TRANSCRIPTIONAL PROFILING OF IMMUNE RESPONSES IN CATTLE EXPERIMENTALLY INFESTED WITH *AMBLYOMMA AMERICANUM* TICKS

A Dissertation

by

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ABSTRACT

Infestation of cattle by Lone Star ticks, *Amblyomma americanum*, leads to damage of hides intended for leather, weight loss, infertility, and potentially death of cattle, which contribute to production losses for farmers. Public concerns regarding chemical residues in food and the environment necessitate development of chemical-free alternative tick controls, such as breeding for tick-resistant phenotypes and developing anti-tick vaccines. Thus, the goal of this study was elucidation of mechanisms that mediate immune responses in cattle infested with *A. americanum* using gene expression techniques.

Methods for isolation of total RNA from bovine tick bite-site biopsies and blood leukocytes were optimized to provide RNA suitable for gene expression studies. Tick bite-site biopsies (6 mm) and blood leukocytes were collected from a total of 13 calves (N=6, Group 4 and N=7, Group 5 calves) during experimental tick infestations to determine *A. americanum* tick-susceptible and -resistant phenotypes. Microarray experiments compared gene expression in tick bite-sites from tick-susceptible, moderately tick-resistant, and highly tick-resistant calves. A total of 35 genes were profiled in tick bite-site biopsies and 12 genes were evaluated in blood leukocytes via gene-specific qRT-PCR assays, and analyzed for each phenotype and for each group of calves as a whole.

Analysis of microarray data revealed differential expression of IL-1R-mediators among the three cattle phenotypes. Expression profiles generated by qRT-PCR for

TLR-mediating genes such as *TLR2*, *TLR4*, *CD14*, and *MyD88* suggest that a MyD88-dependent signaling pathway may mediate the development of acquired immunity in cattle infested with Lone Star ticks. Additionally, increased expression for *IL12*, *IFNγ*, and *TNFα* suggests that a Th1-type cell-mediated reaction may be activated, whereas increased expression of *IL6*, *IL10*, and *IGHG1* supports the involvement of a Th2-type humoral-mediated response at tick bite-sites in cattle infested with at *A. americanum*. Regression analyses identified strong correlations between factors involved in pattern recognition in tick bite-site biopsies, including associations between *TLR4* and *IL1α*, and between *IL1α* and *IL1RN*. In conclusion, this dissertation reports optimal methodology for gene expression studies in tick-infested cattle and provides preliminary data concerning the underlying mechanisms associated with the immune response in Lone Star tick-infested cattle.

DEDICATION

This dissertation is dedicated to my family, including those related by blood and by spirit. My late father, Sonny Brannan, imparted a passion for understanding how things work and a deep appreciation for nature and biology that inspired the resolve to pursue a graduate degree. The delight of my mother, Denise Brannan, empowered me in pursuit to attain an academic degree beyond family expectations. God bless my grandmother, Gale Browning, for her faith and inspiration that lent me the strength to withstand the trials experienced. I deeply appreciate the unwavering support of my three sisters, Haly Brannan, Sheri Brannan, and Annie Brannan, whom are also my closest confidants. I am grateful for the encouragement of my brother, Matthew Brannan, and his wife, Cheri Brannan. Thanks also go to my husband, Aaron Conrad, and his charming family for all their love and inspiration. I am truly fortunate to have two of the best friends anyone could wish for, Marlinda Polanco and Robert Hoyle, who have shared in all my joys and heartaches throughout my pursuit of this degree.

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NOMENCLATURE

qRT-PCR	Quantitative real time reverse-transcriptase polymerase chain reaction
RFLP	Restriction fragment length polymorphism
A. americanum	*Amblyomma americanum* (a.k.a. Lone Star tick)
R. microplus	*Rhipicephalus (Boophilus) microplus* (a.k.a. cattle tick)
USDA-ARS	United States Department of Agriculture, Agriculture Research Station
KBUSLIRL	Knipling-Bushland U.S. Livestock Insects Research Laboratory

TABLE OF CONTENTS

CHAPTER Page

CHAPTER Page

LIST OF FIGURES

FIGURE Page

LIST OF TABLES

CHAPTER I

INTRODUCTION AND

LITERATURE REVIEW

1.1 Goals and Objectives

This study was designed to identify genes that mediate immune responses in cattle infested with Lone Star ticks, *Amblyomma americanum*. Calves were phenotyped for tick-resistance or –susceptibility to Lone Star ticks, as determined by total engorgement weights of female ticks after two experimental *A. americanum* infestations. Skin biopsies from tick bite-sites and blood samples were collected from calves prior to and at time points after experimental tick infestations. Total RNA was extracted from the bovine specimens and reverse transcribed for evaluation of gene expression. Bovine oligonucleotide microarrays were used to identify differentially expressed genes in tick bite-sites among three time points for tick-susceptible, moderately tick-resistant, and highly tick-resistant calves. Gene-specific assays were utilized to profile candidate genes via quantitative real time reverse-transcriptase PCR (qRT-PCR) across multiple time points and tick infestations for each of the three different cattle phenotypes and as a whole for each group of calves. The differentially expressed immune genes from tick-susceptible and -resistant cattle infested with *A. americanum* ticks may be compared to similar gene expression studies with *Rhipicephalus (Boophilus) microplus* tick-infested cattle.

These comparisons may help identify ways to advance anti-tick vaccines and selective breeding strategies to improve tick control programs and overall herd health.

1.2 Background

1.2.1 Tick problem

Ticks are arachnid ectoparasitic arthropods that feed on warm and cold-blooded animals. As vectors of pathogens that cause disease for humans, livestock, and companion animals, tick control is a necessity across the globe (PAROLA and RAOULT 2001). As the most important vector for pathogens affecting livestock, Ixodidae, the family of hard ticks, have an enormous impact on ruminant livestock communities around the world (PETER *et al.* 2005). Ixodid ticks are known to vector bacterial, viral, and protozoan agents, and thus have become a major public health concern since the emergence of Lyme disease. Limited productivity, whether caused directly by tick infestation or by a vectored disease, equals profit losses for cattle producers.

Ixodid tick pests primarily inhabit regions between the latitudes of 35 degrees North and South, corresponding to the prime beef and dairy production areas of the world (SHAW 1970). The annual economic impact of ticks and tick-borne diseases on the livestock industry of the world was estimated near 18 billion dollars (U.S.) in 1997, the most recent estimate available (DE CASTRO 1997). Major items contributing to this cost include, but are not limited to; (1) labor involved in tick control treatments, (2) materials

and equipment for treatment programs, (3) production loss among animals from disease and/or tick burden, and (4) government research, organization, and implementation of strategies for tick control or eradication (SHAW 1970).

1.2.1.1 Tick fever and vector eradication in the United States

Tick fever caused by *Babesia bovis* or *Babesia bigemina* is a major concern for cattle producers within regions inundated with *Rhipicephalus* (*Boophilus) annulatus* and *R. microplus* ticks. These apicomplexan protozoan parasites invade red blood cells of their hosts and cause fever, anemia, and often death in affected cattle. The economic burdens due to fever tick-associated losses have encouraged producers and governments to design strategies for the eradication of cattle ticks.

Federal Authorities in the United States implemented the first cattle tick eradication program in 1907, which was geared toward ridding the country of the Southern cattle tick, *R. annulatus*. Regional campaign districts were each headed by a veterinary surgeon and supported by several veterinary inspectors, stock inspectors, and lay assistants. The stock inspectors systematically covered the districts on horseback, assisted by about a dozen cowboys, roping and inspecting cattle for *R. annulatus* ticks (FRANCIS 1966).

Later in the nineteenth century, the discovery that Texas cattle fever was vectored by *R. annulatus* ticks was monumental for implementation of tick control programs to facilitate eradication efforts (SMITH and KILBORNE 1893). Elimination of

the tick vector offered producers a means to control potential losses from *B. bovis*- and *B. bigemina*-infected cattle. King Ranch, located in Texas, developed the dipping vat in 1889, which became the primary method used for the cattle tick eradication efforts in the United States. Herding cattle through dipping vats filled with tick killing chemicals, also called acaricides, provided an efficient way to treat large herds. The chemical acaricides used in dipping vats were developed at Texas A&M University (FRANCIS and COOPER 1892). Another strategy used for tick eradication was pasture spelling, i.e., vacating tick infested fields during seasons of larval hatching. Eradication of cattle ticks in the United States was declared successful in 1942 and a permanent quarantine zone was established in Texas along the Rio Grande. For several decades the United States remained free of cattle ticks, except for a few occasions when the permanent quarantine zones were temporarily extended. Reports of cattle tick infestations in eight Texas counties in 2009 raised concerns about the emergence of cattle ticks in the United States especially from acaricide-resistant tick populations, however, the temporary quarantine zones in Texas were released in 2011 (ELLIS 2011; HILLMAN 2009).

When the eradication campaign began, white-tailed deer (*Odocoileus virginianus*) were not considered important for the survival and spread of cattle fever ticks in Texas (POUND *et al.* 2010). In Texas, white-tailed deer populations rose from about 200 thousand when cattle tick eradication efforts began to around four million by 2004, and numbers of cattle fever tick outbreaks beyond the permanent quarantine zone increased nearly ten times from 1999 to 2009 (GRAVES 2004; PÉREZ DE LEÓN *et al.* 2010). Since white-tailed deer serve as a competent host for both *R. microplus* and

R. annulatus, pasture spelling was no longer an option because the presence of white-tailed deer allowed the continued existence of tick populations in cattle vacated pastures (POUND *et al.* 2010). The large habitat range maintained by white-tailed deer also permits the spread of cattle ticks into tick free regions. In an attempt to control cattle ticks on white-tailed deer, researchers employed by the United States Department of Agriculture (USDA) developed a four-poster deer feeding device designed to spread acaricide on the neck of feeding deer, which spreads to other parts of the deer body as they groom (GEORGE 2006). Recent molecular studies have identified white-tailed deer infected with *B. bovis*- or *B. bigemina*-like organisms in counties outside of the permanent quarantine zone in Texas (HOLMAN *et al.* 2011; RAMOS *et al.* 2010). Analyses of both *B. bovis*- and *B. bigemina*-like deer isolates determined that there were no genetic distinctions between these parasites and the populations infecting cattle (HOLMAN *et al.* 2011). However, more studies are needed to determine whether these parasites are infective to cattle and the origin of *B. bovis* and *B. bigemina* isolates found in the white-tailed deer sampled in Texas, as these animals could have crossed over or been transported in from Mexico.

1.2.1.2 The impact of Lone Star ticks in the United States

In the United States, Lone Star ticks inhabit the South-Central and Southeastern regions, and may be found along the Atlantic coast as far north as New York and Maine. As *A. americanum* tend to infest livestock in large numbers, these ticks contribute directly

to the debilitation and exsanguination of cattle. Infestations of cattle by large numbers of Lone Star ticks may lead to reduced weight, infertility, decreased milk and meat production, and damaged hides intended for leather; all of which greatly affect the profits available to cattle producers (PETER *et al.* 2005). Furthermore, *Amblyomma* spp. ticks possess long mouthparts that inflict wounds deep enough to sustain secondary bacterial infection and screwworm infestation in tick-infested cattle (LEVOT 1995).

The Lone Star tick is the primary tick pest of veterinary and human importance in the Southern United States (JAMES *et al.* 2001; PETER *et al.* 2005). Human pathogens causing diseases such as tularemia, human monocytic ehrlichiosis (HME), and Southern tick-associated rash illness are known to be transmitted by *A. americanum* ticks (GODDARD and VARELA-STOKES 2009). The latter represents a Lyme-like rickettsial infection caused by *Borrelia lonestari*, the agent suspected to be responsible for the majority of erythema migrans cases reported in the Southern United States (JAMES *et al.* 2001). Cattle sustained infection by *Ehrlichia chaffeensis*, the causative agent of HME, under experimental infestations with *A. americanum* ticks, suggesting that cattle have the potential to serve as a reservoir for this agent and may present a future public health concern (DELOS SANTOS *et al.* 2007). Tick control is a vital component of infectious disease management, and of paramount importance for the welfare of humans, livestock, and companion animals.

1.2.2 Tick control

During the late nineteenth century, the cattle industry experienced a large increase in the demand to feed the growing populations of newly industrialized nations. Efforts aimed at increasing food production for the United States, Africa, and Australia led to the introduction of tick-naïve cattle into tick-infested regions and tick-infested cattle into tick-free regions, causing tick-infestation to become a serious problem. At that time there were few options available for treatment of tick-infested stock, and a lack of scientific knowledge concerning transmission of tick-borne infectious disease agents (SHAW 1970).

1.2.2.1 History of tick control and acaricide development

Early tick deterrents consisted of oil and water emulsions, and included suspensions containing kerosene, cotton-seed oil, fish oil, and/or sulfur. Oil and lard concoctions, smeared on the skin of tick-infested cattle, proved to be efficacious at the time. Beaumont crude oil contained 1½ % sulfur, which offered an improved choice for tick control. However, Beaumont crude oil emulsions scalded the skin of cattle during the hot summer months, and thus were not safe to use on cattle at the times of the year when tick numbers were highest (SMITH and KILBORNE 1893). Mineral oil- and phenol-filled dipping vats were used to treat tick-infested herds as early as 1895. In the 20th century,

the discovery of arsenical compounds revolutionized tick control methods for the cattle industry (ANGUS 1996).

While the use of arsenic-based compounds for ectoparasitic control of sheep date back over a century, South Africa and Australia reported the use of arsenic-based dips to treat tick-infested cattle in 1893 and 1895, respectively (SHAW 1970). The United States Bureau of Animal Industry adopted arsenic-based acaricides as the recommended tick control agents in 1910 for use against cattle tick eradication efforts to rid the country of tick fever (GRAHAM and HOURRIGAN 1977). Arsenical solutions provided a highly effective means of killing ticks, and Tixol® became the best known commercial product for treating tick-infested cattle (SMITH and KILBORNE 1893). However, the limitations required to balance effective arsenical concentrations for tick control with dosage levels considered toxic to humans led to public concerns about arsenical residues in food products (GRAHAM and HOURRIGAN 1977). These factors, coupled with the development of arsenic-resistant tick populations, led to the formulation of organochloride compounds in the mid-1940s (SHAW 1970).

Organochloride compounds were the first synthetic organic pesticides developed for treating tick infestations on cattle. There were three main classes of organochlorides; (1) derivatives of chlorinated ethanes, including DDT (dichlorodiphenyltrichloroethane), DDE (dichlorodiphenyldichloroethane), and DDD (dicofol, methoxyclor); (2) hexachlorocyclohexanes (HCH), including lindane and benzenehexachloride (BHC); and (3) cyclodienes, including chlordane, heptachlor, endrin, tozaphene, aldrin, and dieldrin (TAYLOR 2001). Chlorinated ethanes opened sodium channels along the sensory

and motor nerve fibers of the tick, a mechanism that inhibited sodium conduction and delayed repolarization of the axonal membrane. This delay resulted in repetitive discharge by the nerve axons and caused tick paralysis. HCH compounds bound the γ-amino butyric acid (GABA) receptor of chlorine channels, which also caused repetitive axonal discharge and tick paralysis (TAYLOR 2001). Overuse of these formulations for fly control during the mid-1950s led to the development of HCH-resistant tick populations, and thus HCH compounds were quickly replaced by more effective cyclodienes (LEVOT 1995). Similar to HCHs, cyclodienes also interfered with the GABA receptor and affected calcium in addition to chlorine channels. Organochlorides were pulled from the market due to their persistence in the environment, as these chemicals accumulated in the body fat of animals and resulted in severe consequences for some non-target species (GEORGE *et al.* 2004).

Neutral esters of phosphoric acid were mixed with thio (sulphur) analogues to formulate organophosphates (OPs), a class of acaricide primarily used for control of organochloride-resistant *Rhipicephalus* spp. ticks (SHAW 1970). OPs inhibited acetylcholinesterases (AChE) by mimicking the structure of acetylcholine. This mechanism led to phosphorylation of AChE and blocked the break-down of acetylcholine at the post-synaptic membrane, which induced tick paralysis (TAYLOR 2001). OPs did not persist in the environment after use, although these compounds were the most toxic pesticides known to vertebrates (GEORGE *et al.* 2004). The most widely used OPs on cattle, such as chlorpyrifos, ethion, coumaphos, and chlorfenvinphos, were closely related to lethal nerve gases used in chemical warfare (WARE 2000).

Carbamate compounds were often combined with other active ingredients (GRENIER and GRENIER 1993). Carbamates also blocked AChE via a spontaneously reversible mechanism that does not structurally change the enzyme (TAYLOR 2001). The carbamate compound Carbaryl, albeit carcinogenic, was known for its low level of toxicity to mammals (GRENIER and GRENIER 1993).

Formamidines, such as chlordimeform, clenpyrin, chloromethiuron, and amitraz, were especially effective for tick killing. The alpha-adrenergic octopamine receptor agonist, known as amitraz, caused neuronal hyper-excitability that resulted in tick death (NATHANSON 1985). This compound, also known by the brand name Taktic,® was formulated for the treatment of domestic livestock (GEORGE *et al.* 1998). Widely used in dips, sprays, and pour-on formulations for cattle, Taktic® effectively killed single and multi-host tick species. Trials conducted in Africa determined the efficacy of Taktic® against *Rhipicephalus decoloratus*, *Rhipicephalus evertsi*, *R. appendiculatus*, and *Amblyomma hebraeum* ticks (STANFORD *et al.* 1981), while testing in the United States proved its effectiveness for the control of *R. microplus* populations (GEORGE *et al.* 1998).

Natural pyrethrum from the chrysanthemum plant consisted of a mixture of lipophilic and rapidly metabolized alkaloids, which provided a rapid method for tick killing (CASIDA *et al.* 1983). Natural pyrethrums also exhibited low toxicity for mammals, and thus these acaricide formulations were the most widely used class of acaricide across the globe (TAYLOR 2001). Production of man-made pyrethrin-like

chemical compounds known as synthetic pyrethroids (SPs) offered an efficient alternative to the light sensitive natural pyrethrum formulations.

The first SPs available to treat ticks on cattle were permethrin and fenvalerate (DAVEY and AHRENS 1984). SPs excited cell membranes within the central nervous system of insects and led to extended repolarization, causing tick paralysis. SPs were classified as type I or type II depending upon the presence or absence of an α-cyano moiety (CASIDA *et al.* 1983). Although type II SPs did not induce repetitive discharge, both types of SPs interfered with sodium channels and lead to tick paralysis via delayed axonal repolarization (NARAHASHI 1985). A synergistic relationship was identified between cyano-substituted pyrethroids and several classes of OPs, and resulted in the efficacious treatment of OP-resistant *R. microplus* tick populations at a cheaper cost to producers compared to compounds formulated from SPs alone (SCHNITZERLING *et al.* 1983). Cyano-substituted acaricides, including cyhalothrin, cypermethin, and deltamethrin, were useful against DDT-resistant tick populations, and thus impelled the registration and use of these compounds for tick control (KUNZ and KEMP 1994; NOLAN *et al.* 1979).

The residual effects of pyrethroid-based formulations were prolonged by the addition of a piperonylbutoxide synergist (STENDEL 1985). Additionally, microencapsulation pyrethroid-containing compounds extended the duration of an application, reducing the number of necessary treatments. Flumethrin, an SP/piperonylbutoxide compound, was combined with an ingredient to promote rapid spreading along the skin and hair, and this formulation provided an effective long-lasting

pour-on acaricide for tick control (STENDEL 1985). SP-based acaricides were available as pour-on, spot-on, spray, dip, and ear tag formulations for the control of biting flies, ticks, and lice on domestic livestock (TAYLOR 2001).

Macrocylic lactones, such as avermectin and milbemycin, are systemically functional at low doses. The avermectins consist of ivermectin, doramectin, and eprinodectin, and are derived from the fermentation products of the actinomycete *Streptomyces avermitilis*. The milbemycins consist only of moxidectin, derived from *Streptomyces hygroscopicus aureo lacrimosus*. Macrocylic lactones activate glutamate-gated chlorine channels by binding the GABA receptor, resulting in tick paralysis (MARTIN *et al.* 2002). Since glutamate-gated chlorine channels are only found in invertebrate organisms, these compounds are considered generally safe for use on mammals. Macrocylic lactone-based formulations are currently available for pour-on and subcutaneous injection, and use of these compounds by cattle producers is limited mostly due to their high cost (GEORGE *et al.* 2004).

Acaricide-resistant ticks, contaminated food products, and environmental pollution are still major drawbacks to the use of extensive chemical tick control programs by cattle producers and governments (WILLADSEN 2004). The expenses incurred for discovery, testing, and marketing new classes of chemicals for tick killing, coupled with the rapid development of acaricide-resistant tick populations limit the currently available tick control strategies (WILLADSEN 2004). The overuse of available acaricides not only elevates public concern regarding environmental and food-safety, but directly contributes to the development of acaricide-resistant tick populations. The lack

of reliability for all available acaricide products necessitates the development of alternative tick control methods, such as vaccines and selective breeding programs, for better host protection against ticks (GUERRERO *et al.* 2006).

1.2.2.2 Acaricide resistance in ticks

The development of acaricide-resistant tick populations for every currently available acaricide hinders effective tick control strategies. Monetary losses incurred by cattle producers result in increased costs to food consumers, which will continue to rise unless low-cost alternative tick control strategies are implemented. The selective pressures imposed by the use of pesticides resulted in the development of more than 500 species of acaricide-resistant ticks (GEORGE *et al.* 2004).

The usefulness of some OP and carbamate solutions for tick control was limited by the emergence of OP-resistant tick populations (MILLER *et al.* 2009a). Chlordimeform, an additive used to restore OP efficacy for acaricide-resistant tick populations, was later removed from the market due to carcinogenic properties (GEORGE *et al.* 2004). Additionally, DDT-resistant ticks displayed cross-resistance when treated with SP formulations, and thus use of SPs was limited in *Rhipicephalus* spp. tick-infested regions (MILLER *et al.* 2009b; NOLAN *et al.* 1979). This situation is further compounded by the over-exposure of acaricides by use of continual-dose ear-tag formulations (KUNZ and KEMP 1994).

It appears that the more effective chemicals enhance selection pressures for tick survival and the ticks that survive initial exposure to a distinct class of acaricide are genetically enhanced. Tick populations surviving initial chemical treatments pass acaricide-resistance on to the next generation of ticks, and so on. Furthermore, ticks that develop resistance to a particular class of acaricide more easily develop resistance to alternative chemicals that utilize a similar mode of action for tick killing, as observed with acaricide-resistance to DDT and SP formulations (KUNZ and KEMP 1994). This phenomenon limits the development of new pesticide products, and thus alternative methods for tick control must be established in order to prevent future economic losses to cattle producers.

1.2.2.3 Alternative tick control methods

1.2.2.3.1 Biological control of ticks

Entomopathogenic fungi (EPF) and nematodes (EPN) were evaluated as potential biological tick control agents. EPF are host specific and can penetrate the cuticle of multiple life stages of a targeted tick species. Additionally, the specific virulence of an EPF strain to a particular tick genus or species makes these fungi attractive as potential alternative tick control agents (SAMISH *et al.* 2004).

During off-host field trials, an EPF formulation consisting of a combination of fungal spores from *Metarhizium anisopliae* and *Beauveria bassiana* sprayed onto

pastures was 99% effective at reducing *Ixodes ricinus*, *R. appendiculatus*, and *Amblyomma variegatum* tick populations (KAAYA and MWANGI 1995; KAAYA *et al.* 1996). Populations of *Ixodes scapularis* ticks were reduced up to 90% in residential areas treated with commercially available *M. anisopliae* formulations that were previously registered for use against turf and ornamental plant pests (STAFFORD and KITTRON 2002). When sprayed directly onto tick-infested cattle, *M. anisopliae* EPF formulations reduced *R. microplus* and *R. decoloratus* populations, and increased female tick post-engorgement mortality rates compared to results on untreated calves (CORREIA *et al.* 1998; KAAYA and HASSAN 2000).

As promising as the potential for EPFs as an alternative method for tick control seems, there are drawbacks that must be addressed before these products can reach the commercial market. While application of fungal-based products was similar to that of chemical acaricides, production of these formulations was costly and further limited by a short shelf-life (SAMISH *et al.* 2004). Additionally, the carrier used in preparation of EPF formulations influences the efficacy of the product, and little is known regarding which carrier formulations work best to reduce tick numbers (ÁNGEL-SAHAGÚN *et al.* 2010). Future studies are required to ensure the reliability of EPF agents to invade a targeted tick species, and to evaluate potential effects to non-target arthropods. Other disadvantages of using EPF agents for tick control include their sensitivity to UV irradiation, and the requirement of a highly humid environment for germination and sporulation to occur. The possibility that these environmental factors may contribute to

sub-lethal tick-infections, and thereby cause ticks to develop resistance to EPF agents must also be evaluated prior to marketing.

A Mexican strain of *Steinernema carpocapsae*, a round worm lethal to ticks and other insects, was formulated and sprayed on pastures at a concentration of 50 nematodes per square centimeter. Treating pastures at this dose killed 100% of engorged female *R. annulatus* ticks within five days following experimental placement onto the soil surface (SAMISH *et al.* 1999). EPNs are affected by the same adverse environmental factors as EPFs (low-humidity and UV irradiation), and are further influenced behaviorally by factors such as soil composition, fodder, manure, and ambient temperatures. Additionally, the pathogenicity of EPN formulations was greatly impacted in a species-specific manner by these environmental factors, making EPN agents less appealing than EPF agents as a potential alternative tick control method (SAMISH *et al.* 2004).

1.2.2.3.2 Tick-resistant cattle

Breeding programs for the development of cattle that are less susceptible to the adverse effects from tick infestations offer a much safer alternative to the current chemical tick control programs. Additionally, tick-resistant hosts were reported to carry fewer numbers of ectoparasites compared to tick-susceptible herd-mates infested by the same tick species (AXFORD *et al.* 2000). Johnston and Bancroft (1918) first discovered the innate resistance of cattle to ticks after a local producer reported a cow in his herd as a

"tick killer" in 1908 (JOHNSTON and BANCROFT 1918). By 1915, about half of that producer's herd was of the tick-resistant phenotype and did not sustain more than a few ticks per season under natural conditions. The expression of tick-resistance was characterized by reduced tick weights, delayed engorgement periods, decreased ova production, and fewer numbers of fertile eggs laid by female ticks fed on tick-resistant hosts (BROWN *et al.* 1982a). Reduced tick burdens were observed for tick-resistant cattle without the use of chemical tick controls, and maintained after individuals were moved to other geographical regions (JOHNSTON and BANCROFT 1918).

European *Bos taurus* breeds of cattle were reported to suffer greatly from debilitation and death when infested by cattle ticks, while *Bos indicus* breeds of cattle remained relatively unaffected when infested by the same tick species (FRANCIS 1966). Thus, tick-resistance is considered directly proportional to the amount of *Bos indicus* influence, and cattle comprised of at least 38% Brahman (*Bos indicus*) blood seldom display notable tick burdens (FRANCIS 1966). Pure and cross-bred *Bos indicus* cattle have a natural immune-based advantage over *Bos taurus* breeds when exposed to ticks, and thus an understanding of the immune responses elicited by *Bos indicus* and *Bos taurus* cattle could provide a powerful tools for combating acaricide-resistant tick populations (RIEK 1962).

Tick-resistant *Bos indicus* cattle also demonstrated resistance to the protozoan agents that cause babesiosis, also known as tick fever (FRANCIS 1966). Unfortunately, the naturally tick-resistant *Bos indicus* cattle mature more slowly, and are arguably inferior beef and milk producers compared to the more tick-susceptible *Bos taurus*

breeds. The greater profit potential of maintaining a high-producing pure-bred *Bos taurus* herd was limited by the requirement for higher quality feed, sheltering, and tick control methods for these cattle, and thus cattlemen throughout tropical and sub-tropical regions benefited from cross-breeding their herds with *Bos indicus* lines (SHAW 1970).

In Texas, the King Ranch used a cross-bred Shorthorn (*Bos taurus*)-Brahman (*Bos indicus*) bull to determine the potential of Brahman traits to improve the resiliency of Shorthorn beef stock (BRIGGS 1958). The Shorthorn-Brahman bull was mated to pure-bred Shorthorn cows, and when fed on adequate pasture these progeny were so productive that King Ranch management proceeded with the plan to breed Brahman traits into their Shorthorn stock. One offspring sired by the Shorthorn-Brahman bull was left intact and sired a bull that King Ranch named Monkey, that became the foundation sire for the Santa Gertrudis breed (BRIGGS 1958).

The Santa Gertrudis was the first cattle breed developed in the United States, and was named in honor of the land grant between Captain Richard King and the Crown of Spain, as this agreement initially established King Ranch (BRIGGS 1958). Santa Gertrudis cattle exhibited a greater tolerance to the hot temperatures, high humidity, and ectoparasites in Texas compared to the original pure-bred Shorthorn lines and produced better beef compared to the pure *Bos indicus* lines (BRIGGS 1958).

The Santa Gertrudis Breeders International, organized in 1951, designated the stock at King Ranch as the breed's foundation herd. All modern Santa Gertrudis stock are considered 5/8 Shorthorn and 3/8 Brahman. These cattle are deep cherry red in color

and, based on conformation, acceptable for beef production. The development of this breed showed that an alternative approach to tick control, i.e., host resistance, was reliably passed to successive generations by the deliberate breeding of tick-resistant individuals without prior knowledge of the specific genetic factors that confer tick-resistance. However, the ability to breed cattle with known heritable factors associated with tick-resistance will offer producers a more permanent solution to tick control that requires few supplemental resources or finances.

The development of tick-resistant lines may also facilitate an alternative approach for tick eradication campaigns in temperate regions plagued by heavy infestations of cattle ticks (FRISCH 1999). The Belmont Adaptaur, a *R. microplus* tick-resistant *Bos taurus* line of cattle, was developed at the National Cattle Breeding Station of the Commonwealth Scientific and Industrial Research Organization (CSIRO) at Belmont, Queensland in Australia. These cattle were bred by crossing Hereford-Shorthorn (HS) cattle to individuals that carried ½ *Bos indicus* (¼ Africander and ¼ Brahman) and ½ *Bos taurus* (¼ Hereford and ¼ Shorthorn) blood (FRISCH 1994). The researchers at Belmont selected breeding pairs based on growth, and indirectly selected for genes conferring tick-resistance to produce cattle that displayed a high level of tick-resistance when infested with *R. microplus* ticks. The Adaptaur Association of Australia was the first cattle breed association to require evaluation of a calf for tick-resistance as a requirement for registration.

A major gene for tick-resistance was proposed to be carried by Belmont Adaptaur cattle (FRISCH 1994). This gene, thought to be a dominant heritable trait, was

suggested to provide life-long absolute immunity to cattle ticks. Selection for this gene had the potential to offer selective breeding for tick-resistance, a low-cost means that would increase profits for cattle producers (FRISCH 1999). Unfortunately, the rush to commercialize the Belmont Adaptaur breed compromised the potential identification of this major gene for tick-resistance by eliminating the individuals thought to carry this gene, as these cattle were targeted for sale, and thus were eliminated from future experimental cross-breeding studies (FRISCH *et al.* 2000).

The high degree of genetic variability in immune responses for individual cattle, especially across different breeds, presents difficulty for the identification of genetic mechanisms that contribute to tick-resistance (FRISCH 1999). Without knowledge concerning the genetic mechanisms that confer tick-resistance in cattle, producers are unable to selectively breed for these genes while simultaneously selecting for equally desirable production traits (WILLADSEN 2004). Furthermore, the development of anti-tick vaccines is limited by the lack of information about the immune factors that mediate tick-resistance in cattle.

1.2.2.3.3 Anti-tick vaccines

Market forces have created conflict between the demand for high producing cattle for human consumption, and the need for livestock more tolerant of high temperatures and parasites. The high productivity of tick-susceptible *Bos taurus* breeds is limited by the pressing public interest for the reduction in use of chemical acaricides for tick control

(WILLADSEN *et al.* 1995). Furthermore, the decline in effectiveness for nearly all available acaricide formulations resulted in a more intense search for low-cost and environmentally friendly tick control alternatives. Vaccines offer a simple and cost-effective tick control option that lacks the withdrawal period required before use of cattle after application of tick control chemicals, and thus this alternative is appealing to producers (FRISCH 1999). Another advantage of vaccines is that they may be administered to any animal at any location, whereas acaricide treatments require a facility equipped with dipping vats or chutes to accommodate chemicals formulated for pour-on and spray-on applications.

Moreover, a combination of vaccination and breeding for tick-resistant cattle may present a feasible chemical-free tick control strategy. Contrary to the high short-term mortality of ticks observed with acaricide use, vaccines offer long-term tick control with the disadvantage of reducing immediate kill tick mortality rates. However, use of both acaricides and anti-tick vaccines as an integrated pest control method in sub-tropical regions, where both immediate and long-lasting tick control are crucial for herd survival, has the potential to reduce the amount of tick-related production losses (WILLADSEN *et al.* 1995).

Effective vaccine antigens elicit a host immune response (WILLADSEN 2004). Damaged tick gut cells found in adult *R. microplus* ticks fed on calves previously vaccinated with tick gut cell homogenates derived from partially-fed female ticks led to the development of the first anti-tick vaccine (KEMP *et al.* 1989; WILLADSEN *et al.* 1995). Ticks fed on vaccinated calves exhibited increased mortality, decreased fertility

rates, and turned red in color, a characteristic due to hemolymph leakage that resulted from damage to the gut cells (KEMP *et al.* 1989). Vaccination trials with tick gut homogenates eventually led to a novel concept for immunization using a "concealed antigen" (WILLADSEN *et al.* 1993). This concealed antigen is not known to stimulate an immune response in vaccinated cattle during natural tick infestations, and thus the down-side to its use as a vaccines is that natural tick exposure will not boost the response and periodic re-vaccinations are required to maintain adequate levels of protective immunity in cattle (WILLADSEN 2004). However, vaccination of cattle with a concealed antigen produced antibodies to a factor on tick gut cells that was associated with an essential function for tick survival, and inhibition of this factor by bovine antibodies resulted in gut damage in feeding ticks (KEMP *et al.* 1989).

Vaccines that make use of a concealed antigen were developed and commercialized for use in Australia and Cuba (FRISCH 1999). The identification of distinct concealed tick-antigens was achieved by experimental vaccination of cattle with tick gut homogenates followed by successive purifications and re-vaccinations (WILLADSEN *et al.* 1993). The first concealed antigen used for vaccination trials, Bm86, is an 89 kDa glycoprotein located on tick gut digest cells. Bm86 was named for the species of origin and the year the antigen was first discovered, to wit, *Rhipicephalus* (*Boophilus) microplus* and 1986 (WILLADSEN *et al.* 1993). Later, inoculation of cattle with recombinant forms of this antigen, rBm86, produced bovine antibody levels comparable to vaccinations with the native Bm86 antigen.

Involved in the glycosylation of cell surface proteins for protection against proteolysis, the exact function of Bm86 in the tick gut cells is still unclear. However, this protein shares structural similarity to a series of epidermal-growth-factor-like elements (WILLADSEN 1997). Bovine IgG_1 antibodies were reported to bind Bm86 on tick gut cells during blood-meal acquisition, and this mechanism resulted in cellular lysis that caused damage to ticks, resulting in fluid leakage into the tick hemolymph (WILLADSEN 1997). Bovine IgG_1 antibodies were also able to fix complement on *R. microplus* derived midgut membranes and the humoral response in vaccinated Hereford, as determined by measurement of IgG_1 antibody levels, correlated with host protection against *R. microplus* tick-infestation (JACKSON and OPDEBEECK 1990). These findings led to vaccination trials to determine the efficacy of Bm86 vaccination of cattle for tick control in the field.

Use of the Bm86 vaccine during field trials resulted in weight gains and reduced tick burdens for vaccinated cattle compared to unvaccinated controls under the same conditions (DE LA FUENTE *et al.* 2007). TickGARD, the Bm86 vaccine developed by CSIRO with Biotech Australia and Hoescht Animal Health, became available in the summer of 1994 (WILLADSEN *et al.* 1995). In Australia, vaccination with TickGARD reduced tick numbers on vaccinated cattle by 56% and egg production by 72% in a single tick generation (DE LA FUENTE *et al.* 2007). Additionally, TickGARD vaccination resulted in an average calf weight gain of 18.6 kg more than the average gain for unvaccinated controls managed under the same conditions (JONSSON *et al.* 2000).

The recombinant Bm86 (rBm86) vaccine Gavac™, expressed using a *Pichia pastoris* yeast vector, was later manufactured by Heber Biotec, Cuba (RODRIGUEZ *et al.* 1995). In Cuba, livestock husbandry is government controlled and this regulation of cattle handling likely contributes to the success with use of the Gavac™ vaccine. Acaricide use was reduced by 87%, saving the Cuban cattle industry an estimated six million dollars (DE LA FUENTE *et al.* 2007). Gavac™ trials in Mexico reduced the number of acaricide treatments from 24 to eight per year and the average tick burden per animal from 100 to less than 20. The numbers of reported babesiosis cases (1.9 per 1000 animals) in Cuba compared to the numbers of cases accounted for in the previous year (54 per 1000 animals) were drastically reduced following Gavac™ vaccination (DE LA FUENTE *et al.* 2007). Gavac™ vaccination of cattle given in two initial doses, administered four weeks apart, was reported to confer similar protection to the use of three doses administered as one initial vaccine followed by two boosters at four and seven weeks (VARGAS *et al.* 2010). Additionally, bovine antibody titers after administration of two initial Gavac™ vaccinations were sufficient to cause reduction in female tick engorgement weights and reproductive capacities. Despite the potential advantage offered by Gavac™ vaccination, such as reduced tick numbers, engorgement weights, and fertility, chemical tick controls were still required to prevent heavy tick burdens for Bm86 vaccinated cattle (DE LA FUENTE *et al.* 2007).

A second concealed antigen, Bm91, was reported to enhance the efficacy of Bm86 vaccines (WILLADSEN *et al.* 1996). Bm91 shares amino acid sequence similarity with the mammalian angiotensin converting enzyme (JARMEY *et al.* 1995).

Angiotension, a carboxypeptidase that maintains fluid and electrolyte homeostasis, aids in the regulation of blood pressure and may influence reproduction. Located on the surface of both salivary glands and gut cells, the specific enzymatic function of the Bm91 antigen in ticks is unknown (JARMEY *et al.* 1995). A total of three vaccination trials using Hereford calves revealed that addition of Bm91 did not impair the effects of the initial Bm86 vaccine (WILLADSEN *et al.* 1996). These trials also determined that antibody inhibition of either antigen, Bm86 or Bm91, was independent from the other. Furthermore, use of a combined antigen vaccine resulted in a significantly higher reduction of tick numbers than vaccination with Bm86 alone (WILLADSEN *et al.* 1996). Other proteins found to cause tick mortality by RNAi experimentation, such as ubiquitin and subolesin, were less effective against *R. microplus* and *R. annulatus* ticks than vaccination with Bm86 (ALMAZÁN *et al.* 2010).

The potential for vaccination with Bm86 and its homologues in other tick species to protect cattle was also evaluated. Both Bm86 (Gavac^{TM}) and a *R. annulatus* derived ortholog, Ba86, more effectively reduced tick burdens in cattle infested with *R. annulatus* ticks than those infested with *R. microplus* ticks (CANALES *et al.* 2009). Interestingly, the efficacy of Bm86 vaccination was higher for reduction of *R. annulatus* tick burdens on cattle than the Ba86 homologue. This phenomenon was thought to occur due to cross-reactive epitopes that result from the high sequence similarity among Bm86 homologues, and may explain in part the differences in efficacy observed for these two tick species fed on vaccinated cattle (CANALES *et al.* 2009).

Serum from TickGARD vaccinated cattle reacted strongly when exposed to both native Bm86 and several recombinant homologues derived from *R. decoloratus*, Bd86-1 and Bd86-2, on tick midgut membranes (ODONGO *et al.* 2007). A specific IgG_1 host response, as determined by enzyme-linked immunosorbent assay (ELISA), resulted in a reduction of tick numbers (45%) for *R. decoloratus* fed on Bm86 vaccinated cattle versus unvaccinated controls (DE VOS *et al.* 2001). Additionally, the reproductive capacity of *R. decoloratus* ticks fed on Bm86 vaccinated cattle was reduced by 70%, as determined by smaller engorgement and total egg weights, compared to ticks fed on unvaccinated controls.

Oddly, Bm86 vaccination was efficacious at controlling tick species other than those of the *Rhipicephalus* genus. The total number of *Hyalomma dromedarii* engorging nymphs fed on Bm86 vaccinated cattle was reduced (95%), and total weight of surviving ticks decreased (55%) (DE VOS *et al.* 2001). Similarly, total weights for feeding *Hyalomma anatolicum anatolicum* nymphs were reduced overall (50%) when fed on Bm86 vaccinated cattle. A similar study showed a 89% reduction in *H. dromedarri* nymphs fed on Bm86 vaccinated cattle (RODRÍGUEZ-VALLE *et al.* 2012). In contrast, cattle vaccinated with the *H. anatolicum anatolicum* derived Bm86 homolog, rHaa86, showed only a 20% reduction in tick numbers (JEYABAL *et al.* 2010).

Another peculiarity of the Bm86 vaccine is that it provided anti-tick protection for non-bovine host species. Vaccination of dogs with Bm86 resulted in reduced viability and reproductive potential for the brown dog tick, *Rhipicephalus sanguineus* (PEREZ-PEREZ *et al.* 2010). Similar to use in cattle, the production of antibodies in

response to Bm86 vaccination in dogs was confirmed by a specific ELISA. Remarkably, Bm86 vaccination reduced numbers of hematophagous poultry red mites, *Dermanyssus gallinae*, infesting chickens (HARRINGTON *et al.* 2009). Furthermore, Bm86-specific IgY antibody production was confirmed by ELISA in sera from vaccinated chickens (HARRINGTON *et al.* 2009). This is interesting since poultry red mite infestations are extremely hard to treat for chicken producers because the currently available acaricides may only be applied to empty coops and easily contaminate eggs.

Conversely, use of TickGARD or GAVAC did not have an effect on numbers of *Rhipicephalus appendiculatus*, *Amblyomma variegatum*, or *Amblyomma cajennense* ticks fed on vaccinated cattle (DE VOS *et al.* 2001; RODRÍGUEZ-VALLE *et al.* 2012). In another study, no differences were reported for any tick parameters measured between control and vaccinated cattle challenged with *R. appendiculatus* ticks and supports the previously observed inefficacy of TickGARD to protect cattle from this tick species (ODONGO *et al.* 2007). In spite of the disadvantages of Bm86 vaccination, the usefulness of Bm86-derived vaccines for the control of ticks and other hematophagous ectoparasites was demonstrated for cattle, other agriculturally important animal species, and companion animals.

