# Can Levamisole Upregulate the Equine Cell Mediated Macrophage (M1) Dendritic Cell (DC1) CD4 T-helper 1 (Th1) CD8 T-cytotoxic 1 (Tc1) Immune Response in Vitro?

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ABSTRACT

Equine Protozoal Myeloencephalitis (EPM) is arguably the most common and costly equine neurologic diseases nationwide. The national seroprevalence is >50%, but only 0.5-1% of all horses develops disease during their lifetimes. Some EPM affected horses have decreased immune response. A cell-mediated immune response has been shown to be protective for development of EPM after infection with *Sarcocystis neurona* in mouse models. Levamisole has been proposed as an adjunctive therapy for EPM to upregulate the cell-mediated immune response based on positive results in other species, but there are very limited studies in equids. We hypothesized that levamisole will upregulate the equine cell-mediated macrophage (M1) dendritic cell (DC1) CD4 T-helper 1 (Th1) CD8 Tc1 immune response in vitro.

The first aim was to determine optimal conditions and effects of levamisole on cellular proliferation. Equine PBMCs were harvested from ten horses seronegative for *S. neurona*. The cells were cultured alone, or with one of the mitogens: concanavalin A (ConA) or phorbol 12-myristate 13-acetate and ionomycin (PMA/I), or with a combination of the above mitogens and levamisole at several conditions. Cellular proliferation was assessed using a colorimetric bromodeoxyuridine ELISA assay.

The second aim was to determine the ability of levamisole, under optimized conditions, to upregulate the M1 DC1 CD4Th1 CD8 Tc1 response in vitro based on activation and function. PBMCs from the same 10 horses were cultured with each of the following: no stimulation, conA, and levamisole with and without ConA. To determine proliferation of each specific subset, cells were labeled with a fluorescent dye, CellTrace. Proliferation was determined based on dye dilution using flow cytometry. To determine the effects of levamisole on the specific immune response, cell subsets were labeled with fluorescent antibodies for cell surface markers (CD4, CD8, CD21, CD172a, CD14) and dendritic and macrophage activations markers (MHC Class II, CD86). Induction of T-regs was based on FoxP3 expression. Immune phenotypes were determined based on intracellular cytokine expression (IFNɣ, IL4, IL10).

Study results indicate that levamisole alone did not significantly alter PBMC proliferation compared to the response of unstimulated cells. Cells cultured with either ConA or PMA/I resulted in a statistically significant increase ($P<0.05$) in proliferation compared to unstimulated cells. Cells cultured with ConA and levamisole at 1µg/mL resulted in a significant decrease ($P<0.05$) in proliferation compared with cells cultured with ConA alone.

Flow cytometry data failed to elucidate the specific immune phenotype that is affected by levamisole. Subjectively, there appeared to be a trend for inceased IFNɣ production by CD14 and CD172a positive cells (macrophages and dendritic cells) and a decrease in IFNɣ production by CD4 and CD8 positive cells (T-lymphocytes).

These results demonstrate that levamisole downregulates ConA stimulated PBMC proliferation. Based on these in vitro results, further studies to determine the effectiveness of levamisole on modulating the equine immune system in vivo and to more specifically evaluate the immune cell subets affected by levamisole are warranted.

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List of Abbreviations

ANOVA: Analysis of variance

BBB: Blood brain barrier

BrdU: Bromodeoxyuridine

CD: Cluster of differentiation

CFSE: Carboxyfluorescein succinimidyl ester

CMI: Cell mediated immunity

CPM: Counts per minute

CNS: Central Nervous System

ConA: Concanavalin A

COX: Cyclooxygenase

CSF: Cerebrospinal fluid

DAMP: Damage-associated molecular pattern

DC: Dendritic cell

DMSO: Dimethylsulfoxide

ELISA: Enzyme-linked immunosorbent assay

EPM: Equine Protozoal Myeloencephilits

FBS: Fetal bovine serum

GM-CSF: Granulocyte-macrophage colony-stimulating factor

I: Ionomycin

IFAT: Immunofluorescent antibody test

IFN: Interferon

Ig: Immunoglobulin

IL: Interleukin

IP: Intraperitoneally

LPS: Lipopolysaccharide

M: Macrophage

MHC: Major histocompatibility complex

MAPK: MAP kinase

NFkB: Nuclear factor kappa B

NK: Natural Killer Cells

NO: Nitric oxide

NSAID: Non-steroidal anti-inflammatory drug

PAMP: Pathogen-associated molecular pattern

PBMC: Peripheral blood mononuclear cell

PBS: Phosphate buffered saline

PCR: Polymerase chain reaction

PMA: Phorbol myrisate acetate

PRR: Pattern recognition receptors

PWM: Pokeweed mitogen

PYR: Pyrimethamine

Rt-PCR: Reverse transcriptase polymerase chain reaction

SAG: Surface antigen

SCID: Severe combined immunodeficiency

SDZ: Sulfadiazine

Tc: T cytotoxic

Th: T helper

TLR: Toll-like receptor

TNFα: Tumor necrosis factor alpha

Treg: T regulatory

# Chapter 1. Introduction

## 1.0 Literature Review

## 1.1 Equine Protozoal Myeloencephalitis

### 1.1.1 Clinical Signs

Equine Protozoal Myeloencephalitis (EPM) presents as progressive neurologic disease. Clinical signs are typically multifocal, often asymmetric in nature and vary depending on the specific area of the central nervous system (CNS) where lesions develop. The causative organism, *Sarcocystis neurona*, can infect any part of the CNS: brain, brainstem, spinal cord, or a combination of all of these areas.(1-3) However, despite the fact that clinical signs can vary greatly, a discussion of "typical" clinical signs is warranted.

Typically, early signs of EPM include tripping or stumbling that may be erroneously interpreted as multifocal lameness of musculoskeletal origin. These signs will usually progress to ataxia, weakness, and muscle wasting. The disease can progress acutely over a course of days, or more chronically over weeks to months.(1, 3, 4)

General physical examination of affected horses is often unremarkable though some degree of muscle atrophy may be observed. Temperature, pulse and respiratory rate are usually within normal limits. Horses are generally bright and alert, though affected horses may be dull, lethargic, or exhibit behavioral abnormalities if lesions are present within the brain. Neurologic examination often shows asymmetric weakness, spasticity, and/or ataxia in multiple limbs depending on the exact location of the lesion(s). Diffuse or multifocal hypoalgesia may be noted, as may cranial nerve deficits such as dysphagia, dyspnea, head tilt, and, most commonly, facial nerve paralysis.(1-3)

Differential diagnoses for EPM vary and depend on the clinical signs that the individual horse is exhibiting. Common differentials that may be considered are Cervical Vertebral Instability (Wobblers), Equine Herpesvirus Myeloencephalopathy, Equine Motor Neuron Disease, Polyneuritis equi, West Nile Virus, etc.(2)

1.1.2 Life Cycle:

Equine Protozoal Myeloencephalitis is most commonly caused by the protozoan *Sarcocystis neurona*. Another protozoan, *Neospora hughesi*, has also been shown to cause EPM in horses.(5) However, *S. neurona* is most prevalent in the majority of the United States and has been more extensively studied. Therefore, *S. neurona* will be the focus of this document. *S. neurona* is a member of the Apicomplexan phylum. It has a two host life cycle with the North and South American opossum (*Didelphis virginiana* and *D. albiventris*) serving as definitive hosts.(6) Many other mammals can serve as intermediate hosts including skunks, raccoons, and armadillos. In North America, the raccoon appears to be most important due to their far-ranging natural habitat and the prevalence of *S. neurona*-like sarcocysts in their muscles as well as the success in completing the *S. neurona* life cycle in the laboratory setting.(1) The horse is considered an aberrant host. Briefly, the definitive host, the opossum, becomes infected after feeding on infected intermediate host muscle. The intermediate or aberrant host is then infected by ingesting feed that has been contaminated with opossum feces containing sporocysts.(6)

Opossums ingest sarcocysts while consuming infected muscle of the intermediate host. The sarcocyst wall breaks down during digestion of the muscle and releases bradyzoites. The bradyzoites then invade the lamina propria of the intestine. Here, sexual reproduction occurs resulting in sporulated oocysts. Each oocyst contains two sporocysts which contain four sporozoites. Oocysts and sporocysts are excreted in the opossum feces and remain viable in the environment for over a year. These oocysts and sporocysts are then ingested by the intermediate or aberrant host. In the intermediate host, the

sporocysts break down and undergo asexual reproduction creating schizonts and merozoites which migrate to the muscle to form sarcocysts.(4)

When oocysts or sporocysts are ingested by an aberrant host, such as the horse, the sporocysts excyst and release sporozoites. The sporozoites then invade the vascular endothelium of the horse and asexual reproduction occurs. Only schizonts and merozoites are typically found in equine tissues.(7) It is unknown how they infect the CNS of the horse. As *S. neurona* can infect endothelial cells and there is an experimental model that uses infected peripheral leukocytes to establish infection, it is suspected that either endothelial cells or peripheral leukocytes may be a mechanism for entry into the CNS.(8, 9) Here, they invade neurons and microglial cells where they continue to undergo schizogony.(7, 10)

1.1.3 Pathogenesis:

It is suspected that all horses are susceptible to infection by *S. neurona*, however, only a small percentage of horses that become infected will develop EPM. It is not clear what causes some horses to develop clinical disease. Several factors have been proposed including inoculum dose, parasite strain, stress, and decreased immune function. However, evidence in the literature is conflicting.

One study by Saville and colleagues used a case control design to identify risk factors for the development of EPM.(11) They identified the following risk factors: age, season, prior EPM diagnosis on the premises, opossums on the premises, health events prior to admission, and racing or showing as a primary use. While some of these factors suggest environmental conditions that may increase risk (such as presence of opossums and time of year), others suggest possible host differences may predispose the horse to developing clinical disease (such as health events, age, and racing or showing as a primary use).

Another study by Sofaly and colleagues showed that there was a dose effect on development of clinical signs.(12) In this study, weanlings were transported to induce stress and were assigned to different treatment groups to be inoculated orally with different numbers of *S. neurona* sporocysts. They were

monitored for 5 weeks for induction of antibodies as well as the development of clinical signs. The results indicated that seroconversion occurred more rapidly and more consistent clinical signs were observed in the group with the highest inoculum.

In contrast, Saville et al., failed to demonstrate more severe clinical signs, worsening lesions, or decreased time to seroconversion when comparing horses subjected to a second transport after inoculation to control horses who were only subjected to one transport immediately prior to inoculation.(13)

Similarly, in another study, horses that were treated with high doses of dexamethasone after inoculation with *S. neurona* sporocysts failed to develop more severe histological changes in the CNS on necropsy compared to horses infected and not treated with dexamethasone.(14) The horses treated with dexamethasone did, however, develop other systemic disease including pneumonia, laminitis, and colic while the untreated horses did not.

These studies suggest a complicated relationship with infection with *S. neurona* and development of disease.

It appears that once a naïve horse is exposed to *S. neurona* sporocysts after oral inoculation, dissemination of tissues occurs rapidly. In a study performed by Elitsur and colleagues, 6 naïve ponies were orally inoculated with *S. neurona* sporocysts. Ponies were euthanized and necropsied at various points following inoculation. Parasites were isolated in the mesenteric lymph nodes on day 1, 2 and 7 post-inoculation, in the liver on days 2, 5, and 7 post-inoculation, and in the lungs on days 5, 7, and 9 post-inoculation. Lesions consistent with EPM were apparent histologically in the central nervous system of ponies on days 7 and 9 post-inoculation.(15) Neurologic signs were not noted in any pony at any point during the study. Only the pony euthanized on day 9 developed serum antibodies to *S.*

*neurona*, and none of the ponies developed cerebrospinal fluid (CSF) antibodies during the short study period.(15)

1.1.4 Immune Response Overview:

A brief discussion of the important components of the cell mediated and humoral immune response to pathogens with particular attention to the cell types, cell surface markers, and cytokines follows. See Figure 1 for a basic overview of the different branches of immunity.

Activation of an immune response begins with recognition of invading pathogens. This is accomplished by the innate immune response. Typical cell types included in innate immunity include macrophages, dendritic cells, neutrophils, and natural killer cells (NK). There are two major pathways involved in the recognition of invading pathogens. The first system recognizes signals from the invading microorganism, pathogen-associated molecular patterns (PAMPs). The second recognizes signals from endogenous sources such as dead or dying tissues and cells, damage-associated molecular patterns (DAMPs). These signals are recognized by sentinel cells of the innate immune response, including macrophages and dendritic cells, when they bind to pattern recognition receptors (PRRs) located on the cell membrane. Microorganisms exhibit great diversity; however, there are some common elements necessary to their survival that are highly conserved among microbial species but are not found in mammalian tissues. These include peptidoglycans, lipoteichoic acids, and lipopolysaccharide (LPS). These PAMPs are recognized by a variety of receptors, PRRs, found on the cell membrane, in the cytosol, or in cytoplasmic vesicles. Some PRRs are soluble and found circulating in the blood. The most important of these PRRs is the toll-like receptor (TLR). TLRs are located both on the cell membrane and within the cytoplasm. Cell surface TLRs recognize lipoproteins and LPS, whereas the cytosol TLRs recognize bacterial nucleic acid. TLR 9 and 11 are the most important when considering protozoal disease.(16, 17)

Once the PRRs are activated by binding to their microbial targets, a signaling cascade is initiated within the cell resulting in an inflammatory response and activation of the adaptive immune response. Macrophages and neutrophils are signaled to release lysosomal enzymes and increase phagocytic activity. The macrophages involved in this process are pro-inflammatory and release TNFα, neutrophil chemotactic factors, and nitric oxide (NO) and are called M1 cells. The signaling cascade initiated by activation of PRRs also involves the activation of molecules such as nuclear factor kappa B (NFkB) and MAP kinase (MAPK) by the adaptor protein MyD88. These transcription factors initiate the production of interleukin 1 (IL1), interleukin 6 (IL6), tumor necrosis factor alpha (TNFα), and interferon beta (IFN β). Activation of PAMPs and DAMPs begin production of cytokines which activate the adaptive immune response.(16, 17)

In addition, NK cells use a slightly different mechanism of identifying foreign invaders. These cells look for expression of major histocompatibility complex class I (MHC Class I). This is a highly conserved molecule that is not present on microorganisms. Dying or infected cells reduce their expression of MHC Class I also activating NK cells. The lack of MHC Class I identifies a cell as "non-self" and therefore a target for the immune system.(16, 17)

When these signaling pathways are activated, production of a second round of several different cytokines begins. Chemokines to attract other immune cells to the area and enzymes to break down invaders and damaged cells such as NO, and cyclooxygenase 2 (COX 2) are also produced inducing the expression of inflammatory lipids such as prostaglandins and leukotrienes. TNFα acts on the vascular endothelium in conjunction with IL1, pro-inflammatory cytokines influencing the innate immune response, to make vessels dilate and become more permeable. They prolong inflammation by a positive feedback loop increasing production of TNFα and IL1 as well as COX 2.(17)

The job of the innate immune response is to quickly and non-specifically destroy invading pathogens at the point of invasion. If this process fails, the adaptive immune response takes over. The shift between an innate response and an adaptive immune response is mediated by cytokine production and antigen presentation to T cells. Based on the combination of these two signals, a specific immune response can be elicited: the humoral or cytotoxic immune response. Dendritic cells (DCs), macrophages, and B cells are all capable of processing and presenting antigen, though dendritic cells are the most efficient. Immature dendritic cells and macrophages express low levels of MHC Class II molecules, but expression increases once they are exposed to antigen and maturation is induced. As these cells mature in response to antigen or inflammatory cytokines, they also increase their expression of co-stimulatory molecules, such as CD86.

