INFLUENCE OF SURFACE PROTEIN V6 REGION OF EQUINE INFECTIOUS ANEMIA VIRUS ON CYTOKINE GENE EXPRESSION

A Thesis

by

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ABSTRACT

Equine infectious anemia virus (EIAV) is a member of the lentivirus group of the family *Retroviridae*. EIAV encodes a highly glycosylated SU (surface) protein with interspersed conserved and variable regions. The variable regions are thought to play a key role in virulence determination and virus neutralization. The role of SU in virulence is thought to be based on induction of cytokine gene expression upon binding of the virus to permissive cells leading to clinical signs that are associated with the infection. The proposed experiments will look specifically at one variable region (V6) of SU. My hypothesis was that V6 plays a major role in virulence by inducing cytokine induction upon binding. I used two EIAV strains with distinct phenotypes (virulent EIAV17 and avirulent EIAV19) to test the contribution of V6 to increased cytokine induction. The findings of these studies will aid in the long term goal of vaccine development and potential treatments for EIAV and other retroviruses.

DEDICATION

To my family and friends for their unwavering support.

To my kids, Gabbie and Mitch, who motivated and encouraged me to pursue my goals.

To Tom Leggin who believed in me when I didn't believe in myself, who cheered me on when things got hard, and loved me unconditionally. I am so blessed you chose to be my dad.

To my mom who sacrificed everything to give me a chance to achieve my dreams. You gave me roots and wings and everything I needed in between to be successful. There are not words to convey my eternal gratitude and love for you.

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CHAPTER I

INTRODUCTION

1.1 History of Equine Infectious Anemia

Equine Infectious Anemia Virus (EIAV) was first identified in France in 1843 and recorded again in 1861. By 1870, the virus had spread throughout Europe, Russia, part of Africa and most of Asia. In the first decade of the 1900's, Japan had the first recorded epidemics with over 300 horses dying per year of the infection. EIAV was assigned a viral etiology (classified at a filterable agent) in 1904 which made it the first animal virus to be so classified {Montelaro et al., 1993; Leroux et al., 2004; Issel et al., 1993}.

The first reported case of EIAV in North America was in Wisconsin in 1888. The virus spread to Wyoming in the early 1890's, leading to the first recorded epidemic in North America in 1901. By the late 1900's, the virus had spread to 10 states that coincided with the annual cattle drives from Texas to Minnesota and Montana. A period of quiescence followed, leading many to believe that the virus was no longer a threat to the horse population. The virus was reported in New England in 1935 and a researcher confirmed the presence of EIAV in 29 states by 1941 via horse-inoculation test. The silent spread surprised many in the horse industries since there was still very limited knowledge of the pathogenesis of the virus. The second major outbreak in North America occurred in 1947 at a race-track in New Hampshire. Seventy-seven

Thoroughbred horses died or were euthanized. The outbreak was traced to a horse brought from Florida. That outbreak was probably the single most important incident to raise public awareness about the devastating effects of EIAV and prompted new research and regulations to help safeguard horses from infection {Clark et al., 2003}.

By the 1960's, EIAV was recognized as a major virus impacting equids as widespread epidemics were occurring at racetracks in Maryland, Florida, Washington, and Illinois. Most epidemics were explosive in nature and more than one horse died prior to the disease being detected. In 1965, the United States Livestock Sanitary Association along with other national veterinary and horse industry groups developed a positive uniform plan for quelling the disease. The "Prospectus on Equine Infectious Anemia with Guidelines" was instrumental in halting the spread by the latter part of 1966 {Clark et al., 2003}.

In 1970, Dr. Leroy Coggins of Cornell University developed the first accurate laboratory test for diagnosing the disease {Nakajima et al., 1972; Montelaro et al., 1993}. Previously, diagnosis was confirmed using the horse inoculation test which involved taking the blood of a sick horse and injecting it into a healthy horse and waiting to see if the healthy horse developed symptoms {Montelaro et al., 1993; Issel et al., 1993}. The testing was labor and time intensive and resulted in more infected horses. The development of the "Coggins Test" was a huge breakthrough for diagnosis and disease control. The test is an agar gel immunodiffusion test which detects antibodies to the

virus {Nakajima et al., 1972; Montelaro et al., 1993; Issel et al., 1993}. In 1973, the USDA designated the Coggins Test the official test for determining the presence of EIAV, established a procedure for certifying a network of laboratories qualified to run the test, and prohibited interstate travel of EIAV positive horses except for transport to a slaughterhouse, quarantine facility or research facility. A quarantine directive was issued for positive horses requiring the infected animal to be housed in approved quarters until death or euthanasia. Many in the horse industry fought the directive since inapparent carriers were testing positive but not showing signs of the disease. The directive and disease control programs have proven effective at decreasing the incidence of disease nationwide. In Florida, the incidence of new cases decreased from 12% in 1970 to 0.016% in 2000. This downward trend is a direct result of effective disease control activities, strict enforcement of EIA regulations, and strong support of the equine industry. Until a vaccine becomes available in this country, control programs are the only viable option to reduce the risk of transmission from virus-infected equines {Clark et al., 2003; Montelaro et al., 1993}.

For many decades after its discovery, research on EIAV was restricted due to lack of reliable *in vitro* systems for the isolation, propagation, and quantitation of the virus {Montelaro et al., 1993; Issel et al., 1993}. In the late 1960's, Japanese researchers discovered that EIAV could be propagated *in vitro* in mixed cultures of equine leukocytes {Montelaro et al., 1993}. The research then focused on the kinetics of viral

persistence in EIAV-infected horses with the discovery of the phenomenon of antigenic variation of EIAV in horses manifesting the chronic stages of disease.

Studies in the early 1970's were focused on developing and evaluating the efficacy of a live attenuated EIAV vaccine in protecting against antigenically distinct strains of virus. Early observations in vaccine evaluation demonstrated that horses progressing from chronic EIAV to the inapparent stage of infection were resistant to the development of disease after trial experimental challenges with EIAV {Taylor et al, 2010}. Unfortunately, it was shown that attenuated EIAV vaccine was not able to provide broad protection against challenge with variants of EIAV {Li et al., 2003; Montelaro et al., 1996}. As a result, most countries shifted their focus from immunoprophylaxis of the equine population to identification and isolation of infected animals. Only China and Cuba have large-scale vaccine programs with an attenuated strain. The large scale application of the attenuated EIAV vaccine has been hailed a success in China since it was able to prevent and/or control EIA infections with no observable reversion or vertical transmission {Wang et al., 2010}. However, the use of an attenuated vaccine eliminates the usefulness of the Coggins test and prevents vaccinated horses from entering countries that require a negative EIA antibody test for entry {Montelaro et al., 1993}.

The focus of research changed direction in the late 1970's and early 1980's with the advancement of molecular techniques that allowed the virus to be characterized. In

1976, it was discovered the virus is a retrovirus and a member of the lentivirus subfamily {Montelaro et al., 1993}. Also critical was the development of an experimental infection and disease model in Shetland ponies. Over the past decade, one facet of EIAV research has focused on mapping the genome and understanding the pathogenesis of infection. An interesting area of study is the discovery of virulence factors that may lead to new preventative and therapeutics measures to eliminate the incidence of new infections in the future. {Tagmyer et al., 2008; Lim et al., 2005, Craigo et al., 2007; Covaleda et al., 2010; Howe et al., 2005}

1.2 Virus Replication and Pathogenesis

EIAV is a macrophage-tropic lentivirus of the family *Retroviridae*. Much of the pathogenesis of EIAV is directly related to the replication cycle of a retrovirus. EIAV replication starts with attachment to the cell surface and endocytosis of the infectious particle. Release of the viral core to the cytoplasm is rapidly followed by reverse transcription of the RNA genome. This process is accomplished by the virally encoded reverse transcriptase (RT) that is present in the viral core. The product of reverse transcription is a molecule of linear double stranded DNA that remains associated with the core, most notably with the virally encoded integrase (IN). After entry into the nucleus, successful integration of the EIAV genome into the host genome is required for production of new virions. The process of integration has important biological consequences. The viral genome is replicated along with cellular DNA, thus is passed to daughter cells during cell division. In the absence of transcription the virus is latent,

thus infected cells are not targets of the immune response. In addition, maintenance of the provirus is not effected by treatments with antiretroviral drugs. Thus EIAV causes a persistent, life-long infection in equids for which there is no treatment or cure. Virus elimination can be achieved only by strict isolation or euthanasia of infected animals.

EIAV is spread via mechanical transmission of infected blood. EIAV is found throughout the world with the highest prevalence in geographical areas with warm climates; hence the common name for the disease is swamp fever {Montelaro et al., 1993}. Natural transmission is via insect vectors such as biting flies but transmission may also occur from contaminated needles and blood products and/or improper sterilization of instruments between horses. EIAV is also transmitted transplacentally and via colostrum and milk. {Montelaro et al., 1993}

Three distinct stages of infection (acute, chronic and inapparent) are commonly recognized. The acute stage usually occurs in the first 30 days post-infection and is characterized by marked viremia, high fever, anorexia, and thrombocytopenia. Clinical signs are nonspecific and depend on the age, health, and species of the animal. Foals are more susceptible than adults and donkeys and mules rarely develop clinical signs. Mild cases in horses may produce a fever that lasts less than 24 hours while severely affected horses can develop a high refractory fever and anorexia along with depression, weakness, and ataxia. The acute stage is rarely fatal in naturally infected animals. Following the acute infection, most infected animals progress to the chronic stage. The

chronic stage is the hallmark of EIAV and is characterized by recrudescing episodes of prominent viremia, anorexia, edema, pancytopenia, hemorrhages, diarrhea, glomerulonephritis, and lethargy. Each episode lasts approximately 5 days and the time intervals between episodes increase over the first year post infection. The development of clinical signs and the severity of disease are dependent on age and health of the animal as well as the strain and dose of the infecting virus. After the first year, infected animals often become clinically quiescent, lifelong inapparent carriers. An animal in the inapparent stage has gained immunological control of the virus, but remains infectious to naïve animals. Clinical signs may reoccur if the animal becomes stressed or develops a concurrent illness. {Equine, 2009; Tagmyer et al., 2008; Montelaro et al., 1993}.

Equine macrophages are the predominant cell type for replication and viral spread in the infected animal. The unique nature of macrophages and their role in the immune response impact EIAV pathogenesis. The highest levels of EIAV are found in liver, spleen, lymph nodes, and bone marrow in the acute and chronic stages of the disease, as these tissues are rich in tissue macrophage {Harrold et al., 2000}. The kidneys and peripheral blood also contain virus during the acute stage but at much lower levels. EIAV infects peripheral blood mononuclear cells (PBMCs) but viral replication and release do not occur until the cells have differentiated into mature macrophages {Maury, 1994}. Studies have shown that infection of normal and immune compromised foals results in an initial viremia that reaches maximum titers of 10^4-10^5 $TCID_{50}$ per ml at 10-

12 days post-infection {Perryman et al.,1988}. In cases of chronic EIA, the quantity of virus during viremic episodes ranges from 10^3-10^6 $TCID_{50}$ per ml {Montelaro et al., 1993}. When an animal is afebrile viral antigen and viral DNA are difficult to detect in tissue and PBMC's; levels of virus are reduced to <1-10^3 $TCID_{50}$ per ml {Montelaro et al., 1993}. Latently infected macrophages serve as a viral reservoir for recurrent disease episodes. *In vitro* EIAV infection has a cytopathic effect on macrophages which may correlate *in vivo* with leucopenia, anemia, and thrombocytopenia. **{**Lim et al., 2005; Payne et al., 2004; Covaleda et al., 2010; Sellon et al.,1992; Craigo et al.,2007; Chung et al., 2004; Tornquist et al., 1997}

1.3 EIAV Particle and Genome Organization

Genetically, EIAV is most similar to other retroviruses in the lentivirus group. These include feline immunodeficiency virus (FIV), visna-maedi of sheep and goats, simian immunodeficiency virus (SIV), and human immunodeficiency virus (HIV). EIAV is a somewhat unique lentivirus in that symptoms appear quickly, within 1-4 weeks post infection, rather than months or years later. Thus EIAV has served as a model for lentivirus persistence, pathogenesis and vaccine development {Tagmyer et al., 2008; Taylor et al, 2010}.

