MODULATION OF THE HOST IMMUNE RESPONSE BY *IXODES SCAPULARIS* OR *IXODES PACIFICUS*, NORTH AMERICAN VECTORS OF LYME BORRELIOSIS

By

GEORGE BERNARD SCHOELER

Bachelor of Science
Boise State University
Boise, Idaho
1989

Master of Science
University of California
Berkeley, California
1992

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Thesis approved:

Thesis Advisor

Dean of the Graduate College

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NOMENCLATURE

BSA	bovine serum albumin
Con A	concanavalin A
CPM	counts per minute
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
IFN-γ	interferon –gamma
IL	interleukin
LPS	lipopolysaccharide
mg	milligram
ml	milliliter
ng	nanogram
PBS	phosphate buffered saline
pg	picogram
S.E.	standard error
SGE	salivary gland extract
SGSP	salivary gland soluble protein
S.I.	stimulation index
TNF-α	tumor necrosis factor-alpha
μg	microgram

CHAPTER I

INTRODUCTION

Overview

Lyme borreliosis, or Lyme disease, was first recognized in the United States in 1975, after a mysterious outbreak of juvenile arthritis occurred near Old Lyme, Connecticut (Centers for Disease Control and Prevention, 1996). In 1982, Burgdorfer and coworkers conclusively identified the spirochete nature of the infection, and the association of the organism with ticks of the genus *Ixodes* (Burgdorfer *et al.*, 1982). The etiologic agent was isolated by Barbour (1984), and Johnson *et al.* (1984) subsequently described the organism as a new *Borrelia* species, named *Borrelia burgdorferi* (Barbour, 1984, Johnson *et al.*, 1984). Since the discovery of the etiologic agent, the incidence of Lyme borreliosis has increased yearly in the United States and this infection has emerged as a disease of global human and veterinary public health importance. Lyme borreliosis is currently the most commonly reported vector-borne disease in the United States (Centers for Disease Control and Prevention, 1999).

Lyme borreliosis is caused by several species of spirochetes that have been grouped into the *Borrelia burgdorferi sensu lato* complex (Baranton *et al.*, 1992, Nocton and Steere, 1995). In North America, the *B. burgdorferi sensu stricto* genospecies is the most common cause of Lyme borreliosis (Akins *et al.*, 1998). Since the implementation of a standardized case definition in 1991, greater than 90% of cases have been reported from the northeast and north central United States (Centers for Disease Control and Prevention, 1999). The remaining cases are reported from the West Coast, in particular areas of northern California (Centers for Disease Control and Prevention, 1996).

Borrelia burgdorferi is maintained in nature via complex enzootic cycles which involve rodents and ticks of the *Ixodes ricinus* complex of species, including *I. scapularis* and *I. pacificus* in the United States, *I. ricinus* in Europe, and *I. persulcatus* in Asia (Dekonenko *et al.*, 1988, Dizij and Kurtenbach, 1995, Lane *et al.*, 1991, Spielman *et al.*, 1985). In the eastern and mid-western United States, where the disease is most prevalent, *Ixodes scapular*is is the principal maintenance vector of the spirochete within cycles involving wild rodent reservoirs, primarily the white footed mouse, *Peromyscus leucopus* (Lane *et al.*, 1991, Levine *et al.*, 1985). In these areas, *I. scapularis* is the principal vector to humans, and infection of humans occurs primarily through the bite of infected nymphs (Lane *et al.*, 1991).

In the western United States, *I. pacificus* is the principal vector of *B. burgdorferi* to humans (Clover and Lane, 1995, Lane and Lavoie, 1988). The preferred host of immature *I. pacificus* is the western fence lizard, *Sceloporus occidentalis* (Arthur and Snow, 1968, Lane and Lavoie, 1988). Lizards, in contrast to rodents, are not susceptible to *B. burgdorferi* infection, and recently a borreliacidal factor was discovered in the blood of *S. occidentalis*, which destroys spirochetes in the midgut of feeding *I. pacificus* nymphs (Lane and Quistad, 1998). The enzootic cycle of *B. burgdorferi* in this region is supported by dusky-footed woodrats, *Neotoma fuscipes*, and a non-human biting tick, *Ixodes neotomae* (Brown and Lane, 1992). Thus, those *I. pacificus* immatures that feed on infected woodrats, instead of lizards, may be responsible for transmitting the infection to humans. In support of this, epidemiological studies conducted in northern California have demonstrated that the majority of human cases of Lyme borreliosis are reported during the peak activity period of nymphal *I. pacificus* (Clover and Lane, 1995).

Lyme borreliosis is a multisystem illness with systemic symptoms and the potential for neurologic, rheumatologic, and cardiac involvement occurring in combinations over a period of months to years (Anonymous, 1995). In humans, Lyme borreliosis generally occurs in stages with different clinical manifestations at each stage. Early infection, stage one, is characterized by localized erythema migrans, rash caused by spirochetes disseminating in the skin; regional lymphadenopathy; and, may be accompanied by fever as well as other flu-like symptoms (Steere, 1989). Within days or weeks following infection, spirochetes may spread in the patient's blood or lymph to many sites, resulting in stage two, disseminated infection. Disseminated infection is often associated with involvement of the skin, nervous system, and musculoskeletal system (Steere, 1989). These symptoms include secondary annular skin lesions, headache, joint stiffness and pain, debilitating malaise, and fatigue. The third stage of infection, termed late or persistent infection, is characterized by episodes of arthritis lasting as long as a year or more. Typically, only one or a few large joints are affected. Nervous system involvement including encephalopathy, neuropathy, and dementia may be encountered during this stage (Steere, 1989).

If diagnosed and treated during the early stages, the infection generally resolves with little or no complications. However, if untreated, a debilitating chronic form of the disease manifests itself, which does not respond well to treatment. Additionally, problems in diagnosing the disease may complicate efforts to provide appropriate treatment for the infected patient. Methods to reduce or prevent the number of cases of Lyme borreliosis, including the development of second-generation vaccines and other control measures, are needed.

Since the discovery of the causative agent, studies of Lyme borreliosis have encompassed vector ecology, epidemiology, molecular biology, pathogenesis, and treatment (Jones *et al.*, 1995, Lane *et al.*, 1991, Sigal, 1997, Spielman *et al.*, 1985, Steere, 1989). However, few studies have addressed the effects of host immune responses to the feeding tick, on spirochete transmission and establishment in the host (Wikel, 1996b, Wikel and Bergman, 1997).

Complex immunological interactions occur at the tick-host-pathogen interface (Wikel and Bergman, 1997, Zeidner *et al.*, 1996). Host anti-tick immune responses and tick countermeasures directed against these responses are important elements of the relationship between the host, the feeding tick, and any pathogens that may be transmitted during tick feeding. A greater understanding of the immunology of the tick-host interface will contribute significantly to understanding the transmission of *B. burgdorferi.* Determining the affects of repeated exposure to the tick vector on host immunity, and comparing immune responses elicited by closely related vector ticks, may help identify those tick factors that modulate host immune responses. Identifying and characterizing these responses and the responsible factors may provide a general strategy to reduce infection by other tick-borne pathogens.

Specific and Acquired Host Immunity

Unlike most blood-feeding arthropods, which typically feed on the host for brief periods, ixodid ticks attach to the host and feed for two to three days to almost three weeks, depending on the life stage of the tick (Sonenshine, 1991). During the extended

period required to obtain a blood meal, the tick is continuously exposed to elements of both innate and specific acquired host immune responses (Wikel, 1996a).

Innate immunity includes components of the immune system that are designed to provide an immediate response to protect the host from injury or infection. Innate immune responses do not require previous contact by the host to the infectious agent (Janeway and Travers, 1997). Components of innate immunity include natural barriers to infectious organisms such as the skin, chemicals contained in saliva, tears and sweat, as well as cellular components such as phagocytic cells. The epithelia that make up the skin and linings of the body's internal tubular (respiratory tract, gastroinstestinal tract, etc.) structures provide mechanical, chemical and microbiological barriers to invading microorganisms (Fearon and Locksley, 1996). Mechanical aspects include epithelial cells joined by tight junctions and the movement of mucus by cilia that prevent bacteria from colonizing on the epithelial surface. Fatty acids of the skin, tears, and saliva provide chemical protection against invading microorganisms, and normal flora of the epithelial surface may produce antibacterial proteins that provide further protection against invading pathogens.

Invading organisms that are able to penetrate the mechanical, chemical and microbiological barriers are subject to further components of the innate immune response. The complement system, comprising a series of serum proteins, is important in both innate and acquired immune responses (Taylor *et al.*, 1998). Of these, the alternative pathway of complement activation can be regarded as an innate humoral response. The spontaneous cleavage of C3 to C3b in plasma, and the binding of C3b to bacteria that lack complement regulatory proteins leads to the activation of terminal

complement components that can destroy bacteria (Fearon and Locksley, 1996, Taylor *et al.*, 1998). In addition, factors produced during the activation of complement are chemotactic to immune effector cells, serving to attract these cells to the site of infection (Janeway and Travers, 1997).

Cells involved in innate immunity include phagocytes, natural killer cells, and granulocytes. (Janeway and Travers, 1997, Robertson, 1998). There are two major types of phagocytic cells, neutrophils and macrophages. Neutrophils are phagocytic cells that are not antigen presenting cells. Neutrophils attracted to sites of infection secrete essential bacteriostatic and toxic substances that kill phagocytosed pathogens. Macrophages function in phagocytozing and ultimately destroying pathogens in tissues (Janeway and Travers, 1997). Receptors on macrophages function in antigen uptake and presentation, contributing to the induction of a specific, adaptive immune response (Fearon and Locksley, 1996, Medzhitov and Janeway, 1998).

Cytokines are soluble mediators that orchestrate cellular interactions of the immune system. They are produced by a wide variety of cell types, such as phagocytic cells, lymphocytes, natural killer cells, epithelial cells and fibroblasts (Janeway and Travers, 1997). Cytokines have a wide range of biological activities. These activities include: stimulating or suppressing cell growth and differentiation; inducing antibody class switch; serving as chemoattractants for immune effector cells; and, inducing inflammatory responses (Janeway and Travers, 1997). Cytokines produced by immune effector cells are important in all phases of immune response; they function in both innate and specific acquired immunity. Cytokines secreted by macrophages in response to infection include Interleukin (IL)-1, IL-6, IL-8, IL-12, and tumor necrosis factor (TNF)-α

(VanderMeide and Schellekens, 1996). Phagocytes also release a variety of immune mediators in response to infectious agents including prostaglandins, oxygen radicals, nitric oxide, and platelet activating factor. The effect of specific cytokines and mediators produced by phagocytes, and the activation of complement inflammatory mediators contributes to an inflammatory response. Inflammation is characterized by pain, redness, heat, and swelling (Janeway and Travers, 1997). In addition to specific activated complement factors, neutrophils are directed to the site of infection by IL-8. Interleukin-12 activates natural killer cells to produce interferon (IFN)-γ, which induces the differentiation of CD4+ T cells into T helper (Th)-1 cells (Gumperez and Parham, 1995). Interleukin-1, IL-6, and TNF–α, released by activated macrophages, induce a variety of responses including the activation of hepatocytes to synthesize acute-phase proteins and bone marrow to release neutrophils. These cytokines also raise the body temperature by acting on the hypothalamus, fat cells, and muscle cells (VanderMeide and Schellekens, 1996). Acute-phase proteins also help activate T and B cells in response to infection (Janeway and Travers, 1997).

Specific acquired immunity is characterized as the responses of antigen-specific B and T lymphocytes, resulting in a variety of effector functions as well as the development of immunological memory (Janeway and Travers, 1997). As in innate immune responses, cytokines and other immune factors mediate cell differentiation and cell attraction to the site of infection. Acquired immunity is directed toward a specific antigen, in contrast to innate immunity, which involves immune responses that are not antigen-specific, do not require previous exposure to the antigen, and do not result in immune memory (Janeway and Travers, 1997). Many elements of innate immunity are

also involved in acquired immune responses and dictate the nature of the acquired immune response.

T lymphocytes are critical in the functioning of acquired immune responses. T lymphocytes can be divided into two main classes, cytotoxic (CD8) T cells that kill virus-infected cells, and CD4 T cells, which help activate other cells, such as B cells and macrophages (Janeway and Travers, 1997). Differentiation of naïve CD4 T lymphocytes is influenced by cytokines secreted in the early stages of infection (Scott, 1993). Naïve CD4 T cells differentiate into one of two types of effector cells, Th-1 or Th-2 cells; Th-2 cells may function to activate B lymphocytes to produce antibody while Th-1 cells may function in macrophage activation. Differentiation is driven in response to cytokine stimulation of the naïve CD4 cells. For example, CD4 T cells stimulated with IFN-γ and IL-12, produced by natural killer cells and macrophages, differentiate into Th-1 cells; CD4 cells stimulated by IL-4 and IL-6 will develop into Th-2 cells (Paul and Seder, 1994). CD8 T cells also have the ability to secrete cytokines typical of Th-1 and Th-2 cells (Scott, 1993).

The type of T cell response elicited by antigenic stimulation is important in determining whether the adaptive immune response will be predominately a cell-mediated, or an antibody (humoral) response. In reality, most immune responses consist of a mixture of both cell-mediated and antibody responses. In addition, the CD4 T cell subsets can regulate the growth and effector functions of one another. Interleukin-10, produced by Th-2 cells, inhibits the development of Th-1 cells by acting on antigen-presenting cells, while IFN-γ, produced by activated Th-1 cells, can prevent the activation of Th-2 cells (Mosmann and Coffman, 1989, Scott, 1993).

Tick-Host Immunology

To maintain the complex feeding interface over the extended interval required for the tick to obtain a blood meal, tick salivary glands produce and secrete a variety of materials. These include vasodilatory, anti-inflammatory, anti-itch (kininases), and immunomodulatory molecules that counter host defensive responses directed against the feeding tick, help maintain blood flow to the feeding site, and inhibit the itch/pain response in the host (Ribeiro and Mather, 1998, Wikel and Bergman, 1997). While the molecules introduced into the bite site by the tick are essential to ensure successful feeding, many of these molecules are immunogenic (Wikel, 1996a), inducing a variety of immune responses within the host.

Host immune responses to tick feeding vary based on the tick-host association, the genetic background of the host, life stage of the tick, intensity of the infestation, and previous exposure of the host to tick feeding (Wikel, 1996a). Acquired resistance to tick infestation occurs in some natural hosts (Dizij and Kurtenbach, 1995); although it has been most extensively characterized in laboratory animals and bovines (Wikel, 1996b). Laboratory animal species often develop stronger acquired resistance than do natural hosts (Ribeiro, 1989). However, this is not always the case since immunological reactions of BALB/c mice to *I. ricinus* are reportedly similar to those observed in nature (Mbow *et al.*, 1994a). Specifically, BALB/c mice do not develop resistance to *I. ricinus* nymphs or larvae (Christe *et al.*, 1998, Mbow *et al.*, 1994a). Even in the absence of acquired resistance to feeding, tick infestation does stimulate host immune responses (Borsky *et al.*, 1994, Ganapamo *et al.*, 1995). Therefore, murine models provide valuable information on tick-host immunity because of the large number of different inbred strains

available, their well-characterized immune responses, and the availability of immunological reagents (Wikel and Bergman, 1997).

Tick feeding stimulates host immunoregulatory and effector pathways involving antigen presenting cells, T and B lymphocytes, cytokines, homocytotrophic and circulating antibodies, complement, granulocytes and a multitude of other biologically active molecules (Wikel, 1996a). Wikel and Bergman (Wikel and Bergman, 1997) have proposed a model to describe the interactions between the feeding tick and the host immune system. Salivary antigens, introduced by the tick into the feeding site, are processed by antigen presenting cells (APC) including Langerhans cells in the epidermis, macrophages in the dermis, and dendritic cells in the draining lymph nodes and spleen. After phagocytosis by APCs, tick immunogens are processed by the cells and presented on the APC surface in the context of the class II major histocompatability complex (MHC). Presentation in this manner allows recognition by specific T helper cells and B cells, leading to the production of both circulating and homocytotrophic tick-antigen specific antibodies.

Activated Th-1 cells mediate delayed hypersensitivity responses through the release of cytokines that activate effector cells, including macrophages. In addition, delayed hypersensitivity is the mechanism by which cutaneous basophil hypersensitivity responses to tick feeding occur. T-cells and macrophages attracted to the feeding site can produce cytokines, including IL-1β, IL-2, IL-4, IL-6, IL-10, IFN-γ, and TNF–α, which induce inflammation at the bite site, stimulate responses of other immune cells, and attract other cells to the feeding site. In addition, the alternate pathway of the complement cascade is initiated, causing the release of chemotactic factors that help to

attract more inflammatory cells to the feeding site. Basophils and eosinophils, attracted by complement chemotactic factors, infiltrate the feeding site. These cells, along with resident mast cells, are degranulated, releasing histamine and other inflammatory factors (Wikel and Bergman, 1997).

Tick feeding induces cell-mediated immune responses resulting in inflammation at the bite site, as well as humoral responses resulting in the production of anti-tick antibodies directed against salivary antigens. Innate and acquired immune responses at the bite site are detrimental to tick feeding since immune effector cells are attracted to the feeding site, which attempt to eliminate the tick source of the inflammation. Anti-tick antibodies neutralize components of the saliva, reducing the ability of these components to counter the host immune response (Wikel, 1999b). Furthermore, immune effector cells attracted to the feeding site may produce mediators that induce blood clotting within small vessels, thus restricting blood flow to the feeding site (Janeway and Travers, 1997).

Host immune responses at the feeding site result in multiple immune mechanisms directed against the feeding tick. These immune responses can reduce numbers of feeding ticks, decrease blood meal size, reduce numbers and viability of eggs produced, lengthen feeding times, and may ultimately lead to death of the feeding tick (Wikel, 1996a). Host immune responses could also make the feeding site less favorable for pathogens introduced by the tick, since many of the immune mechanisms directed against feeding ticks are similar to those directed against pathogens that they transmit (Wikel, 1996b).

Tick Modulation of Host Immunity

Given the array of responses generated by the host against the feeding tick, it is not surprising that ticks have evolved mechanisms to modulate host immune responses directed against them while feeding (Wikel, 1996a). Tick saliva contains molecules with antihaemostatic, vasodilatory, anti-inflammatory, and immunosuppressive properties (Ribeiro, 1995). Molecules that inhibit coagulation and enhance vasodilation also contribute to the formation of the tick-feeding site while anti-inflammatory and immunosupressive proteins reduce host immune responses directed against feeding ticks (Wikel and Bergman, 1997).

Tick-induced immunomodulation can reduce both acquired and innate host immune responses directed against feeding ticks; this suppression impairs their ability to respond to any antigen. For example, Wikel (1982) demonstrated that primary antibody responses were suppressed in guinea pigs following infestation with *Dermacentor andersoni* (Wikel, 1982b), while Ribeiro (1989) showed that the saliva of *I. scapularis* introduced during tick feeding can inhibit complement binding, prevent generation of the anaphylotoxin C3a, and inhibit the cleavage of complement component C3 into it's active form, C3b (Ribeiro, 1989).

Other studies have demonstrated that T lymphocyte responses are suppressed during tick feeding. Concanavalin A-stimulated proliferative responses of lymphocytes from guinea pigs, infested with *D. andersoni*, were reduced when compared to noninfested animals, and the reduction was greater during initial infestations than during subsequent infestations (Wikel, 1982b). Concanavalin A mimics the actions of antigens on T lymphocytes and stimulates T cell proliferation *in vitro* (Sharon, 1983). Lymph

node cells of C3H/HeJ mice infested one and four times with two male and two female *Rhipicephalus sanguineus* showed a decrease of 77.9% and 82.4%, respectively, in proliferative responses to Con A when compared to cells from uninfested animals (Ferreira and Silva, 1999). In contrast to lymph node cells, splenocytes from the same mice showed no significant change in proliferative responses, when compared to uninfested control animals (Ferreira and Silva, 1999). The Con A-stimulated proliferative response of splenic lymphocytes from BALB/c mice repeatedly infested with *I. ricinus* was unchanged after the primary and secondary infestations, and then increased above those from noninfested mice after the third and fourth infestations (Dusbabek *et al.*, 1995). In contrast, B cell responses to LPS and pokeweed mitogen-induced proliferation were suppressed after each of four infestations (Dusbabek *et al.*, 1995). Borsky *et al.* (1994) reported reduced responsiveness of splenocytes to Con A after a primary infestation with *I. ricinus* nymphs. Responsiveness increased to levels above control value after the second infestation, and then decreased again following the third exposure (Borsky *et al.*, 1994). Finally, T cells collected from lymph nodes of *I. ricinus* infested BALB/c mice at nine days post-infestation had a suppressed *in vitro* response to Con A (Ganapamo *et al.*, 1996b).

Reduced *in vitro* proliferation of lymphocytes in response to mitogens demonstrates that molecules in tick saliva suppress the activity of these immune effector cells, thus reducing their ability to initiate immune responses. Saliva or salivary gland extracts from various tick species contain molecules that influence Con A-stimulated lymphocyte proliferation (Bergman *et al.*, 1995, Bergman *et al.*, 1998, Ferreira and Silva, 1999, Urioste *et al.*, 1994). Protein in the saliva *of I. scapularis* reduces *in vitro*

proliferation of mouse lymphocytes to Con A (Urioste *et al.*, 1994). Splenocytes from C57BL/6 and C3H/H mice, incubated in the presence of dilutions of *I. dammini* (=*I. scapularis* (Oliver *et al.*, 1993)) saliva, had significantly decreased proliferative responses to Con A (Urioste *et al.*, 1994). Proliferative responses were suppressed up to 70-80%, when compared to that of cells not exposed to tick saliva (Urioste *et al.*, 1994). Salivary gland extracts prepared daily from feeding *D. andersoni* females suppressed *in vitro* lymphocyte responsiveness to Con A and enhanced proliferation in the presence of LPS for lymphocytes from uninfested mice (Ramachandra and Wikel, 1992) and cattle (Ramachandra and Wikel, 1995). Subsequent studies identified a 36-kDa soluble protein from *D. andersoni* salivary glands to be responsible for suppression of Con A-induced lymphocyte proliferation (Bergman *et al.*, 1998).

As previously stated, cytokines play a central role in regulation of host immune responses (Kroemer *et al.*, 1993). The fact that ticks can influence host cytokine production is not surprising (Wikel, 1996c). Ticks suppress host macrophage and T-cell cytokine production, specifically Th-1cytokines, in a manner that reduces development and manifestation of resistance to tick feeding (Wikel and Bergman, 1997). Salivary gland extracts from unmated female *D. andersoni* adults suppressed IL-1β and TNF–α elaboration from macrophages and IL-2 and IFN-γ production from T-lymphocytes (Ramachandra and Wikel, 1992). In BALB/c mice repeatedly infested with *I. ricinus* nymphs, IL-4 levels from lymph nodes draining the attachment site remained high throughout three infestations, while IFN-γ levels were low up until the third infestation, at which time the levels increased (Ganapamo *et al.*, 1995). Urioste, *et al.* (1994) demonstrated that *I. scapularis* saliva down-regulated the production of IL-2 by T-cells

and nitric oxide production by macrophages (Urioste *et al.*, 1994). Tick suppression of macrophage and T-lymphocyte elaboration of cytokines reduces the effectiveness of host immune responses directed against the feeding tick, which increases the likelihood of successful feeding (Wikel, 1996c).

These studies demonstrate that tick saliva, introduced during tick feeding, suppresses both innate and acquired immune responses directed against the feeding tick, thus enhancing the ability of the tick to successfully obtain a blood meal. Innate and acquired immune responses suppressed by feeding ticks include: complement, required for cellular attraction to the feeding site; macrophage and Th-1 cytokine responses, required for inflammatory and cell-mediated responses; and antibodies, which may neutralize essential components in tick saliva. Host immune responses to tick infestation and the countermeasures introduced by ticks to suppress these immune responses are summarized in Table 1.

Table 1. Host responses to tick feeding and tick countermeasures to host immune responses. ***Adapted from Wikel and Bergman, 1997.***

Host Immune Response Components to Tick Feeding	*Tick Countermeasures to Host Immune Response*	*Type of Immunity*	*References*
Complement (alternative, mannan-binding lectin, and classical pathways)	Inhibition of chemotactic factors and C3b/C5b deposition	Acquired and Innate	Ribeiro, 1987, Ribeiro and Spielman, 1986
Macrophages Production of pro-inflammatory/ immunoregulatory cytokines IL-1 and TNF-α	Reduction of IL-1 and TNF-α production	Innate	Ramachandra and Wikel, 1992, Ramachandra and Wikel, 1995
T cells Proliferation Cytokine production (IL-2, IL-4, IL-5, and IFN-γ)	Inhibition of mitogen-induced proliferation. Suppression of elaboration (or mRNA) of IL-2, IL-4, IL-5, and IFN-γ	Acquired	Ganapamo *et al.*, 1995, Ganapamo *et al.*, 1996b, Ramachandra and Wikel, 1992, Wikel, 1982b
Antibodies Production of circulating or homocytotrophic antibodies	Suppression of antibody responses.	Acquired	Fivaz, 1989, Wikel, 1985

Although tick modulation of host immunity likely arose to facilitate feeding, reducing host innate and specific acquired immune responses against the feeding tick may enhance tick-transmission of pathogens since many of the immune mechanisms directed against feeding ticks are similar to those directed against tick-transmitted pathogens (Wikel, 1996b, Wikel and Bergman, 1997, Zeidner *et al.*, 1996).

Immunity to *B. burgdorferi.*

Infection with *B. burgdorferi* causes a multi-system inflammatory ailment in humans, domestic and laboratory animals, although the mechanisms of disease pathology are not well understood (Sigal, 1997). Host immune responses contribute to the pathogenesis of Lyme arthritis and other aspects of Lyme borreliosis in humans and animal species (Sigal, 1997). Complex interactions between spirochetes and immune responses of various host species, and their role in human and animal disease, have been described. Specific immune responses against *B. burgdorferi* infection include phagocytosis of spirochetes by macrophages, production of antibody by B cells, T cell proliferation, and cytokine production by activated T helper and cytotoxic T cells (Sigal, 1997).

The infection of inbred mouse strains with *B. burgdorferi* parallels many aspects of the disease spectrum seen in infected humans (Brown and Reiner, 1998). BALB/c mice experimentally infected with *B. burgdorferi* develop mild lesions and symptoms of borreliosis that resemble human disease (Barthold *et al.*, 1990). In contrast, C3H/HeJ mice develop moderate to severe disease (Barthold, 1991). C3H mice strains, including the C3H/HeN strain used in these studies, develop severe arthritis and, when injected

intradermally, are more permissive to *B. burgdorferi* dissemination and harbor higher numbers of spirochetes within their tissues (Yang *et al.*, 1994). Thus, the genotype of murine models determines their immune response capabilites and resultant pathologic changes induced by *B. burgdorferi* infection. When inoculated intraperitoneally with *B. burgdorferi* at 3 days of age, C3H/He, SWR, C57BL/6, SJL, and BALB/c mice all develop arthritis after 30 days. If inoculated at 3 weeks of age, only C3H and SWR mice develop severe arthritis, and at 12 weeks old, the arthritis is less severe in C3H mice than that observed after inoculation at 3 weeks of age (Sigal, 1997). This suggests that host immunity might play an important role in the outcome of infection in mice.

Virtually all studies conducted to determine host immune responses and disease pathology in laboratory mice strains have involved needle inoculation of *B. burgdorferi* into susceptible hosts. Clearly, tick inoculation and needle inoculation induce different anti-*B. burgdorferi* immune responses (Golde *et al.*, 1994, Sigal, 1997). These differences in immune responses are thought to be due to the differential expression of outer surface proteins by spirochetes in the tick and mammalian host environments. *Borrelia burgdorferi* undergoes significant alterations in antigenic composition as it passes between its arthropod and mammalian hosts (Akins *et al.*, 1998). Upon initiation of engorgement by a *B. burgdorferi*-infected tick, expression of outer surface protein (Osp) A decreases and OspC, which is not expressed in the unfed tick, increases. Other proteins recently reported to be differentially expressed by *B. burgdorferi* spirochetes include EppA, p35, p37, the OspE homologue p21, the OspF homologues BbK2.10 and pG, and Lp6.6 (Akins *et al.*, 1998). These membrane protein changes contribute to the observed differences in host immune responses to tick-transmitted versus needle-

inoculated *B. burgdorferi* (Roehrig *et al.*, 1992). In addition, consideration must be given to tick factors that modulate host defenses.

