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GENETIC IMMUNIZATION IN THE HORSE: THE POTENTIAL FOR ENHANCED IMMUNE RESPONSES WITH DEACYLATED POLYETHYLENEIMINE (PEI) AND IMMUNOSTIMULATORY CYTOKINES AS VACCINE ADJUVANTS

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GENETIC IMMUNIZATION IN THE HORSE: THE POTENTIAL FOR ENHANCED
IMMUNE RESPONSES WITH DEACYLATED POLYETHYLENEIMINE (PEI) AND
IMMUNOSTIMULATORY CYTOKINES AS VACCINE ADJUVANTS

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Agriculture
at the University of Kentucky

By

Deborah Lee Even

Lexington, Kentucky

Co-Directors: Dr.Charles J. Issel, Professor of Veterinary Science
and Dr.R. Frank Cook, Associate Professor of Veterinary Science

Lexington, Kentucky

1995

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ABSTRACT OF DISSERTATION

GENETIC IMMUNIZATION IN THE HORSE: THE POTENTIAL FOR ENHANCED IMMUNE RESPONSES WITH DEACYLATED POLYETHYLENEIMINE (PEI) AND IMMUNOSTIMULATORY CYTOKINES AS VACCINE ADJUVANTS

DNA vaccines in larger animals, such as horses, are generally less effective and elicit significantly weaker immune responses, than in small animal model systems. To provide optimal protection against pathogenic microorganisms, the induction of both humoral and cellular immune responses from DNA vaccination may be necessary. One limitation to DNA immunization in the horse is the difficulty in generating high levels of antigen-specific antibody and CTL responses. Previous work in the laboratory has demonstrated that expression constructs containing native sequences encoding the surface unit (SU) envelope glycoprotein (pCiSU) of the Equine Infectious Anemia Virus (EIAV) are ineffective at stimulating immune responses in the horse. This was attributed to an unusual codon-usage bias of the EIAV genome that significantly limits the expression of SU sequences. Optimizing the codon usage of pCiSU (pSYNSU) in DNA vaccines stimulated low-titer immune responses in inoculated ponies. Another plausible explanation for the reduced effectiveness of these DNA vaccines may be transfection deficiency and low level expression elicited by plasmid vectors in the horse. These studies investigated if the addition of a cationic polymer, deacylated polyethyleneimine (PEI), and/or codon optimized molecular immune-stimulatory cytokines could augment the relatively weak immunogenicity of pSYNSU in DNA vaccination of horses/ponies. Administration of DNA in formulation with PEI resulted in the robust production of very long-lived humoral (15 months after vaccination) responses and induced cell-mediated IFN- γ responses five days after immunization.. Additionally, the co-expression of a family of IL-15 cytokines expanded the repertoire of T cell recognition to SU-specific peptides, in terms of lymphoproliferation. DNA vaccination incorporating one IL-15 family member, IL-15 (SSLSS) significantly enhanced serum antibody levels of IgG_A and IFN- γ mRNA expression levels. These responses were distinctly different from results seen with vaccinates that received 'naked' pSYNSU DNA vaccines. It is evident from these vaccine studies that PEI can enhance DNA vaccine-elicited antibody and CTL-associated responses in the horse and IL-15 (SSLSS) can dramatically augment these responses. These results demonstrate an important role for PEI in promoting the longevity of immune responses to genetic immunization, which has not been reported previously in any large animal model.

KEYWORDS: DNA Vaccination, Horse, Cytokines, Polyethyleneimine, Immune Responses

Deborah Lee Even

December 16, 2011

GENETIC IMMUNIZATION IN THE HORSE: THE POTENTIAL FOR ENHANCED
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December 16, 2011

DEDICATION

This dissertation is dedicated in honor of my mother, Sherry Grace Even, and in memory of my father, David Charles Even. Without the endless love and support of my parents, this representative chapter of my life would not have been possible. They instilled in me the drive to forge on through all my trials and tribulations and taught me that even the largest tasks can be accomplished when approached one step at a time. Although my father has been deceased for a couple of years, I know he is very proud of me and is cheering me on from heaven. I love and miss him very much.

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Chapter I: Background and Literature Review

“It's clear that prevention will never be sufficient. That's why we need a vaccine that will be safe.” Luc Montagnier

I. Introduction

Since the demonstration in the early 1990's that immune responses are inducible by inoculation of plasmid DNA expression vectors encoding foreign antigens (Tang, DeVit et al. 1992), there has been considerable interest in using this technology to develop vaccines. DNA vaccination involves the direct introduction of a plasmid or DNA sequence encoding the antigen(s) against which an immune response is sought into appropriate tissues. The effectiveness of this technique is dependent on the in situ production of antigen after introduction of DNA. This approach offers a number of potential advantages over traditional approaches, including the stimulation of both B- and T-cell responses, improved vaccine stability, the absence of any infectious agent, and the relative ease of large-scale manufacture. As proof of principle of this genetic-based approach, immune responses in humans and animals have been generated using genes from a variety of viral, bacterial, parasitic, and tumor agents (Cox, Zamb et al. 1993; Ulmer, Donnelly et al. 1993; Wang, Ugen et al. 1993; Sedegah, Hedstrom et al. 1994). Scientific advances in immunology, molecular biology, and infectious disease pathogenesis have revolutionized this radical new approach to vaccination, and much focus has shifted toward the development of strategies to further augment immune responses to any number of infectious microorganisms using DNA vaccination.

Advantages of Gene-Based Vaccine Protocols

Genetic or DNA vaccines offer a number of potential advantages over traditional approaches ranging from the fact that they are (1) easy to engineer and manipulate at the molecular level, (2) capable of expressing highly modified or even completely novel antigens, (3) inexpensive to manufacture, and (4) are intrinsically stable, thereby reducing the necessity for maintenance of a “cold chain” during distribution. One of the major advantages of using DNA expression vectors as immunogens is the capability to

stimulate both humoral and cell-mediated arms of the immune system. In contrast, most conventional inactivated vaccine products predominantly induce antibody responses (Tang, DeVit et al. 1992; Ulmer, Donnelly et al. 1993; Xiang, Spitalnik et al. 1994). DNA vaccines are also inherently safe, possessing none of the risks associated with conventional vaccines, such as the possibility of incomplete inactivation in the case of “killed” vaccines or a reversion to virulence associated with live-attenuated products. In addition, one of the early fears that DNA vaccines would integrate into the genome inducing tumors or other metabolic disorders has not been realized after almost two decades of extensive research involving very large numbers of experimental animals (Kutzler and Weiner 2008).

Processing of Antigens Expressed from Plasmid DNA

To produce specific DNA components for genetic vaccination studies, target genes are inserted into molecular expression vectors, with transcription of these sequences usually regulated by a strong constitutive mammalian promoter (Wolff, Malone et al. 1990; Cox, Zamb et al. 1993; Ulmer, Donnelly et al. 1993). Upon injection of genetic vaccines, plasmid DNA is endocytosed into nearby cells, and genes are subsequently transcribed into RNA after nuclear translocation. Single stranded mRNA is then exported into the cytoplasm, where it is bound by ribosomes, translated into protein, and modified in the ER and Golgi Apparatus. One important potential advantage of DNA vaccinations compared with alternative recombinant technologies, such as exogenous antigen production in bacteria, yeast, or baculovirus systems is that the expressed proteins will contain the correct post-translational modifications for the species being vaccinated. Some of these proteins may then be routed to cellular proteosomes where they are degraded into multiple peptides. Subsequent binding of these peptides to major histocompatibility complexes (MHC) class I molecules occurs in the cytoplasm, and results in insertion of the MHC I/peptide antigen complexes into the plasma membrane of the cell, with the peptide bound to the extracellular portion of the molecule. In this bound form, the peptide can interact with T lymphocytes, such as CD8⁺ cytotoxic T lymphocytes (CTL), to stimulate specific cellular immune responses. Therefore, DNA

immunization and antigen processing through the MHC I pathway may favor the induction of cell-mediated immunity (CMI).

DNA Vaccine Delivery Methods

A number of different methods have been used to introduce DNA into animal tissues. The two most common methods involve intradermal (i.d.) or intramuscular (i.m.) injection with a hypodermic needle or gene gun delivery, although tattooing and electroporation are also viable methods to introduce DNA antigen into animal tissue. Electroporation has gained popularity in recent years as a technique to facilitate DNA delivery *in vivo* to antigen presenting cells and enhance immune responses to DNA. Several alternative delivery methods, such as topical administration of plasmid DNA to the eye (Daheshia, Kuklin et al. 1997) and vaginal mucosa (Lewis and Babiuk 1999) and aerosol instillation of naked DNA on nasal and mucosal surfaces (Lewis and Babiuk 1999) have also been used to deliver plasmid DNA, although with marginal success. The injection of DNA alone into a tissue, without chemical agents or physical force, has resulted in gene expression in skin (Choate and Khavari 1997), muscle (Wolff, Malone et al. 1990), liver (Hickman, Malone et al. 1994; Budker, Zhang et al. 1996; Zhang, Vargo et al. 1997), or into the airways (Meyer, Thompson et al. 1995).

The simplicity of delivering immunizations containing only plasmid DNA, as well as the safety associated with the technique makes this an appealing vaccination approach. An ideal DNA vaccine will initiate prompt stimulation of both humoral and cellular arms of the immune system, with minimal doses of the vaccine, and enable a sustainable immunity over time.

Challenges with DNA Vaccination

Unfortunately, a major limitation of DNA vaccines is that while they generally produce strong protective immune responses in small rodent animal models, results are generally much less favorable in large animals including humans (Manoj, Babiuk et al. 2004; van Drunen Littel-van den Hurk, Babiuk et al. 2004; Forde 2005). The primary reason for this is that expression of selected antigens *in vivo* by immunization with unprotected or naked plasmid vector DNA is an extremely inefficient process. Possible

reasons for this poor efficacy of DNA vaccines in large animals may be reduced transfection efficiency, low level gene expression, and suboptimal protein production elicited by some plasmid vectors. Some of the barriers encountered by using DNA as a vaccine approach stem from the large size of the plasmids and genes and the hydrophilic tendencies of the DNA constructs. This poses an issue with the transfection of plasmids into some target cells.

DNA is also extremely susceptible to degradation by cellular nucleases and extracellular enzymes. Although it is difficult to quantitate, it is believed that 90% of the DNA is washed away by the bloodstream or degraded by nucleases, prior to cellular uptake. Nucleic acids distributed by the circulatory system do not appear to be taken up by cells but instead are rapidly degraded, becoming undetectable in most organs by two days post inoculation (Parker, Borellini et al. 1999). Of the remaining DNA in the cytoplasm, less than 1% may be translocated into the nucleus (Parker, Borellini et al. 1999). Intact DNA is necessary for nuclear translocation and subsequent transcription and translation into protein.

Even if DNA molecules survive to enter a cell, their translocation from the cytoplasm to the nucleus is a “perilous journey” (Lechardeur and Lukacs 2006). The cytoskeleton often impedes diffusion of large DNA molecules through the cytoplasm thereby increasing the probability of nuclease degradation (Dauty and Verkman 2005) such that in some cell types 50% of all unprotected DNA is degraded within two hours (Lechardeur, Sohn et al. 1999). Furthermore, for DNA molecules larger than 250 bp, entry into the nucleus requires active transport via the nuclear pore complex or disassembly of the nuclear membrane prior to cell division (Lechardeur and Lukacs 2006). Finally, once inside the nucleus, access of exogenous DNA molecules to the optimum transcriptional microenvironment may be restricted by high-affinity interactions with nuclear proteins such as scaffold attachment factor-A (SAF-A) (Baiker, Maercker et al. 2000; Mearini, Nielsen et al. 2004).

EIAV pSU and pSYNSU in DNA Vaccination

Because of the poor intrinsic efficiency of conventional genetic immunization techniques, the presence of additional factors diminishing either cellular uptake or

expression level of an experimental DNA vaccine may completely abolish detectable immune responses, especially in large animals. This was demonstrated in ponies inoculated with plasmid DNA (pCi Native SU) containing native sequences, encoding the surface unit (SU) envelope glycoprotein of equine infectious anemia virus (EIAV) (Zhou, Cook et al. 2002). Although a single i.m. inoculation containing 100µg of this plasmid was sufficient to induce easily detectable immune responses to pCi native SU DNA in mice, no responses were apparent in ponies after three i.m. doses containing 1.5mg pCi native SU (Zhou, Cook et al. 2002). Native viral sequences encoding SU are poorly expressed in mammalian systems, probably because of an usual codon-usage bias, possession of multiple RNA splice sites, and the presence of motifs with homology to adenosine-rich instability elements (Zhou, Cook et al. 2002). Removal of these inhibitory features using the techniques of codon optimization and synthetically produced DNA sequences (pCi SYNSU) results in a 30-fold increase in SU expression in transfected cell cultures (Cook, Cook et al. 2005).

Furthermore, humoral immune responses were detectable in some ponies following just two i.d. inoculations with 250µg plasmid DNA (pCi SYNSU) containing codon optimized SU sequences (Cook, Cook et al. 2005). However, despite a significant increase in expression levels and immunogenicity compared to native SU sequences, immune responses to SU in pCi SYNSU vaccinated ponies were substantially lower than those typically found in EIAV infected equids. In addition, these responses failed to provide measurable protection from infection or disease following challenge with a virulent virus strain (Cook, Cook et al. 2005), clearly demonstrating the need for major improvements in immunogenicity before genetic vaccines against a wide range of pathogenic organisms are suitable for routine usage in large animals such as the horse.

Equine DNA Vaccine against West Nile Virus

Although the DNA vaccine platform has driven significantly weaker immune responses in large animals compared with mice, a successful, commercially available, plasmid-based vaccine (West Nile-Innovator[®], Fort Dodge Animal Health, Overland Park, KS) is currently licensed in the United States for use against West Nile Virus (WNV) for horses. This DNA vaccine was developed in conjunction with scientists at

the Centers for Disease Control and Prevention (Davis, Chang et al. 2001) and encodes the viral pre-membrane (prM) and envelope (E) proteins. The efficacy of this vaccine, a single 1 mg plasmid DNA dose protects horses from challenge with a virulent strain of WNV (Davis, Chang et al. 2001), results from some highly unusual and fortuitous properties. Following synthesis in transfected cells, the two proteins aggregate to form prM-E particles that are both highly immunogenic and contain viral neutralizing epitopes. Another advantageous characteristic of this system is that protection against WNV is relatively easy to achieve because it is conferred by low serum titers of virus neutralizing antibodies. In the original studies of Davis et al. (Davis, Chang et al. 2001), serum antibodies in DNA vaccinated horses to WNV were barely detectable by immunoblotting at a dilution of 1:25, and the geometric mean neutralizing antibody titer prior to challenge in the four recipient horses were 1:5, 1:20, 1:20, and 1:40 (Davis, Chang et al. 2001). Although protective in this specific case, these levels of antibody reactivity might be considered 'low-titer' humoral responses in most experimental situations. Therefore, this demonstration of a successful development of a DNA vaccine against WNV in the horse represents an exception and not the rule.

Correlates of Immune Protection against Viral Infection

Unlike WNV, obvious correlates of immune protection are unknown against many other equine diseases, such as Equine Herpes Virus- 1 or EIAV. This has impeded the development of prophylactic vaccines, especially against retroviral disease, where safe and effective vaccination strategies seem elusive. In humans, protection against HIV is associated with the induction of CTL (Gallimore, Cranage et al. 1995; Rowland-Jones, Tan et al. 1997), CD4+ lymphocyte (Rosenberg, Billingsley et al. 1997), chemokine (Garzino-Demo, DeVico et al. 1998) and/or antibody responses (Burton 1997). Other factors, such as high titer antibody responses or the induction of CMI responses may be necessary to provide adequate protection against some equine pathogens.

An ideal goal for the design of a vaccine against EIAV may be the production of long-lived host immune responses that provide protection against diverse strains of EIAV introduced through natural routes of infection. In EIAV infected animals, the emergence of non-neutralizing antibodies and virus-specific CD8+ CTL correspond with the

clearance of primary infectious plasma viremia (McGuire, Fraser et al. 2002). While this may resolve acute disease, the virus mutates rapidly to evade immune surveillance. Therefore, a broadening of humoral and cellular immune responses may be necessary to recognize an increased number of viral epitopes. In HIV infected individuals, expansion of the T cell repertoire to recognize multiple CTL epitopes has been shown after acute infection (Yu, Addo et al. 2002). Similarly, in horses after infection with an attenuated strain of EIAV, increased numbers of CTL and T-helper cell epitopes within the viral envelope glycoproteins were identified, indicative of an expansion of immune responses in these animals (Tagmyer, Craigo et al. 2007). Furthermore, the induction of neutralizing antibody (nAb) responses from DNA immunization may be a key element in the successful development of a protective gene-based vaccine against EIAV, especially since broadly reactive nAb responses correlate with virus control during infection in horses (Hammond, Cook et al. 1997; Hammond, Li et al. 2000; Belshan, Baccam et al. 2001; Sponseller, Sparks et al. 2007). Recently, In SCID foals, the protective effects of broadly nAb responses against EIAV infection was demonstrated (Taylor, Leib et al. 2011). However, since the precise correlates of protection against natural EIAV infection are still unknown, a number of vaccine strategies have been investigated to broaden equine immune responses and provide protection against disease.

Cationic Polymers and Lipids in DNA Vaccination

A frequently used approach to enhance the efficacy of plasmid DNA administration for genetic vaccination or gene therapy experiments involves the utilization of liposomal or polymer compounds. These can both protect DNA from nuclease degradation and improve uptake by target cells. A number of these compounds have been tested in both large and small animal systems (Klavinskis, Barnfield et al. 1999; Ochiya, Takahama et al. 1999; Thomas and Klibanov 2002; Liu, Fong et al. 2003; Thomas and Klibanov 2003; Anderson, Peng et al. 2004; Thomas, Lu et al. 2005). In horses, the proprietary cationic lipid DMRIE-DORE (Vical, San Diego, CA) enhanced the induction of humoral responses in a genetic vaccination experiment involving plasmid expression vectors encoding rabies glycoprotein (Fischer, Minke et al. 2003). Unfortunately, these improved responses were relatively short-lived, and anti

glycoprotein G antibody titers declined almost 75% in some animals within 14 days (Fischer, Minke et al. 2003).

The cationic polymer, polyethyleneimine (PEI) can significantly enhance cellular transfection and improve DNA translocation to the nucleus, both in vitro and in vivo (Boussif, Lezoualc'h et al. 1995; Pollard, Remy et al. 1998; Godbey, Wu et al. 1999). In genetic engineering experiments involving the transfection of lung tissues, PEI promoted 10- to 100-fold greater reporter gene expression than many cationic lipid, including DC-cholesterol:DOPE or DMRIE:DOPE (Densmore, Orson et al. 2000). Therefore, high molecular mass polyethylenimines (PEIs) represent a very promising and readily commercially available class of compounds for enhancing the efficiency of DNA delivery in vivo (Diebold, Kursa et al. 1999; Thomas and Klibanov 2002; Manoj, Babiuk et al. 2004).

However, commercial preparations of PEIs contain residual N-acyl groups that severely restrict the ability of these molecules to bind DNA and facilitate its delivery into cells. Removal of these N-acyl groups from linear 25kDa PEI by acid hydrolysis increased the ability of this polymer to mediate transfection of DNA in vitro by 21-fold and produced a 10,000 fold enhancement in the delivery of plasmid DNA in an *in vivo* mouse model (Thomas, Lu et al. 2005). However, the efficacy of fully deacylated preparations of PEI has yet to be evaluated in large animal models. Therefore, deacylated PEI was utilized in a DNA vaccination regimen with EIAV SYNSU plasmid DNA (pSYNSU) to determine its effect on enhancement of gene delivery in the equine, compared to naked DNA alone. With all of the obvious advantages to using PEI as a gene delivery system, toxicity in small animals has been a major concern seen in some model systems. However, because of the vastly increased body mass in large animals, this is not predicted to be a significant problem in the horse.

Immunostimulatory Molecules to Augment DNA Vaccine-Elicited Immune Responses

The hypothesis that co-administration of immunostimulatory molecules will enhance immune responses to gene-based vaccines has been studied extensively, especially in small animal models (Scheerlinck 2001; Manoj, Babiuk et al. 2004). Provision of these immunostimulatory agents in protein form involves high production

and purification costs with the result that many researchers rely on *in vivo* expression of exogenously delivered genes encoding these molecules. Examples of these immunomodulatory molecules include cytokines (GM-CSF (Xiang and Ertl 1995), IL-12 (Kim, Nottingham et al. 1999; Sin, Kim et al. 1999)) and chemokines (eg. CCL2, CCL3, reviewed in (Luther and Cyster 2001)) that can enhance or modify specific immune responses elicited by DNA immunization. Genes encoding co-stimulatory molecules, such as B7.2, CD80, or CD86, may aid in the recruitment of antigen presenting cells and help induce more robust immune responses against a selected panel of antigens recognized as immunodominant in the context of natural infection. Cytokine adjuvants, including IL-2, IL-7, and IL-15 have also been extensively utilized in gene-based immunization as immunomodulatory elements to augment humoral and cellular immune responses to antigens expressed from plasmid DNA in mice and monkeys.

Numerous cytokines have been shown to significantly modulate the inflammatory process. They can act as pro-inflammatory mediators and anti-inflammatory mediators. Moreover, cytokines can stimulate the proliferation of a variety of immune cell types, thereby enabling memory immune responses to pathogens. The production of effector molecules or cellular receptors can also be induced by cytokines. Cytokines assist the immune response in determining the ideal response to pathogens and the magnitude of the response, by signaling immune cells, such as T cells and macrophages, to travel to sites of infection and also by stimulating them to produce more cytokines (Murphy, Travers et al. 2007). In the vaccine treatment groups described in this dissertation, specific cytokines were incorporated into pSYNSU DNA vaccination regimens with PEI to evaluate as immunomodulators in horses and ponies on humoral and CMI responses.

There is now a substantial body of published evidence that certain cytokines act as potent molecular adjuvants for enhancing immune responses to DNA vaccines in at least some, albeit predominately small, animal models. It is now important to determine if equine homologs of these cytokines can produce similar improvements in the immunogenicity of DNA vaccines in the horse, where the potential exposure to nucleases are likely to be greatly enhanced more so than in any mouse or even non-human primate system and the transfection efficiency of plasmid DNA is expected to be reduced. The cytokines selected for this study include the equine homologs of IL-2, IL-7 and IL-15. In

addition, IL-12 was used in combination with IL-18, as these cytokines act synergistically to enhance IFN- γ production (Micallef, Ohtsuki et al. 1996; Ahn, Maruo et al. 1997).

IL-2 as a Vaccine Adjuvant

IL-2 is a member of a common γ -chain (γ_c) family of cytokines that share structural and functional properties, with IL-7 and IL-15, which all consist of a bundle of four alpha helices. Signaling of IL-2 involves a three subunit receptor expressed by lymphocytes, composed of specific IL-2 receptor alpha and beta chains and a common gamma chain (Robb, Munck et al. 1981; Leonard, Depper et al. 1982; Sharon, Klausner et al. 1986; Teshigawara, Wang et al. 1987; Tsudo, Kozak et al. 1987; Takeshita, Ohtani et al. 1992). Members of this family all signal through the same common gamma chain (Takeshita, Ohtani et al. 1992). When IL-2 binds to its receptor, numerous downstream signaling pathways, such as MAPK, PI-3K, or STAT-5, are activated that cause increased transcription of pro-inflammatory mediators (Friedmann, Migone et al. 1996). Upon receptor interaction, IL-2 stimulates the growth, differentiation, and survival of antigen-specific cytotoxic T lymphocytes.

IL-2 is a leukocytotrophic hormone and has been well documented as a T-cell growth factor (Smith 1988). Expression of IL-2 is limited to T cells. It is produced in response to antigenic or mitogenic stimulation in the host (Cantrell and Smith 1984; Smith 1988). IL-2 is secreted after antigen binds to the T-cell receptor, which causes an upregulation in IL-2 receptor (IL-2R) expression. The IL-2/IL-2R interaction stimulates the growth, differentiation and survival of antigen-selected CTL by activating the expression of specific genes (Stern and Smith 1986; Beadling, Johnson et al. 1993; Beadling and Smith 2002). IL-2 directly induces T cell proliferation and potentiates a very robust cellular response in the initial stages of T cell activation. It also contributes significantly to T-cell immunological memory by expanding the population of antigen-specific T cell populations (Yuan, Gallardo et al. 2006). In addition to its role in T-cell proliferation, IL-2 regulates the magnitude of the immune response by inhibiting T cell expansion via induction of the synthesis of FAS and its ligand, FasL (Shrikant and Mescher 2002; Blattman, Grayson et al. 2003). A cascade of intracellular events is

initiated by the binding of Fas to FasL, which ultimately results in the apoptotic cell death (Shrikant and Mescher 2002; Blattman, Grayson et al. 2003).

IL-2/Immunoglobulin G1 (IL-2/Ig) Fusion Construct as a Vaccine Adjuvant

IL-2 has been tested as an adjuvant in many DNA vaccine systems. Simultaneous immunization with plasmid vectors encoding this interleukin has augmented humoral and cellular immune responses to a number of DNA vaccines expressing HIV-1 gp120 (Xin, Hamajima et al. 1998), hepatitis C virus core protein (Geissler, Gesien et al. 1997), hepatitis B virus surface antigen (Chow, Huang et al. 1997), and HSV type 2 glycoprotein D (Sin, Kim et al. 1999). DNA vaccines co-expressing IL-2/Ig were substantially more effective than native IL-2 at inducing immune responses in mice (Barouch, Santra et al. 1998), presumably because the half-life of expressed cytokine fusion construct and avidity was increased, thereby enhancing its potency. IL-2/Ig also modulated cell-mediated responses to SHIV DNA vaccination in monkeys and induced some protective effects against SHIV virus challenge (Barouch, Craiu et al. 2000; Barouch, Santra et al. 2000). Additionally, co-expression of IL-2/Ig with measles DNA vaccination in monkeys also enhanced cellular immunity and facilitated some protection from viral challenge (Premenko-Lanier, Rota et al. 2003). DNA vaccines incorporating IL-2/Ig has also stimulated mucosal and systemic humoral and CMI against SHIV in macaques (Wang, Bertley et al. 2004). In additional DNA vaccine studies, IL-2/Ig induced broad and robust HIV-1 specific murine IFN- γ and CMI responses, more so than with the DNA antigen expression construct alone (Aggarwal, Kumar et al. 2005).

The aforementioned trials in mice and monkeys suggest that the strategy of using IL-2/Ig plasmid could be a highly effective method for the induction of cellular immune responses in the horse to DNA vaccination. To date, only plasmid DNA encoding native IL-2 has been inoculated in horses as part of a DNA vaccine study against EAV (Giese, Bahr et al. 2002). Although low level neutralizing antibodies were detectable for one year post immunization, the fact that no Equine Arteritis Virus (EAV) DNA only immunization group was included to evaluate the relative benefits of IL-2 and the DMRIE-DOPE adjuvant detracts from the value of this study. Therefore, the approach of

using IL-2/Ig in the horse has not been investigated prior to the studies described in this dissertation

IL-15 as a Vaccine Adjuvant

Interleukin-2 and IL-15 share an extensive amount of structural and some functional similarities, despite a lack of significant amino acid homology (Waldmann 2006). Interleukin-15 plays an important role early in the course of innate immune responses (Ohteki 2002). IL-15 mRNA is synthesized by activated monocytes and mononuclear phagocytes (Grabstein, Eisenman et al. 1994), but its expression is detectable in a broad range of cell and tissue types (Grabstein, Eisenman et al. 1994). In contrast to IL-2, IL-15 is not produced by activated peripheral blood T lymphocytes (Grabstein, Eisenman et al. 1994).

Signaling of IL-15 and IL-2 through the same β and γ chain receptor subunits (IL-2R/IL-15R $\beta\gamma/\gamma_c$ chains) explains some of the functional similarities between these cytokines (Carson, Giri et al. 1994; Giri, Ahdieh et al. 1994). However, IL-15 utilizes its own specific receptor alpha chain for signaling. This alpha chain selectively binds to IL-15 with an affinity, over 1000-fold higher than that observed between IL-2 and its alpha chain receptor (Giri, Kumaki et al. 1995; de Jong, Farner et al. 1996).

The production of antibodies from plasma B cells is facilitated by both IL-2 and IL-15, and they can also both affect natural killer cells by stimulating proliferation and differentiation (Waldmann and Tagaya 1999; Waldmann 2006). Similarly to IL-2, IL-15 is also a T cell growth factor. Initially, the two cytokines were thought to be interchangeable for stimulating immune responses, but they were later discovered to have very distinct functions (Grabstein, Eisenman et al. 1994; Armitage, Macduff et al. 1995). The most profound functional differences between these two cytokines are seen in their effects in adaptive immunity. While IL-2 has more of a role in preventing autoimmunity by eliminating self-reactive T cells and also in maintaining regulatory T (Treg) cell populations in the thymus, IL-15 is required for the survival of CD4⁺ and CD8⁺ T-lymphocytes important in the adaptive immune response. IL-15 is also important for inducing the activation and proliferation of T cells and the costimulation of B cells with the CD40 B-cell receptor (Grabstein, Eisenman et al. 1994; Armitage, Macduff et al.

1995). However, one of the most distinct roles of IL-15 is its influence in maintaining memory CD8⁺ T cell populations in the absence of antigenic stimulation and promoting the expansion of these cell populations upon subsequent encounters with the antigens (Lai, Gelfanov et al. 1999). IL-15 is an important survival factor that stimulates T cell proliferation and prevents apoptosis, primarily by exerting its effects on effector memory CD8⁺ T cells (Kanai, Thomas et al. 1996). In addition, IL-15 can also stimulate APCs directly, thereby activating a downstream signaling cascade of cytokine induction and secretion of IL-12 and IFN- γ which further activate APCs and enhance CD8⁺ T cell stimulation (Kutzler, Robinson et al. 2005). This powerful cytokine also plays a role in innate immune responses by stimulating cytokine production and natural killer (NK) cell proliferation *in vivo*.

IL-15 is very efficient at stimulating immunological memory in rodent and primate model systems (Sprent and Surh 2001; Kaech, Wherry et al. 2002; Rocha and Tanchot 2004), although there are no reports concerning the efficacy of this cytokine on memory T cell responses to DNA vaccines in very large animals, such as horses. This has been demonstrated in mice and monkeys (Kim, Trivedi et al. 1998; Sin, Kim et al. 1999; Xin, Hamajima et al. 1999; Bolesta, Kowalczyk et al. 2006; Boyer, Robinson et al. 2007; Rosati, Valentin et al. 2008; Dubie, Maksaarekul et al. 2009; Hu, Chen et al. 2010; Eickhoff, Vasconcelos et al. 2011). In monkey models, IL-15 induced antigen-specific polyclonal CD8⁺ T cell responses and increased the frequency of SIV-specific CD8⁺ T cell responses against SIV DNA vaccination (Dubie, Maksaarekul et al. 2009). It has also been shown to strongly induce vaccine-specific IFN- γ producing CD4⁺ and CD8⁺ effector cells and protection from ongoing SHIV infection with plasmid gag vaccination (Boyer, Robinson et al. 2007). Furthermore, in rhesus monkeys, cellular immune responses were enhanced by the co-inoculation of IL-15 DNA with pHIV gag DNA facilitating control of SIV viremia, and IL-15 also increased memory recall responses to SIV antigens (Rosati, Valentin et al. 2008). IL-15 has also been shown to increase the frequency of effector memory CD8⁺ T cells with HIV DNA vaccination in monkeys (Li, Qi et al. 2010).

In mice, IL-15 has been reported to produce similar effects, as seen in monkeys, and significantly promote the expansion of CD8⁺ memory T cell pools and enhance

CD8⁺ T cell function independent of CD4⁺ T cell help (Bolesta, Kowalczyk et al. 2006). IL-15 co-delivery with DNA vaccination also has been shown to increase the number of antigen-specific IFN- γ producing total and CD8⁺ T cells (Hu, Chen et al. 2010; Eickhoff, Vasconcelos et al. 2011) in mice. Since the induction of immunologic memory and the establishment and maintenance of memory CD8⁺ T cells may be critical for protection against infectious microorganisms (Li, Demirci et al. 2001; Tan, Ernst et al. 2002), IL-15 was predicted to be a powerful molecular adjuvant for incorporation in equine gene-based vaccine studies. Therefore, its immunomodulatory effects on SYNSU DNA vaccines (formulated with PEI) were investigated in the horse.

Sushi IL-15 as a Vaccine Adjuvant or an Antagonist

In DNA vaccine studies with the IL-15 cytokine, soluble component regions of the IL-15 receptor alpha chain were observed to suppress the induction of arthritis in mouse model systems (Ruchatz, Leung et al. 1998) and also prolong the survival rate of certain transplanted heart tissues (Smith, Bolton et al. 2000). Further analysis of the alpha chain region of IL-15 revealed a common motif known for protein-protein interactions in the extracellular region (Wei, Orchardson et al. 2001). This motif of approximately 60 amino acids, known as the Sushi domain, is prevalent on a number of protein-binding molecules and contains four cysteine residues forming two disulfide bonds (Bottenus, Ichinose et al. 1990; Kato and Enjoji 1991; Ranganathan, Male et al. 2000). Work done by Wei et al., 2001, has determined that the IL-15 receptor alpha is dependent on the Sushi domain for binding and protein functionality (Wei, Orchardson et al. 2001) and these domains have been identified on many different protein-binding molecules. In vivo, IL-15 is normally presented on the cell surface as a complex formation with IL-15R α , instead of being secreted as a soluble cytokine (Nguyen, Salazar-Mather et al. 2002; Schluns, Stoklasek et al. 2005; Van Belle and Grooten 2005).

Trans-presentation of cell-bound IL-15 to NK and T cells ensues and subsequent recognition by the cellular receptors for the IL-15 β/γ chains results in survival and proliferation effects. Interaction of IL-15 and its alpha-receptor have been shown to dramatically improve the biological activity of soluble IL-15 and induce strong and specific memory CD8⁺ T cell and NK cell responses in mice (Rubinstein, Kovar et al.

2006). Because the Sushi domain is contained within IL-15R α , this motif might contribute significantly to the superagonist effects seen with IL-15/IL-15R α complexes in Rubenstein et al., 2006. In light of these studies, an equine IL-15 Sushi construct was generated for administration in DNA vaccination studies in horses.

IL-7 as a Vaccine Adjuvant

The immunostimulatory effects of IL-7 on equine immune responses to pSYNSU immunization were also investigated. IL-7 has a non-redundant role in lymphopoiesis, and has been coined the ‘Master Regulator of T-cell Homeostasis’ (Fry and Mackall 2001). Furthermore, in contrast to many other interleukins, IL-7 is not produced by T cells, B cells, or NK cells. However, it is produced by a wide array of cell types, such as dendritic cells (Kroncke, Loppnow et al. 1996), keratinocytes (Heufler, Topar et al. 1993), hepatocytes (Sawa, Arima et al. 2009), neurons and endothelial cells (Watanabe, Ueno et al. 1995). Stromal cells located in the thymus and red bone marrow secrete IL-7 (Sudo, Ito et al. 1989; Watanabe, Ueno et al. 1995), and it plays many important roles in differentiating pluripotent hematopoietic stem cells into lymphohoid progenitor cells, as well as inducing proliferation of all cells in the lymphoid lineage (Collins and Dorshkind 1987; Hunt, Robertson et al. 1987; Whitlock, Tidmarsh et al. 1987; Ogawa, Nishikawa et al. 1988).

The IL-7 cytokine can have dominant effects in both B cell and T cell development in different species. It has been described as an important cytokine in early B cell development in bone marrow (Baird, Gerstein et al. 1999), and it is largely known as a pre-B-cell growth factor, derived from the stromal cells of bone marrow (Namen, Lupton et al. 1988; Goodwin, Lupton et al. 1989). In mice, IL-7 seems to affect both types of lymphocyte development, while it is indispensable for T cell development in humans. Although IL-7 is not required for human B cell development, it can have modulatory effects. The development of CTL responses are also strongly influenced by IL-7 in some model systems. In vivo, this cytokine appears to also be able to drive immune responses towards a T_H2 pathway. It can also influence T-lymphocyte differentiation and is an important factor in maintaining viability of embryonic and early adult T cells (Murray, Suda et al. 1989; Wiles, Ruiz et al. 1992). The survival of T cells

is promoted by the IL-7-dependent activation of anti-apoptotic proteins (Kim, Lee et al. 1998; Opferman, Letai et al. 2003) and the inhibition of pro-apoptotic proteins (Khaled, Li et al. 2002; Li, Jiang et al. 2004; Pellegrini, Bouillet et al. 2004).

As stated above, interleukin-7 is unique and its function in the immune system cannot be replicated by any other cytokine. Primary T-cell development in humans is largely impacted by IL-7 (Puel, Ziegler et al. 1998). When it is absent in gene knockout mouse models, B-cells and T-cells are unable to survive and regenerate, thereby demonstrating a critical role for IL-7 in lymphopoiesis (Grabstein, Waldschmidt et al. 1993; Peschon, Morrissey et al. 1994; Bhatia, Tygrett et al. 1995; von Freeden-Jeffry, Vieira et al. 1995). In work done by Bolotin et al. and Fry et al., IL-7 serum levels were shown to be inversely correlated with the numbers of lymphocytes present in human and murine model systems (Bolotin, Annett et al. 1999; Fry, Connick et al. 2001). Clearly, this cytokine profoundly influences the development of immune responses in mammals, but the effects of IL-7 on B cell and T cell lymphopoiesis in horses are unknown. However, based on the well established importance of IL-7 in mice and humans, it was investigated as a potential immunomodulatory cytokine in equine DNA vaccination with pSYNSU and PEI.

IL-12 as a Vaccine Adjuvant

The cytokine IL-12 is also known as natural killer cell stimulatory factor (NKSF) and enhances NK cells and T-lymphocyte activity. Largely B-cells and some T-cells produce IL-12, but it is also secreted by activated monocytes, macrophages, dendritic cells, peripheral lymphocytes, and some lymphoblastoid cells in an immune response to antigenic stimulation (D'Andrea, Rengaraju et al. 1992; Macatonia, Hosken et al. 1995; Kato, Hakamada et al. 1996; Reis e Sousa, Hieny et al. 1997). Secretion of IL-12 influences the development of T-lymphocytes during antigen priming by eliciting the maturation of type I T-cells from uncommitted T_H0 cells, which can skew the immune response towards a T_H-1 profile (Kobayashi, Fitz et al. 1989; Gately, Wolitzky et al. 1992; Robertson, Soiffer et al. 1992; Germann, Gately et al. 1993). The bioactive form of IL-12 is a heterodimeric 70 kiloDalton (kDa) glycoprotein, composed of 2 unrelated glycoproteins of approximately 40 kDa mol wt (p40) and 35 kDa mol wt (p35) that are

covalently linked together by disulfide bonds (Podlaski, Nanduri et al. 1992). For the SYNSU DNA vaccine trials described in his dissertation, IL-12 subunits were connected by a flexible linker domain and administered with pSYNSU + PEI to investigate effects on immune responses in equids. Production of a biologically active form of equine IL-12 in which the p35 and p40 subunits are linked by a “flexible” glycine/serine rich chain has been described previously (McMonagle, Taylor et al. 2001). A similar codon optimized equine IL-12 construct was synthesized for use in the preliminary DNA vaccine study.

Synergy of IL-12 and IL-18 in DNA vaccination

IL-12 functions cooperatively by binding to interleukin-18 (IL-18) to induce CMI responses, through the IL-18 receptor (Micallef, Ohtsuki et al. 1996; Kohno, Kataoka et al. 1997; Tominaga, Yoshimoto et al. 2000). IL-18 alone is unable to enhance IFN- γ production by NK cells (Walker, Aste-Amezaga et al. 1999), however, synergistically with IL-12, both cytokines stimulate gamma interferon (IFN γ) production (Ahn, Maruo et al. 1997; Barbulescu, Becker et al. 1998; Chang, Segal et al. 2000). Interleukin-18 is a 24 kDa nonglycosylated protein, produced during an acute immune response by immature dendritic cells and activated macrophages, and it acts as a pro-inflammatory cytokine by inducing IFN- γ production (Okamura, Tsutsi et al. 1995). Due to the synergistic nature of both cytokines for function, they were used in combination in a pilot DNA vaccination trial.

Summary

In summary, genetic vaccines have stimulated robust immune responses against many pathogen and tumor antigens in small rodent models. However, with the exception of highly specialized cases, such as the DNA vaccine against WNV, results in larger animal species have generally been disappointing. This is most likely attributed to increased nuclease activity, higher blood volumes that dilute plasmid DNA, and decreased transfection efficiency of DNA into target cells. Consequently, it is predicted that immune responses to gene-based vaccines in very large animals, such as horses, will be enhanced significantly by improving the uptake of the component nucleic acids by target host cells and/or by protecting them from degradation by nucleases. Furthermore, it has been demonstrated that immune responses to genetic vaccines are also enhanced by co-administration of certain immunostimulatory molecules, including the IL-2, IL-7, IL-12, IL-15, and IL-18 cytokines.

II. Hypothesis and Specific Aims

We hypothesized that complex formation with a completely deacylated form of the cationic polymer PEI would improve the intracellular uptake of nucleic acid based expression vectors and/or their resistance to in vivo nucleases and consequently result in significantly enhanced adaptive immune responses to gene-based vaccinations in large animal systems, including the horse. Additionally, we predict, based on a multitude of experimental results from different animal species, that immune responses to gene-based vaccines in horses will be further augmented by co-administration of expression vectors encoding equine homologs of IL-2, IL-7, IL-15, or combinations of IL-12 and IL-18.

Correlates of immune protection against EIAV are unknown; however, recent work suggests that a broadening of humoral or cellular immune responses may afford protection against disease. The most immunogenic viral protein expressed by EIAV is the gp90 or Surface Unit (SU) glycoprotein. The SU glycoprotein is not only highly immunogenic, but contains one of the very few CTL epitopes identified in horses as being specifically bound by MHC of animals possessing the ELA-A1 haplotype. Therefore, Codon optimized nucleic acid sequences encoding the extensively characterized Surface Unit envelope glycoprotein of EIAV (pSYNSU) were used as the source of a model antigen to test these hypotheses,

Initial testing of these hypotheses was conducted under three Specific Aims:

- (1) A pilot study with ponies possessing the ELA-A1 haplotype to examine potential toxic effects of PEI and determine if humoral and/or cellular immune responses are enhanced relative to pSYNSU alone, by immunizations with pSYNSU and PEI, with or without the co-administration of plasmid vectors encoding equine IL-2 + IL-15 or IL-15.
- (2) Assuming support for this hypothesis is provided by the pilot study: Investigate enhancement of immune responses to pSYNSU DNA vaccinations by PEI along with a more comprehensive panel of plasmid expression vectors encoding equine homologs of IL-2, IL-7, IL-15, and IL-12 in conjunction with IL-18 in horse/pony populations with diverse ELA haplotypes.

(3) Investigate anamnestic humoral and cell-mediated memory recall responses 15 months after four DNA immunizations in animals from different vaccine groups following re-inoculation with pSYNSU and/or PEI.

CHAPTER II: MATERIALS AND METHODS

2.1. Interleukin construction, modifications and analysis

Molecular Cloning of Native Equine IL-2 in pCi

Plasmid DNA (pIL-2) containing the complete native equine IL-2 coding sequence including the N-terminal 20 amino acid signal peptide was obtained from Dr D. W. Horohov (Gluck Equine Research Center). These sequences have been previously characterized at the nucleotide level and the protein they encode expressed and found to be biologically active in terms of stimulating equine lymphocyte proliferation in a dosage dependent manner (Vandergriff and Horohov 1993). The restriction endonuclease sites flanking the Equine IL-2 (IL-2) sequences in pIL-2 were not compatible with the strategy of simple excision and ligation into the mammalian plasmid expression vector pCi (Promega Corporation, Madison WI). Therefore, equine IL-2 sequences were amplified in conjunction with the Expand High Fidelity PCR system (Roche Molecular Biochemicals, Indianapolis, IN) using the oligonucleotide primers native IL-2 F and native IL-2 R (Figure 2.1., IDT, Coralville, IA). In addition to the *XhoI* restriction endonuclease recognition site, the native IL-2 F primer contained a consensus Kozak motif immediately upstream from the initiation codon while a UGA termination codon was inserted into primer native IL-2 R prior to the *EcoRI* site (Figure 2.1). The reactions (50µl) were prepared as recommended by the manufacturer with 1.5mM MgCl₂ and subjected to 35 cycles of melting at 94°C for 10 s. annealing at 54°C for 30 s with an extension at 68°C for 120 s. The amplified product was digested with *XhoI/EcoRI*, separated by agarose gel electrophoresis and visualized by ethidium bromide staining. Following electrophoresis the single visible DNA band was excised from the gel, extracted using Agar Ace (Promega) and ligated into *XhoI/EcoRI* digested pCi. The ligated products were used to transform One Shot ® Stbl3 Chemically Competent *E. coli* cells (Invitrogen, Carlsbad, CA). The resultant pCi vector DNA encoding equine IL-2 (pCiIL-2) was purified using the Pure Yield™ Plasmid Midi Prep System (Promega) and sequenced to ensure that disruptions to the open reading frame had not been introduced by either PCR or molecular cloning procedures, using a Thermo Sequenase Cy5.5 Dye Terminator kit (GE Healthcare UK Limited, Little Chalfont, United Kingdom).

Oligonucleotide primers for these reactions consisted of the forward and reverse PCR primers along with IL-2 SEQ1 and IL-2 SEQ2 (Figure 2.1)

Figure 2.1

Oligonucleotide Primers for Molecular Cloning and Modification of Equine Interleukins

A) Equine IL-2

Native IL-2 (F)

CAGCTCCAG-GCCACC-ATGTACAGGATGCAACTCCTGTCTT
XhoI Kozak

Native IL-2 (R)

AGGAATTCTCACGTCAGTGTTGAGATGATGCTTTGA
EcoRI

Native IL-2 (R) Psp1406

GTCAACGTTCGTCAGTGTTGAGATGATGCTTTGA
Psp1406

SYN IL-2 (F)

CAGCTCGAGA-GCCACC-ATGTACCGGATGCAGCTGCTG
XhoI Kozak

SYN IL-2 (R)

GTCAACGTTGGTCAGGGTGCTGATGATGCTCTGGC
Psp1406

IGHC1: Molecular cloning and Site-Directed Mutagenesis

IGHC1 (F) Psp1406

 N V Q E
GTCAAAC-GTT-CAG-GAG-GCCTCCACCACCGCCCCGAAGGTCTTC
Psp1406

IGHC1 (R)

ATAAGAATGCGGCCGCTCATTTACCCGGGTTCTTGGAGACGTTCTT
NotI

IGHC1 ΔC1Q (F)

CAGGACTGGCTGTCAGGAAAGGCGTTCGCGTGTGCGGTCAACAACCAAGCCCTCCCA

IGHC1 ΔC1Q (R)

TGGGAGGGCTTGTTGTTGACCGCACACGCGAACGCCTTTCCTGACAGCCAGTCCTG

Figure 2.1 con't.

