ASSESSMENT OF THE ABILITY OF TWO DIFFERENTLY CONSTRUCTED EQUINE VIRAL VACCINES TO INDUCE SYSTEMIC INFLAMMATION

by

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(Under the Direction of David John Hurley)

ABSTRACT

Equine vaccines generally induce good serum antibody titers, and help control infectious diseases. Vaccine must induce an inflammatory response to initiate the adaptive immune response. However, the nature of the productive inflammatory response is not yet clear. Further, vaccinations can be associated with undesired inflammatory outcomes at the time of vaccination.

In this study, we measured immediate inflammatory activation induced by two commercial equine vaccines relative to environmental controls over the first 48 hours after vaccination. We measured TNF-α and the prosataglandins, PGF2α and PGE2, using enzyme-linked immunosorbent assays. We observed changes in the levels of both TNF-α and the prostaglandins relative to vaccination, but we found that there was no significant change in either relative to the environmental controls or the other vaccine. These studies suggest that neither assay will provide a sufficient test for vaccine induced systemic inflammatory activation.

INDEX WORDS: Systemic inflammation, vaccination, TNF-α ,PGF2α ,PGE2

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DEDICATION

This thesis is dedicated to my family who have always encouraged me to achieve my dreams, and who have supported me since the beginning of my studies. I also wish to recognize my major professor, Dr. David Hurley, who has always encouraged me to achieve my goals, and without whom it would have been impossible for me to complete my studies for this degree. Finally, a most important acknowledgement to the Almighty, without his blessings I cannot imagine where I would stand today.

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CHAPTER 1

INTRODUCTION

Purpose of the study

Vaccines function by establishing a set of conditions in the local tissue that mimic the consequences of an infection. The vaccine must contain activities that initiate the recognition of "danger" related to the agent, "damage" that would be caused by colonization or intracellular growth, and provide a sustained source of antigen that can be sampled and delivered to secondary lymphoid tissues to drive activation and maturation of adaptive immune responses.

A critical part of the function of a vaccine is the establishment of a local inflammatory activation. These inflammatory processes drive many of the tissue level events that are required to successfully induce and sustain a vaccine driven immune response. For this purpose many nonspecific immunostimulants are incorporated into vaccine formulation to enhance body's native or acquired defense system [1, 2]. However, strong inflammatory activation that becomes a systemic process can be the cause of vaccine side-effects or initiate damage leading to vaccine associated illness.

Some anecdotal reports of colic and lameness following vaccination in horses have circulated among veterinarians over the past several years. Colic is a disease that is characterized by local and systemic inflammation. Lameness can also involve inflammatory processes. It appeared to us that it would be worthwhile to assess systemic inflammatory activation by vaccines immediately after their delivery, and see if we could demonstrate any

pattern of inflammatory activation that might provide a link between the inflammation observed and the pathogenesis of disease sequellae.

.In this study, we compared two vaccines that reflect the two extremes in currently licensed vaccine technology (the very classic killed antigen vaccine adjuvanted with Aluminum salts, and a non-replicating virus vector containing defined genes that produces microbial protein in host cells that was stabilized and enhanced with carbopol) for the level and type of systemic inflammatory events that occur in the first two days after vaccination. We used a broad set of systemic inflammatory monitors, as we did not have a clear idea of which of these would provide sensitive or timely probes of vaccine induced systemic inflammation.

Systemic inflammation is associated with the response to infectious diseases. As the vaccination process is designed to mimic the early stages of infection to direct the induction of adaptive immunity, part of the consequences of vaccination is activation of the inflammatory process. Conventional vaccines contain whole micro-organisms, either in the form of killed pathogens or in the form of attenuated, live agents. In either case, components of these agents are recognized in the tissues as part of the vaccination process. Thus, inflammatory interactions between pathogens and the hosts that have been well characterized must be used as models in our investigation of vaccine induced systemic immunity.

Pathogens interact with host cells through a highly conserved group of products that represent their danger in the form of pathogen associated molecular patterns (PAMP) and which are recognized by the host using receptors that recognize the patterns, such as the Toll-like receptor (TLR) family [3].The interaction between PAMP and their receptors are the cellular level regulators of inflammatory processes. Exploitation of these processes is part of the mechanism of successful vaccine action, but may also provide the basis for systemic

inflammatory activation that produces side-effects of vaccines. Many families of PAMP recognition systems are present in the body of the horse. A few will be described here.

Systemic inflammation that is associated with sugar recognition has been described in the pathogenesis of infection and disease in the horse. Mannose-binding lectins (MBL) act as opsonins and are ubiquitous components of the innate immune system identified in a wide range of animal species [4, 5]. MBL acts in concert with MBL-associated serine protease-2 (MASP) to activate complement. Recently it was shown that MBL:MASP activity is reduced in horses with a variety of disease conditions when compared to healthy horses [4].

Endotoxemia is a common problem in foals and many horses with gastrointestinal diseases [6].Circulating endotoxin causes a strong pro-inflammatory activation in the horse through the binding of TLR4 [7]. A cascade of systemic inflammatory products is produced, released, and circulate in the horse with endotoxemia, and these often lead to shock and death. Endotoxin may also cause local activation of inflammatory cascades that lead to the release of inflammatory products into the circulation and have systemic effects [8].

Gram-positive bacteria also signal their dangerous nature through a number of types of PAMP. Their cell wall contains peptidoglycan (PGN) that binds to TLR2 and initiates the production of pro-inflammatory genes [9, 10].Complement, particularly complement protein 3, also binds to peptidoglycan and cleaves into active fragments initiating pro-inflammatory mediator production.[Tizzard's Veterinary Immunology textbook,] PGN can initiate an inflammatory cascade that produces systemic inflammatory mediators, particularly TNF-α and prostaglandins. Gram-positive bacteria can also produce protein toxins that interact with leukocytes to activate cytokine cascades, including the well-studied staphylococcal enterotoxin

B (SEB). SEB binds to leukocytes and induces a storm of cytokine production leading to systemic inflammation [11].

Adjuvants have also been shown to modulate inflammatory activation. Alum has been shown to enhance the activation of IL-1 production and release, to activate complement protein 3 to its active fragments, to enhance prostaglandin production and to induce the production of other cytokines and chemokines [12, 13]. Oil components have been shown to activate inflammatory processes both locally and systemically. Mineral oil is a strong inducer of IL-4 [14]. Oil from the *Quillaja saponaria* tree has been shown to induce the production of interferons and to favor the development of cell mediated immunity [15].The systemic processes involved with this cytokine activation are not clearly established.

Therefore, we plan to utilize what has been learned about induction of systemic inflammation, both in our choice of measurements and in our measurement models, to evaluate systemic inflammatory activation immediately associated with vaccination. We have significant experience measuring inflammatory function in horses. In previous studies an association between systemic inflammation and the pathogenesis of colic and laminitis has been demonstrated [16]. In this study, we conducted a clinical trial to improve our understanding of two of commercially available vaccine technologies available for horses in the market today. We wanted to measure the capacity of the vaccines to induce systemic inflammatory responses that may underlie the adverse response occasionally observed after vaccination during the first 48 hours.

In this trial, we compared a classical alum adjuvanted killed viral vaccine and a poxvirus vectored, carbopol containing viral vaccine for their capacity to induce systemic inflammatory activation. To provide a more comprehensive picture of the inflammatory pattern associated with

vaccination, we measured several inflammatory mediators IL-1β, IL-6, TNF-α, COX-2, IL-8, IFN-γ, MMP-9, RANTES, type 1 IFN, and other mediators including: reactive oxygen species production by isolated neutrophils, prostaglandins (PGF2α, PGE2) production by in whole blood cultures, secretion of TNF-α in whole blood cultures, and production of tissue factor by isolated monocytes. We wished to compare the change in the expression of these inflammatory mediators in the vaccinated groups with the environmental controls as a measure of systemic inflammatory status over the first 48 hours after vaccination.

CHAPTER 2

LITERATURE REVIEW

Role of TNF-α, PGF2α, PGE2 and vaccine adjuvants in inflammation

A. Tumor necrosis factor α

1. General background about tumor necrosis factor α

Tumor necrosis factor-α (TNF-α) was originally identified in 1975 as an endotoxin-induced glycoprotein, which caused hemorrhagic necrosis of sarcomas (as the name suggests) that had been transplanted into mice [17]. Later, it was recognized as a critical inflammatory cytokine that strongly induced fever as a component of systemic inflammation, and it was recognized to have a prime role in inflammation [18]. Though many cells produce TNF-α, it is produced in large quantities by tissue macrophages and mast cells very early in inflammation. This secreted TNF-α acts through two different families of receptors. Both are members of the tumor necrosis factor receptor super family, TNF-R1 and TNF-R2. TNF-R1 is considered to be the more important of these two receptors. TNF-R1 mediates a majority of the biological effects associated with TNF-α stimulation [19]. TNF-α plays a pivotal role in the immune defense against foreign pathogens. In addition, when the body is faced with a strong stimulus, hyper-activation of the TNF-α signaling cascade can result in the development of inflammatory damage and the development of autoimmune diseases [20]. Normally, TNF-α is not detectable in the blood of healthy individuals, but elevated serum and tissue levels are found during periods of inflammation, and during acute infections [21, 22]. TNF-α is one of the very few cytokines that

have a different set of effects when acting at tissue level or at the systemic level. Hence, it is often referred to as a "pleomorphic cytokine".

Following the acute affects of TNF-α, at either the tissue level or the systemic level, during inflammation the body produces a neutralizing response to TNF-α. The serum half-life of TNF-α is for 6-20 min [23]. Neutralization of TNF-α is partly mediated by production of nitric oxide and hydrogen peroxide that activate metalloproteinases (a family of zinc-dependent endopeptidases). These enzymes are involved in removing tumor necrosis factor receptors, and binding and degrading TNF-α itself [24].It has been documented that TNF-α is cleared from the serum primarily by the kidney and the liver [25].

2. TNF-α at the tissue level

At the tissue level, TNF-α is responsible for the "cardinal signs" of inflammation. These are: heat, swelling, pain and redness. During local tissue level inflammation, TNF-α induces pro-inflammatory vascular endothelial changes, initiates a cascade of cytokines, resulting in increase leukocyte adhesion, transendothelial migration, and vascular leakage and promotes thrombosis [18]. TNF-α induces endothelial cells to propagate inflammation by displaying distinct spatial, temporal and anatomical pattern of combinations of the adhesion molecules; E-selectin, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) [26, 27]. TNF-α in combination with other cytokines, IL-8, IL-1β, MCP-1 (Monocyte chemotactic protein-1) and IP-10 (Interferon-inducible protein 10), mediate the recruitment of leukocytes to the site of danger or damage [28]. TNF-α induces the expression of cyclo-oxygenase-2 and the production of prostanoids, particularly PGI 2, resulting in vasodilatation [29]. This is ascribed to the cause of redness and heat at the site of inflammation, by increasing local blood flow. An increase in vascular permeability causes trans-endothelial escape of fluid

and macromolecules resulting in edema or swelling. During inflammation, TNF-α can synergize with the actions of other mediators of inflammation. It has been reported that interaction between lipid mediators, particularly prostaglandins, and TNF-α stimulate human neutrophil activation leading to an increase in the production of superoxide radicals [30].In addition, TNF-α also mediates the secretion of other cytokines including, IL-1 and IL-6.Therefore, this cascade of inflammatory activation is often termed, "cytokine dependent cytokine biosynthesis" [31].

3. TNF-α at the systemic level

At the systemic level, TNF-α serum levels correlate with the severity of infection or damage in the tissues [32]. TNF-α is secreted early in inflammation, but is rapidly degraded in the serum. Its been documented that when the endotoxin (LPS) is injected intravenously, that TNF-α levels peaks around 90 minutes after infusion, and return to the basal levels about 4-6 hours post-infusion [31]. When an animal is exposed to a high dose of recombinant human TNF-α, the result is shock syndrome and severe tissue damage. These findings are very similar to those found in septic shock syndrome [23]. Another systemic effect of TNF-α is the modulation of the endothelial antithrombic phenotype to a thrombic phenotype, by the induction of pro-coagulant proteins, including tissue factor, and the down-regulation the expression of anticoagulant protein, including thrombomodulin. This phenotype promotes intravascular thrombosis [33].Hence, at the systemic level excessive levels of TNF-α can trigger micro vascular thrombosis and cause capillary leakage leading to decreased cardiac output, a reduction in blood pressure, and the induction of shock. TNF-α toxicity causes a syndrome manifested by hypotension, acidosis, oliguria, and hemorrhagic necrosis in vital organs (multi organ failure).The most dramatically affected are the kidney and lung. Another effect of TNF-α is

disseminated intravascular coagulopathy (DIC) [31].High levels of TNF-α in circulation correlate with increased risk of serious complications and mortality [34].

4. TNF-α and acute phase proteins

TNF-α, in concert with the pro-inflammatory cytokines IL-1 and IL-6, act on liver cells to induce a class of proteins, called acute-phase proteins. Acute phase proteins have longer lifetimes in the circulation than cytokines (about 24-48 hours), and have proven to be good intermediate term measures of systemic inflammation. These acute phase proteins include; complement proteins, protease inhibitors (a1-antitrypsin, a1-chymotrypsin, and a2 microglobulin), clotting factors, and C-reactive protein. In the horse, the best studied acute phase proteins are; serum amyloid A (SAA) and hapatoglobin. A rise in serum amyloid-A concentration during severe acute inflammation attracts neutrophils, monocytes and T-cells to the site of infection or damage. Hepatoglobin binds to iron molecules and makes them unavailable to microbes, thus inhibiting bacterial proliferation. This effect of Heptoglobulin indirectly causes anemia by reducing the availability of iron for development of new red blood cells.

5. TNF-α, TNFR-1 and shock

During many gram negative bacterial infections the lipopolysaccharide (LPS) from bacterial cell wall is a potent stimulus for the induction of TNF-α synthesis [31].Its been documented that the lethal effects of endotoxin is predominantly related to excessive production of TNF-α and its release into the circulation [35].TNF-α as the primary mediator of the lethal outcome was confirmed by studies using TNFR-1 deficient mice. The lethal signal was ablated when the binding of TNF-α to its receptor was not possible. These TNFR-1 lacking mice were resistant to the dangerous outcomes to both LPS and staphylococcal enterotoxin-B [36].In mice

bearing TNFR-1, TNF-α production increased to such an extent that it escaped to systemic circulation following local infection, or when the infection entered the bloodstream, resulting in septicemia and septic shock syndrome. Once in systemic circulation it took just few minutes for TNF-α to act on its receptors causing lethal effects, including shock syndrome. TNF-α alone was enough to initiate the metabolic, hemodynamic, and pathological sequelae of septic shock syndrome [23]. Moore and co-workers found that when the serum TNF-α was high due to septicemia, a more severe outcome (generally death) should be expected in foals [37].

Acute, high-level TNF-α exposure results in a syndrome of shock, tissue injury, capillary leakage, hypoxia, pulmonary edema, and multiple organ failure. This may ultimately result in mortality [23].Bacterial infections induce TNF-α which has been implicated in the formation of granulomas. This occurs through the induction of a member of the small inducible gene family, MCP-1(Monocyte chemotactic protein-1) by endothelial cells under the influence of TNF-α [38]. On the other hand chronic exposure to TNF-α by any route results in cachexia, dehydration, depletion of whole-body protein and lipid leading to severe weight loss [23]. So mitigating the effect of TNF-α can reduce or block many of the pathological effects associated with severe inflammation, including shock and vital organ dysfunction [39].

B. Prostaglandins, PGF2α and PGE2

1.A general description of the prostaglandin family

The prostaglandins represent a family of lipid derived pro-inflammatory mediators produced by cells at the site of inflammation. Cells can be activated at the site of inflammation by sugar-binding proteins that recognize; mannose, n-acylated sugars (the scavenger receptor), and asialoglycoproteins to activate the enzymatic conversion of fats into long acting inflammatory mediators. Binding of these sugar-binding receptors promotes increased

respiratory and metabolic activity in these cells [40]. During this stimulated state, these cells produce lipid inflammatory mediators including, prostanoids prostaglandins ,thromboxanes, prostacyclins, radical oxygen species, matrix metalloproteinase enzymes, chemokines and cytokines [41]. Prostanoids are strong mediators of inflammation, and drugs that block their production (non-steroidal anti-inflammatory drugs) are the most commonly used medicines in the world [42].

Arachidonic acid, a 20 carbon polyunsaturated fatty acid, gives rise to the eicosanoid family of mediators. These including: the prostaglandins, the thromboxanes, the leukotrienes and a host of inflammatory metabolites derived from theses products [43]. The type of prostaglandin produced upon stimulation depends on the activity of two enzymes, the cyclo-oxygenase (COX) 1 and 2 enzymes. The enzyme, COX-1 functions primarily in constitutive production of prostaglandin products that are components of homeostatic processes. In contrast, the enzyme, COX-2 is inducible.COX-2 is activated as part of the response to pro-inflammatory stimulation [44, 45]. Several different prostaglandins can be produced by macrophages and endothelial cells when the COX-2 enzyme pathway is activated [46]. COX-2 and another family of enzymes, lipoxygenase, are the two major pathways for metabolism of arachadonic acid.

The release of arachadonic acid is required for production of prostaglandins and leukotrienes [47]. The action of phospholypase A2 on membrane phospholipids releases arachadonic acid, and allows the production of prostanoids (prostaglandins and thromboxane A2) by COX-2 [48]. In addition, arachadonic acid is also a substrate of the 5-lipoxygenase enzyme for the production of leukotreines. Both prostanoids and leukotrienes are important in the regulation of acute inflammation.

2. Prostaglandins in physiological regulation

The metabolic products of the arachidonic acid, particularly PGE2, regulate cellular functions both at the level of physiological homeostasis and by participation in pathological alterations in physiological control [49]. During acute inflammation, PGE2 and PGI2 act as vasodilators. They augment vascular permeability of the capillary bed, in conjunction with histamine and bradykinin, leading to edema [48]. Another product of arachadonic acid metabolism, PGF2α, is an important regulator of reproduction.PGF2α influences luteolysis, ovarian function, luteal maintenance of pregnancy and parturition.PGF2α has also been associated with pathological conditions in acute and chronic inflammation in cardiovascular and rheumatic diseases [50].