Characterization of bovine factors that contribute to tick-resistance and tick-susceptibility by comparing gene expression will provide insight concerning the immune responses elicited in cattle infested with ticks. These findings may lead to the identification of immune mechanisms to facilitate design of novel adjuvants, and thus the improvement of anti-tick vaccines. Furthermore, the identification of biological

mechanisms mediating host immunity will help pinpoint genetic markers for selective breeding programs. The combination of breeding phenotypically tick-resistant cattle and vaccine advancement has the potential to reduce, or even eliminate, use of chemical tick controls.

1.2.3 Immune mediation

1.2.3.1 Host defense in response to A. americanum *infestation*

The dynamic equilibrium allowing for the survival of both the tick-infested host and the feeding ectoparasite is continually maintained by interaction between host immunological mechanisms and tick-secreted factors (WILLADSEN *et al.* 1993). These exchanges are species-specific for both ticks and hosts, and the identification of the mechanisms that mediate bovine immune responses to *A. americanum* ticks is important for an accurate characterization of these dynamic interactions.

Inflammation triggered by infestation of Lone Star ticks on resistant hosts recruited basophils into the skin at sites of tick attachment by an antibody-mediated mechanism (BROWN *et al.* 1982a). It is still unclear whether degranulation of basophils at tick attachment sites is activated by secreted factors in tick saliva, antigen-antibody complexes bound to the basophils, or an anaphylatoxin, or if several of these mechanisms are triggered simultaneously (BROWN *et al.* 1982a). Anaphylactic degranulation of local basophils in the skin of tick-resistant guinea pigs released

histamine, and injection of anti-histamines at tick attachment sites reversed the expression tick-resistance for these hosts (BROWN and ASKENASE 1985). Use of anti-basophil antibodies eliminated basophils in sera from *A. americanum*-infested guinea pigs, which directly abolished the expression of tick-resistance and demonstrated the requirement for basophils for tick-resistance (BROWN *et al.* 1982a).

Other basophil-dependent inflammatory mediators were thought to contribute to tick-resistance by attracting eosinophils, granule-associated proteases, and factors that influence T-cell proliferation (BROWN *et al.* 1982a). *A. americanum*-resistant guinea pigs displayed higher levels of basophils and eosinophils, and concentrations of these cells in peripheral blood correlated with increased numbers of tick bites on these hosts. Although white blood cell counts remained equal between primary and secondary tick exposures, concentrations of both basophils and eosinophils peaked sooner during the secondary tick challenge and this suggests that these cells play a key role in mediation of acquired immune responses (BROWN *et al.* 1983).

Basophil degranulation at tick attachment sites contributed to decreased tick weights and prolonged engorgement periods for female ticks fed on Lone Star tick-resistant guinea pigs (BROWN *et al.* 1982a). Passive transfer of serum from tick-resistant to tick-susceptible guinea pigs infested with *A. americanum* ticks successfully passed on tick-resistance, as characterized by cutaneous basophil-mediated inflammation at tick attachment sites (BROWN *et al.* 1982b). Measurement of IgG_1 antibodies in guinea pigs using specific ELISA assays while conducting these passive transfer experiments demonstrated that tick-resistance was mediated by IgG_1 antibodies. Elimination of tick-

resistance via B-lymphocyte depletion in guinea pig hosts further corroborated the importance of this antibody-mediated mechanism for the expression of tick-resistance (BROWN *et al.* 1982b).

The previously described cutaneous basophil hypersensitivity elicited in guinea pig host infested with Lone Star ticks was confirmed in cattle after three successive experimental tick-infestations using 50 adult male/female *A. americanum* pairs (BROWN *et al.* 1984). Tick numbers did not vary significantly among the three tick challenges. However, Holstein calves developed cutaneous lesions similar to those observed from tick-resistant guinea pig hosts after the initial tick infestation. Furthermore, female ticks fed on tick-resistant cattle exhibited a significant reduction in weight and laid fewer eggs than ticks fed on tick-susceptible calves (BROWN *et al.* 1984). Previously sensitized cattle exhibited intense grooming behaviors within 30 minutes after re-exposure to Lone Star ticks, suggesting that these cattle acquired immunological specificity to salivary components secreted by feeding ticks. In contrast to the observations from tick-resistant guinea pig hosts, basophils concentrations were not increased in peripheral blood from tick-infested cattle (BROWN *et al.* 1984). However, these cells comprised a large proportion of immune infiltrates (~19%) in the dermis at tick attachment sites collected 48 hours after the primary tick exposure and these infiltrates were observed in tick attachment sites only six hours after the secondary tick challenge, and this suggests that basophils play an important role in the expression of Lone Star tick-resistance in cattle (BROWN *et al.* 1984). Interestingly, basophils from cattle were circular in appearance with a relatively small cytoplasm that contained poorly-defined tightly-packed

metachromatic granules, while basophils from guinea pigs appeared oval with well-defined and loosely aligned metachromatic granules (BROWN *et al.* 1984). Although basophil morphology differed between guinea pig and cattle hosts, cutaneous basophil-associated tick-resistance was demonstrated for both host species, supporting the hypothesis that resistance to *A. americanum* ticks is likely mediated by comparable immune factors in bovine and rodent hosts.

In contrast to the basophil-mediated resistance to Lone Star ticks, a mast cell-dependent mechanism was important for tick-resistance in *R. microplus* tick-infested cattle (RIEK 1959). The differences observed between tick attachment sites from *R. microplus* and *A. americanum* tick-infested cattle were suggested to be caused by species-specific salivary antigens secreted upon tick attachment, as cattle ticks have a one-host life cycle and Lone Star ticks use three hosts to complete their life cycle (RIEK 1962). It is also possible that immune responses in cattle infested by different tick species are influenced by the depth of the wounds created during tick attachment (LATIF *et al.* 1990). Ticks classified as *Rhipicephalus* spp. have short mouthparts, and thus attach superficially to the epidermal layer of skin (MOOREHOUSE and TATCHELL 1966). In contrast, Lone Star ticks, a species of the *Amblyomma* genus of ticks, possess long mouthparts that anchor deep through the epidermis and into the dermis of the host (ARTHUR 1970). These different modes of tick-attachment, and the array of immunomodulatory molecules secreted in the saliva of cattle ticks versus Lone Star ticks, likely contribute to distinct mechanisms for expression of tick-resistance in bovine hosts.

1.2.3.2 Host immunomodulation by ticks

Tick saliva contains molecules that help to maintain the prolonged attachment to a host required for tick-feeding and disease transmission. In the course of forming the feeding lesion, secreted bioactive salivary antigens suppress the physiological mechanisms of host immune defenses and allow the tick to imbibe a blood meal (NUTTALL and LABUDA 2004). Tick salivary antigens promote Th2 cytokine production in tick-infested hosts by inhibiting dendritic cell function, altering cytokine profiles, and influencing histamine and leukocyte responses (BROSSARD and WIKEL 2005). Immunosuppressive proteins in tick saliva interfered with complement components, pro-inflammatory cytokines, and antigen presenting cells, and affected the activation of both T and B cells (HOVIUS 2009).

Ixodes scapularis and *Ixodes ricinus* ticks transmit the Lyme disease causing agent *Borrelia burgdorferi*, and thus human health concerns facilitated efforts to obtain knowledge regarding the immunomodulatory molecules of these ticks (HOVIUS *et al.* 2007). Complement interacting components, such as C3 convertase proteins, affect the deposition of complement factors and subsequent opsonization of antigens, resulting in immunosuppression (HOVIUS *et al.* 2008b). The *I. scapularis* anti-complement (ISAC) factor, a C3 convertase protein, was identified as a salivary component that inhibited complement activity to rabbit erythrocytes in human serum (VALENZUELA *et al.* 2000). In addition, the *I. scapularis* salivary protein 20 (Salp20) impaired opsonization and deposition of host immune complexes on tick antigens, and thereby inhibited

complement activation (TYSON *et al.* 2007). Paralogues of ISAC, IRAC I and IRAC II, from the saliva of *I. ricinus* ticks inhibited activation of the alternative complement pathway by an undefined mechanism (SCHROEDER *et al.* 2007). Anti-complement alternative pathway assays evaluated IRAC I and IRAC II activity in the serum of six mammalian and two avian host species, and these two tick salivary factors had similar effects on complement activity in serum from humans and dogs (SCHROEDER *et al.* 2007). Furthermore, evaluation of complement activity in human, pig, dog, horse, sheep, rat, pheasant, and pigeon serum found that IRAC I more strongly inhibited activation of the alternative complement pathway in sheep, pig, and horses whereas IRAC II more powerfully inhibited complement activation in pheasants (SCHROEDER *et al.* 2007). Interestingly, neither IRAC I nor IRAC II affected complement activation in serum from rats or pigeons. Analyses of these findings indicate that IRACI and IRACII target complement inhibition in a host-specific manner.

Several inhibitors of T cell function were identified from the saliva of *I. scapularis* ticks. Salivary protein 15 (Salp15) was shown to bind the dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) receptor on human dendritic cells, leading to impaired Toll-like receptor signaling and activation of $CD4^+$ T cells (HOVIUS *et al.* 2008a). Salp15 also bound to the OspC protein of *B. burgdorferi* and this interaction protected this spirochete from the antibody-mediated killing mechanisms of murine hosts during disease transmission (RAMAMOORTHI *et al.* 2005). Furthermore, RNAi-mediated knockdown of Salp15 in *I. scapularis* tick salivary glands resulted in a reduction of *B. burgdorferi* spirochetes in the skin of tick-infested

mice. This important finding indicated the involvement of a tick salivary protein for pathogen transmission in a tick-infested host. Interestingly, two inhibitors of $CD8^+$ cytotoxic T cells, Sialostatin L and the IL-2 binding protein, were also identified (Hovius et al. 2008).

Similar T cell inhibitors were identified in the saliva of other tick species. Interestingly, prostaglandin E_2 from *R. microplus* ticks inhibited proliferation of bovine T lymphocytes (INOKUMA *et al.* 1994). A novel immunosuppressant identified from *Haemaphysalis longicornis* ticks, HL-p36, prevented T cell activation and production of IL-2 in both bovine peripheral mononuclear cells and mouse splenocytes (KONNAI *et al.* 2009). Homologues of HL-p36 were identified from several other tick species, including *A. americanum*, *A. cajennense,* and *Dermacentor andersoni* (ALJAMALI *et al.* 2009b; KONNAI *et al.* 2009). An *I. ricinus* immunosuppressor (Iris) inhibited expression of Th1-type pro-inflammatory cytokines and promoted expression of Th2-type cytokines in mouse T cells and macrophages (LEBOULLE *et al.* 2002). Additionally, a Th2-type immune response was observed in BALB/c mice after subcutaneous injection of dendritic cells that were previously incubated with *I. ricinus* tick saliva, as confirmed by analyses of Th2-mediating cytokines via competitive PCR (MEJRI and BROSSARD 2007). Saliva from *R. sanguineus* ticks also induced a Th2-type response in mice by promoting expression of TLR-2 on murine dendritic cells, a mechanism that impaired maturation of these cells (OLIVEIRA *et al.* 2010). This over-expression of TLR-2 induced expression of IL-10, which inhibited the production of Th1-mediating cytokines such as IL-12 and TNFα .

Other factors present in tick saliva were reported to target B cells, neutrophils, dendritic cells, anti-microbial peptides, and pro-inflammatory mediators. The B cell inhibitory protein (BIP), isolated from *I. ricinus*, bound the OspA and OspC proteins of *B. burgdorferi in vitro* and inhibited B cell proliferation in mice, an interaction thought to also affect Toll-like receptor signaling (HANNIER *et al.* 2004). An *I. ricinus* derived leukotriene binding protein (Ir-LBP) inhibited neutrophil function in rabbit blood leukocytes and delayed the inflammatory response (BEAUFAYS *et al.* 2008). Specific antibody production, for both IgM and IgG, in BALB/c mice infested with female *I. ricinus* ticks, was impaired after stimulation with bovine serum albumin (MENTEN-DEDOYART *et al.* 2008). Also isolated from *I. ricinus* ticks, Sialostatin L specifically inhibited the action of anti-microbial peptides in mice, such as cathepsin L and cathepsin C (KOTSYFAKIS *et al.* 2006). Furthermore, Sialostatin L blocked activation of human cathepsins L and V, proteases that mediate inflammation by limiting edema and the migration of granulocytes.

Salivary gland extracts (SGE) from *A. variegatum, R. appendiculatus, Dermacentor reticulatus,* and *I. ricinus* ticks were shown to bind several chemokines in human and mouse sera, including chemokine (C-X-C motif) ligand 8 (CXCL8 or IL-8), chemokine (C-C motif) ligand 2 (CCL2 or MCP-1), chemokine (C-C motif) ligand 3 (CCL3 or MIP-1α), chemokine (C-C motif) ligand 5 (CCL5 or RANTES), and chemokine (C-C motif) ligand 11 (CCL11 or eotaxin), as determined by specific ELISA assays (HAJNICKÁ *et al.* 2005; VANČOVÁ *et al.* 2010). Inhibition of these chemokines may limit the ability of the host to recruit macrophages and other lymphocytes into the

skin at sites of tick attachment. Furthermore, SGEs from several tick species were analyzed for their binding specificity to several host wound healing factors in cell lines of humans and mice using specific ELISA assays (HAJNICKÁ *et al.* 2011). SGEs derived from *I. ricinus* and *I. scapularis* bound platelet-derived growth factor, whereas SGEs from *D. reticulatus* and *R. appendiculatus* ticks targeted transforming growth factor beta, fibroblast growth factor 2, and hepatocyte growth factor (HAJNICKÁ *et al.* 2011). Interestingly, SGEs from *A. variegatum* ticks bound to all four of the growth factors evaluated, and this contributed to the hypothesis that immune responses of hosts infested by different species of ticks may be influenced by the depth of tick bites. Bites from *A. variegatum* ticks extend through the epidermis and anchor into the dermis of the host, and thus explain why these ticks may inhibit more host wounding factors compared to ticks that attach more superficially to hosts, as is the case with *R. appendiculatus* ticks.

Immunomodulation of host mechanisms allows ticks to remain attached to a host for the period of time required to obtain a blood meal. Interestingly, some tick-infested hosts were shown to mediate immune responses that affect tick feeding and reproduction (WIKEL and ALLEN 1977). As such, ticks fed on tick-resistant hosts either do not feed to repletion, take longer to do so, and may become less fertile.

1.2.4 Gene expression studies in cattle infested with **R. microplus** *ticks*

Molecular exchanges between a tick and host upon tick-attachment influence the balance between successful feeding for the tick and protective immunity for the host (BAGNALL

et al. 2009). These interactions are regulated by proteins in response to increased or decreased expression of genes, and thus gene expression studies using microarray and quantitative real time reverse transcriptase polymerase chain reaction (qRT-PCR) techniques have identified genes involved in tick-resistance and -susceptibility in cattle infested with *R. microplus* ticks. Microarray analysis of tick attachment sites from Shorthorn-Hereford calves infested with *R. microplus* ticks that exhibited high and low levels of tick-resistance, as determined by standard mean tick counts from five weekly larval tick-infestations, identified several genes involved in the bovine immune response that were associated with calves exhibiting high levels of tick-resistance and included genes involved in calcium and chemokine signaling (WANG *et al.* 2007b). More specifically, expression of chemokine receptor (*CCR1*) and chemokine ligand (*CCL26*) were decreased in tick attachment sites from calves with high levels of tick-resistance 24 hours after tick-infestation (WANG *et al.* 2007b). A later study evaluated gene-specific expression via qRT-PCR in tick attachment sites from six Holstein-Friesian (tick-susceptible) and six Brahman (tick-resistant) calves, and reported up-regulated expression for *CCR1* and *CCL26* in samples from the tick-susceptible phenotype. These analyses suggest that factors involved in chemokine signaling may be associated with host-susceptibility to cattle ticks (Piper et al 2008).

A subsequent study compared gene expression in tick attachment sites from six Holstein-Friesian (tick-susceptible) calves and six Brahman (tick-resistant) calves using an Affymetrix Gene Chip bovine microarray platform (KONGSUWAN *et al.* 2008). This expression array represented a total of 11,255 unique *Bos taurus* gene identities

(UniGene assembly 4.0) and utilized over 100 control probes. All calves had previous tick exposure under natural conditions, were experimentally challenged with 10,000 *R. microplus* larvae weekly for five weeks, and phenotypes were confirmed by standard tick counts (KONGSUWAN *et al.* 2008). Analyses of tick attachment sites, biopsied 24 hours after the fourth tick infestation, identified genes associated with biological processes such as immunity, signal transduction, and cell structure (KONGSUWAN *et al.* 2008). Further analysis of factors determined by microarray analyses as more highly expressed in the tick-resistant phenotype via the major biological pathways from Kyoto encyclopedia of genes and genomes (KEGG (KANEHISA *et al.* 2012)) identified genes associated with cell-mediated immunity and structural integrity (KONGSUWAN *et al.* 2008). Increased expression of factors including the major histocompatibility (MHC) class II molecule, complement components, B lymphocyte attracting chemokines, and immunoglobulin genes suggested that a Th1-type cell-mediated immune response was elicited in *R. microplus* tick-resistant cattle. Additionally, increased expression of collagen genes and other cellular matrix components in tick attachment sites from *R. microplus* tick-resistant calves was thought to aid in strengthening the epidermal barrier in order to deter tick attachment (KONGSUWAN *et al.* 2008). Analyses of fifteen candidate genes selected from the microarray experiments via qRT-PCR indicated that Toll-like receptor signaling and pro-inflammatory mechanisms were up-regulated in tick attachment sites from *R. microplus*-susceptible Holstein-Friesian calves compared to tick-resistant Brahman cohorts (KONGSUWAN *et al.* 2008).

To explore systemic immune factors that influence differential responses between tick-resistant and tick-susceptible calves blood leukocytes were collected from similarly challenged Brahman and Holstein-Friesian calves on three occasions during experimental tick infestations with 10,000 *R. microplus* larvae weekly over seven weeks (PIPER *et al.* 2009). Cattle phenotypes were confirmed by standard tick counts. Gene expression was compared in blood leukocytes using the Affymetrix bovine arrays described above and revealed that the majority of the differentially expressed genes between the two cattle phenotypes were involved in cellular and metabolic processes (PIPER *et al.* 2009). However, nearly 7% of the genes identified as up-regulated in samples from tick-resistant calves were associated with immune functions. Hematological evaluation found that tick-resistant Brahman calves maintained higher numbers of circulating CD4+ and γδ T cells while tick-susceptible Holstein-Friesian calves retained higher levels of circulating IgG_1 antibodies (PIPER *et al.* 2009). Gene-specific qRT-PCR assays identified increased expression of cytokines, such as *IL2*, *IL2Rα*, *TNFα*, and *CCR1*, in blood leukocytes from tick-resistant Brahman cattle compared to tick-susceptible Holstein-Friesian cohorts. These findings suggest that a systemic Th1-type response may be important for cattle resistant to *R. microplus* ticks (PIPER *et al.* 2009).

A combined histological and gene expression study evaluated the expression of cell adhesion molecules in tick attachment sites from tick-susceptible Holstein (N=12; *Bos taurus*) and tick-resistant Nellore (N=12; *Bos indicus*) yearling steers infested with *R. microplus* ticks under field conditions (CARVALHO *et al.* 2010). Tick attachment site

biopsies were collected after removal of an adult female *R. microplus* tick at times when tick infestations were high and low; indicated by the number of ticks counted on a tick-susceptible Holstein animal so that a tick count that totaled $\geq$ 140 ticks per calf was considered high, and a tick count $\leq$ 40 ticks was considered low (CARVALHO *et al.* 2010). Additionally, a biopsy was collected from a tick-free region of each steer prior to tick infestations. Histological analysis of tick attachment sites showed that both tick-susceptible Holstein and tick-resistant Nellore cattle showed similar numbers of neutrophils present at tick feeding lesions. However, more eosinophils were observed in tick attachment sites from tick-resistant Nellore steers compared to sites from tick-susceptible Holstein cohorts. In tick attachment sites from both breeds, basophil numbers were increased compared to biopsies taken from tick-free regions and basophil numbers were significantly higher in tick attachment sites from Nellore steers compared to those from Holstein calves, suggesting that basophils may be important for the expression of bovine tick-resistance.

Gene expression profiles for tick attachment sites from both Holstein and Nellore calves via qRT-PCR indicated that expression of several adhesion molecules, such as vascular cell adhesion molecule-1, intercellular adhesion molecule-1, P-selectin, and E-selectin, was decreased compared to sites biopsied from tick-free regions of these calves (CARVALHO *et al.* 2010). In contrast, the expression of leukocyte adhesion glycoprotein-1 was increased in tick attachment sites from Holstein cattle compared to those taken from Nellore steers. Expression of E-selectin was increased in tick attachment sites from Nellore calves during times of high tick-infestations compared to

tick attachment sites from Holstein cohorts (CARVALHO *et al.* 2010). E-selectin is expressed by endothelial cells for recruitment of basophils and T cells, including memory and γδ T cells, to sites of injury or infection such as the wound originating upon tick attachment. These findings corroborate the previous hypothesis that a Th1-type cell-mediated mechanism may play an important role in bovine tick-resistance to *R. microplus* infestations (CARVALHO *et al.* 2010).

Genes that may contribute to innate breed differences between *R. microplus* tick-resistant and -susceptible calves were investigated by comparison of tick attachment sites from three Holstein-Friesian (12 months of age) and three Brahman (20 months of age) calves of comparable maturity exposed to ticks under experimental conditions. To compensate for breed-associated differences in tick-resistance and -susceptibility, the Holstein-Friesian heifers were infested with 10,000 *R. microplus* larvae whereas Brahman calves were challenged with 50,000 tick larvae on a weekly basis for six weeks (CONSTANTINOIU *et al.* 2010). During week seven of the tick trial, all calves were infested with 20,000 *R. microplus* larvae. Duplicate tick attachment site biopsies were collected, one biopsy for gene expression experiments and one biopsy for histological analysis, prior to the initial tick-infestation, 24 hours post primary infestation (PPI), and 24 hours after weeks one, three, and seven PPI (CONSTANTINOIU *et al.* 2010).

Histological analyses of biopsies revealed that tick-naïve Brahman cattle displayed significantly higher concentrations of T cells, including $CD4^+$, $CD8^+$, $CD25^+$, and γδ T cells, compared to biopsies from tick-naïve Holstein-Friesian heifers (CONSTANTINOIU *et al.* 2010). Immunohistochemical labeling of cells in tick attachment

biopsies from tick-resistant calves revealed that a distinct band of γδ T cells formed around the tick-feeding lesion for all samples collected after week one PPI (CONSTANTINOIU *et al.* 2010). Additionally, γδ T cells were present in higher numbers for tick attachment biopsies from tick-resistant Brahman calves compared to those sampled from tick-susceptible Holstein-Friesian cohorts. Numbers of $CD4^+$ T cells at tick feeding lesions increased significantly in biopsies from tick-susceptible cattle after week one and for both breeds after week seven PPI. In contrast, concentrations of $CD8^+$ T cells significantly decreased in tick attachment sites from both breeds after weeks one and three PPI, whereas numbers of these cells were increased for biopsies collected during week seven (CONSTANTINOIU *et al.* 2010). Overall, numbers of $CD8^+$ T cells were higher in tick attachment sites from Brahman calves for all time points measured. Furthermore, granulocytes infiltrated the areas closest to tick mouthparts in feeding lesions from both breeds, however, total granulocyte numbers remained higher in biopsies from the tick-susceptible Holstein-Friesian calves throughout the trial (CONSTANTINOIU *et al.* 2010). The presence of granulocytes, mainly neutrophils, in the gut of feeding ticks suggests that these cells may be a key source of nutrients for feeding *R. microplus* ticks (CONSTANTINOIU *et al.* 2010). These observations led to the hypothesis that bovine resistance to *R. microplus* ticks was represented by a less intense immune reaction and effective reduction of cell migration into sites of tick attachment, thereby limiting the availability of granulocytes to feeding ticks (CONSTANTINOIU *et al.* 2010).

A similar study biopsied larval tick attachment sites from six Holstein-Friesian (12 months of age) and three Brahman (18 months of age) calves experimentally infested with 10,000 *R. microplus* larvae weekly for seven weeks (PIPER *et al.* 2010). Standard tick counts confirmed the tick-susceptible and -resistant phenotypes. Skin biopsies were collected in duplicate, one for gene expression experiments and one for histological analysis, 24 hours after the tick infestation at week four (PIPER *et al.* 2010). Additionally, three tick-naïve Holstein-Friesian and three tick-naïve Brahman calves from tick-free regions of Australia were biopsied to compare innate differences in gene expression between tick-susceptible and -resistant cattle (PIPER *et al.* 2010).

To further identify mechanisms that may contribute to the high level of tick-resistance in Brahman cattle, microarray experiments using the previously described Affymetrix platform were combined with histological analysis to compare tick-free biopsies and *R. microplus* larval attachment sites (PIPER *et al.* 2010). In biopsies from tick-naïve Brahman calves compared to those from tick-naïve Holstein-Friesian calves, genes with increased expression were involved in the oxidative reduction (PIPER *et al.* 2010). In contrast, genes up-regulated in biopsies from tick-naïve Holstein-Friesian calves were associated with complement components, immunoglobulin heavy chains (IgG_1 and IgG_{2a}), and the immunoglobulin lambda light chain variable region (PIPER *et al.* 2010).

Genes up-regulated in tick attachment sites from tick-infested Holstein-Friesian calves compared to biopsies from the tick-naïve Holstein-Friesian cattle were involved with biological mechanisms including response to wounding, immune response, defense

response, chemotaxis, and cell adhesion (PIPER *et al.* 2010). Oxidative reduction was the only biological process identified by functional analysis of the differentially expressed genes between tick-attachment sites and biopsies from tick-naïve Brahman calves (PIPER *et al.* 2010). Analysis of these microarray data suggest that the oxidative reduction pathway may contribute an innate advantage for the expression of tick-resistance in Brahman cattle infested with *R. microplus* ticks (PIPER *et al.* 2010).

Comparison of tick attachment sites from tick-infested Holstein-Friesian and Brahman calves identified commonly associated biological pathways such as cell communication and extracellular matrix receptor interaction (PIPER *et al.* 2010). However, the genes associated with these pathways differed between the two cattle phenotypes. Up-regulated genes in tick attachment sites from tick-infested Holstein-Friesian cattle compared to those from Brahman cohorts were associated with complement, coagulation, and cytokine-cytokine receptor interaction, whereas focal adhesion was represented by up-regulated genes in sites from Brahman calves (PIPER *et al.* 2010). Furthermore, genes including collagen types I and II, procollagen C-endopeptidase enhancer, osteoglycin, and glutathione peroxidase-7 showed increased expression in tick attachment sites from tick-infested Brahman compared to Holstein-Friesian calves (PIPER *et al.* 2010). Increased expression of collagen genes in tick-resistant calves suggests that resistance to *R. microplus* ticks could be mediated by mechanisms that remodel extracellular matrices (PIPER *et al.* 2010). It was suggested that genetic mechanisms that modify the cell matrix and limit the immune responsiveness for

tick-resistant Brahman may contribute to feeding inhibition for *R. microplus* ticks fed on these cattle.

Histological analyses of the tick attachment sites collected from Holstein-Friesian calves showed signs of a cutaneous cellular response, as indicated by acanthosis, hyperkeratosis, and hyperplasia of the epidermis (PIPER *et al.* 2010). Leukocyte migration, mononuclear cell infiltration, edema, and collagen degeneration were also evident in the dermis of tick attachment sites from the tick-susceptible Holstein-Friesian calves (PIPER *et al.* 2010). In contrast, only one of the six tick attachment sites from the tick-resistant Brahman calves showed signs of a cellular response, and those changes were mild (PIPER *et al.* 2010). These histological observations further support the hypothesis that a decreased immune response at sites of tick attachment may play an important role for the expression of tick-resistance in Brahman cattle. Similar gene expression studies to evaluate the underlying genetic and immune mechanisms in *A. americanum* tick-infested cattle are not available to date, however, studies have reported associations between bovine MHC genes and resistance to Lone Star ticks.

1.2.5 Genetic analysis of MHC in A. americanum *infested cattle*

Major histocompatibility complex genes likely play an important role in bovine immunity to ticks (KONGSUWAN *et al.* 2008). MHC genes produce receptor proteins that are expressed on antigen presenting cells for the recognition of foreign antigens

(TROWSDALE 1995). Once processed by an antigen presenting cell, antigenic peptides are loaded onto MHC receptors and displayed on the cell surface for presentation to T cells (TROWSDALE 1995). Class I MHC receptors are expressed on all nucleated cells and generally present peptides processed from intracellular antigens to T lymphocytes bearing the $CD8^+$ co-receptor. On the other hand, class II MHC receptors are only found on antigen presenting cells and generally display peptides derived from extracellular antigen to $CD4^+$ T helper cells. Upon activation by an MHC-II bound antigen, $CD4^+$ T helper cells produce cytokines and surface markers that attract B cells and regulate the classes of antibodies produced (TROWSDALE 1995). Cattle MHC genes, known as bovine leukocyte antigens (BoLA), are located within three classes of closely-linked and highly polymorphic gene clusters on bovine chromosome 23 (BRINKMEYER-LANGFORD *et al.* 2009).

In mammals, activated T helper lymphocytes are known to initiate up to three types of immune reactions. A type 1 (Th1) immune response leads to expression of interferon-γ (IFN-γ), tumor necrosis factor alpha (TNF-α), and interleukin 2 (IL-2), whereas a type 2 (Th2) T helper reaction leads to the expression interleukin 4 (IL-4), interleukin 5 (IL-5), and increased production of IgE antibodies (DEL PRETE *et al.* 1991; MOULIN *et al.* 2000). The third known immune response mediated by T helper cells, the type 17 (Th17), was characterized by expression of cytokines such as IL-17 and IL-23, and reported to play a role in chronic inflammatory reactions (AGGARWAL *et al.* 2003). It should be mentioned that T cell populations are generally heterogeneous and consist of multiple subsets of effector cells that secrete different combinations of cytokines, and

thus these cells are influenced by the microenvironment for polarization into a specific T helper subset (OPENSHAW *et al.* 1995).

Elucidation of the roles played by BoLA genes and their influence on T helper responses in tick-infested cattle may help determine the functions of particular cell types, such as basophils and eosinophils, for immune responses elicited by tick infestation in tick-resistant and -susceptible cattle. The USDA Agricultural Research Service (ARS) Knipling-Bushland United States Livestock Insects Research Laboratory (KBUSLIRL) located in Kerrville, Texas developed a database to archive genomic DNA correlated with phenotypic data from *A. americanum* tick-susceptible and -resistant cattle (UNTALAN *et al.* 2007). Data were collected over a three year period from calves sired by three different bulls. Two of the bulls were full siblings from a Red Poll cow (*Bos taurus*) bred to a Simmental bull (*Bos taurus*) and were initially selected to sire a herd to investigate horn fly-resistance, a study conducted in concordance with the tick-resistance investigation (UNTALAN *et al.* 2007). The third bull used in this breeding program was of the Simbrah breed (*Bos taurus/Bos indicus*).

The offspring calves from mixed breed heifers bred to these bulls were used to evaluate correlations between BoLA genes and tick-resistance or -susceptibility (UNTALAN *et al.* 2007). Each of the calves was phenotyped as tick-resistant or -susceptible after exposure to two artificial tick infestations (UNTALAN *et al.* 2007). For the first tick infestation, calves eight months of age were infested with 500 nymphs and 30 adult male/female pairs of *A. americanum* ticks while restrained in stanchions to prevent tick removal via grooming. Upon completion of the initial tick infestation,

calves were rested for several weeks prior to being infested with another 30 adult male/female pairs of Lone Star ticks. Once detached, the engorged female ticks were weighed individually and tick weights were totaled for each individual animal. Ranking of total tick weights from 100 calves determined tick-resistant and -susceptible phenotypes; tick-susceptible calves represented by the upper-quartile (highest tick weights), tick-resistant calves comprised of the lower quartile (lowest tick weights), and the two middle quartiles were excluded from the study (UNTALAN *et al.* 2007).

Genomic DNA was collected from the 25 tick-resistant and 25 tick-susceptible calves and used for analysis of the BoLA region of bovine chromosome 23 via a polymerase chain reaction restriction fragment length polymorphism method (UNTALAN *et al.* 2007). This approach allowed mapping of four microsatellite alleles; three located within the BoLA class II gene cluster, and one positioned outside of the BoLA region (UNTALAN *et al.* 2007). Analysis of microsatellite mapping in DNA from tick-resistant and -susceptible calves determined that BoLA genes within the class II-DRB region were associated with the tick-resistant phenotype (UNTALAN *et al.* 2007). This DNA analysis further identified a correlation between allele 174 of the BoLA-DRB3 region and tick-resistance, which warranted additional investigation of polymorphisms within the BoLA-DRB3 region (UNTALAN *et al.* 2007).

Quantitative trait loci (QTL) mapping of the above described microsatellites found a strong correlation between the BoLA-DRB3*4401 allele and the tick-resistant phenotype (UNTALAN *et al.* 2007). Further analysis of the BoLA-DRB3-Exon 2 sequence from tick-resistant calves using several PCR amplified clones revealed a

significant correlation (P=0.011) with allele DRB3*4401 and this phenotype (UNTALAN *et al.* 2007). These correlations suggest a role for BoLA-DRB3 genes for the expression of tick-resistance for calves infested with *A. americanum* ticks. It must be noted that the updated bovine genome sequence (Btau_4.0) demonstrated considerable differences within the BoLA region compared to the assembly used for the investigation described above (Btau_3.1), and thus any associations between BoLA genes and tick-resistance determined using the latter bovine genome sequence require re-evaluation for confirmation (BRINKMEYER-LANGFORD *et al.* 2009).

1.2.6 Tick genomic libraries and gene expression in feeding ticks

The genomic resources available for gene expression studies in cattle, represented by an expressed sequence tag (EST) library comprised of small cDNA clones (generally 200-500 nucleotides), enable the discovery of mechanisms that mediate host immunological mechanisms elicited in response to tick-infestation (JENSEN *et al.* 2007). The most current release for the *Bos taurus* gene index, BtGI 13, contains more than 100,000 ESTs that correspond to over 2,000 unique gene transcripts (http://compbio.dfci.harvard.edu/cgi-bin/tgi/gimain.pl?gudb=cattle [accessed on 01/18/2013]).

In contrast, the genomic resources for gene expression studies in ticks are limited, as only four genomes from more than 800 different tick species have been sequenced to date. The number of unique gene transcripts from all four of the currently sequenced tick genomes, which include *A. variegatum, I. scapularis, R. appendiculatus,*

and *R. microplus,* totals 87 (WANG *et al.* 2007a). Moreover, nearly half (39/87) of these tick genes were derived from the *R. microplus* genome, BmiGI release 2.1 (http://compbio.dfci.harvard.edu/cgi-bin/tgi/gimain.pl?gudb=b_microplus [accessed on 01/18/2013]). Comparative genomic analysis of an EST library containing over 13,000 *R. microplus* transcripts suggested that many of the gene index entries for this tick species have no homologues in other sequenced tick genomes (WANG *et al.* 2007a).

Independent expression libraries constructed from transcripts identified in the salivary glands of some tick species enabled gene expression analyses via suppressive subtractive hybridization (SSH) screenings, and thus the identification of tick factors expressed during feeding. Screening of cDNA clones by the SSH technique using an EST library derived from *R. microplus* tick salivary gland identified increased expression of over 500 genes in feeding *R. microplus* ticks compared to unfed ticks (LEW-TABOR *et al.* 2010). Up-regulated genes were associated with functions such as protease inhibition, histamine binding, and glycosylation, and these mechanisms were suggested to be important factors during tick feeding.

A cDNA library containing 1,024 unique salivary transcripts from *R. sanguineus* ticks was constructed and used to identify factors with increased expression during tick feeding, including cement proteins, mucins, metalloproteases, protease inhibitors, lipocalins, anti-microbial peptides, and immunomodulators (ANATRIELLO *et al.* 2010). Over-expression of immunomodulatory factors in the first few days of tick feeding, such as dendritic cell evasions, p36 immunosuppressants, defensins, lipocalins and thrombin

inhibitors, suggests that these factors play a role in immune-modulation during the host-attachment phase of tick feeding (ANATRIELLO *et al.* 2010).

Microarrays compared gene expression in the salivary glands from *R. microplus* tick larvae prior to host attachment and in early adult ticks fed on *Bos taurus* and *Bos indicus* calves (RODRIGUEZ-VALLE *et al.* 2010). Genes up-regulated in ticks fed on the tick-resistant *Bos indicus* calves compared to ticks fed on tick-susceptible *Bos taurus* cattle included thrombin, protease inhibitors, histamine, immunoglobulin binding proteins, and oxidative stress mediators (RODRIGUEZ-VALLE *et al.* 2010). This study demonstrated that the salivary transcriptomes of feeding *R. microplus* ticks change in a host specific manner, suggesting that ticks may sense which host immune factors require mediation for successful feeding upon host attachment.

Another SSH analysis using an EST library constructed using 1,754 cDNA clones isolated from the salivary glands of feeding *A. cajennense* ticks five days after attachment to rabbit hosts identified genes associated with serpins and cysteine protease inhibitors, factors primarily involved in mediating host blood coagulation mechanisms (BATISTA *et al.* 2008). Expression of immunomodulatory factors including ferritin, defensins, and the Toll-like pathway mediator, TRAF6, was increased in salivary glands from the feeding *A. cajennense* ticks (BATISTA *et al.* 2008). Interestingly, factors expected to be found, such as histamine-binding proteins, cement proteins, and angiotensin-converting enzyme, as identified by EST analysis of salivary glands from feeding *I. scapularis* and *D. andersoni* ticks (ALARCON-CHAIDEZ *et al.* 2007; RIBEIRO *et*

al. 2006), were not detected in this EST analysis of salivary glands from feeding *A. cajennense* ticks (BATISTA *et al.* 2008).

A study that combined the SSH approach with high throughput sequencing analyzed *A. americanum* ticks that had attained appetence by exposure to feeding stimuli while enclosed in a mesh pocket to prevent host attachment identified 40 genes up-regulated immediately prior to feeding (MULENGA *et al.* 2007). These 40 genes included histamine-binding proteins, stress and immune response mediators, transporter polypeptides, insulin-like growth factors, and regulators of extracellular matrix. Half of these genes were conserved in other tick species, and this suggests that a number of molecular mechanisms utilized by ticks to facilitate host attachment and feeding could be maintained across species (MULENGA *et al.* 2007).

Silencing of several genes that produce insulin-like growth factor proteins (IGFBP) via RNAi-mediated knockdown prevented *A. americanum* ticks from feeding to full engorgement (MULENGA and KHUMTHONG 2010). IGFBPs are involved in the secretion of prostacylin, a potent vasodilator and anti-coagulant, and are conserved among a variety of tick species, which makes these genes attractive antigens for the development of multi-tick vaccines. Knockdown of one IGFBP gene, IGFBP-rP1, resulted in a 47% Lone Star tick mortality rate within the first five days of tick feeding (MULENGA and KHUMTHONG 2010). Similarly, RNAi-mediated silencing of the *A. americanum* CD147 receptor gene impeded tick feeding and caused the characteristic color change observed in *R. microplus* ticks fed on Bm86 vaccinated cattle (MULENGA *et*

al. 2007). Although silencing of IGFBP genes and CD147 affected tick feeding, knockdown of these genes did not prevent ticks from attaching to the hosts.

An EST library containing over 3,000 salivary gland transcripts from female *A. americanum* ticks was assembled and used to construct a microarray platform consisting of 1,145 five unique gene sequences (ALJAMALI *et al.* 2009a). Microarrays compared gene expression in the salivary glands of female ticks fed on sheep during various stages of feeding including unfed, early fed (< 50 mg), partially fed (50-200 mg), fast feeding (300-550 mg), and fully engorged (ALJAMALI *et al.* 2009b). The progression from unfed to the early feeding stage was marked by the largest difference in gene expression, and almost entirely composed of up-regulated genes including housekeeping genes, p36 immunosuppressants, and protease inhibitors. Expression of histamine binding proteins and a few protease inhibitors increased in salivary glands from partially fed ticks compared to those from early fed ticks, whereas expression of some p36 immunosuppressants, protease inhibitors, mitochondrial genes, and ribosomal genes were decreased (ALJAMALI *et al.* 2009b). Mitochondrial genes coding for transport-associated proteins and genetic factors involved in protecting ticks against host reactive oxygen responses were up-regulated in salivary glands from fast feeding ticks compared to those from partially fed ticks (ALJAMALI *et al.* 2009b). Furthermore, progression from partially fed to the fast feeding stage was characterized by decreased expression of nearly all p36 immunosuppressants, histamine binding proteins, and protease inhibitors. All up-regulated genes in the salivary glands from fast feeding ticks compared to partially fed tick were involved in reproduction and no changes in gene expression were

observed between salivary glands from fast feeding and fully engorged ticks (ALJAMALI *et al.* 2009b). Analyses of gene expression of feeding *A. americanum* ticks suggest that ticks modulate the host immune response from the attachment phase until the rapid engorgement phase of feeding and may be vulnerable to host immunity during the fast feeding stage.

New antigens for anti-tick vaccines are in great demand. Understanding the genetics involved in tick feeding and host responses to tick attachment would provide key insight regarding the complex interactions that occur at the tick-host interface. Once deciphered, these genetic factors may be used for breeding high-producing tick-resistant cattle and developing multi-tick vaccines, which will provide cattle producers with reliable alternatives to chemical tick control.

CHAPTER II

MATERIALS AND METHODS

2.1 Animals and Sample Collection

Cattle were bred at the USDA-ARS KBUSLIRL, Kerrville, Texas to study bovine resistance to horn flies and Lone Star ticks, and these studies were conducted in concordance (UNTALAN *et al.* 2007). The calves used to optimize skin biopsy collection and storage, and RNA isolation techniques in this current study were sired by the three tick-resistant bulls from the breeding program described above (Chapter I). The calves sampled for gene expression studies herein were sired by a progeny, named Grover, of one of the Red Poll X Simmental bulls previously described, and was also of the tick-resistant phenotype.

Tick-susceptible or -resistant phenotyping of calves in this study was done by staff at the USDA-ARS KBUSLIRL by two successive artificial tick infestations, as previously described (UNTALAN *et al.* 2007). During the primary tick infestation, calves were exposed to 500 nymphs and two sets of 30 male/female pairs of Lone Star ticks. One set of male/female pairs of ticks was used to determine phenotypes of the calves, and the second set was utilized for collection of tick bite-site biopsies. Ticks were placed into socks that were glued onto the shaved flanks of the calves, and each calf was restrained in a stanchion to prevent tick-removal via grooming. Calves were rested for

several weeks after completion of the initial infestation and then re-infested with two sets of 30 adult male/female *A. amblyomma* tick pairs for the secondary tick exposure.