Oligonucleotide Primers for Molecular Cloning and Modification of Equine Interleukins

IGHC1 ΔFc (F)
CAGCTCCTGAGCTCGAGGGAGGGCCTTC

IGHC1 ΔFc (R)
GAAGGCCCTCCCTCGAGCTCAGGAGCTG

Confirmatory Sequencing Primers

Equine IL-2 SEQ 1
ACAGGATGCAACTCCTGTCTTGCAT

Equine IL-2 SEQ 2
CTCTCGAAAGATATCAAGGAATTAATGAGCA

IGHC1 168
CCTGCAGTCCTCAGGGTTCTACTCC

IGHC1 474
CCCTGATGTCAAGTTCAACTGGTAC

B) Equine IL-15

SYN R to S (plus strand)
CATCCTGGCCAACAGCTCCCTGAGCTCGAACGGC

SYN R to S (minus strand)
GCCGTTTCGAGCTCAGGGAGCTGTTGGCCAGGAT

SYNSushi IL-15 L+1*

*Bam*H1

gga tcc gga ggc gga gga agc ggc ggc ggg agt ggc ggt ggc gga tct ggc ctg cag aac tgg cag gac gtg
atc a

*Bcl*I

SYNSushi IL-15 L+2*

*Bam*H1

gga tcc gga ggc gga gga agc ggc ggc ggg agt ggc ggt ggc gga tct ggc ggt ctg cag aac tgg cag gac
gtg atc a

*Bcl*I

*oligonucleotides were synthesized as double stranded “mini genes” although only the plus strand is shown

Molecular Cloning of a Native IL-2 and Equine Immunoglobulin gamma 1 Heavy Chain Fusion Construct

To prolong the known short (15-30min) half-life *in vivo* of interleukin 2 (Malek and Castro 2010) an in-frame fusion construct between IL-2 and the equine immunoglobulin gamma 1 heavy chain IGHC1 (GenBank Accession Number AJ300675 kindly donated by Dr Bettina Wagner, Baker Institute for Animal Health, Cornell University, Ithaca, NY) was produced. The IGHC1 amino acid sequence contains motifs completely homologous with the high affinity Fc receptor and C1q binding sites identified on human IgG molecules (Duncan and Winter 1988; Duncan, Woof et al. 1988). As with IL-2/IgG fusion constructs developed for use in other animal systems, the putative Fc receptor and C1q binding sites were mutated to prevent the immunoligand from directing antibody dependent cell-mediated cytotoxicity and complement-directed cytotoxicity (Zheng, Steele et al. 1995; Barouch, Santra et al. 1998; Barouch and Letvin 2000; Barouch, Santra et al. 2000). Specific nucleotide mutations were introduced into IGHC1 using the QuickChangeTM site-directed mutagenesis kit (Stratagene, La Jolla, CA) in conjunction with the oligonucleotide primer pairs IGHC1 ΔFc F/R and IGHC1 ΔC1q F/R (Table). The separate reactions (50μl) featuring these primer pairs were prepared as recommended by the manufacturer and subjected to 16 cycles of melting at 95°C for 30 s, annealing at 64°C for 60 s and 68°C for 11 min. The successful introduction of specific mutations into the Fc and C1q binding sites was confirmed by nucleotide sequencing using primers IGHC1 168 and IGHC1 474 respectively (Figure 2.1). Amplification by PCR of native IL-2 and IGHC1 ΔFc, ΔC1q sequences to produce an IL-2/IgG fusion construct was achieved using the primers Native IL-2 F/Native IL-2 R Psp1406 and IGCH1 F Psp1406/IGHC1 R (Figure 2.1) respectively. The Native IL-2 R Psp1406 primer was designed with the six nucleotide recognition sequence for the restriction endonuclease *Psp1406*. Similarly, this recognition sequence was included within the 5' terminus of IGHC1 F Psp1406. In addition, six additional nucleotides were included within the 5' terminus of this primer so that when combined with the Psp1406 site the sequence asparagine (N), valine (V), glutamine (Q) and glutamic acid (E) was encoded producing the large hydrophilic, hydrophobic, hydrophilic, hydrophilic amino acid motif commonly observed within the variable-diversity-joining (VDJ) domains of

immunoglobulins (Landolfi 1991). PCR conditions for the Native IL-2 F/Native IL-2 R Psp1406 primer pair comprised of an initial denaturation step of 94°C for 60 s followed by 30 cycles of 94°C for 10 s, 52°C for 30 s and 68°C for 2 min. Similar conditions were employed for the IGCH1 F Psp1406/IGHC1 R primer pair except the annealing temperature was 60°C for 30 s. Following electrophoresis in 0.8% agarose gels the resultant PCR products were extracted using AgarAce and ligated via a three-way reaction into *XhoI/NotI* digested pCi to create native IL-2/Ig.

Synthesis of Codon Optimized Equine IL-2 and molecular cloning of IL-2 and Equine Immunoglobulin gamma 1 Heavy Chain Fusion Construct (IL-2/Ig)

Each codon in native IL-2 was evaluated and if necessary modified to conform to the codon-usage bias of highly expressed equine and human genes as described by Cook et al. (2005) (Cook, Cook et al. 2005). The redesigned sequences were synthesized *in vitro* and inserted into the pCi mammalian expression vector (GenScript Corp. Piscataway, NJ) using *XhoI/NotI* restriction endonuclease sites. The codon optimized IL-2/IGHC1ΔFc, ΔC1q fusion construct was produced by PCR amplification of codon optimized IL-2 using the SYNIL-2 F/SYNIL-1 R primer pair (Figure 2.1). Reaction conditions consisted of an initial denaturation step of 94°C for 60 s followed by 30 cycles of 94°C for 10 s, 63°C for 30 s and 68°C for 2 min. The resultant PCR product was digested with *XhoI* and *Psp1406*, separated by electrophoresis in 0.8% agarose and extracted using AgarAce. This purified fragment was substituted for native IL-2 sequences in native IL-2/Ig using the *XhoI* and *Psp1406* sites to create codon optimized IL-2/Ig.

Synthesis of Codon Optimized Equine IL-7 (IL-7)

The molecular cloning, nucleotide sequence characterization and expression of native equine IL-7 was previously reported from this laboratory (Cook, Cook et al. 2008). Although four isoforms of equine IL-7 generated by alternative RNA splicing events are present in normal horse lymphoid tissue only the largest molecular weight form was selected for these experiments because compared to the other variants it induces the highest proliferative responses in ex vivo horse PBMC (Cook, Cook et al. 2008). To

investigate if codon optimization could improve the expression level of native IL-7 a synthetic version of the coding sequence (IL-7) was designed and manufactured as described for codon optimized IL-2 (GenScript Corp.).

Synthesis of Codon Optimized Equine IL-12 (IL-12)

IL-12 is a heterodimeric cytokine composed of disulphide linked p35 and p40 subunits. The fact the the intact molecule is encoded by two genes differentially expressed in various cell types poses difficulties for the design of IL-12 expression-based molecular constructs. In addition, to ensure that synthesis of the subunits occurs in equimolar quantities following transfection, care must be taken to avoid the intracellular formation of homodimeric p40 molecules, as these act as antagonists of IL-12 p70 activity (Ling, Gately et al. 1995). These problems have been circumvented by production of equine IL-12 from expression vectors containing p35 and p40 cDNAs linked by sequences encoding a glycine/serine rich “flexible” chain to form a single open reading frame. The resultant fusion molecule was shown to be biologically active when conditioned medium from mammalian cells transiently transfected with this construct stimulated gamma interferon production in cultures of horse lymph node derived mononuclear cells (McMonagle, Taylor et al. 2001). The amino acid sequence of the equine IL-12 fusion construct tested as a DNA vaccine adjuvant, including the length of the glycine/serine rich linker chain, was as described previously (McMonagle, Taylor et al. 2001). The nucleic acid sequence encoding the equine IL-12 fusion product was codon optimized, as described above, to maximize its intracellular expression potential.

Synthesis of Codon Optimized Equine IL-15 (IL-15)

Native equine IL-15 was molecularly cloned and sequenced in this laboratory (Cook unpublished). However, protein expression was poor expressed *in vitro* and conditioned medium obtained from human embryonic kidney 293 (HEK293, CRL-1573, ATCC Manassas, VA) cells transiently transfected with mammalian expression vectors encoding native equine IL-15 failed to stimulate proliferative activity in *ex vivo* equine PBMC cultures (Cook unpublished). Therefore, a codon optimized version of equine IL-15 was designed in which the unusually long (48 amino acids) signal peptide is replaced

with that from human tissue plasminogen activator (TPA, (Berg and Grinnell 1991). The modified equine IL-15 nucleic acid sequences were synthesized commercially (The Midland Certified Reagent Company, Midland, TX). During the molecular characterization of native equine IL-15 cDNA nucleotide position 363 was automatically read as Guanine (G) in one sequencing reaction whereas Cysteine (C) was present in others. This resulted in a predicted arginine (R) residue (AGG) at amino acid position 121 instead of serine (S) (AGC). Therefore, equine IL-15 was initially thought to either possess serine, arginine, lysine, serine, serine (SRLSS) or (SSLSS) at amino acid positions 120 to 124. It was not immediately obvious the SRLSS variant was a sequencing artifact, because there is extensive conservation of the LSS motif in IL-15 molecules from many different mammalian species. Therefore, the preceding two amino acids are subject to variation. The codon optimized version of the SRLSS variant was submitted for gene synthesis (IL-15 (SRLSS)), however when it was discovered that this clone was poorly expressed following transient transfection in HEK293 cells R121 was replaced by S using site directed mutagenesis (QuickChangeTM, Stratagene) in combination with the SYN R to S oligonucleotide primer pair ((Figure 2.1), IDT) to generate IL-15 (SSLSS).

Synthesis of Codon Optimized Equine Sushi IL-15 (Sushi IL-15)

With the advent of relatively inexpensive gene synthesis technology it is becoming increasingly feasible to generate genetic constructs encoding novel molecules. The biological activity *in vitro* of human IL-15 is increased 10-fold by covalently attaching the sushi domain from the human IL-15 receptor α chain, via a glycine/serine rich linker to the amino-terminus (Mortier, Quemener et al. 2006). Production of an equivalent equine IL-15 fusion molecule was initiated by identification of sequences homologous to the sushi domain of the human IL-15 receptor α chain in the horse genome (Broad Institute Genome Sequencing Platform, (Wade, Giulotto et al. 2009). In the design of Sushi IL-15 the homologous equine sushi sequences were codon optimized and attached as described previously (Mortier, Quemener et al. 2006) via a 20 amino acid glycine/serine rich linker molecule to the amino terminus of codon optimized IL-15 (SSLSS). However, the signal peptide from human TPA was incorporated into this

equine fusion protein instead of that from the human IL-15 receptor α chain signal peptide used in the human sushi IL-15 construct (Mortier, Quemener et al. 2006). The Sushi IL-15 construct was synthesized and incorporated into the gWiz mammalian expression vector (Gene Therapy Systems Inc, San Diego, CA) by GenScript. The extended glycine/serine linker chain variants Sushi IL-15 L+1 and Sushi IL-15 L+2 were produced by substitution of double stranded adapter oligonucleotides ((Figure 2.1.), GenScript) using unique BamH1 and Bcl1 restriction endonuclease sites. The predicted molecular weight of the mature equine Sushi IL-15 fusion protein is 21686.11 Da assuming complete removal of the TPA signal peptide.

Synthesis of Codon Optimized Equine IL-18

The nucleotide sequence and molecular cloning of native equine IL-18 has been described previously (Nicolson, Penha-Goncalves et al. 1999). This cytokine is synthesized as a biologically inactive precursor protein that only becomes active and secreted from the cell following cleavage with caspase 1 (Fantuzzi and Dinarello 1999). Therefore, secretion of functional IL-18 is reliant on cellular injury and apoptosis. To overcome this problem the mature form of equine IL-18 was fused with the signal peptide from human TPA. A similar strategy has shown that following expression of the resultant recombinant gene, a biologically active form of this equine interleukin is secreted from transiently transfected mammalian cell cultures (O'Donovan, McMonagle et al. 2004). A codon optimized version of equine IL-18 containing the TPA signal peptide (IL-18) was synthesized and inserted into the pCi mammalian expression vector by GenScript.

Expression of Recombinant Horse Interleukins Following Transient Transfection in HEK 293 Cell Cultures

Following molecular cloning into mammalian expression vectors all native and synthetic horse interleukins described above were transfected in triplicate into HEK 293 cell cultures (American Type Culture Collection) maintained in 6-well culture plates (Becton Dickinson and Company, Franklin Lakes, NJ). For transfection of each well, a total of 2 μ g of plasmid DNA was combined with 20 μ g Lipofectamine 2000 (Invitrogen)

in 500µl OptiMEM (Invitrogen). The resultant DNA/cationic lipid complexes were added to HEK 293 cells according to the manufacturer's instructions and incubated at 37°C in 5% CO₂ for 72h after which cell culture fluids were harvested, aliquoted into 0.5ml amounts and stored at -80°C prior to analysis. The volume of each 0.5ml aliquot was reduced to 35µl using a 3K Amicon Ultra Centrifugal Filter Unit (Millipore, Cork, Ireland), as recommended by the manufacturer and dried using a Centrivap Concentrator (Labconco, Kansas City Mo). It was then resuspended in 10µl "cracking buffer" (3.55M β-mercaptoethanol, 10% sodium dodecyl sulfate [SDS], 25% glycerol in 0.5M Tris/HCl pH 6.8) and heated for 10 min at 95°C. The denatured protein samples were separated electrophoretically in a 12% SDS-polyacrylamide gel (Laemmli, 1970) using a Bio-Rad mini-Protein[®] II Dual Slab gel system (Bio-Rad). To examine intracellular expression in the case of IL-18, Brefeldin A (Sigma-Aldrich, St Louis, MO) was added (10µg/ml) to the cell cultures at 64h post transfection and incubation continued at 37°C in 5% CO₂ for a further 8h. After completion of the incubation period the cells were washed in PBS and lysed by the addition of 500µl 1X Reporter Gene Assay Lysis Buffer (Roche Applied Science) containing an EDTA-free protease inhibitor cocktail (Complete, Mini, EDTA-free, Roche Applied Science). Following lysis for 30min at room temperature insoluble material was removed by centrifugation at 16,000xg for 5 min, the protein concentration determined using the Bio-Rad protein reagent (Bio-Rad, Hercules, CA) and 100µg of each lysate separated electrophoretically as outlined above.

In all cases after SDS-PAGE, proteins were transferred onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH) using a Mini-Trans-Blot[®] Electrophoretic Transfer System (Bio-Rad) and the membranes blocked with PBS containing 5% non-fat milk (Bio-Rad) for 2h and then incubated for 1h with 0.5µg/ml (in PBS containing 1% non-fat milk) of the appropriate polyclonal antiserum. The antisera used for expression analysis were as follows: goat anti equine IL-2 (anti-eqIL-2 R&D Systems, Minneapolis, MN); rabbit anti human IL-7 (Antigenix America Inc, Huntington Sta, NY); goat anti human IL-12 (Antigenix); rabbit anti human IL-15 (Antigenix) and goat anti human IL-18 (R&D Systems). Bound antibodies were detected using horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG (h+l) or rabbit anti-goat IgG (Bethyl Laboratories Inc,

Montgomery, TX) diluted 1:500 in PBS containing 1% non-fat milk and visualized with TMB stabilized substrate (Promega).

The biologically active form of IL-18 is reliant on cellular degradative changes such as apoptosis because cleavage of the signal peptide is mediated by a caspase-1. Therefore, to overcome this specific requirement the signal peptide sequences were replaced with those from human TPA in the synthetic equine IL-18 coding sequence. To determine if this sequence was cleaved, Brefeldin A was added to IL-18 transfected HEK 293 cultures and the cell lysates compared by immunoblot analysis using goat anti human IL-18 to proteins secreted into the medium from equivalently transfected non-Brefeldin-A treated cells. Equine TPA-IL-18 contains no potential N-linked glycosylation sites (Figure 3.10). Therefore, the full-length, non-cleaved variant of this protein is predicted to have a molecular weight of 22 k Da whereas removal of the TPA signal peptide reduces the size of the mature form to 18 kDa (Vector NTI).

Proliferative Assays Using Conditioned Medium from HEK Cells Transfected with Native and Synthetic Variants of Equine IL-2, IL-7 and IL-15

Conditioned medium collected 72h following separate transfections of HEK 293 cells with native IL-2, IL-2, native IL-2/Ig, IL-2/Ig, native IL-7, IL-7, native IL-15, IL-15 (SRLSS), IL-15 (SSLSS), Sushi IL-15, Sushi IL-15 L+1 or Sushi IL-15 L+2 was harvested, filtered (0.22µM) to remove cellular debris and stored at -80°C prior to analysis. PBMC were isolated from donor horses by discontinuous density gradient centrifugation (Ficoll-Paque™ Plus, GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and resuspended in RPMI 1640 (Invitrogen) maintenance medium containing 5% fetal equine serum (CELLect™, MP Biomedicals United States, Solon, OH), 100 units/ml penicillin, 100µg/ml streptomycin (Invitrogen) and 55µM 2-mercaptoethanol (Invitrogen) at 2×10^6 cells/ml. Filtered culture fluids from transfected and mock transfected cells (pCi without an insert) were diluted in RPMI maintenance medium and added (100µl/well) to 96-well plates (Becton Dickinson and Company). Finally, 100µl PBMC plus sub-stimulatory amounts of concanavalin A (Sigma-Aldrich) at a final concentration of 2µg/ml were added to each well and the plates incubated at 37°C in 5% CO₂ for 72h. In addition, to media from mock transfected cells, controls consisted of PBMC incubated

with and without concanavalin A in the presence of RPMI maintenance medium. All assays were performed in triplicate. Following the 72h incubation period 1 μ Ci of tritiated (3 H)-thymidine (MP Biomedicals, Inc, Irvine, CA) was added to each well in a volume of 50 μ l RPMI maintenance medium and incubation continued for an additional 8h. PBMC were harvested onto glass fiber filters (TomTec, Orange, CT) and [3 H]-thymidine incorporation determined by liquid scintillation counting (Wallac 1205 Betaplate, Turku, Finland). The proliferative responses were expressed in the form of a stimulation index (SI) representing the average [3 H] thymidine incorporation of replicate PBMC cultures incubated with culture fluids from cells transfected with horse interleukin constructs divided by the average [3 H] thymidine incorporation of replicate PBMC cultures incubated in the presence of culture fluids from mock transfected cells. The PBMC plus culture fluids from mock transfected cells, PBMC plus concanavalin A and PMBC without concanavalin A all incorporated only low levels of [3 H] thymidine, (< 750cpm per 2×10^5 cells).

2.2. Experimental Subjects

Animals

All horses and ponies utilized in the following vaccine trials were maintained on pasture at the Department of Veterinary Science's Maine Chance Farm, Lexington, Ky, in accordance with the Guide for the Care and Use of Agricultural Animals in Agricultural Research. The animal handling protocols and experimental procedures were approved by the University of Kentucky Institutional Animal Care and Use Committee (IACUC). Thorough veterinary exams were conducted on all animals to exclude animals with evidence of illness, trauma, lameness, weight loss, or abnormal coat shedding. Vaccinations for rabies and tetanus toxoid (Merck, Sharp and Dohme, Rahway, NJ) were administered yearly, as well as routine treatment with Ivermectin (Merck) for intestinal parasites. Six ponies possessing the ELA-A1 haplotype were used in the first study (mean age 5.7 years; range, 4-10 years) (Table 2.1). Thirty horses or ponies with diverse ELA haplotypes (Table 2.2) were used in the second study (mean age 4.8 years; range, 3-16 years).

Assessment of Physical Responses to DNA Immunization

Physical responses at the injection sites and in the surrounding area were monitored after each DNA immunization for redness, heat, soreness, and swelling. Discoloration or redness of the skin was visually assessed as an indication of infection. The evaluation of heat was determined by touching the skin to monitor for warmth radiating from the skin. Soreness was determined by touching the skin and monitoring sudden flinching or adverse behavioral reactions, such as pinning back of the ears, as an indication of pain. Swelling at and around the injection sites was measured in mm.

Sample Collection and Processing

Serum samples were collected prior to each inoculation and weekly thereafter for eight weeks in the pilot study (Figure 5.1.), for four weeks after V1-V4 (Figure 5.2), and weekly or monthly after V5 (Figure 5.3.) until SU-specific antibodies were no longer detectable by ELISA or immunoblotting. Samples were centrifuged at 2500 rpm in a MSE Mistral 3000i for ten min at 22°C to allow for serum separation, divided into single-use aliquots, and stored at -20°C for use in serological assays.

Heparinized blood samples were collected aseptically by jugular venipuncture from each inoculated horse/pony, prior to inoculation, seven days, and 14 days, for the pilot study (Figure 5.1.) and V1-V4 (Figure 5.2). Following the V5, heparinized blood was collected prior to inoculation and five days post inoculation (Figure 5.3.). Peripheral blood mononuclear cells (PBMC) were purified by overlaying the buffy coat on Ficoll-Paque PlusTM (Amersham Biosciences, Piscataway, NJ) for density gradient centrifugation. PBMC were washed in sterile PBS, pH 7.2 (Gibco, Grand Island, NY) and enumerated using a ViCell-XR instrument (Beckman Coulter, Miami, FL). PBMC aliquots were cryopreserved in c-RPMI 1640 (Gibco) media supplemented with 40% fetal equine serum (FES, Sigma-Aldrich, St. Louis, MO) and 10% dimethyl sulfoxide (DMSO, Sigma) in 2 ml Nalgene® cryogenic vials (Nalgene Co., Rochester, NY). Cells were frozen overnight at -80°C in Nalgene freezing containers and held in liquid nitrogen for storage until needed for lymphocyte proliferation and cell stimulation assays.

ELA-A Typing

PBMC from all horses and ponies were typed with an antibody-mediated, complement-dependent, microcytotoxicity assay, using previously described alloantisera specific for haplotypes expressed on the ELA-A locus (Bailey 1980; Bailey 1983; Bailey, Antczak et al. 1984). This locus includes the serological haplotypes recognized by the International ELA Workshops namely A1–A10, A14, A15, A19. Antisera were available to determine A1–A10 haplotypes, in addition to the less well characterized W11.

Six ponies possessing the ELA-A1 haplotype were identified for the initial pilot study, using this technique (Table 2.1). ELA typing was also utilized to assess the diverse haplotypes of 30 horses and ponies utilized in the expanded preliminary study (Table 2.2). Horses and ponies were assigned to ten different pSYNSU immunization groups, with three animals per group, such that no two animals possessing the same ELA haplotype were placed together in the same treatment group (Table 2.2).

Table 2.1

ELA Typing of DNA Immunized Ponies from the Pilot Study

Animal Identification	ELA-Type
B33	A1/A5
D47	A1/A8
B29	A1/A9
D49	A1/A3
9954	A1/A3
D55	A1/A7

The ELA haplotpye for each pony was determined as described by Bailey et al., 1984.

Table 2.2**ELA haplotype determination of animals in the expanded DNA vaccine study**

Vaccine Group	Animal ID	MHC Type
Naked DNA	600	A1/A2
	F37	A6/A7
	F41	A5/A10
PEI	F36	A8/A10
	C06	A1/A3
	C10	A2
IL-2/Ig	D07	A1/
	D15	A3/A10
	E13	A6/A9
IL-7	G22	A9
	G23	A2/A7
	G30	A4/A5
IL-12 + IL-18	G21	A1/A2
	G26	A4
	G35	A5/A9
IL-15 (SRLSS)	E03	A3/A5
	E08	A5/W11
	D77	A8/A9
IL-15 (SRLSS) + IL-2/Ig	309	A5/A6
	F84	A2/A9
	F85	A3/W11
IL-15 (SSLSS)	311	A5/W11
	D16	A2
	G75	A2/A10
IL-15 (SSLSS) + IL-2/Ig	E05	A2
	E12	W11
	E15	A3/A5
Sushi IL-15	F06	A2/A5
	F18	A1
	G04	A4/A10

The ELA haplotpye for each horse/pony was determined as described by Bailey et al., 1984.

2.3. DNA Immunization Protocols

Formulation of Polyethyleneimine for DNA Immunization

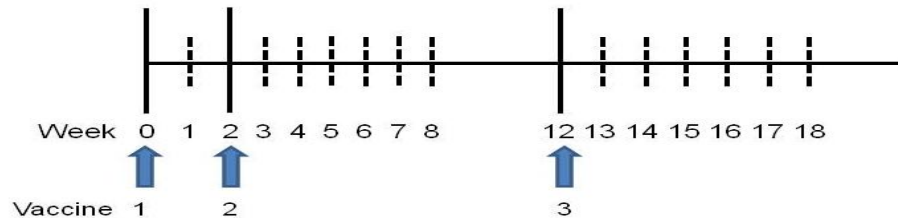
The linear, cationic polymer polyethyleneimine (PEI) (MW 25,000) was obtained from Polysciences, Inc, Warrington, PA. PEI (1.2 g). It was deacylated by refluxing with 24% wt/vol HCl at 110°C for 96hr with complete removal of acyl groups confirmed using NMR spectroscopy conducted by Professor Jurgen Rohr, Department of Pharmaceutical Sciences, University of Kentucky, Lexington, Ky. Each DNA plasmid used for immunization was formulated with PEI at a PEI nitrogen (N):DNA phosphate (P) molar ratio of 10:1 in sterile, nuclease-free, endotoxin-free water.

Immunization Protocol for ELA-A1 Pilot Study

For the first DNA vaccine trial (pilot study), 250 µg pSYNSU suspended in 500 µl nuclease-free water was administered intradermally (i.d.) in the neck of each pony, near the prescapular lymph node at 0 (V1), 2 (V2), and 12 (V3) weeks (Table 2.3). Prior to DNA inoculation, blood samples were collected from the ponies, and sera was examined for the presence of EIAV specific antibodies by immunoblot analysis to ensure that these animals were seronegative. In the initial pilot study, two ponies (B29, D47) received three i.d. injections at weeks 0, 2, and 12 (Figure 2.2), each consisting of 250 µg pSYNSU formulated with PEI (Table 2.3). As a control and to verify a previous study (Cook et al., 2005) demonstrating weak immune responses to SU following multiple inoculations with unprotected plasmid DNA, a single animal (B33) received three i.d. inoculations of pSYNSU at the same intervals (Figure 2.2, Table 2.3). In addition, two horses (D55, 9954) received three equivalent i.d. inoculations with pSYNSU + PEI along with pIL-2/Ig (250 µg/dose) and pIL-15 (SRLSS) (Figure 2.2, Table 2.3). It was unknown at the start of this study that equine IL-15 (SRLSS) expression was lower in transiently transfected HEK293 cells than equine IL-15 (SSLSS). However, when the significantly superior biological activity of the SSLSS variant was discovered an additional horse, D49, was included in the initial pilot study and co-inoculated with three doses (at weeks 0, 2, and 12) of pSYNSU (250 µg) plus pIL-15 (SSLSS) (250 µg) (Figure 2.2, Table 2.3).

Figure 2.2

Schedule of DNA Immunization and Sample Collection for ELA-A1 Ponies in the Pilot Study



DNA vaccines were administered at 0 (V1), 2 (V2), and 12 weeks (V3), as indicated by the arrows. Heparinized blood samples were collected immediately prior to each immunization and two weeks thereafter. Serum samples were collected at weeks 0-8 and 12-18.

Table 2.3**Animal Assignments and pSYNSU Immunization Groups for the Pilot Study**

pSYNSU Immunization Group	Animal Number	Inoculum
Naked DNA	B33	pSYNSU
PEI	B29, D47	pSYNSU and PEI
IL-15 (SSLSS)	D49	pSYNSU, pIL-15 (SSLSS) and PEI
IL-15 (SRLSS) + IL-2/Ig	D55, 9954	pSYNSU, pIL-2/Ig, pIL-15 (SRLSS) and PEI

All i.d. injected plasmid DNAs were formulated in endotoxin-free, nuclease-free, sterile water. All experimental subjects received four i.d. inoculations at 0 (V1), 2 (V2), and 12 weeks (V3). Each plasmid was used at a concentration of 250 µg per dose and, unless otherwise stated, formulated with fully deacylated, linear PEI at an N:P ratio of 10:1

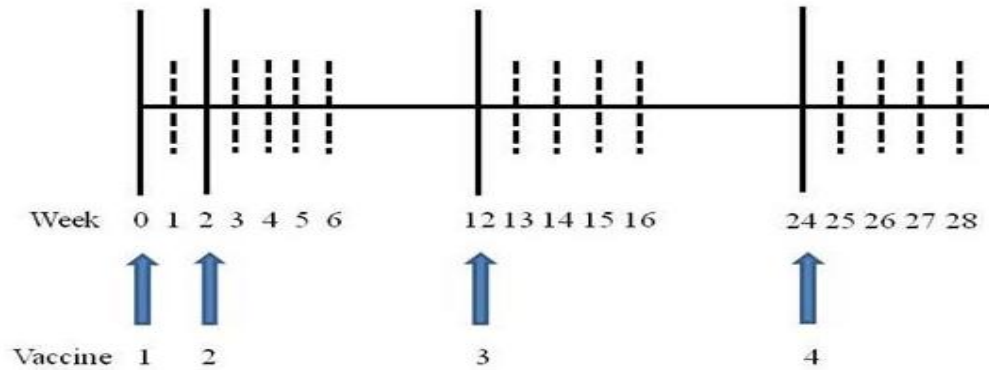
Immunization Protocol for Animals with Diverse ELA Haplotypes

Every animal received a series of four intradermal inoculations near the prescapular lymph node on alternating sides of the neck at 0 (V1), 2 (V2), 12 (V3), and 24 (V4) weeks (Figure 2.3). Each animal was inoculated with 500 µg pSYNSU DNA immunizations in 1 ml sterile, nuclease-free water. Control ponies were immunized with the SYNSU plasmid (Table 2.5). All nine remaining groups received pSYNSU DNA vaccines formulated with 1225 µg PEI (N:P ratio of 10:1) per SYNSU or 500 µg cytokine expression plasmid (Table 2.5). One vaccine group only received pSYNSU immunizations in formulation with PEI (Table 2.5). Cytokine recipient vaccine groups also received vaccines expressing pIL-2/Ig, pIL-7, pIL-12 + pIL-18, pIL-15 (SRLSS), pIL-15 (SRLSS) + pIL-12/Ig, pIL-15 (SSLSS), pIL-15 (SSLSS) + pIL-2/Ig, or pSushi IL-15 (Table 2.5).

Blood samples were collected prior to each immunization and up to four weeks after V3 and V4 (Figure 2.3), for serum analysis by Western blot and ELISA. Heparinized blood samples were also collected prior to each immunization and at one week after each inoculation for isolation of PBMC for cellular assays (Figure 2.3).

Figure 2.3

Schedule of Vaccination and Blood Collection for animals with divergent ELA haplotypes administered pSYNSU DNA Vaccine Protocols



DNA vaccines were administered at 0 (V1), 2 (V2), 12 (V3), and 24 (V4) weeks.
Heparinized blood was collected for PBMC isolation at weeks 0, 2, 3, 12, 13, 24, and 25.
Serum samples were collected at weeks 0-6, 12-16, and 24-28.

Table 2.5**Animal Assignments and pSYNSU Immunization Groups for the 30 Horse DNA Vaccination Trial**

pSYNSU Immunization Group	Animal Number	Inoculum
Naked DNA	600, F37, F41	pSYNSU
PEI	C06, C10, F36	pSYNSU and PEI
IL-2/Ig	D07, D15, E13	pSYNSU, pIL-2/Ig and PEI
IL-15 (SRLSS)	D77, E03, E08	pSYNSU, pIL-2/Ig, pIL-15 (SRLSS) and PEI
IL-15 (SSLSS)	311, D16, G75	pSYNSU, pIL-15 (SSLSS) and PEI
Sushi IL-15	F06, F18, G04	pSYNSU, pSushi IL-15 and PEI
IL-7	G22, G23, G30	pSYNSU, pIL-7, and PEI
IL-12 + IL-18	G21, G26, G35	pSYNSU, pIL-12 + IL-18 and PEI
IL-15 (SRLSS) + IL-2/Ig	309, F84, F85	pSYNSU, pIL-2/Ig, pIL-15 (SRLSS) and PEI
IL-15 (SSLSS) + IL-2/Ig	E05, E12, E15	pSYNSU, pIL-2/Ig, pIL-15 (SSLSS) and PEI

All i.d. injected plasmid DNAs were formulated in endotoxin-free, nuclease-free, sterile water. All experimental subjects received four ID inoculations at 0 (V1), 2 (V2), 12 (V3), and 24 weeks (V4). Each plasmid was used at a concentration of 500 µg per dose and, unless otherwise stated, formulated with fully deacylated, linear PEI at an N:P ratio of 10:1.

Immunization protocol for animals possessing diverse ELA haplotypes maintained for long-term observation

After V4, it was only possible to retain fifteen animals, representative of eight different pSYNSU immunization groups, for long term observation (Table 2.6). These horses or ponies were administered a fifth vaccination (V5) consisting of 500 µg pSYNSU (naked DNA group) in 1 ml sterile, nuclease-free water or 500 µg pSYNSU + PEI (all other pSYNSU immunization groups) (1225 µg) in 1 ml sterile, nuclease-free water, 15 months after V4 (Figure 2.4). Blood samples were collected prior at week 90, prior to V5, and weekly/monthly thereafter until SU-specific antibodies were no longer detectable by immunoblotting (Figure 2.4).

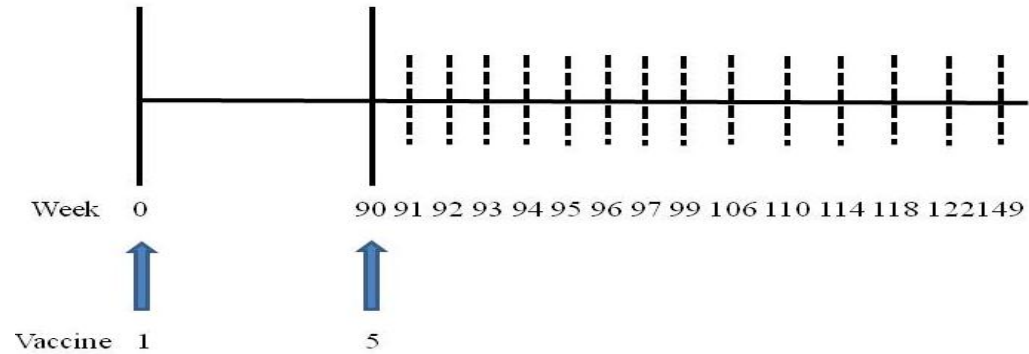
Table 2.6**Animal Assignments and pSYNSU Immunization Groups for V5 recipients**

pSYNSU Immunization Group	Animal Number
naked DNA	600, F37, F41
PEI	C10, F36
IL-2/Ig	D07
IL-15 (SRLSS)	D77, E03, E08
IL-15 (SSLSS)	311, D16, G75
Sushi IL-15	F06, F18, G04
IL-7	G22, G23
IL-12 + IL-18	G26, G35

All i.d. injected plasmid DNAs were formulated in endotoxin-free, nuclease-free, sterile water. Each experimental subject retained after V4 received a fifth ID inoculation at 90 weeks (V5). pSYNSU was used at a concentration of 500 µg per dose and formulated with fully deacylated, linear PEI at an N:P ratio of 10:1 to all animals except for those in the naked DNA group.

Figure 2.4

Schedule of Vaccination and Blood Collection from horses or ponies receiving a fifth Vaccination (V5)



V5 was administered at week 90 to the animals retained for long-term observation. Heparinized blood was collected for PBMC isolation at weeks 90 and 91. Serum samples were collected at week 90 and weekly/monthly thereafter (indicated by the dashed lines), until antibody responses were no longer detectable.

2.4. Analysis of immune responses

Immunoblot Analysis

Gradient-purified EIAV_{PV} (Rwambo, Issel et al. 1990) or antigens derived from transfected cell cultures were separated by SDS-PAGE and electrophoretically transferred to supported nitrocellulose membranes (Bio Rad, 0.2 microns) through wet transfer with a Mini-Trans-Blot[®] Electrophoretic Transfer System (Bio-Rad Laboratories). Non-specific binding of proteins to the nitrocellulose membranes was blocked by Blotting-Grade Blocker, non-fat dry milk (Bio Rad). A reference anti-EIAV polyclonal antiserum was prepared from an EIAV-infected horse by Sheila Cook (MSc). Horseradish peroxidase (HRP)-conjugated antibodies (rabbit anti-Mouse IgG, rabbit anti-horse IgG, or goat anti-mouse IgG) were purchased from Sigma. The TMB Stabilized Substrate (3,3', 5,5'-tetramethylbenzidine (Promega)) was used in Western blots as a color development substrate for localization of horseradish peroxidase-conjugated antibodies.

Description of SU-specific peptides and generation of peptide pools

Forty-four 20mer peptides overlapping by 10 amino acid residues (generously provided by Dr. R.C. Montelaro and described in Tagmyer et al., 2008 and Table 2.7 (Tagmyer, Craig et al. 2008)), representing the entire EIAV_{PV} SU (GenBank accession no AF016316) were synthesized in the Biomedical Research Support Facility Peptide Synthesis Core Center of the University of Pittsburgh, using a Chemtech model 396 Omega synthesizer. The SU-specific peptides produced at the University of Pittsburgh were dissolved in 100% DMSO and maintained at a final concentration of 2 mg/ml. Individual 20mer peptides were combined into seven different pools (I-VII) for the lymphocyte proliferation assays (Table 2.8). For the Real-Time RT-PCR assays, SU-specific peptides were assembled into all even numbered peptides (Even), all odd numbered peptides (Odd), and a composite pool consisting of all 44 overlapping peptides (Total). The RW-12 peptide was also utilized in lymphocyte proliferation assays with PBMC from ELA-A1 ponies in the pilot study, since it contained a CTL epitope recognized by animals with this genetic haplotype (McGuire, Leib et al. 2003).

Table 2.7

Peptide sequences of 44 20mer peptides overlapping by 10 amino acid residues, representing the entire EIAV_{PV} (GenBank accession no AF016316) SU and the RW-12 peptide

gp90 peptide	Peptide sequence*
1	MVSIAFYGGIPGGISTPITQ
2	PGGISTPITQQSEKSKCEEN
3	QSEKSKCEENTMFQPYCYNN
4	TMFQPYCYNNDSKNSMAESK
5	DSKNSMAESKEARDQEMNLK
6	EARDQEMNLKEESKEEKRRN
7	EESKEEKRRNDWWKIGMFL
8	DWWKIGMFLCLAGTTGGIL
9	CLAGTTGGILWWYEGLPQQH
10	WWYEGLPQQHYIGLVAIGGR
11	YIGLVAIGGRLNGSGQSNAI
12	LNGSGQSNAIECWGSFPGCR
13	ECWGSFPGCRPFQNYFSYET
14	PFQNYFSYETNRSMHMDNNT
15	NRSMHMDNNTATLLEAYHRE
16	ATLLEAYHREITFIYKSSCT
17	ITFIYKSSCTDSDHCQEYQC
18	DSDHCQEYQCKKVNLNSSDS
19	KKVNLNSSDSSNPVRVEDVM
20	SNPVRVEDVMNTTEYWGFKW
21	NTTEYWGFKWLECNQTENFK
22	LECNQTENFKTILVPENEMV
23	TILVPENEMVNINDTDTWIP
24	NINDTDTWIPKGCNETWARV
25	KGCNETWARVKRCPIDILYG
26	KRCPIDILYGIHPIRLCVQP
27	IHPIRLCVQPPFFLVQEKGI

Table 2.7 con't.

gp90 peptide	Peptide sequence*
28	PFFLVQEKGIANSTRIGNCG
29	ANSTRIGNCGPTIFLGVLED
30	PTIFLGVLEDNKGVVVRGNYT
31	NKGVVVRGNYTACNVSRLLKIN
32	ACNVSRLLKINRKDYTGIIYQV
33	RKDYTGIIYQVPIFYTCNFTN
34	PIFYTCNFTNITSCNNEPII
35	ITSCNNEPIISVIMYETNQV
36	SVIMYETNQVQYLLCNNNNS
37	QYLLCNNNNSNNYNCVVQSF
38	NNYNCVVQSFGVIGQAHLEL
39	GVIGQAHLELPRPNKRIRNQ
40	PRPNKRIRNQSFNQYNCSIN
41	SFNQYNCSINNKTETELETWKL
42	NKETELETWKLVKTSGITPLP
43	VKTSGITPLPISSEANTGLI
44	ISSEANTGLIRHKRDFGISA
RW-12	RVEDVTNTAEYW

Peptide sequences of 44 20mer peptides overlapping by 10 amino acid residues, representing the entire EIAV_{PV} (GenBank accession no AF016316) SU (generously provided by Dr. R.C. Montelaro and described in Tagmyer et al., 2008 (Tagmyer, Craig et al. 2008)) and the EIAV RW-12 peptide (McGuire, Leib et al. 2003). The SU-specific peptides produced at the University of Pittsburgh were dissolved in 100% DMSO and maintained at a final concentration of 2 mg/ml.

Table 2.8

EIAV SU Peptide Pools

A.

I	II	III	IV	V	VI	VII
1	3	5	7	9	11	13
15	17	19	21	23	25	27
29	31	33	35	37	39	41
43	2	4	6	8	10	12
14	16	18	20	22	24	26
28	30	32	34	36	38	40
42	44	1	17	33	6	22

B.

Odd Pool: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43

C.

Even Pool: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44

D.

Total Pool: Peptides 1-44

Forty-four peptides comprising the entire extracellular region of EIAV Env (SU) were divided into seven peptide pools (A), Odd Pool (B), Even Pool (C), or Total Pool (D). The peptides (generously provided by R.C. Montelaro) were synthesized at the University of Pittsburgh and by GenScript and described in Tagmyer et al., 2008 and Table 3.4.

[³H]-thymidine incorporation assay

Lymphocyte proliferation assays were performed using cryopreserved PBMC to assess the total S-phase activity by measuring the uptake of [³H]-thymidine on stimulated cells (Hammond et al., 1997). Isolated PBMC (2×10^5 cells/ml) were plated in triplicate wells of 96-well flat bottomed, polystyrene plates (TPP, Trasadingen, Switzerland). For the ELA-A1 pilot study, PBMC cultures collected prior to V1 (week 0) and at 2 weeks post V3 (week 14) were analyzed. However, for the expanded study, PBMC cultures collected at week 0, 1 week post V3 (week 13), 1 week post V4 (week 25), and 1 week post V5 (week 91) were analyzed. Lymphocytes were stimulated with media alone, Concanavalin A (Con A) (Sigma) at 10 µg/ml, gradient purified EIAV_{PV} at 10 µg/ml, or EIAV_{PV} peptide pools 1-7 at 21 µg/ml (for the expanded study) for 72 hours at 37°C in humidified 5% CO₂ incubator. PBMC were then pulsed with 1 µCi of [³H]-thymidine (MP Biomedicals, Solon, Ohio) for the final 18 hours. Total nuclear DNA was collected using a Tomtec harvester (J/B Industries, Inc., IL) onto glass fiber filter pads for liquid scintillation counting (Wallac Inc., Gaitheisburg, Md) to determine [³H]-thymidine incorporation. The average corrected counts per minute (CCPM) was determined by subtracting the background for the instrument. A stimulation index (SI) was used to determine EIAV-specific lymphocyte proliferative responses, by using the dividend of the mean CCPM value of [³H]-thymidine of replicate stimulated PBMC cultures for each treatment over the mean CCPM value of [³H]-thymidine of replicate unstimulated PBMC cultures. Con A was used as a positive control, and the results were only considered valid if Con A stimulation indexes were greater than 10.

Determination of DMSO and Cryopreservation Effects on Viability and Lymphoproliferation of Equine PBMC Cultures

Heparinized blood samples were obtained from the long-term EIAV infected horse, D64, and processed by Ficoll-Paque density gradient centrifugation to isolate PBMC populations. Optimization experiments were conducted on either freshly collected or cryopreserved PBMC populations maintained in liquid nitrogen. To evaluate the effect of cryopreservation on the subsequent proliferative ability of equine PBMC, a comparative study was conducted with freshly collected and cryopreserved PBMC.

PBMC populations (2×10^5) collected from an EIAV infected horse, D64, were harvested and cryopreserved for two days. Additional blood was collected two days later and PBMC were harvested to run in a parallel assay with the cryopreserved PBMC. The overall cell viability, in terms of trypan blue exclusion, was determined by a ViCell cell viability analyzer after PBMC isolation for the freshly isolated cells and after thawing for the cryopreserved cell population. Differences in lymphoproliferation to mitogenic Con A (10 μ g/ml), EIAV_{PV} (10 μ g/ml) and SU-specific peptide pool (21 μ g/ml) stimulation were also assessed. Cell populations of 2×10^5 PBMC were incubated with stimulants for 72 h and subsequently labeled with [³H]-thymidine for 18 h, in a standard [³H]-thymidine incorporation assay. In addition, the blastogenic potential of fresh and frozen lymphocytes was observed microscopically. Each peptide pool was used at a concentration of 21 μ g/ml, resulting in a final DMSO concentration of 1.05% per well.

In vitro PBMC Stimulation

All cryopreserved PBMC used in the in vitro stimulation assays were thawed in cRPMI 1640 supplemented with 10% FES. Cells were pelleted by centrifugation at 300 G x 10 minutes and resuspended in medium (c-RPMI 1640 supplemented with FES (2.5% for Real-Time RT-PCR assays and 5% for lymphoproliferation assays), 100 U/ml penicillin/streptomycin [Sigma], 55 μ M 2-mercaptoethanol [GIBCO, Grand Island, NY]) and enumerated on the ViCell-XR instrument. For all lymphocyte proliferation assays, 2×10^5 PBMC were plated as four replicates in a 96-well plate format for each treatment (200 μ l total volume of medium). For the Real-Time RT-PCR assays, 3×10^6 PBMC were plated in single wells in a 24-well plate format for each treatment (one ml total volume of medium).

For the pilot study, sufficient cryopreserved PBMC stocks collected prior to V1 (Pre-vacc.) and at two weeks post V3 (Post-vacc.) were available from B33, B29, D47, D49, 9954, and D55 to determine if detectable amounts of IFN- γ mRNA were produced following incubation with the SU RW-12 peptide, which interacts specifically with the ELA-A1 MHC class I antigen (McGuire, Leib et al. 2003). PBMC cultures were >90% viable upon thawing and maintained viability throughout the assays, as measured by trypan blue exclusion. To stimulate PBMC cultures for the lymphocyte proliferation

assay, PBMC were incubated with 1, 5, and 10 µg/ml gradient purified EIAV_{PV} or 10 µg/ml Con A as a positive control for 72 h. Unstimulated PBMC cultures were used as a negative control to account for background stimulation for each pony. PBMC were assayed in four replicates in complete RPMI 1640 medium (5% fetal equine serum, 1% penicillin/streptomycin, 1% L-glutamine, 55µM β-mercaptoethanol). To determine IFN-γ mRNA expression levels after PBMC stimulation, PBMC were incubated with 10 µg/ml RW-12 peptide, or phorbol 12-myristate 13-acetate (PMA; 25ng/ml; Sigma) and ionomycin (1µM; Sigma) as a positive control for cell stimulation. Untreated PBMC cultures were used as negative control wells.

