KATHRYN E. LEDIN
Characterization of vertebrate host immune responses to the lone star tick *Amblyomma americanum* (Acari: Ixodidae)
(Under the direction of DONALD E. CHAMPAGNE)

*Amblyomma americanum*, the lone star tick, is common throughout the southeastern United States and is known or suspected to vector the emerging pathogens *Ehrlichia chaffeensis*, *E. ewingii*, and *Borrelia lonestari*. In order to address the deficit of literature on immunomodulation of vertebrate hosts by *A. americanum*, we developed a murine model *of A. americanum* infestation and compared various immune parameters of infested and control animals.

We found that infestation with nymphal *A. americanum* caused reductions in the proportions of $CD^+$T cells and $CD8^+$ T cells in the spleens, lymph nodes, and tick feeding lesions in mice, but no significant changes in T cell proliferation to mitogen or antigen. Infestation consistently increased the proportions of Type 2 cytokine positive cells and cytokine secretion and reduced IL-$2^+$ cells and IL-2 secretion in mitogen and antigen-stimulated spleen and lymph node cell cultures and cells taken directly from the bite site. In most experiments, infestation increased proportions of IFN$\gamma^+$ cells and/or secretion of IFN$\gamma$ in antigen- or mitogen-stimulated or directly assayed cells, which did not fit the Type 2 cytokine polarization model observed in other tick infestation studies. In infested mice, B cell populations were either unaffected or decreased in spleens and increased in lymph nodes, and levels of non-specific serum immunoglobulins ($IgG_1$ and $IgG_{2a}$) were decreased. Although some tick-induced changes were apparent on day 4, when ticks had just begun rapid engorgement, the greatest modulation of responses of infested mice was measured on day 7, when ticks had just finished feeding, and day 10, after all ticks had detached.

The research presented in this dissertation is the first mouse model of *A. americanum* infestation, the first description of lymphocyte immunomodulation by *A. americanum*, the first study to measure tick modulation of antigen-specific changes using a transgenic host, the first study of tick immunomodulation by flow cytometry of intracellular cytokine production, and the first study to use flow cytometry to describe cellular responses at the tick bite site.

INDEX WORDS: *Amblyomma americanum*, Ixodidae, Tick, Immunomodulation, Infestation, T cell, Cytokine

# CHARACTERIZATION OF VERTEBRATE HOST IMMUNE RESPONSES TO THE LONE STAR TICK *AMBLYOMMA AMERICANUM* (ACARI: IXODIDAE)

by

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# DEDICATION

This dissertation is dedicated to my husband, who knows how to make me laugh.

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# CHAPTER 1

## INTRODUCTION AND LITERATURE REVIEW

*Amblyomma americanum* (L., 1758), the lone star tick, is common throughout the southeastern United States from central Texas north to Missouri and east to the Atlantic coast (Hair & Bowman 1986). This species has high population densities and a broad host range; in some areas, more than 90% of ticks found infesting mammals and birds are *A. americanum* (Clymer *et al*. 1970). All stages of the *A. americanum* life cycle are known to parasitize larger hosts, including humans (Sonenshine 1979). *Amblyomma americanum* is known or thought to be the principal vector of *Ehrlichia chaffeensis*, the agent of human monocytic ehrlichiosis, *Ehrlichia ewingii*, the agent of canine granulocytic ehrlichiosis, and *Borrelia lonestari*, the agent of southern tick-associated rash illness (Anderson *et al*. 1993, Murphy *et al*. 1998, Burkot *et al*. 2001). Although *A. americanum* is an important vector of these emerging human pathogens, one of the tick species most likely to parasitize humans, and an important pest of livestock and wildlife (Hair & Bowman 1986, Stromdahl *et al*. 2001), deficiencies in our understanding of this species remain. Although a significant amount of research was done on host resistance to *A. americanum* in earlier decades (Brown & Knapp 1980, Jaworski *et al*. 1990), little is known of the effects of infestation on specific cell populations and signaling molecules involved in the immune response.

Studies of tick vectors other than *A. americanum* have looked at the effects of infestation, salivary gland extracts, and saliva on host responses *in vivo* and *in vitro*. The

assays used in nearly all of these experiments measured two basic cellular responses: cell proliferation and cytokine production. Over two decades of research, a large body of evidence for systemic, non-specific T cell inhibition by tick feeding or salivary products has accumulated. However, many basic questions still remain about the roles of various cell populations and the identity of the cytokine producing cells. Also, there is almost no information on the local effects of infestation at the site of tick attachment in the skin or the effects of tick infestation on antigen-specific responses, both of which might influence host susceptibility to tick-borne pathogens. The research presented in this dissertation attempts to answer some of these questions, advance our understanding of the interaction of *A. americanum* with its hosts, and suggest directions for future studies in the field of tick-host interaction.

## Tick biology and feeding

### Classification

Ticks and mites are members of Order Acari in the Class Arachnida, a class that also includes spiders and scorpions. Ticks are in the Suborder Ixodida (Metastigmata), and can be characterized as obligate blood-sucking, large bodied, parasitiform members of Acari which have the unique features of a spiracular plate between the third and fourth coxae, a dentate hypostome, and a sensory organ on the first leg tarsi (Sonenshine 1991). There are approximately 840 described species of ticks which are divided into 19 genera belonging to 3 families: Ixodidae, the hard ticks (13 genera, 670 species); Argasidae, the soft ticks (5 genera, 169 species); and Nuttalliellidae, a monotypic family (1 genus, 1 species) (Keirans *et al.* 1992). The hard ticks are divided into two groups: Prostriata, composed of the genus *Ixodes* only, which can mate on or off the host, and Metastriata, containing all other genera, which only mate after the female has attached to the host

(Sonenshine 1991). In North America, 13 species of hard ticks are responsible for the majority of tick bites and tick-transmitted illnesses to humans, companion animals, and livestock. In addition to the direct injury, blood loss, and irritation caused by tick feeding, these North American ixodids vector the bacterial, rickettsial, viral, and protozoan pathogens causing Lyme disease, Rocky Mountain spotted fever, monocytic, granulocytic, and other ehrlichioses, tularemia, Powassan virus, and babesiosis (Sonenshine 1993).

***Amblyomma americanum***

In the genus *Amblyomma*, there are approximately 100 described species, all of which are 3-host ticks (the larvae, nymphs, and adults feed on three individual hosts, which may or may not be of the same species). *Amblyomma* species are found in tropical and subtropical areas of both hemispheres, although the largest number of species is found in South America (Keirans, 1992). According to morphology and 18S rDNA sequences, *Amblyomma* are monophyletic (Sonenshine 1991, Black *et al.* 1997), but there is still some controversy over 16S rDNA sequence results (Black & Piesman 1994). *Amblyomma americanum* (L.) was the first tick described in the territory that became the United States, by Thomas Salmon in 1754 (Hair & Bowman 1986). *A. americanum* is known to prefer warm and humid habitats (Hair & Bowman 1986, Jackson *et al.* 1996), therefore its typical range is from the central states (Missouri, Oklahoma) south to Texas, and east from Florida to the mid-Atlantic states, although it is often recorded as far north as New England, and its range undoubtedly extends into Mexico (Bishopp & Trembley 1945, Anderson & Magnarelli 1980, Ginsberg *et al.* 1991). Seasonal distribution of *A. americanum* finds the three stages to be active over the warmer months, with a one to two

year life cycle (Hair and Bowman 1986). Larvae hatch in mid summer and are active until mid fall, usually feeding and then overwintering as nymphs. The nymphs become active in early spring and continue host-seeking until mid fall. Nymphs feeding in the spring can molt to adult stage and reproduce by mid summer, whereas nymphs feeding later can overwinter to begin activity as adults the following spring (Hair and Bowman 1986, Jackson *et al.* 1996). This species is infamous for its broad host range, which includes most large animals for all stages of tick and many small mammals and birds for the immature ticks (Cooley & Kohls 1944). Preferred hosts for all stages are bovines and deer (Bishopp & Trembley 1945); at times, tick infestations can become heavy enough to disfigure and kill deer fawns (Sonenshine 1979).

**Ixodid life cycle**

Although all ticks are hematophagous ectoparasites, the digestive and reproductive physiology of the tick families is very different. Soft ticks are short-term feeders that take 5-30 minute blood meals and then retreat to their host's nest. Hard ticks attach to the body of their host and require several days or weeks to complete feeding. The hard tick life cycle is composed of three stages: larva, nymph, and adult. Each takes one large blood meal and then molts to the next stage (or in the case of the adult female, reproduces and dies). The feeding process of each stage is similar although duration and amount of blood vary according to stage and species of tick. A 3-host hard tick, such as *A. americanum*, can spend months off the host between life cycle stages, so that its life cycle lasts for one or more years, whereas a 1-host hard tick such as *B. microplus* can complete its entire life cycle, with its mouthparts attached to its host, from larva to gravid female in 21 days (Sonenshine 1991).

**Feeding**

The following information has been summarized from Coons *et al*. (1986) and Sonenshine (1991). A hard tick attaches to its host by inserting its mouthparts through the host skin and secreting cement around the feeding lesion. Hard tick mouthparts consist of external palps, serrated chelicerae, and a barbed hypostome, all attached to the cylindrical, flexible, basis capituli. During attachment, the chelicerae move horizontally to cut through the host epidermis and into the dermis, and hypostome is inserted into the resulting lesion. The palps remain spread apart on the host skin outside the lesion. Saliva flows out of the salivary ducts in the basis capituli through the feeding canal formed between the channel on the dorsal surface of the hypostome and the ventral surfaces of the cheliceral sheaths. This feeding canal is used for both blood feeding and salivation, which are alternated by raising or lowering the V-shaped pharyngeal valve. The hypostome anchors the tick into the feeding lesion initially but is followed by salivary cement within 5-30 minutes of attachment. Once the mouthparts have been inserted into host tissue, an ixodid tick will spend at least 72 hours in a slow feeding phase during which it secretes additional cementing saliva around the feeding lesion, feeds intermittently, and increases cuticle area to accommodate the impending blood meal. This preparatory phase continues until cuticle growth is completed, and is followed by a rapid feeding phase lasting 12-24 hours, during which the tick ingests the majority of the blood meal and secretes excess fluids back into the host until it has fully engorged with blood. During rapid feeding, intracellular digestion of the blood meal is reduced due to the large influx of host material, which accumulates in the ventriculus and paired caecae of the midgut. Depending on the location of the tick mouthparts and the composition of the hematoma surrounding the attachment site, the blood meal can also consist of lymph

and tissue fluid (Koch *et al.* 1974). This material will be digested over the next 3-4 weeks until molting to the next life cycle stage or oviposition occurs. The specifics of *Amblyomma americanum* feeding are as follows: the average six-legged larva feeds for 4-7 days, ingesting 1.2 µl of blood. The eight-legged nymph feeds for 4-7 days, ingesting 20 µl of blood. The adult female feeds for 8-16 days, ingesting up to 2 ml of blood and returning up to 50% of the blood meal fluid to the host as saliva *(*Sauer & Hair 1972). A 4 mg unfed female will increase to 100-300 mg during slow feeding, and then ascend to a final weight of 600-900 mg during rapid feeding in last 12-48 hours (Sauer *et al.* 1979). Like other metastriate ticks, *A. americanum* females must mate during feeding in order to enter rapid engorgement (Coons *et al.* 1986). The adult male typically ingests less than 2 µl of blood per feeding, and is the only stage that can feed multiple times, usually attaching to the host with each mating partner (Sauer & Hair 1972).

**Salivary glands**

The largest paired glands in the tick body, the salivary glands are the primary site of osmoregulation in ixodid ticks, as well as the location of the host-infecting stage of many tick-borne pathogens (Sonenshine 1991). The salivary glands are grape-like clusters composed of hundreds of acini. Each acinus has one large central cell and several smaller peripheral cells. Metastriate ticks have three types of granular acini and one type of agranular acini. Although salivary glands of unfed ticks are small and appear homogenous, during feeding they increase in size tremendously due to dramatic growth, differentiation, and new cellular activity within the acini. Type I acini are agranular and are located adjacent to the anterior and middle portions of the main salivary duct. These agranular acini are thought to function in secretion of hygroscopic solutions for active

water absorption by unfed ticks. Type II, III, and IV granular acini are found in clusters along the length of the main salivary duct and all of its branches. Type II acini have A cells that make cement, B cells which might produce paralysis toxin, and C cells which are a source of anticoagulant in at least one species (Walker & Fletcher 1987). Type III acini have D and E cells that also make cement, including the 90 kDa protein common to several Ixodid genera (Jaworski *et al.* 1990), and F cells which carry out the osmoregulatory function of the salivary glands. F cells secrete granules during the first two days of attachment, then switch to building a labyrinth of intracellular membranes for secretion of excess water and ions during blood meal concentration. F cell labyrinths are most extensive during rapid engorgement and are responsible for making the salivary glands the most important osmoregulatory organ in ixodid ticks. Type IV acini are only found in male ixodids and have G cells that secrete a product that reduces male spermatophore adhesion to facilitate transfer to the female genital pore during mating.

**Composition of saliva**

Ixodid salivary glands have thousands of acini of three types or more and increase more than 25 times in volume during feeding (Walker *et al.* 1985, McSwain *et al.* 1982). The heterogeneous composition of saliva includes pharmacoactive and immunoactive compounds, immunoreactive compounds, cement for attachment to the host, and excess fluid from the blood meal (Fawcett *et al.* 1986). Salivary glands of female and male *A. americanum* are known to change in protein and lipid composition over time (McSwain *et al.* 1982, Shipley *et al.* 1993, Sanders *et al.* 1996). Composition of secreted saliva of *A. americanum* has been correlated with physical changes in salivary gland cells; larger quantities of granular material in type II and III salivary acini and increased protein

content of saliva were observed during the same earlier stages of feeding (McSwain *et al.* 1992). *Amblyomma* salivary gland RNA synthesis, adenylate cyclase activity, cyclic nucleotide phosphodiesterase, and Na/K ATPase activity are associated with protein secretion or generation of water flow and increase with feeding (Shelby *et al.* 1987, Schramke *et al* 1984, McMullen *et al* 1983, Kaufman *et al.* 1976, Sauer *et al*. 2000). Much of the protein content of salivary glands is deposited in early feeding stages as cement, which is composed of antigenic and non-antigenic polypeptides, lipoproteins, and glycoproteins (Sonenshine 1991). In later stages of *A. americanum* feeding, the protein content of saliva and granular material in the salivary glands was markedly diminished, and salivary gland structure acquired an extensive interconnected membrane network such as that associated with fluid transport (McSwain *et al.* 1992).

Investigations into gene expression and protein and lipid composition of *A. americanum* salivary glands have provided evidence that salivation is a dynamic process. Differential gene expression over time resulted in 30 proteins of 14 to 136 kDa in early feeding (less than 100mg) and at least 8 new proteins in later feeding (>300 mg) (Oaks *et al.* 1991). Seven early salivary proteins of *A. americanum* were identified by antibodies from infested animals as immunoreactive; based on size and cross-reactivity, six were shared with *D. variabilis* and three were also shared with *I. scapularis* (Jaworski *et al.* 1989). The shared 90 and 23 kDa products may be components of attachment cement, because they were secreted during very early stages of feeding and are similar in size to putative cement components of other tick species' saliva (Jaworski *et al.* 1990).

**Pharmacological activity of tick saliva**

Like other blood feeding arthropods, ticks have a variety of pharmacologically active molecules in their saliva that are able to temporarily alter the normal host response to injury so that the tick can complete feeding (Table 1.1). Hard ticks are known to possess several different antihemostatic and vasoactive salivary factors: an apyrase, to break down ATP and ADP released by ruptured cells at the bite site and thus prevent platelet activation, a vasodilator, and a clotting inhibitor. Hard ticks also have high concentrations of prostaglandin $E_2$ ($PGE_2$) in their saliva. *Amblyomma americanum* is unusual among hard tick species and hematophagous arthropods in general for having no known salivary apyrase and very high concentrations of $PGE_2$ (Champagne 1994).

**Table 1.1.** Pharmacologically active components in the saliva of ixodid ticks.

| Genus, species | Salivary compound | Function |
|---|---|---|
| *Amblyomma americanum* | $PGE_2$ (469 ng/ml)<br>$PGF_{2\alpha}$ (>50 ng/ml)<br><br>16 kDa protein<br>$PLA_2$ 55kDa<br>Platelet-activating factor AH | Vasodilation (Ribeiro *et al.* 1992)<br>$PGE_2$ also inhibits Th1 cytokine production, activation of macrophages, neutrophils, platelets, and mast cells<br>Anticoagulant vs. factor Xa and thrombin (Zhu *et al*. 1997a)<br>Hemolytic, anti-platelet aggregation (Zhu *et al*. 1997b)<br>Anti-inflammatory, anti-platelet (Bowman, pers. comm.) |
| *Boophilus microplus* | $PGE_2$ (153 ng/ml) | Vasodilation (Dickinson *et al.* 1976, Higgs *et al.* 1976) |
| *Dermacentor andersoni* | Non-specific activator of C5 | Induces host inflammatory cell chemotactic response (Gordon & Allen 1991) |
| *Ixodes ricinus* | Protease | Anti-complement vs. C3 (Lawrie & Nuttall 2001) |
| *Ixodes scapularis* | Apyrase, $PGE_2$, anticoagulant<br>Anti-bradykinin<br>Anti-anaphylatoxic 18.5 kDa protein | Antiplatelet aggregation, vasodilation & anti-platelet aggregation, anticoagulant vs. factor X<br>Prevents pain (Ribeiro *et al.* 1985)<br>Prevents inflammation from mast cell degranulation and edema (Ribeiro & Spielman 1986), anti-complement vs. C3 (Valenzuela *et al.* 2000) |
| *Rhipicephalus appendiculatus* | Histamine-binding proteins | Suppress inflammation (Paesen *et al*. 1999) |
| *Rhipicephalus sanguineus* | Anti-histamine | Suppresses inflammation (Brossard *et al*. 1979) |

Prostaglandins are important bioactive molecules common to many animal phyla including vertebrates, arthropods and mollusks. *Boophilus microplus*, *I. scapularis*, and *A. americanum* have been shown to secrete $PGE_2$ in their saliva during feeding (Dickinson *et al.* 1976, Higgs *et al.* 1976, Ribeiro *et al.* 1985, 1992). *A. americanum* salivary prostaglandin concentrations are estimated to be 373-565 ng/ml $PGE_2$ and 63-300 ng/ml $PGF_{2a}$, higher than the $PGE_2$ concentrations reported for saliva of other tick species including *B. microplus* (100-183 ng/ml) and *I. scapularis* (61-133 ng/ml) (Dickinson *et al.* 1976, Higgs *et al.* 1976, Ribeiro *et al.* 1985, 1992).

The ixodid saliva $PGE_2$ concentrations are one or more orders of magnitude greater than $PGE_2$ concentrations measured in inflammatory exudates of vertebrate lesions; this may be related to the presumed function of tick prostaglandins as vasodilators and immunosuppressive compounds during feeding (Ribeiro *et al.* 1992), since the miniscule quantities necessary for regulating water and ion transport are produced outside of the salivary glands (Pedibhotla *et al.* 1995). The irony of prostaglandin pharmacological effects on the host is that *A. americanum* depends on the host blood meal to supply the necessary precursor, arachidonic acid, for $PGE_2$ and $PGF_{2a}$ synthesis (Bowman *et al.* 1995a, 1995b). In fact, changing the blood lipid composition of the host animal through diet alters arachidonic acid assimilation, salivary gland lipids, and saliva prostaglandin content in feeding *A. americanum* (Madden *et al.* 1996).

Control of prostaglandin production in *A. americanum* has been attributed to phospholipase $A_2$ ($PLA_2$), a pharmacologically active molecule with hemolytic, antiplatelet aggregation and anticoagulant effects (Zhu *et al.* 1997b). Zhu *et al.* associated the hemolytic activity of *A. americanum* with a 55 kDa protein that had the same molecular weight and biochemical characteristics as PLA2 (Zhu *et al.* 1997b). The

anticoagulant (affecting factor Xa and thrombin) activity of *A. americanum* saliva was associated with a different, 16 kDa protein that was not sensitive to PLA2 inhibitors (Zhu *et al.* 1997a).

## Modulation of immune responses by ticks

### Resistance

Interest in immunomodulation of vertebrate hosts by tick vectors originated in studies of tick resistance, when investigators were trying to determine how certain hosts developed resistance to tick feeding, which components of the immune system were responsible for this resistance, and how a tick circumvented resistance in order to feed on its "natural hosts" (Trager 1939). At that time, the goal was to identify the salivary components that counteracted host resistance in order to create a vaccine so that the host could recognize and disable those components (Kemp *et al.* 1986). Research on host responses was focused on inflammation at the feeding lesion and anti-tick antibodies (Bowessidjaou *et al*. 1977, Brown & Knapp 1980). Although there was evidence that cell-mediated, antibody, and complement-dependent mechanisms were all part of the tick-resistant host response (summarized in Wikel 1992), similar skin responses occurred in both resistant and susceptible animals (den Hollander & Allen 1985, Mbow *et al.* 1994a). Neither was cytokine upregulation correlated with resistance, which was increased by administration of exogenous IL-2 in rabbits (Schorderet & Brossard 1994) but not by endogenous IL-2 in BALB/c mice (Ganapamo *et al.* 1996b). Resistance was associated with genetic background in cattle (George *et al.* 1985), but not in mice (Christe *et al.* 1999). An anti-tick vaccine (Bm86) developed for use against *Boophilus microplus* infestation of cattle did not affect feeding and could not control natural tick

populations without acaricide (Dalton & Mulcahy 2001). Although components of resistance were identified, no single factor was found that would guarantee host resistance sufficient to lower tick infestations below economic thresholds (Wikel 1996b).

With the discovery of the potentiating effects of sand fly saliva on *Leishmania* infection (Titus and Ribeiro 1988), and Saliva-Activated Transmission (SAT) of Thogoto virus by *R. appendiculatus* and *A. variegatum* ticks (Jones *et al.* 1989, 1992), the focus of research on tick infestation has shifted to include infection-enhancing consequences of immunomodulation. For some tick species, a disease model is available to test how infestation affects transmission and pathogenesis, for example, *Ixodes scapularis* feeding on Lyme disease-susceptible and resistant mouse strains (Zeidner 1996; described below). Because many *Ixodes* species have rodent hosts and transmit rodent pathogens, immunomodulation by this genus has been studied more than other tick genera. Similar research on other tick genera has progressed more slowly due to challenges with host husbandry or pathogen culture. The absence of information on *A. americanum* is due to the difficulty of infesting rodent hosts and its relatively recent transition to important vector status in North America; however, immunomodulation by *Amblyomma* species from other geographical regions is also almost completely unstudied.

**Immunomodulation**

Investigators have demonstrated a variety of immunomodulatory effects of tick salivary gland extracts and tick infestation (Appendix 1). A small number of the salivary components causing these effects have been characterized (Table 1.2). Tick feeding and salivary gland extracts have been shown to suppress both innate (nonspecific) and adaptive (specific) immune responses. In general, both *in vitro* and *in vivo* studies have

found that tick feeding suppresses T cell proliferation and production of the Type 1 set of T cell cytokines, while enhancing the production of Type 2 T cell cytokines. Various effects have been reported for other immune cell types as well.

Table 1.2. Immunomodulatory components identified in tick saliva

| Genus, species | Salivary protein size | Action |
|---|---|---|
| *Amblyomma americanum* | 12.6 kDa | Inhibits macrophage migration (Jaworski *et al.* 2001) |
| *Dermacentor andersoni* | 36 kDa | Suppresses proliferation to Con A (Bergman *et al.* 2000) |
| *Dermacentor reticulatus* | Unknown | Inhibits IL-8-induced chemotaxis of granulocytes (Hajnicka *et al.* 2001) |
| *Ixodes ricinus* | 5 kDa<br>65 kDa<br>90 kDa, 94 kDa | Suppresses proliferation to Con A (Urioste *et al.* 1994)<br>Immunogenic (Ganapamo *et al.* 1997, Mejri *et al.* 2001)<br>Immunogenic, increase IL-4 secretion (Mejri *et al.* 2001) |
| *Ixodes scapularis* | unknown<br>43 kDa | Binds IL-2 (Gillespie *et al.* 2001)<br>Unknown (Zeidner *et al.* in press) |
| *Rhipicephalus sanguineus* | <3 kDa, 3-10 kDa | Suppress proliferation to Con A (Ferreira & Silva 1998) |

**Effects on innate immune responses**

Local inflammation and immune activity at the feeding lesion is a well-recognized feature of tick infestation (Mbow *et al.* 1994a, Nuttall 1998). The inflammatory response to tick attachment is similar to one that would accompany any type of skin injury: rapid infiltration of innate immune cells accompanied by increases in soluble factors promoting chemotaxis, vasodilation, and edema. Epidermal-resident cells involved in this response include keratinocytes, Langerhans cells, dendritic cells, tissue macrophages, and mast cells (Wikel 1996a). Additional immune cells migrating to the injury site include neutrophils, eosinophils, basophils, monocytes, and natural killer cells. Many of these cells are able to respond quickly because they are already present in the tissue; they are considered components of the innate response because they recognize

indicators of injury or infection, not specific antigens, and do not develop a "memory" of previous exposure. These cells can also guide the recruitment and development of subsequent, specific immune responses (Abbas *et al*. 2000).

Langerhans cells, dendritic cells, tissue macrophages, and monocytes are all phagocytic cells that process antigens and present them to T cells. These antigen-presenting cells (APCs) are now known to possess pattern recognition receptors (PRRs) that are stimulated by contact with components of bacteria and viruses, such as lipopolysaccharide (LPS), flagellin, and unmethylated DNA (reviewed in Jankovic *et al.* 2001). The APCs' responses to microbial pathogen patterns appear to influence the phenotype of the T cell they subsequently activate. Unlike tissue macrophages, activated Langerhans cells and dendritic cells can migrate to local lymphoid organs to prime the resident T cells, influencing the phenotype of a larger number of cells. Ninety percent of the epidermis is composed of keratinocytes, which can function as limited, resident APCs, presenting antigen to and secreting cytokines that influence dendritic epidermal T cells (Wikel 1996a). Strong evidence for macrophage immunomodulation by sand fly saliva and feeding has led investigators to look for similar effects of tick feeding on APCs, however, the results of tick experiments have been less conclusive. *Rhipicephalus sanguineus* saliva, *I. scapularis* saliva, *I. ricinus* salivary gland extract (SGE), and *R. appendiculatus* SGE downregulated macrophage production of nitric oxide (Urioste *et al.* 1994, Kopecky & Kuthejlová 1998, Kuthejlová *et al*. 2001, Gwakisa *et al.* 2001). *Rhipicephalus sanguineus* saliva also inhibited macrophage activation by IFNγ, but did not inhibit antigen presentation by macrophages (Ferreira & Silva 1998). Increasing numbers of monocytes (Langerhans cells, dendritic cells, or macrophages) and

neutrophils were observed in the skin of mice during their first, second, and third infestations with *I. ricinus* (Mbow *et al.* 1994a).

Neutrophils are phagocytic cells that migrate rapidly to injury sites, also have pattern recognition receptors, and can secrete a variety of pro-inflammatory cytokines (Abbas *et al.* 2000). These cells were prevented from phagocytosis and superoxide production by *Ixodes scapularis* saliva (Ribeiro 1990). Natural killer (NK) cells are lymphocytes that produce and respond to cytokines in the same way as Type 1 T cells, but are considered part of the innate immune system because they do not need specific activation or Major Histocompatibility Complex (MHC) signaling (explained below) to kill infected cells. Interferon gamma (IFNγ) production and cytotoxicity of NK cells were inhibited by salivary gland extracts from *Dermacentor reticulatus* and *I. ricinus* (Kubes *et al.* 1994, Kopecky & Kuthejlová 1998).

Mast cells, basophils, and eosinophils mediate immediate hypersensitivity reactions and release a number of pro-inflammatory factors, anti-parasitic compounds, and Type 2 cytokines (Abbas *et al*. 2000). These cells can be activated by IgE antibody to a previously seen antigen, anaphylatoxic complement components, or mediators released by injured cells. Activated mast cells and basophils release histamine immediately, which increases vasodilation, edema, and leukocyte recruitment. These cells also participate with eosinophils and neutrophils in the late phase response, 2-4 hours after the initial antigen exposure. High numbers of mast cells, basophils, and eosinophils have been observed in the skin of mice multiply infested with *Dermacentor variabilis* and *I. ricinus* (den Hollander & Allen 1985, Mbow *et al.* 1994a). Increases in eosinophils were also observed in mice infested with *Haemaphysalis longicornis* (Ushio *et al.* 1995). Overall, mice rarely became resistant to feeding by any tick species, unlike

other species of rodents and lagamorphs (Brossard *et al.* 1979, Brown & Askenase 1982, Ferreira & Silva 1998, Christe *et al.* 1999). Lack of resistance by mice could be related to their low numbers of circulating basophils, which allows *Ixodes scapularis* to feed readily even without salivary antihistamine (Ribeiro 1987).

Specialized anti-inflammatory factors in the saliva of each tick species might be adaptations to host range, because the antihistamine in *Rhipicephalus sanguineus* saliva ameliorates the consequences of basophil and mast cell activation in its larger hosts (Brossard *et al.* 1982). Histamine release was significantly upregulated in sheep repeatedly infested with *R. evertsi evertsi*, suggesting a hypersensitivity reaction although resistance to ticks was not apparent (Neitz *et al.* 1993).

In addition to cellular innate responses, vertebrate hosts have a number of soluble factors that adhere to microbial cell surfaces and label the microbes (opsonize) for destruction by phagocytic and granulocytic cells. The most significant of these soluble factors is complement, which is an important effector in both innate and acquired immunity. The components of complement not only opsonize cells and trigger immediate hypersensitivity reactions, but can also lyse cells by forming a membrane attack complex (Abbas *et al*. 2000). Saliva of several tick species has been found to have activity against portions of the complement cascade – both *Ixodes ricinus* and *I. scapularis* saliva has activity against the complement component C3 (Valenzuela *et al.* 2000), while *Dermacentor andersoni* saliva is active against C5 (Gordon & Allen 1991). The presence of these complement proteins stimulates phagocytosis by neutrophils and macrophages. Downregulation of these complement components could prevent degranulation by mast cells and basophils at the site of infestation and formation of the

membrane attack complex, which can be activated by anti-tick antibodies in the blood meal to lyse tick midgut cells during engorgement (Kemp *et al*. 1989).

**Effects on cytokines associated with innate immune responses**

One method of intercellular communication common to all cells capable of protein export is the secretion of cytokines. These soluble proteins are used extensively for communication among all cells of the innate and specialized immune systems. Cytokines produced by macrophages, keratinocytes, and other cells of the innate immune system at the injury site and dendritic cells and Langerhans cells at the secondary lymphoid organ strongly influence the response of proximal T cells (Jankovic *et al.* 2001). The primary sources of most cytokines associated with the innate response are the mononuclear phagocytes (macrophages, dendritic cells, and Langerhans cells), although NK cells, T cells, mast cells, keratinocytes, and other cell types also participate in cytokine signaling (Abbas *et al.* 2000). Mononuclear phagocytes produce a variety of cytokines in response to bacterial or viral molecules or stimulation by antigen specific cells. Initial production of the pro-inflammatory cytokines tumor necrosis factor alpha (TNF-$\alpha$) and interleukin 1 beta (IL-1$\beta$) increases inflammation at the injury site by recruiting and activating leukocytes. This is quickly followed by production of interleukin 12 (IL-12), which is important to the response against intracellular pathogens because it promotes production of interferon gamma (IFN$\gamma$), differentiation of naïve T cells to Type 1 phenotype, and cytolytic activity by T cells and NK cells. Interferon gamma from T and NK cells further enhances production of IL-12 and TNF-$\alpha$. Responses to TNF-$\alpha$ and IL-1$\beta$ are enhanced by interleukin 6 (IL-6), which promotes systemic inflammation. In addition to these pro-inflammatory, anti-intracellular

pathogen cytokines, cells associated with the innate immune response produce the anti-inflammatory cytokines interleukin 10 (IL-10) and transforming growth factor beta (TGF-β). Interleukin 10 downregulates activated macrophages by inhibiting IL-12, TNF-α, and expression of the APC costimulatory molecules necessary for T cell activation, while TGF-β suppresses proliferation and activation of macrophages, T cells, and B cells (Abbas *et al*. 2000).

Studies of various tick vectors have looked for similarities to the sand fly system, where feeding or saliva down-regulate macrophage production of the proinflammatory cytokines IL-12, TNF-α and IL-1, and upregulate production of anti-inflammatory cytokines IL-10 and TGF-β (Kamhawi 2000). Results of tick experiments have been mixed: production of IL-1β, TNF-α and IL10 by mouse lymphocytes was reduced by exposure to SGE from *D. andersoni* or *R. appendiculatus* (Ramachandra & Wikel 1992, Gwakisa *et al.* 2001) unaffected by infestation with *I. scapularis* (Schoeler *et al.* 1999), and enhanced by infestation with *I. ricinus* (Mbow *et al.* 1994c, Ganapamo *et al.* 1996a). Increased epidermal production of TNF-α and IL-1α during *I. ricinus* infestation correlated with increases in cell populations associated with the innate response (Mbow *et al.* 1994a, 1994c). *Rhipicephalus appendiculatus* SGE also suppressed IL-6 (Fuchsberger *et al.* 1995). Results of tick experiments measuring IL-12 were also mixed: IL-12 production was reduced by repeat infestations or saliva from *R. sanguineus* (Ferreira & Silva 1999), but infestation with *I. pacificus* or injection of *I. scapularis* saliva upregulated IL-12 mRNA (Zeidner *et al.* in press). Human lymphocyte production of the pro-inflammatory cytokine IL-8, which promotes chemotaxis of granulocytes and neutrophils, was reduced *in vitro* by exposure to SGE from *Amblyomma variegatum*, *Dermacentor reticulatus*, *Haemaphysalis inermis*, *I. ricinus*, and *R. appendiculatus*

(Hajnicka *et al*. 2001). Anti-inflammatory cytokines have been less studied; although IL-10 and TGF-β production were upregulated by *R. sanguineus* infestations and saliva (Ferreira & Silva 1999), IL-10 was downregulated by *R. appendiculatus* SGE. In most tick species, the production of IL-10 has only been studied in the context of T cell cytokines. In *A. americanum* a homologue of the proinflammatory cytokine Macrophage Migration Inhibitory Factor has been reported (Jaworski *et al.* 2001), but there is no other information on the effect of this species on cytokines of the innate immune system.

**Specific immune responses: T cells, B cells, and antibodies**

In addition to macrophages and other cells that have pattern recognition receptors for microbial molecules, vertebrates possess T and B lymphocytes, which have receptors that are specific to particular protein antigens. These T cell receptors (TCRs) and B cell receptors (BCRs) can recognize more than $10^9$ different antigens, a much larger and more specific repertoire than the PRRs of cells of the innate immune system (Abbas *et al.* 2000). Each T or B cell has specificity for just one protein antigen, which is advantageous for two reasons; firstly, the migrating lymphocyte will ignore all non-target antigens, and secondly, when it contacts its specific antigen, it can proliferate to create an army of identical cells to provide a very specific immune defense. This clonal expansion of B and T cells is integral to successful response by the specific immune system, and can be measured by proliferation assays.

B lymphocytes share similarities with antigen-presenting innate immune cells; in addition to their BCRs, they also possess PRRs, and can respond to a pathogen directly (Möller 1999). B cells can function as APCs; they are able to take in whole protein antigens, process them, and display them on their cell surface. What sets activated B

cells apart from other immune cells is their ability to make soluble antibodies, which are released to circulate through the body. These antibodies bind to specific antigens, labeling them for destruction or activating other responses of immune cells. B cell antibodies are specific because they are secreted BCRs; each B cell makes antibodies against only one protein antigen, although these antibodies can be made in different isotypes that correspond to the timing and location of antigen.

T lymphocytes can be separated into two main groups according to function, expression of cell surface molecules (cluster of differentiation or CD molecules), and recognition of major histocompatibility complex (MHC) molecules. In general, helper T cells express CD4 and recognize MHC II, while cytotoxic T cells express CD8 and recognize MHC I. MHC molecules are used by cells to display peptides to T cells. These peptides can be portions of self proteins or foreign antigens, and are processed differently for MHC I and MHC II display, which provide two different systems of surveillance for infectious organisms. In general, cytosolic peptides normally in the cell or from pathogens infecting the cell are displayed by MHC I, while fragments of peptides endocytosed by patrolling phagocytic cells are displayed by MHC II. Nearly all nucleated cells express MHC I, while only professional antigen-presenting cells (mononuclear phagocytes and B cells) express MHC II. $CD4^+$ helper T cells interact with MHC $II^+$ APCs to stimulate phagocytosis and killing of infected cells and antibody production. $CD8^+$ cytotoxic T cells kill infected cells displaying foreign antigen in conjunction with MHC I. Most T cells are MHC restricted; they can only respond to antigens that are bound to MHC molecules.

**Effects on Type 1 T cell cytokines: IFNγ and IL-2**

T cell cytokines were initially categorized according to the cytokine patterns prevalent in $CD4^+$ T helper cells (Th1, Th2), but it is now apparent that the same cytokine patterns are produced by $CD8^+$ cytotoxic T cells (Li *et al.* 1997). Cytokine production by a T cell indicates the properties of the cell (Type 0, 1, or 2) as well as influencing the microenvironment around it (Morel & Oriss 1998). Since T cells that have committed to Type 1 or Type 2 rarely change their commitment (Kelso 1998), ability to produce Type 1 or 2 cytokines has been used as an indicator of cell type. Type 1 T cells are essential for a protective response against intracellular infections and any other microbes that are controlled through phagocytosis. Type 2 T cells are an integral component of the defense against parasites such as helminths and arthropods. Differentiation of naïve Type 0 cells into Type 1 or Type 2 subsets now appears to be controlled by dendritic cell signaling (Jankovic *et al.* 2001). Pathogens detected by PRRs on dendritic cells stimulate secretion of IL-12 and pro-inflammatory cytokines, which drive T cells to Type 1. Lack of IL-12, plus increases in the costimulatory signals B7-1 and B7-2 (additional to binding of the main T cell receptor), appear to commit T cells to Type 2 (Jankovic *et al.* 2001). These T cell subsets can be classified by the cytokines and receptors they express: Type 1 T cells produce the cytokines IFNγ, IL-2, and TNF-α; they have a receptor for IL-12 but lose their receptor for IFNγ. Type 2 T cells produce the cytokines IL-4, IL-5 and IL-13, and lack the IL-12 receptor completely. Both subsets produce IL-10, although levels are typically higher in Type 2 T cells. Type 0 T cells are typically young cells that have not committed to Type 1 or 2; Type 0 can produce IL-2 and IL-4 but not IFNγ. The paradigm of producing Type 1 cytokines against intracellular pathogens and Type 2 cytokines against extracellular pathogens, which distinguishes between resistant and

susceptible responses to *Leishmania* (Lohoff *et al*. 1998), was applied to studies of immune modulation by ticks.

In general, exposure to tick infestation, saliva, or salivary gland extract (SGE) reduced Type 1 cytokine production in all strains of mice (Christe *et al.* 1999). The number of feeding ticks (5-45) and stage did not make a significant difference in levels of IFNγ production by cells of infested mice (Christe *et al.* 1999, Mejri *et al.* 2001). However, some investigators found that cells from BALB/c mice, but not C3H mice, upregulated levels of IFNγ and IL-2 cytokines or mRNA when infested with *I. ricinus* or *I. scapularis* (Mbow *et al.* 1994b, Ganapamo *et al.* 1995, 1996a, Zeidner *et al.* in press). These findings contrast with the stereotype of BALB/c mice as "Type 2 responders" and C3H mice as "Type 1 responders". In a few studies, investigators were able to determine the type of T cell producing cytokine; they observed only $CD4^+$ cells producing IFNγ, while mostly $CD4^+$ and a few $CD8^+$ cells produced IL-2 (Ganapamo *et al.* 1996a, Zeidner *et al* 1997).

$CD4^+$ T cells, $CD8^+$ T cells, and NK cells produce IFNγ, which is essential for control of many microbial infections because of its broad activity in promoting phagocytic killing of pathogens. IFNγ upregulates expression of MHC I and MHC II and antigen processing by APCs. It increases production of the opsonizing antibody IgG2a and activation of phagocytic cells, and contributes to endothelial changes that permit migration of immune cells from the circulation to the site of infection. Infestation with *Ixodes ricinus, I. scapularis,* or *Rhipicephalus sanguineus,* and SGE or saliva of *Dermacentor andersoni, I. ricinus, I. scapularis,* or *R. sanguineus* downregulated production of IFNγ (Ramachandra & Wikel 1992, Ganapamo *et al.* 1995, Zeidner *et al* 1997, Ferreira & Silva 1998, 1999, Christe *et al.* 1999, Kopecky *et al.* 1999, Schoeler *et*

*al.* 1999, Zeidner *et al.* in press). In contrast, infestation with *D. andersoni* did not significantly change production of IFNγ (Macaluso and Wikel 2001).

Interleukin 2 is produced by and increases proliferation of activated, antigen-specific $CD4^+$ and $CD8^+$ T cells. Interleukin 2 also upregulates production of IFNγ and IL-4, and proliferation and activation of B cells and NK cells. Infestation with *D. andersoni*, *I. scapularis* or *R. sanguineus*, and exposure of mouse cells to SGE from *D. andersoni*, *I. ricinus*, *I. scapularis*, or *R. sanguineus* downregulated production of IL-2 (Ramachandra & Wikel 1992, Urioste *et al.* 1994, Ganapamo *et al.* 1996a, Zeidner *et al* 1997, Ferreira & Silva 1998, 1999, Schoeler *et al.* 1999, Macaluso and Wikel 2001).

**Effects on Type 2 T cell cytokines: IL-4, IL-5, and IL-10**

The main function of Type 2 T cells is to direct the mast cell and eosinophil response against parasites. This response, the immediate hypersensitivity reaction, depends on IgE from antigen-specific B cells, which have switched to non-opsonizing antibody isotypes IgE and $IgG_1$ in response to IL-4 from Type 2 cells. Type 2 cells also recruit eosinophils to late phase reactions. Interleukin 4 is produced by $CD4^+$ T cells, activated mast cells, and basophils. The other role of IL-4 is perpetuation of the Type 2 response and antagonism of IFNγ action on macrophages and B cell antibody isotype switching. Interleukin 5 is produced by $CD4^+$ T cells and activated mast cells, and promotes increases in eosinophil differentiation and activation, B cell proliferation, and production of IgA antibody.

In general, infestation or SGE increased production of Type 2 cytokines in all strains of mice tested, for all stages of ticks (Christe *et al.* 1999, Mejri *et al.* 2001). Infestation with *D. andersoni*, *I. pacificus*, *I. ricinus*, *I. scapularis*, or *R. sanguineus*, or

SGE from the three *Ixodes* species, upregulated production of IL-4 and IL-10 (Ganapamo *et al.* 1995, 1996b, Christe *et al.* 1998, Zeidner *et al* 1997, Ferreira & Silva 1999, Kopecky *et al.* 1999, Schoeler *et al.* 1999, Macaluso and Wikel 2001, Mejri *et al.* 2001, Zeidner *et al.* in press). *R. sanguineus* SGE was not assayed for effect on IL-4, but upregulated IL-10 (Ferreira & Silva 1998). Feeding of the same three *Ixodes* species or SGE from *I. pacificus*, *I. scapularis*, or *R. appendiculatus* upregulated production of IL-5 (Fuchsberger *et al.* 1995, Ganapamo *et al.* 1996b). In contrast, lymphocyte production of IL-4 was not influenced by exposure to *D. andersoni* SGE (Wikel 1996b). Both $CD4^+$ and $CD8^+$ T cells were observed to produce IL-4, but only $CD4^+$ cells produced IL-10 during the first infestation, although other cell types produced IL-10 in later infestations (Ganapamo *et al.* 1996b, Zeidner *et al* 1997).

One study found that IL-4 mRNA progressively increased during primary infestation, with a rapid increase in expression at 7 days post-attachment (Mejri *et al.* 2001). Application of exogenous anti-IL-4 antibodies or use of cells from IL-4-deficient mice did not alter the Th2 response to *I. ricinus* infestation or SGE, so it is possible that the observed Type 1/Type 2 polarization is due to a saliva component that inhibits development of the Th1 response, possibly PGE2 (Christe *et al.* 1998). Again, results from several studies using BALB/c mice did not follow this trend, but instead found low production of IL-4 mRNA in this "Type 2 responder" (Mbow *et al.* 1994b, Zeidner *et al* 1997).

