THE EFFECTS OF STAPHYLOCOCCAL ENTEROTOXIN B ON EQUINE T CELLS

by

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(Under the Direction of R. Jeff Hogan)

ABSTRACT

Staphylococcus aureus enterotoxins and their impact on T cells in humans and mice are well described. *S. aureus* prevalence has not only increased in veterinary clinics, but is a relatively common isolate in horses, more so than in other animal species. Yet the role of superantigens in infection is unknown. With this in mind, the effects of staphylococcal enterotoxin B (SEB) on equine T cells were examined. Using an in vitro assay, dose responses and the kinetics of equine T cell proliferation were determined. Equine MHC class II was found to be important for SEB-mediated equine T cell stimulation. Using RT-PCR, the presence of SEB induced cytokine production (i.e. IFN-γ and IL-10) in equine T cells, but additional studies are needed. Taken together, these data suggest that virulence factors such as SEB when expressed may alter the clinical disease associated with *S. aureus* infection in horses.

INDEX WORDS: Superantigens, Staphylococcal Enterotoxin B (SEB), Equine T cell Proliferation, Equine MHC Class II Interaction

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DEDICATION

This thesis is dedicated to the memory of Michael A. Johnson whose enthusiastic support and guidance were critical in my undergraduate research experience through the Student Undergraduate Research Program and my return as a graduate student. This is also dedicated to the memory of Silky whose loyalty and friendship were unwavering.

Dedication is also extended to Walter and Frieda, my parents, who provided understanding and patience throughout the process. Dedication also goes to Bryan for his never-ending enthusiasm and optimistic assistance during the most crucial stage.

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CHAPTER 1

INTRODUCTION

Superantigens are proteins produced by pathogens such as bacteria, mycoplasma, and viruses that stimulate large numbers of T cells. The most extensively studied superantigens are bacterial toxins, specifically staphylococcal enterotoxins, that bind non-specifically to the variable region of the beta chain of T cell receptors (1). For a superantigen to induce activation of T cells, binding to class II MHC molecules on antigen presenting cells is necessary. Due to the extensive range of T cell receptor beta chain regions they bind, superantigens are capable of activating large numbers of T cells in a short period of time. The terminology “cytokine storm” has been used to describe the enormous quantity of cytokines produced by the suddenly large population of activated T cells. Production of these cytokines may result in circulatory shock characterized by a potentially lethal drop in blood pressure.

Although previous studies have examined the impact of *Staphylococcus aureus* enterotoxins on human T cells, very little information is available regarding their effect on equine T cells. Given the increased prevalence of *S. aureus* in veterinary clinics, it is important to understand the effect of this organism and its virulence factors on domestic animal species. The species distribution of coagulase-positive staphylococci in a wide variety of animal species has been well characterized and includes, but is not limited to, dogs, horses, cats, goats, and cows (2, 3). There is also evidence of enterotoxin production by *Staphylococcus* strains that previously were presumed not to be enterotoxigenic (4). In response to bacterial invasion, the B cells of the humoral (Th2) arm of the immune response proliferate and produce large amounts of antibodies that target pathogens for elimination. In contrast, the cell-mediated immune response utilizes T cells that recognize and destroy host cells that have been damaged by the pathogen. Th1 cytokines, such as interleukin (IL)-2, interferon gamma (IFN-γ), and IL-12, stimulate these cell-mediated responses and may suppress certain aspects of the humoral response. In contrast, Th2 cytokines, such as IL-4, IL-5, and IL-10, may stimulate the humoral response and suppress cell-mediated immune responses.

The interactions among Staphylococcal enterotoxin B (SEB), MHC class II, and T cell receptor's of humans and mice have been well studied. For example, toxic shock syndrome toxin-1 (TSST-1) binding to MHC class II molecules can be affected by the peptide bound to the MHC molecule. However, SEB is peptide independent, implying that the length and sequence of the MHC-bound peptide is not critical in the presentation of SEB to T cells. This allows SEB to be more flexible and perhaps more effective as a toxin, and thereby stimulate the large number of T cells that are responsible for the "cytokine storm" (5).

Given the disease it causes in humans, its stability, and multiple routes of administration (e.g. contaminated food, water, and aerosol), SEB may be used in terrorism or biological warfare (6). For this reason, great efforts have been made to develop more sensitive and more rapid diagnostic tests, such as the dissociation-enhanced lanthanide fluorescent immunoassay, to better characterize and control epidemics (6). In vivo and in vitro research to describe SEB-induced changes in gene expression patterns in human peripheral blood mononuclear cells identified a subset of genes that can be used as a predictor of exposure to SEB (7).

Treatment of patients with SEB-induced disease is impeded by the acquired resistance of *S. aureus* to antibiotics. Furthermore, there is compelling evidence that methicillin-resistant strains of *S. aureus* have extended beyond the hospital environment to the community (8). In an effort to overcome these problems, there is increasing interest in the potential use of urokinase, an enzyme that inhibits the growth of *S. aureus* both directly by cytolysis and indirectly by abrogating the neutralizing effect of staphylokinase on the bacteriocidal activities of human neutrophil peptides (9). Specifically, urokinase-type plasminogen activator is a serine protease that has fibrinolytic effects, promotes the homing of leukocytes to inflammatory sites, has anti-microbial properties when complexed with human neutrophil peptides, and blocks the binding of *S. aureus* staphylokinases (9). Additional research has revealed that a nontoxic mutant of Staphylococcal enterotoxin C (SEC) can protect against *S. aureus* infection by inhibiting IFN-γ production and up-regulating IL-10 production in vitro (10). Data from the same study also showed that serum transfer between mice yielded protection against *S. aureus* infection, indicating the possibility of SEC-specific neutralizing antibodies (10). Despite many challenges, a vaccine against SEB has been developed using a Venezuelan equine encephalitis virus vector, but its efficacy is not yet known (11). To date, the majority of research efforts have been directed towards combating SEB in humans, again illustrating the need to extend the focus to other animal species.

The purpose of this research project was to determine whether or not equine T cells, like human and murine T cells, proliferate and/or secrete cytotoxins in response to staphylococcal enterotoxins, in particular SEB. Based upon the similarities in the structure of equine and human T cell receptors, it was hypothesized that SEB would induce proliferation of equine T cells and the production of cytokines. To determine if SEB activates equine T cells, experiments were first performed to identify the concentration of SEB required to induce proliferation of equine T cells and the time course associated with those effects. We determined that equine T cells not only require lower concentrations of SEB to induce proliferation, indicating a higher sensitivity to SEB than murine T cells, but also require additional time to proliferate after exposure to SEB (48 hours for equine T cells versus 24 hours for murine T cells).

Having identified the appropriate concentrations and time required for proliferation, experiments were then performed to determine whether equine T cells required MHC class II for SEB-induced stimulation to occur. Using an antibody specific for equine MHC class II molecules, it was clear that SEB must interact with MHC class II for efficient stimulation of equine T cells. Finally, studies were completed to evaluate SEB-induced production of specific classes of cytokines. Based upon human and mouse studies, SEB treatment was expected to cause equine T cell proliferation and increase the expression of Th2 cytokines such as IL-4, IL-5 and IL-10 while decreasing the production of Th1 cytokines such as IL-2 and IFN- γ (12-16). These data would support the view that SEB induces a switch from Th1-type to Th2-type cytokines, and this mechanism is most likely responsible for the immunoregulatory effect that SEB-reactive T cells experience. Preliminary quantitative RT-PCR experiments suggested that SEB induces an increase in production of IFN- γ and a decrease in IL-10; additional studies are needed to determine if these responses are consistent with equine T cells. Moreover, future studies are needed to determine if virulence factors such as SEB play an important and previously unrecognized role in the clinical disease associated with *S. aureus* infection in horses.

CHAPTER 2

LITERATURE REVIEW

Overview of Staphylococcus aureus

Since its discovery in pus from surgical abscesses by the surgeon Sir Alexander Ogston in Aberdeen, Scotland in 1880, *Staphylococcus aureus* has grown to be a great concern in both community and hospital settings worldwide (17). Approximately 500,000 patients each year contract a staphylococcal infection in hospitals in the United States. *Staphylococcus aureus,* a facultative anaerobe and gram-positive coccus, grows in grape-like clusters when viewed through a microscope with characteristic large, round, golden-yellow colonies indicative of its name. *S. aureus* is catalase positive, i.e., capable of producing catalase, thereby converting hydrogen peroxide to water and oxygen which allows for differentiation of staphylococci from enterococci and streptococci. *S. aureus* can also be identified as being coagulase-positive with its ability to produce coagulase and induce clot formation, yet some strains lack the ability to do so preventing correct identification and proper treatment (18).

Virulence Factors of Staphylococcus aureus

Staphylococcus aureus is the most common cause of “staph” infections and can cause illnesses ranging from minor skin infections and abscesses, to life-threatening diseases such as pneumonia, meningitis, osteomyelitis, endocarditis, toxic shock syndrome (TSS), and septicemia. Not only is *S. aureus* commonly present on skin and in the nose of humans, about 20% of the population are long-term carriers (19). Proteins secreted by staphylococci can cause disease, and determination of the antigenic composition aids in strain classification. Depending on the strain, *S. aureus* is capable of secreting a variety of toxins, which can be categorized into three groups and are usually associated with specific diseases. The first group contains Pyrogenic toxin superantigens (PTSAgs) which have superantigen activities capable of inducing TSS. This group is the most studied of the three groups and includes the toxin TSST-1 and staphylococcal enterotoxins (SE) A, B, C1, C2, C3, D, E, G, H, I, which can cause food poisoning and sepsis. The second group includes exfoliative toxins that are associated with the disease called staphylococcal scalded-skin syndrome (SSSS) commonly seen in children. The third group encompasses other toxins, namely alpha-toxin, beta-toxin, delta-toxin, and several bicomponent toxins, that act on cell membranes.

Enterotoxins are protein toxins released by a microorganism in the intestine and, more specifically, are commonly chromosomally encoded exotoxins. Characteristics of enterotoxins include being heat stable, having a low molecular weight, being water-soluble, having cytotoxic effects, and being capable of killing cells by altering mucosal epithelial cell membranes due to pore formation. The pores activated by these enterotoxins can lead to increased permeability of the apical membrane of intestinal mucosal cells to chloride ions. Activation of these membrane pores results in increased intracellular concentrations of either cAMP or calcium ions. The pore formation has a direct effect on the osmolarity of the luminal contents of the intestines, as the increase in permeability leads to leakage of chloride ions into the lumen followed by sodium and water movement. This leads to a secretory diarrhea within a few hours of ingesting enterotoxins. Several microbial organisms, such as *Staphylococcus aureus* and *Escherichia coli,* contain the enterotoxins needed to create such an effect. The particular toxin of focus for the purpose of this study is the superantigen staphylococcal enterotoxin B.

Superantigens are one of two types of Type I exotoxins, the second type being heat-stable enterotoxins. Superantigens, being secreted proteins, are capable of binding cell surface receptors thereby stimulating intracellular signaling pathways and exhibit highly potent lymphocyte-transforming (mitogenic) activity towards T lymphocytes (20-22). Compared to a normal antigen-induced T cell response in which .001-.0001% of the body's T cells are activated, superantigens are capable of activating up to 20% of T cells (23). This results in a massive immune response that is not specific to any particular epitope on the superantigen. Since one of the fundamental strengths of the adaptive immune system is its ability to target antigens with high specificity, superantigens produce an immune response that is effectively useless and may help microbes evade detection by the immune system (21, 22).

Clinical Disease Caused by Staphylococcus aureus and Staphylococcal Enterotoxins

Disease pathogenesis in humans and mice has been well described for *Staphylococcus aureus*. As previously mentioned, *S. aureus* is capable of causing various types of diseases, including superficial infections such as folliculitis, carbuncles, boils, abscess formation; deep seated infections such as osteomyelitis, bronchopneumonia, empyema, endocarditis, meningitis; bacteremia and hospital acquired infections (24). More serious toxigenic diseases include food poisoning, TSS, and scalded skin syndrome (24).

Diagnostic laboratories are essential in the identification process with respect to outbreaks and new strains of *S. aureus*. In addition to traditional biochemical or enzyme-based tests and culturing, advances in technology have enabled the development of more reliable and rapid techniques for the identification and characterization of clinical isolates of *S. aureus* (25). These diagnostic tools support infection control strategies to not only limit disease transmission, but to also ensure proper use of antibiotics. These techniques include real-time PCR or quantitative PCR, both of which are now employed in clinical laboratories (25-28). Using today’s tools, it is possible to distinguish viable from dead *S. aureus*, as propidium monoazide selectively penetrates dead cells (29).

The treatment of choice for *S. aureus* infection is penicillin; nevertheless penicillin-resistance is extremely common. Gentamicin has also been used in combination therapy to treat more serious infections, such as endocarditis (30, 31), but this antibiotic may cause kidney damage (32). When penicillin was introduced in 1943, antibiotic resistance was extremely rare. By 1950, 40% of hospital *S. aureus* isolates were penicillin resistant, and this percentage had reached 80% by 1960 (33). Since the 1990s, Methicillin-resistant *Staphylococcus aureus* (MRSA) prevalence in hospitals increased dramatically and is now endemic (34). Community acquired MRSA has also become a significant problem (35-37) as humans may contract MRSA from their pets (38) and domesticated animals may contract MRSA from their owners via reverse zoonosis (39, 40).

The mechanism underlying resistance to methicillin is mediated via the *mec* operon which is part of the staphylococcal cassette chromosome *mec* (SCC*mec*). Resistance is conferred by the *mecA* gene that codes for an altered penicillin-binding protein (PBP2a or PBP2') and has a lower affinity for β-lactam antibiotics such as the penicillins, carbapenems, and cephalosporins, and results in resistance to all β-lactam antibiotics. Patients with MRSA infections were treated effectively with the glycopeptide vancomycin until glycopeptide resistance was developed with the acquisition of the *vanA* gene. The *vanA* gene codes for an enzyme that produces an alternative peptidoglycan to which vancomycin will not bind. Vancomycin-intermediate *S. aureus* (VISA) was reported first in Japan in 1996 (41) and the first case of *S. aureus* resistant to glycopeptide antibiotics was reported in 2002 (42). As of 2005, three cases of vancomycin-resistant *S. aureus* (VRSA) infection have been reported in the United States (43).

Spread of *S. aureus*, including MRSA, is primarily through human-to-human contact, although infection can be spread through domesticated animals (38). Hand washing is effective in preventing the transmission of *S. aureus* and the use of disposable aprons and gloves reduces skin-to-skin contact reducing the risk of transmission. In the last several years, there have been numerous cases of *S. aureus* infections in hospitals in the United States and the spread of this organism has been attributed, in part, to the inadequate hygiene of healthcare workers. Being an extremely resilient bacterium, *S. aureus* is capable of surviving merely on polyester for nearly three months. This fact invokes great concern given the frequency with which polyester is present in the hospital setting, e.g., privacy curtains (44).

Transmission of *S. aureus* occurs easily amongst the hands of healthcare workers, seemingly healthy patients carrying a benign or commensal strain of the pathogen, and other uninfected patients. Outside of the hospital, one of the most important and commonly overlooked means of community-associated MRSA infection and transmission is during sexual contact (45). Hospitals have now adopted a variety of methods to help prevent additional spread of this pathogen, such as using alcohol as a topical sanitizer along with quaternary ammonium, routine cleaning, and nonflammable alcohol vapor in CO_2 NAV-CO_2 systems. It is interesting to note that essential oils, such as tea tree oil, have potent antibacterial properties and that vaporizing these oils into the atmosphere significantly reduces airborne bacterial counts and MRSA infections (46-48).

Immune Response to Staphylococcal Enterotoxins

To date, bacterial superantigens include a family of 40 secreted protein toxins produced by *Staphylococcus aureus* and *Streptococcus pyogenes* (49-52). Unrelated superantigens are also produced by *Mycoplasma arthriditis mitogen* (7) and *Yersinia pseudotuberculosis mitogen* (52). Currently, 20 serologically distinct staphylococcal superantigens have been described comprising TSST-1, the staphylococcal enterotoxins (SE) A-E, G-J, and the staphylococcal enterotoxin-like (enterotoxicity unproven) toxins (SEls) K-R and U, and SEl-U2 and Sel-V (52). Most staphylococcal superantigens are encoded by accessory genetic elements such as prophages, transposons, plasmids, and pathogenicity islands (52). Being mobile, these elements are not uniformly distributed among clinical isolates, and significant variation is seen with regions differing between strains most like resulting from horizontal transfer (52).

Little is known regarding the regulation of staphylococcal superantigens. It is known that SEB is expressed at very high levels by some strains, but most staphylococcal superantigens are expressed at very low levels under normal culture conditions (52). In *S. aureus*, the *agr* (accessory gene regulator) system is the best known regulator which is a quorum-sensing system activated at high cell densities (52). Activation of *agr* results in the upregulation of most superantigens, but not SEA, which is constitutively expressed in strains carrying the *sea* gene (52). It still has not been determined whether or not *seb* and *tst-1* genes are activated by the transcription factor SarA (52). Recent studies have suggested that staphylococcal superantigen-like protein (*ssl*) genes are regulated by stress conditions caused by excess cytoplasmic iron (52).

TSST-1, for example, binds to Vβ 15, 16 in mice and to Vβ 2 in humans (22, 51, 53). SEB, on the other hand, is one of the most versatile superantigens and is capable of binding to Vβ 3, 7, 8.1-8.3, 11, 17 in mice and to Vβ 3, 12, 14, 15, 17, 20 in humans (53). The majority of the genes encoding superantigens are located in close proximity to each other on mobile elements of bacterial genomes, namely plasmids or pathogenicity islands (50). The operon described as the enterotoxin gene cluster has been found to be most common in most superantigen-producing bacterial strains (50).

