DISSERTATION

TICK SALIVARY GLAND PROTEINS (SALPS) AS ANTIGENS FOR VACCINE DEVELOPMENT TO DECREASE SPIROCHETE LOAD IN A MURINE MODEL OF LYME BORRELIOSIS

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ABSTRACT OF DISSERTATION

TICK SALIVARY GLAND PROTEINS (SALPS) AS ANTIGENS FOR VACCINE DEVELOPMENT TO DECREASE SPIROCHETE LOAD IN A MURINE MODEL OF LYME BORRELIOSIS

Lyme disease, caused by *Borrelia burgdorferi*, is the most common tick-borne illness in the United States and selected regions of Eurasia. Members of the *Ixodes ricinus* complex of ticks are the vectors for *B. burgdorferi*, with *I. scapularis* being the primary vector in North America. Lyme disease occurs along the east coast as well as the upper Midwest, with the majority of cases occurring in the northeastern regions of the United States. The number of cases of Lyme disease in the U.S. has increased over the last 20 years with 28,921 cases reported in 2008. Prevention of Lyme disease for humans is currently focused on use of repellants and tick removal, and there is currently no vaccine available.

The goal of this dissertation is to test the hypothesis that vaccinating mice with highly immunomodulatory tick salivary proteins in a context that shifts the immune response of the host from a Th2 polarized response, which is normal for tick feeding (Ferreira and Silva 1999), to a Th1 response would block transmission of *B. burgdorferi* by infected ticks. To these ends the objectives of this research were to: 1) Generate adenoviral vaccine vectors containing the tick salivary gene of interest to drive the Th1 response (Chapter 2), 2) test the adenovirus constructs in a murine model for their ability to induce a Th1 shift in cytokines and a subsequent reduction or block of tick-transmitted

B. burgdorferi infection (Chapter 3), and 3) identify more potential tick salivary genes for vaccine antigens utilizing DNA vaccine methodology.

Tick saliva contains a wide range of physiologically active molecules that are critical for effective attachment and engorgement of the tick. While taking a blood meal, hard ticks attach to their vertebrate hosts for several days and introduce saliva, together with pathogens into the skin. During this period, it is necessary for the tick to enhance blood flow, circumvent the host immune response and prevent healing of the feeding site to continue to imbibe blood from the feeding pool created by the tick. It has been demonstrated that immunizing the host with molecules from tick saliva can negatively affect not only tick feeding, but pathogen transmission as well, thereby protecting the host from pathogen transmission.

Seven molecules from *I. scapularis* were investigated in either an adenoviral vaccine vector or in a DNA vaccine vector for their ability to block tick-transmitted *B. burgdorferi* infection in a murine model. Complete prevention of transmission was not observed, but a 60% reduction of spirochete load was observed using a combination of molecules in an adenoviral vector.

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Introduction

Ticks (subphylum *Chelicerata*); class Arachnida; subclass *Acari*; superorder *Parasitiformes*; order *Ixodida*) are obligate blood-feeding ectoparasites of global medical and veterinary importance. Ticks live on all continents of the world (Steen et al. 2006). There are approximately 899 species of ticks; the majority are ectoparasites of wildlife and approximately 10% of these are recognized as disease vectors or for their ability to cause direct damage through blood feeding (Jongejan and Uilenberg 2004). Ticks transmit a greater variety of viruses, bacteria and protozoa than any other blood feeding arthropod (Dennis and Piesman 2005) and are second only to mosquitoes in terms of their medical and veterinary impact (Sonenshine 1991). Other forms of injury attributed to ticks include anemia, dermatosis and toxicosis leading to paralysis. Worldwide there is growing concern because tick-borne infectious diseases are emerging and resurging (Telford and Goethert 2004; Walker 1998; Walker 2005).

Some of the most significant tick-borne diseases of humans and animals and their tick vectors are shown in Table 1.1. The causative agents of these diseases include bacteria (both extracellular and intracellular), viruses, and piroplasm protozoans. The success of ticks as vectors of disease causing agents can be attributed to a number of factors including wide host range, feeding on multiple hosts as well as the mechanism and length of time required to blood feed. The long life span (1-2 years) of most hard

ticks also enhances vector capability because it provides sufficient time for ticks to become a reservoir host. Both trans-stadial and trans-ovariole mechanisms of pathogen transmission are documented in the *Ixodida*. Thus, in certain species, both immature stages and adult ticks are competent vectors.

Table 1.1. Disease agents transmitted by ticks

Disease	**Causative Agent**	**Primary Tick Vectors**
Tularemia	*Franciscella tularemia*	*Amblyomma americanum, D. variabilis, D. andersonii, D. reticulatus,I. ricinus, Rhipicephalus sanguinius,*
Human monocytic ehrlichiosis	*Ehrlichia chaffeensis*	*A. americanum*
Heartwater	*Ehrlichia ruminantium*	*A. variegatum, A. hebraeum*
Bovine anaplasmosis	*Anaplasma marginale*	*Boophilus* spp.
Bovine babesiosis	*Babesia bigemmina, B. bovis*	*Boophilus* spp.
Colorado tick fever	Coltivirus	*D. andersonii*
Rocky mountain spotted fever	*Rickettsia rickettsii*	*Dermacentor variabilis, D. andersonii, R. sanguinius*
Northern Asian Tick Typhus (Siberian tick typhus)	*Rickettsia siberica*	*D. marginatus, D. silvarium, D. nuttalli*
Tick paralysis	Salivary toxin	*D. andersoni, D. variabilis, I. holocylus*
Crimean Congo hemorrhagic fever	*Bunyaviridae*	*Hyalomma marginatum, H. anatolicum, H. rufipes, H. marginatus*
Kyasanur forest disease	*Flaviviridae*	*H. spinigera*
Lyme disease	*Borrelia burgdorferi* sensu stricto	*Ixodes ricinus* complex
Human babesiosis	*Babesia microti* *B. divergens*	*I. scapularis* *I. ricinus*
Human tick-borne ehrlichiosis	*Ehrlichia spp./Anaplasma phagocytophilum*	*I. ricinus* *I. scapularis*

Tick-borne encephalitis	*Flaviviridae*	*I. ricinus, I. persulcatus, D. marginatus*
Epizootic bovine abortion	Agent not established	*Ornithodorus coriaceus*
Relapsing fever	*Borrelia duttonii, B. crociduriae*	*Ornithodoros* spp.,
Canine ehrlichiosis	*E. canis*	*R. sanguineus*
East Coast fever	*Theileria parva*	*R. appendiculatus*
Boutonneuse fever	*Rickettsia conorii*	*Rhipicephalus sanguineus, R. appendiculatus, Haemaphysalis leachi*

The *Ixodes ricinus* species complex comprises a group of ticks that are distributed in almost all geographic regions of the world and includes a number of species of significance to human health because they vector tick-borne encephalitis virus, rickettsiae, piroplasma and the *Borrelia* spirochete (Delaye et al. 1997). This complex includes *Ixodes scapularis* (black-legged or deer tick), vector of Lyme disease (LD) in the US, *I. pacificus* (western black-legged tick) from southern Canada to northern Mexico and *I. ricinus* and *I. persulcatus,* vectors of *Borrelia* in the Palearctic and Oriental regions (Xu et al. 2003). LD is the most common vector borne disease in the United States. Despite federal, state and local efforts to prevent and control LD, a total of 28,921 cases were reported in 2008 (Centers for Disease Control and Prevention 2009) representing a 3-fold increase since 1991 (CDC 2002). The average direct and indirect medical expenses, which includes physicians visits, serological testing, and therapies associated with Lyme disease patient care, are estimated at $2,970 and $5,202 respectively, which translates to an nationwide estimated annual economic impact of approximately US $203M dollars (in 2002 dollars) (Zhang et al. 2006).

Rhipicephalus (Boophilus) microplus (hereafter *Boophilus*), the tropical or southern cattle tick, has colonized most of the world's tropical and sub-tropical countries (McCosker 1979; Murrell et al. 2001) and is the most economically important *Boophilus* species. *B. microplus* is a vector of the protozoan (*Babesia bovis* and *B. bigemina*) and bacterial (*Anaplasma marginale*) organisms which cause bovine babesiosis and anaplasmosis ('tick fever'), respectively. The tick-disease complex of *Boophilus* spp.-*Babesia* spp.-*Anaplasma marginale* is probably the most important affecting world-wide livestock production (deCastro 1977), leading to severe economic losses in milk and beef production and restriction in trafficking of animals, costing more than US $2.5B annually. Chemical treatments (acaricides) are relied on for tick control, however tick resistance to synthetic pyrethroid, organophosphate and amitraz acaricides is widespread (Foil et al. 2004). Control of cattle ticks is required to minimize production losses and the beef industry incurs more than US $200 million in annual losses due to the impact of ticks and tick-borne diseases and costs of treatment to ensure compliance with regulatory protocols for intrastate, interstate and international livestock movement (Playford 2005).

Other species of ticks that are of medical or veterinary importance include *Rhipicephalus appendiculatus* (brown ear tick) which vectors *Theileria parva*, the causative agent of East Coast fever. In eastern and southern Africa, this disease severely limits cattle production. The tropical bont tick, *Amblyomma variegatum* and the bont tick, *A. hebraeum* are also of medical and veterinary importance because they are the primary vectors of *Ehrlichia ruminantium* which causes Heartwater, a vascular leak syndrome inducing rapid death in cows, sheep and goats. Heartwater is one of the more important ruminant diseases in sub-Saharan Africa and Madagascar, and has recently

appeared on a few islands in the Caribbean. The lone star tick, *Amblyomma americanum* is also of increasing importance due to changes in its geographical distribution, discovery of new pathogens for which it is a vector, and increased frequency of transmission of those zoonotic infectious agents to humans (Childs and Paddock 2003). *Amblyomma americanum* is the vector of *E. chaffeensis* which causes human ehrlichiosis. Multiple species of *Dermacentor* have also been implicated as major disease vectors in the United States and elsewhere. *Dermacentor andersonii* and *D. variabilis*, the Rocky mountain wood tick and the American brown dog tick, vector Rocky Mountain spotted fever, a disease caused by *Rickettsia rickettsii*.

All ticks share the same basic developmental pattern; the egg hatches into a six-legged larva, which molts to an eight-legged nymph. Depending on the species, there may be one or multiple nymphal molts before the final molt to an eight-legged adult. Ticks, with rare exception, are obligate blood feeders at all life stages but are considered to be nonpermanent parasites as that they must find a new host each time they feed. Tick life cycles are defined by the number of hosts upon which a species will feed. Argasid ticks feed on multiple hosts over a lifetime, even within a life stage and their most common hosts are generally small nesting vertebrates such as birds and bats. In contrast, ixodid ticks will molt to the next life stage after each feeding on a host. In the Ixodidae, a mated female will deposit a single, large egg batch, and die shortly thereafter. The eggs hatch into larvae, which begin active questing for a host. In "three-host" species such as *Ixodes*, *Amblyomma* and some species of *Dermacentor*, larvae will attach and feed for three to seven days. Once fully engorged, the larvae will drop off the host, molt to a nymph, and will then search for a new host. The nymph will feed for three to eight days,

drop off the host, molt to an adult, and seek a new host for a third time. The most common hosts of immature ixodid ticks are small mammals, ground dwelling birds and lizards. Adult ixodid ticks tend to feed on larger mammals such as deer, livestock, dogs and humans.

Depending on the species of tick, mating may occur on or off the host post feeding. Some *Hyalomma* and *Rhipicephalus* species do not drop off after larval feeding, but instead molt on the host. This is considered a two-host life cycle. *Boophilus, Margaropus*, and some species of *Dermacentor* exhibit a one-host life cycle in which all stages of the tick remain on the host from the first attachment until drop off as mated females. Both of these life cycles of ticks are more common when the host is a larger vertebrate, particularly seen in cattle.

Once a questing tick finds a host, and a suitable site to feed on the host, hard ticks penetrate the host skin with their chelicera and secrete a cement-like substance that helps to prevent detachment. Ticks imbibe the blood that pools in the wound site created by the mouthparts then alternating cycles of feeding and salivating (Gregson 1967). Hard ticks have an interesting conundrum in that the number of days it takes a tick to obtain a successful blood meal is also the number of days that the immune system of the host has to recognize and mount a challenge to the tick. To feed to repletion, a tick must be able to circumvent the host immune response in a temporally appropriate manner. This is accomplished by a diverse array of tick secreted salivary proteins.

Host Immune Response

It has been demonstrated that different vertebrate hosts of ticks have varying immune responses to the bite and the salivary components injected into the host during feeding. The study of these components has been a focus for fast-paced and fascinating research. One of the goals for much of this intense study is to identify possible vaccine candidates for species-specific, anti-tick vaccines. Dissecting the differing immune responses of vertebrate hosts, both in the field and in the laboratory, has been crucial to understanding how to design vaccine for human or animal use.

Many tick-host relationships are characterized by the acquisition of resistance to tick feeding which develops as a result of repeated tick infestations (Willadsen 1980). Acquired resistance was first observed and described by Trager in 1939, who demonstrated that guinea pigs in which *Dermacentor variabilis* had fed upon became resistant to tick infestation over successive feedings (Trager 1939). This acquired resistance is characterized by reduced engorgement, increased duration of feeding, blocked molting and death of engorging ticks (Wikel 1996).

In nature, the primary reservoir for the causative agent of Lyme disease, *Borrelia burgdorferi*, is *Peromyscus leucopus*, the white-footed mouse, which is also the most common host for immature stages of *Ixodes scapularis*, the vector of *B. burgdorferi* (Donahue et al. 1987; Lane et al. 1991; Piesman and Spielman 1979). *P. leucopus* has been shown to be very tolerant of *I. scapularis* feeding and not to develop acquired resistance. Previously exposed *P. leucopus* can show a tendency of reduced tick feeding success, as measured by molting success; but these results were not statistically significant (Hazler and Ostfeld 1995). In this same laboratory study, *Peromyscus*

maniculatus, a mostly western species, demonstrated a significant increase in molting success and higher tick densities per host. *P. gossypinus*, a mouse with a southeastern range, also demonstrate high levels of tolerance to *I. scapularis* feedings (Galbe and Oliver 1992). Given that *Peromyscus* mice are the primary reservoir of Lyme disease, it is not surprising that these mice are amenable to repeated feeding by *Ixodes* ticks and develop very little resistance to salivary components.

Mus musculus has also been shown to be a tolerant host for *Ixodes* ticks. Experiments with outbred mice actually demonstrate higher molting success of *I. scapularis* in subsequent feedings (Galbe and Oliver 1992). BALB/C mice (Wikel et al. 1997) and C3H mice (Narasimhan et al. 2007) have also been shown to be tolerant to repeated feedings, with no measurable antibody titers to tick salivary components. However, Wikel et al. (1997) demonstrated that despite the ability of *I. scapularis* to successfully feed on BALB/C mice, transmission efficiency of *B. burgdorferi* was significantly decreased after multiple prior feedings with pathogen-free ticks, although to date no other research group has been able to repeat these results.

The guinea pig, *Cavia porcellus*, demonstrates a completely different immune response as seen in a murine model. The immune response of this unnatural host of *I. scapularis* to the tick has been studied in depth (Narasimhan et al. 2007). Salivary glands from *I. scapularis* ticks which had been fed on mice for 66 hours were homogenized and injected into naïve guinea pigs. These guinea pigs were subsequently challenged with pathogen-free *I. scapularis*, and no significant decrease in tick engorgement weight was seen, nor was the duration of feeding lengthened. However, when pathogen-free ticks were allowed to feed on a second group of guinea pigs for a period of exactly 24 hours,

four separate times, subsequent challenge with new ticks showed a highly significant decrease in tick engorgement weight and rapid rejection of ticks within 24-48 hours. As transmission of *B. burgdorferi* has been shown to only become efficient after 2 days of feeding (Piesman et al. 1987), this rejection is quite remarkable. Erythema at the site of tick attachment was also observed within guinea pigs in this study. When skin was biopsied from this site, a large influx of inflammatory cells was seen, predominantly, basophils and mast cells, the hallmark of delayed-type hypersensitivity, were seen. Interestingly, robust humoral responses to tick saliva were not detected in these guinea pigs. However, given that the strongest anti-tick reaction was at the 24-48 hour mark, this observation is not completely unreasonable. When microarray data from mRNA and RT-PCR were analyzed from these 24-hour immune guinea pigs, trends in expression of certain genes was observed. The trend was specifically observed in genes related to the tick establishing the feeding pool, such as protease inhibitors, serpins, histamine binding proteins and Salp14. These results may prove to be beneficial moving forward into vaccine development.

Acquired anti-tick immunity also develops in rabbits. Given that lagomorphs are a more natural host for ticks in North America, the study of this immune response is more applicable to vaccine design. It has been observed in our lab and others that rabbits can only be used, at the most, two separate times for feeding adult ticks in ear bags as subsequent tick feedings will result in lower tick engorgement rates and longer feeding times. When this robust response was analyzed, a strong humoral response was seen, with the serum reacting to *I. scapularis* salivary antigens (Narasimhan et al. 2007). The same study also analyzed the protective ability of passively transferred antibody to C3H

mice. When rabbit serum was passively delivered to C3H mice, little difference was seen in engorgement weight and spirochete loads within the ticks recovered from both test and control groups. However, a significant decrease was seen in the transmission of *B. burgdorferi* to the test group compared to the serum control group.

The immune response that is involved in acquired anti-tick immunity tends to be a combination of cell-mediated and humoral immunity (Galbe and Oliver 1992). Cell-mediated immunity typically involves migration of basophils, neutrophils and eosinophils to the tick bite site, with the migration being mediated by T cells and antibodies specific for tick salivary components (Allen 1973; Brown and Askenase 1983). Cell mediators released by the influx of immune cells include serotonin, heparin, kinins, prostaglandins, and histamines, which may be responsible for tick rejection from the feeding site (Galbe and Oliver 1992).

A serosurvey was conducted in humans in Rhode Island from 1991-2000 to ascertain whether or not humans generate a humoral antibody response to repeated tick bites (Burke et al. 2005). Interestingly, of 1490 residents tested, 17% reported itching associated with tick bite, one of the hallmarks of acquired anti-tick immunity. Acquisition of Lyme disease increased from the 15% average to 25-31% in individuals who reported no tick-associated itch. The incidence of Lyme disease decreased to 13% in individuals who reported 3 episodes of tick-associated itch, and to 10% in those with greater than 4 episodes. This suggests that individuals with persistent tick-associated itch are less likely to develop Lyme disease, as a delayed-type hypersensitivity response generated by these individuals will reduce the incidence of Lyme disease in the individual.

Given the knowledge from the animal model work and the indication of acquired anti-tick immunity in humans, as well as the fact that humans are an unnatural host for *I. scapularis*, anti-tick vaccine development for humans may be feasible, albeit difficult. Current research in animal models that exhibit anti-tick immunity is exploring which specific gene products these animals are reacting to, and what the level of response is. Developing a multi-unit vaccine against ticks for human use utilizing the information gleaned from these molecular studies may prove to be highly effective in preventing transmission of not only Lyme disease, but also Anaplasmosis, Babesiosis and Tick-Borne Encephalitis, all of which are transmitted to humans by *I. scapularis*.

Tick strategies to overcome host immunity

Hemostasis after tissue injury is the first host response which must be overcome by ticks; and is prevented by a mixture of several compounds present in tick saliva (Ribeiro 1989; Ribeiro 1995), This mixture contains multiple physiologically active substances including anticoagulants and vasodilators; as well as inhibitors of platelet aggregation induced by the presence of collagen, arachidonic acid, adenosine diphosphate (ADP), or thrombin (Andersen et al. 2005; Champagne 2005; Ribeiro 1995; Ribeiro and Francischetti 2003; Valenzuela 2004). ADP is released by injured cells and dense granules of activated platelets and is one of the most important physiological mediators for the recruitment and aggregation of platelets and the formation of platelet plugs (Benoit and Dogne 2003). Hematophagus arthropods have evolved the ability to block this ADP-mediated activation of platelets through several different salivary proteins (Ribeiro 1995). Apyrase, an enzyme which hydrolyzes ADP, has been identified in

almost all blood-feeding insects examined (Valenzuela et al. 1998). Ornithodorin has been identified from *Ornithodoros moubata*, and in hard ticks boophilin has been characterized from *Boophilus microplus*. Ixolaris (Francischetti et al. 2002) and penthalaris (Francischetti et al. 2004) are two novel Kunitz-type serine-protease inhibitors from *I. scapularis* which have anticoagulant properties similar to host tissue factor pathway inhibitor (TFPI). TFPI functions by binding FX and FXa, inhibiting the formation of the serine protease factor IIA/tissue factor complex that ultimately catalyzes the formation of thrombin (Riewald and Ruf 2002). FXa is also capable of binding to effector cell protease receptor-1 inducing vascular permeability and leukocyte exudation. Another molecule identified in *I. scapularis* is Salp14, which is a non-Kunitz-type anticoagulant involved in blocking the intrinsic pathway of coagulation by inhibiting factor Xa (Das et al. 2001; Narasimhan et al. 2002). Recent studies have demonstrated that the serine protease inhibitors from the Kunitz family also have a strong role in preventing inflammation and tissue injury as well as promoting tissue remodeling (Shigetomi et al. 2010).

