IMMUNE SELECTION IN THE CNS: CONSEQUENCES OF SIV GAG ESCAPE FROM MHC CLASS I-MEDIATED CONTROL

by
Sarah Evelyn Beck

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Abstract:

Immune pressure exerted by host factors including MHC class I-mediated cytotoxic T cell control affects HIV disease progression and drives the development of viral escape mutations; nonetheless, the relationship between host immunity and HIV central nervous system (CNS) disease remains poorly understood. The simian immunodeficiency virus (SIV) macaque model recapitulates key features of HIV infection including development of AIDS and CNS disease. To investigate host factors regulating SIV CNS disease progression, we compared the incidence of SIV encephalitis and the effects of MHC class I allele expression on the development of CNS disease in pigtailed macaques versus rhesus macaques. Species-specific differences in susceptibility to SIV disease demonstrated that host factors are critical to SIV CNS disease progression. We extended these studies by examining CNS pathogenesis in pigtailed macaques expressing the MHC-I allele *Mane-A1*084:01:01*, which confers resistance to SIV-induced CNS disease in pigtailed macaques and induces viral development of prototypic escape (K165R) in the immunodominant SIV Gag KP9 epitope. Insertion of the Gag K165R escape mutation into molecularly cloned SIV/17E-Fr resulted in reduced viral replication compared to wildtype SIV/17E-Fr *in vitro*. To investigate viral fitness *in vivo*, we inoculated six *Mane-A1*084:01:01* expressing macaques: three with SIV/17E-Fr K165R, and three with wildtype, parental SIV/17E-Fr. We found lower CSF, but not plasma, viral loads in animals inoculated with SIV/17E-Fr K165R versus those inoculated with wildtype, and although the escape mutation K165R was genotypically stable in the plasma, it rapidly reverted to wildtype Gag KP9 in both CSF and in microglia. To induce Gag KP9-specific CTL immune pressure, we vaccinated two *Mane-A*084:01:01*

expressing pigtailed macaques with Gag KP9 peptide loaded onto a virus-like particle (VLP) vaccine. Both animals developed robust KP9-specific tetramer responses to vaccination, and upon challenge with SIV/17E-Fr, developed lower viral replication in the CSF but not the plasma. These data clearly demonstrate that viral fitness in the CNS is distinct from the periphery. As therapeutic vaccination strategies to enhance CTL responses against HIV Gag could promote HIV escape, it is vital that we understand the consequences of viral escape on CNS disease.

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I: Introduction

HIV Background

Over three decades ago, a new immunosuppressive syndrome decimated communities of young men nationwide. Patients were succumbing to a spectrum of diseases unusual in this population of young, otherwise seemingly healthy individuals. These included cryptococcal meningitis, tuberculosis, toxoplasmosis, and neoplasia including lymphoma and Kaposi's sarcoma. As the only common link amongst these initial cases was a history of homosexual activity, sexual transmission was suspected.

Shortly thereafter, the causative agent was discovered to be the human immunodeficiency virus (HIV), an RNA virus in the family *Retroviridae* (genus *Lentivirus*). The immunosuppressive syndrome, characterized by plummeting CD4+ T cell counts, was termed Acquired Immunodeficiency Syndrome (AIDS). Since the 1980s, the virus has spread worldwide, affecting people regardless of sexual orientation, race, or socioeconomic status. Worldwide, over 36 million people have died as the result of HIV infection (WHO fact sheet, http://www.who.int/), and HIV/AIDS continues to be an important public health issue today. In 2012, an estimated 35.3 million people worldwide were living with HIV infection (WHO). In the United States alone, approximately 50,000 people are newly infected every year (CDC 2010 survey, www.cdc.gov). Of particular concern to Johns Hopkins Hospital, Maryland has the third highest rate of HIV diagnosis of states/territories in the nation, with Baltimore-Towson ranking as the sixth highest diagnosis rate of any major metropolitan area (http://phpa.dhmh.maryland.gov/). As of 2011 (the last survey done), Maryland has approximately 27,710 individuals living with HIV and/or AIDS (http://phpa.dhmh.maryland.gov/).

Viral structure and genes

HIV is a roughly spherical virion roughly 120 nm in diameter containing two copies of the positive sense RNA genome within a protein capsid surrounded by a lipid bilayer envelope. It is classified in the family *Retroviridae* due to its unique use of reverse transcription for viral replication in a host cell. In particular, HIV is further classified as a *Lentivirus* (taken from *lente*, [Latin for slow]). Lentiviruses are unique amongst retroviruses, not only because of the characteristic protracted course of disease often seen in hosts, but also because the genome encodes for several accessory and regulatory proteins. In addition, lentiviruses are able to infect and replicate in some types of non-dividing cells.[1] Besides HIV in humans, this genus includes viruses that affect multiple species, including feline immunodeficiency virus (FIV), equine infectious anemia virus, caprine arthritis encephalitis virus (CAEV), visna/maedi virus, and simian immunodeficiency virus (SIV). In humans there are two main subtypes of HIV: HIV-1, which is most closely related to viruses found in West African chimpanzees and gorillas, and HIV-2, which is related to viruses found in another African primate, the sooty mangabey. HIV-1 is the more common and more pathogenic of the two subtypes, and it is the most common subtype seen in the United States.

As with other retroviruses, the majority of the HIV-1 genome encodes for the structural genes *gag* (group-specific antigen), *pol* (polymerase), and *env* (envelope glycoproteins). In addition, HIV-1 contains six accessory/regulatory genes: *vif*, *vpr*, *vpu*, *rev*, *nef*, and *tat*. Tat and Rev are gene regulatory proteins necessary for successful transcription and RNA export from the nucleus.[1, 2] The rest are accessory proteins that

alter host cell surface marker expression, affect viral infectivity, and aid in viral nucleoprotein transport.

In total, the HIV-1 genome has 9 open reading frames, and the mature virion is comprised of 15 proteins and the diploid viral genome. The *gag* gene is translated into a precursor polyprotein that is then cleaved by viral protease into: matrix (MA), capsid (CA), nucleocapsid (NC), and p6. CA forms the conical core of the virus that surrounds the genomic RNA, NC, enzymes, and accessory proteins. MA is integral in virion assembly, serving both to target Gag and Gag-Pol precursor proteins to the cell membrane as well as incorporating Env glycoproteins into the viral particles. NC serves to bind the genomic RNA and deliver it to the developing virion. The final component of the polyprotein Gag is p6, which incorporates the accessory protein Vpr during virion assembly and helps mediate efficient virion release from the cell membrane.

The *pol* gene encodes for three Pol protein products: protease (PR), reverse transcriptase (RT), and integrase (IN). The virion that buds from an infected cell is immature, containing Gag-Pol polyprotein precursors. Larger precursor proteins must be cleaved by PR into their protein components (MA, CA, NC, PR, RT, and IN) before the virion fully matures to an infectious form. RT, the enzyme that is a feature of all retroviruses, contains both RNA-dependent and DNA-dependent DNA polymerase functionalities. Following the creation of double stranded viral genomic DNA by RT, IN is responsible for integration of this viral DNA into the host chromosome.

The *env* gene encodes the precursor protein gp160, which is cleaved by a cellular protease into gp120/surface (SU) and gp41/transmembrane (TM) domains. Together, gp120 and gp41 form the glycoprotein spicules on the virion envelope that is responsible

for cellular receptor/coreceptor interactions as well as membrane fusion, with gp41 anchoring the complex within the virion envelope.

Viral Life Cycle

The life cycle of HIV-1 is one of the most complex of viruses, with extensive exploitation of existing cellular machinery.[1, 3] Over the last three decades, this life cycle has been studied considerably, and although we still lack a comprehensive understanding of all of the nuances, an understanding of this life cycle has illuminated many potential targets for pharmaceutical intervention.

Cell entry and viral uncoating

The life cycle of HIV-1 starts with entry into the host cell by fusion mediated by trimers of the Env proteins gp120 and gp41. Host cell CD4+ receptors and coreceptors such as CCR5 and CXCR4 are recognized by gp120. This binding prompts a conformational change in gp41, which subsequently reveals the domain that mediates fusion of the viral envelope with the cell membrane. After cell entry, the virus undergoes uncoating, in which the remaining viral components transition to the reverse transcriptase complex and the capsid is lost.[3]

Reverse transcription and the pre-integration complex

Mediated by RT, reverse transcription is the process in which HIV-1, like other members of the family *Retroviridae*, converts the single stranded RNA genome into double stranded proviral DNA. The process is initiated by cellular tRNA, which binds to the primer binding site (PBR) in the HIV genome, priming the generation of a DNA/RNA hybrid. An RNaseH domain on RT then digests away the RNA portion of this

hybrid, leaving a minus strand short segment of DNA known as the minus-strand strong stop DNA. The minus-strand strong stop DNA performs first strand transfer by jumping from the 5' to the 3' end of the viral RNA, and the newly synthesized DNA strands hybridizes to the complementary long terminal repeat (LTR) region on the RNA priming minus-strand DNA synthesis. The original viral RNA is partially digested away by RNaseH, leaving fragments of viral RNA that are then used to prime plus strand DNA synthesis at the central polypurine tract (PPT). After the cellular tRNA is degraded by RNaseH, plus-strand synthesis is completed with two termination sites: one at the end of the minus-strand and the other at the central termination sequence (CTS), leaving a central "flap" of DNA that is important for the next step--transport of the DNA into the nucleus. Second strand transfer occurs when the PBS on the minus-strand hybridizes with the PBS on the plus-strand, allowing elongation to continue.

Once reverse transcription is complete, the newly made double stranded viral DNA is associated with RT, IN, MA, Vpr, and DNA-binding protein in the pre-integration complex (PIC). The size of the highly compacted PIC is believed to be approximately 28nm.[3] The PIC is transported to the nucleus in a process that is incompletely understood, but is believed to involve Vpr, IN, MA, and the aforementioned DNA "flap".[1, 3]

Integration and Recombination

Once in the nucleus, IN cleaves two bases from the 3' end of both strands of the linear viral DNA, creating a "sticky end". IN makes the same cleavage in chromosomal DNA, and the ends are joined with the aid of cellular DNA repair enzymes. In order for a replication competent virus to be produced, the viral genome must be successfully

integrated into the host DNA. The integrated viral genome may be either latent or transcriptionally active, which is partially determined by the location of integration in the host genome. There are also three other possibilities, none of which result in a replication competent virus: 1) the ends of the viral DNA may join to form a 2-long terminal repeat (LTR) circle, 2) homologous recombination may result in a 1-LTR circle, or 3) the viral DNA may recombine to form a rearranged circle.

Gene expression and RNA transport

The viral LTR contains promoter elements, the initiator, TATA-box, and sites to help position RNA polymerase II so that transcription is initiated; however, efficient elongation cannot occur without the action of the viral protein Tat. The transcriptional transactivator viral protein Tat greatly increases the rate of viral transcription by binding the transactivation response region (TAR) at the 5' end of the viral genome and recruiting host positive transcriptional elongation factor b (P-TEFb). HIV transcription produces multiple complex transcripts, some of which contain introns and cannot exit the nucleus by normal host pathways. Whereas the intronless multiply spliced transcripts can exit the nucleus via normal export pathways, the HIV protein Rev is responsible for transporting intron-containing unspliced and partially spliced viral RNA from the nucleus by binding the Rev-responsive element (RRE) in the *env* sequence, allowing host nuclear export machinery to bind.

Virion assembly, budding, and maturation

Once viral proteins have been synthesized, determinants in the Gag precursor protein Pr55Gag are responsible for localization at the plasma membrane via the MA domain, genomic encapsidation via the NC domain, association with Env glycoproteins

via MA, and stimulation of budding via p6. Maturation of the virus particle occurs after budding when viral PR cleaves Gag and GagPol precursor proteins to generate the progeny proteins.

Clinical Features and Management of HIV-1/AIDS

We now know that HIV-1 is transmitted through the exchange of bodily fluids, such as blood, semen, vaginal secretions, and milk. Within the US, the most common forms of transmission are unprotected sex and sharing needles during intravenous drug use (www.cdc.gov). The risk of transmission is dependent on a number of factors, one of the most important of which is plasma viral load; if the viral load is less than 400 copies/μL, either due to successful antiretroviral treatment or immunologic control, there is a very low risk of transmission to another individual.[4-6] Other infectious genital infectious diseases, such as Herpes simplex-2 (HSV-2) viral infection[7, 8] and bacterial vaginosis[9] have been associated with an increased risk of HIV acquisition. Conversely, medical male circumcision has been shown to reduce the risk of heterosexual acquisition of HIV.[10]

Clinical signs begin approximately 2-4 weeks after HIV-1 infection. Typically these consist of "flu-like" or "mononucleosis-like" symptoms, consisting of fever, body aches, lymphadenopathy, sore throat, rash, and headache. Because of the similarity of these symptoms to flu, most individuals do not immediately seek medical care. This stage is termed primary HIV infection. Shortly after infection there is a brief period of rapid viral replication with peak viremia occurring approximately two weeks post infection.[11, 12] Immediately following this period of peak viremia, both humoral and cellular

responses to HIV-1 can be detected[13-17], followed by partial control of viral replication and decreased levels of viremia. From two to eight weeks post infection, there is also a precipitous decline in CD4+ T cells in the blood. As viral replication drops, the CD4+ T cells are able to partially rebound. At this time, the disease is considered to have moved into the clinical latency stage where virus continues to replicate without causing symptoms of disease. This asymptomatic stage may last anywhere from months to years depending on a number of host factors.

Unfortunately, without a cure for HIV infection, individuals infected with HIV face a lifelong dilemma of managing a chronic disease. Without pharmacologic intervention, viral load in almost all individuals will eventually rise with a corresponding drop in CD4+ cells in peripheral blood. Once the CD4+ T cells are at or below 200 cells/μL blood, the patient is considered to have progressed to AIDS. Intense research over the years has developed numerous antiretroviral drugs targeting different stages of the viral life cycle, and the combination of several of these drugs has been successful in controlling viral replication when patients adhere to the treatment regimen. Antiretroviral therapy (ART) has advanced to the point where infected individuals can achieve a high quality of life and a normal lifespan; unfortunately, ART is expensive and also associated with toxicity and emergence of resistant viruses.

Host response to early HIV-1 infection (humoral and cell-mediated)

Even at the start of the HIV pandemic, researchers were able to detect both humoral[13, 14] and cellular[15, 16] immune responses during the peak of plasma viremia in HIV-1 infection. Humoral immunity is the production of antibodies against an

antigen by plasma cells (effector B cells). “Neutralizing” antibodies are those that are able to prevent the biologic or pathologic effect of an antigen, such as preventing a virus from entering a cell, while “binding” antibodies are those that bind to the antigen and tag it to activate other cells of the immune system. Antibodies are developed against a variety of combinations of protein isoforms derived from the precursor polyproteins Gag, Env, or Pol[18]; however, the functional antibody isotopes seem to mostly be limited to those against a component of Env. The first antibodies produced are IgM isotype against the Env protein p41 before undergoing isotype switching to IgG and IgA.[18] Although these initial antibodies produced during acute viremia are successfully able to bind virus, they do not have a significant effect on plasma viral loads and are therefore not responsible for the initial control in viremia seen after the acute spike.[19] In natural HIV-1 infection, evidence suggests that neutralizing antibodies are not produced at the soon enough and in sufficient levels to account for the drop in HIV-1 replication seen after acute viremia.

The cellular arm of host immunity has been identified as a major player in controlling early HIV-1 replication. Cellular immunity involves the activation and production of virus-specific CD8+ cytotoxic T lymphocytes (CTLs), macrophages, and natural killer (NK) cells and the release of cytokines. Cell-mediated immunity (CMI) is one of the most important defenses the host has against viral infection. Evidence has shown that CMI is crucial to partially controlling early viral replication, as HIV-specific cytotoxic T cell responses are temporally associated with a decrease in viral load.[17, 20, 21] Also, the magnitude of the host’s CMI response to HIV-1 infection is inversely correlated with viral load.[21]

For T cells to become activated as CTLs, they must interact with cellular histocompatability proteins, which are transmembrane glycoproteins expressed on the surface of host cells. There are three categories of major histocompatibility (MHC) genes in vertebrates: class I, II, and III. Class I proteins present intracellular antigens on the cell surface, and are therefore important in host defense against intracellular pathogens, such as viruses or obligate intracellular bacteria. Class II proteins present extracellular antigens, and so are most important in host defense against extracellular pathogens. As almost all cells in the body are susceptible to viral infection, all nucleated cells express MHC class I markers on the surface of the cell. MHC class II markers are typically only present on professional antigen presenting cells (APCs), such as macrophages, dendritic cells, and B cells. Class III genes encode for proteins that have other varied functions in the immune system.

The MHC class I is a heterodimer consisting of an alpha chain with 3 domains (α_1- α_3) and β2-microglobulin (encoded by a different gene). The α_3 domain extends across the membrane, anchoring the complex in the cellular membrane. Domains α_1 and α_2 form a pocket, in the center of which is the peptide-binding groove, which can bind and present peptides 8-10 amino acids long. Ubiquitinated proteins within the cytoplasm of the cell are processed by the proteasome and shuttled to the endoplasmic reticulum (ER) lumen by the transporter associated with antigen processing (TAP). Once there, the peptide fragment is loaded by a polyprotein complex (including TAP, calreticulin, calnexin, Erp57, and tapasin) onto the still-assembling MHC class I glycoprotein. The peptide-MHC complex leaves the ER and moves to the Golgi via the secretory pathway, which transports it to the cell surface.

In a healthy cell, MHC class I will present peptides derived from cellular protein fragments that are produced by normal protein turnover ("self" antigens), which are subsequently ignored by immune surveillance T cells. When a host cell is infected with a virus, viral proteins are produced in the cytosol by cellular machinery during viral replication, and peptides derived from these viral proteins are loaded in MHC class I complexes and subsequently recognized by immune surveillance T cells. Specifically, the viral peptide in the context of MHC class I is recognized by the T cell receptor (TCR) on the surface of the naïve CD8+ T cell. Similar to antibodies, TCRs are comprised of proteins of the immunoglobulin superfamily. Most TCR molecules are comprised of α and β chains, each of which contains variable and constant regions. Like antibodies, diversity is created via V(D)J recombination, allowing for a huge range of antigen recognition. In order for the naïve T cell to recognize an antigen and become activated, the surface protein CD8 must bind the constant region of MHC class I. This close interaction allows for peptide recognition and activation of naïve CD8+ T cells into effector CTLs.

Both MHC classes I and II are polygenic, encoded by multiple genes. MHC class I is encoded by three different loci, so each individual will express at least three different MHC class I molecules on all cells. In addition, they are extremely polymorphic with many amino acid variations in the α_1 and α_2 chains (the dimer forming the peptide-binding grove), so each locus has many alleles. These alleles are also codominant. It is highly likely that any given individual will be heterozygous at each locus, therefore most individuals actually express six different MHC class I molecules (class I haplotype). This ensures that each individual will express multiple MHC alleles with different range of

peptide binding specificities, allowing for a wide range of peptide recognition, not only within one individual, but within the population as a whole. In humans, MHC class I is called human leukocyte antigen (HLA), and the three α-chain genes are HLA-A, HLA-B, and HLA-C. One of the most important host factors of disease resistance is an individual's unique array of major histocompatibility alleles. This is especially relevant to HIV-1 pathobiology, as certain MHC class I alleles have been associated with decreased or increased progression to AIDS.

Host determinants of disease progression and outcome

On average, the typical HIV-infected patient progresses to AIDS approximately one decade from the time of infection[22]. However, there is considerable variation from patient to patient. From the start of the AIDS epidemic, it quickly became apparent that host factors enabled some individuals to progress slower to AIDS than others. The term "long term nonprogressors" (LTNPs) typically refers to the small subset of HIV-1 patients that have stable CD4+ counts without ART and a correspondingly benign course of disease over a long period of time.[23-26] LTNPs can represent anywhere from 2-5% of the total HIV-1 population.[25, 26] An even smaller group of individuals are able to completely control viral replication (<50 copies/mL); this group, comprising approximately only 0.3% of the HIV-1 infected population, is termed "elite suppressors" or "elite controllers".[23, 26] In addition, some HIV-1 negative people (usually sex workers or partners of HIV-1 infected individuals) are termed "highly-exposed HIV seronegatives" because they never show evidence of infection despite repeated

exposures.[27-29] All of these different subgroups have been crucial in studying the potential mechanisms of effective host immunity against HIV-1 infection.