To determine the tick-susceptible or -resistant phenotype for each calf, engorged female ticks were weighed individually once detached. Total tick weights were calculated for the 30 female ticks from each calf and ranked to determine cattle phenotypes; tick-susceptible calves had the highest tick weights (upper quartile), tick-resistant calves had the lowest tick weights (lower quartile), and calves with tick weights in the middle two quartiles were considered to exhibit a moderate level of tick-resistance.

2.1.1 Group 1 calves

A total of 14 calves, approximately six months old and sired by the three bulls described above, were identified as 3W, 152W, 155W, 164W, 178W, 187W, 196W, 198W, 229W, 232W, 236W, 241W, 664W, and 665W (Table 2.1). Tick bite-site biopsies were collected from these 14 calves prior to and on days 1, 2, 3, 4, 5, 6, 7, 10, and 14 during the two tick-infestations of the phenotyping trial described above. After careful removal of a female tick, skin punch biopsies (6 mm) were obtained from sites of tick attachment (tick bite-sites) on the shaved flanks of these calves, and cut in half using a single edged razor blade. One 3-mm portion was flash frozen on dry ice and stored at -80^{o} C until processed. The other 3-mm portion was formalin fixed for histological analysis.

Table 2.1 Phenotyping of calves for tick-resistance by artificial infestation with *Amblyomma americanum* ticks in this study

Calf ID	1° Total tick weight[a]	2° Total tick weight[a]	Phenotype[b]
Group 1 calves			
236W	20.03	3.66	R
229W	19.04	5.04	R
664W	19.31	11.14	R
187W	20.11	14.59	R
196W	18.19	14.87	R
198W	21.53	15.46	M
164W	22.94	15.58	M
155W	19.01	16.84	M
241W	18.70	17.22	M
3W	17.53	17.74	M
232W	19.97	18.00	S
178W	22.24	19.39	S
152W	21.78	19.69	S
665W	21.77	20.07	S
Group 4 calves			
808	23.60	4.49	R
802	20.20	11.17	R
801	20.39	11.16	R
803	22.72	11.57	M
805	19.47	11.84	M
804	23.62	12.95	M
Group 5 calves			
903	16.62	9.51	R
904	21.51	10.59	R
902	21.64	10.80	M
907	20.37	12.61	M
905	19.59	16.61	M
901	21.59	19.66	S
906	21.27	20.44	S

[a] Total tick weights for each calf are the sum of weights of 30 adult, fully-engorged female ticks after natural detachment from the calf.

[b] Phenotypes were determined by ranking total tick weights from the secondary tick infestation and are represented as (R) for highly tick-resistant, (M) for moderately tick-resistant, and (S) for tick-susceptible calves.

Blood was collected by venous puncture of the jugular vein into vacutainer tubes (8 ml) containing the blood anti-coagulant ethylenediaminetetraacetic acid (EDTA). Vacutainer tubes were inverted gently several times before the blood was mixed with 30 ml TRI Reagent® BD (Molecular Research Center, Cincinnati, OH) in a 50 ml conical tube and frozen at -80^{o} C. Blood samples were obtained prior to and on days 3, 7, 10, and 14 during the two previously described tick infestations.

2.1.2 Group 2 calves

Blood was collected from the draining jugular vein of two calves (#7742 and #8334) immediately after slaughter at Rosenthal Meat Center, Texas A&M University, College Station, TX into PAXgene™ blood RNA tubes (Becton, Dickinson and Company, Franklin Lakes, NJ) and 7 ml vacutainer tubes with EDTA for use with the LeukoLOCK™ total RNA isolation system (Applied Biosystems, Carlsbad, CA). All tubes containing blood were inverted several times to mix well at time of collection. The PAXgene™ Blood RNA tubes were incubated at room temperature (RT) for 2 hours prior to storage at -20^{o} C until use.

For an initial evaluation of the LeukoLOCK™ total RNA isolation system (Applied Biosystems) 7 ml of blood was collected instead of the optimal 9 ml vacutainer tubes with EDTA, as suggested by the manufacturer's instructions (Applied Biosystems). The blood sample tube/LeukoLOCK™ filter apparatus was assembled by inserting the transfer spike into the top of the tube containing blood, and connecting to

the inlet port of the LeukoLOCK™ filter via a white slip connector. An 18 gauge needle was attached to the outlet port of the LeukoLOCK™ filter and inserted into a new 7 ml vacutainer tube, providing both the vacuum source to pull the blood through the LeukoLOCK™ filter to isolate the leukocytes, and to dispose of unwanted blood products in a contained waste receptacle. The leukocytes on the filters were washed with 3 ml phosphate-buffered saline (PBS) provided in the kit (Applied Biosystems) by injection through the inlet port of the LeukoLOCK™ filter via syringe, and the PBS waste was collected in a biological waste container. A new syringe containing 3 ml RNAlater® (Applied Biosystems) was similarly used to stabilize the filter-bound leukocytes for storage until processing for RNA isolation. All leukocyte containing Leukolock filters were stored at -20^{o} C until use.

2.1.3 Group 3 calves

Ten calves, approximately six months old and sired by the three bulls described above, were sampled on day 5 of the secondary tick infestation of a phenotyping trial. Calves were identified as 01K, 03K, 04K, 05K, 159K, 165K, 178K, 196K, 215K, and 232K. Blood was drawn from the jugular vein into 9 ml vacutainer EDTA tubes, passed through LeukoLOCK™ filters and the leukocyte-bound filters stored at -20^{o} C until use, as described above.

To assess the utility of freezing biopsies in a 4 M guanidinium isothiocyanate (GITC) two (6 mm) skin punch biopsies from tick bite-sites were collected from each

calf, as previously described. One biopsy sample from each calf was flash frozen on dry ice and the other specimen was placed into 1 ml GITC solution (4 M GITC, 20 mM sodium acetate, 0.5% sarkosyl, and 0.1 mM dithiothreitol, sterile filtered). The GITC solution was prepared by dissolving 0.164 g sodium acetate (Sigma Aldrich, St. Louis, MO) and 47.28 g guanidine thiocyanate (EMD Chemicals, Gibbstown, NJ) in 55 ml nuclease free water prior to adding 5 ml of a pre-mixed 10% sarkosyl (IBI Scientific, Peosta, IA) solution containing 2 mM of dithiothreitol (EMD Chemicals, Gibbstown, NJ). The GITC solution was brought to a final volume of 100 ml using nuclease free water, pH adjusted to 5.5, and sterile filtered before storage at RT until use. All skin biopsy samples were kept frozen at -80^{o} C until use.

2.1.4 Group 4 calves

Six half-sibling bull-calves approximately six months of age, all sired by Grover, were sampled as described above. Calves were identified as 801 through 805, and 808 (Table 2.1). Tick bite-site biopsies and blood samples were collected prior to and on days 3, 4, 5, 6, 7, 10, and 14 during the second tick infestation of a phenotyping trial only. Skin punch biopsies were placed into 1 ml GITC solution and flash frozen on dry ice. Blood was drawn into 9 ml vacutainer tubes containing EDTA and processed through LeukoLOCK™ filters, as described above.

2.1.5 Group 5 calves

Seven half-sibling heifer calves, approximately six months of age and of the same parentage as Group 4 calves, were sampled. Calves were identified as 901 through 907 (Table 2.1). Tick bite-site biopsies and blood samples were collected prior to and on days 3, 5, 8, and 10 during the first and second tick infestations of the phenotyping trial in the same manner described above. Specimens were also obtained prior to and on days 1, 2, 3, 5, 8, and 10 of a third tick infestation using two sets of 30 male/female pairs.

2.2 RNA Extraction from Bovine Skin Biopsies

2.2.1 RNA extraction with the RNeasy® Mini Kit

Prior to RNA extraction, all frozen skin tissue biopsies were ground to a powder in a liquid-nitrogen chilled stainless steel Biopulverizer^{TM} (Biospec Products, Bartlesville, OK). The 3-mm bovine skin punch biopsies (Group 1) were homogenized by repeated passage through an 18 gauge needle in 600 µl buffer RLT containing 40 mM dithiothreitol, according to the RNeasy® Mini Kit handbook (Qiagen, Valencia, CA). Homogenized samples were centrifuged for 3 min at 16,000 relative centrifugal force (rcf) and the lysate transferred to a new microcentrifuge tube. One volume of 70% ethanol was added to the lysate that was mixed by pipetting prior to the addition of each

sample to an RNeasy® mini spin column in a 2 ml collection tube. Spin columns were centrifuged at 8,000 rcf for 15 sec and the flow-through discarded. Next, 700 µl buffer RW1 was added prior to column centrifugation for 15 sec at 8,000 rcf, and the flow-through was again discarded. The columns were washed with 500 µl buffer RPE, centrifuged for 15 sec at 8,000 rcf, and the flow-through discarded. The columns were washed again with 500 µl buffer RPE, centrifuged for 2 min at 8,000 rcf, and the flow-through discarded. Next, each column was transferred to a new 2 ml collection tube and centrifuged for 1 min at 8,000 rcf. The column-bound RNA was eluted with 50 µl nuclease-free water after each spin column was transferred to a new 1.5 ml microcentrifuge tube. Tubes were centrifuged at 8,000 rcf for 1 min and the flow-through, which contained the purified total RNA, was treated with DNase 1 (Invitrogen, Carlsbad, CA).

According to the DNase 1 (Invitrogen) instructions, 1 µl 10X DNase 1 reaction buffer and 1 µl DNase 1 (Amplification grade, 1 U/ml) were added with sufficient nuclease-free water to reach a 10 µl reaction volume for each µg of RNA, and reactions were scaled up linearly for larger amounts of RNA. DNase 1 reactions were incubated at RT for 15 min prior to inactivation by the addition of 1 µl 25 mM EDTA, and then heated at 65^{o} C for 10 min. The RNA for each sample was re-purified by the addition of 350 µl buffer RLT and 250 µl 100% ethanol, and then the DNase 1-treated RNA was transferred to an RNeasy® mini column. Columns were centrifuged at 8,000 rcf for 15 sec and the flow-through discarded. The RNA was washed twice with buffer RPE, as

previously described, and then re-eluted using 50 μl nuclease-free water and stored at -80^{o} C until use.

2.2.2 Evaluation of GITC for biopsy storage

In order to test whether storage of biopsy specimens in 4 M GITC improved total RNA isolation and to optimize the extraction of high quality RNA from bovine skin, duplicate punch biopsies (6 mm) were obtained from each of the 10 Group 3 calves. One biopsy from each calf was flash frozen after being placed into 1 ml GITC solution (described above) and the other biopsy was flash frozen on dry ice without GITC. Total RNA was isolated from calves 03K and 232K samples (N=4) using the RNeasy® Mini Kit (Qiagen), as described above. RNA was isolated from biopsies collected from calves 01K, 04K, 05K, 159K, 165K, 178K, 196K, and 215K (N=16) using a modified TRI Reagent® method followed by purification on RNeasy® mini columns.

2.2.3 Evaluation of a modified TRI Reagent® RNA extraction method

The pulverized 6-mm bovine specimens from the latter sample set (N=16) of Group 3 calves described above were each added to 2.5 ml TRI Reagent® (Molecular Research Center, Cincinnati, OH), and homogenized by repeated passage through an 18 gauge needle. The homogenate was evenly divided between two 1.5 ml microcentrifuge tubes. The supernatant was separated by centrifugation at 14,000 rcf for 5 min and the aqueous

phase, containing RNA, was then extracted with 1-bromo-3-chloropropane (BCP; Sigma Aldrich, St. Louis, MO; 0.1 ml/1 ml aqueous phase). Next, the aqueous phase was extracted with BCP and TRI Reagent® (0.2 ml/1 ml aqueous phase) prior to a final extraction with BCP alone (0.1 ml/1 ml aqueous phase). The aqueous phase of the last extraction was mixed with an equal volume of 70% ethanol before application to RNeasy® Mini columns in a 2 ml collection tube. Columns were centrifuged for 15 sec at 8,000 rcf and the flow-through discarded. Columns were then washed, as described above and in accordance with the RNeasy® Mini Kit instructions (Qiagen). For each column, purified total RNA was eluted with 50 µl nuclease-free water. The eluted RNA from both columns for each sample was combined, and then stored frozen at -80^{o} C until use. Since these samples were not intended for subsequent gene expression experiments, DNase I treatment of the RNA was omitted.

2.2.4 Extraction of RNA from skin biopsies for gene expression studies

The tick bite-site biopsies (6 mm) collected from Group 4 calves (Table 2.1) during the second infestation and Group 5 calves (Table 2.1) during three tick-infestations were each pulverized prior to homogenization in 2.5 ml TRI Reagent®. RNA was extracted using the modified TRI Reagent® method and purified on RNeasy® mini columns, as described above. Total RNA bound to the RNeasy® columns was DNase-treated with the RNase-free DNase Set (Qiagen) after an initial wash with 350 µl buffer RW1. Each column was treated with 70 µl buffer RDD and 10 µl DNase 1 stock solution, provided

with the RNase-free DNase Set (Qiagen), and incubated at RT for 15 min. All columns were washed again with 350 µl buffer RW1, and then washed twice with 500 µl buffer RPE, as described above. Purified total RNA from each column was eluted with 50 µl nuclease-free water and the eluates from the two columns for each sample were combined to reach 100 µl total of RNA prior to storage at -80^{o} C until use.

2.3 RNA Extraction from Bovine Whole Blood Leukocytes

2.3.1 Extraction of samples stabilized in TRI Reagent BD®

Bovine blood samples from Group 1 calves, stabilized in TRI Reagent BD,® were each thawed at RT and 800 µl 5N acetic acid was added prior to RNA extraction. The blood/TRI Reagent BD®/acetic acid mixture was transferred to a new 50 ml conical tube containing 4 ml of 1-bromo-3-chloropropane (BCP), shaken vigorously, and then incubated at RT for 5 min. This modification of substituting the less toxic BCP instead of chloroform is an option in the manufacturer's instructions (Molecular Research Center). Tubes were centrifuged at 10,500 rcf for 25 min at 4^{o} C before the RNA enriched aqueous phase was transferred to a new 50 ml conical containing 20 ml 2-propanol (Sigma-Aldrich, St. Louis, MO). Tubes were inverted several times to ensure the contents were mixed well, incubated at RT for 10 min, and then centrifuged at 10,500 rcf for 10 min at 4^{o} C. The supernatant was decanted and the RNA pellet resuspended by vortexing in 1 ml 75% ethanol. The RNA/ethanol mixture was

transferred to a 1.5 ml microcentrifuge tube and centrifuged at 12,000 rcf for 10 min at 4^{o} C before the ethanol was aspirated from the tube with a pipette. RNA pellets were air dried following a second centrifugation at 12,000 rcf for 5 min at 4^{o} C. The RNA was resuspended with 30 µl nuclease-free water and stored at -80^{o} C.

2.3.2 Leukocyte RNA extraction by PAXgene™ and LeukoLOCK™ methods

The frozen blood samples collected into PAXgene™ blood RNA tubes (Becton, Dickinson and Company) from Group 2 calves were thawed at RT. Instructions for the E.Z.N.A® Blood RNA Kit (Omega Bio-Tek, Norcross, GA) were followed to extract RNA. The PAXgene™ stabilized blood was transferred to a 15 ml conical tube and centrifuged at 4,000 rcf for 10 min at 4^{o} C. The supernatant was discarded and the pellet was resuspended by vortexing in 300 µl TRK lysis buffer, provided in the E.Z.N.A® Blood RNA Kit (Omega Bio-Tek). Protease treatment was performed by adding 590 µl nuclease-free water and 12.5 µl proteinase K (250 µg/ml, BioRad, Hercules, CA) to each RNA sample before incubation on a shaker at 55^{o} C for 10 min. The proteinase K-treated lysate was transferred to a homogenizer column, supplied with the E.Z.N.A® Blood RNA Kit (Omega Bio-Tek), and centrifuged at 13,000 rcf for 3 min at RT. The flow-through was transferred to a new 1.5 ml microcentrifuge tube prior to adding 450 µl 100% ethanol (Sigma-Aldrich). The ethanol-enriched lysate was transferred to a HiBand RNA column in a 2 ml collection tube, provided by the E.Z.N.A® Blood RNA Kit (Omega Bio-Tek). The column was centrifuged at 13,000 rcf for 1 min and the flow-

through discarded with the collection tube. Next, the column was placed into a new collection tube, washed once with 500 µl wash buffer I, and twice with 600 µl wash buffer II (Omega Bio-Tek). Following each wash, the column was centrifuged at 13,000 rcf for 1 min and the flow-through discarded. Next, the column was spun dry for 2 min at 13,000 rcf. Total RNA was eluted from the HiBand RNA column with 50 µl nuclease-free water, and stored at -80^{o} C.

The RNAlater®-stabilized leukocyte preparations on the LeukoLOCK™ filters from Group 2 calves were thawed at RT. While filters thawed, 2.5 ml lysis/binding solution concentrate was pH adjusted with 1.4 ml pH adjustment buffer for each LeukoLOCK™ filter, per manufacturer's instructions (Applied Biosystems). Once thawed, a 3 ml syringe with the plunger retracted was attached to the inlet port of each filter, and used to expel the RNAlater® into a waste container. Next, a 3 ml syringe with 2.5 ml pH adjusted lysis binding solution was attached to the inlet port of each filter to lyse the leukocytes, while flushing the lysate into a 15 ml conical for RNA extraction. Residual cell lysate was collected twice from each filter by disconnecting the syringe, retracting the plunger, reconnecting the syringe to the inlet port, and depressing the plunger to expel the remaining lysate into each respective 15 ml conical tube. The lysate was protease treated by the addition of 2.5 ml nuclease-free water and 25 µl proteinase K, provided in the LeukoLOCK™ kit (Applied Biosystems). Each tube was incubated for 5 min at RT with shaking while the tube containing RNA binding beads, supplied in the LeukoLOCK™ kit (Applied Biosystems), was resuspended by vortexing. Next, 50 µl RNA binding beads and 2.5 ml 2-propanol (Sigma-Aldrich) were added to

each protease-treated lysate. Each tube was incubated with intermittent mixing for 5 min at RT, and centrifuged at 2,000 rcf for 3 min before the supernatant was discarded. The RNA bound beads were then resuspended by vortexing in 600 µl wash solution 1 (LeukoLOCK™ kit, Applied Biosystems) prior to being transferred to a 1.5 ml microcentrifuge processing tube. Each 15 ml conical tube was then rinsed with another 600 µl wash solution 1, which was transferred to its respective microcentrifuge tube. Each tube was centrifuged at 16,000 rcf for 15 sec at RT and the supernatant discarded. The RNA binding bead pellet was resuspended by vortexing in 750 µl wash solution 2/3, provided in the kit (Applied Biosystems). Tubes were centrifuged at 16,000 rcf for 15 sec at RT and the supernatant was aspirated using a pipette. The RNA-bound bead pellet was air-dried for 2-3 min to allow any residual alcohol from the wash solution 2/3 to evaporate before DNase 1 (Applied Biosystems) treatment.

A TURBO™ DNase master mix was prepared by adding 296 µl 1X LeukoLOCK™ DNase buffer to 4 µl TURBO™ DNase (20 U/µl) for each RNA sample, according to manufacturer's instructions (Applied Biosystems). Each sample was treated with 300 µl TURBO™ DNase master mix, vortexed briefly, and incubated for 10 min at RT. The DNase-treated RNA was re-bound to the RNA binding beads by the addition of 300 µl lysis binding solution (Applied Biosystems), which was not pH adjusted, and 300 µl 2-propanol (Sigma-Aldrich) prior to incubation for 3 min at RT. The RNA binding beads were pelleted by centrifugation at 16,000 rcf for 15 sec at RT. The supernatant was carefully aspirated and the pellet washed twice with 750 µl wash solution 2/3 (Applied Biosystems) by centrifugation at 16,000 rcf for 15 sec and 1 min,

respectively. Tubes were centrifuged again briefly to facilitate removal of residual wash solution 2/3 by aspiration with a pipette before air-drying the RNA binding beads for 3 min. RNA was released from the RNA binding beads by adding 100 µl of elution solution (Applied Biosystems) and vortexing before centrifugation at 16,000 rcf for 2 min at RT. The RNA-containing supernatant was transferred to a new microcentrifuge tube and stored at -80^{o} C until use.

2.3.3 Validation of the LeukoLOCK™ total RNA isolation system

To validate the application of the LeukoLOCK™ total RNA isolation system for the extraction of RNA from bovine blood, the leukocyte-bound LeukoLOCK™ filters stabilized in RNAlater® from the 10 Group 3 calves described above were thawed. Filters were flushed and the leukocytes lysed, proteinase K treated, DNase treated, washed, and total RNA eluted in the same manner described above for the LeukoLOCK™ RNA isolation kit method.

2.3.4 RNA extraction from blood leukocytes for gene expression analyses

Blood samples collected from the Group 4 calves during the second *A. americanum* tick infestation and from the Group 5 heifer calves over three tick infestations were passed through LeukoLOCK™ filters and stabilized with RNAlater®. The stabilized leukocyte-

bound filters were thawed and processed for RNA isolation as described above for the LeukoLOCK™ kit method. Extracted RNA was stored at -80^{o} C.

2.4 Quantity and Quality of Isolated RNA

Total RNA was quantified on a Nanodrop® ND 1000 spectrophotometer (Thermo Scientific, Wilmington, DE). The quality of the isolated RNA was assessed by capillary electrophoresis on an Agilent® 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) using an Eukaryote Total RNA Nano Series II chip and Agilent® 2100 Bioanalyzer Expert software, version 2.5. RNA integrity was assessed by evaluation of the RNA integrity number (RIN; Schroeder et al., 2006) and electropherogram plots provided by the Agilent® 2100 Bioanalyzer Expert software (Agilent Technologies), which computes a RIN based on the electropherogram and other parameters to assign a score from 1 to 10. RNA with a RIN score of 10 is considered completely intact RNA, and samples with an RIN score of 1 represent fully degraded RNA. The presence of discrete 18S and 28S rRNA peaks on an electropherogram with low noise between the peaks is indicative of high quality RNA. Statistical testing was performed using SPSS software (Version 16.0; SPSS Inc., Chicago, IL). Independent t-tests with a calculated *P*-value less than or equal to 0.05 were considered statistically significant.

2.5 Quantitative Real Time RT-PCR in Skin Biopsies and Blood Leukocytes from Group 4 Calves

A panel of 35 genes with a putative association with the tick-susceptible or tick-resistant phenotype in cattle was selected to screen the blood and biopsy RNA samples from Group 4 calves (HAMMERLE-FICKINGER *et al.* 2010; KONGSUWAN *et al.* 2008; PIPER *et al.* 2008; PIPER *et al.* 2009; STRANDBERG *et al.* 2005; WANG *et al.* 2007b).

Previously published (Table 2.2) and newly designed gene-specific primers (Table 2.3), designed with Oligo 6 primer software (Molecular Biology Insights, Cascade, CO), were used. Candidate genes included major histocompatibility complex class II DR beta-chain (*MHC II-DRB3*), NLR family pyrin domain containing 3 (*NLRP3*), TNFR-SF1A-associated via death domain (*TRADD*), interleukin 1 alpha (*IL1α*), interleukin 2 receptor alpha (*IL2Rα*), interleukin 6 (*IL6*), interleukin 8 (*IL8*), interleukin 10 (*IL10*), interleukin 12 subunit p 40 (*IL12p40*), tumor necrosis factor receptor-associated factor 3 (*TRAF3*), caspase 8 (*Casp8*), tumor necrosis factor alpha (*TNFα*), myeloid differentiation factor 88 (*MyD88*), chemokine (C-C motif) receptor 1 (*CCR1*), chemokine (C-C motif) ligand 2 (*CCL2*), Toll-like receptor 4 (*TLR4*), cluster of differentiation factor 14 (*CD14*), complement component 1qA (*C1Q*), Toll-like receptor 9 (*TLR9*), interferon gamma (*IFNγ*), interleukin 18 (*IL18*), Toll-like receptor 2 (*TLR2*), tumor necrosis factor receptor-associated factor 6 (*TRAF6*), Toll-like receptor 5 (*TLR5*), nucleotide-binding oligomerization domain containing 2 (*NOD2*), chemokine (C-C motif) ligand 26 (*CCL26*), caspase 1 (*CASP1*), interleukin 1 beta (*IL1β*), nuclear

factor kappa 1 (*NFκB*), dendritic cell protein HFLB5 (*HFLB5*), immunoglobulin gamma heavy chain (*IGHG1*), interleukin 1 receptor type II (*IL1R2*), interleukin receptor antagonist (*IL1RN*), interleukin receptor accessory protein (*IL1RAP*), and interleukin 23 (*IL23*).

A standard curve was generated for each gene specific qRT-PCR primer pair to assess the amplification efficiency and dynamic range for the assay. Template cDNA was prepared in duplicate from each RNA sample from Group 4 calves by reverse transcription using 500 ng total RNA.

Table 2.2 Previously published primers used for gene expression profiling in this study.

Gene name	**Gene Symbol**	**Forward/Reverse primer**	**Product length (bp)**	**Reference**
Interleukin 1 beta	IL1B	5′ gat gat gac ctg gaa gcc att 3′ 5′ tttt cac tgc ctc ctc cag at 3′	185	Strandberg 2005
Tumor necrosis factor-a	TNF	5′ ctg gtt cag aca ctc agg tcc t 3′ 5′ gag gta aag ccc gtc agc a 3′	183	Strandberg 2005
nuclear factor kappa 1	NFKB1	5′ ctg gaa gca cga atg aca ga 3′ 5′ gct gta aac atg agc cgt acc 3′	207	Strandberg 2005
Myeloid differentiation primary response gene 88	MYD88	5′ gcc gcc tgt cgc tct t 3′ 5′ gtc gct gcc agt cgt cc 3′	150	Strandberg 2005
Toll-like receptor 2	TLR2	5' agc act tca acc ctc cct tt 3' 5' gaa tca gaa tgg cag cat ca 3'	216	Strandberg 2005
Interleukin 6	IL6	5' ctg ggt tca atc agg cga 3' 5' cag cag gtc agt gtt tgt gg 3'	206	Strandberg 2005

Table 2.2 Continued

Gene name	Gene Symbol	Forward/Reverse primer	Product length (bp)	Reference
Chemokine (C-C motif) ligand 2	CCL2	5′ ccc tcc tgt gcc tgc tac t 3′ 5′ tca agg ctt tgg agt ttg gt 3′	284	Wang 2007
Chemokine (C-C motif) receptor 1	CCR1	5′ ctg ctg gtg atg att gtc tg 3′ 5′ tgc tct gct cac act tac gg 3′	191	Wang 2007
Dendritic cell protein HFL-B5	HFL-B5	5′ aca ggt gta ttg tgc gag cat 3′ 5′ gca gta ggc caa gtg aat caa3′	194	Wang 2007
Toll-like receptor 5	TLR5	5′ aac gct ttg ctc aaa cac ct 3′ 5′ acc ctc tga tgg act gat gc 3′	301	Piper 2008
Complement component 1, qA	C1QA	5′ att gaa agg cac caa agg c 3′ 5′ ttc tgg tac acg ttc tcc tgg 3′	144	Hammerle-Fickinger 2009
Interleukin 2 receptor alpha	IL2RA	5' tgc taa gag cat ccc gac 3' 5' gct tgg agg act ggg cta 3'	204	Piper 2009
Interleukin 8	IL8	5' ctg tgt gaa gct gca gtt ct 3' 5' tag gca gac ctc gtt tcc at 3'	180	Piper 2009
Interleukin 10	IL10	5' ctt gtc gga aat gat cca gt 3' 5' tct ctt gga gct cac tga ag 3'	209	Piper 2009
Interleukin 18	IL18	5' agc aca ggc ata aag atg gc 3' 5' tgg ggt gca tta tct gaa ca 3'	254	Piper 2009
Interferon, gamma	IFNG	5' gtg ggc ctc tct tct cag aa 3' 5' gat cat cca ccg gaa ttt ga 3'	234	Piper 2009
CD14 molecule	CD14	5' ggt gct acc cga tgt gtc tg 3' 5' aag gga ttt ccg tcc aga gt 3'	191	Kongsuwan 2009

Table 2.3 Primers designed and used for gene expression profiling in this study.

Gene name	Gene symbol	Accession number[a]	Forward/reverse primer[b]	Product (bp)
Toll-like receptor 4	TLR 4	AB056444.1	5' cag agc cga tgg tgt atc tt 3' 5' ctc cca ctc cag gta ggt gt 3'	168
Toll-like receptor 9	TLR 9	NM_183081.1	5' gga gga ccg caa gga 3' 5' gtg cta ttc ggc tgt cgt 3'	229
MHC class II DR beta-chain	BoLA-DRB3	NM_001012680.2	5' gac gag ccc tat cac agt aga at 3' 5' aga gca gac cca gaa cga a 3'	95
Interleukin 12p40	IL12B	NM_174356.1	5' tgc tta ttg agg tcg tgg t 3' 5' gag aag tag gaa tgc ggg 3'	193
Interleukin 1 alpha	IL1A	NM_174092.1	5' gag gac tga ggc tac tat ctg tg 3' 5' tgt gtg tcc acg gct tat t 3'	118
Nucleotide-binding oligomerization domain containing 2	NOD2	NM_001002889.1	5' caa gca gcc tcg tgg gt 3' 5' aac cag aca cac agg aaa cca 3'	123
NLR family, pyrin domain containing 3	NLRP3	NM_001102219.1	5' acc cac ctt tac cta c 3' 5' cag cag cag tgt gat gtg a 3'	134
Caspase 1	CASP1	XM_002692921.1	5' ttg ccc tta tta tct 3' 5' tca cat cca cct tgt atc c 3'	117
Caspase 8	CASP8	NM_001045970.2	5' agg agg gct act cta a 3' 5' tgc ttg tca ttc ggt aaa ctg 3'	93
TNF receptor-associated factor 3	TRAF3	XM_582595.3	5' aca tca aag acg aca 3' 5' gca ggt ccg aag tat cca 3'	54
TNF receptor-associated factor 6	TRAF6	NM_001034661.1	5' caa gag aat acc cag tcg ca 3' 5' gca cag agt cat aca gag gca 3'	109
TNFRSF1A-associated via death domain	TRADD	NM_001045896.1	5' aag ggg agg tcg gtc a 3' 5' aca aga ggt cag cag tag ca 3'	86

Table 2.3 Continued

Gene name	Gene symbol	Accession number[a]	Forward/reverse primer[b]	Product (bp)
Chemokine (C-C motif) ligand 26	CCL26	NM_001205635.1	5′ gct gtg ata ttc acc acc aag 3′ 5′ gtt gct ggg ctc tga gtt t 3′	97
Interleukin 1 receptor accessory protein	IL1RAP	NM_001076155.1	5' gaa ctc aga ctg taa agg tgg t 3' 5' gcg aga gtc ctt cag gta a 3'	152
Interleukin 1 receptor, type II	IL1R2	NM_001046210.1	5' tcc gtg cct ctc atc tt 3' 5' atg gac aca ttg ttt acg agt 3'	200
Interleukin 1 receptor antagonist	IL1RN	NM_174357.2	5' tcc acg gtt acc taa tct gtc 3 '5' cag caa cta att ggt tat tcc tc 3'	153
Interleukin 23	IL-23A	NM_001205688.1	5' gcc tcc ttc tcc gtc tc 3' 5' agg tct ggg tgt cat cct t 3'	129
Immunoglobulin heavy constant gamma 1	IGHG1	BC146168.1	5' atc acc ctg tga ctg ttg cc 3' 5' gcg ttc ccg aga ttg tga g 3'	101

[a] Accession numbers were used to locate respective mRNA target sequences in the NCBI database
[b] Primers were designed from the accessed mRNA sequences with Oligo 6 software (Molecular Biology Insights)

A reverse transcription master mix was prepared using 2.5 μl RT buffer, 2.5 μl random hexamer primers, 1 μl dNTPs, 1.25 μl of RNase inhibitor, and 1 μl Multiscribe™ RT enzyme for each reaction; all reagents listed were provided with the High Capacity cDNA Reverse Transcription kit with RNase inhibitor (Applied Biosystems). Next, 8.25 μl of the RT master mix and nuclease-free water were added to each RNA aliquot in a 25 μl reaction. Reverse transcription reactions were heated to 25° C for 10 min, 37° C for 2 hr, and 85° C for 5 min before cooling to 4° C in an ABI

2720 thermal cycler (Applied Biosystems). The cDNA preparations were diluted 1:4 with yeast tRNA (25 ng/µl), which was prepared from a 25 mg/ml yeast tRNA stock (Invitrogen).

Template cDNA (10 ng) was amplified in a 20 µl qRT-PCR reaction using 200 nM sequence specific primers and 1X SYBR GreenER PCR master-mix (Invitrogen) in a MicroAmp® optical 96-well reaction plate (Applied Biosystems), and sealed with MicroAmp® optical adhesive film. All reactions were run in duplicate on an ABI 7900HT Sequence Detection System (Applied Biosystems) using the manufacturer's default thermal cycling conditions. Amplification data, captured with the ABI Prism SDS (v 2.4) program, was normalized in Microsoft Excel using the log transformed geometric mean of three reference genes, Ribosomal protein L19 (*RPL19*), ATPase Ca++ transporting, type 2C and member 1 (*ATP2C1*), and cytochrome c oxidase subunit 7C (*Cox7C*). Relative quantitation was performed with the ΔΔCt method (LIVAK and SCHMITTGEN 2001) and graphs were generated based on fold change differences in expression relative to average values for day 0 samples for each gene specific expression profile. Fold change differences in expression are reported as the average fold change observed minus one, since average expression values at day 0 were set to a threshold of one.

A total of eight genes including *TLR2*, *TLR4*, *CD14*, *TLR5*, *NLRP3*, *IFNγ*, *MHCII-DRB3*, and *IL18* were assessed by qRT-PCR using RNA derived from whole blood leukocytes from Group 4 calves. For this study, the number of phenotypically distinct animals, i.e., moderately (N=3) and highly tick-resistant (N=3) calves, were too

small to make meaningful statistical inferences (Department of Statistics, Texas A&M University). Therefore, these data are reported without statistical comparisons between the phenotypes, however, differential expression of 5-fold or higher between the two phenotypes are reported as notable.

2.6 Microarray Comparison of Tick Bite-Site Biopsies from Group 5 Calves

2.6.1 cDNA synthesis

RNA from tick bite-site biopsies from Group 5 calves on days 0, 5, and 10 of the first and second tick infestations were concentrated to 3-5 µg in a 10 µl volume using RNeasy® MinElute columns (Qiagen). Total volumes were adjusted to 100 µl with nuclease-free water before 350 µl buffer RLT (Qiagen) and 250 µl 100% ethanol (Sigma-Aldrich) was added to each RNA sample. Each sample was then mixed by pipetting several times and transferred to an RNeasy® MinElute spin column in a 2 ml collection tube. Each column was centrifuged at 8,000 rcf for 15 sec at RT and the flow-through discarded, along with the collection tube. After transfer to a new 2 ml collection tube, each column was washed with 500 µl buffer RPE (Qiagen) and centrifuged at 8,000 rcf for 15 sec at RT. The column-bound RNA was then washed with 80% ethanol prior to centrifugation at 8,000 rcf for 2 min at RT. Next, each column was transferred to a new 2 ml collection tube, and centrifuged at 8,000 rcf for 5 min at RT to dry the column-bound RNA. The total RNA was then eluted from the spin columns with 18 µl

nuclease-free water, and the concentration of each sample was determined using a Nanodrop® ND 1000 spectrophotometer (Thermo Scientific). All samples yielded sufficient quantities for 5 μg microarray hybridization except 902-Day 0, 905-Day 10, and 907-Day 10, which only had sufficient RNA for 3 μg per reaction.

Total RNA (3-5 μg) was added to 0.2 ml PCR tubes that contained sufficient nuclease-free water to equal 10 μl. To prime transcripts for fluorescent tagging during cDNA synthesis, 1 μl of RT primer for the respective Cy fluor (Cy 3 or Cy 5), provided in the 3DNA Array Detection 350™ Kit (Genisphere LLC., Hatfield, PA), was added prior to incubation of the reactions at 80^{o} C for 10 min. All tubes were placed on ice and 9 μl of prepared reverse transcription master mix (4 μl Super Script 5X first strand buffer, 2 μl 0.1 M dithiothreitol, 1 μl dNTPs, 1 μl RNase inhibitor, 1 μl Super Script II reverse transcription enzyme; Genisphere) was added to each reaction. Reverse transcription was performed at 42 ^{o} C for 2 hr in a thermal cycler (model 2720; Applied Biosystems). Reactions were stopped with 3.5 μl sodium hydroxide and EDTA solution (0.5 M NaOH/50 mM EDTA) and then the RNA was denatured by incubation at 65^{o} C for 15 min. The reverse transcription reactions were neutralized using 5 μl 1 M Tris-HCL solution, pH 7.5 and stored at -80^{o} C until use.

2.6.2 Microarray slide preparation

Bovine oligonucleotide (60-70 mer) microarrays containing over 13,000 unique transcripts (EVERTS *et al.* 2005) were purchased from the laboratory of Harris A. Lewin,

PhD, Director of the Institute for Genomic Biology, Department of Animal Sciences, University of Illinois at Urbana-Champaign, Urbana, IL. Microarray slides were labeled with R, M, or S to represent cattle phenotypes, i.e., highly tick-resistant (R), moderately tick-resistant (M), or tick-susceptible (S) calves; the last digit (1-7) of the calf identification number; and a letter to denote the day of sample collection being hybridized to the array, day 0 (A), day 5 (B), and day 10 (C) (Table 2.4).

Each slide was rehydrated by holding array side down over a 50^{o} C water bath for 10 sec. After hydration, each slide was placed onto a 65^{o} C heat block, array side up, to snap-dry for 5 sec and then placed on a laboratory tissue for 1 min to cool. This re-hydration process was repeated five times for each microarray slide. Next, the slides were UV cross-linked using a Stratagene UV Stratalinker® 1800 (Agilent Technologies) at 6000 μJ X 100. Immediately after cross-linking the slides were washed in 1% sodium dodecyl sulfate (SDS) for 5 min, dipped 10 times in sterile water, and rinsed for 3 min in 100% ethanol (Sigma-Aldrich). Each slide was then transferred to a 50 ml conical tube and centrifuged at 200 rcf for 3 min to dry. Dry microarray slides were then stored in a slide box, inside a desiccator, at RT until use.

2.6.3 Microarray hybridizations

Microarray hybridization reactions were prepared by mixing the two opposing, Cy 3 or Cy 5, dye-labeled 28.5 μl cDNA reactions for each calf and day of collection.

Table 2.4 List of microarray experiments for Group 5 calves

Day of sample collection	Calf identification	Phenotype	Cy-color tag 1° infestation	Cy-color tag 2° infestation
Group A				
Day 0	903	R	Cy 3-Green	Cy 5-Red
Day 0	904	R	Cy 5-Red	Cy 3-Green
Day 0	902	M	Cy 3-Green	Cy 5-Red
Day 0	905	M	Cy 5-Red	Cy 3-Green
Day 0	907	M	Cy 3-Green	Cy 5-Red
Day 0	901	S	Cy 5-Red	Cy 3-Green
Day 0	906	S	Cy 3-Green	Cy 5-Red
Group B				
Day 5	903	R	Cy 5-Red	Cy 3-Green
Day 5	904	R	Cy 3-Green	Cy 5-Red
Day 5	902	M	Cy 5-Red	Cy 3-Green
Day 5	905	M	Cy 3-Green	Cy 5-Red
Day 5	907	M	Cy 5-Red	Cy 3-Green
Day 5	901	S	Cy 3-Green	Cy 5-Red
Day 5	906	S	Cy 5-Red	Cy 3-Green
Group C				
Day 10	903	R	Cy 3-Green	Cy 5-Red
Day 10	904	R	Cy 5-Red	Cy 3-Green
Day 10	902	M	Cy 3-Green	Cy 5-Red
Day 10	905	M	Cy 5-Red	Cy 3-Green
Day 10	907	M	Cy 3-Green	Cy 5-Red
Day 10	901	S	Cy 5-Red	Cy 3-Green
Day 10	906	S	Cy 3-Green	Cy 5-Red

Empty reaction tubes were rinsed with 73 µl 1X Tris-EDTA buffer, which was added to the respective dual-labeled cDNA reaction. The 2X hybridization buffer (3DNA Array Detection 350™ Kit; Genisphere) was fully thawed and then resuspended by heating to 70° C for 10 min. Each microarray hybridization mixture was prepared by adding 2 µl

LNA dT blocker, 218 µl nuclease-free water, and 290 µl of the 2X hybridization buffer, all supplied with the 3DNA kit (Genisphere), to each cDNA reaction mixture. The microarray hybridization mixtures were incubated at 80° C for 10 min and kept warm in an Agilent G2545A hybridization oven (Agilent Technologies) at 60° C until use.

Hybridization chambers (Agilent Technologies) were assembled and pre-warmed at 60° C prior to insertion of a gasket slide (Agilent Technologies). Each cDNA hybridization mixture (510 µl) was pipetted onto a gasket slide inside the hybridization chamber immediately prior to insertion of the respective microarray slide, array side down on top of the gasket slide. Each microarray/gasket slide assembly, and the reaction mixture between them, was then secured in the hybridization chamber by tightening the clamps on both slides. Each assembled chamber was rotated and tapped softly on the edges to dislodge air bubbles before being placed, in a balanced manner on the rotator, in the hybridization oven. All microarrays were hybridized with the rotator set at 4 rcf for 16 hr at 60° C.

Wash buffers (2X saline-sodium citrate (SSC)/0.2% SDS, 2X SSC, and 0.2X SSC) were prepared from stock solutions (Agilent Technologies) using nuclease-free water, and warmed to 42° C in a water bath while arrays were hybridized. The hybridization chambers were disassembled and the microarray slides dislodged from the gasket slides while submerged in the 2X SSC/0.2% SDS wash buffer at 42° C using plastic forceps. The microarray slides were washed for 15 min in the warm 2X SSC/0.2% SDS buffer, 15 min in warm (42° C) 2X SSC buffer, and 15 min in 0.2X SSC buffer at RT on a rotator. Microarray slides were centrifuged at 200 rcf for

2 min immediately after the final wash, and placed in the incubation oven at 58° C to keep warm while the capture reagent master mix was prepared.

The Cy dye capture reagents were thawed for 20 min at RT and then heated to 55° C for 10 min. The 2X hybridization buffer was thawed and heated to 70° C for 10 min. The capture reagent hybridization mix was prepared for each microarray slide by adding 2.5 µl Cy 3 capture reagent, 2.5 µl Cy 5 capture reagent, 20 µl of nuclease-free water, and 25 µl resuspended 2X hybridization buffer, all supplied in the 3DNA kit (Genisphere), before incubation at 80° C for 10 min. Cover slips for the array slides were washed in 1% SDS, rinsed in nuclease-free water, dipped 10 times in 100% ethanol, and dried with laboratory tissue prior to use.

