ************************************************************


r02120 provirus (12)  241 EECVADSAVILQNNTFGTVIDWAPRGQFYHNCTGQTQFCPSALVSPTVDSDLTENLDKHK 300
r02120 Env mRNA v1    241 EECVADSAVILQNNTFGTVIDWAPRGQFYHNCTGQTQFCPSALVSPTVDSDLTENLDKHK 300
r02120 Env mRNA v2    151 EECVADSAVILQNNTFGTVIDWAPRGQFYHNCTGQTQFCPSALVSPTVDSDLTENLDKHK 210
                          ************************************************************


r02120 provirus (12)  301 HKKLQSFYPWIWGEKGMSTPRPKMISPVFGPEHPELWRLTVASYRLRIWSGNQTIETRDY 360
r02120 Env mRNA v1    301 HKKLQSFYPWIWGEKGISTPRPKMISPVFGPEHPELWRLTVASYRLRIWSGNQTIETRDY 360
r02120 Env mRNA v2    211 HKKLQSFYPWIWGEKGMSTPRPKMISPVFGPEHPELWRLTVASYRLRIWSGNQTIETRDY 270
                          ***************.********************************************


r02120 provirus (12)  361 KPFYSINLNSSLTVPLQSCVKPPYMLVIGNIVIKPDSQTITCENCRLFTCIDSTFDWQHR 420
r02120 Env mRNA v1    361 KPFYSINLNSSLTVPLQSCVKPPYMLVIGNIVIKPDSQTITCENCRLFTCIDSTFDWQHR 420
r02120 Env mRNA v2    271 KPFYSINLNSSLTVPLQSCVKPPYMLVIGNIVIKPDSQTITCENCRLFTCIDSTFDWQHR 330
                          ************************************************************


r02120 provirus (12)  421 ILLVRAREGVWIPVSMDRPWEASPSIHILTEVLKGVLSRSKRFIFTLIAVIMGLIAVTAT 480
r02120 Env mRNA v1    421 ILLVRAREGVWIPVSMDRPWEASPSIHILTEVLKGVLSRSKRFIFTLIAVIMGLIAVTAT 480
r02120 Env mRNA v2    331 ILLVRAREGVWIPVSMDRPWEASPSIHILTEVLKGVLSRSKRFIFTLIAVIMGLIAVTAT 390
                          ************************************************************


r02120 provirus (12)  481 ASVAGVALHSSVQTVSFVDNWQKNSTRLWNSQSGIDQKLANQINDLRQTVIWMGDRLMSL 540
r02120 Env mRNA v1    481 ATVAGVALHSSVQTVSFVDNWQKNSTRLWNSQSGIDQKLANQINDLRQTVIWMGDRLMSL 540
r02120 Env mRNA v2    391 ASVAGVALHSSVQTVSFVDNWQKNSTRLWNSQSGIDQKLANQINDLRQTVIWMGDRLMSL 450
                          *.************************************************************


r02120 provirus (12)  541 EHRFQLQCDWNTSDFCITPQVYNESKHHWDMVRRHLQGREDNLTLDISKLKEQIFEASQS 600
r02120 Env mRNA v1    541 EHRFQLQCDWNTSDFCIIPQVYNESKHHWDMVRRHLQGREDNLTLDISKLKEQIFEASQS 600
r02120 Env mRNA v2    451 EHRFQLQCDWNTSDFCITPQVYNESKHHWDMVRRHLQGREDNLTLDISKLKEQIFEASQS 510
                          **************** ****************************************


r02120 provirus (12)  601 HLNIVPGAEALDQVAKNLYELNPTTWIKSIGNSTAINFGIMCLCLISLFLVCWTSRRILR 660
r02120 Env mRNA v1    601 HLNIVPGAEALDQVAKNLYELNPTTWIKSIGNSTAINFGIMCLCLIGLFLVCWTSRRILR 660
r02120 Env mRNA v2    511 HLNIVPGAEALDQVAKNLYELNPTTWIKSIGNSTAINFGIMCLCLISLFLVCWTSRRILR 570
                          ********************************************.*************


r02120 provirus (12)  661 QNRENEQAFIAMAHLYRGKGRENVAGSQGP 690
r02120 Env mRNA v1    661 QNRENEQAFIAMAHLYRGKGRENVAGSQGP 690
r02120 Env mRNA v2    571 QNRENEQAFIAMAHLYRGKGRENVAGSQGP 600
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Appendix 1 Figure S2: Alignment of captured SERV-K1 Env mRNA to SERV-K1 provirus. Protein alignment of the predicted amino acid sequence of chromosome 12 provirus and two mRNA variants captured from rhesus macaque r02120 is shown. Matches at each amino acid position are indicated with asterisks (*), while mismatches are indicated with periods (.). Mismatches in each sequence are highlighted in yellow.

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CERV-KOLD35587     1 GAGGCTTTACACTTATTAGCAAAGGAACAATTAGAAAAGGGACATATTGAGCCTTCATTTTCGCCTTGGAATTCTCCTGTGTTTGTAATTCAGAAAAAATCCGGCAGATGGCGCATGCTG  120
HERV-KOLD35587     1 GAGGCTTTACACTTATTAGCAAAGGAACAATTAGAAAAGGGACATATTGAGCCTTCATTTTCGCCTTGGAATTCTCCTGTGTTTGTAATTCAGAAAAAATCCGGCAGATGGCGCGTGCTG  120
SERV-K1 chr.11     1 GAGGCTTTACATTTATTAGCAAAGGAACAATTAGAAAAGGGACACATTGAGCCTTCATTCTCGCCCTGAAATTCTCCTGTATTTGTAATTCAGAAAAAATCAGGCAAATGGCATATGTTA  120
Guereza            1 GAGGCTTTACACTTATTAGCAAAGGAACAATTAGAAAAGGGACACATTGTGCCTTCATTCTCACCCTGGAATTCTCCTGTATTTGTAATTCAGAAAAAATCAGGCAAATGGCGTATGTTA  120
SERV-K1 chr.12     1 GAGGCTTTACATTCATTAGCAAAGGAACAATTTAAAAAGGACACATTGAGCCTTCATTCTCACCCTGGAATTCTCCTGTATTTGTAATACAGAAAAAATCAGGCAAATGGCGTATGTTA  120
Grivet             1 GAGGCTTTACATTCATTAGCAAAGGAACAATTAGAAAAGGGACACATTGAGCCTTCATTCTCACCCTGGAATTCTCCTGTATTTGTAATTAAGAAAAAATCAGGCAAGTGGCGTATGTTA  120
SERV-K1 chr.5      1 GAGGCTTTACATTTATTAGCAAAGGAACAGTCAGAAAAGGGACACACTGAGGCATCATTTTCGCCCTGGAATTCTCCTGTATTTGTAATTCAGAAAAAATCAGGCAGATGGCGTATGTTA  120
CERV-K60           1 GAGGCTTTACATTTATTAGCAAATGAACAGTTAGAAAAGCGACATATTGAGCCTTCATTCTCGCCTTGGAATTCTCCTGTGTTTGTAATTCAGAAGAAATCCAGCAAATGGCGTATGTTA  120
CERV-K102          1 GAGGCTTTACATTTATTAGCAAATGAACAGTTAGAAAAGGGTCATATTGAGCCTTCATTCTCGCCTTGGAATTCTCCTGTGTTTGTAATTCAGAAGAAATCAGGCAAATGGCGTATGTTA  120
HERV-K104          1 GAGGCTTTACATTTATTAGCAAATGAACAGTTAGAAAAGGGTCATATTGAGCCTTCATTCTCGCCTTGGAATTCTCCTGTGTTTGTGATTCAGAAGAAATCAGGCAAATGGCGTATGTTA  120
HERV-K50D          1 GAGGCTTTACATTTATTAGCAAATGAACAGTTAGAAAAGGGTCATATTGAGCCTTCATTCTCGCCTTGGAATTCTCCTGTGTTTGTGATTCAGAAGAAATCAGGCAAATGGCGTATGTTA  120
HERV-K50B          1 GAGGCTTTACATTTATTAGCAAATGAACAGTTAGAAAAGGGTCATATTGAGCCTTCATTCTCGCCTTGGAATTCTCCTGTGTTTGTAATTCAGAAGAAATCAGGCAAATGGCGTATGTTA  120
HERV-K106          1 GAGGCTTTACATTTATTAGCAAATGAACAGTTAGAAAAGGGTCATATTGAGCCTTCATCCTCGCCTTGGAATTCTCCTGTGTTTGTAATTCAGAAGAAATCAGGCAAATGGCGTATGTTA  120
HERV-K101          1 GAGGCTTTACATTTATTAGCAAATGAACAGTTAGAAAAGGGTCATATTGAGCCTTCGTTCTCACCTTGGAATTCTCCTGTGTTTGTAATTCAGAAGAAATCAGGCAAATGGCGTATGTTA  120
HERV-K103          1 GAGGCTTTACATTTATTAGCAAATGAACAGTTAGAAAAGGGTCACATTGAGCCTTCGTTCTCACCTTGGAATTCTCCTGTGTTTGTAATTCAGAAGAAATCAGGCAAATGGCGTATGTTA  120
CERV-K100          1 GAGGCTTTACATTTATTAGCAAATGAACAGTTAGAAAAGGGTCATATTGAGCCTTCGTTCTCACCTTGGAATTCTCCTGTGTTTGTAATTCAGAAGAAATCAGGCAAATGGCATACGTTA  120
HERV-K10           1 GAGGCTTTACATTTATTAGCAAATGAACAGTTAGAAAAGGGTCACATTGAGCCTTCGTTCTCACCTTGGAATTCTCCTGTGTTTGTAATTCAGAAGAAATCAGGCAAATGGCATACGTTA  120
HERV-K108          1 GAGGCTTTACATTTATTAGCAAATGAACAGTTAGAAAAGGGTCATATTGAGCCTTCGTTCTCACCTTGGAATTCTCCTGTGTTTGTAATTCAGAAGAAATCAGGCAAATGGCGTATGTTA  120
HERV-K109          1 GAGGCTTTACATTTATTAGCAAATGAACAGTTAGAAAAGGGTCATATTGAGCCTTCGTTCTCACCTTGGAATTCTCCTGTGTTTGTAATTCAGAAGAAATCAGGCAAATGGCGTATGTTA  120
HERV-K113          1 GAGGCTTTACATTTATTAGCAAATGAACAGTTAGAAAAGGGTCATATTGAACCTTCGTTCTCACCTTGGAATTCTCCTGTGTTTGTAATTCAGAAGAAATCAGGCAAATGGCGTATGTTA  120
HERV-K36           1 GAGGCTTTACATTTATTAGCAAATGAACAGTTAGAAAAGGGTCATATTGAGCCTTCGTTCTCACCTTGGAATTCTCCTGTGTTTGTAATTCAGAAGAAATCAGGCAAATGGCGTATGTTA  120
HERV-K115          1 GAGGCTTTACATTTATTAGCAAATGAACAGTTAGAAAAGGGTCATATTGAGCCTTCGTTCTCACCTTGGAATTCTCCTGTGTTTGTAATTCAGAAGAAATCAGGCAAATGGCGTATGTTA  120
HERV-K102          1 GAGGCTTTACATTTATTAGCAAATGAACAGTTAGAAAAGGGTCACATTGAGCCTTCGTTCTCACCTTGGAATTCTCCTGTGTTTGTAATTCAGAAGAAATCAGGCAAATGGCGTATGTTA  120
HERV-K50A          1 GAGGCTTTACATTTATTAGCAAATGAACAGTTAGAAAAGGGTCACATTGAGCCTTCGTTCTCACCTTGGAATTCTCCTGTGTTTGTAATTCAGAAGAAATCAGGCAAATGGCGTATGTTA  120
                     ********** * ********* ***** *   ****  * ** * **   * ** *  ** ** ** ********** ***** **  **** *****  ***  ****    * *

CERV-KOLD35587   121 ACCGACTTAAGAGCCGTTAATGCTGTAATTCAACCCATGGGGGCTCTCCAACCTGGGTTGCTGTCTCCGGTCATGATCCCCAAAGATCGGCCTTTAGTTATAATTGATCTGAAGGATTGC  240
HERV-KOLD35587   121 ACCGACTTAAGAGCCGTTAATGCTGTAATTCAACCCATGGGGGCTCTCCAACCTGGGTTGCTCTCTCCGGTCATGATCCCCAAAGATCGGCCTTTAGTTATAATTGATCTGAAGGATTGC  240
SERV-K1 chr.11   121 ATGGACTTAAGAGCCGTAAATGCTGTAATTCAACCCACGGGGCCTCTTCAACCCGGACTGCCCTCTCCGGCCATGATCCCCAAAAGACTGGCCTTTAATTATAATTGATCTTAAGGATTGC  240
Guereza          121 ATGGACTTAAGAGCCATAAAGCGTGTAATTCAACCCATGGGGCCTCTCCAACCTGGATTGCCCTCTCCGGCCATGATCCCAAAAGACTGGCCTTTAATTATAATTGATCTTAAGGATTGT  240
SERV-K1 chr.12   121 ATGGACTTAAGAGCCGTAAATGCTGTAATTCAACCCATGGGGCCTCTTCAACCCGGATTGCCCTCTCCGGCCATGATCCCAAAAGACTGGCCTTTAATTATAATTGATCTTAAGGATTGC  240
Grivet           121 ATGGACTTAAGAGCCGTAAATGCTGTAATTCAACCCATGGGGCCTCTTCAACCCGGATTGCCCTCTCCGGCCATGATCCCAAAAGACTGGCCTTTAATTATAATTGATCTTAAGGACTGC  240
SERV-K1 chr.5    121 ACCGACTTAAGAGCCATAAATGCTGTAATTCAACCCATGGGGCCTCTTCAACCCGGATTGCCCTCCCAGCCACGATTCCAAAAGACTGGCCTTTAATTCTAATTGATCTGAAGGACTGC  240
CERV-K60         121 ACTGACTTAAGGGCGTAAATGCTGTAATTCAACCCATGGGGCCTCTCCAACCTGGGTTGCCCTCTCCGGCCATGATCCCAAAAGATTGGCCTTTAATTATAATTGATCTAAAGGATTGC  240
CERV-K102        121 ACTGACTTTAGGGCCGTAAACGCCGTAATTCAACCCATGGGGCCTCTCCAACCTGGGTTGCCCTCTCTGGCCATGATCCCAAAAGACTGGCCTTTAATTATAATTGATCTAAGGATTGC  240
HERV-K104        121 ACTGACTTAAGGGCCGTAAACGCCATAATTCAACCCATGGGGCCTCTCCAACCTAGGTTGCCCTCTCGGCCATGATCCCAAAAGATTGGCCTTTAATTATAATTGATCTAAGGATTGC  240
HERV-K50D        121 ACTGACTTAAGGGCCGTAAACGCCATAATTCAACCCATGGGGCCTCTCCAATCTGGGTTGCCCTCTCCGGCCATGATCCCAAAAGATTGGCCTTTAATTATAATTGATCTAAGGATTGC  240
HERV-K50B        121 ACTGACTTAAGGGCCGTAAACGCCGTAATTCAACCCATGGGGCCTCTCCAACCCGGGTTGCCCTCTCCGGCCATGATCCCAAAAGATTGGCCTTTAATTATAATTGATCTAAGGATTGC  240
HERV-K106        121 ACTAACTTAAGGGCCGTAAACGCCGTAATTCAACCCATGGGGCCTCTCCAACCCGGGTTGCCCTCTCCGGCCATGATCCCAAAAGATTGGCCTTTAATTATAATTGATCTAAGGATTGC  240
HERV-K101        121 ACTGACTTAAGGGCCGTAAACGCCGTAATTCAACCCATGGGGCCTCTCCAACCCGGGTTGCCCTCTCCGGCCATGATCCCAAAAGATTGGCCTTTAATTATAATTGATCTAAGGATTGC  240
HERV-K103        121 ACTGACTTAAGGGCTGTAAACGCCGTAATTCAACCCATGGGGCCTCTCCAACCCGGGTTGCCCTCTCCGGCCATGATCCCAAAAGATTGGCCTTTAATTATAATTGATCTAAAGGACTGC  240
CERV-K100        121 ACTGACTTAAGGGCTGTAAACGCCGTAATTCAACCCATGGGGCCTCTCCAACCCGGGTTGCCCTCTCCGGCCATGATCCCAAAAGATTGGCCTTTAATTATAATTGATCTAAAGGATTGC  240
HERV-K10         121 ACTGACTTAAGGGCTGTAAACGCCGTAATTCAACCCATGGGGCCTCTCCAACCCGGGTTGCCCTCTCCGGCCATGATCCCAAAAGATTGGCCTTTAATTATAATTGATCTAAAGGATTGC  240
HERV-K108        121 ACTGACTTAAGGGCTGTAAACGCCGTAATTCAACCCATGGGGCCTCTCCAACCCGGGTTGCCCTCTCCGGCCATGATCCCAAAAGATTGGCCTTTAATTATAATTGATCTAAAGGATTGC  240
HERV-K109        121 ACTGACTTAAGGGCTGTAAACGCCGTAATTCAACCCATGGGGCCTCTCCAACCCGGGTTGCCCTCTCCGGCCATGATCCCAAAAGATTGGCCTTTAATTATAATTGATCTAAAGGATTGC  240
HERV-K113        121 ACTGACTTAAGGGCTGTAAACGCCGTAATTCAACCCATGGGGCCTCTCCAACCTGGGTTGCCCTCTCCAGCCATGATCCCAAAAGATTGGCCTTTAATTATAATTGATCTAAGGATTGC  240
HERV-K36         121 ACTGACTTAAGGGCTGTAAACGCCGTAATTCAACCCATGGGGCCTCTCCAACCTGGGTTGCCATCTCCAGCCATGATCCCAAAAGATTGGCCTTTAATTATAATTGATCTAAAGGATTGC  240
HERV-K115        121 ACTGACTTAAGGGCTGTAAACGCCGTAATTCAACCCATGGGGCCTCTCCAACCCGGGTTGCCCTCTCCAGCCATGATCCCAAAAGATTGGCCTTTAATTATAATTGATCTAAGGATTGC  240
HERV-K102        121 ACTGACTTAAGGGCTGTAAACGCCGTAATTCAACCCATGGGGCCTCTCCAACCTGGGTTGCCCTCTCCAGCCATGATCCCAAAAGATTGGCCTTTAATTATAATTGATCTAAAGGATTGC  240
HERV-K50A        121 ACTGACTTAAGGGCTGTAAACGCCGTAATTCAACCCATGGGGCCTCTCCAACCTGGGTTGCCCTCTCCAGCCATGATCCCAAAAGATTGGCCTTTAATTATAATTGATCTAAAGGATTGC  240
                     *   **** ** **  * ** **  ************ **** **** *** * *  ***  ** *  * ** *** ** *****  ******** ** ********** ** **