Once introduced into the host, *B. burgdorferi* spirochetes induce both cell-mediated and humoral immune responses (Sigal, 1997). *Borrelia burgdorferi* elicits an antibody response in humans and animals infected with the spirochete (Sigal, 1997). Sera from patients and animals infected by needle inoculation with *B. burgdorferi* may contain antibody which is reactive against a number of proteins expressed by the spirochete, including OspA and B and flagellin (p41) (deSilva *et al.*, 1998, Roehrig *et al.*, 1992). Sera from infected mice are borreliacidal to spirochetes grown *in vitro* (Sadziene *et al.*, 1993). Passive transfer of small amounts of immune sera to naïve mice protects them from challenge with spirochetes grown in culture (Barthold and Bockenstadt, 1993).

Protection has also been shown with immune sera from naturally infected humans and dogs when passively transferred to laboratory rodents (Barthold *et al.*, 1995, Callister *et al.*, 1993). The antibody response to *B. burgdorferi* transmitted by tick inoculation is characterized by the lack of antibody responses to OspA and OspB in the early stage of *B. burgdorferi* infection (deSilva *et al.*, 1998). This humoral immunity is not capable of eliminating infection in the persistently infected serum donor (deSilva *et al.*, 1998), Antibody functions as an opsonin and an activator of complement in the immune response to *B. burgdorferi* (Aydintug *et al.*, 1994, Benach *et al.*, 1984). *Borrelia burgdorferi* can activate complement via both the classical and alternative pathways, but spirochetes are resistant to complement bactericidal activity in the absence of specific anti-*B. burgdorferi* antibody (Kochi *et al.*, 1991).

Animal studies suggested that T cells are involved in protective responses to *B. burgdorferi*. However, T cells may also be involved in the pathogenesis of Lyme borreliosis (Sigal, 1997). *Borrelia burgdorferi* has significant effects on T lymphocytes including T cell proliferation, cytokine production, and cytotoxic responses of T cells *in vitro* and *in vivo* (Sigal, 1997). CD4 T cells are involved in controlling needle-inoculated *B. burgdorferi* infections in mice (Keane-Myers and Nickell, 1995b). Depletion of CD4 T cells causes an increase, while depletion of CD8 T cells results in a decrease, in the severity of infection (Keane-Myers and Nickell, 1995b). T cells of infected C3H mice produce IFN-γ when exposed to needle-inoculated spirochetes while spleen cells of C3H mice produce more IL-2 and IFN-γ and less IL-4 than BALB/c spleen cells exposed to *B. burgdorferi* (Keane-Myers and Nickell, 1995a, Ma *et al.*, 1994). T cell clones from humans infected with *B. burgdorferi* produce Th-1 cytokines, including IL-2, GM-CSF, IFN-γ, but not IL-4 or IL-5, indicating a Th-1 pattern of cytokine production (Yssel *et al.*, 1991), which polarizes toward a cell-mediated immune response.

Fc-mediated phagocytosis of *B. burgdorferi* by polymorphonuclear cells, as well as Fc- and non-Fc mediated phagocytosis by macrophages was demonstrated (Benach *et al.*, 1984, Montgomery *et al.*, 1994). Cytokines elaborated by macrophages after exposure to *B. burgdorferi* include IL-1β, TNF–α, IL-12, and interferon-β (Sigal, 1997). However, the role of phagocytic cells and macrophage-produced cytokines in the development or resistance to disease in humans and animals is not clearly understood.

Studies in humans and animal models demonstrate that host immune responses to *B. burgdorferi* infection have both protective and pathogenic roles during infection.

Humoral immunity plays a protective role; however *B. burgdorferi* antigen-antibody immune complexes have been found in the patients spinal fluid, indicating a potentially proinflammatory role (Sigal, 1997). Phagocytosis by macrophages and other cells helps to eliminate spirochetes from infected hosts, but macrophages also contribute to the pathogenesis of disease in hamsters (DuChateau *et al.*, 1996). Th-1 or macrophage-associated cytokines may inhibit *B. burgdorferi* infection (Zeidner *et al.*, 1996). However, anti-IL-12 causes a decrease in peak antibody levels and an increase in the number of *B. burgdorferi* spirochetes isolated from ear punch biopsies of infected mice. Because anti-IL-12 causes a decrease in the level of Th-1 cells, it is thought that Th-1 cells may be involved in the pathogenesis of arthritis (Anguita *et al.*, 1996). Regardless of the role that host immunity has on the development of disease in *B. burgdorferi* infected hosts, tick-induced modulation of host immunity may play an important role in the transmission and establishment of *B. burgdorferi* infection in susceptible hosts by influencing a wide variety of host immune responses. Modulation of host immune responses by the feeding tick, including transient changes in T cell and macrophage cytokines, may serve to polarize toward a Th-2 type response. This polarization may enhance the feeding ability of the tick by reducing cell-mediated immunity at the feeding site, thus reducing inflammation. This reduction in inflammatory responses at the feeding site may also enhance the transmission and establishment of *B. burgdorferi* or any tick-transmitted disease-causing agent.

Tick-Induced Modulation of *B. burgdorferi* Infection

Disease causing agents have evolved methods to modulate host immune responses directed against them. Pathogens are capable of suppressing host immunity, and as previously stated, ectoparasitic arthropods have also evolved strategies to modulate host immune responses directed against them. Mechanisms used by pathogens to circumvent host defenses include interfering with antigen processing, reducing pro-inflammatory cytokines, altering the ratio of Th-1 and Th-2 cells and their respective cytokines, selectively targeting local immune responses, and blocking complement activation (Kotwal, 1996, Marrack and Kappler, 1994) as previously stated. Many of these mechanisms are the same as those used by ticks to modulate host immune responses directed at them while feeding (Table 1). The implications of tick-mediated immunosuppression on transmission and establishment of tick-borne pathogens is being realized (Zeidner *et al.*, 1996).

Recent studies have investigated how tick-induced immunosuppression of host immune responses affects transmission and establishment of *B. burgdorferi*. Zeidner, *et al.* (1996) showed that modifying cytokine levels modifies disease expression in mice infected by tick-transmitted spirochetes. C3H/HeJ mice previously reconstituted with TNF–α, IL-2, and IFN-γ during the course of tick feeding are protected from infection, and the cytokines are not directly responsible for the killing of spirochetes. These results suggest that Th-1 or macrophage associated cytokines may reduce the severity, or perhaps prevent, B. *burgdorferi* infection (Zeidner *et al.*, 1996). These results differ from those of Keane-Myers (1995a) which suggest that IL-4 enhances host resistance to needle-inoculated *B. burgdorferi* in C3H/HeN mice while increases in IFN-γ production

were associated with an increased spirochete burden in tissues and susceptibility to arthritis. The contrast in these studies suggests the importance of natural routes of infection (tick-transmitted) when examining host responses to Lyme borreliosis, and the importance of host immune responses directed against feeding ticks on transmission and establishment of *B. burgdorferi* infection. In a separate study, tick saliva and *B. burgdorferi* spirochetes introduced during *I. scapularis* feeding suppressed cytokine release by T-cells and macrophages, thus reducing the T-cell response needed to respond to spirochetes after their introduction into host skin (Zeidner *et al.*, 1996). Results of these studies support the hypothesis that tick-induced suppression of cytokines enhances the ability of the tick to feed and contributes to transmission of *B. burgdorferi.* However, further studies are needed to fully address the interaction between tick infestation, specific salivary components, and *B. burgdorferi*, in relation to transmission and establishment of host infection.

Tick-Host Immunology and Pathogen Transmission

Because hosts develop resistance to tick feeding following repeated infestations (Wikel, 1996a), it is not surprising that infestation with uninfected ticks can stimulate protection against pathogen transmission during subsequent feeding by the same tick species. Rabbits previously infested with uninfected *D. andersoni* are resistant to infection with tick-borne *Francisella tularensis* (Bell *et al.*, 1979). In addition, following four successive infestations of BALB/c mice with pathogen-free *I. scapularis*, transmission of *B. burgdorferi* by infected *I. scapularis* nymphs was reduced almost six-fold (Wikel *et al.*, 1997). Only 16.7% of repeatedly infested mice became infected with

B. burgdorferi, while 100% of naive mice given one exposure to infected ticks became infected with *B. burgdorferi*. These data suggest that acquired host immune responses, developed following repeated exposure to pathogen-free ticks, are able to neutralize tick-induced immunosuppression and thus reduce the ability of the tick to feed and transmit *B. burgdorferi*.

Host immune responses can counteract tick-induced immunosuppression after repeated exposure to feeding ticks (Wikel *et al.*, 1997), and that counteracting tick-induced host immunosuppression reduces pathogen transmission. However, the immunological basis for the observed reduction of tick-induced immunosuppression has not been determined. The immunological basis for reducing pathogen transmission by the tick may involve a decrease in tick-induced immunosuppression by the repeatedly infested host, which may reduce tick feeding success, and consequently the ability of the tick to successfully transmit a pathogen.

Scope and Significance of Present Study

Experiments conducted in this study were designed to investigate immune responses developed by inbred laboratory mouse strains during the course of repeated infestations with *I. scapularis*, the principal vector of *B. burgdorferi* in the United States. Further experiments were conducted to compare these immune responses to those developed by the vector of *B. burgdorferi* in the western United States, *I. pacificus*. Understanding tick-induced changes in host immunity over the course of repeated infestations, and comparing host immune responses to the closely related species, *I. scapularis* and *I. pacificus*, may aid in discovering the tick factors that modulate host

immune responses. Identifying and characterizing these tick-introduced molecules may lead to new strategies for controlling *B. burgdorferi* infection, as well as other tick-borne pathogens.

One control method may involve the creation of vaccines that stimulate responses directed against tick factors that modulate host immunity. An anti-immunosuppressant vaccine might provide protection against *B. burgdorferi* by reducing the ability of the tick to modulate the host's immune response, thus reducing the ability of the tick to feed and successfully transmit *B. burgdorferi*. This vaccine may also provide protection against other tick-borne infectious agents since the same factors might facilitate transmission and establishment of a variety of tick-borne infectious agents.

Immunological interactions between the tick and host involve both acquired and innate host immune responses, and the introduction of a variety of substances in tick saliva, which are designed to counteract host immune responses and to sustain blood flow at the feeding site. Modulation of host immune responses during tick feeding appears to be a balance between the reduction of the immune responses that limit successful feeding, and maintenance of sufficient immunocompetence for survival of the host. Altering the immune response at the feeding site establishes an "immunologically-privileged" area that could facilitate tick feeding and consequently enhance transmission of tick-borne pathogens.

Previous studies, cited above, demonstrated that ticks modulate immune responses directed against them. Specifically, the modulation of cytokine, and lymphocyte *in vitro* proliferative responses, has been reported for some tick-host associations. In many of these studies, suppression of these immune responses induced

after initial exposure to the feeding tick becomes less intense after repeated exposures to the same tick species. This reduction of tick-induced immunomodulation suggests that host immune responses, developed after repeated tick exposure, might reduce or eliminate those tick factors that modulate host cytokine and lymphocyte proliferative responses.

Few studies to date have characterized changes in host cytokine or lymphocyte *in vitro* proliferative responses immediately after each exposure of a series of infestations, and no studies have examined changes induced after repeated infestations with *I. scapularis*. While host immune responses, including cytokine and lymphocyte responses, have been assessed after single infestations with *I. scapularis*, nothing is known regarding how host immunity is affected by infestation with *I. pacificus*. A thorough characterization of host immune responses developed in murine hosts after exposure to these important vector species is critical in understanding the complex immunological relationships at the tick-host interface. Identifying and characterizing these interactions will provide a greater understanding of the role pathogens play in the complex immunological interrelationship that exists between the host and the feeding tick.

The hypothesis upon which the first specific aim of this study is based is that the magnitude of host cytokine and *in vitro* lymphocyte proliferative responses, developed after initial exposure to pathogen-free *I. scapularis* nymphs, are reduced after repeated infestations with the same tick species.

First specific aim. Define tick-induced modulation of host immune responses during the course of one to four repeated infestations with pathogen-free *Ixodes scapularis* nymphs, by a) assessing tick-induced modulation of host T lymphocyte and

macrophage cytokines and, b) tick-induced modulation of *in vitro* lymphocyte proliferative responses.

The hypothesis upon which the second specific aim of this study is based is that the closely related vectors of Lyme borreliosis in the U.S., *I. pacificus* and *I. scapularis,* modulate host immune responses in a similar manner.

Second specific aim. Define tick-induced modulation of host immune responses after infestation with pathogen-free *Ixodes pacificus* nymphs by examining tick-induced T lymphocyte and macrophage cytokine responses of C3H/HeN mice infested with pathogen-free *I. scapularis* or *I. pacificus* nymphs.

The third specific aim of this study is based on the hypothesis that *I. pacificus* salivary gland soluble proteins modulate *in vitro* lymphocyte proliferative responses in a similar manner as *I. scapularis*.

Third specific aim. Determine the affects of *I. scapularis*, *I. pacificus*, and *D. andersoni* SGSP on *in vitro* proliferative responses of T-lymphocytes from BALB/c and C3H/HeN mice. The objective in investigating this third specific aim is to compare *in vitro* proliferative responses of lymphocytes from noninfested BALB/c and C3H/HeN mice, exposed to SGSP derived from *I. scapularis*, *I. pacificus*, and *D. andersoni*.

The final hypothesis is that BALB/c and C3H/HeN mice, repeatedly infested with *D. andersoni* nymphs, develop acquired immune responses that significantly affect the ability of nymphs to successfully feed and molt to the adult stage.

Fourth specific aim. Evaluate tick biology parameters of *D. andersoni* nymphs after repeated infestations of BALB/c and C3H/HeN mice.

CHAPTER II

MATERIALS AND METHODS

Experiments were conducted to study the effects of repeated infestations with pathogen-free ticks, or tick salivary gland soluble proteins, on various immune responses of BALB/c or C3H/HeN mice. In the first series of experiments, systemic cytokine responses of these inbred mice strains were compared after one to four exposures to pathogen-free *I. scapularis* nymphs. Mice were infested with 10 pathogen-free nymphs each, and ticks were allowed to feed for until replete, or for a maximum of 5 days. A 14-day tick-free period was maintained between infestations. Following each series of infestations, spleens were harvested from tick-infested and uninfested mice. Lymphocytes and splenic macrophages were cultured to induce cytokine production from each of these cell types. Cytokine concentrations were then determined in culture supernatants using antigen-capture ELISA. Differences in lymphocyte and macrophage cytokine concentrations were then compared for each infestation of the two mouse strains.

The effect of repeated infestations with pathogen-free *I. scapularis* nymphs on *in vitro* lymphocyte proliferative responses of C3H/HeN mice was determined. Mice were again infested one to four times with 10 pathogen-free *I. scapularis* nymphs each, with a 14-day tick free period maintained between infestations. Following each infestation in the series, splenocytes were cultured with concanavalin A (Con A) *Eschericia coli* lipopolysaccharide (LPS), or salivary gland soluble proteins (SGSP) derived from *I. scapularis* females, and differences in lymphocyte *in vitro* proliferative responses were determined between infested and non-infested mice, for each infestation.

Ixodes pacificus is the primary vector of Lyme borreliosis in the western United States (Lane *et al.*, 1991). Because of its importance in the ecology of this disease, and since no studies to date have examined immune responses to this tick species, experiments were conducted to examine the effect of *I. pacificus* infestation on host immune responses. C3H/HeN mice were infested once or twice with either pathogen-free *I. scapularis* or *I. pacificus* nymphs in a similar manner as discussed above. After each infestation, lymphocyte and macrophage cytokine responses were compared in culture supernatants using antigen capture ELISA.

To further investigate host immune responses to *I. pacificus*, *in vitro* proliferative responses of splenocytes from BALB/c and C3H/HeN mice, never previously exposed to ticks, were exposed to salivary gland soluble proteins derived from *I. scapularis*, *I. pacificus*, and *Dermacentor andersoni* females. *In vitro* proliferative responses of unstimulated lymphocytes, and lymphocytes stimulated with Con A, were compared to establish the effects of salivary gland proteins of these three tick species on lymphocyte responses of mice.

Experimental Animals

Female BALB/c and C3H/HeN, between eight and twelve weeks in age, weighing 20-25 g, were used in these studies. Mice were obtained from Jackson Laboratories, Bar Harbor, Maine or Charles River Laboratories, Wilmington, Massachusetts. Mice were housed at the Laboratory Animal Resources facility of the School of Veterinary Medicine, Oklahoma State University, Stillwater, Oklahoma. Mice were maintained at 22°C and were provided a commercial diet and water *ad libitum.*

Maintenance and care of all mice conformed to the U. S. Public Health Service Policy of Humane Care and Use of Laboratory Animals by Awardee Institutions and the National Institute of Health Principles for Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training. A staff veterinarian directs the Laboratory Animal Resources Unit at Oklahoma State University.

Tick Colonies

Ixodes scapularis adults were obtained from a colony maintained in the laboratory of Dr. Stephen Wikel, Oklahoma State University, Stillwater, OK. *Ixodes pacificus* adults were obtained as a generous gift from Dr. Robert S. Lane, Division of Insect Biology, University of California, Berkeley, California and maintained according to the following protocol. Adults of both species were fed separately on sheep. Fertilized females were stored in 16-ml glass vials with a screen lid in desiccators over a saturated potassium sulfate solution, which is used to maintain relative humidity at approximately 97% within the desiccators. After oviposition and larval eclosion, pathogen-free nymphs were obtained by feeding larvae on either BALB/c or C3H/HeN mice. Mice were confined in cylindrical hardware cloth tubes and approximately 250-500 larvae, not counted, applied to the face and shoulders of each mouse using a fine paint brush. Confinement within the hardware cloth tube was required to prevent grooming of larvae prior to their attachment to the mouse. After approximately three to four hours of confinement, mice were released and held in standard mouse cages with stainless steel floors, over water. Water in each cage was checked daily for detached ticks. Engorged larvae were removed and placed into vials to allow for molting to the

nymphal stage. All life stages of *I. pacificus* or *I. scapularis* were held at 22°C with a 14:10 light: dark photoperiod in desiccators over potassium sulfate solution. *Dermacentor andersoni* adults and nymphs were obtained from the tick production facility, Department of Entomology and Plant Pathology, Oklahoma State University, Stillwater, OK. *Dermacentor andersoni* adults and nymphs were housed in an identical manner, as were the *Ixodes* species used, however, they were stored in desiccators over saturated potassium nitrate solution, which maintains relative humidity at approximately 93%.

Infestation

Mice were infested by confining ticks to a capsule secured to the back of the mouse. The capsules consisted of 1.5 milliliter polypropylene microcenterfuge tubes (Fisher Scientific, Pittsburgh, PA), cut in half, and secured to the closely clipped fur on the back of each mouse with a four to one mixture (w/w) of calophonium (rosin) (Sigma Scientific, St. Louis, MO) and beeswax. Capsules were applied to control animals in the same manner and the same number of times as for the tick-exposed mice. A new cutaneous infestation site was used for each tick exposure.

Each group of experimental, tick-infested, mice was infested with 10 pathogen-free nymphs each. Nymphs of *I. scapularis* or *I. pacificus* were allowed to feed until fully engorged or for a maximum of five days. This time period was utilized because previous work has shown that >95% of nymphs of both species complete feeding within five days on laboratory mice. Because of their larger size and longer feeding period to engorge, *D. andersoni* nymphs were allowed to feed until replete, or for a

maximum of seven days. A 14-day tick-free period was maintained between each infestation in experiments involving repeated exposure to pathogen-free nymphs.

On the day that ticks detached, or were removed from mice, ticks were weighed to the closest tenth of a milligram and then placed within vials and held in desiccators as previously described. Ticks were held until molting had occurred, or tick death, for those ticks that failed to molt. Weighing of ticks and storage to determine molting ability allowed the comparison of various tick biology parameters including: percentage of ticks that successfully fed (number recovered from each mouse divided by the number applied x 100); their weight after feeding; duration of feeding; and, viability as determined as the percentage of nymphs that successfully molted to the adult stage. Assessment of these parameters was used to determine if host immune responses affect feeding success of the nymphs.

Tissue Culture Medium

Base medium used for culturing of splenocytes for stimulation of macrophage and T cell cytokines, as well as for *in vitro* lymphocyte proliferation assay, was RPMI 1640 (Life Technologies Inc., Gaithersburg, MD) containing 2 mM glutamine, 100 units/milliliter penicillin, and 100 micrograms/milliliter streptomycin. Media were sterilized by passage through a 0.22-micrometer filter. Heat inactivated (56°C, 30 minutes) fetal bovine serum (Hyclone Laboratories, Logan, UT) was added to base medium at a final concentration of 10% for lymphocyte cultures and 5% for splenic macrophage cultures.

Splenocytes

To prepare splenocytes for lymphocyte or macrophage cytokine induction, or *in vitro* lymphocyte proliferation assays, single cell suspensions were prepared from tick-infested or uninfested control mice. Spleens were removed from each mouse under sterile conditions. Pressing the entire spleen between the ground glass edges of sterile microscope slides (Fisher Scientific, Pittsburgh, PA) mechanically disrupted the spleen capsule and resulted in a suspension of lymphocytes and macrophages. Slides used for this purpose were precleaned by washing in running water, dried, washing in 70% ethanol, drying, and autoclaving. The resulting cell suspension was filtered through sterile cell strainers (Fisher Scientific, Pittsburgh, PA) to remove connective tissue. Cell suspensions were then washed twice in RPMI 1640 media by centrifugation at 150 x g for 10 minutes at 4°C. Cells were re-suspended in two milliliters of RPMI and then counted using a hemocytometer (Fisher Scientific, Pittsburgh, PA). To count cells, 90 microliters of Turk's solution [0.01 g gentian violet (Mallinckrodt) Paris, KY, 3 ml glacial acetic acid and 97 ml distilled water] was placed into a well of a 96-well flat bottomed tissue culture plate (Fisher Scientific, Pittsburgh, PA). Ten microliters of the cell suspension was then placed into the Turk's solution, making a final dilution of 1:10. The hemocytometer was then charged with enough of the resulting solution at which the cells could be easily counted. Cells were counted in two diagonally placed large squares, containing 16 smaller squares, on the hemocytometer. Cells present in the two-milliliter suspensions were determined by taking the cell count from the two large squares, dividing by two (to get cells per milliliter), multiplying by two (cells suspended in two milliliters), multiplying by 10 (dilution factor), and then multiplying by 10^4. The cell

count in each suspension was finally adjusted to 5.0 x 10^6 cells/ml for the *in vitro* lymphocyte proliferation assay, and 2.5 x 10^6 cells/ml for the lymphocyte cytokine induction assays.

Macrophages were enriched from total splenocytes by adherence to glass, according to the methods of Ramachandra and Wikel (1992). Spleen cells were suspended at a concentration of 1 x 10^7 cells per ml in antibiotic-supplemented RPMI 1640 medium containing 20% heat-inactivated fetal bovine serum. Cell suspensions were placed into a 100-millimeter diameter sterile glass Petri dish (Fisher Scientific, Pittsburgh, PA), and incubated for one hour at 37°C in a humidified 5% CO_2 atmosphere. Non-adherent cells were removed by gentle agitation and flushing of medium. Ten milliliters of fresh antibiotic-supplemented RPMI medium containing 20% heat-inactivated fetal bovine serum were added and cells cultured for an additional hour prior to removal of medium as described above. Ten milliliters of a 1:5,000 dilution of versene (Life Technologies Inc., Gaithersburg, MD, USA) was added to each Petri plate for five minutes at 37°C to detach cells. Cells were collected in versene solution by gently scraping the bottom of the petri dish using a tissue scraper (Fisher Scientific, Pittsburgh, PA). Harvested cells were pelleted by centrifugation at 150 x g for 10 minutes at 4°C and washed three times in serum-free, antibiotic-supplemented RPMI 1640 medium before being resuspended in antibiotic supplemented RPMI 1640 medium containing 5% fetal bovine serum. Macrophages were suspended at a final concentration of 5 x 10^5 cells per milliliter.

Lymphocyte Cytokine Induction

Induction of the T lymphocyte derived cytokines was performed as modified from the methods of Ramachandra and Wikel, (1992). Lymphocytes were obtained from spleens taken during the course of repeated infestations, as previously described. Lymphocytes were suspended at a concentration of 2.5×10^6 cells per milliliter in serum and antibiotic-supplemented RPMI 1640 media and dispensed at one milliliter per well in a 24 well flat-bottomed tissue culture plate (Fisher Scientific, Pittsburgh, PA). Each well received 5 micrograms/ml of Con A (Calibiochem, La Jolla, CA), in a volume of 20 microliters, prior to incubation for 48 hours at 37°C in a humidified 5% CO_2 atmosphere. Supernatants were collected in one-milliliter samples, placed into individually labeled 1.5-ml cryovials (Fisher Scientific, Pittsburgh, PA) and frozen at –20°C until used in the cytokine assays.

Macrophage Cytokine Induction

Macrophage cell suspensions were obtained from splenocyte suspensions prepared from infested, one to four times, and uninfested control animals as previously described. Macrophages were suspended at 5×10^5 cells per milliliter in antibiotic-supplemented RPMI medium containing 5% fetal calf serum and cultured at one milliliter per well in a 24 well flat bottom culture plate (Fisher Scientific, Pittsburgh, PA). For the first trial of both the BALB/c and C3H/HeN mice in the first series of experiments, macrophages were stimulated with 2.5 micrograms per milliliter, in a volume of 20 microliters, of *Escherichia coli* LPS (Sigma Chemical Co., St. Louis, MO). Cells were incubated for 18 hours for IL-1β production and 48 hours for TNF-α cytokine

production, prior to collection of supernatants. Because very low amounts of macrophage-derived cytokines were obtained in the first experimental trials utilizing repeatedly infested BALB/c and C3H/HeN mice, during subsequent experiments macrophages were primed with 1000 units per milliliter of recombinant IFN-γ (PharMingen Inc., San Diego, CA) in a volume of 20 µl of RPMI 1640 containing 100-units/ml penicillin, 100-micrograms/ml streptomycin. Cells were incubated for 24 hours at 37°C in a humidified 5% CO_2 atmosphere. After this initial incubation, five micrograms per milliliter of *E. coli* LPS, in a volume of 20 ml of RPMI 1640 containing 100-units/ml penicillin, 100-micrograms/ml streptomycin, was added to each well and macrophages were incubated for an additional eight hours prior to collection of supernatants. Macrophage supernatants were collected in one-milliliter samples, placed into individually labeled 1.5-ml cryovials (Fisher Scientific, Pittsburgh, PA) and frozen at –20°C until used in the cytokine assays.

Cytokine Assays

Lymphocyte and macrophage cytokine concentrations in culture supernatants were quantitated by antigen capture enzyme-linked immunosorbent assay (ELISA), using Opt-EIA ELISA kits or matched cytokine detection and capture antibody pairs, purchased from PharMingen Inc. (San Diego, CA) or from Endogen Inc. (Woburn, MA) for the IL-1β assay, as previously described (Zeidner *et al.*, 1997).

ELISA protocols were modified for each cytokine assay from protocols provided by the manufacturer. Detailed protocols for each cytokine analyzed are provided in Appendix I. The general procedures for the ELISAs conducted are as follows. One

hundred microliters of capture (primary) antibody, (diluted to the appropriate working dilution in the specific coating buffer for each assay) was applied to each well of a 96-well microtitration plate (Immunolon-2, Dynatech Laboratories, Chantilly, VA for the IL-2 and IL-4 assays, and Nunc Maxisorp Immunoplate, Nalge Nunc Int., Denmark, for all other cytokine assays). Plates were then incubated overnight. After incubation, plates were washed with phosphate buffered saline (PBS, appendix I) containing tween-20 (PBS/Tween) (0.05%, v/v) (polyoxyethylenesorbitan monolaurate, Sigma, St. Louis, MO). Each well was then blocked with either PBS containing 3% (w/v) bovine serum albumin (BSA) (Fluka Chemie AG, Switzerland) or PBS containing 10% (v/v) fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT) for the specified time at room temperature. After blocking, plates were washed again with PBS/Tween. Fifty or 100 microliters per well, depending on assay protocol, of either recombinant cytokine standards (PharMingen Inc., San Diego, CA) or culture supernatants were placed into wells, and plates were incubated for the specified amount of time according to the assay protocol. Wells containing only PBS/BSA (or PBS/FBS) served as blank controls. After washing with PBS/Tween, 100 microliters of biotinylated detection, secondary, antibody were applied into each well and plates incubated for the specified time period. Plates were again washed with PBS/Tween. One hundred microliters of streptavidin-horseradish peroxidase (HRPO), or avidin-HRPO conjugate, diluted in PBS/BSA or PBS/FBS, were then added to each well and plates incubated. After washing with PBS/Tween, substrate solution (ABTS or TMB substrate, see Appendix II) was added to each well and plates were incubated for color development. When Opt-EIA kits were utilized, adding 50 microliters of 2N sulfuric acid to each well stopped color

development. Plates were read at 405 or 450 nanometers, depending on protocol, using an automatic microplate reader (model no. EL311, Biotek Instruments, Winsooki, VT).