The EIAV virion (Figure 1.1) is approximately 100nm in diameter and consists of a lipid enveloped encasing an oblong core that contains two copies of single stranded RNA {Montelaro et al., 1993}. The EIAV surface projections are composed of two *env*-

encoded glycoproteins, a heavily glycosylated protein of 90kDa (gp90) and a membrane spanning 45kDa glycoprotein (gp45) {Montelaro et al., 1993}. EIAV gp90 is now commonly referred to as the surface unit (SU) glycoprotein of the virus and gp45 the transmembrane (TM) glycoprotein {Montelaro et al., 1993}. EIAV SU and TM molecules associate via noncovalent interactions thus SU can be lost during virus purification{Montelaro et al., 1993} The EIAV matrix (MA) protein is located on the cytoplasmic side of the membrane and serves as the bridge between the cytoplasmic tail of TM and the capsid (CA) protein that forms the viral core {Montelaro et al., 1993}.

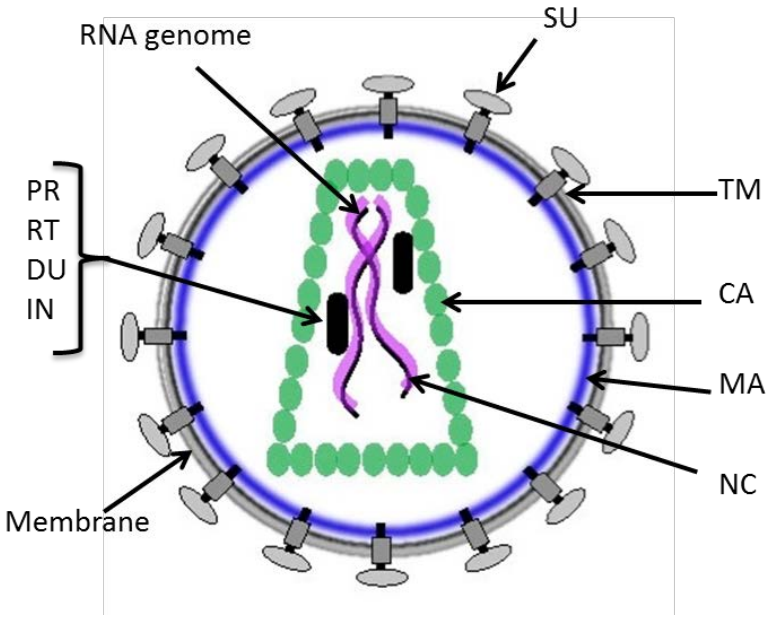

Figure 1.1: EIAV virion. Abbreviations: LTR, long terminal repeat; MA- matrix protein; CA- capsid protein; NC- nucleocapsid protein; PR, protease; RT- reverse transcriptase; DU- dUTPase; IN- integrase; SU- surface unit glycoprotein; TM- transmembrane glycoprotein.

Packaged inside the core are two copies of genomic RNA in association with the basic nucleocapsid (NC). Also present in the core are a few copies each of virally encoded protease (PR), RT and IN.

EIAV genome is 8.2kb which is the smallest and simplest of all lentiviral genomes making it a useful model to study lentiviral replication and pathogenesis (Figure 1.2) {Montelaro et al., 1993; Leroux et al., 2004}. Lentiviral transcription is regulated by long terminal repeats (LTR) {Leroux et al., 2004}. LTR sequences are a key component in cell tropism and virulence with clear differences between fibroblast culture adapted and field strains {Payne et al., 1999; Payne et al., 2004; Maury et al., 2005; Maury et al., 1998; Maury et al., 2007; Montelaro et al. 1993}. Transcription of the viral genome generates an unspliced mRNA from which the Gag and Pol polyproteins are produced. This unspliced mRNA is also packaged into new virions. The *env* gene products (SU and TM) are produced from a singly spliced mRNA. A family of multiply spliced mRNAs encodes the nonstructural proteins Tat, Rev and S2.

1.3.1. Viral Proteins

1.3.1.1 Gag

The Gag polyprotein is the precursor molecule to the MA, CA and NC proteins. Gag polyproteins are synthesized in the cytoplasm and transported to the plasma membrane where they form immature budding particles {Puffer et al., 1998}. The assembly of Gag precursor proteins (Pr^{55}) on the plasma membrane is essential in that this process drives assembly and virus budding from the host cells {Leroux et al., 2004; Provitera et al.,

2000}. Maturation of the virion to an infectious particle occurs either during or immediately after budding when the Gag polyproteins are processed by the virus-encoded protease {Puffer et al., 1998; Provitera et al, 2000}.

1.3.1.2 Pol

The EIAV Gag-Pol precursor ($Pr180^{gag/pol}$) is encoded from unspliced mRNA. This longer polyprotein is synthesized via a ribosomal frame shift at a site upstream of the Gag stop codons. $Pr180^{gag/pol}$ is important as it includes the enzyme reverse transcriptase (RT), which is essential for the synthesis of viral DNA from the RNA genome. $Pr180^{gag/pol}$ processes both precursor polyproteins, and the virally encoded dUTPase (DU) (essential for replication in non-dividing monocyte-derived macrophages)and the integrase (IN) responsible for integrating the dsDNA copy of the retroviral genome into the host chromosome {Leroux et al., 2004; Lichtenstein et al., 1995; Threadgill et al. 1993}.

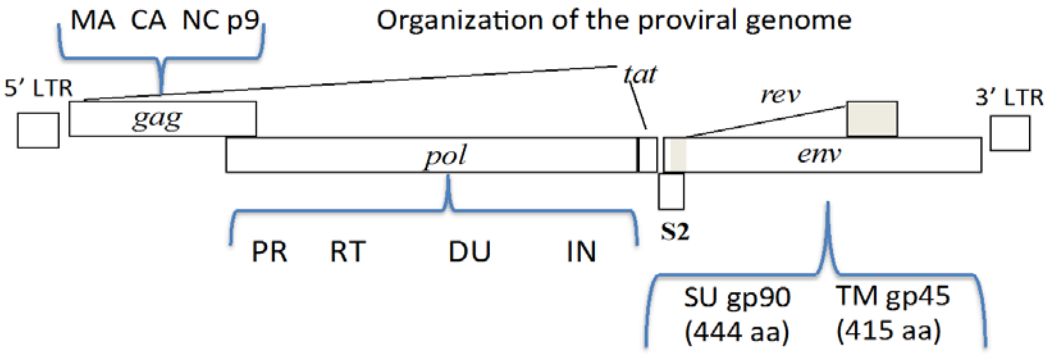

Figure 1.2: Organization of the EIAV proviral genome. Abbreviations: LTR, long terminal repeat; MA- matrix protein; CA- capsid protein; NC- nucleocapsid protein; PR, protease; RT- reverse transcriptase; DU- dUTPase; IN- integrase; SU- surface unit glycoprotein; TM- transmembrane glycoprotein.

1.3.1.3 Env

EIAV *env* gene encodes the SU and TM envelope glycoproteins. The SU protein is highly glycosylated with pathogenic strains, such as $EIAV_{WY}$, containing at least 17 potential N-linked glycosylation sites in their SU coding sequences {Cook et al., 2013}. Approximately half of the molecular weight of EIAV SU is composed of glycosylated residues {Cook et al., 2013}. Many of the potential N-linked glycosylation sites in SU are subject to change during the course of the infection {Leroux et al., 2001}. SU also contains neutralizing epitopes, as demonstrated by mouse monoclonal antibodies, and is often referred to as the principal neutralizing domain or PND {Ball et al., 1992; Cook et al., 2013; Hussain et al., 1998}. SU proteins mediate attachment and entry into permissive cells, and are major targets of the immune response. The host receptor for EIAV is ELR-1, a member of the tumor necrosis factor family of receptor proteins

{Cook et al, 2013; Ball et al., 2005; Zhang et al, 2005}. *In vitro* experiments have demonstrated that EIAV-receptor entry is mediated by a low-pH endocytic pathway {Cook et al., 2013; Brindley et al., 2008; Jin et al., 2005}.

Throughout the course of infection, initial and recrudescing, SU is able to undergo significant changes in sequence without compromising function. The selective force driving the evolution of SU is the host immune system {Cook et al., 2013}. EIAV was the first lentivirus to be associated with "antigenic drift" as each distinct febrile episode in an infected equid is caused by genetically and antigenically distinct SUs {Cook et al., 2013; Leroux et al., 1997, 2001; Craigo et al., 2010}. SU variation can be striking and viral quasispecies or swarms emerge in the infected host {Leroux et al., 2004; Craigo et al., 2007; Craigo et al., 2009}. Based on virus neutralization studies, the development of *env* quasispecies is responsible for the discrete febrile episodes typical of EIA. Each febrile episode is associated with antigenic variants that evade established immune surveillance {Craigo et al., 2010}. Comparisons of SU sequences reveal the presence of discrete variable and conserved regions within the protein {Payne et al., 1987}.

1.3.1.4. Regulatory Proteins

EIAV encodes 3 regulatory proteins: Tat, Rev, and S2. Tat interacts with the LTR through the trans-activation response element (TAR) and is essential for efficient transcription {Rosin-Arbesfeld et al., 1998}. Rev is a regulator of viral gene expression acting post-transcriptionally to allow the essential translocation of unspliced and

partially spliced mRNA from the nucleus to the cytoplasm {Leroux et al., 2004}. S2 is a cytoplasmic protein that may interact with the Gag protein but whose exact role has not been determined {Covaleda et al., 2010; Leroux et al., 2004}.

1.3.1.4.1 Tat

The transactivator of transcription (Tat) is expressed from a spliced mRNA. Tat exon 1 derives from the 5'end of the genome while the location of exon 2 is between the *pol* and *env* genes. Tat enhances the elongation efficiency of the host RNA polymerase {Cook et al., 2013}. EIAV Tat functions in a similar manner to HIV-1 Tat. It interacts with the nascent mRNA transcript by binding to a short RNA hairpin structure called the trans-activation responsive (TAR) element. This interaction serves as a scaffold for additional cellular proteins with the end result being phosphorylation of the C-terminus of RNA polII, enhancing its elongation efficiency by 100 to 1000-fold {Leroux et al., 2004}.

1.3.1.4.2 S2

S2 is a 65 amino acid protein synthesized from a multiply spliced mRNA. The S2 coding region begins in between the *pol-env* intergenic region and overlaps the N-terminus of *env* {Leroux et al., 2004; Covaleda et al., 2010}. The S2 protein is found in all isolates of EIAV; its presence is highly conserved in the genome suggesting its importance in the virus replication cycle {Leroux et al., 2004}. S2 is mainly localized in the cytoplasm and is thought to interact with the EIAV Gag precursor {Covaleda et al., 2010}. S2 is not required for viral replication *in vitro* in equine cell lines including equine monocyte

derived macrophages (eMDM) which are the primary target for replication {Leroux et al., 2004; Covaleda et al., 2010}. However, the importance of S2 in *in vivo* viral pathogenesis has been consistently demonstrated in the Shetland pony model {Leroux et al., 2004; Covaleda et al., 2010; Fagerness et al., 2006}. Also, it has been demonstrated that pro-inflammatory cytokine expression is increased in equine macrophages that have been infected with $EIAV_{WY}$ as compared to macrophages that have been infected with a mutant EIAV that is lacking S2 {Covaleda et al., 2010; Cook et al., 2013}.

1.3.1.4.3 Rev

Rev, regulator of expression of viral proteins, acts post-transcriptionally by allowing nuclear export of partially spliced and unspliced RNA from the nucleus to the cytoplasm {Cook et al., 2013; Leroux et al., 2004}. Rev, also produced from a multiply spliced mRNA, has 2 exons. Exon 1 is located near the 5'-end of the *env* gene while exon 2 overlaps the TM coding region. Rev is essential for expression of structural proteins and thus virus production {Leroux et al., 2004}. Rev binds to a highly structured region of the EIAV genome called the rev response element (RRE). The RRE is present in unspliced and singly spliced mRNAs and multiple copies of Rev bind this region, Rev binding suppresses splicing and facilitates passage of viral mRNA across the nuclear membrane {Leroux et al., 2004; Cook et al., 2013}. Thus Rev regulates its own expression. In the absence of Rev, viral mRNAs are spliced to form Tat and Rev mRNAs. As the level of Rev in the cell increases, viral mRNA splicing is suppressed {Leroux et al., 2004}.

1.4 EIAV SU and its Variable Regions

EIAV SU (Figure 1.3) has 8 variable regions, V1-V8, and changes in these regions have been shown to allow the virus to evade or escape neutralizing antibodies {Tagmyer et al., 2008; Souza et al., 2009; Craigo et al., 2007}. In early studies, Ball et al. localized virus neutralizing epitopes to variable regions of SU {Ball et al., 2005}. Tagmyer et al. showed that V3 and V4 contained major neutralization epitopes, with V3 the principal neutralization domain {Tagmyer et al., 2008}. Studies by Souza et al. identified 2 neutralizing epitopes in V3, one in V5 and possibly one in V6. In vaccine trials, some protection against EIAV challenge can be measured when horses are challenged with homologous, but not heterologous, Env bearing viruses {Craigo et al., 2007; Li et al., 2003}. Thus successful vaccine development for lentiviruses must account for both preexisting and post-vaccination accumulations of Env mutations in the virus population {Tagmyer et al., 2008; Payne et al., 2004; Li et al., 2003}.