**Effects on cell proliferation**

After a T cell is presented with antigen by an APC, it undergoes proliferation (clonal expansion) in order to generate a large group of cells that have the same antigen

specificity. Proliferation of cells can also be studied with mitogens, which are non-specific, polyclonal activators of lymphocytes. Concanavalin A (Con A) and phytohemagglutin (PHA) stimulate T cell responses, while lipopolysaccharide (LPS) stimulates B cell responses. The ability of whole cell suspensions to proliferate in response to these mitogens can provide a preliminary assessment of the functionality of the two main sets of lymphocytes. Although tick infestation can increase numbers of B and $CD4^+$ T lymphocytes (Zeidner *et al.* 1997), nearly all studies found that infestation or exposure to tick SGE impaired lymphocytes' ability to proliferate to T cell mitogens, while responses to B-cell mitogens were unaffected or increased. These results suggested that the decreases in production of Type 1 cytokines observed during *in vivo* or *in vitro* exposure to the same ticks could be due to decreased or impaired populations of Type 1, $CD4^+$ T cells.

Lymphocyte proliferation in response to the T-cell mitogens Con A and PHA was decreased following incubation with saliva or salivary gland extracts of *D. andersoni*, *I. ricinus*, *I. scapularis*, *R. sanguineus*, and *Boophilus microplus* (Fivaz 1989, Wikel 1982, Wikel & Osburn 1982, Ramachandra & Wikel 1992, 1995, Inokuma *et al.* 1994, Urioste *et al.* 1994, Ferreira & Silva 1998, Inokuma *et al.* 1998). Infestation with any of these tick species or *Amblyomma variegatum* also impaired the proliferative response of host lymphocytes to Con A or PHA (Wikel 1982, Borsky *et al.* 1994, Koney *et al.* 1994, Ganapamo *et al.* 1996b, Ferreira & Silva 1999, Inokuma *et al.* 1998, Macaluso and Wikel 2001). Infestation with *R. sanguineus* suppressed proliferation to T-cell mitogens equally in several strains of mice, indicating that the response did not depend on host genetic background (Ferreira & Silva 1998).

Lymphocyte proliferation in response to the B cell mitogen LPS was unaffected or enhanced during infestation with *D. andersoni* or *I. ricinus*, or exposure to SGE of *D. andersoni* (Wikel 1982, Ramachandra & Wikel 1992, 1995, Ganapamo *et al.* 1996b). Suppression of proliferation to T cell mitogens and lack of effect or enhancement of proliferation to B cell mitogens have been remarkably consistent findings from studies of most species of tick vectors. Only one study found that *I. ricinus* infestation slightly increased proliferation to the T cell mitogens Con A and PHA (Dusbabek *et al.* 1995), and two studies found that *B. microplus* infestation and *I. ricinus* infestation decreased proliferation to the B cell mitogen LPS (Inokuma *et al.* 1993, Dusbabek *et al.* 1995).

Although infestation decreased T cell proliferation to mitogens, it did not completely abrogate proliferation to specific antigens. Exposure to tick SGE without mitogens increased proliferation of lymphocytes from hosts that had been infested with *D. andersoni* or *I. ricinus*, but had no effect on uninfested mice, indicating that SGE did not act as a non-specific mitogen (Wikel & Osburn 1982, Borsky *et al.* 1994, Dusbabek *et al.* 1995, Ganapamo *et al.* 1995, 1997). SGE, but not integument extract, from female *I. ricinus* stimulated lymphocytes from hosts infested with other stages of *I. ricinus*, but not hosts infested with *Amblyomma hebraeum* (Ganapamo *et al.* 1997), so the proliferative response was specific to tissue and to tick species. This experimental design did not test differences in response to specific antigens, because uninfested mice would not have a specific response to SGE antigens. Only one study, using keyhole limpet hemocyanin (KLH)-specific T cells, has examined changes in response to a specific antigen and found that *Rhipicephalus sanguineus* saliva did not inhibit KLH antigen presentation by murine splenocytes but did suppress KLH-specific T cell proliferation (Ferreira & Silva 1998)

Investigations of the relationship between cytokines and the proliferative response to SGE antigens found that *in vitro* IL-4 production was positively correlated with increases in SGE-stimulated proliferation by lymphocytes from infested mice (Ganapamo *et al.* 1995, Mejri *et al.* 2001). However, there were no correlations between lymphocyte proliferation to SGE and decline of IFNγ production or treatment with recombinant IL-2 (Schorderet & Brossard 1994, Ganapamo *et al.* 1995).

**Effects on antibody production**

B cells are stimulated to secrete antibodies by contact with microbes that correspond to their PRRs or by contact with antigen-specific $CD4^+$ helper T cells. The secreted antibodies bind to a specific antigen and label it for response by phagocytic or granulocytic cells. Unlike T cells, few B cells circulate; their residence in lymphoid organs delays B cell exposure to antigen until after T cells and cells of the innate immune system have already begun to respond. Each B cell makes antibodies against one specific protein antigen, although these antibodies can be made in different isotypes that correspond to the timing and location of antigen. The three most prevalent circulating antibody isotypes have different functions. Immunoglobulin M (IgM) is made early and has lower affinity but more binding sites; IgG is made later, but has higher affinity; and IgE is structurally specific for binding to receptors on the cells mediating immediate hypersensitivity. In addition, subclasses of IgG antibodies have different abilities to opsonize microbes and activate the complement system. For example, in mice $IgG_{2a}$ is opsonizing while $IgG_1$ is not (Morel & Oriss 1998). Cytokines can amplify B cell proliferation and selectively promote switching to different antibody isotypes and subclasses.

Typically, specific IgM antibodies are made within 5 days of exposure to the antigen, but specific IgG antibodies are not present in high quantities in the serum until 2 weeks after exposure (Abbas *et al.* 2000). In general, infested hosts had decreased primary antibody (IgM) responses, and total and specific antibody titers were decreased or unaffected, although the effect on anti-tick antibodies varied. Anti-tick antibodies are associated with host resistance (Wikel 1992), so it is in the interest of the tick to prevent their increase. Anti-tick antibodies decreased with repeated infestations of *B. microplus* and *D. andersoni* (Wikel & Osburn 1982, Inokuma *et al.* 1993), and were unchanged during infestations of *R. evertsi evertsi* (Njau *et al.* 1990). Anti-tick antibody titers increased with repeated infestations of *I. ricinus in vivo*, but decreased in response to tick antigens *in vitro* (Borsky *et al.* 1994, Dusbabek *et al.* 1995). IgM antibody to T-dependent antigens (not tick-derived) decreased during and after multiple infestations of *D. andersoni*, *R. appendiculatus*, *R. evertsi evertsi*, but stayed unchanged following infestation with *R. zambeziensis* (Wikel 1985, Fivaz 1989, Njau *et al.* 1990). The Type 2 polarization of the immune response was confirmed by detection of $IgG_1$ but not $IgG_{2a}$ specific anti-tick antibodies in BALB/c mice infested with *I. ricinus* nymphs (Christe *et al*. 2000, Mejri *et al.* 2001). There is evidence that immunoglobulins ingested by ticks enter the tick circulatory system as intact proteins and are removed by specific immunoglobulin binding proteins (IGBPs) (Wang & Nuttall 1994, Jasinskas *et al.* 2000), possibly because their presence is detrimental to the feeding ticks (Wang *et al.* 1998, Rechav & Nuttall 2000). Introduced into the host circulation through the bite site, these IGBPs might reduce immunoglobulin concentrations in the host as well.

**Effects of tick infestation or salivary gland extract on infection**

Most tick-borne pathogens are intracellular and require a Type 1 response for protective immunity (Preston & Jongejan 1999). The observations that tick infestation, salivary gland extracts, or saliva often suppress Type 1 responses and enhance Type 2 responses suggests that tick immunomodulation might facilitate pathogen transmission and establishment in the host. Experiments evaluating the effects of infestation or salivary gland extract on infections *in vitro* or *in vivo* have observed that cytokine responses are even more polarized towards Type 2 than with tick exposure alone, and exogenous reconstitution of a Type 1 environment could ameliorate infection. *Rhipicephalus sanguineus* saliva deactivated IFNγ-assisted macrophage killing of *T. cruzi* parasites in a dose-dependent manner and enhanced parasite burden in IFNγ-free cultures (Ferreira & Silva 1998). *Rhipicephalus sanguineus* saliva also significantly inhibited IFNγ-induced NO production by macrophages in a dose-dependent manner that correlated with deactivation of parasite killing (Ferreira & Silva 1998). Incubation of *R. sanguineus* saliva with *T. cruzi*-stimulated cells significantly suppressed C3H/HeJ macrophage and splenocyte production of IL-12 while significantly enhancing macrophage production of TGF-β (Ferreira & Silva 1999). *Rhipicephalus sanguineus* saliva also inhibited IFNγ production in response to splenocytes cultured with *Trypanosoma cruzi* (Ferreira & Silva 1998). *Ixodes ricinus* SGE reduced IFNγ production and enhanced IL-10 production of *Borrelia afzelii*-stimulated BALB/c splenocytes (Kopecky *et al.* 1999). As previously mentioned, *Ixodes scapularis* infestation also increased polarization of Type 1 and Type 2 cytokine responses in susceptible (C3H), but not resistant (BALB/c) mice (Zeidner *et al.* 1997). Exogenous supplementation of TNF-α, IFNγ or IL-2 at the time of infestation suppressed spirochete

transmission by *I. scapularis* (Zeidner *et al.* 1996). Neutralization of IL-4 prior to infestation decreased spirochete load in target organs (Zeidner *et al.* in press). *B. burgdorferi*-primed dendritic cells administered prior to tick infestation reversed Th2 polarization of susceptible C3H/HeJ mice (Zeidner *et al.* 1997). The exception to the Type 1/ Type 2 paradigm was the observation that *Rhipicephalus appendiculatus* salivary gland extract, Con A, PMA, specific antigen stimulation, and exogenous IL-2 all increased host lymphocyte susceptibility to invasion with *Theileria parva*; pretreatment of cells with bovine anti-IL-2R antibody prevented the increase due to IL-2, while IFNγ exposure had no effect (Shaw *et al.* 1993).

## Diseases vectored by *Amblyomma americanum*

With the exception of Lyme borreliosis, theileriosis, and anaplasmosis, vaccines are not yet available for most tick-transmitted pathogens, so disease prevention relies entirely on personal protective measures such as wearing repellant and use of acaricides on tick habitats and hosts. Contact between humans and ticks has become more frequent due to increases in tick and host populations and human development and recreation in tick habitats (Standaert *et al.* 1995). This is especially worrisome considering the implication of *A. americanum* as the known or suspected vector of three emerging diseases in the United States, human monocytic ehrlichiosis, canine granulocytic ehrlichiosis, and southern Lyme disease (Anderson *et al.* 1993, James *et al.* 2001, Paddock *et al*. 2001). Historically, *A. americanum* was considered to be one of 5 species vectoring Rocky Mountain spotted fever (RMSF) (Burgdorfer 1969, Sonenshine 1979), and was known to carry a rickettsial pathogen in Arkansas (Burgdorfer 1975). *A. americanum* was also implicated as a vector of Tularemia and Q fever, and was thought

to cause tick paralysis (Sonenshine 1979). In addition, *A. americanum* was thought to vector Lyme disease (Schulze *et al.* 1984, Ouellette *et al.* 1997) or a similar spirochetal illness because of its erythema-causing bite (Kirkland *et al.* 1997). Recent evidence refutes the role of *A. americanum* as a vector of RMSF (Burgdorfer 1998), but suggests that it may vector both rickettsial and spirochetal pathogens infecting humans.

***Ehrlichia chaffeensis***

Although *A. americanum* had long been notorious in the southeast for its aggressive host seeking and irritating bite, it became nationally famous in 1993 for its role in vectoring *Ehrlichia chaffeensis*, an emerging human pathogen. A series of outbreaks of unexplained rickettsial illnesses in 1986-1990 that were seronegative for Rocky Mountain spotted fever (RMSF) and other known human diseases but seropositive for *Ehrlichia canis*, a pathogen of dogs, attracted the attention of public health workers (Maeda *et al.* 1987, Petersen *et al.* 1989). In 1991, the causative organism was isolated from samples taken from army recruits at Fort Chaffee, Arkansas, and found to be a new species of *Ehrlichia*, subsequently named *Ehrlichia chaffeensis* (Dawson *et al.* 1991, Anderson *et al.* 1991). Soon thereafter, *Ehrlichia chaffeensis* was detected by polymerase chain reaction (PCR) in seven pools of adult *A. americanum* and one adult *Dermacentor variabilis* tick collected from the locations of *E. chaffeensis* cases (Anderson *et al.* 1992a, 1993). Additional surveys of *A. americanum* found *E. chaffeensis* infection rates of 0.9%-28.8% in ticks collected from Georgia north to Connecticut and west to Missouri (Roland *et al.* 1998, Steiner *et al.* 1999, Ijdo *et al.* 2000, Stromdahl *et al.* 2000, Whitlock *et al.* 2000). Ten and twelve-year retrospective serosurveys documented highly significant temporal and geographic correlations between

*A. americanum* infestation and *E. chaffeensis*-reactive serum antibodies in white-tailed deer in Georgia and other southeastern states as early as 1982 (Lockhart *et al.* 1995, 1996). Cell culture isolation of *E. chaffeensis* organism from white-tailed deer, and transmission of *E. chaffeensis* infection between deer by infestation with *A. americanum* additionally confirmed the role of these animals as host and vector of the pathogen (Ewing *et al.* 1995, Lockhart *et al.* 1997a, Davidson *et al.* 2001). Further corresponding to the host preference of *A. americanum, E. chaffeensis*-reactive antibodies or PCR-positive sequences were found in raccoons, opossums, and coyotes, but not rodents (Lockhart *et al.* 1997b, Lockhart *et al.* 1998, Comer *et al.* 2000, Kocan *et al.* 2000).

***Ehrlichia ewingii***

Another new *Ehrlichia, E. ewingii,* which causes canine granulocytic ehrlichiosis, is apparently vectored by *A. americanum* and possibly several other species of human-biting ticks (Anderson *et al.* 1992b, Murphy *et al.* 1998). This pathogen has been reported to infect immunocompromised humans. Its true prevalence in ehrlichiosis patients may be concealed by similarity of symptoms and cross-reactivity in clinical assays designed for other *Ehrlichia* species (Buller *et al.* 1999, Carpenter *et al.* 1999, Paddock *et al.* 2001). The ability of *A. americanum* to carry this pathogen and the spirochete discussed below raises the possibility of this vector simultaneously transmitting multiple infections to its hosts.

***Borrelia lonestari***

After more than two decades of reports of an erythema chronicum migrans-like rash following the bite of *A. americanum* from regions where Lyme disease is rare

(Patterson *et al.* 1979, Schulze *et al.* 1984, Campbell *et al.* 1995, Kirkland *et al.* 1997), researchers have finally been able to associate *A. americanum* infestation with the transmission of a spirochete to a human patient (James *et al.* 2001). Numerous collections of *Amblyomma americanum* ticks throughout their normal distribution had been found infected with spirochetes that were not conclusively identifiable as *Borrelia burgdorferi* (summarized in Stromdahl *et al.* 2001). Attempts to isolate the spirochete were unsuccessful, but a PCR assay was developed to identify infected ticks (Barbour *et al.* 1996). Isolation and sequencing of DNA from the *A. americanum* spirochete revealed it to be a new species, *Borrelia lonestari*, possibly related to the agent of bovine borreliosis, *Borrelia theileri* (Barbour *et al.* 1996, Rich *et al.* 2001). In 1999, *Borrelia lonestari* DNA sequences were successfully amplified from an attached *A. americanum* female and its bite site on a Lyme-disease negative patient with symptoms of erythema, providing evidence of the association of this vector, pathogen, and human disease (James *et al.* 2001).

## Objectives of the dissertation

The research presented in this dissertation attempts to advance our understanding of the way that infestation with *A. americanum* affects host immune responses. The interaction between *A. americanum* and its hosts is a dynamic process that develops over time after initial attachment. Because *A. americanum* salivary glands change in volume, secretion rate, lipid content, protein content and composition of secreted saliva over time (McSwain *et al.* 1982, McSwain *et al.* 1992, Shipley *et al.* 1993, Sanders *et al.* 1996), we chose to measure the effect of *in vivo* infestation for our experiments, using BALB/c mice as our model host. In order to assess changes in the host during this prolonged

interaction, we designed our experiments to examine host responses at four time points, two during infestation and two shortly after all ticks had detached. Although mice are infrequent natural hosts of *A. americanum* nymphs, we chose to use BALB/c mice because they are common models for tick infestation studies and are thought to approximate the human response to other blood-feeding vectors (Chen *et al.* 1998).

Our first objective for this study was to characterize the effects of *A. americanum* infestation on mitogen-stimulated systemic and local cellular immune responses by looking at cell proliferation and cytokine production by cells from the spleens and lymph nodes of infested and control mice. Based on those results, our second objective was to identify these immune cells and specifically the cytokine-producing cells that were affected by infestation. Our third objective was to determine whether similar changes in immune responses took place in the skin at the site of tick attachment, also by quantifying cell populations and cytokine expression. Our fourth and final objective was to measure infestation-induced changes in host responses to a surrogate antigen as a model for host responses to a pathogen transmitted by feeding ticks.

# CHAPTER 2

# CHANGES IN PROLIFERATIVE, CYTOKINE, AND ANTIBODY RESPONSES OF BALB/c MICE INFESTED WITH *AMBLYOMMA AMERICANUM* [1]

[1] Ledin, K.E., S. Singh, and D.E. Champagne. To be submitted to *Journal of Medical Entomology*.

## Abstract

Successful infestation of BALB/c mice with nymphal *Amblyomma americanum* made it possible to study host responses to *A. americanum* feeding. Proliferative and cytokine responses of murine splenocytes were compared between groups of *A. americanum*-infested and uninfested mice. Splenocyte proliferation in response to the B cell mitogen LPS was suppressed by more than 60% in infested mice, but their response to the T cell mitogen Con A was not affected. Cytokine levels in infested animals were clearly polarized towards a Type 2 response. Type 1 cytokine production was suppressed by as much as 59% (IFNγ) and 87% (IL-2) in infested mice. Interferon gamma (IFNγ) production by splenocytes of infested mice was suppressed on days 4 and 7 post-infestation, and interleukin-2 (IL-2) production by lymphocytes from the spleen and lymph nodes of infested mice was suppressed on all days measured. In contrast, on days 7, 10, and 14 post-infestation, infested mice produced 4 to 124-fold more than the control levels of the Type 2 cytokines IL-4, IL-10, and IL-13. Systemic (spleen) and local (draining lymph nodes) responses were similar for all cytokines except IFNγ. Immunoglobulin antibody subtype ratios were also polarized to Type 2. Adherent cell production of the cytokines IL-12, IL-6, TNF-α, and IL-10 was also measured and found to be not significantly different between infested and control mice.


## Introduction

Hematophagous arthropods are known to affect their mammalian hosts' physiological responses to blood feeding in a variety of ways. The pharmacological effects of tick infestation on vasodilation, platelet activity, and clotting responses have been described for several important tick vectors, as have the immunological effects on

leukocyte proliferation and cytokine production (reviewed in Ribeiro 1995, Wikel 1996b).

Infestation and *in vitro* studies to evaluate immunomodulation have been performed on at least eleven important Ixodid tick vectors: *Rhipicephalus appendiculatus, R. evertsi, R. sanguineus, Ixodes pacificus, I. ricinus, I. scapularis, Haemaphysalis longicornis, Dermacentor andersoni, D. reticulatus, Boophilus microplus,* and *Amblyomma variegatum* (Inokuma *et al.* 1998, Neitz *et al.* 1993, Ushio *et al.* 1995, Wikel 1996b, 1999). In general, leukocytes from mammals infested with ticks exhibit decreased proliferation in response to T cell mitogens, lower levels of Type 1 cytokines, and higher levels of Type 2 cytokines. *In vitro* exposure of mammalian lymphocytes to tick salivary gland extracts produces a similar polarization of Type 1/Type 2 responses. Evidence suggests that such a polarization of responses might make a host more susceptible to tick-borne pathogens (Zeidner *et al.* 1996, 1997). However, *Amblyomma americanum,* one of the most common human-biting ticks in the American Southeast, and the known or suspected vector of *Ehrlichia chaffeensis, E. ewingii*, and *Borrelia lonestari* (Anderson *et al*. 1993, Murphy *et al*. 1998, Burkot *et al*. 2001), has not been examined for its ability to affect host immune responses.

We developed a murine model of *A. americanum* infestation, and tested the effects of infestation on mouse splenocyte responses *ex vivo*. We evaluated the effects of *A. americanum* infestation on lymphocyte proliferation, cytokine production, antibody isotype ratio, and adherent cell cytokines. In order to compare the local and systemic effects of tick feeding, we looked at cytokine production by lymphocytes taken from the lymph nodes draining the bite site and from the spleen. Although mice are infrequent natural hosts of *A. americanum* nymphs, the inbred BALB/c mouse strain has been

shown to be a good model of the human response to other blood-feeding vectors (Chen *et al.* 1998), and has often been used to measure the effects of tick infestation and tick salivary gland extracts, so results of this study can be compared to data in the literature available on other tick species.

## Materials and Methods

### Mice

Eight week old female BALB/c mice (Jackson Laboratories, Bar Harbor, Maine) were provided with food and water *ad libitum* and housed at 22°C in the Animal Resource Facility of the College of Arts and Sciences, University of Georgia, Athens, GA. All animal procedures were reviewed and approved by the University of Georgia Institutional Animal Care and Use Committee (AUP No. A970180 and A2001-10203).

### Ticks

*Amblyomma americanum* nymphs (Tick Rearing Facility, Oklahoma State University, Stillwater, OK) were housed in a ≥ 75% humidity chamber at room temperature for a minimum of one month before feeding on mice. Nymphs were obtained from a laboratory colony that fed as larvae on pathogen-free rabbits. Larvae were assumed to be pathogen-free because no transovarially-transmitted pathogen is known from this tick species. Further, a sample of nymphs analyzed at the Viral and Rickettsial Zoonoses branch of the Centers for Disease Control (Atlanta, GA) using the PCR methods of Anderson *et al*. (1992a), Paddock *et al*. (1997), and Sumner *et al*. (1997) was negative for DNA from any *Ehrlichia* species.

**Tick infestation**

Tick containment capsules were prepared by trimming the bottom 0.5 ml from 1.5 ml Eppendorf tubes. Mice were anesthetized by isoflurane inhalation to produce recumbent sedation. A 2 $cm^2$ area was shaved on the back of each mouse and two capsules were glued on to it with an 80% gum rosin (Sigma): 20% beeswax compound (Nelson & Kozub 1980).

Two to six hours after capsule application, mice were briefly anesthetized as above and fifteen nymphs were placed in each capsule (=thirty nymphs per mouse). To provide additional barriers to tick escape, infested mice were held in a double cage enclosure, with the standard mouse cage on a platform over water inside of a larger rat cage with a filter lid. Control mice received identical handling and containment regimens but no ticks. Mice were inspected daily for tick detachment and capsule integrity. In each of two trials, three infested and two control mice were sacrificed at each of four time points, 4, 7, 10, and 14 days post-infestation, to harvest spleens and draining lymph nodes for immunological studies. The trial was repeated once, giving a total of N=24 for infested and N=16 for control mice.

The four time points when mice were sacrificed were chosen on the basis of the feeding physiology of *A. americanum* nymphs (Sauer & Hair 1972). Samples taken at 4 days post-attachment cover the period of attachment and slow feeding preceding rapid engorgement. Samples from day 7 measure host responses when nearly all nymphs have completed rapid engorgement and detached from the host. Samples from day 10 provide data 3 days after rapid engorgement, and samples from day 14 allow the mice one week to recover from tick feeding. Ticks were allowed to feed until repletion or removed at the time that mice were sacrificed if still attached. Replete nymphs were washed with 0.5 %

sodium hypochlorite, 70% ethanol, and nanopure water, and housed at ≥ 75% humidity until they molted to adult stage.

### Lymphocyte preparation

Three infested and two control mice were dissected aseptically at 4, 7, 10, and 14 days post-infestation to harvest spleens and inguinal lymph nodes following modified methods of Ramachandra and Wikel (1992). Briefly, spleens were disrupted by grinding between the frosted edges of two sterile glass microscope slides to prepare a single cell suspension in tissue culture medium RPMI 1640 supplemented with 200 mM L-Glutamine, 100 mM sodium pyruvate, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml gentamycin, and 10% heat-inactivated fetal calf serum (Life Technologies, Rockville, MD). Inguinal lymph nodes were disrupted by first pulling apart with two sterile 25g x 1 1/2 inch needles and then grinding with the plunger of a sterile 5 cc syringe. Cells were washed three times in the medium by repeated centrifugation and resuspension. An aliquot of each sample was stained with trypan blue, and viable cells were counted with a hemacytometer.

## Enrichment of splenic adherent cell populations

Mice were dissected aseptically to harvest spleens following modified methods of Ramachandra and Wikel (1992). Spleens were disrupted to prepare a single cell suspension in RPMI medium as described above. Cells were washed twice in RPMI 1640 medium by repeated centrifugation and resuspension. Adherent cells were enriched from the total splenocyte population by adherence to plastic following modified methods of Schoeler *et al.* (1999). Splenocytes were incubated in sterile 100 x 15 mm petri dishes

with RPMI medium supplemented with 20% heat-inactivated fetal calf serum (FCS) for one hour at 37°C, 5% $CO_2$. Media and non-adherent cells were removed following gentle agitation and replaced with fresh RPMI with 20% FCS pre-warmed to 37°C; adherent cells were incubated for an additional hour. Media and remaining non-adherent cells were decanted, and adherent cells were detached by incubating with 5 ml of a 1:5000 dilution of Versene (Life Technologies, Rockville, MD) for 5 minutes at 37°C, 5% $CO_2$. Adherent cell suspensions were washed twice and resuspended in RPMI with 5% FCS. An aliquot of each sample was stained with trypan blue, and viable cells were counted with a hemacytometer.

**Lymphocyte proliferation assay**

Splenocyte cell suspensions were adjusted to 5 X $10^6$ cells/ml, and 100 µl of each suspension (5 X $10^5$ cells) was dispensed per well in a 96 well microtiter plate. Each cell suspension was assayed in triplicate for proliferative response to stimulation with different concentrations (2.5, 5.0, and 10.0 µg per ml) of the mitogens concanavalin A (Con A) or lipopolysaccharide (LPS) (Sigma, St. Louis, MO). Negative controls for stimulation received medium only. After 54 hours of incubation at 37°C, 5% $CO_2$, cells were pulsed with one µCi of methyl-$^3$H thymidine (Amersham Life Science, Piscataway, NJ), per well and incubated for 18 hours further. Cells were harvested using an automated cell-harvester (Skatron Inc., Sterling, VA). Incorporation of radioactivity was determined with a liquid scintillation counter (Beckman Instruments, Fullerton, CA). Mean counts per minute (CPM) ± SE were determined for each treatment.

**Stimulation of lymphocytes for cytokine production**

One ml (5 X $10^6$ cells) of each lymphocyte suspension was incubated with 1.0 μg/ml Con A in a 24 well plate (total volume two ml). Control wells prepared from each cell suspension received 1 ml of medium only. After 48 hours, contents of wells were centrifuged at 12,000 rpm for 5 minutes to remove cellular debris, then frozen at –70°C until used for quantification of secreted cytokine production.

**Stimulation of adherent cells for cytokine production**

Adherent cell suspensions were adjusted to 5 X $10^5$ cells/ml, and 500 μl of each suspension was cultured in quadruplicate with 1000 U/ml of recombinant interferon gamma (IFNγ) (BD PharMingen) in a 24 well plate for 2 hours. Following priming with IFNγ, cells were incubated with 5.0 μg/ml LPS (total volume 1 ml). After 18 hours, the contents of two wells of each suspension were harvested for tumor necrosis factor alpha (TNF-α) quantification, and again at 48 hours, contents of the remaining two wells were harvested for quantification of interleukin 12 (IL-12), IL-6, and IL-10. Harvested well supernatants were centrifuged at 12,000 rpm for 5 minutes to remove cellular debris, then frozen at –70°C until used for quantification of secreted cytokine production.

**Cytokine quantification**

Supernatants were assayed for levels of Type 1 (IFN- γ, IL-2, IL-12), Type 2 (IL-4, IL-6, IL-10, IL-13), and inflammatory (TNF-α) cytokines by a commercial antigen-capture ELISA (BD PharMingen, San Diego, CA, and R&D Systems, Minneapolis, MN) according to manufacturer's protocols. Adherent cell supernatants collected at 18 hours

were assayed for TNF-α, while supernatants collected at 48 hours were tested for IL-12, IL-6, and IL-10. The following capture and detection monoclonal antibody pairs were used: IFNγ, clones R4-6A2 and XMG1.2; IL-2, JES6-1A12 and JES6-5H4; IL-12, C15.6 and C17.8; IL-4, BVD4-1D11 and BVD6-24G2; IL-6, MP5-20F3 and MP5-32C11; IL-10, JES5-2A5 and SXC-1; IL-13, 38213.11 and polyclonal detection antibody; TNFα, G281-2626 and MP6-XT3. Each sample was assayed in duplicate and absorbance values were expressed in pg/ml as determined by comparison with a standard curve obtained from known quantities of recombinant cytokine standards (BD PharMingen and R&D Systems). Limits of detection of the capture antibodies used were IFNγ, 15.625-4000 pg/ml; IL-2, 31.25-4000 pg/ml; IL-12, 62.5-4000 pg/ml; IL-4, 15.625-1000 pg/ml; IL-6, 250-4000 pg/ml; IL-10, 125-4000 pg/ml; IL-13, 15.625-4000 pg/ml, and TNFα, 31.25-2000 pg/ml. Supernatants from lymph node and spleen cell cultures were initially assayed at a 1:2 dilution for all cytokines with the exception of spleen cell secretion of IFNγ, which was assayed at a 1:5 dilution. Dilutions were subsequently adjusted by 1-2 steps to bring supernatant cytokine concentrations within the limits of detection by capture antibodies. Typically, supernatants from control mice were re-tested at full strength for IL-4, IL-10, and IL-13, while supernatants from infested mice were diluted 1:4 or 1:8. Supernatants were re-tested at full strength, 1:2, or 1:10 dilutions for IFNγ and full strength or 1:4 dilutions for IL-2. Supernatants from adherent cell cultures were within the ranges of capture antibody detection at dilutions of 1:4 for IL-12 and TNFα and 1:2 for IL-6.

**Immunoglobulin quantification**

Whole blood was collected by severing the brachial artery at the time of dissection. To encourage clot formation, samples were held at room temperature for one hour and then stored at 4°C overnight. After 24 hours, samples were centrifuged at 10,000 rpm for 5 minutes to remove cellular components, and serum samples were frozen at –20°C until used for quantification of immunoglobulins. Supernatants were assayed for levels of immunoglobulin G2a ($IgG_{2a}$) and $IgG_1$ total antibody by a commercial antigen-capture ELISA (BD PharMingen, San Diego, CA) according to manufacturer's protocols. The following capture and detection monoclonal antibody pairs were used: $IgG_{2a}$, clones R11-89 and R19-15 and $IgG_1$, A85-3 and A85-1. Each sample was assayed in duplicate and absorbance values were expressed in ng/ml as determined by comparison with a standard curve obtained from known quantities of immunoglobulin standards (Calbiochem, La Jolla, CA). Limits of detection of the capture antibodies used were $IgG_{2a}$, 0.781-25 pg/ml and $IgG_1$, 0.195-6.25 pg/ml. Serum samples were assayed at 1:2000 and 1:4000 dilutions for $IgG_{2a}$ and a 1:16000 dilution for $IgG_1$.

**Data and statistical analysis**

Data were summarized on Excel spreadsheets (Microsoft, Redmond, WA) and imported into Statview (SAS Institute, Cary, NC) for hypothesis testing. ANOVA was used to determine whether proliferation and cytokine levels in each group of mice changed significantly over time. Student's t-test was used to determine significant differences in cell proliferation, cytokine production, and immunoglobulin levels between control and infested mice for each sampling interval. Simple regression was used to test

for a dose-response effect between the number of ticks feeding and each outcome variable. Significance was set at $p < 0.05$.

## Results

### Infestation

Twenty-four mice were each infested with 30 *A. americanum* nymphs, for a total of 720 nymphs (Table 1.1). Of these 720 nymphs, 78% attached and fed (range 15-30 nymphs / mouse). All tick attachment took place within 2 hours of infestation. All nymphs allowed to feed to repletion detached in 4-11 days, 97% of them within 7 days of attachment. Ninety-two percent of fed nymphs molted to adult stage.

Table 2.1: Feeding success of *A. americanum* nymphs infesting BALB/c mice.

| Number of nymphs | | Percent |
|---|---|---|
| placed | 720 | 100% |
| attached | 561 | 78% of placed |
| replete | *422 | *100% of attached |
| molted | *389 | *92% of replete |
| female | *288 | *74% of molted |

*Does not include 139 nymphs removed from mice sampled on day 4.

Gross increases in the sizes of spleens and lymph nodes of infested mice were observed on days 7, 10, and 14 post-attachment. Spleens of infested mice showed the most enlargement on day 10, at which time they were approximately 5.4-fold larger in volume than spleens of control mice (Fig. 2.1). Spleens and lymph nodes of infested and control mice did not appear visibly different in size on day 4 post-attachment.

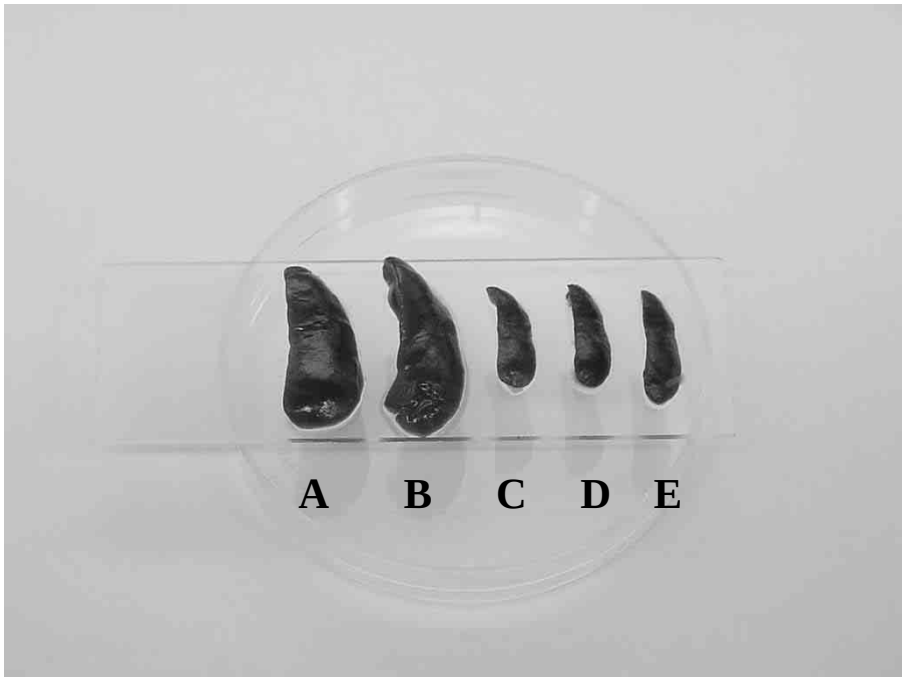


Figure 2.1. Enlargement of spleens of infested mice. Spleens from day 10 shown: from infested mice (A, B); from control mice (C, D); from a naïve mouse (E). Shown 1x magnification.

Although there was variation in the number of ticks feeding on individual mice (15-30 nymphs / mouse), simple linear regression determined that there was no dose-response effect between the number of ticks feeding and differences in splenocyte proliferation or cytokine production by that mouse (data not shown).

**Splenocyte proliferation**

Suppression of the B cell proliferative response in infested mice began during the rapid engorgement phase of feeding between day 4 and day 7, and did not return to control levels within one week of tick detachment (Fig. 2.2C). Specifically, proliferation

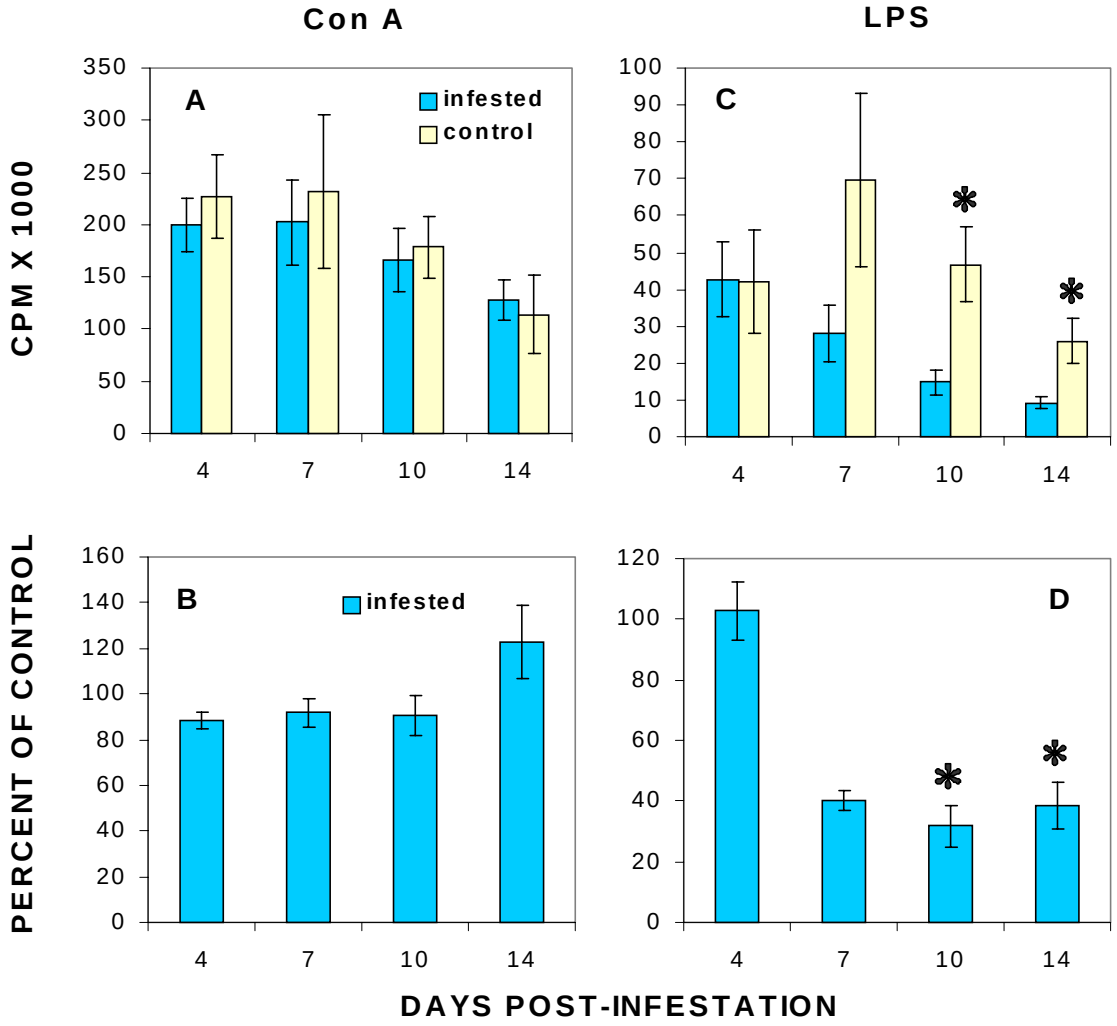


Figure 2.2. Spleen cell proliferation as counts per minute (CPM) (A, C) and percent of control (B, D) in response to 72 hours incubation with 5.0 µg/ml of the T cell mitogen Con A (A, B) or the B cell mitogen LPS (C, D). Error bars indicate ± one SE. *= $p < 0.05$

in response to LPS, a B cell mitogen, was suppressed by 60% relative to control mice on day 7 post-infestation, and became significantly decreased on the last two sampling days, with 78% suppression on day 10 and 72% suppression on day 14 (Fig.2.2D).

Proliferation in response to Con A, a T cell mitogen, was not significantly suppressed in infested mice at any of the four sampling times, and average proliferation was never less than 89% of the average for control mice ($p \geq 0.55$) (Fig.2.2A, B). Proliferative responses of control mice to either mitogen did not change significantly over time, although there was large variation in control values. To standardize results among points with different control values, proliferation data were also represented as percent of control (Figure 2.2B, D). Incubating splenocytes with lower or higher concentrations of LPS and Con A did not change the pattern of results but produced lower or higher overall counts per minute (CPM) (data not shown).

**Cytokine production**

Production of the Type 1 cytokines IFNγ and IL-2 in response to Con A was significantly suppressed in infested mice (Fig. 2.3). Interleukin-2 was consistently suppressed, while IFNγ production was lower in splenocytes but higher in cells from the draining lymph nodes.

Interferon gamma secretion was lower overall in splenocytes from infested mice and significantly suppressed on day 4 (35% less than control) and day 7 (59% less than control) post-infestation (Fig. 2.3A). Although IFNγ production by lymph node cells from infested mice was also suppressed on day 4 post-infestation (72% less than control), it surpassed levels in control mice on day 7 (6.8-fold), day 10 (19-fold), and day 14 (5.6-fold) (Fig. 2.3C). However, IFNγ was the only cytokine measured that was produced in

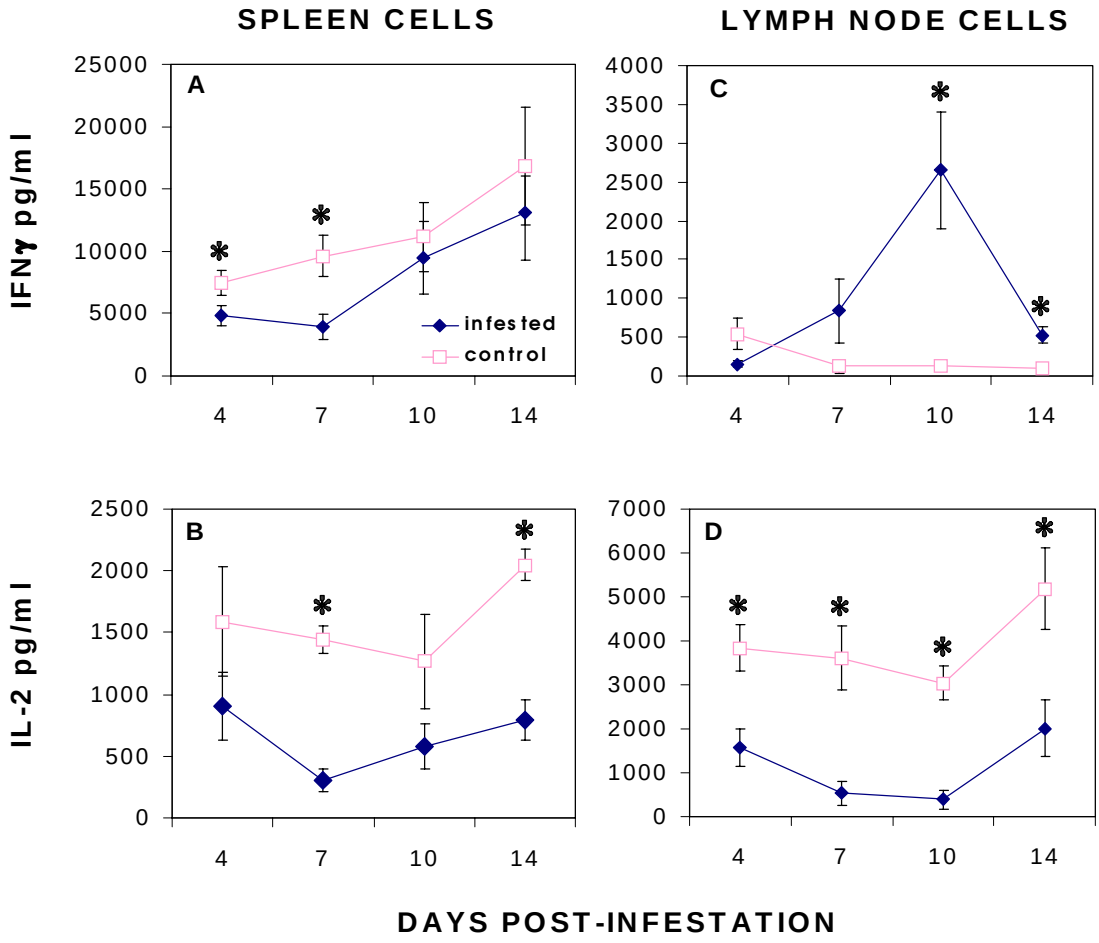


Figure 2.3. Secretion of Type 1 cytokines by spleen (A, B) and draining lymph node (C, D) cells after 48 hours incubation with 1.0 µg/ml ConA. Error bars indicate ± one SE. *=$p<0.05$

lower concentrations by lymph node cells than by spleen cells; production by lymph node cells from either group of mice was never more than 28% of splenocyte IFNγ at any time point.