Based on the specific cytokine production of the dendritic cell, T cells will be stimulated in one of three ways (Figure 1): towards a humoral or T helper 2 (Th2) response, towards a cell mediated or T helper 1 (Th1) response, or towards tolerance or a T regulatory (Treg) response. A Treg response is typically stimulated when self-antigen or non-pathogenic antigen is spontaneously processed by the dendritic cell. If a T cell recognizes this antigen, the DC or macrophage will produce IL10 which stimulates a Treg response. In response to secretion of IL12 by the DC or macrophage, Th1 cells will be activated resulting in a cell mediated immune response. These DCs are referred to as DC1. Alternatively, if a DC or macrophage secretes IL1 and IL6, Th2 cells will be activated and a humoral immune response will be initiated. These DCs are referred to as DC2 cells.(16, 17)

Different types of lymphocytes can be identified by their characteristic cell surface markers. These are known as cluster of differentiation (CD) molecules. The major lymphocytes of interest for our study are B-cells, CD4 lymphocytes, and CD8 lymphocytes. CD4 is present only on Th cells, and CD8 is present on T cytotoxic (Tc) cells. Th cells can be further identified as Th1 or Th2 based on the cytokines they produce

and which arm of the immune system they trigger. CD4 Th1 cells are signaled by secretion of IL12 and subsequently the Th1 cells will begin to produce IFNɣ. These cells then activate CD8 Tc1 cells.(16, 17)

CD4 Th2 cells are activated by secretion of IL1 and IL6 promoting a Th2 response. The activated Th2 cells then begin to produce IL4. A Treg response is signaled by the production of IL10, which promotes Treg differentiation and production of more IL10. Treg cells also use a unique specialized transcription factor, FoxP3, which is responsible for the production of IL10 and TGFβ.(18, 19)

The cytokines IL6, TGFβ, and IL23 promote activation of CD4 Th17 cells. In response, these cells produce IL17, upregulate neutrophil mediated inflammation, and are potent B cell helpers. Th17 cells have the ability to readily switch to Th1 cells and may be a transient stage in the development of T cells. They may eventually convert into IFNɣ producing Th1 cells. It is also possible that Th17 cells and Treg cells are alternate fates of the same lineage.(16, 17)

The cell surface molecules CD5 and CD21 are B cell markers used in other species. CD5 is an adhesion molecule and receptor for CD72. It recognizes common bacterial molecules as well as immunoglobulin and DNA. CD5 is present on particular subset of B cells from a fetal liver or omentum lineage (B1 cells) rather than a myeloid origin like conventional B cells (B2 cells). In horses, CD5 is present on approximately 80% of T lymphocytes as well as NK cells and at very low levels on mature B2 lymphocytes.(20) The surface receptor CD21, on the other hand, has been shown to bind to equine B lymphocytes almost exclusively. The binding pattern suggests that only one population of lymphocytes, B cells, are stained.(21) CD21 is a complement receptor that binds C3d. When antigen with C3d binds to CD21, a signal is sent through the CD19 molecule that lowers the threshold for B cell activation 100 fold.(17)

1.1.5 Immune Response to *Sarcocystis neurona*:

A robust cell mediated immune (CMI) response, as evidenced by a Th1 response and IFNɣ production, is important in resistance to many protozoal and intracellular pathogens. Several models in rodents and horses support the role of the CMI response to *S. neurona* as well. Several studies have been performed demonstrating the necessity of CMI for protection against other protozoal organisms, such as *Toxoplasma gondii*, and some have shown that the protozoan itself is able to induce suppression of the host immune system.

1.1.5a Evidence in Mice:

Several studies in mice have begun to elucidate the protective immune response to *S. neurona*. One of the earliest studies performed demonstrated that mice could be infected with *S. neurona*, but that the immune status of the mouse determined whether or not encephalitis would develop. Mice that did not have functional T cells would develop encephalitis, whereas immunocompetent and severe combined immunodeficiency (SCID) did not.(22) Because of this, it was suspected that T cells and the innate immune response may be playing a vital role in protection from the development of disease.

An additional study in SCID mice demonstrated the dependence on IFNɣ for protection. Sellon et al. demonstrated that *S. neurona* infected SCID mice developed disease when IFNɣ was depleted. Infection of SCID mice with *S. neurona* followed by eliminating IFNɣ with an anti-IFNɣ antibody resulted in the development of neurologic disease like that of immunocompetent mice. However, when only depleting the NK cell population of SCID mice, neurologic disease failed to develop.(23) This suggests that while IFNɣ is vital for protection from disease, NK cells do not seem to play an important role.(23) Therefore, it is possible that another innate immune cell type that produces IFNɣ may be responsible for protection from disease.

Dubey, Lindsay, and colleagues performed several early studies demonstrating the critical role of IFNɣ as well as identifying some of the pathologic changes induced by *S. neurona* infection in those mice.

Infection of IFNɣ knockout mice have shown that the protozoa initially divides in the intestinal tissues before the mice become parasitemic 1-8 days post infection.(10, 24, 25) IFNɣ knockout mice develop fulminant neurologic disease after infection, while immunocompetent mice do not.(10, 26, 27) These studies begin to suggest that T cells and IFNɣ are important to protect against disease after infection with *S. neurona* indicating an important role for the cell mediated immune response.

Witonsky et al. demonstrated later that the protective immune response to *S. neurona* in immunocompetent mice was mediated by both CD4 and CD8 cells, but placed more importance on the CD8 response.(28) Immunocompetent mice developed an increase in CD44/CD8 memory T cell population but did not develop disease. In contrast, IFNɣ knockout mice did develop disease, but did not develop the same increase in memory T cell population.(27) Furthermore, infecting CD8 deficient mice resulted in lethal encephalitis while a memory T cell response did not develop.(29) These mice had functional CD4 T cells and yet were unable to prevent the development of disease after infection with *S. neurona*.

The importance of the cell mediated immune response was supported in another study utilizing B cell deficient mice. In this study, the B cell deficient mice did not develop encephalitis even though no antibodies were produced in response to infection. They did, however, develop an increase in CD4 and CD8 cells in response to infection.(30) These results suggest that a humoral immune response is not necessary for protection against the development of encephalitis after infection with *S. neurona*.

1.1.5b Evidence in Horses:

One of the earliest studies demonstrating the importance of the CMI response in immunity towards EPM was performed by Tournquist and colleagues in 2001.(31) In this experiment, peripheral blood mononuclear cell (PBMC) proliferation and the immunophenotype in horses naturally infected with *S. neurona* that had developed clinical disease of EPM was compared to PBMC responses from horses that

were seropositive for *S. neurona* but asymptomatic and to horses that were both seronegative and asymptomatic. Using a [$^3$H] thymidine incorporation assay, it was determined that symptomatic horses had a significantly decreased proliferation index to Con A stimulation as well as to stimulation with *S. neurona* antigen compared to both seropositive-asymptomatic and seronegative-asymptomatic horses.(31)

In addition, immunophenotyping of PBMCs was performed by flow cytometric analysis of cell subtypes. Monoclonal antibodies to CD4, CD8, and CD172a, a marker for granulocytes, were used. Symptomatic horses had a lower mean percentage of circulating CD4+ lymphocytes than seropositive-asymptomatic horses and seronegative-asymptomatic horses. There was no significant difference in CD8+ lymphocytes or CD172a+ monocytes between the groups.(31)

The Tornquist study, while suggesting that the cell mediated immune response is reduced in EPM horses, did not elucidate whether the decreased immune response was pre-existing or if it was a result of the infection itself. A later study by Spencer and colleagues demonstrates decreased parasite specific proliferation by EPM affected horses. This supports that the idea that the protozoa itself decreased parasite specific immune responses.(32)

In this study, PBMCs were harvested from 43 horses negative to serum and CSF EPM Western Blot and 28 horses with clinical signs of EPM and Western Blot positive CSF samples for antibodies against *S. neurona*. Lymphocytes were plated in 96-well plates with either SnSAG1 or 5µg/mL concanavalin A (ConA). Unstimulated controls were plated with media only. Proliferation was assessed by [$^3$H] thymidine incorporation assay and reported as mean counts per minute (CPM). Results showed EPM positive horses had significantly lower SnSAG1 induced proliferation compared to EPM negative horses (mean CPM were 3,344.6 and 8,538.4, respectively). No statistically significant difference in unstimulated or ConA induced proliferation was discovered between the groups; however, the power of

the study was very low (0.403) which may have explained the lack of significance. More information may have been gleaned if an experimental group of EPM western blot serum positive horses without clinical signs were included in the study.(32)

In addition, lymphocytes from these horses were stimulated with SnSAG1 and harvested after 12, 24, 48, and 72 hours to assess for the presence of IFNɣ using reverse transcriptase polymerase chain reaction (Rt-PCR). No IFNɣ was detectable at any time point in EPM positive horses while, in EPM negative horses, IFNɣ was detectable by 12 hours in some horses and by 24 hours in all samples.(32)

A follow-up study performed by this group showed that EPM affected horses had a statistically significant decrease in IFNɣ expression after 48 hours and an increase in IL4 expression by PBMCs after 72 hours of culture with SnSAG1.(33) Similar groups to the previous study were used (21 EPM negative horses as determined by CSF Western Blot and lack of clinical signs, and 21 EPM positive horses as determined by CSF Western Blot and presence of compatible clinical signs). Cytokine production was determined by Rt-PCR. No significant difference was found for the cytokines: TNFα, IL2, or IL6.(33)

Interestingly, slightly differing results were found in a study performed by Yang and colleagues.(34) In this study, PBMCs were isolated from 22 horses with at least a presumptive diagnosis of EPM. Horses were considered to have a diagnosis of EPM if they exhibited characteristic clinical signs on neurologic examination, were positive for *S. neurona* antibodies on Western Blot or SAG1 analysis of serum or CSF, responded to treatment for EPM, or had characteristic lesions on necropsy. Twenty seropositive horses without clinical signs of EPM were used as a control group. Leukocyte proliferative responses to stimulation with 1µg/mL Pokeweed mitogen (PWM), 1µg/mL or 5µg/mL ConA, 20ng/mL phorbol myrisate acetate (PMA) and 10pg/mL ionomycin (I), and live *S. neurona* merozoites were compared. Proliferation was measured based on a [$^3$H]-thymidine incorporation assay. Changes in proliferation were compared to unstimulated controls and reported as delta counts per minute. PBMCs were also

isolated and percentage of cell subsets were determined based on cell surface staining and flow cytometric analysis.(34)

Significant results from this study showed that horses with EPM had significantly lower proliferative response to stimulation with PMA/I than non-EPM affected horses. The response to PWM was not significantly different between groups. However, unlike the study performed by Spencer et al., no significant difference was found in the proliferative response to ConA between EPM and non-EPM affected horses. Like previous studies, a non-significant difference in antigen specific response between EPM and non-EPM affected horses was elucidated. However, when blood was stored overnight and analyzed the following day, EPM affected horses actually had an increased antigen specific proliferative response compared to non-EPM affected horses.(34) It is unclear why this may be the case.

For immune subset percentages, it was found that EPM affected horses had an increased percentage of CD4+ cells. However, the percentage of CD8+ cells, CD5+ cells, and monocytes were not significantly different between groups. EPM affected horses had a slight, but significant, increase in neutrophils compared to non-EPM affected horses.(34)

This study further supports a possible role of parasite induced decreased immune responses as evidenced by the decreased proliferative response of PBMCs from EPM affected horses to non-specific stimulation by PMA/I as well as to *S. neurona* merozoites. Further, an increase in percentage of CD4+ lymphocytes in EPM affected horses may also play a role, though the mechanisms for why and how this change occurs is unclear. It is also unknown if these changes are a result of infection of the horses by *S. neurona*, or if the changes are present before, and possibly a prerequisite for, infection.

Slightly conflicting results were obtained in a study performed by Witonsky and colleagues.(35) The objective of their study was to determine whether horses experimentally infected with *S. neurona* developed suppressed immune responses. Horse were experimentally infected with *S. neurona* and

PBMCs were enriched from blood. Lymphocyte proliferation in response to stimulation with ConA, PMA/I, PWM, and *S. neurona* merozoites was compared to healthy controls. They also compared IFNɣ production in infected horses versus controls.

As opposed to earlier studies in naturally infected horses, no decreased proliferation of lymphocytes was present in response to *S. neurona* specific stimulation. However, like the previously mentioned study by Witonsky et al., there was a significant decrease in proliferation to non-specific stimulation by PMA/I as soon as 2 days post-infection. In addition, a trend towards decreased IFNɣ production by infected horses compared to controls was identified, though the difference was not significant.(35)

There are several possibilities why the responses to antigen specific proliferative response was different in this case compared to the others. For one, the method of infection was different in the experimentally infected horses in this study compared to previous studies by others. In this study, whole blood was collected and PBMCs were isolated and infected with *S. neurona* merozoites, then autotransfused back to the horse. Previous studies by others relied on oral inoculation of the parasite,(12, 13, 15, 36, 37) or by direction intravenous infusion of merozoites.(36) Using infected PBMCs as a mode of infection may result in a longer time until development of altered immune responses after infection since this method of administration may effectively "hide" the merozoites from the immune system. Also, in this study, horses were infected with a SnSAG 1+ strain, but the strain of *S. neurona* used as mitogen did not express SnSAG 1. This could result in less significant results since the antigen may not be as specific as it would be if both strains were the same. Also, by bypassing the normal method of infection, it is possible that the same alterations in immune response seen in naturally infected horses does not occur to the same degree in experimentally infected horses.

Another study by Lewis and colleagues(38) suggested that horses with clinical EPM may have decreased ability of antigen presenting cells to activate CD4 and CD8 cells. For this study, horses were

experimentally infected with *S. neurona* and their in vitro response to mitogen and *S. neurona* merozoites were evaluated. They showed that, while response of PBMCs to PMA/I was increased in EPM positive versus EPM negative horses, antigen specific induced proliferation was decreased in affected versus unaffected horses. This suggests that EPM horses may have decreased ability to process and present *S. neurona* specific antigen while being at a heightened state of activation to non-specific antigen. Additionally, proliferation and apoptosis of cells were intermittently affected based on infection status.(38)

Another study investigated the effects of oral and intravenous inoculation with *S. neurona* (WSU-1 strain) sporocysts or merozoites, respectively, on SCID horses compared to immunocompetent horses.(36) Two immunocompetent and 2 SCID Arabians were infected orally with sporocysts and 4 immunocompetent and 1 SCID Arabian were infected intravenously. The horses were monitored for development of neurologic signs, parasitemia, seroconversion, and CSF antibody production. PCR was used to detect the presence of *S. neurona* in blood and tissues.

Immunocompetent horses remained healthy and antibody titers against *S. neurona* were detectable in the serum and CSF by day 30 post-infection. Four of 6 immunocompetent horses developed neurologic signs at days 28, 42, 50, and 289 post-infection. Immunocompetent horses did not have detectable parasites in the blood 3 hours post-infection. *S. neurona* was not detected in peripheral tissues in any infected immunocompetent horse; however, immunocompetent horses that developed neurologic signs had positive CNS tissue samples by PCR and/or culture at post mortem examination.(36)

SCID horses, on the other hand, remained seronegative to *S. neurona* at all time points. While no SCID horses were PCR positive for *S. neurona* in the blood, *S. neurona* was cultured from the blood for all horses at varying time points. The protozoa was also detected by both PCR and culture from peripheral tissues at post-mortem examination, but were negative by both PCR and culture on CNS samples.

Histopathology on SCID affected horses showed findings consistent with bronchopneumonia in one SCID horses and mild multifocal hepatic necrosis in another SCID horse. Another SCID horse had evidence of moderate follicular lymph node necrosis.(36)

The results of this study suggest that a competent immune system is necessary to control parasitemia and prevent peripheral infection with *S. neurona*, but is apparently not necessary to prevent invasion of the CNS, and perhaps may be required. Two possible explanations are proposed. First, it is possible that an intact innate immune response is sufficient to prevent CNS infection with *S. neurona*. Second, it is possible that T- or B- cells are necessary for the protozoa to gain access to the central nervous system and subsequently cause disease. This second option may be less likely since the authors of this study report that administering anti-IFNɣ to SCID mice results in the development of severe fulminant development of neurologic signs after inoculation with *S. neurona* merozoites despite the lack of T and B cells.(36)

1.1.6 Diagnosis:

There are many challenges to diagnosing EPM due to the high seroprevalence rate but low incidence of disease in the United States. The only method for definitive diagnosis is by identifying characteristic lesions and parasites within the central nervous system.

Clinical antemortem diagnosis begins by identifying compatible clinical signs, ruling out other possible causes of the clinical signs, and then proving exposure of the horse to *S. neurona* or *N. hughesi*.

Equine Protozoal Myeloencephalitis should be considered a differential diagnosis in any horse presenting with neurologic signs. Since any area of the nervous system can be affected in horses with EPM, it can present similar to many other neurologic diseases. Therefore, the first step to diagnosis is performing a thorough neurologic examination and identifying a neuro-anatomical localization that explains the clinical signs. Once this is accomplished, a list of compatible differential diagnosis should be

made. Appropriate diagnostic testing to rule out other differentials and to prove exposure of the horse to *S. neurona* must then be performed. It is beyond the scope of this thesis to discuss appropriate diagnostic testing to rule out other neurologic disease; however, the following is a discussion about diagnostic testing for EPM due to *S. neurona*.

1.1.6a Immunodiagnostic testing:

Several serological tests are available to measure antibody production against *S. neurona*; however, positive results of these tests alone are of minimal value to the clinician because of the high rate of exposure. Cut-off values for each test has been developed in order to help increase the probability of true EPM cases compared to exposure; however, the development of semi-quantitative tests and tests capable of detecting intrathecal antibody production has improved the value of these tests.

Use of intrathecal antibody production, C-value, antigen specific antibody quotient to accurately diagnose EPM cases:

While detection of antibodies in serum alone is adequate to prove exposure, finding antibodies within the CSF can greatly increase the likelihood that active infection is present within the central nervous system. However, the detection of antibodies within the CNS alone insufficient to prove intrathecal antibody production since there is normally a certain rate of diffusion of antibodies from the peripheral blood into the CNS. When the blood-brain-barrier (BBB) is intact, this happens at a constant, predictable rate. Therefore, by comparing the antibody levels in the serum to that in the CSF, we can then predict whether or not there has been active intrathecal antibody production.(39) If the integrity of the BBB is questioned, on the other hand, a simple comparison of serum to CSF ratios may not be adequate. In this case, comparison of the ratio of antigen specific antibody to other proteins provide a much more accurate estimation of intrathecal antibody production.(39, 40) Blood contamination of the sample may

also produce falsely elevated results. Performing a cytological examination of the fluid or comparing albumin levels to antigen specific antibodies can help determine if blood contamination has occurred.