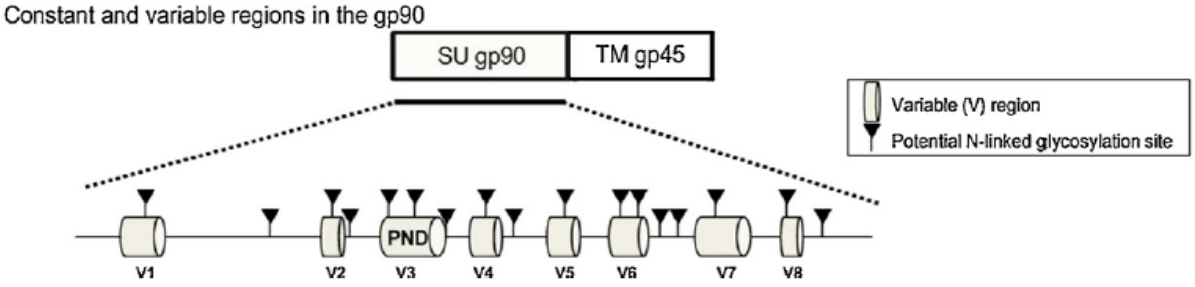

Figure 1.3- Constant and variable regions in the gp90. Diagram showing the localization of variable regions within SU (gp90) along with potential N-linked glycosylation sites and neutralizing determinants (including the principal neutralizing determinant PND) in a fibroblast-cell adapted variant of $EIAV_{wy}$ (Cook et al., 2013)

1.4.1 SU Sequence Variation and Virulence

In addition to roles in immune escape and avoidance, EIAV *env* variation has a measurable effect on virus virulence. This was demonstrated by Fuller and Payne in a Shetland pony model using molecular clones of EIAV. They showed that infection with virus stocks derived from clones differing in only in *env* sequences resulted in distinct disease outcomes {Payne et al., 1994; Payne et al., 1998}. The $EIAV_{17}$ virus stock is acutely virulent, usually producing severe disease within 10 days post-infection. In contrast the $EIAV_{19/17LTR}$ virus stock lacks acute virulence, replicating only to very low titers in ponies. These two viruses have identical LTRs, *gag*, *pol* and *S2* sequences, and only differ in their *env* regions (Appendix I, Figure 1). Further, when viruses chimeric in the *env* region were generated, a virus with its SU region derived from $EIAV_{17}$ and its TM derived from $EIAV_{19}$ ($EIAV_{17SU/19TM}$) produced acute disease; while virus with SU derived from $EIAV_{19}$ and TM from $EIAV_{17}$ ($EIAV_{19SU/17TM}$) did not {Payne et al., 2004}. $EIAV_{17}$, $EIAV_{19/wyoLTRs}$ and the chimeric *env* viruses replicated to similar levels in cultured equine monocyte-derived macrophages (eMDM) suggesting that their intrinsic replication rates might not account for the observed differences in virulence properties {Covaleda et al, 2010}. These results focused attention on SU sequences.

1.4.2 SU Sequence Variation and Cytokine Expression

1.4.2.1 *In vitro* Studies

In a study using the $EIAV_{17}$ and $EIAV_{19}$ virus stocks, Lim et al. showed that $EIAV_{17}$, but not $EIAV_{19}$, elicits the induction of pro-inflammatory cytokines when cultured equine

monocyte derived macrophage (eMDM) are treated with virus for short periods of time{Lim et al., 2005}. $EIAV_{17}$ exposure significantly increased expression of interleukin (IL)-1α, IL-1β, IL-6, IL-10 and tumor necrosis factor (TNF)-α whereas avirulent $EIAV_{19}$ failed to induce expression of these cytokines significantly above control levels. Maximum levels of cytokine induction were measured within 0.5 to 1 hour post infection, strongly implicating virus binding and/or entry as the initial triggers of altered cytokine gene expression.

Use of a mouse diarrhea model demonstrated an additional biologic activity of the $EIAV_{17\ SU}$ protein (and its V6 peptide) {Ball et al., 2005}. SU_{17} and SU_{19} proteins were given to mouse pups and the pups scored for diarrhea over a period of 12 hours. SU_{17} induced significant dose dependent diarrhea in the model while SU_{19} did not {Ball et al., 2005}. Use of synthetic peptides in the mouse diarrhea model revealed that the V6 region of SU_{17}, but not SU_{19} was induced a dose dependent diarrhea. Thus SU_{17} and SU_{19}, and their V6 peptides have distinct biological activities in a mouse diarrhea model for viral enterotoxins {Ball et al., 2005}.

1.5 SU Variable Region 6 (V6)

The studies described above lead Payne and Fuller to further investigate the contribution of the V6 region of EIAV SU to altered disease expression (virulence phenotype). Thus they generated viruses with chimeric SU proteins. The chimeric SU proteins targeted V6 from $EIAV_{17SU}$ and $EIAV_{19SU}$. The SU proteins differ at 8 of 13 amino acids in the V6

region, with other amino acid differences scattered throughout SU. The amino acid changes are non-conservative and V6 is the most divergent region between the two SU proteins. By creating chimeric clones that specifically target the V6 region from virulent and avirulent viruses, the hypothesis that V6 plays a role in virulence was tested. The V6 chimeric viruses were constructed to replace the 19V6 sequence with the V6 from $EIAV_{17}$ (Appendix I, Figure 2). This virus is referred to as $EIAV_{17V6/19SU}$. The V6 region of $EIAV_{19}$ was likewise placed into the background of the $EIAV_{17}$ env gene. This construct is called $EIAV_{19V6/17}$. These two viruses share identical LTRs, gag, pol, S2 and TM. $EIAV_{17}$ differs from $EIAV_{19V6/17}$ by ~13 amino acids. $EIAV_{19SU}$ differs from $_{EIAV17V6/19SU}$ by ~ 13 amino acids.

The results of the V6 swaps were subtle, but show the sequence of the V6 region does impact virulence phenotype (Appendix I, Figure 2). Four out of four $EIAV_{17}$ infected ponies experienced febrile episodes that began on, or before, day 11 post-infection. . The maximum recorded temperatures were 102°F to 106°F for $EIAV_{17}$ infected ponies and febrile episodes were accompanied by thrombocytopenia {Fuller, unpublished}. Placing the V6 sequence from $EIAV_{19SU}$ in the context of $EIAV_{17}$ resulted in febrile episodes at 11 and 12 dpi in two ponies, a febrile episode at 35 dpi in an third animal and no febrile episode prior to day 40 in the final pony. Among the 4 ponies infected with $EIAV_{19V6/17}$, 2 had normal body temperatures through day 25 post-infection and 2 animals reached 104°F to 105°F, all displayed mild thrombocytopenia. In contrast, neither of the 2 ponies infected with the $EIAV_{17V6/19SU}$ viral construct experienced a

febrile episode through 40 days post infection and both maintained a normal platelet counts {Fuller, unpublished}. The clinical course of these two animals was indistinguishable from $EIAV_{19SU/17TM}$ infected Shetland ponies {Payne et al., 2004}.

Peripheral blood mononuclear cells were collected from Shetland ponies infected with $EIAV_{17}$, $EIAV_{17V6/19SU}$ and $EIAV_{19V6/17SU}$. PBMC were collected at 12, 14, 15 and 17 days post-infection and gene expression levels of IL-8, IL-1α, IL-1β, and TNF-α were measured by quantitative PCR. Figure 1.4 shows a summary of this data {Covaleda, unpublished}. A review of the gene expression patterns shows that of the three infecting virus stocks, $EIAV_{17}$ has a unique profile. Most notably, IL-1α IL-1β and IL-8 gene expression levels are decreased in the $EIAV_{17}$ infected ponies. This is reflected in the overall averages, and was also consistent for individual ponies. While the absolute decreases are modest (a maximum of 2-fold) the overall trend is in sharp contrast to the trend of increased levels of IL-1α and IL-1β gene expression levels in ponies infected with either $EIAV_{19V6/17}$ (moderately virulent) or $EIAV_{17V6/19SU}$ (avirulent). Thus the $EIAV_{17\ SU}$ sequence is unique and V6 sequences contribute to the virulence phenotype. However, V6 alone is not sufficient to change the avirulent $EIAV_{19SU/17TM}$ to the high virulence phenotype of $EIAV_{17}$ and is not the only contributor to cytokine gene expression levels.

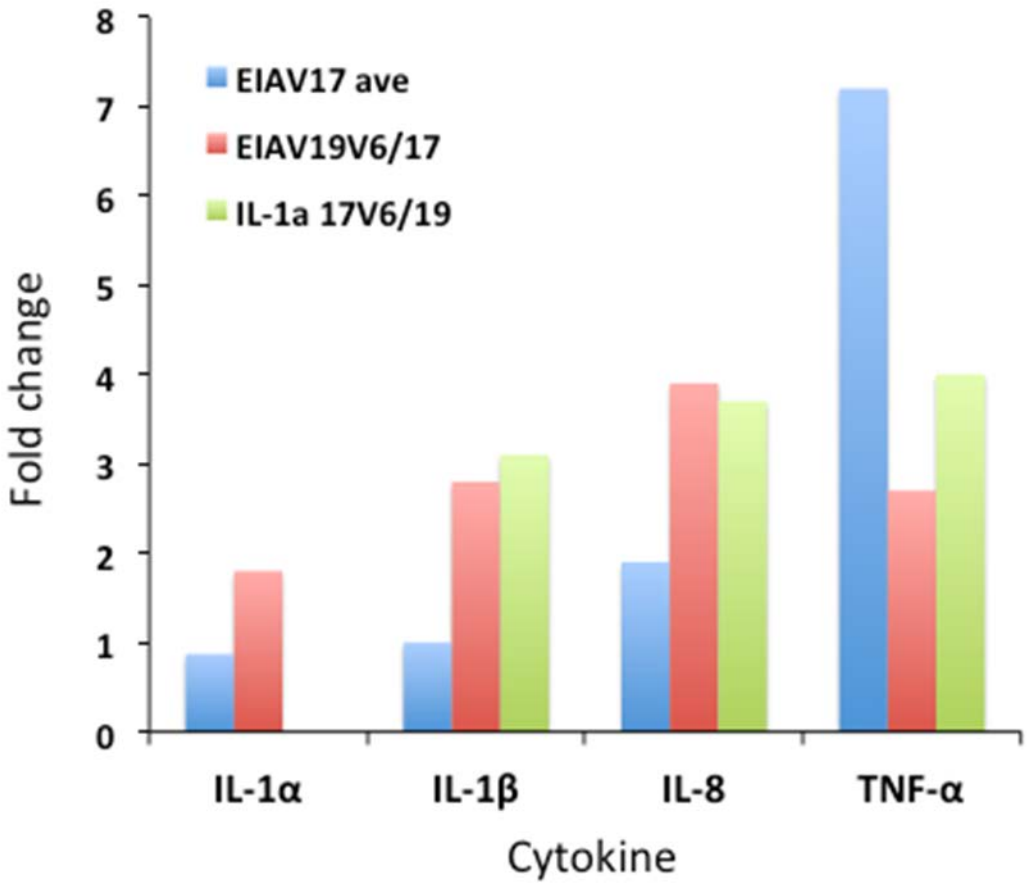

Figure 1.4- Average changes in gene expression during EIAV infection. The data for $EIAV_{17}$ and $EIAV_{19V6/17}$ represents averages of 4 animals. $EIAV_{17V6/19SU}$ data are average values from 2 ponies. All qPCR assays were done in triplicate. Statistical significance was not determined due to the low number of animals used.

The largest increases in TNF α, IL-8, and IL1β were in the 2 ponies infected with EIAV $_{17V6/19SU}$, neither of which experienced a febrile episode. It appears that avirulent EIAV $_{17V6/19SU}$ may be associated with higher expression levels of proinflammatory cytokine genes than moderately virulent EIAV $_{19V6/17}$; however it is not possible to determine statistical significance of this observation, given the low numbers.