For the expanded 30 horse/pony study, PBMC were incubated for 24 h at 37°C in humidified 5% CO₂ incubator to allow the PBMC to recover from the thaw before treatment with any stimulants. To stimulate PBMC cultures for the lymphocyte proliferation assay, PBMC were incubated with 21 µg/ml of each peptide pool 1-7, 10 µg/ml gradient purified EIAV_{PV} or 10 µg/ml Con A as a positive control for 72 h. For the Real-Time RT-PCR experiments, PBMC cultures were incubated with 21 µg/ml of each Odd, Even, or Total peptide pool. The total concentration of each peptide pool used to stimulate PBMC (21 µg/ml) was consistent with previous work (Tagmyer, Craig et al. 2008). PBMC were treated with medium alone, or medium supplemented with DMSO (1.05%), Con A (10 µg/ml), or each peptide pool. For the optimization experiments, PBMC cultures were inoculated with individual peptides 20 or RW-12 at a concentration of 10 µg/ml, described in Tagmyer et al., 2007 (Tagmyer, Craig et al. 2007). Con A mitogen was used as a positive control, and lymphoproliferative responses to peptide pools were only examined if SI values for Con A were greater than 20, as this was consistent with SI values observed following Con A stimulation of fresh, non-cryopreserved PBMC cultures.

Effects of Priming with Equine IL-2 on Lymphoproliferative Responses

Experiments were also conducted to ‘prime’ the lymphocyte populations in the PBMC cultures to determine if this would augment proliferative responses to SU-specific peptide pools. Recombinant equine IL-2 was added to 2×10^5 PBMC cultures at concentrations of 0.1-1.0 ng/ml for 72 h and media was removed by careful aspiration

and replaced with either media containing 21 µg/ml of each peptide pool or 0.1-1.0 ng/ml IL-2 as positive controls. For negative control wells, PBMC were initially stimulated with IL-2 (0.1-1.0 ng/ml) and re-supplemented with media only after the media change. The viability of PBMC cultures varied between 87-91%. Stimulation indexes were calculated by dividing the mean of replicate wells for peptide stimulated PBMC populations by the mean of their respective media control (non-peptide stimulated PBMC). An SI of 2 or above was statistically determined to be a significant increase in lymphocyte proliferation.

Effects of Priming with Gradient Purified EIAV or Con A on Lymphoproliferative Responses

The priming potential of gradient purified EIAV_{PV} or Con A on PBMC from D64 was also determined. The 2×10^5 PBMC cultures were incubated with 0, 2, 4, 6, 8, or 10 µg/ml concentrations of EIAV_{PV} or Con A at 10 µg/ml. After 72 h, the media was changed and cells were supplemented with fresh media containing peptide pool 5 or 8 (21 µg/ml). Peptide pool 5 was utilized because previous results suggested that PBMC from D64 exhibited the highest proliferative responses to stimulation with this pool (data not shown), and peptide pool 8 was selected because it was composed of newly synthesized peptides from GenScript with a known purity.

PBMC from D64 (EIAV long-term infected carrier) were incubated with 10 µg/ml virus for 0-5 days in vitro and then incubated with either media, virus, Con A at 10 µg/ml, or peptide pools 1-7 at 21 µg/ml for 1-7 more days, after media change to remove virus. In a parallel assay, cells were also incubated with EIAV_{PV} or individual peptide pools alone for 1-7 days. RNA was isolated at each of these time points, reverse transcribed into cDNA, and real time RT-PCR was performed.

RNA isolation and quantification

Total cellular RNA was extracted from PBMC in culture after 24 h or 96 h with MagMAX™- 96 Total RNA Isolation Kit, according to manufacturer's instructions with a MagMAX™ Express Magnetic Particle Processor. RNA quantifications were

performed with a NanoDrop ND-1000 at OD260 nm wavelength with 1 µl of each RNA sample.

Relative Quantification of Cytokine Gene Expression using TaqMan® Real-Time RT-PCR

Real Time RT-PCR assays were performed to examine relative levels of IL-2 and IFN-γ gene expression from PBMC in response to stimulation with each peptide pool. For relative quantification of cytokine mRNA expression of IL-2 and IFN-γ, the reverse transcription reaction was performed with 0.5 µg of total RNA using RT random primers and a MultiScribe™ reverse transcriptase (High Capacity cDNA Reverse Transcription kit with RNase inhibitor [Applied Biosystems, Foster City, CA]) according to the manufacturer's instructions. The reactions were incubated at 25°C for 10 min, 37°C for 120 min and 85°C for 5 min (optimized and unpublished method by Y.Y. Go and Dr. Udeni Balasuriya, University of Kentucky). The resultant cDNA was diluted 1:1 with nuclease-free water to allow for measurement of multiple cytokine genes by real-time RT-PCR. Samples of cDNA were stored at -20°C until further analysis by Real-Time RT-PCR.

The Real-Time RT-PCR reactions were set up using the CAS-1200 (Corbett Life Science) automated PCR setup machine. For cytokine gene expression, equine specific intron-spanning IFN-γ, IL-2, and beta-glucuronidase (β-GUS) specific primer/probe sets (Breathnach, Sturgill-Wright et al. 2006) were utilized. Briefly, PCR amplification was carried out using a 10µl reaction mixture containing 4.5 µl of diluted cDNA, 0.5 µl of 20× assay mix for the primer/probe set of interest (Applied Biosystems, Foster City, CA) and 5 µl of TaqMan® Gene Expression Master Mix (Applied Biosystems, Foster City, CA). Samples were incubated in duplicate wells at 95°C for 10 min followed by 40 cycles at 95°C for 15 s and 60°C for 60 s using an Applied Biosystems 7500 Fast Real-Time PCR System.

LinReg analysis was used to analyze the quantitative RT-PCR data by determining a baseline fluorescence, with a baseline subtraction (Ramakers, Ruijter et al. 2003), followed by analysis with the $\Delta\Delta C_T$ method (Livak and Schmittgen 2001) where $\Delta\Delta C_T = (\text{mean IFN}\gamma C_T - \text{mean } \beta\text{-GUS } C_T)_{\text{stimulant of interest}} - (\text{mean IFN}\gamma C_T - \beta\text{-GUS } C_T)_{\text{media}}$ ("the calibrator") to assess relative quantification of gene expression.

A stably expressed reference gene, beta-glucuronidase (B-GUS), was used as an internal control to determine variations in RNA levels between samples. The calibrator used was the mean ΔC_T of the media control for each animal for each individual gene. The results are calculated as $2^{-\Delta\Delta C_T}$, expressed as relative quantity (RQ), and represent fold change increases in relative gene expression.

Determination of Serum Immunoglobulins to EIAV SU-specific IgG_A and IgG_T

Murine monoclonal antibodies to equine IgG_A and IgG_T were used in an ELISA format to determine the endpoint titers of these IgG subclasses induced by each of the DNA immunization protocols (Lunn, Holmes et al. 1995; Lunn, Holmes et al. 1996; Soboll, Horohov et al. 2003). Serum samples were analyzed from each immunized individual at weeks 0, 3, 12-16, and 24-28 weeks post V1 for quantitative levels of EIAV SU-specific IgG_A and IgG_T antibodies, using an ELISA assay that has been previously described (Lunn, Soboll et al. 1999).. Statistical analysis of the ELISA data was performed by Xia Yu and Keith Kohrs at the University of Kentucky, Department of Statistics, using the mixed procedure analysis with SAS. Briefly, gradient purified EIAV_{PV} at 2 µg/ml was coated onto 96-well polystyrene flat bottom Immulon Microtiter plates (Thermo Fisher Scientific) in 0.05M carbonate/bicarbonate buffer (pH 9.6) and incubated overnight at 4°C. Subsequently, the plates were washed with PBS/0.05% IGEPAL (CA-630 Sigma-Aldrich, St. Louis, MO) and blocked with 5% Blotting-grade Blocker Serum (Biorad) in ELISA wash buffer supplemented with 0.05% IGEPAL (CA-630 Sigma-Aldrich, St. Louis, MO) for 2 h at 37°C and washed again. Serum samples diluted 1:40 and 1:80 in ELISA wash buffer were incubated in single wells for 30 min at 22°C. Each plate included positive control serum samples (from EIAV infected horse) incubated at 1:40 and 1:80. Plates were washed with PBS/0.05% IGEPAL and incubated for 1 h at 22°C with murine monoclonal antibodies specific for anti-equine IgG_A ((clone CVS48), Sheoran et al, 1998) and IgG_T ((clone CVS38), Sheoran et al, 1998) for 30 min at 22°C. Following washes with PBS/0.05% IGEPAL, plates were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG antibody (Sigma-Aldrich) for 30 min at 22°C. Plates were washed again, developed by the addition of substrate *o*-phenylene-diamine hydrochloride (Sigma Fast tablet sets, Sigma-Aldrich), and stopped

by the addition of 3N HCl. Colorimetric changes were measured at A_{450} by a spectrophotometer, to determine relative serum antibody concentrations.

Virus Neutralization Assay

An virus-specific neutralization assay was used to measure antibody concentrations against EIAV in horse serum, to assess the loss of infectivity through reaction of the virus with specific antibodies. The EIAV neutralization assay was performed by Dr. R. Frank Cook and Sheila Cook (MSc) according to a published method (Cook et al, 1995). Two dilutions of horse serum were incubated for 3 weeks with 100 tissue culture infectious doses (TCID₅₀) of EIAV_{PV}. The level of neutralizing antibody present was determined by the highest serum dilution that could render reverse transcriptase activity undetectable by radiolabelling.

Statistics

The mixed procedures of SAS program was used to analyze all EIAV SU-specific IgG_A and IgG_T titers determined in the ELISA assay, IFN- γ expression levels in the pilot study, and the entirety of the lymphocyte proliferation data (analyzed by Xia Yu and Keith Kohrs, from the Department of Statistics at the University of Kentucky). For the lymphocyte proliferation assay, corrected counts per minute (CCPM) were compared relative to media controls to determine positive proliferation over time in response to DNA vaccination. Proliferation results to treatments were assessed at each time point and between vaccination time points, for both vaccination groups and for all 30 individual animals. Statistically significant differences were determined at the level of $P < 0.05$. Descriptive statistics (Mann 1995) were used to analyze IL-2 and IFN- γ mRNA expression levels after four and five immunizations, with a minimal level of significance determined to be an RQ value of 2.

Chapter III: Results

3.1: Recombinant Equine Interleukins

Equine Interleukin Construction and Analysis

As a result of the codon modifications made to pCiIL-2, the G + C content was increased from 46.2% in native IL-2 to 59.1% in the synthetic variant (Table 3.1). The predicted molecular weight of the equine IL-2/IGHC1 fusion protein is 54817.22 (Vector NTI) for the precursor form and 52512.70 for the mature species assuming removal of the 20 amino acid signal peptide. An alignment between the IL-2 sequences is shown in Table 3.2. The 36% GC content of native equine IL-7 cDNA was increased to 58.2% by codon optimization. An alignment between these sequences is shown in Table 3.3. Codon optimization of the equine IL-12 sequences changed the G/C content from 52.4% with the native sequences to 53.1% in the synthetic gene (Table 3.4). The G/C content of the codon optimized equine IL-18 construct was increased 15.1% compared to native sequences encoding this interleukin (Table 3.5). The process of codon optimization also increased the G/C content of the IL-15 (SRLSS) and IL-15 (SSLSS) from 35% to 55.8% (Table 3.6). However, addition of the Sushi domain and linker molecules (L+1 and L+2) to the IL-15 (SSLSS) construct did not alter the amino acid sequence of this interleukin (Table 3.7).

Figure 3.1

Comparison between Native and Codon Optimized Equine IL-2 Nucleotide Sequences

		1		50
Native IL-2	(1)	-----	ATGTACAGGATGCAACTCCTGTCTTGCATCGCACTAACTCTTG	
IL-2	(1)	AGCCACC	ATGTACCGGATGCAAGCTGCTGAGCTGCATCGCCCTGACCCTGG	
Consensus	(1)		ATGTAC GGATGCA CT CTG TGCATCGC CT AC CT G	
		51		100
Native IL-2	(44)		CAGTCTTGCAAACAGTGCACCTACTTCAAGCTCTAAGAGGGAAACACAG	
IL-2	(51)		CCGTGCTGGCCAACAGCGCCACCAGCAGCAGCAAGCGGGAGACCCAG	
Consensus	(51)		C GT CT GC AACAG GC CC AC AGC AAG GGGA AC CAG	
		101		150
Native IL-2	(94)		CAACAACCTGAAGCAATTACAGATGGAATTAAAGTTGCTTTTGGGAAGGAGT	
IL-2	(101)		CAGCAGCTGAAGCAGCTGCAGATGGA CCTGAAGCTGCTGCTGGAGGGCGT	
Consensus	(101)		CA CA CTGAAGCA T CAGATGGA T AAG TGCT TGGA GG GT	
		151		200
Native IL-2	(144)		TAAATAACAAGAATCCCAAACCTCTCCAAGATTCTCACATTTAAAATTA	
IL-2	(151)		GAACAACAACAAGAACCCCAAGCTGAGCAAGATCTGACCTTCAAGATCA	
Consensus	(151)		AA AA AACAAGAA CCAA CT CAAGAT CT AC TT AA AT A	
		201		250
Native IL-2	(194)		ACATGCCCAAGAAGGCCACAGAATTGAAACATCTTCAGTGTCTAGAGAGAA	
IL-2	(201)		ACATGCCCAAGAAGGCCACCAGGCTGAAAGCACCTGCAGTGCCTGGAGGAG	
Consensus	(201)		ACATGCCCAAGAAGGCCAC GA TGAA CA CT CAGTG CT GA GA	
		251		300
Native IL-2	(244)		GAACTCAAACCTCTGGAGGAATGCTAAAAAAGTTTCTCTCGAAAGATAT	
IL-2	(251)		GAGCTGAAGCCCCTGGAGGAGATGCTGAAGAAGTTCTGAGCAAGGACAT	
Consensus	(251)		GA CT AA CC CTGGAGGA ATGCT AA AACTT CT AA GA AT	
		301		350
Native IL-2	(294)		CAAGGAATTAAATGAGCAAATCAATGTAACAGTTCTGGGACTAAAGGGGT	
IL-2	(301)		CAAGGAGCTGATGAGCAAATCAACGTGACCGTGCTGGGCCTGAAGGGCA	
Consensus	(301)		CAAGGA T ATGAGCAA ATCAA GT AC GT CTGGG CT AAGGG	
		351		400
Native IL-2	(344)		CTGAAACAAAGATTTCACATGTGAATATGATAATGAGACAGAAACAATTGTA	
IL-2	(351)		GCGAGACCCGGTTTCACCTGCAGGTACGACAACGAGACCGAGACCATCGTG	
Consensus	(351)		GA AC G TTCAC TG GA TA GA AA GAGAC GA AC AT GT	
		401		450
Native IL-2	(394)		GAATTTCTGAACAAATGGATTACCTTTTGTCAAAGCATCATCTCAACACT	
IL-2	(401)		GAGTTCTGAACAAGTGGATTACCTTTCTGCCAGAGCATCATCAGCACCT	
Consensus	(401)		GA TT CTGAACAA TGGAT ACCTT TG CA AGCATCATC AC CT	
		451		
Native IL-2	(444)		GACG	
IL-2	(451)		GACC	
Consensus	(451)		GAC	

The additional seven nucleotides and the 5' terminus of IL-2 represent a consensus Kozak motif.

Figure 3.2

Equine IL-2 and IL-2/Immunoglobulin (Ig) Fusion Protein Constructs

		1	50
Native IL-2	(1)	MYRMQLLSICIALTLAVLANSAPTSSSKRETQQQLKQLQMDLKLLLEGVNN	
IL-2	(1)	MYRMQLLSICIALTLAVLANSAPTSSSKRETQQQLKQLQMDLKLLLEGVNN	
IL-2-Ig	(1)	MYRMQLLSICIALTLAVLANSAPTSSSKRETQQQLKQLQMDLKLLLEGVNN	
IgG-Fc/C1q	(1)	-----	
IGHC1	(1)	-----	
Consensus	(1)	MYRMQLLSICIALTLAVLANSAPTSSSKRETQQQLKQLQMDLKLLLEGVNN	
		51	100
Native IL-2	(51)	NKNPKLSKILTFKINMPKKATELKHLQCLEEELKPLEEMLKNFLSKDIKE	
IL-2	(51)	NKNPKLSKILTFKINMPKKATELKHLQCLEEELKPLEEMLKNFLSKDIKE	
IL-2-Ig	(51)	NKNPKLSKILTFKINMPKKATELKHLQCLEEELKPLEEMLKNFLSKDIKE	
IgG-Fc/C1q	(1)	-----	
IGHC1	(1)	-----	
Consensus	(51)	NKNPKLSKILTFKINMPKKATELKHLQCLEEELKPLEEMLKNFLSKDIKE	
		101	150
Native IL-2	(101)	LMSNINVTVLGLKGSETRFTCEYDNETETIVEFLNKWITFCQSIISTLT	
IL-2	(101)	LMSNINVTVLGLKGSETRFTCEYDNETETIVEFLNKWITFCQSIISTLT	
IL-2-Ig	(101)	LMSNINVTVLGLKGSETRFTCEYDNETETIVEFLNKWITFCQSIISTLT	
IgG-Fc/C1q	(1)	-----	
IGHC1	(1)	-----	
Consensus	(101)	LMSNINVTVLGLKGSETRFTCEYDNETETIVEFLNKWITFCQSIISTLT	
		151	200
Native IL-2	(150)	-----	
IL-2	(150)	-----	
IL-2-Ig	(151)	VQEASTTAPKVFALAPGCGTTS DSTVALGCLVSGYFPEPVKVS WN S G S L T	
IgG-Fc/C1q	(1)	---ASTTAPKVFALAPGCGTTS DSTVALGCLVSGYFPEPVKVS WN S G S L T	
IGHC1	(1)	---ASTTAPKVFALAPGCGTTS DSTVALGCLVSGYFPEPVKVS WN S G S L T	
Consensus	(151)	ASTTAPKVFALAPGCGTTS DSTVALGCLVSGYFPEPVKVS WN S G S L T	
		201	250
Native IL-2	(150)	-----	
IL-2	(150)	-----	
IL-2-Ig	(201)	SGVHTFPSVLQSSGFYSLSSMVTVPASTWTSETYICNVVHAASNFKVDKR	
IgG-Fc/C1q	(48)	SGVHTFPSVLQSSGFYSLSSMVTVPASTWTSETYICNVVHAASNFKVDKR	
IGHC1	(48)	SGVHTFPSVLQSSGFYSLSSMVTVPASTWTSETYICNVVHAASNFKVDKR	
Consensus	(201)	SGVHTFPSVLQSSGFYSLSSMVTVPASTWTSETYICNVVHAASNFKVDKR	
		251	300
Native IL-2	(150)	-----	
IL-2	(150)	-----	
IL-2-Ig	(251)	IEPIPDNHQKVCDSKCPKCPAPELEGGPSVFIFPPNPKDTLMITRTPEV	
IgG-Fc/C1q	(98)	IEPIPDNHQKVCDSKCPKCPAPELEGGPSVFIFPPNPKDTLMITRTPEV	
IGHC1	(98)	IEPIPDNHQKVCDSKCPKCPAPELEGGPSVFIFPPNPKDTLMITRTPEV	
Consensus	(251)	IEPIPDNHQKVCDSKCPKCPAPELEGGPSVFIFPPNPKDTLMITRTPEV	
		301	350
Native IL-2	(150)	-----	
IL-2	(150)	-----	
IL-2-Ig	(301)	TCVVVDVSQENPDVKFNWYMDGVEVRTATTRPKEEQFNSTYRVVSVLRIQ	
IgG-Fc/C1q	(148)	TCVVVDVSQENPDVKFNWYMDGVEVRTATTRPKEEQFNSTYRVVSVLRIQ	
IGHC1	(148)	TCVVVDVSQENPDVKFNWYMDGVEVRTATTRPKEEQFNSTYRVVSVLRIQ	
Consensus	(301)	TCVVVDVSQENPDVKFNWYMDGVEVRTATTRPKEEQFNSTYRVVSVLRIQ	

Figure 3.2 con't.

		351	400
Native IL-2	(150)	-----	-----
IL-2	(150)	-----	-----
IL-2-Ig	(351)	HQDWLSGKAFAFACAVNNQALPQPIERTITKTKGRSQEPQVYVLAPHPDELS	
IgG-Fc/C1q	(198)	HQDWLSGKAFAFACAVNNQALPQPIERTITKTKGRSQEPQVYVLAPHPDELS	
IGHC1	(198)	HQDWLSGKEFKCKVNNQALPQPIERTITKTKGRSQEPQVYVLAPHPDELS	
Consensus	(351)	HQDWLSGK F C VNNQALPQPIERTITKTKGRSQEPQVYVLAPHPDELS	
		401	450
Native IL-2	(150)	-----	-----
IL-2	(150)	-----	-----
IL-2-Ig	(401)	KSKVSVTCLVKDFYPPEINIEWQSNQPELETKYSTTQAQDSDGSYFLY	
IgG-Fc/C1q	(248)	KSKVSVTCLVKDFYPPEINIEWQSNQPELETKYSTTQAQDSDGSYFLY	
IGHC1	(248)	KSKVSVTCLVKDFYPPEINIEWQSNQPELETKYSTTQAQDSDGSYFLY	
Consensus	(401)	KSKVSVTCLVKDFYPPEINIEWQSNQPELETKYSTTQAQDSDGSYFLY	
		451	490
Native IL-2	(150)	-----	-----
IL-2	(150)	-----	-----
IL-2-Ig	(451)	SKLSVDRNRWQQGTTFTCGVMHEALHNHYTQKNVSKNPGK	
IgG-Fc/C1q	(298)	SKLSVDRNRWQQGTTFTCGVMHEALHNHYTQKNVSKNPGK	
IGHC1	(298)	SKLSVDRNRWQQGTTFTCGVMHEALHNHYTQKNVSKNPGK	
Consensus	(451)	SKLSVDRNRWQQGTTFTCGVMHEALHNHYTQKNVSKNPGK	

Predicted amino acid sequences (single letter code) of native equine IL-2, codon optimized equine IL-2, and an equine IL-2/Immunoglobulin heavy chain (IGHC1) fusion protein (IL-2-Ig). Codon optimization had no effect on the predicted amino acid sequence of equine IL-2. Comparison of IL-2-Ig, IGHCI following site directed mutagenesis (IgG-Fc/C1q) and IGHCI (GenBank accession number AJ300675) demonstrates relative positions of L to E along with EFKCK to AFACA substitutions to inactivate high affinity Fc receptor and C1q binding sites respectively.

Figure 3.3

Comparison Between Native IL-7 and Codon Optimized IL-7 Nucleotide Sequences

A

		1	50
Native IL-7	(1)	ATGTTCCATGTTTCTTTAGGTATATCTTTGGAATTCCTCCCCTGATCCT	
IL-7	(1)	ATGTTCCACGTGAGCTTCAGGTACATCTTCGGCATCCCCCCTGATCCT	
Consensus	(1)	ATGTTCCA GT TT AGGTA ATCTT GG AT CC CCCCTGATCCT	
		51	100
Native IL-7	(51)	TGTTCTGT TGCCAGTAGCATCGTCTGACTGTGATATTGAAGGTAAAGATG	
IL-7	(51)	GGTGTCTGTGCCCCGTGGCCAGCAGCGACTGCGACATCGAGGGCAAGGACG	
Consensus	(51)	GT CTG TGCC GT GC GACTG GA AT GA GG AA GA G	
		101	150
Native IL-7	(101)	GCAAAGAATATCAGAATGTTCTAATGATCAGCATCGATGAATGGACAGC	
IL-7	(101)	GCAAGGAGTACCAGAACGTGCTGATGATCAGCATCGACGAGCTGGACAGC	
Consensus	(101)	GCAA GA TA CAGAA GT CT ATGATCAGCATCGA GA TGGACAGC	
		151	200
Native IL-7	(151)	ATGATAGAAATTGATAGCAATTGCCTGAAATAAGAATCTAACTTTTTTAA	
IL-7	(151)	ATGATCGAGATCGACAGCAAC TGCCTGAACAACGAGAGCAACTTTCTCAA	
Consensus	(151)	ATGAT GA AT GA AGCAA TGCCTGAA AA GA AACTT TT AA	
		201	250
Native IL-7	(201)	AAACATTCA TGTGATGATAATAAGGAACCTCTGTTTTTAAATC GTGCTG	
IL-7	(201)	GAAGCACAGCTGCGACGACAACAAGGAGCCCCTGTTCC TGAACAGAGCCG	
Consensus	(201)	AA CA TG GA GA AA AAGGA CC CTGTT T AA G GC G	
		251	300
Native IL-7	(251)	CTCGCAAGATGAGGCAATTTCTTAAAATGAATATCAGTGAGGATTTCAAT	
IL-7	(251)	CCCGGAAGATGAGGCA GTTCTGAAGATGAACATCAGCGAGGAC TTCAAC	
Consensus	(251)	C CG AAGATGAGGCA TT CT AA ATGAA ATCAG GAGGA TTCAA	
		301	350
Native IL-7	(301)	CTCCACTTAGCAAGAGTTTCA CAGGGCACATTAAAGCTGTTGAACTGCAC	
IL7	(301)	CTGCACCTTGCCAGAGTGAGC CAGGGCACCTGAAGCTGCTGAACTGCAC	
Consensus	(301)	CT CAC T GC AGAGT CAGGGCAC T AAGCTG TGA ACTGCAC	
		351	400
Native IL-7	(351)	CACCAAGGGTAAAGGAAGAAAACCACCTTCTCTAGGTGAAGCCCCAACCCA	
IL-7	(351)	CACCAAGGGCAAGGGCAGGAAGCCCCCAGCCTGGGCGAGGCCCAAGCCCA	
Consensus	(351)	CACCAAGGG AA GG AG AA CC CC CT GG GA GCCCA CCCA	
		401	450
Native IL-7	(401)	CTAAGAAATTGGAAGAGAAATAATCTTTAAAGGAACAGAA AAAACAGAA T	
IL-7	(401)	CCAAGAACTTGGAGGAGAAACAAGAGCC TGAAGGAGCAGAA GAAGCAGAAC	
Consensus	(401)	C AAGAA TGGA GAGAA AA T AAGGA CAGAA AA CAGAA	
		451	500
Native IL-7	(451)	GACTTGTGTTTCCTAAAGATACTACTACAAAAGATAAAAAC TTGT TGGAA	
IL-7	(451)	GACCTGTGCTTTCCTGAAGATCTGCTGCAGAAGATCAAGACCTGCT TGGAA	
Consensus	(451)	GAC TGTG TTCCT AAGAT CT CT CA AAGAT AA AC TG TGGAA	
		501	531
Native IL-7	(501)	TAAAATTTTGAGGGACGCTAAAGAACACTGA	
IL-7	(501)	CAAGATCTTGAGGGACGCCAAGGAGCACTGA	
Consensus	(501)	AA AT TGAGGGACGC AA GA CACTGA	

Figure 3.3 con't.

B

		1	50
Native IL-7	(1)	MFHVSFRYIFGIPPLILVLLPVASSDCDIEGKDGKEYQNVLMISIDELDS	
IL-7	(1)	MFHVSFRYIFGIPPLILVLLPVASSDCDIEGKDGKEYQNVLMISIDELDS	
Consensus	(1)	MFHVSFRYIFGIPPLILVLLPVASSDCDIEGKDGKEYQNVLMISIDELDS	
		51	100
Native IL-7	(51)	MIEIDSNCLNNE SNFFKKHSCDDNKEPLFLNRAARKMRQFLKMNISED FN	
IL-7	(51)	MIEIDSNCLNNE SNFFKKHSCDDNKEPLFLNRAARKMRQFLKMNISED FN	
Consensus	(51)	MIEIDSNCLNNE SNFFKKHSCDDNKEPLFLNRAARKMRQFLKMNISED FN	
		101	150
Native IL-7	(101)	LHLARVSQGT LKLLNCTTKGKGRKPPSLGEAQPTKNLEENKSLKEQKKQN	
IL-7	(101)	LHLARVSQGT LKLLNCTTKGKGRKPPSLGEAQPTKNLEENKSLKEQKKQN	
Consensus	(101)	LHLARVSQGT LKLLNCTTKGKGRKPPSLGEAQPTKNLEENKSLKEQKKQN	
		151	177
Native IL-7	(151)	DLCFLKILLQKIKTCWNKILRDAKEH-	
IL-7	(151)	DLCFLKILLQKIKTCWNKILRDAKEH-	
Consensus	(151)	DLCFLKILLQKIKTCWNKILRDAKEH	

(A) Nucleotide sequence. (B) Predicted amino acid sequence represented by the single letter code

Figure 3.4

Equine IL-12 p40 and p35 Fusion Constructs

A

		1	50
Native IL-12	(1)	-----ATGGGT CACCAGTGGT TGGT CTCTCC TGGTTT TCCC	
IL-12	(1)	GAATTCAGCCACC ATGGGA CACCAGTGGC TGGT GCTGAGT TGGTT C TCCC	
Consensus	(1)	ATGGG CACCAGTGG TGGT CT TGGTT TCCC	
		51	100
Native IL-12	(38)	TGGT TT TGCTGGCGTC TCC CT CATGGC CAT ATGGGA ACTGGAGAA AGAT	
OptIL12	(51)	TGGT GC TGCTGGCCAG TCC ACTGATGGC TAT TGGGAGCTGGAGAA GGAC	
Consensus	(51)	TGGT TGCTGGC TCC CT ATGGC AT TGGGA CTGGAGAA GA	
		101	150
Native IL-12	(88)	GTGTAT GTTGTAGAAT TGGATTGGTACCCTGATGCCCTGGAGAAATGGT	
OptIL12	(101)	GTGTAC GTGGTGGAGC TGGATTGGTATCCAGACGCTCCAGGCAGATGGT	
Consensus	(101)	GTGTA GT GT GA TGGATTGGTA CC GA GC CC GG GA ATGGT	
		151	200
Native IL-12	(138)	GGTCTC ACCTGCAATAC CCTGAAGAAGAGGCATCACCTGGACCTCGG	
OptIL12	(151)	GGT GCTGACATGCAACACT CCCGAGGAAGAGGGATCACTTGGACATCCG	
Consensus	(151)	GGT CT AC TGCAA AC CC GA GAAGA GG ATCAC TGGAC TC G	
		201	250
Native IL-12	(188)	CCCAGAG CAATGAGGTCTTAGGCTCTGGCAAACCTTGACCATCCAAGTC	
OptIL12	(201)	CTCAGTC CAATGAAGTGC TGGGCAGCGGCAAGACACTGACAAATTCAGGTG	
Consensus	(201)	C CAG CAATGA GT T GGC GGCAA AC TGAC AT CA GT	
		251	300
Native IL-12	(238)	AAAGAGTTTGGAGATGCTGGC TGGTACACCTGT CACAAGGAGGC GAGGT	
OptIL12	(251)	AAGGAGTTTGGGGATGCC GGA TGGTACACCTGC CAT AAGGGAGGA GAGGT	
Consensus	(251)	AA GAGTTTGG GATGC GG TGGTACACCTG CA AA GGAGG GAGGT	
		301	350
Native IL-12	(288)	TCTGAG CCATTCTCACCTGCTGCTTCACAAGAAAGGAAGATGGAATTTGGT	
IL-12	(301)	GCTGTC CCACAGTCACCTGCTGCTGCACAAGAAAGAGGATGGGATTTGGT	
Consensus	(301)	CTG CCA TCACCTGCTGCT CACAAGAA GA GATGG ATTTGGT	
		351	400
Native IL-12	(338)	CCACTGAC ATTTTAAAAGAC CAGAAAGAATCCAA AATAAGACCTTTCTA	
IL-12	(351)	CCACTGAT ATTTCTGAAAGAT CAGAAAGAATCCAA GATAAGACATTTCTG	
Consensus	(351)	CCACTGA ATT T AAAGA CAGAA GAATCCAA AATAAGAC TT CT	
		401	450
Native IL-12	(388)	AAATGTGAGGCCAAAGAAAT TATTCGGACGT TTCACATGCTGGTGGCTGAC	
IL-12	(401)	AAATGTGAGGCCAAAAAC TATAGCGGAAGG TTCACTTGT TGGTGGCTGAC	
Consensus	(401)	AAATGTGAGGC AA AA TAT CGGA G TTCAC TG TGGTGGCTGAC	
		451	500
Native IL-12	(438)	AGCAATCAG TACTGATT TGAAATTCAGTGT CAAAAGCAGCAGAGGTTTCT	
IL-12	(451)	TGCCATCTC TACTGATCTGAA GTTTTCCGTGAAGTCCAGCCGGAAGCA	
Consensus	(451)	GC ATC TACTGAT TGAA TT GT AA CAGC G GG C	
		501	550
Native IL-12	(488)	CTGACCCCCGAGGGGTGACGTGTGGAGCAGCGACACTCTCCGCAGAGAGG	
IL-12	(501)	GC GAT CCTAGGGAGTGACATGTGGGGCCGCCACTCTGTCTGCCGAGAGG	
Consensus	(501)	GA CC G GG GTGAC TGTGG GC GC AC CT TC GC GAGAGG	
		551	600
Native IL-12	(538)	GT CAGC GTGGACGACAGGGAGTA TAAGAA GTACACGGTGGAGTGT CAGGA	
IL-12	(551)	GTGTCT GTGGACGACCGGGAGTA CAAGAA ATACACTGTGGAGTGT CAGGA	
Consensus	(551)	GT GTGGACGAC GGGAGTA AAGAA TACAC GTGGAGTGT CAGGA	

Figure 3.4 con't.

		601		650
Native IL-12	(588)	GGGCAGTGCCTGCCCCGCCGCCGAGGAGAGCCTGCCATTGAGATCGTGG		
IL-12	(601)	GGGGAGTGCCTGTCCC GCCGCCGAGAGAGCCTGCCAATCGAAATCGTGG		
Consensus	(601)	GGG AGTGC TG CC GCCGCCGAGAGAGCCTGCC AT GA ATCGTGG		
		651		700
Native IL-12	(638)	TGGATGCTGTTTACAAGCTCAAGTATGAAAACTACACCAGCGGCTTCTTC		
IL-12	(651)	TGGATGCTGTGCATAAGCTGAAGTATGAAAAATATACCTCTGGCTTCTTT		
Consensus	(651)	TGGATGCTGT CA AAGCT AAGTATGAAAA TA ACC GGCTTCTT		
		701		750
Native IL-12	(688)	ATCAGGGACATCATCAAACAGACCCGCCAAGAACCTGCAGCTGAAAGCC		
IL-12	(701)	ATCCGGGATATTATCAAGCCTGACCCCCCTAAGAACCTGCAGCTGAAACC		
Consensus	(701)	ATC GGGA AT ATCAA CC GACCC CC AAGAACCTGCAGCTGAA CC		
		751		800
Native IL-12	(738)	ATTAAAGAATTCTCGGCAGGTGGAGGTGAGCTGGGGAGTACCCGAGACCT		
IL-12	(751)	TCGAAGAATAGCCGCAGGTGGAGGTGAGTGGGGAATATCCAGAGACTT		
Consensus	(751)	T AAGAAT CG CAGGTGGA GT AG TGGGA TA CC GAGAC T		
		801		850
Native IL-12	(788)	GGAGCACCCACATTCCCTACTTCTCCCTGACATTTCTCTATTTCAGGTCAG		
IL-12	(801)	GGAGCACCCACATAGTTATTTCTCTCTGACTTTTAGTATTTCAGGTGCAG		
Consensus	(801)	GGAGCACCCACAT TA TTCTC CTGAC TT TATTTCAGGT CAG		
		851		900
Native IL-12	(838)	GGCAAGAACAAGAAAGGAAAGGAAAGACAGACTCTTTCATGGATGAGACTTC		
IL-12	(851)	GGCAAGAACAAGAAAGAGAGAAAGGATAGGCTGTTCATGGATGAGACATC		
Consensus	(851)	GGCAAGAACA AA GA AG AA GA AG CT TTCATGGATGAGAC TC		
		901		950
Native IL-12	(888)	AGCCACAGTACATGCCACAAGGATGGCCAGATCCGTGTCCAAGCCAGGG		
IL-12	(901)	CGCCACTGTGACTTGCCACAAGGACGGGCAGATTAGAGTGCAGGCTCGGG		
Consensus	(901)	GCCAC GT AC TGCCACAAGGA GG CAGAT G GT CA GC GGG		
		951		1000
Native IL-12	(938)	ACCGCTACTACAGCTCATCCCTGGAGCGAATGGGCATCCGTATCCTGCACT		
IL-12	(951)	ACCGCTACTATTCTTCAGTGGAGCGAGTGGGCTTCTGTGAGCTGCTCT		
Consensus	(951)	ACCGCTACTA TC TGGAGCGA TGGGC TC GT CTGC T		
		1001		1050
Native IL-12	(988)	GGTGGCGGTGGCTCGGGCGGTGGTGGATCCGTGGCGGCGGATCTAGGAA		
IL-12	(1001)	GGCGGCGGAGGATCTGGAGGGGGCGGAGCGCGGCGGAGGCTCCCGAA		
Consensus	(1001)	GG GGCGG GG TC GG GG GG GG CGG GGCGG GG TC GGAA		
		1051		1100
Native IL-12	(1038)	CCTCCCCACAGCCACACCAGGCCAGGAATGTTTCAGTGCCTCAACCACT		
IL-12	(1051)	CCTGCTTACAGCTACCCCTGGACCAGGCATGTTTCAGTGTCTGAATCATA		
Consensus	(1051)	CCT CC ACAGC AC CC GG CCAGG ATGTT CAGTG CT AA CA		
		1101		1150
Native IL-12	(1088)	CCCAAACTGCTGTGAGGACCGTACGCAACACGCTTCAAGAGGCCAGGCAA		
IL-12	(1101)	GTCAAGATCTGCTGTGAGGACGTAGCAACACTCTGCAAGAGGCCAGACAG		
Consensus	(1101)	CA AA CTGCTGTGAGGAC GT AGCAACAC CT CAGAAGGCCAG CA		
		1151		1200
Native IL-12	(1138)	ACCCTAGAATTCTACTCTTGCACCTCTGAAGAGATCGATCATGAGGATAT		
IL-12	(1151)	ACCCTGAGTTTTACTCTTGCACATCCGAGGAGATCGATCATGAAGACAT		
Consensus	(1151)	ACCCT GA TT TACTC TGCAC TC GA GAGATCGATCATGA GA AT		
		1201		1250
Native IL-12	(1188)	CACAAAAGACAAGAGCAGCACCGTGGCGCCTGCCTCCCCCTGGAACCTCG		
IL-12	(1201)	CACAAAAGACAAGTCTAGCACGTGGCGCCTGCCTGCCACTGGAACCTGG		
Consensus	(1201)	CACAAA GACAAG AGCAC GTGGC GCCTGCCT CC CTGGAAC T G		

Figure 3.4 con't.

		1251		1300
Native IL-12	(1238)	CCCCAACGAGAGT	TGCC	CTGGCTTC
IL-12	(1251)	CCCCAACGAGTCC	TGT	CTGGCTTC
Consensus	(1251)	CCCC AACGAG	TG CTGGCTTC	AG GAGATC TTT AT AC AAT
		1301		1350
Native IL-12	(1288)	GGGAGT	TGCCTGAC	CCCCGGAAAGGC
IL-12	(1301)	GGATCC	TGCCTGACTCC	AGGCAAAGC
Consensus	(1301)	GG	TGCCTGAC CC GG AA GC	TTC ATGATGAC CTGTG CT
		1351		1400
Native IL-12	(1338)	TAGCAG	CATCTATGAGGAC	TGAAGATGTAC
IL-12	(1351)	GTCTTC	CATCTATGAGGAC	TGAAGATGTAT
Consensus	(1351)		CATCTATGAGGAC	TGAAGATGTA CAGGTGGAGTTCAA GCCA
		1401		1450
Native IL-12	(1388)	TGAATGCCAAGCTGT	TGAT	AGATCCTCAGAGG
IL-12	(1401)	TGAATGCCAAGCTG	CTGAT	TGACCCACAGCGC
Consensus	(1401)	TGAATGCCAAGCTG	TGAT GA CC CAG G	CAGATCTTTCTGGA GAG
		1451		1500
Native IL-12	(1438)	AACATGCTGACAGCCAT	TGACAAGCTGATGC	CAGGCCCTGAACTTCAACAG
IL-12	(1451)	AACATGCTGACTGCCAT	CGACAAGCTGATGC	CAGGCCCTGAACTTCAACAG
Consensus	(1451)	AACATGCTGAC GCCAT	GACAAGCTGATGC	CAGGCCCTGAACTT AACAG
		1501		1550
Native IL-12	(1488)	TGAGACT	TGTGCCACAAAGGCC	TCCCTTGAAGGACTGGATTTTTAT
IL-12	(1501)	TGAAACAGTGCCT	TCAGAAAGCCT	TCCCTGGAGGGCCTGGATTTTTAT
Consensus	(1501)	TGA AC GTGCC CA AAGCC	TCCCT GA GG CTGGATTTTTTA	AAAA
		1551		1600
Native IL-122	(1538)	CTAAAGTCAAGCTCTGCAT	CTTCTTCATGCCTTC	CAGAATCCGCGCAGTG
IL-12	(1551)	CTAAAGTGAACTGTGCAT	CTTGCTGCATGCCTTC	CGCATTAGAGCTGTG
Consensus	(1551)	CTAAAGT AA CT TGCAT CT CT	CATGCCTTC G AT G GC GTG	
		1601		1647
Native IL-12	(1588)	ACCATCAACAGGATGATGGGCTA	TCTGAATGCTTCCTAA	-----
IL-12	(1601)	ACCATCAATAGAATGATGGGCTA	CCTGAATGCTTCCTGAGCGGCCGC	
Consensus	(1601)	ACCATCAA AG ATGATGGGCTA	CTGAATGC TCCT A	

(A) Nucleotide sequence of a published equine IL-12 p40/p35 fusion construct (GenBank Accession Number AF401989) compared with equivalent codon optimized (Opt) sequences. Predicted amino acid sequences (single letter code) demonstrating codon optimization had no effect on the predicted amino acid sequence of the equine IL-12 fusion protein.

Figure 3.5

Comparison Between Native and Codon Optimized Equine IL-18 Nucleotide Sequences

A

		1		50
Native IL-18	(1)	-----ATGGCTGCTGGACCAGTAGAAGACAATTGCATTAGCT		
IL-18	(1)	GAATTCAGCCACCATGGACGCCATGAAGCGCGCCTGTGCTGCGTGTCTGC		
Consensus	(1)	ATGG GC G TGC T		
		51		100
Native IL-18	(38)	TGGTGGAAATGAAATTATTGACAACACACTTTACTTTGTAGCTGAAAAC		
IL-18	(51)	TGCTGTGTGGCGCCGTGTGTTGTGTCCTATCTCAGGAAATCCACGCCCCG		
Consensus	(51)	TG TG T TTG C CA T A T G C		
		101		150
Native IL-18	(88)	GATGAAACCTTGAATCAGATTACTTTGGCAGGCTTGAACCTAAACTCTC		
IL-18	(101)	TTCAGGAGGGGAGCTAGATACTTCGGCAGACTGGAGCCTAAGCTGAGCAT		
Consensus	(101)	A G A A T C G G C		
		151		200
Native IL-18	(138)	AATCATACGAAATTTGAACGACCAAGTTCCTCTCATTTAACAGGGAAATC		
IL-18	(151)	CATTAGGAATCTGAACGACAGGTGCTGTTCATCAACCAGGGCAACCGAGC		
Consensus	(151)	AT A AC A T TC TCA A A C		
		201		250
Native IL-18	(188)	AACCTGTGTTTTGAGGATATGCCTGATTCTGATTGTACAGACAACGGCACCC		
IL-18	(201)	CTGTGTTTCGAGGACATGCCCCGACGCGACTGCACCGACAATGCCCTCTCAG		
Consensus	(201)	T GA A C C C		
		251		300
Native IL-18	(238)	CAGACCGTATTTATCATATATATGTATAAAGATAGCCTCACTAGAGGTCT		
IL-18	(251)	ACCGTGTATTATTTATCTATATGTATAAGGATTCCCTGACCAAGGCTGGC		
Consensus	(251)	T TTATC A A CA G		
		301		350
Native IL-18	(288)	AGCGGTAAACCATCTCTGTGAAGTGTGAGAAAAAGTCTACTCTCTCTGTGA		
IL-18	(301)	CGTGACTATCTCCGTGAAATGCGAGAAGACTTCCACCCTGAGCTGCAAGA		
Consensus	(301)	G G A C C AGA C C CT C A		
		351		400
Native IL-18	(338)	AGAACAAAATTATTTTCTTTAAGGAAATGAGTCCTCCTGAGAATATCAAT		
IL-18	(351)	ACAAGATCATCTCTTTTAAAGAGATGAGTCCACTGAGAACATTAACGAT		
Consensus	(351)	A AA A AT TT AG A CCT A A TA C AT		
		401		450
Native IL-18	(388)	GATGAAGGAAATGACATCATATTCTTTTCAGAGAAGTGTTCCAGGACATGA		
IL-18	(401)	GAAGGCAACGATATTATCTTCTTTTCAGAGATCTGTGCCAGGACACGACGA		
Consensus	(401)	GA G AT ATC T TT A A GA		
		451		500
Native IL-18	(438)	TGATAAGATACAGTTTGTAGTCTTCACTGTATAAAGGATACTTTCTAGCTT		
IL-18	(451)	CAAGATCCAGTTCGAATCTTCCTGTACAAGGATATTTTCTTGGCCTGCG		
Consensus	(451)	A A TC A A T T		
		501		550
Native IL-18	(488)	GTGAAAAGAGAATGATCTTTTCAAACTCATTTTGAAGAGAAAGGATGAA		
IL-18	(501)	AGAAGGAGAACGACCTGTTCAGCTGATTCTGAAGGAGAAAGGACGAGAAC		
Consensus	(501)	A A A A T T T G A A A GA A		
		551		600
Native IL-18	(538)	AATGGGGATAAATCTGTAATGTTCACTGTTCAAAACCAAGAACTAG-----		
IL-18	(551)	GGCGATAAATCTGTGATGTTTACAGTGCAGAACCAAGAACTGAGCGGCCCGC		
Consensus	(551)	G A T T A A A G		

Figure 3.5 con't.

