ABSTRACT

Title of Document:	ROLE OF SELECT *BORRELIA BURGDORFERI*-INDUCIBLE TICK GENE-PRODUCTS IN PATHOGEN PERSISTENCE WITHIN THE VECTOR
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Lyme disease, also known as Lyme borreliosis, a common vector-transmitted illness across the Unites States and Europe, is caused by the pathogen *Borrelia burgdorferi*, which is transmitted by *Ixodes scapularis* ticks. While ticks are known to transmit a diverse set of bacterial, protozoan and viral disease agents, there are only limited investigations addressing how *Ixodes* immune responses influence the survival or persistence of specific pathogens within the tick. In North America, *I. scapularis* transmits a wide array of human and animal pathogens including a group of pathogenic bacteria, known as *Borrelia burgdorferi* sensu lato complex. Due to the evolutionary divergence from other bacteria, and the possession of a unique cellular structure, *B. burgdorferi* cannot be classified as a conventional Gram-positive or Gram-negative bacteria, instead they are classified as a spirochete. Additionally, key pattern recognition molecules or PAMPs, such as lipopolysaccharides and peptidoglycans, are absent or structurally distinct in *B. burgdorferi.* Thus, the wealth of knowledge generated in other model arthropods, regarding the genesis of host immune responses against classical bacterial pathogens, might not be applicable to *B. burgdorferi*. The primary goal for this dissertation is to characterize components of the tick immune responses that modulate *B. burgdorferi* infection and use this information to better understand specific aspects of tick immunity as well as to contribute to the development of new strategies that interfere with pathogen persistence and transmission. The following aims were addressed: assessment of the expression profile of the *I. scapularis* innate immune transcriptome to identify genes that are induced in the *B. burgdorferi*-infected vector. Next, a select set of pathogen-inducible gene-products was further studied for their possible harmful or beneficial roles in pathogen persistence in the vector. Based on recent findings in other disease vectors as well data generated within this thesis, I particularly focused on characterization of two select sets of *B. burgdorferi*-inducible tick gene-products that are potentially involved in maintenance of gut microbe homeostasis (Dual oxidase and peroxidase) and events linked to phagocytosis (Rho GTPase).

ROLE OF SELECT *BORRELIA BURGDORFERI*- INDUCIBLE TICK GENE-PRODUCTS IN PATHOGEN PERSISTENCE WITHIN THE VECTOR.

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Dedication

To my family who has always encouraged me to follow my dreams and to my friends who have always been here for me.

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List of Abbreviations

µg	microgram
µl	microliter
^{0}C	Degrees Celsius
bp	Base pair
BSA	Bovine serum albumin
BSK	Barbouer Stoener and Kelly media
cDNA	Complement DNA
ml	milliliter
ELISA	Enzyme linked Immunosorbent Assay
GST	Glutathione S-tranferase
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kDa	kilo Dalton
LB	Luria Bertani
mRNA	Messenger RNA
RNAi	RNA Interference
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
rpm	Revolution per min
RT-PCR	Reverse transcriptase-polymerase chain reaction
SDS-PAGE	Sodium Dodecyl Sulphate- Polyacrylamide Gel Electrophoresis
WT	Wild type
DTN	Di-tyrosine network

LPS	Lipopolysaccharides
PG	Peptidoglycan
PAMPs	Pathogen associated molecular patterns
LRR	Leucine-rich repeat
AMP	Antimicrobial Peptides
NOS	Nitric Oxide Synthase
SOD	Super Oxide Synthase
ROS	Reactive Oxygen Species
IMD	Immune Deficiency Pathway
JAK	Janus Kinase
STAT	Signal Transducer and Activator of Transcription

Chapter 1: Immunity-related genes in *Ixodes scapularis*

Introduction

Although there are several hundreds of tick species known to exist (Jongejan et al., 2004), only a handful transmits human diseases. *Ixodes scapularis* is one of the predominant tick species that transmits a wide array of serious human and animal pathogens, including *Borrelia burgdorferi*, the pathogen of Lyme borreliosis (Anderson,1991; Burgdorfer et al.,1982). Our understanding of arthropod innate immune responses, primarily involving the fruit fly and mosquito, has advanced over the past decades (Vilmos and Kurucz, 1998). However, our knowledge of tick immune responses, especially occurrence of immune-related genes and pathways, and specifically how these components respond to invading pathogens, remains under-explored. Notably, many pathogens that persist in, and are transmitted through, ticks are evolutionarily distinct and possess unique structures (Hajdusek et al., 2013). For example, key pattern recognition molecules, or pathogen associated molecular patterns (PAMPs), such as lipopolysaccharides (LPS) and peptidoglycan (PG), are completely absent or structurally different in major tick-borne pathogens, such as in Lyme disease spirochetes (Fraser et al., 1997; Schleifer et al., 1972; Takayama et al., 1987). Thus, the wealth of knowledge generated in other model arthropods, especially regarding the genesis of host immune responses against classical Gram-positive or Gram- negative bacterial pathogens, might not be applicable to tick-borne pathogens like *B. burgdorferi.* The primary goal of this review chapter is to present a general overview of tick immune components, as gathered from the genome sequence and published data, and to discuss their potential for

modulation of infection, with a particular focus on a major tick-borne pathogen, *B. burgdorferi*. A better understanding of *I. scapularis* immune responses to invading pathogens could contribute to the development of new strategies that interfere with relevant pathogen persistence and transmission.

Lyme Disease

Lyme disease or Lyme borreliosis, an emerging infectious disease, caused by the spirochetal bacteria *Borrelia burgdorferi*. Though antibiotic treatment is usually successful, diagnosis of this pathogen is currently difficult and a vaccine to prevent the disease in human is unavailable according to the Center for Disease Control, CDC. If left untreated, individuals can develop severe inflammatory complications including Lyme arthritis and carditis.

The transcriptome of *B. burgdorferi* undergoes many changes throughout the enzootic life cycle. Some proteins are known to be expressed by the bacteria during infection in the murine host while other proteins are expressed during the vector phase of the life cycle. (Kung et al., 2013) Although several proteins were shown to play important roles in supporting *B. burgdorferi* persistence in the host or vector, the precise function of most of these enigmatic gene-products in borrelial biology or infectivity is unknown. (Kung et al., 2013)

I. Scapularis Genome

The *I. scapularis* genome is relatively large, approximately 2.1 Gb in size and contains nearly 70% repetitive DNA (Ullmann et al., 2005). Recently it was completely sequenced by the *I. scapularis* genome project - a partnership between a number of tick research communities and institutions (Hill and Wikel, 2005; Pagel Van Zee et al., 2007). Toward the end of 2008, sequencing centers announced the annotation and release of the whole genome sequence data (IscaW1, 2008; GenBank accession ABJB010000000). The sequence data were derived from purified genomic DNA preparations isolated from an in-bred tick colony and sequenced to approximately 6-fold coverage using a combined whole genome shotgun and clone-based approach. The genome information are organized and displayed by a bioinformatics resource center focused on invertebrate vectors of human disease called VectorBase (www.vectorbase.org), which is funded by the National Institute of Allergy and Infectious Diseases, National Institutes of Health. The *I. scapularis* gene counts included 20,486 high confidence protein-coding genes, 316 non-coding genes and 20,771 transcripts. While the most recent release (IscaW1.3; 2014) reported no modifications of protein-coding loci, it incorporated a new prediction for 285 non-coding RNAs.

Identification of potential *I. scapularis* Innate Immune Related Genes

I. scapularis genome sequence information is now available through several publicly accessible databases (Hill and Wikel, 2005; Pagel Van Zee et al., 2007). In order to generate a comprehensive list of tick immune genes and related pathways, the National Institute of Allergy and Infectious Diseases Bioinformatics Resource Center for *I. scapularis* genome information (www.VectorBase.org) for annotated immune-related genes was initially searched. In addition, the relevant literature for the identification of additional innate immune genes, including those discovered in the ticks, fruit fly, mosquito and mammalian genomes (Ferrandon et al., 2007; Hoffmann et al., 1990; Hoffmann and Reichhart, 2002; Jaworski et al., 2002; Kopacek et al., 2010; Osta et al., 2004; Sonenshine, 1993; Janeway and Medzhitov, 2002; Christophides et al., 2002; Dimopoulos et al., 2000; Dong et al., 2006; Govind and Nehm, 2004; Saul, 2004; Tanji et al., 2007; Tanji and Ip, 2005; Valanne et al., 2011; Yassine and Osta, 2010) was reviewed. The latter information was then used to search for possible *Ixodes* orthologs via BLASTP in the VectorBase database. Additionally, a few potentially immune-related *I. scapularis* genes that were previously identified to be responsive to *B. burgdorferi* infection were also included in the list (Dai et al., 2010; Rudenko et al., 2005; Zhang and Zhu, 2009). In total, 266 genes were identified and, for simplicity, categorized into one of the following major immune pathways or components (number of unique genes is indicated in parentheses): gut-microbe homeostasis (17), agglutination (37), leucine-rich repeat proteins (22), proteases (33), coagulation (11), non-self recognition and signal transduction via Toll, IMD, and JAK-STAT pathways (53), free radical defense (13), phagocytosis (33), and anti-microbial peptides (14). Although this list may not be

comprehensive as additional published data could have been inadvertently overlooked or there may be yet-to-be identified genes involved in tick immune defense, it still represents the majority of genes involved in the tick immune response. In the following sections, these components and pathways are systematically expanded and discussed regarding their occurrence in ticks. Their potential influences on the persistence and transmission of tick-borne pathogens like *B. burgdorferi* are also discussed.

Gut Microbe Homeostasis

The critical importance of gut microbiota in shaping host immunity is described in a number of organisms including model arthropods (Buchon et al., 2013; Dillon and Dillon, 2004; Hooper et al., 2012; Kamada et al., 2013; Round and Mazmanian, 2009; Schuijt et al., 2013). Characterization of gut microbiota in ticks including *I. scapularis* as well as their influence on the persistence of tick-borne pathogens like *B. burgdorferi* has been a focus of a number of recent studies (Clay et al., 2008; Narasimhan et al., 2014; Carpi et al., 2011). Because many of these gut microbes play a beneficial role in the physiology of the host, the immune system must be able to differentiate commensal microbes versus pathogenic microorganisms (Macpherson and Harris, 2004). Though mechanisms that contribute to the microbial surveillance and eliminate pathogens, while tolerating the indigenous microbiota, remain obscure in ticks, these are well studied in many arthropods, particularly in *D. melanogaster* (Buchon et al., 2013). It has been established that immune reactivity in the fly gut ensures preservation of beneficial and dietary microorganisms, while mounting robust immune responses to irradiated pathogens (Buchon et al., 2013). There are at least two models of fly immunity that sense and preserve beneficial bacterial associations, while eliminating potentially damaging associations (Lazzaro and Rolff, 2011). The first is by recognition of non-self molecules (of invading microbes) while the second is the recognition of "danger" signals that are released by damaged host cells. It is also likely that these two models work together to maintain an effective gut microbe homeostasis. Recent studies suggest that dual oxidase (DUOX) and peroxidases enzymes play a key role in this process (Kim and Lee, 2014). While a number of other regulatory molecules may participate in this

process, 17 different genes were classified within *I. scapularis* genome to this pathway, including a single dual oxidase (DUOX) and several peroxidase proteins (**Table 1**).