Both spleen and lymph node cells from infested mice had decreased production of IL-2 throughout infestation (Fig. 2.3B, D). Splenocytes from infested mice produced 43-79% less IL-2 than splenocytes from control mice, and lymph node cells from infested mice lagged by 59-87% in IL-2 production. Differences between infested and control mice were greatest on day 7, after rapid engorgement, and were still significantly elevated one week after tick detachment. Cells from lymph nodes of infested and control mice produced an average of 1.7 times and 2.5 times more IL-2, respectively, than spleen cells from the same mice.

Con A-stimulated production of all Type 2 cytokines measured was upregulated in infested mice, peaking at day 7 post-infestation and declining to control levels by day 14 (Fig.2.4). In both spleen and lymph node cells, the highest production of IL-4, IL-10, and IL-13 was measured on day 7, soon after nymphs had completed rapid engorgement. The pattern of Type 2 cytokine production by stimulated lymph node and spleen cells of infested mice was similar, although increases in lymph node cytokines in infested mice were apparent on day 4, and were greater than changes in spleen cell cytokines. On day 7, production of IL-4 by cells from infested mice peaked at 4-fold higher in spleen cells and 60-fold higher in lymph nodes (Fig. 2.4A, D). Interleukin-10 was upregulated 6.8-fold in spleen cells from infested mice and 32 fold in lymph node cells (Fig. 2.4B, E). IL-13 was 7-fold higher in splenocytes from infested mice and 147-fold higher in lymph node cells (Fig. 2.4C, F). These high levels of Type 2 cytokines in infested mice declined over time and in most cases were no longer significantly different

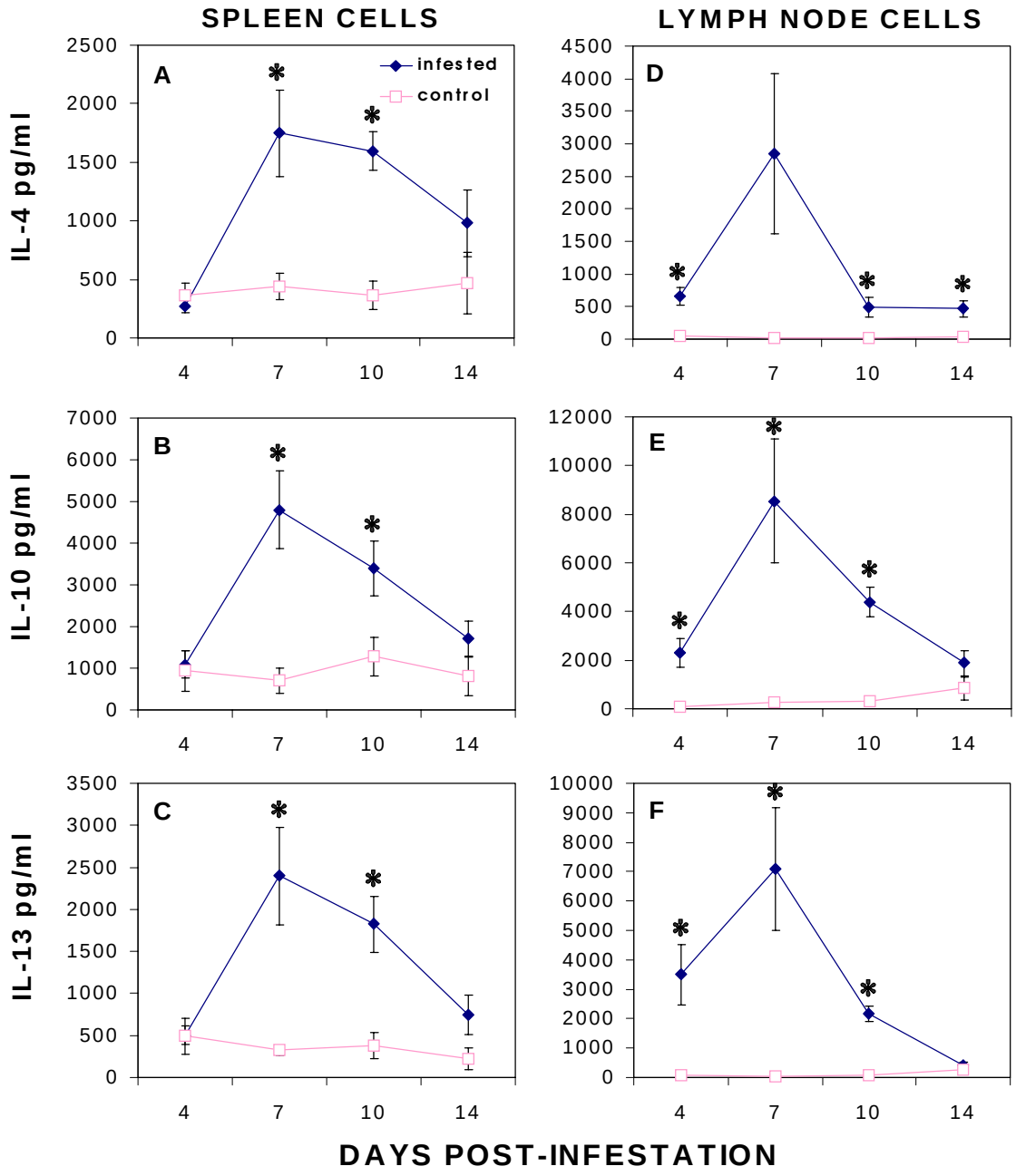


Figure 2.4. Secretion of Type 2 cytokines by spleen (A, B, C) and draining lymph node (D, E, F) cells after 48 hours incubation with 1.0 µg/ml ConA. Error bars indicate ± one SE. *= p <0.05

from cytokine levels in control mice by day 14 post-infestation. Type 2 cytokine production by cells from control mice did not change significantly over time for any of the five cytokines measured. Lymph node cells from infested mice produced an average of 2-3 times more Type 2 cytokines on days 4 and 7 than spleen cells from the same mice.

**Ratio of antibodies associated with Type 1 and Type 2 T cell responses**

The ratio of immunoglobulin isotypes produced by infested mice (Fig. 2.5A) correlated with their T cell cytokine profile. Although amounts of both immunoglobulin isotypes measured were lower in the serum of infested mice, the ratio of the immunoglobulin associated with Type 2 responses, $IgG_1$ to the immunoglobulin associated with Type 1 responses, $IgG_{2a}$ was consistently higher in serum from infested mice on days 4, 7, and 10, returning to control values on day 14. The immunoglobulin ratio in infested mice was 3.6:1 to 5.8:1, while the ratio in control mice was measured as 1.3:1 to 3.1:1. Infested mice produced less of both immunoglobulin isotypes on days 4, 7, and 10 post-infestation (Fig. 2.5B, C). Serum from infested mice contained 27-64% less $IgG_1$ and 62-91% less $IgG_{2a}$ than serum from control mice. Differences in levels of both serum antibodies were greatest on day 10. On day 14, relative levels of both IgGs were reversed, and serum from infested mice had 30-40% more of both antibody subtypes.

**Adherent cell cytokine production**

Levels of IL-12, IL-6, and TNF-$\alpha$ produced by adherent spleen cells were not significantly different between infested and control mice except for IL-12 on day 14

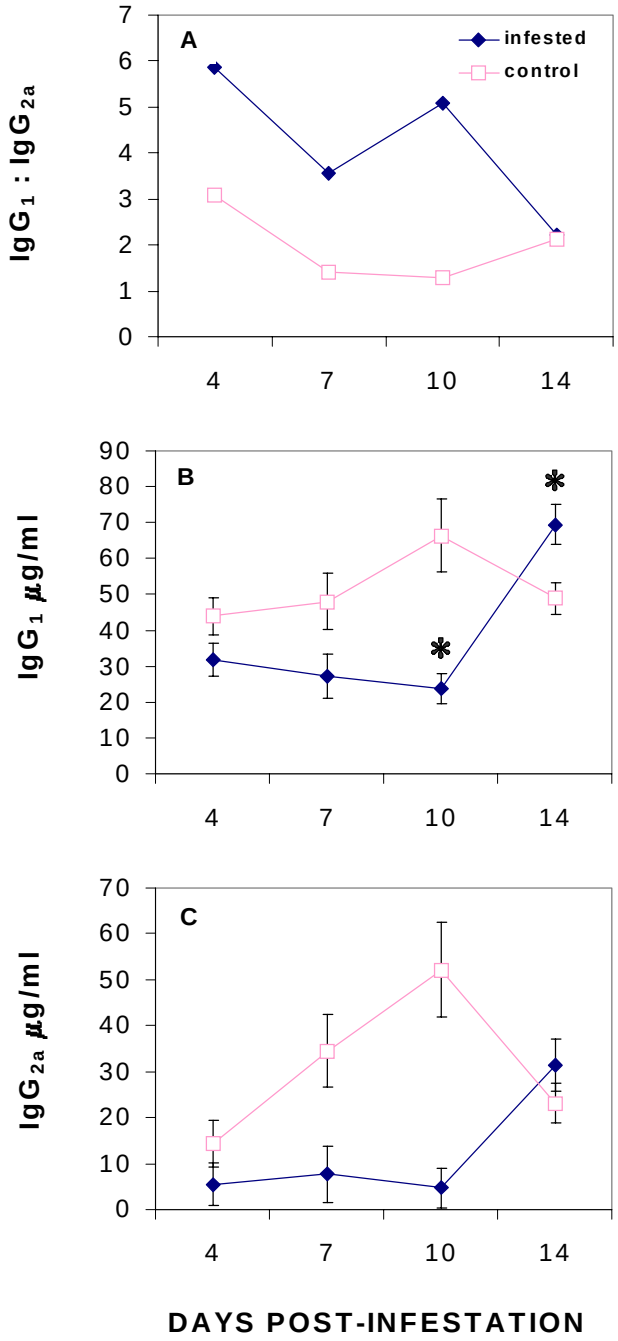


Figure 2.5. Ratio of total serum $IgG_1$ to total serum $IgG_{2a}$ in infested and control mice (A); µg/ml total serum $IgG_1$ (B) and $IgG_{2a}$ (C) in infested and control mice. Error bars indicate ± one SE. *=$p<0.05$

(Figure 2.6). Adherent cells from both groups of mice secreted similar quantities of IL-12 throughout tick feeding, and were only different on day 14, when cells from infested mice produced significantly less IL-12. Levels of TNF-α produced by cells from infested mice were lower than controls on days 4, 10, and 14 but the large variation in control values made differences between the groups insignificant. Differences from one time point to the next in control cytokine concentrations could not be attributed to experimental error but were consistent over two trials. Interleukin-6 production was nearly the same in both groups of mice. Levels of IL-12, TNF-α, and IL-6 cytokines produced by adherent cells were the same order of magnitude as the upper levels of most T cell cytokines measured. At all time points, adherent cells from both infested and control mice produced levels of IL-10 that were below the limit of detection of the ELISA (< 65 pg/ml) (data not shown).

## Discussion

Although small rodents are only occasional natural hosts of nymphal *A. americanum*, we were able to achieve successful feeding on BALB/c mice by confining ticks within capsules on individual hosts. The capsules enhanced attachment and feeding rates by preventing loss of ticks due to host grooming or tick dispersal. Additionally, we observed that *A. americanum* nymphs that had been held for several weeks after molting to nymphal stage were more likely to accept murine hosts than freshly molted nymphs, while the number of ticks placed (5, 15, or 30) did not affect feeding success rates (data not shown). We suggest that the enhanced humidity in our containment system was beneficial for *A. americanum* feeding and development, resulting in feeding and molting

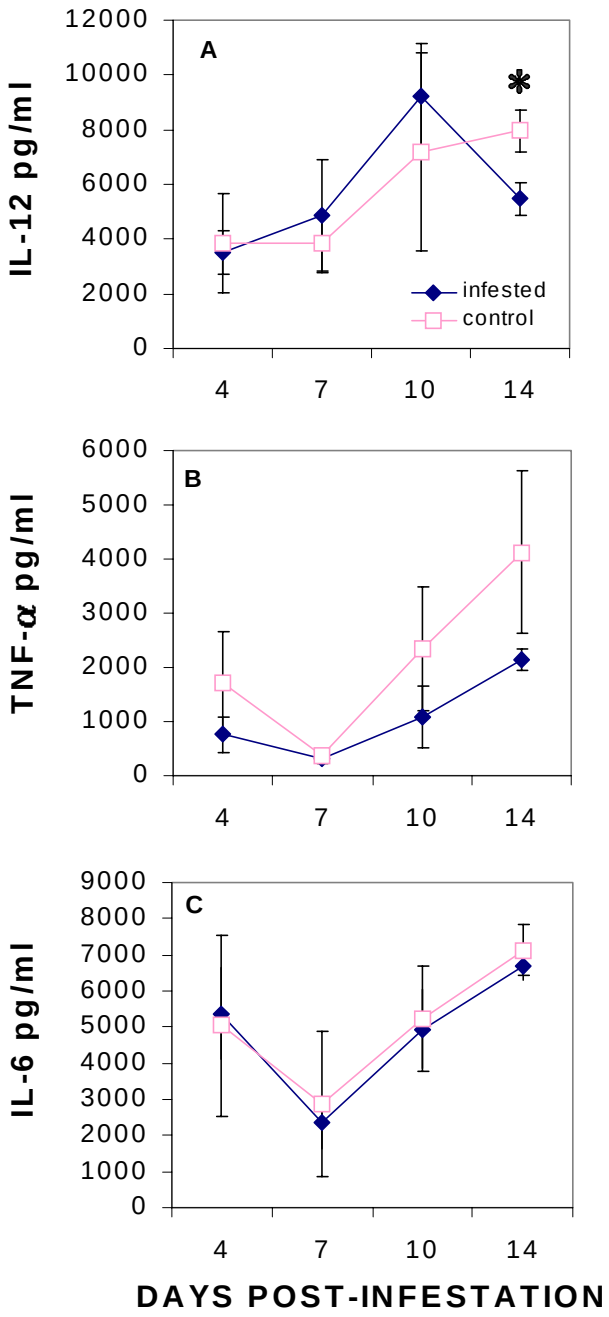


Figure 2.6. Secretion of IL-12 (A), TNF-α (B), and IL-6 (C) cytokines by purified adherent splenocytes after 48 hours incubation with 1000 units of recombinant IFNγ and 5.0 μg/ml LPS. Error bars indicate ± one SE. * = $p < 0.05$

rates comparable to those achieved in studies of less humidity-dependent tick species (Schoeler *et al.* 1999, Macaluso & Wikel 2001).

The gross enlargement of spleens and lymph nodes we observed in infested mice on days 7, 10, and 14 post-infestation were similar to those observed by researchers working with other species of ticks (Mbow *et al*. 1994b, Ganapamo *et al*. 1995, Ferreira & Silva 1999). The large size of the spleen in infested mice was probably due to increased hematopoesis to replace erythrocytes depleted by tick feeding or clearance of damaged erythrocytes. Enlargement of these lymphoid organs could also be due to salivary antigen-stimulated proliferation of immune cells. The nymphs used to infest the mice were obtained from a colony maintained on pathogen-free animals, so it is unlikely that mice were exposed to tick-borne pathogens. Although it was not feasible to test for all tick-borne diseases, PCR analysis of a sample of nymphs indicated no infection with any *Ehrlichia* species; complete blood counts performed in later experiments show no evidence of infection in tick-infested mice (Chapter 3).

In *A. americanum*-infested mice, mitogen-induced T cell proliferative responses were not affected while B cell proliferative responses were significantly suppressed up to a full week post-detachment (Fig. 2.2). Suppression of B cell proliferation became evident on day 7 post-infestation and was significant on day 10 and day 14, with no evidence of recovery by the end of the trial. On day 7, B cell inhibition was not statistically significant due to between-trial variation. Variation in control values could be due to a transient stress response to increased handling during tick detachment on days 5-8, when all mice were being manipulated daily

Our proliferation results differed from *in vivo* and *in vitro* experiments with other tick species and mouse hosts. With *A. americanum* infestation, we found small, non-

significant decreases in T cell proliferation, whereas infestation with or *in vitro* treatment with salivary gland extracts (SGE) of 4 other tick species in the genera *Dermacentor*, *Ixodes*, and *Rhipicephalus* suppressed murine proliferative responses to T cell mitogens by 20% or more (Ramachandra & Wikel 1992, Borsky *et al.* 1994, Urioste *et al.* 1994, Ganapamo *et al.* 1996b, Ferreira & Silva 1998, 1999, Macaluso & Wikel 2001). This effect was not confined to mouse models; infestation with or exposure to SGE of *Amblyomma variegatum*, *Boophilus microplus*, *Dermacentor andersoni*, and three species of *Rhipicephalus* also suppressed T cell proliferative responses in larger animals such as dogs, sheep, and cattle (Fivaz 1989, Wikel 1982, Wikel & Osburn 1982, Neitz *et al.* 1993, Inokuma *et al.* 1993, 1998, Koney *et al.* 1994, Ramachandra & Wikel 1995).

Only one study on *I. ricinus* infestation did not report a suppressive effect on murine T cell proliferation and found that the response to B cell mitogen suppressed by as much as 32% (Dusbabek *et al.* 1995). In all other studies, B cell proliferative responses were either suppressed along with T cell responses, or were unaffected or enhanced by infestation or SGE (Wikel 1982, Ramachandra & Wikel 1992, Inokuma *et al.* 1993, 1998, Ganapamo *et al.* 1996b). The differences between our results and previous research suggest that the effect of *A. americanum* infestation on host responses is different than the effects of other tick species, possibly because of a difference in saliva composition or immunomodulatory mechanism. The changes we observed in B cell proliferation were much greater than those recorded in previous studies and merit further investigation to determine whether infestation reduces B cell populations or responses.

We observed that infestation was associated with decreases in total $IgG_1$ and $IgG_{2a}$ antibodies on days 4, 7, and 10 (Fig. 2.5). In other tick studies, infested hosts had decreased primary antibody responses, and decreased or unaffected titers of total

antibodies and specific antibodies (Wikel 1985, Fivaz 1989, Njau *et al.* 1990). Decreases in overall antibody production could be due to suppression of B cell responses or to salivary immunoglobulin binding proteins used to remove ingested host immunoglobulins (Wang & Nuttall 1994, Jasinskas *et al.* 2000).

Downregulating B cell responses might reduce antibody production by the host; anti-tick antibodies are associated with host resistance (Wikel 1992), so it is in the interest of the tick to prevent their increase. Typically, specific IgG antibodies are not present in high quantities in the serum until 2 weeks after exposure (Abbas *et al.* 2000). We noticed increases in $IgG_1$ and $IgG_{2a}$ antibodies in infested mice on day 14, probably due to production of specific antibodies to tick antigens. These increases in antibodies might also reflect recovery from immunosuppression; however, this conclusion is contradicted by the continued suppression of B cell proliferation (Fig. 2.2). Although *A. americanum* infestation appears to reduce immunoglobulin production in general, its effect on specific antibody production would have to be measured at later times post-infestation, using a system that could compare specific antibody production to an antigen that was recognized by both infested and control mice.

The results of our T cell cytokine experiment indicate what cytokines are produced by mitogen-stimulated cells from infested and control mice. Cytokine production is correlated with cell phenotype, and can be used to infer the nature of the active cellular environment (Type 1 or 2) in the lymphoid organs (Morel & Oriss 1998). *Amblyomma americanum*-infested BALB/c cytokine responses showed Type 2 > Type 1 polarization similar to that previously observed in murine responses to other tick species, with high levels of IL-4, IL-10, and IL-13 and low levels of IFNγ and IL-2 (Ramachandra & Wikel 1992, Zeidner *et al.* 1997, Ferreira & Silva 1998, 1999, Christe *et al.* 1999,

Kopecky *et al.* 1999, Schoeler *et al.* 1999, Macaluso & Wikel 2001, Mejri *et al.* 2001). Relative to studies of BALB/c mice infested with *Dermacentor andersoni* and *Ixodes scapularis* nymphs, we found larger differences in production of all cytokines between control and infested mice, although the cytokine levels measured in our experiment were similar to or lower than levels reported in studies with other tick species (Zeidner *et al.* 1997, Schoeler *et al.* 1999, Macaluso & Wikel 2001). In our experiment, primary infestation with *A. americanum* upregulated secretion of the Type 2 cytokines IL-4 and IL-10 as much or more than had been measured in studies of *D. andersoni* and *I. scapularis*. The significant downregulation of IL-2 and IFNγ secretion that we measured during primary infestation was not observed in other tick studies, although they used the same, inbred BALB/c mouse strain as the experimental hosts. The difference in cytokine secretion between studies using the same strain of mice suggests that Type-2 polarized cytokine production is not an automatic response to all ectoparasites, and there is an *A. americanum*-specific effect on murine cytokine responses.

All of the cytokines we measured were significantly affected at the time that most ticks had completed rapid engorgement on day 7 except for lymph node production of IFNγ and IL-4. On day 7, lymph node production of IL-4 was extremely high in infested mice but not significant due to between-trial variation ($p=0.053$). The upregulation of Type 2 cytokines IL-4, IL-10, and IL-13 continued for at least 3 days after most ticks had detached, and suppression of IL-2 production persisted for at least one week post-detachment in both lymph node and spleen cells, indicating that infestation had both local and systemic effects that persisted beyond the mechanical injury of tick feeding. Although IFNγ and IL-4 are thought to be crossregulatory (Morel & Oriss 1998), we observed that IFNγ production was suppressed only during early stages of infestation and

engorgement (day 4 in lymph node cells, days 4 and 7 in splenocytes). This preceded IL-4 upregulation, suggesting that *A. americanum* infestation affects other, earlier mediators of IFNγ levels in infested mice.

Although we did not observe any effects on overall T cell proliferation, changes in cytokine production during and after tick feeding indicate that T cell subsets of infested mice should be investigated more closely. In particular, the patterns of IL-2, IL-4, IL-10, and IL-13 production suggest that infested mice have more numerous or more active Type 2 T cells than Type 1 T cells in their draining lymph nodes on days 4-10 post infestation and in their spleens on days 7-14 post infestation. Although both $CD4^+$ helper T cells and $CD8^+$ cytotoxic T cells are known to produce Type 1 and Type 2 cytokines (Mosmann *et al.* 1989, Sad *et al.* 1997), it was not possible to identify cellular sources of cytokines with the assays used in this study; a different assay will be necessary to evaluate changes in the specific contributions of T cells to cytokine production during infestation. The differences in timing and direction of IFNγ response between spleen and lymph node cells of infested mice merit further investigation as well. Also, cytokine responses to mitogen should be interpreted cautiously, because they are not always representative of responses to a specific antigen, such as those secreted by a pathogen. Therefore, it would be useful to find a model of antigen-specific T cell responses to determine how those are affected by infestation.

Isotype switching to $IgG_1$ is promoted by IL-4 and associated with Type 2 cytokine responses, while switching to $IgG_{2a}$ is induced by IFNγ and associated with Type 1 responses (Morel & Oriss 1998). The $IgG_1$: $IgG_{2a}$ ratio we measured in infested mice was 2-4 fold higher than the response of control mice (Fig. 2.5) and 1.5-2 fold the ratio expected in healthy mammals (Abbas *et al.* 2000), consistent with the Type 2 polarization

of the immune response. This Type 2 isotype polarization has also been observed in BALB/c mice infested with *B. burgdorferi*-infected and uninfected *I. ricinus* nymphs (Christe *et al*. 2000, Mejri *et al*. 2001).

Evidence of *A. americanum* modulation of T cell cytokines prompted us to look for changes in responses leading to T cell activation. Antigen-presenting cells (APCs) secrete cytokines that initiate T cell responses (Abbas *et al.* 2000). We were not able to detect significant differences between the ability of adherent cells from infested and control mice to produce IL-12, IL-6, or TNF-α during the time of tick feeding (Fig. 2.6). If infestation downregulated Type 1 T cell responses by affecting APCs, changes in APC-derived cytokines would precede T cell responses. However, the only significant difference occurred in IL-12 on day 14, when T cells from infested mice were returning to normal responses. The ability of their adherent cells to produce IL-12 in culture did not prevent infested mice from developing a Type 2 cytokine response. Modulation of APCs by tick infestation also seems to have a species-specific component; SGE from *D. andersoni* suppressed TNF-α and IL-1β production by cells from BALB/c mice, while infestation with *I. scapularis* suppressed TNF-α but not IL-1β (Ramachandra & Wikel 1992, 1995, Schoeler *et al.* 1999). In C3H mice, *R. sanguineus* infestation suppressed IL-12 production but did not affect macrophage presentation of specific antigen to T cells, and *I. scapularis* infestation did not affect production of either TNF-α or IL-1β (Ferreira & Silva 1998, 1999, Schoeler *et al.* 1999).

Reduction of the host Type 1 response could benefit feeding ticks by decreasing inflammation in response to tick salivary antigens, thereby allowing more efficient feeding. In addition, reduction in Type 1 cytokines, especially IFNγ, can make vertebrate hosts more susceptible to infection by microbial pathogens (Romagnani 1997). Sand fly

saliva inhibits Type 1 responses by suppressing macrophage activity, and has been shown to enhance transmission of intracellular *Leishmania* parasites to the mammalian host (Titus & Ribeiro 1988, Theodos & Titus 1993). *Ixodes scapularis* feeding exacerbated Type 2 > Type 1 cytokine polarization and increased infection in mice susceptible to the extracellular pathogen *B. burgdorferi*; however, supplementing Type 1 cytokines restored resistance to *B. burgdorferi* (Zeidner *et al.* 1997). Depending on how *A. americanum* infestation affects different aspects of B and T cell function, including responsiveness of Type 1 cells to antigen presentation, cytokine secretion, and antibody production, infestations might prevent adequate cellular and humoral responses against pathogens such as *E. chaffeensis*, *E. ewingii*, or *B. lonestari*. Until animal models are developed to study host immune responses during pathogen transmission by *A.americanum*, the findings of this experiment and further studies will be useful to infer the potential effects of *A. americanum* infestation on host responses to disease.

# CHAPTER 3

# CHANGES IN CELL POPULATIONS AND CELL PHENOTYPES IN BALB/c MICE INFESTED WITH *AMBLYOMMA AMERICANUM*[1]

[1] Ledin, K.E., S. Singh, S. Kumar, and D.E. Champagne. To be submitted to *The American Journal of Tropical Medicine and Hygiene*

## Abstract

*Amblyomma americanum*, the primary vector of *Ehrlichia chaffeensis* and the putative vector of *E. ewingii* and *Borrelia lonestari* in the southeastern United States, has been shown to modulate the proliferative, cytokine, and antibody responses of BALB/c mice. The responses affected are not only important for tick resistance but might also have a significant role in resistance to intracellular bacteria and spirochetes. For this study, we used flow cytometry to identify cell types and quantify cytokine production by specific cell types from infested and control mice. We found that infestation with *A. americanum* nymphs significantly decreases proportions of $CD4^+$ cells and $CD8^+$ cells in both spleens and draining lymph nodes. Proportions of B cells ($CD19^+$) and macrophages ($CD14^+$) were unaffected or slightly upregulated by infestation. Infested mice had significantly smaller populations of $CD4^+$IL-$2^+$ cells, and significantly larger populations of IL-$4^+$ cells than control mice. Reduction of important T cell populations could have consequences in host response to not only tick-borne illnesses, but also to a variety of other intracellular pathogens.


## Introduction

Nearly all studies of immune interactions between ticks and their vertebrate hosts have demonstrated an effect of tick feeding, salivary gland extract, or saliva on cellular responses. Differential proliferation in response to mitogens and the ability to produce Type 1 or 2 cytokines have been used to indirectly determine which cell types are affected by exposure to tick feeding or saliva components. Cell-depletion experiments indicated that $CD4^+$ T cells were important in controlling the proliferative response following secondary exposure to tick antigens (Ganapamo *et al*. 1996b, 1997).

Only one study has used flow cytometry to document proliferative responses of $CD4^+$, $CD8^+$, and B cells in mice infested with pathogen-free ticks (Zeidner *et al*. 1997). In C3H/HeJ mice, B cell numbers were decreased by infestation with *I. scapularis*, while $CD4^+$ and $CD8^+$ cell populations remained unchanged (Zeidner *et al*. 1997). Our previous observations of suppression of the proliferative response to LPS and lower overall antibody titers in infested mice suggested that numbers or functions of B cells were also affected by *A. americanum*. In this study, we used flow cytometry to directly measure the impact of infestation on $CD4^+$ and $CD8^+$ T cell and B cell populations in the spleen and draining lymph nodes.

In many different tick-host systems there is indirect evidence that ticks downregulate the host's Type 1 T cells, which are known to be necessary for protection against intracellular infections and to enhance phagocytosis of microbes (Romagnani 1997). However, both $CD4^+$ helper T cells and $CD8^+$ cytotoxic T cells produce Type 1 and Type 2 cytokine profiles, even though their roles in immune responses are very different (Mosmann *et al*. 1986, Li *et al*. 1997). How tick infestation affects cytokine production by $CD4^+$ and $CD8^+$ T cells is unknown. Cell-depletion experiments have shown that $CD4^+$ T cells are needed in spleen cell suspensions from infested mice to produce the cytokines IFNγ, IL-2, IL-4, IL-10, TNF-α, and GM-CSF. $CD8^+$ cells are needed to produce IL-2, IL-4, and IL-10 in response to mitogen (Ganapamo *et al*. 1996a, Zeidner *et al*. 1997). In our previous experiments, proliferation of splenic T cell populations was not affected by *A. americanum* infestation, but the cytokine profiles of infested mice were significantly polarized, with high levels of Type 2 cytokine production by cells from the spleens and lymph nodes. Our second objective for this

study was to evaluate the contribution of T cell subtypes to cytokine production by measuring intracellular cytokine expression of spleen and lymph node cells.

## Materials and Methods

### Ticks

*Amblyomma americanum* nymphs were acquired from the Oklahoma State University Tick Rearing Facility and housed as described previously (Chapter 2).

### Mice

Female BALB/c mice over 8 weeks of age were purchased from Jackson Laboratories and housed in the Animal Resources Facility as described previously (Chapter 2). All animal procedures were reviewed and approved by the University of Georgia Institutional Animal Care and Use Committee (AUP No. A970180 and A2001-10203).

### Infestation

Anesthetized mice had tick-containment capsules attached to them with rosin/beeswax glue as described previously (Chapter 2). Twenty-four mice were infested with 15 *A. americanum nymphs* in each capsule, for a total of 30 nymphs per mouse. Sixteen control mice did not receive nymphs but were otherwise handled identically to the infested mice. In each of two trials, three infested mice and two control mice were sacrificed at each of four time points: 4 days, 7 days, 10 days, and 14 days after tick attachment. Mice were checked daily for tick detachment. Replete nymphs were removed and stored as described previously (Chapter 2).

**Complete blood count**

At the time of spleen and lymph node dissection, 250 µl of blood was collected from the brachial artery and immediately deposited into a 1.5 ml ethylene diamine tetra-acetic acid (EDTA)-coated vial (Becton Dickinson, Franklin Lakes, NJ) to prevent coagulation of the sample. Samples were delivered within 2 hours of collection to the Clinical Pathology laboratory at the University of Georgia School of Veterinary Medicine for complete blood counts to evaluate mice for anemia and gross irregularities in differential leukocyte counts. Observed values were compared to referenced erythrocyte and leukocyte values for BALB/c mice (Moore 2000).

**Lymphocyte preparation**

Mice were sacrificed and dissected to remove the spleen and inguinal (draining) lymph nodes. As previously described (Chapter 2), spleens were disrupted between two sterile frosted glass slides into RPMI media. Lymph nodes were dissected from surrounding tissue and perforated with two 25 gauge x 1.5 inch sterile needles, then disrupted by grinding against the bottom of the plastic petri dish with the distal end of the plunger of a sterile 5 ml syringe. Cells were centrifuged and rinsed twice with RPMI media and then counted with a hemacytometer to determine number of mononuclear cells per ml. A portion of the spleen cells and all of the lymph node cells were suspended to a concentration of $5 \times 10^6$/ml.

**Cell surface molecule staining**

Immunolabeling of cell surface molecules was performed as follows: An aliquot of each skin cell suspension was centrifuged to remove media and then erythrocytes were lysed by suspending cells in nanopure $H_20$ for 10 seconds followed by immediate addition of 10x phosphate-buffered saline (PBS) at pH 7.0 to make an isotonic 1x PBS solution. Each cell suspension was transferred in 200 µl aliquots to a 96-well V-bottom cell culture plate (Corning Inc., Corning, NY). The plate was spun at 1500 rpm for 7 minutes to remove PBS. Cells were re-suspended and incubated overnight at 4°C in dilutions of fluorochrome-conjugated antibodies for cell surface molecules: fluorescein isothiocyanate (FITC) anti-CD4, clone GK1.5; allophycocyanin (APC) anti-CD8, clone 53-6.7; R-phycoerythrin (R-PE) anti-CD19, clone 1D3; R-PE anti-CD14, clone rmC5-3; and FITC anti-MHC II ($I\text{-}A^d$), clone 39-10-8 (BD PharMingen, San Diego, CA). All antibodies were diluted 1:1000 with PAB staining buffer (PBS with 0.1% sodium azide, 0.1% bovine serum albumin (BSA) (Sigma, St. Louis, MO)). Antibodies, antibody dilutions, and stained cells were always kept in the dark and at 4°C or on ice. A control sample was prepared with rat isotype-matched control antibodies for each fluorochrome (rat $IgG_1$, clone R3-34; rat $IgG_{2a}$, clone R35-95, BD PharMingen) to check for non-specific staining. Single- and triple- stained control samples of all antibodies were also prepared to facilitate setting detectors and compensation on the FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). After staining overnight at 4°C, cells were washed twice with 200 µl PAB and stored in PAB at 4°C for 1-4 days until read with the FACSCalibur.

**Intracellular cytokine staining**

An aliquot of each spleen and lymph node cell suspension from each mouse was incubated with 50ng/ml phorbol myristate acetate (PMA), 500ng/ml ionomycin (Sigma, St. Louis, MO.), and 1µl/ml brefeldin A (GolgiPlug™, BD PharMingen) in RPMI for 6 hours at 36°C, 5% $CO_2$. Cell suspensions were centrifuged to remove media, lysed to remove red blood cells, and transferred to a 96-well V-bottom plate as described above. The plate was spun to remove PBS. Cells were re-suspended and pre-incubated for 5 minutes at 4°C in Fc Block™ (anti-CD16/CD32 FcγIII/II receptor antibody, clone 2.4G2; BD PharMingen). Two fluorochrome-labeled antibodies for cell surface molecules, FITC anti-CD4 and APC anti-CD8, or the appropriate controls, diluted as described above, were added to the wells and the plate was incubated for 45 minutes at 4°C. Cells were washed twice with 200 µl PAB per well for 7 minutes at 4°C at 1500 rpm and then incubated for 10 minutes at 4°C with 100 µl Cytofix/Cytoperm™ Buffer (BD PharMingen) following the protocol of the Cytofix/Cytoperm™ kit (BD PharMingen). Cells were washed twice with 200 µl Perm/Wash™ solution (BD PharMingen) and then incubated overnight at 4°C in R-PE-labeled anti-IL-2, clone S4B6; anti-IL-4, clone 11B11; anti-IL-10, clone JES5-16E3; or anti-IFNγ, clone XMG1.2 antibody diluted 1:100 with Perm/Wash™ solution. Cells were washed twice with 200 µl Perm/Wash™ solution, resuspended in PAB, and kept at 4°C for 1-4 days until read with the FACSCalibur. Isotype-matched control antibodies (rat $IgG_{2a}$, clone R35-95; rat $IgG_1$, clone R3-34, BD PharMingen) were used to prepare single and triple-stained control samples concurrently with samples from individual mice.

### Flow cytometry

Cell samples were loaded into 12x75mm polystyrene culture tubes (Fisher, Pittsburgh, PA) and data on 10,000 events (or, rarely, the total cell number in each sample if less than 10,000) were acquired with a Becton Dickinson FACSCalibur. Samples were analyzed by size, granularity, and fluorescence with CellQuest software (BD PharMingen) to identify leukocyte subpopulations. Isotype-matched, single, and multiply stained controls were used to set detectors, compensation, and standard quadrants for separating cell types.

### Data and statistical analysis

Flow cytometry and complete blood count data were exported to Microsoft Excel (Microsoft, Redmond, WA) spreadsheets for analysis by ANOVA and individual pair-wise t-tests for significance at the $p < 0.05$ level with the Statview program (SAS Institute, Cary, NC).

## Results

### Infestation

*Amblyomma americanum* feeding results were similar to those previously reported in Chapter 2. Eighty two percent of nymphs attached to mice, most within 2 hours of placement in containment capsules. More than 95% of nymphs allowed to remain attached completed feeding between day 5 and day 7 post-infestation. Ninety eight percent of engorged nymphs molted to the adult stage.

**Complete blood count**

A complete blood count of samples taken during mouse dissection demonstrated a mild, normocytic (normal size RBC), polychromic (color range indicative of normal RBC age distribution), regenerative (normal hematopoetic replacement) anemia in infested mice. Infested mice had decreased packed cell volume ($\bar{x}$=32.2%), red blood cell numbers (5.6 x $10^6$/µl), and hemoglobin levels ($\bar{x}$=9.0g/dl) relative to control mice and referenced values for normal mice on day 4 when ticks were beginning rapid engorgement and day 7, when ticks had completed rapid engorgement. The red blood cell parameters of corpuscular volume and hemoglobin concentration were lower in infested mice at the time that ticks were beginning rapid engorgement on day 4 (37.0 fl and 24.8g/dl, respectively), but were not different from control mice or reference values for the rest of the study. Non-significant decreases in platelet populations on days 4 and 7 ($\bar{x}$=566 x $10^3$/ul) corresponded with observations of several days of decreased hemostasis during and after rapid engorgement. A differential count of leukocytes did not identify any significant differences between control and infested populations of lymphocytes, neutrophils, basophils, or eosinophils (data not shown). Leukocyte counts of control and infested mice were normal relative to reference values, and no signs of infection were visualized in blood smears, suggesting that neither group of mice was responding to infection with a pathogenic organism.

**Quantification of cell populations from lymphoid organs**

Three cell populations which could be differentiated on the basis of cell surface molecule expression, $CD4^+$ T cells, $CD8^+$ T cells, and $CD19^+$ B cells, were found to compose more than 90% of all leukocyte-gated cells in spleen and lymph node samples.

At all time points measured, infested mice had proportionately fewer $CD4^+$ and $CD8^+$ cells in samples from either lymphoid organ, except splenic $CD4^+$ cells on day 14 (Fig. 3.1). In both spleens and lymph nodes, $CD4^+$ and $CD8^+$ T cell populations were significantly decreased on day 7, just after most ticks had completed rapid engorgement. Significant decreases in lymph node $CD4^+$ cells from infested mice were evident on day 4, preceding decreases in splenic $CD4^+$ populations. Decreases in $CD8^+$ cells in lymph nodes and spleens of infested mice persisted until day 10 and day 14, respectively. $CD4^+CD8^+$ double-positive cells were present in very small numbers (less than 1%), and were not found to be significantly different between infested and control mice in either organ at any time point.

Splenic $CD19^+$ B cell proportions were not significantly different in infested and control mice (Figure 3.2A). Lymph node B cell proportions were significantly increased in infested mice only on day 7 post-infestation (Fig. 3.2B). Increases in B cell proportions did not completely compensate for decreases in the two T cell types, leaving differences of 6-8% in total lymph node lymphocytes on days 4-14 and 4-13% in total spleen lymphocytes on days 7-14 post-infestation, which represent proportional increases in $CD4^-CD8^-CD19^-$ cells of unknown lineage in those organs (data not shown).