		1		50
Native IL-18	(1)	-----ATGGCTGCTGGACCAGTAGAAGACAATTGCATTAGCT		
IL-18	(1)	GAATTCAGCCACCATGGACGCCATGAAGCGCGCCTGTGCTGCGTGTCTGC		
Consensus	(1)	ATGG GC G TGC T		
		51		100
Native IL-18	(38)	TGGTGGAATGAAATTATTGACAACACACTTTACTTTGTAGCTGAAAAC		
IL-18	(51)	TGCTGTGTGGCGCCGTGTGTTGTGTCCTATCTCAGGAAATCCACGCCCCG		
Consensus	(51)	TG TG T TTG C CA T A T G C		
		101		150
Native IL-18	(88)	GATGAAACCTTGAATCAGATTACTTTGGCAGGCTTGAACCTAAACTCTC		
IL-18	(101)	TTCAGGAGGGGAGCTAGATACTTCGGCAGACTGGAGCCTAAGCTGAGCAT		
Consensus	(101)	A G A A T C G G C		
		151		200
Native IL-18	(138)	AATCATACGAAATTTGAACGACCAAGTTCCTTCATTAAACCAGGGAAATC		
IL-18	(151)	CATTAGGAATCTGAACGACAGGTGCTGTTCATCAACCAGGGCAACCGAGC		
Consensus	(151)	AT A AC A T TC TCA A A C		
		201		250
Native IL-18	(188)	AACCTGTGTTTTGAGGATATGCCTGATTCTGATTGTACAGACAACGGCACCC		
IL-18	(201)	CTGTGTTTCGAGGACATGCCCCGACAGCGACTGCACCGACAATGCCCTCAG		
Consensus	(201)	T GA A C C C		
		251		300
Native IL-18	(238)	CAGACCGTATTTATCATATATATGTATAAAGATAGCCTCACTAGAGGTCT		
IL-18	(251)	ACCGTGTATTATTATCTATATGTATAAGGATTCCCTGACCAGAGGCGCTGGC		
Consensus	(251)	T TTATC A A CA G		
		301		350
Native IL-18	(288)	AGCGGTAACCATCTCTGTGAAGTGTGAGAAAAAGTCTACTCTCTCTGTGA		
IL-18	(301)	CGTGACTATCTCCGTGAAATGCGAGAAGACTTCCACCCTGAGCTGCAAGA		
Consensus	(301)	G G A C C AGA C C CT C A		
		351		400
Native IL-18	(338)	AGAACAAAATTATTTCTTTAAAGGAAATGAGTCCTCCTGAGAATATCAAT		
IL-18	(351)	ACAAGATCATCTCTTTTAAAGAGATGAGTCCAAGTGAAGACATTAACGAT		
Consensus	(351)	A AA A AT TT AG A CCT A A TA C AT		
		401		450
Native IL-18	(388)	GATGAAGGAAATGACATCATATTCTTTTCAGAGAAGTGTTCAGGACATGA		
IL-18	(401)	GAAGGCAACGATATTATCTTCTTTTCAGAGATCTGTGCCAGGACACGACGA		
Consensus	(401)	GA G AT ATC T TT A A GA		
		451		500
Native IL-18	(438)	TGATAAGATACAGTTTGTAGTCTTCACTGTATAAAGGATACTTTCTAGCTT		
IL-18	(451)	CAAGATCCAGTTCGAATCTTCCTGTACAAGGGATATTTTCTGGCCTGCG		
Consensus	(451)	A A TC A A T T		
		501		550
Native IL-18	(488)	GTGAAAAGAGAATGATCTTTTCAAACTCATTTTGAAGAAAAGGATGAA		
IL-18	(501)	AGAAGGAGAACGACCTGTCAAGCTGATTCTGAAGGAGAAAGGACGAGAAC		
Consensus	(501)	A A A A T T T G A A A GA A		
		551		600
Native IL-18	(538)	AATGGGGATAAATCTGTAATGTTCACTGTTCAAAACCAAGAACTAG-----		
IL-18	(551)	GGCGATAAATCTGTGATGTTTACAGTGCAGAACCAAGAACTGAGCGGCCCGC		
Consensus	(551)	G A T T A A A G		

Figure 3.5 con't.

B

Native IL-18 IL-18	MAAGPVEDNCISLVEMKFIIDNTLYFVAENDENLESDYFGRLEPKLSIIRN -MDAMKRGLCCVLLLCGAVFVSPSQEIHARFRRGARFYFGRLEPKLSIIRN
Native IL-18 IL-18	LNDQVLFINQGNQPVFEDMPDSDCTDNAPQTVFIIYMYKDSLTRGLAVTI LNDQVLFINQGNQPVFEDMPDSDCTDNAPQTVFIIYMYKDSLTRGLAVTI
Native IL-18 IL-18	SVKCEKTSTLSCKNKIISFKEMSPPENINDEGNDIIFQRSVPGHDDKIQ SVKCEKTSTLSCKNKIISFKEMSPPENINDEGNDIIFQRSVPGHDDKIQ
Native IL-18 IL-18	FESSLYKGYFLACEKENDLFLKLILKEKDENGDKSVMFTVQNQN FESSLYKGYFLACEKENDLFLKLILKEKDENGDKSVMFTVQNQN

(A) Nucleotide sequence. (B) Predicted amino acid sequence represented by the single letter code.

Figure 3.6

Comparison Between Native Equine IL-15 and Codon Optimized Equine IL-15 Nucleotide Sequences

A

		1		50
Native IL15	(1)	ATGA GAATT TT GAAACCAT ATTT GAGA AGTACT TGCATCCAGTGCTACTT		
IL15	(1)	---- GAATTC - GCCACCAT GGAC GCGA ----- TGAAGCGGGGGCT - CTG		
Consensus	(1)	GAATT G ACCAT G GA TG A C G GCT CT		
		51		100
Native IL15	(51)	GTGTTTACTTCTGAACAGTCATTTT TTAACT GAGGCTGGCATTTCATGTCT		
IL15	(39)	CTGTGTGTGTC ----- TCCTGTGT ----- GGAGCTGTC - TTCGTGAGT		
Consensus	(51)	TGT T T C TC T T T G GCTG C TTC TG T		
		101		150
Native IL15	(101)	TCATTTTGGGCTGTATCAGTGCAGGTCTTCCTAAAA CAGAG GCAA ACT CTGG		
IL15	(76)	CC - TTCCCA GGAGAT CCAC CGCT AGGT TCCG CCGCGGCGCCCGCAA - CTGG		
Consensus	(101)	C TT G G CA AGGT C C GCAA CTGG		
		151		200
Native IL15	(151)	CAGGATGTAATAAGTGATTGAAAAGAATTGAAGATCTTATTCATCTAT		
IL15	(124)	CAGGACGTGATT TTCC GATCTCAAGCGTATCGAGACCTCATCCAGTCTAT		
Consensus	(151)	CAGGA GT AT GAT T AA G AT GA GA CT AT CA TCTAT		
		201		250
Native IL15	(201)	ACATGTTGATGCCACTTTATATACTGAAAGCGATGCTCATCCCAGTTGCA		
IL15	(174)	TCATGTGATGCCACCCGTACACCGAGTCCGACGCGCACCCCAGCTGCA		
Consensus	(201)	CATGT GATGCCAC T TA AC GA CGA GC CA CCCAG TGCA		
		251		300
Native IL15	(251)	AAGTAGCAGCGATGAAGTGCTTTCTCCTGGAGTTACATGTTATTTTGCA T		
IL15	(224)	AGGTGCCGCCATGAAGTGCTTCTGCTGGAGCTGCACGTGATCCTGCA C		
Consensus	(251)	A GT GC GC ATGAAGTGCTT CT CTGGAG T CA GT AT TGCA		
		301		350
Native IL15	(301)	GAGTCAGAAACGAGGACATTAAGGAAACAGTAGAAACCTTATCATCCT		
IL15	(274)	GAGAGCAGAAATGAAGACATCAAGAGACGGTCGAGAACCTGATCATCCT		
Consensus	(301)	GAG CAGAAA GA GACAT AA GA AC GT GA AACCT ATCATCCT		
		351		400
Native IL15	(351)	AGCAAACAGCAGCTTATCTCTAATGGGAATGTTACAGAATCTGGATGCA		
IL15	(324)	GGCCAACAGCCGGCTGAGCTCGAACGGCAACGTACTGAGTCAGGCTGCA		
Consensus	(351)	GC AACAGC G T TC AA GG AA GT AC GA TC GG TGCA		
		401		450
Native IL15	(401)	AAGAGTGTGAGGAACTGGAGGAAAAAAATATTAAGAATTTTGCAGAGT		
IL15	(374)	AGGAGTGCAGGAACTGGAGGAAAAAAACATAAAGAATTCCCTCAGTCT		
Consensus	(401)	A GAGTG GAGGAACTGGAGGAAAA AA AT AA GAATT T CAG T		
		451		486
Native IL15	(451)	TTTGTACATATCGTGCAAATGTTTCATCAACCCCTCT		
IL15	(424)	TTTGTGCAATCGTTCAAATGTTTCATCAATCCGTCC		
Consensus	(451)	TTTGT CA ATCGT CAAATGTTTCATCAA CC TC		

Figure 3.6 con't.

B

		1		50
Native IL15	(1)	MRI <u>IKPYLRSTCIQCYLC</u> LLINSHFLTE <u>AG</u> IHVFI LGCISAGLP <u>KTE</u> <u>AMW</u>		
IL15ss	(1)	---MDAMKRGLCCVLLLCGAVFVSPS-QE-----IHARFRRGARNW		
IL15sr	(1)	---MDAMKRGLCCVLLLCGAVFVSPS-QE-----IHARFRRGARNW		
Consensus	(1)	MDAMKRGLCCVLLLCGAVFVSPS QE IHARFRRGARNW		
		51		100
Native IL15	(51)	QDVISDLKRIEDLIQSIHV DATLYTESDAHP SCKVAAMKCF LLELHVILH		
IL15ss	(38)	QDVISDLKRIEDLIQSIHV DATLYTESDAHP SCKVAAMKCF LLELHVILH		
IL15sr	(38)	QDVISDLKRIEDLIQSIHV DATLYTESDAHP SCKVAAMKCF LLELHVILH		
Consensus	(51)	QDVISDLKRIEDLIQSIHV DATLYTESDAHP SCKVAAMKCF LLELHVILH		
		101		150
Native IL15	(101)	ESRNEDIKETVENLIILANS <u>SL</u> SSNGNVTESGCKECEEELEEKNIKEFLQS		
IL15ss	(88)	ESRNEDIKETVENLIILANS <u>SL</u> SSNGNVTESGCKECEEELEEKNIKEFLQS		
IL15sr	(88)	ESRNEDIKETVENLIILANS <u>RL</u> SSNGNVTESGCKECEEELEEKNIKEFLQS		
Consensus	(101)	ESRNEDIKETVENLIILANS <u>SL</u> SSNGNVTESGCKECEEELEEKNIKEFLQS		
		151		162
Native IL15	(151)	FVHIVQMFINPS		
IL15ss	(138)	FVHIVQMFINPS		
IL15sr	(138)	FVHIVQMFINPS		
Consensus	(151)	FVHIVQMFINPS		

(A) Nucleotide sequences for only the SSLSS variants are shown. (B) Predicted amino acid sequence represented by the single letter code for Native IL15 and both SRLSS and SSLSS variants of IL15. The two potential cleavage sites within the signal peptide of Native IL-15 are italicized and underlined.

Figure 3.7

Amino Acid Content of Codon Optimized IL-15 Constructs

		1	50
IL15	(1)	MDAMKRGLCCVLLLCGAVFVSPSQEIHARFRRGAR	-----
Sushi IL15	(1)	MDAMKRGLCCVLLLCGAVFVSPSQEIHARFRRGARSITCPTPTSVEHADI	
Sushi IL15 L+1	(1)	MDAMKRGLCCVLLLCGAVFVSPSQEIHARFRRGARSITCPTPTSVEHADI	
Sushi IL15 L+2	(1)	MDAMKRGLCCVLLLCGAVFVSPSQEIHARFRRGARSITCPTPTSVEHADI	
Consensus	(1)	MDAMKRGLCCVLLLCGAVFVSPSQEIHARFRRGARSITCPTPTSVEHADI	
		51	100
IL15	(36)	-----	-----
Sushi IL15	(51)	RVKSYNLSSKERYICNSGFKRKAGTSSLTECVLNETTNLAHWTTPNLKCI	
Sushi IL15 L+1	(51)	RVKSYNLSSKERYICNSGFKRKAGTSSLTECVLNETTNLAHWTTPNLKCI	
Sushi IL15 L+2	(51)	RVKSYNLSSKERYICNSGFKRKAGTSSLTECVLNETTNLAHWTTPNLKCI	
Consensus	(51)	RVKSYNLSSKERYICNSGFKRKAGTSSLTECVLNETTNLAHWTTPNLKCI	
		101	150
IL15	(36)	-----NWQDVISDLKRIEDLIQSIHVDATLYTE	
Sushi IL15	(101)	SGGSGGGGSGGGSGGGGS--LQNWQDVISDLKRIEDLIQSIHVDATLYTE	
Sushi IL15 L+1	(101)	SGGSGGGGSGGGSGGGGS--LQNWQDVISDLKRIEDLIQSIHVDATLYTE	
Sushi IL15 L+2	(101)	SGGSGGGGSGGGSGGGGSGLQNWQDVISDLKRIEDLIQSIHVDATLYTE	
Consensus	(101)	SGGSGGGGSGGGSGGGGSGLQNWQDVISDLKRIEDLIQSIHVDATLYTE	
		151	200
IL15	(64)	SDAHPSCKVAAMKCFLLELHVILHESRNEDIKETVENLIILANSSLSSNG	
Sushi IL15	(149)	SDAHPSCKVAAMKCFLLELHVILHESRNEDIKETVENLIILANSSLSSNG	
Sushi IL15 L+1	(150)	SDAHPSCKVAAMKCFLLELHVILHESRNEDIKETVENLIILANSSLSSNG	
Sushi IL15 L+2	(151)	SDAHPSCKVAAMKCFLLELHVILHESRNEDIKETVENLIILANSSLSSNG	
Consensus	(151)	SDAHPSCKVAAMKCFLLELHVILHESRNEDIKETVENLIILANSSLSSNG	
		201	237
IL15	(114)	NVTESGCKECEEELEEKNIKEFLQSFVHIVQMFINPS-	
Sushi IL15	(199)	NVTESGCKECEEELEEKNIKEFLQSFVHIVQMFINPS-	
Sushi IL15 L+1	(200)	NVTESGCKECEEELEEKNIKEFLQSFVHIVQMFINPS-	
Sushi IL15 L+2	(201)	NVTESGCKECEEELEEKNIKEFLQSFVHIVQMFINPS-	
Consensus	(201)	NVTESGCKECEEELEEKNIKEFLQSFVHIVQMFINPS	

Amino acid (single letter code) content of codon optimized (SSLSS variant) of equine IL-15 (IL15), Sushi/equine IL-15 fusion construct (SushiIL15) and two extended glycine/serine rich “flexible linker” variants of Sushi/equine IL-15 (Sushi IL15 L+1, Sushi IL15 L+2)

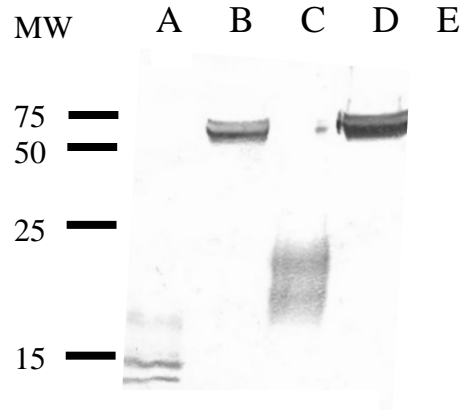
Expression of Native and Codon Optimized Versions of Equine IL-2 and Equine IL-2/IGHC1 Fusion Constructs

Culture fluids harvested from HEK 293 cells transiently transfected with native IL-2, native IL-2/Ig, IL-2 and IL-2/Ig were analyzed for the presence of IL-2 or the IL-2/IGHC1 fusion protein by immunoblotting with a polyclonal equine IL-2 antiserum. Equivalent culture fluids from mock transfected HEK 293 cells provided the negative controls (Figure 3.8). At least two protein products with a potential third more weakly stained band were detectable in cell culture fluids from HEK 293 cells transiently transfected with IL-2 (Figure 3.8). IL-2 contains two potential N-linked glycosylation sites; therefore, it is likely the lower form may constitute the non-glycosylated form of the molecule, whereas the higher apparent molecular weight species are differentially glycosylated. As expected, it appeared that higher levels of IL-2 were secreted from IL-2 transfected cells (Figure 3.8), although larger apparent molecular weights species were detected than those expressed from native equine IL-2 sequences. The diffuse staining pattern observed in the equine IL-2 species derived from the codon optimized gene suggests the presence of differential glycosylation (Figure 3.8). Therefore, the proteins secreted from HEK 293 cells transiently transfected with native IL-2 and synthetic IL-2 were treated with N-glycosidase F. In contrast to the two diffuse staining protein species detected in untreated material expressed from codon optimized IL-2, there was just a single predominant band with similar migrational characteristics to native IL-2 derived from the native equine sequences after treatment with N-glycosidase F (Figure 3.9).

Protein bands consistent with this size and reactive with the equine IL-2 antiserum were detectable in the culture fluids from native IL-2/Ig and codon optimized IL-2/Ig transfected cells (Figure 3.8). In contrast to proteins expressed from native IL-2 and codon optimized IL-2, those derived from the fusion constructs possessed very similar apparent molecular weights (Figure 3.8), suggesting mRNAs containing the IGHCI1 sequences shared the same intracellular destiny following transcription. Although the differences were not dramatic, amounts of the IL-2/Ig fusion protein secreted from codon optimized pIL-2/Ig transfected cells were consistently higher in all three separate transfection experiments (data not shown) than that from cells transfected with native pIL-2/Ig .

Figure 3.8

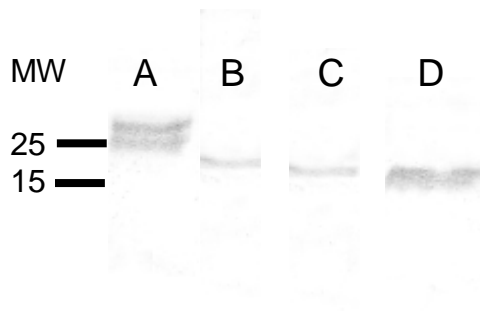
Immunoblot Analysis of Native and Codon Optimized Equine IL-2 Expression



HEK 293 cells were transfected with native pIL-2 (A), native pIL-2/Ig (B), codon optimized pIL-2 (C) and codon optimized pIL-2/Ig (D). At 72 h post transfection, cell culture fluids were harvested, processed and analyzed by immunoblot using a polyclonal equine IL-2 antiserum. Cell culture fluids from mock transfected HEK 293 cells were used as a negative control (E)

Figure 3.9

Deglycosylation of Equine IL-2 Derived from Native IL-2 and Codon Optimized Sequences



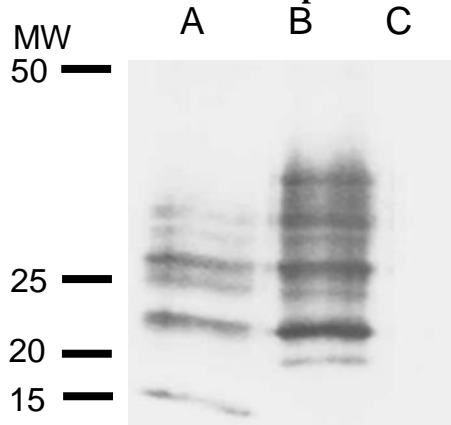
Cell culture fluids harvested at 72 h following transfection of HEK 293 cells with native or codon optimized versions of equine pIL-2 were processed, treated with PNGaseF and analyzed by immunoblotting using a polyclonal equine IL-2 antiserum. Secreted glycosylated forms of codon optimized IL-2 (A) and native IL-2 (B) were compared to PNGaseF treated native IL-2 (C) and codon optimized IL-2 (D).

Expression of Native and Codon Optimized Versions of Equine IL-7

Culture fluids harvested from HEK 293 cells transiently transfected with native pIL-7 and codon optimized pIL-7 were analyzed by immunoblotting with a polyclonal rabbit antiserum against human IL-7. The multiple protein products shown in (Figure 3.10) with apparent molecular weights in excess of 17,000 Da almost certainly represent differentially glycosylated forms of this interleukin. Although a weakly stained product with a molecular weight consistent with the non-glycosylated form of IL-7 is present in cell-culture medium from codon optimized pIL-7 transfected cells, there was no equivalent detectable product secreted from cells receiving the native sequences (Figure 3.10). Instead, a lower molecular weight product was observed (Figure 3.10). While aberrant migrational effects in PAGE might explain this result, similar findings were observed in conditioned media from each of the triplicate transfections (data not shown). In addition to reducing the potential for alternative RNA splicing events, codon optimization also appeared to promote a small increase in expression as evidenced by the relative reactivity between native IL-7 and codon optimized IL-7 conditioned media preparations to anti human IL-7 (Figure 3.10). Furthermore, as observed with the synthetic IL-2, codon optimization of IL-7 coding sequences promoted more extensive glycosylation (Figure 3.10).

Figure 3.10

Immunoblot Analysis of Native and Codon Optimized Equine IL-7 Expression.



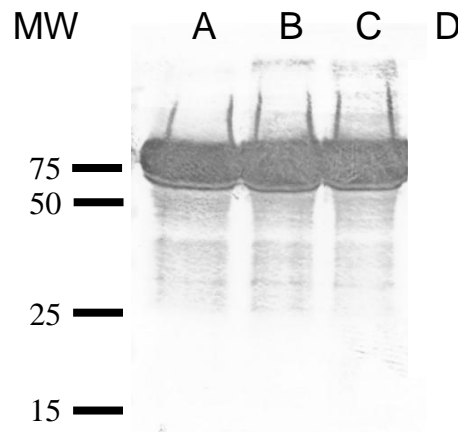
HEK 293 cells transfected with native pIL-7 (A) or codon optimized pIL-7 (B) at 72 h post transfection cell culture fluids were collected and analyzed by immunoblot using a polyclonal rabbit antiserum against human IL-7. Cell culture fluids from mock transfected HEK 293 cells were used as a negative control (C).

Expression of Codon Optimized Equine IL-12

Costs prohibited synthesis of an equivalent fusion protein comprised of native sequences for comparison purposes, but immunoblot analysis using goat anti human IL-12 antiserum demonstrated that secreted equine IL-12 was easily detectable in conditioned medium from codon optimized pIL-12 transfected cells (Figure 3.11). The migrational characteristics of the fusion protein in PAGE appeared identical to previously published results (McMonagle, Taylor et al. 2001) and were consistent with a predicted molecular weight of 57933.71 (Vector NTI) assuming removal of the putative 22 amino acid signal peptide.

Figure 3.11

Immunoblot Analysis of Codon Optimized Equine IL-12 Fusion Construct Expression



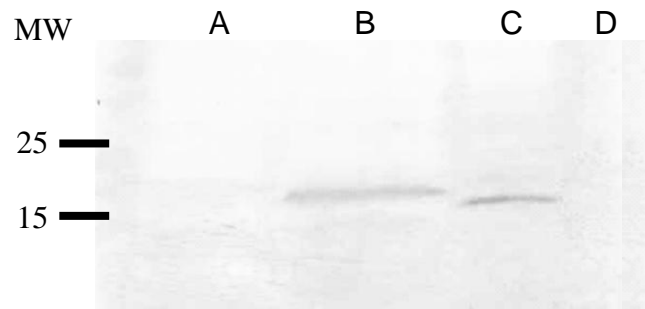
Three separate transfection experiments (A, B, C) were conducted using the codon optimized equine pIL-12 fusion construct in HEK 293 cells. At 72 h post transfection, cell culture fluids were collected and analyzed by immunoblot using a polyclonal goat antiserum against human IL-12. Cell culture fluids from mock transfected HEK 293 cells were used as a negative control (D).

Expression of Codon Optimized Equine IL-18

A lower apparent molecular weight anti IL-18 reactive species was detected in conditioned medium compared to Brefeldin A treated cell lysates by immunoblot analysis, showing that the appropriate cleavage of the TPA signal peptide occurred in proteins expressed from the codon optimized pIL-18 construct (Figure 3.12). Furthermore, the apparent molecular weights of the proteins binding IL-18 antibodies in Brefeldin A lysates and conditioned medium were consistent with sizes of approximately 22 and 18 kDa, respectively (Figure 3.12).

Figure 3.12

Immunoblot Analysis of Codon Optimized Equine IL-18 Expression



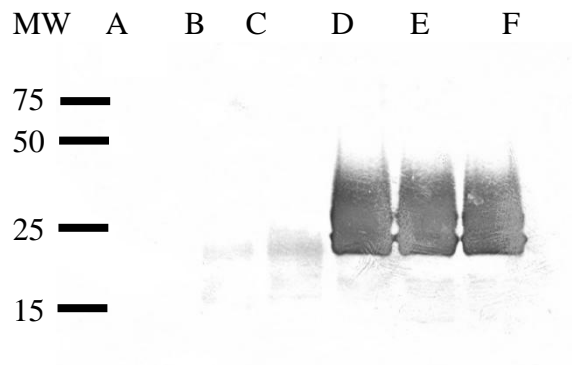
Separate transfections of HEK 293 cells with codon optimized pIL-18 were conducted with and without the addition of Brefeldin A. At 72 h post transfection, lysates from Brefeldin A treated cells (B) and culture fluids from untreated cells (C) were collected and analyzed by immunoblot using a polyclonal goat antiserum against human IL-18. Cell lysates from mock transfected Brefeldin A treated HEK 293 cells (A) and culture fluids untreated mock transfected cells were used as negative controls (D).

Expression of Equine IL-15 Based Molecular Clones

Culture fluids harvested from HEK 293 cells transiently transfected with native pIL-15 and the codon optimized pIL-15 constructs SRLSS, SSLSS, Sushi IL-15, Sushi IL-15 L+1 and Sushi IL-15 L+2 were analyzed by immunoblotting with a polyclonal rabbit antiserum against human IL-15. There was no detectable expression following transient transfection of HEK 293 cells of native pIL-15, as determined by immunoblot analysis of either Brefeldin A treated cell lysates (data not shown) or conditioned medium (Figure 3.13). However, a protein product was detectable in supernatants from pIL-15 (SRLSS) transfected cells (Figure 3.13). Protein expression was detected at higher levels in supernatants from the pIL-15 (SSLSS) transfected cells (Figure 3.13). The predicted molecular weight of the mature form of equine IL-15 is approximately 13 kDa (Vector NTI); therefore, it is very likely that the larger, more predominant species detected in IL-15 (SRLSS) and IL-15 (SSLSS) are glycosylated (Figure 3.13). Dramatic increases in IL-15 protein expression levels were detected by immunoblot analysis in conditioned medium from HEK 293 cells transfected with pSushi IL-15, pSushi IL-15L+1 and pSushi IL-15L+2 transfected HEK 293 cells (Figure 3.13). These 22-24 kDa protein products were also observed by Ponceau S staining of the nitrocellulose membrane following electrophoresis, but they were not observed in mock transfected cells or cells receiving native pIL-15, pIL-15 (SRLSS) or pIL-15 (SSLSS) (Figure 3.14). It is very likely that the two major species and the diffuse staining higher molecular weight species represent differentially glycosylated forms of the fusion protein. The addition of one (Sushi IL-15 L+1) or two (Sushi IL-15 L+2) additional glycine residues to the “flexible” linker did not increase the apparent expression of the sushi-equine IL-15 fusion protein (Figures 3.13, 3.14).

Figure 3.13

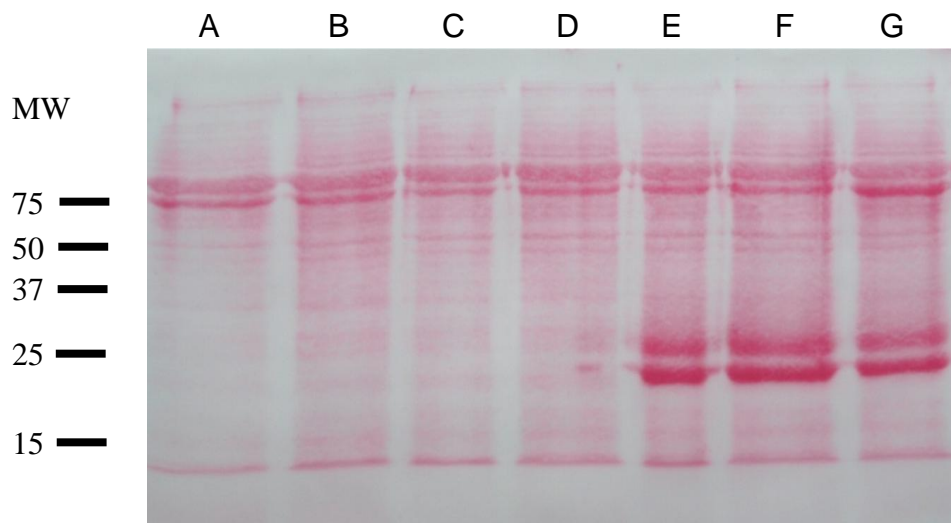
**Immunoblot Analysis of Native, Codon optimized, and Sushi Fusion Construct
Equine IL-15 Expression Levels**



HEK 293 cells were transfected with native pIL-15 (B), codon optimized pIL-15 (SRLSS) (C) codon optimized pIL-15 (SSLSS) (D), Sushi pIL-15 (E) Sushi pIL-15 L+1 (F) and Sushi pIL-15 L+2 (G). At 72 h post transfection, cell culture fluids were collected and analyzed by immunoblot using a polyclonal rabbit antiserum against human IL-15. Cell culture fluids from mock transfected HEK 293 cells were used as a negative control (A).

Figure 3.14

**Proteins Present in Conditioned Medium Following Transfection of HEK 293 Cells
with pCi Vectors Encoding IL15 or SushiIL15 Fusion Proteins**



Ponceau S stained nitrocellulose membrane showing processed conditioned medium from mock HEK 293 cells (A) or with pCi vectors encoding native IL-15 (B), codon optimized

IL-15 (SRLSS) (C) codon optimized IL-15 (SSLSS) (D), Sushi IL-15 (E) Sushi IL-15 L+1 (F) and Sushi IL-15 L+2 (G).

Biological Activity of Native and Codon Optimized Versions of Equine IL-2 and Equine IL-2/IGHC1 Fusion Constructs

The biological activity present in conditioned medium from HEK 293 cells transfected with native pIL-2, codon optimized pIL-2, native pIL-2/Ig, and codon optimized pIL-2/Ig was measured in terms of proliferative activity on horse PBMC cultures. As predicted, culture fluids from cells transfected with pIL-2 or pIL-2 stimulated significant increases in lymphocyte proliferation of equine PBMC cultures (Table 3.1). Higher proliferative activity was observed at medium dilutions of 1:10 rather than 1:4. However, it is clear from these results that codon optimization at best only resulted in an incremental rather than a wholesale improvement in the expression of equine IL-2.

The conditioned medium harvested from HEK 293 cells transiently transfected with the native equine pIL-2/Ig fusion construct induced lower proliferation levels in horse PBMC than media from cells transfected under similar conditions with native pIL-2 or codon optimized pIL-2 (Table 3.1). However, codon optimization of sequences encoding equine pIL-2 in the codon optimized pIL-2/Ig construct appeared to increase expression relative to native pIL-2/Ig (Figure 3.2), resulting in a >2-fold increase in the PBMC SI at the 1:10 dilution (Table 3.1).

Table 3.1
IL-2 Cytokine-Stimulated Proliferation of Equine PBMC in Vitro

Plasmid Construct	1:4 Dilution	1:10 Dilution
native IL-2	29.2	41.1
codon optimized IL-2	24.6	44.3
native IL-2/Ig	9.0	4.8
codon optimized IL-2/Ig	13.7	11.5

Proliferation of horse PBMC cultures in response to conditioned medium from HEK 293 cells transiently transfected with native pIL-2, codon optimized pIL-2, native pIL-2/Ig, and codon optimized pIL-2/Ig were determined by [³H]-thymidine incorporation. Results are expressed in the form of a stimulation index with culture fluids used at final dilutions of 1:4 and 1:10.

Biological Activity of Native and Codon Optimized Versions of Equine IL-7

Despite the differences in IL-7 expression levels, biological activity in terms of proliferative effects on horse PBMC cultures between native IL-7 and codon optimized IL-7 conditioned medium at dilutions of 1:10 was indistinguishable, with each construct producing SIs of 5.5.

Biological Activity of Native and Codon Optimized Versions of Equine IL-15

Conditioned medium from native equine pIL-15 transfected cells failed to induce proliferative responses in horse PBMC cultures during co-stimulation assays with concanavalin A (Table 3.2). However, low proliferative activity in equine PBMC was stimulated by conditioned medium from pIL-15 (SRLSS) transfected cells (Table 3.2). Although, in terms of protein production this codon optimized synthetic gene conferred improvements compared with native sequences, higher levels of expression were obtained (Figure 3.6) with a concomitant 5-fold increase in horse PBMC proliferative activity present in conditioned media from cells transfected with pIL-15 (SSLSS) (Table 3.2). The very high expression level observed for the sushi-equine IL-15 fusion protein was mirrored by its biological activity with proliferative activity on horse PBMC cultures, readily detectable at dilutions of 1:1280 in conditioned medium from pSushi IL-15 transfected HEK 293 cells (Table 3.2). The addition of one (Sushi IL-15 L+1) or two (Sushi IL-15 L+2) additional glycine residues to the “flexible” linker did not increase either the biological activity (Table 3.2.) of the sushi-equine IL-15 fusion protein. Therefore, increasing the linker length within the fusion protein does not appear to confer additional advantages at least as measured by the in vitro assays employed in this study.

Table 3.2**In Vitro Biological Activity of Equine IL-15 Constructs**

Dilution	IL15	15 _{SR}	15 _{SS}	Sushi15	Sushi15 _{L+1}	Sushi15 _{L+2}
1:4	1.0	2.1	ND	ND	ND	ND
1:10	1.0	1.0	2.1	4.4	4.1	4.0
1:20	1.0	1.0	2.1	6.0	5.8	5.5
1:40	ND	ND	1.6	6.0	4.8	6.1
1:80	ND	ND	1.6	6.2	7.0	6.6
1:160	ND	ND	1.3	6.6	6.0	6.9
1:320	ND	ND	1.2	5.4	5.4	5.9
1:640	ND	ND	1.0	5.6	4.8	5.4
1:1280	ND	ND	1.0	5.2	3.8	4.6

Proliferation of horse PBMC in response to addition of cell culture fluids from cells transfected with native equine pIL-15 (IL-15), pIL-15 (SRLSS) (15_{SR}), pIL-15 (SSLSS) (15_{SS}), pSushi IL-15 (Sushi15), pSushi IL-15 L+1, (Sushi15_{L+1}) and pSushi IL-15 L+2, (Sushi15_{L+2}) were determined by [³H]-thymidine incorporation. Results are expressed in the form of a stimulation index with culture fluids used at final dilutions of 1:4 to 1:1280. ND = Not Done.

3.2: Assessment of Immune Responses to DNA Vaccination in ELA-A1 Possessing Ponies

Physical Responses of ELA-A1 Ponies to Intradermal Inoculation with pSYNSU Immunizations

Injection sites were monitored for inflammatory responses in terms of redness, swelling, heat, or soreness after each intradermal inoculation with plasmid DNA vaccines. Inflammatory reactions reported during this preliminary trial were transient. At no time did inoculation with naked pSYNSU elicit physical responses in B33. Mild to moderate side effects, pertaining to heat and swelling at inoculation sites, were noted from all five ponies receiving PEI in the DNA vaccine regimen. Local reactions were more pronounced after each inoculation among recipients of the intradermal injections containing cytokines (D49, 9954, and D55), than among the two recipients receiving DNA injections without cytokines (D47 and B29). The presence of heat after immunization was determined subjectively by touching the injection sites. Heat was noted in three ponies after V1 and in all five ponies after V2 and V3 with vaccines containing PEI. While firm nodules at the injection sites were observed in all five ponies receiving vaccine regimens with PEI, DNA vaccines with cytokines caused increased swelling of the skin after each vaccination. Firm nodules were detected in ponies D47 and B29, inoculated with PEI and antigen, for three weeks after V1 and for two weeks after V2 and V3 (Table 3.3). While no redness was detected at the injection sites in these ponies, they both responded with flinching of the skin when any pressure was applied to the nodules. In contrast, ponies in the pSYNSU immunization group with IL-15 and/or IL-2/Ig exhibited increased side effects. All three ponies receiving cytokine expression plasmids developed large nodules at all of the injection sites that persisted for approximately four weeks after each inoculation (Table 3.3). The diameter of swelling at injection sites in D49 ranged from 11-53 mm after V1 and 15-80 mm after V2 and V3 (Table 3.3). Similarly, nodules approximately 65-82.5 mm in diameter were measured in ponies 9954 and D55 after each inoculation but decreased in size significantly four weeks later (Table 3.3). The swelling that formed at the injection sites caused the ponies to flinch and pin their ears back when the nodules were touched, which was indicative of

soreness from the immunizations. However, no evidence of redness or purulent discharge consistent with bacterial infections was observed at any injection site.

Table 3.3
Physical Responses of ELA-A1 Ponies to pSYNSU Immunizations

Horse	Vaccination	Duration of heat (weeks)	Swelling (mm)	Duration of swelling (weeks)
D33	V1	None	None	None
	V2	None	None	None
	V3	None	None	None
D47	V1	2	14-30	3
	V2	2	25	2
	V3	2	25	2
B29	V1	2	12-32	3
	V2	2	10-25	2
	V3	2	10-25	2
D49	V1	None	11-53	4
	V2	2	16-80	4
	V3	2	15-78	4
9954	V1	None	10-65	4
	V2	2	16-80	4
	V3	2	15-78	4
D55	V1	1	11-81	4
	V2	2	25-82.5	3
	V3	2	15-78	4

Physical responses to vaccination were monitored weekly until they completely subsided. Heat was determined by warmth of the skin to the touch. The swelling represents maximal and minimal measurements assessed in mm after each vaccination.

Immunoblot Reactivity Following pSYNSU Immunizations

As predicted from previous studies (Cook, Cook et al. 2005), antibodies were not detectable in immunoblot assays following three i.d. inoculations with ‘naked’ or unprotected SYNSU plasmid DNA (Figure 3.15). A similar lack of responsiveness was observed in one (D47) pSYNSU + PEI recipient (Figure 3.15). Although antibody responses were visible in the second member of this group (B29) following just two immunizations, these declined rapidly with no evidence of boost to the third vaccination (Figure 3.15). However, antibodies were detectable after two immunizations in ponies receiving pSYNSU + PEI, pIL-2/Ig, pIL-15 (SRLSS) or pSYNSU + PEI, pIL-15 (SSLSS). These levels increased significantly at three weeks post V3, although there was evidence of a progressive decline after this time point (Figures 3.16-3.18).

Equine IgG Subclass-Specific ELISA

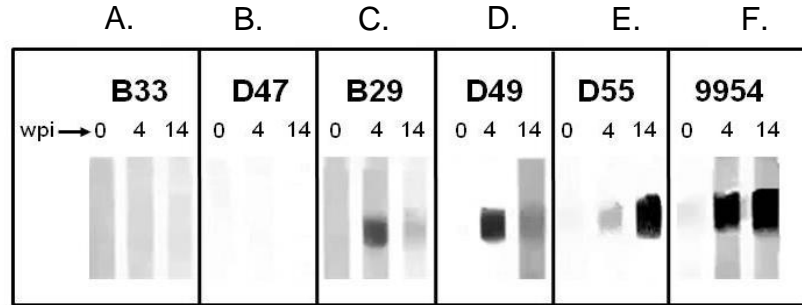
Equine IgG_A and IgG_T subclasses induced following pSYNSU immunization were monitored by Enzyme-Linked Immunosorbant Assays (ELISA). Not surprisingly, there was a general concordance between the IgG_A ELISA data and the results from the immunoblot assays. Amounts of IgG_A did not rise above background levels (Figure 3.19.) in B33, inoculated with naked pSYNSU, or in D47 (Figure 3.20), inoculated with pSYNSU + PEI. Serological responses detected in the second pSYNSU + PEI vaccinee (B29) two weeks after V2 were short-lived, with no increase in antibody levels detected following the third immunization (Figure 3.21). The highest IgG_A responses were observed in the pSYNSU + PEI, pIL-2/Ig, pIL-15 (SRLSS) recipient 9954 (Figure 3.16.). This antibody subclass increased almost 3-fold above the pre-immunization base-line value at one week post V2 (Figure 3.16.), although these levels did not persist and declined 50% by three weeks post V2. In contrast to B29, 9954 was responsive to further antigenic stimulation with levels of IgG_A to SYNSU increasing about 2-fold three weeks post V3 (week 15) (Figure 3.16.). At this peak, amounts of IgG_A attained approximately 70% of the level detected in serum from a long-term EIAV infected horse showing the typical, very strong humoral reactivity to SYNSU.

A very similar pattern of reactivity was observed in the second member (D55) (Figure 3.17) of the IL-2/Ig + IL-15 (SRLSS) group and in D49 (Figure 3.18), an IL-15

(SSLSS) recipient. Nonetheless, these responses were a significant improvement, compared with animals receiving pSYNSU without PEI or cytokines (B33, (Cook, Cook et al. 2005)). The IgG_T subclass was not detectable in sera from any of the vaccinated animals, including the high IgG_A responders 9954 and D55, with the notable exception of the pIL-15 (SSLSS) recipient D49 (Figure 3.18).

Figure 3.15

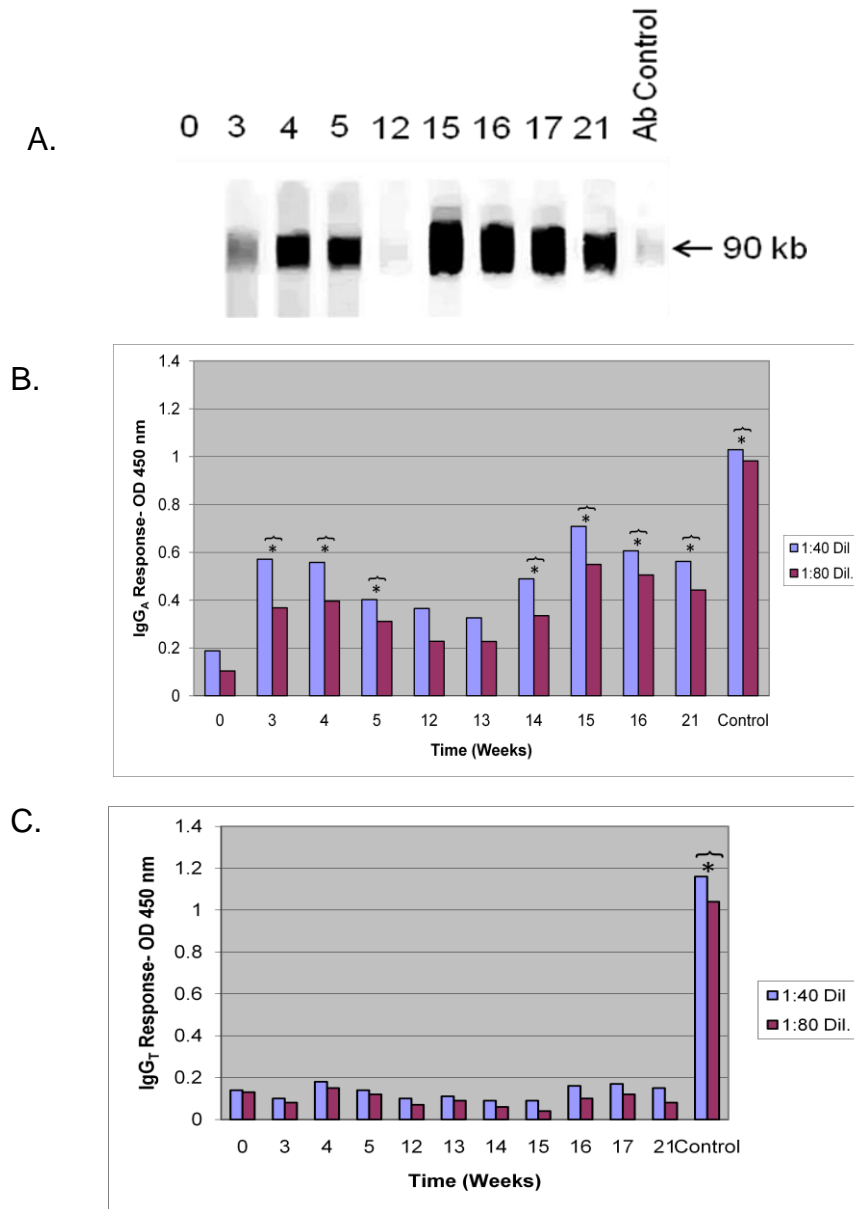
Analysis of SU-Specific Serum Antibody Reactivity from ELA-A1 Ponies on IgG Immunoblot



Serum samples collected at 0, 4 and 14 weeks after immunization from ponies B33 (A.), D47 (B.), B29 (C.), D49 (D.), D55 (E.) and 9954 (F.) were diluted 1:20 and analyzed by immunoblot analysis against SDS-disrupted EIAV_{PV} for detection of SU-specific antibodies.

Figure 3.16

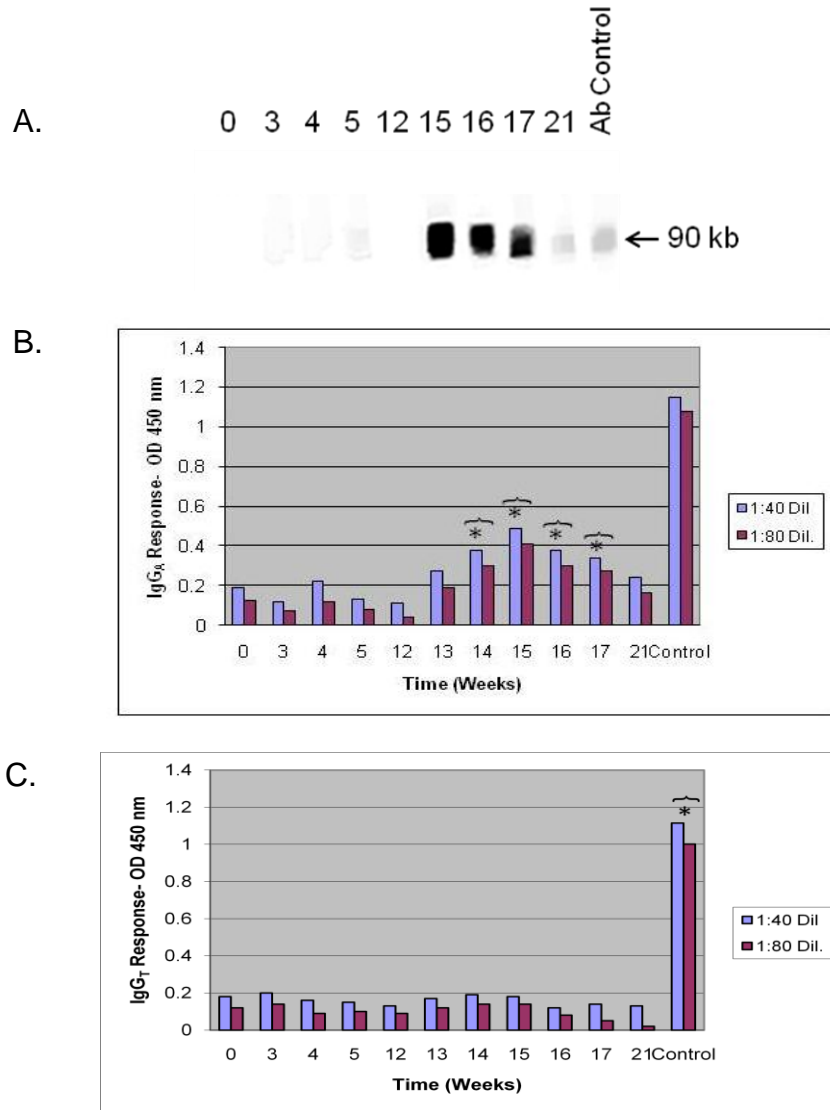
Analysis of SU-Specific Serum Antibody Reactivity from Pony 9954 on IgG Immunoblot (A.), IgG_A ELISA (B.) and IgG_T ELISA (C.)



