# ENVELOPE DETERMINANTS OF EIAV VACCINE PROTECTION AND THE EFFECTS OF SEQUENCE VARIATION ON IMMUNE RECOGNITION

by

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Developing an effective lentiviral vaccine has been an elusive goal, largely due to immune evasion mechanisms of lentiviruses. Lentiviral envelope (Env) proteins pose a major obstacle to vaccine development due to extreme antigenic variation, but research with *equine infectious anemia virus* (EIAV) has indicated Env to be a primary determinant of vaccine efficacy. We have developed an attenuated EIAV vaccine capable of protecting horses from disease after homologous challenge. However, when variant challenge strains with divergent Env proteins are utilized, vaccine protection is incrementally decreased with increasing divergence from the homologous Env. ***I hypothesize there may be Env-specific immune responses associated with vaccine protection and believe antigenic variation may have profound effects on immune recognition.*** Utilizing thymidine incorporation and chromium release assays on PBMC from vaccinated horses challenged with the homologous strain, I identified broadly reactive regions of Env recognized by T-helper and CTL cells. With PBMC from vaccinated ponies challenged with divergent EIAV strains I identified Env-specific immune responses associated with vaccine protection from disease. Although I was unable to correlate antibody neutralization with protection, I found one T-helper and eight CTL peptide responses associated with vaccine protection. Three of the CTL peptides were located in variable domains of Env. To understand the effects of variation on immune recognition, I utilized sera and PBMC from vaccinated and

variant infected ponies to analyze the cross-reactivity of humoral and cellular immune responses. Due to limited proliferative activity, I was unable to fully analyze the effects of Env variation on lymphoproliferation; however, CTL analysis indicated Env variation had profound effects on immune recognition in EIAV vaccinated and infected ponies. The most remarkable effects of Env variation were observed in *in vitro* neutralization assays, where there was no detectable cross-reactivity of neutralizing antibodies. Collectively, these results indicate that eliciting key immune responses to more conserved regions of the EIAV Env may be crucial in developing an effective EIAV vaccine. The peptide-specific responses identified in this dissertation could serve as important targets for future EIAV vaccines and this analysis may be a valuable complement to ongoing work in SIV and HIV.

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# 1.0 INTRODUCTION TO EQUINE INFECTIOUS ANEMIA VIRUS, IMMUNOLOGY AND VACCINE DEVELOPMENT

## 1.1 STRUCTURE OF EIAV

Equine infectious anemia virus (EIAV) is a member of the lentivirus genus in the Retroviridae family of viruses and shares genetic and structural similarity with human (HIV), simian (SIV) and feline (FIV) immunodeficiency viruses. With an RNA genome of 8.2kb, EIAV is the smallest of the known lentiviruses (75). All lentiviruses contain *gag*, *pol* and *env* genes which encode for the structural and enzymatic proteins of the virus. EIAV *gag* encodes the matrix (p15), capsid (p26), nucleocapsid (p11) and p9 proteins (Fig 1.1). While p9 is critical for virion release from the plasma membrane of the infected macrophage, the other Gag proteins provide the structure of EIAV (20). The hallmark of every lentivirus is the bullet shaped capsid, which encloses the nucleocapsid encased RNA genome. Surrounding the capsid, are matrix proteins which underlie the lipid bilayer and interact with the Env proteins. The lipid bilayer, obtained during budding from infected macrophage, is studded with the *env* encoded proteins, gp90 and

gp45. Gp90 is the surface protein responsible for binding the ELR1 receptor on equine macrophage (142) and is non-covalently linked to gp45, the membrane spanning protein.

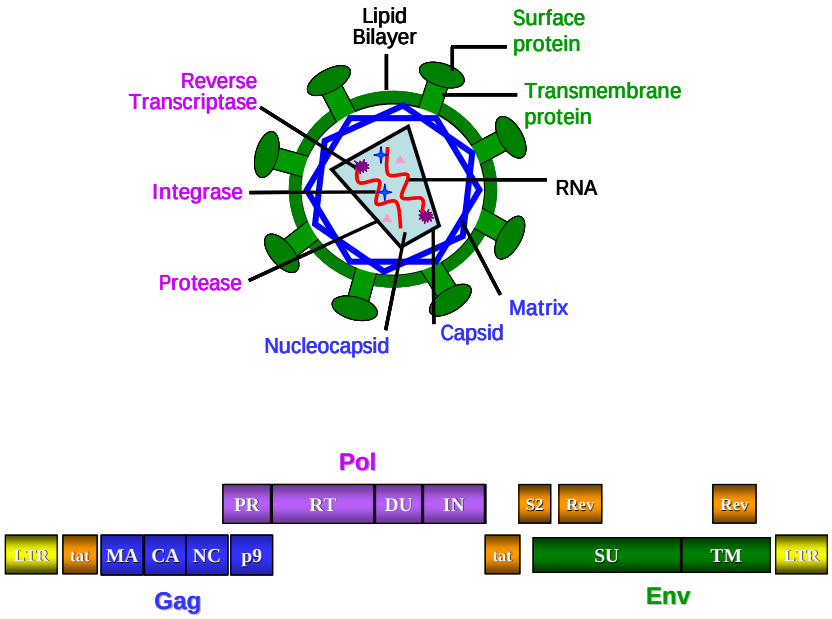


**Figure 1.1 EIAV virion and genomic organization.**

EIAV *pol* encodes the enzymatic proteins needed for productive viral infection. Reverse transcriptase-RNaseH (p66) is essential for the conversion of viral RNA into DNA. RT lacks a proofreading enzyme; hence, numerous mutations are introduced in the viral genome every time the RNA is reverse transcribed. These mutations allow the virus to rapidly evolve and evade the host immune responses. Because EIAV infects non-dividing macrophages, there is a limited supply of dTTP, an essential nucleotide for DNA synthesis. Macrophage must synthesize RNA for protein production; therefore there is an abundant supply of dUTP. EIAV *pol* encodes for a dUTPase (p15) that is capable of converting dUTP to dUMP and PPi, which is an essential step in the *de novo* synthesis of dTTP (71). Once the viral genome is converted to DNA, viral

integrase (p30) incorporates the DNA into the genome of the macrophage. EIAV, like all lentiviruses, relies on host cell machinery to transcribe the DNA to messenger RNA for protein translation. Many of the viral proteins are translated as polyproteins and need to be cleaved before a mature virion may assemble. EIAV encodes a protease (p12) capable of cleaving the viral polyproteins into the functional proteins required for virus assembly.

In addition to the proteins coded by *gag, pol* and *env*, EIAV has three accessory proteins: Tat, Rev and S2, making EIAV the least complex lentivirus. Tat and Rev are common to all lentiviruses; Tat functions as a transcription activator, while Rev regulates RNA splicing and RNA transport from the nucleus (135). The function of S2 is currently not defined (75), but studies have shown that knocking out S2 down-regulates *in vivo* replication without altering *in vitro* viral kinetics (79, 81).

## 1.2 CLINICAL COURSE OF EIAV INFECTION

While genetically similar to other lentiviruses, the clinical course of EIAV is dramatically different from its lentiviral relatives. While HIV, SIV and FIV are all primarily lymphotropic viruses transmitted through direct contact with infected individuals, EIAV is a macrophage tropic virus that infects equids through horse fly bites or contaminated needles. Infection with HIV, SIV or FIV cause similar clinical progressions that entail a slow, progressive degenerative disease that culminates in the collapse of the immune system and eventual death of the host to opportunistic infection (3). Conversely, EIAV produces a rapid and dynamic infection that ultimately leads to a life-long asymptomatic phase (97).

Two months post exposure to EIAV, infected horses develop an acute episode of disease characterized by fever, thrombocytopenia, anemia and viremia (Fig 1.2). Sequencing of plasma RNA from experimentally infected ponies during the acute episode reveals the original EIAV inoculum (77). The equine immune system gains control of the acute episode causing a drop in viral load without eradicating the virus. Eventual EIAV evasion of the immune responses allows for rapid viral replication which triggers another febrile episode, marking the beginning of the chronic phase of disease. The hallmark of EIAV infection is the chronic phase where infected horses cycle through recurrent disease episodes, characterized by fever, thrombocytopenia, and peak plasma viremia (97). Each disease episode can be attributed to a new viral quasispecies that was capable of escaping host immune control and that is distinct from the preceding episode (77). Infected horses continue cycling with chronic disease for approximately one year, after which an inapparent carrier phase is achieved.

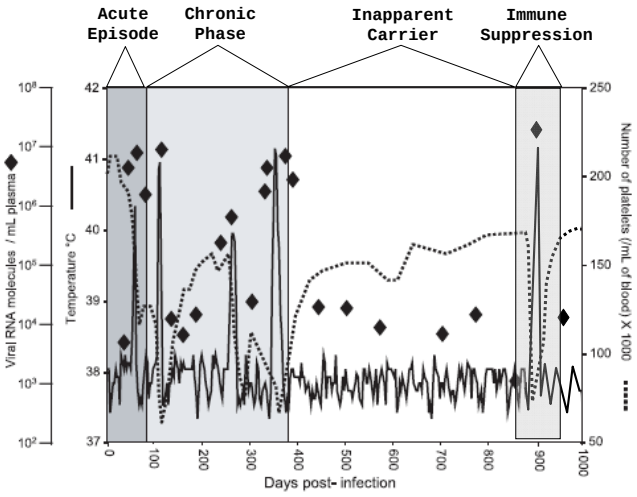


**Figure 1.2 Clinical course of EIAV infection.** Figure adapted from Leroux et al. 2004. Vet. Res. 35:485-512 (75).

Inapparent carriers of EIAV strictly control virus replication and are free from further disease episodes for the remaining live span of the horse. The inapparent stage of disease cannot be attributed to attenuation of the virus, as whole blood transfers from inapparent carriers to naïve horses induce disease in recipient animals. Additionally, stressing or immune suppressing inapparent carrier horses can cause a recrudescing disease with a distinct quasispecies from the last febrile episode, indicating that viral evolution is maintained during the inapparent stage, and suggesting that there is strict immunological control of EIAV (64, 89, 97). Greater than 90% of horses infected with EIAV advance to inapparent carriers, which is in stark contrast to SIV, where less than 5% of experimentally infected monkeys are nonprogressors (48). In addition to gaining immunologic control of EIAV, inapparent carrier horses are resistant to further EIAV challenge, demonstrating natural, prophylactic immunity (97) that is desirable for a lentiviral vaccine.

## 1.3 HOST IMMUNE RESPONSE TO VIRAL INFECTIONS

The host immune response to viral infections is complex, involving multiple cellular reactions between distinct cell types. For the purpose of this dissertation, only the adaptive immune responses of T-helper cells, cytotoxic T-lymphocytes and neutralizing antibodies will be discussed. The immunology described here is adapted from the text of Retroviruses and also Immuno Biology 5 (3, 66).

Upon infection of a naïve host, viruses are transferred to the nearest lymphatic organ. In the case of blood borne viruses, such as EIAV, the virions are transported to the spleen where they are engulfed by professional antigen presenting cells (APC), such as dendritic cells (DC) and macrophages. The APC process the pathogen and present antigenic peptides bound to major histocompatibility complex (MHC) molecules on the cell surface. Cells of the adaptive immune system capable of recognizing the MHC:peptide complexes bind the APC and become activated effector cells (Fig 1.3).

There are two major classes of MHC molecules able to present antigenic peptides. Class I MHC molecules bind both ends of a short antigenic peptide (~9-10 amino acids) for presentation to CD8+ T-lymphocytes. These peptides are derived from intracellular pathogens, such as viruses. For MHC II molecules, the peptides are derived from extracellular pathogens taken up by cells through various forms of endocytosis. MHC II molecules present antigenic peptides in a binding cleft. The binding cleft is capable of recognizing peptides of variable length, with typical lengths exceeding 13 amino acids. Naïve CD4+ T-lymphocytes bind peptides complexed with MHC II.

MHC:peptide recognition is not sufficient to drive T-cell activation, co-stimulatory signals from the APC must be received by the binding T-lymphocyte for full activation to occur. The first co-stimulatory interaction, which initiates T-cell activation, involves B7 proteins on APC interacting with CD28 on T-cells. The activation signal is completed by the T-cell CD40 ligand binding to the APC CD40 receptor. Once activated, T-cells begin producing IL2, a necessary cytokine for T-cell expansion. The interaction between CD40 and CD40 ligand also causes the APC to produce more B7, thereby prolonging T-cell activation. These co-stimulatory signals are sufficient for activating CD4+ T-cells into effector T-helper cells (Th). Effector Th

cells “help” other cells of the adaptive immune system through cytokine release and APC activation (Fig 1.3).

APC presenting viral peptides in the context of MHC I molecules interact with CD8 T-cells. The same co-stimulatory events occur as for CD4 T-cell activation, but due to the destructive nature of effector CD8 T-cells, further co-stimulation is required for the differentiation of CD8 T-cells to cytotoxic T-lymphocytes (CTL). The additional co-stimulation can be achieved by two separate means. If the CD8 cells are bound to DC, which have natural intrinsic co-stimulatory activity, the DC can stimulate the CD8 to synthesize IL-2, and drive their own proliferation and differentiation. If the CD8 cells recognize antigen on weakly co-stimulating cells, effector Th must activate the same APC to trigger an increased expression of co-stimulatory molecules on the APC. The APC are then capable of activating CD8 T-cells to make their own IL-2.

Once activated, effector CTLs circulate and identify virally infected cells through peptide:MHC I complexes. Binding of the CTL T-cell receptor to the peptide:MHC I complex on virally infected cells permit other co-receptors to crosslink and form an immune synapse, which allows for the targeted release of lytic granules from the CTLs to the infected cell (Fig 1.3). The lytic granules contain cytolytic proteins capable of destroying virally infected cells. After the deadly delivery, the CTLs disengage and move to the next virally infected target cell. By destroying virally infected cells, CTLs help the immune system control viral replication.

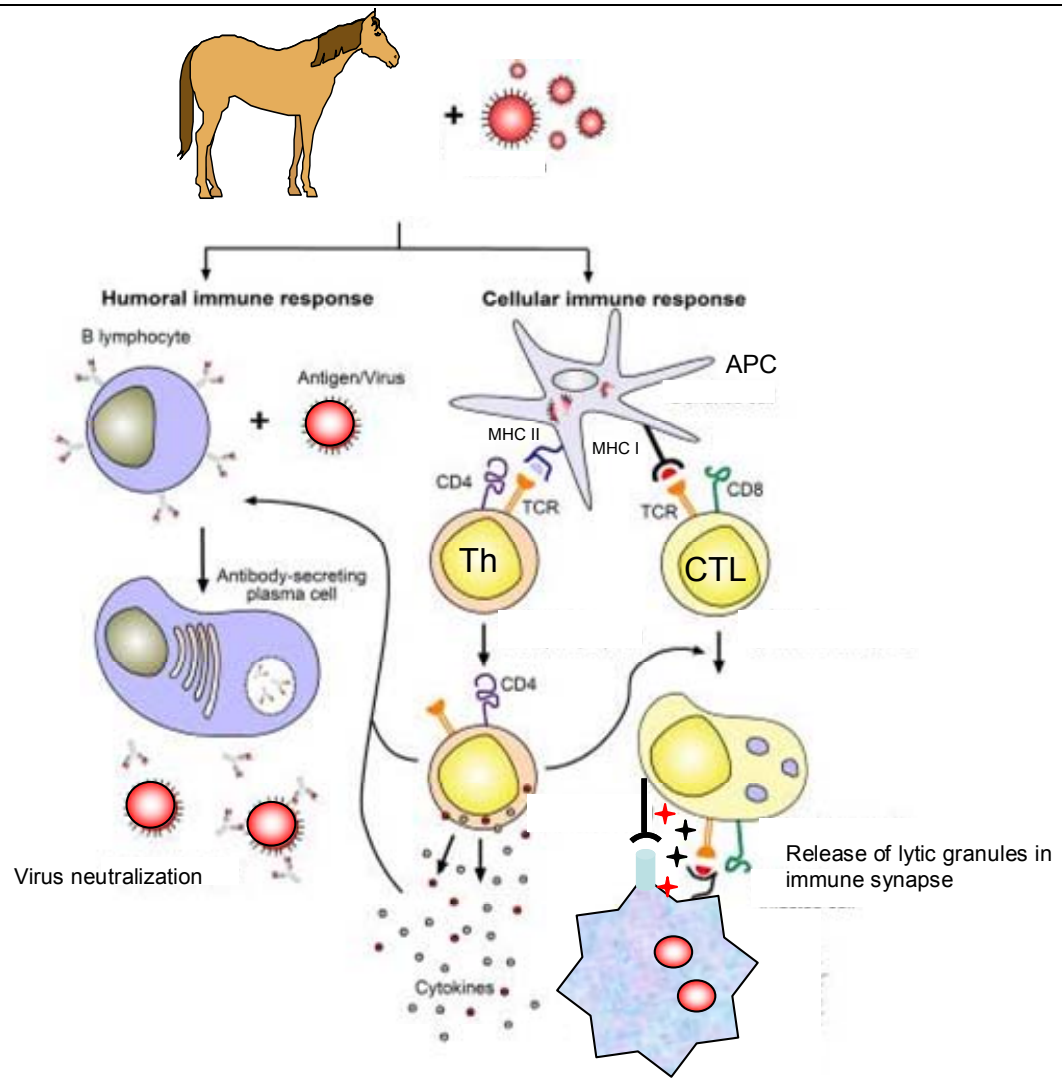


**Figure 1.3 Host immune response to viral infection.** After activation by antigen presenting cells (APC), T-helper cells (Th) cells provide the necessary cytokines and signals to activate cytotoxic T-lymphocytes (CTL) and B lymphocytes. CTL recognize and destroy virally infected cells; B lymphocytes produce antibodies to bind whole virus and viral antigens. Adapted from www.influenzareport.com/ir/images/image27.jpg

In addition to the cellular immune response, adaptive immunity also includes a humoral response which involves the production of antigen specific antibodies by B-lymphocytes. B-cells are uniquely adapted to bind soluble molecules through their cell-surface immunoglobulin, IgM. B-cells internalize soluble antigens and display peptides on MHC II molecules, allowing effector Th cells to bind and drive B-cell activation and differentiation. Th activated B-cells undergo immunoglobulin class switching and produce vast amounts of antigen specific antibodies. Some antibodies are capable of coating the virus to allow for better uptake by phagocytic cells. Phagocytic cells express receptors for the Fc portion of the antibody. The Fc receptors bind and engulf the antibody coded antigen in a process termed opsonization. Other antibodies trigger the complement system, which may directly destroy the pathogen or enhance opsonization. For viral infections, the most desirable antibody response is the development of neutralizing antibodies, which bind the virus and prevent it from entering new target cells (Fig 1.3).

With the aid of Th cells, neutralizing antibodies and CTLs are capable of halting viral infection and removing the pathogen from circulation. After clearing the infection, memory T and B cells continue to circulate in the host for extended periods, sometimes for the remaining life span of the host. When the same pathogen is encountered again, a faster, more efficient immune response is mounted by the host's memory cells to prevent re-infection and disease. These are the immune responses that make vaccination possible. Instead of causing illness with a live pathogen, vaccines use non-pathogenic forms of infectious agents to mount immune responses with lasting memory. Then, if the pathogen is encountered by the vaccinated host, the memory B and T cells can produce an appropriate immune response to preclude infection.

## 1.4 HOST IMMUNE RESPONSE TO EIAV INFECTION

CTL are effective at controlling lentiviral acute episodes, while neutralizing antibodies develop later in infection to aid the CTL in controlling viral replication and spread. The CTL and neutralization response develop with the aid of Th cells. Unfortunately, for most lentiviral infections, CD4+ Th cells are the primary target for infection (66). While the CTL and neutralizing antibody responses are successful at controlling replication, they are incapable of clearing the virus from the host. In the case of HIV, SIV and FIV, rapid mutations allow the lentivirus to escape host immune control and infect new CD4+ Th cells, ultimately leading to the collapse of the immune system. Unlike its lentiviral relatives, EIAV is eventually controlled by the horse's immune system.

There has been limited exploration into specific immune responses that control EIAV infection in horses. Like other lentiviral infections, control of the acute episode is associated with the appearance of CTL (90) and non-neutralizing antibodies (109, 122). CTL cells fluctuate throughout the course of infection, where as antibodies plateau after six months post infection (52). Neutralizing antibodies, originally thought to develop two months post infection, have recently been detected after the resolution of the acute episode (93). These antibodies, which are directed against a principal neutralization domain (PND) in gp90 (76, 77), continue to increase in titer and breadth of specificity throughout the first year of infection (12, 52, 60, 104). Each disease episode during the chronic phase of EIAV infection corresponds to viral escape of host immune control. The new quasispecies, which is distinct from the previous febrile episode, is brought under control by new type specific neutralizing antibodies. Immune responses to

EIAV eventually prevail, controlling viral replication and disease for the remaining lifespan of the infected horse without ever clearing the virus.

Attempts to understand EIAV immune control have led to the identification of CTL and T-helper (Th) epitopes in the Gag and Pol proteins of the virus (22, 46, 93, 94). Vaccines aimed at inducing these epitope-specific immune responses have not been effective at preventing infection or disease (45, 119). While the humoral response to gp90 has been extensively investigated, the cellular immune response to Env has remained virtually unexplored. To date, no immune correlates of protection have been revealed for EIAV or any other lentivirus.

## 1.5 LENTIVIRAL VACCINE DEVELOPMENT

The development of successful vaccines in the past has been based on empiric observations, without a firm understanding of appropriate immune responses. The most cited example is Edward Jenner using cowpox to inoculate individuals against smallpox infection (114). Jenner's success sparked a wave of vaccine development that has brought many deadly diseases such as rabies, influenza, plague, typhoid, measles, and diphtheria under control. With such success, it was anticipated that the same techniques could be used to develop an effective HIV vaccine.

Most vaccines against viral infections, such as smallpox and flu, elicit neutralizing antibodies capable of preventing virus entry into target cells. Therefore, the first HIV vaccines to be tested focused on evoking a neutralizing antibody response using recombinant Env proteins. Most of these trials failed in phase 1 and 2 testing (107). Extensive research demonstrated that HIV is capable of evading neutralizing antibodies through various

mechanisms, including N-linked glycosylation of the Env proteins (117), conformational masking of receptor-binding sites (73), and escape mutations (118, 138). Failure of the empiric approach in developing an effective HIV vaccine led to the exploration of immune mechanisms necessary for protective immunity.

Natural immunity to an infectious agent is desirable when attempting to identify key immune responses required for vaccine protection. Unfortunately, less than 5% of individuals infected with HIV are long-term nonprogressors, meaning their immune response is capable of controlling HIV replication and the individual is free of clinical symptoms of AIDS for greater that 10 years after infection (39). In need of a system to identify correlates of protective immunity, focused turned to the SIV macaque model system.

### 1.5.a SIV vaccine development

SIV is the "gold standard" for studying perspective AIDS vaccines in preclinical studies. Numerous vaccine strategies have been explored with SIV, including attenuated virus, whole-inactivated virus, viral vectors, DNA, and subunit vaccines. Only the use of attenuated SIV vaccines has offered complete or near-complete protection from homologous or heterologous virulent challenge (5, 37, 38, 70, 95, 102, 103, 127, 139). A comparison of attenuated vaccine strategies against other SIV vaccine strategies reveals that 95% of attenuated SIV vaccines lower viral loads by more than 3 logs, where as only 7% of other immunization strategies are capable of similar efficacy (70).

Attempts to correlate immune responses with SIV vaccine protection have proven less than successful. Neutralizing antibodies are the focus of an ongoing debate. Several researchers have found a protective role for neutralizing antibodies in SIV protection (85, 86, 115), while

others have been unable to correlate neutralization with protection (95, 102, 103, 127). With no clear association between protection and neutralizing antibodies, focus has been shifting to the CTL response. Like neutralizing antibodies, the role of CTL cells can not definitively be correlated with SIV protection (67, 87, 124, 127). Frustrated with a lack of correlates of protection, there is now a concerted effort by the Live Attenuated Consortium of the International AIDS Vaccine Initiative to identify the mechanisms of protection conferred by attenuated SIV vaccines in monkeys (70).

An additional aspect of SIV that makes it an attractive model for HIV studies is the ability to manipulate the SIV genome to express HIV Env instead of SIV Env. SHIV, as it is known, is capable of establishing infection in nonhuman primates without causing disease. During the course of infection, HIV specific antibodies develop which are capable of neutralizing the parental HIV strain (98). HIV Env specific immune responses in non-human primates can therefore be studied with the SHIV system. Similar to SIV vaccines, the best protection from heterologous challenge was obtained with attenuated SHIV vaccines. While sterilizing immunity was not achieved with any of the vaccines, lower viral set points and slower disease progression was observed in some of the vaccinated animals. Like SIV, studies with SHIV have yet to reveal any correlates of vaccine immunity (15).

### 1.5.b EIAV vaccine development

To understand immune mechanisms responsible for controlling lentiviral infections, non-primate lentivirus, such as EIAV and FIV, may also be utilized. Experimentation with these lentiviruses offers dual benefits in contributing to a better understanding of lentiviral protection while also aiding in the development of effective veterinary vaccines. The extensive research with FIV

actually led to the first USDA approved, commercially available lentiviral vaccine against in 2002 (134). Reported efficacy in FIV vaccinated kittens is 84%, compared to the 90% infection rate in control cats (1). While Fel-O-Vax FIV demonstrates that protective lentiviral vaccines are possible, the greatest benefit of FIV and EIAV vaccine research may be the observation of a remarkable spectrum of vaccine efficacy, ranging from apparent sterilizing immunity to severe disease enhancement.

EIAV is particularly interesting to study because of its unique clinical course of disease. EIAV is the only lentiviral system where the natural host develops disease then gains immunologic control of the evolving pathogen. In addition, inapparent carrier horses of EIAV are resistant to further virulent challenge. The natural immunologic control and prophylactic immunity of inapparent carrier horses suggests that an effective vaccine against EIAV can be developed. In fact, the Chinese routinely vaccinate all their horses with an attenuated strain of EIAV. Unfortunately, no independent evaluation of the Chinese vaccine has been performed to confirm the efficacy against preventing infection and disease (98). Concerns over reversion to virulence of attenuated vaccines, and the lack of a marker to differentiate infected verse vaccinated horses led researchers to investigate other strategies for vaccination (Table 1.1).

**Table 1.1 Summary of EIAV vaccines and their protective efficacies.**

| VACCINE | DESCRIPTION | # OF ANIMALS | CHALLENGE TYPE | # INFECTED | CLINICAL SIGNS | REFERENCE |
|---|---|---|---|---|---|---|
| **Inactivated Whole Virus** | formalin-inactivated | 12 | homologous | 0/12 | asymptomatic | Issel et al. 1992 (65) |
| | | | secondary heterologous | 12/12 | typical (3), none (9) | |
| **Subunit** | LLgp - lectin affinity purified envelope glycoproteins | 4 | homologous | 0/4 | asymptomatic | Issel et al. 1992 (65) |
| | | 4 | heterologous | 4/4 | enhancement (2), typical (1), asymptomatic (1) | |
| **Recombinant** | rgp90 - baculovirus-expressed recombinant gp90 | 4 | homologous | 0/4 | asymptomatic | Wang et al. 1994 (136), Raabe et al. 1998 (116) |
| | | 17 | heterologous | 17/17 | enhancement (5), typical (5), asymptomatic (3) | |
| **Attenuated** | EIAVΔDU - deletion in the dUTPase coding region | 2 | homologous | 2/2 | typical | Lichtenstien et al. 1995 (83), Hammond et al. 1999 (54) |
| **Particulate** | gpEIAV covalently coupled to glutaraldehyde-activated iron oxide beads | 4 | homologous | 4/4 | delayed disease | Hammond et al. 1999 (51) |
| **Attenuated** | EIAVΔS2 - two stop codons introduced into S2 | 9 | homologous | 0/9 | asymptomatic | Li et al. 2003 (79) |
| **Pepitde** | gp90 specific lipopeptide | 3 | homologous | 3/3 | less severe disease | Ridgely et al. 2003 (119) |
| **DNA** | codon optimized SU | 4 | homologous | 4/4 | typical | Cook et al. 2005 (28) |
| **Attenuated** | EIAVD9 - EIAVΔS2 with 9 base pair deletion in S2 | 12 | homologous | 0/12 | asymptomatic | Craigo et al. 2007 (32) |
| | | 24 | heterologous/homologous Env | 24/24 | typical (9), asymptomatic (15) | Craigo et al. 2007 (36) |

Outside of the attenuated vaccines, one of the first vaccination approaches tested for EIAV was a formaldehyde inactivated form of the virion (65). The inactivated EIAV, produced from a cell adapted strain of the virus, was used to immunize 12 ponies. Upon challenge with the homologous EIAV strain, none of the 12 ponies became infected or developed signs of disease. One hundred days after homologous challenge, the ponies were challenged a second time with a pony-virulent cell adapted strain of EIAV ($EIAV_{PV}$). All 12 ponies became infected after this heterologous challenge, and three developed classic signs of equine infectious anemia (EIA). Nine of the 12 vaccinated ponies remained asymptomatic after heterologous challenge, suggesting some degree of protection was offered by the formalin inactivated EIAV vaccine.

Upon analysis of neutralizing antibodies in the vaccinated ponies, there was no detectable correlation between vaccine protection and neutralizing antibody development (65).

A subunit vaccine composed of lectin affinity-purified EIAV Env was compared to the protection achieved with the formalin inactivated EIAV. Eight ponies were vaccinated with the subunit vaccine; four were protected from homologous challenge, while the other four became infected after $EIAV_{PV}$ challenge. Two of the $EIAV_{PV}$ challenged ponies demonstrated enhanced febrile episodes. PBMC from ponies immunized with the subunit vaccine had diminished EIAV-specific proliferation responses compared to PBMC from horses vaccinated with inactivated EIAV. These findings indicate that eliciting a cellular immune response may be an important component of an effective vaccine (65).

A third trial vaccination utilized recombinant EIAV gp90 and yielded surprising results. A baculovirus-expressed recombinant gp90 was used to vaccinate 17 ponies (116, 136). Following heterologous challenge with virulent $EIAV_{PV}$, the 17 ponies became infected and demonstrated a range of clinical disease presentation. Three of the vaccinated ponies remained asymptomatic, five developed typical EIA signs and nine demonstrated enhanced clinical disease characterized by early onset of acute episode with severe thrombocytopenia. While a correlation between the severity of disease and level of viral RNA in the plasma was established, there was no definitive association between enhancement and serum antibody reactivity (116). At first it was thought that the macrophage tropism of EIAV led to the enhancement of disease, but this was unfounded as a recombinant FIV vaccine also caused enhanced disease after virulent challenge (128). Unlike EIAV, FIV is a predominantly lymphotropic virus.

Various other EIAV vaccine strategies were attempted with little success at protecting from infection or disease. A particulate virus aimed at targeting gradient purified EIAV into the

phagocytic pathway to elicit a cell mediated response failed to protect ponies from infection, but did delay the onset of clinical disease and viremia (51). A lipopeptide vaccine containing a immune dominant Env epitope transiently induced a CTL response, however, each horse became infected and developed low grade fevers after virulent challenge (119). A DNA vaccine containing codon optimized gp90 was able to induce EIAV-specific antibodies and lymphoproliferation, but was unable to protect ponies from infection or disease when challenged with $EIAV_{PV}$ (28).

Research aimed at elucidating the mechanisms of EIAV replication, persistence and pathogenicity inadvertently assisted in vaccine development. In an attempt to understand the requirement of dUTPase, an EIAV DU knockout was constructed and tested *in vitro* and *in vivo*. Deleting DU resulted in delayed *in vitro* replication in bone marrow derived macrophage, the natural targets of EIAV, but the knockout had no effect on *in vitro* replication kinetics in fetal equine kidney cells (FEK), which are permissive for EIAV replication (83). The role of DU in i*n vivo* replication kinetics was explored by infecting Shetland ponies with EIAVΔDU. There was a 10-100 fold decrease in viral loads compared to $EIAV_{PV}$ infected ponies. Despite lower viral loads, EIAVΔDU infected ponies mounted similar antibody responses to $EIAV_{PV}$ infected ponies. To determine if the immune responses elicited by EIAVΔDU were protective, two EIAVΔDU infected ponies were challenged with $EIAV_{PV}$. The two ponies developed signs of classical EIA, indicating that the immune responses elicited by EIAVΔDU were not protective (54).

Investigating the role of S2 in EIAV replication led to another attenuated vaccine candidate. While the role of the accessory proteins Tat and Rev are clearly understood, the function of the S2 protein is not. In an effort to understand the mechanism of action, S2 mutants

of EIAV were developed. *In vitro* replication kinetics of the EIAVΔS2 mutant in equine macrophages was equivalent to the parental EIAV (81). When used to infect ponies, EIAVΔS2, like EIAVΔDU, had reduced *in vivo* replication kinetics and also produced virus specific immune responses (80). To determine if these ponies were protected from virulent challenge, the EIAVΔS2 infected ponies were challenged with $EIAV_{PV}$. Apparent sterilizing immunity was achieved as each pony was protected from infection and disease (79), which is in stark contrast to EIAVΔDU infected ponies, where challenge by $EIAV_{PV}$ produced disease. Protective immunity correlated with the development of a mature Env-specific immune response, as measured by antibody titer, avidity index and conformational ratio. Like previous EIAV vaccines, there was no correlation between *in vivo* immune protection and *in vitro* serum neutralizing titer. However, this vaccine proved that attenuated EIAV vaccines can elicit protective immunity even against a rigorous virulent EIAV challenge.

## 1.6 IMMUNE MATURATION TO ENVELOPE PROTEINS IN EIAV AND SIV INFECTED ANIMALS

In an effort to identify correlates of lentiviral protective immunity, independent studies with EIAV and SIV were conducted yielding similar results. An antigen specific immune maturation to EIAV and SIV was revealed during an immunological analysis of longitudinal serum from EIAV infected horses (52) and SIV infected monkeys (23, 25). Quantitative and qualitative antibody measurements with serum antibody displayed an increased immune maturation to the Env proteins of both viruses (Fig 1.4). The quantitative measurement endpoint titer revealed that

Env specific antibodies titers increase during the first 2-3 months post infection, before maintaining an indefinite steady state level in infected hosts.