Studies have revealed that DUOX plays an essential role in gut mucosal immunity and homeostasis (Baeet al., 2010; Deken et al., 2013). DUOX, a member of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase NOX family (Geiszt and Leto, 2004), has previously been shown to be a key source of local microbicidal reactive oxygen species (ROS) production within the fly gut (Kim and Lee, 2014). Targeted depletion of DUOX production in flies has resulted in the overproduction of commensal gut bacteria, rendering the fly susceptible to infection (Buchon et al., 2013; Kim and Lee, 2014). As originally discovered in *Caenorhabditis elegans* (Edens et al., 2001), in addition to ROS generation, DUOX function is also implicated for catalysis of protein cross-linking that contribute to maintenance of gut microbiota in *Anopheles gambiae* (Kumar et al., 2010). In mosquitoes, DUOX along with a specific heme peroxidase, catalyzes the formation of an acellular molecular barrier, called the dityrosine network (DTN), that forms in the luminal space along the gut epithelial layer during feeding (Kumar et al., 2010). Formation of the DTN decreases the gut permeability to various different immune elicitors protecting the gut microbiota, both commensal and pathogenic species. Another recent study revealed that an ovarian dual oxidase is essential for insect eggshell hardening through the production of H_2O_2 that ultimately promotes protein crosslinking (Dias et al., 2013). Further studies into how DUOX-peroxidase systems maintains gut microbiota in *I. scapularis* could give novel insight into how pathogens that

are transmitted though ticks are able to evade the immune system and persist within the vector.

Table 1: Putative Gut-microbe homeostasis proteins found in *I. scapularis*

Annotation	Accession Number
Dual Oxidase	ISCW007865
Phospholipid-hydroperoxide glutathione peroxidase, putative	ISCW019584
Phospholipid-hydroperoxide glutathione peroxidase, putative	ISCW022517
Glutathione peroxidase, putative	ISCW020571
Peroxidase	ISCW017070
Peroxidase	ISCW022537
Glutathione peroxidase, putative	ISCW008495
Oxidase/peroxidase, putative	ISCW002528
Phospholipid-hydroperoxide glutathione peroxidase, putative	ISCW015098
Oxidase/peroxidase, putative	ISCW017368
Oxidase/peroxidase, putative	ISCW005828
Peroxidase	ISCW024650
Glutathione peroxidase, putative	ISCW020569
Oxidase/peroxidase, putative	ISCW018825
Peroxidase	ISCW013159
Thioredoxin peroxidase, putative	ISCW013767
Glutathione peroxidase, putative	ISCW001759

Agglutination

Agglutination, the biological phenomenon in which cells or particles clump together, has been described within various different tick species (Kibuka-Sebitosi, 2006; Uhlir et al., 1996). A group of carbohydrate-binding proteins called lectins (Grubhoffer et a., 1997; Grubhoffer et al., 2004) could be a key mediator of the process, which are often produced in a tissue specific manner in the arthropods, especially in the gut, hemocytes or fat bodies (Grubhoffer et al., 2004; Grubhoffer et al., 2005). Agglutination of pathogens by lectins, which also function as host recognition receptors for pathogen associated molecular patterns (Dam and Brewer, 2010), has been reported in many arthropod vectors including mosquitoes and tsetse flies, where they play an important role in the pathogen-host relationship (Abubakar et al., 1995; Abubakar et al., 2006; Barreau et al., 1995; James, 2003). While lectins can function as signaling factors for the maturation of the African trypanosome, or as various lytic factors (Abubakar et al., 1995; Abubakar et al., 2006), they act as agonists of development of the malarial parasites within the vector (Barreau et al., 1995; James, 2003) in mosquitoes. Although tick lectins, particularly those in hard ticks (*Ixodidae*), have not been studied as extensively as other arthropod lectins, previous reviews have summarized the available information on lectins of the tick *I. ricinus* (Grubhoffer et al., 2004; Grubhoffer and Jindrak; 1998). Since most lectins isolated from arthropods are the ones from the hemocoel, most studies have focused on their localization or haemagglutinating activity in the haemolymph (Sonenshine, 1993; Kuhn et al., 1996). In *I. ricinus*, such activity was characterized as a Ca^{2+} dependent binding activity (Grubhoffer et al., 2004). An 85 kDa lectin, produced by the granular haemocytes and basal laminae surrounding the haemocoel, was identified to

have a strong binding affinity for sialic acid (Grubhoffer et al., 2004). This immunoreactivity supports the idea that lectins may function as a recognition molecule of the immune system in ticks, implying that lectins could influence the persistence of tick-borne pathogens like *B. burgdorferi*. In fact, the hemocytes in *I. ricinus* can also phagocytize *B. burgdorferi* through the coiling method, which has previously been thought to be a lectin-mediated process (Grubhoffer and Jindrak, 1998). Specifically, two agglutinins/lectins were isolated from the gut, one 65 kDa and the other 37 kDa in size; the former being the main agglutinin with a binding affinity for mucin while the latter protein was found to have a strong affinity for a specific glucan (Grubhoffer et al., 2004; Grubhoffer and Jindrak, 1998). In addition, it is also suggested that the gut agglutinin is a potential LPS-binding protein that in co-operation with other digestive enzymes could affect the persistence of gram-negative bacteria and spirochetes that pass through the gut lumen (Uhlir et al., 1996; Grubhoffer et al., 2004). In addition to hemolymph and gut, lectin activities are also documented in the salivary gland; a 70 kDa protein has been identified as being responsible for the hemagglutinating activity in this organ (Grubhoffer et al., 2004). It is thus possible that lectin or related function in the salivary glands could influence pathogen transmission. In fact, a tick mannose-binding lectin inhibitor that is produced in the salivary glands has been shown to interfere with the human lectin compliment cascade significantly impacting the transmission and survival of *B. burgdorferi* (Schuijt et al., 2011). Taken together, it is likely that lectins play a role in immunity of *I. scapularis* that encodes at least 37 lectins or related proteins in the genome (**Table 2**).

Table 2: Putative Agglutination proteins found in *I. scpularis*

Annotation	Accession Number
Ferritin	ISCW015079
Beta-galactosidase	ISCW000651
Ubiquitin associated domain containing protein	ISCW023764
Chitin Bindin Peritrophin A	ISCW006076
Beta-galactosidase precursor	ISCW019676
galectin, putative	ISCW008553
Manose binding ER-golgi comparment lectin	ISCW016179
Ixoderin Precursor	ISCW002664
Ixoderin Precursor	ISCW022063
Ixoderin B	ISCW013797
Ixoderin B	ISCW003711
Hemelipoglycoprotein precursor	ISCW012423
Ferritin	ISCW023334
galectin, putative	ISCW020268
Beta-galactosidase	ISCW019681
Beta-galactosidase precursor	ISCW019677
Beta-galactosidase precursor	ISCW019679
Beta-galactosidase precursor	ISCW016637
Hemelipoglycoprotein precursor	ISCW024299
fatty acyl-CoA elongase, putative	ISCW010899
Galectin	ISCW020268
Ixoderin Precursor	ISCW024686
Hemelipoglycoprotein precursor	ISCW012424
Sodium/proton exchanger	ISCW008652
C-Type Lectin, Putative	ISCW010467
Ixoderin Precursor	ISCW013746
double sized immunoglobulin g binding protein A	ISCW021766
Galectin	ISCW008553
Hemelipoglycoprotein precursor	ISCW021704
Beta-galactosidase precursor	ISCW019678
Lectin, Putative	ISCW012623
Galectin	ISCW020586
Hemelipoglycoprotein precursor	ISCW014675
Hemelipoglycoprotein precursor	ISCW021709
Ixoderin Precursor	ISCW012248
Ubiquitin associated and SH3 domain containing protein B	ISCW021035
galectin, putative	ISCW020586

Leucine Rich Repeats

Leucine-rich repeat (LRR) proteins have previously been shown to occur in more than 2000 proteins including Toll-like receptors throughout the plant and animal kingdom, and are thought to play an essential role in host defense (Bell et al.,2003; Enkhbayar et al., 2004; Boman and Hultmark, 1981; Kobe and Kajava, 2001). LRR proteins typically contain 20-29 amino acid residues (with repeats ranging from 2-42) that are involved in protein-protein interactions in diverse cellular locations and biological functions. The biological significance of LRR containing proteins in ticks remains unknown. The *I. scapularis* genome encodes at least 22 potential LRR proteins (**Table 3**). Unlike ticks, roles of LRR proteins in the immunity of the other arthropods including blood meal seeking arthropods are relatively well characterized (Povelones et al., 2009; Povelones et al., 2011). For example, *Anopheles gambiae*, which contains LRR proteins, such as LRIM1 and APL1C, has been identified as a potent antagonist of malarial parasites and has been shown to limit *Plasmodium* infection by activating a complement like system (Povelones et al, 2009; Povelones et al., 2011; Baxter et al., 2010; Fraiture et al., 2009). *Manduca sexta,* which contains an LRR protein categorized as leureptin, has been shown to bind lipopolysaccharide and is involved in hemocyte responses to bacterial infection (Zhu et al., 2010). Further studies into how tick LRR containing proteins contribute to the vector immunity and influences pathogen persistence is warranted.

Table 3: Leucine-rich repeat (LRR) proteins

Annotation	Accession Number
Lumicans	ISCW001027
LRR (in flii) interacting protein	ISCW016609
Lumicans	ISCW005645
F-Box/ LRR protein, putative	ISCW000110
F-Box/ LRR protein, putative	ISCW010598
F-Box/ LRR protein, putative	ISCW010597
F-Box/ LRR protein, putative	ISCW010599
LRR protein, putative	ISCW008095
LRR protein	ISCW014626
F-Box/ LRR protein, putative	ISCW010347
F-Box/ LRR protein, putative	ISCW005273
LRR and NACHT domain containing protein	ISCW004678
LRR and NACHT domain containing protein	ISCW001292
LRR protein	ISCW012038
F-Box/ LRR protein, putative	ISCW016452
F-Box/ LRR protein, putative	ISCW018961
F-Box/ LRR protein, putative	ISCW013925
LRR containing G-protein coupled receptor	ISCW015788
F-Box/ LRR protein, putative	ISCW018134
F-Box/ LRR protein, putative	ISCW008236
LRR protein, putative	ISCW003174

Proteases/Protease Inhibitors

A number of immune cascades that serve to recognize and control invading pathogens depend on activity of specific proteases or protease inhibitors (Janeway and Medzhitov, 2002; Sojka et al., 2011). Proteases, specifically serine proteases, have previously been shown to be a key regulating molecule for several of these immune response pathways including coagulation, antimicrobial peptide synthesis and melanization of pathogens (Janeway and Medzhitov, 2002; Gorman and Paskewitz, 2001; Jiravanichpaisal et al., 2006). Such a serine protease-dependent cellular response manifests through the rapid activation of immune pathways in response to the pathogen detection (Hoffmann et al., 1999; Fujita, 2002) as demonstrated by the coagulation pathway of the horseshoe crab. Activation of this pathway has been shown to be controlled by three serine proteases, factor C, factor B, and a pro-clotting enzyme (Tokunaga et al., 1987). When LPS is present, clotting factors that are stored within hemocytes are readily released into the hemolymph that ultimately results in the immobilization of the invading pathogen.