Inflammation can be typically defined by the triple response of Lewis: pain, redness and heat, with redness and heat being due to vasodilatation. Tick saliva contains immunomodulatory compounds that prevent inflammatory reactions from disrupting the feeding process by creating an environment that allows for blood flow without inducing pain (Francischetti et al. 2005; Wikel 1999; Wikel and Alarcon-Chaidez 2001). *In vivo* and *in vitro* modulation of T cell cytokine production by tick saliva or during tick infestation has been described. Macrophage production of the inflammatory cytokines IL-1 and TNF-α is decreased in the presence of salivary gland extracts from different tick

species (Gwakisa et al. 2001; Ramachandra and Wikel 1992). Dendritic cell production of IL-12 and TNF-alpha is also strongly decreased in the presence of tick saliva (Cavassani et al. 2005; Sa-Nunes et al. 2007). Lymphocyte production of IL-2 and IFN-γ are diminished in the presence of *D. andersoni* salivary gland extracts (Ramachandra and Wikel 1992). Tick saliva inhibited Con A-induced (Cavassani et al. 2005) and OVA-induced IL-2 production by T cells (Sa-Nunes et al. 2007). An *I. ricinus* immunosupressor (Iris) has been shown to inhibit the production of several inflammatory cytokines (Gillespie et al. 2001; Leboulle et al. 2002). An IL-2 binding protein was described in *I. scapularis* saliva, which decreases the availability of this cytokine and affects T-cell proliferation (Gillespie et al. 2001). In addition, splenocytes from mice infested with *I. scapularis* nymphs presented decreased production of IL-2 and IFN-γ and increased production of IL-4 and IL-10 when stimulated with concanavalin A (Schoeler et al. 1999; Zeidner et al. 1997), suggesting that tick infestation polarizes the adaptive immune response to a Th2 profile (Ferreira and Silva 1999), which may prevent adequate immune recognition of pathogens during transmission (Wikel 1999). Despite the extensive literature on the tick modulation of cytokine production, the characterization of molecules controlling such activities is still in the early stages, but increasing numbers of these molecules have been characterized in the last few years.

Vasodilatation may seemingly be beneficial to the tick and the development of the feeding pool; however, it instigates immune cascades that may ultimately prevent successful feeding. Controlled vasodilatation is therefore in the tick's best interest. Prostaglandin E_2 (PGE_2) has been identified as a vasodilator essential in keeping the blood pool available to the tick (Ribeiro et al. 1985), and also has a profound regulatory

effect on host immunity. PGE_2 from *I. scapularis* is also an inhibitor of dendritic cell maturation and function. *In vitro* work with bone marrow derived dendritic cells demonstrated dose-dependent inhibition of IL-12 and TGF-β when exposed to PGE_2 (Sa-Nunes et al. 2007). PGE_2 from *B. microplus* has been demonstrated to inhibit mononuclear cell response to artificial mitogens, theoretically down regulating further IL-2 production by T cells (Inokuma et al. 1994). Sialostatin L and sialostatin L2 have been identified in *I. scapularis* (Kotsyfakis et al. 2008; Kotsyfakis et al. 2007; Kotsyfakis et al. 2006) and are functional cystatins which have anti-enzymatic activity against a limited number of vertebrate papain-like cysteine proteases, specifically capthesin L and S (Kotsyfakis et al. 2007). The effect of these type 2 cystatins in tick feeding is the reduction of the inflammation and proliferation of cytotoxic T lymphocytes (CTL) (Kotsyfakis et al. 2006). Further research with sialostatin L indicates that it has the potential to reduce LPS-induced TNF-α and IL-12 production by inhibiting maturation of dendritic cells. In a murine autoimmune encephalitis model, this action of sialostatin L significantly prevented disease symptoms, potentially serving as a new therapy for autoimmue disease (Sa-Nunes et al. 2009). Sialostatins have also been identified in *I. ricinus* (Chmelar et al. 2010) and *Ornithodoros moubata* (Salat et al. 2010), indicating they may induce a broad range of down-regulating effects on host immunity after tick bite which may potentially effect pathogen transmission delivered by ticks. Salp25D has been identified in *I. scapularis* as an amine-binding protein capable of countering the inflammatory and vasoactive amines produced in the inflammation response (Das et al. 2001). Salp25D has a high homology to existing invertebrate and vertebrate glutathione peroxidases. Reactive oxygen species (ROS) produced in the vertebrate host of ticks

during normal metabolism and are capable of causing cellular damage (Singh and Shichi 1998). During injury, ROS attract neutrophils to the wound, allowing for healing to begin. Glutathione, glutathione peroxidase and glutathione reductase act together to counter the negative effects of ROS on tissues (Steiling et al. 1999). This is a beneficial molecule for a feeding tick to express as it will limit the host's attempts to heal the feeding site of the tick. Pain and itch responses are also critical to overcome to prevent host awareness of the tick, and *I. scapularis* saliva contains a multitude of proteins to counteract these host defenses. Of interest is a carboxypeptidase from *I. scapularis* which destroys bradykinin preventing pain and edema (Ribeiro and Mather 1998). Bradykinin is a cystatin produced during the intrinsic pathway of blood coagulation (Millan 1999), inducing the release of TNF-α by neutrophils, stimulating the release of IL-1β and IL-6 from various cell types, which leads to hyperalgesia (Cunha et al. 1991; Cunha et al. 1992).

The ability of *I. scapularis* to inhibit complement activity by binding C3 convertase of the alternative complement cascade in the host thus blocking the production of inflammatory anaphylactic toxins is mediated by a protein termed Isac (Valenzuela et al. 2000). Isac acts in a similar manner to known regulators of complement activation, decay accelerating factor and factor H, although it belongs to a new class of complement regulatory molecules. Its action is involved in the inhibition of the interaction of factor B with C3b (Valenzuela et al. 2000). Two similar proteins have been isolated and characterized from *I. ricinus*, and it seems that all ticks in the *I. ricinus* complex analyzed to date have related anticomplement proteins (Daix et al. 2007). One of the more well studied salivary proteins from *I. scapularis* is termed Salp15. Salp15 has the capability to

inhibit $CD4^+$ T cell activation via repression of calcium fluxes triggered by T cell receptor ligation which translates to lower production of IL-2 (Anguita et al. 2002), a cytokine critical in promoting growth of both B and T cells as well as an inflammatory T cell response from naïve T cells. Salp15 is also capable of down regulating CD25 expression on T cells (Anguita et al. 2002), which results in poor IL-2 signaling as CD25 is the high affinity subunit of the IL-2 receptor. Salp15 is a ligand of CD4, preventing the activation of $CD4^+$ T cells (Garg et al. 2006) compromising the host immune response by causing conformational changes to T cells which negatively affect intracellular signaling and consequently inhibits cell propagation and proliferation (Ashish et al. 2008).

This is just a brief look into the both broad and deep literature base that is focused on specific salivary proteins of ticks and their function. Certainly, ticks have evolved a complex, and sometimes redundant, myriad of proteins to mask their presence while feeding on vertebrate hosts to ensure a successful and complete blood meal. Ticks have also evolved a number of molecules with dual function as a means to facilitate their ability to take a blood meal while inducing complex changes to host immunity in this process. The pathogens which are transmitted by ticks have also evolved to take advantage of the immunomodulation that the tick drives, enhancing vector competence (Wikel 1999).

Pathogen-vector interactions

The complexity of vector-borne disease also involves the interplay between the vector and the infectious agent. A well-defined example of an interaction between *B.*

burgdorferi and *I. scapularis* is that of OspA-TROSPA. OspA is an outer surface protein of *B. burgdorferi* which has been shown to be highly upregulated during infection of the tick (de Silva et al. 1996). OspA-deficient spirochetes fail to colonize and survive within *I. scapularis*, and cannot bind to the tick gut (Yang et al. 2004). The tick midgut is the primary site where *B. burgdorferi* persists, especially in the long time period between tick blood meals (de Silva and Fikrig 1995). Persistence in the midgut by *B. burgdorferi* must be accomplished by some kind of pathogen-host contact, and that contact involves TROSPA. Tick receptor for OspA has been defined as the point of contact in the tick midgut for OspA (Pal et al. 2004a). This study showed that TROSPA is predominantly localized in the intercellular spaces and luminal surface of the gut, especially along tight junctions between microvilli. Immunofluoresence studies showed that there is a colocalization of *B. burgdorferi* and TROSPA at these sites. The expression of TROSPA in the tick is not at a constant level. It is most highly expressed in the immature larval and nymphal stages compared to the adult stage, and is downregulated after a blood meal, paralleling OspA expression by *B. burgdorferi*. This temporal expression of TROSPA most likely has to do with transtadial transmission of *B. burgdorferi* and spirochete detachment from the midgut for transmission to a new host, respectively. It was previously thought that *B. burgdorferi* had little influence on the vector, but this study showed that ticks infected with *B. burgdorferi* expressing OspA had higher levels of TROSPA expression compared to uninfected ticks (Pal et al. 2004a). It is obvious that this interaction is critical for the spirochete life cycle and maintenance in the invertebrate host.

Another pathogen transmitted by *I. scapularis*, *Anaplasma phagocytophilum*, utilizes a different salivary protein to infect the tick's salivary glands. Unlike *B. burgdorferi*, which colonizes the midgut of the tick, *A. phagocytophilum* resides in the salivary glands until tick feeding commences (Telford et al. 1996), at which point the bacterium then migrates to the vertebrate host and infects granulocytes for further dissemination (Hodzic et al. 2001). Acquisition of *A. phagocytophilum* by the tick begins at two days post attachment, when the bacterium moves through the midgut to the salivary glands (Hodzic et al. 1998). Sukumaran, et al. observed a temporal relationship in the up regulation of Salp16 in the salivary glands from unfed *A. phagocytophilum* infected ticks; which was not seen in unfed and uninfected ticks at 24 hours post-attachment. When Salp16 expression was silenced in *I. scapularis* via RNAi, *A. phagocytophilum* acquisition was reduced 10-fold. Using Salp16 silenced ticks, Sukumaran, et al. further determined that Salp16 was not required for maintenance of infection or transmission of infection, just establishment of infection of the salivary glands (Sukumaran et al. 2006).

Pathogen-vector interactions most certainly extend beyond colonization of the invertebrate host into the transmission of the pathogen into the vertebrate host. It has been observed that the pharmacologic effects of tick saliva can enhance pathogen transmission (Gillespie et al. 2000). This can be observed indirectly, such as through the actions of Isac blocking the complement cascade, consequently inhibiting the membrane attack complex; or directly such as the ability of Salp15 to bind OspC on the surface of *B. burgdorferi* (Ramamoorthi et al. 2005). When salivary gland lysate is inoculated concurrently with *B.* burgdorferi the spirochete load as measured in the target organs of

heart, bladder and joint is much higher (Zeidner et al. 2002). Expression of Salp15, a tick salivary protein known to inhibit T cell activation, is up regulated in *B. burgdorferi* infected tick salivary glands during engorgement, to the point of a 13-fold higher expression, when compared to uninfected ticks. This expression is also specific to *B. burgdorferi*. To determine if Salp15 might interact with *B. burgdorferi* a gel overlay assay showed binding to OspC (Ramamoorthi et al. 2005). OspC is up regulated at the same point in tick feeding when OspA is down regulated, and facilitates migration to the salivary gland and infection of the vertebrate host (Grimm et al. 2004; Pal et al. 2004b). When spirochetes are preincubated with Salp15 prior to infection in mice, a higher spirochete load is seen in joints, skin and bladder as compared to spirochetes alone. *B. burgdorferi* preincubated with Salp15 is also more resistant to antibody-mediated killing as compared to spirochetes alone, and this protection allows for re-infection of previously immune mice (Ramamoorthi et al. 2005).

A more recently described interaction between *I. scapularis* and *B. burgdorferi* involves sialostatin L2 from the saliva of the tick. Co administration of sialostatin L2 with needle infected *B. burgdorferi* exacerbates skin infection of *Borrelia* in a murine model at a six-fold increase of spirochetes as measured by qPCR (Kotsyfakis et al. 2010). Like Salp15, sialostatin L2 is strongly induced during feeding, suggesting that it is important to successful tick feeding during the period in which spirochetes are being transmitted (Kotsyfakis et al. 2010). The mechanism of the facilitation of the increase of spirochetes within the skin has yet to be elucidated, as well as additional sialostain L2 targets outside of cathepsin L and S (see above).

The molecular interactions between infectious agents and arthropod vectors for colonization of the vectors have been elucidated for other diseases as well. Galactins play an important role for infection and survival of *Leshmania major* in the *Phlebotomus paptasi* midgut; and is considered the key protein for vector competence (Kamhawi et al. 2004). Studies in *Plasmodium berghei*, a species that infects rodents, have shown that the sporozoite-specific transmembrane protein TRAP (thrombospondin-related anonymous protein) is essential for sporozoite gliding, cell invasion and *in vivo* infectivity (Sultan et al. 1997). Sporozoite capacity to invade host cells is mechanistically related to their ability to glide on solid substrates, both activities depending on TRAP. Sporozoite invasion into mosquito salivary glands and the rodent liver, as well as penetration into human HepG2 and hamster CHO cells were all partially impaired by loss-of-function mutations in TRAP (Matuschewski et al. 2002).

Anti-Borrelia vaccine strategies

The first Lyme disease vaccine was licensed as LYMErix in December of 1998 and was withdrawn from the market February 26, 2002. This vaccine utilized recombinant OspA of *B. burgdorferi* as the immunogen. The theory behind this was that the vaccine recipient generated antibodies to OspA, and when a tick took a blood meal from a vaccinated individual, *B. burgdorferi* located in the tick midgut expressing OspA was opsonized and subsequently lysed, thus clearing infection from tick midgut. However, as previously discussed, OspA is downregulated upon uptake of a blood meal. If OspA antibodies encounter *B. burgdorferi* after this downregulation, the spirochetes

survive as they are now expressing OspC (de Silva et al. 1996). The efficacy rate of the vaccine was initially defined as 76% (Nigrovic and Thompson 2007), and there are many theories behind this relatively low rate, and most likely is due to a combination of causes; one theory being that given the genetic variability of OspA in different isolates of *B. burgdorferi*, antibodies generated to the recombinant OspA may not recognize variation within the protein sequence of other isolates (Nigrovic and Thompson 2007). Another cause is the fact that OspA is a concealed antigen, and antibodies act in the tick midgut, not in the host, therefore the natural boosting of the immune response from the infectious agent in the host's bloodstream is removed, resulting in low antibody titers over a course of time (Thomas and Fikrig 2002). An initial series of 3 inoculations over 12 months were necessary to reach the 76% efficacy rate; with only 2 inoculations lowering the efficacy to 40%, and patient compliance was low in response to receiving annual boosters (Brenner 2006). Other reasons may include genetic variability of vaccine recipients. It has been shown that OspA recognition in mice is dependent on Toll-like receptor (TLR) 1 and 2 (de Silva et al. 1996). Some individuals who received the vaccine had intrinsic defects in TLR-mediated lipoprotein signaling and could not mount an appropriate innate immune response involving macrophages; and subsequently had low levels of antibody (Alexopoulou et al. 2002). It has been shown that in humans, the ability of that person to generate an effective humoral response declines with age (Nordin and Makinodan 1974), so another possibility of reduced efficacy of this vaccine is the age of the vaccine recipients.

A serious issue that surrounded LYMErix was the safety of the vaccine. Treatment-resistant Lyme disease has long been an issue with *B. burgdorferi* infection.

This is defined as a case where an infected individual was treated with a full course of doxycyline, but still presents arthritis-like symptoms months to years' post initial infection. One factor involved in this issue is that these individuals tend to have a HLA-D4 genotype. This genotype predisposes carriers to rheumatoid-like arthritis (Gross et al. 1998) and these same individuals show high levels of autoantibody to OspA in the synovium (Nocton et al. 1994). Therefore when these individuals were given recombinant OspA, debilitating autoimmunity issues arose in some patients, but causality has been extremely difficult to prove (Nigrovic and Thompson 2007). The rest of the adverse events, a total of 92.6%, were described as not serious, and were no different than the typical adverse events to all vaccines (Lathrop et al. 2002). However, 26.8% of vaccine recipients, compared to 8.3% of controls, experienced these milder reactions. A media blitz ensued, and coined those who had suffered from adverse reactions as "vaccine victims". On December 14, 1999 the first class action lawsuit against the manufacturer, SmithKlineBeecham, was filed, and others followed. At this point the FDA met and decided to require the manufacturer to provide more vaccine safety and efficacy data, but did not remove the vaccine from public use. In 2001 what was already low levels of vaccine sales began to drop further, and the manufacturer, now GlaxoSmithKline, chose to withdraw the vaccine from the market. All class action lawsuits were settled on July 9th 2003 (Nigrovic and Thompson 2007).

Anti-tick and anti-vector vaccine strategies

It has been observed that people who develop a hypersensitivity immune reaction to the bite of *I. scapularis* appear to acquire Lyme disease less frequently than people

who do not experience an immune reaction (Burke et al. 2005). Experimentally, it has been demonstrated that antibodies generated against mosquito midgut lysates lowered vector competence, reducing transmission of malarial parasites (Lal et al. 2001), while antibodies to a sandfly midgut galectin eliminated sandfly vectored infections of leishmania in membrane feeding studies (Kamhawi et al. 2004). Bites from uninfected sandflies provide protection for mice against cutaneous leishmaniasis (Kamhawi et al. 2000), and seroconversion of humans against sandflies correlates with development of protective immunity to leishmaniasis (Gomes et al. 2002). Given this evidence, it is feasible an anti-vector vaccine could not only interrupt feeding, but also block transmission of pathogens by arthropod vectors.

The concept of identifying and characterizing tick protective antigens has become a fast-growing area of research since the vaccines for the control of *Rhiphicephalus (Boophilus) microplus*, TickGARD in Australia and GAVAC Plus in Cuba, designed to limit tick populations transmitting bovine Babesiosis entered the market in the early 1990s (de la Fuente and Kocan 2003; Willadsen 2004). The target explored in this anti-tick vaccine research was the midgut. The tick midgut is a relatively simple structure of a single layer of epithelial cells that sits on top of a thin basal lamina. The luminal surface of the midgut is covered with microvilli, while the distal plasma membrane is highly folded until feeding commences, allowing for distension of the midgut. All digestion of the blood meal is intracellular, with the meal being transported across cellular membranes by phagocytosis and micropinocytosis. This is a very slow process and enables the fed ticks to hold large quantities of the original meal for months to years (Sonenshine 1991). This absence of proteolytic enzymes in the midgut is ideal for survival of ingested

pathogen (Sonenshine 1991). The anti-*Boophilus microplus* vaccine is based on recombinant Bm86, a tick midgut antigen which is expressed on the surface of gut cell. The greatest effect of this vaccine is upon female fecundity. The number of ticks surviving to engorgement is reduced by 65% and the average weight of ticks which managed to engorge is reduced by 33%. 86% of those surviving ticks showed visible damage. Not surprisingly, the egg laying capacity of surviving female ticks, as measured by the conversion of the weight of engorged female ticks into eggs, is reduced from an average of 54% to 19% (Willadsen et al. 1989). This is considered to be a "concealed" antigen vaccine as antibody titers are not boosted by tick infestation, so sustained tick control does require booster vaccinations (Willadsen and Jongejan 1999).

Other explorations of tick midgut antigens as vaccine candidates include the P27/30 antigen from *Haemaphysalis longicornis*, a tick in East Asia and Australia which vectors theileriosis caused by *Theileria sergenti/buffeli/orientalis* among grazing cattle. In this study, the recombinant P27/30 (rP27/30) expressed in Escherichia coli was used to immunize mice and mice were challenge-infested with ticks at different developmental stages of the same species. Immunization did stimulate a specific protective anti-tick immune response in mice, demonstrated by the statistically significant longer pre-feeding periods in adult ticks, and significantly longer feeding periods in both larval and adult ticks. On the other hand, only larval ticks exhibited low attachment rates (31.1%) (You 2005). This vaccine attempt demonstrated only intermediate success at blocking tick infection.

Sialostatin L2 is considered to be a "silent" antigen and has shown potential for an anti-*Ixodes* vaccine. As mentioned above, this molecule is a potent inhibitor of capthesin

L and S, and is greatly up regulated in the salivary gland during tick feeding, as opposed to sialostatin L, which is down regulated in both the midgut and salivary gland (Kotsyfakis et al. 2007). It is considered a silent antigen due to the fact that antibody to sialostatin L2 is undetectable by Western analysis at physiological levels seen in the saliva in a murine model (Kotsyfakis et al. 2008), although when injected into mice at superphysiological levels, anti-sialostatin L2 antibody can be detected (Kotsyfakis et al. 2007). When this molecule is silenced in *I. scapularis* female ticks by injecting dsRNA, the recovered females showed an 80% decrease in sialostatin L2 transcript levels. 40% of all the silenced ticks which were allowed to attach to rabbits were unable to feed and died, with obvious inflammation at the bite site of the dead ticks. The remaining 60% of ticks that did attach and attempt to feed displayed a reduction in engorged weight (60mg compared to 170 mg from control ticks) and a 70% inhibition of egg-laying (Kotsyfakis et al. 2007). The rabbits that had been fed upon by the silenced ticks were maintained and clean, wild-type female ticks were allowed to feed on these rabbits two weeks after the first infestation. The attached ticks fed poorly and were unable to engorge, and an inflammatory response was visible at the tick attachment site. Rabbits from the control ticks allowed normal and complete feeding of the clean, wild-type female ticks (Kotsyfakis et al. 2007). To test the viability of sialostatin L2 as a vaccine candidate, 100 ug of recombinant sialostatin L2 was injected into guinea pigs four times during a two week period. Two weeks after the final injection, twenty nymphal ticks were placed on vaccinated mice and allowed to feed. Early rejection was observed in the vaccine group at a rate of three times higher than observed in the control group. The ticks that did manage to remain attached triggered apparent signs of inflammation at 72 hours post-

attachment, and displayed a statistically significant increase in feeding duration and a decrease in final engorgement weight, both of which are considered signs of less successful tick feeding (Kotsyfakis et al. 2008).

Another molecule which has been explored for vaccine potential is Salp15. Given the inhibitory effect of Salp15 on $CD4^+$ T cells and the molecule's ability to bind OspC, it is a likely candidate. When mice were passively immunized with anti-Salp15 rabbit serum, the number of spirochetes in the heart and joint was significantly reduced, and 40% of mice were fully protected three weeks post challenge with 10^3 spirochetes (Dai et al. 2009). When anti-OspA antibody was added to anti-Salp15, the reduction was increased to 70% protection at 7 days in skin. The researchers then combined anti-Salp15 antibodies with anti-OspC antibodies, and achieved a 80% protection rate at 21 days (Dai et al. 2009), demonstrating a relatively high effect on *B. burgdorferi* transmission.