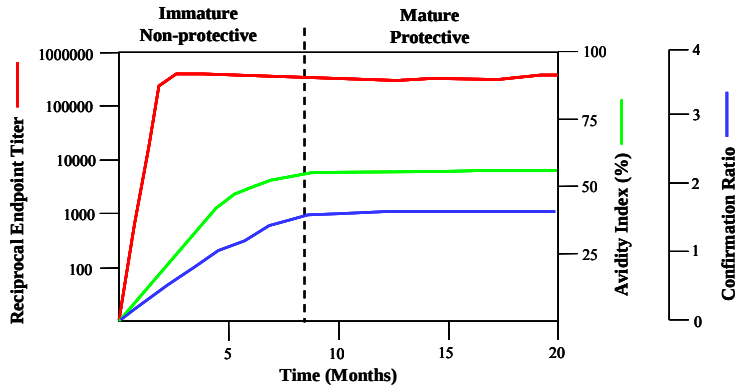


**Figure 1.4 Qualitative and quantitative antibody measurements.** Reciprocal endpoint titer is measured with a standard ConA ELISA. The avidity index is measured by comparing the binding of primary antibody to gradient purified EIAV after washing with or without 8M Urea. Avidity indices >30% are indicators of mature immunity. The confirmation ration reflects a comparison of primary antibody binding to gradient purified EIAV versus denatured EIAV. A ratio >1.0 represents predominant antibody reactivity with conformational epitopes.

A prolonged evolution of specific antibody parameters was revealed using three different qualitative measurements: avidity index, conformational ratio, and neutralization titer. For both EIAV and SIV, initial antibody binding to the virus is weak, but begins to increase in avidity over 6-8 months before reaching steady state levels. Unlike the avidity, the progression of antibody conformational dependence is opposite for these two lentiviruses. EIAV Env specific antibodies initially recognize predominantly linear epitopes, but mature to identify predominantly conformational epitopes (Fig 1.4); SIV Env specific antibodies maintain high conformational ratios, but throughout the course of infection, the ratio begins to decline. For

both EIAV and SIV, the evolution of conformational epitope recognition evolves in parallel with increasing antibody avidity.

A general broadening of neutralizing antibodies was observed for both EIAV and SIV infections. However, the levels of neutralizing antibodies fluctuate in both infections, making them unreliable indicators of mature immune responses. Therefore, measurements of avidity and conformational ratio of serum antibodies serve as reliable indicators of immune maturation to EIAV and SIV infection, while levels of Env specific antibodies and neutralizing antibodies do not accurately track the maturation process (99).

## 1.7 IMMUNE MATURATION IN VACCINATED ANIMALS

For both EIAV and SIV, the best protection from infection was achieved with attenuated vaccines. Interestingly, both systems required six months to develop protective immunity after vaccination (99) indicating a close correlation between immune maturation and vaccine protection. Analysis of SIV vaccinated monkeys established that protective vaccine immunity is associated with mature antibody responses. Non-protected vaccinated monkeys or monkeys that developed enhanced disease after virulent challenge demonstrated characteristic immature immune responses (25). The wide spectrum of vaccine efficacy observed in EIAV offered researchers the same opportunity to explore immune responses elicited by different vaccines.

To discern antibody correlates of protective immunity and disease enhancement a comparative analysis was performed on seven EIAV vaccines that demonstrated a spectrum of protective efficacy. The study included day of challenge serum from horses/ponies vaccinated

with either the attenuated viruses (EIAVΔS2 and EIAVΔDU), formalin-inactivated EIAV, or gp90 subunit vaccines (lentil-lectin purified Env (LLgp), HPLC purified gp90 (Vgp90), and two recombinant baculovirus gp90 (rgp90)) (54). Antibody titer, neutralization titer, avidity index and conformational ratio were all measured in the vaccinated animals. No single antibody parameter absolutely correlated with vaccine protection or enhancement of disease (Fig 1.5).

Contrary to the immune maturation model, where protected animals developed high titer antibodies with strong avidity to conformational epitopes, the EIAV vaccinated animals protected from disease did not exhibit all the necessary qualitative measurements (Fig 1.5). While the antibody titer and conformational ratio of symptomatic animals were consistent with the maturation model, not all of the protected animals developed high avidity antibody responses. Additionally, no virus neutralizing activity was observed at day of challenge in any of the vaccinated animals. Overall, it was established that all of the *in vitro* humoral immune parameters identified by the maturation model to lentiviral infection were not absolutely required for the protective immune response generated by this cohort of vaccines. Interestingly, the EIAVΔS2 vaccine, which provided apparent sterilizing immunity, satisfied the criteria of the immune maturation model (54).

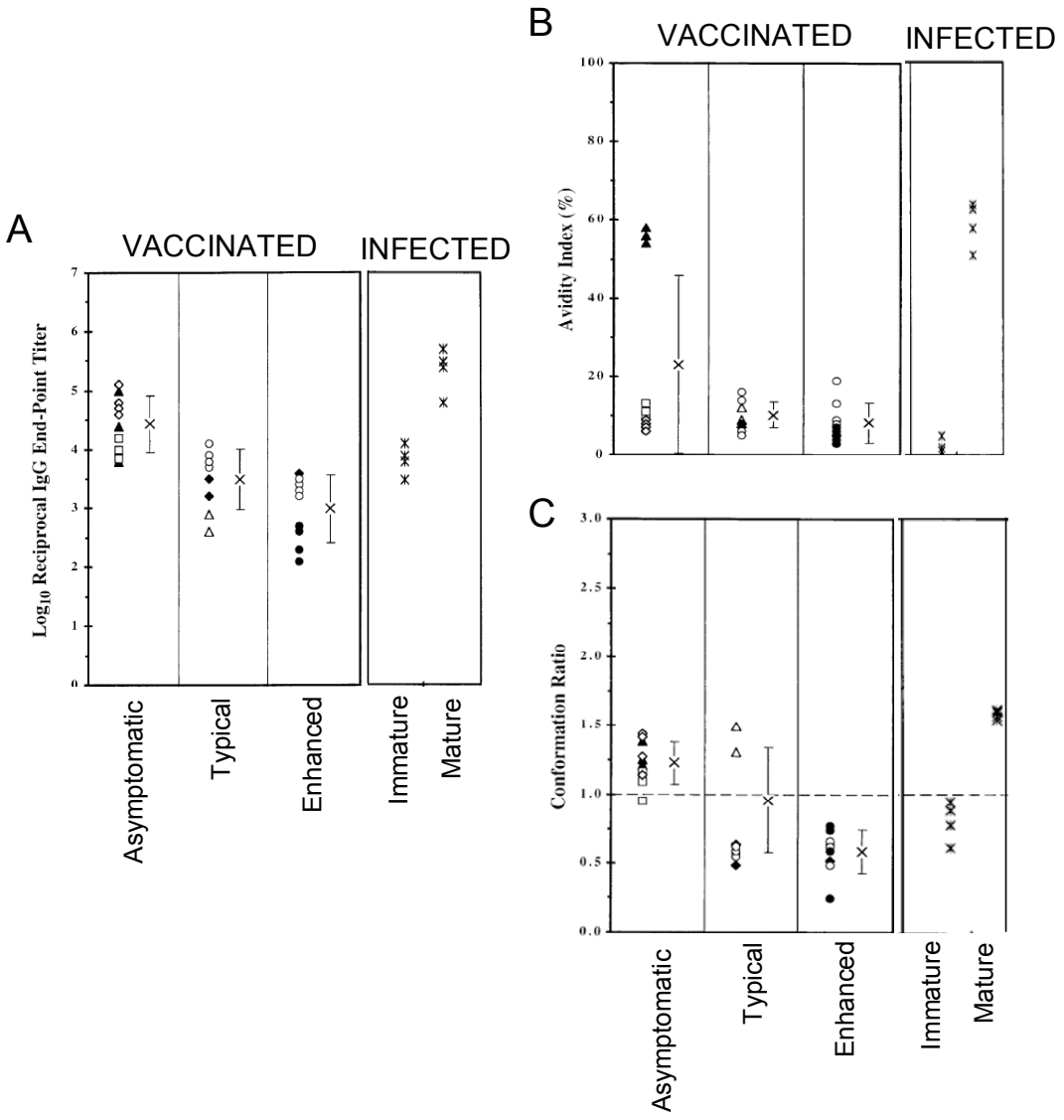


**Figure 1.5 Antibody titer and conformation ratio as indicators of vaccine protection.** A mature immune response in infected ponies is characterized by high endpoint titer (A) and high avidity (B) to conformational epitopes (C). The antibody profile of Ponies vaccinated with EIAVΔS2 (▲), EIAVΔDU (Δ), inactivated whole virus (□), lentil-lectin purified Env (◊), HPLC purified gp90 (♦), or recombinant baculovirus gp90 (○ and ●) were analyzed to determine if there were antibody correlates of vaccine efficacy and enhancement. Figure adapted from Hammond et al. 1999. Virology 262:416-430 (54).

In addition to illustrating a correlation between vaccine protection and antibody endpoint titer and conformation, the comparative analysis exposed an apparent dependence of vaccine efficacy on EIAV Env proteins. The conformational ELISAs revealed marked differences in antibody specificities which could be traced to the nature of the Env proteins in the different EIAV vaccines. The attenuated EIAV vaccines, which offered the best protection from infection and disease, displayed natural, oligomeric viral glycoproteins. The Env proteins of the attenuated EIAV strains were identical to virulent virus, and were therefore able to elicit antibodies to conformational epitopes, much like natural EIAV infection. Fixing EIAV with formaldehyde caused the envelope to be rigid and non-dynamic, potentially masking some antibody epitopes, thereby lowering the conformational ratio and perhaps reducing the protective efficacy compared to the attenuated vaccines. For the recombinant vaccines, the lentil-lectin purification process used to purify Env was mild and allowed for conformational epitopes to be displayed, while the harsh HPLC purification of the Vgp90 vaccine lead to non-native glycoprotein structures and resulted in the recognition of only linear antibody epitopes. Finally, recombinant gp90 produced in baculovirus infected insect cells was only able to produce linear epitopes, most likely due to the differences of insect protein production compared to mammalian protein production. LLgp offered the best protection from disease out of the recombinant vaccines, while the rgp90 caused severe enhancement of disease (54). Taken together, these observations emphasize the importance of using native viral glycoproteins in lentiviral vaccine preparations.

## 1.8 EIAV ENVELOPE PROTEINS

The EIAV Env, presented as a trimer on the virion surface, is composed of a surface glycoprotein (gp90) and a transmembrane glycoprotein (gp45) (Fig 1.6). The non-covalently linked proteins are anchored in the lipid bilayer through the membrane spanning domain of gp45. The cytoplasmic tail of gp45 is the most conserved portion of the two Env proteins, while gp90 is the most variable protein of the entire virus. The error prone lentiviral RT permits the development of mutations in the Env proteins, which rapidly accumulate enabling EIAV to escape host immune control. In addition to escape mutations, Env employs glycosylation to mask the virus from the immune system. The envelope proteins are heavily glycosylated with 18 predicted N-linked glycosylation sites on gp90 and four on gp45. With these immune evasion techniques, EIAV breaks free of immune control, rapidly replicates and induces new febrile episodes. Longitudinal sequencing analysis of plasma viral RNA from a chronically infected pony revealed that Env mutations are not randomly distributed. Instead, eight variable regions (V1 to V8) were identified where amino acid sequences were more than 30% divergent from the consensus sequence (77).

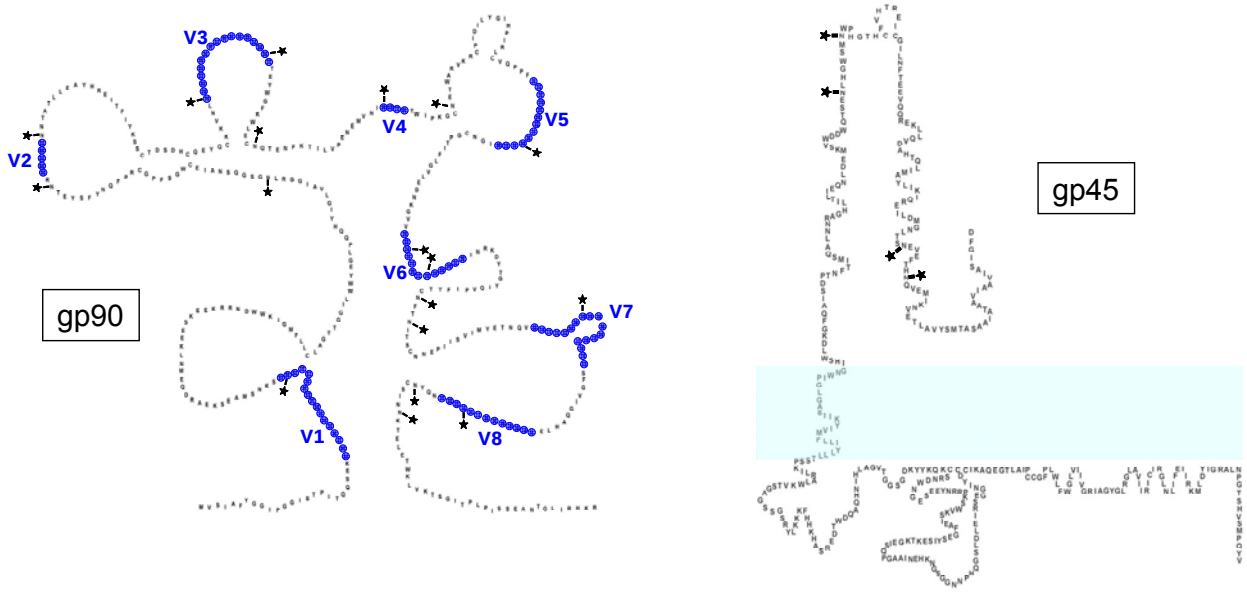


**Figure 1.6 EIAV Env glycoproteins.** EIAV Env is composed of a surface glycoprotein, gp90, non-covalently linked to a transmembrane glycoprotein, gp45. N-linked glycosylation sites are indicated with a *. The predefined variable domains of gp90 are highlighted in blue and labeled V1-V8. The shaded box on gp45 indicates the lipid bilayer. Adapted from Tagmyer et al. 2007. JGV 88:1324-1336 (132).

The gp90 protein serves as the target for neutralizing antibodies. Longitudinal serum neutralization analysis against homologous virus from a chronically infected pony revealed that EIAV Env evolution during persistent infection leads to an increasingly neutralization resistant phenotype (60). Three neutralization epitopes were identified in gp90 using monoclonal antibodies (12), two of these epitopes span V3 and compose the principal neutralization domain (PND) while the third is in the V5 region. As expected, a pony infected with an EIAVΔPND mutant did not develop a measurable neutralization titer. However, after immune suppression with dexamethasone, neutralizing antibodies were detected in the EIAVΔPND infected pony,

demonstrating that V3 is not required for the development of a neutralization response (33).

With the extensive variability and rapid ability to evade host immune responses, Env poses a major obstacle the development of any lentiviral vaccine (18). The previously described work with EIAV and SIV established an immune maturation to the Env proteins during infection and vaccination. Additionally, EIAV vaccine efficacy was shown to be highly dependent on the conformation of Env. For these reasons, I hypothesize that Env is a primary determinant of vaccine efficacy, and that effective vaccines must elicit appropriate immune responses to conserved regions of the viral envelope. To test this hypothesis, a new attenuated vaccine, $EIAV_{D9}$, was developed to test the effects of Env variation on vaccine efficacy.

## 1.9 $EIAV_{D9}$ VACCINE

The EIAVΔS2 was the most effective vaccine at preventing infection and disease after virulent challenge, however, as with all other attenuated lentiviral vaccines, reversion to virulence remained a major concern (98). The original EIAVΔS2 vaccine was developed by introducing two stop codons into the S2 gene of the infectious molecular clone $EIAV_{UK}$ (81). The mutations did not disrupt the second exon of *tat*, the initiation codon of *env*, or the putative *rev*-response element (RRE). The insertion of the first stop codon introduced a novel *Spe I* site in S2 that was not found in the parental S2, discriminating EIAVΔS2 from the parental $EIAV_{UK}$ (32, 79). Due to rapid evolution of lentiviral genomes and the ability to easily overcome two stop codons, a new EIAVΔS2 mutant was developed to minimize the potential reversion to virulence. Overlapping PCR mutagenesis techniques were utilized to create $EIAV_{D9}$ which has a nine base

pair deletion in EIAVΔS2 (32). The deletion conserved the two stop codons of EIAVΔS2, and also did not affect *tat*, *env* or the *rev*-response element (Fig 1.7).

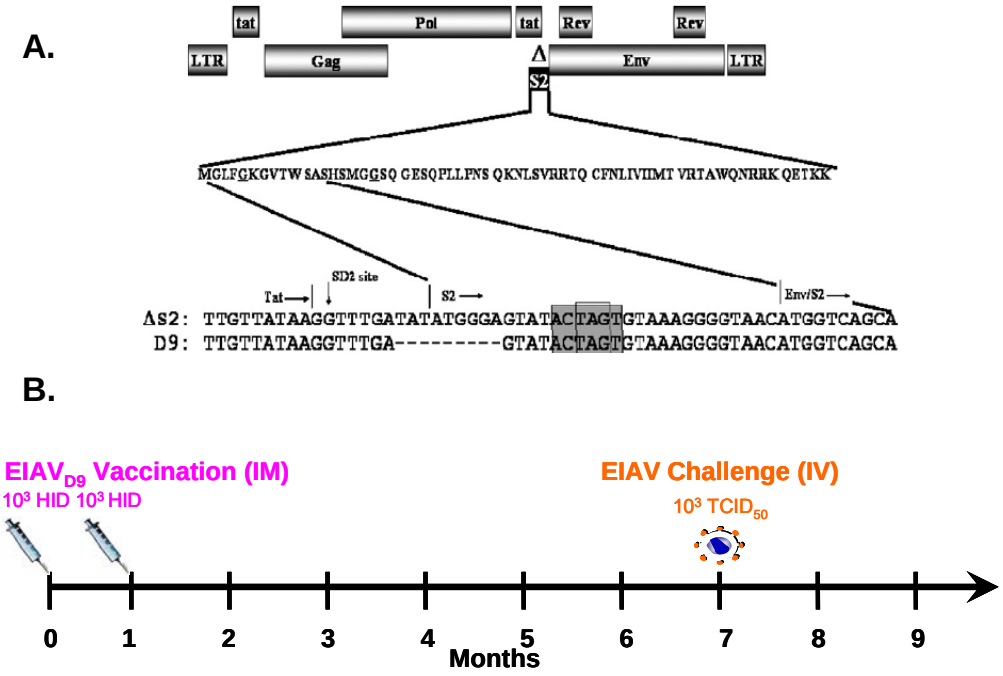


**Figure 1.7 EIAV$_{D9}$ vaccine and immunization strategy.** (A) The EIAV$_{D9}$ vaccine was developed by deleting nine amino acids from the EIAVΔS2 construct. The shaded box indicates one of the two preserved stop codons of EIAVΔS2. (B) EIAV$_{D9}$ is administered in two intramuscular doses 30 days apart. Seven months after the first immunization, the animals are intravenously challenged with virulent EIAV. HID = horse infectious dose, TCID$_{50}$ = tissue culture infectious dose. Adapted from Craigo et al. 2007. Vaccine 25:834-845 (32).

Twelve horses were immunized with EIAV$_{D9}$. None of the twelve immunized hoses became infected after homologous challenge with EIAV$_{PV}$, indicating apparent sterilizing immunity (32). Since EIAV$_{UK}$ is a molecular clone of EIAV$_{PV}$, the two are considered to be homologous (29). Each vaccinated horse developed antibody responses consistent with the

immune maturation model and indicated that a mature immune response was achieved at the time of challenge. To test the apparent "sterilizing" immunity of the 12 vaccinated horses, a 14 day dexamethasone treatment was used to immune suppress each immunized horse two months after $EIAV_{PV}$ challenge. Before dexamethasone treatment, all 12 horses were free of EIA and detectable plasma viremia, as measured by Real-time RT-PCR. Immune suppression caused six of the 12 horses to develop EIA, and post-dexamethasone treatment revealed 50% of the horses were infected with $EIAV_{PV}$. Despite not achieving "sterilizing" immunity, the $EIAV_{D9}$ vaccine did prevent disease and apparent infection before immune suppression, making $EIAV_{D9}$ valuable in discerning the contribution of Env to vaccine efficacy.

Towards this goal, Env variants of EIAV were developed to be used as challenge strains for the $EIAV_{D9}$ vaccine. The majority of Env variation takes place in gp90, with little variation occurring in gp45 (77), therefore the focus of the trial involved variation of the gp90 protein. Plasma samples were obtained from a pony chronically infected with $EIAV_{PV}$ to isolate variant viruses during sequential disease episodes. From this study, three variants were isolated that diverged from the $EIAV_{PV}$ gp90 by 6, 10 and 13% at the amino acid level (Fig 1.8).

The gp90 of each variant was placed into the backbone of $EIAV_{UK3}$. In developing $EIAV_{UK}$ from $EIAV_{PV}$, a 68 base pair duplication in the 3'LTR and an arginine at position 103 instead of a tryptophan in the second exon of *rev* were introduced (27). For $EIAV_{UK3}$ clone, the duplication was deleted, and the tryptophan was restored, making $EIAV_{UK3}$ equivalently pathogenic as $EIAV_{PV}$ in horses. Therefore, $EIAV_{UK3}$ was used to construct the variant Env EIAV challenge strains. Ponies infected with the three Env challenge strains EV0, EV6, and EV13 developed EIA and sustained viral loads (36).

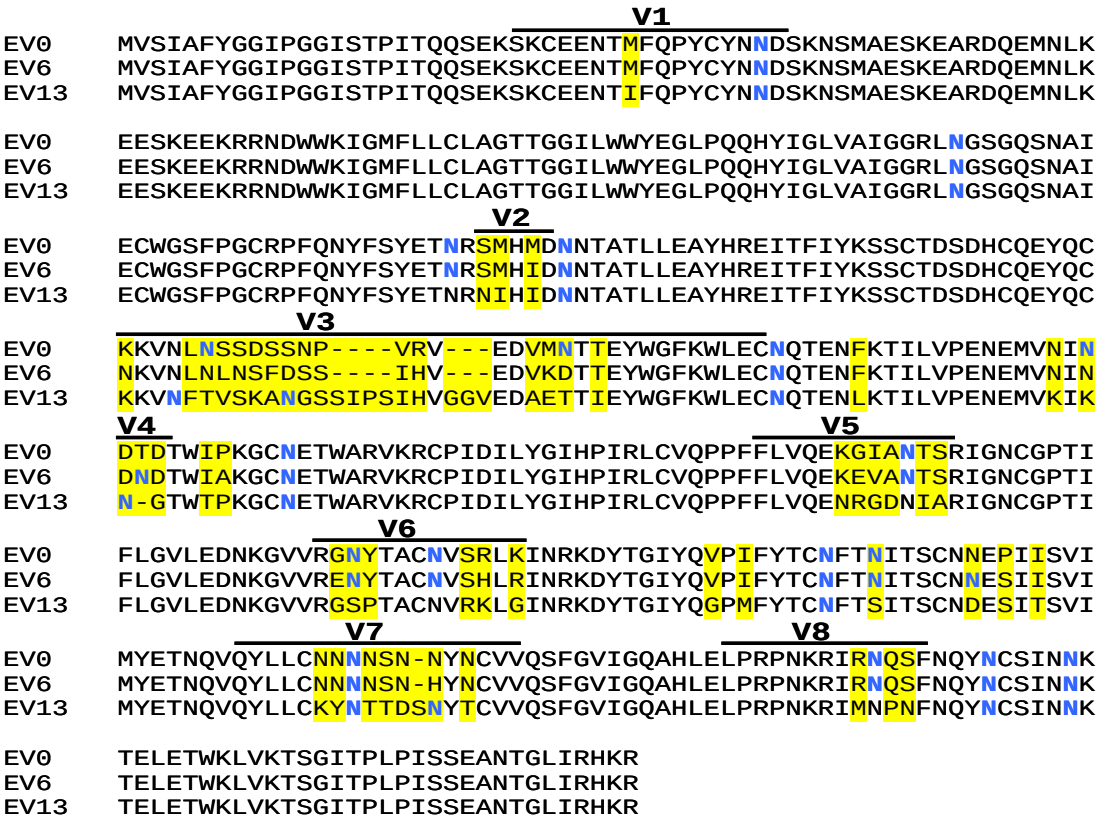


**Figure 1.8 Alignment of EIAV variant gp90 proteins.** Divergent amino acids between the three variant challenge strains are highlighted in yellow. The predefined variable domains are labeled V1-V8, potential N-linked glycosylation sites are in **blue**.

To test the contribution of Env to vaccine efficacy, 24 ponies were immunized with $EIAV_{D9}$ and subsequently challenged 7 months post vaccination with EV0 ($EIAV_{UK3}$), EV6 (6% variant Env) or EV13 (13% variant Env). By 120 days post challenge, one EV0, three EV6 and five EV13 ponies developed EIA, demonstrating a clear inverse correlation between Env divergence and vaccine protection (36) (Fig 1.9).

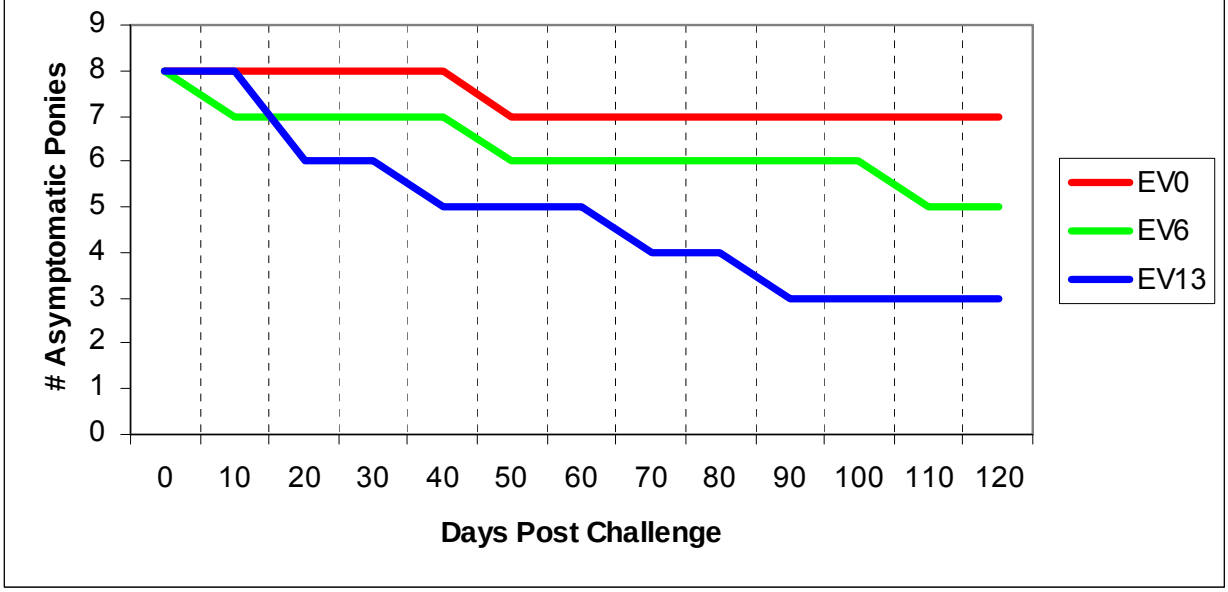


**Figure 1.9 Disease outcome of variant challenged $EIAV_{D9}$ vaccinated ponies.**

As Env is the only variant protein in the three different challenge strains, there must be Env-specific immune responses associated with vaccine protection from disease. Differentiating the responses between the protected and non-protected ponies would provide valuable insight into the appropriate humoral and cellular immune responses necessary for vaccine induced immunity to EIAV. Identifying Env-specific immune responses could also help to address the effects of Env variation on immune recognition while also aid in the development of more effective lentiviral vaccines by developing new vaccine strategies to target the specified immune responses.

## 2.0 HYPOTHESIS AND SPECIFIC AIMS

While many vaccines against infectious diseases have been developed through empiric methodologies, it is clear that understanding specific immune responses will be necessary to develop an effective lentiviral vaccine. The best way to dissect protective anti-lentiviral immune responses is through animal vaccine model systems. While SIV has been the "gold standard" for identifying key immune responses for HIV vaccine development, researchers have fallen short of finding immune correlates of vaccine protection. However, the $EIAV_{D9}$ vaccine, in combination with the different challenge strains provides a unique opportunity to identify key determinants of vaccine induced protective immunity. Additionally, the three challenge strains allow for the investigation of the effects of natural Env sequence variation on immune recognition. By analyzing the immune responses of protected and non-protected vaccinated ponies after challenge, ***I hypothesize there may be Env-specific cellular and humoral immune responses associated with vaccine protection from disease. Additionally, I believe that the natural Env sequence variation between the three challenge strains will alter both the cellular and humoral immune responses in infected and vaccinated ponies.***

**The specific aims addressed in this dissertation are:**

**SPECIFIC AIM 1: Define Env peptide-specific cellular reactivity of the EIAV reference strain.** A necessary foundation for defining determinants of vaccine protection is an understanding of the humoral and cellular immune responses to the Env proteins of the vaccine and the homologous challenge strain. Previous work identified the V3 and V4 regions as integral to neutralizing antibody recognition, but the cellular immune response to Env have remained unexplored. The first objective is to utilize day of challenge PBMC from 12 $EIAV_{D9}$ vaccinated horses and PBMC from two $EIAV_{PV}$ infected horses to identify broadly reactive Env specific Th and CTL peptide responses. Cellular responses will be analyzed using thymidine incorporation to measure Th proliferation and chromium release to measure CTL cytolytic activity. These data will provide a basis for identifying cellular immune responses associated with vaccine protection and provide a reference for analyzing the effects of amino acid variation on immune recognition.

**SPECIFIC AIM 2: Identify humoral and cellular immune determinants associated with vaccine protection from disease.** Challenging 24 $EIAV_{D9}$ vaccinated ponies with divergent Env strains demonstrated that Env is a primary determinant of vaccine efficacy. The vaccine trial provides the unique opportunity to elucidate immune responses associated with vaccine protection from disease. Day of challenge PBMC from the 24 vaccinated ponies will be used to analyze Env specific Th proliferation and CTL cytolytic activity to identify Env peptide-specific immune responses associated with vaccine protection from disease. Additionally, day of challenge and four week post challenge sera will be used to analyze vaccine-specific and challenge strain-specific neutralizing antibodies to determine if a relationship exists between

neutralization activity and protective efficacy. These data will provide valuable insight into lentiviral vaccine protection and may aid in the development of more effective EIAV vaccines.

**SPECIFIC AIM 3: Analyze the effects of natural Env variation on immune recognition in EIAV infected and vaccinated ponies.** Lentiviruses utilize numerous escape mechanisms to evade the host immune responses. Among the escape mechanisms is antigenic variation of immune epitopes. To examine the effects of natural Env variation, we will analyze the humoral and cellular immune responses of ponies infected with each of three challenge strains. Sera from infected ponies will be used to measure the level of cross-reactivity between the three variant strains, and also to map the neutralization specificity in Env. PBMC from ponies infected with each variant will be used to map Env peptide-specific Th and CTL responses and to analyze the effects of amino acid variation on cellular immune recognition. Additionally, sera and PBMC from $EIAV_{D9}$ vaccinated ponies will also be used to define the effects of variation on vaccine induced immunity. Collectively, these data will help to provide a better understanding of Env variation on immune recognition, which may have profound effects on vaccine efficacy.

With the $EIAV_{D9}$ vaccine trials, we are poised to identify critical immune determinants necessary for vaccine protection from disease and how natural Env variation may affect immune responses elicited by protective vaccines. These data may provide solid groundwork for further investigation of immune responses associated with vaccine protection. Additionally, this work may help to develop more effective EIAV vaccines. Understanding the immune responses and effects of Env variation in the EIAV model system, could possibly aid in the investigation of protective immune responses in other lentiviral systems.

# 3.0 ENVELOPE-SPECIFIC T-HELPER AND CTL RESPONSES ASSOCIATED WITH PROTECTIVE IMMUNITY TO EQUINE INFECTIOUS ANIMEMIA VIRUS

This chapter was modified from:

Tara L. Tagmyer, Jodi K. Craigo, Sheila J. Cook, Charles J. Issel, Ronald C. Montelaro. Envelope-specific T-helper and cytotoxic T-lymphocyte responses associated with protective immunity to equine infectious anemia virus. (2007). Journal of General Virology. 88:1324-1336.

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## 3.1 INTRODUCTION

*Equine infectious anemia virus* (EIAV) is a lentivirus of equids that shares genetic and structural similarities with HIV-1, but causes a unique disease process among the known lentiviruses (97). While human and other animal lentiviruses cause progressive degenerative or immune suppressive disease, EIAV infection of horses is characterized by dynamic recurring disease cycles in the first year of infection. Following this chronic stage of disease, most infected horses progress to a long term inapparent carrier state in which disease and virus replication is strictly controlled for the life span of the horse. This inapparent state is not attributable to attenuation of the infecting virus, as whole blood transfers from inapparent carriers can induce disease in naïve recipient horses and immune suppression of inapparent horses can induce a recurrence of disease, indicating the potential virulence of the infecting EIAV (64, 89, 97). In addition, inapparent carriers of EIAV appear to be highly resistant to additional exposure to diverse and highly virulent strains of EIAV, suggesting that the immune response in inapparent carriers not only controls the existing infection, but provides a high level of prophylactic immunity (97). Thus, the EIAV system is useful in examining the natural immunologic control of a pathogenic lentivirus infection, despite the array of persistence mechanisms employed by these viruses.

During the past 20 years, we have evaluated a number of experimental EIAV vaccines, with the results revealing that EIAV-specific immune responses are a double-edged sword that can protect or enhance viral disease and infection upon virus challenge of experimentally vaccinated horses (34, 54, 65, 79, 83, 116, 136). As observed in similar studies of experimental vaccines in the SIV and FIV systems, attenuated EIAV vaccines have to date produced the highest level of vaccine immunity against virulent EIAV challenge (15). Interestingly, however,

these attenuated lentiviral vaccines do not achieve optimum protective immunity until six months post inoculation, indicating a progressive maturation of virus-specific immunity. In addition to the protective immunity observed in EIAV inapparent carriers, the mature immunity elicited by attenuated EIAV vaccines provides a complementary model for defining critical immune correlates of enduring broadly protective vaccine immunity to a lentiviral infection.