3. The role of prostaglandins in inflammation

Studies conducted using a mouse model demonstrated that the inducible prostaglandins produced by COX-2 activation, PGE_2 and PGF2α gradually increase in concentration over the first 24 hrs of acute inflammation [51]. This increase in inducible prostaglandin response was not accompanied by an increase in the constitutive prostanoids produced by COX-1. When LPS was given to rats intravenously, it resulted in an increase the production of PGE2, and the induction of fever [52]. Similar results were obtained when S*higella* endotoxin was injected into mice. An increase in the circulating levels of thromboxane B2 and prostaglandin F2α (PGF2α), PGE2, PGD2, 6- keto-PGF1α (a stable metabolite of PGI2) were observed at 24 hours after treatment [53]. PGE2, a central mediator of fever and is inactivated in peripheral tissues, primarily in the lungs and liver via carrier-mediated cellular uptake and enzymatic oxidation [52]. Similarly, PGF2α is rapidly metabolized in the lungs, liver and kidney into inactive metabolites that are excreted through the urine [50].

It has been demonstrated that PGE2 stimulates macrophages to express inflammatory chemokines and cytokines.PGE2 synergistically augment LPS induced expression of interleukin-1β and interleukin-6 genes in mouse macrophages [54]. However, PGE2 inhibited LPS-induced TNF-α production. It appears that PGE2 functions by activation of adenylate cyclase, leading to an increase camp. This increase in cAMP allows for activation of cAMP-dependent protein kinase that results in down-regulation of LPS-induced TNF-α production in liver cells [48].

PGF2α, PGE2, and PGI have a critical role in eliciting acute inflammation and its related pathology. Understanding the time course of production of prostaglandins and their appearance in the circulation after an inflammatory insult is a pathway to determining the potential severity and duration of that insult. Prostaglandins have an important role in modulating the expression of inflammatory cytokines and modulating their impact during an inflammatory event. In this study, we monitored the production of prostaglandins PGF2α and PGE2 and their endogenous and induced production by circulating leukocytes in whole blood cultures following vaccination as part of our assessment of the severity of systemic inflammation associated with two very differently constructed vaccines. We hoped that the outcomes of these assessments might provide a basis for interpretation of the relationship between systemic inflammatory activation and productive adaptive immune responses to vaccine, or the unwanted inflammatory consequences of vaccines.

C. Vaccine adjuvants

1. General description of adjuvants and their function

The term adjuvant is derived from a Latin term '*adjuvare'* which means '*to help*'. That certain non-specific agents, when associated with antigen that provide the specific targets for adaptive immune responses in a vaccine, can improve those immune responses was recognized

many decades ago by Ramon and Glenny [55]. In their studies, they employed inorganic salts, specifically aluminum hydroxide, to improve the responses of horses and guinea pigs to diphtheria and tetanus toxoids [56].

Since then, many adjuvants have been formulated with the common goal of eliciting stronger immune responses to vaccines. These adjuvants appear to differ in their mode of action, but the actual mode of action of most is currently unknown. It is known that adjuvants can be used to boost the adaptive arm of immune response, and to aid in the generation of effective immunological memory [57]. It is believed that some adjuvants modulate the immune response by modulating the maturation or activation of dendritic cell (DC). There is some evidence that adjuvants can alter the migration, maturation, capacity for antigen presentation, and/or the expression of co-stimulatory molecules on DC. Recently aluminum hydroxide (alum) and the oil-in-water emulsion, MF59, have shown to enhance monocyte differentiation into DCs, by promoting the acquisition of a mature DC phenotype and increased expression of MHC class II and CD86 molecules [58]. In turn, stimulated DC provides a "context" for activation of T and/or B cell mediated immunity. Some adjuvants increase the concentration of MHC molecules on DC, while others alter the balance of cytokine production responsible for inducing the development of Th1 or Th2 responses to antigen [59]. Some adjuvants act directly on T or B cells, stimulating their proliferation and promoting their conversion into memory cells that are critical to duration of immunity [56].There are adjuvants, including alum salts, that fix complement component 3 (C3) as a critical element in their function. Mice deficient in C3 do not have an enhanced immune response to alum adjuvanted vaccines [60].These mice may show deficiency in eliciting good immune response. for this class of adjuvant .

2. Common types of adjuvants

The majority of adjuvants used in commercial vaccines are intrinsic adjuvants. Intrinsic adjuvants are a component of the immunogen preparation, such as cell wall components of bacteria that are part of a bacterin preparation. In some vaccines, including those that contain attenuated live or heat-killed viruses or bacteria, microbial components that activate Toll-like receptors (TLR) are part of the vaccine preparation. Most of the time, these natural adjuvants have been shown to be safe in human vaccine formulation, and have been used experimentally with promising results. On the other hand, another class of adjuvants - the extrinsic adjuvants, are components that are not native to the vaccine organisms added to enhance the immune response. Extrinsic adjuvants often have many limitations on their use, and are added as part of the design to elicit a specific immune response. Among the extrinsic adjuvants that are widely used in human vaccines, only the aluminum salt precipitates, commonly known as alum, is contained in licensed products. [56].

There are significant differences in the common use of adjuvants in human and animal vaccines. First, the only adjuvant that has been licensed for human use in North America is the family of aluminum salts. Second, many different extrinsic adjuvants are commercially available for use in animal vaccines, including aluminum salt compounds, oil in water, water in oil, saponin (*Quillaja saponaria*) derived adjuvants, and at least one cytokine based adjuvant [61]. There are far more vaccines sold based on aluminum salt adjuvants than all other extrinsic adjuvants combined.

3. Alum based vaccines

Alum is most widely approved adjuvant in current use. It is included in many animal vaccines, and is the only adjuvant type used in human vaccines. Aluminum salts come under

subclass of immunopotentiators, classified originally as signal-1 facilitators, which includes ‘‘depot adjuvants.In spite of demonstration that aluminum salts function as adjuvants, the exact mechanisms by which alum enhances the immune response is still largely unknown [13]. Some hold the opinion that alum primarily functions by adsorption of antigen and allowing its slow, equilibrium based release of antigen into the tissue from a depot to extend the exposure of the body to antigen and provide an orderly response [62].

Alum is also an irritant particle that has been shown to activate innate responses both *in vitro* and *in vivo.* The pathway of innate activation has been shown empirically to promote a Th2-biased response and induce increased levels of antibody, primarily IgG1 antibody. Alum induces IL-4-secreting cells in the spleen and polarizes the immune response towards a Th2. [63]. Alum adjuvants have also been shown to increase titers of IgE. It is interesting to note that the preferential stimulation of Th2 by alum is not a function of direct activation of DC. This is confirmed by the unbiased enhancement of cytokine responses *in vitro* by aluminum salts [12].

Alum is not recognized by any members of the TLR family. This supports the finding of Malherbe and co-workers that the depot-forming vaccine adjuvants may not require TLR activation to induce activation of T helper cells that promote a strong immune response [64]. Most recent findings suggest that Alum acts through NLR (nucleotide-binding domain leucine-rich repeat-containing) protein NLRP3 and its adaptor which may activate the inflammasome and caspase-1 [65].There is induced secretion of IL-1β, IL-18, and IL-33 suggesting that activation of the NLRP3-inflammasome may be a common mechanism of action of particulate adjuvants, including alum, and QuilA and chitosan [13].This also suggests that mode of action of alum is not directly through different TLRs.

4. Carbopol based vaccines

Another compound that has been used in animal vaccines is carbopol.Carbopol is a water soluble acrylic acid polymer resin. Carbopol has been used primarily as a stabilizer of protein structures in some designs and as a stabilizer and adjuvant in others. The class of alkyl–polyacrylate esters have shown to be efficient in triggering strong mucosal responses against inactivated Newcastle disease virus (iNDV), inactivated influenza virus strain MRC-11 (iMRC-11), and haemagglutinin/neuraminidase subunits of influenza virus strain A/Texas (HA/NA) [66]. The use of carbopol resulted in secretion of more IgA antibody in mouse studies of viral immunity [66]. In sheep, carbopol adjuvanted vaccines induced higher protective antibody titers than an oil adjuvanted vaccine containing the same antigen preparation of exopolysaccharide from *Staphylococcus aureus*. Yet, there was no significant difference in the level of local or systemic inflammation between the oil adjuvanated vaccine and the carbopol adjuvanted vaccine in the sheep [67]. Similarly, it was documented in pigs that carbopol was less likely to induce vaccine associated pathology than comparable vaccine utilizing oil-in water adjuvant [68].

5 .Efficacy and toxicity of adjuvants

Despite the seminal role of adjuvants in vaccine development, only a few families of adjuvants are regularly incorporated into animal vaccines. In contrast, the literature is full of experimental adjuvants that show promise to stimulate strong protective immunity, but have not been proven to induce an acceptable level of toxicity. A strong triggering of proinflammatory receptors in the innate immune network can lead to the development of the toxic effects, including systemic inflammation [69]. Aluminum adjuvants have been shown to be generally safe and effective for more than half a century; however, these adjuvants have also been

associated with severe local reactions such as erythema, subcutaneous nodules and contact hypersensitivity [70].

The narrow line between an effective adjuvant and a toxic compound in the real world is often discovered only by testing in animals. The difference largely depends on empirical proof that the adjuvant selected is truly compatible with the species in which it is used. Different levels of efficacy and toxicity after vaccination using the same antigen with different adjuvants in same species have been reported in the past [67].

6. Contemporary adjuvant systems.

Recently, a new-generation of vaccines that have been produced in viral vectors designed to induce intra-cellular protein production without viral replication have been developed. The most widely used of these vectors is the canarypox virus in the development of mammalian vaccines. Canarypox virus can be readily grown in avian cells, but does not replicate in mammalian cells [71]. However, the production of proteins by the canarypox vector includes a number of poxvirus proteins that function as adjuvants in these vaccines. This is particularly true for the pox early proteins. In this study, we utilized canarypox vectored carbopol containing viral vaccine and alum adjuvanted killed viral vaccine. Thus, this study provides an important chance to assess a comparison between the most classical adjuvant system and a very recently introduced vaccine with both intrinsic and extrinsic adjuvant with respect to the level of systemic inflammatory activation that they drive. To our knowledge, this is a comparison that has never previously been undertaken and should help in designing safe vaccine delivery system in sensitive animals including horses.

CHAPTER 3

THE BROADER STUDY CONTEXT

A.A summary of additional inflammatory monitoring carried out by others in our laboratory.

This section briefly describes the important findings from the broader set of inflammatory measurements carried out under this study by my colleagues. To provide a larger view of the place of the assessments that I conducted and report in this thesis, I have included a summary of the findings from additional assessments carried out using the same horses and sampling schedule. In this section, I will provide information about the macroscopic signs of systemic inflammation provided by the measurement of clinical signs, and the results of assessments of radical oxygen species production by isolated neutrophils, tissue factor production by isolated neutrophils, and the expression of inflammatory gene measured from the mRNA harvested from isolated total leukocyte populations.

The clinical signs monitored in this study included: temperature, heart rate, respiration rate, bowel sounds, vaccine site reaction, and attitude. A composite clinical score was compiled based on summing the values of the change from basal value for each measurement at each time point monitored. The basal score of all horses in the study was 0, and was based on the values obtained at day -6, -3 and 0.The six-hour observation revealed that the vectored vaccine induced significantly stronger clinical response than the killed vaccine. This was based on greater site reaction scores for the vectored vaccine. The responses recorded at 12 hours after vaccination indicated a further polarization of the clinical signs, with more evidence of fever (and higher fevers), more site reactions, and evidence of increased bowel sounds in the horses given the

vectored vaccine. None of the scores represented severe problems, but all were clear and could be documented. The horses receiving the killed vaccine also developed clinical signs of systemic inflammation. Most occurred at 12 hours after vaccination, and were limited to fever and site reactions to the vaccine. No additional clinical indications of systemic inflammation were observed later than 12 hours after vaccination.

To examine the potential mechanistic processes by which these clinical scores were generated, our group measured a large set of cellular and molecular indicators of systemic inflammation. These indicators were based on: 1) evidence of an *in vivo* response - levels of TNF-α, PGE2, and PGF2α in unstimulated whole blood cultures, and evidence of changes in cellular function utilizing induced TNF-α, PGE2, and PGF2α production in whole blood cultures (this data the will be reported in detail within this thesis), 2),evidence of changes in cellular activation and function measured as the unstimulated or induced production of induced radical oxygen species by isolated neutrophils, and production of tissue factor by isolated monocytes, and 3) changes in cell activation on the gene level (changes in RNA message expression).We examined the changes in these indicators relative to vaccination. Using study, we attempted to develop models that illustrated the correlations between changes in the cellular or molecular indicators and the observed clinical signs. We also attempted to build a case for which of these inflammatory functions were contributing to the observed clinical signs and providing the central basis for development of systemic inflammatory activation.

At the cellular level, we observed no indication of enhanced or depressed production of radical oxygen species (either endogenous or induced) by isolated neutrophils from horses vaccinated with either vaccine relative to controls [73]. All three groups had roughly equivalent endogenous levels of ROS production, on average, over the course of the experiment. The

induced levels of ROS production were very consistent relative to endogenous production both for individual animals within the study and as the mean of the groups. There was considerable animal-to-animal variation in both the endogenous and inducible production of ROS by the horses in this study. This may have masked small changes in cellular function. An explanation for this may be the result of the small sample size and the diversity in the distribution in age, housing and breed of the horses included in the study.

Similarly, we observed no indication of either enhanced or depressed production of tissue factor (TF) by cultured mononuclear cells from the horses in the study due to vaccination with either vaccine. All three groups of horses had rather consistent levels of inducible TF production, but there was considerable variability among individual horses, as we observed with ROS production.

This study suggests that the general activation level and overall response capacity of both monocytes and neutrophils were not significantly affected by delivery of either vaccine. These *in vitro* measurements are indirect and offer more a snapshot of the overall activation level of circulating cells and their total capacity to respond to strong stimuli than describing any specific outcome *in vivo*. They are useful in that they offer a way to see changes in immediate past inflammatory activation in the animal and to predict when a loss or increase in response capacity is likely to be a feature of the horse's response in the immediate future.

Statistical assessment of the expression of the cytokine genes for IL-1β, IL-6, IL-8, RANTES, IP-10, IFN-gamma, TNF-α, COX-2, INF−α, and INF-β from the total circulated leukocyte population derived directly from the blood samples of the horses relative to vaccination with either the vectored vaccine or the killed vaccine demonstrated significant activation relative to controls for most of the genes at 6 and 12 hours after vaccination. There

was differential activation of these genes by the two vaccines relative to each other as well. The type 1 interferon genes, IFN-α and IFN-β, the IFN-γ gene had higher levels of expression in the circulating leukocytes from the horses in the killed vaccine group than those in the vectored vaccine group, and expression of the IP-10 gene was significantly higher in circulating leukocytes from the horses in the vectored vaccine group than those in killed vaccine group at 6 and 12 hours after vaccination.

Again, this summary of the results from the other components of the project is provided to allow the reader a broader context for understanding my contribution. The results presented in the body of this thesis represent my work, but were only a component of the larger project in which I was involved. I do not wish to leave the impression that I conducted the full body of the work involved in the project as a whole. However, I do not believe that it is fair to the reader to receive my findings totally out of the context of the larger study.

CHAPTER 4

MATERIALS AND METHODS

A. Horses

Twenty five horses were selected from three pools of available horses owned by units of the College of Veterinary Medicine or College of Agriculture and Environmental Sciences after screening to determine that they had normal basal inflammatory responses. Five were chosen from the herd owned by the College of Veterinary Medicine and 20 were selected from herds owned by the College of Agriculture and Environmental Sciences. All the horses were housed on pasture in Athens, Georgia, USA. The horses ranged in age between 2 and 23 years old. The horses were of several breeds; Thoroughbred, Quarter Horse, Missouri Fox Trotter, Paint and Appaloosa. Five were male and twenty were female. All the horses were vaccinated for Rabies and West Nile viruses within the previous year.

B. Blood samples

Blood was collected from the jugular vein after preparation of the skin to minimize potential contamination. Twenty ml of blood was collected for each sampling. Samples were collected at seven different time points; days -6,-3, 0 pre-vaccination, and 6, 12, 24, and 48 hours post-vaccination. The samples were collected using heparin as an anticoagulant.

C. Vaccines

Two commercial vaccines were assessed in this study. An alum adjuvanted killed viral vaccine (killed vaccine), and a Canarypox vectored carbopol containing viral vaccine (vectored vaccine).The vaccines were purchased from a commercial supplier and all of the doses of each

vaccine were from the same manufacturer's lot. The vaccines were handled and stored according to the manufacturer's label directions. The vaccines were delivered by one person with the rest of the research team blinded to the treatment of each horse.

D. Biological stimulants utilized in the in vitro assessments

Whole blood was stimulated in microcentrifuge tubes using the stimulants listed to assess the level of TNF-α, PGF2α, and PGE2 produced in culture.Lipopolysaccharide from *E.coli* O111: B4 (LPS, List Biological Laboratories, Inc.) at 1, 10 and 100 pg/ml ,peptidoglycan from *Staphylococcus aureus* (PGN, Biochemika,fluka) at 10, 100 and 1000 ng/ml , staphylococcal enterotoxin B (SEB, Calbiochem, CA) at 1000 ng/ml, and phorbol 12-myristate 13 acetate (PMA, Sigma, St. Louis, MO) at 10^{-7}M were utilized in these assays [72]. For measurement of endogenous secretion of TNF-α, PGF2α, and PGE2, whole blood that was not stimulated with any of the above was utilized.