For all ELISAs conducted, each dilution of recombinant cytokine standard or culture supernatant was assayed in triplicate. To determine the concentration of cytokines in culture supernatants, standard curves were constructed using recombinant cytokines. Optical density values from doubling dilutions of recombinant cytokine standards ranging from 2000 to 15.6 picograms per milliliter were obtained. The mean of the three-optical density values for each recombinant cytokine dilution was determined. The concentration and mean optical density values of the recombinant standard dilutions were log-transformed and a linear regression constructed to produce a regression equation for each assay performed. Cytokine concentrations were determined from the corresponding supernatant optical density values, using the regression equation calculated for each assay. Concentrations were then multiplied by the appropriate factor to determine the amounts of each cytokine assayed per milliliter of supernatant tested. Percent change was calculated by subtracting the control value from the infested value, dividing by the control value, then multiplying by 100.

Lymphocyte Proliferation Assay

To determine the effects of *I. scapularis* SGSP, Con A, and LPS on *in vitro* proliferation of repeatedly infested and control, uninfested, C3H/HeN mouse splenocytes, an assay was modified from the procedures of Bergman *et al.* (1995). C3H/HeN mice, infested from one to four times with pathogen-free *I. scapularis* nymphs, or uninfested control animals, were sacrificed post-infestation, as outlined in the experimental design

section. Three tick infested and three uninfested control animals were utilized in each infestation group. After each infestation series, spleens were removed from each mouse as described above. Resulting splenocytes were suspended at 5.0 x 10^6 cells/ml in RPMI medium supplemented with 100-units/ml penicillin, 100-micrograms/ml streptomycin, and 10% fetal bovine serum. *Ixodes scapularis* SGSP, prepared as discussed below, was diluted in sterile PBS to 2.0, 1.0, or 0.5 μg in 50 μl and Con A or LPS was diluted in sterile PBS to 0.5 or 0.1 μg in 50 μl. Fifty microliters of each dilution of SGSP, Con A, LPS, or sterile PBS was placed in triplicate into a sterile 96-well flat bottom, tissue culture microtiter plate (Corning, Corning NY). An equal volume of two-fold concentrated RPMI 1640, supplemented with antibiotics and 20% FBS, was then added to each well. Plates were incubated 24 hr at 37°C in a humidified 5% carbon dioxide atmosphere to assess sterility, prior to adding murine splenocytes.

Splenocytes (100 μl containing 5.0 x 10^5 cells) were added to each well and plates were incubated at 37°C in a humidified 5% carbon dioxide atmosphere. After 56 hours of incubation, one μCi of methyl-^{3}H-thymidine with a specific activity of 248 GBq/mmol (NEN Life Science Products, Boston, MA) was added to each well, in a volume of 20 microliters of antibiotic-supplemented RPMI lacking serum. Following addition of radiolabeled thymidine, cells were cultured for an additional 18 hours. The amount of label incorporated was determined by immobilizing the nucleic acid in each well onto glass fiber filter, using an automated cell harvester (Brandel, Gaithersburg, MD). Filters were allowed to dry overnight and then placed in six-ml plastic, screw cap scintillation vials (Wheaton, VWR Scientific Products, Suwanee, GA) and covered with two ml biodegradable counting scinillant (Amersham, Arlington Heights, IL). The

incorporation of methyl-tritiated thymidine radioactivity on the filters was determined by use of a liquid scintillation spectrophotometer (Beckman Instruments, Palo Alto CA). Each sample was assayed in triplicate. Mean counts per minute ± standard error of the mean were determined for each treatment.

To determine how SGSP prepared from *I. scapularis*, *I. pacificus*, or *D. andersoni* affects *in vitro* proliferative responses of normal mouse splenocytes and splenocytes stimulated with 0.1 μg/well of Con A, the *in vitro* lymphocyte proliferation assay was modified according to the following procedures. Ten BALB/c and ten C3H/HeN mice, never exposed to feeding ticks, were sacrificed and splenocytes prepared as discussed above. The final concentration of splenocytes in cell suspensions prepared from each mouse was 5.0×10^6 cells/ml. One hundred microliters of antibiotic-supplemented RPMI 1640 containing 10% FBS were added into each well of a 96-well tissue culture plate. Plates were incubated at 37°C in a humidified 5% CO_2 environment for 24 hours to assess sterility. After preparation of spleen cell suspensions from mice, splenocytes (100 μl containing 5.0×10^5 cells) were added to each well in a 96-well tissue culture plate. Salivary gland soluble proteins from each tick species were diluted to a final concentration of 2.0, 1.0, 0.5, and 0.25 μg in 20 μl in sterile PBS. Concanavalin A was diluted to 0.1 μg in 20 μl in sterile PBS, or antibiotic-supplemented RPMI 1640 lacking serum. To assess lymphocyte responses of normal murine splenocytes, 20 μl of each of the four dilutions of the SGSP from each tick species were added into each well. Each dilution was assayed in triplicate. Cells incubated with sterile PBS served as unstimulated controls. To assess lymphocyte responses of Con A-stimulated splenocytes, 0.1 μg of Con A, diluted in 20 μl antibiotic-supplemented RPMI

1640, lacking serum, was added in triplicate into wells containing the various dilutions of SGSP from each tick species. Cells were incubated for two hours prior to the addition of Con A. Cells incubated with sterile RPMI 1640 alone served as unstimulated controls, while cells incubated with Con A alone served as Con A-stimulated controls. Cells were incubated for 56 hours at 37°C in a humidified 5% CO_2 environment prior to addition of radiolabeled thymidine as discussed above. Incorporation of label was determined as previously discussed. The counts per minute of unstimulated cells were compared to cells cultured with the four dilutions of SGSP from each tick species, while the counts per minute of cells cultured with Con A only were compared to cells stimulated with Con A and SGSP from each tick species.

To test the viability of cells cultured over the 74-hour incubation period, as well as to determine if SGSP was toxic to lymphocytes, the trypan blue exclusion method was utilized. One well containing the highest dilution of SGSP from each tick species, as well as one well containing cells alone, was randomly chosen from a culture plate containing cells from each mouse strain. One hundred microliters of each cell suspension was removed from the culture plate and placed into a well on a 96-well flat-bottomed culture plate containing 100 µl of trypan blue (Sigma, St. Louis, MO) solution (0.2% w/v in PBS). After thorough mixing of the suspensions, ten microliters of the suspension was placed onto a clean microscope slide, and a cover slip applied. One hundred cells were counted from each suspension, and the percentage of viable cells determined for each tick SGSP preparation, as well as for cells cultured in complete media only.

Salivary Gland Soluble Proteins (SGSP)

Adult *I. scapularis, I. pacificus*, and *D. andersoni* were fed on sheep for four to six days, as described by Patrick and Hair (1976). On the day they were removed from sheep, partially engorged female ticks were washed sequentially with deionized water, 70% ethanol, 3% hydrogen peroxide, and deionized water to remove blood and other surface contaminants. To remove salivary glands, ticks were sliced in half using a single edged razor blade and then placed into sterile phosphate buffered saline. Viewing tissues through a dissecting microscope, salivary glands were removed from each half of the body cavity and placed into clean sterile PBS. Salivary glands from approximately 100 ticks of each species were removed and pooled, and then frozen at -20°C in sterile PBS until used to make the SGSP preparations.

To prepare SGSP, salivary glands of each species were combined and homogenized in an ice-cold glass homogenizer in the fluid that glands were frozen in post-dissection. Particulate material was removed from the homogenate by centrifugation at 18,400 x g (13,500 rpm, JA-17 rotor, Beckman, Fullerton, CA) for 45 min at 5°C. The 18,400 x g supernatant was then centrifuged at 105,000 x g (50,000 rpm, TL100.3 rotor, Beckman) for one hour at 5°C. The protein content of the supernatant, SGSP, was determined by microtiter plate bicinchoninic acid assay of Smith *et al.* (1985) (BCA Protein Assay, Pierce, Rockford, IL), using a bovine serum albumin (2 mg/ml, in a 0.9% aqueous NaCl soultion, Pierce, Rockford, IL) as standard. The standard was diluted with PBS to protein concentrations of 50, 100, and 150 μg per ml. Salivary gland soluble proteins were diluted from 1:20 to 1:640. Fifty parts of Solution A were mixed with one part solution B to obtain the working reagent for BCA protein assay. A total of 250

microliters of working reagent was deposited into each well of a flat bottom, 96 well plate (Corning, New York, NY). Twenty microliters of each standard dilution (1, 2, and 3 μg total protein) and SGSP dilution were added per well in triplicate. Twenty microliters per well of PBS added in triplicate served as blank. The plate was covered and agitated on a vortex shaker (Scientific Industries, Bohemia, NY) for 2-3 minutes. The plate was incubated at 60°C for 30 minutes and optical densities determined at 562 nm using an automatic microplate reader. Optical density values for each sample and standard were averaged and protein content of SGSP determined by linear regression. Salivary gland soluble protein was diluted to a final concentration of 0.2 mg/ml for the *I. scapularis* preparation, 1.6 mg/ml for the *I. pacificus* preparation, and 0.9 mg/ml for *D. andersoni* preparation. All SGSP preparations were diluted to their final concentration in PBS, filter-sterilized (sterile 0.22 μm centrifugal filter, Millipore), and frozen at –20°C until use.

Data analysis

Tick Biology Parameters. For experiments involving one to four infestation of mice with *I. scapularis* or *D. andersoni* nymphs, Kolmolgorov – Smirnov (Steel *et al.*, 1997) tests were used to compare the duration of feeding and the weight of nymphs recovered in the first through fourth infestation groups for both trials conducted on either of the mice strains. For experiments involving repeated infestations of C3H/HeN mice to *I. scapularis,* or *I. pacificus* nymphs, Kolmolgorov - Smirnov tests were used to compare the weight of both species of nymphs recovered in the first and second infestation groups for both trials. For all experiments involving tick feedings, Chi-square tests were used to

compare the duration of feeding and the proportion of ticks that successfully molted to the adult stage for each exposure group.

In vitro proliferative responses of lymphocytes of mice repeatedly infested with *I. scapularis* nymphs. Counts per minute of unstimulated cells and cells exposed to Con A, LPS, and SGSP from mice exposed to pathogen-free ticks and control mice not exposed to ticks were measured. Stimulation indices were calculated by dividing the counts per minute of stimulated cells (Con A, LPS, or SGSP treated) by that of unstimulated cells. For each tick exposure group (1, 2, 3 and 4 infestations) counts per minute and stimulation indices of tick-exposed mice were compared to those from unexposed mice using the Kruskal-Wallis test (Steel *et al.*, 1997).

The Kruskal-Wallis test was used to compare counts per minute and stimulation indices between all four control mouse groups and all four-tick infestation groups separately. When a Kruskal-Wallis test indicated a significant difference ($p \leq 0.05$), six pair-wise comparisons (1 vs 2, 1 vs 3, 1 vs 4, 2 vs 3, 2 vs 4, 3 vs 4) were performed using a normalized rank distribution (Gibbons, 1971). The significant, two-tailed, alpha and corresponding Z-value, was calculated using the methods of Dunn to ensure an overall alpha not greater than 0.05 per six comparisons (Marascuilo and Serlin, 1988).

***In vitro* proliferative responses of splenocytes exposed to SGSP.**

Two effects (tick species and SGSP concentration) were evaluated for lymphocytes collected from each mouse strain. ANOVA was used if the data passed tests verifying that it met assumptions of parametric statistical analysis (e.g. homogeneity of variance and lack of significant correlation between mean and variance) (Steel *et al.*,

1997). Square root and fourth root transformations were used to transform the data to meet assumptions of parametric statistical analysis. The non-parametric Kruskal-Wallis test (Steel *et al.*, 1997) was used if transformed data still did not meet assumptions of parametric statistical analysis.

The Sheffe F test was used for pairwise posthoc comparisons if ANOVA indicated a significant difference (Steel *et al.*, 1997). When a Kruskal-Wallis test indicated a significant difference, pairwise posthoc comparisons were performed using a normalized rank distribution (Gibbons, 1971). The significant (two-tailed) alpha (and corresponding Z-value) was calculated using the methods of Dunn to ensure an overall alpha not greater than 0.05 per group of comparisons (Marascuilo and Serlin, 1988).

Cytokine Assays. For experiments conducted to assess cytokine responses of BALB/c and C3H/HeN mice repeatedly infested with pathogen-free *I. scapularis* nymphs, mean cytokine concentrations for control and experimental mice exposed one to four times to pathogen-free ticks were calculated to describe the cytokine levels for each tick-exposed and corresponding non-exposed control group. To test for significant differences between cytokine levels in control versus tick-infested mice, the Kruskal-Wallis test was used (Steel *et al.*, 1997).

In experiments conducted to assess the response of C3H/HeN mice to *I. pacificus* and *I. scapularis* nymphs, mean cytokine concentrations for control and experimental mice exposed once or twice to pathogen-free *I. scapularis* or *I. pacificus* were calculated to describe the cytokine levels for each tick-exposed and corresponding non-exposed control group. To test for significant differences between cytokine levels in control versus mice infested by either tick species, the Kruskal-Wallis test was used (Steel *et al.*,

1997). When the Kruskal-Wallis test indicated a significant difference, three pairwise comparisons of cytokine levels, control vs *I. pacificus*, control vs *I. scapularis*, *I. pacificus* vs *I. scapularis,* were performed using a normalized rank distribution (Gibbons, 1971). The significant, two-tailed, alpha and corresponding Z-value, was calculated using the methods of Dunn to insure an overall error rate not greater than 0.05 per three pairwise comparisons (Marascuilo and Serlin, 1988). In all statistical analyses, significance was assessed at the $p \leq 0.05$ level.

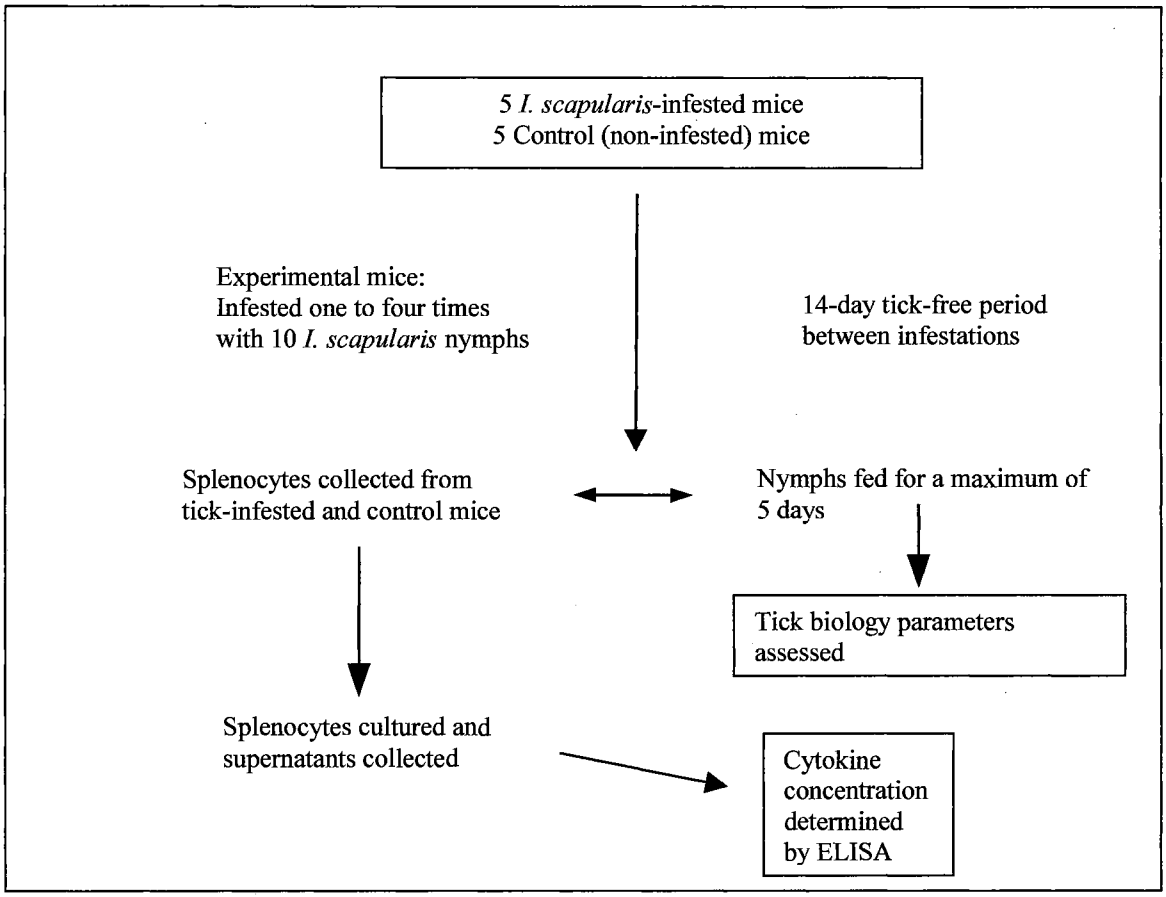
Experimental Design

Specific Aim I. Define tick-induced modulation of host immune responses during the course of repeated infestations with pathogen-free *Ixodes scapularis* nymphs.

- Tick-induced modulation of T lymphocyte and macrophage cytokines during the course of repeated infestations with pathogen-free *I. scapularis* nymphs.

This experiment was conducted to investigate how repeated infestations with pathogen-free *I. scapularis* nymphs affects lymphocyte and macrophage cytokine production in BALB/c and C3H/HeN mice. As outlined in figure 1, female BALB/c or C3H/HeN mice, with no history of tick exposure, were divided into four groups, each group consisting of five infested, experimental, mice and five uninfested, control mice.

Figure 1. Infestation and assay series for experiments to measure cytokine responses of tick infested vs. noninfested BALB/c and C3H/HeN mice.



Experimental mice were infested one to four times with 10 pathogen-free *I. scapularis* nymphs. After each series of infestations, splenocytes were collected from each mouse, and macrophage and T lymphocyte cytokine concentrations compared between tick-infested and noninfested mice. Each series of infestations was performed twice for each mouse strain to increase the sample size of the mice for which immunological parameters were assessed.

Mice were monitored daily to ensure that secondary infections, which may bias the results of the cytokine elaboration assays, were not seen at tick feeding sites on the mice. In the experience of investigations in this laboratory, secondary infections rarely occur at tick feeding sites in studies of this kind. Additionally, once ticks complete feeding or are removed, the 14-day tick-free period results in healing of the bite sites and re-growth of fur causes feeding capsules to become dislodged, allowing mice access to the feeding sites for grooming.

To determine if host immune responses affects feeding success of *I. scapularis* nymphs, the following calculations were made at the end of each infestation: the percentage of ticks that successfully fed; their weight after feeding; viability; and, the percentage of nymphs molting to adults. These parameters were determined for ticks recovered from each mouse to ascertain if host immune responses affected the feeding success of nymphs collected from each mouse.

Following a 14-day tick-free period, the remaining experimental mice were re-infested with *I. scapularis* nymphs, and the infestation regime repeated. This pattern of infestation was continued for the remaining groups of mice (figure 1), until all

experimental and control mice had been assayed for cytokine production from T lymphocytes and macrophages.

Since cytokines produced by T lymphocytes and macrophages regulate a variety of immune responses, their presence or absence may greatly influence the host response to tick feeding. Tick feeding can suppress both host macrophage and T lymphocyte cytokines that are important components of both innate and acquired immune responses (Wikel and Bergman, 1997, Zeidner *et al.*, 1996). To this end, cytokine responses of naïve BALB/c and C3H/HeN mice versus mice repeatedly exposed to tick infestation, were analyzed to determine the effects that repeated infestation with *I. scapularis* nymphs has on the production of cytokines within and between each mouse strain. Table 2 lists the specific cytokines that were assayed in this series of experiments; the cells that produce the specific cytokines; and, the primary function of each cytokines in the host immune response.

To test for the production of T lymphocyte derived cytokines (IL-2, IL-4 IL-10, and IFN-γ), lymphocytes from spleens of tick infested or control mice were tested *in vitro* for their ability to produce cytokines upon Con A stimulation, and splenic macrophages harvested from experimental and control mice were evaluated for their ability to produce IL-1β and tumor necrosis factor (TNF)-α in response to *E.coli* LPS and recombinant IFN-γ. Cytokines in lymphocyte and macrophage culture supernatants were quantitated by antigen capture ELISA, as described above. A dose-response curve was established relative to the normal concentration of the cytokines to be assayed for each cytokine investigated.

Table 2. Cytokines to be assayed for, including the cells that produce them and selected examples of their function in host immune responses. ***Adapted from Janeway and Travers, 1997.***

Cytokine producing cell	*Cytokines to be assayed*	*Cytokine Function*
Macrophage	Interleukin-1 (IL-1β)	IL-1β: Stimulation of macrophages and lymphocytes
	Tumor Necrosis Factor-α (TNF-α)	TNF-α: Induces local inflammation
T Lymphocyte	Interleukin-2 (IL-2)	IL-2: Induces T-cell proliferation
	Interleukin-4 (IL-4)	IL-4: B and T cell proliferation
	Interleukin-10 (IL-10)	Il-10: B and T cell proliferation
	Interferon-γ (IFN-γ)	IFN-γ: Macrophage activation

- Tick-induced modulation of *in vitro* lymphocyte proliferative responses during the course of repeated infestations with pathogen-free *I. scapularis* nymphs.

These experiments were conducted to investigate how repeated infestations with pathogen-free *I. scapularis* nymphs affects *in vitro* lymphocyte proliferative responses of C3H/HeN mice. C3H/HeN mice were compared in this series of experiments because preliminary experiments conducted demonstrated that this inbred strain produces more intense immune responses against *I. scapularis* than do BALB/c mice. As discussed for the previous experiment, and outlined in figure 1, female C3H/HeN mice, with no history of tick exposure, were divided into four groups, each group consisting of three infested, experimental, mice and three uninfested, control, mice. Experimental mice were infested one to four times with 10 pathogen-free *I. scapularis* nymphs each, and nymphs were allowed to feed to repletion, or for a maximum of five days. After each series of infestations, splenocytes were collected from each mouse, and *in vitro* proliferative responses of lymphocytes to Con A, *E. coli* LPS, and *I. scapularis* SGSP compared between tick-infested and noninfested mice. Each series of infestations was performed twice to increase the sample size of the mice for which immunological parameters were assessed. A previously discussed, mice were monitored daily to ensure that secondary infections, which could bias the results of the *in vitro* lymphocyte proliferation assays, were not seen at tick feeding sites on the mice. In addition, to determine if host immune responses affects feeding success of *I. scapularis* nymphs, tick biology parameters were assessed for ticks recovered after each infestation, as previously discussed. Following a 14-day tick-free period, the remaining experimental mice were re-infested with *I.*

scapularis nymphs, and the infestation regime repeated. The pattern of infestations was continued for the remaining groups of mice (figure 1), until all experimental and control mice had been assayed for *in vitro* lymphocyte responses.

Specific Aim II. Define tick-induced T lymphocyte and macrophage cytokine responses of C3H/HeN mice infested with pathogen-free *I. pacificus* nymphs

Lyme borreliosis, caused by infection with the spirochete *B. burgdorferi*, is the most commonly reported arthropod transmitted disease in the United States (Centers for Disease Control and Prevention, 1999). *Ixodes scapularis* and *I. pacificus* are the primary vectors of *B. burgdorferi* in the United States and *I. ricinus* is the primary vector in Europe (Lane *et al.*, 1991). Previous studies have shown that infestation with *I. ricinus* and *I. scapularis* modulates host T lymphocyte cytokine responses to a Th-2 profile in inbred laboratory mice (Christe *et al.*, 1999). Because little is known concerning host immune responses to *I. pacificus* infestation, this series of experiments was conducted to compare cytokine responses of C3H/HeN mice to repeat infestations with *I. scapularis* and *I. pacificus* nymphs. As mentioned above, C3H/HeN mice were chosen because previous studies demonstrated that this mouse produces stronger and more consistent cytokine responses to *I. scapularis* than BALB/c mice. Additionally, C3H mouse strains, including the C3H/HeN strain to be used in these studies, develop severe arthritis, while BALB/c mice are more resistant to the development of clinical disease (Barthold *et al.*, 1990). Further, when injected intradermally, susceptible C3H mice are more permissive than resistant BALB/c mice to *B. burgdorferi* dissemination and harbor higher numbers of spirochetes within their tissues (Yang *et al.*, 1994).

As outlined in figure 2, female C3H/HeN mice were divided into groups of five *I. scapularis* infested, five *I. pacificus* infested, and five uninfested, control, animals. Experimental, tick exposed, mice were infested one or two times with 10 pathogen-free *I. pacificus* or *I. scapularis* nymphs. Nymphs were allowed to feed to repletion, or for a period of five days. After each series of infestations, splenocytes were collected from each mouse, and macrophages and T cells were cultured with mitogens as described above, prior to determining cytokine concentrations by antigen capture ELISA. Cytokine concentrations were then determined for mice infested with each tick species and for uninfested control mice. Infestations were performed twice for each tick species to increase the sample size of the mice for which immunological parameters are assessed. As mentioned previously, mice were monitored daily to ensure that secondary infections did not develop in any of the mice used in the experiment. Table 3 lists the specific cytokines that were assayed for in this series of experiments; the cells that produce the specific cytokines; and, the primary function of the cytokines in the host immune response.

To test for the production of T lymphocyte and macrophage derived cytokines, lymphocytes from spleens of *I. scapularis*-infested, *I. pacificus*-infested, or uninfested control mice were tested *in vitro* for their ability to produce cytokines upon Con A stimulation, and splenic macrophages harvested from tick infested and uninfested mice were evaluated for their ability to produce IL-1β, IL-6, and tumor necrosis factor (TNF)-α in response to *Escherichia coli* LPS and recombinant IFN-γ stimulation. Cytokine concentrations were determined using antigen capture ELISA as previously discussed. At the end of each infestation, tick biology parameters, described above, were analyzed

from each tick species to determine if repeated exposure affected tick-feeding success of either *I. pacificus* or *I. scapularis* nymphs.

Figure 2. Infestation series for exposure of mice to pathogen-free *Ixodes scapularis* or *I. pacificus* nymphs to assess tick biology parameters of replete nymphs recovered and to determine concentrations of lymphocyte and macrophage cytokines in tick-infested and non-infested animals.