In a dark room the capture reagent master mix (50 µl) was pipetted onto the array portion of each microarray slide immediately before carefully placing a cover slip over the area. Each prepared slide was inserted into a 50 ml conical tube containing 200 µl of nuclease-free water to prevent over drying of the microarray, and incubated for 4 hr at 58° C in the hybridization oven without rotating. The cover slips were removed in warm 2X SSC/0.2% SDS buffer and microarray slides washed as described above. After the final centrifugation at 200 rcf for 2 min, the microarray slides were dipped in dye saver (Genisphere) to protect the captured dye signal from fading until scanned.

2.6.4 Microarray scanning and statistical analysis

Array slides were scanned with an Axon 4100 scanner manually set to a Cy 3/Cy 5 dye ratio of 1.0. Microarray image files were aligned to the bovine oligo microarray template provided with the slides and the signal intensity ratio for each feature was captured. Template aligned feature data were exported to a text file for each microarray image, and subsequently analyzed with Genespring version GX10 (Agilent Technologies). A custom microarray technology was constructed to interpret these bovine microarray slide images in the Genespring program. To generate this tool the *Bos taurus* genome was chosen for a two-color generic microarray platform, and the image file from the microarray M5B was chosen as a template for this data set. The oligo name column of the text file was chosen as the feature identifier. Background corrected means for each dye signal were used to determine the intensity of the signal ratio expressed for each feature.

Due to the small sample size, microarray experiments were analyzed based on phenotype (tick-susceptible, N=2; moderately tick-resistant, N=3; highly tick-resistant, N=2) and grouped by day of sample collection to yield a list of differentially expressed genes between days 0-5, days 5-10, and days 0-10 for each of the three cattle phenotypes. Using the technology devised above for the bovine oligo microarrays in Genespring GX10, a time course interpretation was generated by incorporating the data from all 21 microarray experiments (Table 2.2). Dye swaps were indicated and the raw expression data were threshold set to 11 prior to normalization using the lowess method

(QUACKENBUSH 2002). To increase the stringency of the experiments, lowess normalized data were further processed by selection for genes expressing a background corrected signal ranked above 20 percentile. Genes were further selected for 70 % expression intensity in one of the three grouping (day of collection) conditions.

Statistical analysis was performed using an independent t-test and corrections for false discovery rates were omitted. The lists of differentially expressed genes was exported to an Excel file and the oligo probe identifiers were manually searched for the respective accession number in the template file provided with the bovine oligo microarrays. Each accession number for a differentially expressed gene with a fold change ≥ 2 was then searched in the NCBI database using the basic local alignment search tool (ALTSCHUL *et al.* 1990) to determine the gene name and associated function.

Gene ontology analysis was performed, using the official gene symbol identified by the BLAST accession number, with DAVID Bioinformatics Resources 6.7 (HUANG *et al.* 2009a; HUANG *et al.* 2009b) to identify biological processes and Kyoto encyclopedia of genes and genomes pathways associated with the lists of up- and down-regulated genes generated by microarray analyses described above (KEGG; (KANEHISA *et al.* 2012). Genes not currently annotated in the DAVID database were excluded from gene ontology analysis.

2.6.5 qRT-PCR follow-up of 11 candidate genes

Six candidate genes that were identified as differentially expressed in one or more of the three cattle phenotypes by microarray analyses, such as Interleukin 1 alpha (*IL1α*), Interleukin 1 receptor accessory protein (*IL1RAP*), Interleukin 1 receptor 2 (*IL1R2*), Interleukin 1 receptor antagonist (*IL1RN*), Immunoglobulin heavy chain constant 1 (*IGHG1*), and Toll-like receptor 5 (*TLR5*), were chosen for microarray validation and gene-specific analysis via qRT-PCR. Primers for these six genes were previously described (Section 2.5, Tables 2.2 and 2.3). An additional five genes including Interleukin 1 beta (*IL1β*), Toll-like receptor 4 (*TLR4*), Interleukin 6 (*IL6*), and Interleukin 12 (*IL12*), and Interleukin 23 (*IL23*) were selected from the group of 35 candidate genes profiled via qRT-PCR in samples from Group 4 calves, also previously described (Section 2.5, Tables 2.2 and 2.3), for further evaluation in samples from Group 5 calves. Thus, a total of 11 candidate genes were profiled across three tick infestations in tick bite-site biopsies, and six of the 11 genes were also evaluated in blood leukocytes from Group 5 calves.

Template cDNA was prepared and qRT-PCR reactions were performed as described above (Section 2.5). Captured amplification data was normalized using the log transformed geometric mean for two of the previously described reference genes, *RPL19* and *ATP2*, and relative quantitation values were calculated using the with the ΔΔCt method (LIVAK and SCHMITTGEN 2001), as described above. Graphs depicting fold change differences in expression relative to day 0 of the first tick infestation were

generated for the three tick infestations using the average expression values for Group 5 calves as a whole, and for each phenotype.

2.7 Gene to Gene Interactions in Tick Bite-Site Biopsies from Group 5 Calves

Relative quantitation (RQ) values from qRT-PCR profiles in tick bite-site biopsies from Group 5 calves for each of the 11 candidate genes evaluated were utilized for regression curve estimations to determine the likelihood of gene to gene interactions. The average RQ values from the seven calves (N=7), at each collection time throughout the three tick infestations (17 total), were used to prepare scatterplots and to calculate R^2. Regression analysis was also performed using the average RQ values from each cattle phenotype (N=2 tick-susceptible, N=3 moderately tick-resistant, and N=2 highly tick-resistant) and RQ values from each calf individually. Gene to gene interaction was predicted based on high regression R^2 values ($R^2 \geq 0.8$) and F scores, resulting in statistical significance ($P = 0.0001$).

CHAPTER III

RESULTS

3.1 Optimization of Sample Collection and RNA Extraction

*3.1.1 Isolation of RNA from tick bite-site biopsies**

The quantity and quality of total RNA isolated from tick bite-site biopsies from cattle in this study were compared using two different methods, as previously described (BRANNAN *et al.* 2013). The greatest quantity of total RNA was obtained from bovine 6-mm tick bite-site biopsies using the modified TRI Reagent® method compared to the 3-mm biopsy specimens extracted with the RNeasy® Mini Kit. An average of 0.763 ± 0.505 μg/mm^3 (range 0.272 to 1.394 μg/mm^3) total RNA was obtained from the former and this yield was significantly higher ($P = 0.001$) than the 0.014 ± 0.009 μg/mm^3 average amount (range 0.007-0.031 μg/mm^3) obtained from the 3-mm biopsy specimens with the RNeasy® kit (Table 3.1).

High quality RNA, as determined by Bioanalyzer chip analysis, was isolated from bovine tick bite-site biopsies using the modified TRI Reagent® method (Figure 3.1). This method yielded an average RIN of 7.69 ± 0.99, which was

*Data reported in section 3.1.1 is reprinted with permission from "Evaluation of methods for the isolation of High Quality RNA from bovine and cervine hide biopsies" by Jaime L. Brannan, Patricia J. Holman, Pia U. Olafson, John H. Pruett, and Penny K. Riggs, 2013. *The Journal of Parasitology*, 99 (1), 19-23, Copyright [2013] by Allen Press Inc.

significantly increased ($P = 0.011$) compared to the average RIN score of 5.39 ± 2.02 for RNeasy® Mini Kit extracted samples (Figure 3.1A-B), as described previously (BRANNAN *et al.* 2013).

The utility of a 4 M guanidinium isothiocyanate (GITC) solution for freezing and subsequent storage of bovine tick bite-site biopsy samples was also evaluated, as previously described (BRANNAN *et al.* 2013). Use of 4 M GITC did not result in significant differences in total RNA yields (Table 3.2) or RIN scores (Figure 3.1C) for either of the two RNA extraction techniques evaluated.

Table 3.1 Yield and quality of total RNA isolated from bovine tick bite-site biopsies by two different methods.

RNA Isolation Method	**Sample (N, mm^3)**	**Average RNA Yield ($\mu g/mm^3$) $\pm$ STDV**	**Average RIN[a] $\pm$ STDV**
RNeasy® Mini Kit	Bovine (N=9, 99 mm^3)	0.014 $\pm$ 0.009†	5.39 $\pm$ 2.02‡
Modified TRI Reagent®	Bovine (N=7, 198 mm^3)	0.763 $\pm$ 0.505†	7.69 $\pm$ 0.99‡

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[a] RNA integrity number (RIN) was calculated using Agilent® 2100 Bioanalyzer.

† Independent t-tests for significance ($P \leq 0.05$) were performed with SPSS 16.0 software ($P = 0.001$).

‡ Independent t-tests for significance ($P \leq 0.05$) were performed with SPSS 16.0 software ($P = 0.011$).

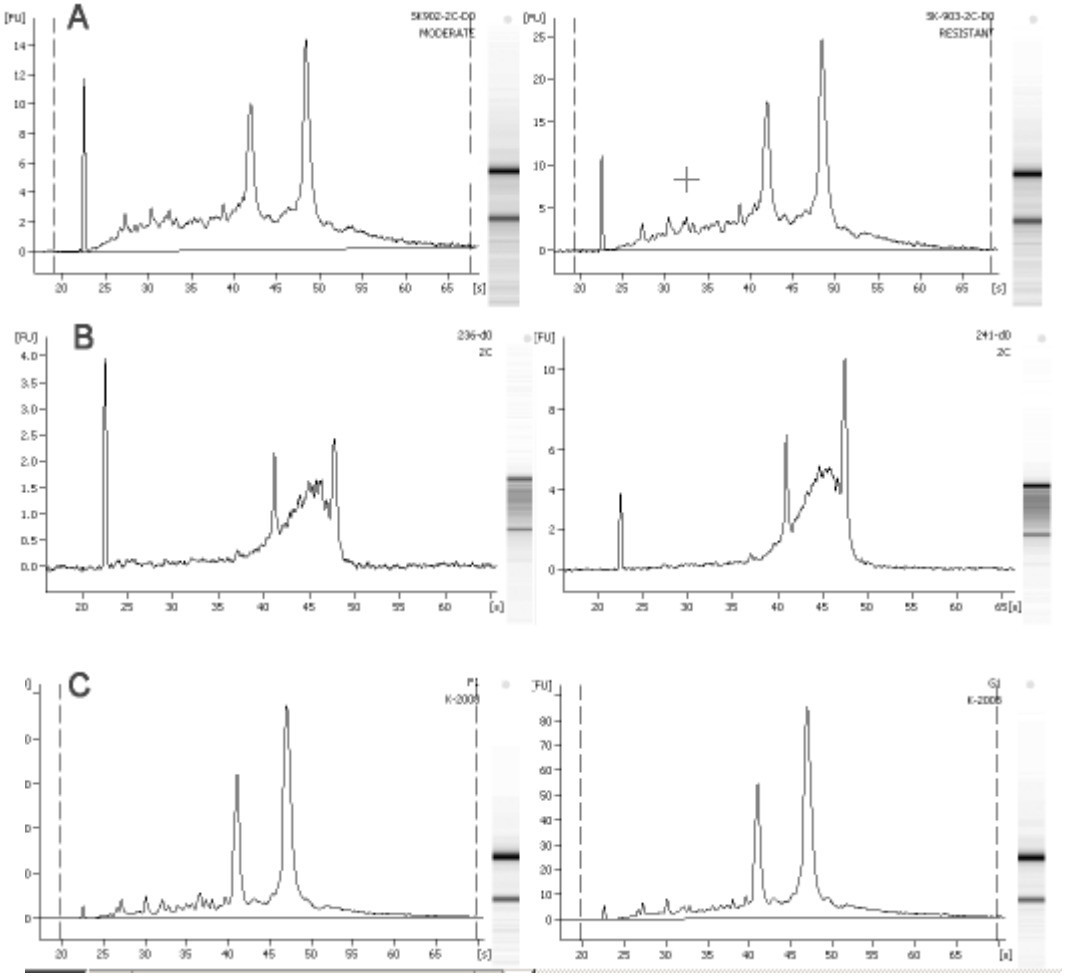

Figure 3.1 Representative electropherograms and gel images of total RNA extracted from bovine tick bite-site biopsies by two different methods. Skin biopsy-derived total RNA (A) from calves 902 and 903 using the modified TRI Reagent® method, (B) from 2 calves 236 and 241 using the RNeasy® Mini Kit, (C) from two biopsies from calf 01 either flash frozen (FF) or flash frozen in GITC (GITC) using the modified TRI Reagent® method. The X and Y axes represent time (seconds) and fluorescence (units of fluorescence), respectively. *Reprinted with permission from "Evaluation of methods for the isolation of High Quality RNA from bovine and cervine hide biopsies" by Jaime L. Brannan, Patricia J. Holman, Pia U. Olafson, John H. Pruett, and Penny K. Riggs, 2013. *The Journal of Parasitology*, 99 (1), 19-23, Copyright [2013] by Allen Press Inc.

Table 3.2 Yield and quality of total RNA isolated from bovine tick bite-site biopsies frozen with or without 4 M GITC.

		Treatment at Collection			
		Flash Frozen Average ± STDV		Flash Frozen + GITC Average ± STDV	
RNA Isolation Method	**Sample (N)**	**Total RNA (μg/mm²)**	**RIN[a]**	**Total RNA (μg/mm²)**	**RIN[a]**
RNeasy® Mini Kit	2	0.014† ± 0.005	3.85‡ ± 1.77	0.019† ± 0.004	3.90† ± 1.98
TRI Reagent®	8	0.186† ± 0.048	8.59‡ ± 0.47	0.187† ± 0.048	8.30† ± 0.83

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[a] RNA integrity number (RIN) was calculated using Agilent® 2100 Bioanalyzer.

† Independent t-tests for significance ($P \leq 0.05$) were performed with SPSS 16.0 software ($P = 0.001$).

‡ Independent t-tests for significance ($P \leq 0.05$) were performed with SPSS 16.0 software ($P = 0.011$).

However, extraction of tick bite-site biopsies using the modified TRI Reagent® method resulted in significantly higher RNA yields ($P = 0.001$) and RIN scores ($P = 0.0001$ and $P = 0.001$) compared to those processed with the RNeasy® Mini Kit (Table 3.2), as described previously (BRANNAN *et al.* 2013). Furthermore, high quality RNA was obtained from samples processed with TRI Reagent® regardless of whether 4 M GITC was used or not (Figure 3.1C).

3.1.2 Isolation of RNA from blood leukocytes

When three different RNA isolation methods were evaluated for use with frozen bovine whole blood or isolated blood leukocytes, the highest concentration of total RNA was obtained using PAXgene® Blood RNA tubes. An average of 6.88 μg total RNA was obtained, which was significantly higher ($P = 0.001$) than the 1.64 μg of RNA isolated using TRI Reagent® BD (Table 3.3). The LeukoLock™ total RNA isolation system yielded an average of 5.62 μg of total RNA, which was also significantly higher ($P = 0.010$) than the average for TRI Reagent® BD extracted RNA, and this amount was not significantly different from the average yield obtained with PAXgene® Blood RNA tubes (Table 3.3).

The highest quality of total RNA from frozen blood leukocytes was obtained using the LeukoLock™ total RNA isolation system, and this method yielded a significantly higher ($P = 0.017$) average RIN of 7.7 ± 0.14 than the 4.33 ± 2.69 average RIN obtained with the TRI Reagent® BD method (Figure 3.2A-B). RIN scores were not detectable for PAXgene® isolated RNA (Figure 3.2C).

Table 3.3 Yield and RIN for total RNA isolated from bovine blood leukocytes with three different methods.

RNA isolation method Number of samples	**Volume collected (ml)**	**Average total RNA (μg) ± STDV**	**Average RIN[a] ± STDV**
Tri Reagent BD N=10	8	1.64†,‡ ± 1.56	3.43§ ± 2.02
LeukoLock N=2	7	5.62† ± 1.99	7.7§ ± 0.14
PAXgene N=2	2.5	6.88‡ ± 0.99	ND[b]

[a] RNA integrity number (RIN) was calculated using Agilent® 2100 Bioanalyzer.

[b] ND, not detected.

† Independent t-tests for significance ($P \leq 0.05$) were performed with SPSS 16.0 software ($P = 0.010$).

‡ Independent t-tests for significance ($P \leq 0.05$) were performed with SPSS 16.0 software ($P = 0.001$).

§ Independent t-tests for significance ($P \leq 0.05$) were performed with SPSS 16.0 software ($P = 0.017$).

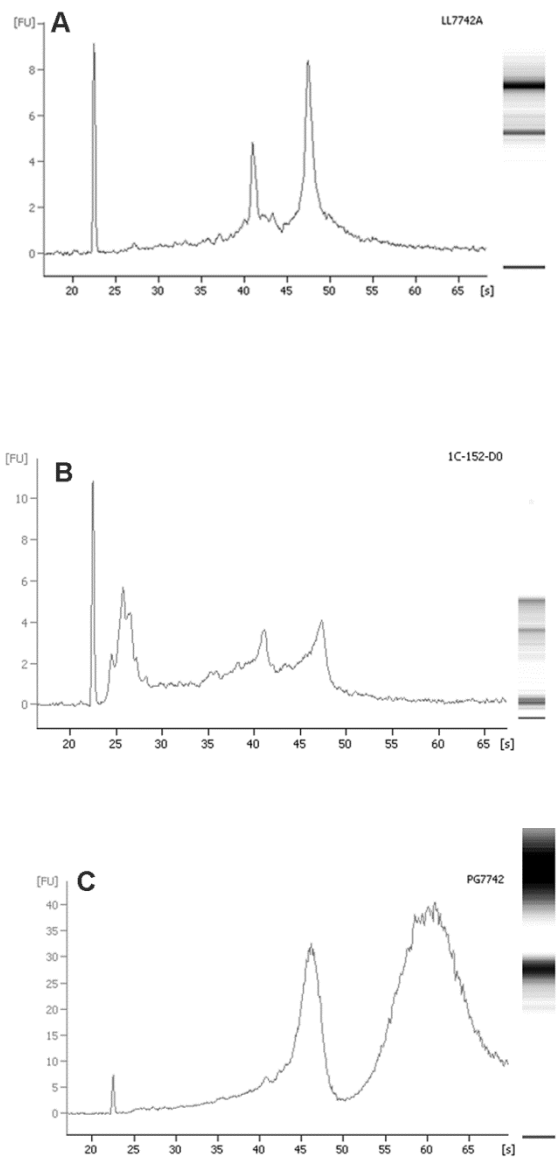

Figure 3.2 Representative electropherograms and gel images of total RNA extracted from bovine blood leukocytes using three different methods. Blood leukocyte-derived RNA (A) using the LeukoLOCK™ Total RNA Isolation system, (B) using TRI Reagent® BD, (C) using PAXgene® Blood RNA tubes. The X and Y axes represent time (seconds) and fluorescence (units of fluorescence), respectively.

3.2 qRT-PCR Analysis of 35 Candidate Genes in Tick Bite-Site Biopsies and Blood Leukocytes from Group 4 Calves

3.2.1. Gene expression profiles in tick bite-site biopsies and blood leukocytes by phenotype

Tick bite-site biopsies from the highly tick-resistant (HR) phenotype showed notably increased expression (fold change $\geq$ 5) over those from the moderately tick-resistant (MR) phenotype for *TLR4*, *CD14*, *NLRP3*, and *IFNγ* (Figures 3.3 and 3.4). In general, this occurred on days 4, 5, and 6 of the tick infestation and ranged from 5- to 100-fold. Gene expression for *CD14* was notably higher in biopsies from HR calves compared to those from MR cohorts by 5- to 20-fold on days 3 and 10 (Figure 3.3), and rose similarly for *IFNγ* by 50- to 100-fold on days 3, 7, and 10 of the tick infestation (Figure 3.4). Gene expression in blood leukocytes from HR calves revealed 10- to 80-fold rises over MR cohorts for *TLR5* on days 4, 5, 6, 7, and 14, while the moderately tick-resistant phenotype was increased 10- to 30-fold higher for *IFNγ* on days 4, 5, and 10 compared to those from highly tick-resistant calves (Figure 3.4). No notable differences in blood leukocytes were observed between the two phenotypes for expression of *TLR4*, *CD14*, or *NLRP3* (Figure 3.3).

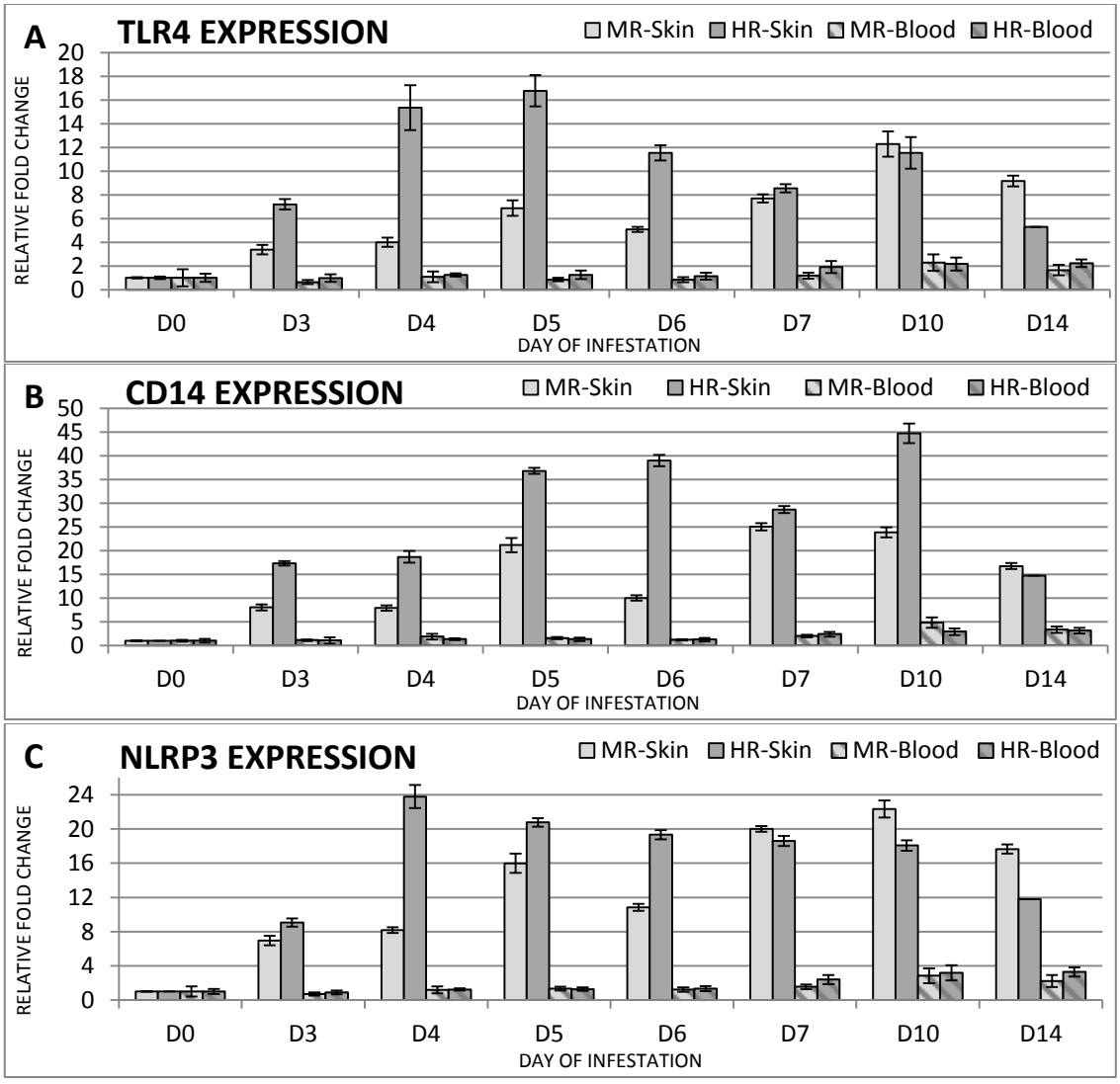

Figure 3.3 Relative gene expression by qRT-PCR for (A) *TLR4*, (B) *CD14*, and (C) *NLRP3* in bovine tick bite-site biopsies (Skin) and blood leukocytes (Blood). Specimens were collected from moderately (MR) and highly (HR) tick-resistant Group 4 calves during the second experimental tick infestation of a phenotyping trial. Fold changes are relative to the average of the biological replicates (n=3) for each phenotype at day 0 and error bars indicate standard error of the mean.

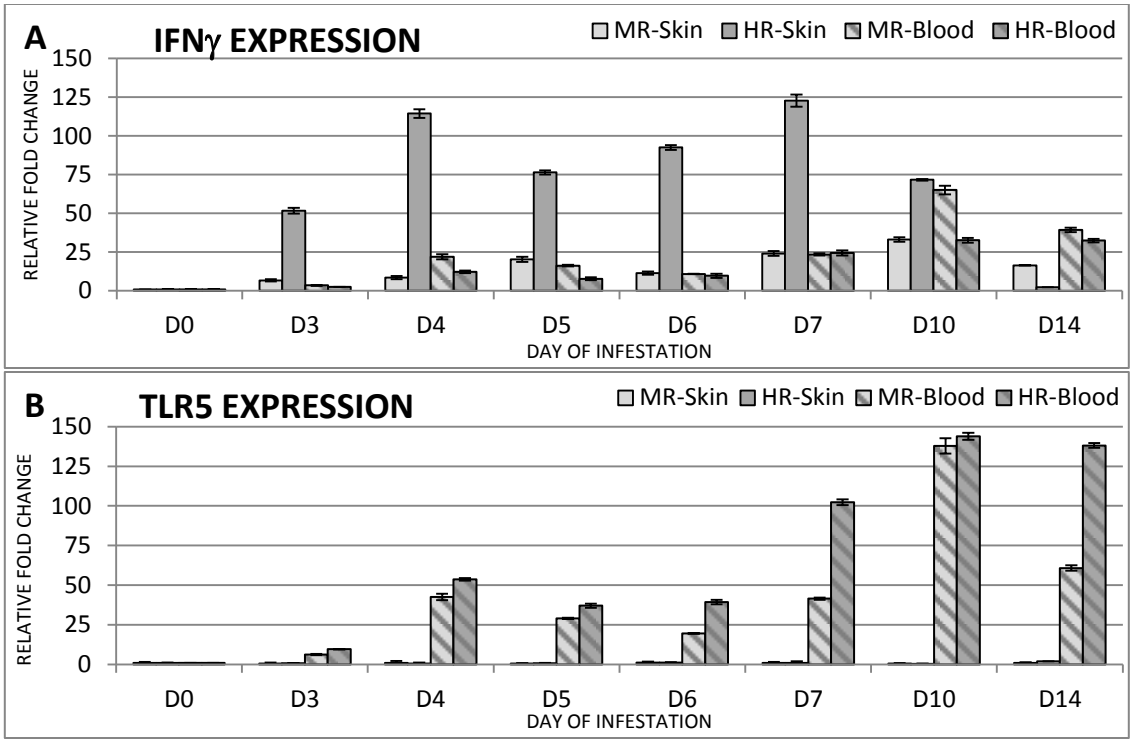

Figure 3.4 Relative gene expression by qRT-PCR for (A) *IFNγ* and (B) *TLR5* in bovine tick bite-site biopsies (Skin) and blood leukocytes (Blood). Specimens were collected from moderately (MR) and highly (HR) tick-resistant Group 4 calves during the second experimental tick infestation of a phenotyping trial. Fold changes are relative to the average of the biological replicates (n=3) for each phenotype at day 0 and error bars indicate standard error of the mean.

No notable differences in gene expression were found in tick bite-site biopsies or blood leukocytes for *IL18* or *MHCII-DRB3* (data not shown).

Similarly, expression for *IL6*, *IL8*, *IL1RN*, and *IGHG1* in tick bite-site biopsies from HR calves was notably higher compared to those from MR cohorts (Figure 3.5). These increases ranged 100- to 8,000-fold and were generally found on days 3, 4, 5, 6, 7, 10 and 14 of the tick infestation, with the exception that *IGHG1*expression was notably higher in biopsies from MR calves on days 10 and 14 compared to those from HR cohorts (Figure 3.5D).

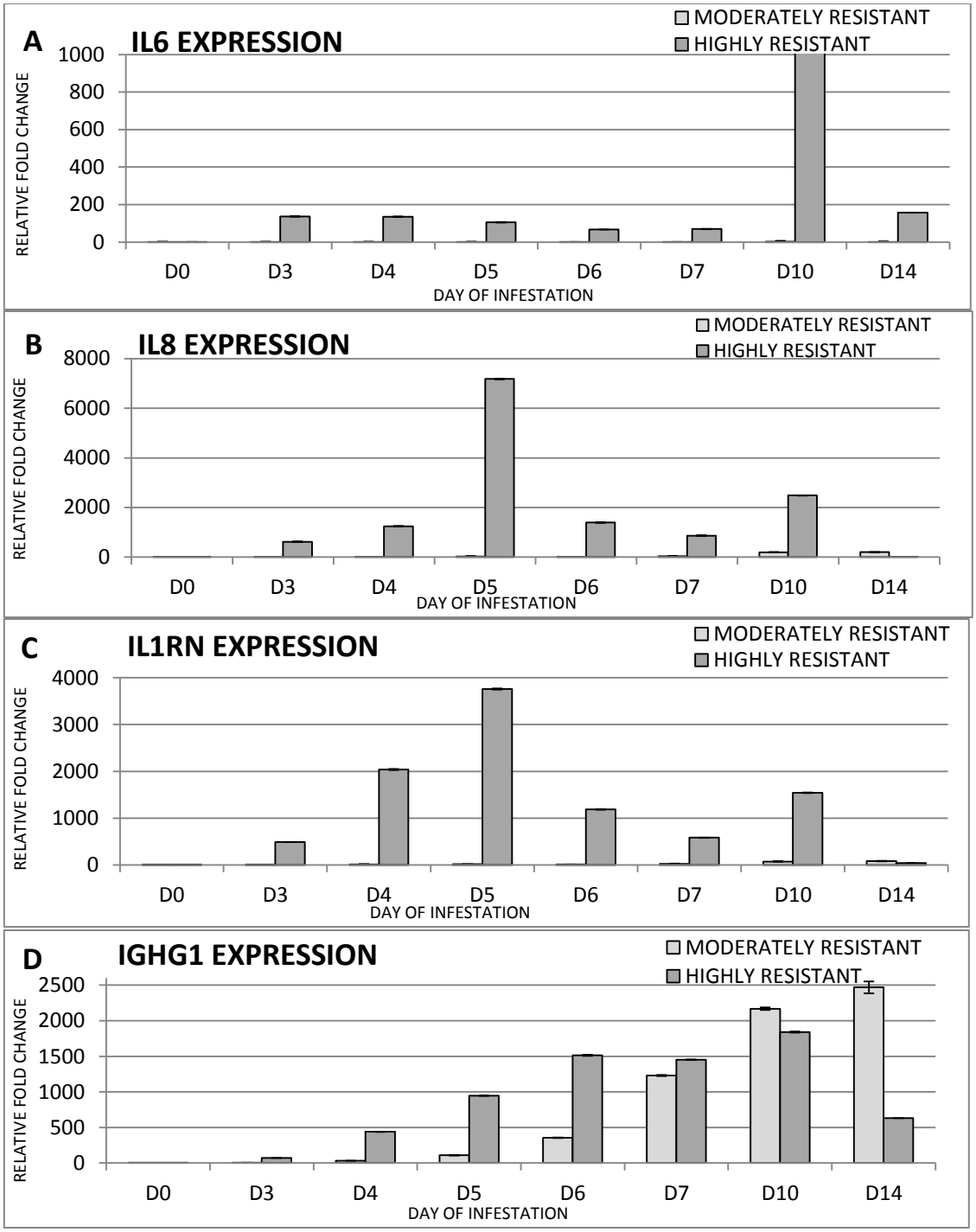

Figure 3.5 Relative gene expression by qRT-PCR for (A) *IL6*, (B) *IL8*, (C) *IL1RN*, and (D) *IGHG1* in tick bite-site biopsies. Specimens were collected from moderately (MR) and highly (HR) tick-resistant Group 4 calves during the second experimental tick infestation of a phenotyping trial. Fold changes are relative to the average of the biological replicates (n=3) for each phenotype at day 0 and error bars indicate standard error of the mean.

The largest gene expression differences observed in tick bite-site biopsies between HR and MR cattle were an 8,000-fold increase on day 5 for *IL8*, a 4,000-fold increase on day 5 for *IL1RN*, a 1,000-fold increase on day 6 for *IGHG1*, and a 1,000-fold increase on day 10 for *IL6* (Figure 3.5A-D). However, *IGHG1* expression increased 2,000-fold in biopsies from MR calves on day 14 compared to those from HR cohorts (Figure 3.5).

Gene expression in tick bite-site biopsies from HR calves was notably increased 5- to 15-fold for *IL2Rα*, *IL10*, *CCL2*, and *IL1α* on days 3, 4, and 6 compared to biopsies from MR cohorts (Figure 3.6). These differences were greatest on day 4 for *IL2Rα* and *IL10*, on day 3 for *CCL2*, and occurred only on day 5 only for *IL1α* (Figure 3.6-D).

No notable differences in expression between tick bite-site biopsies from MR and HR cattle were identified on any day of the tick infestation for *TNFα*, *NOD2, Casp1*, *IL18, IL1β*, *IL1R2*, *IL1RAP*, *CCR1, CCL26, CASP8, TRADD, TRAF3, TRAF6, C1Q, IL23, HFLB5, TLR2*, *TLR9*, *IL12p40*, and *NFkB* (data not shown).

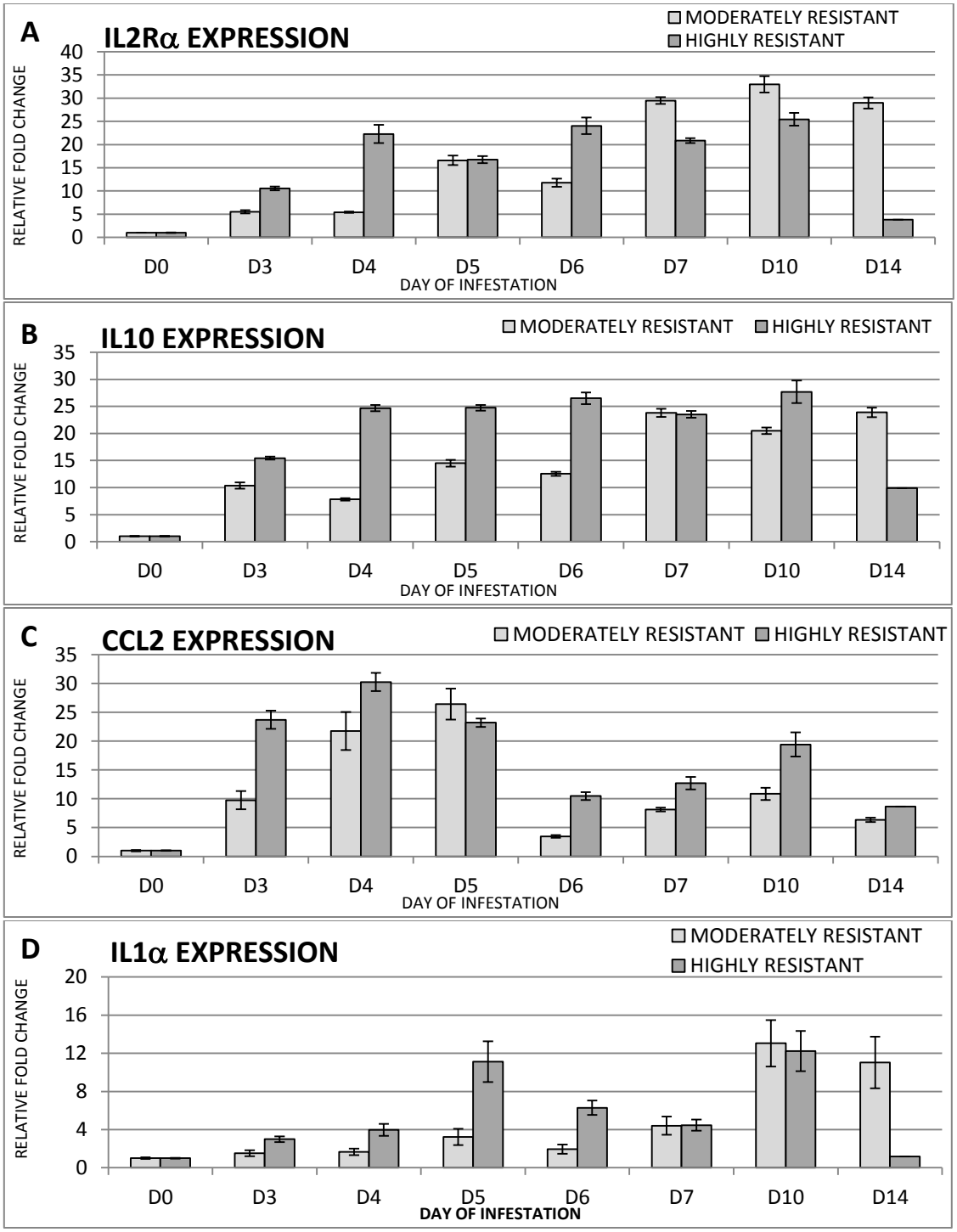

Figure 3.6 Relative gene expression by qRT-PCR for (A) *IL2Rα*, (B) *IL10*, (C) *CCL2*, and (D) *IL1α* in bovine tick bite-site biopsies. Specimens were collected from moderately (MR) and highly (HR) tick-resistant Group 4 calves during the second experimental tick infestation of a phenotyping trial. Fold changes are relative to the average of the biological replicates (n=3) for each phenotype at day 0 and error bars indicate standard error of the mean.

3.2.2 Whole group expression profiles in tick bite-site biopsies and blood leukocytes

Significantly increased expression ($p \leq 0.05$) was observed in tick bite-site biopsies on all days measured with a peak on day 10 of the tick infestation for a total of 13 genes including *MHCII-DRB3*, *IL1RAP*, *NLRP3*, *TRADD*, *IL1α*, *IL2Rα*, *IL6*, *IL8*, *IL10*, *IL12p40*, *TRAF3*, *Casp8*, and *IGHG* (Figures 3.7-3.11). Similar patterns of gene expression were observed in blood leukocytes for two genes, *TLR2* and *TLR5* (Figure 3.7)

In tick bite-site biopsies, the highest increase in gene expression for *MHCII-DRB3* and *NLRP3* occurred on day 10 and ranged 10- to 20-fold (Figure 3.7A-B). In contrast, significantly increases were observed on day 5 and day 7 for *TLR2* expression in these biopsies (Figure 3.7A-C). Gene expression for *TLR5* did not change significantly in these biopsies on any day of the tick infestation (Figure 3.7D). However, expression in blood leukocytes was significantly increased on day 10 by 2-fold for *TLR2*, and 125-fold for *TLR5* (Figure 3.7C-D). No changes were observed for expression of *MHCII-DRB3* and *NLRP3* in blood leukocytes on any day of the tick infestation (Figure 3.7A-B).

Significantly increased gene expression in tick bite-site biopsies on day 10 ranged from 4- to 12-fold for *IL1α* and *IL1RAP*, and from 160- to 300-fold for *IL6* and *IL8* (Figure 3.8A-D). Significantly increased expression was also observed in these biopsies on day 5 with smaller fold changes compared to day 10, except for the 300-fold rise for *IL8* (Figure 3.8A-D).

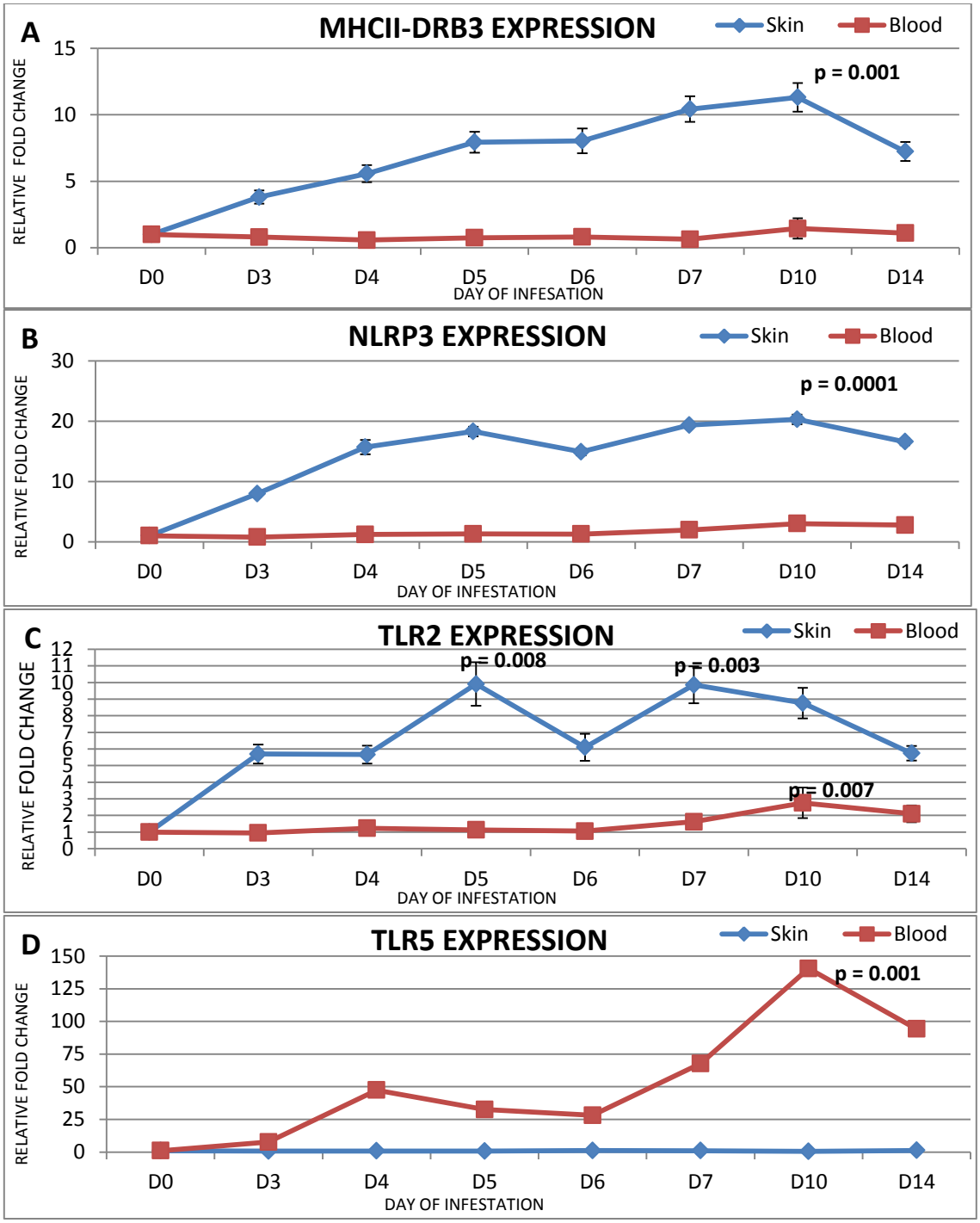

Figure 3.7 Relative gene expression by qRT-PCR for (A) *MHCII-DRB3*, (B) *NLRP3*, (C) *TLR2*, and (D) *TLR5* in bovine tick bite-site biopsies (skin) and blood leukocytes (blood). Specimens were collected from Group 4 calves (n=6) during the second experimental tick infestation of a phenotyping trial. Fold changes are relative to the average of biological replicates at day 0 and error bars indicate standard error of the mean. Significant differences with the highest fold change are indicated with the respective *P*-value above the corresponding data point.