Serum samples collected at 0, 3, 4, 5, 12, (13, 14), 15, 16, 17, and 21 weeks after DNA immunizations from pony 9954 (IL-2/Ig + IL-15 (SRLSS) pSYNSU immunization group) were diluted 1:20 and analyzed by immunoblot analysis against SDS-disrupted EIAV_{PV} for SU-specific antibodies and diluted 1:40 and 1:80 for analysis by ELISA for IgG_A and IgG_T antibody levels. Significant increases ($P < 0.05$) after V2 and V3 are denoted (*), as determined by the Mixed Procedures of SAS.

Figure 3.17

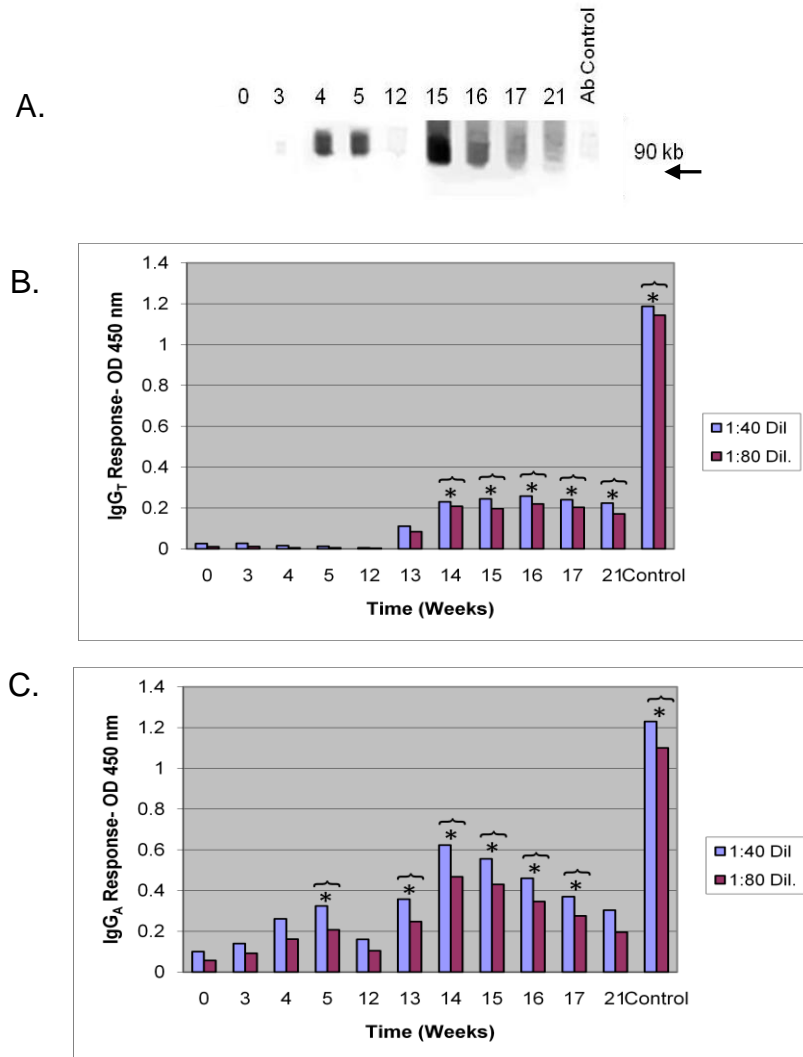
Analysis of SU-Specific Serum Antibody Reactivity from Pony D55 on IgG Immunoblot (A.), IgG_A ELISA (B.) and IgG_T ELISA (C.)



Serum samples collected at 0, 3, 4, 5, 12, (13, 14), 15, 16, 17, and 21 weeks after DNA immunizations from pony D55 (IL-2/Ig + IL-15 (SRLSS) pSYNSU immunization group) were diluted 1:20 and analyzed by immunoblot analysis against SDS-disrupted EIAV_{PV} for SU-specific antibodies and diluted 1:40 and 1:80 for analysis by ELISA for IgG_A and IgG_T antibody levels. Significant increases ($P < 0.05$) after V2 and V3 are denoted (*), as determined by the Mixed Procedures of SAS.

Figure 3.18

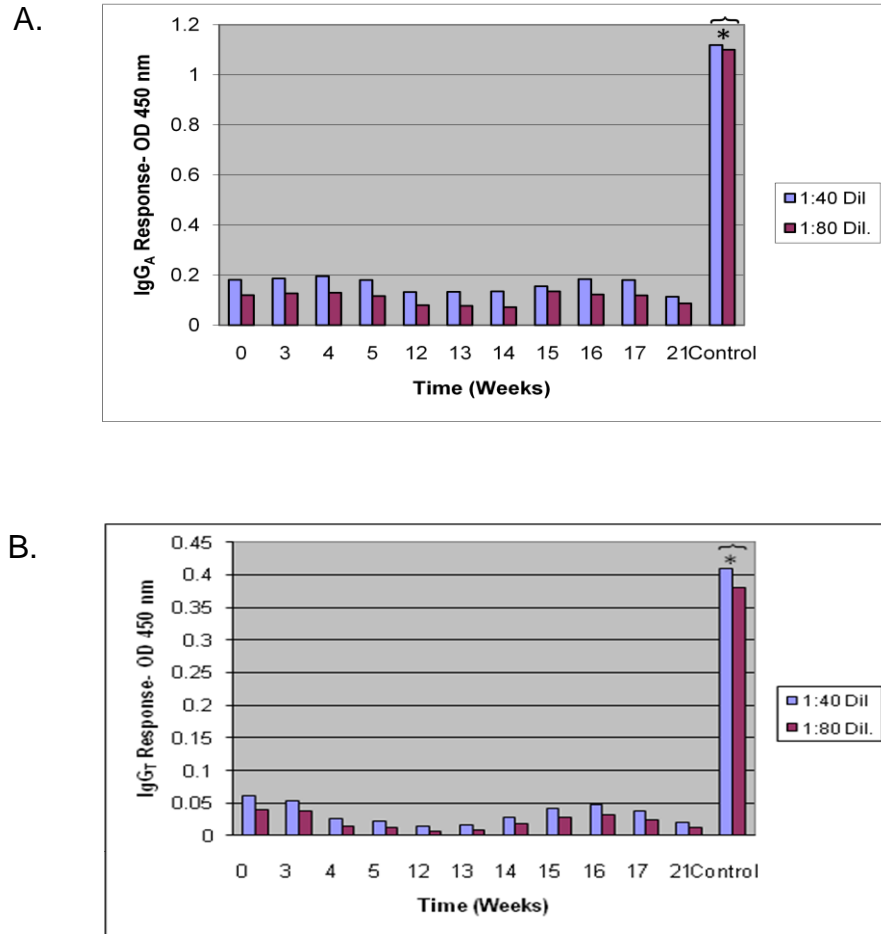
Analysis of SU-Specific Serum Antibody Reactivity from Pony D49 on IgG Immunoblot (A.), IgG_A ELISA (B.) and IgG_T ELISA (C.)



Serum samples collected at 0, 3, 4, 5, 12, (13, 14), 15, 16, 17, and 21 weeks after DNA immunizations from pony D49 (IL-15 (SSLSS) pSYNSU immunization group) were diluted 1:20 and analyzed by immunoblot analysis against SDS-disrupted EIAV_{PV} for SU-specific antibodies and diluted 1:40 and 1:80 for analysis by ELISA. Significant increases ($P < 0.05$) after V2 and V3 are denoted (*), as determined by the Mixed Procedures of SAS.

Figure 3.19

Analysis of SU-Specific Serum Antibody Reactivity from Pony B33 on IgG_A ELISA (A.) and IgG_T ELISA (B.)

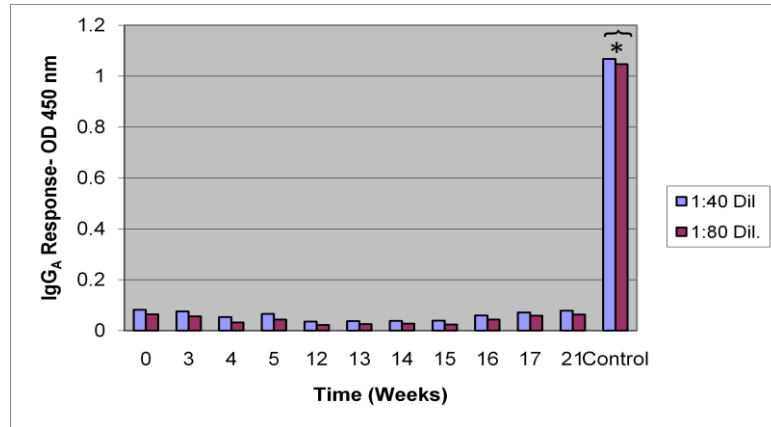


Serum samples collected at 0, 3, 4, 5, 12, 13, 14, 15, 16, and 21 weeks after DNA immunizations from pony B33 (naked DNA pSYNSU immunization group) were analyzed by ELISA against EIAV_{PV} for SU-specific IgG_A and IgG_T antibodies.

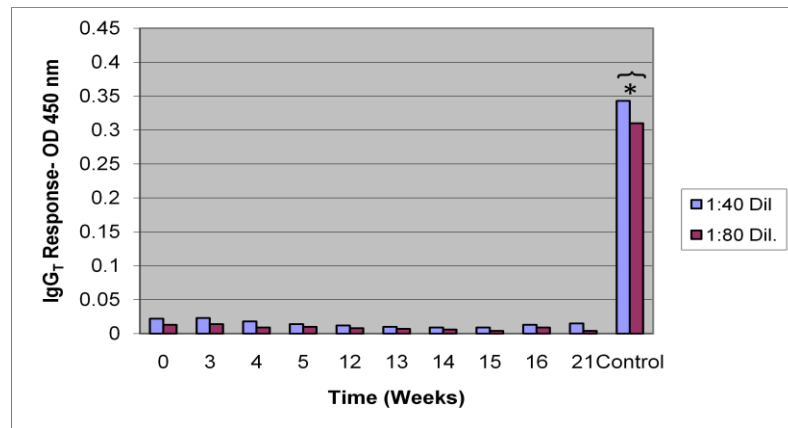
Figure 3.20

Analysis of SU-Specific Serum Antibody Reactivity from Pony D47 on IgG_A ELISA (A.) and IgG_T ELISA (B.)

A.



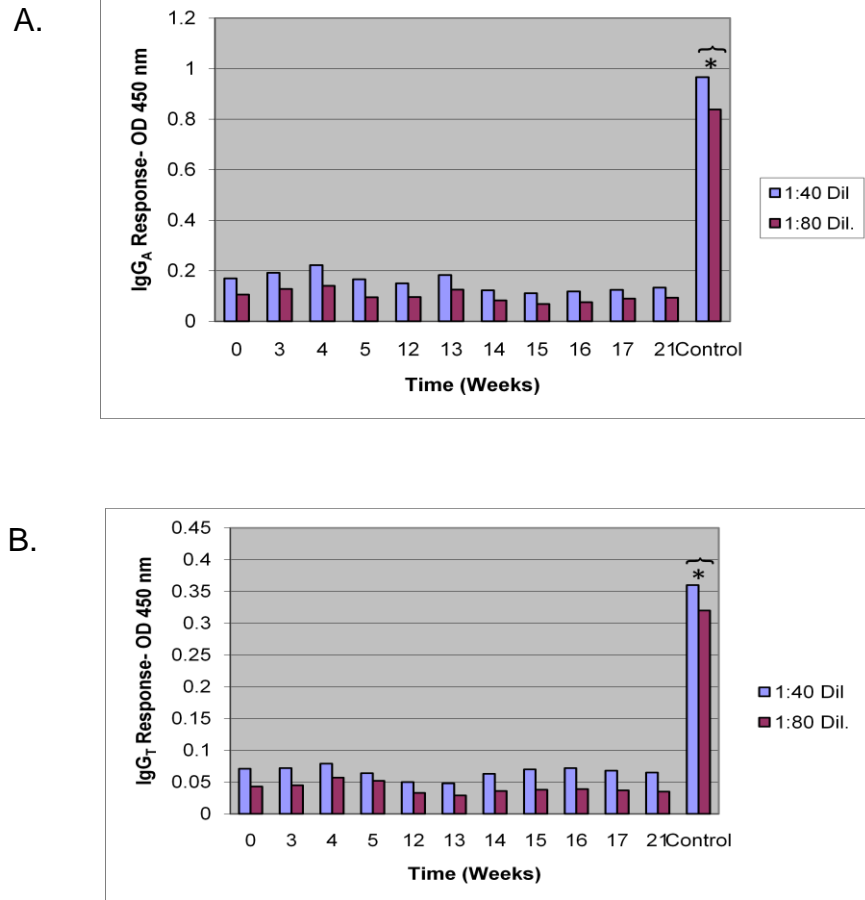
B.



Serum samples collected at 0, 3, 4, 5, 12, 13, 14, 15, 16, and 21 weeks after DNA immunizations from pony D47 (PEI pSYNSU immunization group) were analyzed by ELISA against EIAV_{PV} for SU-specific IgG_A and IgG_T antibodies.

Figure 3.21

Analysis of SU-Specific Serum Antibody Reactivity from Pony B29 on IgG_A ELISA (A.) and IgG_T ELISA (B.)



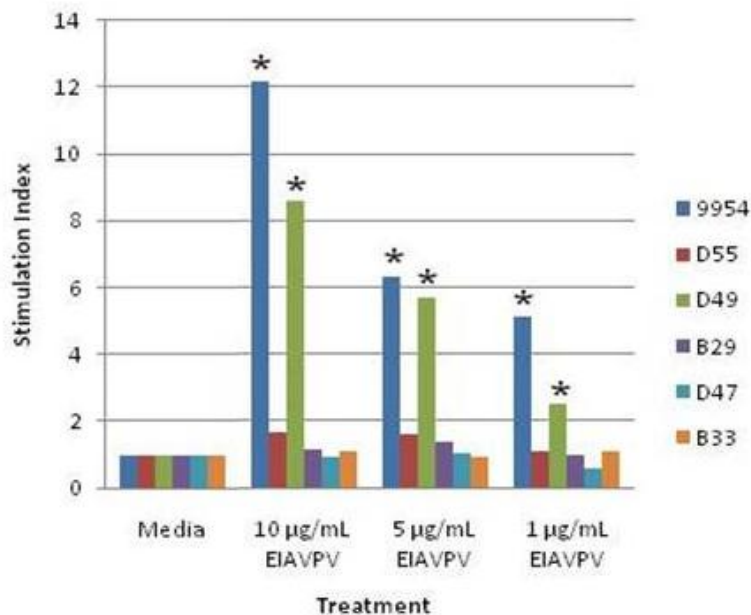
Serum samples collected at 0, 3, 4, 5, 12, 13, 14, 15, 16, and 21 weeks after DNA immunizations from pony B29 (PEI SYNSU immunization group) were analyzed by ELISA against EIAV_{PV} for SU-specific IgG_A and IgG_T antibodies.

SU-specific Lymphocyte Proliferative Responses Induced by Gradient Purified EIAV pSYNSU Immunization Groups

Induction of ex vivo SU-specific T-cell proliferative responses were measured using PBMC isolated from each pony two weeks following the third immunization. The relative levels of lymphoproliferation were compared in the form of a stimulation index (SI) representing [^3H] - thymidine incorporation of gradient-purified EIAV antigen stimulated cultures divided by [^3H] - thymidine incorporation of unstimulated cultures. No significant increases in lymphocyte proliferation were detected in PBMC cultures from the naked DNA or PEI pSYNSU immunization groups. However, significant lymphoproliferative activity to EIAV antigen was detectable in two of the cytokine recipients, 9954 and D49, in a dose-dependent manner (Figure 3.22).

Figure 3.22

Lymphocyte Proliferation of ELA-A1 Ponies in Response to in Vitro Stimulation



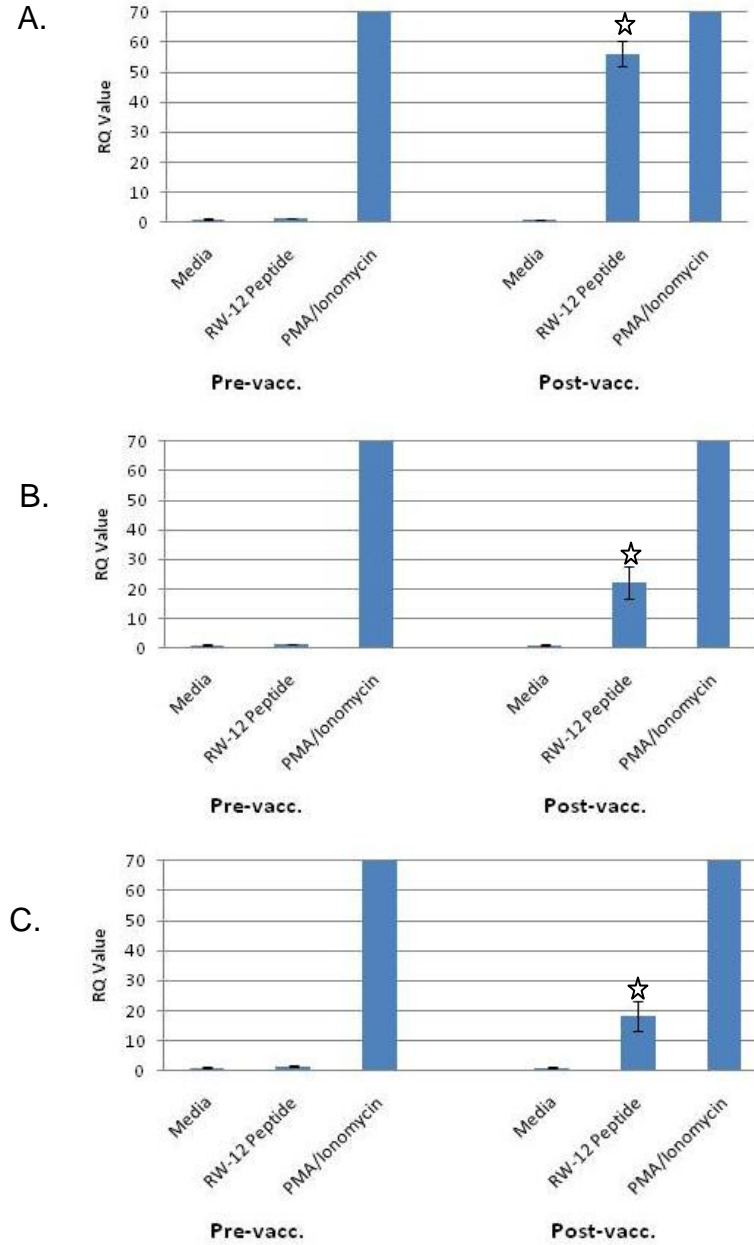
PBMC collected from ELA-A1 ponies at 2 weeks after the third pSYNSU immunization were stimulated in vitro with concentrations of EIAV_{PV} ranging from 10, 5, and 1 µg/ml or Con A (10 µg/ml) for 72 h. PBMC were labeled for 18 h with [^3H]-Thymidine. Significant interactions ($P<0.05$) from unstimulated controls are marked by stars.

Gamma Interferon Expression following Stimulation of PBMC with the SU RW-12 Peptide Antigen

Prior to PBMC incubation with RW-12 peptide or PMA/Ionomycin treatments, the viability of cryopreserved PBMC for each animal was determined to be between 90-95% with a Vi-Cell cell viability analyzer. PBMC populations collected from each pony prior to the first immunization (pre-vacc.) and two weeks after the third immunization (post-vacc.) were incubated with the RW-12 peptide (McGuire, Leib et al. 2003) (10 µg/ml) for 48 h at 37° C prior to RNA isolation, cDNA synthesis, and quantitation of IFN-γ mRNA by real-time RT-PCR. PBMC populations from each time point incubated in vitro without RW-12 peptide were used as negative controls. Positive control wells to measure maximum IFN-γ expression were treated with PMA (25 ng/ml) and ionomycin (1 µM). IFN-γ mRNA expression levels were calculated based on RQ values using the media control for each sample as a calibrator and equine β-GUS as a housekeeping gene. No significant increases in IFN-γ mRNA levels, relative to the media control, were observed in PBMC incubated with the RW-12 peptide that were collected from the naked DNA pSYNSU recipient (B33) or PEI pSYNSU recipients (B29 and D47) (data not shown). However, IFN-γ expression was significantly elevated by incubation with this peptide in post V2 samples collected from cytokine recipient ponies, 9954, D55, and D49 (Figure 3.23). The most dramatic (56-fold) increases in IFN-γ expression were observed in 9954 (Figure 3.23).

Figure 3.23

IFN- γ mRNA Expression Levels of PBMC from ELA-A1 Ponies Inoculated with pSYNSU Immunizations Following In Vitro Stimulation with the RW-12 Peptide



PBMC from Pony 9954 (A), D55 (B), and D49 (C) were collected. Results are expressed as the relative quantification (RQ) mean (+S.E.) values. Significant differences ($P < 0.05$) between PBMC collected prior to V1 (Pre-vacc.) and two weeks after V3 (Post-vacc.) are indicated (☆).

3.3: Assessment of Humoral Immune Responses to pSYNSU Immunization in Horses and Ponies Possessing Diverse ELA-Haplotypes

Physical Responses following DNA Vaccination

Adverse reactions to genetic immunization were assessed in each of the 30 horses and ponies, after vaccination (V1-V4). Heat was detected at the injection sites of all inoculated animals after each immunization. Vaccines expressing naked DNA antigen, without PEI, elicited mild physical responses in ponies after each of the DNA vaccinations. While soft nodules were noted ranging in average diameter from 65-114 mm, the average duration of swelling only persisted for 1-2 weeks in these vaccinates; heat from the skin nodules was also observed for similar durations of time (Table 3.4). Although vaccination with PEI + pSYNSU resulted in a comparable duration of heat released from the injection sites and swelling of skin nodules, the persistence of swelling was evident for 4-5 weeks after each immunization (Table 3.4). This suggestive of enhanced inflammatory responses to DNA vaccination in these animals. However, the addition of cytokines to the PEI + pSYNSU vaccine further augmented the physical responses to vaccination that were observed for most vaccinates. For recipients of pIL-7, and pIL-12 + pIL-18, heat and swelling were noted for several more weeks after V1 and V2, than after V3 and V4 in these animals (Table 3.4). A similar trend was observed in horses from the IL-15 (SRLSS) group, with average skin nodules ranging in size from 63-96 mm after each vaccination (Table 3.4). In contrast, vaccines expressing pIL-2/Ig and pIL-15 (SSLSS) + IL-2/Ig caused increased swelling of the skin after each vaccination (Table 3.4). Members of these vaccine groups demonstrated comparable physical responses to vaccination with duration of heat detection (3-5 weeks) and size of swelling in the skin (73-144 mm in diameter), although the average duration of swelling persisted for longer periods of time after vaccination with pIL-15 (SRLSS) + pIL-2/Ig (Table 3.4). Interestingly, immunization with DNA vaccines expressing pIL-15 (SSLSS), pSushi IL-15, or pIL-15 (SRLSS) evoked the largest physical responses in these horses, with very large skin nodules, ranging in diameter from 106-143 mm, after each intradermal inoculation (Table 3.4). Local reactions in these recipients were extremely sore and warm to the touch, but exhibited no signs of redness or purulence.

Table 3.4

Physical Responses of 30 Horses or ponies to Different pSYNSU DNA Immunization Protocols

pSYNSU Immunization Group	Vaccination	Duration of Heat (weeks)	Mean Swelling (mm)	Mean Duration of Swelling (weeks)
naked DNA	V1	3	81	2
	V2	3	101	1
	V3	1	65	1
	V4	2	114	2
PEI	V1	3	65	5
	V2	2	79	4
	V3	3	67	5
	V4	3	86	4
IL-2/Ig	V1	4	119	7
	V2	4	116	6
	V3	5	92	6
	V4	3	73	4
IL-7	V1	4	76	5
	V2	6	121	6
	V3	3	91	3
	V4	2	98	3
IL-12 + IL-18	V1	6	97	9
	V2	5	100	7
	V3	2	106	3
	V4	2	97	3
IL-15 (SSLSS)	V1	3	106	5
	V2	2	109	4
	V3	3	133	5
	V4	2	118	4
IL-15 (SSLSS) + IL-2/Ig	V1	5	87	8
	V2	6	86	7
	V3	3	86	8
	V4	4	144	6
IL-15 (SRLSS)	V1	3	108	6
	V2	3	121	3
	V3	3	133	4
	V4	1	124	3
IL-15 (SRLSS) + IL-2/Ig	V1	4	99	6
	V2	4	131	9
	V3	7	153	4
	V4	5	105	7
Sushi IL-15	V1	4	133	5
	V2	6	124	3
	V3	3	143	2
	V4	4	140	4

Physical responses to vaccination were monitored weekly after V1-V4, until they completely subsided. Heat was determined by warmth of the skin to the touch. Average values for duration of heat and size and duration of swelling of vaccine group members are represented.

Serological Responses to DNA Immunization

Humoral immune responses following immunization with pSYNSU or pSYNSU + PEI, with and without co-administration of immunostimulatory cytokine plasmids, were evaluated by immunoblot analysis and ELISA to detect IgG_A and IgG_T subtypes. The initial immunization did not elicit a significant difference in the specific SU antibody levels among any of the individuals in the ten pSYNSU immunization groups; however, after the second immunization, antibody responses were detectable in 13 of the 30 animals. None of the horses or ponies inoculated with the naked pSYNSU vaccine or vaccines expressing pIL-12/pIL-18 or pSushi IL-15 elicited antibody responses to antigen after the second injection (Figure 3.28, 3.33).

Overall, the antibody responses in vaccinates receiving naked DNA immunizations were decreased in reactivity to EIAV_{PV} on immunoblot analysis, compared to immunizations containing PEI. A minimum of three vaccinations were required before humoral immune responses were detected on immunoblot analysis, and these responses were short-lived being undetectable by week 24 at time of V4 (Figure 3.24). All of the ponies in the naked DNA group responded to V4 with apparent boosts in antibody response levels (Figure 3.24). These were greatly diminished by four weeks after V4 and not detectable when serum antibody levels were re-examined 26 weeks after V4 (Figure 3.24).

In the ELA-A1 pilot study, low-level reactivity was seen in one of two recipients following three immunizations with pSYNSU and PEI. In contrast, all three recipients in the expanded pilot study responded with detectable antibody responses to DNA vaccination containing pSYNSU and PEI, although variation between individuals with C06 and F36 showed higher reactivity at most time points examined than C10 (Figure 3.25). All members of the PEI vaccine group responded after V3, however, responses in C10 were relatively weak (Figure 3.25). Nonetheless, these antibody responses were still detectable 12 weeks later at time of V4 in all three animals (Figure 3.25). Despite the differences in antibody levels between individuals in the PEI group after V3 and V4, all three animals maintained strong, long-lived, anamnestic responses to V4 in all animals (Figure 3.25). Even in C10, the lowest antibody responder, humoral immune responses to SYNSU, just as with the high responder F36, were still detectable nine months after

V4 (Figure 3.25). This demonstrated the stimulation of long-term SU-specific antibody production in the horse. Unfortunately, C06 could not be retained in the trial, although it might be predicted from the intensity of the reaction at three weeks post V4 that he too would have detectable SU antibody responses at week 60 (Figure 3.25).

Although all three horses in the IL-2/Ig pSYNSU immunization group responded with antibody production to pSYNSU, there was considerable variation between individuals with diverse ELA haplotypes (Figure 3.26). Reactivity was easily detectable in E13 and D07, but only after V4, whereas robust antibody responses in D15 were observed by one week post V2 (Figure 3.26). SU-specific antibody responses from the IL-2/Ig pSYNSU immunization group were weaker than humoral responses detected from the PEI pSYNSU immunization group. Only D07 was retained for long-term observation, and antibody responses were still detectable at week 60, with an equivalent intensity to that detected at week 27 (Figure 3.26).

Although responses varied between individuals, the IL-2/Ig, IL-7, IL-12 + IL-18, IL-15 (SRLSS) + IL-2/Ig, and IL-15 (SSLSS) + IL-2/Ig pSYNSU immunization groups demonstrated similar overall responses to the PEI pSYNSU immunization group after V3 and V4 (Figures 3.26-3.30). Almost all of the animals in these groups showed improved responses compared to the naked DNA pSYNSU vaccinates, and 55% of the subjects tested had SU-specific antibodies at 35 weeks post V4 (Figures 3.26-3.30). Ponies in the IL-7 pSYNSU immunization group exhibited considerable variation between individuals in antibody response to DNA vaccination, with G22 exhibiting low antibody response levels (Figure 3.27). Although G23 and G30 elicited higher humoral immune responses, there was no qualitative indication of improvement in antibody expression over animals from the PEI pSYNSU immunization group (Figure 3.27). Equine sera from the IL-12 + IL-18 pSYNSU immunization group were also analyzed by immunoblot analysis for the presence of SYNSU-specific antibody production. Although, all of the vaccinated animals responded with antibody production by two weeks post V3 (Figure 3.28), there was no qualitative indication of improvement in immune responses over responses detected in the PEI pSYNSU immunization group. A sustained response was also only evident in one animal, G26, at six months after V4 (Figure 3.28).

In animals co-immunized with pIL-15 (SRLSS), SU-specific antibodies were detectable in E08 following V2 and after V3 in E03 and D77 (Figure 3.31). Strong anamnestic responses were seen in E08 after V2, V3, and V4, or after V3 and V4 in the cases of E03 and D77 (Figure 3.31). Furthermore, antibodies against SU were still present in serum samples from these recipients at 36 weeks post V4 (Figure 3.31), and the intensity of the reactions appeared similar to those seen in horses or ponies receiving pSYNSU + PEI (Figure 3.25).

From animals in the IL-15 (SRLSS) + IL-2/Ig pSYNSU immunization group, SU-specific serum antibodies were detectable by immunoblotting from horse 309 following V2 or after V3 in E12 and E15 (Figure 3.29). Anamnestic responses were also observed in all members of this group post V4 (Figure 3.29). However, the level of humoral reactivity was below that encountered in the pSYNSU + PEI vaccinated animals (Figure 3.25). Furthermore, SU reactive antibodies were only minimally apparent in pony F41 at 36 weeks post V4 (Figure 3.25).

Similar responses were observed in immunoblot analysis of serum samples collected from horses that received vaccines containing pIL-2/Ig + pIL-15 (SSLSS) (Figure 3.30). Once again, none of the animals in this group possessed the ELA-A1 haplotype (Table 2.2) and SU-specific humoral immune responses were lower than responses seen in pSYNSU + PEI recipients at each of the time points analyzed (Figure 3.25). However, SU reactivity was still visible at 36 weeks post V4 in all three horses (Figure 3.30).

In contrast, all animals [none of which possessed the ELA-A1 haplotype, Table 2.2] administered vaccines containing pIL-15 (SSLSS) in the absence of pIL-2/Ig had detectable SU-specific humoral responses in immunoblot analysis just after V2 and showed strong anamnestic responses following both V3 and V4 (Figure 3.32). Furthermore, all the members of this group remained seropositive to SU at 36 weeks post V4 (Figure 3.32).

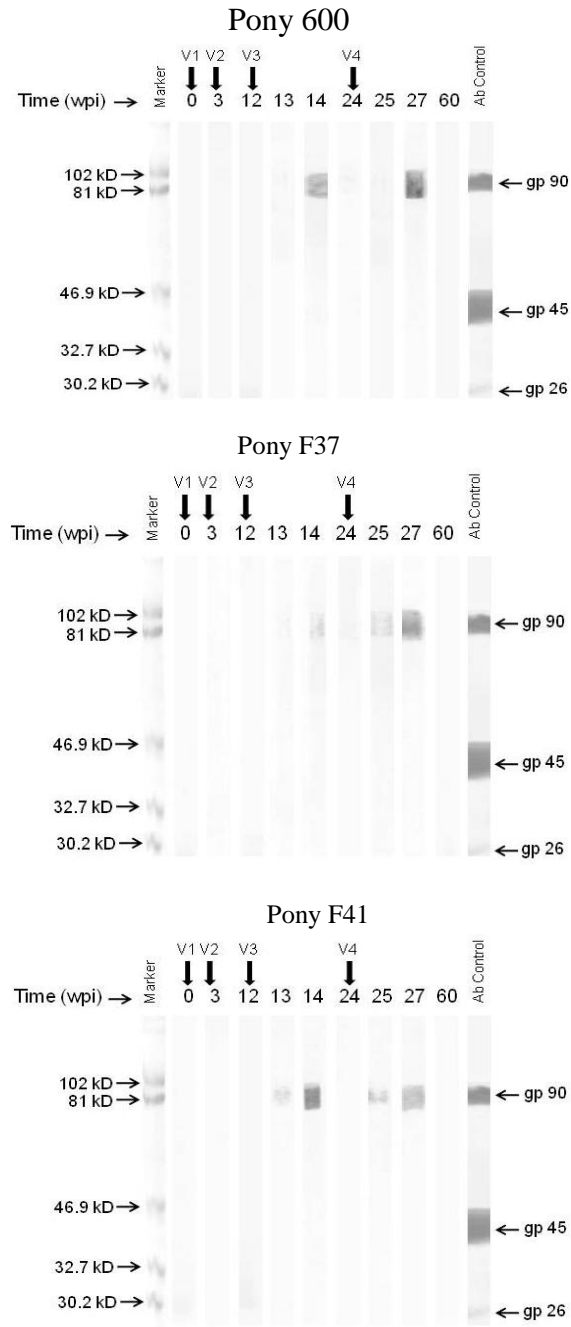
The results from the in vitro assays demonstrate that the pSushi IL-15 construct is expressed at high levels in transfected HEK293 cell cultures. Therefore, it was predicted that this modified form of IL-15 might have very powerful adjuvant effects when simultaneously administered with pSYNSU + PEI. However, humoral immune

responses to SU in this pSYNSU immunization group appeared to be very similar to those observed in the naked pSYNSU recipients (Figure 3.33). Antibodies to SU were not detectable in immunoblots until after V3, and while there was evidence of anamnestic responses following V4, these declined rapidly (Figure 3.33).

Unfortunately, it was not possible to retain all vaccinates beyond 36 weeks post V4. However, of the 25 animals that were retained, 16 elicited notable, sustained antibody responses against SYNSU. Surprisingly, humoral responses were still detectable eight months after V4, by Western blot analysis. These long-term antibody levels were only detected in animals that had received PEI and were not evident in ponies from the naked DNA pSYNSU immunization group. Furthermore, differences in response levels between vaccinated animals receiving PEI or PEI and cytokine expression constructs were not discernible, thereby demonstrating the influence of PEI on the maintenance of long-lived antibody production with DNA vaccination.

Figure 3.24

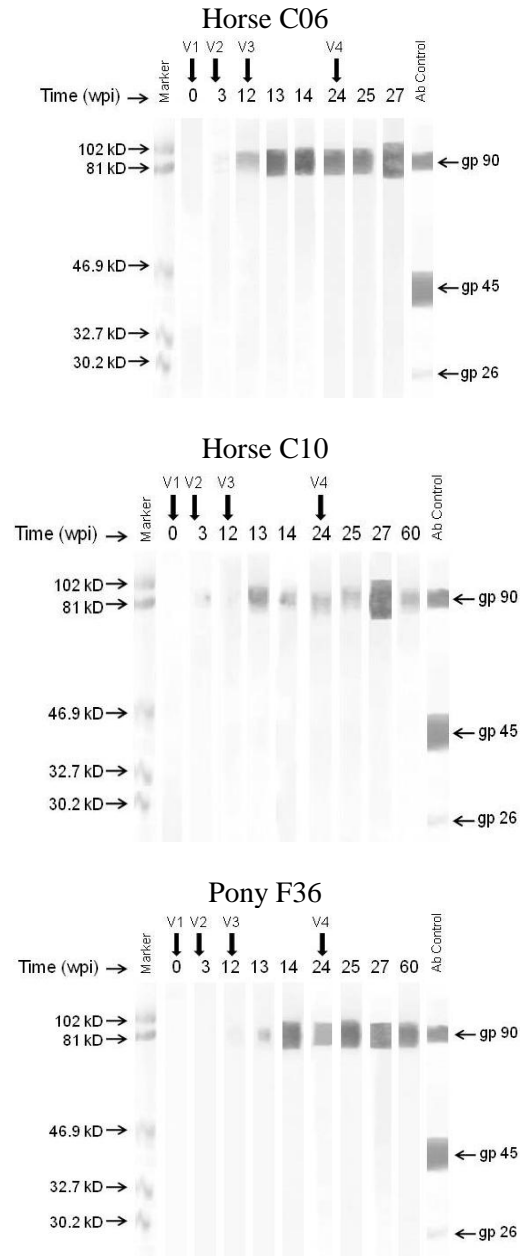
Analysis of SU-Specific Serum Antibody Reactivity from Immunized Ponies in the Naked DNA pSYNSU Immunization Group on IgG Immunoblot



Serum samples collected at 0, 3, 12, 13, 14, 24, 25, 27, and 60 weeks from the naked DNA pSYNSU immunization group were diluted 1:20 and analyzed by immunoblot analysis against SDS-disrupted EIAV_{PV}. A reference anti-EIAV horse serum diluted 1:20 was used as antibody control.

Figure 3.25

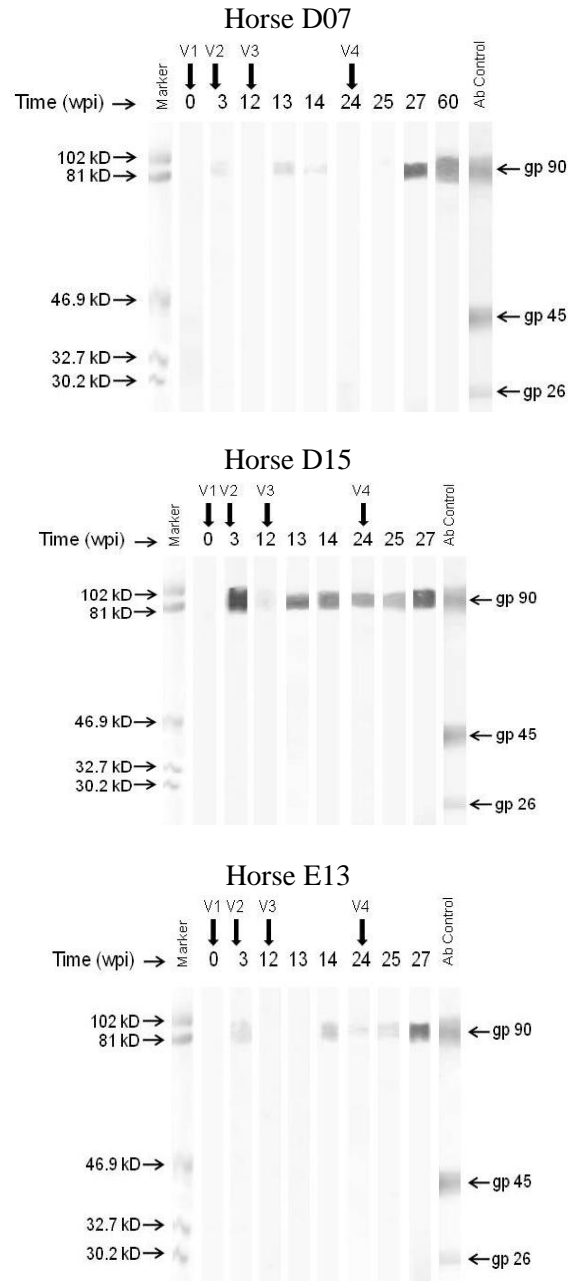
**Analysis of SU-Specific Serum Antibody Reactivity from Immunized Ponies in the
PEI pSYNSU Immunization Group on IgG Immunoblot**



Serum samples collected at 0, 3, 12, 13, 14, 24, 25, 27, and 60 weeks after from the PEI pSYNSU immunization group were diluted 1:20 and analyzed by immunoblot analysis against SDS-disrupted EIAV_{PV}. A reference anti-EIAV horse serum diluted 1:20 was used as antibody control.

Figure 3.26

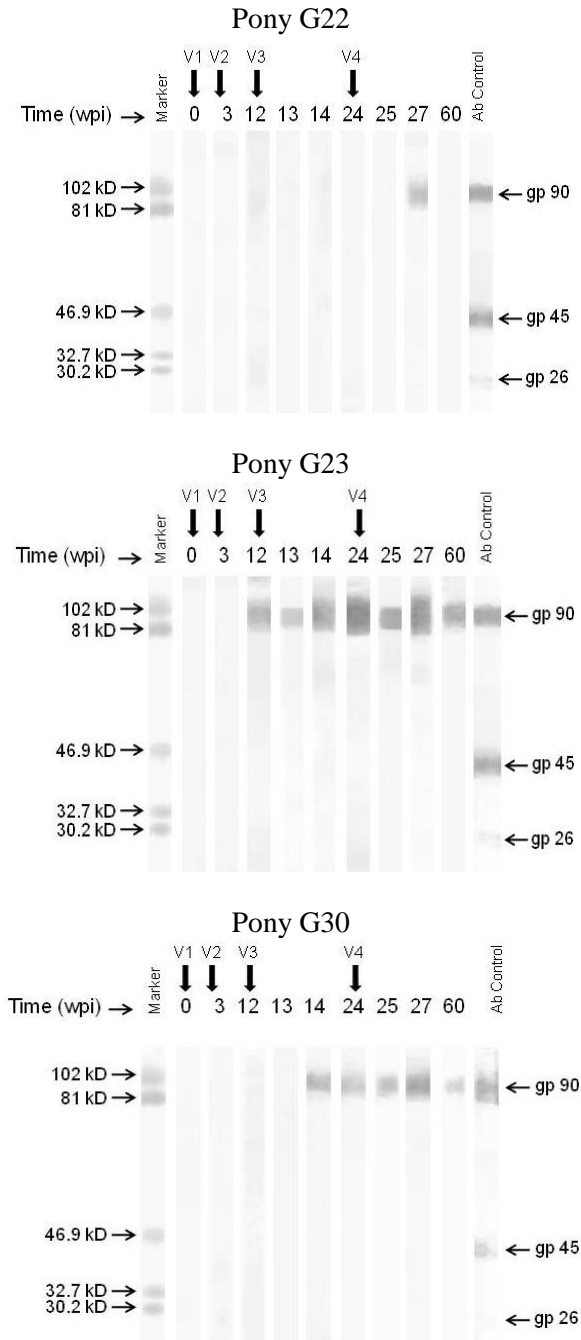
Analysis of SU-Specific Serum Antibody Reactivity from Immunized Ponies in the IL-2/Ig pSYNSU Immunization Group on IgG Immunoblot



Serum samples collected at 0, 3, 12, 13, 14, 24, 25, 27, and 60 weeks from the IL-2/Ig pSYNSU immunization group were diluted 1:20 and analyzed by immunoblot analysis against SDS-disrupted EIAV_{PV}. A reference anti-EIAV horse serum diluted 1:20 was used as antibody control.

Figure 3.27

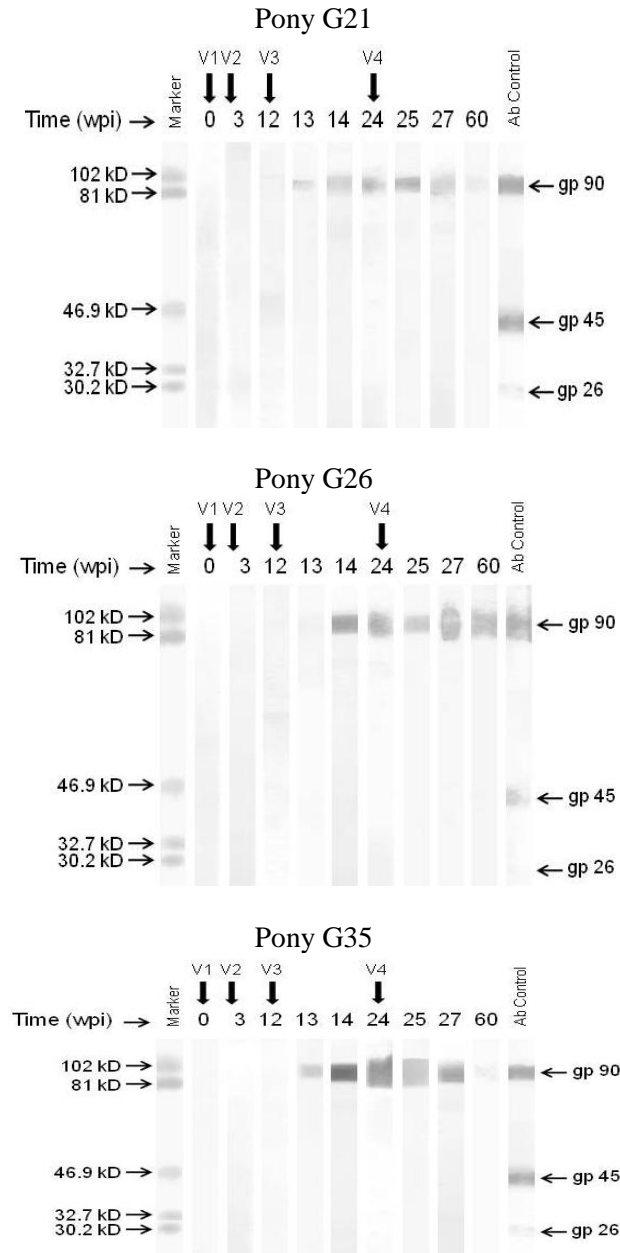
Analysis of SU-Specific Serum Antibody Reactivity from Immunized Ponies in the IL-7 pSYNSU Immunization Group on IgG Immunoblot



Serum samples collected at 0, 3, 12, 13, 14, 24, 25, 27, and 60 weeks from the IL-7 pSYNSU immunization group were diluted 1:20 and analyzed by immunoblot analysis against SDS-disrupted EIAV_{PV}. A reference anti-EIAV horse serum diluted 1:20 was used as antibody control.