One of the earliest genetic factors of HIV resistance discovered was the importance of coreceptors in transmission. As mentioned previously, HIV requires CD4 surface marker expression as well the presence of a chemokine coreceptor for the virus to enter the cell. This requirement for coreceptor binding explains the tropism that HIV shows for certain types of cells; for example, T cell tropic strains of HIV preferentially use the coreceptor CXCR4 (fusin), while macrophage tropic strains use CCR5.[30] Studies have also found CCR5 tropic viruses are prevalent in acute or asymptomatic infections while CXCR4 or dual tropic viruses (R5X4) are prevalent in progressive disease.[31-34] These observations have led to the conclusion that R5-tropic viruses are responsible for initiating infection within an individual. The importance of CCR5 in HIV transmission is further supported by the discovery of deletions in the CCR5 gene (CCR5Δ32) in some highly exposed HIV-seronegative individuals. Individuals that are homozygous for CCRΔ32 have nearly complete protection from infection, while individuals heterozygous for CCRΔ32 show delayed progression to AIDS.[35-37] Although the CCRΔ32 mutation is present at a relatively high frequency (5-14%) in European-descent populations, it is not found at all in African, Asian, and American Indian populations.[38] Therefore, although compelling, the CCR5Δ32 mutation only explains a small percentage of host resistance to HIV-1 infection.

There is a great deal of data pointing to CTL-mediated MHC class I dependent immunologic pressure as a crucial mechanism for HIV control. *HLA-B*5701* and *HLA-B*27* MHC class I alleles in particular are historically overrepresented in LTNP and ES

groups.[23, 24, 39-41] The importance of the role of HLA haplotype in HIV-1 resistance was further confirmed with the International HIV Controllers Society genome-wide association study (GWAS), in which over 300 single nucleotide polymorphisms (SNPs) with genome-wide association to HIV progression were identified only within MHC-I.[42] In addition, specific residues associated with immunologic control of HIV were localized to the peptide-binding groove of the MHC molecule. Multiple GWAS studies have been published to date, and the only significant associations found thus far have involved the *HLA* and chemokine regions of the genome, with *HLA* being the primary region affecting control of viral loads.[43]

Viral antigenic variation

Ultimately, cell-mediated immunity and other host mechanisms are unable to eradicate HIV from the host. This is because HIV-1, like other retroviruses, is able to accrue massive genetic and antigenic variation that allows it to evade control from the host's immune system, ART, or vaccine therapy. The is largely due to the fact that continuous replication occurring during the clinically latent phase using error-prone reverse transcriptase (RT) allows for huge numbers of point mutations to accumulate. This low replication fidelity is because RT lacks proofreading ability that cellular polymerases have. Although initial estimates from *in vitro* were higher[44], the error rate of HIV-1 is most likely somewhere around 1.4-3 x 10^{-5} errors per base pair, per replication cycle.[45]

Although many mutations in the HIV genome occur as a result of the high error rate of RT during viral replication, there are other ways HIV-1 generates genomic

diversity. One of the best known and most important is APOBEC3G, a member of the APOBEC family of cellular proteins that induces mutations in the HIV genome by deaminating cytosine residues to uracil in the minus-strand of viral DNA, resulting in a G to A mutation.[45, 46] Another major source of genetic variation in HIV-1 is viral recombination.[45, 47]. As with all other retroviruses, HIV-1 co-packages two viral RNA genomes in the capsid of one virion. During generation of viral DNA during reverse transcription, RT has been shown to be able to switch templates between co-packaged RNA at any point in the HIV genome, creating recombinant viruses. A single host cell must first be co-infected with more than one genetically distinct HIV and a virion must be packaged with one RNA genome from each distinct virus before true recombinant viruses can be created. Viral recombination is considered by some to have more of an evolutionary influence than mutation for several reasons: mutations are more likely to be harmful than beneficial, recombination can create new diversity by shuffling pre-existing mutations within a population and allows different areas of the viral genome to evolve independently, and recombination can combine beneficial mutations from different viral genetic lines into one.[45]

Of particular interest to this project, viruses may escape CTL-mediated killing by accruing mutations that interfere with TCR recognition of viral peptide in the context of the MHC class I molecule. From the viral perspective, evasion from immune pressure may be accomplished by introducing viral peptide mutations that interfere with binding in the MHC peptide groove or mutations outside the epitope that alter the way that peptides are processed in the proteasome, thus affecting the ability of MHC class I to effectively present peptide for T cell recognition.[48]

Mapping of CTL responses to HIV-1 epitopes has identified functional CTLs that recognize and respond to Gag epitopes (especially those localized to HIV Gag p24 [capsid]), are associated with decreased viral load.[49-55] Furthermore, *HLA* alleles associated with slower or decreased progression to AIDS, such as *HLA-B*5701*, recognize peptides in Gag.[50, 55, 56] Although *HLA-B*5701*, *B*27*, and other *HLA* alleles are found in slow and non-progressors, not every individual that expresses these alleles will display this phenotype. This may be partially explained by the fact that individuals with "protective" alleles that go on to progressive disease do not have highly functional CTL responses (4 or more cytokines) against Gag p24.[49]

There are several hypotheses that might explain specifically why Gag is so crucial to immunologic control of HIV-1. Not only is Gag very immunogenic, but also evidence suggests that Gag-specific CTLs are able to respond and kill viral infected cells sooner than CTLs directed against other HIV-1 epitopes, such as Env or Nef.[57] This may be due in part to the large relative abundance of gag proteins within the infected cell (~ 400 molecules/cell compared to ~12 molecules/cell for RT).[58] The HIV-1 *gag* region of the genome is also a highly conserved region that encodes for main structural proteins.[56] The conserved nature of the Gag sequence implies that this is a region of the HIV-1 genome that does not tolerate mutations well.

The "fitness" of a virus is defined as the ability of that virus to successfully replicate when compared to a similar virus (e.g., two different strains), while the "fitness cost" refers to reduced replication as the result of escape mutations. Several lines of evidence provide support for the hypothesis that escape mutations in the Gag polyprotein, especially the capsid region, incur a fitness cost to the virus. Escape mutations found in

regions of Gag that are restricted by HLA molecules are frequently associated with decreased viral load.[40, 59-61] Also, in the macaque model of SIV infection, multiple studies have shown that escape viral mutations to epitopes in the capsid protein revert back to wildtype in the absence of MHC class I/CTL-mediated immune pressure.[62-64] For some mutations, looking at the protein structure of capsid in the region of the escape mutation has helped to explain subsequent fitness cost. Structural analysis of HIV-1 p24 has shown that the canonical escape mutation T242N within the HLA-B*57/5801-restricted Gag epitope TW10 reduces viral fitness because Thr-242 is critical for nucleating and stabilizing helix 6 in the capsid protein.[60]

Protective *HLA* alleles and viral escape mutations are not always associated with decreased disease progression. One possible explanation to this has been the observation that many viral escape mutations are accompanied by "compensatory" mutations that partially or completely restore viral fitness.[60, 61, 65-68] For example, escape within the *HLA-B*5701*-restricted Gag epitope TW10 is associated with several additional mutations within the CypA-binding loop of the capsid, and these mutations are more likely to be found in *HLA-B*5701*-positive individuals with progressive disease.[61]

NeuroAIDS and HAND

Shortly after HIV-1 was identified as the cause of AIDS, physicians recognized that individuals with AIDS frequently demonstrated varying degrees of neurocognitive impairment (NCI). At that time, neurocognitive disorders were generally divided into two categories: 1) HIV-associated dementia (HAD), and 2) minor cognitive/motor disorder (MCMD).[69] Under current nosology, the broad spectrum of neurologic disease

associated with HIV-1 infection is referred to as HIV-associated neurocognitive disorders (HAND), which is further subdivided into HAD, MC/MD, and asymptomatic neurocognitive impairment (ANI).[69] The most severe manifestation, still generally referred to as HAD, is characterized by moderate to severe cognitive impairment that results in marked difficulty performing activities of daily living.[69] Before the advent of effective ART, HAD was recognized as a relatively common condition in HIV-infected individuals, especially those with low CD4+ counts and high viral load. After introduction of effective ART, the incidence of HAD has drastically decreased along with the incidence of AIDS.[69, 70] Unfortunately, mild and moderate forms of HAND have persisted despite successful ART, and amongst the mildest forms of HAND, the overall incidence of NCI has actually increased in the post-ART era compared to the pre-ART era.[70] The 2010 CHARTER study reported that overall 52% of HIV-1 infected individuals had some level of NCI; of these, only 7% were diagnosed with HAD, 12% had mild NCI, and 33% had evidence of asymptomatic NCI.[70]

Despite the recognition of chronic neurologic disease as an important manifestation of HIV infection, the pathogenesis of HAND in successfully ART-treated individuals continues to be elusive. There are numerous possible explanations, including the legacy effect (viral or inflammatory-mediated damage prior to initiation of therapy), immune restoration disorder, host genetic factors, and poor CNS penetrance of ART.[71, 72] Most likely, HAND is the end result of multiple pathophysiologic factors that contribute to a complex neurobehavioral outcome. A recent Multicenter AIDS Cohort Study GWAS was unsuccessful in finding an impact of relatively common genetic variations on the risk for developing HAND.[71] Furthermore, the clinical observations

that define HAND are not pathognomonic for HIV-1 infection, and a fairly large proportion of HIV seronegative individuals fit these criteria, implicating co-morbidities in addition to HIV damage.[71] The continued high prevalence of HAND in the post-ART era is especially concerning because these individuals have poorer clinical outcomes, with higher mortality, lower quality of life, and worse adherence to treatment.[72]

CTLs and NeuroAIDS

Although some systemic effects of HIV-1 infection likely affect the nervous system, the virus itself directly infects the CNS with productive infection of microglia and perivascular macrophages and nonproductive infection of astrocytes.[73-75] Characteristic histopathologic lesions of HIV-1 associated encephalopathy include multifocal giant cell encephalitis (MGCE), characterized by perivascular macrophages and multinucleated giant cells harboring replicating HIV-1, as well as a progressive diffuse leukoencephalopathy (PDL) with myelin loss, astrocyte proliferation, and histiocytic parenchymal infiltrates.[76] One theory is that mild and moderate forms of HAND are at least in part due to chronic neuroinflammation as the result of persistent HIV-1 replication.[71, 72]

Given the importance of CTLs in host immunologic control of early HIV-1 infection in the periphery, CTLs also likely play an important role in the pathogenesis of HIV-associated neurologic disease. Indeed, perivascular CD8+ T cells have been demonstrated in the brains of patients with HIV-associated encephalitis (HIVE)[77-80], which suggests that CTLs may be important in controlling HIV replication in the brain.

CTLs directed against HIV-1 are also believed to infiltrate the brain early in infection, as CD8+ T cells are found in the brains of individuals that have not yet developed disease.[80] These findings are also supported by findings from primate models of neuroAIDS. Rhesus macaques (*Macaca mulatta*) infected with a macrophage tropic strain of simian immunodeficiency virus showed massive increases in the number of CD8+ T cells in the brain as well as increased expression of cytolytic enzymes (granzymes, perforin, IFNγ).[81-83] On the other hand, it is possible that CTL-mediated viral control may be acting as a double-edge sword by contributing to neuropathology through cytotoxic granule release and CTL-mediated neuronal death.[77]

As the CNS is one of the most difficult systems to monitor disease progression in detail due to limited accessibility, there are significant challenges in studying HIV pathogenesis in the brains of human patients. For this reason, primate models of retroviral-associated encephalitis have been invaluable in studying neuroAIDS in depth and longitudinally.

Simian immunodeficiency virus

Simian immunodeficiency virus (SIV) is the most closely related retrovirus to HIV. HIV-1 is believed to have arisen from SIV found in chimpanzees (SIV_{cpz}) as the result of at least three independent cross-species transmission events, most likely from exposure to infected blood from bushmeat.[84] Similarly, HIV-2 is believed to have arisen from SIV in West African sooty mangabeys (SIV_{sm}).[85, 86] Despite high circulating viremia, SIVs in these natural hosts do not generally cause disease.[87] Shortly after the AIDS epidemic in people began, a similar syndrome was observed in

several different groups of captive Asian macaques[87], including a small group of rhesus macaques.[88, 89] These Asian animals, which were not endemically infected with SIV, turned out to be infected with SIV_{mac}, which was later found to be closely related to HIV-2 and HIV_{sm}.[85] It was quickly realized that Asian macaques were susceptible to SIV and develop an immune syndrome very similar to HIV/AIDS while the natural African primate hosts generally do not. SIV is estimated to have been in African primates for over 32,000 years, so it is highly likely that host adaptation (host and virus coevolution) is responsible for its low pathogenicity in host species.[90] Since the discovery of SIV, a variety of different SIV strains in different primate species have been used as animal models to recapitulate various features of HIV infection in humans.

Primate models of SIV-associated encephalitis

Animal models are particularly useful in studying the neuropathogenesis of HIV because of CNS sample availability at any time point throughout infection. Most primate models of AIDS have used rhesus macaques (*Macaca mulatta*), and most primate models of neuroAIDS have used either $SIV_{mac}239$ or $SIV_{mac}251$, both of which are R5-tropic (like almost all SIV strains) and cause acute viremia.[91] Both viruses are also capable of inducing meningitis, although only $SIV_{mac}251$ causes encephalitis and glial activation in the neuropil characteristic of human HIVE.[91] Although $SIV_{mac}251$ provides an excellent model for HIV neuropathogenesis, there are several limitations: only approximately one quarter of animals infected will actually progress to develop encephalitis, and the time frame is long, taking two years or more, for the development of AIDS.[91] For this reason, several accelerated models of SIVE have been established.

In one model, classic retroviral encephalitis can be induced in up to 90% of rhesus macaques by using monoclonal antibodies to deplete CD8+ T cells.[92-94] This model also serves to highlight the importance of CTL immunologic control in the development of HIVE, although it is somewhat limited because entire subsets of immune cells (CD8+ T cells and NK cells) are eliminated. Another method that researchers have used to reliably induce SIV-encephalitis (SIVE) is to develop neurotropic (able to infect cells in the CNS) and neurovirulent (able to induce neuropathology) strains of SIV. Macrophage tropism has long been known to play an important part in the development of HIVE[95, 96], although studies have shown that macrophage tropism alone is not sufficient to induce the development of neurologic disease.[97] Studies with recombinant SIV indicate that portions of the *env* gene impart macrophage tropism, but that complete *env*, *nef*, and 3'LTR sequences are necessary for neurovirulence.[97, 98]

The Retrovirus laboratory at Johns Hopkins University developed another primate model of accelerated neuroAIDS by dual inoculating pigtailed macaques with a neurovirulent molecular clone (SIVmac/17E-Fr) and an immunosuppressive swarm (SIV/DeltaB670). Using this combination, 70-90% of pigtailed macaques develop classic retroviral encephalitis and all animals develop AIDS-defining criteria within three months.[99, 100] Although most studies of SIV pathogenesis have been done using rhesus macaques, pigtailed macaques have proven to develop more rapid, more severe neurologic disease when compared with rhesus macaques given the same SIV inoculum.

Neuroprotective host factors and viral escape in pigtailed macaques

Another reason that nonhuman primate models of HIV pathogenesis have proved invaluable is macaque genetic complexity and associated wide range of host responses to viral infection. Macaques have a larger number of MHC class I alleles than humans, and some alleles have been identified that are associated with altered progression to AIDS.[101] In rhesus macaques, the MHC class I alleles *Mamu-A*01* and *–B*17* have been correlated with decreased viral load and increased survival time.[102-105] The immunodominant SIV epitope restricted by *Mamu-A*01* (CM9, CTPYDINQM$_{181–189}$) is in the same highly conserved region of Gag capsid as HIV TW10, which is restricted by *HLA-B*57*.[106-108] This provides an invaluable tool to study CTL-mediated immunologic control; however, the supply of rhesus macaques for research is limited, and it has become increasingly difficult to obtain *Mamu-A*01*-positive rhesus for research for a variety of reasons.[109, 110]

Pigtailed macaques also show similar MHC class I allele-associated protection. Expression of the allele *Mane-A1*084:01:01* (formerly *Mane-A*10*) is associated with lower viral load and this protein presents the immunodominant Gag capsid epitope KP9 (KKFGAEVVP$_{164-172}$), a region that overlaps with HIV Gag KF11 (an immunodominant epitope recognized by *HLA-B*5701*).[110, 111] Furthermore, MHC class I-mediated immune pressure at that epitope rapidly induces the formation of a canonical lysine to arginine escape mutation (K165R). Using the accelerated model of neuroAIDS in pigtailed macaques, our lab has demonstrated a neuroprotective effect of MHC class I allele expression; animals that express *Mane-A1*084:01:01* are much less likely to develop SIV encephalitis (SIVE) than animals that do not.[112, 113] This is the first time that HIV/SIV-induced organ-specific disease outcome has been shown to be altered by

MHC class I expression. This finding helps explain the fact that, although all dual-inoculated animals go on to develop high viral loads and progress to AIDS, a small proportion of animals consistently do not develop encephalitis.

Interestingly, *Mane-A1*084:01:01* positive macaques with the highest CNS viral loads developed the prototypic K165R Gag escape as previously found in the plasma in pigtailed macaques inoculated with SIV*mac*251[112], raising the question of how large a role escape mutations play in the development of SIVE. Longitudinal sequencing of plasma and CSF in these animals also showed SIV K165R Gag escape prevalence increases over time, and that this is driven by KP9-specific CTL pressure.[113] In addition, SIV K165R Gag escape mutations were archived in latent proviral DNA reservoirs, including the brain in animals receiving HAART that suppressed viral replication.[113] Replication-competent SIV Gag K165R escape mutations were present in the resting CD4+ T cell reservoir in HAART-treated SIV-infected macaques, indicating that escape develops during the decaying phases of viremia and then can persist in latent reservoirs, including the brain.[113]

Project goals

Despite the provocative relationships between MHC class I alleles and the development of AIDS, associations between the expression of MHC class I alleles and HIV-induced organ-specific disease outcomes, including HAND, have not been investigated. In addition, the relationship between CTL escape and HIV CNS disease remains poorly understood. It is likely that viral fitness in the CNS is distinct from fitness in the periphery given numerous studies that have shown neurovirulent HIV and SIV

contain signature changes in many genes that differ from virus replicating in the periphery.[95, 97, 114] In addition, it is likely that viral escape can arise in the CNS independent of the periphery because different selective pressures on virus evolution exist in the CNS versus the periphery. The overarching goal of the proposed studies was to establish the foundation for novel immunomodulatory therapies targeting the CNS by gaining an understanding of how host genetics and manipulation of cell-mediated immune responses alters progression of SIV-induced CNS disease.

A large part of understanding host genetics and disease resistance is understanding species differences in disease progression. Chapter II focuses on the inherent differences and similarities between rhesus and pigtailed macaques within the confines of the same accelerated CNS disease paradigm. In a retrospective study, we directly compared disease progression in 44 pigtailed macaques versus 29 rhesus macaques that were dual-inoculated with SIV/17E-Fr (neurovirulent clone) and SIV/DeltaB670 (immunosuppressive swarm). Despite the emergence of pigtailed macaques as an increasingly popular animal model to study HIV pathogenesis, few other groups have looked at the inherent differences between this species and Indian-origin rhesus macaques (the most frequently used animal model historically). We found that SIV infection results in faster progression to AIDS in pigtailed macaques and a greater decline in CD4+ lymphocytes compared to rhesus macaques. In addition, pigtailed macaques have consistently higher viral loads in both plasma and cerebrospinal fluid than rhesus macaques and more frequently develop SIVE (as measured by the presence of multinucleated giant cells and perivascular histiocytic cuffing on histopathologic examination). We also investigated the comparative roles that protective MHC class I

allele expression plays in each species, and we found that *Mane-A1*084:01:01* expression in pigtailed macaques and *Mamu-A*01* expression in rhesus are both associated with decreased progression to SIVE, although the effect is stronger in pigtailed macaques.