Protease inhibitors that control a variety of proteolytic pathways are also known to play important roles in arthropod immunity (Kanost, 1999). A group of serine protease inhibitors, called serpins, have been the focus of many recent studies that demonstrated the critical contribution of these proteins in the regulation of inflammation, blood coagulation and complement activation in mammals (Kanost, 1999). Serpins are also known to contribute in immunity and physiology in arthropods, as shown in mosquitoes (Gulley et al., 2013) and flies (Reichhart et al., 2011). A detailed

characterization of serpins in ticks, including that in *I. scapularis,* has been published by Mulenga et al. (Mulenga et al., 2009). Interestingly, most were differentially expressed in guts and salivary glands of unfed and partially fed ticks (Mulenga et al., 2009). It is speculated that ticks could utilize some of these serpins to manipulate host defense to facilitate tick feeding and disease transmission. The role of tick serpins in the physiology and immunity in the vector waits further investigation. More recently, a novel serpin, named IRS-2, was described in *I. ricinus* (Chmelar et al., 2011). IRS-2 was shown to inhibit cathepsin G and chymase thereby inhibiting host inflammation and platelet aggregation and was also thought to act as modulators of vascular permeability. Although the extent that serpins play a role in host microbe interactions remains unknown, studies also explored their potentials as target antigens for development of a tick vaccine (Muleng et al., 2001).

Table 4: Putative Proteases/ Protease inhibitor proteins found in *I. scapularis*

Annotation	Accession Number
Serpin 4 precursor	ISCW023622
Serpin 2 precursor	ISCW010422
Serpin 2 precursor	ISCW018607
Serpin 1 precursor	ISCW023618
Serpin 7 precursor	ISCW024109
PAP associated domain containing protein	ISCW014870
Serpin 7 precursor	ISCW009616
Serine proteinase inhibitor serpin-3	ISCW015204
Heparan sulfate 2-O sulfotransferase, putative	ISCW000208
Secreted Salivary gland peptide	ISCW023621
Serpin	ISCW016489
Hypothetical protein	ISCW017929
Secreted Salivary gland peptide	ISCW023620
Serpin 4 precursor	ISCW023623
Protein disulfide isomerase 1	ISCW002080
Alkaline phosphatase	ISCW023785
Alkaline phosphatase	ISCW003801
Alkaline phosphatase	ISCW004677
Hypothetical protein	ISCW021544
Zinc metalloprotease	ISCW008637
Zinc metalloprotease	ISCW005798
Zinc metalloprotease	ISCW005687
Zinc metalloprotease	ISCW005854
Serpin 8 precursor	ISCW014652
Serpin	ISCW014100
Zinc metalloprotease	ISCW012815
Conserved hypothetical protein	ISCW006169
Serpin 8 precursor	ISCW015349
Zinc metalloprotease	ISCW021286
Serpin 2 precursor	ISCW021417
Serpin 2 precursor	ISCW014779
Secreted serine protease	ISCW014551
Alkaline phosphatase	ISCW000162

Coagulation

Injury, or presence of microbes in arthropods, could result in the induction of two major proteolytic pathways - coagulation and melanization (Theopold et al., 2004). Key enzymes for these processes, which crosslink the clot or induce the proteolytic cascade similar to the vertebrate clotting cascade, include transglutaminase and phenoloxidase. Studies of the horseshoe crab have provided a breakthrough in our understanding of the coagulation pathway in arthropods (Theopold et al., 2004). The coagulation pathway is characterized by a rapid sequence of highly localized serine proteases, which culminates in the generation of thrombin; this process is tightly regulated to ensure excessive clot formation does not occur (Crawley et al., 2011). *I. scapularis* genome encodes for at least 11 genes that may be part of the coagulation pathway (**Table 5**) although how this pathway controls wound healing or effects microbial survival remains unknown. Notably, while *I. scapularis* genome lacks genes related to the melanization (phenoloxidase) pathway, phenol oxidase activity was detected in the hemolymph of the soft ticks, *Ornithodoros moubata* (Kadota et al., 2002).

Table 5: Putative Coagulation proteins found in *I. scapularis*

Annotation	Accession Number
Proclotting enzyme precursor	ISCW013112
Thrombin inhibitor	ISCW000427
Proclotting enzyme precursor	ISCW000320
Proclotting enzyme precursor	ISCW011206
Proclotting enzyme precursor	ISCW001322
Keratinocyte transglutaminase	ISCW019475
Proclotting enzyme precursor	ISCW011961
Proclotting enzyme precursor	ISCW003779
Proclotting enzyme precursor	ISCW010999
Prostate-specific transglutaminase	ISCW009303
Prostate-specific transglutaminase	ISCW011739

Non-self recognition and signal transduction pathways

Three major pathways, namely Toll, immune deficiency (IMD), and Janus kinase (JAK)- signaling transducer activator of transcription (STAT) pathways, contribute to the activation of immune responses in arthropods, as detailed earlier (Hoffmann and Reichhart, 2002; Govind and Nehm, 2004; Tanji et al., 2007; Tanji and Ip, 2005; Valanne et al., 2011; Belvin and Anderson, 1996; De Gregorio et al., 2002; Kaneko and Silverman, 2005; Liu et al., 2012; Rawlings et al., 2004; Souza-Neto et al., 2009; Xi et al., 2008; Zambon et al., 2005). While Toll pathways are activated in the presence of bacterial, viral, and fungal pathogens, the IMD pathway is induced upon the presence of Gram- negative bacteria. The arthropod JAK-STAT pathway, analogous to a cytokine-signaling pathway in mammals (Shuai et al., 1993), is also known to be activated in the presence of bacterial or protozoan pathogens (Liu et al., 2012; Buchon et al., 2009; Gupta et al., 2009). The Toll pathway is most extensively studied in *Drosophila* where nine Toll receptors are encoded in the genome (Valanne et al., 2011). Cell wall components in gram-positive bacteria stimulate this pathway. The precise fungal component that induces specific Toll is not well defined. Stimulation of the Toll pathway leads to cleavage of the protein Spatzle, which eventually leads to the activation of the NF-κB transcription factor family members, Dif and Dorsal, which are homologous to mammalian c-Rel and RelA resulting in the production of different antimicrobial peptides (Christophides et al.,2002; Hetru et al., 2003; Irving et al., 2001). Research in *Drosophila* has shown that gram-positive bacteria induce the Toll pathway leading to toll specific anti-microbial peptides (AMP), such as drosomycin (Zhang and Zhu, 2009). While the roles of Toll pathways in *I. scapularis* remain obscure, at least 33 genes

potentially belong to this pathway (**Table 6**). The IMD pathway, on the other hand, is activated by the peptidoglycan molecules present on the surface of Gram-negative bacteria, which is recognized by host cells via peptidoglycan recognition receptors (Ferrandon et al., 2007). This recognition leads to the activation of an adaptor protein and further downstream signaling molecules, activating transcription factor Relish, a compound Rel-Ank protein homologous to mammalian p100 and p105, ultimately resulting in the production of AMPs (Ferrandon et al., 2007; Matova and Anderson, 2006). Although the tick genome is known to encode at least 20 potential genes from this pathway (**Table 6**), similar to Tolls, how the IMD pathway affects gram-negative pathogens including *B. burgdorferi* is unknown. *I. scapularis* encodes many representative genes from both pathways (**Figure 1**). A critical and common aspect in the response reflected by both the Toll and the immune deficiency pathways, through the recognition of non-self, is the ability to induce a specific AMP to combat microbial infections. It is also thought that these two pathways can work synergistically to activate the expression of the same AMP (Tanji et al., 2007).

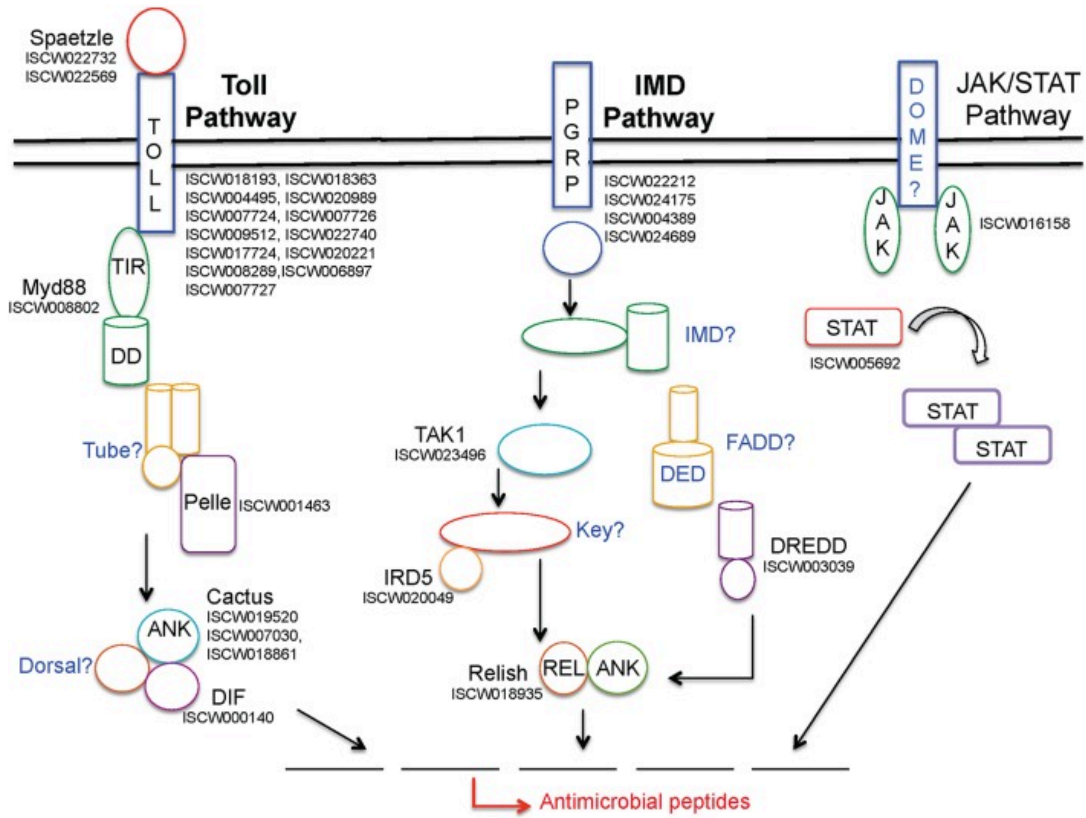

Figure 1: Schematic representation of *I. scapularis* immune-related genes potentially representing three major signaling pathways that lead to anti-microbial responses. While annotated tick genes from each pathway are shown in black font, the apparently missing genes are indicated in blue.