Figure 3.28

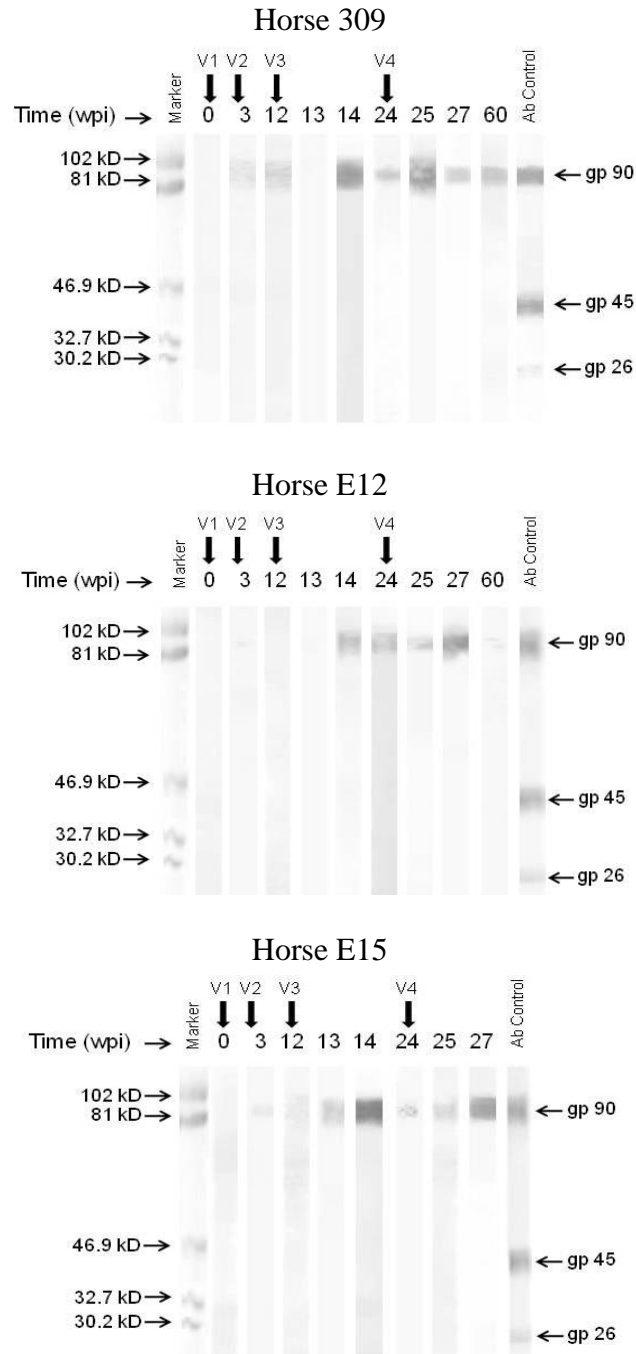
Analysis of SU-Specific Serum Antibody Reactivity from Immunized Ponies in the IL-12 + IL-18 pSYNSU Immunization Group on IgG Immunoblot



Serum samples collected at 0, 3, 12, 13, 14, 24, 25, 27, and 60 weeks from the IL-12 + pIL-18 pSYNSU immunization group were diluted 1:20 and analyzed by immunoblot analysis against SDS-disrupted EIAV_{PV}. A reference anti-EIAV horse serum diluted 1:20 was used as antibody control.

Figure 3.29

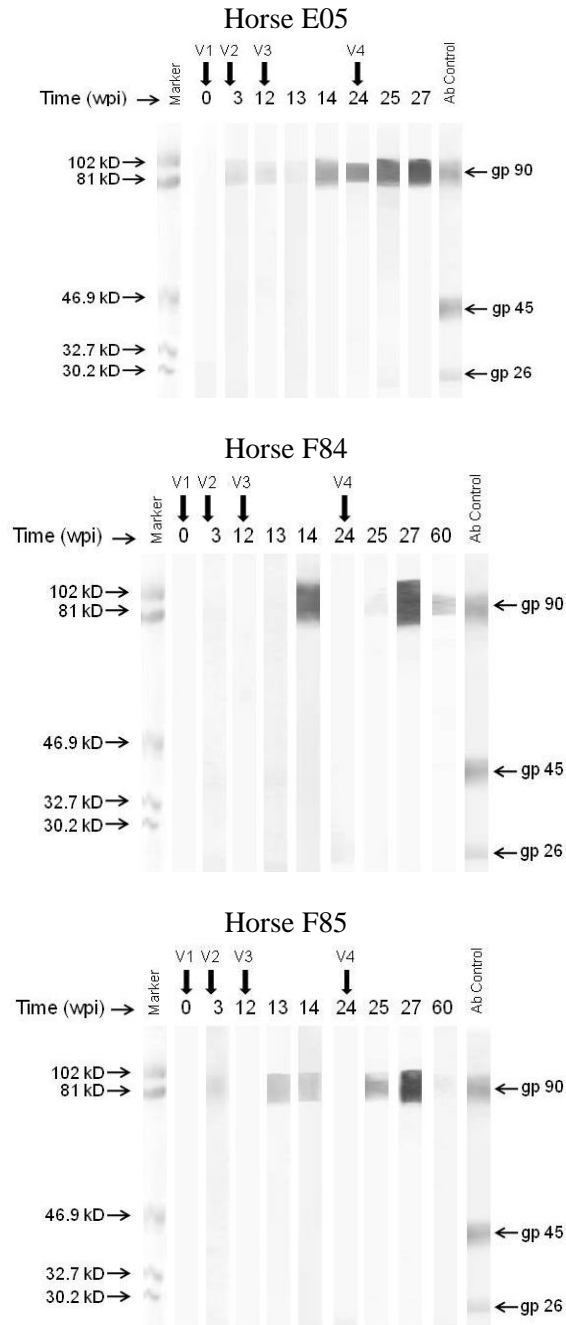
Analysis of SU-Specific Serum Antibody Reactivity from Immunized Ponies in the IL-15 (SRLSS) + IL-2/Ig pSYNSU Immunization Group on IgG Immunoblot



Serum samples collected at 0, 3, 12, 13, 14, 24, 25, 27, and 60 weeks from the IL-15 (SRLSS) + IL-2/Ig pSYNSU immunization group were diluted 1:20 and analyzed by immunoblot analysis against SDS-disrupted EIAV_{PV}. A reference anti-EIAV horse serum diluted 1:20 was used as antibody control.

Figure 3.30

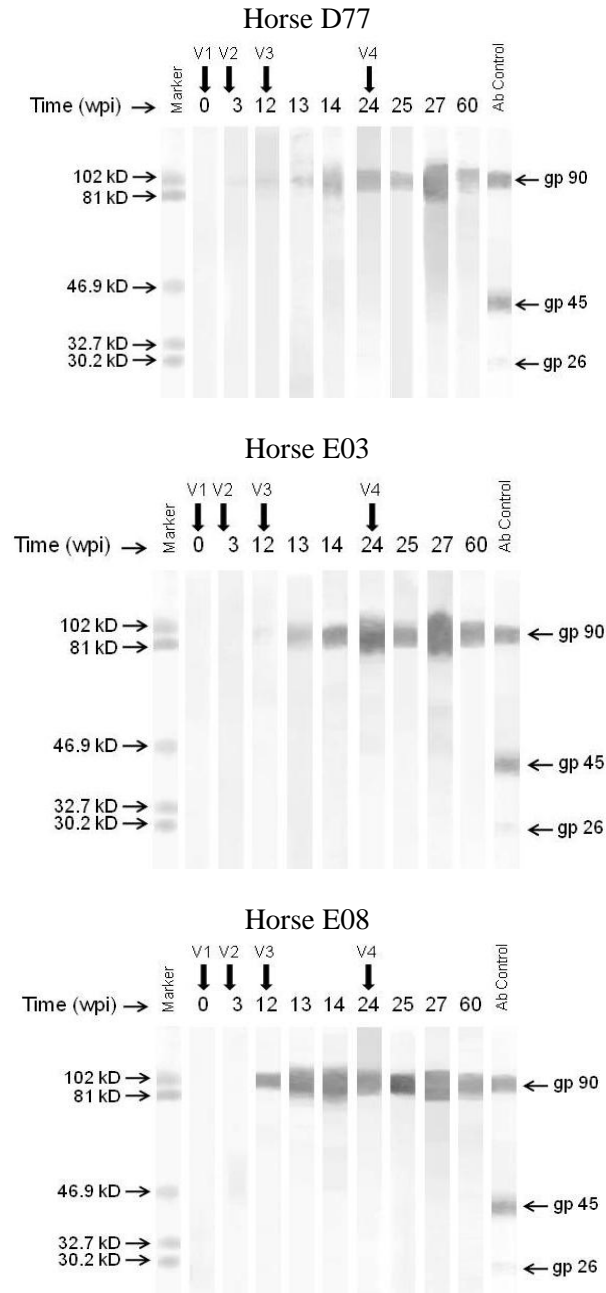
Analysis of SU-Specific Serum Antibody Reactivity from Immunized Ponies in the IL-15 (SSLSS) + IL-2/Ig pSYNSU Immunization Group on IgG Immunoblot



Serum samples collected at 0, 3, 12, 13, 14, 24, 25, 27, and 60 weeks from the IL-15 (SSLSS) + IL-2/Ig pSYNSU immunization group were diluted 1:20 and analyzed by immunoblot analysis against SDS-disrupted EIAV_{PV}. A reference anti-EIAV horse serum diluted 1:20 was used as antibody control.

Figure 3.31

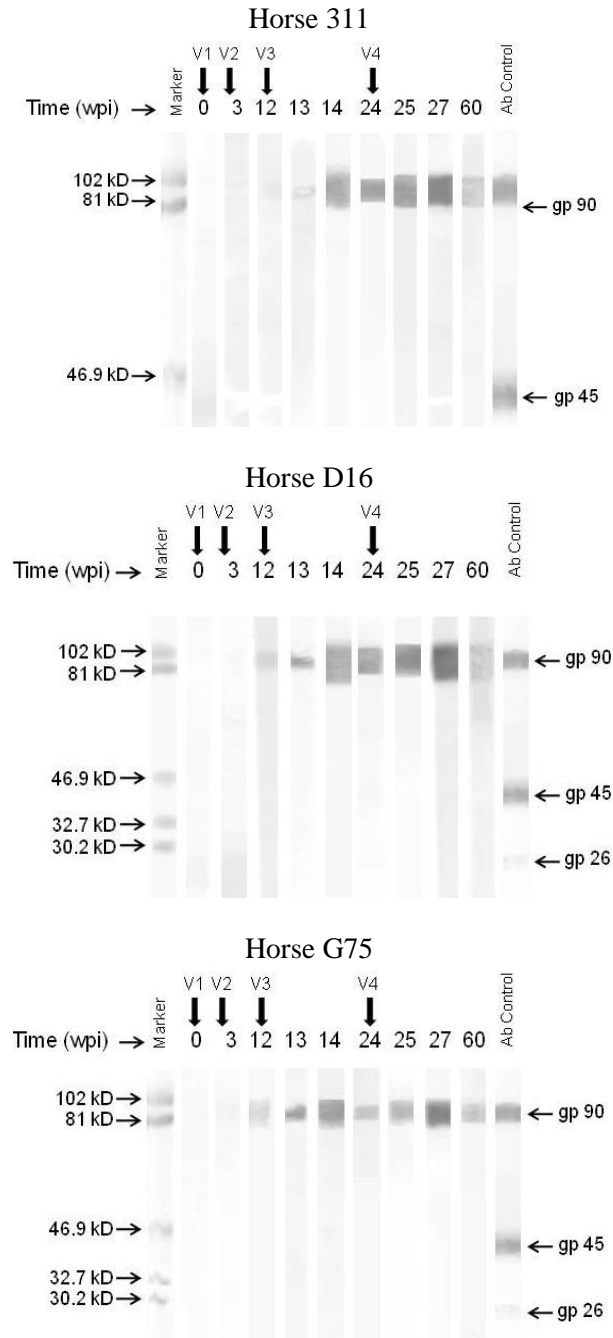
Analysis of SU-Specific Serum Antibody Reactivity from Immunized Ponies in the IL-15 (SRLSS) pSYNSU Immunization Group on IgG Immunoblot



Serum samples collected at 0, 3, 12, 13, 14, 24, 25, 27, and 60 weeks from the IL-15 (SRLSS) pSYNSU immunization group were diluted 1:20 and analyzed by immunoblot analysis against SDS-disrupted EIAV_{PV}. A reference anti-EIAV horse serum diluted 1:20 was used as antibody control.

Figure 3.32

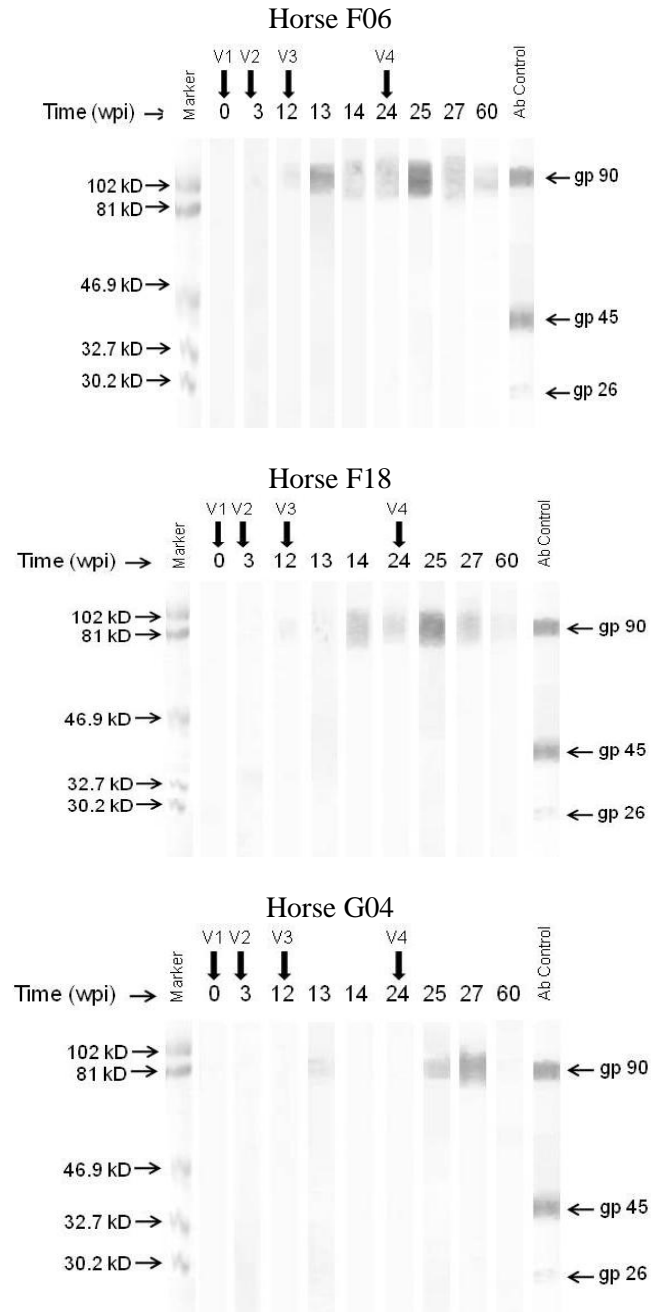
Analysis of SU-Specific Serum Antibody Reactivity from Immunized Ponies in the IL-15 (SSLSS) pSYNSU Immunization Group on IgG Immunoblot



Serum samples collected at 0, 3, 12, 13, 14, 24, 25, 27, and 60 weeks from the IL-15 (SSLSS) pSYNSU immunization group were diluted 1:20 and analyzed by immunoblot analysis against SDS-disrupted EIAV_{PV}. A reference anti-EIAV horse serum diluted 1:20 was used as antibody control.

Figure 3.33

Analysis of SU-Specific Serum Antibody Reactivity from Immunized Ponies in the Sushi IL-15 pSYNSU Immunization Group on IgG Immunoblot



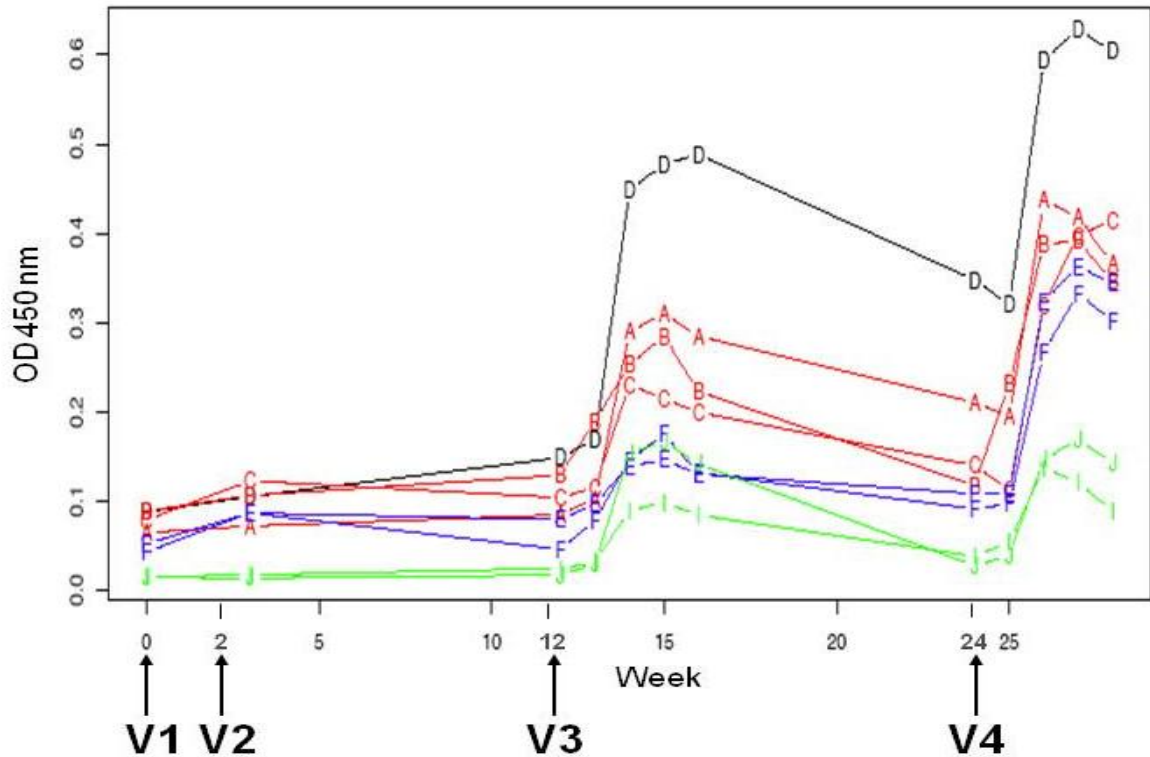
Serum samples collected at 0, 3, 12, 13, 14, 24, 25, 27, and 60 weeks from the Sushi IL-15 pSYNSU immunization group were diluted 1:20 and analyzed by immunoblot analysis against SDS-disrupted EIAV_{PV}. A reference anti-EIAV horse serum diluted 1:20 was used as antibody control.

Induction of IgG_A and IgG_T Specific Responses by Different pSYNSU Immunization Protocols

One month prior to V3, ponies from the IL-7 and IL-12 + IL-18 pSYNSU immunization groups received their yearly rabies vaccination. This was not expected to have any impact on SU-specific humoral responses after V3. However, high levels of serum IgG_A responses were detected in all six ponies, prior to immunization, because of aberrant reactivity to cellular proteins (from virus preparation) in the EIAV_{PV} capture antigen (data not shown). Therefore, only the remaining eight pSYNSU immunization groups were included in the statistical analysis of the IgG_A ELISA data after V3 and V4. Based on statistical similarities ($P > 0.05$) of mean IgG_A responses between pSYNSU immunization groups over time (after V3 and V4), four statistically similar groups were identified. In general, statistical analysis of the IgG_A ELISA data mirrored the qualitative results from immunoblotting. The Sushi IL-15 pSYNSU immunization group was indistinguishable at all time points from the naked DNA pSYNSU immunization group, with the lowest overall levels of IgG_A (Figure 3.34.). Significantly higher ($P < 0.05$) IgG_A responses after V3 and V4 were observed in the PEI, IL-2/Ig, and IL-15 (SRLSS) pSYNSU immunization groups (Figure 3.34.). The IL-2/Ig + IL-15 (SRLSS) and IL-2/Ig + IL-15 (SSLSS) pSYNSU immunization groups also had higher IgG_A levels than pSYNSU vaccinated animals, but differed from the subjects in group 2 in having significantly greater responses following V3 (Figure 3.34). However, the highest IgG_A titers were observed in the pIL-15 (SSLSS) vaccinates. Significant responses ($P < 0.05$), identified by the Mixed Procedures of SAS, initially occurred two weeks post V3 and continued through V4, with significant differences still detected at the final week 28 time point (Figure 3.34). In contrast to enhanced levels of IgG_A, the ELISA results determined that there were no detectable levels of IgG_T present in serum samples collected from any of the DNA vaccinated horses and ponies at any time point.

Figure 3.34

IgG_A ELISA Reactivity of pSYNSU DNA Immunization Treatment Groups



Average serum IgG_A reactivity of each pSYNSU immunization group was determined by ELISA at 1:40 dilutions. Serum was analyzed at 0, 3, 12, 13, 14, 15, 16, 24, 25, 26, 27, and 28 weeks from individual DNA vaccinated animals and represented as the mean for each pSYNSU immunization group. Individual points are indicated by a letter on the graph. Statistical analysis was performed by Xia Yu at the University of Kentucky, Department of Statistics, using SAS PROC MIXED. Vaccine groups that were not statistically different from one another were subcategorized into four groups, as indicated by black (D=IL-15 (SSLSS), red (A=PEI, B=IL-2/Ig, C=IL-15 (SRLSS)), blue (E=IL-15 (SRLSS), F=IL-15 (SSLSS)), and green (I=naked DNA, J=Sushi IL-15) lines.

Neutralizing Antibody Responses

To determine if SU-specific antibodies induced by different pSYNSU immunization protocols possessed viral neutralizing activity, serum samples collected prior to immunization, at two weeks post V3, and at three weeks post V4 were analyzed in a homologous neutralization assay against EIAV_{PV}. Neutralizing antibody activity was undetectable in all of the serum samples when measured against a viral dose of 100TCID₅₀, although this was detectable in serum samples from an EIAV_{PV} infected control animal (data not shown).

3.4: Analysis of Cellular Immune Responses in DNA Vaccinated Horses and Ponies Possessing Diverse ELA-Haplotypes

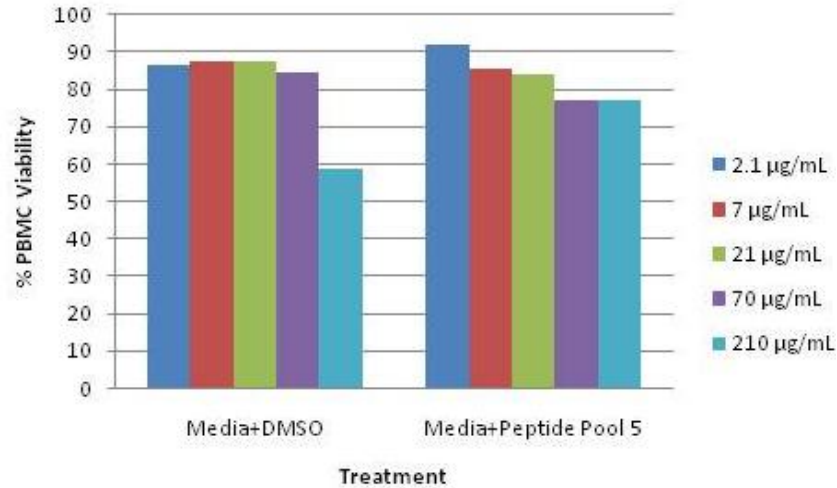
Optimization of Lymphoproliferative Responses to SU-specific Peptide Pools

(i) Effect of DMSO

Since DMSO has a potential for toxicity in cell culture, the effect of this amphipathic solvent on equine PBMC was determined. Incubation of equine PBMC from an EIAV infected control horse (D64) for 3 days in increasing amounts of DMSO, from 0% to 10.5%, decreased the viability of live cells significantly in culture with straight DMSO and peptide pool 5, reconstituted in DMSO. Pool 5 was selected for this optimization assay because it contained peptide 20 with the RVED epitope. Cell viability was compromised only in high concentrations of DMSO (3.5-10.5%), while concentrations of DMSO below 3.5% did not induce cell death (Figure 3.35). In view of the toxicity of DMSO, it is not surprising that lymphocyte proliferation following stimulation with 21 µg/ml concentrations of peptide pool 5 declined as DMSO concentrations increased. However, a DMSO concentration of 1.05% had the opposite effect and increased the background level of lymphocyte proliferation compared to PBMC incubated in media alone (Figure 3.35).

Figure 3.35

Effects of Increasing DMSO and Peptide Pool 5 Concentrations on PBMC Viability in Vitro



PBMC cultures from an EIAV infected horse (D64) were incubated with 2.1-210 µg/ml DMSO or Peptide Pool 5 for 72 h. Cell viability was determined by a Vi-Cell Cell Viability Analyzer.

(ii) Fresh vs. cryopreserved PBMC

To evaluate the effect of cryopreservation on the subsequent proliferative ability of equine PBMC, a comparative study was conducted with freshly isolated and cryopreserved PBMC from an EIAV infected horse, D64. In terms of trypan blue exclusion, the overall cell viability of fresh PBMC as determined by a ViCell counter was 97.6%, compared with 92.9% for cryopreserved PBMC. In addition to the 4.7% decrease in overall cell viability, significant differences ($P < 0.05$) were evident with mitogenic stimulation by Con A between the mean proliferative response of cryopreserved ($15,001 \pm 1417$ ccpm) and fresh PBMC ($21,647 \pm 2475$ ccpm). Despite this difference, a similar blastogenic potential of fresh and frozen lymphocytes was observed microscopically. Furthermore, no significant differences ($P > 0.05$) in lymphoproliferation were detected in response to EIAV_{PV} and SU-specific peptide pool stimulation between fresh ($17,418 \pm 1143$ for EIAV_{PV} stimulation and $13,709 \pm 1303$ for peptide pool stimulation) and frozen ($17,190 \pm 857$ for EIAV_{PV} stimulation and $13,261 \pm 1172$ for peptide pool stimulation) cell populations, as measured by the incorporation of [³H]-thymidine.

(iii) Comparison of lymphoproliferative responses to peptides from different manufacturers

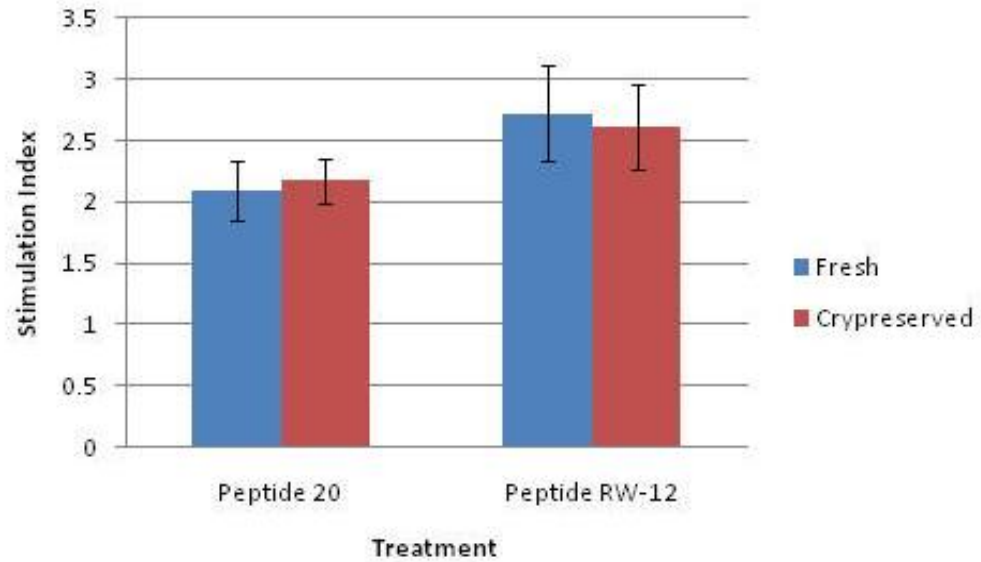
Although a majority of the peptides utilized in these studies was synthesized at the University of Pittsburgh, replacement peptides and the RW-12 peptide were produced by GenScript. Representative peptides from each manufacturer were selected to compare their stimulatory potentials on equine PBMC in vitro. The peptides received from the University of Pittsburgh were HPLC-purified and confirmed by mass spectrometry, but of unspecified purity, while the peptides synthesized by GenScript were at least 85% pure. Optimization studies with these individual peptides were initially performed on PBMC from an EIAV infected horse, D64, prior to use in lymphoproliferative assays with PBMC from DNA vaccinated animals. Since D64 possessed the ELA-A1 haplotype,

increased reactivity to peptides containing the RVED epitope was expected. This epitope, contained within peptide 20, was synthesized at the University of Pittsburgh (Peptide Synthesis Facility), while a custom RW-12 peptide, also containing this epitope, (McGuire, Fraser et al. 2004) was synthesized by GenScript (Table 2.7).

The effects on lymphoproliferation of fresh and cryopreserved PBMC, collected at equivalent time points, from D64 were evaluated in response to peptides 20 and RW-12. Lymphocyte proliferation responses to the peptides were comparable in D64 with SI values of 2 for peptide 20 and 2.7 for RW-12 stimulation (Figure 3.36). These results were very similar for fresh and cryopreserved PBMC (Figure 3.36). To account for the effect of size differences between peptides 20 and RW-12 on PBMC stimulation, a similar experiment was also performed with equivalent numbers of molecules, based on the molecular weight of each peptide. A peptide molecular weight (MW) calculator from www.Biopeptide.com was used to calculate the MW of each peptide. PBMC from D64 were incubated with 2.45 M of each peptide with the extent of lymphocyte proliferation evaluated by [³H]-Thymidine incorporation as outlined above. Once again, the stimulation indexes (2.2 for peptide 20 and 2.6 for RW-12 peptide) were very similar, suggesting the source of the peptide used in this study was not a significant factor in determining the outcome of the results

Figure 3.36

Lymphocyte Proliferation of Fresh and Cryopreserved Equine PBMC Cultures to Peptides 20 and RW-12 in Vitro



Mean stimulation indexes representing lymphocyte proliferation responses of fresh and cryopreserved PBMC cultures from an EIAV infected horse (D64) to 72 h stimulation with 21 µg/ml peptide 20 or peptide RW-12.

(iv) Effects of priming with equine IL-2 on lymphoproliferative responses

Experiments were also conducted to determine if pre-incubation of PBMC cultures with recombinant equine IL-2 would augment proliferative responses to SU-specific peptide pools after subsequent incubation. Interestingly, IL-2 at concentrations of 0.1 ng/ml (low) and 1.0 ng/ml (high) doses induced lymphoproliferative responses in the positive control cells, SI=3.12 and SI=2.94, respectively, compared with cells that were not treated with this cytokine. However, no differences were observed between media controls and the addition of peptides at any of the IL-2 concentrations tested (data not shown).

(v) Effects of priming with gradient purified EIAV or Con A on lymphoproliferative responses

Similar experiments were performed to test the priming potential of gradient purified EIAV_{PV} or Con A on PBMC from D64. Cells stimulated with virus or Con A alone responded in a dose-dependent manner, however, addition of peptides produced no additional proliferative activity (data not shown). Similarly, when PBMC were initially incubated with peptide pool 5 or 8 at 21 µg/ml and then stimulated with varying concentrations of Con A, the same results were observed. Lymphocyte populations proliferated in a dose-dependent response to Con A stimulation and not to peptide pools (data not shown). It was determined from the optimization assays that the lymphoproliferation to peptide pools could not be enhanced by pre-stimulation of PBMC with antigen-specific (EIAV) or mitogenic (Con A or IL-2) stimulants. Therefore, based on these optimization studies, lymphocyte proliferation assays with equine PBMC from pre- and post-vaccinated horses and ponies were conducted using cryopreserved cell populations incubated in vitro with peptide pools at 21 µg/ml.

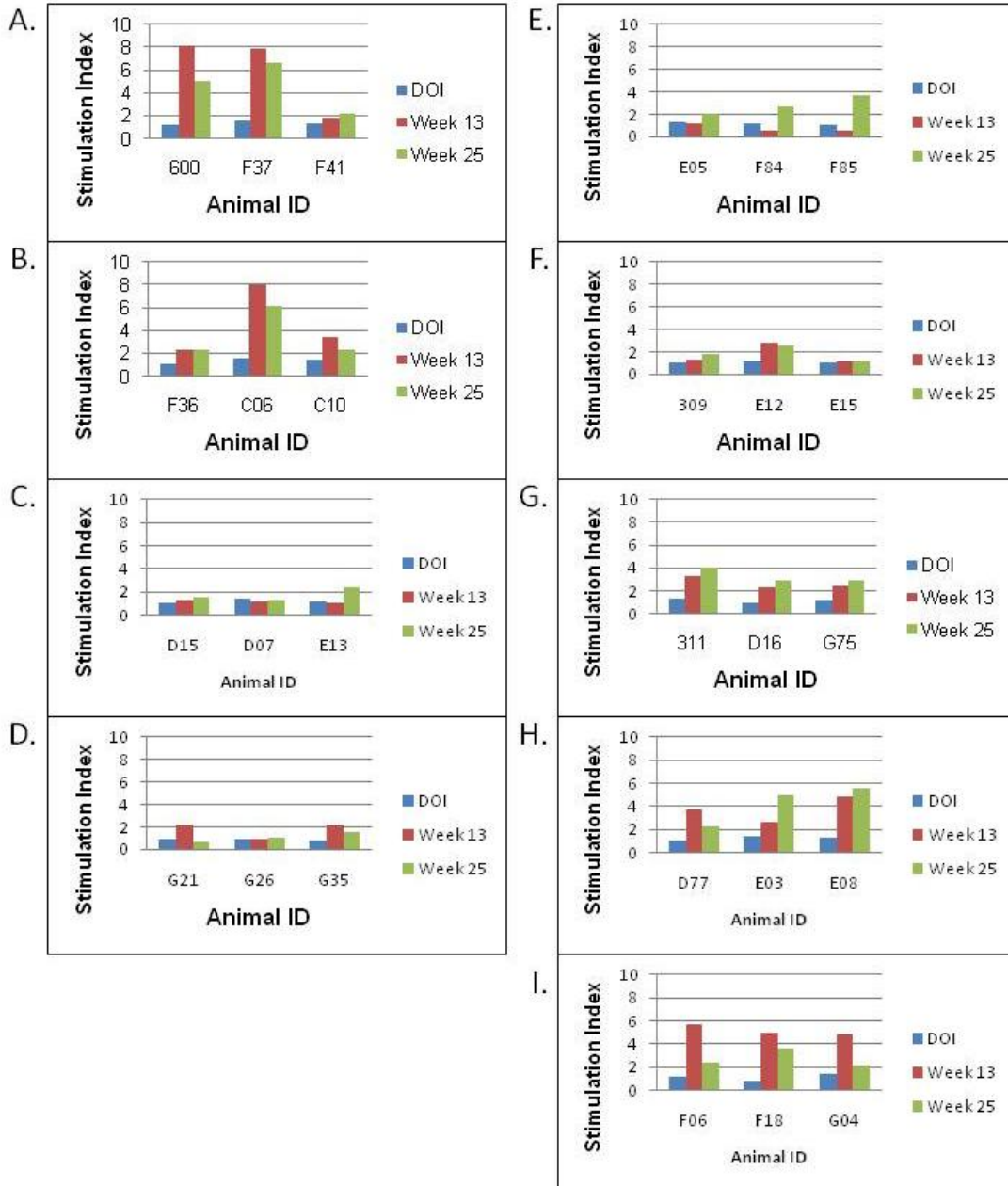
Effects of Gradient Purified EIAV_{PV} on Lymphoproliferative Responses of PBMC Cultures from DNA Vaccinated Animals

Cryopreserved PBMC collected at preinoculation and one week after the third and fourth inoculations, from all 30 horses and ponies in the 10 pSYNSU immunization groups, were tested concurrently for their ability to proliferate in vitro to SU-specific

peptide pools (21 µg/ml) or gradient purified EIAV_{PV} (10 µg/ml). Significant lymphoproliferative responses were induced following immunization with pSYNSU, although as predicted with diverse animal groups, there was considerable variation between individual horses and ponies (Figure 3.37). The stimulation indexes to EIAV_{PV} were as high in 2/3 ponies from the pSYNSU immunization group as they were in the pSYNSU immunization groups, PEI, IL-15 (SRLSS), IL-15 (SSLSS), and IL-2/Ig + IL-15 (SSLSS), following V3 and V4 (Figure 3.37). This demonstrated that in contrast to humoral immunity, short-term lymphoproliferative responses were not improved by either PEI or any of the cytokine adjuvants at the doses used in this study (Figure 3.37). Proliferative responses from members of the Sushi IL-15 pSYNSU immunization group were also similar to the naked DNA pSYNSU immunization group (Figure 3.37). In contrast, no significant lymphocyte proliferation was observed in animals receiving IL-7 and only very low proliferative levels were detected in the IL-2/Ig, IL-12 + IL-18, and IL-15 (SRLSS) + IL-2/Ig pSYNSU immunization groups (Figure 3.37).

Figure 3.37

Lymphoproliferative Responses of PBMC from DNA Vaccinates to EIAV_{PV}



Lymphocyte Proliferation Responses to EIAV_{PV} from animals in the A. naked DNA, B. PEI, C. IL-2/Ig, D. IL-12 + IL-18, E. IL-15 (SSLSS) + IL-2/Ig, F. IL-15 (SRLSS) + IL-2/Ig, G. IL-15 (SSLSS), H. IL-15 (SRLSS), and I. Sushi IL-15 pSYNSU immunization groups in the form of stimulation indexes. All represented stimulation indexes at 13 and 25 weeks of 2 or above have been determined to be statistically significant ($P < 0.05$), by SAS PROC MIXED from Xia Yu at the University of Kentucky, Department of Statistics.

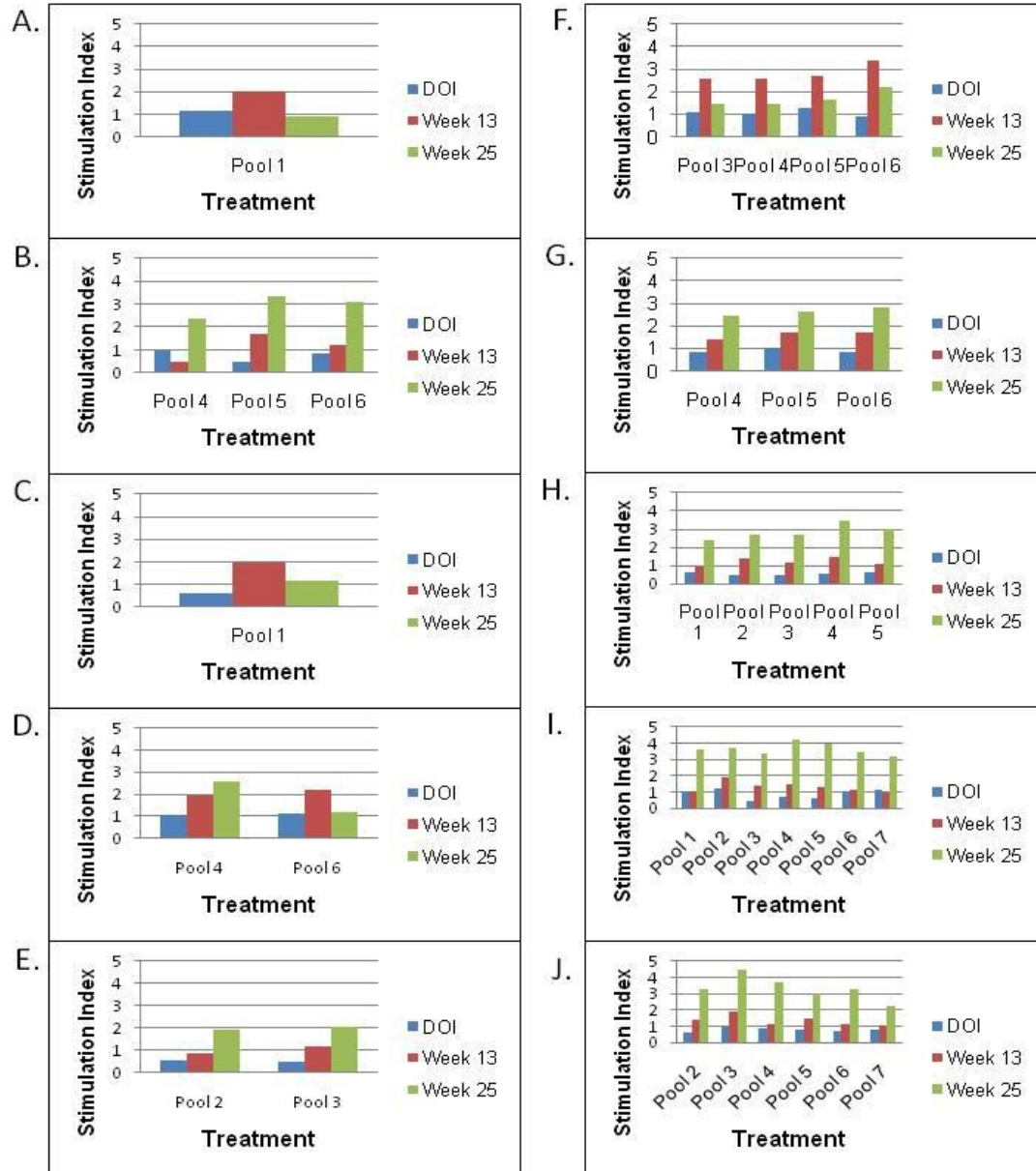
Effects of EIAV SU-specific Peptide Pools on Lymphoproliferative Responses of PBMC Cultures from DNA Vaccinated Animals

Preliminary short-term proliferation assays were performed by using EIAV SU-specific peptide pools as stimulants in a standard thymidine-incorporation lymphoproliferation assay. Significant lymphoproliferative responses, in cells collected one week after both V3 (week 13) and V4 (week 25), were detected in all animals receiving IL-15 (SRLSS) and in two of the three animals receiving IL-15 (SSLSS) (Figure 3.38). However, the stimulation indexes in pIL-15(SSLSS), pIL-15 (SRLSS), and pSushi IL-15 recipients did not exceed those in the pSYNSU immunized animals, 600 and F37 (Figure 3.38).

Animals in the IL-7, IL-2/Ig, IL-15 (SSLSS) + IL-2/Ig, and IL-15 (SRLSS) + IL-2/Ig pSYNSU immunization groups did not display any proliferative activity in response to SU-specific peptide pools (data not shown). Of the remaining groups, PBMC proliferation from the immunized horses and ponies at weeks 13 and 25 was generally low and variable among pSYNSU immunization group members (Figure 3.38). Significant [³H]-thymidine incorporation was detected to one of the pools tested in one of the three pSYNSU (F41) and pIL-12 + pIL-18 (G35) vaccinates (Figure 3.38). Two of the three animals receiving pIL-15 (SSLSS) demonstrated significant proliferative responses to two peptide pools (Figure 3.38). In contrast, two of the three Sushi IL-15 recipients and all three members of the IL-15 (SRLSS) pSYNSU immunization group displayed significant lymphoproliferative responses to multiple pools (Figure 3.38).

Figure 3.38

Lymphoproliferative Responses of PBMC from DNA Vaccinates to EIAV SU-specific peptide pools



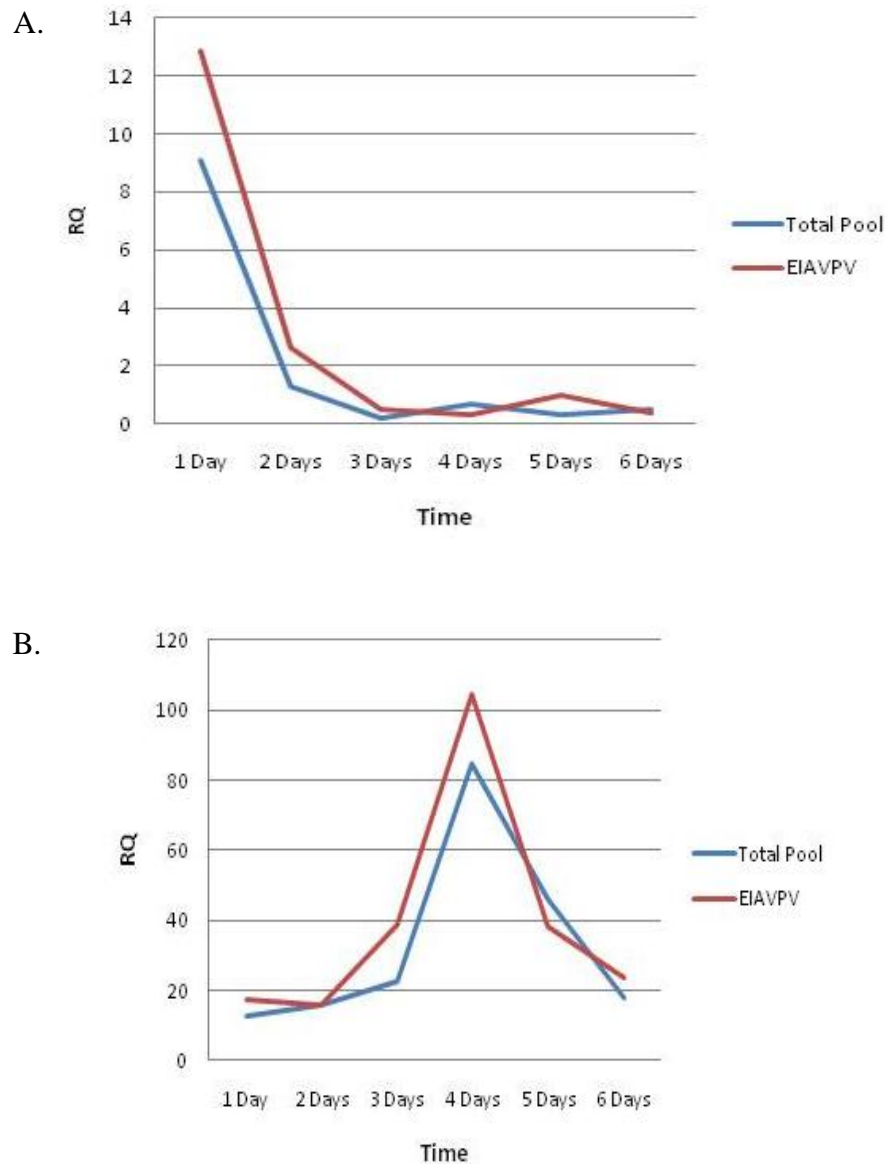
Lymphocyte Proliferation Responses to EIAV SU peptide pools from DNA vaccinates: A. F41, B. C06, C. G35, D. 311, E. D16, F. F06, G. F18, H. D77, I. E03, and J. E08 in the form of stimulation indexes. All represented stimulation indexes of 2 or above at Weeks 13 and 25 were determined to be statistically significant ($P < 0.05$), by SAS PROC MIXED from Xia Yu at the University of Kentucky, Department of Statistics.