Superantigens are produced intracellularly by the bacteria and are extracellularly released upon infection as mature toxins (54). Sequences of these toxins are relatively conserved among different subgroups, and, more importantly, their 3D structures are similar, resulting in similar functional effects among different groups (55, 56). Crystallography has revealed that enterotoxins are compact, ellipsoidal proteins sharing a characteristic two-domain folding pattern comprising an NH2-terminal β barrel globular domain known as the oligosaccharide/ oligonucleotide fold that binds the major histocompatibility complex class II (MHC class II), a long α-helix that diagonally spans the center of the molecule, and a COOH terminal globular domain that binds the T cell Receptor (TCR) (55, 57).

Superantigens bind first to the MHC Class II and then coordinate to a TCR with the appropriate specific Vβ motif, and a preference has been shown for the HLA-DQ form of the MHC II molecule in humans (21, 50, 56). Binding to the MHC II α-chain allows the superantigen to be properly oriented to coordinate with the TCR. As a result, it is less common for superantigens to attach a polymorphic β-chain mediated by a zinc ion coordination complex between three superantigen residues and a highly conserved region of the HLA-DR β chain (56). Several staphylococcal superantigens, such as SEA and SEB, are capable of cross-linking MHC molecules by binding to both the α and β chains. Cross-linking of these molecules stimulates massive cytokine expression, release of antigen presenting cells (APCs), and induction of costimulatory molecule production which allows APCs to bind and activate additional T cells (55, 56).

The T cell binding region of the superantigen interacts with the Vβ chain of the TCR. A given superantigen is capable of activating a large proportion of the T cell population by binding to more than one of the 18 different types of Vβ regions in humans for which there are 18 families or types (58) and 22 families for mice (57, 59). In horses, there are 9 families with TCR Vβ2, 12 being the most commonly represented (60), similar to Vβ4, 6, 12 being most common in humans (61) and Vβ 6, 8 most common in mice (62). The variability that occurs among people with respect to frequency of different T cell region types explains the variation seen in strength of response to certain superantigens.

Of the five possible groupings for superantigens, SEB belongs to Group II. Superantigens of Group II interact with the Vβ region using mechanisms that are conformation-dependent and these interactions are mostly independent of specific Vβ amino acid side-chains. Since the presentation of SEB is peptide independent, the length of the SEB peptide is not critical, a fact that may facilitate the ability of SEB to stimulate the maximal number of T cells and result in excessive cytokine production (5). The biological strength of the superantigen, specifically its ability to stimulate T cells, is determined by its affinity for the TCR. Superantigens with the highest affinity for the TCR elicit the strongest response, and streptococcal mitogenic exotoxin Z-2 (SMEZ-2) is currently the most potent superantigen yet discovered (63).

Cross-linking of the MHC and the TCR by the superantigen induces a signaling pathway that results in T cell proliferation and the production of cytokines. It has been demonstrated that the normal signaling pathway responsible for T cell activation is impaired in superantigen-activated T cells when concentrations of Zap-70, a member of the protein-tyrosine kinase family that initiates T cell signaling, are reduced (64). Fyn, a member of the Src family of tyrosine kinases, is activated by a tyrosine kinase leading to the adaptive induction of anergy, a response that is in contrast to activation of leukocyte-specific protein tyrosine kinase (Lck) (65). Furthermore, both protein kinase C and protein tyrosine kinase pathways are activated resulting in up-regulation of proinflammatory cytokine production (66). There is evidence that this alternative signaling pathway may impair the calcium/calcineurin and Ras/MAPkinase pathways slightly, while still allowing a significant inflammatory response to occur (65).

Stimulation of antigen presenting cells and T cells by superantigen elicits a response that is primarily inflammatory in nature. This Th1 type response stimulates the production of the signature proinflammatory cytokines such as IL-1, IL-2, IL-6, TNF-α, and IFN-γ (66). It is the chaotic and massive release of these cytokines, a response that is termed a "cytokine storm," that results in symptoms ranging from rashes and fever to multi-organ failure, coma and death (49, 50). Deletion or anergy of activated T cells after infections is the result of production of IL-10 from prolonged exposure to the toxin. Production of IL-10 induces down-regulation of IL-2, MHC Class II, and costimulatory molecules on the surface of antigen presenting cells which produce memory cells that do not respond to antigen stimulation (67, 68). The non-responsive memory cells result from cytokine-mediated suppression of T cells and MHC cross-linking. This leads to signal pathway activation which suppresses hematopoiesis and upregulates Fas-mediated apoptosis (69).

The results of other studies indicate that *S. aureus* itself is capable of being internalized by and inducing apoptosis of bovine mammary epithelial cells (70). Furthermore, there is evidence that IL-2R beta is critical in mice for the normal pattern of programmed cell death that occurs in response to SEB exposure via Fas-mediated apoptosis (71). In essence, the cytokines released by stimulated T cells result in suppression of T cells and the loss of these cells accounts for the damage that occurs to the host's tissues and organ systems. As a result, the immune system is ultimately compromised as the host's cells become anergic or are deleted, even if the initial inflammatory response was not devastating.

The indirect effects of superantigens are evident during food poisoning, when superantigen-producing bacteria release a heat-resistant toxin. As little as 20-35 μg of superantigen are capable of inducing vomiting and gastrointestinal toxicity (50). Moreover, superantigens stimulate recruitment of neutrophils to infection sites in a T cell-independent fashion by activating monocytes, production of TNF-α, and increased expression of adhesion molecules that recruit additional leukocytes to the site. The end result is tissue damage due to the excessive inflammatory response (72). It is also important to note that immune system function can be impaired further when both endotoxins and superantigen(s) are released in a bacterial infection (73). Several important diseases have been linked to superantigen production, including toxic shock syndrome, Kawasaki disease, eczema, Guttate psoriasis, rheumatoid arthritis, diabetes mellitus, and scarlet fever (50).

One goal of treatment is to remove the bacteria that produce superantigens. This is accomplished primarily through the use of antibiotics which are commonly used in conjunction with vasopressors and fluid resuscitation (22). Antibodies produced in response to some superantigens can prevent T cell activation by neutralizing the superantigens (74). Synthetic antibodies and peptides that block the interaction of superantigens with the MHC class II molecule have been developed with the goal of preventing T cell activation (74). Furthermore, immunosuppressants such as corticosteroids have been used to prevent T cell activation and the release of cytokines (73).

Reverse Zoonotic Transmission

The majority of research has focused upon the effects of superantigens on humans and mice with little known about the impact of this agent on other animal species. With the increasing prevalence of multi-drug resistant and superantigen encoding strains of *S. aureus* in animal and veterinary hospitals, and the close contact that occurs between companion animals and their owners, there is an increasing need to investigate the effects of staphylococcal enterotoxins on domesticated animals. Not only has it been demonstrated that transmission of *S. aureus* occurs as the result of human-to-human and human-to-animal contact, but also from animal to human (38). Although the prevalence of *S. aureus* and staphylococcal enterotoxins has been studied in dogs and cats (2, 4, 39, 75), there are few such studies regarding horses.

Extensive movement of horses, such as thoroughbreds and standardbreds, occurs commonly between the United States, Canada and Europe; therefore, colonization and infection may be more widespread than recognized (76). *S. aureus* is a concern as an equine pathogen because horses may be a community reservoir of MRSA and a source of infection or reinfection for humans (76). It is also important to consider the large number of horses in North America and the frequent close contact between people and horses. Moreover, due to the cost of veterinary care and treatment, a significant number of *S. aureus* cases may go undiagnosed and untreated allowing for further spread of the bacterium.

SEB is one of the most common *S. aureus* superantigens not only in humans but also in domesticated animals (77). Therefore investigating the effect of this superantigen in horses is highly relevant and clinically significant. Furthermore, it was recently shown that horses are not only highly sensitive to bacterial superantigens, but that toxic shock syndrome toxin-1 can induce clinical symptoms similar to those that occur in humans, namely pneumonia and shock

(75). Based upon these facts and the structural similarities between human and equine T cell receptors, the purpose of the present study was to investigate the effects of staphylococcal enterotoxin B on equine T cells.

CHAPTER 3

MATERIALS AND METHODS

ConA and SEB

Concanavalin A (ConA) was obtained from Vector Labs (Cat. No. NC9025063) and was diluted to a stock concentration of 1000 μg/ml for later use at 0.5, 1, 2, 5, and 10 μg/ml.

Highly purified staphylococcal enterotoxin B was obtained from Toxin Technology, Inc (Cat. No. BT 202) and was diluted from 1 μg/ml stock concentration to desired concentrations (i.e. 2.5, 5, 10, 20, and 50 μg/ml) in PBS or medium as defined below. The concentration of SEB used for the murine experiments was based on previous studies (5).

Antibodies

Murine antibodies used for flow cytometry were purchased from BD Bioscience and diluted in staining wash buffer (SWB, 0.1% sodium azide, 2% fetal calf serum) according to manufacturer's specifications; working concentrations of each antibody were determined prior to use. Antibodies used included: PerCP Rat Anti-Mouse CD8a (Cat. No 553036) used at a final dilution of 1:200, PE Rat Anti-Mouse CD4 (Cat. No. 553730) used at a final dilution of 1:400, and Fc blocking antibody, Purified Rat Anti-Mouse CD16/CD32 (Cat. No. 553142) used at a final dilution of 1:200.

Equine antibodies used for flow cytometry were diluted according to manufacturer's recommendations; optimal final dilutions of each antibody were confirmed by titration. Mouse Anti-Equine CD8 was purchased from Affinity BioReagents (Cat. No. MA1-82731) and used at a 1:200 dilution per the manufacturer's suggestions. Mouse Anti-Equine CD4 was purchased from AbD Serotec (Cat. No. MCA1078) and used at a 1:100, an Anti-SARS M protein IgG1 monoclonal antibody 283C was used at a 1:400 concentration (AnaSpec Inc., Cat. No. NR-621) and mAb anti-MHC Class II was obtained from Novus Biologicals (Cat. No. NB100-64955) and used at a 1:50 concentration. Cells incubated with either anti-MHC class II antibody or the SARS isotype control were incubated for 1hr at 37°C/5% CO_2 (Nuaire Air-Jacketed Incubator with HEPA filter) and then treated with the optimal concentration of SEB (2.5 mg/ml). Goat serum was used for blocking (2% in SWB) in all experiments with equine cells. Alexa Fluor® 647 goat anti-mouse IgG (H + L) was used for staining the unconjugated equine CD4 and CD8 antibodies and was obtained from Molecular Probes (Cat. No. A21235).

Preparation of Murine Splenocytes

A single cell suspension of BALB/c mouse splenocytes was used for the comparative study. Briefly, spleens were collected in a 50 ml conical tube containing 10 ml of Hanks' Balanced Salt Solution (HBSS) (1X) (Gibco Cat. No. 14170-112) and prepared by pressing through a 70 µM cell strainer with the plunger from a 3 ml syringe. The cell strainer was then rinsed with HBSS to bring the volume to 50 ml to ensure collection of the maximum number of splenocytes. Cells were centrifuged (Eppendorf 5810R) at 450 x*g* for 8 min to pellet the cells, and the fluid was decanted. Next, 3 ml of warm Gey’s solution (4.15 g NH_4Cl, 0.5 g $KHCO_3$, 0.5 ml 0.5% Phenol Red, ddH_2O to final volume of 500 ml) was added per spleen and the cells were incubated at room temperature for 5 minutes. Cells were then washed with 40-45 ml of HBSS, centrifuged at 450 x*g* for 8 min to pellet the cells and the supernatant decanted.

Cells were resuspended in 10 ml of HBSS per spleen and cell number was determined via Trypan blue dilution (1:20) and counted on a hemocytometer. Cells (excluding non-labeled controls) were then labeled with carboxy-fluorescein diacetate, succinimidyl ester (CFSE) from a working stock solution containing 0.5 μM CFSE (Vybrant CFDA SE Cell Tracer Kit from Invitrogen, Cat. No. V12883) to track cell division via dye inheritance by daughter cells. Cells were suspended in the HBSS/CFSE solution at 1x10^7 cells/ml in a 15 ml polypropylene tube and incubated for 10 minutes at room temperature in the dark. Cells were then washed 3 times with HBSS (without CFSE), counted, and suspended in Complete Tumor Cell Medium (CTM) at 1x10^6 cells/ml.

A 1.0 liter batch of CTM consisted of the following: 50 ml fetal calf serum, 500 ml bottle of S-MEM (Gibco Cat. No. 11380-037), 7.5 g dextrose (Sigma Cat. No. G7021), 75 ml essential amino acids (50x) (Gibco Cat. No. 11130-051), 140 ml non-essential amino acids (100x) (Gibco Cat. No. 11140-050), 100 ml sodium pyruvate (100mM, 100x) (Gibco Cat. No. 11360-070), 100 ml L-glutamine (200mM, 100x) (Gibco Cat. No. 25030081), 8.5 g sodium bicarbonate (Sigma Cat. No. S-5761), 500 mg gentamycin (Sigma Cat. No. G-1264), 600 mg penicillin G (Gibco Cat. No. 860-1830MJ), 1 g streptomycin sulfate (Sigma Cat. No. S-9137), 34 μl 2-mercaptoethanol (Fisher Cat. No. 034461-100), and the pH was adjusted to 7.0 with 10N NaOH.

Unlabelled (control) and CFSE labeled cells were plated at 2 ml/well (1x10^6 cells/ml) in 24-well plates with 1 plate per day for days 1-4. Treatments of ConA or SEB were then added at the indicated concentrations and the cells were incubated overnight at 37°C/5% CO_2 and stained for flow cytometry on days 1-4. All assays were performed in triplicate.

Preparation of Equine Peripheral Blood Mononuclear Cells (PBMCs)

Heparinized equine whole blood samples were obtained from healthy adult horses on The University of Georgia’s equestrian team (Table 1). Four 10 ml samples were collected in BD Vacutainer® tubes (Cat. No. 366480) from each horse. Isolation of peripheral blood mononuclear cells was carried out according to the manufacturer's specifications (Histopaque 1077, Sigma-Aldrich Procedure. No.1077) and as previously described (78). Once isolated, lymphocytes were suspended in 10 ml of CTM, cell number was determined, cells were CFSE-labeled as previously described and plated in multiple 96-well plates. Cells in individual plates were studied each day for days 2-4. Treatments of ConA, SEB, anti-MHC class II, and/or Anti-SARS M protein IgG1 monoclonal antibody were then added at the optimal concentrations. After an overnight incubation at 37°C/5% CO_2, the cells were stained for flow cytometry on days 1-4 in triplicate.

Flow Cytometry

Cells were stained with the appropriate antibodies on days 1-4 for mouse and 2-4 for horse experiments. For mouse splenocytes, FC block (anti-CD16/32) was applied at 50 μl/well (1:100 dilution) and incubated for 10 minutes on ice in the dark. This was followed by centrifugation at 450 x*g* for 3 minutes, the addition of 100 μl/well of the mixture of CD4/CD8 antibodies, and incubated for 20 minutes on ice in the dark. After washing 3 times with SWB, cells were suspended in 200 μl of SWB and stored at 4°C in the dark until signals were acquired on the BD LSR II using BD FACSDiva software v5.0.2.

For equine PBMCs, the primary CD4 or CD8 antibodies were applied with a 20 minute incubation on ice in the dark, followed by blocking with goat serum (2%) for 10 minutes on ice in the dark. Anti-CD4/CD8 was detecting using a secondary Alexa Fluor® 647 antibody. Equine cells were washed and suspended as described for murine cells. A strict gating strategy was used to identify singlets and to exclude clumps of cells. Cell numbers and frequencies were determined based on data output by the BD FACSDiva software and cell counts obtained using a hemocytometer.

Statistical Analysis

Statistical analysis was performed for all of the data collected to determine significance in proliferation of T cells due to exposure of SEB relative to controls. Cell counts, concentration data, flow cytometry data or data from other aspects of the study were compiled in Microsoft Excel with average number of cells, differences, standard deviation, and percent proliferation calculated. Graphs of the number of cells vs. treatment and percent proliferation vs. treatment were created. Frequency values of CD4+ or CD8+ T cell proliferation are the percent of the parent population generated by BD FACSDiva™ v6.1 software from flow cytometric analysis. Statistical significance with $p < 0.05$ was generated by ANOVA with SigmaStat® 3.1 software.

Table 1. Horses used in study for blood sampling.

Horse # (Current Study)	Horse	Breed	Age	Sex	Medical Conditions	Treatment	Sample Date(s)
1	Kentucky Baroness	Thoroughbred Mare	15	F	N/A*	N/A***	5/13/08, 6/27/08, 7/7/08
2	Orlando	Warmblood Gelding	12	M	N/A*	N/A***	9/29/08, 12/9/08
3	Blue	Thoroughbred Gelding	13	M	Minor Lameness**	N/A***	9/29/08, 12/9/08
4	Teddy	Thoroughbred Gelding	6	M	N/A*	N/A***	9/29/08, 12/9/08
5	Oscar	Warmblood Gelding	15	M	Chronic Lameness**	N/A***	9/29/08, 11/5/08, 12/9/08
6	Tupelo	Warmblood Gelding	10	M	Chronic Lameness**	N/A***	9/29/08
7	Win-she-wrote	Thoroughbred Mare	17	F	N/A*	N/A***	11/5/08
8	Tiara	Arabian Mare	14	F	N/A*	N/A***	3/31/09, 5/19/09
9	Travis	Quarter Horse Gelding	13	M	N/A*	N/A***	3/31/09, 5/19/09
10	Davis	Oldenburg Gelding	9	M	N/A*	N/A***	5/27/09
11	Hampton	Thoroughbred Gelding	10	M	N/A*	N/A***	5/27/09

*No remarkable health issues within the last two years. Horses are ridden daily and are in good health which was confirmed prior to blood collection.

