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Modeling host responses to tick feeding and tick-borne flavivirus evolution and dispersal.

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Modeling host responses to tick feeding and tick-borne flavivirus evolution and dispersal.

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Dedication

To Michelle, my chief supporter, and to Galen and Myla, who enrich my life; and to Jesus, who makes the love we share and the work we do both possible and worthwhile.

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Abstract: Ixodid ticks are hematophagous arthropods that feed on host animals for an extended period of time. To facilitate feeding, ticks secrete a complex mixture of salivary molecules that inhibit host responses such as coagulation, complement, itch responses, and immune responses. Ticks act as vectors for an extremely broad range of human and animal pathogens. Many tick-borne pathogens are significant public health risks throughout the world. In addition, even un-infected ticks cause significant economic losses in the livestock industry. Seminal studies have shown that animals with prior exposure to un-infected ticks can develop an immune response that reduces successful feeding and pathogen transmission by infected ticks in subsequent infestations. Despite the importance of the host response, very few studies have looked at the *in vivo* response to ticks at the level of the skin. In the following studies, the murine cutaneous response during initial and subsequent infestations with *Ixodes scapularis* and *Dermacentor andersoni* nymphs was characterized using PCR-array, microarray, histopathology, and protein-detection methods. These studies show a pro-inflammatory

innate-like immune response characterized by cytokines IL-1b, IL-6, IL-10, chemotaxis of neutrophils and monocytes, wound healing responses, anti-microbial peptides, reactive oxygen species, and C-type lectins. Some down-regulation of transcription during primary infestation was indicated. During secondary infestations, these responses intensified and more cell types infiltrated the bite site. A Th2-type response was suggested for *D. andersoni* but a mixed Th1/Th2 response was suggested for *I. scapularis*. The host response to *I. scapularis* was much more activated during primary infestations than that to *D. andersoni*, possibly because of differences in the mode of tick attachment. Comparison of these host responses to published literature suggests the cutaneous response to ticks can be characterized as a wound healing response. In a final study, a model of tick-borne flavivirus evolution and dispersal was refined. This study suggests that Powassan encephalitis virus has been in North America for about 12,000 years, and that it will become a more significant public health risk as deer and tick populations increase in the United States.

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Chapter 1: Introduction

PART 1: AN INTRODUCTION TO TICKS.

What are ticks?

Ticks are blood-feeding arthropods that parasitize nearly every species of terrestrial vertebrate. They form temporary associations with host animals for mating and feeding, but spend the majority of their lives unassociated with host animals. Tick anatomy (Fig. 1.1) consists of the body with attached legs and a poorly developed head structure termed the capitulum. The capitulum is arrayed with palps, chelicerae, and hypostome. The palps serve as sensory organs and do not participate directly in feeding. The chelicerae are sharp cutting appendages used to initiate the feeding site. The hypostome is a long projection with backward pointing denticles that aid in anchoring the tick to the host. The food canal is formed by the chelicerae and hypostome, allowing saliva to flow to the host and blood, lymph, cells, and digested tissues to return to the feeding tick. In adults, the dorsal surface of the body is covered by the scutum, a shield-shaped structure that covers the entire dorsal surface in males but only the first third in females. This distinction allows easy determination of the sexes in most species of ticks. The anal opening is in the midline on the posterior ventral surface of the body; in females, the genital opening is anterior to the anal opening. Nymphal and adult ticks have four pairs of legs, while larval ticks have three.

Tick classification and evolution.

Ticks are in phylum Arthropoda, class Arachnida, subclass Acari, order Parasitiformes, suborder Ixodida, superfamily Ixodoidea. Thus they are more related to

spiders and mites then they are to insects. Within the Ixodoidea, there are three families: Ixodidae (hard ticks), Argasidae (soft ticks), and Nuttalliellidae. Nuttalliellidae appears to be the basal group, followed by Argasidae and finally Ixodidae [1]. The Ixodidae are divided into the prostriate lineage containing the genus *Ixodes* and the metastriate lineage containing the subfamilies Bothriocrotoninae, Amblyomminae, Haemaphysalinae, Rhipicephalinae, and Hyalomminae. It is important to note the genus *Dermacentor* falls in the metastriate lineage within the Rhipicephalinae [2] (Fig. 1.2). Due to the extremely broad distribution of ticks it is believed that they first evolved before the break up of Pangea, more than 210 million years ago [3]. Additional support for this hypothesis comes from phylogenetic studies showing that holothyrid mites, a group with a modern-day distribution limited to Gondwanaland, is the closest sister group to the Ixodida [4]. Recently, new samples led to improved systematics for the Nuttalliellidae, yielding the most recent hypothesis that ticks evolved in the Permian 260-270 million years ago using therapsid reptiles as hosts [1].

Tick life cycle and basic behavior.

Ticks have four life stages: egg, larva, nymph, and adult (Fig 1.3). Each stage, except the egg, requires a blood meal to progress to the next stage. Most tick species utilize three separate hosts for the larval, nymphal, and adult stages, although some utilize only one or two hosts (examples include *Hyalomma marginatum* and *Rhipicephalus (Boophilus) microplus*). Mated, fully fed females oviposit many thousands of eggs and then die. In most species, ticks spend the majority of their lives apart from host animals. They can regulate water balance during these periods of fasting by secreting a hygroscopic fluid from the salivary gland that captures water vapor from the air. The hydrated fluid can then be ingested. Interestingly, the lower limit of relative humidity

conducive to tick survival (~75%) is directly related to the ability of this fluid to capture water vapor [5]. Most tick species molt and enter diapause off the host. In addition to these physiological functions, unfed ticks often aggregate in response to assembly pheromones, most often purine compounds such as guanine in tick excrement. These loose aggregations may increase mate and host finding success, as well as grouping ticks in suitable locations for survival. Feeding behavior is initiated by ticks crawling to the end of grass stems or other structures and raising their front pair of legs in the air. This is called questing. Various host factors such as CO_2, NH_3, and odors from the skin increase tick activity and act as chemoattractants [6]. In addition, attraction-aggregation-attachment (AAA) pheromone, secreted by ticks already feeding on a host, attracts other ticks to the feeding site. Males, who are thought to initiate and prepare the feeding area for incoming females and immatures, most often secrete AAA pheromone. Mating behavior is controlled by the sequential activity of attractant sex pheromone, mounting sex pheromone, and genital sex pheromone that attract and orient excited males to receptive females [6]. Mating occurs on the host in the metastriate lineages, and either on or off the host in prostriate lineages.

Tick feeding.

After a suitable host and feeding site have been located, ticks use their sharpened chelicerae to cut the skin and initiate the feeding lesion. The hypostome and a salivary secretion termed attachment cement are used to anchor the tick during feeding. Blood feeding occurs slowly until immediately before detachment, when they enter a rapid feeding phase. Cues for entering the rapid feeding phase are not well understood, but mating plays a role as unmated females do not enter this phase [7]. Feeding represents a drastic physiological change from a long-term fasting state to rapid engorgement of large

quantities of highly nutritious blood. This necessitates large changes in tick water balance, salivary gland secretions, gut activity, and cuticle synthesis. Interestingly, ticks use their salivary glands to re-introduce excess fluids into the host, effectively concentrating the blood meal [8]. The salivary glands contain three types of acini, termed types I-III. Type I is believed to secrete hygroscopic fluids for water balance in the unfed state [8], while types II and III undergo significant morphological changes during feeding, making them the likely source of salivary gland secretions after attachment [9]. These significant, dynamic changes in salivary gland morphology combined with changes in salivary proteins during feeding [10] suggest that salivation is a dynamic process modulated across feeding and perhaps in response to host and pathogen cues as well. The overall repertoire of putative secreted proteins identified in tick saliva is large: 470 were identified in *Ixodes scapularis* [11], while >210 were identified in *Dermacentor andersoni* [12] based on salivary transcriptome analyses. Many of these putative secreted molecules form large families, which have been speculated to increase feeding success through antigenic variation [11]. The number of salivary proteins and aspects of antigenic variation are important because ticks feed for an extended period of time. General feeding times for hard ticks are 3-4 days for larvae, 3-5 days for nymphs, and 7-14 days for adults. Thus tick salivary proteins must inhibit or overcome the whole gamut of host responses including innate and adaptive immunity to successfully feed.

Basic biology of the ticks used in my projects.

      Ixodes scapularis is a three-host tick that belongs to the prostriate lineage of hard ticks. Its range in the United States encompasses the area from Texas to Florida north to Canada (Fig. 1.4). Isolated populations also exist in Wisconsin and Minnesota. In the northern part of its range, *I. scapularis* larvae hatch and feed in late summer. Engorged

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larvae molt and overwinter, and the resulting nymphs feed in late spring or early summer. Adults feed in the late summer or fall, with engorged females laying eggs that hatch late the following summer, completing the cycle. The late emergence of larvae is believed to be important for the transmission of tick-borne pathogens that are not transovarially maintained because the larvae feed on a cohort of host animals already exposed to potentially infected nymphs that fed earlier in the year [13]. This staggered feeding schedule may explain the increased success of pathogens such as *Borrelia burgdorferi* in the northern Unites States, since warmer temperatures in the south likely change this feeding pattern. In Europe, maintenance of tick-borne encephalitis virus in tick populations is related to the synchronous feeding of larvae and nymphs that allows transmission between co-feeding ticks [14]. Immature stages of *I. scapularis* feed on a wide range of small animals, but perhaps the best documented is the white-footed mouse, *Peromyscus leucopus*. Adult *I. scapularis* are fairly host specific, feeding largely on white-tailed deer (*Odocoileus virginianus*). *O. virginianus* populations have risen enormously over the last hundred years due to the regulation of hunting and the removal of predators [15], and with them the population of *I. scapularis* has also increased. This has been dramatically demonstrated on Monhegan Island off the coast of Maine that eradicated deer in 1999. Tick populations dropped from 6-17 adults per hectare to 0.67/hectare by 2003 [16]. These considerations strongly suggest the population and range of *I. scapularis* is modulated by the population of deer. *I. scapularis* has been shown to transmit the causative agents of Lyme disease, anaplasmosis, babesiosis, and Powassan encephalitis. All of these diseases are significant public health risks in the United States. The increase in *I. scapularis* population and range is already reflected in increased incidence of Lyme disease; other *I. scapularis*-borne pathogens will likely

follow suit. Interestingly, nymphal *I. scapularis* have been shown to be the primary vectors of Lyme disease to humans [17].

Dermacentor andersoni is a three-host metastriate tick confined to the Rocky Mountains and northwestern United States (Fig. 1.4). In Colorado, seasonal host seeking activity of *D. andersoni* adults spanned the months from March-June, with the peak in April. Temperatures between 5-20°C and relative humidity >20% were necessary for tick activity [18]. In western Montana, larval host-seeking behavior occurred between mid-June and September, while nymphal host-seeking behavior spanned April-mid August. Larval and nymphal ticks were shown to feed on *Peromyscus maniculatus* (deer mouse), *Spermophilus lateralis* (ground squirrel), *Neotoma cinerea* (woodrat), and *Eutamias spp.* (chipmunks), although *S. lateralis* and *N. cinerea* were most commonly infested [19]. Thus *D. andersoni* nymphs feed before larvae each year, similar to *I. scapularis* with similar potential to maintain non-transovarially transmitted pathogens. Adult *D. andersoni* likely feed on large animals such as ungulates and are believed to be the most common vector of disease to humans [18]. Within a localized habitat, *D. andersoni* have been shown to be associated with shrubs such as rose and Saskatoon, and clumps of grass [20]; others have reported that grasses near shrubs or rocks were the most common questing sites for adult *D. andersoni* [21]. *D. andersoni* has been shown to transmit Colorado tick fever virus (CTFV), Powassan encephalitis virus (POWV), *Anaplasma marginale*, *Francisella tularensis*, and *Rickettsia rickettsii*. It can also cause tick paralysis, a tick saliva-induced toxicosis.

I. scapularis and *D. andersoni* thus represent hard ticks from divergent lineages that occupy different ranges, and transmit different diseases with the exception of Powassan encephalitis. Feeding *I. scapularis* penetrate deeply into the skin with the

hypostome and secrete a minimal amount of attachment cement, while *D. andersoni* barely passes through the epidermis and secrete a large amount of cement. Very little overlap (approximately 6% sequence similarity; Dr. Francisco Alarcon-Chaidez, pers. comm.) in the salivary transcriptome between these two species has been observed [11,12]. These differences in biology, range, behavior, and diseases transmitted are surprising since they fill such similar ecological roles. Of particular interest are the determinants of vector competency between these ticks. While this trait is undoubtedly multifactorial, an important aspect of transmission success is the host response to tick feeding [22,23,24].

PART 2: HOST RESPONSES TO TICK FEEDING.

Early studies.

In 1939, Trager published his observations that guinea pigs infested with *Dermacentor variabilis* larvae developed resistance to subsequent infestations as indicated by greatly reduced feeding success. Larval feeding sites in naïve guinea pigs showed little or no inflammatory reaction, while guinea pigs with prior exposure to ticks showed an extensive inflammatory reaction, epithelial thickening, and edema [25]. This was the first time the response of a host animal had been shown to reduce the success of tick feeding, and sparked much interest in host determinants of anti-tick immunity.

Host resistance to tick feeding.

The acquisition of resistance to tick feeding has been demonstrated in a wide range of tick-host interactions. In general, greater resistance is observed in laboratory

animals than in natural hosts. For example, little or no resistance was measured in dogs (a natural host) infested with *Amblyomma cajennense* or *Rhipicephalus sanguineus* ticks, while guinea pigs rapidly developed resistance to *R. sanguineus* [26,27]. In addition, BALB/c mice infested with *Ixodes ricinus* did not develop measurable resistance after three infestations [28]. While BALB/c mice are laboratory animals, mice are a natural host of immature *Ixodes* spp. ticks. In contrast, dogs (an unnatural host in this case) were able to develop resistance to *Ixodes scapularis* ticks [29]. Donkeys, horses, sheep, and rabbits have also been shown to develop resistance to various tick species [30,31,32]. Cattle develop resistance to tick feeding, both through direct tick exposure and immunization with tick antigens [33,34]. However, resistance in cattle is variable depending on the breed and tick in question [35].

Specific aspects of the host response have been implicated in the expression of tick resistance. Lymph node cells from tick-resistant guinea pigs adoptively transferred to naïve animals conferred resistance similar to donor animals, but this response was not observed when serum was transferred [36]. However, later studies showed that tick resistance could be adoptively transferred to naïve animals through serum from guinea pigs or rabbits infested with adults [37,38]. This is likely because adults stimulate the production of much higher titer anti-tick antibodies. Other basic studies showed the administration of cyclophosphamide or methotrexate blocked the acquisition of resistance in guinea pigs infested with *D. andersoni* larvae [39,40]. These studies made a strong case that the host immune response was mediating resistance, with T-cells playing a greater role than the humoral response.

Other aspects of the host response are important in resistance to ticks. Treatment of guinea pigs with cobra venom factor, which depletes complement titers, did not block

the acquisition of tick resistance during primary infestations but did block the expression of resistance during secondary infestations (i.e., ticks were not rejected when complement titers were low) [41]. A further study showed that C4 deficient guinea pigs could acquire and express resistance to *D. andersoni* larvae at the same level as wild type guinea pigs [42], suggesting that the alternative pathway of complement was required for the expression of resistance in guinea pigs. The histamine receptor antagonists promethazine and metiamide administered together reduced the expression of resistance to the level of naïve animals, but did not block the acquisition of resistance in guinea pigs infested with *D. andersoni* larvae [43]. Antibody Fc receptors have been shown to be required for the expression of resistance to *A. americanum* in guinea pigs [44] and *Haemaphysalis longicornis* in mice [45]. IgE and mast cells were required for the expression of resistance in mice infested with *Haemaphysalis longicornis* [46]. However, mast-cell deficient mice were able to develop resistance to *D. variabilis*, apparently by basophils compensating for the missing cell type [47]. Indeed, the importance of basophils in tick-rejection was suggested very early [40], and confirmed recently using mice that conditionally lack basophils [45]. Finally, molecules important in forming the epidermal barrier were found to be increased in cattle breeds that express higher resistance to ticks [48].

Early host responses to tick feeding.

For successful feeding, ticks must inhibit early host defenses such as pain, itch, coagulation, and complement. A metallo dipeptidyl carboxypeptidase that degrades bradykinin, an important mediator of pain, has been identified in *I. scapularis* saliva [49]. *I. ricinus* expresses a lipocalin that specifically binds to leukotriene B4 with nano-molar affinity [50]. Eicosanoids such as leukotrienes and prostaglandins are known for causing

hyperalgesia at sites of inflammation [51]. Although the importance of histamine in the expression of host resistance to ticks has already been mentioned, additional work has characterized its role at the feeding lesion. Overall, histamine release is believed to disrupt feeding and initiate grooming behavior in host animals, both of which would be detrimental to the feeding tick [52]. Histamine injected into the skin near the feeding site of *Rhipicephalus microplus* larvae caused a proportion of larvae to detach early in the feeding response, but had little effect after ticks were firmly attached [53]. Using an *in vitro* feeding apparatus, the addition of histamine or serotonin to the feeding media caused a significant reduction in salivation and feeding by *D. andersoni* adults [54]. Interestingly, both histamine binding proteins and histamine release factors have been identified in tick saliva [55,56,57,58]. These apparently contradictory roles for tick saliva have been elegantly explained by experiments showing that histamine release factors are only necessary later in the feeding process during the rapid engorgement phase. Thus ticks initially secrete histamine-binding proteins to avoid host detection, but later secrete histamine release factors to increase blood delivery during the rapid feeding phase. Despite these fascinating manipulations of histamine biology, some animals including humans develop pruritus following tick bites [59].

The coagulation cascade results in the production of fibrin that polymerizes to form a clot. This occurs by the extrinsic pathway, based on the activation of FVII by tissue factor, and the intrinsic pathway, which is initiated by the activation of FXII by negatively charged surfaces. Both pathways converge at the formation of activated FX and result in the formation and cross-linking of fibrin-based blood clots. It is thought that both pathways work together to amplify the coagulation response *in vivo*. All blood-feeding arthropods have evolved mechanisms to inhibit coagulation and allow feeding to

occur. *D. andersoni* saliva has been shown to increase both the prothrombin time and the activated partial thromboplastin time by inhibiting FV and FVII [60]. Two kunitz-domain containing proteins isolated from *I. scapularis* saliva, pentholaris and ixolaris, inhibit the activated FVII/tissue factor complex. Interestingly, both these proteins appeared to use FX as a scaffold to increase their inhibitory activity since the IC50 for both proteins was greatly reduced in the presence of FX [61,62]. Proteins in the salp14 family from *I. scapularis* inhibit the intrinsic pathway of coagulation by blocking FX activation [63]. In addition to these proteins that block the coagulation cascade, tick saliva has been shown to degrade any blood clots that did form, by direct fibrinolysis [64] or by activation of plasminogen and the endogenous fibrinolytic pathway [65].

The complement cascade is a set of evolutionarily conserved proteins that act in a sequential manner to attack pathogen membranes. Various complement fragments such as C3a, C5a, and C3b act in opsonization of pathogens and as chemo-attractants for host immune cells. Complement activation can occur through the mannose-binding lectin pathway (MBL which binds carbohydrates), the classical pathway (which binds antibody Fc receptors), and the alternative pathway (activated by the tick-over or auto-activation of C3). The classical and MBL pathways use the same C3 convertase, C4bC2, while the alternative pathway uses C3bBb as the C3 convertase. The formation of the C3 convertase activates a positive feedback loop that results in the formation of the membrane-attack complex [66]. Initial studies on the interaction between host complement and tick feeding suggested the alternative but not classical or MBL complement pathway was important for the expression of tick resistance in guinea pigs infested with *D. andersoni* [41]. Inhibitory activity toward the alternative complement pathway was found in *I. scapularis* saliva [67]. Interestingly, the host range of adult *I.*

ricinus, *I. hexagonus*, and *I. uriae* was shown to correlate to the complement neutralization capacity of saliva from each tick species [68]. Further work on the anti-complement activity of *I. scapularis* saliva using HPLC and N-terminal sequencing led to the discovery of *I. scapularis* anti-complement (Isac) protein. Isac was shown to inhibit alternative pathway activity by blocking the deposition of C3 and the activation of factor B [69]. Isac was later shown to be a member of a larger anti-complement protein family along with salp20, a molecule that destabilized the alternative C3 convertase by binding to properdin, a positive regulator of complement activation [70,71]. Anti-complement molecules similar to Isac were identified from the saliva of *I. ricinus* and named *I. ricinus* anti-complement (IRAC) I and II. These molecules also inhibited the deposition of C3, but blocked the association of C3 and factor B, and the stability of the C3-B complexes rather than the activation of factor B [72]. Finally, an *I. scapularis* protein that inhibits the mannose-binding lectin complement activation pathway was identified, although the specific mechanism has not been reported [73]. Thus, tick saliva has been shown to inhibit both MBL and alternative complement activation pathways, with functional significance for resistance and host-competence.

Bite site histopathology.

Every interaction between the tick and host involves the skin. The skin is the first host organ to be damaged, and plays a major role throughout the feeding process. The skin is increasingly recognized to have a significant role in immunity, acting as a sentinel organ that also shapes the ensuing immune response [74]. Anatomically, the skin is divided into two compartments, the epidermis and dermis. The barrier function of the epidermis is maintained by keratinocytes, while keratinocytes, lymphocytes, and langerhans cells play a role responding to epidermal invasion [74]. The dermal

compartment is much more heterogeneous, with lymphocytes, macrophages, mast cells, natural killer cells, fibroblasts, and multiple types of dendritic cells [74]. In addition, lymphatic and vascular channels allow the migration of many additional cell types in and/or out of the dermis. Thus the skin presents a complex array of resident and circulating cells that participate in homeostasis, immunosurveillance, and immune responses. In the case of tick feeding, the cutaneous response represents both the initiation and effector functions of the host[1].

Soon after attachment, feeding ticks must interact with host immune cells infiltrating the tick bite site. These cells can release antimicrobial peptides, reactive oxygen species, cytokines, and chemokines to potentiate the developing immune response. Early descriptions of *D. variabilis* larval feeding lesions on guinea pigs showed a distinct lack of cellular infiltrate during primary infestation. Secondary infestation lesions were defined by edema, epidermal thickening, and a dense inflammatory infiltrate consisting of neutrophils, eosinophils, and polyblasts (small mononuclear cells) [25]. Later studies using *D. andersoni* larvae and guinea pigs showed a similar pattern, although some edema and basophils were present late in the primary infestation; secondary infestation was marked by intense infiltration of basophils, eosinophils, and neutrophils [40]. Guinea pigs infested with *I. holocyclus* larvae showed transient neutrophil infiltration and sustained mononuclear response on primary infestation, and a sustained mononuclear response and progressive increases in basophil numbers during secondary infestation [75]. Basophils, eosinophils, and mononuclear cells dominated feeding lesions from guinea pigs infested with *R. sanguineus* adults,

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[1] This paragraph is drawn from the author's paper Heinze DM et al. (2012). Parasites & Vectors 2012, 5:26 doi:10.1186/1756-3305-5-26. This is an open access journal.

whereas dogs showed primarily neutrophils and mononuclear cells. Interestingly, only guinea pigs developed resistance to tick feeding, suggesting a role for basophils in resistance and neutrophils in tolerance to tick feeding [76]. Cattle infested with *Hyalomma a. anatolicum* developed resistance after one infestation. Macroscopically, this was associated with the development of epidermal vesiculation. Microscopically, infiltration of neutrophils and mononuclear cells with small numbers of basophils and eosinophils characterized primary infestations. On secondary infestation, significant increases in basophils, eosinophils, and mononuclear cells were evident, although neutrophils were still a dominant cell type [77]. Immunophenotyping the anti-tick cellular response in sheep infested with *Hyalomma a. anatolicum* revealed increases in CD8$^+$ and γδ T-cells and reduced MHC II+ cells on primary infestation, while increases in CD8$^+$, CD4$^+$, γδ T-cells, MHC II+, B cells, and CD1+ antigen presenting cells were seen on secondary infestation [78]. Mice infested with *I. ricinus* nymphs or *D. variabilis* larvae showed eosinophils, basophils, neutrophils, and monocytes, although basophils could only be demonstrated by transmission electron microscopy [28,47]. The inflammatory infiltrate in mice infested with *I. scapularis* nymphs was similar, consisting of lymphocytes, neutrophils, and eosinophils with a marked pauci-cellular zone near the tick hypostome on primary infestation. On secondary infestation this pauci-cellular zone was absent, and macrophages were prominent [79]. Humans also develop an inflammatory response to tick feeding. The intensity of the inflammatory infiltrate correlated to a past history of exposure to ticks [79]. Resistance reactions in animals range from tick death to reductions in fed weights, molting success, or fecundity. There is no data suggesting human resistance reactions causing tick death, but there have not been any controlled experiments that could have measured more subtle expressions of

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resistance. Feeding lesions in humans show eosinophils, neutrophils, mononuclear cells, perivascular infiltrates, granulomatous reactions, fibrin thrombi, dermal edema, vasculitis, and dermal necrosis [80]. This list reflects the lack of control over primary/secondary infestations and tick species in humans since these were not designed studies but pathological investigations of patient samples, but suggests that animal data reflects the human picture. Interestingly, studies in guinea pigs infested with *D. andersoni* larvae have shown that tick cement and salivary molecules are present after tick attachment for at least 10 days [81]. This means there is extensive exposure of the host immune system to tick antigens even after feeding and the final aspects of the wound healing response in the skin may be similar to expelling a foreign body. A few themes emerge from these studies, such as the greatly increased inflammatory infiltrate on secondary exposures and the importance of granulocytes such as basophils and eosinophils in animals that develop resistance to tick feeding. The importance of mast cells and basophils in host resistance to tick feeding has already been described. Interestingly, there is little understanding of their exact role at the tick bite site or of tick countermeasures that modulate their function beyond the histamine and leukotriene inhibitors mentioned above.

Interaction between innate immune cells and tick saliva.

Despite the implication of neutrophils in host tolerance to tick feeding, several studies have shown that tick saliva modulates neutrophil function. *I. scapularis* saliva was shown to decrease neutrophil aggregation in response to anaphylatoxin, reduce phagocytosis of *Borrelia burgdorferi* (but not group B streptococci), inhibit zymosan-induced superoxide release and decrease N-formyl-methionyl-leucyl-phenyl-alanine stimulated β-glucuronidase secretion [82,83]. Two proteins with homology to cysteine-

containing domains of a disintegrin and metalloprotease with thrombospondin motifs (ADAMTS) proteases were identified from *I. scapularis* saliva. These molecules were designated ISL929 and 1373 and shown to reduce neutrophil β_2 integrin expression and superoxide release. Mice immunized with ISL929 and 1373 recruited more neutrophils to tick feeding lesions and showed a reduced tick-transmitted *Borrelia burgdorferi* load compared to BSA-immunized mice [83]. Salp25, an immunodominant antigen from *I. scapularis,* was shown to function as an antioxidant capable of reducing H_2O_2 [84]. These studies indicate that tick saliva targets distinct neutrophil functions, and suggests that the neutrophil may be more important in host immunity to ticks and tick-borne pathogen transmission than previously appreciated.

The macrophage is a versatile cell in the innate immune system, capable of phagocytosis, pathogen killing, antigen presentation, and secretion of inflammatory modulators. Not surprisingly, tick saliva can inhibit many macrophage functions. *Ex vivo* mouse splenic macrophages from mice infested for five days with *I. pacificus* or *I. scapularis* nymphs stimulated with lipopolysaccharide showed a trend toward increased production of IL-1β, IL-6, and TNF-α, although only IL-1β and TNF-α from *I. pacificus* infested animals increased significantly [85]. Saliva from *R. sanguineus* females fed for 6-7 days did not impair macrophage antigen presentation *in vitro*, but did inhibit IFN-γ induced trypanocidal activity and NO production from trypomastigote-infected macrophages [86]. *D. variabilis* saliva was shown to increase basal and platelet-derived growth factor induced migration of the macrophage cell line IC-21. However, saliva suppressed zymosan particle phagocytosis [87]. Thus, tick saliva can modulate macrophage cytokine production, phagocytosis, and pathogen killing, while migration and antigen presentation are unchanged or increased. In addition, a homologue of

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mammalian macrophage migration inhibitory factor (MIF) was identified in *A. americanum* saliva. Immunization of rabbits with tick MIF increased the feeding time of female *A. americanum* ticks [88]. However, mammalian MIF is a potent pro-inflammatory cytokine that counter-acts the immunosuppressive effects of glucocorticoids [89] making it difficult to speculate on the true function of tick MIF.

Dendritic cells (DCs) are extremely important cells in host defense, bridging the gap between innate and adaptive immunity by capturing antigens and presenting them to lymphocytes in the context of costimulatory receptors and cytokines that influence the ensuing adaptive response. Langerhans cells (epidermal DCs) have been shown to capture tick salivary proteins in the skin [90] and present them to lymphocytes [91]. Depletion of skin DCs using ultra-violet light reduced the acquisition and expression of tick-resistance in guinea pigs infested with *D. andersoni* larvae [92]. These early studies showed the importance of DCs as antigen presenting cells in host immunity to ticks, and turned the focus of research towards saliva-induced modulation of DC function. Saliva from female *R. sanguineus* fed for 7 days was shown to inhibit the differentiation of mouse bone marrow cells into DCs when cultured with GM-CSF and IL-4. Those DCs that did differentiate expressed lower levels of co-stimulatory molecules CD80 and CD86 in comparison to DCs differentiated without saliva. This was demonstrated even when lipopolysaccharide (LPS) was added to the media. DCs differentiated with or without saliva were pulsed with keyhole limpet hemocyanin (KLH) and injected into mouse footpads. Six days later, lymph node cells were harvested and stimulated with KLH *ex vivo*. Lymph node cells from mice injected with saliva-treated DCs showed significant reductions in INF-γ, IL-10, and TGF-β secretion, while KLH-induced proliferation was unchanged [93,94]. Thus tick saliva can inhibit the differentiation and costimulatory

activity of dendritic cells. *R. sanguineus* saliva-treated immature murine bone-marrow derived DCs showed reduced migration towards the chemokines Regulated and Normally T-cell Expressed and Secreted (RANTES), MIP-1α, and MIP-1β in a boyden microchamber assay. When saliva was mixed with the chemokines and untreated DCs were used, tick saliva inhibited DC migration towards MIP-1α but not MIP-1β, MIP-3β, or RANTES, suggesting that tick saliva bound or inactivated MIP-1α. Interestingly, migration of DCs *in vivo* was not influenced by tick saliva [94]. However, another group showed that saliva from *I. ricinus* females fed for 6 days reduced migration of DCs from the skin to lymph node [95], suggesting differences between tick species or the assays used may influence this response. A number of studies have reported that stimulated DCs treated with saliva show, in general, reductions in TNF-α, IL-6, IL-12, and increases in IL-10 [96,97,98,99,100]. Several explanations for these results have been described. *I. scapularis* saliva has been shown to contain significant concentrations of prostaglandin E2 that decreases DC secretion of IL-12 and TNF-α [99]. In addition, Salp15, an *I. scapularis* salivary protein, has been shown to bind to DC sign, a lectin receptor on DCs, decreasing IL-6, IL-12, and TNF-α in a Raf-1/MEK dependent manner [100]. These two processes may act together to inhibit DC cytokine production. Alternatively, *I. ricinus* saliva decreased phosphorylation of signaling intermediates in the MAPK and NF-κB pathways in DCs stimulated with lipoteichoic acid (LTA). TNF-α secretion by LTA or *B. afzelii* stimulated DCs was reduced in the presence of saliva and/or MAPK, NF-κB, or PI3K inhibitor, suggesting a connection between these signaling pathways and TNF-α secretion. IL-10 secretion was increased in DCs treated with LTA and saliva in a protein-kinase A dependent manner [96]. In addition to these cytokines, *I. ricinus* saliva inhibited LPS but not *B. afzelii*-induced type-I interferon release, while tick saliva

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interfered with type-I interferon signaling in both cases based on reductions in STAT-1 phosphorylation [96]. These studies suggest multiple and potentially overlapping mechanisms behind saliva-induced modulation of DC cytokine production. Besides modulation of cytokine production, tick saliva also interferes with DC antigen presentation and uptake. Lymph node DCs isolated from mice intradermally injected with *I. ricinus* saliva or PBS once a day for four days and on the fourth day with a peptide from the LCMV glycoprotein were co-cultured with LCMV glycoprotein specific CD4+ T cells. DCs from saliva treated mice stimulated significantly less T-cell IL-2, and polarized the T-cell response toward a Th2 profile, demonstrated by reductions in IFN-γ and IL-17 and increases in IL-4 [95]. Finally, *I. rininus* saliva reduced murine DC phagocytosis of *B. afzelii* spirochetes [97]. In summary, DCs have been shown to take up and present tick salivary antigens and are indispensible in host immunity to ticks. However, tick saliva clearly interferes with every step of DC function including antigen uptake, differentiation of naïve DCs, migration, expression of costimulatory molecules, cytokine production, and antigen presentation. Nevertheless, some aspects of the interaction between tick saliva and DCs remain to be elucidated *in vivo*.

Interaction between adaptive immune cells and tick saliva.

Tick feeding or tick salivary antigens have been shown to modulate many functions of the adaptive immune response. Broadly, tick feeding has been shown to modulate lymphocyte cytokine profiles toward a Th2 response, inhibit antigen or mitogen-induced T-cell proliferation, and reduce antigen-specific antibody responses. Increases in IL-4 and IL-10, and decreases in IL-2 and IFN-γ were measured in *ex vivo* ConA stimulated splenocytes from mice infested with *I. scapularis* nymphs compared to uninfested mice [101]. Similar responses were observed in mice infested with *D.*

andersoni nymphs, although decreases in LFA-1 and VLA-4 were also recorded [102]. These results were supported by experiments using ConA stimulated *ex vivo* lymphocytes from mice treated with saliva from *I. ricinus* [103] or *R. sanguineus* [104] and peripheral blood mononuclear cells from humans treated with saliva from *I. ricinus* [105]. Clonotypic CD4+ T-cells expressing a T-cell receptor specific for influenza hemaglutinin (HA) were adoptively transferred to *I. scapularis* [106] or *D. andersoni* [107] infested mice that were also intradermally injected with HA. These CD4+ T cells were shown to develop the capacity to secrete IL-4 in response to HA, providing *in vivo* evidence of IL-4 secretion from CD4+ T cells exposed to tick saliva. A sphingomyelinase was isolated from *I. scapularis* saliva with homology to a dermonecrotic protein from *Loxosceles intermedia* (Brown Recluse spider). This protein was found to induce IL-4 secretion by CD4+ T cells in the same clonotypic T-cell system [108], suggesting that specific salivary molecules may induce host Th2 responses. In addition to modulating cytokine profiles, tick feeding has been shown to inhibit antigen and mitogen-induced T-cell proliferation. Peripheral blood mononuclear cells from *Bos indicus* or *B. taurus* were stimulated with ConA in the presence or absence of salivary gland extract from female *D. andersoni* ticks fed for 1, 2, 3, …, or 9 days. ConA induced proliferation was inhibited by 20% by extract from ticks fed for 1, 2, 3, or 4 days, but was inhibited 70-90% by extract from ticks fed for 5 or more days. Interestingly, saliva potentiated LPS-induced mononuclear cell proliferation with a similar profile of activity from different salivary gland extracts [109]. Saliva from 6-7 day fed female *R. sanguineus* ticks inhibited ConA-induced proliferation of splenic T-cells from mice infested with *R. sanguineus*. However, saliva did not interfere with antigen presentation between KLH loaded macrophages and KLH-sensitive lymphocytes, but did inhibit their proliferation [86]. Lymph node T cells

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from *I. ricinus* infested mice treated with ConA and increasing concentrations of saliva showed an initial increase and then decrease in proliferation and IL-4 release as saliva concentration increased. T cells from naïve mice showed a dose-dependent decrease. Reduced *ex vivo* antigen-specific proliferation was observed in T cells from mice immunized with sheep red blood cells (SRBC) and tick saliva compared to mice injected with SRBC alone [103]. *I. ricinus* saliva or salivary gland extract inhibited the proliferation of human peripheral blood mononuclear cells in response to ConA or LPS [105]. Salivary gland extract from *D. anderoni* inhibited the proliferation of mouse splenocytes treated with ConA. Fractionation of the salivary gland extract based on molecular weight showed that this inhibition was dependent on molecules greater than 30kDa and less than 3kDa [110]. A subsequent study isolated a 36-43kDa protein dubbed P36 that inhibited ConA-induced proliferation of mouse splenocytes by 40% [111]. These studies show that saliva from many different tick species can inhibit the antigen or mitogen-induced proliferative response of multiple different animal species, suggesting that this is an important, conserved function of tick saliva. Interesting mechanistic insight into this process has come from studies on the *I. scapularis* immunosuppressant Salp15. Salp15 was shown to inhibit T-cell proliferation and IL-2 production in mouse T-cells stimulated with anti-CD3. This phenotype could be rescued by adding IL-2 to the culture medium, suggesting that the reduction in proliferation was in part due to diminished IL-2 production. Subsequently, the carboxy-terminal portion of Salp15 was shown to interact with domains 1-2 of CD4. This interaction changes the conformation of CD4, reducing the ability of the src-family tyrosine kinase LCK to associate with the cytoplasmic tail of CD4. This reduces downstream signaling through the TCR complex, inhibiting IL-2 production. Salp15 has also been shown to inhibit

adaptive immune responses dependent on antigen specific T-cells *in vivo* [112]. Thus, tick salivary proteins may target specific functions of T lymphocytes to aid successful blood feeding.

Tick feeding has also been shown to modulate B-cell functions, although there is much less literature covering this subject than T-cells. As mentioned previously, adoptive transfer of serum from guinea pigs infested with *D. andersoni* adults (but not nymphs) to naïve guinea pigs conferred resistance to subsequent infestations [38], suggesting that antibodies and the B-cell response are important in the host response to tick feeding. B-cells from guinea pigs injected with SRBCs during primary or secondary infestation with *D. andersoni* larvae showed a significantly lower anti-SRBC IgM response than B-cells from uninfested animals [113]. Similarly, mice infested with *I. ricinus* adults were injected with BSA in QuilA adjuvant five days after infestation. Levels of anti-BSA IgG and IgM were significantly lower in tick-infested mice compared to tick-free mice, a reduction that was not dependent on changes in T-cell populations [114]. *Ixodes ricinus* saliva inhibited LPS or ConA-induced B-cell activation (measured by increases in CD69) and IL-10 secretion. In addition, saliva inhibited LPS but not ConA stimulated B-cell proliferation [115]. These studies demonstrate the importance of B-cells in the host response to ticks and indicate that tick saliva can inhibit antigen-specific antibody production and mitogen-stimulated B-cell proliferation.

Tick salivary proteins neutralize host cytokines, chemokines, and growth factors.

In addition to modulating the activity of specific cell types, tick saliva can directly interact with host molecules such as cytokines, chemokines, and growth factors, neutralizing or modulating their activity. An early study demonstrated that salivary gland extracts from *D. reticulatus*, *A. variegatum*, *R. appendiculatus*, *Haemaphysalis inermis*

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and *I. ricinus* ticks could bind and inhibit the ELISA-based detection of human IL-8. Salivary gland extracts were fractionated using liquid chromatography and the fraction with peak anti-IL-8 activity was shown to inhibit the chemotaxis of human granulocytes in response to IL-8 [116]. Anti-CCL3 activity was identified in *R. sanguineus* using radiolabeled CCL3 and chemical cross-linking to probe a salivary gland cDNA library. The identified protein was named evasin-1 and shown to be highly specific for CCL3 (MIP-1α), CCL4 (MIP-1β), and CCL18 (MIP-4) [117]. Interestingly, the structure of evansin-1 has been determined and it is not homologous to any known chemokine binding protein. The selectivity of binding appears to be determined by the N-terminal residues of the chemokine [118]. Further work by the same group identified two additional chemokine binding proteins, evasin-3, which binds CXCL1 and CXCL8, and evasin-4, which binds CCL5 (RANTES) and CCL11 (eotaxin) [119]. Using an ELISA-based method, salivary gland extracts from male or female *A. variegatum* ticks inhibited detection of chemokines CXCL8 (IL-8), CCL2 (MCP-1), CCL3, CCL4, CCL5, and CCL11. The highest activity was against CCL11. Interestingly, the activity of female salivary gland extract appeared to decline as feeding progressed for most chemokines, while it increased in male extracts, suggesting the male may aid female feeding activity [120]. A similar study demonstrated that salivary gland extracts from 5-6 day fed male or female *R. appendiculatus* bound to CXCL8, CCL2, CCL3, CCL3, CCL5, and CCL11 with no significant differences between sexes. They went on to compare the binding of 3-4 day fed male and female *I. ricinus* salivary gland extract. Interestingly, female extract bound CCL2 and CCL3 but not CCL5 or CCL11, while male extract bound all four chemokines, again supporting the possibility that for some tick species the male salivary secretions may be important to the feeding female. This study also states that

saliva of *D. reticulatus*, *I. ricinus*, *R. appendiculatus*, and *A. variegatum* bind to IL-2 and IL-4, although the data is not shown [121]. Supporting this report, an IL-2 binding activity was demonstrated in *I. scapularis* saliva [122]. Salivary gland extract of a number of tick species has been shown to bind and inhibit the activity of growth factors. Salivary gland extract from *A. variegatum* bound TGF-β1, PDGF, FGF-2, and HGF; *D. reticulatus* and *R. appendiculatus* bound TGF-β1, FGF-2 and HGF; *I. ricinus* and *I. scapularis* bound PDGF. Interestingly, salivary gland extract from tick species that bound PDGF inhibited cell proliferation and induced modifications of the cytoskeleton *in vitro* [123]. These results suggest that tick saliva can block chemoattractants for neutrophils, macrophages, eosinophils, and T-cells, bind vitally important cytokines such as IL-2 and IL-4, and inhibit the activity of growth factors important in host wound healing.

This discussion has highlighted the very impressive capacity of tick saliva to inhibit host responses. This activity protects the tick from discovery (and hence removal) by blocking mediators of pain and itch. It also inhibits early host responses including coagulation, complement, and histamine release that serve important homeostatic roles and are also intertwined with the initiation of the immune response. The inhibitory activity also covers the function of neutrophils, macrophages, dendritic cells, lymphocytes, and chemical messengers and effector molecules of these cells. In addition to these functions, tick feeding appears to induce a Th2 polarization of the immune response, which is speculated to represent a subversion of the immune response away from a more protective Th1 response. Although there is interest in developing potential pharmaceutical agents from tick salivary proteins, the primary driver for the research described above has been its relation to tick control and tick-borne pathogen

transmission. For this reason, tick-borne pathogen transmission at the tick-host-pathogen interface will be the topic of the following sections.

PART 3: INTERACTIONS BETWEEN THE TICK, PATHOGEN, AND HOST.

Ticks aid tick-borne pathogen transmission.