1.6 Hypothesis and Objectives

The goal of my research project was to examine the viruses described above for their role in increased cytokine/chemokine gene expression in cultured macrophages. It was hypothesized that V6 plays a major role in virulence by inducing cytokine induction upon binding of macrophages. Thus, there would be a positive correlation between the presence of V6 from a virulent strain and cytokine production. It was also hypothesized that cultured macrophages could be used as a surrogate for animal studies. If so, the results obtained from *in vitro* studies would be repeatable in an *in vivo* model.

The specific objective of this study was to:

1. Determine the role of the V6 region on cytokine expression in cultured eMDM
    a. Construct 2 chimeric viruses specifically isolating the V6 region using site-directed mutagenesis and PCR;
    b. Measure cytokine cDNA levels of IL-8, IL-1α, IL-1β, and TNF-α in eMDM infected with either virulent $EIAV_{17}$ or avirulent $EIAV_{19}$ using QPCR;
    c. Measure cytokine cDNA levels of IL-8, IL-1α, IL-1β, and TNF-α in eMDM infected with $EIAV_{17V6/19SU}$ or $EIAV_{19V6/17SU}$ using QPCR.

CHAPTER II

MATERIALS AND METHODS

2.1 Animals

Six outbred horses (#2000, #2230, #91, #2153, #2165, and Eli) owned by Texas A&M University were used in this study. Horses were housed on a pasture at Veterinary Medical Park which is an AAALAC-accredited facility on the campus of Texas A&M University. Horses were cared for according the *Guide for the Care and Use of Laboratory Animals.* All horses had an annual Coggins test to ensure EIA negative status, were current on all required vaccinations and were in good health at the time of the blood collection. All procedures were approved by the Institutional Animal Care and Use Committee.

2.2 Virus Production and Purification

Molecular clones for $EIAV_{17}$ and $EIAV19_{WyoLTR}$ were constructed by the Payne laboratory as previously described {Payne et al., 1998; Payne et al., 2004}. The Payne laboratory used those molecular clones to construct 2 chimeric viruses specifically isolating the V6 region (Figure A1) using PCR. The constructs consisted of swaps of a 28 amino acid region encompassing V6. To create a chimeric virus with the V6 region of $EIAV_{17}$ onto $EIAV19_{WyoLTR}$ ($EIAV_{17V6/19SU}$), PCR was used to generate 3 products: EIAV 19SU 5' (424bp), EIAV 19SU 3' (993bp) and 17V6 (229 bp). The three products were joined via overlap extension PCR to generate a chimeric 1.5 kb SU fragment

containing unique *Sph1* and *BstEII* sites at its 5' and 3' ends respectively. This 1.5 kb fragment was cloned into pDONR201 and following sequencing confirmation the fragment was excised by restriction digestion with *SphI/BstEII* and moved into *Sph1/BstEII* digested $EIAV_{19/WyoLTR}$ to generate the final infectious provirus, $EIAV_{17V6/19SU}$. The final clone was again verified by the SU region sequencing.

The chimeric $EIAV_{19V6/17SU}$ virus was also constructed by generating a chimeric *env* fragment by overlap extension PCR. In this instance the V6 region was derived from $EIAV_{19}$ and the 5' and 3' flanking fragments derived from $EIAV_{17}$. The resulting 1.5 kb fragment was cloned into pDONR201 and sequence verified. A 720 bp *HindIII* fragment containing the V6 swap was then purified and ligated into a *HindIII* digested and gel purified $EIAV_{17\Delta BamHI}$ ($EIAV_{17}$ lacking a 2.6 kb *BamHI* fragment from the *gag-pol* region) provirus. PCR screening was used to identify recombinant clones and sequencing was performed to identity a construct with the *HindIII* fragment in the desired direction. $EIAV_{19V6/17\Delta BamHI}$ was then digested with *BamHI* and ligated to a gel purified *BamHI* fragment from $EIAV_{17}$ to regenerate a complete infectious provirus containing the V6 swap. Colonies with the correct orientation of the *BamHI* fragment were identified and verified by sequencing. This two-step cloning procedure was used simply to facilitate gel purification and recovery of the desired fragments.

Plasmid constructs of complete EIAV proviruses and chimeras were transfected into D17 (canine) cells with a calcium phosphate transfection kit (Invitrogen). Culture supernatants were collected at 48, 72, and 96 hours post-transfection. Culture supernatants were tested for the presence of reverse transcriptase (RT) using [^{3}H]TTP

{Payne et al., 2004}. RT values greater than 3 times the values of the uninfected controls were considered positive for viral growth. One-milliliter aliquots of positive D17 cell supernatants were then passed onto eMDM to generate virus stocks. eMDM culture supernatants were collected 3, 7, and 13 days post infection and tested for the presence of RT. Virus stocks were concentrated and cell-secreted molecules removed from clarified culture supernatants by ultrafiltration against at least 10 volumes of fresh complete MEMα using a Vivaflow 200 tangential-flow ultrafiltration unit (Sartorius corp. Edgewood, NY) with a MWCO of 100kDa {Lim et al., 2005}. Lim et al. have reported that ninety-six percent of non-viral proteins and cytokines with molecular weights between 7 to 75 kDa are removed under these conditions {Lim et al., 2005}. RT activity of the concentrated viral stocks was measured post-filtration and the cpm/ml was calculated. Culture supernatant of mock-infected eMDM was collected and filtered under identical conditions and used as a control medium (CM) for the experimental infections. Virus stocks were normalized based RT activity and the amount of SU protein determined via Western blot.

2.3 eMDM Culture

Whole blood was collected from EIA-negative horses into an anticoagulant citrate dextrose (ACD) solution blood collection bottle (The Metrix Company, Dubuque, Iowa). As described by Lim, blood was centrifuged at 1900 x g for 30 minutes to separate the buffy coat layer which was then centrifuged through a HybriMax histopaque cushion (d= 1.077 g/cm^3; Sigma, St. Louis, MO) to separate peripheral blood mononuclear cells

(PBMC's) {Lim et al., 2003} . PBMC's were washed four times with Dulbecco's Ca2+ and Mg2+ - free phosphate buffered saline (PBS) (Sigma, St. Louis, MO), 5% adult horse serum (endotoxin and EIA tested, Invitrogen/Gibco, Carlsbad, CA) and 1% penicillin-streptomycin solution (Sigma, St. Louis, MO). PBMC's were resuspended in Modified Eagles Medium alpha (MEMα) with 10% horse serum, 15 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin to a concentration of 12 X 10^6 cells/ml. One-ml aliquots of the cell suspension were added to each of the 12-wells of a Poly-D-Lysine plate (Becton Dickinson Labware) and incubated at 37 °C in 5% CO_2. Non adherent cells and blood products were removed 24 hours after plating by washing with warmed (35^o C) 1X PBS. The plates were returned to the incubator for 72 hours to allow for monocyte maturation into macrophages. Maturation was determined by daily visual inspection of the cells in each plate.

2.4 Immunoblotting

To determine the amounts of SU protein relative to RT activity for the four virus stocks, 60,000 cpm of RT activity were pelleted by centrifugation at 13000 x g for 2 h at 4 ºC. Viral pellets were resuspended in RIPA buffer, separated by 10% SDS-PAGE, transferred to nitrocellulose and analyzed by Western blotting. Primary antibody to EIAV SU was a mouse monoclonal antibody specific to EIAV SU (gp90(A)-86) obtained from NIH AIDS Research and Reference Reagent Program {Hussain et al., 1987} and used at a dilution of 1/200 in Tris-buffered saline and 0.05% Tween 20 (TBS-T) plus 5% non-fat dry milk. After overnight incubation at 4 ºC, membranes were rinsed

three times for five minutes in TBS-T and then incubated for 1 h at room temperature with goat anti-mouse HRP at a dilution 1:3000 (Pierce). The membrane was rinsed again and incubated with SuperSignal West Femto maximum sensitivity substrate (Pierce) for 4 minutes and imaged using x-ray film.

2.5 Infection of eMDM

Five treatment groups were used: $EIAV_{17}$, $EIAV_{19}$, $EIAV_{17V6/19SU}$, $EIAV_{19V6/17SU}$, and media/uninfected control. Cells collected at four time points were tested for each treatment group: 30 minutes post infection (mpi), 1 hour post infection (hpi), 2 hpi, and 4 hpi. Previous studies by Lim and colleagues showed cytokine induction within 30 mpi with minimal differences past the 4hpi time point {Lim et al., 2005}. Cultured eMDM were treated with equal amounts of virus and incubated in a 37°C incubator with 5% CO_2. At each time point, media was removed and the cells were lysed with RLT buffer from the Qiagen RNeasy Kit (QIAGEN, Valencia, CA.). Lysates were collected after the addition of the buffer and stored at -80^{o} C until ready for RNA isolation.

2.6 RNA Isolation and cDNA Synthesis

RNA was isolated from lysates using the Qiagen RNeasy Mini Kit (QIAGEN, Valencia, CA.) according to the manufacturer's instructions for total isolation of RNA from animal cells. In order to eliminate DNA contamination, the extracted RNA was treated with 1µl of DNase 1 (Invitrogen, Carlsbad, CA). RNA was quantified using a Nanodrop

Spectrophotometer ND1000 (Thermo Scientific, Wilmington, DE). RNA was stored at -80° C until used for cDNA synthesis.

cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA). Briefly, 2 µl of 10X RT Buffer, 1 µl of 100mM dNTP mix, 2 µl Random Primers, 1 µl Reverse Transcriptase, and nuclease-free H_2O was added to 1µg of RNA for a total volume of 20 µl. The reaction conditions used were as follows: 25°C for 10 minutes, 37°C for 120 minutes, and 85°C for 5 minutes. cDNA was stored at -20° C.

2.7 qPCR

Taqman primer and probe sets, obtained from Applied Biosystems, were designed for each cytokine (See Table 2.1) and extensively validated {Allen et al., 2007; Payne and Covaleda unpublished}. qPCR reactions contained Custom Taqman Gene Expressions Assay Mix containing primers and probes (Applied Biosystems, Carlsbad, CA), Taqman Universal PCR Master Mix containing Taq, dNTPs, and buffer (Applied Biosystems, Carlsbad, CA), 1.2 µl of cDNA and nuclease-free water for a total volume of 10 µl per reaction. All qPCR measurements were run on ABI 7900HT real time PCR system using 384 well plates. Each reaction was run in triplicate. Amplification was carried out using the following parameters: 1 cycle at 50^{o} C for 2 minutes, 1 cycle at 95^{o} C for 10 minutes, followed by 40 cycles of 95^{o} C for 15seconds and 55^{o}C for 20 seconds.

Changes in the gene expression level of 4 cytokines/chemokines: IL-8, IL-1α, IL-1β, and TNF-α were determined.

Table 2.1. Primer and probe sequences used for Taqman PCR

Primer Name	Primer Sequence
Equine IL-8 forward	5'-GCCACACTGCGAAAACTCA-3'
Equine IL-8 reverse	5'-GCACAATAATCTGCACCCACTTTG-3'
Equine IL-8 probe	5'-ACGAGCTTTACAATGATTTC-3' (FAM6)
Equine IL1-α forward	5'-CAATATCTTGCGACTGCTGCATTAA-3'
Equine IL1-α reverse	5'-CTCTTCTGATGTATAAGCACCCATGT-3'
Equine IL1-α probe	5'-ACGCAGTGAAATTT-3' (FAM6)
Equine IL1-β forward	5'-TGTACCTGTCTTGTGGGATGAAAG-3'
Equine IL1-β reverse	5'-GCTTTTCCATTTTCCTCTTTGGGTAA-3'
Equine IL1-β probe	5'-CCTACAGCTGGAGACAGT-3' (FAM6)
Equine TNF-α forward	5'-TTCTCGAACCCCAAGTGACG-3'
Equine TNF-α reverse	5'-GCTGCCCCTCGGCTT-3'
Equine TNF-α probe	5'-ATGTTGTAGCAAACCC-3' (FAM6)
18s forward	5'-AAACGGCTACCACATCCAA-3'
18s reverse	5'-TCGGGAGTGGGTAATTTGC-3'
18s probe	5'-AAGGCAGCAGGCGC-3' (FAM6)

2.8 Data Analysis

qPCR data was collected and analyzed with the SDS 2.3 software which is designed for the ABI 7900HT real time PCR system. The software detects and determines the

threshold cycle (Ct), the level at which the fluorescence gives a signal over the background, and displays it as the linear portion of the amplified curve. The Ct value was used to quantify gene expression using the $2^{-\Delta\Delta Ct}$ method, also known as the comparative threshold cycle (Ct) or ΔΔ Ct method (Livak and Schmittgen, 2001). The value for each sample was normalized relative to the expression of the 18S rRNA gene as described in Applied Biosystems User Bulletin No. 2 (P/N 4303859). Changes in gene expression mediated by each virus (treatment) were compared to the media control (untreated). Fold changes in gene expression were also compared between treatments. A twofold change from baseline was considered to be significant. Statistical analysis was completed with the assistance of the Texas A&M Department of Statistics. Data was analyzed first with the Shapiro-Wilk test to ensure normal distribution and then with the Levine test to ensure equal variances. A linear mixed model to determine the statistical significance of the cytokine/chemokine gene expression in respect to each virus and time point (Appendix II).