Subolesin, a salivary molecule which has homology to existing endopeptidases, has been identified in *I. scapularis, I ricinus, D. marginatus, D. variabilis, A. americanum, R. microplus, Hy. M. marginatun,* and *H. punctata* (Almazan et al. 2005a; Almazan et al. 2003; de la Fuente et al. 2006). The tick protective antigen, also known as 4D8, was discovered by cDNA expression library immunization (ELI) and analysis of expressed sequenced tags (EST) in a mouse model of tick infestations for identification of cDNAs protective against *I. scapularis* (Almazan et al. 2003). The gene and protein sequences were found to be conserved in invertebrates and vertebrates and the gene was expressed in all tick developmental stages and in adult tissues of several tick species, suggesting a conserved function for 4D8 (Almazan et al. 2005a). Further investigation

utilizing RNAi of subolesin in feeding ticks caused degeneration of the gut, salivary and reproductive tissues, and further showed a >90% reduction in oviposition in all tick species tested indicating a critical role of this endopeptidase in the tick life cycle (de la Fuente et al. 2006). Immunization with recombinant *I. scapularis* subolesin reduced larval, nymphal and adult tick infestations at approximately 71% efficacy supporting the use of this molecule for development of anti-tick vaccines (Almazan et al. 2005a; Almazan et al. 2005b).

Cement proteins secreted in saliva by ticks function to keep the mouthparts embedded in the host's dermis until feeding is complete. When recombinant forms of a cement protein from *R. appendiculatus* were injected into guinea pigs and hamsters which were then challenged by ticks, the animals developed a strong humoral and delayed-type hypersensitivity (DTH) reaction to the tick bite (Trimnell et al. 2002). This reaction resulted in death of immature and adult engorged ticks in hamster, guinea pig and rabbit models with several tick species including *I. ricinus* (Trimnell et al. 2005; Trimnell et al. 2002). When the cement protein as a vaccine was explored in a tick-borne encephalitis virus (TBEV) mouse model with *I. ricinus* ticks, only 48% of experimental mice supported virus transmission to nymphs in a cofeeding model compared to 95% in controls. Only 16% of the nymphs from the experimental mice became infected through cofeeding, compared to 51% in controls. Furthermore, 46% of vaccinated mice survived an otherwise fatal TBEV infection compared to 15% of control mice (Labuda et al. 2006).

Leishmania has also seen success in transmission-blocking anti-vector vaccines based upon salivary molecules beginning with maxidilian from *Lytzomyia longipalpis*.

Maxidilian is a vasodilator which greatly exacerbates disease when coinoculated with *Leishmania major* (Morris et al. 2001). When synthetic maxadilian was injected subcutaneously into mice with complete Freund's adjuvant, and boosted with maxidilian alone two weeks later, in subsequent challenge with *L. major* alone or *L. major* plus sandfly saliva, vaccinated mice had cutaneous lesions 3- to 5-fold smaller than control mice and lesions healed 15 days faster than controls. The parasite burden was also markedly decreased (Morris et al. 2001). Valenzuela, et al immunized mice with a single protein isolated by SDS-PAGE named SP15 from *Phlebotomus papatasi* and challenged mice with *L. major* plus salivary gland homogenate (SGH) and demonstrated significantly smaller lesions and lower parasite load nine weeks postinoculation compared to controls (Valenzuela et al. 2001). A DNA vaccine of SP15 produced consistent results plus intense humoral and DTH reactions, with the protective effect of the vaccine lasting 3 months post vaccination (Valenzuela et al. 2001). In a hamster model of visceral leishmaniasis, Gomes et al found a specific cDNA that induced DTH from *L. longipalpis* named LJM19. Hamsters vaccinated intradermally with LGM19 and subsequently challenged with *L. infantum* plus (SGH) showed a marked and significant survivorship from visceral leishmaniasis (Gomes et al. 2008). Also, LJM19-immunized hamsters maintained a low parasite load that correlated with an overall high IFN-γ/TGF-β ratio and inducible NOS expression in the spleen and liver up to 5 months postinfection. Importantly, a DTH response with high expression of IFN-γ was also noted in the skin of LJM19-immunized hamsters 48 h after exposure to uninfected sand fly bites. Induction of IFN-γ at the site of bite could partly explain the protection observed in the viscera of LJM19-immunized hamsters through direct parasite killing and/or priming of anti-

Leishmania immunity (Gomes et al. 2008). These results further reinforce the concept of using components of arthropod saliva in vaccine strategies to induce a strong DTH and/or IFN-γ response against vector-borne diseases.

Adenovirus vector vaccines and induction of immune responses

Successful vaccine design resulting in protection against disease, particularly intracellular pathogens, is directly related to the concentration of cytotoxic T lymphocytes (CTL) generated in response to the vaccine (Jin et al. 1999; Schmitz et al. 1999; Shiver et al. 2002). Live viral vectors, including those based on Adenovirus (Ad), modified vaccinia virus Ankara (MVA), canarypox, vesicular stomatitis virus, and herpes virus have all been reported to elicit protective T-cell-mediated immunity (CMI). A robust CTL response suppresses viral replication as a result of infected cell destruction and subsequently may prevent the emergence of mutant viruses as well (Gomez-Roman and Robert-Guroff 2003; Robinson 2002). The ability of Ad to induce mucosal immunity (Gallichan and Rosenthal 1996), directly infect antigen-presenting dendritic cells (Jooss et al. 1998; Kirk et al. 2001a; Kirk et al. 2001b; Kirk and Mule 2000), induce 5-10 fold more CTL than MVA and others (Casimiro et al. 2003a; Yang et al. 2003), and elicit higher protective effectiveness on a per-CTL basis (Shiver et al. 2002), gives Ad vectors an advantage over other viral vectors. When modified for vaccine delivery, recombinant Ad does not down regulate MHC molecules as compared to wild-type Ad (Zhong et al. 1999). Infection of dendritic cells (DCs) with a recombinant Ad vector expressing a gene of interest can subsequently present viral proteins on the cell surface via major histocompatibility complex (MHC) I expression, which will drive formation of $CD8^+$

cytotoxic T-lymphocytes (CTL) (Zhong et al. 1999). These vectors generate a helper T cell type I (Th1) response and may also induce high-titer antibody responses against encoded proteins (Sullivan et al. 2000). Another benefit of Ad vectors is that they do not integrate into the host genome (Kreppel and Kochanek 2004). Ad-based vaccines have been delivered orally, intranasally, and by injection. Ad virions do not have an envelope, rendering them more stable than other vectors. In addition, Ad vectors can be lyophilized and stored without losing their structural integrity (Gomez-Roman and Robert-Guroff 2003). Furthermore, liquid formulations of Ad vector vaccines are stable, can be stored for long periods of time, and can be delivered to inclement conditions such as high or freezing temperatures (Evans et al. 2004).

However, the drawbacks of Ad vectors, particularly first-generation Ad vectors, are considerable. Most of the first-generation, and a number of second-generation Ad vectors are derived from Ad serotype 5. To create vaccine vectors, the early genes for E1 (E1A or E1B) were deleted, which have a variety of functions in the viral life cycle and are the primary transforming proteins of the virus (Flint and Shenk 1997). It was initially believed that deletion of the regions made the virus incapable of replicating its DNA. However the effects of deletion of either E1A or E1B on viral replication *in vitro* can be overcome by infecting with a high multiplicity of virus (Jones and Shenk 1979; Mittereder et al. 1994). Most first-generation vectors are also deleted for the E3 region. E3 is not required for growth in tissue culture and its deletion provides additional room for insertion of foreign genes. E3 encodes a variety of proteins that act to inhibit innate and adaptive immune responses to the virus during productive infections (Lichtenstein et al. 2004). While the anti-inflammatory and immune inhibitory effects of E3 are of clear

importance for the growth of the wild-type virus, the roles for E3 in protecting Ad vector-transduced cells from innate and adaptive immune responses are less clear. E3, or certain E3 proteins, have been found to be protective in certain situations (Harrod et al. 1998; Ilan et al. 1997) but not in others (Gantzer et al. 2002; Schowalter et al. 1997).

Other drawbacks include the fact that Ad vectors, especially first-generation vectors, are highly inflammatory in spite of the evidence that natural Ad infections may not be very inflammatory, in part due to the use of a cytomegalovirus (CMV) promoter (Lichtenstein and Wold 2004). The immunologic response to first-generation Ad vector vaccines is limited by pre-existing immunity of the vaccinee to Ad (Casimiro et al. 2003b; Yang et al. 2003). It has been reported that 40% to 97% of humans have neutralizing antibodies to Ad5 (Chirmule et al. 1999; Vogels et al. 2003) the most widely used serotype for gene transfer vectors, and that two-thirds of humans studied have lympho-proliferative responses against Ad (Chirmule et al. 1999).

There are numerous mechanisms by which pre-existing immunity interferes with Ad vectored vaccines but the simplest is the presence of neutralizing antibody followed by CMI elimination of Ad infected APC. Several approaches have been proposed to overcome the barrier of pre-existing anti-vector immunity. The most straightforward approach is to increase the vector vaccine dose, but as mentioned above, there is evidence that increasing vaccine doses can allow the otherwise replication-deficient virus to replicate and also may increase induction of undesired CMI responses (Barouch et al. 2003). This was unfortunately observed in the death of an 18-year old boy who was undergoing Ad based gene therapy and was given a dose of 3.8×10^{13} virus particles and subsequently suffered hepatotoxicity and acute inflammatory response due to the

activation of innate immunity (Raper et al. 2003). Therefore, investigators using first-generation Ad vector vaccines either use the approach of a naked DNA prime and an Ad vector boost (Yang et al. 2003), or chimeric Ad vectors bearing epitopes from rare Ad subtypes, such as Ad35, or even from different species (Thorner et al. 2006; Zhi et al. 2006). The question does rise whether receiving an Ad-based vaccine for one disease would terminate the use of Ad vector immunization in the individual for all other Ad-based vaccines? Further work developing second-generation Ad vectors has begun to overcome these limitations, and may provide solutions to the issues mentioned above.

Use of DNA vaccines

In 1990, a seminal study showed that the injection of a DNA plasmid in mouse muscle resulted in a significant expression of the protein encoded by the plasmid (Wolff et al. 1990). Starting with this discovery, various antigens encoded by plasmids have been successfully used to induce the production of antibodies (Cox et al. 1993; Tang et al. 1992) and cytotoxic T lymphocytes (Ulmer et al. 1993), demonstrating the potential of this strategy for DNA vaccination and gene therapy. Progress in this field has resulted in the development and the marketing of three veterinary DNA vaccines since 2005. Two of them are authorized for use in the United States: one targets the West Nile virus infection in horses (Davidson et al. 2005) and the other targets canine malignant melanoma (Bergman et al. 2006). The third vaccine, authorized for use in Canada in salmon, is directed against the infectious hematopoietic necrosis virus (Garver et al. 2005).

The mechanisms of immune stimulation by DNA vaccines have not clearly elucidated. Studies demonstrate that the quantity of antigen produced in vivo after DNA inoculation is in the nanogram to picogram range. Given the relatively small amounts of protein synthesized by DNA vaccination, a likely explanation for the efficient induction of a broad-based and sustained immune response is the immune-enhancing properties of the DNA itself. Unmethylated CpG sequences that are present in certain plasmids are recognized by Toll-like receptor 9 (TLR9), which leads to an adjuvant effect through the activation of the innate immune system (Sato et al. 1996). However, studies have shown that TLR9 or CpG are not necessarily essential for the induction of innate immunity after the injection of a DNA vaccine (Babiuk et al. 2004; Spies et al. 2003). In fact, CpG can play a role independently of TLR9 in primary B cells (Zhu et al. 2009) and TANK-binding kinase-1 signaling pathways can be activated by dsDNA *in vitro* (Peters et al. 2002; Stetson and Medzhitov 2006) and *in vivo* (Shirota et al. 2009). dsDNA can therefore serve as an adjuvant and since it induces T-cell responses, dsDNA is essential for the immunogenicity of DNA vaccines (Ishii et al. 2008).

There are at least three mechanisms by which the antigen encoded by plasmid DNA is processed and presented to elicit an immune response. One possibility is direct priming by somatic cells (myocytes or any MHC class II-negative cells); another is direct transfection of professional APCs; and thirdly, cross-priming in which plasmid DNA transfects a somatic cell and/or professional APC and the secreted protein is taken up by other professional APCs and presented to T cells (Gurunathan et al. 2000).

The advantages of DNA vaccines as compared to current vaccines are worth consideration in vaccine design. DNA vaccines mimic the effects of live attenuated

vaccines in their ability to induce major histocompatibility complex (MHC) class I-restricted $CD8^+$ T-cell responses (Gurunathan et al. 2000). Also, DNA vaccines can be manufactured in a relatively cost-effective manner and stored with relative ease, eliminating the need for a "cold chain" (Gurunathan et al. 2000). The use of the DNA approach has also promised to overcome the safety concerns associated with live vaccines, being the reversion to an infectious status (Ruprecht 1999). This approach may also avoid the risks of killed vaccines, as seen with the tainting of a polio vaccine with live polio virus due to production error (Offit 2005). DNA vaccines have experienced a recent resurgence of interest due to technical improvements; specifically, gene optimization strategies, improved RNA structural design, novel formulations of adjuvants and more effective delivery approaches (Kutzler and Weiner 2008). The use of species-specific codon optimization has shown to increase protein production on a per-cell basis which can lead to enhanced T-cell responses (Cheung et al. 2004; Frelin et al. 2004; Ramakrishna et al. 2004), and antibody induction (Cheung et al. 2004; Yadava and Ockenhouse 2003). Also, this methodology allows for multiple antigens to be targets in various combinations, often within the same delivery vector (Carvalho et al. 2009). This may be particularly useful in diseases such as Lyme disease where the infectious agent or vector of disease expresses various antigens during different life cycle stages. In comparison to viral vectored vaccines, DNA vaccines also have an advantage in that they do not induce anti-vector immunity in the host (Ledgerwood and Graham 2009).

The disadvantages of DNA vaccines are the number of safety concerns which have been raised about the use of DNA vaccines. These include the possibility that these vaccines may integrate into the host genome, increasing the risk of malignancy by

activating oncogenes or inactivating tumor suppressor genes. DNA vaccines may induce immune responses against transfected cells, thereby triggering the development of autoimmune disease. Another concern is the possibility of inducing antigen tolerance rather than immunity, and/or stimulating the production of cytokines that alter the host's ability to respond to other vaccines and resist infection (Klinman et al. 1997). Plasmids can also persist at the site of injection for many months. They can also be found far from the original site of injection, possibly carried by transfected lymphocytes or macrophages (Martin et al. 1999). Concerns that DNA vaccines might promote the development of autoimmune diseases arise from the immunostimulatory activity of CpG motifs in the plasmid backbone. It has been known for many years that bacterial DNA can induce the production of anti–double-stranded-DNA auto-antibodies in normal mice and accelerate the development of autoimmune disease in lupus-prone animals (Gilkeson et al. 1995; Gilkeson et al. 1993; Steinberg et al. 1990). Balancing these safety concerns is the observation that toxicity has not been reported among normal animals treated with therapeutic doses of DNA vaccines. In addition, hundreds of human volunteers have been exposed to plasmid DNA vaccines without serious adverse consequences (Gurunathan et al. 2000). According to www.clinicaltrials.gov, there are currently 615 DNA vaccine trials underway. Trials include Dengue virus, HIV, metastatic breast cancer, pandemic influenza, and several focused on safety and efficacy of novel vaccine vectors and delivery mechanisms in humans.

Conclusions

As research continues to expand into the interactions between the spirochete, the host and the tick, more molecules of interest will be defined. The importance of tick saliva in host immune regulation, specifically down regulation of the inflammation response, has been a focus of much research since the mid-1980s. The full effects of this down regulation are seen most predominantly in a tick's natural host. As previously discussed, there is a very specific interaction between a natural vertebrate host of a tick and the tick species' ability to continuously feed to repletion on said host. Evasion of recognition by the host's immune response is critical to the fitness of a tick species. Ticks are thought to have evolved saliva that inhibits the cutaneous immune response of their most common hosts (Ribeiro 1987). The classes of components in tick saliva include enzymes involved in preventing clotting of blood, enzyme inhibitors of coagulation enzymes, host protein homologues which inhibit different arms of the immune response, immunoglobulin-binding proteins, and cytokine expression modulators such as Salp15 (Steen et al. 2006).

As is the purpose of any research into infectious disease, it is the hope that the small steps made with each publication will advance our ability to treat and control disease. The knowledge of the delicate and specific interactions between pathogen and vector and vector and vertebrate host can advance the goal of treating and controlling disease. Knowing that there are specific components necessary in tick midgut and the surface of *B. burgdorferi* to maintain the pathogen throughout molting and blood meals could provide new targets for vaccination. Previous attempts to vaccinate against OspA proved successful in the lab and clinical trials, if not in real-life due to economic

limitations. Perhaps blocking of TROSPA could reduce the morbidity caused by Lyme disease. The vaccine against Bm86, a midgut protein of *Rhipicephalus* (*Boophilus) microplus*, has proven to be very successful in cattle. More promising targets include those involved in the tick saliva-pathogen interaction as these occur in the vertebrate's bloodstream. It would be ideal to block the spirochete's ability to mask itself with salivary proteins by generating a host immune response to the tick salivary proteins. Also, utilizing the molecules in the saliva which allow for successful feeding in ticks via immune evasion as vaccine immunogens could prove to be highly effective. Given that most individuals aren't aware of a tick bite, vaccination could prove to be the best route for prevention of Lyme disease.

Chapter 2

Construction of adenoviral vectored *Ixodes scapularis* salivary protein targeted vaccines

Introduction

Adenoviridae are nonenveloped viruses with a 30- to 40-kb linear double-stranded DNA genome that has been extensively studied and developed for vaccination and gene therapy applications. Replication deficient adenovirus vectors (Ad vectors) have several advantages, including the ability to package large quantities of DNA, ease of production and broad cell tropism (Wilson 1996). First-generation Ad vectors have been designed from wild-type Adenovirus serotype 5 by deleting the E1 and E3 regions. E1, comprised of E1A and E1B, is the first region of the virus to be expressed in wild-type infection. Its function involves replication of the virus as E1 contains the primary transforming proteins of the virus and packaging machinery (He et al. 1998). E1A activates viral transcription and re-programming of cellular gene expression to provide an optimal environment for viral replication (Flint and Shenk 1989). E1A also promotes entry into and passage through the cell (Frisch and Mymryk 2002). The E1 deletion does keep intact the packaging signals for virus production, which is critical for viral encapsulation (Bett et al. 1994). Due to the removal of genes critical in replication of Ad, production of the Ad vector virus can therefore only occur in a cell culture line derived from human embryonic kidney cells that was transformed to contain the E1 region of the adenovirus genome, known as HEK-293 cells (Graham et al. 1977). It was initially believed that the

deletion of both E1A and E1B would make the virus incapable of replication, but it has more recently been shown that this deficiency can be overcome when cells are infected at a high multiplicity (Marienfeld et al. 1999). The E3 region encodes a variety of proteins that act to inhibit innate and adaptive immune responses to the virus during productive infections (Lichtenstein et al. 2004). E3 is not required for growth in tissue culture and its deletion provides additional room for insertion of foreign genes (Schaack 2005).

Initial work with adenovirus as a vector for vaccines or gene therapy was limited as there was no simple procedure for generating vectors with both E1 and E3 deletions (Berkner 1992). Bett et. al. had observed that the adenoviral genome has the ability to circularize in infected cells, and chose to exploit this phenomenon to generate infectious, circular Ad genomes that can be propagated as bacterial plasmids (Bett et al. 1994). In this method, the foreign DNA is inserted into a small shuttle plasmid that contains the left-most end of the Ad genome including the inverted terminal repeat (ITR), the packaging signal and a multiple cloning site in place of the E1 for foreign DNA insertion. The second plasmid contains the entire Ad genome modified to be non-infectious via deletion of E3, packaging genes and sigma factors (Bett et al. 1994). Due to the deletion of the E1 region, complementation of these gene products from HEK-293 cells is required for viral propagation. Co-transfection of HEK-293 cells with the two plasmids will result in homologous recombination between overlapping Ad sequences, but this occurs at a very low level. Ng et. al. further refined this system by increasing the efficiency of recombination of the two plasmids by 20-30 fold by substituting Cre/*lox*P mediated recombination for homologous recombination (Ng et al. 1999). The same group further enhanced the efficiency of recombination 100-fold by inserting a head to

head ITR in the shuttle plasmid (Ng et al. 2000b). Further manipulations of the two-plasmid rescue system utilizes the yeast recombinase FLP/*frt* which is equally efficient for the two plasmid system, and serves as an alternative to the Cre/*lox*P system when the latter is undesirable for vector construction (Ng et al. 2000a).

Adenovirus elicits protective T-cell-mediated immunity (CMI), which suppresses viral replication as a result of infected cell destruction and subsequently may prevent the emergence of mutant viruses (Gomez-Roman and Robert-Guroff 2003; Robinson 2002). Ad vectors have the ability to induce mucosal immunity (Gallichan and Rosenthal 1996), directly infect antigen-presenting dendritic cells (Jooss et al. 1998; Kirk et al. 2001a; Kirk et al. 2001b; Kirk and Mule 2000) and elicit high protective effectiveness on a per-CTL basis (Shiver et al. 2002). When modified for vaccine delivery, recombinant Ad does not down regulate MHC molecules as compared to wild-type Ad (Zhong et al. 1999). Infection of DCs with a recombinant Ad vector expressing a gene of interest can subsequently present viral proteins on the cell surface via MHC I expression, which will drive formation of $CD8^+$ CTL (Zhong et al. 1999). These vectors generate a Th1 response and may also induce high-titer antibody responses against encoded proteins (Sullivan et al. 2000). These benefits of Ad vectors in combination with the flexibility of the Ad vector system lend us to believe that this delivery mechanism for salivary proteins from *I. scapularis* as vaccine antigens will be highly effective and push the host to a strong CTL and humoral immune response against the selected antigens, therefore warrants further research as to the utility of these vaccine vectors for an anti-tick vaccine.