There are two methods currently used to identify intrathecal antibody production, the Goldman-Witmer coefficient (C-value), and the antigen specific antibody index.(40) The C-value compares the ratio of serum:CSF total IgG to the ratio of serum:CSF antigen specific antibody. The antibody index compares serum and CSF albumin to serum and CSF antigen specific antibody. In this way, we can predict whether there is intrathecal antibody production rather than just increased diffusion across the BBB or blood contamination.

1.1.6b Serological Tests:

There are several serological tests that are currently available. They are the Western Blot, the Immunofluorescent Antibody Test (IFAT), and two Surface Antigen enzyme-linked immunosorbent assays (ELISAs), the SAG 1, 5, 6, and the SAG 2, 4/3. In addition, a polymerase chain reaction (PCR) assay is also available.

The first diagnostic test developed was the Western Blot. Unfortunately, the original test had a low specificity since cross reaction with other protozoa could result in false positive test results. A modified western blot that uses bovine serum with antibodies against *S. cruzi* to "block" cross-reacting antigens has since been developed. The Western Blot uses whole merozoites as antigen and therefore the test will detect antibodies that are directed towards any *Sarcocystis* species, even non-pathogenic ones. By exposing the immunoblots to bovine *S. cruzi* IgG prior to the equine test sample, non-specific proteins are "blocked" which reduces false positive results.(41) This test has improved the sensitivity (89%) and specificity (69%), but other more accurate tests are available. The use of cut-off values that have been established for the test is also designed to help eliminate the possibility of false positive results due to cross-reactivity. Interpretation of the western blot and the modified western blot is qualitative relying

on subjective evaluation of colored bands. These tests require extensive experience to interpret correctly.

The IFAT is another test that is available for EPM diagnosis. Whole merozoites are immobilized on a glass slide/well and the slide/well is exposed to serum from the test subject. If present in the sample, antibodies bind to the surface antigen of the merozoite on the slides/well. A secondary fluorophore-linked antibody binds to the IgG from the serum in the sample and fluorescence is visually assessed. Like the western blot, the IFAT uses whole *S. neurona* merozoites as antigen. The IFAT available from University of California, Davis uses a surface antigen (SAG) 1 expressing strain. Also like the western blot, the IFAT is a subjective test that requires experience by the person interpreting the results. The IFAT, however, is subject to false positive results by cross reaction with *S. fayeri* antibodies, a protozoan to which horses are often exposed but which does not cause disease. The reported sensitivity and specificity vary and depend on the sample and control populations used in the study.(42, 43) In one study comparing IFAT results to the western blot and modified western blot in neurologic and non-neurologic horses, the IFAT had a reported sensitivity of 89% and the specificity was reportedly 100% in this patient population.

The newest available tests are the surface antigen (SAG) ELISAs. These tests detect antibodies to specific surface antigens that are immunogenic and associated with *S. neurona*. These antigens are expressed as recombinant proteins in *Escherichia coli*. However, antigenic diversity has been demonstrated in different strains of *S. neurona*, and this must be considered when choosing a test.(44, 45)

One ELISA test available for EPM diagnosis is for antibodies produced against SAG 1, 5, and 6. The first surface antigen that was discovered and developed for testing was the SAG 1.(46) Since then, it has since been shown that several strains of *S. neurona* do not express this surface antigen. Another study

performed by Crowdus and colleagues has suggested that the surface antigen SAG 5 is expressed in some strains that do not express SAG1 and that these two surface antigen are mutually exclusive.(45) In this same study, however, one tested strain that had been isolated in a sea otter with encephalitis expressed neither of these surface antigen and instead expressed a sequence that likely encodes a novel SAG.(45) An identical SAG was isolated in a subsequent study evaluating the genetic diversity of *S. neurona* strains in sea otters. This SAG was named SAG 6 by the authors.(47)

The results of these studies suggests that neither SAG 1, 5, or 6 are required for survival or CNS penetration of *S. neurona* and that antigenic diversity must be taken into account when selecting and interpreting tests. The SAG 1, 5, 6 ELISA attempts to minimize the effects of surface antigen diversity in *S. neurona* species by including all three surface antigen. By including multiple surface antigen in the test, it may also allow for differences in the immune response of individual horses. Limited studies are available evaluating the validity of this test.

Another ELISA has been developed detecting antibodies against SAG 2 and a chimeric protein SAG 4/3. This test was developed based on studies showing that surface antigen 2, 3, and 4 are highly conserved among *S. neurona* species, including several strains causing natural infection in horses, and show no cross-reactivity with other common parasites.(44, 48, 49) In sea otters, there may be cross reactivity with this test and exposure to *S. falcatula*, however, it has been shown that horses do not develop an antibody response to this protozoa and therefore cross-reactivity is not a concern.(37)

1.1.7 Treatment:

Several treatments for EPM are available. Anti-protozoal medications are the mainstay of treatment and adjunctive therapies consist of anti-inflammatories and immunomodulating drugs.

1.1.7a Folate Inhibiting Drugs:

The combination of sulfadiazine and pyrimethamine (SDZ/PYR) was one of the earliest drug therapies for horses with EPM. The combination of these two drugs inhibits folate metabolism in the parasite. Sulfadiazine acts by competing with para-aminobenzoic acid as a substrate for the enzyme dihydropteroate synthase (not present in mammalian cells) which incorporates para-aminobenzoic acid into dihydropterioic acid, the immediate precursor of folic acid. Pyrimethamine inhibits dihydrofolate reductase activity which is necessary for purine and pyrimidine nucleotide synthesis. Pyrimethamine effects mammalian dihydrofolate reductase as well, but is more active against the parasite. The combination of these two drugs causes the inability of the protozoa to synthesize nucleic acids and amino acids which results in death of the organism. Unfortunately, these drugs can inhibit folate metabolism in the host resulting in bone marrow suppression and anemia, leukopenia, and fetal abnormalities. The treatment protocol requires a long duration of treatment to achieve clinical resolution, with 3 months being the standard minimum treatment duration.(2) Absorption of pyrimethamine can be inhibited by dietary folate, therefore, it is best to fast the patient for 2 hours prior to administration and keep the horse fasted for 2 hours after administration.(50)

The typical dosage regimen is 1 mg/kg pyrimethamine and 22 mg/kg sulfadiazine once daily. This dosing regimen has shown to achieve CNS concentrations of pyrimethamine of 0.02-0.1 µg/mL and sulfonamide of 2-8 µg/mL.(51, 52) Pyrimethamine and sulfadiazine are available as an FDA approved suspension called ReBalance®[b].

1.1.7b Triazine Drugs:

Two such drugs have been approved for use in horses for treatment of EPM, diclazuril and ponazuril. These drugs are suspected to target the apicoplast organelle, a chloroplast organelle that is involved with messaging in the nucleus of the organism. These drugs are considered to be coccidiostatic, though

efficacy studies between coccidiostatic and coccidiocidal drugs show that the two provide similar outcomes.(1, 53, 54)

Ponazuril:

Ponazuril is a metabolite of toltrazuril, another anti-coccidial drug that is used in pigs and lambs. Ponazuril has been shown to inhibit development and asexual reproduction in *S. neurona*.(53) In horses, therapeutic levels of ponazuril are achieved by 7 days after beginning treatment.(55) Because of this, using a loading dose of 15 mg/kg has been recommended to achieve therapeutic concentrations more quickly. Administering ponazuril concurrently with 2 ounces of vegetable oil may increase the absorption of the drug (Furr, unpublished data).

When dosed at 5 mg/kg orally, steady state concentrations of 0.16 +/- 0.06 μ/mL in the CSF were achieved.(55) In one study by Furr and colleagues, the efficacy of the drug was found to be 60% and a relapse rate to be 8% with a treatment course of 28 days in a study population of 101 horses.(56) Treatment success is thought to be improved with a longer course of treatment, and it is generally recommended to continue treatment as long as signs of improvement exist. Ponazuril is currently available as an FDA approved paste called Marquis®[c].

Diclazuril:

Similar to ponazuril, diclazuril is a triazine drug that has broad spectrum anticoccidial activity thought to exert its effects on the apiclast or mitochondria of the organism. Its chemical composition is similar to ponazuril. In a study performed by Hunyadi and colleagues, therapeutic levels of diclazuril were attained in the CSF of horses treated with a dose of 0.5 mg/kg which is half of the recommended dose.(57) Diclazuril is absorbed quickly after administration and a loading dose is not necessary.

A study by Pusterla and colleagues determined that foals fed a daily low dose of diclazuril (0.5mg/kg) from age 4 weeks to 12 months had a reduced monthly seroconversion rates (6-37%) compared to untreated foals (53-82%) at the same farm. This may be explained by a decrease rate of infection of foals treated with the daily low dose diclazuril.(58)

The current recommended dose of diclazuril is 1 mg/kg orally once daily. It is available as an FDA approved pellet called Protazil®[d].

1.1.7c Decoquinate:

Decoquinate is a quinolone anticoccidial drug used to treat intestinal coccidiosis in poultry, cattle, sheep, and goats. It inhibits coccidial respiration by interfering with electron transport from ubiquinone to cytochrome c in the mitochondria. A study by Lindsay and colleagues showed that decoquinate has cidal effects against *S. neurona* in vitro at a concentration of 240 nM when treated for 10 days.(59) A clinical field study performed by Ellison and colleagues showed that 93.6% of horses (132 of 141) receiving decoquinate combined with levamisole after a presumptive diagnosis of EPM improved clinically after treatment. The same study showed that 89.3% of horses receiving decoquinate-levamisole combined treatment showed a drop in serum titer to SAG 1, 5, 6 four to six weeks after treatment.(60) These results are promising, though several flaws exist in this study. For example, EPM was only a presumptive diagnosis in these cases and diagnosis was based solely on the presence of clinical signs and serum antibody titers against SAG 1, 5, 6. No CSF was collected in these horses, and there was no standardization of categorization of clinical signs. Whether or not other neurologic diseases were considered and ruled out was not disclosed and the use of other ancillary treatments were not reported. The patient population was derived from many different referring veterinarians and therefore standardization of diagnosis and treatment is highly unlikely. However, the results are interesting and more investigation is necessary.

Decoquinate is available either alone in combination with levamisole in products known as Orogin or Oroquin 10 from Pathogenes, Inc, though currently they do not have FDA approval.

1.1.7d Supportive Therapies:

In some cases of EPM, additional supportive therapies may be warranted. Non-steroidal anti-inflammatory drugs (NSAIDs) such as phenylbutazone or flunixin meglumine can help reduce transient worsening of clinical signs when initiating therapies. In severe cases, the use of corticosteroids may also be considered, though steroid treatment should be limited to the short-term since it is unknown how they may affect the immune response and clearance of *S. neurona*. Anti-oxidant therapies such as vitamin E and free-radical scavengers such as dimethylsulfoxide (DMSO) may also be used, though there is limited experimental evidence of a benefit specifically in EPM affected horses.(3)

As described previously, there is evidence suggesting that horses become susceptible to EPM when immunocompromised. Therefore, immunomodulators have been used by some clinicians to help treat EPM. These products include killed *Propionibacterium acnes*, mycobacterial wall extract, inactivated parapox ovis virus, and transfer factor. Another immunomodulatory drug that has been used for its anecdotal benefits include levamisole, the subject of this research project. No current studies exist on the benefit of these drugs in the treatment of EPM. However, there has been evidence in other species that levamisole may modulate the immune system. Minimal experimental evidence is available to show the effects of levamisole on the equine immune system.

1.2 Levamisole:

Levamisole is a synthetic phenylimidazolthiazole drug that was first introduced in 1966 as an anthelmintic. Since then, it has been used as an immunomodulatory drug in the treatment of various diseases in humans, such as atopic disease, leprosy, and colorectal cancer. Experimental studies investigating the role levamisole plays in modulating the immune system have produced conflicting

results, some showing enhancement of the cell mediated immune response, others showing inhibition of the immune response, and still others showing no effect.(61-65) However, in clinical trials, levamisole has consistently been shown to improve outcomes in patients with certain types of colorectal cancer.(66-73) Despite this, the mechanism of action of levamisole is still being determined.

1.2.1 Evidence in Rodents:

An early study in mice demonstrated that the effects of levamisole may be due to T-cell mediated feedback suppression on the humoral immune response.(61) Mice (BALB/c, lacking a thymus) were either injected with levamisole at 2.5 mg/kg or 10 mg/kg at various time points before challenge with intraperitoneal injection of sheep erythrocytes (experimental group) or saline (control group). Spleens were harvested four days after challenge and splenic plaque forming cells were counted. Their results show that mice receiving levamisole at 10 mg/kg 4 or 6 days prior to challenge had suppressed IgM antibody responses to intraperitoneal sheep erythrocyte injection compared to controls. This effect was not seen in mice administered the lower dose of levamisole or when administered either dose of levamisole 8 days prior to challenge. In fact, though they do not show the data, the 2.5mg/kg dose of levamisole appeared to enhance the antibody response, while lower doses had no effect.(61)

When the kinetics of the antibody response was investigated, levamisole treated mice had a peak response 12 hours earlier than control mice. The numbers of plaque forming units were slightly, though significantly, increased compared to controls at their respective peaks. Yet, at the 4 day time point, the number of plaque forming units in mice receiving levamisole was much lower than control mice. The number of plaque forming units of treated mice was similar to those of control mice by day 6.(61)

Interestingly, this study also showed an effect of antigen dose on the levamisole mediated response. In mice that were administered $2 \times 10^7$ sheep erythrocytes intraperitoneally, levamisole administration at 10mg/kg increased the number of splenic plaque forming cells by up to 200%. Mice that were

administered 2 x $10^8$ sheep red blood cells intraperitoneally showed a decreased of splenic plaque forming cells by 50%.(61)

Further, this study also provides evidence that T cells are important in the mitigation of the levamisole induced suppression of the IgM response. Untreated mice were administered splenic cells from levamisole treated mice. The recipient mice were than administered sheep erythrocytes as before. These mice had a significantly decreased humoral immune response compared to control mice. However, when recipient mice were also given anti-Thy 1.2 antiserum plus complement, the IgM suppression was eliminated.(61) Since T cells and not B cells are depleted after treatment with anti-Thy 1.2 antiserum, this provides evidence that T cells, and not B cells, are mediators of the suppression of the IgM response.

Similar to the earlier experiment, however, enhancement of the IgM response (instead of suppression) was observed when lower doses of treated mouse splenic cells were administered as well as when lower doses of sheep erythrocytes were used.(61)

This study is interesting for several reasons. It demonstrates that T cells play a role in modulating the humoral immune response in mice. However, it also clearly demonstrates that this response is affected by several variables including: levamisole dose, timing of administration, antigen dose, and time after antigen administration. Not only was suppression of an IgM response either achieved or not achieved, but enhancement of the response occurred depending on the conditions.

A study performed in 2000 used Brown Norway rats (high levels of IgE and relatively deficient cell mediated immunity) to determine the effects of levamisole on Th1/Th2 cytokine balance.(63) In their experiment, an optimal dose of levamisole, 25mg/kg, was determined and this dose was then used to treat mice for 7 days. To determine the optimal dose of levamisole, mice were injected intraperitoneally with levamisole at several doses ranging from 0.625mg/kg to 25mg/kg once daily for one week. Serum

IgE and IFNɣ levels were measured daily. The results showed a dose dependent induction of serum IFNɣ and suppression of IgE.(63)

Following the determination of an optimal dose of levamisole, rats were administered 25mg/kg levamisole intraperitoneally (IP) once daily. Blood samples were taken on days 0, 3, 5, 8, and 12 to measure serum IgG and IFNɣ levels. Groups of two rats were killed at each time point and their spleens were harvested to determine cytokine gene expression (IL12, IL18, and IFNɣ). The results indicated that levamisole treatment results in an increase in serum IFNɣ levels and IFNɣ gene expression, and a suppression of serum IgE levels and IL4 gene expression by day 3 after levamisole treatment. This increase in IFNɣ production was associated with an increase in IL18, but not IL12, gene expression.(63)

Additionally, the effect of levamisole on ameliorating the vigorous autoimmune response these mice exhibit after administration of mercuric chloride were investigated. The rats were injected intraperitoneally with 25mg/kg levamisole on days -2 through +5 and blood samples were taken at 5 day intervals for 20 days. Two rats from the experimental group and control group were euthanized at each time point to measure splenic cytokine gene expression. Levamisole treated rats exhibited a 2-log reduction in maximum serum IgE levels accompanied by a marked reduction in splenic IL4 gene expression.(63)

The results of this study show an increase in IL18 gene expression which is produced by macrophages and monocytes. Therefore, unlike the previous study, this finding supports the involvement of monocytes/macrophages in the levamisole induced shift from a humoral towards a cell mediated immune response.

1.2.2 Evidence in Humans:

A study performed by Chen and colleagues further support that levamisole may enhance a Th1 response by affecting the activation and maturation of monocyte derived dendritic cells.(62) In this study,

dendritic cells from healthy donors were isolated from peripheral blood mononuclear cells (PBMCs) by positive selection using anti-CD14+ microbeads. The cells were then cultured with granulocyte macrophage-colony stimulating factor and IL4. These cells were then cultured for 48 hours with 1µM levamisole, lipopolysaccharide (LPS), a combination of levamisole and LPS, or with media alone. Cytokine production (IL12 p40 and IL10) was then evaluated. Results showed that levamisole slightly enhanced DC secretion of these cytokines both alone and with LPS stimulation.(62) Whether or not these results would be clinically significant is unknown.