**No additional health issues within the last two years.

***Received only normal treatments including annual vaccinations, every other month de-worming and occasional sedation for teeth floating.

****Equestrian team horse blood samples were collected 1 week prior to competition or 1 week following competition to avoid results influenced by anti-inflammatory treatment.

CHAPTER 4

RESULTS

Proliferation of Murine T cells after Exposure to ConA or SEB

To establish a baseline for comparison to the equine T cells, murine splenocytes were labeled with CFSE to track cell division and measure proliferation over time after stimulation with either ConA or SEB. The splenocytes were cultured with 2 µg/ml ConA or 50 µg/ml SEB until the indicated time points (D1, D2, D3, D4) when they were stained with CD4 and CD8 antibodies for flow cytometry. The gating strategy for flow cytometric analysis is shown in Figure 1. This strategy selects only single cells to eliminate the possibility of doublets or clumps of cells that might skew results by omitting stronger fluorescence than single cells. Representative results of stimulation of murine T cells by ConA or SEB are shown in the histogram in Figure 2 with peaks indicative of proliferation increasing at the indicated time points. Marked cellular proliferation occurred with both ConA and SEB treatment relative to control over the 4 day time span. The magnitude of cellular proliferation from ConA treatment is greater than with SEB treatment.

Absolute T cell numbers and frequency of T cells were calculated based on cell counts and flow cytometry data. Absolute CD4+ and CD8+ T cell numbers of cells are presented in Figures 3 and 4, respectively. Incubation of cells with ConA resulted in an 8-fold expansion of CD4+ T cells, whereas SEB induced a 2-fold expansion of CD4+ T cells relative to negative controls (Figure 3). Incubation with ConA resulted in a 16-fold expansion of CD8+ T cells, while SEB resulted in a 3-fold expansion of these cells relative to negative controls (Figure 4).

The frequency of CD4+ T cells in the population over the four days was not changed by incubation with ConA, but decreased after SEB stimulation relative to negative controls (Figure

5). In contrast, the frequency of CD8+ T cells over the four days after ConA stimulation doubled, but this frequency did not changed after SEB stimulation relative to negative controls (Figure 6).

To determine if changes in cell numbers or frequencies were due to differences in cell viability, live versus dead cell counts were taken on days 1-4 immediately prior to antibody staining using trypan blue. A ratio of approximately 80 living to 20 dead cells was maintained throughout the study (Table 2).

Kinetics and Proliferation of Equine Peripheral Blood Mononuclear Cells after Exposure to ConA or SEB

Equine peripheral blood mononuclear cells (PBMCs) were labeled with CFSE to track cell division and measure proliferation over time after incubation with ConA or SEB. Cells were stained with antibodies for CD4+ or CD8+ T cell populations on days 1-4 post-treatment and the gating strategy used for flow cytometric analysis (Figure 7) selected only single cells. The effects of incubation with ConA or SEB on equine T cells are summarized in Figures 8-10 with peaks indicative of increasing proliferation over time. Samples from a total of eleven animals were collected over the duration of the study with details seen in Table 1.

CD4+ equine PBMCs cultured with 2 µg/ml ConA, 10 µg/ml SEB, or 50 µg/ml SEB proliferated on days 1-4 when compared to negative controls (Figure 8). Robust proliferation was observed with ConA. Of the two SEB concentrations, best proliferation was observed with 10 µg/ml SEB. Contour maps depicting the kinetics of equine CD4+ T cell proliferation are presented in Figure 9. In these maps, proliferation is evident for both ConA and SEB treated cells relative to negative controls with the lower concentration of SEB treatment resulting in the most visible proliferation (Figure 9).

Further experiments were carried out to examine the effects of even lower concentrations of SEB on equine T cells. Contour map depictions of equine CD4+ T cell proliferation with lower concentrations of SEB are presented in Figure 10. Equine PBMCs were cultured with 2 µg/ml ConA, 5 µg/ml SEB, 10 µg/ml SEB or 20 µg/ml SEB proliferated on days 2-4 when compared to negative controls (Figure 10). There is evidence of marked proliferation of both ConA and SEB treated cells relative to negative controls over the 3 days of study (days 2, 3, and 4 after treatment). The best proliferation was observed with 5 µg/ml of SEB at day 4(Figure 10). Due to the delay in the response of equine cells to SEB, data were gathered only for days 2-4 in subsequent experiments with day 1 omitted.

ConA stimulation resulted in an increase in the absolute number of CD4+ T cells whereas there was no such response to SEB at either 10 µg/ml or 20 µg/ml SEB (Figure 11). The lowest concentration of SEB (5 µg/ml) resulted in an increase in the number of CD4+ T cells over time, with the highest number observed on day 5 (Figure 11). The frequency of CD4+ T cells incubated with ConA increased nearly double whereas there was no such response to SEB (Figure 12). As occurred with the murine cells, the ratio of live to dead cells was maintained throughout the study (Table 3). Collectively, these data are in agreement with results obtained using murine cells. In both systems CD4+ T cell numbers increased over time best seen with the lowest concentration of SEB (5 µg/ml).

Both equine CD4+ and CD8+ cell proliferated significantly after exposure to ConA or SEB (Figures 13 and 14, Table 4). Optimal proliferation of CD4+ T cells occurred with 2.5 µg/ml of SEB (Figure 13) with equine T cells observed as being more sensitive to lower concentrations of SEB than murine T cells (2.5 vs. 50 µg/ml, respectively). There appears to be no effect of either ConA or SEB on the CD8+ T cells of horse 8 in Figure 14. This was not the case with horses 9 and 10 and was not duplicated again with the same horse (8) in Figure 18 which all exhibited CD8+ T cell proliferation in response to SEB treatment (Figures 14, 16, 18).

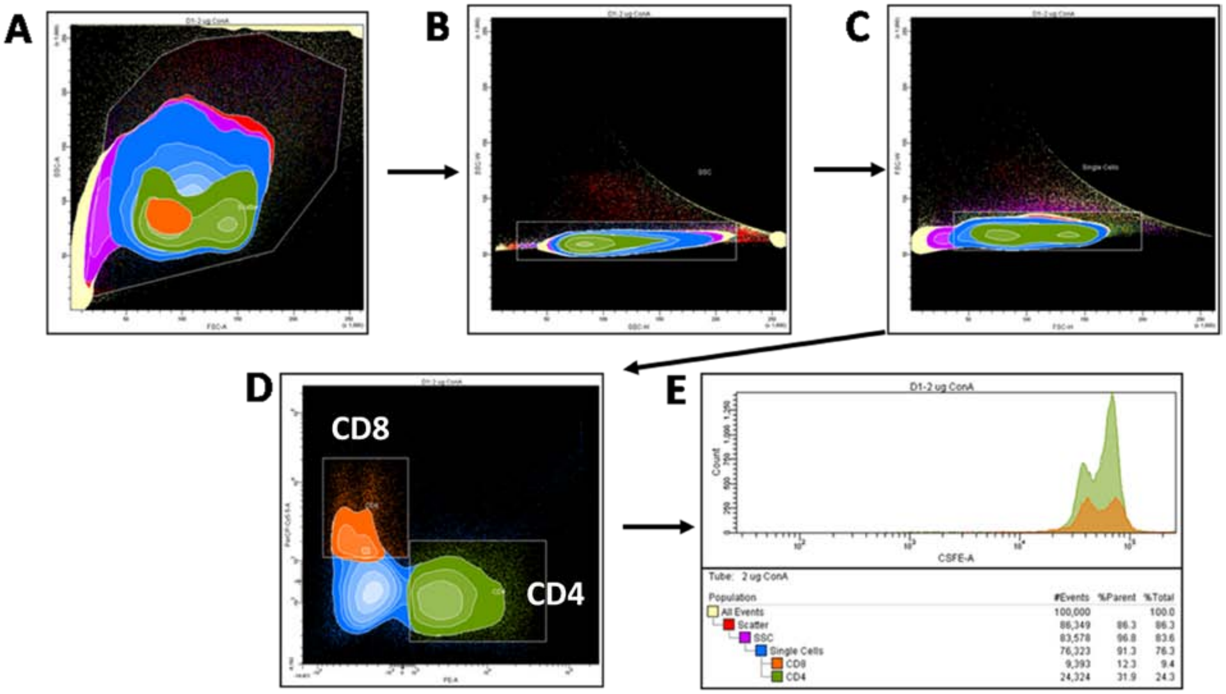
Proliferation in both equine CD4+ T cells and CD8+ T cells after exposure to ConA or SEB in multiple horses can be seen in Figures 15 and 16 and is statistically significant (Table 4). Contour map representation of the kinetics of equine CD4+ T cell proliferation via CFSE labeling in horses 10 and 8 (labelled H2 and H3 in Figure 15, respectively) reveal marked proliferation for both horses following ConA and SEB treatment relative to negative controls over a 4 day time span post-treatment with ConA or SEB. In Figure 16, contour map representation of the kinetics of equine CD8+ T cell proliferation via CFSE labeling in horses 10 and 8 (labelled H2 and H3 in Figure 15, respectively) can be seen. Unlike the response of no CD8+ T cell proliferation in Figure 14 for horse 8, significant proliferation is seen in the same horse with both ConA and SEB treatment relative to negative controls (Figure 16). This might be explained by a memory response from exposure to an SEB producing *S. aureus* strain between blood collection points, but this could not be confirmed at the time of the study and should be further investigated.

In Figures 13 and 14, we measured which T cells were proliferating in the population using a CD4 or CD8 antibody. When comparing data from the same animal, proliferation among the negative population (shown in blue) in Figure 14 more than likely corresponds to the CD4+ T cell population in Figure 13 and vice versa with the negative population in Figure 13 corresponding to the CD8+ T cell population in Figure 14. It is also important to note the variability in response seen among the animals, especially in CD8+ T cells proliferative response. This is not surprising because we examined a highly outbred population of horses (see Table 1).

Effects of Blocking MHC Class II on Proliferation of Equine CD4+ and CD8+ T Cells Stimulated with ConA or SEB

To investigate whether or not SEB binding the MHC class II molecule is required for T cell proliferation, equine PBMCs were cultured with either 2 μg/ml ConA or 2.5 μg/ml SEB in the presence or absence of anti-MHC class II antibody or isotype control until the indicated time points (D2, D3, D4) when they were stained with a CD4 or CD8 antibody for flow cytometric analysis. As previously observed, marked proliferation is seen with both ConA and SEB treatment relative to negative controls over a 4 day time span. In this initial experiment, the presence of the anti-MHC class II antibody dramatically reduced proliferation of T cells in response SEB relative to isotype control (Figures 17-18).

Initial findings supported results obtained in other species and suggest that MHC class II binding is required for T cell proliferation. However, due to the outbred nature of horses, it was unclear if these data were unique to the animal (horses 8 and 9 in Figures 17-18). To further examine if MHC class II molecule binding is consistently required to induce equine T cell proliferation, multiple horse samples were examined. As previously observed, marked proliferation is seen with both ConA and SEB treatment relative to negative controls in addition to statistically significant (Table 5) reduction of proliferation via MHC Class II antibody treatment relative to isotype control in horses 8 and 9 (H2 and H3 in Figure 19, respectively) and in horses 10 and 11 (H4 and H5 in Figure 20, respectively).



Tube: 2 ug ConA

Population	#Events	%Parent	%Total
All Events	100,000		100.0
Scatter	86,349	86.3	86.3
SSC	83,578	96.8	83.6
Single Cells	76,323	91.3	76.3
CD8	9,393	12.3	9.4
CD4	24,324	31.9	24.3

Figure 1. Murine model gating strategy. CD4+ and CD8+ T cell populations among splenocytes were selected based on prior gating on singlets to rule out the possibility of excessive fluorescence omitted by doublets or clumps of cells to ignore dead cell debris. Figure 1a represents all murine splenocytes, Figure 1b and 1c show selection of singlets based on SSC-W versus SSC-H and FSC-W versus FSC-H gating, Figure 1d shows isolation of a representative CD4+ T cell population in green and CD8+ T cell population in orange, and Figure 1e is a histogram representation of both CD4+ and CD8+ population with corresponding population frequency data below generated by BD FACSDiva™ v6.1 software.

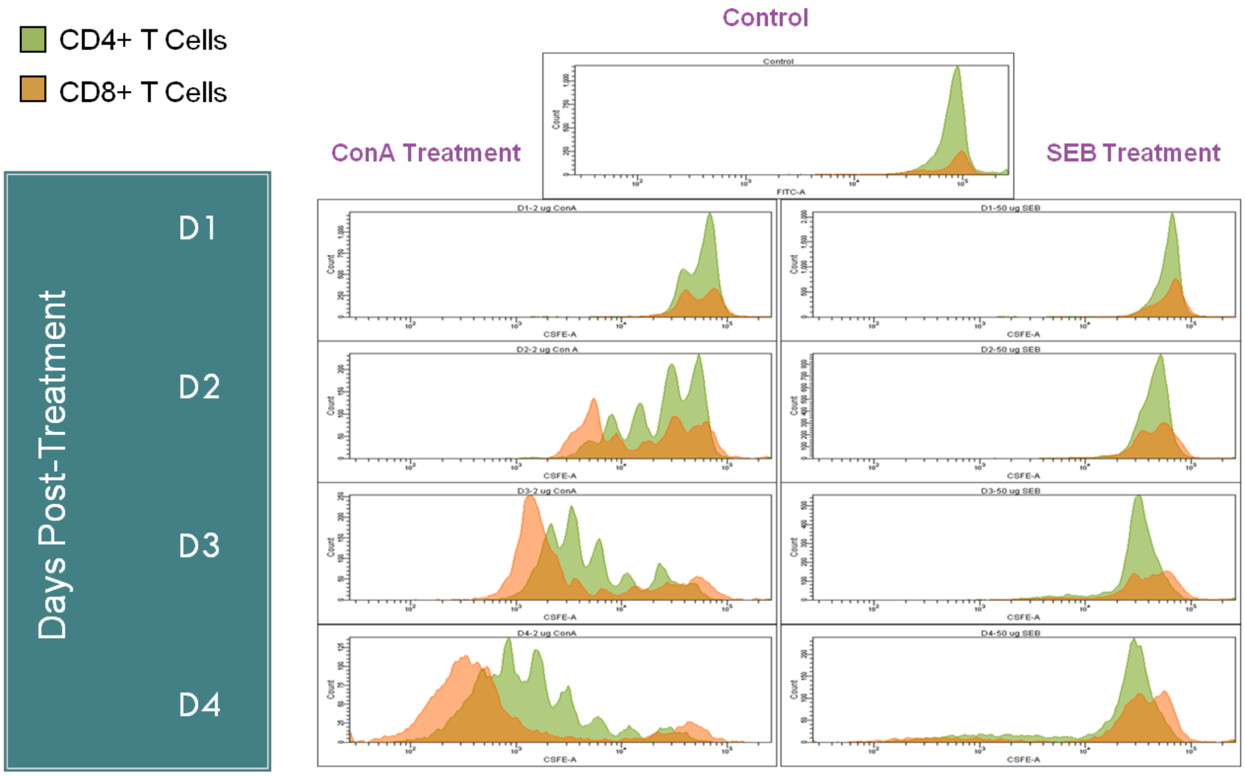


Figure 2. Proliferation of murine T cells after exposure to ConA or SEB. Histogram representation of the kinetics of CD4+ and CD8+ T cell proliferation via CFSE labeling and peaks indicative of cell division. Mouse splenocytes were cultured with 2 µg/ml ConA or 50 µg/ml SEB until the indicated time points (D1, D2, D3, and D4) when they were stained with CD4 and CD8 antibodies for flow cytometry. Data are shown using Figure 1 gating strategy. Marked proliferation is seen with both ConA and SEB treatment relative to negative controls for both CD4+ (shown in green) and CD8+ (shown in orange) T cell populations over a 4 day time span post-treatment.

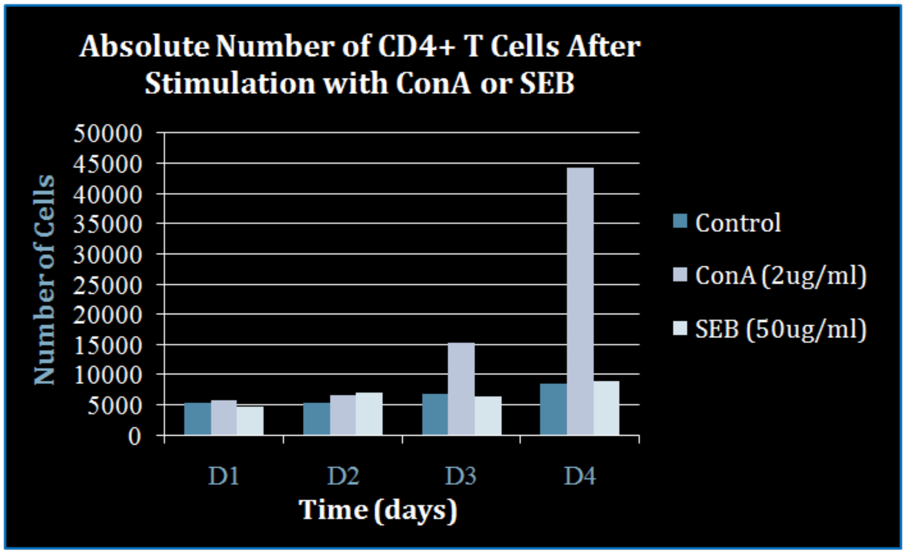


Figure 3. Absolute number of murine CD4+ T cells after exposure to ConA or SEB. CD4+ T cell numbers among splenocytes were recorded in triplicate over a 4 day time span (D1, D2, D3, and D4) post-treatment with SEB or ConA. ConA stimulation resulted in an 8-fold expansion of CD4+ T cells in contrast to SEB stimulation which resulted in a 2-fold expansion of CD4+ T cells relative to negative controls. Absolute numbers were calculated using hemocytometer data, live cell gating and frequencies determined by CD4+ and CD8+ T cell gating strategy.