Methods and Materials

Genes of interest: Salp14, Salp15, Salp25A and Salp25D were obtained as cDNA plasmids from Erol Fikrig. Isac was obtained from a cDNA library made from *I. scapularis* salivary glands at 68+ hours post-attachment during tick feeding (courtesy of Andrias Hojgaard).

Virus generation with AdMax system: All Salp genes were amplified from their respective plasmids using primers with BamHI and EcoRI recognition sites built into their 5' and 3' ends respectively (Table 2.1). Amplicons were digested according to manufacturer's protocols with BamHI and EcoRI (New England Biolabs, Ipswich, MA) and ligated into corresponding overhanging ends on the carrier plasmid pDC515 from the AdMax system (Microbix, Toronto, Ontario, Canada). pDC515 carrying the genes of interest, or without genes of interest for AdEmpty control, were transformed into *E. coli* Top10F' cells (Invitrogen, Carlsbad, CA), and positive colonies were determined through PCR screening. Positive colonies were grown overnight in 250 ml of Luria-Burtani (LB) broth supplemented with ampicillin and plasmids recovered with a Maxi Kit (Qiagen, Valencia, CA). The helper plasmid, pBHGFrtΔE1,E3FLP was also transformed into Top10F' cells and grown overnight with plasmid recovery by maxiprep.

Table 2.1: Primers used to amplify genes of interest for Ad construct generation. Recognition sites for restriction enzymes are in lower case while priming sites are in upper case.

Salp14EcoR1senADMAX	aagaattcgccaccATGGGGTTGACCGGAACCATGC
Salp14BamH1AsenADMAX	ttggatccTCATAAGTTTTTCTCCTGATCTTTC
Salp15EcoR1senADMAX	aagaattcgccaccATGGAATCTTTCGTCGCAATGAAGG
Salp15BamH1AsenADMAX	ttggatccCTAACATCCGGGAATGTGGCCAACGC
Salp25AEcoR1senADMAX	aagaattcgccaccATGAAGCTTGTTTTAAGCTTGGCCG
Salp25ABamH1AsenADMAX	ttggatccCTAAAAGCCACTTATTTCAGTACAA
Salp25DEcoR1senADMAX	aagaattcgccaccATGGGTCCCCTGAACCTCGGCGATC
Salp25DBamH1AsenADMAX	ttggatccTCAGTCCATGGTTGTTCGGAGGTAG
OspAEcoRISenADMAX	aaGAATTCgccaccatgaaaaaatatttattgggaatagg
OspABamH1AsnADMAX	ttggatccGATGAAATTAAAAACGCTTTAAAA
Salp15EcoRV ADEASY	gaatgatatcATGGAATCTTTCGTCGCAATG
Salp15 SalI ADEASY	gaatgtcgacACATCCGGGAATGTGGCCAACGC
Salp15NotI ADEASY	gaatgcggccgcATGGAATCTTTCGTCGCAATG
Salp15NotIReverse ADEASY	gaatgcggccgcACATCCGGGAATGTGGCCAACGC
Salp14EcoRV ADEASY	gaatgatatcATGGGGTTGACCGGAACCATG
Salp14SalI ADEASY	gaatgtcgacTAAGTTTTTCTCCTGATCTTTCTTTG
Salp14NotIReverse ADEASY	gaatgcggccgcTAAGTTTTTCTCCTGATCTTTCTTTG
Salp25AEcoRv ADEASY	gaatgatatcATGAAGCTTGTTTTAAGCTTG
Salp25ASalI ADEASY	gaatgtcgacAAAGCCACTTATTTCAGTACAATTTG
Salp25DEcoRV ADEASY	gaatgatatcATGGGTCCCCTGAACCTCGGC
Salp25DSalI ADEASY	gaatgtcgacGTCCATGGTTGTTCGGAGGTAGTTCT
OspAEcoRV ADEASY	gaatgatatcATGAAAAAATATTTATTGGG
OspANotI ADEASY	gaatgcggccgcGTAATTTCAACTGCTGACCCC
AdEasy Shuttle forward sequencing	CTCACGGGGATTTCCAAGTC
AdEasy Shuttle reverse sequencing	ATGCAGTCGTCGAGGAATTG

Transfection with the two-plasmid rescue system was performed with a 3:5 ratio of helper plasmid to carrier plasmid in the presence of salmon sperm DNA, which helps precipitate the helper and carrier plasmids without interfering with the function. (Sigma, St. Louis, MO) using a calcium chloride transfection kit per manufacturers recommendations (Invitrogen, Carlsbad, CA). HEK-293 cells (ATCC, Manassas, VA) were grown in Minimal Essential Media (MEM) containing Earle's salts (Invitrogen,

Carlsbad, CA) supplemented with 10% FBS (Atlas Biologicals, Fort Collins, CO) 10 µM nonessential amino acids (Lonza,)100 µM sodium pyruvate and 1.5% sodium bicarbonate (Invitrogen, Carlsbad, CA) and at all times kept below passage 15. Cells were seeded onto a 6-well plate and allowed to grow to confluence. 500 µl of the transfections were added drop wise to each well of HEK-293 cells and allowed to incubate for 16 hours at 37°C. The media containing the transfection was removed and an agarose overlay of 2% Noble Agar (BD Biosciences, San Jose, CA), MEM (Invitrogen, Carlsbad, CA), and 20% FBS (Atlas Biologicals, Fort Collins, CO) was placed on top of the cells and allowed to incubate for 10 days. Plaques were pulled as plugs from transfected cells and triturated in 1 ml HEK-293 media and placed on fresh HEK-293 cells at 60% confluence over 4 wells of a 6 well plate. Cells were observed daily for cytopathic effects (CPE), and fresh media was added on day three, with the supernatant saved for virus purification as well as on day six. A 1 ml aliquot of the pooled supernatant was subjected to DNA extraction to confirm the presence of the respective Salp gene via sequencing of the gene of interest using the original primers used for gene isolation (Table 2.1) and BigDye 3.1 (Applied Biosystems, Carlsbad, CA) sequencing reaction mix at a 1:8 dilution. Sequences were obtained on a 3130xl Genetic Analyzer (Applied Biosystems, Carlsbad, CA). Sequences were analyzed with SeqMan from the DNASTAR Lasergene package (DNASTAR, Madison, WI). Supernatants which were positive for the gene of interest were then considered crude virus isolate and were placed on fresh HEK-293 cells at 80% confluence. After substantial CPE was apparent, infected cells were harvested by scraping, concentrated by centrifugation and lysed by 3 cycles of freeze-thaw in a dry ice-methanol bath. Cell debris was pelleted and

virus was purified by consecutive banding on a CsCl step gradient consisting of 1 ml of CsCl at 1.4 g/ml and 2 ml of CsCl at 1.25 g/ml in phosphate-buffered saline (PBS) with an SW40 rotor at 36,000 rpm for 50 min, followed by an isopycnic gradient consisting of 1.35 g/ml CsCl in PBS and centrifugation at 65,000 rpm overnight with a VTi65 rotor. Virus was collected from both gradients by side puncture of the tubes. Collected virions were diluted 3 times with virion dilution buffer consisting of 10mM Tris pH 8, 100mM NaCl, 0.1 mg/ml BSA and 50% glycerol and frozen at -80°C. Prior to dilution 10 ul of collected virus stock was diluted 3x with water and the A_{260} was measured. One A_{260} is equivalent to 10^{12} viral particles and there are approximately 50-100 particles per plaque-forming unit (Kanegae et al. 1994). The final titer of purified virus was $1x10^{10}$ PFU/ml for each of the four salivary gland constructs.

Virus generation with AdEasy system: Salp14, Salp15, Salp25A, and Salp25D were amplified out of respective cDNA plasmids using restriction linked primers as listed in Table 1. Genes were cloned into pShuttle-IRES-hrGFP-1 (Stratagene, La Jolla, CA) vector with corresponding ends. Confirmation of proper direction and correct gene being inserted into the shuttle vector was performed by sequencing using the primers listed in Table 1 and BigDye 3.1 (Applied Biosystems, Carlsbad, CA) sequencing reaction mix at a 1:8 dilution. Sequences were obtained on a 3130xl Genetic Analyzer (Applied Biosystems, Carlsbad, CA). Sequences were analyzed with SeqMan from the DNASTAR Lasergene package (DNASTAR, Madison, WI). AdSalp-pShuttle clones in the correct orientation were utilized to make AdSalp constructs per manufacturer's recommendations with the AdEasy Adenoviral Vector System (Stratagene, La Jolla, CA). Briefly, BJ5183 *E. coli* cells were co-transformed with Salp-pShuttle_IRES-hrGFP-1

linearized plasmid and pAdEasy-1 supercoiled plasmid, with pUC18 DNA in a separate reaction as a control. Recovered colonies were tested for recombinant Ad plasmid by growing overnight in 3 ml LB- kanamycin. A 2 ml aliquot was subjected to plasmid mini preparation with Qiaquick miniprep kit (Qiagen, Valencia, CA) and recovered plasmid was digested with PacI (New England Biolabs, Ipswich, MA) visualized on a 0.8% agarose gel. Plasmid preps which demonstrated a larger band as compared to controls were assumed to be positive recombinants. Recombinant AdSalp plasmids were then transformed into XL-10 Gold *E.coli* cells. Recovered colonies were then grown in 500 ml LB-kanamycin broth and plasmid isolation was performed using a maxi kit (Qiagen, Valencia, CA). The recombinant plasmids were then prepared for transfection with the ViraPack transfection kit (Stratagene, La Jolla, CA) and subsequently placed onto HEK-293 cells at 70% confluency and allowed to grow for 7 days with addition of media on the second and fifth day. Cells were then washed with PBS and scraped into fresh PBS. The cell suspension was subjected to four rounds of freeze-thaw in a dry ice-methanol bath. Cellular debris was pelleted and recovered supernatant was considered primary virus stock. The titer of the primary virus stock for all four genes of interest was $2x10^8$ PFU/ml. 2 ml of primary virus stock was used to infect 80% confluent HEK-293 cells for virus amplification. After substantial CPE was apparent, infected cells were harvested by scraping, concentrated by centrifugation and lysed by 4 cycles of freeze-thaw in a dry ice-methanol bath. Cell debris was pelleted and virus was purified by consecutive banding on a CsCl step gradient consisting of 1 ml of CsCl at 1.4 g/ml and 2 ml of CsCl at 1.25 g/ml in phosphate-buffered saline (PBS) with an SW40 rotor at 36,000 rpm for 50 min, followed by an isopycnic gradient consisting of 1.35 g/ml CsCl in PBS

and centrifugation at 65,000 rpm overnight with a VTi65 rotor. Virus was collected from both gradients by side puncture of the tubes. Collected virions were diluted 3 times with virion dilution buffer consisting of 10mM Tris pH 8, 100mM NaCl, 0.1 mg/ml BSA and 50% glycerol and frozen at -80°C. Prior to dilution 10 ul of collected virus stock was diluted 3x with water and the A_{260} was measured. $0.75x10^{10}$ PFU/ml of virus for each of the four salivary constructs was purified. HEK-293 cells at 70% confluency were infected with AdSalp15GFP, AdSalp25AGFP and AdSalp25DGFP and 5 days after infection cells were visualized under fluorescent microscopy to see if GFP was present (Fig. 2.2).

Western analysis of Ad constructs: Fresh HEK-293 cells at 70-80% confluence were infected with $1x10^9$ PFU/ml of AdSalp constructs. When CPE was visible, cells were scraped and lysed by 3 cycles of freezing-thawing. The lysis product was concentrated using Centriplus Centrifugal Filter Devices (Millipore, Billerica, MA) and run on a 1 well 12% Tris-Gly acrylamide gel (Invitrogen, Carlsbad, CA) and transferred to a nitrocellulose membrane per manufacturer's instructions. Membranes were cut into strips and probed with goat anti-Ad5 antibody or guinea pig anti-Salp15antibody or anti-Salp25D antibody (courtesy of Erol Fikrig). Both AdSalp15 and AdSalp25D infected HEK-293 cells showed reaction with corresponding antibody at the expected size as compared to recombinant Salp15 and recombinant Salp25D controls (data not shown).

BMDC isolation and infection: To confirm the ability of AdSalp15 to infect murine cells, bone marrow derived dendritic cells (BMDCs) were generated as previously described (Inaba et al. 1992). Briefly, femurs were harvested from C3H/HeJ mice and placed into media comprised of MEM, (Invitrogen, Carlsbad, CA) supplemented with

10% FBS (Atlas Biologicals, Fort Collins, CO). The ends of femurs were removed with a scalpel and bone marrow was flushed out with a 22 gauge needle into fresh media. Cells were triturated and pelleted at 1200 rpm for 5 minutes. Cells were washed 2x in HBSS (Invitrogen, Carlsbad, CA) supplemented with 2% FBS (Atlas Biologicals, Fort Collins, CO). Cells were resuspended in fresh media, and enumerated on a hemacytometer. The cell number was adjusted to $1x10^6$/ml in MEM, 10% FBS, and 100ng/ml recombinant GM-CSF (Invitrogen, Carlsbad,CA) and plated in a 24-well plate. At day 7 cells were removed from the cell culture plate using Nozyme (Sigma-Aldrich, St. Louis, MO) and infected with 1 x 10^9 PFU of AdSalp15 at 37°C for 1 hour. BMDCs were recovered by centrifugation t 1200 RPM for 5 minutes and resuspended in BMDC medium without GM-CSF. After 24 hour incubation at 37°C, cells were removed from the cell culture plate with Nozyme, washed 2X in PBS, and incubated with anti-Salp15 antibody from guinea pigs (courtesy Erol Fikrig) for 1 hour at 37°C. Cells were washed 3X with PBS, and incubated with anti-Guinea pig FITC labeled IgG for 1 hour at 37°C. Recovered cells were visualized on a Zeiss Axioscope (Fig. 2.1)

Murine antibody production: To determine if the Ad constructs generated were capable of generating an antibody response in mice, C3H/HeJ mice were injected subcutaneously with $1x10^9$ PFU/ml of virus. Blood was collected on day 14 post injection by cheek punch. Serum was analysed by Western blot assay as detailed above for determination of antibody production to recombinant Salp15, Salp25A and Salp25D (data not shown).

Results

We first attempted to generate high-titer Ad constructs for Salp14, Salp15, Salp25A and Salp25D using the Microbix AdMax system. Although PCR amplification of Salp14 was successful, attempts to clone the fragment into the shuttle vector pDC515 were unsuccessful and further work in the AdMax system with Salp14 was discontinued. We did generate AdSalp15 and AdSalp25D that produced immunogenic Salp15 and Salp25D proteins as shown by Western analysis from infected HEK-293 cells. We also probed the cell lysate from HEK-293 cells with anti-Ad5 antibody and detected adenovirus proteins for AdSalp15, AdSalp25A and AdSalp25D. The constructs were also immunogenic as demonstrated by infection of BMDCs and cell surface staining with anti-Salp15 and anti-Salp25D antibody (Fig. 2.1). We did not have availability of anti-Salp25A antibody and were not able to perform similar analysis of AdSalp25A. Similar results were seen when Stratagene's AdEasy system was utilized. Salp14 proved to be difficult again to subclone into the shuttle vector pShuttle-IRES-hrGFP-1. We did see production of immunogenic Salp15 and Salp25D proteins as determined by Western analysis from HEK-293 cells and infection of BMDCs (data not shown). We also saw production of GFP in BMDCs as the AdEasy constructs were made with a GFP tag present (Fig. 2.2), indicating production of viral proteins by the host cells.

Figure 2.1: BMDC a) infected with AdSalp15 and stained with anti-Salp15 antibody b) infected with AdSalp15 and stained with anti-Ad5 antibody c) uninfected control

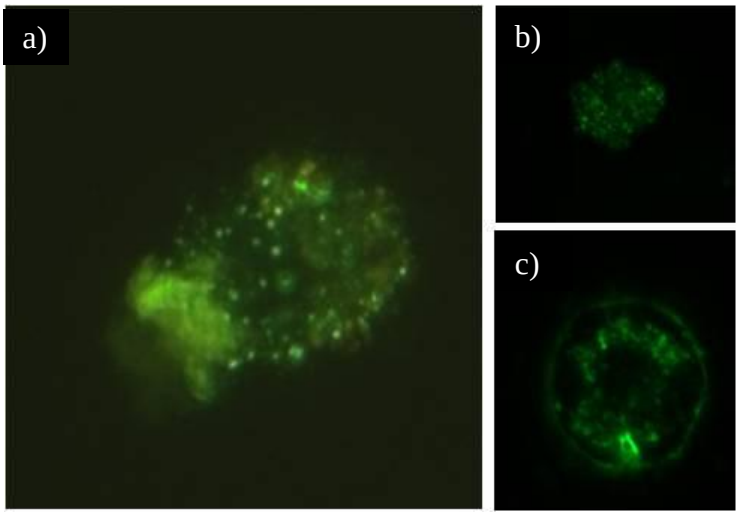
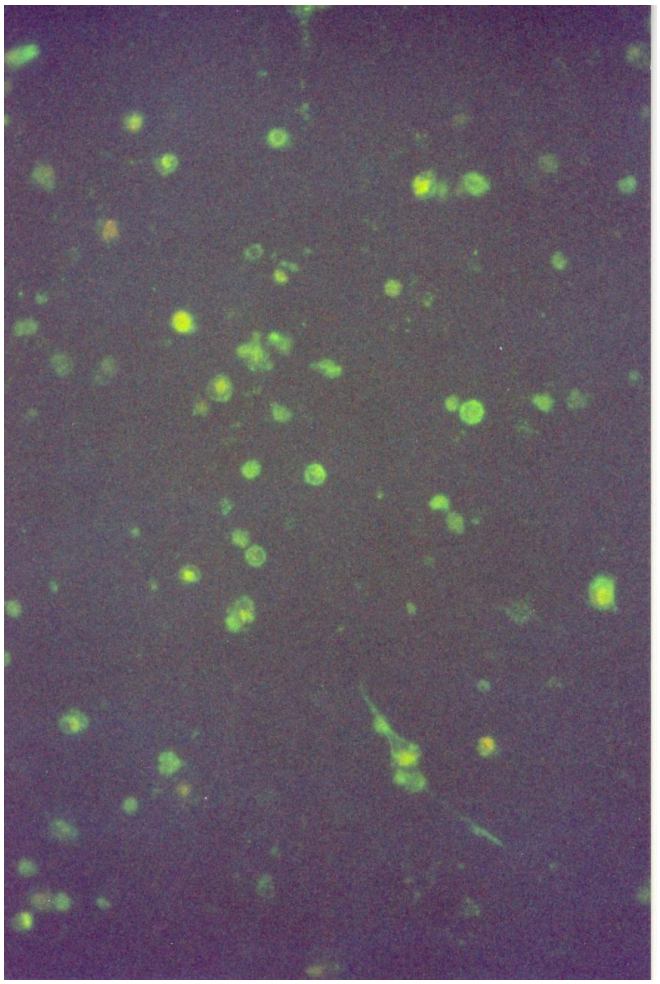


Figure 2.2: AdSalp15GFP infected BMDCs



The low titer ($1x10^{10}$ PFU/ml) produced after amplification of primary viral stocks became a limiting factor in utilizing both the AdMax and the AdEasy constructs. When mice were injected with $1x10^{9}$ PFU/ml of Ad constructs we could not detect antibody to recombinant Salp15, Salp25A or Salp25D. It was not feasible to inject a higher titer of virus into mice as we were limited by the volume we were able to inject subcutaneously.

Discussion

Given the utility of Ad vectors and the flexibility of the system, we chose to generate Ad vectors to four *I. scapularis* salivary proteins with known or putative functions in modulating the host's immune system. Salp14 is a non-Kunitz-type anticoagulant involved in blocking the intrinsic pathway of coagulation by inhibiting factor Xa (Das et al. 2001; Narasimhan et al. 2002). Salp25A is a putative antioxidant, with no known homology to existing sequences in GenBank (Das et al. 2001). Salp25D has a high homology to existing invertebrate and vertebrate glutathione peroxidases. Reactive oxygen species (ROS) are produced in the vertebrate host of ticks during normal metabolism and are capable of causing cellular damage (Singh and Shichi 1998). During injury, ROS attracts neutrophils to the wound, allowing for healing to begin. Glutathione, glutathione peroxidase and glutathione reductase act together to counter the negative effects of ROS on tissues (Steiling et al. 1999). This is a beneficial molecule for a feeding tick to express as it will limit the host's attempts to heal the feeding site of the tick, allowing for a longer period to feed.

Salp15 has the capability to inhibit CD4+ T cell activation via repression of calcium fluxes triggered by T cell receptor ligation which translates to lower production

of IL-2 (Anguita et al. 2002), a cytokine critical in promoting growth of B and T cells as well as an inflammatory T cell response from naïve T cells. Salp15 can also interfere with IL-2 signaling by down regulating CD25 expression on T cells (Anguita et al. 2002), as CD25 is the high affinity subunit of the IL-2 receptor. Salp15 further compromises the host immune response by binding to CD4 and causing conformational changes which negatively affect intracellular signaling and consequently inhibiting cell propagation (Ashish et al. 2008). When spirochetes are preincubated with Salp15 prior to infection in mice, a higher spirochete load is seen in joints, skin and bladder as compared to spirochetes alone and *B. burgdorferi* is more resistant to antibody-mediated killing. Also, the presence of Salp15 allows for re-infection of mice previously immune to *B. burgdorferi* infection (Ramamoorthi et al. 2005).