In chapter III we examined the specific effects that KP9 CTL-mediated immunologic control has on viral escape and disease outcome in our well characterized pigtailed macaque model. We produced a molecularly cloned SIV/17E-Fr K165R Gag escape mutant virus by inserting the K165R mutation into the neurovirulent clone, SIV/17E-Fr using site-directed mutagenesis to understand the potential fitness cost of this escape mutation as it pertains to neurovirulence. The first aim in this study was to determine the relative fitness of SIV/17E-Fr K165R Gag escape compared to wildtype SIV/17E-Fr. To compare the *in vitro* fitness of these viruses, we performed competition assays in multiple primary cell lines. Competition assays in CEMx174 cells (hybrid T/B-cell human cell line susceptible to HIV/SIV infection) as well as primary cultured microglia, macrophages and lymphocytes, and demonstrated that, 1) the cloned SIV/17E-Fr K165R virus replicates successfully in all cell lines tested, and 2) there appears to be a fitness cost to escape, as SIV/17E-Fr K165R replicated at lower levels and more slowly than wildtype SIV/17E-Fr.

We expanded these studies *in vivo* by inoculating pigtailed macaques with molecularly cloned SIV and longitudinally tracked viral load, plasma CD4+ levels, and reversion of Gag K165R to wildtype Gag KP9. In total, six pigtailed macaques that expressed the allele *Mane-A1*084:01:01* were inoculated: three with wildtype SIV/17E-Fr, and three with SIV/17E-Fr K165R. We found that there was no difference between

plasma viral loads in animals inoculated with a virus containing wildtype Gag KP9 or escape Gag K165R, but animals inoculated with SIV/17E-Fr K165R had a slightly lower CSF viral load than those inoculated with wildtype SIV/17E-Fr. Most interestingly, reversion to wildtype SIV/17E-Fr was noted at day 7 p.i. in the CSF only in two animals inoculated with SIV/17E-Fr K165R, suggesting that viral fitness in the CNS is different from the periphery.

The second aim in chapter III was to longitudinally track *in vivo* Gag and Tat peptide-mediated CTL recognition, reversion, and compensatory mutations, and determine if there was a shift in CTL focus from the immunodominant SIV Gag epitope in *Mane-A1*084:01:01* animals inoculated with SIV/17E-Fr K165R. We found that these animals shifted their functional CTL response from Gag KP9 to a co-immunodominant epitope in SIV Tat (KVA10), although there was no evidence of escape at the new locus. In addition, we found no evidence of compensatory mutations in the region of Gag KP9, in contrast to what was reported by Reece et al in a similar animal model.[115]

In continuation with our ongoing characterization of KP9 escape in *Mane-A1*084:01:01* positive pigtailed macaques, we tested a vaccine with the purpose of stimulating cell-mediated immunity against Gag KP9 in the hopes of decreasing SIV-associated neurologic disease. In collaboration with Raphael Viscidi at the Johns Hopkins School of Public Health, we primed, boosted, and challenged two monkeys with a novel virus like particle (VLP) vaccine platform[116] that presents SIV Gag KP9. Both animals successfully developed detectable Gag KP9 tetramer-positive CTL responses before challenge with the neurovirulent clone SIV/17E-Fr. Although there was no difference between the plasma viral load, the vaccinated animals showed lower longitudinal CSF

viral loads than control animals, further suggesting CTL-mediated viral control is different in the CNS when compared with the periphery.

Conclusions

Although new antiretroviral drugs continue to be developed, no antiretroviral therapy has been able to provide a cure. Unfortunately, vaccine trials have met with limited success. To date, nonhuman primates remain the best animal model of HIV/SIV to study either prophylactic or therapeutic vaccines. Although any preventative vaccine will likely require multiple approaches to be maximally successful at preventing and controlling infection, studies in primates have suggested that therapeutic vaccines will need to target Gag in order to be successful in controlling chronic viremia.[117] This work shows that SIV Gag specific CTL-mediated immunologic control of viral infection is important to organ-specific pathogenesis, targeted immune pressure drives viral escape that has a detrimental effect on viral replication, and that immunologic pressure on a highly conserved epitope in Gag is unique in the CNS when compared to the periphery. As therapeutic vaccination strategies to enhance CTL responses against HIV Gag could promote HIV escape, it is vital that we understand the consequences of viral escape on disease progression, especially in the CNS. Our studies have significantly improved our understanding of how CMI alters progression of SIV-induced neurologic disease, establishing the foundation for novel immunomodulatory therapies targeting the CNS in HIV infection.

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II. Exploring Differences in Species Susceptibility to SIV Encephalitis

Introduction

Given the similarities between simian immunodeficiency virus (SIV) and HIV, SIV/macaque models have been valuable for elucidating HIV pathogenesis, testing therapeutics, and vaccine development. The most widely used species for SIV studies include rhesus macaques (*Macaca mulatta*) and pigtailed macaques (*Macaca nemestrina*). Although SIV inoculation of Indian-origin rhesus macaques is the most common and best characterized primate model of HIV infection, limited availability of these animals has prompted interest in alternate macaque species for SIV studies.[1] In particular, pigtailed macaques have gained in popularity as a primate model of HIV pathogenesis because of their larger size, tractable temperament, and susceptibility to SIV infection.[2] Previous studies have also suggested not only that SIV infection in pigtailed macaques more closely recapitulates HIV infection in humans, but that pigtailed macaques are also more likely to progress to SIV-associated encephalitis than rhesus macaques.[3, 4]

In parallel with the discovery that certain MHC class I alleles in humans were associated with altered progression from subclinical infection to AIDS[5], similar relationships were identified in SIV/macaque models of HIV. MHC class I alleles of rhesus macaques (*Macaca mulatta*) in particular have been well characterized[6-9] with discovery of MHC class I alleles, such as *Mamu-B*17*, *Mamu-A*07*, and the most studied, *Mamu-A1*001*, that are associated with increased viral control and decreased progression to AIDS.[9-14] Like humans and rhesus macaques, individual MHC class I alleles in pigtailed macaques that present SIV peptides to cytotoxic T cells have been characterized.[15-17] Of these, the pigtailed macaque MHC class I allele Mane-

A*084:01 (formerly named *Mane-A*10*) has been associated with lower peak viral load and decreased progression to AIDS in animals inoculated with SIVmac251.[2] In our well-characterized model of HIV CNS disease in pigtailed macaques inoculated with both SIV/17E-Fr and SIV/DeltaB670, we have found that *Mane-A*084:01* is a neuroprotective allele that is associated with decreased progression to SIV CNS disease independent of influencing plasma viral load.[15, 16] Interestingly, *Mane-A*084:01* recognizes an immunodominant SIV Gag epitope KP9 that is homologous to the HIV Gag epitope KF11, which is restricted by *HLA-B*57:01* in humans.[17, 18]

Few studies have directly compared SIV disease progression between rhesus and pigtailed macaques[2, 19, 20], and no studies have reported in detail whether the induction of SIV CNS disease varies between these two species. Although advances in HIV treatment have dramatically reduced the incidence of HIV-associated dementia (HAD), neurocognitive defects in treated HIV infected individuals persist, manifest as less severe HIV-associated neurocognitive deficits (HAND).[21] SIV/macaque models will be essential for establishing the causes of HAND.

The SIV pigtailed macaque model developed at Johns Hopkins recapitulates key features of HIV-associated neurologic disease, including high viral load in the cerebrospinal fluid (CSF), encephalitis[4, 22, 23], as well as PNS damage [24, 25], and therefore serves as the basis for comparison with disease progression in rhesus macaques inoculated with the same SIV combination. To investigate whether host factors regulate SIV CNS disease progression, we compared the course of disease and the incidence of SIV encephalitis (SIVE) in rhesus and pigtailed macaques that were dual-inoculated with

both SIV/DeltaB670, an immunosuppressive swarm, and SIV/17E-Fr, a neurovirulent clone.[4, 22]

Methods

Animal studies

This retrospective study included 44 pigtailed macaques (*Macaca nemestrina*) and 29 rhesus macaques (*Macaca mulatta*) that were intravenously inoculated with SIV/DeltaB670 (50 AID50) and SIV/17E-Fr (10,000 AID50) as previously described.[4] Animals were perfused with sterile saline at euthanasia to remove blood and circulating virus from brain. Animals were MHC class I typed by sequence-specific primer (SSP)-PCR.[15] By design, pigtailed macaques were euthanized at approximately 84 days post-inoculation or at the onset of AIDS-defining illness (whichever came first) given consistent progression to AIDS at this timepoint. Initially, 3 rhesus macaques were euthanized at day 84 post-inoculation to compare directly with pigtailed macaque studies. As these three rhesus macaques did not develop either AIDS or CNS disease, the subsequent 26 rhesus macaques were euthanized at the onset of AIDS-defining illness, which ranged from 36 to 560 days PI. Animals were euthanized if any two of the following occurred: weight loss greater than 15% of baseline, clinical signs of organ-specific disease (i.e., CNS, lung, etc.), intractable diarrhea, or opportunistic infection. All animal procedures in this study were performed according to the principles set forth by the Institutional Animal Care and Use Committee at Johns Hopkins University and the National Research Council's Guide for the care and use of laboratory animals.

Quantitation of SIV RNA

To quantitate ongoing viral replication in infected animals, SIV RNA in plasma, CSF, and tissues was measured by qRT-PCR using primers in the SIV gag region, as previously described.[16, 26] Briefly, the primers used to detect unspliced viral RNA were: forward primer, SGAG03, 5'-CAGGGAAIIAAGCAGATGAATTAG-3'; reverse primer, SGAG04, 5'-GTTTCACTTTCTCTTCTGCGTG-3'; and probe, pSUS05, 5'-(6-carboxyfluorescein [FAM])ATTTGGATTAGCAGAAAGCCTGTTGGAG (6-carboxytetramethylrhodamine [TAMRA])-3' with cycle conditions of 50°C for 30 min, 94°C for 15 min to reverse transcribe RNA, which was followed by 45 cycles of PCR at 94°C for 15 s, 55°C for 15 s, and 60°C for 30 s (detection limit 100 copies/mL).[16]

Histopathology

Sections of brain were scored for the presence of encephalitis as previously described.[27] SIV-associated encephalitis was defined as multifocal perivascular accumulations of macrophages and multinucleated giant cells and glial nodules. Hematoxylin and eosin stained sections of frontal and parietal cortex, basal ganglia, thalamus, midbrain, and cerebellum were scored in a blinded fashion. Mild encephalitis was classified as those animals who had an average of less than 10 macrophage-rich perivascular cuffs, moderate encephalitis scores were those animals with an average of 10 to 30 perivascular cuffs, and severe encephalitis were animals with an average of more than 30 perivascular cuffs.[27]

Immunohistochemical staining and quantitative image analysis

Macrophage activation and infiltration in the brain was quantified by immunohistochemistry using a primary antibody against CD68 (KP-1, diluted 1:2,000, DAKO, Carpinteria, CA) on standardized coronal sections of brain including basal ganglia and frontal cortex. Immunohistochemical staining and standardized sampling was done as previously described.[28] Twenty adjacent fields in the subcortical white matter subjacent to the cingulate gyrus were captured at 200X magnification (an area of ~3 mm^2) using a Nikon DS-Ri1 digital camera (Nikon, Tokyo, Japan). These images were binarized and the area fraction of immunopositive pixels was calculated using Nikon Elements to enumerate the area occupied by CD68 positive macrophages.

Statistical analysis

Survival curves for pigtailed macaques and rhesus macaques up to day 84 post-inoculation were compared by the Gehan-Breslow-Wilcoxon test. Similarly, groupwise comparisons of plasma and CSF viral load, CD4+ T cell counts, and CSF IL-6 and CCL2, and terminal tissue viral load were performed using the two sample nonparametric Mann-Whitney t-test. The Fisher's exact test (one-sided) was used to identify associations between MHC class I allele expression versus development of SIV CNS disease.

Results

Accelerated progression to AIDS with a greater decline in CD4+ lymphocytes in pigtailed macaques compared to rhesus macaques

All pigtailed macaques developed AIDS-defining criteria by approximately 84 days p.i. with a steady decline in CD4+ T cells from day 45 onwards (Fig. 1a). In contrast, rapid progression to AIDS in rhesus macaques was much less common; although four rhesus macaques progressed rapidly (<60 days p.i), AIDS developed in most rhesus macaques from 120 to 350 days p.i.

To compare kinetics of immunosuppression after SIV infection, we measured CD4+ T cell counts in blood longitudinally in all animals, revealing a greater decline from baseline in CD4+ T cells in pigtailed macaques compared to rhesus macaques (Fig. 1b). When measured at day 84 p.i., rhesus macaques had the same absolute CD4+ T cell count as pigtailed macaques, however rhesus macaques had a significantly lower terminal absolute CD4+ lymphocyte count than pigtailed macaques (P=0.0003, Mann-Whitney t-test). The nadir median CD4+ cell count in rhesus was reached at >300 days p.i., consistent with a more protracted course of disease progression in rhesus macaques than pigtailed macaques.

Pigtailed macaques consistently developed higher viral loads in both plasma and cerebrospinal fluid than rhesus macaques

SIV viral load was measured in both the plasma and CSF of all animals throughout the course of infection (Fig 2a,b). Pigtailed macaques had a significantly higher viral load than rhesus at day 84 p.i. in both plasma (Fig 2c; P=0.0002, Mann-

Whitney t-test) and CSF (Fig 2d; *P*=0.0093, Mann-Whitney t-test). Even with the extremely long study duration of rhesus macaques compared to pigtailed macaques, terminal viral loads were higher in pigtailed macaques than in rhesus macaques in the plasma (*P*=0.0092, Mann-Whitney t-test) and CSF, where the difference approached significance (*P*=0.0619, Mann-Whitney t-test).

Pigtailed macaques developed SIV-induced CNS disease more frequently than rhesus macaques

To quantify the incidence and severity of SIV-induced CNS disease, the CNS of all animals were scored for the presence of classic SIV encephalitis defined by presence of perivascular cuffs of macrophages, multinucleated giant cells and scattered glial nodules. Pigtailed macaques trended to develop SIV encephalitis more frequently than rhesus macaques. (Table 2; *P*=0.0686, one-sided Fisher's exact test). Correspondingly, pigtailed macaques had higher levels of CCL2 (MCP-1) in the CSF than rhesus macaques during both acute (Fig 3b; *P*=<0.0001 at d7 p.i, Mann-Whitney t-test) and chronic phases of infection (*P*=0.046 at d84 p.i., Mann-Whitney t-tests). In addition, when terminal CSF CCL2 levels were compared, CCL2 was significantly higher (*P*=0.045, Mann-Whitney t-test) in pigtailed macaques compared to rhesus macaques. CSF IL-6 levels in pigtailed macaques were slightly higher than rhesus macaques in acute (*P*=0.13 at d7 p.i., Mann-Whitney t-test) and chronic (*P*=0.22 at d84 p.i., Mann-Whitney t-test) stages of infection, but IL-6 levels were the same between pigtailed and rhesus macaques terminally (*P*=0.92, Mann-Whitney t-test).

Comparable terminal tissue SIV RNA levels in pigtailed macaques and rhesus macaques

To compare extent of viral replication in peripheral tissues and the CNS at terminal timepoints, we measured SIV RNA levels in the spleen and basal ganglia (Fig 4a,b) from rhesus macaques and pigtailed macaques. While viral RNA levels were higher in both the spleen and basal ganglia of pigtailed macaques compared to rhesus, there was not a significant difference between the two groups.

MHC class I alleles play an important role in the development of SIV-encephalitis in both pigtailed macaques and rhesus macaques

To determine whether host genetics contribute to the progression of neurologic disease across macaque species, we genotyped all animals for expression of MHC class I alleles previously associated with lower plasma viral loads including *Mamu-A1*001* in rhesus macaques and *Mane-01*084:01:01* in pigtailed macaques. 5 of 29 (17%) of rhesus macaques expressed *Mamu-A1*001*; 14 of 44 (32%) of the pigtailed cohort expressed *Mane-01*084:01:01* (Table 3). In pigtailed macaques, *Mane-01*084:01:01* expression was not associated with altered plasma or CSF viral loads (Fig 5b,d). In contrast, rhesus that expressed *Mamu-A1*001* had significantly lower terminal plasma viral load (Fig 5a; P=0.0190, Mann-Whitney), but viral load in the CSF was not associated with this MHC class I allele (Fig 5c; P=0.3710, Mann-Whitney). However, both rhesus macaques that expressed *Mamu-A1*001* and pigtailed macaques expressing *Mane-01*084:01:01* had lower levels of SIV RNA (P=0.0107 and P=0.0568, respectively, Mann-Whitney) in the brain consistent with MHC class I-mediated neuroprotection (Fig 5e and 5f).

Discussion

Rhesus macaques and pigtailed macaques have proven to be valuable animal models of HIV due to their susceptibility to SIV; however, few studies have directly compared SIV outcomes in these two different macaque species. Over the last 20 years, we have studied a large number of pigtailed and rhesus macaques that were inoculated with the same SIV combination, allowing us to compare retrospectively a large number of animals. Based on our observations and the observations of others[2, 4], we hypothesized that rhesus macaques would progress to AIDS more slowly and develop SIVE less frequently. In addition, we hypothesized that the immunodominant MHC class I allele *Mamu-A1*001* would show similar neuroprotective effects in rhesus macaques as the immunodominant allele *Mane-A1*084:01:01* has been shown in pigtailed macaques.[15]

As expected, pigtailed macaques progressed to disease more rapidly than rhesus macaques, shown by shorter survival, higher longitudinal plasma and CSF viral loads, and a greater drop in CD4+ cells before d84 p.i. in pigtailed macaques compared to rhesus. At terminal time points, plasma and CSF viral loads (P=0.0092 and P=0.062 respectively, Mann-Whitney) as well as SIV RNA levels in spleen and brain (P=17 and P=0.23 respectively, Mann-Whitney) were lower in rhesus macaques than pigtailed macaques. With this in mind, it is not surprising that our cohort of pigtailed macaques died or were euthanized due to AIDS-defining illness much earlier than rhesus. It was initially suspected that rhesus macaques would have a relatively similar course of infection as pigtailed macaques; however, when the first three animals (12L, 21M, and

23M) were dual-inoculated and then euthanized at the time that pigtailed macaques have progressed to AIDS, none of these animals had developed encephalitis or AIDS-defining illnesses. Thus, subsequent rhesus macaques were allowed to progress until the development of AIDS-defining illness, which in many animals did not occur until after 200 days post inoculation.

Consistent with previous findings in both rhesus and pigtailed macaques[2, 19, 29-31], we found decreases in CD4+ T cell levels in both species after SIV inoculation; however, there was a much greater decrease in CD4+ T cells from baseline in pigtailed macaques. Pigtailed macaques also showed a classic pattern of acute CD4+ T cell decrease followed by a slight rebound then a progressive loss of CD4+ T cells over time. Conversely, rhesus macaques had an initial drop that was maintained within the 84-day post-inoculation time span. The median CD4+ T cell levels then continued to decline over time as animals progressed past 500 days, indicating again that rhesus macaques progressed more slowly compared to pigtailed macaques, even with a relatively virulent inoculation.

Consistent with our hypothesis, we showed that rhesus macaques did not develop neurologic disease as frequently as pigtailed macaques. With the dual-inoculum of SIV/17E-Fr and SIV/B670, 64% of pigtailed macaques developed SIVE compared to 42% of rhesus, although the mean time to euthanasia for rhesus macaques (201.3 days) was substantially longer than pigtailed macaques (79.7 days). We also compared several well-defined markers of SIV CNS disease, CSF IL-6 and MCP-1, both of which predict the development of SIVE.[27] Pigtailed macaques had higher IL-6 and MCP-1 in the CSF at acute (d10 p.i.) and chronic (d42 p.i. and beyond) time points. Terminal CSF IL-6

levels in rhesus were the same as in pigtailed macaques, consistent with prolonged disease progression in rhesus macaques. Terminal CSF MCP-1 levels were still significantly higher in pigtailed macaques than rhesus.