Optimization studies with initial priming of EIAV-infected (D64) PBMC with EIAV_{PV} followed by subsequent stimulation with SU-specific total peptide pool

Stimulation of PBMC from D64 with EIAV_{PV} or the total peptide pool for 1-6 days resulted in significant increases in IL-2 and IFN- γ expression (Figure 3.39). IL-2 expression was significantly upregulated after a one day incubation with virus or peptide pools, while IFN- γ expression was not observed until a four day incubation with virus or peptide pools (Figure 3.39). Initial priming of PBMC with virus did not enhance cytokine expression in response to SU-specific total peptide pool stimulation for any of the time points examined (data not shown). Based on the peak IL-2 and IFN- γ cytokine expression levels, it was concluded that cell stimulation experiments with DNA vaccinated PBMC would utilize stimulation with peptide pools alone for one day to measure IL-2 expression and four days to evaluate IFN- γ mRNA levels.

Figure 3.39

Real Time RT-PCR Analysis of IL-2 and IFN- γ Expression in EIAV Infected (D64) PBMC after Total Peptide Pool or EIAV_{PV} In Vitro Stimulation



Relative quantification (RQ) of IL-2 (A.) or IFN- γ (B.) gene expression in equine PBMC from EIAV infected D64 in response to incubation with total peptide pool or EIAV_{PV} in vitro for 1-6 days.

SU-specific IFN- γ and IL-2 mRNA Expression in PBMC Cultures from pSYNSU Vaccinates

Preliminary Real-Time RT-PCR assays were conducted to determine IL-2 and IFN- γ mRNA expression levels of PBMC from selected pSYNSU immunization groups after in vitro stimulation with SU-specific peptide pools. Cryopreserved PBMC, collected at time of inoculation (V1) and one week post V4, were evaluated from 12 horses and ponies in four pSYNSU immunization groups; naked DNA, PEI, IL-15 (SSLSS) and Sushi IL-15. Con A mitogen was used as a positive control, and negative control wells contained 1.05% DMSO, equivalent with the final concentration present in each peptide pool.

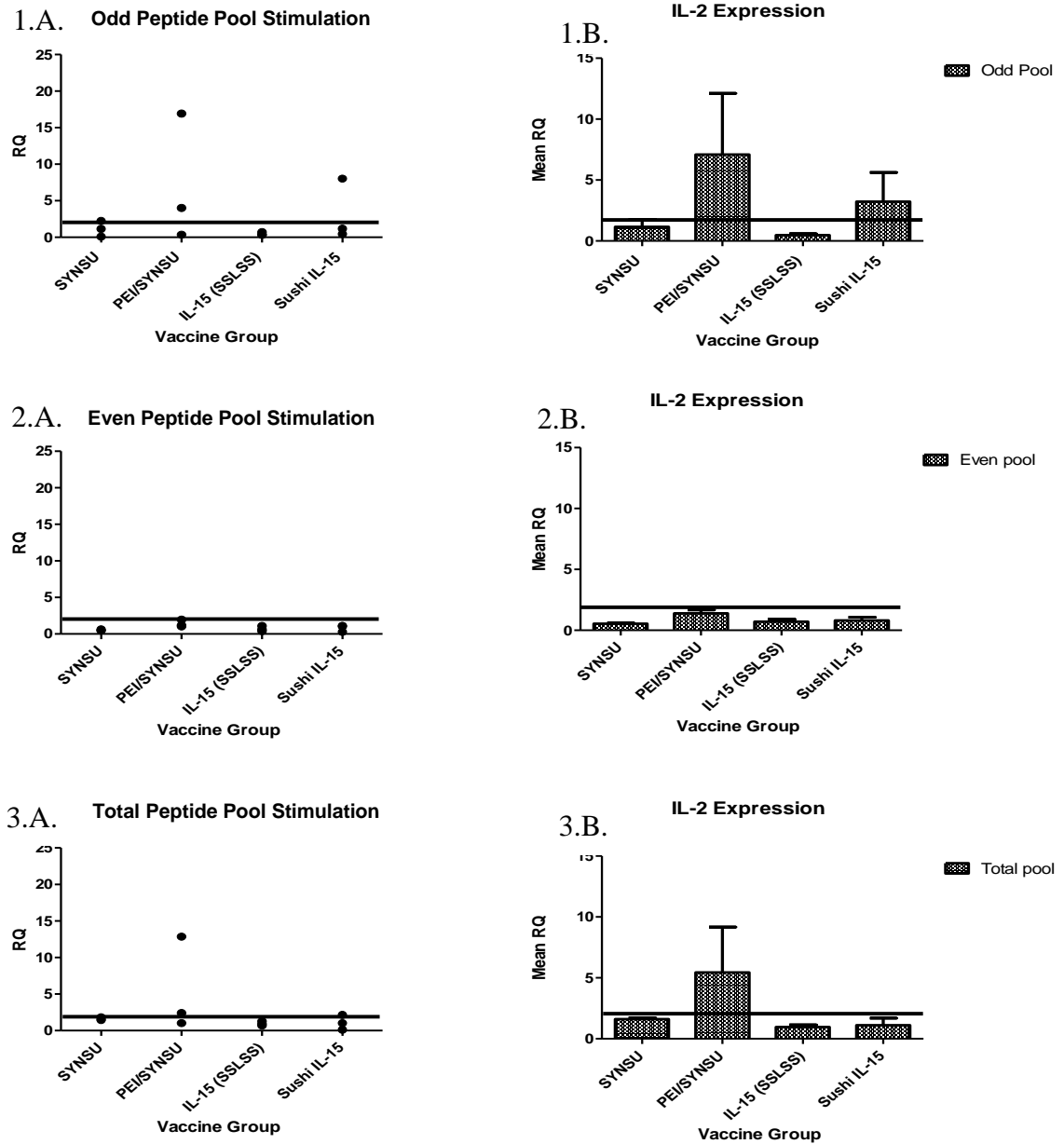
A relative quantification (RQ) value of 2.0 was defined as the lower limit of cytokine expression. As expected, there was no indication of increased IL-2 or IFN- γ expression in preimmunization PBMC from any of the 12 horses or ponies when they were stimulated with each peptide pool (data not shown). Stimulation with SU-specific peptide pools did not elicit IL-2 expression in the naked DNA pSYNSU immunization group (mean RQ values between 0.5-1.6 for odd, even, and total pools), after 24 h stimulation in vitro (Figure 3.40). In the PEI pSYNSU immunization group, IL-2 expression in F36 and C10 increased by 17.5- and 4.8-fold in response to stimulation with the odd numbered peptide pool along with 13.9- and 2.5-fold increases to the complete peptide pool (Figure 3.40). The RQ values for the third member of the group (C06) remained below 2.0 (Figure or Table) (Figure 3.40). Interestingly, IL-2 expression was not detected in the IL-15 (SSLSS) pSYNSU immunization group (mean RQ values between 0.4 and 0.9) and was only detected (an 8-fold increase) following stimulation with the odd numbered SU-peptide pool in one member F06 of the Sushi IL-15 pSYNSU immunization group (Figure 3.40).

Levels of IFN- γ mRNA present after stimulation of equine PBMC by SU-specific peptide pools for 96 h were also investigated in these four pSYNSU immunization groups one week post V4. As expected from the analysis of IL-2 expression, IFN- γ levels were not detected in PBMC collected prior to immunization, upon incubation with any of the peptide pools (data not shown). However, when peptide pools were incubated in vitro with equine PBMC from the pSYNSU vaccinated group, 5- and 6.5-fold increases of

IFN- γ expression levels were detected in two ponies (F37, F41) to odd pool stimulation (Figure 3.41). There was also a mean 3.7-fold change in IFN- γ expression over unstimulated PBMC for the group (Figure 3.41). In contrast to the naked DNA pSYNSU immunization group, peptide stimulation of lymphocyte cultures from animals in the PEI pSYNSU immunization group resulted in 10.1- and 5.4-fold changes in IFN- γ expression levels to odd and total peptide pools, respectively (Figure 3.41). A similar trend in IFN- γ expression was detected in the Sushi IL-15 pSYNSU immunization group to stimulation with peptide pools, with mean fold changes for the group between 3.4 and 5.7 in response to all three peptide pools (Figure 3.41). Interestingly, 12.3- to 24.4-fold increases in IFN- γ expression levels to odd, even, and total peptide pool stimulation were detected for all three horses in the group (Figure 3.41). While G75 was a low responder to odd peptide pool stimulation, IFN- γ levels were still 2.4-fold higher than unstimulated PBMC (Figure 3.41).

Figure 3.40

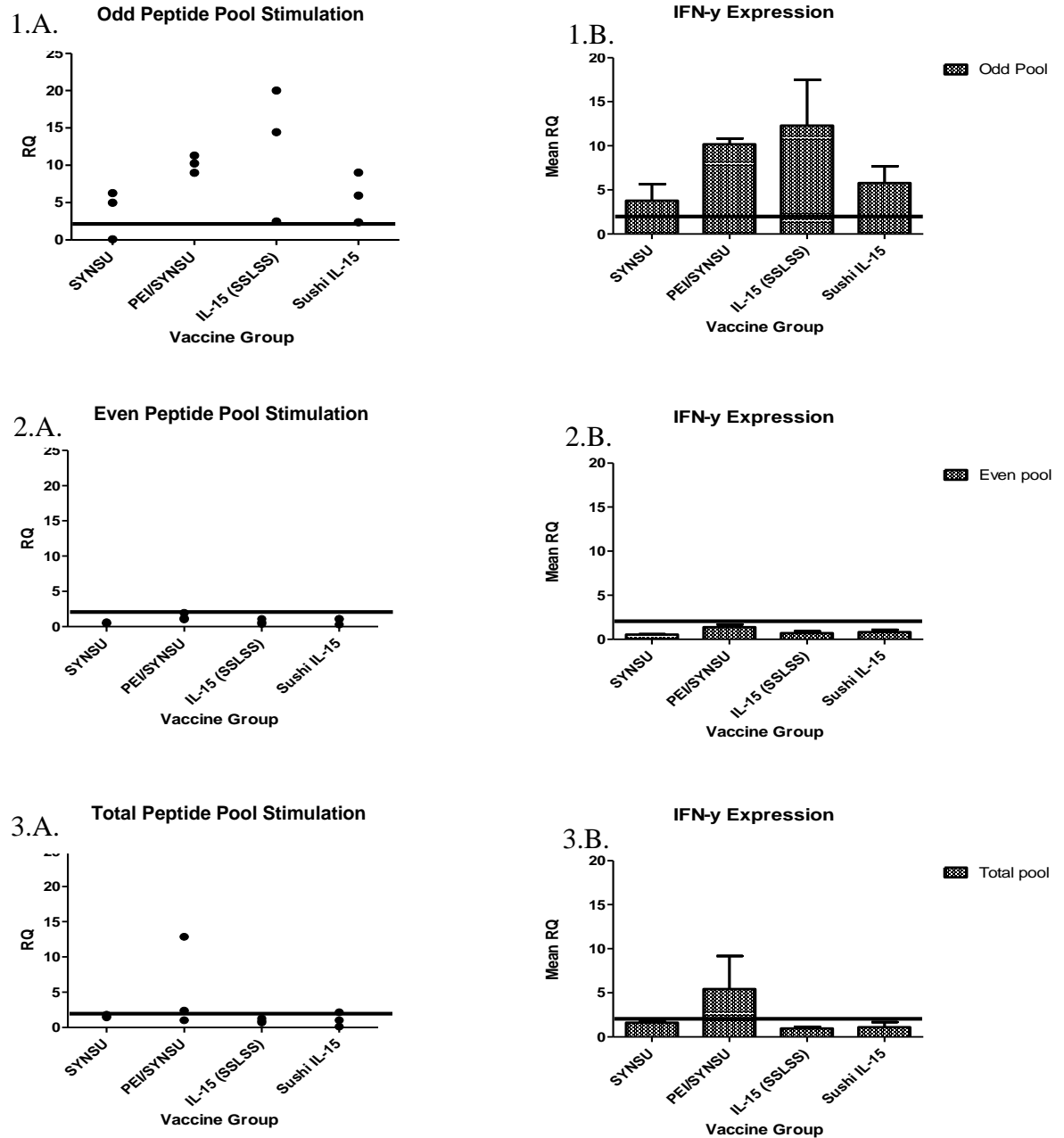
Real Time RT-PCR Analysis of IL-2 Expression in Equine PBMC from pSYNSU immunization groups after V4



Relative quantification (RQ) of IL-2 gene expression in equine PBMC from DNA pSYNSU immunization groups after V4 in response to incubation with peptide pools in vitro: 1.A. RQ of Odd pool, 1.B. Mean RQ of odd pool, 2.A. RQ of Even Pool, 2.B. Mean RQ of even pool, 3.A. RQ of Total pool, 3.B. Mean RQ of total pool

Figure 3.41

Real Time RT-PCR Analysis of IFN- γ Expression in Equine PBMC from pSYNSU Immunization Groups after V4



Relative quantification (RQ) of IFN- γ gene expression in equine PBMC from pSYNSU immunization groups after V4 in response to incubation with peptide pools in vitro: 1.A. RQ of Odd pool, 1.B. Mean RQ of odd pool, 2.A. RQ of Even Pool, 2.B. Mean RQ of even pool, 3.A. RQ of Total pool, 3.B. Mean RQ of total pool

3.5: DNA immunization protocols and maintenance of long-term immune memory in animals

Physical Responses to pSYNSU Immunization after V5

After administration of V5, heat was detectable in all 15 animals for 2-3 weeks and swelling of the skin was noted for 3-4.5 weeks in all vaccinates (Table 3.5). However, differences in the sizes of skin swelling were very evident between groups. Members of the IL-7 and IL-15 (SRLSS) pSYNSU immunization groups demonstrated weak physical responses to vaccination, with the average diameter of skin nodules ranging in size from 85-86 mm (Table 3.5). In contrast, moderate areas of swelling (111 mm) at the injection sites were observed in both animals in the IL-12 + IL-18 group (Table 3.5). The largest inflammatory reactions were observed in the naked DNA, PEI, and the IL-15 (SSLSS) pSYNSU immunization groups, with swelling ranging from 122-127 mm in diameter (Table 3.5).

Table 3.5

**Physical Responses of Horses or ponies to Different pSYNSU DNA
Immunization Protocols after V5**

pSYNSU Immunization Group	Duration of Heat (Weeks)	Average Swelling (mm)	Average Duration of Swelling (Weeks)
naked DNA	2	137	3
PEI	3	128	4
IL-7	3	85	4
IL-12 + IL-18	3	111	5
IL-15 (SSLSS)	2	122	3
IL-15 (SRLSS)	2	86	4

Physical responses to vaccination were monitored weekly after V5, until they completely subsided. Heat was determined by warmth of the skin to the touch. The average size and duration of swelling and the duration of heat represent averages among pSYNSU immunization group members.

Humoral Immune Responses to pSYNSU Immunization (V5)

(i.) Immunoblot Analysis

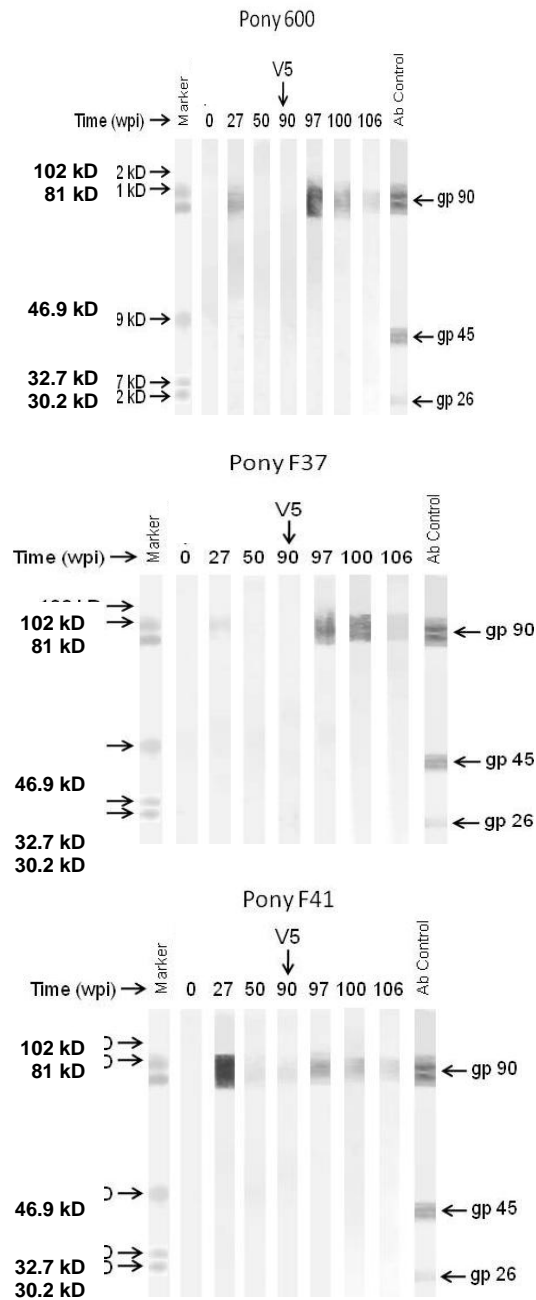
Antibodies to SU were undetectable by immunoblot assay in Ponies 600 and F37 and only barely visible in F41 at week 90, immediately prior to V5 (Figure 3.42). This fifth pSYNSU immunization without PEI induced easily detectable anamnestic responses in all three recipients (Figure 3.42). However, subsequent serum samples collected at 10 and 16 weeks post V5 suggested that these responses were subject to a rapid decline, as judged by their relative intensities (Figure 3.42).

In contrast to the naked DNA pSYNSU immunization group, SU-specific antibodies were readily apparent at 60 weeks post V4 in the two remaining members of the PEI pSYNSU immunization group (C10 and F36) (Figure 3.43). Despite the presence of these very long-lived antibody responses, both animals had clearly defined anamnestic responses to V5 (Figure 3.43). Furthermore, in the case of C10 that was retained for long-term observations, SU antibodies were still readily apparent at 59 weeks post V5 (Figure 3.43). A very similar result was seen with horses or ponies in the IL-15 (SRLSS) (Figure 3.44) or IL-15 (SSLSS) (Figure 3.45) pSYNSU immunization groups. SU-specific antibodies were still detectable in all these vaccinates at 66 weeks post V4, and all animals, with the exception of G75, had anamnestic responses to V5 (Figures 3.44-3.45).

The only other ponies that could be retained for the study were two in the IL-7 pSYNSU immunization group (G22 and G23) and two ponies in the IL-12 + IL-18 pSYNSU immunization group (G26 and G35). Considerable individual variation was observed in these animals. Antibodies against SU were present in G23 and G26 immediately prior to V5 (Week 90) (Figures 3.46-3.47), but not in G22 or G35 (Figures 3.46-3.47). Although all four ponies showed some evidence of an anamnestic response to V5, this varied in intensity (Figures 3.46-3.47). Furthermore, SU antibodies were not readily apparent in G22 or G35 at 59 weeks post V5, while they were clearly present by immunoblot analysis at this time point in G23 and G26 (Figures 3.46-3.47).

Figure 3.42

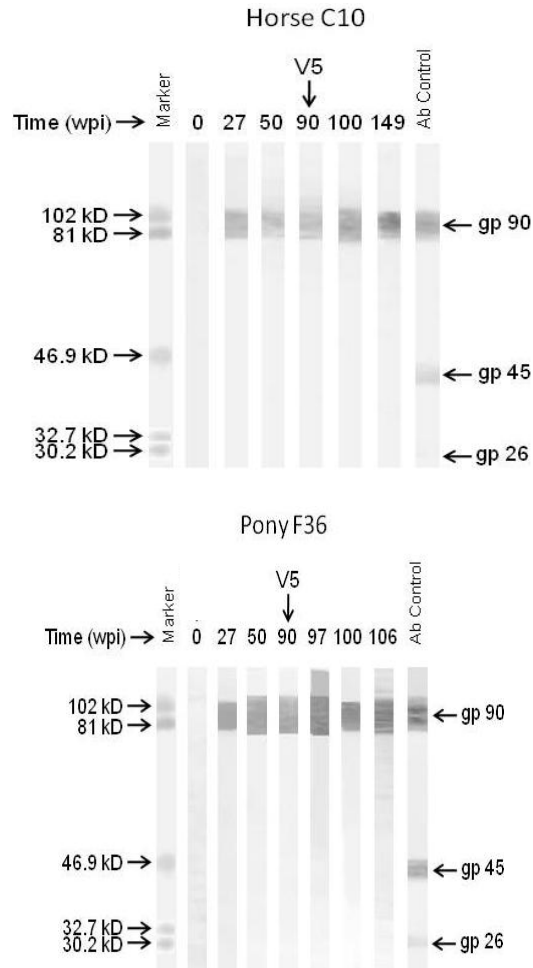
Analysis of SU-Specific Serum Antibody Reactivity from Immunized Ponies in the Naked DNA pSYNSU Immunization Group on IgG Immunoblot



Plasma samples collected at 0, 27, 50, 90, 97, 100, and 106 weeks after immunization from the naked DNA pSYNSU immunization group (600, F37, F41) were diluted 1:20 and analyzed by immunoblot analysis against SDS-disrupted EIAV_{PV}. A reference anti-EIAV horse serum diluted 1:20 was used as antibody control.

Figure 3.43

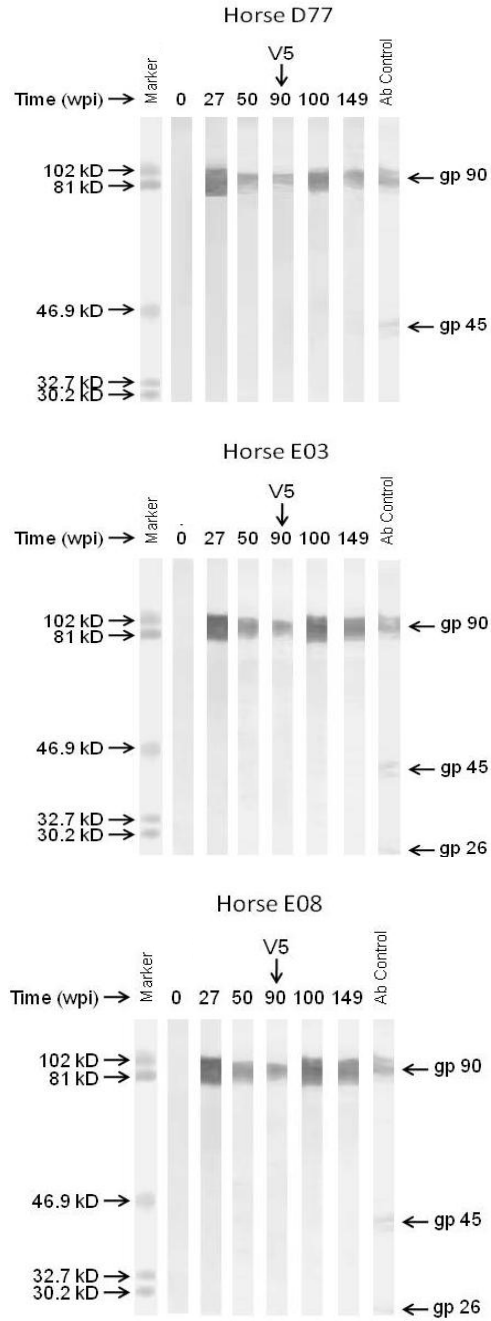
Analysis of SU-Specific Serum Antibody Reactivity from Immunized Ponies in the PEI pSYNSU Immunization Group on IgG Immunoblot



Plasma samples collected at 0, 27, 50, 90, 97 (for F36), 100, 106 (for F36), and 149 (for C10) weeks after immunization from the PEI pSYNSU immunization group (C10, F36) were diluted 1:20 and analyzed by immunoblot analysis against SDS-disrupted EIAV_{PV}. A reference anti-EIAV horse serum diluted 1:20 was used as antibody control

Figure 3.44

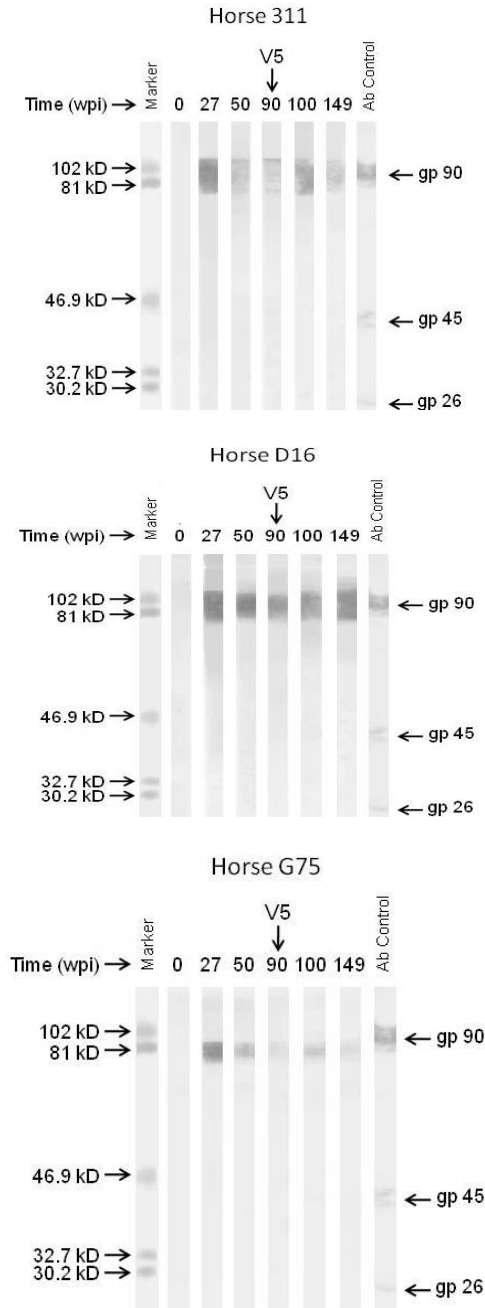
Analysis of SU-Specific Serum Antibody Reactivity from Immunized Ponies in the IL-15 (SRLSS) pSYNSU Immunization Group on IgG Immunoblot



Plasma samples collected at 0, 27, 50, 90, 100, and 149 weeks after immunization from the IL-15 (SRLSS) pSYNSU immunization group (D77, E03, E08) were diluted 1:20 and analyzed by immunoblot analysis against SDS-disrupted EIAV_{PV}. A reference anti-EIAV horse serum diluted 1:20 was used as antibody control

Figure 3.45

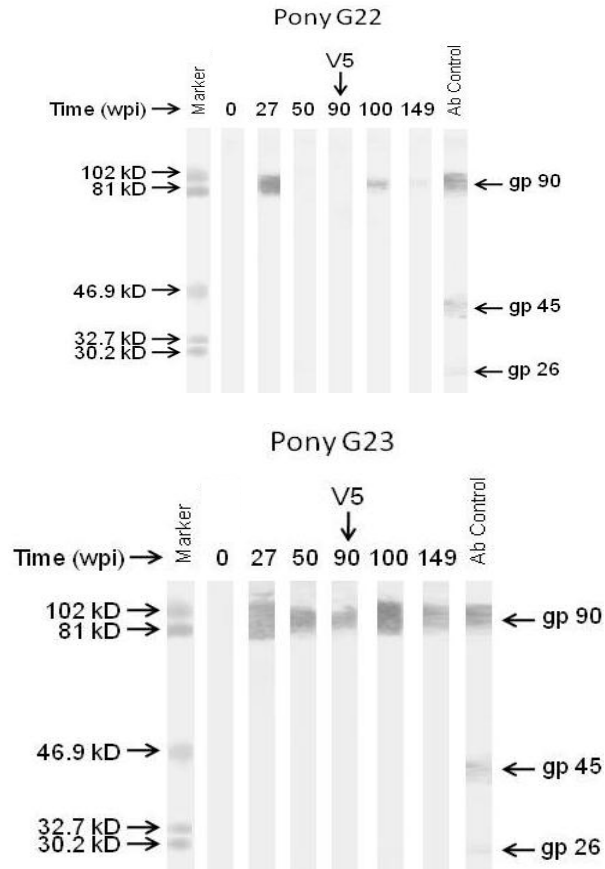
Analysis of SU-Specific Serum Antibody Reactivity from Immunized Ponies in the IL-15 (SSLSS) pSYNSU Immunization Group on IgG Immunoblot



Plasma samples collected at 0, 27, 50, 90, 100, and 149 weeks after immunization from the IL-15 (SSLSS) pSYNSU immunization group (311, D16, G75) were diluted 1:20 and analyzed by immunoblot analysis against SDS-disrupted EIAV_{PV}. A reference anti-EIAV horse serum diluted 1:20 was used as antibody control

Figure 3.46

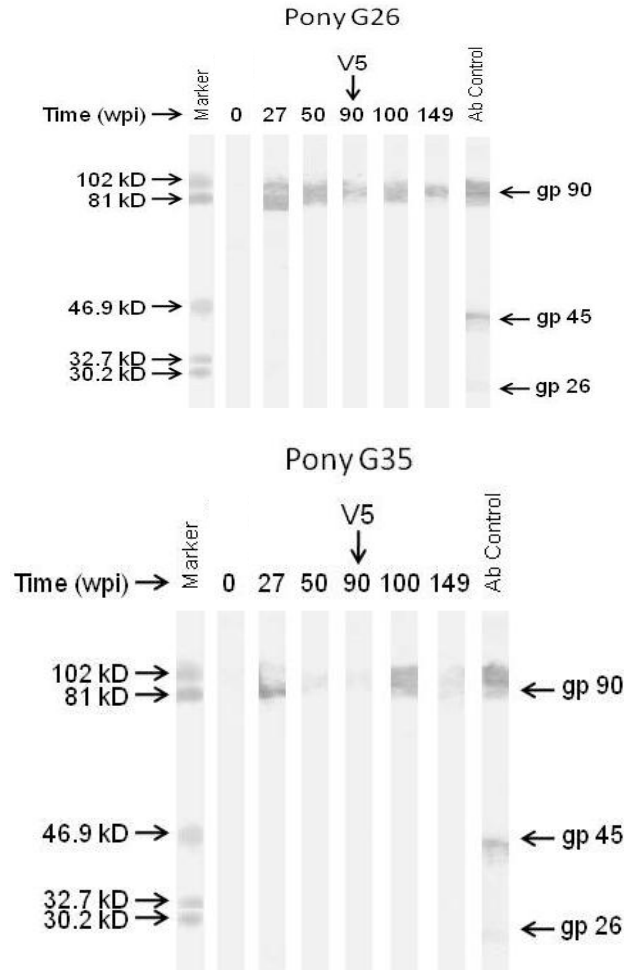
Analysis of SU-Specific Serum Antibody Reactivity from Immunized Ponies in the IL-7 pSYNSU Immunization Group on IgG Immunoblot



Plasma samples collected at 0, 27, 50, 90, 100, and 149 weeks after immunization from the IL-7 pSYNSU immunization group (G22, G23) were diluted 1:20 and analyzed by immunoblot analysis against SDS-disrupted EIAV_{PV}. A reference anti-EIAV horse serum diluted 1:20 was used as antibody control.

Figure 3.47

Analysis of SU-Specific Serum Antibody Reactivity from Immunized Ponies in the IL-12 + IL-18 pSYNSU Immunization Group on IgG Immunoblot



Plasma samples collected at 0, 27, 50, 90, 97 (for F36), 100, 106 (for F36), and 149 (for C10) weeks after immunization from the IL-12 + IL-18 pSYNSU immunization group (G26, G35) were diluted 1:20 and analyzed by immunoblot analysis against SDS-disrupted EIAV_{PV}. A reference anti-EIAV horse serum diluted 1:20 was used as antibody control

(ii.) Analysis of neutralizing antibodies

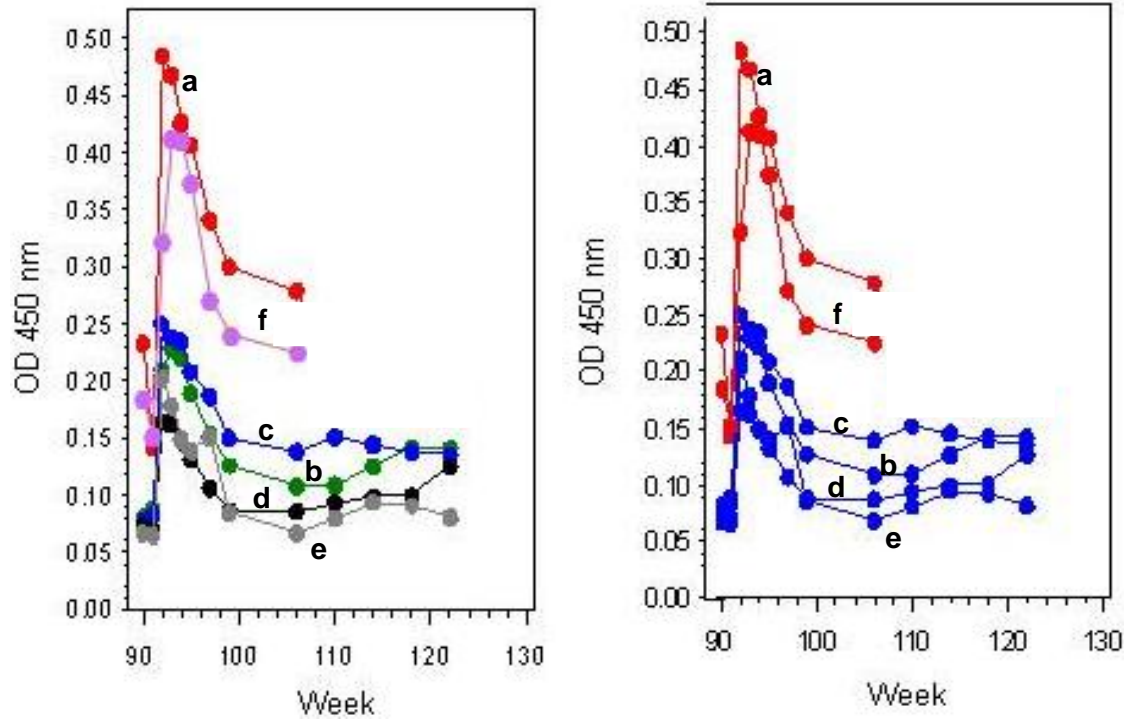
To investigate if antibodies induced by immunization with pSYNSU or pSYNSU + PEI possessed viral neutralizing activity, serum samples collected two weeks post V5 were analyzed in a homologous neutralization assay against EIAV_{PV}. No neutralizing activity was detected in these samples when measured against a viral dose of 100 TCID₅₀ (data not shown).

(iii.) Quantitation by IgG_A ELISA of V5 induced anamnestic humoral responses

Specific anti-SU IgG_A end-point titers in serum samples pre- and post-V5 were measured by ELISA. Statistical analysis of the data was performed by Xia Yu at the University of Kentucky, Department of Statistics, using the mixed procedure with Sas. The IgG_A ELISA results supported the immunoblot analysis, demonstrating that all SYNSU DNA immunization protocols tested were capable of inducing very long-lived (>1 year) memory B-cell populations, as evidenced by the anamnestic responses post V5 (Figure 3.48). Furthermore, the highest relative anamnestic responses were observed in the naked DNA and PEI pSYNSU immunization groups (P<0.05) (Figure 3.48), suggesting the potentiating effects of IL-15 (SSLSS) seen after V3 and V4 are not permanent, but may instead depend on the co-administration of this cytokine.

Figure 3.48

IgG_A ELISA Reactivity of pSYNSU Immunization Groups after V5



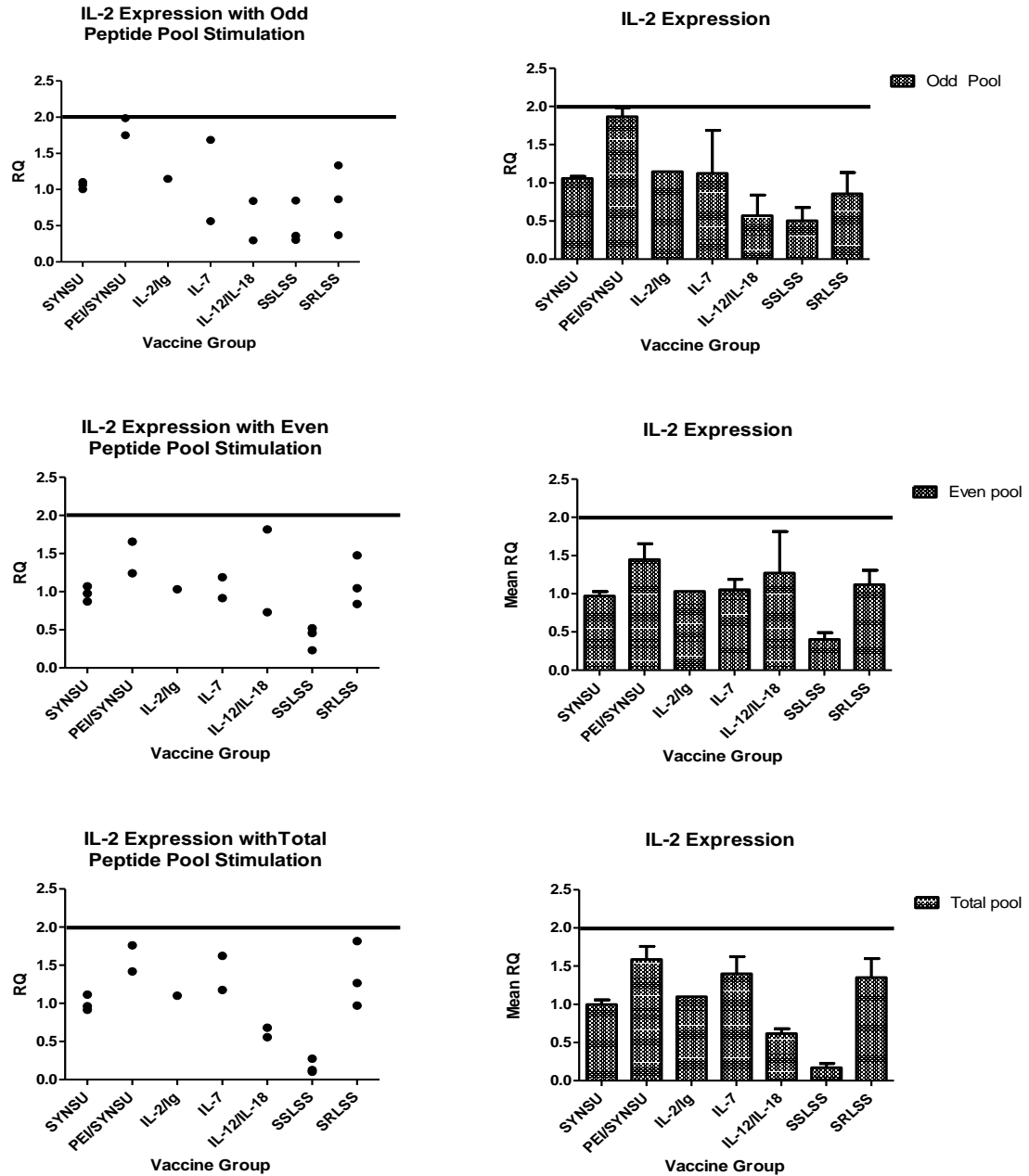
Average serum IgG_A reactivity at 1:40 dilution of pSYNSU immunization groups after V5, as determined by ELISA. Serum was collected at 0, 12, 90, 91, 92, 93, 94, 95, 97, 99, 106, 110, 114, 118, and 122 weeks from the following pSYNSU immunization groups: (a) PEI, (b) IL-15 (SRLSS), (c) IL-15 (SSLSS), (d) IL-7, (e) IL-12 + IL-18, and (f) naked DNA.

IFN- γ mRNA Expression Levels in PBMC Populations post V5

PBMC collected at 5 days post V5 were incubated with SU-specific odd number, even number, and a total pool consisting of all 44 overlapping peptides encompassing SU. Amounts of IFN- γ or IL-2 mRNA were determined by real-time RT-PCR. There was no significant increase in IL-2 mRNA expression ($RQ < 2.0$) in PBMC stimulated with SU-specific peptide pools in any of the pSYNSU immunization groups (Figure 3.49). However, significant increases in IFN- γ mRNA production were observed in SU-peptide pool stimulated PBMC in both remaining pSYNSU + PEI recipients, all three members of the IL-15 (SRLSS) pSYNSU immunization group, and in one horse (311) in the IL-15 (SSLSS) pSYNSU immunization group (Figure 3.50). Furthermore, low but significant ($RQ > 2.0$) increases in IFN- γ mRNA expression were induced in SU-peptide pool stimulated PBMC collected from animals in the IL-7 and IL-12 + pIL-18 pSYNSU immunization groups (Figure 3.50). In contrast, IFN- γ mRNA production was not enhanced in PBMC from any of the naked DNA pSYNSU immunization group members following incubation with SU peptide pools, suggesting this plasmid construct without PEI does not induce strong CTL responses (Figure 3.50).

Figure 3.49

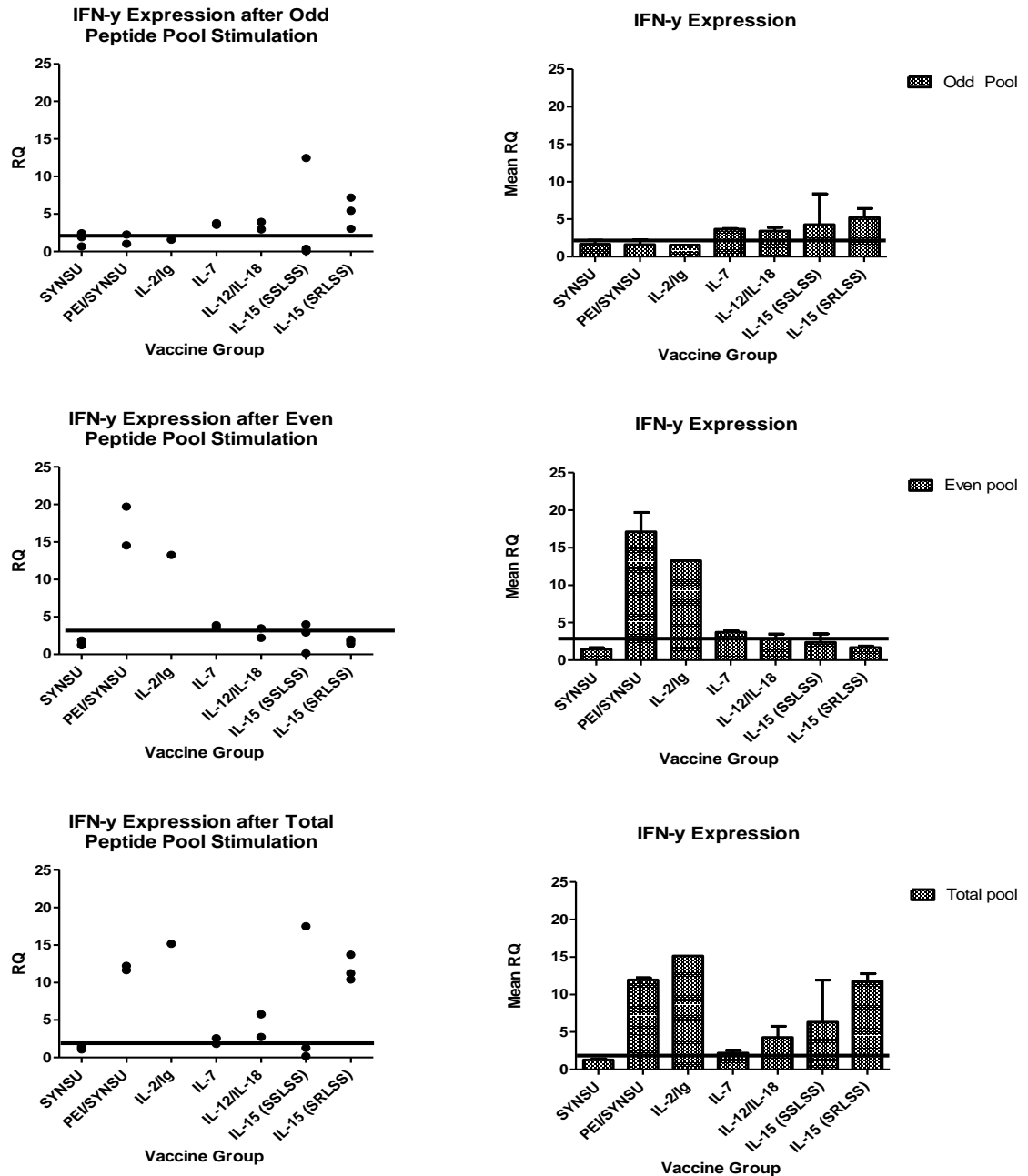
Real Time RT-PCR Analysis of IL-2 Expression in Equine PBMC from pSYNSU Immunization Groups after V5



Relative quantification (RQ) of IL-2 gene expression in equine PBMC from pSYNSU immunization groups after V5 in response to incubation with peptide pools in vitro: 1.A. RQ of Odd pool, 1.B. Mean RQ of odd pool, 2.A. RQ of Even Pool, 2.B. Mean RQ of even pool, 3.A. RQ of Total pool, 3.B. Mean RQ of total pool.

Figure 3.50

Real Time RT-PCR Analysis of IFN- γ Expression in Equine PBMC from pSYNSU Immunization Groups after V5



Relative quantification (RQ) of IFN- γ gene expression in equine PBMC from pSYNSU immunization groups after V5 in response to incubation with peptide pools in vitro: 1.A. RQ of Odd pool, 1.B. Mean RQ of odd pool, 2.A. RQ of Even Pool, 2.B. Mean RQ of even pool, 3.A. RQ of Total pool, 3.B. Mean RQ of total pool.