CHAPTER III

RESULTS

3.1 Analysis of Viral Stocks

Stocks for each of the viruses under study were generated by primary transfection into D17 cells following by a single passage onto eMDM. The development of cytopathic effects (data not shown) and RT assays of culture supernatants (Table 3.1) showed that the newly constructed V6 chimeric viruses were replication competent.

Table 3.1. Reverse transcriptase titers: Virus stocks

Virus Sample	CPM/ML
$EIAV_{17}$	1.3×10^6
$EIAV_{19}$	5.8×10^5
$EIAV_{17V6/19SU}$	6.9×10^5
$EIAV_{19V6/17}$	2.8×10^5

Equal amounts of each virus stock (based of cpm/ml) were concentrated by centrifugation and analyzed by Western blot using a monoclonal antibody to a conserved SU epitope (Figure 3.1). A visual examination of the blot showed that each stock contained approximately the same amount of SU per cpm. Thus eMDM were infected with equivalent cmp of each virus stock.

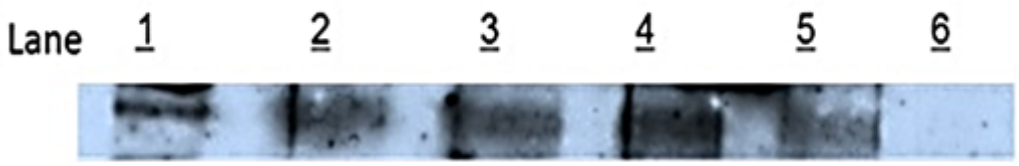

Figure 3.1. Western blot to detect SU protein. SU was detected with a mouse monoclonal antibody against a conserved SU epitope present in the four virus stocks. Lane 1, molecular weight markers. Lane 2, $EIAV_{17}$. Lane 3, $EIAV_{19}$. Lane 4 $EIAV_{17V6/19SU}$. Lane 5 $EIAV_{17V6/19SU}$. Lane 6, eMDM control media. Similar amounts of SU protein are detected in each lane.

3.2 qPCR Results

The expression of IL1-α, IL1b, IL8, TNF-α and 18S rRNA (housekeeping gene) were measured for each sample. All qPCR assays were run in triplicate-for a total of 1800 reactions. Each 384-well plate contained all the genes and time points for one horse and each plate was run one time. The 18S ribosomal RNA was the baseline to which all other genes were compared. The raw data are provided in Appendix II. Gene expression for TNF-α was not determined in four of the six horses so no results for TNF-α were included in the data sets or in the statistical analysis.

3.3 Statistical Analysis

Statistical analyses was performed to more rigorously examine the data. The model chosen for analysis was a linear mixed model. This model was chosen because it takes into account random effects as well as fixed effects. For this model, the fixed and

random effects are assumed to have a normal distribution. In our model, the horse was the random effect and the virus and time points were the fixed effects.

$$Y_{ijk} = \mu + horse_i + virus_j + time_k$$

A Shapiro-Wilk test was used in order to determine if the residuals were normally distributed (as required by the linear mixed model). The analysis indicated that the data from 5 of the 6 horses had normally distributed residuals and we could proceed with the data analysis using the linear mixed model. These data are shown in Appendix II.

3.4 Data Analysis

Figures 3.2-3.4 show the change in gene expression for each treatment for each horse through the time course of the experiment. For each horse, the control media responses were set to 1 (baseline). When graphed in this manner it is evident that there were considerable differences in responses among horses, as expected for outbred animals. For example, expression of IL8, as a result of $EIAV_{17}$ treatment was increased by 3.5 fold at 0.5 h for horse #2153 while remaining at baseline for horse #2000. IL1-a expression was even more variable between individual horses. With $EIAV_{17}$ treatment the expression of IL-1a was increased by 14-fold (at 0.5 h) for horse Eli but again remained at baseline levels for horse #2000. The data for expression of IL-1b reveals similar differences between horses, with $EIAV_{17}$ treatment inducing a 10-fold increase in gene expression for horse Eli at the 1 h timepoint while #2000 shows no change above baseline. Though responses for each horse are variable, it does appear that treatment with $EIAV_{17}$ caused the greatest fold-changes in expression for each of the three genes

assayed, in accordance to data previously reported by Lim {Lim et al., 2005}. Examining individual horse responses for the other three treatments did not reveal any obvious pattern.

While there was significant variation between horses, making individual comparison difficult, a sufficient number of horses were used to allow for a comparison of the mean fold change at each time point, as shown in Figure 3.5.

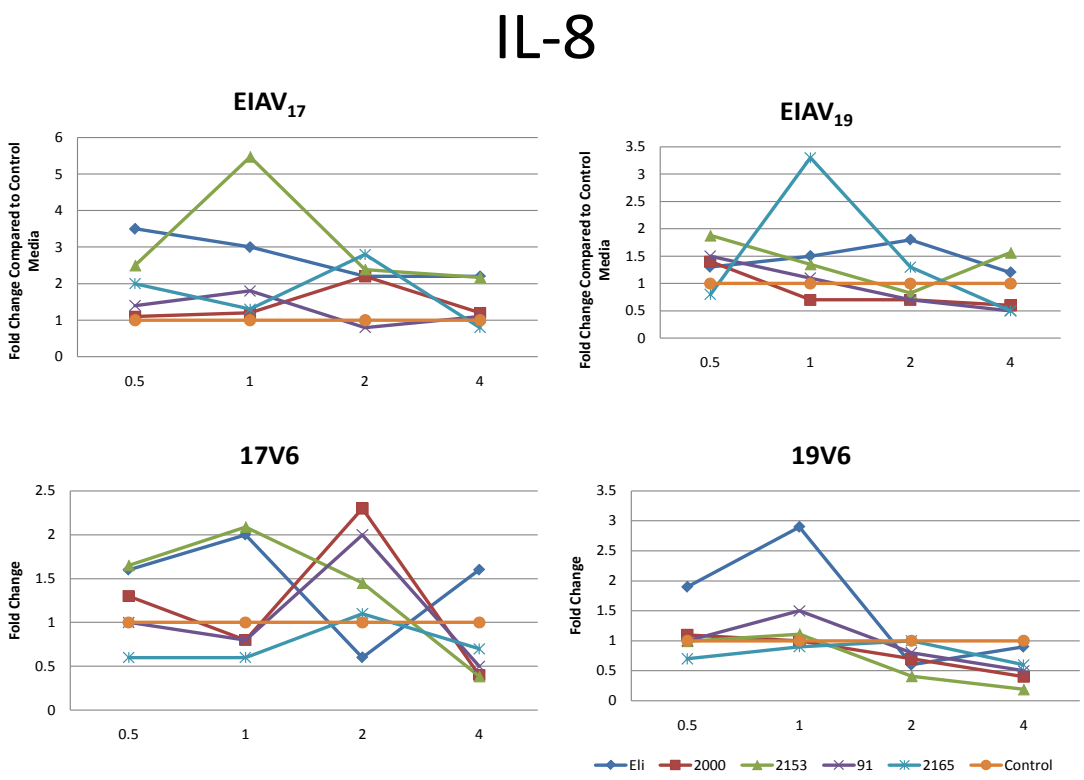

Figure 3.2- Change in IL-8 gene expression. Change in gene expression for each virus treatment for each horse through the time course of the experiment. The control media responses were set to 1 (baseline) for each horse.

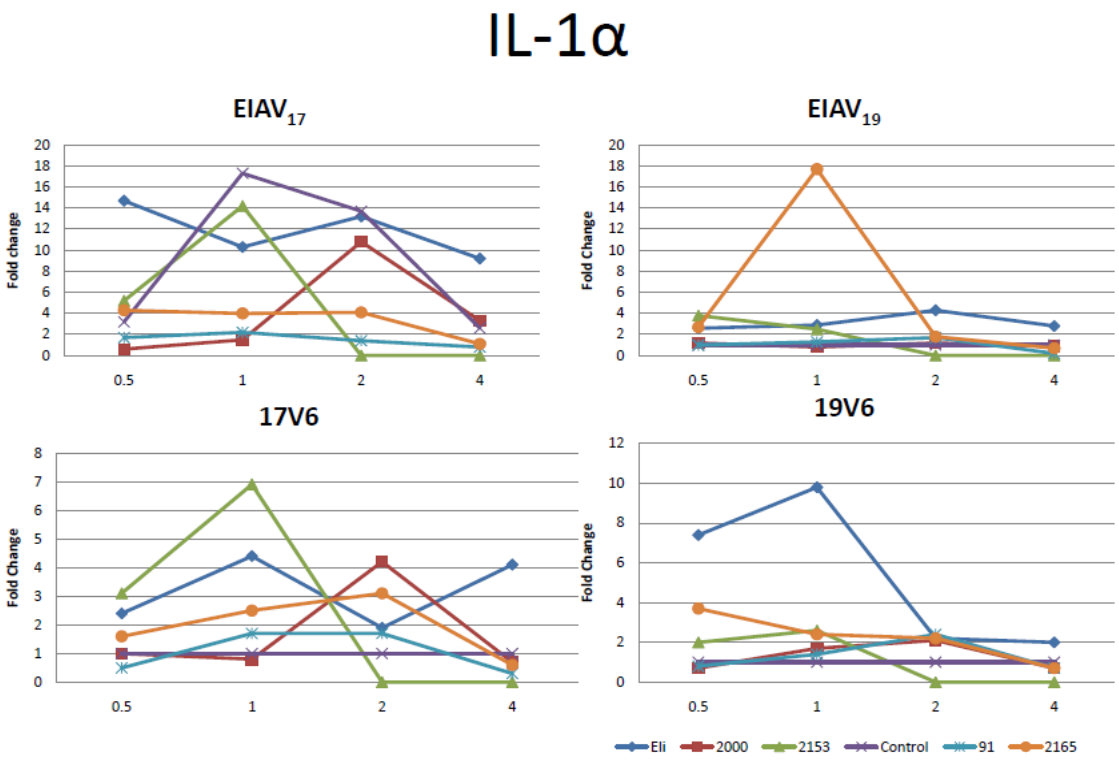

Figure 3.3- Change in IL-1α gene expression. Change in gene expression for each virus treatment for each horse through the time course of the experiment. The control media responses were set to 1 (baseline) for each horse.

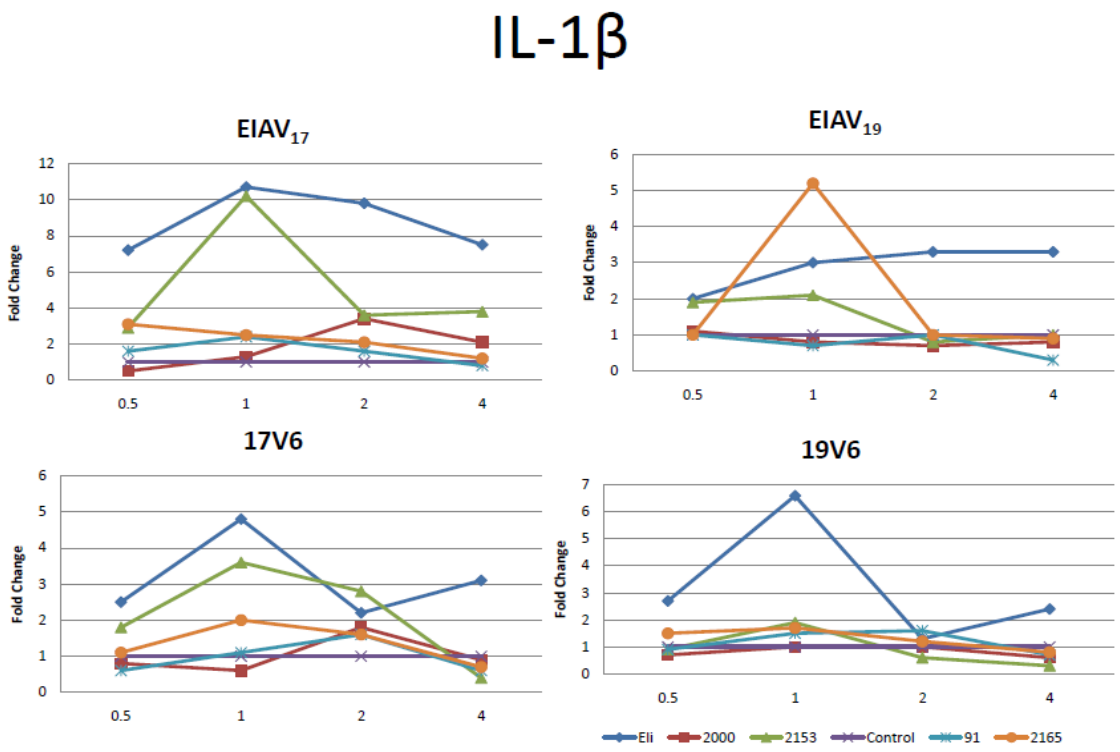

Figure 3.4- Change in IL-1β gene expression. Change in gene expression for each virus treatment for each horse through the time course of the experiment. The control media responses were set to 1 (baseline) for each horse.