$CD14^+$ macrophages and monocytes made up a very small percentage of total cells in spleens (~2%) and lymph nodes (~0.7%) (Figure 3.2C, D). There were slightly more $CD14^+$ cells in the spleens of infested mice than in control mice at all time points measured (Fig. 3.2C). Proportions of $CD14^+$ lymph node cells were not different between the two groups of mice until day 14, when the number of $CD14^+$ cells appeared to suddenly increase in control mice, probably due to experimental error such as sample contamination with another antibody (Fig. 3.2D). $CD14^+$ spleen cells from infested mice

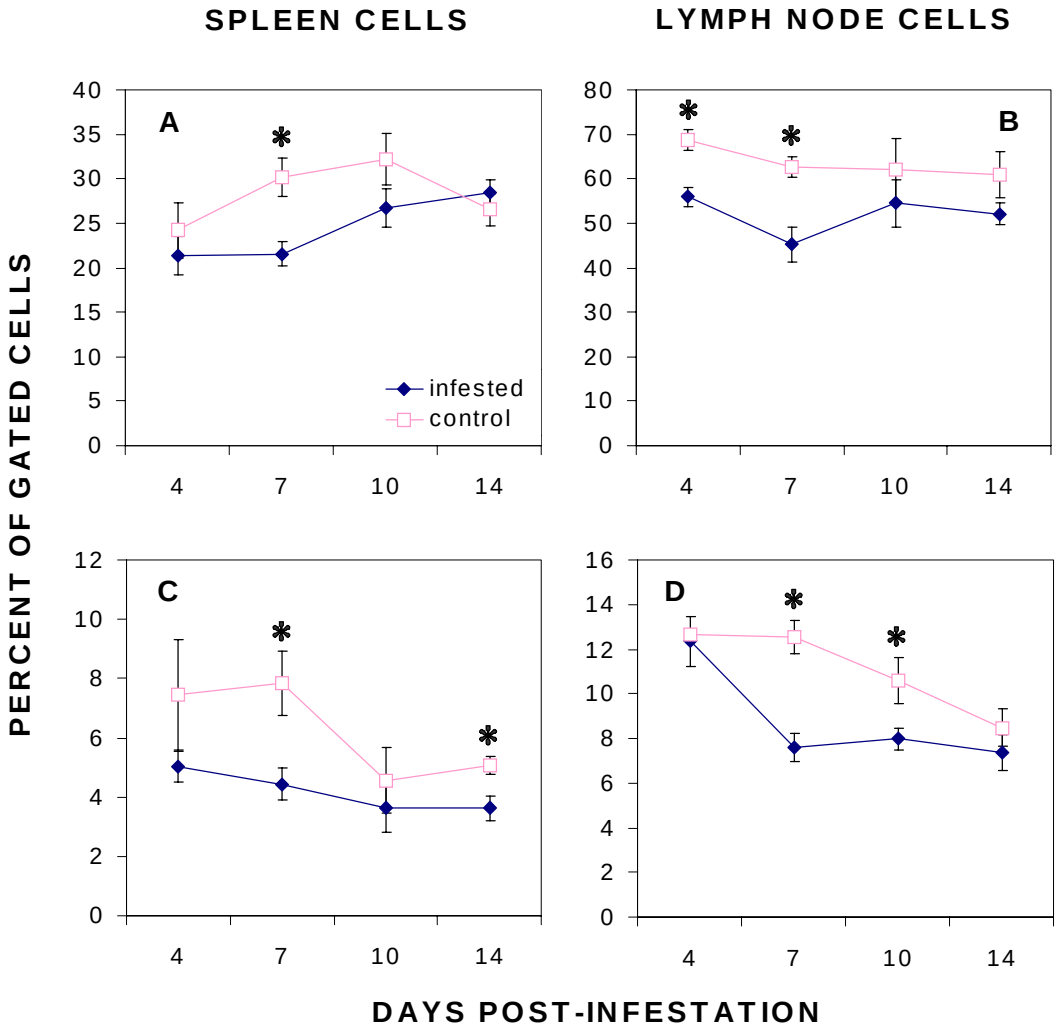


Figure 3.1. Relative proportions of, and changes in $CD4^+$(A, B) and $CD8^+$ (C, D) cells in spleens and lymph nodes of infested and control mice. Error bars indicate ± one SE. * = $p < 0.05$

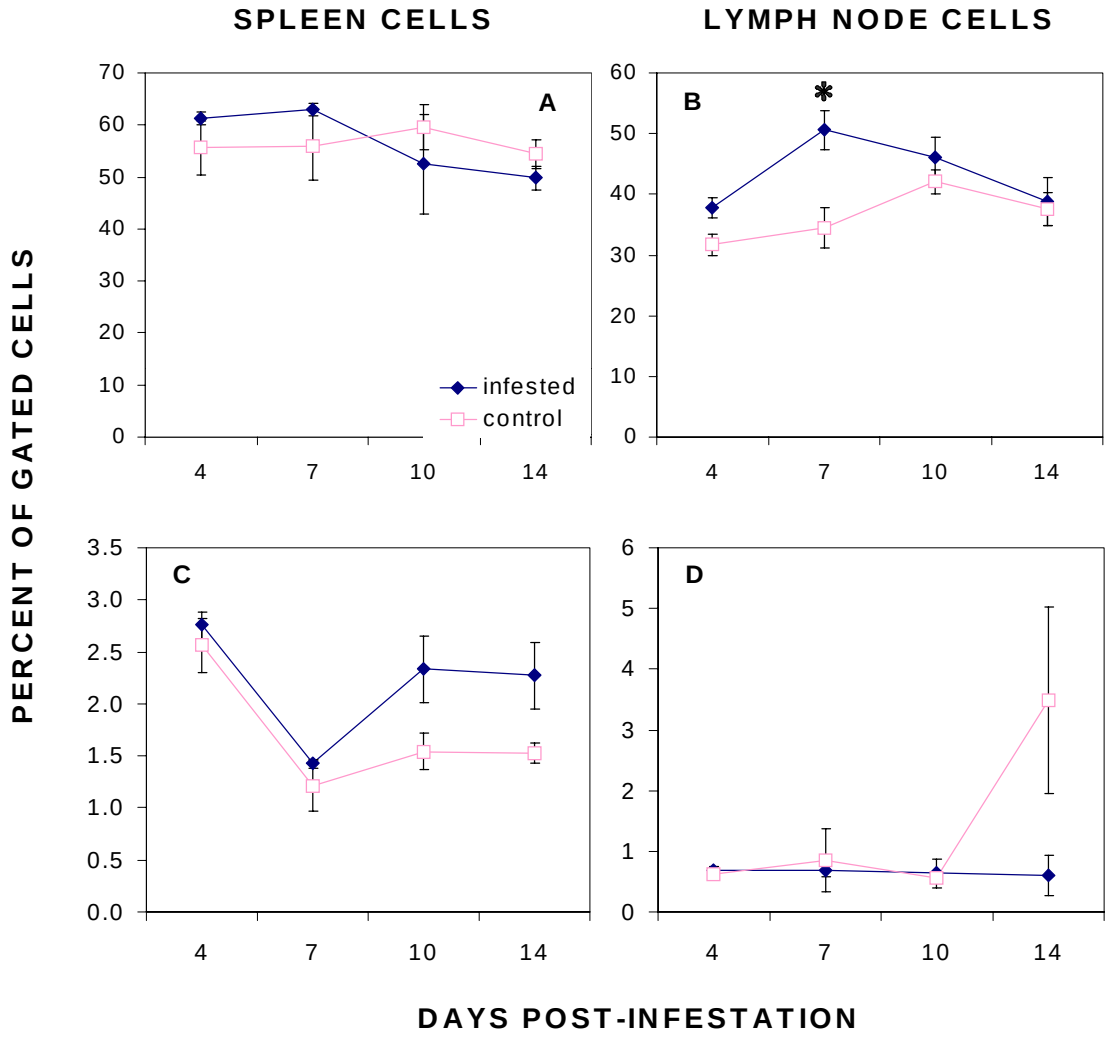


Figure 3.2 Relative proportions of, and changes in $CD19^+$ (A, B) and $CD14^+$ (C, D) cells in spleens and lymph nodes of infested and control mice. Error bars indicate ± one SE. * = $p < 0.05$

showed increases in MHC $II^+$ fluorescence intensity at all time points measured, with significantly higher MHC $II^+$ fluorescence intensity on day 10 post-infestation (data not shown).

**Mitogen-stimulated intracellular cytokine expression**

After ticks completed feeding, cell samples from infested mice produced fewer interleukin-2-positive cells ($IL\text{-}2^+$) in response to mitogen than samples from control mice (Fig. 3.3). Decreases in $CD4^+IL\text{-}2^+$ cells were significant in spleen samples from infested mice on day 7 and the lymph node samples of infested mice on day 10 post-infestation (Fig. 3.3A, B). $CD4^+$ cells were the main $IL\text{-}2^+$ cell type, making up an average of 80% of the $IL\text{-}2^+$ cells in the spleen samples and 85% of the $IL\text{-}2^+$ cells in the lymph node samples. Spleen samples from infested mice also had significantly fewer $CD8^+$ $IL\text{-}2^+$ cells, although $CD8^+$ $IL\text{-}2^+$ cells from lymph nodes were unaffected (Fig. 3.4C, D). On average, 10% of $IL\text{-}2^+$ cells from spleens and lymph nodes of control mice were $CD8^+$. 11% of $IL\text{-}2^+$ cells from the spleen and 5% of $IL\text{-}2^+$ cells from the lymph nodes were neither $CD4^+$ nor $CD8^+$ ($CD4^-CD8^-$). Differences in proportions of $CD4^-CD8^-$ $IL\text{-}2^+$ cells between infested and control mice were 0.6% or less.

Proportions of $IFN\gamma^+$ spleen and lymph node cells were not significantly different between infested and control mice (Fig. 3.4A, B). Proportions of $CD4^+IFN\gamma^+$ cells in infested mice appeared to be slightly higher in spleen samples on days 7, 10, and 14 and in lymph node samples on days 7 and 10 post-infestation, but differences were too small to be significant. An average of 43% percent of $IFN\gamma^+$ cells from spleens were $CD4^+$ and 33% of $IFN\gamma^+$ cells were $CD8^+$, while in lymph node samples the averages were 27% $CD4^+$ and 57% $CD8^+$.

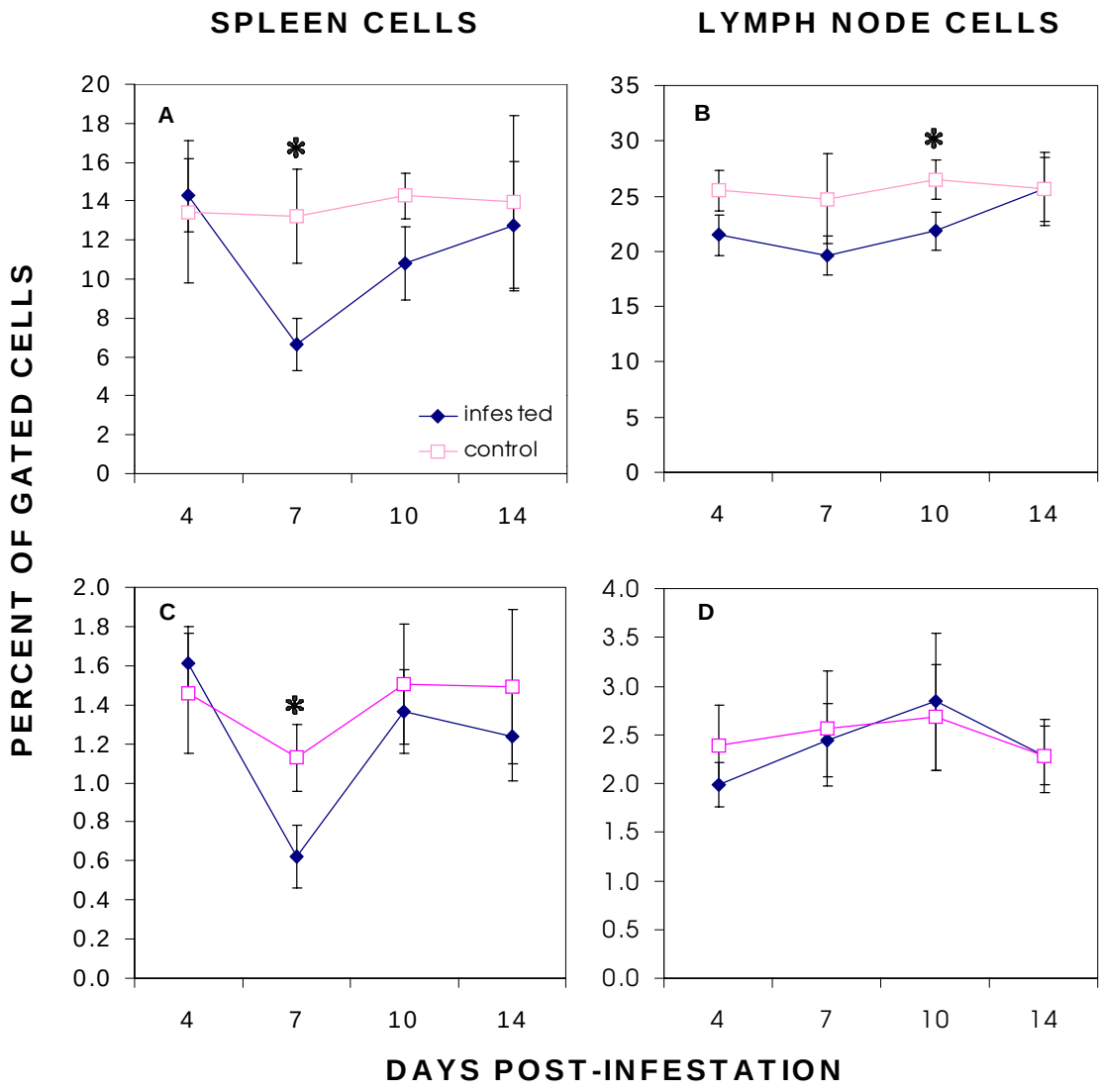


Figure 3.3 Relative proportions of, and changes in IL-2 producing $CD4^+$ cells (A, B) and $CD8^+$ cells (C, D) in spleens and lymph nodes of infested and control mice. Error bars indicate ± one SE. * = $p < 0.05$

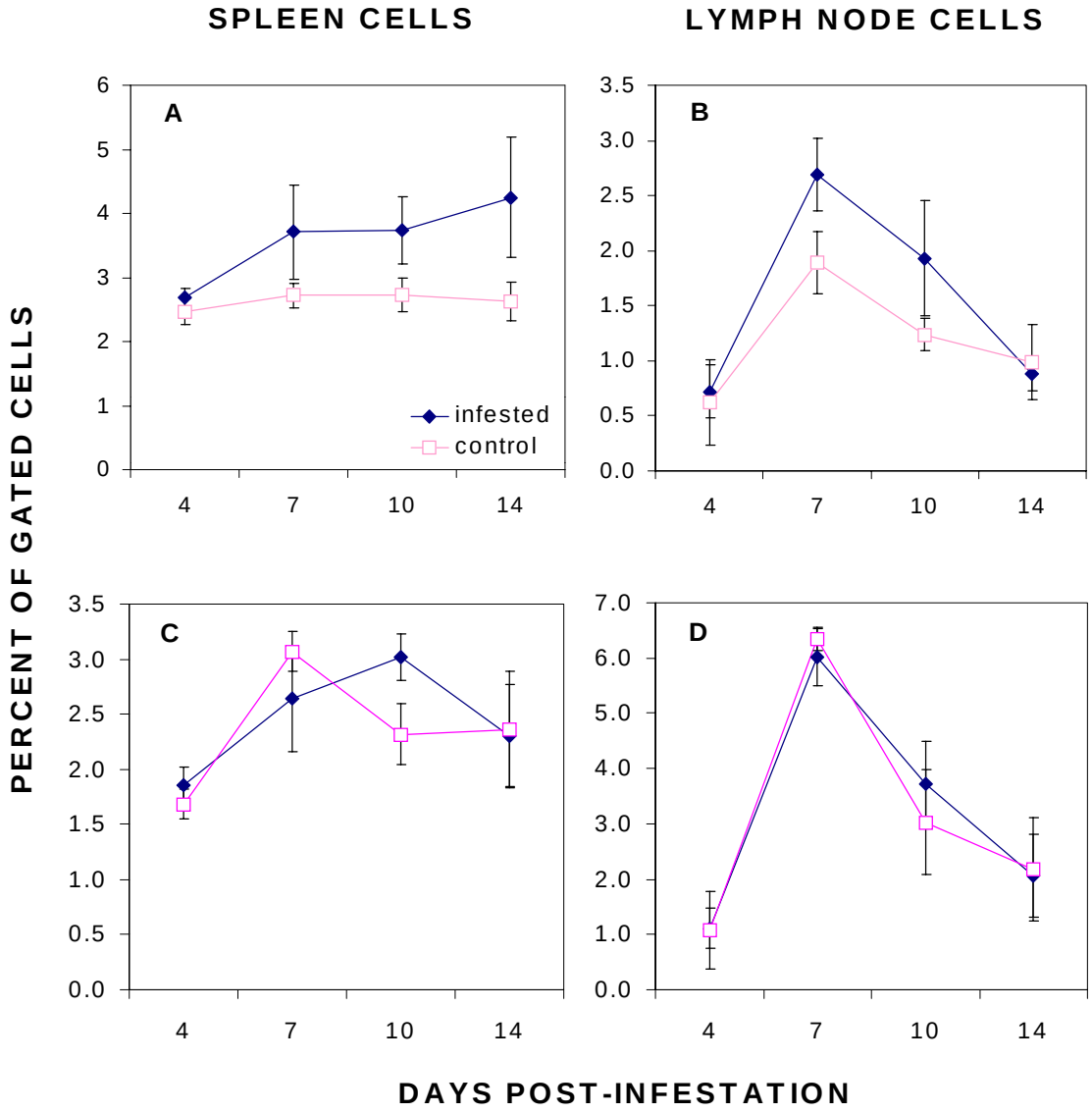


Figure 3.4 Relative proportions of, and changes in IFNγ producing $CD4^+$ cells (A, B) and $CD8^+$ cells (C, D) in spleens and lymph nodes of infested and control mice. Error bars indicate ± one SE. * = $p < 0.05$

Mitogen-stimulated spleen and lymph node cells sampled from infested mice had significantly higher proportions of Type-2 cytokine producing cells than were found in control mice (Fig. 3.5). Although the total numbers of IL-$4^+$ and IL-$10^+$ cells were less than 5%, infested mice had significantly more IL-$4^+$ cells in spleen samples on days 7 and10 post-infestation, and in lymph node samples on day 10 post-infestation (Fig 3.5A, B). IL-$4^+$ cells from lymph nodes of infested mice also appeared to increase on day 7, but the difference was not significant due to large variation in both infested and control samples. These increases in IL-$4^+$ cells occurred right after most ticks finished rapid engorgement and persisted until all ticks had detached from infested hosts. Infested mice had significantly more IL-$10^+$ spleen cells on days 4 and 10 post-infestation, but were not significantly different from control mice in proportions of IL-$10^+$ lymph node cells (Fig. 3.5C, D). In spleens and lymph node samples of both groups of mice, the majority of IL-$4^+$ and IL-$10^+$ cells were $CD4^-CD8^-$. Only about 22% of the spleen cells and 34% of the lymph node cells positive for these cytokines were $CD4^+$. Very few $CD8^+$ cells produced these Type 2 cytokines; less than 12% of IL-$4^+$ cells, and less than 6% of IL-$10^+$ cells were $CD8^+$ in either group of mice.

In flow cytometry samples of both organs from both groups of mice, the percent of cells positive for Type 1 cytokines was substantially higher than the percent of cells positive for Type 2 cytokines. The percent of total IL-$2^+$ cells was 12.4% and 23.8% for spleens and lymph nodes, respectively; in the same samples, IFN$\gamma^+$ cells were 7.4% and 5.4% of the total cells. In contrast, the percent of IL-$4^+$ cells from spleens and lymph nodes was 1.7% and 1.1%, and the percent of IL-$10^+$ cells from the same organs was 1.9% and 0.7%.

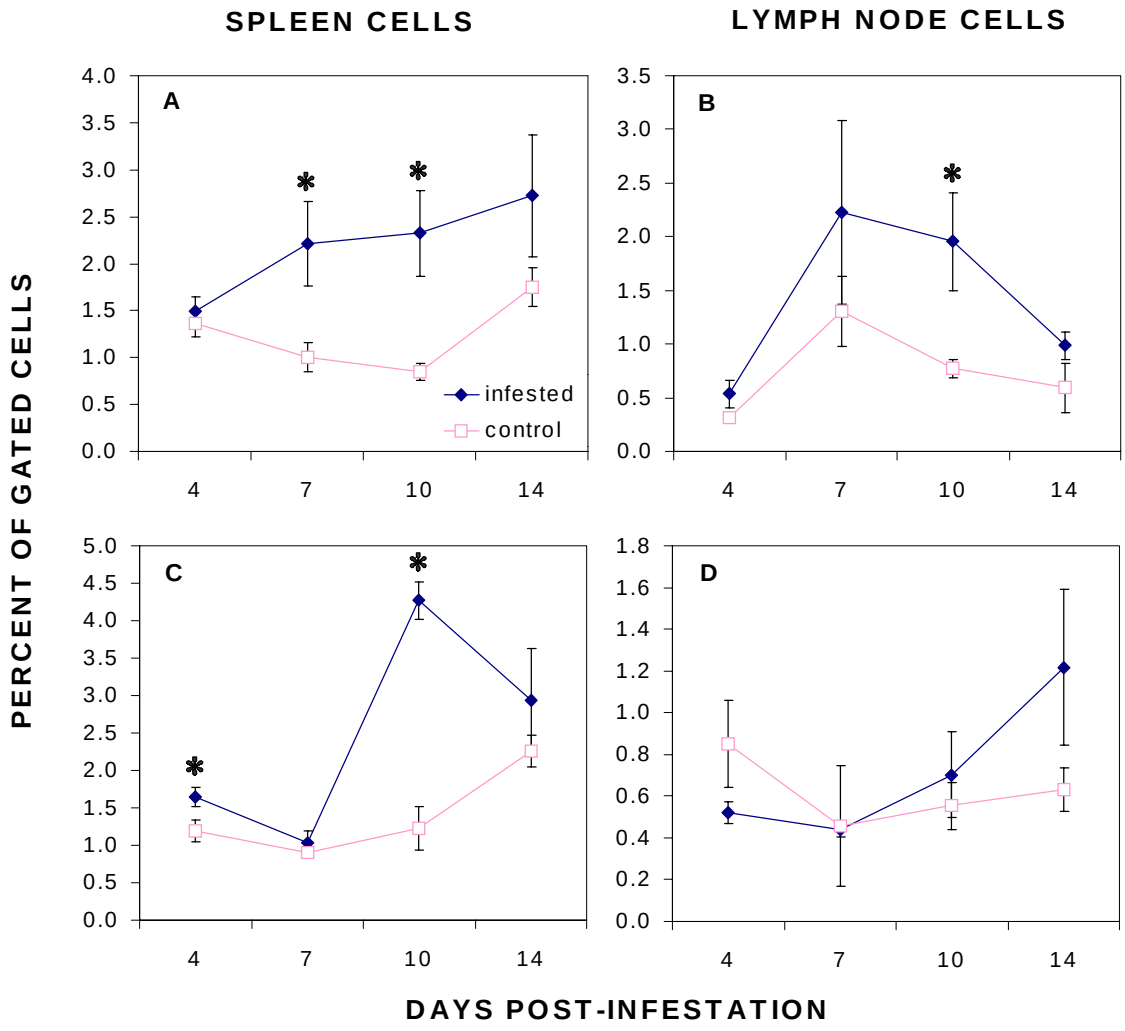


Figure 3.5. Relative proportions of total IL-4$^{+}$ (A, B) and IL-10$^{+}$ (C, D) producing cells in spleens and lymph nodes of infested and control mice. Error bars indicate ± one SE. * = $p < 0.05$

## Discussion

Complete blood counts were done in an attempt to determine if the spleen enlargement observed in infested BALB/c mice on days 7, 10, and 14 post-infestation could be attributed to response to infestation, anemia, or infection. Infested mice were slightly anemic on all four days measured, with cell counts and RBC indices indicative of a replacing anemia (Aiello *et al*. 1998), suggesting that spleen enlargement was primarily due to hematopoesis. Complete blood count results also showed no changes in blood lymphocyte counts or other evidence of infection, supporting our premise that immune changes documented in *A. americanum*-infested mice were not responses to a tick-transmitted pathogen. The enlargement of spleens and lymph nodes have also been documented in other infestation experiments using ticks from laboratory colonies (Mbow *et al*. 1994b, Ganapamo *et al*. 1995, Ferreira & Silva 1999), which suggests that they are a general response to feeding ticks.

$CD4^+$ and $CD8^+$ T cell populations, which are essential for acquired immune responses to many pathogens, were substantially decreased by *A. americanum* infestation. Suppression of helper T cell ($CD4^+$) populations was manifested earlier in lymph nodes local to the bite site than in the spleens of infested mice, as would be expected when dendritic cells from the feeding lesion are activating T cells in the draining lymph nodes before a systemic response occurs (Jankovic *et al*. 2001). Cytotoxic T cell ($CD8^+$) responses show the effect of infestation later and longer than $CD4^+$ cells, presumably because one of the pathways of $CD8^+$ cell activation is through $CD4^+$ cell help. Measured increases in B cell proportions in infested mice did not make up for decreases in the two T cell types; the average differences between total lymphocyte populations (the sum of all $CD4^+$, $CD8^+$, and $CD19^+$ cells) in infested and control mice were as large as the

significant differences measured in T cell proportions, indicating that the effect was specific to T cells. These decreases in T cell populations of infested mice do not correlate with our earlier findings of no significant difference in proliferative response to T-cell mitogens.

Splenic B cell populations were not significantly different between infested and control mice, although we had previously observed suppressed proliferation to the B cell mitogen LPS on days 7, 10, and 14 post-infestation. The lack of significant decreases in proportions of B cells sampled directly from the spleens of infested mice suggests that suppression of proliferation and decreased non-specific antibody production were due to functional inhibition of B cells, such as an inability to respond to the mitogen, rather than decreases in lymphocyte numbers. The B cell populations of draining lymph nodes were increased in infested mice, showing that there was no suppression of B cell populations local to the bite site.

Only one other tick infestation study has directly measured changes in $CD4^+$, $CD8^+$, and B lymphocyte populations in uninfected mice (Zeidner *et al*. 1997). This study used anti-$CD45^+$/B220 to identify B cell populations, but this antibody also binds some $CD8^+$ T cells (BD PharMingen 2000). Zeidner *et al*. (1997) found that in C3H/HeJ mice, B cell numbers were decreased 14% by infestation with *I. scapularis*, while $CD4^+$ and $CD8^+$ cell populations remained unchanged. Infestation with ticks carrying *Borrelia burgdorferi* caused a 109% increase in B cells, and an 88% increase in $CD4^+$ cells, without significantly affecting $CD8^+$ populations. Our results indicate that *A. americanum* infestation of BALB/c mice significantly decreases $CD4^+$ and $CD8^+$ populations, and leaves B cells unaffected. The differences between *I. scapularis* and *A. americanum* effects on cell populations could be attributed to the strain of mouse used,

but can also be interpreted to support our contention that *A. americanum* has a species-specific effect on host cellular responses.

Suppression of $CD4^+$ and $CD8^+$ cell populations, like that caused by the HIV virus, can lead to vulnerability to opportunistic infections. The systemic suppression of cell populations affects both resistance to and recovery from infections, and could be a reason for the susceptibility of heavily tick-infested animals to infections (Gillespie 1998).

Antigen presenting cells are known to control T cell differentiation to Type 1 or Type 2, and can regulate responses to pathogens and parasites; however, mature $CD14^+$ macrophages have only a small role in this process because they are non-migratory (Jankovic *et al*. 2001). Immature, circulating $CD14^+$ monocytes enter tissues on a regular basis but are only heavily recruited to sites of inflammation (Abbas *et al*. 2000). Therefore, it is not surprising that $CD14^+$ cell populations in lymph nodes and spleens were not significantly affected by infestation. The slight increase observed in splenic $CD14^+$ cells could be due to an increase in peripheral blood monocytes settling in the spleens of infested mice. $CD14^+$ tissue macrophage populations will be much more important in regulating the responses at the feeding lesion, and further research on this cell type should focus on the host skin. Further research on the infestation response of antigen-presenting cells in lymphoid organs should look at $CD11c^+$ or other surface molecules expressed by Langerhans cells and dendritic cells.

Although we did not measure MHC $II^+$ antigen presenting cell populations other than macrophages and B cells, we observed that MHC II fluorescence intensity increased in splenic $CD14^+$ and B cells of infested mice. Increase in fluorescence intensity is a crude indicator of increased surface expression, suggesting that MHC II is upregulated in

macrophages of infested mice. This observation does not contradict the *in vitro* finding that tick saliva did not impair macrophage presentation of antigen to antigen-specific cells (Ferreira & Silva 1998). Our previous research did not find significant differences in cytokines produced by adherent cells of infested and control mice, but MHC $II^+$ expression is another aspect of the APC response that merits further investigation.

Our data on intracellular cytokine expression of IL-2 and IL-4 correspond to the pattern of cytokine production we measured previously with ELISA; in both assays, IL-2 was suppressed and IL-4 was upregulated in both spleens and lymph nodes of infested mice. For both cytokines, the largest differences in cytokine production or positive cells were usually seen on day 7 and/or day 10 post-infestation, and significant differences in proportions of positive cells were correlated with significant differences in cytokine production. The similar pattern of results in the IL-2 and IL-4 assays suggests that changes in cytokine production can be attributed to changes in the proportions of cytokine-secreting cells. Therefore, IL-2 suppression by *A. americanum* could be due to a different mechanism than the direct suppression by an IL-2 binding protein found in the saliva of *Ixodes scapularis* (Gillespie *et al*. 2001).

In this experiment, we found that $IFN\gamma^+$ cell populations were not decreased in infested mice as would be expected during Type 1/Type 2 cytokine polarization. The slight increase in $IFN\gamma^+$ cells in samples from spleens and lymph nodes of infested mice contradicted our previous finding (Chapter 2) of downregulation of IFNγ production by mitogen-stimulated spleen cells from infested mice. Although increased levels of IFNγ secretion by lymph node cells (Chapter 2) might be correlated with proportions of $IFN\gamma^+CD4^+$ cells (Fig. 3.4B), our flow cytometry results suggest that differences in IFNγ secretion reflect changes in cell function rather than cell number. Other researchers have

also reported equivocal results with IFNγ production by infested BALB/c mice (Zeidner *et al*. 1997, Schoeler *et al*. 1999, Macaluso & Wikel 2001).

The proportions of cells making IL-10 in this study did not correspond to secreted levels of IL-10 in our previous results (Chapter 2). The highest levels of IL-10 secreted by both spleen and lymph node cells were measured on day 7 post-infestation; on the same day, the proportions of IL-$10^+$ cells were nearly identical in both organs in both groups of mice. Although our earlier cytokine ELISAs measured substantial quantities of IL-10 produced in response to Con A (as much as 8000 pg/ml), the number of IL-$10^+$ cells was very low (less than 5% of gated cells), suggesting that production of this cytokine also does not correlate with the number of positive cells.

Mitogen-stimulated cytokine production does not precisely reflect a T cell's cytokine production *in situ*; however, it can be used to determine the phenotype (Type 0, 1, or 2) of the T cells sampled at a given point in time (Kelso 1998). Typically, Type 0 cells produce IL-2 and IL-4, Type 1 cells produce IFNγ and IL-2, and Type 2 cells produce IL-4 and IL-10 (Abbas *et al*. 2000). In our flow cytometry results, the numbers of cells positive for intracellular cytokines were not reflective of amount of cytokine measured by ELISA – few cells were positive for IFNγ or IL-10 although more pg/ml of those cytokines were produced in culture. Mitogen-stimulated cell suspensions from infested and control mice had more IL-$2^+$ cells than any other cytokine-positive cells, suggesting that most cells were Type 0. The low numbers of $IL4^+$ cells and IL-$10^+$ cells we found relative to IL-$2^+$ and IFN$\gamma^+$ also suggest that more cells were Type 1 than Type 2. This finding could be due to technical limitations of flow cytometry; several studies have reported that Type 1 cytokine positive cells were easier to detect than Type 2 cells, possibly because PMA stimulation preferentially enhanced synthesis of IFNγ and IL-2

and inhibited IL-4 (Cartwright *et al*. 1999, Luttmann *et al*. 1999, Yawalkar *et al*. 2000). Reconciling this finding with our cytokine data on upregulation of Type 2 cytokines will require further research.

Cell-depletion experiments have shown that $CD4^+$ T cells are needed for splenocyte cultures from *I. ricinus*-infested mice to produce the cytokines IL-2, TNF-α, and GM-CSF in response to mitogen (Ganapamo *et al*. 1996a). In *I. scapularis*-infested C3H/HeJ mice, antibody depletion of $CD4^+$ splenocytes abolished production of IL-2 and IL-10, and significantly reduced production of IFNγ and IL-4, whereas depletion of $CD8^+$ splenocytes reduced IL-2, IL-4, and IL-10 production, and had no effect on IFNγ (Zeidner *et al*. 1997). In our study, $CD4^+$ cells were the most significant population of $IL\text{-}2^+$ cells, while most $IFN\gamma^+$ cells were $CD4^+$ or $CD8^+$. Of the very small numbers of cells making IL-4 and IL-10, most were CD4-CD8-, and almost none were $CD8^+$. The only strong correlation we were able to make between cell type and cytokine production were $CD4^+IL2^+$ cells, which have been found to be the substantial majority of $IL\text{-}2^+$ cells in other systems also (Prussin & Metcalfe 1995). Although we can approximately compare the proportions of $CD4^+$ cells from each mouse group that were cytokine positive, future research on the contribution of cell types to cytokine production should test sorted $CD4^+$ and $CD8^+$ cells.

The low numbers of $CD4^+$ positive cells expressing other cytokines could be due to PMA stimulation, which has been shown to decrease $CD4^+$ expression in human peripheral blood samples by as much as 90% after 4 hours incubation (Hennessy *et al*. 2001). However, it is unlikely that $CD4^+$ expression was unaffected in $IL\text{-}2^+$ cells and suppressed in the other cytokine positive cells. In other systems, IFNγ is produced mainly by cytotoxic $CD8^+$ T cells (Prussin & Metcalfe 1995, Mascher *et al*. 1999); further

research is needed explain why infestation affects $CD4^{+}IFN\gamma^{+}$ but not $CD8^{+}IFN\gamma^{+}$ cells. Also, the identity of the $IFN\gamma^{+}$, IL-$4^{+}$ and IL-$10^{+}$ $CD4^{-}CD8^{-}$ cells remains to be determined. These cells may be T cells with a gamma delta TCR ($\gamma\delta$ T cells), which can produce these cytokines and are $CD4^{-}CD8^{-}$. Other $CD4^{-}CD8^{-}$ cells may also have a role in cytokine secretion during *A. americanum* infestation. Natural killer cells produce IFNγ, neutrophils and macrophages secrete IL-10, and B cells have been found to produce IL-2, IL-4, IL-6, IL-10 in quantities equivalent to those made by T cells (Abbas *et al*. 2000, Romani et al. 1997, Harris *et al*. 2000). Since B cells are the major cell type present in lymphoid organs, their contribution to the total cytokine production should be investigated as well.

An additional area that should be addressed in future studies of *A. americanum* infestation is the expression of cytokines at the bite site, where cell phenotypes could influence the host's earliest responses to pathogens transmitted by ticks. Data from draining lymph nodes suggests a mixed response, with upregulation of both IFNγ and IL-4 and suppression of IL-2, but T cell populations are much smaller in the skin (Wikel 1996a).

# CHAPTER 4

# CELLULAR RESPONSES TO *AMBLYOMMA AMERICANUM* INFESTATION AT THE BITE SITE[1]

[1] Ledin, K.E., S. Kumar, S. Singh, and D.E. Champagne. To be submitted to *Parasite Immunology*.

## Abstract

*Amblyomma americanum*, the lone star tick, is a common human-biting tick found throughout the southeastern United States, where it is the primary vector of *Ehrlichia chaffeensis* and the putative vector of *E. ewingii*, and *Borrelia lonestari*. In this study, we investigated responses to *A. americanum* infestation in the immediate vicinity of the bite site to determine if they were correlated with the systemic downregulation of T cell populations and Type 2 polarization of cytokine responses that we had previously measured in spleen and lymph node cells from infested BALB/c mice. We quantified cell populations and cytokine expression with flow cytometry, finding that proportional representation of $CD14^+$ cells increased, $IFN\gamma^+$, $IL\text{-}4^+$ and $IL\text{-}10^+$ cells fluctuated, and $CD4^+$, $CD8^+$, and $IL\text{-}2^+$ cells decreased at the bite site of infested mice in comparison to skin samples from control mice. Using immunohistochemistry, we observed increases in the total numbers of $CD4^+$, $CD14^+$, $IL\text{-}2^+$, and $IL\text{-}4^+$ cells *in situ* in the dermis of the bite site. These complementary methods allowed us to characterize the cellular environment at the bite site as well as assessing the intensity of the response to infestation. To our knowledge, this is the first use of flow cytometry to describe cellular responses at the site of tick infestation.


## Introduction

As a physical barrier to infection and the matrix for a complex network of many immune cells, the skin can be considered the largest immune organ in the body (Abbas *et al*. 2000). Among the many populations of immune cells present in the skin during its response to injury or infection are resident Langerhans cells, dendritic cells, keratinocytes, dendritic epidermal T cells, and tissue macrophages; and infiltrating

neutrophils, eosinophils, basophils, monocytes, natural killer cells, and T cells (Wikel 1996a). These cells are capable of a variety of responses to arthropod infestation or salivary products. Most research on skin responses to tick infestation has focused on inflammatory responses conferring resistance to reinfestation. Histological analyses of primary infestations show infiltration of neutrophils, eosinophils, red blood cells, macrophages/monocytes, and mast cells around the feeding lesion (Brown & Knapp 1980, Mbow *et al*. 1994a). In subsequent infestations of both resistant and non-resistant hosts, numbers of mast cells, eosinophils, and basophils are increased, a classical sign of an immediate hypersensitivity response (Abbas *et al*. 2000). The composition of this cellular infiltration is probably due to a combination of the normal host response to injury, the host response to previously encountered tick antigens, and the pharmacomodulatory components of tick saliva. However, the cells present at and recruited to the feeding lesion can also be considered to be the first line of defense against any pathogens transmitted by the feeding tick.

Cytokine responses at the tick bite site have only been described for infestation with one vector species, *Ixodes ricinus* (Mbow *et al*. 1994b, c). Lymphocyte types and cytokine producing cells were compared between primary and subsequent infestations by *in situ* hybridization and immunohistochemistry, but not related to systemic changes in mouse immune responses.

In our previous experiments, we observed that mice infested with *A. americanum* had reduced T lymphocyte populations and Type 2-polarized cytokine responses in the spleen and draining lymph nodes for up to one week after tick detachment. We hypothesized that systemic immunomodulation would be preceded by similar, local effects at the infestation site. Using BALB/c mice, we developed a method to quantify

cellular responses in the skin at the bite site and compared the results to immunohistochemistry data from bite site samples from the same mice. These skin cells were not stimulated with either antigen or mitogen, so their responses are a direct representation of their phenotype within the host animal.

## Materials and Methods

### Ticks

*Amblyomma americanum* nymphs were acquired from the Oklahoma State University Tick Rearing Facility and housed as described previously (Chapter 2).

### Mice

As described previously (Chapter 2), female BALB/c mice over 8 weeks of age were purchased from Jackson Laboratories and housed in the Animal Resources Facility. All animal procedures were reviewed and approved by the University of Georgia Institutional Animal Care and Use Committee (AUP No. A970180 and A2001-10203).

### Infestation

Anesthetized mice had tick-containment capsules attached to them with rosin/beeswax glue as described previously (Chapter 2). Twenty-four mice were infested with 15 *A. americanum nymphs* in each capsule, for a total of 30 nymphs per mouse. Sixteen control mice did not receive nymphs but were otherwise handled identically to the infested mice. In each of two trials, three infested mice and two control mice were sacrificed at each of four time points: 4 days, 7 days, 10 days, and 14 days after tick

attachment. Mice were checked daily for tick detachment. Replete nymphs were removed and stored as described previously (Chapter 2).

**Bite site dissection and cell preparation**

Mice were sacrificed by cervical dislocation and tick-containment capsules and remaining ticks were removed. Skin dissection and cell incubation procedures were a modification of Belkaid *et al*. (1996). The skin surface under and around the capsules was rinsed twice with 70% ethanol over a 5-minute period. The skin was allowed to dry for 15 minutes, and then the capsule-area skin was dissected and placed dermal-side down in 1 ml of Hank's Balanced Salt Solution (HBSS) (Life Technologies, Rockville, MD) with 15% heat-inactivated fetal calf serum (FCS) (Life Technologies) and 1mg/ml collagenase/dispase (Roche Molecular Biochemicals, Mannheim, Germany). Skin samples were incubated in a 24-well plate at 36°C, 5% $CO_2$for 1 hour. The dermal sides of skin samples were scraped with a sterile scalpel blade, and additional cells removed by grinding the dermal surface, in HBSS, against the bottom of a plastic petri dish with the distal end of the plunger of a sterile 5 ml syringe. Cells were centrifuged and rinsed once with HBSS media and once with tissue culture medium RPMI 1640 supplemented with 200 mM L-Glutamine, 100 mM sodium pyruvate, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml gentamycin, and 10% FCS (Life Technologies). Washed cells were re-suspended in RPMI with 10% FCS. An aliquot of the cells was stained with trypan blue to verify viability and counted with a hemacytometer to determine number of total cells per ml. Cells were suspended to a concentration of $5 \times 10^6$ cells/ml.

**Cell surface molecule staining**

Immunolabeling of cell surface molecules on cells taken directly from the skin was performed following modified procedures of Belkaid *et al*. (1996). An aliquot of each skin cell suspension was centrifuged to remove media and then erythrocytes were lysed by suspending cells in nanopure $H_20$ for 10 seconds followed by immediate addition of 10x phosphate-buffered saline (PBS) at pH 7.0 to make an isotonic 1x PBS solution. Each cell suspension was transferred in 200 µl aliquots to a 96-well V-bottom cell culture plate (Corning Inc., Corning, NY). The plate was spun at 1500 rpm for 7 minutes to remove PBS. Cells were re-suspended and incubated overnight at 4°C in dilutions of fluorochrome-conjugated antibodies for cell surface molecules: fluorescein isothiocyanate (FITC)- anti-CD4, clone GK1.5; allophycocyanin (APC) anti-CD8, clone 53-6.7; and R-phycoerythrin (R-PE) anti-CD14, clone rmC5-3 (BD PharMingen, San Diego, CA). All antibodies were diluted 1:1000 with PAB staining buffer (PBS with 0.1% sodium azide, 0.1% bovine serum albumin (BSA) (Sigma, St. Louis, MO)). Antibodies, antibody dilutions, and stained cells were always kept in the dark and at 4°C or on ice. A control sample was prepared with rat isotype-matched control antibodies for each fluorochrome (rat $IgG_1$, clone R3-34; rat $IgG_{2a}$, clone R35-95, BD PharMingen) to check for non-specific staining. Single- and triple- stained control samples were also prepared to facilitate setting detectors and compensation on the FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). After staining overnight at 4°C, cells were washed twice with 200 µl PAB and stored in PAB at 4°C for 1-4 days until read with the FACSCalibur.

**Intracellular cytokine staining**

Immunolabeling of surface molecules and intracellular cytokines was performed following modified procedures of Belkaid *et al*. (1996). In order to allow cells to accumulate the cytokines they were producing at the time of dissection, an aliquot of each skin cell suspension was incubated in 1µl/ml of the protein transport inhibitor GolgiPlug™ (BD PharMingen) in RPMI for 12 hours at 36°C (Kamhawi, pers.com.). Cell suspensions were centrifuged to remove media, lysed to remove red blood cells, and transferred to a 96-well V-bottom plate as described above. The plate was spun and PBS removed. Cells were re-suspended and pre-incubated for 5 minutes at 4°C with Fc Block™ (anti-CD16/CD32 FcγIII/II receptor antibody, clone 2.4G2, BD PharMingen). Two fluorochrome-labeled antibodies for cell surface molecules, FITC anti-CD4 and APC anti-CD8, or the appropriate controls (BD PharMingen), diluted as described above, were added to the wells and the plate was incubated for 45 minutes at 4°C. Cells were washed twice with 200 µl PAB per well for 7 minutes at 4°C at 1500 rpm and then incubated for 10 minutes at 4°C with 100 µl Cytofix/Cytoperm™ Buffer (BD PharMingen) following the protocol of the Cytofix/Cytoperm™ kit (BD PharMingen). Cells were washed twice with 200 µl Perm/Wash™ solution (BD PharMingen) and then incubated overnight at 4°C with R-PE-conjugated anti-IL-2, clone S4B6; anti-IL-4, clone 11B11; anti-IL-10, clone JES5-16E3; or anti-IFNγ, clone XMG1.2 antibody diluted 1:100 with Perm/Wash™ solution. Cells were washed twice with 200 µl Perm/Wash™ solution, resuspended in PAB, and held at 4°C for 1-4 days until read with the FACSCalibur. Isotype-matched control antibodies (rat $IgG_{2a}$, clone R35-95; rat $IgG_1$,

clone R3-34, BD PharMingen) were used to prepare single and triple-stained control samples concurrently with samples from individual mice.

**Flow cytometry, analysis, statistics**

Cell samples were loaded into 12 x 75mm polystyrene culture tubes (Fisher, Pittsburgh, PA) and data on 10,000 events (or, rarely, the total cell number in each sample if less than 10,000) were acquired with a FACSCalibur flow cytometer (Becton Dickinson). Isotype-matched, single, and multiply stained controls were used to set detectors, compensation, and standard quadrants for separating cell types. Samples were analyzed by size, granularity, and fluorescence with CellQuest™ software (Becton Dickinson) to identify leukocyte subpopulations. Following the methods of Belkaid *et al*. (1996), intracellular cytokine samples were acquired using 2-color analysis, but only PE-anti-cytokine antibody data were used for statistics. Data were exported to Microsoft Excel (Microsoft, Redmond, WA) spreadsheets for analysis by ANOVA and individual pair-wise t-tests for significance at the $p<0.05$ level with the Statview program (SAS Institute, Cary, NC). Data were analyzed as number of positive cells per 10,000 and adjusted by the number of cells harvested from the mouse to determine number of positive cells per bite site.

**Immunohistochemistry**

One infested and one control mouse was sacrificed at each of four time points: 4 days, 7 days, 10 days, and 14 days after tick attachment. Tick-containment capsules and remaining ticks were removed. The skin surface under and around the capsules was rinsed twice with 70% ethanol over a 5-minute period. The skin was allowed to dry for

15 minutes, and then the capsule-area skin was dissected, bisected, and placed into Histo-Prep® tissue capsules (Fisher Scientific, Pittsburgh, PA). Tissue capsules were immersed in liquid nitrogen for 30 seconds and then frozen at –70°C until used for cryosectioning.

Tissue samples were thawed to –20°C, cut to uniform shape, and embedded in Tissue-Tek® OCT Compound (Sakura Finetek, Torrance, CA) on cutting platforms. A Leica CM1800 Cryostat was used to cut 10 μm sections of embedded tissue. Sections were transferred to Gold Seal® Rite-On® micro slides (Becton Dickinson, location) and allowed to dry for 1 hour at room temperature. Sections were fixed by serial saturation in increasing concentrations of acetone, spending 3 minutes each in 60%, 70%, 80%, and 90% acetone.