Based on a comparison of the quantitative and qualitative properties of lentiviral Env-specific antibodies, we have defined an association between immature/non-protective or enhancing and mature/protective virus-specific immunity in the EIAV/horse (54), SIV/monkey (23, 25), and SHIV/monkey (24) vaccine systems. Thus, we propose that lentiviral vaccines must sufficiently drive this immune maturation to achieve protective efficacy. Additionally, we hypothesize that the EIAV Env is a major determinant of vaccine efficacy and that the maturation to protective immunity is associated with a redirection of host immune responses from immunodominant variable Env domains to relatively immunorecessive conserved Env domains.

There is to date only limited characterization of cellular and humoral immune responses associated with enduring protective EIAV immunity. Clearance of the acute viremia typically correlates with the appearance of CTL (90) and non-neutralizing antibodies (109, 122). While levels of CTL fluctuate throughout the disease course, neutralizing antibodies generally do not appear until two months post-infection and increase in titer and breadth of specificity during the first year post-infection (12, 52, 60, 104). Recently, however, Mealey *et al* have shown the presence of neutralizing antibodies immediately after the resolution of acute disease, perhaps suggesting an earlier role for neutralizing antibodies in controlling EIAV replication in experimentally infected horses (93). During the chronic phase of EIAV infection, there is a high

propensity for the selection of antigenic variants of EIAV that are able to temporarily escape the existing neutralizing antibodies directed to Env proteins or CTL responses directed to Gag protein epitopes (22, 60, 63, 94). The prevalence of antibody and CTL antigenic variants is consistent with a role for these specific immune responses in controlling EIAV infection. McGuire and colleagues have thoroughly examined the specificity of CTL responses to EIAV Gag and Pol proteins to identify broadly reactive Th and CTL epitopes in these proteins (22, 60, 63, 94). However, vaccines based on these broadly reactive T-cell epitopes have failed to elicit protective immunity against EIAV challenge (45), indicating that immune responses to other viral proteins, such as Env, may be an important component to the establishment of protective immunity to EIAV.

To explain the observed requirement of maturation for protective immunity, we have proposed that the progression towards protection is associated with recognition of conserved immunorecessive epitopes of the Env proteins. A necessary foundation for testing this hypothesis is a detailed characterization of humoral and cellular epitopes in the Env proteins that are recognized during protective immunity. We have previously examined in detail the neutralizing epitopes of EIAV Env proteins and the effects of sequence variation on viral neutralization properties (12, 33, 60). In contrast to the detailed studies of Env neutralization determinants, there have been limited studies of the Th and CTL epitopes of EIAV Env proteins. Thus, in the current study we have used 12 horses immunized with a highly protective attenuated EIAV vaccine and two experimentally infected asymptomatic carrier horses, with the infecting virus strain in both groups of horses expressing identical Env proteins, to elucidate regions of EIAV Env that are broadly recognized by Th and CTL. The results of these studies for the first time provide a comprehensive mapping of Env specific Th and CTL peptides associated with

enduring protective immunity to EIAV and provide a foundation for determining the effects of Env variation on immune recognition.

## 3.2 MATERIALS AND METHODS

### 3.2.a Experimental subjects

All equine procedures were conducted at the Gluck Equine Research Center of the University of Kentucky according to protocols approved by the University of Kentucky IACUC. Twelve horses were vaccinated with the live attenuated $EIAV_{D9}$ vaccine (31). Serological typing of the MHC I locus was performed by the University of Kentucky Equine Blood Typing Laboratory using established techniques for the 11 A-locus antigenic specificities of the equine lymphocyte alloantigen (ELA-A) (Table 3.1) (9, 10). Direct sequencing of the DRA and DQA MHC II loci was performed using previously published protocols (4, 44). C23, C66 and B81 were not MHC II typed due to lack of cells. After allowing the horses seven months to establish a mature immune response to the vaccine, blood was drawn from each horse, and Peripheral Blood Mononuclear Cells (PBMC) were isolated over a Histopaque gradient. On the following day, the vaccinated horses were subsequently challenged intravenously with a reference virulent $EIAV_{PV}$ using a low dose multiple exposure challenge to mimic the predominant natural route of EIAV infection by horse fly bites (79).

Two naive horses were challenged in parallel with three times $10^1$ HID (horse infectious dose) as positive infection controls for the $EIAV_{PV}$ low dose multiple exposure challenge. Blood was obtained from these horses at regular intervals, including after each EIAV febrile episode.

PBMC used in these experiments were obtained during asymptomatic stages of infection for these two horses. Five naive horses were included in the study as uninfected controls. All purified PBMC were resuspended in autologous serum and 10% DMSO, and cryopreserved in liquid nitrogen.

### 3.2.b Production of synthetic peptides

Eighty-five 20-mer peptides overlapping by 10 residues representing the entire $EIAV_{UK}$ (GeneBank AF0161316) Env were synthesized in the Biomedical Research Support Facilities Peptide Synthesis Core of the University of Pittsburgh using an Advanced Chemtech Model 396 Omega Synthesizer. All peptides were HPLC purified and confirmed by mass spectrometry. The peptides were dissolved at 2mg/ml in 100% DMSO and stored at -80°C until further use. Pools of 9-10 peptides were constructed in a standard matrix such that each peptide was represented in two pools. The pools were used to stimulate PBMC from the vaccinated and control horses in lymphoproliferation assays, and to pulse target cells for chromium release assays. The design of the matrix was such that reactive peptides were indicated by a proliferative or a cytolytic response to the two distinct pools containing the peptide. Reactive peptides were then assayed individually to determine their reactivity in the separate assays (121).

### 3.2.c Lymphoproliferation assays

PBMC from the 12 vaccinated, two infected, and five uninfected horses were tested for recognition of EIAV envelope peptides in a standard seven-day thymidine incorporation assay, as previously described (46) with minor modifications. Cryopreserved PBMC from the vaccinated, challenged and naïve horses were >80% viable upon thawing and maintained

viability throughout the assays, as measured by trypan blue exclusion. PBMC, at 2 X $10^5$ cells/well, were assayed in six replicates in complete RPMI-1640 (10% fetal equine serum, 1% penicillin/streptomycin, 1% L-glutamine, 55uM β-mercaptoethanol). In initial experiments, we observed relatively high lymphoproliferation backgrounds in PBMC in the absence of peptide stimulation, apparently resulting from *in vitro* amplification of vaccine or challenge virus infection in the cultured cells. The addition of AZT (5uM) (Sigma) to the PBMC upon plating, and its replenishment every two days, abrogated virus production and eliminated the observed background lymphoproliferation (data not shown). Cell viability assays with trypan blue exclusion and MTT (Roche) staining indicated there were no deleterious effects of AZT on PBMC viability. Pokeweed Mitogen (PWM) and acetone-extracted EIAV (AE-EIAV) were used as positive proliferative controls throughout the assays at 2.5ug/ml and 10ug/ml respectively. The peptide pools were used at 20ug/ml, and individual peptides were used at 10ug/ml. DMSO was added to the media control wells to match the DMSO concentration in the peptide wells. The PBMC were incubated with PWM for 48 hours, acetone-extracted EIAV for four days, and the peptides for six days prior to labeling with 0.75uCi [$^3$H]thymidine (Amersham Biosciences). The cells were incubated an additional 16-18 hours with [$^3$H]thymidine before being harvested and quantified for $^3$H incorporation by liquid scintillation counting. Stimulation indices (SI) were calculated by dividing the mean counts per minute (c.p.m.) of stimulated cells by the mean c.p.m. of non- stimulated cells. A positive response to the peptide pools was SI>1. A positive response to the individual peptides was set at least 2X the naïve PBMC stimulation, SI>2.5. Reactive peptides were defined as having >2.5 SI in greater than 50% of the horses, and a p-value <0.05 in the Wilcoxon Ranked Sums Test.

### 3.2.d MHC Blocking Assay

To determine the MHC specificity of the observed peptide-specific lymphoproliferation, a blocking assay was performed using anti equine MHC I (H58A) and MHC II (EqT2) antibodies (VMRD). Preliminary blocking assays showed the optimal antibody concentration for MHC I and MHC II to be 10ug/ml. The optimal concentration for the IgG control was 5ug/ml. The antibodies were incubated with PBMC (2 x $10^6$ cells/ml) for one hour at 37°C, 5% $CO_2$ before stimulation with peptides at 20ug/ml. After one hour of peptide stimulation in the presence of antibody, the cells were washed three times with 1X PBS 1% horse serum (HS) and resuspended in 200ul of RPMI complete. A standard seven day thymidine incorporation assay was then performed as described above. Proliferation was considered MHC II-restricted when the stimulation index of the MHC II antibody treated PBMC was decreased by >50% compared to the stimulation index of PBMC treated with IgG or MHC I antibodies. Since MHC II molecules present peptides to CD4 Th cells, it can be inferred that MHC II restricted peptides cause Th proliferation.

### 3.2.e CTL assays

Standard chromium release assays previously employed for the identification of EIAV CTL epitopes in Gag and Pol were used for these studies, with minor modifications (53, 90, 91, 119, 143). PBMC from the vaccinated or infected horses were expanded *in vivo* for 7-10 days with either 2.5ug/ml PWM or 10ug/ml gradient purified EIAV in complete RPMI supplemented with 20U rhIL2 (Hoffmann-La Roche INC.). After expansion, the PWM stimulated cells were labeled with 100uCi $^{51}Cr$ ($Na^{51}CrO$) (MP) for one hour at 37°C, 5% $CO_2$. Cells were washed

four times with 1X PBS 1% HS and 30,000 cells/well were plated prior to peptide pulsing. Peptide pools were used at a final concentration of 20ug/ml, and individual peptides were used at a final concentration of 10ug/ml. The target cells were pulsed for one hour at 37°C, 5% $CO_2$. EIAV-stimulated cells were used as effector cells at a 20:1 E:T ratio. After the effector cell addition, the cells were incubated 12-16 hrs prior to being harvested. Following this incubation, 25ul of cell supernatant was added to 175ul of OptiPhase SuperMix scintillation fluid (Perkin Elmer) and analyzed for $^{51}$Cr release with a MicroBeta reader (Perkin Elmer). Maximum $^{51}$Cr release was determined by plating $^{51}$Cr-labeled target cells with the non-ionic detergent Nonidet P-40 to lyse cells. Background spontaneous lysis was determined by plating $^{51}$Cr-labeled target cells with 0.1ml of medium alone. Percent specific lysis was calculated as follow:

($^{51}$Cr release in peptide wells - spontaneous $^{51}$Cr release) x 100/ ($^{51}$Cr release by NP-40- spontaneous $^{51}$Cr release) = % Specific lysis

Reactive peptides were defined as causing >10% specific lysis in >50% of the reactive horses and have a p-value <0.05 in the Mann-Whitney Test.

## 3.3 RESULTS

### 3.3.a Experimental subjects

The 12 vaccinated horses were subjects of a vaccine trial to test the efficacy of an attenuated vaccine strain, $EIAV_{D9}$, containing an engineered nine-base pair deletion and two termination codons in the viral S2 gene. Each vaccinated horse seroconverted by 35 days post infection, and Env-specific serological assays performed on longitudinal serum samples confirmed the

establishment of mature immune responses to the vaccine strain of virus by six months post-infection (31). Before the horses were challenged with the reference virulent $EIAV_{PV}$ strain, whole blood was drawn, and PBMC were isolated for use in cellular immune assays. All 12 horses were protected from virulent virus challenge as evidenced by lack of EIA signs and detectable challenge virus in plasma. Two naive horses were experimentally infected with $EIAV_{PV}$ to serve as infection and virulence controls for the $EIAV_{PV}$ challenge. PBMC from these $EIAV_{PV}$-infected horses were isolated after at least six months post infection, when mature EIAV Env-specific immune responses were documented. PBMC from five naïve horses were included in the lymphoproliferation assays to establish appropriate background levels.

### 3.3.b Proliferation in response to the synthetic peptide pools

Preliminary proliferation assays were performed using PWM or AE-EIAV as stimulants in a standard thymidine incorporation lymphoproliferation assay. All the horses responded well to PWM, with stimulation indices (SI) above 50 (Table 3.1). In response to AE-EIAV, all horses displayed a positive proliferation with SI values ranging from 3-140, presumably reflecting the variation in virus-specific immune responses among this outbred population of horses (Table 3.1). Proliferation in response to PWM and AE-EIAV demonstrated that the PBMC maintained proliferative capabilities following cryopreservation and thawing. Based on these preliminary observations, the PWM and AE-EIAV were used as positive controls for lymphoproliferation in parallel assays to the peptide stimulations.

**Table 3.1 Preliminary cellular data from vaccinated and infected horses.**

| | | | MHC I | MHC II | | Stimulation Indices (‡) | | % Specific Lysis (§) |
|---|---|---|---|---|---|---|---|---|
| | Horse (*) | Age | ELA-A (†) | DRA | DQA | PWM | AE EIAV | Envelope Pool |
| D9 Vaccinated Horses | C13 | yearling | A2/A3 | 101 | 301/401 | 178 +/- 91 | 12 +/- 3 | 9 +/- 7 |
| | C15 | yearling | A2/W11 | 101/301 | 301 | 132 +/- 34 | 3 +/- 1 | 5 +/- 7 |
| | C55 | 4 years | A4/A7 | 101/201 | 201/1301 | 67 +/- 13 | 19 +/- 14 | 18 +/- 3 |
| | B81 | 10 years | A1/A11 | ND(‖) | ND | 105 +/- 36 | 10 +/- 4 | 15 +/- 7 |
| | C9 | yearling | A3/A5 | 301/501 | 301/1301 | 136 +/- 46 | 3 +/- 2 | negative |
| | C16 | yearling | A1/A3 | 301/501 | 301/1301 | 176 +/- 18 | 43 +/- 15 | 11 +/- 8 |
| | C66 | 3-4 years | A2/A5 | ND | ND | 53 +/- 23 | 20 +/- 5 | 15 +/- 12 |
| | C62 | 19 years | A2/W11 | 101/301 | 201/1301 | 161 +/- 25 | 12 +/- 6 | 26+/- 15 |
| | C22 | yearling | A2/A8 | 201/301 | 201/701 | 163 +/- 83 | 24 +/- 11 | 11 +/- 5 |
| | C23 | yearling | W11/A3 | ND | ND | 294 +/- 81 | 18 +/- 4 | 1.3 +/- 1.5 |
| | B61 | 5 years | A2/A10 | 101 | 301/501 | 134 +/- 15 | 6 +/- 2 | 4 +/- 2 |
| | 266 | 10 years | A2/A3 | 101 | 401 | 283 /- 9 | 7 +/- 5 | negative |
| PV Challenged Horses | D64 | 6 years | A2/A9 | 101 | 501 | 156 +/- 70 | 27 +/- 12 | 2 +/- 0 |
| | D58 | 3 years | A2/A5 | 101/301 | 101/301 | 135 +/- 40 | 133 +/- 80 | negative |

(*) Horses were vaccinated with the live attenuated D9 vaccine. PBMC were obtained from each vaccinated horse at seven months post-vaccination. Challenged horses were infected with $EIAV_{PV}$ and PBMC were obtained after 13 months post-challenge.

(†) Horses were ELA-A typed at the University of Kentucky Equine Blood Typing Laboratory to establish the heterogenity of the group.

(‡) PWM and AE EIAV were used to stimulate cells in the thymidine incorporation assay. Stimulation indices are reported as the mean +/- SD.

(§) A pool of gp90 peptides was used to pulse target cells in the chromium release assay. Specific lysis is reported as the mean +/- SD.

(‖) There were not sufficient PBMC to determine the haplotype

An inclusive scan of all potential T-helper peptides was conducted using a peptide matrix strategy. Eighty-five peptides (20-mers overlapping by 10 amino acids), encompassing the $EIAV_{UK}$ Env proteins gp90 (SU) and gp45 (TM), were synthesized and used in pools of 9-10 peptides (Figure 3.1A) to stimulate PBMC from the vaccinated and infected horses in a standard thymidine incorporation assay and also to label target cells for the chromium release assay. Figure 3.1B is a representative graph of the matrix results for the lymphoproliferation assay. An SI>1.0 was used as the cutoff for determining positive proliferation.

A

| | I | II | III | IV | V | VI | VII | VIII | IX |
|---|---|---|---|---|---|---|---|---|---|
| A | 1 | 3 | 5 | 7 | 9 | 11 | 13 | 15 | 17 |
| B | 19 | 21 | 23 | 25 | 27 | 29 | 31 | 33 | 35 |
| C | 37 | 39 | 41 | 43 | 45 | 47 | 49 | 51 | 53 |
| D | 55 | 57 | 59 | 61 | 63 | 65 | 67 | 69 | 71 |
| E | 73 | 75 | 77 | 79 | 81 | 83 | 85 | 2 | 4 |
| F | 6 | 8 | 10 | 12 | 14 | 16 | 18 | 20 | 22 |
| G | 24 | 26 | 28 | 30 | 32 | 34 | 36 | 38 | 40 |
| H | 42 | 44 | 46 | 48 | 50 | 52 | 54 | 56 | 58 |
| I | 60 | 62 | 64 | 66 | 68 | 70 | 72 | 74 | 76 |
| J | 78 | 80 | 82 | 84 | | | | | |

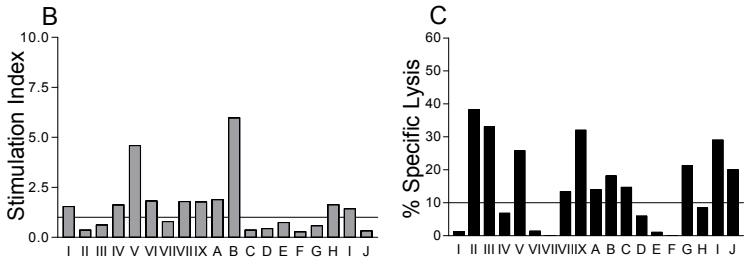


**Figure 3.1 Proliferation and CTL lysis with EIAV Env peptide matrix. A.** A panel of sequential overlapping Env 20-mer peptides encompassing gp90 and gp45 were synthesized and pooled into a matrix format. **B.** Representative graph of the reactivity of individual matrix pools in stimulating PBMC for the lymphoproliferation assay. A proliferative response to the peptide pools is defined as an SI>1, as indicated by the solid line on the graph. **C.** Representative graph of the cytolytic reactivity of individual matrix pools in labeling target PBMC in the chromium release assay. A positive CTL response to the peptide pools was defined as Specific Lysis >10%, as indicated by the solid line.

Two independent assays were conducted on PBMC from each horse for a total of 28 assays. If a peptide was identified as positive (SI>1) in eight out of the 28 assays (~30%), the peptide was subsequently tested individually to determine peptide-specific proliferative properties (Table 3.2). Based on these criteria, 21 peptides were identified and tested individually (Table 3.2). An additional five peptides were also chosen (#23, #60, #73, #74 and

#77) because the corresponding peptide pools produced high stimulation indices in PBMC from several horses, but not in the minimum of eight horses.

**Table 3.2 $EIAV_{UK}$ peptides and peptide matrix results.**

| | | Matrix Hits(†) | |
|---|---|---|---|
| GP90 Peptides | Peptide Sequence(*) | Proliferation(‡) | CTL(§) |
| 1 | MVSIAFYGGIPGGISTPITQ | **9** | 1 |
| 2 | PGGISTPITQQSEKSKCEEN | 4 | 1 |
| 3 | QSEKSKCEENTMFQPYCYNN | 3 | **4** |
| 4 | TMFQPYCYNNDSKNSMAESK | **8** | 2 |
| 5 | DSKNSMAESKEARDQEMNLK | 6 | **4** |
| 6 | EARDQEMNLKEESKEEKRRN | 5 | 1 |
| 7 | EESKEEKRRNDWWKIGMFLL | **12** | 3 |
| 8 | DWWKIGMFLLCLAGTTGGIL | 2 | 2 |
| 9 | CLAGTTGGILWWYEGLPQQH | **12** | **4** |
| 10 | WWYEGLPQQHYIGLVAIGGR | 4 | 2 |
| 11 | YIGLVAIGGRLNGSGQSNAI | **10** | 3 |
| 12 | LNGSGQSNAIECWGSFPGCR | 5 | 2 |
| 13 | ECWGSFPGCRPFQNYFSYET | 4 | 3 |
| 14 | PFQNYFSYETNRSMHMDNNT | 5 | 2 |
| 15 | NRSMHMDNNTATLLEAYHRE | 6 | 2 |
| 16 | ATLLEAYHREITFIYKSSCT | 5 | 2 |
| 17 | ITFIYKSSCTDSDHCQEYQC | **10** | **4** |
| 18 | DSDHCQEYQCKKVNLNSSDS | 3 | 2 |
| 19 | KKVNLNSSDSSNPVRVEDVM | **9** | 1 |
| 20 | SNPVRVEDVMNTTEYWGFKW | 3 | 1 |
| 21 | NTTEYWGFKWLECNQTENFK | 4 | **4** |
| 22 | LECNQTENFKTILVPENEMV | 4 | 2 |
| 23 | TILVPENEMVNINDTDTWIP | 6 | **4** |
| 24 | NINDTDTWIPKGCNETWARV | 2 | 1 |
| 25 | KGCNETWARVKRCPIDILYG | **15** | 3 |
| 26 | KRCPIDILYGIHPIRLCVQP | 1 | **4** |
| 27 | IHPIRLCVQPPFFLVQEKGI | **17** | **4** |
| 28 | PFFLVQEKGIANTSRIGNCG | 2 | **4** |
| 29 | ANTSRIGNCGPTIFLGVLED | **14** | 3 |
| 30 | PTIFLGVLEDNKGVVRGNYT | 2 | 3 |
| 31 | NKGVVRGNYTACNVSRLKIN | 4 | 3 |
| 32 | ACNVSRLKINRKDYTGIYQV | 2 | **4** |
| 33 | RKDYTGIYQVPIFYTCNFTN | 6 | 2 |
| 34 | PIFYTCNFTNITSCNNEPII | 3 | 3 |
| 35 | ITSCNNEPIISVIMYETNQV | **14** | **4** |
| 36 | SVIMYETNQVQYLLCNNNNS | 2 | 3 |
| 37 | QYLLCNNNNSNNYNCVVQSF | 2 | 1 |
| 38 | NNYNCVVQSFGVIGQAHLEL | 2 | 2 |
| 39 | GVIGQAHLELPRPNKRIRNQ | 1 | **4** |
| 40 | PRPNKRIRNQSFNQYNCSIN | 2 | **4** |
| 41 | SFNQYNCSINNKTELETWKL | 2 | **4** |
| 42 | NKTELETWKLVKTSGITPLP | 7 | 1 |
| 43 | VKTSGITPLPISSEANTGLI | 2 | 3 |
| 44 | ISSEANTGLIRHKRDFGISA | 3 | 3 |

| | | Matrix Hits(†) | |
|---|---|---|---|
| GP45 Peptides | Peptide Sequence(*) | Proliferation(‡) | CTL(§) |
| 45 | RHKRDFGISAIVAAIVAATA | 2 | **4** |
| 46 | IVAAIVAATAIAASATMSYV | 4 | 3 |
| 47 | IAASATMSYVALTEVNKIME | 3 | 3 |
| 48 | ALTEVNKIMEVQNHTFEVEN | **10** | 3 |
| 49 | VQNHTFEVENSTLNGMDLIE | 0 | 3 |
| 50 | STLNGMDLIERQIKILYAMI | **10** | 3 |
| 51 | RQIKILYAMILQTHADVQLL | 0 | 2 |
| 52 | LQTHADVQLLKERQQVEETF | 7 | 3 |
| 53 | KERQQVEETFNLIGCIERTH | 1 | **4** |
| 54 | NLIGCIERTHVFCHTGHPWN | 4 | 3 |
| 55 | VFCHTGHPWNMSWGHLNEST | 4 | 1 |
| 56 | MSWGHLNESTQWDDWVSKME | 7 | 1 |
| 57 | QWDDWVSKMEDLNQEILTIL | 1 | 3 |
| 58 | DLNQEILTILHGARNNLAQS | **11** | 3 |
| 59 | HGARNNLAQSMITFNTPDSI | 4 | 3 |
| 60 | MITFNTPDSIAQFGKDLWSH | 7 | 1 |
| 61 | AQFGKDLWSHIGNWIPGLGA | 4 | 3 |
| 62 | IGNWIPGLGASIIKYIVMFL | 2 | **4** |
| 63 | SIIKYIVMFLLIYLLLTSSP | 4 | 3 |
| 64 | LIYLLLTSSPKILRALWKVT | 4 | **4** |
| 65 | KILRALWKVTSGAGSSGSRY | 4 | 3 |
| 66 | SGAGSSGSRYLKKKFHHKHA | **11** | 3 |
| 67 | LKKKFHHKHASREDTWDQAQ | 2 | 3 |
| 68 | SREDTWDQAQHNIHLAGVTG | **11** | **4** |
| 69 | HNIHLAGVTGGSGDKYYKQK | 2 | 1 |
| 70 | GSGDKYYKQKCSRNDWNGES | **9** | 3 |
| 71 | CSRNDWNGESEEYNRRPKSW | 3 | 3 |
| 72 | EEYNRRPKSWVKSIEAFGES | 4 | 3 |
| 73 | VKSIEAFGESYISEKTKGEI | 6 | 1 |
| 74 | YISEKTKGEISQPGAAINEH | 6 | 2 |
| 75 | SQPGAAINEHKNGSGGNNPH | 2 | 2 |
| 76 | KNGSGGNNPHQGSLDLEIRS | **11** | **4** |
| 77 | QGSLDLEIRSEGGNIYDCCI | 5 | 2 |
| 78 | EGGNIYDCCIKAQEGTLAIP | 3 | 1 |
| 79 | KAQEGTLAIPCCGFPLWLFW | **9** | 2 |
| 80 | CCGFPLWLFWGLVIIVGRIA | 1 | 3 |
| 81 | GLVIIVGRIAGYGLRGLAVI | **8** | 2 |
| 82 | GYGLRGLAVIIRICIRGLNL | 3 | 3 |
| 83 | IRICIRGLNLIFEIIRKMLD | **10** | 2 |
| 84 | IFEIIRKMLDYIGRALNPGT | 5 | 2 |
| 85 | YIGRALNPGTSHVSMPQYV | 3 | 2 |

(*) 20-mer peptides overlapping by 10 amino acids were synthesized by the Biomedical Research Support Facilities Peptide Synthesis Core of the University of Pittsburgh.

(†) Matrix Hits sums the number of times that a peptide was indicated in the cellular assays using the matrix pools.

(‡) For the proliferation assay, a peptide had to be indicated eight or more times (shaded) to be considered for individual ananlysis.

(§) For the CTL assay, a peptide had to be indicated four times (shaded) to be considered for individual analysis.

### 3.3.c Peptide-specific lymphoproliferation

To clearly delineate broadly reactive peptides from non-reactive peptides, the 26 peptides identified from the peptide matrix assays were used to stimulate the PBMC from the same panel of vaccinated and infected horses. Background proliferation in response to these peptides was established by stimulating PBMC from five naïve horses with the 26 peptides. The average background stimulation index was $\leq$ 0.9. We defined a reactive peptide as 1) having a stimulation index >2.5 (greater than two times the background) in seven of the 14 horses and 2) having a p-value < 0.05 in the Wilcoxon Ranked Sums Test.

Overall, the PBMC from the vaccinated and infected horses proliferated in response to a variety of peptides (Figure 3.2). Of the 26 tested peptides, 23 peptides were positive for proliferation in seven of the 14 horses (Figure 3.3A). Only peptides #17, #48 and #58 did not cause proliferation in 50% of the horses, and were therefore not considered reactive. For the 23 peptides that were positive for proliferation in $\geq$50% of the horses, the Wilcoxon Ranked Sums Test was used to establish the statistical significance of the SI values. This test takes into consideration the number of positive events and also the magnitude of stimulation. The ranks 1, 2 and 3 were defined as SI of 2.5-5, 5-7.5 and >7.5, respectively (Figure 3.2). All 23 peptides that caused stimulation in seven of the 14 horses also had p-values < 0.05, and were thus considered to be reactive (Figure 3.3A).

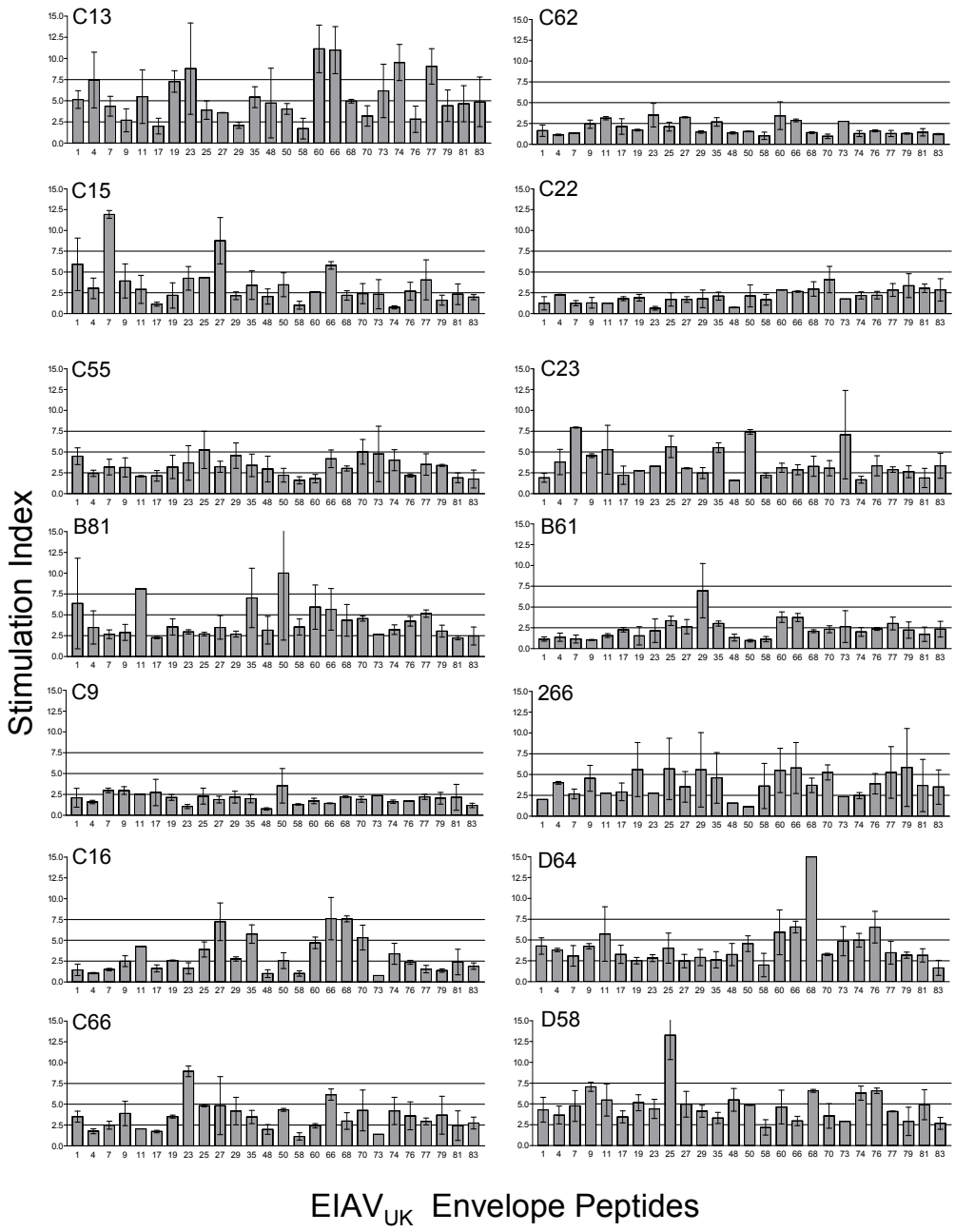


**Figure 3.2 Proliferation with individual Env peptides.** Individual peptides indicated by the peptide matrix pool analysis (Table 3.2) were assayed in a standard thymidine incorporation assay against PBMC isolated from the 14 horses. A proliferative response to the individual peptides is defined as an SI > 2.5. Graphs are averages of three individual assays. The ranks 1, 2 and 3 were defined as a SI 2.5-5, 5-7.5, >7.5, respectively.

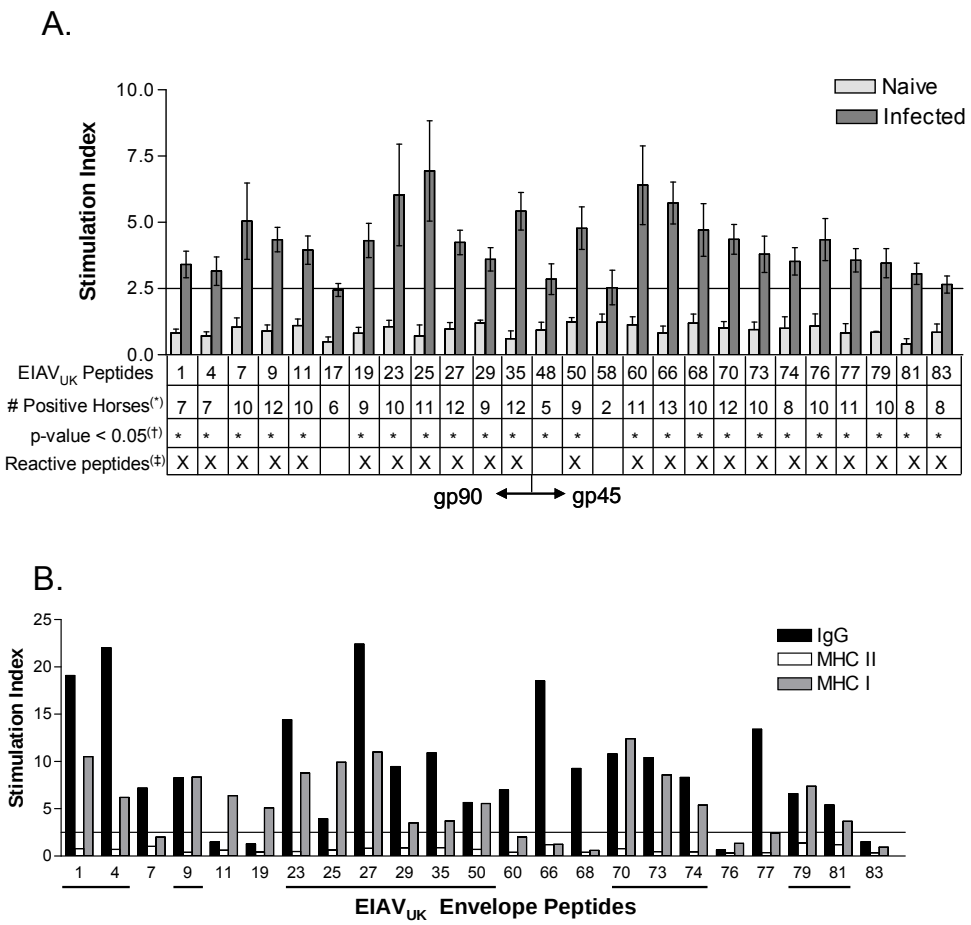


**Figure 3.3 Identification and characterization of reactive T-Helper peptides.** **A.** A panel of five naïve horses was used to establish background proliferation with the 26 peptides. The average background was a stimulation index of 0.9. (*) Individual horses were positive for the peptide if the peptide caused a SI > 2.5. (†) The p-value was determined using the Wilcoxon Ranked Sums Test. (‡) Reactive peptides were defined by two criteria: 1) a peptide causing proliferation in seven of the 14 horses tested and 2) the peptide having a p-value < 0.05 in the Wilcoxon Ranked Sums Test. **B. MHC blocking assay.** PBMC were either blocked with MHC I, MHC II or IgG antibody prior to stimulation with the broadly reactive peptides. A decrease in stimulation >50% compared to the stimulation index of PBMC treated with IgG or MHC I antibody is considered significant. CD4 restricted peptides are underlined.