Recently, Klatt *et al* performed a retrospective study that compared SIVmac239 infection in rhesus macaques versus pigtailed macaques. Although pigtailed macaques developed AIDS more rapidly than rhesus macaques, they did not have a correspondingly higher viral load, in contrast to our study.[2] Previously, Polacino *et al* compared $SHIV_{SF162\ P4}$ infection in pigtailed and rhesus macaques, and concluded that pigtailed macaques had significantly higher peak and setpoint plasma viral loads as well as a significantly higher proportion of animals that showed persistent viremia.[20] Similar to the findings of Polacino *et al*, our group of SIV-inoculated pigtailed macaques had higher viral loads in plasma and CSF than rhesus macaques. Additional important differences between our study and previous reports include persistent viremia with much higher plasma viral loads, and uniform progression to AIDS-defining illness in all pigtailed macaques in a relatively short time frame. This is likely due to the high virulence of the inoculum used in our cohort. All animals in our cohort were dual-inoculated with both a macrophage-tropic, neurovirulent clone as well as an immunosuppressive swarm, as opposed to single inoculation with cloned SIVmac239.

In a study comparing neuropathogenesis of rhesus and pigtailed macaques inoculated with a simian-human immunodeficiency virus (SHIV)(KU-2), 21 of 22 pigtailed macaques failed to develop SHIV-associated neurologic disease with productive CNS viral replication; however, this was believed to be due to the inherent failure of the SHIV to replicate in pigtailed macaque macrophages, while the virus was macrophage

tropic in rhesus, leading to effective CNS viral replication in most animals.[32] Macrophage tropism has been well established as a pre-requisite for SIV or SHIV strains to cause lentiviral-associated neurologic disease in either rhesus or pigtailed macaques.[32-35] This is the first study to directly compare the pathogenesis of rhesus and pigtailed macaques challenged with a very neurovirulent SIV inoculum.

To compare the effects of host genetics on disease progression, we focused on the most well characterized MHC class I allele in each animal: *Mamu-A1*001* in rhesus and *Mane-A*084:01:01* in pigtailed macaques. Proportions of macaques that expressed each allele in our population is similar to what has been reported for each species, although there is a large amount of variation in *Mamu-A1*001* expression between different wild and captive populations of rhesus macaques. 31.8% of the pigtailed macaques in this study expressed *Mane-A*084:01:01*, closely following the 32.1% reported previously[36]; 18.5% of our rhesus expressed *Mamu-A1*001*, although the range of reported frequency of *Mamu-A1*001* expression varies from <1-33% depending on the population and country of origin, with Indian-origin rhesus having a much higher *Mamu-A1*001* prevalence compared to Chinese-origin animals.[13, 37, 38]

We had hypothesized that *Mamu-A1*001* in rhesus macaques would demonstrate similar neuroprotective effects as has been reported with *Mane-A*084:01:01* in pigtailed macaques. In this study, expression of the *Mamu-A1*001* allele in rhesus was associated with a lower plasma viral load, which is consistent with reported findings.[11-13, 39] Surprisingly, this difference seen in the periphery did not translate to the CSF, in which levels of viral RNA were not different between animals that expressed the allele from those that did not. We nonetheless found lower levels of viral RNA and less

inflammation in the brains of rhesus expressing *Mamu-A1*001*, indicating that *Mamu-A1*001* is neuroprotective. This indicates greater MHC class I-mediated viral control in the brain compared to the periphery, underscoring the unique nature of T cell-mediated immunity in the central nervous system. There was no difference in plasma or CSF viral loads of pigtailed macaques that expressed *Mane-A*084:01:01*. Nonetheless, both SIV RNA and inflammation in the brain were lower in pigtailed macaques that express *Mane-A*084:01*.

This study illustrates the marked differences in SIV disease progression that genetically distinct species have in response and outcome to an identical neurovirulent inoculum. In addition, the lack of appreciable difference in plasma viral loads between pigtailed macaques and rhesus macaques at acute infection (d10 p.i. P=0.16, Mann-Whitney) implies that the intrinsic/innate immune responses are not playing a major role in species differences. Rather, adaptive, in particular cell-mediated, immunity is the major determinant of species differences in disease outcome to SIV. Clearly, the relationship between MHC class I alleles and disease outcome is complex and multifactorial, and no single MHC class I allele will define the whole immunologic outcome. Thus, deeper and more comprehensive MHC class I haplotyping of these animals will be critical to fully understanding the relationship between host genetics and viral immunity. This is also an important consideration during vaccine development—these findings suggest that vaccination against one single epitope is unlikely to be completely effective. If we are to understand the differences between individual responses to HIV-1 infection, we must try to understand the differences we see in the best models available to us, namely, primate models of lentiviral disease.

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II: Figures

Pigtailed macaques				Rhesus macaques			
Animal ID	**Days PI**	**SIVE**	*Mane-01*084:01:01*	**Animal ID**	**Days PI**	**SIVE**	*Mamu-A*001*
A1P005	46	+	-	**19K**	36	+	-
T4363	48	+	+	**20L**	47	+	-
PCf2	50	+	-	**20E**	49	+	-
72T	57	+	-	**110-93**	56	+	-
PYv1	60	+	-	**21L**	84	-	+
PWe2	60	+	-	**14K***	85	+	-
PNl2	62	+	-	**12L***	85	-	-
A1P014	66	+	-	**21M***	86	-	-
BM03	67	+	-	**23M***	87	-	
18242	72	+	-	**32M**	90	+	-
CC33	73	+	-	**P408**	119	+	-
PBm2	77	+	+	**13H**	121	+	-
11U	77	+	+	**S246**	122	+	-
BK09	82	+	-	**10M**	130	-	
BI55	82	+	-	**15M**	133	+	-
PDi2	83	+	-	**3I**	178	+	-
01P006	83	+	-	**12M**	214	-	-
01P005	83	-	+	**12H**	230	-	-
T4364	84	-	-	**P407**	238	-	+
PEi2	84	-	-	**22K**	240	-	-
PAj2	84	-	-	**526-91**	261	-	-
BP41	84	+	-	**257-92**	295	-	-
BP33	84	-	+	**13U**	327	-	+
A1P003	84	-	+	**16G**	339	-	-
03P003	84	+	-	**451-93**	380	-	-
A1P018	85	-	+	**332-93**	489	-	+
02P004	85	-	-	**AQ04**	532	-	-
18292	85	+	+	**24G**	554	-	-
17850	85	+	-	**3H**	560	-	+
A2P005	86	+	+				
A1P016	86	-	+				
394	86	+	-				
PSk2	87	-	-				
18031	87	+	-				
389	87	+	-				
PUi2	88	-	+				
708	88	+	-				
17834	92	-	+				
387	92	+	-				
PJk2	93	-	+				
713	93	-	-				
18033	94	+	-				
715	94	-	-				
PRk2	98	-	+				

Table 1. MHC I allele genotyping and presence of SIV-associated encephalitis at death/euthanasia. A total of 44 pigtailed macaques and 29 rhesus macaques were dual inoculated with SIV/B670 and SIV/17E-Fr. The initial three rhesus macaques (*) that were dual-inoculated were euthanized at d84 prior to the development of AIDS.

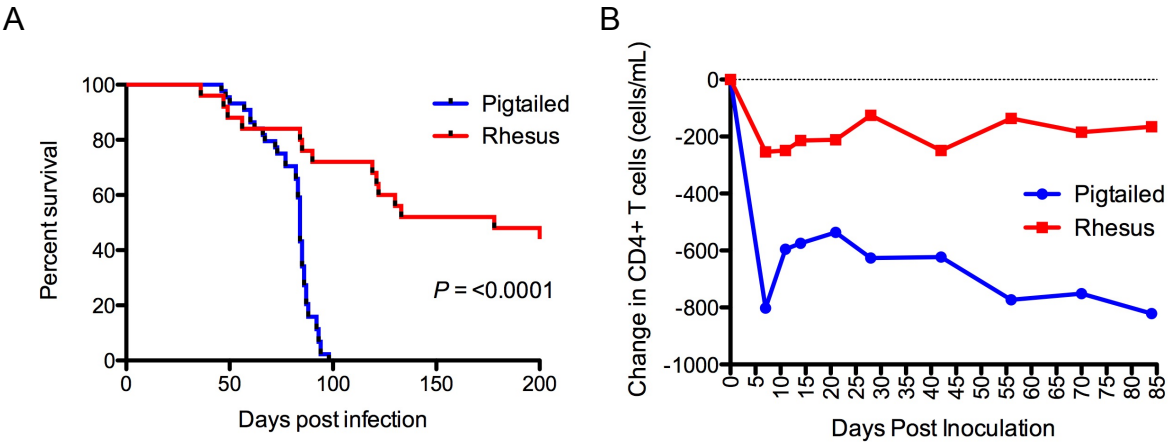

Figure 1. Survival and CD4+ T cell loss in pigtailed macaques and rhesus macaques. SIV-inoculated rhesus macaques (red) survived significantly longer than pigtailed macaques (blue) (A). After SIV-inoculation, pigtailed macaques (blue) had a greater drop in CD4 T cells than rhesus macaques (red) as measured in absolute change from prebleed baseline (B). *P* value for the survival curves were compared using the Gehan-Breslow-Wilcoxon Test.

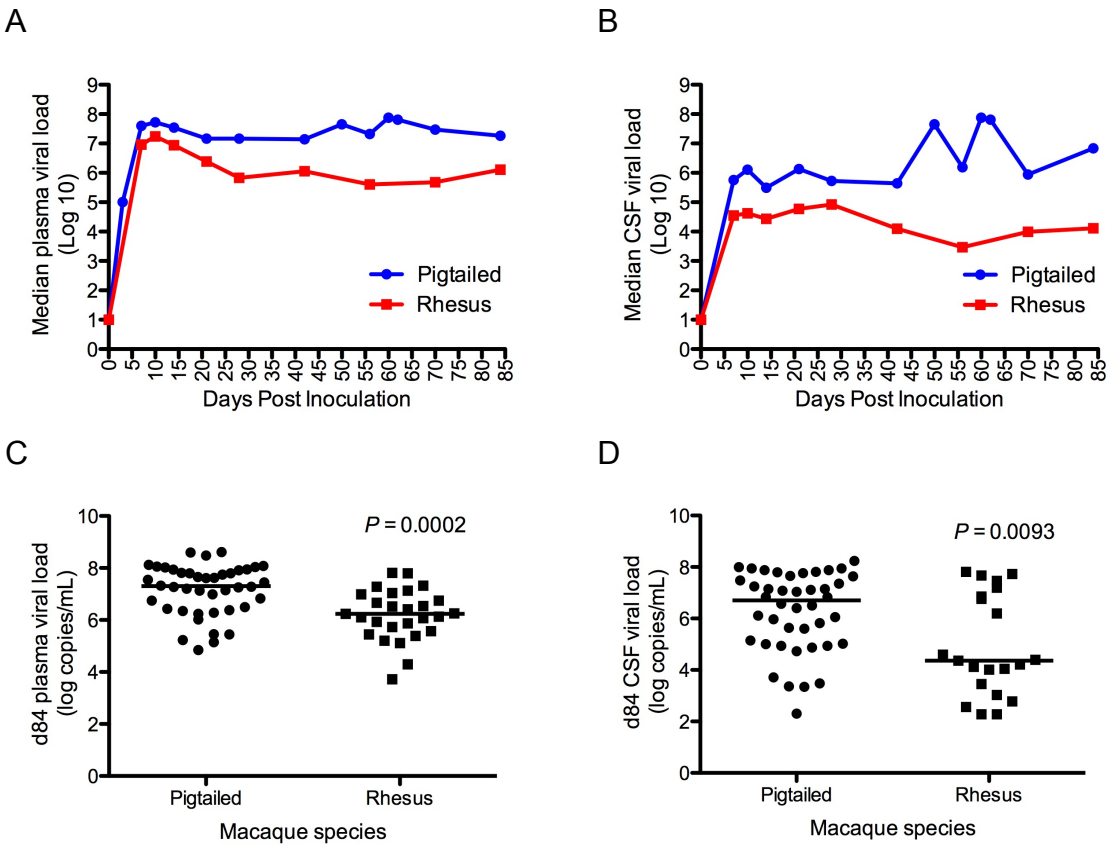

Figure 2. Comparative viral replication between pigtailed and rhesus macaques in plasma, CSF, and tissues. The viral load in both plasma (A) and CSF (B) was higher in pigtailed macaques (blue) compared to rhesus macaques (red). By d84 p.i., viral load in the plasma (C) and CSF (D) were significantly lower in rhesus macaques compared to pigtailed macaques. *P* values for groupwise comparisons (C,D) were determined using the Mann Whitney test. Horizontal lines represent the median.

Species	SIVE -	SIVE +	Total
Pigtailed	16	28	44
Rhesus	15	11	26
Totals	31	39	70

Table 2. The incidence of SIV-associated encephalitis in pigtailed macaques compared to rhesus macaques. Pigtailed macaques are more likely to develop SIV-associated encephalitis (SIV-E) than rhesus macaques based on histopathologic scoring ($P = 0.0686$, one-sided Fisher's exact test).

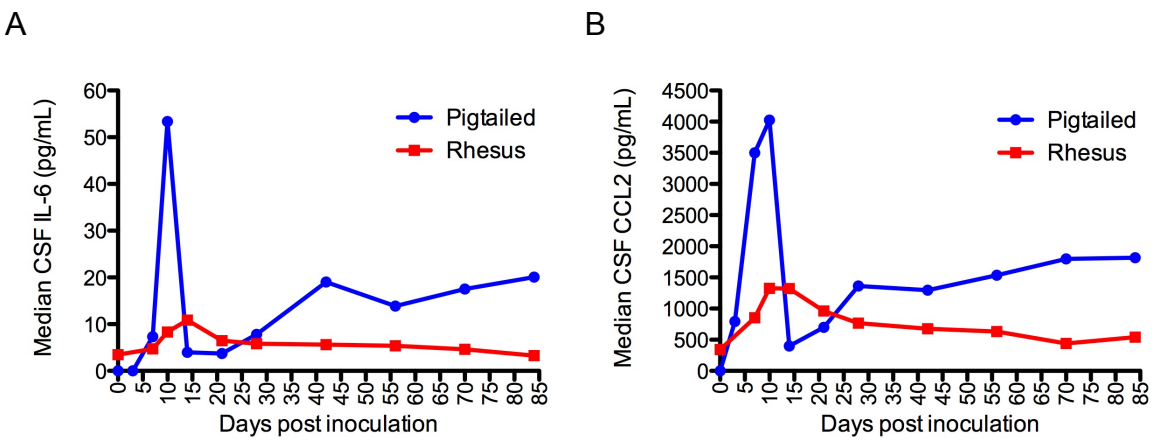

Figure 3. Longitudinal pro-inflammatory markers in the CSF in pigtailed versus rhesus macaques. CSF levels of IL-6 (A) and MCP-1 (CCL2) (B) were higher at acute, asymptomatic, and chronic/terminal phases of disease in pigtailed macaques (blue) compared to rhesus macaques (red).

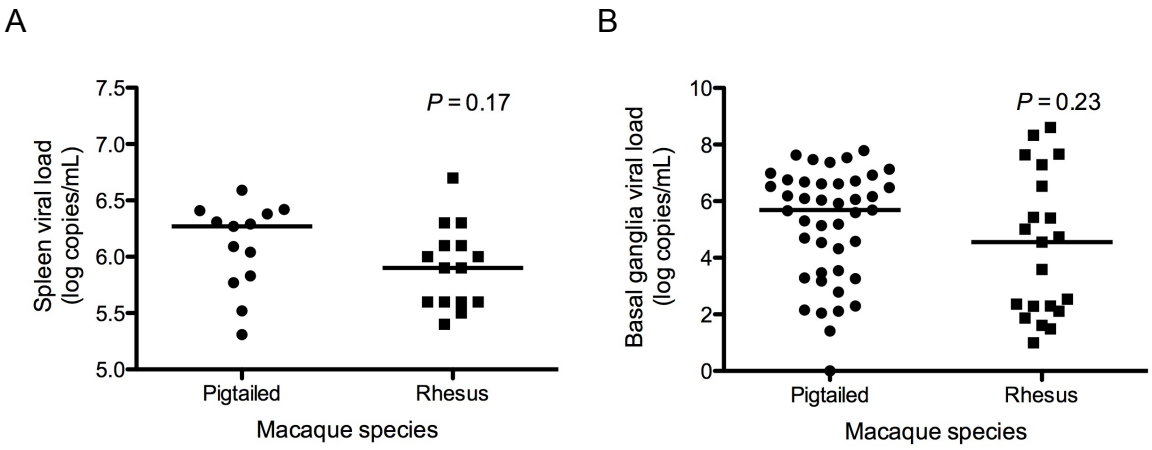

Figure 4. Comparative terminal tissue viral loads in pigtailed and rhesus macaques. Viral load in the spleen (A) and basal ganglia (B) were higher in pigtailed macaques, although these differences are not statistically significant. *P* values for groupwise comparisons were determined using the Mann Whitney test. Horizontal lines represent the median.

Rhesus macaques				Pigtailed macaques			
*Mamu-A*001*	SIVE -	SIVE +	Total	*Mane-A1*084:01:01*	SIVE -	SIVE +	Total
POS	5	0	5	POS	9	5	14
NEG	9	11	20	NEG	7	23	30
Total	14	11	25	Total	16	28	44

Table 3. The effect of host genetics on development of SIVE in pigtailed and rhesus macaques. Both *Mane-A1*084:01:01* in pigtailed macaques and *Mamu-A*001* in rhesus macaques were neuroprotective, in that expression of these alleles was associated with failure to develop encephalitis. This association was stronger in pigtailed macaques (P = 0.0113, one-sided Fisher's exact) compared to rhesus macaques (P = 0.0377, one-sided Fisher's exact), although expression of the neuroprotective MHC class I alleles significantly decreased the risk of progression to SIV CNS disease in both species.

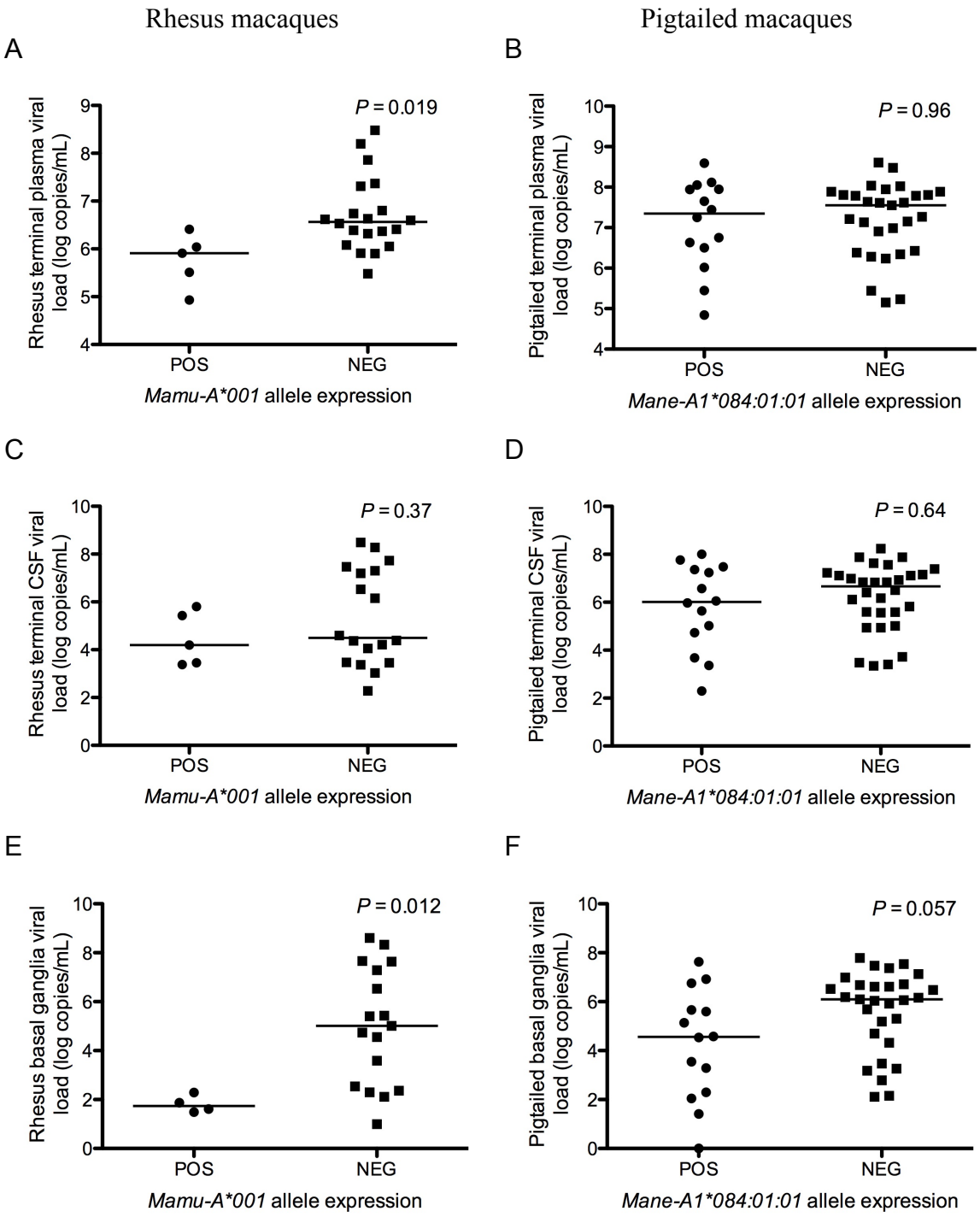

Figure 5. The effect of host genetics on plasma, CSF, and brain viral loads in pigtailed and rhesus macaques. Terminal viral load levels segregated by MHC class I

allele expression were compared in plasma (A), CSF (C), and basal ganglia (E) in pigtailed and rhesus macaques (B,D, and F respectively). Although differences in terminal plasma and CSF viral loads were variable, immunodominant MHC class I alleles in both species were associated with decreased brain viral load. *P* values for groupwise comparisons were determined using the Mann Whitney test. Horizontal lines represent medians.