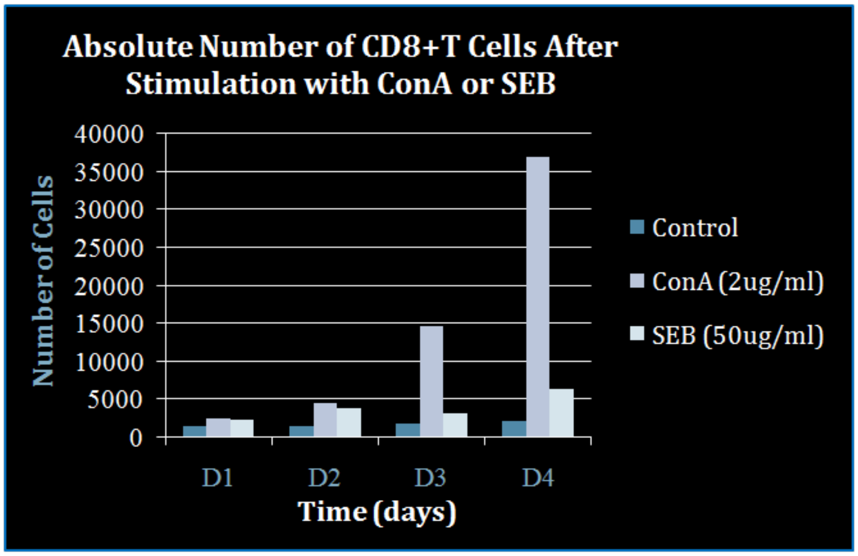


Figure 4. Absolute number of murine CD8+ T cells after exposure to ConA or SEB. CD8+ T cell numbers among splenocytes were recorded in triplicate over a 4 day time span (D1, D2, D3, and D4) post-treatment with SEB or ConA. ConA stimulation resulted in a 16-fold expansion of CD8+ T cells in contrast to SEB stimulation which resulted in a 3-fold expansion of CD8+ T cells relative to negative controls. Absolute numbers were calculated using hemocytometer data, live cell gating and frequencies determined by CD4+ and CD8+ T cell gating strategy.

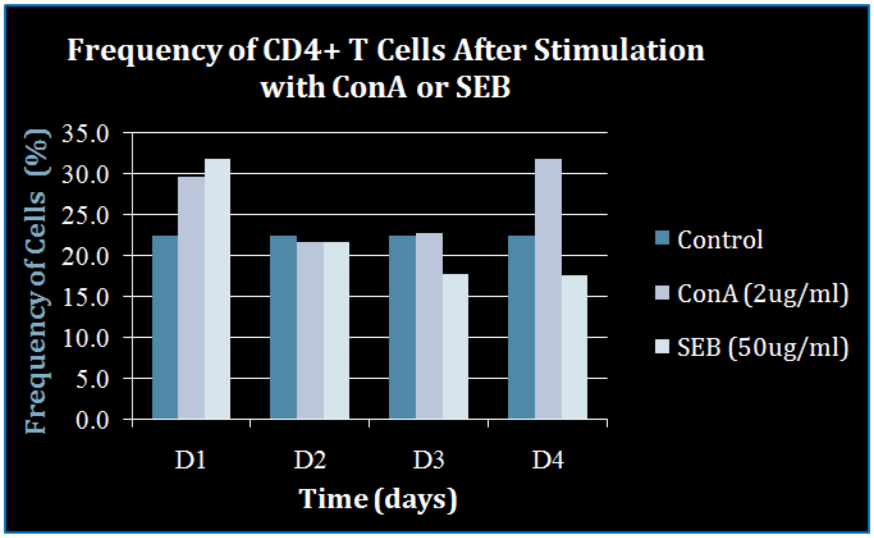


Figure 5. Frequency of murine CD4+ T cells after exposure to ConA or SEB. CD4+ T cell frequency among splenocytes was recorded in triplicate over a 4 day time span (D1, D2, D3, and D4) post-treatment with SEB or ConA. Frequency of CD4+ T cells following ConA stimulation did not show marked change over time, but CD4+ T cell frequency decreased following SEB stimulation relative to negative controls. Frequency values represent the percent of the parent population and were obtained using BD FACSDiva™ v6.1 software.

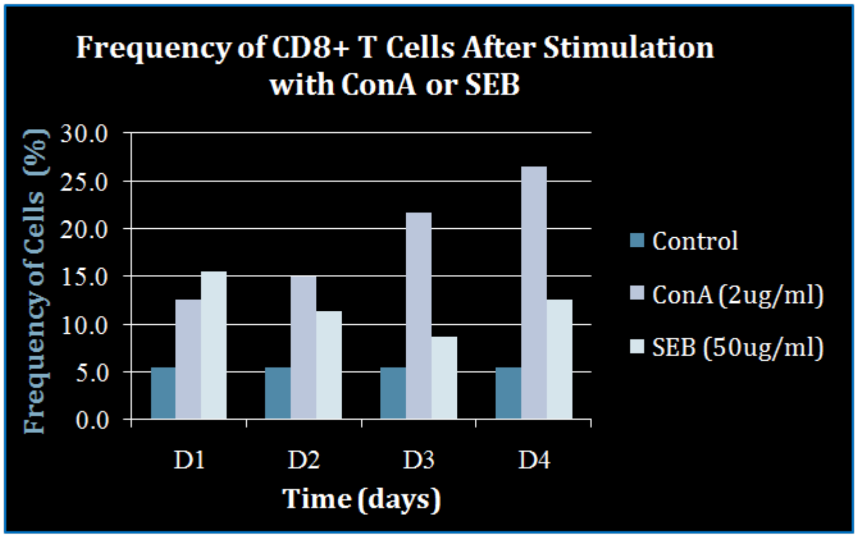
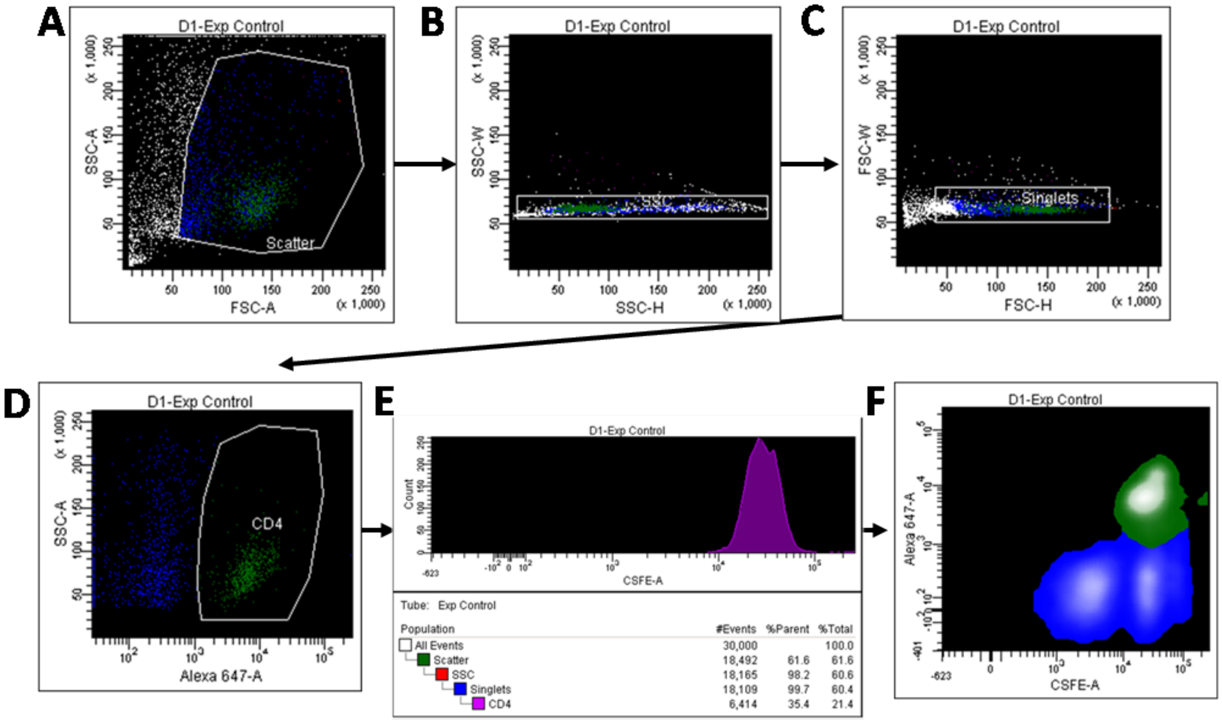


Figure 6. Frequency of murine CD8+ T cells after exposure to ConA or SEB. CD8+ T cell frequency among splenocytes was recorded in triplicate over a 4 day time span (D1, D2, D3, and D4) post-treatment with SEB or ConA. Frequency of CD8+ T cells following ConA stimulation doubled over time, but CD 8+ T cell frequency stayed approximately the same following SEB stimulation relative to negative controls. Frequency values represent the percent of the parent population and were obtained using BD FACSDiva™ v6.1 software

Table 2. Average of live vs. dead murine cells after stimulation with ConA or SEB

	Control	ConA (2ug/ml)	SEB (50ug/ml)
D1 Live/Dead	16/1	13/1	10/1
D2 Live/Dead	16/3	20/3	22/3
D3 Live/Dead	20/7	45/5	24/4
D4 Live/Dead	25/6	93/24	34/10

Note: Numbers obtained from hemacytometer cell counts.



Tube: Exp Control

Population	#Events	%Parent	%Total
☐ All Events	30,000		100.0
🟩 Scatter	18,492	61.6	61.6
🟥 SSC	18,165	98.2	60.6
🟦 Singlets	18,109	99.7	60.4
🟪 CD4	6,414	35.4	21.4

Figure 7. Equine model gating strategy. CD4+ and CD8+ T cell populations among lymphocytes were selected based on prior gating on singlets to rule out the possibility of excessive fluorescence omitted by doublets or clumps of cells to ignore dead cell debris. Figure 7a represents all equine lymphocytes, Figure 7b and 7c show selection of singlets based on SSC-W versus SSC-H and FSC-W versus FSC-H gating, Figure 7d shows isolation of a representative CD4+ T cell population, Figure 7e is a histogram representation of the CD4+ population with corresponding population frequency data below generated by BD FACSDiva™ v6.1 software, and Figure 7f is a contour map display of the same CD4+ population shown in Figures 7d and 7e with the positive population shown in green. Data shown are from Horse 1 (Table 1).

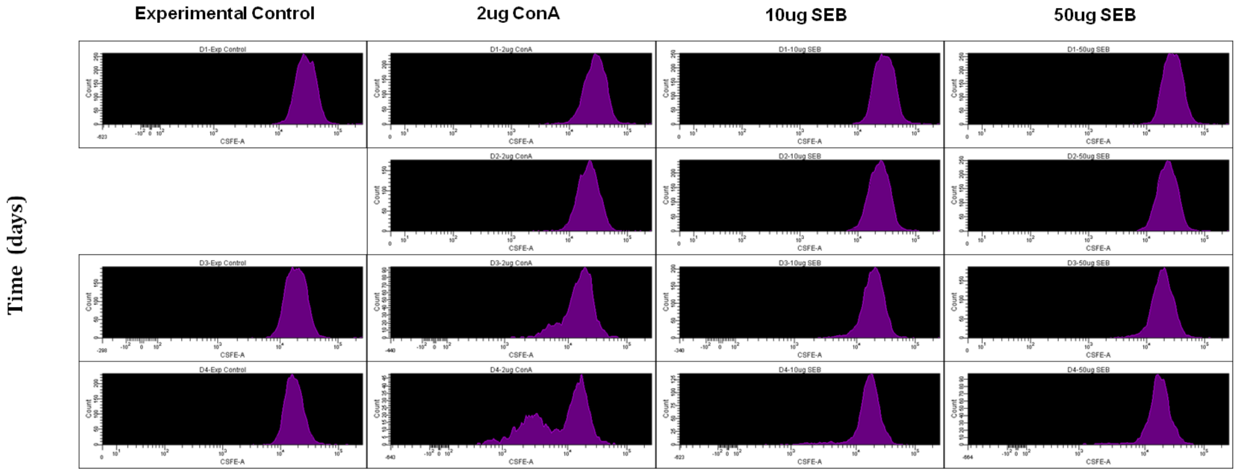


Figure 8. Proliferation of equine CD4+ T cells after exposure to ConA or SEB. Histogram representation of the kinetics of equine CD4+ T cell proliferation via CFSE labeling. Equine PBMCs were cultured with 2 μg/ml ConA, 10 μg/ml SEB, or 50 μg/ml SEB until the indicated time points (D1, D2, D3, D4) when they were stained with a CD4 antibody for flow cytometry. Marked proliferation is seen with both ConA and SEB treatment relative to negative controls over a 4 day time span post-treatment with ConA or SEB with best proliferation observed using 10 μg/ml for SEB. Data shown are from Horse 1 (Table 1).

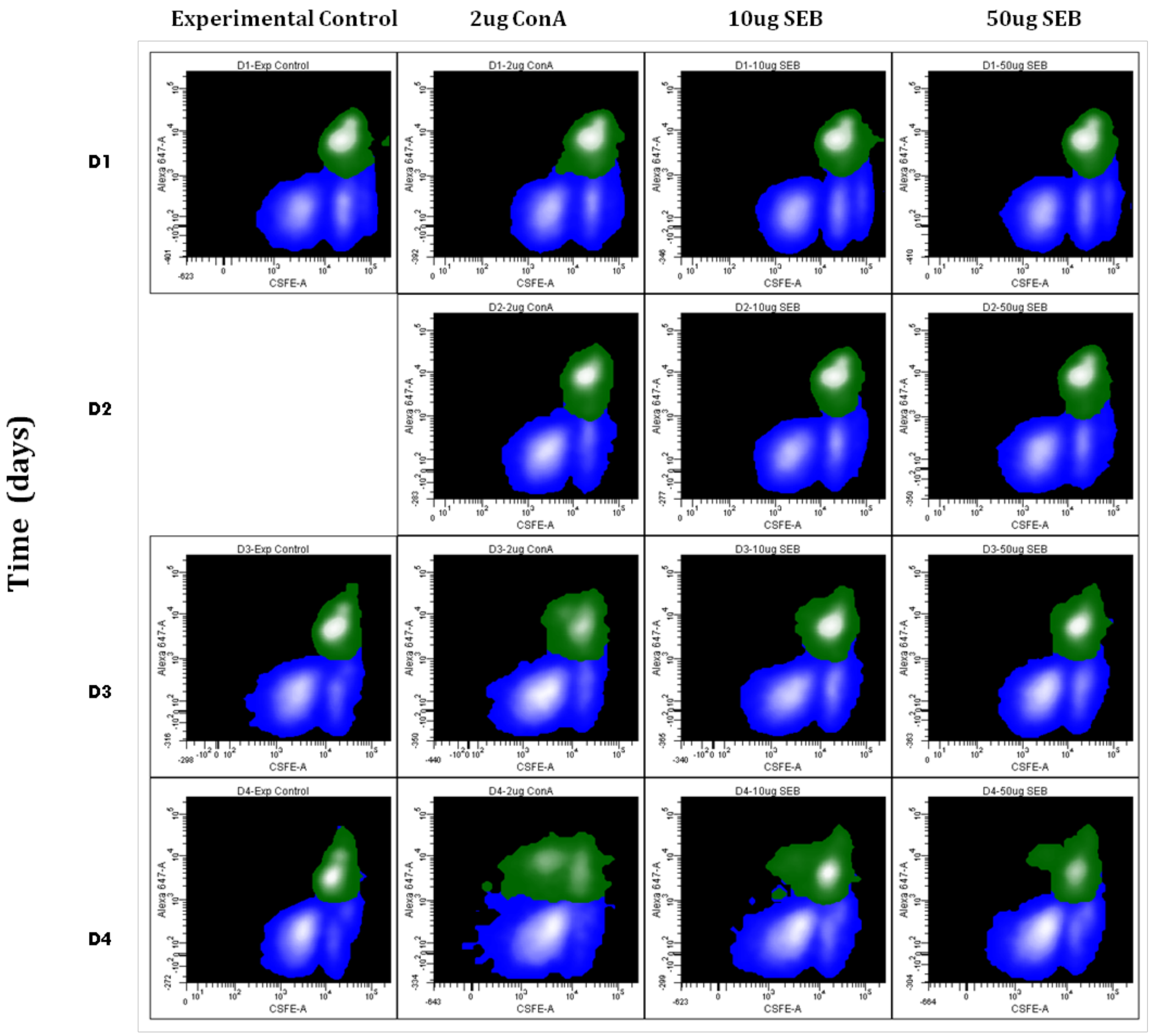


Figure 9. Proliferation of equine CD4+ T cells after exposure to ConA or SEB. Contour map representation of the kinetics of equine CD4+ T cell proliferation (shown in green) via CFSE labeling. Equine PBMCs were cultured with 2 µg/ml ConA, 10 µg/ml SEB, or 50 µg/ml SEB until the indicated time points (D1, D2, D3, D4) when they were stained with a CD4 antibody for flow cytometry. Marked proliferation is seen with both ConA and SEB treatment relative to negative controls over a 4 day time span post-treatment with ConA or SEB with best proliferation observed using 10 µg/ml for SEB. Day 2 experimental control samples could not be collected due to

insufficient cell numbers. Note: Data in this figure correspond to Figure 8. Data shown are from Horse 1 (Table 1).