### 3.3.d T-Cell specificity of peptide specific lymphoproliferation

Since Th cells recognize peptides presented by MHC II receptors, blocking assays were performed to determine if the peptides were CD4-dependent. Horse D64 was chosen for the depletion assay because PBMC from this horse responded well in the previous proliferation assays. Monoclonal antibodies specific for MHC I or MHC II antigens or control IgG were incubated with the cells prior to stimulation with the peptides. A standard seven-day proliferation assay with $^{3}$H-thymidine was then performed to determine the effects of the antibodies on peptide stimulation. A 50% decrease in SI of the cells incubated with the MHC II-specific antibody compared to the SI of cells incubated with the MHC I-specific or control IgG antibody indicated that the peptide was MHC II-restricted, indicating the peptide is CD4-dependent. Fourteen of the 23 peptides were determined to be CD4-restricted with this assay (Figure 3.3B). Five of the peptides demonstrated a pattern that indicated both CD4 and CD8 cells were proliferating in response to those peptides. Peptides #7, #60, #66, #68, and #77 all decreased in SI when MHC I or MHC II antibodies were used. Further studies are needed to verify the results. Peptides #11, #19, #76 and #83 failed to produce significant stimulation in this assay; therefore, we were unable to determine the CD4/CD8 restriction of these peptides (Figure 3.3B).

### 3.3.e Cytolytic response to the synthetic peptide pools

Preliminary CTL assays were conducted to determine the cytolytic capabilities of the PBMC from the panel of vaccinated and infected horses against a pool of sequential Env 20-mer peptides representing the entire gp90 protein sequence. PBMC from the 12 vaccinated horses

displayed cytolytic activity to the envelope peptide pool with specific lysis values ranging from 0-30% (Table 3.1). The PBMC samples from the two infected horses failed to display significant CTL activity against the Env peptide pool at the six month post infection time point.

To identify reactive CTL peptide peptides, the envelope peptide pool matrix was again utilized to screen CTL activity of PBMC from four of the vaccinated horses. Figure 3.1C is a representative graph of the matrix results for the CTL assay. Four horses were used to identify potentially positive peptides. The cutoff for a positive cytolytic response was set at 10% specific lysis, and each peptide had to cause lysis in all four assays to be tested individually. With these criteria, 20 peptides were chosen for further analysis as individual peptides (Table 3.2). Additionally, peptides #50, #52 and #54 were chosen because they caused high specific lysis in three of the four CTL assays.

### 3.3.f Peptide specific CTL responses

To determine which peptides were recognized by CTL cells, the 23 peptides identified with the peptide matrix were tested individually in chromium release assays (Figure 3.4). Of the 12 vaccinated horse PBMC samples, eight had clear CTL activity against individual envelope peptides. PBMC from horses C15, 266 and C9 did not have detectable CTL to the individual peptides at the day of challenge time point, and were therefore not used to determine which peptides were reactive. Due to insufficient B61 PBMC, this horse was also excluded from determining reactive peptides. Specific lysis of PBMC from untreated and non reactive horses was typically < 2.0%, so a 10% specific lysis cutoff was set for determining which peptides were positive.

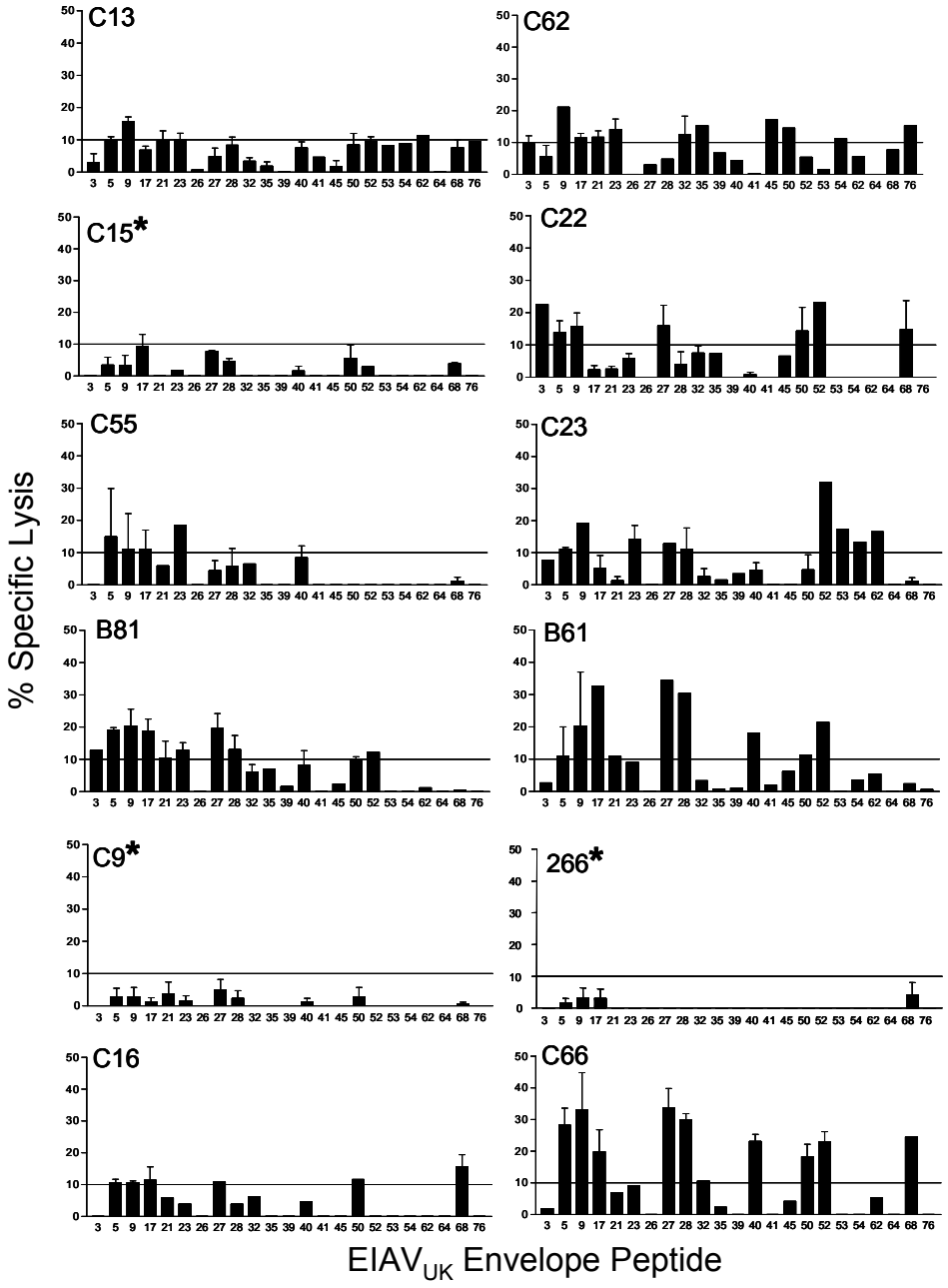


**Figure 3.4 CTL lysis with individual peptides.** Individual reactive peptides identified in the matrix assay (Table 3.2), were used to assay cytolytic activity of PBMC from the 12 vaccinated horses in a standard chromium release assay. A specific lysis >10% was considered positive. Graphs are averages of three individual assays. * indicate horses that were used as non-reactive horses for determining broadly reactive peptides.

The eight horses that responded to the peptides were used to determine which peptides were reactive. For a peptide to be considered reactive, it had to 1) cause 10% specific lysis in four of the eight responder horses, and 2) have a p-value < 0.05 in the Mann-Whitney test. Of the 23 peptides tested, only seven peptides (#5, #9, #17, #23, #27, #50 and #52) caused >10% specific lysis in four or more of the reactive horses (Figure 3.5). The remaining 16 peptides had less than 5% specific lysis in this cohort of horses. The Mann-Whitney test was used to determine the significance of the specific lysis in the responder horses compared to non responder horses. Peptides #5, #9, #17, #23, #27, #50 and #52 had p-values < 0.05 (Figure 3.5), and were subsequently considered to be reactive. Although peptide #28 did have a p-value < 0.05, it was not considered reactive because only three horses responded to that peptide.

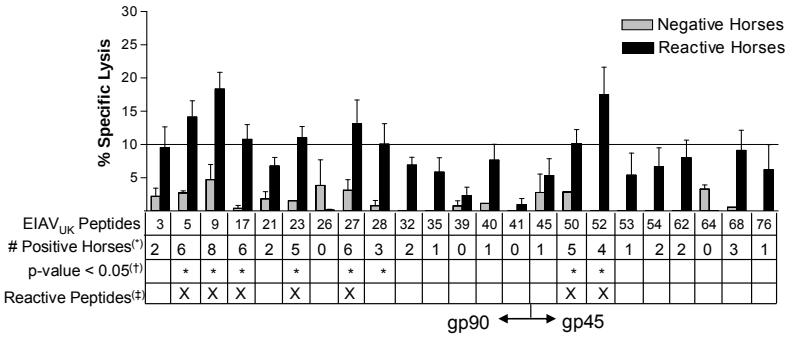


**Figure 3.5 Identification of reactive CTL peptides.** The reactive CTL peptides were identified using a panel of eight vaccinated horses that demonstrated detectable levels of CTL activity. The remaining three non responding horses were used to establish the background specific lysis for the peptides. (*) Individual horses were positive for the peptide if the peptide caused specific lysis >10%. (†) The p-value was determined using the Mann-Whitney test. (‡) Reactive peptides were defined by two criteria: 1) a peptide causing significant specific lysis in four of the eight reactive horses and 2) a peptide having a p-value < 0.05 in the Mann-Whitney test.

## 3.4 DISCUSSION

The goal of the current study was to use synthetic peptide methodologies to characterize in detail the specificity of EIAV envelope-specific cellular immune responses associated with the protective immunity produced in horses inoculated with an attenuated viral vaccine (12 horses) and in asymptomatic carrier horses (two horses). The results of these comprehensive analyses reveal for the first time the overall specificity of Th and CTL responses to EIAV envelope that are recognized in horses with protective immunity. These data provide a necessary foundation to examine the evolution of cellular immune responses associated with the progressive maturation of host immunity to a protective stage, to evaluate the role of specific cellular epitopes in the control of EIAV infection, and to evaluate experimental Env-based vaccines designed to optimize the immunogenicity of these reactive cellular immune epitopes. In this regard, these studies will provide an important complement to similar studies by McGuire and colleagues that have focused on the nature and protective role of cellular immune responses to EIAV Gag proteins (22, 45, 46, 93, 94).

For a peptide to be considered broadly reactive, the peptide must be presented by the majority of expressed MHC alleles. Two MHC II loci, which present peptides to Th cells, were typed in this study, and nine of the eleven typed horses had distinct DRA/DQA combinations. C9 and C16 both shared DRA*101/DRA*301 and DQA*301/DQA*1301, but still differed in their proliferative response to the individual peptides. Therefore, it can be reasoned that they differ at another MHC II locus. Allelic restriction at the less polymorphic DRA allele is not evident with the 23 reactive peptides. At the more polymorphic DQA allele, seven different haplotypes were observed. The dominant allele of the horses typed was DQA*301, with six of the eleven horses expressing that allele. None of the 23 reactive peptides look to be DQA*301

restricted as horses with and without DQA*301 reacted to the same peptides. Peptides 29, 68 and 74 appear not to be presented by DQA*301, but seem to be widely presented by the remaining alleles. The next dominant allele was DQA*1301, which was expressed in four of the eleven horses typed. Peptides 76 and 83 are the only two peptides excluded from Th recognition in animals expressing DQA*1301. On closer examination it is found that only horses with DQA*301, 401 and 501 can recognize peptide 76; and horses expressing DQA*101, 401 and 701 can recognize peptide 83. With less than half the expressed DQA alleles recognizing these two peptides, they are not broadly reactive. While peptides 79 and 81 are not presented by the dominant DQA*301 and DQA*1301, they are considered to be broadly reactive because they seem to be presented by the remaining five alleles. Peptides 7, 9, 11, 19, 23, 25, 27, 35, 60, 66, 70 and 77 appear to be presented by four or more (>50%) of the DQA alleles, and are thereby broadly reactive. Of the 23 identified reactive peptides, it appears that 17 are broadly reactive across a majority of DQA haplotypes (Figure 3.6).

CTL cells recognize peptides presented by MHC I molecules. Only the ELA-A locus has been defined in horses, and it is less polymorphic than the human counterpart. The lack of defined ELA-A types may be due to the serological reagents used in the typing process (21). Additionally, there are problems with serological typing such as allele mismatching. It was reported that target cells from one horse with an ELA-A5 haplotype were not lysed by memory CTL from another horse with the ELA-A5 haplotype, indicating that the serological reagents used for haplotyping can non-specifically recognize more alleles than originally thought (143). Until a more discriminating MHC I typing procedure is developed, we cannot definitively assign ELA-A allelic restriction to the defined peptides. It does appear that peptide 50 may not be broadly reactive, in that only one horse without the A2 allele recognizes that peptide. The

remaining six peptides are recognized by a range of diverse ELA-A types, and can therefore be considered broadly reactive (Figure 3.6).

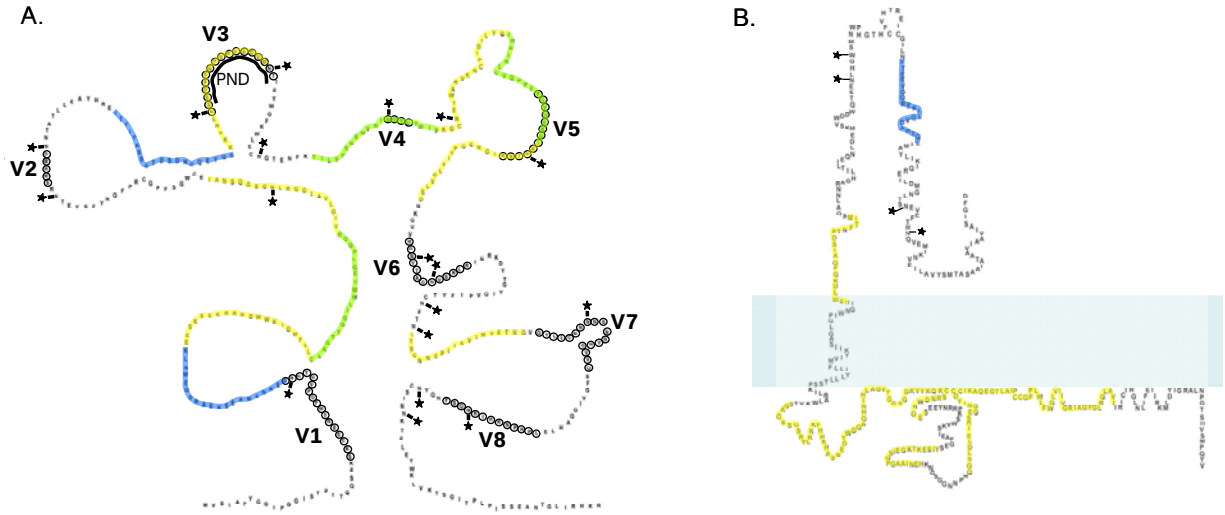


**Figure 3.6 Location of broadly reactive peptides in the Env proteins. A. gp90 protein.** Schematic model of gp90 adapted from Ball *et al* (12). Variable domains in the Env sequence are circled and labeled V1-V8. N-Linked glycosylation sites are marked with a star. The PND is the principal neutralization domain. The T-helper peptides are highlighted in yellow, and the CTL peptides are highlighted in blue. Overlapping CTL and T-helper peptides are highlighted in green. **B. gp45 protein.** Schematic model of gp90 adapted from Montelaro et *al* (97). The shaded box represents the lipid bilayer of the viral envelope differentiating the ectodomain (top) and intracytoplasmic tail (bottom) of gp45. N-linked glycosylation sites are marked with a star. The T-helper peptides are highlighted in yellow and the CTL peptides are highlighted in blue. Overlapping CTL and T-helper peptides are highlighted in green.

Of the 17 broadly reactive T-helper peptides, nine were located in the gp90 protein, and eight in the gp45 protein. Gp90, which is the more variable portion of envelope, has eight defined variable domains which are assumed to occupy the outer exposed loops on a 3D structure of the protein, like the HIV-1 gp120 protein (77). Of the nine broadly reactive T-helper peptides from the gp90 sequence, four are located in these variable domains (Figure 3.6A). Peptide 19 is in V3, peptide 23 is in V4, and peptides 27 and 29 are both in V5. Only two of the five CTL peptides in this protein are contained in variable domains. Peptide 23 is located in the V4 domain and peptide 27 in the V5 domain. The principal neutralization domain (PND), along with other major neutralization antibody determinants (V4 and V5), are located in the variable loops of gp90. These antibody determinants have considerable overlap with T-helper and/or CTL peptides. The remaining T-helper and CTL peptides are in more conserved regions of gp90 that are shielded by the variable loops and are perceived to be immunorecessive.

There are no predetermined variable domains in the gp45 protein, but it is accepted that the cytoplasmic tail is more conserved than the ectodomain. Only one T-helper peptide (peptide 60) is found in the ectodomain, along with the only CTL peptide of this protein (peptide 52). The remaining seven T-helper peptides are found in the putative intracytoplasmic tail (Figure 3.6B).

Lentiviral envelope proteins are some of the most heavily glycosylated proteins characterized to date. The role of N-linked glycosylation in antigen presentation has yet to be established. The EIAV gp90 protein has 18 predicted N-linked glycosylation sites, yet the majority of the identified broadly reactive T-helper and CTL peptides lack potential glycosylation sites. Additionally, gp45 contains four potential N-linked glycosylation sites, and none of them are included in the identified cellular peptides. These data are consistent with the

concept that lentiviruses sequester glycosylated Env sequences from cellular recognition, as observed for the SIV Env protein (123).

With the current characterization of T-helper and CTL peptides of EIAV gp90 and gp45 associated with protective immunity, we now have the necessary foundation to examine the evolution of these cellular immune responses during maturation to protective immunity, and to evaluate these peptides as correlates of protective vaccine immunity.

## 3.5 ACKNOWLEDGMENTS

I would like to thank Jodi Craigo for organizing the vaccine trial and Timothy Sturgeon for technical assistance with the development of the CTL assay. Additionally, I would like to thank Sheila Cook and Chuck Issel at the University of Kentucky for performing all of the animal procedures.

# 4.0 ENVELOPE-SPECIFIC TH AND CTL DETERMINANTS OF EIAV VACCINE PROTCTION FROM DISEASE

This chapter was modified from:

Tara L. Tagmyer, Jodi K. Craigo, Sheila J. Cook, Deborah L. Even, Charles J. Issel, Ronald C. Montelaro. Determinants of EIAV vaccine protection and the effects of sequence variation on immune recognition. Submitted to Journal of Virology.

## 4.1 INTRODUCTION

*Equine infectious anemia virus* (EIAV) is a macrophage tropic, equine lentivirus that has been used extensively as a model for HIV-1 persistence and pathogenesis and for AIDS vaccine development (15, 36, 52, 54, 59, 76, 97-99, 108). Compared to other progressively degenerative lentiviral infections, EIAV is characterized by three distinct phases of infection: acute, chronic and inapparent. By two months post EIAV exposure, most horses experience an acute disease episode characterized by high fever, drop in platelets, and high viral load. The horse then enters a chronic stage of infection characterized by recurrent disease episodes, which typically progress to the inapparent carrier stage of disease for the lifespan of the animal (97). During the inapparent phase, the horse has gained immunologic control of the virulent and constantly evolving lentivirus, as demonstrated by the fact that whole blood transfers from inapparent carriers to naïve horses can cause an acute episode within two months (64, 89, 97). Additionally, if the inapparent carrier is immune suppressed or stressed, there can be recrudescing disease associated with a new viral quasispecies. Inapparent carriers of EIAV appear to be resistant to additional exposure to EIAV variant strains, indicating the horse has gained a high level of prophylactic immunity (97). This type of immunity is especially desirable for lentiviral vaccines, which makes EIAV a valuable model system for studying vaccine protection.

Immune control of acute EIAV viremia has typically been associated with the appearance of cytotoxic T lymphocytes (CTL) (90) and non-neutralizing antibodies (90, 109, 122). Recently, virus-specific neutralizing antibodies have been found to develop after the resolution of the acute episode (93), and continue to increase in titer and breadth of specificity throughout the first year of infection (12, 52, 60). We have previously identified V3 and V4 of the surface

envelope (Env) glycoprotein (gp90) as major neutralization epitopes, with V3 containing the principal neutralization domain (PND) of EIAV (12, 60, 76, 77). However, there has been only limited characterization of cellular immune responses to EIAV Env that are associated with protective immunity. McGuire and colleagues have thoroughly examined Gag and Pol specific T-helper (Th) and CTL responses (22, 46, 93, 94), but vaccines based on these broadly reactive immune determinants has failed to elicit a protective immune response against EIAV challenge (45).

Previous work with EIAV, SIV, and SHIV has shown there is a progressive maturation of virus-specific immunity in response to these various attenuated lentiviral vaccines (23-25, 54). A comparison of quantitative and qualitative lentiviral Env-specific antibodies reveals a correlation between an immature immune response with non-protection or enhancement, and a mature immune response with protective vaccine immunity. Based on these earlier studies, we have proposed that EIAV Env is a primary determinant of vaccine efficacy and that an effective vaccine must be able to elicit broad humoral and cellular immune responses to conserved Env determinants.

Previously, we described a highly effective attenuated EIAV vaccine ($EIAV_{D9}$) that protects 100% of horses from disease by homologous virulent challenge (32). In that study, we were able to identify regions of EIAV gp90 and gp45 envelope proteins that were broadly recognized by CTL and Th cells from protected horses with diverse MHC haplotypes (132). It has always been assumed that Env variation poses a major obstacle to lentiviral vaccine protection; therefore, vaccine strategies have focused on the more conserved Gag proteins to elicit protective immune responses. To directly examine the role of Env variation on vaccine efficacy, 24 ponies were vaccinated with the highly effective $EIAV_{D9}$ vaccine (32, 36). Seven

months post vaccination, the ponies were challenged with either a homologous Env strain (EV0), or variant Env strains with 6% (EV6) or 13% (EV13) divergence from the homologous Env. During the 120 day observation period, one EV0, three EV6, and five EV13 ponies developed signs of equine infectious anemia (EIA), clearly revealing an inverse correlation between protection from disease and divergence from the homologous Env (36). In light of the marked differences in vaccine protection observed against the variant Env challenge viruses, we hypothesized that analyses of vaccine immunity to the variant Env proteins could reveal important correlates of vaccine protection. Thus, the goal of the current study was to use this panel of experimentally immunized ponies displaying different levels of vaccine protection to evaluate potential immune correlates of protective immunity by in depth analyses of vaccine induced Env-specific neutralizing antibody responses, lymphoproliferative responses, and cellular cytotoxicity responses.

## 4.2 MATERIALS AND METHODS

### 4.2.a Experimental vaccinations and challenge

All equine procedures were conducted at the Gluck Equine Research Center of the University of Kentucky according to protocols approved by the University of Kentucky IACUC. Twenty-four ponies were vaccinated with the previously described attenuated $EIAV_{D9}$ vaccine (32, 36, 79). Serological typing of the MHC I locus was performed as previously described (9, 10). Direct sequencing of the DRA and DQA MHC II loci was performed using previously published protocols (Table 4.1) (4, 44).

**Table 4.1 Summary of $EIAV_{D9}$ vaccinated ponies.**

| | Pony | Sex | Age | MHC I | MHCII | | Acute Disease |
|---|---|---|---|---|---|---|---|
| | | | | | DRA | DQA | |
| **EV0 Challenged** | A27 | F | 7 | A9/W11 | 101/101 | ND | |
| | C27 | F | 5 | W11/W11 | 101/JBH11 | 301/1501/1301[b] | |
| | C28 | F | 5 | A10/A10 | 101/101 | 301/1301 | 49 dpc |
| | C34 | F | 5 | A8/A10 | 101/201 | 201/201 | |
| | E31 | M | 3 | A1/A3 | 101/101 | 301/401 | |
| | E33 | M | 3 | A9/A9 | 101/101 | 701/701 | |
| | E34 | F | 3 | A1/A9 | 101/JBH11 | 301/1201/1301 | |
| | E35 | F | 3 | A1/A1 | 201/JBH11 | 301/701 | |
| **EV6 Challenged** | 558 | M | 12 | A2/A9 | 101/301 | 301/1301 | 113dpc |
| | A32 | F | 7 | A9/A9 | 101/301 | 301/1301 | |
| | A33 | M | 7 | A9/A9 | 101/101 | 301/401/1301 | 4 dpc |
| | C29 | M | 5 | A3/A9 | 101/101 | 701/701 | |
| | C33 | F | 5 | A1/A1 | 201/JBH11 | 1001/1201 | |
| | C36 | M | 5 | A1/A1 | 101/JBH11 | 301/1201 | 47 dpc |
| | D31 | M | 4 | A2/A9 | 101/101 | 301/301 | |
| | D44 | M | 4 | A2/A9 | 101/301 | 301/1501 | |
| **EV13 Challenged** | 886 | F | 9 | A5/A6 | 101/101 | 1301/1301 | 70 dpc |
| | B22 | M | 6 | ND[a] | 101/101 | 301/401 | 14 dpc |
| | B27 | M | 6 | A5/A6 | 101/101 | 1101/1101 | 36 dpc |
| | D34 | M | 4 | A5/A9 | 201/201 | 701/1001/1301 | |
| | D38 | M | 4 | A2/A3 | 101/101 | 1501/1501 | 85 dpc |
| | D39 | M | 4 | A1/A2 | 101/201 | 201/201 | 17 dpc |
| | D40 | M | 4 | A2/A2 | 101/101 | 601/1501 | |
| | E30 | M | 3 | A9/W11 | 101/101 | 301/301 | |

[a] Due to insuffient PBMC, MHC typing was not performed

[b] Three MHC II sequences including the 1303 allele for DQA are not uncommon. See Fraiser and Bailey, 1998.

Serum and plasma were obtained from the vaccinated ponies at regular intervals throughout the trial. At seven months post vaccination, blood was drawn from each pony, and PBMC were isolated and cryopreserved in autologous serum and 10% DMSO for future use in cellular immune assays. On the following day, the vaccinated ponies (8 per group) were intravenously challenged with $10^3$ median tissue culture infectious doses ($TCID_{50}$) of EV0, EV6 or EV13 (8 ponies/group) (36). The challenge viruses were derived from the same reference proviral clone and differed only in the gp90 segment of the Env gene. The EV0 Env is homologous to $EIAV_{D9}$ vaccine strain; the EV6 and EV13 gp90 proteins diverge from EV0 by 6% and 13% in their

respective amino acid sequences. The EV0, EV6, and EV13 were shown to be neutralization distinct, each being inactivated by immune serum from ponies infected with the homologous, but not heterologous, virus strain. Temperature and platelet counts were taken for 120 days post challenge as previously described to monitor disease progression (36). During the observation period, one EV0 challenged pony, three EV6 challenged ponies, and five EV13 challenged ponies developed clinical signs of equine infectious anemia (EIA). The remaining 15 ponies were protected from disease (Table 4.1).

### 4.2.b Neutralizing antibody assay

The development of neutralizing antibodies to EV0, EV6, and EV13 at DOC and 4 weeks post challenge (4wpc) was assessed in an ELISA-based infectious center assay as previously described (52). Briefly, $10^5$ fetal equine kidney (FEK) cells were plated in 24 well plates and incubated 18-24 hours at 37°C. Two-fold dilutions of heat inactivated serum (56°C, 1 hour) from DOC and 4wpc were added to 100 infectious units of EV0, EV6, or EV13. After one hour incubation, the serum-virus supernatant was added to the FEK cells in triplicate. The following day, an overlay of 0.8% carboxymethyl cellulose was added to the cells to prevent the virus from spreading throughout the culture. The cultures were incubated an additional seven days at 37° before being fixed with 3.7% formaldehyde and permeabilized with 1% Triton X-100. A reference α-EIAV primary serum antibody was used at 1:200, and secondary antibody, horseradish peroxidase-conjugated goat anti-horse IgG (United States Biochemical Corp.) was used at 1:5000. The labeled cells were stained with 3-amino-9-ethyl-carbazole (Sigma) in a sodium acetate buffer (pH 5.5) supplemented with $H_2O_2$, and visualized with a dissecting microscope. The 50% neutralization titers of each serum sample were determined by linear

regression analysis of the log10 reciprocal dilution versus the number of apparent foci. A neutralization titer of 1:25 was used as the cutoff for a positive response.

### 4.2.c Production of variant synthetic peptides

EIAV Env specific peptides (sequential 20-mers overlapping by 10 residues) were synthesized in the Biomedical Research Support Facilities Peptide Synthesis Core of the University of Pittsburgh using an Advanced Chemtech Model 396 Omega Synthesizer. Variant specific peptides were designed using EV6 clone #567IVC8 and EV13 clone #567p10 (35, 76). Previously described UK peptides served as the EV0 peptides, since EV0 and UK have identical Env amino acid sequences (132). Seventeen EV6 specific peptides and 26 EV13 specific peptides were synthesized to account for the amino acid sequence differences with EV0 (Table 4.2). All peptides were dissolved at 2mg/ml in 100% DMSO and stored at -80°C. To construct variant specific matrix pools, the variant peptides were substituted for their EV0 counterpart and added to the conserved EV0 peptides in a matrix format as previously described (132).

**Table 4.2 Variant specific Env peptides.**

| | EV0 Peptide Sequence | EV6 Peptide Sequence | EV13 Peptide Sequence |
|---|---|---|---|
| 1 | MVSIAFYGGIPGGISTPITQ | | |
| 2 | PGGISTPITQQSEKSKCEEN | | |
| 3 | QSEKSKCEENTMFQPYCYNN | | QSEKSKCEENTIFQPYCYNN |
| 4 | TMFQPYCYNNDSKNSMAESK | | TIFQPYCYNNDSKNSMAESK |
| 5 | DSKNSMAESKEARDQEMNLK | | |
| 6 | EARDQEMNLKEESKEEKRRN | | |
| 7 | EESKEEKRRNDWWKIGMFLL | | |
| 8 | DWWKIGMFLLCLAGTTGGIL | | |
| 9 | CLAGTTGGILWWYEGLPQQH | | |
| 10 | WWYEGLPQQHYIGLVAIGGR | | |
| 11 | YIGLVAIGGRLNGSGQSNAI | | |
| 12 | LNGSGQSNAIECWGSFPGCR | | |
| 13 | ECWGSFPGCRPFQNYFSYET | | |
| 14 | PFQNYFSYETNRSMHMDNNT | PFQNYFSYETNRSMHIDNNT | PFQNYFSYETNRNIHIDNNT |
| 15 | NRSMHMDNNTATLLEAYHRE | NRSMHIDNNTATLLEAYHRE | NRNIHIDNNTATLLEAYHRE |
| 16 | ATLLEAYHREITFIYKSSCT | | |
| 17 | ITFIYKSSCTDSDHCQEYQC | | |
| 18 | DSDHCQEYQCKKVNLNSSDS | DSDHCQEYQCNKVNLNLNSF | DSDHCQEYQCKKVNFTVSKA |
| 19 | KKVNLNSSDSSNPVRVEDVM | NKVNLNLNSFDSSIHVEDVK | KKVNFTVSKANGSSIPSIHVGGVEDAE |
| 20 | SNPVRVEDVMNTTEYWGFKW | DSSIHVEDVKDTTEYWGFKW | NGSSIPSIHVGGVEDAETTIEYWGFKW |
| 21 | NTTEYWGFKWLECNQTENFK | DTTEYWGFKWLECNQTENFK | TTIEYWGFKWLECNQTENLK |
| 22 | LECNQTENFKTILVPENEMV | | LECNQTENLKTILVPENEMV |
| 23 | TILVPENEMVNINDTDTWIP | TILVPENEMVNINDNDTWIA | TILVPENEMVKIKNGTWTP |
| 24 | NINDTDTWIPKGCNETWARV | NINDNDTWIAKGCNETWARV | KIKNGTWTPKGCNETWARV |
| 25 | KGCNETWARVKRCPIDILYG | | |
| 26 | KRCPIDILYGIHPIRLCVQP | | |
| 27 | IHPIRLCVQPPFFLVQEKGI | IHPIRLCVQPPFFLVQEKEV | IHPIRLCVQPPFFLVQENRG |
| 28 | PFFLVQEKGIANTSRIGNCG | PFFLVQEKEVANTSRIGNCG | PFFLVQENRGDNIARIGNCG |
| 29 | ANTSRIGNCGPTIFLGVLED | | DNIARIGNCGPTIFLGVLED |
| 30 | PTIFLGVLEDNKGVVRGNYT | PTIFLGVLEDNKGVVRENYT | PTIFLGVLEDNKGVVRGSPT |
| 31 | NKGVVRGNYTACNVSRLKIN | NKGVVRENYTACNVSHLRIN | NKGVVRGSPTACNVRKLGIN |
| 32 | ACNVSRLKINRKDYTGIYQV | ACNVSHLRINRKDYTGIYQV | ACNVRKLGINRKDYTGIYQG |
| 33 | RKDYTGIYQVPIFYTCNFTN | | RKDYTGIYQGPMFYTCNFTS |
| 34 | PIFYTCNFTNITSCNNEPII | PIFYTCNFTNITSCNNESII | PMFYTCNFTSITSCNDESIT |
| 35 | ITSCNNEPIISVIMYETNQV | ITSCNNESIISVIMYETNQV | ITSCNDESITSVIMYETNQV |
| 36 | SVIMYETNQVQYLLCNNNNS | | SVIMYETNQVQYLLCKYNTT |
| 37 | QYLLCNNNNSNNYNCVVQSF | QYLLCNNNNSNHYNCVVQSF | QYLLCKYNTTDSNYTCVVQSF |
| 38 | NNYNCVVQSFGVIGQAHLEL | NHYNCVVQSFGVIGQAHLEL | DSNYTCVVQSFGVIGQAHLEL |
| 39 | GVIGQAHLELPRPNKRIRNQ | | GVIGQAHLELPRPNKRIMNP |
| 40 | PRPNKRIRNQSFNQYNCSIN | | PRPNKRIMNPNFNQYNCSIN |
| 41 | SFNQYNCSINNKTELETWKL | | NFNQYNCSIN NKTELETWKL |
| 42 | NKTELETWKLVKTSGITPLP | | |
| 43 | VKTSGITPLPISSEANTGLI | | |
| 44 | ISSEANTGLIRHKRDFGISA | | |

### 4.2.d Lymphoproliferation assays

DOC PBMC from the 24 vaccinated ponies were tested for recognition of EIAV envelope peptides in a standard seven-day thymidine incorporation assay, as previously described (132). Briefly, DOC PBMC from 22 of the 24 vaccinated ponies were >80% viable upon thawing and maintained viability throughout the assays, as measured by trypan blue exclusion. DOC PBMC from ponies B22 and D34 were not viable and therefore not used in further cellular assays. C36 had abnormal DOC PBMC which yielded nonspecific hyper-reactivity in the cellular assays, and therefore was excluded from this study. PBMC were plated at 2 X $10^5$ cells/well in complete RPMI-1640 (10% fetal equine serum, 1% penicillin/streptomycin, 1% L-glutamine, 55uM β-mercaptoethanol) with 5uM AZT (Sigma) and stimulated in triplicate wells with 2.5ug/ml Pokeweed Mitogen (PWM), 20ug/ml peptide matrix pools, or 10ug/ml individual peptides. The initial scan for potentially reactive peptides utilized challenge strain specific matrix pools to account for possible differences in T-cell recognition and reactivity. AZT was replenished every other day in 5ul of media. Triplicate media control wells were set up with DMSO to match the DMSO concentration in the peptide pool and peptide wells. The PBMC were incubated for 48 hours with PWM, and for six days with the peptide pools or peptides prior to labeling with 0.75uCi $^3$H-thymidine (Amersham Biosciences). After 16-18 hour incubation, the cells were harvested and quantified for $^3$H-thymidine incorporation by liquid scintillation counting. Stimulation indices (SI) were calculated by dividing the mean counts per minute (c.p.m.) of stimulated cells by the mean c.p.m. of non-stimulated cells. A positive response to the peptide matrix pools was SI>1 and SI>2.5 for individual peptides. Broadly reactive peptides were identified as peptides that caused positive proliferation in >50% of the ponies and that were presented by the majority of the MHC II molecules.