Examination of Figure 3.5 shows the average changes in genes expression level (versus control media) for each virus. For the three genes measured, the fold-increases in expression were the most dramatic when cells were treated with $EIAV_{17}$. Expression of IL-1a increased 3 fold at 0.5 h, rose to 6 fold by 1 h and dropped back to 3 fold by 4 hours. A similar pattern was seen for IL-1b gene expression. Gene expression of IL-8 gene was 2-fold at 0.5 h with the maximum fold-increase (2.5 fold) at 1 hpi. Treatment of eMDM with the $EIAV_{19}$ virus stock resulted in much more modest changes in gene

expression. The expression of IL-1a was the most effected by $EIAV_{19}$ treatment; IL-1a mRNA levels were 2-fold above the control at 0.5 hpi and transiently rose to 5-fold at 1 hpi before falling to background levels by 4 hpi. IL-1b expression rose only marginally, to 2-fold over background levels at 1 hpi but then dropped by 2 hpi. The IL-8 data suggests some slight level of increase with $EIAV_{19}$ treatment, but never reaches 2-fold. Somewhat unexpectedly, the two chimeric viruses showed similar effects on cytokine gene expression, and appeared to be no different than the $EIAV_{19}$ virus. Expression of IL-1α was increased for all four virus infections compared to control media at 0.5 hpi indicating that cytokines are induced early, and that changes in gene expression are may result from virus or entry. Gene expression levels then dropped over the course of the experiment, returning to background levels for EIAV19 and the two chimeric viruses.

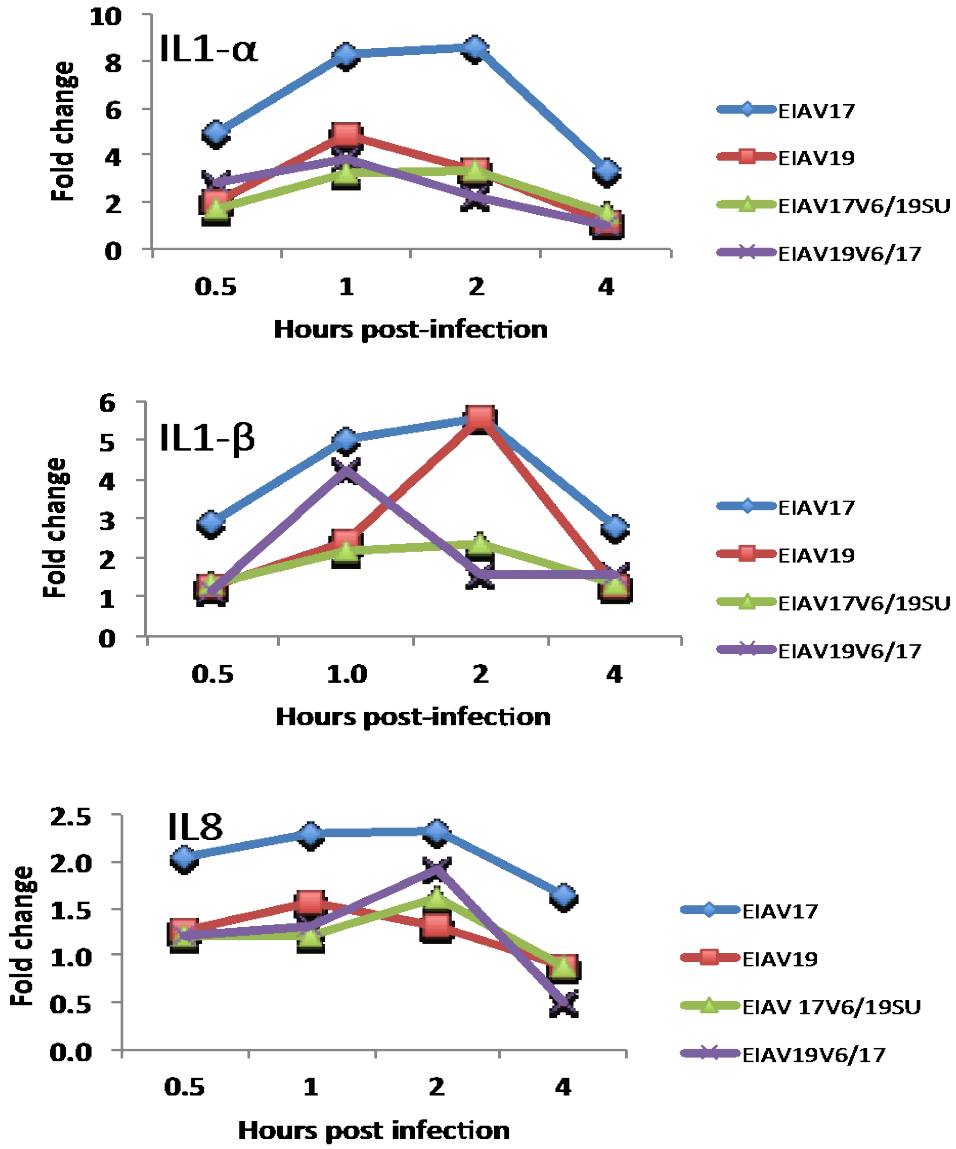

Figure 3.5- Average changes in gene expression levels. Mean fold changes in gene expression (relative to mock infected control). Values are averages for all donor horses. eMDM infected with 17b (■), 19 (▲), 17V6 (><), 19V6 (>●<).

CHAPTER IV

SUMMARY

EIAV is a lentivirus in the family *Retroviridae* that infects equids including horses, donkeys, and mules. EIAV infection is rarely fatal but results in a persistent life-long infection for which there is no treatment or cure. Because there is no vaccine or preventative therapy, horses that test positive for EIAV must be quarantined for life, cannot travel, and are often euthanized to limit the spread of the virus. The virus was discovered in 1904 but was not actively controlled until 1975 when a diagnostic test, Coggin's test, was available and testing became widely accepted. Today, EIAV is most frequently diagnosed in apparently healthy equids because of required surveillance testing when animals are moving interstate, going to congregation points, and at transfer of ownership. {Issel, 2010}

The outcome of an infection with EIAV is determined by a number of factors including the host immune system, the virus, and environmental factors. Some viral strains will greatly impact one species but have no effect in another. Infected animals may shed high levels of virus that is transmitted quickly to surrounding susceptible animals particularly if the infected animal is stressed or immunosuppressed. The most common route of transmission is via mechanical transfer from one equid to another. The major determinants of viral transfer are the level of virus in the blood and the amount transferred. The vector with the highest potential of transmission is man because large

amounts are transferred via blood transfusion and biological agents. Insect vectors, including horse flies, dear flies, and stable flies, are frequently blamed as a common source of transmission. The actual risk of transmission is extremely low even from horse with a high viremia because of the small amount of blood that is transferred from each insect. {Issel, 2010}

Once a horse is infected, it will develop a persistent, recrudescing viremia with variable clinical signs. A typical pattern is that an animal that is initially infected will develop a high fever with concurrent anemia, thrombocytopenia, and weight loss. Clinical signs are mediated by pro-inflammatory cytokines (IL-1α, IL-1β, IL-6, IL-8, and TNFα) when tissue associated viral burdens reach threshold levels. The acute disease usually resolves within a few weeks and the animal enters the chronic phase of the disease with reoccurrence of clinical signs over the course of a year with increased periods of latency between clinical signs. Initial control of EIAV infections is mediated via cell mediated immunity with the humoral immune response requiring six to eight months to mature. Immune control is established only after the immune system has co-evolved with the virus to a fully mature state {Craigo et al., 2010}. Once the viral replication has been controlled, the animal will remain free of clinical disease until a variant virus emerges that can evade immunological surveillance {Mealey et al., 2004}. The viral replication does not stop as evidenced by the production of disease when blood is transmitted from an inapparent carrier to a naïve host. {Tornquist et al., 1997}

EIAV has the ability to create quasispecies which produce escape mutants with the ability to evade the host immune system and induce additional clinical disease. Serologic and genetic characterizations of the evolution of EIAV quasispecies during chronic disease have demonstrated a close correlation between changes in viral neutralization specificity and variations in the sequence of the viral *env* glycoproteins, specifically in the gp90 region {Craigo et al., 2010}. The evolution of the viral quasispecies is continuous and the natural variation observed in the quasispecies is a major challenge to vaccine development {Craigo et al., 2010}. Ultimately, the capacity for the EIAV envelope to evolve and evade immune surveillance profoundly effects the ability of the animal to control the virus and impacts our ability to develop effective vaccines and therapeutics. {Craigo et al., 2013}

The goal of the current study was to understand the role of one specific variable region in the complex SU protein and its impact on virulence. By looking at cytokine induction in cells infected with variant viruses, we can determine if a specific region may have role in the overall virulence of the virus. Then comparing data obtained *in vitro* with the *in vivo* pony data, we can clearly elucidate that the results may not be fully extrapolated into an *in vivo* model. The current study using, QPCR as a method of detection, expands on current studies by Covaleda and the Payne lab {Covaleda et al., 2010}. The study uses QPCR techniques that were validated by Allen and demonstrated by Covaleda {Allen et al., 2007; Covaleda et al., 2010}.

Cytokines are small proteins that act as signaling molecules to regulate inflammation and control cellular functions. Cytokines are a diverse group of pro- or anti-inflammatory factors that are grouped into families based on their structural homology or the structure of their receptors. Chemokines are a subset of cytokines that induce cell migration. Chemokines belong to two catagories based on their biological activities: maintenance of homeostasis through immune surveillance or induction of inflammation. The binding of a cytokine or chemokine to a cell sets off a cascade of events that regulate cell function such as cell adhesion, phagocytosis, cytokine secretion, cell activation, cell proliferation, apoptosis, angiogenesis, and proliferation. All the cytokines included in this study are inflammatory mediators which would be activated during a viral infection. {Ramesh et al., 2013; Tornquist et al., 1997}

Interleukin- 1 (IL-1) consists of two molecules- IL-1 α and IL-1β. IL-1 alpha and IL-1 beta are cytokines that are primarily secreted by macrophages and have distinct roles in antibody production. They have two main functions in that they assist leukocytes to migrate through vessel walls to the site of infection and acts on the thermoregulatory centers of the brain to raise the body temperature.

IL- 8, also known as CXCL8, is secreted by macrophages, as well as other cells, upon phagocytosis of foreign material. It has 2 primary functions: induce chemotaxis and induce phagocytosis. It is also associated with inflammation and has been demonstrated to be overexpressed in chronic conditions such as cancer and hepatitis C virus infection.

The role of IL-8 in EIAV has yet to be determined but it may have opposing antiviral and proviral effects between acute and chronic disease states. {Koo et al., 2006}

TNF-alpha is an inflammatory cytokine that produces a myriad of responses in the body. Activation of TNF-α directly inhibits erythropoiesis and may be a primary contributor of the anemia associated with EIAV infection {Morceau et al., 2009}. Our interest in this cytokine stems from its roll in chemotaxis and the induction of neutrophil proliferation as well as its role on fever induction. Previous studies {Lim et al., 2005} demonstrated significant changes in the same cytokine/chemokines used for these experiments. I used that data as a baseline to test my hypothesis that the V6 region of virulent EIAV17 is a key contributor to cytokine/chemokine stimulation post infection.

Studies have clearly demonstrated that there is a significant increase in proinflammatory cytokines upon infection with virulent EIAV. It is unclear why induction of TNF-α was so variable between the horses used in the *in vitro* studies. Given that the animals are outbred and not genetically identical, some variation is expected. However, given that TNF-α is strong contributor to the clinical signs expected with acute EIAV infection and a strong factor in the induction of anemia, it is surprising that there was not some consistency between the horses.