E. Induction of TNF-α production in whole blood cultures

Whole blood stimulation assays were performed in sterile microcentrifuge tubes to determine the impact of vaccination on the induced production of TNF-α.50 μl of LPS was added to 450 μl of whole blood to yield final concentrations of 10 pg/ml, 100pg/ml and 1000 pg/ml in the culture. In parallel, PGN was added to tubes of whole blood at 1 ng/ml, 10 ng/ml, and 100 ng/ml per ml, and PMA was added to tubes of whole blood at a concentration of 10^{-7} M. Control tubes were prepared by 50 μl of PBS to similar tubes of whole blood. The samples were incubated at 37°C for 5.5 hours on a shaker. After incubation the microcentrifuge tubes were centrifuged at 8000rpm for 8 minutes at room temperature. The plasma was removed and transferred to new, sterile microfuges tubes for storage at -80°C.

F. Induction of prostaglandin production in whole blood cultures

To induce the production of prostaglandins whole blood was incubated with each stimulus in microcentrifuge tubes. LPS was added to whole blood at a final concentration of 1ng/ml, SEB at a final concentration in whole blood at 1μg/ml, and PMA at a final concentration of 10^{-7}M in whole blood. Control tubes were prepared by adding PBS to the same volume of whole blood. The samples were incubated at 37°C for 21 hours on a shaker. After the incubation the tubes were centrifuged at 8000 rpm for 8 minutes. Half of the plasma collected was subjected to immediate processed for the prostaglandin assays, and the rest of the plasma was stored at -80°C for future experiments.

G. TNF-α assay

To measure the level of TNF-α in plasma, we utilized an enzyme-linked immunosorbent assay (ELISA) based on a commercially available pair of antibodies recognizing equine TNF-α. Immunlon 4HBX 96 well plates (Thermo, MA USA) were coated with polyclonal antibody, PETNFAI (Endogen) at 1mg/ml in pH 9.6 carbonate buffer overnight at 4^{o}C.The plates were washed with phosphate buffered saline (PBS) containing 0.05% tween 20 (wash buffer).The plates were blocked with 1% bovine serum albumin added to PBS (Blocking Buffer) by incubation at room temperature for 1 hour. To conduct the assay, frozen plasma samples, positive and controls were thawed at room temperature, diluted at 1:5 in sample buffer (1% BSA in wash buffer) and incubated at 37^{o} C for 1 hr. Recombinant equine TNF-α (Endogen) was used as a standard by serially diluting the protein from 2500pg/ml to 39.05 pg/ml in sample buffer. The standards, plasma that was spiked with TNF-α, and the experimental samples were placed in quadruplicate well of the ELISA plates. The plates were incubated at 37^{o} C for 2 hours. The plates were washed three times with wash buffer. Biotin labeled polyclonal antibody PETNFABI

(Endogen) at 0.25 mg/ml was added to all wells of the plate, and the plate was incubated for 90 minutes at 37^{o} C. The plates were washed four times with wash buffer. A preparation of avidin-HRP (BD Biosciences, Pharmingen, San Diego, CA) at a 1:5000 dilution in sample buffer was added to each well. The plate was incubated at 37^{o} C for 1 hr. The plate was washed four times with wash buffer. ABTS, (2 component kit from KPL ,MD,USA) a peroxidase substrate containing 2,2'-Azino-di-(3-ethylbenzthiazoline-6-sulphonate), and hydrogen peroxide diluted from a fresh 30% stock to a final concentration of 0.03% was added to each well (100µl) of the plate. The plate was incubated for 30 minutes, or until intense color developed in the positive controls (which ever was shorter), in the dark to allow color development. Plates were read using an ELISA plate reader (Dynex MRX II, Chantilly, VA) at 405nm when the highest concentration of the standard well in different plates showed strong color development; this yielded a reading between 1.3-1.6 OD in most cases.

H. Prostaglandin assay

PGF2α and PGE2 were measured using ACE competitive Enzyme Immunoassay kits from Cayman chemicals (Ann Arbor, MI, USA).Plasma from induced whole blood samples were utilized as the samples for this assay. On the day the assay was performed, 100 µl of plasma was added to 900 µl of methanol, vortexed for 30 s, and the methanol was removed by evaporation in an evacuated centrifuge (Centravap, Labconco, Kansas City, MO).Concentrations of PGE_2 and PGF2α were determined using the appropriate ELISA kits and the concentrations of samples were determined relative to the standards provided in the kit.

I. Statistical analysis

The endogenous and induced TNF-α and prostaglandin activity results were analyzed using the Two-way ANOVA routine with 1og transformed (log_e for TNF-α and PGE2, whereas

log $_{10}$ for PGF2α) and ranked data in SAS V9 software (Cary, NC, USA).We wished to assess the effect of time after vaccination and vaccine group on the level of TNF-α or prostaglandin secretion relative to the clinical measurers of systemic inflammation. The effects of time and vaccine group were assessed to determine if they were independent or interactive co-variables with respect to clinical systemic inflammatory response.

Therefore, we included time and vaccine group as an interaction term in each of the iterative models used to assess the data. Two different two-way ANOVA protocols were utilized to determine the better fit relative to the heterogeneous variance between the treatment groups and with respect to the outlier data. In one model, $\log_{e \text{ or } 10}$ transformed data was assessed, and in the other ranked data, using a non-parametric test, was used. Each was run under SAS by with PROC MIXED or PROC RANK routines, respectively. We constructed a two-way ANOVA model predicting dependent variables of concentration of (Y) TNF-α or Prostaglandins PGE2, PGF2α in the whole blood cultures for both endogenous and induced levels on the vaccine group or other biological stimulants (X, independent variables). Thus, we generated a clear picture of the role of the TNF-α and prostaglandin responses of the 25 horses. The distribution of each set of quantitative variables was tested for normality. As the data that did not have a normal distribution, it was log transformed before analysis. The outcome of transformed data was reported as least square means (LSMEANs) plus or minus the standard deviation (SD) of the log transformed values. Additionally, for TNF-α assays , a second Two-way ANOVA model based on time and vaccine group using the ranked data to generate median values for a nonparametric model of the dependent variable, TNF-a, was tabulated for comparison. The level of significance was set at $P<0.1$ in both models due to small sample size.

CHAPTER 5

RESULTS

This thesis will report the findings for the level of TNF-α and prostaglandin, both endogenous and induced, from whole blood cultures that represent components of a larger study. We will compare these finding with the rest of the data generated by the whole study in the discussion, but this set of data will be treated as an independent investigation for the purposes of this thesis. This thesis will address how well endogenous and induced TNF-α and prostaglandin assessments from whole blood cultures function as reporters of vaccine induced systemic inflammatory activation.

The results presented here will comprise a detailed report the levels of TNF-α and prostaglandin produced in whole blood cultures relative to the vaccine period in this study. The data will be split by time relative to vaccination. The data collected at 6, 3 and 0 days pre-vaccination will be analyzed both across the three groups by day and across the groups as a pool of the three days to provide an assurance that the three groups were fairly divided. As the assessment of the pooled and day 0 data indicated that there were no significant differences among the three groups prior to vaccination, we presented the day 0 data as baseline. A detailed summary of this statistical analysis is presented in Appendix A and B. The reminder of the data, collected after vaccination, will be presented as time individually relative to baseline. Both the TNF-α and prostaglandin data will reflect two types of information from whole blood cultures. The first, endogenous level of inflammatory activation based on the quantity of TNF-α or prostaglandin measured in the plasma from unstimulated whole blood. The second, inducible

production of TNF-α or prostaglandin based on measurement of plasma from whole blood cultures stimulated with lipopolysaccharide (LPS, for both TNF-α and prostaglandin), peptidoglycan (PGN, for TNF-α), staphylococcal enterotoxin B (SEB, for prostaglandin), and phorbol myristate acetate (PMA, for both TNF-α and prostaglandin). As previously reviewed, LPS, PGN and SEB are microbial triggers of inflammation and were used to reflect the effect of the vaccines on the ability of blood cells to mount an immune response. PMA is an intracellular stimulator of signaling through maximal activation of protein kinase C. PMA drives a maximal response and is used as a "yard stick" of inflammatory capacity.

The raw data will be summarized in line graphs. Each vaccine groups and the control group will be shown as an individual line with the standard error of the mean of each data point included as an error bar. The raw data was log transformed and ranked for assessment by two way ANOVA. Significance was accepted at $P<0.1$ because of the small sample size. A summary of the ANOVA findings are presented in Appendix A and B. In the graphs presented in the body of this thesis, significant findings for log transformed comparisons are indicated with a *, and those established using ranked data are indicated with a #. When both assessments concur, both a * and a # will be displayed.

A. TNF-α measurements.

1. Endogenous production of TNF-α in the whole blood cultures comparing the groups receiving vectored and killed vaccines relative to the controls.

We measured the endogenous production of TNF-α in whole blood cultures for each group of horses by ELISA. The raw data was calculated in pg/ml by comparison to a standard curve using equine recombinant TNF-α.The data represented in the graph below presents the mean and standard error of the mean for the measurements at each assessment point within each

group. Significance was determined using either $\log_e$ transformed data or ranked data by ANOVA and is represented using the sign convention described above.

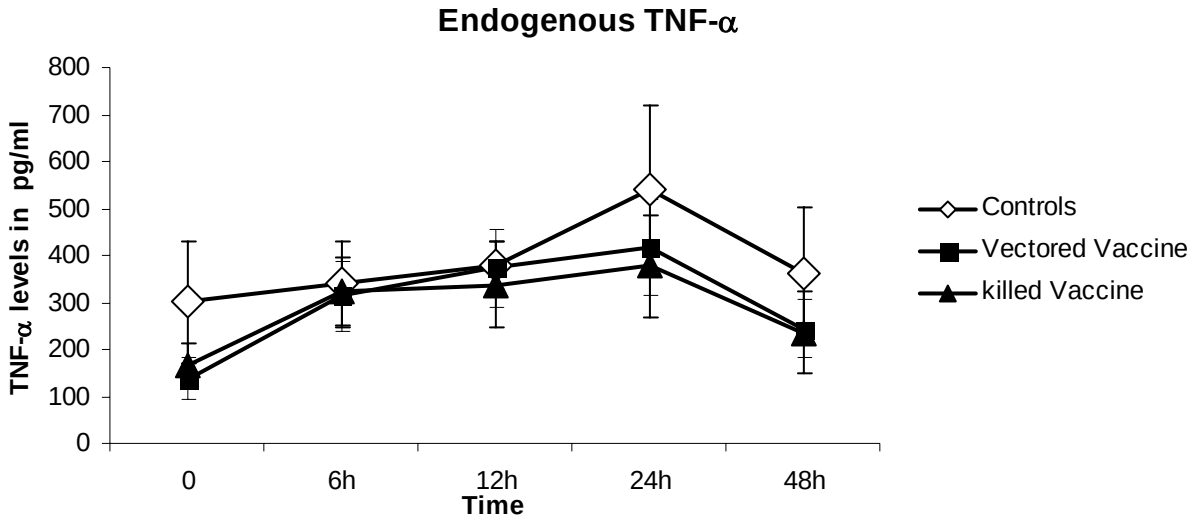

Figure 1. Endogenous production of TNF-α, in pg/ml by the three groups, during the post vaccination in the plasma from unstimulated whole blood cultures.

The statistical assessments indicated that both the vectored vaccine group and the killed vaccine group were not significantly different from the control group for the endogenous production of TNF-α during either the baseline period, or after vaccination. A complete summary of the statistical analysis can be found in Table 1 of Appendix A.

2. A comparison of induced production of TNF-α in the whole blood cultures after stimulation with lipopolysaccharide (LPS) between vaccinates and controls.

To assess for differences in blood cells to respond to inflammatory stimuli that act through TLR4, we added LPS to blood cultures to stimulate production of TNF-α. We measured the LPS induced production of TNF-α for each group of horses by ELISA. The data represented

in the graphs below presents the mean and standard error of the mean for the measurements at each assessment point within each group. Significance was determined using either $\log_e$ transformed data or ranked data by ANOVA and is represented using the sign convention described above.

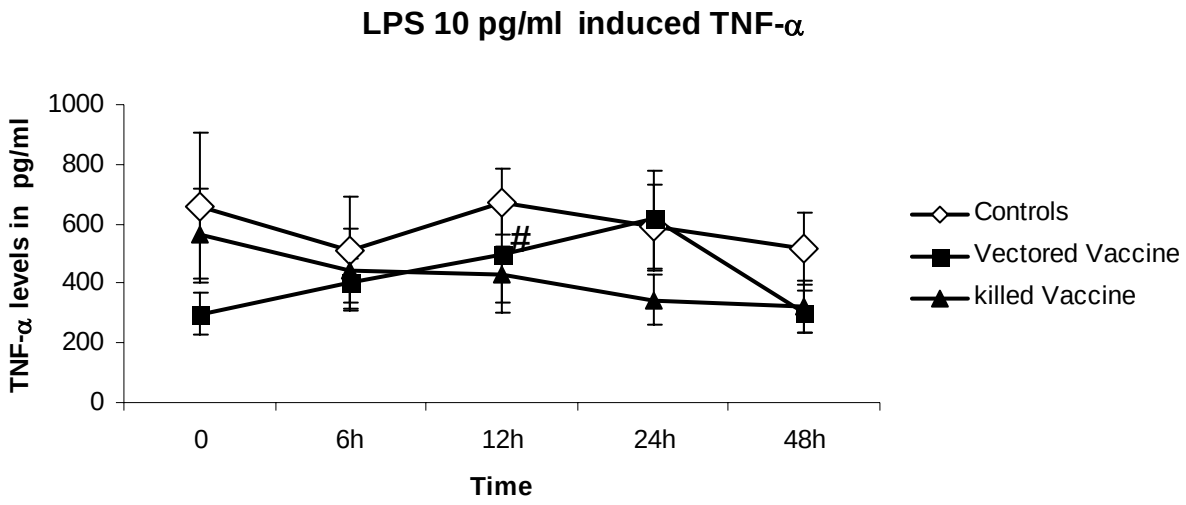

Figure 2. LPS 10pg/ml induced production of TNF-α following vaccination by the three groups of horses in the study. LPS was added to the blood cultures at a final concentration of 10pg/ml.

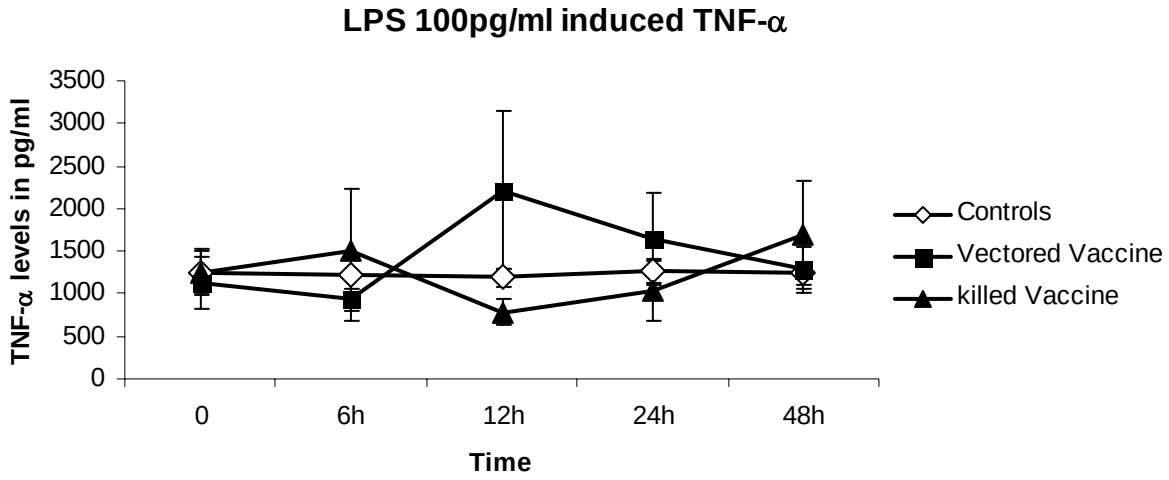

Figure 3. LPS 100pg/ml induced production of TNF-α following vaccination by the three groups of horses in the study. LPS was added to the blood cultures at a final concentration of 100pg/ml.

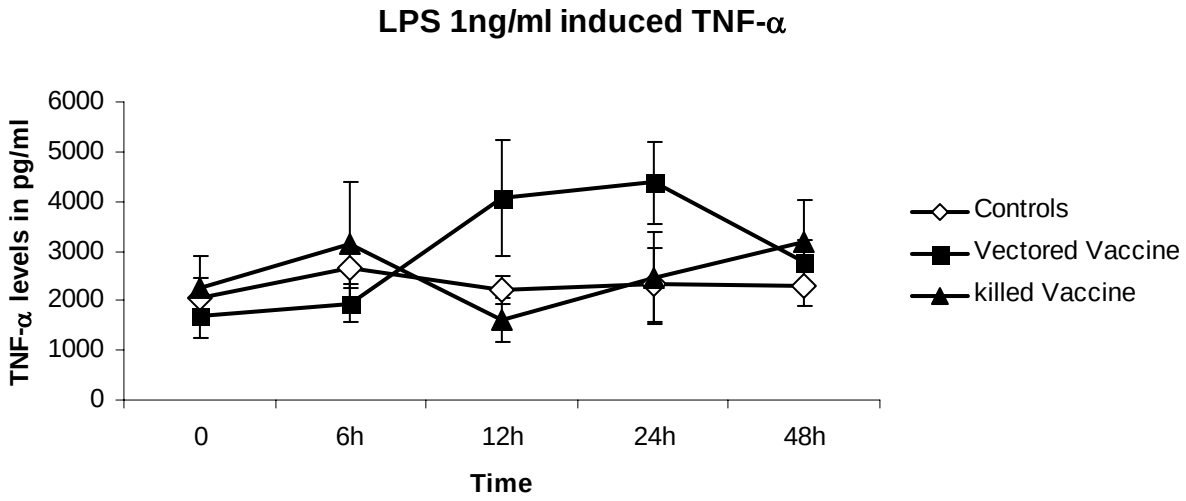

Figure 4. LPS 1ng/ml induced production of TNF-α following vaccination by the three groups of horses in the study. LPS was added to the blood cultures at a final concentration of 1ng/ml.