III. The Consequences of SIV Gag Escape from MHC Class I-mediated Control

Introduction:

Entering the fourth decade of the HIV-1 epidemic, efforts to find an effective vaccine strategy continue to be frustrating given mixed results.[1-4] Studies of HIV-infected individuals categorized as long term nonprogressors and elite controllers have shown an overrepresentation of certain major histocompatibility complex (MHC) class I alleles, including *HLA-B*5701* and *HLA-B*27*, that suggest cytotoxic T lymphocyte (CTL)-mediated MHC class I dependent immunologic pressure is a crucial mechanism for HIV control.[5-8] A recent genome-wide association study of HIV progression conducted by the International HIV Controllers Society confirmed this by showing that over 300 single nucleotide polymorphisms (SNPs) with association to HIV disease progression were identified only within the MHC.[9] The discovery of a link between expression of certain protective MHC class I alleles and decreased progression to AIDS has stressed the importance of understanding host genetics and MHC class I-mediated immunologic control of viral replication when considering vaccine design.

Shortly after HIV-1 was identified as the cause of AIDS, physicians recognized that individuals with AIDS frequently demonstrated varying degrees of HIV-associated neurocognitive disorders (HAND) that ranged from HIV-associated dementia (HAD) to asymptomatic neurocognitive impairment (ANI).[10] With widespread availability of effective antiretroviral therapy, the challenges of clinical management of HIV-infected individuals have shifted from treating life-threatening opportunistic infections to managing a chronic disease in an increasingly aging HIV-infected population. Consequently, although the incidence of HAD has decreased with the advent of effective ART, the cumulative prevalence of HAND has remained unchanged because of a relative

increase in the mild to moderate manifestations.[11] This trend is especially disturbing because individuals with HAND overall tend to have worse clinical outcome with higher rates of mortality and lower adherence to treatment.[12]

Cytotoxic lymphocytes likely play an important role in the pathogenesis of HIV-associated neurologic disease. Perivascular CD8+ T cells have been demonstrated in the brains of patients with HIV-associated encephalitis (HIVE)[13-16], even before the development of clinical neurologic signs.[16] In addition, rhesus macaques (*Macaca mulatta*) depleted of CTLs through the use of anti-CD8 monoclonal antibodies and then infected with simian immunodeficiency virus (SIV) have an extremely high incidence of SIV-associated encephalitis (SIVE).[17-19]

Given the importance of CTLs in the pathogenesis of HIV CNS disease, it is surprising that so few studies have examined the relationship between protective MHC class I allele expression and the development of lentiviral-associated neurologic disease. Using a well-characterized accelerated model of SIVE in pigtailed macaques (*Macaca nemestrina*)[20-22], our group has shown that the expression of the MHC class I allele *Mane-A1*084:01:01* (formerly *Mane-A*10*) is neuroprotective; animals that lack this allele are 2.5 times more likely to develop SIVE and have significant CNS SIV replication.[23] Correspondingly, longitudinal CSF and plasma SIV RNA sequencing revealed the development of a canonical escape in SIV Gag KP9 (K165R) following the emergence of *Mane-A1*084:01:01*/Gag KP9 tetramer-specific CTLs, demonstrating that CTL-mediated immunologic pressure was driving the escape.[24] Also, the SIV Gag K165R escape mutation was archived in latent proviral DNA and was present in resting

CD4+ T cells, indicating that the escape mutation could persist in latent reservoirs such as the brain.[24]

In this study, we engineered the Gag K165R point mutation into the neurovirulent molecular clone SIV/17E-Fr. Escape mutations in HIV and SIV are typically associated with a loss of fitness, and indeed, the development of Gag K165R escape in *Mane-A1*084:01:01*-positive pigtailed macaques has been shown by others to be associated with decreased plasma viral load.[25] The main goal of this study was to evaluate the potential fitness cost of SIV Gag K165R, an escape mutation that develops as the result of CTL-mediated immune pressure associated with the known neuroprotective MHC class I allele *Mane-A1*084:01:01*. We show that inoculation of *Mane-A1*084:01:01*-positive pigtailed macaques with SIV/17E-Fr K165R resulted in lowered CSF viral loads compared to macaques inoculated with wildtype SIV/17E-Fr. Reversion to wildtype SIV Gag KP9 in animals inoculated with SIV/17E-Fr K165R also only occurred in the CSF. Finally, we utilized a novel VLP-based vaccine platform to induce targeted CTL-mediated immune pressure on Gag KP9, and found that vaccinated animals challenged with wildtype SIV/17E-Fr have a lower viral load in the CSF when compared to unvaccinated controls. Plasma viral levels remain unaffected in any of these studies, suggesting that *Mane-A1*084:01:01*-associated CTL control is especially crucial to control of viral replication in the nervous system.

Methods:

Generation of the escape neurovirulent clone SIV/17E-Fr K165R

We constructed an infectious SIV molecular clone containing the K165R escape mutation (Figure 1) by designing synthetic oligonucleotides with an A to G point mutation to modify the KP9 segment of Gag in the SIV/17E-Fr plasmid to K165R using the QuikChange Site-directed Mutagenesis kit (Stratagene, Agilent Technologies©). Briefly, the KP9 sequence was excised from pUC19 SIV 17E-Fr and inserted into a smaller plasmid, pDSRed N1, for site-directed mutagenesis. After the sequence was confirmed by direct sequencing, the insert was excised with the restriction enzyme Sbf1 and re-ligated back into the original plasmid. Stocks for *in vitro* and *in vivo* experiments were generated by transfecting this plasmid into CEMx174 cells.

Primary cell isolation and culture

Primary pigtailed macaque lymphocytes and macrophages were obtained from heparinized peripheral whole blood collected from adult macaques (healthy donors or animals in this study). PBMCs were isolated by discontinuous density gradient centrifugation with 25% Percoll, and subsequently cultured to select for either lymphocytes or macrophages as previously described.[26, 27] Briefly, peripheral blood lymphocytes were cultured by stimulating 1 x 10^7 uninfected PBMC in R10 (RPMI supplemented with 10% FBS, gentamicin (50 mg/ml), 2 mM glutamine,10 mM HEPES buffer) containing PHA (2μg/mL) and IL-2 (10 U/mL) for 48 hours, then cells were washed and resuspended at a concentration of 2 x 10^6 cells/ml in R10 with IL-2. Primary macrophages were cultured in 20% macrophage differentiation media (RPMI supplemented with 20% human serum, gentamycin, glutamine, HEPES, and macrophage

colony stimulating factor [100U]) for 7 days, then washed and resuspended in 10% macrophage differentiation media (with 10% human serum).

Microglia were isolated from macaque cerebral cortex as previously described.[28] Briefly, fresh cortical tissue was minced and trypsinized with 0.25% trypsin, DNase (50 μg/mL), and gentamycin (50 μg/mL) in DMEM [4.5g/L glucose]). Trypsinized tissue was filtered into a clean 50mL conical tube using 183 μM sterile mesh, washed, resuspended in sterile PBS, and centrifuged with Percoll for 35 minutes at 15,000 rpm in an Oakridge tube. The microglia were then removed from beneath the myelin layer and filtered through a 40μm mesh. Cells were plated in DMEM with 10% FBS in 25-cm^2 flasks overnight, washed, and cultured for 5 days in DMEM supplemented with 5% FBS, 5% giant-cell-growth-tumor conditioned media (OriGen, Gaithersburg, MD), 1mM sodium pyruvate, and 50 μg of gentamicin/mL.

In vitro viral replicative fitness assays

Primary macaque cells (lymphocytes, macrophages, and microglia) and CEMx175 cells were infected in duplicate with either SIV/17E-Fr (MOI=0.05), SIV/17E-Fr K165R (MOI=0.05) in 6 well plates or 25-cm^2 flasks. After 24 hours, the cells were washed three times with PBS to remove virus and then cultured in appropriate media (see above) for 15 days. Culture supernatants were subsequently collected at 48-hour intervals for p27 ELISA (Advanced Bioscience Laboratories, Rockville, MD) and viral RNA isolation.

Animal studies

A total of 10 male juvenile *Mane-A1*084:01:01*-positive pigtailed macaques (*Macaca nemestrina*) were used in this study. MHC class I expression by pigtailed macaques was evaluated by sequence specific PCR or Roche 454 Titanium pyrosequencing.[24] Six macaques were intravenously inoculated with either SIV/17E-Fr or SIV/17E-Fr K165R (groups 1 and 2, Table 1) at a dose of AID50 10,000. Two additional macaques (group 3, Table 1) were intravenously dual-inoculated with intravenously inoculated with SIV/DeltaB670 (50AID50) and SIV/17E-Fr (AID50 10,000) as previously described.[20] The final two macaques (group 4, Table 1) were primed then boosted in approximately two-week intervals with 250 μg KP9-loaded VLP administered intradermally then intramuscularly (quadriceps muscle) until a Gag KP9-specific tetramer response was achieved, than challenged with SIV/17E-Fr (AID50 10,000). For all animals, blood and CSF samples were taken approximately every 14 days, and animals were perfused with sterile saline when euthanatized at day 84 post inoculation. These studies were approved by the Johns Hopkins University Institutional Animal Care and Use Committee.

Quantitation and sequencing of SIV RNA

SIV RNA was measured in the plasma and CSF by real-time reverse transcription PCR (qRT-PCR) using primers directed against SIV Gag as previously described.[24] PCR was performed on cDNA generated from the plasma, CSF, and cultured cell supernatant with the SIV Gag KP9-specific primers (forward) 5_-CACGCAGAAGAGAAAGTGAA-3_ and (reverse) 5_GTTCCTCGAAT(AG)TC(GT)GAT CC-3_ using Platinum PCR supermix

(Invitrogen) and specific cycle conditions (94°C for 2 min; 30 cycles of 94°C for 15 s, 56°C for 30 s, 72°C for 1 min; and a final extension of 72°C for 8 min).
PCR product was cloned (TOPO TA) and colonies were selected for sequencing as described.[24] Sequences were aligned and analyzed using Geneious 5.0.2 software.

Measuring SIV Gag KP9 specific CD3+/CD8+ T cells

Leukocytes in 0.1 mL whole blood were stained with allophycocyanin (APC)-conjugated *Mane-A1*084:01:01*/Gag KP9 tetramer at a 1:500 dilution for 30 minutes at room temperature in the dark, followed by counterstaining with anti-CD3-PE (BD, Cat #552127), anti-CD4-PerCP-Cy™5.5 (BD, Cat # 552838), and anti-CD8-FITC (BD, Cat #557085) conjugated antibodies for 30 minutes at room temperature in the dark. Erythrocytes were lysed and leukocytes were fixed with 2 mL FACS lysing solution (BD, Cat #349202) for 20 minutes at room temperature, washed twice, and resuspended in 0.5 mL FACS buffer. At least 20,000 CD4 negative, CD3 positive, CD8 positive, tetramer positive cells were acquired using an LSRFortessaTM flow cytometer, and analysis was performed using FlowJo (Tree Star Inc., Ashland, OR). The percentage of tetramer positive CTLs were normalized to uninfected control animals.

Generation of the Gag KP9-specific virus-like particle (VLP) vaccine

The virus-like particle (VLP)-based vaccine was composed of bovine papillomavirus L1 capsid protein that was genetically engineered to contain 8 glutamic acids and a cysteine in a surface exposed loop of the protein with Gag KP9 covalently linked to the particle.[29] The negatively charged glutamic acids served as docking sites

for protein/peptide antigens with an NH2 tag of 8 positively charged arginines and flanking cysteines. The SIV Gag KP9 antigen was covalently linked to the particle by an oxidation-reduction reaction.

VLP vaccination

Blood and CSF were collected from two *Mane-A1*084:01:01*-positive pigtailed macaques (group 4) three times at one-week intervals before vaccination (prebleed samples). Following the final prebleed sample collection, both macaques were intradermally administered with 100 μg (in 0.1 μL buffered saline) SIV Gag KP9-VLP and monitored (day 0). Both animals were boosted intradermally on days 14 and 28, but due to sample preparation the dose was later determined to be suboptimal. On days 42, 56, 84, and 98 both animals were subsequently boosted with VLP by both intradermal (100 μg) and intramuscular (250 μg in 0.25 μL buffered saline) routes. Blood and CSF were collected on days 14, 28, 42, 56, 62, 70, 84, and 98 post VLP prime at day 0. Because SIV Gag KP9 specific CTL responses were consistently observed by tetramer FACS analysis by this point, VLP boosts were discontinued. Animals were challenged with SIV/17E-Fr 7 days after the final VLP boost.

Results:

Kinetics of viral replication *in vitro*

Replication of wildtype SIV/17E-Fr and mutant SIV/17E-Fr K165R were directly compared *in vitro* in the lymphocyte cell line CEMx174, macaque peripheral blood

lymphocytes, macrophages, and microglia (figure 2). The goals of these in vitro studies were to 1) confirm that the mutant virus construct SIV/17E-Fr K165R was replication competent *in vitro*, and 2) compare the replicative fitness of SIV/17E-Fr K165R versus the wildtype clone SIV/17E-Fr. Previous studies using SHIV strains have shown rapid reversion of the Gag K165R mutation to wildtype KP9 when inoculated into animals that do not mount an immunologic response against the KP9 epitope, suggesting that K165R mutation incurs a fitness cost.[25]

By infecting the HIV/SIV permissible hybrid B/T cell line CEMx174, we showed that the molecular clone SIV/17E-Fr K165R was able to replicate successfully, but produced less virus compared to wildtype SIV/17E-Fr (Figure 2a), suggesting decreased replicative fitness. Since CEMx174 cells are a transformed cell line, we also extended the competition assays in primary macaque cells isolated from blood (lymphocytes and macrophages). Since the backbone of the mutant virus is a macrophage-tropic SIV clone[27], the Gag escape mutant was expected to have similar macrophage tropism. As expected, SIV/17E-Fr K165R was also replication competent in primary lymphocytes and macrophages, but similar to the findings in CEMx174 cells, had slower growth compared to the parental virus containing wildtype Gag KP9 (Figure 2b,c).

As a neurovirulent clone, wildtype SIV/17E-Fr also replicates readily in microglia.[28] As an *in vitro* surrogate measure of viral replication in the brain, we isolated and infected macaque microglia. Although the viral growth curve of SIV/17E-Fr K165R was more similar to wildtype SIV/17E-Fr in infected primary microglia than in primary lymphocytes or macrophages, escape virus still demonstrated lower peak virus and a slower growth curve (Figure 2d). Lower *in vitro* viral replication regardless of cell

type was consistent with the premise that the Gag K165R mutation incurs a general fitness cost.

Kinetics of viral replication *in vivo*

To determine whether fitness cost of the Gag K165R mutation demonstrated *in vitro* was seen *in vivo*, we inoculated three *Mane-A1*084:01:01*-positive pigtailed macaques with SIV/17E-Fr K165R (group 2) and compared SIV replication with three *Mane-A1*084:01:01*-positive pigtailed macaques inoculated with wildtype SIV/17E-Fr (group 1). These studies were terminated at 84 days post inoculation (PI) and longitudinal viral loads in the plasma and cerebrospinal fluid (CSF) were compared (Figure 3). Longitudinal plasma viral loads in animals inoculated with mutant SIV/17E-Fr K165R were nearly identical to those animals inoculated with wildtype SIV/17E-Fr (Figure 3a). In contrast, the median CSF viral load of animals inoculated with SIV/17E-Fr/K165R was lower than those inoculated with SIV/17E-Fr after acute infection (Figure 3b). These findings illustrate that the fitness cost of SIV Gag mutation may selectively alter CNS replication without altering SIV replication in the periphery.

In addition, we inoculated two pigtailed macaques expressing the MHC class I allele *Mane-A1*084:01:01* with both the mutant SIV clone 17E-Fr K165R and the immunosuppressive swarm SIV/B670 (group 3) to compare directly with the rapid SIV CNS model developed at Johns Hopkins University that utilizes dual infection with17E-Fr and SIV/B670 to induce SIV CNS disease.[20, 22] However, SIV *env* sequencing revealed that the SIV clone 17E-Fr K165R was rapidly overwhelmed by SIV/B670 strains, minimizing the detectable biologic effect of SIV/17E-Fr K165R (data not shown).

Differences in compartmental viral genotype selection

Cloning and sequencing of SIV *gag* in both the peripheral (plasma) and CNS (CSF) compartments in animals inoculated with SIV/17E-Fr K165R (group 2) was used to track reversion from mutant Gag K165R to wildtype Gag KP9. Since we inoculated pigtailed macaques that express *Mane-A1*084:01:01* that would mount constant immunologic pressure against reversion to Gag KP9, we did not expect to see significant reversion to wildtype. Indeed, previous studies that challenged *Mane-A1*084:01:01*-positive pigtailed macaques with a natural SIV isolate containing the Gag K165R mutation showed maintenance of the escape mutation.[25] As expected, plasma genotyping of SIV *gag* in the three animals inoculated with SIV/17E-Fr K165R showed SIV Gag K165R stability up to day 28 post inoculation (figure 4a,b); however, CSF genotyping showed consistent reversion to wildtype at the Gag KP9 locus starting as early as 7 days post inoculation in both animals genotyped (figure 4c,d). SIV was successfully cultured from the microglia of one of these animals showed a similar pattern of reversion, with 25% of virus genotyped having reverted to SIV Gag KP9 wildtype (data not shown). We were unable to culture virus from the microglia of the remaining two animals.

The development of specific CTL responses against immunologic epitopes in SIV Gag and Tat were longitudinally tracked using ELISPOT assays. Although evidence suggests that Gag KP9 is the immunodominant epitope recognized by *Mane-A1*084:01:01*, we hypothesized that CTL-mediated immunologic pressure could switch to an alternate epitope in the absence of Gag KP9 in the inoculum. Indeed, we found a

strong IFNγ response to Tat KVA10, an epitope also shown to be restricted by *Mane-A1*084:01:01*.[30] Interestingly, one macaque inoculated with only wildtype SIV/17E-Fr showed an equally high Tat KVA10 response as those inoculated with SIV/17E-Fr K165R (data not shown). This indicated that there is a consistent immunologic response to Tat KVA10 in *Mane-A1*084:01:01*-positive SIV infected animals, implying that Tat KVA10 is a codominant epitope whose effect is best seen when the strong Gag KP9 response disappears. Sequencing of the Tat KVA10 locus in day 14 plasma failed to show escape in response to increased Tat epitope CTL-mediated immune pressure (data not shown).