CERV-KOLD35587   241 TTTTTTACCGTTCCTCTGGCAAAACAGGATTTTGAAAAATTTGCTTTTACTATACCAGCCATAAATAATAAAGAACCAGCCACCAGGTTTCAGTGGAAAGTGTTGCCTCAGGGAATGCTT  360
HERV-KOLD35587   241 TTTTTTACCGTTCCTCTGGCAAAACAGGATTTTGAAAAATTTGCTTTTACTATACCAGCCATAAATAATAAAGAACCAGCCACCAGGTTTCAGTGGAAAGTGTTGCCTCAGGGAATGCTT  360
SERV-K1 chr.11   241 TTTTTTACCATTCCTCTGGCAGAGCAGGATTGTGAAAAATTTGCCTTTACTATACCAGCCATAAAAAATAAAGAACCAGCCACCAGGTTTCAGTGGAAAGTATTACCTCAGGGAATGCTT  360
Guereza          241 TTTTTTACCATTCCTCTGGCAGAGCAAGATTGTGAAAAATTTGCCTTTACTATACCAGCCATAAATAATAAAGAACCAGCCACCAGGTTTCAGTGGAAAGTTTTACCTCAAGGAATGCTT  360
SERV-K1 chr.12   241 TTTTTTACCATTCCTCTGGCAGAGCAAGATTGTGAAAAATTTGCCTTTACTATACCAGCCATAAATAATAAAGAACCAGCCACCAGGTTTCAGTGGAAAGTACTACCTCAGGGAATGCTT  360
Grivet           241 TTTTTTACCATTCCTCTGGCAGAGCAAGATTGTGAAAAATTTGCCTTTACTATACCAGCCATAAATAATAAAGAACCAGCCACCAGATTTCAGTGGAAAGTACTACCTCAGGGAATGCTT  360
SERV-K1 chr.5    241 TTTTTTACCATTCCTCTGGCAGAGCAGGATTGTGAAAAATTTGCTTTTACTATACCAGCCGTAAATAATAAAGAACCAGCCACCAGGTTTCAGTGGAAAGTGTTACCTCGGGGAATGCTT  360
CERV-K60         241 TTTTTTACCATCCCCTCTGGCGGAGCCGGATTGCGAAAAATTTGCCTTTACTATACCAGCCATAAATAATAAAGAACCAGCCACCAGGTTTCAGTGGAAAGTGTTACCTCAGGGAATGCTT  360
CERV-K102        241 TTTTTTACCATCCCCTCTGGCGGAGCAGGATTGCGAAAAATTTGCCTTTACTATACCAGCCATAAATAATAAAGAACCAGCCACCAGGTTTCAGTGGAAAGTGTTACCTCAGGGAATGCTT  360
HERV-K104        241 TTTTTTACCATCCCCTCTGGCGGAGCAGGATTGCGAAAAATTTGCCTTTACTATGCAGCCATAAATAATAAAGAACCAGCCACCAGGTTTCAGTGGAAAGTGTTACCTCAGGGAATGCTT  360
HERV-K50D        241 TTTTTTACCATCCCCTCTGGCGGAGCAGGATTGCGAAAAATTTGCCTTTACTATACCAGCCATAAATAATAAAGAACCAGCCACCAGGTTTCAGTGGAAAGTGTTACCTCAGGGAATGCTT  360
HERV-K50B        241 TTTTTTACCATCCCCTCTGGCAGAGCAGGATTGTGAAAAATTTGCCTTTACTATACCAGCCATAAATAATAAAGAACCAGCCACCAGGTTTCAGTGGAAAGTGTTACCTCAGGGAATGCTT  360
HERV-K106        241 TTTTTTACCATCCCCTCTGGCAGAGCAGGATTGTGAAAAATTTGCCTTTACTATACCAGCCATAAATAATAAAGAACCAGCCACCAGGTTTCAGTGGAAAGTGTTACCTCAGGGAATGCTT  360
HERV-K101        241 TTTTTTACCATCCCCTCTGGCAGAGCAGGATTGTGAAAAATTTGCCTTTACTATACCAGCCATAAATAATAAAGAACCAGCCACCAGGTTTCAGTGGAAAGTGTTACCTCAGGGAATGCTT  360
HERV-K103        241 TTTTTTACCATCCCCTCTGGCAGAGCAGGATTGCGAAAAATTTGCCTTTACTATACCAGCCATAAATAATAAAGAACCAGCCACCAGGTTTCAGTGGAAAGTGTTACCTCAGGGAATGCTT  360
CERV-K100        241 TTTTTTACCATCCCCTCTGGCAGAGCAGGATTGTGAAAAATTTGCCTTTACTATACCAGCCATAAATAATAAAGAACCAGCCACCAGGTTTCAGTGGAAAGTGTTACCTCAGGGAATGCTT  360
HERV-K10         241 TTTTTTACCATCCCCTCTGGCAGAGCAGGATTGTGAAAAATTTGCCTTTACTATACCAGCCATAAATAATAAAGAACCAGCCACCAGGTTTCAGTGGAAAGTGTTACCTCAGGGAATGCTT  360
HERV-K108        241 TTTTTTACCATCCCCTCTGGCAGAGCAGGATTGTGAAAAATTTGCCTTTACTATACCAGCCATAAATAATAAAGAACCAGCCACCAGGTTTCAGTGGAAAGTGTTACCTCAGGGAATGCTT  360
HERV-K109        241 TTTTTTACCATCCCCTCTGGCAGAGCAGGATTGTGAAAAATTTGCCTTTACTATACCAGCCATAAATAATAAAGAACCAGCCACCAGGTTTCAGTGGAAAGTGTTACCTCAGGGAATGCTT  360
HERV-K113        241 TTTTTTACCATCCCCTCTGGCAGAGCAGGATTGTGAAAAATTTGCCTTTACTATACCAGCCATAAATAATAAAGAACCAGCCACCAGGTTTCAGTGGAAAGTGTTACCTCAGGGAATGCTT  360
HERV-K36         241 TTTTTTACCATCCCCTCTGGCAGAGCAGGATTGTGAAAAATTTGCCTTTACTATACCAGCCATAAATAATAAAGAACCAGCCACCAGGTTTCAGTGGAAAGTGTTACCTCAGGGAATGCTT  360
HERV-K115        241 TTTTTTACCATCCCCTCTGGCAGAGCAGGATTGCGAAAAATTTGCCTTTACTATACCAGCCATAAATAATAAAGAACCAGCCACCAGGTTTCAGTGGAAAGTGTTACCTCAGGGAATGCTT  360
HERV-K102        241 TTTTTTACCATCCCCTCTGGCAGAGCAGGATTGCGAAAAATTTGCCTTTACTATACCAGCCATAAATAATAAAGAACCAGCCACCAGGTTTCAGTGGAAAGTGTTACCTCAGGGAATGCTT  360
HERV-K50A        241 TTTTTTACCATCCCCTCTGGCAGAGCAGGATTGCGAAAAATTTGCCTTTACTATACCAGCCATAAATAATAAAGAACCAGCCACCAGGTTTCAGTGGAAAGTGTTACCTCAGGGAATGCTT  360
                     ********* * *********  * *  ****  ************ ****** *** **** **** ********************** ** **********  * ****  *********

CERV-KOLD35587   361 AATGGTCCAACTATTTGCCAGACTTTTGTAGCTCAAGCTCTTCAACCAGTTAGAGAC-AGTTTTCAGACTCTTATATCATTCATTATGTTGATGATATTTTGTGTGCTACAGAAAGGAGA  479
HERV-KOLD35587   361 AATGGTCCAACTATTTGCCAGACTTTTGTAGCTCAAGCTCTTCAACCAATTAGAGAC-AGTTTTCAGACTCTTATATCATTCATTATGTTGATGATATTTTGTGTGCTACAGAAAGGAGA  479
SERV-K1 chr.11   361 AATAGTCCAACTATTTGTCATACTTTTGTAGGTCAAGTCCTTCAACCAGTTAGAGACAAATTTTCAGATTGTTCTATCGTTCACTATATTCATGCTATTTTATGTGCTGCAGAAACAAGA  480
Guereza          361 AATAGTCCAACTATTTGTCAAACTTTGTAGGTCAAGTCCTTCAACCAGTTAGAGACAAATTTTCAGATTGTTATATCATTCACTATATTGATGATATTTTATGTGCTGCAGAAACAAGA  480
SERV-K1 chr.12   361 AATAGTCCAACTATTTGTCAAACTTTCGTAGGTCAAGTCCTTCAACCAGTTAGAGACAAATTTTCAGATTGTTATATCATTCACTATATTGATGATATTTTATGTGCTGCAGAAACAAGA  480
Grivet           361 AATAGTCCAACTATTTGTCAAACTTTCGTGGGTCAAGTCCTTCAACCAGTTAGAGAAAAATTTTCAGATTGTTATATCATTCACTATATTGATGATATTTTATGTGCTGCAGAAACAAGA  480
SERV-K1 chr.5    361 AATAGTCCAACTATTTGTCAAACTTTCGTAGGTCAAGTTCTTCAACCAGTTAGAGACAAATGTTCCGATTGTTACATCATTCACTATATTGATGCTATTTTATGTGCTGCACAAACAAGA  480
CERV-K60         361 AATAGTCCAACTATTTGTCAGATTTTGTAGGTCGAGCTCTTCAACCAGTTAGAGACAAGTTTTCAGACTGTTATTTTATTCATTATTTTGATGATATTTTATGTGCTGCAGAAACGAAA  480
CERV-K102        361 ACTAGTCCAACTATTTGTCAGACTTTTGTAGGTCGAGCTCTTCAAACAGTTAGAGACAAGTTTTCAGACTGTTATATTATTCATTATATTGATGATATTTTATGTGCTGCAGAAACGAGA  480
HERV-K104        361 AATAGTCCAACTATTTGTCAGACTTTTGTAGGTCGAGCTCTTCAACCAGTTAGAGACAAGTTTTCAGACTGTTATATTATTCATTATATTGATGATATTTTATGTGCTGCAGAAACGAAA  480
HERV-K50D        361 AATAGTCCAACTATTTGTCAGACTTTTGTAGGTCGAGCTCTTCAACCAGTTAGAGACAAGTTTTCAGACTGTTATATTATTCATTATATTGATGATATTTTATGTGCTGCAGAAACGAAA  480
HERV-K50B        361 AATAGTCCAACTATTTGTCAGACTTTTGTAGGTCGAGCTCTTCAACCAGTTAGAGAAAAGTTTTCAGACTGTTATATTATTCATTATATTGATGATATTTTATGTGCTGCAGAAACGAAA  480
HERV-K106        361 AATAGTCCAACTATTTGTCAGACTTTTGTAGGTCGAGCTCTTCAACCAGTTAGAGAAAAGTTTTCAGACTGTTATATTATTCATTATATTGATGATATTTTATGTGCTGCAGAAACGAAA  480
HERV-K101        361 AATAGTCCAACTATTTGTCAGACTTTTGTAGGTCGAGCTCTTCAACCAGTTAGAGAAAAGTTTTCAGACTGTTATATTATTCATTATATTGATGATATTTTATGTGCTGCAGAAACGAAA  480
HERV-K103        361 AATAGTCCAACTATTTGTCAGACTTTTGTAGGTCGAGCTCTTCAACCAGTTAGAGAAAAGTTTTCAGACTGTTATATTATTCATTATATTGATGATATTTTATGTGCTGCAGAAACGAAA  480
CERV-K100        361 AATAGTCCAACTATTTGTCAGACTTTTGTAGGTCGAGCTCTTCAACCAGTGAGAGAAAAGTTTTCAGACTGTTATATTATTCATTATATTGATGATATTTTATGTGCTGCAGAAACGAAA  480
CERV-K10         361 AATAGTCCAACTATTTGTCAGACTTTTGTAGGTCGAGCTCTTCAACCAGTGAGAGAAAAGTTTTCAGACTGTTATATTATTCATTATATTGATGATATTTTATGTGCTGCAGAAACGAAA  480
HERV-K108        361 AATAGTCCAACTATTTGTCAGACTTTTGTAGGTCGAGCTCTTCAACCAGTTAGAGAAAAGTTTTCAGACTGTTATATTATTCATTGTATTGATGATATTTTATGTGCTGCAGAAACGAAA  480
HERV-K109        361 AATAGTCCAACTATTTGTCAGACTTTTGTAGGTCGAGCTCTTCAACCAGTT-----AAAGTTTTCAGACTGTTATATTATTCATTATATTGATGATATTTTATGTGCTGCAGAAACGAAA  475
HERV-K113        361 AATAGTCCAACTATTTGTCAGACTTTTGTAGGTCGAGCTCTTCAACCAGTTAGAGACAAGTTTTCAGACTGTTATATTATTCATTATATTGATGATATTTTATGTGCTGCAGAAACGAAA  480
HERV-K36         361 AATAGTCCAACTATTTGTCAGACTTTTGTAGGTCGAGCTCTTCAACCAGTTAGAGAAAAGTTTTCAGACTGTTATATTATTCATTATATTGATGATATTTTATGTGCTGCAGAAACGAAA  480
HERV-K115        361 AATAGTCCAACTATTTGTCAGACTTTTGTAGGTCGAGCTCTTCAACCAGTTAGAGAAAAGTTTTCAGACTGTTATATTATTCATTATATTGATGATATTTTATGTGCTGCAGAAACGAAA  480
HERV-K102        361 AATAGTCCAACTATTTGTCAGACTTTTGTAGGTTGAGCTCTTCAACCAGTTAGAGAAAAGTTTTCAGACTGTTATATTATTCATTATATTGATGATATTTTATGTGCTGCAGAAACGAGA  480
HERV-K50A        361 AATAGTCCAACTATTTGTCAGACTTTTGTAGGTCGAGCTCTTCAACCAGTTAGAGAAAAGTTTTCAGACTGTTATATTATTCATTATATTGATGATATTTTATGTGCTGCAGAAACGAGA  480
                     * * ************** ** * *** ** * *  **  ****** ** *        * * *** ** * **   *  **** * * ** *** ****** ****** ** ***  * *


CERV-KOLD35587   480 GACAAATTAATTGACTGTTACACATTTCTGCAGACAGAGGTTGCAAACGCAGGACTGACGACAGCATCTGATAAGATTCAAACCTCTACTCCTTTCCATTACTTGGAAATGCAGGTGGAG  599
HERV-KOLD35587   480 GACAAATTAATTGACTGTTACACATTTCTGCAGACAGAGGTTGCAAACGCAGGACTGACGACAGCATCTGATAAGATTCAAACCTCTACTCCTTTCCATTACTTGGAAATGCAGGTGGAG  599
SERV-K1 chr.11   481 GACAAATTAATTGACTGTTACACATTTCTGCAAGCAGAGGTTGCCAACGCAGGACTAAAAATAGCATCTGATAAGATCCAAACCTCTACTCCTTTCATTATTTAGGGATGCAGATAGAA  600
Guereza          481 GAAAGATTAATTGACTGTTACACATTTCTGCAAGCAGAGGTTGCCAGCGCAGGACTAACAATAGCATCTGATAAGATCCAAACCTCTGATCCTTTTCATTATTTAGGGATGCAAATAGAA  600
SERV-K1 chr.12   481 GAAAAATTAATTGACTGTTACACATTTCTGCAAGCAGAGGTTGCCAACGCAGGACTAACAATAGCATCTGATAAGATCCAAACCTCTGCTCCTTTTCATTATTTAGGGATGCAGATAGAA  600
Grivet           481 GAAAAATTAATTGACTGTTATACATTTCTGCAAGCAGAGGTTGCCAACGCAGGACTAACAATAGCATCTGATAAGATCCAAACCTCTGCTCCTTTTCATTATTTAGGGATGCAGATAGAA  600
SERV-K1 chr.5    481 GACAAATGAATTGACTGTTACACATTTCTGCAAGCAGAGGTTGCCAACGCAGGACTGACAAGAGCATCTGATAAGATTCAGACCTCTACTCCTTTCATTATTTAGGGATGCAGATAGAA  600
CERV-K60         481 GATAAATTAATTGACTGTTATACATTTCTGCAAGCAGAGGTTGCCAATGCAGGACTGGCAATAGCATCTGATAAGATCCAAACCTCTACTCCTTTTCATTATTTAGGGATGCAGATAGAA  600
CERV-K102        481 GATAAATTAATTGACTGTTACACATTTCTGCAAGCAGAGGTTGCCAACGCAGGACTGGCAATAGCATCTGATAAGATCCAAACCTCTACTCCTTTTCATTATTTAGGGATGCAGATAGAA  600
HERV-K104        481 GATAAATTAATTGACTGTTATACATTTCTGCAAGCAGAGGTTGCCAACGCAGGACTGGCAATAGCATCTGATAAGATCCAAACCTCTACTCCTTTTCATTATTTAGGGATGCAGATAGAA  600
HERV-K50D        481 GATAAATTAATTGACTGTTATACATTTCTGCAAGCAGAGGTTGCCAACGCAGGACTGGCAATAGCATCTGATAAGATCCAAACCTCTACTCCTTTTCATTATTTAGGGATGCAGATAGAA  600
HERV-K50B        481 GATAAATTAATTGACTGTTATACATTTCTGCAAGCAGAGGTTGCCAATGCTGGACTGGCAATAGCATCTGATAAGATCCAAACCTCTACTCCTTTTCATTATTTAGGGATGCAGATAGAA  600
HERV-K106        481 GATAAATTAATTGACTGTTACACATTTCTGCAAGCAGAGGTTGCCAATGCTGGACTGGCAATAGCATCTGATAAGATCCAAACCTCTACTCCTTTTCATTATTTAGGGATGCAGATAGAA  600
HERV-K101        481 GATAAATTAATTGACTGTTATACATTTCTGCAAGCAGAGGTTGCCAGTGCTGGACTGGCAATAGCATCTGATAAGATCCAAACCTCTACTCCTTTTCATTATTTAGGGATGCAGATAGAA  600
HERV-K103        481 GATAAATTAATTGACTGTTATACATTTCTGCAAGCAGAGGTTGCCAATGCTGGACTGGCAATAGCATCTGATAAGATCCAAACCTCTACTCCTTTTCATTATTTAGGGATGCAGATAGAA  600
CERV-K100        481 GATAAATTAATTGACTGTTATACATTTCTGCAAGCAGAGGTTGCCAATGCTGGACTGGCAATAGCATCCGATAAGATCCAAACCTCTACTCCTTTTCATTATTTAGGGATGCAGATAGAA  600
HERV-K10         481 GATAAATTAATTGACTGTTATACATTTCTGCAAGCAGAGGTTGCCAATGCTGGACTGGCAATAGCATCCGATAAGATCCAAACCTCTACTCCTTTTCATTATTTAGGGATGCAGATAGAA  600
HERV-K108        481 GATAAATTAATTGACTGTTATACATTTCTGCAAGCAGAGGTTGCCAATGCTGGACTGGCAATAGCATCTGATAAGATCCAAACCTCTACTCCTTTTCATTATTTAGGGATGCAGATAGAA  600
HERV-K109        476 GATAAATTAATTGACTGTTATACATTTCTGCAAGCAGAGGTTGCCAATGCTGGACTGGCAATAGCATCTGATAAGATCCAAACCTCTACTCCTTTTCATTATTTAGGGATGCAGATAGAA  595
HERV-K113        481 GATAAATTAATTGACTGTTATACATTTCTGCAAGCAGAGGTTGCCAATGCTGGACTGGCAATAGCATCTGATAAGATCCAAACCTCTACTCCTTTTCATTATTTAGGGATGCAGATAGAA  600
HERV-K36         481 GATAAATTAATTGACTGTTATACATTTCTGCAGGCAGAGGTTGCCAATGCTGGACTGGCAATAGCATCTGATAAGATCCAAACCTCTACTCCTTTTCATTATTTAGGGATGCAGATAGAA  600
HERV-K115        481 GATAAATTAATTGACTGTTATACATTTCTGCAAGCAGAGGTTGCCAGTGCTGGACTGGCAATAGCATCTGATAAGATCCAAACCTCTACTCCTTTTCATTATTTAGGGATGCAGATAGAA  600
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HERV-K102        481 GATAAATTAATTGACTGTTATACATTTCTGCAAGCAGAGGTTGCCAATGCTGGACTGGCAATAGCATCTGATAAGATCCAAACCTCTACTCCTTTTCATTATTTAGGGATGCAGATAGAA  600
HERV-K50A        481 GATAAATTAATTGACTGTTATACATTTCTGCAAGCAGAGGTTGCCAATGCTGGACTGGCAATAGCATCTGATAAGATCCAAACCTCTACTCCTTTTCATTATTTAGGGATGCAGATAGAA  600
                     ** * ** ************ ***********  ********** *  ** ******    * ******* ******** ** *******  ****** ***** ** *  *****  * **