### 4.2.e CTL assays

DOC PBMC were used to assay CTL activity in a standard chromium release assay as previously described (132). Briefly, DOC PBMC from the vaccinated ponies were expanded *in vivo* for 7-10 days with either 2.5ug/ml PWM (target cells) or 10ug/ml gradient purified EIAV (effector cells) in complete RPMI supplemented with 200U rhIL2 (Hoffmann-La Roche INC.). The target cells were labeled with 100uCi $^{51}Cr$ ($Na^{51}CrO$) (MP) for one hour at 37°C, 5% $CO_2$, and washed four times with 1X PBS, 1% horse serum. About 30,000 target cells/well were pulsed with either 20ug/ml of the peptide matrix pools or 10ug/ml of individual peptides for two hours at 37°C, 5% $CO_2$. EIAV-stimulated effector cells were added at a 20:1 E:T ratio and incubated 12-16 hrs prior to being harvested. A 25ul sample of cell supernatant was added to 175ul of OptiPhase SuperMix scintillation fluid (Perkin Elmer) and analyzed for $^{51}Cr$ release with a MicroBeta reader (Perkin Elmer). Maximum $^{51}Cr$ release was determined by plating $^{51}Cr$-labeled target cells with the non-ionic detergent Nonidet P-40 to lyse cells. Background spontaneous lysis was determined by plating $^{51}Cr$-labeled target cells with 0.1ml of medium alone. Percent specific lysis was calculated as follows:

($^{51}Cr$ release in peptide wells - spontaneous $^{51}Cr$ release) x 100/ ($^{51}Cr$ release by NP-40-spontaneous $^{51}Cr$ release) = % Specific lysis

A positive response to the peptide matrix pools was SL>5% and SL>10% for individual peptides. Broadly reactive peptides were identified as inducing a CTL response in >50% of the responder ponies, and being presented by the majority of MHC I alleles.

### 4.2.f Statistical analysis

To determine the statistical relevance of peptide responses in the group of protected ponies compared to the non-protected ponies, a two tailed Students T-test with unequal variances was preformed. P-values <0.05 were considered to be statistically significant. To determine if there was a correlation between amino acid divergence and loss of CTL reactivity, Excel's CORREL function was used where array one was divergence from EV0 and array two was CTL response of the individual ponies. A correlation coefficient r-value of +/- 1 signifies perfect correlation.

## 4.3 RESULTS

### 4.3.a Neutralizing antibody response

To determine if neutralizing antibodies raised against the $EIAV_{D9}$ vaccine were associated with vaccine protection from disease, the level of neutralizing activity to the vaccine envelope was measured with DOC sera from each vaccinated pony. The 24 vaccinated ponies were grouped according to those protected from disease and those that developed equine infection anemia (EIA) after virulent virus challenge. The neutralizing antibody response elicited by the vaccine was quantified using DOC sera and the EV0 challenge strain, which had a homologous envelope to the $EIAV_{D9}$ vaccine. At DOC, only eight of the 24 vaccinated ponies had detectable neutralizing antibodies to EV0 (Fig 4.1A).

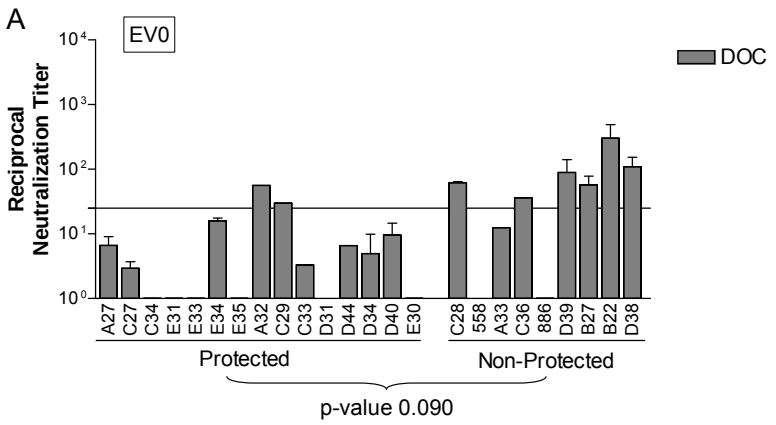


**Figure 4.1A Neutralizing antibody response elicited by the $EIAV_{D9}$ vaccine at day of challenge.** A positive neutralization response was set at 1:25 titer. The p-value was obtained using the Student's T-test.

Only two ponies with detectable neutralizing antibodies were protected, while the other six ponies with EV0-specific neutralizing antibodies at DOC were not protected from disease after virulent challenge. While the data initially appeared to indicate that the non-protected ponies developed better neutralizing antibody responses compared to protected ponies, there was in fact no statistical difference between the neutralizing activity of the protected ponies compared to the non-protected ponies (Student's T-test p-value = 0.090). Based on these assays, it appeared that EV0 specific neutralizing activity elicited by the $EIAV_{D9}$ vaccine was not associated with protective vaccine immunity.

The contribution of challenge strain-specific neutralizing antibodies to protection from disease was analyzed by measuring the level of neutralization in four week post challenge sera against the specific challenge strain for each vaccinated pony (Fig 4.1B).

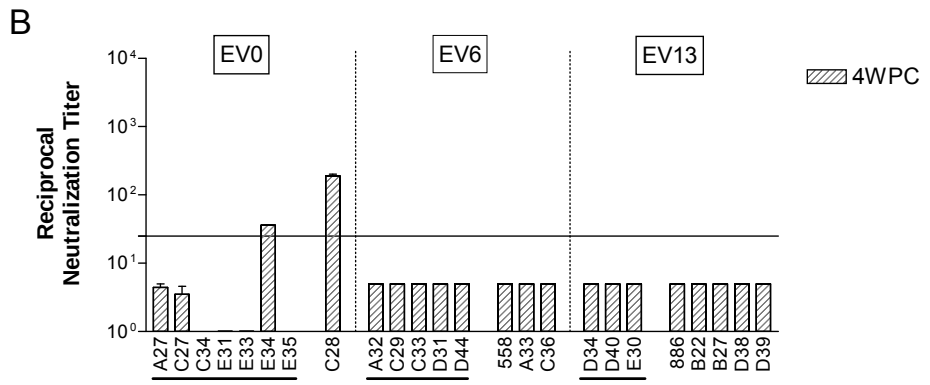


**Figure 4.1B Challenge strain-specific neutralizing antibody response at four weeks post challenge.** The ponies were grouped according to challenge strain. Underlined ponies were protected from disease.

Only two ponies, E34 and C28, had detectable challenge strain-specific neutralizing antibodies at 4wpc; no detectable challenge strain-specific neutralizing antibodies were measured in the remaining 22 ponies. Based on the current in vitro neutralization assay, an association between vaccine protection from disease and neutralizing activity to the respective challenge virus at 4wpc was not evident.

### 4.3.b Proliferation response to an $EIAV_{D9}$ Env-specific peptide pool

Day of challenge (DOC) PBMC from each pony were used in proliferation and CTL assays to determine if Env-specific immune responses elicited by the $EIAV_{D9}$ vaccine could be associated with protection from disease. The overall Env-specific proliferative response elicited by $EIAV_{D9}$ was measured in PBMC from each vaccinated pony in a $^3$H-thymidine incorporation assay against a comprehensive Env peptide pool. The comprehensive Env pool contained sequential EV0 Env peptides spanning the entire gp90 protein. The 21 ponies with viable DOC PBMC had

variable proliferative responses to the comprehensive Env pool, as expected from an outbred population of ponies (Fig 4.2A).

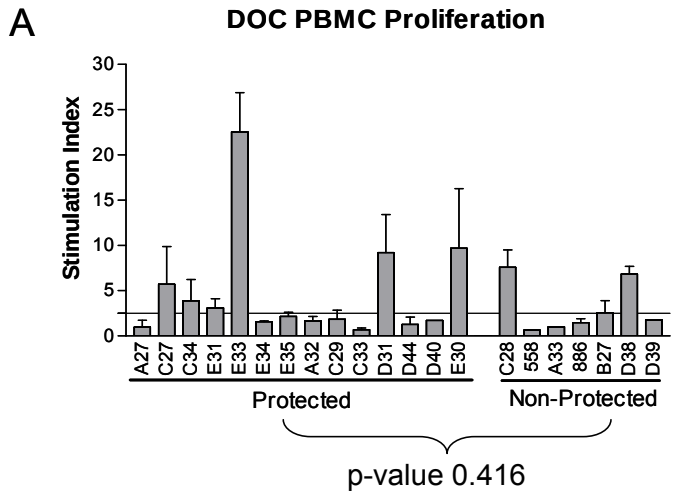


**Figure 4.2A Env-specific proliferation responses of vaccinated ponies.** Proliferation against a comprehensive Env peptide pool spanning the entire EV0 gp90 was tested against day of challenge PBMC from vaccinated ponies in a thymidine incorporation assay. A SI = 2.5 was the cutoff for a positive response. P-value was obtained with the Student's T-test.

There was no statistical difference between the proliferation responses of the protected ponies compared to the non-protected ponies (Student's T-test p-value = 0.416), therefore a more detailed analysis of peptide-specific proliferation was performed to determine if a relationship existed between Env-specific proliferation and protection from virulent EIAV challenge.

### 4.3.c Env peptide specific proliferation

Challenge strain specific peptide matrix pools were tested against the PBMC from the 21 vaccinated ponies in a thymidine incorporation assay to execute an inclusive scan of all Env peptides. Specific peptides were chosen from the matrix pools to be tested individually if they were reactive (SI>1.0) in 11 or more of the 21 ponies tested. Following these criteria, 33 EV0 specific peptides were indicated for individual analysis (data not shown).

To identify peptide specific proliferation that was potentially associated with protective immunity, the 33 EV0 specific peptides indicated by the peptide matrix were tested against DOC PBMC from the 21 vaccinated ponies in a thymidine incorporation assay. Peptides 17 and 61 caused a positive proliferation response (SI>2.5) in 11 or more of the 21 ponies (Fig 4.2B), and were presented by the majority of the represented MHC II alleles. Therefore, these vaccine Env specific peptides were considered to be broadly reactive in this group of ponies.

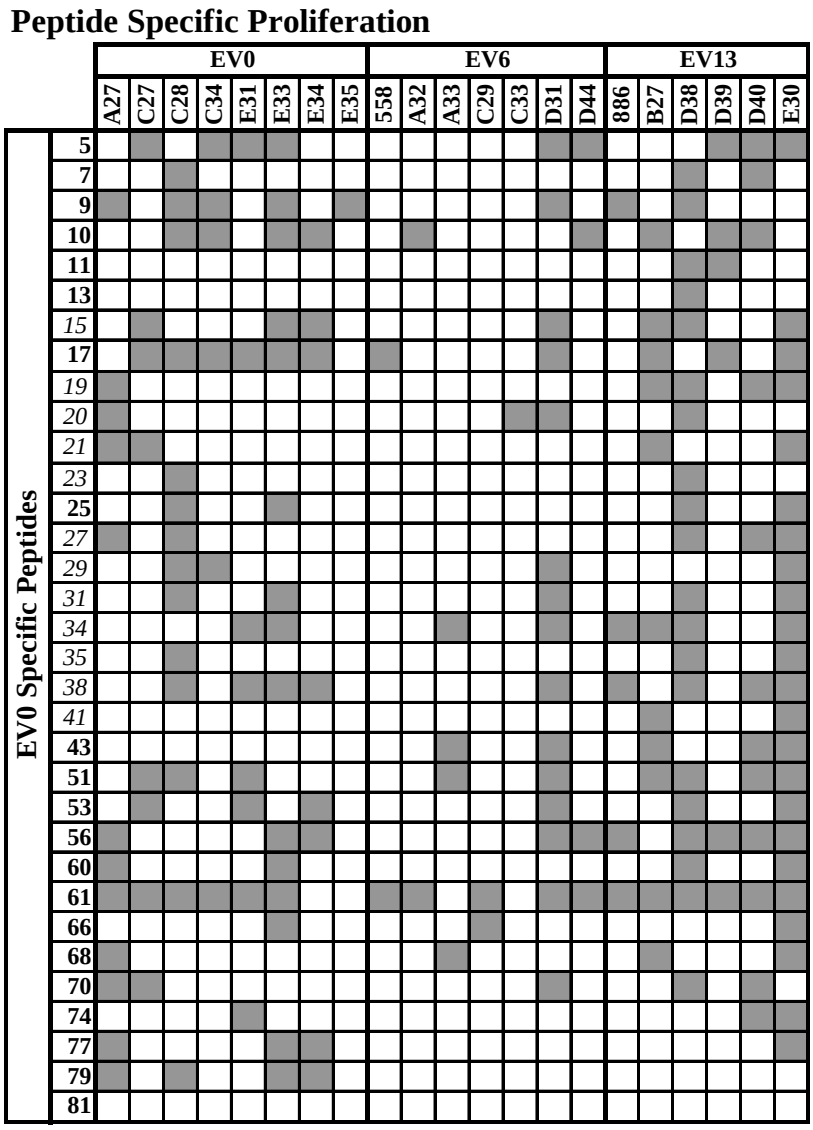


**Figure 4.2B Env peptide-specific proliferation in vaccinated ponies.** Thymidine incorporation was used to measure proliferation of DOC PBMC from the 21 $EIAV_{D9}$ vaccinated ponies in response to peptides indicated by the matrix pools. Variant peptides are *italicized,* conserved peptides are **bold**. A shaded block identifies ponies positively proliferating (SI>2.5) to specific peptides.

Peptide specific proliferation responses potentially associated with vaccine protection were identified by grouping the ponies according to protection or progression to disease after virulent EIAV challenge. For each peptide, the SI of the 14 protected ponies were averaged and the SI of the seven non-protected ponies were averaged (Fig 4.3A). A clear separation between the two groups of ponies was observed for peptides 5, 10, and 53. A more thorough analysis of these peptides was achieved by plotting the SI values of the individual ponies within the two groups (Fig 4.3B). The only statistically significant difference between the individual peptide responses of the protected and non-protected ponies was for peptide 5 (Student's T-test p-value 0.0156).

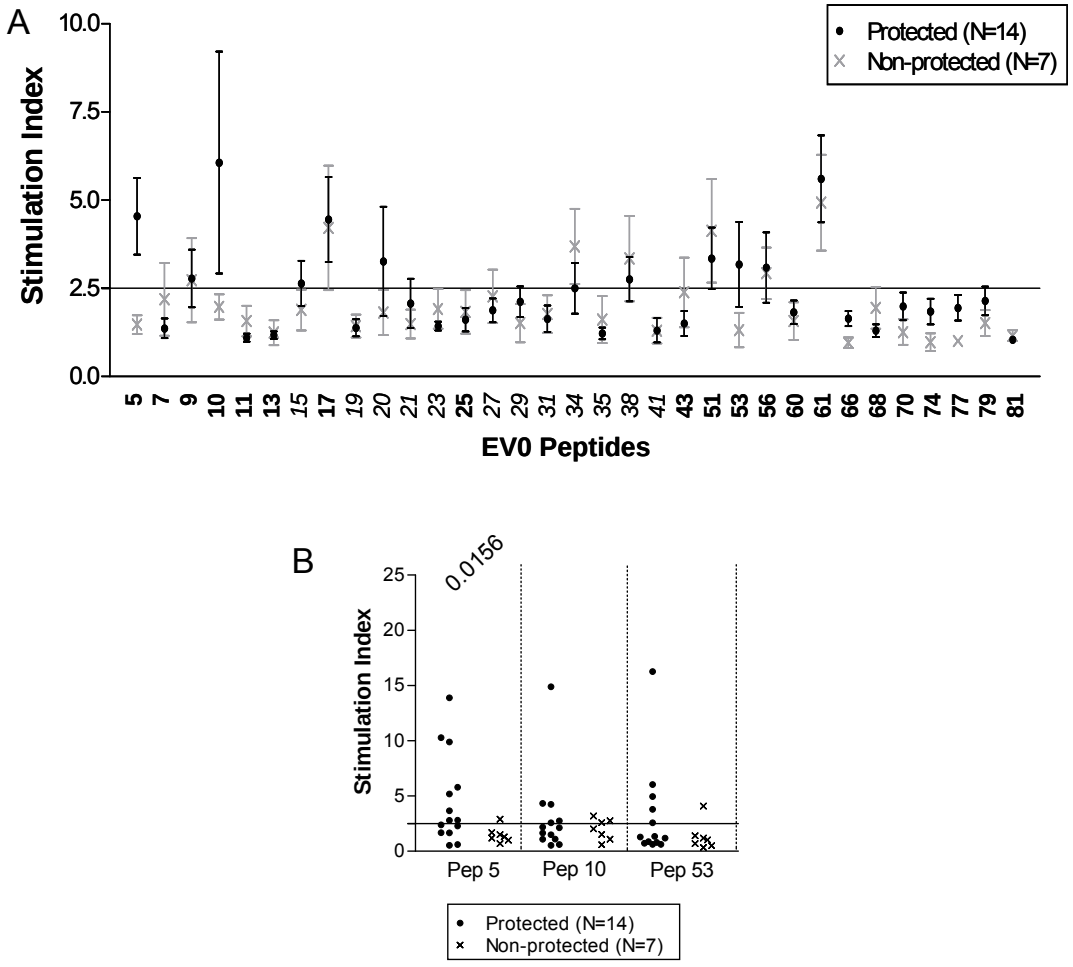


**Figure 4.3 Peptide specific proliferation responses in protected and non-protected ponies.** (A) The stimulation indices for each peptide from the thymidine incorporation assay were averaged for the 14 protected ponies and the seven non-protected ponies to highlight differences in the peptide responses. Variant peptides are *italicized*, conserved peptides are **bold**. (B) Specific reactivity for each pony to the peptides of interest in the thymidine incorporation assay. P-values from the Student's T test are listed for each statistically significant peptide.

### 4.3.d Peptide specific CTL reactivity

The Env-specific CTL response was examined to determine if the $EIAV_{D9}$ vaccine elicited peptide-specific cytotoxic responses associated with vaccine protection from disease after virulent challenge. In an initial evaluation, the CTL responses to a comprehensive Env pool of peptides was tested in DOC PBMC from the 21 vaccinated ponies (Fig 4.4A). Cytolytic activity was detected in 12 of the 21 vaccinated ponies at DOC, presumably reflecting natural fluctuations in virus specific CTL activity during persistent EIAV infection (52). Of these 12 ponies, seven were protected from disease and five developed EIA after virulent EIAV challenge. Differences in cytolytic activity between the protected and non protected ponies in response to the Env peptide pool was not statistically significant (Student's T-test p-value = 0.676).

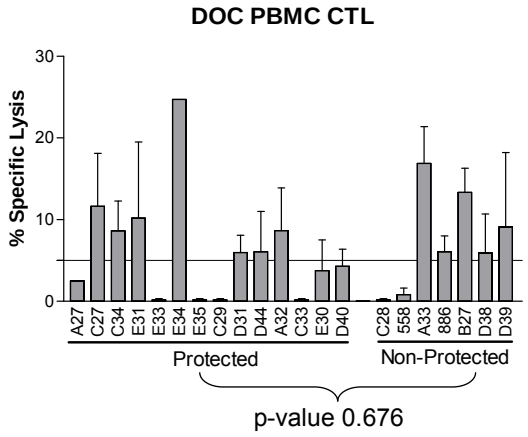


**Figure 4.4A Env-specific CTL responses of vaccinated ponies.** CTL lysis of day of challenge PBMC from vaccinated ponies pulsed with a comprehensive peptide pool spanning the entire EV0 gp90 was measured in a chromium release assay. A %SL = 5 was the cutoff for a positive response. P-value was obtained using the Student's T-test.

A more comprehensive study to identify individual peptide responses potentially associated with vaccine protection from disease was performed next. The DOC PBMC from the 12 vaccinated ponies with detectable CTL activity were tested in a chromium release assay against challenge strain-specific peptide matrix pools to identify potentially reactive CTL peptides (data not shown). From this analysis, 20 EV0 were identified for individual analysis. Peptide specific CTL responses elicited by the vaccine were identified by testing the EV0 peptides against the DOC PBMC of the 12 vaccinated ponies in a chromium release assay. Of the 20 EV0 peptides, peptides 8, 9, 17, 18, 20, 21, 27, 32, 36, 38, 52 and 62 were reactive (>10% SL) in >50% of the ponies tested (Fig 4.4B).

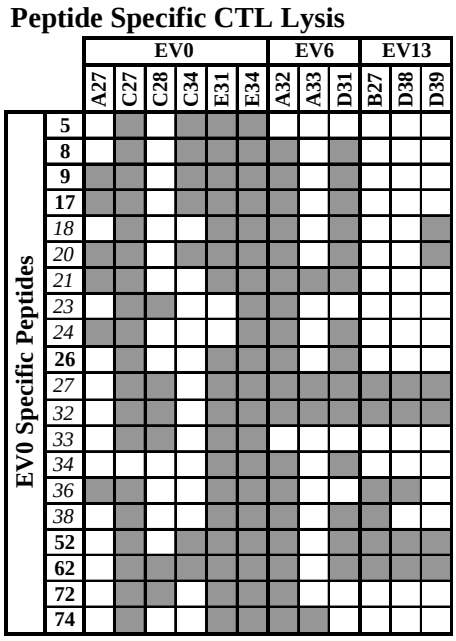


**Figure 4.4B Env peptide-specific CTL responses of vaccinated ponies.** Chromium release assays were used to measure CTL lysis of DOC PBMC from the 12 vaccinated ponies in response to peptides indicated by the matrix pools. Variant peptides are *italicized*, conserved peptides are **bold**. A shaded block represents a positive cytolytic peptide response (SL>10%) by the pony. E:T ratio was 20:1.

These peptides, with the exception of peptides 18 and 21, were presented by the majority of MHC I alleles present in this cohort of ponies, and were therefore considered to be broadly reactive. Peptides 18 and 21 appeared to be restricted to the A1, A9, and W11 alleles, and therefore not broadly reactive. The remaining EV0 peptides failed to generate appropriate cytolytic responses in more than six of the vaccinated ponies; for this reason, they were not considered to be broadly reactive.

### 4.3.e CTL responses associated with protective immunity

Peptide-specific CTL responses potentially associated with protective vaccine immunity were identified by grouping the vaccinated ponies according to disease development. For this comparison, the %SL of the seven protected ponies and five non-protected ponies were averaged for each reactive peptide (Fig 4.5A). There was an apparent demarcation between the two groups of ponies for peptides 5, 8, 9, 17, 18, 20, 21, 24, 26, 32, 36, and 38. Closer analysis revealed a statistically significant difference between the protected and non-protected ponies for peptides 5, 8, 9, 17, 20, 24, 26, and 38 (T-test p-values ranged from 0.0007 to 0.0344) (Fig 4.5B). There was no statistical significance between the %SL of the protected and non-protected ponies for peptide 36. Because peptides 18 and 21 were MHC restricted, reactivity to these peptides segregate with the MHC alleles, not with protection. Therefore, these peptides were not associated with vaccine protection from disease. From this study, it appeared that peptides 5, 8, 9, 17, 20, 24, 26, and 38 were associated with protective vaccine immunity.

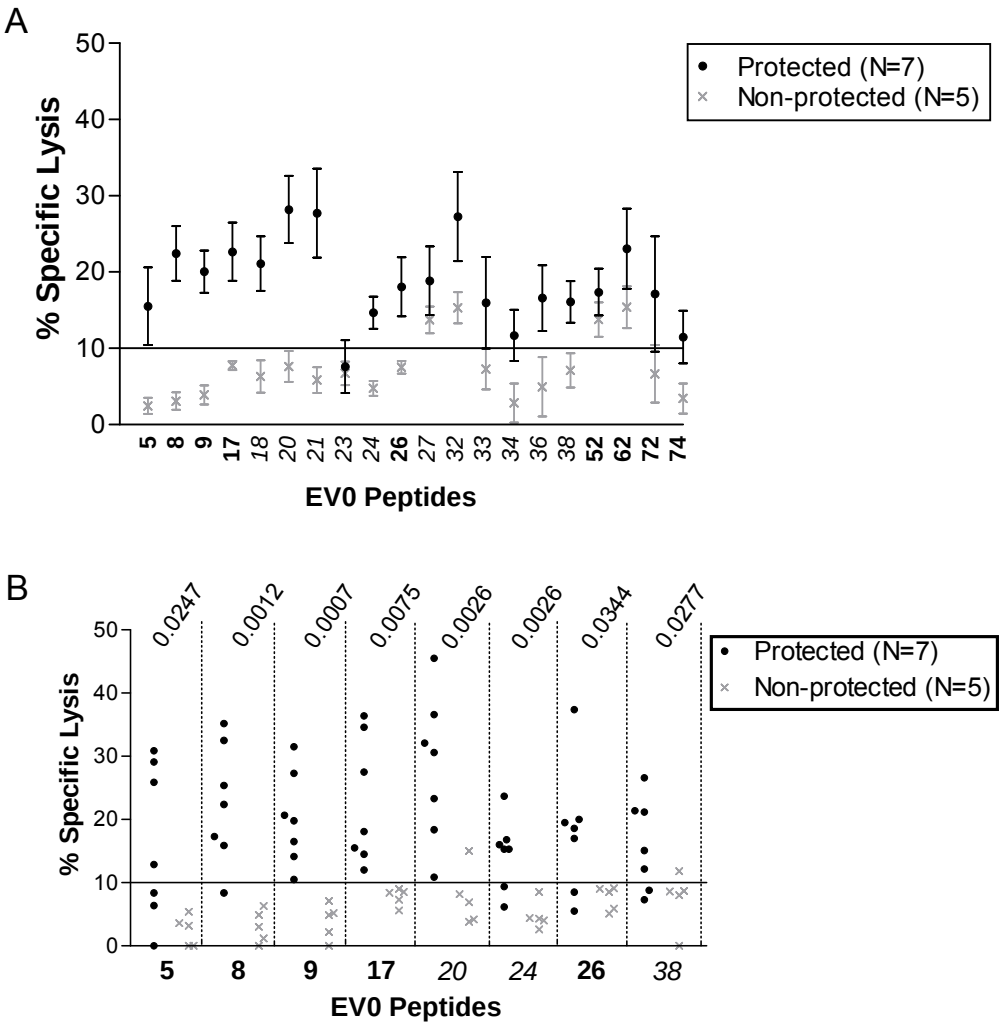


**Figure 4.5 Peptide specific CTL responses in protected and non-protected ponies.** (A) The %SL for each peptide in the chromium release assay was averaged for the seven protected ponies and the five non-protected ponies to highlight the different peptide responses. Variant peptides are *italicized*. (B) Specific reactivity for each pony to the peptides of interest in the chromium release assay. P-values from the Student's T test are listed for each statistically significant peptide. E:T = 20:1.

## 4.4 DISCUSSION

Using EIAV variants with divergent Env proteins to challenge $EIAV_{D9}$ vaccinated ponies provided the unique opportunity to address the effects of defined Env variation on vaccine efficacy, and also to identify potential determinants of protective immunity. The study by Craigo et al. (2007) (36) definitively revealed Env as a primary determinant of lentiviral vaccine efficacy, indicating a significant inverse relationship between the extent of challenge-strain Env divergence from the vaccine strain and the observed vaccine protection. In the current study, we have taken advantage of this unique vaccine trial to characterize the humoral and cellular immune responses to Env that may be associated with vaccine protective immunity. Neutralizing antibody titers, Th proliferation, and CTL lysis of the $EIAV_{D9}$ vaccinated ponies protected from disease were compared to those that were not protected to evaluate potential determinants of vaccine immunity. For the first time, Th and CTL envelope peptides associated with protective EIAV vaccine immunity have been identified.

Like other lentiviral model systems (HIV, SIV and SHIV), there is considerable debate about the role of neutralizing antibodies in protective immunity. Various studies with EIAV, like SIV, have failed to establish a correlation between neutralizing antibody development and vaccine protection (26, 32, 50, 54, 102, 103). While a range of studies have shown that passive immunization with neutralizing antibodies can protect against HIV or SHIV challenge (85, 86, 115), there have been other studies demonstrating that passively administered immune serum cannot protect animals from SIV infection (6, 95, 102, 103, 127). When the neutralizing antibody titers of the 24 vaccinated ponies were measured against the homologous Env strain, EV0, only eight ponies had detectable neutralization titers at DOC, but these responses could not

be correlated with vaccine protection from disease. At four weeks post challenge, two of the 24 vaccinated ponies had challenge strain-specific neutralizing antibodies. Both ponies were challenged with EV0; one pony was protected from disease, while the other pony developed EIA after challenge. Because an *in vitro* assay was used to measure neutralization titers, the possibility that EV6 and EV13 neutralizing antibodies were present in the vaccinated ponies but undetectable in our neutralization assay could not be discounted. However, with the current assay, we were unable to correlate a neutralization response with vaccine protection from disease.

With no established relationship between antibody neutralization and vaccine protection, the cellular immune response elicited by $EIAV_{D9}$ was analyzed. Effective vaccines against viruses that cause chronic infection, such as Epstein-Barr virus and cytomegalovirus, elicit polyfunctional Th and CTL responses (55). During HIV infection, there is a skewing of the Th response, such that CD4+ T-cells with proliferative capabilities are destroyed while CD4+ T-cells secreting IFNγ are abundant (113). Targeting an appropriate Th proliferation response is therefore a desirable quality for lentiviral vaccines. The thymidine incorporation assay for PBMC proliferation identified one Env-specific peptide which was associated with vaccine protection from disease, and the amino acid sequence of this peptide was conserved across the three variant challenge strains. As equine-specific reagents become available to study the cytokine profile of the Th cells, it will be of interest to study the cytokine response evoked by the $EIAV_{D9}$ vaccine to determine if there is an association with vaccine immunity.

The proliferation assay also distinguished broadly reactive regions of EIAV Env. A group of peptides was recently described as broadly reactive in 12 $EIAV_{D9}$ vaccinated horses demonstrating protective immunity from disease (132). One of the peptides identified in the

current trial, peptide 61, overlaps with a previously described broadly reactive peptide, peptide 60. Additionally, when the two $EIAV_{D9}$ trials are combined, peptides 9, 17, 27 and 70 can be described as broadly reactive peptides in the two groups of ponies. It should be noted that ponies having the same DRA/DQA alleles in this study do not always have the same peptide specific proliferation responses. This may be attributed to the third MHC II allele, DRB. There is not complete sequence analysis for DRB, therefore it was not characterized in these ponies (43).

As with a Th response, a polyfunctional CTL response is regarded as necessary for a protective lentiviral vaccine (57). In HIV, various studies have shown a correlation between CTL levels and long-term nonprogressors (56, 120), and control of acute (16, 72) and chronic viremia (105). Additionally, in the SIV model of infection, depletion of CD8+ T-cells results in loss of immune control (67, 87, 124). As of yet, CTL peptides correlating with HIV or SIV vaccine protection have not been identified. The bulk of HIV and SIV CTL research has relied upon IFNγ and tetramer staining, but it is becoming increasing clear that determining the functional diversity of the CTL response is equally important to quantifying the CTL response using these techniques (107, 141). In the current study, a functional chromium release assay was utilized to test PBMC from 12 vaccinated ponies with measurable DOC CTL activity to identify potentially protective CTL peptide specific responses elicited by the $EIAV_{D9}$ vaccine. Broadly reactive CTL peptides elicited by the $EIAV_{D9}$ vaccine were previously described (132). Again, without a more discriminating assay to type the ELA-A allele, firm MHC restrictions cannot be assigned to identified peptides (132, 143). However, the peptides identified in the current vaccine trial do not seem to be MHC restricted and confirm four of the broadly reactive peptides from the previous trial, peptides 9, 17, 27, and 52 (132). Additionally, when data from the two $EIAV_{D9}$ trials are combined, peptides 5, 32 and 62 are also found to be broadly reactive.