Ticks have been shown to facilitate pathogen transmission to susceptible hosts. This is believed to be mediated by salivary molecules that modulate host responses into patterns favorable for pathogen transmission and establishment. This has been shown for a wide range of tick-pathogen-host interactions. Guinea pigs infested with uninfected *R. appendiculatus* were injected with tick-borne encephalitis virus with and without salivary gland extract (SGE) from partially fed female *I. ricinus*. Four times as many uninfected ticks became infected with virus when injected with SGE, a process later termed Saliva-Activated Transmission (SAT). In addition, 67% of guinea pigs injected with virus and SGE developed viremia, while only 30% developed it with virus alone. Thus specific components of saliva facilitated the infection of guinea pigs and the transmission of virus to the feeding ticks [124]. Facilitation of transmission has also been shown for tick-borne bacteria. Mice were injected with *B. lusitaniae* or *B. burgdorferi* with or without SGE from *I. ricinus* (the vector of *B. lusitaniae*) or *I. scapularis* (the vector of *B. burgdorferi*). Interestingly, higher *Borrelia* loads were measured in mice injected with SGE from the natural vector [125]. This indicated that salivary proteins facilitate *Borrelia* transmission and that vector-specific tick-*Borrelia* interactions may govern this process. Another study demonstrated accelerated proliferation and significant increases in *Francisella tularensis* when injected into mice with SGE from *I. ricinus* ticks [126]. Thus, tick saliva

can facilitate the transmission and establishment of both viral and bacterial pathogens. While the exact mechanisms are unknown, it is postulated that the modulation of host immunity described above may influence these processes. In some cases, tick-borne pathogens can modulate the salivary secretions of the tick to its own benefit. *Anaplasma phagocytophilum* has been shown to induce the expression of salp16 in *I. scapularis* ticks during feeding. Interestingly, feeding ticks lacking salp16 could acquire *A. phagocytophilum* normally, but the bacteria were not able to invade the salivary glands [127]. Another study showed that *B. burgdorferi* induced the expression of salp15 in *I. scapularis* ticks. Salp15 bound to the outer surface protein C of *B. burgdorferi* and protected it from antibody-mediated killing *in vitro*. Mice injected with *B. burgdorferi* and salp15 had a higher pathogen load than mice infected with *B. burgdorferi* alone [128]. These studies show that tick-borne pathogens can modulate the vector environment to facilitate their intra-vector survival and transmissibility. The extent to which tick-borne pathogens co-opt the immunomodulatory arsenal of the tick is an unknown, and presents many fascinating questions.

The timing of tick-borne pathogen transmission.

Ticks transmit an extremely broad range of pathogens, including protozoa, bacteria, and viruses. These disparate pathogens have developed an intricate interaction with the tick vector that determines when and in what environment the pathogen will be deposited in the host skin during feeding. Studies with *B. burgdorferi* suggest that upon initiation of tick feeding, spirochetes multiply in the gut. At some point, these spirochetes develop into a motile form that escapes the tick gut, invades the salivary glands, and ultimately invades the host skin [129]. The signals that control this process are poorly understood. Because of the extensive reactivation phase, *B. burgdorferi*

spirochetes are not transmitted until 24 hrs. after attachment, and peak transmission is not observed until 48-72 hrs. [130]. At the other end of the spectrum, mice were infected with Powassan encephalitis virus when infected ticks were in contact with the mouse for only 15 min., suggesting that this virus was already present in the salivary glands before attachment and feeding began [131]. Tick-borne encephalitis virus follows a similar pattern, being transmitted within 1 hr and probably earlier [132]. Other tick-borne pathogens are deposited in the skin sometime between the very rapid kinetic of the tick-borne flaviviruses and the slower kinetic of *Borrelia ssp.* However, information on transmission kinetics for many tick-borne pathogens is lacking.

How do ticks become infected?

The prevailing dogma for many years held that arthropod vectors became infected when feeding on parasitemic hosts. Thus, the primary determinant of infection was the number of pathogens present per unit volume of vertebrate blood. This train of thought influenced the search and identification of the reservoir hosts for many vector species. However, in 1987 a novel method of pathogen transmission between ticks was demonstrated. This study showed efficient transmission of thogoto virus between infected adult and uninfected nymphal ticks while feeding on guinea pigs that never developed detectable viremia. This phenomena was termed "co-feeding transmission." Surprisingly, co-feeding transmission was actually slightly less efficient when hamsters were used, despite the high viremia they developed [133]. These results were extended by another group that used two individual "chambers" to contain ticks on a guinea pig. TBEV-infected adult ticks were placed in chamber 1 along with 50 uninfected nymphs, and 50 uninfected nymphs were placed in chamber 2. Efficient transmission of TBEV was demonstrated between the infected adults and nymphal ticks feeding in both chamber

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1 and 2 even in the absence of detectable viremia [134]. These studies resulted in a paradigm shift from the previous dogma, suggesting that ticks could potentially act as both vectors and reservoirs of a pathogen, with the vertebrate acting as a bridge to connect the two with little requirements for vertebrate susceptibility. The situation appears to be, in reality, a complex mixture of both the old and new dogmas. *Rhipicephalus sanguineus* ticks could be infected by feeding on dogs inoculated with *Rickettsia conorii*, suggesting that these bacteria could be acquired from bacteremic blood. However, these infected adult ticks and uninfected nymphs were then fed on naïve dogs, and the co-feeding transmission rate was 90-100%. The success of co-feeding transmission was greatly reduced when dogs with prior exposure to *R. conorii*-infected *R. sanguineus* were used [135]. Co-feeding transmission has also been demonstrated for *B. burgdorferi*, although the close proximity of infected and uninfected ticks appeared to be a more important factor for this pathogen [136]. Thus, the mechanism of tick-borne pathogen maintenance in nature is a complex process. Most (if not all) tick-borne pathogens are maintained across molting periods (trans-stadial) and many are transmitted from infected adult females to eggs (trans-ovarial). Amplification of infected arthropods can occur through feeding on infected animals with high parasitemia, but may more often occur through a co-feeding mechanism. This significantly complicates the process of defining the vertebrates most important in maintaining tick-borne pathogens, since the factors involved in host immunology and tick saliva that drive co-feeding transmission have not been well characterized. At present, a localized infection of the skin and the migration of infected cells such as dendritic cells and macrophages between tick feeding sites are the leading hypothesis [137].

The importance of the host response: prior exposure to ticks can protect from tick-borne disease.

The strongest line of evidence supporting the importance of the host response to tick feeding in tick-borne pathogen transmission is a body of literature indicating that the host response to tick bites can reduce subsequent pathogen acquisition from infected ticks. Only 36% of rabbits with prior exposure to uninfected *D. andersoni* died when infested with *F. tularensis*-infected ticks compared to 100% of rabbits without prior exposure to ticks [138]. Another study focused on tick-borne bacteria demonstrated that only 16.7% of BALB/c mice infested 4 times with uninfected *I. scapularis* nymphs were infected by *B. burgdorferi* infected ticks, in contrast to 100% of naïve animals [23]. A study among residents of Block Island, Rhode Island demonstrated that tick-associated itch was correlated with protection from Lyme disease, suggesting these results are not limited to laboratory animals [139]. Other studies indicate that this phenomenon is not limited to tick-borne bacteria. *Bos taurus* oxen resistant to *R. apendiculatus* ticks exhibited mild disease when infested with *Theileria parva* infected ticks, while naïve animals became severely ill or died [140]. Mice immunized with a tick-cement antigen from *R. appendiculatus* were infested with TBEV-infected *I. ricinus* adults and uninfected nymphs. Significant reductions in co-feeding transmission were demonstrated. These mice were then challenged with an intra-peritoneal injection of TBEV. Immunized mice with prior exposure to TBEV-infected ticks showed a survival similar to a single dose of a commercially available TBEV vaccine [24]. These results show that the host response to uninfected ticks can protect host animals from tick-borne bacteria, protozoa, and viruses. The influence of the host response on tick-borne viral transmission is surprising since these viruses are likely transmitted immediately after

attachment, before the host has had time to respond. TBEV and *Borrelia ssp.* have been detected in liquid saliva and tick cement, but the individual contributions of each to pathogen transmission are unknown [132]. How the host response modulates the establishment of these pathogens is largely unknown. It is interesting to note that the host response can, in some cases, modulate co-feeding transmission rates with potentially profound impact on the amplification of infected ticks in nature.

PART 4: THE IMPACT OF TICKS ON HUMANS AND LIVESTOCK AND TICK CONTROL STRATEGIES.

The burden of ticks and tick-borne disease.

World wide, mosquitoes surpass ticks in terms of causing disease in humans. However, ticks transmit more species of pathogens than any other known vector group. In the United States, ticks are the most important vector of disease to humans; in 2010, 22,561 confirmed human cases of Lyme disease were reported, in comparison to 1,021 cases of West Nile Virus (WNV) [141]. Ticks and tick-borne disease are also a major problem for the livestock industry. Tick infestations alone can significantly retard animal growth, reduce milk production, and potentially lead to death. Tick infestations reduce the value of hides by 20-30% [142]. The estimated global costs of ticks and tick-borne disease were 13.9-18.7 billion US dollars per year in 1997 and are undoubtedly higher now [143]. In the United States, millions of dollars are spent every year to eliminate infestations of livestock and wild animals with *R. (B.) microplus* and related ticks. This effort is believed to save the US livestock industry a minimum of 3 billion US dollars annually [144]. In many developing countries, ticks pose a major economic threat and hinder the development of an otherwise lucrative livestock industry [145]. For these

reasons, efficient, effective, and economical tick control strategies are desperately needed for reducing the global burden of ticks and tick-borne diseases.

Tick control strategies.

The cornerstone of present tick control strategies is the use of a range of acaricides. These products have suffered extensively from the development of resistant ticks, usually within 10 years of the introduction of a new compound. The cost of developing new compounds is very high and the perceived market and lifespan of a new product is too small to stimulate significant efforts toward new chemical developments [145]. In addition to these challenges, acaricides must meet strict safety requirements for the user and animal with minimal contamination of animal products. Other methods of tick control, based on the development of successful anti-tick immunity are possible. One solution is to use livestock strains with high natural resistance to ticks. This approach, while successful in controlling ticks, suffers from the difficulty of selecting for both tick immunity and other desirable traits such as milk production simultaneously. Another strategy was to develop anti-tick vaccines. Only one recombinant anti-tick vaccine is presently available, marketed as TickGARD in Australia and Gavac in Cuba. These vaccines are both based on the Bm86 antigen. Surprisingly, this antigen was identified from the gut of *R. (B.) microplus*, based on the hypothesis that host antibodies could damage the tick after being ingested. In addition, "concealed" antigens offered the possibility of producing immunity in cattle otherwise highly susceptible to ticks. Controlled trials suggested that the Bm86 vaccine could reduce the tick yield from a standard infestation by 90%. In field studies after release, vaccination has been shown to greatly reduce the use of acaricides. This apparent success has to be tempered by the limited range of tick species the vaccine covers, and the demonstration of geographical

variants, even of the target species, that are not controlled [146]. Thus, vaccination strategies need improved antigen targets that cross-react with a broad range of ticks and are less likely to suffer from antigenic escape. A number of other tick antigens have been investigated with some promising results. Many of these antigens are "exposed" in the sense that host animals naturally contact these proteins during tick feeding. While most of these antigens show promise in causing tick mortality, it is exciting to note that some of them have been shown to protect hosts from tick-borne disease or block co-feeding transmission even when ticks can successfully feed. Thus, different antigens may be optimal for blocking pathogen transmission versus tick feeding, and suggest that a cocktail of tick antigens (and potentially even pathogen antigens as well) may prove to be the most effective strategy [147]. This may be especially true in the case of Lyme Disease, after the only licensed vaccine was voluntarily removed from the market due to low public demand after concerns the vaccine might cause an auto-immune arthritis. Despite many safety reviews that did not find a scientific basis for this claim, the formation of class-action lawsuits and anti-vaccine advocacy groups precluded any continued development [148]. The inclusion of tick antigens might strengthen Lyme vaccines and provide a route for avoiding public concern over the arthitogenic potential of the present primary antigen, OspA.

PART 5: SPECIFIC AIMS.

A gap in knowledge.

In this chapter, I have discussed the literature showing tick saliva as a unique cocktail of molecules that modulate host defenses to facilitate successful blood feeding

and pathogen transmission. These effects are mediated by the pharmacological action of tick saliva on host defenses and they contribute to the earliest aspects of pathogenesis of these infections. Even though tick-saliva is believed to modulate host responses into patterns favorable for tick-borne pathogen establishment, little effort has gone into understanding the status of the host response in the skin at the specific times that different pathogens are believed to be transmitted. Additionally, while work is ongoing on vaccine candidates, little work has been done to characterize the correlates of protective immunity in terms of both anti-tick and anti-transmission responses. Work in this area has been further hampered by the lack of *in vivo* studies and experimental work focused on the skin, the relevant organ for both tick feeding and tick-borne pathogen transmission. This is a significant impediment to our understanding of the tick-host interface and tick-borne pathogen transmission. Because of the significant public health and economic burden of ticks and tick-borne disease more effective control strategies are urgently needed.

Goals.

The initial **long-term goal** for this research was to understand both the spectrum and temporal patterns of host responses to tick feeding. As time has passed, the long-term goal for this project has evolved into building putative models of the host response to tick feeding. A model, however crude, gives one something to test and a framework for future hypothesis. The **objective** was to identify tick-induced changes in host gene expression over the time-course of tick engorgement and correlate these changes with histological analysis of the bite site during primary and repeated infestations with *I. scapularis* and *D. andersoni* nymphs. As these projects expanded, a secondary objective became to use phylogenetic methods on all available full-length open reading frame

sequences of tick-borne flaviviruses to understand their evolution and dispersal. The results of both of these objectives are models relevant to ticks and tick-borne disease.

Specific aim 1a: Gene expression profiling of the host response to tick feeding.

<u>Hypothesis:</u> Host genes differentially expressed during blood feeding are key components in the host response to infestation and expression profiles will differ during primary and repeated infestations.

<u>Approach:</u> A PCR- or microarray-based approach was proposed to characterize patterns of host gene expression at the bite-site during the course of primary and secondary infestations with *I. scapularis* and *D. andersoni* nymphs.

Specific aim 1b: Histological analysis of tick bite sites.

<u>Hypothesis:</u> Pathological analysis of the feeding lesions of nymphal *I. scapularis* and *D. andersoni* will identify important differences between these tick species and allow a correlation with the gene expression data.

<u>Approach:</u> Tissue samples will be fixed using various fixatives or frozen as needed. Tissues will be embedded in paraffin or freezing media and carefully sectioned to allow

analysis at the site of hypostome entry into the skin. Slides will be stained with various stains as needed and read according to general pathology.

Specific aim 2: Characterize the evolution and dispersal of the tick-borne flaviviruses.

Hypothesis: Tick borne flaviviruses evolved and dispersed in a clinal fashion beginning in far-eastern Eurasia and spreading across Asia and then into Europe, as proposed by Zanotto and colleagues [149].

Approach: All available full-length open reading frame sequences of tick-borne flaviviruses will be analyzed using phylogenetic methods such as neighbor-joining, genetic distance, maximum likelihood, and Bayesian analysis (as implemented in BEAST software).   These estimates will be correlated with geographical distance between specific isolates. Finally, a time to most recent common ancestor analysis will be used to estimate the dates of viral divergence events and this information compared with available geological records to piece together a model of tick-borne flavivirus dispersal.
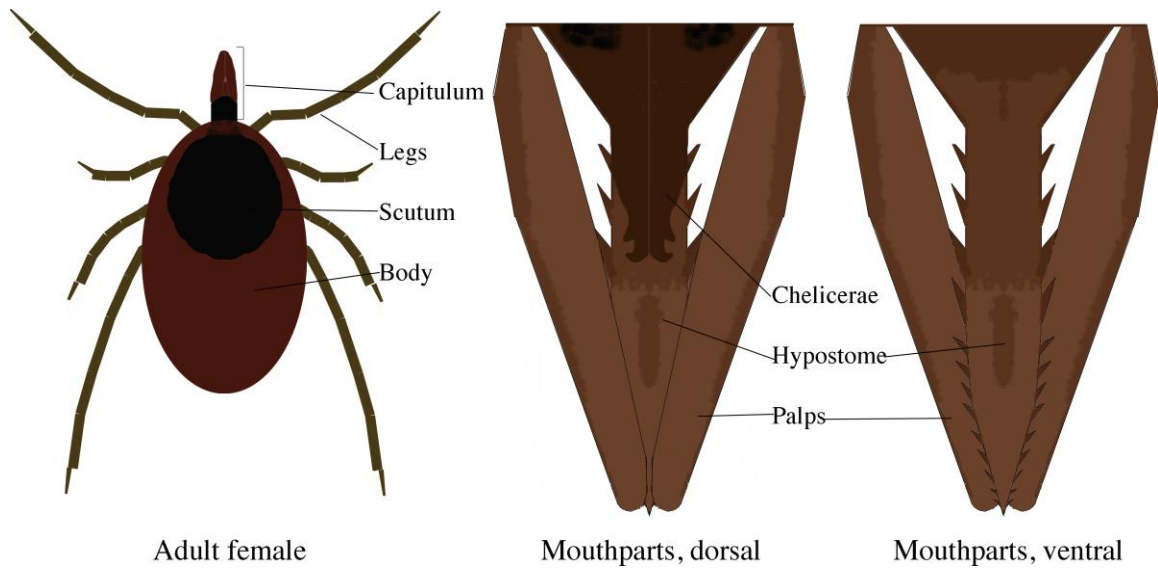
Figure 1.1: Tick anatomy.

Adult female tick (far left) and a close-up views of the dorsal (middle) and ventral (far right) aspects of the mouthparts.
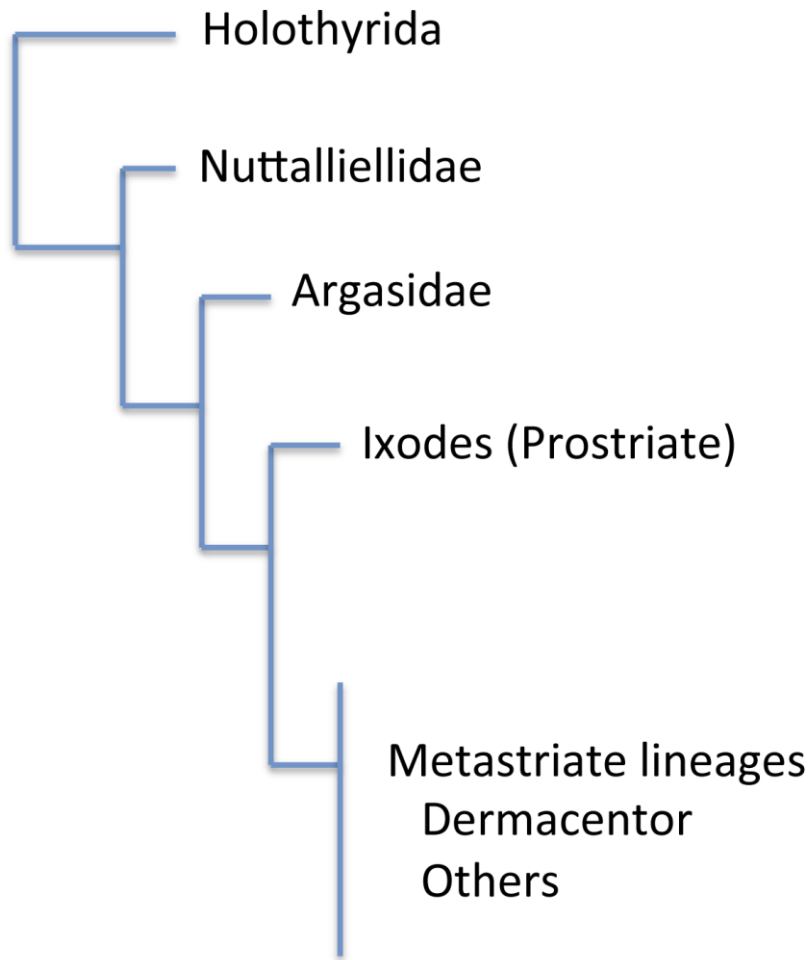
Figure 1.2: Basic tick phylogeny.

This tick phylogeny is hand-drawn and is only meant to show the relationships between major tick families based on current understanding of their phylogeny. It shows the presently hypothesized evolution from Holothyrid mites through early ticks (Nuttalliellidae) to present day soft and hard ticks. It also shows the clear split between prostriate and metastriate lineages of hard ticks.
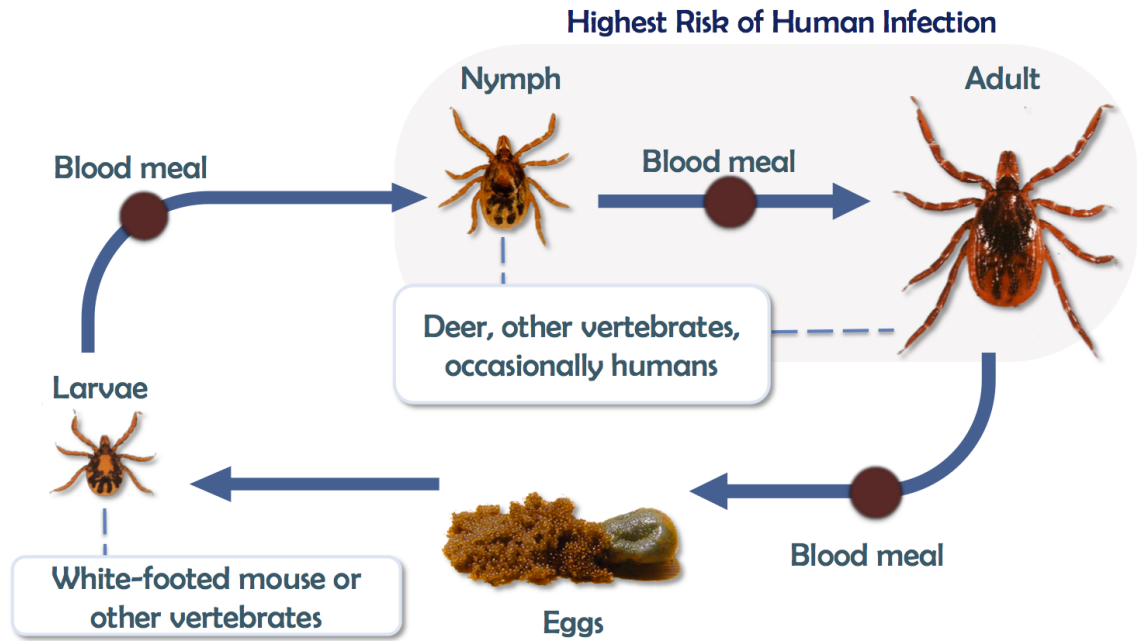
Figure 1.3: Tick life cycle.

This figure shows the life cycle of *I. scapularis*, including common host animals and stages that are likely to transmit infection to humans. The life cycle of *D. andersoni* is very similar with slight variations in the host animals used and the risk of human infection is thought to be highest for the adult stage. This figure is courtesy of Dr. Francisco Alarcon-Chaidez and has never been published in any format before this dissertation.
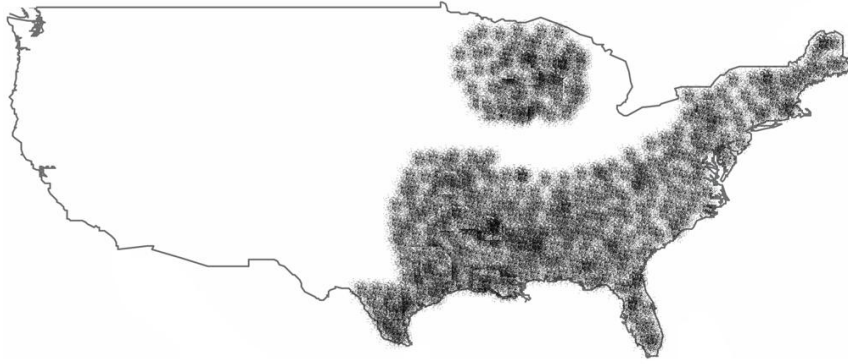
Ixodes scapularis
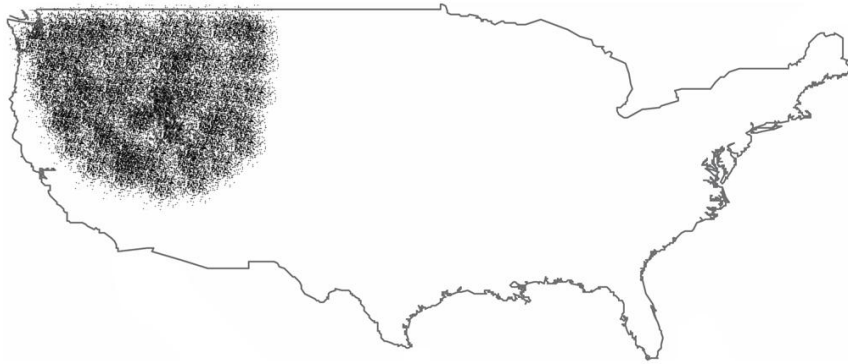
Dermacentor andersoni

Figure 1.4: The ranges of *I. scapularis* and *D. andersoni*.

These maps show the approximate ranges within the United Sates of the two tick species used in this dissertation. *I. scpaularis* is limited to the southern states from central US to the east coast, where its range extends northwards to Maine. There is also an isolated population in Wisconsin/Minnesota. *D. andersoni* is limited to the north western US.

Chapter 2: Methods[2]

TICKS.

Pathogen-free tick colonies were maintained in our laboratory as described [150]. All life cycle stages were kept in sterile glass vials with mesh tops in desiccators at 22°C containing saturated solutions of K_2SO_4 or KNO_3 to obtain 97% or 93% relative humidity for *I. scapularis* or *D. andersoni* respectively, with a 16:8 hour photoperiod. For routine colony maintenance adult and/or nymphal ticks were fed on New Zealand white rabbits and nymphs and/or larvae were fed on mice.

ANIMALS.

BALB/c mice used in this study were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were cared for in accordance with guidelines of the Committee on Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources National Research Council, Washington, DC).

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[2]This chapter is a compilation of the methods in two of the author's papers: Heinze DM et al. (2012). Parasites & Vectors 2012, 5:26 doi:10.1186/1756-3305-5-26 and Heinze DM, et al. PLoS One. 2012;7(10):e47301. Both of these journals are open access.

TIME COURSE INFESTATIONS.

To perform time course infestations, 6-8 week old female BALB/c mice were placed in individual restrainers or anesthetized with a 150μL intraperitoneal injection containing 10mg/mL ketamine (Fort Dodge Animal Health, Fort Dodge, IA) and 1mg/mL xylazine (Phoenix Pharmaceutical, St. Joseph, MO) in PBS (Gibco, Life Technologies, Carlsbad, CA) and infested with pathogen-free nymphal ticks. *Ixodes scapularis* ticks exhibited high appetence and 8-10 ticks were needed to ensure attachment of 4-5 ticks, while *D. andersoni* exhibited low appetence and 20 ticks were required to ensure attachment of 1-2 ticks. After only one or two rounds of experimental infestation, it was noticed that all the successful ticks attached either to the ears or face, with a slight preference for ears in *I. scapularis* and a strong preference for ears in *D. andersoni.* Hence, in all subsequent infestations, ticks were placed in the ears of mice to increase feeding success and improve experimental results by normalizing the location of tick attachment. Ticks were allowed to attach for approximately one hour and unattached ticks were discarded. Mice were then removed from restraints (if used) and housed individually. Secondary infestations involved two rounds of infestation. Mice were infested with nymphal ticks that were allowed to complete their feeding cycle (4-5 days). Fourteen days after the last primary infestation tick dropped off the animals, mice were re-infested with nymphal ticks using the same protocol described above. For tissue harvesting, infested mice were euthanized by CO_2 inhalation or ketamine/xylazine (200υL IP injection) anesthesia followed by cervical dislocation and skin biopsies with attached ticks were harvested using 4mm biopsy punches (Premier Products Co., Plymouth Meeting, PA). For *I. scapularis,* bite sites were analyzed at 1, 3, 6, 12, 48, 72, and 96 hr post-infestation (hpi) during primary infestations, and 12, 48, 72, and 96hpi

during secondary infestations. For *D. andersoni,* bite sites were analyzed at 12, 48, 96, and 120hpi during primary infestations, and at 120hpi during secondary infestations. These time points were chosen to look at early (12hpi), mid (48hpi), late (72 or 96hpi), and detachment (96 or 120hpi) periods of feeding. The very early time points for *I. scapularis* were pursued because this tick transmits viruses of interest to the laboratory group which are likely transmitted very early after attachment. *Dermacentor andersoni* nymphs feed for a longer period that *I. scapularis,* thus the 120hpi and 96hpi time point represents the very end of the feeding period for each tick species, respectively. Three mice were measured at each time point in all micro-array or PCR-array experiments; controls consisted of 3 similarly housed but tick-free mice. The number of biological replicates at each time point was determined by the minimum needed for generating reasonable statistics and the economic status of the laboratory. A post-experiment power analysis using the PowerAtlas [151] and inputting experimentally generated p-values with a significance level at p=0.05 suggested an average power of 0.49. This rather low power should to be tempered by the consistency across different comparisons that could not be factored into this simple power analysis. Biopsies were stored in RNAlater (Ambion) at -20°C. The Institutional Animal Care and Use Committee of the University of Texas Medical Branch approved all animal experiments (protocols #0907054 and 0901003).

RNA ISOLATION.

Ticks were removed from all skin biopsies before RNA extraction. Tissue samples were homogenized individually in 1 mL Trizol (Life Technologies, Carlsbad, CA) using an Ultra-Turrax T8 (Ika, Wilmington, NC) tissue disperser. After 5 min

incubation, 200μL chloroform (Fisher Scientific, Waltham, MA) was added and the samples incubated for 3 min. Samples were centrifuged at 12,000 × g, 4°C for 15 min, and the aqueous phase was retained. One volume 70% ethanol (Acros Organics) was added and the samples applied to RNeasy micro kit (Qiagen) columns. The RNeasy protocol was then followed, including the in-column DNase digestion step. I have found that obtaining high purity RNA is facilitated by adding one extra wash step with buffer RW1 and two extra wash steps with buffer RPE. All samples were eluted in nuclease-free water. After extraction, RNA was quantitated spectrophotometrically using a NanoDrop ND-1000 (NanoDrop Technologies, DE). All samples were required to read greater than 1.8 on both A_{260}/A_{280} and A_{260}/A_{230} ratios. For subsequent microarray analysis, quality of the purified RNA was assessed by visualization of 18S and 28S RNA bands using an Agilent BioAnalyzer 2100 (Agilent Technologies, CA). Resulting electropherograms were used in the calculation of the 28S/18S ratio and the RNA Integrity Number, which was greater than 6.8 in all samples [152]. For subsequent real-time PCR analysis, RNA integrity was determined by denaturing (formaldehyde) agarose gel electrophoresis followed by staining with Sybr Gold stain (Invitrogen). Visualization of clear ribosomal bands indicated minimal degradation. Eluted RNA samples were aliquoted and stored at -80°C until use.

HOST GENE EXPRESSION PROFILING USING AFFYMETRIX GENECHIPS.

Total RNA (500ng) was converted to cRNA for microarray analysis using the Ambion MessageAmp™ Premier RNA Amplification Kit (Life Technologies Corporation, CA) according to manufacturer's instructions. Total fragmented cRNA (10υg) was hybridized to the Affymetrix GeneChip Mouse Genome 430A 2.0 array

according to the manufacturer's (Affymetrix, CA) conditions. The chips were washed and stained in a GeneChip Fluidics Station 450 and fluorescence detected with an Affymetrix-7G Gene Array scanner using the Affymetrix GeneChip Command Console software (AGCC1.1). For *I. scapularis* experiments, gene expression changes in comparison to tick-free mice were identified using Partek Genomics Suite (Partek, MO) following the default gene expression workflow. For *D. andersoni* experiments, the resulting CEL files were uploaded to iReport™ (Ingenuity Systems), and the default data analysis was pursued. The resulting values were then filtered for p-values $\leq$ 0.05 and a fold change $\leq$ −1.5 or $\geq$ +1.5.

HOST GENE EXPRESSION PROFILING USING PATHWAY-SPECIFIC PCR ARRAY ANALYSIS.

For the later time point study in *I. scapularis*, host cutaneous gene expression was assessed at each time point using three commercially available RT^2 Profiler PCR Arrays (SAbiosciences, now Qiagen). Arrays were chosen to measure biological pathways related to T-helper cell differentiation (PAMM-073), wound healing (PAMM-013), and signal transduction (PAMM-014). Each 96-well array contains 84 test and five housekeeping genes. Each array also included controls to assess genomic DNA contamination, RNA quality, and general qRT-PCR performance (for more information, see http://www.sabiosciences.com/). For each array, 1μg total RNA purified from skin biopsies was converted into cDNA using the RT^2 First strand kit (Qiagen). Template cDNAs were mixed with RT^2 SYBR Green/Fluorescein qPCR Master Mix (Qiagen) and loaded onto the array using an 8-channel pipette. Arrays were analyzed on an iCycler iQ5 real-time PCR System (Bio-Rad) under standard cycling conditions (10 min at 95°C,

15 s at 95°C, 1 min 60°C for 40 cycles, and an 80-cycle 55-95°C melt curve). The instrument's software was used to calculate the threshold cycle (C_t) values for all molecules analyzed. Fold-changes in gene expression between test and control mice were calculated using the ΔΔCt method. For each included gene, individual measurements that were below the threshold selected ($C_t > 34$) were excluded from further analysis. This was done to reduce the impact of stochastic variations in rare transcripts on the calculated fold change and its associated p-value. Data normalization was based on correcting all C_t values for the average C_t values of the invariant endogenous control genes hypoxanthine guanine phosphoribosyl transferase (Hprt) and heat shock protein 90 alpha (Hsp90ab1). These genes were selected based on analysis of tested housekeeping genes in geNorm [153]. Statistical significance was assessed using LIMMA (linear models in micro-array analysis) in HTqPCR, an R-based program designed for real-time PCR array data analysis [154]. Statistical comparisons were generated for all time points vs. uninfested controls, between time-points (i.e., 12hr-48hr, 48hr-72hr, etc.), and between infestations (e.g. 12hr primary vs. 12hr secondary, etc.). Data sets were filtered with the following criteria: fold change ≥ 3 or ≤ -2 with an adjusted p-value ≤ 0.01.

GENE ONTOLOGY ANALYSIS.

Gene ontology analysis was conducted on the resulting lists of significantly modulated genes. For microarray analysis, the lists of significant genes at each time point were culled based on two criteria: one, genes had to have an existing Entrez ID, and two, each gene was represented only once. For PCR-array analysis, two main lists were created, one that contained all significant genes at any time point in the primary

infestation, and a similar list for the secondary infestation. Each of these lists were divided into up- and downregulated genes and these sublists individually submitted to the Database for Annotation, Visualization, and Integrated Discovery (DAVID) website using the Affymetrix GeneChip Mouse Genome 430A 2.0 array as a background for microarray studies or all genes measured for PCR-arrays [155,156]. The functional annotation chart and functional annotation clustering tools were used to assess enriched gene ontology terms. In particular, the functional annotation clustering tool groups similar gene ontology terms into clusters with a mathematically-generated ranking score. These clusters can then be named based on the gene ontology terms in each cluster, giving a concise over-view of the array data. These clusters were an invaluable tool for guiding subsequent literature searches on the exact function of the modulated genes. For microarray studies, gene expression data was also entered into Ingenuity Pathway Analysis software (Ingenuity Systems, Redwood City, CA).

VALIDATION OF ARRAY DATA.

For each study, array results were validated by an additional experiment. For each experiment, a different list of gene targets were identified to validate. These lists usually contained a group of genes significantly modulated in the specific experiment, plus a few others believed to be important in host responses to tick feeding that I wanted to double-check. In each case, a completely separate set of mice was infested with ticks and tissues harvested as described above. For the *I. scapularis* microarray experiment, gene targets (table 2.1) were validated at 1, 3, 6, and 12 hpi. For the *I. scapularis* PCR-array experiment, gene targets (table 2.2) were validated at 48 and 96hpi during primary infestations and 48 and 72hpi during secondary infestations. For both *I. scapularis*

validations, 4 mice were tested per time-point. For the *D. andersoni* microarray experiment, gene targets (table 2.3) were validated at 12, 48, 96, and 120hpi during primary infestations and 120hpi during secondary infestations using 3 mice/time point. Pre-optimized primer pairs were purchased from Qiagen for the *I. scapularis* experiments. The primer sequences are proprietary information of Qiagen. For the *D. andersoni* microarray validation, primers were purchased from Integrated DNA Technologies; these primer sequences are provided in table 2.5. Primers were mixed with RT^2 SYBR green qPCR master mix (Qiagen) and aliquoted into iCycler iQ PCR plates (Bio-Rad) using an epMotion 5075 automated pipetting system (Eppendorf). Plates were sealed and stored at −20°C until use. For each real-time PCR plate, the RT^2 First Strand Kit (Qiagen) was used to convert 1μg total RNA into cDNA, which was then loaded onto PCR plates using the epMotion 5075 automated pipetting system (Eppendorf). In some cases, the epMotion system was not functioning properly and the plates were loaded with primer and/or cDNA using an 8-channel pipette. These plates were analyzed on an iCycler iQ5 real-time PCR instrument (Bio-Rad) with the following cycling protocol: 10 min at 95°C; 15 s at 95°C, 1 min 60°C for 40 cycles, and an 80-cycle (+0.5°C/cycle) 55-95°C melt curve. Every plate included hypoxanthine guanine phosphoribosyl transferase (Hprt) and heat shock protein 90 alpha (Hsp90ab1) as endogenous control genes, and 'no template' and 'no first strand' controls. HTqPCR, an R-based program for real-time PCR data analysis [154], was used to analyze data using the delta-delta Ct method for gene expression normalization and measurement, and the linear models in microarray analysis (LIMMA) package for statistical comparisons between infested and tick-free mice.

QUANTITATIVE REAL-TIME PCR ANALYSIS OF MOUSE LYMPH NODES AFTER *D. ANDERSONI* FEEDING.

Draining cervical/auricular lymph nodes were harvested from mice used for validation of the *D. andersoni* microarray data set during secondary infestations at 120hpi. Quantitative real-time PCR analysis was undertaken using the gene targets in table 2.4. These results were compared against lymph nodes from tick-free mice. RNA was isolated and analyzed by PCR as described above.

CYTOKINE ANALYSIS.

For the *I. scapularis* PCR-array experiment, the relative concentrations of interleukin-1β (IL-1β), IL-3, IL-4, IL-6, IL-10, IL-17A, interferon-γ (IFN-γ), and monocyte chemoattractant protein-1 (MCP-1 or CCL2) at the tick bite site were quantified using an 8-analyte bioplex assay and the Bioplex 200 system (Bio-Rad). Samples represented two time points (4 mice/time point) in the primary and secondary infestations (48hr and 96hr primary, and 48hr and 72hr secondary). Biopsies were removed from storage at -80°C and immediately homogenized in 1mL protein extraction buffer containing 0.5% BSA (Fisher Scientific), 0.1% Igepal-630 (Sigma), and 1% Halt protease inhibitor (Thermo Scientific) in PBS. Homogenates were centrifuged at 20,000 x g at 4°C for 20 min. The resulting supernatants were divided into aliquots and stored at -80°C until use. Standards, blanks, and samples were analyzed in duplicate according to the manufactures' instructions. Analyte concentrations were determined from the standard curve(s) by analysis of mean fluorescent intensity values using the Bio-Plex

Manager™ 6.0 software. Individual time points were compared to controls using a 2-tailed T-test in Prism (GraphPad Software, Inc.).

ARRAY DATA SUBMISSIONS.

Array data was made publicly available through Gene Expression Omnibus.

Ixodes scapularis PCR-array data under accession number GSE33345.

Ixodes scapularis microarray data under accession number GSE39100.

Dermacentor andersoni microarray data will be public when the related manuscript is published.

HISTOPATHOLOGY ANALYSIS.

Skin biopsies with attached ticks were harvested from infested mice as described above and fixed in zinc fixative (BD Pharmingen) or 10% neutral buffered (phosphate) formalin (Fisher Scientific) for 48hrs. After fixation, samples were transferred to 70% ethanol (Acros Organics) and stored at 4°C until processing. In some cases, tissues were treated for 2 hours with decal (Decal Chemical Corp, Tallman, NY) in an attempt to improve sectioning. Tissues were embedded in paraffin and 5μM sections were cut. Sections from the skin at the site of the entry of the hypostome were stained with hematoxylin and eosin. Stained slides were read with guidance from Dr. Judith Aronson and Dr. Brent Kelly.

R COMMANDS FOR DATA ANALYSIS.

This section is not meant to be a primer on using R for microarray or quantitative real-time PCR data analysis. However, some basic commands were very frequently used and are included here. R is a free language designed for statistical computing. It can be downloaded from < http://cran.r-project.org> . HTqPCR, the R package used in quantitative real-time PCR analysis can be downloaded by opening R and typing the following commands:

>source("http://bioconductor.org/biocLite.R")
>biocLite("HTqPCR")

Each necessary package is loaded into R by using the following generic command:

>library("package")

Within each package, a user's guide can be accessed using the command:

>openVignette()

These user's guides are invaluable. For the quantitative real-time PCR analysis, the package "HTqPCR" was used. The basic commands for analysis are as follows:

>path <- "/Documents and Settings/… "

This command to defines the directory containing the experimental data files. In this folder, each PCR data file must be saved as an individual .txt file containing the cycle thresholds, corresponding gene names, and sample names. In addition, a separate template file named "files.txt" must contain the name of each .txt data file and the experimental condition represented by each file. HTqPCR uses this second file to define the test and control groups in the analysis.

>files <- read.delim(file.path(path, "files.txt"))

This command loads the template file.

>raw <- readCtData(files=files$File, path=path, n.features=89, format="plain", column.info=list(feature=2, type=3, position=1, Ct=4))

This command loads the raw data. The specific number after "n.features" defines the number of genes on each plate. "Feature" (gene names), "type" (test or housekeeping gene), "position" (gene location), "Ct" (cycle threshold), etc. are specific to each experiment and are defined by an integer corresponding to the column in which the information is contained in the raw data files (the left-most column is "1").

>d.norm <- normalizeCtData(raw, norm="deltaCt", deltaCt.genes=c("Hprt1", "Hsp90ab1"))

This command normalizes the raw data using the deltaCt method. The argument "deltaCt.genes=c("gene"…)" allows you to specify which housekeeping genes are to be used in normalizing the data.

The next few commands refer to running the LIMMA test. First, a matrix in which each experimental condition is contained in a column is defined. Then, these column names are used to set up contrasts to be statistically tested. LIMMA is a powerful tool for making multiple statistical comparisons at the same time. To do this, a contrast is entered for each test desired. Here are the commands:

>design <- model.matrix(~0+files$Treatment)

This gives the default matrix based on the template file.