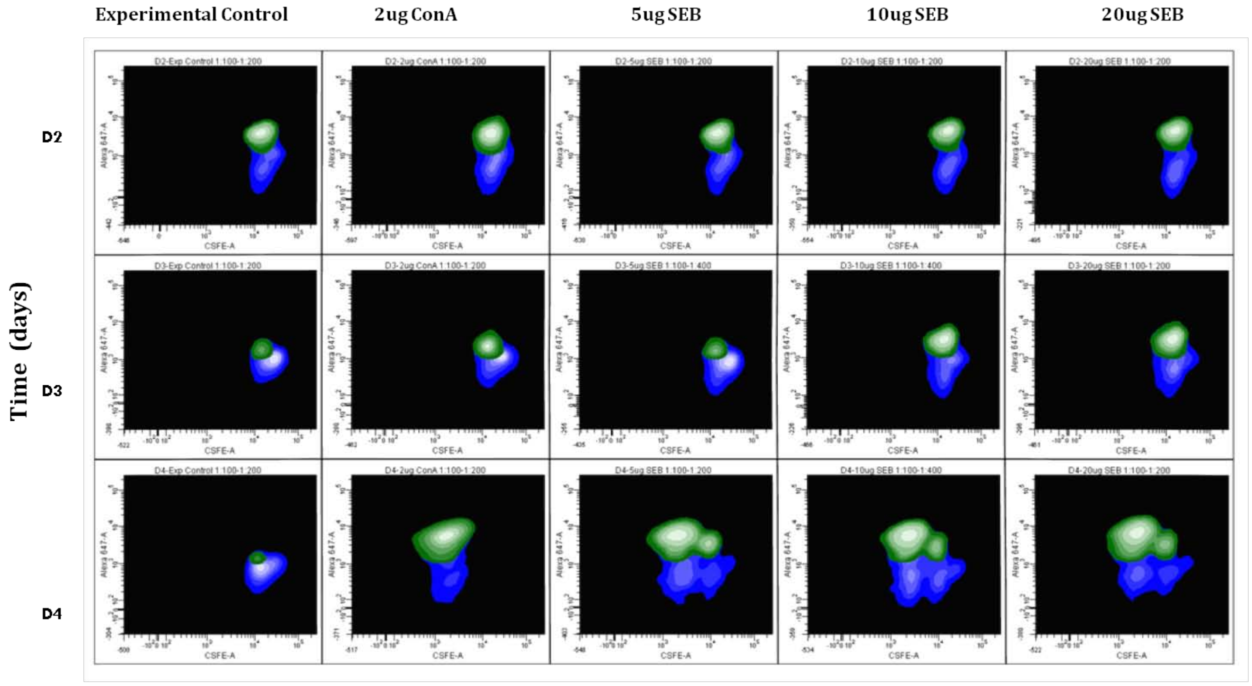


Figure 10. Proliferation of equine CD4+ T cells after exposure to ConA or SEB. Contour map representation of the kinetics of equine CD4+ T cell proliferation (shown in green) via CFSE labeling. Equine PBMCs were cultured with 2 µg/ml ConA, 5 µg/ml SEB, 10 µg/ml SEB, or 20 µg/ml SEB until the indicated time points (D2, D3, D4) when they were stained with a CD4 antibody for flow cytometry. Marked proliferation is seen with both ConA and SEB treatment relative to negative controls over a 4 day time span post-treatment with ConA or SEB. Highest proliferation was observed using 5 µg/ml for SEB. Day 1 was omitted due to slow response of equine T cells to SEB. Data shown are from Horse 1 (Table 1).

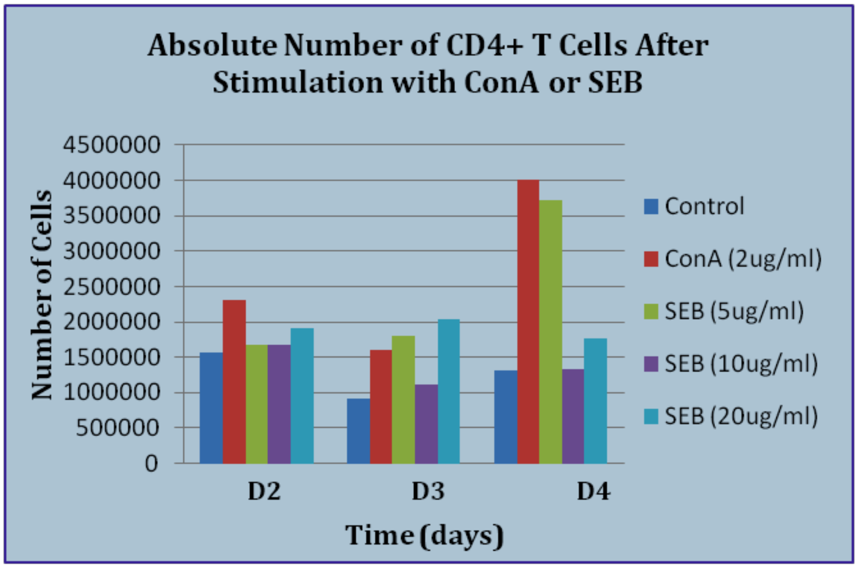


Figure 11. Absolute number of equine CD4+ T cells after exposure to ConA or SEB. Equine CD4+ T cell numbers were recorded at the indicated days post-treatment with SEB or ConA (D2, D3, D4). ConA stimulation resulted in an increase of CD4+ T cell numbers. This is in contrast to SEB stimulation, which resulted in no change of CD4+ T cell numbers at 10 µg/ml and 20 µg/ml of SEB relative to negative controls. The lowest concentration of SEB (5 µg/ml) reveals a trend similar to the mouse model with CD4+ T cell numbers increasing over time. Note: Data in this figure correspond to Figure 10. Data shown are from Horse 1 (Table 1).

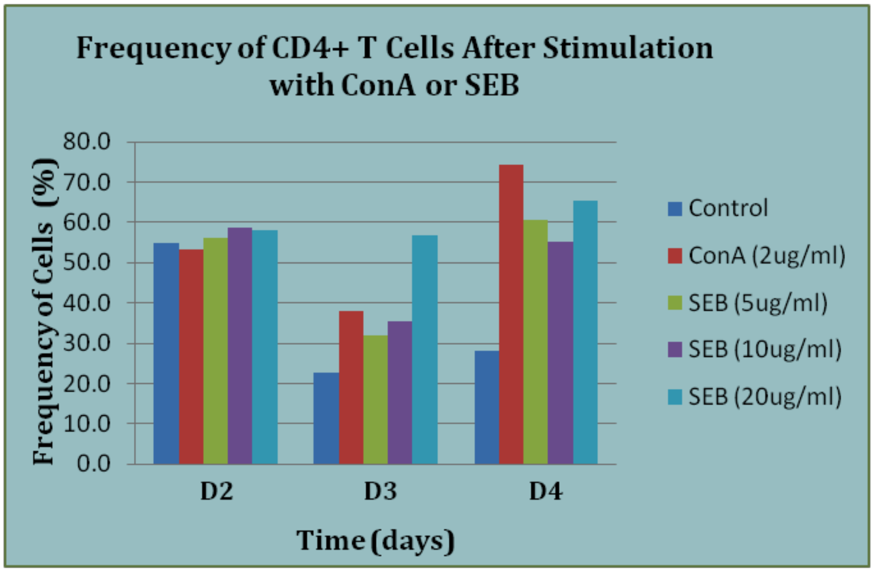


Figure 12. Frequency of equine CD4+ T cells after exposure to ConA or SEB. Equine CD4+ T cell numbers were recorded at the indicated days post-treatment with SEB or ConA (D2, D3, D4). Frequency of CD4+ T cells with ConA doubled while frequency stayed approximately the same with SEB relative to negative controls. Note: Data in this figure correspond to Figure 10. Data shown are from Horse 1 (Table 1).

Table 3. Average of live vs. dead equine cells after stimulation with ConA or SEB

	Control	ConA (2ug/ml)	SEB (5ug/ml)	SEB (10ug/ml)	SEB (20ug/ml)	SEB (50ug/ml)
D2 Live/Dead	19/4	29/4	20/2	19/7	22/3	29/7
D3 Live/Dead	27/4	28/6	31/7	21/12	24/8	20/9
D4 Live/Dead	31/6	36/6	41/10	16/9	18/10	17/9

Note: Data correspond to Figures 10-12 and are from Horse 1 (Table 1).

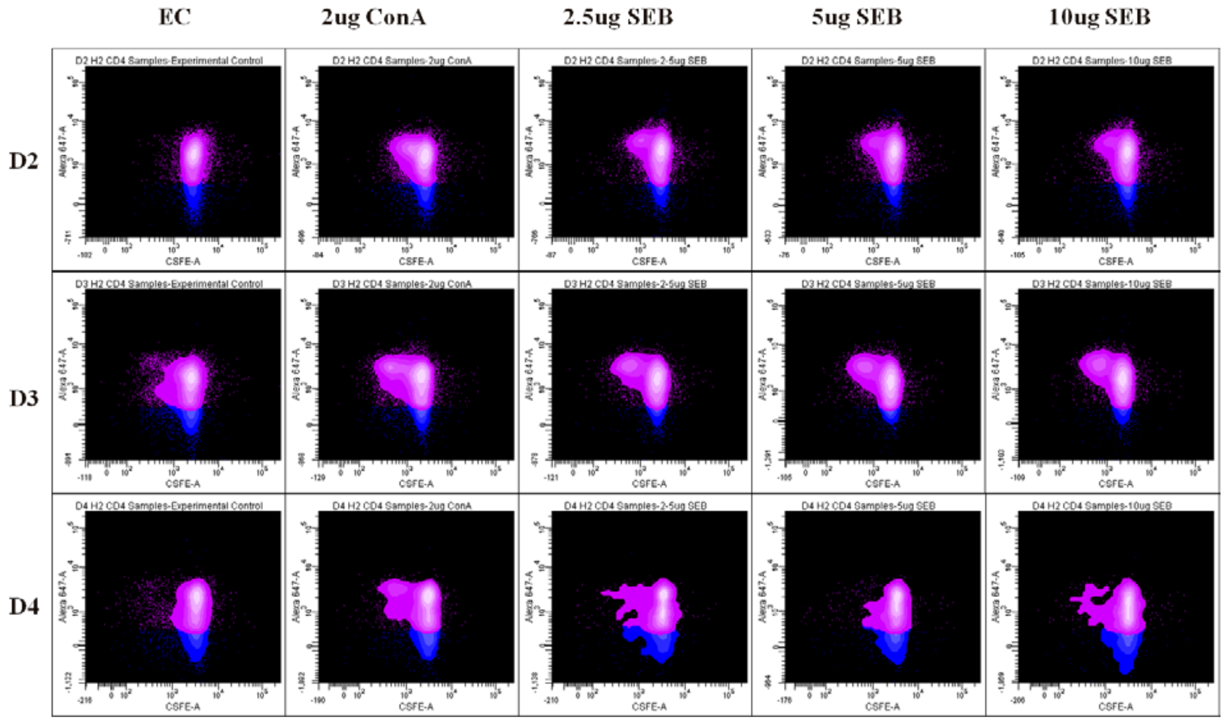


Figure 13. Proliferation of equine CD4+ T cells after exposure to ConA or SEB. Contour map representation of the kinetics of equine CD4+ T cell proliferation (shown in pink) via CFSE labeling. Equine PBMCs were cultured with 2 µg/ml ConA, 2.5 µg/ml SEB, 5 µg/ml SEB, or 10 µg/ml SEB until the indicated time points (D2, D3, D4) when they were stained with a CD4 antibody for flow cytometry. Marked proliferation is seen with both ConA and SEB treatment relative to negative controls over a 4 day time span post-treatment. Highest proliferation was observed using 2.5 µg/ml for SEB in CD4+ T cells. Data shown are from Horse 8 (Table 1).

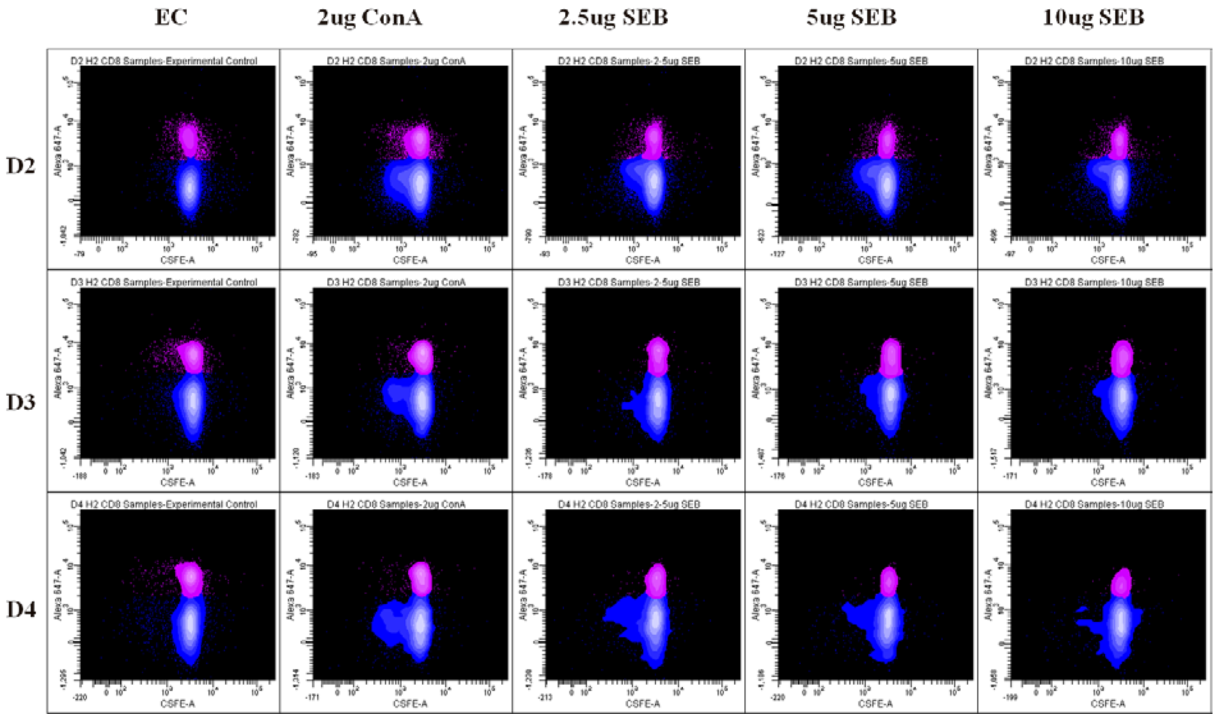


Figure 14. Proliferation of equine CD8+ T cells after exposure to ConA or SEB. Contour map representation of the kinetics of equine CD8+ T cell proliferation (shown in pink) via CFSE labeling. Equine PBMCs were cultured with 2 μg/ml ConA, 2.5 μg/ml SEB, 5 μg/ml SEB, or 10 μg/ml SEB until the indicated time points (D2, D3, D4) when they were stained with a CD8 antibody for flow cytometry. Proliferation of CD8+ T cells from this animal in this experiment appears to remain unchanged with both ConA and SEB treatment relative to negative controls. Data shown are from Horse 8 (Table 1).

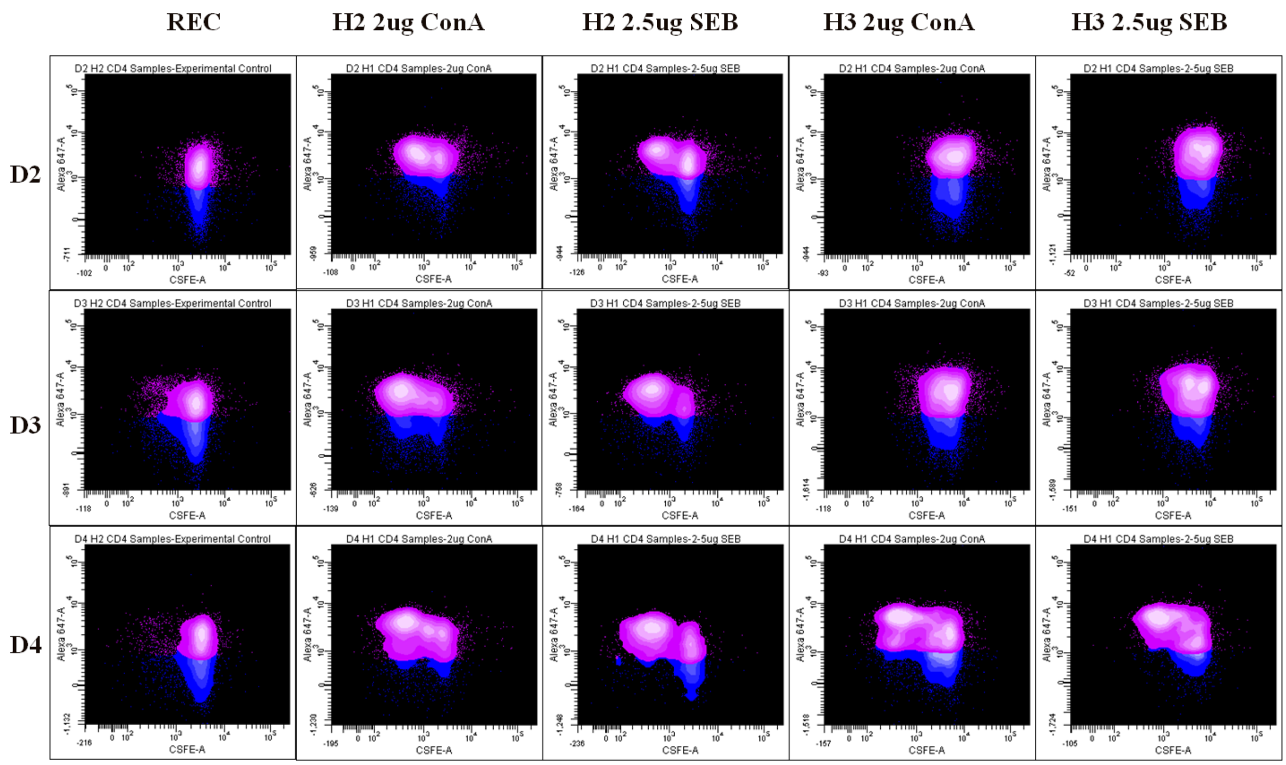


Figure 15. Proliferation of equine CD4+ T cells after exposure to ConA or SEB in multiple horses. Contour map representation of the kinetics of equine CD4+ T cell proliferation via CFSE labeling in horses 10 and 8 (H2 and H3 in figure, respectively). Equine PBMCs were cultured with 2 μg/ml ConA or 2.5 μg/ml SEB until the indicated time points (D2, D3, D4) when they were stained with a CD4 antibody for flow cytometry. Marked CD4+ T cell proliferation is seen in both horses with both ConA and SEB treatment relative to negative controls over a 4 day time span post-treatment.