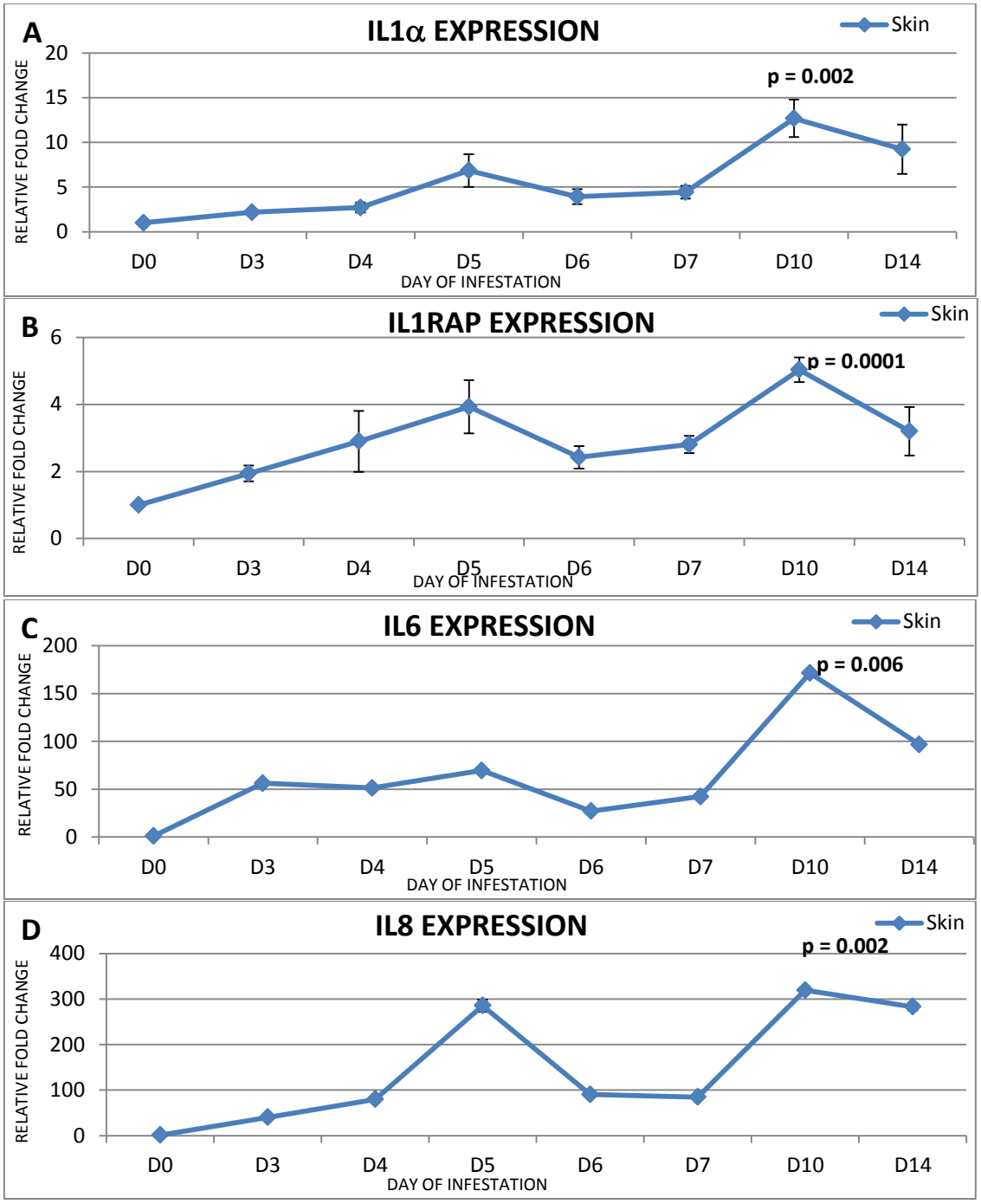

Figure 3.8 Relative gene expression by qRT-PCR for (A) *IL1α,* (B) *IL1RAP*, (C) *IL6*, and (D) *IL8* in bovine tick bite-site biopsies. Specimens were collected from Group 4 calves (n=6) during the second experimental tick infestation of a phenotyping trial. Fold changes are relative to the average of biological replicates at day 0 and error bars indicate standard error of the mean. Significant differences with the highest fold change are indicated with the respective *P*-value above the corresponding data point.

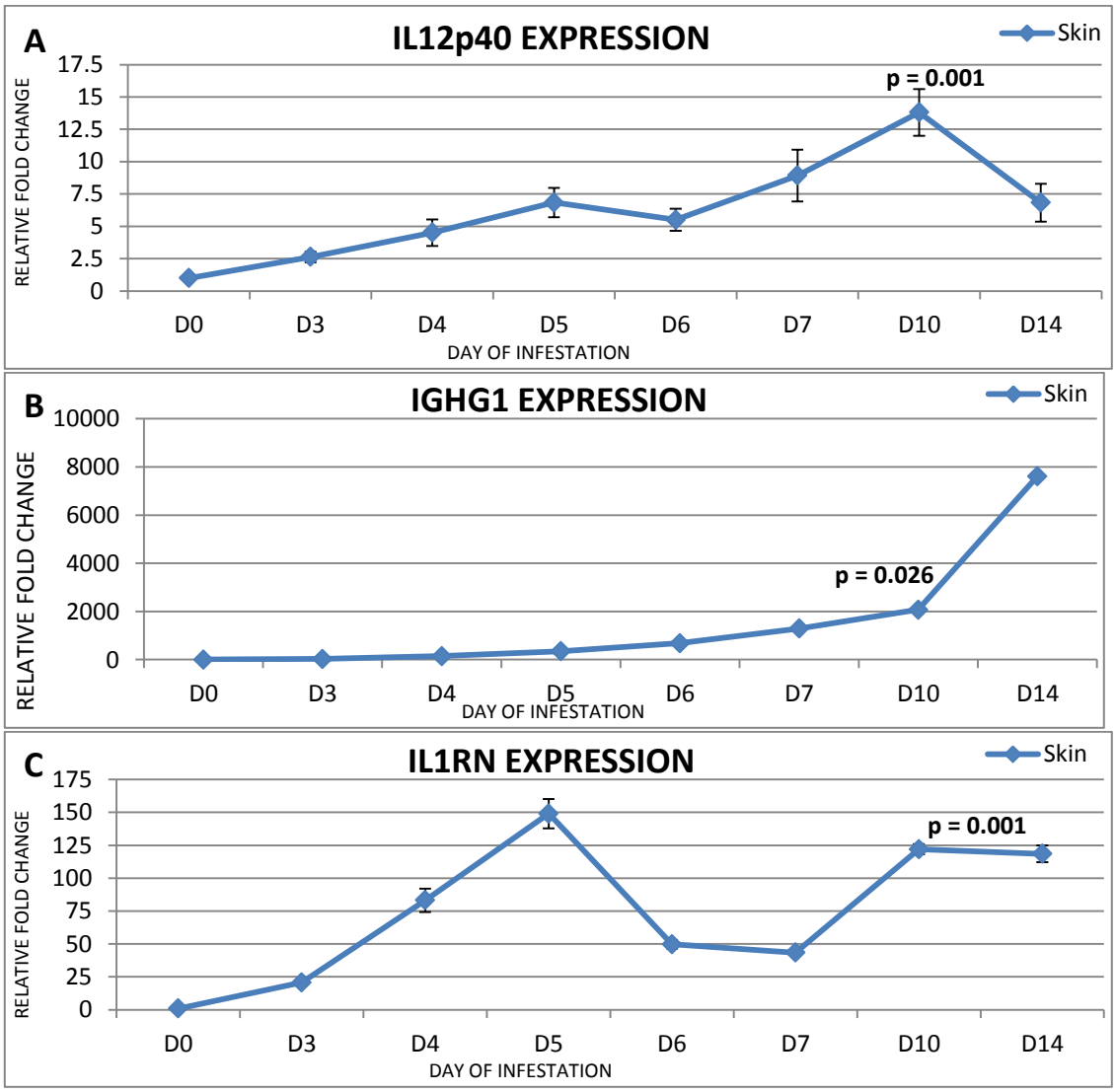

Figure 3.9 Relative gene expression by qRT-PCR for (A) *IL12p40*, (B) *IGHG1*, and (C) *IL1RN* in bovine tick bite-site biopsies. Specimens were collected from Group 4 calves (n=6) during the second experimental tick infestation of a phenotyping trial. Fold changes are relative to the average of biological replicates at day 0 and error bars indicate standard error of the mean. Significant differences with the highest fold change are indicated with the respective *P*-value above the corresponding data point.

Significantly increased gene expression for *IL12p40*, *IGHG1*, and *IL1RN* in tick bite-site biopsies was observed on day 10 of the tick infestation (Figure 3.9A-C). Expression of *IL12p40* in these biopsies was significantly increased on all days measured and reached a 13-fold peak on day 10 (Figure 3.9A). Due to the high variability in gene expression in these biopsies, indicated by large standard deviations (data not shown), the 8,000-fold peak on day 14 for *IGHG1* was not significantly up-regulated. However, the 2,000-fold increase on day10 of the tick infestation was significantly increased (Figure 3.9B). Similarly, *ILIRN* expression in these biopsies was not significantly up-regulated at the highest observed peak on day 5, but was significantly increased by 12-fold on day 10 (Figure 3.9C).

In tick bite-site biopsies, expression rose significantly each day of the tick infestation and peaked on day 10 for five genes, including *IL2Rα*, *IL10*, *TRAF3*, *Casp8*, and *TRADD* (Figures 3.10 and 3.11). Significantly increased peaks on day 10 ranged 20- to 30-fold for *IL2Rα* and *IL10* in these biopsies (Figure 3.10). Although expression in these biopsies for *TRAF3*, *Casp8*, and *TRADD* was low overall, significant peaks on day 10 ranged 1- to 5-fold (Figure 3.11A-C).

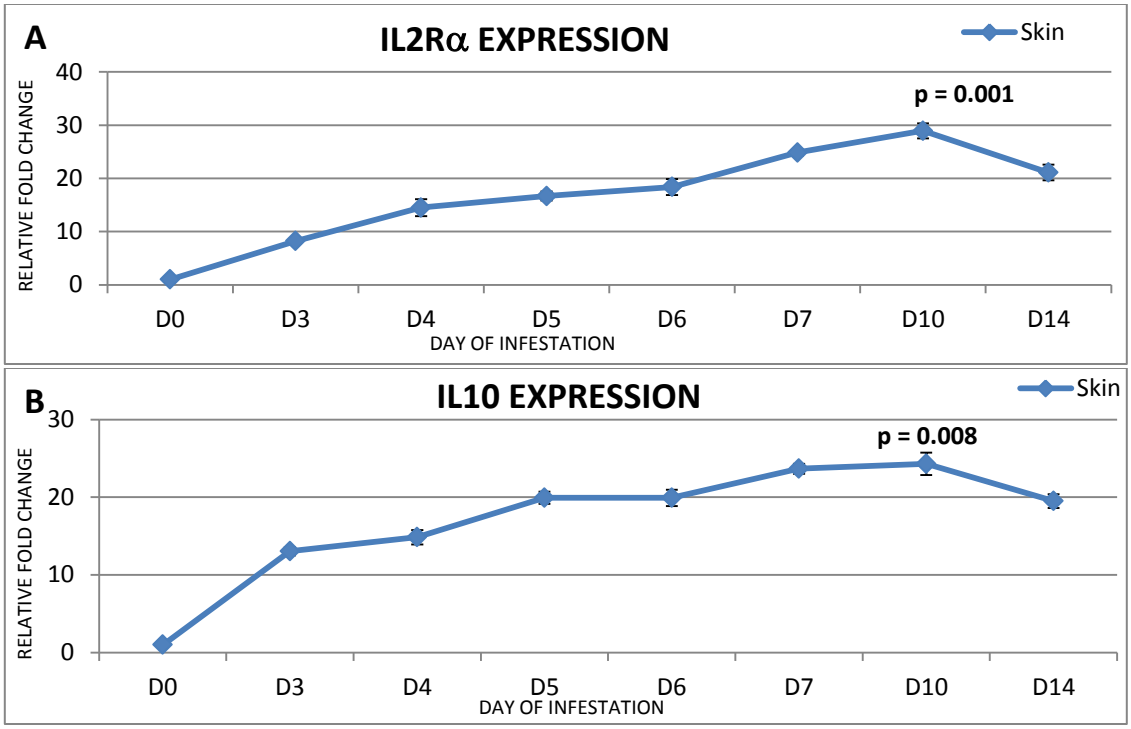

Figure 3.10 Relative gene expression by qRT-PCR for (A) *IL2Rα* and (B) *IL10* in bovine tick bite-site biopsies. Specimens were collected from Group 4 calves (n=6) during the second experimental tick infestation of a phenotyping trial. Fold changes are relative to the average of biological replicates at day 0 and error bars indicate standard error of the mean. Significant differences with the highest fold change are indicated with the respective *P*-value above the corresponding data point.

Significantly increased expression in tick bite-site biopsies peaked on day 5 for four genes, including *TNFα*, *MyD88*, *CCR1*, and *CCL2* (Figure 3.12A-D). Significantly increased expression for *TNFα* in these biopsies rose by 2-fold on day 5 only, whereas expression of *MyD88* was significantly increased on all days measured (Figure 3.12A-B). Significantly increased expression of *CCR1* and *CCL2* in these biopsies peaked on day 5 and ranged from 25- to 70-fold (Figure 3.12C-D).

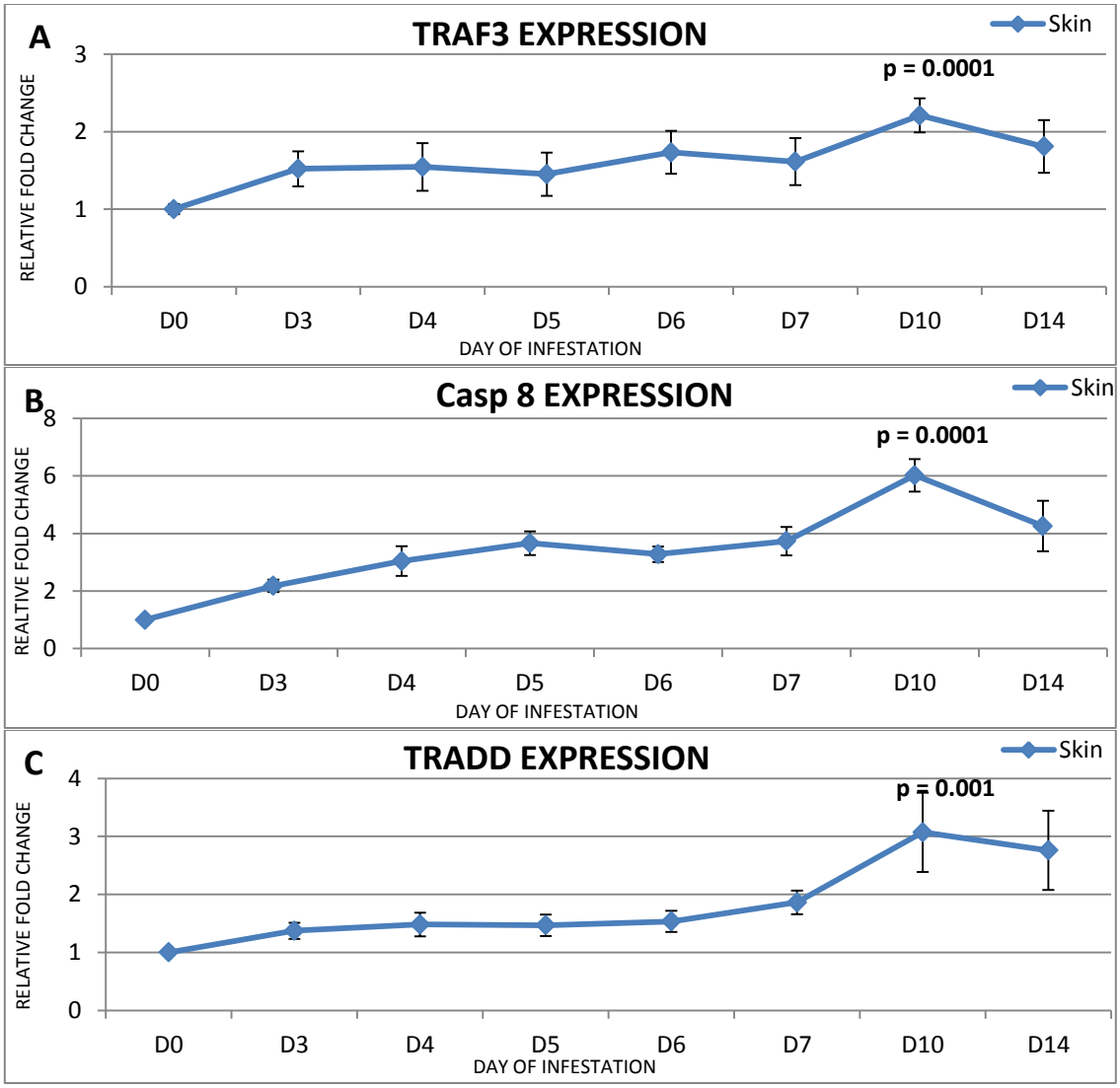

Figure 3.11 Relative gene expression by qRT-PCR for (A) *TRAF3*, (B) *Casp8*, and (C) *TRADD* in bovine tick bite-site biopsies. Specimens were collected from Group 4 calves (n=6) during the second experimental tick infestation of a phenotyping trial. Fold changes are relative to the average of biological replicates at day 0 and error bars indicate standard error of the mean. Significant differences with the highest fold change are indicated with the respective *P*-value above the corresponding data point.

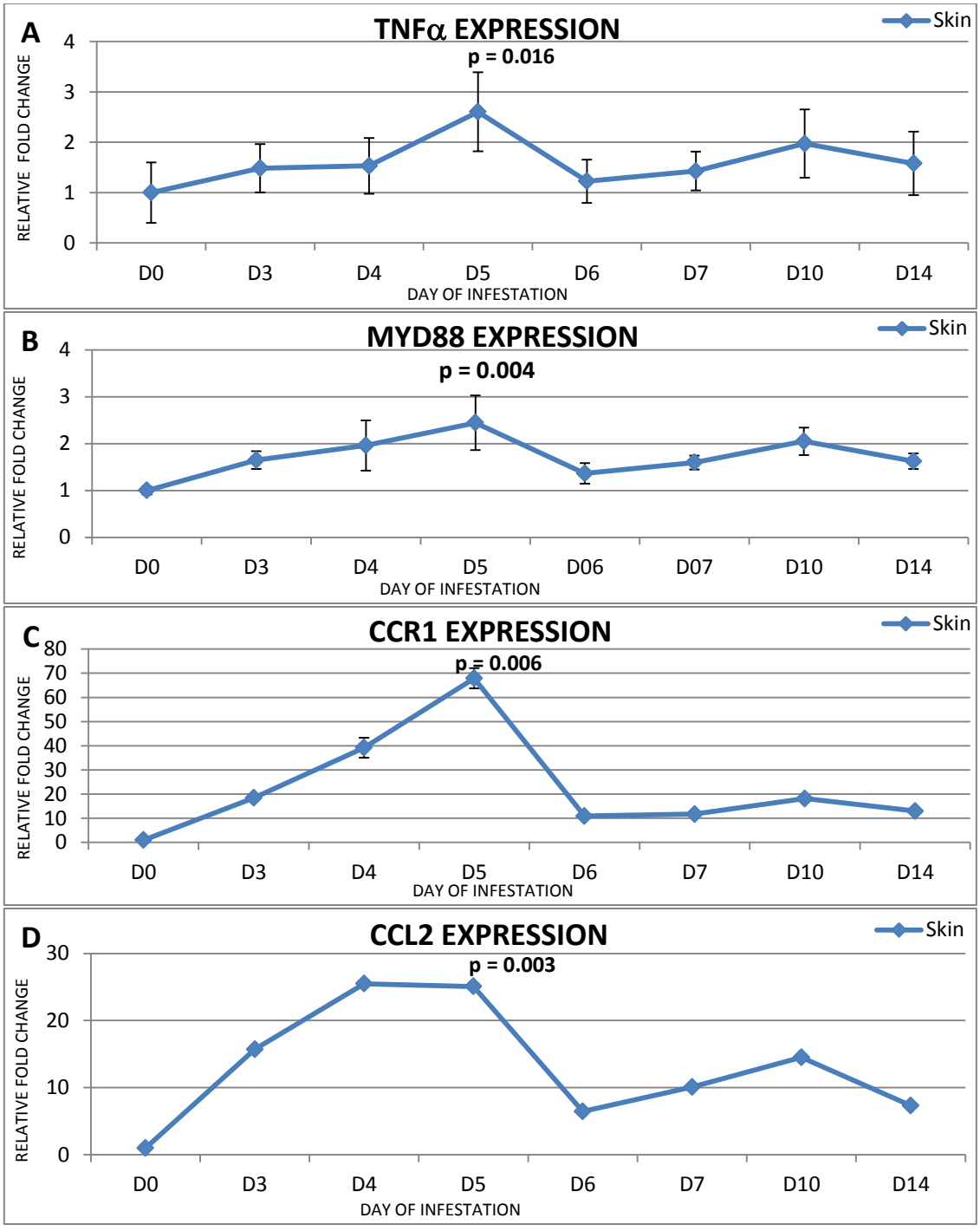

Figure 3.12 Relative gene expression by qRT-PCR for (A) *TNFα,* (B) *MyD88*, (C) *CCR1*, and (D) *CCL2* in bovine tick bite-site biopsies. Specimens were collected from Group 4 calves (n=6) during the second experimental tick infestation of a phenotyping trial. Fold changes are relative to the average of biological replicates at day 0 and error bars indicate standard error of the mean. Significant differences with the highest fold change are indicated with the respective *P*-value above the corresponding data point.

Significantly up-regulated expression for *TLR4* and *CD14* in tick bite-site biopsies ranged 10- to 30-fold on days 5 and 10 (Figure 3.13A-B). No significant changes were observed in blood leukocytes for *TLR4* and *CD14* (Figure 3.13A-B).

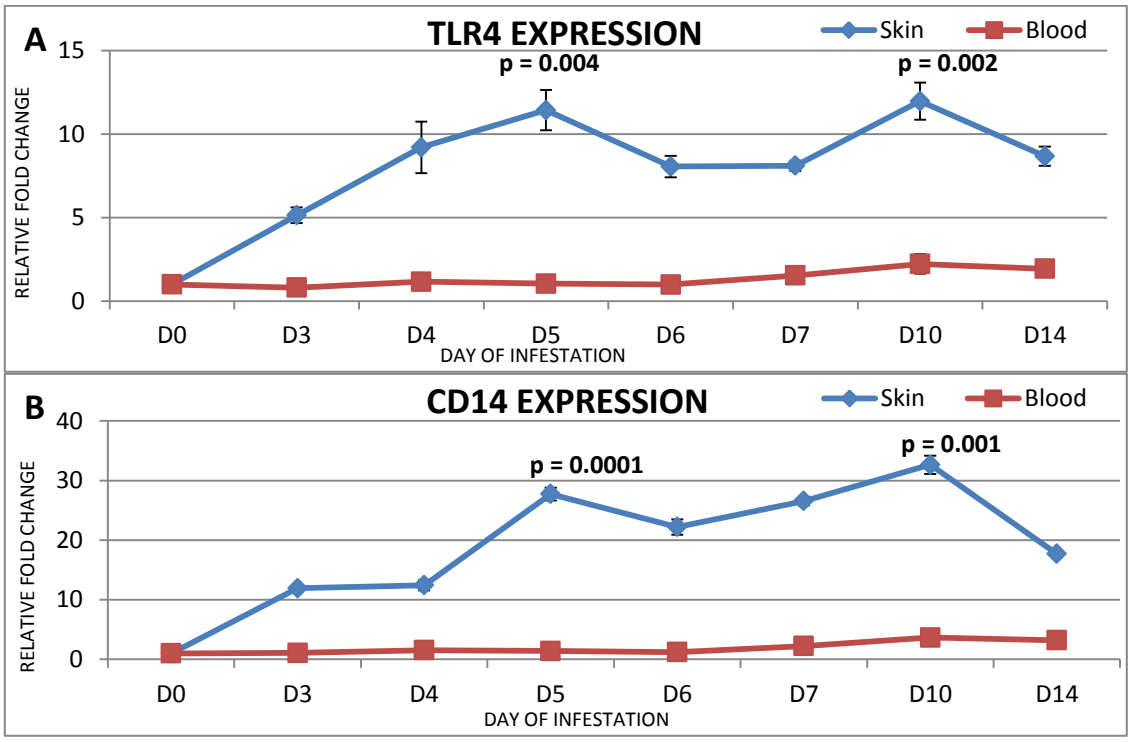

Figure 3.13 Relative gene expression by qRT-PCR for (A) *TLR4* and (B) *CD14* in bovine tick bite-sites (Skin) and blood leukocytes (Blood). Specimens were collected from Group 4 calves (n=6) during the second experimental tick infestation of a phenotyping trial. Fold changes are relative to the average of biological replicates at day 0 and error bars indicate standard error of the mean. Significant differences with the highest fold change are indicated with the respective *P*-value above the corresponding data point.

Expression of four genes, *IFNγ*, *IL18*, *C1Q*, and *TLR9*, in tick bite-site biopsies was significantly increased on all days measured and peaked on day 7 (Figures 3.14 and 3.15). Expression of *IFNγ* in these skin biopsies was significantly up-regulated by 50-fold on day 7, whereas expression of this gene in blood leukocytes was significantly up-regulated by 45-fold on day 10 (Figure 3.14A). Significantly increased expression of *IL18* in tick bite-site biopsies peaked at 3-fold on day 7, whereas no significant changes were found in blood leukocytes for this gene (Figure 3.14B).

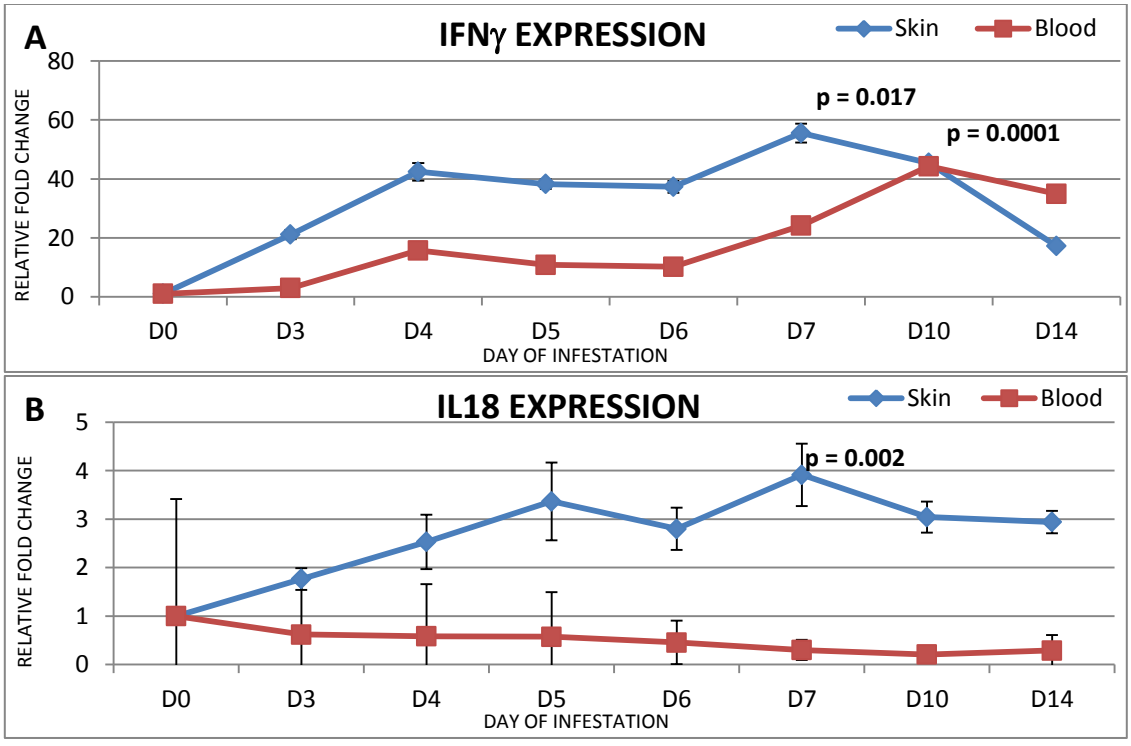

Figure 3.14 Relative gene expression by qRT-PCR for (A) *IFNγ* and (B) *IL18* in bovine tick bite-site biopsies (Skin) and blood leukocytes (Blood). Specimens were collected from Group 4 calves (n=6) during the second experimental tick infestation of a phenotyping trial. Fold changes are relative to the average of biological replicates at day 0 and error bars indicate standard error of the mean. Significant differences with the highest fold change are indicated with the respective *P*-value above the corresponding data point.

Gene expression for *C1Q* and *TLR9* in tick bite-site biopsies was significantly increased on all days measured and peaked on day 7 by 13-fold and 8-fold, respectively (Figure 3.15-B).

In tick bite-site biopsies, no significant differences in expression were identified on any day measured for a total of nine genes, including *TRAF6*, *NOD2*, *CCL26*, *CASP1*, *IL1β*, *IL1R2*, *IL23*, *NFκB*, and *HFLB5* (data not shown).

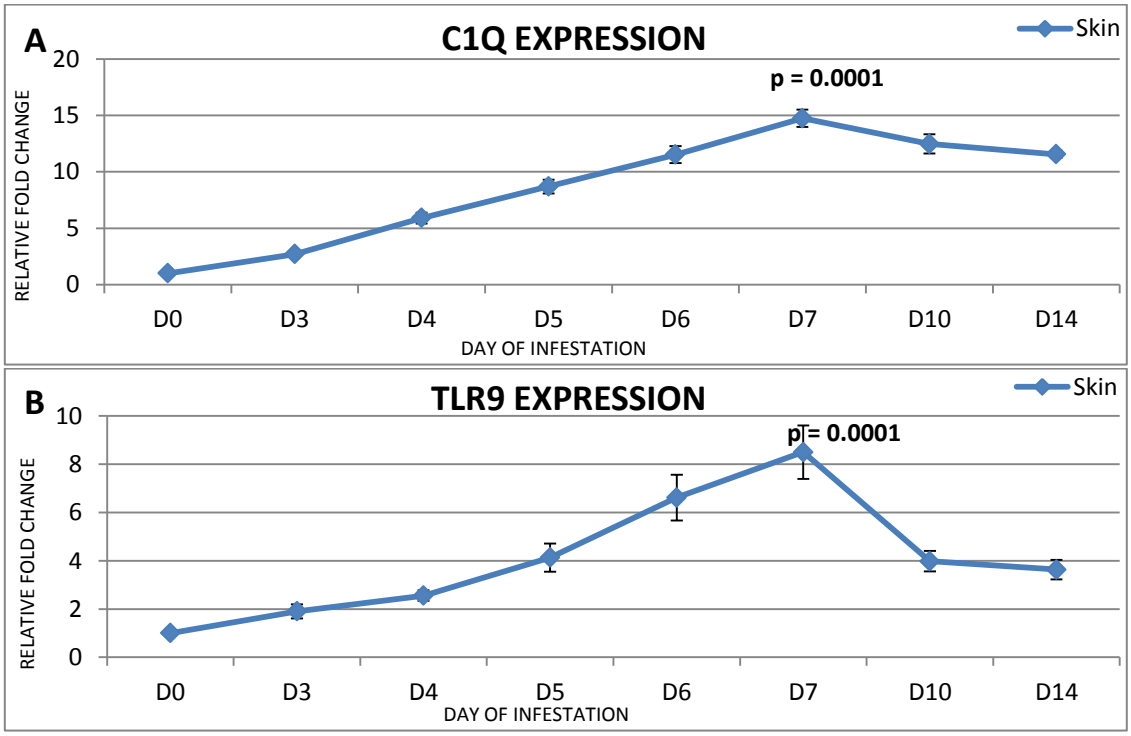

Figure 3.15 Relative gene expression by qRT-PCR for (A) *C1Q* and (B) *TLR9* in bovine tick bite-site biopsies. Specimens were collected from Group 4 calves (n=6) during the second experimental tick infestation of a phenotyping trial. Fold changes are relative to the average of biological replicates at day 0 and error bars indicate standard error of the mean. Significant differences with the highest fold change are indicated with the respective *P*-value above the corresponding data point.

3.3 Differential Gene Expression by Phenotype in Group 5 Calves

Total RNA from tick bite-site biopsies collected from Group 5 calves on days 0, 5, and 10 of the first and second tick infestations (Section 2.1.5) was reverse transcribed for comparison by microarray experiments (Section 2.6), as previously described. A Cy3 dye bias that likely resulted from the high temperatures and ozone in Texas led to an abnormal amount of green background in the image files. This dye bias may have interfered with the background subtraction method used to determine intensity ratios for each feature, and may have affected the data obtained.

3.3.1 Differential gene expression in tick bite-site biopsies from highly tick-resistant (HR) calves

Microarrays compared gene expression in tick bite-site biopsies from two highly tick-resistant (HR) calves. Analyses of microarray experiments revealed a total of 55 genes (21 up-regulated and 34 down-regulated) differentially expressed between days 0 and 5. Gene ontology analysis of up-regulated genes showed factors associated with the defense response, antimicrobial activity, metal ion binding, glycoproteins, and cell membranes, whereas down-regulated genes were associated with ATP binding, cell projection, and the regulation of peptidase activity (DAVID Bioinformatics Software). Further analysis using the Kyoto encyclopedia of genes and genomes (KEGG) identified pathways, and those associated with up-regulated genes included complement, coagulation, endocytosis, calcium signaling, and cytokine-cytokine signaling. In

contrast, KEGG pathways identified from analysis of down-regulated genes included Fc-γ-mediated phagocytosis, oxidative phosphorylation, and apoptosis (DAVID Bioinformatics Software).

A total of 125 differentially expressed genes (49 up-regulated and 76 down-regulated) were identified in biopsies from HR cattle between days 5 and 10, as determined by microarray analysis. Gene ontology analysis of up-regulated genes found factors involved in the oxidative stress response and antioxidant activity, whereas factors associated with receptor activity and RNA modification were identified from analysis of down-regulated genes (DAVID Bioinformatics Software). KEGG pathways associated with the up-regulated genes included natural killer cell cytotoxicity, and apoptosis, while KEGG pathways such as endocytosis, focal adhesion, complement, and coagulation were associated with down-regulated genes (DAVID Bioinformatics Software).

A total of 84 differentially expressed genes (26 up-regulated and 58 down-regulated) were identified between days 0 and 10 in biopsies from HR calves, as determined by microarray analysis of tick bite-site biopsies from HR calves. Gene ontology analysis of up-regulated genes did not identify any associated biological processes. However, analysis of down-regulated genes was similar to the findings reported between days 5 and 10, and revealed factors associated with receptor signaling and RNA modification. KEGG pathways associated with up-regulated genes included natural killer cell cytotoxicity and cytokine-cytokine signaling, whereas focal adhesion, complement, and coagulation were associated with down-regulated genes.

Table 3.4 Genes associated with immune responses differentially expressed in tick bite-site biopsies from highly tick-resistant Group 5 calves as determined by microarray analysis.

Gene name	Gene symbol	Accession number	Associated immune function	Gene regulation		
				D0-D5[a]	D5-D10[b]	D0-D10[c]
Complement component C1Q	C1QB	NM_001046599.1	complement cascade	up	down	-
Complement factor H	CFH	XR_028692.1	complement cascade	-	down	down
Tumor necrosis factor receptor superfamily 1B	TNFRSF1B	NM_001040490.1	defense response	up	down	-
Lactoferrin	LTF	NM_180998	defense response	up	down	-
S100 calcium binding protein A8	S100A8	XM_868934.2	antimicrobial peptide	up	down	-
Lactoperoxidase	LPO	NM_173933.2	response to ROS	-	up	up
Immunoglobulin heavy chain gamma 1	IGHG1	BC146168.1	B cell response	up	down	-
C-X-C motif chemokine ligand 13	CXCL13	NM_001015576.2	B lymphocyte chemoattractant	-	up	down
Cluster of differentiation 96	CD96	NM_001035072.1	T and NK cell adhesion	down	up	-
Cluster of differentiation 244	CD244	NM_001192350.1	NKC mediated cytotoxicity	-	up	up
Interleukin 1 receptor, type II	IL1R2	NM_001046210.1	IL1 binding	-	down	down
Actin related protein 2/3 complex, subunit 5-like	ARPC5L	NM_001034737.1	Fc-γ-R mediated phagocytosis	down	-	-

[a] Gene regulation was up-regulated (up) or down-regulated (down) on day 5 compared to day 0.
[b] Gene regulation was up-regulated (up) or down-regulated (down) on day 10 compared to day 5.
[c] Gene regulation was up-regulated (up) or down-regulated (down) on day 10 compared to day 0.

Table 3.4 shows 12 differentially expressed genes associated with immune function in tick bite-site biopsies from highly tick-resistant Group 5 calves, as determined by analyses of microarray experiments.

3.3.2 D*ifferential gene expression in tick bite-site biopsies from moderately tick-resistant (MR) calves*

Gene expression in tick bite-site biopsies from three moderately tick-resistant (MR) calves was compared by microarrays. A total of 418 (244 up-regulated and 174 down-regulated) differentially expressed genes were identified between days 0 and 5. Gene ontology analysis identified factors associated with up-regulated genes including acetylation, focal adhesion, apoptosis, immune effector regulation, response to wounding, T cell activation, anti-apoptosis, Ig-mediated response, B cell immunity, antioxidant activity, and IL1 receptor signaling (DAVID Bioinformatics Software). Similarly identified factors represented by down-regulated genes included amino acid phosphorylation, transcriptional regulation, and secreted proteins. KEGG pathways associated with up-regulated genes included natural killer cell cytotoxicity, F-c-γ-mediated phagocytosis, B cell signaling, and Nod-like receptor activity, however, the only KEGG pathway associated with down-regulated genes was Toll-like receptor signaling (DAVID Bioinformatics Software). KEGG pathways shared by different up- and down-regulated genes included endocytosis, cytokine-cytokine signaling, transforming growth factor beta (TGF-β) activation, calcium binding, complement, and coagulation (DAVID Bioinformatics Software).

A total of 234 (58 up-regulated and 176 down-regulated) genes were differentially expressed in biopsies from MR calves between days 5 and 10, as determined by the microarray experiments. Functional analysis of up-regulated genes identified factors associated with glycoproteins, cell membrane proteins, and signaling components, while mediators of phosphoproteins, cell signaling, acetylation, focal adhesion, blood vessel development, defense response, complement cascade, response to bacterium, anti-apoptosis, and acute inflammation were associated with down-regulated genes (DAVID Bioinformatics Software). KEGG pathways associated with up-regulated genes included endocytosis and Toll-like receptor signaling, whereas natural killer cell cytotoxicity, Fc-γ-mediated phagocytosis, and Nod-like receptor signaling were associated with down-regulated genes (DAVID Bioinformatics Software). Complement and cell adhesion pathways were commonly represented by different up- and down-regulated genes.

A total of 261 (134 up-regulated and 127 down-regulated) genes were differentially expressed in biopsies from MR cattle between days 0 and 10, as determined by the previously described microarray experiments. Gene ontology analysis of up-regulated genes revealed associations with cell signaling, acetylation, calcium binding, focal adhesion, T cell activation, and complement control, whereas analysis of down-regulated genes identified factors associated with the activation of amino acid phosphorylation, ATP binding, and nucleotide binding (DAVID Bioinformatics Software). KEGG pathways associated with up-regulated genes included Fc-γ-mediated

Table 3.5 Genes associated with immune responses differentially expressed in tick bite-site biopsies from moderately tick-resistant Group 5 calves as determined by microarray analysis.

Gene name	Gene symbol	Accession number	Associated immune function	Gene regulation		
				D0-D5	D5-D10	D0-D10
Immunoglobulin heavy chain gamma 1	IGHG1	BC146168.1	B cell response	up	down	down
Peptidoglycan recognition protein	PGLYRP1	NM_174573.2	Toll-like receptor signaling	up	down	up
C-X-C motif chemokine ligand 5	CXCL5	NM_174300	IL1/TNF signaling	up	down	-
Interleukin 1 receptor accessory protein	IL1RAP	NM_174300	IL1/TNF signaling	up	down	-
Alpha-2-macroglobulin	A2M	XM_591612.3	IL1/TNF signaling	up	down	-
Heat shock protein 90 beta member 1	HSP90B1	NM_174700.2	Stress response	up	down	-
Lactoferrin	LTF	NM_180998.2	defense response	up	down	-
Interleukin 1 receptor antagonist	IL1RN	NM_174357.2	IL1/TNF signaling	-	down	-
Fc epsilon RI	FCER1G	NM_174537.2	B cell response	up	down	-
Cluster of differentiation 276	CD276	XM_864408.2	B cell response	up	down	up
Heat shock protein 1	HSPD1	XM_001249790.1	B cell response	up	-	up
nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	NFKBIA	NM_001045868.1	B cell response	up	down	up
Complement component 4 binding protein B	C4BPB	NM_174253	Complement cascade	down	up	-
Heat shock protein binding protein 1	HSPBP1	NM_001046131.1	Stress response	down	up	-
TIR domain-containing adapter molecule 2	TICAM2	NM_001046456.1	Toll-like receptor signaling	down	up	-

[a] Gene regulation was up-regulated (up) or down-regulated (down) on day 5 compared to day 0.
[b] Gene regulation was up-regulated (up) or down-regulated (down) on day 10 compared to day 5.
[c] Gene regulation was up-regulated (up) or down-regulated (down) on day 10 compared to day 0.

phagocytosis, complement, coagulation, natural killer cell cytotoxicity, and Nod-like signaling. In contrast, KEGG pathways such as B cell and Fc-ε-R1 signaling were represented by down-regulated genes (DAVID Bioinformatics Software). Commonly associated pathways, represented by different up- and down-regulated genes, included leukocyte transendothelial migration, endocytosis, and cell adhesion.