>colnames(design) <- c("experimental condition 1", "experimental condition 2")

This command re-names the columns in the default matrix to names meaningful for an individual experiment. Some caution is required here, because it will not re-order the columns; look at the column order in the default matrix and rename each column as needed in the order they appear in the default matrix.

>contrasts <- makeContrasts(experimental condition 1-experimental condition 2, levels=design)

This command defines the contrasts to be tested. Be careful, the contrasts are always set up as "test-control."

>qDE.limma <- limmaCtData(d.norm, design=design, contrasts=contrasts)

>qDE.limma

These commands execute the test and provide the data. Other arguments defining the number of technical and biological replicates in the experiment can be used. Data can be copied out of R into excel or saved directly using permutations of the following command:

>write.table(qDE.limma, file="filename.txt")

Tables.

Table 2.1: Gene list for quantitative real-time PCR validation of the *I. scapularis* microarray study.

Banf1	Fn1	Il2	Socs1
C1qb	Foxp1	Il4ra	Stat6
Ccl2	IFN-γ	Jak1	Vapb
Ccl7	IL-10	Muc1	Vwf
Ccr5	IL-1β	Myb	Hprt1
Clec4e	IL-3	Saa1	Hsp90ab1
Ctse	IL-4	Sele	No RT
Cxcl5	IL-6	Serpina3n	No temp

Table 2.2: Gene list for quantitative real-time PCR validation of the *I. scapularis* PCR-array study.

Ccl2	IL-1β	Jak2	Tbx21
Ccl7	IL-3	Mmp13	Actb
Ccr5	IL-4	Rorc	Gadph
Clec7a	IL-6	Sele	Gusb
Cxcl5	Itgal	Sell	Hprt1
Gata3	Itgam	Selp	Hsp90ab1
IFN-γ	Itgb1	Socs1	No RT
IL-10	Itgb2	Stat6	No temp

Table 2.3: Gene list for quantitative real-time PCR validation of the *D. andersoni* microarray study.

Arg1	Cxcl5	il4	S100a9
Ccl12	Hdc	Il6	sele
Ccl6	Hprt	Irak4	Serpine1
Ccl7	Hsp90ab1	krt16	Socs3
Ccr1	ifng	krt6b	Stat3
Chi3l1	il10	Map3k6	Wnk1
Clec10a	il12a	Mt1	No RT
Clec4e	Il1b	ptges	No temp

Table 2.4: Gene list for quantitative real-time PCR analysis of mouse lymph nodes after secondary infestation with *D. andersoni* for 120hrs.

Actb	Il12a	Stat3
Foxp3	Il17a	Stat4
Gapdh	Il1b	Stat5a
Gata3	Il4	Stat6
Hprt	Il6	Tbx21
Hsp90ab1	RORgt	Tgfb1
Ifng	Socs3	No RT
Il10	Stat1	No temp

Table 2.5: List of gene targets and primer sequences used in the *D. andersoni* quantitative real-time PCR validation of the microarray data and also in quantitative real-time PCR analysis of lymph nodes at 120hpi during secondary infestations.

Gene name	Primer 1 (5'-3')	Primer 2 (5'-3')
RORgt	Ccgctgagagggcttcac	Tgcaggagtaggccacattac
Gapdh	gtggagtcatactggaacatgtag	aatggtgaaggtcggtgtg
Tbx21	caagaccacatccacaaacatc	Ttcaaccagcaccagacag
Arg1	Agtgttgatgtcagtgtgagc	Gaatggaagagtcagtgtggt
Stat4	Caactcctctgtcaccatgt	cgtcaaagctatgtccagtga
Chi3l1	Ccatcaaagccataagaacgc	ccagaaacaccaacctgaaga
Hdc	Gaccgaatcacaaaccacag	tctacctccgacatgccaa
Irak4	Cagcagtagttgaggttcacg	Acacccaaatctgacatctaca
Stat1	gacttcagacacagaaatcaactc	Ttgacaaagaccacgcctt
Stat6	agttcttcctgcttccgatg	Gccaccatcagacaaatacttc
Tgfb1	cgtggagtttgttatctttgctg	Gacgtcactggagttgtacg
Stat3	Agctcctcagtcacgatca	gttcaagcacctgacccttag
Ptges	Caggaatgagtacacgaagcc	Gtattacaggagtgacccagatg
Il12a	ctctcgttcttgtgtagttcca	acagatgacatggtgaagacg
Foxp3	Ctgtcttccaagtctcgtctg	Ctggtctctgcaggtttagtg
Lcn2	Cctgtgcatatttcccagagt	Ctacaatgtcacctccatcctg
Il1b	Cgagatttgaagctggatgc	Tgacagtgatgagaatgacctg
Il17a	gagcttcccagatcacagag	Agactacctcaaccgttcca
Il10	Atggccttgtagacaccttg	Gtcatcgatttctcccctgtg
Sele	Cctgattgttttgaacctagacg	Cgtcctcattgctctacttgt
Stat5a	Cgcttgattcttttcagtgaca	Tgagaacacccgcaatgag
Ccl6	Gaagtgtcttgaaagccttgatg	Agaaactccaagactgccat
Hprt	Aacaaagtctggcctgtatcc	Ttccctggttaagcagtacag
Serpine1	ggctgagatgacaaaggct	Tcacaagtctttccgaccaag
Il6	Gcaagtgcatcatcgttgttc	Agtcggaggcttaattacacat
Gata3	gtccccattagcgttcctc	Ccttatcaagcccaagcgaa
Ifng	Agataatctggctctgcagga	Gtcattgaaagcctagaaagtctg
Hsp90ab1	Cctgaaaggcaaaggtctcc	Ccaccctgctctgtactact
Clec10a	gaccaaggagagtgctagaag	tgactgagttcctgcctct
Wnk1	Aaaggcatggttcaaaaggtc	Gcagatctaccgtcgagtga
Ccr1	Aggaactggtcaggaataatagc	Caaaggcccagaaacaaagtc
Il4	Tgatgctctttaggctttccag	Cagagactctttcgggcttt
Clec4e	Cttatggtggcacagtcctc	Agtggcaatgggtggatg
S100a9	ccatcagcatcatacactcctc	Tggaagcacagttggcaa
Map3k6	Gatttccggggccatatactg	Cacagagacatcaagggagac
Krt6b	Ctgcttttgtacgcttgttga	Gacagcatcattggagagagg
Ccl7	Tttgtttcttgacatagcagcat	Tctcactctctttctccacca
Cxcl5	Gatccagacagacctccttct	ttgatcgctaatttggaggtga
Mt1	gctcttcttgcaggaggtg	Tcacttactccgtagctccag
Krt16	cagctcattctcgtacttggtc	Tcaaagactacagcccctact
Actb	Cgatggaggggaatacagc	Tctttgcagctccttcgtt
Socs3	ggaaacttgctgtgggtga	Gagatttcgcttcgggacta
Ccl12	Ggaggcatagaagtgtggaaa	agagacactggttcctgact

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Chapter 3: Early immunologic events at the *Ixodes scapularis* tick-host interface[3]

INTRODUCTION.

Emerging and re-emerging diseases transmitted by blood feeding arthropods are significant global public health problems. Ticks transmit the greatest variety of microbial pathogens of any blood feeding arthropod, including bacteria (e.g., spirochetes, rickettsiae, ehrlichiae, anaplasma), protozoans (e.g., *Babesia*), and viruses [157]. Infectious agents transmitted by ticks are delivered to the vertebrate host together with saliva at the bite site. Tick salivary glands produce a complex repertoire of bioactive molecules that creates an immunologically privileged microenvironment facilitating blood feeding and pathogen transmission [158]. Salivation is not a continuous process during blood feeding [159] and the repertoire of salivary proteins changes during the course of feeding [10,11]. These temporal patterns allow the saliva proteins of the tick to "prime" the feeding site to different degrees prior to introduction of infectious agents. Not all tick-borne pathogens are transmitted at the same time during the feeding process. Tick-borne encephalitis virus was observed to be transmitted within the first few hours of attachment/feeding, while *B. burgdorferi* was observed to be transmitted between 24 and 48 hours post tick attachment/feeding [130,131,132]. It is likely that the temporal expression of immunomodulatory tick salivary proteins secreted into the bite site depends

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[3] This chapter has been published as Heinze DM, et al. PLoS One. 2012;7(10):e47301. PLoS One is an open access journal.

on the pathogen it transmits. In this study, tick-induced changes in cutaneous gene expression were measured at 1, 3, 6, and 12 hours post infestation (hpi) with *I. scapularis* nymphs using Mouse Genome microarrays. In addition, histopathological analysis of tick bite sites at the same time points were also conducted and correlated, where possible, to the microarray data. This information may reveal immunomodulation at the tick-host interface induced by tick saliva that facilitates tick-borne virus transmission.

RESULTS AND DISCUSSION:

Microarray analysis of host immune responses to early tick feeding.

The immune response at the tick-host interface was investigated at 1, 3, 6 and 12 hours post nymphal tick infestation (hpi) using microarrays. Significantly modulated genes increased across time (Figure 3.1a), reflecting the development of host responses as the infestation progressed. A higher percentage of modulated genes were shared with adjacent than distant time points (Figure 3.1b). The specific gene expression profiles were similar between 1 and 3 hours, but showed appreciable change between 3 and 6 hours, and again between 6 and 12 hpi (Figure 3.1c). These results suggest that cutaneous responses undergo rapid changes in gene expression profiles in the early stages of tick feeding.

Significantly modulated genes were divided into up and downregulated lists at each time point and submitted to the Database for Annotation, Visualization and Integrated Discovery (DAVID) bioinformatics database. The functional annotation-clustering tool was used to group similar terms together. These clusters were then named according to the gene ontology terms in each cluster (Table 3.2). At 1 and 3 hpi, the only

significantly up-regulated cluster was "post-translational modification: ubiquitin, isopeptide;" no significantly downregulated clusters were observed. Changes in gene expression at 1 and 3 hpi were related to signaling pathways such as NF-κB and cation homeostasis, suggesting pro-inflammatory pathways are already activated. At 6 hpi, clusters related to cytoskeletal elements, keratinocyte migration, cell signaling, transcription, and immune responses were prominent. At 12 hpi, cell cycle, cytoskeletal elements, and immune response clusters were observed. Immune response clusters differed between 6 and 12 hpi. At 6 hpi, anti-microbial responses, immunoreceptor signaling, and negative regulation of myeloid differentiation were significant, while at 12 hpi, inflammation and chemotaxis were significant. These results suggest a rapid pro-inflammatory evolution of the early host response beginning soon after attachment that progress from early inflammatory signaling and pre-programmed anti-microbial responses to the infiltration of innate immune cells by 12 hpi.

Cytoskeletal changes.

At both 6 at 12 hpi, the most significantly upregulated gene ontology clusters were related to components of the cytoskeleton such as intermediate filaments. A closer look at these genes revealed many keratin intermediate filament transcripts. Keratin intermediate filaments have been shown to protect epithelial tissues from mechanical and non-mechanical stresses, modulate apoptosis, regulate some aspects of skin pigmentation, and control keratinocyte migration during the process of wound healing [160,161,162,163]. Because the initiation of the feeding lesion necessitates significant local damage to epithelial tissues, these ontology terms likely reveal early epithelial attempts to close the wound. Interestingly, Krt6, a gene upregulated at both 6 and 12 hpi has already been shown to be upregulated in keratinocytes at wound margins as early as 6

hrs post injury, and to pair with Krt16/17 during wound healing [163]. The up-regulation of all these genes at 12 hpi in this study supports the possibility these gene ontology clusters describe a nascent wound healing response at the tick bite site.

Transcription factors and cell signaling pathways.

A number of gene ontology clusters were related to signaling pathways and downstream targets such as transcription factors. Upregulated clusters at 6 hpi were transcription/transcription factors and protein phosphatase, while downregulated clusters included G-protein signaling domain. While many transcription factors were modulated, the largest group belonged to members of the activator protein-1 (AP-1) transcription factor family. AP-1 is composed of homo- or hetero- dimers of Fos, Jun, Atf3, Fra, Maff, and potentially other proteins. It can activate an extremely broad range of processes that include oncogenesis, regulation of bone homeostasis, and immunity. In the skin, loss of JunB leads to a systemic lupus erythematosus-like phenotype, while loss of both c-Jun and JunB leads to postnatal death. An inducible deletion of c-Jun and JunB in the skin leads to a psoriasis-like phenotype characterized by joint inflammation, hyperkeratosis, and epidermal infiltrates [164]. Thus, AP-1 members play a significant role in regulating epidermal inflammatory responses. The microarray data suggests that AP-1 may be activated through the mitogen-activated protein kinase (MAPK) pathway, based on the up-regulation of Mak3k6 and negative regulators of the MAPK pathway such as dual-specificity phosphatases (DUSP). In addition to AP-1, members of the NF-κB family such as Nfkbia and Nfkbiz were also upregulated. While both of these genes encode proteins classified as NF-κB inhibitors, Nfkbiz has been shown to induce the transcription of IL-6 following NF-κB activation via IL-1/TLR signaling [165]. Additional support for NF-κB activation in this study includes the upregulation of other

NF-κB target genes such as IL-1β and CCL2. It is possible the upregulation of Nfkbia represents the activation of a negative feedback loop on NF-κB signaling.

G-protein signaling domain was the primary downregulated cluster related to signaling. Two groups of G-protein signaling modulators were represented: regulators of G-protein signaling (RGS) and G-protein coupled receptor kinases (GRK). Both of these groups act to dampen GPCR activity. RGS molecules accomplish this by enhancing the intrinsic GTPase activity of activated Ga subunits [166] while GRK proteins phosphorylate the active GPCR, making it a high-affinity target for arrestin binding which blocks G-protein binding and activation [167]. Thus, the down-regulation of these molecules may potentiate GPCR signaling at the tick bite site.

Immune response.

A number of clusters in the gene ontology analysis at 6 hpi were related to the immune response. These were cation homeostasis, anti-microbial response, negative regulation of myeloid cell differentiation, and B-cell, T-cell, and Toll-like receptor signaling. Within the cation cluster were transcripts for genes involved with iron, zinc, calcium, and proton transport or regulation. In particular, lactotransferritin, metallothionein 1, and metallothionein 2 have been shown to function in regulating reactive oxygen species production and scavenging [168,169]. While some of the genes in this cluster are related calcium transport and may function in cell signaling, it is likely that regulating the oxidative status of the tissues near the bite site is the primary function of these genes.

Genes of interest in the anti-microbial cluster were beta-3 defensin (Def3b) and peptidoglycan recognition protein (Pglyrp1). Defensins are small positively charged cysteine-rich peptides with antimicrobial activity; interestingly, epithelial tissues but not

neutrophils were the primary sources of mouse beta-defensins [170]. Def3b has wide spectrum anti-microbial activity against bacteria [171], fungi [172], and viruses [173]. Pglyrp1 has been shown to enhance intracellular killing of bacteria in neutrophils [174]. Thus, early host responses to tick feeding include up-regulation of potent anti-microbial proteins that could impact the transmission of tick-borne pathogens.

Genes within the negative regulation of myeloid cell differentiation and B-cell, T-cell and Toll-like receptor signaling clusters included the transcription factors and signaling intermediates mentioned above (see Transcription factors and cell signaling pathways heading).

At 12 hpi, the only gene ontology cluster related to the immune response was inflammation and chemotaxis. The genes in this cluster were cytokines, chemokines, and related molecules. Chemokines are small peptides that are potent activators and chemoattractants for leukocytes, and play an important role at the sites of inflammation. Overall, nymphal tick feeding induced the expression of chemokines specific for neutrophil (Cxcl1 and 5) and monocyte (Ccl2, 6, 7, and 12) recruitment. In addition, Cxcl14 was upregulated, a chemokine specific for dendritic cell precursors [175] but without a defined function in the skin [176]. Increasing evidence suggests that small inflammatory mediators such as leukotrienes, prostaglandins, platelet activating factor, and complement initiate chemotaxis to sites of inflammation. This initial response is amplified by cytokine production that drives chemokine synthesis [177]. The microarray results support the up-regulation of Il1b, Il6, and C1qb that may interact with the chemokine profile to maintain and amplify the chemotactic response. While the sequence of events could not be defined in this study, the gene expression profile strongly

suggests the recruitment of neutrophils and monocytes to the bite site, in agreement with our histological observations (see below).

Ingenuity pathways analysis.

All significantly modulated genes were submitted to Ingenuity Pathways Analysis (IPA). This analysis has some advantages over DAVID in that it takes into account the fold change associated with each modulated transcript when determining biological significance. The most significant pathway in this dataset was related to acute inflammation and immune cell recruitment, supporting the DAVID analysis. The interactions between genes in this pathway were mapped over time (Figure 3.2), showing temporal increases in gene modulation in this pathway. In addition, all the genes modulated at any time point in the inflammatory response pathway were plotted to show temporal changes in gene expression (Figure 3.3). These data suggests that early tick feeding is characterized by an inflammatory response from the earliest time point that intensifies as feeding continues.

Validation of microarray data

Based on the significance of chemotaxis and inflammation in the gene ontology analyses, a list of chemokines, cytokines, inflammatory molecules, and genes in the ontology term "viral reproduction" (GO:0016032) was chosen for validation by real-time PCR (Table 3.1). Specific validation targets were chosen by significant fold change in the array data or previously identified genes of interest in host anti-tick responses [178]. All significantly modulated genes at any time point in the validation experiment are shown in figure 3.4. While exact fold changes did not correlate, trends of up or down regulation were well preserved between the array and validation studies. Ctse, Foxp1,

IL-6, and Muc1 were modulated in the array but were not validated. Other genes such as Ifng, IL-3, Jak1, Stat6, and Vwf were not modulated in either study. Significant increases in chemokine gene expression begin at 1 hpi and intensify as time progresses, supporting the importance of chemotaxis seen in the array. Up-regulation of IL-1β, IL-10, and IL-4ra along with the general inflammation group supports early inflammatory changes at the bite site. While genes in the viral reproduction group showed some significant modulation, changes between time points were not consistent, casting some doubt on the biological significance of these differences.

Histopathology

Concurrently with gene expression measurements, histopathological analysis of the bite site was pursued to see if morphological changes could be correlated with array data. Resident cells such as keratinocytes, fibroblasts, dendritic cells, and mast cells initially sense cutaneous tissue injury or antigenic stimuli. These cells release rapidly synthesized or pre-formed pro-inflammatory and chemotactic molecules including histamine and eicosanoids. These molecules can stimulate cytokine production and endothelial cells to mobilize Weibel-Palade bodies containing pre-formed selectins to the cell surface. While these early events cannot be measured at the transcriptional level, the histopathological analysis clearly shows tissue damage from the insertion of the hypostome and degranulating mast cells (Figure 3.S1) as early as 1 hpi. Minor inflammatory changes consisting of a few inflammatory cells and a small amount of eosinophilic material near the tick hypostome were also observed. By 3 hpi, inflammatory cells were readily evident, the eosinophilic material near the hypostome was much more pronounced, and the tissue architecture had the appearance of streaming

toward the bite site, even in hypodermal muscle layers. This appearance suggests that ticks may initially insert the hypostome deeply and then retract it, pulling deeper tissues towards the epidermis. These changes intensify at 6 hpi, leading to a very intense, neutrophil dominated inflammatory lesion by 12 hpi. Also visible at 12 hpi were potential areas of myositis, muscle necrosis, and increased congestion in blood vessels near the hypostome (Figure 3.5).

Early immunologic response to tick bites:

The early appearance of pro-inflammatory changes in transcription and histopathology was unexpected. Previous studies in our laboratory had suggested a minimal early host response [178], supporting many studies that have shown that tick salivary components are capable of inhibiting nearly every aspect of cell recruitment. *Ixodes ricinus* saliva contains leukotriene B4 binding proteins that have been shown to reduce neutrophil migration [50], histamine binding proteins have been described from *R. appendiculatus* saliva [179], and numerous tick anti-complement molecules have been described [69,71,73]. The release of histamine, eicosanoids, and complement fragments are likely some of the earliest events in the chemotactic cascade. In addition, *I. scapularis* saliva has been shown to downregulate neutrophil beta-2 integrins, reduce phagocytic efficiency, and inhibit intracellular killing of *B. burgdorgeri* [180]. The reduction in intracellular killing may be explained by salivary proteins that block super-oxide formation [83] and detoxify reactive oxygen species [181]. Tick salivary proteins have also been shown to bind human IL-8 [116] and chemokines such as CXCL8 [182]. These studies show that tick saliva can inhibit later events in the chemotactic cascade and also effector functions of neutrophils. Against this backdrop, the present study shows

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that leukocyte (such as neutrophil) chemotaxis and pro-inflammatory gene transcription was initiated before 3 hpi. Thus, despite the impressive arsenal of inhibitory tick salivary proteins, the host is able to mount a surprisingly timely immune response. Studies in mice with labeled neutrophils (enhanced GFP expression under the control of the lysozyme M promoter) have shown that neutrophils migrate into sites of sterile cutaneous injury as soon as 20 minutes post-injury. Neutrophil numbers then increased rapidly for 2 hrs when a plateau-phase was reached [183]. In a similar model using a larger wound (6mm) and EGFP- labeled neutrophils, influx was measurable at 4 hrs and did not plateau until 2-3 days post wounding [184]. These studies suggest that neutrophil chemotaxis into sites of cutaneous injury was initiated within 20 minutes, but the subsequent kinetics and final concentration of neutrophils may depend on other factors such as the size of the wound. In this study, very few neutrophils were visible at the bite site by 1 hpi. It should be noted that this is 1 hour after apparent tick attachment and hence represents the maximum length of attachment. Even so, it seems likely that the very early phase of neutrophil recruitment to nymphal tick bite sites is slower than that reported to sterile cutaneous wounds. At 3 hpi, appreciable neutrophils were present, and their numbers increased as time progressed, suggesting the plateau phase may not be reached during the time scale of the experiment.

CONCLUSIONS.

Gene-expression analysis reveals modulation of some pro-inflammatory genes at 1 and 3 hpi that intensifies to include genes related to antimicrobial responses, regulation of reactive oxygen species, wound healing, and signaling through AP-1, NF-κB, MAPK,

and G-protein pathways at 6 hpi. At 12 hpi, chemokines and cytokines consistent with a neutrophil-dominated immune response are prominent. Histopathology showed mast cell degranulation by 1 hpi and increasing neutrophil influx beginning at 3 hpi, supporting the array data. These results suggest early cutaneous host responses to tick feeding are more pro-inflammatory than expected, and highlight the importance of neutrophils and related pathways in tick engorgement and tick-borne viral transmission.

Tables.

Table 3.1: **Gene list for real-time PCR validation**

Banf1	Fn1	Il2	Socs1
C1qb	Foxp1	Il4ra	Stat6
Ccl2	IFN-γ	Jak1	Vapb
Ccl7	IL-10	Muc1	Vwf
Ccr5	IL-1β	Myb	Hprt1
Clec4e	IL-3	Saa1	Hsp90ab1
Ctse	IL-4	Sele	No RT
Cxcl5	IL-6	Serpina3n	No temp

Table 3.2: **Gene ontology clusters from DAVID analysis**.

Clusters from upregulated genes	
6 hpi	12hpi
Cytoskeleton, intermediate filament, keratin filament, non-membrane bound organelle	Cytoskeleton: intermediate filament
Transcription factor, regulation of transcription, DNA binding	Keratin, intermediate filament
Epithelial development, keratinocytes, cytoskeleton	DNA replication
Transcription and RNA metabolism	DNA repair
Protein phosphatase	Cytoskeleton organization: intermediate filament
Cation homeostasis	Epidermis development, hair follicle cycle
Anti-microbial response	Inflammation, chemotaxis
Negative regulation of myeloid cell differentiation	Epithelial development
B-cell, T-cell, and Toll-like receptor signaling	Cell cycle
	Nucleolus, membrane-enclosed lumen

Clusters from downregulated genes	
6 hpi	12hpi
Secreted: signal peptide, disulfide bond, glycosylation	Skeletal system development
Armadillo repeat-containing proteins	Regulation of transcription, DNA binding
	Internal side of plasma membrane, organelle organization
Von Willebrand factor C domain	
G protein signaling domain	
Extracellular matrix	
Skeletal system: ossification	
Cell and cell-cell adhesion	
Blood vessel development	
Transmembrane, glycoprotein	
Golgi, cytoplasmic vesicle, clathrin coated vesicle	

Figures.
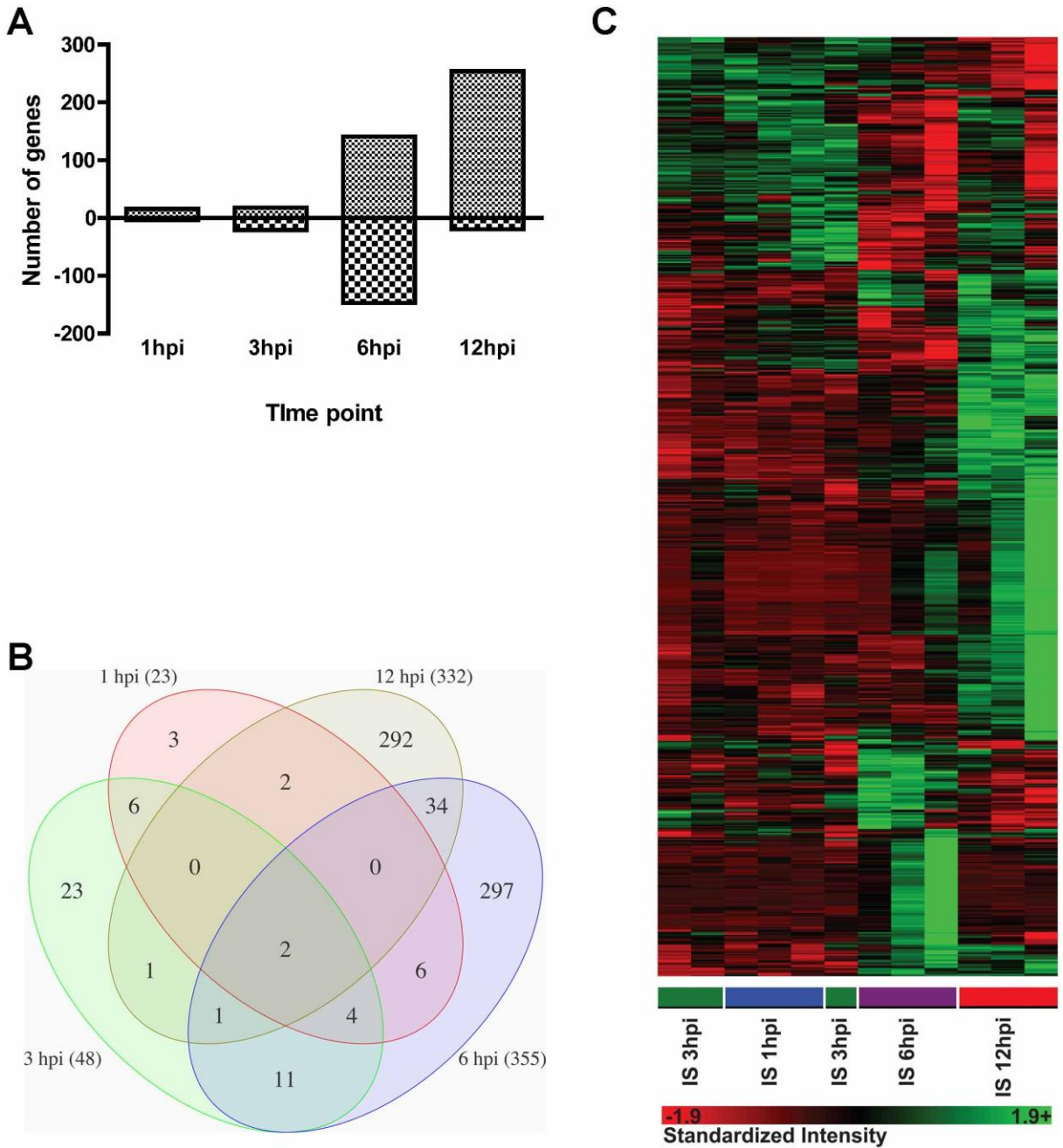
Figure 3.1: An overview of gene expression profiles from tick bite sites at 1, 3, 6, and 12 hpi.

A: the total number of up- or downregulated genes at each time point; B: Venn diagram showing overlap of significantly modulated genes between time points; C: Heat map showing temporal changes in gene expression profiles.

1hpi

3hpi

FOS

CD44 (includes EG:100330801)

HSP90B1

SOCS3*

CD28*

LUM*

JUNB

PRMT2

TRAFD1

B3GNT2

IL6

ATF3

ELF3

NFKBIA*

HSF1 (includes EG:15499)

MSR1 (includes EG:20288)

NRIP1

ESR2

MARCO

Ceacam1

IL36RN

VTN

GAB1

IL1B

ESR1

ZC3H12A

CCL13

IFNGR1

KITLG*

DUSP1

IGF1

NFE2L2

EGR1

XDH

CXCL2

71
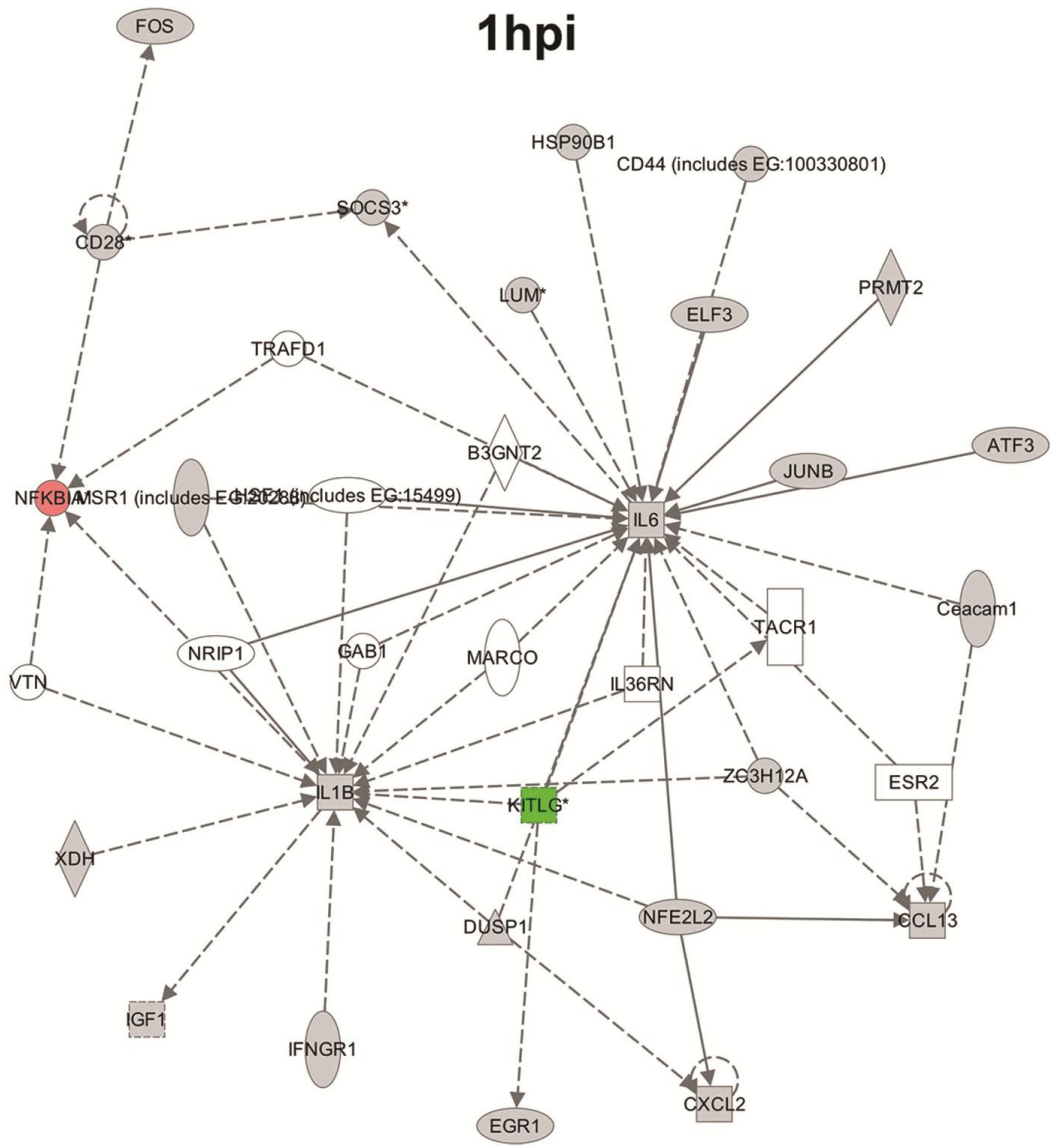
6hpi
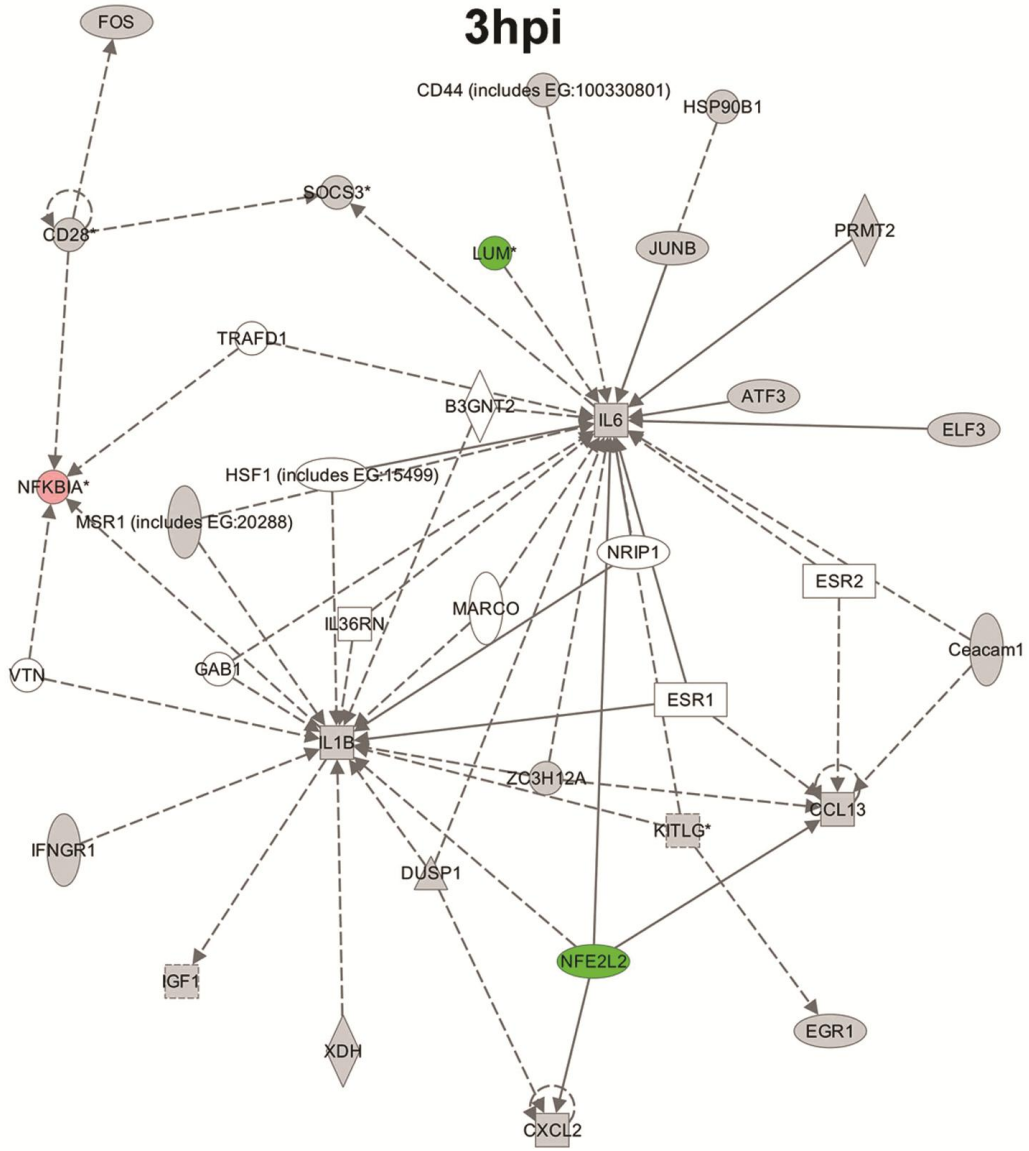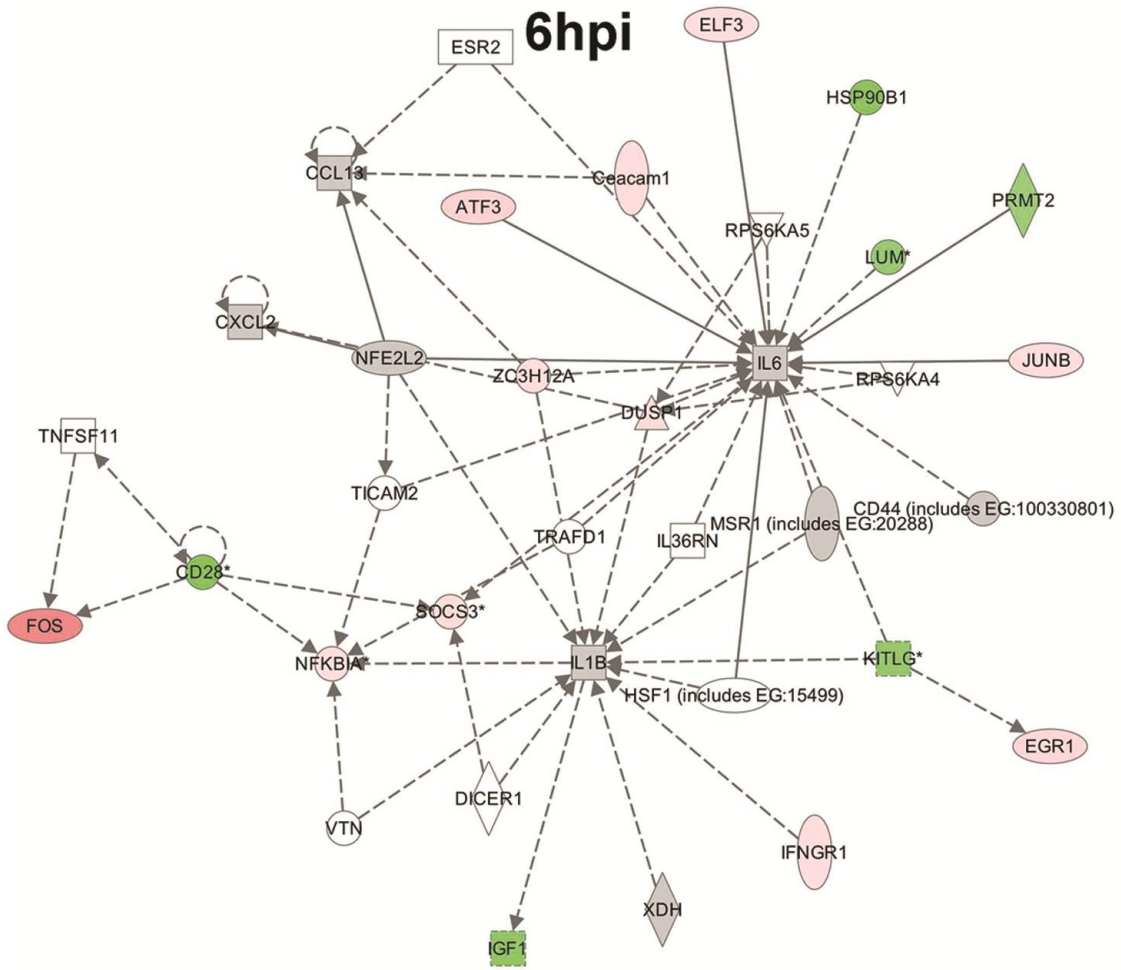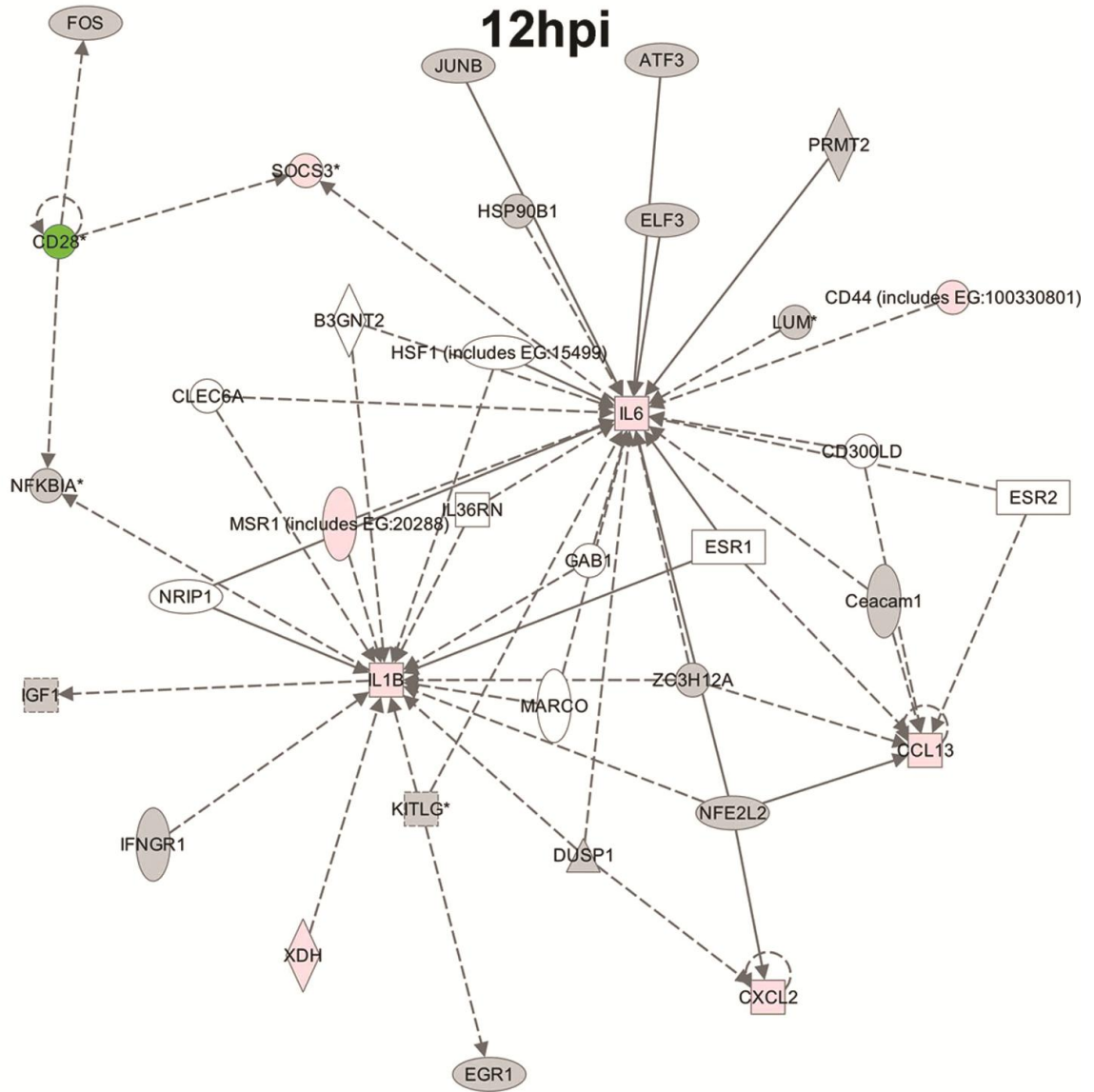
Figure 3.2: IPA analysis, acute inflammation pathway.