In defining CTL peptide responses associated with vaccine protection from disease, our analysis revealed a statistically significant association between protection from disease and CTL responses to peptides 5, 8, 9, 17, 20, 24, 26 and 38. In an independent study, lipopeptides corresponding to the amino acid sequences of EIAV gp90 peptides 20 and 21 were used to vaccinate three horses. While the vaccine did not prevent infection, it was capable of reducing the severity of disease, indicating that the CTL responses associated with these peptides can have some protective effect (119). The cytolytic response to peptides 5, 8, 9, 17, 20, 24, 26 and 38 can be targeted in future lentiviral vaccine trials to enhance protective immune responses.

Five (peptides 5, 8, 9, 17, and 26) of the eight CTL peptides associated with vaccine protection from disease were found in more conserved regions of gp90 (Fig 4.6), with each challenge strain having identical amino acid sequences for these five peptides. Additionally, there were no N-linked glycosylation sites found in these five peptides. The three other CTL peptides were variable between the three challenge strains. CTL peptide 20 spanned the V3 region of gp90, peptide 24 included the V4 region, and peptide 38 contained part of the V7 region. The variant challenge strains each had unique amino acid sequences for these peptides and were also variable in the number and placement of N-linked glycosylation sites. The strain specific peptides along with the variant peptides identified in the matrix analysis will be tested against these PBMC to determine if the amino acid changes altered the observed cytolytic activity.

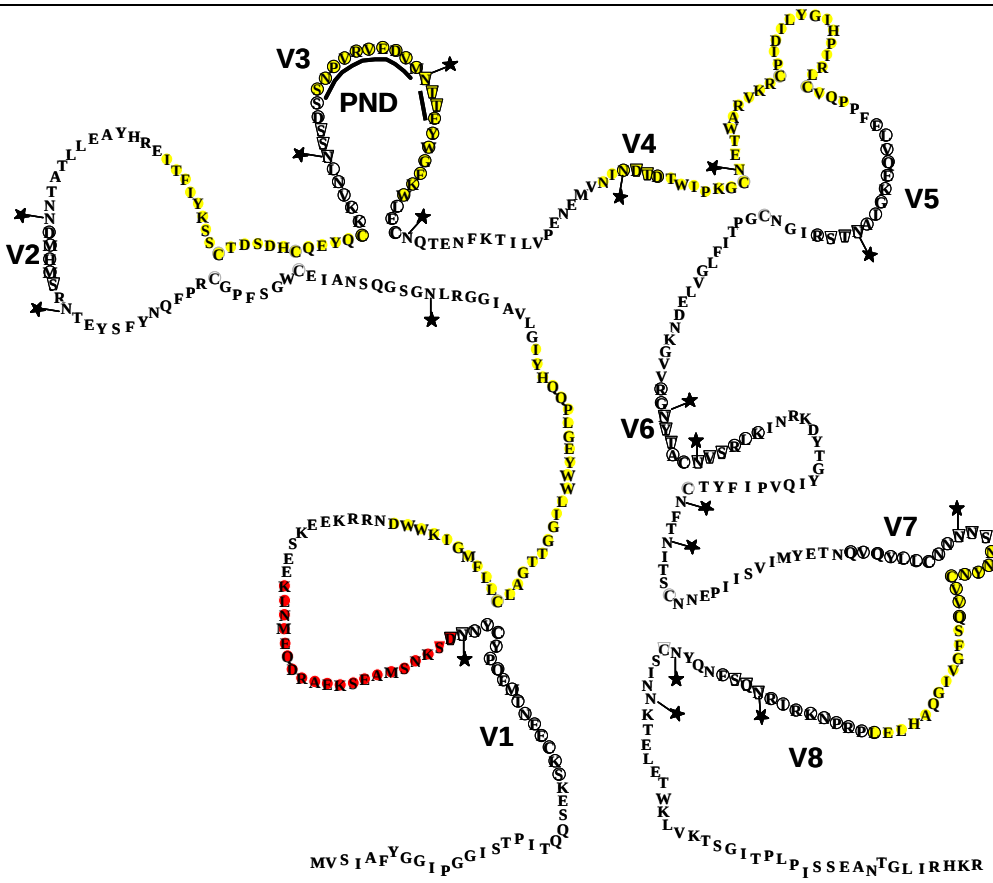


**Figure 4.6 Env specific peptide responses associated with vaccine protection.** Schematic model of gp90 adapted from Ball et al. (1992). Variable domains are circled and labeled V1-V8. N-linked glycosylation sites are marked with a star. PND is the principal neutralization domain. CTL peptides associated with protection are highlighted in yellow. Proliferation and CTL peptide highlighted in red.

In conclusion, the current studies have used the EIAV vaccine system to evaluate potential Env-specific immune responses associated with the development of protective vaccine immunity. The reactive Th and CTL Env peptides identified here as being associated with vaccine protection may serve as critical determinants of vaccine efficacy and should be targeted in the design of new immunization strategies for EIAV vaccine development.

## 4.5 ACKNOWLEDGMENTS

I would like to thank Jodi Craigo for organizing the vaccine trial and Shannon Barnes and Timothy Sturgeon for helping with the extensive amount of blood processing. Additionally, I would like to thank Debbie Even for performing the MHC I, and Sheila Cook and Chuck Issel for conducting the animal procedures.

# 5.0 EFFECTS OF NATURAL ENVELOPE VARIATION ON IMMUNE RECOGNITION IN EIAV INFECTED AND VACCINATED PONIES

Portions of this data were published in:

Tara L. Tagmyer, Jodi K. Craigo, Sheila J. Cook, Deborah L. Even, Charles J. Issel, Ronald C. Montelaro. Determinants of EIAV vaccine protection and the effects of sequence variation on immune recognition. Submitted to Journal of Virology.

AND

Jodi K. Craigo, Baoshan Zhang, Shannon Barns, Tara L. Tagmyer, Sheila J. Cook, Charles J. Issel, Ronald C. Montelaro. Envelope variation as a primary determinant of lentiviral vaccine efficacy. (2007). PNAS PMID: 17846425.

## 5.1 INTRODUCTION

*Equine infectious anemia virus* (EIAV) is a macrophage tropic lentivirus that infects equids through horsefly bites (97). While genetically and structurally similar to other lentiviruses (HIV, SIV, FIV), EIAV infection induces a dramatically different disease course characterized by three distinct phases: acute, chronic and inapparent. By two months post infection, horses develop an acute episode of disease characterized by fever, thrombocytopenia, and high viral loads. CTL responses control virus replication after the acute episode, but envelope (Env) variation of EIAV allows the virus to escape immune control and produce a second febrile episode, which initiates the chronic phase of disease. During the chronic phase, an infected horse cycles through recurrent disease episodes for approximately one year, after which, full immunological control is typically attained, and the infected horse becomes an inapparent carrier of EIAV. The inapparent phase of disease is due to host immune control and cannot be attributed to viral attenuation, as immune suppressing or stressing an infected horse will cause a recrudescing disease episode with a viral quasispecies distinct from the previous febrile episode (64, 89, 97). Although viral replication is strictly controlled in inapparent carriers of EIAV, whole blood transfers to naïve horses result in acute EIA in recipient horses. Additionally, inapparent carriers of EIAV are resistant to further EIAV challenge, demonstrating natural prophylactic immunity (97).

The limited characterization of equine immune responses to EIAV has revealed that virus-specific CTLs are associated with control of the acute episode, but these responses fluctuate throughout the course of disease (52, 90). Both T-helper and CTL immune epitopes have been described for the Gag and Pol proteins of EIAV (22, 46, 93, 94), but vaccines based on eliciting these immune responses have failed to protect ponies from virulent challenge (45, 119). Recently, broadly reactive Env peptides were identified in vaccinated ponies protected

from homologous challenge, but their role in vaccine protection has yet to be established (132). Using the same vaccine but challenging immunized ponies with EIAV strains containing divergent Env proteins proved Env to be a primary determinant of vaccine efficacy (36). Additionally, analysis of peptide specific cellular responses identified one Th and eight CTL Env specific peptides associated with vaccine protection from disease (131).

While neutralizing antibodies have been detected after the acute episode of EIAV (93), they typically do not develop until two months post infection (52, 60). Initial peptide mapping studies revealed that neutralizing antibodies bind three specific areas of gp90, which were later mapped to the V3 and V5 regions of Env (12, 77). The two peptides encompassed by V3 compose the principal neutralization domain (PND) of EIAV. Studies with a natural PND deletion mutant, EIAVΔPND, revealed that after immune suppression, neutralizing antibodies developed against EIAV without the PND, suggesting that other areas of gp90 could serve as targets for the neutralizing antibodies (33). Further studies revealed that the V3 and V4 regions of Env confer neutralization specificity to EIAV in ponies chronically infected with EIAV (59, 60).

Recent cellular and humoral epitope mapping studies of the Env proteins have focused on the reference strain $EIAV_{PV}$ or the $EIAV_{D9}$, both of which share the same Env proteins. To begin understanding the effects of Env variation on immune reactivity, variant strains of EIAV with divergent gp90 proteins were constructed from viral isolates of a pony chronically infected with $EIAV_{PV}$ (32). Sequence analysis was performed on sequential fevers to determine the level of amino acid divergence in the Env proteins from the $EIAV_{PV}$ infecting strain (35, 76). Viral clones from the IV febrile episode and pre-immune suppression serum yielded gp90 proteins 6% and 13% divergent from the infecting $EIAV_{PV}$ strain. The gp90 of each variant strain was placed

into the backbone of the infectious molecular clone $EIAV_{UK3}$, which shares a homologous Env with $EIAV_{PV}$ (36). To determine the pathogenesis of each variant, groups of four ponies were infected with EV0 (homologous $EIAV_{PV}$ Env), EV6 (6% divergent), or EV13 (13% divergent). Each variant produced a febrile episode by 45 days post infection, with four ponies developing chronic disease.

We hypothesize that the immune responses to each variant Env EIAV challenge strain would alter the observed immune responses associated with the $EIAV_{D9}$ vaccinate (131, 132). To test this hypothesis, PBMC and serum from the variant infected ponies were tested in the cellular and humoral assays to identify regions of the variant Env that evoked an immune response. PBMC from the EV6 and EV13 infected ponies were also tested against EV0 peptides to determine if the amino acid differences in EV0 would alter the observed cellular immune responses to the strain specific peptides. Sera from the infected ponies were tested against the three different challenge strains to determine the extent of cross-reactivity of the neutralizing antibodies. Additionally, the serum was used against chimeric constructs to verify the target region of Env for neutralizing antibodies. To understand the effects of Env variation on immune recognition in vaccinated ponies, PBMC from the $EIAV_{D9}$ vaccinated ponies challenged with EV6 and EV13 were tested against EV6 or EV13 specific peptides, respectively. Sera from $EIAV_{D9}$ vaccinated ponies were also tested against all three variant challenge strains to determine the extent of cross-reactivity induced by the vaccine. The results of these studies suggest that Env variation can have profound effects on the immune recognition in infected and vaccinated ponies.

## 5.2 MATERIALS AND METHODS

### 5.2.a Experimental pony infections with variant isolates

All equine procedures were conducted at the Gluck Equine Research Center of the University of Kentucky according to protocols approved by the University of Kentucky IACUC. Twelve ponies were infected with $10^3$ tissue culture infectious doses ($TCID_{50}$) of either EV0, EV6, or EV13 (four ponies per variant EIAV) as previously described (36). EV6 and EV13 diverged from the EV0 gp90 amino acid sequence by 6% and 13% respectively (c.f. Fig 1.8). Serum and blood were obtained at regular intervals throughout the infections, with peripheral mononuclear cells (PBMC) being isolated from the blood and cryopreserved in autologous serum and 10% DMSO for future use in cellular immune assays. Regular temperature and platelet counts were taken throughout the course of infection to monitor disease development. EV0 infected pony 9959 was euthanized at 178 days post infection (dpi) along with EV6 infected ponies #10 (65dpi) and #50 (130dpi) due to severe disease. The remaining ponies were euthanized at nine months post infection (mpi) after developing mature immune responses to the infecting EIAV strain. Viral loads were sustained above $10^3$ RNA copies/ml of plasma in each infected pony (36). PBMC and serum were utilized from the $EIAV_{D9}$ variant challenge trial (36) to examine the effects of Env variation on immune recognition.

### 5.2.b Lymphoproliferation assays

PBMC from six months post infection were tested against variant-specific Env peptides in a standard seven-day thymidine incorporation assay, as previously described (132). As the immune system requires 6-8 months to develop a mature immune response (52, 99), PBMC and

serum were taken six months after infection for immune analysis. Two of the EV6 infected ponies were euthanized before six months post infection and were not included in the cellular assays. PBMC from ten of the 12 infected ponies were >80% viable upon thawing and maintained viability throughout the assays, as measured by trypan blue exclusion. PBMC were plated at 2 X $10^5$ cells/well in complete RPMI-1640 (10% fetal equine serum, 1% penicillin/streptomycin, 1% L-glutamine, 55uM β-mercaptoethanol) with 5uM AZT (Sigma) and stimulated in triplicate wells with 2.5ug/ml Pokeweed Mitogen (PWM), 20ug/ml of a comprehensive Env peptide pool, 20ug/ml matrix pools, or 10ug/ml of individual peptides. The comprehensive pool contained variant strain specific peptides spanning the entire gp90 protein. AZT was replenished every other day in 5ul of media. Triplicate media control wells were set up with DMSO to match the DMSO concentration in wells containing Env peptides. The PBMC were incubated for 48 hours with PWM, and for six days with the Env peptides prior to labeling with 0.75uCi [$^3$H]thymidine (Amersham Biosciences). After 16-18 hour incubation, the cells were harvested and quantified for $^3$H incorporation by liquid scintillation counting. Stimulation indices (SI) were calculated by dividing the mean counts per minute (c.p.m.) of stimulated cells by the mean c.p.m. of non- stimulated cells. A positive response to peptide pools was SI>1 and SI>2.5 for individual peptides.

**5.2.c CTL assays**

PBMC after six months post infection were used to assay CTL activity in a standard chromium release assay as previously described (132). Briefly, PBMC from the 10 infected ponies were expanded *in vivo* for 7-10 days with either 2.5ug/ml PWM (target cells) or 10ug/ml gradient purified EIAV (effector cells) in complete RPMI supplemented with 200U rhIL2 (Hoffmann-La

Roche INC.). The target cells were labeled with 100uCi $^{51}Cr$ ($Na^{51}CrO$) (MP) for one hour at 37°C, 5% $CO_2$, and washed four times with 1X PBS containing 1% horse serum. Approximately 30,000 target cells/well were pulsed for two hours at 37°C, 5% $CO_2$ with 20ug/ml of a comprehensive variant-specific Env peptide pool, 20ug/ml of peptide matrix pools, or 10ug/ml of individual Env peptides (131). EIAV-stimulated effector cells were added at a 20:1 E:T ratio and incubated 12-16 hrs prior to being harvested. A 25ul sample of cell supernatant was added to 175ul of OptiPhase SuperMix scintillation fluid (Perkin Elmer) and analyzed for $^{51}Cr$ release with a MicroBeta reader (Perkin Elmer). Maximum $^{51}Cr$ release was determined by plating $^{51}Cr$-labeled target cells with the non-ionic detergent Nonidet P-40 to lyse cells. Background spontaneous lysis was determined by plating $^{51}Cr$-labeled target cells with 0.1ml of medium alone. Percent specific lysis was calculated as follows:

($^{51}Cr$ release in peptide wells - spontaneous $^{51}Cr$ release) x 100/ ($^{51}Cr$ release by NP-40-spontaneous $^{51}Cr$ release) = % Specific lysis

A positive response to peptide pools was SL>5% and SL>10% for individual peptides.

**5.2.d Neutralizing antibody assay**

The development of neutralizing antibodies to EV0, EV6, and EV13 throughout the course of infection was measured using an ELISA-based infectious center assay as previously described (52). Briefly, $10^5$ fetal equine kidney (FEK) cells were plated in 24 well plates and incubated 18-24 hours at 37°C. Two-fold dilutions of heat inactivated serum (56°C, 1 hour) were added at 100 infectious units of EV0, EV6, or EV13. After one hour incubation, the serum-virus supernatant was added to the FEK cells in triplicate. The following day, an overlay of 0.8% carboxymethyl

cellulose was added to the cells to prevent the virus from spreading throughout the culture. The cultures incubated an additional seven days at 37° before being fixed with 3.7% formaldehyde and permeabilized with 1% Triton X-100. Primary α-EIAV serum was used at 1:200, and secondary antibody, horseradish peroxidase-conjugated goat anti-horse IgG (United States Biochemical Corp.) was used at 1:5000. The labeled cells were stained with 3-amino-9-ethyl-carbazole (Sigma) in a sodium acetate buffer (pH 5.5) supplemented with $H_2O_2$, and visualized with a dissecting microscope. The 50% neutralization titers of each serum sample were determined by linear regression analysis of the reciprocal dilution versus the number of apparent foci. A neutralization titer of 1:25 was used as the cutoff for a positive response.

**5.2.e Development of chimeric viruses**

Chimeric viruses were constructed between EV0 and EV6 by utilizing the two HindIII sites in gp90 to switch the V3 to V4 region between the two strains. Attempts to make similar chimeric constructs between EV0 and EV13 did not yield viable virus. PCR products containing the V3 to V4 regions were synthesized using a three step overlapping PCR reaction with variant strain-specific primers (Fig 5.1, Table 5.1). PCR A: The first Hind III site to V4 region of the donor strain was amplified (~240bp) using primer 1 and strain specific primer 2 with donor strain DNA. PCR B: The V4 to second Hind III site was amplified (~513bp) using primer 4 and variant specific primer 3 with backbone strain DNA. Standard PCR conditions were used on the first round of PCR, followed by Qiagen PCR Clean-Up.

**Table 5.1 Primers for chimeric clone construction.**

| Primer | Primer Sequence | Specificity | Chimera[a] |
|---|---|---|---|
| **EV-1 FWD** | GCTACATTATTAGAAGCTTATCATAGGGAG | universal | all |
| **EV-4 RVS** | GGTTAAAGCTTTGGTTCCTTATTCT | universal | all |
| **EV0-2 RVS** | CCAGGTATCAGTATCATTGATATTTACC | EV0 V4 | EV6v0 and EV13v0 |
| **EV6v0-3 FWD** | AATGATACTGATACCTGGATAGCTAAGG | EV0 V4 with EV6 backbone sequence | EV6v0 |
| **EV13v0-3 FWD** | AATGATACTGATACCTGGACACCTAAGG | EV0 V4 with EV13 backbone sequence | EV13v0 |
| **EV6-2 RVS** | CCAGGTATCATTATCATTGATATTTACC | EV6 V4 | EV0v6 |
| **EV0v6-3 FWD** | AATGATAATGATACCTGGATACCTAAGG | EV6 V4 with EV0 backbone sequence | EV0v6 |
| **EV13-2 RVS** | CCAGGTACCATTTTTGATTTTTACC | EV13 V4 | EV0v13 |
| **EV0v13-3 FWD** | CAAAAATGGTACCTGGATACCTAAGG | EV13 V4 with EV0 backbone sequence | EV0v13 |

[a] chimera naming = receptor strain with donor V3/V4

The second reaction was an asymmetric PCR adapted from a previously described protocol (137). Primer 2 and 3 share overlapping sequences that allow for limited self priming when the PCR products from PCR A and B are combined. Therefore 3ul each of PCR A and PCR B were combined in a 50ul standard PCR reaction without primers and double $MgCl_2$ (3mM). The reaction cycled 16 times at 95°C for 60sec, 55°C for 60sec and 72°C for 60sec, after which the third PCR was conducted using 20ul of PCR 2 with the addition of 1ul each of primer 1 and primer 4. New PCR buffer, $MgCl_2$, dNTP and Taq were added to make up a 50ul reaction. The third PCR cycled through 92°C for 60sec, 55°C for 60sec and 72°C for 3min 25 times and finished with 72°C for 15min. The final PCR product (~750bp) was run on a 1% agarose gel and purified with the Qiagen Clean-up kit.

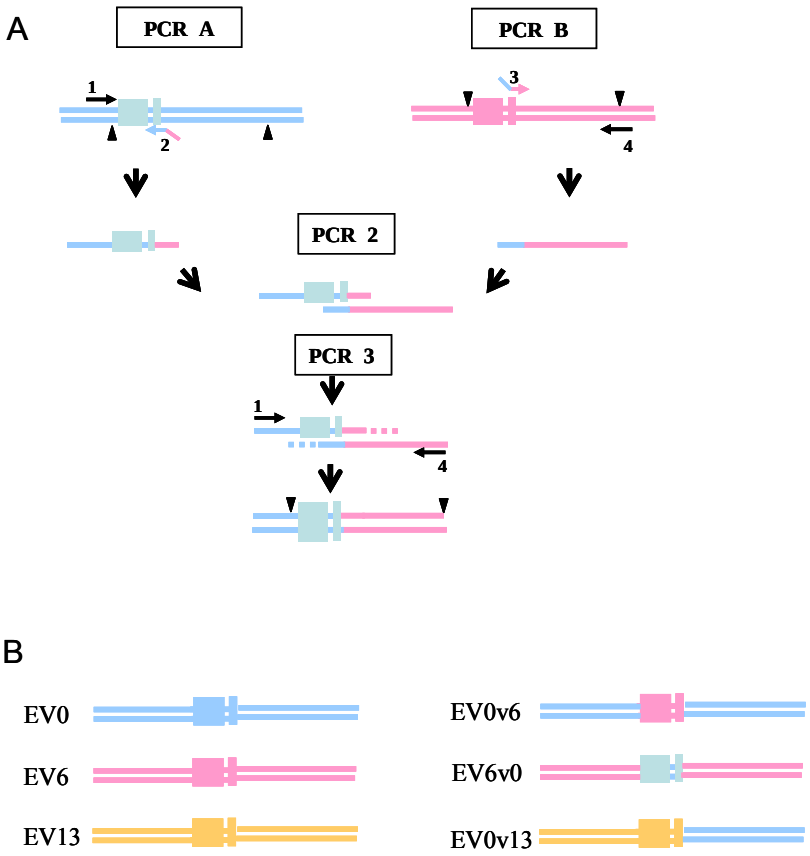


**Figure 5.1 PCR strategy and chimeric clones.** (A) Three PCR reactions were conducted to obtain the V3-V4 region of each variant EIAV strain. The first PCR reaction separately amplified the Hind III to V3 region and the V4 to Hind III region. Self-priming of PCR product A and B occurred during the second PCR step. The third PCR then amplified the entire region using primers 1 and 4. Conserved PCR primers are in black, the variable PCR primers are colored to match the variant DNA. The large block represents V3, the small block represents V4, and the triangles represent the HindIII sites. (B) Schematic representation of the parental DNA and chimeric clones. EV0v13 was obtained from Chengqun Sun.

The PCR products were digested with Hind III; EV0 and EV6 were digested with Hind III and shrimp alkaline phosphotase (SAP). The digested backbone DNA was phenol chloroform extracted and ethanol precipitated prior to ligation with the PCR products. Ligations were performed with T4 ligase at 23°C for 4 hours before transformation into *dam-/dcm-* competent *E. coli* (New England BioLabs). Miniprep cultures were grown for DNA isolation and sequence analysis.

### 5.2.f Transfection of chimeric clones

After sequence analysis, suitable chimeric clones were transfected into equine dermal cells (ED) using Lipofectamine$^{TM}$ 2000 (Invitrogen). ED cells were plated in 6 well plates at $2x10^5$ cells in 2ml eMEM the day before transfection. For the transfection, 2.5ug of chimeric DNA was incubated for 30min at room temperature with 500ul OptiMem and 6.25ul of Lipofectamine$^{TM}$ 2000, following the manufacturer's protocol. The DNA was added to ED cells and incubated for 4 weeks at 36°C, 5% $CO_2$. To quantify the amount of virus production, supernatant samples were taken every 3-5 days for micro-RT analysis as previously described (77). The $TCID_{50}$ of the supernatant from the transfected cells was determined using the previously described infectious center assay (52). The supernatants were aliquoted and stored at -80°C for future use.

## 5.3 RESULTS

### 5.3.a Clinical progression of EIAV Env variant infected ponies

Each pony infected with the EIAV variant Env strains developed acute EIA episodes by 43 days post infection (Table 5.2). Four ponies, 9955, #10, #50 and 9959, developed chronic EIA and cycled through multiple fevers. EV0 infected pony 9955 cycled through five additional fevers before being euthanized at 178dpi due to severe disease. EV6 infected ponies, #10 and #50 were also euthanized at day 65 and 130, respectively, due to the severity of their disease. EV13 infected pony 9959 cycled through four additional fevers before becoming an inapparent carrier of EIAV. Ponies 009 and A26 each had one atypical fever more than 130 days after the acute episode; the remaining ponies were asymptomatic after the acute episode for the 9 month observation period. It has been established that immune responses to EIAV evolve from an immature, ineffective response to a mature protective response by 6-8 months post infection (52, 99), therefore, PBMC from >6mpi were isolated for Th and CTL analysis. Because ponies #10 and #50 were euthanized before developing a mature immune response, they were not used in the cellular assays.

**Table 5.2 Disease and immune response of variant infected ponies.**

| | Pony | Febrile episodes | Proliferation[b] | CTL[c] | Neutralizing Antibodies[d] |
|---|---|---|---|---|---|
| **EV0 Infected** | A23 | 14dpi[a] | - | - | + |
| | 9913 | 43dpi | - | - | + |
| | 9955 | 28dpi, 106dpi, 111-112dpi, 173-174dpi, 177dpi, 178dpi † | + | - | + |
| | 9961 | 12dpi | + | - | + |
| **EV6 Infected** | #10 | 16dpi, 49dpi, 58dpi, 65dpi † | ND[e] | ND | + |
| | #50 | 17dpi, 63dpi, 99-100dpi, 124dpi, 126dpi, 130dpi † | ND | ND | + |
| | B32 | 16dpi | + | + | + |
| | 956 | 19dpi | + | + | + |
| **EV13 Infected** | 009 | 13-14dpi, 267dpi | + | + | + |
| | 9959 | 14dpi, 49dpi, 74dpi, 105-106dpi, 132-133dpi | + | - | + |
| | A26 | 14dpi, 247dpi | + | - | + |
| | B023 | 14dpi | + | + | + |

† Pony euthanized

a dpi = days post infection

b Proliferation in response to comprehensive Env peptide pool in PBMC from 6-9 months post infection

c CTL in reponse to comprehensive Env peptide pool in PBMC from 6-9 months post infection

d Infecting strain neutralizing antibody development through 9 month observation period

e Ponies euthanized before 6 months post infection, assays were not performed

### 5.3.b Env-specific PBMC proliferation 6 months post infection

To measure the proliferation response of the variant infected ponies, PBMC from >6mpi were used in a thymidine incorporation assay with a variant Env-specific comprehensive peptide pool. PBMC from the EV0 infected ponies underwent limited proliferation in response to the EV0-Env peptide pool (Fig 5.2). Since EV0 shares a homologous Env with $EIAV_{D9}$, for which cellular immune peptides were previously mapped (131, 132), cellular analysis outside of the comprehensive peptide pool was not conducted on these four EV0 infected ponies. The PBMC from the two EV6 infected ponies demonstrated low proliferation responses to the EV6-Env peptide pool. All four of the EV13 infected ponies developed an EV13-Env specific proliferation response by 6mpi, with ponies 9959 and A26 demonstrating the highest

proliferation responses out of the ten infected ponies. The remaining two EV13 infected ponies had similar proliferation responses to the EV6 infected ponies.

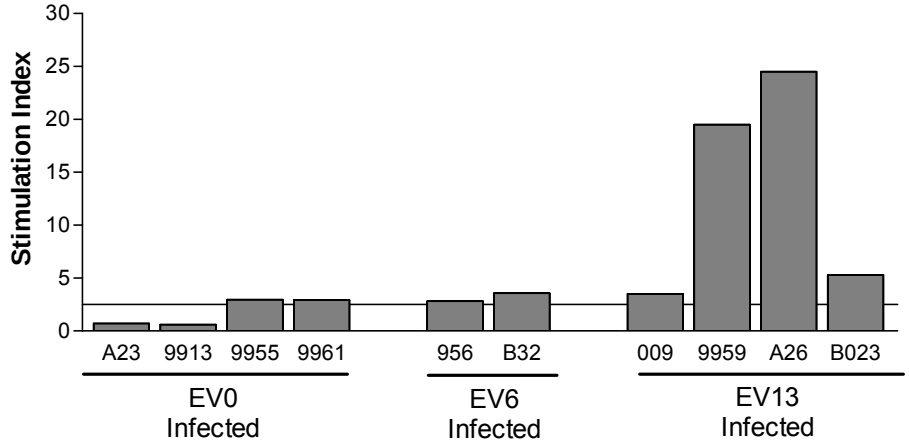


**Figure 5.2 Proliferation responses to variant Env-specific peptide pools.** PBMC from >6mpi were utilized in the thymidine incorporation assay against comprehensive variant Env-specific peptide pools. A proliferation cutoff was set at SI>2.5.

For the EV6 and EV13 infected ponies, variant Env-specific peptide matrix pools were used in the lymphoproliferation assay to scan for potentially reactive proliferative peptides. Twelve EV6 peptides and ten EV13 peptides were indicated in the thymidine incorporation assay using Env-specific peptide matrix pools. To determine the reactivity of each peptide, the indicated peptides were tested individually in the proliferation assay, and the responses were averaged for the two EV6 infected ponies (Fig 5.3A) and the four EV13 infected ponies (Fig 5.3B). PBMC from the EV6 infected ponies proliferated in response to EV6 peptides 7, 9, 25, 27, 35, 43, 49 and 84. For the EV13 infected ponies, there was a positive proliferative response

to EV13 peptides 19, 24, 25, 30, 35, 78 and 84. Due to the limited number of ponies, we could not establish the broadly reactive nature of the identified peptides.

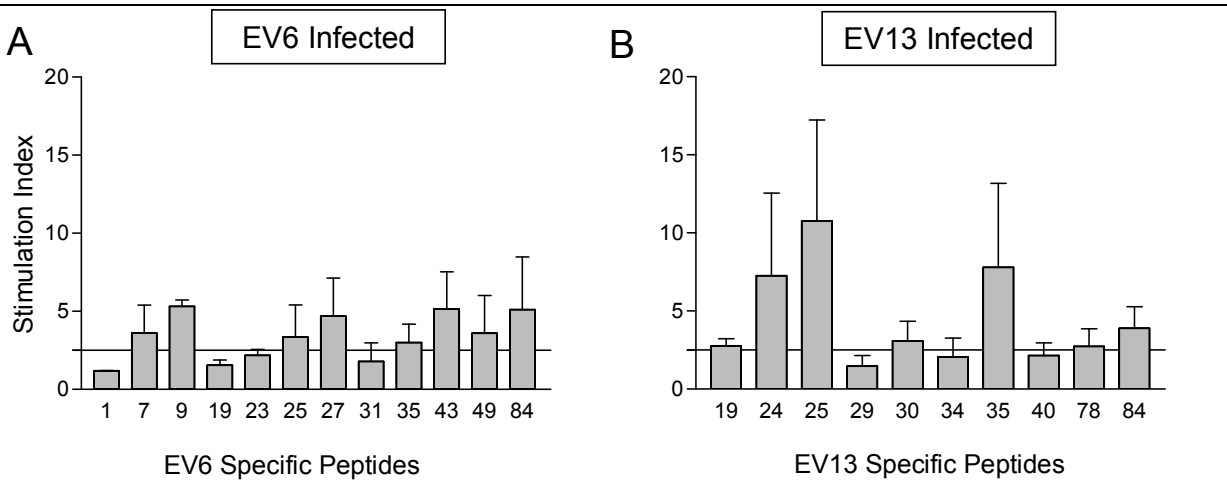


**Figure 5.3 Variant peptide-specific proliferation responses in EV6 and EV13 infected ponies.** PBMC from EV6 and EV13 infected ponies were isolated >6mpi and utilized in the thymidine incorporation assay against matrix indicated, EV6 (A) or EV13 (B) specific peptides, respectively. A positive proliferation cutoff response was set at SI>2.5.

### 5.3.c Effects of Env variation on PBMC proliferation in variant infected ponies

To determine if Env variation affected the proliferation responses elicited during infection with EV6 or EV13, the EV0 peptides corresponding to the variant matrix-indicated peptides were used in the lymphoproliferation assay with PBMC from EV6 and EV13 infected ponies. Due to the limited number of ponies infected with each variant EIAV strain, statistically relevant conclusions could not be established; however, trends in proliferation were noted. Five of the twelve EV6 peptides indicated in the matrix analysis were divergent from EV0, and four of the EV0 peptides (peptides 19, 23, 27 and 31) did not appear to alter PBMC proliferation compared

to the EV6 peptides (Fig 5.4A). For peptide 35, the amino acid differences between EV0 and EV6 seemed to suppress the proliferation response the EV0-35, as SI=1.4 for this peptide while EV6-35 SI=3.

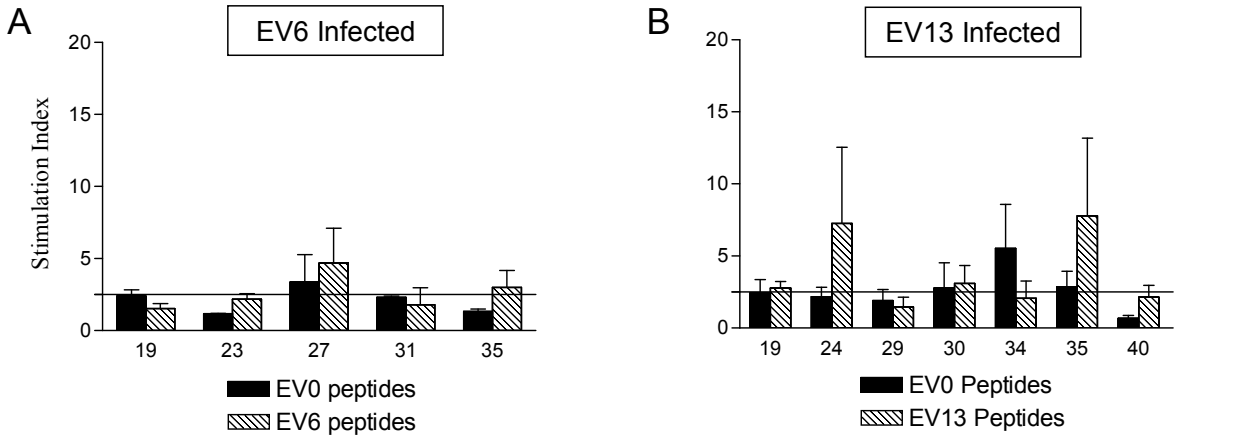


**Figure 5.4 Effects of variation on PBMC proliferation in variant EV6 and EV13 infected ponies.** EV0 peptides that diverged from matrix indicated EV6 or EV13 peptides were tested against PBMC from EV6 (A) and EV13 (B) infected ponies in the thymidine incorporation assay. A positive proliferation cutoff was set at SI>2.5.