Fixed slides were air-dried for 20 minutes and then stained according to the manufacturer's protocol for the TSA™ Biotin System (NEN™ Life Science Products, Boston, MA). All procedures except primary antibody incubation were carried out at room temperature, and all were separated by three 5-minute washes in PBS/Tween 20® (EM Science, Gibbstown, NJ). Briefly, slides at room temperature were blocked with 0.5% Blocking Reagent (NEN™) for 30 minutes, quenched for endogenous peroxidases with 0.04% $H_2O_2$ for 10 minutes, and then incubated overnight at 4°C in primary antibody at a 1:100 dilution. Primary antibodies were chosen based on likelihood of detecting positive cells as determined by flow cytometry of similar skin samples. Antibodies used were: anti-CD4, clone RM4-5; anti-CD14, clone rmC5-3; anti-IL-2, clone JES6-5H4; anti-IL-4, clone BVD6-24G2; secondary antibody for CD14, polyclonal anti-rat Ig. After anti-CD14 labeled slides were incubated in secondary antibody for 1 hour, slides were treated with streptavidin-horseradish peroxidase at 1:100 in TSA

Blocking Reagent for 30 minutes (SA-HRP), biotinyl tyramide amplification reagent at 1:50 in PBS, and SA-HRP for an additional 30 minutes. Antibody binding was visualized with diaminobenzidine (Sigma, St. Louis, MO) staining; color development was monitored by microscopy so the reaction could be stopped with $H_2O$ when development was sufficient. Slides were counterstained in Harry's hematoxilin (Sigma), dried, and mounted with coverslips using GelMount (Biomedia Corporation, Foster City, CA).

### Cell quantification

Bright-field microscopy with an Olympus CH30 Biological Microscope (Olympus America Inc., Melville, NY) was used to identify DAB-stained $CD4^+$, $CD14^+$, IL-$2^+$, and IL-$4^+$ cells in the dermal regions of skin sections. Stained cells were counted at 400x magnification in three fields per section from one infested and one control mouse for each of the four sampling dates. The area of skin evaluated per field was 0.159 $mm^2$. Average counts of positive cells for each antibody were compared between control and infested mice and between sampling dates.

## Results

### Infestation

*Amblyomma americanum* feeding results were similar to those previously reported for BALB/c mice (Chapter 2). Approximately 79% of nymphs placed in containment capsules attached and fed on mice. More than 98% of nymphs allowed to feed to repletion completed feeding between day 5 and day 7 post-infestation.

**Flow cytometric analysis of cell surface molecule expression**

The number of cells expressing the surface molecules CD4, CD8, and CD14 were different in skin samples from the tick bite sites of infested animals than in skin samples from the same region on control mice (Fig. 4.1). Proportions of $CD4^+$ and $CD8^+$ T cells per 10,000 cells sampled from the bite site were somewhat lower than numbers of the same cells in skin samples from control mice on days 10 and 14 post infestation, when all ticks had finished feeding (Fig. 4.1A, C), although the total numbers of $CD4^+$ and $CD8^+$ cells at the bite site were slightly higher overall than those in the skin of control mice ($CD4^+$ p= 0.0135, $CD8^+$ p=0.0318) (Fig 4.1B, D). The number of $CD14^+$ cells at the bite site was significantly higher overall than the number of $CD14^+$ cells in samples from control mice (p=0.0002), with significant differences between the groups on days 4, 7, and 14. (Fig. 4.1C, F). The number of unlabeled cells at the bite site also increased on days 10 and 14 post-infestation (data not shown). In both infested and control mice, the majority of labeled cells in each sample of 10,000 cells were $CD4^+$ ($\bar{x}$=209), with were fewer $CD14^+$ cells ($\bar{x}$=77) and $CD8^+$ cells ($\bar{x}$=48%).

**Flow cytometric analysis of intracellular cytokine expression**

Cells sampled from the bite sites of infested mice had different cytokine profiles than cells from the same skin region of control mice (Fig. 4.2, 4.3). Proportions and total numbers of $IFN\gamma^+$ cells at the bite site were significantly increased on days 4 and 7 post-infestation, spanning the time that most ticks completed rapid engorgement (Fig. 4.2A, 4.3A). $IL\text{-}4^+$ cells were also more numerous at the bite site on days 4 and 7 post-infestation (Fig. 4.2C, 4.3C). Both $IL\text{-}2^+$ and $IL\text{-}10^+$ cells at the bite site were present in larger numbers on days 4 and 7 but decreased proportionately on day 10 post-infestation,

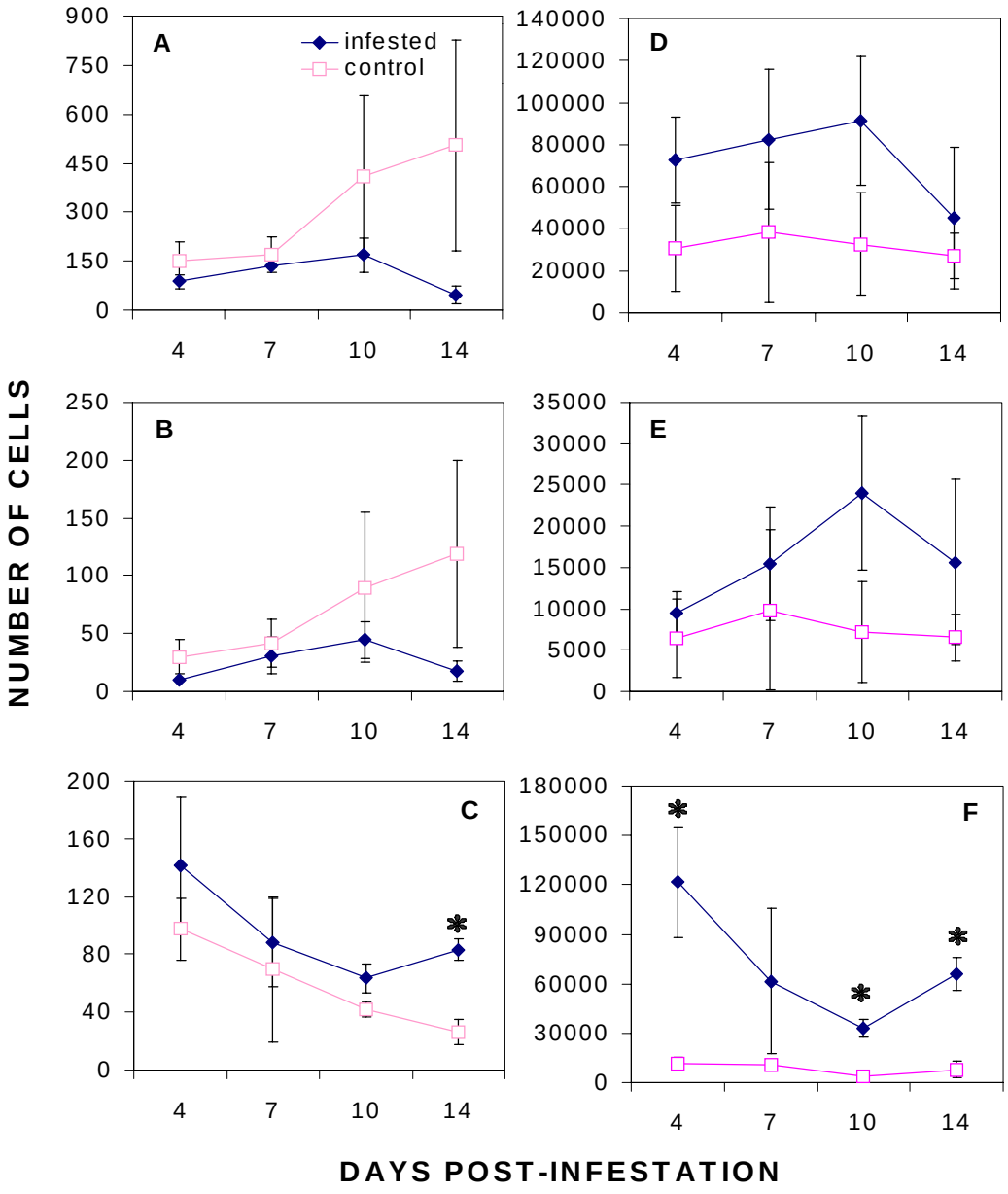


Figure 4.1. Numbers of $CD4^+$ (A, B), $CD8^+$ (C, D), and $CD14^+$ (E, F) cells from the bite site of infested and control mice. Numbers of $CD4^+$ (A), $CD8^+$ (C), and $CD14^+$ (E) cells per 10,000 cells as acquired by flow cytometry. Total numbers of $CD4^+$ (B), $CD8^+$ (D), and $CD14^+$ (F) cells present in the skin sample from the bite site. Error bars indicate ± one S.E. *=$p<0.05$

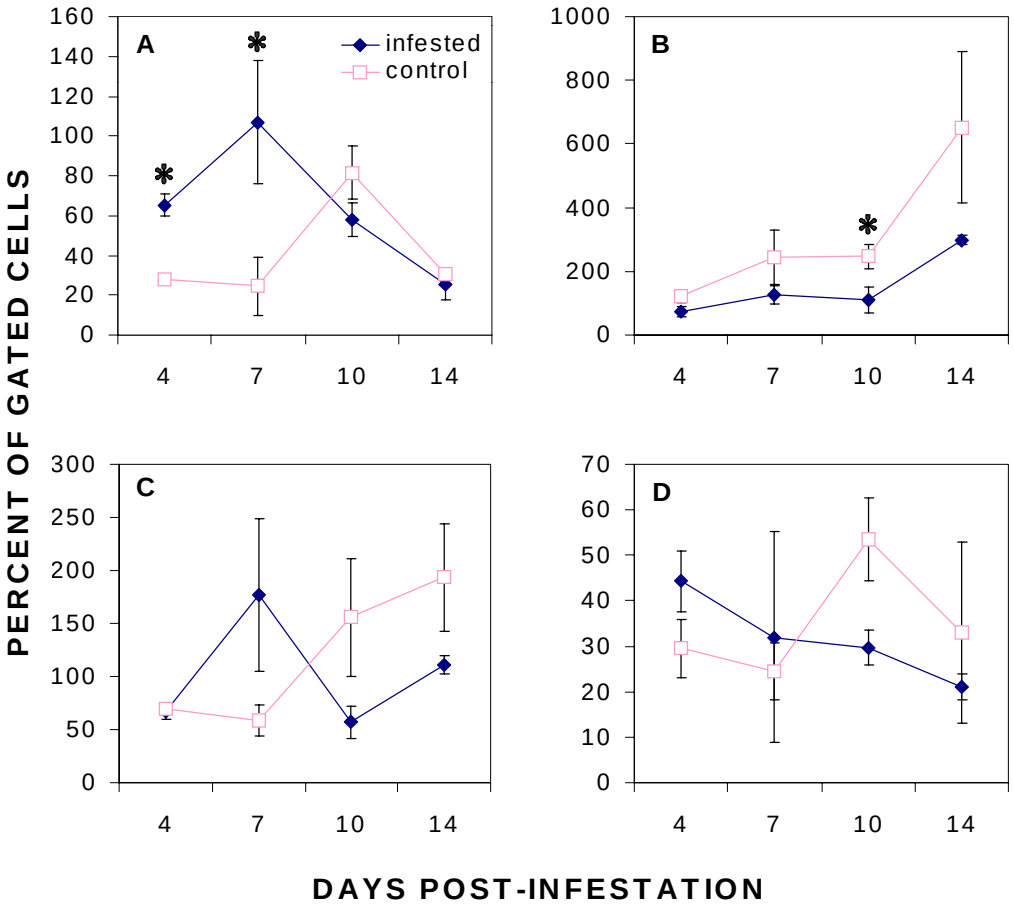


Figure 4.2. Numbers of bite site cells per 10,000 expressing the Type 1 cytokines IFNγ (A) and IL-2 (B) and the Type 2 cytokines IL-4 (C) and IL-10 (D) in infested and control mice. Error bars indicate ± one S.E. *=$p<0.05$

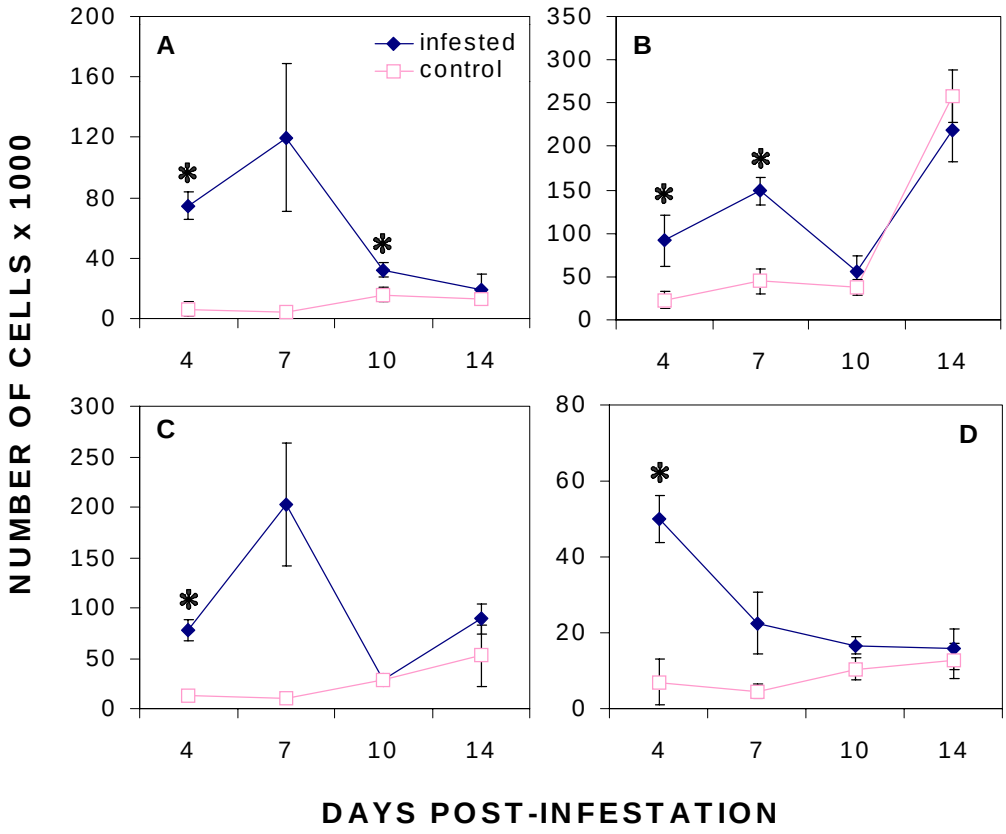


Figure 4.3. Total numbers of cells per bite site expressing the Type 1 cytokines IFNγ (A) and IL-2 (B) and the Type 2 cytokines IL-4 (C) and IL-10 (D) in infested and control mice. Error bars indicate ± one S.E. *=$p<0.05$

when all ticks had completed feeding (Fig. 4,3B, D, 4.2B, D). Proportions of IL-2$^+$ cells at the bite site were lower throughout infestation than IL-2$^+$ cells from the skin of control mice (Fig. 4.2B) (ANOVA $p < 0.001$), although numbers of IL-2$^+$ cells were slightly higher in samples from infested mice than from controls. In general, skin samples from both infested and control mice had more IL-2$^+$ cells per 10,000 cells ($\bar{x}$=234) than IFN$\gamma^+$ ($\bar{x}$=53), IL-4$^+$ ($\bar{x}$=111), or IL-10$^+$ ($\bar{x}$=33) cells.

**Immunohistochemistry**

Skin sections taken from the bite sites of infested mice were 0.5-1.0 mm thicker and had more cells positive for the cell surface markers and intracellular cytokines of interest than sections from the same region of skin on control mice. Bite site skin sections had increased dermal thickness due to more numerous cells in that tissue layer (Fig. 4.4). In sections from infested mice on days 10 and 14 post-infestation, after all ticks had detached, inflammation extended from the epidermis to the muscle layer in the lower dermis. Counts of all DAB-stained cells were higher in infested than control mice (Table 4.1). Differences between sections from infested and control mice were apparent on day 4 post-infestation for CD14$^+$ cells, on day 7 for CD4$^+$ and IL-2$^+$ cells, and on day 10 for IL-4$^+$ cells. CD14$^+$ cells had the highest counts overall, and IL-2$^+$ cells the lowest counts, of all DAB-stained cells. CD14$^+$ cells increased in bite site sections on day 4 post infestation, when ticks were just beginning rapid engorgement, and were substantially higher than CD14$^+$ counts in control sections on days 7 and 14 post-infestation (Fig. 4.4B, C, E, F). We also observed that CD14$^+$ cells in the dermis of the bite site appeared in clusters on day 7 but were evenly distributed throughout the tissue similarly to other labeled cells on day 14 post-infestation (Fig 4.4A, D, 4.5A, D). We did not have

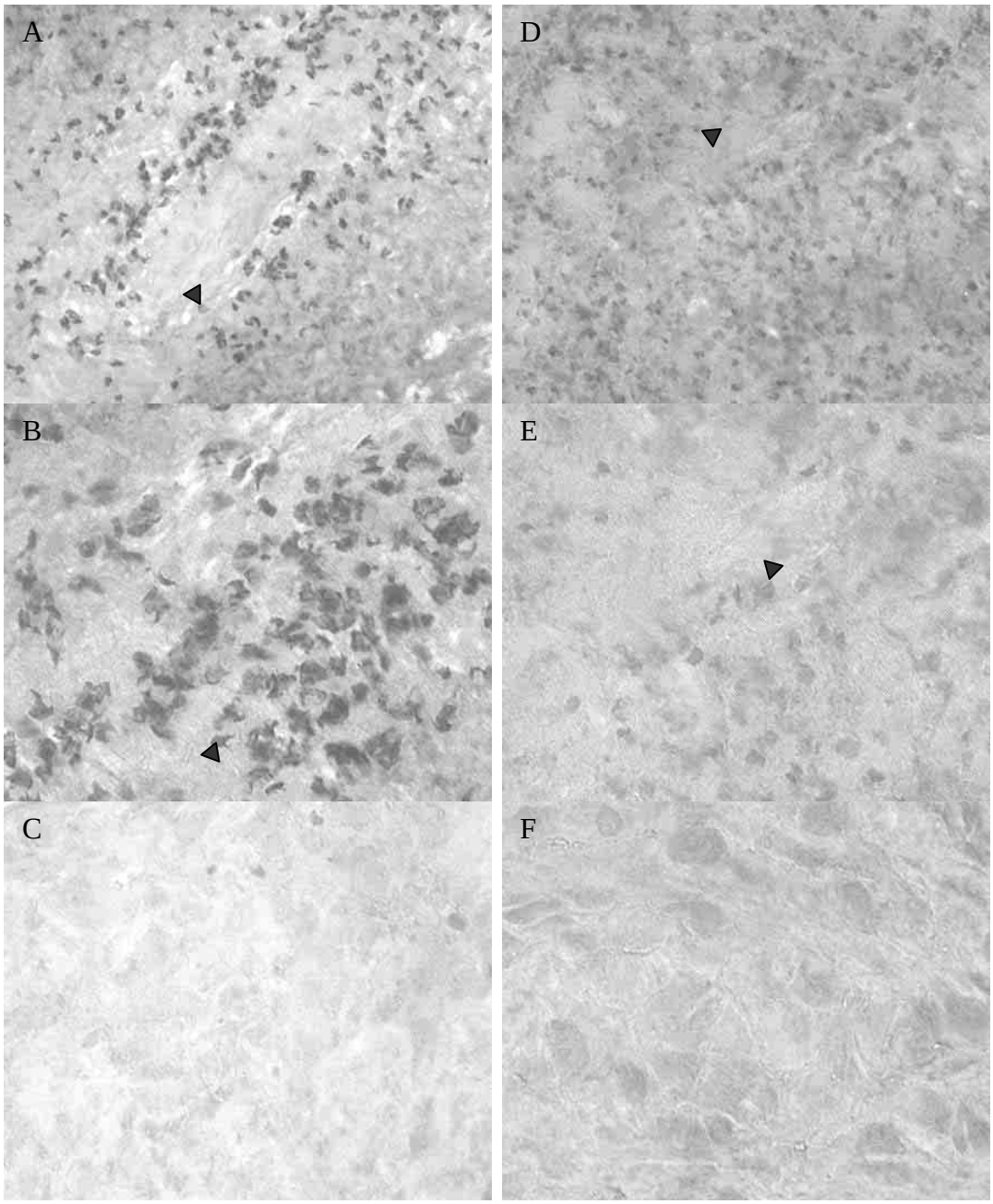


Figure 4.4. Distribution of $CD14^+$ cells at the bite site of an infested BALB/c mouse on day 7 at 200x (A) and 400x (B) and day 14 at 200x (D) and 400x (E). Anti-CD14-stained skin samples from a control mouse at 400x on day 7 (C) and day 14 (F). Arrowheads indicate one $CD14^+$ cell.

Table 4.1. Average number of DAB-stained $CD4^+$, $CD14^+$, IL-$2^+$, or IL-$4^+$ cells counted in three 0.159 $mm^2$ dermal regions of skin sections from one infested and one control mouse, ± s.e.

| | Day 4 | Day 7 | Day 10 | Day 14 |
|---|---|---|---|---|
| Infested $CD14^+$ | 8.00 ± 1.41 | 128.67 ± 2.16 | ND | 113.00 ± 3.54 |
| Control $CD14^+$ | 2.33 ± 0.41 | 1.00 ± 0.71 | 2.00 ± 0.0 | 2.00 ± 0.71 |
| Infested $CD4^+$ | 0.33 ± 0.41 | 5.67 ± 1.78 | 8.33 ± 1.78 | 84.67 ± 5.40 |
| Control $CD4^+$ | 0.00 | 0.00 | 2.00 ± 1.41 | 0.00 |
| Infested $IL2^+$ | 2.33 ± 0.41 | 5.67 ± 0.41 | 10.33 ± 1.78 | 41.67 ± 6.01 |
| Control $IL2^+$ | 3.00 ± 0.0 | 2.00 ± 0.0 | 0.00 | 1.00 ± 0.71 |
| Infested $IL4^+$ | 0.00 | 0.00 | 53.33 ± 4.32 | 52.67 ± 10.98 |
| Control $IL4^+$ | 0.00 | 0.67 ± 0.41 | 0.00 | 0.00 |

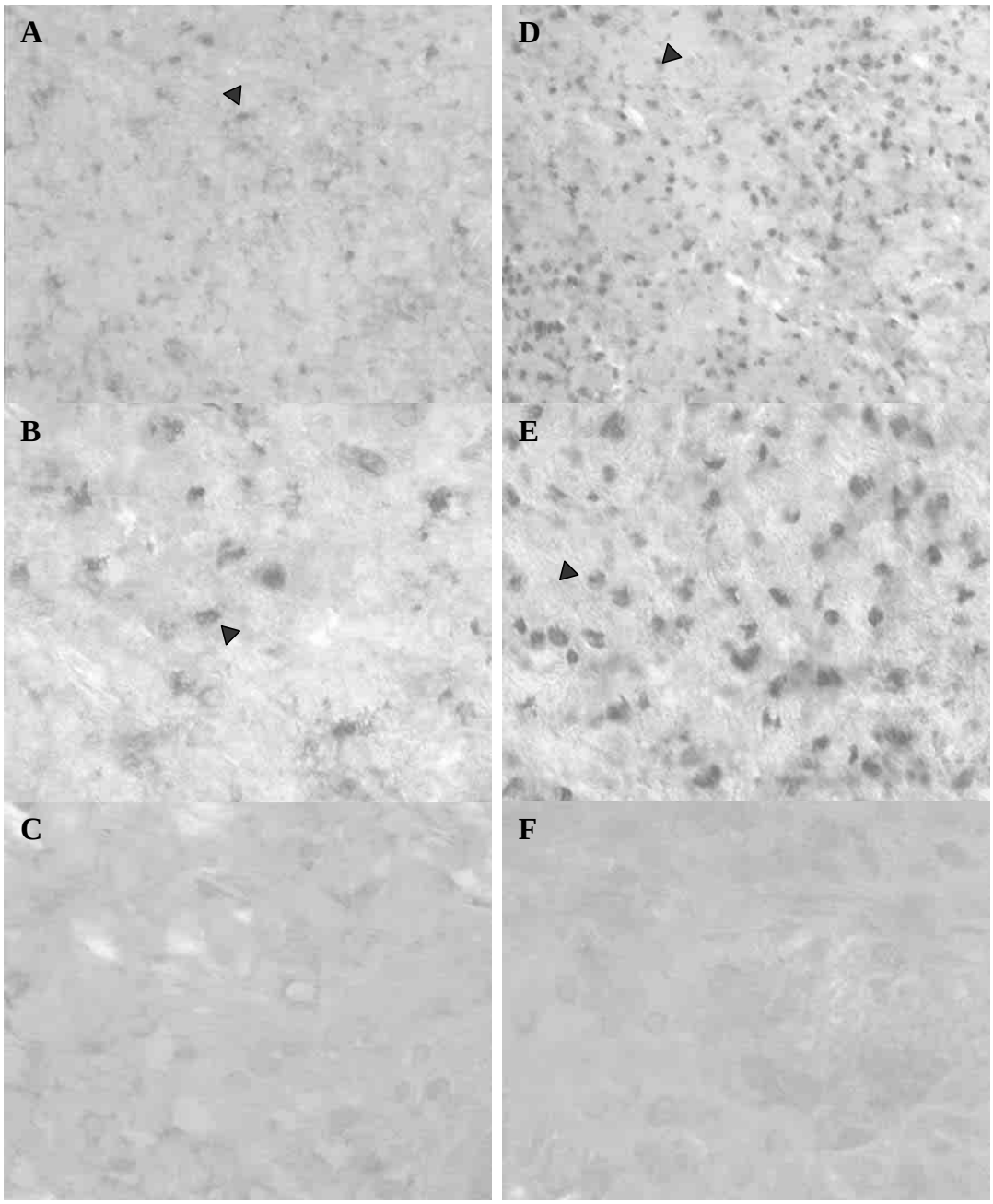


Figure 4.5. Distribution of $CD4^+$ cells at the bite site of an infested BALB/c mouse on day 7 at 200x (A) and 400x (B) and day 14 at 200x (D) and 400x (E). Anti-CD4-stained skin samples from a control mouse at 400x on day 7 (C) and day 14 (F). Arrowheads indicate one $CD4^+$ cell.

sufficient tissue to detect $CD14^+$ cells in bite site sections from day 10. $CD4^+$, $IL\text{-}2^+$, and $IL\text{-}4^+$ cells appeared to be evenly distributed throughout the dermis of the bite site on all days sampled (Fig.4.5, 4.6, 4.7). Counts of DAB-stained $CD4^+$ and $IL\text{-}2^+$ cells increased in bite site sections on day 7, just after most ticks completed rapid engorgement (Fig. 4.5B, C, 4.6A, B), and continued to increase up to day 14 post-infestation, when all ticks had been detached for at least 4 days (Fig. 4.5E, F, .4.6C, D). $IL4^+$ cells were not increased in bite site sections on day 7 (Fig. 4.7A, B) but were more numerous on days10 and 14 post-infestation, after all ticks had finished feeding (Fig.4.7C, D). In bite site sections, $IL4^+$ cells were five times as numerous as $IL\text{-}2^+$ cells on day 10, and remained slightly more in number than $IL\text{-}2^+$ cells on day 14 post-infestation (Table 4.1). On day 10 post-infestation, bite site samples also had six-fold more $IL\text{-}4^+$ cells than $CD4^+$ cells. It was not possible to quantify unstained cells with this method.

## Discussion

When an infected tick attaches to a host, the pathogens it transmits must first pass through the host skin at the bite site before disseminating to cause systemic infection. The effectiveness of the complex immune environment of the skin as a barrier to pathogens may be compromised by tick infestation (Nuttall 1998). Infestation with *A. americanum* has been shown to alter cellular responses in systemic and local lymphoid organs (Chapters 2, 3). Results from this study suggest that responses at the bite site are affected as well. The two methods used in this study, flow cytometry and immunohistochemistry, provided complementary information about the composition of the cellular environment at the bite site. With flow cytometry, it was possible to quantify cells of interest in a three-dimensional volume of skin and describe the phenotype of the

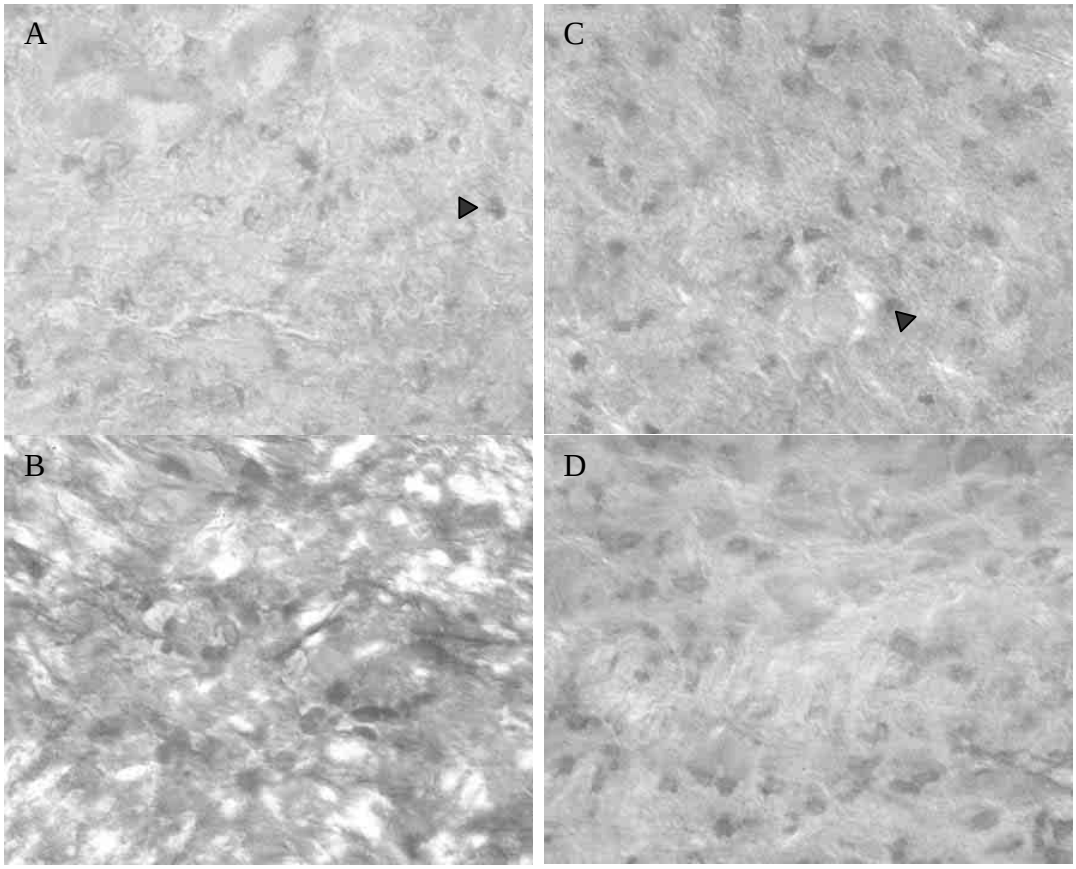


Figure 4.6. IL-$2^+$ cells at the bite site of an infested BALB/c mouse at 400x on day 7 (A) and day 14 (B). Anti-IL-2-stained skin samples from a control mouse at 400x on day 7 (C) and day 14 (D). Arrowheads indicate one IL-$2^+$ cell.

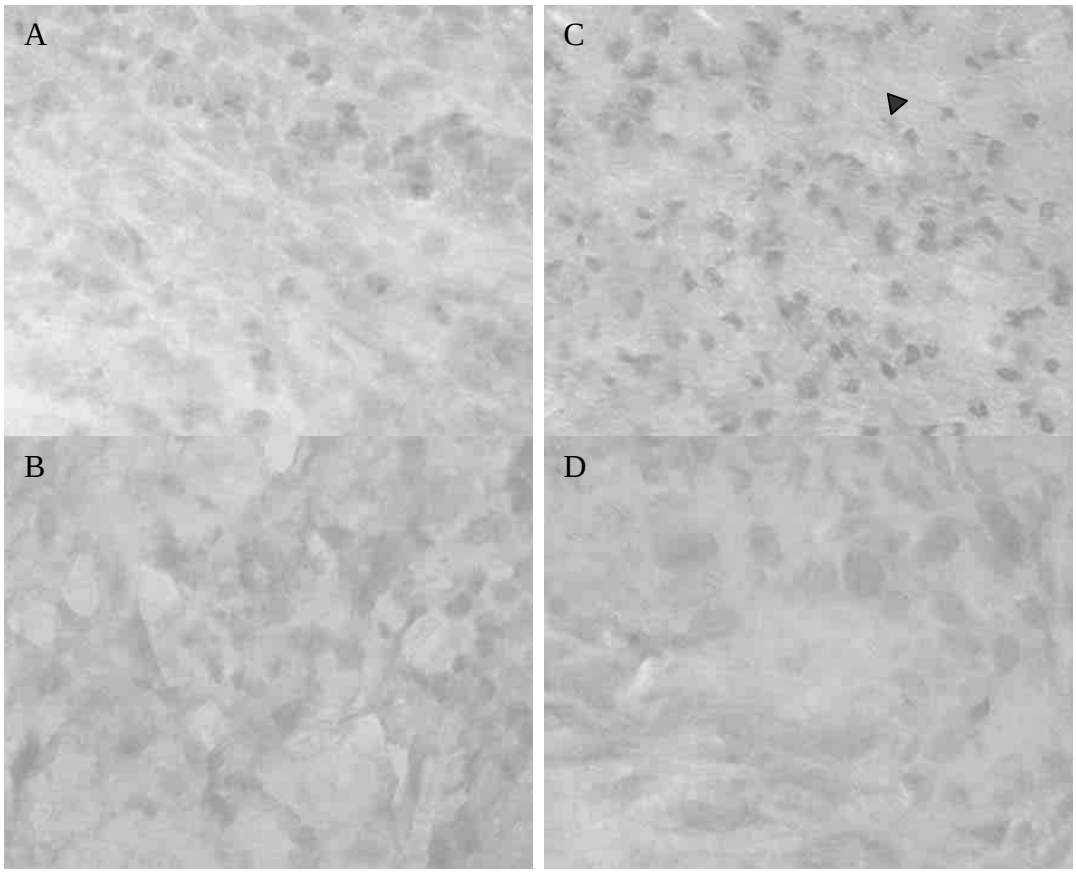


Figure 4.7. IL-4$^{+}$ cells at the bite site of an infested BALB/c mouse at 400x on day 7 (A) and day 14 (B). Anti-IL-4-stained skin samples from a control mouse at 400x on day 7 (C) and day 14 (D). Arrowhead indicates one IL-4$^{+}$ cell.

response by comparing numbers of cell types among samples obtained from infested and control mice. Immunohistochemistry allowed quantification of cells of interest in two-dimensional sections as well as qualitative assessment of the location and distribution of labeled cells. Using these techniques, we measured cell surface molecule-expression and cytokine-positive cells in order to compare the phenotype of the cells at the tick bite site to the same skin area in control mice.

The surface molecule CD14 is expressed by macrophages and monocytes, which are innate immune cells that reside in and infiltrate skin and other tissues during immune responses (Abbas et al. 2000). $CD14^+$ cells can process foreign antigens and present them to $CD4^+$ T cells, and might have an important role in determining T cell responses in non-lymphoid tissues through antigen presentation, cytokine secretion, and co-stimulatory molecules (Jankovic *et al*. 2001). A homologue of human Macrophage Migration Inhibitory Factor (MMIF), a T cell cytokine that increases macrophage populations at sites of inflammation, was recently identified in salivary gland and midgut tissues of *A. americanum* (Jaworski *et al*. 2001). In keeping with the finding that recombinant *A. americanum* MMIF inhibited migration of human macrophages *in vitro* (Jaworski *et al*. 2001), we observed that *A. americanum* infestation of mice increased dermal macrophage/monocyte populations *in vivo*. It is not clear whether the MMIF homologue of *A. americanum* was present in the saliva during infestation, or if its presence enhanced tick feeding or was an inadvertent attractant for monocytic cells.

Numbers of $CD14^+$ cells at the bite site of infested mice were consistently higher than those in the skin of control mice (Figure 4.1E, F). Increased numbers of $CD14^+$ cells were also counted in skin sections with immunohistochemistry (Table 4.1), supporting the conclusion that the populations of $CD14^+$ cells at the bite site were much larger than

in control mice. The average proportions of $CD14^+$ cells in the skin at the bite site were similar to proportions of $CD14^+$ cells in the spleens and higher than proportions of $CD14^+$ cells in the lymph nodes of BALB/c mice in our previous study (Fig. 2.2). Our previous study did not find differences in proportions of $CD14^+$ cells in spleens and lymph nodes of infested and control mice (Chapter 3), although the proportions of $CD14^+$ cells were similar to those found in the skin. The results of these two studies suggest that this cell type is affected by infestation locally at the bite site but not systemically in the lymphoid organs.

The number of $CD4^+$ and $CD8^+$ T cells present in the skin is usually low. Although some T cells will infiltrate skin during immune responses, they are typically greatly outnumbered by the influx of neutrophils and other innate immune cells (Wikel *et al*. 1996b). Although counts of $CD4^+$ cells in immunohistochemistry sections from the bite site increased on days 7, 10, and 14 post-infestation, they were never as numerous as $CD14^+$ cells (Table 4.1). Using flow cytometry, we observed that the numbers of both $CD4^+$ and $CD8^+$ T cells at the bite site were higher relative to the numbers of the same cells in the skin of control mice over time (Fig. 4.1B, D), but proportions of these cells declined (Fig.4.1A, C) concurrently with increases in dermal inflammation observed in immunohistochemistry sections (Fig. 4.4), suggesting that populations of other inflammatory cells were recruited to the bite site in higher numbers than T cells. In contrast, $CD4^+$ and $CD8^+$ cells in the skin of control mice increased over sample times, possibly because inflammatory cells decreased after the initial irritation of attaching the capsules subsided. The large numbers of unstained inflammatory cells in the dermis of the bite site (Fig. 4.4) were most likely populations of neutrophils, eosinophils, and erythrocytes observed in previous histological studies of the feeding lesion created by *A.*

*americanum* (Brown & Knapp 1980). Relatively small populations of lymphocytes were observed in primary infestations of *A. americanum* and *I. ricinus* (Brown & Knapp 1980, Mbow *et al*. 1994c). $CD4^+$ and $CD8^+$ T cells are important in antigen-specific delayed-type hypersensitivity reactions (DTH) (Abbas *et al*. 2000), and proportions of both cell types would probably increase with re-infestation when cells recognized and responded to tick antigens, as was observed in secondary and tertiary infestations with *Ixodes ricinus* (Mbow *et al*. 1994c).

The cytokine environment of the bite site may have an important influence on host responses to pathogens introduced by feeding ticks. Increases in IFNγ can upregulate macrophage activation and expression of MHC II molecules, while increases in IL-4 can inhibit macrophage activation (Abbas *et al*. 2000). Interferon-gamma and MHC II-antigen complexes are important for promoting Type 1 T cell responses (Jankovic *et al*. 2001) and the development of delayed hypersensitivity responses, both of which can be protective against intracellular infections (Belkaid *et al*. 2000). However, large amounts of soluble antigen, such as that present in tick saliva, introduced in the absence of macrophage-activating microbial structures, can induce Type 2 responses (Jankovic *et al*. 2001).

Flow cytometry of bite site skin indicated that infestation did not affect the two Type 1 cytokines the same way: $IL\text{-}2^+$ cells were decreased by infestation, while $IFN\gamma^+$ cells were increased (Fig. 4.2). These results echoed our previous findings of consistent, significant decreases in $IL\text{-}2^+$ cells and IL-2 secretion by mitogen-stimulated cells from spleens and lymph nodes and subtle increases in $IFN\gamma^+$ cells and IFNγ secretion by cells from lymph nodes of infested BALB/c mice (Chapter 2, Chapter 3). Increased counts of DAB-stained $IL\text{-}2^+$ cells in bite site sections did not contradict our flow cytometry

findings; numbers of DAB-stained IL-$2^+$ cells remained small (Table 4.1), suggesting that IL-$2^+$ cells made up only a small proportion of the total response. A similar study on mice infested with *Ixodes ricinus* was able to identify very small numbers of IFN$\gamma^+$ and IL-$4^+$ cells in sections from primary infestations but no IL-$2^+$ cells until the secondary and tertiary infestations (Mbow *et al*. 1994c).

Flow cytometry of bite site samples indicated that the number of cells expressing Type 2 cytokines increased somewhat during infestation and then decreased after all ticks had detached (Fig. 4.2). There were similarities in the pattern of IL-4 production in skin to that previously observed in the lymph nodes and spleen cells of infested BALB/c mice; in all assays, IL-4 was upregulated on day 7, when most ticks had just completed rapid engorgement (Fig.4.2, 2.4, 3.4). Interestingly, IL-$4^+$ cells were found greater numbers in skin samples from infested and control mice than in mitogen-stimulated cultures of spleens and lymph nodes in previous experiments (Chapter 3). Numbers of IL-$4^+$ cells at the bite site were greater than IFN$\gamma^+$ cells on days 4,7, and 14 post-infestation and equal to IFN$\gamma^+$ cells on day 10 (Fig. 4.2). Counts of DAB-stained IL-$4^+$ cells were higher than counts of IL-$2^+$ cells on days 10 and 14 in bite site sections, also suggesting that the bite site had a Type 2 cytokine environment (Table 4.1).

Three of the four cytokine-positive cells measured with flow cytometry were present in relatively greater numbers at the bite site during tick attachment and feeding, but were more rare at the bite site than in control skin on days 10 and 14 post-infestation. This post-infestation decrease in IL-$4^+$, IL-$10^+$, and IFN$\gamma^+$ cells at the bite site could be due to proportionate increases in other cells migrating to the bite site after all ticks had detached.

In addition to T cells, there are several different innate cell types in the skin that can also produce what are considered Type 1 and Type 2 cytokines: natural killer cells and mast cells secrete IFNγ, mast cells secrete IL-4, and macrophages, monocytes, mast cells, keratinocytes, and neutrophils produce IL-10 (Wikel *et al*. 1996b, Romani *et al*. 1997, Abbas *et al*. 2000). The changes in proportions of cytokine-producing cells during infestation could be due to a variety of cell types, but autofluorescence of skin cell populations prepared for intracellular cytokine staining precludes labeling with more than one fluorochrome-conjugated antibody per sample. Sorting by cell surface markers prior to incubation with protein transport inhibitor would permit separate analysis of cytokine production by each cell population.

As indicated in reports of protection against pathogen transmission by pre-exposure to uninfested vectors (Wikel *et al*. 1997, Belkaid *et al*. 2000), the response to natural challenge can be entirely different from laboratory models of vector-borne disease. Evidence of changes in cellular responses at the bite site of *A. americanum* infestation in our study suggests that pathogens transmitted by this vector may encounter a more permissive environment for host infection. Infestation with *B. burgdorferi* infected *I. scapularis* nymphs polarizes host responses further towards Type 2 than infestation with pathogen free nymphs (Zeidner *et al*. 1997). Alternatively, the presence of pathogen-associated microbial structures, which are lacking in tick saliva, may provide the stimulus for Type 1 antigen-presenting cell responses (Jankovic *et al*. 2001). Also, repeated infestations may lead to the development of a delayed-type hypersensitivity response in the host skin at the bite site, which can be protective against intracellular pathogens (Belkaid et al. 2000). An interesting application of flow cytometry will be to compare the development of protective or permissive responses at the site of tick

infestation during natural transmission of a tick-borne pathogen to naïve and previously tick-infested mice.

# CHAPTER 5

# EFFECTS OF *AMBLYOMMA AMERICANUM* INFESTATION ON ANTIGEN-SPECIFIC CELLULAR RESPONSES[1]

[1] Ledin, K.E., S. Kumar, S. Singh, and D.E. Champagne. To be submitted to *Applied and Experimental Acarology*.

## Abstract

The effects of *Amblyomma americanum* infestation were examined in a BALB/c-TgN (DO11.10) 10Loh model to evaluate tick-induced modulation of antigen-specific responses to OVA peptide. Infestation with *A. americanum* nymphs did not affect proliferative response to this specific antigen, but infestation decreased proportions of antigen-specific cells, $CD4^+$ cells, and $CD19^+$ cells in spleens and lymph nodes of BALB/c-TgN mice. In response to specific antigen, spleen and lymph node cells from infested mice also had increased production of the Type 2 cytokines IL-4, IL-10, and IL-13, and reduced production of the Type 1 cytokine IL-2 but not IFNγ. Increases in proportions of antigen-specific cytokine-producing cells were associated with increases in cytokine secretion for Type 2 cytokines but not Type 1 cytokines. In this antigen-specific model, *A. americanum* infestation enhanced Type 2 cytokine responses in conjunction with suppression of the major lymphocyte subsets.