This diagram shows the interactions between genes and the temporal increase in expression of genes in the acute inflammation and immune cell recruitment pathway from the IPA analysis. Upregulated genes are orange/pink, downregulated genes are green, and unchanged or unsignificant genes are grey.
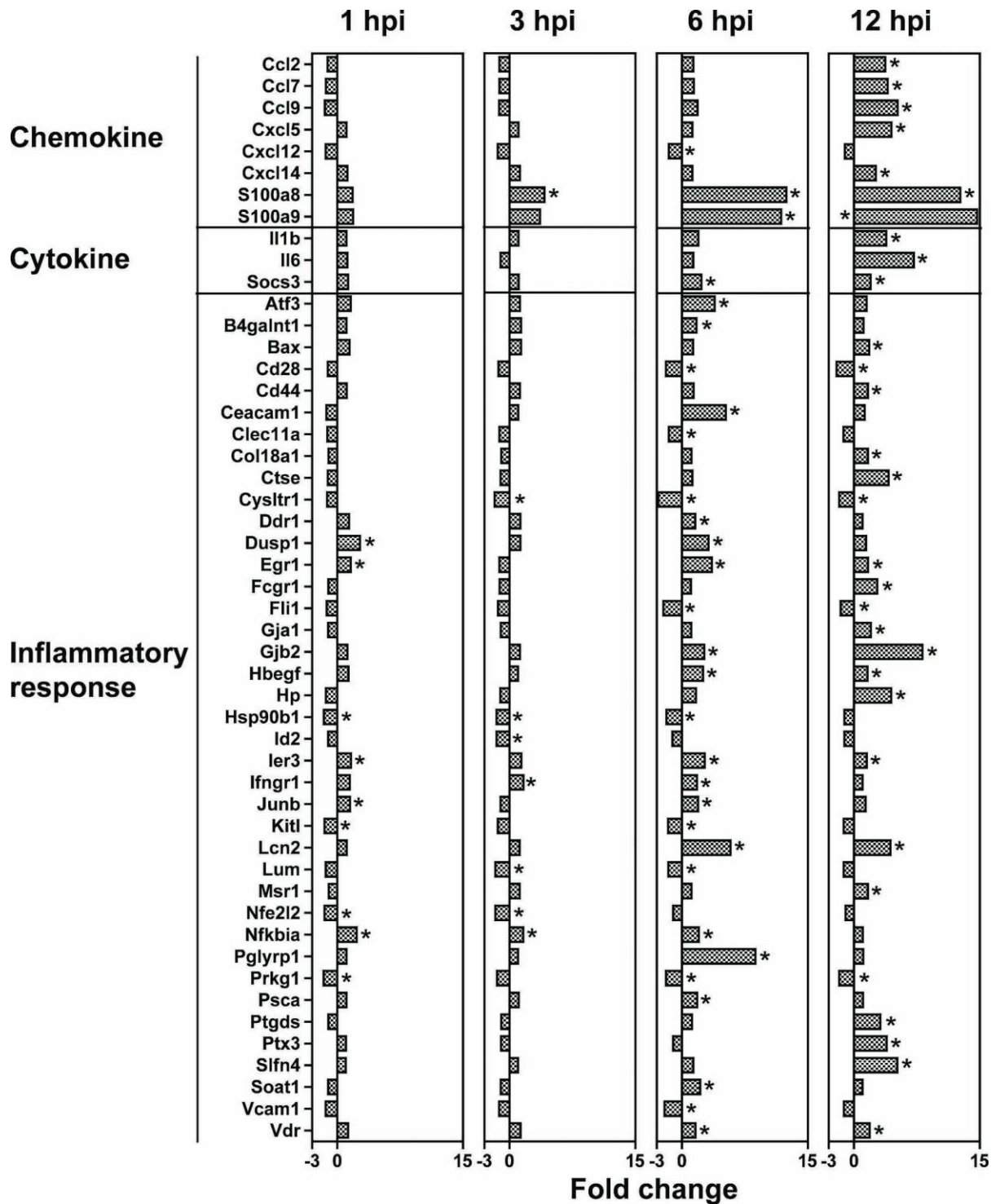
Figure 3.3: Genes in IPA pathway inflammatory response.

All genes significantly modulated at any time point in the inflammatory response pathway from the IPA analysis were plotted showing temporal changes in gene expression. Genes significant at each time point are marked with an asterisk.
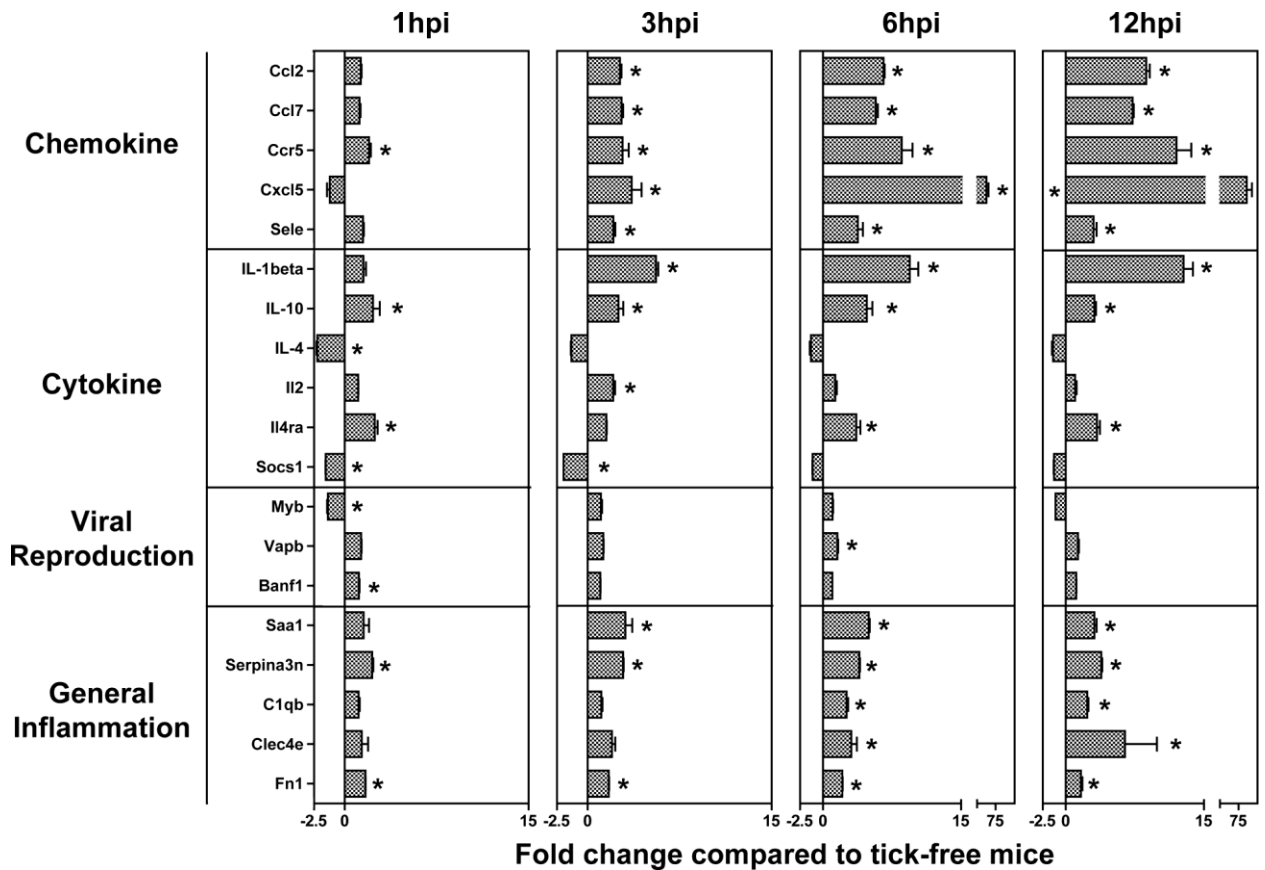
Figure 3.4: **Real time PCR validation of array data.**

Validation targets were chosen based on significant fold change in the array study at 12 hpi or genes previously identified as important in host anti-tick responses. All significantly modulated genes at any time point in the validation study are plotted. Significant changes in gene expression at individual time points are marked with an asterisk.
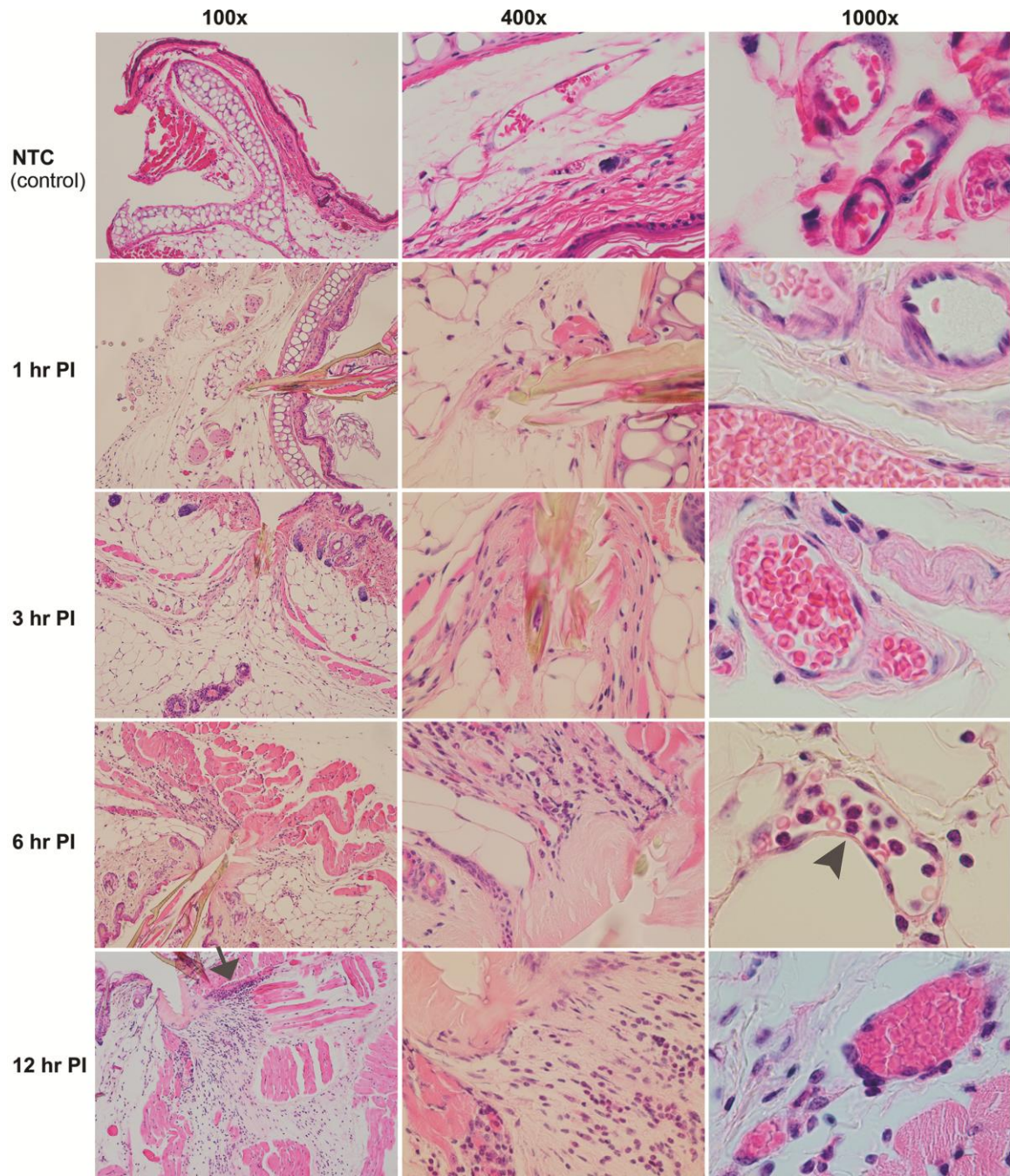
Figure 3.5: Histopathology of Ixodes scapularis nymphal bite sites at 1-12hpi.

The sections were stained with hematoxylin and eosin (methods). The arrowhead marks a marginating neutrophil at 6 hpi 1000x, while the arrow marks areas of putative myositis/muscle necrosis at 12 hpi 100x. The 100x and 400x pictures include the tip of the hypostome. The 1000x images show differences in blood vessels near the bite site.
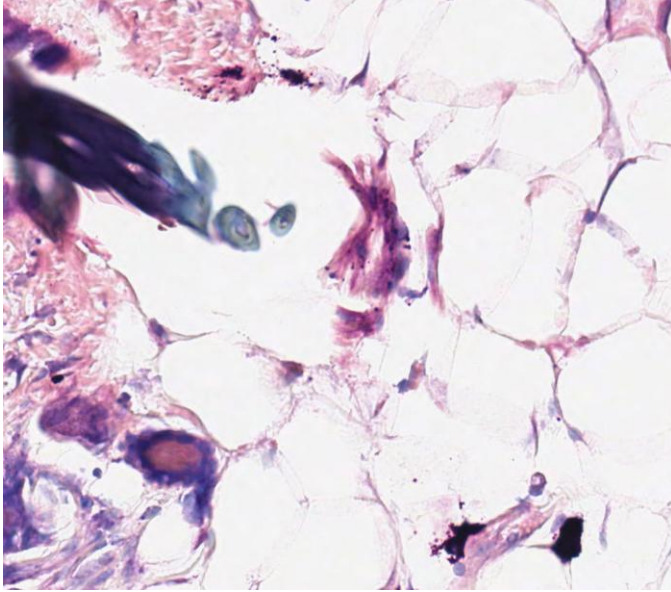
Figure 3.S1: Histopathology of Ixodes scapularis nymphal bite sites at 1 hpi.

Sections were stained with Geimsa. Mast cells appear as collections of dark purple granules.

Chapter 4: Transcriptional profiling of the murine cutaneous response during initial and subsequent infestations with *Ixodes scapularis* nymphs[4]

INTRODUCTION

The process of tick feeding activates a highly complex sequence of events at the bite site that facilitate the acquisition of a blood meal and create a suitable microenvironment for pathogen transmission and establishment [185]. These events are governed by an array of salivary molecules secreted by the tick and the responses of the host to those molecules. It is a dynamic relationship with outcomes ranging from successful tick engorgement and potential pathogen transmission to tick rejection and greatly reduced pathogen acquisition. A critical factor that controls this variability is the host response to tick feeding. Laboratory animals with prior exposure to ticks may be significantly protected from pathogen acquisition from infected ticks [23]; after a single feeding with *D. variabilis*, rabbits develop an anti-tick immunity that greatly reduces successful blood feeding during future infestations [25]. These observations suggest the host response to infestation may yield vital insights for tick and tick-borne disease control.

In an effort to understand the spectrum and temporal patterns of the *in vivo* host response to ticks, a PCR-array based approach was used to characterize the patterns of cutaneous bite-site gene expression during the course of primary and secondary infestations of mice with *I. scapularis* nymphs. Gene expression was measured at 12, 48,

72, and 96 hpi during primary and secondary infestations. Expression data was then validated at the mRNA and protein level using additional quantitative real-time PCR and bioplex assay.

RESULTS

In this study, Balb/cJ mice were infested with nymphal *I. scapularis* ticks and the expression of 233 gene transcripts were measured at the bite site lesion during primary and secondary infestations. These results revealed a distinct expression profile in naïve mice that was markedly different from that observed following a secondary infestation. Based on the selection criteria for differentially regulated genes (see Chapter 2: Methods), 35 genes were identified that differed in expression during primary infestation (17 upregulated and 18 downregulated) and 138 genes that differed during secondary infestation compared to uninfested control mice. The total numbers of differentially expressed genes when compared to control mice are illustrated in Figure 4.1. Fold changes ranged from negative 24-fold (leptin, secondary infestation at 12 hr post-infestation (p.i.)) to over 3000-fold (Cxcl5, secondary infestation at 96 hpi). Results from the primary infestation did not show any significant changes in gene expression at 12 hpi when compared to control mice. At 48 hpi, however, significant modulation of gene expression was observed which gradually reduced towards the end of the feeding period (96 hpi). As expected, many additional genes were modulated during secondary infestation. Overall, numbers of upregulated genes remained fairly stable across different time points as well as within each infestation scheme while a more variable response was observed for downregulated or unresponsive genes (Fig. 4.1). Statistical evaluation using

linear models in microarray analysis (LIMMA) did not show any significant changes in expression between time points within an infestation scheme; however, significant results were obtained when comparing expression levels between primary and secondary infestations (Fig. 4.2).

Gene ontology (GO) analysis was undertaken to assess potential biological functions represented in the lists of modulated genes (Tables 4.1, 4.2, and 4.3). GO-terms enriched from genes upregulated during the primary infestation (Table 4.1) clustered into two categories: host response and biomineral formation. In the host response category, the majority of GO-terms were related to chemotaxis, cytokines, immune response, and cellular location while a single term was observed in the category of biomineral formation. This category deals with all aspects of development of hard tissues that consist mainly of inorganic compounds and small amounts of organic matrices. Genes that were downregulated during the primary infestation were enriched for GO-terms that fell into two clusters: nucleotide metabolism/transcription and Similar Expression to Fibroblast Growth Factor and IL-17Rs or SEFIR (an IL-17 receptor domain) (Table 4.2). The former category contained terms related to gene expression and metabolic processes while the SEFIR category contains domains known to participate in IL-17RA signaling independently of classic Toll/IL-1R (TIR) structures including MyD88 and TRIF [186].

In contrast with the primary infestation, upregulated transcripts during secondary exposure were enriched for GO-terms related to a wide array of categories as shown in Table 4.3. From this data, four major categories are evident: cytokine, chemotaxis, immune cell signaling and activation, and leukocyte adhesion. Other groups of enriched terms included T-cell regulation and cell surface molecules while the remaining terms

clustered into a number of minor categories related to putative secreted, hematopoietic lineage, inflammation, protein-protein interactions (sushi domain), activation peptide, and tyrosine kinase phosphorylation. On the other hand, only GO terms 'negative regulation of cell proliferation' (GO_BP:0008285) and SEFIR (IPR013568:SEFIR) were significantly enriched from genes downregulated in the secondary exposure.

Modulation of gene expression during primary infestations.

While gene ontology allows assessment of inapparent biological processes in a list of genes, it does not allow direct comparison between time points or infestations at the gene level. To facilitate this, all 233 genes measured were divided into individual groups based on shared characteristics of the translated protein (shared family, pathway, or function). These groups and the genes in each group is shown in table 4.4. Genes modulated during the primary infestation are shown in Figure 4.3. Upregulated genes that were consistently expressed during the course of tick feeding included cytokines Il10, Il6, and Il1b, chemokines Ccl2, Ccl7, Cxcl1, Cxcl2, and Cxcl5, pattern recognition receptor Clec7a, modulator of inflammation prostaglandin-endoperoxide synthase 2 (Ptgs2 or Cox2), extracellular matrix (ECM) proteases Mmp9, 10, and 13, and the adhesion molecules L selectin (Sell), and β-2 integrin (Itgb2).

Among downregulated genes, the most notable were members of the IL-17 receptor family (Il17rb, Il17rc, and Il17re), which were consistently downregulated during the entire feeding process while the pro-inflammatory cytokines Il17a, Il17c, Il17d, and Il17f were not expressed. Other downregulated genes were T-cell molecules Cd28, Gata3, and retinoic acid-related orphan nuclear hormone receptor C (Rorc), DNA repair molecule telomerase reverse transcriptase (Tert), basement membrane/ECM

structural components Hapln1 and Lama1, neural adhesion molecule Ncam2, mitogenic pathway member Jun, WNT pathway members Jun and Lef1, hedgehog pathway members Bmp2 and Bmp4, and retinoic acid pathway member Hoxa1.

Modulation of gene expression during secondary infestations.

During the secondary infestation, Th1 and Th2 cytokines joined those upregulated on primary exposure. Interleukin-17 receptors remained downregulated, while Il2ra and Il4ra were upregulated. The expression profile of chemokines and PRR was similar to the primary infestation with the addition of Ccl1. Cytokine signaling molecules Jak2, Myd88, Syk, Socs1, and Socs3 were upregulated. The CD40 ligand (Cd40lg) joined Ptgs2 in the modulators of inflammation group. Many T-cell markers were upregulated along with Th1 and Th2 cytokines; however, transcriptional regulators important for CD4 T cell differentiation such as Tbx21, Gata3, and Rorc were unchanged or downregulated. The only exception was Forkhead box P3 (Foxp3), which was upregulated along with the cytokine Il10, suggesting the possible involvement of T regulatory cells [187] (Fig. 4.4A). All three selectins were upregulated, although Selp was only upregulated at 12 hpi. Integrins β-2, α-M, α-L, and α-4 were upregulated while α-2 was downregulated. Cadherins and integrin binding molecules were downregulated with the exception of Syk and Icam1. Anti-apoptotic molecule Bcl2l1 and DNA repair molecule Tert were downregulated while pro-apoptotic molecule Fasl was upregulated. ECM proteases were strongly upregulated, but members of the BM/ECM structural molecule and ECM protease inhibitor groups were down regulated. With the exception of a few matricellular

molecules, ECM interacting molecules, and growth factors, all the remaining groups were downregulated (Fig. 4.4B).

Array result validation.

Based on the results of PCR array analysis as well as other studies reported in literature, twenty-five genes potentially involved in the host response to tick infestation were selected and further verified using quantitative real-time PCR (see table 4.5 for a gene list). Gene expression was determined at 48 and 96 hpi for the primary infestation, and 48 and 72 hpi for the secondary exposure. Twenty of the twenty-five genes tested showed a profile highly consistent with the PCR array results (Fig. 4.5, table 4.6). In contrast, five genes (Il3, Gata3, Rorc, Tbx21 and Selp) showed variable patterns of modulation. In particular, Il3 up-regulation was detected at 96 hpi in the primary infestation. Down-regulation of Gata3 was significant only in the secondary infestation while Rorc down-regulation was apparent at all time points but not significant. Lastly, Tbx21 expression was upregulated at 48 hpi in the secondary infestation and Selp up-regulation was not detected.

Regulation of protein expression may occur at many points between transcription and the production of functional protein. For this reason, the copies of an individual gene transcript may not reflect the expression of protein. To provide support for the transcriptome profile data, an 8-analyte bioplex assay was utilized to measure protein expression by select cytokine and chemokine genes at the bite site (Fig. 4.6). Analytes were chosen based on differentially modulated or biologically important molecules from the array data offered in bioplex format. Cytokines IL-1β, IL-4, IL-6, IFN-γ, and

chemokine CCL-2 were significantly upregulated in agreement with the array and validation experiments. Interleukin-3, IL-10, and IL-17a showed similar but non-significant regulation. In order to directly compare protein and mRNA levels, fluorescent intensity values from the bioplex assay were converted to fold change over control sample fluorescence (Fig. 4.7). With the exception of low-abundance transcripts, these results suggest mRNA expression profiling at the tick-host interface could detect differences that correlate to the levels of expressed protein and can be a powerful tool for high-throughput functional analysis of the host cutaneous response to tick feeding.

DISCUSSION

Primary infestation.

During tick feeding, the cutaneous environment responds to skin injury by initiating innate defense mechanisms, shaping the ensuing adaptive immune response, and accommodating effector responses of adaptive immunity. In contrast, the feeding tick secretes an arsenal of salivary molecules that pharmacologically inhibit potentially unfavorable host responses [185]. The late initiation of host responses during primary infestation compared to secondary infestation is a striking example of tick-induced suppression of the host response. Early events at the bite site become measurable by 48 hpi and include up-regulation of Clec7a, a lectin pattern recognition receptor. *I. scapularis* SALP15 has been shown to modulate dendritic cell (DC) function through the lectin receptor DC-SIGN [100]. Together, these results suggest lectin pattern-recognition receptors may be important in initiation and modulation of anti-tick immunity. Ligation

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of Clec7a induces the up-regulation of Cxcl2 and Il10 [188], molecules that were also upregulated in this study. Tick-induced expression of Il10 has been previously reported and may represent a method of immune evasion by dampening pro-inflammatory responses [101,189].

Other cytokines upregulated early in the host response were Il1b and Il6. These are both potent pro-inflammatory cytokines suggesting a balance between anti-inflammatory Il10 and pro-inflammatory Il1b and Il6 during the early host response to ticks. The presence of Il1b and Il6 at the bite site is supported by previous studies [105,190] and the concomitant up-regulation of Icam1, Ptgs2 (COX2), Cxcl1, Cxcl2, Cxcl5, and Mmp13, molecules known to be induced by these cytokines [191,192,193]. The CXCL chemokines detected in this study are potent chemoattractants for neutrophils, although they have also been shown to attract monocytes and mast cells [194]. Ccl2 and Ccl7 were originally described as macrophage chemotactic proteins (MCP) 1 and 3, reflecting their primary role as chemoattractants for macrophages, but they are also known to recruit basophils, eosinophils, NK cells, and DCs [194]. Recruitment of these cells into the bite site could be facilitated by the up-regulation of Sell and Itgb2. These results suggest a model of immune activation during primary infestation where Clec7a initiates neutrophil chemotaxis and anti-inflammatory cytokine production. Increased production of Il1b and Il6 by unknown mechanisms could play a role in promoting up-regulation of chemokines specific for neutrophils and macrophages, which in turn produce matrix metalloproteinases (MMP) and prostaglandins. Neutrophils are known to be present at the bite-site [79], but their role in anti-tick immunity is not well understood. Based on the previous identification of *I. scapularis* salivary proteins (ISL929 and 1373) that reduce super-oxide formation and expression of β-2 integrins in neutrophils treated

with TNF-α [83], it is reasonable to assume that they are important components of anti-tick immunity. These changes suggest reduced neutrophil ability to respond to tissue insult and to destroy phagocytosed infectious agents.

Matrix metalloproteinases have a wide range of potential functions at the tick bite-site. MMP cleavage of ECM components exposes cryptic sites that have been associated with increased migration of leukocytes to the inflammatory focus; cleavage can also release bioactive molecules from the ECM. *I. scapularis* has been shown to possess a large family of salivary serine-protease inhibitors (serpins) that may be important in inhibiting host responses [195]. Immunization of rabbits with a serpin from *I. ricinus* resulted in increased tick mortality and reduced weight and fecundity in female ticks [196]. Since MMPs degrade and inactivate endogenous serpins [197], it is reasonable to hypothesize that MMPs contribute to host immunity by degrading tick-secreted serpins. MMPs also aid in angiogenesis and wound healing, processes that are inhibited by tick feeding [198]. Gene ontology gives general support to this analysis of the primary infestation. Significant terms from genes upregulated during primary infestation clustered into host response and biomineral formation groups. The host response category was dominated by chemokine, chemotaxis, cytokine, and immune response terms, although none of these terms were specific for any cell type. GO analysis also supported the role of upregulated genes as secreted molecules acting in the extracellular space. Analysis of downregulated genes during primary infestation identified nucleotide metabolism/transcription and SEFIR domain (IPR013568: SEFIR, an IL-17R domain) as significant. These terms are of interest for CD4 T cell differentiation (discussed below), and support the possibility that tick feeding suppresses

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transcription during primary infestation. This is a potential mechanism behind the late induction of host responses during primary infestation.

Secondary infestation.

A second exposure to feeding by *I. scapularis* nymphs resulted in a faster and stronger host response as shown in Figures 3A and B. In contrast to the primary infestation, very significant gene modulation was evident by 12 hpi. The genes modulated during primary infestations were also modulated during secondary infestations and were, in general, the genes with the highest fold-changes. Thus, the genes upregulated during the primary infestation form a core host-response that drives anti-tick immunity even on repeated exposure.

Migration.

The migration of cells into an inflammatory focus is an important aspect of host immunity [194]. Resident cells must recognize skin damage by the feeding tick and secrete factors that enhance the recruitment of immune effectors to the bite site. Gene ontology analyses of upregulated genes during the secondary infestation strongly support the important role of chemotaxis in the anti-tick immune response. Specific GO terms suggested the migration of neutrophils, monocytes, other leukocytes, and lymphocytes into the bite site (Table 4.3). The up-regulation of Ccl1 was the only observed change in chemokine expression between primary and secondary infestation. Interestingly, this chemokine has been shown to attract Th2 and T regulatory cells [194]. Other upregulated genes known to support cell migration included selectins, integrins, and the integrin ligand Icam1. While a number of alpha chain integrins were upregulated, the

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only beta chain upregulated was β-2.   In support of previous reports that *I. scapularis* saliva inhibited endothelial cell expression of P selectin [199], the present study showed only minimal up-regulation of Selp that was not supported by later validation.

Cytokines.

Many additional cytokines were modulated during the secondary infestation when compared to the primary exposure (Fig. 4.4A). These transcripts group together to form the cytokine cluster on gene ontology analysis, lending formal support to their importance in the anti-tick response.   In particular, Il4 and Il13 were upregulated; these cytokines can be produced by Th2 cells, but also by basophils, eosinophils, and mast cells. Basophils have been shown to be indispensible for anti-tick immunity in models of infestation where acquired resistance occurs [45,200], and their migration into the bite site was supported by the up-regulation of CCL chemokines and Il3, which are chemotactic factors for basophils [201]. It is interesting to note the significant increase in expression of Il4 and Il13 during the secondary exposure despite the down regulation of Il25, an important inducer of type-2 immunity [202].   In contrast to these type-2 cytokines, up-regulation of Ifng and Il27 could be due to the presence of T cells, Th1 cells, NK cells, and antigen presenting cells [203,204]. The up-regulation of Ifng is surprising in light of previous reports of suppression by tick saliva [189], although negligible increases in expression have been previously reported in BALB/c mice infested with *I. scapularis* [101]. The mechanisms behind Ifng and Il4 up-regulation were strong enough to overcome the down-regulation of Il18, a known inducer of both cytokines [191]. Up-regulation of colony stimulating factors 2 (GM-CSF or Csf2) and 3 (G-CSF or Csf3) and Il3 suggests that tick feeding may stimulate increased

hematopoiesis and/or myelopoiesis. This possibility was supported by the gene ontology analysis (Table 4.3), previous reports of extramedullary erythropoiesis in tick-infested mice [205], and the down-regulation of Il17d, an inhibitor of hematopoietic progenitor colony formation [206]. Finally, the present study also supports previously reported repression in the expression of tumor necrosis factor family members by tick salivary molecules [100,207]. In summary, the cytokine profile during secondary infestation presents a complex interplay between inducers and repressors of type 1 and type 2 immunity.

T-cells.

Th2 polarization of the cytokine response to tick feeding has been thoroughly documented by *in vitro* and *in vivo* studies [185]. For this reason, the modulation of genes associated with T-cell and helper T-cell differentiation was characterized. During primary infestation, classic T-cell markers such as Cd3, Cd4, and Cd8 did not significantly change, suggesting early T-cell involvement is minimal. Interestingly, the expression of co-stimulatory molecule Cd28 was downregulated, which could be due to a lack of CD4 T-cell activation at the bite site, or the migration of CD28-expressing cells out of the skin. Genes related to Th17 differentiation, including the transcription factor Rorc, Il17, and the Il17 receptors were either unchanged or downregulated, despite the high levels of Il1b and Il6. Most genes related to Th2 development were unchanged with the exception of Gata3, which was downregulated. Gata3 is an important transcription factor in Th2 development. Transcripts related to Th1 and T reg development were unchanged. These results suggest that during primary infestation of mice with *I.*

scapularis nymphs, the cutaneous environment is not strongly polarized (or polarizing) toward any helper T-cell sub-set.

On secondary infestation, the up-regulation of T-cell markers Cd2, Cd3, Cd4, and Cd8 suggested T-cell involvement at the bite-site. However, the polarization of CD4 T-cells remained equivocal. While Th2 cytokines Il4 and Il13 were upregulated, Gata3 remained repressed (Fig. 4.4A). Similarly, the Th1 cytokine Ifng was upregulated, but Il12 and Tbx21 remained unresponsive. Th17-related transcripts were downregulated or unchanged. Interestingly, Fopx3 and Il10 were upregulated, supporting a possible role for T regulatory cells at the bite site. In summary, results from the secondary exposure strongly suggests that Th17 involvement at the bite-site is unlikely, while the remaining data shows a mixed Th1/Th2 cytokine profile and suggests the involvement of T regs. Failure to produce a polarized CD4 T cell response was also observed when keyhole limpet haemocyanin (KLH)-specific T cells were stimulated with KLH-loaded DCs in the presence of *Rhipicephalus sanguineus* tick saliva [94]. This implies that non-polarized CD4 T cell responses may be a common trait of anti-tick immunity and also supports the present results at the protein/cellular level. Sialostatin L, an *I. scapularis* salivary protein, suppressed IL-17 production by lymph node cells during the induction of experimental autoimmune encephalomyelitis in mice [208]. In the present study, significant Th17 suppression was observed (Fig. 4.3, 4.4A) even from a *naïve* state, supporting the possibility that tick saliva contains potent suppressors of Th17 immunity.

Signaling.

Another focus of the present study was to uncover novel signaling pathways activated at the tick bite-site. Surprisingly, most genes related to the signaling pathways

tested were either downregulated or unresponsive. Immunoreceptor signaling was a significant exception. Gene ontology results showed that the largest gene cluster was related to immune cell signaling and activation. This is consistent with the rest of the results and suggests immunoreceptor signaling as a potential major pathway induced by tick feeding. However, no modulation of signal transducers and activators of transcription (STAT) or NF-κB pathway molecules was observed. The lack of STAT modulation in this study was surprising since STAT molecules are important effector molecules of cytokine signaling that induce their own expression [209]. Modulation or silencing of the NF-κB pathway could be significant because of its vital role in the induction and regulation of immunity [210]. These results paired with the increase of SOCS transcripts suggest that the tick bite site is characterized by both suppression and activation of immunoreceptor signaling.

Gene ontology analysis of the downregulated genes during secondary infestation showed only two significant terms: negative regulation of cell proliferation (GO_BP: 0008285) and SEFIR (IPR013568). This suggests the genes downregulated during secondary infestation do not fit into a common theme for GO enrichment. However, many groups and pathways were qualitatively downregulated. Transcripts related to integrin binding and neural adhesion were downregulated, indicating potential impairment of cell migration or adhesion-related signaling at the tick bite site. Increased cell turnover was suggested by the down-regulation of genes encoding anti-apoptotic and DNA repair molecules. The down-regulation of BM/ECM structure and ECM protease inhibitor groups combined with the up-regulation of ECM proteases suggest significant modulation of ECM components. In addition to these groups, genes in classical pathways such as mitogenic, WNT, hedgehog, stress, and metabolism were

downregulated. The WNT signaling pathway regulates a variety of cellular processes including cell proliferation, migration, and tissue morphogenesis. In canonical signaling, WNT stabilizes β-catenin that acts as a transcriptional co-activator by interacting with Lef/T-cell transcription factors (Tcfs) to regulate WNT target gene expression. Non-canonical signaling, on the other hand, is calcium-dependent and leads to activation of c-jun N-terminal kinase which plays a role in cell proliferation, differentiation, and apoptosis [211]. In addition to its role in developmental biology, the Hedgehog pathway has been shown to play a role in regulating regenerating cell populations [212]. Since cell proliferation, regeneration, and morphogenesis are involved in wound healing, epithelial maintenance, and hair follicle cycling, tick feeding may influence these processes. However, it is unclear whether this is a result of tick-saliva induced repression or a consequence of the inflammatory process at the bite-site lesion. In this regard, the infestation protocol avoided the use of capsules or any device to restrain the ticks during feeding that might have influenced the inflammatory reaction. In either case, the present results qualitatively suggest the tick bite-site is characterized in part by the suppression of signaling molecule transcription.

CONCLUSIONS

This study supports a model of the tick-host interface where tick saliva inhibits gene transcription, Th17 immunity, and signal transduction molecule up-regulation. In contrast, the host senses infestation through lectin PRRs and is primarily focused on the recruitment and subsequent activation of immune cells. During primary infestation, neutrophils and macrophages are recruited, while many additional cell types are recruited

during secondary infestation. Host effector responses include a mixed Th1/Th2 CD4 T cell response, innate effector functions, a highly proteolytic environment, and increased cell turn-over. These responses are dampened by the action of T regulatory cells, Socs, and Il10.

To my knowledge, this is the first report of *in vivo* transcriptome profiling at the *I. scapularis* tick-host interface. The results suggest tick feeding may activate favorable host responses such as the inhibition of gene transcription, down-regulation of signaling molecules, and up-regulation of inhibitors of inflammation while repressing unfavorable responses such as Th17 immunity. The mixed Th1/Th2 profile reported here is a novel finding that implies greater complexity to the host cutaneous response than previously reported. This study will allow the rational design of further work probing *in vivo* mechanisms at the tick-host-pathogen interface.

Tables.

Table 4.1: Gene ontology results from genes upregulated during primary infestation.

Significant gene ontology terms from genes upregulated at any time point during primary infestation of mice with *I. scapularis* nymphs are shown grouped into clusters based on the functional annotation clustering tool available from DAVID. Terms in the host response cluster have been grouped by similarity.

Cluster	Term	PValue
Host response	Chemokine activity	0.005
	Chemokine receptor binding	0.005
	Chemokine signaling pathway	0.014
	Small chemokine, interleukin-8-like	0.0031
	CXC chemokine	0.047
	Cytokine	0.0009
	Cytokine activity	0.0041
	Cytokine-cytokine receptor interaction	0.015
	NOD-like receptor signaling pathway	0.001
	Extracellular region part	0.025
	Extracellular space	0.0036
	Secreted	0.0097
	Immune response	0.008
	Inflammatory response	0.0012
	Defense response	0.0003
	Response to wounding	0.0039
	Taxis	0.0007
	Chemotaxis	0.0007
	Locomotory behavior	0.0007
	Behavior	0.006
Biomineral formation	Biomineral formation	0.024

Table 4.2: Gene ontology results from genes downregulated during primary infestation.

Significant gene ontology terms from genes downregulated at any time point during primary infestation of mice with *I. scapularis* nymphs are shown grouped into clusters based on the functional annotation clustering tool available from DAVID.

Cluster	Term	PValue
Nucleotide metabolism and transcription	Cell morphogenesis	0.039
	DNA binding	0.020
	Positive regulation of biosynthetic process	0.025
	Positive regulation of cellular biosynthetic process	0.022
	Positive regulation of gene expression	0.046
	Positive regulation of macromolecule biosynthetic process	0.022
	Positive regulation of macromolecule metabolic process	0.042
	Positive regulation of nitrogen compound metabolic process	0.014
	Positive regulation of nucleobase, nucleoside, nucleotide and nucleic-acid metabolic process	0.010
	Positive regulation of RNA metabolic process	0.032
	Positive regulation of transcription	0.036
	Positive regulation of transcription from RNA polymerase II- promoter	0.025
	Positive regulation of transcription, DNA-dependent	0.032
	Regulation of RNA metabolic process	0.025
	Regulation of transcription	0.046
	Regulation of transcription from RNA polymerase II promoter	0.027
	Regulation of transcription, DNA-dependent	0.027
	Sequence-specific DNA binding	0.018
	Tissue morphogenesis	0.026
SEFIR domain	IPR013568:SEFIR (IL-17R domain)	0.026

Table 4.3: Gene ontology results from genes upregulated during secondary infestation.

Significant gene ontology terms from genes upregulated at any time point during secondary infestation of mice with *I. scapularis* nymphs are shown grouped into clusters based on the functional annotation clustering tool available from DAVID.

Cluster	Terms	PValue	Cluster	Terms	PValue
Putative Secreted	Disulfide bond	0.00002	**Immune cell signaling and activation**	Positive regulation of signal transduction	0.0032
	Signal peptide	0.0038		Positive regulation of cell communication	0.005
	Signal	0.0043		Four-helical cytokine, core	0.0061
	Glycoprotein	0.013		Regulation of cell activation	0.0063
Cytokine	Immune response	0.00001		Regulation of peptidyl-tyrosine phosphorylation	0.0071
	Cytokine	0.00026		Regulation of leukocyte proliferation	0.008
	Cytokine activity	0.0013		Regulation of T cell activation	0.008
	Extracellular space	0.0019		Positive regulation of peptidyl-tyrosine phos.	0.011
	Cytokine-cytokine receptor interaction	0.0054		Regulation of lymphocyte activation	0.014
Hematopoietic lineage	Hematopoietic cell lineage	0.0011		Regulation of leukocyte activation	0.014
	T-cell	0.0032		Regulation of hematopoiesis by cytokines	0.019
	Regulation of hematopoiesis by cytokines	0.019		Regulation of mononuclear cell proliferation	0.019
Inflammation	Inflammatory response	0.027		Regulation of lymphocyte proliferation	0.019
	Defense response	0.027		Regulation of protein kinase cascade	0.02
Chemotaxis	Chemotaxis	0.0024		Positive regulation of T cell activation	0.03
	Locomotory behavior	0.0024		Regulation of cellular localization	0.033
	Taxis	0.0024		Positive regulation of protein kinase cascade	0.033
	Leukocyte adhesion	0.017		Regulation of protein modification process	0.034
	Cell chemotaxis	0.026		Regulation of cellular protein metabolic process	0.034
	Leukocyte chemotaxis	0.026		Positive regulation of immune system process	0.034
	Neutrophil chemotaxis	0.026		Regulation of chemokine production	0.039
	Leukocyte migration	0.032		Regulation of chemokine biosynthetic process	0.039
Cell surface molecules	Monocyte and its Surface Molecules	0.0025		Positive regulation of cell activation	0.045
	Cell adhesion molecules (CAMs)	0.0071		Positive regulation of protein modification process	0.047
	External side of plasma membrane	0.01		Regulation of T cell proliferation	0.047
	Cell surface	0.021	**Tyrosine phosphorylation**	Regulation of peptidyl-tyrosine phosphorylation	0.0071
T-cell regulation	Positive regulation of T cell activation	0.03		Positive regulation of peptidyl-tyrosine phos.	0.011
	Regulation of cellular localization	0.033	**Leukocyte adhesion**	Monocyte and its Surface Molecules	0.0025
	Regulation of alpha-beta T cell differentiation	0.039		Cell adhesion molecules (CAMs)	0.0071
	Positive regulation of alpha-beta T cell diff.	0.039		Neutrophil and Its Surface Molecules	0.0082
Sushi domain	domain:Sushi 2	0.036		Leukocyte adhesion	0.017
	domain:Sushi 1	0.036		Adhesion Molecules on Lymphocyte	0.019
	domain:EGF-like	0.036		Natural killer cell mediated cytotoxicity	0.021
Activation peptide	Propeptide:Activation peptide	0.048			

Table 4.4: Gene groups used to allow comparisons between time points.