CERV-KOLD35587   600 GAAATGAAAATTAAACCACAAAAAATAGAAATAAGAAAAGACACATTAAAAACATTAAATGACTTTCAAAAATTGCTAGGAGATATTAATTGGATTCAGCCAACTCTAGGCATCTCTACT  719
HERV-KOLD35587   600 GAAACGAAAATTAAACCATAAAAAATAGAAATAAGAAAAGACACATTAAAAACATTAAATGACTTTCAAAAATTGCTAGGAGATATTAATTGGATTCAGCCAACTCTAGGCATCTCTACT  719
SERV-K1 chr.11   601 AATAGAAAAATTAAGCCACAAAAAATAGAAATAAGAAAAGACACATTAAAAACATTAAATGACTTTCAGAAATTGTTAGGCGATATTAATTGGATTCGGCCAACTCTAGGCATTCCTACT  720
Guereza          601 AATAGAAAAATTAAGCCACAAAAAATAGAAATAAGAAAAGATGCATTAAAAACATTAAATGATTTTCAAAAATTGTTAGGTGATATTAATTGGATTCGGCCAACTCTAGGCATTCCTACT  720
SERV-K1 chr.12   601 AGTAGAAAAATTAAGCCACAAAAAATAGAAATAAGAAAAGATACTTTAAAAACTTAAATGACTTTCAAAAATTGTTAGGCGATATTAATTGGATTCGGCCAACTCTAGGCATTCCTACT  720
Grivet           601 AATAGAAAAATTAAGCCACAAAAAATAGAAATAAGAAAAGATACATTAAAAACATTAAATGACTTTCAAAAATTGTTAGGCGATATTAATTGGATTCGGCCAACTCTAGGCATTCCTACT  720
SERV-K1 chr.5    601 ATTAGAAAATTTAAGCCACAAAAAATAGAAATAA-----GACACATTAAAAACACCAAATGACTTTCAGAAATTGCTAGGCGATATTAATTGGATTCGGCCAACTCTAGGCATTCCTACT  715
CERV-K60         601 AATAGAAAAATTAAGCCACAAAAAATAGAAATAAGAAAAGACACATTAAAAACACTAAATGATTTTCAAAAATTGCTGGGAGATATTAATTGGATTCGGCCAACTCTAGGCATTCCTACT  720
CERV-K102        601 AATAGAAAAATTAAGCCACAAAAAATAGAAATAAGAAAAGACACATTAAAAACACTAAATGATTTTCAAAAATTGCTGGGAGATATTAATTGGATTCGGCCAACTCTAGGCATTCCTACT  720
HERV-K104        601 AATAGAAAAATTAAGCCACAAAAAATAGAAATAAGAAAATACACATTAAAAACACTAAATGATTTTCAAAAATTGCTGGGAGATATTAATTGGATTCGGCCAACTCTAGGCATTCCTACT  720
HERV-K50D        601 AATAGAAAAATTAAGCCACAAAAAATAGAAATAAGAAAATACACATTAAAAACACTAAATGATTTTCAAAAATTGCTGGGAGATATTAATTGGATTCGGCCAACTCTAGGCATTCCTACT  720
HERV-K50B        601 AATAGAAAAATTAAGCCACAAAAAATAGAAATAAGAAAAGACACATTAAAAACACTAAATGATTTTCAAAAATTACTAGGAGATATTAATTGGATTCGGCCAACTCTAGGCATTCCTACT  720
HERV-K106        601 AATAGAAAAATTAAGCCACAAAAAATAGAAATAAGAAAAGACACATTAAAAACACTAAATGATTTTCAAAAATTACTAGGAGATATTAATTGGATTTGGCCAACTCTAGGCATTCCTACT  720
HERV-K101        601 AATAGAAAAATTAAGCCACAAAAA-TAGAAATAAGAAAAGACACATTAAAAACACTAAATGATTTTCAAAAATTACTAGGAGATATTAATTGGATTCGGCCAACTCTAGGCATTCCTACT  719
HERV-K103        601 AATAGAAAAATTAAGCCACAAAAAATAGAAATAAGAAAAGACACATTAAAAACACTAAATGATTTTCAAAAATTACTAGGAGATATTAATTGGATTCGGCCAACTCTAGGCATTCCTACT  720
CERV-K100        601 AATAGAAAAATTAAGCCACAAAAAATAGAAATAAGAAAAGACACATTAAAAACACTAAATGATTTTCAAAAATTACTAGGAGATATTAATTGGATTCGGCCAACTCTAGGCATTCCTACT  720
HERV-K10         601 AATAGAAAAATTAAGCCACAAAAAATAGAAATAAGAAAAGACACATTAAAAACACTAAATGATTTTCAAAAATTACTAGGAGATATTAATTGGATTCGGCCAACTCTAGGCATTCCTACT  720
HERV-K108        601 AATAGAAAAATTAAGCCACAAAAAATAGAAATAAGAAAAGACACATTAAAAACACTAAATGATTTTCAAAAATTACTAGGAGATATTAATTGGATTCGGCCAACTCTAGGCATTCCTACT  720
HERV-K109        596 AATAGAAAAATTAAGCCACAAAAAATAGAAATAAGAAAAGACACATTAAAAACACTAAATGATTTTCAAAAATTACTAGGAGATATTAATTGGATTTGGCCAACTCTAGGCATTCCTACT  715
HERV-K113        601 AATAGAAAAATTAAGCCACAAAAAATAGAAATAAGAAAAGACACATTAAAAACACTAAATGATTTTCAAAAATTACTAGGAGATATTAATTGGATTCGGCCAACTCTAGGCATTCCTACT  720
HERV-K36         601 AATAGAAAAATTAAGCCACAAAAAATAGAAATAAGAAAAGACACATTAAAAGCACTAAATGATTTTCAAAAATTACTAGGAGATATTAATTGGATTCGGCCAACTCTAGGCATTCCTACT  720
HERV-K115        601 AATAGAAAAATTAAGCCACAAAAAATAGAAATAAGAAAAGACACATTAAAAACACTAAATGATTTTCAAAAATTACTAGGAGATATTAATTGGATTCAGCCAACTCTAGGCATTCCTACT  720
HERV-K102        601 AATAGAAAAATTAAGCCACAAAAAGTAGAAATAAGAAAAGACACATTAAAAACACTAAATGATTTTCAAAAATTACTAGGAGATATTAATTGGATTCGGCCAACTCTAGGCATTCCTACT  720
HERV-K50A        601 AATAGAAAAATTAAGCAACAAAAAATAGAAATAAGAAAAGACACATTAAAAACACTAAATGATTTTCAAAAATTACTAGGAGATATTAATTGGATTCGGCCAACTCTAGGCATTCCTACT  720
                         *  *** **** * * ***** **********      *  * ******  *   ******* ***** *****  * ** ****************  ****************  *****

CERV-KOLD35587   720 TATGCCATGTCAAATTTGTTCTCTATCTTAAGAGGGGATCCAGAATTGAATAGTAAAAGAACATTAACTCCAGAAGCAACTAAAGAAATTGAATTAGTTGAAGAAAAAATTCGGTCAGCA  839
HERV-KOLD35587   720 TATGCCATGTCAAATTTGTTCTCTATCTTAAGAGGGGATCCAGAATTGAATAGTAAAAGAACATTAACTCCAGAAGCAACTAAAGAAATTGAATTAGTTGAAGAAAAAATTCGGTCAGCA  839
SERV-K1 chr.11   721 TATGCCATGTCAAATTTGTTCTCTATCCTTAGAGGAGACCCAGACTTAAATAGTAAAAGAATATTAACCCCAGAGGCAACAAAAGAAATTAAATTAGTGGAAGAAAAAATTCAGTCAGCG  840
Guereza          721 TATGCCATGTCAAATTTGTTCTCTATCCTAAGAGGAGATCCAGACTTAAATAGTAGGAGAATATTAACCCCAGAGGCAACAAAAGAAATTAAATTAGTGGAAGAAAAAATTCAGTCAGCG  840
SERV-K1 chr.12   721 TATGCCATGTCAAATTTGTTCTCTATCCTAAGAGGAGATCCAGACTTAAATAGTAAAAGAATATTAACCCCAGAGGCAACAAAAGAAATTAAATTAGTGGAAGAAAAAATTCAGTCAGCG  840
Grivet           721 TATGCCATGTCAAATTTGTTCTCTATCCTAAGAGGAGATCCAGACTTAAATAGTAAAAGAATATTAACCCCAGAGGCAACAAAAGAAATTAAATTAGTGGAAGAAAAAATTCAGTCAGCG  840
SERV-K1 chr.5    716 TATGCCATGTCAAATTTGTTCTCTATCCTAAGAGGAGACCCAGACTTAAACAGTAAAAGAATATTAACCCCAGAGGCAACAAAAGAAATTAAATTAGTGGAAGAAAAATTCAGTCAGTG  835
CERV-K60         721 TATGCCATGTCAAATTTGTTCTCTATCTCAAGAGGAGACTCAGACTTAAATAGTAAAAGAATGTTAACCCCAGAGGCAACAAAAGAAATTAAATTAGTGGAAGAAAAAATTCAGTCAGCG  840
CERV-K102        721 TATGCCACGTCAAATTTGTTCTCTATCTTAAGAGGAGACTCAGACTTAAATAGTAAAAGAATGTTAACCCCAGAGGCAACAAAAGAAATTAAATTAGTGGAAGAAAAAATTCAGTCAGCA  840
HERV-K104        721 TATGCCATGTCAAATTTGTTCTCTATCTTAAGAGGAGACTCAGACTTAAATAGTAAAAGAATGTTAACCCCAGAGGCAACAAAAGAAATTAAATTAGTGGAAGAAAAAATTCAGTCAGTG  840
HERV-K50D        721 TATGCCATGTCAAATTTGTTCTCTATCTTAAGAGGAGACTCAGACTTAAATAGTAAAAGAATGTTAACCCCAGAGGCAACAAAAGAAATTAAATTAGTGGAAGAAAAAATTCAGTCAGTG  840
HERV-K50B        721 TATGCCATGTCAAATTTGTTCTCTATATTAAGAGGAGACTCAGACTTAAATAGTAAAAGAATATTAACCCCAGAGGCAACAAAAGAAATTAAATTAGTGGAAGAAAAAATTCAGTCAGCG  840
HERV-K106        721 TATGCCATGTCAAATTTGTTCTCTATCTTAAGAGGAGACTCAGACTTAAATAGTAAAAGAATGTTAACCCCAGAGGCAACAAAAGAAATTAAATTAGTGGAAGAAAAAATTCAGTCAGCG  840
HERV-K101        720 TATGCCATGTCAAATTTGTTCTCTATCTTAAGAGGAGACTCAGACTTGAATAGTAAAAGAATATTAACCCCAGAGGCAACAAAAGAAATTAAATTAGTGGAAGAAAAAATTCAGTCAGCG  839
HERV-K103        721 TATGCCATGTCAAATTTGTTCTCTATCTTAAGAGGAGACTCAGACTTAAATAGTAAAAGAATATTAACCCCAGAGGCAACAAAAGAAATTAAATTAGTGGAAGAAAAAATTCAGTCAGCG  840
CERV-K100        721 TATGCCATGTCAAATTTGTTCTCTATCTTAAGAGGAGACTCAGACTTAAATAGTCAAAGAATATTAACCCCAGAGGCAACAAAAGAAATTAAATTAGTGGAAGAAAAAATTCAGTCAGCG  840
HERV-K10         721 TATGCCATGTCAAATTTGTTCTCTATCTTAAGAGGAGACTCAGACTTAAATAGTCAAAGAATATTAACCCCAGAGGCAACAAAAGAAATTAAATTAGTGGAAGAAAAAATTCAGTCAGCG  840
HERV-K108        721 TATGCCATGTCAAATTTGTTCTCTATCTTAAGAGGAGACTCAGACTTAAATAGTAAAAGAATGTTAACCCCAGAGGCAACAAAAGAAATTAAATTAGTGGAAGAAAAAATTCAGTCAGCG  840
HERV-K109        716 TATGCCATGTCAAATTTGTTCTCTATCTTAAGAGGAGACTCAGACTTAAATAGTAAAAGAATGTTAACCCCAGAGGCAACAAAAGAAATTAAATTAGTGGAAGAAAAAATTCAGTCAGCG  835
HERV-K113        721 TATGTCATGTCAAATTTGTTCTCTATCTTAAGAGGAGACTCAGACTTAAATAGTAAAAGAATGTTAACCCCAGAGACAACAAAAGAAATTAAATTAGTGGAAGAAAAAATTCAGTCAGCA  840
HERV-K36         721 TATGCCATGTCAAATTTGTTCTCTATCTTAAGAGGAGACTCAGACTTAAATAGTAAAAGAATGTTAACCCCAGAGGCAACAAAAGAAATTAAATTAGTGGAAGAAAAAATTCAGTCAGCG  840
HERV-K115        721 TATGCCATGTCAAATTTGTTCTCTATCTTAAGAGGAGACTCAGACTTAAATAGTAAAAGAATATTAACCCCAGAGGCAACAAAAGAAATTAAATTAGTGGAAGAAAAAATTCAGTCAGCA  840
HERV-K102        721 TATGCCATGTCAAATTTGTTCTCTATCTTAAGAGGAGACTCAGACTTAAATAGTAAAAGAATATTAACCCCAGAGGCAACAAAAGAAATTAAATTAGTGGAAGAAAAAATTCAGTCAGCG  840
HERV-K50A        721 TATGCCATGTCAAATTTGTTCTCTATCTTAAGAGGAGACTCAGACTTAAATAGTAAAAGAATATTAACCCCAGAGGCAACAAAAGAAATTAAATTAGTGGAAGAAAAAATTCAGTCAGCG  840
                     **** ** ******************     ****** **  **** ** ** ***    ****  ****** ******  **** ********* ********** ******