Dendritic cells that had been treated with LPS or levamisole were then added to purified populations of naïve CD4+ T cells to determine the effect on activation of T cells. In this study, levamisole did induce sufficient maturation of DCs to promote a Th1 immune response as evidenced by increased production of IFNɣ, though suppression of a Th2 response as evidenced by a decreased production of IL5 was not also seen. However, levamisole treatment did not increase CD4 Th1 cell proliferation as assessed by thymidine incorporation.(62)

Another group of researchers studied the effects of levamisole on monocyte and lymphocyte cytotoxicity, activation, and proliferation; induction of proteins by cytokines; and expression of tumor-associated antigens. PBMCs were isolated using density gradient centrifugation and cultured for 48 hours with and without 1µM levamisole, 10ng/mL LPS, or a combination of levamisole and LPS.

Levamisole had no consistent effect on the evaluated IFNɣ-induced proteins or on production of tumor necrosis factor. Levamisole also had no effect on monocyte or natural killer cell cytotoxicity or proliferation, or on the activation or proliferation of T lymphocytes.(64) Based on this study, no immunomodulatory effects of levamisole on healthy individuals could be detected. However, this does not rule out the possibility that levamisole may have an effect on an individual with a dysfunctional immune system.

### 1.2.3 Evidence in Horses:

Limited studies on the immunomodulatory effects of levamisole have been performed in horses. One study performed by Krakowski and colleagues, however, was conducted on the effect of nonspecific immunostimulation of pregnant mares with levamisole or 1,3/1,6 glucan on immunoglobulin levels in colostrum and selected indices of cellular and humoral immunity in foals.(74) In this study, 6 unvaccinated mares were injected 3 times with levamisole at 2.5 mg/kg at 7 day intervals beginning 4-6 weeks prior to the anticipated foaling date. This group was compared to a group injected with 1,3/1,6 glucan and a group of control mares. It was determined that the colostrum of mares immunostimulated with levamisole or 1,3/1,6 glucan had a higher IgG and IgG(T) compared to unstimulated mares. Furthermore, the immunity of foals from immunostimulated mares was higher than controls. Foals of treated mares had a greater phagocytic activity of polymorphonuclear cells and greater phagocytic index and destructive ability of neutrophils. These increases were present even before the foals were allowed access to colostrum.(74)

### 1.2.4 Effects of Levamisole Breakdown Products:

As illustrated above, there is conflicting evidence of whether and how levamisole modulates the immune system. Two studies performed by Hanson and colleagues were performed to determine if different levamisole breakdown products were formed depending on storage and culture conditions, and if so, whether or not these different breakdown products affect the immune system differently.(75, 76)

First, the investigators looked at whether storing levamisole at difference conditions or making it fresh immediately prior to use effected the lymphocyte proliferative response to ConA. Levamisole was either made fresh, stored at 37°C for 2 weeks, or stored at 4°C for two weeks prior to each experiment. The levamisole was then added to cell cultures of mouse spleen cells which were stimulated with ConA.

Proliferation as assessed using a [$^3$H]-thymidine incorporation assay. In this experiment, both freshly prepared solutions of levamisole and levamisole stored for 2 weeks at 4°C at a pH of 7.5-8 increased the lymphocyte proliferative response to Con A. The increase was significantly higher with the levamisole stored for 2 weeks at 4°C. They found that levamisole alone (without concurrent stimulation with ConA) was mitogenic, though the effect was synergistic with ConA.(76)

Next, the study looked at the stability of levamisole at different storage conditions. To determine the stability of levamisole during storage, levamisole was stored for two weeks either at a temperature of 4°C or 37°C and at a pH of 6, 7, 7.5, or 8. During this time, aliquots were removed and analyzed by high pressure liquid chromatography. When levamisole was stored for 2 weeks at 37°C at a pH of 7 or 7.5, levamisole decomposed to form three different breakdown products: Product 1 [3-(2-mercaptoethyl)-5-phenyl imidazolidine-2-one], Product 2 [6-phenyl-2,3-dihydroimidaso (2,1-b) thiazole], and Product 3 [bis (3-(-2-oxo-5-phenylimidazolidin-1-yl)ethyl) disulfide]. At a pH of 8, only product 2 was formed; at a pH of 6, levamisole did not breakdown. When levamisole was stored for 2 weeks at 4°C, only products 1 and 2 were formed and only at pH of 8.(76)

Finally, each of the degradation products were purified and tested to elucidate their effects on mouse splenic lymphocyte proliferation (Figure 2). They found that Product 1 inhibited the lymphocyte proliferative response to ConA. Product 2 increased the proliferative response at a concentration of between 1.0 and 10.0 µg/mL but was not mitogenic alone, whereas concentrations greater than 10.0 µg/mL were inhibitory. Product 3 had no effect on ConA stimulation with concentrations lower than 10.0 µg/mL, but significantly inhibited the ConA proliferative response at a concentration of 10.0 µg/mL. Product 3 was mitogenic alone at concentrations between 2.0 and 10.0 µg/mL.(76)

These results are interesting because they may help elucidate the reasons behind the widely varying outcomes from studies investigating levamisole's effects on the immune system. It is also interesting to

note that levamisole is mostly metabolized to Product 2 in vivo. This could be relevant to future studies wishing to decrease the variation in results of investigation into levamisole's immunomodulating properties.

In their second study, Hanson and colleagues determined that Product 1 and Product 2 are each formed within 4 hours of culture during a 72 hour period both with and without lymphocytes present. Product 1 was found to not be present at inhibitory levels, while Product 2 was formed in concentrations high enough to be stimulatory. Product 2, however was not present when cultured at a pH of 7, and when levamisole was cultured with lymphocytes maintained at a pH of 7, no stimulatory effect was observed. Product 3 was not found to be present at any conditions during the 72 hours culture period. These results suggest that the stimulatory effects of levamisole is primarily due to the effects of Product 2 and that the effects of levamisole on cell culture may be affected by both culture conditions as well as the method of preparing and/or storing levamisole prior to use.

Chapter 2. Study Design:

Because levamisole is being used empirically by practitioners in the treatment of EPM and because few studies have been performed evaluating the effects of levamisole in the horse, we undertook this experiment to further investigate the potential role for levamisole as an immunomodulatory agent in horses. To this end, we developed a two part experiment with two goals:

Aim 1a. Determine the optimal conditions and effects of levamisole on cellular proliferation in vitro. Culture freshly isolated equine peripheral blood mononuclear cells (PBMCs) with different mitogens and levamisole at different conditions known to inhibit or stimulate proliferation. Assess proliferation using a commercially available colorimetric bromodeoxyuridine (BrdU) assay.

Aim 1b. Determine the ability of levamisole to upregulate the cell-mediated immune response in vitro based on cellular activation and function. Culture freshly isolated equine PBMCs with mitogens and

mitogens + levamisole (at optimized conditions). Assess cellular activation of subsets based on staining cells with surface markers (CD4, CD8, CD21, CD172a, CD14), dendritic cell and macrophage activation markers (MHC class II, CD86), T-regulatory (FoxP3) and immune phenotype based on cytokine production (IFNɣ, IL4, IL10).

We hypothesized that levamisole would upregulate the equine cell-mediated macrophage (M1) dendritic cell (DC1) CD4 T-helper 1 (Th1) CD8 T-cytotoxic 1 (Tc1) immune response in vitro.

## 2.1 Materials and Methods

The study was performed in two parts. The first aimed to determine whether levamisole at different concentrations and conditions to promote the formation of the previously mentioned breakdown products could affect the proliferative response of PBMCs to mitogen stimulation. To do this, cells were cultured with levamisole at two different concentrations and two different conditions with unstimulated cells, cells stimulated with ConA and cells stimulated with PMA/I. Proliferation was assessed using a commercially available BrdU proliferation assay. Proliferation of each combination was compared to unstimulated cells and the proliferation of cells stimulated with levamisole in addition to mitogen was compared to mitogen stimulation alone.

The second aim of the study was to evaluate the specific immune phenotype that was affected by levamisole stimulation. To do this, levamisole at the concentration and condition that had the largest effect on proliferation during the first part of the study was used. Cells were cultured with levamisole, mitogen, levamisole and mitogen together, or left unstimulated with media only. Cells were labeled with fluorochrome conjugated antibodies for CD21, CD4, CD8, CD14, and CD172a. Intracellular cytokine staining was performed using antibodies for IFNɣ, IL4, and IL10. Cells were labeled with antibodies for MHC Class II and CD86 to assess the activation status of macrophages and dendritic cells. A fluorochrome conjugated antibody for FoxP3 was used to assess the activation status of Treg cells. Cell

subset percentages, cytokine production, and activation status were measured using flow cytometry and samples stimulated with levamisole or mitogen were compared to unstimulated cells. Samples stimulated with levamisole and mitogen were compared to mitogen stimulation alone.

We hypothesized that levamisole would upregulate a M1 DC1 CD4 Th1 CD8 Tc1 immune response in vitro.

2.1.1 Horses:

Adult horses in the Blacksburg area were tested for serum antibody titers for *S. neurona* SAG 1, 5, 6. Testing was performed at a commercial laboratory, Pathogenes, Inc. Serum was collected from horses in the school herd, as well as from local client owned herds. Horses who had an antibody titer <4 were negative and were considered for enrollment in the study.

All horses were up to date on vaccinations before and during the study period. They were vaccinated once yearly against Eastern and Western Equine Encephalitis Virus, West Nile Virus, Tetanus, and Rabies and twice yearly against Equine Herpes Virus, Equine Influenza Virus, and Potomac Horse Fever. They were dewormed regularly.

One horse, Amber, was treated intermittently during the study period for chronic, recurrent uveitis/conjunctivitis, including NSAID therapy. She had an enucleation between the first part of the study and the second part of the study. Another horse, Frenchy, was treated for a laceration one month prior to the second part of the study with treatment including NSAID and antibiotic therapy. A third horse was treated for cellulitis approximately one month prior to the second part of the study, including NSAID and antibiotic therapy.

The general outline of the experimental design is depicted in Figure 3.

2.1.2 Aim 1a:

In order to determine whether levamisole at different concentrations and stored at different conditions shown to promote the development of specific degradation products had an effect on PBMC proliferation or on mitogen induced proliferation, a 5-bromo-2-deoxyuridine (BrdU) proliferation assay[e] was used.

2.1.2a Peripheral Blood Mononuclear Cell Enrichment:

The experiment was run in three groups of 3, 3, and 4 horses on separate days. Samples were collected and processed on the same day. The enrichment procedure was performed as previously described by Witonsky and colleagues.(77) Blood was drawn from the left jugular vein of each horse into lithium heparin blood tubes. The samples were kept at room temperature until all blood for the day was collected and processed. The blood was diluted 2:1 with phosphate buffered saline (PBS) and gently mixed. 15mL centrifuge tubes were then filled with 5mL Lymphoprep™[f] and 10mL of the diluted blood was carefully layered on top of the Lymphoprep™. Each tube was centrifuged at 1500 rpm for 30 minutes at 23°C. The buffy coat from each sample was aspirated and transferred to a fresh tube. The sample was then washed two times with PBS. After washing, cells were counted by taking a 10µL sample, adding trypan blue, and then loading the cells into a hemocytometer. They were then diluted to a concentration of $2 \times 10^6$ cells per mL in complete media (RPMI media with L-glutamine, Hepes buffer 25mM, 10% heat inactivated fetal bovine serum, 1% penicillin and 1% streptomycin) and plated in 100µL aliquots in 96 well round bottom plates at a concentration of $2 \times 10^5$ cells per well.

2.1.2b Culture Conditions:

In order to determine the effects of levamisole stored at different conditions proven to promote the production of particular breakdown products, cells were either left unstimulated, or stimulated with one of the conditions below (concentrations listed are final concentrations in each well):

- 1 µg/mL levamisole stored at 4° for two weeks (Product 2)

- 10 μg/mL levamisole stored at 4° for two weeks (Product 2)
- 1 μg/mL levamisole freshly made (Product 1)
- 10 μg/mL levamisole freshly made (Product 1)
- ConA 5μg/mL
- Con A and 1 μg/mL levamisole stored at 4° for two weeks (Product 2)
- Con A and 10 μg/mL levamisole stored at 4° for two weeks (Product 2)
- Con A and 1 μg/mL levamisole freshly made (Product 1)
- Con A and 10 μg/mL levamisole freshly made (Product 1)
- PMA 20μg/mL and I 10pg/mL (PMA/I)
- PMA/I and 1 μg/mL levamisole stored at 4° for two weeks (Product 2)
- PMA/I and 10 μg/mL levamisole stored at 4° for two weeks (Product 2)
- PMA/I and 1 μg/mL levamisole freshly made (Product 1)
- PMA/I and 10 μg/mL levamisole freshly made (Product 1)

Cells were cultured at 37°C with 5% $CO_2$ for 72 hours total.

2.1.2c Measurement of Proliferation:

Twelve hours prior to harvest, BrdU from a commercially available BrdU proliferation assay was added to each well which would be incorporated into dividing cells. BrdU is a thymidine analog that is incorporated into the genome during DNA synthesis. As more cellular division takes place, more BrdU will be incorporated into the DNA. At the end of the incubation period, the plates were harvested. Anti-BrdU antibody was added to each well, followed by a substrate (both available in the kit) which caused a colorimetric reaction that is read by an ELISA reader. The magnitude of the absorbance for the developed color is proportional to the quantity of BrdU incorporated into cells, which is a direct indication of cell proliferation.

The absorbance was determined at a wavelength of 370nm. Background absorbance was determined using a reference wavelength of 492nm and subtracted.

Absorbance of wells with cells stimulated at the conditions mentioned previously were compared to unstimulated cells and the results were recorded as a change in proliferation. Cells cultured with levamisole in addition to a mitogen were also compared to cells stimulated by mitogen alone and analyzed for statistical significance.

The condition that maximally affected cell proliferation was selected for use in aim 1b of the study.

2.1.3 Aim 1b:

Blood was drawn from the same 10 horses. Samples were taken and processed on the same day and the experiment was run in three batches as before. Peripheral blood mononuclear cells were enriched as described previously. Cells were counted and diluted to concentration of $5 \times 10^6$ per mL then plated into 96 well round bottom plates in 100µL aliquots.

2.1.3a Culture Conditions:

The concentration and storage condition of levamisole was determined from Aim 1a. The condition of levamisole that resulted in the most significant difference in proliferation in the first experiment was selected to be used in this second experiment. Cells were stained with CellTrace™ Violet prior to plating. Samples were cultured with media only (unstimulated), with mitogen (5µg/mL ConA) only, with mitogen (5µg/mL ConA) and levamisole (1µg/mL made fresh), and with levamisole (1µg/mL made fresh) alone.

Cells were cultured at 37°C with 5% $CO_2$ for 72 hours total.

2.1.3b Cell Surface Marker, Activation Marker, and Intracellular Cytokine Staining:

Intracellular cytokine staining was performed using a commercially available kit, Cytofix/Cytoperm™, according to the manufacturer's instructions.[g]

Twelve hours prior to harvesting, the protein transport inhibitor brefeldin A was added to each sample at a concentration of 1µL per 1mL of cell culture. This resulted in the accumulation of cytokines and other proteins within the golgi apparatus. The samples were then returned to the incubator until harvest.

Upon harvesting, antibodies for cell surface (CD21, CD4, CD8, CD14, CD172a) and activation markers (MHC Class II, CD86, FoxP3) were added to the samples which were incubated at 4°C for 20 minutes (see Table 1 for specific markers and cytokines). The samples were then washed twice and fixed and permeabilized with a solution of formaldehyde and saponin (supplied with the commercially available Cytofix/Cytoperm kit). The samples were incubated at 4°C for an additional 30 minutes. Cells were again washed with a commercially available solution of fetal bovine serum (FBS) and saponin. Antibodies against intracellular cytokines diluted in the commercially available FBS and saponin solution were added to each well. The samples were incubated again for 30 minutes and subsequently washed with the FBS/saponin solution and resuspended in PBS. The samples were stored overnight at 4°C and analyzed the next day using a FacsAria flow cytometer.

Percentages of each cell subset (CD4, CD8, CD21, CD14, and CD172a), as well as percentages of each cell subtype producing IFNɣ, IL4, and IL10 were determined. Percentages of CD14 and CD172a positive cells expressing activation markers MHC Class II and CD86 were determined. Percentages of CD4 cells expressing FoxP3 was determined. Changes in the percentages of each cell subtype, cytokine production, and activation marker expression with each treatment were calculated and analyzed for statistical significance.

2.2 Statistical Analysis

### 2.2.1 BrdU Data

A mixed model analysis of variance (ANOVA) was used to analyze the proliferation data for statistical significance comparing treated samples to untreated samples. A Freidman Chi Square was used to examine the difference between each levamisole treatment compared to untreated cells, and between mitogen with each levamisole treatment compared to mitogen stimulation alone.

### 2.2.2 Flow Cytometry Data

A mixed model ANOVA was used to analyze the flow cytometry data for statistical significance comparing treated cells to untreated cells, and comparing levamisole treatment with mitogen to mitogen treatment alone.