All 233 genes measured in this study were grouped based on characteristics of the encoded protein. These groups were used to allow comparisons between time points throughout this study.

Cytokine	
Cytokines	Csf2, Csf3, Ifng, Il10, Il12b, Il13, Il15, Il17a, Il17c, Il17d, Il17f, Il18, Il1a, Il1b, Il2, Il21, Il22, Il23a, Il25, Il27, Il3, Il4, Il5, Il6, Lta, Tgfb1, Tnf
Cytokine receptors	Il12rb1, Il12rb2, Il17rb, Il17rc, Il17rd, Il17re, Il23r, Il6ra, Il7r, Il2ra
Chemokines	Ccl1, Ccl2, Ccl20, Ccl22, Ccl7, Cx3cl1, Cxcl1, Cxcl12, Cxcl2, Cxcl5, Cxcl9
Pattern recognition	Clec7a, Tlr4
Cytokine signaling pathway molecules	
JAK/STAT	Jak1, Jak2, Stat3, Stat4, Stat5a, Stat6
Adaptor molecules	Myd88, Tirap, Traf6, Syk, Irf1
SOCS	Socs1, Socs3
Modulators of inflammation	Yy1, Cacybp, Cebpb, Edg1, Ptgs2, Entpd1, Hc, Isg20, Cd40lg
NF-κBPathway:	Ikbkb, Nfkbia, Tank, Nos2, Tnf, Nfkb1
T-cell molecules	
T-Cell	Cd2, Cd247, Cd28, Cd3d, Cd3e, Cd3g, Cd4, Cd5, Cd8a, Foxp3, Gata3, Icos, Ifng, Il10, Il12b, Il12rb1, Il12rb2, Il13, Il17a, Il17c, Il17d, Il17f, Il17rb, Il17rc, Il17rd, Il17re, Il18, Il1b, Il2, Il21, Il22, Il23a, Il23r, Il25, Il27, Il4, Il4ra, Il5, Il6, Il6ra, Nfatc2, Rorc, Stat3, Stat4, Stat5a, Stat6, Tbx21, Tcf7, Tgfb1, Traf6
Th1	Ifng, Il12rb2, Tbx21, Stat4, Il12b, Il2
Th17	Il17a, Il17c, Il17d, Il17f, Il17rb, Il17rc, Il17rd, Il17re, Il1b, Il21, Il22, Il23a, Il23r, Il6, Il6ra, Rorc, Stat3, Tgfb1, Traf6
Th2	Il4, Stat6, Gata3, Il5, Il13, Tcf7, Il25
Treg	Foxp3, Tgfb1, Il10
Apoptosis/DNA repair	
Anti-apoptotic	Bcl2l1, Birc1a, Birc2, Birc3, Birc5, Mdm2, Bcl2
Pro-apoptotic	Bax, Ei24, Igfbp3, Fasl, Fas
DNA repair	Gadd45a, Brca1, Trp53, Tert, Yy1
Cell adhesion molecules	
Selectins	Sele, Sell, Selp
Integrins	Itga2, Itga3, Itga4, Itga5, Itgae, Itgal, Itgam, Itgav, Itgax, Itgb1, Itgb2, Itgb3, Itgb4
Cadherins	Cdh1, Cdh2, Cdh3, Cdh4, Ctnna1, Ctnna2, Ctnnb1
Integrin binding	Col3a1, Col4a3, Ctgf, Icam1, Itga2, Itga3, Itga5, Itgb1, Itgb3, Lamb2, Mmp14, syk, Thbs1, Timp2, Vcam1, Vtn

Extracellular matrix proteins (ECM)	
BM and ECM structural molecules	Col1a1, Col2a1, Col3a1, Col4a1, Col4a2, Col4a3, Col5a1, Col6a1, Entpd1, Fn1, Hapln1, Itgb4, Lama1, Lama2, Lama3, Lamb2, Lamb3, Lamc1, Sparc, Timp1, Timp2, Timp3
ECMProteases:	Adamts1, Adamts2, Adamts5, Adamts8, Mmp10, Mmp11, Mmp12, Mmp13, Mmp14, Mmp15, Mmp1a, Mmp2, Mmp3, Mmp7, Mmp8, Mmp9
ECMProteaseInhibitors:	Col4a3, Timp1, Timp2, Timp3
Matricellular proteins	Spock1, Tnc, Thbs1, Thbs2, Thbs3, Spp1, Sparc, Ctgf, Postn
Neuronal cell adhesion molecules	Ncam1, Ncam2, Cntn1, Syt1
ECM interacting	CD34, Tgfbi, Vtn, Cd44, Pecam1, Vcan, Sgce, Ecm1, Emilin1, Fbln1
Signaling pathways, cell cycle control (WNT, HH, Mitogenic pathway)	
Mitogenic pathway	Egr1, Fos, Jun, Nab2, Cdkn1a, Cdkn1b, Cdkn2a, Cdkn2b, Atf2, Cdk2
WNT pathway	Birc5, Ccnd1, Cdh1, Fgf4, Jun, Lef1, Myc, Tcf7, Vegfa, Wisp1, Pmepa1, Ctnnb1
Hedgehog pathway	Bmp2, Bmp4, En1, Foxa2, Hhip, Ptch1, Wnt1, Wnt2
Growth factors	Vegfa, Fgf4, Igfbp3, Igfbp4, Csf2, Ctgf, Csf3
Stress pathway:	Hsf1, Hspb1, Trp53, Mdm2
Metabolism/insulin related genes	Cebpb, Fasn, Gys1, Hk2, Lep, Nrip1, Odc1, Pparg, Cyp19a1, Greb1, Tfrc
Retinoic acid pathway:	En1, Hoxa1, Rbp1

Table 4.5: List of genes measured by qRT-PCR for validation of PCR array results.

List of genes measured by qRT-PCR for validation of PCR array results.

Gene symbol	Gene Name
Ccl2	Chemokine (C-C motif) ligand 2
Ccl7	Chemokine (C-C motif) ligand 7
Ccr5	Chemokine (C-C motif) ligand 5
Clec7a	C-type lectin domain family 7, member a
Cxcl5	Chemokine (C-X-C motif) ligand 5
Gata3	GATA binding protein 3
IFN-γ	Interferon gamma
IL-10	Interleukin 10
IL-1β	Interleukin 1 beta
IL-3	Interleukin 3
IL-4	Interleukin 4
IL-6	Interleukin 6
Itgal	Integrin alpha L
Itgam	Integrin alpha M
Itgb1	Integrin beta 1 (fibronectin receptor beta)
Itgb2	Integrin beta 2
Jak2	Janus kinase 2
Mmp13	Matrix metallopeptidase 13
Rorc	RAR-related orphan receptor gamma
Sele	Selectin, endothelial cell
Sell	Selectin, lymphocyte
Selp	Selectin, platelet
Socs1	Suppressor of cytokine signaling 1
Stat6	Signal transducer and activator of transcription 6
Tbx21	T-box 21
Actb	Actin, beta
Gadph	Glyceraldehyde-3-phosphate dehydrogenase
Gusb	Glucuronidase, beta
Hprt1	Hypoxanthine guanine phosphoribosyl transferase 1
Hsp90ab1	Heat shock protein 90 alpha (cytosolic), class B member 1

Table 4.6: Direct comparison of PCR array and qRT-PCR validation experiments.

Fold change and p-values obtained from the PCR array and qRT-PCR validation experiments are directly compared. "Primary" refers to primary infestation, while "secondary" refers to the secondary exposure. A "+" marks fold changes calculated from transcripts below the detection limit (Ct ≥ 34); red text denotes p-values ≤ 0.01.

Direct comparison of PCR array and qRT-PCR validation experiments.

gene	48 hr primary array fold change	adj. p value	48 hr primary validation fold change	adj. p value	96 hr primary array fold change	adj. p value	96 hr primary validation fold change	adj. p value
Ccl2	8.85	2.68E-03	8.7	2.43E-10	12.1	4.52E-04	20.5	2.36E-12
Ccl7	7.94	5.06E-04	8.66	6.02E-12	11	1.59E-04	17.7	3.37E-13
Clec7a	3.14	1.47E-03	5.09	5.44E-05	3.9	3.90E-02	8.61	1.95E-05
Cxcl5	121	3.29E-04	208	4.62E-15	68.5	2.43E-03	130	4.21E-11
Gata3	0.372	1.47E-03	0.661	2.21E-02	0.422	9.97E-03	0.716	5.68E-02
IFNg	3.79^+	1.71E-02	1.94^+	1.94E-01	1.5^+	4.50E-01	6.62^+	1.62E-03
IL1b	16.7	1.62E-04	25.3	8.11E-12	20.4	1.45E-03	27.4	1.81E-09
IL10	4.51	1.46E-03	5.71	4.15E-10	5.19	2.74E-02	9.17	1.19E-08
IL3	0.588^+	3.34E-01	2.8^+	3.08E-02	2.75^+	2.68E-01	11.7	1.21E-03
IL4	0.597	9.10E-02	1.04^+	9.58E-01	0.738	4.58E-01	3.85^+	1.80E-01
IL6	17.5	4.58E-04	64.1	1.84E-08	51.1	1.33E-03	165	8.00E-09
Itgal	2.58	1.02E-02	2.46	1.75E-02	1.9	5.54E-02	4	2.56E-03
Itgam	2.7	1.92E-04	2.51	3.22E-07	1.51	1.58E-01	2.68	7.64E-06
Itgb1	1.1	3.49E-01	0.801	6.50E-02	1.02	9.03E-01	1.03	8.53E-01
Itgb2	3.04	1.92E-04	2.66	2.34E-03	2.89	5.85E-03	3.57	1.18E-03
Jak2	1.11	5.38E-01	1.32	1.36E-01	1.17	4.95E-01	2.25	4.12E-04
Mmp13	20.3	6.32E-06	30.1	9.62E-07	6.26	1.43E-03	33	1.21E-05
Rorc	0.249	5.87E-04	0.666	6.32E-01	0.501	9.97E-03	1.12	8.97E-01
Sele	1.87	1.33E-03	14.9	8.01E-03	2.66	3.45E-03	43.8	1.55E-03
Sell	14.9	1.92E-04	11.4	1.58E-05	7.87	2.70E-03	12	5.46E-05
Selp	1.9	2.91E-03	1.51	6.97E-05	1.56	5.13E-02	2.08	1.21E-05
Socs1	0.719	1.90E-01	0.679	4.11E-02	1.31	1.92E-01	1.16	4.77E-01
Stat6	0.826	3.31E-01	0.875	1.46E-01	0.911	6.04E-01	0.983	8.69E-01
Tbx21	0.753^+	3.06E-01	1.36^+	6.26E-01	0.963^+	9.01E-01	3.18^+	6.13E-02

gene	48 hr secondary array fold change	adj. p value	48 hr secondary validation fold change	adj. p value	72 hr secondary array fold change	adj. p value	72 hr secondary validation fold change	adj. p value
Ccl2	99.7	1.68E-05	103	6.37E-18	67.2	6.00E-05	81.6	3.32E-15
Ccl7	95.8	2.08E-06	88.1	4.47E-19	76.6	2.32E-06	90.6	2.06E-15
Clec7a	25.8	5.32E-06	56.3	9.84E-12	27.3	2.32E-06	39	5.04E-09
Cxcl5	2120	1.77E-06	1680	1.16E-18	1460	1.07E-06	2390	1.14E-17
Gata3	0.323	1.48E-04	0.44	5.64E-06	0.333	2.29E-03	0.436	2.34E-04
IFNg	50.7	5.90E-06	121	2.53E-10	33.7	5.85E-05	84.9	1.17E-08
IL1b	74.3	5.32E-06	102	2.26E-15	114	1.45E-06	107	1.32E-13
IL10	248	1.77E-06	343	7.73E-21	191	4.00E-06	239	4.16E-17
IL3	30.1	3.71E-04	74.8	2.83E-11	78	2.37E-04	90.9	2.05E-10
IL4	71.6	5.70E-05	225	3.98E-07	33.1	1.13E-04	234	9.30E-06
IL6	237	5.86E-06	903	5.93E-13	292	4.00E-06	2.8^+	3.64E-01
Itgal	6.58	4.04E-04	11.7	2.71E-07	5.99	5.74E-03	10.5	1.13E-05
Itgam	4.91	1.52E-04	9.43	6.93E-15	4.29	7.90E-03	6.68	8.08E-11
Itgb1	1.01	9.60E-01	1.16	1.88E-01	1.18	4.59E-01	1.21	1.83E-01
Itgb2	7.83	1.37E-05	7.51	3.52E-07	9.68	1.60E-04	7.77	1.09E-05
Jak2	7.02	6.07E-05	10.4	4.01E-11	7.7	8.51E-05	7.69	7.96E-09
Mmp13	69.5	1.37E-05	215	1.69E-10	85.3	1.13E-04	582	5.70E-10
Rorc	0.137	8.06E-05	0.429	2.80E-01	0.196	2.29E-03	0.565	5.46E-01
Sele	4.77	6.94E-04	81.9	4.53E-05	5.92	1.60E-04	116	3.03E-04
Sell	75.5	7.04E-07	105	9.94E-11	59.2	5.29E-05	88.8	5.51E-09
Selp	2.43	5.83E-04	2.41	7.71E-10	1.9	4.97E-02	1.78	1.09E-05
Socs1	2.87	6.06E-04	3.04	2.75E-07	2.29	1.51E-02	1.6	5.42E-02
Stat6	0.856	3.49E-01	0.797	2.93E-02	1.01	9.82E-01	0.563	5.60E-02
Tbx21	2.35^+	5.19E-02	15.6	1.15E-05	1.74^+	1.07E-01	3.82^+	9.16E-02

"+" marks values below detection limit (Ct > 34)
Red text indicates p value ≤ 0.01
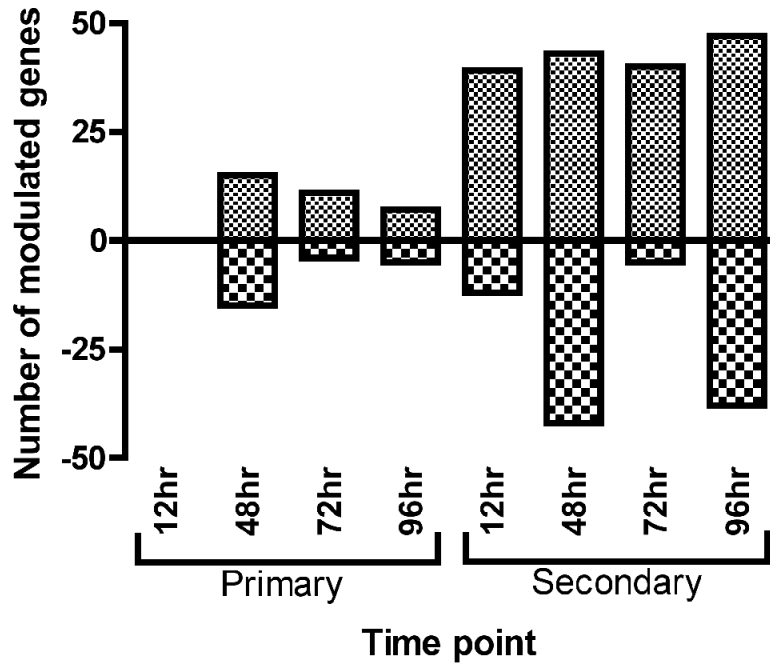
Figure 4.1: Number of modulated genes at each time point compared to tick-free mice.

Number of significantly up and downregulated genes measured at each time point during primary and secondary infestations of mice with *I. scapularis* nymphs compared to tick-free mice.

A

Group | Symbol | Time point

Fold change over primary infestation
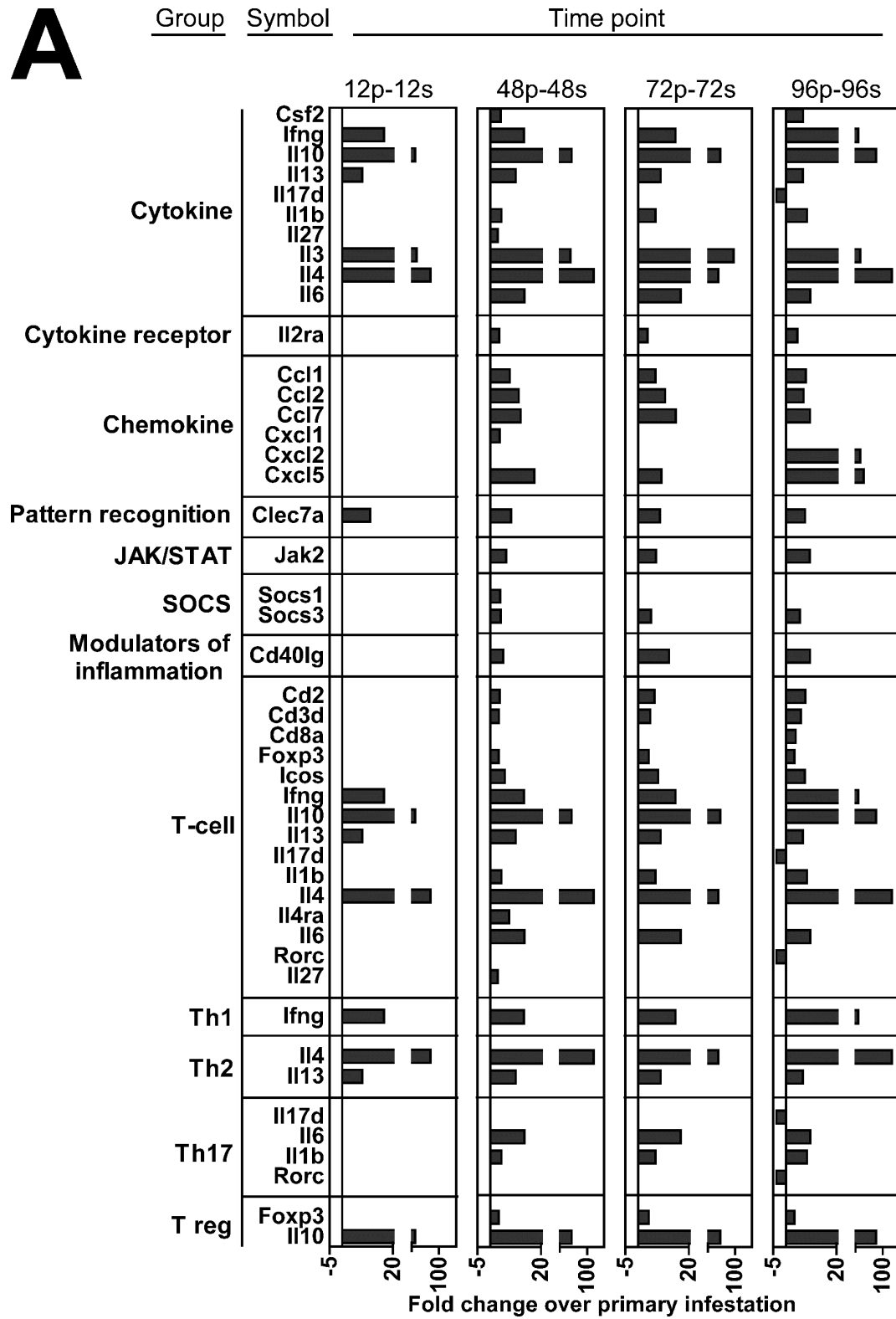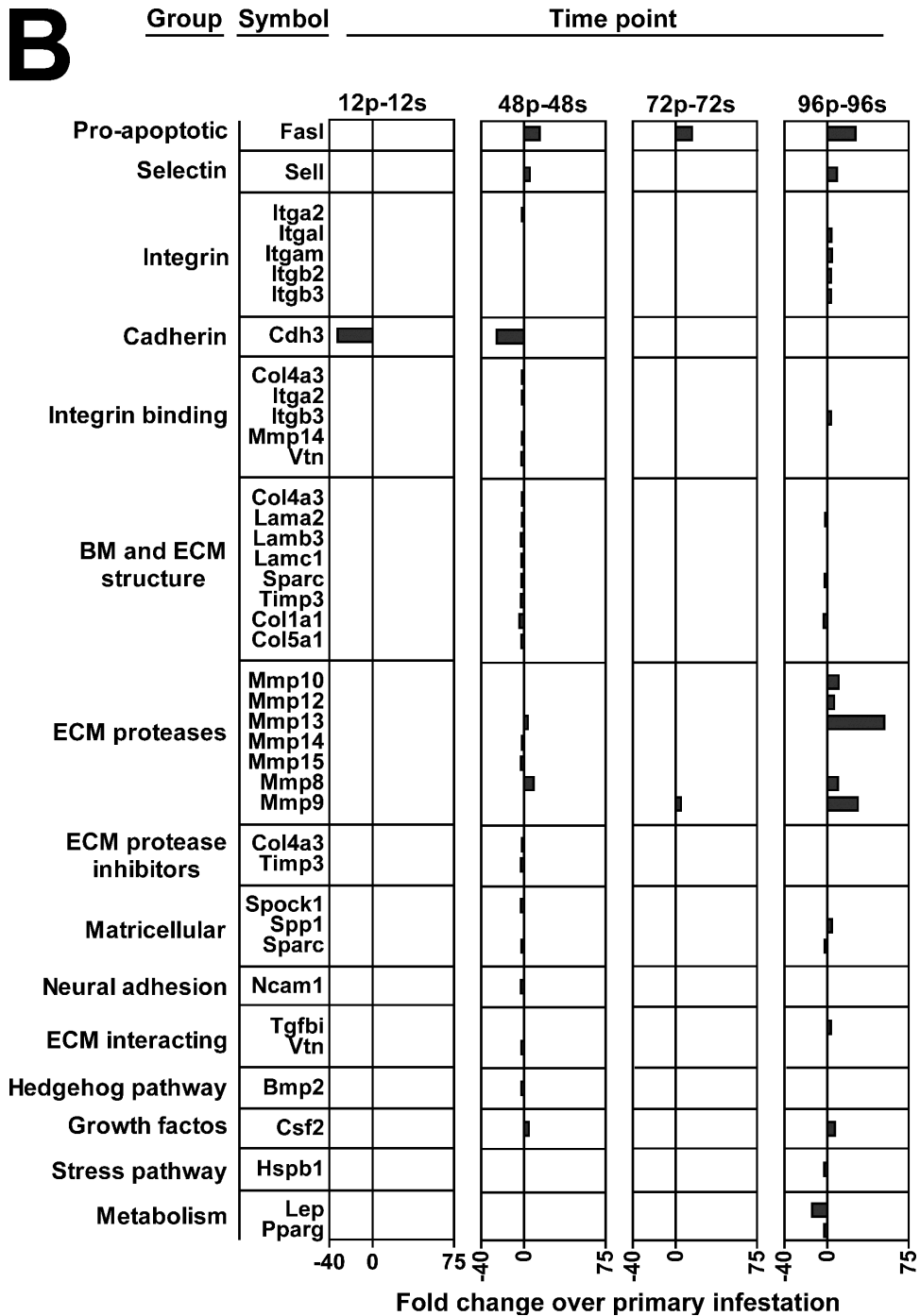
Figure 4.2: Statistical comparison between primary and secondary infestations.

Significant changes in gene expression between primary and secondary infestation were measured using LIMMA and the same filtering criteria as before (Methods). Each column shows the genes significantly modulated over the primary infestation during a secondary exposure of mice to *I. scapularis* nymphs
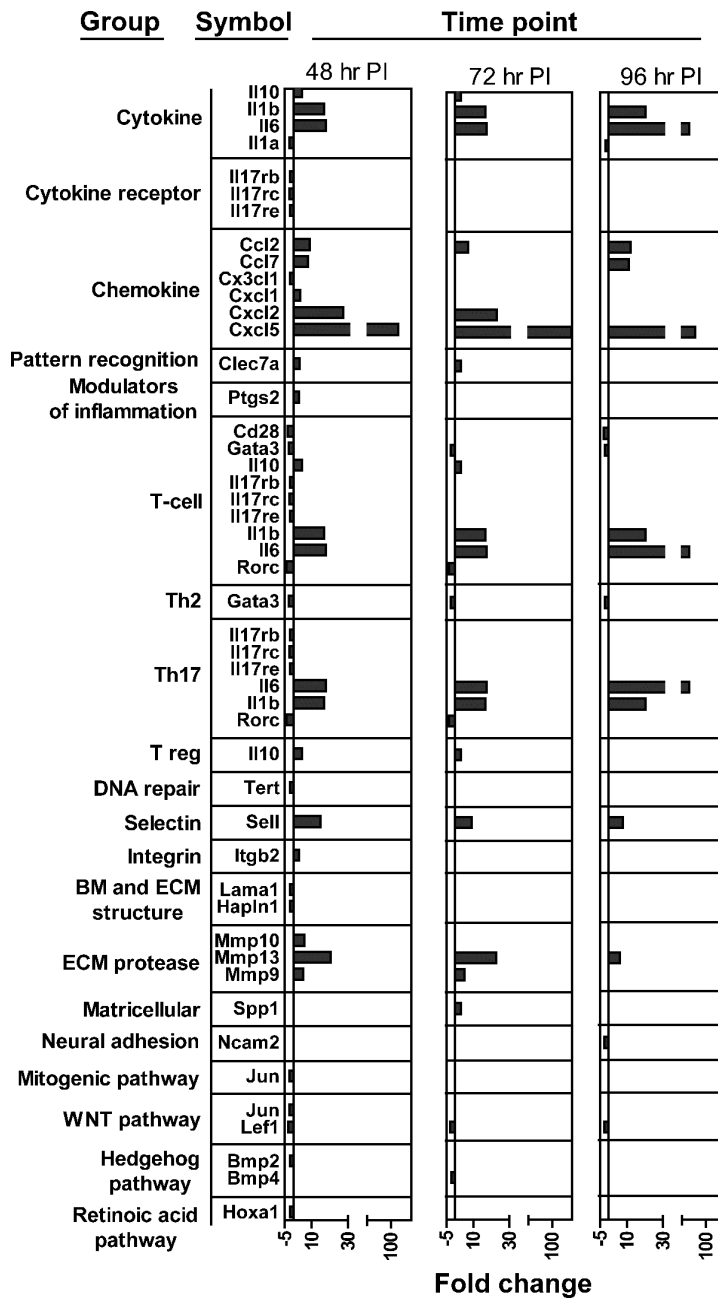
Figure 4.3: Genes modulated during primary infestations of mice compared to tick-free mice.

Gene expression was measured using PCR arrays at 12, 48, 72, and 96 hpi during a primary exposure of BALB/cJ mice to *I. scapularis* nymphs. Significantly modulated genes were divided into biologically meaningful groups (Methods) to allow direct comparison between time points. Official gene symbols and fold changes for all results at 48, 72, and 96 hpi are shown. No significant gene modulation was apparent at 12 hpi

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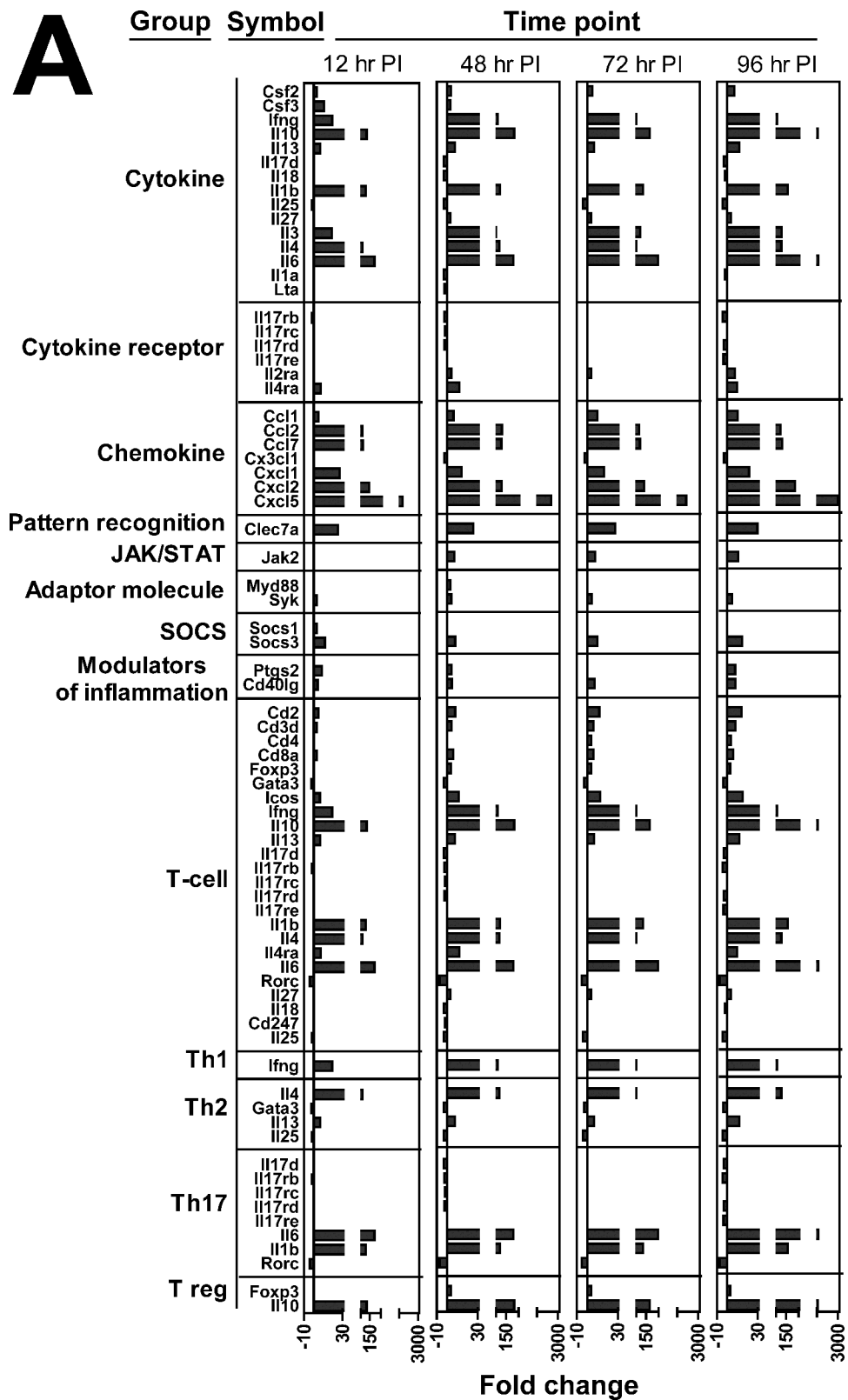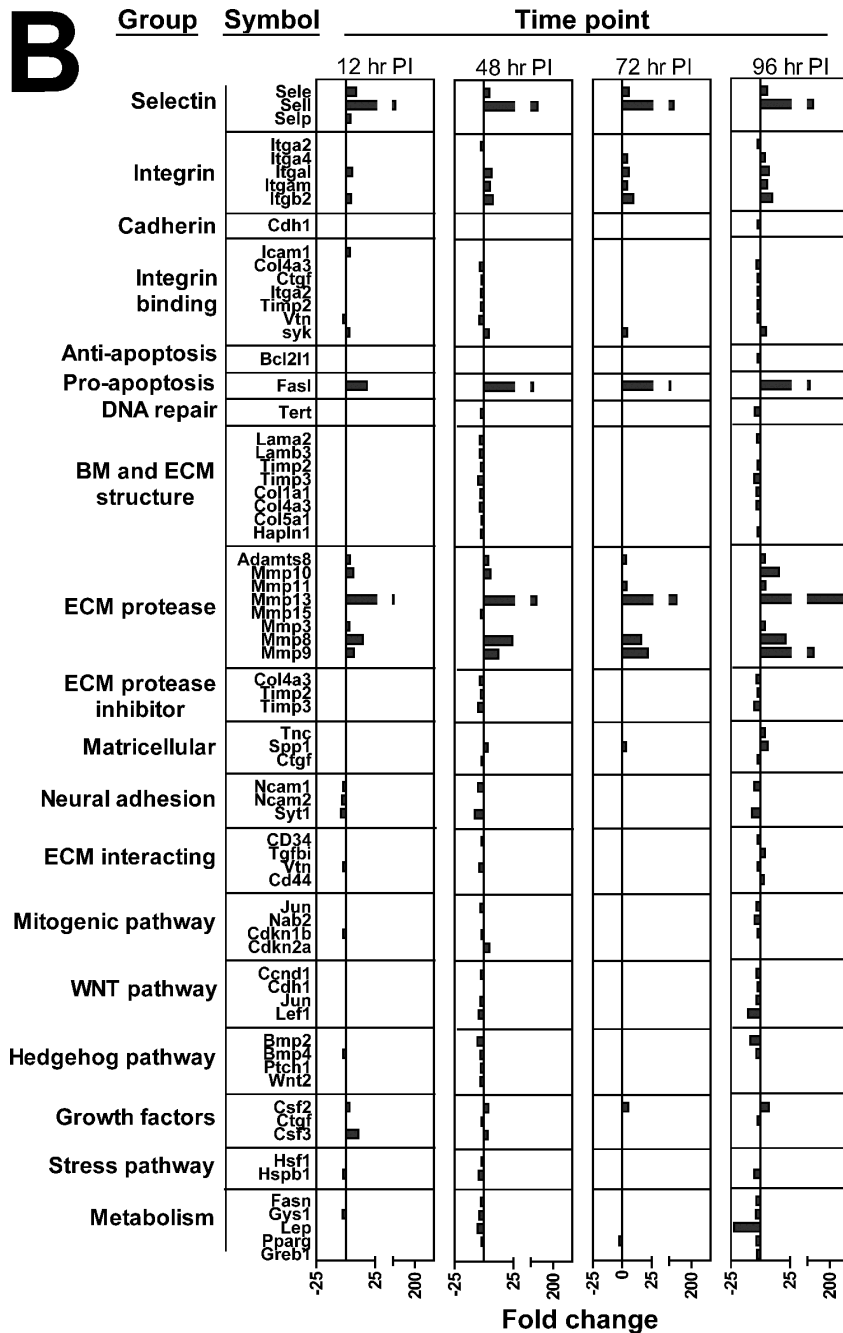
Figure 4.4: Genes modulated during secondary infestations of mice compared to tick-free mice.

Gene expression during a secondary exposure of BALB/cJ mice to *I. scapularis* nymphs was measured using PCR arrays. Significant results were divided into groups as before. Official gene symbols and fold changes are shown at 12, 48, 72, and 96 hr post secondary infestation. The figure is split into sections A and B to aid viewing.

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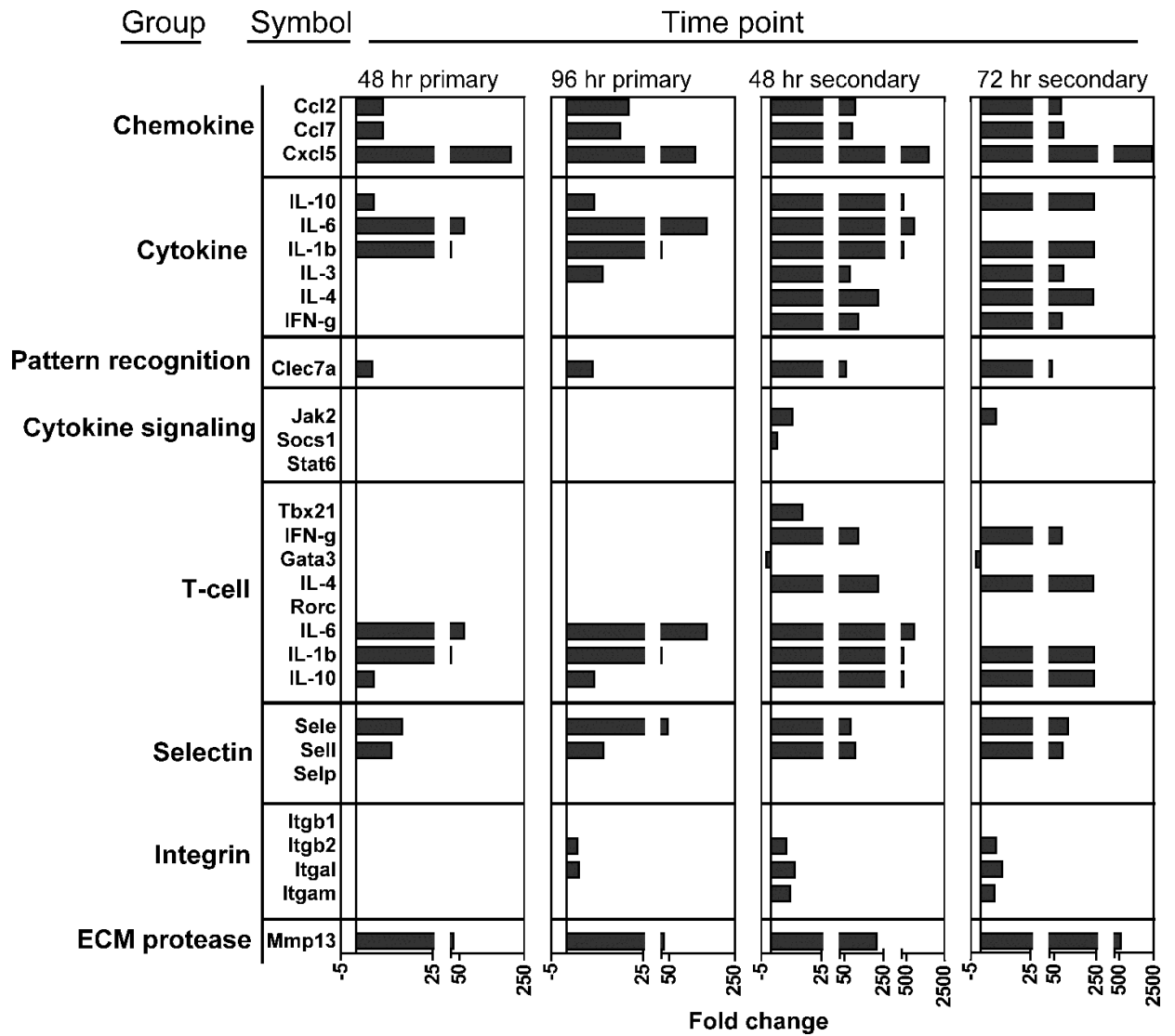
Figure 4.5: Quantitative real-time PCR validation of PCR array data.

Based on the PCR array results, 25 genes were chosen for validation in a separate infestation experiment. Gene expression was measured at 48 and 96 hpi during primary infestations and 48 and 72 hpi during secondary exposures. All significant results are shown. In general, gene modulation supported the array study.

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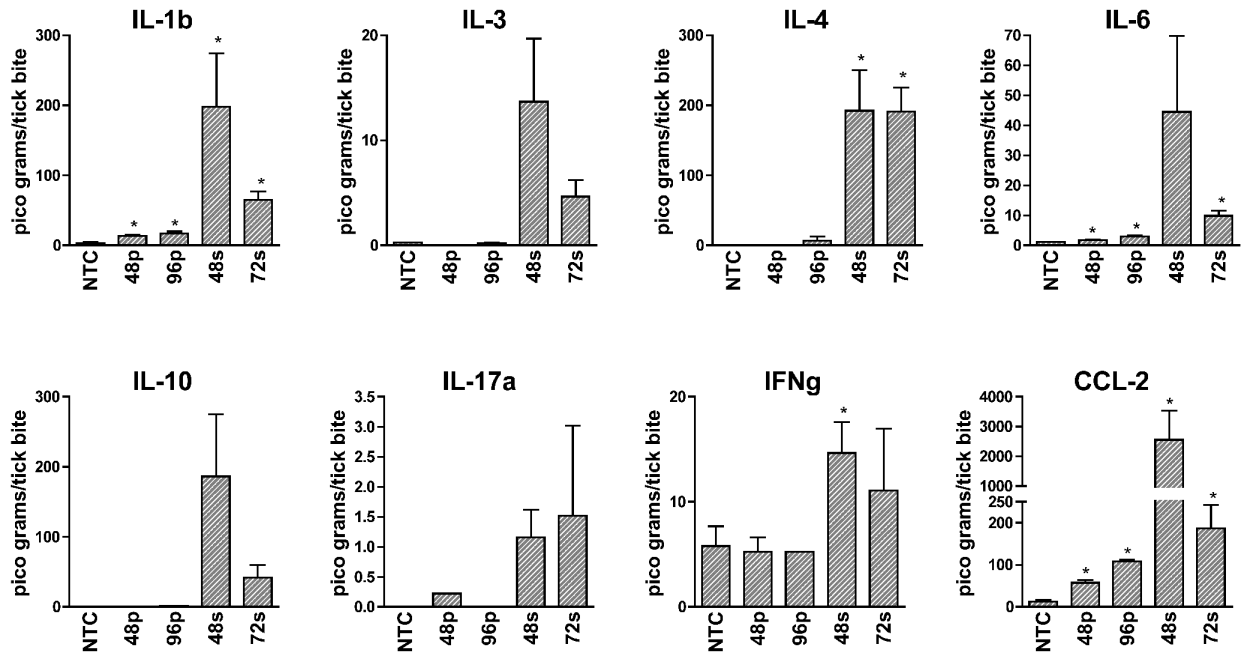
Figure 4.6: Cytokine analysis of tick bite sites during primary and secondary infestations.