It has been observed that people who develop a hypersensitivity immune reaction to the bite of *I. scapularis* appear to acquire Lyme disease less frequently than people who do not experience an immune reaction (Burke et al. 2005). Given this evidence it is feasible an anti-tick vaccine could not only interrupt feeding, but also block transmission of pathogens by ticks. Leishmania has seen success in transmission-blocking anti-vector vaccines based upon salivary molecules beginning with maxidilian from *Lytzomyia longipalpis*. Maxidilian is a vasodilator which greatly exacerbates disease when coincoulated with *Leishmania major* (Morris et al. 2001). When synthetic maxadilian was injected subcutaneously into mice with complete Freund's adjuvant, boosted with maxidilian alone two weeks later, then subsequently challenged with *L. major* alone or *L. major* plus sandfly saliva, vaccinated mice had cutaneous lesions 3- to 5-fold smaller than control mice and lesions healed 15 days faster than controls. The parasite burden was

also markedly decreased (Morris et al. 2001). Valenzuela et al immunized mice with a single protein isolated by SDS-PAGE named SP15 from *Phlebotomus papatasi* and challenged vaccinated mice with *L. major* plus salivary gland homogenate (SGH) and demonstrated significantly smaller lesions and lower parasite load nine weeks post-promastigote challenge compared to controls (Valenzuela et al. 2001). A DNA vaccine of SP15 produced consistent results plus intense humoral and DTH reactions in the host, with the protective effect of the vaccine lasting 3 months post vaccination (Valenzuela et al. 2001). In a hamster model of visceral leishmaniasis, Gomes et al found a specific cDNA that induced DTH from *L. longipalpis* named LJM19. Hamsters vaccinated intradermally with LGM19 and subsequently challenged with *L. infantum* plus (SGH) showed a marked and significant survivorship from visceral leishmaniasis (Gomes et al. 2008). LJM19-immunized hamsters maintained a low parasite load that correlated with an overall high IFN-γ/TGF-β ratio and inducible NOS expression in the spleen and liver up to 5 months post-infection. Importantly, a DTH response with high expression of IFN-γ was also noted in the skin of LJM19-immunized hamsters 48 h after exposure to uninfected sand fly bites. Induction of IFN-γ at the site of bite could partly explain the protection observed in the viscera of LJM19-immunized hamsters through direct parasite killing and/or priming of anti-*Leishmania* cellular immunity (Gomes et al. 2008). These results reinforce the concept of using components of arthropod saliva in vaccine strategies against vector-borne diseases. Although we were not successful at producing high-titer Ad vectored vaccines to *I. scapularis* salivary proteins, we were able to generate humoral immunity to Ad-vectored Salp15 and Salp25D, and we believe this methodology is a feasible approach in developing host immunity to salivary components

introduced during feeding, and potentially blocking transmission of Lyme disease in a murine model. Given this, we will further pursue commercial avenues for generation of Ad constructs containing the genes we feel will produce both humoral and cellular immunity to tick salivary proteins and block transmission of Lyme disease.

Chapter 3

Immunization with adenoviral-expressed salivary gland proteins (SALPs) decreases spirochete load in a murine model of Lyme Borreliosis

Introduction

The black-legged tick, *Ixodes scapularis*, is capable of transmitting several infectious agents including *Borrelia burgdorferi*, *Babesia microti*, and *Anaplasma phagocytophilum* (de la Fuente et al. 2008). *B. burgdorferi sensu lato* is the infectious agent of Lyme disease, the most prevalent tick-borne illness in the United States and certain areas of Eurasia (Fikrig and Narasimhan 2006). The saliva of blood-feeding arthropods, including ticks, is a mixture of pharmacologically active components which are capable of circumventing the host's haemostatic system and altering the inflammatory and immune response of the host (Gillespie et al. 2000; Ribeiro 1995; Wikel 1999). This modulation of the host's response allows for ticks to feed to repletion in the natural host. In the non-natural host, tick feeding may result in immune and allergic responses (Ribeiro 1989; Trager 1939).

Tick saliva is also important in the transmission of tick-borne disease in that it is capable of enhancing pathogen transmission, which has been termed saliva-activated transmission (SAT) (Nuttall and Jones 1991). The SAT phenomenon has been demonstrated for several tick-borne pathogens including Thogoto virus (Jones et al. 1989), tick-borne encephalitis virus (Labuda et al. 1993), *B. afzelii* (Pechova et al. 2002),

B. burgdorferi s.s., and *B. lusitaniae* (Machackova et al. 2006; Zeidner et al. 2002) and *Francisella tularensis* (Krocova et al. 2003). Preventing infection of hosts with *B. burgdorferi* could in theory be prevented by generating a vaccine targeted to tick salivary proteins (de la Fuente et al. 2008; Willadsen 2004). In order to completely block pathogen transmission and reduce successful tick feeding, vaccine design based upon antigen cocktails may be necessary for effective anti-tick vaccines as evidence exists that a mixture of antigens significantly increases protective efficacy (Willadsen 2008).

In this study we chose to explore the use of replication-incompetent Adenovirus (Ad) vectors for vaccine delivery. Ad vectors have a number of advantages, including the capability to induce mucosal immunity (Gallichan and Rosenthal 1996), direct infection of antigen presenting dendritic cells (Jooss et al. 1998; Kirk et al. 2001a; Kirk et al. 2001b; Kirk and Mule 2000), induction of 5-10 fold greater CMI response than other viral-based vectors (Casimiro et al. 2003a; Yang et al. 2003), and higher protective effectiveness on a per-cytotoxic T-cell basis (Shiver et al. 2002). Ad vectors also elicit high-titer antibody responses against encoded proteins (Sullivan et al. 2000). Also, Ad virons do not have an envelope, rendering them more stable than other vectors. Ad vectors can be lyophilized and stored without losing their structural integrity (Gomez-Roman and Robert-Guroff 2003). There are currently no Ad based vaccines on the market, but according to www.clinicaltrials.gov, there are four trials in Phase III and Phase IV development. These trials include renal disease, prostate cancer, angina pectoris, respiratory disease and ovarian cancer.

Methods and Materials

Tick colony: Laboratory-reared, spirochete-free *I. scapularis* and *B. burgdorferi* B31-infected *I. scapularis* nymphal ticks were raised as described previously (Piesman 1993). These ticks were infected with low-passage-number *B. burgdorferi* strain B31 and the rate of infection in this tick colony was 95%.

Mice: Virus-free 5 to 6-week-old C3H/HeJ mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA). The mice were maintained in group cages and were sacrificed at the end of these studies by cervical dislocation.

Virus construction: Salp15, Salp25A, Salp25D and Isac fragments were subcloned from cDNA plasmids into shuttle vector Ad5CMV-K NpA (ViraQuest, North Liberty, IA). Recombinant adenovirus were amplified and purified by $CsCl_2$ ultracentrifugation by ViraQuest. Adenovirus vector constructs lacking inserts (AdEmpty) were acquired as controls (ViraQuest, North Liberty, IA).

PCR detection B. burgdorferi in mice: DNA was extracted from 25 mg of heart, 15 mg of bladder, or individual larval or nymphal *I. scapularis* ticks using a commercial blood and tissue DNA isolation kit per manufacturer's instructions (Qiagen Inc., Santa Clarita, CA, USA). Quantitative PCR for the fliD gene was used for *B. burgdorferi* detection as previously described (Zeidner et al. 2001).

Western Analysis: 5 µg of recombinant Salp15 (courtesy of Andrias Hojgaard), recombinant Salp25A (courtesy of Erol Fikrig) were run in a bucket gel and transferred to a nitrocellulose membrane. The membrane was then loaded into a line blotter and a 1:500 and a 1:1000 dilution of serum from individual mice were run on individual lanes with guinea pig generated anti-Salp15 or anti-Salp25A (courtesy of Erol Fikrig)

polyclonal antibody as controls. The membrane was removed from the line blotter and incubated with 1:5000 anti-IgG mouse antibody. SuperSignal West Femto Maximum Sensitivity Chemiluminescent Substrate (Pierce, Rockford, IL) was utilized for staining and bands were visualized utilizing UVP.

Isolation and infection of BMDCs: Murine bone marrow-derived dendritic cells (BMDCs) were generated as previously described (Inaba et al. 1992). At day 7 cells were removed from cell culture plate using Nozyme (Sigma-Aldrich, St. Louis, MO) and infected with 1 x 10^{10} PFU of AdSalp15 at 37°C for 1 hour. BMDCs were recovered by centrifugation t 1200 RPM for 5 minutes and re-suspended in BMDC medium. After 24 hour incubation at 37°C, cells were removed from the cell culture plate with Nozyme, washed 2X in PBS, and incubated with anti-Salp15 antibody from Guinea pigs (courtesy Erol Fikrig) for 1 hour at 37°C. Cells were washed 3X with PBS, and incubated with anti-Guinea pig FITC labeled IgG for 1 hour at 37°C. Recovered cells were visualized on a Zeiss Axioscope.

Preparation of splenocytes for cytokine production: Spleens from animals were harvested and pooled spleens prepared for stimulation and cytokine production as described previously (Zeidner et al. 1997). 1x 10^6 splenocytes were stimulated with 2 µg of concanavalin A (Con A) and supernatants were collected at 24 h and 48 h post stimulation. Supernatants were harvested and frozen at -80°C until use. IL-2, IL-4, IL-5, IFNγ and TNF concentration was determined with a cytometric bead array (CBA) for Mouse Th1/Th2 cytokines (BD Biosciences, San Jose, CA) on a FACSCaliber flow cytometer (BD Biosciences, San Jose, CA) per manufacturer's instructions. Data was

analyzed with FCAP array software (Soft Flow, Hungary Ltd.) and normalized using supernatants from unstimulated splenocytes.

***B. burgdorferi* B31 needle inoculation challenge:** Three experimental groups of seven mice apiece were given either 100 µl of injection buffer (20mM HEPES, 3% sucrose) for the buffer control, $2x10^{10}$ PFU AdEmpty virus in 100 µl injection buffer, or $1x10^{10}$ of AdSalp15 and AdIsac pooled in 100 µl of injection buffer subcutaneously, between the scapulae along the dorsal midline. Mice were challenged two weeks post-injection with 1000 needle inoculated *Borrelia burgdorferi* B31 spirochetes subcutaneously, between the scapulae along the dorsal midline. Two weeks post-challenge, ear biopsies were taken to determine the number of infected mice (Sinsky and Piesman 1989) and blood collected for Western blot analysis. Six weeks post-challenge, heart and bladder were taken and divided for *Borrelia* culture in Barbour-Stoner-Kelley (BSK) media as well as quantitative PCR for *B. burgdorferi* (Zeidner et al. 2001), blood was collected for Western analysis, and spleens removed for cytokine production assays.

Uninfected *Ixodes scapularis* challenge: Two replicates of three experimental groups of seven mice apiece were given either 100 µl of injection buffer for the buffer control group, $4x10^{10}$ PFU AdEmpty virus in 100 µl injection buffer, or $1x10^{10}$ of AdSalp15, AdSalp25A, AdSalp25D, and AdIsac pooled in 100 µl of injection buffer subcutaneously, between the scapulae. Two weeks post-inoculation five *I. scapularis* ticks were allowed to feed to repletion per mouse. Recovered ticks were weighed post drop-off. Two weeks post tick drop-off, blood was collected. Six weeks post drop off blood was collected and spleens removed for cytokine production assays.

***B. burgdorferi* infected *I. scapularis* challenge:** Four replicates of three experimental groups of seven mice apiece were given either 100 µl of injection buffer (20mM HEPES, 3% sucrose) for the buffer control group, $4x10^{10}$ PFU AdEmpty virus in 100 µl injection buffer, or $1x10^{10}$ of AdSalp15, AdSalp25A, AdSalp25D, and AdIsac pooled in 100 µl of injection buffer subcutaneously, between the scapulae. Two weeks post-inoculation five *B. burgdorferi* B31 infected *I. scapularis* ticks were allowed to feed to repletion per mouse. Recovered ticks were weighed and subsequently cultured to confirm *B. burgdorferi* infection (Dolan et al. 1997). Two weeks post tick drop-off, blood was collected and ear biopsy taken to determine the number of infected mice (Sinsky and Piesman 1989). Six weeks post tick drop-off, blood was collected for Western analysis, and heart and bladder were taken and divided between BSK culture for *Borrelia* and quantitative PCR for *B. burgdorferi* (Zeidner et al. 2001). Spleens were also removed for cytokine production assays.

***B. burgdorferi* infected *I. scapularis* challenge with individual Adenovirus constructs:** Two replicates of four experimental groups of seven mice apiece were given 100 µl of injection buffer (20mM HEPES, 3% sucrose) for the buffer control group, $1x10^{10}$ PFU AdEmpty virus in 100 µl injection buffer, $1x10^{10}$ of AdSalp15 in 100 µl injection buffer, or $1x10^{10}$ of AdIsac in 100 µl of injection buffer subcutaneously, between the scapulae. Two weeks post-inoculation five *B. burgdorferi* B31 infected *I. scapularis* ticks were allowed to feed to repletion per mouse Two weeks post tick drop-off, blood was collected for Western analysis and ear biopsy taken to determine number of infected mice (Sinsky and Piesman 1989). Six weeks post tick drop-off, blood was collected for Western analysis, and heart and bladder were taken and divided between

BSK culture for *Borrelia* and quantitative PCR for *B. burgdorferi* (Zeidner et al. 2001). Spleens were also removed for cytokine production assays.

Xenodiagnostic challenge: Three groups of five mice apiece were given either 100 µl of injection buffer (20mM HEPES, 3% sucrose) for the buffer control group, $2x10^{10}$ PFU AdEmpty virus in 100 µl injection buffer, or $2x10^{10}$ of AdSalp15 and AdIsac pooled in 100 µl of injection buffer subcutaneously, between the scapulae. Two weeks post-inoculation five *B. burgdorferi* B31 infected *I. scapularis* ticks were allowed to feed to repletion per mouse. Recovered ticks were cultured to confirm *B. burgdorferi* infection (Dolan et al. 1997). Two weeks post tick drop-off, blood was collected and ear biopsy taken to determine the number of infected mice (Sinsky and Piesman 1989). Four weeks post drop off 100 uninfected *I. scapularis* larvae were placed on each mouse and allowed to feed to repletion. Ten larvae per mouse were subjected individually to DNA extraction, and 10 were pooled per mouse, and 20 µg of DNA from each tick was utilized for qPCR determination of spirochete number. The remaining larvae were allowed to molt to nymphs, ten of which were also subjected to individual DNA extraction, from which 20 µg of DNA was utilized for qPCR determination of spirochete count. Six weeks post tick drop-off, blood was collected from mice for Western blot analysis, heart and bladder were taken and divided between BSK culture for *Borrelia* and quantitative PCR for *B. burgdorferi*. Three weeks post larval molt, 5 nymphs from each original mouse were placed individually on new, uninfected C3H/HeJ mice to determine the vector competence of ticks from each experimental group. Two weeks post drop-off blood was collected and ear biopsy taken to determine the number of infected mice. Six weeks post tick drop-off, spleens were removed for cell culture and heart and bladder

were taken and divided between BSK culture for *Borrelia* and quantitative PCR for *B. burgdorferi* (Zeidner et al. 2001).

Statistical analysis: The significance of difference of the means was evaluated using oneway analysis of variance with non-parametric Wilcoxan rank sum test with Chi-square approximation of significance. If the oneway analysis was significant, pairwise analysis of treatment groups was also performed.

Results

***In vitro* assays:** Adenovirus (Ad) is capable of directly infecting dendritic cells. When modified for vaccine delivery, recombinant Ad does not down regulate MHC molecules as compared to wild-type Ad (Zhong et al. 1999). Infection of DCs with a recombinant Ad vector expressing a gene of interest can subsequently present viral proteins on the cell surface via MHC I expression, which will drive formation of $CD8^+$ CTL (Zhong et al. 1999). To confirm surface expression of viral constructs of salivary proteins on dendritic cells, BMDCs were harvested and subsequently infected with AdSalp15. Indirect immunofluorescence confirmed cell surface expression of Salp15 (Fig. 3.1). DCs can be matured by adenovirus transduction which can lead to a skewing of T-cell responses toward more effective Th1 profiles (Hirschowitz et al. 2000). To determine the cytokine profile of mice inoculated with Adenovirus vector constructs, 6 mice per experimental group were inoculated with injection buffer (buffer control), AdEmpty cassette, or a combination of AdSalp15, AdSalp25A, AdSalp25D, and AdIsac. Seven days and 14 days post inoculation three mice from each group were sacrificed with spleens and inguinal lymph nodes harvested for cell culture. Cells were stimulated with

ConA and supernatants collected at 24 and 48 hours post-stimulation. Cytokine profiles from supernatants were assayed for IFN-γ, TNF-α, IL-2, IL-4 and IL-5 (Fig. 3.2). A marked increase in IFN-γ was seen in the AdEmpty mice and greater increase seen in the AdSalp infected mice as compared to buffer control at 14 days post inoculation.

Figure 3.1. BMDCs a) infected with AdSalp15 and stained with anti-Salp15 antibody displaying surface expression of viral vectored protein b) infected with AdSalp15 and stained with anti-Ad5 antibody c) uninfected control

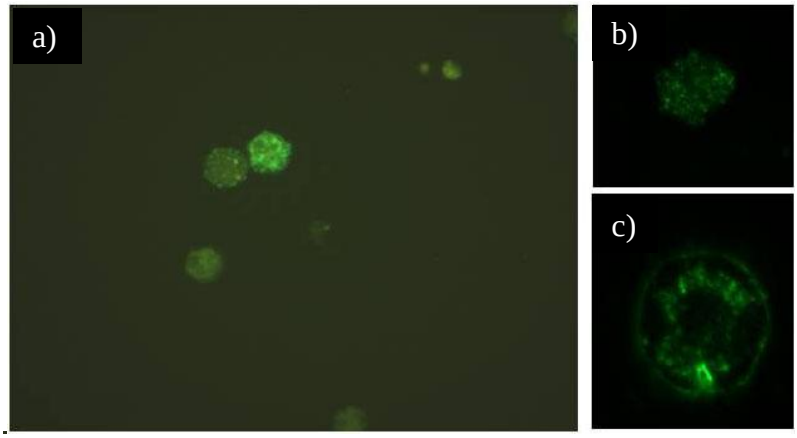
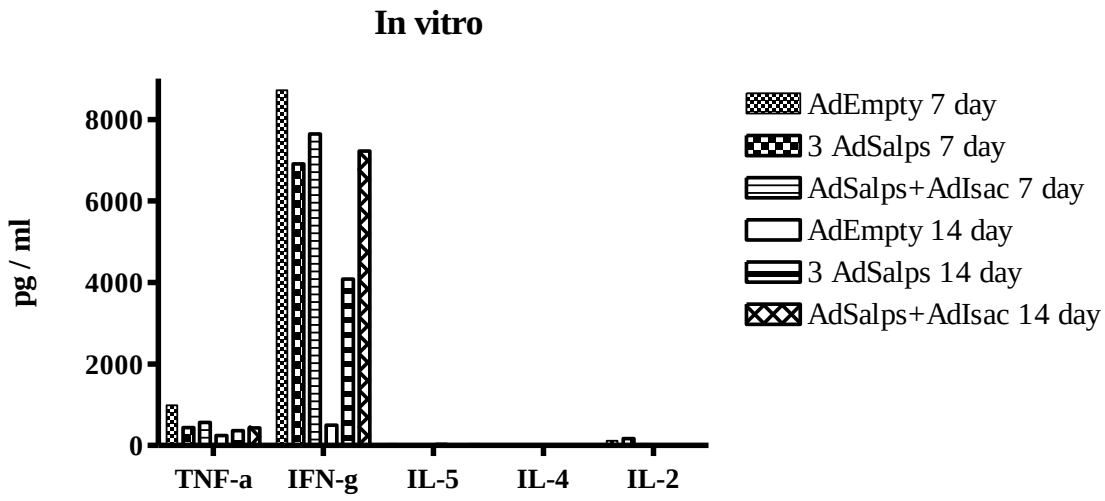


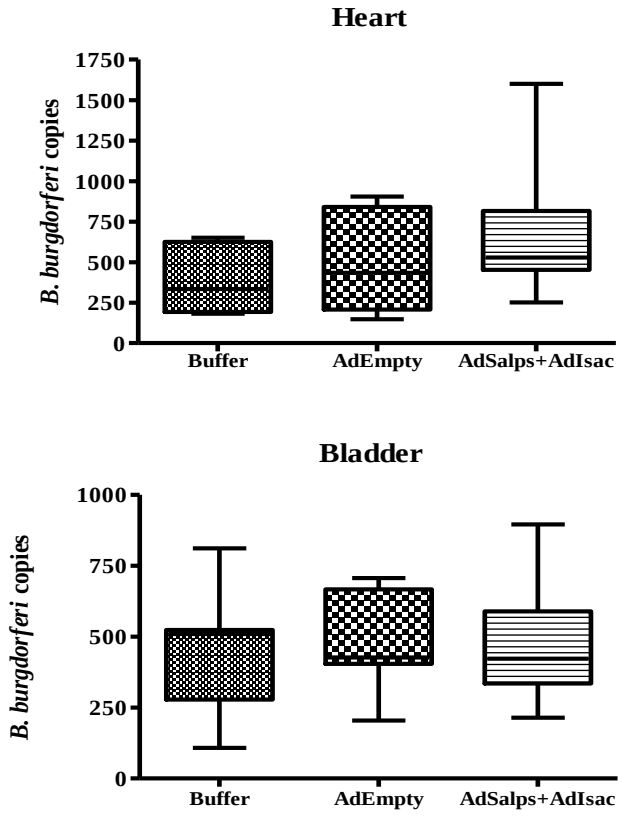
Figure 3.2. Cytokine profiles at 48 hours from cultured spleens harvested at 7 and 14 days post vaccination.