Statistical significance was set at $p<0.05$.

## Chapter 3: Results

### 3.1 Horses

Serum from forty horses from Blacksburg, VA was collected and tested for antibodies against *S. neurona* SAG 1, 5, 6. Of these, 14 horses negative for serum antibody production were identified and 10 were selected for inclusion in the study. One horse was rejected due to old age, one due to movement from the property prior to the study period, and two due to distant location. The final 10 horses included in the study ranged in age from 2 to 24 years old and included 4 Arabians, 2 Warmbloods, 2 Standardbreds, 1 Thoroughbred, and 1 Quarter Horse. There were 7 geldings and 3 mares. The individual horse ages, breeds, and sex are summarized in Tables 2-4.

### 3.2 BrdU Proliferation Assay

Cells were left unstimulated (media only) or stimulated with levamisole, mitogen, or a combination of levamisole and mitogen as described previously. Each treatment was compared to unstimulated cells and levamisole treatment with mitogen was compared to mitogen treatment alone. Results are reported as Δabsorbance calculated by subtracting the absorbance of the unstimulated sample from the absorbance of the treated samples.

There was a statistically significant increase in proliferation with all mitogen treated samples compared to unstimulated samples. This included samples that were treated with levamisole in addition to mitogen stimulation.

Treatment with levamisole at any storage condition and concentration in the absence of a mitogen did not result in a statistically significant difference in proliferation compared to unstimulated samples.

When comparing levamisole treatment combined with mitogen stimulation to mitogen stimulation alone, the only combination that resulted in a statistically significant difference in proliferation was the addition of fresh levamisole 1µg/mL to ConA stimulated cells. This resulted in a significant decrease in proliferation compared to ConA stimulation alone ($p=0.015$). No other combination of levamisole and mitogen resulted in a significant difference in proliferation compared to mitogen stimulation alone.

These results are summarized in Figures 4 and 5. The P-values are summarized in Table 5.

Since Levamisole 1µg/mL freshly made added to ConA stimulated cells resulted in the only significant change in proliferation, this was chosen for the second part of the study to look at the effect of levamisole on percentages of individual cell subsets, activation, and cytokine production.

### 3.3 Flow Cytometry Data

#### 3.3.1 Individual Cell Subsets:

Stimulation with ConA alone resulted in an increase in percentage of CD4 positive cells compared to unstimulated cells, though this increase was not statistically significant (p=0.082). Treatment with ConA and Levamisole resulted in a statistically significant increase in percentage of CD4 positive cells compared to unstimulated cells (p=0.0054). However, adding levamisole to ConA stimulated cells did not result in a statistically significant increase in CD4 positive cells compared to cells stimulated with ConA alone (p=0.2485).

No significant difference in percentages of CD8, CD14, or CD172a positive cells were detected with any treatment compared to unstimulated cells, or when adding levamisole treatment to ConA stimulated cells.

3.3.2 Cytokines (Figure 6):

IFNɣ

Stimulation with ConA or with ConA and levamisole 1 µg/mL freshly made resulted in a statistically significant increase in percentage of double positive cells when looking at cells stained for IFNɣ and CD4 ($p<0.0001$), CD8 ($p<0.0001$), CD14 ($p<0.0001$), or CD172a ($p<0.0001$) cells compared to unstimulated cells.

However, there was no statistically significant difference in percentages of IFNɣ positive cells in any population when comparing levamisole treatment to unstimulated cells, or when comparing levamisole treatment with ConA to ConA stimulation alone.

IL4

Stimulation with ConA or with ConA and levamisole 1 µg/mL freshly made resulted in a statistically significant increase in percentage of double positive cells when looking at cells stained for IL4 and CD4 (ConA p=0.0004/ConALev p=0.0071), CD14 (ConA p<0.0001/ConALev p<0.0001), or CD172a (ConA

p<0.0001/ConALev p<0.0001) cells compared to unstimulated cells. However, the percentage of IL4 and CD8 double positive cells was not significantly different from unstimulated cells when treated with either ConA alone or ConA and levamisole.

There was no statistically significant difference in percentages of IL4 positive cells in any subset population when comparing levamisole treatment to unstimulated cells, or when comparing levamisole treatment with ConA stimulation to ConA stimulation alone.

IL10

Stimulation with ConA or with ConA and levamisole 1 µg/mL freshly made resulted in a statistically significant increase in percentage of double positive cells when looking at cells stained for IL10 and CD4 (ConA p=0.0304/ConALev p=0.0138), CD8 (ConA p=0.0018/ConALev p<0.0001), CD14 (ConA p=0.0009/ConALev p=0.0002), or CD172a (ConA p=0.0007/ConALev p=0.0032) cells compared to unstimulated cells.

Again, there was no statistically significant difference in percentages of IL10 positive cells in any population when comparing levamisole treatment to unstimulated cells, or when comparing levamisole treatment with ConA to ConA stimulation alone.

FoxP3

Stimulation with ConA or with ConA and levamisole 1 µg/mL freshly made resulted in a statistically significant increase in percentage of double positive cells when looking at cells stained for FoxP3 and CD4 (ConA p=0.0436/ConALev p=0.0213) cells compared to unstimulated cells.

There was no statistically significant difference in percentages of FoxP3 and CD4 double positive cells when comparing levamisole treatment to unstimulated cells, or when comparing levamisole treatment with ConA to ConA stimulation alone.

### 3.3.3 Activation Markers

There was a statistically significant difference with ConA stimulation between CD14 MHC class II and unstimulated cells. There were trends (p= 0.0513) for ConA stimulated CD14 CD86 and ConA with levamisole (p=0.06) for a statistically significant increase in CD14 MHC class II and CD14 CD86 expression compared to unstimulated cells.

There was a statistically significant increase in percentages of CD86 and CD172a double positive cells when treated with either ConA alone (p=0.0151) or ConA with levamisole (p=0.0089) compared to unstimulated cells. However, adding levamisole to ConA treated cells did not result in an increase in percentages of double positives compared to ConA stimulation alone (p=0.8099).

P-values for the Flow Cytometry portion of the study are summarized in Table 6.

## Chapter 4: Discussion

The results of the BrdU proliferation experiment suggest that levamisole at a concentration of 1µg/mL made fresh has a suppressive effect on the ConA stimulated proliferation of equine PBMCs. Levamisole was not mitogenic alone. However, the flow cytometry portion of our study failed to elucidate the specific immune phenotype response based on cell surface marker expression (CD21, CD4, CD8, CD172a, or CD14) or cytokine production (IFNɣ, IL4, or IL10) when levamisole was cultured with ConA stimulated PBMCs or with PBMCs alone.

### 4.1 Aim 1a.

In the BrdU portion of our study, levamisole only had a statistically significant effect when used in combination with ConA. No significant difference was observed when PBMCs were cultured alone with levamisole or when cultured with the combination of levamisole and PMA/I. This indicated that levamisole may have more of an effect on T cells or macrophages than other PBMCs since ConA is a

potent mitogen of T cells acting through a T cell receptor in the presence of macrophages while PMA/I is a panleukocyte mitogen acting directly on protein kinase C.(78, 79)

ConA interacts directly with the T cell receptor in order to activate T cells to proliferate. However, the presence of macrophages as a source of IL2 is also necessary. It appears that ConA can render inactive or resting T lymphocytes sensitive to IL2 induced proliferation.(78) PMA/I, on the other hand, induces panleukocyte proliferation by diffusing into the cell and activating protein kinase c directly, subsequently increasing cyclic GMP. This is a calcium dependent reaction and ionomycin is added in order to increase release of calcium into the cytoplasm from intracellular stores.(80) In addition, ionomycin and PMA act synergistically to induce phosphorylation of protein kinase C.

As a possible future investigation to further elucidate the mechanism of levamisole's suppression of ConA induced proliferation, a follow-up study investigating the effects of levamisole using a purified population of T cells without macrophages and supplemented with IL2 would be interesting. If no changes in proliferation were noted when macrophages were depleted from the sample, a conclusion that levamisole primarily exerts its effects on T lymphocyte through macrophages would be supported. If, however, levamisole treatment induced the same decrease in proliferation in a T cell exclusive population, it could be concluded that levamisole exerts its effects on T lymphocytes directly.

Unfortunately, it was not within our budget to use purified breakdown products or to measure our samples for the presence of the specific breakdown products. Therefore, while we used conditions that had previously been reported to promote the production of the specific breakdown products, we cannot be sure of the specific combination of products within our samples. Because of the time constraints, the experiment was run in 3 batches resulting in different batches of fresh levamisole being made on the day of each experiment. While the same person made up each batch of levamisole, it is possible that variations from day to day could have influenced the breakdown products. Also, the levamisole stored

at 4 degrees for 2 weeks was made up prior to the experiment and was used for all the batches run during that week. It is unknown if the mixture of breakdown products within the sample remained constant over that time period. This could be another potential source of variation within the experiment.

4.2 Aim 1b.

The second part of our study failed to show a change in the measured values with levamisole treatment compared to unstimulated cells or compared to cells stimulated with ConA alone. This indicates that a change in the percentages of each cell subset (CD4, CD8, CD21, CD14, CD172a) was not induced with levamisole treatment. ConA treatment alone or in combination with levamisole also did not result in a change of the cell subset percentages, except for CD4+ cells. When levamisole was added to ConA stimulated cells, an increase in CD4+ cells was measured compared to unstimulated cells. Yet, ConA stimulation alone did not result in a significant difference compared to unstimulated cells. However, when comparing ConA with levamisole treatment to ConA treatment alone, a statistically significant difference was not observed in CD4+ cell percentages. Absolute numbers of cells could not be compared because the number of cells counted in each sample varied and depended on a standard collection time. Proliferation based on CellTrace Violet™ was attempted, but the data has not yet been optimized for statistical analysis.

Macrophage and dendritic cells take a long time, up to 7 days, to differentiate from peripheral blood monocytes.(81) Dendritic cells represent only about 1% of PBMCs in the body.(82) In one study, adherent cells obtained from PBMCs from 500mL equine whole blood were cultured for 4 days to yield between 2 x $10^6$ and 8 x $10^6$ immature dendritic cells per 100 mL of blood (2-8%).(83) In another, 6-7 days were needed for differentiation of monocytes to immature dendritic cells.(81) It is possible that culturing our PBMCs for longer and in the presence of granulocyte-macrophage colony-stimulating

factor (GM-CSF) may have resulted in greater numbers of macrophages and dendritic cells. In some cases in which limited numbers of cells were available for analysis, greater number of cells may have allowed us to detect a significant difference in cell percentages, activation markers, or cytokine production more obvious.

Cytokine production was also not affected by levamisole treatment. While treatment with ConA and ConA with levamisole resulted in a statistically significant increase in production by all cell subsets of IFNɣ, IL4 (except by CD8+ cells), IL10, and FoxP3, the addition of levamisole to unstimulated cells or to ConA stimulated cells did not result in a significant change in production of any cytokine.

In this experiment, we attempted to survey several immune cells including those from the innate immune response (macrophages and dendritic cells), the humoral immune response (B cells and Th2 cells), and the cell mediated immune response (Th1 and Tc cells). We did this using cell surface markers as well as intracellular cytokine production.

We chose to look at intracellular cytokine production by blocking protein transport 12 hours to harvesting the cells. We did this to be able to identify which cell types were producing each cytokine. It is possible that this amount of time was insufficient to detect significant differences in cytokine production induced by levamisole. While brefeldin can be toxic to cells if left in culture for more than 12 hours, it would be interesting to test the sample supernatants to determine if the cytokine production over the entire 72 hours of culture was significantly different between treatments. Similarly, culturing the cell for shorter or longer periods of time may also result in a greater effect of treatment that was not detectable at the 72 hour time period. If peak cytokine production occurred before or after the 72 hour mark, we may have missed the opportunity to detect a significant difference in cytokine production by only evaluating this time point.

It is also possible that levamisole treatment may influence the production of cytokines other than the ones tested in our study. For example, in the study performed by Szeto and colleagues, a significant difference in the cytokine gene expression of IL18 in splenic cells was discovered in brown Norway rats treated with 25mg/kg levamisole IP. As this study also suggests, it may be worthwhile to look at samples other than peripheral blood, such as splenic cells.(63)

Finally, the effects of levamisole seem to vary depending on the dose, the timing of administration, the immune status of the host, and whether the study was performed in vivo or in vitro. Further in vivo and in vitro studies investigating the effects of each of these parameters on levamisole mediated response of the equine immune system is warranted. Some studies in mice and humans, in vivo and in vitro results can be conflicting. The study mentioned in the introduction by Kurakata and colleagues showed that there is a possible effect of a systemically produced "serum factor" induced by treatment with levamisole.(61) If that is the case, a greater change in the immune response may be seen after systemic administration of levamisole as opposed to in vitro treatment of PBMCs. It would be worthwhile in a future study to investigate the immune response in horses that have been treated with various doses.

A discussion of our methods and suggestions for future studies is included in the paragraphs to follow.

4.3 Horses:

For our study, we isolated equine PBMCs from whole blood collected from healthy horses who were negative for antibodies to *S. neurona* based on SAG 1, 5, 6 ELISA. While we wished to minimize variables that may affect the results of our study by using healthy horses, it is possible that levamisole may affect healthy and immunocompromised horses differently. It has been shown in mouse and human models that the effects of levamisole can vary depending on the immune status of the individual. For example, while levamisole may return a dysfunctional immune system to normal, it generally will not increase an adequate immune response.(61, 64, 65, 84, 85) As mentioned previously, it has been suggested that

horses that develop clinical EPM after infection with *S. neurona* may be immunocompromised whether by external factors, host factors, or parasite induced factors.(3, 11-13, 31, 86, 87) Therefore, we may see different effects of levamisole on PBMCs from horses that are serologically positive for *S. neurona* and not showing clinical signs as well as from horses who have clinical EPM.

In addition, there was a subjective association between age of horse and result of the SAG 1, 5, 6 with older horses more likely to be negative than younger horses. Forty horses were tested and only 14 (35%) were found to be negative to serum antibodies. The remaining 26 horses (65%) were positive. Of the 14 negative horses, 8 (57%) were 19 years or older. This is a much greater percentage of aged horses than those in the group that tested positive for antibodies to *S. neurona*, 3 out of 26 horses (11%). Therefore, it is possible that these horses may have been undergoing some degree of immunosenescence which could have affected the results of this study. It has been shown that aging horses, as well as humans, exhibit decreased responsiveness to vaccines and increased susceptibility to disease despite the presence of serum antibodies. At the same time, an age related increase in the production of pro-inflammatory cytokines from macrophages has also been demonstrated.(88-91) Therefore, the age of our study population may have affected the results of our SAG 1, 5, 6 ELISA or may have skewed the effects of levamisole on PBMCs from these aged horses. This may also explain the high levels of background proliferation that was observed in unstimulated PBMCs from some horses during some of the BrdU proliferation studies.

The majority of the horses in our study were either Standardbred or Arabian. It is also possible that specific breeds may behave differently and decrease our ability to detect a significant difference. Repeating the study with fewer breeds or with a larger number of horses after performing a power analysis would help minimize this effect.

Furthermore, the experiments in our study were carried out over a 2 year period with SAG 1, 5, 6 testing performed at the beginning of this period and not repeated. It is possible that the immune status of individual horses changed over this course of time resulting in the disparity seen between our BrdU study results and the flow cytometry results. Repeating the study with different populations of horses would be worthwhile. These populations would include younger seronegative horses, seropositive horses without clinical signs, and seropositive horses with clinical signs.

## 4.4 BrdU

The first part of our study looked at the effects of levamisole at different concentrations and stored at different conditions on the proliferation of PBMCs. The proliferation of cells cultured with levamisole at four different conditions (1µg/mL made fresh, 1µg/mL stored for 2 weeks at 4°C, 10µg/mL made fresh, and 10µg/mL stored for 2 weeks at 4°C) were compared to unstimulated cells. Levamisole at each of these conditions was also added to mitogen stimulated cells (ConA and PMA/I) and the proliferation was compared to mitogen stimulation alone. We found that there was a large degree of variability between horses and, as mentioned previously, some horses had a high level of background proliferation. This may be a result of "inflamm-aging" as described previously, or could be a result of variation in the technique used when plating and processing cells. However, the experiment was repeated multiple times and this background proliferation could not be eliminated. One horse, the youngest, consistently appeared to have the least amount of background proliferation. The BrdU proliferation assay has not be previously used in this laboratory. It would be interesting to compare the results of the assay to other techniques such as tritiated thymidine incorporation, or Alamar blue proliferation assays. Repeating the study with a larger number of horses may also help to minimize the effect of the variability between horses and could result in more significant results.

## 4.5 Flow Cytometry

### 4.5.1 CellTrace™ Proliferation Assay

In the second portion of our study, we attempted to measure proliferation based on the incorporation of CellTrace™, a fluorescent dye that is covalently bound to intracellular amines. As the cells divide, the concentration of CellTrace™ within the cell is split between the two daughter cells resulting in a reduction by approximately ½ of the CellTrace™ within the cell. Therefore, it is possible to track the number of divisions by flow cytometry. The mechanism by which this occurs is similar to the more commonly used carboxyfluorescein succinimidyl ester (CFSE); however, CFSE could not be used in our experiment because of the dye combinations used for the cell surface markers. In addition, using this method, we hoped to be able to determine a change in the number of cellular divisions, and therefore the change in proliferation, of the PBMCs when levamisole was added to mitogen stimulated cells. Unfortunately, the CellTrace™ incorporation assay did not produce the results as expected. Normal peaks were not identifiable and the concentration of CellTrace™ within the cells did not appear to decrease by half reliably and the resolution of the generational peaks was poor.