Concentrations of IL-1β, IL-3, IL-4, IL-6, IL-10, IL-17a, IFN-γ, and CCL2 were measured in skin biopsies from tick feeding sites at 48 and 96 hpi primary infestation (48p and 96p) and 48 and 72 hpi secondary infestation (48s and 72s) and compared to normal mouse skin using a two-tailed T-test; * indicates a p-value ≤ 0.05 compared to tick-free mice.
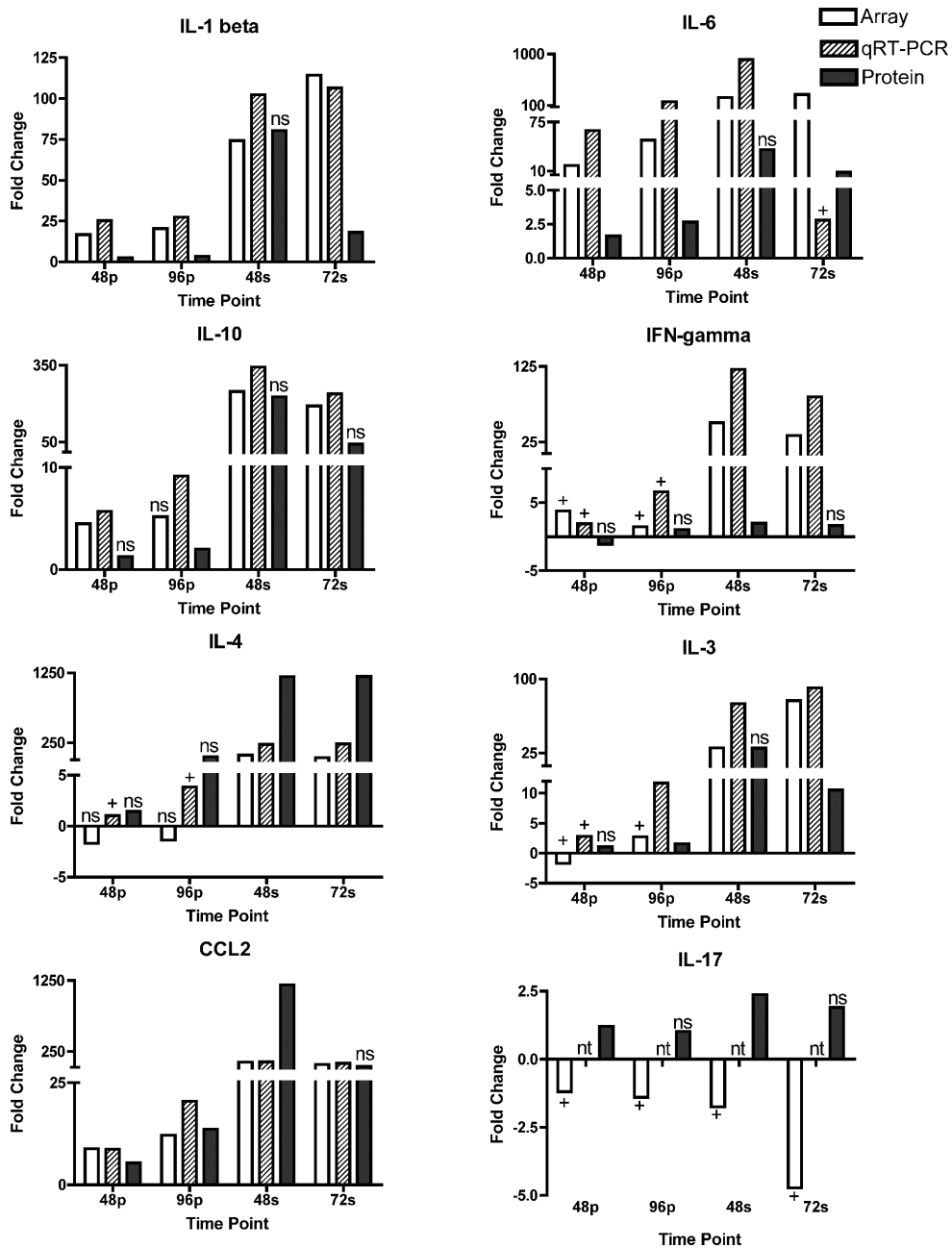
Figure 4.7: Comparison of PCR array, qRT-PCR validation, and protein expression levels.

Gene expression and protein levels were compared by transforming protein expression data into fold change in fluorescence intensity over control samples. "ns" refers to non-significant results (p-value > 0.01 for gene expression and > 0.05 for protein expression); "+" denotes fold changes calculated from transcripts below the detection limit (Ct ≥ 34); "nt" refers to genes not tested.

Chapter 5: Host cutaneous responses to *Dermacentor andersoni* feeding during primary and repeated infestations.

INTRODUCTION.

In the last two chapters, gene expression profiling of the murine cutaneous response to nymphal *I. scapularis* feeding was presented. In this chapter, gene expression profiling of the murine cutaneous response to *D. andersoni* nymphs will be discussed. *D. andersoni* and *I. scapularis* represent divergent phylogenetic lineages of hard ticks [213]. *D. andersoni* ticks make a very superficial lesion in the skin, while *I. scapularis* penetrates deeply into subcutaneous tissue during feeding. *D. andersoni* secretes a large amount of attachment cement that is deposited on top of the epidermis or between layers of the epidermis, while *I. scapularis* secretes much less cement at the level of the dermis and below. Salivary transcriptome analyses of these ticks showed very little overlap in the putative secreted proteins, suggesting that although the sialomes of these ticks have overlapping function they likely differ in their mechanism of action [11,12]. *I. scapularis* and *D. andersoni* also differ in the range of pathogens they transmit, suggesting differences in vectorial capacity. Because of these differences, these tick species are ideal candidates to study conserved and divergent host responses to tick feeding. This will define the core anti-tick response and may also identify host factors that influence vector competence. In this study, Affymetrix GeneChip Mouse Genome 430A 2.0 arrays were used to measure host gene expression in the skin of infested mice at 12, 48, 96, and 120 hours post infestation (hpi) during primary infestations, and 120 hpi during secondary infestations. In addition, pathological analysis of bite-site lesions from

primary infestation time points and quantitative real-time PCR analysis of lymph nodes from 120hpi during the secondary infestation were completed. This final analysis was undertaken in an attempt to define the polarization of murine CD4+ T cells during infestations with nymphal ticks since skin-based gene expression profiling provided no conclusive answer to this particular question.

RESULTS AND DISCUSSION:

The number of up- and down-regulated genes meeting the filtering criteria at each time point is shown in figure 5.1. Although there was a small decrease in the number of upregulated genes between 12 and 48 hpi, the general trend was an increasing number of upregulated genes across time. The downregulated genes show a different profile, with more genes modulated at early and late time points than those in the middle. A list of all genes modulated at any time point in the study was used to generate a heat map (fig. 5.2A). This heat map suggested a similar gene expression profile for the first three time points (12, 48, and 96hpi during primary infestations) that then shifted to a second pattern that was similar between 120hpi in primary and secondary infestations. This suggested that there was a shift in gene expression profiles between early and late time points, as well as between primary and secondary infestations. To provide a higher-resolution analysis of the primary infestation time points, a second heat map was constructed using only the significantly modulated genes in the primary infestation (fig. 5.2B). While this heat map shows some differences between 12, 48, and 96 hpi, the overall similarity seen in the first heat map was preserved. In addition, the dramatic change in expression profile between the first three time points and the 120hpi primary infestation time point

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was maintained. A Venn diagram for the primary infestation time points (fig. 5.3A) shows more uniquely modulated genes at 12hpi and 120hpi than at other time points, again suggesting differences between early and late responses. To explore the patterns observed in the heat maps, all significantly modulated genes from 12, 48, and 96 hpi primary infestation were combined into a single data set. This list, the list of modulated genes at 120 hpi primary infestation, and the list of modulated genes at 120 hpi secondary infestation were used to create a Venn diagram (fig. 5.3B). This analysis revealed 137 genes unique to early primary infestations, 131 genes unique to late primary infestations, 651 genes unique to late secondary infestations, and 57 genes shared between all time points. These lists, along with the lists of genes shared between each group were submitted to the Database for Annotation, Visualization, and Integrated Discovery (DAVID) bioinformatics database using the Affymetrix Mouse Genome 430A 2.0 array as a background list. The functional annotation-clustering tool was used to group similar ontology terms together, and the top ten clusters containing significant ontology terms ($p < 0.05$) were named based on the terms included (table 5.1).

The core response (genes shared between all time points)

Analyzing the shared response between all time points allows the description of a "core" response to tick feeding. Significant gene ontology clusters were chemotaxis and inflammation, cation homeostasis, intermediate filaments and keratinization, carbohydrate binding, and regulation of blood pressure. Genes in the chemotaxis and inflammation clusters were chemokines, cytokines, and anti-microbial molecules. Chemokines *Ccl2*, *Ccl6*, *Ccl7*, *Ccl12*, *Cxcl1*, *Cxcl2*, *Pf4* (platelet factor 4 or *Cxcl4*), and receptor *Ccr1* were upregulated and are consistent with monocyte and neutrophil

migration into the bite site. Additional chemoattractants for neutrophils such as the S100 molecules (calgranulins) and Saa3 (serum amyloid A3) were also upregulated. Antimicrobial proteins such as *Lcn2* (lipocalin 2) and *Ptx3* (pentraxin related gene) that function in iron sequestration and opsonization, respectively, were upregulated. These results combined with the up-regulation of *Il1b* suggest a potent innate-like immune response dominated by macrophages and neutrophils is a hallmark of the anti-tick response. Gene ontology terms in clusters 3 and 6 were related to cations or metal ions. Genes in these clusters related to ion regulation were *Mt1* and *Mt2* (metallothionein); these molecules are involved in regulating copper and zinc, but also are involved in reactive oxygen species scavenging, cell transcription, and immune responses. Studies with metallothionein knockout mice suggest that *Mt1* and *Mt2* play protective roles in inflammatory settings [169,214]. Xanthine dehydrogenase, an enzyme involved in the oxidative metabolism of purines, was also upregulated. Xanthine dehydrogenase may be converted to xanthine oxidase by sulfhydryl oxidation, and xanthine oxidase can be an important source of reactive oxygen species [215]. Thus the generation and scavenging of reactive oxygen species appears to be the primary function of the genes in these clusters. Clusters 4 and 5 were dominated by gene ontology terms related to intermediate filaments and keratinization. Indeed, keratin intermediate filaments *Krt6a*, *Krt6b*, and *Krt16* were upregulated. *Krt16* has been shown to pair with either *Krt6a* or *Krt6b* to form essential intermediate filaments in epithelial wound repair. Specifically, these intermediate filaments are required to support keratinocyte migration into the wound site [162,163]. In addition to these molecules, *Sprr1b* (small proline rich 1b) was upregulated. The encoded protein is a significant component of the cornified cell envelope that maintains a permeability barrier across epithelial tissues [216].

Downregulated genes included a minor collagen (type 11 alpha 1), the cytoskeletal component spectrin, and a little-studied molecule Limch1 (LIM and calponin homology domains 1). These results suggest that a keratin intermediate filament-based wound healing response is activated at the tick feeding lesion; however, it is surprising that other cytoskeletal elements are downregulated, a pattern that is even more striking in the early primary infestation as discussed below. Thus, keratin intermediate filaments but not other cytoskeletal molecules are activated in response to tick bites. Gene ontology cluster 7 was related to carbohydrate binding. The primary molecules in this group were c-type lectin receptors *Clec4d* and *Clec4n*, although other molecules such as *Ccl7*, *Pf4*, and *Ptx3* were also present. C-type lectins are expressed on dendritic cells and macrophages, and play important roles in orchestrating the inflammatory response. Their importance in host responses to tick feeding is strongly suggested by studies showing that the *I. scapularis* salivary protein Salp15 can bind and activate DC-sign (CD209) [100]. CLEC4N (dectin-2) is thought to associate with FcRγ and *to* signal through a Syk-Card9 pathway to activate NF-κB [217]. Ligands include mannose, fungal antigens, and dust mites [218], although others likely exist. The precise role of c-type lectins in tick feeding is unknown, but these results suggest that they may act as important pattern recognition receptors that modulate downstream responses. The final cluster contained terms related to regulation of blood pressure. The primary gene in this group, *Wnk1* (lysine deficient protein kinase 1), is downregulated and it influences blood pressure by regulating salt absorption in the kidney. *Wnk1* also plays a similar role in sweat glands [219]. Upregulated genes in this group include *Hmox1* (heme oxygenase 1), and *Gch1* (GTP cyclohydrolase 1). Hmox1 can play a role in protecting tissues from oxidative damage, while Gch1 can produce either NO or reactive oxygen species depending cofactor

availability. Thus the gene ontology terminology may be misleading here, as the genes appear more related to regulating oxidative status than blood pressure. In summary of this section, these results suggest that a potent innate immune response, regulation of the oxidative status of the skin, a keratin intermediate filament-based wound healing response, and C-type lectin pattern recognition are highly conserved aspects of the host response to ticks throughout primary and secondary exposure.

Early primary infestation

Gene ontology analysis of the early primary infestation revealed a number of clusters related to nucleotide processing such as DNA repair, nucleotide binding, transcription, and mRNA metabolism. A review of the genes represented by these clusters suggested they could be broadly grouped into DNA repair molecules, DNA helicases of the SWI/SNF family, transcription factors, components of the spliceosome, and mRNA metabolism. DNA helicases of the SWI/SNF family are not active helicases, but function as DNA translocases thought to modulate chromatin structure [220]. Thus, most of these molecules can be related to transcription, from chromatin remodeling, transcription factor binding, and RNA splicing, to mRNA metabolism. Interestingly, nearly all of these genes were downregulated, suggesting that transcription is decreased early in the primary infestation. Of the few upregulated transcriptional regulators, *Nfkbia* and *Tsc22d3* were of particular interest because they have been shown to inhibit NF-κB and AP-1 pro-inflammatory pathways [221]. DNA repair molecules were also downregulated. In contrast, molecules in the circadian rhythm cluster, such as *Per1* were upregulated. Overexpression of *Per1* in tumor cells was shown to increase DNA-damage induced apoptosis and significantly decreased cell growth in untreated cells [222]. These

data suggest that some connection between DNA repair, circadian rhythm molecules, and growth arrest in the skin at the bite site may exist. Terms in the final cluster were related to cardiac muscle contraction, although review of these genes, and genes in the highly significant (though un-clustered) GO term actin binding (GO:0003779), revealed molecules related to muscle contraction, actin binding, and cytoskeletal adaptor molecules that may relate signaling events to changes in the cytoskeleton. Cytoskeletal elements have been shown to play vital roles in epithelial wound healing processes by aiding cell migration and wound contracture [223]. Thus, the early host responses were characterized by a reduction in transcription, DNA repair, inhibition of inflammation, potential inhibition of the cell cycle, and down-regulation of cytoskeletal elements. These effects suggest that tick feeding may inhibit early aspects of the wound healing response.

When the genes shared between early and late primary infestation were submitted to DAVID, no significant clusters were observed.

Late primary infestation

The first three clusters (regulation of adhesion, blood vessel development, embryonic development) and clusters 6 and 7 (cell growth, MAPK activity) share similar genes and will be treated together here. In the process of normal epidermal wound healing, repair must take place in the epidermis, dermis, and vasculature to restore normal function. Particularly for the re-epithelialization of the wound, keratinocytes must loose some of their adhesiveness and migrate into the wound area. This process shares similar characteristics to the epithelial-mesenchymal transition (EMT) [224]. In support of a

similar process at the tick bite site, genes known to be involved in EMT such as *Tdgf1* (teratocarcinoma-derived growth factor 1 or Cripto-1), *Cyr61* (cysteine rich protein 61 or CCN1), and *Smad5* (SMAD family member 5, a TGF-β signaling intermediate) were upregulated. While not specific to EMT, the MAPK signaling pathways is known to be important [225], increasing the relevance of cluster 7. Additional upregulated genes that may be involved were *Tnfrsf12s* (tumor necrosis factor receptor superfamily, member 12a), *Junb* (Jun-B oncogene), and *Epgn* (epithelial mitogen). While a specific role for the encoded proteins in EMT has not been delineated, these molecules may be related to TNF-α, AP-1, and growth factor responses are known to be involved [225]. Downregulated molecules included extracellular matrix components *Col1a1* (collagen type I alpha 1) and *Lamb2* (laminin beta 2), calcium ion channel or signaling molecules *Tesc* (tescalcin) and *Pkd2* (polycyctic kidney disease 2), integrin ligand *Edil3* (EGF-like repeats and discoiding I-like domains 3), and growth factor related molecules *Pdgfrb* (platelet derived growth factor receptor beta) and *Tgfb3* (transforming growth factor beta 3). In the final analysis, a convincing case for EMT in wound healing responses during late primary infestations cannot be made. However, changes in adhesion, cell growth, MAPK activity, and the appearance of genes normally associated with embryonic development such as *Tdgf1* are at least suggestive of a related process.

The remaining clusters were nuclear or organelle lumen, RNA processing, positive regulation of transcription, and meiosis or cell cycle. The majority of the genes in these clusters code for RNA binding proteins, RNA processing proteins, and transcriptional activators/transcription factors. An interesting theme among the RNA-related molecules was an interaction with rRNA, implying some interaction with ribosomes. Most of the genes in these clusters were upregulated, contrasting with the

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transcription-related genes early in the primary infestation. Thus, the data suggests transcription is inhibited during early host responses, but then activated near the end of the feeding cycle.

Genes shared between 120 hpi primary and 120 hpi secondary infestation clustered into a number of broad categories such as extracellular region, cell surface, cellular homeostasis, and regulation of phosphorylation. Two clusters were more specific, however. Fatty acid biosynthesis contained three genes, *Fcer1a* (IgE Fc receptor), *Scd3* (stearoyl-coenzyme A desaturase), and *Ch25h* (cholesterol 25-hydroxylase. *Fcer1a* and *Ch25h* were upregulated, while *Scd3* was downregulated. *Fcer1a* encodes the alpha chain of the FcεR, a receptor that binds IgE with high affinity. The receptor is activated by antigen-induced cross-linking, and induces many downstream responses including the release of histamine and the production of prostaglandins D2 and E2 [226]. Supporting this role, genes encoding proteins involved in prostaglandin E synthesis are upregulated at 120 hpi primary infestation, although they are not shared with 120 hpi secondary infestation. *Ch25h* encodes a protein that catalyzes the formation of 25-hydroxycholesterol [227], a molecule that can act as a chemoattractant for migrating cells but also inhibits the function of B cells [228] and can promote the survival of *Listeria monocytogenes* infected cells [229]. *Scd3* is an important enzyme in the synthesis of monounsaturated fatty acids, many of which are subsequently used as components of membrane phospholipids. Interestingly, decreasing fatty acid synthesis inhibited rift valley fever virus replication in mouse embryonic fibroblasts [230], suggesting that fatty acid synthesis may aid viral replication. These genes suggest a role for fatty acid metabolites and the control of fatty acid synthesis at the tick bite site. Finally, the response to wounding cluster contained pro-inflammatory

genes such as *Il6*, *Cxcl5*, and *P2ry12* (purinergic receptor P2Y) and extracellular matrix molecules *Cd44* and *Timp3* (tissue inhibitor of metalloproteinase 3). These genes are involved in inflammation and cell migration, although they are often associated with acute inflammation rather than appearing late in the induction of the immune response as they do here.

In summary, late in the primary infestation the innate immune response remains the major player. Keratin-based wound healing responses and the suggestion of an EMT-type transition driving wound re-epithelialization are present. In addition, transcription appears to be potentiated in contrast to earlier in the primary infestation. Genes shared with the secondary infestation suggest fatty acid metabolism and the response to wounding are important in host responses near the end of the feeding cycle.

Secondary infestation

There were 651 genes modulated only in the secondary infestation. Gene ontology analysis of this group of genes resulted in many clusters related to the immune response. These clusters shared many genes with each other and I will discuss them in relation to what appeared to be the most distinctive feature(s) of each cluster or group of clusters. The first cluster contained GO terms related to inflammatory and defense responses. Genes in this group supported four primary processes: complement, coagulation, Toll-like receptor/IL-1 response, and acute phase response. Clusters 2, 5, 8, and 9 were leukocyte and lymphocyte activation, T cell activation and leukocyte proliferation, regulation of immune cells, and regulation of adaptive immune response, respectively. These clusters shared similar genes, and supported the importance of

protein kinases (SRC family, SYK, and JAK), T-cell costimulation (CD40, CD86, CD3d, and LCK), cytokines, immunoreceptors (cytokine, antibody, C-type lectin, and Toll-like receptors 1 and 4), and signaling intermediates in immunoreceptor signaling. Cluster 3, positive regulation of immune response, contained many similar features. However, two genes strongly related to iron handling in the innate immune response, *Slc11a1* (solute carrier family 11 member 1) and *Hpx* (hemopexin) were upregulated. Cluster 4 contained GO terms related to lysosomes. The largest group of upregulated molecules in this cluster consisted of genes encoding many different cathepsins. Cathepsins are lysosomal proteinases that function primarily in protein turnover in the lysosome [231]. However, additional roles for cathepsins include (but are not limited to) antigen presentation [232], keratinocyte differentiation [232], itch responses [233], and wound healing [234]. The chemotaxis cluster contained chemokines *Ccl24*, *Ccl17*, *Ccl5*, *Ccl4*, *Ccl8*, *Ccl3*, and *Cxcl16* consistent with the migration of lymphocytes, monocytes, neutrophils, eosinophils, and basophils into the bite site. Additional molecules that supported cell migration such as integrins, chemoattractants, and intracellular molecules that regulate changes in cell shape were also upregulated. Cluster 7 contained terms related to regulation of cytokine production. These genes code for proteins that function as immunoreceptors, cytokine signaling intermediates, and cytokines. Finally, the endocytosis cluster contained genes encoding classical endocytotic proteins such as *Clta* (clathrin, light polypeptide) and *Picalm* (phosphatidylinositol binding clathrin assembly protein). Other genes in this cluster were antibody Fc receptors *Fcer1g*, *Fcgr3*, and *Fcgr2b* and lectin recepors *Clec7a*, *CD209a, CD209d, CD209e*, and *Mrc1*. These receptors may function in endocytosis by binding antibodies or carbohydrate moieties and triggering endocytotic machinery. This may be an important method of delivering

tick-derived antigens to the endosomal/lysosomal compartment where they could be processed for antigen presentation.

These results suggest the importance of complement, coagulation, innate immune responses, and adaptive immunity in the host response to *D. andersoni* nymphs. In particular, the importance of Toll-like receptors 1 and 4 and lectin pattern recognition receptors appear to be important aspects of the innate immune response during secondary infestations. Both TLRs and C-type lectins have been shown to be critical pattern recognition receptors for sensing pathogens, initiating and shaping innate and adaptive immune responses [217,235]. Both TLR2 and DC-SIGN (CD209e) have already been shown to be important in anti-tick responses by modulating cytokine responses [98,100]. These studies support the present results and also suggest that the role of these receptors is likely broader than previously reported. Within the adaptive immune response, these results suggest that the capture, processing, and presentation of antigens is an important process during secondary infestations. In particular, cathepsins may be important proteins in this process with possible contributions to wound healing and/or itch responses. The array results also support the co-stimulation and activation of lymphocytes.

Validation

A list of genes was chosen to validate the microarray data (table 5.2**)** using quantitative real-time PCR. Genes were chosen based on significant fold change in the microarray study that were involved in important pathways identified in the gene ontology analysis. Trends of up- and down-regulation were well preserved between the

microarray and validation study (fig. 5.4). In general, more genes had significant fold changes in the microarray than the validation for 12 hpi and 48 hpi, and the validation had more significant fold changes from 96 hpi onwards. At the 120 hpi secondary infestation time point, all the gene targets validated except *Chi3l1* and *Wnk1*. It should be noted that some genes that were significant only at later time points in the array study became significant earlier in the validation study (i.e., *Arg1, Cxcl5*), while some genes that were significant early in the array study did not become significant until later time points in the validation (i.e. *Ccl12, Ccl7, Krt6b, Krt16,* and *S100a9*). Interestingly, the trend of more significant gene modulation at 12 hpi than 48 hpi seen in the array data was upheld in the validation study. In summary, the validation study upheld the microarray study with a few minor differences. Samples used for the validation study were derived from a completely separate infestation experiment than the array study, making it an extremely rigorous test and likely contributing to the small variations seen between data sets.

Lymph node study

The host response to ticks has frequently been reported to be polarized toward the production of Th2 cytokines. However, in the earlier work with *I. scapularis*, the gene expression profile in the skin was consistent with a more mixed response [178]. Similarly, gene expression profiling in the skin at *D. andersoni* bites did not yield a conclusive picture of CD4+ T cell polarization. While *Il4* was present, so were *Il6* and *Il1b*, cytokines more associated with Th17 responses than Th2. To assay the systemic host response, the expression of a panel of genes (table 5.3) related to CD4+ T cell

differentiation was measured in lymph node samples from naïve and 120 hpi secondary infestation mice. Important transcription factors for T regulatory cells (*Foxp3*), Th17 cells (*Rorgt*), and Th1 cells (*Tbx21*) were either downregulated or unchanged between infested and control mice. In contrast, an important transcription factor for Th2 cells (*Gata3*) was upregulated along with Th2 cytokine *Il4*. Thus, the results of this experiment strongly suggest the systemic response to repeated *D. andersoni* nymphal feeding in mice is polarized toward a Th2 profile.

Histology

To examine the histopathology of the bite site, skin biopsies were collected from infested mice at 12, 48, 96, and 120 hpi during primary infestations. These biopsies were embedded in paraffin and carefully sectioned to locate the hypostome entry into the skin. The resulting sections were stained with hematoxylin and eosin (fig. 5.6**).** The bite site is characterized by a large quantity of attachment cement secreted by the tick to anchor it to the host. The amount of cement increases throughout the feeding period, and it contains readily evident layers, suggesting that additional cement is secreted in waves throughout the feeding cycle. The deepest penetration of the hypostome into the skin is very shallow, in most cases barely passing through the epidermis. At 12hpi, a mild inflammatory infiltrate was present in the dermis, especially concentrated at the junction between dermal connective tissue and underlying adipose tissue. A small lesion was located immediately under the hypostome and likely represents the feeding "pool." Surprisingly, at 48 hpi, the number of inflammatory cells had not increased from 12 hpi, perhaps mirroring the reduction in gene expression seen at this time point. The feeding

lesion is very well defined, and extravasated erythrocytes are readily evident around the hypostome. Some thickening of the epidermis is evident. By 96hpi, the inflammatory infiltrate has increased dramatically and the feeding lesion appears to have moved deeper into the dermis. Epidermal thickening and extravasated erythrocytes and leukocytes are present. At 120 hpi, most of the changes observed at 96 hpi appear intensified. The infiltrate is very dense, the epidermis is markedly thickened, the feeding lesion is poorly defined and the dermal tissue near the hypostome appears to be loosing its normal architecture. Extravasated erythrocytes and leukocytes are prominent. In the microarray analysis, the chemotaxis of immune cells into the bite site, and aspects of wound healing responses were especially prominent. The histological analysis supports these basic conclusions, showing an inflammatory infiltrate that increases across time, and significant changes in the epidermal and dermal compartments near the feeding tick. The importance of changes in the epidermal layer in the host response to ticks is not known, however, it is possible that the host attempts to "slough off" the tick by greatly increasing epithelial cell replication. Despite the modulation of genes related to apoptosis, this was not a significant feature of the tissue sections analyzed. In addition, any conclusions about apoptosis based on this data would be tenuous at best since there was no "positive" control with which to compare rates of apoptosis in different cell types.

Conclusions

The microarray data suggests that the chemotaxis of neutrophils, monocytes, and other cell types, the production and scavenging of reactive oxygen species, keratin-based wound healing responses, and C-type lectins are important host responses to tick feeding

conserved across primary and secondary infestations. Later time points in the primary infestation include additional support for wound healing activities including the suggestion of EMT. The secondary infestation is characterized by increases in these same pathways, along with the addition of complement, coagulation, Toll-like receptors, acute phase response, antigen presentation, and the activation of lymphocytes. These results were supported by quantitative real-time PCR validation. The systemic murine response to *D. andersoni* nymphal feeding was polarized toward a Th2 profile, based on real-time PCR analysis of draining lymph nodes from secondary infestations. The histological analysis supported the chemotaxis of neutrophils and monocytes into the bite site, and the marked thickening of the epithelial layers may be the result of the up-regulation of genes related to keratinization and wound healing.

Tables.

Table 5.1. Top ten clusters from DAVID analysis based on lists from Fig. 3B Venn diagram.

The core analysis represents significantly modulated genes shared between early primary (DAP12, 48, 96), late primary (DAP120), and secondary infestations (DAS120). DAP, *D. andersoni* primary; DAS, *D. andersoni* secondary.

Core
1 Chemotaxis, immune response, response to wounding
2 Chemotaxis, cell migration
3 Cation homeostasis
4 Intermediate filament, cytoskeleton organization and structure
5 Keratinization, keratinocyte and epidermal cell differentiation
6 Response to metal ion
7 Carbohydrate binding
8 Regulation of blood pressure
9
10

DAP12-48-96 only
1 Response to DNA damage and cellular stress, DNA repair
2 Intracellular non-membrane-bounded organelle
3 Nucleotide and ATP binding
4 Regulation of transcription, DNA binding
5 Circadian rhythm
6 Chromosome, chromatin binding and organization
7 Nuclear body, nucleoplasm
8 mRNA metabolic process
9 Regulation of transcription
10 Cardiac muscle contraction

DAP120-DAP12-48-96
1 No clusters with significant terms
2
3
4
5
6
7
8
9

DAP120 only
1 Regulation of cell and cell-substrate adhesion
2 Blood vessel development
3 Embryonic development
4 Nuclear or intracellular organelle lumen
5 RNA processing
6 Cell growth
7 MAP kinase activity
8 Positive regulation of transcription and RNA metabolic process
9 Meisis (cell cycle)

DAP120-DAS120
1 Extracellular region
2 Proteinaceous extracellular matrix
3 Cell surface
4 Response to wounding
5 Cellular homeostasis
6 Fatty acid biosynthetic process
7 Regulation of phosphorylation, protein kinase activity
8
9
10

DAS120
1 Inflammatory and defense response
2 Leukocyte and lymphocyte activation, T cell differentiation
3 Positive regulation of immune response, immune response-regulating cell surface receptor signaling pathway
4 Lysosome
5 T cell activation, leukocyte and lymphocyte proliferation
6 Chemotaxis
7 Regulation of cytokine production
8 Regulation of immune cells
9 Regulation of adaptive immune response
10 Endocytosis

DAS120-DAP12-48-96
1 Keratinocyte, epidermal cell differentiation
2 Rhythmic process

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Table 5.2: Gene list for quantitative real-time PCR validation of the *D. andersoni* microarray study.

Arg1	Cxcl5	il4	S100a9
Ccl12		Il6	sele
Ccl6	Hprt	Irak4	Serpine1
Ccl7	Hsp90ab1	krt16	Socs3
Ccr1	ifng	krt6b	Stat3
Chi3l1	il10	Map3k6	Wnk1
Clec10a	il12a	Mt1	No RT
Clec4e	Il1b	ptges	No temp

Table 5.3: Gene list for quantitative real-time PCR analysis of mouse lymph nodes after secondary infestation with *D. andersoni* for 120hrs.

Actb	Il12a	Stat3
Foxp3	Il17a	Stat4
Gapdh	Il1b	Stat5a
Gata3	Il4	Stat6
Hprt	Il6	Tbx21
Hsp90ab1	RORgt	Tgfb1
Ifng	Socs3	No RT
Il10	Stat1	No temp

Figures.
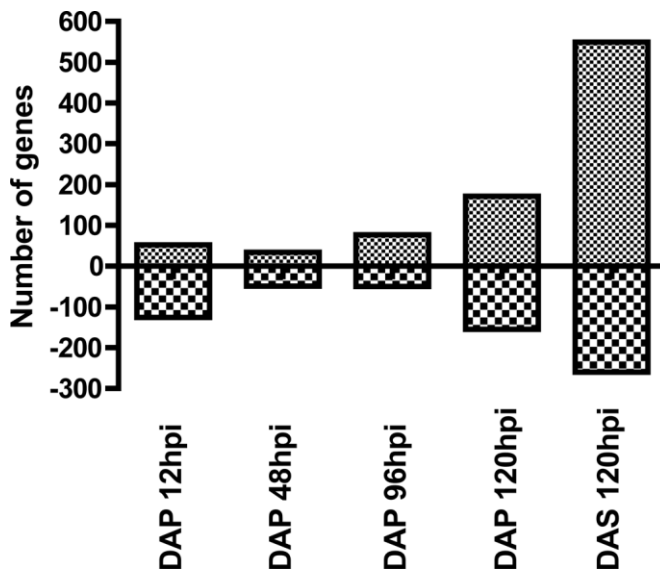
Figure 5.1: Number of up and downregulated genes at each time point in the microarray data.

Mice were infested with *D. andersoni* nymphs and 4mm skin biopsies collected at 12, 48, 96, and 120 hpi during primary infestations and 120 hpi during secondary infestations. Gene expression profiling was measured using Affymetrix mouse genome 430A 2.0 arrays. Significance was assessed using iReports data analysis methods, and results were filtered for genes with fold changes greater than +/-1.5 and p-values less than 0.05. DAP, *D. andersoni* primary; DAS, *D. andersoni* secondary; hpi, hours post infestation.
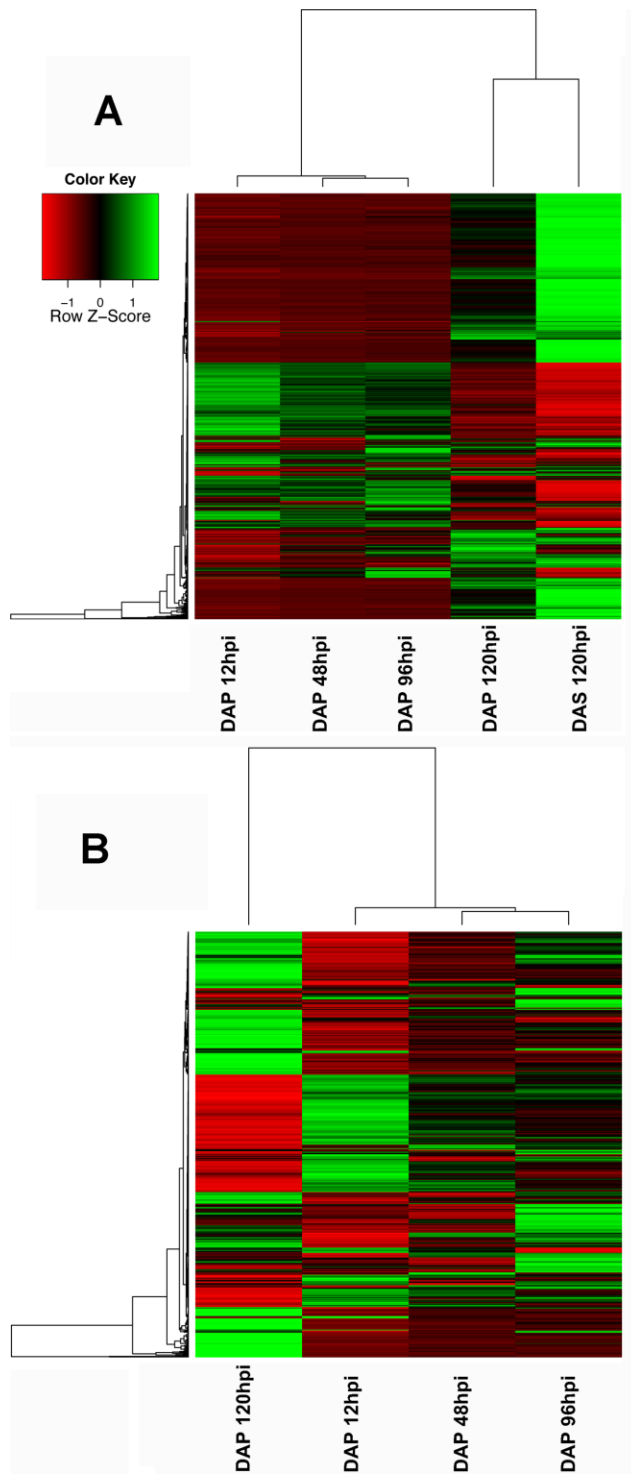
Figure 5.2: Heatmaps showing changes in gene expression across time.

A: changes in genes significant at any time point in the microarray study. B: changes in genes significant during the primary infestation only.
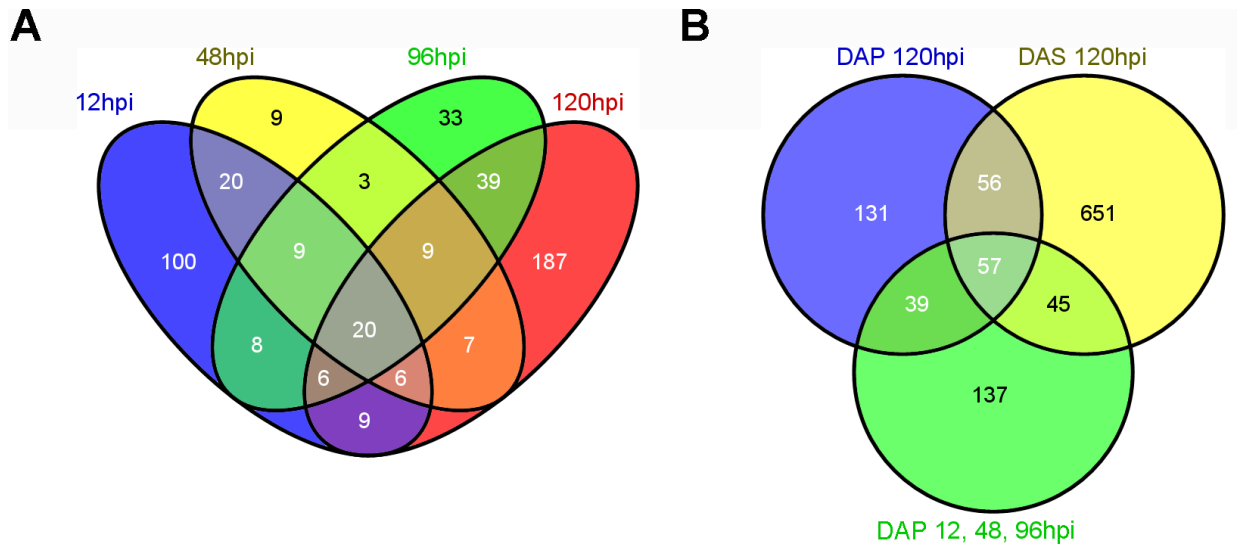
Figure 5.3: Venn diagram showing the overlap of significantly modulated genes between time points in the microarray study.

A: Venn diagram for primary infestation time points. B: Venn diagram for early time points (DAP12, 48, and 96 hpi), later time points (DAP120 hpi), and secondary infestation (DAS120 hpi). The Venn diagram in B was constructed based on variations in gene expression profiles suggested in the heatmaps. All Venn diagrams were created using [236].
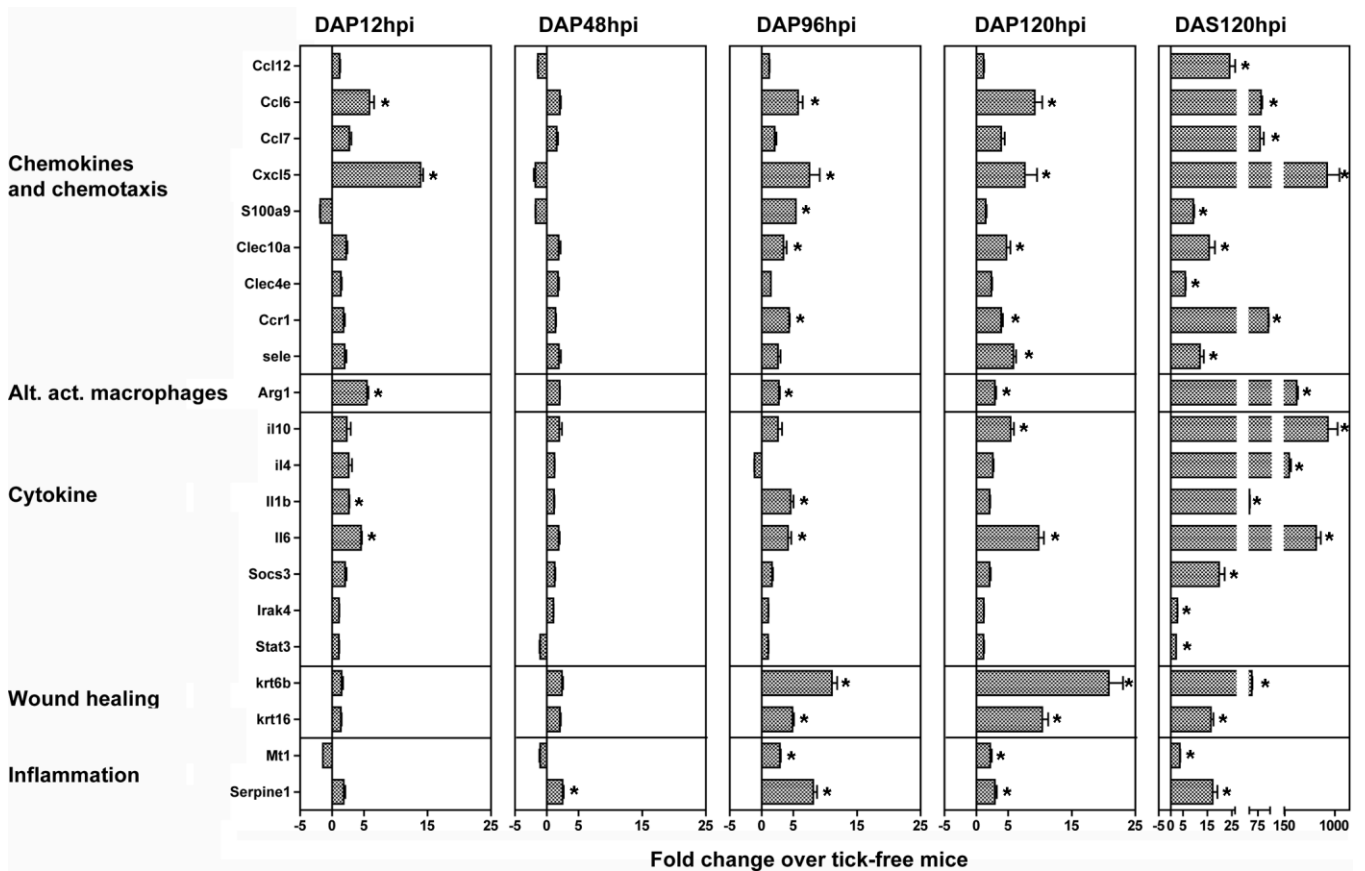
Figure 5.4: Validation of microarray data by quantitative real-time PCR.

A list of genes significantly modulated in the microarray study (Table 5.2) were validated using quantitative real-time PCR on bite site skin samples from a separate infestation experiment. Significance was assessed using LIMMA (linear models in microarray analysis) implemented in HTqPCR, and R based program for qrt-PCR analysis (see methods chapter). All the genes marked with an asterisk were significant (p < 0.05) as compared to tick-free mice.
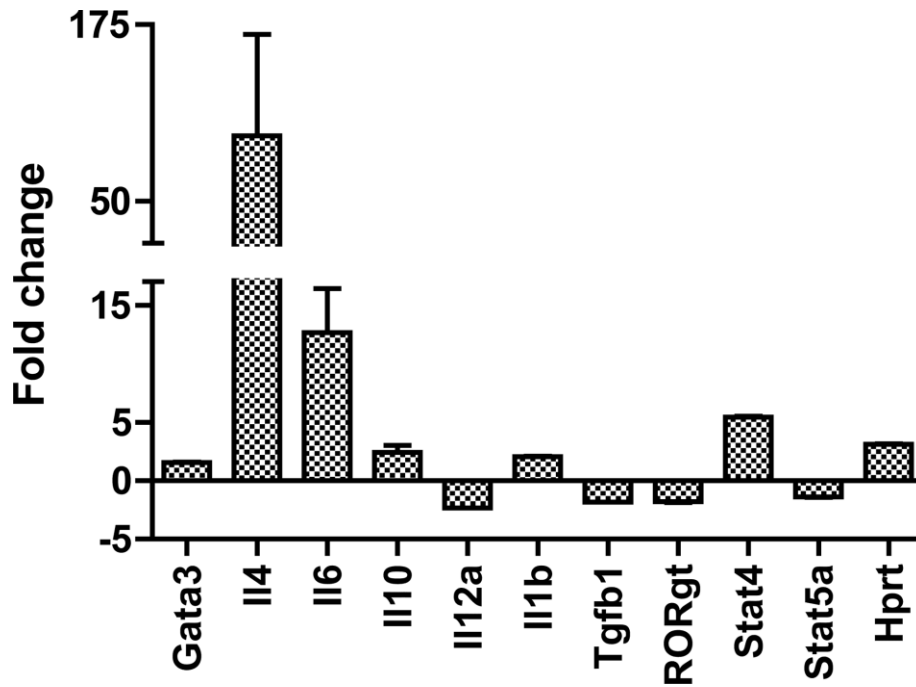
Figure 5.5: Lymph node gene expression profile supports a systemic Th2 response.