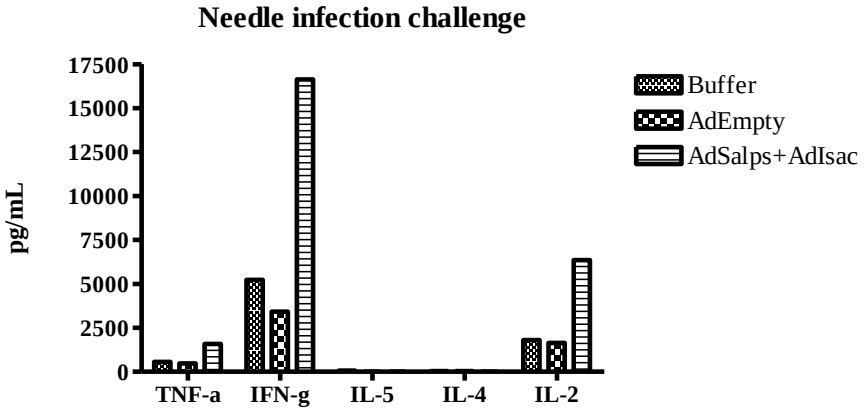
***Borrelia burgdorferi* B31 needle infection challenge:** Evidence exists that vaccination with defined tick antigens can induce significant immunity to tick infestation (Kotsyfakis et al. 2008; Willadsen 2004). As the Ad vector constructs were generated to express tick salivary genes, induce high levels of Th1 associated cytokines and perhaps interfere with tick feeding, it was necessary to determine the effect of Ad inoculation in mice subsequently challenged with *B. burgdorferi* B31 by needle infection without tick feeding. Seven mice each were injected with injection buffer (buffer control), AdEmpty cassette, or AdSalps15, AdSalp25A, AdSalp25D and AdIsac. Two weeks post-injection, mice were challenged with 1000 spirochetes injected subcutaneously. Two weeks post infection, ear biopsies were taken for BSK culture and serum collected. All mice were positive for *B. burgdorferi* culture at two weeks post infection. Western analysis of serum demonstrated production of anti-Salp15 and anti-Salp25D antibody (data not shown). Six weeks post infection heart and bladder were collected for qPCR and BSK culture. No significant difference in spirochete numbers in heart ($p=0.4886$) or bladder ($p=0.9744$) were observed between experimental groups (Fig. 3.3a). At six weeks post infection spleens were collected for cytokine profiles and serum was collected for Western analysis. A significant increase in IFN-γ was observed in mice inoculated with AdSalp15, AdSalp25A, AdSalp25D and AdIsac versus buffer control and AdEmpty cassette at 48 hours post stimulation ($p<0.0001$), as well as TNF-α ($p<0.0001$) and IL-2 ($p<0.0001$) (Fig. 3.3b). Anti-Salp15 and anti-Salp25D antibodies were detected from mice by Western blot analysis (data not shown).

Figure 3.3. Vaccinated mice challenged with needle infection of *B. burgdorferi* a) qPCR results of heart and bladder 6 weeks post infection b) cytokine profiles from spleen 48 hours post stimulation.

a)



b)



Uninfected *Ixodes scapularis* challenge: Adenovirus vectors not expressing genes of interest are capable of inducing multiple inflammatory genes *in vivo* including tumor necrosis factor α (TNF-α), interleukin-6 (IL-6), interferon-γ (IFN-γ), and IL-12 (Muruve 2004). To clarify the effect of AdEmpty cassette versus AdSalp15, AdSalp25A, AdSalp25D and AdIsac on tick feeding as well as cytokine profiles in mice fed upon by ticks not infected with *B. burgdorferi*, two replicates of three groups of 7 mice apiece were inoculated with injection buffer, AdEmpty cassette, or a cocktail of AdSalp15, AdSalp25A, AdSalp25D and AdIsac. Two weeks post inoculation, 5 uninfected *I. scapularis* nymphal ticks were placed on each mouse and allowed to feed to repletion. Recovered ticks were weighed, and no significant difference in tick weight or feeding duration was observed (Table 3.1).

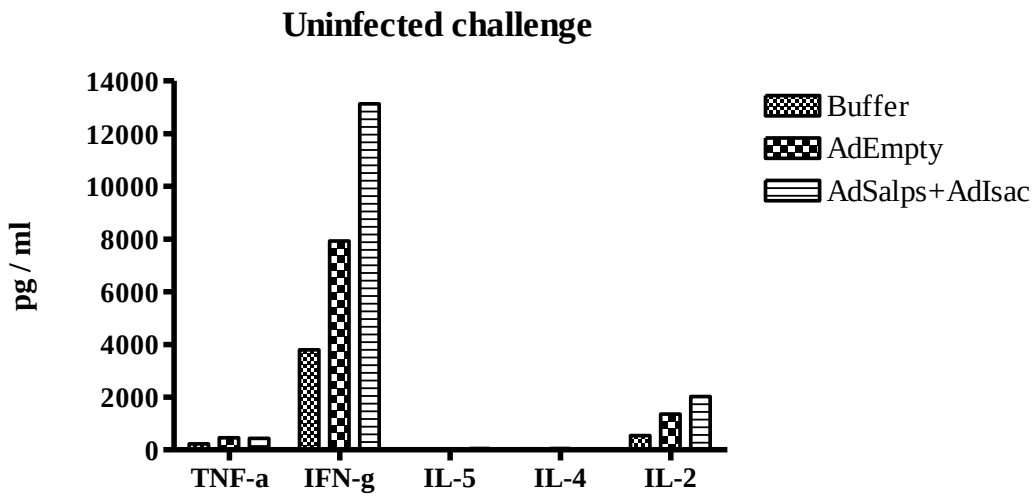
Table 3.1. Weight of recovered ticks in mg

Uninfected tick feeds	Buffer	AdEmpty	AdSalps+AdIsac	
Trial 1	3.75	3.84	3.48	
Trial 2	3.63	3.54	3.51	p=0.3636
Infected tick feeds				
Trial 1	3.87	4.53	3.28	
Trial 2	3.51	2.49	3.43	
Trial 3	3.86	3.25	3.83	p=0.8153

Blood was collected at two weeks for Western blot analysis, demonstrating antibody production to Salp15 (data not shown). Six weeks post drop-off blood was collected, and spleens and inguinal lymph nodes were harvested for cytokine profile assays. Western blot analysis demonstrated antibody production to Salp15 (data not shown). Cytokine profiles were similar to what was seen previously with an increase of IFN-γ was in mice inoculated with AdEmpty cassette versus buffer control, and a greater

increase in IFN-γ inAdSalp15, AdSalp25A, AdSalp25D and AdIsac versus buffer control and AdEmpty cassette at 48 hours post stimulation ($p<0.0001$) as wells as TNF-α ($p=0.0002$) and IL-2 ($p=0.0009$) (Fig. 3.4).

Figure 3.4. Cytokine profiles at 48 hours post stimulation of cultured spleens from vaccinated mice challenged with uninfected *I. scapularis* nymphs.



***B. burgdorferi* infected *I. scapularis* challenge:** It has been hypothesized that to effectively block tick feeding or pathogen transmission by ticks that a vaccine would need to be designed with a cocktail of antigens (Willadsen 2008). To determine the effect of vaccination with a combination of AdSalp15, AdSalp25A, AdSalp25D and AdIsac collectively on transmission of *B. burgdorferi* by *I. scapularis*, four replicates of three groups of 7 mice apiece were inoculated with injection buffer, AdEmpty cassette, or a cocktail of AdSalp15, AdSalp25A, AdSalp25D and AdIsac. Two weeks post inoculation, 5 *B. burgdorferi* infected *I. scapularis* nymphal ticks were placed on each

mouse and allowed to feed to repletion. Recovered ticks were weighed, and no significant difference in tick weight or feeding duration was observed (Table 3.1). Ticks were subsequently pooled by mouse and were cultured in BSK media. Cultures were read at seven days with all tick pools being positive. Ear biopsies of mice were taken at two weeks post drop-off, all of which were positive for *B. burgdorferi*.

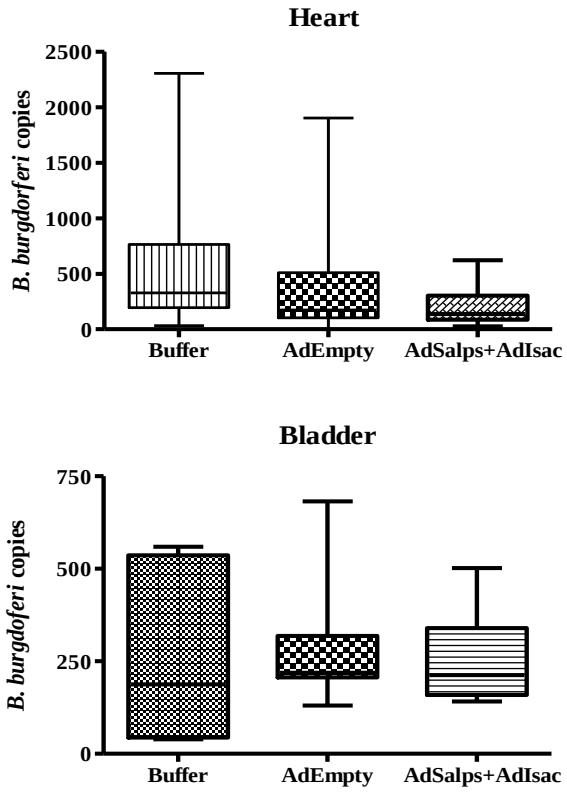
Six weeks post infection, heart and bladder were collected for qPCR and BSK culture. No significant difference in spirochete numbers in the heart ($p=0.0671$) or bladder ($p=0.6105$) (Fig. 3.5a), however an obvious trend was observed with a reduction in spirochetes in the hearts of AdSalp vaccinated mice at a rate of 59.7% as compared to buffer controls. This decrease in spirochete numbers was not observed in the third trial. There was no noticeable difference in spirochete load in between any of the experimental groups for this third trial. Our only conclusion for this result was that due to necessity, a combination of young and old nymphal ticks were utilized for the feed. It was observed that the old ticks, which were placed on the AdSalps +AdIsac group, fed very poorly. Attachment was on average 2 ticks out of 5 placed on the mice and feeding duration was extended by an average of 12 hours. The younger nymphal ticks, however, fed successfully with high attachment rate (4 out of 5 placed ticks) and the feeding duration was as observed for the previous two trials. For this reason, we chose to repeat the trial a fourth time and based upon those results, which were similar to what was observed in the first two trials, and elected to remove the results from this third trial from our final data.

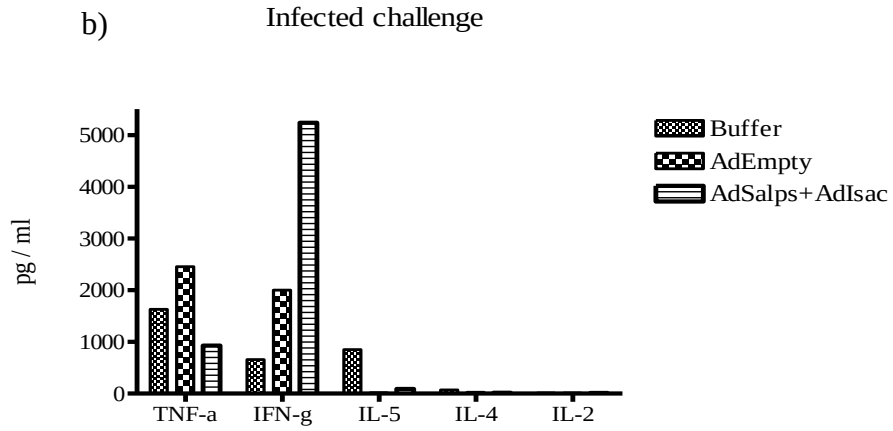
At six weeks post infection spleens and inguinal lymph nodes were collected for cytokine profiles and serum was collected. Cytokine profiles were similar to what was seen previously with an increase of IFN-γ in mice inoculated with AdEmpty cassette

versus buffer control, and a greater increase in IFN-γ inAdSalp15, AdSalp25A, AdSalp25D and AdIsac versus buffer control and AdEmpty cassette at 48 hours post stimulation ($p<0.0001$), as well as TNF-α ($p=0.0036$) ((Fig. 3.5b).

Figure 3.5. Mice vaccinated with a combination of AdSalp15, AdSalp25A, AdSalp25D and AdIsac challenged with *B. burgdorferi* infected *I. scapularis* a) qPCR results of heart and bladder b) cytokine profiles from spleen 48 hours post stimulation.

a)





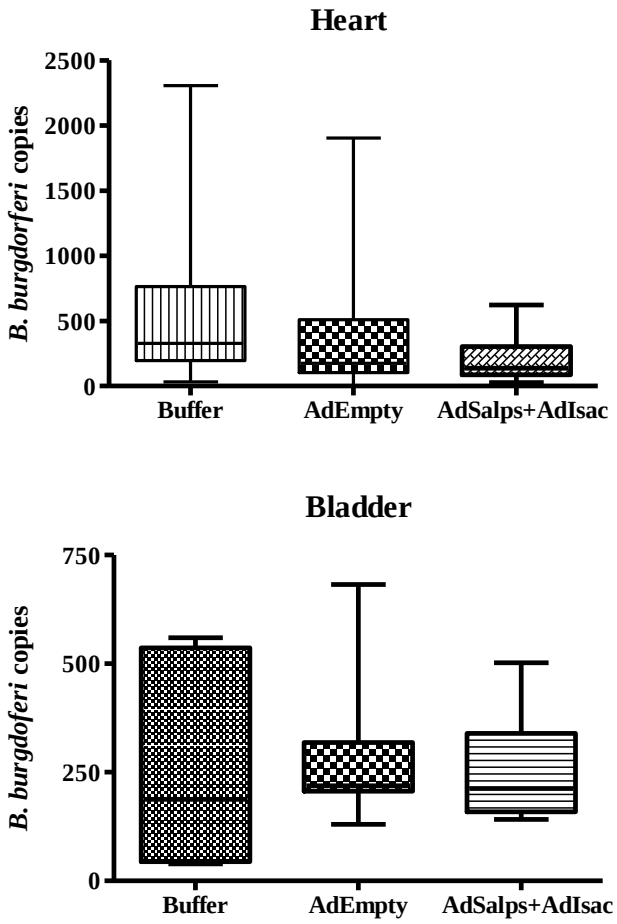
Testing of individual Adenovirus constructs with *B. burgdorferi* infected *I. scapularis*: To elucidate what the effect of AdSalp15 and AdIsac were individually on spirochete load and cytokine profiles two replicates of 7 mice from 4 groups were inoculated with either injection buffer (buffer control), AdEmpty cassette, AdSalp15 or AdIsac. Two weeks post inoculation, 5 *B. burgdorferi* infected *I. scapularis* nymphal ticks were placed on each mouse and allowed to feed to repletion. Ticks were subsequently pooled by mouse and were cultured in BSK media. Cultures were read at seven days with all tick pools being positive. Ear biopsies of mice were taken at two weeks post drop-off, all of which were positive for *B. burgdorferi*.

Six weeks post infection heart and bladder were collected for qPCR and BSK culture. No significant difference was observed in spirochete numbers in heart ($p=0.3537$) or bladder ($p=0.2674$) between any experimental groups. However, a trend was observed in a reduction of the spirochete load in the hearts and bladders of AdIsac vaccinated mice as compared to buffer control, AdEmpty control or AdSalp15 (Fig. 3.6a). The cytokine profiles of AdEmpty and AdSalp15 were similar to that of the buffer control. AdIsac, however, demonstrated a large increase in IFN-γ as compared to all

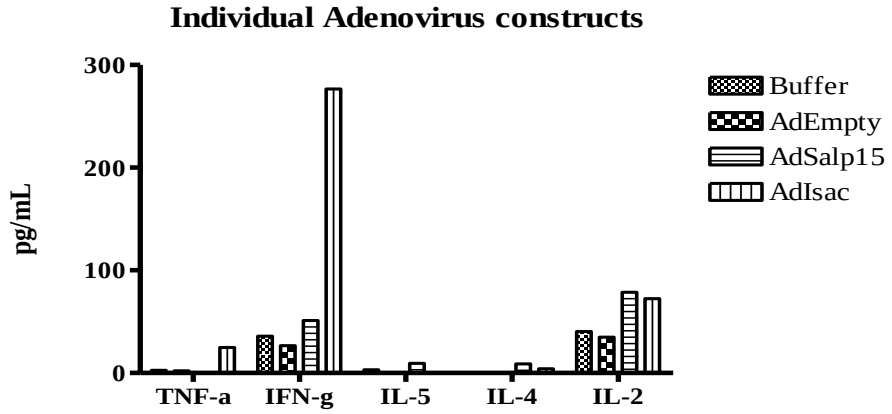
other experimental groups (p=0.0003), as well as TNF-α (p<0.0001) and IL-2 (p<0.0001) (Fig 3.6b).

Figure 3.6. Mice vaccinated with individual Adenovirus constructs challenged with infected tick bite. a) qPCR results from heart and bladder 6 weeks post infected tick feed. b) cytokine profiles from spleen 48 hours post stimulation.

a)



b)

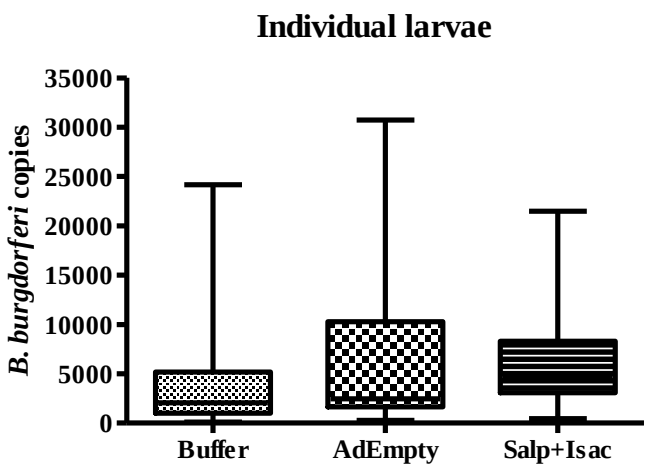


Xenodiagnostic challenge: Given the trend we observed in reduction of spirochete load in the heart (~60%, not statistically significant) of vaccinated mice we wanted to know if this decrease was large enough to interrupt transmission from vaccinated, infected mice to clean larval ticks. Five mice per group were either given injection buffer, AdEmpty cassette virus, or were vaccinated with AdSalp15 and AdIsac. Two weeks post-injection mice were challenged with 5 *B. burgdorferi* B31 infected nymphs. Two weeks after tick drop off, ear biopsies were taken from mice to confirm infection rate of mice, with all mice being positive for *B. burgdorferi*. Blood was also collected and Western analysis confirmed antibodies to Salp15. Four weeks after the infected nymphal feed, 100 uninfected larvae were placed on the mice and allowed to feed to repletion. Approximately 50-80 larvae were recovered per mouse. Ten larvae per mouse were subjected to DNA extraction individually and normalized by quantification of the concentration of DNA. All samples were diluted to a concentration of 4 ng/µl. qPCR analysis of normalized larval DNA indicated no significant difference in spirochete load between the three experimental groups (p=0.0519) (Fig. 3.7a). Six weeks after infected nymphal feed, mice were sacrificed with heart and bladder being taken and divided in half for culture and qPCR analysis. All BSK cultures were positive and heart

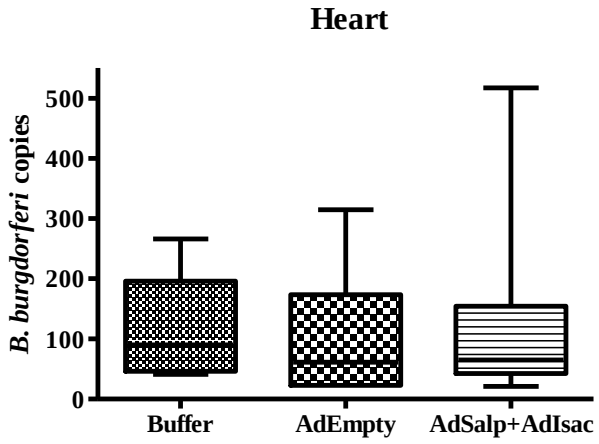
and bladder were not significant for differences in spirochete load (heart=0.8160 and bladder=0.5605) (Fig. 3.7b). Ticks were allowed to molt to nymphs and 10 individual nymphs per mouse were subjected to DNA extraction and qPCR analysis. There was a highly significant difference in *B. burgdorferi* copies within nymphs between all groups ($p<0.0001$), and pairwise comparison of injection buffer control to AdSalp15+AdIsac not being significant ($p=0.8282$) and pairwise comparison of AdEmpty to AdSalp15+AdIsac being highly significant ($p<0.001$) (Fig. 3.7c). Six weeks after molted nymphs were allowed to feed, mice were sacrificed with heart and bladder being taken and divided in half for culture and qPCR analysis. All BSK cultures were positive with the exception of one mouse from the buffer control group and one mouse from the AdSalp15+AdIsac group. Heart and bladder were not significant for differences in spirochete load (heart=0.1843 and bladder=0.8808) (Fig. 3.7d). Cytokine profiles of splenocytes at 48 hours did demonstrate a difference between the experimental groups and the buffer control at 48 hours post stimulation in production of IFN-γ ($p=0.0002$), TNF-α ($p=0.0008$). and IL-2 ($p=0.0013$) (Fig. 3.7e).