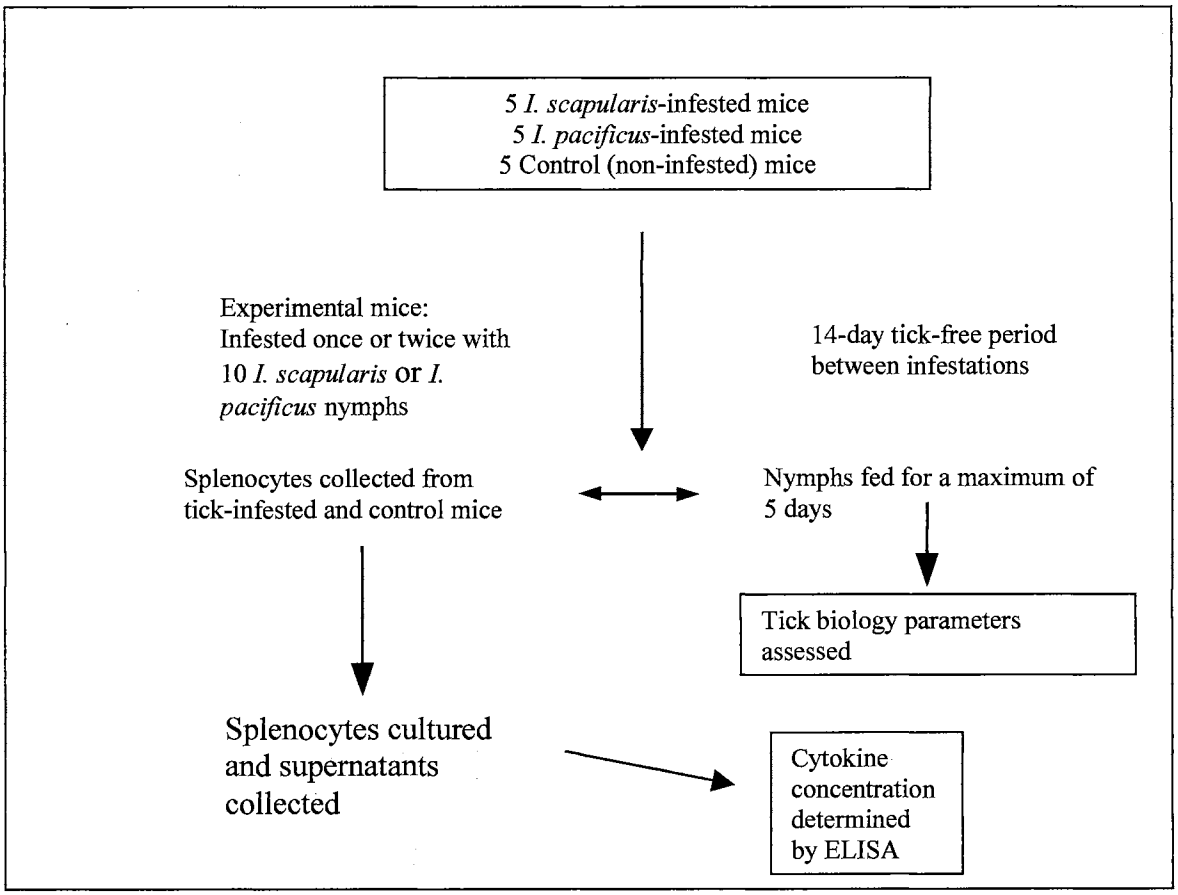


Table 3. Cytokines to be assayed for, including the cells that produce them and selected examples of their function in host immune responses. ***Adapted from Janeway and Travers, 1997.***

Cytokine producing cell	*Cytokines to be assayed*	*Cytokine Function*
Macrophage	Interleukin-1 (IL-1β)	IL-1β: Stimulation of macrophages and lymphocytes
	Tumor Necrosis Factor-α (TNF-α)	TNF-α: Induces local inflammation
	Interleukin-6 (IL-6)	IL-6: B and T-cell proliferation, acute phase protein production
T Lymphocyte	Interleukin-2 (IL-2)	IL-2: Induces T-cell proliferation
	Interleukin-4 (IL-4)	IL-4: B and T cell proliferation
	Interleuking-5 (IL-5)	IL-5: Eosinophil proliferation
	Interleukin-6 (IL-6)	IL-6: B and T-cell proliferation, acute phase protein production
	Interleukin-10 (IL-10)	IL-10: B and T cell proliferation
	Interferon-γ (IFN-γ)	IFN-γ: Macrophage activation

Specific Aim III. Determine the affects of *I. scapularis, I. pacificus* and *D. andersoni* salivary gland soluble proteins on *in vitro* proliferative responses of T-lymphocytes from BALB/c and C3H/HeN mice.

Previous studies have evaluated the effect of saliva or salivary gland-derived proteins from various ixodid tick species on the responses of T lymphocytes *in vitro* (Bergman *et al.*, 1995, Bergman *et al.*, 1998, Dusbabek *et al.*, 1995, Urioste *et al.*, 1994). However, no studies to date have evaluated how salivary gland proteins from *I. pacificus* effect proliferative responses of murine lymphocytes. This experiment was conducted to compare the affects of SGSP from three ixodid tick species on *in vitro* lymphocyte responses of inbred mice not exposed to feeding ticks.

Female BALB/c and C3H/HeN mice of similar ages and weights were divided into groups of ten mice each. Splenocytes were prepared from each mouse and proliferative responses of lymphocytes cultured with dilutions of *I. scapularis*, *I. pacificus*, or *D. andersoni* SGSP determined by *in vitro* lymphocyte proliferation assay. SGSP from each tick species was diluted in sterile PBS to a final concentration of 2.0, 1.0, 0.5, and 0.25 μg/well. Lymphocytes cultured in complete media alone served as unstimulated controls. Proliferative responses of lymphocytes from each mouse strain were compared to cells cultured in media alone.

To determine how SGSP from each tick species affects Con A induced lymphocyte proliferation, cells from each mouse were incubated for two hours in the presence of each of the four dilutions of SGSP from each tick species. After the two-hour incubation, cells were stimulated with 0.1 μg/well of Con A. Cells incubated with Con A alone served as unexposed controls, while cells cultured in media alone served as

unstimulated controls. Proliferative responses of lymphocytes, both Con A stimulated and unstimulated, exposed to SGSP from each tick species were compared to determine the effect of SGSP on lymphocytes from each mouse strain.

Specific Aim IV. Acquired immunity to *D. andersoni* nymphs.

Immunologically based acquired resistance to ticks has been described for many tick-host associations (Brossard *et al.*, 1991, Brossard and Wikel, 1997, Wikel, 1982a, Wikel, 1996a, Wikel *et al.*, 1996). However, some tick-host relationships are characterized by a lack of acquired resistance. For instance, neither BALB/c nor C3H/HeN mice develop resistance after repeated infestations with *I. ricinus* (Christe *et al.*, 1998, Christe *et al.*, 1999, Mbow *et al.*, 1994a).

This experiment was conducted to compare tick biology parameters of *D. andersoni* taken from mice repeatedly infested with *D. andersoni* nymphs with ticks collected from mice repeatedly infested with *I. scapularis* nymphs. BALB/c and C3H/HeN females, of approximately equal size and weight, were divided into groups of four infested and four uninfested control mice each. Groups of mice of both strains were infested from one to four times with ten *D. andersoni* nymphs each. Nymphs were allowed to feed until repletion, or for a maximum of seven days. At the end of each infestation, the following tick biology parameters were determined for each infestation of each mouse strain: the percentage of ticks that successfully fed; their weight after feeding; viability; and, the percentage of nymphs molting to adults. These parameters were determined for ticks recovered from each mouse to ascertain if host immune responses affected feeding success.

Tick biology parameters from each mouse strain were compared to data obtained previously from BALB/c and C3H/HeN mice repeatedly infested with *I. scapularis* nymphs. Comparison of this data was done to determine changes in tick biology parameters in ticks recovered after each infestation indicated the development of acquired immune responses to feeding *D. andersoni* nymphs in either of the mouse strains.

CHAPTER III

RESULTS

Specific aim I: Define tick-induced modulation of host immune responses during the course of repeated infestations with pathogen-free *Ixodes scapularis* nymphs.

- Tick-induced modulation of host T lymphocyte and macrophage cytokines during the course of one to four repeated infestations with pathogen free nymphs.

Repeated infestation of BALB/c and C3H/HeN mice with pathogen-free *Ixodes scapularis* nymphs resulted in significant changes in host cytokine production and an apparent polarization toward a Th-2 cytokine profile. Although changes in cytokine production were induced by infestation with *I. scapularis*, acquired resistance to infestation did not develop in either murine strain. Amounts of both T lymphocyte and macrophage cytokines differed between the two trials; however, the trends in cytokine production for each strain were similar during both trials.

Tick Biology Parameters. Tick biology parameters for both trials of repeated infestations of BALB/c and C3H/HeN mice are provided in Table 4. Few significant differences in tick biology were observed. The mean number of ticks recovered from mice ranged from 6.6 to 9.0, with an average of 8.6 ± 0.3 (mean ± standard error) ticks recovered from BALB/c mice during both trials. The same parameters for ticks recovered from C3H/HeN mice ranged from 8.2 to 9.8 ticks recovered per animal, with

Table 4. Replete nymphs recovered from mice, mean weight ± standard error of recovered nymphs, mean number of days spent feeding, and percent nymphs successfully molting to adults for *I. scapularis* nymphs fed on BALB/c and C3H/HeN mice, trials 1 and 2. In each column, groups indicated by the same letter are not significantly different, p>0.05.

Infestation	Mean tick weight (mg) ± S.E.	Mean feeding duration (days)	% molt	Mean tick weight (mg) ± S.E.	Mean feeding duration (days)	% molt
BALB/C		**Trial 1**			**Trial 2**	
1	3.63 ± 0.09a	4.3 a	87.3a	3.51 ± 0.09a	4.4a	93.7a
2	3.56 ± 0.01a	4.1ab	93.7a	3.42 ± 0.12a	4.6bc	87.4a
3	3.61 ± 0.14a	3.8b	81.8a	3.47 ± 0.12a	4.3c	87.6a
4	3.21 ± 0.15b	3.3c	91.7a	3.47 ± 0.19a	4.3ab	93.3a
C3H/HEN		**Trial 1**			**Trial 2**	
1	3.56 ± 0.08a	4.0b	95.7a	3.25 ± 0.09b	4.5b	91.4a
2	3.86 ± 0.09b	3.6a	96.5a	3.13 ± 0.10b	4.2a	93.5a
3	3.11 ± 0.12b	4.2b	88.8b	3.31 ± 0.11b	3.9a	93.6a
4	3.31 ± 0.18b	4.2b	87.8b	3.82 ± 0.16a	4.1a	85.4a

an average burden of 9.2 ± 0.2 ticks. Mean weights of replete nymphs from BALB/c mice were significantly different only for ticks recovered from mice exposed four times during the first trial, while no significant differences were observed during the second trial. Significant differences were seen in the duration of tick feeding on mice exposed three or four times during the first trial and among the four infestation groups of the second trial. However, these differences did not establish a consistent pattern of increase or decrease in feeding duration (Table 4). No significant differences were seen in the viability (% molt) of engorged nymphs.

Significant differences were only seen in mean weight of ticks recovered from C3H/HeN mice infested once during the first trial, and infested four times during the second trial (Table 4). Significant differences in feeding duration occurred only during the second infestation of the first trial. During the second trial, ticks collected at the end of the first infestation weighed significantly more than those obtained following subsequent infestations. The only significant reductions in viability of ticks recovered from C3H/HeN mice occurred during the third and fourth infestations of the first trial.

Lymphocyte Cytokines. Significant differences in the production of T lymphocyte cytokines of infested mice, when compared to uninfested control mice, were observed for both BALB/c (Table 5) and C3H/HeN (Table 6) mice. Trends in cytokine production were similar for both strains of mice; however, differences in intensity of responses were observed between murine strains and between trials within the same strain.

Depending on the number of infestations of BALB/c mice, the Th-1 cytokines, IL-2 and IFN-γ, were either significantly suppressed, or not significantly different from control values. Specifically, suppression of IL-2 was significant following the first, second, and third tick exposures during the first trial, and following the second and fourth exposures during the second trial. In contrast, IFN-γ was highly variable during infestations of BALB/c mice and did not produce a clear pattern of suppression or enhancement, during either trial. In contrast to the Th-1 cytokines profiles, the Th-2 cytokines, IL-4 and IL-10, were significantly enhanced in BALB/c mice relative to the control values for all but two infestations. Specifically, IL-4 was significantly enhanced for all but mice infested three and four times for the first trial, while IL-10 was significantly enhanced for all infestations (Table 5).

Ixodes scapularis infestations of C3H/HeN mice polarized T lymphocyte cytokine responses in a manner similar to those observed for infested BALB/c mice. Significant suppression of IL-2 was observed for all infestations, except for mice infested three times during the first trial and four times during the second trial. Suppression of IFN-γ was observed at the end of each infestation; however, this suppression was significant following the second and fourth infestations of the first trial and during the second, third, and fourth infestations of the second trial. Levels of IL-4 were significantly enhanced for C3H/HeN mice at the end of each infestation. Likewise, IL-10 was significantly enhanced at the end of each infestation, with the exception of the second exposure during the first trial (Table 6).

Table 5. Mean concentrations and percent change of T lymphocyte cytokines for *I. scapularis*-infested and noninfested (control) BALB/c mice, trials 1 and 2. Asterisk "*" indicates significant difference between cytokine concentrations of infested and control animals at the $p \leq 0.05$ level.

Cytokine	Times Infested	Trial 1 Mean Concentration (± S.E.) ng/ml		Trial 2 Mean Concentration (± S.E.) ng/ml		Percent Change	
		Infested	Control	Infested	Control	Trial 1	Trial 2
IL-2	1	13.3 ± 0.5	12.3 ± 0.2	30.1 ± 0.6	32.8 ± 1.0	+ 8.1	- 8.2
IL-2	2	4.8 ± 0.3	6.9 ± 0.8	24.9 ± 1.2	31.3 ± 0.9	- 30.4	- 20.5 *
IL-2	3	0.7 ± 0.1	3.0 ± 0.3	32.4 ± 0.5	31.2 ± 0.9	- 76.7 *	+ 3.8
IL-2	4	2.5 ± 0.2	3.8 ± 0.5	28.9 ± 1.2	33.5 ± 0.4	- 34.2	- 13.7 *
IL-4	1	1.6 ± 0.3	0.6 ± 0.09	4.2 ± 0.3	0.9 ± 0.1	+ 167.7 *	+ 367.7 *
IL-4	2	1.2 ± 0.9	0.4 ± 0.06	2.9 ± 0.1	0.9 ± 0.05	+ 200.0 *	+ 222.2 *
IL-4	3	0.8 ± 1.0	0.7 ± 0.9	3.0 ± 0.4	1.9 ± 0.2	+ 14.3	+ 57.9 *
IL-4	4	0.7 ± 1.0	0.6 ± 0.1	3.6 ± 0.6	1.2 ± 0.06	+ 16.7	+ 200.0 *
IL-10	1	9.4 ± 1.2	3.0± 0.6	31.4 ± 4.3	12.3 ± 2.8	+ 213.3 *	+ 155.3 *
IL-10	2	1.3 ± 0.2	0.2 ± 0.08	47.1 ± 5.3	17.6 ± 1.7	+ 550.0 *	+ 167.6 *
IL-10	3	2.6 ± 0.8	0.4 ± 0.09	22.5 ± 3.1	11.5 ± 2.2	+ 550.0 *	+ 98.3 *
IL-10	4	1.3 ± 0.4	0.3 ± 0.06	33.3 ± 3.6	9.2 ± 1.0	+ 333.3 *	+ 261.9 *
IFN-γ	1	24.4 ± 3.0	25.5 ± 0.9	167.0 ± 24.4	165.8 ± 28.4	- 4.3	+ 1.2
IFN-γ	2	2.9 ± 0.7	1.8 ± 0.4	116.5 ± 8.7	127.2 ± 14.4	+ 61.1	- 8.4
IFN-γ	3	4.9 ± 1.1	4.1 ± 1.1	97.7 ± 13.7	94.5 ± 13.8	+ 19.5	+ 3.4
IFN-γ	4	1.6 ± 0.3	3.3 ± 1.8	132.6 ± 17.3	99.2 ± 12.4	- 51.5	+ 33.7

Table 6. Mean concentrations and percent change of T lymphocyte cytokines for *I. scapularis*-infested and noninfested (control) C3H/HeN mice, trials 1 and 2. Asterisk "*" indicates significant difference between cytokine concentrations of infested and control animals at the $p \leq 0.05$ level.

Cytokine	Times Infested	Trial 1 Mean Concentration (± S.E.) ng/ml		Trial 2 Mean Concentration (± S.E.) ng/ml		Percent Change	
		Infested	Control	Infested	Control	Trial 1	Trial 2
IL-2	1	4.3 ± 0.5	8.4 ± 0.2	5.7 ± 0.2	8.4 ± 0.8	- 48.8 *	- 32.1 *
IL-2	2	3.8 ± 0.6	8.6 ± 0.2	4.9 ± 0.5	9.0 ± 1.0	- 55.8 *	- 45.6 *
IL-2	3	3.7 ± 0.5	5.1 ± 0.7	5.5 ± 0.4	8.9 ± 0.9	- 27.5	- 38.2 *
IL-2	4	2.3 ± 0.4	4.5 ± 0.3	6.5 ± 0.7	9.9 ± 1.8	- 48.9 *	- 34.3
IL-4	1	1.3 ± 0.3	0.7 ± 0.06	2.5 ± 0.3	0.7 ± 0.1	+ 85.7 *	+ 257.1 *
IL-4	2	2.2 ± 0.2	1.1 ± 0.06	3.4 ± 0.5	0.6 ± 0.07	+ 100.0 *	+ 466.7 *
IL-4	3	1.6 ± 0.08	0.5 ± 0.1	2.2 ± 0.1	0.7 ± 0.04	+ 220.0 *	+ 214.3 *
IL-4	4	1.1 ± 0.2	0.3 ± 0.05	2.8 ± 0.2	0.7 ± 0.1	+ 266.7 *	+ 300.0 *
IL-10	1	22.9 ± 0.9	5.2 ± 1.4	26.5 ± 4.8	11.1 ± 1.9	+ 340.4 *	+ 138.7 *
IL-10	2	19.1 ± 0.9	13.4 ± 1.9	33.0 ± 4.2	8.9 ± 1.3	+ 42.5	+ 270.8 *
IL-10	3	21.2 ± 0.5	10.1 ± 1.0	33.3 ± 1.9	9.6 ± 0.8	+ 109.9 *	+ 246.9 *
IL-10	4	18.2 ± 0.8	8.4 ± 1.1	36.3 ± 4.9	11.2 ± 1.6	+ 116.7 *	+ 224.2 *
IFN-γ	1	122.6 ± 4.6	174.7 ± 16.6	523.2 ± 87.2	880.3 ± 147.3	- 29.8	- 40.6
IFN-γ	2	47.1 ± 2.9	122.4 ± 8.7	599.8 ± 28.3	1033.7 ± 137.8	- 61.5 *	- 42.0 *
IFN-γ	3	87.9 ± 4.7	129.1 ± 19.5	824.1 ± 11.0	1264.8 ± 64.1	- 31.9	- 34.8 *
IFN-γ	4	71.3 ± 8.7	145.3 ± 10.3	596.2 ± 55.4	1055.7 ± 72.3	- 50.9 *	- 43.5 *

Macrophage Cytokines. In contrast to T lymphocyte cytokines, elaboration of IL-1β and TNF-α by splenic macrophages collected from infested mice of either murine strain was highly variable and no clear pattern of polarization could be discerned (Table 7). The only significant differences observed post-infestation of BALB/c mice were for IL-1β at the end of the first (-79.2%), and second infestations (+148.5%) and for TNF-a (-53.5%) after the third infestation of the first trial (Table 7). The only significant difference in macrophage cytokines from C3H/HeN mice was for IL-1β following the fourth infestation (-54.5%) of the second trial (Table 7). Although the percent suppression or enhancement in macrophage cytokines was high following some infestations (Table 7), these difference were not significant at the $p \leq 0.05$ level due to the high degree of variation around the mean, and the relatively small quantities of cytokines produced.

Table 7. Mean concentrations and percent change of macrophage cytokines for *I. scapularis*-infested and noninfested (control) BALB/c and C3H/HeN mice, trials 1 and 2. Asterisk "*" indicates significant difference between cytokine concentrations of infested and control animals at the $p \leq 0.05$ level.

Cytokine	Times Infested	Trial 1 Mean Concentration (± S.E.) pg/ml		Trial 2 Mean Concentration (± S.E.) pg/ml		Percent Change	
		Infested	Control	Infested	Control	Trial 1	Trial 2
BALB/c							
IL-1β	1	179.1 ± 46.0	860.7 ± 230.0	737.8 ± 109.8	595.3 ± 100.8	- 79.2 *	+ 24.0
IL-1β	2	76.6 ± 24.0	30.8 ± 4.1	238.1 ± 83.2	308.2 ± 113.2	+ 148.5 *	- 22.8
IL-1β	3	215.5 ± 40.2	275.0 ± 41.4	882.1 ± 147.8	1163.4 ± 276.6	- 21.6	- 24.2
IL-1β	4	112.9 ± 18.0	173.0 ± 37.5	642.2 ± 82.0	490.7 ± 52.8	- 34.8	+ 30.9
TNF-α	1	107.4 ± 33.4	189.7 ± 31.3	311.2 ± 60.8	447.3 ± 45.1	- 43.4	- 30.4
TNF-α	2	22.2 ± 4.3	35.9 ± 5.6	530.9 ± 157.7	418.1 ± 29.9	- 38.2	+ 27.0
TNF-α	3	75.5 ± 5.5	162.3 ± 5.1	590.9 ± 177.5	1185.1± 395.9	- 53.5 *	- 51.0
TNF-α	4	33.9 ± 15.6	51.5 ± 7.4	367.5 ± 205.0	389.7 ± 157.1	- 34.2	- 5.7
C3H/HeN							
IL-1β	1	351.7 ± 39.4	510.6 ± 87.2	370.2 ± 51.7	246.3 ± 51.5	- 31.1	+ 50.3
IL-1β	2	750.7 ± 64.3	677.9 ± 67.0	386.0 ± 73.5	463.6 ± 73.3	+ 10.7	- 16.7
IL-1β	3	277.0 ± 42.1	221.6 ± 53.1	330.8 ± 63.8	249.2 ± 33.3	+ 25.0	+ 32.7
IL-1β	4	285.9 ± 36.2	204.0 ± 22.2	210.5 ± 52.5	463.1 ± 85.8	+ 40.2	- 54.5 *
TNF-α	1	117.9 ± 3.4	123.0 ± 14.2	157.0 ± 53.4	140.6 ± 49.5	- 4.1	+ 11.6
TNF-α	2	135.0 ± 16.8	127.3 ± 17.9	183.8 ± 39.7	165.1 ± 59.7	+ 6.1	+ 11.3
TNF-α	3	196.3 ± 59.7	315.7 ± 216.2	145.2 ± 70.7	104.6 ± 48.3	- 37.8	+ 38.8
TNF-α	4	108.6 ± 25.9	67.2 ± 39.6	499.3 ± 207.7	649.8 ± 142.0	+ 61.1	- 23.2

- Tick-induced modulation of *in vitro* lymphocyte proliferative responses during the course of repeated infestations with pathogen-free nymphs. *In vitro* proliferative responses of lymphocytes from C3H/HeN mice repeatedly infested with pathogen-free *I. scapularis* nymphs were significantly different than responses of lymphocytes from uninfested mice, when exposed to salivary gland soluble proteins (SGSP) derived from adult *I. scapularis* females. Significant differences between infested and uninfested mice were seen for virtually every infestation at all three dilutions of *I. scapularis* SGSP. In contrast to SGSP responsiveness, proliferation of lymphocytes from repeatedly infested and non-infested mice differed significantly only for cells from once-infested mice cultured with 0.1 μg/well of Con A, but not for cells cultured with 0.5 μg/well of Con A. No differences in proliferation were observed for lymphocytes from infested and non-infested mice exposed to either dilution of LPS.

Tick Biology Parameters. Few significant differences in tick biology parameters of *I. scapularis* nymphs were observed (Table 8). The mean number of *I. scapularis* nymphs recovered from mice ranged from 8.00 to 9.42, with an average of 8.81 ± 0.30 (mean ± standard error) ticks recovered over the four infestations. Mean weights of replete *I. scapularis* nymphs ranged from 3.63 ± 0.10 mg (mean ± standard error) for nymphs recovered after the third infestation to 2.89 ± 0.16 mg for nymphs recovered after the fourth infestation (Table 8). The mean replete weight of nymphs recovered after the fourth infestation was significantly less than that of nymphs recovered after the second and third infestations, but not significantly different than ticks recovered after the first infestation (Table 8). Mean feeding duration of *I. scapularis* nymphs recovered from

Table 8. Replete nymphs recovered from mice, mean weight ± standard error of recovered nymphs, mean number of days spent feeding, and percent nymphs successfully molting to adults for *I. scapularis* nymphs fed on C3H/HeN mice. Groups indicated by the same letter in each column are not significantly different, p>0.05.

Infestation	Ticks recovered	No. of mice	Mean ticks recovered /mouse	Mean tick weight (mg) ± S.E.	Mean feeding duration (days)	% molt
1	219	24	9.1	3.30 ± 0.08ab	4.34 ± 0.60a	95.89a
2	144	18	8.0	3.32 ± 0.10a	4.13 ± 0.59b	90.10a
3	113	12	9.4	3.63 ± 0.10a	4.10 ± 0.60b	93.81a
4	52	6	8.7	2.89 ± 0.16b	4.21 ± 0.67ab	90.38a

C3H/HeN mice ranged from 4.3 days following the first infestation to 4.1 days after the third. Mean feeding duration of ticks following the first infestation was significantly higher than feeding duration of ticks fed following the second and third exposures, but not different from ticks fed following the fourth infestation (Table 8). These differences did not reveal a consistent pattern of increase or decrease in feeding duration (Table 8). No significant differences were seen in viability, measured as percent molt, of engorged nymphs recovered.

***In vitro* lymphocyte proliferation.** No significant differences between lymphocyte responses of infested and control mice were observed for unstimulated cells (cells not exposed to Con A, LPS, or SGSP) from any of the four infestations (Table 9). Additionally, no significant differences were seen in responses of lymphocytes from noninfested and infested animals cultured with Con A (0.5 μg/well) or either dilution of LPS (0.5 or 0.1 μg/well). The only significant difference in lymphocyte responses of cells exposed to Con A or LPS was for cells from once-infested mice exposed to 0.1 μg/well of Con A (Table 9). In this case, the count per minute ± standard error (S.E.) was 17,907 ± 643 for noninfested mice as compared to 25,555 ± 787 for mice infested once with *I. scapularis* nymphs. This resulted in a 42.7% increase in counts per minute, a difference that is significant (Table 9).

For lymphocytes cultured with each of the three dilutions of *I. scapularis* SGSP (2.0, 1.0, and 0.5 μg/well), significant differences in proliferative responses were seen for virtually all infestations and SGSP dilutions (Table 9). For example, lymphocytes cultured with 2.0 and 1.0 μg/well of SGSP, the counts per minute ± S.E. of tick-infested mice were significantly higher for tick-infested mice than those of uninfested mice, for

Table 9. Counts per minute (CPM) ± S.E. of methyl-^{3}H-thymidine incorporation into lymphocytes from C3H/HeN mice repeatedly infested with pathogen-free *I. scapularis* nymphs. Asterisk "*" indicates significant difference between infested and control mice for each infestation within each treatment group. Significance assessed at the $p \leq 0.05$ level.

Treatment	Infestation	Control	Infested	Percentage Change
Unstimulated	1	2973 ± 672	3011± 602	1.3
	2	2006 ± 425	2414 ± 570	20.3
	3	2089 ± 442	2120 ± 441	1.5
	4	1541 ± 220	2029 ± 257	32.0
Con A 0.5 μg	1	133098 ± 20007	131530 ± 16414	- 1.2
	2	114435 ± 13900	87014 ± 11727	-24.0
	3	98850 ± 11160	91664 ± 11558	-7.2
	4	73169 ± 6444	68688 ± 8439	-6.1
Con A 0.1 μg	1	17907 ± 1493	25555 ± 1804	42.7*
	2	15131 ± 2175	16037 ± 1260	6.0
	3	14194 ± 1161	13929 ± 1979	-1.9
	4	10003 ± 876	12118 ± 239	21.1
LPS 0.5 μg	1	50687 ± 6216	50904 ± 6472	0.4
	2	45325 ± 4831	36878 ± 3105	-18.6
	3	31117 ± 2755	28218 ± 2178	-9.4
	4	29850 ± 3725	28667 ± 2256	-4.0
LPS 0.1 μg	1	35914 ± 4830	37518 ± 5094	4.5
	2	21528 ± 2083	19470 ± 1090	-9.6
	3	20346 ± 2235	17180 ± 1410	-15.6
	4	16527 ± 1389	17859 ± 907	8.1
SGSP 2.0 μg	1	1506 ± 62	3028 ± 548	101.1*
	2	1168 ± 155	3595 ± 616	208.0*
	3	1512 ± 188	2743 ± 178	81.4*
	4	876 ± 77	2469 ± 504	181.8*
SGSP 1.0 μg	1	837 ± 61	1772 ± 409	111.7*
	2	798 ± 86	2936 ± 525	267.9*
	3	936 ± 112	1860 ± 167	98.7*
	4	621 ± 106	1761± 378	183.6*
SGSP 0.5 μg	1	1183 ± 194	1708 ± 330	50.1
	2	820 ± 51	2329 ± 568	184.0*
	3	1351 ± 262	2011 ± 340	49.0
	4	698 ± 125	1472 ± 313	111.0*

mice infested once, twice, three, and four times with pathogen-free *I. scapularis* nymphs (Table 9). For lymphocytes cultured with 0.5 μg/well of SGSP, counts per minute were higher for lymphocytes from infested mice, when compared to lymphocytes from uninfested mice for all infestations, the differences were only significant for mice infested twice (182.2%) and mice infested four times (108.5%, table 9). For the cells cultured with all dilutions of SGSP, the increase in proliferative responses ranged from 49% following the third infestation for cells cultured with 0.5 μg/well SGSP, to an increase of 267.9% for cells from twice-infested mice cultured with 1.0 μg/well SGSP.