Gene expression changes between secondary infestation 120 hpi draining lymph nodes and lymph nodes from tick-free mice measured by quantitative real-time PCR. Significance was assessed using LIMMA (linear models in microarray analysis) implemented in HTqPCR, and R based program for qrt-PCR analysis (see methods chapter). All the genes shown were significant ($p < 0.05$).
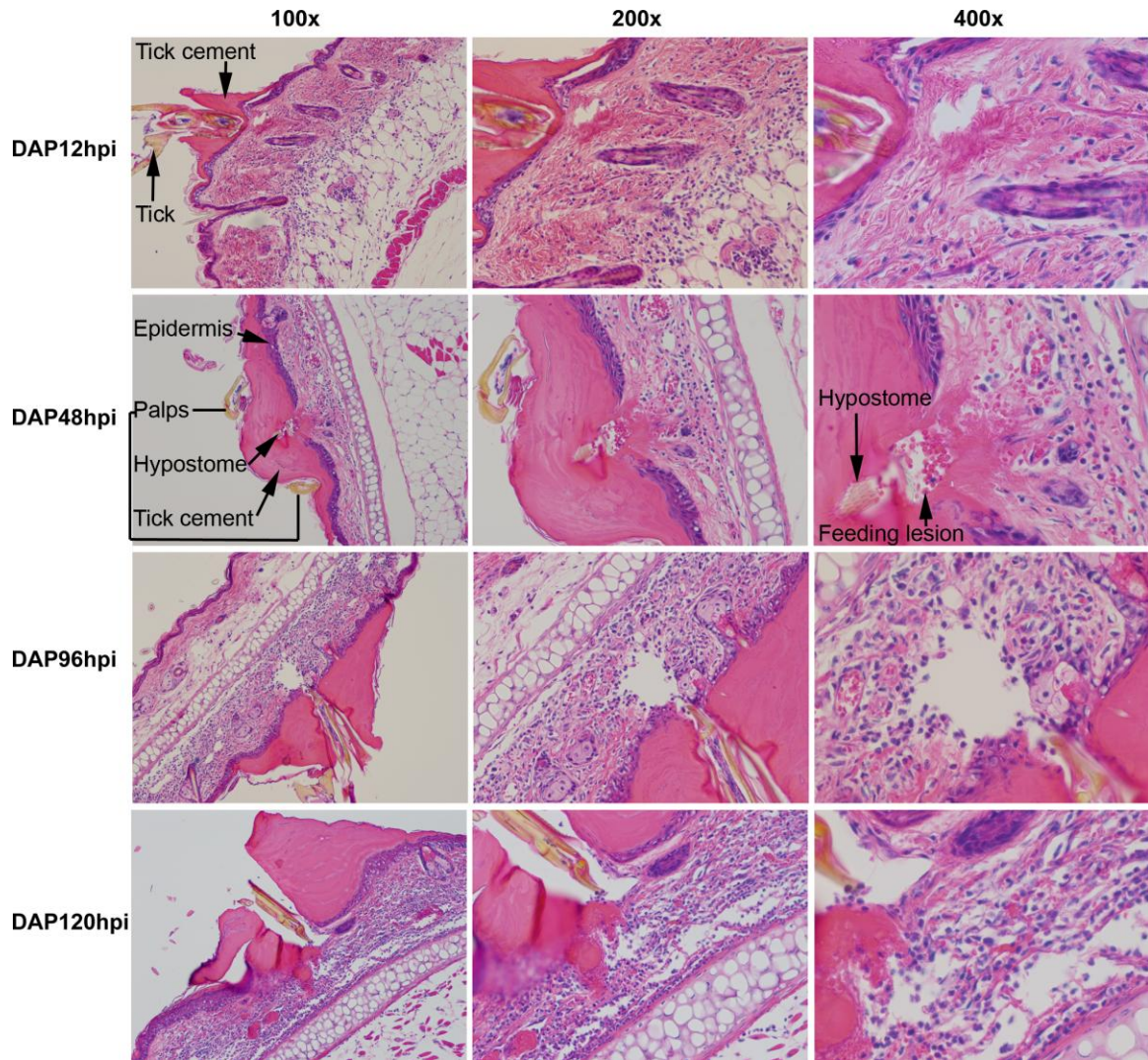
Figure 5.6: Histological analysis of *D. andersoni* bites during primary infestations.

Biopsies from tick feeding sites were fixed in zinc fixative and embedded in paraffin. Paraffin blocks were carefully sectioned and slides showing the entry of the hypostome into the skin were stained with H&E. In the top two rows, relevant structures such as the tick, tick cement, epidermis, palps, and feeding lesion have been labeled. The second row appears different that the others because the orientation of the tick in the block was different.

Chapter 6: Revisiting the clinal concept of evolution and dispersal for the tick-borne flaviviruses using phylogenetic and biogeographic analyses[5]

SUMMARY

Tick-borne flaviviruses (TBF) are widely dispersed across Africa, Europe, Asia, Oceania and North America, and some present a significant threat to human health. Seminal studies on Tick-borne encephalitis viruses (TBEV), based on partial envelope gene sequences predicted a westward clinal pattern of evolution and dispersal across northern Eurasia, terminating in the British Isles. This hypothesis was tested using all available full-length ORF TBF sequences. Phylogenetic analysis was consistent with current reports. However, linear and non-linear regression analysis of genetic versus geographic distance combined with BEAST analysis identified two separate clines suggesting that TBEV spread both east and west from a central point. In addition, BEAST analysis suggested that TBF emerged and dispersed over at least 16,000 years, significantly earlier than previously predicted. Thus climatic and ecological changes may have played a greater role in TBF dispersal than humans.

INTRODUCTION

Flaviviruses are small, enveloped viruses containing a single stranded positive sense RNA genome of approximately 11kb [237]. Based on phylogenetic analysis combined with virus, vector, and host relationships the genus *Flavivirus* was originally divided into four groups comprising two mosquito-associated groups (either *Aedes* spp. or *Culex* spp.), one tick-associated group (*Ixodes* spp. and *Ornithodorus* spp.) and one group for which there is no-known vector [238,239]. Recently, many insect-specific viruses that do not appear to infect vertebrates have been isolated and form a distinct group from the four previously proposed groups [240,241], although some sit within the mosquito-borne groups [242,243].

Tick-borne flaviviruses (TBF) have been sub-divided into mammalian and seabird-associated groups based on their specificity for vertebrate hosts and their associated ticks. Tick-borne flaviviruses known to infect humans include tick-borne encephalitis virus (TBEV), Omsk hemorrhagic fever virus (OHFV), Langat virus, Alkhurma hemorrhagic fever virus (AHFV), Kyasanur forest disease virus (KFDV), and Powassan virus (POWV). TBEV has the largest impact on human health with around 12,000 human cases per year and a case fatality rate ranging from 0.5-20% depending on location [244,245]. POWV is a rare but increasing public health risk in the United States, with around 30-40 clinical cases documented since its identification. This virus is also widespread in the Primorsky region of Russia and although infection with POWV is rarely fatal, over 50% of cases result in permanent neurologic sequelae [246].

TBEV is maintained in a cycle involving ixodid ticks and rodents. Ticks may become infected while feeding on a viremic vertebrate host or when co-feeding with

infected ticks [247]. After acquiring TBEV, a proportion of ticks remain persistently infected for the remainder of their life cycle. The virus is transmitted to subsequent vertebrate and invertebrate hosts via viremic and/or non-viremic transmission and also transovarially to tick progeny [248]. Mathematical modeling of different transmission routes suggests that non-viremic transmission (tick to co-feeding tick) supported by transovarial transmission is sufficient to explain the observed prevalence and persistence of TBEV in natural foci; viremic transmission is of less importance due to the short period of viremia in rodent hosts [249]. Large animals such as deer do not play a role in maintaining or amplifying virus, but are very important as hosts for adult ticks [246]. TBEV is transmitted to humans via the bite of infected ticks, although transmission through unpasteurized milk is also documented [250]. In the case of other tick-borne flaviviruses, human infection through contact with infected animals is reported for Omsk hemorrhagic fever virus and Alkhurma hemorrhagic fever virus [251,252]; no natural human cases have been reported for Langat virus or Kadam virus [253].

POWV was isolated in 1958 from a fatal case of encephalitis in Powassan, Ontario [254]. Molecular epizootiologic studies have shown that it exists in nature as two distinct lineages [255]. Lineage I cycles between *Ixodes cookei* ticks and small or medium sized mammals such as groundhogs (*Marmota monax*) while lineage II cycles in *Ixodes scapularis* ticks and *Peromyscus leucopus* mice [256,257]. Lineage II has been named deer-tick virus (DTV), and shows distinct geographical clustering in New England and the mid-west [257]. POWV has also been isolated in the Primorsky region of Russia; the high level of similarity between Russian and North American isolates and their limited genetic variability resulted in estimates that POWV was introduced into Russia from North America during the past 100 years [258].

Based on differences in the nucleotide and amino acid sequence of the envelope (E) protein, three basic subtypes of TBEV have been delineated: TBEV-Europe (western), TBEV-Siberia, and TBEV-far east (FE-TBEV) [149,259,260]. Previously, Zanotto and colleagues had shown that the rate of non-synonymous nucleotide substitutions in the E-protein gene had a linear correlation with distance in kilometers from an arbitrarily defined focus, suggesting a continuous geographical cline of evolution from far-eastern Eurasia towards Europe [149]. The present phylogenetic study sought to test the clinal hypothesis for the evolution and dispersal of the TBF using all available full-length ORF TBF sequences to provide more comprehensive data and to assess whether or not the previous hypothesis of [149] was robust. Understanding these evolutionary and biogeographic characteristics is important in identifying the determinants of epidemiology and pathogenicity of these viruses. Such studies can also contribute to taxonomic decisions and predictions of their future impact on human health.

METHODS

Viral strains and genome sequences

Full-length genome sequences of tick-borne flaviviruses were downloaded from the United States National Center for Biotechnology Information (NCBI). The resulting list of tick-borne flavivirus (TBF) sequences were culled based on the availability of reasonable data on the date and locality of isolation for each strain. Because of the wide geographical distribution represented in the resulting data set, localities were considered adequate if they were specific to a region (e.g., Primorsky region, Russia). In some instances strains are named after readily identifiable cities; these were used as a location when no other source was available. Tyuleniy virus and Meaban virus were chosen as

outgroup strains based on published phylogenies [245]. Table 1 shows the strains of TBF and the related location data used in this study.

Phylogenetic Analysis

Sequence alignment

Open reading frames for each sequence were identified using MacVector (MacVector Inc., Cary, NC); this information was used to trim sequences to ORFs where necessary to ensure proper translation. The resulting sequences were imported into SeaView [261] and aligned as amino acids using the MUSCLE algorithm [262]. Gap-only sites were deleted, and the resulting alignment trimmed to eliminate UTRs using the sites tool.

Neighbor-joining analysis

A neighbor-joining [263] analysis was conducted using PAUP (Sinauer Associates, Inc. Sunderland, MA) with the following parameters: 1000 bootstrap replicates, randomly broken ties, general time-reversible distance measure with gamma distribution shape parameter of 0.5. The output tree was a bootstrap 50% majority-rule consensus tree.

Maximum likelihood analysis

Model Test [264] was used to estimate the best-fit evolutionary model for the sequence alignment. This model was then entered into PAUP and a maximum-likelihood tree was generated using the Bioportal at the University of Oslo [265]. Bootstrap support was calculated in Garli using the same parameters again using the Bioportal for computational analysis.

Kishino-Hasegawa Test

The test between the NJ tree and the Maximum Likelihood tree was implemented in PAUP* using the default settings in the program.

Beast analysis

Bayesian Markov Chain Monte Carlo (MCMC) as implemented in the BEAST package was used to estimate the time to the most recent common ancestor (tMRCA) for the Tick-borne flaviviruses. Strains were selected on the basis of having a year of collection associated with the sequence. BEAST analysis was performed using a relaxed clock model (uncorrelated lognormal) and the SRD06 nucleotide substitution model [266]. All analyses were run in duplicate to determine convergence on the Bioportal at the University of Oslo. The Bayesian skyline coalescent tree prior was determined to produce the most robust results. The MCMC chain was run for 80 million iterations and convergence was assessed using the TRACER program. A maximum clade credibility tree was produced using Tree Annotator (BEAST package) with the initial 10% of trees removed as burn-in.

Phylogeographical Analysis

Synonymous and non-synonymous substitutions per synonymous or non-synonymous site were measured using MEGA5 [267]. This pair-wise analysis was conducted using the Kumar model (Nei and Kumar 2000). Pair-wise comparisons between POWV LB (NC_003687), OHFV Guriev (AB507800), or TBEV Sofjin-HO (AB062064) and all other sequences excluding outgroup strains were made and plotted versus the distance in kilometers between the point of isolation of the two viruses. These distances were calculated using Google Earth (<http://www.google.com/earth/index.html>). An assumption was made that distances

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should be measured across North America and Russia rather than the shortest path between isolation locations because the shortest path often led across the Arctic, an unlikely route of dispersal. To accommodate this, a basic path between Ontario, Canada and Primorsky Krai, Russia was constructed and all other distances (except those isolates in North America) were computed using this basic pathway. The TBEV Sofjin-HO data-set was divided into 'TBEV-only' and 'TBEV-FE plus TBF' and re-graphed to explore differences in dispersal. Linear and non-linear regression was implemented in GraphPad Prizm (GraphPad Software, Inc. La Jolla, CA).

RESULTS/DISCUSSION

The age of the tick borne flaviviruses and climate considerations

In order to understand the temporal constraints on the origin and dispersal of the tick-borne flaviviruses, BEAST was used to estimate the time to most recent common ancestor (tMRCA) of each lineage. The resulting tree is shown in figure 6.1. The estimated time before the present for the basal node in this analysis (28600 years 95%HPD=16100-44900) is surprising and suggests the relationship between ticks and tick-borne flaviviruses has ancient roots. To my knowledge, this is the first time that full-length ORF sequences have been used to date more ancient lineages in the TBF tree. Estimates of more recent events such as the tMRCA for KFDV/AHFV and the TBEV group are supported by previous work [268,269] suggesting these results are consistent with presently emerging temporal models of TBF evolution. Given the estimated length of time since the origin of the mammalian TBF group (16000-45000 years ago), the

climatic changes that have occurred over this period should be considered as potential factors in the spread and distribution of these viruses. In the Northern Hemisphere, the last glacial maximum was approximately 20,000 years (20 kyrs) ago. After a rapid warming phase (termination), significant oscillations in global climate occurred between 18 and 11.5 kyrs ago. Significant discharges of ice and melt water into the Northern Atlantic Ocean caused two significant cold epochs (Heinrich stadial 1 from 18-15.5 kyr and the Younger Dryas from 13-11.5 kyr ago) punctuated by a period of warmer temperatures (Bølling-Allerød from 15.5-13 kyr) [270]. During this period of time, the Karshi Virus (KSIV)/Royal Farm Virus (RFV), Gadgets Gully Virus (GGYV), and POWV lineages were predicted to have diverged from the remaining TBF. Present climatic conditions were attained 11 kyr ago at the beginning of the Holocene, although some climatic fluctuations have occurred since that time. Ice-core data suggest a widespread cooling event 8.2 kyr ago, associated with dry conditions across the Middle East and Asia [271] that is reflected in changes in pollen assemblages in sediments collected from lake Baikal [272]. From the last glacial maximum to roughly 7 kyr ago, sea level rose about 140m. Sea level proxies such as sedimentation rate and organic and foraminiferal carbon isotopes from cores collected in Singapore suggest sea level rise was relatively constant (~1.8cm/yr) with a short cessation 7.8-7.4 kyr ago [273], while coral-reef proxies in the Caribbean suggest there may have been some periods of rapid sea-level increase (6.5-13.5m over a century or two) at 14.2, 11.5, and 7.6 kyr ago [274]. Under either scenario, the rise in sea level separated Siberia and Alaska 12.7-12.9 kyr ago (11,000 ^{14}C years ago calibrated using [275]) [276], and Britain from mainland Europe roughly 8-6.5 kyr ago [277].

These climatic conditions likely limited northern dispersal of early lineages of mammalian TBF such as KSIV/RFV, GGYV, and POWV into areas free from glacial ice. Interestingly, the location of isolation of AHFV/KFDV and Langat virus are still in southern Asia despite the predicted divergence of these lineages (roughly 10.2 kyr and 7.6 kyr ago, respectively) well after modern climatic conditions were reached. However, this may be due to an incomplete understanding of the ranges of these viral lineages.

Phylogenetic analysis

Phylogenetic trees produced by neighbor-joining, maximum likelihood, and Bayesian methods shared similar topology and were consistent with previous reports [278]. The maximum likelihood and neighbor-joining trees showed equivalent likelihoods as defined using the Kishino-Hasegawa test (K-H test, p<0.0001). In the interest of space, only the BEAST tree is shown (Fig. 6.1). As expected, the POWV lineage showed a deep divergence from the TBF, branching between the GGYV and KFDV lineages with 98% bootstrap support. The POWV lineage then diverged into POWV and DTV groups [255]. The POWV group contained lineages from the United States (POWV64_7062), Canada (POWVLB), and Russia (POWV Nadezdinsk, Partizansk, and Spassk9). Based on tree topology, it appears the United States isolate represents the earliest lineage, followed by the Canadian isolate, while the Russian isolates represent the most recent lineage. These events have 100% bootstrap support in the maximum likelihood tree (Supplemental file 1). This topology suggests the Russian isolates represent a recent introduction into Russia from Canada as has been previously suggested [258].

The TBEV lineage showed a deep split between eastern and western TBEV. The eastern group contained the Siberian/Baltic lineages and FE-TBEV, while the western group contained the sheep encephalitic viruses and W-TBEV. Within the eastern TBEV group, the Siberian/Baltic lineage was the first to diverge, followed by isolates from Irkutsk, and finally FE-TBEV. Within the western TBEV group, the Greek Goat Encephalitis Virus (GGEV)/Turkish Sheep Encephalitis Virus (TSEV) lineage was the first to diverge, followed by the Spanish Sheep Encephalitis Virus (SSEV)/Louping Ill Virus (LIV) lineage, and lastly W-TBEV. All of these nodes had 100% bootstrap support in the maximum likelihood tree, and suggest a well-demarcated split between eastern and western TBEV groups. This has some implications for the clinal dispersal postulated for the TBEV, as discussed below.

Geographic versus genetic distance

Plotting genetic versus geographic distance from POWV LB to all sequences downstream of POWV showed rapid saturation of genetic distance. From the standpoint of POWV LB, all downstream sequences were roughly genetically equidistant from POWV LB, making this comparison uninformative as a measure of TBF dispersal even though non-linear regression gives a high R^2 value (Figure 6.2A). A similar comparison using OHFV Guriev showed rapid saturation of genetic distance for all members of the TBEV group. However, different genetic distances were found for members of the TBF; linear regression of these plots showed a moderate correlation between genetic and geographic distance with a non-significant slope (Figure 6.2B, C). Thus I conclude that outside the TBEV group, little information remains to allow speculation on TBF dispersal

using this analysis method. In keeping with the work of Zanotto et al [149], I also plotted

genetic and geographic distance from TBEV Sofjin-HO which occupied the most ancient

lineage of the FE-TBEV viruses (Figure 6.2D). This graph suggested that a correlation

might exist between genetic and geographic distance for the TBEV group and the TBF

group. These two groupings (TBEV and 'TBF plus FE-TBEV') were plotted separately

and the correlation assessed with linear regression (Figure 6.2E, F). For linear regression

analysis, the Russian POWV isolates were removed from the TBF plus FE-TBEV group

since they may represent recently introduced viruses. The slopes of both lines were

significantly greater than zero and R^2 values were 0.9118 and 0.9839 for TBEV and 'TBF

plus FE-TBEV' respectively. This suggests that genetic distance correlated with

geographic distance for the 'TBF plus FE-TBEV' group as well as the TBEV group.

However, the correlation for the TBEV group suggests a route of dispersal from far

eastern Russia towards Europe whereas the 'TBF plus FE-TBEV' analysis does not.

POWV

The origin and dispersal of GGYV and POWV is controversial because their

present distribution is far from any ancestral or descendant lineages. The phylogenetic

tree strongly suggested these viruses originated in the Old World in southern Asia.

GGYV was isolated from *Ixodes uriae,* a seabird-associated tick, on Gadgets Gully Island

in the southern ocean [279], and therefore may have been widely disseminated by

migratory seabirds. Limited sampling and the mobility of its vertebrate hosts likely

explain the apparent anomaly of the location of isolation of GGYV far from Asia. The

BEAST analysis (Fig. 6.1) predicted the lineage that gave rise to POWV/DTV split from

the TBF 12.3 (95% HPD=7 -19) kyr ago. While this is only an estimate, it is consistent with the possibility that this divergence event may have been due to movement across the Bering land bridge leading to POWV and DTV lineages circulating in the Americas. Reconstruction of climatic conditions in Beringia at this time suggests it was not colder than the present era, and may have even encompassed the postglacial thermal maximum associated with a transition to a *Betula*-dominated tundra [280,281]. It is also interesting to note that this time period is roughly coincident with the second wave of human migrations into North America [282], providing additional evidence that climatic conditions were permissive for the dispersal of POWV. By 11 kyr ago, the Bering land bridge was flooded by the Bering Sea, isolating the ancestral POWV lineage from Old World TBF and limiting any further dispersal to the Americas. Perhaps the greatest detractor from this hypothesis is the lack of documented descendant lineages in North America. It is possible that with further sampling other viruses belonging to this lineage will be identified.   Significant ecological disturbances such as deforestation and unregulated hunting of deer and bison occurred in North America after European colonization. These practices could have caused a drastic reduction in tick populations leading to heavily localized distributions and potential extinction of lineages related to POWV/DTV. At a minimum, these changes would increase the difficulty of obtaining a wider sampling of potential POWV/DTV relatives.

Other scenarios are possible. The relatively close association between GGYV and POWV raises the possibility that early ancestors of POWV were also widely disseminated by seabirds. Lending some support to this speculation are data supporting the dispersal of *Borrelia* spp. by seabirds and associated *Ixodes uriae* with evidence of admixture with terrestrial (mammal-*Ixodes ricinus*) cycles [283]. However, if POWV

were moving freely between the Americas and Siberia one would expect several Russian lineages in the tree. In addition, if TBF movement by seabirds is common then the apparent absence of TBEV in North America is difficult to explain. Another possibility is a very recent (100-300 years ago) introduction of POWV into North America, coincident with significant Russian immigration to Canada and the United States. This is very difficult to reconcile with the present estimates and those of others [257] of the divergence of POWV and DTV. While it is possible that this divergence occurred in Russia, it is more likely due to a New World adaptation of POWV from *Ixodes cookei*/*Ixodes marxii* cycles to *Ixodes scapularis* cycles, or *vice versa* since it is not clear which lineage is ancestral. Any recent introduction scenario also has to explain the lack of TBEV in North America.

The presence of POWV in the Primorsky region of Russia is difficult to explain. Based on the close relationship between the Russian POWV isolates and the original Canadian isolate and the single Russian lineage in the tree the most parsimonious explanation is a single introduction of POWV into Russia. Previous papers have speculated that importing American mink into Russia was responsible for this introduction [258]. Given the Russian presence in Alaska until 1867 it is likely this introduction occurred during this time.

The DTV lineage has diverged to produce two sub-groups, one in Wisconsin and the other in Connecticut/Massachusetts [257]. While the significance of these foci is uncertain, it is interesting to note that the distribution of Lyme disease follows the same profile [284]. The phylogeography of *Borrelia burgdorferi* suggests a wide prehistoric distribution that was reduced to localized refugia in the 19th and early 20th century by deforestation and unregulated deer hunting [285]. The earliest records of *I. scapularis* in

North America come from the 1920s for Naushon Island, Massachusetts [286] and the 1960s for Wisconsin [287]; these areas may represent refugial foci from which deer, ticks, and tick-borne disease are expanding into the United States. This may explain the DTV foci and suggests that the virus will spread beyond these localized areas.

It is important to note this model of POWV/DTV dispersal is based on the presented estimates of the tMRCAs in this lineage and the present tree topology. It is possible that with further sampling other viruses belonging to this lineage would be identified, and these would enable a more accurate analysis of the origin of these viruses. However, given that DTV and POWV are the only two viruses identified in this lineage, a single introduction into the Americas is the most parsimonious explanation given the topology of the trees generated from the samples currently available. If POWV were being regularly transported between Russia and North America one would expect the tree topology to reflect this rather than the distinct Russian and American POWV clades observed. Thus it was postulated that either human involvement or significant ecological change (such as the opening or closing of the Bering Land Bridge) are the primary determinants of dispersal for this lineage of TBF.

TBEV

Northern expansion of the TBF began at least 7 kyr ago, based on the predicted divergence of Langat virus, the last Old World TBF with a location of isolation in southern Asia. According to the present estimates, OHFV, a central Russian virus, diverged around 4.5 kyr ago. Thus it seems reasonable to assert that between these

divergence events a significant northward expansion must have occurred. However, northern expansion before this time frame cannot be ruled out. According to the present analysis, the most recent common ancestor of the TBEV group was present 3.1 (95%HPD=1.8-4.9) kyr ago. This initial divergence separated the eastern from western TBEV groups. It was expected that the tMRCA of nodes within the eastern and western TBEV groups would support the clinal hypothesis of dispersal from far eastern Russia into Europe. However, the divergence of the Siberian/Baltic lineage from the eastern TBEV group and the GGEV/TSEV lineage from the western TBEV group appear almost simultaneously (2.4 95%HPD=1.3-3.7 and 2.4 95%HPD=1.3-3.8 kyr ago) for eastern and western respectively). After the divergence of the Siberian/Baltic lineages from the eastern TBEV group, viruses isolated in Irkutsk were predicted to diverge at 1.8 (95%HPD=1.4-3.8) and 1.4 (95%HPD=1-2.8) kyr ago for TBEV strains 886_84 and 178_79 respectively. After the divergence of the GGEV/TSEV lineage from the western TBEV group, the present analysis predicted the most recent common ancestor of the SSEV/LIV and W-TBEV groups at 1.8 (95%HPD=1-2.8) kyr ago. These temporal considerations do not support a model of TBEV dispersal from eastern Eurasia moving westward, but rather a dispersal beginning in central Russia moving eastwards for the eastern-TBEV group and westwards for the western-TBEV group. The divergence of the sheep encephalitic lineages may have been driven by contact between the forest-associated western TBEV and new hosts such as sheep and goats brought near the forest to graze. LIV was likely introduced into the United Kingdom via transportation of animals to Ireland [288], while W-TBEV has been limited to mainland Europe due to its lack of association with livestock and the formation of the English Channel before the arrival of these lineages in Europe. These results support clinal aspects of the evolution

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of TBEV, but do suggest a modification of the original hypothesis to include a central Eurasian divergence of eastern and western TBEV lineages (Fig. 6.3). This view is attractive because it explains the correlation between genetic and geographic distance while remaining consistent with the phylogenetic tree and predicted times of divergence. Previous calculations postulated the divergence of Siberian and FE-TBEV 1.7-2 kyr ago [289], the arrival of LIV in the British Isles less than 800 years ago [288], and the divergence of TBEV-Oshima and Russian FE-TBEV 260-430 years ago [289]. These numbers are in general support of the present estimates of 2424 (95%HPD=1342-3749), 1020 (95%HPD=530-1628), and 294 (95%HPD=170-456) years ago for these three events.

Conclusion

The topology of the presented trees was consistent with previous reports [278]. However, the inclusion of the tMRCA analysis suggests that TBF comprise a more ancient lineage than expected. This indicates that TBF may evolve more slowly in comparison to mosquito-borne flaviviruses than previous estimates [290]. This is likely due to differences in generation times and duration of persistent vector infection between ticks and mosquitoes, and possibly the impact of humans as amplifying hosts for some mosquito-borne flaviviruses. Early TBF may have disseminated widely in southern Asia because of harsh climatic conditions in the north. Estimates of the tMRCA of the POWV/DTV lineage suggests ancestral lineages may have dispersed across the Bering Land Bridge before its closure at the beginning of the Holocene. Based on the present estimates, Old World TBF lineages show clear northward movement between 7 and 5 kyr

ago, culminating in a central Russian divergence of eastern and western TBEV groups. Dispersal within the TBEV group then progressed eastwards for the eastern TBEV group and westwards for the western TBEV group, producing the clinal pattern observed here and previously [149] but also refining its scope.

Tables.

Table 6.1: Viral strains used in this analysis.

Strain	Geographical Origin	Year of Isolation	Source of Isolate	Accession No.
Neudoerfl	Neudoerfl, Austria	1971	Ixodes ricinus	U27495.1
263	Temelin, czech rep	1987	I. ricinus	U27491.1
Salem	Bodensee, germany	2006	Macaca sylvanus	FJ572210.1
AS33	Amberg, Germany	2005	I. ricinus	GQ266392.1
Hypr	Brno, Czech Rep	1953	Human blood	U39292.1
Toro-2003	Toro, Sweden	2003	I. ricinus	DQ401140.2
K23	Karlsruhe, Germany	1975	I. ricinus	AM600965.1
886-84	Irkutsk, Russia	1984	Clethrionomys rufocanus	EF469662.1
Kolarovo-2008	Kolarovo, Russia	2008	I. pavlovskyi	FJ968751.1
Primorye-332	Mirnyy, Nadezhdinsky, Primorsky	1991	Human blood	AY169390.3
Primorye-212	Vladivostok, Primorsky	1991	Human blood	EU816450.1
Primoye-253	Solovey-Klyuch, Nadezhdinsky, Primorsky	1991	Human blood	EU816451.1
Primoye-270	Mirnyy, Nadezhdinsky, Primorsky	1991	Human blood	EU816452
Kavalerovo	Kavalerovo, russia	1985	human	FJ402885.1
Dalnegorsk	Dalnegorsk, Russia	1973	brain	FJ402886.1
Oshima 5-10	Kamiiso, Japan	1995	dog blood	AB062063.2
178-79	Irkutsk, Russia	1979	I. persulcatus	EF469661.1
Zausaev	Tomsk, Russia	1985	human brain	AF527415.1
Vasilchenko	Novosibirsk, Russia	1969		L40361.3
EK-328	Estonia	1972	I. persulcatus	DQ486861.1
205	Khabarovsk, Russia	1973	unknown	DQ989336.1
Glubinnoe/2004	Glubinnoe, Primorsky, Russia	2004	human brain	DQ862460.1
Svetlogorie	Svetlogor'e, Russia	2008	human brain	GU121642.1
Sofjin-HO	Khabarovsk, Russia	1937	human brain	AB062064.1
TSEV	Gebze area, northwestern Turkey	1960		DQ235151
LI 369/T2	Ayrshire, Scotland, UK	1963	I. ricinus	NC_001809.1
SSEV	Spain (basque region)	1987	sheep	DQ235152
GGEV	Vergina, Greece	1969	Goat brain	DQ235153
KFDV/KFD P 9605	Kyasanur Forest, Shimoga District, Karnataka, India	1957	human blood	AY323490
AHFV	Jeddah, Saudi Arabia	1995	human blood	NC_004355
OHFV/guriev	Omsk region, Russia	1947	human	AB507800
POWV/64-7062	New York, USA	1964	ticks on marmota sp.	HM440563
POWV/DTVWiC08	Spooner, WI, USA	2008	I. scapularis	HM440562
POWV/DTVWiB08	Spooner, WI, USA	2008	I. scapularis	HM440561
POWV/DTVWiA08	Spooner, WI, USA	2008	I. scapularis	HM440560
POWV/DTVMa96	Nantucket, MA, USA	1996	I. scapularis	HM440559
POWV/DTVWi99	Chippewa falls, WI, USA	1999	I. scapularis	HM440558
POWV/Nadezdinsk-1991	Nadezdinsk region, Russia	1991	human blood	EU670438
POWV/partizansk2006	Partizansk region, Russia	2006	human blood	EU543649
POWV/LB	Powassan, Ontario, Canada	1958	human brain	NC_003687
POWV/Spassk-9	Spassk region, Russia	1975	Dermacentor silvarum	EU770575
DTV/ctb30	Conneticut, USA	1995	I. scapularis	AF311056
KSIV Karshi Virus	Beshkent, Uzbekistan	1972	Ornithodorus papillipes	NC_006947
Meaban Virus	Meaban island (French island)	1981	O. maritimus	DQ235144
Tyuleniy Virus	Three Arch Rocks National Wildlife Refuge, OR, USA	1971	I. uriae	DQ235148
Royal Farm Virus	Kabul City, Afghanistan	1968	Argas hermanni	DQ235149
Langat TP21	Kuala Lumpur, Malaysia	1956	I. granulatus	NC_003690
Kadam Virus	Kadam mountain, Nakapiripirit, Karamoja, Uganda	1967	Rhipicephalus pravus	DQ235146
Gadgets Gully Virus	Hurd Pt Macquarie Island	1975	Ix. Uriae	DQ235145
Saumarez Reef Virus	Australia	1977		DQ235150
Primorye-18	Russia	1997	Human blood	GQ228395.1
Primorye-86	Russia	1986	Human brain	EU816455.2
Primorye-90	Russia	1990	Human brain	FJ997899.1
Primorye-2239	Russia	1985	I. persulcatus	HM859895.1
Primorye-633	Russia	1978	Mouse	HM859894.1
Primirye-89	Russia	1987	Human brain	FJ906622.1
Primorye-94	Russia	1994	Human blood	EU816454.1
Primorye-69	Russia	2000	Human blood	EU816453.1

Figures
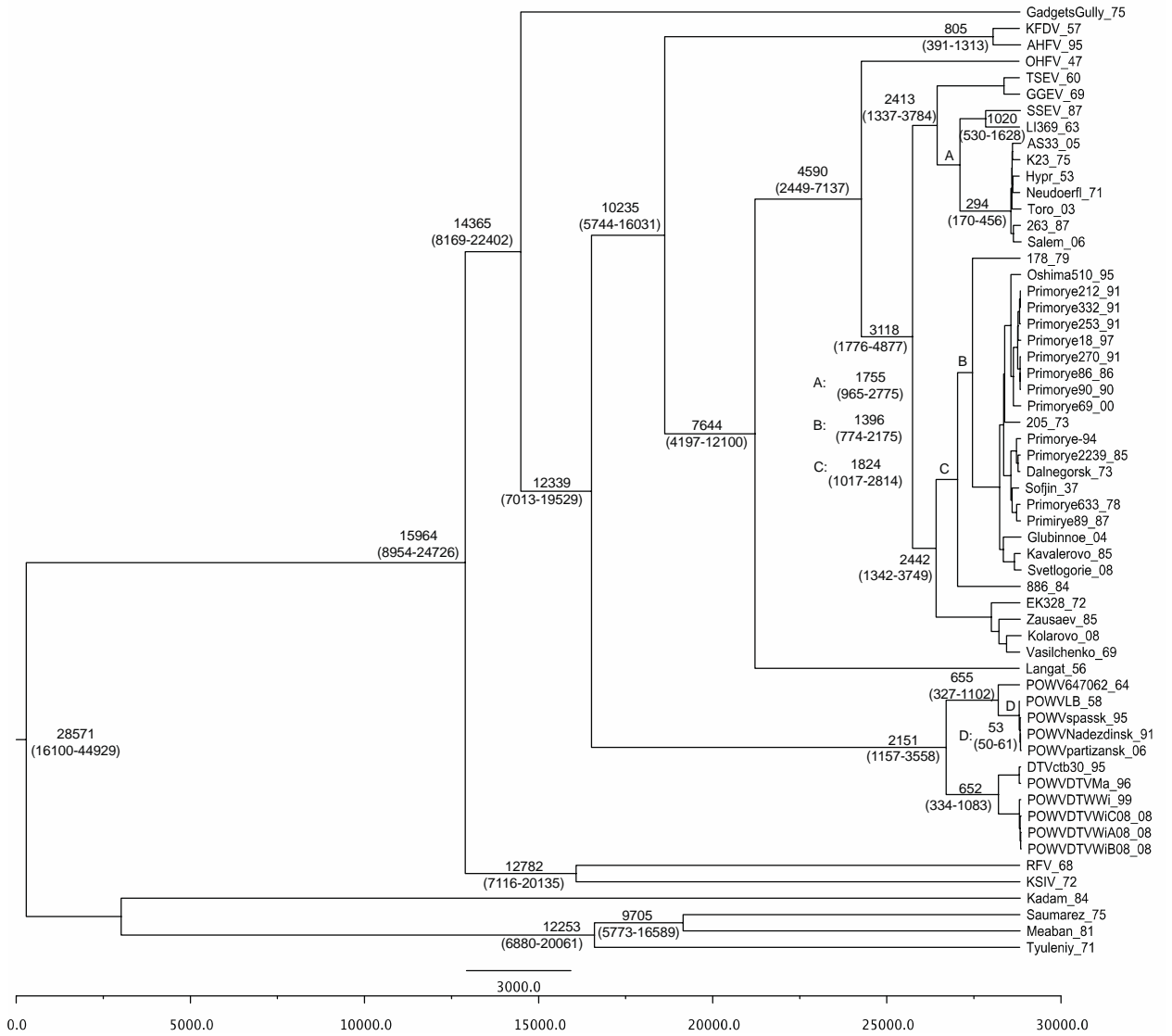
Figure 6.1. Maximum clade credibility tree of the tick-borne flaviviruses.

The node labels indicate the tMRCA with the 95% HPD in brackets. All nodes labeled with the tMRCA had a posterior probability of 1 (data not shown). Strains are labeled as strain name_date of collection.
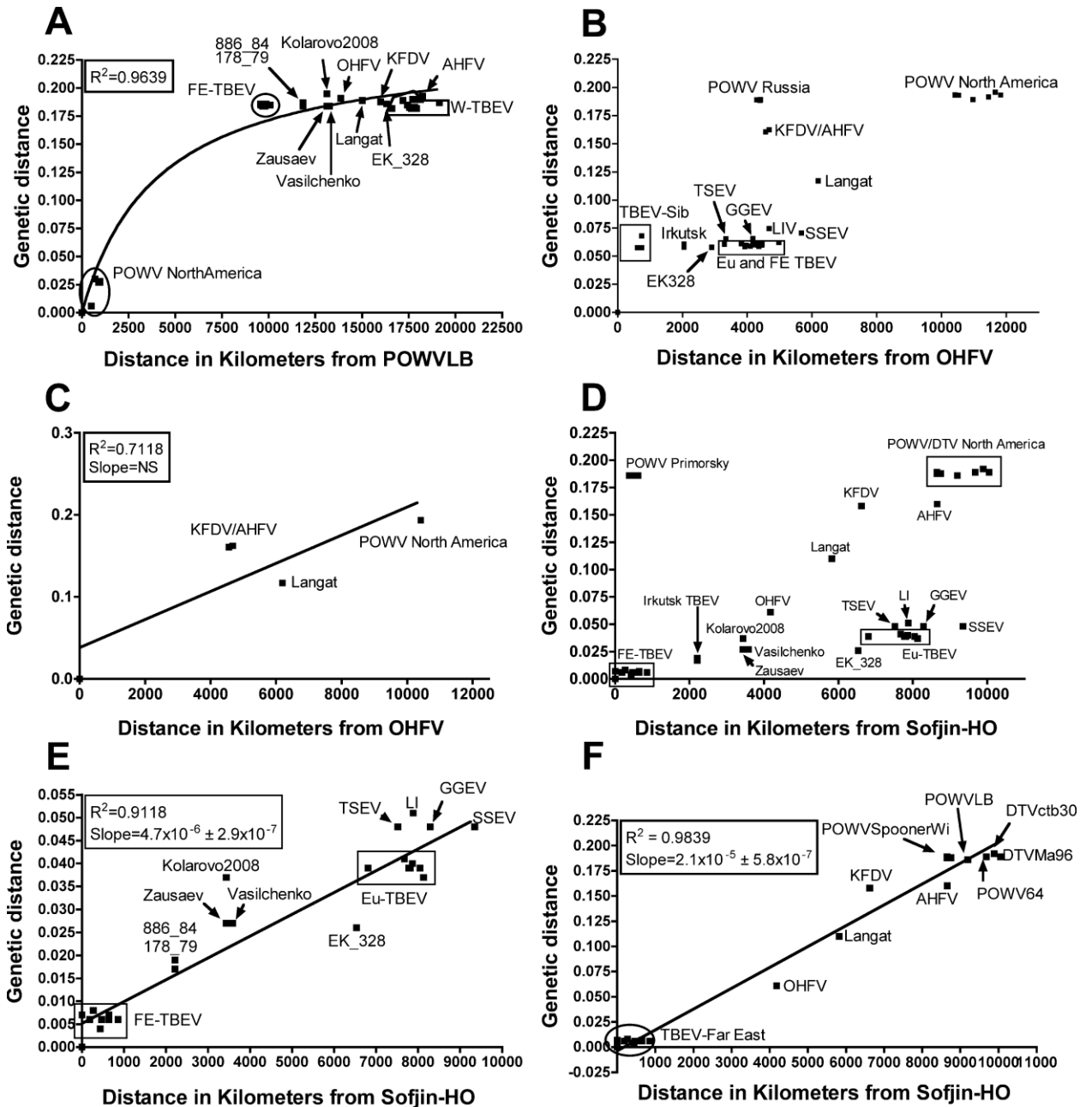
Figure 6.2. **Genetic versus geographic distance between members of the TBF.**

Genetic versus geographic distance between viral isolates was plotted for POWV LB (A), OHFV Guriev (B, C), and TBEV Sofjin-HO (D, E, F). Linear and non-linear regression lines are shown where calculated. For regression, Russian POWV isolates were removed from relevant analysis (A, C, F).
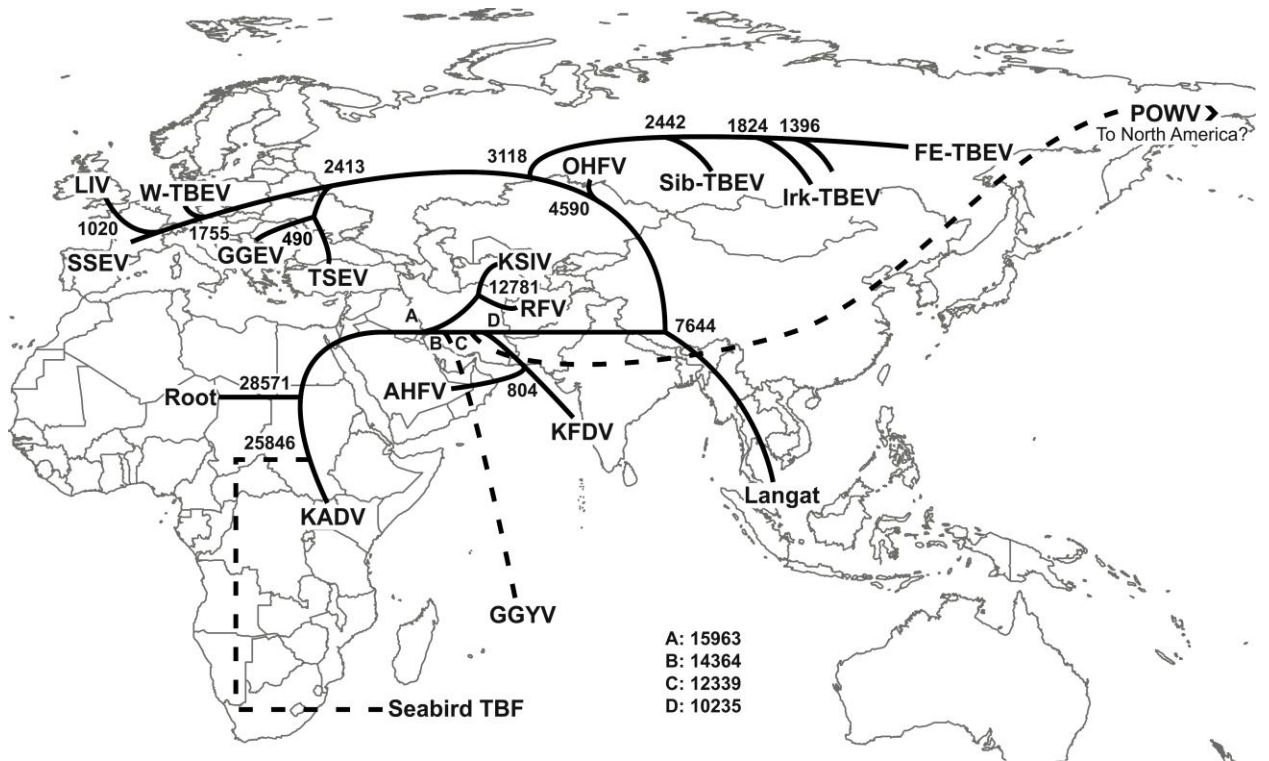
Figure 6.3. A possible model for TBF dispersal.