Mutations that develop outside of an immunodominant viral epitope can partially restore viral fitness, and are termed compensatory mutations. *Mane-A1*084:01:01*-positive pigtailed macaques vaccinated with an SIV/influenza vaccine that rapidly developed SIV Gag K165R escape commonly also subsequently developed a Gag V145A compensatory mutation.[31] Interestingly, we found no evidence of V145A or other compensatory mutations in the region of KP9 in our animals.

Virus-like particle vaccine platform targeting SIV Gag KP9

To boost KP9-specific CTL responses before SIV inoculation that specifically targets the SIV Gag KP9 epitope and thereby accelerate K165R escape in animals inoculated with SIV/17E-Fr, we utilized a unique vaccine platform designed to induce a robust CMI response using a virus-like particle that presented the SIV Gag KP9 peptide. Two *Mane-A1*084:01:01*-positive pigtailed macaques were primed and boosted until a consistent Gag KP9-specific CTL response was observed. We induced a robust CTL

response in both animals, as measured by Gag KP9-tetramer response (Figure 5b). Interestingly, intradermal vaccination had minimal ability to prime and boost an immune response, while the intramuscular route produced a strong response within two weeks of the first boost (day -42 pre-inoculation, Figure 5b).

After establishing that VLP vaccination increases Gag KP9-specific immunologic response, animals were challenged with a single-inoculum of wildtype SIV/17E-Fr. Post-challenge, both animals responded with an early and robust KP9 tetramer-specific anamnestic response in acute infection (Figure 5b), considerably earlier than typically seen in unvaccinated animals at day 56 p.i. (Figure 5a). While VLP vaccination did not measurably alter plasma viral load, both animals had lower viral loads in the CSF compared to control animals (n=3).

Discussion:

For the first time, this study has demonstrated a uniquely CNS compartment-specific fitness cost to viral escape from MHC class I-mediated immunologic pressure. After inserting the canonical SIV Gag escape mutation K165R into the neurovirulent molecular clone SIV/17E-Fr, we were inoculated *Mane-A1*084:01:01*-positive pigtailed macaques with the cloned escape mutant virus and demonstrated decreased viral load in the CSF, but not the plasma. Viral sequencing in these animals revealed reversion to wildtype Gag KP9 only in the CSF and not the plasma, corresponding with decreased CNS fitness of K165R. In a reciprocal experiment we induced targeted immunologic pressure by vaccinating animals with a novel VLP-based construct that focused the CTL

response on Gag KP9. Subsequent challenge with wildtype SIV/17E-Fr resulted in a decreased viral load in the CSF, but not plasma. Together, these findings provide compelling evidence that suggest a CNS-specific loss in viral fitness that is incurred as a result of Gag escape.

VLP Gag KP9 vaccination provided us with a novel way to induce targeted CTL-specific immunologic pressure. VLP particles are especially immunogenic because of their size (40-50 nm), repetitive surface docking sites that present foreign antigen, and their ability to self-adjuvanate.[29, 32-34] VLP vaccine prime and boosts delivered intramuscularly induced a robust KP9-specific CTL response as measured both by the number of *Mane-A1*084:01:01*/KP9 tetramer positive CTLs as well as CTL production of IFNγ and TNFα as measured by intracellular staining FACS (data not shown). Memory phenotype FACS analysis of tetramer positive CTLs also showed that almost all of the KP9 specific memory CD8+ T cell response consisted of effector memory cells, with 42% of tetramer positive cells defined as EM1 transitional memory cells (CD95+/CD28+/CCR7-), 55% defined as EM2 fully differentiated effector memory (CD95+/CD28-/CCR7-), and only the remaining 3% as central memory (CD95+/CD28+/CCR7+). In rhesus macaques, vaccines that are able induce robust effector memory T cell responses are believed to provide better protection against SIV infection than vaccines that produce predominantly central memory T cell responses (lymphoid tissue-based) because of the amount of time necessary for an effective antigen-stimulated response to mount.[35-37] In the future, it would be useful to carry out a similar study with VLP vaccinated pigtailed macaques to evaluate how long the Gag KP9 T cell memory response persists. As a single-epitope vaccine is not likely to effectively

prevent SIV or HIV infection due to the development of escape and/or compensatory mutations, further studies vaccinating with multiple SIV Gag epitopes on a VLP backbone are needed.

Evaluation of CD8+ T cell functional responses to Gag peptide pools by Smith *et al* (2005) first recognized Gag KP9 as an important immunodominant SIV Gag epitope in pigtailed macaques.[38] This is especially relevant because KP9 is a homologue of the Gag epitope KF11, which is recognized by HLA-B57*01.[39] Identification of alleles that were shared amongst animals that responded to Gag KP9 and functional studies to demonstrate binding confirmed that *Mane-A1*084:01:01* was the main SIV Gag KP9 presenting molecule.[38] Furthermore, SIV in *Mane-A1*084:01:01*-positive animals rapidly developed the canonical escape mutation Gag K165R, and these animals had lower levels of SIV RNA in the plasma, suggesting a replicative fitness cost to mutation.[38, 40] When *Mane-A1*084:01:01*-negative macaques were inoculated with SHIV containing the Gag K165R mutation, there was rapid reversion to wildtype KP9, confirming the fitness cost to mutation.[41]

In direct contrast to these findings, our lab found no difference in either plasma viral RNA or CD4+ cell counts between pigtailed macaques that expressed *Mane-A1*084:01:01* and those that did not; however, CSF viral RNA was lower in *Mane-A1*084:01:01*-positive macaques.[23] The main difference between these groups was inoculum: the animals reported on by Smith *et al* were inoculated with SIVmac251 while our animals were dual-inoculated with SIV/DeltaB670 and SIV/17E-Fr in order to maximize the development of SIVE. Data from this study were more consistent with data from our dual-inoculated animals than animals inoculated with SIVmac251. Animals in

this study were all *Mane-A1*084:01:*01-positive and were inoculated with either the wildtype SIV/17E-Fr or escape neurovirulent clone SIV/17E-Fr K165R, and the only difference between the two groups was in CNS viral RNA. These data support the view that the efficacy of antigen-specific CTL control in the CNS clearly differs from the periphery.[42]

Numerous studies in both HIV[43-46] and SIV[31, 47-50] have reported the emergence of compensatory mutations, or mutations that occur outside of an epitope that may partially or completely restore viral replicative fitness. In rhesus macaques that express the MHC class I allele *Mamu-A1*001,* a variety of compensatory mutations emerge concurrent with the development of escape at the *Mamu-A1*001*-restricted Gag epitope CM9 (T182A).[47, 50] In several studies, compensatory mutations in SIV have been shown to restore viral replicative capacity specifically by restoring normal viral packing and assembly.[49, 51] Likewise, pigtailed macaques that express *Mane-A1*084:01:01* will often develop a second Gag mutation after escape from Gag KP9 has occurred 20 amino acids upstream (V145A).[31] Interestingly, we found no evidence of compensatory mutations in our *Mane-A1*084:01:01*-postivie pigtailed macaques, either at V145A or new sites; however, sequences in our study only spanned several hundreds of amino acids before and after Gag KP9, so it is entirely possible that compensatory mutations may have occurred at other regions more distant in Gag.

In addition to SIV Gag SIV, *Mane-A1*084:01:01* recognizes two immunodominant epitopes in SIV Tat: KVA10 (KKETVEKAVA) and KSA10 (KKAKANTSSA).[30] Escape mutations also develop in both Tat epitopes, although there is not a single consensus escape mutation that emerges unlike canonical KP9

escape.[30] We hypothesized that the *Mane-A1*084:01:01*-mediated CTL control would switch in focus from Gag KP9 to one or both of these Tat epitopes animals that were inoculated with SIV/17E-Fr K165R. We found that these animals had a robust early response to Tat KVA10 but not Tat KSA10 (as measured longitudinal by IFNγ ELISPOT and KVA10 or KSA10 tetramer response). At no point in the 84 day study did any animals inoculated with SIV/17E-Fr K165R show a CTL response to Gag KP9. Interestingly, we found an equal response to Tat KVA10 68X (one of the animals inoculated with SIV/17E-Fr), suggesting that, rather than an immunologic switch to Tat KVA10 in the absence of Gag KP9, these animals responded to Tat KVA10 as a codominant epitope.

There are several limitations to a study of this nature. We were limited in sample size given limited availability of macaques. Single-inoculation using only SIV/17E-Fr doesn't reliably cause overt encephalitis, although there is evidence of productive infection in the CNS. None of the animals in this study developed SIVE. Dual-inoculation with SIV/DeltaB670 would be the most ideal way to evaluate CNS pathology; however, we found that the immunosuppressive swarm SIV/DeltaB670 quickly overwhelmed SIV/17E-Fr K165R replication in the brain, making the pathologic effects of our introduction of the Gag K165R mutation in SIV/17E-Fr difficult to evaluate. Since SIV/DeltaB670 is a swarm and not a cloned virus, it was impossible to introduce the Gag K165R mutation into all variants present in the DeltaB670 inoculum.

SIV *env* cloning and sequencing allows us to differentiate between the immunosuppressive swarm and the neurovirulent clone, SIV/17E-Fr. Interestingly viral RNA was 100% SIV/DeltaB670 in the basal ganglia; however, one of the two animals

was 100% SIV/17E-Fr when SIV DNA was sequenced, implying that only the neurovirulent clone SIV/17E-Fr was able to integrate. Full viral genome sequencing would be necessary to rule out the possibility of viral recombination between SIV/Delta B670 and SIV/17E-Fr.

Surprisingly, few studies of HIV or SIV have specifically looked at the association of MHC class I-mediated viral control and CNS outcome.[23, 24] We have found compelling evidence for epitope-specific targeted immune pressure on viral fitness that is CNS-specific. In addition, we developed a vaccine strategy to target CTL-mediated immunologic pressure on this Gag epitope and found subsequent decreased SIV viral load in CNS. As the struggle to find an effective vaccine strategy for HIV-1 continues, it is extremely important to understand the interactions between host immunogenetics and organ specific HIV-induced disease. Our data provide the rationale for the development of a therapeutic vaccine-approach to control HIV-associated system-specific pathology, especially in the CNS.

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III: Figures

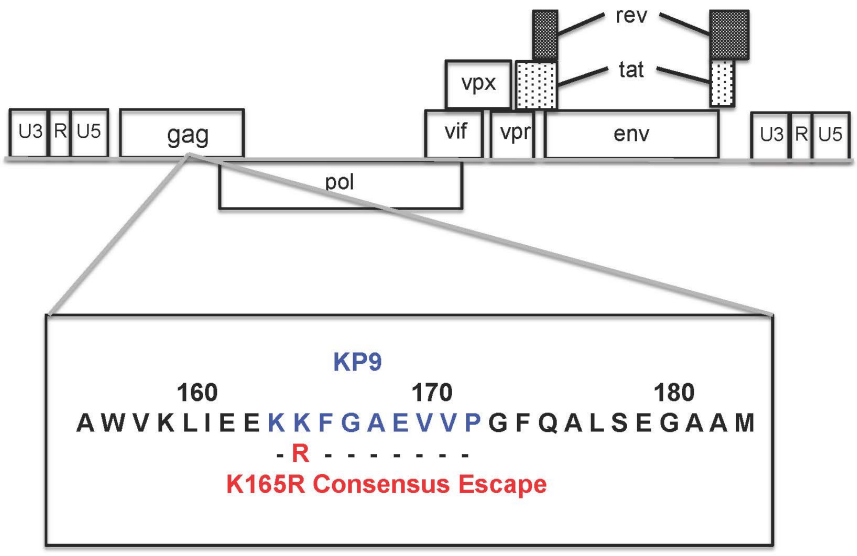

Fig 1. The MHC class I allele *Mane-A1*08401* recognizes KP9, a dominant epitope in SIV Gag.

In response to MHC class I-mediated CTL pressure, *Mane-A1*08401* positive macaques develop a consensus escape mutation in the capsid region of SIV Gag, identified as K165R escape SIV, as the result of a single point mutation lysine to arginine at amino acid 165 of Gag (A→G).

Group	Number of animals	Inoculum			VLP vaccinated
		SIV/17E-Fr	SIV/17E-Fr K165R	SIV/Delta B670	
1	3	+	-	-	-
2	3	-	+	-	-
3	2	-	+	+	-
4	2	+	-	-	+

Table 1. Animal groups in this study. Ten pigtailed macaques were inoculated with SIV/17E-Fr alone, SIV 17E-Fr K165R alone, or SIV/17E-Fr K165R and SIV/DeltaB670 together.

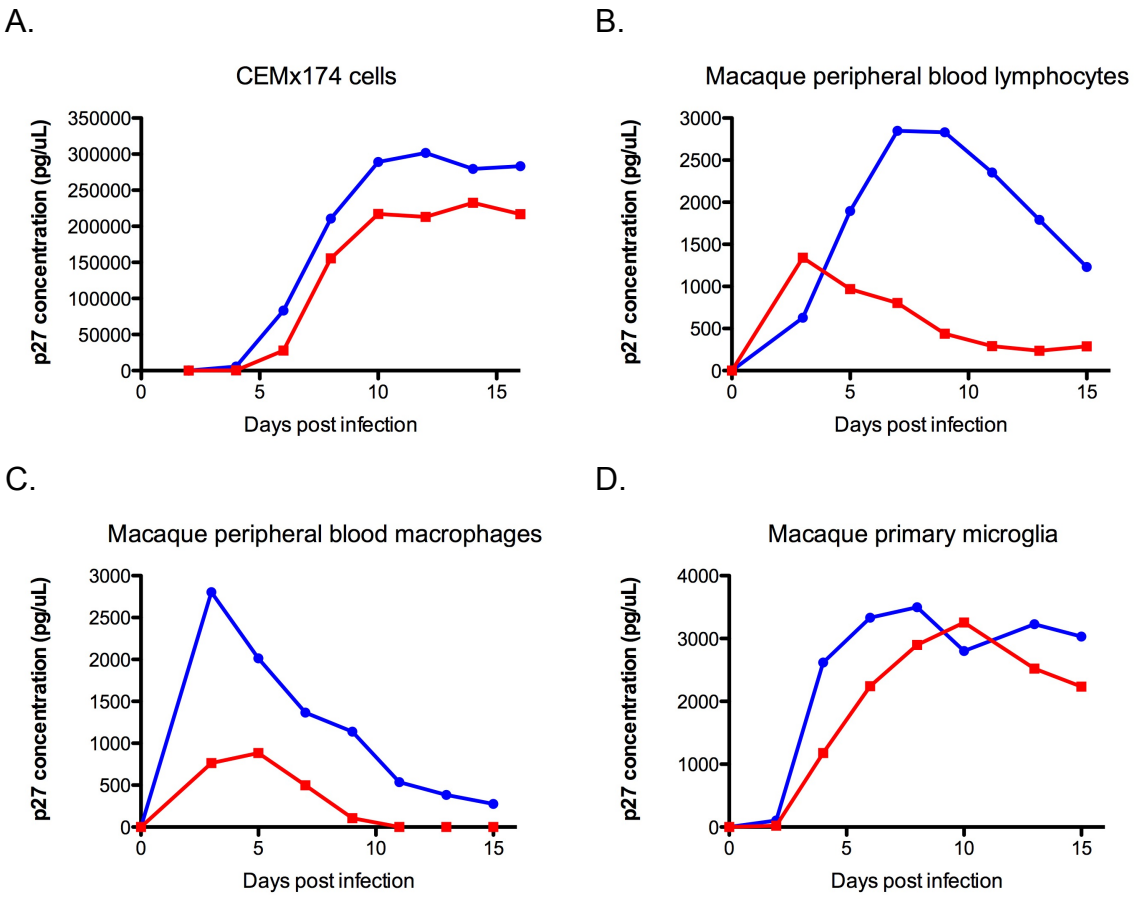

Fig 2. Comparative replication of SIV/17E-Fr K165R vs wildtype SIV/17E-Fr. *In vitro*, replication of SIV/17E-Fr K165R (red) was lower than wildtype SIV/17E-Fr (blue) in the CEMx174 B/T cell hybrid line (A) and in cultured primary lymphocytes (B), macrophages (C), and microglia (D).

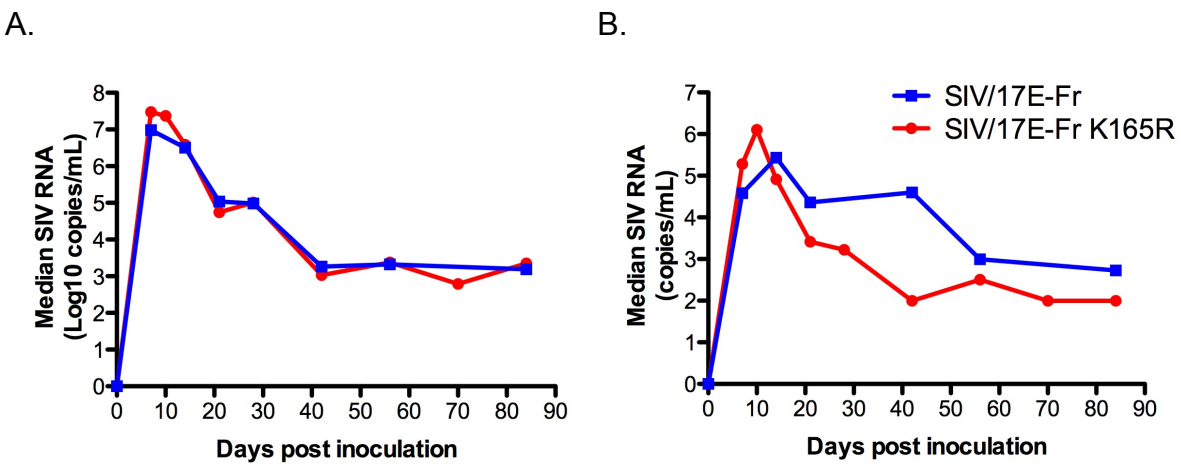

Fig 3. Replicative fitness of SIV/17E-Fr versus SIV/17E-Fr/K165R in *Mane-A1*08401* positive macaques. Viral load in the plasma (A) was similar in *Mane-A1*08401* positive pigtailed macaques infected with SIV/17E-Fr (blue) and SIV/17E-Fr K165R (red). In contrast, CSF viral load in macaques infected with SIV/17E-Fr/K165R was consistently lower than SIV/17E-Fr after acute infection.

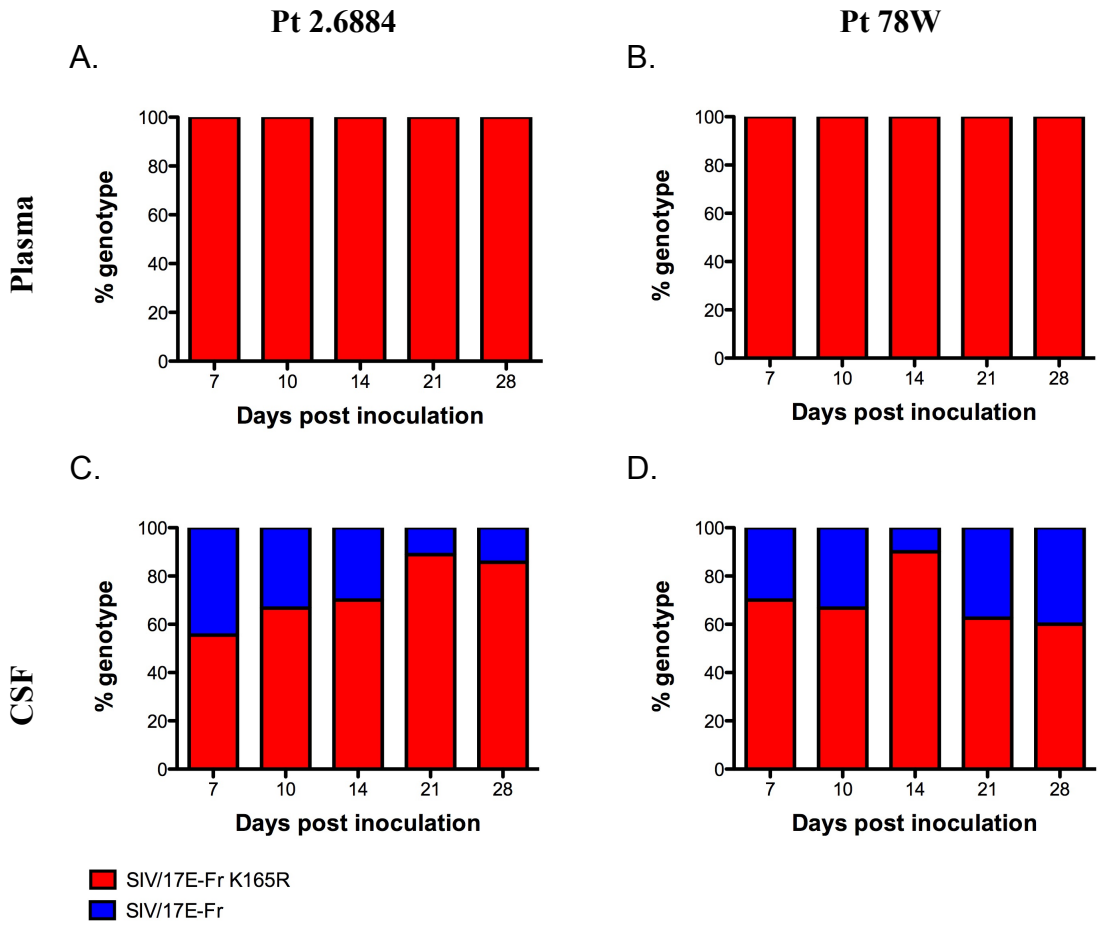

Fig 4. Longitudinal SIV RNA genotyping in the plasma and CSF of two pigtailed macaques inoculated with SIV/17E-Fr K165R.