Table 6: Putative Non-self recognition (Toll, IMD, and JAK-STAT pathways) proteins found in *I. scapularis*

Annotation	Accession Number
Regulator of ubiqutin pathway, putative	ISCW015648
NF-kappaB inhibitor IkappaB, putative	ISCW007030
Peptidoglycan recognition receptor protein	ISCW022212
Embryonic polarity dorsal, putative	ISCW000140
Ankyrin repeat-containing protein	ISCW018861
Peptidoglycan recognition receptor protein	ISCW024175
Netrin receptor DSCAM	ISCW016844
Netrin receptor DSCAM	ISCW016100
Caspase, apoptotic cysteine protease, putative	ISCW003039
Netrin receptor DSCAM	ISCW020406
Peptidoglycan recognition receptor protein	ISCW004389
Netrin receptor DSCAM	ISCW022828
Scavenger receptor class B	ISCW003371
Scavenger receptor class B	ISCW010934
Scavenger receptor class B	ISCW002412
Netrin receptor DSCAM	ISCW003295
UBX domain-containing protein, putative	ISCW011870
NF-kappaB inhibitor IkappaB, putative	ISCW019520
Nuclear factor nf-kappa-B P105 subunit, putative	ISCW018935
Peptidoglycan recognition receptor protein	ISCW024689
N-CAM Ig domain containing protein	ISCW022144
Toll	ISCW018193
Secreted Protein, putative	ISCW021005
Secreted Protein, putative	ISCW024521
Toll	ISCW018363
Serine-threonine protein kinase, plant-type, putative	ISCW001463
Toll	ISCW004495
Toll	ISCW020989
Spatzle	ISCW022569
Fibrinogen	ISCW009412
Tartan protein, putative	ISCW016292
Secreted Protein, putative	ISCW024389
Toll	ISCW007724
Toll	ISCW007726
Myd88, putative	ISCW008802
Fibrinogen	ISCW009412
Toll	ISCW009512
Toll	ISCW022740
Serine/threonine protein kinase	ISCW020049
Toll	ISCW017724

Tartan protein, putative	ISCW021508
Kekkon 1, putative	ISCW018006
Toll	ISCW020221
Tolkin	ISCW022120
Membrane glycoprotein LIG-1, putative	ISCW005558
Slit Protein	ISCW018651
Toll	ISCW008289
Adenylate Cyclase	ISCW012040
Toll	ISCW006897
Fibrinogen	ISCW024309
Toll	ISCW007727
Spatzle	ISCW022732
Fibrinogen	ISCW001478
JAK	ISCW016158
STAT 3	ISCW005692

Free radical defense

Free radicals, such as reactive oxygen species (ROS), which include superoxide radicals (O•2), hydroxyl radicals (•OH), or other compounds, and are able to react with biomolecules and cause damage to DNA, proteins and lipids, play a critical role in cell signaling (Thannickal and Fanburg, 2000). ROS are known to play an important role in arthropod development (Owusu-Ansah and Banerjee, 2009) and they are indispensible in arthropod immunity including activation of specific immune pathways (Bubici et al., 2006; Molina-Cruz et al., 2008; Morgan and Liu, 2011; Pereira et al., 2001). For example, mosquitoes that have previously been infected with *Wolbachia* bacteria were observed to produce much higher levels of ROS (Pan et al., 2012). Nitric oxide (NO), a highly unstable free radical gas, is also another component of free radical defense that has been shown to be toxic to both parasites and pathogens (James, 2003; Wanderska-Nowak, 2004). In insects, NO is known to induce following parasite infection (Davies, 2000; Dimopoulos et al., 1998).

A family of superoxide dismutase (SOD) that catalyzes the conversion of these free radicals to non-toxic O_2 and relatively less toxic hydrogen peroxide (H_2O_2) is responsible for destroying any free radicals generated in the hosts (Landis et al.). Gluthione-S-tranferases (GST) also detoxify stress-causing agents, including toxic oxygen free radical species (Sharma et al., 2004). The gene encoding GSTs are shown to be induced in model arthropods upon oxidative stress and microbial challenge, including infection with *B. burgdorferi* in ticks (Rudenko et al., 2005). Despite these studies, how different free radicals or SOD detoxification participate in pathogen persistence or

clearance within the *I. scapularis*, which encodes at least 13 genes of this pathway (**Table 7**), remains uncharacterized.

Table 7: Putative Free radical defense proteins found in *I. scapularis*

Annotation	Vectorbase Accession Number
Manganese superoxide dismutase	ISCW016585
Ras responsive element binding protein	ISCW009132
Superoxide dismutase Cu-Zn	ISCW012382
Cu 2+/ Zn 2+ Superoxide dismutase SOD1	ISCW011852
Manganese superoxide dismutase	ISCW016737
Manganese superoxide dismutase	ISCW012767
Superoxide dismutase	ISCW018077
Superoxide dismutase	ISCW015027
Superoxide dismutase	ISCW024422
Superoxide dismutase Cu-Zn	ISCW008219
Decarboxylase	ISCW021675
Nitric Oxide Synthase	ISCW018074
Nitric Oxide Synthase Interacting Protein	ISCW017590

Phagocytosis

Phagocytosis, the process by which cells recognize, bind and ingest relatively large particles, is considered to be an evolutionarily conserved cellular response (Walters and Papadimitriou, 1978). Phagocytosis is a major cellular immune response in arthropods that have been mostly studied in model insects (Sideri et al., 2008). This process is mediated by hemocytes, which are primarily present in the hemolymph and also within various organs. Phagocytosis of microbes plays a critical role in arthropod defense. Blocking of phagocytosis in *Drosophila* mutants impairs the fly's ability to survive subsequent bacterial infection (Elrod-Erickson et al., 2000). In ticks, the hemocytes within the hemolymph, have previously been shown to be phagocytic to various pathogens (Inoue et al., 2001). Whereas solid experimental evidence of phagocytosis of *B. burgdorferi* within *I. scapularis* is lacking, certain cell lines derived from ticks have been shown to be phagocytic to spirochetes (Mattila et al., 2007). Further studies into the phagocytic pathway of *I. scapularis* that encodes 33 potentially related genes (**Table 8**) would provide insight into whether or how pathogens such as *B. burgdorferi* are phagocytosed, or how the tick-borne pathogens are able to escape this cellular immune response. *I. scapularis* genome encodes for five small GTPases belonging to Rho family, which amongst other cellular functions, have been shown to play central roles in phagocytosis (Bokoch, 2005; Etienne-Manneville and Hall, 2002).

Table 8: Putative Phagocytosis proteins found in *I. scapularis*

Annotation	Accession Number
Integrin beta-3	ISCW024103
Cadherin- repeats domain containing protein	ISCW013741
Rho GTPase activating protein	ISCW015851
Protocadherin fat	ISCW017319
Thioester containing protein	ISCW020822
Cadherin	ISCW005817
Rho GTPase activating protein	ISCW015201
Protocadherin beta-6	ISCW016805
Integrin alpha-ps	ISCW005672
GTPase Rho	ISCW004349
Rho guanine nucleotide exchange factor	ISCW014238
GTPase Rho	ISCW004348
Protocadherin fat	ISCW016765
Rho GTPase activating protein RICH2	ISCW003282
Integrin alpha	ISCW003186
Integrin beta-3	ISCW010037
GTPase Rho	ISCW006741
Rho GTPase activating protein	ISCW003559
Integrin beta subunit	ISCW008948
p116 Rho-interacting protein	ISCW007121
Integrin alpha repeat domain containing protein	ISCW019648
Rho GTPase activating protein	ISCW018998
Rho guanine exchange factor	ISCW008875
Rho	ISCW002009
Integrin alpha-ps	ISCW022321
Rho GTPase activating protein	ISCW019271
GTPase Rho	ISCW018929
Rho GTPase activating protein	ISCW001560
Rho GDP dissociation inhibitor	ISCW020878
Rho associated kinase	ISCW011682
GTPase Rho	ISCW015794
Integrin beta subunit	ISCW002553
Integrin alpha	ISCW003185

Antimicrobial peptides

The production of antimicrobial peptides (AMPs), a hallmark of systemic humoral immune responses, is an important aspect of host defense in arthropods (Bulet et al., 1999). At least eight different classes of AMPs have been observed in the fruit fly, *Drosophila*. They are mainly produced by fat bodies, are secreted into the hemolymph, and can be further grouped into three different families based on their intended target: gram-negative bacteria, gram-positive bacteria, and fungi (Imler and Bulet, 2005). In arthropods, specific AMPs are produced as a result of activation of Toll, IMD or JNK-STAT pathways against the presence of bacteria, fungus or virus.

AMPs which likely produce classical antimicrobial peptides in the gut and hemocoel (Hynes et al., 2005; Saito et al., 2009) are relatively well studied in ticks. AMPs have been found to be produced in hard ticks, such as *I. scapularis* and *Dermacentor variabilis*, as well as in the soft tick, *Ornithodoros moubata* (Rudenko et al., 2005; Hynes et al., 2005; Saito et al., 2009; Nakajima et al., 2002). A defensin like gene is induced in response to *B. burgdorferi* by *I. ricinus* but is expressed in a tissue-specific manner that is not capable of clearing the infection (Rudenko et al., 2005). The exact role of *I. scapularis*, which encodes for at least 14 AMPs (Table 9), in clearance of tick-borne pathogens remains unclear. In addition, ticks may also produce non-classical AMPs. Although gastric digestion in ticks is primarily intracellular, degradation of blood components, such as hemoglobin, could create peptides with antimicrobial activities (Sonenshine et al., 2005). Whether these fragments would protect against pathogenic bacteria has not currently been reported.

Table 9: Putative Antimicrobial peptides proteins found in *I. scapularis*

Annotation	Accession Number
Putative secreted salivary gland peptide	ISCW005928
Secreted Protein, putative	ISCW018425
Secreted Salivary gland peptide	ISCW002695
TAK 1 putative	ISCW009364
Secreted Salivary gland peptide	ISCW001310
Beta transducin Trp-Asp domain containing protein	ISCW014204
Map Kinase activating death domain protein	ISCW017494
Secreted Salivary gland peptide	ISCW018541
Defensin	ISCW022102
Preprodefensin Putative	ISCW016747
Secreted Salivary gland peptide	ISCW002331
Secreted Salivary gland peptide	ISCW016466
Secreted Salivary gland peptide	AAV63544
Arsenite-resistance protein	ISCW011320

Concluding Remarks

I. scapularis ticks are known to transmit a diverse set of disease agents that range from pathogens of bacterial, protozoan and viral origin. A number of studies have explored the immunomodulatory activities of tick saliva or components of salivary gland on mammalian hosts, or how these activities benefit tick-transmitted pathogens (Hovius et al., 2008; Pal and Fikrig, 2010). However, relatively limited studies have addressed how vector immune responses influence the survival or persistence of specific pathogens within the tick. This is rather surprising given that ticks are known to encode a number of identified immune effector mechanisms including humoral (classical AMPs) and cellular (such as phagocytosis) immune responses, as well as evolutionary conserved signaling molecules or potentially active pathways representing Toll, IMD, or JNK/STAT pathways, their contribution in shaping *I. scapularis* immunity remains largely obscure. Tick-borne pathogens have evolved to be persistent and to be transmitted by a specific tick species. Thus, it is conceivable that these pathogens have co-evolved and developed a successful and intimate relationship with the host. Additionally, to persist successfully within nature, these pathogens must have also evolved specific mechanisms to persist in the vector and evade innate immune insults. For example, when artificially challenged with the Lyme disease pathogen, *I. scapularis* ticks have slow phagocytic responses, and therefore, practically remain immunotolerant against spirochete infection (Johns et al., 2001). In contrast, another hard tick species, *D. variabilis*, when challenged with the same pathogen, generates a rapid and effective increase in phagocytic cells, clearing the infection, and thus is highly immunocompetent against spirochete infection. With the availability of *I. scapularis* genome information, as well as development of robust

functional genomics, and bioinformatics that enables efficient high-throughput genome sequencing, there is an exciting future research into *I. scapularis* immunity. This dissertation presents new research into the special biology of vector-microbial interaction and some aspects of tick immunity.