To evaluate TLR4 induced inflammatory activation, we utilized LPS at the 10 pg/ml, 100pg/ml, and 1ng/ml in whole blood cultures. In our previous studies, we found that the peak

response to LPS varied among individual horses between 10 and 1000 pg/ml [74, 75].In this study, we observed that whole blood cultures stimulated with LPS 10pg/ml were not significantly different during the baseline period among the three groups, but produced significantly less TNF-α when the vectored vaccine group was compared to the control group at 12 hours after vaccination. A summary of the analysis is presented in Table 2 of Appendix A. When higher concentrations of LPS, 100 pg/ml and 1000 pg/ml, were utilized, we did not observe any significant differences between either group of vaccinates or the controls either during the baseline period or after vaccination (Table 3, 4 appendix A).

This analysis indicates that the killed viral vaccine did not cause any change in the ability of blood cells to respond to the LPS compared to control horses. It also suggests that the vectored vaccine may slightly reduce the capacity of blood cells to respond to LPS (significantly at 12 hours after vaccination). The blood cells from all three groups produced TNF-α in response to LPS in these studies and retained their ability to be stimulated through TLR4.

3. A comparison of induced production of TNF-α in the whole blood cultures after stimulation with peptidoglycan (PGN) between the vaccinates and controls.

To assess the response to inflammatory activation driven by the cell wall products of gram positive bacteria, PGN derived from *Staphylococcus aureus* was utilized. PGN signals through the TLR2 receptor on the cell surface [9, 10]. We used three concentration of PGN in these studies;1ng/ml, 10ng/ml and 100ng/ml, to stimulate whole blood cultures. The data represented in the graphs below presents the mean and standard error of the mean for the measurements at each assessment point within each group. Significance was determined using either $\log_e$ transformed data or ranked data by ANOVA and is represented using the sign convention described above.

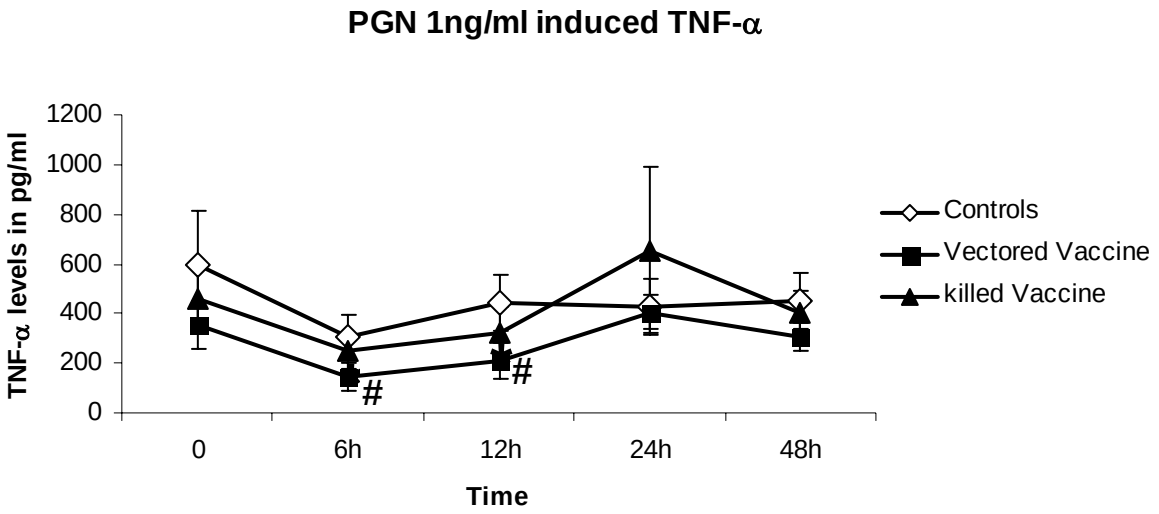

Figure 5. PGN 1ng/ml induced production of TNF-α following vaccination by the three groups of horses in the study. PGN was added to the blood cultures at a final concentration of 1ng/ml.

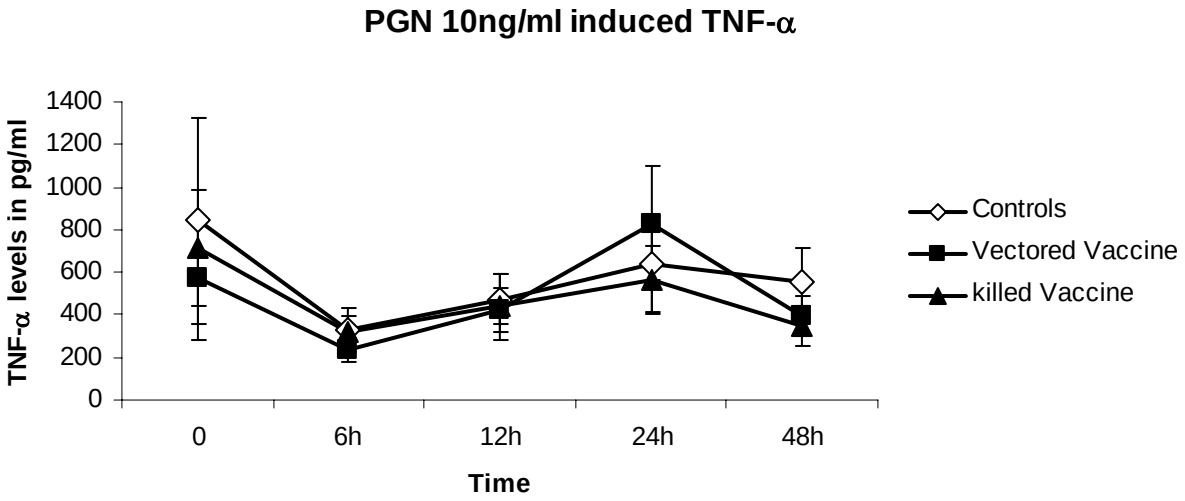

Figure 6. PGN 10ng/ml induced production of TNF-α following vaccination by the three groups of horses in the study. PGN was added to the blood cultures at a final concentration of 10 ng/ml.

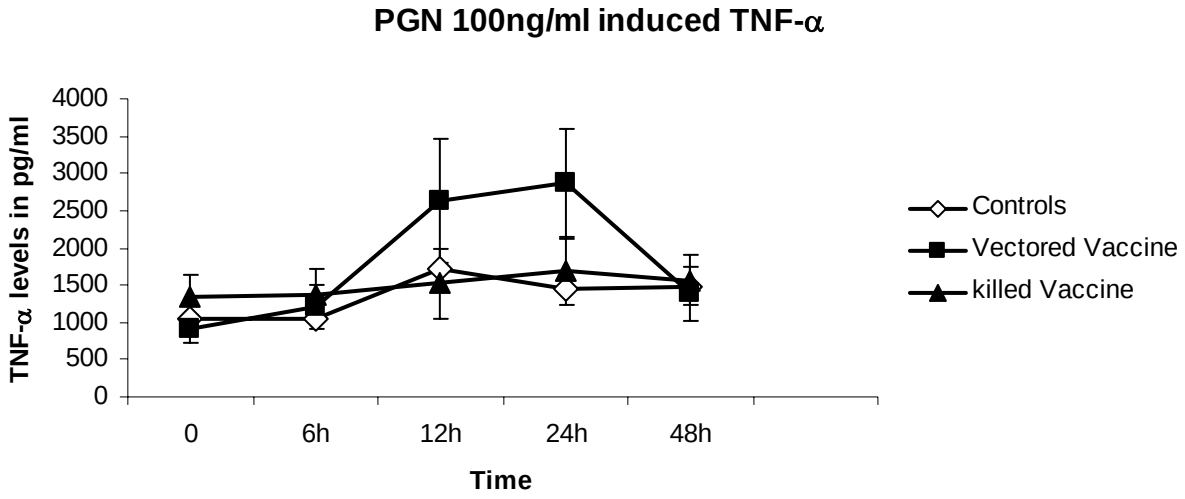

Figure 7. PGN 100pg/ml induced production of TNF-α following vaccination by the three groups of horses in the study. PGN was added to the blood cultures at a final concentration of 100 ng/ml.

An assessment of the responses of the vectored vaccine group relative to the control group indicated that treatment of whole blood with PGN at 1 ng/ml significantly reduced the production of TNF-α at 6 and 12 after vaccination. A complete summary of these analyses can be found in Table 5 of Appendix A. In contrast, when whole blood was stimulated with PGN at 10ng/ml or 100ng/ml, no significant difference in the level of TNF-α produced was observed at any time point. The summary of these analyses can be found in Tables 6 and 7 of Appendix A.

An analysis of the response of the killed viral vaccination group relative to the controls showed no significant difference in the level of TNF-α induced by any of the concentrations of PGN was observed relative to the controls; either during the baseline period or after vaccination. The detailed summary of these analyses can be found in Tables 5, 6 and 7 of Appendix A.

These analyses indicate that the vectored vaccine appeared to slightly reduce the sensitivity of whole blood leukocytes to PGN, with a significant reduction for whole blood

leukocytes stimulated with the lowest dose of PGN (1 ng/ml) observed 12 hours after vaccination. No comparable reduction in PGN response was observed for the horses vaccinated with the killed viral vaccine. All the whole blood cultures demonstrated increased production over the endogenous levels when stimulated with PGN, suggesting that the response through the TLR2 receptor complex was not ablated.

4. A comparison of induced production of TNF-α in the whole blood cultures after stimulation with phorbol myristate acetate (PMA) between vaccinates and controls.

To assess the maximal response to inflammatory activation driven by intracellular signaling "overload", PMA was used to stimulate whole blood cultures. PMA is a potent stimulant of protein kinase C that is a common intermediated in the transduction pathway of external signaling complexes [76]. In previous studies, we optimized the concentration of PMA needed to maximally activate equine leukocytes and determined it to be 10^{-7}M. Therefore, whole blood cultures for both of the groups of vaccinate and controls were stimulated with PMA at 10^{-7}M. The data represented in the graph below presents the mean and standard error of the mean for the measurements at each assessment point within each group. Significance was determined using either $\log_e$ transformed data or ranked data by ANOVA and is represented using the sign convention described above.

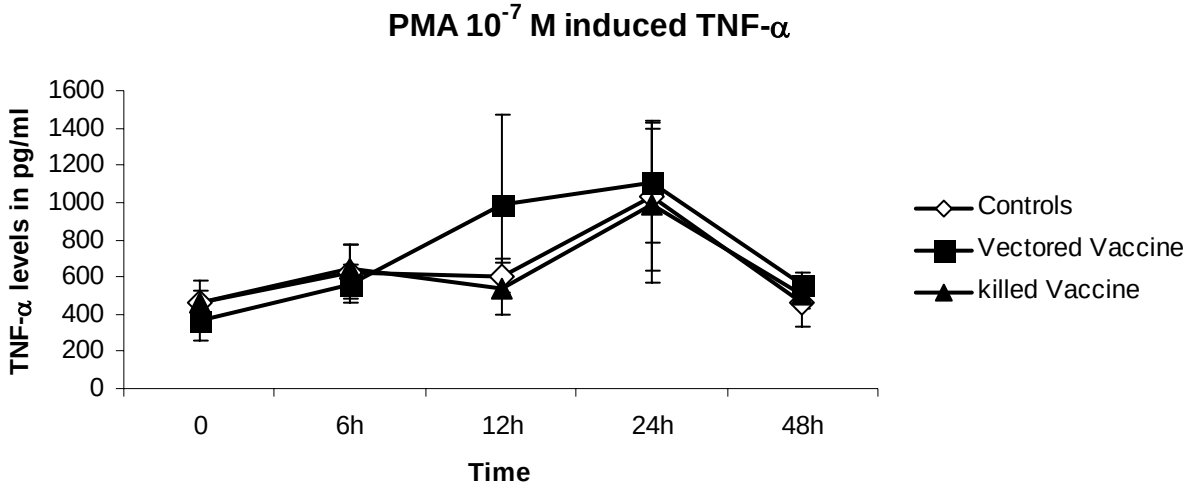

Figure 8. PMA induced production of TNF-α, following vaccination by the three groups of horses in the study. PMA was added to the blood cultures at a final concentration of 10^{-7}M

In our analyses, we observed no significant difference between either group of vaccinates and the control in the level of TNF-α induced by 10^{-7}M PMA. This was true both for analysis of the baseline values and the values obtained after vaccination. A detailed summary of these analyses can be examined in Table 8 of Appendix A. This data also suggests that the intracellular signaling that drives TNF-α production downstream from cell surface receptor activation was not enhanced or diminished by either vaccine over the period assessed, at least with respect to protein kinase C mediated production.

B. Summary of the TNF-α findings

In these studies, endogenous or stimulated whole blood cultures proved to have to great an animal-to-animal variation to be very useful as a monitor of vaccine induced systemic inflammation. While the models appeared to function essentially as designed and did report differences between animals in both endogenous and induced TNF-α production in the whole

blood cultures, a more sensitive monitor of inflammatory activation is required to properly assess vaccine induced systemic inflammatory activation.

C. Prostaglandin (PGF2α and PGE2) measurements.

5. Endogenous production of PGE2 and PGF2 in the whole blood cultures comparing the groups receiving vectored and killed vaccines relative to the controls.

In addition to assessing the production of TNF-α in the whole blood cultures, we measured the production of two prostaglandins, PGF2α and PGE2, as indicators of endogenous and induced inflammation in a parallel set of whole blood cultures. The production of prostaglandin provides a different view of both the inflammatory status, through endogenous production in unstimulated whole blood cultures, and the capacity of blood cells to respond to stimulation relative to vaccination. We utilized LPS, SEB and PMA as the stimulants in these assays to represent inflammatory activation by gram negative or gram positive bacteria, and the capacity to respond to the signaling cascade induced by inflammatory stimulation in general.

The plasma from the whole blood cultures were subjected to methanol extraction, then measured for PGE2 and PGF2α by ELISA. The raw data was calculated in pg/ml by comparison to a standard curve. The data represented in the graphs below presents the mean and standard error of the mean for the measurements at each assessment point within each group. Significance was determined using either log transformed data or ranked data by ANOVA and is represented using the sign convention described above.

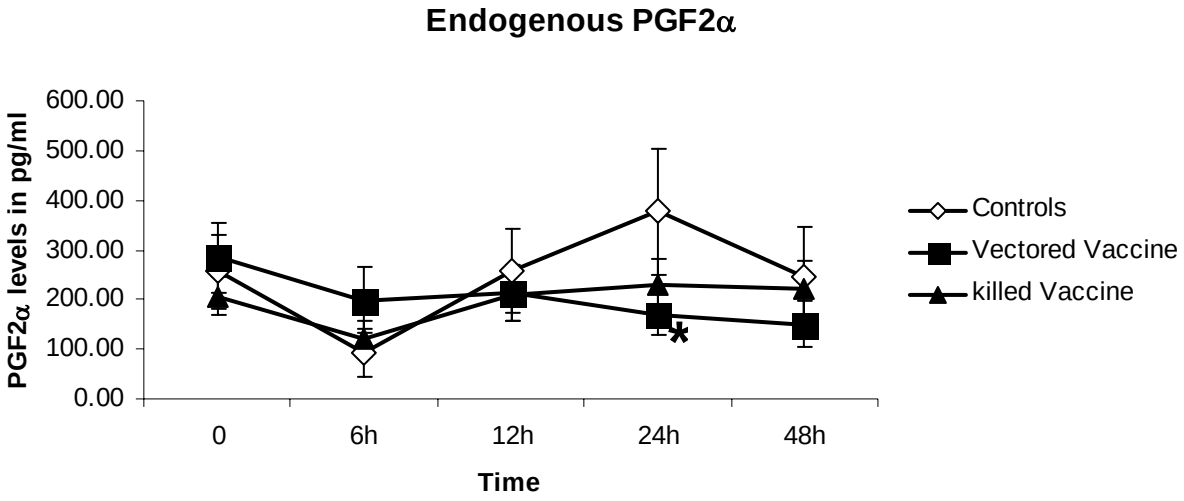

Figure 9. Endogenous production of PGF2α in whole blood cultures from the three groups of horses utilized in this study.

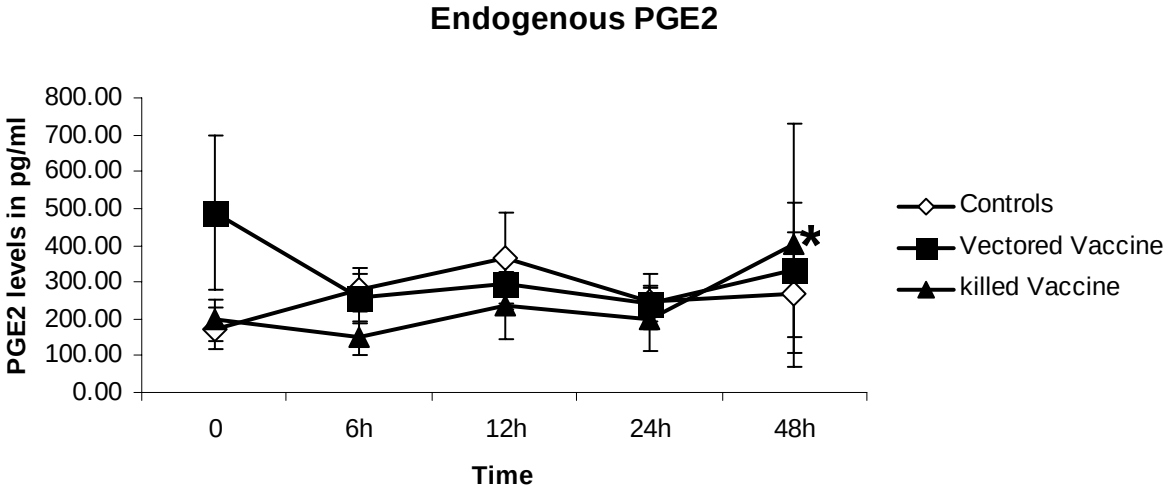

Figure10. Endogenous production of PGE2 in whole blood cultures from the three groups of horses utilized in this study.