Seven of the ten matrix indicated EV13 peptides had variable amino acids from EV0, and three of the EV0 specific peptides appeared to alter the proliferation response compared to the EV13 specific peptides (Fig 5.4B). EV13 infected ponies responded to EV13-24 and EV13-35 better than the EV0 complementary peptides. Conversely, EV13 infected ponies appeared to respond better to EV0-34 compared to EV13-34. The remaining EV0 specific peptides did not seem to alter PBMC proliferation of the EV13 infected ponies.

### 5.3.d Effects of Env variation on PBMC proliferation in $EIAV_{D9}$ vaccinated ponies

To understand the effects of Env variation on PBMC proliferation in vaccinated ponies, DOC PBMC from the $EIAV_{D9}$ vaccinated ponies challenged with EV6 or EV13 were used in the thymidine incorporation assay against variant-specific peptides. Challenge strain-specific peptide matrix pools indicated 11 EV6 and nine EV13 specific peptides for individual analysis in the lymphoproliferation assay. Due to the low peptide specific proliferation responses from the vaccinated ponies, it was difficult to ascertain the effects of amino acid variation on Th recognition. With the exception of peptides 6-34 (Fig 5.5A) and 13-27 (Fig 5.5B), it appeared that proliferation was not affected by the amino acid variations of the selected peptides. The amino acid changes in peptides 6-34 and 13-27 appeared to block the proliferative responses compared to the EV0 complementary peptides, although not to a significant level (T-test p-values 0.0600 and 0.2323, respectively). These data suggest that the sequence variation of these specific peptides does not affect PBMC proliferation, at least within the limits of the thymidine incorporation assay.

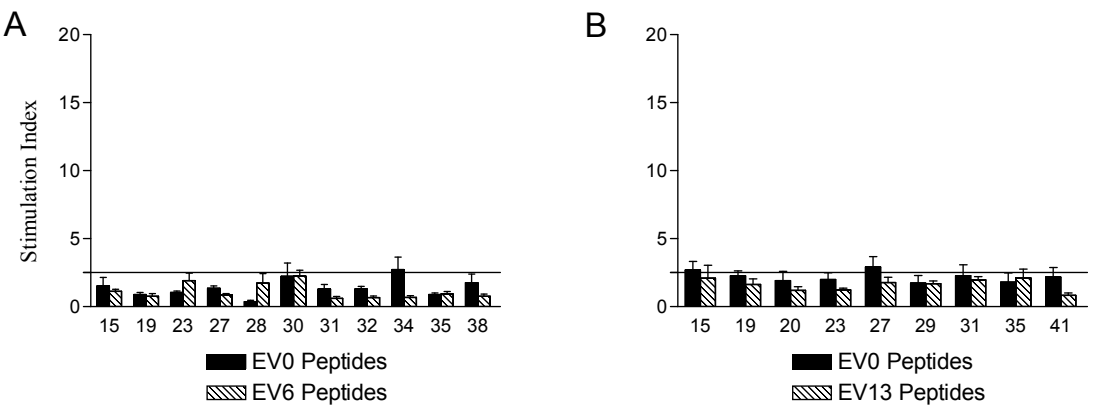


**Figure 5.5 Effects of variation on PBMC proliferation in $EIAV_{D9}$ vaccinated ponies.** Day of challenge PBMC from EV6 and EV13 challenged ponies were tested against EV6 or EV13 specific matrix pools to identify potentially reactive peptides. The EV6 (A) and EV13 (B) peptides were tested against the PBMC in the thymidine incorporation assay to determine the effects of variation on proliferation. Positive response SI>2.5.

### 5.3.e Env-specific cytolytic activity 6 months post infection

The CTL response of the EV0, EV6 and EV13 infected ponies was determined using variant-specific comprehensive Env peptide pools in a chromium release assay with PBMC from >6mpi. The PBMC from the EV0 infected ponies did not have cytolytic activity at the time points tested (Fig 5.6) and were not included in further CTL assays. PBMC from the two EV6 infected ponies demonstrated low cytolytic responses to the EV6-Env peptide pool. Two of the four EV13 infected ponies, 009 and B023, had high CTL responses, while the remaining two did not have cytolytic activity at the time point tested. EV13 infected ponies 9959 and A26 were therefore dropped from further CTL analysis.

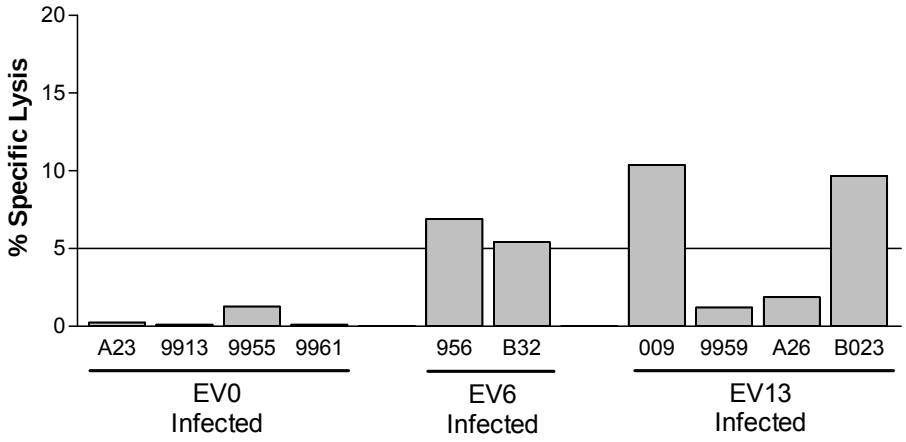


**Figure 5.6 Cytolytic responses to variant Env-specific peptide pools.** PBMC from >6mpi were utilized in the chromium release assay against comprehensive variant Env-specific peptide pools. A positive %SL cutoff was set at %SL>5.

PBMC from the EV6 and EV13 infected ponies were tested against variant Env-specific peptide matrix pools in a chromium release assay to perform an inclusive scan of possible CTL peptides. The matrix analysis identified 16 EV6-specific peptides and ten EV13-specific peptides to be tested individually. The %SL of each peptide for the two EV6 infected ponies were averaged and only peptides 37 and 84 had positive CTL responses (Fig 5.7A). When the %SL of each peptide was averaged for the two EV13 infected ponies with detectable CTL activity, peptides 8, 14, 21, 27 and 63 were positive for cytolytic activity (Fig 5.7B).

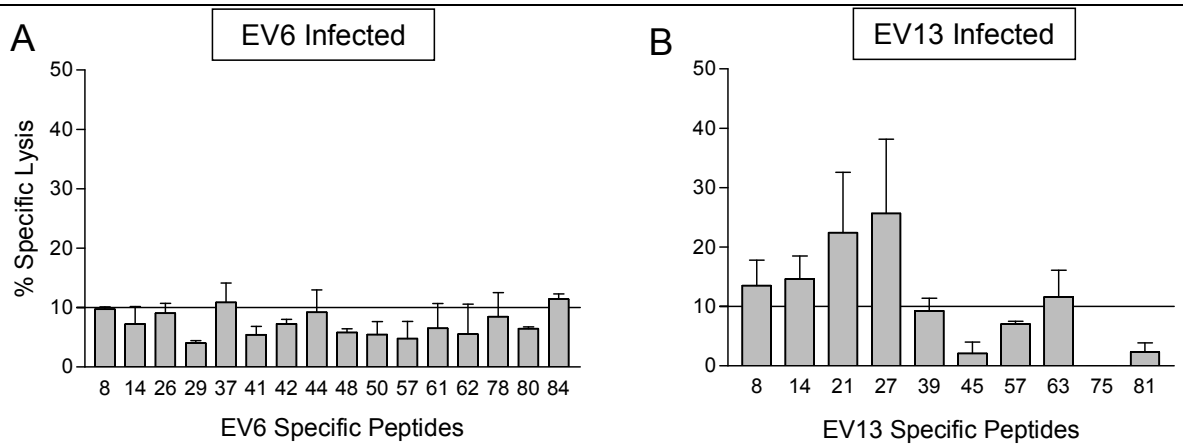


**Figure 5.7 Variant peptide-specific CTL responses in EV6 and EV13 infected ponies.** PBMC from EV6 and EV13 infected ponies were isolated >6mpi and utilized in the chromium release assay against matrix indicated, EV6 (A) or EV13 (B) specific peptides, respectively. A positive cytolytic cutoff response was set at %SL>10.

### 5.3.f Effects of Env variation on CLT lysis in EV6 and EV13 infected ponies

To determine if Env variation affected the CTL response in variant infected ponies, PBMC from EV6 and EV13 infected ponies were tested against EV0-specific peptides in a chromium release assay. Only two of the 16 EV6 matrix-indicated peptides varied from EV0, and four of the ten EV13 matrix-indicated peptides were divergent from EV0. The limited number of infected ponies with CTL reactivity prevented the data from achieving statistical relevance; however, there were noticeable trends in the data. While EV0-14 did not alter cytolytic activity compared to EV6-14, EV0-37 appeared to reduce the level of CTL lysis compared to EV6-37 (Fig 5.8A). The %SL in response to EV0-37 was 5.6 while the response to EV6-37 was %SL=10.9. For the EV13 infected ponies, only EV0-27 appeared to reduced the level of cytolytic activity compared

to the EV13 specific peptides, %SL=2 compared to 25.7% (Fig 5.8B). The remaining divergent peptides did not seem to alter CTL activity.

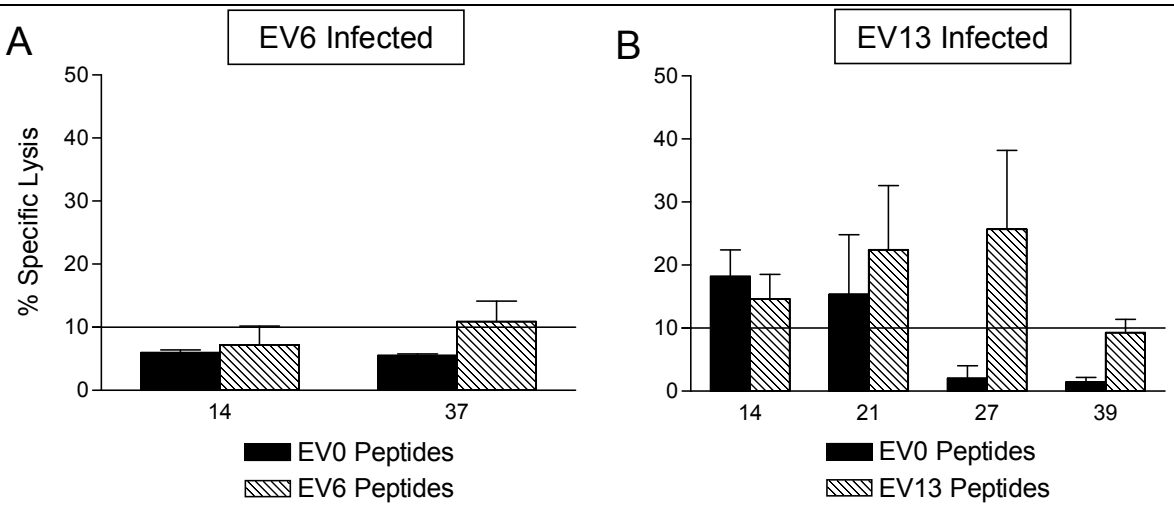


**Figure 5.8 Effects of variation on CTL lysis in variant EV6 and EV13 infected ponies.** EV0 peptides that diverged from matrix indicated EV6 or EV13 peptides were tested against PBMC from EV6 (A) and EV13 (B) infected ponies in the chromium release assay. A positive cytolytic response cutoff was set at %SL>10.

### 5.3.g Effects of Env variation on CLT lysis in $EIAV_{D9}$ vaccinated ponies

To gain better insight into the effects of Env variation on immune recognition in vaccinated ponies, DOC PBMC from the $EIAV_{D9}$ vaccinated ponies challenged with EV6 or EV13 were utilized in a chromium release assay against variant-specific Env peptides. Challenge strain-specific matrix pools identified nine EV6 and 11 EV13 specific peptides in the chromium release assay. Three of the peptides identified with the variant matrices were CTL peptides associated with vaccine protection from disease. To ascertain if these peptides were reactive against DOC

PBMC from the vaccinated ponies, the variant specific peptides were tested against the DOC PBMC (Fig 5.9).

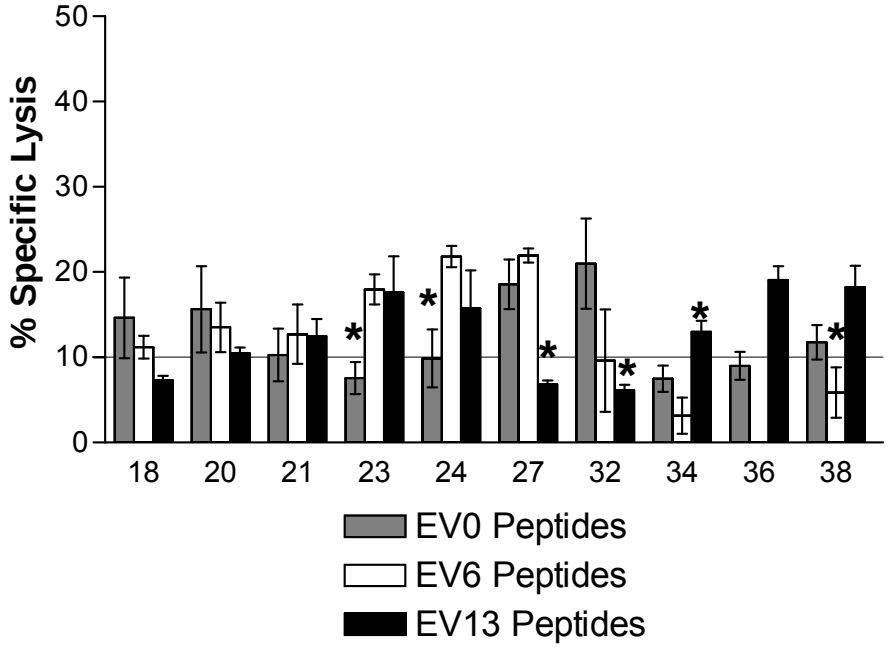


**Figure 5.9 Effects of variation on CTL lysis in $EIAV_{D9}$ vaccinated ponies.** Day of challenge PBMC from EV6 and EV13 challenged ponies were tested against EV6 or EV13 specific matrix pools in the initial screening for reactive peptides. Variant peptides corresponding to amino acid differences between EV0, EV6, and EV13 were used in the chromium release assay against DOC PBMC from EV6 and EV13 challenged ponies. Statistically significant changes in reactivity compared to the EV0 complementary peptide are indicated by with *.

The amino acid variations for peptide 21 did not affect CTL activity. For peptides 18 and 20, the amino acid changes did influence specific lysis; as the sequence diverged from EV0, cytolytic activity was incrementally lost (correlation coefficient r-value -0.9999 and -0.9982,

respectively). With some peptides, such as peptide 32, CTL activity can be completely negated with divergence from EV0-32. While the reduction in CTL response to peptide EV6-32 is not statistically significant, the loss of response to EV13-32 is significant (T-test p-values 0.2134 and 0.0371, respectively). For EV0-27, CTL specificity is lost only against EV13-27 (T-test p-value 0.01); while EV6-27 maintains the same level of CTL activity as EV0-27. The sequence changes in peptide EV6-38 causes a loss of CTL reactivity, but CTL lysis is not hindered with the amino acid differences present in the EV13 variant, indicating sequence variation does not always equate with loss of CTL response. In fact, amino acid changes can lead to gain of CTL recognition, as observed with the variants for peptides 23 and 24. Additionally, the EV13 variants of peptides 34 and 36 also caused a gain of CTL reactivity (T-test p-values 0.0321 and 0.0122, respectively) (Fig 5.9).

**5.3.h Neutralizing antibody response is variant infected ponies**

An ELISA based infectious center assay was used to monitor the development of variant specific neutralizing antibodies in monthly serum samples from each infected pony. Three of the four EV0 infected ponies developed EV0 specific neutralizing antibodies by 90dpi. The titer for 9913 was minimally positive, never exceeding 1:40, while ponies 9961 and 9955 sustained titers ~$1:10^2$ (Fig 5.10A). The fourth EV0 infected pony developed a neutralization titer more slowly compared to the other three EV0 infected ponies, but sustained a comparable titer to 9961 and 9955. EV6 specific neutralizing antibodies were detected in EV6 infected pony 956 by 30dpi (Fig 5.10B), while the remaining EV6 infected ponies did not develop significant titers until 60dpi. Ponies 956 and B32 maintained their titers around $1:10^2$-$10^3$. The titer for pony #50 peaked around $1:10^3$ on day 60, corresponding to the second febrile episode for this pony. While

#50 maintained a neutralization titer at 1:$10^2$, the disease signs were severe, and the pony was euthanized at 130dpi. Pony #10 achieved a similar neutralization titer compared to pony #50, shortly before euthanasia at 65dpi. Three of the four EV13 infected ponies had detectable neutralization titers against EV13 by 90dpi which were maintained above cutoff for the remainder of the observation period (Fig 5.10C). Pony B023 did not develop neutralizing antibodies until 150dpi, but eventually achieved the titers of the other EV13 infected ponies.

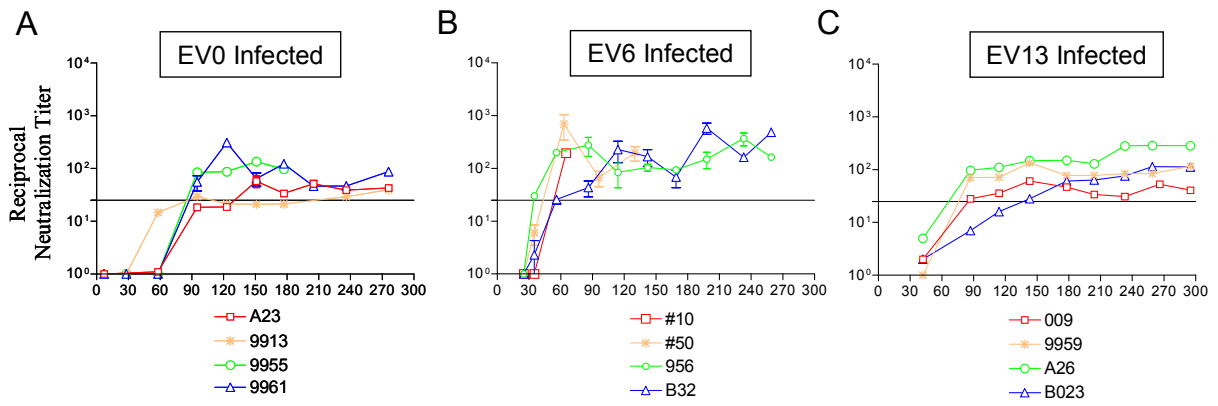


**Figure 5.10 Neutralizing antibody responses in variant infected ponies.** A cell-based ELISA was utilized to measure the development of neutralizing antibodies throughout the course of infection with EV0, EV6, or EV13. A positive neutralization cutoff was set at a titer of 1:25.

### 5.3.i Specificity of neutralizing antibodies

Since these variants were developed from a pony chronically infected with $EIAV_{PV}$ (35, 36, 77), post immune suppression serum from that pony was used in the neutralization assay to determine if cross-reactive antibodies were present. The pony was infected with $EIAV_{PV}$, which shares a homologous Env with EV0; EV6 was developed from an isolate from the fourth febrile episode,

and EV13 was developed from a pre-immune suppression isolate. Post-immune suppression serum from the infected pony had a very high neutralization titer to EV0 and was also capable of neutralizing EV6 (Fig 5.11A). Serum neutralization was detected against EV13, but it did not reach the positive cutoff of 1:25.

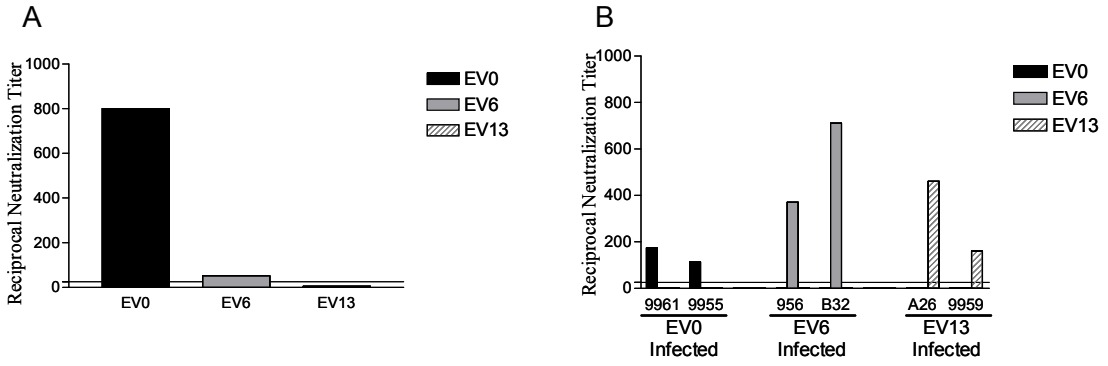


**Figure 5.11 Cross-reactivity of neutralizing antibodies from infected ponies.** (A) Post immune suppression serum neutralization of the pony from which EV6 and EV13 were isolated. (B) Sera from variant infected ponies were tested against the three EIAV Env variant strains and were only able to neutralize homologous virus.

To determine if ponies infected with EV0, EV6, or EV13 developed cross-reactive antibodies, sera from selected ponies were tested against the three challenge strains. Serum samples were chosen from ponies demonstrating a mature immune response with high neutralization titers. Therefore, EV0 infected ponies 9961 (276dpi) and 9955 (151dpi), EV6 infected ponies 956 (233dpi) and B32 (198dpi), and EV13 infected ponies A26 (295dpi) and 9959 (295dpi) were selected for the cross-reactivity studies. With the ELISA based infectious center assay, no cross-reactive neutralizing antibodies were detected against the three variant

strains (Fig 5.11B). The neutralizing antibodies developed during infection with these variant strains were highly strain specific, and incapable of neutralizing the other two variants.

Finally, to determine if EIAV vaccinated ponies developed cross-reactive neutralizing antibodies, DOC and 4wpc sera from $EIAV_{D9}$ vaccinated ponies challenged were tested against the three challenge strains. Sera from the vaccinated ponies were only able to neutralize the homologous Env, EV0. Even in the ponies that became infected by 4wpc, variant challenge strain specific neutralizing antibodies did not develop (Fig 5.12).

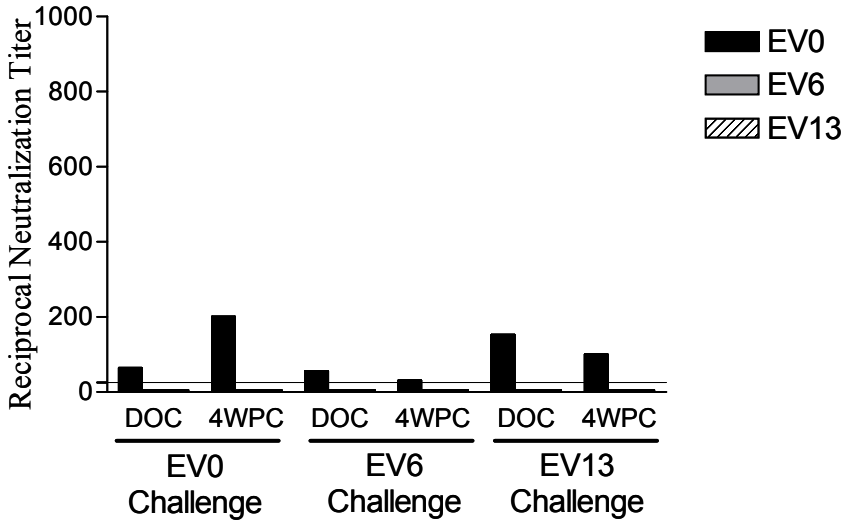


**Figure 5.12 Cross-reactivity of neutralizing antibodies from $EIAV_{D9}$ vaccinated ponies.** DOC and 4wpc sera from each $EIAV_{D9}$ vaccinate pony was tested against each challenge strain to determine the level of cross-reactivity. Only EV0 specific neutralizing antibodies were detected. The graph shows a representative pony from each challenge group.

### 5.3.j Neutralizing antibodies directed against the V3 and V4 region of Env

It was previously established that neutralizing antibodies evolve to recognize the V3 and V4 regions of Env during persistent $EIAV_{PV}$ infection (60, 76, 77). To determine if the V3 and V4 regions of EV0, EV6 and EV13 confer neutralization specificity to each variant Env strain, chimeras were constructed by switching the V3 to V4 region of EV0 and EV6 (c.f. Fig 5.1B). The chimeric constructs between EV0 and EV13 did not produce viable virus, but a colleague in the lab, Dr. Chengqun Sun, developed a construct containing the EV13 N-terminus in the EV0 backbone. This construct contained the V3-V4 region of EV13 and an additional amino acid change at 146 of gp90, where EV13 contained an isoleucine (I) residue and EV0 contained a methionine (M) residue. We felt this amino acid would substitution would not alter the neutralization phenotype and used this clone, termed EV0v13, in the neutralization analysis.

Mature immune serum demonstrating high level neutralization activity was chosen for the analysis of neutralization specificity. Based on these criteria, EV0 infected pony 9961 at 276dpi, and EV6 infected pony B32 at 198dpi, and EV13 infected pony A26 at 295dpi were chosen for the study. The serum from EV0 infected Pony 9961 was capable of equally neutralizing EV0 and EV6v0, with titers just below 1:200 (Fig 13A). In comparison, the neutralization titer against EV0v6 was significantly lower, at 1:40, and there was no neutralization of EV6 with serum from 9961. Serum from EV6 infected Pony B32 had very high neutralization titers against EV6 and EV0v6 (~1:600 and ~1:700, respectively), but was unable to neutralize EV0 or EV6v0. For the EV0v13 chimeric clone, serum from A26 was capable of neutralizing EV13 and EV0v13, albeit to a lesser extent (~1:300 and ~1:150, respectively) (Fig 13B). Serum from EV0 infected pony 9961 was only able to neutralize EV0; the titer to EV0v13

was considerably weaker (~1:170 compared to ~1:30). Collectively, these data indicate the serum neutralizing antibodies detected in these assays are primarily directed against the V3-V4 region of gp90.

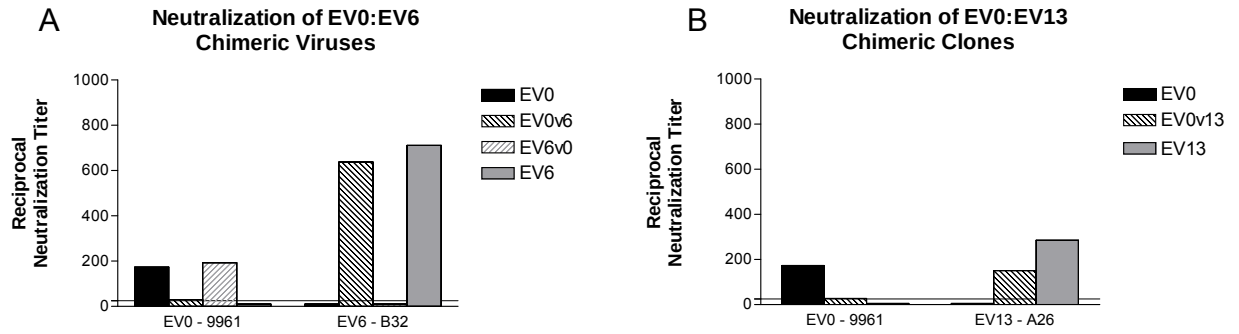


**Figure 5.13 Neutralizing antibodies directed against the V3-V4 region of gp90.** Chimeric viruses were constructed by switching the V3-V4 region of EV0 and EV6 and by placing the N-terminus to V4 region of EV13 into the EV0 backbone. (A) Serum samples from EV0 infected pony 9961 and EV6 infected pony B32 were tested against the EV0:EV6 chimeric constructs in the neutralization assay. (B) Serum samples from EV0 infected pony 9961 and EV13 infected pony A26 were tested against the EV0v13 virus.

## 5.4 DISCUSSION

Lentiviruses escape immune recognition through alterations in key immune epitopes of viral proteins, and the level of viral replication is high enough in untreated individuals that every possible point mutation in the entire viral genome arises on a daily basis (11). These point mutations permit the virus to escape neutralizing antibody and T-cell mediated immune responses allowing the virus to persist in the infected host indefinitely. Due to fitness constraints on many viral proteins, the majority of escape mutations occur in the viral Env. The effects of Env variation in EIAV infected ponies is easily observed during the chronic phase of infection, where escape mutants induce new febrile episodes (76, 77). Viral quasispecies isolated during sequential febrile episodes are distinct from the preceding episode, indicating the virus has escaped immune control to cause disease. Horses eventually gain enduring control of viral replication and become inapparent carriers of EIAV.

With the cellular peptide mapping of the $EIAV_{D9}$ Env proteins in horses and the identification of Env-specific peptide responses associated with vaccine protection from disease, we sought to examine the effects of natural Env variation on these defined immune responses. Since the variant challenge of $EIAV_{D9}$ vaccinated ponies demonstrated Env to be a primary determinant of vaccine efficacy, we hypothesized that the divergent amino acids in the two variant strains would alter the immune responses compared to EV0. Ponies infected with these variants all developed acute episodes, with some becoming chronically infected. In the proliferation assays, strain specific matrix pools were utilized to identify potentially reactive peptides, and some of the peptides identified were divergent from the EV0 peptide sequence. When both EV0 and variant-specific peptides were tested in the thymidine incorporation assay, the effects of variation on PBMC proliferation were minimal, with many of the EV0 peptides not

altering proliferation compared to the homologous variant-peptides. With the limited number of ponies infected with the variant strains of EIAV, we could not conclude if the proliferative responses in each infected pony were cross-reactive with EV0 specific peptides. However, the proliferation to peptides EV0-24, EV0-34 and EV0-35 suggest that Env variation can affect PBMC proliferation in infected ponies. Additionally, the proliferative responses in the $EIAV_{D9}$ vaccinated ponies indicated that the amino acid variations in peptides EV6-34 and EV13-27 were sufficient to alter proliferation. The low level proliferation of the $EIAV_{D9}$ vaccinated ponies prevented a more stringent analysis of the effects of amino acid variation on PBMC proliferation. With this limited data, we were unable to conclusively determine the effects of Env variation on PBMC proliferation; perhaps if a more discriminating assay were used, changes in reactivity could be observed. Additionally, the thymidine incorporation assay only measures T cell proliferation; without assays for equine cytokine expression, we cannot ascertain the effects of Env variation on other Th cell responses.

CTL escape mutations of lentiviral epitopes have been widely reported (22, 40, 47, 78, 96, 111). To understand the effects of amino acid variation on CTL recognition in EIAV infected and vaccinated ponies, PBMC were tested against variant peptides to determine the reactivity. In the EV6 and EV13 variant infected ponies, peptide specific cytolytic activity was only affected by two EV0-specific peptides. However, when PBMC from the $EIAV_{D9}$ vaccinated ponies were used, profound effects on CTL reactivity were observed. The amino acid variation required to alter cytolytic activity did not need to be extensive, as one or two amino acid changes was enough to lose CTL reactivity, as with peptides 32 and 38 (Table 5.3).

**Table 5.3 Variant peptide sequences and the effects on CTL reactivity in vaccinated ponies.**

| Peptide | Peptide Sequence | CTL Reactivity |
|---|---|---|
| EV0-18 | DSDHCQEYQCKKVNLNSSDS | ++ |
| EV6-18 | **********LNSF | + |
| EV13-18 | **********FTV*KA | - |
| | | |
| EV0-20 | SNP------VR-VEDVMNTTEYWGFKW | ++ |
| EV6-20 | DSS------IH-****KD********* | + |
| EV13-20 | NGSSIPSIHVGG***AE**I******* | + |
| | | |
| EV0-21 | NTTEYWGFKWLECNQTENFK | + |
| EV6-21 | D****************** | + |
| EV13-21 | **I***********L* | + |
| | | |
| EV0-23 | TILVPENEMVNINDTDTWIP | - |
| EV6-23 | **********N****A | ++ |
| EV13-23 | **********K*KN-G**T* | ++ |
| | | |
| EV0-24 | NINDTDTWIPKGCNETWARV | - |
| EV6-24 | ****N****A********** | +++ |
| EV13-24 | K*KN-G**T********** | ++ |

| Peptide | Peptide Sequence | CTL Reactivity |
|---|---|---|
| EV0-27 | IHPIRLCVQPPFFLVQEKGI | ++ |
| EV6-27 | **********EV | +++ |
| EV13-27 | **********NRG | - |
| | | |
| EV0-32 | ACNVSRLKINRKDYTGIYQV | +++ |
| EV6-32 | *****H*R********** | - |
| EV13-32 | ****RK*G********** | - |
| | | |
| EV0-34 | PIFYTCNFTNITSCNNEPII | - |
| EV6-34 | **********S** | - |
| EV13-34 | *M*******S*****D*S*T | + |
| | | |
| EV0-36 | SVIMYETNQVQYLLCNNNNS | - |
| EV13-36 | **********KY*TT | ++ |
| | | |
| EV0-38 | NNYNCVVQSFGVIGQAHLEL | + |
| EV6-38 | *H********** | - |
| EV13-38 | DSN*T********** | ++ |

Without knowing critical anchor residues needed for binding the peptides to the MHC I molecules, we cannot determine if the cytolytic response was altered due to decreased binding affinity of the peptide to the MHC I molecule, or the inability of the T-cell receptor to recognize the peptide-MHC complex (30, 112).