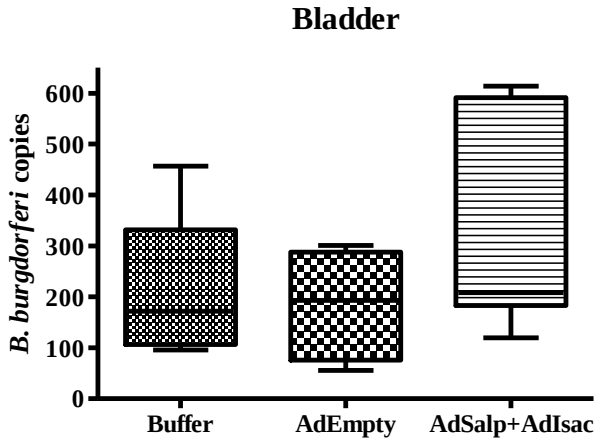
Figure 3.7. Xenodiagnostic challenge: Mice vaccinated with injection buffer, AdEmpty cassette, or AdSalp15+AdIsac constructs challenged with infected tick bite, and subsequently fed upon by clean larval *I. scapularis* tick. a) qPCR results from individual larvae b) qPCR results from heart and bladder 6 weeks post infected nymphal feed c) qPCR results from individual nymphs d) qPCR results from mice fed upon by molted nymphs from clean larval feed e) cytokine profiles from spleen 48 hours post stimulation

a)

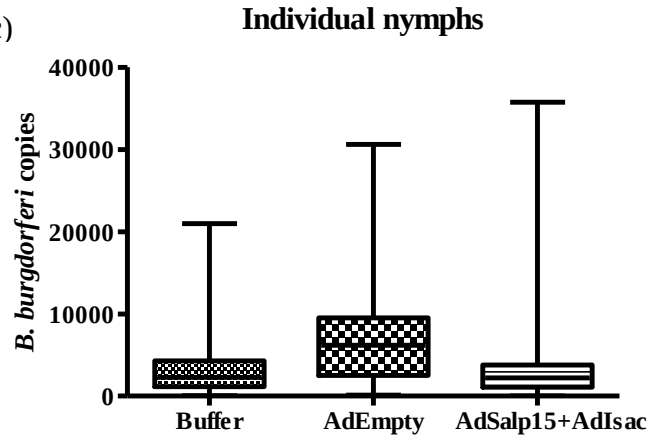

Individual larvae
B. burgdorferi copies
35000
30000
25000
20000
15000
10000
5000
0
Buffer
AdEmpty
Salp+Isac

b)

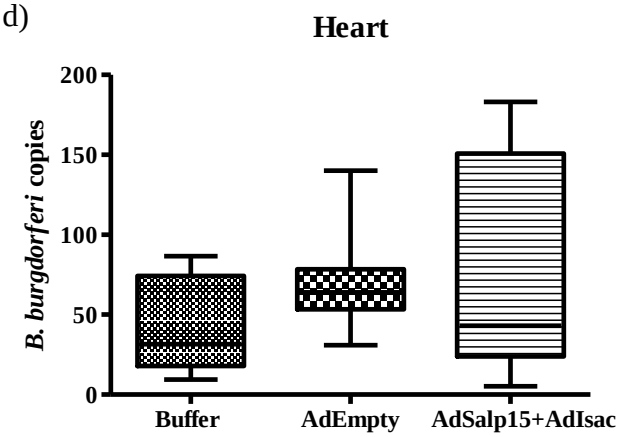

Heart
B. burgdorferi copies
500
400
300
200
100
0
Buffer
AdEmpty
AdSalp+AdIsac

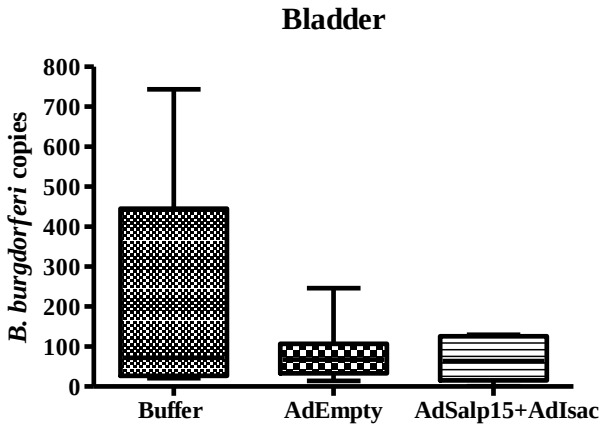

Bladder
B. burgdorferi copies
600
500
400
300
200
100
0
Buffer
AdEmpty
AdSalp+AdIsac

c)

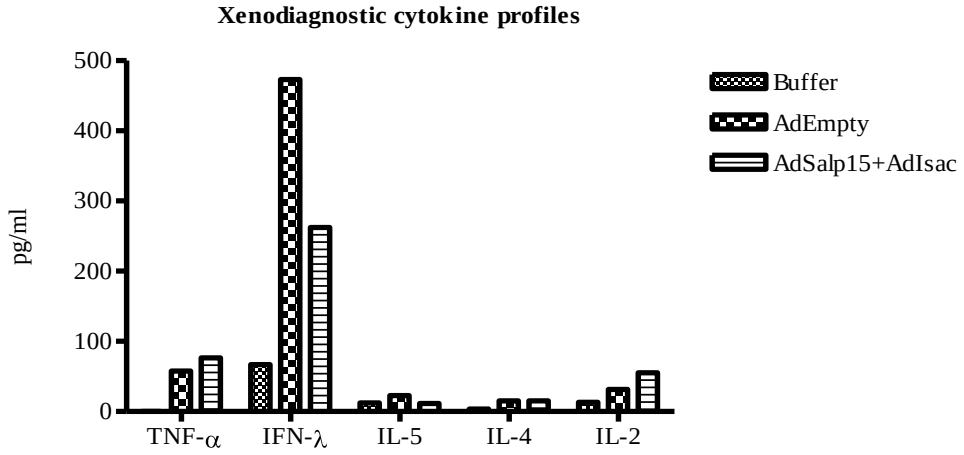

Individual nymphs
B. burgdorferi copies
40000
30000
20000
10000
0
Buffer
AdEmpty
AdSalp15+AdIsac

d)


Heart
B. burgdorferi copies
200
150
100
50
0
Buffer
AdEmpty
AdSalp15+AdIsac


Bladder
B. burgdorferi copies
800
700
600
500
400
300
200
100
0
Buffer
AdEmpty
AdSalp15+AdIsac

e)



Discussion

Vaccines are typically designed to target the antigens of infectious agents. Arthropod-borne disease provides another avenue for vaccine exploration given the invertebrate host's own defenses against the vertebrate host's immune response. Antigens which are directly involved in the tick's ability to acquire a successful blood meal as well as facilitate spirochete transmission to the vertebrate host may serve as excellent candidates in designing and producing a transmission-blocking vaccine (de la Fuente et al. 2008). Tick saliva contains immunomodulatory compounds that prevent inflammation reactions from disrupting the feeding process (Francischetti et al. 2005; Wikel 1999; Wikel and Alarcon-Chaidez 2001). *In vivo* and *in vitro* modulation of the cytokine production by tick saliva or during tick infestation has been demonstrated (Schoeler et al. 1999; Zeidner et al. 1997), polarizing the host immune response to tick bite toward a Th2 response. Given this effect of tick saliva on the immune response of the host, it is our hypothesis that utilizing vaccination with specific *I. scapularis* salivary proteins in a manner which shifts the tick's ability to down regulate the inflammation

response to a response that will allow for inflammation will block transmission of *B. burgdorferi*.

Earlier, unpublished work in our laboratory demonstrated that a host immune response to tick antigens can affect tick feeding. Mice were immunized three times with a combination of recombinant Salp14, Salp15, Salp25A and Salp25D with complete Freund's adjuvant for the first inoculation and incomplete Freund's for the second two inoculations. Two weeks later *B. burgdorferi* B31 infected nymphal ticks were allowed to feed upon the vaccinated mice. A 60% reduction in fed tick weight was observed, but no effect on transmission of *B. burgdorferi* was seen. These results encouraged further exploration of vaccination with tick salivary antigens using novel presentation to attempt to interfere with transmission of *B. burgdorferi*.

We chose to examine four salivary antigens from *I. scapularis*. Salp25A is a putative antioxidant, with no known homology to existing sequences in GenBank (Das et al. 2001). Salp25D is an amine-binding protein capable of countering the inflammatory and vasoactive amines produced in the inflammation response (Das et al. 2001). Salp25D has a high homology to existing invertebrate and vertebrate glutathione peroxidases. Reactive oxygen species (ROS) produced in the vertebrate host of ticks during normal metabolism and are capable of causing cellular damage (Singh and Shichi 1998). During injury, ROS will attract neutrophils to the wound, allowing for healing to begin. Glutathione, glutathione peroxidase and glutathione reductase act together to counter the negative effects of ROS on tissues (Steiling et al. 1999). This is a beneficial molecule for a feeding tick to express as it will limit the host's attempts to heal the feeding site of the tick.

Isac is part of a described family of proteins which interfere in the vertebrate complement cascade. Isac acts in a similar manner to known regulators of complement activation, decay accelerating factor and factor H, although it belongs to a new class of complement regulatory molecules. Its action is involved in the inhibition of the interaction of factor B with C3b (Valenzuela et al. 2000). Two similar proteins have been isolated and characterized from *I. ricinus*, and it seems that all ticks in the *I. ricinus* complex analyzed to date have related anticomplement proteins (Daix et al. 2007).

Salp15 has the capability to inhibit $CD4^+$ T cell activation via repression of calcium fluxes triggered by T cell receptor ligation which translates to lower production of IL-2 (Anguita et al. 2002), a cytokine critical in promoting an inflammatory T cell response from naïve T cells. Salp15 is also capable of down regulating CD25 expression on T cells (Anguita et al. 2002), which results in poor IL-2 signaling as CD25 is the high affinity subunit of the IL-2 receptor. Salp15 is also a ligand of CD4, preventing the activation of $CD4^+$ T cells (Garg et al. 2006) compromising the host immune response by causing conformational changes to T cells which negatively affect intracellular signaling and consequently inhibits cell proliferation (Ashish et al. 2008). Salp15 is also capable of interacting with an outer surface protein from *B. burgdorferi* known as OspC. Salp15 can bind to OspC and effectively coat the spirochete in this molecule (Ramamoorthi et al. 2005). When spirochetes are preincubated with Salp15 prior to infection in mice, a higher spirochete load is seen in joints, skin and bladder as compared to spirochetes alone. *B. burgdorferi* preincubated with Salp15 is also more resistant to antibody-mediated killing as compared to spirochetes alone (Ramamoorthi et al. 2005), and Salp15 binding allow for re-infection of previously immune mice (Ramamoorthi et al. 2005).

Recently published research utilizing passive transfer of immunity to Salp15 from rabbits into mice demonstrated 40% protection from *B. burgdorferi* infection. Active immunization with recombinant Salp15 also demonstrated 40% protection in mice (Dai et al. 2009). We show a 60% reduction in the number of spirochetes using a single dose of Ad vectored Salp proteins including Salp15 and Isac. Although this reduction was not statistically significant, a trend existed which might be resolved with larger sample sizes of mice. When Salp15 and Isac were examined individually, AdSalp15 did not reduce the spirochete burden as compared with injection buffer control or AdEmpty control, but AdIsac showed a trend in the reduction of spirochetes. Although Salp15 is a seemingly obvious target molecule for vaccination given the multitude of effects it has on host immunity and in the transmission of *B. burgdorferi*, in our hands with the current system it does not have an effect in the reduction of spirochetes in the vertebrate host. Isac, however, may be of greater interest, and is worth exploring with other delivery mechanisms or boosting strategies. When the ability of AdSalp15 and AdIsac to stimulate an inflammatory response as measured by IFN-γ and TNF-α levels from cultured splenocytes was measured, we observed a marked increase of these two cytokines as compared to buffer control and AdEmpty control. When AdSalp15 and AdIsac were investigated individually, AdIsac demonstrated the largest increase of inflammatory cytokines (Fig. 3.6b). This result also encourages further work with Isac as an anti-tick vaccine candidate. However, given the results and the time spent on infected tick challenge, a more appropriate early experiment to run with our vaccine candidate antigens would be to have developed an *in vitro* assay for *B. burgdorferi* killing utilizing cell lines that exist for CTL and NK assays. This may have eliminated further work with

Salp25A, Salp25D and Salp15. Also, knowing that Western analysis of antibodies generated to the vaccine antigens is simply a surrogate marker for activation of the immune response, a better experiment to run may have been a cytotoxic assay utilizing derived fibroblasts which are not MHC class restricted to show *in vitro* stimulation of fibroblasts.

A caveat of designing an anti-tick vaccine based on tick proteins is that the sialome of *I. scapularis* has a great deal of redundancy within sequenced mRNAs, and the redundancy is different enough not to be alleles of the same gene (Valenzuela et al. 2002). This variability is also observed with high polymorphism of salivary proteins as analyzed by SDS-PAGE (Wang and Nuttall 1999). Salivary gene variability may derive from divergence of function from duplicated genes over the long evolutionary history of ticks, and may be related to temporal expression of these genes during the long feeding process of hard ticks (Narasimhan et al. 2007; Valenzuela et al. 2002). This diversity could be one reason why we did not see an effect in the reduction of spirochetes in the vertebrate host when vaccinating against tick salivary proteins. The high variability seen within spirochete load in the heart and bladder of infected mice could be due to a variety of factors. *I. scapularis* is known to have a large amount of phenotypic and genetic variation, which is often discontinuously distributed among populations (Norris et al. 1996). Salp15 presents an excellent example of genetic redundancy within the sialome of *I. scapularis*. It likely that there is not a single Salp15 protein, but several Salp15 homologues present in the tick genome as evidence exists the salp15 gene family has undergone a phase of positive selection (Schwalie and Schultz 2009). Phylogenetic analysis of *I. ricinus, I. persulcatus*, *I. pacificus*, and *I. scapularis* demonstrates that there

are ate lest three genes in the Salp15 family and there may be many more (Hojgaard et al. 2009). Isac is also a member of a gene family of anticomplement proteins (Ribeiro et al. 2006; Soares et al. 2005), with more members being added to this family recently (Tyson et al. 2007). Variation within this family may be a result of genetic variation between individual ticks, rather than each tick possessing several members of the gene family however (Tyson et al. 2007). The variation within experimental groups of mice may also be attributable to the genetic diversity of the spirochete as well. It has been shown that the genetic diversity of *B. burgdorferi* spirochetes, which manifests as trait variation, has epidemiological and ecological consequences (Kurtenbach et al. 2006; Wormser et al. 1999). Antigenic heterogeneity of *B. burgdorferi* populations transmitted by single ticks has clearly been demonstrated (Ohnishi et al. 2001) and may complicate elucidating the cause of the high levels of variation seen in this study.

The experimental and clinical experience with Ad vectors has revealed significant host immune responses that limit safety and efficacy *in vivo*. Ad vectors are highly inflammatory in spite of the evidence that natural Ad infections may not be very inflammatory, in part due to the use of a cytomegalovirus (CMV) promoter (Lichtenstein and Wold 2004). First generation Ad vectors efficiently induce adaptive immunity to Ad vectors, eliminating the ability to boost the host with the same Ad vector backbone. The immunologic response to first-generation Ad vector vaccines is also limited by pre-existing immunity of the vaccinee to Ad (Casimiro et al. 2003b; Yang et al. 2003). It has been reported that 40% to 97% of humans have neutralizing antibodies to Ad5 (Chirmule et al. 1999; Vogels et al. 2003) the most widely used serotype for gene transfer vectors, and that two-thirds of humans studied have lympho-proliferative responses against Ad

(Chirmule et al. 1999). Many of these issues have been resolved with second-generation Ad vectors, and we plan future work with these vectors once we have identified candidate genes which will block transmission of *B. burgdorferi*.

Chapter 4

cDNA vaccination of mice with novel *Ixodes scapularis* salivary genes

Introduction

In 1990, a seminal study showed that the injection of a DNA plasmid in mouse muscle resulted in a significant expression of the protein encoded by the plasmid (Wolff et al. 1990). Starting with this discovery, various antigens encoded by plasmids have been successfully used to induce the production of antibodies (Cox et al. 1993; Tang et al. 1992) and cytotoxic T lymphocytes (Ulmer et al. 1993), thereby demonstrating the potential of this strategy for DNA vaccination and gene therapy. Progress in this field has resulted in the development and the marketing of three veterinary DNA vaccines since 2005. Two of them are authorized for use in the United States: one targets the West Nile virus infection in horses (Davidson et al. 2005) and the other targets canine malignant melanoma (Bergman et al. 2006). The third vaccine, authorized for use in Canada in salmon, is directed against the infectious hematopoietic necrosis virus (Garver et al. 2005).

VR1020 (Vical Inc.) is a plasmid which has been extensively used as a DNA vaccine to deliver antigens into animals including humans (Wang et al. 2004). This plasmid was modified to create a high-throughput cloning DNA plasmid to be used as a delivery and expression system of salivary genes from sand flies in animal skin for the purpose of studying immune response in animals to sand fly salivary proteins (Oliveira et al. 2006). For this plasmid, VR2001-TOPO, VR1020 was modified by the addition of

topoisomerases flanking the cloning site to allow for rapid cloning without the creation of new restriction sites. Additionally, the plasmid contains the tissue plasminogen activator signal peptide which increases the probability of producing a secreted protein and mimics the presentation of the antigens that are injected by the sand fly into the skin (Oliveira et al. 2006).

DNA vaccination using VR2001-TOPO with a single molecule from *L. longipalpis*, LJM19, protected hamsters against the fatal outcome of *Leishmania infantum chagasi*, visceral leishmaniasis (Gomes et al. 2008). This protection correlated with a high IFN-γ/TGF-β ratio and inducible nitric oxide synthetase production, indicating a strong inflammatory response allowing for a complete block in infection. A strong delayed-type hypersensitivity response was also observed in the skin of immunized hamsters 48 hours after exposure to sand fly bites (Gomes et al. 2008). The ability of LJM19 to drive such a strong inflammatory response is believed to be the cause for the protective effect of a single molecule (Jesus Valenzuela, personal communication). Given this information we chose to explore several *I. scapularis* molecules in the VR2001-TOPO vector for a similar response in mice.

Methods and Materials

Tick colony: Laboratory-reared, spirochete-free *I. scapularis* and *B. burgdorferi* B31-infected *I. scapularis* nymphal ticks were raised as described previously (Piesman 1993). These ticks were infected with low-passage-number *B. burgdorferi* strain B31 and the rate of infection in this tick colony was 95-98%.

Tick salivary gland extract collection: *I. scapularis* salivary glands were dissected aseptically from female adults at 24 hours, 48 hours, 72 hours and 96 hours post attachment as

previously described (Piesman 1995). Salivary glands were washed two times in sterile phosphate-buffered saline, and ground by mortar and pestle into homogenous extracts in PBS that were pooled from 4 to 6 individual females per day in a volume of 30 µl PBS for each day. The SGE from each day was combined for the final working pool.

Mice: Virus-free, 5 to 6-week-old C3H/HeJ mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA). The mice were maintained in group cages according to IACUC regulations and were sacrificed at the end of these studies by cervical dislocation.

cDNA plasmids and challenge: All cDNA plasmids used in this study were generated by Jesus Valenzuela and colleagues. The plasmid VR2001-TOPO was the vector used for expression (Oliveira et al. 2006). 20 µg of each plasmid was injected in a volume of 10 µl injection buffer (20mM HEPES, 3% sucrose) intradermally at the base of the tail once a week for a three week period. Two weeks after final injection, mice were either sacrificed and spleens were harvested for cyotkine assays or mice were subjected to uninfected or *B. burgdorferi* infected nymphal tick feed.

PCR detection and culture of *B. burgdorferi* in mice: DNA was extracted from 25 mg of heart, or 15 mg of bladder using a commercial blood and tissue DNA isolation kit per manufacturer's instructions (Qiagen Inc., Santa Clarita, CA, USA). Quantitative PCR for the fliD gene was used for *B. burgdorferi* detection as previously described (Zeidner et al. 2001). Ear biopsies were taken from experimental mice and placed into BSK medium to determine the infection status (Sinsky and Piesman 1989).

Preparation of splenocytes for cytokine production: Spleens from animals were harvested and pooled spleens prepared for stimulation and cytokine production as described previously (Zeidner et al. 1997). $1x10^6$ splenocytes were stimulated with 2 µg

of concanavalin A (Con A) or 10 µl of SGE. Supernatants were collected at 24 h and 48 h post stimulation. Supernatants were harvested and frozen at -80°C until use. IL-2, IL-4, IL-5, IFNγ and TNF-α concentration was determined with a cytometric bead array (CBA) for Mouse Th1/Th2 cytokines (BD Biosciences, San Jose, CA) on a FACSCaliber flow cytometer (BD Biosciences, San Jose, CA) per manufacturer's instructions. Data was analyzed with FCAP array software (Soft Flow, Hungary Ltd.) and normalized with unstimulated splenocytes.

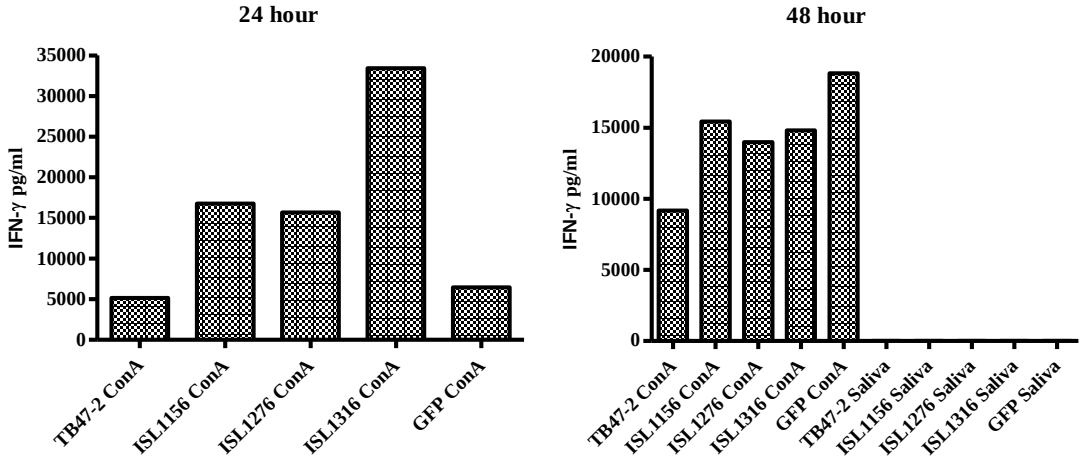
Statistical analysis: The significance of difference between the means was evaluated using one way analysis of variance with non-parametric Wilcoxan rank sum test with Chi-square approximation of significance.