The count per minute of unstimulated cells, and cells exposed to each dilution of mitogen or SGSP, differed between infestations for both control and infested mice within each infestation group. For example, the counts per minute of unstimulated cells from uninfested mice ranged from 2,973 ± 672 (mean ± SE) for cells from control mice of the once-infested group to 1,541 ± 200 for cells from the four-times exposed group (Table 9). For mice exposed to tick feeding, counts per minute of the 0.5 μg/well Con A-exposed cells ranged from 131,530 ± 16,414 to 68,688 ± 8,439 (table 9). Correlation between tick exposure (infested 1, 2, 3, or 4 times) vs. noninfested mice, and *in vitro* response of cells demonstrated that exposure to ticks did not significantly affect how cells responded to Con A or LPS ($p > 0.05$). In contrast, correlation between tick exposure and response to SGSP indicated that tick exposure significantly affected how cells responded to SGSP ($p \leq 0.05$). However, the number of infestations was not correlated to the response of lymphocytes to SGSP ($p > 0.05$).

To determine how each treatment (Con A, LPS, or SGSP) affected the proliferation of lymphocytes, when compared to unstimulated cells (not exposed to

mitogen or SGSP), and to normalize data by describing responses as a ratio between each treatment and the associated unstimulated cells within that treatment group, stimulation indices were calculated for cells cultured from repeatedly infested and uninfested mice. Stimulation indices for infested and noninfested mice for each dilution of Con A, LPS, or SGSP are presented in table 10. No significant differences were seen between stimulation indices of cells from tick-infested or noninfested mice for either dilution of LPS or for the 0.1 μg/well of Con A or (Table 10). The only significant difference in stimulation indices for Con A was seen for cells cultured with 0.5 μg/well Con A following the second infestation (Table 10). In this case the difference between the tick exposed and unexposed mice was –34.6% (Table 10). In contrast, significant differences were observed among stimulation indices of cells from tick-infested or noninfested mice for all three dilutions of SGSP (Table 10). For cells cultured with 1.0 μg/well of SGSP, the percentage difference in stimulation indices of infested mice were significantly higher than control mice for mice infested one, two, three and four times (Table 10). For cells cultured with 2.0 and 0.5 μg/well of SGSP, stimulation indices of repeatedly infested mice were significantly higher than control mice for mice infested two, three, and four times. Stimulation indices from once-infested mice, exposed to either 2.0 or 0.5 μg/well of SGSP, were similarly higher than the stimulation indices of control mice. However, these differences were not significant (Table 10).

Differences in stimulation indices within each treatment (Con A, LPS, or SGSP) between infestation groups (infestations one, two, three, four) were not significant for uninfested control or infested mice cultured with Con A or LPS, or for control mice cultured with any dilution of SGSP (Table 10). In contrast, stimulation indices of cells

Table 10. Stimulation indices ± S.E. of lymphocytes from C3H/HeN mice repeatedly infested with pathogen-free *I. scapularis* nymphs. Asterisk "*" indicates significant difference between stimulation index of infested and control for each infestation within each treatment group. Responses followed by the same letter between infestations for each treatment are not significantly different. Responses not followed by letters are not significantly different between infestations. Significance assessed at the $p \leq .05$ level.

Treatment	Infestation	Control	Infested	Percentage Change
Con A 0.5 μg	1	48.52 ± 4.00	47.00 ± 5.12	- 3.1
	2	63.56 ± 6.65	41.54 ± 4.96	-34.6*
	3	53.02 ± 5.57	46.83 ± 4.00	-11.7
	4	52.77 ± 9.30	37.12 ± 6.84	-29.7
Con A 0.1 μg	1	7.03 ± 0.99	9.66 ± 1.43	37.4
	2	8.39 ± 1.23	8.20 ± 1.34	-2.3
	3	8.91 ± 2.18	8.70 ± 2.40	-2.4
	4	6.90 ± 0.80	6.39 ± 0.70	- 7.4
LPS 0.5 μg	1	19.03 ± 1.85	18.40 ± 2.34	- 4.7
	2	25.57 ± 2.92	19.31 ± 3.49	-24.5
	3	17.08 ± 2.13	15.41 ± 2.05	- 9.8
	4	22.24 ± 4.40	15.49 ± 2.38	-30.4
LPS 0.1 μg	1	13.26 ± 1.11	13.28 ± 1.33	- 0.2
	2	12.62 ± 2.02	10.30 ± 2.00	-14.0
	3	10.92 ± 1.16	9.34 ± 1.40	-14.5
	4	11.87 ± 1.68	9.49 ± 1.18	-20.0
SGSP 2.0 μg	1	0.62 ± 0.11	1.07 ± 0.16	72.6
	2	0.82 ± 0.23	1.67 ± 0. 81	103.6*
	3	0.85 ± 0.14	1.54 ± 0.26	81.2*
	4	0.60 ± 0.06	1.20 ± 0.14	100.0*
SGSP 1.0 μg	1	0.34 ± 0.05	0.58 ± 0.07a	70.6*
	2	0.54 ± 0.14	1.38 ± 0.18b	155.6*
	3	0.50 ± 0.06	1.00 ± 0.15ab	100.0*
	4	0.40 ± 0.02	0.83 ± 0.08ab	107.5*
SGSP 0.5 μg	1	0.43 ± 0.04	0.57 ± 0.06a	32.6
	2	0.50 ± 0.09	0.98 ± 0.04b	96.0*
	3	0.66 ± 0.06	1.01 ± 0.09b	53.0*
	4	0.44 ± 0.02	0.70 ± 0.07ab	59.1*

from infested animals differed between infestation groups for cells cultured with 1.0 or 0.5 μg/well of SGSP, but not for cells cultured with 2.0 μg/well of SGSP. For example, stimulation indices of cells from infested mice, cultured with 1.0 μg/well SGSP, were significantly less for cells from once-infested when compared to twice-infested mice, but did not differ for mice infested three or four times (Table 10). Similarly, stimulation indices of cells from once-infested mice, cultured with 0.5 μg/well SGSP, were significantly less than stimulation indices from mice infested two or three times, but not different from mice infested four times with pathogen-free *I. scapularis* nymphs (Table 10).

For the stimulation indices calculated, correlation between tick exposure, infested (1, 2, 3, or 4 times) vs. noninfested mice, and *in vitro* response of cells, demonstrated that exposure to ticks did not significantly affect how cells responded to Con A or LPS ($p > 0.05$). In contrast, correlation between tick exposure and response to SGSP indicated that tick exposure significantly affected how cells responded to SGSP ($p \leq 0.05$). The number of times infested was not significantly correlated with how the cells responded to Con A, LPS, or SGSP ($p > 0.05$).

Specific Aim II. Define tick-induced modulation of host immune responses after infestation with pathogen-free *Ixodes pacificus* nymphs.

Tick Biology Parameters. The mean number of ticks recovered from each mouse ranged from 8.6 for mice infested once with *I. scapularis* in the first trial to 10.0 for mice exposed once to *I. scapularis* nymphs in the same trial (Table 11). The mean number of ticks recovered from mice was similar for both tick species, and did not differ

significantly. Mean engorged weight of nymphs was significantly less for *I. pacificus* nymphs recovered after the first infestation of the first trial, and after the second infestation of the second trial, as compared to *I. scapularis* nymphs (Table 11). Mean engorged weights for nymphs recovered after the second infestation of the first trial and first infestation of the second trial were similar and did not differ significantly between tick species (Table 11). Mean feeding duration differed significantly between species for the second infestation of the first trial and for both infestations of the second trial (Table 11). Viability of replete nymphs was not significantly different either between or within tick species for any infestation in the first or second trials (Table 11).

Table 11. Mean number of nymphs recovered from mice, mean weight ± standard error of recovered nymphs, mean number of days spent feeding, and percent nymphs successfully molting to adults for *I. scapularis* and *I. pacificus* nymphs fed on C3H/HeN mice, trials 1 and 2. For each trial, groups indicated by the same letter in each column are not significantly different, p>0.05.

Tick Species	Times infested	Ticks recovered	Mean ticks recovered /mouse	Mean tick weight (mg) ± S.E.	Mean feeding duration (days)	% molt
			Trial 1			
I. scapularis	1	86	8.6	3.09 ± 0.11a	4.34a	88.4a
I. pacificus	1	91	9.1	2.75 ± 0.14b	4.49a	86.8a
I. scapularis	2	50	10.0	3.14 ± 0.16a	4.30a	92.0a
I. pacificus	2	47	9.4	3.15 ± 0.21a	4.04b	93.6a
			Trial 2			
I. scapularis	1	93	9.3	3.30 ± 0.13a	4.29a	89.3a
I. pacificus	1	94	9.4	3.35 ± 0.15a	4.01b	90.4a
I. scapularis	2	46	9.2	3.74 ± 0.17a	4.09a	95.6a
I. pacificus	2	44	8.8	2.86 ± 0.19b	4.02b	81.8a

Lymphocyte Cytokines. Significant differences were observed for both tick species in production of T lymphocyte cytokines by tick-infested mice, when compared to uninfested control mice. In addition, significant differences were observed in production of T lymphocyte cytokines between *I. scapularis* and *I. pacificus*-infested mice. Mean concentrations (± standard error) of T lymphocyte cytokines and the percent change of the cytokine concentrations between *I. scapularis*-infested, *I. pacificus*-infested, and non-exposed mice for both series of repeated infestations are presented in Tables 12 and 13.

While the patterns of cytokine production were similar for mice infested with either tick species, differences in intensity of responses occurred. During the first trial, IL-2 production was significantly suppressed for both infestations in *I. pacificus*-infested mice, but not significantly in *I. scapularis*-infested mice, although it was suppressed relative to non-infested mice. During the second trial, IL-2 was suppressed for mice infested with either tick species, but the differences were not significant compared to uninfested controls. Following the first infestation for both trials, IL-2 was suppressed to a greater extent by *I. pacificus* than by *I. scapularis* infestation. Suppression of IL-2 was then similar for mice infested with either species during the second infestations of both trials (Tables 2 and 3). Interferon-γ was significantly suppressed by *I. scapularis* infestation for all but the initial infestation of the first trial. During that first infestation, this difference was not significant when compared to uninfested mice, but was significant when compared to *I. pacificus*-infested mice (Table 12). These findings demonstrate that infestation with either *I. scapularis* or *I. pacificus* suppresses IL-2 relative to uninfested animals; and, *I. pacificus* appears to suppress IL-2 to a greater degree than does *I.*

scapularis. In contrast, *I. scapularis* appears to suppress IFN-γ production more effectively than *I. pacificus* (Tables 12 and 13).

Infestation with either tick species enhanced Th-2 cytokines relative to uninfested controls for virtually all infestations (Tables 12 and 13). Although not all infestations resulted in significant enhancement of Th-2 cytokines, the trend was for those cytokines to be up regulated, relative to responses of uninfested mice, following one or two exposures to either *I. scapularis* or *I. pacificus*. Furthermore, *I. pacificus* is more effective at enhancing the production of Th-2 associated cytokines than *I. scapularis*. Exposure to either tick species up regulated IL-4 production. This up regulation was significant when compared to uninfested control mice for all infestations by *I. pacificus*, but not significant following exposure to *I. scapularis* (Tables 12 and 13). Interleukin-10 was up regulated relative to unexposed control mice during all infestations of mice exposed to either tick species (Tables 12 and 13). As observed for IL-4, this increase in IL-10 was significant only in mice infested with *I. pacificus*. The Th-2 cytokine, IL-6, was significantly up regulated by *I. pacificus* relative to uninfested control mice for all infestations, except the initial exposure of the second trial (Tables 12 and 13). Although during that one infestation, IL-6 induced by *I. pacificus* did not differ significantly from control mice, it differed significantly from the amount of IL-6 produced by *I. scapularis*-infested mice. In contrast to the other Th-2 cytokines, IL-5 production did not show a consistent pattern of up regulation following either *I. pacificus* or *I. scapularis* infestation (Tables 12 and 13).

Table 12. Mean concentrations (nanograms/milliliter) ± standard error and percent change of T lymphocyte cytokines for *Ixodes scapularis*-infested, *I. pacificus*-infested and noninfested (control) C3H/HeN mice, trial one. In each row, groups indicated by the same letter are not significantly different, p>0.05. * Each treatment group consisted of five mice for each infestation.

Cytokine	Times infested	Mean Concentration (ng/ml) ± Standard Error			Percent Change	
		Control *	*I. scapularis* infested *	*I. pacificus* infested *	*I. scapularis* infested	*I. pacificus* infested
IL-2	1	14.1 ± 0.8a	11.3 ± 1.3ab	8.0 ± 0.8b	-19.8	-43.3
IL-2	2	20.3 ± 1.08a	13.3 ± 0.7ab	12.2 ± 0.9b	-34.5	-39.9
IL-4	1	1.6 ± 0.1a	3.3 ± 0.2ab	4.6 ± 0.8b	+106.3	+187.5
IL-4	2	2.0 ± 0.1a	9.9 ± 0.7ab	12.4 ± 1.1b	+395.0	+520.0
IL-5	1	0.3 ± 0.04a	0.3 ± 0.03a	0.6 ± 0.07b	0.0	+100.0
IL-5	2	0.2 ± 0.05a	0.3 ± 0.04ab	0.9 ± 0.2b	+50.0	+350.0
IL-6	1	1.9 ± 0.2a	2.9 ± 0.1ab	4.1 ± 0.3b	+52.6	+115.8
IL-6	2	3.0 ± 0.1a	12.0 ± 0.8ab	18.8 ± 2.0b	+300.0	+527.8
IL-10	1	4.6 ± 0.5a	10.3 ± 0.6ab	21.4 ± 2.3b	+123.9	+365.2
IL-10	2	5.2 ± 1.4a	18.7 ± 3.1ab	27.0 ± 4.4b	+259.6	+419.2
IFN-γ	1	504.5 ± 40.1ab	280.3 ± 22.4a	636.98 ± 21.3b	-44.4	+26.2
IFN-γ	2	678.6 ± 63.7a	247.0 ± 22.6b	378.4 ± 29.1ab	-63.6	-44.2

Table 13. Mean concentrations (nanograms/milliliter) ± standard error and percent change of T lymphocyte cytokines for *Ixodes scapularis*-infested, *I. pacificus*-infested and noninfested (control) C3H/HeN mice, trial two. In each row, groups indicated by the same letter are not significantly different, p>0.05. * Each treatment group consisted of five mice for each infestation, except for the control group of the twice-exposed animals, which contained four mice.

Cytokine	Times infested	Mean Concentration (ng/ml)			Percent Change	
		Control *	*I. scapularis* infested *	*I. pacificus* infested *	*I. scapularis* infested	*I. pacificus* infested
IL-2	1	5.9 ± 0.9a	4.0 ± 0.5a	3.0 ± 0.4a	-32.2	-49.2
IL-2	2	11.2 ±1.6a	5.8 ± 1.0a	6.2 ± 0.8a	-48.2	-44.6
IL-4	1	3.3 ± 0.6a	7.2 ± 1.4ab	14.8 ± 0.6b	+118.2	+348.5
IL-4	2	7.3 ± 0.5a	17.9 ± 2.2ab	20.9 ± 2.0b	+169.0	+186.3
IL-5	1	2.1 ± 0.4a	1.8 ± 0.2a	1.9 ± 0.3a	-14.3	-9.5
IL-5	2	0.9 ± 0.2a	1.3 ± 0.3a	1.8 ± 0.5a	+44.4	+100.0
IL-6	1	4.0 ± 0.3ab	3.1 ± 0.3a	6.7 ± 0.9b	-22.5	+67.5
IL-6	2	6.5 ± 0.8a	9.4 ± 1.1ab	12.3 ± 0.9b	+44.6	+89.2
IL-10	1	19.8 ± 2.4a	35.0 ± 4.2ab	44.2 ± 2.7b	+76.8	+123.2
IL-10	2	20.0 ± 4.5a	37.7 ± 5.4ab	43.3 ± 5.0b	+88.5	+116.5
IFN-γ	1	1317.8 ± 115.4a	836.0 ± 72.7b	1078.8 ± 15.6ab	-36.6	-18.1
IFN-γ	2	915.5 ± 83.8a	411.8 ± 76.1b	609.8 ± 51.8ab	-55.0	-33.4

Macrophage Cytokines. In contrast to T lymphocyte cytokines, cytokine elaboration by splenic macrophages cultured following one or two infestations with either *I. scapularis* or *I. pacificus* nymphs was highly variable both between infestations and tick species with few significant differences observed. Mean concentrations (± standard error) of macrophage cytokines and percent change of cytokine concentrations for infested and control mice are presented in Table 14.

Interleukin-1β was up regulated in mice infested with either tick species for both exposures of the first trial, but only significantly for *I. pacificus*-infested mice (Table 14). In the second trial, IL-1β was up regulated, but not significantly, for *I. pacificus*-infested mice at the end of both exposures (Table 14). These results indicate that *I. pacificus* may up regulate IL-1β production in C3H/HeN mice, but infestation with *I. scapularis* does not appear to stimulate a similar response. Significant differences in TNF-α levels occurred at the end of the second infestation of the first trial, when this cytokine was suppressed by *I. pacificus* and enhanced by *I. scapularis* (Table 14). However, these values did not differ from the levels of TNF-α produced by uninfested control mice. During the second trial, the only significant difference in TNF-α production was up regulation by *I. pacificus* at the end of the second infestation (Table 14). In contrast to IL-1β and TNF-α, no significant differences were observed for IL-6 production.

Table 14. Mean concentrations (picograms/milliliter) ± standard error and percent change of macrophage cytokines for *Ixodes scapularis*-infested, *I. pacificus*-infested and noninfested (control) C3H/HeN mice, trials one and two. In each row, groups indicated by the same letter are not significantly different, p>0.05. * Each treatment group consisted of five mice for each infestation, except for the control group of the twice-exposed animals of trial two, which contained four mice.

Cytokine	Times infested	Mean Concentration (pg/ml)			Percent Change	
		Control *	*I. scapularis* infested *	*I. pacificus* infested *	*I. scapularis* infested	*I. pacificus* infested
			Trial 1			
IL-1β	1	92.4 ± 28.9a	145.6 ± 28.8ab	334.2 ± 42.0b	+57.6	+261.7
IL-1β	2	70.2 ± 19.4a	122.8 ± 21.5ab	450.2 ± 110.6b	+74.9	+541.3
IL-6	1	2368.3 ± 368.3a	2261.7 ± 306.4a	3974.5 ± 608.0a	-4.5	+67.8
IL-6	2	7264.1 ± 1340.7a	7956.5 ± 1076.6a	7273.8 ± 1462.1a	+9.5	+0.1
TNF-α	1	123.1 ± 4.7a	128.3 ± 7.0a	126.8 ± 7.8a	+4.2	+3.0
TNF-α	2	110.7 ± 38.6ab	157.3 ± 83.5a	22.9 ± 7.8b	+42.1	-79.3
			Trial 2			
IL-1β	1	886.6 ± 138.2a	738.8 ± 93.8a	1111.8 ± 190.2a	-16.6	+25.4
IL-1β	2	480.2 ± 104.0a	494.2 ± 82.1a	858.6 ± 310.5a	+2.9	+78.8
IL-6	1	9024.5 ± 825.3a	9060.9 ± 583.7a	7790.3 ± 940.1a	+0.4	-13.7
IL-6	2	8448.6 ± 961.5a	9821.6 ± 2385.9a	14087.8 ± 739.8a	+16.3	+66.7
TNF-α	1	123.8 ± 19.6a	187.6 ± 55.0a	140.8 ± 22.9a	+51.5	+13.7
TNF-α	2	572.0 ± 55.7a	615.4 ± 71.9ab	828.7 ± 35.8b	+7.6	+44.9

Specific Aim III. Determine the effects of *I. scapularis, I. pacificus, and D. andersoni* salivary gland soluble proteins on *in vitro* proliferative responses of T-lymphocytes from BALB/c and C3H/HeN mice.

In vitro proliferative responses of lymphocytes from BALB/c and C3H/HeN mice were significantly suppressed when exposed to salivary gland soluble proteins (SGSP) derived from each tick species. Different patterns of suppression or enhancement in *in vitro* lymphocyte proliferative responses were observed for cells cultured with SGSP of each of the three species. *In vitro* proliferative responses of cells cultured with SGSP and 0.1 μg of Con A were also suppressed when exposed to SGSP from each tick species for virtually every SGSP dilution. Responses of lymphocytes to SGSP, cultured with or without Con A, also differed depending on the strain of mice that the cells were derived from. However, the general patterns of SGSP produced changes in proliferative responses were similar for BALB/c and C3H/HeN mice; the differences between the mouse strains were more quantitative than qualitative.

Proliferative responses of lymphocytes from BALB/c mice, cultured with SGSP alone (no Con A), were suppressed when compared to cells cultured without SGSP for all but one concentration of SGSP, from one of the species (Table 15). This exception was seen with 2.0 μg/well of *I. scapularis* SGSP in which the mean counts per minute were 700.0 ± 79.0 (mean ± S.E.) as compared to the mean count of 604.9 ± 64.5 for cells not exposed to SGSP. This indicated an increase in proliferative response of 15.7% (Table 15). However, in all other cases, SGSP from each species suppressed proliferative responses of lymphocytes, when compared to lymphocytes cultured without SGSP. The

mean counts per minute of cells cultured with SGSP ranged from 384.2 ± 57.4 for cells cultured with 2.0 μg/well of *I. pacificus* SGSP to 600.5 ± 65.6 for cells cultured with 1.0 μg/well of *D. andersoni* SGSP (Table 15). This corresponded to a suppression of proliferative responses of from 36.5% for 2.0 μg/well *I. pacificus* SGSP, to 0.8% for 1.0 μg/well of *D. andersoni* SGSP (Table 15), when compared to cells cultured without SGSP.

Results indicate that for cells cultured with SGSP alone, *I. pacificus* SGSP suppresses *in vitro* proliferative responses to a greater degree ($p < 0.05$) than did either *I. scapularis* or *D. andersoni*, which did not differ significantly ($p > 0.05$). This suppression of proliferative responses was significant only for the highest SGSP concentration (2.0 μg/well) and was not significantly different at the other concentrations of SGSP (Table 15).

For lymphocytes cultured with SGSP and Con A, a different pattern of proliferative responses was observed. Con A-stimulated proliferative responses were suppressed for all concentrations of SGSP derived from *I. scapularis* or *I. pacificus*, when compared to cells cultured with Con A alone (Table 15). This suppression of Con A stimulated responses was not significantly different for cells cultured with *I. scapularis* or *I. pacificus* SGSP (Table 15), while differences between *I. scapularis* and *D. andersoni* were significant at the three lower SGSP concentrations (1.0, 0.5, and 0.25 μg/well).

Table 15. Mean counts per minute (CPM) ± S.E., and percentage change of lymphocytes exposed to SGSP, BALB/c mice.

BALB/c Mice	2.0 μg/well	1.0 μg/well	0.5 μg/well	0.25 μg/well
		Counts per Minute		
		SGSP only		
I. scapularis	700.0 ± 79.0	483.9 ± 52.9	492.5 ± 72.2	417.1 ± 34.5
I. pacificus	384.2 ± 57.4	410.6 ± 45.5	452.3 ± 43.5	463.1 ± 41.7
D. andersoni	504.6 ± 44.0	600.5 ± 65.6	548.6 ± 51.0	534.5 ± 46.2
		cells only, no SGSP = 604.9 ± 64.5		
		SGSP and 0.1 μg/well Con A stimulation		
I. scapularis	4664.9 ± 551.5	8649.7 ± 1313.9	9875.0 ± 1474.4	10434.6 ±1339.7
I. pacificus	8009.0 ± 959.3	11005.1 ± 1989.8	14569.1 ± 2476.0	13838.7 ± 1985.2
D. andersoni	3419.4 ± 750.1	17606.7 ± 2432.1	25382.2 ± 2977.8	27631.0 ± 2421.8
		cells and Con A 0.1 μg/ml (no SGSP) = 18402.5 ± 2351.3		
		cells only, no SGSP = 569.71 ± 59.58		
		Percentage Change		
		SGSP only*		
I. scapularis	15.7b	-27.3a	-18.6a	-31.0a
I. pacificus	-36.5a	-32.1a	-25.2a	-23.4a
D. andersoni	-16.6ab	-0.8a	-9.3a	-11.6a
		SGSP and 0.1 μg/well Con A stimulation†		
I. scapularis	-74.7a	-53.0a	-46.3a	-43.3a
I. pacificus	-56.5a	-40.2ab	-20.8ab	-24.8ab
D. andersoni	-81.4a	-4.3b	37.9b	50.1b

* Percentage change = [(CPM of cells with SGSP – CPM of cells only)/ CPM of cells only] * 100.

† Percentage change = [(CPM of cells with SGSP & Con A – CPM of cells + Con A without SGSP)/ CPM of cells + Con A without SGSP)] * 100. In each column, groups indicated by the same letter are not significantly different, $p>0.05$.

The mean counts per minute (± S.E.) ranged from 14569.1 ± 2476.0 for 0.5 μg/well *I. scapularis* SGSP to 4664.9 ± 551.5 for 2.0 μg/well *I. scapularis* SGSP (Table 15), resulting in suppression of Con A stimulated proliferative responses from 20.8% for 0.5 μg/well *I. pacificus* SGSP to 74.7% for *I. scapularis* SGSP, when compared to lymphocytes cultured with Con A alone.

For lymphocytes exposed to *D. andersoni* SGSP, suppression of Con A stimulated proliferative responses occurred only at the two highest SGSP concentrations. At 2.0 μg/well, the greatest suppression of Con A stimulated responses was observed for *D. andersoni* SGSP, -81.4%, which was not significant from suppression induced by SGSP of the other two species at this concentration. However, the suppression of Con A stimulated proliferative responses was clearly concentration dependent for *D. andersoni* SGSP. At the two lower concentrations, SGSP enhanced Con A induced T lymphocyte proliferation; counts per minute were 25,382 ± 2977.8 and 27631.0 ± 2421.8 for the 0.5 and 0.25 μg/well of Con A respectively, corresponding to an increase in responses from 37.9 to 50.1%, when compared to cells cultured with Con A alone (Table 15).

Results demonstrate that for lymphocytes derived from BALB/c mice, SGSP from *I. pacificus* suppresses *in vitro* lymphocyte proliferation in the absence of Con A to a greater degree than SGSP from the other species. However, for cells cultured with SGSP and Con A, a different pattern occurs. Suppression of Con A-induced *in vitro* lymphocyte proliferative responses was observed for SGSP of all three species at the highest SGSP concentration, and this suppression was not significantly different among the species. At the three lower concentrations, suppression of Con A-stimulated *in vitro* proliferative responses was seen with SGSP from *I. scapularis* and *I. pacificus*. While

responses of cells cultured with SGSP of each of these two species did not differ significantly, *I. scapularis* SGSP suppressed proliferative responses to a greater degree than *I. pacificus* SGSP, and this suppression was significantly different than responses of cells cultured with *D. andersoni* SGSP at these three concentrations. Further, *D. andersoni* SGSP suppressed Con A stimulated proliferative responses only at the two highest concentrations, while at the two lowest concentrations, SGSP enhanced proliferative responses to levels above those of Con A responses alone.

In vitro proliferative responses of lymphocytes from C3H/HeN mice differed when compared to responses of cells from BALB/c mice. Counts per minute of cells from C3H/HeN mice, exposed to SGSP from each tick species, are presented in Table 16. Responses of lymphocytes from C3H/HeN mice, cultured with SGSP alone (no Con A), were suppressed for virtually every concentration of SGSP, when compared to cells cultured without SGSP (Table 15). As observed for BALB/c mice, 2.0 μg/well of *I. scapularis* SGSP did not suppress proliferative responses. The counts per minute (± S.E.) for cells cultured with 2.0 μg/well of *I. scapularis* SGSP was 2250.0 ± 122.4, as compared to cells alone in which the count per minute was 2033.8 ± 193.8. This represented an increase of 10.6% in proliferative response above cells cultured without SGSP (Table 16). Increase in proliferative responses above cells cultured without SGSP was also seen for cells cultured with 0.5 and 0.25 μg/well of *D. andersoni* SGSP. The counts per minute for these cells were 2613.3 ± 366.9 and 2061.2 ± 135.1, representing an increase in proliferative responses of 28.5 and 1.3%, respectively. Similar to responses of cells from BALB/c mice, proliferative responses of cells from C3H/HeN mice, cultured with all dilutions of *I. pacificus* SGSP and the three lower dilutions of *I.*

scapularis SGSP, were suppressed, when compared with responses of unstimulated cells alone (Table 16). The counts per minute of these cells ranged from 384.2 ± 57.4 for cells cultured with 2.0 μg/well of *I. pacificus* SGSP, to 492.5 ± 72.2 for cells cultured with 0.5 μg/well of *I. scapularis* SGSP. This represents a suppression of proliferative responses ranging from 36.5 to 18.6%, respectively, when compared to cells cultured without SGSP (Table 16). For lymphocytes cultured with *D. andersoni*, suppression of proliferative responses was observed only with cells cultured with 2.0 and 1.0 μg/well of SGSP, which corresponds to a suppression of 16.6 and 0.8%, respectively, when compared to cells cultured without SGSP. (Table 16).