CERV-KOLD35587   840 CAAGTAAATAGAATAGATCACTTAGCCCCACTCCAACTTTTGATTTTTGCTACTGCACATTCTCCAACAGGCATTATTGTTCAAAATACAGATCTTGTGGAGTGGTTCTTCCTTCCTCAT  959
HERV-KOLD35587   840 CAAGTAAATAGAATAGATCACTTAGCCCCACTCCAACTTTTGATTTTTGCTACTGCACATTCTCCAACAGGCATTATTGCTCAAAATACAGATCTTGTGGAGTGGTTCTTCCTTCCTCAT  959
SERV-K1 chr.11   841 CAAATAAGTAAAATAGATCCCTTAGCCCCACTCCAACTTTTGATTTTTGCTACTGCACATTCTCCAACAGGCATCATTGTTCAAAATACTGATCTTGTGGAGTGGTCATTCCTTCCTCAC  960
Guereza          841 CAAATAAATAGAATAGATTCTTTAGCCCCACTCCAACTTTTAATTTTTGCCACTGCACATTCTCCAACAGGCATCATTGTTCAAAATACTGATCTTGTAGAGTGGTCATTCCTTCCTCAC  960
SERV-K1 chr.12   841 CAAATAAATAGGATAGATCCTTTAGCCCCACTCCAACTTTTGATTTTTGCTACTACACATTCTCCAACAGGCATCATTATTCAAAACACTGATCTTGTGGAGTGGTCATTCCTTTTCTCAC  960
Grivet           841 CAAATAAGTAGAATAGATCCCTTAGCCCCACTCCAACTTTTAATTTTTGCTACTACACATTCTCCAACAGGCATCATTATTCAAAATACTGATCTTGTGGAGTGGTCATTTCTTCCTCAC  960
SERV-K1 chr.5    836 CAAATAAGTAGAATAGATCCCTTAGCCCCACTCCAACTTTTGATTTTTGCTACTGCACATTCTCCAACAGGCAGCATTGTTCAAAATACTGATCTTGTGGAGTGGTCGTTCCTTCCTCAG  955
CERV-K60         841 CAAATAAATAGAATAGATCCCTTAGCCCCACTCCAACTTTTGATTTTTGCCACTGCACATTCTCCAACAGGCATCATTATTCAAAATACTGATCTTGTGGAGTGGTCATTCCTTCCTCGC  960
CERV-K102        841 CAAATAAATAGAATAGATCCCTTAGCCCCACTCCAACTTTTGATTTTTGCCACTGCACATTCTCCAACAGGCATCATTATTCAAAATACTGATCTTGTGGAGTGGTCATTCCCTCCTCAC  960
HERV-K104        841 CAAATAAATAGAATAGATCCCTTAGCCCCACTCCAACTTTTGATTTTTGCCACTGCACATTCTCCAACAGGCATCATTATTCAAAATACTGATCTTGTGGAGTGGTCATTCCTTCCTCAC  960
HERV-K50D        841 CAAATAAATAGAATAGATCCCTTAGCCCCACTCCAACTTTTGATTTTTGCCACTGCACATTCTCCAACAGGCATCATTATTCAAAATACTGATCTTGTGGAGTGGTCATTCCTTCCTCAC  960
HERV-K50B        841 CAAATAAATAGAATAGATCCCTTAGCCCCACTCCAACTTTTGATTTTTGCCACTGCACATTCTCCAACAGGCATCATTATTCAAAATACTGATCTTGTGGAGTGGTCATTCCTTCCTCAC  960
HERV-K106        841 CAAATAAATAGAATAGATCCCTTAGCCCCACTCCAACTTTTGATTTTTGCCACTGCACATTCTCCAACAGGCATCATTATTCAAAATACTGATCTTGTGGAGTGGTCATTCCTTCCTCAC  960
HERV-K101        840 CAAATAAATAGAATAGATCCCTTAACCCCACTCCAACTTTTGATTTTTGCCACTGCACATTCTCCAACAGGCATCATTATTCAAAATACTGATCTTGTGGAGTGGTCATTCCTTCCTCAC  959
HERV-K103        841 CAAATAAATAGAATAGGTCCCTTAGCCCCACTCCAACTTTTGATTTTTGCCACTGCACATTCTCCAACAGGCATCATTATTCAAAATACTGATCTTGTGGAGTGGTCATTCCTTCCTCAC  960
CERV-K100        841 CAAATAAATAGAATAGATCCCTTAGCCCCACTCCAACTTTTGATTTTTGCCACTGCACATTCTCCAACAGGCATCATTATTCAAAATACTGATCTTGTGGAGTGGTCATTCCTTCCTCAC  960
HERV-K10         841 CAAATAAATAGAATAGATCCCTTAGCCCCACTCCAACTTTTGATTTTTGCCACTGCACATTCTCCAACAGGCATCATTATTCAAAATACTGATCTTGTGGAGTGGTCATTCCTTCCTCAC  960
HERV-K108        841 CAAATAAATAGAATAGATCCCTTAGCCCCACTCCAACTTTTGATTTTTGCCACTGCACATTCTCCAACAGGCATCATTATTCAAAATACTGATCTTGTGGAGTGGTCATTCCTTCCTCAC  960
HERV-K109        836 CAAATAAATAGAATAGATCCCTTAGCCCCACTCCAACTTTTGATTTTTGCCACTGCACATTCTCCAACAGGCATCATTATTCAAAATACTGATCTTGTGGAGTGGTCATTCCTTCCTCAC  955
HERV-K113        841 CAAATAAATAGAATAGATCCCTTAGCCCCACTCCGACTTTTGATTTTTGCCACTGCACATTCTCCAATAGGCATCATTATTCAAAATACTGATCTTGTGGAGTGGTCATTCCTTCCTCAC  960
HERV-K36         841 CAAATAAATAGAATAGATCCCTTAGCCCCACTCCAACTTTTGATTTTTGCCACTGCACATTCTCCAACAGGCATCATTATTCAAAATACTGATCTTGTGGAGTGGTCATTCCTTCCTCAC  960
HERV-K115        841 CAAATAAATAGAATAGATCCCTTAGCCCCACTCCAACTTTTGATTTTTGCCACTGCACATTCTCCAACAGGTATCATTATTCAAAATACTGATCTTGTGGAGTGGTCATTCCTTCCTCAC  960
HERV-K102        841 CAAATAAATAGAATAGATCCCTTAGCCCCACTCCAACTTTTGATTTTTGCCACTGCACATTCTCCAACAGGTATCATTATTCAAAATACTGATCTTGTGGAGTGGTCATTCCTTCCTCAC  960
HERV-K50A        841 CAAATAAATAGAATAGATCCCTTAGCCCCACTCCAACTTTTGATTTTTGCCACTGCACATTCTCCAACAGGTATCATTATTCAAAATACTGATCTTGTGGAGTGGTCATTCCTTCCTCAC  960
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CERV-KOLD35587   960 AGTACAATTAAGACTTTTACATTGTACTTGGATCAAATGGCTACATTAATTGGTCAGGCAAGATTACGAATTATAAAATTGTGTGAAAGTGACCCAGATAAAATCATTGTTCCTTTAAAC 1079
HERV-KOLD35587   960 AGTACAATTAAGACTTTTACATTGTACTTGGATCAAATGGCTACATTAATTGGTCAGGCAAGATTACGAATAATAAAATTGTGTGAAAGTGACTCAGATAAAATCATTGTTCCTTTAAAC 1079
SERV-K1 chr.11   961 AGTACAATTAAGACTTTTACATTGTACTTGGATGAAATAGCTACATTAATTGGTCAGGCAAGATTACAAATAATAAAATTGTGTGGTAATGACCCAGACAAAATAGTTGTTCCCTTAACC 1080
Guereza          961 AGTACAATTAAAACTTTTACATTGTACTTGGATCAAATAGCTACATTAATAGGTCAGACAAGATTACGAATAATAAAATTGTGTGGTAATGACCCAGACAAAATAGTTGTTCCTTAACC 1080
SERV-K1 chr.12   961 AGTACAATTAAGACTTTTACATTGTACTTGGATCGAATAGCTACATTAATTGGTCAGGCAAGATTACGGATAATAAAATTGTGTGGTAATGACCCAGACAAAATAGTTGTTCCTTTAACC 1080
Grivet           961 AGTACAATTAAGACTTTTACATTGTACTTGGATCAAATAGCTACATTAATTGGTCAGGCAAGATTACGAATAATAAAATTATGTGGTAATGACCCAGACAAAATAGTTGTTCCTTAACC 1080
SERV-K1 chr.5    956 AGTACAATTAAGACTTTTACGTTGTACTTGGATCAAATAGCTACATTAATTGGTCAGGCAAGATTACGAATAATAAAATTGTGTGGAAATGACCCAGACAAAATAGTTGTTCCTTTAACC 1075
CERV-K60         961 AGTACAGTTAAGACTTTTACATTGTACTTGGATCAAATAGCTACATTAATTGGTCAGACAAGATTACGAATAATAAAATTATGTGGAAATGACCCAGACAAAATAGTTGTCCCTTTAACC 1080
CERV-K102        961 AGTACAGTTAAGACTTTTACATTGTACTTGGATCAAATAGCTACATTAATTGGTCAGACAAGATTACGAATAATAAAATTATGTGGCAATGACCCAGACAAAATAGTTGTCCCTTTAACC 1080
HERV-K104        961 AGTACAGTTAAGACTTTTACATTGTACTTGGATCAAATAGCTACATTAATTGGTCAGACAAGATTACGAATAATAAAATTATGTGGAAATGACCCAGACAAAATAGTTGTCCCTTTAACC 1080
HERV-K50D        961 AGTACAGTTAAGACTTTTACATTGTACTTGGATCAAATAGCTACATTAATTGGTCAGACAAGATTACGAATAATAAAATTATGTGGAAATGACCCAGACAAAATAGTTGTCCCTTTAACC 1080
HERV-K50B        961 AGTACAGTTAAGACTTTTACATTGTACTTGGATCAAATAGCTACATTAATCGGTCAGACAAGATTACGAATAATAAAATTATGTGGAAATGACCCAGACAAAATAGTTGTCCCTTTAACC 1080
HERV-K106        961 AGTACAGTTAAGACTTTTACATTGTACTTGGATCAAATAGCTACATTAATCGGTCAGACAAGATTACGAATAATAAAATTATGTGGAAATGACCCAGACAAAATAGTTGTCCCTTTAACC 1080
HERV-K101        960 AGTACAGTTAAGACTTTTACATTGTACTTGGATCAAATGGCTACATTAATCGGTCAGACAAGATTACGAATAATAAAATTATGTGGAAATGACCAAGACAAAATAGTTGTCCCTTTAACC 1079
HERV-K103        961 AGTACAGTTAAGACTTTTACATTGTACTTGGATCAAATAGCTACATTAATCGGTCAGACAAGATTACGAATAACAAAATTATGTGGAAATGACCCAGACAAAATAGTTGTCCCTTTAACC 1080
CERV-K100        961 AGTACAGTTAAGACTTTTACATTGTACTTGGATCAAATAGCTACATTAATCGGTCAGACAAGATTACGAATAACAAAATTATGTGGAAATGACCCAGACAAAATAGTTGTCCCTTTAACC 1080
HERV-K10         961 AGTACAGTTAAGACTTTTACATTGTACTTGGATCAAATAGCTACATTAATCGGTCAGACAAGATTACGAATAACAAAATTATGTGGAAATGACCCAGACAAAATAGTTGTCCCTTTAACC 1080
HERV-K108        961 AGTACAGTTAAGACTTTTACATTGTACTTGGATCAAATAGCTACATTAATCGGTCAGACAAGATTACGAATAATAAAATTATGTGGGAATGACCCAGACAAAATAGTTGTCCCTTTAACC 1080
HERV-K109        956 AGTACAGTTAAGACTTTTACACTGTACTTGGATCAAATAGCTACATTAATCGGTCAGACAAGATTACGAATAATAAAATTATGTGGAAATGACCCAGACAAAATAGTTGTCCCTTTAACC 1075
HERV-K113        961 AGTACAGTTAAGACTTTTACATTGTACTTGGATCAAATAGCTACATTAATCGGTCAGACAAGATTACGAATAATAAAATTATGTGGGAATGACCCAGACAAAATAGTTGTCCCTTTAACC 1080
HERV-K36         961 AGTACAGTTAAGACTTTTACACTGTACTTGGATCAAATAGCTACATTAATTGGTCAGACAAGATTACGAATAATAAAATTATGTGGAAATGACCCAGACAAAATAGTTGTCCCTTTAACC 1080
HERV-K115        961 AGTACAGTTAAGACTTTTACACTGTACTTGGATCAAATAGCTACATTAATCGGTCAGACAAGATTACGAATAATAAAATTATGTGGAAATGACCCAGACAAAATAGTTGTCCCTTTAACC 1080
HERV-K102        961 AGTACAGTTAAGACTTTTACACTGTACTTGGATCAAATAGCTACATTAATTGGTCAGACAAGATTACGAATAATAAAATTATATGGAAATGACCCAGACAAAATAGTTGTCCCTTTAACC 1080
HERV-K50A        961 AGTACAGTTAAGACTTTTACACTGTACTTGGATCAAATAGCTACATTAATTGGTCAGACAAGATTACGAATAATAAAATTATGTGGAAATGACCCAGACAAAATAGTTGTCCCTTTAACC 1080
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CERV-KOLD35587  1080 AAGGAACAGGTTAGACAAGCCTTTATCAATTCTGGTGCATGGCAGATTGGTCTTGCTGATTTTGTGGGAATTATTGACAATCATTACCCAAAAACAAAAATCTTCCAGTTTTTAAAATTG 1199
HERV-KOLD35587  1080 AAGGAACAGGTTAGACAAGCCTTTATCAATTCTGGTGCATGGCAGATTGGTCTTGCTGATTTTGTGGGAATTATTGACAATCATTACCCAAAAACAAAAATCTTCCAGTTTTTAAAATTG 1199
SERV-K1 chr.11  1081 AAGGAACAAGTTAGACAAGCCTTTATCAATTCTGGTGCATAGCAGATTGGTCTTGCTGATTTTGTGGGAATTATTGATAATCATTACCCAAAAACAAAAATCTTCCAGTTTTTAAAATTG 1200
Guereza         1081 AAGGAACAAGTTAAACAAGCCTTTATCAATTCTGGTGCATGGCAAATTGGCCTTGCTGATTTTGTGGGAATTATTGATAATCATTACCCAAAAACAAAAATCTTCCAGTTTTTAAAATTG 1200
SERV-K1 chr.12  1081 AAGGAACAAGTTAGGCAAGCCTTTATCAATTCTGGTGCATGGCAGATTGGCCTTGCTAATTTTGTGGGAGTTATTGATAATCATTACCCAAAAACAAAAATCTTCCAGTTTTTAAAATTG 1200
Grivet          1081 AAGGAACAAGTTAGGCAAGCCTTTATCAATTCTGCTGCATGGCAGATTGGCCTTGCTAATTTTGTGGGAGTTATTGATAATCACTACCCAAAAACAAAAATCTTCCAGTTTTTAAAATTG 1200
SERV-K1 chr.5   1076 AAGGAACAAGTTAGACAAGCCTTTATCAGTTCTGGTGCATGGCAGATTGGTCTTGTTGATTTTGTGGGAATTATTGATAATCATTACCCAAAACCAAAAATCTTCCAGTTTTTAAAATTG 1195
CERV-K60        1081 AAGGAACAAGTTAGACAAGCCTTTATCAATTCTGGTGCATGGCAGATTGGTCTTGCTAATTTTGTGGGAATTATTGATAATCATTACCCAAAAACAAAAATCTTCCAGTTCTTAAAATTG 1200
CERV-K102       1081 AAGGAACAAGTTAGACAAGCCTTTATCAATTCTGGTGCATGGCAGATTGGTCTTGCTAATTTTGTGGGAATTATTGATAATCATTACCCAAAAACAAAAATCTTCCAGTTCTTAAAATTG 1200
HERV-K104       1081 AAGGAACAAGTTAGACAAGACTTTATCAATTCTGGTGCATGGCAGATTGGTCTTGCTAATTTTGTGGGAATTATTGATAATCATTACCCAAAAACAAAGATCTTCCAGTTCTTAAAATTG 1200
HERV-K50D       1081 AAGGAACAAGTTAGACAAGCCTTTATCAATTCTGGTGCATGGCAGATTGGTCTTGCTAATTTTGTGGGAATTATTGATAATCATTACCCAAAAACAAAGATCTTCCAGTTCTTAAAATTG 1200
HERV-K50B       1081 AAGGAACAAGTTAGACAAGCCTTTATCAATTCTGGTGCATGGCAGATTGGTCTTGCTAATTTTGTGGGAATTATTGATAATCATTACCCAAAAACAAAGATCTTCCAGTTCTTAAAAATG 1200
HERV-K106       1081 AAGGAACAAGTTAGACAAGCCTTTATCAATTCTGGTGCATGGCAGATTGGTCTTGCTAATTTTGTGGGAATTATTGATAATCATTACCCAAAAACAAAGATCTTCCAGTTCTTAAAACTG 1200
HERV-K101       1080 AAGGAACAAGTTAGACAAGCCTTTATCAATTCTGGTGCATGGCAGATTGGTCTTGCTAATTTTGTGGGAATTATTGATAATCATTACCCAAAAACAAAGATCTTCCAGTTCTTAAAATTG 1199
HERV-K103       1081 AAGGAACAAGTTAGACAAGCCTTTATCAATTCTGGTGCATGGCAGATTGGTCTTGCTAATTTTGTGGGAATTATTGATAATCATTACCCAAAAACAAAGATCTTCCAGTTCTTAAAATTG 1200
CERV-K100       1081 AAGGAACAAGTTAGACAAGCCTTTATCAATTCTGGTGCATGGCAGATTGGTCTTGCTAATTTTGTGGGACTTATTGATAATCATTACCCAAAAACAAAGATCTTCCAGTTCTTAAAATTG 1200
HERV-K10        1081 AAGGAACAAGTTAGACAAGCCTTTATCAATTCTGGTGCATGGCAGATTGGTCTTGCTAATTTTGTGGGACTTATTGATAATCATTACCCAAAAACAAAGATCTTCCAGTTCTTAAAATTG 1200
HERV-K108       1081 AAGGAACAAGTTAGACAAGCCTTTATCAATTCTGGTGCATGGAAGATTGGTCTTGCTAATTTTGTGGGAATTATTGATAATCATTACCCAAAAACAAAGATCTTCCAGTTCTTAAAATTG 1200
HERV-K109       1076 AAGGAACAAGTTAGACAAGCCTTTATCAATTCTGGTGCATGGAAGATTGGTCTTGCTAATTTTGTGGGAATTATTGATAATCATTACCCAAAAACAAAGATCTTCCAGTTCTTAAAATTG 1195
HERV-K113       1081 AAGGAACAAGTTAGACAAGCCTTTATCAATTCTGGTGCATGGCAGATTGGTCTTGCTAATTTTGTGGGAATTATTGATAACCATTACCCAAAAACAAAGATCTTCCAGTTCTTAAAATTG 1200
HERV-K36        1081 AAGGAACAAGTTAGACAAGCCTTTATCAATTCTGGTGCATGGCAGATTGGTCTTGCTAATTTTGTGGGAATTATTGATAATCATTACCCAAAAACAAAGATCTTCCAGTTCTTAAAATTG 1200
HERV-K115       1081 AAGGAACAAGTTAGACAAGCCTTTATCAATTCTGGTGCATGGCAGATTGGTCTTGCTAATTTTGTGGGAATTATTGATAATCATTACCCAAAAACAAAGATCTTCCAGTTCTTAAAACTG 1200
HERV-K102       1081 AAGGAACAAGTTAGACAAGCCTTTATCAATTCTGGTGCATGGCAGATTGGTCTTGCTAATTTTGTGGGAATTATTGATAATCATTACCCAAAAACAAAGATCTTCCAGTTCTTAAAACTG 1200
HERV-K50A       1081 AAGGAACAAGTTAGACAAGCCTTTATCAATTCTGGTGCATGGCAGATTGGTCTTGCTAATTTTGTGGGAATTATTGATAATCATTACCCAAAAACAAAGATCTTCCAGTTCTTAAAACTG 1200
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CERV-KOLD35587  1200 AATACTTGGATTTTACCTAAAATTACTAGACATAAACCTTTAGAAAATGCTCTGACAGTGTTTACTGATGGTTCCAGCAATGGAAAAGCGGCTTACACCAGGCCAAAAGAACGACTCATT 1319
HERV-KOLD35587  1200 ACTACTTGGATTTTACCTAAAATTACTAGACATAAACCTTTAGAAAATGCTCTGACAGTGTTTACTGATGGTTCCAGCAATGGAAAAGCGGCTTACACCAGGCCAAAAGAACGACTCATT 1319
SERV-K1 chr.11  1201 ACTACTTGGATTTTACCTAAAATTACAGACATGAACCTCTAGAAAATGCTCTGACAGTATTTACTGATGGTTCCAGCAATGGAAAAGCAGCTTACACAGGGCCAAAAGAGCGAGTAATC 1320
Guereza         1201 ACTACTTGGATTTTACCTAAAATTACCAGACATAAACCTTTAGAAAATGCTCTGACAGTATTTACTGATGGTTCCAGCAATGGAAAAGCGGCTTACACAGGGCCAAAAGAGCGAGTAATC 1320
SERV-K1 chr.12  1201 ACTACTTGGATTTTACCTAAAATCACCAGACATGAGCCTTTAGAAAATGCTCTGACAGTATTTACTGATGGTTCCAGCAATGGAAAAGCGGCTTACACAGGACCAAAAGAGCGAGTAATC 1320
Grivet          1201 ACTACTTGGATTTTACCTAAAATTACCAGACATGAGCCTCTAGAAAATGCTCTGACAATATTTACTGATGGTTCCAGCAATGGAAAAGCAGCTTACACAGGACCAAAAGAGCGAGTAATC 1320
SERV-K1 chr.5   1196 ACTACTTGGATTTTACCTAAAATTACCAGACATGAACCTTTAGAAAATGCTCTGACAGTATTTACTGATGGTTCCAGCAATGGAAAAGCGGCTTACACAGGGCCAAAAGAGCGAGTAATC 1315
CERV-K60        1201 ACTACTTGGATTCTACCTAAAATTACCAGACACGAACCTTTAGAAAATGCTCTAACAGTATTTAGTGATGGTTCCAGCAATGGAAAAGTGGCTTACACAGGGCCAAAAGAACGAGTAATC 1320
CERV-K102       1201 ACTACTTGGATTCTACCTAAAATTACCAGACGTGAACCTTTAGAAAATGCTCTAACAGTATTTACTGATGGTTCCAGCAATGGAAAAGCGGCTTACACAGGGCCGAAAGAACGAGTAATC 1320
HERV-K104       1201 ACTACTTGGATTCTACCTAAAATTACCAGACATGAACCTTTAGAAAATGCTCTAACAGTATTTACTGATGGTTCCAGCAATGGAAAAGCAGCTTACACAGGGCCGAAAGAACGAGTAATC 1320
HERV-K50D       1201 ACTACTTGGATTCTACCTAAAATTACCAGACATGAACCTTTAGAAAATGCTCTAACAATATTTACTGATGGTTCCAGCAATGGAAAAGCAGCTTACACAGGGCCGAAAGAACGAGTAATC 1320
HERV-K50B       1201 ACTACTTGGATTCTACCTAAAATTACCAGACGTGAACCTTTAGAAAATGCTCTAACAGTATTTACTGATGGTTCCAGCAATGGAAAAGCAGCTTACACAGGGCCGAAAGAACGAGTAATC 1320
HERV-K106       1201 ACTACTTGGATTCTACCTAAAATTACCAGACGTGAACCTTTAGAAAATGCTCTAACAGTATTTACTGATGGTTCCAGCAATGGAAAAGCAGCTTACACAGGGCCGAAAGAACGAGTAATC 1320
HERV-K101       1200 ACTACTTGGATTCTACCTAAAATAACCAGACGTGAACCTTTAGAAAATGCTCTAACAGTATTTACTGATGGTTCCAGCAATGGAAAAGCAGCTTACACAGGGCCGAAAGAACGAGTAATC 1319
HERV-K103       1201 ACTACTTGGATTCTACCTAAAATTACCAGATGTGAACCTTTAGAAAATGCTCTAACAGTATTTACTGATGGTTCCAGCAATGGAAAAGCAGCTTACACAGGACCGAAAGAACGAGTAATC 1320
CERV-K100       1201 ACTACTTGGATTCTACCTAAAATTACCAGACGTGAACCTTTAGAAAATGCTCTAACAGTATTTACTGATGGTTCCAGCAATGGAAAAGCAGCTTACACAGGGCCGAAAGAACGAGTAATC 1320
HERV-K10        1201 ACTACTTGGATTCTACCTAAAATTACCAGACGTGAACCTTTAGAAAATGCTCTAACAGTATTTACTGATGGTTCCAGCAATGGAAAAGCAGCTTACACAGGGCCGAAAGAACGAGTAATC 1320
HERV-K108       1201 ACTACTTGGATTCTACCTAAAATTACCAGACGTGAACCTTTAGAAAATGCTCTAACAGTATTTACTGATGGTTCCAGCAATGGAAAAGCAGCTTACACAGGACCGAAAGAACGAGTAATC 1320
HERV-K109       1196 ACTACTTGGATTCTACCTAAAATTACCAGACGTGAACCTTTAGAAAATGCTCTAACAGTATTTACTGATGGTTCCAGCAATGGAAAAGCAGCTTACACAGGGCCGAAAGAACGAGTAATC 1315
HERV-K113       1201 ACTACTTGGATTCTACCTAAAATTACCAGACGTGAACCTTTAGAAAATGCTCTAACAGTATTTACTGATGGTTCCAGCAATGGAAAAGCAGCTTACACAGGGCTGAAAGAACGAGTAATC 1320
HERV-K36        1201 ACTACTTGGATTCTACCTAAAATTACCAGACGTGAACCTTTAGAAAATGCTCTAACAGTATTTACTGATGGTTCCAGCAATGGAAAAGCAGCTTACACAGGGCCGAAAGAACGAGTAATC 1320
HERV-K115       1201 ACTACTTGGATTCTACCTAAAATTACCAGACGTGAACCTTTAGAAAATGCTCTAACAGTATTTACTGATGGTTCCAGCAATGGAAAAGCAGCTTACACAGGGCCGAAAGAACGAGTAATC 1320
HERV-K102       1201 ACTACTTGGATTCTACCTAAAATTACCAGACGTGAACCTTTAGAAAATGCTCTAACAGTATTTACTGATGGTTCCAGCAATGGAAAAGCAGCTTACACAGGACCGAAAGAACGAGTAATC 1320
HERV-K50A       1201 ACTACTTGGATTCTACCTAAAATTACCAGACGTGAACCTTTAGAAAATGCTCTAACAGTATTTACTGATGGTTCCAGCAATGGAAAAGCAGCTTACACAGGACCGAAAGAACGAGTAATC 1320
                     * ********** ********** *  ***    * *** ************** *** * ***** ************************  ********  * *  *****  ** * **