The maximum clade credibility tree was manually plotted onto geographic space showing the most parsimonious route of dispersal for these viruses based on all complete genomic sequences. Approximate tMRCA dates are shown at all major nodes in the tree.
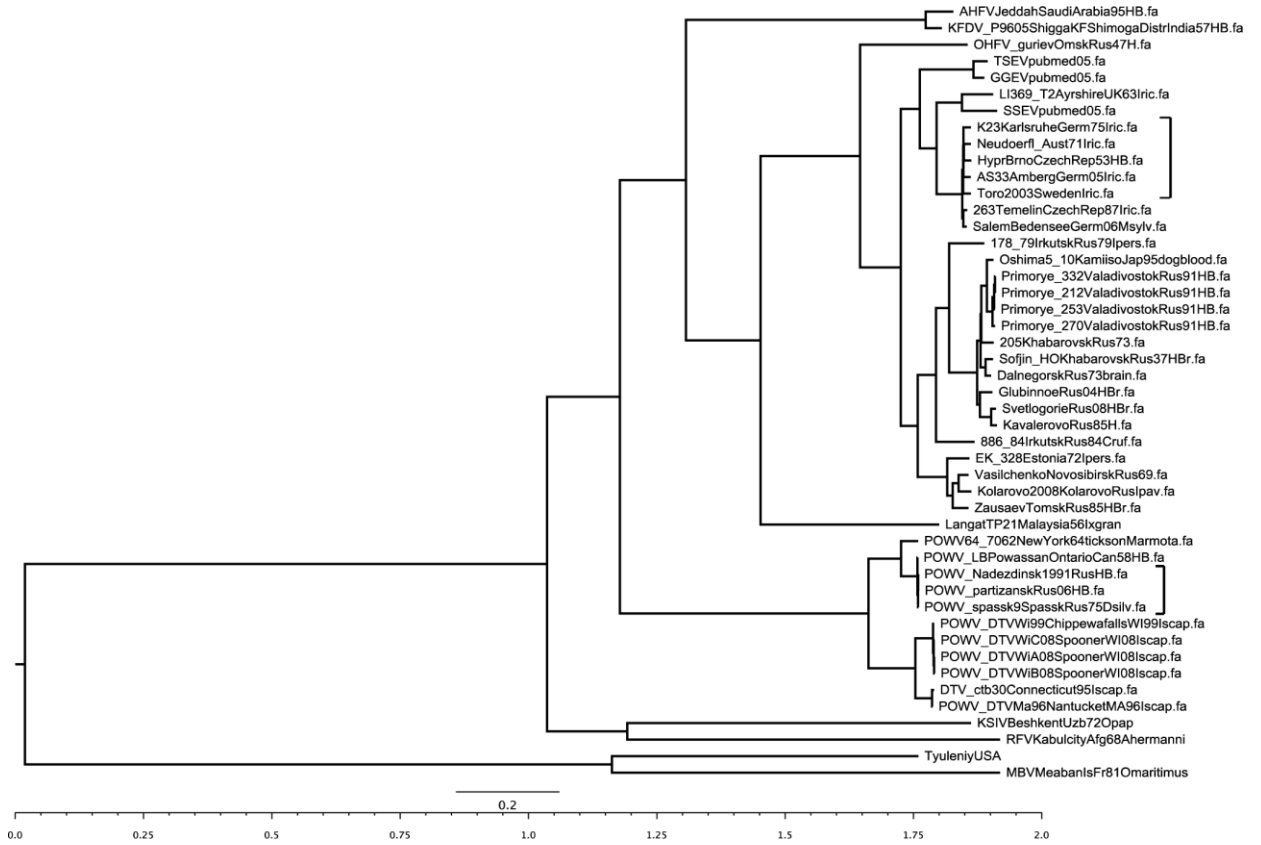
Supplemental file 6.1: Maximum likelihood tree for the TBF.

Black brackets denote areas where bootstrap support was less than 90%.

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Chapter 7: Conclusions

SUMMARY OF MAJOR RESULTS.

Ixodes scapularis

Early changes (1 hpi) at the bite site visible on histological analysis were mast cell degranulation and the mechanical injury caused by the hypostome. The gene expression analysis suggested that post-translational modifications, nitric-oxide mediated signaling, and negative regulation of myeloid cell differentiation were significant. At 3 hpi, minimal changes in the gene expression profile compared with 1 hpi were evident, but definite increases in immune cell recruitment were noted. By 6 hpi, upregulated clusters on gene ontology analysis revealed cytoskeletal changes, transcription, protein phosphatase, cation homeostasis, and antimicrobial responses. Downregulated clusters included G-protein signaling, ECM/adhesion, blood vessel development, transmembrane or secreted molecules, and cytoplasmic vesicle. Histology showed increasing neutrophil influx with other types of immune cells present. Twelve hours after attachment, upregulated clusters were related to cytoskeleton, keratin, DNA replication, epidermal development, inflammation, and cell cycle. Downregulated clusters included skeletal system development, regulation of transcription, and plasma membrane. These results suggest that active wound healing responses are present by 12 hpi and also a dynamic change in transcription-related gene transcripts between 6 hpi (upregulated) and 12 hpi (downregulated). This is supported by the PCR array study, where transcription-related

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genes were downregulated at early (48 hpi) time points. The limited nature of the PCR array study narrows the scope of hypothetical models. During the primary infestation, decreases in transcription, cell signaling, and T-cell activation were suggested. Tick feeding appeared to be sensed by C-type lectins such as *Clec7a*, that stimulated a pro-inflammatory, innate immune response dominated by *Il1b, Il6, Il10*, and chemokines specific for (although not limited to) neutrophils and monocytes. A proteolytic environment and innate effector functions such as prostaglandins were implied by the data. During secondary infestations, the same response was present, although the fold up-regulations were much higher. In addition, signaling molecules such as *Syk, Jak2*, and *MyD88* were upregulated, and a much broader range of cytokines including those associated with Th1, Th2, and T regulatory cell responses. Th17 responses were inhibited, while SOCS (suppressor of cytokine signaling) and T-regulatory cell responses were increased. These anti-inflammatory changes could be due to automatic negative feedback on the inflammatory response, or may be specifically potentiated by components of tick saliva. Thus the overall picture included degranulation of mast cells, migration of neutrophils and monocytes, C-type lectins, keratinization/wound healing responses, innate inflammation (*Il1b, Il6*, and *Il10*), and finally a mixed Th1/Th2 type inflammatory response during secondary infestations (Fig. 7.1).

Dermacentor andersoni

The microarray results were divided into the core response (those genes shared across all time points), early primary infestation (12-96 hpi), late primary infestation (120 hpi), and secondary infestation (120 hpi). The core response showed keratinization,

chemotaxis, C-type lectins, and innate effectors such as reactive oxygen species and anti-microbial peptides were the most conserved aspects of the host response over time. Early primary infestation was characterized by the down-regulation of molecules related to transcription, nucleus, and cytoskeleton, and the up-regulation of circadian rhythm genes. As in the *I. scapularis* data, this suggests the tick may inhibit gene transcriptional activities early in the feeding process. Late primary infestation showed clusters related to transcription, adhesion, blood vessel/embryonic development, cell growth, MAP kinase activity, and cell cycle as significant. These clusters had about equal numbers of up and downregulated genes, making it difficult to say if these responses are potentiated or inhibited. However, there was a marked shift toward up-regulation compared with early primary infestation, suggesting a trend toward activation. Considering these clusters as a whole suggests the possibility of an "EMT-like" phenotype of cells at the bite site. While a true EMT is not thought to occur during wound healing, some aspects of this change are re-capitulated, as potentially seen here. During the secondary infestation, the gene ontology clusters can be related to the immune response. In general, this appears to be a potent innate-like inflammatory response that is set in motion by the adaptive immune system. Acute phase molecules, coagulation factors, and complement were upregulated. Many sensing mechanisms, such as Toll-like receptors, Fc receptors, and C-type lectins were represented, along with signaling intermediates such as src-family kinases, *Jak, Syk*, and *MyD88*. The importance of chemotaxis was highlighted by the great increase in selectins, integrins, chemokines, and integrin ligands. The cytokine milieu was dominated by *Il6, Il1b, Il10, Il4, Tgfb*, and *Csf1*. In addition to these results, wound healing processes and lysosomal proteases were also present. The effector mechanisms

of the anti-tick response were antimicrobial proteins, reactive oxygen species, lipid mediators (prostaglandins, leukotrienes), histamine, coagulation, and complement. In summary, the core response to tick feeding was keratinization, C-type lectins, chemotaxis, and metal ion handling. This core was joined by decreases in transcription and cytoskeletal elements early in the primary infestation. Late primary infestation shows increases in transcriptional machinery and hints at an EMT-like phenomenon. Secondary infestation is characterized by an enormously more intense inflammatory response with a broadened range of immune effectors (Fig. 7.2).

A COMPARISON BETWEEN HOST RESPONSES TO DIFFERENT TICK SPECIES.

Unfortunately, comparing the host response to *I. scapularis* and *D. andersoni* nymphs based on these studies is not straightforward because of the different methods (PCR array or microarray) used in measuring gene expression at corresponding time points. However, microarray data was available for both studies at 12 hpi during primary infestations. The list of significantly modulated genes shared between these two arrays is shown in table 7.1. Interestingly, these shared responses represent genes related to keratinization, chemotaxis, metal ion handling, and C-type lectins, similar to the core response to *D. andersoni*. Thus these appear to be conserved host responses early in the primary infestation, and likely throughout the feeding process. Genes that were not shared were entered into DAVID to get a sense of the functional categories represented by genes unique to the host response to each tick. The top 10 clusters containing significant (p < 0.05) gene ontology terms are shown in table 7.2. Clusters relating to keratinization, DNA metabolism, ATP and nucleotide binding, and nuclear lumen (or

nucleoplasm) were shared between the responses to the two tick species. Clusters for DNA replication, hair cycle, cell cycle, immune response, positive regulation of cellular component organization, and pyrimidine metabolism were unique to *I. scapularis*. Clusters for non-membrane bound organelle, chromatin binding, ubiquitin-dependent protein catabolism, and regulation of transcription were unique to *D. andersoni*. Strikingly, the genes in *I. scapularis* clusters were almost entirely upregulated, while the only cluster with more upregulated genes than downregulated genes in *D. andersoni* was the keratinization cluster. Thus, while similar functional groupings are modulated, the host response to *I. scapularis* appears to be much more activated than that to *D. andersoni*.

In an attempt to compare secondary responses, significantly modulated genes at 96 hpi during a secondary infestation with *I. scapularis* and 120 hpi during a secondary exposure to *D. andersoni* were compared. This comparison suffers from the already mentioned caveat of the PCR array/microarray comparison. The significantly modulated genes shared between these responses are shown in table 7.3. Broadly speaking, these genes represent chemotaxis (chemokines, selectins, integrins, ICAM1), cytokines (*Il6, Il10, Il1b, Il4*), matrix metaloproteases, C-type lectins (including *Syk*), T-cells, Toll-like receptors, and cytokine signaling (*Jak2*, STATs). Thus, these particular responses are highly conserved in the host response to tick feeding during secondary infestations. Lists of unique genes were not analyzed because of the inherent differences in the number of genes tested between PCR and microarrays. Even this list of shared genes is undoubtedly smaller than reality due to the same limitations.

A small number of genes were validated by quantitative real-time PCR at the same time points for both tick species. A comparison of these genes is shown in figure 7.3. Interestingly, at 48hpi during primary infestations, no significant genes were measured for *D. andersoni*, while there were many significantly modulated genes for *I. scapularis*. While some significant modulation is seen in the *D. andersoni* data at 96 hpi, all the fold changes were under 10, while a number of fold changes in the *I. scapularis* response were over 100. This pattern changes during the secondary infestation, when similar fold changes are seen in the host response to both tick species. Perhaps the most dramatic difference between these later data sets is the strong (~100 fold) up-regulation of *Ifng* in the response to *I. scapularis*, while it is not even significantly modulated in the response to *D. andersoni*. This data suggests that the Th1/Th2 polarization of the host response to these two tick species is different. *Dermacentor andersoni* elicits primarily a Th2 response, while *I. scapularis* elicits a mixed response with both Th1 and Th2 cytokines. It has been speculated the Th2 response was a subversion of the host response away from a more protective Th1 response. However, mice develop resistance to *D. andersoni* (as measured by reduced engorgement weights and molting success) [102] but not to *I. scapularis* [101], suggesting that the more Th1-biased response to *I. scapularis* is less protective than the Th2 response to *D. andersoni*. In reality, I suspect that the Th1/Th2 paradigm is not helpful in terms of defining host resistance to ticks, but may well have significant impact on tick-borne pathogen transmission.

In addition to gene expression comparisons, direct histological comparison of *I. scapularis* and *D. andersoni* bite sites could be made at 12 and 48 hpi during a primary infestation (Fig. 7.4). There is a striking difference in the overall morphology of the tick

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bites between these two species. *Ixodes scapularis* uses a minimal amount of attachment cement, which is deposited deep in the feeding lesion, at the level of the dermis and below. In addition, *I. scapularis* penetrates deeply into the host, even passing through the dermis and auricular cartilage. *Dermacentor andersoni*, on the other hand, secretes a copious amount of attachment cement on top or in-between epidermal layers, and appears to embed the hypostome primarily in this cement rather than in the host skin. Indeed, the hypostome barely penetrates through the epidermis. Thus the mechanism of attachment is different between *I. scapularis* and *D. andersoni*. The much more invasive attachment of *I. scapularis* may explain the higher fold changes seen in the gene expression data. However, no qualitative difference is apparent in the number of inflammatory cells at the bite site.

TICK-BORNE FLAVIVIRUS EVOLUTION AND DISPERSAL.

In terms of the evolution and dispersal of the tick-borne flaviviruses, the primary limitation of that work is the relatively small number of full-length ORF sequences available for analysis. Of particular interest to concerns in the United States is the POWV lineage, which still presents difficult questions in terms of its ultimate origins and dispersal. The model presented in chapter 6 is the most parsimonious with the present data, but leaves many questions to be answered about the lack of addition POWV lineages in the USA. For these reasons, ecological studies aimed at discovering additional POWV isolates and possibly new viruses in this lineage would go a long way toward defining the origins and dispersal of these viruses. The recent discovery of a

potentially tick-borne virus in Missouri [291] suggests the idea of uncovering additional tick-borne viruses in the USA is not far fetched.

LIMITATIONS OF GENE EXPRESSION STUDIES.

The primary limitation of the gene expression profiling studies is the complex *in vivo* environment being measured. In particular, the skin is made up of a large number of different cell types, and the diversity of cell types only increases upon tick feeding. For this reason, it is impossible to say with certainty that any particular response is due to a single cell type. Also, tick feeding necessitates the formation of a wound and it is impossible to parse out the host responses specific to wound healing or tick feeding. However, this may be irrelevant in terms of characterizing the host response to tick feeding because the wound healing response is an integral part of it. In fact, the wound healing response may be the primary anti-tick response in the skin and may be precisely what the tick must inhibit to successfully feed.

FUTURE DIRECTIONS.

The host response to ticks.

These studies have suggested a number of host responses that are highly conserved between tick species. These include keratinization, migration of immune cells (particularly neutrophils and macrophages), metal ion handling, and C-type lectins. Using genetically manipulated mice that lack key mediators of these pathways, it may be

possible to assess their mechanistic importance in host resistance to ticks or tick-borne pathogen transmission. In general, mice do not develop strong resistance reactions to ticks. Even though mice have been shown to develop resistance to *D. andersoni*, this phenotype (reductions in fed weights and molting success) may be too vague to precisely define the immune correlates of protection. However, mice with prior exposure to ticks show significant protection from both tick-borne bacteria and viruses [23,24]. This phenotype is quite marked, and should allow characterization of host responses important for blocking pathogen transmission. This information could be vital for screening vaccine candidates for transmission blocking activity. In addition, similar studies might allow characterization of the influence of the host response on the vector capacity of different tick species.

Many open questions remain on the function of immune cells migrating into the bite site. The early appearance of neutrophils combined with the rapid kinetics of tick-borne viral transmission suggests a possible interaction between tick-borne viruses and neutrophils. Other pathogens such as Leishmania use neutrophils as a "Trojan horse" to gain access to DCs and macrophages [292]; this process may be enhanced by parasite-induced delay of apoptosis [293]. Thus, the interaction between tick saliva and neutrophils may be very important in tick-borne pathogen transmission. A simple experiment involving freshly isolated neutrophils in appropriate media with and without tick saliva could measure differences in life span (and potentially other end points such as cytokine profile) induced by salivary molecules. If significant changes are measured, the potential for C-type lectin, TLR, or other signaling pathway involvement could be elucidated. This experiment could be taken a step farther to include a tick-borne

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pathogen as well in case there is synergy between salivary and pathogen neutrophil modulation. It would be important to look for pathogen-infected neutrophils in skin sections from mice infested with infected ticks to be sure neutrophils are a target of *in vivo* infection. Finally, the role of neutrophils in facilitating/inhibiting infection could be assessed by using wild type or neutrophil-depleted mice and measuring the kinetics of infection and course of disease after challenge with an infestation of infected ticks.

Another key question is the function or activation status of neutrophils recruited into the bite site. While it is clear they are recruited in large numbers, it is not known what they do once they arrive. This could be assessed by staining neutrophils in tissue sections from sterile wounds in comparison to tick bites with neutrophil activation markers such as CD66b or myeloperoxidase. Another possibility would be to test the activation status of neutrophils *in vitro* after treatment with LPS with or without saliva. A much more complex approach might involve laser-capture micro-dissection of neutrophil-rich regions of a tick bite and a thorough proteomic analysis using 2-D gels and mass spectrometry. These methods could be applied to other cell types infiltrating the bite site as well. The data in this dissertation suggests that the cells recruited into the bite site are activated at the mRNA level. This can be seen from the upregulation of CD4 and IL-4 or of neutrophil and monocyte specific chemokines and IL-1β or IL-6. Tick salivary molecules, as discussed in the introduction, can modulate cytokine production and inactivate cytokines after release, suggesting tick feeding does modulate the activation status of immune cells. However, the experiments described in this dissertation do not have a "positive" control with which to compare the rate of cell activation without tick saliva, so no definitive conclusions can be made at this time.

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Further experiments looking at protein expression, protein function, and mRNA expression in saliva-exposed vs control activated cells might help answer some of these questions on the function of immune cells after recruitment.

Additional experiments to determine the kinetics of cell recruitment in the skin throughout the tick-feeding period are also important. This is because tick-borne pathogen transmission can occur very late (up to 72hrs for *Borrelia*). The recruitment kinetics could be measured using a time course infestation and staining for each cell type in tissue sections near the hypostome entry into the skin. Protein level detections using bioplex technology or even 2-D gels as mentioned above could also be helpful in defining the network of functional proteins present throughout the feeding process. The microarray and histopathology data suggested increased angiogenesis may be an important process later in the host response, so measuring the increase of endothelial markers could be revealing, especially since endothelial cells are targets of infection by tick-borne pathogens such as *Rickettsia spp* [294].

CONCLUDING REMARKS.

The process of epithelial wound healing has been divided into a number of phases that culminate in tissue repair and restoration of normal function. In a broad sense, these involve the formation of a platelet plug and blood clot, followed by the induction of an inflammatory response, the re-epithelialization and contraction of the wound site, and the remodeling phase [295]. These phases are not discrete and form an overlapping continuum throughout the healing process. Damage to local cells and the activation of platelets release chemoattractants that induce the inflammatory response, but also release

growth factors important for proliferation and re-epithelialization. Microarray analysis of the margins of full-thickness wounds in mice revealed results strikingly similar to those presented in this dissertation, including gene ontology clusters related to chemotaxis, defense response, keratinization, ion transport, and more. The top 50 upregulated genes showed extensive overlap with those reported for *D. andersoni*, while the top 50 downregulated genes showed little overlap [296]. These similarities indicate that the host response to tick feeding can be regarded, in general terms, as a wound healing response. This is in contrast to expression profiling between human patients with atopic dermatitis and normal human skin. Analysis of whole skin showed the up-regulation of a small number of genes that included *Krt6* and *Krt16*, S100 genes, and molecules related to the cornified envelope [297]. Analyses comparing *ex vivo* cultured keratinocytes showed 157 modulated genes [298]. The present studies show some overlap with the first study, but very little with the second. The small number of measured changes in the first study and the lack of significant immune response genes suggests significant differences between the allergic/atopic response in the skin and the response to tick feeding. These comparisons are relevant because the host response to ticks has been compared to a Th2-polarized, allergic-type response. The process of tick attachment necessitates local damage to the skin. For successful feeding, the tick has to maintain this local lesion as a conduit for nutritious blood, and must also remain attached to the host. The tick maintains the lesion through the action of tick saliva, which has an impressive arsenal of immunosuppressive compounds that block or inhibit every phase of the wound healing response. Attachment to the host is mediated by the hypostome and attachment cement; the amount of reliance on these two anchors differs between tick species. The host, on

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the other hand, can reject the tick by destabilizing the attachment, closing the lesion in the skin, or both. Host resistance to tick feeding is evident only after exposure to ticks, suggesting that this process relies on the adaptive immune response. Thus some amount of neutralization of salivary components is likely necessary to facilitate tick rejection. However, it should be noted that mice could develop resistance to *D. andersoni* but not to *I. scapularis*. While this could be related to differences in salivary components, it is quite possible that it is due to the very shallow feeding and superficial attachment of *D. andersoni* that allows the murine host response to more easily destabilize the attachment site or otherwise close the feeding lesion.

The primacy of the wound healing response in the skin comes as a surprise since the tick is secreting such a large array of salivary proteins into the bite. These proteins function as immunomodulators and toxins, but apparently do not drive changes in gene expression patterns large enough to be measured as "response to toxins". However, while I believe the host response to ticks appears most like a wound healing response, there are definite differences. These may be related to the importance of C-type lectins and specific combinations of inflammatory mediators. In addition, there is no such thing as a "recall" response to a wound. Thus the greatly increased speed and magnitude of the secondary response underlines the activity of T-cells and antibodies in the neutralization of salivary proteins. This activity can only be visualized as an active adaptive immune response at the resolution of this study. These points aside, the primary response mode of the skin to damage (or tick bites) appears to be defined by rapid closure and return to normal function. These activities are aided by the adaptive immune response that may

neutralize tick immunomodulators that get in the way, but does not change its basic identity.

Tables.

Table 7.1: **Significantly modulated genes shared between *I. scapularis* and *D. andersoni* 12hpi after during a primary exposure, as measured by microarray, p ≤ 0.05.**

DAP; *Dermacentor andersoni* primary; ISP; *Ixodes scapularis* primary.

Gene symbol	DAP 12hpi FC	ISP 12hpi FC
Krt6b	18.35	44.41
S100a8	7.35	12.69
S100a9	7.06	14.68
Sprr1b	6.36	8.2
Krt16	5.47	10.62
2610528A11Rik	3.94	14.48
Hp	2.5	4.42
Rab18	2	1.83
Ccl9	1.83	5.23
Ms4a6d	1.73	3.89
Nfil3	1.66	2.47
Clec4d	1.64	2.65
Krt6a	1.59	5.2
Ccl7	1.56	4.02
Mt2	1.55	3.08
Rpl39l	1.54	1.89
Acbd3	-1.85	-2.23
Rdx	-1.89	-2.17
Prrx1	-2.01	-2.28
Spnb2	-2.05	-2.15
Dbp	-3.02	-2.09
Xist	-4.19	-2.68
Ddx6	-5.42	-2.25

Table 7.2: **Gene ontology analysis comparing genes unique to *I. scapularis* or *D. andersoni* host response at 12hpi during primary infestation.**

IS 12hpi only	DA 12hpi only
Intermediate filament, keratin filament, cytoskeleton	Non-membrane bound organelle, cytoskeleton
DNA metabolism, DNA repair, response to DNA damage	Chromatin binding, organization, modification
DNA replication, DNA structural changes	DNA metabolism, DNA repair, response to DNA damage
Hair cycle, molting cycle, epidermis development	Cornified envelope, keratinization
Nuclear lumen, organelle lumen	Ubiquitin-dependent protein catabolic process
Cell cycle, cell division, M phase	ATP and nucleotide binding
ATP and nucleotide binding	Macromolecule catabolic process
Immune response, chemotaxis, cytokine, chemokine	Regulation of transcription, transcription factor binding
Positive regulation of cellular component organization	Nucleoplasm
Pyrimidine metabolism	

Table 7.3: **Significantly modulated genes shared between *I. scapularis* and *D. andersoni* infested mice late in a secondary infestation.**

Ixodes scapularis gene expression measured by PCR array, while *D. andersoni* expression measured by microarray. ISS *Ixodes scapularis* secondary; DAS *Dermacentor andersoni* secondary.

Gene	ISS 96hpi FC	DAS 120hpi FC
Cxcl5	3019.3	22.35
Il6	494.56	90.03
Il10	420.71	25.57
Mmp13	308.69	44.82
Cxcl2	254.82	19.26
Il1b	170.86	6.87
Ccl2	105.91	36.64
Ccl7	105.42	30.84
Il4	93.7	7.71
Sell	68.51	3.26
Mmp9	37.88	4
Clec7a	29.79	7.7
Cxcl1	24.36	3.63
Mmp8	21.38	2.16
Icos	15.21	2.03
Socs3	14.79	7.56
Cd2	14.25	2.77
Jak2	11.08	5.47
Itgb2	10.04	6.17
Il4ra	9.85	6.43
Cd3d	8.07	3.16
Ptgs2	7.89	5.75
Itgam	5.93	3.48
Sykb	4.87	4
Tnc	3.96	4.15
Gusb	3.23	2.33
Cd44	3.07	2.83
Mmp12	2.95	5.76
Irf1	2.78	2.11
Tlr4	2.64	2.55
Myd88	2.63	2.57
Icam1	2.33	2.37
Stat5a	2.26	2.1
Il7r	2.2	2.62
Tgfb1	2.2	2.37
Entpd1	1.97	2.14
Selp	1.84	2.29
Itgae	1.66	2.5
Timp3	-5.26	-2.69
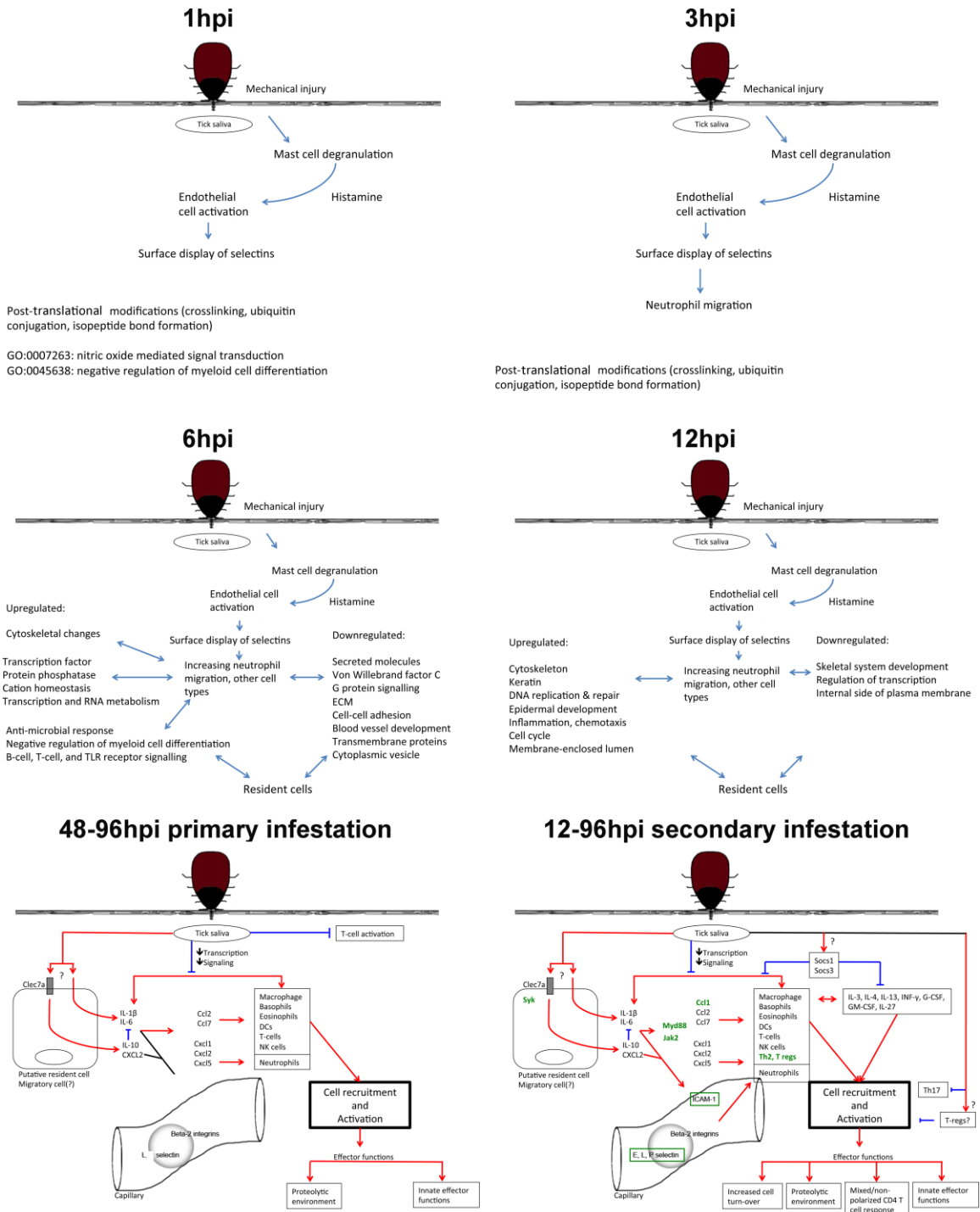
Figure 7.1: **Proposed models of the murine cutaneous host response during primary and repeated infestations with *I. scapularis* nymphs. Red lines are activated pathways; blue are inhibited pathways; green lettering highlights differences between primary and secondary exposures.**

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Core response

Saliva ← Damage → Keratinization

Chemotaxis

C-type lectins

Resident cell

Neutrophil Macrophage → Reactive oxygen species / Antimicrobial peptides

Early primary infestation

Saliva Damage

↑ Non-membrane-bounded organelle
↓ Nuclear body, nucleoplasm
↑ Nucleotide and ATP binding

↑ Circadian rhythm

↓ Cytoskeleton, actin cytoskeleton

↓ DNA damage/repair response
↓ Regulation of transcription, DNA binding
↓ mRNA metabolic process
↓ Regulation of transcription
↓ Chromosome, chromatin binding

Late primary infestation

Saliva Damage

Positive regulation of transcription
Nuclear or intracellular organelle lumen
RNA processing

Regulation of adhesion
Blood vessel development
Embryonic development
↓ Cell growth
MAP kinase activity
↓ Meisis (cell cycle)

Secondary infestation

Keratinization → Saliva Damage ← Wound healing

TLR CTL FcR CytR
Syk MyD88 Lck Hck Lyn Jak2 Btk
Responding cell

AMP ROS
Lipid mediators
Histamine

IL-1b IL-6
IL-10 IL-4
Tgfb M-CSF

Chemokines
Chemotaxis

Antigen uptake, processing & presentation

B cells

T-cell activation

Antibodies

Coagulation factors
Complement

Selectins
ICAM1

Integrins

Blood vessel

Figure 7.2: **Proposed models of the murine cutaneous host response during primary and repeated infestations with *D. andersoni* nymphs.**
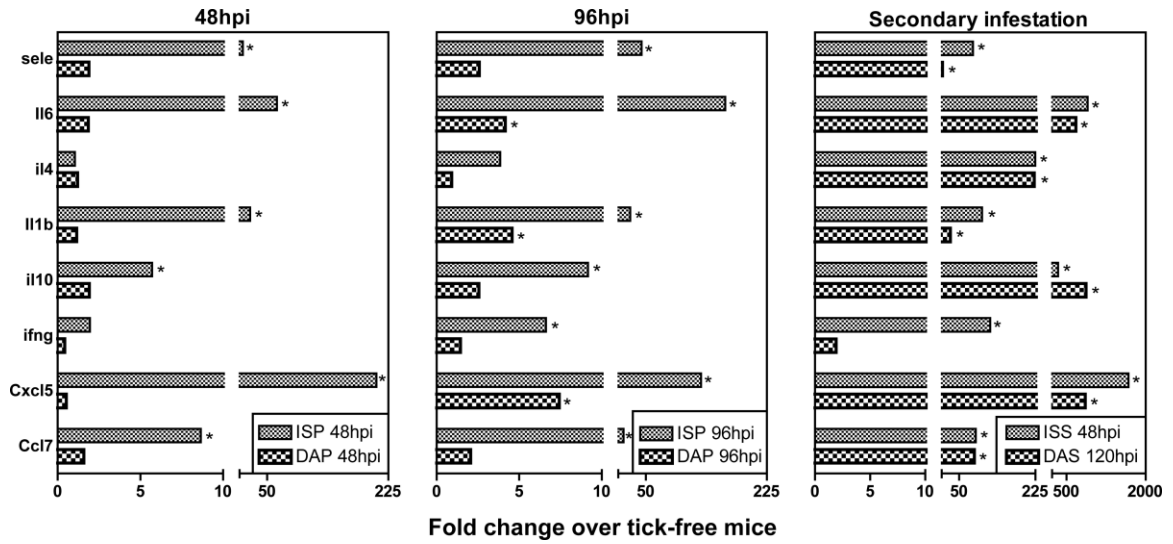
Figure 7.3: **Direct comparison of the cutaneous host response to *D. andersoni* or *I. scapularis* as measured by quantitative real-time PCR.**
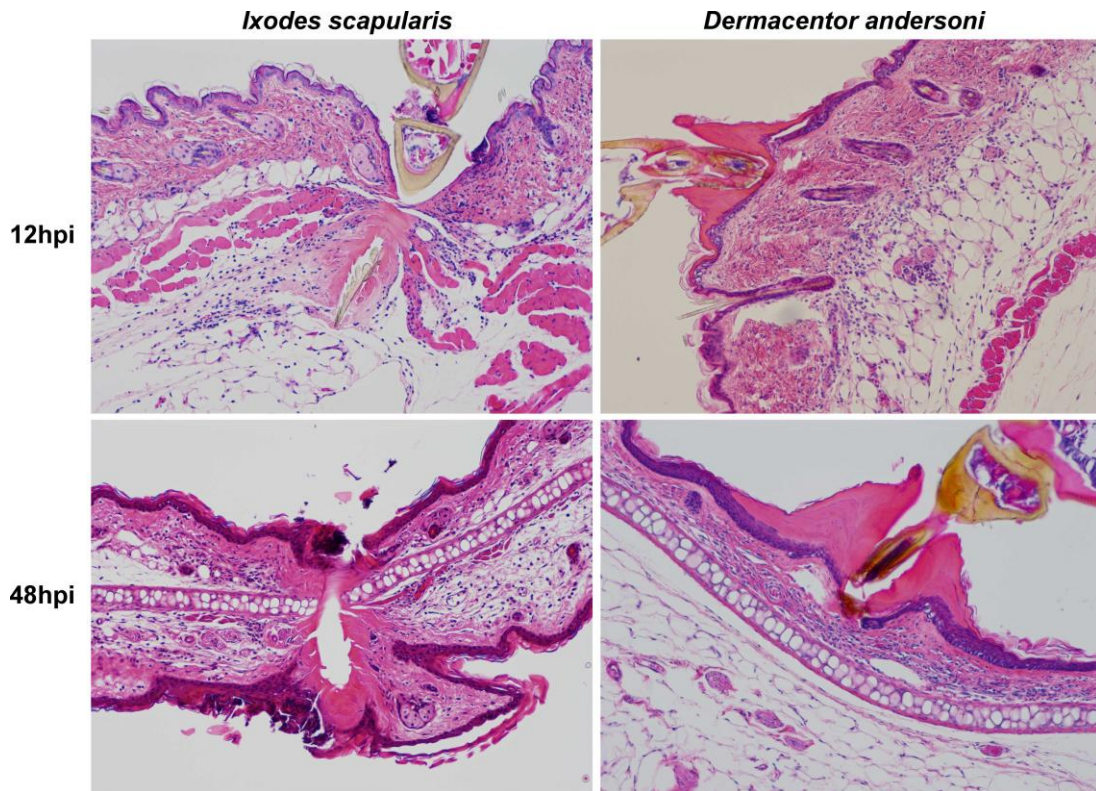
Figure 7.4: **Comparison of bite-site histology between *I. scapularis* and *D. andersoni* at 12 and 48 hpi during primary infestations.**

This figure shows the marked difference in hypostome penetration and cement usage.

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Vita

Dar Heinze was born in Houston, TX on Feb. 13, 1983 to W. Daniel and Judith Heinze. His education through the end of high school took place in a homeschooling environment. He attended Wheaton College in Wheaton, IL where he graduated magna cum laude with a B.M. in flute with elective studies in biology. After graduating from college, he married K. Michelle Young of Boxborough, MA. The following year he worked as a free-lance math tutor for high school and college students and worked for W. Daniel Heinze to review recent developments in renewable energy technology. He was accepted into the MD/PhD program at the University of Texas Medical Branch, in Galveston, TX, where he matriculated in 2007. During the following two years, he rotated in the laboratories of Dr. Mark Evers, Dr. Edward Sherwood, and Dr. Stephen Wikel, where he settled studying host responses to tick feeding. In addition, he was awarded a Truman Graves Blocker scholarship for academic achievement in medical school and scored 252/99 on the USMLE step 1 exam. In the following three years, he was able to secure funding under the "Emerging and Tropical Infectious Disease" training grant, publish three first-author manuscripts, and present a number of oral and poster presentations, two of which received awards. He also stayed active in the MD/PhD program serving on the recruitment committee as a member for two years and as chair for two years. Publications, presentations, and awards are summarized below:

Publications:

Heinze DM, Wikel SK, Thangamani S, Alarcon-Chaidez FJ. Transcriptional profiling of the murine cutaneous response during initial and subsequent infestations with Ixodes scapularis nymphs. *Parasit Vectors*. 2012 Feb 6;5:26. PubMed PMID: 22309607; PubMed Central PMCID: PMC3293053.

Heinze DM, Gould EA, Forrester NL. Revisiting the clinal concept of evolution and dispersal for the tick-borne flaviviruses by using phylogenetic and biogeographic analyses. J Virol. 2012 Aug;86(16):8663-8671. Epub 2012 Jun 6. PubMed PMID: 22674986; PubMed Central PMCID: PMC3421761.

In press: Heinze DM, Carmical JR, Aronson JF, and Thangamani S. Early immunologic events at the tick-host interface. *PLos One*, accepted Sept. 2012.

Posters:

Gene expression profiling of the host immune response to *Dermacentor andersoni*. Heinze D, Alarcon-Chaidez F, Boppana V, and Wikel S. Presented at the Department of Pathology Research Trainee Poster Session, May 2010.

Gene expression profiling of the host response to *Ixodes scapularis*. Heinze D, Alarcon-Chaidez F, Wikel S. Presented at the IHII/McLaughlin Colloquium, UTMB, March 2011.

Early events in the murine cutaneous response to tick (*Ixodes scapularis*) feeding. DM Heinze, JR Carmical, and S Thangamani. Presented at the IHII/McLaughlin Colloquium, UTMB, 13 April 2012.

Early immunologic events at the tick-host interface. Presented at the Department of Pathology Research Trainee Poster Session, May 2012.

Oral Presentations

2010: Gene expression profiling of the host response to the Ixodid tick Dermacentor andersoni. Presented at the Experimental Pathology Research Work in Progress Seminar.

2011: Transcriptional profiling of the cutaneous host response to Ixodes scapularis. Presented at the Experimental Pathology Research Work in Progress Seminar.

2012: Modeling host responses at the tick-host interface. Presented at the Experimental Pathology Research Work in Progress Seminar.

Awards

2008: Truman Graves Blocker Scholarship
2012: James W. McLaughlin Travel Award
2012: Edward S. Reynolds Award

Funding

2010-2012: T32AI007526 "Emerging and Tropical Infectious Diseases"

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This dissertation was typed by Dar Heinze.