## Introduction

The specific immune response is an innovation of the vertebrate immune system that enhances protective responses against infectious organisms. The features of clonal proliferation and immune memory make possible amplification and continuation of specific responses. While a number of bacterial surface molecules and other mitogens will induce non-specific proliferation by interacting with pattern-recognition receptors (PRRs) on a variety of cell types, a peptide fragment from a foreign organism is only recognized by a few, specific T and B cells (Abbas *et al*. 2000). Typically, specific responses take several days or weeks to develop fully, but the population of specific cells

persists for much longer, so the host is better prepared for subsequent challenges with the same antigen. Although innate cellular responses are activated more quickly by PRR recognition of general microbial features, they do not recognize specific antigens, and therefore do not have the specific immune response's capacity for amplification or specific memory (Abbas *et al* 2000).

Somatic recombination of the T cell receptor genes during cell maturation allows T cells to express a large number of different T cell receptors (TCRs) that recognize residues of protein antigens displayed by major histocompatibility complex (MHC) molecules on other cells (Abbas *et al*. 2000). During T cell maturation in the thymus, T cells undergo positive and negative selection to ensure that their TCRs recognize self-MHC molecules and foreign antigen but do not have high avidity for self antigens (Abbas *et al*. 2000). Although few T cells express a particular TCR, antigen-mediated activation of a T cell will lead to clonal proliferation, resulting in multitudes of identical T cells with TCRs specific for that antigen. Naïve T cells are activated with two signals from an antigen-presenting cell; first, an MHC-antigen complex binds to the TCR and the T cell surface molecule CD4 or CD8. Second, costimulatory molecules on the antigen-presenting cell bind to accessory molecules on the T cell. $CD4^+$ T cells recognize antigens displayed by MHC II molecules on antigen presenting cells (APCs), typically dendritic cells, monocytes, and macrophages (Abbas *et al*. 2000).

In our search of existing literature, we found only two previous studies that have looked at the effect of tick exposure on the specific response to antigens. In the first, repeated infestations of cattle with *Amblyomma variegatum* reduced the levels of specific antibodies to OVA peptide on the third and fourth infestations (Inokuma *et al*. 1993). In

the second, exposure of keyhole-limpet hemocyanin (KLH)-specific T cells to saliva of *Rhipicephalus sanguineus in vitro* significantly reduced the ability of these cells to proliferate in response to KLH presented by macrophages (Ferreira & Silva 1998). Many other tick immunomodulation studies have examined the effect of tick exposure on the specific cellular or antibody response to tick antigens (Appendix 1). The results of these experiments merely demonstrated that the sensitization of the host to a variety of tick antigens could be detected upon subsequent exposure. Although tick infestation did not prevent the eventual development of an antigen-specific memory response to tick salivary antigens, no control group was available to compare antigen-specific responses because uninfested hosts do not recognize tick antigens.

In our previous experiments, we observed that infestation of BALB/c mice by *A. americanum* nymphs affects a variety of T cell responses, decreasing the proportions of cells expressing $CD4^+$ and $CD8^+$ surface molecules and increasing mitogen-stimulated production of Type 2 cytokines (Chapter 2). We also found reductions in B cell proliferative responses to LPS and in levels of circulating antibody isotypes. All of these responses are important during exposure to infectious organisms, including pathogens transmitted by ticks. However, pathogens trigger antigen-specific host responses, which can be very different from nonspecific responses to mitogen.

In this experiment, we modeled this interaction by studying the impact of *A. americanum* infestation on peptide-specific responses of a transgenic mouse in which the majority of circulating $CD4^+$ cells bears the same peptide-specific T cell receptor (TCR). We decided to use the model system of BALB/c-TgN (DO11.10) 10Loh mice and OVA peptide from ovalbumin. Because many of the changes we observed previously in

BALB/c mice were in $CD4^+$ cells, we used a mouse model from the same genetic background with a transgenic $CD4^+$ TCR. BALB/c-TgN (DO11.10) 10Loh mice carry an MHC II restricted, rearranged T cell receptor transgene specific for ovalbumin (Murphy *et al*. 1990). The majority of $CD4^+$ T cells in these mice expresses this transgene and therefore responds to the OVA peptide. Selection for this OVA-TCR occurs during T cell maturation, so these OVA-specific cells are naïve T cells, not memory cells generated after exposure to antigen. Our intention was to use the response of these cells to OVA antigen as a model for the response of T cells from naïve mice to first exposure to a pathogen transmitted during tick infestation. Based on the results of our previous experiments, we hypothesized that cells from *A. americanum*-infested BALB/c-TgN mice would show a polarization of cytokine responses to Type 2 and reduction in $CD4^+$ cells, as well as a reduction in antigen-specific proliferation.

## Materials and Methods

### Mice

Eight week old female BALB/c-TgN (DO11.10) 10Loh mice were purchased from Jackson Laboratories (Bar Harbor, ME) or obtained from in-house stocks (UGA Cellular Biology Department) and housed in the Animal Resources Facility as described previously (Chapter 2). All animal procedures were reviewed and approved by the University of Georgia Institutional Animal Care and Use Committee (AUP No. A970180 and A2001-10203).

**Ticks**

*Amblyomma americanum* nymphs were acquired from the Oklahoma State University Tick Rearing Facility and housed as described previously (Chapter 2).

**Infestation**

Anesthetized mice had tick-containment capsules attached to them with rosin/beeswax glue as described previously (Chapter 2). Twenty-four mice were infested with 15 *A. americanum nymphs* in each capsule, for a total of 30 nymphs per mouse. Sixteen control mice did not receive nymphs but were otherwise handled identically to the infested mice. In each of two trials, three infested mice and two control mice were sacrificed at each of four time points: 4 days, 7 days, 10 days, and 14 days after tick infestation. Mice were checked daily for tick detachment. Replete nymphs were removed and stored as described previously (Chapter 2).

**Lymphocyte preparation**

Mice were sacrificed and dissected to remove the spleen and inguinal (draining) lymph nodes. As previously described (Chapter 2), spleens were disrupted between two sterile frosted glass slides into RPMI media. Lymph nodes were dissected from surrounding tissue and disrupted into media by grinding with the plunger of a sterile 5 ml syringe. Cells were centrifuged and rinsed twice with media and then counted with a hemacytometer to determine number of mononuclear cells per ml. A portion of the spleen cells and all of the lymph node cells were suspended to a concentration of $5 \times 10^6$ cells/ml.

**Lymphocyte proliferation assay**

Splenocyte cell suspensions were adjusted to 5 x $10^6$ cells/ml, and 100 µl of each suspension (5 X $10^5$ cells) was dispensed per well in a 96 well microtiter plate. Each cell suspension was assayed in triplicate for proliferative response to stimulation with different concentrations (0.3, 1.0, and 3.0 µM) of OVA peptide (aa 323-339 of chicken ovalbumin protein, sequence SQAVHAAHAEINEAGRE, synthesized at the Molecular Genetics and Instrumentation Facility of the University of Georgia, Athens, GA) or 5.0µg per ml of the mitogens Con A or LPS. Negative controls for stimulation received medium only. After 54 hours of incubation at 37°C, 5% $CO_2$, cells were pulsed with one µCi of methyl-$^3$H Thymidine (Amersham Life Science, Piscataway, NJ) per well and incubated for 18 hours further. Cells were harvested using an automated cell-harvester (Skatron Inc., Sterling, VA). Incorporation of radioactivity was determined with a liquid scintillation counter (Beckman Instruments, Fullerton, CA). Mean counts per minute (CPM) ± SE were determined for each treatment.

**Quantification of secreted cytokine production**

One ml (5 X $10^6$ cells) of each lymphocyte suspension was incubated with 1.0 µM OVA in a 24 well plate (total volume two ml). After 48 hours, contents of wells were centrifuged at 12,000 rpm for 5 minutes to remove cellular debris, and supernatants were frozen at –70°C until used for quantification of secreted cytokine production. Supernatants were assayed for levels of Type 1 (IL-2, IFNγ) and Type 2 (IL-4, IL-10, IL-13) cytokines by antigen-capture ELISA (BD PharMingen, San Diego, CA, and R&D

Systems, Minneapolis, MN) according to manufacturer's protocols. The following capture and detection monoclonal antibody pairs were used: IL-2, clones JES6-1A12 and JES6-5H4; IFNγ, R464A2 and XMG1.2; IL-4, BVD4-1D11 and BVD6-24G2; IL-10, JES5-2A5 and SXC-1; and IL-13, 38213.11 and polyclonal detection antibody. Each sample was assayed in duplicate and absorbance values were expressed in pg/ml as determined by comparison with a standard curve obtained from known quantities of recombinant cytokine standards (BD PharMingen and R&D Systems). Limits of detection of the capture antibodies used were IFNγ, 15.625-4000 pg/ml; IL-2, 31.25-4000 pg/ml; IL-4, 15.625-1000 pg/ml; IL-10, 125-4000 pg/ml; and IL-13, 15.625-4000 pg/ml. Supernatants from lymph node and spleen cell cultures were initially assayed at a 1:2 dilution for all cytokines with the exception of spleen cell secretion of IFNγ, which was assayed at a 1:5 dilution. Dilutions were subsequently adjusted to bring cytokine concentrations within the limits of ELISA detection as previously described (Chapter 2) with the difference that supernatants re-tested for IL-2 were also diluted 1:8.

**Cell surface molecule staining**

Immunolabeling of cell surface molecules was performed as follows: An aliquot of each spleen and lymph node suspension was centrifuged to remove media and then erythrocytes were lysed by suspending cells in nanopure $H_20$ for 10 seconds followed by immediate addition of 10x phosphate-buffered saline (PBS) at pH 7.0 to make an isotonic 1x PBS solution. Each cell suspension was transferred in 200 µl aliquots to a 96-well V-bottom cell culture plate (Corning Inc., Corning, NY). The plate was spun at 1500 rpm for 7 minutes to remove PBS. Cells were re-suspended and incubated in biotinylated

OVA TCR-specific monoclonal antibody KJ1-26 (obtained from Dr. Rick Tarleton, University of Georgia, Athens, GA) diluted 1:100 with PAB staining buffer (PBS with 0.1% sodium azide, 0.1% bovine serum albumin (BSA) (Sigma, St. Louis, MO)) for 45 minutes at 4$^{o}$C. Cells were washed with 200 µl PAB per well for 7 minutes at 4$^{o}$C at 1500 rpm. Cells were re-suspended and incubated overnight at 4$^{o}$C in streptavidin-allophycocyanin (SAv-APC) conjugate (BD PharMingen) diluted to 1:100 in PAB and in combinations of the following fluorochrome-conjugated antibodies for cell surface molecules, diluted 1:1000 in PAB: fluorescein isothiocyanate (FITC) anti-CD4, clone GK1.5; R-phycoerythrin (R-PE) anti-CD19, clone 1D3; R-PE anti-CD14, clone rmC5-3; and FITC anti-MHC II (I-A$^{d}$), clone 39-10-8 (BD PharMingen). Antibodies, antibody dilutions, and stained cells were always kept in the dark and at 4$^{o}$C or on ice. A control sample was prepared with rat isotype-matched control antibodies for each fluorochrome (rat $IgG_1$, clone R3-34; rat $IgG_{2a}$, clone R35-95, BD PharMingen) to check for non-specific staining. Single- and triple- stained control samples of all antibodies were also prepared to facilitate setting detectors and compensation on the FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). After staining overnight at 4$^{o}$C, cells were washed twice with 200 µl PAB and stored in PAB at 4$^{o}$C for 1-4 days until read with the FACSCalibur.

**Intracellular cytokine staining**

Frequency of intracellular cytokine expression by antigen-specific cells was determined by stimulating cells, blocking cytokine export, and immunolabeling cells for surface molecules and intracellular cytokines. An aliquot of each spleen and lymph node

suspension from each mouse was incubated with 1μM OVA peptide and 1μl/ml brefeldin A (GolgiPlug™, BD PharMingen) in RPMI for 6 hours at 36°C. Cell suspensions were centrifuged to remove media, lysed to remove red blood cells, and transferred to a 96-well V-bottom plate as described above. Cells were re-suspended and pre-incubated for 5 minutes at 4°C in Fc Block™ (anti-CD16/CD32 FcγIII/II receptor antibody, clone 2.4G2; BD PharMingen), and then for 45 minutes in biotinylated OVA TCR-specific monoclonal antibody KJ1-26 diluted 1:100 with PAB. Cells were washed with 200 μl PAB per well for 7 minutes at 4°C at 1500 rpm. Cells were re-suspended in SAv-APC at 1:100 and CD4-FITC at 1:1000 or the appropriate control antibodies at 1:1000 in PAB and incubated for 45 minutes at 4°C. Cells were washed twice with 200 μl PAB per well for 7 minutes at 4°C at 1500 rpm and then incubated for 10 minutes at 4°C with 100 μl Cytofix/Cytoperm™ Buffer (BD PharMingen) following the protocol of the Cytofix/Cytoperm™ kit (BD PharMingen). Cells were washed twice with 200 μl Perm/Wash™ solution (BD PharMingen) and then incubated overnight at 4°C in R-PE-labeled anti-IL-2, clone S4B6; anti-IL-4, clone 11B11; anti-IL-10, clone JES5-16E3; or anti-IFNγ, clone XMG1.2 antibody diluted 1:100 with Perm/Wash™ solution. Cells were washed twice with 200 μl Perm/Wash™ solution, resuspended in PAB, and kept at 4°C for 1-4 days until read with the FACSCalibur. Isotype-matched control antibodies fluorochrome (rat $IgG_{2a}$, clone R35-95; rat $IgG_1$, clone R3-34, BD PharMingen) were used to prepare single and triple-stained control samples concurrently with samples from individual mice.

### Flow cytometry analysis

Cell samples were loaded into 12x75mm polystyrene culture tubes (Fisher, Pittsburgh, PA) and data on 10,000 events (or, rarely, the total cell number in each sample if less than 10,000) were acquired with a Becton Dickinson FACSCalibur. Using CellQuest software (BD PharMingen), samples were analyzed by size, granularity, and fluorescence to identify leukocyte subpopulations. Isotype-matched, single, and multiply stained controls were used to set detectors, compensation, and standard quadrants for separating cell types.

### Spreadsheets and statistical analysis

Proliferation, cytokine, and flow cytometry data were summarized on Excel spreadsheets (Microsoft, Redmond, WA) and imported into Statview (SAS Institute, Cary, NC) for hypothesis testing by ANOVA and individual pair-wise t-tests for significance at the $p = 0.05$ level. Simple regression was used to test for a dose-response effect between the number of ticks feeding and each outcome variable.

## Results

### Infestation

*Amblyomma americanum* feeding results were similar to those previously reported for BALB/c mice (Chapter 2). Most nymphs (> 80%) attached within 2 hours of placement on mice. Eighty two percent of attached nymphs allowed to remain attached were able to engorge to repletion. More than 96% of feeding nymphs achieved repletion

between day 5 and day 7 post-infestation. Approximately 81% of engorged nymphs molted to the adult stage.

**Splenocyte proliferation**

Infestation with *A. americanum* nymphs did not have any significant effect on the proliferative responses of splenocytes to specific antigen or to mitogens (Fig. 5.1, 5.2). There were no significant differences in proliferation measured in response to 0.3, 1.0, or 3.0 M OVA peptide (Fig. 5.1A-C), 5.0 mg/ml of the T cell mitogen Con A (Fig. 5.2A), or 5.0 mg/ml of the B cell mitogen LPS (Fig. 5.2B).

**Quantification of cell populations from lymphoid organs**

Infestation affected proportions of antigen-specific cell populations in spleens and lymph nodes (Fig. 5.3). Differences were measured by flow cytometry of cells taken directly from the lymphoid organs without any antigen or mitogen stimulation. In spleens of infested mice, proportions of $CD4^+$ cells with the OVA-specific TCR ($CD4^+KJ1\text{-}26^+$) were reduced on day 7 post-infestation ($p=0.053$), and were significantly less than $CD4^+KJ1\text{-}26^+$ cell populations in control mice on days 10 and 14 post-infestation ($p < 0.05$) (Fig. 5.3A). The decline in antigen-specific cells coincided with the time that most ticks finishing rapid engorgement and continued up to one week after tick feeding. The proportions of $CD4^+$ $KJ1\text{-}26^+$ cells in the lymph nodes of infested

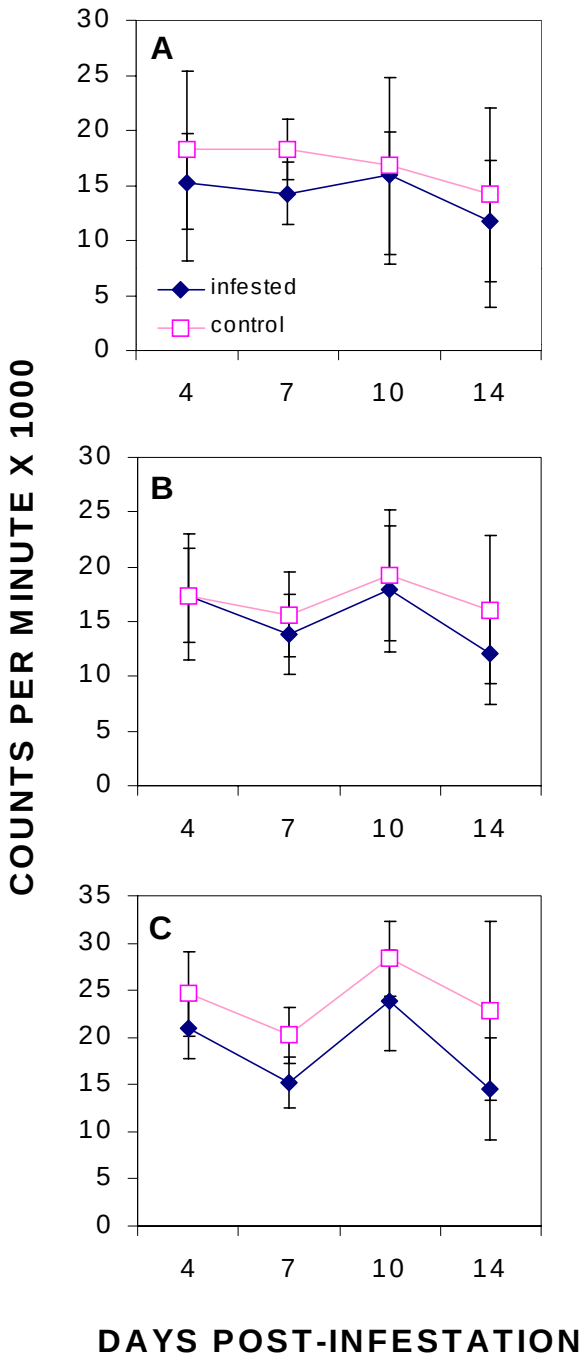


Figure 5.1. Antigen-specific DO11 spleen cell proliferation in response to 72 hour incubation with 0.3 µM (A), 1.0 µM (B), and 3.0 µM (C) OVA peptide, measured in counts per minute (CPM). Error bars indicate ± one SE. *=$p<0.05$

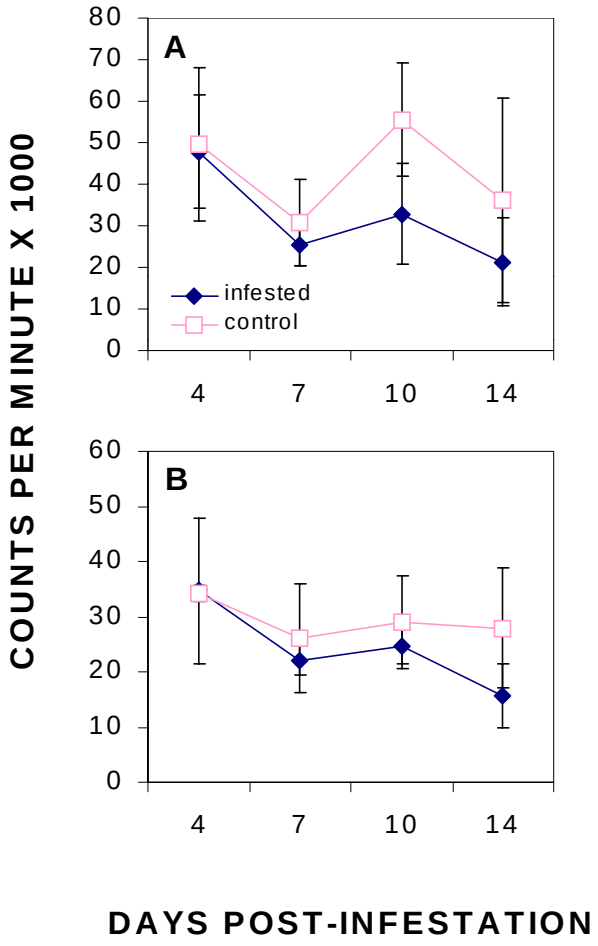


Figure 5.2. DO11 spleen cell proliferation in response to 72 hour incubation with 5.0 µg/ml of the T cell mitogen Con A (A) or the B cell mitogen LPS (B), measured in counts per minute (CPM). Error bars indicate ± one SE. *=$p<0.05$

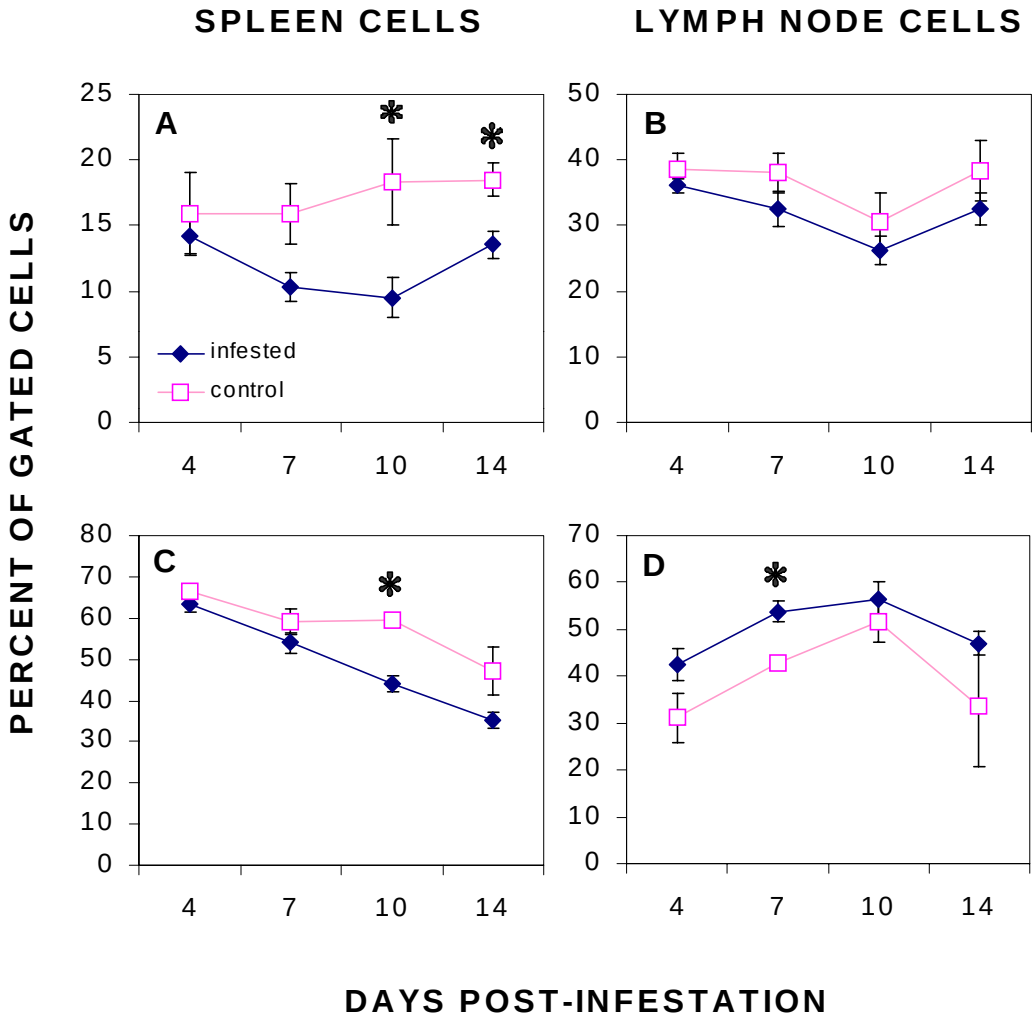


Figure 5.3. Relative proportions of, and changes in $CD4^{+}KJ1\text{-}26^{+}$ (A, B) and $CD19^{+}MHC\ II^{+}$ (C, D) cells in the spleens and draining lymph nodes of infested and control BALB/c-TgN mice. Error bars indicate ± one SE. *=$p<0.05$

mice were also lower overall compared to those in control mice, but not significantly different on any specific day measured (ANOVA p=0.0177).

Infestation also affected non-antigen-specific $CD4^+$ cells. On average, 36% of $CD4^+$ cells in the spleen and 32% of $CD4^+$ cells in the lymph nodes did not have the OVA-specific TCR. Infested mice had fewer non-OVA-specific $CD4^+$ cells ($CD4^+KJ1\text{-}26^-$) in spleens and lymph nodes (ANOVA p=0.0319 and p=0.0504, respectively) than control mice did, with the largest decreases in $CD4^+$ spleen cells on day 10 and $CD4^+$ lymph node cells on day 7 (40.2% and 55.8% less than control, respectively).

Proportions of $CD19^+$MHC $II^+$ B cells were significantly different in spleens and lymph nodes of infested and control mice (Fig. 5.3C, D). Splenic B cells were significantly decreased in infested mice on day 10 post-infestation, when all ticks had detached. However, proportions of B cells were increased in the lymph nodes of infested mice throughout the study and were significantly higher on day 7 post-infestation, when most ticks had just finished rapid engorgement. Expression of MHC $II^+$ fluorescence intensity appeared to be slightly reduced in B cells from spleens and lymph nodes of infested mice.

There were no significant differences in the number of cells expressing $CD14^+$ in spleens or lymph nodes of infested and control mice (data not shown).

**Antigen-stimulated cytokine production**

In response to OVA antigen, spleen cells from infested BALB/c–TgN mice produced less of the Type 1 cytokine IL-2 and spleen and lymph node cells produced mice (Fig. 5.4, 5.5). Antigen-stimulated splenocytes from infested mice produced

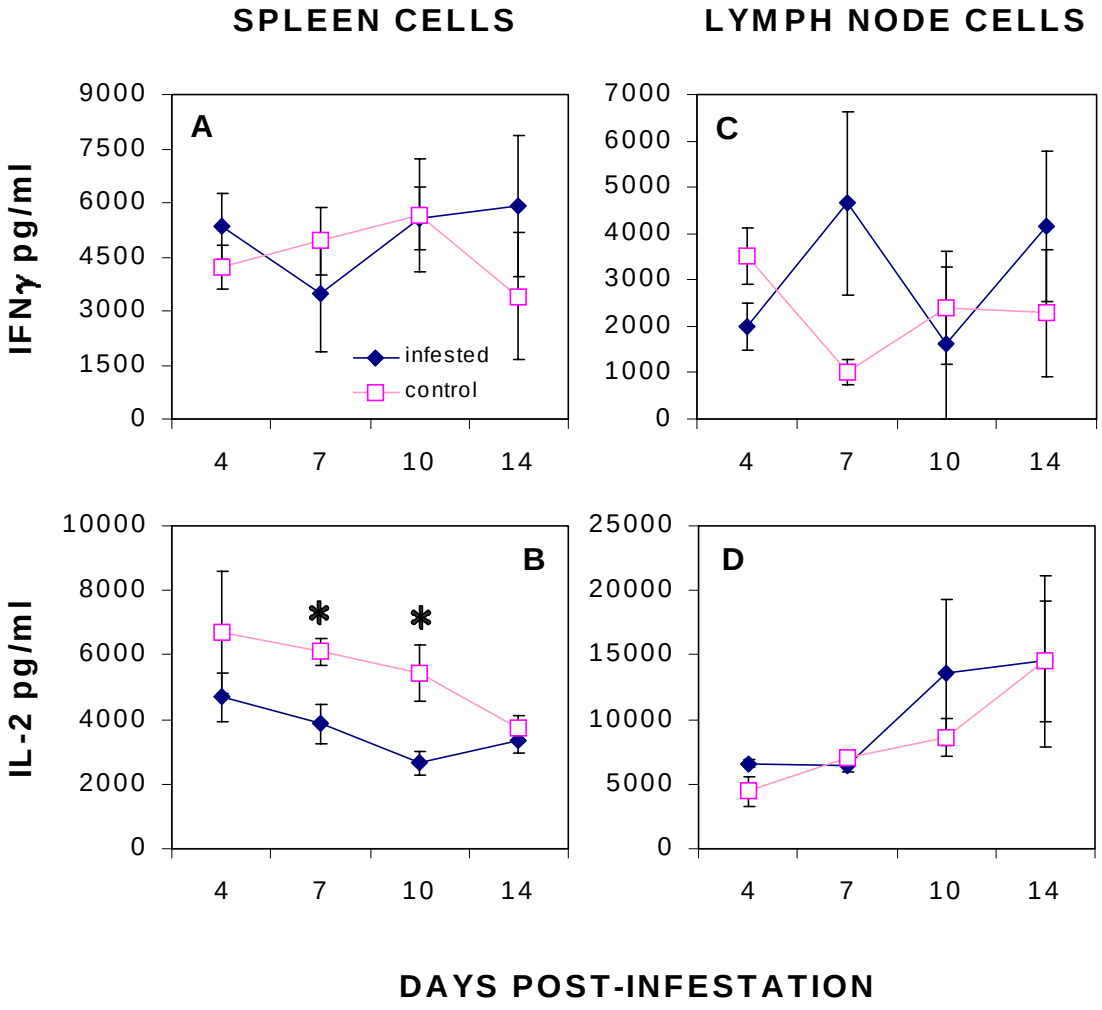


Figure 5.4. Secretion of Type 1 cytokines by spleen (A, B) and draining lymph node (C, D) cells of BALB/c-TgN mice after 48 hr incubation with 1.0 µM OVA peptide. Error bars indicate ± one SE. *= $p<0.05$

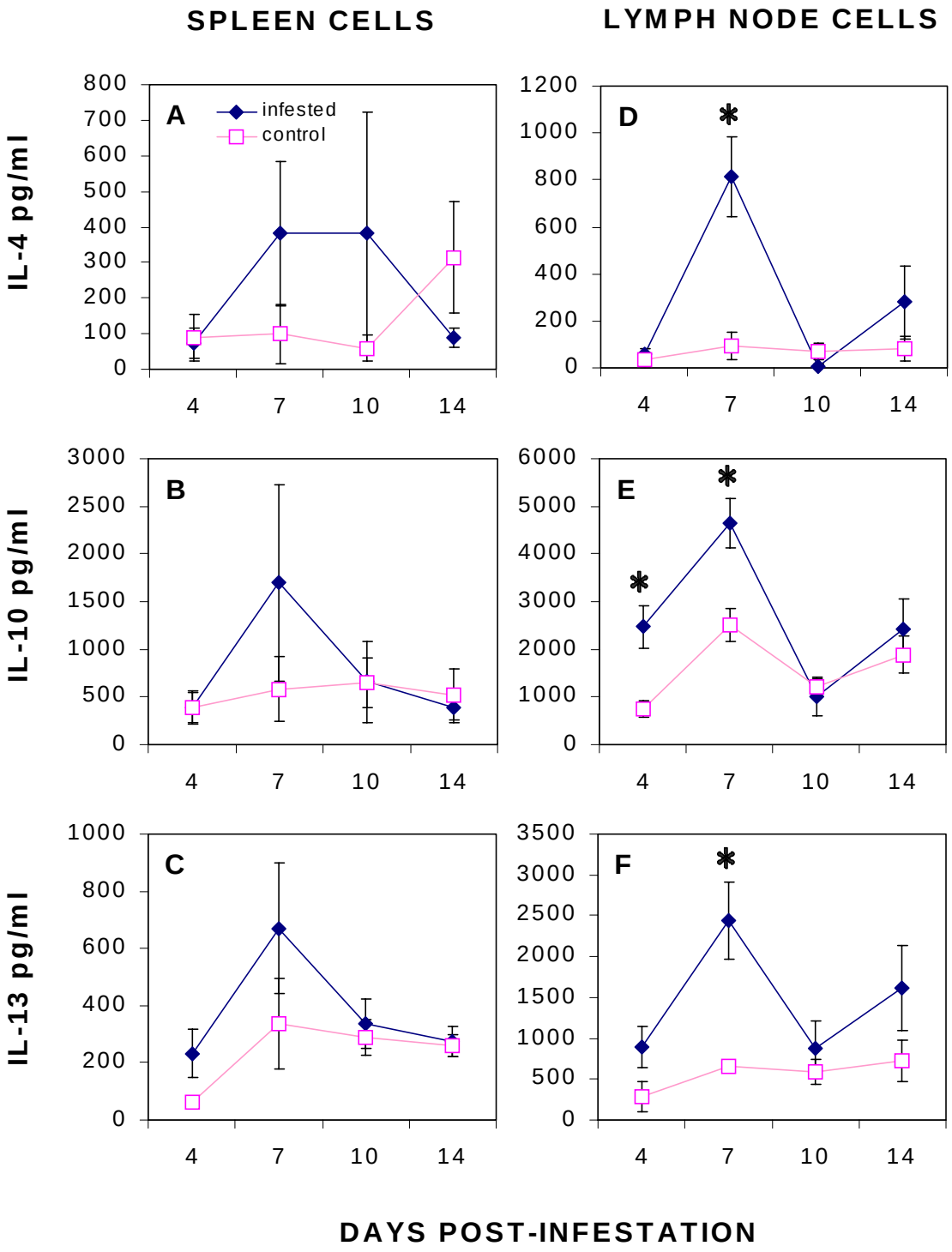


Figure 5.5. Secretion of Type 2 cytokines by spleen (A, B, C) and draining lymph node (D, E, F) cells from BALB/c-TgN mice after 48 hr incubation with 1.0 µM OVA peptide. Error bars indicate ± one SE. *=$p<0.05$

significantly less IL-2 than splenocytes from control mice on days 7 and 10 post infestation (36.6% and 51.2% less, respectively), when ticks were completing rapid engorgement and detaching from their hosts (Fig. 5.4B). There were no significant effects of infestation on antigen-stimulated secretion of IL-2 by lymph node cells (Fig. 5.4D) or on IFNγ secretion by spleen or lymph node cells (Fig. 5.4A, C) on any days more of the Type 2 cytokines IL-4, IL-10, and IL-13 than the same cells from control measured. OVA antigen-stimulated secretion of IL-4, IL-10, and IL-13 by lymph node cells from infested mice was significantly increased, measuring 8.8-fold, 1.9-fold, and 3.7-fold higher, respectively, than quantities secreted by cells from control mice on day 7 post-infestation, just after most ticks completed rapid engorgement (Figure 5.5D, E, F). Antigen-stimulated production of IL-10 by lymph node cells from infested mice was also 3.3-fold higher than in cells from control mice on day 4, when rapid engorgement had just begun. Antigen-stimulated spleen cells of infested mice produced more of all three Type 2 cytokines on day 7 post-infestation, but the differences between infested and control mice were not significant due to the large variation measured in levels of cytokine production. In general, levels of antigen-stimulated secretion of Type 2 cytokines were higher in lymph node cultures than in spleen cell cultures

**Antigen-stimulated intracellular cytokine expression**

Antigen-stimulated cell cultures from spleens and lymph nodes of infested mice had more cytokine-positive cells than cultures from control mice. Stimulation with OVA peptide for 6 hours resulted in higher proportions of OVA antigen-specific ($KJ1\text{-}26^{+}$) cells positive for $IL\text{-}2^{+}$, $IL\text{-}4^{+}$, and $IL\text{-}10^{+}$ cytokines in splenocytes from infested mice

than in splenocytes from control mice (Fig. 5.6, 5.7). On day 10 post-infestation, stimulation with OVA also resulted in significantly more KJ1-26$^+$ IFNγ$^+$, IL-4$^+$, and IL-10$^+$ cells among lymph node cells from infested mice compared to lymph node cells from control mice.

Proportions of KJ1-26$^+$IL-2$^+$ cells were significantly greater in OVA-stimulated splenocytes from infested mice than in OVA-stimulated splenocytes from control mice on days 10 and 14 post infestation, after all ticks had detached (Fig. 5.6B, D). There were no significant differences in proportions of KJ1-26$^+$IL-2$^+$ cells from lymph nodes of infested and control mice, although KJ1-26$^+$IL-2$^+$ cells appeared to be less abundant in infested mouse cell cultures on days 4 and 7 post-infestation. Although proportions of KJ1-26$^+$IFNγ$^+$ cells appeared to be increased in OVA-stimulated spleen cells from infested mice, they were not significantly higher on any day measured (Fig. 5.6A), but cultures from infested mice did produce more KJ1-26$^+$IFNγ$^+$ lymph node cells on day 10 post-infestation (Fig. 5.6C).

Although numbers of cytokine-positive cells were low due to the short period of antigen stimulation, infested mice had higher proportions of KJ1-26$^+$ Type 2 cytokine-producing splenic T cells than control mice (Fig. 5.7). Infested mice had increased proportions of KJ1-26$^+$ splenocytes positive for Type 2 cytokines overall, with significant increases in KJ1-26$^+$IL-4$^+$ cells on day 10 (Fig. 5.7A) and KJ1-26$^+$IL-10$^+$ cells on day 7 post-infestation (Fig. 5.7C). On day 10, infested mice also had significantly more KJ1-26$^+$IL-4$^+$ and KJ1-26$^+$IL-10$^+$ producing lymph node cells than control mice (Fig. 5.7B, D); this elevation in Type 2 cells persisted, but was not significant, on day 14 post-infestation. In general, OVA-stimulated spleen and lymph node suspensions from both

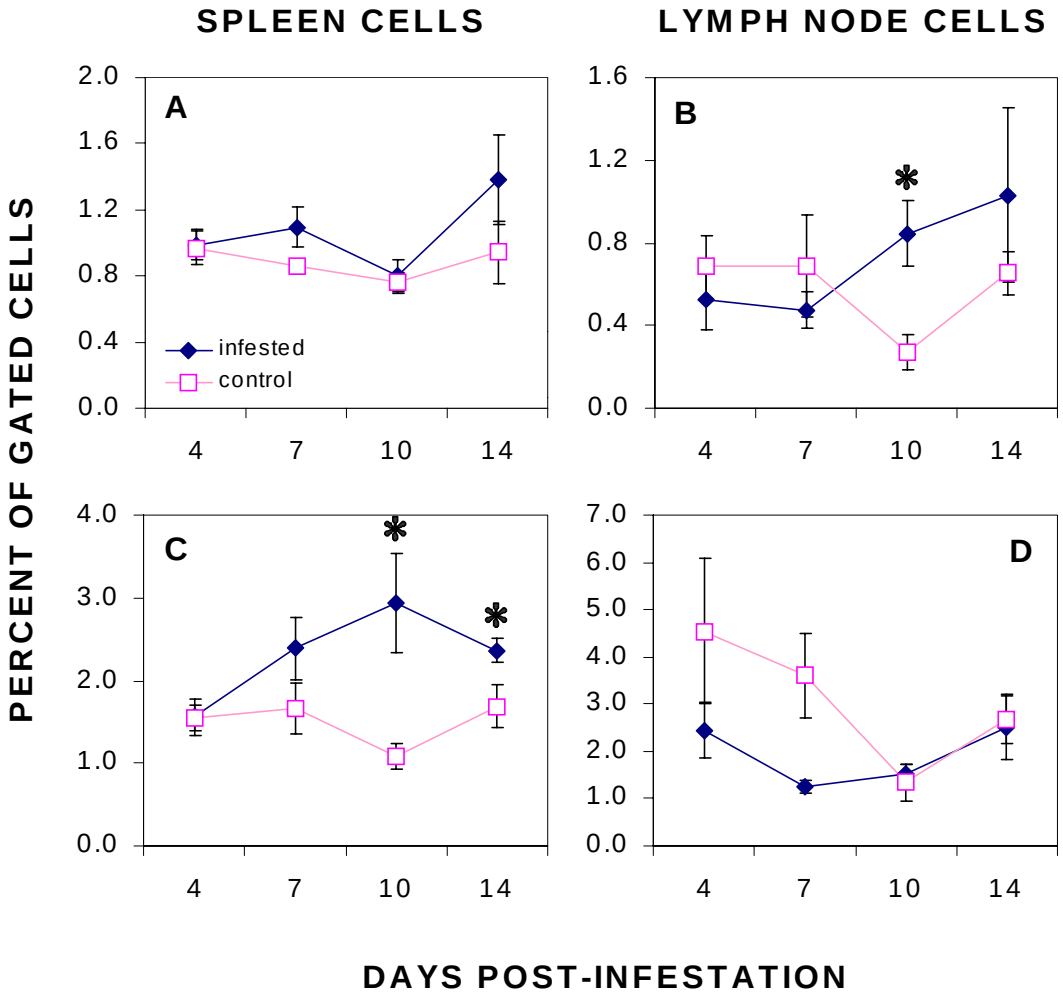


Figure 5.6. Proportions of KJ1-26$^{+}$IFN$\gamma^{+}$ (A, B) and KJ1-26$^{+}$IL-2$^{+}$ (C, D) cells present in lymphocyte populations from the spleen and draining lymph nodes of BALB/c-TgN mice after six hours incubation with 1.0 µM OVA peptide and 1µl/ml GolgiPlug™. Error bars indicate ±• one SE. *=$p<0.05$

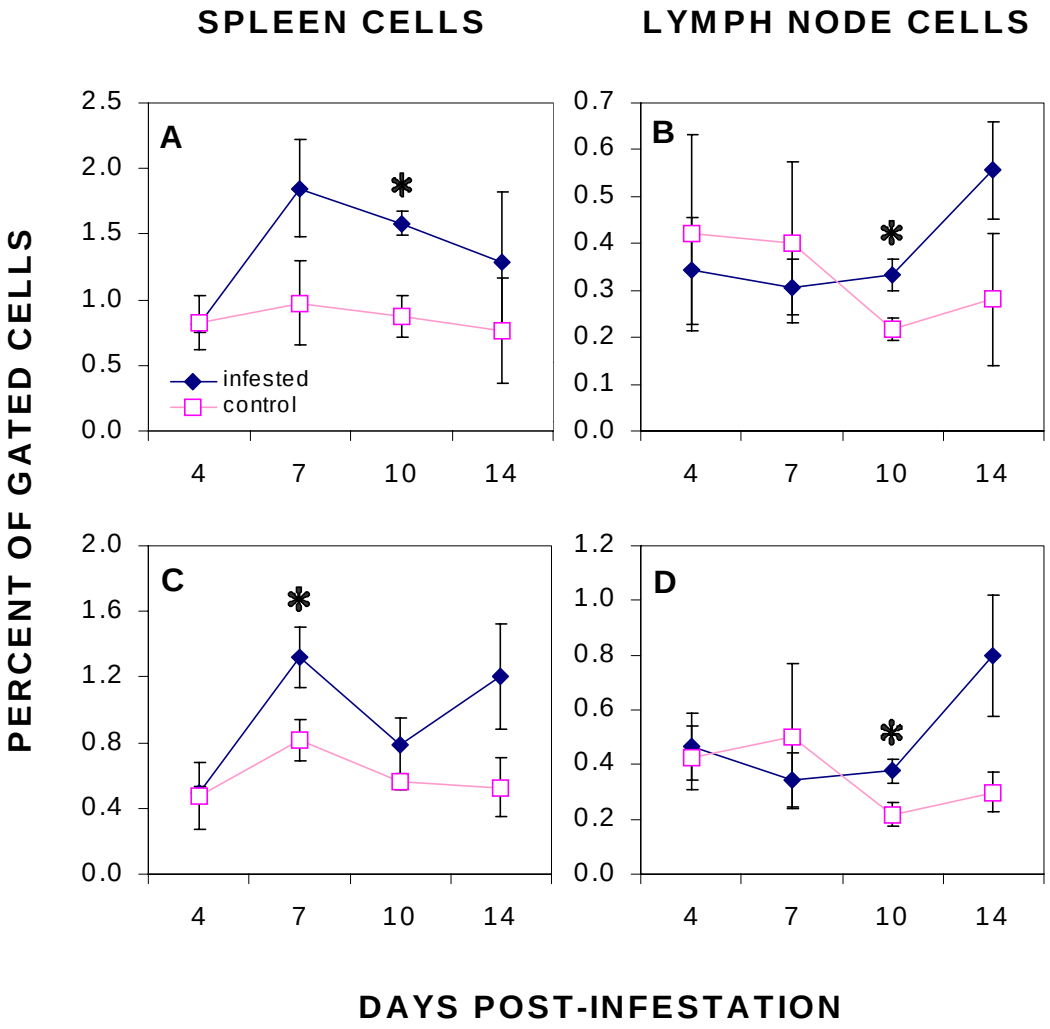


Figure 5.7. Proportions of KJ1-26$^{+}$IL-4$^{+}$ (A, B) and KJ1-26$^{+}$IL-10$^{+}$ (C, D) cells present in lymphocyte populations from the spleen and draining lymph nodes of BALB/c-TgN mice after six hours incubation with 1.0 µM OVA peptide and 1 µl/ml GolgiPlug™. Error bars indicate ± one SE. *=$p<0.05$

groups of mice had higher proportions of IL-$2^+$ cells than other cytokines, while the other cytokine-positive cells occurred in approximately equal proportions (Fig. 5.6, 5.7). The overall average of KJ1-$26^+$IL-$2^+$ cells was 1.90% and 2.49% for spleens and lymph nodes, respectively. In the same samples, the percent of KJ1-$26^+$IFN$\gamma^+$ cells from spleens and lymph nodes was 0.97% and 0.65%, KJ1-$26^+$IL-$4^+$ cells 1.12% and 0.36%, and KJ1-$26^+$IL-$10^+$ cells 0.77% and 0.43%.