Table 3.5 shows 15 differentially regulated genes related to immune function in tick bite-site biopsies from moderately tick-resistant Group 5 calves, as determined by microarray analysis.

3.3.3 Differential gene expression in tick bite-site biopsies from tick-susceptible (S) calves

Gene expression in tick bite-site biopsies from two tick-susceptible (S) calves was compared by microarray analyses. A total of 136 differentially expressed genes (87 up-regulated and 49 down-regulated) were identified between days 0 and 5. Gene ontology analysis of up-regulated genes found mediators of transcriptional regulation and SH3 binding, while analysis of down-regulated genes revealed factors associated with focal adhesion, phosphoproteins, and calcium binding (DAVID Bioinformatics Software). KEGG pathways represented by up-regulated genes included oxidative phosphorylation, Toll-like receptor signaling, Nod-like receptor signaling, natural killer cell cytotoxicity, and signaling of both T and B lymphocytes. In contrast, KEGG pathways associated

with down-regulated genes included focal adhesion and calcium signaling (DAVID Bioinformatics Software).

Microarray analysis of biopsies from S calves identified 107 (47 up-regulated and 60 down-regulated) differentially expressed genes between day 5 and day 10. Gene ontology analysis of up-regulated genes identified factors involved in metal ion binding, phosphorylation, and nucleotide binding, whereas analysis of down-regulated genes revealed mediators of transcriptional regulation, IL-1 receptor signaling, and SH3 binding (DAVID Bioinformatics Software). KEGG pathways associated with up-regulated genes included ubiquitin-mediated proteolysis and endocytosis, whereas cell adhesion, Toll-like receptor signaling, oxidative phosphorylation, and leukocyte transendothelial migration were represented by down-regulated genes (DAVID Bioinformatics Software).

A total of 63 differentially expressed genes (50 up-regulated and 13 down-regulated) were identified between day 0 and day 10 in biopsies from S calves. Gene ontology analysis of up-regulated genes found factors involved in nucleotide binding, while analysis of down-regulated genes revealed mediators of focal adhesion and anti-apoptosis (DAVID Bioinformatics Software). KEGG pathways represented by up-regulated genes included TGF-β signaling, cell adhesion, and apoptosis, whereas focal adhesion, calcium binding, and leukocyte transendothelial migration were associated with down-regulated genes.

Table 3.6 Genes associated with immune responses differentially expressed in tick bite-site biopsies from tick-susceptible calves Group 5 calves as determined by microarray analysis.

Gene name	Gene symbol	Accession number	Gene function	Gene regulation		
				D0-D5	D5-D10	D0-D10
Toll-like receptor 5	TLR5	XM_594146.3	Toll-like receptor signaling	up	down	-
Cluster of differentiation 80	CD80	XM_617543.3	Toll-like receptor signaling	up	down	-
Signal transducer and activator of transcription 4	STAT4	NG_012852	Toll-like receptor signaling	up	down	-
Interleukin 1 alpha	IL1A	DR749242	IL1/TNF signaling	-	down	up

[a] Gene regulation was up-regulated (up) or down-regulated (down) on day 5 compared to day 0.
[b] Gene regulation was up-regulated (up) or down-regulated (down) on day 10 compared to day 5.
[c] Gene regulation was up-regulated (up) or down-regulated (down) on day 10 compared to day 0.

Four differentially expressed genes associated with immunity were identified by microarray analysis of tick bite-site biopsies from tick-susceptible Group 5 calves and are represented in Table 3.6.

3.4 qRT-PCR Follow-Up of Candidate Genes in Tick Bite-Site Biopsies and Blood Leukocytes from Group 5 Calves

3.4.1 Gene expression profiles by phenotype

In tick bite-site biopsies, notable differences (fold change $\geq$ 5) between the tick-resistant and susceptible (HR, MR, and S) cattle phenotypes were identified for *IGHG1* and *Il1β* (Figure 3.16). In general, expression of *IGHG1* in biopsies from the HR phenotype was highest on day 8 and day 10 for all three tick infestations and ranged 25- to 170-fold higher compared to biopsies from MR and S cohorts (Figure 3.16A). However, on day 5 of the primary tick infestation notably increased expression of *IGHG1* in biopsies from MR calves reached 40- and 80-fold more than samples from the HR and S phenotypes, respectively. No notable differences were observed in blood leukocytes among any of the three cattle phenotypes for expression of *IGHG1* (Figure 3.16).

Microarray analysis of tick bite-site biopsies from MR calves revealed that *IGHG1* expression was increased on day 5 and decreased on day 10 (Table 3.5), a pattern consistent with the expression profile generated by qRT-PCR for this gene (Figure 3.16A).

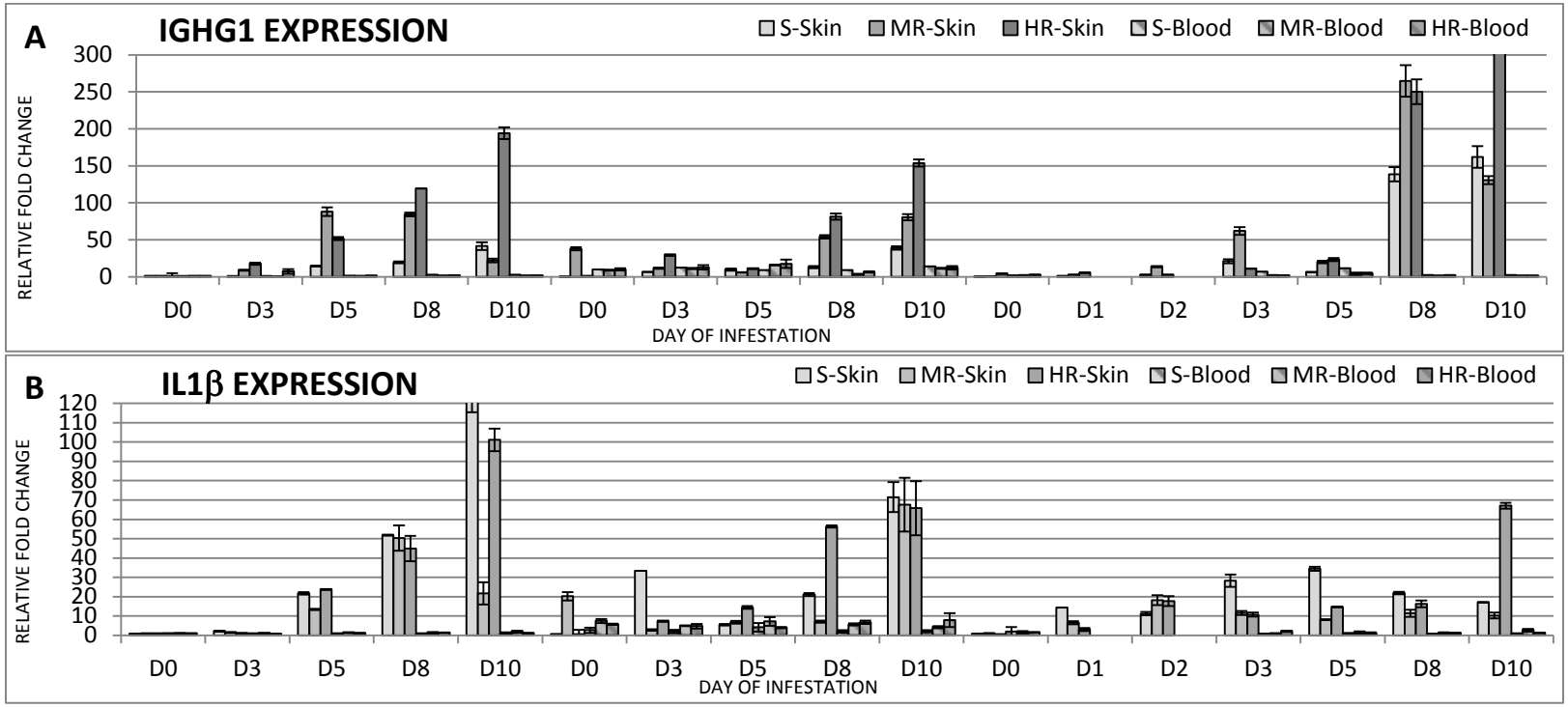

Figure 3.16 Relative gene expression by qRT-PCR for (A) *IGHG1* and (B) *IL1β* in bovine tick bite-site biopsies (Skin) and blood leukocytes (Blood). Specimens were collected from Group 5 tick-susceptible (n=2), moderately tick-resistant (n=3), and highly tick-resistant (n=2) calves during three experimental tick infestations. Fold changes are relative to the average of the biological replicates for each phenotype on day 0 of the first tick infestation and error bars indicate standard error of the mean.

In tick bite-site biopsies from S calves, notably increased expression of *Il1β* on day 10 of the first tick infestation and days 1, 3, and 5 of the third tick infestation ranged 2- to 100-fold higher compared to samples from MR and HR cohorts (Figure 3.16B). On the other hand, *Il1β* expression in biopsies from the HR phenotype was notably increased on day 8 of the second tick infestation and day 10 of the third tick infestation, ranging from 30- to 50-fold higher than samples from MR and S cohorts.

No notable differences in gene expression for *IL23* or *TLR4* were found in tick bite-site biopsies from HR, MR, or S cattle during any of the three tick infestations (Figure 3.17A-B). In contrast, expression of *IL23* was notably increased in blood leukocytes from HR cattle compared to those from S cattle, ranging 8- to 15-fold higher on days 3 and 5 of the second tick infestation (Figure 3.17A). In addition, expression of *IL23* in blood leukocytes of HR cattle was notably increased by 10-fold on day 10 of the second tick infestation, and days 5, 8, and 10 of the third tick infestation when compared to samples from MR and S cohorts. Expression of *TLR4* was notably increased in blood leukocytes from HR calves compared to those from MR and S phenotypes by 5- to 20-fold on days 3, 5, 8, and 10 during the second infestation, and by 5- to 10-fold on days 3 and 5 of the third tick infestation (Figure 3.17B).

Expression of *TLR5* in the tick bite-site biopsies from HR calves was notably higher compared to samples from MR and S cohorts, rising 10-fold on day 10 of the third tick infestation (Figure 3.17C). In blood leukocytes from MR calves,

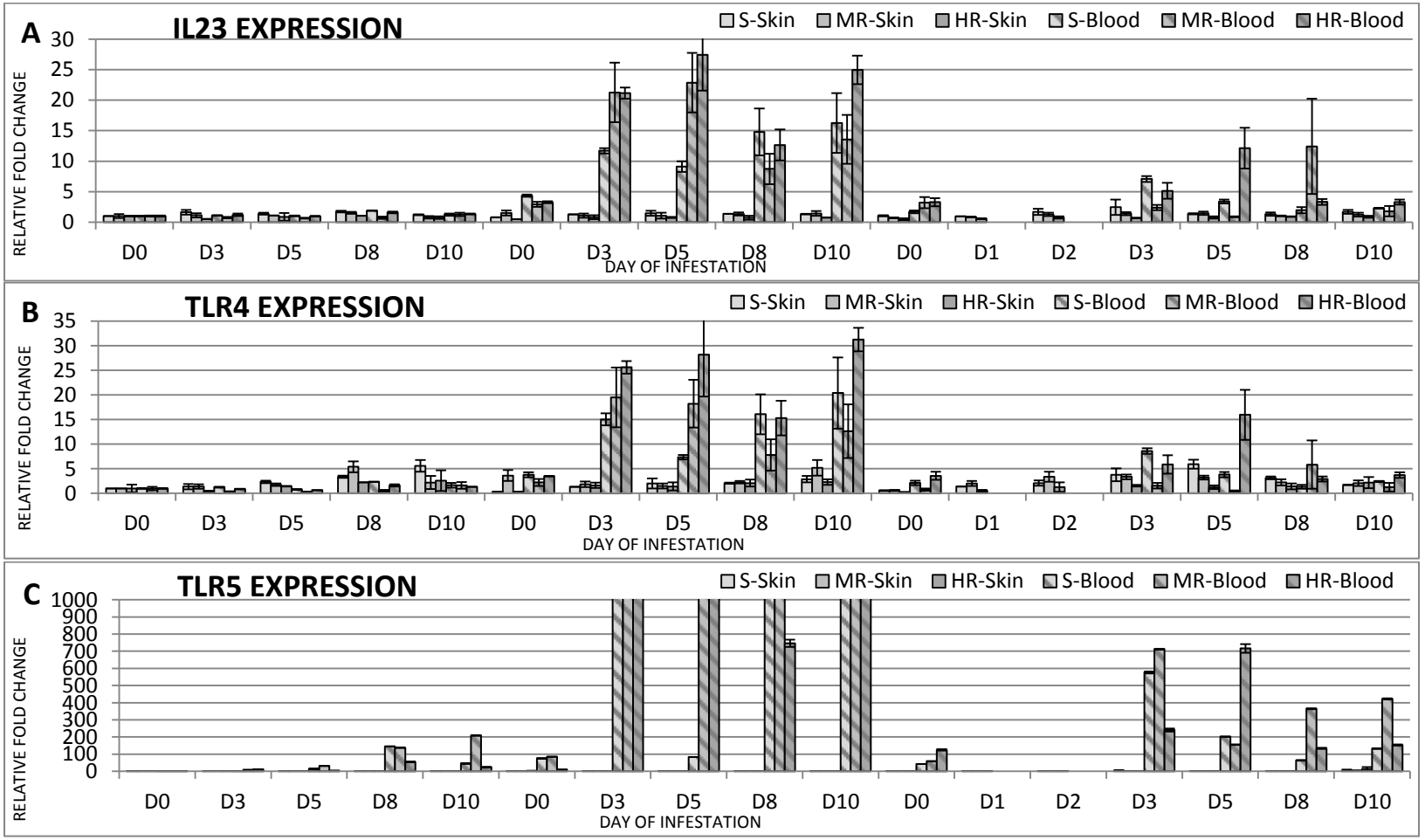

Figure 3.17 Relative gene expression by qRT-PCR for (A) *IL23*, (B) *TLR4*, and (C) *TLR5* in bovine tick bite-site biopsies (Skin) and blood leukocytes (Blood). Specimens were collected from Group 5 tick-susceptible (n=2), moderately tick-resistant (n=3), and highly tick-resistant (n=2) calves during three experimental tick infestations. Fold changes are relative to the average of the biological replicates for each phenotype on day 0 of the first tick infestation and error bars indicate standard error of the mean.

TLR5 expression was notably increased by 2,000- to 10,000-fold on days 3, 5, 8, and 10 of the second tick infestation, and 100- to 500-fold on days 3, 8, and 10 of the third tick infestation when compared to biopsies from S and HR phenotypes (Figure 3.17C).

In general, expression of *IL1α*, *IL1RN*, and *IL6* was notably increased in tick bite-site biopsies from cattle of the S phenotype compared to those from MR and HR cohorts (Figure 3.18). Increased expression of *IL1α* in biopsies from S calves ranged 1- to 10-fold higher on day 10 of the first tick infestation, and days 3, 5, and 8 of the third tick infestation compared to samples from MR and HR calves (Figure 3.18A). Overall, expression values for *IL1α* in tick bite-site biopsies fluctuated over time during the third tick infestation, whereas these values increased over time during the first two tick infestations. Additionally, *IL1α* expression in biopsies of S cattle, as determined by qRT-PCR, was consistent with microarray results (Table 3.6).

During the first tick infestation, expression of *IL1RN* in tick bite-site biopsies from S calves was notably increased up to 200-fold on day 8 compared to biopsies from HR calves, and up to 300-fold on day 10 compared to samples from MR and HR calves (Figure 3.18B). On days 3, 5, and 8 of the third tick infestation, *IL1RN* expression was notably higher in biopsies of S cattle, ranging 25- to 150-fold higher than biopsies from HR cohorts.

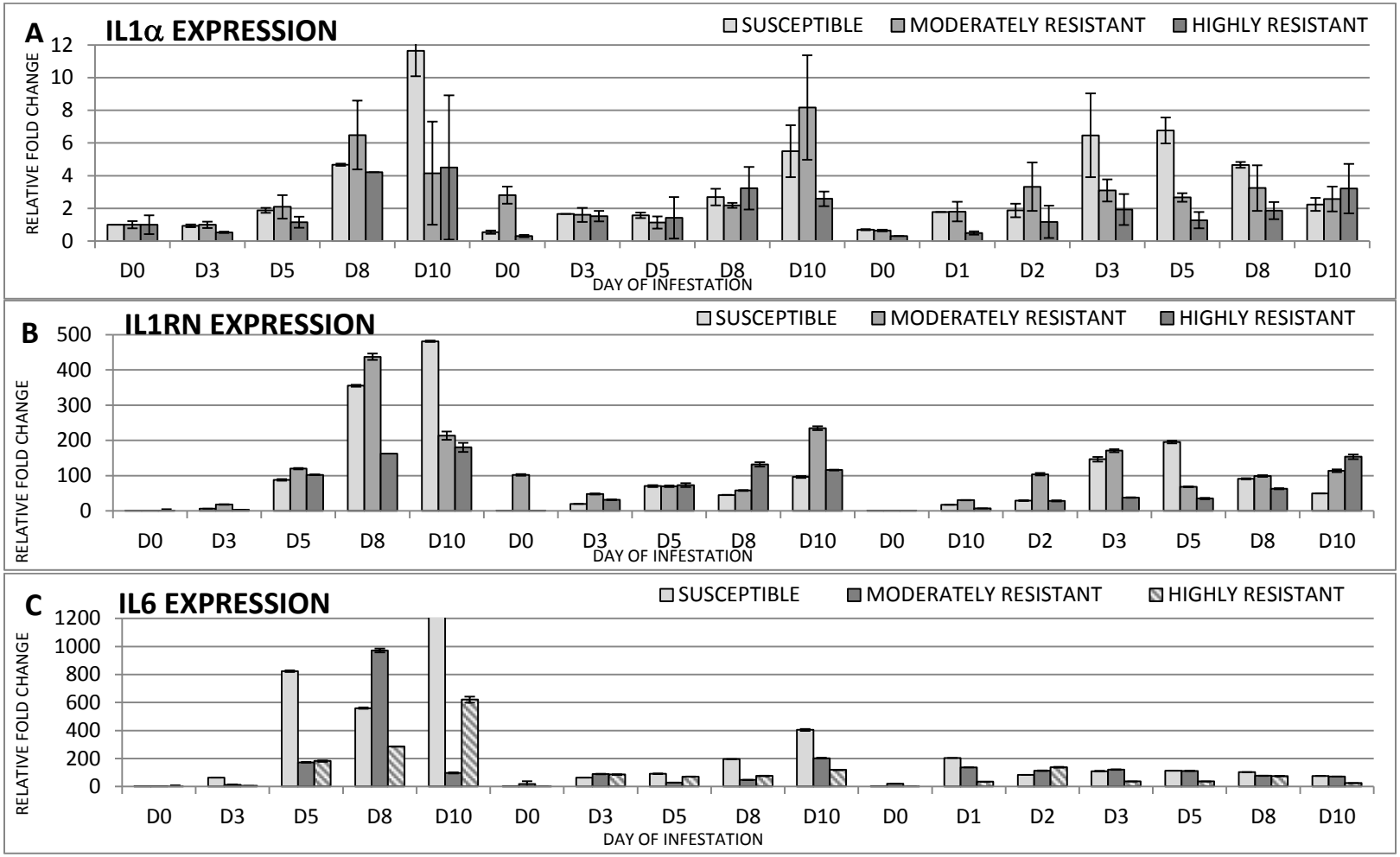

Figure 3.18 Relative gene expression by qRT-PCR for (A) *IL1α*, (B) *IL1RN*, and (C) *IL6* in bovine tick bite-site biopsies. Specimens were collected from Group 5 tick-susceptible (n=2), moderately tick-resistant (n=3), and highly tick-resistant (n=2) calves during three experimental tick infestations. Fold changes are relative to the average of the biological replicates for each phenotype on day 0 of the first tick infestation and error bars indicate standard error of the mean.

In contrast, expression of *IL1RN* in tick bite-site biopsies from HR calves was notably higher on day 8 of the second tick infestation and day 10 of the third tick infestation, ranging 40- to 100-fold compared to biopsies from MR and S calves.

In tick bite-site biopsies of S calves, expression of *IL6* was notably increased by 65- to 1,100-fold on days 5, 7, and 10 of the first tick infestation, days 8 and 10 of the second tick infestation, and day 1 of the third tick infestation compared to biopsies from MR and HR phenotypes (Figure 3.4.1.3C).

No notable differences in expression for *IL1R2* or *IL1RAP* were observed in tick bite-site biopsies from HR, MR, and S cattle over the three tick infestations (data not shown). In addition, no notable differences in *IL12p40* expression among the phenotypes were observed in skin biopsies or blood leukocytes for any of the three tick infestations (data not shown).

3.4.2 Whole group gene expression profiles

In tick bite-site biopsies, similar patterns of gene expression were observed for *IGHG1* and *IL1β* (Figure 3.19). Expression of *IGHG1* in these biopsies was significantly increased ($P \leq 0.05$) more than 50-fold on days 8 and 10 of the first and second tick infestations (Figure 3.19A). Additionally, significant up-regulation of *IGHG1* in these biopsies reached 200-fold on days 8 and 10 of the third tick infestation. In blood leukocytes, expression of *IGHG1* was significantly up-regulated 10- to 20-fold on days 0, 3, and 10 of the second tick infestation (Figure 3.19A). Due to variation in

expression values, as indicated by a high standard deviation, the 15-fold increase observed on day 5 of the second tick infestation in blood leukocytes was not statistically significant ($P = 0.62$).

Significant up-regulation of *IL1β* in tick bite-site biopsies ranged from 20- to 60-fold on days 5, 8, and 10 of the first tick infestation (Figure 3.19B). Although *IL1β* was similarly significantly up-regulated in these biopsies throughout the second tick infestation, gene expression levels on days 5 and 8 were much lower than during the first infestation, ranging 10- to 20-fold with a peak at 60-fold on day 10. During the third tick infestation, *IL1β* expression in these biopsies was significantly increased 10- and 20-fold from day 2 onwards, peaking on day 10. No significant increases for *IL1β* expression were found in blood leukocytes during any of the three tick infestations (Figure 3.19B).

Significant increases were observed in tick bite-site biopsies and blood leukocytes for *IL12p40* and *TLR4* (Figure 3.20). Expression of *IL12p40* in tick bite-site biopsies significantly increased over time and peaked at 2- to 4-fold on day 8 followed by a slight decline on day 10 during the first and third tick infestations (Figure 3.20A). In blood leukocytes, expression of *IL12p40* rose significantly by 3-fold on days 8 and 10 of the second tick infestation (Figure 3.20A).

Expression of *TLR4* in tick bite-site biopsies significantly increased over time and peaked at 2-fold on days 8 and 10 of the first tick infestation, on day 10 of the second tick infestation, and on day 5 of the third tick infestation (Figure 3.20B). In blood leukocytes, expression of *TLR4* was significantly increased on days 3, 5, 8 and 10 of the second tick infestation with a peak of 20-fold on day 3 (Figure 3.20B).

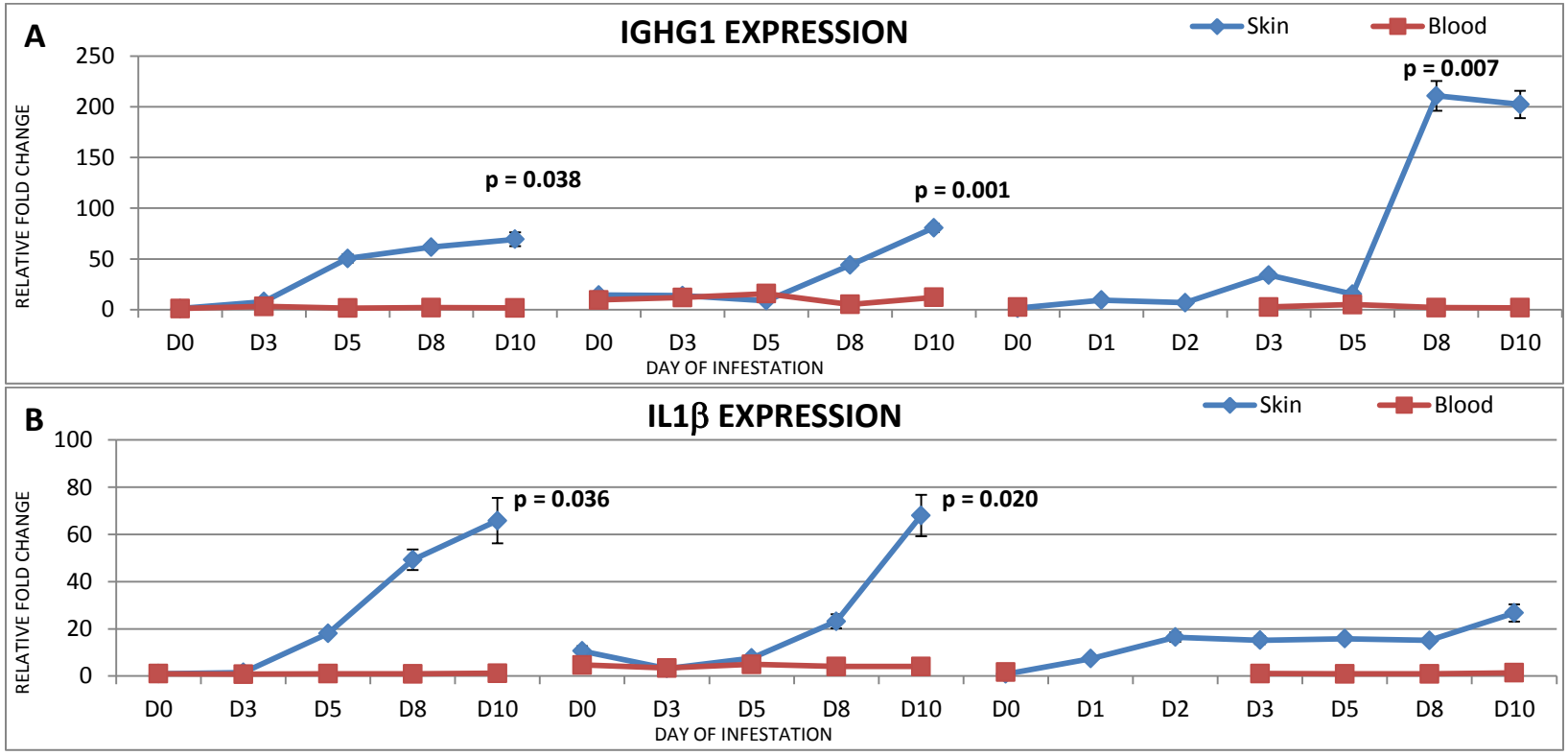

Figure 3.19 Relative gene expression by qRT-PCR for (A) *IGHG1* and (B) *IL1β* in bovine tick bite-site biopsies (Skin) and blood leukocytes (Blood). Specimens were collected from Group 5 calves (n=7) during three experimental tick infestations. Fold changes are relative to the average of the biological replicates on day 0 of the first tick infestation and error bars indicate standard error of the mean. Significant differences with the highest fold change are indicated with the respective *P*-value above the corresponding data point.

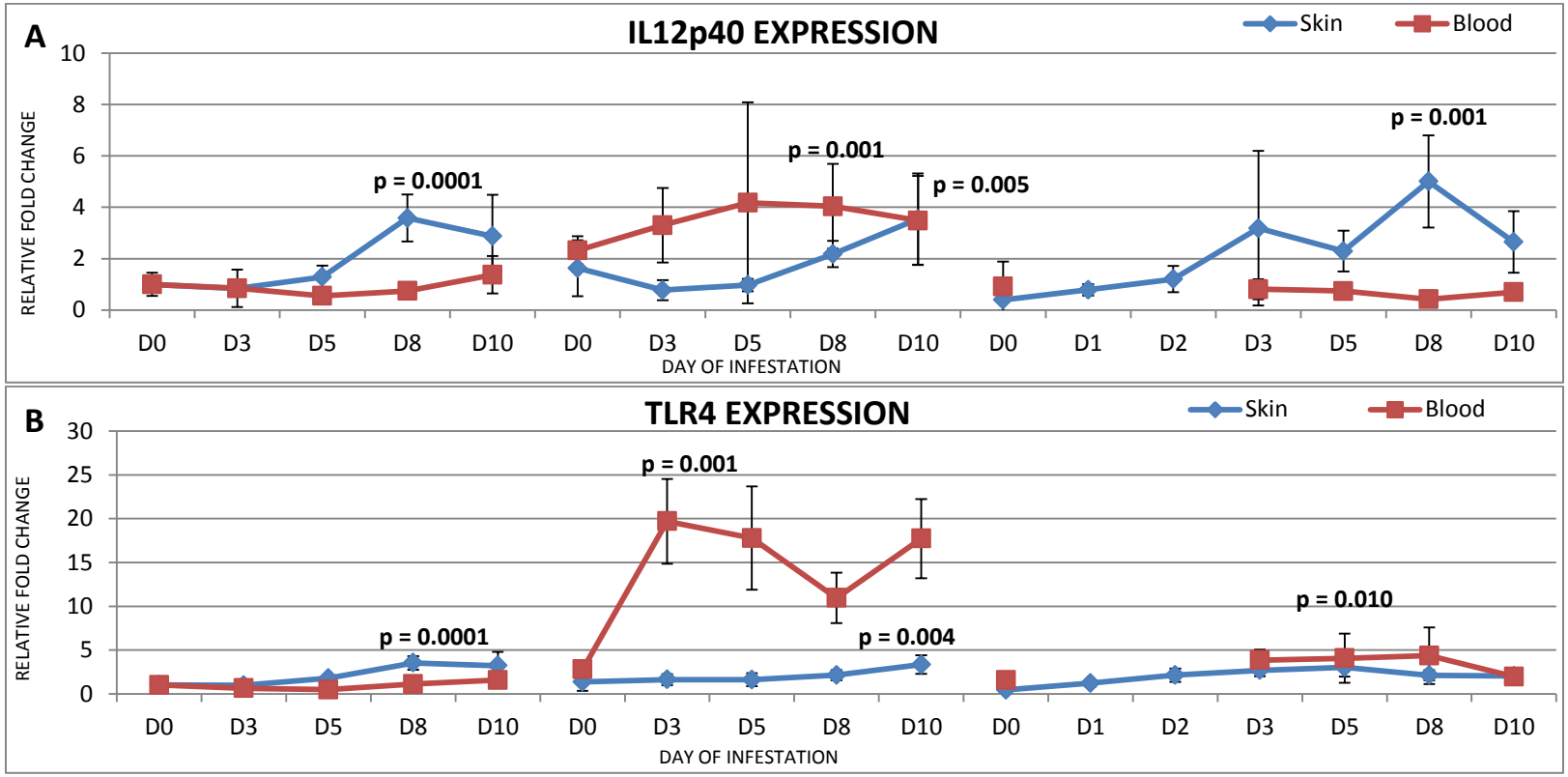

Figure 3.20 Relative gene expression by qRT-PCR for (A) *IL12p40* and (B) *TLR4* in bovine tick bite-site biopsies (Skin) and blood leukocytes (Blood). Specimens were collected from Group 5 calves (n=7) during three experimental tick infestations. Fold changes are relative to the average of the biological replicates on day 0 of the first tick infestation and error bars indicate standard error of the mean. Significant differences with the highest fold change are indicated with the respective *P*-value above the corresponding data point.

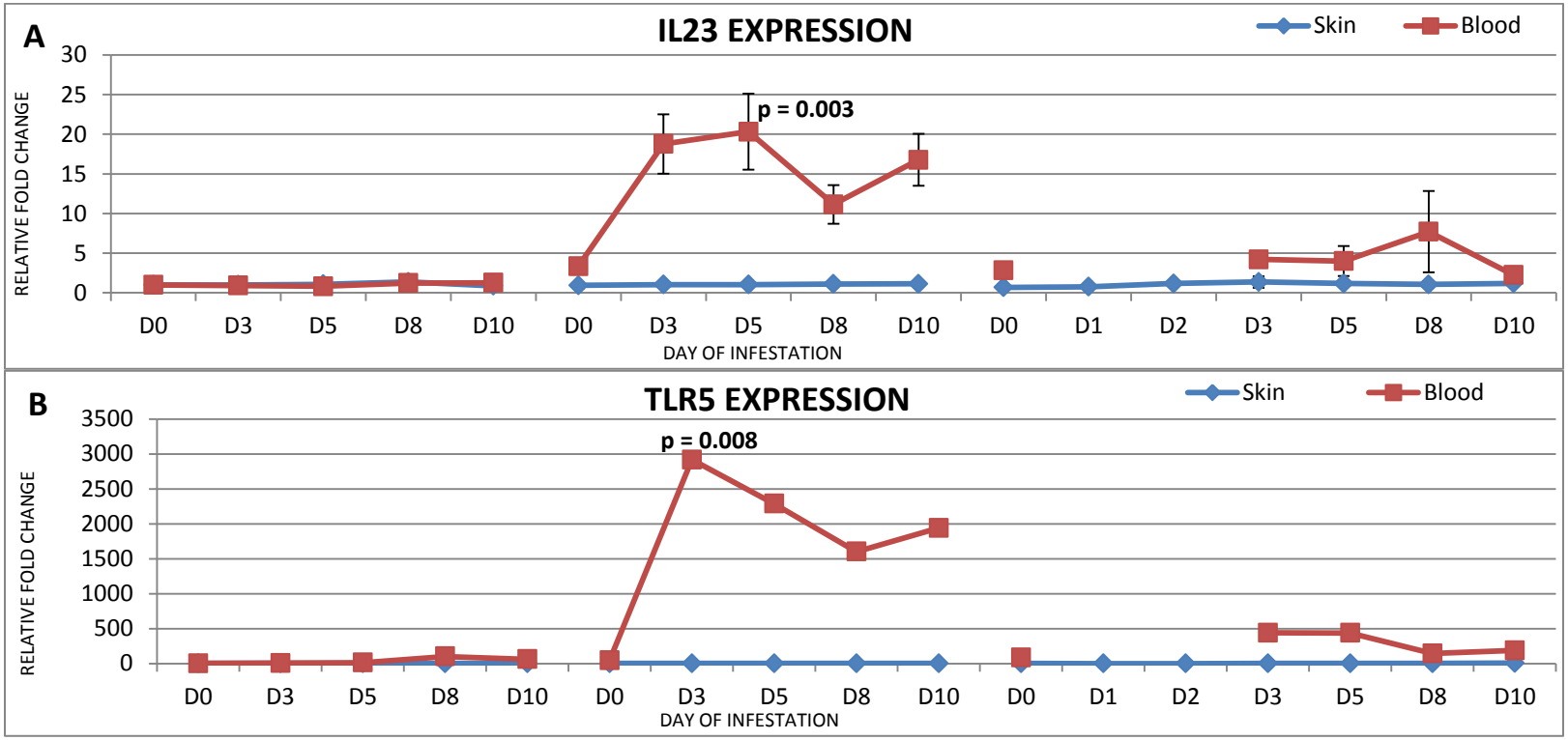

Figure 3.21 Relative gene expression by qRT-PCR for (A) *IL23* and (B) *TLR5* in bovine tick bite-site biopsies (Skin) and blood leukocytes (Blood). Specimens were collected from Group 5 calves (n=7) during three experimental tick infestations. Fold changes are relative to the average of the biological replicates on day 0 of the first tick infestation and error bars indicate standard error of the mean. Significant differences with the highest fold change are indicated with the respective *P*-value above the corresponding data point.

Similar patterns of gene expression were observed for *IL23* and *TLR5* in tick bite-site biopsies and revealed no significant changes during any of the three tick infestations, except for the significant increase of 5-fold on day 10 for *TLR5* during the third tick infestation (Figure 3.21). In blood leukocytes, expression of both *IL23* and *TLR5* showed significant up-regulation on days 3, 5, 8, and 10 of the second tick infestation, and these increases ranged 10- to 20-fold for *IL23* and 1,500- to 3,000-fold for *TLR5* (Figure 3.21).

The patterns of expression for *IL1RN*, *IL6*, and *IL1*α in tick bite-site biopsies were similar and showed significantly increased peaks on days 8 and 10 of the first tick infestation, and on day 10 of the second tick infestation (Figure 3.22). Expression of *IL1RN* in these biopsies was significantly up-regulated by 100- to 300-fold on days 5, 8, and 10 of the first tick infestation (Figure 3.22A). Although *IL1RN* expression was significantly increased in these biopsies during the second tick infestation, there was a distinct reduction compared to the first infestation, ranging 50- to 150-fold on the same days measured. Additionally, significant rises in *IL1RN* expression in these biopsies remained increased by 50- to 100-fold on days 2, 3, 5, 8, and 10 of the third tick infestation (Figure 3.22A).

Expression of *IL6* in tick bite-site biopsies was similarly significantly up-regulated by 300- to 500-fold during the first tick infestation, 50- to 100-fold during the second tick infestation, and 50- and 100-fold during the third tick infestation (Figure 3.22B).

Expression of *IL1α* in tick bite-site biopsies was also similarly significantly increased, however, expression peaked by only 5-fold on day 10 of the first tick infestation and by 4-fold on day 10 of the second tick infestation (Figure 3.22C).

No significant changes were found for expression of *IL1R2* or *IL1RAP* in tick bite-site biopsies from Group 5 calves (data not shown).

3.5 Gene to Gene Interactions in Bite-Site Biopsies from Group 5 Calves

Gene expression correlations in tick bite-site biopsies from Group 5 calves were determined by regression curve analysis and based on a high regression value ($R^2 \geq 0.8$) and statistically significant ($P \leq 0.05$) F score. When expression values for the 11 candidate genes were analyzed in these biopsies for Group 5 calves as a whole (N = 7), the expression of three genes, *IL1β*, *IL1RN*, and *TLR4* correlated with *IL1α* (Table 3.7). Additionally, expression of *IL6* showed a strong correlation ($R^2 = 0.835$) with *IL1RN* (Table 3.7).

Table 3.7 Gene to gene interactions in tick bite-site biopsies from Group 5 calves.

Status (N)	Dependent variable	Independent variable	R squared	F score[a]
Group 5 (N=7)	IL1α	IL1β	0.886	116.726
Group 5 (N=7)	IL1α	IL1RN	0.83	72.258
Group 5 (N=7)	IL1α	TLR4	0.875	104.62
Group 5 (N=7)	IL1RN	IL6	0.835	76.035

[a] All F scores indicate significance with a *P*-value ≤ 0.0001

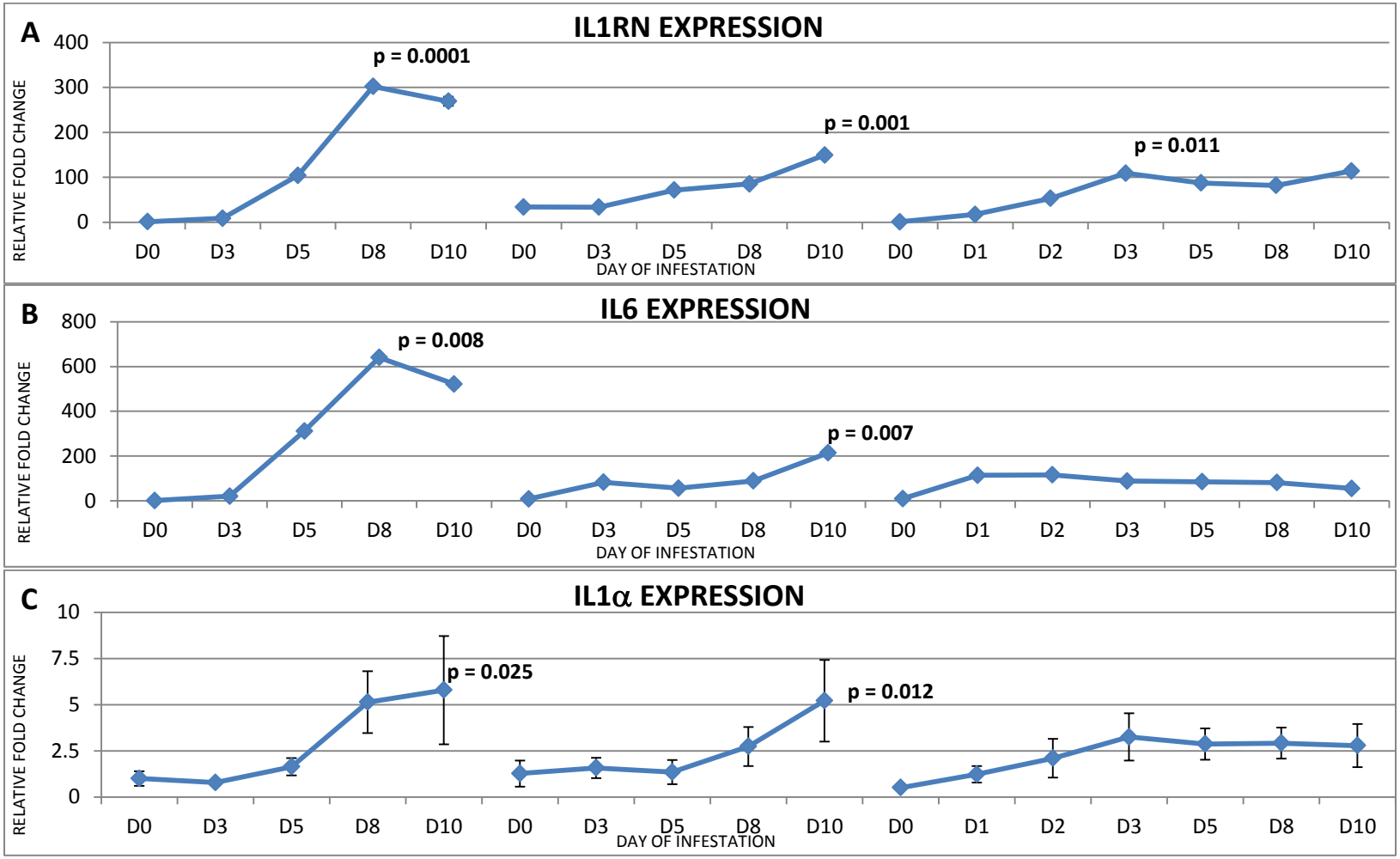

Figure 3.22 Relative gene expression by qRT-PCR for (A) *IL1RN*, (B) *IL6*, and (C) *IL1α* in bovine tick bite-site biopsies. Specimens were collected from Group 5 calves (n=7) during three experimental tick infestations. Fold changes are relative to the average of the biological replicates on day 0 of the first tick infestation and error bars indicate standard error of the mean. Significant differences with the highest fold change are indicated with the respective *P*-value above the corresponding data point.