In macaques inoculated with the cloned escape virus, SIV/17E-Fr K165R, reversion to wildtype SIV Gag KP9 (blue) developed rapidly in the CSF but not the plasma. Viral RNA was isolated from CSF and plasma and viral genotype was established by cloning and sequencing.

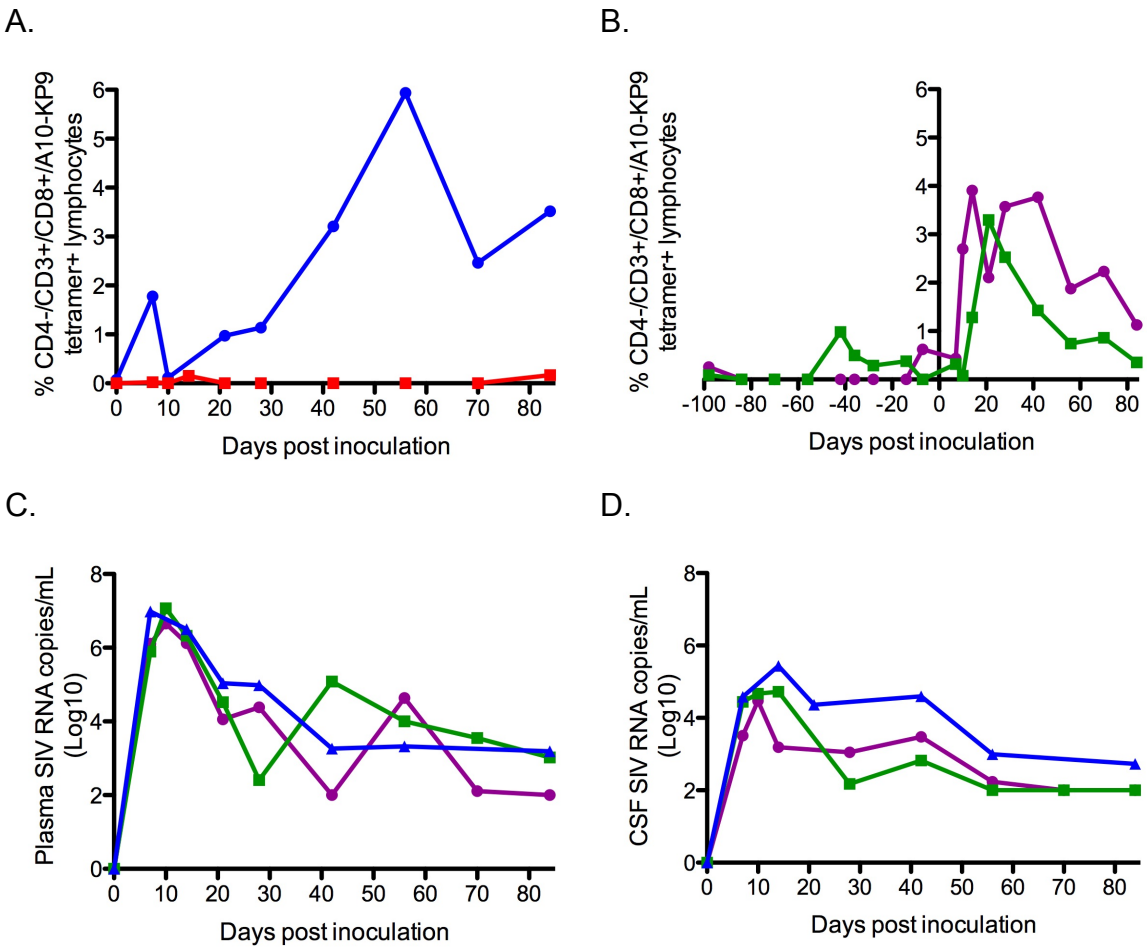

Fig 5. Impact of Gag KP9 vaccination on SIV replication after SIV/17E-Fr challenge. The SIV Gag KP9-specific CTL response in an unvaccinated animal (a) inoculated with SIV/17E-Fr (blue) peaked at day 56 p.i., while animals inoculated with SIV/17E-Fr K165R (red) had no significant KP9 CTL response (n=3, median). In contrast, peak KP9 CTL response in VLP vaccinated animals (purple [22Y] and green [74X] lines) (b) occurred at days 14 and 21 p.i., much earlier than the unvaccinated animals. Plasma viral loads (c) in VLP vaccinated animals (purple and green) challenged with SIV/17E-Fr were similar to the plasma viral load of unvaccinated animals (blue) inoculated with SIV/17E-Fr (n=3, median). However, the CSF viral loads of VLP

vaccinated animals (purple and green) were lower than wildtype controls (blue) after acute infection by greater than one log. CTL response was measured by percentage of CD3+/CD8+/KP9-*Mane-A1*08401* tetramer + lymphocytes. Plasma and CSF viral loads were measured by qRT-PCR.

IV. Summary and Future Directions

Despite encouraging scientific progress made in the last three decades of the HIV/AIDS epidemic in understanding HIV immunity and pathogenesis, clinical vaccine trials have met with mixed success and an effective prophylactic or therapeutic vaccine remains elusive.[1-3] Studies of HIV-infected individuals resistant to AIDS progression, such as long-term nonprogressors and elite controllers, have provided compelling evidence for the importance of host genetics and the influences of MHC class I on host immunity against viral infections.[4-6] Because of remarkable similarities between SIV pathogenesis in Asian-origin macaques and HIV pathogenesis in people, nonhuman primates have become a popular animal to model HIV/AIDS pathogenesis. In addition, nonhuman primate animal models are optimal for studying natural host viral immunity because, like humans, macaques have an extremely high diversity of MHC class I allele expression.[7-9]

Macaques have also become an invaluable resource as a model for stuying the pathogenesis and treatment of neuroAIDS. Varying degrees and manifestations of HIV-associated neurocognitive disorders (HAND) are common and debilitating sequela to HIV infection. Although the incidence of the most severe form of neuroAIDS, HIV-associated dementia, has drastically decreased with the advent of effective antiretroviral therapy, the overall prevalence of neurologic dysfunction has remained unchanged due to an increase in the milder forms of HAND.[10] As the brain is a relatively inaccessible organ to study viral disease pathogenesis, animal models have been crucial to studying HIV-associated brain pathology.

Despite the known importance of host genetics on HIV progression, few have specifically looked at the relationship between host genetics and the development of HIV

associated neurologic disease in great detail.[11] In the series of studies described in this thesis, we compared and contrasted species-specific responses to SIV infection as well as characterized the dramatic effects that Gag specific MHC class I-mediated immunologic pressure has on CNS outcome. In the largest study of its kind, we first compared SIV neuropathogenesis and outcome in 44 pigtailed macaques versus 28 rhesus macaques that were all dual-inoculated with a neurovirulent clone (SIV/17E-Fr) and an immunosuppressive viral swarm (SIV/DeltaB670). Compared to pigtailed macaques, rhesus macaques have a markedly prolonged course of disease progression with less immunosuppression (lower drop in CD4+ T cells), lower viral loads, and decreased progression to SIV-encephalitis (SIVE). For the first time, we also compared head-to-head the effects that MHC class I allele expression has on the development of neuroAIDS in both pigtailed macaques and rhesus macaques, and found that, although there were no differences on CSF viral loads based on MHC class I allele expression, both pigtailed and rhesus macaques that expressed their respective MHC class I alleles had decreased viral loads in the brain.

In the next series of studies, we characterized the effect of SIV Gag KP9 escape (K165R) from the MHC class I allele in pigtailed macaqeus, *Mane-A*084:01:01* on viral fitness. As we had hypothesized, the Gag escape virus SIV/17E-Fr K165R had lower replication *in vitro*, implying that the K165R mutation incurred a fitness cost. However, this fitness cost translated *only* to reduced viral fitness in the central nervous system when we compared pigtailed macaques that were inoculated with wildtype SIV/17E-Fr with macaques inoculated with escape SIV/17E-Fr K165R. Viral loads in the CSF were lower in animals inoculated with SIV/17E-Fr K165R while viral loads in the plasma remained

unchanged compared with wildtype Gag KP9. A surprising finding was that reversion to wildtype Gag KP9 occurred only in the CSF and not the plasma. We then stimulated Gag KP9 epitope-specific immunologic pressure by vaccinating additional pigtailed macaques with a VLP vaccine loaded with Gag KP9, followed by challenge with SIV/17E-Fr. Again, vaccinated animals had lower viral loads in the CSF but not the plasma. These data show that Gag KP9 mutation in the neurovirulent clone SIV.17E-Fr imparts a dramatic fitness cost only in the CNS, suggesting that Gag KP9 may play a crucial role in SIV neuropathogenesis.

Although these studies of MHC class I-mediated effects on the development of SIVE have added to the large body of compelling evidence that host genetics play a vital role in HIV pathogenesis, MHC allele expression represents only one piece of this picture. In addition, we are interested in investigating the importance of T cell receptor (TCR) selection as it pertains to viral immunity. The effectiveness of CTL-mediated immunity is dependent on a highly diverse array of TCR sequences (clonotypes) that can recognize an almost limitless variety of antigens presented on diverse MHC molecules. It then stands to reason that it would be almost impossible for TCR clonotypes to be shared between individuals; however, several of these "public" clonotype responses have been demonstrated in a variety of infectious diseases, including HIV.[12-14] Although TCR diversity would be advantageous because of an increased capacity to respond to viral epitope mutations, an effective CTL response remains dependent upon TCR binding and specificity. The TCR of most T cells comprises two chains, α and β, each of which has three complementarity determining regions (CDRs), of which CDR3 is in direct contact

with the peptide.[15] Sequencing of the CDR3 regions of TCRs from epitope-specific CTLs allows us to evaluate the breadth of TCR clonotypes that respond to that antigen.

Theoretically, multiple TCR clonotypes targeting the same immunodominant epitope might prevent widespread viral escape.[16] Studies of TCR clonotypes that respond to immunodominant HIV Gag epitopes have shown evidence of multiple clonotypes that expand during the course of infection as well as the emergence of Gag-specific clonotypes that exhibit cross-reactivity.[16, 17] TCR clonotype sequencing of *Mane-A*084:01:01*-positive macaques that were vaccinated with vaccines expressing wildtype SIV Gag KP9 then challenged with SIVmac251 or SHIV showed a widely diverse population of TCR clonotypes in SIV Gag KP9-specific T cells.[18] In addition, the vast majority of KP9-specific CTL TCR clonotypes identified after vaccination subsequently disappeared after SIV challenge.[18] Conversely, rhesus macaques were reported to develop a clonal CTL response to vaccine that not only persisted after SHIV challenge but was maintained for several years.[19]

To investigate serial TCR clonotype selection in our SIV model, we isolated mRNA from SIV Gag KP9-specific CTLs from a *Mane-A*084:01:01*-positive pigtailed macaque (68X) that was inoculated with wildtype SIV/17E-Fr by FACS sorting 10,000 to 30,000 CD3 and KP9-tetramer dual-positive lymphocytes using blood samples obtained at multiple time points. cDNA synthesis was performed with the 5' SMARTer RACE (rapid amplification of cDNA ends) cDNA amplification kit (Clontech) and anchored PCR for TCR β chain (*TRB*) gene products was performed using the previously described protocol and primer sequences.[18] Traditional cloning and sequencing of the amplified *TRB* gene products was performed, and the CDR3 amino acid sequences were identified

by the ends of the Vβ regions (identifiable by the amino acid sequence CASS) and the Jβ regions (identified by XFGXG).

To test this technique, we first processed pre-inoculation lymphocytes for TCR clonotype sequences and found that we were able to successfully sequence and identify CDR3 sequences. We identified a very diverse array of TCR clonotypes with very little repeat in clone sequences (Figure 1), which was unsurprising given the lack in selective immunologic pressure. Interestingly, at 84 days post-inoculation (terminal bleed), Gag KP9 specific CTLs showed marked clonotype selection (Figure 2). We only identified three unique *TRB* CTR3 sequences, and of these, two dominated the immune response. This is in contrast to the findings of Smith *et al*, in which no dominant clonotypes emerged at any point post-vaccination or post-challenge. However, this is only one animal, and we now are currently genotyping TCR clones at intermediate time points. In addition, we have sorted Gag KP9-specific CTLs longitudinally from the two pigtailed macaques that were VLP-KP9 vaccinated and challenged with SIV/17E-Fr, which will enable us to compare the clonally expanded CTLs post-vaccination with those that expanded post-challenge.

Another aspect of host adaptive immunity that has been gaining interest in recent years is the potential utility of antibody-dependent cellular cytotoxicity (ADCC) stimulation of natural killer (NK) cells for therapeutic or prophylactic vaccination development.[20, 21] ADCC is a unique immunologic process that involves parts of both the humoral and cell-mediated immune systems by targeting virus-infected cells whose surface antigens have been bound by antibodies that are recognized by Fc receptors (CD16) expressed on the surface of NK cells, macrophages, and other immune cells.

Classic ADCC involves NK cell activation and subsequent killing of the virus-infected cell. The failure of strictly humoral or cell-mediated HIV vaccines to effectively prevent HIV infection has led many to believe that the most effective approach to vaccine design may be creating a vaccine that stimulates both arms of the immune system.[21] Indeed, the presence of ADCC antibodies directed against HIV proteins, especially Env, are associated with slower decline of CD4+ T cells and in slower disease progression.[22, 23]

To confirm the development of ADCC responses by NK cells in our SIV-infected pigtailed macaque model, we measured intracellular cytokine production by macaque NK cells that were stimulated with SIVmac239 Env peptide pools (NIH AIDS reagent program catalog #2322) using previously reported methods.[24] Peripheral blood mononuclear cells were isolated from an uninfected pigtailed macaque and incubated with sera from a chronically infected macaque and SIV Env peptide pools. We measured intracellular IFNγ and TNFα production by CD3-/CD8+/CD159a+ NK cells and found variable IFN-γ and TNF-α production in response to stimulation by multiple SIV Env pools (Figure 3). Although this data is preliminary, we believe that these data suggest that SIV infection in our pigtailed macaque model induces ADCC responses. Ultimately, the goal is to map ADCC response of pigtailed macaques to SIV peptides, not only in Env, but of other SIV proteins as well, with the end goal of developing an ADCC-based vaccine using the VLP platform.

Overall, these studies provide compelling evidence that immunologic pressure on a highly conserved epitope in Gag drives viral escape that has a detrimental effect on viral replication specifically in the CNS, implying CTL-mediated immunity is unique in

the CNS when compared to the periphery. As vaccination strategies to enhance CTL responses against HIV Gag could promote HIV escape, it is imperative that we fully understand the ramifications that therapeutic vaccination could have in the CNS including development of neuroAIDS. Hopefully, our studies have helped improve our understanding of the interplay of host genetics and CTL-mediated viral immunity on the development of SIV-associated neurologic disease with the ultimate goal of developing novel immunomodulatory therapies that could protect the CNS from HIV infection.

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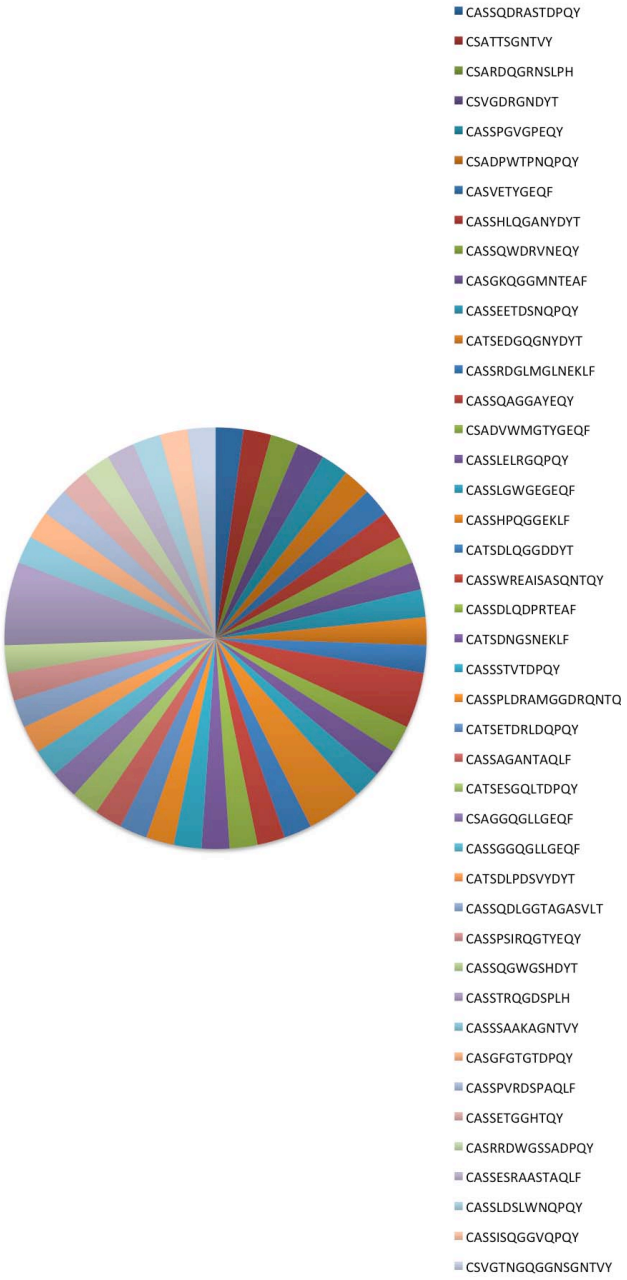
IV: Figures

A.

Length	CDR3	Jβ	#	%
14	CASSQDRASTDPQY	FGPG	1	2.1
11	CSATTSGNTVY	FGEG	1	2.1
13	CSARDQGRNSLPH	FGNG	1	2.1
12	CSVGDRGNDYT	FGSG	1	2.1
7	CASSPGVGPEQY	FGPG	1	2.1
13	CSADPWTPNQPQY	FGDG	1	2.1
11	CASVETYGEQF	FGPG	1	2.1
14	CASSHLQGANYDYT	FGSG	1	2.1
13	CASSQWDRVNEQY	FGPG	1	2.1
14	CASGKQGGMNTEAF	FGQG	1	2.1
14	CASSEETDSNQPQY	FGDG	1	2.1
14	CATSEDGQGNYDYT	FGSG	1	2.1
16	CASSRDGLMGLNEKLF	FGSG	1	2.1
13	**CASSQAGGAYEQY**	FGPG	2	4.3
14	CSADVWMGTYGEQF	FGPG	1	2.1
13	CASSLELRGQPQY	FGDG	1	2.1
13	CASSLGWGEGEQF	FGPG	1	2.1
13	**CASSHPQGGEKLF**	FGSG	2	4.3
13	CATSDLQGGDDYT	FGSG	1	2.1
17	CASSWREAISASQNTQY	FGAG	1	2.1
14	CASSDLQDPRTEAF	FGQG	1	2.1
13	CATSDNGSNEKLF	FGSG	1	2.1
12	CASSSTVTDPQY	FGPG	1	2.1
20	CASSPLDRAMGGDRQNTQY	FGAG	1	2.1
14	CATSETDRLDQPQY	FGDG	1	2.1
13	CASSAGANTAQLF	FGEG	1	2.1
14	CATSESGQLTDPQY	FGPG	1	2.1
13	CSAGGQGLLGEQF	FGPG	1	2.1
14	CASSGGQGLLGEQF	FGQG	1	2.1
14	CATSDLPDSVYDYT	FGSG	1	2.1
17	CASSQDLGGTAGASVLT	FGAG	1	2.1
15	CASSPSIRQGTYEQY	FGPG	1	2.1
13	CASSQGWGSHDYT	FGSG	1	2.1
13	**CASSTRQGDSPLH**	FGNG	3	6.4
14	CASSSAAKAGNTVY	FGEG	1	2.1
13	CASGFGTGTDPQY	FGPG	1	2.1
14	CASSPVRDSPAQLF	FGEG	1	2.1
12	CASSETGGHTQY	FGAG	1	2.1

B.