## Discussion

Although pathogens may initiate host responses by interaction with pathogen recognition receptors (PRRs) on antigen presenting cells (APCs), in many cases host resistance to or recovery from infection depends on the subsequent responses of antigen-specific T cells. Nearly all tick-transmitted pathogens are intracellular, and hosts must respond with Type 1 $CD4^+$ and/or $CD8^+$ T cells in order to resist infection (Preston & Jongejan 1999). In general, some of the pathogenic microbes are phagocytosed by antigen-presenting dendritic cells at their portal of entry into the host, such as the tick feeding site in the skin. These antigen presenting cells migrate to the nearest draining lymph node to present pathogen antigens to the $CD4^+$ T cells (Jankovic *et al*. 2001). Pathogen-specific $CD4^+$ T cells activated by the APC-presented antigens respond by clonal proliferation and cytokine production, which determine subsequent effector responses (Abbas *et al*. 2000). In our previous research (Chapter 2, 3), we found that infestation of BALB/c mice with *A. americanum* nymphs affected T cell cytokine production in response to non-specific stimulation with mitogens; in this study, we found that *A. americanum* infestation had an effect on the responses of a population of antigen-specific T cells to a surrogate antigen.

*Amblyomma americanum* infestation had a significant impact on the antigen-specific cytokine responses, but not on the antigen and mitogen-stimulated proliferative responses, of BALB/c-TgN mice. Although the proliferation of splenocytes from *A. americanum*-infested BALB/c-TgN mice appeared to be slightly decreased in response to all concentrations of antigen or mitogen, it was never significantly different from the proliferative response of control mice (Fig. 5.1, 5.2). In this study, as in our previous experiment (Chapter 2), we did not observe any effect of infestation on the BALB/c T cell proliferative response to Con A mitogen. We also did not observe any difference in the responses of infested and control mice to LPS, although we had seen significant suppression of response to LPS in our previous experiment with BALB/c mice (Chapter 2). Given that BALB/c-TgN mice share the same genetic background with BALB/c mice except for their transformed TCRs, the difference in the B cell responses of the two strains cannot be explained at this time. It is not apparent how differences in T cell characteristics could influence host responses to a thymic-independent (TI) mitogen. In this study, we also did not observe any effect of infestation on T cell proliferative responses to the specific antigen, OVA peptide. Only one previous study has investigated the effect of tick exposure on proliferative response to a surrogate antigen, finding that incubation of keyhole limpet hemocyanin (KLH)-specific T cells *in vitro* with saliva from *Rhipicephalus sanguineus* reduced the proliferative response of those T cells to macrophages pre-incubated with KLH (Ferreira & Silva 1998). Although we used a different method, providing soluble antigen to whole spleen cell suspensions, the lack of difference between responses of cells from our infested and control mice suggests that *A. americanum* infestation did not inhibit antigen-specific T cell proliferation.

In contrast to our proliferation findings, our flow cytometric quantification of lymphoid cell surface markers expressed in spleens and lymph nodes of infested mice adds to the cumulative evidence for overall T cell inhibition by ixodid tick infestation or tick saliva. In infested BALB/c-TgN mice, the proportions of $CD4^+$ cells in the spleen and lymph nodes (Fig 5.3) were reduced nearly as much as they were in our previous experiment with BALB/c mice (Chapter 3). In both mouse strains, the effect of infestation on $CD4^+$ spleen cells began on day 7 post-infestation, but in BALB/c–TgN mice the effect persisted up to one week after ticks had finished feeding (Fig. 5.3). In BALB/c-TgN mice, effects on $CD4^+$ lymph node cells began slightly later than in BALB/c mice but also persisted for the duration of the study. In conjunction with the overall decline in $CD4^+$ cells, the $CD4^+$ $KJ1\text{-}26^+$ specific population was also reduced.

Approximately one third of $CD4^+$ cells from the spleens and lymph nodes of both infested and control mice were not $KJ126^+$. Down-regulation of TCR expression can occur when large quantities of antigen are present, however, we observed this phenomenon in both unstimulated and antigen-stimulated cells. Some of the $CD4^+KJ126^-$ cells could be monocytes and macrophages; however, monocytic cells typically make up less than 5% of the total cell population in lymphoid organs. The proportion of $CD4^+KJ126^-$ cells was smaller in infested mice than in control mice to a similar degree and on the same days that the proportion of $CD4^+KJ126^+$ cells was reduced in infested mice. Given that the responses of the $CD4^+KJ126^-$cells to infestation were similar overall to the responses of $CD4^+KJ126^+$ cells, we suspect that this population consists mainly of $CD4^+$ lymphocytes that have downregulated their TCRs. The reason for this downregulation remains to be determined.

While this overall downregulation of $CD4^+$ cells does not reflect any antigen-specific effects of infestation, it does suggest that cultures of cells from infested mice might contain fewer antigen-specific cells than similar cultures from control mice, so measured differences in antigen-stimulated cytokine production might be affected by the relative numbers of antigen-specific cells. Further investigation is necessary to determine the effects of overall reductions in $CD4^+$ cell populations in infested mice on their responses to tick-borne pathogens.

*Amblyomma americanum* feeding appears to have non-antigen-specific effects on B cells as well as T cells. Although we did not observe an effect of infestation on BALB/c-TgN splenocyte proliferation to the B cell mitogen LPS (Fig. 5.2), we found that infested mice had significantly smaller proportions of splenic B cells than control mice (Figure 3.3). We also found that populations of B cells in the draining lymph nodes of infested BALB/c-TgN mice were increased by similar amounts on day 7 post-infestation to B cells measured previously in infested BALB/c mice (Chapter 3). There are relatively few studies of the impact of tick infestation on B cell responses (Appendix 1); the unusual effects of *A. americanum* infestation on B cells deserve further research.

The lack of effect of infestation on spleen and lymph node $CD14^+$ macrophage and monocyte populations described in Chapter 3 was also observed in this experiment, which found no differences in $CD14^+$ expression on spleen or lymph node cells from infested or control BALB/c–TgN mice. As previously discussed, more dramatic changes in these cells could be expected at the bite site since these cells typically migrate to sites of inflammation (Chapter 4). For future research, it will be interesting to examine the

effects of infestation on motile antigen-presenting cell types more likely to migrate from the bite site into lymph nodes or spleens, such as dendritic cells or Langerhans cells.

The effects of infestation on cytokine secretion by OVA-stimulated cells were very similar to the effects of infestation on mitogen-stimulated cytokine secretion (Chapter 2). Production of Type 2 cytokines was increased in both spleens and lymph nodes of infested mice, and peaked on day 7 post- infestation, just after most ticks had finished rapid engorgement (Fig. 5.5). Even though infestation appeared to have reduced proportions of antigen-specific cells (Fig. 5.3), Type 2 cytokines were still produced in larger quantities by cells from infested BALB/c-TgN mice. The effect of infestation on the antigen-stimulated production of Type 1 cytokines was less clear (Fig. 5.4). Infestation decreased production of IL-2 by spleen cells, but the effect did not continue past day 10 and was not observed in lymph node cells. Infestation did not appear to affect antigen-induced production of IFNγ as consistently as mitogen-induced production was affected (Chapter 2). The results of this study suggest that infestation could change the cytokines produced during antigen-specific responses, possibly generating more of a Type 2 response to the antigen.

The proportions of cytokine-positive cells produced by antigen stimulation of spleen and lymph node cell cultures were very low compared to those observed in our previous experiment using mitogen stimulation (Chapter 3). The lack of cytokine-positive cells in cultures from both infested and control mice was most likely due to the short duration of antigen stimulation. T cells require longer than 6 hours to produce their full response to stimulation with antigen. Our data represent the early stages of the antigen-specific response, and show some evidence of Type 2 polarization in infested

mice, but not nearly as much as was expected. Declines in proportions of cytokine-positive cells from control mice could also be attributed the short period of antigen stimulation.

The effects of infestation on antigen-specific and mitogen stimulation in generating cytokine positive cells were more similar among Type 2 cells than among Type 1 cells. With either method of stimulation, we observed higher proportions of IL-$4^+$ and IL-$10^+$ cells in spleen cell cultures from infested mice on days 7 and/or 10 and in lymph node cultures from infested mice on day 10 post-infestation (Fig. 5.7). Antigen-stimulated spleen and lymph node cultures from infested BALB/c-TgN mice also appeared to have higher proportions of IFN$\gamma^+$ cells (Fig. 5.6) than cell cultures of control mice, as observed in mitogen-stimulated cells from BALB/c mice (Chapter 3). However, proportions of antigen-stimulated IL-$2^+$ cells in spleen and lymph node cultures from BALB/c-TgN mice (Fig. 5.6) were much smaller than we had previously observed in mitogen-stimulated cultures from BALB/c mice (Chapter 3), suggesting that these cultures should have been stimulated with antigen for longer than 6 hours, because cells stimulated for 48 hours secreted a substantial amount of IL-2 (Fig. 5.4).

In this experiment, we examined the effects of *A. americanum* infestation on BALB/c-TgN cellular responses to OVA antigen as a model for host immune responses to a pathogen introduced during feeding by an infected tick. Our results suggest that infestation does not affect proliferation to specific antigens, but can increase the proportions of Type 2 cytokine-positive cells and the secretion of Type 2 cytokines during antigen-specific responses. Type 2 cytokines can create a more permissive environment for infection with intracellular pathogens by downregulating macrophage

anti-microbial responses (Abbas *et al*. 2000). Most crossregulation by Type 2 cytokines occurs by downregulation of IFNγ (Morel & Oriss 1998). However, we observed little effect of infestation on antigen-specific IFNγ$^{+}$ cells or IFNγ production. There is evidence that the exposure of antigen-presenting cells to large amounts of antigen, such as that present in tick saliva, in the absence of microbial structures may result in the development of Type 2 responses in associated T cells (Jankovic *et al*. 2001). However, it is suspected that tick saliva itself contains components that influence host immune responses; a few of these have been identified and shown to affect lymphocyte proliferation and cytokine secretion (Table 1.2). A homologue of the proinflammatory cytokine Macrophage Migration Inhibitory Factor was recently characterized in the salivary gland tissue of *A. americanum* (Jaworski *et al*. 2001), but other salivary components in this vector remain to be identified.

Infestation with infected ticks appears to cause Type 2 responses in pathogen-susceptible hosts (Zeidner *et al*. 1997, 2000, Christe *et al*. 2000), while needle inoculation of the same pathogen engenders mixed Type 1 and Type 2 responses in infected but uninfested hosts (Christe *et al*. 2000). Promotion of Type 2 responses by *A. americanum* infestation in the presence of microbial antigen could be evaluated by testing the effect of infestation on cytokine responses of mice infected with an intracellular pathogen. Reinfestation of host animals with *Ixodes ricinus* has been shown to promote Type 1 delayed-hypersensitivity responses at the bite site (Mbow *et al*. 1994c), which might provide protection against tick-borne pathogens (Wikel *et al*. 1997); it would also be interesting to compare antigen-specific responses between naïve and previously *A. americanum*-infested mice to see if cytokine responses have changed in this manner.

# CHAPTER 6

# CONCLUSIONS

Ixodid ticks, which feed for extended periods of time, modify normal host immune responses in order to facilitate blood meal acquisition (Wikel 1999). Infestation with ixodid ticks has been shown to have effects on not only the microenvironment of the bite site but also on systemic immune responses (Appendix 1), both of which could make the host more susceptible to any pathogens transmitted by the tick during feeding. *Amblyomma americanum*, like other species of ixodid ticks, remains attached to and feeding on its hosts for extended periods of time. Although resistance to *A. americanum* has been studied for several decades, little was known about the effects of infestation on lymphocyte populations and signaling molecules.

We developed a murine model of *A. americanum* infestation and compared various immune parameters between infested and control mice. We first measured the immunomodulatory effects of *A. americanum*, then we attempted to answer more specific questions about the nature of the host response by identifying the affected cell types, comparing systemic responses in the spleen and lymph nodes to responses at the infestation site in the skin, and examining antigen-specific responses as a model for responses to a pathogen introduced during infestation.

Our first objective for this study was to characterize the effects of *A. americanum* infestation on mitogen-stimulated systemic and local cellular immune responses of

BALB/c mice. We found that changes in the immune responses of infested mice began on day 4, when attached ticks were beginning rapid engorgement, and were highest on day 7, when most ticks had completed rapid engorgement, or day 10, when all ticks had detached. In most cases responses were returning to control levels by day 14, a week after ticks completed rapid engorgement. In infested mice, spleen cell proliferation to B cell mitogen was suppressed, but proliferation to T cell mitogen was normal. Mitogen-stimulated spleen cells from infested mice produced less of the Type 1 cytokine IL-2, and more of the Type 2 cytokines (IL-4, IL-10, IL-13) than cells from control mice. Changes in cytokine production by lymph node cells had a similar pattern, which was noticeable earlier. Mitogen-stimulated production of the cytokine IFNγ was the exception; it was suppressed in spleen cells and increased in lymph node cells. The higher ratio of serum $IgG_1$ to $IgG_{2a}$ produced by infested mice supported the characterization of their response as Type 2. Infested mice produced lower levels of both non-specific IgG isotypes than control mice. Mitogen-stimulated adherent cells from infested mice did not produce significantly different amounts of antigen-presenting cell cytokines (IL-12, TNF-α, IL-6, IL-10). The differences between our observations of immune responses to *A. americanum* infestation and other author's results with other ixodid tick species suggest that changes in host responses during infestation are not solely a product of mechanical injury, but are specific to the attached ectoparasite.

Based on these results, we identified the immune cell populations and subsets that were affected by infestation with *A. americanum*. We found that infestation reduced the proportions of $CD4^+$ and $CD8^+$ cells in the spleens and lymph nodes of infested mice. Proportions of B cells were not significantly affected in the spleens of infested mice but were increased in their lymph nodes. There were no significant differences in

proportions of $CD14^+$ cells between the two groups of mice. Mitogen-stimulated cell cultures from the spleens and lymph nodes of infested mice had fewer $IL\text{-}2^+$ cells and more $IFN\gamma^+$, $IL\text{-}4^+$, and $IL\text{-}10^+$ cells. The main cell type producing IL-2 was the $CD4^+$ cell. Both $CD4^+$ and $CD8^+$ cells produced IFNγ, but increases were only seen in $CD4^+IFN\gamma^+$ cells from infested mice. Mitogen-stimulated $CD4^+$, $CD8^+$, and $CD4^-CD8^-$ cells made IL-4 and IL-10; all three cell types contributed to the increase in $IL\text{-}4^+$ and $IL\text{-}10^+$ cells in cultures from infested mice.

Our third objective was to determine how immune responses in the skin at the bite site were affected by infestation. We took cells directly from the site and did not culture them with mitogens or antigen. Comparing flow cytometry and immunohistochemistry results, we found that both numbers and proportions of $CD14^+$ cells were increased in the skin of infested mice at the bite site compared to skin from the same region in control mice. Proportions of $CD4^+$ and $CD8^+$ cells decreased at the bite site compared to skin from control mice, probably due to the influx of other inflammatory cell types. Infestation also affected cytokine production in the skin, increasing the proportions of $IL\text{-}4^+$ and $IFN\gamma^+$ cells and reducing the proportions of $IL\text{-}2^+$ cells at the bite site relative to equivalent skin on control mice.

Our fourth and final objective was to measure infestation-induced changes in the responses of the most affected cell type, $CD4^+$ T cells, to a specific antigen. For this objective, we used mice that were engineered to have large populations of antigen-specific $CD4^+$ T cells. Spleen cells from infested mice had slightly, but not significantly, lower proliferation to either specific antigen or to mitogens than control mice. Infestation non-specifically reduced the proportions of $CD4^+$antigen-specific T cells in the spleen and lymph nodes and the proportions of B cells in the spleens of infested mice, while

proportions of B cells in the lymph nodes of infested mice were increased. Antigen-stimulated spleen cells from infested mice secreted less of the Type 1 cytokine IL-2 and more of the Type 2 cytokines IL-4, IL-10, and IL-13. Antigen-stimulated lymph node cells from infested mice also secreted more of the Type 2 cytokines, but there were no significant differences in the Type 1 cytokines IL-2 and IFNγ. In contrast, antigen-stimulated spleen cell cultures from infested mice had higher proportions of all cytokine-positive (IFNγ+, IL-$2^+$, IL-$4^+$, and IL-$10^+$) antigen-specific cells than cultures from control mice. Antigen-stimulated lymph node cell cultures from infested mice also had increased proportions of IFN$\gamma^+$, IL-$4^+$, and IL-$10^+$ cells relative to control mice, but the proportion of IL-$2^+$ cells was decreased.

The results of our studies on spleen, lymph node, and skin cells from BALB/c and BALB/c-TgN mice show that infestation with *A. americanum* has broad effects on unstimulated, stimulated, and antigen-specific responses of $CD4^+$ T cells, $CD8^+$ T cells, and B cells. These three classes of lymphocytes are essential components of the antigen-specific immune response: $CD4^+$ T helper cells direct responses of macrophages, $CD8^+$ T cells and B cells; $CD8^+$ cytotoxic T cells kill infected cells; and B cells produce specific antibodies that label infected cells or pathogens for destruction by other immune cells (Abbas *et al*. 2000). Type 1 $CD4^+$ and $CD8^+$ T cells are especially important for acquired immune responses to intracellular pathogens (Romagnani 1997). Most tick-borne pathogens are intracellular bacteria, protozoa, or viruses, but Type 1 T cell responses are also protective against the extracellular *Borrelia burgdorferi* (Sonenshine 1993, Zeidner *et al*. 1996). Type 1, IFNγ-producing $CD4^+$ cells are needed for protective responses to infection with *Ehrlichia chaffeensis, Cowdria ruminantium, Anaplasma marginale, Rickettsia* spp., *Babesia* spp., *Theileria annulata,* and tick-borne viruses (Barnewall &

Rikihisa 1994, Preston & Jongejan 1999). $CD8^+$ cytotoxic T cells are necessary for protective responses against *Theileria parva*, *Rickettsia* species, tick-borne viruses and probably also *Cowdria ruminantium* (Totté *et al*. 1999, McKeever *et al*. 1999, Walker *et al*. 2001).

Although Type 1 responses are protective against most vector-borne pathogens, many studies of immunomodulation by tick and insect vectors have reported polarization towards a Type 2 response (Wikel 1999). Reduction of the host Type 1 response might benefit ticks by decreasing inflammation in response to tick salivary antigens, thereby allowing more efficient feeding. Based on a new understanding of dendritic cell signaling to T cells (Jankovic *et al*. 2001), the Type 2 polarization of the immune response by uninfected ticks could be attributed to the large volume of tick salivary antigens stimulating T cells in the absence of activation of dendritic cell pattern recognition receptors by microbial antigens. However, infestation with pathogen-transmitting ticks also results in Type 2 polarization of host responses (Zeidner *et al*. 1997, Christe *et al*. 2000). The presence of specific components in tick saliva that interact with host cytokines or influence their secretion (Gillespie *et al*. 2001, Mejri *et al*. 2001) suggests that cytokine modulation is a tick countermeasure against host defenses.

We found that infestation with *A. americanum* consistently increased the proportions of Type 2 cytokine-positive cells and the levels of Type 2 cytokine secretion in mitogen and antigen-stimulated lymphoid cell cultures and cells taken directly from the bite site. In nearly every case, infestation also reduced proportions of $IL\text{-}2^+$ cells and IL-2 secretion in the same cell samples. However, in most of our experiments, infestation either did not affect or increased the proportions of Type 1 cytokine $IFN\gamma^+$ cells and/or secretion of IFNγ in response to specific antigen or mitogen. Other authors

have found stable or increased IFNγ production by cells from mice infested with *D. andersoni*, *I. ricinus*, and *I. scapularis* (Mbow *et al*. 1994c, Zeidner *et al*. 1997, Schoeler *et al*. 1999, Macaluso & Wikel 2001). In contrast, infestations with pathogen-transmitting *I. scapularis* have been shown to downregulate IFNγ production by host cells (Zeidner *et al*. 1997, 2001).

The observation that spleen, lymph node, and skin cell cultures from infested mice have fewer IL-$2^+$ cells and produce less IL-2 in response to specific antigen could be explained in several different ways. One explanation is that in infested mice, more cells are committed to the Type 2 phenotype, which is unable make IL-2 (Morel & Oriss 1998). If more cells are already committed to the Type 2 phenotype, then there are fewer cells available for Type 1 responses; in general, clonal proliferation to a specific antigen would quickly increase the number of responding cells, but IL-2 is the main cytokine stimulating clonal proliferation (Abbas *et al*. 2000). Another reason for IL-2 downregulation could be the observed increase in Type 2 cytokines, which can have a cross-regulatory effect on Type 1 cells. For instance, IL-10 can downregulate IL-2 and IFNγ in intracellular infections (Redpath *et al*. 2001). A third possibility is that the decrease in proportions of $CD4^+$ cells seen in all samples from infested mice might reflect true reductions in $CD4^+$ populations, which would affect IL-2 more than other cytokines measured because nearly all IL-2 producing cells are $CD4^+$. The reason for the observed reductions in proportions of $CD4^+$ and $CD8^+$ cells in infested mice are currently unexplained, and deserve further investigation. A fourth reason for reduced populations of IL-$2^+$ cells could be the down-regulation of IL-2 production, similar to that occurring during chronic illnesses (Abbas *et al*. 2000), in order to prevent apoptosis by cells continuously exposed to tick salivary antigens; it is not clear if tick exposure lasts long

enough to cause this effect. A final reason for IL-2 downregulation could be the presence of an IL-2 binding protein in the saliva of *A. americanum*, like that found in the saliva of *I. scapularis* (Gillespie *et al*. 2001). However, this unlikely because the cytokine-producing cells were washed several times, and were not exposed to high concentrations of saliva during their 6-48 hour incubation with mitogen or antigen.

Few studies have reported effects of tick infestation on B cell responses. We observed a mixture of effects in infested mice: splenic B cell populations or responses were suppressed, lymph node B cell populations increased, and levels of serum $IgG_1$ and $IgG_{2a}$ antibodies were decreased. Specific antibodies produced by B cells can provide a protective response against the tick borne pathogens *Ehrlichia chaffeensis*, *Borrelia burgdorferi*, and *Babesia species*; against the latter two pathogens, $IgG_2$ antibodies are particularly important (Schmitz *et al*. 1992, Brown & Palmer 1999, Winslow *et al*. 2000). We did not continue the study long enough to measure differences in specific antibodies in BALB/c-TgN mice, which would take 2 weeks to develop and require exposure to OVA antigen for induction, but this or a similar experiment would provide interesting data. Also, we are curious to know if the majority of IL-$4^+$ and IL-$10^+$ cells, which were $CD4^-CD8^-$, are B cells, since recent research suggests that B cells can produce some cytokines in quantities as large as T cells, and might also have a role in T cell differentiation (Harris *et al*. 2000).

The variety of immune responses that were affected by *A. americanum* infestation, and in particular the effects on $CD4^+$ T cells, B cells, and cytokine production in an antigen-specific system, suggest that infestation could affect a host's ability to mount an effective specific immune response against microbial infection. There are reports that tick infestation or tick saliva enhances Type 2 responses in laboratory models

of disease (Zeidner *et al*. 1997, 2000, Ferreira & Silva 1998, Christe *et al*. 2000). To our knowledge, a comparison of disease progression with and without concurrent *A. americanum* infestation has not yet been made; this is the logical next step for defining the effect of this tick on specific immune responses of hosts. Experiments could include comparisons of antigen presentation, cell proliferation, cytokine production, and antibody production to pathogen antigens between *A. americanum*-infested and control hosts, as well as quantification of the disease organism and symptoms of infection in relevant host tissues.

APPENDIX A: Summary of tick immunomodulation experiments ↑=increased ↓=decreased 0=no change
Shaded areas: response characteristic of $T_H2$>$T_H1$ polarization
Results of multiple infestations expressed with one symbol unless infestations produced different effects.

SGE=salivary gland extract (*in vitro* exposure), PBL=peripheral blood lymphocytes, PBMC=peripheral blood mononuclear cells, ConA=Concanavalin A, PHA=phytohemagglutinin, LPS=lipopolysaccharide, PWM=pokeweed mitogen, Ag=antigen, Ig= immunoglobulin, IFNγ=interferon gamma, IL=interleukin, TGFβ=transforming growth factor beta, NO=nitric oxide, NK=natural killer cell, OVA=ovalbumin, SRBC=sheep red blood cells, BSA=bovine serum albumen, mϕ=macrophage, TNF=tumor necrosis factor, ICAM-1=intracellular adhesion molecule 1, GMCSF=granulocyte/macrophage colony stimulating factor

| Tick species | Hosts and cells used | Method of exposure | T cell responses<br>Proliferation to T cell mitogens ConA & PHA<br>Ag-specific proliferation<br>Cytokines: IFNγ, IL-2, IL-4, IL-5, IL-10, IL-13, TGFβ | B cell responses<br>Proliferation to B cell mitogens LPS & PWM<br>Ab responses<br>Complement responses | Macrophage responses<br>Cytokines: IL-1, IL-12, TNF, IL-6, IL-10, TGFβ<br>killing mechanism: NO<br>NK and other cell responses | Reference |
|---|---|---|---|---|---|---|
| *A. variegatum, D. reticulatus, Ha. inermis, I. ricinus, R. appendi-culatus* | Human<br>Blood granulocytes | SGE | | | ↓IL-8 secretion<br>↓IL-8 binding to IL-8R<br>↓granulocyte chemotaxis | Hajnicka *et al*. 2001 |
| *A. variegatum* | Cattle<br>PBL | Infest x4 | ↓ proliferation to ConA | | | Koney *et al*. 1994 |
| *B. microplus* | Cattle<br>PBL | Infest x4<br><br>SGE | 0↓↓↓ proliferation to ConA, PHA<br>0↓↓↓ % T cells in total lymphocyte count<br>↓ proliferation to PHA | ↓↓↓↓ proliferation to LPS<br>000↓ %B cells in total lymphocyte count<br>00↓↓ Ig to OVA | | Inokuma *et al*. 1993 |
| *D. andersoni* | Cattle<br>PBL | Infest x4 | 00↓↓ proliferation to PHA<br>00↑↑ tick Ag-specific proliferation | ↓tick-specific serum Igs | | Wikel & Osburn 1982 |
| " | Guinea pig<br>lymphocytes | Infest x1 | ↓↓ proliferation to ConA | 00 proliferation to LPS | | Wikel 1982 |
| " | Guinea pig<br>splenocytes | Infest x1 | | ↓ IgM response to SRBC (T cell-dependent ) | | Wikel 1985 |
| " | BALB/c mice<br>splenocytes | Infest x2 | ↓↓ proliferation to ConA<br>00 IFNγ ↑↑ IL-4<br>0↓ IL-2 ↑↑ IL-10 | | | Macaluso & Wikel 2001 |

| | | | | | | |
|---|---|---|---|---|---|---|
| " | BALB/c mice<br>splenocytes<br>PE macrophages | SGE | ↓ proliferation to ConA<br>↓ IFNγ<br>↓ IL-2 | ↑ proliferation to LPS | ↓ IL-1<br>↓ TNF | Ramachandra & Wikel 1992 |
| " | BALB/c mice<br>splenocytes | SGE | ↓ proliferation to ConA due to 36-43 kDa saliva fraction | ↑ proliferation to LPS | | Bergman *et al*. 1995 |
| " | Cattle<br>PBMC | SGE | ↓ proliferation to ConA | ↑ proliferation to LPS | ↓ IL-1 (LPS)<br>↓ TNF (LPS) | Ramachandra & Wikel 1995 |
| *D. reticulatus* | Human<br>PBMC | SGE | | | ↓ human NK cell cytotox. Restored by IFNα2 | Kubes *et al*. 1994 |
| *Ha. longicornis* | WBB6F1 mice | Infest x1 | ↑ IL-5 | | ↑ eosinophils | Ushio *et al*. 1995 |
| *I. pacificus* | C3H/HeN mice<br>mRNA<br>splenocytes<br>lymph node cells | Infest x1 | ↑ IFNγ ↑ IL-4<br>0 IL-10<br>0 IL-5 | | ↑ IL-12 | Zeidner *et al*. in press |
| " | C3H/HeN mice<br>mRNA<br>lymph node cells | SGE | ↑ IFNγ 0 IL-4<br>0 IL-10<br>0 IL-5 | | 0 IL-12 | Zeidner *et al*. in press |
| *I. ricinus* | BALB/c mice<br>splenocytes | Infest x3 | ↓↑↓ proliferation to ConA | ↑tick-specific serum Igs | | Borsky *et al*. 1994 |
| " | BALB/c mice<br>skin | Infest x3 | | | 0↑↑ basophils<br>0↑↑ eosinophils<br>0↑↑ degranulated mast cells<br>↑ monocytes ↑ neutrophils | Mbow *et al*. 1994a |
| " | BALB/c mice<br>mRNA<br>lymph & skin | Infest x3 | ↑↑↑ IFNγ ↑↑↑ IL-4<br>0↑↑ IL-2 | | | Mbow *et al*. 1994b |
| " | BALB/c mice<br>skin | Infest x3 | CD4>CD8 | No B cells detected | ↑ IL-1α (ConA) ↑ TNFα<br>↑ ICAM-1 | Mbow *et al*. 1994c |
| | Rabbits<br>PBL | Infest x2<br>Treat with IL-2 | ↑ proliferation to SGE; less in IL-2 treated rabbits | ↑ tick-specific serum Ig; more in IL-2 treated rabbits | | Schorderet & Brossard 1994 |
| " | BALB/c mice<br>splenocytes | Infest x4 | 00↑↑ proliferation to ConA<br>0↑↑↑ proliferation to PHA | ↓↓0↓ proliferation to LPS<br>0↓↓↓ proliferation to PWM<br>0000 tick-specific serum Ig<br>↓↓↓↓ Ig generation by B cells in vitro | 00↓ mast cells | Dusbabek *et al*. 1995 |
| " | BALB/c mice<br>lymphocytes | Infest x3 | ↑↑↑proliferation to SGE<br>↓↓↑ IFNγ ↑↑↑ IL-4 | | | Ganapamo *et al*. 1995 |
| " | BALB/c mice<br>T cells | Infest x3 | ↑ IL-2 ↑ GM-CSF<br>↓ IL-2 w. ↑SGE | | ↑ TNF | Ganapamo *et al*. 1996a |
| " | BALB/c mice<br>$CD4^+$T cells, B cells | Infest x1 | ↓ proliferation to ConA<br>↑ tick Ag-specific proliferation<br>↑ IL-10<br>↑ IL-5 | ↑ proliferation to LPS | | Ganapamo *et al*. 1996b |

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|---|---|---|---|---|---|---|
| " | BALB/c mice<br>CBA mice<br>C3H mice<br>C57BL/6 mice<br>DBA mice<br>FVB mice<br>SJL mice | Infest x3<br>Infest x1<br>Infest x3<br>Infest x3<br>Infest x1<br>Infest x1<br>Infest x1 | ↓↓↓ IFNγ ↑↑↑ IL-4 ↑↑↑ IL-13<br>0 IFNγ ↑ IL-4<br>0↓↓ IFNγ ↑↑↑ IL-4 0↑↑ IL-13<br>↓↓↓ IFNγ ↑↑↑ IL-4 0↑↑ IL-13<br>↓ IFNγ ↑ IL-4<br>↓ IFNγ ↑ IL-4<br>↓ IFNγ ↑ IL-4 | | | Christe *et al*. 1999 |
| " | BALB/c mice<br>Lymphocytes, serum | Infest x1 | ↓ IFNγ ↑ IL-4 | ↓ $IgG_{2a}$ ↑IgE | | Christe *et al*. 2000 |
| " | BALB/c mice<br>lymphocytes | Infest x1 | ↓ IFNγ ↑ IL-4 | ↑ tick-specific serum $IgG_1$, no $IgG_{2a}$ | | Mejri *et al*. 2001 |
| " | BALB/c mice<br>lymphocytes | SGE<br>65kDa protein | ↑ proliferation to SGE by $CD4^+$T cells | | | Ganapamo *et al*. 1997 |
| " | BALB/c mice<br>splenocytes | SGE | | | ↓ NO (LPS) ↓ IFNγ (LPS)<br>↓ NK cytotoxicity (LPS) | Kopecky & Kuthejlová 1998 |
| " | BALB/c mice<br>splenocytes | SGE | | | ↓ IFNγ (LPS) ↑ IL-10<br>anti-IL-10 restores response | Kopecky *et al*. 1999 |
| " | BALB/c mice<br>macrophages | SGE | | | ↓ NO (LPS) ↓superoxide | Kuthejlová *et al*. 2001 |
| *I. scapularis* | BALB/c mice<br>C3H/HeJ mice<br>splenocytes | Infest x1 | ↓ IFNγ ↑ IL-4<br>↓ IL-2 ↑ IL-10 | | | Zeidner *et al*. 1997 |
| " | BALB/c mice<br>splenocytes<br>macrophages | Infest x4 | 00↑0 IFNγ ↑↑↑↑ IL-4<br>0↓0↓ IL-2 ↑↑↑↑ IL-10 | | 00↓0 IL-1β 00↓0 TNFα | Schoeler *et al*. 1999 |
| " | C3H/HeN mice<br>splenocytes<br>macrophages | Infest x4 | ↓ IFNγ ↑ IL-4<br>↓ IL-2 ↑ IL-10 | | 00↑0 IL-1β 0↑00 TNFα | Schoeler *et al*. 1999 |
| " | C3H/HeN mice<br>mRNA<br>splenocytes<br>lymph node cells | Infest x1 | ↑ IFNγ ↑ IL-4<br>↑ IL-10<br>↑ IL-5 | | 0 IL-12 | Zeidner *et al*. in press |
| " | C3H/HeN mice<br>mRNA<br>lymph node cells | SGE | ↑ IFNγ ↑ IL-4<br>↑ IL-10<br>↑ IL-5 | | ↑ IL-12 | Zeidner *et al*. in press |
| " | T-hybridomas | saliva | ↓ IL-2 by tick Ag-specific cells | | | Ribeiro *et al*. 1985 |
| " | Rabbit serum + RBC | saliva | | ↓ alternative complement pathway<br>↓ C3b deposition | | Ribeiro 1987 |
| " | Rat<br>PE neutrophils | Saliva | | | ↓ neutrophil responses | Ribeiro *et al*. 1990 |
| " | C57BL/6 mice<br>splenocytes<br>PE macrophages | saliva | ↓ proliferation to ConA, PHA due to ≥5kDa trypsin-sensitive saliva fraction<br>↓ IL-2 | | ↓ NO (LPS) | Urioste *et al*. 1994 |
| *R. appendi-culatus* | Rabbit skin & splenocytes | Infest x4 | ↓ proliferation to ConA (intradermal)<br>↓ proliferation to PHA | ↓↓ Anti-BSA titers | | Fivaz 1989 |

| | | | | | | |
|---|---|---|---|---|---|---|
| " | Human<br>PBL | SGE | | | ↓ TNFα ↓ IL-1α ↓ IL-1β<br>↓ IFNα ↓IL-5 ↓IL-6<br>↓IL-7 ↓IL-8 | Fuchsberger *et al*. 1995 |
| " | Murine macrophage cell line | SGE | | | ↓ TNFα (LPS) ↓IL-10<br>↓ IL-1α (LPS) ↓NO | Gwakisa *et al*. 2001 |
| *R. evertsi evertsi* | Rabbit | Infest x1 | | 0 tick-specific serum Ig<br>↓ IgM response to BSA<br>↓ response to SRBC<br>(T cell-dependent ) | | Njau *et al*. 1990 |
| " | Sheep<br>PBL and basophils | Infest x4 | ↓ proliferation to PHA<br>↓ proliferation to SGE<br>0 % T cells in total lymphocyte count | 0 non-specific Ig production | 0 basophils 0 eosinophils<br>↑ 10x histamine-basophils | Neitz *et al*. 1993 |
| *R. sanguineus* | Dog<br>PBL | Infest x2 | ↓ proliferation to ConA, PHA, PWM; trypsin-sensitive | ↓ proliferation to LPS | | Inokuma *et al*. 1998 |
| " | C3H/HeJ mice splenocytes | saliva | ↓ proliferation to ConA due to 3-10 kDa & <3 kDa saliva fraction<br>↓ Ag-specific proliferation (KLH)<br>↓ IFNγ ↑ IL-10<br>↓ IL-2 | | ↓ mϕ activation by IFNγ<br>↓ NO induced by IFNγ | Ferreira & Silva 1998 |
| " | C3H/HeJ mice splenocytes<br>PE macrophages | Infest x4 | ↓ proliferation to ConA<br>↓ IFNγ ↑ IL-4<br>↓ IL-2 ↑ IL-10 | | ↓ IL-12<br>↑ TGFβ | Ferreira & Silva 1999 |

# REFERENCES


Abbas, A.K., Lichtman, A.H., and Pober, J.S. 2000. Cellular and Molecular Immunology, 4th edition. W.B. Saunders Company. Philadelphia, PA.

Aiello, S.E. (ed.). 1998. The Merck Veterinary Manual. Merck & Co., Inc. Whitehouse Station, NJ. Pp. 8-17.

Anderson, J.F., and Magnarelli, L.A. 1980. Vertebrate host relationships and distribution of ixodid ticks (Acari: Ixodidae) in Connecticut, U.S.A. J. Med. Entomol. 17: 314-323.

Anderson, B.E., Dawson, J.E., Jones, D.C., and Wilson, K.H. 1991. *Ehrlichia chaffeensis*, a new species associated with human ehrlichiosis. J. Clin. Microbiol. 29: 2838-2842.

Anderson, B.E., Sumner, J.W., Dawson, J.E., Tzianabos, T., Greene, C.R., Olson, J.G., Fishbein, D.B., Olsen-Rasmussen, M., Holloway, B.P., George, E.H., and Azad, A.F. 1992a. Detection of the etiologic agent of human ehrlichiosis by polymerase chain reaction. J. Clin. Microbiol. 30: 775-780.

Anderson, B. E., Greene, C.E, Jones, D.C., and Dawson, J.E. 1992b. *Ehrlichia ewingii* sp. nov., the etiologic agent of canine granulocytic ehrlichiosis. Int. J. Syst. Bacteriol. 42: 299-302.

Anderson, B.E., Sims, K.G., Olson, J.G., Childs, J.E., Piesman, J.F., Happ, C.M., Maupin, G.O., and Johnson, B.J.B. 1993. *Amblyomma americanum*: a potential vector of human ehrlichiosis. Am. J. Trop. Med. Hyg. 49: 239-244.

Barbour, A.G., Maupin, G.O., Teltow, G.J., Carter, C.J., and Piesman, J. 1996. Identification of an uncultivable *Borrelia* species in the hard tick *Amblyomma americanum*: possible agent of a Lyme disease-like illness. J. Infect. Dis. 173: 403-409.

Barnewall, R.E., and Rikihisa, Y. 1994. Abrogation of gamma interferon-induced inhibition of *Ehrlichia chaffeensis* infection in human monocytes with iron transferrin. Infect. Immun. 62: 4804-4810.

Belkaid, Y., Jouin, H., and Milon, G. 1996. A method to recover, enumerate, and identify lymphomyeloid cells present in an inflammatory dermal site. A study in laboratory mice. J. Immunol. Methods. 199: 5-25.

Belkaid, Y., Valenzuela, J.G., Kamhawi, S., Rowton, E., Sacks, D.L., and Ribeiro, J.M.C. 2000. Delayed-type hypersensitivity to *Phlebotomus papatasi* sand fly bite: an adaptive response induced by the fly? Proc. Natl. Acad. Sci. U.S.A. 97: 6704-6709.

Bergman, D.K., Ramachandra, R.N., and Wikel, S.K. 1995. *Dermacentor andersoni*: salivary gland proteins suppressing T-lymphocyte responses to concanavalin A *in vitro*. Exp. Parasitol. 81: 262-271.

Bergman, D.K., Palmer, M.J., Caimano, M.J., Radolf J.D., and Wikel, S.K. 2000. Isolation and molecular cloning of a secreted immunosuppressant protein from *Dermacentor andersoni* salivary gland. J. Parasitol. 86: 516-525.

Bishopp, F.C., and Trembley, H.L. 1945. Distribution and hosts of certain North American ticks. J. Parasitol. 31: 1-54.

Black, W.C. IV, and Piesman, J. 1994. Phylogeny of hard- and soft-tick taxa (Acari: Ixodida) based on mitochondrial 16S rDNA sequences. Proc. Natl. Acad. Sci. U.S.A. 91: 10034-10038.

Black, W.C. IV, Klompen, J.S., and Keirans, J.E. 1997. Phylogenetic relationships among tick subfamilies (Ixodida: Ixodidae: Argasidae) based on the 18S nuclear rDNA gene. Mol. Phylogenet. Evol. 7: 129-144.

Borsky, I., Hermánek, J., Uhlír, J., and Dusbábek, F. 1994. Humoral and cellular immune response of BALB/c mice to repeated infestations with *Ixodes ricinus* nymphs. Int. J. Parasitol. 24: 127-132.

Bowessidjaou, J., Brossard, M., and Aeschlimann, A. 1977. Effects and duration of resistance acquired by rabbits on feeding and egg laying in *Ixodes ricinus*. Experientia. 33: 528-530.

Bowman, A.S., Sauer, J.R., Neese, P.A., and Dillwith, J.W. 1995a. Origin of arachidonic acid in the salivary glands of the lone star tick, *Amblyomma americanum*. Insect Biochem. Molec. Biol. 25: 225-233.

Bowman, A.S., Sauer, J.R., Zhu, K., and Dillwith, J.W. 1995b. Biosynthesis of salivary prostaglandins in the lone star tick, *Amblyomma americanum*. Insect Biochem. Molec. Biol. 25: 735-741.

Brossard, M., and Girardin, P. 1979. Passive transfer of resistance in rabbits infested with adult *Ixodes ricinus* L: humoral factors influence feeding and egg laying. Experientia. 35: 1395-1397.

Brossard, M., Monneron, J.P., and Papatheodorou, V. 1982. Progressive sensitization of circulating basophils against *Ixodes ricinus* antigens during repeated infestations of rabbits. Parasite Immunol. 4: 355-361.

Brown, S.J., and Knapp, F.W. 1980. *Amblyomma americanum*: sequential histological analysis of adult feeding sites on guinea pigs. Exp. Parasitol. 49: 303-318.

Brown, S.J., and Askenase, P.W. 1982. Blood eosinophil and basophil responses in guinea pigs parasitized by adult *Amblyomma americanum* ticks. Am. J. Trop. Med. Hyg. 31: 593-598.