Analysis of genes in tick bite-site biopsies from S cattle identified strong correlations between expression of *IL1α* and three genes, *IL1β*, *IL1RAP*, and *TLR4* (Table 3.8). Additionally, expression of *IL1RAP* correlated with *TLR4* in biopsies from S calves (Table 3.5.2). In biopsies from MR calves, the expression of *IL1β* and *TLR4* correlated with *IL1α* (Table 3.8). A strong correlation between expression of *IL1α* and three genes, *IL1β, TLR4,* and *IL1RN*, was identified in biopsies from HR calves (Table 3.8). Furthermore, expression of *IL1β* correlated with *IL1RN* in biopsies from HR calves (Table 3.8).

Table 3.8 Gene to gene interactions in tick bite-site biopsies from tick-susceptible, moderately tick-resistant, and highly tick-resistant Group 5 calves.

Status[a] (N)	Dependent variable	Independent variable	R squared	F score[b]
S (N=2)	IL1α	IL1β	0.784	54.411
S (N=2)	IL1α	IL1RAP	0.837	76.951
S (N=2)	IL1α	TLR4	0.835	76.166
S (N=2)	IL1RAP	TLR5	0.905	143.627
MR (N=3)	IL1α	IL1β	0.935	215.208
MR (N=3)	IL1α	TLR4	0.802	60.631
HR (N=2)	IL1α	IL1β	0.794	57.968
HR (N=2)	IL1α	IL1RN	0.863	94.771
HR (N=2)	IL1α	TLR4	0.859	91.456
HR (N=2)	IL1β	IL1RN	0.841	79.262
HR (N=2)	IL1RN	TLR4	0.815	65.9

[a] Tick-susceptible (S), moderately tick-resistant (MR), highly tick-resistant (HR).
[b] All F scores indicate significance with a *P*-value $\leq$ 0.0001.

In tick bite-site biopsies from calf 906 of the S phenotype, the expression of four genes, *IL1β*, *IL1RAP*, *IL1RN*, and *TLR4*, showed strong correlations to *IL1α* (Table 3.9). Additional correlations were identified for two genes, *IL1RN* and *TLR4*, with expression of *IL1RAP*, and between expression of *IL1RN* and *TLR4* (Table 3.9). No correlations in gene expression were identified by in biopsies from calf 901 of the S phenotype.

Correlations were identified in tick bite-site biopsies from calf 905 of the MR phenotype between *IGHG1* and *IL12p40*, and for *IL1α* with two genes, *IL1β* and *TLR4* (Table 3.9). In biopsies from MR calf 902, a correlation was identified between expression of *IL1RN* and *IL12p40* (Table 3.9). Interactions identified in biopsies from calf 907 of the MR phenotype included expression of *IL1α* with three genes, *IL1β, IL1RN*, and *TLR4* (Table 3.9). Additionally, correlations between *IL1β* and two genes, *IL1RN* and *IL6,* and between *IL1RN* and *IL6* were identified in biopsies from calf 907 of the MR phenotype (Table 3.9).

Correlations between expression of *IL1α* and three genes, *IL1β, IL1RN*, and *TLR4*, were identified in tick bite-site biopsies from calves 903 and 904 of the HR phenotype (Table 3.9). In biopsies from HR calf 904, expression of *IL1β* was correlated with two genes, *IL1RN* and *IL6*, and expression of *IL1α* was correlated with *IL6* (Table 3.9). In biopsies from HR calf 903, expression of *IL1RN* was correlated with *TLR4* (Table 3.9).

Table 3.9 Gene to gene interactions in tick bite-site biopsies from individual Group 5 calves.

Status[a] (calf ID)	Dependent variable	Independent variable	R squared	F score[b]
S (906)	IL1α	IL1β	0.781	53.422
S (906)	IL1α	IL1RAP	0.873	103.038
S (906)	IL1α	IL1RN	0.804	61.635
S (906)	IL1α	TLR4	0.838	77.458
S (906)	IL1RAP	IL1RN	0.871	101.682
S (906)	IL1RAP	TLR4	0.874	104.158
S (906)	IL1RN	TLR5	0.828	71.972
MR (902)	IL1α	IL1β	0.87	100.133
MR (902)	IL1α	IL12p40	0.906	143.771
MR (902)	IL1α	TLR4	0.799	59.507
MR (902)	IL1RN	IL12p40	0.87	100.392
MR (905)	IGHG1	IL12p40	0.861	92.683
MR (907)	IL1α	IL1β	0.914	159.242
MR (907)	IL1α	IL1RN	0.922	177.199
MR (907)	IL1α	TLR4	0.841	79.615
MR (907)	IL1β	IL1RN	0.978	669.016
MR (907)	IL1β	IL6	0.847	83.15
MR (907)	IL1RN	IL6	0.824	70.357
HR (903)	IL1α	IL1β	0.827	71.559
HR (903)	IL1α	IL1RN	0.911	152.789
HR (903)	IL1α	TLR4	0.889	120.065
HR (903)	IL1RN	TLR4	0.812	64.631
HR (904)	IL1α	IL1β	0.849	84.225
HR (904)	IL1α	IL1RN	0.844	81.251
HR (904)	IL1α	IL6	0.828	72.286
HR (904)	IL1α	TLR4	0.882	112.14
HR (904)	IL1β	IL1RN	0.952	294.65
HR (904)	IL1β	IL6	0.841	79.126

[a] Tick-susceptible (S), moderately tick-resistant (MR), highly tick-resistant (HR).
[b] All F scores indicate significance with a *P*-value $\leq$ 0.0001.

CHAPTER IV

DISCUSSION

Ixodid (hard) ticks consist of a capitulum, a false head bearing mouthparts, attached to a fused thorax and abdomen that gives the tick body a flattened oval-shaped appearance (STAFFORD 2007). The capitulum possesses a pair of extrasensory palps, a pair of chelicerae, and the hypostome on a base (basis capitulum), and these features are used for species identification. The chelicerae are scalpel-like projections used to cut into the host's skin prior to insertion of the barbed hypostome to facilitate host attachment (ANDERSON and MAGNARELLI 2008; STAFFORD 2007). To secure attachment, Ixodid ticks secrete cement into the host skin that hardens around the hypostome, forming an anchor for the parasite and sealing off the feeding lesion to promote siphoning of blood and prevent leakage (ANDERSON and MAGNARELLI 2008; ARTHUR 1970; STAFFORD 2007).

Ticks with long hypostomes that possess relatively large backwards-facing teeth, as is the case with *Amblyomma* spp. ticks, require little cement to secure host attachment since the hypostome anchors deep through the epidermis and into the dermis of the host (ARTHUR 1970; JAWORSKI *et al.* 1992; LATIF *et al.* 1990; MOOREHOUSE and TATCHELL 1966). On the other hand, ticks with short mouthparts, such as *Rhipicephalus* spp. ticks, must secrete additional amounts of cement to compensate for the more superficial mode of attachment, as the hypostome of these ticks only penetrates the epidermal layer of the skin. The antigenic components of tick cement were reported to be conserved among

many species ticks, and thus the amount of cement secreted, along with the depth of anchored tick mouthparts seem to be key factors for eliciting host immune mechanisms (JAWORSKI *et al.* 1992). Many variables that affect the host response to tick attachment are influenced by tick cement such as the number of components secreted, the quantity and antigenicity of each component, the amount of contact surface area between the tick and host, and the depth of the penetrating mouthparts (ANDERSON and MAGNARELLI 2008; LATIF *et al.* 1990). Once anchored to the host, ticks maintain an intimate association that involves fluid exchange between tick and host for several days to several weeks (JAWORSKI *et al.* 1992; WIKEL 1996).

In a life cycle analysis of laboratory maintained tick colonies female *Amblyomma americanum* ticks completed feeding in 10-15 days with an average engorgement period of 12 days (TROUGHTON and LEVIN 2007). Additionally, female ticks generally reached engorgement weights over 100 times greater compared to pre-attachment weights, and imbibed enough blood to triple that number during the engorgement period. However, much of the blood consumed by ticks was returned, along with tick salivary secretions, during periods of regurgitation (ANDERSON and MAGNARELLI 2008; BROWN 1988). Initial tick salivary secretions inhibited blood clotting, suppressed inflammatory mediators, dilated local capillaries, and digested host tissues to allow hemorrhage for the formation of the blood pool. During tick feeding, blood ingestion alternates with tick salivation to maintain inhibition of host immune mechanisms, and to allow regurgitation of midgut components for removal of waste from the tick (ANDERSON and MAGNARELLI 2008; ARTHUR 1970; MOOREHOUSE and TATCHELL 1966).

Tick-secreted factors inhibit an array of host immune mechanisms including antigen presenting cells, complement components, T cells, B cells, macrophages, anti-microbial proteins, inflammatory mediators, wound healing factors, and Toll-like receptors (WILLADSEN *et al.* 1993). Inhibition of host immunity allows the prolonged attachment of a tick to its host in order to obtain a blood meal, and host immunomodulation is mediated in a species-specific manner. A large majority of the currently characterized host response mediators were identified from the salivary glands of *Ixodes scapularis*, as these ticks transmit the agent causing Lyme disease (HOVIUS 2009). However, several host immunomodulatory molecules were identified from other tick species, including *Haemaphysalis longicornis*, *Amblyomma variegatum*, *Amblyomma cajennense*, and *A. americanum* (ALJAMALI *et al.* 2009a; ALJAMALI *et al.* 2009b; KONNAI *et al.* 2009).

One study analyzed T cell responses in bovine peripheral mononuclear cells and mouse splenocytes and reported that the HL-p36 immunosuppressant from the salivary glands of *H. longicornis* ticks inhibited the production of interleukin 2 (IL-2), thereby preventing activation and proliferation of T cells (KONNAI *et al.* 2009). This is important because zoonotic disease agents, such as *Ehrlichia chaffeensis* and *Anaplasma bovis*, were identified in the salivary glands of *H. longicornis* ticks collected from grazing cattle in Korea after antibodies to *E. chaffeensis* were identified in blood from United States soldiers and Korean patients suffering from febrile illnesses (LEE and CHAE 2009). In an investigation to determine the importance of T helper cells for the clearance of *E. chaffeensis*, inbred mice that lacked genes for the major

histocompatibility receptor II (*MHCII*) and cluster of differentiation 4 (*CD4*) were experimentally infected with *E. chaffeensis* (GANTA *et al.* 2004). Interestingly, mice that lacked the CD4 gene experienced delayed clearance of *E. chaffeensis* compared to clearance in wild type mice, while mice that lacked the MHCII gene, which eliminated both CD4 and CD8 T cells, did not clear *E. chaffeensis* infections. Presuming that T cells are similarly required for the elimination of *E. chaffeensis* in cattle, inhibition of T cell activation by tick immunosuppressants may prevent bovine, murine, and other mammalian hosts from mounting an immune response sufficient for clearance of *E. chaffeensis* or other disease agents.

A total of five homologues of HL-p36 were identified in salivary glands from two *Amblyomma* spp. ticks, *Amblyomma cajennense* and *A. americanum* ticks (ALJAMALI *et al.* 2009a). Interestingly, p36 immunosuppressant genes of *A. americanum* were differentially regulated as ticks fed on sheep, as determined by microarray analysis of salivary glands from unfed, partially fed, fast feeding, and replete female Lone Star ticks (ALJAMALI *et al.* 2009b). Expression of p36 immunosuppressants was highest during host attachment and as ticks entered the early feeding stage, whereas expression of immunosuppressant genes decreased several days later as ticks progressed into the partially fed stage. Furthermore, expression of p36 immunosuppressants was completely diminished when ticks began the rapid engorgement phase of feeding, a time at which only genes affecting reproduction were up-regulated (ALJAMALI *et al.* 2009b). Much remains to be discovered concerning the capacity of *A. americanum* ticks to modulate bovine host immune mechanisms. Nevertheless, the potential for *A. americanum*-derived

p36 immunosuppressants to inhibit T cell responses in bovine and other agriculturally important hosts is likely an important factor for tick feeding and disease transmission.

Salivary gland extracts (SGEs) from ticks were reported to inhibit the function of several host immune factors, although the specific inhibitory molecules that mediate these mechanisms remain uncharacterized. Specific ELISA binding assays were used to evaluate the anti-chemokine activity of SGEs from *A. variegatum*, *Dermacentor reticulatus*, and *Ixodes ricinus* in serum from humans and mice (HAJNICKÁ *et al.* 2005). Evaluation of cytokine activity for interleukin 8 (IL-8), monocyte chemotactic protein (MCP-1), macrophage inflammatory protein (MIP-1α), regulated upon activation normal T-cell expressed and secreted protein, eotaxin, interleukin 2 (IL-2) and interleukin 4 (IL-4) revealed that tick SGEs interacted with host cytokines in a species- and sex-specific manner (HAJNICKÁ *et al.* 2005). Exposure to SGEs reduced detectable cytokine levels for six of the seven previously described factors for both male and female *D. reticulatus* (HAJNICKÁ *et al.* 2005). Furthermore, SGEs from male *I. ricinus* reduced activity of all seven cytokines evaluated, whereas female tick SGEs inhibited only three, including IL-8, IL-2, and IL-4. Dose-dependent responses between SGEs and five of the seven cytokines evaluated, including IL-8, MCP-1, MIP-1α, IL-4, and eotaxin, were similarly identified from both male and female *A. variegatum* ticks (HAJNICKÁ *et al.* 2005). These factors mediate inflammation by recruitment and activation of immune cells, such as neutrophils, T cells, eosinophils, and basophils to sites of injury, as would occur with tick bites. Thus, it was suggested that tick SGEs

affect the migration, activation, and proliferation of host immune cells (HAJNICKÁ *et al.* 2005).

The specificity of tick SGEs for binding host wound healing factors from several tick species was evaluated in mammalian epithelial and fibroblast cell lines from humans, mice, pigs, and hamsters (HAJNICKÁ *et al.* 2011). SGEs from *Ixodes ricinus* and *Ixodes scapularis* ticks bound the platelet-derived growth factor while SGEs from *D. reticulatus* and *Rhipicephalus appendiculatus* bound transforming growth factor beta (TGFβ), fibroblast growth factor 2, and hepatocyte growth factor. Interestingly, SGEs from *A. variegatum* ticks bound all four of the growth factors evaluated and this may be explained in part by the long mouthparts of *Amblyomma* spp. ticks, as the hypostome of these ticks penetrates into the dermis of the host and the deep wounds created may require mediation of more host factors by ticks to obtain a blood meal.

Histological differences between tick-attachment sites from an *Amblyomma* spp. tick, *A. variegatum*, and a *Rhipicephalus* spp. tick, *R. appendiculatus*, were evaluated and *A. variegatum* bite lesions on the ears of tick-naïve rabbits 48 hours after tick attachment were large and marked by inflammation, swelling, necrosis, skin sloughing, and serous exudate, while *R. appendiculatus* bite lesions showed few cellular changes (LATIF *et al.* 1990). Bite lesions from *A. variegatum* ticks displayed ten times more cellular infiltration than lesions from *R. appendiculatus* ticks, and were characterized by a pool of neutrophils underneath tick mouthparts (LATIF *et al.* 1990). Neutrophils were the most abundant cell type infiltrating *A. variegatum* bite lesions followed by fewer numbers of eosinophils and mononuclear cells, whereas *R. appendiculatus* bite lesions

were predominantly infiltrated by mononuclear cells and showed only a few neutrophils and eosinophils (LATIF *et al.* 1990). Rabbits that were previously exposed to either tick species displayed clearly demarked lesions with heavy cellular infiltration, epithelial cell necrosis, degenerated leukocytes, and eosinophilic degranulation when re-infested by *A. variegatum* ticks. However, when similarly tick-sensitized rabbits were re-infested with *R. appendiculatus* ticks, only slight epidermal thickening and cellular infiltration were observed (LATIF *et al.* 1990). This one directional cross-resistance was thought to arise in part from the host response elicited by the deeper penetration of mouthparts by *A. variegatum* ticks compared to the more superficial bites of *R. appendiculatus*. Many studies to date focused on the host response to ticks within the first few hours to days after tick attachment. Despite the knowledge gained, ticks remain attached to a host for several weeks and the host response could vary during this time (WIKEL and BERGMAN 1997).

In order to accurately characterize the dynamic interactions that occur at the tick-host interface, identification of the mechanisms that mediate the host immune response during tick feeding is crucial. Previous studies designed to identify key cellular mediators in the skin of *A. americanum* tick-resistant cattle and laboratory animals focused on elucidating the relationship between IgG_1 antibodies and basophils (BROWN 1982; BROWN and ASKENASE 1985; BROWN *et al.* 1984; BROWN *et al.* 1982a; BROWN *et al.* 1982b). Some investigations characterized the importance of other cell types, such as mast cells and eosinophils, for the expression of host resistance to *A. americanum* ticks (BROWN 1985; BROWN *et al.* 1983; RIEK 1962).

This current investigation utilized gene expression techniques to profile bovine immune responses in tick bite-site biopsies and blood leukocytes from cattle experimentally infested with Lone Star ticks. Degraded RNA negatively affects the efficiency of microarray hybridizations and qRT-PCR assays, and thus quality assessment of total RNA isolates prior to performing gene expression experiments is imperative. Optimization of sample collection and total RNA extraction techniques from bovine tick-bite site biopsies was therefore an important first aim in this current study (BRANNAN *et al.* 2013). To accomplish this aim, the first step was to determine an effective method to pulverize the cattle skin biopsies. The robust nature of cattle hide, which is comprised of substances such as collagen, mucoid components, adipose tissue, and ribonucleases (RNases), presents a particular challenge for RNA extraction. Thus, cattle hide necessitates a unique technique for tissue maceration in order to extract intact RNA (BURTON and REED 1956). In other animal species, skin punch biopsies were pulverized with a Mikro-Dismembrator (Sartorius Stedim Biotech, Aubagne, France), bead beater, homogenizer, or by grinding in a mortar and pestle (BERGLUND *et al.* 2007; BRUNING *et al.* 2011; MENZIES and INGHAM 2006; WOOD *et al.* 2009). Previously, bovine tick attachment site biopsies were cut into pieces, wrapped in aluminum foil, frozen in liquid nitrogen, and pulverized with a hammer on a frozen metal block (BAGNALL *et al.* 2009; PIPER *et al.* 2010). This study found that macerating the bovine skin tissue in a liquid nitrogen-chilled Biopulverizer^{TM} prevented the introduction and activation of RNases, as evidenced by the high quality RNA obtained (BRANNAN *et al.*

2013). The success of this technique was presumed to be due to limited sample manipulation and better containment of frozen bovine skin biopsy specimens.

The next step was to find an effective method for total RNA extraction from the pulverized bovine tissue. A modified TRI Reagent® method outperformed the RNeasy® Mini Kit for both yield and quality of total RNA from bovine skin biopsies (BRANNAN *et al.* 2013). Since the tissue samples were similarly biopulverized prior to RNA extraction and the total RNA was purified on RNeasy® mini columns after the RNA extraction step for both methods, the tissue lysis reagent used to separate the RNA from other cellular components seemed to be the critical difference affecting the quality of the total RNA isolated. Cattle hide is water-proof due to a coating of mucoid components, such as hyaluronic acid and chondroitin sulfate, over the densely packed collagen fibers (BURTON and REED 1956). Since these waterproofing components must be removed before hide tanning chemicals can penetrate the tissue, they likely require removal prior to isolation of high quality total RNA, suggesting that the tissue lysis reagent used for total RNA extraction from cattle skin is vital. Adipose tissue, another major component of cattle skin, is also problematic for the extraction of intact RNA due to its high triglyceride content (HEMMRICH *et al.* 2009). Use of a TRI Reagent® equivalent for tissue lysis yielded 10 times more total RNA from human adipose tissue compared to extraction with the RNeasy® Mini Kit (HEMMRICH *et al.* 2009). The isolation of total RNA from difficult tissues, such as cattle skin, appears to benefit from the presence of guanidinium chloride, a strong deproteinizing agent found in TRI Reagent® formulations.

The ubiquitous presence of RNases in skin tissue compromises the integrity of total RNA obtained, and thus research has focused on finding methods of RNA isolation that resolve this issue (HOLLEY *et al.* 1961; TABANICK and FREED 1961). Use of a single-step RNA isolation technique yielded high quality total RNA from pancreatic tissue, which is also RNase-rich (CHIRGWIN *et al.* 1979). Since the development of TRI Reagent®, a solution consisting of acid guanidinium, phenol, and chloroform, among other components, isolation of intact RNA from RNase-rich tissues has improved (CHOMCZYNSKI and SACCHI 1987). Furthermore, intact total RNA was successfully isolated from human and mouse skin punch biopsies, confirmed by 1% formaldehyde gel electrophoresis or Bioanalyzer chip analysis, using TRI Reagent® or its equivalents (JENSEN *et al.* 2012; MURO *et al.* 2003; RIGGS *et al.* 2005). This current study further demonstrates that the use of TRI Reagent® for tissue lysis facilitates isolation of high quality total RNA from biopulverized bovine skin biopsies (BRANNAN *et al.* 2013).

The use of 4 M guanidinium isothiocyanate (GITC) to protect bovine skin biopsies from degradation by RNases during frozen storage was investigated in this study (BRANNAN *et al.* 2013). The half-life of RNases was reduced to 10 seconds or less when solutions containing 4 M or higher concentrations of GITC were used (MILLER and BOLEN 1978). However, use of 7.5 M GITC resulted in protein contamination of isolated total RNA, which affected downstream analyses (ZSINDELY *et al.* 1970). When applied to the surface of mouse epidermis, GITC improved the quality of RNA isolated from mouse skin biopsies (RIGGS *et al.* 2005). However, no difference in the integrity of RNA was observed from bovine skin biopsies frozen with or without GITC in this study

(BRANNAN *et al.* 2013). The lack of difference in quality of RNA from samples frozen with or without GITC suggests that the handling of the samples at collection in this study was effective in preventing RNA degradation, as evidenced by the high quality of total RNA obtained. Once again, the yield and RIN scores for samples extracted with TRI Reagent® were significantly higher than those extracted with the RNeasy® Mini Kit, further corroborating it as the optimal method for processing bovine skin biopsies (BRANNAN *et al.* 2013).

In addition to RIN scores, an electropherogram is generated when analyzing RNA by capillary electrophoresis using a Bioanalyzer chip. The electropherogram may also be examined visually to detect the quality of RNA analyzed. High quality RNA is depicted by the presence of well-defined peaks for 18S and 28S ribosomal RNA (rRNA) subunits with low noise between the two peaks, as seen in samples extracted herein with the TRI Reagent® technique (BRANNAN *et al.* 2013). Low molecular weight noise between the peak representing small RNAs and the 18S rRNA peak may cause a slight reduction in the RIN score for high quality RNA. However, noise observed between the 18S and 28S rRNA peaks indicates RNA degradation and results in a lowered RIN value, as depicted for RNA isolated with the RNeasy® Mini Kit in this study (BRANNAN *et al.* 2013). Total RNA suitable for gene expression studies from skin samples must be intact, as determined by a minimum RIN score of 6.5, and free of protein, RNase, and genomic DNA contamination (BECKER *et al.* 2010; BRUNING *et al.* 2011; FLEIGE and PFAFFL 2006; SRA *et al.* 2005). Since the quantity of total RNA required for these experiments is small (typically 5 μg or less), total RNA isolated from bovine skin

biopsies with the TRI Reagent® technique in this study was more than sufficient for the reproduction of experimental data (BRANNAN *et al.* 2013).

Optimization of methods to collect biopsies from tick-infested cattle and isolate total RNA from the specimens is imperative to conduct reliable gene expression experiments for the investigation of bovine tick-resistance. Experimental tick infestations required for the collection of bovine tick bite-site biopsies are difficult and expensive to replicate, and this necessitates a dependable methodology for the isolation of high quality RNA from these samples. Thus, it was shown that using a liquid nitrogen pre-chilled Biopulverizer™ to macerate bovine skin biopsies followed by RNA extraction with a modified TRI Reagent® method isolated ample amounts of high quality total RNA suitable for gene expression studies (BRANNAN *et al.* 2013).

Total RNA isolation from blood samples was also optimized to obtain quality RNA for gene expression studies to examine the bovine systemic immune response to tick infestation. In this study, the best quality total RNA, as determined by Bioanalyzer chip analysis, was obtained from bovine blood leukocytes with the LeukoLOCK™ total RNA isolation system. The mean RIN from LeukoLOCK™ extracted RNA in this study was consistent with previously reported results that used the same method to isolate total RNA from bovine blood leukocytes (HAMMERLE-FICKINGER *et al.* 2010). .

The highest yield of total RNA in this current study was obtained with PAXgene® BloodRNA tubes, although the isolated RNA was of extremely poor quality, as evidenced by no visible peaks for 18S and 28S rRNA and no detectable RIN score. The high yield with PAXgene® BloodRNA tubes was unexpected since the 2.5 ml

volume used was much less than the 7-8 ml blood required for total RNA isolation with TRI Reagent® BD and LeukoLOCK™ methods. Use of TRI Reagent® BD for total RNA isolation from frozen bovine blood resulted in low yields and poor quality RNA. The isolation of poor quality RNA from PAXgene® BloodRNA tubes may be due to use of the E.Z.N.A Blood RNA Kit instead of the PAXgene® RNA extraction kit available from the same manufacturer. The major differences between these two RNA extraction kits are that diethylpyrocarbonate (DEPC)-treated water is used during the proteinase K digestion step for the E.Z.N.A Blood RNA Kit, while a buffer containing proprietary ingredients is provided with the PAXgene® RNA extraction kit, and the RNA-containing supernatant after proteinase K treatment is separated by use of a homogenizer column with the former kit, whereas separation was achieved by centrifugation with the latter kit. Thus, it is presumed that the use of DEPC water was not sufficient for removal of all proteinaceous material from the RNA, which may have affected the quality assessment. It should be pointed out that variation in the yield of total RNA may also be independent of the method used and fluctuations in yield from animal to animal occur, as the number of available leukocytes could be influenced by environmental and stress conditions (DHABHAR *et al.* 1996).

Isolation of RNA from blood leukocytes with the LeukoLOCK™ system yielded significantly higher amounts of total RNA than samples processed with TRI Reagent® BD. However, the yield of total bovine RNA obtained with the LeukoLOCK™ system in this current study was low compared to a previous report using the same method to extract RNA from bovine blood leukocytes (HAMMERLE-

FICKINGER *et al.* 2010). The difference in yield observed between this study and previously published findings may be due to the freezing of samples prior to RNA isolation herein versus the use of freshly collected blood in the other, as frozen storage of tissues may affect the quantity and quality of total RNA obtained (HOLLAND *et al.* 2003; PEREZ-NOVO 2005).

RNA isolation from bovine whole blood with TRI Reagent® BD was expected to yield high quality total RNA from a one-step extraction method while protecting the transcripts from degradation by RNases since TRI Reagent® BD contains GITC. High quality total RNA was also expected from extraction of whole blood with PAXgene® BloodRNA tubes since blood was collected directly into a tube containing a proprietary RNA stabilizing additive and a cationic detergent to prevent *in vitro* RNA degradation. The quality of total RNA isolated with PAXgene® BloodRNA tubes or TRI Reagent® BD was possibly affected by the presence of red blood cell components in the whole blood lysates (FLEIGE and PFAFFL 2006). A similar study determined the best method for obtaining total RNA from bovine blood samples that require frozen storage was by removal of the red blood cell components prior to freezing blood leukocytes in a TRI Reagent® BD equivalent (HAMMERLE-FICKINGER *et al.* 2010).

The LeukoLOCK™ total RNA isolation system utilizes filters that isolate blood leukocytes from hemoglobin and other blood cell components. The leukocyte containing filters are then washed with phosphate buffered saline to remove residual red blood cell constituents prior to stabilization in RNALater® to protect transcripts from degradation by RNases. According to the manufacturer's unpublished observations, there were no

differences in gene expression values between total RNA extracted from blood leukocytes bound on LeukoLOCK™ filters that were processed immediately or stored frozen, as analyzed by qRT-PCR (http://www.invitrogen.com/site/us/en/home/References/Ambion-Tech-Support/rna-isolation/tech-notes/isolate-rna-from-white-blood-cells-captured-by-a-novel-filter-sy.html [accessed on 02/21/2013]). Storage times and conditions may also affect the quality of RNA isolated from blood (FLEIGE and PFAFFL 2006). Furthermore, unknown factors such as exogenous contaminants, RNases, hemoglobin, phenol, and other organic compounds may lead to tissue-matrix-effects that interfere with reverse transcription and other downstream applications (FLEIGE and PFAFFL 2006). Since degraded RNA affects the efficiency and Ct values of qRT-PCR assays, total RNA from blood leukocytes with a minimum RIN score of 5 is recommended for reliable qRT-PCR performance (FLEIGE and PFAFFL 2006). In this study, high quality RNA was isolated from blood leukocytes that were obtained using the LeukoLOCK™ total RNA isolation system and stored frozen, further corroborating the utility of this method for the isolation of high quality RNA from frozen bovine blood leukocytes.

The goal in this current study was to identify mechanisms of bovine immunity elicited by cattle in response to infestation by *A. americanum* ticks in anticipation of elucidating genetic factors that contribute to tick-resistance. Tick bite-site biopsies and blood leukocytes were collected from two groups of calves during experimental Lone Star tick infestations, and gene expression was evaluated by microarray and qRT-PCR techniques. Phenotyping of cattle determined that Group 4 consisted of three moderately

tick-resistant and three highly tick-resistant calves that were sampled during the second tick infestation of a phenotyping trial, while Group 5 contained two tick-susceptible, three moderately tick-resistant, and two highly tick-resistant calves from which specimens were obtained throughout three tick infestations.

A total of 35 genes associated with bovine immunity to ticks, as reported by previous studies of *Rhipicephalus (Boophilus) microplus* infestations in tick-susceptible and -resistant cattle (KONGSUWAN *et al.* 2008; PIPER *et al.* 2008; PIPER *et al.* 2009; WANG *et al.* 2007b) or identified by microarray analyses of one of the three cattle phenotypes infested with *A. americanum* ticks in this current study, were selected for gene-specific expression profiling via qRT-PCR in tick bite-site biopsies from Group 4 calves. Eight of these 35 candidate genes were also evaluated in blood leukocytes from Group 4 calves. Microarray experiments compared global gene expression in tick bite-site biopsies on days 0, 5, and 10 from Group 5 calves during the first and second tick infestations for each cattle phenotype in this study. Analysis of microarrays identified genetic pathways that may influence bovine immune responses to Lone Star ticks and a total of six differentially expressed genes associated with immune mechanisms were selected for gene-specific analysis by qRT-PCR in tick bite-site biopsies from Group 5 calves in this study. An additional five genes of interest were selected based on increased expression, as determined by qRT-PCR profiles of tick bite-site biopsies from Group 4 calves, and were also evaluated by qRT-PCR in tick bite-sites from Group 5 calves. A total of six genes from the 11 candidates described above were profiled in blood leukocytes collected from Group 5 calves.

Of the 35 genes evaluated by qRT-PCR in tick bite-sites from Group 4 calves, 13 genes were significantly increased ($p = \leq 0.05$) on day 10 of the second tick infestation including major histocompatibility complex class II subunit DRB3 (*MHCII-DRB3*), interleukin 1-receptor accessory protein (*IL1RAP*), NOD-like receptor family, pyrin domain containing 3 (*NLRP3*), Tumor necrosis factor receptor type 1-associated DEATH domain protein (*TRADD*), interleukin 1 alpha (*IL1α*), interleukin 2-receptor alpha (*IL2Rα*), interleukin 6 (*IL6*), interleukin 8 (*IL8*), interleukin 10 (*IL10*), interleukin 12 subunit p40 (*IL12p40*), TNF receptor-associated factor 3 (*TRAF3*), Caspase 8 (*CASP8*), and immunoglobulin heavy chain gamma-1 (*IGHG1*). Interestingly, the observed increased expression of these 13 immune genes on day 10 coincided with the decreased expression of p36 immunosuppressant genes in feeding *A. americanum* ticks as they progressed from a partially fed stage into the rapid engorgement phase (ALJAMALI *et al.* 2009b). Since the ticks used in this study secure host attachment over the first few days after host exposure, and then feed slowly for five to seven days, it is speculated that female ticks enter the rapid engorgement phase of feeding between days 8 and 10 of the tick infestation. The increased expression observed for genes such as *IGHG1*, *IL6*, *IL2Rα*, *IL10*, *IL12*, and *MHCII-DRB3* suggest that both T cells and B cells may play a role in mediating the bovine host immune response to Lone Star tick infestation. This hypothesis is consistent with previous reports of associations between tick-resistance and bovine immune responses, as both cell-mediated and antibody-mediated mechanisms were implicated (ALLEN 1989; BROWN *et al.* 1984; DE CASTRO and NEWSON 1993; KONGSUWAN *et al.* 2010; KONGSUWAN *et al.* 2008; NASCIMENTO *et*

al. 2010; PIPER *et al.* 2008; PIPER *et al.* 2010; PIPER *et al.* 2009; RIEK 1959; RIEK 1962; WANG *et al.* 2007b).

Correlating with the increased expression of *IGHG1* in Group 4 calves on day 10 of the second tick infestation, *IGHG1* expression was also highest in tick bite-site biopsies on day 10 of all three tick infestations from Group 5 calves. Presuming that *IGHG1* correlates with circulating levels of IgG_1 antibodies, gene expression patterns for this gene in the bovine biopsies herein were unexpected. Considering the shorter lag-time in the IgG_1 antibody response to sensitized antigen (JANEWAY 2008), a peak in expression for *IGHG1* would be expected sooner than day 10 for the second and third tick infestations. However, heavy chain regions of IgG_1 antibodies are comprised of one variable and three constant subunits, and *IGHG1* encodes only one of the three constant subunits. Thus, expression of *IGHG1* may not serve as the best indicator of IgG_1 antibody production (JANEWAY 2008). The significance of increased IgG_1 is that production of this antibody indicates the involvement of a Th2-type humoral-mediated immunity, and thus could implicate the importance of this response in cattle infested by Lone Star ticks, which could be of use for vaccine development. The cutaneous response in mice infested with *Ixodes scapularis* ticks showed a mixed Th1/Th2 cytokine profile during the acquired immune response, a phenomenon contributed to the potential activation of T regulatory cells (HEINZE *et al.* 2012). Furthermore, *Ixodes ricinus* tick saliva inhibited the migration and maturation of murine dendritic cells *in vivo* and *in vitro*, leading to the failure of Th1 or Th17 polarization and the promotion of a Th2 response (SKALLOVÁ *et al.* 2008).

Recently, IgG-allergen complexes in mice were shown to interact with basophils to release platelet activating factor for increased vascular permeability, and these complexes also bound to murine basophil Fc-γ-RII-III cell surface receptors more efficiently than to surface receptors on monocytes, macrophages, dendritic cells, NK cells, neutrophils, and eosinophils (TSUJIMURA *et al.* 2008). Additionally, basophil depletion of mice infested with *H. longicornis* abolished the expression of tick-resistance, and thereby established the importance of these cells for tick-specific acquired immune resistance (WADA *et al.* 2010). Basophils were present in higher numbers at *R. microplus* attachment sites from tick-resistant Nellore cattle, however, IgG_1 antibody levels were higher in peripheral blood from tick-susceptible Holstein-Friesian calves (CARVALHO *et al.* 2010; PIPER *et al.* 2010). Furthermore, the expression of tick-resistance varied among cattle exposed to different tick species, as was observed between the three-host tick, *A. americanum*, and the one-host tick, *R. microplus* (RIEK 1959; RIEK 1962). Degranulation of basophils at tick attachment sites from guinea pigs displaying resistance, as determined by evidence of cutaneous basophil hypersensitivity (CBH) response, to *A. americanum* ticks were considered important for tick-resistance (BROWN and ASKENASE 1985). The CBH response observed for tick-resistant guinea pigs was further confirmed in tick-resistant cattle infested with *A. americanum* ticks (BROWN *et al.* 1984) and in this current study, expression of *IGHG1* was highest in tick bite-site biopsies from the highly tick-resistant phenotype compared to moderately tick-resistant and tick-susceptible Group 5 calves for 9 of the 17 time points measured over the three tick infestations.

Furthermore, CBH responses contributed to gut damage in ticks fed on guinea pigs on two occasions during tick-infestation, once during the tick attachment phase and once at a time that coincides with the rapid engorgement phase of tick feeding. The latter point is presumed to be a time when ticks are most susceptible to damage by IgG_1-mediated basophil degranulation due to the down-regulation of immunosuppressant genes upon entering the rapid engorgement stage of feeding (BROWN and ASKENASE 1985). Basophils infiltrating tick bite-sites from Lone Star tick-infested cattle were absent from peripheral blood samples during expression of the CBH response, however, basal levels returned after tick engorgement was complete (BROWN *et al.* 1984). Basophils were also reported to play a critical role in initiating systemic IgE-mediated allergic chronic inflammation in mice, although much is still unknown about this mechanism (OBATA *et al.* 2007).

Similar to a CBH response, passive cutaneous anaphylaxis (PCA) was dependent upon interaction between Fc-γ-RIII receptors and mast cells at the site of allergen contact in mouse skin (ARIMURA *et al.* 1990). A proposed model for PCA described an immediate hypersensitivity response involving degranulation of mast cells that were stimulated by allergen crossing the skin barrier in previously sensitized hosts (TSUJIMURA *et al.* 2008). However, mast cell degranulation was insufficient for elimination of the allergen and basophils were recruited into the skin, initiating a state of chronic inflammation. The insufficiency of mast cells to mediate anti-tick immunity was evidenced by the lack of tick-resistance after basophil depletion in mice using basophil-restricted diphtheria toxin receptors, and thereby leaving mast cells unaffected (WADA *et*

al. 2010). As thus, it could be presumed that CBH reactions in cattle are mediated by similar mechanisms, and inadequate antigen elimination induced by prolonged host attachment during tick feeding may lead to the recruitment basophils into the skin at tick attachment sites to mediate anti-tick immunity. It was reported that guinea pig IgG_1 antibodies activated complement via the alternative pathway and complement component C5 was chemotactic for basophils, and these factors were reported to play a role in the local host response to Lone Star ticks (ALLEN 1989; BROWN 1985; BROWN and ASKENASE 1985; BROWN *et al.* 1984; BROWN *et al.* 1982a; BROWN *et al.* 1983; WIKEL 1979).

Complement activation was implicated in the mediation of CBH reactions when depletion of complement activity using cobra venom abolished tick-resistance, a mechanism that also reduced the amount of basophils in tick bite lesions of tick-resistant guinea pigs infested by *Dermacentor andersoni*, a three-host tick (ALLEN 1989; WIKEL and ALLEN 1977). Both IgG_1 and IgG_2 antibody isoforms were reported to fix complement in cattle, however, IgG_1 antibodies more efficiently activated complement compared to IgG_2 (MCGUIRE *et al.* 1979). In this current study, expression of the gene encoding the complement component, q subcomponent (*C1Q*), in tick bite-site biopsies from the six Group 4 calves peaked on day 7 during the second tick infestation. This peak in expression suggests that activation of complement factors may play a role in mediating the bovine acquired immune response to Lone Star ticks. Bovine host IgG_1 antibodies may bind to tick antigens, and could interact with C1q via Fc receptors to initiate the classical complement pathway. However, further studies are necessary to

elucidate the role these antibody-mediated complement mechanisms play in mediating a Th2 type immune response in *A. americanum* tick-infested cattle.

The expression of two cytokine genes, *IL6* and *IL10*, further support the involvement of a Th2 humoral-mediated response in cattle infested with *A. americanum* ticks. IL-6 is a key mediator of host immune responses to foreign antigens that is produced by fibroblasts, macrophages, dendritic cells, T and B cells, endothelial cells, and keratinocytes (AKIRA *et al.* 1993; DIEHL and RINCÓN 2002). IL-6 is released by keratinocytes during delayed-type hypersensitivity reactions and is thought to regulate the transition from the innate neutrophil-dominated response to the acquired macrophage-dominated response during chronic inflammation (JANEWAY 2008; KAPLANSKI *et al.* 2003). In addition, IL-6 induced expression of IL-4 and inhibited expression of IFNγ in mice, which promoted differentiation of $CD4^+$ T cells into a Th2-type subset (RINCÓN *et al.* 1997). In this current study, expression of *IL6* in tick bite-site biopsies from six Group 4 calves was highest on day 10 of the second tick infestation. In skin biopsies from the highly tick-resistant Group 4 calves, *IL6* expression was 1000-fold higher on day 10 compared to the moderately tick-resistant phenotype, suggesting that IL-6 may play a role in mediating the acquired immune response in Lone Star tick-resistant cattle. Presuming that IL-6 also promotes Th2 differentiation in cattle as in mice, these observations would be expected and suggest that IL-6 may contribute to a Th2-mediated acquired response in Lone Star tick-resistant cattle.

Consistent with previously discussed gene expression supporting a role for Th2-mediated acquired immunity for tick-resistant cattle in this current study, expression

for *IL10* was notably higher in tick bite-site biopsies from highly tick-resistant Group 4 calves compared to moderately tick-resistant cohorts on days 3, 4, 5, 6, and 10 of the second tick infestation. Additionally, expression of *IL10* in tick bite-sites from all six Group 4 calves was significantly up-regulated on day 10 of the second tick infestation. In contrast to results reported in this study, expression of *IL10* was increased in tick attachment sites from *R. microplus* tick-susceptible Holstein-Friesian calves compared to those taken from tick-resistant Brahman cohorts (PIPER *et al.* 2008). The differences in association of tick-resistance and -susceptibility with IL-10 may be explained in part by differences in bovine host responses elicited by a three-host tick, *A. americanum*, and a one-host tick, *R. microplus*, which may be due to differences in bite depth for these tick species, as discussed above. Tick saliva from another three-host tick, *Dermacentor variabilis*, preferentially mediated a Th2 cytokine profile in a murine macrophage cell line, as evidenced by increased expression of IL-4 and IL-10 (KRAMER *et al.* 2011).