A portion of this chapter has been published previously:

Smith AA and Pal U. Immunity-related genes in *Ixodes scapularis*- perspectives from genome information in "Lyme Disease: Recent Advances and Perspectives" hosted by Dr(s) Catherine Ayn Brissette, Tanja Petnicki-Ocwieja in Frontiers in Microbioligy, (In press) 2014.

Chapter 2: Identification of *B. burgdorferi*-inducible immune related genes in ticks

Introduction

Lyme disease, a common vector-transmitted illness, is caused by the pathogen *B. burgdorferi* senso lato complex. Successful maintenance of this pathogen in the natural infection cycle requires two hosts; a vertebrate host, for example *Peromyscus leucopus*, the white-footed mouse in North America, as well as an arthropod vector, *I. scapularis* or the deer tick. Transmission occurs when *I. scapularis* takes a blood meal on a vertebrate host during which the bacteria are deposited into the skin. From there, the spirochete can disseminate to various different internal organs including the heart and joints. Humans are incidental hosts and if infected and left untreated, can develop severe inflammatory complications including Lyme arthritis, carditis and a variety of serious neurological complications.

Ticks are known to transmit a diverse set of bacterial, protozoan, and viral disease agents yet there is only limited investigation addressing how vector immune responses influence the survival or persistence of specific pathogens within the tick. This is rather surprising given that ticks are known to encode components of a number of immune effector mechanisms, including humoral (classical AMPs) or cellular (phagocytosis) immune responses as well as evolutionary conserved signaling molecules or potentially active pathways (Toll, IMD, or JNK/STAT). Tick-borne pathogens have evolved to persist and be transmitted by a specific tick species. *I. scapularis* transmits a wide array

of human and animal pathogens including *B. burgdorferi*. Due to the evolutionary divergence from other bacteria, and the possession of a unique cellular structure, *B. burgdorferi* cannot be classified as a conventional Gram-positive or Gram-negative bacterial species. Additionally, key pattern recognition molecules or PAMPs, such as lipopolysaccharides and peptidoglycans, are absent or structurally distinct, respectively, in *B. burgdorferi.* Thus, the wealth of knowledge generated in other model arthropods, regarding the genesis of host immune responses against classical bacterial pathogens, might not be applicable for *B. burgdorferi*. As described in Chapter 1, a list of tick immune genes and related pathways was generated through a comprehensive analysis of the recently sequenced *I. scapularis* genome data that are available through several publicly accessible databases was performed. To accomplish this, the National Institute of Allergy and Infectious Diseases Bioinformatics Resource Center (www.VectorBase.org) for annotated *I. scapularis* immune-related genes was initially searched. In addition, the relevant literature to identify additional innate immune genes, including those discovered in related tick species, or in fruit fly, mosquito and mammalian genomes was reviewed. The latter information was then used to search for possible *I. scapularis* orthologs via BLASTP (NCBI) against the VectorBase database (www.vectorbase.org). In total, 266 genes were identified and categorized into one of the following nine major immune pathways or components (number of unique genes): gut-microbe homeostasis (17), agglutination (37), leucine-rich repeat (LRR) proteins (21), proteases (33), coagulation (11), non-self recognition and signal transduction via Toll, IMD, and JAK-STAT pathways (55), free radical defense (13), phagocytosis (33), and anti-microbial peptides (14).

In this chapter the results of experiments that tested the expression of these genes when *I. scapularis* was infected with *B. burgdorferi* are reported.

Methods

Gene expression in B. burgdorferi infected versus uninfected ticks

Primers were designed using Invitrogen OligoPerfect software. All primers were designed to have the same annealing temperature of 60 ^{o} C and amplified to similar sizes (~ 200 bp). Primers were specifically designed to expand more than one exon to ensure genomic DNA contamination would not affect any further experiments. Group of two C3H mice were injected with *B. burgdorferi* B31 isolate (10^5 cells/mouse). Infection was confirmed by Western blotting with mouse serum. A parallel group of non-infected age-matched mice were maintained. After infection was established for roughly 2 weeks, naïve *I. scapularis* nymph ticks were placed in both groups of mice and removed after 48 hours of feeding. Total RNA was extracted from 20 ticks using TRIzol reagent (Invitrogen). Samples were further treated with RNase free DNase 1 and reverse transcribed into cDNA to further reduce DNA contamination.

The relative levels of each gene-specific transcript were assessed by quantitative PCR (qPCR). Each primer pair was tested for both efficiency and non-specific amplification by melt curve analysis. Any primers showing non-specific amplification were redesigned or discarded from analysis. The amplification cycle consisted of an initial denaturation step at 95° C for 5 min followed by 45 cycles each at 95° C for 10 sec, 60° C for 20 sec and 72° C for 30 sec with a final melt curve analysis: 55° C for 30 sec, increase 0.5^{o} C per cycle to 95° C. The amplification was performed in an iQ5 real-time thermal cycler using SYBR Green Supermix (Roche). For expression screening, 15 candidate genes were assayed in each 96 well PCR plate with duplicate wells of cDNA

(infected or uninfected samples) and negative (no template) control. Transcript levels of individual genes were assessed in both infected and uninfected samples using the $2^{-\Delta\Delta Ct}$ method, normalized against *β–Actin* and presented as a fold increase in gene expression.

Results

A sensitive quantitative RT-PCR (qRT-PCR) was used to compare transcript levels of 266 genes in both *B. burgdorferi* infected ticks as well as in naïve ticks to identify any potential genes that plays a role in either the persistence or clearing of *B. burgdorferi*. Total RNA was isolated and qRT-PCR was preformed using gene specific primers. Analysis of qRT-PCR data revealed that 20 genes were up-regulated, 53 genes down-regulated and 194 genes non-regulated in the presence of *B. burgdorferi.* The 73 genes that were found to be differentially expressed are listed in **Table 10**, where genes annotated in red were up-regulated and those in blue were down-regulated.

Discussion

The overall purpose of this experiment was to test the comprehensive list of potential immune related genes presented in Chapter 1. The hypothesis was that those genes that are differentially regulated in the presence of the pathogen might play an essential role in the persistence of the bacteria within the vector. While there may be many other gene products that aren't differentially expressed that are key to the immune regulation of the vector, which could affect the pathogens persistence, it was decided to focus on those genes that appear to be specifically induced by *B. burgdorferi.* Since tools have already been developed to knockdown the expression of genes via RNA interference the focus of this dissertation is on determining the role of the up-regulated genes in the pathogenesis and persistence of *Borrelia.* Based on our literature search, of the 20 up-regulated genes, two genes, Dual Oxidase/Peroxidase and a Rho GTPase were found to be the most rational targets that warranted further characterization.

The first pathway studied is the one potentially involve in maintenance of gut microbe homeostasis. Gut microbiota serve a critically important function in shaping host immunity in a number of organisms, including model arthropods. The goal is to further characterize the role of the *I. scapularis* Dual Oxidase/Peroxidase genes (those that were up-regulated) in the persistence of the bacteria. This is the subject of Chapter 3 and Chapter 4. The second pathway studied was phagocytosis, starting with the most strongly enhanced gene within the pathway Rho GTPase. This is the subject of Chapter 5.

Table 10: Differentially expressed genes

Annotation	Pathway	Fold Change
Dual Oxidase	Gut Microbe Homeostasis	2.62
Oxidase/peroxidase, putative	Gut Microbe Homeostasis	3.75
Oxidase/peroxidase, putative	Gut Microbe Homeostasis	2.00
Ferritin	Agglutination	15.7
Beta-galactosidase	Agglutination	4.15
Ubiquitin associated domain containing protein	Agglutination	2.79
Chitin Bindin Peritrophin A	Agglutination	2.17
Galectin	Agglutination	- 2.09
Hemelipoglycoprotein precursor	Agglutination	-2.25
Beta-galactosidase precursor	Agglutination	-2.46
Lectin, Putative	Agglutination	-2.55
Galectin	Agglutination	-2.79
Hemelipoglycoprotein precursor	Agglutination	-3.42
Hemelipoglycoprotein precursor	Agglutination	-3.91
Ixoderin Precursor	Agglutination	-8.04
Ubiquitin associated and SH3 domain containing protein B	Agglutination	-22.29
Lumicans	Leucine-Rich Repeats	-2.61
F-Box/ LRR protein, putative	Leucine-Rich Repeats	-3.33

F-Box/ LRR protein, putative	Leucine-Rich Repeats	-3.99
F-Box/ LRR protein, putative	Leucine-Rich Repeats	-2.31
LRR protein, putative	Leucine-Rich Repeats	-2.59
F-Box/ LRR protein, putative	Leucine-Rich Repeats	-5.23
F-Box/ LRR protein, putative	Leucine-Rich Repeats	-5.84
Serpin 2 precursor	Protease/Protease-Inhibitors	-9.15
Serpin 1 precursor	Protease/Protease-Inhibitors	-2.18
Secreted Salivary gland peptide	Protease/Protease-Inhibitors	-2.96
Serpin	Protease/Protease-Inhibitors	-5.58
Hypothetical protein	Protease/Protease-Inhibitors	-2.10
Serpin 4 precursor	Protease/Protease-Inhibitors	-3.33
Zinc metalloprotease	Protease/Protease-Inhibitors	-4.10
Thrombin inhibitor	Coagulation	-2.03
Proclotting enzyme precursor	Coagulation	-2.03
Proclotting enzyme precursor	Coagulation	2.14
Proclotting enzyme precursor	Coagulation	-2.25
Proclotting enzyme precursor	Coagulation	-18.6
Prostate-specific transglutaminase	Coagulation	-2.09
NF-kappaB inhibitor IkappaB, putative	Toll, IMD, and JAK-STAT pathways	3.18
Peptidoglycan recognition receptor protein	Toll, IMD, and JAK-STAT pathways	106.00