In our analysis of the data, we observed that endogenous PGF2α production was significantly decreased in whole blood cultures from the vectored vaccine group relative to the control group at 24 hours after vaccination. None of the other comparisons between the vectored

viral vaccine group and the control group demonstrated significant differenced either in baseline or after vaccination. The details of this analysis are summarized in Table 9 of Appendix B. In contrast, endogenous PGF2α production by whole blood cultures from the killed vaccine group was not different than the control group either at baseline, or at any time after vaccination. A detailed summary of this analysis can be examined in Table 9 of Appendix B.

The analysis of PGE2 production in whole blood cultures revealed a problematic variability when comparing the killed vaccine group with the control group for the baseline samples. We observed significantly lower endogenous production of PGE2 in the whole blood cultures for the killed viral vaccine group on day -3 of the baseline measurements relative to the controls. However, there was no difference in the pooled day -6, -3 and 0 PGE2 values between either vaccine group or the controls. A detailed summary of this analysis can be examined in Table 10 of Appendix B. Similarly, after vaccination, we observed significantly lower endogenous production of PGE2 in the whole blood cultures for the killed viral vaccine group relative to the controls at 48 hours after vaccination. A detailed summary of these analyses can be examined in Table 10 of Appendix B.

When we compared the vectored viral vaccine group with the control group we observed no significant difference in the endogenous production of PGE2 in whole blood cultures. A detailed summary of these analyses can be examined in Table 10 of Appendix B.

6. A comparison of induced production of PGE2 and PGF2α in the whole blood cultures after stimulation with LPS between vaccinates and controls.

To assess for differences in blood cells to respond to inflammatory stimuli that act through TLR4, we added LPS to blood cultures to stimulate production of prostaglandin [77, 78]. We measured the LPS induced production of prostaglandin for each group of horses by ELISA.

The raw data was calculated in pg/ml by comparison to a standard curve. The data represented in the graphs below presents the mean and standard error of the mean for the measurements at each assessment point within each group. Significance was determined using either log transformed data or ranked data by ANOVA and is represented using the sign convention described above.

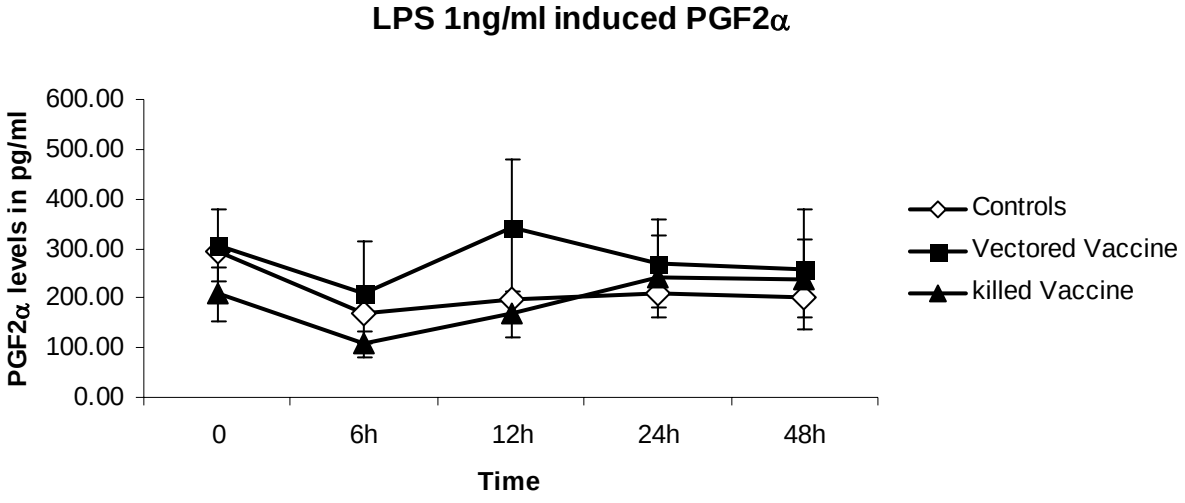

Figure 11. LPS induced production of PGF2α in whole blood cultures from the three groups of horses utilized in this study. The final concentration of LPS in the cultures was 1ng/ml.

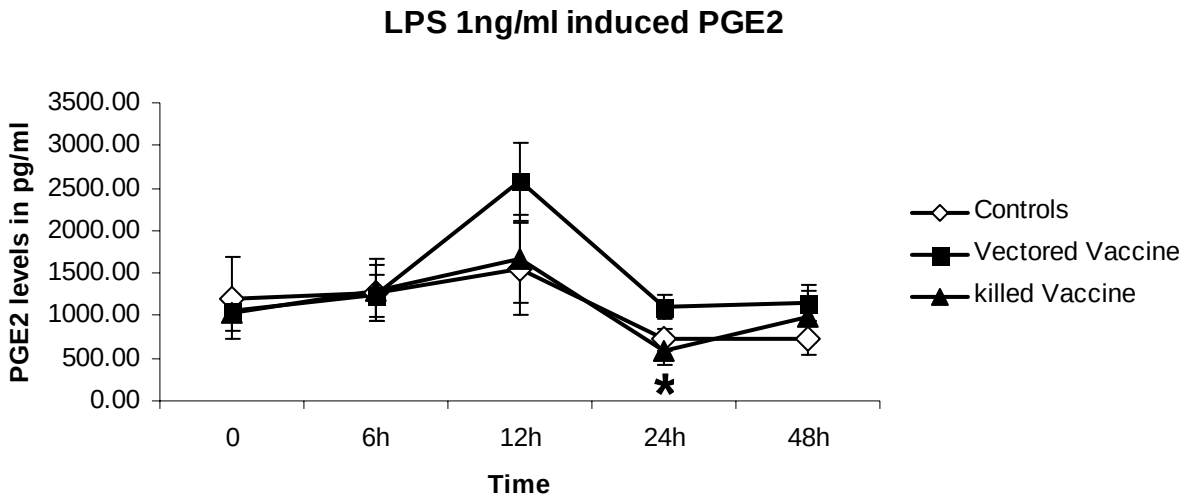

Figure 12. LPS induced production of PGE2 in whole blood cultures from the three groups of horses utilized in this study. The final concentration of LPS in the cultures was 1ng/ml.

Our analysis did not reveal any significant difference in PGF2α secretion in whole blood cultures stimulated with LPS either during the baseline period, or after vaccination when either vaccine group was compared to the control group. A detailed summary of these analyses can be examined in Table 11 if appendix B.

In contrast, significantly less PGE2 was released from LPS stimulated whole blood cultures from the killed viral vaccine group than the control group at 24 hours after vaccination. No other significant differences between the killed viral vaccine group and the controls either during the baseline period or after vaccination. A detailed summary of these analyses can be examined in Table 12 of Appendix B.

No significant difference in LPS induced PGE2 secretion in whole blood cultures between the vectored viral vaccine group and the control group were observed either during the baseline period or after vaccination. A detailed summary of these analyses can be examined in Table 12 of Appendix B.

In summary, there is no evidence that the vectored vaccine altered the capacity of blood leukocytes to respond to LPS through TLR4 and produce prostaglandin. In contrast, the killed vaccine appeared to slightly suppress PGE2 production, and the suppression was significant at 24 hours after vaccination.

7. A comparison of induced production of PGE2 and PGF2α in the whole blood cultures after stimulation with Staphylococcal enterotoxin B (SEB) between vaccinates and controls.

SEB is a protein toxin, produced by some pathogenic staphylococcal bacterial strains, that interacts with MHC class II and T cell receptor constant chain molecules [79]. SEB stimulates mononuclear leukocytes to produce high levels of a large number of cytokines, referred to as a cytokine storm. This cytokine storm is a strong inducer of inflammatory activation. To mimic the response to gram positive bacterial septicemia, we used SEB at 1 μg/ml in whole blood cultures to induce the production of prostaglandin. We measured the SEB induced production of prostaglandin for each group of horses by ELISA. The raw data was calculated in pg/ml by comparison to a standard curve. The data represented in the graphs below presents the mean and standard error of the mean for the measurements at each assessment point within each group. Significance was determined using either log transformed data or ranked data by ANOVA and is represented using the sign convention described above.

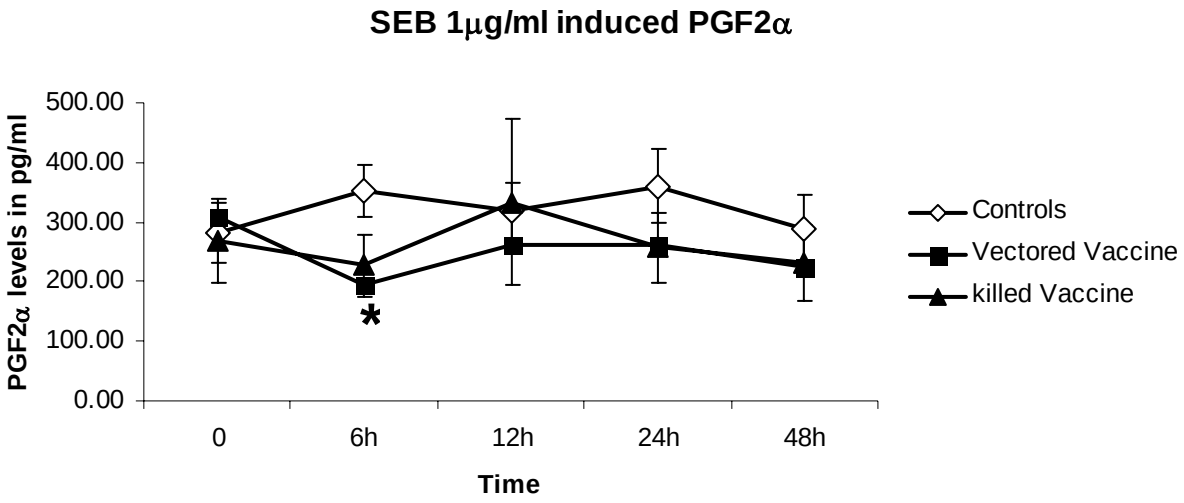

Figure 13. SEB induced production of PGF2α in whole blood cultures from the three groups of horses utilized in this study. The final concentration of SEB in the cultures was 1 μg/ml

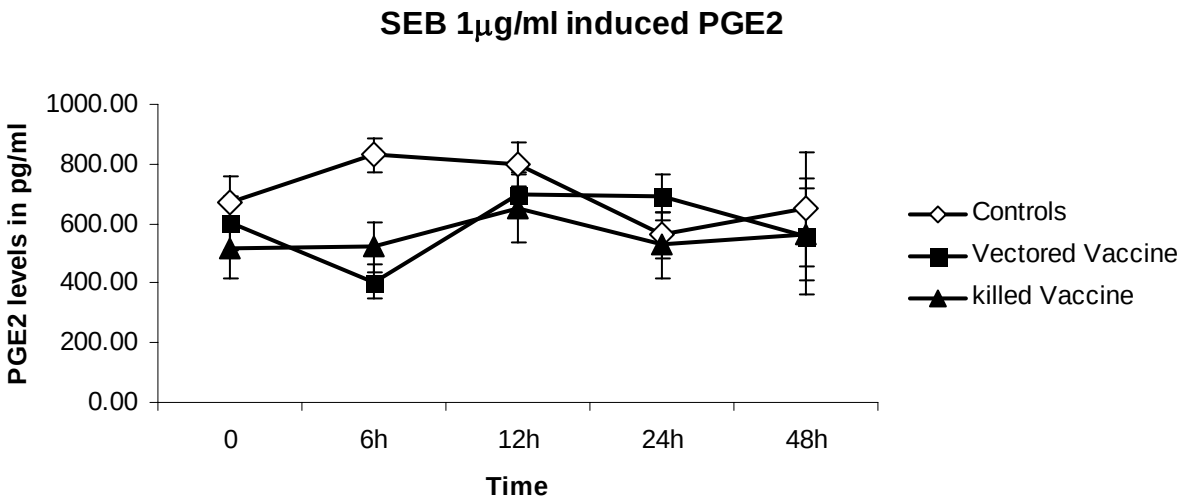

Figure 14. SEB induced production of PGE2 in whole blood cultures from the three groups of horses utilized in this study. The final concentration of PMA in the cultures was 1 μg/ml.

An analysis of SEB stimulated whole blood culture results showed the vectored, viral vaccine group produced significantly lower levels PGF2α at 6 hours after vaccination than the

control group. No other significant differences between the vectored viral vaccine group and the control group with respect to SEB induced PGF2α productions were observed either during the baseline period or after vaccination. A detailed summary of the analyses is presented in Table 13 of Appendix B.

In contrast, SEB induced production of PGF2α by whole blood cultures from the killed viral vaccine group were not significantly different than the control group for any comparisons made during the baseline period or after vaccination. A detailed summary of these analyses can be examined in Table 13 of Appendix B.

We measured no significant differences in SEB induced PGE2 production in whole blood cultures from either of the vaccine groups relative to the controls either during the baseline period or after vaccination. A detailed summary of these analyses can be examined in Table 14 of Appendix B.

In summary, a slight reduction in PGF2α production by the vectored vaccine group after vaccination was observed, reaching significance at 6 hours after vaccination. No such reduction was observed in whole blood cultures stimulated with SEB relative to vaccination.

8. A comparison of induced production of PGE2 and PGF2α in the whole blood cultures after stimulation with PMA between vaccinates and controls

PMA was utilized to induce maximal signaling in blood leukocytes and drive downstream production of inflammatory mediators. PMA induces production of both PGE2 and PGF2α in whole blood culture that is significantly greater than endogenous levels. To mimic the response to cell activation that utilizes protein kinase C based signaling, we used PMA at 10^{-7}M in whole blood cultures to induce the production of prostaglandin. We measured the PMA induced production of prostaglandin for each group of horses by ELISA. The raw data was

calculated in pg/ml by comparison to a standard curve. The data represented in the graphs below presents the mean and standard error of the mean for the measurements at each assessment point within each group. Significance was determined using either log transformed data or ranked data by ANOVA and is represented using the sign convention described above.

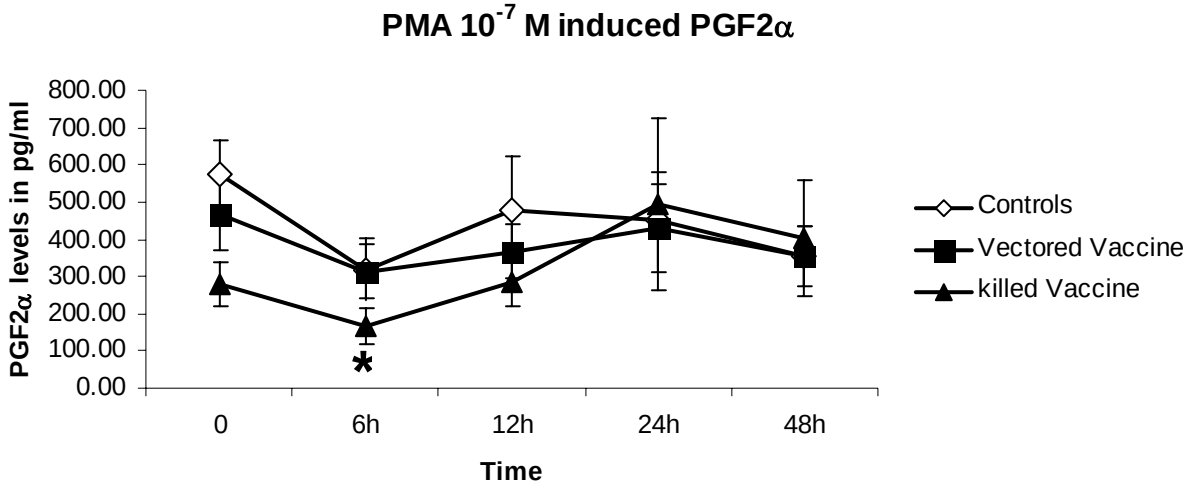

Figure 15 PMA induced production of PGF2α in whole blood cultures from the three groups of horses utilized in this study. The final concentration of PMA in the cultures was 10^{-7}M

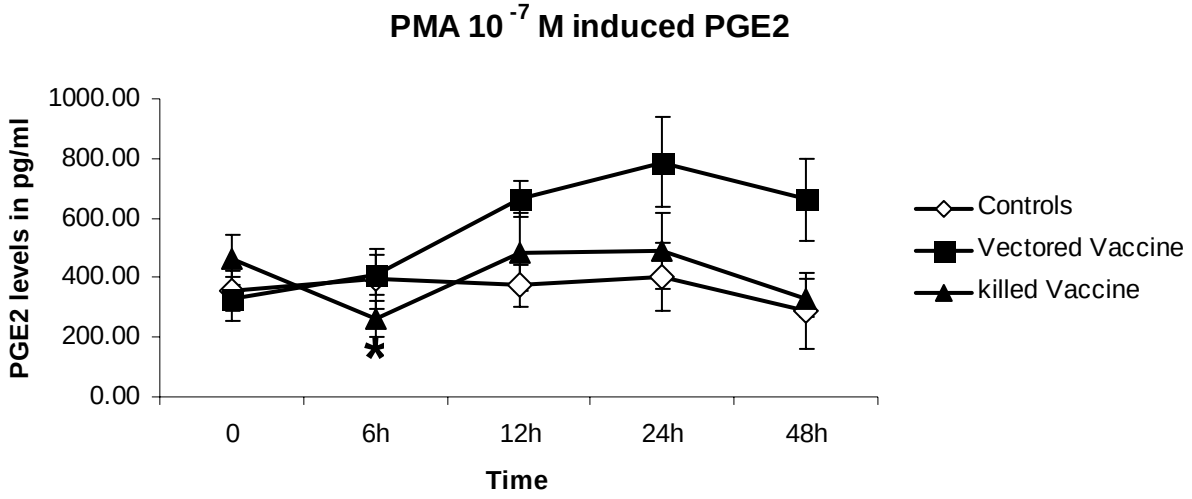

Figure 16 PMA induced production of PGE2 in whole blood cultures from the three groups of horses utilized in this study. The final concentration of PMA in the cultures was 10^{-7}M

Our analysis indicated that PMA stimulated whole blood cultures from the killed viral vaccine group produced significantly more PGF2α than the controls at the time of vaccine in the baseline period, and significantly less PGF2α than the controls at 6 hours after vaccination. No other significant differences in PMA induced PGF2a production was observed between the killed viral vaccine group and the control group in this study. A detailed summary of these analyses can be examined in Table 15 of appendix B.