Results indicate that for lymphocytes from C3H/HeN mice cultured with SGSP alone, *I. pacificus* SGSP suppressed *in vitro* proliferative responses to a greater degree than did SGSP from either of the other two species ($p<0.05$), and that responses of cells cultured with *I. scapularis* SGSP were significantly lower than responses of cells cultured with *D. andersoni* SGSP ($p<0.05$). Analyses of effects of SGSP from each species showed that proliferative responses of cells cultured with the two highest concentrations (2.0 μg/well and 1.0 μg/well) of *I. pacificus* SGSP were significantly different ($p<0.05$) than responses of cells cultured with *I. scapularis* of *D. andersoni* SGSP, which did not differ significantly ($p>0.05$, Table 16). At 0.5 μg/well SGSP, responses of cells cultured with *I. scapularis* or *I. pacificus* SGSP did not differ from each other ($p>0.05$), but did differ from cells cultured with *D. andersoni* SGSP ($p<0.05$, Table 16). At the lowest concentration, 0.25 μg/well, responses of cells cultured with *I. scapularis* were different than responses of cells cultured with *D. andersoni* ($p<0.05$), but neither of these differed from proliferative responses of cells cultured with *I. pacificus* SGSP ($p>0.05$, Table 16).

Similar to results observed for BALB/c mice, C3H/HeN lymphocytes cultured with SGSP and Con A showed a different pattern of proliferative responses, when compared to cells cultured with SGSP alone. Con A stimulated *in vitro* proliferative responses of lymphocytes from C3H/HeN mice were suppressed by all concentrations of *I. scapularis* SGSP, but only by the two highest dilutions, 2.0 μg/well and 1.0 μg/well, of the *I. pacificus* SGSP, and only with the 2.0 μg/well dilution of *D. andersoni* SGSP. Counts per minute (± S.E.) of cells cultured with *I. scapularis* SGSP and Con A ranged from 4829.9 ± 327.4 for cells exposed to 2.0 μg/well *I. scapularis* SGSP, to 10434.6 ± 1339.7 for cells cultured with 0.25 μg/well *I. scapularis* SGSP (Table 16). These responses corresponded to a decrease in Con A-stimulated proliferative responses from 65.5 to 22.6%, when compared to Con A stimulation alone (Table 16). The greatest suppression in Con A stimulated proliferative responses was seen with 2.0 μg/well *I. pacificus* SGSP, in which counts per minute were 794.1 ± 94.6, corresponding to a suppression of 99.5%, when compared to cells cultured with Con A alone (Table 16). Suppression of Con A-stimulated responses were seen only with the two higher SGSP dilutions for cells cultured with *I. pacificus* SGSP, while the two lowest dilutions resulted in an increase in proliferative responses of 8.1 and 2.7%, respectively, when compared to cells exposed only to Con A (Table 16). For cells cultured with *D. andersoni* SGSP, proliferative responses were suppressed only at the 2.0 μg/well concentration, resulting in a decrease of 86.5% when compared to responses of cells cultured only with Con A (Table 16). Con A-stimulated proliferative responses of lymphocytes exposed to the other three *D. andersoni* SGSP dilutions were increased from 30.7 to 105.1%, as compared to responses of Con A stimulation only (Table 16).

At all concentrations of SGSP, Con A-stimulated *in vitro* proliferative responses of lymphocytes from C3H/HeN mice cultured with *I. scapularis* SGSP were significantly different than responses of cells cultured with *D. andersoni* SGSP ($p<0.05$, Table 16). For cells cultured with 2.0 μg/well SGSP, cells exposed to *I. pacificus* and *D. andersoni* SGSP, did not differ significantly, but did suppress Con A-stimulated responses significantly greater than cells exposed to *I. scapularis* SGSP (Table 16). In contrast, cells cultured with the three lowest concentrations of *I. pacificus* and *I. scapularis* SGSP did not differ significantly ($p>0.05$, Table 16). When exposed to the 1.0 and 0.25 μg/well concentration of *I. scapularis* and *I. pacificus* SGSP, Con A stimulated proliferative responses were not significantly different from each other ($p>0.05$), but were significantly different from responses of cells exposed to *D. andersoni* SGSP ($p<0.05$, Table 16). At the concentration of 0.5 μg/well, the responses of cells exposed to *I. scapularis* SGSP differed significantly from cells cultured with *D. andersoni* SGSP ($p>0.05$, Table 16), but not from cells exposed to *I. pacificus* SGSP.

Responses of lymphocytes from both strains of mice, cultured with SGSP alone, indicate that *I. pacificus* SGSP suppresses *in vitro* lymphocyte proliferation to a greater degree than does SGSP from each of the other species. In addition, while *I. scapularis* SGSP enhances proliferative responses at the highest dilution, at the three lower dilutions proliferative responses are suppressed, when compared to cells not exposed to SGSP. Responses of lymphocytes from BALB/c mice, exposed to *D. andersoni* SGSP, were suppressed for all concentrations of SGSP, but lymphocyte responses of cells from C3H/HeN mice were suppressed only at the two highest concentrations of SGSP.

For cells cultured with SGSP and Con A, a different pattern occurs. Suppression of *in vitro* lymphocyte proliferative responses was observed for SGSP of all three species at the highest SGSP concentration. This suppression was significantly greater for cells cultured with *I. pacificus* or *D. andersoni* SGSP. At the three lower concentrations, suppression of *in vitro* proliferative responses occurred only with SGSP from *I. scapularis* and this suppression was significantly different than responses of cells cultured with *D. andersoni* SGSP. Responses of cells cultured with *I. scapularis* and *I. pacificus* SGSP did not differ significantly at the three lower SGSP concentrations, but did differ from *D. andersoni* at 1.0 μg/well and 0.25 μg/well. Furthermore, *D. andersoni* SGSP suppressed Con A stimulated proliferative responses only at the highest SGSP concentration, while at the three lowest concentrations, SGSP enhanced proliferative responses to levels significantly above those of Con A alone.

Table 16. Mean counts per minute (CPM) ± S.E., and percentage change of lymphocytes exposed to SGSP, C3H/HeN mice.

C3H/HeN mice	2.0 μg/well	1.0 μg/well	0.5 μg/well	0.25 μg/well
	Counts per Minute			
	SGSP only			
I. scapularis	2250.0 ±122.4	1597.8 ± 155.4	1474.0 ± 149.4	1404.6 ± 115.9
I. pacificus	702.3 ± 64.0	1015.9 ± 105.7	1461.8 ± 204.6	1570.5 ± 142.2
D. andersoni	1811.7 ± 156.5	1858.8 ± 138.6	2613.3 ± 366.9	2061.2 ± 135.1
	cells only, no SGSP = 2033.8 ± 193.8			
	SGSP and 0.1 μg/well Con A stimulation			
I. scapularis	4829.9 ± 327.4	8649.7 ± 1313.9	9875.0 ± 1474.4	10434.6 ±1339.7
I. pacificus	794.1 ± 94.6	11005.1 ± 1989.8	14569.1 ± 2476.0	13838.7 ± 1985.2
D. andersoni	1824.1 ± 182.1	17606.7 ± 2432.1	25382.2 ± 2977.8	27631.0 ± 2421.8
	cells and Con A 0.1 μg/ml (no SGSP) = 13474.6 ± 1304.5			
	cells only, no SGSP = 2021.9 ± 198.5			
	Percentage Change			
	SGSP only*			
I. scapularis	10.6b	-21.4b	-27.5a	-30.9a
I. pacificus	-65.5a	-50.0a	-28.1a	-22.8ab
D. andersoni	-10.9b	-8.6b	28.5b	1.3b
	SGSP and 0.1 μg/well Con A stimulation†			
I. scapularis	-64.2a	-35.8a	-26.7a	-22.6a
I. pacificus	-99.5b	-18.3a	8.1ab	2.7a
D. andersoni	-86.5b	30.7b	88.4b	105.1b

* Percentage change = [(CPM of cells with SGSP – CPM of cells only)/ CPM of cells only] * 100.

† Percentage change = [(CPM of cells with SGSP & Con A – CPM of cells + Con A without SGSP)/ CPM of cells + Con A without SGSP)] * 100. In each column, groups indicated by the same letter are not significantly different, p>0.05.

To determine if changes in lymphocyte proliferative responses were due to potential toxic effects of SGSP on cells, trypan blue dye exclusion was assessed. Results indicated that SGSP of each of the tick species was not toxic to lymphocytes of either mouse strain. For lymphocytes derived from BALB/c mice, cells not exposed to SGSP were 88% viable after the 72-hour incubation period. Cells exposed to the highest dilution of SGSP, 2.0 μg/well, from each tick species were assayed to determine if SGSP from any of the species was toxic to cells. Lymphocytes from BALB/c mice, exposed to *I. scapularis* SGSP, were 90% viable while those cultured with *I. pacificus* and *D. andersoni* SGSP were 91 and 93% viable, respectively. None of these differences were significant. Results for cells from C3H/HeN mice were similar and did not indicate a toxic effect of SGSP from any of the tick species. For lymphocytes from C3H/HeN mice cultured without SGSP, 86% of the cells were viable after 72 hour incubation period. Cells exposed to *I. scapularis* SGSP were 90% viable, and those cultured with *I. pacificus* and *D. andersoni* SGSP were 89 and 91% viable, respectively. Again, none of these differences were significant, indicating that SGSPs were not toxic to lymphocytes of either mouse strain.

Specific Aim IV: Evaluate tick biology parameters of *D. andersoni* nymphs after repeated infestations of BALB/c and C3H/HeN mice.

To determine if BALB/c or C3H/HeN mice acquire resistance to repeated infestations with *D. andersoni*, each mouse strain was infested one to four times with pathogen-free *D. andersoni* nymphs. This experiment was performed since neither

mouse strain acquired resistance to either *I. scapularis* or *I. pacificus* nymphs. Tick biology parameters for ticks recovered from each strain of mouse are presented in Table 17.

The mean number of ticks recovered after repeated infestations of BALB/c mice decreased significantly after the initial infestation. The mean number of ticks recovered after the first infestation was 9.13 and decreased to 5.25 and 4.25 following the second and third infestations, respectively. Following the fourth infestation, only 1.25 ticks per mouse were recovered, which was significantly less than the number of ticks recovered following the first, second, or third infestations (Table 17).

The mean weight (± S. E.) of ticks recovered from BALB/c mice after the second and third infestations was significantly less than after the first and fourth infestations, ranging from 24.88 ± 1.07 mg after the first infestation, 23. 78 ± 2.82 and 18.98 ± 2.14 after the second and third infestations, respectively, to a low of 17.50 ± 1.53 mg after the second infestation. Mean feeding duration increased from an average of 6.45 days after the first infestation to 6.86 days following the second infestation, after which it dropped slightly to a mean of 6.71 and 6.80 days following the third and fourth infestations, respectively. This increase in feeding duration following the second and third infestations was significantly different than the duration following the first infestation, but not different than that seen for the fourth infestation (Table 17). Finally, the molting success (percentage molt) ranged from 76.2 % for ticks recovered after the second infestation to 100% for ticks recovered after the fourth infestation. This difference in molting success was significantly less after the second infestation than that seen after the first infestation, but not significantly different following the other infestations (Table 17).

For nymphs recovered after infestations of C3H/HeN mice, the mean number of ticks recovered decreased significantly after each of the four infestations and ranged from 8.50 ticks per mouse recovered after the first infestation, to 0.75 ticks recovered after the fourth infestation (Table 17). The mean weight (± S. E.) of nymphs recovered after each infestation ranged from 25.25 ± 0.96 mg following the first infestation to 15.07 ± 7.46 mg after the fourth infestation. The mean weight of recovered ticks was significantly different between the first and second infestations, but the mean weights of ticks recovered after the third or fourth infestation did not differ significantly from the mean engorgement weight after the second infestation (Table 17).

The mean feeding duration of nymphs infesting C3H/HeN mice ranged from 6.49 days following the first infestation to 7.00 days following the fourth. This increase in feeding duration was significantly different following the first and second infestation; feeding duration following the second infestation did not differ from mean duration observed following the third or fourth infestations (Table 17). Finally, the percentage of nymphs that successfully molted to the adult stage following repeated infestations of C3H/HeN mice ranged from 83.7% following the second infestation to 100% following the fourth and did not differ significantly between infestations (Table 17).

Table 17. Replete nymphs recovered from mice, mean weight ± standard error of recovered nymphs, mean number of days spent feeding, and percent nymphs successfully molting to adults for *D. andersoni* nymphs fed on BALB/c and C3H/HeN mice. Ten nymphs applied per mouse. In each column, groups indicated by the same letter are not significantly different, p>0.05.

Infestation	Ticks recovered	No. of mice	Mean ticks recovered /mouse	Mean tick weight (mg) ± S.E.	Mean feeding duration (days)	% molt
BALB/c						
1	73	8	9.13a	24.88 ± 1.07a	6.45a	95.9a
2	42	8	5.25b	17.50 ± 1.53b	6.86b	76.2b
3	17	4	4.25b	18.98 ± 2.14b	6.71b	88.2ab
4	5	4	1.25c	23.78 ± 2.82a	6.80ab	100.0ab
C3H/HeN						
1	68	8	8.50a	25.25 ± 0.96a	6.49a	91.5a
2	49	8	6.13b	16.74 ± 1.40b	6.71b	83.7a
3	11	4	2.75c	20.38 ± 3.33ab	6.64ab	90.9a
4	3	4	0.75d	15.07 ± 7.46ab	7.00ab	100.0a

CHAPTER IV

DISCUSSION

Complex immunological interactions occur at the tick-host interface (Wikel, 1996b). These interactions influence the ability of the tick to successfully feed, and consequently its ability to transmit pathogens such as *B. burgdorferi* (Wikel and Bergman, 1997). Tick feeding stimulates a wide variety of host immune responses involving antigen-presenting cells, T and B lymphocytes, cytokines, homocytotropic and circulating antibodies, complement, granulocytes, and other immunologically active molecules (Borsky *et al.*, 1994, Brossard *et al.*, 1991, Wikel, 1982a, Wikel, 1996a). Anti-tick immune responses may lead to the development of acquired resistance to repeated exposures to tick infestation. Immunologically based acquired resistance to ticks has been described for some tick-host associations (Brossard *et al.*, 1991, Brossard and Wikel, 1997, Wikel, 1996a, Wikel *et al.*, 1996), while others are characterized by a lack of acquired resistance. Even though acquired resistance to tick feeding may not occur, tick infestation induces immune response changes in the host (Borsky *et al.*, 1994, Ganapamo *et al.*, 1995).

It is well established that ixodid ticks have developed countermeasures to reduce host immune responses directed against them while feeding (Wikel and Bergman, 1997). Tick-induced immunomodulation involves: inhibiting the activity of complement components; impairing natural-killer cell function; reducing antibody responses; suppressing *in vitro* proliferative responses of T lymphocytes; and, modulating cytokine responses of macrophages and T-cells (Wikel and Bergman, 1997, Wikel *et al.*, 1996).

The purpose of this study was to evaluate immune responses of inbred laboratory mice to repeated infestations with *I. scapularis* nymphs, and to compare responses of mice to either infestation, or salivary gland proteins, of *I. scapularis*, *I. pacificus* or *D. andersoni*. The first objective evaluated macrophage and T lymphocyte cytokine responses of two strains of mice repeatedly infested with *I. scapularis* nymphs. Then, using the mouse strain that responded most vigorously to tick feeding, *in vitro* proliferative responses were assessed in repeatedly infested animals. To compare immune responses developed against *I. pacificus* or *I. scapularis* nymphs, the next objective evaluated macrophage and T lymphocyte cytokine responses of mice repeatedly infested with each of these species. To further analyze the differences in immune responses developed in murine hosts *to I. pacificus* or *I. scapularis*, the next objective of this study evaluated *in vitro* proliferative responses of lymphocytes from BALB/c and C3H/HeN mice cultured with salivary gland proteins from *I. scapularis* and *I. pacificus* females, and compared these to the responses of lymphocytes cultured with salivary proteins of *D. andersoni* females. The final objective of this study evaluated biological parameters of ticks repeatedly infesting BALB/c and C3H/HeN mice, to determine if acquired resistance developed in these mice strains to repeated feeding of *D. andersoni* nymphs

To evaluate macrophage and T lymphocyte cytokine responses of mice repeatedly infested with *I. scapularis*, BALB/c and C3H/HeN mice were infested from one to four times with pathogen-free nymphs. Results showed that neither mouse strain developed acquired resistance to tick feeding. These patterns are consistent with those reported for

mice infested with other members of the *Ixodes ricinus* complex (Christe *et al.*, 1998, Mbow *et al.*, 1994a, Wikel *et al.*, 1997). The effects of host acquired resistance to tick infestation are often very dramatic with significant reduction in engorgement weights and increased mortality of feeding ticks (Wikel, 1982a, Wikel, 1996a). The host species has an impact on whether or not acquired resistance develops (Ribeiro, 1989). While mice do not develop acquired resistance to *I. scapularis* nymphs, guinea pigs infested with the same species develop resistance to subsequent infestations (Das *et al.*, 1998, Wikel *et al.*, 1997). Weights of *I. scapularis* nymphs obtained from guinea pigs sensitized by three repeated infestations averaged only 0.86 ± 0.69 (mean ± S.D.) milligrams, while the weights of nymphs obtained from naive animals averaged 3.24 ± 0.24 milligrams (Das *et al.*, 1998).

A few significant differences were seen in selected tick biology parameters after some infestations. However, these differences were not consistent with what has been previously reported for host acquired resistance to tick infestation (Das *et al.*, 1998, DenHollander and Allen, 1985). Differences in engorgement weights observed during this study may be due to differential sex ratios of nymphs applied to each mouse, as it is not possible to distinguish sex in nymphal ticks, and engorged nymphs that molt to adult female or male ticks are not of equal weight (Christe *et al.*, 1998). Other factors may have caused the significant differences observed in tick biology parameters. Tick feeding can be influenced by tick age, environmental factors, season (Balashov, 1972, Branagan, 1974, Gladney and Drummond, 1970, Sweatman, 1970), or by murine strain infested (Randolph, 1979). Differences in tick biology parameters observed in this study were not

consistent with the development of acquired resistance to infestation by either murine strain.

To our knowledge, this is the first study to examine cytokine responses in BALB/c or C3H/HeN mice at the termination of repeated infestations with *I. scapularis* nymphs. Although neither mouse strain developed acquired resistance to *I. scapularis*, clear differences were seen in T lymphocyte cytokine responses between the two mouse strains. Distinct changes in cytokine production were observed between infested and non-infested mice, which were comparable for both strains. The Th-2 lymphocyte cytokines were enhanced in both mouse strains and pro-inflammatory Th-1 lymphocyte cytokines were either significantly suppressed or did not differ from non-infested control mice.

At the end of each infestation, T lymphocyte cytokine responses of both murine strains were highly polarized toward a Th-2 lymphocyte cytokine pattern. Although the polarization profiles were similar, the magnitudes of change differed between infested and control mice of the two strains. The Th-1 cytokines IL-2 and IFN-γ were either suppressed or did not differ significantly from controls. However, the Th-2 cytokines IL-4 and IL-10 were significantly enhanced relative to control values for virtually every infestation of the two murine strains. While the magnitude of these differences in T lymphocyte cytokines varied between the two trials for both murine strains, the trends in production of these cytokines were consistent for both strains during both trials. Differences in cytokine production by each murine strain during the first and second trials may be related to feeding differences in nymphs used for the infestations. Although ticks were maintained under identical conditions and obtained from the same colony, it is

well established that even ticks from the same egg mass feed differently (Balashov, 1972). Furthermore, seasonal differences impact tick feeding (Balashov, 1972). Due to the complexities of maintaining tick colonies and the amount of time required to conduct one series of repeat infestations, the two trials for each murine strain were conducted at different times of the year. Although nymphs used for each infestation were age-matched, and syngeneic mice of the same age were used, the differences in the intensity of cytokine responses between the two trials may reflect tick physiology as well as the inherent variability encountered in biological systems. While differences in intensity of cytokine responses were observed between trials for both BALB/c and C3H/HeN mice, both trials, for both strains, clearly indicated a polarization toward a Th-2 cytokine profile.

Polarization of cytokine responses by feeding ticks toward a Th-2 pattern has been reported for other tick-host associations. C3H/HeJ mice, infested once with pathogen-free *I. scapularis* nymphs, showed a suppression of IL-2 and IFN-γ beginning four days post-infestation and returning to levels similar to uninfested mice by day 12 post-infestation (Zeidner *et al.*, 1997). Production of IL-4 was greatly up regulated in mice exposed to pathogen-free nymphs, reaching a peak by day six post-infestation and remained elevated throughout the 12-day period. Likewise, IL-10 was also up regulated in mice exposed to pathogen-free nymphs; levels were highest at the tenth day post infestation and then decreased toward baseline levels by the twelfth day post-infestation (Zeidner *et al.*, 1997). These results, when taken with the findings of this study, demonstrate that the bias towards a Th-2 cytokine profile is evident after one infestation

with *I. scapularis* nymphs, and continues, as shown here, at least through the course of four repeated infestations.

Axillary and brachial lymph node $CD4^+$ T cells of BALB/c mice, infested one to three times with *I. ricinus* nymphs, were assessed for their ability of to produce IL-2, IL-4, IL-5, IL-10 and IFN-γ following *in vitro* stimulation with Con A (Ganapamo *et al.*, 1995, Ganapamo *et al.*, 1996a, Ganapamo *et al.*, 1996b). The comparisons were made nine days after the initiation of infestation. High levels of IL-4 and low levels of IL-2, IL-5, IL-10, and IFN-γ were observed. An increase of IL-2, IL-5, and IL-10 was found between the first and third infestation, indicating polarization of cytokine responses toward a Th-2 pattern. Results of this and previous studies demonstrate that infestation with *I. scapularis* or *I. ricinus* polarizes the T cell response towards a Th-2 pattern, while diminishing expression of Th-1 responses.

T lymphocytes are important as regulators and effectors of host immunity to ticks (Wikel, 1996b). Suppression of Th-1 and enhancement of Th-2 cytokine responses by the feeding tick could affect the expression of host acquired resistance. Reduced responses affecting T and B-lymphocytes would diminish both delayed type hypersensitivity responses, as well as immunoregulatory responses to tick antigens introduced while feeding. Additionally, reduced IFN-γ production would impair macrophage activation (Wikel, 1996a). An important aspect of this polarization toward a Th-2 response is the observation that both IL-4 and IL-10 were greatly enhanced in both mouse strains following repeated tick exposure. Both of these Th-2 cytokines inhibit Th-1 cell growth and differentiation (O'Farrell *et al.*, 1998, O'Garra, 1998); therefore, an increase in IL-4 and IL-10 levels may contribute to the suppression of Th-1 responses,

possibly resulting in a reduction in the expression of acquired resistance. Th-1 responses have been shown to be an essential element of acquired resistance in various tick-host associations (Wikel, 1996a).

Likewise, IL-4 and IL-10 have been demonstrated to be important in regulating immune responses. The development of Th-2 cells has been ascribed to the exposure of naive CD4+ T cells to IL–4 at the onset of an immune response (O'Garra, 1998), while IL-10 is a key inhibitor of many aspects of the inflammatory response (O'Farrell *et al.*, 1998). Both IL-4 and IL-10 inhibit macrophage activation and the production of pro-inflammatory cytokines and chemokines (O'Garra, 1998); and, IL-10 has also been recently shown to directly regulate the proliferation of macrophages (O'Farrell *et al.*, 1998). The observed up regulation of IL-4 due to tick feeding could lead to the development of a Th-2 response, resulting in a reduction of Th-1 responses. Additionally, IL-10 reduces macrophage-regulated pro-inflammatory responses, as well as the ability of the macrophage to present antigen to sensitized lymphocytes. High levels of IL-10 may also impact Langerhans cells (LC), which are important in the expression of acquired resistance to tick infestation (Nithiuthai and Allen, 1985), by suppressing LC migration and antigen presentation, further reducing immune effector components necessary to induce acquired resistance to tick infestation and respond to tick-transmitted disease-causing agents.

In contrast to the bias of the immune response towards a Th-2 lymphocyte cytokine profile, macrophage cytokine elaboration from BALB/c and C3H/HeN mice following repeated infestations with *I. scapularis* nymphs, was highly variable both between and within the murine strains, and few significant differences were observed.

The extremely low levels of macrophage cytokines seen during the first trials may have contributed to the lack of detectable differences. Lymphocyte cytokines were reported as nanograms per milliliter, while macrophage cytokines were given in picograms per milliliter (Tables 5-7). During the second trials, we attempted to increase production of macrophage cytokines by priming them with recombinant IFN-γ. This treatment did not reduce the variability in cytokine levels for either mouse strain. Consequently, no clear patterns emerged regarding modulation of macrophage cytokines. In previous studies, differences in IL-1β and TNF-α were detected. Mbow *et al.* (1994), observed that keratinocytes in the region of the bite site of *I. ricinus*-infested BALB/c mice contained mRNA for IL-1 and TNF-α, and more IL-1 and TNF-α positive mononucelar cells infiltrated the dermis during the second and third infestations (Mbow *et al.*, 1994b). Macrophages obtained from BALB/c mice, never exposed to ticks, were inhibited in their ability to produce IL-1β and TNF-α when exposed to salivary gland extracts prepared from feeding *D. andersoni* females (Ramachandra and Wikel, 1992).

Results of the experiments of this study clearly showed that T lymphocyte cytokine responses were polarized toward a Th-2 profile with a concurrent suppression of Th-1 cytokines. This occurred after the first infestation of both mice strains, and continued throughout the course of the four exposures. To further evaluate host immune responses to repeated infestations with *I. scapularis*, our next step was to evaluate *in vitro* lymphocyte proliferative responses of repeatedly infested mice. Since earlier results showed that cytokine responses of C3H/HeN mice to tick infestation were more vigorous and consistent than those of BALB/c mice, this portion of the study was conducted using only C3H/HeN mice. Similar to the first study, mice were infested one to four times with

pathogen-free *I. scapularis* nymphs. At the end of each series of infestations, *in vitro* proliferative responses of lymphocytes, harvested from infested and noninfested control mice, were determined.

In agreement with the results of the tick biology parameters assessed following repeated *I. scapularis* infestations of mice in the earlier portion of this study, no evidence of acquired resistance was observed following any infestations of C3H/HeN mice during this portion of our study. In addition, few significant changes in lymphocyte proliferative responses were observed for Con A or LPS stimulated lymphocytes following infestation. In contrast, significant differences in lymphocyte responses to *I. scapularis* SGSP were observed between repeatedly infested and uninfested mice. *Ixodes scapularis* SGSP suppressed the proliferation of lymphocytes from uninfested mice, while proliferation was significantly increased in mice infested with *I. scapularis* nymphs when compared to uninfested mice. This increase was evident after the initial infestation and continued throughout the four infestations with pathogen-free nymphs.

Our results confirm results of other studies that have reported that inbred laboratory mice do not develop acquired resistance to repeated infestations with ticks of the *I. ricinus* complex of species (Christe *et al.*, 1998, Mbow *et al.*, 1994a, Wikel *et al.*, 1997), including infestation with *I. scapularis* nymphs. The host species has an impact on whether or not acquired resistance develops (Ribeiro, 1989). Rabbits become progressively resistant when infested repeatedly with adult *I. ricinus* (Schorderet and Brossard, 1993). Similarly, guinea pigs infested with *I. scapularis* develop resistance to successive infestations (Das *et al.*, 1998, Wikel *et al.*, 1997). As a further example of the importance of the host species in the development of acquired resistance, dogs do not

develop resistance to repeated infestations with *R. sanguineus*, while guinea pigs develop resistance with repeated exposures to the same species (Ferreira and Silva, 1999).