IL-10 was reported to reduce chronic inflammation by inhibition of Th1-type immunomodulatory factors including IL-1, TNF-α, IL-6, IL-8, IL-12, IFN-γ, IL-2, and MHC class II receptors in human cell lines, and thus has become a critical immune regulator between the inflammatory and the humoral immune responses (BEJARANO *et al.* 1992; CASSATELLA *et al.* 1993; CAUX *et al.* 1994; DE WAAL MALEFYT *et al.* 1991; DE WAAL MALEFYT *et al.* 1993; FIORENTINO *et al.* 1991). In mice, IL-10 was reported to amplify the effects of IL-4 and thereby enhance Th2-mediated immunity by promoting proliferation of natural killer (NK) cells, production of IgG antibodies from B cells,

expression of Fc receptors on monocytes/macrophages, and activation of CD8+ T cells (CARSON *et al.* 1995; CHEN and ZLOTNIK 1991; DEFRANCE *et al.* 1992; GO *et al.* 1990; ROUSSET *et al.* 1992; TAN *et al.* 1993; TE VELDE *et al.* 1992). In this current study, microarray analysis of bovine tick bite-site biopsies from tick-susceptible, moderately tick-resistant, and highly tick-resistant phenotypes revealed an up-regulation of genes associated with natural killer cell cytotoxicity and B cell responses for all 3 phenotypes. Presuming that IL-10 acts to limit Th1-type responses and promote antibody activity in cattle, as reported in human and mouse models, analysis of microarray data in this study suggests that increased *IL10* expression may influence immunomodulatory factors involved in a Th2-type acquired immune response in Lone Star tick-infested cattle. The T helper responses discussed above are mediated by MHCII presentation of antigen to T cells, and as such MHC genes may influence tick-resistance and -susceptibility in cattle (KONGSUWAN *et al.* 2008).

The MHC homologue in cattle, known as bovine leukocyte antigen (BoLA), is reported to influence cattle growth, reproduction, milk yield, and disease resistance (ACOSTA-RODRÍGUEZ *et al.* 2005; DIETZ *et al.* 1997; MARTINEZ *et al.* 2006; PARK *et al.* 1993; TAKESHIMA and AIDA 2006; TEALE *et al.* 1999; TEMPLETON *et al.* 1988; VAN EIJK *et al.* 1992). Analysis of restriction fragment length polymorphisms (RFLP) in dairy cattle revealed associations between BoLA class II DRB3 alleles and increased production of IgM, IgG_2, and complement activity, and decreased numbers of mononuclear cells (DIETZ *et al.* 1997). In this current study, expression of *MHCII-DRB3* in tick bite-site biopsies from all six Group 4 calves rose significantly each day until

reaching a peak on day 10 of the second tick infestation, supporting the hypothesis that acquired immune responses elicited in cattle infested with Lone Star ticks are likely influenced by BoLA class II DRB3 genes.

Previously, an analysis of microsatellite markers in cattle of mixed origin infested with *R. microplus* ticks revealed that alleles in the BoLA class II DRB3 region correlated with increased tick numbers, and therefore were associated with tick-susceptibility (ACOSTA-RODRÍGUEZ *et al.* 2005). In contrast, RFLP analysis of the BoLA class II DRB3 region in Gyr x Holstein cattle infested with *R. microplus* larvae identified several BoLA class II DRB3 alleles associated with low tick numbers, and thus associated these alleles with the tick-resistant phenotype (MARTINEZ *et al.* 2006). Additionally, RFLP analysis of tick-susceptible and -resistant calves infested with *A. americanum* ticks identified an association between BoLA-DRB3 allele *4401 and the tick-resistant phenotype (UNTALAN *et al.* 2007). It should be mentioned, however, that analyses of BoLA alleles performed using the Btau 3.1 bovine genome assembly should be reevaluated for confirmation due to the significant differences found between Btau 3.1 and the most recent bovine genome assembly, Btau 4.0 (BRINKMEYER-LANGFORD *et al.* 2009). While this should not discount the existence of associations between BoLA class II DRB3 alleles and tick-susceptibility or -resistance, the specific alleles identified are no longer considered accurate and require further studies to substantiate the previously reported allele-specific associations.

Once activated by MHCII-mediated presentation of antigen, T lymphocytes express high affinity IL-2Rα on the surface to promote differentiation and proliferation

of the specific T helper subsets discussed above (JANEWAY 2008). In this current study, expression of *IL2Rα* in tick bite-site biopsies from the six Group 4 calves was similar to observations for *MHCII-DRB3*, rising significantly each day until reaching a peak on day 10 of the second tick infestation. This supports the hypothesis that T cells play a role in the bovine acquired immune response elicited by *A. americanum* infestation. Furthermore, *IL2Rα* expression was notably higher in tick bite-sites from highly tick-resistant Group 4 calves on days 3, 4, and 6 compared to the moderately tick-resistant cohorts, further suggesting that T cell activation may be involved in mediating in the local expression of tick-resistance in Lone Star tick-infested cattle. In contrast, expression of *IL2Rα* was significantly higher in peripheral blood leukocytes from tick-resistant *R. microplus* tick-infested cattle compared those taken from tick-susceptible cohorts, which also supports a role for IL-2Rα in the systemic response to tick infestation (PIPER *et al.* 2009).

Differentiation of mammalian naïve T cells into a Th1 subset is initiated by dendritic cell expression of IL-12, which promotes cell-mediated immunity by inducing secretion of pro-inflammatory factors including IFN-γ, IL-2, TNF-α, and TGF-β (AKIRA *et al.* 2001; JANEWAY 2008; TIZARD 2004). In early activated dendritic cells of mice, secretion of IL-12 initiated the development of a Th1 type cell-mediated response (LANGENKAMP *et al.* 2000). However, prolonged activation of murine dendritic cells reduced IL-12 production, and led to preferential differentiation of a Th2-type response (KALIŃSKI *et al.* 1999; REIS E SOUSA *et al.* 1999). This change in T helper subsets mediated by early and protracted activation of dendritic cells could contribute to the

recruitment of basophils during chronic inflammatory reactions, such as CBH and PCA, discussed above. In bovine peripheral blood leukocytes treated with SGEs from *B. microplus* ticks, expression of Th1-mediating cytokines including IFN-γ, TNF-α, and IL-12 were decreased in a time-and dose-dependent manner during an LPS-induced immune response compared to similarly stimulated control samples (BRAKE and PÉREZ DE LEÓN 2012).

In this current study, expression of *IL12p40* significantly increased in tick bite-site biopsies from the six Group 4 calves on all days measured during the second tick infestation and peaked on day 10, suggesting that a local Th1 response may also be active in *A. americanum* tick-infested cattle. If maturation of dendritic cells is similarly mediated in cattle as in mice and contribute to a Th2 response, then a reduction in *IL12p40* expression would be expected at day 10. However, IL-12 is encoded by two genetically distinct subunits, p35 and p40, of which the latter is known to be used by additional immune pathways (JANEWAY 2008; OPPMANN *et al.* 2000), and thus the possibility that prolonged Th1 activation may contribute to a Th2-type immune response in tick-infested cattle should not be disregarded. The expression of a single subunit may not directly correlate with expression of the functional IL-12 protein and the mechanism by which IL-12 production is decreased in mature dendritic cells of mice and cattle remains to be characterized.

In cattle, IFN-γ was reported to mediate Th1 differentiation, and influence the production of IgM and IgG_2 antibodies from B cells (ESTES *et al.* 1994). IFN-γ mediates T lymphocyte migration, and thus was originally called the macrophage activating

factor. Differentiation into a Th1 subset is regulated by IFN-γ-stimulation of immunoglobulin production from B cells and promotion of NK cell function in human T cells (Schroder 2004). In this current study, increased expression of *IFNγ* in tick bite-site biopsies from the six Group 4 calves peaked on day 7 of the second tick infestation, suggesting that NK cell- and Th1-mediated immune responses may be important for the acquired local response in cattle infested by Lone Star ticks. Additionally, *IFNγ* expression was higher in tick bite-site biopsies of highly tick-resistant calves compared to moderately tick-resistant cohorts during the first week. This was unexpected given the reports of *IFNγ* inhibition by IL-10 in human CD4+ cell lines (DE WAAL MALEFYT *et al.* 1993). However, these gene interactions could differ in bovine CD4+ cells, other cells infiltrating tick bite-sites, and in vivo versus results reported from *in vitro* studies.

Natural killer cell activity was up-regulated on days 5 and 10 in tick bite-sites from all three phenotypes for Group 5 calves, as determined by microarray analysis, and this suggests that IFN-γ may work in concert with IL-10 and other factors to enhance the function of bovine natural killer cells during tick infestation. Furthermore, *IFNγ* expression in blood leukocytes for all six Group 4 calves was also significantly increased during the first week of the second tick infestation. Although overall *IFNγ* expression was lower in blood leukocytes with a peak on day 10 instead of day 7 compared to observations from tick bite-sites, this finding supports the hypothesis that IFN-γ may play a role in both the local and systemic bovine responses to Lone Star tick infestation.

In this current study, a significant increase in expression of *TNF-α* in tick bite-site biopsies was observed on day 5 of the second tick infestation for the six Group 4 calves. As a Th1 promoting cytokine produced mainly by activated macrophages and T cells, *TNF-α* mediates leukocyte migration during acute phase responses by inducing the expression of adhesion molecules and MHC antigen receptors (DUSTIN *et al.* 1986; JANEWAY 2008; OSBORN *et al.* 1989; POBER *et al.* 1986; TIZARD 2004; WALSH *et al.* 1991). Microarray analyses of tick bite site biopsies from moderately tick-resistant and tick-susceptible Group 5 calves in this study showed adhesion factors were up-regulated on day 5 and down-regulated on day 10, suggesting that TNF-α may influence cell adhesion factors during the bovine local immune response elicited by Lone Star ticks. This gene was not profiled in blood leukocytes from cattle in this current study, however, evaluation of peripheral blood leukocytes from tick-resistant and -susceptible cattle infested with *R. microplus* ticks found that *TNFα* expression was significantly higher in blood from tick-resistant cattle compared to samples from tick-susceptible cohorts, and thus may also be important for the systemic response mediating the expression of tick-resistance (PIPER *et al.* 2009).

Innate mechanisms of immunity influence acquired immunity, and thus the roles of innate signaling factors in bovine immune responses to ticks were explored in this study. Initial recognition of foreign antigens by sentinel cells is mediated by the activation of pattern recognition receptors (PRRs) (JANEWAY 2008). The most well-studied PRRs are the mammalian Toll-like receptors (TLRs), a family of highly-conserved transmembrane proteins capable of binding to molecular patterns on

pathogens (TIZARD 2004). However, other PRRs such as the interleukin-1 receptor (IL-1R) are also highly conserved among mammals, and share a conserved cytoplasmic domain with TLRs, known as Toll-IL1 receptor (TIR) domain (ADACHI *et al.* 1998). The extracellular domains of mammalian PRRs differ and allow for recognition of a greater variety of patterns displayed on the surface of pathogens and foreign antigens (AKIRA *et al.* 2001; BECKER and O'NEILL 2007; SIMS 2002). Activation of multiple PRRs simultaneously influences the specificity for cytokine production, and this regulates the development of T helper cell responses (MOSER and MURPHY 2000; PULENDRAN *et al.* 2001).

In this current study, a strong correlation between *IL1*α and *TLR4* was identified in tick bite-site biopsies from five of the seven Group 5 calves, suggesting a relationship may exist between these two pathways in response to *A. americanum* tick-infestation. Moreover, gene expression for *TLR4* and its co-receptor, *CD14*, was similarly increased in tick bite-site biopsies from Group 4 and Group 5 calves with peaks occurring on days 5 and 10 for all tick infestations evaluated, suggesting that TLR-4 may be involved in mediating innate and acquired local immune responses to Lone Star ticks. In contrast, expression of *TLR4* was decreased, while *CD14* expression was increased in murine macrophages following exposure to *D. variabilis* tick saliva (KRAMER *et al.* 2011), which supports the hypothesis that host immunomodulation by tick salivary molecules is mediated in a species-specific manner.

Mammalian TLR-4 generally recognizes lipid proteins, however, this factor may also be activated by an array of host ligands including extracellular matrix fragments,

fibrogen, endogenous heat shock proteins, β-defensins, high mobility group box 1, hyaluronic acid, heparin sulfate, and other danger associated molecular patterns (DAMPs) from injured and dying cells, as would occur during wounding inflicted by tick attachment (AKIRA and TAKEDA 2004; BARRAT and COFFMAN 2008; JIANG *et al.* 2005; TIZARD 2004). Interestingly, genes encoding heat shock and calcium binding proteins were up-regulated on day 5 and down-regulated on day 10 in tick bite-site biopsies from highly and moderately tick-resistant Group 5 calves, as determined by microarray analyses in this current study. Furthermore, activation of ligand-bound TLR-4 on dendritic cells of human patients with cutaneous systemic sclerosis promoted the production of pro-inflammatory cytokines (VAN BON *et al.* 2010). Thus, it appears that TLR4-mediated signaling may play a role in mediating the host inflammatory responses in Lone Star tick-infested cattle as follows.

Once stimulated, both TLRs and IL-1Rs recruit MyD88 to initiate signaling pathways that lead to the activation of transcription factors, which produce an array of cytokines (AKIRA and TAKEDA 2004; O'NEILL and GREENE 1998). Studies in mice reported that MyD88 signaling is essential for IL-1- and some TLR-mediated responses (ADACHI *et al.* 1998; TAKEUCHI *et al.* 2000). Moreover, dendritic cells from MyD88 deficient mice did not respond to activation by several PRR ligands, including IL-1, IL-18, peptidoglycan, and CpG-DNA. Coinciding with expression of patterns observed for *TLR2* and *TLR4* in tick bite-site biopsies from calves in this current study, expression of *MyD88* in biopsies from the six highly and moderately tick-resistant Group 4 calves was significantly increased on all days measured and peaked on day 5 during the second

tick infestation. Analyses of these expression data suggest that both *TLR2* and *TLR4* signal via a MyD88-dependent pathway and may play a role in mediating the bovine local response to Lone Star ticks. Analysis of tick attachment site biopsies from *R. microplus* tick-infested cattle reported significantly higher expression of *TLR2* and *MyD88* in biopsies from tick-susceptible Holstein-Friesian calves compared to tick-resistant Brahman calves (KONGSUWAN *et al.* 2008; PIPER *et al.* 2008). Furthermore, a Th2 immune response was preferentially mediated by TLR-2 activation in a murine macrophage cell line after exposure to *D. variabilis* tick saliva (KRAMER *et al.* 2011). These findings suggest that TLR2-mediated signaling may be important for polarization of the local bovine immune response to three-host ticks, such as *A. americanum* and *D. variabilis*. However, more studies are necessary to determine how TLR-2 mediating factors affect the immune responses of cattle infested with different species of ticks.

Molecules that signal downstream of MyD88 in IL-1R- and TLR-mediated pathways and lead to activation of the transcription factor NFkB include IL-IR-associated kinases, transforming growth factor-β-activated kinases (TAK), TAK-binding proteins, and tumor necrosis factor-receptor-associated factor 6 (TRAF6) (AKIRA and TAKEDA 2004). In this current study, expression of *TRAF6* and *NFkB* in tick bite-site biopsies from Group 4 calves did not change significantly throughout the second tick infestation and was not differentially expressed between moderately and highly tick-resistant cohorts. Thus, no link between these factors mediating cell signaling and increased expression of cytokines in the local response of these cattle to *A. americanum* tick-infestation was found in this study. In contrast, expression of both *NFkB* and *TRAF6*

was significantly increased in tick attachment sites from tick-susceptible Holstein-Friesian calves infested with *R. microplus* ticks compared to sites taken from tick-resistant Brahman calves (KONGSUWAN *et al.* 2008; PIPER *et al.* 2008).

In this current study, *TLR2* expression in tick bite-site biopsies from the six Group 4 calves was significantly increased on all days measured with peaks on day 5 and day 10 of the second tick infestation. *TLR2* was similarly expressed in blood leukocytes from the six Group 4 calves in this study, but was much lower and peaked only on day 10. Analysis of expression profiles for *TLR2* in this study suggests the possible involvement of TLR-2-mediated signaling in both local and acquired immune responses of Lone Star tick-infested cattle. Interestingly, TLR-2 activation stimulated proliferation and cytokine production from bovine γ/δ T cells (WESCH *et al.* 2011). Previously, tick-resistant Brahman cattle infested with *R. microplus* ticks had higher numbers of γ/δ T cells infiltrating tick attachment sites compared to biopsies from tick-susceptible Holstein-Friesian calves (CONSTANTINOIU *et al.* 2010), and this may explain in part the higher expression of *TLR2* in bite-site biopsies compared to blood leukocytes in this current study. However, higher concentrations of circulating γ/δ T cells were also found in blood leucocytes from *R. microplus* tick-resistant Brahman compared to Holstein-Friesian calves (PIPER *et al.* 2009).

TLR5 and *TLR9* were evaluated in tick bite-site biopsies from *A. americanum* tick-infested calves in this current study due to previously suggested associations in *R. microplus* tick-susceptible cattle (PIPER *et al.* 2008). Up-regulation of *TLR5* and *TLR9* expression was found in tick attachment sites from *R. microplus*-susceptible calves

compared to sites without ticks attached, but not between tick-attachment and non-attachment sites from tick-resistant calves (Piper 2008). Similar to the previously reported results from *R. microplus* infestations, microarray analyses of tick bite-site biopsies from *A. americanum* tick-susceptible Group 5 calves in this current study revealed that expression of *TLR5* was up-regulated on day 5 and down-regulated on day 10, although analysis of *TLR5* expression via qRT-PCR in tick bite-sites from Group 4 and Group 5 calves showed no changes in expression. This lack of correlation between microarray and qRT-PCR results was unexpected, but this may have occurred due to the probability of identifying false positives during microarray analyses, a pitfall that will be discussed below.

In contrast, *TLR5* expression in blood leukocytes was increased with a peak on day 10 for the six tick-resistant Group 4 calves during the second tick infestation and was similarly up-regulated in blood from the Group 5 calves (two tick-susceptible and five tick-resistant) during the second tick infestation this current study, suggesting that TLR-5 may play a role in the bovine systemic response to *A. americanum* tick infestations. The differences between the local and systemic expression of *TLR5* in this study are not clear.

In contrast to the results from a previous study that showed an association between *TLR9* expression and the tick-susceptible bovine phenotype (PIPER *et al.* 2008); expression of *TLR9* was significantly increased in tick bite-site biopsies from the six tick-resistant Group 4 calves on all days measured with a peak on day 7 during the second tick infestation in this current study. Analyses of these results suggest that TLR-9

may play a role in the local bovine acquired immune response to *A. americanum* tick infestation.

It has been argued that PAMP recognition by TLRs required a secondary signal via detection of a DAMP by another class of PRR, such as Nod-like receptors (NLRs) and Rig-like helicases, to initiate an inflammatory response (ISHII *et al.* 2001; MARTINON *et al.* 2006). These classes of PRRs were reported to recognize both microbial PAMPs and endogenous DAMPs in the cytoplasm of cells, leading to activation of inflammasomes for the recruitment of caspases, which mediate cell survival and death (BECKER and O'NEILL 2007). Inflammasomes are particularly important for host inflammatory responses to wounding, as would occur during tick attachment. An inflammatory response results from recruitment of Casp-1 and subsequent cleavage of pro-IL-1β and pro-IL-18 mRNA into mature forms, a mechanism largely dependent upon activation of the NLRP3-inflammasome (MARTINON *et al.* 2009). In this current study, expression of *NLRP3* and *IL18* in tick bite-site biopsies from the six Group 4 calves was up-regulated during the second tick infestation with a peak on day 10 and day 7, respectively. Analyses of these results suggest that the NLRP3-inflammasome may be involved in mediation of the bovine local immune response to *A. americanum* infestation.

Expression of another factor in the NLRP3-inflammasome pathway, *IL1β*, in tick bite-site biopsies from the seven Group 5 calves was significantly increased on day 10 of the first and second tick infestations, yet no significant differences in expression were observed in biopsies from the six Group 4 calves. Also, no significant differences

for *IL1β* expression were found in blood leukocytes from Group 4 or Group 5 calves, which was unexpected since IL-1β is the secreted IL-1R ligand and primarily produced by leukocytes (LEE *et al.* 2009). Interestingly, suppression of IL-1β was reported to occur in laboratory animals infested by several tick species, including *R. appendiculatus* and *D. andersoni* (FUCHSBERGER *et al.* 1995; RAMACHANDRA and WIKEL 1992; RAMACHANDRA and WIKEL 1995). Tick immunomodulatory molecules could potentially target or indirectly affect factors that mediate the NLRP3-inflammasome, such as IL-1β, *Casp1* and *NFkB*, and may explain in part the lack of differential expression observed for these genes in tick bite-site biopsies and blood leukocytes from Lone Star tick-infested cattle in this study.

Although distinct genes encode IL-1α and IL-1β, both produce a 31 kDa cytoplasmic ligand that initiates signaling by binding the IL-1 receptor (BLACK *et al.* 1988; DINARELLO 1996; MOSLEY *et al.* 1987). IL-1α is stored as a pre-formed molecule in human keratinocytes and epithelial cells involved in maintenance of the skin barrier and released in response to wounding, as would occur upon tick attachment (LEE *et al.* 2009). In transgenic mice, keratinocyte stimulation by exogenous IL-1α induced a strong cutaneous inflammation (GROVES *et al.* 1995). In this current study, microarray analyses of tick bite-site biopsies from tick-susceptible Group 5 calves revealed increased expression for *IL1α* days 5 and 10, an observation supported by qRT-PCR analyses in these biopsies. Furthermore, expression of *IL1α* in tick bite-site biopsies from the six Group 4 calves was up-regulated on all days measured with a peak on day 10 of the second tick infestation, and was similarly expressed during the first and second

tick infestations for the seven Group 5 calves. These observations suggest a role for IL-1α in mediating the bovine local immune response to Lone Star ticks, as expected. Expression of *IL1α* was not detectable in blood leukocytes from Group 4 or Group 5 calves, which was expected since IL-1α is the membrane-bound ligand for IL-1R, and therefore not secreted into the bloodstream (LEE *et al.* 2009).

Factors such as IL-1RAP, IL-1RN, and IL-1R2 are involved in the regulation of IL-1R signaling, and the co-receptor IL-1RAP is required for signal initiation following ligand and receptor binding (HUANG *et al.* 1997). Expression for *IL1RAP* was expected to appear similar to patterns for *IL1α* or *IL1β*, and expression of this gene in tick bite-site biopsies from the six Group 4 calves was similar to observations for *IL1α* with a significantly increased peak on day 10 of the second tick infestation. Microarray analyses also showed up-regulation of *IL1RAP* on day 5 and down-regulation on day 10 in tick bite-site biopsies from moderately tick-resistant Group 5 calves. In contrast, no significant changes for expression of *IL1RAP* in tick bite-site biopsies from the seven Group 5 calves were identified by qRT-PCR. The factors that may affect gene expression among the two different groups of calves and contribute to the observed inconsistencies in the results reported in this study will be discussed below.

A decoy receptor, encoded by *IL1R2*, was reported to regulate IL-1 induced inflammation via an IL-4 dependent mechanism in human leukocytes (COLOTTA *et al.* 1993). Since IL-1R2 functions to limit inflammation, expression of this gene was expected to be similar to observations for *IL1α*. However, expression of *IL1R2* in tick bite-site biopsies from Group 4 and Group 5 calves via qRT-PCR revealed no significant

changes. In contrast, microarray analyses of tick bite-site biopsies from highly tick-resistant Group 5 calves found down-regulation of *IL1R2* on day 5 and day 10. As mentioned above, inconsistencies between microarray and qRT-PCR data will be discussed below, however, interpretation of these data suggests that IL-1R2 does not likely contribute to immune responses elicited by cattle infested with Lone Star ticks.

The final IL-1R-mediating gene evaluated in this study, *IL1RN*, encodes an IL-1 receptor antagonist (IL-1RA) that inhibits signaling by blocking access of IL-1α and IL-1β to IL-1R in murine thymocytes (SECKINGER *et al.* 1987). As reviewed by Perrier *et al.* 2006, IL-1RA is secreted by various cell types in the skin such as epithelial cells and keratinocytes, and the balance of this antagonist with IL-1 influenced inflammation and disease susceptibility in both humans and mice (AREND 2002; IWAKURA 2002; PALMER *et al.* 2007; PERRIER *et al.* 2006). In this current study, microarray analyses of tick bite-site biopsies from moderately tick-resistant Group 5 calves revealed down-regulation of *IL1RN* expression on day 10, whereas qRT-PCR analysis revealed up-regulation in the local response on day 10. The higher fold change observed for qRT-PCR profiles in tick bite-site biopsies from the seven Group 5 calves on day 10 of the first tick infestation compared to the fold change on day 10 of the second tick infestation likely contributed to the down-regulation for *IL1RN* identified via microarrays. Furthermore, the expression ratios between the first and second tick infestations for *IL1RN* were confirmed by analysis of qRT-PCR data, and thereby corroborate the microarray results reported in this study. Expression of *IL1RN* in tick bite-site biopsies from the six Group 4 calves was similar to the patterns observed for Group 5 calves,

with the significantly increased peak on day 10 of all three tick infestations. Correlations between *IL1α* and *IL1RN* expression were identified in tick bite site-biopsies from four of the seven Group 5 calves in this study, and the strongest correlations occurred in biopsies from the highly tick-resistant phenotype. Analyses of these results suggest that expression of *IL1RN* may be influenced by *IL1α* in tick bite-sites and could play a role in mediating the local inflammatory response for Lone Star tick-resistant cattle.

Apoptotic inflammasomes, or apoptosomes, recruit initiator caspases including Casp-8, -10, -2, and -9 to activate executioner caspases such as Casp-3, -6, and -7 to mediate programmed cell death (MARTINON *et al.* 2009). The tumor necrosis factor receptor 1 (TNFR-1) was reported to bind an adapter protein, known as the TNFR-SF1A-associated via death domain (TRADD), and facilitate an association between the fas-associated death domain and caspase-8 during apoptosis in human cell lines (MICHEAU and TSCHOPP 2003). Furthermore, the lymphotoxin-β receptor on the surface of human epithelial and fibroblast cells mediated apoptosis via a TNFR-associated mechanism that recruited the tumor necrosis factor receptor-associated factor 3 (TRAF-3) in a ligand-dependent manner (KUAI *et al.* 2003). In this current study, *Casp8* expression was significantly increased in tick bite-site biopsies from all six Group 4 calves on all days measured with a peak on day 10 of the second tick infestation. Similar patterns of expression were also observed for *TRAF3* and *TRADD* in tick bite-sites from Group 4 calves, suggesting that apoptosis is involved in the acquired immune response elicited in Lone Star tick-infested calves. It is unclear how mechanisms of apoptosis may affect the expression of tick-resistance, however, one possibility is that these factors may

be up-regulated to maintain the integrity of immune responses by removing cells that no longer function properly.

Non-canonical inflammasome mechanisms have been reported to process pro-IL-1β into its functional form by alternate factors, such as proteinase 3, cathepsin B, elastase, caspase-11, and caspase-8, and activation of this pathway via the last factor was associated with TLR-4 activation in mice (KAYAGAKI *et al.* 2011; MAELFAIT *et al.* 2008; NETEA *et al.* 2010). In this current study, expression of *Casp8* in tick bite-site biopsies from the six Group 4 calves was similar to that observed for *TLR4*, with a significant peak in expression on day 10 of the second tick infestation. Additionally, *IL1β* was similarly expressed, suggesting that an alternate inflammasome mechanism may contribute to bovine immune responses to *A. americanum* ticks.

Tick lectins were reported to bind host factors including hyaluronic acid, sialic acid, and LPS in tick hemocytes, and were suggested to activate host complement cascades (GRUBHOFFER *et al.* 2004; KUHN *et al.* 1996; UHLÍŘ *et al.* 1996). Furthermore, lectin pattern recognition receptors mediated the innate inflammatory response in mice infested with *I. scapularis* (HEINZE *et al.* 2012). The formation of a caspase-8 inflammasome complex following recognition of the fungal agent *Candida albicans* by a lectin protein initiated Th1- and Th17-type immune mechanisms in human dendritic cells (GROSS *et al.* 2006; LEIBUNDGUT-LANDMANN *et al.* 2007). The activation of Th17 cells from naïve and memory human CD4+ T cells is initiated by interleukin 23, a factor formed when subunits of IL-23p19 associate with IL-12p40 (HARRINGTON *et al.* 2005; OPPMANN *et al.* 2000). To assess the possible involvement of a Th17-type response in

this current study the expression of *IL23* was evaluated in tick bite-site biopsies, and gene expression did not change in biopsies from Group 4 or Group 5 calves. In contrast, expression of *IL23* in blood leukocytes from the seven Group 5 calves was significantly up-regulated on all days measured during the second tick infestation, suggesting the possibility of systemic activation of bovine memory T cells in response to infestation by Lone Star ticks. Although no evidence of an association between Th17-type immunity and *A. americanum* tick-resistance is reported to date, polarization of this pathway was inhibited in acquired cutaneous responses of mice infested with *I. scapularis* and *I. ricinus* ticks (HEINZE *et al.* 2012; SKALLOVÁ *et al.* 2008).

Chemokines are a family of chemotactic cytokines that promote inflammation and regulate the migration of circulating leukocytes into the tissue at sites of injury or infection, as would occur with tick bites (JANEWAY 2008; TIZARD 2004). IL-8 is a chemokine ligand that recruits neutrophils and is produced by the activation of TLRs on macrophages, mast cells, and epithelial cells (TIZARD 2004; UTGAARD *et al.* 1998; WOLFF *et al.* 1998). Accumulation of pre-formed IL-8 in specialized vesicles of endothelial cells, known as Weibel-Palade bodies, was reported to serve as an innate memory mechanism for inflammatory responses (WOLFF *et al.* 1998). In this current study, overall expression for *IL8* in tick bite-site biopsies from the six Group 4 calves revealed significant up-regulation on day 5 and day 10 of the second tick infestation. Furthermore, expression of *IL8* in tick bite-site biopsies of highly tick-resistant Group 4 calves was notably increased compared to moderately tick-resistant cohorts, suggesting

that innate memory mechanisms may contribute to tick-resistance in cattle infested by *A. americanum*.

Expression of *CCL2* was decreased in murine macrophages treated with saliva from *D. variabilis* ticks (KRAMER *et al.* 2011). CCL-2 is a chemokine ligand produced by human T cells, fibroblasts, keratinocytes, endothelial cells, macrophages, and mast cells to attract monocytes and memory T cells to sites of injury or infection (CARR *et al.* 1994; JANEWAY 2008; TIZARD 2004). Due to a previously reported association between *CCL2* expression and *R. microplus*-infested tick-susceptible cattle (PIPER *et al.* 2008), this gene was evaluated in tick bite-site biopsies in this current study. In contrast to the previous report, expression of *CCL2* in biopsies from the six tick-resistant Group 4 calves in this study was significantly up-regulated on all days measured with a peak occurring on days 4 and 5 of the second tick infestation, suggesting the possibility that increased numbers of memory T cells occur at sites of tick attachment in *A. americanum* tick-infested cattle.

Chemokine receptors, expressed on the surface of monocytes and T cells, initiate the recruitment and transendothelial migration of these cells into sites of tissue injury or infection (JANEWAY 2008; TIZARD 2004; WEBER *et al.* 2001). Expression of the chemokine receptor, CCR1, was previously reported to have an association with tick-susceptible cattle infested with *R. microplus* ticks, and therefore was evaluated herein. In this current study, expression of the chemokine receptor *CCR1* in tick bite-site biopsies from the six tick-resistant Group 4 calves was significantly increased on all days measured with a peak on day 5 of the second tick infestation. Analyses of expression for

this gene supports the likelihood that chemokines mediate an influx of T cells at tick bite-sites from cattle infested by Lone Star.

In this current study, the key limitation of the experiments conducted was the small sample size, as Group 4 contained three highly tick-resistant and three moderately tick-resistant calves and Group 5 contained two tick-susceptible, two highly tick-resistant, and three moderately tick-resistant calves. Previously, an estimation of sample size across seven sets of cDNA microarray data revealed that evaluations for outbred populations required a sample size five times larger than was needed for analyses of inbred lines, and this was due to the high variation of genetic influence on gene expression (WEI *et al.* 2004). Use of a less-sensitive false positive rate and a more stringent fold-change cutoff during analyses of microarray data may compensate in part for a small sample size, and thus a two-fold cutoff and no correction for false discovery rates were utilized during analyses of microarray data in this current study. To further compensate for the low number of available biological replicates in this study, microarray experiments compared gene expression across three time points for each cattle phenotype instead of across the three phenotypes for each time point. These actions may have increased the likelihood of obtaining false positives with microarray results, and thus could have contributed to the inconsistencies in gene expression observed between microarray and qRT-PCR methods in the results reported above.

Although qRT-PCR has become the gold standard for validation of microarray experiments, data obtained with these two methods may not always agree. Differences in factors including probe sequences, and thus target locations, between the two methods

were reported to have the greatest impact on variability in the gene expression data obtained (CANALES *et al.* 2006).

In this current study, RNA for microarray experiments was reverse transcribed with oligo-dT primers because these primers bound the poly (A) tail of mRNA to make full-length cDNA copies since the oligonucleotide probes printed on the microarrays used in this study were biased towards the 3′ end of the target cDNA. On the other hand, random primers were used to measure gene-specific expression via qRT-PCR since these primers bound multiple sections of sequence to replicate short copies of overlapping cDNA fragments, and produce the largest number of amplicons for each gene-specific assay.

There also lies an inherent limitation of both methods for detecting weakly expressed genes (CANALES *et al.* 2006). Due to use of a non-specific intercalating dye that bound double-stranded DNA, a melting curve analysis and standard curve was generated for each qRT-PCR assay in this study to assess gene-specificity, amplification efficiency, and dynamic range.

The failure of this study to find noteworthy immunological differences between tick-susceptible and tick-resistant phenotypes may be due to the limitations described above. It may also be that the phenotyping process utilized in this current study was not suited for such a small number of animals, which is likely a primary factor contributing to the lack of substantial findings between the bovine phenotypes evaluated in this study.

Nevertheless, this current study did provide preliminary data for future investigations to elucidate important factors involved in the mediation of bovine tick-

resistance to *A. americanum*. Interpretation of the data reported in this dissertation suggests that factors involved in pattern recognition may influence the development of a Th2 humoral-associated response in Lone Star tick-infested cattle. Increased expression of factors that may contribute to a local Th2 response in these cattle occurred primarily on day 10 of tick infestations. Knowledge of how factors such as complement components and mediators of IgG_1 antibody interactions may affect the engorgement and reproductive capacity of ticks fed on tick-resistant hosts is imperative to the development of alternative tick control methods. Furthermore, studies to further elucidate the roles for innate immune mechanisms for mediation of a Th2 response in tick-infested cattle may provide information leading to optimal adjuvant design, which could also facilitate the advancement of anti-tick vaccines.

CHAPTER V

CONCLUSIONS

This study offers novel insight concerning the immune responses of cattle infested with Lone Star ticks, *Amblyomma americanum*. This information reveals genetic and cellular processes involved in the bovine immune response to the primary tick pest of the Southern United States. This knowledge will facilitate future studies to elucidate more specific interactions within and among the immune mechanisms likely to be involved in mediating immune responses elicited in tick-infested cattle reported in this study.

A goal met by this study was to empirically determine optimal protocols for the isolation of high quality RNA from bovine tick bite-site biopsies and blood leukocytes in sufficient quantities for gene expression studies to be carried out. The methodology herein will facilitate future molecular immunological investigations of cattle and may enable investigations using other agriculturally important ruminant species.

In this study, it was anticipated that mechanisms associated with the tick-resistant phenotype would be identified, as the phenotyping method used herein previously contributed to the identification of alleles in the BoLA class II region associated with resistance to Lone Star ticks (UNTALAN *et al.* 2007). However, that analysis evaluated microsatellite DNA markers for 25 tick-susceptible and 25 tick-resistant calves, whereas this current study evaluated gene expression from a total of 13 calves, and only two were the tick-susceptible phenotype. In this study, the size of the sample group was the primary limiting factor and the results presented herein indicate that future studies

should phenotype a larger group of cattle and focus on comparisons between the highly tick-resistant and tick-susceptible phenotypes. Due to the small number of phenotypically distinct calves sampled in this study, the focus became identification of genetic and immune pathways elicited in cattle infested with *A. americanum* ticks. The results presented in this study implicate the activation of genes encoding pattern recognition receptors and ligands, cytokines, chemokine receptors and ligands, complement component C1q, and immunoglobulin γ heavy chain subunit-1 in tick-infested cattle.

Results presented in this study indicate that activation of Toll-like receptors, TLR-2 and TLR-4, via a MyD88-dependent mechanism may play a role in mediating the bovine local immune response to Lone Star ticks. Both TLR-2 and TLR-4 have been reported to recognize host cellular components released by injured cells, and the bites of Lone Star ticks lead to deep lesions at sites of tick attachment in cattle (AKIRA *et al.* 2001; LEVOT 1995). Stimulation of TLR-2 and TLR-4 in bovine keratinocytes and immune cells from tick-resistant and -susceptible calves may identify distinct cytokine profiles based on phenotype and could lead to knowledge with potential to help improve vaccine antigens.

Furthermore, a strong correlation was found between expression of TLR4 and the IL-1R ligand, IL-1α, in tick bite-sites from calves in this study. This is noteworthy since synergistic and regulatory relationships have been reported between TLRs and other PRRs and the idea that the group of activated PRRs, as a whole, regulate the array of cytokines produced, which influences the ratio of dendritic cells to T cells and initiates

the differentiation of T helper cell subsets (AKIRA *et al.* 2001; LIU *et al.* 2001; MOSER and MURPHY 2000; PULENDRAN *et al.* 2001). Currently, a variety of PRR ligands including lipids, carbohydrates, nucleic acids, lipoproteins, peptidoglycans, flagellin, toxins, ATP, and helicases are used as vaccine adjuvants, as these mechanisms stimulate innate immune responses that directly influence acquired immunity (BEUTLER 2009; IWASAKI and MEDZHITOV 2010; KAWAI and AKIRA 2010; MARASKOVSKY *et al.* 2009; MARRACK *et al.* 2009; MCKEE *et al.* 2010; MOSCA *et al.* 2008; TAKEDA and AKIRA 2005). Future research to evaluate and characterize relationships between different PRRs in tick-infested cattle may lead to improved anti-tick vaccines.

Results provided in this study suggest that innate inflammasome pathways may be active in the bovine local response to Lone Star ticks. Since functional IL-1β and IL-18 proteins require cleavage of the pro- IL-1β and - IL-18 mRNA transcripts by Caspase-1 and formation of the NFkB signaling complex is mediated by several subunits, future research should focus on quantitation of protein expression. Measurement of active caspase-1, IL-1β, IL-18, and NFkB at tick bite-sites from tick-resistant and -susceptible cattle could help characterize the importance of inflammasome mechanisms for cattle resistant to Lone Star ticks.

The generation of T lymphocyte helper cell subsets and effector cytokines influenced by innate immune receptors and activation of inflammasomes lead to adaptive immune responses to ticks. Thus, a better understanding of how these mechanisms are mediated will greatly impact the design of vaccines to induce bovine host protective mechanisms. Differentiation into a Th1 subset is initiated by dendritic

cell expression of IL-12 and promotes cell-mediated immunity by secreting IFN-γ, IL-2, TNF-α, and TGF-β, while differentiation into a Th2 subset to facilitate humoral-mediated immunity is directed by expression of IL-4, IL-5, IL-10, and IL-13 (AKIRA *et al.* 2001; JANEWAY 2008; TIZARD 2004). Results presented herein reveal increased expression for *IL12*, *IFNγ,* and *TNFα* and suggest that a Th1 type response may be active in tick-infested calves during the first week of tick infestation. However, increased expression of *IL6*, *IL10*, and *IGHG1* on day 10 suggests that a Th2 type humoral-mediated response may also occur in cattle infested with Lone Star ticks. Both are consistent with previous reports of immune responses elicited in the skin of *A. americanum*- resistant guinea pigs, as those suggested the involvement of both humoral-mediated and cell-mediated mechanisms in response to ticks (BROWN 1982).

This study identified increased expression of the IL-1R ligand, IL-1α associated with Lone Star tick infestation. It is known that skin injuries result in the release of IL-1α, inducing expression of cytokines and chemokines in keratinocytes, and adhesion molecules in endothelial cells (GROVES *et al.* 1996; GROVES *et al.* 1992; MURPHY *et al.* 2000; PICKER *et al.* 1990). Cytokines and chemokines stimulated by activation of the IL-1R pathway via IL-1α recruited CLA^+ memory T cells to the site of injury in humans exhibiting atopic dermatitis, and these cells preferentially mediated a Th-2 type response by selective apoptosis of Th1 memory cells (AKDIS *et al.* 2003). A future direction for characterization of factors important for bovine tick-resistance would be identification and enumeration of memory T cells in tick bite-site biopsies from tick-resistant versus -susceptible cattle, as this may elucidate the importance of Th2-mediated

immunity for expression of bovine tick-resistance. Furthermore, immunohistochemical labeling of memory T cells, along with chemokine ligands and receptors, in tick bite-site biopsies might serve to characterize the involvement of chemokines for the local immune response in tick-resistant versus -susceptible cattle.

A unique chemokine gene identified in cattle was reported to share a high degree of sequence similarity to *CCR1*, and thus was named CCR1-like (*CCR1L*) (WIDDISON *et al.* 2010). This gene was speculated to have arisen from a gene duplication event and cattle having more copies of *CCR1L* could express higher amounts of CCR1. Future research to characterize copy number variation and breed specific expression of CCR1 may identify an association between CCR1 haplotypes and tick-resistance in cattle.

Increased expression for the gene encoding a heavy chain γ subunit of the immunoglobulin IgG_1 was identified in the local response of cattle infested with Lone Star ticks. Cytokines IL-4 and IL-13 are reported to mediate production of IgG_1 antibodies from bovine B cells whereas IFN-γ mediates IgG_2 antibody production (ESTES *et al.* 1994; ESTES *et al.* 1995). Evaluation of the primary bovine Th2-mediating cytokines, *IL4* and *IL13*, should be pursued in future studies as gene expression profiles for these cytokines may help implicate the involvement of a Th2 type response in the expression of tick-resistance. Furthermore, future research should measure IgG_1 antibodies in tick bite-site biopsies and peripheral blood samples using techniques such as immunohistochemistry and ELISA, as enumeration of IgG_1 antibodies could possibly demonstrate a relationship between the expression of *IGHG1*, or other genes encoding subunits of this antibody, and the production of IgG_1 antibodies in cattle during tick

infestations. It is possible that expression of a gene encoding a variable region subunit of IgG_1 may provide a better evaluator of IgG_1 production for gene expression experiments.

Increased expression of *IL10* in the local bovine response to *A. americanum* tick infestation was shown in this study. Interestingly, polymorphisms in the promoter region of the human *IL10* gene are associated with reduced expression of this gene following stimulation of peripheral blood leukocytes *in vitro* (TURNER *et al.* 1997). Presuming *IL10* functions similarly in cattle, future research to investigate DNA polymorphisms of this gene in tick-susceptible and tick-resistant calves could help elucidate an association between this gene and tick-resistance.

In conclusion, novel knowledge about genetic mechanisms involved in the bovine immune response to Lone Star ticks was generated in this study. This knowledge will provide a basis for future research, potentially leading to information that may improve anti-tick vaccines and breeding programs to offer better tick control strategies that benefit the cattle industry, and may be applicable to other tick and host species.

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