An analysis of the production of PGF2α in whole blood cultures from vectored vaccine group stimulated with PMA demonstrated no significant difference from the control group either during the baseline period or after vaccination. A detailed summary of these analyses can be examined in Table 15 of Appendix B.

In addition, production of PGE2 by PMA stimulated whole blood cultures from the killed viral vaccine group was significantly lower than the control group at 6 hours after vaccination. No other significant differences were observed in PGE2 production between the killed viral

vaccine group and the control group either during the baseline period or after vaccination. A detailed summary of the analyses can be examined in Table 16 of Appendix B. It is of interest to note that both PGF2α and PGE2 were significantly reduced at 6 hours after vaccination for the killed viral vaccine group relative to controls.

We observed no significant difference between the amount of PGE2 produced by whole blood cultures stimulated with PMA for the vectored viral vaccine group and the control group either during the baseline period or after vaccination. A detailed summary of these analyses can be examined in Table 16 of Appendix B.

D. summary of the prostaglandin finding

In this study we measured both endogenous levels of prostaglandin production from whole blood cultures, and increased levels of prostaglandin after stimulation with LPS, SEB or PMA in individual animals. The level of animal-to-animal variation made it difficult to achieve a high enough level of sensitivity in these assays to make them very useful as monitors of vaccine induced systemic inflammation.

CHAPTER 6

DISCUSSION

Vaccines are essential tools in veterinary medicine. There are a staggering number of vaccines available to the veterinarian designed to help control infectious diseases of both production and companion animals. There are dozens of commercial vaccines for the horse, with new vaccines being introduced on a regular basis. In general, research into the actions of vaccines has focused on the capacity of a vaccine to drive an adaptive immune response. That is, do the vaccines induce antibody, evidence of cell-mediated immunity, or both, that results in protection from the development of disease symptoms after challenge.

In this study, we have approached a different vaccine issue. While there have been many cases of immediate responses to vaccines reported as untoward incidents and side-effects, no previous studies to our knowledge have attempted to broadly and systematically measure the systemic inflammatory consequences of the delivery of vaccines during the period before the development of an adaptive immune response. This thesis focused on the measurement of two indicators of systemic inflammation, endogenous and induced secretion of TNF-α and prostaglandins in whole blood cultures. Each of these measures is simple and utilizes widely available commercial reagents. If either had provided a clear and reproducible indicator of vaccine associated systemic inflammation they would have provided tools that could be used by essentially any laboratory world-wide to conduct similar studies.

Our assessment of the systemic inflammatory responses associated with both the killed viral vaccine containing the widely used and established adjuvant, alum, and the new technology

poxvirus vectored vaccine indicated that neither whole blood culture method provided enough sensitivity nor animal to animal reproducibility to serve as clear monitors for vaccine induced systemic inflammation. We observed some individual points where significant decreases in endogenous or induced production of either TNF-α or prostaglandin corresponded with clinical signs of systemic inflammation in the horses. However, we generally saw more discord than concordance between the clinical observations and the points of significance in these two assays.

A. An overview of vaccine issues related to systemic inflammation

About 200 years ago, Edward Jenner first discovered that deliberately injecting healthy patients with material from a patient with cowpox could protect them from developing the symptoms of smallpox. This process was named vaccination, after the Latin word for cow. Although the vaccination process imparted most individuals a high level of protection from small pox, the use of live cowpox virus could cause illness in some of those receiving the vaccination. While Jenner had clear evidence that vaccination protected against small pox, the process was subject to intense debate due to the fact that cowpox could cause infection and even death in some patients who received it. Thus a classic quest began; to find absolutely safe and absolutely effective vaccines.

Over the past 200 years, many new vaccine technologies have been developed. We have gone from using from crude, whole-pathogen preparations as the state of the art, to developing molecularly defined, subunit and vectored vaccines, including genetically engineered protein expression, hybred organisms and designed chimeras. The use of viral vectors as antigen delivery vehicles, and the injection of naked DNA hold the promise of new vaccines that yield highly effective immunity without any demonstrable of side effects [80, 81].

Vaccines containing primarily killed virus with immune enhancing adjuvants have been developed. These vaccines induce high and lasting titers of circulating antibody. They are highly effective in controlling some infectious diseases [82, 83].In addition, the attenuation of many pathogens to avirulent forms has allowed the development of live agent vaccines that induce a broad based immune response including both the development of protective antibodies and evidence of cell mediated protection.

Both killed and live vaccines must elicit protection by mimicking localized inflammation similar to that caused by the pathogen against which protection is desired. Unfortunately, the exact nature and scope of the most productive inflammatory response required to translate local interaction with a pathogen and the development of adaptive immunity has not been fully defined. Further, detrimental effects may result from the development of stronger or more lasting local inflammatory processes, or the development of a severe systemic inflammatory response. Among the more rare consequences of a systemic inflammatory response induced by vaccination are the development of systemic inflammatory response syndrome (SIRS) or generalized immunosupression[40].

B .Project summary and critical findings

The contents of this thesis represent a detailed analysis of the changes in the levels of circulating TNFα and two prostaglandins, PGF2α and PGE2 relative to the administration of two vaccines of very different construction. The systemic inflammatory response to each of the vaccines was evaluated relative to a series of environmental controls, and assessed relative to a three point measurement of the basal level of inflammatory activation of each horse, measured on day -6, -3 and immediately prior to vaccination. We also assessed the level of systemic inflammatory activation of each vaccine relative to the other in these studies. The data reported

here represents only one component of a much larger study that included the assessment of clinical signs (temperature, respiratory rate, bowel sounds, change in attitude feed intake and colic score) reactive oxygen species, tissue factor (procoagulant protein, CD-142), and the level of gene expression of inflammatory mediators IL-1β, IL-6, IL-8, RANTES, IP-10, IFN-gamma, TNF-α, INF-alpha, INF-beta, COX-2, over first 48 hours following vaccination.

We found that both the vaccines; killed vaccine, and the vectored carbopol containing viral vaccine, induced no significantly increase the endogenous secretion of TNF-α in whole blood cultures at any of the post vaccination time points (0, 6, 12, 24 or 48 hours) compared to the environmental controls. When we analyzed the COX2 associated prostaglandins, the vectored vaccine group had a significantly lower endogenous production of PGF2α compared to the control horses at 24 hours post-vaccination. All other time points showed no significant difference between the killed vaccinates and controls. When we compared the levels of PGE2, we found that the horses receiving the killed vaccine had significantly lower circulating levels than the environmental controls at 48 hours after vaccination. Further, when we evaluated the level of prostaglandins induced by stimulating with SEB, LPS, and PMA in whole blood cultures, we measured significantly lower levels of prostaglandin produced by cells from both the vectored vaccine group, and the killed virus vaccine groups at 12, 24 and 48 hours after vaccination than we measured in the controls. However, we never measured an increase in production of these prostaglandins by the vaccinated horses.

The following is a summary of the significant findings of the other components of this project to be used as a reference point in interpreting the findings presented within this thesis. When these findings are combined with the components of the study reported within this thesis, we can clearly see that both the killed viral vaccine and the vectored vaccine induce significant

systemic inflammatory activation. The pattern of systemic inflammatory activation had a similar time course for both vaccines relative to the controls, but the pattern of inflammatory activation was not identical.

Discriminating features in the pattern of inflammatory activation established by other components of this study for the two vaccines include:

1) The values for the levels of circulating TNF-α and the COX2 associated prostaglandins did not have a tight concordance with the clinical signs of inflammation induced by the two vaccines. Endogenous TNF-α measured after vaccination with either vaccine was either lower than that of the controls, or was the same. Further, the two vaccines induced differential levels and time courses of clinical signs after vaccination, but the level of endogenous TNF-α production for the two vaccine groups was not different. The measurement of induced TNF-α in whole blood cultures also failed to show concordance with the development of clinical signs. Similarly, the COX2 associated prostaglandins measured in this study showed individual points where either endogenous or induced levels were lower than the control. No increase in the prostaglandin level of in vaccine groups relative to the control group were observed after vaccination. So, the measurement of prostaglandin did not show concordance with the development of clinical signs either. Therefore, the measurement of these two inflammatory mediators did not prove useful in monitoring the induction of systemic inflammatory responses after delivery of the two vaccines in this study.
2) An assessment of gene expression by circulating leukocytes that reflect inflammatory activation after vaccination indicated that there was an increase in the level TNF-α m-RNA expression in the circulating leukocytes from the killed viral vaccine group relative

to the control group at 6 and 24 hours after vaccination. However, the same analysis revealed that the vectored vaccine group never demonstrated an increase in TNF-α message during the study relative to the controls, but showed a decline in the expression of TNF-α message in circulating leukocytes at 48 hours after vaccination relative to the control group. This suggests that TNF-α may be differentially induced by the two vaccines. It should be noted that no increase in endogenous or inducible TNF-α production in the whole blood cultures was ever observed during this study.

3) The expression of COX2 genes by leukocytes isolated from the circulation of the killed virus vaccine group was also higher than the control group at 6, 12 and 24 hours after vaccination. Similarly, enhanced COX2 gene expression was observed in the leukocytes isolated from the circulation of vectored vaccine group relative to the controls at 12 and 24 hours after vaccination. Thus, enhanced systemic expression of the COX2 gene may be a general feature of the vaccine induced systemic inflammatory response, but it appears to follow the appearance of clinical signs. It should be noted that no increase in endogenous or induced prostaglandin production in the whole blood cultures was ever observed during this study.

An interesting discordance was observed in these studies. The killed vaccine appeared to induce an increase in TNF-α message in the same circulating leukocytes that failed to deliver increased levels of TNF-α protein in our whole blood cultures. One explanation of this may be that there is not a direct relationship with production of TNF-α message and the appearance of TNF-α protein in the medium or circulation. TNF-α is produced as a membrane bound cytokine and requires the activation of a protease to cleave it from the membrane into its active, circulating form [84]. In contrast, we know of no similar explanation for the discord between our

measurements of the COX2 gene and endogenous or induced prostaglandin levels, except that COX2 produces PGI first and the metabolism of PGI to PGE2 or PGF2α may not have been complete in our whole blood cultures.

The elements common to inflammatory activation observed in close concordance between the two vaccines appear to represent the most basic and universal tissue level interactions with vaccines. We believe that these features are likely to be found as a consequence of most vaccines, and that they may play a role in the translation of innate to adaptive responses to vaccines. In contrast, the differential features that distinguish the systemic inflammatory response of the two vaccines tested in this study, particularly the differential activation of type 1 interferon and IP-10 genes, may reflect the unique interactions of the elements of each vaccine in the tissues.

When one considers the difference in biological pathway that the body uses to "address" each vaccine, it is not hard to imagine how these differences occur. The killed, alum adjuvanted vaccine interacts primarily with the surface of cells and is taken up by pinocytosis or phagocytosis by tissue macrophages and epithelial cells. In contrast, the vectored viral vaccine enters the target cells using poxvirus receptors and drives the intracellular production of the vaccine genes. The processing of vaccine components is different on the level of how vaccine materials traffic inside the responding cells, and the types of potential cellular recognition of danger, damage and antigen. Thus, there are probably many differences in tissue level activation, that we have not yet even imagined a way to monitor, that are important to the differences in both inflammatory activation and its translation into an adaptive immune response that are yet to discover.

B. Insights from these studies that may help determine the differential role of adjuvant in inducing systemic inflammation.

The present study was designed to compare two differently constructed commercial vaccines for their capacities to induce systemic inflammatory activation. While this was an important goal, it leaves many interesting questions without answers. At this point, we can not clearly ascribe to any component or components of the vaccines a specific role in inducing systemic inflammation. This study did not address the specific role of the adjuvant, and the antigen that are present as a result of antigen production in the induction of systemic inflammatory changes. It would be desirable from the standpoint of vaccine design and testing to have specific information about the capacity of each of these components in the induction of systemic inflammation.

Assuming that each of the individual vaccine components from this study were freely available to us and we were not limited in our access to appropriate horses, we believe that the following experiment would provide substantial insight into the role of each component of the vaccine in the induction of systemic inflammation. The experiment would be set up as shown in the table below.

Group	**No Adjuvant**	**Carbopol**	**Alum**
No virus	Environmental controls	carbopol only	Alum only
Killed virus	Killed virus only	Killed virus+ carbopol	Killed vaccine as tested in the thesis

Vectored antigen	Vectored antigen only	Vectored vaccine as tested in thesis	Vectored antigen + Alum

In this experiment we will utilize 90 horses (10 per group) that have been qualified for the experiment using the same selection and blocking criteria as in the studies conducted for this thesis. We will perform the same set of clinical and laboratory assessments, both to generate the baseline and post-vaccine data.

In this experiment, we will be able to establish a series of statistical contrasts base on a 3x3 Latin square and a fully interactive model. The data generated will allow for individual comparison of each antigen and adjuvant directly to the controls. In addition, each combination of antigen and adjuvant would be represented for comparison with the formulation of the commercial vaccines. By conducting multi-variant analysis and looking for evidence of concordance between adjuvant and/or antigen mediated inflammation and that represented by the "vaccine" combinations of antigen and adjuvant, we should be able to assemble a clear picture of the relative and, possibly, absolute contribution of each component to the development of immediate (at least within the first 24 hours after vaccination) systemic inflammatory activation on the macroscopic and microscopic level.

With this data in hand, it will be possible to design better and more accurate tools for the assessment of both vaccine components and whole vaccines relative to their safety. Thus, a better balance of efficacy and safety in equine vaccines can be achieved. Hopefully, my laboratory will have the opportunity to do these experiments in the near future.

CHAPTER 7

CONCLUSION

In this thesis, I analyzed two families of inflammatory responses of circulating blood cells in whole blood cultures; the endogenous and induced production of the cytokine TNF-α and COX2 associated prostaglandins (PGF2α, PGE2) for the first two days following vaccination in this study. Our attempts to assess the activation of systemic inflammatory activation by measuring TNF-α protein release in whole blood cultures after vaccination did not prove to have sufficient sensitivity or reproducibility (with respect to animal-to-animal variation) to function as a good monitor of vaccine induced systemic inflammatory activation. While we measured some significant changes in the level of both endogenous and induced TNFα secretion in whole blood cultures during the experiment, they did not show a concordance with the development of clinical signs of inflammatory activation.

Similarly, the secretion of COX2 associated prostaglandins in the whole blood cultures did not prove either a sensitive or reproducible enough to be a good monitor of vaccine induced systemic inflammatory activation in these studies. While individual difference in endogenous and induced secretion of prostaglandin by the two vaccine groups relative to the control group was observed, they always reflected a decline in secretion and did not show concordance with the observed clinical signs of inflammation. Thus, neither of these prostaglandin assay systems appears to be a suitable monitor of vaccine induced systemic inflammatory activation in their present form.

Some assessment from the larger study, of which this thesis represents a component, showed both high levels of significant enhancement, and good concordance with the observation of clinical signs. The expression of messenger RNA for genes of inflammatory mediators was increased relative to the expression of clinical signs, including both TNF-α and COX2. Unfortunately, we were unable to show the concomitant levels of TNF-α protein or the prostaglandins we measured in this study, as that would have yielded a simpler and less expensive method for conducting these assessments with commercially available tools accessible by all researchers world-wide. For now, the variability of "home grown" primers, probes and PCR reaction systems will add an additional level of difficulty to reproducing these finding between laboratories, and in extending them to other vaccines. Differential gene expression that discriminated between the vaccine groups was also demonstrated. This provides hope we will be able to reconstruct the responses in experiments utilizing both the vaccines and their individual components in the future, and eventually learn why different vaccines have different inflammatory side-effects.

The original aim of this study was to quantitatively measure systemic inflammatory activation that resulted from vaccination with commercial vaccines during the period between vaccination and the second day after vaccination, when most of the untoward incidents related to vaccines are reported. We utilized a model that compared two vaccines of very different construction, a killed, alum adjuvanted viral vaccine, and a vectored viral vaccine, in the hope that they would generate patterns that were as different as their construction. We measured the changes relative to unvaccinated controls kept in the same environment at the same time to allow us to see the effect of uncontrollable factors that impact systemic inflammation relative to the

study. Further, we took three baseline measurements for each animal to assure that we had a clear and unchanging baseline that indicated a fair distribution of animals in the study.

Therefore, we conclude that it is possible to monitor vaccine induced systemic inflammatory activation, particularly utilizing mRNA assessment of circulating cells, but it is not yet possible to dissect the inflammatory activation necessary for adaptive immune activation from that which causes untoward incidents associated with vaccines.

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APPENDIX-A

ENDOGENOUS AND INDUCED PRODUCTION OF TNF-α BY VACCINE GROUP AND TIME.

The effects of vaccination on endogenous and induced TNF-α from whole blood cultures were evaluated using the Two-way ANOVA routine in SAS V9 software (Cary, NC, USA). The effects of time and vaccine group were assessed to determine if they were independent or interactive co-variables with respect to the induction of a systemic inflammatory response. The effect of time and vaccine group on the level of TNF-α measured by ELISA in the plasma collected from the whole blood cultures is represented in the tables

Tables - Two-way ANOVA was run under SAS by with either PROC MIXED (for $\log_e$ transformed data) or PROC RANK (for rank sorted data) routines to generate an understanding of the relationship between treatment, time and the production of TNF-α. For the PROC MIXED model, the raw TNF-α secretion, calculated in pg/ml, was $\log_e$ transformed. For the other model, PROC RANK (a non-parametric test), the data was progressively ranked from highest to lowest value. PROC RANK was used to comparison TNF-α production of the two vaccination groups with the controls relative to time after vaccination. Significance was accepted if $p < 0.1$.