One possible explanation for the poor resolution of the generational peaks was that the concentration of CellTrace™ was too low resulting in the inability to differentiate reliable between peaks. In the study comparing CFSE and CellTrace™ as proliferation assays it was found that resolution between the division peaks was poor especially after several divisions. They explained two possible causes for this: first, they suggested that dye was being transferred from one cell to another resulting in intermediate quantities of dye instead of halving of the dye that would normally be expected with cellular division. In their study, they found that in vitro culturing of cells did result in some degree of dye transfer between cells.(92) Perhaps culturing cells at a lower concentration could help minimize this effect. A second explanation was that autoflourescence of cells may be interfering with fluorescence of the stained cells resulting in a decreased resolution of divisional peaks. In order to negate the effects of autoflourescence, higher concentrations of dye were used to increase the mean fluorescence intensity

of stained cells. This resulted in an improvement in the resolution of the divisional peaks. In this study, double (20µM) and quadruple (40µM) concentrations of CellTrace were used without significantly affecting the viability of cells.(92)

After the addition of CellTrace™ and the incubation period, we also lost a large quantity of the PBMCs that we began with. Several possibilities were explored and attempts were made to minimize this loss. For example, it was suggested that cells could have been lost during quenching with a large volume of media with 1% protein and during the subsequent washes. Initially this was done in 50mL centrifuge tubes, and since the concentration of cells was very low, the pellets were very small and cells may have been lost from the pellet while pouring out the supernatant. Therefore, smaller, 15 mL centrifuge tubes were used.

The possibility that the CellTrace™ dye was cytotoxic at higher concentrations was also investigated as a cause for the lost PBMCs. Subjectively, it appeared that there were more non-viable cells present after the CellTrace™ incubation when counted under a microscope with trypan blue staining. In order to attempt to minimize the possible toxicity, we decreased the final working concentration of CellTrace™ used from the recommended 5µM to 1µM solution. This may have helped decrease cells lost to toxicity, however, recoveries were still less than expected. While a reference for the appropriate concentration of CellTrace™ Violet for equine PBMCs could not be found, there have been studies performed comparing CellTrace™ Violet to the more commonly used CFSE. In two such studies, no significant difference in viability was found between CFSE and CellTrace™ Violet at various concentrations.(92, 93) However, the researchers in the first study used complete media with 10% FBS as the staining media instead of PBS in order to minimize cellular toxicity.(92) In the second study using the protocol described in the product manual (which was the same protocol used in our experiment), no significant difference in cell viability was found.(93) In our experiment, PBS was used as a staining medium and the addition of complete media with only 1% FBS was used to quench excess dye at the end of the staining

period. It is possible that using complete media with 10% FBS as either the staining media or quenching media may have resulted in less cytotoxicity and greater cell recoveries.

In order to mitigate possible cytotoxicity from the CellTrace™, lower concentrations were used. While this seemed to modestly improve our recoveries, we still lost a significant number of cells during the CellTrace™ staining step, sometimes resulting in a reduction of 1/3 to 1/2 of our cell concentration. If the CellTrace™ was indeed causing toxicity to our PBMCs, it is also possible that it affected the results of the rest of our study. We may have seen more significant differences in our cell subset populations and cytokine production if the CellTrace™ Violet stain had not been present. In the future, repeating this experiment with another proliferation dye such as CFSE, repeating the experiment without the addition of a proliferation dye, or performing further experiments to optimize the concentration and technique when using the CellTrace™ Violet proliferation dye could provide interesting results.

In the same study mentioned previously, it was noted that CellTrace™ Violet resulted in decreased resolution of cell divisions compared to CFSE when high concentrations of the dye were used.(92) Therefore, trials using lower concentrations of CellTrace™ Violet to determine an optimal dye concentration may improve the resolution of our peaks which would provide us with more meaningful data.

Another possible explanation for the lack of distinct peaks during our experiment is that the flow rate during analysis was too high. According to the manufacturer provided protocol, low flow rates should be used during analysis in order to separation of distinct generational peaks. Alternatively, an Attune® Acoustic Focusing Cytometer could be used at any flow collection rate.

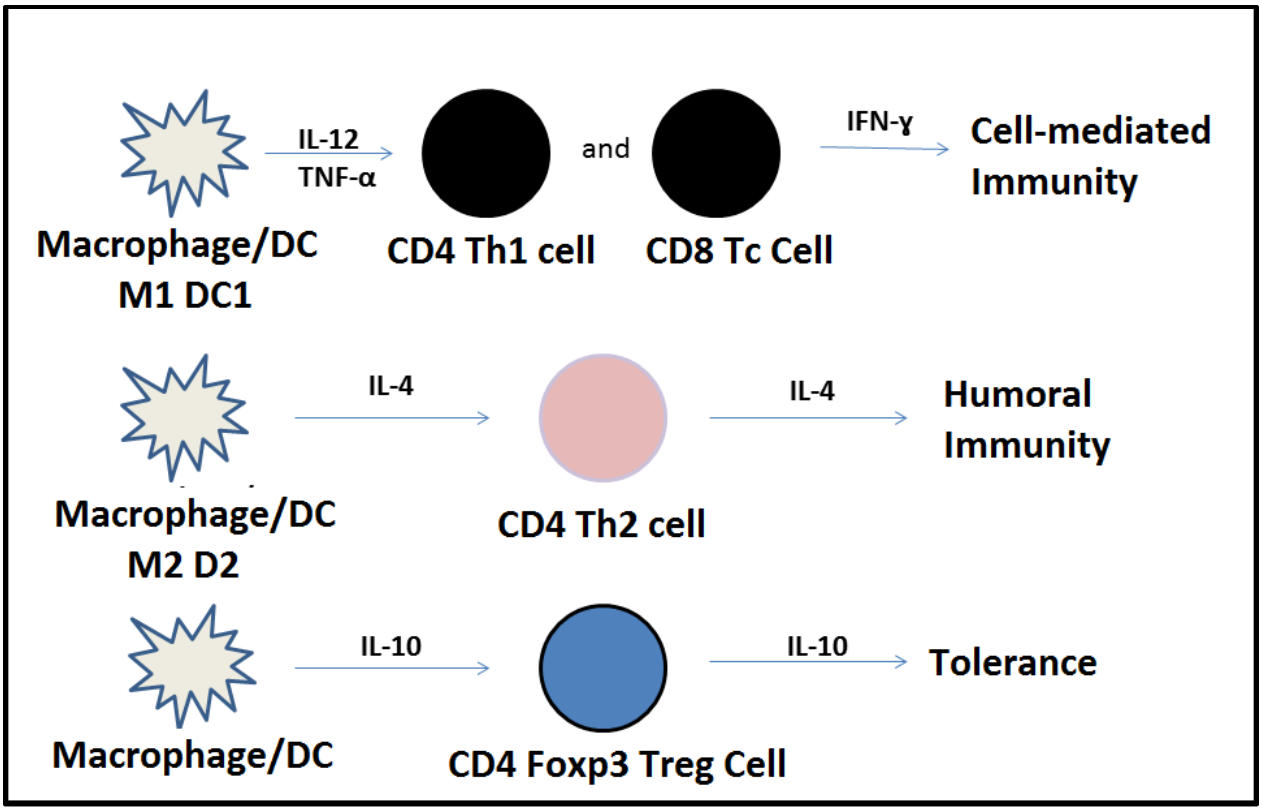


**Figure 1.** Basic overview of the different immune responses.

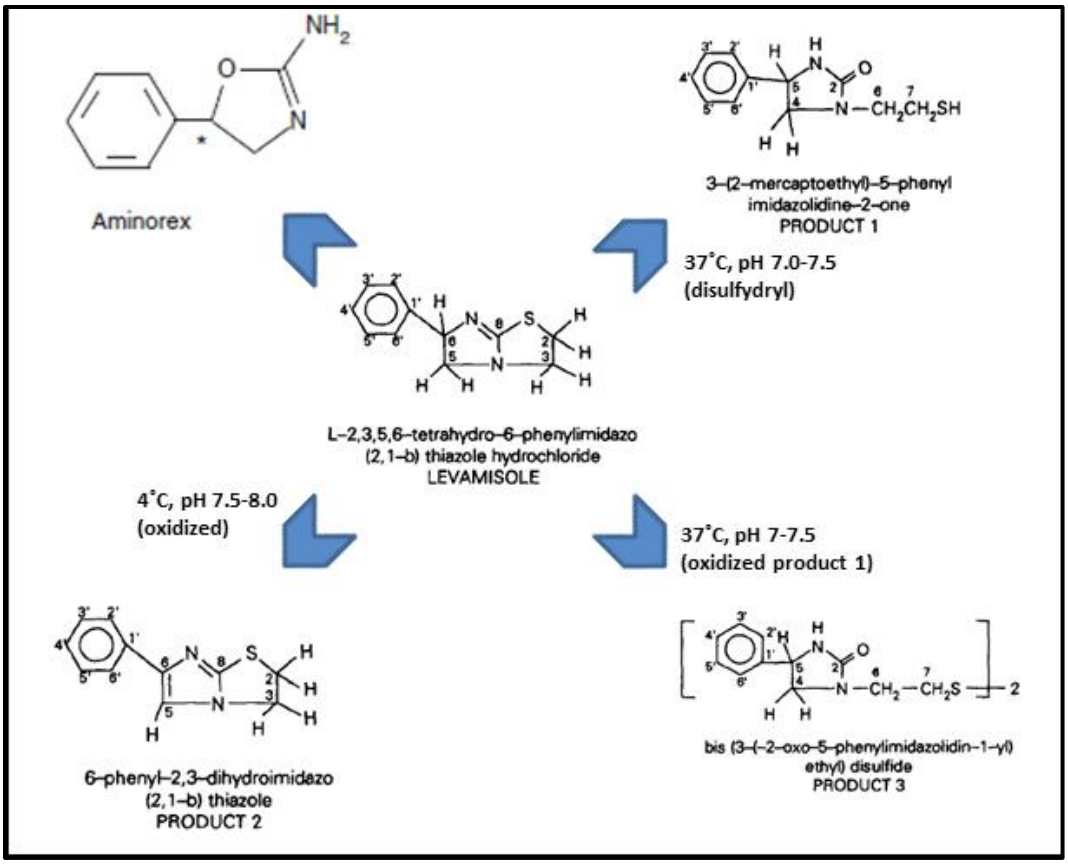


**Figure 2.** Different breakdown products of levamisole at different conditions. (Adapted from Hanson and colleagues.(76))

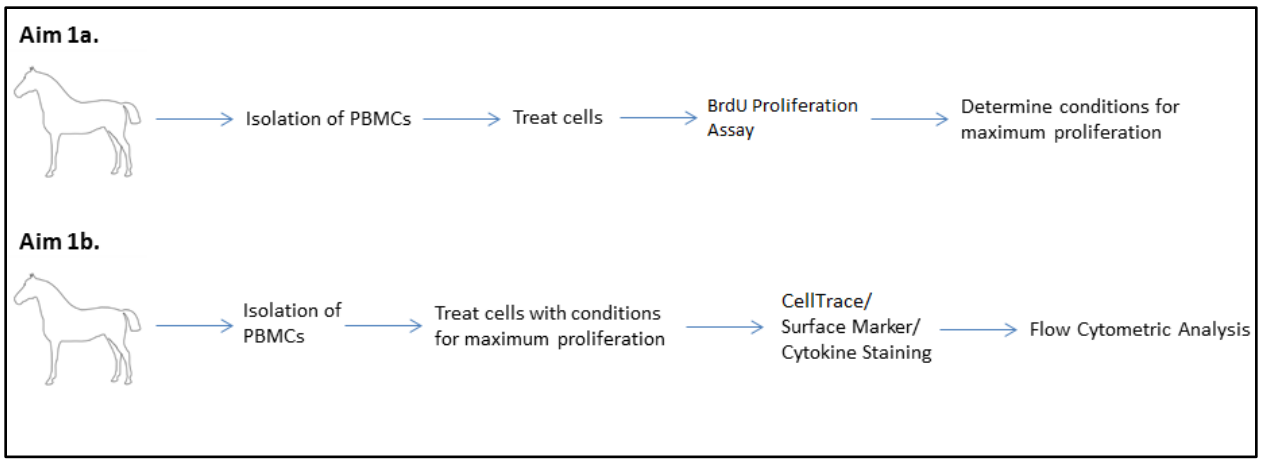


**Figure 3**. General outline of experimental design.

| Cell Type | Surface Marker | Activation | Cytokines |
|---|---|---|---|
| Macrophages | CD172a | MHC Class II, CD86 | IFNɣ, IL4, IL10 |
| Dendritic Cells | CD14 | MHC Class II, CD86 | IFNɣ, IL4, IL10 |
| CD4 Lymphocytes | CD4 | FoxP3 | IFNɣ, IL4, IL10 |
| CD8 Lymphocytes | CD8 | | IFNɣ |
| B Lymphocytes | CD21 | | |

**Table 1.** Cell surface makers, activation markers and cytokines used for flow cytometric analysis.

| Horse | Owner/Location | Age | Sex | Breed | SAG 1, 5, 6 Results |
|---|---|---|---|---|---|
| Classic | APSC Smithfield | 21 | G | Arabian | 2, 2, 2 |
| JR | APSC Smithfield | 23 | G | Arabian | 2, 2, 4 |
| Khuzy | APSC Smithfield | 16 | G | Arabian | 2, 2, 2 |
| Raad | APSC Smithfield | 22 | G | Arabian | 2, 2, 2 |
| Amber | OVPI CMMID | 19 | M | Standardbred | 2, 2, 2 |
| Frenchy | OVPI CMMID | 19 | M | Standardbred | 2, 2, 4 |
| Roxette | OVPI CMMID | 19 | M | Warmblood | 4, 4, 4 |
| Duke | Huffman | 2 | G | Warmblood | 2, 4, 2 |
| Ruby | OVPI CMMID | 11 | M | Quarter Horse | 2, 2, 2 |
| Slew | OVPI CMMID | 19 | G | Thoroughbred | 2, 2, 2 |

**Table 2**. Location, age, sex, and breed of horses negative for SnSAG 1, 5, 6 used in the study.

| Horse | Owner/Location | Age | Sex | Breed | SAG 1, 5, 6 Results |
|---|---|---|---|---|---|
| Moe | Maxwell | 10 | G | Walking Horse | 2, 2, 2 |
| Ruby | Maxwell | 5 | M | Walking Horse X Quarter Horse | 2, 2, 2 |
| Flo | Reeder | 4 | M | Arabian Cross | 2, 2, 2 |
| Mack | Jones | 30 | G | Welsh Cross | 2, 2, 2 |

**Table 3.** Location, age, sex, and breed of horses negative for SnSAG 1, 5, 6 not used in the study.

| Horse | Owner/Location | Age | Sex | Breed | SAG 1, 5, 6 Results |
|---|---|---|---|---|---|
| Rosie | OVPI CMMID | 15 | M | Quarter Horse | 8, 4, 16 |
| Latin | OVPI CMMID | 16 | M | Standardbred | 8, 8, 8 |
| Mint | OVPI CMMID | 15 | M | Thoroughbred | 8, 4, 4 |
| Lindsay | OVPI CMMID | 19 | M | Quarter Horse | 4, 4, 8 |
| Ellie | OVPI CMMID | 24 | M | Appaloosa | 8, 8, 8 |
| Brownie | APSC Smithfield | 10 | M | Quarter Horse | 2, 8, 4 |
| Rosie | APSC Smithfield | 8 | M | Quarter Horse | 2, 8, 4 |
| Paloma | APSC Smithfield | 14 | M | Warmblood | 8, 8, 8 |
| Royal | APSC Smithfield | 20 | M | Quarter Horse | 4, 2, 8 |
| Lilly | APSC Smithfield | 11 | M | Thoroughbred | 8, 8, 8 |
| Nita | APSC Smithfield | 13 | M | Quarter Horse | 8, 8, 8 |
| Ducky | APSC Smithfield | 8 | M | Quarter Horse | 4, 4, 8 |
| Jazz | APSC Smithfield | | M | Quarter Horse | 8, 4, 2 |
| Georgia | APSC Smithfield | 17 | M | Quarter Horse | 4, 8, 4 |
| Maggie | Reeder | 6 | M | Arabian x Draft | 16, 8, 8 |
| Yankee | Reeder | 9 | G | Arabian Cross | 16, 4, 16 |
| Rudy | Funk | 13 | G | Lipizzaner | 32, 16, 64 |
| Nada | Funk | 10 | G | Lipizzaner | 2, 4, 8 |
| Brandy | Snider | 13 | M | Quarter Horse | 4, 8, 8 |
| Cajun | Snider | 10 | G | Draft Cross | 32, 64, 32 |
| Toby | Snider | 11 | G | Walking Horse | 64, 64, 64 |
| Rosie | Snider | 13 | M | Pony | 8, 8, 8 |
| Dillon | Maxwell | 16 | G | Walking Horse Cross | 2, 8, 8 |
| Denali | Reuss (@ Reeder) | 10 | M | Paint | 8, 8, 8 |
| Weedy | Jones | 12 | G | Arabian Cross | 4, 8, 8 |
| Desi | Golden (@ Funk) | 13 | M | Quarter Horse | 64, 8, 4 |