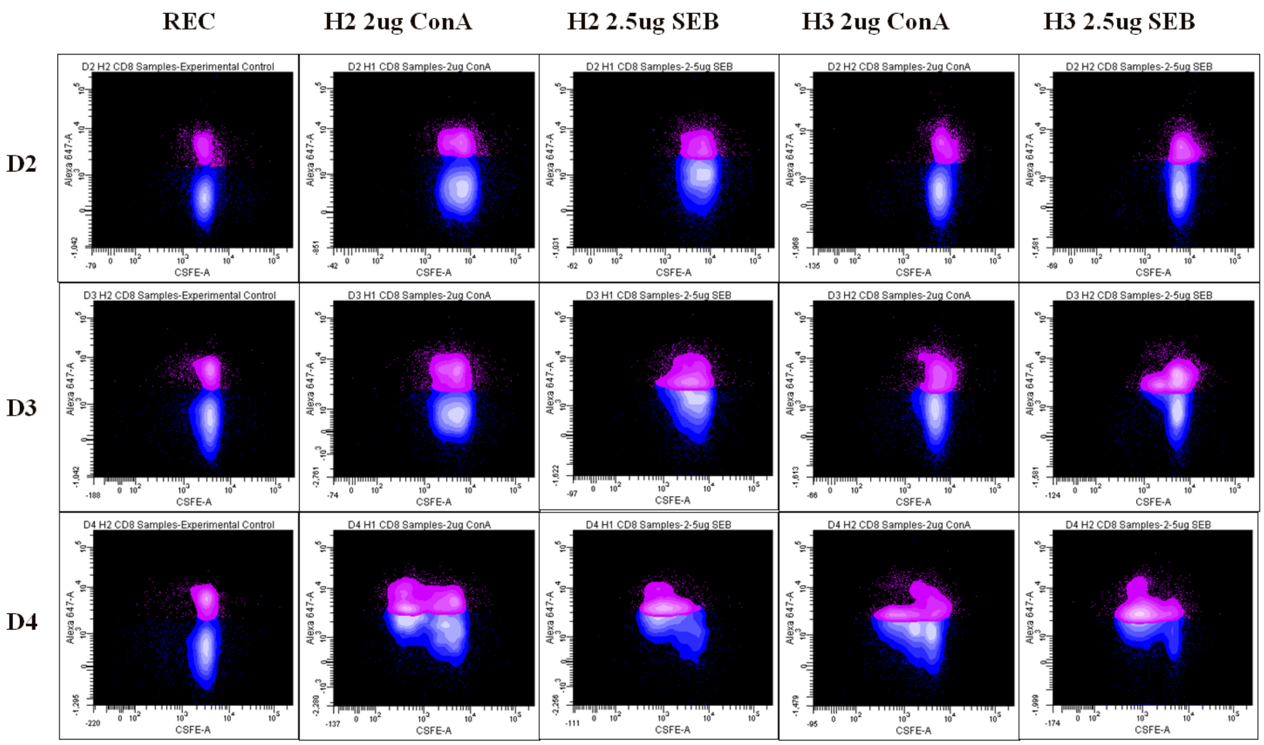


Figure 16. Proliferation of equine CD8+ T cells after exposure to ConA or SEB in multiple horses. Contour map representation of the kinetics of equine CD8+ T cell proliferation via CFSE labeling in horses 10 and 8 (H2 and H3 in figure, respectively). Equine PBMCs were cultured with 2 µg/ml ConA or 2.5 µg/ml SEB until the indicated time points (D2, D3, D4) when they were stained with a CD8 antibody for flow cytometry. Marked CD8+ T cell proliferation is seen in both horses with both ConA and SEB treatment relative to negative controls.

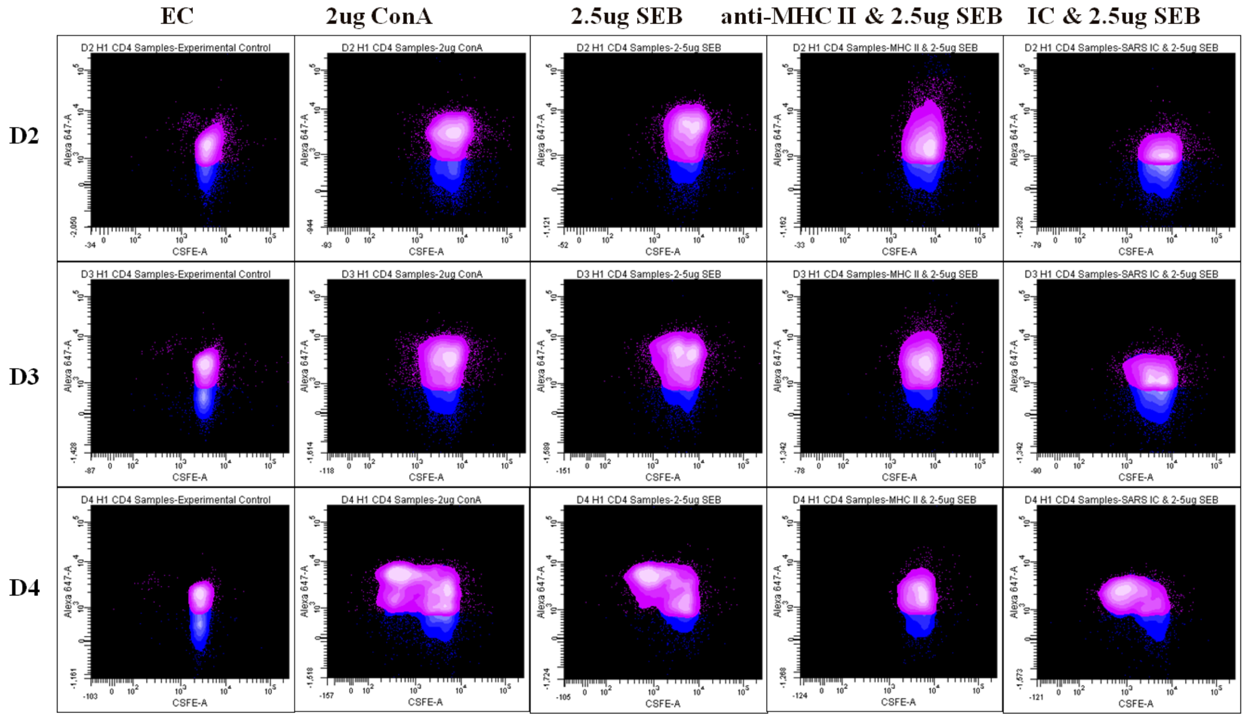


Figure 17. Proliferation of equine CD4+ T cells after exposure to ConA or SEB in the presence of an anti-equine MHC class II antibody. Contour map representation of the kinetics of equine CD4+ T cell proliferation via CFSE labeling. Equine PBMCs were cultured with either 2 µg/ml ConA or 2.5 µg/ml SEB. Where indicated, cells were pre-incubated with either anti-MHC class II antibody or isotype control (IC) for 1 hr prior to the addition of 2.5 µg/ml SEB. At the indicated time points (D2, D3, D4), cells were stained with an anti-CD4 antibody and/or Alexa Fluor 647 antibody for flow cytometry. Marked proliferation is seen with both ConA and SEB treatment relative to negative controls over a 4 day time span post-treatment. A reduction in proliferation significant reduction of proliferation was seen in cultures treated with MHC class II antibody when compared to isotype control. Data shown are from Horse 8 (Table 1).

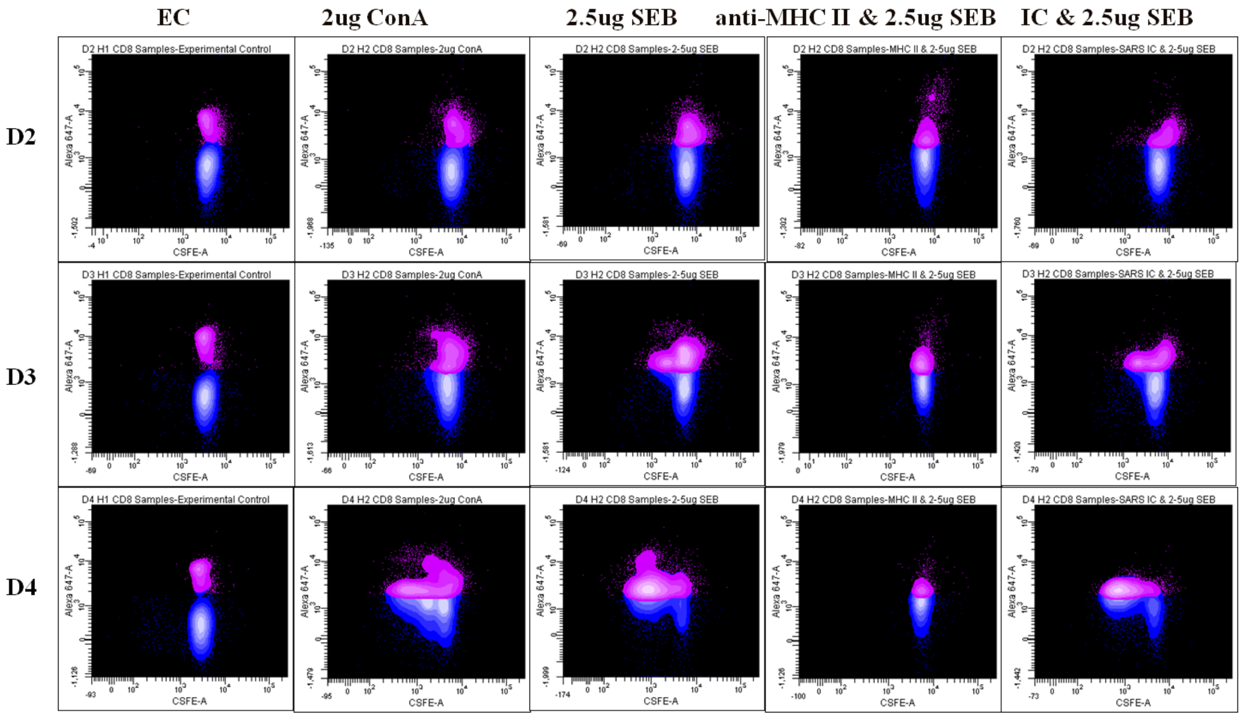


Figure 18. Proliferation of equine CD8+ T cells after exposure to ConA or SEB in the presence of an anti-MHC class II antibody. Contour map representation of the kinetics of equine CD8+ T cell proliferation via CFSE labeling. Equine PBMCs were cultured with either 2 µg/ml ConA or 2.5 µg/ml SEB. Where indicated, cells were pre-incubated with either anti-MHC class II antibody or isotype control (IC) for 1 hr prior to the addition of 2.5 µg/ml SEB. At the indicated time points (D2, D3, D4), cells were stained with an anti-CD8 antibody and/or Alexa Fluor 647 antibody for flow cytometry. Marked proliferation is seen with both ConA and SEB treatment relative to negative controls over a 4 day time span post-treatment. A reduction in proliferation significant reduction of proliferation was seen in cultures treated with MHC class II antibody when compared to isotype control. Data shown are from Horse 9 (Table 1).

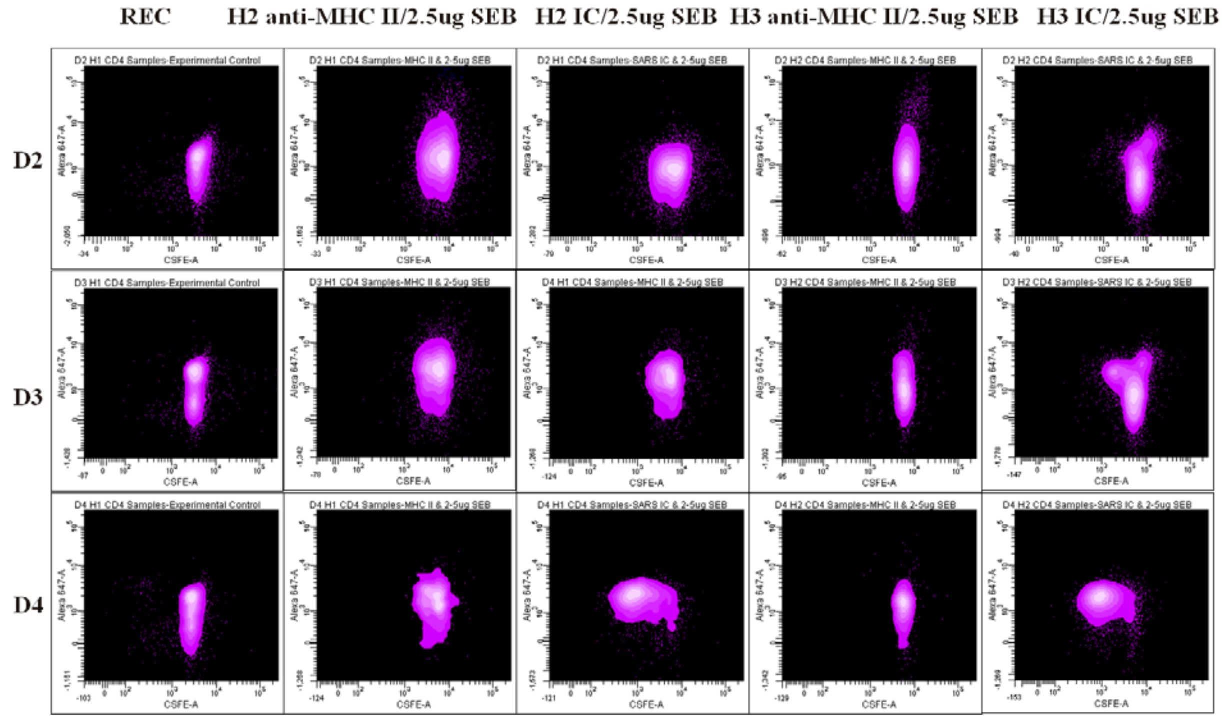


Figure 19. Proliferation of equine T cells from multiple animals after exposure to SEB in the presence of MHC class II-specific-antibodies. Contour map representation of the kinetics of equine T cell proliferation via CFSE labeling in horses 8 and 9 (H2 and H3 in figure, respectively) with gating on lymphocytes. Equine PBMCs were cultured with anti-MHC class II antibody or isotype control (IC) for 1 hr and then 2.5 µg/ml SEB until the indicated time points (D2, D3, D4) when they were stained with Alexa Fluor 647 antibody for flow cytometry. Marked proliferation is seen in both horses with SEB treatment relative to negative (representative, REC) controls over a 4 day time span post-treatment with significant reduction of proliferation via MHC class II antibody relative to isotype control.

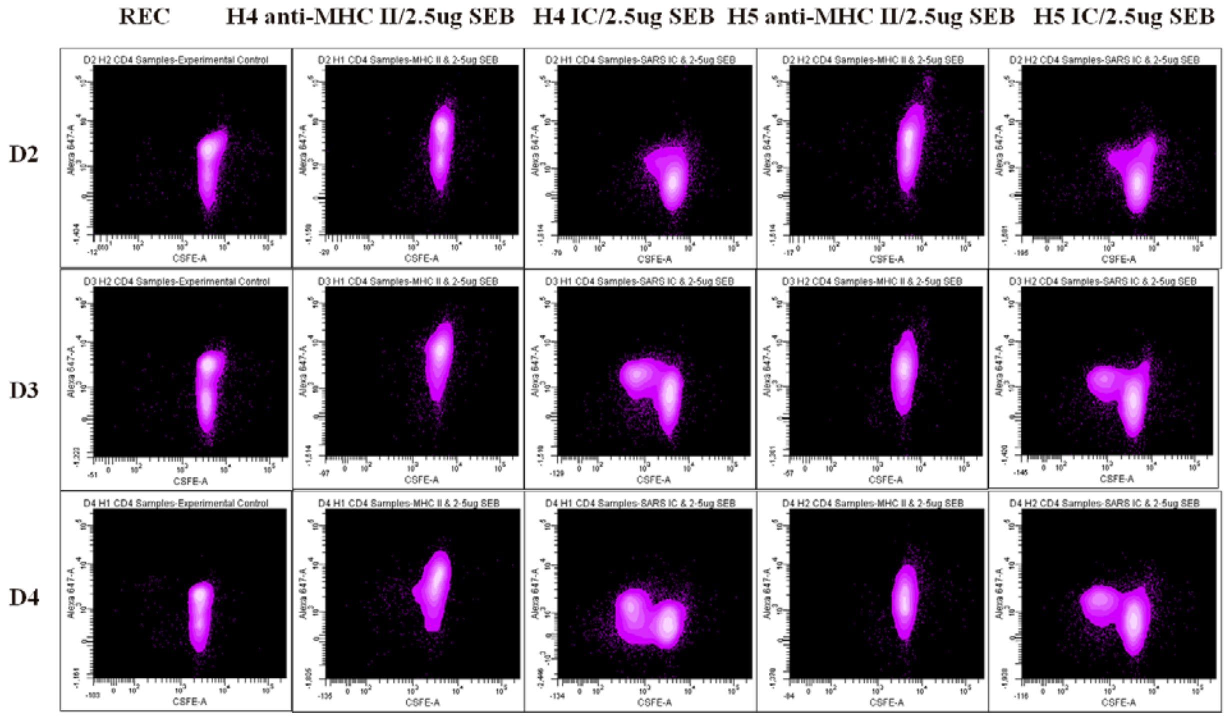


Figure 20. Proliferation of equine T cells from multiple animals after exposure to SEB in the presence of an equine-specific MHC class II antibody. Contour map representation of the kinetics of equine T cell proliferation via CFSE labeling in horses 10 and 11 (H4 and H5 in figure, respectively) with gating on lymphocytes. Equine PBMCs were cultured with anti-MHC class II antibody or isotype control (IC) for 1 hr and then 2.5 μg/ml SEB until the indicated time points (D2, D3, D4) when they were stained with Alexa Fluor 647 antibody for flow cytometry. Marked proliferation is seen in both horses with SEB treatment relative to negative (representative, REC) controls over a 4 day time span post-treatment with significant reduction of proliferation via MHC class II antibody relative to isotype control.