My data revealed that the V6 chimeric viruses did not induce cytokine/chemokine expression levels above the avirulent $EIAV_{19}$ virus. The results showed that there is not

a strong correlation between the V6 region alone and cytokine induction, but did demonstrate that the V6 region is a component in the overall virulence of $EIAV_{17}$. Additional studies involving the V6 region and other SU or TM regions will help understand the contribution of the variable regions to the induction of disease pathogenesis and clinical signs in horses. The ultimate goal is to determine specific regions of the genome that can be targeted to create an effective vaccine against the virus that clearly distinguishable from natural infection.

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APPENDIX I

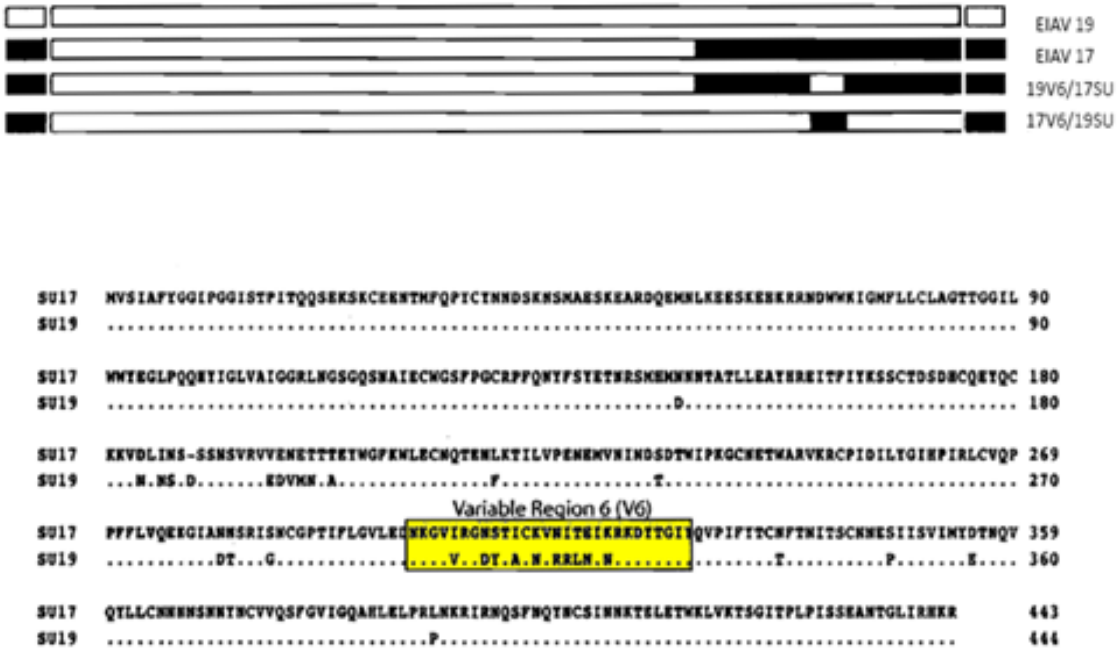

Figure A1- Top is a schematic of the 4 clones described in this proposal. The black boxes represent sequences derived from the virulent Wyoming strain of EIAV. The white boxes represent the nt sequences derived from avirulent culture adapted EIAV. Bottom is a comparison of SU amino acids. The EIAV 17 sequence is shown above; EIAV19 residues that differ are shown below. The highlighted region represents the 28 amino acid region that is swapped in the chimeric viruses. Figures obtained from Dr. Payne's NIH grant.

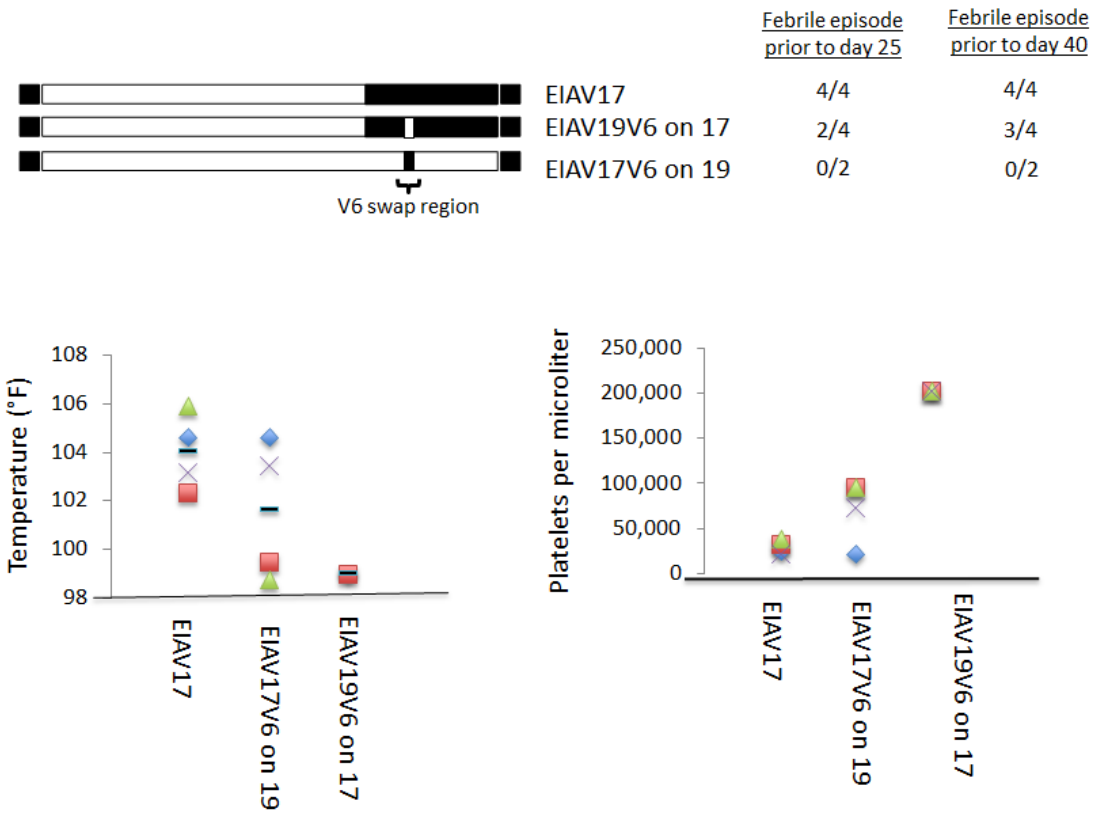

Figure A2- EIAV proviral constructs with the V6 swap region (above). *In vivo* temperature and platelet data (Fuller; 2011-2013).

APPENDIX II

Statistical Analysis

Data was analyzed using a linear mixed model. This model was chosen for this data analysis because the model takes into account random effects as well as fixed effects. The fixed and random effects are assumed to have a normal distribution. In our model, the horse was the random effect and the virus and time points as the fixed effects.

$$Y_{ijk} = \mu + horse_i + virus_j + time_k$$

Before we could analyze the data sets with the model, we first performed a Shapiro-Wilk test in order to determine if the residuals were normally distributed. If they were not, we would not be able to determine statistical significance of the results. When we looked at the data from all six horses, we found that the residuals were not normally distributed (See Figure A3 and Figure A4). This was attributed to outliers skewing the data.

In order to obtain a data set that we could use to find statistical significance, we discarded the data from Horse 2230 and repeated the Shapiro-Wilk using the data from the remaining five horses. This data set was determined to have normally distributed residuals (See Figure A5 and Figure A6) and we could proceed with the data analysis using the linear mixed model.

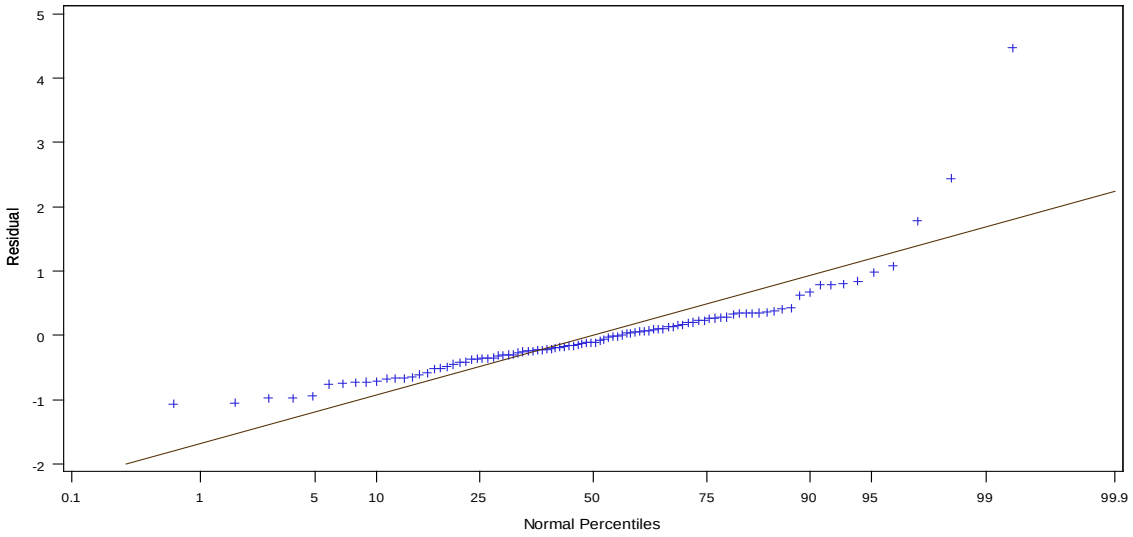

Figure A3- This plot shows that the residuals are not normally distributed; they should fall on a straight line. This plot was created using data from all six horses.

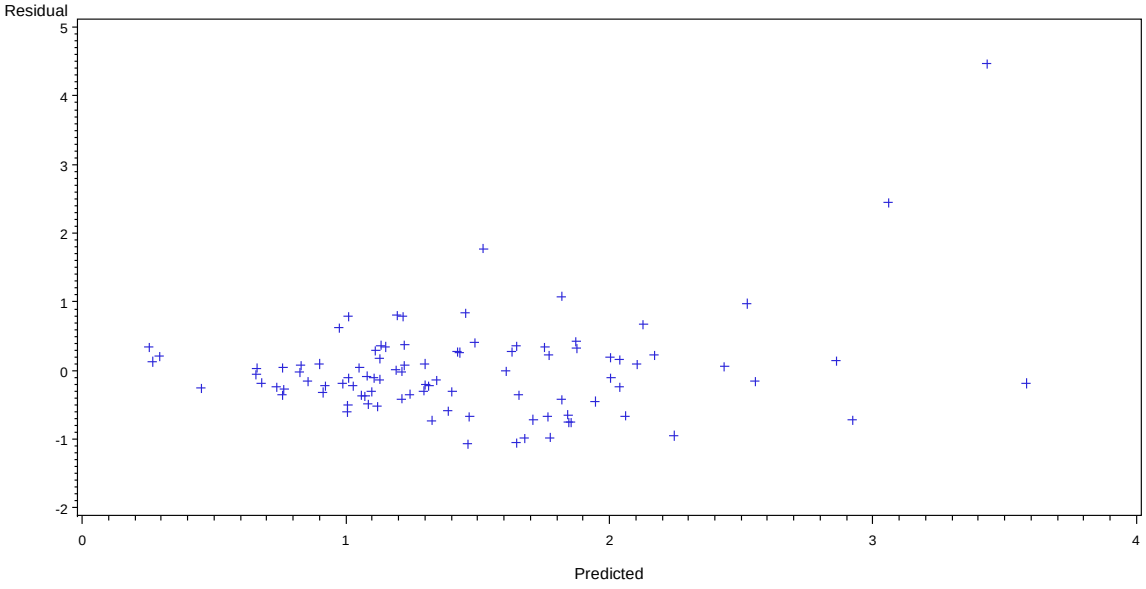

Figure A4- This plot shows how the residuals do not have constant variance because the scatterplot has a discernable trumpet pattern. This plot is created using data from all six horses. This pattern is created by outliers specifically from data from Horse 2230.