Although acquired resistance to *I. scapularis* was not evident in repeatedly infested C3H/HeN mice, differences were seen in proliferative responses of splenic lymphocytes from mice exposed to feeding ticks, when compared to uninfested control mice. Specifically, lymphocytes from repeatedly infested mice, exposed to each of three dilutions of *I. scapularis* SGSP, showed significantly increased proliferative responses. These differences were significant following each infestation for the 2.0 μg/well and 1.0 μg/well dilution of SGSP, and following the second and fourth infestations for the 0.5 μg/well dilution of SGSP. Although the differences following the first and third infestations were not significant for this dilution, the trend was similar to that of cells exposed to the other dilutions of SGSP.

In contrast, the proliferative responses of cells from infested and noninfested mice, cultured with Con A or LPS, were significantly different only following the first infestation for cells cultured with 0.1 μg/well Con A. No other significant differences were observed in cells exposed to Con A or LPS following any of the infestations, suggesting that prior exposure to feeding *I. scapularis* nymphs does not affect proliferative responses of splenic lymphocytes exposed to these mitogens.

Correlation analysis revealed that tick exposure did not affect how cells responded to either Con A or LPS ($p > 0.10$). Correlation analyses between tick exposure and proliferative responses demonstrated that exposure to ticks significantly affected how cells responded to SGSP ($p < 0.0001$), while the number of tick exposures did not affect the magnitude of the response to SGSP ($p > 0.10$). Changes in *in vitro* proliferative

responses to SGSP were independent of how many times mice were infested. Furthermore, the results of the comparisons of stimulation indices of uninfested and repeatedly infested mice are consistent with comparisons based on counts per minute for cells cultured with Con A, LPS or SGSP.

The results of this study demonstrate that proliferative responses of lymphocytes from C3H/HeN mice, exposed to the mitogens Con A or LPS, are not affected by infestation with *I. scapularis*. However, exposure to *I. scapularis* feeding significantly affects the proliferative response of lymphocytes exposed to *I. scapularis* SGSP. One infestation is sufficient to significantly increase the proliferative responses of cells cultured with SGSP, and the magnitude of the SGSP induced response observed after the first exposure does not differ significantly from the magnitude of responses observed after the second, third, and fourth infestation.

Differences were observed in the counts per minute between uninfested mice for each of the four infestations, for each of the mitogens or SGSP. In a similar manner, differences were observed between infested mice for each mitogen and SGSP for each of the four infestations (Table 9). These differences were not unexpected, since mice from successive infestations were of different ages when lymphocytes were prepared and cultured, and it is well established that changes in both functional and phenotypic profiles of T lymphocytes occur in rodents of different ages (Linton *et al.*, 1997).

In contrast to our results, infestation with other tick species has been shown to significantly affect mitogen-stimulated *in vitro* lymphocyte proliferative responses. Concanavalin A-stimulated proliferative responses of lymphocytes from guinea pigs, infested with *D. andersoni*, were reduced when compared to noninfested animals, and the

reduction was greater during an initial infestation than during a subsequent infestation (Wikel, 1982b). Lymph node cells of C3H/HeJ mice infested one and four times with two male and two female *R. sanguineus* showed a decrease of 77.9% and 82.4%, respectively, in proliferative responses to Con A when compared to cells from uninfested animals (Ferreira and Silva, 1999). In contrast to lymph node cells, splenocytes from the same mice showed no significant change in proliferative responses, when compared to uninfested control animals (Ferreira and Silva, 1999). The Con A-stimulated proliferative response of spleen lymphocytes from BALB/c mice repeatedly infested with *I. ricinus* was unchanged after the primary and secondary infestations, and then increased above those from noninfested mice after the third and fourth infestations (Dusbabek *et al.*, 1995). In contrast, B cell responses to LPS and pokeweed mitogen were suppressed after each of four infestations (Dusbabek *et al.*, 1995). In a similar study, Borsky *et al.* (1994) reported reduced responsiveness of splenocytes to Con A after a primary infestation with *I. ricinus* nymphs. Responsiveness increased to levels above control value after the second infestation, and then decreased again following the third exposure (Borsky *et al.*, 1994). Finally, T cells collected from lymph nodes of *I. ricinus* infested BALB/c mice at nine days post-infestation had a suppressed *in vitro* response to Con A. Our results do not show a change in splenic lymphocyte responsiveness to Con A or LPS between infested and uninfested mice after repeated exposures to *I. scapularis* nymphs.

Concanavalin A mimics the actions of antigens on T lymphocytes and stimulates T cell proliferation *in vitro* (Sharon, 1983). Saliva or salivary gland extracts from various tick species contain molecules that influence Con A-stimulated lymphocyte proliferation (Bergman *et al.*, 1995, Bergman *et al.*, 1998, Ferreira and Silva, 1999,

Urioste *et al.*, 1994). Splenocytes from C57BL/6 and C3H/H mice, incubated in the presence of dilutions of *I. dammini* (=*I. scapularis (Oliver et al., 1993)*) saliva, had significantly decreased proliferative responses to Con A (Urioste *et al.*, 1994). Proliferative responses were suppressed up to 70-80%, when compared to that of cells not exposed to tick saliva (Urioste *et al.*, 1994). Salivary gland extracts prepared daily from feeding *D. andersoni* females suppressed *in vitro* lymphocyte responsiveness to Con A and enhanced proliferation in the presence of LPS for lymphocytes from uninfested mice (Ramachandra and Wikel, 1992) and cattle (Ramachandra and Wikel, 1995). Subsequent studies identified a 36-kDa soluble protein from *D. andersoni* salivary glands to be responsible for suppression of Con A-induced lymphocyte proliferation (Bergman *et al.*, 1998).

Our results, in agreement with those of other studies, demonstrate that lymphocytes from tick exposed animals respond differently to tick-derived antigens, when compared to lymphocytes from uninfested control animals. For example, *D. andersoni* salivary gland extract enhanced *in vitro* lymphocyte proliferation of lymphocytes from tick resistant guinea pigs when compared to noninfested animals (Wikel *et al.*, 1978). Lymphocytes from rabbits or BALB/c mice, cultured in the presence of salivary gland or integumental extracts, had significantly enhanced responsiveness (Borsky *et al.*, 1994, Schorderet and Brossard, 1993). Similar results were reported by Dusbabek *et al.* (1995), for *I. ricinus* infested BALB/c mice (Dusbabek *et al.*, 1995). Results of these and the present study demonstrate that prior exposure to tick salivary or whole tick extract antigens significantly stimulates the antigen-specific *in vitro* proliferative responses of lymphocytes.

T lymphocytes have both immunoregulatory and effector functions that are essential for maintaining the integrity of the immune system. Therefore, it is not surprising that ticks have evolved substances to modulate T lymphocyte responses (Wikel, 1999a). Modifying T lymphocyte responses could affect host immune responses not only to the feeding tick, but to tick-transmitted infectious agents as well, since many of the immune responses directed against the feeding tick are those that respond to tick-transmitted pathogens (Wikel and Bergman, 1997).

These first experiments evaluating host immunity to *I. scapularis* show that infestation reduces inflammatory responses directed against the feeding tick by suppressing pro-inflammatory Th-1 cytokine production, while enhancing the production of anti-inflammatory Th-2 cytokines. These reductions in inflammatory responses at the tick-feeding site probably enhance the ability of the tick to successfully feed. Additionally, while lymphocyte responses to T cell or B cell mitogens are not affected by repeated exposure *to I. scapularis* infestation, lymphocyte responses to salivary gland proteins are clearly affected by prior exposure to *I. scapularis*. Possibly, T lymphocytes that have previously encountered tick salivary gland antigens, are activated by subsequent exposure to these antigens, explaining why *in vitro* proliferative responses of cells from infested mice are significantly greater than responses of cells from naïve mice (Tables 9 and 10). Recognition of salivary antigens by previously sensitized T lymphocytes may enhance Th-2 cytokine production; this enhanced production of Th-2 cytokines would then suppress Th-1 cytokine production (O'Garra, 1998), leading to reduction in inflammatory responses directed against the feeding tick.

The changes in cytokine and *in vitro* T lymphocyte responses observed for *I. scapularis* infestations lead us to hypothesize that similar changes in immune responses may be induced in laboratory mice by repeated infestations with the closely related species, *I. pacificus*. *Ixodes scapularis* and *I. pacificus* are members of the *I. ricinus* species complex; these species are the most important vectors of *B. burgdorferi* to humans in the United States (Lane *et al.*, 1991). These species are somewhat similar in their ecology, having broad host ranges and occupying somewhat similar habitat types (Lane *et al.*, 1991). Therefore, because of their close relationship, and their importance as vectors of Lyme borreliosis, the next series of experiments were conducted to compare immune responses to these two species. In this regard, our first objective was to evaluate macrophage and T lymphocyte cytokine responses of C3H/HeN mice after infestations with each of these species.

Differences in cytokine responses were observed between tick-infested and non-infested mice, and between mice infested with *I. scapularis* or *I. pacificus* nymphs. Pro-inflammatory Th-1 T lymphocyte cytokines were suppressed and Th-2 cytokines were enhanced for nearly all infestations with *I. scapularis* or *I. pacificus* nymphs. Furthermore, infestation with *I. pacificus* nymphs results in more pronounced cytokine responses, either suppression or enhancement for virtually every cytokine evaluated, than infestation with *I. scapularis* nymphs. An exception was IFN-γ. *Ixodes scapularis* suppressed IFN-γ production more effectively than did infestation with *I. pacificus* nymphs. These patterns of cytokine responses occurred in the absence of host acquired resistance to either tick species.

As discussed previously, host acquired resistance to tick infestation has an immunological basis, and often has a profound affect on tick biology (Wikel, 1996a). However, as our results previously demonstrated, some tick-host associations do not result in the development of acquired resistance, notably BALB/c and C3H/HeN mice repeatedly infested with *I. scapularis*, or BALB/c mice infested with *I. ricinus* (Christe *et al.*, 1998, Christe *et al.*, 1999, Mbow *et al.*, 1994a). The only tick biology differences observed between *I. scapularis* and *I. pacificus* infesting C3H/HeN mice, were that *I. pacificus* nymphs fed more quickly, and weighed less upon completion of engorgement, than *I. scapularis* nymphs. These differences may be attributable to sexual dimorphism of engorged nymphs, which cannot be ascertained at the time of infestation with unfed nymphs. Engorged nymphs of the closely related species, *I. ricinus*, which molt to adult females or males, are not of equal weight (Christe *et al.*, 1998).

Although acquired resistance did not develop, tick infestation modulated cytokine production by host macrophages and T- lymphocytes. Systemic cytokine responses of mice infested with either tick species are greatly polarized, following each infestation, toward a Th-2 pattern. Specifically, the Th-1 cytokines IL-2 and IFN-γ were suppressed, while levels of the Th-2 cytokines IL-4, IL-5, IL-6 and IL-10 were enhanced for virtually every infestation by either tick species. Polarization of cytokine responses was similar for mice infested with either tick species; however, differences in intensity of responses were observed in mice infested with either *I. scapularis* or *I. pacificus* nymphs. Although not all differences were statistically significant, the trends in lymphocyte cytokine production clearly show that for almost every cytokine analyzed, *I. pacificus* infestation suppresses or enhances cytokine production to a greater degree than does infestation with

I. scapularis nymphs. An exception to this trend was observed for IFN-γ, in which *I. scapularis* infestation suppresses IFN-γ production more effectively than does infestation with *I. pacificus*.

Even though the trends in cytokine responses during each infestation were similar for both trials, the magnitude of cytokine polarization differed between the two trials. These differences may be attributed to variations in the feeding of nymphs used in the two trials as well as the fact that the two trials were conducted at different times of the year. These differences are not unexpected, since as discussed previously, a variety of factors can influence tick feeding (Balashov, 1972, Branagan, 1974, Gladney and Drummond, 1970, Sweatman, 1970). Even ticks derived from the same egg mass feed differently (Balashov, 1972). Due to the time required to perform the two infestations of each trial and the coordination of those two trials with rearing of both tick species, the two trials were performed approximately six months apart. While differences in the magnitude of cytokine responses were observed between the two trials, both trials clearly indicated a similar pattern of modulation of cytokine responses by each tick species.

The polarization of cytokine responses observed in mice infested by *I. scapularis* or *I. pacificus* are in agreement with our results involving repeated infestations of mice with *I. scapularis*, where we established that suppression of Th-1 cytokines, and up regulation of Th-2 cytokines, continued through four repeated infestations in the absence of evident acquired host resistance by either BALB/c or C3H/HeN mice. In a different study, C3H/HeJ mice, infested once with *I. scapularis* nymphs, showed a 55% suppression of IL-2 and 26% suppression of IFN-γ beginning four days post-infestation and returning to levels similar to those of uninfested mice by the twelfth day post-

infestation (Zeidner *et al.*, 1997). Furthermore, production of IL-4 was greatly up regulated in mice exposed to pathogen-free *I. scapularis* nymphs, reaching a peak of 723% of control mice by day six post-infestation and remained elevated throughout the 12-day period. In the same study, IL-10 was also up regulated to the highest level at day-10 post infestation, approximately 450% of uninfested mice, and then decreased toward baseline levels by the twelfth day post-exposure (Zeidner *et al.*, 1997).

Cytokine changes following tick feeding has been reported for other tick-host associations, as discussed earlier. Cytokine production by axillary and brachial lymph node $CD4^+$ T cells taken from BALB/c mice infested one to three times with 15 *I. ricinus* nymphs were compared following *in vitro* stimulation with Con A (Ganapamo *et al.*, 1995, Ganapamo *et al.*, 1996a, Ganapamo *et al.*, 1996b). Nine days after the beginning of infestation, high levels of IL-4 and low levels of IL-2, IL-5, IL-10, and IFN-γ were observed. Increases of IL-2, IL-5, and IL-10 occurred between the first and third infestations, suggesting a Th-2 polarization of cytokine responses. In addition, salivary gland extract (SGE) from partially fed female *I. ricinus* suppressed IFN-γ and enhanced IL-10 production by lymphocytes of BALB/c mice stimulated by *E. coli* LPS (Kopecky *et al.*, 1999). Both IL-2 and IFN-γ production by T lymphocytes from uninfested BALB/c mice were reduced by exposure to SGE of *Dermacentor andersoni* females (Ramachandra and Wikel, 1992). Furthermore, C3H/HeJ mice infested with adult *Rhipicephalus sanguineus* had a Th-2 polarization of cytokine responses (Ferreira and Silva, 1999). Results of those studies and data presented here demonstrate that tick infestation, or salivary gland components, polarize T cell responses toward a Th-2 pattern, while diminishing expression of Th-1 cytokines.

Modulation of cytokine production could affect expression of host-acquired resistance and immune responses to tick-borne disease causing agents. Reduced Th-1 responses would diminish delayed type hypersensitivity to tick salivary gland antigens, which contribute to the cellular influx at the attachment sites of certain host species (Mosmann and Coffman, 1989, Wikel, 1996b). Furthermore, diminished Th-1 responses would also reduce cytotoxic T cell responses and the production of antibody as a result of reduced help for B cells. Impaired elaboration of IFN-γ would decrease activation of macrophages (Wikel, 1996a). Both IL-4 and IL-10 were greatly enhanced in mice infested with either species of tick. These Th-2 associated cytokines inhibit Th-1 cell growth and differentiation (O'Farrell *et al.*, 1998, O'Garra, 1998) and they are possibly a factor in the observed cytokine polarization. Polarization to a Th-2 cytokine profile may contribute to the lack of acquired resistance observed for infestations by either tick species evaluated here.

In contrast to the bias of the immune response towards a Th-2 lymphocyte cytokine profile, and similar to that seen with repeated *I. scapularis* infestations of BALB/c or C3H/HeN mice, macrophage cytokine elaboration from C3H/HeN mice following infestations with *I. scapularis* or *I. pacificus* nymphs, was highly variable, and few significant differences were observed. However, as discussed earlier, changes in macrophage cytokines following exposure to feeding ticks or tick salivary gland extracts have been reported. Mbow, *et al.* (1994b) observed that keratinocytes in the region of the bite site of *I. ricinus*-infested BALB/c mice contained mRNA for IL-1 and TNF-α, and more IL-1 and TNF-α positive mononucelar cells infiltrated the dermis during the second and third infestations (Mbow *et al.*, 1994b). In a study involving exposure of normal

macrophages from BALB/c mice to *D. andersoni* salivary gland extract, Ramachandra and Wikel (1992), demonstrated that salivary gland extract inhibited production of both IL-1β and TNF-α (Ramachandra and Wikel, 1992). Reducing pro-inflammatory macrophage cytokines could be advantageous to the feeding tick; since reduced levels of IL-1β can impair activation of T lymphocyte-mediated immunoregulatory and effector pathways. Lowered TNF-α levels can decrease B and T lymphocyte activation and resistance to infection.

Reducing inflammatory and cell-mediated responses directed toward the feeding tick may facilitate tick-transmission of pathogens, since immune mechanisms that could inhibit feeding ticks are the same innate and acquired immune responses involved in immunity to disease causing agents (Wikel and Bergman, 1997). Zeidner *et al.* (1996), established that reconstituting the cytokine environment modifies development of disease in mice infested by spirochete infected ticks. C3H/HeJ mice passively reconstituted with TNF-α, IL-2, and IFN-γ are protected from infection, suggesting that Th-1 and/or macrophage cytokines may prevent *B. burgdorferi* infection (Zeidner *et al.*, 1996). In a separate study, *B. burgdorferi* spirochetes introduced during *I. scapularis* feeding on Lyme borreliosis susceptible C3H/HeJ mice resulted in up-regulation of IL-4 and IL-10 and down-regulation of IL-2 and IFN-γ (Zeidner *et al.*, 1997). These studies suggest that tick-mediated polarization of the host immune response towards a Th-2 cytokine pattern contributes to establishment of infection with *B. burgdorferi.* However, in a study involving needle inoculation of *B. burgdorferi*, passive administration of recombinant murine IL-4 to C3H/HeN mice on days two, five and eight post-inoculation of spirochetes resulted in early control of infection (Keane-Myers *et al.*, 1996). This

protective role of IL-4 reported by Keane-Myers *et al.* (1996) might be related to differences resulting from tick versus needle inoculation of spirochetes. Results of these studies demonstrate that tick-mediated polarization of host cytokine responses is important both for the tick to successfully feed, as well as to transmit *B. burgdorferi.*

To our knowledge, this is the first study to evaluate cytokine responses of inbred mice to infestation with *I. pacificus*. Our results, and results of other studies, demonstrate that infestation with ticks of the *I. ricinus* complex of species polarizes host cytokine responses toward a Th-2 profile, while suppressing production of pro-inflammatory Th-1 cytokines. In addition, we have shown that *in vitro* lymphocyte proliferative responses are affected by previous exposure of the host to *I. scapularis* infestation. Changes in *in vitro* lymphocyte responses have also been reported for infestation of inbred mice with *I. ricinus* (Borsky *et al.*, 1994, Dusbabek *et al.*, 1995). However, nothing is known of how exposure to *I. pacificus* affects lymphocyte proliferative responses. For that reason, the next series of experiments was conducted to compare *in vitro* proliferative responses of lymphocytes from BALB/c and C3H/HeN mice exposed to salivary gland proteins from *I. scapularis* and *I. pacificus*. Responses of cells exposed to salivary gland proteins were then compared to responses of cells exposed to salivary proteins derived from a tick species, *D. andersoni*, which reportedly induces acquired immune responses in infested hosts.

Our results demonstrate that different patterns of suppression or enhancement of *in vitro* lymphocyte proliferative responses were observed for lymphocytes cultured with *I. scapularis*, *I. pacificus*, or *D. andersoni* salivary gland soluble proteins (SGSP). The responses of cells cultured with SGSP also differed between the mouse strains.

Additionally, Con A stimulated proliferative responses of lymphocytes from each mouse strain showed different patterns of suppression or enhancement when cultured with SGSP of the various tick species.

For cells cultured with *I. scapularis* or *I. pacificus* SGSP, similar trends in lymphocyte proliferative responses were observed for both mouse strains. *In vitro* proliferative responses of lymphocytes cultured with SGSP alone (not exposed to Con A), from both mouse strains were suppressed to the greatest degree in cells cultured with *I. pacificus* SGSP. For cells cultured with 2.0 μg/well *I. scapularis* SGSP, an increase in proliferative responses was observed when compared to cells cultured without SGSP. This was seen for both mice strains. In contrast to this enhanced response, the three lower *I. scapularis* SGSP dilutions suppressed *in vitro* proliferative responses in a similar manner as was seen for cells exposed to *I. pacificus* SGSP. These results suggest that SGSP from both *Ixodes* species affects *in vitro* proliferative responses in a similar manner.

In contrast, SGSP derived from *D. andersoni* affects *in vitro* lymphocyte proliferative responses of lymphocytes from each mouse strain differently. For example, proliferative responses of lymphocytes from BALB/c were suppressed for each dilution of *D. andersoni* SGSP. This suppression was less than that seen for cells exposed to SGSP of either *Ixodes* species. A different pattern was seen for lymphocytes from C3H/HeN mice. Responses of C3H/HeN lymphocytes, exposed to *D. andersoni* SGSP, were suppressed only for the 2.0 and 1.0 dilution; at the two lower dilutions, proliferative responses were enhanced when compared to responses of cells alone. Therefore, it appears that *D. andersoni* SGSP affects lymphocyte responses less effectively at lower

concentrations than SGSP of the two *Ixodes* species in which responses do not differ as dramatically at the various SGSP concentrations.

In contrast to responses of lymphocytes not exposed to Con A, responses of lymphocytes cultured with Con A were suppressed to a greater degree in cells cultured with *I. scapularis* SGSP than *I. pacificus* SGSP. In both mouse strains, *I. scapularis* SGSP suppressed Con A stimulated responses at each SGSP dilution. In contrast, all concentrations of *I. pacificus* inhibited Con A stimulated responses of lymphocytes from BALB/c mice, but responses were suppressed only at the two highest concentrations in lymphocytes from C3H/HeN mice.

Concanavalin A-stimulated responses of lymphocytes exposed to *D. andersoni* SGSP were similar for both mouse strains at the highest SGSP dilution. At the 1.0 μg/well concentration, responses of BALB/c lymphocytes were only slightly suppressed relative to control values, where responses of lymphocytes from C3H/HeN mice were enhanced. Finally, at the two lower concentrations, 0.5 μg/well and 0.25 μg/well, Con A-stimulated proliferative responses were enhanced compared to control cells and this enhancement of responses is greatest in cells from C3H/HeN mice.

Our results demonstrate that SGSP from each tick species affect lymphocyte proliferative responses differently. For cells not exposed to Con A, *I. pacificus* SGSP suppresses proliferative responses the greatest, followed by *I. scapularis* and then *D. andersoni* SGSP. This pattern of responses is seen in lymphocytes derived from each mouse strain. A different pattern is seen for Con A-stimulated proliferative responses. For lymphocytes exposed to Con A, *I. scapularis* SGSP suppresses proliferative responses the greatest, followed by *I. pacificus* SGSP. Interestingly, *D. andersoni* SGSP,

while suppressive at the highest concentration, appears to enhance proliferative responses at the lower SGSP concentrations. Clearly, affects of *D. andersoni* SGSP are more concentration dependent, especially at the lower concentration, than those of SGSP of the two *Ixodes* species; concentrations greater than 2.0 μg/well may prove more effective at suppressing Con A stimulated responses.

The fact that lymphocytes from each of the mouse strains respond differently, when exposed to *I. scapularis* SGSP, is not surprising given the differences in cytokine responses of the same mouse strains that were observed after repeated infestation of *I. scapularis* nymphs, during our first series of experiments. *In vitro* proliferative responses, in agreement with the cytokine results, suggest that C3H/HeN mice respond more vigorously to tick infestation, or tick-derived molecules present in SGSP, than do BALB/c mice.

To our knowledge, this is the first study to examine non-mitogen induced *in vitro* responses of lymphocytes cultured with tick salivary gland derived molecules. Other studies have examined the effects of salivary gland compounds on Con A-stimulated proliferative responses. Concanavalin A mimics the actions of antigens on T lymphocytes and stimulates T cell proliferation *in vitro* (Sharon, 1983). Saliva or salivary gland extracts from different tick species contain compounds that influence Con A-stimulated lymphocyte proliferation (Bergman *et al.*, 1995, Bergman *et al.*, 1998, Ferreira and Silva, 1999, Urioste *et al.*, 1994). For example, Con A-induced proliferative responses of splenocytes from C57BL/6 and C3H/H mice, exposed to various dilutions of *I. scapularis* saliva, were suppressed up to 70-80%, when compared to that of cells not exposed to tick saliva (Urioste *et al.*, 1994). Salivary gland compounds derived daily

from feeding *D. andersoni* females suppressed *in vitro* lymphocyte responsiveness to Con A for lymphocytes from mice (Ramachandra and Wikel, 1992) and cattle never exposed to tick infestation (Ramachandra and Wikel, 1995).

In addition to the affects seen when culturing cells in the presence of tick derived SGSP, tick feeding has been shown to affect mitogen-stimulated *in vitro* lymphocyte proliferative responses. Concanavalin A-stimulated responses of lymphocytes from *D. andersoni*-infested guinea pigs were suppressed, when compared to noninfested animals. This reduction was greater during initial infestations than during subsequent infestations (Wikel, 1982b). Lymph node cells of C3H/HeJ mice infested one and four times with adult *R. sanguineus* showed a decrease in proliferative responses to Con A when compared to cells from uninfested animals (Ferreira and Silva, 1999) while splenocytes from the same mice showed no significant change in proliferative responses, when compared to uninfested animals (Ferreira and Silva, 1999). The Con A-stimulated response of splenocytes from BALB/c mice repeatedly infested with *I. ricinus* was unchanged after a primary and secondary infestation, and then was enhanced after the third and fourth infestations (Dusbabek *et al.*, 1995). In a similar study, Borsky *et al.* (1994) reported reduced responsiveness of splenocytes to Con A after one infestation with *I. ricinus* nymphs. Responsiveness increased to levels above control value after the second infestation, and then decreased again following the third exposure (Borsky *et al.*, 1994). Finally, T cells collected from lymph nodes of *I. ricinus* infested BALB/c mice at nine days post-infestation had a suppressed *in vitro* response to Con A.

Tick saliva contains many pharmacologically active compounds (Champagne and Valenzuela, 1996, Ribeiro, 1995, Sauer *et al.*, 1996). Some of these compounds are

involved in modulating the host's response to the feeding tick (Wikel, 1996b, Wikel and Bergman, 1997). The immunomodulatory affects of tick saliva affect both innate and specific adaptive immune responses (Kopecky *et al.*, 1999). For example, saliva from *I. scapularis* blocked the alternative pathway of complement activation (Ribeiro, 1987) and inhibited the activity of anaphylatoxin (Ribeiro and Spielman, 1986). Salivary gland extracts of *D. andersoni* inhibited Con A-induced IFN-γ and IL-2 production from BALB/c lymphocytes (Ramachandra and Wikel, 1992). Salivary gland extract of *I. ricinus* markedly suppressed LPS-induced production of IFN-γ by mouse splenocytes while up regulating the production of IL-10 (Kopecky *et al.*, 1999). Finally, SGE derived from *Rhipicephalus appendiculatus* suppressed a number of cytokines (IFN-α, IFN-γ, IL-1, IL-5, IL-6, IL-7, and IL-8) produced by human leukocytes (Fuchsberger *et al.*, 1995). These results, and those of the present study, demonstrate that tick saliva, or salivary gland compounds, modulate a variety of responses involved in host immunity to tick feeding.