CERV-KOLD35587  1320 GAAACTCAATATCACTGGGCTCAAAGAGCAGAGTTGGTTGCTGTCATTATAGTGTTACAAGATTTTAATCAGCCTATTAACATTGTATCAGATTCTGCATATGTAGTACAGGCTACAAAG 1439
HERV-KOLD35587  1320 GAAACTCAATATCACTGGGCTCAAAGAGCAGAGTTGGTTGCTGTCATTACAGTGTTACAAGATTTTAATCAGCCTATTAACATTGTATCAGATTCTGCATATGTAGTACAGGCTACAAAG 1439
SERV-K1 chr.11  1321 AAAACTCAATATCAATCGGCTCAAAGGGCAGAGTTGGTTGCAGTCATTACAGTGTTACAAGATTTTAAGCAACCTGTTAATATTATATCAGATTCTGCATATGTAGTACAGGCTACAAGG 1440
Guereza         1321 AAAACTCAATATCAATCAGCTCAAAGGGCAGAGTTGGTTGCAGTCATTACAGTGTTAGAAGATTTTAATCAACCTGTTAATATTATATCAGATTCTGCATATGTAGTACAGGCTACAAGG 1440
SERV-K1 chr.12  1321 AAAACTCAGTATCAATCAGCTCAAAGAGCAGAGTTGGTTGCAGTCATTACAGTGTTACAAGATTTTAATCAACCTGTTAATATTATATCAGATTCTGCATATGTAGTACAGGCTACAAGG 1440
Grivet          1321 AAAACTCAGTATCAATCAGCTCAAAGGGCAGAGTTGGTTGCAGTCATTACAGTGTTACAAGATTTTAATCAACCTGTTAATATTATATCAGATTCTGCATATGTAGTACAGGCTACAAAG 1440
SERV-K1 chr.5   1316 AAAACTCAATATCAATCG--ATATTGGGCAGAGTTGGTTGCAGTCATTACAGTGTTACAAGATTGTAATCAACCTGTTAATATTGTATCAGATTCTGCATATGTAGTACAGGCTACAAGG 1433
CERV-K60        1321 AAAACTCCATATTAATCGGCTCAAAGAGCAGAGTTGGTTGCAGTCATTACAGTGTTACAAGATTTTGATCAACCTATCAATATTATATCAGATTCTGCATATGTAGTACAGGCTACAAGG 1440
CERV-K102       1321 AAAACTCCGTATCAATCGGCTCAAAGAGCAGAGTTGGTTGCAGTCATTACAGTGTTACAAGATTTTGACCAACCTATCAATATTATATCAGATTCTGCATATGTAGTACAGGCTACAAGG 1440
HERV-K104       1321 AAAACTCCATATCAATCAGCTCAAAGAGCAGAGTTGGTTGCAGTCATTACAGTGTTACAAGATTTTGACCAACCTATCAATATTATATCAGATTCTGCATATGTAGTACAGGCTACAAGG 1440
HERV-K50D       1321 AAAACTCCATATCAATCAGCTCAAAGAGCAGAGTTGGTTGCAGTCATTAAAGTGTTACAAGATTTTGACCAACCTATCAATATTATATCAGATTCTGCATATGTAGTACAGGCTACAAGG 1440
HERV-K50B       1321 AAAACTCCATATCAATCTGCTCAAAGAGCAGAGTTGGTTGCAGTCATTACAGTGTTACAAGATTTTGACCAACCTATCAATATTATATCAGATTCTGCCTATGTAGTACAGGCTACAAGG 1440
HERV-K106       1321 AAAACTCCATATCAATCAGCTCAAAGAGCAGAGTTGGTTGCAGTCATTACAGTGTTACAAGATTTTGACCAACCTATCAATATTATATCAGATTCTGCATATGTAGTACAGGCTACAAGG 1440
HERV-K101       1320 AAAACTCCATATCAGTCGGCTCAAAGAGCAGAGTTGGTTGCAGTCATTACAGTGTTACAAGATTTTGACCAACCTATCAATATTATATCAGATTCTGCCTATGTAGTACAGGCTACAAGG 1439
HERV-K103       1321 AAAACTCCATATCAATCGGCTCAAAGAGCAGAGTTGGTTGCAGTCATTACAGTGTTACAAGATTTTGACCAACCTATCAATATTATATCAGATTCTGCATATGTAGTACAGGCTACAAGG 1440
CERV-K100       1321 AAAACTCCATATCAATCGGCTCAAAGAGACGAGTTGGTTGCAGTCATTACAGTGTTACAAGATTTTGACCAACCTATCAATATTATATCAGATTCTGCATATGTAGTACAGGCTACAAGG 1440
HERV-K10        1321 AAAACTCCATATCAATCGGCTCAAAGAGACGAGTTGGTTGCAGTCATTACAGTGTTACAAGATTTTGACCAACCTATCAATATTATATCAGATTCTGCATATGTAGTACAGGCTACAAGG 1440
HERV-K108       1321 AAAACTCCATATCAATCGGCTCAAAGAGCAGAGTTGGTTGCAGTCATTACAGTGTTACAAGATTTTGACCAACCTATCAATATTATATCAGATTCTGCATATGTAGTACAGGCTACAAGG 1440
HERV-K109       1316 AAAACTCCATATCAATCGGCTCAAAGAGCAGAGTTGGTTGCAGTCATTACAGTGTTACAAGATTTTGACCAACCTATCAATATTATATCAGATTCTGCATATGTAGTACAGGCTACAAGG 1435
HERV-K113       1321 AAAACTCCATATCAATCGGCTCAAAGAGCAGAGTTGGTTGCAGTCATTACAGTGTTACAAGATTTTGACCAGCCTATCAATATTATATCAGATTCTGCATATGTAGTACAGGCTACAAGG 1440
HERV-K36        1321 AAAACTCCATATCAATCGGCTCAAAGAGCAGAGTTGGTTGCAGTCATTACAGTGTTACAAGATTTTGA------TATCAATATTATATCAGATTCTGCCTATGTAGTACAGGCTACAAGG 1434
HERV-K115       1321 AAAACTCCATATCAATCAGCTCAAAGAGCAGAGTTGGTTGCAGTCATTACAGTGTTACAAGATTTTGACCAACCTATCAATATTATATCAGATTCTGCATATGTAGTACAGGCTACAAGG 1440
HERV-K102       1321 AAAACTCCATATCAATCGGCTCAAAGAGCAGAGTTGGTTGCAGTCATTACAGTGTTACAAGATTTTGACCAACCTATCAATATTATATCAGATTCTGCATATGTAGTACAGGCTACAAGG 1440
HERV-K50A       1321 AAAACTCCATATCAATCGGCTCAAAGAGCAGAGTTGGTTGCAGTCATTACAGTGTTACAAGATTTTGACCAACCTATCAATATTATACAGATTCTGCATATGTAGTACAGGCTACAAGG 1440
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CERV-KOLD35587  1440 GATGTTGAGACAGCCCTAATCAAATATAGTATAGATGATCAGTTAAACCAGCTGTTTAATATGTTACAACAAACTGTAAGAAAAAGAAATTTCCCATTTTATGTTACTCATATTCGAGCA 1559
HERV-KOLD35587  1440 GATGTTGAGACAGCCCTAATCAAATATAGTATAGATGATCAGTTAAACCAGCTGTTTAATATGTTACAACAAACTGTAAGAAAAAGAAATTTCCCATTTTATGTTACTCATATTCGAGCA 1559
SERV-K1 chr.11  1441 GATGTTGAGACAGCTCTAATTAAATATAGCATGGATGATCAGTTAAACCAGCTGTTCAATTTACTACAACAAACTGTAAGAAAAAGGAATTTCCCATTTTATATTACTCATATTCGAGCA 1560
Guereza         1441 GATGTTGAGACAGCTCTAATTAAATATAGCATGGATGATCAGTTAAACCAGCTATTCACTTTGTTACAACAAACTGTAAGAAAGAGAAATTTCCCATTTTATATTACTCATATTCGAGCA 1560
SERV-K1 chr.12  1441 GATGTTGAGACAGCTCTAATTAAATATAGCATGGATGATCAGTTAAACCAGTTGTTTAATTTATTACAACAAACTGTAAGAAAAAGAATTTCTCATTTTATATTACTCATATTCGAGCA 1560
Grivet          1441 GATGTTGAGACAGCTCTAATTAAATATAGCATGGATGATCAGTTAAACCAGTTGTTCAATTTATTACAACAAACTGTAAGAAAAAGGAATTTCCCATTTTATATTACTCATATTCGAGCA 1560
SERV-K1 chr.5   1434 GATGTTGAGACAGCTCTAATTAAATACAGCATGGATGATCAGTTAAACCAGCTGTTCAATTTATTACAACAAACTGTAAGAAAAAGGAATTTCCCATTTTATATTACTGGTATTCGAGCA 1553
CERV-K60        1441 GATGTTGAGACAGCTCTAATTAAATATAGCATGGACGATCAGTTAAACCAGCTATTCAATTTATTACAACAAACTGTAAGAAAAAGAAACTTCCCATTTTATATTACTCATATTCGAGCA 1560
CERV-K102       1441 GATGTTGAGACAGCTCTAATTAAATATAGCATGGATGATCAGTTAAACCAGCTATTCAATTTATTACAACAAACTGTAAGAAAAAGAAATTTCCCATTTTATATTACTCATATTCGAGCA 1560
HERV-K104       1441 GATGTTGAGACAGCTCTAATTAAATATAGCATGGATGATCAGTTAAACCAGCTATTCAATTTATTACAACAAACTGTAAGAAAAAGAAATTTCCCATTTTATATTACTCATATTCGAGCA 1560
HERV-K50D       1441 GATGTTGAGACAGCTCTAATTAAATATAGCATGGATGATCAGTTAAACCAGCTATTCAATTTATTACAACAAACTGTAAGAAAAAGAAATTTCCCATTTTATATTACTCATATTCGAGCA 1560
HERV-K50B       1441 GATGTTGAGACAGCTCTAATTAAATATAGCATGGATGATCAGTTAAACCAGCTATTCAATTTATTACAACAAACTGTAAGAAAAAGAAATTTCCCATTTTATATTACTCATATTCGAGCA 1560
HERV-K106       1441 GATGTTGAGACAGCTCTAATTAAATATAGCATGGATGATCAGTTAAACCAGCTATTCAATTTATTACAACAAACTGTAAGAAAAAGAAATTTCCCATTTTATATTACTCATATTCGAGCA 1560
HERV-K101       1440 GATGTTGAGACAGCTCTAATTAAATATAGCATGGATGATCAGTTAAACCAGCTATTCAATTTATTACAACAAACTGTAAGAAAAAGAAATTTCCCATTTTATATTACTCATATTCGAGCA 1559
HERV-K103       1441 GATGTTGAGACAGCTCTAATTAAATATAGCATGGATGATCAGTTAAACCAGCTATTCAATTTATTACAACAAACTGTAAGAAAAAGAAATTTCCCATTTTATATTACTCATATTCGAGCA 1560
CERV-K100       1441 GATGTTGAGACAGCTCTAATTAAATATAGCATGGATGATCAGTTAAACCAGCTATTCAATTTATTACAACAAACTGTAAGAAAAAGAAATTTCCCATTTTATATTACTCATATTCGAGCA 1560
HERV-K10        1441 GATGTTGAGACAGCTCTAATTAAATATAGCATGGATGATCAGTTAAACCAGCTATTCAATTTATTACAACAAACTGTAAGAAAAAGAAATTTCCCATTTTATATTACTCATATTCGAGCA 1560
HERV-K108       1441 GATGTTGAGACAGCTCTAATTAAATATAGCATGGATGATCAGTTAAACCAGCTATTCAATTTATTACAACAAACTGTAAGAAAAAGAAATTTCCCATTTTATATTACAACATATTCGAGCA 1560
HERV-K109       1436 GATGTTGAGACAGCTCTAATTAAATATAGCATGGATGATCAGTTAAACCAGCTATTCAATTTATTACAACAAACTGTAAGAAAAAGAAATTTCCCATTTTATATTACTCATATTCGAGCA 1555
HERV-K113       1441 GATGTTGAGACAGCTCTAATTAAATATAGCATGGATGATCAGTTAAACCAGCTATTCAATTTATTACAACAAACTGTAAGAAAAAGAAATTTCCCATTTTATATTACTCATATTCGAGCA 1560
HERV-K36        1435 GATGTTGAGACAGCTCTAATTAAATATAGCATGGATGATCAGTTAAACCAGCTATTCAATTTATTACAACAAACTGTAAGAAAAAGAAATTTCCCATTCTATATTACTCATATTCGAGCA 1554
HERV-K115       1441 GTTGTTGAGACAGCTCTAATTAAATATAGCATGGATGATCAGTTAAACCAGCTATTCAATTTATTACAACAAACTGTAAGAAAAAGAAATTTCCCATTTTATATTACTCATATTCGAGCA 1560
HERV-K102       1441 GATGTTGAGACAGCTCTAATTAAATATAGCATGGATGATCAGTTAAACCAGCTATTCAATTTATTACAACAAACTGTAAGAAAAAGAAATTTCCCATTTTATATTACTCATATTCGAGCA 1560
HERV-K50A       1441 GATGTTGAGACAGCTCTAATTAAATATAGCATGGATGATCAGTTAAACCAGCTATTCAATTTATTACAACAAACTGTAAGAAAAAGAAATTTCCCATTTTATATTACTCATATTCGAGCA 1560
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CERV-KOLD35587  1560 CATACTAATTTATCAGAGCCTTTAACTAAAGCAAATGAACAAGTTGACTTGCTAGTATCATCTGCATTCCTGGAAGCACAAGAACTTCATGCCTTGACTCATGTAAATGCAACAGGACTA 1679
HERV-KOLD35587  1560 CATACTAATTTACCAGAGCCTTTAACTAAAGCAAATGAACAAGTTGACTTGCTAGTATCATCTGCATTCCTGGAAGCACAAGAACTTCATGCCTTGACTCATGTAAATGCAACAGGACTA 1679
SERV-K1 chr.11  1561 CACACTAATTTACCAGGACCTTTAACTAAAGCAAATGAACAAGCTGACTTACTGGTATCCTCTGCATTCATAAAAGCACAAGAACTTCATGCTTTGACTCATGTAAATGCAGCAGGGTTA 1680
Guereza         1561 CACACTAATTTACCAGGACCTTTAACTAAAGCTAATGAAAAGGCTGACTTACTGGTATCCTCTGCATTCATAAAAGCACAAGAACTTCATGCTTTGACTCATGTAAATGCAGCAGGGTTA 1680
SERV-K1 chr.12  1561 CACACTAATTTGCCAGGACCTTTAACTATAGCAAATGAACAAGCTGACTTACTGGTATCCTCTGCATTCATAAAAGCACAAGAACTTCATGCTTTGACTCATGTAAATGCAGCAGGGTTA 1680
Grivet          1561 CACACTAATTTACCAGGACCTTAAACTATAGCAAATGAACAAGCTGACTTACTGGTATCCTCTGCATTCTTAAAAGCACAAGAACTTCATGCTTTAACTCATGTAAATGCAGCAGGGTTA 1680
SERV-K1 chr.5   1554 CACACTAATTTACCGGGACCTTTAACTAAAGGAAATGAACAAGCTGACTTACTAGTATCATATGCATTCATAAAAGCATAAGAACTTCATGTTTTAACTCATGTAAATGCAGCAGGGTTA 1673
CERV-K60        1561 CACACTAATTTACCAGGGCCTTTGACTAAAGCAAATGAACAAGCTGACTTACTGGTGTCATCTGCATTCATAAAAGCACAAGAACTTCATGCTTTGACTCATGTAAATGCAGCAGGATTA 1680
CERV-K102       1561 CACACTAATTTACCAGGGCCTTTGACTAAAGCAAATGAACAAGCTGACTTACTGGTATCATCTGCATTCATAAAAGCACAAGAACTTCATGCTTTGACTCATGTAAATGCAGCAGGATTA 1680
HERV-K104       1561 CACACTAATTTACCAGGGCCTTTGACTAAAGCAAATGAACAAGCTGACTTACTGGTATCATCTGCATTCATAAAAGCACAAGAACTTCATGCTTTGACTCATGTAAATGCAGCAGGATTA 1680
HERV-K50D       1561 CACACTAATTTACCAGGGCCTTTGACTAAAGCAAATGAACAAGCTGACTTACTGGTATCATCTGCATTCATAAAAGCACAAGAACTTCATGCTTTGACTCATGTAAATGCAGCAGGATTA 1680
HERV-K50B       1561 CACACTAATTTACCAGGGCCTTTGACTAAAGCAAATGAAGAAGCTGACTTACTGGTATCATCTGCACTCATAAAAGCACAAGAACTTCATGCTTTGACTCATGTAAATGCAGCAGGATTA 1680
HERV-K106       1561 CACACTAATTTACCAGGGCCTTTGACTAAAGCAAATGAAGAAGCTGACTTACTGGTATCATCTGCACTCATAAAAGCACAAGAACTTCATGCTTTGACTCATGTAAATGCAGCAGGATTA 1680
HERV-K101       1560 CACACTAATTTACCAGGGCCTTTGACTAAAGCAAATGAACAAGCTGACTTACTGGTATCATCTGCACTCATAAAAGCACAAGAACTTCATGCTTTGACTCATGTAAATGCAGCAGGATTA 1679
HERV-K103       1561 CACACTAATTTACCAGGGCCTTTGACTAAAGCAAATGAACAAGCTGACTTACTGGTATCATCTGCACTCATAAAAGCACAAGAACTTCATGCTTTGACTCATGTAAATGCAGCAGGATTA 1680
CERV-K100       1561 CACACTAATTTACCAGGGCCTTTGACTAAAGCAAATGAACAAGCTGACTTACTGGTATCATCTGCACTCATAAAAGCACAAGAACTTCATGCTTTGACTCATGTAAATGCAGCAGGATTA 1680
HERV-K10        1561 CACACTAATTTACCAGGGCCTTTGACTAAAGCAAATGAACAAGCTGACTTACTGGTATCATCTGCACTCATAAAAGCACAAGAACTTCATGCTTTGACTCATGTAAATGCAGCAGGATTA 1680
HERV-K108       1561 CACACTAATTTACCAGGGCCTTTGACTAAAGCAAATGAACAAGCTGACTTACTGGTATCATCTGCACTCATAAAAGCACAAGAACTTCATGCTTTGACTCATGTAAATGCAGCAGGATTA 1680
HERV-K109       1556 CACACTAATTTACCAGGGCCTTTGACTAAAGCAAATGAACAAGCTGACTTACTGGTATCATCTGCACTCATAAAAGCACAAGAACTTCATGCTTTGACTCATGTAAATGCAGCAGGATTA 1675
HERV-K113       1561 CACACTAATTTACCAGGGCCTTTGACTAAAGCAAATGAACAAGCTGACTTACTGGTATCATCTGCACTCATAAAAGCACAAGAACTTCATGCTTTGACTCATGTAAATGCAGCAGGATTA 1680
HERV-K36        1555 CACACTAATTTACCAGGGCCTTTGACTAAAGCAAATAAACAAGCTGACTTACTGGTATCATCTGCACTCATAAAAGCACAAGAACTTCATGCTTTGACTCATGTAAATGCAGCAGGATTA 1674
HERV-K115       1561 CACACTAATTTACCAGGGCCTTTGACTAAAGCAAATGAACAAGCTGACTTACTGGTATCATCTGCACTCATAAAAGCACAAGAACTTCATGCTTTGACTCATGTAAATGCAGCAGGATTA 1680
HERV-K102       1561 CACACTAATTTACCAGGGCCTTTGACTAAAGCAAATGAACAAGCTGACTTACTGGTATCATCTGCACTCATAAAAGCACAAGAACTTCATGCTTTGACTCATGTAAATGCAGCAGGATTA 1680
HERV-K50A       1561 CACACTAATTTACCAGGGCCTTTGACTAAAGCAAATGAACAAGCTGACTTACTGGTATCATCTGCACTCATAAAAGCACAAGAACTTCATGCTTTGACTCATGTAAATGCAGCAGGATTA 1680
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CERV-KOLD35587  1680 AAAAATAAATTTGACATCACATGGAAACAGGCAAAAAATATTGTACAACATTACACCCAGTGTCAGATTCCA---CACCTGCCCACTCAGGAGGCAGGAGTTAATCCCAGAGGTCTGTGT 1796
HERV-KOLD35587  1680 AAAAATAAATTTGACATCACATGGAAACAGGCAAAAAATATTGTACAACATTACACCCAGTGTCAGATTCCA---CACCTGCCCACTCAGGAGGCAGGAGTTAATCCCAGAGGTCTGTGT 1796
SERV-K1 chr.11  1681 AAAAACAAATTTAATGTCACATGGAAACAGGCAAAAGATATAGTACAACATTGCACCCAGTGTCAAGTACTA---CACCTGCCCACTCAAGAGGCAGGAGTTAATCCCAGAGGTCTGTGT 1797
Guereza         1681 AAAAACAAATTTGATGTCACATGGAAACAGGCAAAAAATATTGTACAACATTGTACCCAGTGTCAAGTACTA---CACCTGCCCACCCAAGAGGCAGGAGTTAACCCCAGAGGTCTGTGT 1797
SERV-K1 chr.12  1681 AAAAACAAATTTGATGTCACATGGAAACAGGCAAAAGATATTGTACGACATTGCACCCAGTGTCAAGTACTA---CACCTGCCCACTCAAGAGGCAGGAGTGAATCCCAGAGGTCTGTGT 1797
Grivet          1681 AAAAACAAATTTGATGTCACATGGAAACAGGCAAAAGATATTGTACAACACTGCACCCAGTGTCAAGTACTA---CACCTGCCCACTCAAGAGGCAGGAGTTAATCCCAGAGGTCTGTGT 1797
SERV-K1 chr.5   1674 AAAAACAAATTTGATGTCACTTGGAAACAGGCAAAAGATATTGTACAACCCTGCACCCAATGTTAAGTCCTA---CACCTGCCCACTCAAGAGGCAGGAGTTAATCCCAGAGGTCTGTGT 1790
CERV-K60        1681 AAAAACAAATTTGATGTCACATGGAAACAGGCAAAAGATATTGTACAACATTGCACCCAGTGTCAAGTCTTA---CACCTGCCCACTCAAGAGGCAGGAGTTAATCCCAGAGGTCTGTGT 1797
CERV-K102       1681 AAAAACAAATTTGATGTCACATGGAAATAGGCAAAAGATATTGTACAACATTGCACCCAGTGTCAAGTCTTA---CACCTGCCCACTCAAGAGGCAGGAGTTAATCCCAGAGGTCTGTGT 1797
HERV-K104       1681 AAAAACAAATTTGATGTCACATGGAAACAGGCAAAAGATATTGTACAACATTGCACCCAGTGTCAAGTCTTA---CACCTGCCCACTCAAGAGGCAGGAGTTAATCCCAGAGGTCTGTGT 1797
HERV-K50D       1681 AAAAACAAATTTGATGTCACATGGAAACAGGCAAAAGATATTGTACAACATTGCACCCAGTGTCAAGTCTTA---CACCTGCCCACTCAAGAGGCAGGAGTTAATCCCAGAGGTCTGTGT 1797
HERV-K50B       1681 AAAAACAAATTTGATGTCACATGGAAACAGGCAAAAGATATTGTACAACATTGCACCCAGTGTCAAGTCTTA---CACCTGCCCACTCAAGAGGCAGGAGTTAATCCCAGAGGTCTGTGT 1797
HERV-K106       1681 AAAAACAAATTTGATGTCACATGGAAACAGGCAAAAGATATTGTACAACATTGCACCCAGTGTCAAGTCTTA---CACCTGCCCACTCAAGAGGCAGGAGTTAATCCCAGAGGTCTGTGT 1797
HERV-K101       1680 AAAAACAAATTTGATGTCACATGGAAACAGGCAAAAGATATTGTACAACATTGCACCCAGTGTCAAGTCTTA---CACCTGCCCACTCAAGAGGCAGGAGTTAATCCCAGAGGTCTGTGT 1796
HERV-K103       1681 AAAAACAAATTTGATGTCACATGGAAACAGGCAAAAGATATTGTACAACATTGCACCCAGTGTCAAGTCTTA---CACCTGCCCACTCAAGAGGCAGGAGTTAATCCCAGAGGTCTGTGT 1797
CERV-K100       1681 AAAAACAAATTTGATGTCACATGGAAACAGGCAAAAGATATTGTACAACATTGCACCCAGTGTCAAGTCTTA---CACCTGCCCACTCAAGAGGCAGGAGTTAATCCCAGAGGTCTGTGT 1797
HERV-K10        1681 AAAAACAAATTTGATGTCACATGGAAACAGGCAAAAGATATTGTACAACATTGCACCCAGTGTCAAGTCTTA---CACCTGCCCACTCAAGAGGCAGGAGTTAATCCCAGAGGTCTGTGT 1797
HERV-K108       1681 AAAAACAAATTTGATGTCACATGGAAACAGGCAAAAGATATTGTACAACATTGCACCCAGTGTCAAGTCTTA---CACCTGCCCACTCAAGAGGCAGGAGTTAATCCCAGAGGTCTGTGT 1797
HERV-K109       1676 AAAAACAAATTTGATGTCACATGGAAACAGGCAAAAGATATTGTACAACATTGCACCCAGTGTCAAGTCTTA---CACCTGCCCACTCAAGAGGCAGGAGTTAATCCCAGAGGTCTG--- 1789
HERV-K113       1681 AAAAACAAATTTGATGTCACATGGAAACAGGCAAAAGATATTGTACAACATTGCACCCAGTGTCAAGTCTTA---CACCTGCCCACTCAAGAGGCAGGAGTTAATCCCAGAGGTCTGTGT 1797
HERV-K36        1675 AAAAACAAATTTGATGTCACATGGAAACTGGCAAAAGATATTGTACAACATTGCACCCAGTGTCAAGTCTTA---CACCTGCCCACTCAAGAGGCAGGAGTTAATCCCAGAGGTCTGTGT 1791
HERV-K115       1681 AAAAACAAATTTGATGTCACATGGAAACAGGCAAAAGATATTGTACAACATTGCACCCAGTGTCAAGTCTTA---CACCTGCCCACTCAAGAGGCAGGAGTTAATCCCAGAGGTCTGTGT 1797
HERV-K102       1681 AAAAACAAATTTGATGTCACATGGAAACAGGCAAAAGATATTGTACAACATTGCACCCAGTGTCAAATCTTA---CACCTGCCCACTCAAGAGGCAGGAGTTAATCCCAGAGGTCTGTGT 1797
HERV-K50A       1681 AAAAACAAATTTGATGTCACATGGAAACAGGCAAAAGATATTGTACAACATTGCACCCAGTGTCAAATCTTA---CACCTGCCCACTCAAGAGGCAGGAGTTAATCCCAGAGGTCTGTGT 1797
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CERV-KOLD35587  1797 CCTAATGCGTTATGGCAAATGGATGTCACACATGTACCTTCAGTTGGAAAATTGTCATTTCTCCATGTGACAGTTGATGCTTATTCACATTTTGTAAGGGCAAACTGCCAGACAGGAGAA 1916
HERV-KOLD35587  1797 CCTAATGCGTTATGGCAAATGGATGTCACACATGTACCTTCAGTTGGAAAATTGTCATTTCTCCATGTGACAGTTGATGCTTATTCACATTTTGTAAGGGCAAACTGCCAGACAGGAGAA 1916
SERV-K1 chr.11  1798 CCTAATGCATTATGGCAAATGGATGTTACACATGTACCTTCATTTGGAAAATTATCATATGTTCATGTAACAGTTGATACTTATTCACATTTCATATGGGCAGCCTGCCAGACAGGAGAA 1917
Guereza         1798 CCTAATGCCTTATGGCAAATGGATGTTACCCATGTACCTTCATTTGGAAAATTATCATATGTTCATGTAACAGTTAATACTTATTCACATTTCATATGGGCAACATGCCAGACAGGAGAA 1917
SERV-K1 chr.12  1798 CCTAATGCATTATGGCAAATGGATGTTACCCATGTACCTTCATTTGGAAAATTATCATATGTTCATGTAACAGTTGATACTTATTCACATTTCATATGGGCAACATGCCAGACAGGAGAA 1917
Grivet          1798 CCTAATGCATTATGGCAAATGGATGTTACCCATGTACCTTCATTTGGAAAATTATCATATGTTCATGTAACAGTTGATACTTATTCACATTTCATATGGGCAACATGCCAGACAGGAGAA 1917
SERV-K1 chr.5   1791 CCTAACGCATTATGGCAAATGTATGTTACACATGTACCTTCATTTGGAAAATTATCATACGTTCATGAAACAGTTGATACTTATTCACATTTCATATGGGCAACCTGCTAGACAGGAGAA 1910
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HERV-K106       1798 CCTAATGCATTATGGCAAATGCATGTCACGCATGTACCTTCATTTGGAAGATTATCATATGTTCATGTAACAGTTGATACTTATTCACATTTCATATGGGCAACTTGCCAAACAGGAGAA 1917
HERV-K101       1797 CCTAATGCATTATGGCAAATGGATGTCACGCATGTACCTTCATTTGGAAGATTATCATATGTTCATGTAACAGTTGATACTTATTCACATTTCATATGGGCAACTTGCCAAACAGGAGAA 1916
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HERV-K50A       1798 CCTAATGCATTATGGCAAATGGATGTCACGCATGTACCTTCATTTGGAAGATTATCATATGTTCACGTAACAGTTGATACTTATTCACATTTCATATGGGCAACTTGCCAAACAGGAGAA 1917
                                                  *** ** ************ ****** *** **     * ** *  ** *** ** **************  ** *****   *** * *********