Results

Cyotkine profiles of mice vaccinated with cDNA constructs: To determine the potential for individual molecules from *I. scapularis* saliva to drive a strong IFN-γ response, we vaccinated mice over a three week period with cDNA constructs, including a GFP expressing control construct. Mice were allowed to rest for two weeks at which point mice were sacrificed and spleens removed for cytokine profile assays. Cultured spleens were stimulated with either ConA or *I. scapularis* salivary gland extract (SGE). Stimulation with SGE showed no demonstrable change in IFN-γ levels as compared to controls. This could be due to the possibility that we didn't have the correct stimulatory protein at a high enough concentration to push a cytokine response, or perhaps the antigen wasn't expressed well *in vivo*. A third possibility is that perhaps the SGE used is actually immunosuppressive *in vitro*. ConA stimulation however showed a spike in IFN-

γ production from ISL1156 and ISL1276 vaccinated mice, and a large spike in ISL1316 vaccinated mice as compared to GFP controls at 24 hours post stimulation (Fig. 4.1).

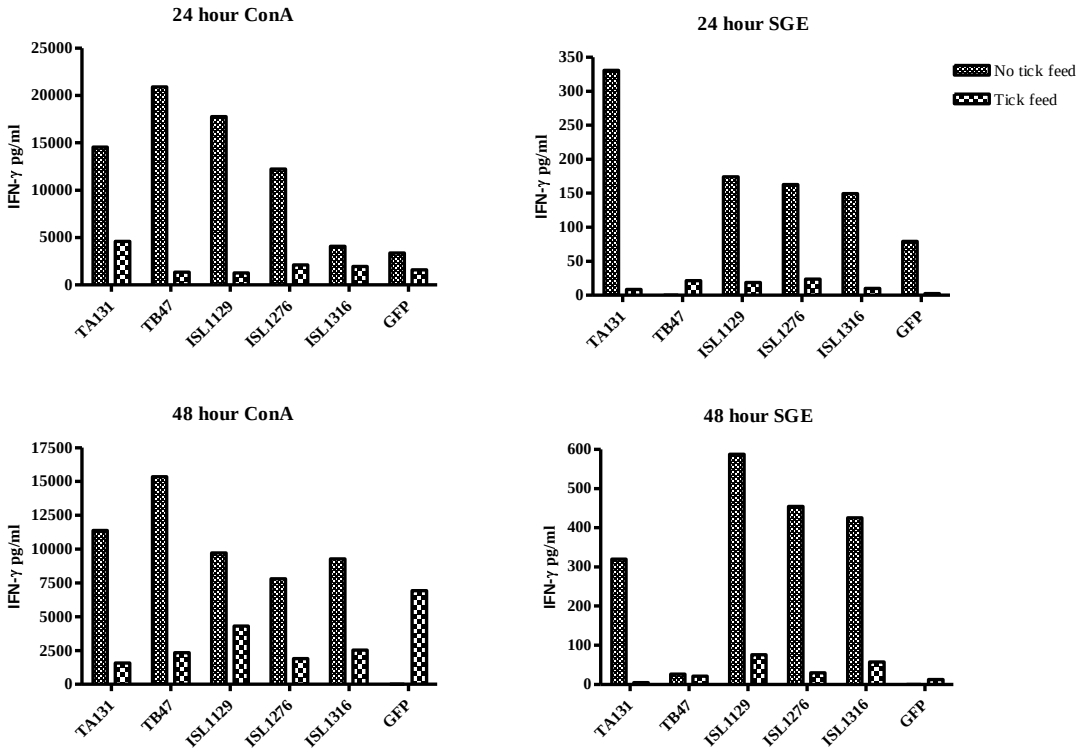
Figure 4.1: IFN-γ profiles of splenocytes from mice vaccinated with cDNA constructs 48 hours post stimulation with either ConA or *I. scapularis* saliva.



The effect of uninfected tick feed on the cytokine profiles of vaccinated mice became a matter of interest. The same vaccination protocol was followed as before, but two weeks after inoculations, mice were split into two groups, one which was sacrificed and spleens removed for culture, and the other which was subjected to uninfected, nymphal *I. scapularis* feeding. Two weeks after drop off was complete, mice were sacrificed and spleens removed for cell culture, and were stimulated either by ConA or *I. scapularis* SGE. Tick feeding appeared to only confound the results as the GFP controls had similar levels of IFN-γ stimulation as the cDNA constructs (Fig. 4.2). This is perhaps due to the fact that subjecting the mice to tick feed exposed them to many molecules that confounded our ability to discern the effect of tick feeding, given that tick feeding results in a decrease in IFN-γ production. Mice that were not subjected to tick feeding showed an increase in IFN-γ production that was dependant on which molecule

was being examined (Fig. 4.2). TA131, ISL1129, ISL1276 and ISL1316 all showed a marked increase in IFN-γ at 24 and 48 hours whether the splenocytes were stimulated with ConA or SGE. TB47 followed the trend observed in the first experiment with high production at 24 hours, but low production at 48 hours (Fig. 4.2).

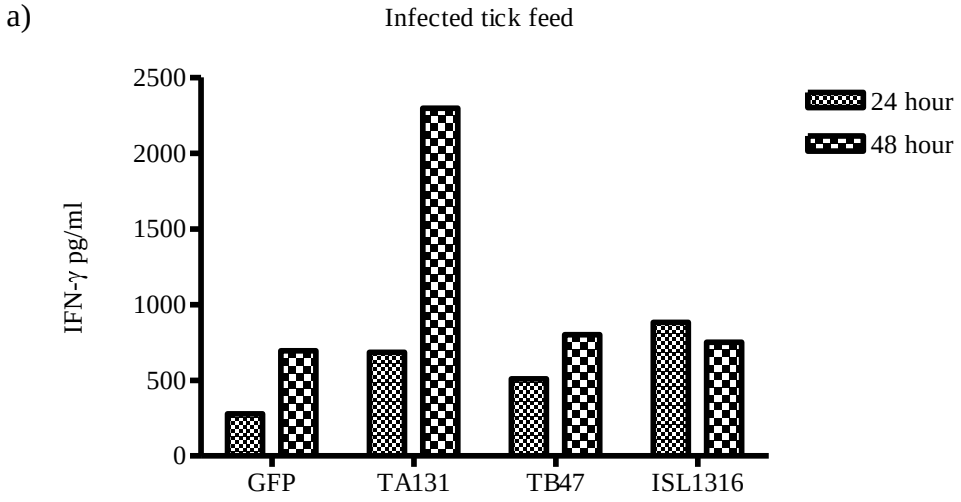
Figure 4.2: IFN-γ profiles of splenocytes from mice vaccinated with cDNA constructs 24 and 48 hours post stimulation with ConA.



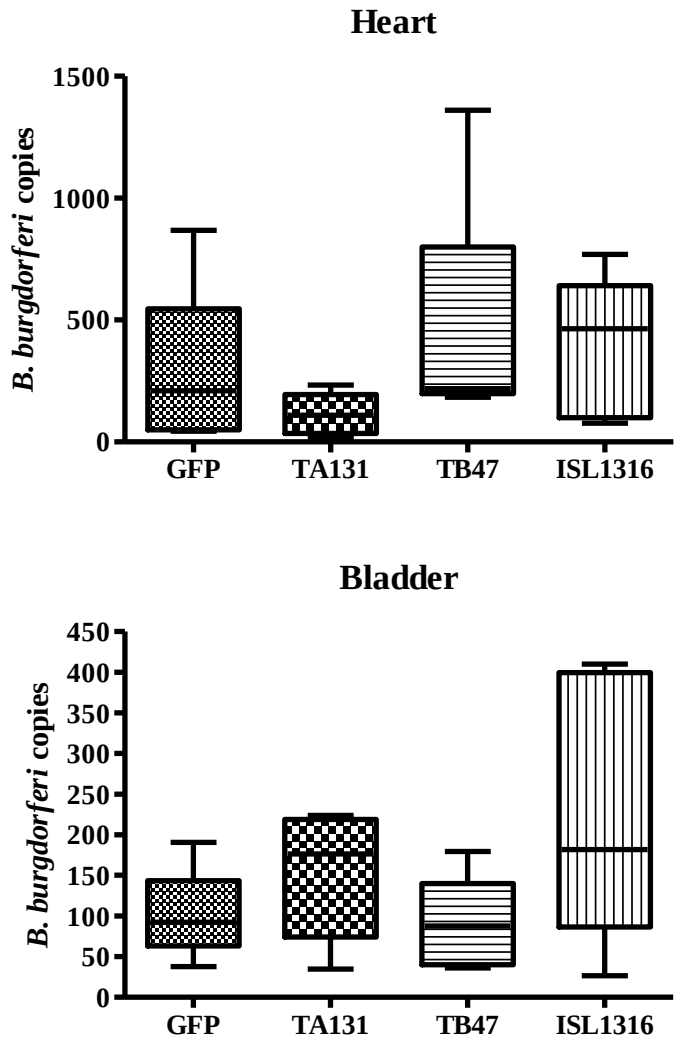
Efficacy of cDNA construct immunization in blocking tick-transmitted *B. burgdorferi*: Given the ability of selected salivary molecules from *I. scapularis* to induce an IFN-γ response, these molecules were tested as immunogens for their ability to block tick-transmitted *B. burgdorferi*. Mice were vaccinated with GFP control, TA131, TB47 or ISL1316 over a three week period. Two weeks after the last inoculation, *B. burgdorferi* infected nymphal *I. scapularis* ticks were placed on each mouse and allowed

to feed to repletion. Two weeks after drop off of feeding ticks, ear biopsies of mice were taken to confirm infection by BSK culture. All mice were positive by culture for *B. burgdorferi*. Six weeks after tick drop off, mice were sacrificed and heart and bladder were taken for qPCR and BSK culture; as well as spleens for cell culture and cytokine profiles. The IFN-γ response seen in the mice vaccinated with salivary cDNA constructs as compared to GFP control was similar to previous experiments, with the exception of TA131 at 48 hours post stimulation, which was markedly increased (Fig. 4.3a). Heart and bladder from all mice were positive by BSK culture for *B.* burgdorferi. qPCR analysis of heart and bladder showed no significant difference in spirochete numbers as compared to GFP controls (heart p=0.2262, bladder p=0.3577) (Fig. 4.3b).

Figure 4.3: a) IFN-γ profiles of splenocytes from mice vaccinated with cDNA constructs and subsequently challenged with *B. burgdorferi* infected *I. scapularis* nymphs 48 hours post stimulation with ConA. b) qPCR results from heart and bladder of challenged mice.



b)



Discussion

It has been demonstrated that different vertebrate hosts of ticks have varying immune responses to the bite and the salivary components injected into the host during feeding. Many tick-host relationships are characterized by the acquisition of resistance to tick feeding which develops as a result of repeated tick infestations (Willadsen 1980). Acquired resistance was first observed and described by Trager in 1939, who demonstrated that guinea pigs in which *Dermacentor variabilis* had fed upon became resistant over successive feedings (Trager 1939). This acquired resistance is

characterized by reduced engorgement, increased duration of feeding, blocked molting and death of engorging ticks (Wikel 1996). It has been demonstrated that animals preexposed to ticks were protected from tularemia (Bell et al. 1979), and Lyme disease (Nazario et al. 1998), and vaccination with a tick salivary cement protein protected mice against lethal tick-borne encephalitis virus (Labuda et al. 2006).

The protective effect of salivary proteins is also observed in other arthropod-borne disease. Preexposure to mosquito saliva through bites led to partial protection against *Plasmodium berghei* infection (Donovan et al. 2007). Immunization with the molecules PpSP15 and maxidilan, from *Phlebotomus paptasi* and *Lutzomyia longipalpis* respectively, protect against *Leishmania major* infection in mice (Morris et al. 2001; Valenzuela et al. 2001). More recently, DNA vaccination using VR2001-TOPO with a single molecule from *L. longipalpis*, LJM19, protected hamsters against the fatal outcome of *Leishmania infantum chagasi*, visceral leishmaniasis (Gomes et al. 2008). This protection correlated with a high IFN-γ/TGF-β ratio and inducible nitric oxide synthetase production, indicating a strong inflammatory response allowing for the block in infection. A strong delayed-type hypersensitivity response was also observed in the skin of immunized hamsters 48 hours after exposure to sand fly bites (Gomes et al. 2008).

We chose to test six different tick salivary molecules in the DNA vaccination vector VR2001-TOPO in the ability of the molecules to generate a strong IFN-γ response in mice, as well as the ability of these molecules to block tick-transmitted *B. burgdorferi* infection. TB131 has been identified as a Salp9 homolog, with Salp9 being a anticoagulant (Narasimhan et al. 2002). TB47 is also a homolog of Salp9 and Salp 11, which is Factor Xa inhibitor, similar to Ixolaris (Francischetti et al. 2002). ISL1156 is a

putative protease inhibitor; ISL1276 is a putative secreted histamine binding protein; and ISL1316 is a carboxypeptidase (Valenzuela 2004), and ISL1129 is currently an unidentified molecule.

Cytokine profiles of the six molecules demonstrated a strong IFN-γ response from TA131, TB47 and ISL1129 with ConA stimulation as compared to a control plasmid containing the GFP gene. (Fig. 4.1 and 4.2). When cultured splenocytes were stimulated with SGE, TA131, ISL1129, ISL1276 and ISL1316 showed a strong up regulation of IFN-γ as compared to GFP controls (Fig. 4.2). The SGE we used was acquired over a 4 day feeding period to ensure we captured the entire protein profile of saliva as it is likely to have a temporal expression pattern during the course of feeding (Valenzuela 2004; Valenzuela et al. 2002). When mice were vaccinated with TA131, TB47 and ISL1316 and subsequently challenged with infected ticks, there was no effect on spirochete numbers in heart or bladder as compared to GFP controls, with the exception of a trend seen with TA131 for decreased spirochete load in the heart (Fig. 4.3b). This was a disappointing result given the high levels of IFN-γ produced in mice vaccinated with these genes.

We are currently planning future experiments involving the TA131, TB47, ISL1129 and ISL1316 using the cDNA constructs in conjunction with a prime-boost strategy with recombinant protein from the same genes. Evidence exists in the literature that this technique leads to increased amounts of neutralizing antibodies and higher cytotoxic T lymphocyte responses (Kutzler and Weiner 2008). We are also currently contracting with ViraQuest to produce Adenovirus constructs of these four genes. It is our hypothesis that a vaccine designed with the correct combination of different salivary

molecules and presented in the correct manner will block tick-transmitted *B. burgdorferi* infection.

Chapter 5

Conclusions

The results of this dissertation expand the knowledge base of the effect of vaccination with specific *Ixodes scapularis* salivary proteins on the transmission of *Borrelia burgdorferi* in a murine model of Lyme disease. The results suggest that a few of the salivary proteins examined have the ability to decrease the spirochete load in mice as compared to controls.

The second chapter, entitled "Construction of Adenoviral vectored *Ixodes scapularis* salivary protein targeted vaccines" focused on building adenoviral constructs expressing Salp14, Salp15, Salp25A and Salp25D. Previous work demonstrated the contribution of Salp14 as an anticoagulant (Das et al. 2001; Narasimhan et al. 2002) and Salp25D as an amine binding protein, which helps counter the host immune response, (Das et al. 2001) for *I. scapularis* in obtaining a successful bloodmeal. Salp15 is a well-studied molecule and contributes greatly to successful feeding in *I. scapularis* by the ability to inhibit CD4+ T cell activation, down regulate IL-2 production, and inhibit CD25 expression on T cells, thus interfering with IL-2 activation of T cells (Anguita et al. 2002). Salp15 also binds OspC on the surface of *B. burgdorferi* effectively masking the spirochete in this highly immunomodulatory tick protein, and assisting in transmission of the spirochete (Ramamoorthi et al. 2005). With the exception of Salp14, the genes for these proteins were cloned from cDNA copies and successfully inserted into the adenoviral genome. However, attempts to generate a viral titer high enough to

vaccinate mice at the target titer of $1x10^{10}$ PFU/ml was unsuccessful and commercial avenues for virus production were then explored.

Chapter three entitled "Immunization with adenoviral-expressed salivary gland proteins (SALPs) decreases spirochete load in a murine model of Lyme Borreliosis" focused on testing the hypothesis that presenting highly immunomodulatory tick salivary proteins in a context that shifts the immune response of the host from a Th2 polarized response, which is normal for tick feeding (Ferreira and Silva 1999), to a Th1 response would block transmission of *B. burgdorferi* by infected ticks. To drive the Th1 response in mice, an adenovirus vaccine vector was utilized as these vectors push a strong inflammatory response by their own accord (Sullivan et al. 2000). Adenoviral constructs containing Salp15, Salp25A, Salp25D and Isac, an anticomplement protein from *I. scapularis* (Valenzuela et al. 2000), were generated and a series of experiments designed to test the ability of these constructs to block tick-transmission of *B. burgdorferi* were commenced.

The need to understand the effect of vaccination on *B. burgdorferi* infection without the presence of tick saliva was addressed by vaccinating mice and challenging with needle infection of cultured *B. burgdorferi*. When all four adenoviral constructs, AdSalp15, AdSalp25A, AdSalp25D and AdIsac were inoculated into mice and compared to injection buffer control and adenovirus vector without an inserted gene (AdEmpty), there was no measureable difference seen in the spirochete burden of the target tissues of heart and bladder in mice. This result confirmed what was expected, that the adenoviral constructs directed toward tick proteins had no effect on *B. burgdorferi* infection without presence of tick saliva.

Previous, unpublished research from our laboratory indicated that mice immunized three times with a combination of Salp14, Salp15, Salp25A and Salp25D demonstrated a 60% reduction in fed tick weight, but had no effect on transmission of *B. burgdorferi*. When mice were vaccinated with AdSalp15, AdSalp25A, AdSalp25D and AdIsac and challenged with uninfected *I. scapularis* nymphs, no difference in fed tick weight was observed. Cytokine profiles from vaccinated mice demonstrated a strong Th1 response in up regulation of IFN-γ and TNF-α as compared to buffer and AdEmpty controls.

Vaccinated mice were then subjected to *B. burgdorferi* infected tick feeds to see if there was an effect on transmission of the spirochete. The cytokine profiles from the vaccinated mice mimicked what was previously seen, confirming the shift from a Th2 to a Th1 response. The spirochete burden in the heart of vaccinated mice was 60% lower than the burden in the heart of buffer control mice. Although this result was not statistically significant, the trend does exist, and a larger sample size could resolve the lack of significance. When AdSalp15 and AdIsac were tested individually against controls for the ability to reduce spirochete burden, AdSalp 15 did not generate a noticeable Th1 response in vaccinated mice and did not reduce the spirochete load in the heart as compared to AdIsac. AdIsac showed a strong Th1 response and a non-significant trend toward lower spirochete load in the heart of vaccinated mice.

Given the 60% decrease seen in spirochete burden seen in vaccinated mice, the question arose was to whether this was enough of a decrease to effect transmission from mice infected by tick bite to clean, larval ticks. Mice were vaccinated with buffer control, AdEmpty or AdSalp15+AdIsac and subsequently challenged with infected

nymphs. Mice were allowed to rest for four weeks, and then infested with clean *I. scapularis* larval ticks. Individual larval ticks were subjected to qPCR analysis of spirochete load and no significant difference existed between experimental groups. Five weeks after larvae dropped off of the mice, and had molted to nymphs, individual nymphs were tested by qPCR for spirochete load. A significant difference was observed between the groups, with the nymphs from AdSalp15+AdIsac vaccinated mice having a lower spirochete load. These nymphs were allowed to feed on clean mice and did not demonstrate any difference in ability to transmit *B. burgdorferi* to naïve mice.

An experimental variable which confounded the results was the use of all four constructs simultaneously in most experiments, outside of the individual vaccination with AdSalp15 and AdIsac. AdSalp25A and AdSalp25D seemingly had no effect on the host immune response or tick-transmitted infection of *B. burgdorferi*. AdSalp15 appeared to dampen the effect of AdIsac in its ability to decrease the spirochete load in the heart of mice infected by tick bite. Isac as a vaccine antigen may certainly hold merit, and further exploration with this protein in different delivery systems is warranted for future research.

The fourth chapter is entitled "cDNA vaccination of mice with novel *Ixodes scapularis* salivary genes" and describes a series of experiments with tick salivary genes in a DNA vaccine vector performed with collaborators from the NIH. Six different cDNA constructs were tested for the ability to produce a strong IFN-γ response. Previous research using this specific cDNA vector for DNA vaccination showed a complete block of *Leishmania infantum chagasi* infection when a single molecule from *Lytzomyia longipalpis* was inoculated (Gomes et al. 2008). Protection against infection showed a

strong correlation to a high IFN-γ/TGF-β profile seen in mice. Of the six cDNA constructs tested, TA131, TB47, ISL1129 and ISL1316 showed a strong up regulation of IFN-γ in mice. When mice were vaccinated with TA131, TB47 and ISL1316 and subsequently challenged with infected ticks, there was no effect on spirochete numbers in heart or bladder as compared to GFP controls, with the exception of a trend seen with TA131 for decreased spirochete load in the heart. This preliminary work will lead to further study of TA131, TB47, ISL1129 and ISL1316 in an adenoviral vector, and as a DNA vaccine with a recombinant protein prime-boost.

The goal of this dissertation was to explore several tick salivary molecules for the ability to serve as vaccine antigens in an anti-tick, *B. burgdorferi* transmission-blocking vaccine. Through this work, we have gained a better insight into the vaccine potential of a few molecules, while eliminating several other molecules. Although much work remains in developing an anti-tick vaccine, every potential antigen identified could lead us closer to having an effective tool in controlling Lyme disease and improving public health.

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