It is established that N-linked glycosylation can affect antibody recognition, but glycosylation may also interfere with peptide processing and presentation on MHC I molecules (17, 41, 62, 130). While it was desirable to determine the role of N-linked glycosylation in CTL recognition, it was not possible to access the effects of glycosylation on cytolytic activity with this panel of peptides. First, heavily glycosylated areas of Env were excluded from cellular recognition, potentially indicating that N-linked glycosylation may inhibit cytolytic activity. Second, instead of gaining/losing N-linked glycosylation sites in the variant peptides, many of the glycosylation sites were shifted up or down stream. The peptides that did gain/lose a glycosylation site did not offer a clear role of glycosylation in CTL recognition. Again, without

knowing critical anchor residues, it cannot be determined if cytolytic activity was affected by the glycosylation site or by the changing of a critical anchor residue.

The most profound effects of Env variation on immune recognition were observed in the neutralization response. In ponies infected with the variant strains, only strain-specific neutralizing antibodies were detected that were unable to cross-neutralize the other variant EIAV strains. Using chimeric constructs, neutralizing specificity of EV0 and EV6 mapped to the V3 to V4 region of gp90, as previously reported for $EIAV_{PV}$ (59, 60). The chimeric construct containing the EV13 N-terminus half in the EV0 backbone was less clear, as serum neutralization sensitivity from an EV13 infected pony was not fully restored against EV0v13. While serum from the EV13 infected pony was capable of neutralizing EV0v13, the neutralization titer was approximately half of the titer of EV13. This observation has previously been reported (60), and has been attributed to the dominance of the neutralization resistant phenotype, indicating areas outside of V3-V4 may also contribute to Env neutralization resistance.

The lack of cross-neutralization between the variant infected ponies was a surprising result, as there was minimal variation between the three strains. It is assumed that inapparent carrier ponies of EIAV are protected from further EIAV challenge due to broadly cross-neutralizing antibodies. Even $EIAV_{D9}$ vaccinated ponies protected from virulent challenge against EV6 or EV13 did not have detectable cross-reactive neutralizing antibodies at day of challenge or four weeks post challenge. These results may suggest either neutralizing antibodies do not play an important role in protection, or our *in vitro* neutralization assay cannot detect the cross-reactive neutralizing antibodies. The later explanation is most likely correct as *in vitro* assays do not always correlate 100% with *in vivo* responses.

These studies demonstrate that very little Env variation is sufficient to alter cellular and humoral immune responses in EIAV infected and vaccinated ponies. Additional studies need to be performed with more animals to gain better insight into the true effects of Env variation on immune recognition. These studies do suggest that more conserved regions of Env should be targeted in future vaccine trials, as variations in CTL and neutralizing antibody epitopes may ablate the protective vaccine response.

## 5.5 ACKNOWLEDGMENTS

I would like to thank Baoshan Zhang for providing the variant DNA and also for assisting in the design and development of the chimeric clones. I would like to thank Chengqun Sun for providing the EV0-EV13 chimeric construct used to map neutralization sensitivity. Additionally, I would like to thank Jodi Craigo for organizing the variant infections, and Shannon Barnes and Tim Sturgeon for assisting with the blood processing.

# 6.0 SUMMARY AND FUTURE DIRECTIONS

The extensive research and public health programs aimed at combating HIV have been unable to weaken the deathly grip of the AIDS epidemic. HIV continues to infect millions of individuals worldwide, with five million new HIV infections in 2005. While sub-Saharan Africa continues to be to most affected area, Eastern Europe and Central Asia have witnessed the steepest rate increase in HIV infections (2). Although antiretroviral drugs have improved the length and quality of life for HIV infected individuals in developed countries, the best hope for stopping the global spread of new HIV infections lies in the development of a protective vaccine. Empiric approaches have largely failed in developing HIV vaccines, proving that a better understanding of protective immunity is essential to developing an effective lentiviral vaccine. Due to the complexities in conducting human trials for HIV vaccine studies, lentiviral vaccine research must rely on animal models systems to unravel the immune responses necessary for protective immunity.

While the SIV/monkey system has been the "gold standard" for evaluating protective vaccine immunity, non-human primate models have also proven to be beneficial at elucidating appropriate anti-lentiviral immune responses. FIV and EIAV have added tremendous insight in

the development of lentiviral vaccines by demonstrating major pitfalls of potential vaccines, including vaccine induced antibody enhancement of disease (128, 136) and the inability to achieve sterilizing immunity (32). While sterilizing immunity is the ultimate goal of lentiviral vaccine research, it is becoming increasingly clear that that level of protection may not be a possibility. Even when apparent sterilizing immunity was achieved against homologous challenge, we demonstrated through immune suppression that the EIAV vaccinated horses were indeed infected with the challenge strain (32). If sterilizing immunity is not possible, a vaccine capable of limiting virus replication and preventing disease would be beneficial in halting the spread of new infections. Unfortunately, we were unable to protect ponies from disease against challenge strains which were just 6% or 13% divergent in gp90 (36). This does not bode well for HIV vaccine research as lentiviruses may diverge in the surface glycoprotein by more than 30-40% (3).

The $EIAV_{D9}$ vaccine trial also illustrated that Gag and Pol specific immune responses alone may not be sufficient in protecting vaccinated ponies from disease after virulent challenge. This is problematic as many of the current trial HIV vaccines only contain the more conserved Gag and Pol proteins. We cannot conclude that Gag and Pol specific immune responses are not necessary for protective vaccine efficacy, just that these specific responses are not sufficient to protect EIAV vaccinated ponies from disease after challenge. Up to recently, Env antigens have been neglected as viable vaccine candidates due to the extensive variability of the proteins. However, we have identified Env specific immune responses that were significantly associated with vaccine protection from disease. These peptide based responses should be evaluated in more vaccine trials to determine their true correlation with vaccine protection. These peptide

specific responses may serve as valuable vaccine targets that could potentially protect vaccinated ponies from disease or infection.

## 6.1 SUMMARY OF RESULTS AND POTENTIAL IMPLICATIONS

Understanding key immune responses necessary for vaccine protection has proven to be an elusive goal for many lentiviral researchers. Some argue that neutralizing antibodies (85, 86, 115) and CTL responses (67, 87, 124) are correlated with vaccine protection in SIV and SHIV, but both claims have been refuted in other studies (95, 102, 103, 127). Now there is a consorted effort by the Live Attenuated Consortium of the International AIDS Vaccine Initiative to identify the mechanisms of protection conferred by attenuated SIV vaccines in monkeys (70). The work presented in this dissertation may be a valuable complement to ongoing SIV and SHIV research as we have utilized the EIAV system to map protective Env immune responses associated with vaccine protection from disease.

### 6.1.a No detectable association between neutralizing antibodies and vaccine protection from disease

The $EIAV_{D9}$ vaccine is capable of eliciting strain specific neutralizing antibodies, however, the antibodies were unable to recognize EV6 or EV13 (131). Additionally, in ponies infected with each challenge strain, the neutralization response was highly strain specific and incapable of cross-neutralizing the other variants. A caveat of our neutralization study is the use of an *in vitro* neutralization assay to measure antibody responses. It is well established that *in vitro* responses do not always correlate with *in vivo* responses. In this regard, we cannot be absolutely certain of

the lack of correlation between neutralizing antibodies and protection, or that cross-reactive antibodies were not produced in the vaccinated and infected ponies.

Additionally, our neutralization assay only detects the ability of antibodies to bind infectious virus and prevent entry into target cells. Outside of neutralization, there maybe other antiviral antibody mechanisms, such as activation of the complement cascade (101), antibody-dependent cell-mediated cytotoxicity (68) and opsonophagocytosis by macrophage (133), that may contribute to viral clearance and reduce infection of new cells. In fact, the broadly neutralizing HIV antibody b12 functions better when the antibody has an intact Fc (58). The Fc portion of antibody allows for complement activation and opsonophagocytosis. The observation that b12 function is enhanced with intact Fc indicates that other antibody mechanisms of action may be contributing to the documented neutralization. Current neutralization assays only measure the ability of an antibody to block entry into target cells. Therefore, new methods to detect different antibody mechanisms of protection need to be developed in addition to a more reliable neutralization assay.

### 6.1.b Env-peptide specific cellular immune responses associated with protective immunity

While we were unable to link neutralizing antibody development with vaccine efficacy, our studies with EIAV have identified one Th and eight CTL peptides apparently associated with vaccine protection from disease after virulent challenge (131). An initial mapping of broadly reactive cellular peptides identified 17 Th and six CTL peptides in $EIAV_{D9}$ vaccinated horses protected from homologous challenge (132). The fact that more of these peptides were not associated with vaccine protection from disease after heterologous challenge suggests that broadly reactive responses do not always correlate with protective immune responses. This may

be a potential pitfall for HIV vaccine development, as many of the broadly reactive immune responses reported on at the AIDS Vaccine 2007 Conference (Seattle, WA) from ongoing HIV vaccine trials may not protect the vaccinated individuals from infection or disease. While we have identified specific peptide responses statistically correlated with vaccine protection, only 21 ponies were involved in the trial. Therefore, it would be beneficial to test the identified peptides in more EIAV vaccinated ponies with different disease outcomes to strengthen the statistical relevance of the study.

The Th peptide and five of the CTL peptides associated with vaccine protection from disease identified in the study were located in more conserved regions of the Env that had identical peptide sequences for the three variant challenge strains. This offers hope in the development of a lentiviral vaccine, as it was previously believed immune responses to Env would be ineffective due to the ability of the virus to develop escape mutants to evade the host immune response (18). Since many of the escape mutations that develop during lentiviral infection revert back to wild type in the absence of selective pressure (40, 47, 78, 111) indicates there is a certain amount of fitness cost to the virus with each mutation. Due to fitness constraints, there are portions of Env that must remain conserved for the protein to function properly (110). Vaccines need to drive immune responses to these regions of virus in order to be efficacious. Since the three variant challenge strains in our studies were developed from an ancestral species, we cannot say that the peptides identified would be conserved across other strains of EIAV. Performing a gp90 alignment between EV0 and a natural isolate of EIAV that is >40% divergent, $EIAV_{PA}$, demonstrates that there is little variation in the amino acid sequences of peptides associated with protective vaccine immunity (Fig 6.1). The majority of the peptides have >70% conservation of the amino acid sequences. The only exception is

peptide 5, where only 45% of the peptide sequence was conserved. From the level of conservation in the peptides associated with vaccine protection, it may be suggested that these peptides are in regions integral to Env structure and function, and should be targeted in future vaccine trials.

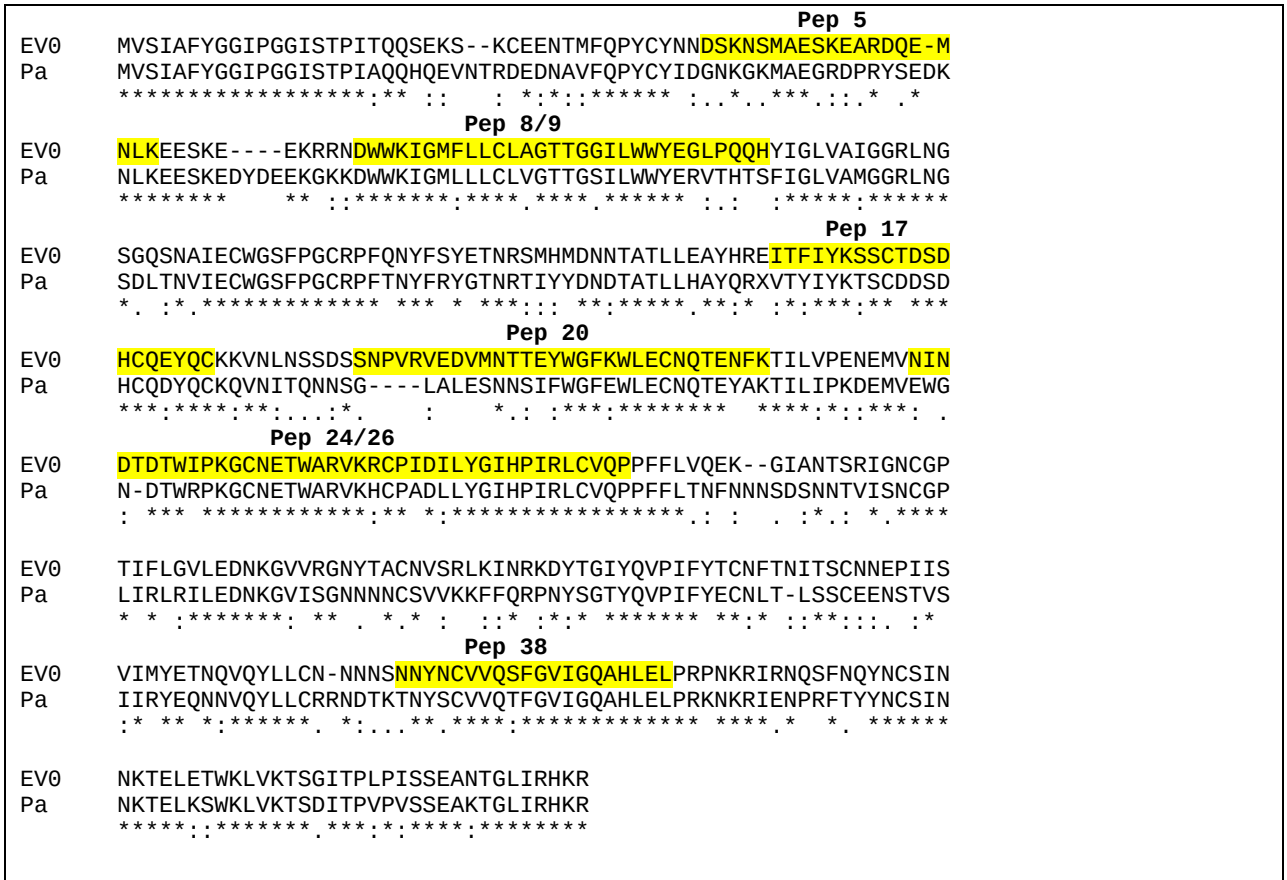


**Figure 6.1 Alignment of gp90 proteins from EV0 and EIAV$_{Pa}$.** EIAV$_{Pa}$ is a natural isolate of EIAV. The CTL peptides associated with vaccine protection from disease are labeled and highlighted in yellow. The amino acid sequence of EIAV$_{Pa}$ in these specific regions is >70% conserved compared to EV0, with the exception of peptide 5.

### 6.1.c Env variation has profound effects on immune recognition

Three of the eight CTL peptides associated with vaccine protection from disease were in predefined variable domains of gp90, specifically the V3, V4, and V7 domains. The cytolytic responses to these three peptides were altered when the complementary variant peptides were utilized in the CTL assay. Peptide 20, which spanned the V3 region, was the most divergent of the CTL peptides, yet each variant was still recognized in the CTL assay, albeit at progressively lower levels (131). The amino acid differences for peptide 20 spanned the entire 20 amino acid sequence. CTL epitopes are typically nine to ten amino acids long; therefore, each potential CTL epitope in peptide 20 contained at least one variable amino acid, suggesting that the $EIAV_{D9}$ vaccine is capable of eliciting CTL responses to epitopes not present in the original inoculum. In addition, most of the CTL and Th responses identified in ponies infected with the three different variant strains were not altered by amino acid variations within specific peptides. Taken together, these data indicate that while Env variation may have profound effects on some immune recognition, the cellular immune response to EIAV is capable of recognizing different versions of the same peptide, as previously reported in an HIV model with Nef epitopes (84).

## 6.2 PEPTIDE STRATEGIES TO ELUCIDATE MINIMAL CELLULAR EPITOPES

It is established that a single amino acid change is enough to abrogate the CTL response to a specific peptide (140) by either decreasing T-cell receptor affinity or by changing critical anchor residues in the peptide (30, 112). Without knowing the residues required for the equine MHC I

molecule to bind peptides, we cannot determine why cellular reactivity to a specific peptide is altered in response to variant peptides. To better understand the effects of amino acid variation on cellular reactivity, the minimal cellular epitope should be defined for each reactive peptide. Then, more in depth studies may be conducted to determine the MHC anchor residues and how amino acid variation affects immune recognition.

Some of the minimal peptide epitopes may be inferred by closer examination of the reactive peptides. If two overlapping peptides are both reactive in a CTL assay, such as peptides 8 and 9, it may be inferred that the epitope is contained in the ten amino acid overlap of the two peptides. Since CTL epitopes are 9-10 amino acids in length, a new ten amino acid peptide containing the region of overlap could be constructed for use in the CTL assay (Table 6.1). In cases where an individual peptide is reactive and the surrounding peptides are not, such as peptide 26, it may be inferred that the central ten amino acids of the reactive peptide contain the minimal epitope. To confirm, consecutively smaller peptides of the 20-mer could be constructed and tested in the cellar assays to identify optimal epitopes. By defining the optimal epitopes, we would be in a better position to describe the effects of Env variation on immune recognition, and how the variation affected peptide presentation and recognition.

**Table 6.1 Peptide strategies to elucidate minimal cellular epitopes.**

| Peptide | Original Peptide Sequence | New Peptide Sequences | Implications[†] |
|---|---|---|---|
| 8<br>9 | DWWKIGMFLLCLAGTTGGIL<br>CLAGTTGGILWWYEGLPQQH | CLAGTTGGIL | (+) peptide contains epitope<br>(-) two separate epitopes for pep 8 and 9 |
| 26 | KRCPIDILYGIHPIRLCVQP | RCPIDILYGIHPIRLCVQ<br>CPIDILYGIHPIRLCV<br>PIDILYGIHPIRLC<br>IDILYGIHPIRL<br>DILYGIHPIR | (+) peptide contains epitope<br>(-) deletion knocked out important amino acid for peptide recognition, previous peptide contained entire epitope |

[†] Potential results if peptides were used in the cellular assays. (+) indicates a positive response in the assay, (-) indicates a negative response in the assay. These results do not include all potential outcomes, just most likely.

## 6.3 PEPTIDE STRATEGIES TO EXAMINE THE EFFECTS OF N-LINKED GLYCOSLYATION ON CELLULAR RECOGNITION

Lentiviruses employ N-linked glycosylation on surface Env proteins to shield the virion from host immune responses. Because areas of heavy N-link glycosylation are often excluded from cellular immune recognition (123, 131, 132), we were unable to fully examine the effects of glycosylation on lymphoproliferation or CTL lysis. While there were glycosylation sites present in some of our identified Env peptides, the effects of glycosylation on cellular immune recognition could not be determined because the complementary variant Env peptides also had glycosylation sites that were shifted either up- or down-stream in the peptide sequence. Additionally, in the peptides that lost glycosylation sites, it was impossible to determine if the different immune responses observed in the cellular assays were due to glycosylation or due to changes in anchor residues attaching the peptide to the MHC molecule.

To clearly examination the affects of glycosylation on cellular recognition the minimal epitope of a peptide containing an N-linked glycosylation site must first be identified. After identifying the minimal epitope, three forms of the peptide with different modifications to the glycosylation signal could be constructed for use in the cellular assays. The first peptide would be the actual sequence encoded by the EIAV *env* gene (nonglycosylated peptide). The second peptide would be a glycosylated analogue synthesized using a commercially available derivative of asparagine (N) bearing a N-acetyl-D-glucosamin moiety (62) (glycosylated peptide). While the addition of a glycosyl group has decreased CTL recognition for some peptides, reactivity to other glycosylated peptides has been unaffected (61). The third peptide would change the asparagine (N) to an aspartic acid (D) residue to represent the naturally processed form of the

peptide (deglycosylated peptide). During peptide processing, a mammalian deglycosylation enzyme converts asparagine to aspartic acid to remove the glycosyl group (42, 130). Researchers have found better CTL reactivity to some deglycosylated peptides from HIV (42), Hepatitis C virus (126), and lymphocytic choriomeningitis virus (LCMV) glycoproteins (61, 62).

The three synthetic peptides could then be used in cellular immune assays to determine the affects of N-linked glycosylation on immune reactivity. Recognition of the nonglycosylated peptide, without reactivity to the glycosylated or deglycosylated peptides, could indicate gp90 is not glycosylated or post-translationally modified at this specific site (41). Recognition of the glycosylated peptide indicates N-linked glycosylation does affect cellular immune reactivity of the specific peptide. Recognition of the deglycosylated peptide could indicate glycosylation does not affect cellular reactivity, but the glycosyl group must first be removed before proper recognition of the peptide can occur. Conversely, failure to recognize the deglycosylated peptide may indicate that asparagine is a critical anchor residue for MHC binding. Recognition of all three peptides may imply that the specific glycosylation signal present in the peptide is not always glycosylated and numerous forms of the peptide may exist in the context of gp90. Experimenting with these types of peptides could provide a better understanding of the affects of N-linked glycosylation on cellular immune recognition.

## 6.4 NEW ASSAYS TO CHARACTERIZE THE CYTOKINE PROFILE OF TH AND CTL CELLS

Aside from determining the minimal epitopes of the peptides and the effects of variation on the immune responses, additional studies to understand the cytokine profile of the reactive Th and CTL cells would be beneficial towards understanding the immune responses required for vaccine efficacy. While the cellular assays employed in this dissertation measured the proliferation of Th and the cytolytic activity of CTL, it is now known that polyfunctional Th and CTL responses are required for successful vaccines against chronic infection (57). While other lentiviral systems are capable of routinely measuring cytokine responses, such as IFNγ, TNFα and IL2, these techniques are not possible with EIAV due to the lack of equine specific antibodies for the appropriate cytokines. In respect to measuring the Th response, these techniques are far superior and offer more reliable results compared to the proliferation assay. Having these and other related antibodies could make flow cytometry and ELISpot possible.

While I attempted to develop a flow cytometry assay for Th proliferation, the procedure was hampered by the lack of specific antibodies, and the results from non-specific stains such as CSFE did not correlate with the thymidine incorporation assay. Flow cytometry and ELISpot assays would improve the cellular assays in many ways, including utilizing less cells, more reproducible results, decreasing the amount of time, and allow us to better compare our results with other lentiviral systems, such as HIV and SIV. Equine specific antibodies and reagents are currently in development and, when available, can be used to develop improved assays for lymphoproliferation.

## 6.5 ARE THE ENV PEPTIDE-SPECIFIC IMMUNE RESPONSES SUFFICIENT FOR PROTECTIVE IMMUNITY?

As stated above, only 21 ponies were available to characterize the Th and CTL responses associated with vaccine protection from disease. While this is an excellent starting point, with so few ponies, we cannot definitively conclude that the identified peptide specific responses are sufficient for vaccine protective efficacy. As new vaccine trials are developed, PBMC from the vaccinated ponies should be tested against the Th and eight CTL peptides to determine if there is a definite correlation with vaccine efficacy. Additionally, vaccines could be developed to target these specific responses to determine if they are sufficient for protection from virulent challenge.

### 6.5.a Peptide based vaccines to induce specific cellular responses associated with protective immunity

To determine if the peptide specific immune responses identified in these studies are sufficient for vaccine protection, peptide based vaccines could be utilized that target the specific cellular immune responses. There are several advantages to using peptide based vaccines which include, targeting specific cellular immune response, incorporating multiple cellular epitopes, and using numerous variants of a peptide epitope to account for variability and MHC restriction (13, 88). Despite the numerous advantages, peptide based vaccines to date have been ineffective at mounting lasting cellular or humoral immune responses capable of protecting animals from lentiviral challenge (14). In EIAV, a gp90 peptide was able to transiently induce CTL responses; however, the immunized ponies were not protected from disease or infection after virulent

challenge (119). A SHIV peptide vaccine was capable of eliciting cellular immune responses that protected monkeys from disease but not infection (100).

In spite of these and other similar results, researchers have continued to investigate the potential benefits of peptide based vaccines for HIV. Recently, a multivalent HIV peptide vaccine elicited CD4 and CD8 effector memory cells in mice. This study demonstrated that adding a lipid group to the peptide enhanced the immunogenicity of the vaccine compared to non-lipidated peptides, and negated the need for an adjuvant. The immune responses elicited in this trial were broadly reactive against numerous HIV-1 subtypes, demonstrating that peptide vaccines may provide protection from challenge (7).

There is a potential pitfall for peptide based vaccines: "original antigenic sin". While most commonly associated with antibody responses, "original antigenic sin" has been found to affect CTL responses as well (69, 74, 92). Early escape mutants of poorly conserved CTL epitopes would not allow for the recognition of variant epitopes in different challenge strains (140). One way to limit the effects of original antigenic sin is to develop peptide vaccines that encompass all variants of potential T-cell epitopes (PTE). PTE peptides have recently been used to demonstrate that T-cells are capable of recognizing Nef variants early in infection, even before the peptide sequence is present in the circulating virus in the infected host (84). This suggests that vaccine induced T cells may be able to recognize epitopes both in and outside of the subtypes represented in the vaccine.

### 6.5.b Chimeric challenge of $EIAV_{D9}$ vaccinated ponies to determine which immune responses are necessary for vaccine protection

Aside from peptide based vaccines, a new $EIAV_{D9}$ vaccine trial could be conducted with a chimeric Env EIAV challenge strains to determine specific immune responses required for vaccine efficacy. Nearly 100% of the $EIAV_{D9}$ vaccinated ponies challenged with EV0 were protected from disease, whereas the majority of EV13 challenged ponies developed equine infectious anemia (32, 36). Due to the prominent phenotypical differences between the two strains, EV0 and EV13 would be optimal for constructing chimeric challenge strains. Only three of the eight CTL peptides associated with vaccine protection from disease had different amino acid sequences between EV0 and EV13; the Th peptide and remaining CTL peptides were in more conserved region on Env. One variant CTL peptide was in V3, one was in V4, and the final one was in V7. It was previously found that there was better success at making replication competent chimeric viruses when the V3 and V4 regions were switched together. Therefore, chimeric challenge strains could be constructed by placing the N-terminus to the V4 region of EV13 into the EV0 backbone, and also by switching the V4 to C-terminus half of EV13 into the EV0 backbone (EV0N13 or EV0C13, respectively) (Table 6.2).

**Table 6.2 Chimeric viruses for challenging $EIAV_{D9}$ vaccinated ponies.**

| CHALLENGE STRAIN | OUTCOME | IMPLICATIONS |
|---|---|---|
| EV0  | Protected from disease | Contains protective epitopes |
| EV13  | Not protected from disease | Does not contain protective epitopes |
| EV0C13  | Protected from disease | N-term EV0 epitopes necessary |
| | Not protected from disease | C-term EV0 epitopes or combination of N and C-term EV0 epitopes necessary |
| EV0N13  | Protected from disease | C-term EV0 epitopes necessary |
| | Not protected from disease | N-term EV0 epitopes or combination of N and C-term EV0 epitopes necessary |

$EIAV_{D9}$ vaccinated ponies challenged with EV0N13 not protected from disease would indicate that the cellular epitopes present in the N-terminus of EV0 are important for vaccine protection. Conversely, if the ponies are protected, it would indicate that the cellular epitopes in the C-terminus of EV0 are important for vaccine efficacy. Depending on the results, new chimeric challenge strains could be made with smaller substituted regions to clearly define the minimal epitopes of Env that are necessary for vaccine efficacy.

## 6.6 DETERIMINING THE ROLE OF NEUTRALIZING ANTIBODIES IN VACCINE PROTECTION

In lentiviral vaccine research, the role of neutralizing antibodies in protective immunity is an ongoing debate. While several researchers have found a protective role for neutralizing

antibodies in SIV protection (85, 86, 115), others have been unable to correlate neutralization with protection (95, 102, 103, 127). In our studies, we were unable to correlate vaccine protection from disease with the development of neutralizing antibodies. The above proposed chimeric challenge viruses would help to analyze the contribution of neutralizing antibodies to vaccine protection. Since the strain specific neutralizing antibodies to EV0 and EV13 are not cross-reactive, the EV0N13 chimeric challenge strain, which contains the EV13 PND, would help determine if neutralizing antibodies are important for vaccine protection. If EV0N13 vaccinated ponies are protected from disease, it could potentially indicate that neutralizing antibodies are not necessary for vaccine protection. Alternatively, if the ponies are not protected, it maybe inferred that the neutralization response is essential for protective efficacy. The data would need to be analyzed in the context of the CTL response to determine the contribution of each of arm of the immune system. New challenge strains could then be developed to further dissect the appropriate immune responses required for protective vaccine efficacy.

## 6.7 CONTRIBUTION OF GAG AND POL IN VACCINE PROTECTION

While our $EIAV_{D9}$ variant challenge trial identified Env as a primary determinant of vaccine efficacy, immune responses to other viral proteins may be necessary for complete vaccine protection. Gp90 was the only divergent protein in the challenge strains compared to the $EIAV_{D9}$ vaccine, making the challenge strains homologous to $EIAV_{D9}$ in terms of the Gag and Pol proteins. Even against this homologous challenge, the Gag and Pol specific immune responses were unable to protect the vaccinated ponies from disease. It cannot be conclude that the Gag and Pol specific responses were not necessary for immune protection, as determined for

the Env-specific immune responses, only that Gag and Pol specific immunity was insufficient to protect the ponies from disease. To determine the contribution of the Gag and Pol specific immune responses in vaccine protection from disease, similar studies that parallel the $EIAV_{D9}$ variant challenge trial could be performed where divergent Gag and Pol proteins can be placed in the homologous backbone to develop new challenge strains.

The Montelaro lab has several EIAV isolates with divergent Gag proteins, and is in the process of placing these Gag variants into the EV0 backbone to use as challenge strains for $EIAV_{D9}$. If all of the ponies are protected from disease after virulent challenge, this would indicate that Gag-specific immune responses do no contribute to vaccine protection. However, if protective efficacy is lost, as observed in the Env variant challenge, Gag-specific immune responses could be associated with vaccine protection. Depending on the outcome of the variant Gag challenge, mapping experiments with Gag-specific peptides could be performed to identify peptide specific immune responses associated with vaccine protection from disease. Similar studies could also be performed using Pol variants of EIAV. These types of studies would aid in the development of more effective EIAV vaccines and provide a better understanding of the immune responses needed for a protective lentiviral vaccine.

## 6.8 INVESTIGATING THE EFFICACY OF POLYVALENT AND CONSENSUS ENV VACCINES

While sterilizing immunity is desirable for a lentivirus vaccine, our current understanding of protective lentiviral immunity is not sufficient to develop such an effective vaccine. A vaccine capable of decreasing viral load and protecting from disease maybe a beneficial alternative to

sterilizing immunity and may aid in halting the spread of HIV to uninfected individuals. While the $EIAV_{D9}$ vaccine was incapable of producing sterilizing immunity, improvements could be made to the vaccine provide better protective efficacy of the vaccine. One potential way to induce better protection from disease would be to use a polyvalent attenuated $EIAV_{D9}$ vaccine or a consensus Env $EIAV_{D9}$ vaccine. Polyvalent vaccines incorporate divergent repeats of the same antigen and/or a number of different antigens to elicit a protective immune response against different primary isolates. Consensus vaccines are synthetic protein sequences based on the viral population (88). While such techniques have been successful in preventing pneumococcus, polio and influenza (129); HIV researchers have found limited success in protecting polyvalent vaccinated animals from heterologous challenge (8, 19, 49, 74, 82, 106, 125). While these types of vaccines are unable to elicit sterilizing immunity, they are capable of increasing the breadth of the immune response while increasing the time till the onset of disease.

To construct a polyvalent vaccine, attenuated EV6 and EV13 viruses could be constructed by deleting the S2 protein as performed in $EIAV_{D9}$. Then $EIAV_{D9}$ with the new $EV6_{D9}$ and $EV13_{D9}$ strains could be used to inoculate ponies in our standard vaccination protocol. Additionally, a consensus sequence could be constructed from the three variant Env sequences and placed into the $EIAV_{D9}$ backbone for immunization. An inoculum composed of equal parts EV0, EV6 and EV13 could then be used to challenge the polyvalent or consensus vaccinated ponies to evaluate the protective efficacy of the immunization. This challenge strategy more closely mimics natural exposure to lentiviruses, which are not single isolates, but a quasispecies. As the polyvalent inoculum has not been tested against the $EIAV_{D9}$ vaccine, it would be of interest to determine the protective efficacy of the $EIAV_{D9}$ vaccine against this challenge. In the variant challenge trial of $EIAV_{D9}$ vaccinated ponies, the ponies were

challenged with a very high dose of each variant strain. Protective efficacy may be increased by more closely mimicking the natural exposure inoculum of EIAV.

Much of the research performed with HIV polyvalent vaccines has been performed in mice or other rodent model systems, where challenge with HIV is not possible. EIAV has several advantages over the HIV rodent model system when discerning the effects of polyvalent or consensus vaccines. First, the EIAV system has a reliable vaccination strategy with challenge strains that are 100% infectious in ponies. Second, since the vaccination procedure is performed in the natural host of the virus, we are able to challenge the vaccinated ponies with virulent EIAV to determine the protective efficacy against infection and diseases. Third, a reference for the protective efficacy of the polyvalent or consensus vaccines has already been established by challenging the $EIAV_{D9}$ vaccinated ponies with the three individual variants used to develop the polyvalent and consensus vaccines (36). Therefore, the EIAV system is in a unique position to help demonstrate the value of polyvalent or consensus vaccination strategies.

## 6.9 CONCLUSION

While SIV maybe the "gold standard" for testing HIV vaccine strategies, the work described here proves that EIAV can be extremely beneficial in elucidating appropriate lentiviral immune responses necessary for vaccine protection from disease. Using the attenuated $EIAV_{D9}$ vaccine, we were able to show for the first time that Env is a primary determinant of vaccine efficacy, and demonstrated that specific immune responses elicited by the $EIAV_{D9}$ vaccine are associated with protection from disease. With our assays, it appears that the cellular responses to Env-specific peptides are more crucial to protective immunity than the humoral immune responses. We also

demonstrate that Env variation has profound effects on CTL recognition and neutralizing antibody responses. The majority of the cellular peptides identified are located in more conserved regions, making them optimal targets for future EIAV vaccine trials.

SIV researchers have spent years attempting to meaningfully correlate neutralizing antibodies or CTL responses with vaccine protection from disease. Only now is there a concerted effort at elucidating immune responses associated with vaccine protection (70). With EIAV, we have shown Env-specific immune responses significantly associated with vaccine protection from disease. We have also demonstrated Env to be an important determinant of vaccine efficacy, and are in a position to evaluate the role of Gag immune responses in vaccine protection. These findings further establish EIAV as a powerful lentivirus model system that can identify key vaccine induced immune responses that have so far evaded SIV and HIV researchers. Further research with EIAV will yield important information on lentiviral vaccine immunity that will undoubtedly aid in the development of an effective HIV vaccine.

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