15	CASRRDWGSSADPQY	FGPG	1	2.1
15	CASSESRAASTAQLF	FGEG	1	2.1
14	CASSLDSLWNQPQY	FGDG	1	2.1
14	CASSISQGGVQPQY	FGDG	1	2.1
17	CSVGTNGQGGNSGNTVY	FGEG	1	2.1

Figure 1. Preliminary TCR sequencing of lymphocytes from a *Mane-A*084:01:01*-positive pigtailed macaque confirmed our technical ability to sequence TCR clonotypes. Unsurprisingly, TCR clonotypes pre-infection are very diverse.

A.

Length	CDR3	Jβ	#	%
12	CASSTGSYQPQY	FGDG	1	4.8
12	CASSSPVLHDYT	FGSG	12	57.1
17	CASSPERTGSTGASVLT	FGAG	8	38.1

B.

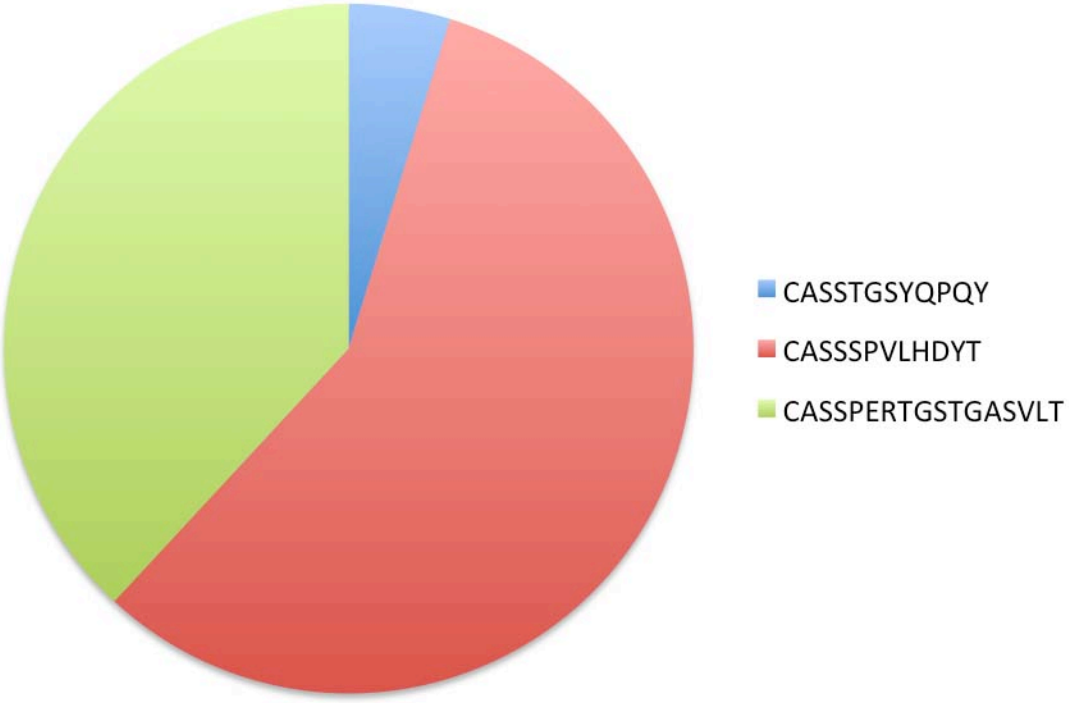


Figure 2. Development of epitope-specific TCR selection in an SIV infected pigtailed macaque. CRD3 TCR sequencing of SIV Gag KP9-specific CTLs in a *Mane-A*084:01:01*-positive pigtailed macaque inoculated with SIV/17E-Fr showed clear TCR clonotype selection at 84 days p.i. with two clonotypes emerging as the dominiant TCR sequences.

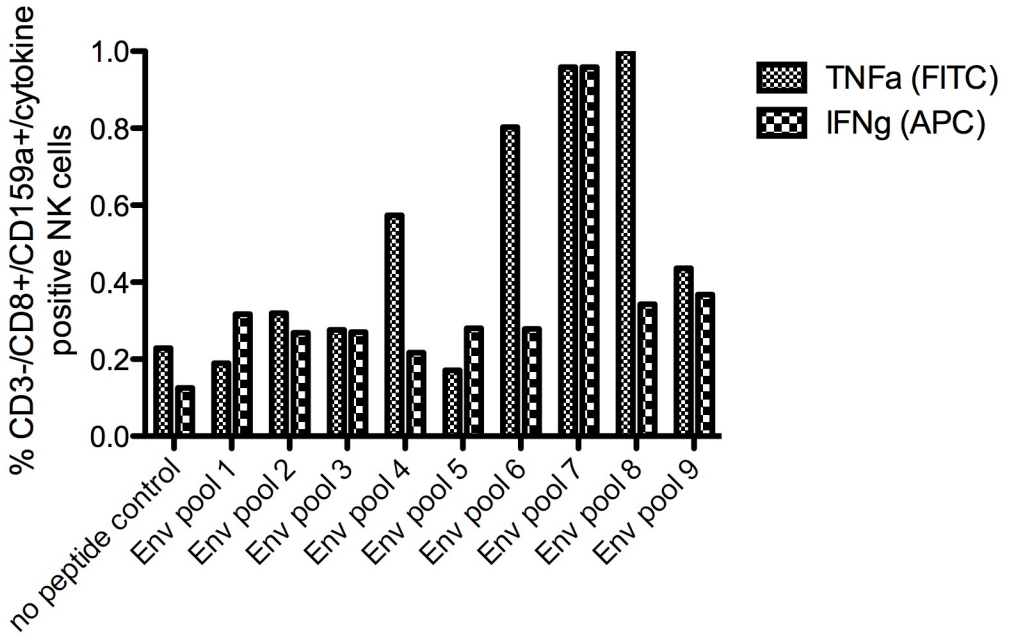


Figure 3. ADCC response in pigtailed macaques. Stimulation of naïve macaque natural killer cells with serum from a chronically infected animal resulted in variable TNFα and IFNβ production in response to SIVmac239 Env peptide pools, consistent with an ADCC response.

Sarah E. Beck
January 30, 1980
sbeck8@jhmi.edu

Objectives

Completion of my PhD studying host genetic resistance to SIV encephalitis in the Cellular and Molecular Medicine graduate program at Johns Hopkins University and board certification in anatomic veterinary pathology by the American College of Veterinary Pathologists. Becoming a principal investigator studying infectious disease pathogensis.

Education

Virginia Maryland Regional College of Veterinary Medicine Blacksburg, VA
Doctor of Veterinary Medicine August 2003 – May 2007

- Class rank: 18/89; Cumulative grade point average: 3.68/4.00
- Public & Corporate Veterinary Medicine track
- Concentration of studies: Pathology, Public Health, Epidemiology, and Food Animal Medicine & Surgery

University of Maryland College Park, MD
Bachelor's degree in Marine Biology August 1998 – May 2002

- Cumulative Grade point average: 4.00/4.00
- Major Research: Phylogenetic study of three right whale populations (2000 - 2002)

Relevant Experience

Johns Hopkins School of Medicine Baltimore, MD
Department of Molecular and Comparative Pathobiology
Pathology postdoctoral fellow July 2008 – Present
Studying in a four year program aimed at providing diagnostic anatomic veterinary pathology and basic research training

- Designated Senior Pathology Resident from July 2011 – July 2012, providing advice and supervision for junior pathology residents
- Perform gross necropsy examinations on submitted animals from sources including: the JHU research colony, the National Aquarium in Baltimore, the Baltimore Zoo, and a variety of small and large animal private practices
- Examine histologic sections from necropsy specimen tissues and complete case reports under the supervision of ACVP boarded veterinary pathologists
- Examine biopsy specimens and complete diagnostic case reports under the supervision of boarded veterinary pathologists
- Participate in bi-weekly morning rounds by presenting cases submitted for necropsy
- Coursework: Introduction to Pathology (JHU Medical School second year course), LAM/PATH Integrated Problem Solving (680.702), Comparative Medicine Research Seminar Series (680.700), Comparative Pathology Conference (680.711), Molecular Biology- Fundamentals and Protocols (200.702)

Johns Hopkins School of Medicine Baltimore, MD
Cellular and Molecular Medicine
Graduate student, PhD candidate August 2009 – Present
Studying pathobiology in the Cellular and Molecular Medicine graduate program with the

goal of attaining a PhD.

- Coursework: Fundamental Virology (260.804), Topics in CMM (ME250.706), Research in CMM (ME800.801), Molecular Biology and Genomics (ME260.709), CMM core discussion (ME800.703), Genetics (ME260.708), Translational Rearch in NeuroAIDS & Mental Health (ME200.701), HIV Biology (ME250.714), Great Experiments in Biology (ME260.812).

Aberdeen Veterinary Clinic Aberdeen, MD

Associate Veterinarian June 2007 – June 2008

Practice primarily small animal medicine in a non-emergency mixed-animal practice in Maryland

- Performed wellness examinations and promoted preventative medicine through the use of vaccinations and routine testing
- Diagnostically worked up medical cases then provided a plan, diagnosis, and prognosis to discuss with clients
- Provided day ICU care for admitted patients
- Performed non-orthopedic canine and feline surgical procedures (spays, neuters, mass removals, etc.)

United States Public Health Service Bethesda & Poolesville, MD

Junior COSTEP, NIH May 2005 – August 2005

Participated in the Junior COSTEP program at the National Institutes of Health with Pathology Services. Pathology Services provide a complete post-mortem exam of any deceased animal that are property of the NIH. This included a range of species: mice, rats, sheep, macaques, owl and squirrel monkeys, frogs, zebra fish, and New Zealand white rabbits.

- Performed necropsies and recorded findings on a variety of species
- Recorded histopathology findings and formulated independent diagnoses
- Completed and submitted case write-ups on two clinical cases to the Wednesday slide conference at the Armed Forces Institute of Pathology and hand selected the submitted slides
- Submitted a case report to the Journal of Laboratory Animal Medicine on a case of pancreatic amyloidosis in a rhesus macaque with type II diabetes
- Prepared specially stained slides and EM photos of pancreas for the aforementioned case
- Presented my findings and conclusions in weekly rounds after examining slides of current cases

Junior COSTEP, NIHAC May 2004 – August 2004

Participated in the Junior COSTEP program at the National Institutes of Health, Animal Center. The Animal Center is a research and quarantine center for new and old world primates, rabbits, laboratory mice, dogs, pigs, sheep, and other species used for research.

- Gained familiarity with lab animal medicine and attended weekly veterinary meetings
- Performed dental cleanings on primates and monitored them while on inhalant anesthesia
- Drew blood and preformed TB tests on primates for periodic health screens
- Administered medications to primates on a daily basis
- Assisted with emergency surgeries and performed porcine castrations
- Accompanied the veterinarians on daily health checks

Virion Systems Inc. Rockville, MD

Molecular Biologist May 2002 – August 2003

Virion is a biotech company that primarily uses the cotton rat as an animal model for various human viral and bacterial diseases, including respiratory syncytial virus, herpes-2 virus, influenza, and tuberculosis.

- Worked primarily with BSL-2 infectious agents
- Designed and performed the herpes-2 virus animal studies
- Ran the subsequent cytokine analysis studies
- Collected tissue (heart and lung block, turbinates, spleen, kidneys, liver, stomach, and brains) for animal protocols
- Attained technical skills including: PCR, RNA and DNA extraction, RTPCR, cDNA synthesis, and limited tissue culture

Peer reviewed publications

Kelly KM, Tocchetti C, Lyashkov A, Bedja D, Graham G, **Beck SE**, Pate KA, Queen SE, Adams RJ, Paolocci N, Mankowski JL. CCR5 Inhibition Prevents Cardiac Dysfunction in the SIV/Macaque model of HIV. *Journal of the American Heart Association*. (accepted February 2014).

Dorsey JL, Mangus LM, Oakley JD, **Beck SE**, Kelly KM, Queen SE, Metcalf Pate KA, Adams RJ, Marfurt CF, Mankowski JL. Loss of Corneal Sensory Nerve Fibers in SIV-infected Macaques: An Alternate Approach to Investigate HIV-induced PNS Damage. *American Journal of Pathology*. (accepted February 2014).

Kelly KM, **Beck SE**, Pate KA, Queen SE, Dorsey JL, Adams RJ, Avery LB, Hubbard W, Tarwater PM, Mankowski JL. Neuroprotective maraviroc monotherapy in simian immunodeficiency virus-infected macaques: reduced replicating and latent SIV in the brain. *AIDS*. 2013. Oct 29. [Epub ahead of print].

Wenzel AR, Wack AN, **Beck SE**, Bronson E. Pathology in practice. Nasal and nasopharyngeal polyps. *Journal of the American Veterinary Medical Association*. 2012. 241(7): 885-7.

Hadfield CA, Clayton LA, Clancy MM, **Beck SE**, Mangus LM, Montali RJ. Proliferative thyroid lesions in three diplodactylid geckos: *Nephrurus amyae*, *Naphrurus levis*, and *Oedura marmorata*. *Journal of Zoo and Wildlife Medicine*. 2012. 43(1): 131-40.

Del Carmen Martino-Cardona M, **Beck SE**, Brayton C, Watson J. Eradication of Helicobacter spp. by using medicated diet in mice deficient in functional natural killer cells and complement factor D. *Journal of the American Association for Laboratory Animal Science*. 2010. 49(3): 294-9.

Gaines CA, Hare MP, **Beck SE**, and Rosenbaum HC. Nuclear markers confirm taxonomic status and relationships among highly endangered and closely related right whale species. *Proceedings of the Royal Society B*. 2005. 272: 533-542.

Abstracts

Beck SE, Queen SE, Mankowski JL. Consequences of immune selection in a pigtailed macaque model of SIV CNS disease. 12th International Symposium on Neurovirology/2013 Conference on HIV in the Nervous System, October-November 2013.

Beck SE, Queen SE, Mankowsi JL. Immune selection in a pigtailed macaque model of SIV CNS disease: costs and consequences. 20th Conference on Retroviruses and Opportunistic Infections, March 2013.

Beck SE, Queen SE, Mankowski JL. Consequences of immune selection in a pigtailed macaque SIV model of HIV CNS disease. 63rd Annual Meeting of the American College of Veterinary Pathologists, December 2012.

Beck SE, Queen SE, Mankowski JL. In vivo and in vitro fitness of a cloned SIV escape mutant in a pigtailed macaque model of HIV CNS disease. 62nd Annual Meeting of the American College of Veterinary Pathologists, December 2011.

Beck SE, Agarwal JR, Matsui W. Targeting multiple myeloma cancer stem cells through the liver X receptor and retinoid X receptor pathways. 61st Annual Meeting of the American College of Veterinary Pathologists, October 2010.

Beck SE, Wack AN, Montali RJ. Fungal dermatitis in a captive population of Panamanian golden frogs (*Atelopus zeteki*) from 2006 to 2009. 60th Annual Meeting of the American College of Veterinary Pathologists, December 2009.

Martino-Cardona MC, **Beck SE**, Brayton C, Watson J. Dietary treatment eradicated *Helicobacter* sp. from young mice deficient in NK cells. 60th Annual Meeting of the American Association of Laboratory Animal Science. November 2009.

Metcalf Pate KA, **Beck SE**, Montali RJ, and Scorpio DG. Persistent wasting and hypoproteinemia in a rhesus macaque: an unusual final diagnosis. 60th Annual Meeting of the American Association of Laboratory Animal Science. November 2009.

Oral presentations

Beck SE, Queen SE, Mankowski JL. Costs and conseqeunces of immune selection of SIV in the CNS: Impact of an SIV CTL escape mutation on viral fitness. Department of Molecular and Comparative Pathobiology Year in Review, May 2012.

Beck SE, Brennan KK, Mankowski JL. Case Presentation: Encephalitis in a Titi Monkey. Primate Pathology Pre-conference Workshop. 61st Annual Meeting of the American College of Veterinary Pathology, October 2010.

Beck SE, Hadfield C, Southard T, Montali RM. 2009. "Severe proliferative epicarditis with mesothelial inclusions in a silver arowana (*Osteoglossum bicirrhosum*)". AAZV 16th Annual Zoo and Wildlife Pathology Workshop. Tulsa, OK.

Beck SE. 2009. "Case report: viral infection in an Eastern box turtle." Comparative Medicine Research Seminar Series, Johns Hopkins, Baltimore, MD.

Beck SE. 2007. "Bilaterally symmetrical necrotizing encephalopathy in a 7-month old kitten." 35th Annual Southeastern Veterinary Pathology Conference, Tifton, GA.

Beck SE and Hare MP. 2002. "Determining the true number of right whale species." Honors thesis defense, University of MD, College Park, MD.

Teaching activities

Johns Hopkins School of Medicine (680.701). LAM/PATH Integrated Problem Solving (LIPS), 2hr lecture "Acute and Chronic inflammation". September 2013.

Johns Hopkins School of Medicine (300.713). Pathology for Graduate students: Basic mechanisms, 1hr lecture "Inflammation". August 2013.

Johns Hopkins School of Public Health (187.620.01). Environmental Toxicological Pathology, 2hr lecture "Toxicologic Pathology of the Female Reproductive System". April 2010, 2011, 2012, 2013.

Johns Hopkins School of Medicine (680.701). Comparative Pathobiology & Genetically Engineered Mice, 1hr lecture "Murine Models of Prostate Cancer". May 2010.

Johns Hopkins School of Medicine (300.713). Pathology for Graduate students: Basic mechanisms, Mouse Necropsy lab instruction. September 2008.

Research support

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07/01/2012 – 06/30/2013
Immune selection in the CNS: Costs and Consequences
Co-investigator: Joseph Mankowski
Role: Principal Investigator

2T32RR007002-35 (C. Zink)
07/01/2010 - 06/30/2015
Training Veterinarians for Careers in Biomedical Research
PI: Christine Zink
Research mentor: Joseph Mankowski, DVM, PhD, DACVP
Role: PhD Candidate / Graduate Student

Honors and Awards

- Young Investigator Award, 20th Conference on Retroviruses and Opportunistic Infections (2013)
- Intersociety Council for Pathology Information, Inc. Trainee Travel Award recipient (2012)
- Graduate student/resident ACVP Travel Award recipient (2012 annual meeting)
- Member of the Maryland Veterinary Medical Association (2007-current)
- Member of the American Veterinary Medical Association (2007-2009)
- Salisbury Award in Veterinary Medicine (2007)
- Member of the Phi Zeta chapter of the VMRCVM--top 25% of the class (2007)
- Public/Corporate Liaison officer for the Public Veterinary Practice Club (2005 - 2006)
- Member of the Student Chapter of the American Veterinary Medical Association (2003-2007)
- Member of the Public Veterinary Practice Club (2003-2007)
- Student member of the Virginia Veterinary Medical Association (2003-2007)
- Honors Citation in Biology (2002) for research on right whale populations with Dr. Matt Hare
- Howard Hughes Fellowship (2000 - 2002)
- Member of the Golden Key International Honour Society (2002)