Caspase, apoptotic cysteine protease, putative	Toll, IMD, and JAK-STAT pathways	6.16
Secreted Protein, putative	Toll, IMD, and JAK-STAT pathways	2.08
Toll	Toll, IMD, and JAK-STAT pathways	7.18
Myd88, putative	Toll, IMD, and JAK-STAT pathways	10.79
Fibrinogen	Toll, IMD, and JAK-STAT pathways	4.84
Toll	Toll, IMD, and JAK-STAT pathways	2.55
Tolkin	Toll, IMD, and JAK-STAT pathways	7.15
Spatzle	Toll, IMD, and JAK-STAT pathways	-4.58
Toll	Toll, IMD, and JAK-STAT pathways	-2.15
Spatzle	Toll, IMD, and JAK-STAT pathways	-2.00
Fibrinogen	Toll, IMD, and JAK-STAT pathways	-4.63

Tartan protein, putative	Toll, IMD, and JAK-STAT pathways	-5.59
Secreted Protein, putative	Toll, IMD, and JAK-STAT pathways	-2.56
Toll	Toll, IMD, and JAK-STAT pathways	-3.62
N-CAM Ig domain containing protein	Toll, IMD, and JAK-STAT pathways	-7.07
NF-kappaB inhibitor IkappaB, putative	Toll, IMD, and JAK-STAT pathways	-4.56
Scavenger receptor class B	Toll, IMD, and JAK-STAT pathways	-3.92
Regulator of ubiqutin pathway, putative	Toll, IMD, and JAK-STAT pathways	-10.15
GTPase Rho	Phagocytosis	19.29
Rho GTPase activating protein	Phagocytosis	2.65
Cadherin- repeats domain containing protein	Phagocytosis	-2.72
Protocadherin fat	Phagocytosis	-11.13
Cadherin	Phagocytosis	-3.16
Rho guanine nucleotide exchange factor	Phagocytosis	-3.54

Rho GTPase activating protein RICH2	Phagocytosis	-4.77
Integrin beta-3	Phagocytosis	-2
p116 Rho-interacting protein	Phagocytosis	-18.21
Rho GTPase activating protein	Phagocytosis	-7.71
Integrin alpha-ps	Phagocytosis	-11.71
GTPase Rho	Phagocytosis	-2.64
Integrin alpha	Phagocytosis	-2.90
Defensin	Antimicrobial Peptides	12.57
Secreted Salivary gland peptide	Antimicrobial Peptides	17.71
Beta transducin Trp-Asp domain containing protein	Antimicrobial Peptides	-3.12
TAK 1 putative	Antimicrobial Peptides	-3.20

Chapter 3: Role of a dual oxidase-peroxidase system in *B. burgdorferi* infection and transmission through ticks

Introduction

Lyme disease, the most prevalent tick-borne infection in the United States, is caused by the spirochete bacterium, *B. burgdorferi* (Stanek et al., 2012; Steere, 2001). The pathogen thrives in nature through an intricate infectious cycle, including an arthropod vector, *I. scapularis*, and a vertebrate host, primarily wild rodents (Radolf et al., 2012). *I. scapularis* engorges upon the host dermis, usually for several days, and during this feeding process, pathogens like *B. burgdorferi* can efficiently transit between the arthropod and mammalian hosts (Piesman et al., 1990; Tyson and Piesman, 2009). Once acquired by ticks, the pathogen remains in the vector gut throughout the intermolt period until the next blood meal when a fraction of the spirochetes exit the gut, enter the hemocoel, and finally invade the salivary glands (de Silva et al., 2009; Pal and Fikrig, 2010). Once in the glands, the bacteria can then be transmitted to a new host. Thus, maintenance of the pathogen in the enzootic cycle requires its successful persistence through multiple developmental stages of the arthropod as well as its coordinated dissemination through tick tissues to a new host.

The gut environment of blood feeding arthropods including that of the feeding tick (Dong et al., 2006; Munderloh and Kurtti, 1995; Yassine and Osta, 2010; Soneshine, 1993), which pathogens like *B. burgdorferi* encounters upon arrival from an infected host, is likely to be hostile. As spirochetes adapt to survive in the luminal spaces of the

gut, the bacteria must avoid the intense digestive activities of gut epithelial cells and at the same time bypass innate immune defense mechanisms (Kopacek et al., 2010). Although gastric digestion in ticks is primarily intracellular, degradation of blood components, such as hemoglobin, could create peptides with antimicrobial activities (Soneshine et al., 2005). In addition, a number of studies in model arthropods illustrated the existence of diverse and potent pathogen recognition molecules, which in turn induce the Toll (against bacterial, viral, and fungal pathogens) or immune-deficiency (against Gram- bacteria) pathways (Govind and Nehm, 2004; Hoffmann and Reichhart, 2002; Tanji et al., 2007; Tanji and Ip, 2005; Valanne et al., 2011). How these pathways operate in *I. scapularis,* especially their activation following *B. burgdorfe*ri invasion remains obscure. Nevertheless, ticks likely produce classical antimicrobial peptides in the gut (Hynes et al., 2005) even though their role in tick immunity remains enigmatic; for example, although a defensin-like gene is up-regulated in *Ixodes* ticks following infection with *B. burgdorferi,* it is unable to clear the infection (Rudenko et al., 2005). Earlier studies also reported the development of annotated catalogs of organ-specific transcripts (Anerson et al., 2008; Ribeiro et al., 2006). Despite these studies, the precise mechanism by which *B. burgdorferi* survives in the gut or evades the tick innate immune system requires further investigation.

Replication of pathogens or commensal bacteria within the gut during blood meal engorgement by arthropods could activate epithelial immunity; thus, arthropods must develop a strategy to maintain the gut homeostasis. A previous study demonstrated that a molecular barrier, termed dityrosine network (DTN), is formed within the gut of a blood-

sucking arthropod, *Anopheles gambiae,* during the uptake of a blood meal and surrounds the gut epithelial layer (Kumar et al., 2010). The formation of the DTN decreases the gut permeability to immune elicitors, thereby protecting the beneficial gut microbiota, which in turn, supports the invading pathogens like *Plasmodium*. The DTN in mosquitoes is dependent upon two separate enzymes, dual oxidase (Duox) and a heme peroxidase, which catalyze the cross-linking of tyrosines (Kumar et al., 2010). Dual oxidase is a prominent member of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) family and a transmembrane protein that has previously been shown to be a source of local reactive oxygen species (ROS), which play an important role in maintenance of mucosal immunity (Donko et al., 2005). In *Drosophila*, targeted depletion of Duox and the subsequent decrease in ROS production leads to uncontrolled proliferation of gut bacteria and also renders the fly more susceptible to microbial infection (Ha et al., 2005). Similarly, expression of Duox in airway epithelial cells promotes antimicrobial defenses (Geiszt et al., 2003).

A search in the *I. scapularis* genome database indicated that the arthropod encodes for a single Duox and at least 16 potential peroxidases. Considering an important yet less understood role of Duox and specific peroxidase enzymes in formation of an epithelial barrier influencing the host-microbe homeostasis (Kumar et al., 2010; Ha et al., 2005; Bae et al., 2010), we sought to investigate how these enzymes affect persistence of a major tick-borne pathogen, *B. burgdorferi*. These studies contribute to a better understanding of specific aspects of tick immunity as well as help in the development of new strategies that interfere with pathogen persistence in an enzootic

infectious cycle.

Methods

Borrelia burgdorferi, mice, and ticks

A low passage infectious isolate of *B. burgdorferi*, clone B31-A3, was used in this study (Elias et al., 2002). Four- to six-week-old C3H/HeN mice were purchased from the National Institutes of Health. Animal experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee and Institutional Biosafety Committee. *I. scapularis* ticks used in this study originated from a colony that is maintained in our laboratory.

RNA isolation and PCR

The oligonucleotide primer sequences for the quantitative RT-PCR analysis of tick gene expression or measurement of bacterial load are indicated in Table 11 found on page 64. For analysis of *Duox* and *peroxidase* expression, the naïve nymphal ticks or nymphs microinjected with dsRNA were allowed to feed on naïve or *B. burgdorferi*-infected C3H/HeN mice (25 ticks/mouse) as detailed (Kariu et al., 2011; Kariu et al., 2013). The ticks were collected at various time points of feeding or as post-fed (PF) nymphs. Individual ticks were processed separately. Total RNA was extracted from ticks using TRIzol (Invitrogen), and treated with RNase-free DNaseI (Qiagen), and then reverse transcribed to cDNA using the AffinityScript cDNA Synthesis kit (Stratagene). The relative transcript levels of the target genes in ticks were assessed by quantitative RT-PCR (qRT-PCR) (Yang et al., 2009). To test the efficiency and exclude non-specific amplification of the primer pairs, the qRT-PCR amplification in each well was followed by melt-curve analysis. The amplification was performed in an iQ5 real-time thermal

cycler (Bio-Rad) using SYBR Green Supermix (Bio-Rad). The amplification cycle consisted of an initial denaturation step at 95° C for 5 min followed by 45 cycles each at 95° C for 10 sec, 60° C for 20 sec and 72° C for 30 sec with a final melt curve analysis: 55° C for 30 sec, increase 0.5^{o} C per cycle to 95° C. Transcript levels of individual genes were calculated using the $2^{-\Delta\Delta Ct}$ method and further normalized against tick *β-actin* transcripts (Yang et al., 2009). For assessment of levels of *B. burgdorferi* or gut bacterial numbers in ticks, the *flaB* or *16S* transcript levels, respectively, were quantitated using qPCR (Yang et al., 2009).

RNA interference

RNA interference of target tick genes was accomplished using published procedures (Kariu et al., 2013; Pal et al., 2004). Tick cDNA was prepared as described above and used as a template to amplify various fragments of the open reading frames of the corresponding target genes, *duox* (accession number *ISCW007865*) or peroxidases (accession numbers *ISCW017368* and *ISCW002528*, referred to herein as *peroxidase 002528* and *peroxidase 017368*). A fragment of the *gfp* gene was also amplified as a control. The primers used to amplify the specific DNA fragments are listed in Table 11. The *duox*, *peroxidase*, and *gfp* amplicons were separately cloned into the corresponding restriction sites of the L4440 double T7 script vector, and the dsRNA was synthesized and purified using a commercial kit (MEGAscript RNAi kit, Ambion, Inc.). Five µl of the dsRNA (2 µg/µl) was loaded into capillary tubes and microinjected into the gut of unfed nymphs (25 ticks/group). The injected nymphs were kept overnight in an incubator and used for feeding on mice. Fully engorged ticks were individually processed for

assessment of gene silencing as well as pathogen levels using qRT-PCR analysis. Primers that bind upstream and downstream of the target cDNA encompassing the dsRNA sequence were used for the qRT-PCR, as indicated in Table 11.