Because T lymphocytes are essential for maintaining the integrity of the immune system, and are critical in the host's response to the feeding tick (Wikel and Bergman, 1997), it is not surprising that ticks have evolved substances to modulate T lymphocyte responses (Wikel, 1999a). The fact that SGSP from *I. scapularis* and *I. pacificus* affect lymphocyte proliferative responses differently is not surprising, since cytokine responses elicited by infestation of mice with these species are different. Both *I. scapularis* and *I. pacificus* are competent vectors of *B. burgdorferi* (Burgdorfer *et al.*, 1985, Lane *et al.*, 1994, Lane *et al.*, 1991, Piesman, 1993). Based solely on the modulation of host cytokine profiles and non-Con A-induced lymphocyte proliferative responses, *I. pacificus*

would be predicted to be a more efficient vector of *B. burgdorferi* than *I. scapularis*. This difference is because the polarization of cytokines toward a Th-2 profile is stronger in mice infested with *I. pacificus* nymphs than mice infested with *I. scapularis* nymphs, and non-mitogen induced *in vitro* lymphocyte proliferative responses are suppressed more strongly by *I. pacificus* SGSP than *I. scapularis* SGSP. However, based on epidemiological data, *I. scapularis* is a much more important vector of *B. burgdorferi* in the United States than is *I. pacificus*. While the immunological interactions between the vector, pathogen, and host are important in understanding the dynamics of pathogen transmission, factors other than these interactions are more important in the epidemiology of Lyme borreliosis. Of key importance is the ecology of the tick-reservoir host association in Lyme borreliosis endemic areas of the United States (Burgdorfer *et al.*, 1982, Lane *et al.*, 1991, Levin *et al.*, 1999, Spielman *et al.*, 1985)

To our knowledge, this is the first study to compare *in vitro* proliferative responses of inbred mice to salivary gland products of three different ixodid ticks, and the first study to evaluate salivary gland proteins from *I. pacificus*. These findings contribute to our understanding of tick-host immunological relationships, and further our understanding of the interaction between *B. burgdorferi* and its tick vectors.

As demonstrated in this study, repeated exposures to ticks of the *I. ricinus* complex of species does not induce acquired immune responses in inbred laboratory mice. However, acquired resistance to *I. ricinus* has been reported in a natural host for this tick, the bank vole *Clethrionomys glareoulus* (Dizij and Kurtenbach, 1995). Clearly, the tick-host relationship has an impact on whether or not acquired resistance develops (Ribeiro, 1989). For example, while we and others have observed that BALB/c mice do

not develop resistance to ticks of the *I. ricinus* species complex, this mouse strain does develop resistance to *D. variabilis* infestation, expressing it during the third and fourth infestations (DenHollander and Allen, 1985). Therefore, to determine whether inbred laboratory mice develop resistance to infestation by a species of tick not a member of the *I. ricinus* species complex, the final experiment of this study were conducted to evaluate tick biology parameters of mice infested with *D. andersoni* nymphs, to determine if acquired resistance developed in either of these strains of mice.

Changes in tick biology parameters occurred in both mouse strains following each infestation with *D. andersoni* nymphs. These changes are most pronounced when examining the mean number of ticks recovered per mouse after each infestation. The mean number of ticks recovered declined steadily following the initial infestation and was significantly less following each infestation of C3H/HeN mice, and three of the four infestations of the BALB/c mice. This suggests that fewer ticks were able to successfully attach and feed after each subsequent infestation. However, those ticks that did successfully attach were able to feed and molt successfully as demonstrated by comparing the mean weights and percentage molt of ticks recovered after each infestation. Mean weight (± S.E.) did not drop consistently after each infestation, and was only significantly decreased from the first infestation following the second infestation in each mouse strain (Table 17). However, the variability of the mean weight increased following each infestation, suggesting that ticks in each subsequent infestation did not feed equally well. Similarly, the viability (percentage molt) of ticks that successfully fed did not drop consistently following each successive infestation, and was only significantly different between infestations for BALB/c mice (Table 17). Finally,

the mean feeding duration observed following the second infestation was significantly greater than that of the first infestation for both mouse strains. After the second infestation, this difference was significantly different from the initial infestation only following the third infestation of the BALB/c mice (Table 17).

These results suggest that acquired resistance developed in both mouse strains following the initial infestation with *D. andersoni* nymphs. This resistance apparently affected the ability of the ticks to attach to mice that were previously infested, as our observations were that the ticks were viable and continued to crawl within the capsule for a few days after applied. Thus, ticks applied to previously tick-exposed mice either refused to, or were unable to, attach to the host, which explains the successive decrease in the number of ticks recovered after each infestation.

As discussed previously, host acquired resistance to tick infestation is expressed by reduced engorgement weights, longer feeding periods, decreased egg production, and death of feeding ticks (Wikel and Bergman, 1997). While we did not see a consistent decrease in mean engorgement weights following each infestation, the variability of mean engorgement weight increased after each infestation, suggesting that not all ticks from each infestation fed equally well. We did observe increased feeding times after the initial infestation, further contributing to our conclusion that acquired resistance to *D. andersoni* developed in both mouse strains.

As mentioned in the preceding sections, the development of acquired resistance varies depending on the tick-host association. For example, rabbits become progressively resistant when infested repeatedly with adult *I. ricinus* (Schorderet and Brossard, 1993), while, as we have seen, mice do not develop resistance to successive infestations with

this species. Guinea pigs infested with *I. scapularis* develop resistance to successive infestations (Das *et al.*, 1998, Wikel *et al.*, 1997), but again, laboratory mice do not develop acquired resistance. A final example of the importance of the tick-host relationship in the development of acquired resistance, dogs do not develop resistance to repeated infestations with *R. sanguineus*, while guinea pigs do develop resistance to repeated exposures to this species (Ferreira and Silva, 1999).

Of interest in this study is the observation that ticks exposed to previously infested mice either would not, or could not attach and successfully feed. While ticks remained viable and walking within capsules for up to two days following application to the host, after each successive infestation, more ticks would remain inside capsules but did not feed. However, those that were able to attach did successfully feed since a consistent drop in mean engorgement weights was not seen following each subsequent infestation. Therefore, some host factor possibly interfered with the tick's ability to attach to the host, thus preventing successful feeding. Further studies should be conducted to determine what these factors are, and how they are expressed by the repeatedly infested animal.

Experiments presented here have further defined the immunological relationship between two laboratory mouse strains and the two principal Lyme borreliosis vectors in the United States. Acquired resistance to these two species, *I. scapularis* or *I. pacificus* did not develop in either mouse strain. In contrast, acquired resistance to repeated infestations of a different tick species, *D. andersoni*, was evident following repeated exposures in both mouse strains.

Although acquired resistance was not evident in either BALB/c or C3H/HeN mice following repeated infestations with either *I. scapularis* or *I. pacificus*, changes in immune responses were observed in both mice strains. Repeated infestations with *I. scapularis* nymphs polarized lymphocyte cytokine profiles towards a Th-2 pattern, while suppressing pro-inflammatory Th-1 cytokine responses. Macrophage cytokine responses, however, were variable following repeated infestations of the mouse strains. This pattern of cytokine responses, while evident in both mouse strains, was more pronounced for C3H/HeN mice. This pattern of cytokine responses was also seen in C3H/HeN mice following infestation with *I. pacificus* nymphs; infestation with *I. pacificus* led to more pronounced cytokine responses for all cytokines assayed, except for IFN-γ. Our results clearly indicate that infestations with these two tick species significantly alters the host cytokine response towards an anti-inflammatory Th-2 response.

In vitro lymphocyte proliferative responses were also changed following repeated infestations with *I. scapularis* nymphs. While mitogen-stimulated responses were unaffected, responses of lymphocytes from previously infested mice to *I. scapularis* salivary gland proteins was significantly different than responses of lymphocytes collected from uninfested control animals. Finally, *in vitro* proliferative responses of lymphocytes from BALB/c and C3H/HeN mice were assessed for lymphocytes exposed to salivary gland proteins from *I. scapularis*, *I. pacificus*, and *D. andersoni*. Results suggested that lymphocytes from each mouse strain respond differently to these proteins. *In vitro* proliferative responses of lymphocytes cultured with *I. pacificus* proteins were suppressed to a greater degree than responses of cells cultured with salivary proteins of

the other species. In contrast, Con A-stimulated *in vitro* proliferative responses were suppressed more by *I. scapularis* salivary proteins than by those of the other species.

Findings of this study contribute to our understanding of the host-vector-pathogen relationships of Lyme borreliosis and potentially to other tick-borne diseases. Understanding the immunological responses induced by closely related vector species may aid in discovering the tick factors that modulate host immune responses, which assist the tick in both successful feeding and transmission of tick-borne pathogens. Understanding the immunological responses induced by various tick species may aid in discovering the tick factors that modulate host immune responses, which assist the tick in both successful feeding and transmission of tick-borne pathogens

An effective strategy for preventing disease caused by tick-borne pathogens could be based upon inhibiting those tick factors that modulate host immunity. Such a vaccine could circumvent the need to develop vaccines against each individual tick-transmitted pathogen. Further research should focus on identifying the salivary gland produced molecules that are responsible for modulating host immune responses directed against infestations with these, and other tick species.

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APPENDICES

APPENDIX I

Buffers and Media Compositions

Phosphate Buffered Saline (PBS, 0.15 M, pH 7.2)

1. Dissolve the following in 900 ml of distilled water.

Potassium phosphate (monobasic KH_2PO_4)	0.20 g
Sodium phosphate (dibasic, Na_2HPO_4)	1.16 g
Sodium chloride (NaCl)	8.00 g
Potassium Chloride (KCl)	0.20 g

2. Bring the final volume to one liter. Adjust pH to 7.2.

Rosewell Park Memorial Institute (RPMI) 1640 media with L-glutamine

1. Dissolve one packet of RPMI 1640 medium (with 200 mM L-glutamine) powder (Life Technologies Inc., Gaithersburg, MD) in 900-ml triple distilled water in a 2000-ml flask. Place a Teflon coated stir bar at the bottom of the flask and place on a magnetic stirrer.
2. Add 2.0 g of sodium bicarbonate ($NaHCO_3$).
3. Stir all components until completely dissolved.
4. Adjust pH to 7.1 with 1 N HCl.
5. Bring the total volume to one liter.
6. Filter sterilize using 0.22 μm filter with bell fitting (Millipore, Bedford, MA).

APPENDIX II

Enzyme-Linked Immunoabsorbent Assay (ELISA) reagents and recipes.

Sodium Carbonate-Bicarbonate Coating Solution (0.1 M, pH 9.5)

1. Dissolve the following in 100 ml-distilled water.
   Sodium bicarbonate (NaHCO3) 0.840 g
   Sodium carbonate (Na2CO3) 0.356 g
2. Adjust pH to 9.5, if necessary.

Sodium Carbonate-Bicarbonate Coating Solution (0.05 M, pH 9.6)

1. Dissolve the following in 100 ml-distilled water.
   Sodium bicarbonate (NaHCO3) 0.293 g
   Sodium carbonate (Na2CO3) 0.159 g
2. Adjust pH to 9.6, if necessary.

Sodium Phosphate Coating Solution (0.1 M, pH 6.0)

1. Dissolve the following in 100 ml-distilled water.
   Sodium phosphate (dibasic, Na_2HPO_4) 1.42 g
2. Adjust pH to 6.0 with 6N HCl.

3% Bovine Serum Albumin Assay Diluent

1. Dissolve the following in 100 ml-phosphate buffered saline.
   Immunoassay-grade Bovine Serum Albumin
   (Fluka Chemie AG, Switzerland) 3.00 g
2. pH should be at 7.0. Use within 30 days of preparation.

4% Bovine Serum Albumin Assay Diluent

1. Dissolve the following in 100 ml-phosphate buffered saline.
   Bovine Serum Albumin (Fluka Chemie AG, Switzerland) 4.00 g
2. pH should be at 7.0. Use within 30 days of preparation.

10% Fetal Bovine Serum Assay Diluent

1. Add 10 ml of sterile, heat inactivated (56°C for 30 minutes) fetal bovine serum (Hyclone Laboratories, Logan UT) to 100 ml-phosphate buffered saline.
2. Adjust pH to 7.0, if necessary. Use within 30 days of preparation.

Phosphate Buffered Saline/0.05% Tween-20 Wash Buffer

1. Add 500 µl of Tween-20 (polyoxyethylenesorbitan monolaurate, Sigma, St. Louis,

MO) to 1000 ml of phosphate buffered saline in a 2000 ml flask.
2. Place a Teflon coated stir bar at the bottom of the flask and place on a magnetic stirrer.
3. Stir solution for approximately 1 hour to ensure complete incorporation of Tween-20 into PBS.

50 mM TRIS Wash Buffer

1. Add the following to 1000 ml of distilled water in a 2000 ml flask.

Tris base	6.06 g
Tween-20	2.00 ml

2. Place a Teflon coated stir bar onto the bottom of the flask and place on a magnetic stirrer. Stir for approximately one hour to ensure all components have dissolved.
3. Adjust pH to 7.9 - 8.1 with 1N NaOH or 1N HCl.

ABTS Substrate Solution.

1. Prepare 500 ml of phosphate citrate buffer.
   a. Add the following to 500 ml of distilled water.

Citric acid (monohydrate)	10.5 g
Sodium phosphate (dibasic, Na_2HPO_4)	14.2 g

   b. Adjust pH to 5.0.
2. Add 150 mg of 2, 2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium (Sigma, St. Louis, MO) to 500-ml phosphate-citrate buffer.
3. Aliquot 11 ml into individual vials. Store at –20°C until use.
4. Within 20 minutes of use, thaw ABTS substrate solution. Add 10 µl of 30% hydrogen peroxide (H_2O_2) per 11-ml aliquot and vortex. Dispense 100 µl per well.

TMB Substrate Solution.

1. TMB (tetramethylbenzene) substrate solutions A and B (PharMingen, San Diego, CA). Allow solutions to come to room temperature prior to use.
2. Mix equal volumes of substrate solution A (H_2O_2 in a buffered solution) with solution B (aqueous solution of methanol and dimethyl sulfoxide) into a glass container and vortex.
3. Add 100 µl of substrate solution into each well.

2 N Sulfuric Acid Stop Solution.

1. Add 5.55 ml H_2SO_4 to 99.45 ml-distilled water.
2. Add 50 µl of stop solution into each well.

0.18 N Sulfuric Acid Stop Solution.

1. Add 0.98 ml H_2SO_4 to 99.02 ml-distilled water.
2. Add 100 µl of stop solution into each well.

APPENDIX III

Enzyme-Linked Immunosorbent Assay Protocols

Modified Tumor Necrosis Factor-alpha (TNF-α) ELISA Protocol (PharMingen, San Diego, CA)

Plate: Immunolon-2 (Dynatech Laboratories, Chantilly, VA)

1) Dilute purified anti-TNF-α capture antibody to 12 μg/ml in sodium phosphate coating solution.
2) Add 100 μl per well.
3) Seal plate and incubate at 37°C for 1 hour, then at 4°C overnight.
4) Wash ≥ 4 times with PBS/Tween.
5) Block non-specific binding by adding 200 μl assay diluent per well.
6) Seal plate and incubate at room temperature for 30 minutes.
7) Wash ≥ 3 times with PBS/Tween.
8) Add standards and samples diluted in assay diluent at 100 μl per well.
9) Seal plate and incubate overnight at 4°C.
10) Wash ≥ 4 times with PBS/Tween.
11) Dilute biotinylated anti-TNF-α detecting antibody to 3.0 μg/ml in assay diluent. Add 100 μl/well.
12) Seal plate and incubate at room temperature for 1 hour.
13) Wash ≥ 6 times with PBS/Tween.
14) Dilute streptavidin-HRP (PharMingen, San Diego, CA) conjugated enzyme 1:1000 in assay diluent. Add 100 μl per well.
15) Seal plate and incubate at room temperature for 30 minutes.
16) Wash ≥ 8 times with PBS/Tween.
17) Add 100 μl substrate solution into each well. Incubate at room temperature for color development. Read plate at 45 and 60 minutes.
18) Read OD at 405 nm.

Solutions

Binding Solution: 0.1M Na_2HPO_4 solution, pH 6.0.
Wash Buffer: PBS/Tween.
Assay Diluent: 3% BSA/PBS.
Substrate Solution: ABTS Substrate.
Standards: Concentration: 10 μg at 100 μg/ml

1. Add 95 μl of PBS/3% BSA to 5 μl aliquot (250 ng in 100 μl = 2.5 ng/μl = 2500 pg/μl)
2. Take 8 μl of 250 ng/100 μl and add to 992 μl PBS/3% BSA (8 μl = 20 ng = 20000 pg/ml)
3. Take 100 μl of 20,000 pg/ml and add 900 μl PBS/3% BSA (1:10 dilution of 20000 pg/ml) = 2000 pg/ml

4. Do doubling dilutions of 2000 pg/ml to generate samples for creation of a standard curve.

Modified Interleukin-1 beta (IL-1β) ELISA Protocol
(Endogen Inc., Woburn, MA)

Plate: Nunc Maxisorp Immunoplate (Nalge Nunc Inc., Denmark)

1) Add 100 µl/well of diluted coating antibody (dilute in Phosphate Buffered Saline, pH 7.4)
2) Cover the plate and incubate overnight at room temperature, 20-25°C.
3) Empty the plate of coating solution. Blot on paper towels or other absorbent material.
4) Add 200 µl/well of assay diluent.
5) Cover the plate and incubate 1 hour at room temperature, 20-25°C.
6) Wash the plate 3 times with wash buffer, then blot on paper towels or other absorbent material.
7) Determine the standard curve points you wish to run.
8) Make the appropriate standard dilutions in assay buffer or your culture medium.
9) Make sure your samples have been diluted to fall within the range of your standard curve.
10) Add 50 µl/well of diluted biotin-labeled detecting antibody.
11) Add 50 µl/well of diluted standards and test samples.
12) Cover the plate and incubate for 2 hours at room temperature, 20-25°C.
13) Wash the plate 3 times with wash buffer, then blot on paper towels or other absorbent material.
14) Dilute enzyme 1:1000 in assay diluent.
15) Add 100 µl/well of diluted enzyme conjugate.
16) Cover the plate and incubate for 30 minutes at room temperature, 20-25°C.
17) Wash the plate 3 times with wash buffer, then blot.
18) Add 100 µl/well of TMB Substrate Solution and incubate at room temperature, 20-25°C, for 30 minutes.
19) Stop the reaction with 100 µl/well of 0.18-M sulfuric acid Stop Solution.
20) Read the plate at 450 nm.

Solutions
Coating Buffer: PBS, pH 7.4
Assay Diluent: PBS with 4% BSA.
Wash Buffer: 50mM TRIS, 0.2% Tween-20.
Stop Solution: 0.18M H_2SO_4.
Recombinant Standards: To make standards (range 2000 pg/ml – 15.1 pg/ml):
1. Add 990 µl of PBS/3% BSA to 10 µl aliquot (100,000 pg in 1000 µl = 100 pg/ml).
2. Take 20 µl of 100 pg/ml and add to 980 µl PBS/3% BSA (20 µl = 2 ng = 2000 pg/ml).
3. Do doubling dilutions of 2000 pg/ml to generate samples for creation of a standard curve.

Modified Interleukin-2 (IL-2) ELISA Protocol
(PharMingen, San Diego, CA)

Plate: Immunolon-2 (Dynatech Laboratories, Chantilly, VA)

1) Dilute purified anti-IL-2 capture antibody to 4 μg/ml in binding solution.
2) Add 50 μl per well.
3) Incubate at 4°C overnight.
4) Wash ≥ 4 times with PBS/Tween.
5) Block non-specific binding by adding 200 μl assay diluent per well.
6) Seal plate and incubate at room temperature for 30 minutes.
7) Wash ≥ 3 times with PBS/Tween.
8) Add standards and samples diluted in assay diluent at 100 μl per well.
9) Seal plate and incubate overnight at 4°C.
10) Wash ≥ 4 times with PBS/Tween.
11) Dilute biotinylated anti-IL-2 detecting antibody to 2 μg/ml in assay diluent. Add 100 μl/well.
12) Seal plate and incubate at room temperature for 1 hour.
13) Wash ≥ 6 times with PBS/Tween.
14) Dilute strepavidin-HRP (PharMingen) conjugated enzyme 1:1000 in assay diluent. Add 100 μl per well.
15) Seal plate and incubate at room temperature for 30 minutes.
16) Wash ≥ 8 times with PBS/Tween.
17) Add 100 μl substrate solution into each well. Incubate at room temperature for color development. Read plate at 45 and 60 minutes.
18) Read OD at 405 nm.

Solutions

Binding Solution: 0.05 carbonate-bicarbonate buffer.
Wash Buffer: PBS/Tween.
Assay Diluent: 3% BSA/PBS.
Standards: Concentration: 100 μg/ml

1. Add 990 PBS/3% BSA to 10 μl tube (0.01 μg/10 μl) = 10 ng or 10,000 pg /ml
2. Take 200 μl of 10,000 pg/ml and add to 800 μl PBS/3% BSA = 2,000 pg/ml
3. Do doubling dilutions of 2000 pg/ml to generate samples for creation of a standard curve.

Modified Interleukin-4 (IL-4) ELISA Protocol
(PharMingen, San Diego, CA)

Plate: Immunolon-2 (Dynatech Laboratories, Chantilly, VA)

1) Dilute purified anti-IL-4 capture antibody to 4 μg/ml in binding solution.
2) Add 50 μl per well.
3) Incubate at 4°C overnight.
4) Wash ≥ 4 times with PBS/Tween.
5) Block non-specific binding by adding 200 μl assay diluent per well.
6) Seal plate and incubate at room temperature for 30 minutes.
7) Wash ≥ 3 times with PBS/Tween.

8) Add standards and samples diluted in assay diluent at 100 µl per well.
9) Seal plate and incubate overnight at 4°C.
10) Wash ≥ 4 times with PBS/Tween.
11) Dilute biotinylated anti-IL-4 detecting antibody to 2 µg/ml in assay diluent. Add 100 µl/well.
12) Seal plate and incubate at room temperature for 1 hour.
13) Wash ≥ 6 times with PBS/Tween.
14) Dilute strepavidin-HRP (Pharmingen) conjugated enzyme 1:1000 in assay diluent. Add 100 µl per well.
15) Seal plate and incubate at room temperature for 30 minutes.
16) Wash ≥ 8 times with PBS/Tween.
17) Add 100 µl substrate solution into each well. Incubate at room temperature for color development.
18) Read plate at 45 and 60 minutes. Read OD at 405 nm

Solutions
Binding Solution: 0.05 carbonate-bicarbonate buffer.
Wash Buffer: PBS/Tween.
Assay Diluent: 3% BSA/PBS.
Standards: Concentration: 50 µg/ml
1. Add 990 PBS/3% BSA to 10 µl tube (0.01 µg/10 µl) = 10 ng or 10,000 pg /ml
2. Take 200 µl of 10,000 pg/ml and add to 800 µl PBS/3% BSA = 2,000 pg/ml
3. Do doubling dilutions of 2000 pg/ml to generate samples for creation of a standard curve.

OPT-EIA KIT ELISA PROTOCOL
(PharMingen, San Diego, CA)

Protocol for assays for Interlekin-5, 6, and 10

Plate: Nunc Maxisorp Immunoplate (Nalge Nunc Inc., Denmark)
1) Add 100 µl/well of diluted capture antibody (dilute in coating solution, pH 9.5). Dilute 1:250 (48 µl Ab to 11.95 ml coating solution, for 1 plate).
2) Cover the plate and incubate overnight at room temperature, 20-25°C.
3) Wash 3 times with wash buffer. Blot on paper towels or other absorbent material.
4) Add 200 µl/well of assay diluent.
5) Cover the plate and incubate 1 hour at room temperature, 20-25°C.
6) Wash the plate 3 times with wash buffer, then blot on paper towels or other absorbent material.
7) Determine the standard curve points you wish to run.
8) Make the appropriate standard dilutions in assay diluent or your culture medium.
9) Add 100 µl of each standard to appropriate wells.
10) Cover plate and incubate at room temperature (20-25°C) for 2 hours.
11) Wash the plate 5 times with wash buffer, then blot on paper towels or other absorbent material.
12) Add 100 µl of working detector to each well.

13) Cover the plate and incubate for 1 hour at room temperature, 20-25°C.
14) Wash the plate 7 times with wash buffer, then blot on paper towels or other absorbent material.
15) Add 100 µl/well of TMB Substrate Solution and incubate (without plate cover) at room temperature, 20-25°C, for 30 minutes, in the dark.
16) Stop the reaction with 50 µl/well of stop solution.
17) Read the plate at 450 nm, 30 minutes after the addition of stop solution.

Solutions
Coating Solution: 0.1 M carbonate-bicarbonate, pH 9.5.
Assay Diluent: PBS with 10% FBS, pH 7.0.
Wash Buffer: PBS with 0.05% Tween-20.
Stop Solution: 2 N sulfuric acid (H_2SO_4).
Working Detector: Dilute biotinylated detection Ab 1:250 (48 µl in 11.90 ml) in assay diluent. Within 15 minutes prior to use, add avidin-HRP reagent to diluted detection Ab (1:250 or 48 µl in 11.95 ml).
Standards: To make standards (range 1000 pg/ml – 15.1 pg/ml):
Add 20 µl reconstituted standard to 2,980 µl assay diluent (= 1000 pg/ml). Add 500 µl 1000 pg/ml to 500 µl assay diluent (= 500 pg/ml). Continue doubling dilutions down to 15.6 pg/ml.
Reconstitution of lyophilized standard: Reconstitute each vial with 1.0 ml deionized H_2O. Allow the standard to equilibrate for at least 15 minutes before making dilutions. After reconstitution, aliquot standard stock at 50 µl/vial and freeze at <-70°C.

OPT-EIA Kit for Interferon-gamma (IFN-γ)
(PharMingen, San Diego, CA)

Plate: Nunc Maxisorp Immunoplate (Nalge Nunc Inc., Denmark)
1) Add 100 µl/well of diluted capture antibody (dilute in coating solution, pH 9.5). Dilute 1:250 (48 µl Ab to 11.95 ml coating solution, for 1 plate).
2) Cover the plate and incubate overnight at room temperature, 20-25°C.
3) Wash 3 times with wash buffer. Blot on paper towels or other absorbent material.
4) Add 200 µl/well of assay diluent.
5) Cover the plate and incubate 1 hour at room temperature, 20-25°C.
6) Wash the plate 3 times with wash buffer, then blot on paper towels or other absorbent material.
7) Determine the standard curve points you wish to run.
8) Make the appropriate standard dilutions in assay diluent or your culture medium.
9) Add 50 µl of each standard or sample to appropriate wells.
10) Add 50 µl of working detector to each well.
11) Cover the plate and incubate for 2 hours at room temperature, 20-25°C.
12) Wash the plate 5 times with wash buffer, then blot on paper towels or other absorbent material.
13) Add 100 µl of enzyme reagent (Avidin-HRP) to each well.

14) Seal plate and incubate for 30 minutes at room temperature, 20-25°C.
15) Wash the plate 10 times with Wash Buffer, then blot on paper towels or other absorbent material.
16) Add 100 µl/well of TMB Substrate Solution and incubate (without plate cover) at room temperature, 20-25°C, for 30 minutes, in the dark.
17) Stop the reaction with 50 µl/well of stop solution.
18) Read the plate at 450 nm, 30 minutes after the addition of stop solution.

Solutions

Coating Solution: 0.1 M carbonate-bicarbonate, pH 9.5.
Assay Diluent: PBS with 10% FBS, pH 7.0.
Wash Buffer: PBS with 0.05% Tween-20.
Stop Solution: 2 N H_2SO_4.
Working Detector: Dilute biotinylated detection Ab 1:125 in assay diluent.
Enzyme Reagent: Dilute enzyme reagent (Avidin-HRP)1:250 in assay diluent.
Standards: To make standards (range 2000 pg/ml – 15.1 pg/ml):
Add 20 µl reconstituted standard to 1,630 µl assay diluent (= 2000 pg/ml). Add 500 µl 2000 pg/ml to 500 µl assay diluent (= 1000 pg/ml). Continue doubling dilutions down to 15.6 pg/ml generate samples for creation of a standard curve.
Reconstitution of lyophilized standard: Reconstitute each vial with 1.0 ml deionized H_2O. Allow the standard to equilibrate for at least 15 minutes before making dilutions. After reconstitution, aliquot standard stock at 50 µl/vial and freeze at <-70°C.

VITA

George Bernard Schoeler

Candidate for the Degree of

Doctor of Philosophy

Thesis: MODULATION OF THE HOST IMMUNE RESPONSE BY *IXODES SCAPULARIS* OR *IXODES PACIFICUS*, NORTH AMERICAN VECTORS OF LYME BORRELIOSIS.

Major Field: Entomology

Biographical:

Education: Received Bachelor of Science (Major: Biology) from Boise State University, Boise, Idaho in May 1989. Received Master of Science (Major: Entomology) from University of California, Berkeley, California in May 1992. Completed requirements for the Doctor of Philosophy degree at Oklahoma State University in May 2000.

Experience: Medical Entomologist, Medical Service Corps, United States Navy, February 1992 to present. Graduate Research Assistant, Department of Entomological Sciences, University of California, August 1989, to February 1992. Research Technician, United States Department of Agriculture, Agricultural Research Service, Caldwell, Idaho, September 1986 to July 1989.

Professional Memberships: Entomological Society of America, Acarological Society of America, United States Naval Institute.