**Table 4.** Location, age, sex, and breed of horses positive for SnSAG 1, 5, 6 and not used in the study.

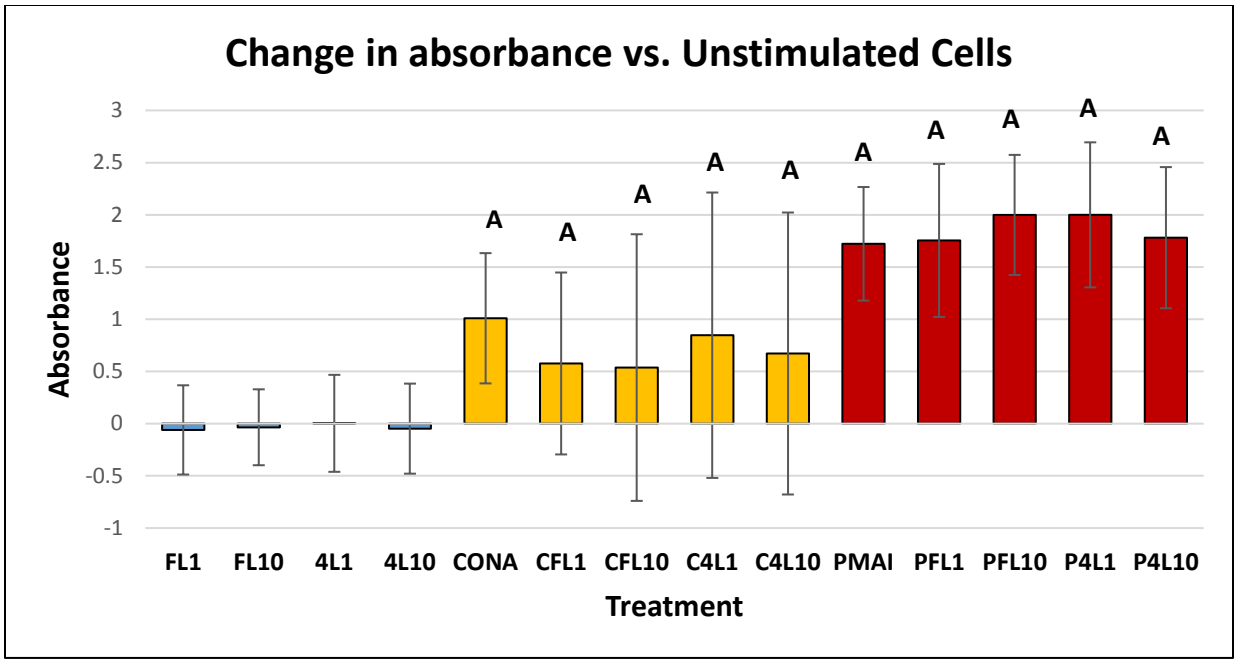


**Figure 4.** Change in absorbance of treated cells compared to untreated cells. Statistically significant differences compared to unstimulated cells are marked with an "A". The error bars represent standard deviation.

Treatments are indicated as follows:

**FL1** = 1 µg/mL levamisole freshly made, **FL10** = 10 µg/mL levamisole freshly made, **4L1** = 1 µg/mL levamisole stored at 4°C for 2 weeks, **4L10** = 10 µg/mL levamisole stored at 4°C for 2 weeks, **ConA** = Concanavalin A (Con A) 5µg/mL alone, **CFL1** = Con A and 1 µg/mL levamisole freshly made, **CFL10** = Con A and 10 µg/mL levamisole freshly made, **C4L1** = Con A and 1 µg/mL levamisole stored at 4°C for 2 weeks, **C4L10** = Con A and 10 µg/mL levamisole stored at 4°C for 2 weeks, **PMAI** = Phorbol myrisate acetate 20µg/mL and ionomycin 10pg/mL (PMA/I), **PFL1** = PMA/I and 1 µg/mL levamisole freshly made, **PFL10** = PMA/I and 10 µg/mL levamisole freshly made, **P4L1** = PMA/I and 1 µg/mL levamisole stored at 4°C for 2 weeks, **P4L10** = PMA/I and 10 µg/mL levamisole stored at 4°C for 2 weeks

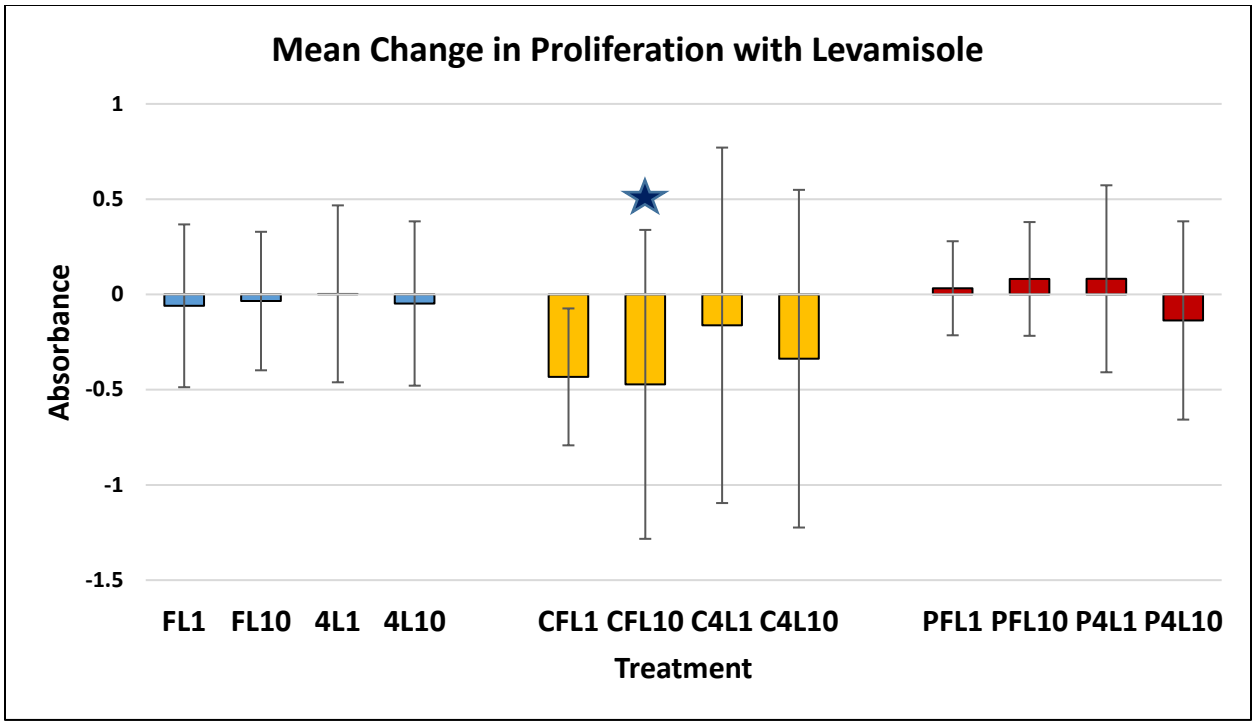


**Figure 5.** Mean change in proliferation with levamisole treatment compared to: unstimulated cells (yellow), ConA (5 µg/mL) stimulated cells (green), or PMA/I (20 µg/mL/10pg/mL) stimulated cells (red). A statistically significant decrease in proliferation was discovered with levamisole 1µg/mL freshly made when added to ConA stimulated cells compared to ConA stimulation alone and is marked with a star.

Treatments are indicated as follows:

**FL1** = 1 µg/mL levamisole freshly made, **FL10** = 10 µg/mL levamisole freshly made, **4L1** = 1 µg/mL levamisole stored at 4°C for 2 weeks, **4L10** = 10 µg/mL levamisole stored at 4°C for 2 weeks, **ConA** = Concanavalin A (Con A) 5µg/mL alone, **CFL1** = Con A and 1 µg/mL levamisole freshly made, **CFL10** = Con A and 10 µg/mL levamisole freshly made, **C4L1** = Con A and 1 µg/mL levamisole stored at 4°C for 2 weeks, **C4L10** = Con A and 10 µg/mL levamisole stored at 4°C for 2 weeks, **PMAI** = Phorbol myrisate acetate 20µg/mL and ionomycin 10pg/mL (PMA/I), **PFL1** = PMA/I and 1 µg/mL levamisole freshly made, **PFL10** = PMA/I and 10 µg/mL levamisole freshly made, **P4L1** = PMA/I and 1 µg/mL levamisole stored at 4°C for 2 weeks, **P4L10** = PMA/I and 10 µg/mL levamisole stored at 4°C for 2 weeks

| Versus Unstimulated Cells | P value |
|---|---|
| **ConA** | 0.0347 |
| **ConA + Levamisole 1µg/mL Fresh** | 0.0296 |
| **ConA + Levamisole 10µg/mL Fresh** | 0.0087 |
| **ConA + Levamisole 1µg/mL Stored 4°** | <0.0001 |
| **ConA + Levamisole 10µg/mL Stored 4°** | <0.0001 |
| | |
| **PMA/I** | <0.0001 |
| **PMA/I + Levamisole 1µg/mL Fresh** | <0.0001 |
| **PMA/I + Levamisole 10µg/mL Fresh** | <0.0001 |
| **PMA/I + Levamisole 1µg/mL Stored 4°** | <0.0001 |
| **PMA/I + Levamisole 10µg/mL Stored 4°** | <0.0001 |
| | |
| **Levamisole 1µg/mL Fresh** | 1.0000 |
| **Levamisole 10µg/mL Fresh** | 1.0000 |
| **Levamisole 1µg/mL Stored 4°** | 1.0000 |
| **Levamisole 10µg/mL Stored 4°** | 1.0000 |
| | |
| **Versus ConA Stimulated Cells** | |
| **ConA + Levamisole 1µg/mL Fresh** | 0.0374 |
| **ConA + Levamisole 10µg/mL Fresh** | 1.000 |
| **ConA + Levamisole 1µg/mL Stored 4°** | 1.000 |
| **ConA + Levamisole 10µg/mL Stored 4°** | 0.9881 |
| | |
| **Versus PMA/I Stimulated Cells** | |
| **PMA/I + Levamisole 1µg/mL Fresh** | 0.9984 |
| **PMA/I + Levamisole 10µg/mL Fresh** | 0.8982 |
| **PMA/I + Levamisole 1µg/mL Stored 4°** | 0.9736 |
| **PMA/I + Levamisole 10µg/mL Stored 4°** | 0.9755 |

**Table 5.** Summary of P-values for BrdU Proliferation Assay. Significance was set at P<0.05.

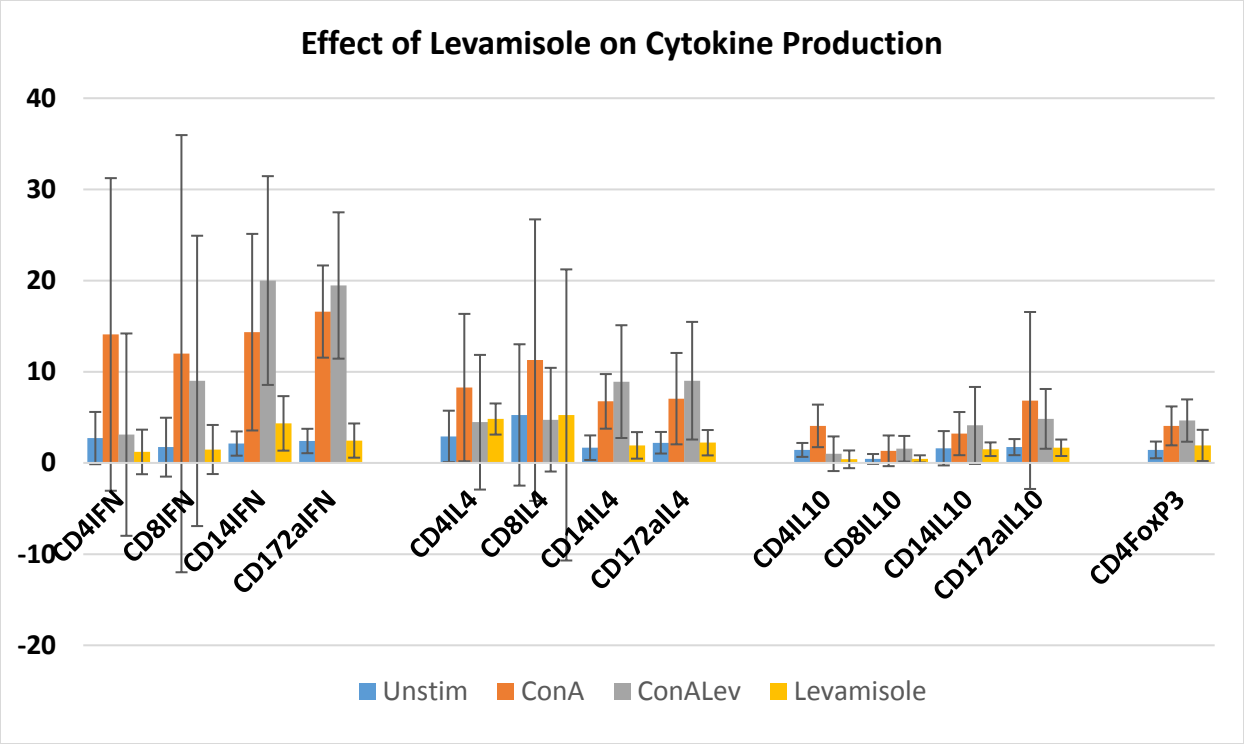


**Figure 6.** Mean percentage of cells producing cytokines compared in each group of cells. Percentages of cells producing IFNɣ is indicated in the first group, percentages of cells producing IL4 is indicated in the second group, and percentages of cells producing IL10 is indicating in the third group. Percentages of cells expressing FoxP3 is indicated in the last group. The blue bar represents unstimulated cells, the orange represents ConA stimulated cells, the purple bar represents ConA and levamisole stimulated cells, and the yellow bar represents cells stimulated with levamisole only.

| | ConA vs. Unstimulated | ConA + Levamisole vs. Unstimulated | Levamisole vs. Unstimulated | ConA + Levamisole vs. ConA |
|---|---|---|---|---|
| **CD4IFNg** | <0.0001 | <0.0001 | 0.886 | 0.9191 |
| **CD8IFNg** | <0.0001 | <0.0001 | 0.976 | 0.86 |
| **CD14IFNg** | <0.0001 | <0.0001 | 0.2505 | 0.6314 |
| **CD172aIFNg** | <0.0001 | <0.0001 | 0.993 | 0.2664 |
| **CD4IL4** | 0.0004 | 0.0071 | 0.4147 | 0.2799 |
| **CD8IL4** | 0.1205 | 0.7621 | 0.9723 | 0.2186 |
| **CD14IL4** | <0.0001 | <0.0001 | 0.9991 | 0.4378 |
| **CD172aIL4** | <0.0001 | <0.0001 | 0.9925 | 0.1492 |
| **CD4IL10** | 0.0304 | 0.0138 | 0.9377 | 0.7393 |
| **CD8IL10** | 0.0018 | <0.0001 | 0.9835 | 0.1482 |
| **CD14IL10** | 0.0009 | 0.0002 | 0.7867 | 0.5548 |
| **CD172aIL10** | 0.0007 | 0.0032 | 0.9795 | 0.5768 |
| **CD4FoxP3** | 0.0436 | 0.0213 | 0.8796 | 0.7546 |
| **CD4** | 0.082 | 0.0054 | 0.9815 | 0.2485 |
| **CD8** | 0.5674 | 0.7556 | 0.499 | 0.7814 |
| **CD21** | 0.5239 | 0.4017 | 0.6062 | 0.8448 |
| **CD14** | 0.9823 | 0.8688 | 0.3879 | 0.7474 |
| **CD172a** | 0.9914 | 0.9535 | 0.4479 | 0.8529 |
| **CD14MHCII** | 0.109 | 0.1483 | 0.9035 | 0.8742 |
| **CD14CD86** | 0.0513 | 0.7229 | 0.7984 | 0.1183 |
| **CD14MHCIICD86** | 0.0001 | 0.0008 | 0.3353 | 0.5353 |
| **CD172aMHCII** | 0.7865 | 0.5621 | 0.3081 | 0.736 |
| **CD172aCD86** | 0.0151 | 0.0089 | 0.9938 | 0.8099 |
| **CD172aMHCIICD86** | 0.0003 | <0.0001 | 0.2806 | 0.2629 |

**Table 6.** Summary of P-values for Flow Cytometry analysis of cell subsets and intracellular cytokine production. Significance was set at P<0.05.