Infection studies

C3H mice were infected with spirochetes (10^5 spirochetes/mouse) in order to assess the RNA interference-mediated reduction of *duox* or *peroxidase* expression affected *B. burgdorferi* acquisition by nymphal ticks. Following 14 days of infection, the corresponding Duox, peroxidase, or GFP dsRNA preparations were administrated to nymphs, and the microinjected ticks were placed on *B. burgdorferi*-infected mice (25 ticks/mouse). Groups of ticks were forcibly detached from the mice following 48 hours of feeding, while the others were allowed to feed to repletion and were collected. The *flaB* transcripts were detected using qRT-PCR. At least three independent experiments were performed and 6-10 ticks were recovered from each mouse. Each tick was analyzed individually for measurement of target transcripts or pathogen burden using qRT-PCR.

Confocal immunofluorescence microscopy

For confocal microscopy (30), tissues from engorged ticks were fixed for 24 h at 4°C in 4% (w/v) paraformaldehyde in PBS followed by infiltration with 30% sucrose. Cryosectioning was performed at -20°C using a cryotome, and 5 μm thick sections were incubated in PBS with 5% goat serum and 0.5% Tween 20. The sections were then labeled with mouse monoclonal antibodies against dityrosine (Genox Corporation), rabbit

anti-peroxidase antibodies (Sigma), or isotype control (mouse or rabbit IgG, Sigma) followed by Alexa-488-labeled goat anti-mouse or Alexa-568-labeled goat anti-rabbit IgG (Invitrogen). The slides were washed using PBS with 0.05% Tween-20, stained with DAPI (Invitrogen), and imaged with a LSM 510 laser confocal microscope (Zeiss).

Assays for nitric oxide synthase activity

The NOS activity of the tick tissues was analyzed using a commercial kit (Ultrasensitive Colorimetric Assay kit, Oxford Biomedical Research). Whole tick samples were homogenized in PBS under the ice and centrifuged briefly. The protein concentration in the collected supernatant was measured. The NOS activity was detected using an equal amount of protein (40 μg) from groups of *duox*, *peroxidase*, or *gfp* knockdown ticks, and the OD values at 540 nm were calculated using a standard curve consisting of serial dilutions of nitrite from 0-100 μM.

Bioinformatics and statistical analysis

Unless indicated otherwise, protein annotation and searches were executed using the VectorBase (www.vectorbase.org) database. Analyses of protein families and domains were performed using the Pfam (http://pfam.sanger.ac.uk/) and CDD (http://www.ncbi.nlm.nih.gov/structure/cdd/cdd.shtml) databases. Sequence alignment analysis was performed using Cobalt Constraint-based Multiple Protein Alignment Tool (www.ncbi.nlm.nih.gov/tools/cobalt/). Results are expressed as the mean ± standard error (SEM). Statistical significance of differences observed between experimental and

control groups were analyzed using GraphPad Prism version 4.0 (GraphPad Software, CA). A two-tailed Student's *t*-test was utilized to compare the mean values, and $p < 0.05$ was considered significant.

Results

Expression of dual oxidase during early tick feeding coincides with the replication of bacteria in the gut, which features a distinct dityrosine network (DTN)

Ixodid ticks are known to harbor a diverse set of gut bacteria (van Overbeck et al., 2008; Moreno et al., 2006). To further study the role of the Duox in the genesis of a DTN and protection of invading pathogens like *B. burgdorferi*, replication of the gut bacterial population during feeding was assessed. To accomplish this, naïve mice (3 animals/group) were parasitized with *B. burgdorferi*-infected nymphal ticks (15 ticks/mouse), and partially fed ticks were removed at various time points from 6-48 hours of feeding. Batches of harvested ticks were subjected to total RNA or DNA isolation. Total bacterial numbers were determined by qPCR analyses using whole tick genomic DNA extracts as a template and generic bacteria-specific 16S primers, while expression of *duox* was determined using qRT-PCR. The results showed that bacterial numbers in the feeding tick gut peaks around 20 hours during engorgement (**Figure 2A**). Although up regulation of *duox* occurred at multiple time points during feeding, the earliest peak was recorded at 20 hours of feeding (**Figure 2B**), which temporally coincided with the peak bacterial numbers in feeding ticks. These data suggested that the induction of *duox* during the initial hours of feeding was potentially in response to gut microbe replication. A similar tick feeding study to assess whether *B. burgdorferi* presence within the gut also induced *duox* expression was then performed. The results showed that the ticks that parasitized *B. burgdorferi*-infected mice contained at least three-fold higher levels of *duox* transcripts in comparison to the ticks that fed on naïve mice (**Figure 2C**). Confocal

immunofluorescence microscopy was employed to assess whether a DTN structure could also be localized in feeding ticks. To accomplish this, samples of gut and a control organ (salivary gland) were removed from 24-48 hour fed ticks, and frozen tissue sections were labeled with a mouse monoclonal antibody generated specifically against dityrosine linkage, or isotype control antibodies. The data showed specific binding of dityrosine antibodies to the tick gut, indicating presence of a DTN, while no reactivity was recorded for the salivary glands (**Figure 2D**) or in gut tissues incubated with isotype control antibodies.

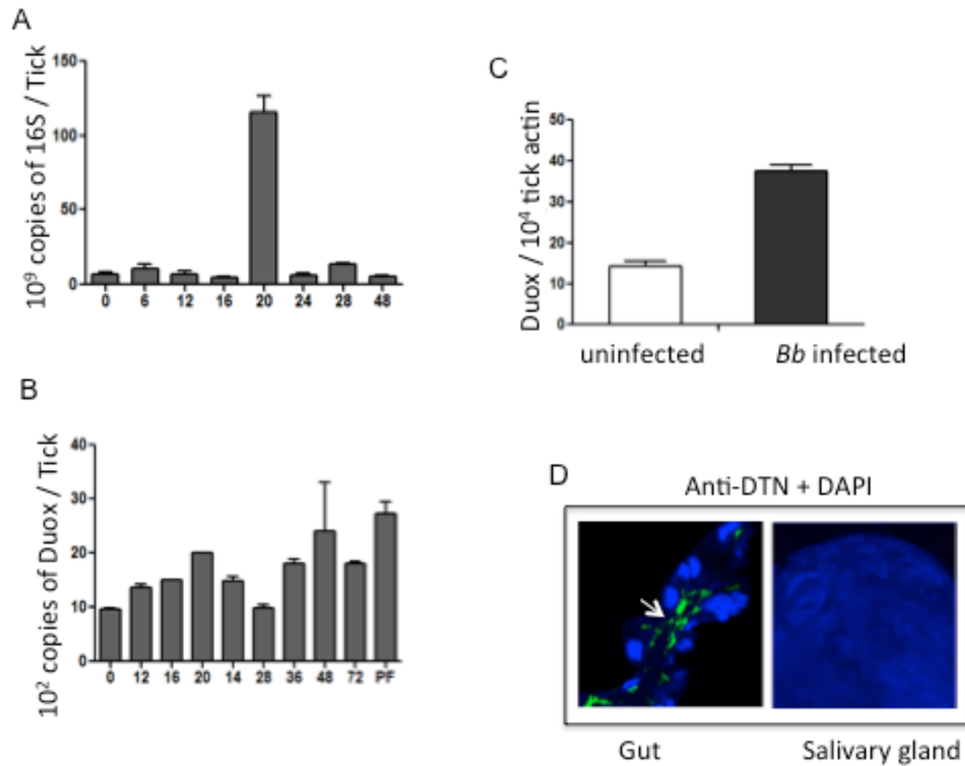

Fig. 2. Proliferation of bacteria in feeding ticks coincides with expression of *dual oxidase* (*Duox*) in the gut, which contained a detectable dityrosine network. (**A**) Proliferation of gut bacterial population within feeding ticks. Naïve nymphal ticks were allowed to parasitize naïve mice and were collected at different times after placement on the mice. Bacterial numbers were measured by qPCR detection of *16S* rRNA levels. (**B**) Expression of *Duox.* Naïve nymphal ticks were collected at different times of feeding on mice or as fully engorged post-fed (PF) ticks, and *Duox* transcript levels were measured by qRT-PCR. Mean ± SEM of three independent infection experiments is presented. (**C**) *B. burgdorferi* infection in ticks further enhances *duox* transcript levels. Naïve nymphal ticks were allowed to parasitize uninfected or *B. burgdorferi* (*Bb*)-infected mouse and collected at 48 hours after placement on mice, and Duox transcript levels were measured by qRT-PCR. (**D**) The existence of a dityrosine network (DTN) within the tick gut. Feeding ticks were collected at 24 hours following their placement on mice, and confocal immunofluorescence staining of tick tissue sections (gut and salivary glands) was performed using isotype control IgG or dityrosine antibodies (labeled green). The nuclei in tick tissues were labeled with DAPI (blue). Arrow indicates the DTN in the lumen adjacent to gut cells.

Duox knockdown reduced B. burgdorgeri persistence in ticks

The observation that the replication of gut microbes peaked at 20 hours of feeding, which also coincided with *duox* expression and formation of the DTN in the gut, suggested that RNAi-mediated knockdown of *duox* could interfere with the occurrence of a DTN and affect the persistence of *B. burgdorferi* in the gut. Because *duox* is a relatively large gene (5418 bp), five different dsRNA preparations that encompass various regions of the gene were generated (**Figure 3A**) and empirically tested for their ability to silence target gene expression. One of them (dsRNA2) was selected based on its efficiency in knocking down *duox* expression significantly (**Figure 3B**, $p < 0.01$) and used in all subsequent studies. Whether reduction of *duox* expression affects formation of the DTN and whether this influences *B. burgdorferi* persistence was tested using dsRNA-injected ticks that fed on *B. burgdorferi*-infected mice. It was found that the appearance of the DTN formation was less obvious in Duox-knockdown ticks (**Figure 3C**), which also retained significantly less *B. burgdorferi* burden when compared to control (GFP dsRNA-injected) ticks (**Figure 3D**, $p < 0.01$). Similarly, qPCR analysis using 16S rRNA levels of total gut bacteria also showed an apparent decrease in Duox-knockdown ticks, however, the decrease was not found to be significant (**Figure 3E,** $p < 0.14$) Together, these results suggest that Duox plays a beneficial role for the Lyme disease pathogen while feeding ticks acquire it from infected hosts.