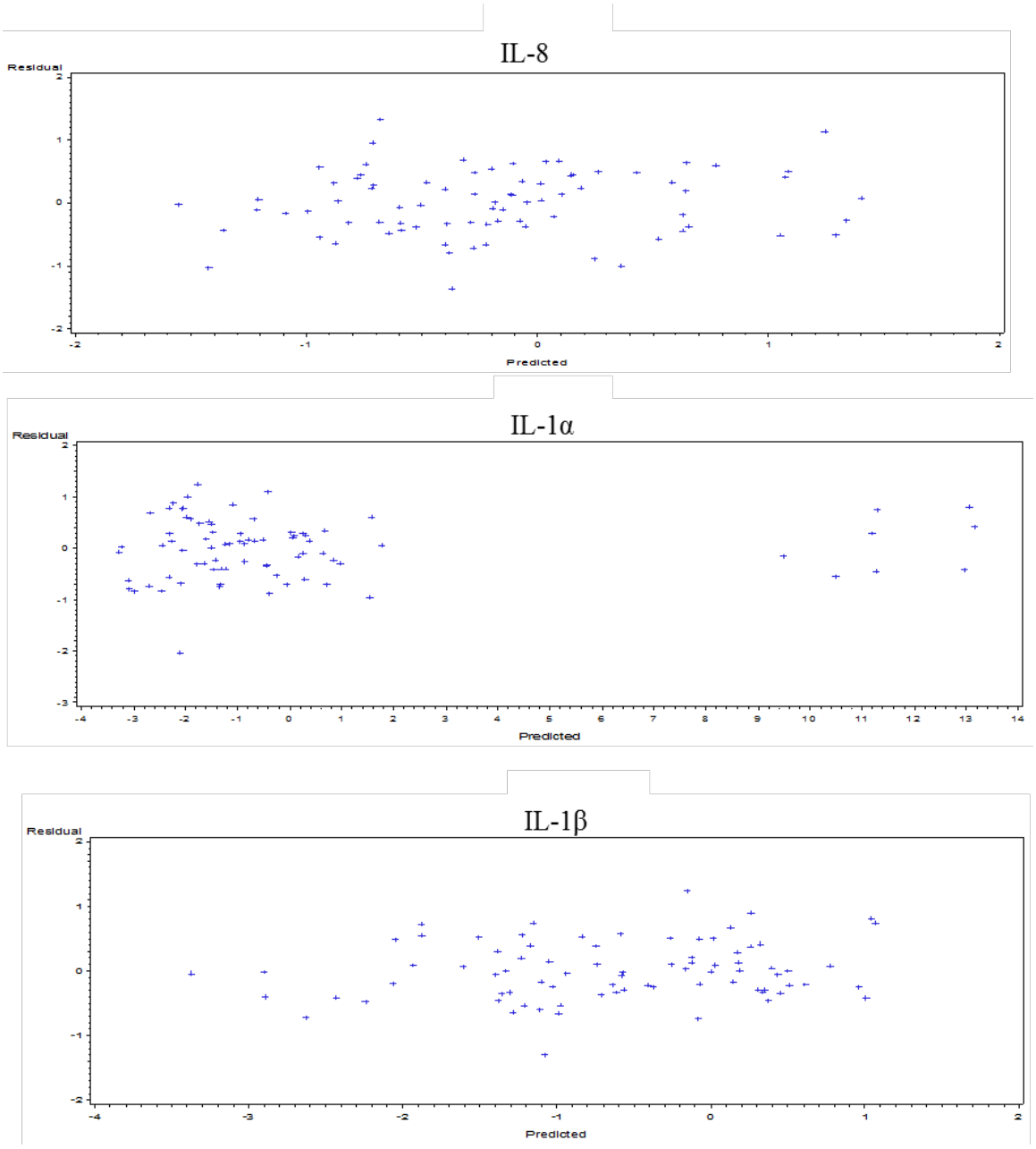

Figure A5- There is no discernable pattern in the residuals, so you conclude that they have constant variance. These plots were obtained using data from 5 horses and omitted the data from Horse 2230.

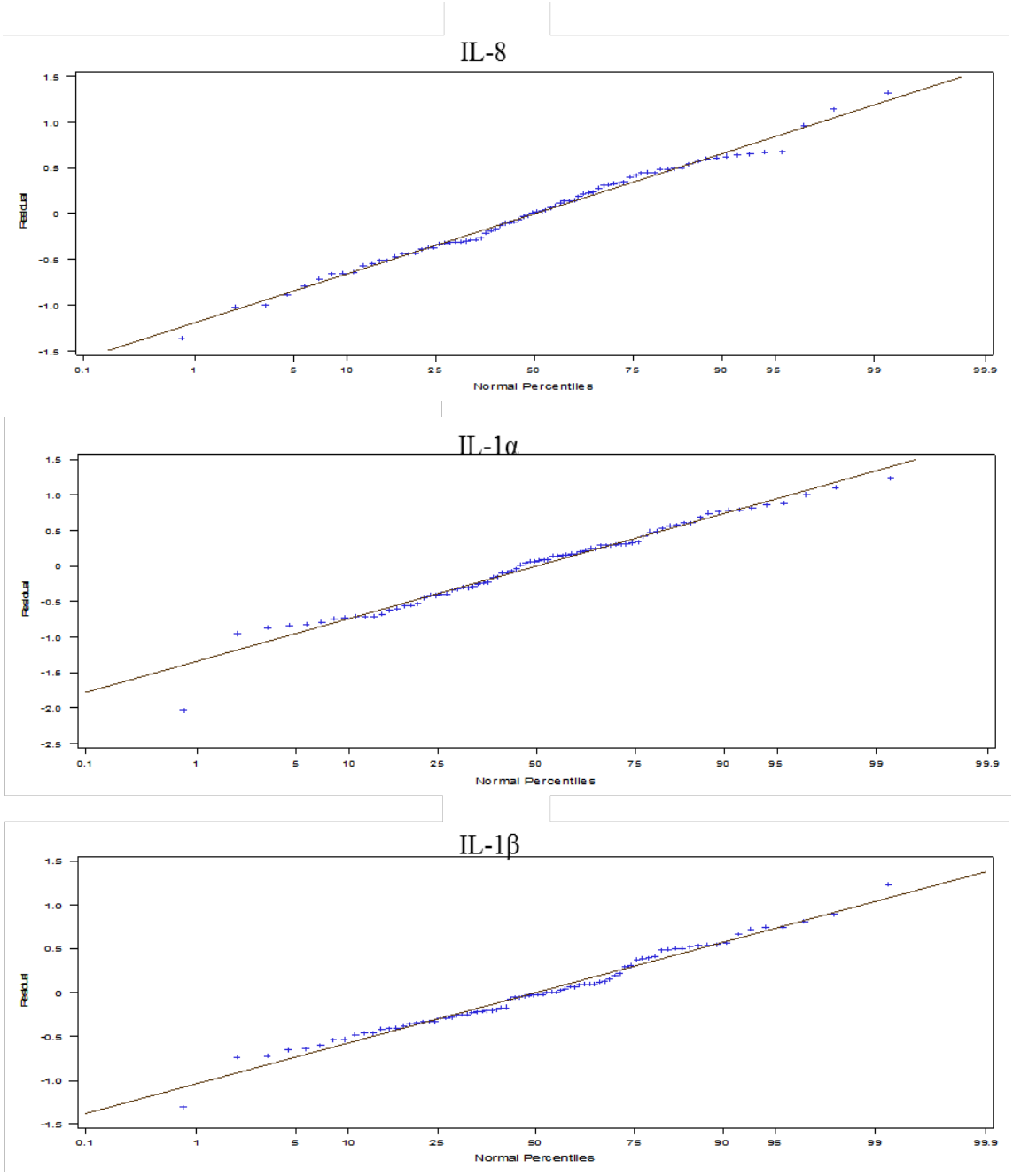

Figure A6- The residuals fall along a straight line, so you conclude that they have a normal distribution. These plots were obtained using data from 5 horses and omitted the data from Horse 2230.

qPCR Results:

Once it was determined that the data was normally distributed and the residuals had a constant variance, thereby accepting the null hypothesis, the data was analyzed using the linear mixed model. First the model determined the least squares means of each virus, which is the group means after having controlled for a covariate. Then the model looks at the differences of least square means between each group. There we look at the adjusted p-value to determine if the viruses are related or have a significant difference. A significant difference is determined by a p-value of < 0.05. The least square means and differences of least square means were determined for each cytokine/chemokine gene expression.

Table A1- Least Means Squares to Determine Statistical Significance of Cytokine/Chemokine Gene Expresion:

Data for IL-8:

Least Squares Means

Effect	Virus	Estimate	Standard Error	DF	t Value	Pr > |t|
Virus	EIAV17V6	0.01180	0.1992	12	0.06	0.9537
Virus	EIAV17b	-0.8690	0.1992	12	-4.36	0.0009
Virus	EIAV19	-0.1280	0.1992	12	-0.64	0.5324
Virus	EIAV19V6	0.3088	0.1992	12	1.55	0.1470

Differences of Least Squares Means

Effect	Virus	-Virus	Estimate	Standard Error	DF	t-Value	Pr > |t|	Adjustment	Adj P
Virus	EIAV17V6	EIAV17b	0.8809	0.2157	12	4.08	0.0015	Tukey-Kramer	0.0072
Virus	EIAV17V6	EIAV19	0.1398	0.2157	12	0.65	0.5290	Tukey-Kramer	0.9141
Virus	EIAV17V6	EIAV19V6	-0.2970	0.2157	12	-1.38	0.1937	Tukey-Kramer	0.5358
Virus	EIAV17b	EIAV19	-0.7410	0.2157	12	-3.43	0.0049	Tukey-Kramer	0.0222
Virus	EIAV17b	EIAV19V6	-1.1779	0.2157	12	-5.46	0.0001	Tukey-Kramer	0.0007
Virus	EIAV19	EIAV19V6	-0.4369	0.2157	12	-2.03	0.0657	Tukey-Kramer	0.2324

For IL-8, it was determined that the 17b virus had a significantly different expression level than the other 3 viruses. 17V6, 19V6, and 19 did not significantly differ in expression level when compared to each other.

Data for IL-1α:

Least Squares Means

Effect	Virus	Estimate	Standard Error	DF	t-Value	Pr > |t|
Virus	EIAV17V6	0.5083	1.2282	12	0.41	0.6863
Virus	EIAV17b	-0.6819	1.2282	12	-0.56	0.5889
Virus	EIAV19	0.4749	1.2282	12	0.39	0.7058
Virus	EIAV19V6	0.4766	1.2282	12	0.39	0.7048

Differences of Least Squares Means

Effect	Virus	_Virus	Estimate	Standard Error	DF	t-Value	Pr > |t|	Adjustment	Adj P
Virus	EIAV17V6	EIAV17b	1.1902	0.2719	12	4.38	0.0009	Tukey-Kramer	0.0043
Virus	EIAV17V6	EIAV19	0.03340	0.2719	12	0.12	0.9043	Tukey-Kramer	0.9993
Virus	EIAV17V6	EIAV19V6	0.03170	0.2719	12	0.12	0.9091	Tukey-Kramer	0.9994
Virus	EIAV17b	EIAV19	-1.1568	0.2719	12	-4.25	0.0011	Tukey-Kramer	0.0053
Virus	EIAV17b	EIAV19V6	-1.1585	0.2719	12	-4.26	0.0011	Tukey-Kramer	0.0053
Virus	EIAV19	EIAV19V6	-0.00170	0.2719	12	-0.01	0.9951	Tukey-Kramer	1.0000

Data for IL-1β:

Least Squares Means

Effect	Virus	Estimate	Standard Error	DF	t-Value	Pr > |t|
Virus	EIAV 17V6	-0.4892	0.3871	12	-1.26	0.2303
Virus	EIAV 17b	-1.4978	0.3871	12	-3.87	0.0022
Virus	EIAV 19	-0.3155	0.3871	12	-0.82	0.4309
Virus	EIAV 19V6	-0.2450	0.3871	12	-0.63	0.5386

Differences of Least Squares Means

Effect	Virus	_Virus	Estimate	Standard Error	DF	t-Value	Pr > |t|	Adjustment	Adj P
Virus	EIAV17V6	EIAV 17b	1.0086	0.2539	12	3.97	0.0018	Tukey-Kramer	0.0087
Virus	EIAV 17V6	EIAV 19	-0.1736	0.2539	12	-0.68	0.5070	Tukey-Kramer	0.9012
Virus	EIAV 17V6	EIAV 19V6	-0.2441	0.2539	12	-0.96	0.3552	Tukey-Kramer	0.7730
Virus	EIAV 17b	EIAV 19	-1.1822	0.2539	12	-4.66	0.0006	Tukey-Kramer	0.0027
Virus	EIAV 17b	EIAV 19V6	-1.2527	0.2539	12	-4.93	0.0003	Tukey-Kramer	0.0017
Virus	EIAV 19	EIAV 19V6	-0.07050	0.2539	12	-0.28	0.7860	Tukey-Kramer	0.9921

For IL-1α and IL-1β, the data shows a significant difference in expression in 17b compared to the other 3 viruses. The 17V6, 19V6, and 19 viruses had no significant differences when compared to each other.