CERV-KOLD35587  1917 AATACTTCCCATGTTAAAAGACATTTAGTATCTTGTTTTGCTGTCATGGGAGTTCCAGAAAAAATTAAAACGCATAATGGGCCAGGATACTGTAGTAAGACATTTCAAAAATTCTTAAAT 2036
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SERV-K1 chr.11  1918 AGTACTTCCCATGTTAAAAAACACTTATTATCTTGCTTTGCTGTAATGGGAGTTCCAGAAAAAATTAAAACTGACAATGGACCAAGATATTGTAGTAAAGCTTTCCAAAAATTCTTAAAT 2037
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SERV-K1 chr.12  1918 AGTACTTCCCATGTTAAAAAACATTTATTATCTTGTTTTGCTGTAATGGGAGTTCCAGAAAAAATTAAAACTGACAATGGACCAGGTTATTGTAGTAAAGCTTTCCAAAAATTCTTAAAT 2037
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                     * ****************** *** * * * * ***     ** * ********************** *****    ***** *** * ** ********  * ** ************** *
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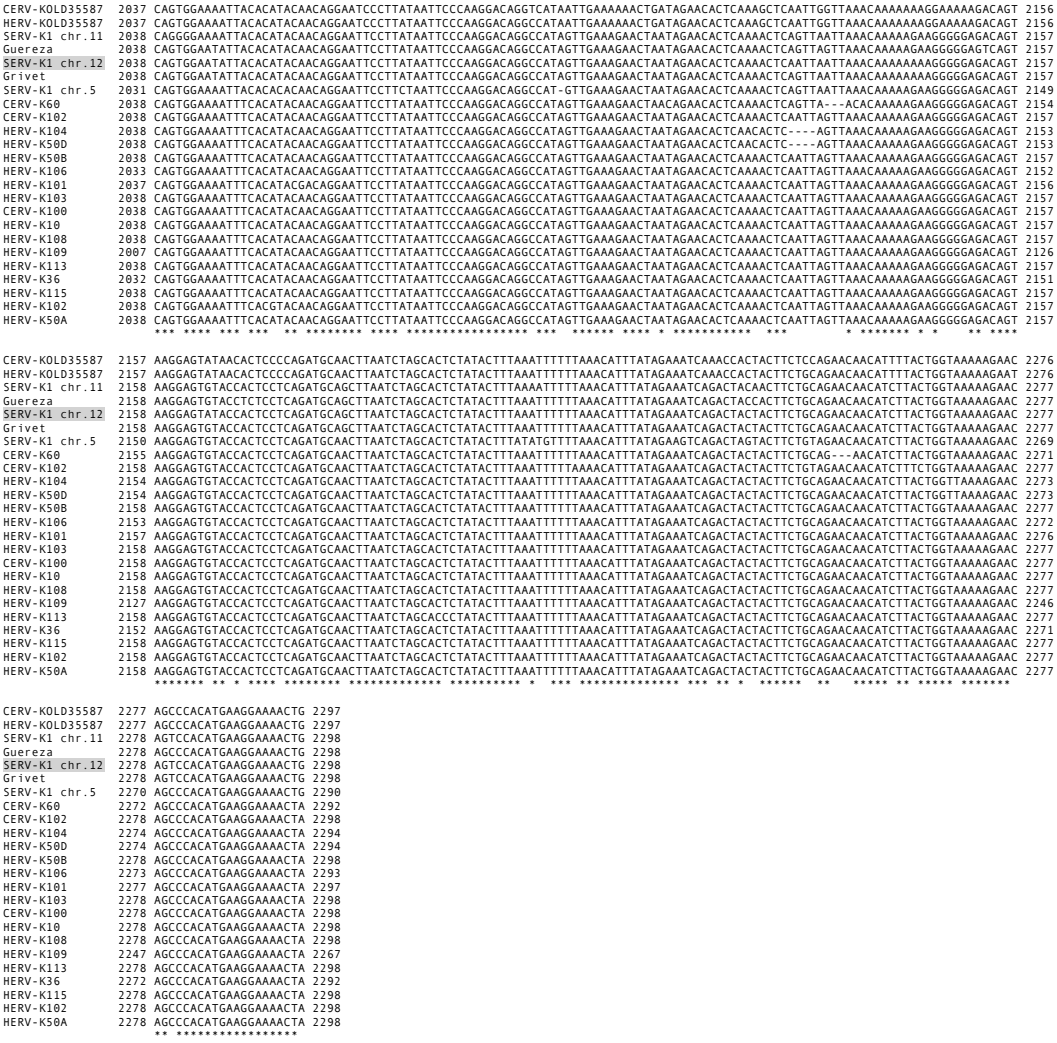
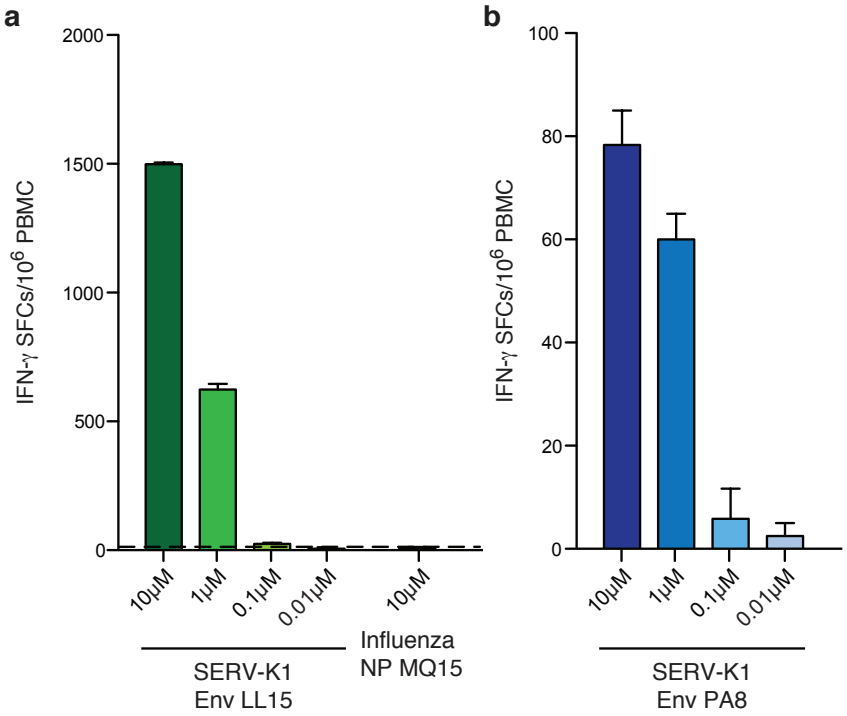
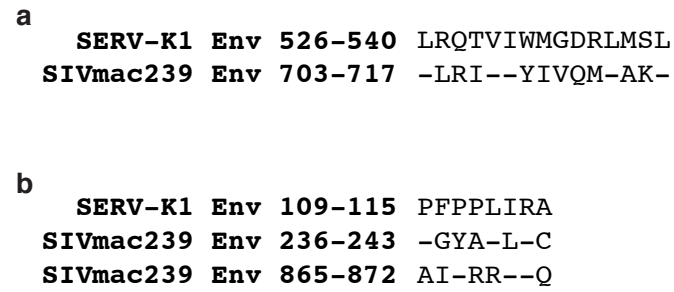


Appendix 1 Figure S3: Alignment of SERV-K to primate ERVs. Nucleotide alignment showing part of the *pol* locus of SERV-K from rhesus macaque r02120 aligned to various ERVs from hominids (humans, chimpanzees) and old world monkeys. Matches at each amino acid position are indicated with asterisks (*). The SERV-K1 locus described here is highlighted in grey. CERV = chimpanzee endogenous retrovirus; HERV = human endogenous retrovirus. Sequences shown here were used to construct phylogram in Figure 1d.