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Appendix 1. Detailed Methods

Peripheral Blood Mononuclear Cell Enrichment Protocol

1. Remove Lymphoprep™[h] from the refrigerator and allow to come to room temperature. Make sure phosphate buffered saline without calcium or magnesium (PBS)[i] is at room temperature as well. Some PBS will also need to remain in the refrigerator or on ice to be used cold later.
2. Collect required amount of blood into lithium heparin vacutainer tubes.
3. Gently invert the blood tubes to mix and pool the blood samples into 50mL centrifuge tubes, using multiple tubes if the volume is too great. Note the total volume of blood per horse.
4. Prepare a blood smear on a slide.
5. Dilute the blood with twice the volume of PBS making a 1:2 dilution of blood to PBS. Gently invert the tube to mix the sample.
6. Transfer 5mL Lymphoprep™ to 15mL centrifuge tubes. One tube will be needed for every 10mL of blood/PBS solution.
7. Gently invert the 50mL centrifuge tubes and transfer 10mL of the blood/PBS solution to each 15mL centrifuge tube containing Lymphoprep™. This must be done carefully by slowly layering the blood/PBS on top of the Lymphoprep™.
    a. Slightly inverting the 15mL centrifuge tube while slowly layering the blood/PBS will help ensure minimal disruption of the interface.
8. Spin the samples at 1500rpm for 30 minutes at 23°C. Set acceleration to 4 or 5 and set the brake at 0.
9. Once the centrifugation is complete, remove the tubes carefully without disturbing the buffy coat.
10. Aspirate and discard most of the plasma and PBS at the top of the tube.
11. Slowly and carefully with low suction, remove the buffy coat without disturbing the interface and transfer to another 50mL centrifuge tube. Note the volume of the buffy coat recovered.
12. Make a 1:2 dilution of buffy coat to cold PBS for the first wash.
13. Spine the new 50mL centrifuge tubes at 1300rpm for 10 minutes at 4°C with acceleration and brake at 7 or 8.
14. Once they are done spinning, remove the tubes carefully without disturbing the pellet. Pour off the supernatant and resuspend the pellet in 10mL cold PBS. Transfer the suspension to a 15mL centrifuge tube for the next wash.
15. Spin these tubes at 1300rpm for 5 minutes at 4°C with acceleration and brake as above.
16. Remove from the centrifuge, discard supernatant and thoroughly resuspend pellet in 5 or 10mL cold PBS.
17. Transfer 10uL of the suspension to a microcentrifuge tube and mix with an equal volume of trypan blue.
18. Spin the 15mL centrifuge tubes in the centrifuge as above.
19. While the tubes are spinning, transfer a small volume of the trypan blue stained cells to a hemocytometer slide and count the cells to determine the total number of cells in the tube.
20. Calculate the volume of media needed to resuspend the cells to achieve a concentration appropriate for the assay being performed.
    a. BrdU Proliferation assay: $2 \times 10^6$ cells per mL
    b. Flow Cytometry: $5 \times 10^6$ cells per mL

21. Once the cells have finished spinning, discard the supernatant and resuspend the pellet in the required volume of cold complete RPMI media[j] with 10% FBS to achieve the appropriate cell concentration.
22. Plate 100uL of the cell suspension per well of 96 well round bottom plates and add the appropriate mitogen according to the requirements each assay. See the appropriate protocol below for instructions.
23. Incubate the cells at 37°C with 5% $CO_2$ for the required amount of time for the assay being performed. See the appropriate protocol for further instruction.

BrdU (5-bromo-2'-deoxyuridine) Colorimetric Proliferation Assay Protocol[k]

1. Isolate, plate, and culture cells according to the Peripheral Blood Mononuclear Cell (PBMC) enrichment protocol.
2. Cell suspensions should be plated at a concentration of 2 x $10^5$ cells per well in triplicate and stimulated with the following conditions (final concentrations of mitogens listed):
    a. Unstimulated (100µL media + 100µL cell suspension)
    b. Levamisole 1µg/mL prepared fresh (100µL levamisole + 100µL cell suspension)
    c. Levamisole 10µg/mL prepared fresh (100µL levamisole + 100µL cell suspension)
    d. Levamisole 1µg/mL stored for 2 weeks at 4°C (100µL levamisole + 100µL cell suspension)
    e. Levamisole 10µg/mL stored for 2 weeks at 4°C (100µL levamisole + 100µL cell suspension)
    f. Concanavalin A[l] (ConA) 5µg/mL (100µl ConA + 100µL cell suspension)
    g. ConA 5µg/mL + Levamisole 1µg/mL prepared fresh (100µL mitogen mixture + 100µL cell suspension)
    h. ConA 5µg/mL + Levamisole 10µg/mL prepared fresh (100µL mitogen mixture + 100µL cell suspension)
    i. ConA 5µg/mL + Levamisole 1µg/mL stored for 2 weeks at 4°C (100µL mitogen mixture + 100µL cell suspension)
    j. ConA 5µg/mL + Levamisole 10µg/mL stored for 2 weeks at 4°C (100µL mitogen mixture + 100µL cell suspension)
    k. Phorbol myrisate acetate 20ng/mL[m] and Ionomycin 10pg/mL (PMA/I) (100µl PMA/I + 100µL cell suspension)
    l. PMA/I 20ng/mL/10pg/mL + Levamisole 1µg/mL prepared fresh (100µL mitogen mixture + 100µL cell suspension)
    m. PMA/I 20ng/mL/10pg/mL + Levamisole 10µg/mL prepared fresh (100µL mitogen mixture + 100µL cell suspension)
    n. PMA/I 20ng/mL/10pg/mL + Levamisole 1µg/mL stored for 2 weeks at 4°C (100µL mitogen mixture + 100µL cell suspension)
    o. PMA/I 20ng/mL/10pg/mL + Levamisole 10µg/mL stored for 2 weeks at 4°C (100µL mitogen mixture + 100µL cell suspension)
3. Culture cells for 72 hours at 37°C and 5% $CO_2$.
4. Twenty-four hours prior to harvesting, add 20µL BrdU labeling solution per well.
    a. BrdU labeling solution is provided as a 1000x concentrated solution. Dilute the concentrated BrdU labeling solution 1:100 with sterile complete culture media prior to use.
5. After a total of 72 hours culture time, harvest the cells.
6. Centrifuge the plates at 300g at 23°C for 10 minutes. Remove supernatant by flicking off or aspirating.
7. Add 200µL FixDenat solution to each well. Do not resuspend cells.
    a. FixDenat solution is provided ready to use.
8. Incubate cells for 30 minutes at room temperature.
9. Remove FixDenat solution by thoroughly flicking off and tapping the plate.
10. Add 100µL anti-BrdU-POD working solution to each well.

    a. Anti-BrdU-POD is provided as a powder. Dilute the contents of the bottle in 1.1mL double distilled water. For 10 minutes and mix thoroughly.
    b. Prior to use, the stock solution must then be further diluted 1:100 with the provided antibody dilution solution.
11. Incubate cells for 90 minutes at room temperature.
12. Remove the antibody concentrate thoroughly by flicking off and tapping the plate.
13. Wash each well 3 times with 200µL Washing Buffer Solution.
    a. Washing Buffer is provided as a 10x concentrated solution. Dilute the Washing Buffer 1:10 with double distilled water prior to use.
14. Thoroughly remove Washing Buffer Solution after each wash by flicking off and tapping.
15. Add 100µL Substrate Solution to each well.
    a. Substrate Solution is provided ready to use.
16. Incubate the plate at room temperature to allow color development.
17. Analyze each plate with an ELISA reader at 5 minutes and in 5 minute increments for 30 minutes total.
    a. Absorbance should be analyzed at a wavelength of 370nm.
    b. A reference wavelength of 492nm should be included.
18. After the 30 minute analysis, add 25 µL of stop solution (1M $H_2SO_4$) to each well and mix thoroughly.
    a. Absorbance should be analyzed at a wavelength of 450nm.
    b. A reference wavelength of 690nm should be included.
    c. Measurement must be carried out within 5 minutes of adding the stop solution.

CellTrace™ Violet Proliferation Assay[n]

1. Obtain peripheral blood mononuclear cells (PBMCs) per protocol at a concentration of 1 x $10^6$/mL in PBS at room temperature.
2. Prepare CellTrace™ stock solution immediately prior to use by adding 20uL DMSO to one vial of CellTrace™ reagent. Mix well.
3. Add 0.2 µL of the CellTrace™ solution per mL of cells for a final working solution of 1 µM CellTrace™. Gently, but thoroughly, mix the cells.
4. Incubate the cells at room temperature or 37°C for 20 minutes protected from light.
5. After the incubation period, add 5 times the original staining volume of complete RPMI culture medium containing at least 1% FBS to the cells and gently mix (ex. Add 15mL media with at least 1% FBS for every 3mL cell suspension for a total of 18mL). Incubate for 5 minutes.
    a. This removes any free dye remaining in the solution.
6. Centrifuge the cells at 1300 rpm for 5 minutes at room temperature to pellet the cells.
    a. Pour off the supernatant.
    b. Resuspend the cells in fresh prewarmed complete RPMI culture media with 10% FBS at a concentration appropriate for assay being performed.
    c. Count cells to ensure appropriate cell numbers and concentrations.
    d. Centrifuge to pellet cells and resuspend in appropriate amount of complete RPMI culture media if the concentration needs to be adjusted.
7. Incubate the cells for at least 10 minutes before flow cytometric analysis to allow the CellTrace™ reagent to undergo acetate hydrolysis.
8. Plate cells and add appropriate mitogens and culture as required for the experiment.
9. Proceed normally with cell surface and cytokine staining.
10. Approximate fluorescence excitation/emission maxima: CellTrace™ Violet: 405/450 nm.

CellTrace™ can be used with aldehyde-based fixative such as paraformaldehyde.

CellTrace™ reagent covalently binds to cells and will not wash out after permeabilization.

Cell Surface Marker and Intracellular Cytokine Staining Protocol Using Cytofix/Cytoperm™ Fixation/Permeabilization Kit with GolgiPlug™ Protein Transport Inhibitor Containing Brefeldin A[o]

1. Obtain peripheral blood mononuclear cells at a concentration of 5 x $10^6$ per mL.
2. Plate 200 µL cells in 96 well round bottom plate. Add appropriate mitogens and culture for required length of time (72 hours).
3. 12 hours prior to harvesting, add 1 µL GolgiPlug™ (brefeldin A) per 1 mL cells, or 0.2 µL per 200 µL cell culture.
    a. Dilute 0.2 µL GolgiPlug™ in 20 µL complete culture media for each well.
    b. Add 20 µL of the above solution per well.
4. Continue incubation for 12 hours and then harvest.
5. Centrifuge plates at 1300 rpm at 4°C for 5 minutes to pellet cells.
    a. Discard supernatant.
6. Resuspend cells in 50 µL of staining solution in cold PBS. (See end of protocol for staining for our panels.)
7. Incubate at 4°C for 20 minutes protected from light.
8. Wash cells 2 times.
    a. Add 125 µL cold PBS to wells and centrifuge at 1300 rpm at 4°C for 5 minutes.
    b. Discard supernatant.
    c. Add 200 µL cold PBS to well and centrifuge as before.
    d. Discard supernatant.
9. If secondary antibody is needed (panels 6-11), follow steps below:
    a. Resuspend pellets in 50 µL of staining solution in cold PBS. (See end of protocol for concentrations.)
    b. Incubate at 4°C for 20 minutes protected from light.
    c. Wash cells 2 times.
        i. Add 125 µL cold PBS to wells and centrifuge at 1300 rpm at 4°C for 5 minutes.
        ii. Discard supernatant.
        iii. Add 200 µL cold PBS to well and centrifuge as before.
        iv. Discard supernatant.
10. Thoroughly resuspend cells in 100 µL of Fixation/Permeabilization solution.
    a. Fixation/Permeablilization solution is supplied as a ready to use solution.
11. Incubate for 20 minutes at 4°C protected from light.
12. Wash cells two times in Perm/Wash buffer.
    a. Centrifuge plates at 1300 rpm at 4°C for 5 minutes.
    b. Discard supernatant.
    c. Add 200 µL cold Perm/Wash buffer to each well and resuspend cells.
        i. Perm/Wash buffer is supplied as a 10x concentrated solution. Dilute the solution 1:10 with distilled water prior to use.
    d. Centrifuge plates at 1300 rpm at 4°C for 5 minutes.
    e. Discard supernatant.
    f. Repeat c., d., and e.
13. Resuspend cells in 50 µL of staining solution in Perm/Wash buffer. (See end of protocol for staining for our panels.)
14. Incubate at 4°C for 30 minutes protected from light.

15. Wash cells two times in Perm/Wash buffer.
    a. Add 125 µL Perm/Wash buffer to each well.
    b. Centrifuge plates at 1300 rpm at 4°C for 5 minutes.
    c. Discard supernatant.
    d. Add 200 µL cold Perm/Wash buffer to each well and resuspend cells.
    e. Centrifuge plates at 1300 rpm at 4°C for 5 minutes.
    f. Discard supernatant.
16. Thoroughly resuspend cells in 100-125 µL PBS.
17. Samples are now ready for flow cytometric analysis.

Staining Panels:

Panel 1.
- CD21

Panel 2.
- CD4
- CD8
- IFNɣ

Panel 3.
- CD4
- CD8
- IL4

Panel 4.
- CD4
- FoxP3
- IL4

Panel 5.
- CD8
- IL10

Panel 6.
- CD14
- CD86
- MHC Class II
- IFNɣ

Panel 7.
- CD14
- CD86
- MHC Class II
- IL4

Panel 8.
- CD14
- CD86
- MHC Class II
- IL10

Panel 9.
- CD172a
- CD86

MHC Class II
IFNɣ

Panel 10.

CD172a
CD86
MHC Class II
IL4

Panel 11.

CD172a
CD86
MHC Class II
IL10

List of Antibodies:

| Marker | Flourochrome | Concentration per 5 x $10^5$ cells | Isotype | Manufacturer | Catalog Number |
|---|---|---|---|---|---|
| **CD4** | FITC | 3.75 µL/well | IgG1 Mouse Anti-Horse | AbD Serotec | MCA1078F |
| **CD4** | PE | 3.75 µL/well | IgG1 Mouse Anti-Horse | AbD Serotec | MCA1078PE |
| **CD8** | PE | 5 µL/well | IgG2a Mouse Anti-Horse | AbD Serotec | MCA1080PE |
| **CD14** | Unconjugated | 0.5 µL/well | IgG1 Mouse Anti-Equine | Wagner Laboratory | Clone 105 |
| **CD21** | PE | 5 µL/well | IgG1 Mouse Anti-Human | BD Pharmingen™ | 557327 |
| **CD86** | PE | 10 µL/well | IgG2b Mouse Anti-Human | BD Pharmingen™ | 555665 |
| **CD172a** | Unconjugated | 0.5 µL/well | IgG1 Mouse Anti-Bovine | Kingfisher Biotech, Inc | WS0567B-100 |
| **MHC Class II** | FITC | 0.25 µL/well | IgG1 Mouse Anti-Horse | AbD Serotec | MCA1085F |
| **FoxP3** | AlexaFluor® 488 | 10 µL/well | IgG2a Rat Anti-Human | eBioscience® | 53-4776 |
| **IFNɣ** | AlexaFluor® 647 | 0.5 µL/well | IgG1 Mouse Anti-Bovine | AbD Serotec | MCA1783A647 |
| **IL4** | AlexaFluor® 647 | 0.25 µL/well | IgG1 Mouse Anti-Equine | Wagner Laboratory | Clone 13G7 |
| **IL10** | AlexaFluor® 647 | 0.25 µL/well | IgG1 Mouse Anti-equine | Wagner Laboratory | Clone 165-2 |
| **Secondary** | PerCP-ef® 710 | 0.25 µL/well | IgG1 Rat Anti-Mouse | eBioscience® | 46-4015 |

Endnotes:

[a] Cytofix/Cytoperm™ Fixation/Permeabilization Kit with GolgiPlug™ Protein Tranport Inhibitor Containing Brefeldin
[b] ReBalance®: Pegasus Laboratories, Inc. Pensacola, FL, USA
[c] Marquis®: Merial, Inc. Duluth, GA, USA
[d] Protazil®: Merck Animal Health. Summit, NJ, USA
[e] Cell Proliferation ELISA, BrdU (colorimetric): Roche Life Science GmbH. Mannheim, Germany
[f] Lymphoprep™: Stemcell Technologies, Inc. Vancouver, BC, Canada
[g] Cytofix/Cytoperm™: BD Biosciences. San Jose, CA, USA
[h] Lymphoprep™: Stemcell Technologies™, Vancouver, BC, Canada. Catalog Number: 07801
[i] PBS, 1x: Corning, Manassas, VA, USA. Catalog Number: 210-040-CV
[j] RPMI 1640, 1x: Corning, Manassas, VA, USA. Catalog Number: 10-041-CV
[k] Cell Proliferation ELISA, BrdU (colorimetric): Roche Life Science GmbH. Mannheim, Germany. Catalog Number: 11 647 229 001
[l] Concanavalin A Lyophilized: Sigma-Aldrich®. St Louis, MO, USA. Catalog Number: C0412
[m] PMA: Sigma-Aldrich®. St Louis, MO, USA. Catalog Number: P8139 1mg
[n] CellTrace™ Violet Cell Proliferation Assay: Life Technologies™, Inc. Carlsbad, CA, USA. Catalog Number: C34557
[o] Cytofix/Cytoperm™ Fixation/Permeabilization Kit with GolgiPlug™ Protein Tranport Inhibitor Containing Brefeldin