Table 4. Equine CD4+ and CD8+ T cell frequency after exposure to ConA or SEB in multiple animals

	Horse #8 CD4+			Horse #8 CD8+		
Time	**EC**	**2ug ConA**	**2.5ug SEB**	**EC**	**2ug ConA**	**2.5ug SEB**
D2	54.8	61.2**	70.3***	29.0	25.1*	29.0
D3	58.4	65.0**	72.6***	40.3	35.1**	37.6*
D4	72.2	73.4*	84.8***	39.2	38.1*	37.1*
	Horse #9 CD4+			**Horse #9 CD8+**		
Time	**EC**	**2ug ConA**	**2.5ug SEB**	**EC**	**2ug ConA**	**2.5ug SEB**
D2	77.4	78.9	78.4	26.3	23.0*	29.5*
D3	82.7	82.0	84.3*	29.9	29.9	43.7**
D4	81.5	86.1*	90.5**	34.0	18.1***	28.0**
	Horse #10 CD4+			**Horse #10 CD8+**		
Time	**EC**	**2ug ConA**	**2.5ug SEB**	**EC**	**2ug ConA**	**2.5ug SEB**
D2	64.5	83.0***	76.7***	29.3	33.5**	36.2**
D3	70.6	85.5***	85.2***	32.9	35.5*	39.5***
D4	76.3	73.5*	81.5**	34.9	36.1*	40.5***
	Horse #11 CD4+			**Horse #11 CD8+**		
Time	**EC**	**2ug ConA**	**2.5ug SEB**	**EC**	**2ug ConA**	**2.5ug SEB**
D2	67.7	78.3***	71.2**	46.4	52.6**	51.1*
D3	66.6	74.8**	77.9***	47.7	37.9***	57.2***
D4	68.3	71.5*	77***	43.2	35.1***	44.6

Note: All values represent the percent of CD4+ or CD8+ T cell frequency of the parent population generated by BD FACSDiva™ v6.1 software from flow cytometric analysis.
*Values are statistically significant with $p < 0.05$ generated by ANOVA with SigmaStat® 3.1 software
**Values are statistically significant with $p < 0.0001$ generated by ANOVA with SigmaStat® 3.1 software
***Values are statistically significant with $p < 0.00005$ generated by ANOVA with SigmaStat® 3.1 software

Table 5. Equine CD4+ and CD8+ frequency and MHC class II antibody blocking in multiple animals after exposure to ConA or SEB

	Horse #8 Total Lymphocytes		
Time	**2.5ug SEB**	**anti-MHC II Ab**	**Isotype Control**
D2	30.4	22.2*	29.6
D3	25.7	14.2**	26.7
D4	10.6	3.0*	9.7
	Horse #9 Total Lymphocytes		
Time	**2.5ug SEB**	**anti-MHC II Ab**	**Isotype Control**
D2	35.3	18.4**	34.8
D3	21.5	9.1**	22.5
D4	24.2	4.7**	21.3
	Horse #10 Total Lymphocytes		
Time	**2.5ug SEB**	**anti-MHC II Ab**	**Isotype Control**
D2	31.8	23.4*	30.3
D3	28.9	19.1**	30.3
D4	25.1	13.4**	24.2
	Horse #11 Total Lymphocytes		
Time	**2.5ug SEB**	**anti-MHC II Ab**	**Isotype Control**
D2	38.4	17.1**	35.7
D3	27.8	7.4**	25.8
D4	19.4	4.0**	18.5

Note: All values represent the percent frequency of total lymphocytes generated by BD FACSDiva™ v6.1 software from flow cytometric analysis.

*Values are statistically significant with $p < 0.005$ generated by ANOVA with SigmaStat® 3.1 software

**Values are statistically significant with $p < 0.00005$ generated by ANOVA with SigmaStat® 3.1 software

CHAPTER 5

DISCUSSION

For the duration of the study, we chose to use cell counts via hemocytometer to determine cell viability and CFSE to track cell division. CFSE labels microtubules and assumes equal dispersion of microtubules, therefore the fluorescence expression is halved with each division allowing easy tracking of proliferation over time on a logarithmic scale. Other methods of measuring cell proliferation include the quantitation of DNA synthesis by measuring tritiated thymidine (3H-thymidine) or bromodeoxyuridine (BrdU) uptake or by measuring changes in total DNA content with DNA specific dyes such as Hoechst 33258. Proliferation may also be measured by quantitation of the reduction of the intracellular environment by tetrazolium salt reduction or alamarBlue™ reduction. No method is without its flaws. Without an appropriate gating strategy, CFSE will not correctly isolate the particular single cells of interest. With tritiated thymidine, the number obtained for cells diving may not be accurate given incorporation into mitochondrial versus genomic DNA. When measuring metabolism, cells that are dying are also metabolically active yielding inaccurate data.

In the present study, exposure of murine T cell to either ConA or SEB resulted in the proliferation of CD4+ and CD8+ T cells. The mitogenic effect of ConA was exemplified by the increase in the absolute number of cells; incubation with ConA resulted in an 8-fold expansion of CD4+ T cells and a 16-fold expansion of CD8+ T cells (Figures 3, 4). The responses of the murine cells to SEB was similar, albeit of a less magnitude; the CD4+ T cells expanded 2-fold and CD8+ T cells expanded 3-fold (Figures 3, 4). These results were expected because ConA is thought to stimulate all T cells regardless of TCR usage. However, SEB tends to stimulate cells expressing Vβ 3, 7, 8.1-8.3, 11, 17 in mice (53).

The changes in the frequency of CD4+ T cells differed for the two stimuli, with SEB treatment resulting in a decrease in frequency while there was no change in response to ConA (Figure 5). In contrast, the frequency of CD8+ T cells doubled in response to ConA while there was no apparent effect of SEB (Figure 6). There are a several possible explanations for the lack of CD8+ T cell response observed. Consistent with previous studies is clonal deletion and clonal anergy with anergy of naive and memory T cells by superantigen related to cytokine production (79, 80). Another possibility for the CD8+ T cell observation, though less likely and not supported by the live vs. dead cell counts (Table 2), is apoptosis. In bulk culture, comparison of live vs. dead cells showed no dramatic change throughout the duration of the experiment indicative of good cell viability (Table 2). Overall these data indicate that stimulation of murine T cells with ConA results in a more accelerated rate of proliferation in comparison to a less accelerated rate seen in murine T cells stimulated with SEB.

The responses of equine PBMCs to incubation with either ConA or SEB were different than those of murine cells, but are statistically significant (Table 4). For example, equine T cells proliferated at much lower concentrations of SEB (2.5 μg/ml) than those required for murine T cells (50 μg/ml) (Figures 8-16). Furthermore, the proliferative response to SEB took approximately twice as long to be evident in equine T cells (48 hours) than in murine T cells (24 hours) (Figures 8-10). Additionally, the equine CD4+ T cell response to either ConA or SEB was more pronounced than the CD8+ T cell response when compared to unstimulated cells (controls) (Figures 8-16).

The number of unstimulated equine CD4+ T cells (negative control) changed little over time over the four days (Figure 12). This is more than likely due to the number of dead cells being replaced by new cells. In contrast, CD4+ T cell numbers were dramatically increased by day 4 in response to ConA. While incubation of cells with SEB at high concentrations (10 µg/ml and 20 µg/ml) resulted in little change in CD4+ T cells numbers, a substantial increase occurred in response to 2.5 µg/ml. In fact, the increase in CD4+ T cell numbers induced by exposure to this concentration of SEB was nearly equivalent to ConA treatment. These results demonstrate that ConA and SEB induce proliferation of equine T cells, and that these cells are more sensitive to SEB than murine cells. Furthermore, neither SEB nor ConA caused significant changes in cell viability (Table 2).

Both equine CD4+ and CD8+ cells proliferated significantly after exposure to ConA or SEB (Figures 13 and 14, Table 4). There appears to be no effect of either ConA or SEB on the CD8+ T cells of horse 8 in Figure 14. This was not the case with horses 9 and 10 and was not duplicated again with the same horse (8) in Figure 18 which all exhibited CD8+ T cell proliferation in response to SEB treatment (Figures 14, 16, 18). This might be explained by a memory response from exposure to an SEB producing *S. aureus* strain between blood collection time points, but this could not be confirmed at the time of the study and should be further investigated.

When comparing data from the same animal, proliferation among the negative population (shown in blue) in Figure 14 more than likely corresponds to the CD4+ T cell population in Figure 13 and vice versa with the negative population in Figure 13 corresponding to the CD8+ T cell population in Figure 14. This remained consistent throughout the duration of the experiment suggesting that although the animals examined are outbred and diverse; their T cell repertoires are similar.

For equine T cells, the response to both ConA and SEB is slower in comparison to murine T cells which suggests that this is an inherent property of equine cells compared to mouse cells. It is important to note the variability in response seen among the animals, especially in CD8+ T cells proliferative response. This is not surprising because we examined a highly outbred population of horses (see Table 1). Furthermore, the experiment could only be carried out until day 4 without adding additional cell culture media which would alter the concentrations of SEB or ConA. T cell proliferation beyond day 4 not only reveals increased cell death due to lack of nutrients, but also decreased proliferation with the loss of APCs and their ability to present SEB to T cells. Further investigation of apoptosis would require 7-Aminoactinomycin D (7-AAD) staining, additional experiments, and access to additional animals.

To examine whether or not SEB requires binding to the MHC class II molecule to stimulate proliferation of equine T cells, an equine-specific anti-MHC class II antibody was added to the culture supernatants. The results were compared to those obtained with a SARS IgG1 isotype control. Equine PBMCs were cultured for 48-96 hours in the presence or absence of ConA or SEB. As shown in Figure 17, little proliferation was observed in the experimental control (EC). However, treatment with ConA resulted in marked proliferation of CD8+ T cells. The bulk of proliferation mediated by ConA was observed at 96 hours (D4), and these data are supported by absolute cell numbers (Figure 12). SEB (2.5 μg/ml) resulted in marked proliferation as indicated by a decrease in fluorescence intensity by day 4 post-treatment.

To investigate whether MHC class II is involved in the proliferation of equine CD8+ T cells, an anti-MHC class II antibody or isotype control was added to cells for 1 hour prior to the addition of SEB. As shown in Figure 17, cells receiving the isotype control proliferated similar to SEB alone. In contrast, cells treated with the anti-MHC class II antibody failed to proliferate. These data along with data in Figures 18-20 suggest that the response of equine cells to SEB treatment requires interaction with MHC class II as previously reported for humans and mice and is statistically significant (Table 5). As previously shown, MHC class II blocking is adequate because it doesn't appear that SEB can bind directly to the TCR in mouse T cells without the presence of class II molecules (81). It has been demonstrated that not only does the anti-MHC II antibody block the interaction of SEB and MHC class II, but it apparently recognizes an MHC epitope that is expressed by most equine (Figs. 17-20). Future studies still need to be carried out to map the specific region of binding by SEB to the MHC class II molecule.

Equine T cell cytokine production was examined as a result of SEB and ConA stimulation. Data from this study support that Th1 type cytokine production was increased as seen by an increase in IFN- γ mRNA via quantitative RT-PCR and a reduction in Th2 type cytokine production represented by a decrease in IL-10 mRNA (data not shown). This data is not consistent with other studies describing an overall class switch from Th1 to Th2 type cytokines. Additional experiments are needed to determine if this effect is reproducible, to examine various time points after SEB treatment, and to examine other cytokine types produced such as TNF-α and IL-2 to determine if the immune response is skewed to a Th2 response as previously demonstrated (68, 69).

CHAPTER 6

CONCLUSION

Spread of *Staphylococcus aureus* including MRSA occurs through human-to-human, human-to-animal, and animal-to-human contact (38). Although previous studies have examined the effects of staphylococcal enterotoxin B on human and murine T cells, little information is available with respect to the effects this superantigen has on other animal species, equine in particular. Infection with *S. aureus* strains producing TSST-1 has been associated with toxic shock syndrome in humans and similar clinical signs have been found in horses following pulmonary infection (40). A major concern is the possibility that horses may serve as a community reservoir of MRSA and source of infection or reinfection in humans. Due to the large North American horse population and the frequent close contact between humans and horses, there a great need to examine the effects of *S. aureus* and its virulence factors on horses.

The purpose of this study was to determine whether or not equine T cells, like human and murine T cells, proliferate and/or secrete cytotoxins in the presence of staphylococcal enterotoxins, in particular staphylococcus enterotoxin B. Based on the structural similarities of equine versus human T cell receptors, our hypothesis was that the presence of SEB should result in the proliferation of equine T cells and the production of Th2 cytokines.

Data from the mouse model experiments support that both ConA and SEB stimulate CD4+ and CD8+ murine T cells. Specifically, it appears that ConA stimulates a more robust response than SEB. This was reflected in the number of T cells seen with ConA CD4+ T cells expanding 8 fold versus ConA CD8+ T cells expanding 16 fold and SEB CD4+ T cells expanding 2 fold versus ConA CD8+ T cells expanding 3 fold. Frequency of CD4+ T cells with

ConA did not show marked change, but with SEB the frequency decreased. Frequency of CD8+ T cells with ConA doubled, but frequency stayed approximately the same with SEB.

Data from the equine model time course experiments reveal that lower concentrations of SEB better stimulate equine T cells than murine T cells (2.5 μg vs. 50 μg respectively). Moreover, measureable proliferation in response to SEB stimulation takes longer in equine T cells compared to murine T cells (48 hours vs. 24 hours respectively). Also, the CD4+ equine T cell response resulting from SEB or ConA stimulation is more robust than the CD8+ T cell response. These data suggest that equine T cells respond strongly to the presence of SEB and are more sensitive to SEB indicated by more significant proliferation at lower concentrations.

It was also necessary to investigate whether or not SEB requires interaction with MHC class II to stimulate equine T cell proliferation and production of cytokines. Data from this study support that equine CD4+ and CD8+ T cell proliferation was severely reduced with blocking by an MHC class II antibody relative to the isotype control. Additional studies should be conducted mapping the specific binding of SEB to the region on the MHC class II molecule in addition to the half-life of the MHC class II antibody.

Finally, equine T cell cytokine production was examined as a result of SEB stimulation. Additional experiments are needed to determine if this effect is reproducible, to examine various time points after SEB treatment, and to examine other cytokine types produced such as TNF-α and IL-2 to determine if the immune response is skewed to a Th2 response as previously demonstrated (68, 69). All of these data taken together strongly support that SEB stimulates equine T cell proliferation and cytokine production and that this stimulation requires the MHC class II molecule. This indicates the potential of an anti-MHC class II antibody as a therapeutic in treating staphylococcal enterotoxin B infection.

References

1. Baker MD & Acharya KR (2004) Superantigens: structure-function relationships. *Int J Med Microbiol* 293(7-8):529-537.
2. Biberstein EL, Jang SS, & Hirsh DC (1984) Species distribution of coagulase-positive staphylococci in animals. *J Clin Microbiol* 19(5):610-615.
3. Shimizu A*, et al.* (1991) Characteristics of Staphylococcus aureus isolated from lesions of horses. *J Vet Med Sci* 53(4):601-606.
4. Becker K*, et al.* (2001) Enterotoxigenic potential of Staphylococcus intermedius. *Appl Environ Microbiol* 67(12):5551-5557.
5. Hogan RJ, VanBeek J, Broussard DR, Surman SL, & Woodland DL (2001) Identification of MHC class II-associated peptides that promote the presentation of toxic shock syndrome toxin-1 to T cells. *J Immunol* 166(11):6514-6522.
6. Smith DR, Rossi CA, Kijek TM, Henchal EA, & Ludwig GV (2001) Comparison of dissociation-enhanced lanthanide fluorescent immunoassays to enzyme-linked immunosorbent assays for detection of staphylococcal enterotoxin B, Yersinia pestis-specific F1 antigen, and Venezuelan equine encephalitis virus. *Clin Diagn Lab Immunol* 8(6):1070-1075.
7. Hammamieh R, Bi S, Das R, Neill R, & Jett M (2004) Modeling of SEB-induced host gene expression to correlate in vitro to in vivo responses. *Biosens Bioelectron* 20(4):719-727.