Appendix 1 Figure S4: ELISPOT titration of SERV-K1 Env-specific T cell responses. (A) Titration of SERV-K1 Env LL15-specific CD4+ T cell response in r02120 (1 week prior to SIV infection) using decreasing concentrations of peptide. Influenza nucleoprotein 15-mer MQ15 is included as a negative control. **(B)** Titration of SERV-K1 Env PA8-specific CD8+ T cell response in r02120 (20 weeks post-SIV infection) using decreasing concentrations of peptide.



Appendix 1 Figure S5: Alignments of SERV-K1 Env T cell epitopes to SIVmac239 Env peptides. (A) Amino acid alignment of r02120 CD4+ T cell epitope SERV-K1 Env LL15 to SIVmac239 Env LL15. This is the only SIVmac239 Env peptide with 10 or less mismatches to SERV-K1 Env LL15. **(B)** Amino acid alignment of r02120 CD8+ T cell epitope SERV-K1 Env PA8 to SIVmac239 Env PC8 and AQ8. These are the only SIVmac239 Env peptides with 5 or less mismatches to SERV-K1 Env PA8. For both (a) and (b), matches to the SERV-K1 Env peptide are shown as dashes.

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Appendix 2: Tertiary mutations stabilize CD8+ T lymphocyte escape-associated compensatory mutations following transmission of simian immunodeficiency virus.

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Author contributions

The author created the SIVmac239-2V, SIVmac239-2V-P, and SIVmac239-P viruses, and performed and made figures for the experiments shown in Figure 4B, C, and E.

I. The problem and scientific approach

Reversion of particular CD8+ T cell escape mutations is well documented following human immunodeficiency virus (HIV) transmission between HLA-discordant individuals and is dependent upon the fitness cost of the escape mutation. Escape mutations that exact a high fitness cost often revert following HIV transmission to an HLA-discordant individual, while those that impose a low fitness cost do not (*1*) (*2*) (*3*). Two epitopes targeted by CD8+ T cell responses associated with long-term HIV control, HLA-B*27-bound $_{Gag263\text{-}272}$ KK10 and HLA-B*57-bound $Gag_{240-249}$ TW10, lie within a conserved region of Gag, and escape mutations within these epitopes reduce viral fitness. However, while the codon 242 threonine-to-asparagine (T242N) escape mutation within $Gag_{240-249}$ TW10 reverts readily following transmission to an HLA-disparate host, the R264K escape mutation within $Gag_{263-272}$ KK10 does not (*3*) (*4*). This finding is surprising given that the R264K mutation reduces viral replicative fitness to a significantly greater extent than the T242N mutation (*5*). Compensatory mutations that maintain viral fitness while allowing for intra-epitopic variation have been described for both $Gag_{263-272}$ KK10 and $Gag_{240-249}$ TW10, and the difference in reversion kinetics between these epitopes is likely dependent on these compensatory mutations (*4*) (*6*) (*7*).

In simian immunodeficiency virus mac239 (SIVmac239)-infected rhesus macaques, the Mamu-A1*001:01-bound CD8+ T cell epitope $Gag_{181-189}$ CM9 is analogous to $Gag_{263-272}$ KK10 and $Gag_{240-249}$ TW10 in that it lies within a conserved region of Gag and has associated compensatory mutations that increase the fitness of viral variants containing $Gag_{181-189}$ CM9 escape mutations. We, and others, have previously shown that the canonical $Gag_{181-189}$ CM9 escape mutation T182A is

frequently linked with the flanking downstream mutation I206V and reverts following transmission to Mamu-A1*001:01-negative rhesus macaques (*8*) (*9*) (*10*). Additionally, the I206V compensatory mutation is consistently associated with a subsequent upstream mutation, I161V. This mutation is not necessary for the emergence of viruses with the T182A escape mutation, but it is believed to further increase viral fitness following mutations at both T182 and I206.

II. Materials, Methods, & Results

A. Pre-existing compensatory mutations I161V and I206V do not facilitate rapid $Gag_{181-189}$ CM9 escape

We hypothesized that preexisting I161V and I206V compensatory mutations would facilitate rapid, acute-phase escape within the conserved $Gag_{181–189}$ CM9 CD8+ T cell epitope upon transmission to Mamu-A1*001:01-positive rhesus macaques. To test this hypothesis, we engineered an SIVmac239 variant harboring the I161V and I206V compensatory mutations associated with $Gag_{181–189}$ CM9 escape (SIVmac239-2V) by site-directed mutagenesis of SIVmac239 plasmid DNA using the QuikChange XL kit (Stratagene, La Jolla, CA) and generated a virus stock as previously described (*2*). Following intravenous infection of a Mamu-A1*001:01-positive rhesus macaque, r98003, with 100 ng Gag p27CA, SIVmac239-2V replicated efficiently, peaking at 3.96×10^7 viral RNA (vRNA) copies/ml plasma at 10 days post-infection (d.p.i.), as measured by quantitative reverse transcription-PCR (RT-PCR) using previously described techniques (*11*) (Appendix 2 Figure 1A). This level of viral replication is consistent with a large body of data collected from wild-type SIVmac239-infected Mamu-A1*001:01-positive rhesus macaques (*12*) (*13*). Additionally, major histocompatibility complex (MHC) class

I tetramer staining of freshly isolated peripheral blood mononuclear cells (PBMC) revealed that SIVmac239-2V elicited a strong CD8+ T cell response against $Gag_{181-189}$ CM9, with 3.5% of all circulating CD8+ T cells targeting this epitope at 14 d.p.i. (Appendix 2 Figure 1A). Importantly, this $Gag_{181-189}$ CM9-specific CD8+ T cell response remained at high frequency in r98003 through 15 weeks p.i. (w.p.i.).

Next, we monitored viral evolution within *gag* by performing Roche/454 pyrosequencing on plasma samples as described previously (*8*). Unexpectedly, instead of observing rapid, acute-phase escape of $Gag_{181-189}$ CM9, we detected high-frequency reversions of both the I161V and I206V compensatory mutations (Appendix 2 Figure 1B). The frequency of revertant viruses in plasma increased through 8.5 w.p.i., when 98.3% of plasma virus sequences were wild-type SIVmac239 at I206 and 84% were wild-type SIVmac239 at I161. Subsequently, we detected two $Gag_{181-189}$ CM9 escape mutations, C181G and T182A, only after the I206V compensatory mutation had reverted almost entirely to wild type, with I206V present in only 1.7% of all pyrosequencing reads (Appendix 2 Figure 1B). The C181G mutation is rare but is a previously described $Gag_{181-189}$ CM9 escape mutation that also associates with the I161V and I206V compensatory mutations in *Mamu-A1*001:01*-positive, SIVmac239-infected rhesus macaques (*8*) (*9*). These three mutations increased in frequency together and were tightly linked, with 87% of sequences containing one or both escape mutations also harboring the I206V compensatory mutation (data not shown). While we failed to observe rapid acute-phase CD8+ T cell escape, r98003 did manifest the earliest $Gag_{181-189}$ CM9 escape mutations of nine *Mamu-A1*001:01*-positive rhesus macaques that we have investigated previously via Roche/454 pyrosequencing (*8*) (Appendix 2 Figure 1C).

B. The SIV compensatory mutations Gag I161V and I206V do not always revert following transmission to Mamu-A1*001:01-negative rhesus macaques.

Given the surprising reversion of the compensatory mutations following infection of a naive host, we next investigated additional animals infected with SIVmac239 harboring the pre-engineered I161V and I206V mutations. Friedrich et al. previously described three rhesus macaques infected with 3XSIVmac239, a virus encoding the $Gag_{181–189}$ CM9 escape mutation T182A and the two compensatory substitutions I161V and I206V (*2*). We Roche/454 pyrosequenced virus from plasma samples obtained from these three animals, spanning the first 8 months of infection, to make direct comparisons with virus sequences from r98003 and to glean additional insight into why SIVmac239-2V reverted at positions I161V and I206V before $Gag_{181–189}$ CM9 escape. Two of these rhesus macaques were Mamu-A1*001:01-negative (r98020, r96115), and one was Mamu-A1*001:01-positive (r97089). The 3XSIVmac239 circulating in r97089 retained all three (I161V, T182A, and I206V) pre-engineered mutations, most likely due to low-frequency Mamu-A1*001:01-restricted, $Gag_{181–189}$ CM9-specific CD8+ T cells (*14*) (Appendix 2 Figure 2A). In contrast, 3XSIVmac239 evolution diverged between the two Mamu-A1*001:01-negative animals. The 3XSIVmac239 circulating in r96115 reverted completely to wild type at all three positions (I161, T182, and I206) by 24 w.p.i., supporting our data from r98003 (Appendix 2 Figure 2B). Surprisingly, 3XSIVmac239 in r98020 reverted completely to wild type at position T182 by 24 w.p.i. but maintained the compensatory mutations I161V and I206V in 100% of sequence reads through 30 w.p.i. (Appendix 2 Figure 2C). This finding is in contrast to our data from r98003 and r96115,

in which compensatory mutations I161V and I206V reverted in the absence of a $Gag_{181–189}$ CM9 escape mutation (Appendix 2 Figures 1B and 2B).

C. The downstream Gag mutation A312P stabilizes SIV containing compensatory mutations I161V and I206V in the absence of a $Gag_{181–189}$ CM9 escape mutation.

The divergent viral evolution between r98003, r96115, and r98020 raised the possibility that a tertiary mutation was maintaining viral fitness while allowing for the retention of the I161V and I206V compensatory mutations. To test this hypothesis, we analyzed Gag sequences from a cohort of 25 chronically SIV-infected rhesus macaques, including r98003, r97089, and r98020, at various time points postinfection (11 *Mamu*-A1*001:01-positive, 14 Mamu-A1*001:01-negative macaques) using consensus Sanger sequencing as previously described (*15*). We found that viral sequences from five of seven SIVmac239-infected, Mamu-A1*001:01-positive animals contained mutations I161V, T182A/C, I206V, and a downstream mutation, Gag A312P (Appendix 2 Figure 3A). Virus from the two remaining Mamu-A1*001:01-positive animals (r98001 and r99084) harbored the A312P mutation alone, suggesting that this mutation can precede the I161V, T182A/C, and I206V mutations. In contrast, none of these mutations were observed in Mamu-A1*001:01-negative animals (Appendix 2 Figure 3A). Thus, it appeared that A312P was associated with $Gag_{181–189}$ CM9 escape. Next, we examined the association of A312P with the maintenance of the I161V and I206V compensatory mutations following transmission of our engineered viruses to naive hosts. In two out of the three Mamu-A1*001:01-positive animals infected with 3XSIVmac239 (r97089 and r97035), the A312P mutation arose following infection, which again suggested that A312P stabilizes the $Gag_{181–189}$ CM9 escape-associated compensatory mutations

(Appendix 2 Figure 3B). In the two Mamu-A1*001:01-negative animals infected with 3XSIVmac239 (r98020 and r96115), A312P was present only in r98020, where the compensatory mutations persisted in the absence of $Gag1_{81-189}$ CM9 escape. In r98003, the Mamu-A1*001:01-positive animal infected with SIVmac239-2V, the lack of A312P was associated with reversion of the compensatory mutations to wild type (Appendix 2 Figure 3C).

To further test if the tertiary mutation A312P would stabilize the I161V and I206V compensatory mutations during reversion of $Gag_{181-189}$ CM9 epitope mutations, we infected a Mamu-A1*001:01-negative animal, r00033, with virus from rh1937 that contained the T182C $Gag_{181-189}$ CM9 escape mutation, the I161V and I206V compensatory mutations, and the tertiary A312P mutation (Appendix 2 Figure 3A, D). In agreement with our previous observations, the A312P mutation stabilized the I161V and I206V compensatory mutations, while the T182C escape mutation reverted to wild type (Appendix 2 Figure 3D). Cumulatively, these data suggest that A312P is necessary for the maintenance of I161V and I206V compensatory mutations in the absence of $Gag_{181-189}$ CM9 escape.

D. Gag A312P is a bona fide compensatory mutation.

We observed that A312P consistently associated with the $Gag_{181-189}$ CM9 escape-associated compensatory mutations, but we wanted to further assess the compensatory characteristics of the A312P mutation. We first investigated the possibility that A312P could be a CD8+ T cell escape mutation but found no evidence of T cells targeting this region of Gag. Using *in silico* binding algorithms (http://www.cbs.dtu.dk/services/NetMHC/) we found that no 8-, 9-, or 10-mer peptides

between SIVmac239 Gag residues 301 and 323 are predicted to bind Mamu-A1*001:01 with physiologically relevant affinity (50% inhibitory concentration [IC50] ≤ 500 nM) (*16*). In addition, we detected no CD8+ T cell responses to the A312 region by a gamma interferon (IFN-γ) enzyme-linked immunosorbent spot assay (ELISPOT) in 7 Mamu-A1*001:01-positive rhesus macaques with detectable $Gag_{181–189}$ CM9-specific CD8+ T cell responses, 4 of which also had circulating virus harboring the A312P mutation (Appendix 2 Figure 4A, red symbols). These results, in combination, support the conclusion that CD8+ T cell pressure does not drive the emergence of A312P in Mamu-A1*001:01-positive animals.

Next, we performed an *in vitro* SIVmac239 replication assay to compare the growth kinetics and levels of stability of viruses containing the I161V, I206V, and A312P mutations. We engineered two SIVmac239 mutants (in addition to SIVmac239-2V) containing the I161V, I206V, and A312P mutations together (SIVmac239-2V-P) or the A312P mutation alone (SIVmac239-P). We infected separate cultures of staphylococcal-enterotoxin B-stimulated rhesus macaque CD4+ T cells with 2 x 10^7 virus particles of either SIVmac239 or a mutant virus in complete medium containing 100 IU interleukin 2 (IL-2)/ml and cultured them for 7 days. On days 7 and 14, virus-containing cell supernatant was diluted 1:4 with medium and used to infect additional activated CD4+ T cells. We compared the frequencies of infected targets for each mutant virus on days 14 and 21 by intracellular Gag p27 antibody staining, normalizing to the frequency of SIVmac239-infected targets (Appendix 2 Figure 4B). Mutant viruses showed replication kinetics similar to that of SIVmac239 over the first 14 days but exhibited a higher replicative capacity by day 21. We Sanger sequenced the mutant viruses from our

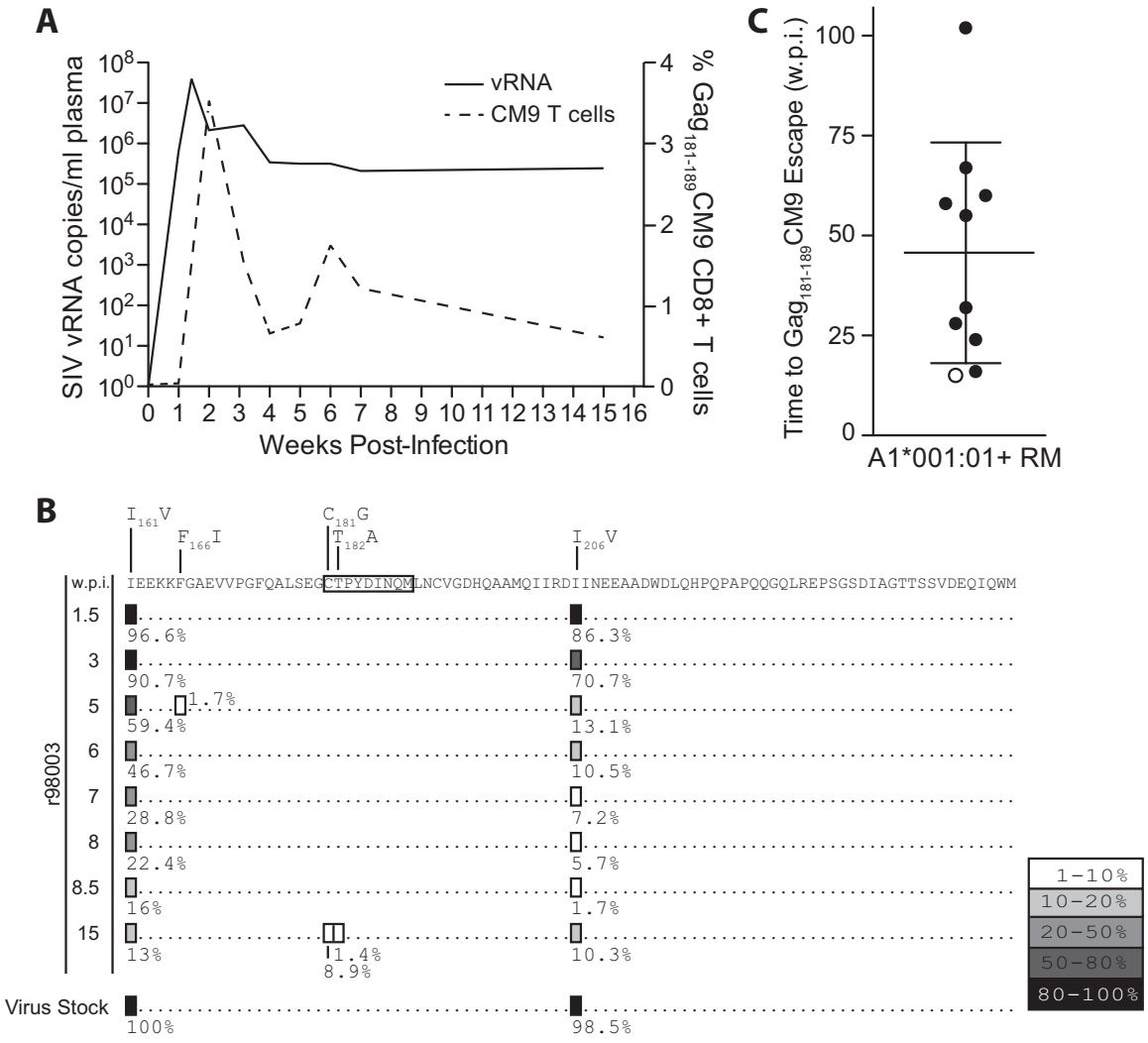
replication assay at day 14 and found that SIVmac239-2V, but not SIVmac239-2V-P, showed a prominent double peak indicating reversion of the I206V mutation (Appendix 2 Figure 4C).