For all our tables below.

1. Cabopol containing canarypox vectored viral vaccine will be referred as vectored vaccine .
2. Alum adjuvanted killed viral vaccine will be referred as killed vaccine.
3. Environmental controls will be referred as controls.

Table 1-Endogenous secretion of TNF-α prior to and after vaccination was compared for each of the vaccinated groups against the control groups. Significance was accepted if p <0.1.and labeled as * against the comparison groups

Comparison of the vectored vaccine and control using log-transformed values

	Least square means +/- standard deviation		P-value
	Control	vectored vaccine	
Day -6	6.0+/-0.41	6.0+/-0.29	0.9595
Day-3	6.0+/-0.41	5.8+/-0.29	0.5904
T 0	5.3+/-0.41	4.3+/-0.29	0.0532*
T 6	5.6+/-0.41	5.5+/-0.29	0.7987
T12	5.9+/-0.41	5.7+/-0.29	0.6416
T24	6.1+/-0.41	6.0+/-0.31	0.8707
T48	5.7+/-0.41	5.3+/-0.31	0.5214

Comparison of the alum adjuvanted, killed vaccine and control using log-transformed values

	Least square means +/- standard deviation		P-value
	Control	killed vaccine	
Day -6	6.0+/-0.41	5.8+/-0.29	0.6699
Day-3	6.0+/-0.41	5.5+/-0.31	0.2817
T 0	5.3+/-0.41	5.0+/-0.31	0.6380
T 6	5.6+/-0.41	5.5+/-0.29	0.7923
T12	5.9+/-0.41	5.4+/-0.29	0.3175
T24	6.1+/-0.41	5.6+/-0.29	0.3976
T48	5.7+/-0.41	5.1+/-0.33	0.2945

Comparison of the vectored vaccine and control using ranked data

	Median		P-value
	Control	Vectored vaccine	
Day -6	420.28	334.33	0.6500
Day-3	438.00	317.31	0.5500
T 0	178.41	81.60	0.3100
T 6	420.28	246.38	0.7400
T12	363.42	312.84	0.5800
T24	335.24	366.79	0.7400
T48	253.85	263.93	0.7000

Comparison of the killed, alum adjuvanted vaccine and control using ranked data

	Median		P-value
	Control	Killed vaccine	
Day -6	420.28	324.68	0.5300
Day-3	438.00	289.58	0.2300
T 0	178.41	165.79	0.5800
T 6	420.28	278.05	0.7900
T12	363.42	290.50	0.4400
T24	335.24	275.50	0.3200
T48	253.85	170.19	0.3500

Interpretation: No significant difference was observed for either the mean endogenous TNF-α production for either group of vaccinates relative to the control.

Table 2- A comparison of lipopolysaccharide (10 pg/ml) induced secretion of TNF-α by the two groups of vaccinates relative to the controls. Significance was accepted if p <0.1.and labeled as * against the comparison groups

Whole blood cultures stimulated with lipopolysaccharide (LPS) were prepared at three points before vaccination and at four point following vaccination. Plasma from the whole blood cultures stimulated with LPS at 10pg/ml is reported here.

Comparison of the vectored vaccine and control using log-transformed values for stimulation with LPS at 10pg/ml.

	Least square means +/- standard deviation		P-value
	Control	Vectored vaccine	
Day -6	6.3+/-0.38	6.4+/-0.27	0.8312
Day-3	6.2+/-0.38	5.8+/-0.28	0.3263
T 0	6.1+/-0.38	5.4+/-0.27	0.1183
T 6	6.0+/-0.38	5.8+/-0.27	0.7106
T12	6.4+/-0.38	5.7+/-0.27	0.1052
T24	6.2+/-0.38	6+/-0.27	0.6188
T48	6.1+/-0.38	5.6+/-0.28	0.2313

Comparison of the killed vaccine and control using log-transformed values for stimulation with LPS at 10pg/ml.

	Least square means +/- standard deviation		P-value
	Control	Killed vaccine	
Day -6	6.3+/-0.38	6+/-0.27	0.6095
Day-3	6.2+/-0.38	5.7+/-0.27	0.3028
T 0	6.1+/-0.38	6+/-0.27	0.8229
T 6	6.0+/-0.38	5.7+/-0.27	0.5583
T12	6.4+/-0.38	6+/-0.28	0.3651

T24	6.2+/-0.38	5.6+/-0.27	0.1871
T48	6.1+/-0.38	5.6+/-0.28	0.2667

Comparison of the vectored vaccine and control using ranked values for stimulation with LPS at 10pg/ml.

	Median		P-value
	Control	Vectored vaccine	
Day -6	435.70	414.27	0.7700
Day-3	479.34	266.05	0.1100
T 0	614.46	247.73	0.1300
T 6	494.67	328.00	0.8600
T12	736.36	317.78	0.0800*
T24	646.10	426.13	0.7800
T48	389.87	335.56	0.1400

Comparison of the killed vaccine and control using ranked values for stimulation with LPS at 10pg/ml.

	Median		P-value
	Control	Killed vaccine	
Day -6	435.70	425.55	0.5700
Day-3	479.34	367.54	0.3200
T 0	614.46	436.55	0.8200
T 6	494.67	278.78	0.6500
T12	736.36	451.36	0.1800
T24	646.10	238.23	0.1800
T48	389.87	259.48	0.1300

Interpretation: The analysis of the rank data indicates a significant difference in the amount of TNF-α induced by treatment of whole blood with 10 pg/ml of LPS comparing the vectored viral

vaccinate group with the control group at 12 hours post-challenge (p = 0.08) .No other significant interactions were observed.

Table 3- A comparison of lipopolysaccharide (100 pg/ml) induced secretion of TNF-α by the two groups of vaccinates relative to the controls.

Whole blood cultures stimulated with LPS were prepared at three points before vaccination and at four point following vaccination. Plasma from the whole blood cultures stimulated with LPS at 100 pg/ml is reported here.

Comparison of the vectored vaccine and control using log-transformed values for stimulation with LPS at 100 pg/ml

	Least square means +/- standard deviation		P-value
	Control	Vectored vaccine	
Day -6	7.1+/-0.41	6.8+/-0.29	0.5168
Day-3	7.1+/-0.41	6.5+/-0.29	0.2651
T 0	6.9+/-0.41	6.6+/-0.29	0.5308
T 6	7.0+/-0.41	6.5+/-0.29	0.2978
T12	7.0+/-0.41	7.1+/-0.29	0.8019
T24	7.0+/-0.41	7.0+/-0.29	0.9995
T48	6.9+/-0.41	6.9+/-0.29	0.9383

Comparison of the killed vaccine and control using log-transformed values for stimulation with LPS at 100 pg/ml

	Least square means +/- standard deviation		P-value
	Control	Killed vaccine	
Day -6	7.1+/-0.41	6.9+/-0.29	0.7334

Day-3	7.1+/-0.41	6.9+/-0.29	0.7044
T 0	6.9+/-0.41	6.9+/-0.29	0.9999
T 6	7.0+/-0.41	6.6+/-0.29	0.4528
T12	7.0+/-0.41	6.5+/-0.29	0.3332
T24	7.0+/-0.41	6.6+/-0.29	0.4510
T48	6.9+/-0.41	7.1+/-0.29	0.7502

Comparison of the vectored vaccine and control using ranked values for stimulation with LPS at 100 pg/ml

	Median		P-value
	Control	Vectored vaccine	
Day -6	1197.68	978.94	0.5500
Day-3	878.66	488.45	0.3600
T 0	1389.17	778.64	0.6800
T 6	995.11	612.45	0.3500
T12	952.11	1314.85	0.9800
T24	968.89	905.88	0.9000
T48	1106.69	1061.88	0.9000

Comparison of the killed vaccine and control using ranked values for stimulation with LPS at 100 pg/ml.

	Median		P-value
	Control	Killed vaccine	
Day -6	1197.68	1182.92	0.7200
Day-3	878.66	1065.06	0.7700
T 0	1389.17	951.21	0.9600
T 6	995.11	804.23	0.3800
T12	952.11	652.61	0.2900
T24	968.89	692.03	0.3300
T48	1106.69	1121.56	0.8100

Interpretation: No significant difference in the level of TNF-α induced by 100 pg/ml LPS were observed when each of the vaccine groups was compared to the controls.

Table 4- A comparison of lipopolysaccharide (1000 pg/ml) induced secretion of TNF-α by the two groups of vaccinates relative to the controls.

Whole blood cultures stimulated with LPS were prepared at three points before vaccination and at four point following vaccination. Plasma from the whole blood cultures stimulated with LPS at 1000 pg/ml is reported here.

Comparison of the vectored vaccine and control using log-transformed values for stimulation with LPS at 1000 pg/ml

	Least square means +/- standard deviation		P-value
	Control	Vectored vaccine	
Day -6	8+/-0.37	7.6+/-0.26	0.4321
Day-3	7.8+/-0.37	7.5+/-0.26	0.4410
T 0	7.6+/-0.37	7.1+/-0.26	0.3328
T 6	7.8+/-0.37	7.4+/-0.26	0.3150
T12	7.7+/-0.37	7.9+/-0.26	0.5595
T24	7.6+/-0.37	8.2+/-0.26	0.1587
T48	7.7+/-0.37	7.8+/-0.26	0.7510

Comparison of the killed vaccine and control using log-transformed values for stimulation with LPS at 1000 pg/ml

	Least square means +/- standard deviation		P-value
	Control	Killed vaccine	
Day -6	8.0+/-0.37	7.7+/-0.26	0.5724

Day-3	7.8+/-0.37	7.8+/-0.26	0.9302
T 0	7.6+/-0.37	7.5+/-0.26	0.8063
T 6	7.8+/-0.37	7.3+/-0.26	0.2045
T12	7.7+/-0.37	7+/-0.26	0.1518
T24	7.6+/-0.37	7.4+/-0.26	0.7385
T48	7.7+/-0.37	7.8+/-0.26	0.7513

Comparison of the vectored vaccine and control using ranked values for stimulation with LPS at 1000 pg/ml

	Median		P-value
	Control	Vectored vaccine	
Day -6	2367.48	2116.28	0.3000
Day-3	2416.11	1879.00	0.5900
T 0	1757.81	1091.78	0.2000
T 6	2440.42	1748.03	0.2200
T12	2417.85	3120.24	0.6600
T24	1526.07	4062.78	0.1300
T48	2454.32	2489.70	0.6900

Comparison of the killed vaccine and control using ranked values for stimulation with LPS at 1000 pg/ml.

	Median		P-value
	Control	Killed vaccine	
Day -6	2367.48	2254.13	0.5700
Day-3	2416.11	2414.60	0.9600
T 0	1757.81	1388.01	0.5700
T 6	2440.42	1604.69	0.2700
T12	2417.85	1412.22	0.2900
T24	1526.07	1752.40	0.8600
T48	2454.32	2133.72	0.7300

Interpretation: No significant difference was observed between either vaccine group and the control with respect to production of TNF-a in whole blood cultures stimulated with 1000 pg/ml of LPS.

Table 5- A comparison of peptidoglycan (PGN, 1ng/ml) induced secretion of TNF-α by the two groups of vaccinates relative to the controls.

Whole blood cultures stimulated with PGN were prepared at three points before vaccination and at four point following vaccination. Plasma from the whole blood cultures stimulated with PGN at 1 ng/ml is reported here. Significance was accepted if $p < 0.1$.and labeled as * against the comparison groups

Comparison of the vectored vaccine and control using log-transformed values for stimulation with PGN at 1 ng/ml.

	Least square means +/- standard deviation		P-value
	Control	Vectored vaccine	
Day -6	5.8+/-0.42	5.6+/-0.31	0.6702
Day-3	5.9+/-0.42	5.9+/-0.29	0.9309
T 0	6.1+/-0.42	5.4+/-0.29	0.2227
T 6	5.6+/-0.42	4.4+/-0.31	0.0248
T12	5.9+/-0.42	5.1+/-0.31	0.0935*
T24	5.9+/-0.42	5.9+/-0.29	0.9820
T48	6.0+/-0.42	5.5+/-0.29	0.4168

Comparison of the killed vaccine and control using log-transformed values for stimulation with PGN at 1 ng/ml

	Least square means +/- standard deviation		P-value
	Control	Killed vaccine	
Day -6	5.8+/-0.42	5.1+/-0.31	0.1740
Day-3	5.9+/-0.42	6.0+/-0.31	0.8750
T 0	6.1+/-0.42	5.7+/-0.29	0.5080
T 6	5.6+/-0.42	5.3+/-0.31	0.5670
T12	5.9+/-0.42	5.5+/-0.31	0.3857
T24	5.9+/-0.42	5.8+/-0.29	0.8660
T48	6.0+/-0.42	5.7+/-0.29	0.6773

Comparison of the vectored vaccine and control using ranked values for stimulation with PGN at 1 ng/ml.

	Median		P-value
	Control	Vectored vaccine	
Day -6	339.31	213.99	0.3400
Day-3	417.25	363.72	0.9200
T 0	380.65	228.58	0.3400
T 6	264.66	95.21	0.0700*
T12	396.51	143.22	0.0900*
T24	442.59	314.61	0.9800
T48	396.69	315.02	0.3900

Comparison of the killed vaccine and control using ranked values for stimulation with PGN at 1 ng/ml

	Median		P-value
	Control	Killed vaccine	
Day -6	339.31	204.75	0.2100
Day-3	417.25	434.19	0.8300
T 0	380.65	331.45	0.7000
T 6	264.66	211.66	0.3800
T12	396.51	183.98	0.3200

T24	442.59	298.99	0.6100
T48	396.69	348.79	0.6200

Interpretation: The analysis indicates that whole blood cultures from horses receiving the vectored vaccine produced less TNF-α than the controls when stimulated with PGN at 1 ng/ml at both 6 and 12 hrs after vaccination.

Table 6- A comparison of PGN (10 ng/ml) induced secretion of TNF-α by the two groups of vaccinates relative to the controls.

Whole blood cultures stimulated with PGN were prepared at three points before vaccination and at four point following vaccination. Plasma from the whole blood cultures stimulated with PGN at 10 ng/ml is reported here.

Comparison of the vectored vaccine and control using log-transformed values for stimulation with PGN at 10 ng/ml

	Least square means +/- standard deviation		P-value
	Control	Vectored vaccine	
Day -6	6.1+/-0.49	5.9+/-0.34	0.7695
Day-3	6.1+/-0.49	6.2+/-0.34	0.8489
T 0	6.2+/-0.49	5.6+/-0.34	0.3487
T 6	5.5+/-0.49	5.2+/-0.34	0.5531
T12	6+/-0.49	5.2+/-0.34	0.1760
T24	6.2+/-0.49	6.3+/-0.34	0.8125
T48	6.1+/-0.49	5.7+/-0.34	0.4763

Comparison of the killed vaccine and control using log-transformed values for stimulation with PGN at 10 ng/ml

	Least square means +/- standard deviation		P-value
	Control	Killed vaccine	
Day -6	6.1+/-0.49	5.6+/-0.34	0.3736
Day-3	6.1+/-0.49	6.1+/-0.34	0.9433
T 0	6.2+/-0.49	6.1+/-0.34	0.8417
T 6	5.5+/-0.49	5.5+/-0.34	0.9826
T12	6+/-0.49	5.6+/-0.36	0.5562
T24	6.2+/-0.49	6+/-0.34	0.7554
T48	6.1+/-0.49	5.8+/-0.36	0.5679

Comparison of the vectored vaccine and control using ranked values for stimulation with PGN at 10 ng/ml

	Median		P-value
	Control	Vectored vaccine	
Day -6	502.34	398.38	0.7700
Day-3	452.51	458.18	0.8800
T 0	380.65	171.29	0.3100
T 6	352.92	216.38	0.3700
T12	541.15	350.12	0.7900
T24	350.78	635.60	0.8300
T48	433.41	316.16	0.4000

Comparison of the killed vaccine and control using ranked values for stimulation with PGN at 10 ng/ml

	Median		P-value
	Control	Killed vaccine	
Day -6	502.34	293.31	0.4300
Day-3	452.51	501.71	0.9200
T 0	380.65	419.09	0.8500

T 6	352.92	293.68	0.8500
T12	541.15	204.29	0.6500
T24	350.78	380.81	0.6500
T48	433.41	267.54	0.2300

Interpretation: No significant difference in the level of TNF-α induced by 10 ng/ml of PGN was observed with either of the vaccine treatments relative to the controls.

Table 7- A comparison of PGN (100 ng/ml) induced secretion of TNF-α by the two groups of vaccinates relative to the controls.

Whole blood cultures stimulated with PGN were prepared at three points before vaccination and at four point following vaccination. Plasma from the whole blood cultures stimulated with PGN at 100 ng/ml is reported here.