Lymphocyte Proliferation Responses in PBMC Populations post V5

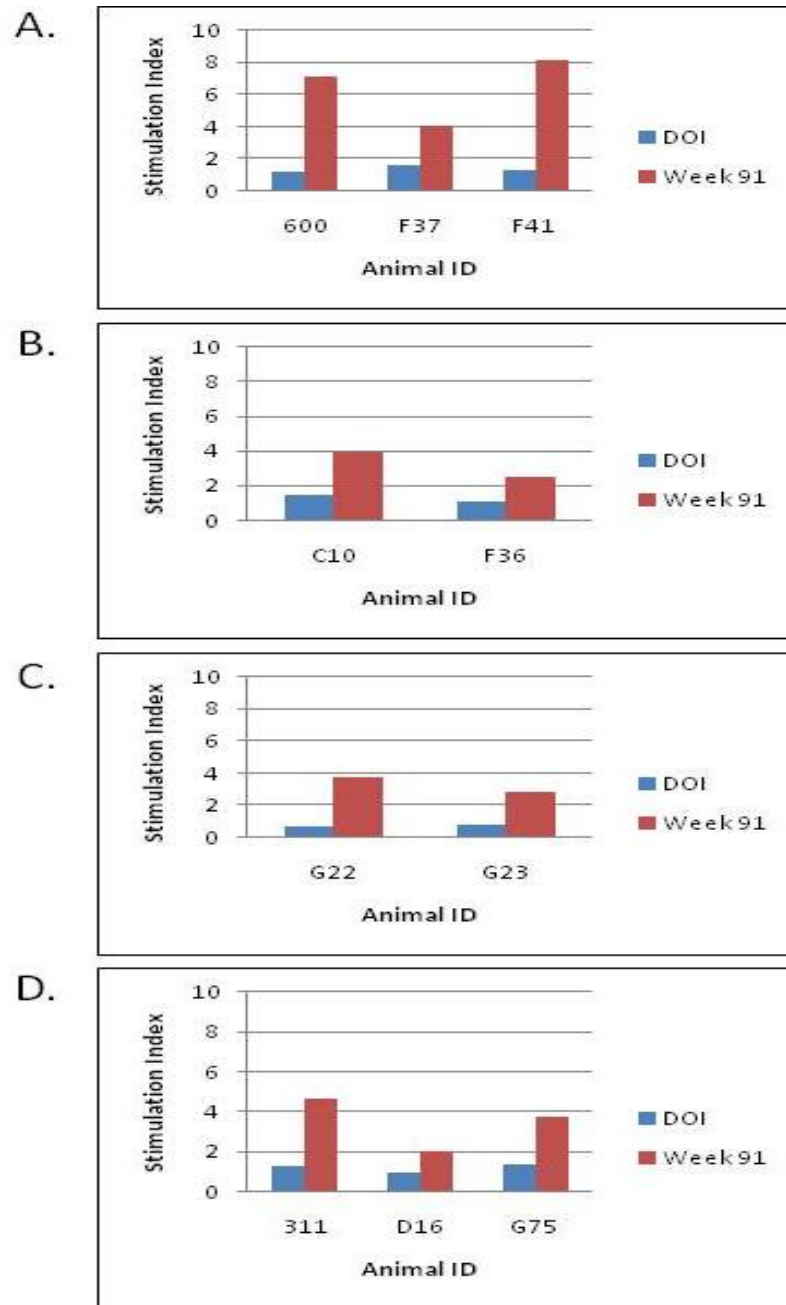
Despite no detectable enhancement in IFN- γ expression, PBMC harvested 5 days post V5 from all of the naked DNA pSYNSU vaccinates did exhibit lymphocyte proliferative responses. PBMC from all members of this group (600, F37, F41) had significant SI levels >2.0 when incubated with gradient purified virus (Figure 3.51), while pony F37 responded to peptides within four separate pools (4, 5, 6, 7) and pony 600 responded to peptides within pools 3 and 7 (Figure 3.52).

Lymphocyte proliferation responses to gradient purified preparations of EIAV_{PV} were also detected in the PEI (C10, F36), IL-7 (G22, G23), and IL-15 (SSLSS) pSYNSU immunization group members (Figure 3.51). However, SI values in these animals did not exceed those observed in the naked DNA pSYNSU immunization group (Figure 3.51). Lymphocyte proliferative responses (SI < 2.0) could not be detected in the IL-12 + IL-18 and IL-15 (SRLSS) pSYNSU immunization group members (Figure 3.51.).

In addition to two of the pSYNSU recipients mentioned above (600, F37) lymphocyte proliferative responses (SI > 2.0) to the SU peptide pools were observed in both members of the PEI, IL-7, and one member of the IL-15 (SSLSS) (G75) pSYNSU immunization group (Figure 3.52). Furthermore, despite the lack of reactivity against gradient purified EIAVPV, two horses in the IL-15 (SRLSS) pSYNSU immunization group also demonstrated weak reactivity to some SU peptide pools (Figure 3.52). However, in contrast to the broad reactivity observed following V4 (Figure 3.38), this was much more restricted post V5 (E03 reacted to SU pools 3 and 5, whereas D77 reacted to pools 1 and 2; Figure 3.52), suggesting some loss in responsiveness. Interestingly, the opposite was apparent in the IL-7 pSYNSU immunization group, as both ponies exhibited low but detectable lymphocyte proliferation to all seven peptide pools (Figure 3.52)

Figure 3.51

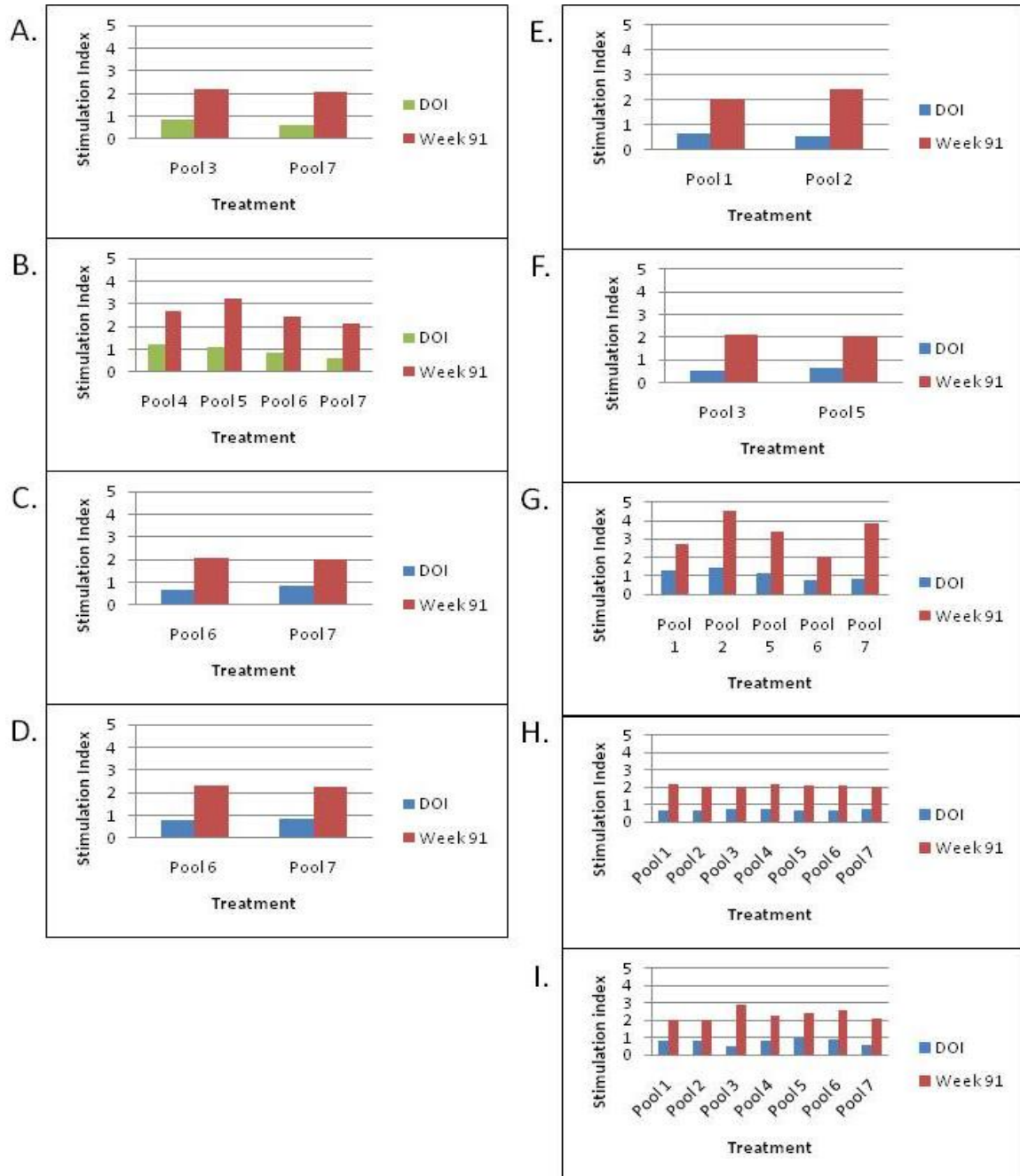
Lymphoproliferative Responses of PBMC from pSYNSU Vaccinates to EIAV_{PV}



Lymphocyte proliferation responses to EIAV_{PV} from animals in the A. naked DNA, B. PEI, C. IL-7, and D. IL-15 (SSLSS) pSYNSU immunization groups in the form of stimulation indexes at Week 0 and Week 91. All represented lymphoproliferation data at week 91 are statistically significant ($P < 0.05$).

Figure 3.52

Lymphoproliferative Responses of PBMC from pSYNSU Vaccinates to EIAV SU-specific peptide pools



Lymphocyte proliferation responses to EIAV SU peptide pools from DNA vaccinates at Week 0 and Week 91: A. 600, B. F37, C. C10, D. F36, E. D77, F. E03, G. G75, H. G22, I. G23 in the form of stimulation indexes. All represented lymphoproliferation data at week 91 are statistically significant ($P < 0.05$).

Chapter IV: Discussion

DNA vaccination has shown great promise and offers a novel approach for stimulating protective humoral and cell-mediated immune responses against a number of viral, protozoal, and bacterial pathogens. Initially, DNA vaccines were investigated because of their potential to induce CTL, as a consequence of antigen expression within cells of vaccinated animals. In fact, this technology has been demonstrated to effectively induce CTL in mice, monkeys, and humans. However, the full potential of DNA vaccines has yet to be realized in all species. A crucial limitation of naked DNA vaccines in larger animals, such as the horse, is its weak immunogenicity. Many of the immune responses elicited by DNA vaccination are not as potent as those induced by other vaccine methods. The reasons for the reduced efficacy of these vaccines are not known, but may involve lower distribution and uptake of DNA and/or differences in how antigens expressed from DNA vaccines are processed and presented in large animals.

Cell-mediated immune responses are believed to play a key role in providing protection against some pathogenic infections in large animals. While DNA vaccines have frequently been reported to generate humoral immune responses in the horse, the induction of cellular immunity is generally not indicated in the literature. Despite the limitations of this technology, the successful application of an equine DNA vaccine against West Nile Virus fueled the DNA vaccine platform in the development of strategies to produce robust immune responses to immunization and better understand the nature of protective immunity against other equine pathogens. It is clear that improvements need to be implemented for this technology to be widely adopted for commercial application in veterinary medicine.

Many potential approaches have been explored to optimize DNA vaccine-induced immunity. In this dissertation, we have focused on and adapted three main successful strategies from murine and non-human primate model systems to enhance the immunogenicity of DNA vaccination, using EIAV SYNSU as an antigen expression system. These strategies include: (1) the use codon optimization of plasmid DNA to increase in vivo expression levels; (2) the formulation of DNA with a cationic polymer, PEI, to protect DNA from nuclease degradation and facilitate cellular transfection; and

(3) the incorporation of immunostimulatory cytokine plasmids to the SYNSU DNA vaccines to further modulate immune responses. PEI is reported to facilitate a 1000-fold increase in cellular transfection of DNA in genetic engineering experiments in vitro in mice. Surprisingly, prior to this work, the adjuvant effects of PEI on vaccination had not been tested in horses or any other species. Therefore, the use of PEI as an adjuvant for the equine DNA vaccination studies described in this work represents a novel and practical application for this polymer. Additionally, while the adjuvant effects of many different cytokines on DNA vaccinations have been demonstrated in other species, the results have been modest when used in horses (Reviewed in Weiner et al., 2008, Nature Reviews). Thus, this work sought to develop comprehensive DNA vaccine approaches that would bolster equine immune responses to immunization.

A key finding of this dissertation work is that plasmid DNA vaccines, encoding the SYNSU antigen expression construct, formulated with PEI generates cellular and long-lived humoral immune responses in the horse, with relatively low doses (250-500 µg) of plasmid. In contrast, previous work has demonstrated that weak lymphoproliferative and moderate antibody responses can be generated in ponies against codon optimized EIAV envelope sequences with multiple, high (1.5 mg) doses of DNA vaccines (Cook, Cook et al. 2005). We also show that the addition of codon optimized IL-15 gene constructs further augment IgG_A antibody responses, expand lymphoproliferative responses, and increase the expression of IFN-γ mRNA levels with in vitro stimulation of PBMC from immunized animals. This may facilitate the development of novel, safer and more effective vaccine adjuvants for use in DNA vaccination of horses against other pathogens.

4.1. In Vitro Effects of Codon Optimization

4.1.1. Effect of codon optimization on the expression levels of cytokine constructs

Codon optimization has become a commonly utilized approach to improve the immunogenicity of DNA vaccines. This technique alters the codon bias of a gene without changing the original amino acid sequence of the protein (Kim, Oh et al. 1997; zur Megede, Chen et al. 2000). Thus, it can result in the enhanced expression of cloned

genes and production of larger quantities of the foreign protein in the immunized individual. The successfulness of codon optimization in stimulating strong immune responses has been demonstrated in several experimental animal models (Nagata, Uchijima et al. 1999; Egan, Charini et al. 2000). When the immunogenic effects of synthetic genes were compared to those of wild-type genes in mammalian DNA immunization trials, the codon-optimized genes elicited robust immune responses *in vivo*, compared to low or moderate responses generated by wild-type genes (Uchijima, Yoshida et al. 1998; Stratford, Douce et al. 2000).

While the approach of codon optimization of antigen and cytokine expression constructs has been commonly utilized in mouse, monkey, and human DNA vaccine models, it has not been used extensively in the horse. Many equine lentivirus sequences and native cellular genes are poorly expressed *in vivo* when utilized with DNA vaccination techniques. This may correspond with low level protein expression *in vitro* (Cook, Cook et al. 2005). However, work done in our laboratory has shown that codon optimization of a lentivirus sequence encoding for EIAV SU markedly improved *in vitro* protein expression, compared to native SU gene sequences, in DNA vaccine studies (Cook, Cook et al. 2005). DNA immunization with codon optimized SU gene sequences also enhanced humoral and lymphoproliferative responses in the horse (Cook, Cook et al. 2005).

As a strategy to further enhance equine immune responses to DNA vaccination, we also generated a number of synthetic cytokine plasmids, to use concurrently with the codon optimized antigen expression construct SYNSU. Due to codon bias, a careful balance needed to be maintained between codon optimization and G/C content optimization in the synthesis of these constructs. Codon optimization can affect negative cis acting sites such as A/T and G/C-rich stretches, which may negatively influence expression. Therefore, prior to producing synthetic constructs, thorough computational analyses were conducted to compare equine gene sequences to highly expressed sequences across many different species to determine modifications which might maximize expression in the immunized host. Although ‘optimized codons’ for protein synthesis may be different depending on the species, most mammals have very similar codon usage (Nakamura, Gojobori et al. 2000).

Native equine cytokine coding sequences for IL-2, IL-7, and, in particular, IL-15 resemble lentivirus sequences in being A/T rich. Therefore, gene sequences for equine IL-2/Ig, IL-7, and three variants of IL-15 (SRLSS, SSLSS, and Sushi), with different in vitro protein expression levels, were modified to conform to a codon usage bias that is similar to highly expressed genes in other mammalian species, including the horse. In addition, gene sequences for IL-12 and IL-18 were also codon-optimized

Although codon optimization also improved expression of equine IL-2, IL-2/Ig, and IL-7, compared to native sequences, the effects were more modest. This demonstrates that A/T rich sequences trending towards an unusual codon-usage bias are not always synonymous with very low protein expression levels. The presence of secondary structural motifs, alternative or cryptic RNA splice sites, and/or adenosine rich instability elements may have more of an impact on protein expression.

This work has also demonstrated that IL-15 sequences are relatively easy to manipulate, resulting in vastly different effects. Codon optimization, along with replacement of native peptide leader sequences, was essential to generate detectable expression of the equine IL-15, in the context of the pCi vector. A ‘family’ of IL-15 plasmids were created that represent low (IL-15 (SRLSS)), moderate (IL-15 (SSLSS)), and high (Sushi IL-15) level expression constructs. A single amino acid substitution at residue 121, changing the sequence from SRLSS to SSLSS, produces significant effects on the expression of this cytokine. Since higher expression levels were detected with the SSLSS variant, it is likely that an arginine residue was assigned to amino acid position 121 in equine IL-15 because of an automatic base-calling error and that serine is the correct amino acid at this site. However, by far the most unexpected results were seen with Sushi IL-15 in vitro expression levels. The attachment of the human TPA and Sushi domain to the IL-15 (SSLSS) plasmid resulted in dramatic enhancement of protein expression in HEK 293 transfected cells compared to expression levels seen for IL-15 (SSLSS).

Gene sequences for IL-12 and IL-18 were also codon-optimized. Moderate levels of the synthetic IL-12 (p40/p35) fusion protein were detected in culture supernatants from transfected HEK 293 cell cultures by immunoblotting, thereby demonstrating that this modified construct was secreted from cells. The synthetic IL-18 construct was also

detected in conditioned medium from HEK 293 transfected cells by immunoblotting. The lower apparent molecular weight protein secreted from transfected cells, compared to the intracellular product, provided strong evidence that the TPA signal peptide was cleaved prior to being exported from transfected cells.

The level of protein expression for the synthetic codon optimized IL-12 (p40/p35) fusion construct was not compared with native sequences. However, immunoblot analysis suggested that significant amounts of this fusion protein were secreted into culture fluids from codon optimized pIL-12 transfected HEK 293 cell culture. Cleavage of the signal peptide in the IL-18 protein was also confirmed by immunoblot analysis of conditioned medium from the synthetic equine pIL-18 transfected HEK293 cell cultures. Furthermore, the fact that these cultures were actively dividing and not showing obvious signs of apoptosis suggests that unlike native IL-18, cleavage of the replacement signal peptide from IL-18 was caspase-1 independent.

Our results show that compared to the native sequences, codon optimization leads to the enhanced expression of the IL-2/Ig, IL-7 and IL-15 cytokine expression constructs. However, it is not known if the differences in synthetic or native cytokine expression levels are attributed to modifications in transcriptional regulation or alterations in the nuclear translocation of mRNAs. Studies by other groups have shown that altering the codon usage bias of gag mRNAs removed multiple inhibitory sequences, eliminated export via the exportin-1 nuclear export pathway, enabled a Rev/RRE-independent expression of HIV-1 Gag, and subsequently increased protein expression, unlike wild-type gag mRNAs which are Rev/RRE dependent for expression (Graf, Bojak et al. 2000; Kotsopoulou, Kim et al. 2000). However, these alternatives were not investigated as part of this dissertation work.

4.1.2. Effect of codon optimization on the biological activity of cytokine constructs

In contrast to native equine IL-15, both codon optimized IL-15 forms, SSLSS and SRLSS, are biologically active in vitro. However, the IL-15 (SRLSS) is significantly less active at inducing lymphoproliferation of equine PBMC than IL-15 (SSLSS). This result mirrors the expression pattern observed by immunoblotting for these constructs. Surprisingly, the Sushi IL-15 construct induced proliferation of equine PBMC, even at a 1:1280 dilution of cell culture supernatant. By comparison, the biological activity of

human IL-15 was only increased 10-fold in vitro by the covalent attachment of the sushi domain from the human IL-15R α chain (Mortier, Quemener et al. 2006). Therefore, this level of enhancement in protein expression represents another novel finding of this study.

Although bio-assays were not performed on the protein expressed from codon optimized IL-12, this construct is identical at the amino acid level to that described by McMonagle et al., 2001, and has been previously shown to be biologically active (McMonagle, Taylor et al. 2001). Biological activity has also been previously demonstrated for the equine IL-18 construct (O'Donovan, McMonagle et al. 2004). Therefore, it was not tested in these assays.

4.1.3. Effect of codon optimization on differential glycosylation of cytokine constructs

Interestingly, proteins expressed from codon optimized pIL-2 and to a lesser extent codon optimized pIL-7 had different migrational characteristics in terms of apparent higher MW in SDS-PAGE, compared with the equivalent proteins derived from native sequences. Differential glycosylation is the most likely explanation for these differences. This laboratory has shown the four potential N-linked glycosylation sites contained within equine IL-7 are probably utilized, and that PNGase F treatment of supernatants from cells transfected with this construct resulted in a single predominant band (Cook, Cook et al. 2008). Equine IL-2 contains two potential N-linked glycosylation sites. The increased shift in molecular weight and multiple banding patterns observed with codon optimized IL-2 were clearly shown to be from differential glycosylation by treatment with PNGase F. This finding suggests that altering the codon usage bias of mRNA may influence its intracellular processing, transport and final destination. There are no published accounts describing the effects of codon optimization on the glycosylation of IL-2. Similarly, codon optimization may also affect the glycosylation pattern of equine IL-7, causing the upward shift in apparent molecular weight visualized in vitro.

Alternatively, the lower molecular weight species observed for codon optimized IL-7 may be produced as a result of an RNA splicing event. Many mammalian expression vectors, including pCi (Promega), which contain the human cytomegalovirus (CMV) major immediate-early gene enhancer/promoter, also possess a downstream

chimeric intron. The presence of this structure flanking the multiple cloning site generally increases expression of ligated exogenous sequences (Brinster, Allen et al. 1988; Choi, Huang et al. 1991; Palmiter, Sandgren et al. 1991). Therefore, because of this arrangement, the 5' splice donor site in the pCi vector may become joined by spliceosomal reactions to one of many potential cryptic splice acceptor sites (An A/G motif preceded by pyrimidine-rich tracts) present in native horse IL-7 sequences (Cook, Cook et al. 2008). Although further studies are required to confirm this hypothesis, modern codon optimization algorithms are designed to eliminate most of the cryptic splice donor or acceptor sites present in native sequences. This is, however, a plausible explanation for the apparent absence of an equivalent protein species in culture fluids from synthetic IL-7 transfected cells.

Examination of the native equine IL-15 coding sequences revealed they were A/T rich (65%), with an aberrant codon-usage bias similar to that seen in EIAV. Furthermore, the unusually long (48 amino acids) signal peptide contains two proteolytic sites. While cleavage at the second site results in the mature secreted form of IL-15, utilization of the first site causes the resultant 19 amino acid longer variant of the protein to be retained in the cell (Kurys, Tagaya et al. 2000). Therefore, in addition to codon optimization for expression in equine cell-types, a process that increased the G/C content to 55.8%, the native signal peptide was replaced with that from human TPA. Since the predicted molecular weight of the mature form of equine IL-15 is approximately 13 kDa (Vector NTI), it is likely that the larger predominant species detected by immunoblotting is glycosylated.

In addition to the very high protein expression observed with Sushi IL-15 from transfected cell cultures, multiple band sizes were also observed on immunoblot analysis. This could be representative of different Sushi IL-15 species, which may be attributed to differential patterns of glycosylation. It should be noted that in addition to three potential N-linked glycosylation sites within equine IL-15, two additional sites occur within the Sushi domain.

4.1.4. Potential applications for the Sushi domain attachment to gene sequences

The overexpression of Sushi IL-15 in vitro raised the question of whether this phenomenon is specific to this particular cytokine construct, or whether it is a general phenomenon. More specifically, could the TPA sequence and Sushi domain be linked to pSYNSU, other cytokine expression constructs, or indeed any other ORF, to substantially enhance subsequent protein expression? If this were a general effect, then the addition of TPA and the equine IL-15R α chain protein coding Sushi domain to alternative sequences would be a very powerful tool in situations where high-level expression of polypeptide was required. This could potentially be utilized as a gene therapy tool. Alternatively, it could also be used for the increased production of cytokines or other proteins in cell culture, useful for drug or enzyme studies. However, many questions remain to be answered: (1) Do the TPA and/or Sushi sequences act as downstream enhancers?, (2) Do the TPA and Sushi elements make the mRNA more stable?, (3) Is the protein overexpression induced from post-transcriptional effects, such as more efficient nuclear export to the rER and Golgi complex or increased translational effects? .

4.2. SYNSU DNA Vaccination Trials

4.2.1. Preliminary investigation of the immunogenicity of PEI and cytokine expression constructs on SYNSU DNA vaccination in ELA-A1 expressing ponies

Individually, the IL-2/Ig and IL-15 plasmid expression constructs have been shown to enhance the immunogenicity of DNA vaccines in mice and monkeys. DNA vaccines expressing an IL-2/Ig plasmid further augmented immune responses in both species, compared to native plasmid IL-2 (Barouch, Santra et al. 1998; Barouch, Craiu et al. 2000; Barouch, Santra et al. 2000). The combination of IL-2/Ig + IL-15/Ig was also found to result in significant enhancement of immune responses in the mouse model over either cytokine alone (Ferrone, Perales et al. 2006). However, these cytokines have not been previously investigated in equine DNA vaccination.

Therefore, a small pilot study was initiated to determine if PEI, IL-2/Ig, and IL-15 could improve the immunogenicity of DNA vaccines in the horse, using EIAV SYNSU as a model antigen expression system. Several ponies were screened and only those that possessed the ELA-A1 haplotype were selected to be vaccine recipients. We expected

that these ponies would all recognize the RW-12 CTL epitope in the pSYNSU DNA vaccines (McGuire, Leib et al. 2003). There is a danger of introducing bias into the results when such small group sizes are utilized, especially since individual variation in immune responses is a common problem in large animal vaccine studies. However, we hoped that a common ELA type might lessen variability in responses to immunization. Although it was difficult to differentiate whether ponies were high or low responders to the DNA vaccines, the data clearly indicated that the addition of cytokines to the vaccine regimen augmented humoral and cellular immune responses.

In the only published information about potential strategies to enhance immune responses to DNA vaccination in horses, rabies DNA vaccination was formulated with the cationic lipid DMRIE–DOPE [*N*-(1-(2, 3-dimyristyloxypropyl)-*N*, *N*-dimethyl-(2-hydroxyethyl) ammoniumbromide/dioleoyl phosphatidylethanolamine] to examine the effects on anti-rabies serological responses induced in the horse (Fischer, Minke et al. 2003). They demonstrated protection could improve anti-rabies serological responses in this species (Fischer, Minke et al. 2003; Minke, Fischer et al. 2006). However, alternative cationic polymers, such as PEI, had not been evaluated, even though for some applications, this secondary amine was shown to be superior to DMRIE-DOPE (Densmore, Orson et al. 2000). Therefore, the immunogenic effects of PEI on equine immune responses to SYNSU DNA vaccinations were examined in this trial.

Naked DNA pSYNSU immunizations elicited weak humoral and cell-mediated immune responses in the horse. These results are entirely consistent with previous findings (Cook, Cook et al. 2005). In contrast, serological responses to SU were detectable after the second immunization in one of the pSYNSU + PEI recipients (B29), suggesting the cationic polymer may potentially augment immune responses to DNA vaccines in at least some animals. However, significant enhancement of SU-specific antibody responses was observed in animals immunized with IL-2/Ig + pIL-15 (SRLSS) (D55, 9954) or with IL-15 (SSLSS) (D49) pSYNSU, receiving 250 µg doses of each plasmid DNA.

In addition to induction of antibodies against SU, there is also strong evidence for the existence of SU-specific CMI responses in the ELA-A1 possessing ponies receiving cytokine plasmids. Significant lymphoproliferative responses to *in vitro* incubation with

gradient purified EIAV_{PV} was observed in PBMC harvested two weeks post V3 from 9954 and D49. Furthermore, significant increases in IFN- γ mRNA levels were induced by incubation with the RW-12 peptide in PBMC isolated from all three cytokine recipients. These results strongly suggest that just three immunizations with pSYNSU + PEI + pIL-2/Ig + pIL-15 (SRLSS) or pSYNSU + PEI + pIL-15 (SSLSS) stimulated both humoral and CMI responses, whereas, in this pilot study, no SU-specific reactivity was detectable following three immunizations with naked pSYNSU.

There is always a danger with very small experimental group sizes that results can be unduly influenced by unusually high or low responders. However, this preliminary data provided strong encouragement to proceed with more extensive studies, a high priority of which was to confirm these preliminary findings. In addition, as stated previously, the pilot study was initiated prior to the discovery of the effects of the SRLSS and SSLSS motifs on equine IL-15 expression. Therefore, it was not known if the enhanced immune responses seen in 9954 and D55 were the result of synergistic effects between IL-2/Ig and IL-15 (SRLSS) or if this variant of IL-15 was expressed at such low levels that it was not biologically active in vivo.

4.2.2. PEI and cytokine expression constructs as pSYNSU vaccine adjuvants in horses and ponies with divergent MHC haplotypes

Results from the preliminary DNA vaccine trial in ponies suggested that the addition of PEI to plasmid DNA did not enhance immune responses in all DNA immunized animals. However, significant increases in immune responses against the SYNSU antigen were detected following delivery of the IL-2/Ig + IL-15 (SRLSS) and IL-15 (SSLSS) plasmid constructs in ponies possessing the ELA-A1 MHC haplotype. The objective of this study was to compare the effects of PEI and selected immunostimulatory molecular adjuvants with a DNA vaccine comprised only of pSYNSU in a diverse population of animals possessing many of the known different ELA haplotypes. An important aim of the second immunization trial was to confirm the results of the pilot study demonstrating enhancement of immune responses by pSYNSU + PEI + pIL-2/Ig + pIL-15 (SRLSS) or pSYNSU + PEI + pIL-15 (SSLSS), compared to inoculations with pSYNSU or pSYNSU + PEI. In addition, possible synergistic effects

between pIL-2/Ig and pIL-15 (SSLSS) were investigated. The effects of pIL-2/Ig, pIL-15 (SRLSS), and pIL-15 (SSLSS) constructs on immune responses were also individually investigated, along with IL-7, IL-12 + IL-18, and Sushi IL-15.

Due to the availability of animals and the design of the vaccine trial, three horses or ponies with different MHC haplotypes were assigned to each group. We had hoped that the formulation of pSYNSU DNA vaccines with PEI, with or without cytokine plasmids, would generate similar humoral or cellular immune responses amongst group members, despite the divergent genetic composition of the animals. However, differences were still observed between individual members in many of the vaccine groups in the intensity of immune responses elicited. Some animals were much higher or lower responders than others. For future studies, it may be possible to replace serological ELA-typing methods with the analysis of polymorphic microsatellites (Tseng, Miller et al. 2010). This recently developed technique has the advantage in that it provides information about MHC class II alleles, in addition to MHC class I. Therefore, the use of this assay should provide a more rational basis on which to interpret humoral and CMI responses to DNA or any other vaccination strategy.

4.2.3. Antigen expression vector or immune-modulator-driven humoral responses

A potentially significant difference in the second immunization trial was the decision to increase the amount of pSYNSU per dose to 500 µg from 250 µg used previously (Cook, Cook et al. 2005) and in the pilot study. It was anticipated that this might induce more uniform immune responses between the individual members of a vaccine group. Although the 2-fold increase in DNA induced no apparent improvements in immune responses for all three recipients compared with previous studies (B33, (Cook, Cook et al. 2005)) during the first three immunizations with naked pSYNSU, it did appear to have a significant effect when plasmid DNA was complexed with PEI.

In mice, single IM injections of plasmid DNA between 10 and 100 µg doses have generated vigorous immune responses to a multitude of antigens (Ulmer, Donnelly et al. 1993; Davis and Whalen 1995; Davis, Brazolot Millan et al. 1997; Fu, Guan et al. 1998). Also, in horses, two 250 µg i.d. immunizations of plasmid DNA expressing the VSV G protein induced neutralizing antibody responses against VSV (Cantlon, Gordy et al.

2000), and four to five doses of pCi SYNSU at 250 µg elicited immune responses in ponies (Cook, Cook et al. 2005). In addition, two 500 µg i.d. injections of bovine herpesvirus-1 glycoprotein D plasmid DNA induced neutralizing antibody responses in cattle (van Drunen Littel-van den, Braun et al. 1998). Furthermore, it is not uncommon for very high doses (2-5 mg) of plasmid DNA to be used in the DNA vaccination of monkeys (Barouch, Powers et al. 2005; Boyer, Robinson et al. 2007; Rosati, Valentin et al. 2008). Therefore, the 500 µg dose of each plasmid, used for DNA immunizations, was within a reasonable dose range of efficacy that has stimulated immune responses in other animal models.

In contrast to the pilot study, humoral immune responses were easily detectable in all three pSYNSU + PEI recipients following the second or third immunization. Furthermore, when measured over the duration of the trial, IgG_A antibody responses in the PEI pSYNSU immunization group equaled or exceeded those detected in many of the animals receiving cytokine expression vectors. This includes animals in the pIL-2/Ig + pIL-15 (SRLSS) pSYNSU immunization group that appeared so successful in the pilot study. Antibody responses of this magnitude had not been previously observed in animals immunized with pSYNSU. Therefore, it is plausible that antigen expression from PEI complexes containing 250 µg of pSYNSU is below the threshold required to trigger immune responses in a majority of the horse population. It is also presumed that at this plasmid dose, co-immunization with cytokine expression constructs, such as pIL-2/Ig + pIL-15 (SRLSS) will significantly augment the development of immune responses. In the initial pilot study with animals possessing the ELA-A1 haplotype, very strong antibody responses to SU were observed in the two ponies (9954 and D55) co-immunized with IL-2/Ig + IL-15 (SRLSS). Weak responses were also detected in ponies receiving 250 µg doses of pSYNSU DNA vaccines formulated with PEI, similar to those seen in ponies immunized with naked SYNSU DNA. However, a very different picture emerged during the second IL-2/Ig + IL-15 (SRLSS) co-immunization experiment, in horses that possessed diverse ELA haplotypes. By increasing the dose of pSYNSU in each vaccine to 500 µg, robust humoral and cellular responses were detected in all three animals receiving vaccines formulated with PEI. These responses greatly surpassed those detected from the horses immunized with 500 µg doses of IL-2/Ig + IL-15 (SRLSS).

This suggests that the immune responses elicited after DNA immunization may be dependent on the plasmid dose.

It is possible that a 2-fold increase in pSYNSU complexed with PEI may generate enough antigen to induce immune responses independently of the majority of cytokines utilized in this second trial, at least at the single DNA dose level of cytokine expression vectors tested. Therefore, the enhanced immune responses may be antigen driven and not cytokine driven. In fact, in terms of humoral immunity, only pIL-15 (SSLSS) induced consistently higher responses than PEI in conjunction with 500 µg of pSYNSU.

Interestingly, animals receiving immunizations formulated with PEI maintained very long-lived humoral responses after four and five DNA immunizations, in comparison to the naked DNA group. This is a very significant finding. The production of robust, anamnestic antibody responses, from the inclusion of PEI in DNA vaccine regimens, may be important to confer protection against some pathogenic microorganisms.

Consequently, these results demonstrate for the first time that the addition of PEI can significantly enhance immune responses to DNA vaccines in the horse. Furthermore, these responses are augmented by the administration of specific equine cytokine expression vectors, such as IL-15 (SSLSS).

4.2.4. Dose effects of antigen or cytokine expression constructs on immune responses

There may be complex relationships between immune responses, the dosage of an antigen expression vector, and the augmentation of these responses by molecular adjuvants, such as cytokines. These relationships need to be more fully investigated in future studies. For example, high levels of antigen expression may overwhelm or act antagonistically against the effects of exogenously expressed cytokines with the possible exception of IL-15 (SSLSS). Therefore, titration of the antigen expression vector may need to be conducted in vivo to fully evaluate the potential beneficial effects of molecular adjuvants.

In addition, titration of putative molecular adjuvants is also required. This is clearly demonstrated in the case of IL-15 (SSLSS) and Sushi IL-15. While IL-15 (SSLSS) enhanced the immunogenicity of pSYNSU + PEI, pSushi IL-15 appeared to produce antagonistic effects against those stimulated by the cationic polymer and

suppress humoral responses, such that they were indistinguishable from the naked DNA pSYNSU immunization group. As Sushi IL-15 demonstrated very strong lymphoproliferative potential in vitro, it is likely that local overexpression of this molecule may account for its ability to suppress humoral responses in vivo, rather than structural changes affecting for example receptor signaling.

Since the efficacy of IL-15 as a vaccine adjuvant in maintaining anamnestic, high-avidity T cell responses to antigens expressed from DNA vaccination has been well documented in rodent and monkey models, similar results were anticipated in the horse. However, the variability shown in humoral and cellular immune responses to the IL-15 family members was unexpected. Different expression levels of equine IL-15 can affect in vitro biological activity and might exhibit similar in vivo effects to promote or antagonize immune responses to pSYNSU. To maximize the adjuvant effects of IL-15 in genetic vaccines, it will be critical to determine the appropriate dose for administration. Co-immunization of rhesus monkeys with various IL-15 doses in an influenza DNA system produced very different immune effects to influenza DNA immunization (Yin, Virology, 2009). In that study, low dose (250 µg) co-expression of IL-15 enhanced CD4⁺ and CD8⁺ T cell proliferation, IFN-γ production, and central memory T cell levels in proliferating CD8⁺ T cells (Yin, Virology, 2009), while high doses of IL-15 (4 mg) produced inhibitory effects (Yin, Virology, 2009). This work demonstrated the importance of optimizing cytokine adjuvants for gene-based vaccination experiments and may be applicable to many other species. Conducting initial titrations on cytokine doses, prior to use in DNA vaccination, may also help increase the overall immunogenicity of this technique in the horse.

In the studies described in this dissertation, plasmid IL-15 family members augmented humoral and cellular immune responses. In particular, the co-expression of IL-15 (SSLSS) increased SU-specific IgG_A antibody responses and IFN-γ expression levels after V4. The high IgG_A levels were indicative of a T_H1-type response, defined by the cytokine production of IFN-γ and IL-2 (Mosmann and Sad 1996). Cell-mediated immunity has been associated with increased levels of TH1-type cytokine production from CD4⁺ T cells. Plasmid IL-15 (SSLSS) may act as a potent immunomodulatory element to augment T-cell responses to DNA vaccination. Furthermore, the low level

expression construct, IL-15 (SRLSS), when co-expressed with pSYNSU + PEI DNA vaccines, expanded the T cell recognition repertoire for lymphoproliferative responses. This was observed, to a lesser extent, with the Sushi IL-15 vaccine group after V4. Although immunization with the IL-15 constructs produced expanded lymphoproliferative responses in vitro to SU-specific peptide pools, they did not augment short-term lymphocyte proliferative responses to EIAV_{PV} stimulation. Interestingly, the IL-7 vaccine group also demonstrated an expansion in epitope recognition after V5. If confirmed in future equine studies, this could have a profound effect on vaccine design for larger animals. The ability to respond to multiple epitopes could be extremely important in the case of pathogens, such as lentiviruses, that have intrinsically high mutation rates.

While lower doses of IL-15 may be more effective at eliciting robust immune responses in DNA immunization studies, the opposite may be true for some of the other cytokines used in this study. For example, in rhesus monkeys, at least four multigenic HCV DNA vaccinations, co-delivering 800 µg of hIL-7 plasmid via electroporation, was required to increase HCV-specific T cell responses, within a 5 month time frame (Park, Song et al. 2010). Furthermore, in a tumor model using LCMV infected mice, 10 µg of exogenous recombinant IL-7 protein administered daily for two weeks was necessary to augment and sustain anti-tumor responses (Pellegrini, Calzascia et al. 2009). Therefore, it is possible the 500 µg dose level of pIL-7 plasmid DNA was not sufficient to enhance antibody and all CMI responses to PEI + pSYNSU. However, future experiments to evaluate pIL-7 dose levels would also need to be conducted using a range of pSYNSU + PEI concentrations.

No significant lymphocyte proliferation was observed in animals receiving IL-7 and only very low proliferative levels were detected in the IL-2/Ig, IL-12 + IL-18, and IL-15 (SRLSS) + IL-2/Ig vaccine groups. It is difficult to believe that all animals in these groups were inherently low responders and so it is possible that immunization with these cytokine plasmids (at the 500 µg/ml dose level), exerts inhibitory effects on short-term (one week post vaccination) lymphocyte proliferative responses to EIAV_{PV} in vitro. The effectiveness of these cytokines have been shown in DNA vaccine studies with other

species, therefore, it would be worthwhile to determine if different doses of DNA could also enhance the immunogenicity of DNA vaccines in horses.

4.2.5. Timing of molecular adjuvants

In addition to dosage considerations, effects on immune responses have also been observed with the timing of cytokine administration in other animal models. The administration of pIL-2/Ig, either before or coincident with an HIV antigen expression vector, suppressed DNA vaccine-elicited immune responses, relative to vaccination without pIL-2/Ig DNA (Barouch, Santra et al. 1998). However, the injection of this cytokine two days after inoculation with the HIV plasmid DNA substantially augmented antibody, proliferative, and CTL responses (Barouch, Santra et al. 1998). Barouch et al., 2000 has also shown that the administration of plasmid IL-2/Ig subsequent to DNA vaccination amplifies antigen-specific immune responses, compared to the simultaneous delivery of cytokine and antigen expression constructs (Barouch, Craiu et al. 2000). A plausible explanation is that the IL-2 receptor was upregulated in these cells by the initial DNA vaccination, which enabled a more robust response after IL-2/Ig delivery. Since IL-2 exerts direct effects on T cells, the timing of cytokine delivery in relation to immune priming might be critical. The same might be true for other molecular cytokine adjuvants.

Antigen presenting cells and T cells receive signals by pro-inflammatory molecules that stimulate adjuvant effects before, during, and/or after antigenic presentation to T cells by MCH complexes. In a comprehensive study with various pro-inflammatory cytokines, proper timing of the adjuvant cytokine DNA relative to the antigen was paramount in determining the magnitude of the immune response and the clinical effects in mice (Ferrone, Perales et al. 2006). Future experiments need to be conducted in the horse to determine whether immune responses to DNA vaccination can be augmented by administering antigen and cytokine expression plasmids concurrently or at different time frames. These optimizations may also depend on the antigen expression system utilized. The immune system might first need to be primed with a specific antigen prior to cytokine amplification to achieve optimal enhancement of immune

responses. This might be critical to optimize the magnitude of the immune responses in vivo and improve the overall immunogenicity of DNA vaccines in horses.

Summary

Only limited reports on the immunomodulatory effects of cytokines adjuvants in the DNA vaccination of horses are available in the literature. The present study differs from previous reports of DNA vaccination in horses in several ways. First, we used the cationic polymer PEI as a known transfection agent *in vitro* to greatly improve the weak immunogenicity of pSYNSU in equine DNA vaccination and compared it to the effects induced by pSYNSU alone. The only previous study with a cationic lipid, DMRIE-DOPE, in rabies DNA vaccination of horses showed no comparison of the immune responses to a 'naked' DNA vaccine group or indication of enhanced cellular responses in vaccinates (Fischer, Minke, 2003). In a later report, a molecular adjuvant, GM-CSF, administered with EHV-1 expression constructs and DMRIE-DOPE, were shown to produce potent neutralizing antibody responses when compared to naked DNA (Minke, Fischer et al. 2006). However, the effects of DMRIE-DOPE on the immunogenicity of EHV-1 DNA without GM-CSF were not tested in that model system. Our studies showed that both arms of the adaptive immune response were induced by PEI, relative to DNA only. Second, we have shown that PEI with or without IL-15 can simultaneously modulate multiple immune parameters, such as antibody, lymphocyte proliferation, and IL-2 and IFN- γ cytokine secretion activity. PEI alone can also significantly impact the onset, intensity, and duration of antigen-specific serological responses, although this may be dependent on the dose level of the pSYNSU antigen expression vector. Third, we have compared the adjuvant properties of a comprehensive panel of plasmid cytokines on immune responses to DNA vaccination and have demonstrated significant augmentation with the IL-15SSLSS construct. Although the effects of native IL-2, IL-6, and IL-12 have been previously investigated in independent DNA vaccine studies, they either affected only humoral responses or were not concluded to be effective molecular adjuvants. The administration of 200 μ g of native pIL-2 in an EAV gene-based protocol elicited substantial humoral responses, however the immunogenic effects of plasmids encoding EAV antigens in the absence of pIL-2 were not investigated (Giese, Bahr et al. 2002). Therefore, this was not a true test of cytokine efficacy. The gene gun approach has also been utilized with influenza DNA vaccines co-expressing IL-6 to enhance

immunity in horses, but the immune responses were primarily directed towards a Th2-type response (Soboll, Horohov et al. 2003). In addition, IL-12 (500ug) has been tested as a molecular adjuvant with *Rhodococcus* and EIAV (p15/p26) DNA vaccine protocols (500ug of each plasmid), with no apparent augmentation in immune response (Mealey et al., 2007). Fourth, we have demonstrated very long-lived, anamnestic memory B and T cell immune responses resultant from pSYNSU DNA vaccination of horses and ponies.

Chapter V: Conclusions

This study has confirmed that gene codon optimization can affect the post-translational modification of the encoded antigen, and documented that the addition of the TPA signal peptide and the sushi domain from equine IL-15R α dramatically enhances IL-15 expression in vitro. In addition, for the first time, DNA vaccination of horses has been demonstrated to induce potent CMI responses, in conjunction with robust antibody responses, simply by the formulation of plasmid DNA with PEI. In the immunogenicity trials, PEI induced strong IFN- γ and IL-2 mRNA expression levels, high anamnestic IgG_A-specific antibody responses, and moderate lymphoproliferation to pSYNSU in all animals after three and four immunizations. This indicates that PEI improved levels of in vivo plasmid transfection and pSYNSU gene expression, compared to controls with equivalent numbers of immunizations. However, many questions remain. The dose level of the antigen expression plasmid clearly plays a major role as shown by differences in results in pSYNSU + PEI recipients in the pilot (250 μ g) and secondary (500 μ g) immunization studies. Indeed multiple exposures to naked DNA eventually results in an improved immune response as seen in the naked DNA pSYNSU immunization group post V5. Therefore, additional experiments will be required to evaluate the dose of both the antigen expression vector and vectors encoding potential molecular adjuvants such as cytokines. Although IL-2/Ig + IL-15 (SRLSS) and IL-15 (SSLSS) augmented responses to immunization with 250 μ g pSYNSU + PEI, only the latter produced significant effects when the dose of pSYNSU + PEI was increased to 500 μ g. Interestingly, at this higher dose of antigen expression vector, IL-15_{SSLSS} and Sushi IL-15 had completely opposite effects on humoral immune responses following co-immunization with 500 μ g of pSYNSU + PEI

Taken together, these are very important findings, which have not been previously demonstrated for gene-based vaccines in horses. Furthermore, the fact that co-immunization with some cytokine molecular adjuvants appears to expand epitope recognition in cellular immune responses to DNA vaccination may if confirmed, have

significant implications for the design of vaccines against pathogens with high mutational capacity such as EIAV and HIV-1.

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