Brown, W.C., and Palmer, G.H. 1999. Designing blood-stage vaccines against *Babesia bovis* and *B. bigemina*. Parasitol. Today. 15: 275-281.

Buller, R. S., Arens, M., Hmiel, S.P., Paddock, C.D., Sumner, J.W., Rikihisa, Y., Unver, A., Gaudreault-Keene, M., Manian, F.A., Liddell, A.M., Schmulewitz, N., and Storch, G.A. 1999. *Ehrlichia ewingii*, a newly recognized agent of human ehrlichiosis. N. Eng. J. Med. 341: 148-155.

Burgdorfer, W. 1969. Ecology of tick vectors of American spotted fever. Bull. Wld. Hlth. Org. 40: 375-381.

Burgdorfer, W. 1975. A review of Rocky Mountain spotted fever (tick-borne typhus), its agent, and its tick vectors in the United States. J. Med. Entomol. 12: 269-278.

Burgdorfer, W. 1998. Ecological and epidemiological considerations of Rocky Mountain spotted fever and scrub typhus. In: Walker, D.H. (ed.), Biology of Rickettsial Diseases. CRC Press. Boca Raton, FL. Pp. 33-50.

Burkot, T.R., Mullen, G.R., Anderson, R., Schneider, B.S., Happ, C.M., and Zeidner, N.S. 2001. *Borrelia lonestari* DNA in adult *Amblyomma americanum* ticks, Alabama. Emerg. Infect. Dis. 7: 471-473.

Campbell, G.L., Paul, W.S., Schriefer, M.E., Craven, R.B., Robbins, K.E., and Dennis, D.T. 1995. Epidemiologic and diagnostic studies of patients with suspected early Lyme disease, Missouri, 1990-1993. J. Infect. Dis. 172: 470-480.

Carpenter, C. F., Gandhi, T.K., Kong, L.K., Corey, G.R., Chen, S-M., Walker, D.H., Dumler, J.S., Breitschwerdt, E., Hegarty, B., and Sexton, D.J. 1999. The incidence of ehrlichial and rickettsial infection in patients with unexplained fever and recent history of tick bite in central North Carolina. J. Infect. Dis. 180: 900-903.

Cartwright N., Demaine A., Jahromi M., Sanders H., and Kaminski E.R. 1999. A study of cytokine protein secretion, frequencies of cytokine expressing cells and IFN-G gene polymorphisms in normal individuals. Transplantation. 68: 1546-1552.

Champagne, D.E. 1994. The role of salivary vasodilators in bloodfeeding and parasite transmission. Parasitol. Today. 10: 430-433.

Chen, Y.L., Simmons, F.E.R., and Peng, Z.K. 1998. A mouse model of mosquito allergy for study of antigen-specific IgE and IgG subclass responses, lymphocyte proliferation, and IL-4 and IFN-gamma production. Int. Arch. Allergy Immunol. 116: 269-277.

Christe, M., Rutti, B., and Brossard, M. 1998. Susceptibility of BALB/c mice to nymphs and larvae of Ixodes ricinus after modulation of the IgE production with anti-interleukin-4 or anti-interferon-γ monoclonal antibodies. Parasitol. Res. 84: 388-393.

Christe, M., Rutti, B., and Brossard, M. 1999. Influence of the genetic background and parasite load of mice on the immune response developed against nymphs of *Ixodes ricinus*. Parasitol. Res. 85: 557-561.

Christe, M., Rutti, B., and Brossard, M. 2000. Cytokines (IL-4 and IFN-gamma) and antibodies (IgE and IgG2a) produced in mice infected with *Borrelia burgdorferi* sensu stricto via nymphs of *Ixodes ricinus* ticks or syringe inoculations. Parasitol Res. 86: 491-496.

Clymer, B.C., Howell, D.E., and Hair, J.A. 1970. Animal hosts of economically important ticks (Acarina) in east central Oklahoma. Ann. Entomol. Soc. Amer. 63: 612-614.

Comer, J.A., Nicholson, W.L., Paddock, C.D., Sumner, J.W., and Childs, J.E. 2000. Detection of antibodies reactive with *Ehrlichia chaffeensis* in the raccoon. J. Wildl. Dis. 36: 705-12.

Cooley, R.A., and Kohls, G.M. 1944. The genus *Amblyomma* (Ixodidae) in the United States. J. Parasitol. 30: 77-111.

Coons, L.B., Rosell-Davis, Rosemarie, and Tarnowksi, B.I. 1986. Bloodmeal digestion in ticks. In: Sauer, J.R., and Hair, J.A. (eds.). Morphology, Physiology, and Behavioral Biology of Ticks. John Wiley & Sons. New York, New York. Pp. 248-279.

Dalton, J.P., and Mulcahy, G. 2001. Parasite vaccines – a reality? Vet. Parasitol. 98: 149-167.

Davidson, W.R., Lockhart, J.M., Stallknecht, D.E., Howerth E.W., Dawson, J.E., and Rechav, Y. 2001. Persistent *Ehrlichia chaffeensis* infection in white-tailed deer. J. Wild. Dis. 37: 538-546.

Dawson, J.E., Anderson, B.E., Fishbein, D.B., Sanchez, J.L., Goldsmith, C.S., Wilson, K.H., and Duntley, C.W. 1991. Isolation and characterization of an Ehrlichia sp. from a patient diagnosed with human ehrlichiosis. J. Clin. Microbiol. 29: 2741-2745.

den Hollander, N., and Allen, J.R. 1985. *Dermacentor variabilis*: acquired resistance to ticks in BALB/c mice. Exp. Parasitol. 59: 118.

Dickinson, R.G., O'Hagan, J.E., Schotz, M., Binnington, K.C., and Hegarty, M.P. 1976. Prostaglandin in the saliva of the cattle tick *Boophilus microplus*. Aust. J. Exp. Biol. Med. Sci. 54: 475-486.

Dumler, J.S., and Bakken, J.S. 1995. Ehrlichial diseases of humans: emerging tick-borne infections. Clin. Infect. Dis. 20: 1102-1110.

Dusbabek, F., Borsky, I., Jelinek, F., and Uhlir, J. 1995. Immunosuppression and feeding success of *Ixodes ricinus* nymphs on BALB/c mice. Med. Vet. Entomol. 9: 133-140.

Ewing, S.A., Dawson, J.E., Kocan, A.A., Barker, R.W., Warner, C.K., Panciera, R.J., Fox, J.C., Kocan, K.M., and Blouin, F.F. 1995. Experimental transmission of *Ehrlichia chaffeensis* (Rickettsiales: Ehrlichieae) among white-tailed deer by *Amblyomma americanum* (Acari: Ixodidae). J. Med Entomol. 32: 368-374.

Fawcett, D.W., Binnington, K., and Voight, W. 1986. The cell biology of the ixodid tick salivary gland. In Sauer, J.R., and Hair, J.A. (Eds.) Morphology, Physiology, and Behavioral Biology of Ticks. Horwood. Chichester, England. Pp. 22-45.

Ferreira, B.R., and Silva, J.S. 1998. Saliva of *Rhipicephalus sanguineus* tick impairs T cell proliferation and IFN-γ-induced macrophage microbicidal activity. Vet. Immunol. Immunopath. 64: 279-293.

Ferreira, B.R., and Silva, J.S. 1999. Successive tick infestations selectively promote a T-helper 2 cytokine profile in mice. Immunology. 96: 434-439.

Fivaz, B.H. 1989. Immune suppression induced by the brown ear tick *Rhipicephalus appendiculatus* Neumann, 1901. J. Parasitol. 75: 946-952.

Fuchsberger, N., Kita, M., Hajnická, V., Imanishi, J., Labuda, M., and Nuttall, P.A. 1995. Ixodid tick salivary gland extracts inhibit production of lipopolysaccharide-induced mRNA of several different human cytokines. Exp. Appl. Acarol. 19: 671-676.

Ganapamo, F., Rutti, B., and Brossard, M. 1995. *In vitro* production of interleukin-4 and interferon-γ by lymph node cells from BALB/c mice infested with nymphal *Ixodes ricinus* ticks. Immunology. 85: 120-124.

Ganapamo, F., Rutti, B., and Brossard, M. 1996a. Cytokine production by lymph node cells from mice infested with *Ixodes ricinus* ticks and the effect of tick salivary gland extracts on IL-2 production. Scand. J. Immunol. 44: 388-393.

Ganapamo, F., Rutti, B., and Brossard, M. 1996b. Immunosuppression and cytokine production in mice infested with *Ixodes ricinus* ticks: a possible role of laminin and interleukin-10 on *in vitro* responsiveness of lymphocytes to mitogens. Immunology. 87: 259-263.

Ganapamo, F., Rutti, B., and Brossard, M. 1997. Identification of an *Ixodes ricinus* salivary gland fraction through its ability to stimulate CD4 T cells present in BALB/c mice lymph nodes draining the tick fixation site. Parasitology. 115: 91-96.

George, J.E., Osburn, R.L., and Wikel, S.K. 1985. Acquisition and expression of resistance by *Bos indicus* and *Bos indicus* x *Bos taurus* calves to *Amblyomma americanum* infestation. J. Parasitol. 71: 174-182.

Gillespie, J.R. 1998. Animal Science. Delmar Publishers. Albany, NY. Pp. 448-453, 675-677.

Gillespie, R.D., Dolan, M.C., Piesman, J., and Titus, R.G. 2001. Identification of an IL-2 binding protein in the saliva of the Lyme disease vector tick, *Ixodes scapularis*. J. Immunol. 166: 4319-4327.

Ginsberg, H.S., Ewing, C.P., O'Connell, A.F. Jr., Bosler, E.M., Daley, J.G., and Sayre, M.W. 1991. Increased population densities of *Amblyomma americanum* (Acari: Ixodidae) on Long Island, New York. J. Parasitol. 77: 493-495.

Gordon, J.R., and Allen, J.R. 1991. Nonspecific activation of complement factor 5 by isolated *Dermacentor* andersoni salivary antigens. J. Parasitol. 77: 296-301.

Gwakisa, P., Yoshihara, K., Long To, T., Gotoh, H., Amano, F., and Momotan, E. 2001. Salivary gland extract of *Rhipicephalus appendiculatus* ticks inhibits *in vitro* transcription and secretion of cytokines and production of nitric oxide by LPS-stimulated JA-4 cells. Vet. Parasitol. 99: 53-61.

Hair, J.A., and Bowman, J.L. 1986. Behavioral ecology of *Amblyomma americanum* (L.). In: Sauer, J.R., and Hair, J.A. (eds.). Morphology, Physiology, and Behavioral Biology of Ticks. John Wiley & Sons. New York, New York. Pp. 406-427.

Hajnicka, V., Kocakova, P., Slavikova, M., Slovak, M., Gasperik, J., Fuchsberger, N., and Nuttall, P.A. 2001. Anti-interleukin-8 activity of tick salivary gland extracts. Parasite Immunol. 23: 483-489.

Harris, D.P., Haynes, L., Sayles, P.C., Duso, D.K., Eaton, S.M., Lepak, N.M., Johnson, L.L., Swain, S.L., and Lund, F.E. 2000. Reciprocal regulation of polarized cytokine production by effector B and T cells. Nat. Immunol. 1: 475-482.

Hennessy, B., North, J., Deru, A., Llewellyn-Smith, N., and Lowdell, M.W. 2001. Use of Leu3a/3b for the accurate determination of CD4 subsets for measurement of intracellular cytokines. Cytometry. 44: 148-152.

Higgs, G.A., Vane, J.R., Hart, R.J., Potter, C., and Wilson, R.G. 1976. Prostaglandins in the saliva of the cattle tick, *Boophilus microplus* (Canestrini) (Acarina, Ixodidae). Bull. Entomol. Res. 66: 665-670.

Ijdo, J.W., Wu, C., Magnarelli, L.A., Stafford, K.C. III, Anderson, J.F., and Fikrig, E. 2000. Detection of *Ehrlichia chaffeensis* DNA in *Amblyomma americanum* ticks in Connecticut and Rhode Island. J. Clin. Microbiol. 38: 4655-4656.

Inokuma, H., Kerlin, R.L., Kemp, D.H., and Willadsen, P. 1993. Effects of cattle tick (*Boophilus microplus*) infestation on the bovine immune system. Vet. Parasitol. 47: 107-118.

Inokuma, H., Kemp, D.H., and Willadsen, P. 1994. Prostaglandin $E_2$ production by the cattle tick (*Boophilus microplus*) into feeding sites and its effect on the response of bovine mononuclear cells to mitogen. Vet. Parasitol. 53: 293-299.

Inokuma, H., Aita, T., Ohno, K., and Onishi, T. 1998. Effects of infestation by *Rhipicephalus sanguineus* on lymphocyte blastogenic responses to mitogens in dogs. J. Vet. Med. Sci. 60: 1013-1016.

Jackson, L.K., Gaydon, D.M., and Goddard, J. 1996. Seasonal activity and relative abundance of *Amblyomma americanum* in Mississippi. J. Med. Entomol. 33: 128-131.

James, A.M., Liveris, D., Wormser, G.P., Schwartz, I., Montecalvo, M.A., and Johnson, B.J.B. 2001. *Borrelia lonestari* infection after a bite by an *Amblyomma americanum* tick. J. Infect. Dis. 183: 1810-1814.

Jankovic, D., Liu, Z., and Gause, W.C. 2001. Th1- and Th2-cell commitment during infectious disease: asymmetry in divergent pathways. Trends Immunol. 22: 450-457.

Jasinskas, A., Jaworski, D.C., and Barbour, A.G. 2000. *Amblyomma americanum*: specific uptake of immunoglobulins into tick hemolymph during feeding. Exp. Parasitol. 96: 213-221.

Jaworski, D.C., Needham, G.R., Sherif, N., Muller, M.T., and Simmen, F.A. 1989. Characterization of the gene products from cDNA expression libraries of the salivary glands of unfed and feeding lone star ticks. In: Borovsky, D., and Spielman, A. (eds) Host Regulated Developmental Mechanisms in Vector Arthropods. University of Florida Press. Vero Beach, Florida. Pp. 238-247.

Jaworski, D.C., Muller, M.T., Simmen, F.A., and Needham, G.R. 1990. *Amblyomma americanum*: identification of tick salivary gland antigens from unfed and early feeding females with comparisons to *Ixodes dammini* and *Dermacentor variabilis*. Exp. Parasitol. 70: 217-226.

Jaworski, D.C., Jasinskas, A., Metz, C.N., Bucala, R., and Barbour, A.G. 2001. Identification and characterization of a homologue of the pro-inflammatory cytokine Macrophage Migration Inhibitory Factor in the tick, *Amblyomma americanum*. Insect Mol. Biol. 10: 323-331.

Jones, L.D., Hodgson, E., and Nuttall, P.A. 1989. Enhancement of virus transmission by tick salivary glands. J. Gen. Virol. 70: 1895-1898.

Jones, L.D., Kaufman, W.R, and Nuttall, P.A. 1992. Modification of the skin feeding site by tick saliva mediates virus transmission. Experientia. 48: 779-782.

Kamhawi, S. 2000. The biological and immunomodulatory properties of sand fly saliva and its role in the establishment of *Leishmania* infections. Microbes Infect. 2: 1765-1773.

Kaufman, W.R., Diehl, P.A., and Aeschlimann, A.A. 1976. Na,K-ATPase in the salivary glands of the ixodid tick *Amblyomma hebraeum* (Koch) and its relation to the process of fluid secretion. Experientia. 32: 986-987.

Keirans, J.E. 1992. Systematics of the Ixodida (Argasidae, Ixodidae, Nuttalliellidae): an overview and some problems. In: Fivaz, B., Petney, T., and Horak, I. (eds.) Tick Vector Biology: Medical and Veterinary Aspects. Springer-Verlag. New York, New York. Pp. 1-21.

Kelso, A. 1998. Cytokines: principles and prospects. Immunol. Cell Biol. 76: 300-317.

Kemp, D.H., Agbede, R.I.S., Johnston, L.A.Y., and Gough J.M. 1986. Immunization of cattle against *Boophilus microplus* using extracts derived from adult ticks: feeding and survival of the parasite on vaccinated cattle. Int. J. Parasitol. 16: 115-120.

Kemp, D.H., Pearson, R.D., Gough, J.M., and Willadsen, P. 1989. Vaccination against *Boophilus microplus*: localization of antigens on tick gut cells and their interaction with the host immune system. Exp. Appl. Acarol. 17: 43-58.

Kirkland, K.B., Klimko, T.B., Meriwether, R.A., Schriefer, M., Levin, M., Levine, J., MacKenzie, W.R., and Dennis, D.T. 1997. Erythema migrans-like rash illness at a camp in North Carolina – a new tick-borne disease? Arch. Int. Med. 157: 2635-2641.

Kocan, A., Crowder Levesque, G., Whitworth, L.C., Murphy, G.L., Ewing, S.A., and Barker, R.W. 2000. Naturally occurring *Ehrlichia chaffeensis* infection in coyotes from Oklahoma. Emerg. Infect. Dis. 6: 477-480.

Koch, H.G., Sauer, J.R., and Hair, J.A. 1974. Concentration of the ingested meal in four species of hard ticks. Ann. Entomol. Soc. America. 67: 861-866.

Koney, E.B., Morrow, A.N., Heron, I., Ambrose, N.C., and Scott, G.R. 1994. Lymphocyte proliferative responses and the occurrence of dermatophilosis in cattle naturally infested with *Amblyomma variegatum*. Vet. Parasitol. 55: 245-256.

Kopecky, J., and Kuthejlová, M. 1998. Suppressive effect of *Ixodes ricinus* salivary gland extract on mechanisms of natural immunity *in vitro*. Parasite Immunol. 20: 169-174.

Kopecky, J., Kuthejlová, M., and Pechová, J. 1999. Salivary gland extract from *Ixodes ricinus* ticks inhibits production of interferon-γ by the upregulation of interleukin-10. Parasite Immunol. 21: 351-356.

Kuthejlová, M., Kopecky, J., Stepanova, G., and Macela, A. 2001. Tick salivary gland extract inhibits killing of *Borrelia afzelii* spirochetes by mouse macrophages. Infect. Immun. 69: 575-578.

Kubes, M., Fuchsberger, N., Labuda, M., Zuffová, E., and Nuttall, P.A. 1994. Salivary gland extracts of partially fed *Dermacentor reticulatus* ticks decrease natural killer cell activity *in vitro*. Immunology. 82: 113-116.

Lawrie, C.H., and Nuttall, P.A. 2001. Tick-host interactions: the anti-complement activity in *Ixodes ricinus*. The Royal Entomological Society International Symposium. University of Aberdeen. September 10-12, 2001

Lee, E.H., and Rikihisa, Y. 1996. Absence of tumor necrosis factor alpha, interleukin-6 (IL-6), and granulocyte-macrophage colony-stimulating factor expression but presence of IL-1β, IL-8, and IL-10 expression in human monocytes exposed to viable or killed *Ehrlichia chaffeensis*. Infect. Immun. 64: 4211-4219.

Li, L., Sad, S., Kagi, D., and Mosmann, T.R. 1997. CD8 Tc1 and Tc2 cells secrete distinct cytokine patterns *in vitro* and *in vivo* but induce similar inflammatory reactions. J. Immunol. 158: 4152-4161.

Lockhart, J.M., Davidson, W.R., Dawson, J.E., and Stallknecht, D.E. 1995. Temporal association of *Amblyomma americanum* with the presence of *Ehrlichia chaffeensis* reactive antibodies in white-tailed deer. J. Wild. Dis. 31: 119-124.

Lockhart, J.M., Davidson, W.R., Stallknecht, D.E., and Dawson, J.E. 1996. Site-specific geographic association between *Amblyomma americanum* (Acari: Ixodidae) infestations and *Ehrlichia chaffeensis*-reactive (Rickettsiales: Ehrlichieae) antibodies in white-tailed deer. J. Med. Entomol. 33: 153-158.

Lockhart, J.M., Davidson, W.R., Dawson, J.E., Stallknecht, D.E., and Howerth E.W. 1997a. Isolation of *Ehrlichia chaffeensis* from wild white-tailed deer (*Odocoileus virginianus*) confirms their role as natural reservoir hosts. J. Clin. Microbiol. 35: 1681-1686.

Lockhart, J.M., Davidson, W.R., Dawson, J.E., and Stallknecht, D.E. 1997b. Natural history of *Ehrlichia chaffeensis* in the Piedmont physiographic province of Georgia. J. Parasitol. 83: 887-894.

Lockhart, J.M., Davidson, W.R., Stallknecht, D.E., and Dawson, J.E. 1998. Lack of seroreactivity to *Ehrlichia chaffeensis* among rodent populations. J. Wild. Dis. 34: 392-396.

Lohoff, M., Gessner, A., Bogdan, C., and Röllinghoff, M. 1998. The Th1/Th2 paradigm and experimental murine leishmaniasis. Int. Arch. Allergy Immunol. 115: 191-202.

Luttmann, W., Sengler, C., Herzog, V., Balkow, S., Matthys, H., and Virchow, J.C. Jr. 1999. Differential modulation of interleukin-4 and interleukin-13 secretion from human peripheral blood mononuclear cells. Immunology Letters. 69: 225-231.

Macaluso, K.R., and Wikel, S.K. 2001. *Dermacentor andersoni*; effects of repeated infestations on lymphocyte proliferation, cytokine production, and adhesion-molecule expression by BALB/c mice. Ann. Trop. Med. Parasitol. 95: 413-427.

Madden, R.D., Sauer, J.R., Dillwith, J.W., and Bowman, A.S. 1996. Alteration of arachidonate levels in tick salivary glands by dietary modification of host blood lipids. Arch. Ins. Biochem. Physiol. 31: 53-72.

Maeda, K., Markowitz, N., Hawley, R.C., Ristic, M., Cox, D., and McDade, J. 1987. Human infection with *Ehrlichia canis*, a leukocytic rickettsia. N. Engl. J. Med. 316: 853-856.

Mascher, B., Schlenke, P., and Seyfarth, M. 1999. Expression and kinetics of cytokines determined by intracellular staining using flow cytometry. J. Immunol. Methods. 223: 115-121.

Mbow, M.L., Christe, M., Rutti, B., and Brossard, M. 1994a. Absence of acquired resistance of nymphal *Ixodes ricinus* L. ticks in BALB/c mice developing cutaneous reactions. J. Parasitol. 80: 81-87.

Mbow, M.L., Rutti, B., and Brossard, M. 1994b. IFNg, IL-2, and IL-4 mRNA expression in the skin and draining lymph nodes of BALB/c mice repeatedly infested with nymphal *Ixodes ricinus* ticks. Cell. Immunol. 156: 254-261.

Mbow, M.L., Rutti, B., and Brossard, M. 1994c. Infiltration of $CD4^+$ $CD8^+$ T cells, and expression of ICAM-1, Ia antigens, IL-1$\alpha$ and TNF-$\alpha$ in the skin lesion of BALB/c mice undergoing repeated infestations with nymphal *Ixodes ricinus* ticks. Immunology. 82: 596-602.

McKeever, D.J., Taracha, E.L.N., Morrison, W.I., Musoke, A.J., and Morzaria, S.P. 1999. Protective immune mechanisms against *Theileria parva*: evolution of vaccine development strategies. Parasitol. Today. 15: 263-267.

McMullen, H.L., Bantle, J.A., Essenberg, R.C., and Sauer, J.R. 1983. Changes in the cyclic nucleotide phosphodiesterase activity in the salivary glands of female *Amblyomma americanum* (L.) ticks during feeding. Insect Biochemistry. 13: 585-592.

McSwain, J.L., Essenberg, R.C., and Sauer, J.R. 1982. Protein changes in the salivary glands of the female lone star tick, *Amblyomma americanum*, during feeding. J. Parasitol. 68: 100-106.

McSwain, J.L., Essenberg, R.C., and Sauer, J.R. 1992. Oral secretion elicited by effectors of signal transsduction pathways in the salivary glands of *Amblyomma americanum* (Acari: Ixodidae). J. Med. Entomol. 29: 41-48.

Mejri, N., Franscini, N., Rutti, B., and Brossard, M. 2001. Th2 polarization of the immune response of BALB/c mice to *Ixodes ricinus* instars, importance of several antigens in activation of specific Th2 subpopulations. Parasite Immunol. 23: 61-69.

Möller, G. 1999. Receptors for innate pathogen defence in insects are normal activation receptors for specific immune responses in mammals. Scand. J. Immunol. 50: 341-347.

Moore, D.M. 2000. Hematology of the mouse (*Mus musculus*). In: Feldman, B.F., Zinkl, J.G., and Jain, N.C. (eds.) Schalm's Veterinary Hematology, 5th edition. Lippincott Williams & Wilkins. Philadelphia, PA. Pp. 1219-1224.

Morel, P.A., and Oriss, T.B. 1998. Crossregulation between Th1 and Th2 cells. Crit. Rev. Immunol. 18: 275-303.

Mosmann, T.R., and Coffman, R.L. 1989. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annu. Rev. Immunol. 7: 145-173.

Murphy G.L., Ewing S.A., Whitworth L.C., Fox J.C., and Kocan A.A. 1998. A molecular and serologic survey of *Ehrlichia canis*, *E. chaffeensis*, and *E. ewingii* in dogs and ticks from Oklahoma. Vet Parasitol. 79: 325-339.

Murphy, K.M., Heinberger, A.B., and Loh, D.Y. 1990. Induction by antigen of intrathymic apoptosis of $CD4^{+}CD8^{+}$TCRlo thymocytes in vivo. Science. 250: 1720-1723.

Neitz, A.W., Gothe, R., Pawlas, S., and Groeneveld, H.T. 1993. Investigations into lymphocyte transformation and histamine release by basophils in sheep repeatedly infested with *Rhipicephalus evertsi evertsi* ticks. Exp. Appl. Acarol. 17: 551-559.

Nelson, W.A., and Kozub, G.C. 1980. *Melophagus ovinus* (Diptera, Hippoboscidae – evidence of local mediation in acquired resistance of sheep to keds. J. Med. Entomol. 17: 291-297.

Njau, B.C., Nyindo, M., and Mutani, A. 1990. The interaction between the immune response of rabbits to heterologous antigens and a primary infestation with *Rhipicephalus evertsi evertsi*. Vet. Res. Commun. 14: 113-122.

Nuttall, P. 1998. Displaced tick-parasite interactions at the host interface. Parasitology. 116: S65-S72.

Oaks, J.F., McSwain, J.L., Bantle, J.A., Essenberg, R.C., and Sauer, J.R. 1991. Putative new expression of genes in ixodid tick salivary gland development during feeding. J. Parasitol. 77: 378-383.

Ouellette, J., Apperson, C.S., Howard, P., Evans, T.L., and Levine, J.F. 1997. Tick-raccoon associations and the potential for Lyme disease spirochete transmission in the coastal plain of North Carolina. J. Wild. Dis. 33: 28-39.

Paddock, C.D., Sumner, J.W., Shore, G.M., Bartley, D.C., Elie, R.C., McQuade, J.G., Martin, C.R., Goldsmith, C.S., and Childs, J.E. 1997. Isolation and characterization of *Ehrlichia chaffeensis* strains from patients with fatal ehrlichiosis. J. Clin. Microbiol. 35: 2496-2502.

Paddock, C.D., Folk, S.M., Shore, G.M., Machado, L.J., Huycke, M.M., Slater, L.N., Liddell, A.M., Buller, R.S., Storch, G.A., Monson, T.P., Rimland, D., Sumner, J.W., Singleton, J., Bloch, K.C., Tang, Y.W., Standaert, S.M., and Childs, J.E. 2001. Infections with *Ehrlichia chaffeensis* and *Ehrlichia ewingii* in persons coinfected with human immunodeficiency virus. Clin. Infect. Dis. 33: 1586-1594.

Paesen, G.C., Adams, P.L., Harlos, K., Nuttall, P.A., and Stuart, D.I. 1999. Tick histamine-binding proteins: isolation, cloning, and three-dimensional structure. Mol. Cell. 3: 661-671.

Patterson, J.W., Fitzwater, J.E., and Connell, J. 1979. Localized tick bite reaction. Cutis. 24: 168-169.

Pedibhotla, V.K., Sarath, G., Sauer, J.R., and Stanley-Samuelson, D.W. 1995. Prostaglandin biosynthesis and subcellular localization of prostaglandin H synthase activity in the lone star tick, *Amblyomma americanum*. Insect Biochem. Molec. Biol. 25: 1027-1039.

Petersen, L.R., Sawyer, L.A., Fishbein, D.B., Kelley, R.W., Thomas, R.J., Magnarelli, L.A., Redus, M., and Dawson, J.E. 1989. An outbreak of ehrlichiosis in members of an army reserve unit exposed to ticks. J. Infect. Dis. 159: 562-568.

Preston, P.M., and Jongejan, F. 1999. Protective immune mechanisms to ticks and tick-borne diseases of ruminants. Parasitol. Today. 15: 255-258.

Prussin, C., and Metcalfe, D.D. 1995. Detection of intracytoplasmic cytokine using flow cytometry and directly conjugated anti-cytokine antibodies. J. Immunol. Methods. 188: 117-128.

Ramachandra, R.N., and Wikel, S.K. 1992. Modulation of host-immune responses to ticks (Acari: Ixodidae): effect of salivary gland extracts on host macrophages and lymphocyte cytokine production. J. Med. Entomol. 29: 818-826.

Ramachandra, R.N., and Wikel, S.K. 1995. Effect of *Dermacentor andersoni* (Acari: Ixodidae) salivary gland extracts on *Bos indicus* and *B. taurus* host lymphocytes and macrophages: *in vitro* cytokine elaboration and lymphocyte blastogenesis. J. Med. Entomol. 32: 338-345.

Rechav, Y., and Nuttall, P.A. 2000. The effect of male ticks on the feeding performance of immature stages of *Rhipicephalus sanguineus* and *Amblyomma americanum* (Acari: Ixodidae). Exp. Appl. Acarol. 24: 569-578.

Redpath, S., Ghazal, P., and Gascoigne, N.R.J. 2001. Hijacking and exploitation of IL-10 by intracellular pathogens. Trend Microbiol. 9: 86-92.

Ribeiro, J.M.C. 1987. *Ixodes dammini*: salivary anti-complement activity. Exp. Parasitol. 64: 347-353.

Ribeiro, J.M.C., Makoul, G.T., Levine, J., Robinson, D.R., and Spielman, A. 1985. Antihemostatic, antiinflammatory, and immunosuppressive properties of the saliva of a tick, *Ixodes dammini*. J. Exp. Med. 161: 332-344.

Ribeiro, J.M.C., and Spielman, A. 1986. *Ixodes dammini*: salivary anaphylatoxin inactivating activity. Exp. Parasitol. 62: 292-297.

Ribeiro, J.M.C., Weiss, J.J., and Telford III, S.R. 1990. Saliva of the tick *Ixodes dammini* inhibits neutrophil function. Exp. Parasitol. 70: 382-388.

Ribeiro, J.M.C., Evans, P.M., McSwain, J.L., and Sauer, J.R. 1992. *Amblyomma americanum*: characterization of salivary prostaglandins $E_2$ and $F_{2\alpha}$ by PR-HPLC/bioassay and gas chromatography-mass spectrometry. Exp. Parasitol. 74: 112-116.

Rich, S.M., Armstrong, P.M., Smith, R.D., and Telford, S.R. III. 2001. Lone star tick-infecting borreliae are most closely related to the agent of bovine borreliosis. J. Clin. Microbiol. 39: 494-497.

Roland, W.E., Everett, E.D., Cyr, T.L., Hasan, S.Z., Dommaraju, C.B., and McDonald, G.A. 1998. *Ehrlichia chaffeensis* in Missouri ticks. Am. J. Trop. Med. Hyg. 59: 641-643.

Romagnani, S. 1997. The Th1/Th2 paradigm. Immunol. Today. 18: 263-266.

Romani, L., Mencacci, A., Cenci, E., Spaccapelo, R., DelSoro, G., Nicoletti, I., Trinchieri, G., Bistoni, F., and Puccetti, P. 1997. Neutrophil production of IL-12 and IL-10 in candidiasis and efficacy of IL-12 therapy in neutropenic mice. J. Immunol. 158: 5349-5356.

Sad, S., Krishnan, L., Bleackley, R.C., Kagi, D., Hengartner, H., and Mosmann T.R. 1997. Cytotoxicity and weak CD40 ligand expression of $CD8^+$ type 2 cytotoxic T cell restricts their potential B cell helper activity. Eur. J. Immunol. 27: 914-922.

Sanders, M.L., Scott, A.L., Glass, G.E., and Schwartz, B.S. 1996. Salivary gland changes and host antibody responses associated with feeding of male lone star ticks (Acari: Ixodidae). J. Med. Entomol. 33: 628-634.

Sauer, J.R., and Hair, J.A. 1972. The quantity of blood ingested by the lone star tick (Acarina: Ixodidae). Ann. Entomol. Soc. America. 65: 1065-1068.

Sauer, J.R., Needham, G.R., McMullen, H.L., and Morrison, R.D. 1979. Cyclic nucleotides, calcium and salivary fluid secretion in ixodid ticks. Recent Adv. Acarol. 1: 365-373.

Sauer, J.R., Essenberg, R.C., and Bowman, A.S. 2000. Salivary glands in ixodid ticks: control and mechanism of secretion. J. Insect Physiol. 46: 1069-1078.

Schmitz, J.L., Schell, R.F., Callister, S.M., Lovrich, S.D., Day, S.P., and Coe, J.E. 1992. Immunoglobulin G2 confers protection against *Borrelia burgdorferi* infection in LSH hamsters. Infect. Immun. 60: 2677-2682.

Schoeler, G.B., Manweiler, S.A., and Wikel, S.K. 1999. *Ixodes scapularis*: effects of repeated infestations with pathogen-free nymphs on macrophage and T lymphocyte cytokine responses of BALB/c and C3H/HeN mice. Exp. Parasitol. 92: 239-248.

Schorderet, S., and Brossard, M. 1994. Effects of human recombinant interleukin-2 on resistance, and on the humoral and cellular response of rabbits infested with adult *Ixodes ricinus* ticks. Vet. Parasitol. 54: 375-387.

Schramke, M.L., McNew, R.W., Schmidt, S.P., Essenberg, R.C., and Sauer, J.R. 1984. Changes in dopamine-sensitive adenylate cyclase activity in salivary glands of the female lone star tick, *Amblyomma americanum* (L.), during feeding. Insect Biochem. 14: 595:600.

Schulze, T.L., Bowen, G.S., Bosler, E.M., Lakat, M.F., Parkin, W.E., Altman, R., Ormiston, B.G., and Shisler, J.K. 1984. *Amblyomma americanum*: a potential vector of Lyme disease in New Jersey. Science. 224: 601-603.

Shaw, M.K., Tilney, L.G., and McKeever, D.J. 1993. Tick salivary gland extract and interleukin-2 stimulation enhance susceptibility of lymphocytes to infection by *Theileria parva* sporozoites. Infect. Immun. 61: 1486-1495.

Shelby, K.S., Bantle, J.A., and Sauer, J.R. 1987. Biochemical differentiation of lone star tick, *Amblyomma americanum* (L.), salivary glands: effects of attachment, feeding, mating. Insect Biochem. 17: 883-890.

Shipley, M.M., Dillwith, J.W., Bowman, A.S., Essenberg, R.C., and Sauer, J.R. 1993. Changes in the lipids of the salivary glands of the lone star tick, *Amblyomma americanum*, during feeding. J. Parasitol. 79: 834-842.

Sonenshine, D.E. 1979. Ticks of Virginia (Acari: Metastigmata). Research Division Bulletin 139. Virginia Polytechnic Institute and State University. Blacksburg, Virginia. Pp. 1-41.

Sonenshine, D.E. 1991. Biology of Ticks, Vol.1. Oxford University Press. New York, New York. Pp. 1-66, 119-188.

Sonenshine, D.E. 1993. Biology of Ticks, Vol.2. Oxford University Press. New York, New York. Pp. 107-319.

Standaert, S.M., Dawson, J.E., Schaffner, W., Childs, J.E., Biggie, K.L., Singleton, J. Jr., Gerhardt, R.R., Knight, M.L., and Hutcheson, R.H. 1995. Ehrlichiosis in a golf-oriented retirement community. N. Engl. J. Med. 333: 420-425.

Steiner, F.E., Pinger, R.R., and Vann, C.N. 1999. Infection rates of *Amblyomma americanum* (Acari: Ixodidae) by *Ehrlichia chaffeensis* (Rickettsiales: Ehrlichieae) and prevalence of *Ehrlichia chaffeensis*-reactive antibodies in white-tailed deer in southern Indiana, 1997. J. Med. Entomol. 36: 715-719.

Stromdahl, E.Y., Randolph, M.P., O'Brien, J.J., and Gutierrez, A.G. 2000. *Ehrlichia chaffeensis* (Rickettsiales: Ehrlichieae) infection in *Amblyomma americanum* (Acari: Ixodidae) at Aberdeen Proving Ground, Maryland. J. Med. Entomol. 37: 349-356.

Stromdahl, E.Y., Evans, S.R., O'Brien, J.J., and Gutierrez, A.G. 2001. Prevalence of infection in ticks submitted to the human tick test kit program of the U.S. Army Center for Health Promotion and Preventive Medicine. J. Med. Entomol. 38: 67-74.

Sumner, J.W., Nicholson, W.L., and Massung, R.F. 1997. PCR amplification and comparison of nucleotide sequences from the *groESL* heat shock operon of *Ehrlichia* species. J. Clin. Microbiol. 35: 2087-2092.

Theodos, C.M., and Titus, R.G. 1993. Salivary gland material from the sand fly *Lutzomyia longipalpis* has an inhibitory effect on macrophage function in vitro. Parasite Immunol. 15: 481-487.

Titus, R.G., and Ribeiro, J.M.C. 1988. Salivary gland lysates from the sand fly *Lutzomyia longipalpis* enhance *Leishmania* infectivity. Science. 239: 1306-1308.

Totté, P., Bensaid, A., Mahan, S.M., Martinez, D., and McKeever, D.J. 1999. Immune responses to *Cowdria ruminantium* infections. Parasitol. Today. 15: 290-294.

Trager, W. 1939. Acquired immunity to ticks. J. Parasitol. 25: 57-81.

Urioste, S., Hall, L.E., Telford III, S.R., and Titus, R. 1994. Saliva for the Lyme disease vector, *Ixodes dammini*, blocks cell activation by a nonprostaglandin E2-dependent mechanism. Journal of Experimental Medicine. 180: 1077-1085.

Ushio H, Hirota S, Jippo T, Higuchi S, Kawamoto K, Kitamura Y, and Matsuda H. 1995. Mechanisms of eosinophilia in mice infested with larval *Haemaphysalis longicornis* ticks. Immunology. 84: 469-75.

Valenzuela, J.G., Charlab, R., Mather, T.N. and Ribeiro, J.M.C. 2000. Purification, cloning, and expression of a novel salivary anticomplement protein from the tick, *Ixodes scapularis*. J. Biol. Chem. 275: 18717-18723.

Walker, A.R., Fletcher, J.D., and Gill, H.S. 1985. Structural and histochemical changes in the salivary glands of *Rhipicephalus appendiculatus* during feeding. Int. J. Parasitol. 15: 81-100.

Walker, A.R., and Fletcher, J.D. 1987. Histology of digestion in nymphs of *Rhipicephalus appendiculatus* fed on rabbits and cattle naïve and resistant to the ticks. Int. J. Parasitol. 17: 1393-411.

Walker, D.H., Olano, J.P., and Feng, H-M. 2001. Critical role of cytotoxic T lymphocytes in immune clearance of rickettsial infection. Infect. Immun. 69: 1841-1846.

Wang, H., and Nuttall, P.A. 1994. Excretion of host immunoglobulin in tick saliva and detection of IgG-binding proteins in tick haemolymph and salivary glands. Parasitology. 109: 525-530.

Wang, H., Paesen, G.C., Nuttall, P.A., and Barbour, A.G. 1998. Male ticks help their mates to feed. Nature. 391. 753-754.

Whitlock, J.E., Fang, Q.Q., Durden, L.A., and Oliver, J.H. Jr. 2000. Prevalence of *Ehrlichia chaffeensis* (Rickettsiales: Ehrlichieae) in *Amblyomma americanum* (Acari: Ixodidae) from the Georgia coast and barrier islands. J. Med. Entomol. 37: 276-280.

Wikel, S.K. 1982. Influence of *Dermacentor andersoni* infestation on lymphocyte responsiveness to mitogens. Ann. Trop. Med. Parasitol. 76: 627-632.

Wikel, S.K. 1985. Effects of tick infestation on the plaque-forming cell response to a thymic dependent antigen. Ann. Trop. Med. Parasitol. 79: 195-198.

Wikel, S.K. 1992. Immunological strategies for suppression of vector arthropods: novel approaches in vector control. Bull. Soc. Vector Ecol. 17: 10-19.

Wikel, S.K. 1996a. Immunology of the skin. In Wikel, S.K. (ed.) The Immunology of Host-Ectoparasitic Arthropod Relationships. CAB International. Wallingford, England. Pp. 1-29.

Wikel, S.K. 1996b. Immunology of the tick-host interface. In Wikel, S.K. (ed.). The Immunology of Host-Ectoparasitic Arthropod Relationships. CAB International. Wallingford, England. Pp. 204-231.

Wikel, S.K. 1999. Tick modulation of host immunity: an important factor in pathogen transmission. Int. J. Parasitol. 29: 851-859.

Wikel, S.K., and Osburn, R.L. 1982. Immune response of the bovine host to repeated low-level infestations with *Dermacentor andersoni*. Ann. Trop. Med. Parasitol. 76: 405-414.

Wikel, S.K., Ramachandra, R.N., Bergman, D.K., Burkot, T.R., and Piesman, J. 1997. Infestation with pathogen-free nymphs of the tick *Ixodes scapularis* induces host resistance to transmission of *Borrelia burgdorferi* by ticks. Infect. Immun. 65: 335-338.

Winslow, G.M., Yager, E., Shilo, K., Volk, E., Reilly, A., and Chu, F.K. 2000. Antibody-mediated elimination of the obligate intracellular bacterial pathogen *Ehrlichia chaffeensis* during active infection. Infect. Immun. 68: 2187-2195.

Yawalkar N., Hunger R.E., Pichler W.J., Braathen L.R., and Brand, C.U. 2000. Human afferent lymph from normal skin contains an increased number of mainly memory / effector $CD4^+$ T cells expressing activation, adhesion and co-stimulatory molecules. Eur J Immunol. 30: 491-497.

Zeidner, N., Dreitz, M., Belasco, D., and Fish, D. 1996. Suppression of acute *Ixodes scapularis*-induced *Borrelia burgdorferi* infection using tumor necrosis factor-α, interleukin-2, and interferon-γ. J. Infect. Dis. 173: 187-195.

Zeidner, N., Mbow, M.L., Dolan, M., Massung, R., Baca, E., and Piesman, J. 1997. Effects of *Ixodes scapularis* and *Borrelia burgdorferi* on modulation of the host immune response: induction of a TH2 cytokine response in Lyme disease-susceptible (C3H/HeJ) mice but not in disease-resistant (BALB/c) mice. Infect. Immun. 65: 3100-3106.

Zeidner, N., Dolan, M.C., Massung, R., Piesman, J., and Fish, D. 2000. Coinfection with *Borrelia burgdorferi* and the agent of human granulocytic ehrlichiosis suppresses IL-2 and IFNγ production and promotes an IL-4 response in C3H/HeJ mice. Parasite Immunol. 22: 581-588.

Zeidner, S., Rutherford, J.S., Schoeler, G.B., Schneider, B.S., Dolan, M.C., Kimsey, R., and Wikel, S.K. In press. Neutralization of IL-4 counteracts tick modulation of host immunity and reduces *Borrelia burgdorferi* infection in Lyme disease susceptible mice.

Zhu, K., Sauer, J.R., Bowman, A.S., and Dillwith, J.W. 1997a. Identification and characterization of anticoagulant activities in the saliva of the lone star tick, *Amblyomma americanum* (L.). J. Parasitol. 83: 38-43.

Zhu, K., Dillwith, J.W., Bowman, A.S., and Sauer, J.R. 1997b. Identification of hemolytic activity in the saliva of the lone star tick (Acari: Ixodidae). J. Med. Entomol. 34: 160-166.