Finally, we generated structural models of the SIVmac239 capsid (CA) hexamer as previously described (*17*) and assessed locations and potential interactions of the I161, T182, I206, and A312 amino acid residues (Appendix 2 Figure 4D, top). The I161 residue is found in helix 1 of CA, and its side chain protrudes into helix 4, where the I206 residue is located, supporting the observed linkage between the I161V and I206V mutations. Importantly, the A312 residue lies within CA helix 9, near the I206-containing helix 4 of the adjacent CA monomer (Appendix 2 Figure 4D, bottom). Thus, SIVmac239 CA structural data suggest that the A312P mutation may influence the intermolecular interactions and stability of the CA hexamer. In support of this hypothesis, we consistently detected higher levels of intracellular Gag p27 CA in cells infected with viruses containing the A312P mutations (Appendix 2 Figure 4E). Taken together, our structural and *in vitro* sequencing data strongly support our conclusion that A312P is a tertiary compensatory mutation that stabilizes SIVmac239 expressing the $Gag_{181–189}$ CM9-associated compensatory mutation.

III. Discussion

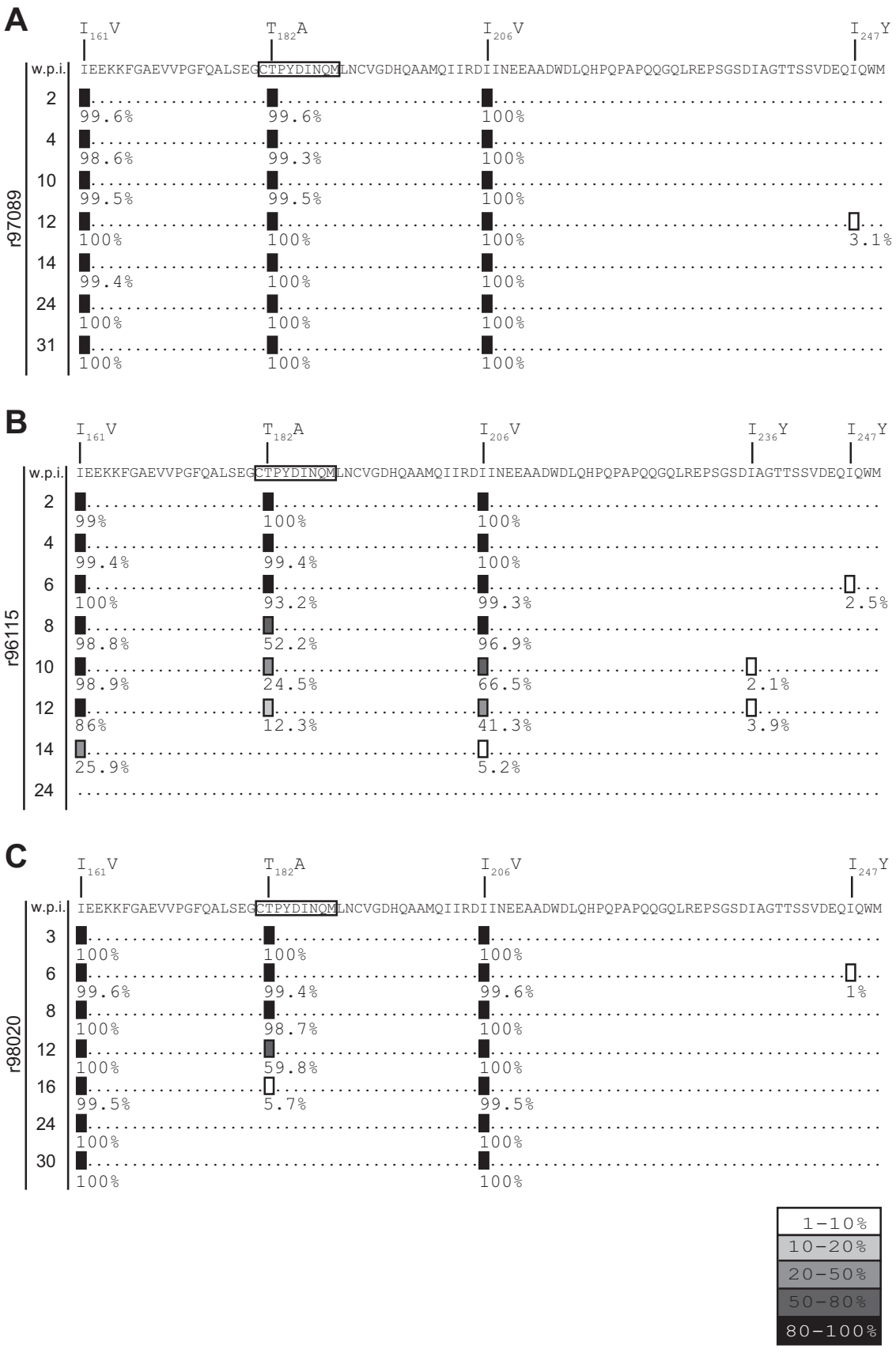
The association of A312P with the I161V and I206V compensatory mutations has not previously been described in studies of $Gag_{181–189}$ CM9 escape but is not altogether an unexpected finding. In fact, the data presented herein contribute to a growing body of evidence that HIV and SIV variation within Gag is highly organized, both in terms of mutation kinetics and constrained patterns of escape (*18*) (*19*) (*20*). These structural

restraints have contributed to the identification of Gag as a premier target for T cell-based HIV vaccine design. As HIV circulates in human populations, there is concern that CD8+ T cell epitopes will be "lost" as escape substitutions accumulate in transmitted viruses. While the finding that such escape mutations frequently revert in the absence of selecting HLA alleles ameliorates this concern, the persistence of compensatory mutations in transmitted viruses may facilitate rapid escape when the virus next encounters CD8+ T cells restricted by the selecting HLA molecule. However, our data indicate that transmitted compensatory mutations do not necessarily facilitate rapid, acute-phase immune escape from CD8+ T cell responses. Further study of additional compensatory mutations, in terms of their frequency in circulating HIV strains and their effect on post-transmission CD8+ T cell responses, is warranted to fully understand their impact on the adaptation of HIV to HLA molecules present at high frequency within the human population.

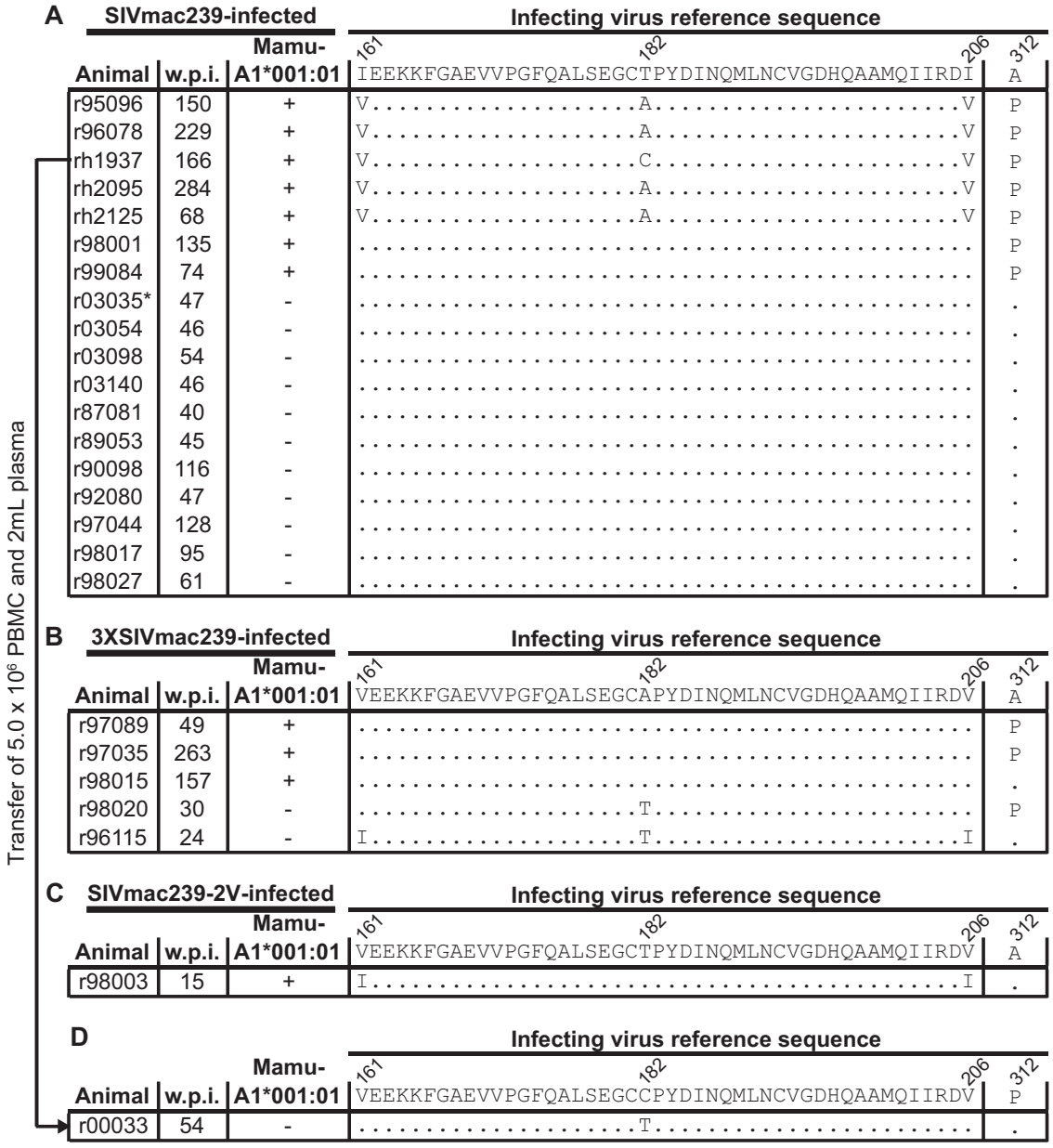


Appendix 2 Figure 1: Compensatory mutations in SIVmac239-2V revert rapidly to wild type despite a $Gag_{181–189}$ CM9-specific $CD8_{TL}$ response. (A) Replication of the engineered SIVmac239 mutant SIVmac239-2V in r98003 was monitored in plasma over the first 15 weeks postinfection (w.p.i.). During the same period, the magnitude of the $Gag_{181–189}$ CM9-specific $CD8_{TL}$ response was assessed by Mamu-A1*001:01/$Gag_{181–189}$ CM9 tetramer staining. **(B)** The $Gag_{181–189}$ CM9 region from the SIVmac239-2V-infected, Mamu-A1*00101-positive rhesus macaque r98003 was Roche/454 pyrosequenced at the indicated w.p.i. Each consensus sequence indicates all mutations present in 1% or more of total sequence reads (limit of detection). Amino acid substitutions are shown above the reference sequence; the frequencies of the mutations are shown both as percentages and as shaded boxes according to prevalence, as indicated by the key at the bottom right. Boxed amino acids within the reference sequence comprise the $Gag_{181–189}$ CM9

epitope. The consensus sequence of the SIVmac239-2V virus stock used to infect r98003 is shown below the sequences from r98003. **(C)** Times to $Gag_{181–189}$ CM9 escape (as defined by the first of two consecutive time points showing amino acid substitutions within the epitope at a frequency of >1% of total sequence reads) in nine MamuA1*00101-positive rhesus macaques (filled circles) and animal r98003 (open circle). The means and standard deviations of times to $Gag_{181–189}$ CM9 escape are shown.

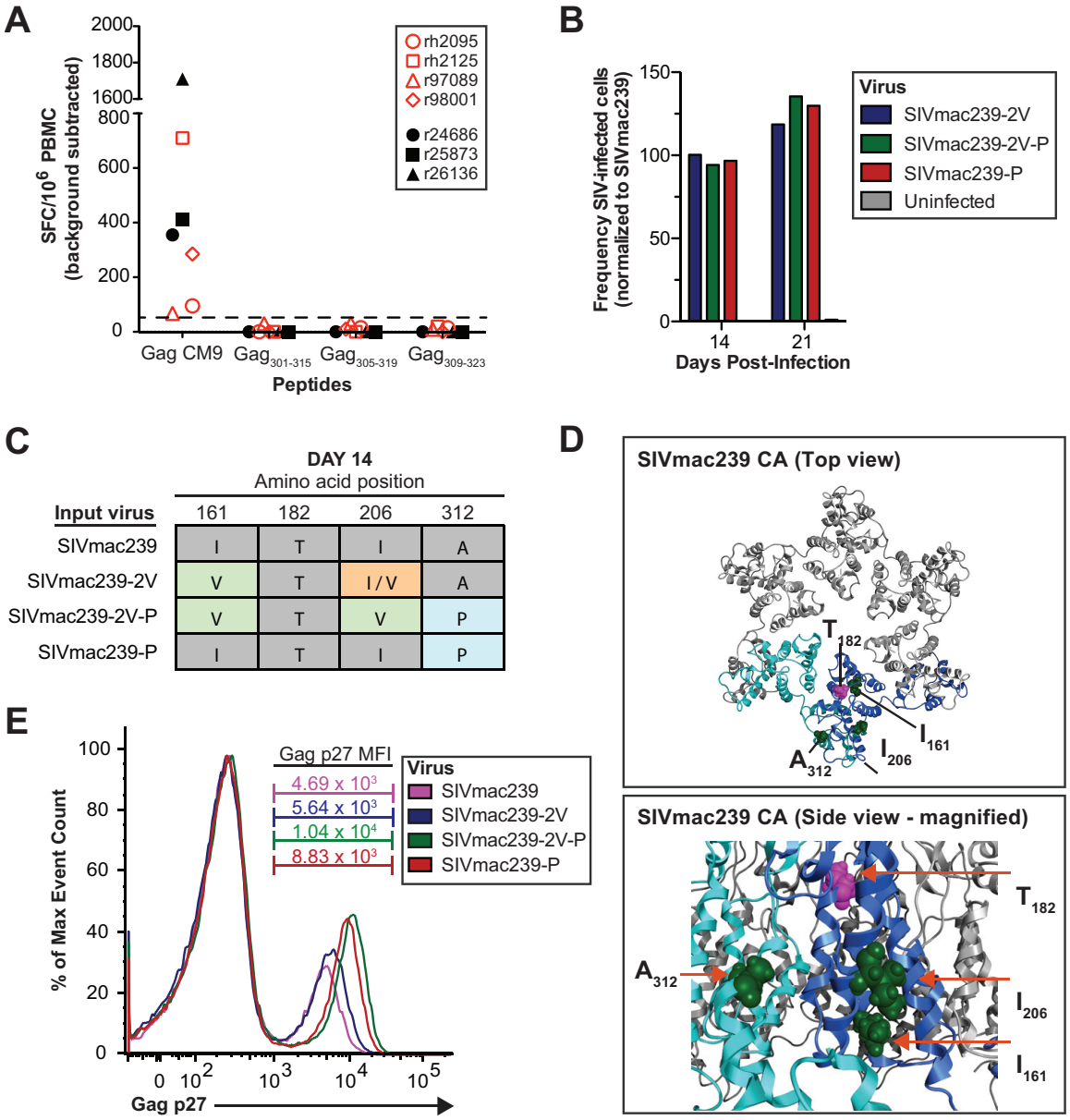

A
I161V
T182A
I206V
I247Y
w.p.i.
IEEKKFGAEVVPGFQALSEGCTPYDINQMLNCVGDHQAAMQIIRDIINEEAADWDLQHPQPAPQQGQLREPSGSDIAGTTSSVDEQIQWM
r97089
2
99.6%
99.6%
100%
4
98.6%
99.3%
100%
10
99.5%
99.5%
100%
12
100%
100%
100%
3.1%
14
99.4%
100%
100%
24
100%
100%
100%
31
100%
100%
100%
B
I161V
T182A
I206V
I236Y
I247Y
w.p.i.
IEEKKFGAEVVPGFQALSEGCTPYDINQMLNCVGDHQAAMQIIRDIINEEAADWDLQHPQPAPQQGQLREPSGSDIAGTTSSVDEQIQWM
r96115
2
99%
100%
100%
4
99.4%
99.4%
100%
6
100%
93.2%
99.3%
2.5%
8
98.8%
52.2%
96.9%
10
98.9%
24.5%
66.5%
2.1%
12
86%
12.3%
41.3%
3.9%
14
25.9%
5.2%
24
C
I161V
T182A
I206V
I247Y
w.p.i.
IEEKKFGAEVVPGFQALSEGCTPYDINQMLNCVGDHQAAMQIIRDIINEEAADWDLQHPQPAPQQGQLREPSGSDIAGTTSSVDEQIQWM
r98020
3
100%
100%
100%
6
99.6%
99.4%
99.6%
1%
8
100%
98.7%
100%
12
100%
59.8%
100%
16
99.5%
5.7%
99.5%
24
100%
100%
30
100%
100%
1-10%
10-20%
20-50%
50-80%
80-100%

Appendix 2 Figure 2: Consensus sequences of the $Gag_{181–189}$ CM9 region in rhesus macaques infected with 3XSIVmac239. The $Gag_{181–189}$ CM9 regions from three rhesus macaques infected with 3XSIVmac239 were Roche/454 pyrosequenced at the indicated w.p.i. **(A)** r97089 (Mamu-A1*001:01 positive); **(B)** r96115 (MamuA1*001:01 negative); **(C)** r98020 (Mamu-A1*001:01 negative). Each consensus sequence indicates all mutations present in 1% or more of total sequence reads (limit of detection). Amino acid substitutions are shown above the reference sequence; the frequencies of the mutations are shown both as percentages and as shaded boxes according to prevalence, as indicated by the key at the bottom right. Boxed amino acids within the reference sequence comprise the $Gag_{181–189}$ CM9 epitope.



Appendix 2 Figure 3: The Gag A312P mutation stabilizes the compensatory mutations Gag I161V and I206V. Consensus Sanger sequences spanning Gag I161 through I206 and the tertiary mutation site Gag A312 at the indicated w.p.i. are shown. **(A)** Eighteen SIVmac239-infected rhesus macaques (seven Mamu-A1*001:01 positive and eleven Mamu-A1*001:01 negative). *, r03035 was infected with the SIVmac239 variant B*008:01 8 SIVmac239 described in (*21*). **(B)** Five 3XSIVmac239- infected rhesus macaques (three Mamu-A1*001:01 positive and two Mamu-A1*001:01 negative). **(C)** One Mamu-A1*001:01-positive, SIVmac239-2Vinfected rhesus macaque. **(D)** One Mamu-A1*001:01-negative rhesus

macaque infected by transfer of plasma and PBMC from rh1937 at 166 w.p.i. (see the arrow). Reference sequences for respective infecting SIV strains are shown across the top.



Appendix 2 Figure 4: Gag A312P is a tertiary compensatory mutation stabilizing SIVmac239 CA expressing I206V. (A) Seven Mamu-A1*00101-positive rhesus macaques infected with SIV were screened for T cell responses against three overlapping 15-mer peptides spanning the A312 region by IFN-γ ELISpot. Animals harboring SIV with the A312P mutation are represented with open red symbols. SFC, spot-forming cells. The dashed line at 50 SFC is the limit of detection for this assay. **(B)** Frequency of Gag p27 CD4 T cell targets infected with engineered mutant viruses in vitro. Values were normalized to the frequency of Gag p27 targets infected with SIVmac239. **(C)** Table showing Sanger sequencing results from vRNA taken at day 14 of the in vitro replication assay. **(D)** Structural modeling of SIVmac239 capsid,

showing positions of the I161, T182, I206, and A312 amino acid residues. (Top) Top view of the SIVmac239 CA hexamer, with one monomer colored light blue and a second monomer colored dark blue. (Bottom) Magnified side view of the potential interaction between A312 (within the light-blue monomer) and I206 (within the dark-blue monomer). **(E)** Representative Gag p27-staining histogram, showing the mean fluorescent intensity of intracellular Gag p27-fluorescein isothiocyanate (FITC) staining for each virus at day 21 of our in vitro replication assay. Results are indicative of separate assays performed with two different Gag p27-specific antibodies.

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