8. Fey PD, *et al.* (2003) Comparative molecular analysis of community- or hospital-acquired methicillin-resistant Staphylococcus aureus. *Antimicrob Agents Chemother* 47(1):196-203.

9. Jin T, Bokarewa M, & Tarkowski A (2005) The role of urokinase in innate immunity against Staphylococcus aureus. *Microbes Infect* 7(9-10):1170-1175.

10. Hu DL, *et al.* (2005) A mutant of staphylococcal enterotoxin C devoid of bacterial superantigenic activity elicits a Th2 immune response for protection against Staphylococcus aureus infection. *Infect Immun* 73(1):174-180.

11. Lee JS, *et al.* (2002) Immune protection against staphylococcal enterotoxin-induced toxic shock by vaccination with a Venezuelan equine encephalitis virus replicon. *J Infect Dis* 185(8):1192-1196.

12. Goettelfinger P, Lecerf F, Berrih-Aknin S, & German-Fattal M (2001) Cytokine deviation induced by intrathymic injection of staphylococcal enterotoxin B (SEB). *Eur Cytokine Netw* 12(3):487-500.

13. Plaza R, Rodriguez-Sanchez JL, & Juarez C (2007) Staphylococcal enterotoxin B in vivo modulates both gamma interferon receptor expression and ligand-induced activation of signal transducer and activator of transcription 1 in T cells. *Infect Immun* 75(1):306-313.

14. Noel C, Florquin S, Goldman M, & Braun MY (2001) Chronic exposure to superantigen induces regulatory CD4(+) T cells with IL-10-mediated suppressive activity. *Int Immunol* 13(4):431-439.

15. Yoh K, *et al.* (2000) Cytokines and T-cell responses in superantigen-related glomerulonephritis following methicillin-resistant Staphylococcus aureus infection. *Nephrol Dial Transplant* 15(8):1170-1174.

16. Tsao SM, Hsu CC, & Yin MC (2006) Meticillin-resistant Staphylococcus aureus infection in diabetic mice enhanced inflammation and coagulation. *J Med Microbiol* 55(Pt 4):379-385.

17. Anonymous (1984) Classics in infectious diseases. "On abscesses". Alexander Ogston (1844-1929). (Translated from eng) *Rev Infect Dis* 6(1):122-128 (in eng).

18. Matthews KRR, J.; Gillespie, B.E.; Luther, D.A.; Oliver, S.P. (1997) Identification and Differentiation of Coagulase-Negative Staphylococcus aureus by Polymerase Chain Reaction *Journal of Food Protection* 60(6):3.

19. Kluytmans J, van Belkum A, & Verbrugh H (1997) Nasal carriage of Staphylococcus aureus: epidemiology, underlying mechanisms, and associated risks. (Translated from eng) *Clin Microbiol Rev* 10(3):505-520 (in eng).

20. Cleary PP, *et al.* (1998) High-frequency intracellular infection and erythrogenic toxin A expression undergo phase variation in M1 group A streptococci. (Translated from eng) *Mol Microbiol* 28(1):157-167 (in eng).

21. Schlievert PM (1982) Enhancement of host susceptibility to lethal endotoxin shock by staphylococcal pyrogenic exotoxin type C. (Translated from eng) *Infect Immun* 36(1):123-128 (in eng).

22. Llewelyn M & Cohen J (2002) Superantigens: microbial agents that corrupt immunity. (Translated from eng) *Lancet Infect Dis* 2(3):156-162 (in eng).

23. Hongmin Li AL, Daisuke Tsuchiya, Lukas Leder, Xavier Ysern, Patrick M. Schlievert, Klaus Karjalainen, and Roy A. Mariuzza. (1998) Three-Dimensional Structure of the Complex between a T Cell Receptor b Chain and the Superantigen Staphylococcal Enterotoxin B. *Immunity* 9:10.

24. Bohach GA, Fast DJ, Nelson RD, & Schlievert PM (1990) Staphylococcal and streptococcal pyrogenic toxins involved in toxic shock syndrome and related illnesses. (Translated from eng) *Crit Rev Microbiol* 17(4):251-272 (in eng).

25. Oliveira DC & de Lencastre H (2002) Multiplex PCR strategy for rapid identification of structural types and variants of the mec element in methicillin-resistant Staphylococcus aureus. (Translated from eng) *Antimicrob Agents Chemother* 46(7):2155-2161 (in eng).

26. Andriesse GI, van Rijen M, Bogaers D, Bergmans AM, & Kluytmans JA (2009) Comparison of two PCR-based methods and conventional culture for the detection of nasal carriage of Staphylococcus aureus in pre-operative patients. (Translated from Eng) *Eur J Clin Microbiol Infect Dis* (in Eng).

27. Kobayashi N, *et al.* (2009) Rapid and sensitive detection of methicillin-resistant Staphylococcus periprosthetic infections using real-time polymerase chain reaction. (Translated from eng) *Diagn Microbiol Infect Dis* 64(2):172-176 (in eng).

28. Wellinghausen N, Siegel D, Gebert S, & Winter J (2009) Rapid detection of Staphylococcus aureus bacteremia and methicillin resistance by real-time PCR in whole blood samples. (Translated from Eng) *Eur J Clin Microbiol Infect Dis* (in Eng).

29. Kobayashi H, Oethinger M, Tuohy MJ, Hall GS, & Bauer TW (2009) Improving clinical significance of PCR: Use of propidium monoazide to distinguish viable from dead staphylococcus aureus and staphylococcus epidermidis. (Translated from Eng) *J Orthop Res* (in Eng).

30. Korzeniowski O & Sande MA (1982) Combination antimicrobial therapy for Staphylococcus aureus endocarditis in patients addicted to parenteral drugs and in

nonaddicts: A prospective study. (Translated from eng) *Ann Intern Med* 97(4):496-503 (in eng).

31. Bayer AS*, et al.* (1998) Diagnosis and management of infective endocarditis and its complications. (Translated from eng) *Circulation* 98(25):2936-2948 (in eng).

32. Cosgrove SE*, et al.* (2009) Initial low-dose gentamicin for Staphylococcus aureus bacteremia and endocarditis is nephrotoxic. (Translated from eng) *Clin Infect Dis* 48(6):713-721 (in eng).

33. Chambers HF (2001) The changing epidemiology of Staphylococcus aureus? (Translated from eng) *Emerg Infect Dis* 7(2):178-182 (in eng).

34. Johnson AP*, et al.* (2001) Dominance of EMRSA-15 and -16 among MRSA causing nosocomial bacteraemia in the UK: analysis of isolates from the European Antimicrobial Resistance Surveillance System (EARSS). (Translated from eng) *J Antimicrob Chemother* 48(1):143-144 (in eng).

35. Vardakas KZ, Matthaiou DK, & Falagas ME (2009) Incidence, characteristics and outcomes of patients with severe CA-MRSA pneumonia. (Translated from Eng) *Eur Respir J* (in Eng).

36. Reygaert W (2009) Methicillin-resistant Staphylococcus aureus (MRSA): prevalence and epidemiology issues. (Translated from eng) *Clin Lab Sci* 22(2):111-114 (in eng).

37. Reygaert W (2009) Methicillin-resistant Staphylococcus aureus (MRSA): molecular aspects of antimicrobial resistance and virulence. (Translated from eng) *Clin Lab Sci* 22(2):115-119 (in eng).

38. Oehler RL, Velez AP, Mizrachi M, Lamarche J, & Gompf S (2009) Bite-related and septic syndromes caused by cats and dogs. (Translated from eng) *Lancet Infect Dis* 9(7):439-447 (in eng).

39. Sanchez S (2008) What Are the Risks of Contracting MRSA From Domesticated Animals? in *Medscape Infectious Diseases* (Medscape Infectious Diseases © 2008 Medscape).

40. Holbrook TC, *et al.* (2003) Toxic shock syndrome in a horse with Staphylococcus aureus pneumonia. (Translated from eng) *J Am Vet Med Assoc* 222(5):620-623, 601-622 (in eng).

41. Blot SI, Vandewoude KH, Hoste EA, & Colardyn FA (2002) Outcome and attributable mortality in critically Ill patients with bacteremia involving methicillin-susceptible and methicillin-resistant Staphylococcus aureus. (Translated from eng) *Arch Intern Med* 162(19):2229-2235 (in eng).

42. Hiramatsu K, *et al.* (1997) Methicillin-resistant Staphylococcus aureus clinical strain with reduced vancomycin susceptibility. (Translated from eng) *J Antimicrob Chemother* 40(1):135-136 (in eng).

43. Chang S*, et al.* (2003) Infection with vancomycin-resistant Staphylococcus aureus containing the vanA resistance gene. (Translated from eng) *N Engl J Med* 348(14):1342-1347 (in eng).

44. Neely AN & Maley MP (2000) Survival of enterococci and staphylococci on hospital fabrics and plastic. (Translated from eng) *J Clin Microbiol* 38(2):724-726 (in eng).

45. Cook HA, Furuya EY, Larson E, Vasquez G, & Lowy FD (2007) Heterosexual transmission of community-associated methicillin-resistant Staphylococcus aureus. (Translated from eng) *Clin Infect Dis* 44(3):410-413 (in eng).

46. Warnke PH, *et al.* (2009) The battle against multi-resistant strains: Renaissance of antimicrobial essential oils as a promising force to fight hospital-acquired infections. (Translated from Eng) *J Craniomaxillofac Surg* (in Eng).

47. Hammer KA, Carson CF, & Riley TV (2008) Frequencies of resistance to Melaleuca alternifolia (tea tree) oil and rifampicin in Staphylococcus aureus, Staphylococcus epidermidis and Enterococcus faecalis. (Translated from eng) *Int J Antimicrob Agents* 32(2):170-173 (in eng).

48. Doran AL, Morden WE, Dunn K, & Edwards-Jones V (2009) Vapour-phase activities of essential oils against antibiotic sensitive and resistant bacteria including MRSA. (Translated from eng) *Lett Appl Microbiol* 48(4):387-392 (in eng).

49. Buonpane RA, Moza B, Sundberg EJ, & Kranz DM (2005) Characterization of T cell receptors engineered for high affinity against toxic shock syndrome toxin-1. (Translated from eng) *J Mol Biol* 353(2):308-321 (in eng).

50. Alouf JE & Muller-Alouf H (2003) Staphylococcal and streptococcal superantigens: molecular, biological and clinical aspects. (Translated from eng) *Int J Med Microbiol* 292(7-8):429-440 (in eng).

51. Brouillard JN, *et al.* (2007) Crystal structure of the streptococcal superantigen SpeI and functional role of a novel loop domain in T cell activation by group V superantigens. (Translated from eng) *J Mol Biol* 367(4):925-934 (in eng).

52. Fraser JD & Proft T (2008) The bacterial superantigen and superantigen-like proteins. (Translated from eng) *Immunol Rev* 225:226-243 (in eng).

53. Herman A, Kappler JW, Marrack P, & Pullen AM (1991) Superantigens: mechanism of T-cell stimulation and role in immune responses. (Translated from eng) *Annu Rev Immunol* 9:745-772 (in eng).

54. Sriskandan S, Faulkner L, & Hopkins P (2007) Streptococcus pyogenes: Insight into the function of the streptococcal superantigens. (Translated from eng) *Int J Biochem Cell Biol* 39(1):12-19 (in eng).

55. Petersson K, Forsberg G, & Walse B (2004) Interplay between superantigens and immunoreceptors. (Translated from eng) *Scand J Immunol* 59(4):345-355 (in eng).

56. Mehindate K, *et al.* (1995) Cross-linking of major histocompatibility complex class II molecules by staphylococcal enterotoxin A superantigen is a requirement for inflammatory cytokine gene expression. (Translated from eng) *J Exp Med* 182(5):1573-1577 (in eng).

57. Papageorgiou AC, Tranter HS, & Acharya KR (1998) Crystal structure of microbial superantigen staphylococcal enterotoxin B at 1.5 A resolution: implications for superantigen recognition by MHC class II molecules and T-cell receptors. (Translated from eng) *J Mol Biol* 277(1):61-79 (in eng).

58. Kimura N, Toyonaga B, Yoshikai Y, Du RP, & Mak TW (1987) Sequences and repertoire of the human T cell receptor alpha and beta chain variable region genes in thymocytes. (Translated from eng) *Eur J Immunol* 17(3):375-383 (in eng).

59. Chou HS, Nelson CA, Godambe SA, Chaplin DD, & Loh DY (1987) Germline organization of the murine T cell receptor beta-chain genes. (Translated from eng) *Science* 238(4826):545-548 (in eng).

60. Schrenzel MD, Watson JL, & Ferrick DA (1994) Characterization of horse (Equus caballus) T-cell receptor beta chain genes. (Translated from eng) *Immunogenetics* 40(2):135-144 (in eng).

61. Rosenberg WM, Moss PA, & Bell JI (1992) Variation in human T cell receptor V beta and J beta repertoire: analysis using anchor polymerase chain reaction. (Translated from eng) *Eur J Immunol* 22(2):541-549 (in eng).

62. Bill J, Appel VB, & Palmer E (1988) An analysis of T-cell receptor variable region gene expression in major histocompatibility complex disparate mice. (Translated from eng) *Proc Natl Acad Sci U S A* 85(23):9184-9188 (in eng).

63. Arcus VL*, et al.* (2000) Conservation and variation in superantigen structure and activity highlighted by the three-dimensional structures of two new superantigens from Streptococcus pyogenes. (Translated from eng) *J Mol Biol* 299(1):157-168 (in eng).

64. Watson AR & Lee WT (2006) Defective T cell receptor-mediated signal transduction in memory CD4 T lymphocytes exposed to superantigen or anti-T cell receptor antibodies. (Translated from eng) *Cell Immunol* 242(2):80-90 (in eng).

65. Choi S & Schwartz RH (2007) Molecular mechanisms for adaptive tolerance and other T cell anergy models. (Translated from eng) *Semin Immunol* 19(3):140-152 (in eng).

66. Bradley GS & Teresa K (2005) Staphylococcal enterotoxins: A purging experience in review, Part I1 1 Editor's Note: Part II of this article will appear in the December 15, 2005 issue of CMN (Vol. 27 No. 24). 27(23):179-186.

67. Lussow AR & MacDonald HR (1994) Differential effects of superantigen-induced "anergy" on priming and effector stages of a T cell-dependent antibody response. (Translated from eng) *Eur J Immunol* 24(2):445-449 (in eng).

68. Miller C, Ragheb JA, & Schwartz RH (1999) Anergy and cytokine-mediated suppression as distinct superantigen-induced tolerance mechanisms in vivo. (Translated from eng) *J Exp Med* 190(1):53-64 (in eng).

69. Yamaguchi M, Nadler S, Lee JW, & Deeg HJ (1999) Induction of negative regulators of haematopoiesis in human bone marrow cells by HLA-DR cross-linking. (Translated from eng) *Transpl Immunol* 7(3):159-168 (in eng).

70. Wesson CA, *et al.* (1998) Staphylococcus aureus Agr and Sar global regulators influence internalization and induction of apoptosis. (Translated from eng) *Infect Immun* 66(11):5238-5243 (in eng).

71. Suzuki H, Hayakawa A, Bouchard D, Nakashima I, & Mak TW (1997) Normal thymic selection, superantigen-induced deletion and Fas-mediated apoptosis of T cells in IL-2 receptor beta chain-deficient mice. (Translated from eng) *Int Immunol* 9(9):1367-1374 (in eng).

72. Diener K, Tessier P, Fraser J, Kontgen F, & McColl SR (1998) Induction of acute inflammation in vivo by staphylococcal superantigens I: Leukocyte recruitment occurs independently of T lymphocytes and major histocompatibility complex Class II molecules. (Translated from eng) *Lab Invest* 78(6):647-656 (in eng).

73. Jabara HH & Geha RS (1996) The superantigen toxic shock syndrome toxin-1 induces CD40 ligand expression and modulates IgE isotype switching. (Translated from eng) *Int Immunol* 8(10):1503-1510 (in eng).

74. Erlandsson E, *et al.* (2003) Identification of the antigenic epitopes in staphylococcal enterotoxins A and E and design of a superantigen for human cancer therapy. (Translated from eng) *J Mol Biol* 333(5):893-905 (in eng).

75. Middleton JR, *et al.* (2005) Surveillance of Staphylococcus aureus in veterinary teaching hospitals. (Translated from eng) *J Clin Microbiol* 43(6):2916-2919 (in eng).

76. Weese JS, *et al.* (2005) Methicillin-resistant Staphylococcus aureus in horses and horse personnel, 2000-2002. (Translated from eng) *Emerg Infect Dis* 11(3):430-435 (in eng).

77. Rossi RE & Monasterolo G (2004) Prevalence of serum IgE antibodies to the Staphylococcus aureus enterotoxins (SAE, SEB, SEC, SED, TSST-1) in patients with persistent allergic rhinitis. (Translated from eng) *Int Arch Allergy Immunol* 133(3):261-266 (in eng).

78. Figueiredo MD, Vandenplas ML, Hurley DJ, & Moore JN (2009) Differential induction of MyD88- and TRIF-dependent pathways in equine monocytes by Toll-like receptor agonists. (Translated from eng) *Vet Immunol Immunopathol* 127(1-2):125-134 (in eng).

79. Lee WT & Vitetta ES (1992) Memory T cells are anergic to the superantigen staphylococcal enterotoxin B. (Translated from eng) *J Exp Med* 176(2):575-579 (in eng).

80. Blankson JN, Loh DY, & Morse SS (1995) Superantigens and conventional antigens induce different responses in alpha beta T-cell receptor transgenic mice. (Translated from eng) *Immunology* 85(1):57-62 (in eng).

81. Qasim W, Kehoe MA, & Robinson JH (1991) Does staphylococcal enterotoxin B bind directly to murine T cells? (Translated from eng) *Immunology* 73(4):433-437 (in eng).