Comparison of the vectored vaccine and control using log-transformed values for stimulation with PGN at 100 ng/ml

	Least square means +/- standard deviation		P-value
	Control	Vectored vaccine	
Day -6	7.1+/-0.4	7.2+/-0.28	0.7541
Day-3	7.3+/-0.4	7.1+/-0.28	0.7441
T 0	6.9+/-0.4	6.6+/-0.28	0.6156
T 6	6.8+/-0.4	6.9+/-0.28	0.9082
T12	7.3+/-0.4	7.4+/-0.28	0.8081
T24	7.0+/-0.4	7.4+/-0.28	0.3520
T48	7.1+/-0.4	6.8+/-0.28	0.5326

Comparison of the killed vaccine and control using log-transformed values for stimulation with PGN at 100 ng/ml

	Least square means +/- standard deviation	P-value

	Control	Killed vaccine	
Day -6	7.1+/-0.4	7.2+/-0.28	0.7766
Day-3	7.3+/-0.4	7.4+/-0.28	0.8448
T 0	6.9+/-0.4	7+/-0.28	0.8482
T 6	6.8+/-0.4	6.9+/-0.28	0.9555
T12	7.3+/-0.4	7+/-0.28	0.5433
T24	7+/-0.4	7+/-0.28	0.9658
T48	7.1+/-0.4	7.1+/-0.28	0.8862

Comparison of the vectored vaccine and control using ranked values for stimulation with PGN at 100 ng/ml

	Median		P-value
	Control	Vectored vaccine	
Day -6	1127.53	1797.82	0.4000
Day-3	1342.53	1801.51	0.9400
T 0	1093.95	803.27	0.5300
T 6	1132.95	904.68	0.8700
T12	1150.11	1680.50	0.5800
T24	892.42	3065.85	0.2800
T48	1213.61	1092.83	0.7000

Comparison of the killed vaccine and control using ranked values for stimulation with PGN at 100 ng/ml

	Median		P-value
	Control	Killed vaccine	
Day -6	1127.53	1426.01	0.4700
Day-3	1342.53	2051.30	0.6000
T 0	1093.95	1263.81	0.7900
T 6	1132.95	826.69	0.8500
T12	1150.11	1186.98	0.8500
T24	892.42	1323.14	0.8100
T48	1213.61	1629.42	0.9800

Interpretation: No significant difference in the level of TNF-α induced by 100 ng/ml of PGN was observed with either of the vaccine treatments relative to the controls.

Table 8- A comparison of phorbol myristate acetate (PMA, 10^{-7}M) induced secretion of TNF-α by the two groups of vaccinates relative to the controls.

Whole blood cultures stimulated with PMA were prepared at three points before vaccination and at four point following vaccination. Plasma from the whole blood cultures stimulated with PMA at 10^{-7}M is reported here.

Comparison of the vectored vaccine and control using log-transformed values for stimulation with PMA at 10^{-7}M

	Least square means +/- standard deviation		P-value
	Control	Vectored vaccine	
Day -6	6.3+/-0.32	6.6+/-0.23	0.5418
Day-3	6.3+/-0.32	6.3+/-0.23	0.91156
T 0	6+/-0.32	5.6+/-0.23	0.3675
T 6	6.3+/-0.32	6.2+/-0.23	0.6985
T12	6.3+/-0.32	6.3+/-0.23	0.8415
T24	6.6+/-0.32	6.7+/-0.23	0.8132
T48	5.9+/-0.32	6.3+/-0.23	0.3678

Comparison of the killed vaccine and control using log-transformed values for stimulation with PMA at 10^{-7}M

	Least square means +/- standard deviation	P-value

	Control	Killed vaccine	
Day -6	6.3+/-0.32	6.3+/-0.23	0.8962
Day-3	6.3+/-0.32	6.2+/-0.23	0.6582
T 0	6.0+/-0.32	6.0+/-0.23	0.9800
T 6	6.3+/-0.32	6.2+/-0.23	0.7839
T12	6.3+/-0.32	5.9+/-0.23	0.2377
T24	6.6+/-0.32	6.4+/-0.23	0.5099
T48	5.9+/-0.32	6.1+/-0.23	0.6353

Comparison of the vectored vaccine and control using ranked values for stimulation with PMA at $10^{-7}M$

	Median		P-value
	Control	Vectored vaccine	
Day -6	600.10	754.42	0.7700
Day-3	515.76	564.12	0.7000
T 0	410.34	244.44	0.3200
T 6	624.36	487.89	0.6600
T12	642.26	349.13	0.6800
T24	921.57	767.86	0.9400
T48	412.25	532.57	0.4500

Comparison of the killed vaccine and control using ranked values for stimulation with PMA at $10^{-7}M$

	Median		P-value
	Control	Killed vaccine	
Day -6	600.10	519.81	0.6800
Day-3	515.76	433.82	0.4800
T 0	410.34	504.36	0.8800
T 6	624.36	719.66	0.9400
T12	642.26	291.77	0.3600
T24	921.57	379.90	0.5000
T48	412.25	468.34	0.7300

Interpretation: No significant difference in the level of TNF-a induced by 10^{-7}M PMA was observed with either of the vaccine treatments relative to the controls.

APPENDIX-B

AN ANALYSIS OF ENDOGENOUS AND INDUCED PRODUCTION OF PGF2α AND PGE2 PRODUCED BY TWO GROUPS OF VACCINATES COMPARED WITH A GROUP OF CONTROLS

The effects of vaccination on endogenous and induced prostaglandin production in whole blood cultures were evaluated using the Two-way ANOVA routine in SAS V9 software (Cary, NC, USA). The effects of time and vaccine group were assessed to determine if they were independent or interactive co-variables with respect to the induction of a systemic inflammatory response. The effect of time and vaccine group on the level of prostaglandin measured by ELISA in the plasma collected from the whole blood cultures is represented in the tables

Tables - Two-way ANOVA was run under SAS by with either PROC MIXED (for $\log_{10/e}$ transformed data) routine to generate an understanding of the relationship between treatment, time and the production of prostaglandin. For the PROC MIXED model, the raw prostaglandin secretion, calculated in pg/ml, was $\log_{10/e}$ transformed. Significance was accepted if $p < 0.1$

For all the tables below

1. Cabopol containing canarypox vectored viral vaccine will be referred as vectored vaccine.
2. Alum adjuvanted killed viral vaccine will be referred as killed vaccine.
3. Environmental controls will be referred as control.

Table 9- Endogenous secretion of PGF2α prior to and after vaccination was compared for each of the vaccinated groups against the control groups. Significance was accepted if p <0.1.and labeled as * against the comparison groups

Comparison of the vectored vaccine and control using log-transformed values

	Least square means +/- standard deviation		P-value
	Control	Vectored vaccine	
Day -6	2.12±0.20	2.14±0.14	0.9129
Day-3	2.28±0.20	2.09±0.14	0.4286
T 0	2.33±0.20	2.21±0.14	0.6082
T 6	1.63±0.20	1.85±0.14	0.3617
T12	2.33±0.20	2.16±0.14	0.4827
T24	2.49±0.20	2.09±0.14	0.0988*
T48	2.29±0.20	2.19±0.14	0.6731

Comparison of the killed vaccine and control using log-transformed values

	Least square means +/- standard deviation		P-value
	Control	Killed vaccine	
Day -6	2.12±0.20	2.09±0.14	0.9261
Day-3	2.28±0.20	2.14±0.14	0.5692
T 0	2.33±0.20	2.24±0.14	0.6966
T 6	1.63±0.20	1.85±0.14	0.3664
T12	2.33±0.20	2.19±0.14	0.5524
T24	2.49±0.20	2.14±0.14	0.1532
T48	2.29±0.20	2.15±0.14	0.5596

Interpretation- Whole blood cultures from the horses vaccinated with the vectored vaccine produced significantly less PGF2α endogenously than the controls at 24 hours after vaccination (p=0.0988)

Table 10- Endogenous secretion of PGE_2 prior to and after vaccination was compared for each of the vaccinated groups against the control groups. Significance was accepted if p <0.1.and labeled as * against the comparison groups

Comparison of the vectored vaccine and control using log-transformed values

	Least square means +/- standard deviation		P-value
	Control	Vectored vaccine	
Day -6	5.13±0.90	5.2±0.64	0.9482
Day-3	5.35±0.90	5.23±0.64	0.9114
T0	4.74±0.90	5.38±0.64	0.5610
T06	5.52±0.90	4.99±0.64	0.6326
T12	5.41±0.90	5.15±0.64	0.8104
T24	5.06±0.90	5.04±0.64	0.9864
T48	4.83±0.90	4.66±0.64	0.8755

Comparison of the killed vaccine and control using log-transformed values

	Least square means +/- standard deviation		P-value
	Control	Killed vaccine	
Day -6	5.13±0.90	4.12±0.64	0.3608
Day-3	5.35±0.90	3.33±0.64	0.0677*
T0	4.74±0.90	4.62±0.64	0.9133
T06	5.52±0.90	3.84±0.64	0.1286
T12	5.41±0.90	4.07±0.64	0.2254

T24	5.06±0.90	3.97±0.64	0.3234
T48	4.83±0.90	2.65±0.64	0.0495*

Interpretation- Whole blood cultures from the horses vaccinated with the, killed viral vaccine produced less endogenous PGE2 than the controls horses both on day -3 prior to vaccination, and at 48 hours following vaccination. This suggests that the 10 horses in the killed vaccine group were potentially intrinsically less capable of producing PGE2.

Table 11-Lipopolysaccharide (LPS, 1ng/ml) induced secretion of PGF2α prior to and after vaccination was compared for each of the vaccinated groups against the control groups

Comparison of the vectored vaccine and control using log-transformed values after LPS stimulation with 1 ng/ml

	Least square means +/- standard deviation		P-value
	Control	Vectored vaccine	
Day -6	2.45±0.20	2.25±0.14	0.4275
Day-3	2.38±0.20	2.23±0.14	0.5483
T0	2.37±0.20	2.25±0.14	0.6166
T06	2.14±0.20	1.85±0.14	0.2331
T12	2.20±0.20	2.16±0.14	0.8620
T24	2.18±0.20	2.2±0.14	0.9330
T48	2.14±0.20	2.03±0.14	0.6677

Comparison of the killed vaccine and control using log-transformed values after stimulation with LPS at 1 ng/ml.

	Least square means +/- standard deviation		P-value
	Control	Killed vaccine	
Day -6	2.45±0.20	2.33±0.14	0.6233
Day-3	2.38±0.20	2.16±0.14	0.3846
T0	2.37±0.20	2.11±0.14	0.2976
T06	2.14±0.20	1.78±0.14	0.1460
T12	2.2±0.20	2.01±0.14	0.4376
T24	2.18±0.20	2.08±0.14	0.6740
T48	2.14±0.20	2.02±0.14	0.6443

Interpretation- Whole blood cultures stimulated with LPS at 1 ng/ml did not demonstrate any significant difference in the secretion of PGF2α when either vaccination group was compared with the controls

Table 12-Lipopolysaccharide (LPS, 1ng/ml) induced secretion of PGE2 prior to and after vaccination was compared for each of the vaccinated groups against the control groups Significance was accepted if $p < 0.1$.and labeled as * against the comparison groups

Comparison of the vectored vaccine and control using log-transformed values after LPS stimulation with 1 ng/ml

	Least square means +/- standard deviation		P-value
	Control	Vectored vaccine	
Day -6	6.99±0.54	6.91±0.38	0.9122
Day-3	6.94±0.54	6.56±0.38	0.5686
T0	6.81±0.54	6.89±0.38	0.9004
T06	7.01±0.54	6.96±0.38	0.9422
T12	7.18±0.54	7.70±0.38	0.4309
T24	6.52±0.54	6.95±0.38	0.5066
T48	6.35±0.54	6.61±0.38	0.6979

Comparison of the killed vaccine and control using log-transformed values after stimulation with LPS at 1 ng/ml

	Least square means +/- standard deviation		P-value
	Control	Killed vaccine	
Day -6	6.99±0.54	6.79±0.38	0.7684
Day-3	6.94±0.54	6.79±0.38	0.8228
T0	6.81±0.54	6.67±0.38	0.8332
T06	7.01±0.54	6.82±0.38	0.7700
T12	7.18±0.54	7.07±0.38	0.8580
T24	6.52±0.54	4.71±0.38	0.0065*
T48	6.35±0.54	6.35±0.38	0.9942

Interpretation- Whole blood cultures from the horses vaccinated with the killed viral vaccine produced less induced PGE2 than the controls horses at 24 hours following vaccination.

Table 13- Staphylococcal enterotoxin (SEB, 1 ug/ml) induced secretion of PGF2α prior to and after vaccination was compared for each of the vaccinated groups against the control groups Significance was accepted if $p < 0.1$.and labeled as * against the comparison groups

Comparison of the vectored vaccine and control using log-transformed values after SEB stimulation with 1 ug/ml

	Least square means +/- standard deviation		P-value
	Control	Vectored vaccine	
Day -6	2.34±0.23	2.07±0.16	0.3372
Day-3	2.32±0.23	2.26±0.16	0.8219
T0	2.3±0.23	2.37±0.16	0.7972
T06	2.47±0.23	1.98±0.16	0.0823*

T12	2.36±0.23	2.06±0.16	0.2861
T24	2.37±0.23	2.24±0.16	0.6296
T48	2.22±0.23	2.16±0.16	0.8504

Comparison of the killed vaccine and control using log-transformed values after stimulation with SEB at 1 ug/ml

	Least square means +/- standard deviation		P-value
	Control	Killed vaccine	
Day -6	2.34±0.23	2.08±0.16	0.3537
Day-3	2.32±0.23	2.12±0.16	0.4903
T0	2.30±0.23	2.31±0.16	0.9697
T06	2.47±0.23	2.15±0.16	0.2581
T12	2.36±0.23	2.24±0.16	0.6718
T24	2.37±0.23	2.22±0.16	0.5899
T48	2.22±0.23	2.11±0.16	0.7107

Interpretation- Whole blood cultures from the horses receiving the vectored vaccine that were stimulated with SEB at 1 ug/ml induced significantly lesser secretion of PGF2α than the control horses at 6 hours after vaccination (p=0.0823)

Table 14- Staphylococcal enterotoxin (SEB, 1 ug/ml) induced secretion of PGE2 prior to and after vaccination was compared for each of the vaccinated groups against the control groups

Comparison of the vectored vaccine and control using log-transformed values after SEB stimulation with 1 ug/ml

	Least square means +/- standard deviation		P-value
	Control	Vectored	

		vaccine	
Day -6	6.05±0.46	5.98±0.32	0.8926
Day-3	6.27±0.46	6.24±0.32	0.9542
T0	6.27±0.46	6.27±0.32	0.9983
T06	6.58±0.46	5.92±0.32	0.2393
T12	6.58±0.46	6.50±0.32	0.8945
T24	6.19±0.46	6.47±0.32	0.6201
T48	6.13±0.46	5.55±0.32	0.3028

Comparison of the killed vaccine and control using log-transformed values after stimulation with SEB at 1 ug/ml

	Least square means +/- standard deviation		P-value
	Control	Killed vaccine	
Day -6	6.05±0.46	6.03±0.32	0.9631
Day-3	6.27±0.46	6.19±0.32	0.8796
T0	6.27±0.46	6.11±0.32	0.7727
T06	6.58±0.46	6.14±0.32	0.4297
T12	6.58±0.46	6.32±0.32	0.6445
T24	6.19±0.46	5.88±0.32	0.5700
T48	6.13±0.46	5.42±0.32	0.2030

Interpretation- Whole blood cultures stimulated with SEB at 1 ug/ml did not demonstrate any significant difference in the secretion of PGE2 when either vaccination group was compared with the controls

Table 15- Phorbol myristate acetate (PMA, 10^{-7}M) induced secretion of PGF2α prior to and after vaccination was compared for each of the vaccinated groups against the control groups

Comparison of the vectored vaccine and control using log-transformed values after PMA stimulation with 10^{-7}M

	Least square means +/- standard deviation		P-value
	Control	Vectored vaccine	
Day -6	2.51±0.18	2.44±0.12	0.7711
Day-3	2.56±0.18	2.53±0.12	0.8953
T0	2.74±0.18	2.50±0.12	0.2623
T06	2.43±0.18	2.34±0.12	0.6726
T12	2.60±0.18	2.44±0.12	0.4634
T24	2.58±0.18	2.40±0.12	0.3959
T48	2.49±0.18	2.4±0.12	0.6659

Comparison of the killed vaccine and control using log-transformed values after stimulation with PMA at 10^{-7}M

	Least square means +/- standard deviation		P-value
	Control	Killed vaccine	
Day -6	2.51±0.18	2.40±0.12	0.6142
Day-3	2.56±0.18	2.34±0.12	0.3204
T0	2.74±0.18	2.32±0.12	0.0524*
T06	2.43±0.18	1.98±0.12	0.0365*
T12	2.60±0.18	2.30±0.12	0.1580
T24	2.58±0.18	2.37±0.12	0.3194
T48	2.49±0.18	2.31±0.12	0.4014

Interpretation- Whole blood cultures from the horses receiving the killed vaccine that were stimulated with PMA at 10^{-7}M induced significantly less secretion of PGF2α than the control horses at both time 0 (just prior to receiving the vaccine, p=0.0524)) and 6 hours after vaccination (p=0.0365)

Table 16- Phorbol myristate acetate (PMA, 10^{-7}M) induced secretion of PGE2 prior to and after vaccination was compared for each of the vaccinated groups against the control groups Significance was accepted if p <0.1.and labeled as * against the comparison groups

Comparison of the vectored vaccine and control using log-transformed values after PMA stimulation with $10^{-7}M$

	Least square means +/- standard deviation		P-value
	Control	Vectored vaccine	
Day -6	5.82±0.51	5.83±0.36	0.9924
Day-3	6.03±0.51	5.93±0.36	0.8735
T0	5.80±0.51	5.28±0.36	0.4059
T06	5.81±0.51	5.71±0.36	0.8830
T12	5.80±0.51	6.46±0.36	0.3004
T24	5.83±0.51	6.40±0.36	0.3615
T48	5.24±0.51	6.27±0.36	0.1035

Comparison of the killed vaccine and control using log-transformed values after stimulation with PMA at $10^{-7}M$

	Least square means +/- standard deviation		P-value
	Control	Killed vaccine	
Day -6	5.82±0.51	5.92±0.36	0.8733

Day-3	6.03±0.51	6.07±0.36	0.9501
T0	5.80±0.51	5.8±0.36	0.9996
T06	5.81±0.51	4.61±0.36	0.0587*
T12	5.80±0.51	5.65±0.36	0.8072
T24	5.83±0.51	5.76±0.36	0.9182
T48	5.24±0.51	5.48±0.36	0.7001

Interpretation- The killed vaccine group produced less PGE2 after incubation with 10^{-7}M PMA than the controls at 6 hours after vaccination (p=0.0587).