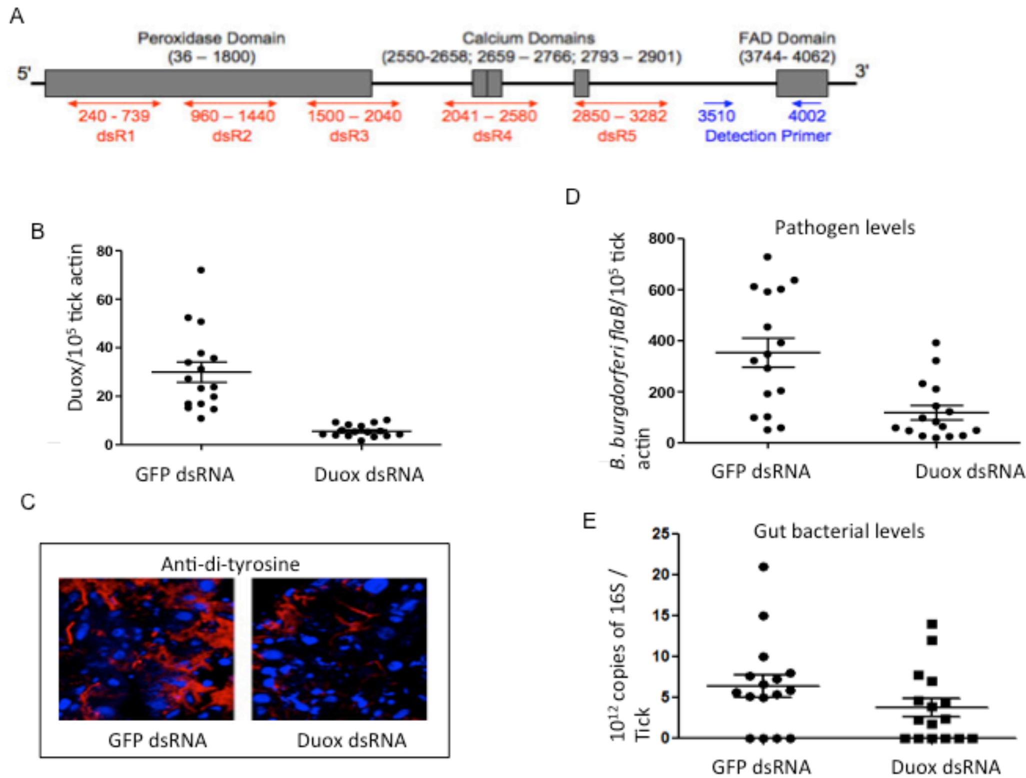

Fig. 3. Knockdown of *Duox* impairs DTN formation and influences persistence of *B. burgdorferi* in feeding ticks. (**A**) Schematic representation of *Duox* open reading frame showing regions targeted for RNA interference. Nucleotide positions of identifiable domains, as identified by NCBI conserved domain database search (gray boxes), regions encompassing dsRNA constructs (dsR1-dsR5, red), and detection primers (blue), are shown. Out of these dsRNA constructs tested, dsRNA2 (spanning nucleotide positions 960-1440) was most effective and used for subsequent studies. (**B**) Knockdown of *Duox* transcripts induced by RNA interference. Nymphal ticks (25/group) were injected with Duox dsRNA or control GFP dsRNA and fed on naïve mice, collected at 48h after onset of feeding, isolated guts were processed for measurement of *Duox* transcripts. Left panel donates RT-PCT analysis using primers spanning dsR1 region within peroxidase domain of Duox, which indicated a dramatic reduction of corresponding transcripts in dsR2-injected ticks. Right panel showed quantitative RT-PCR analysis using detection primers against *Duox* (Fig. 2A), and the results normalized against tick *β-actin*. Each circle represents an individual tick that was processed and analyzed separately. Compared to ticks microinjected with GFP dsRNA, ticks injected Duox dsRNA experienced significant downregulation of *Duox* expression ($p < 0.01$). (**C**) *Duox* knockdown interferes with the formation of the DTN. After 60 hours of feeding, ticks were collected, and the guts were dissected. DTN formation was detected using anti-dityrosine antibodies (red) and a nuclear stain, DAPI (blue) and imaged under a confocal microscope. (**D**) Knockdown of *Duox* expression reduces *B. burgdorferi* persistence in engorged ticks. Ticks were microinjected with Duox dsRNA or GFP dsRNA and allowed to parasitize mice that were infected with *B. burgdorferi*. After feeding, ticks were collected and *B. burgdorferi* burden in ticks was detected by measuring *flaB* transcripts and normalized with tick *β-actin*. Each dot represents an individual tick sample. Compared to ticks microinjected with GFP dsRNA, *B. burgdorferi* burden in ticks was significantly reduced after injection of Duox dsRNA ($p < 0.01$).(**E**) Knockdown of *Duox* expression reduces gut bacterial levels in engorged ticks. Ticks were microinjected with Duox dsRNA or GFP dsRNA and allowed to parasitize mice. After feeding, ticks were collected and *B. burgdorferi* burden in ticks was detected by measuring 16s genomic copies per tick. Each dot represents an individual tick sample. (ns, $p < 0.14$)

Identification of a gut peroxidase involved in DTN formation

Since previous studies suggested that DTN formation could require participation of a specific gut peroxidase (Kumar et al., 2010), the tick gut was labeled using an anti-peroxidase antibody. The results showed that peroxidase labeling in the gut readily co-localized with the DTN labeling (**Figure 4A**). A further search for the existence of a gut peroxidase that is potentially involved in DTN formation was conducted. The *Ixodes* genome encodes for at least 16 potential peroxidase proteins. RT-PCR analyses indicated that 11 out of 16 peroxidases showed dramatic yet variable levels of transcripts in feeding ticks (**Figure 4B**). Whether their expression is modulated during spirochete infection was assessed by comparing corresponding mRNA levels in ticks that fed on *B. burgdorferi*-infected versus naïve mice. The data showed that, while expression of a majority of these peroxidases remains unaltered in the presence of *B. burgdorferi*, two of them (annotated as *ISCW017368* and *ISCW002528*) displayed dramatic induction when spirochetes are present in feeding ticks (**Figure 4C**, $p < 0.01$). Subsequently, a measurement of transcript levels of both of these genes suggested their induction at various different time points of tick feeding (**Figure 4D and 4E**), while mRNA levels of *ISCW002528* peak between 6-12 hours, ISCW017368 was most dramatically expressed between 24-36 hours.

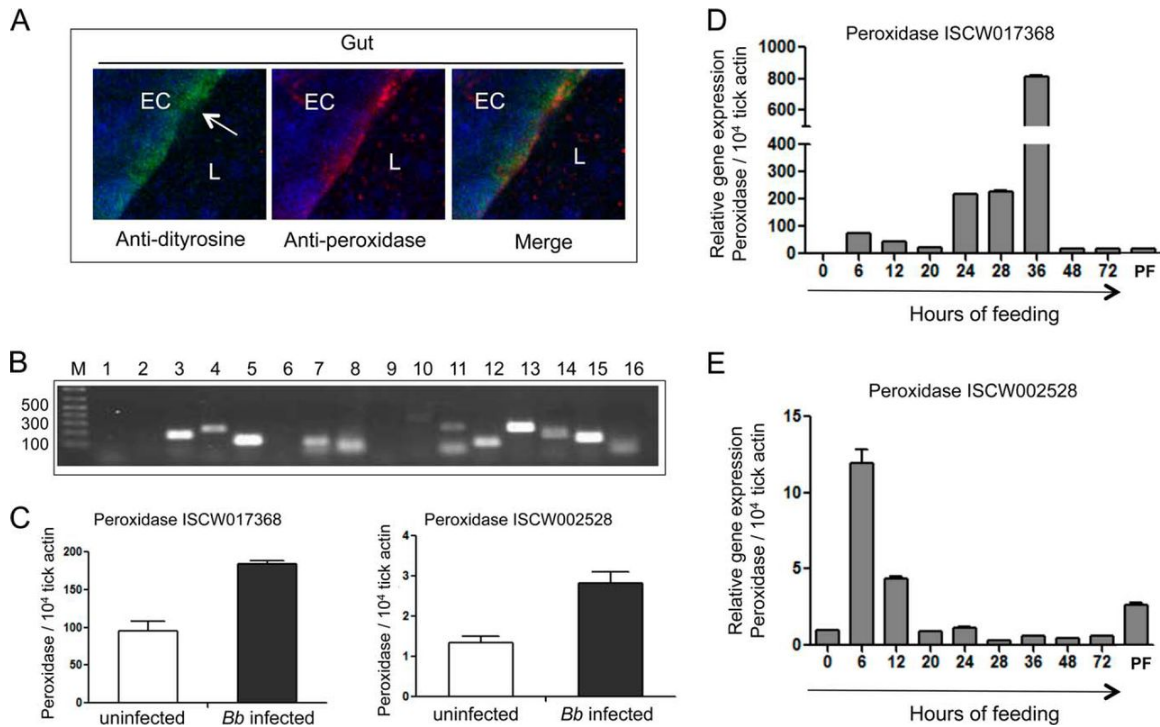

Fig. 4. Localization and expression of gut peroxidases. (**A**) Colocalization of peroxidase and DTN in fed tick gut. Confocal immunofluorescence staining of tick gut at 60 hours of feeding was performed using dityrosine antibodies (Green), peroxidase antibodies (Red), and DAPI. Arrow indicates DTN; EC, epithelial cell; L, luminal space. (**B**) Expression of 16 annotated tick peroxidases during feeding. Groups of ticks were collected at 48 hours of feeding and expression of the following 16 peroxidase genes (lane numbers denoted in parentheses) in feeding ticks was detected by RT-PCR: *ISCW013767* (1), *ISCW018825* (2), *ISCW017070* (3), *ISCW005828* (4), *ISCW022517* (5), *ISCW013159* (6), *ISCW008495* (7), *ISCW002528* (8), *ISCW001759* (9), *ISCW017368* (10), *ISCW022537* (11), *ISCW015098* (12), *ISCW019584* (13), *ISCW024650* (14), *ISCW020571* (15), *ISCW020569* (16) (**C**) Identification of two peroxidases that are upregulated during *B. burgdorferi* infection in ticks. Ticks were allowed to feed on naïve mice or *B. burgdorferi*-infected mice, collected at 48 hours of feeding and processed for qRT-PCR using gene-specific primers, as indicated in Supplementary Table 1. While expression of most of the peroxidases did display detectable alteration (data not shown), *peroxidase ISCW017368* and *peroxidase ISCW002528* were significantly upregulated in ticks that fed on *B. burgdorferi*-infected mice, compared to those that fed on naïve mice ($p < 0.01$). (**D**) and (**E**) Kinetics of peroxidase expression. Naïve nymphal ticks were collected at different times of feeding on mice or as fully engorged post-fed (pf) ticks, and transcript levels of *ISCW017368* (panel D) and *ISCW002528* (panel E) were measured by qRT-PCR. Mean ± SEM of three independent infection experiments is presented.

Selective silencing of peroxidase ISCW017368 influences DTN formation and B. burgdorferi acquisition by ticks

Because two of the 16 potential peroxidase enzymes, ISCW017368 and ISCW002528, were induced upon spirochete infection of the ticks, an RNAi-mediated knockdown strategy was used to assess their relative importance in DTN formation and the subsequent persistence of *B. burgdorferi*. dsRNA preparations targeting each gene were constructed to accomplish this. Injected ticks displayed dramatic down-regulation of corresponding genes as compared to control (GFP dsRNA-injected) ticks (**Figure 5A**). Unlike *ISCW002528*-silenced ticks or control ticks, *ISCW017368*-knockdown ticks reflected an obvious impairment of DTN integrity (**Figure 5B**). Accordingly, silencing of *ISCW017368* significantly influenced levels of *B. burgdorferi* in ticks (**Figure 5C**, $p<0.01$) while *ISCW002528* knockdown had no apparent effects (**Figure 5C**, $p > 0.05$), in comparison to corresponding controls. Together, these data suggest that the peroxidase ISCW017368, as well as Duox, play an important role in the formation of the DTN and influence the persistence of Lyme disease pathogens within ticks.

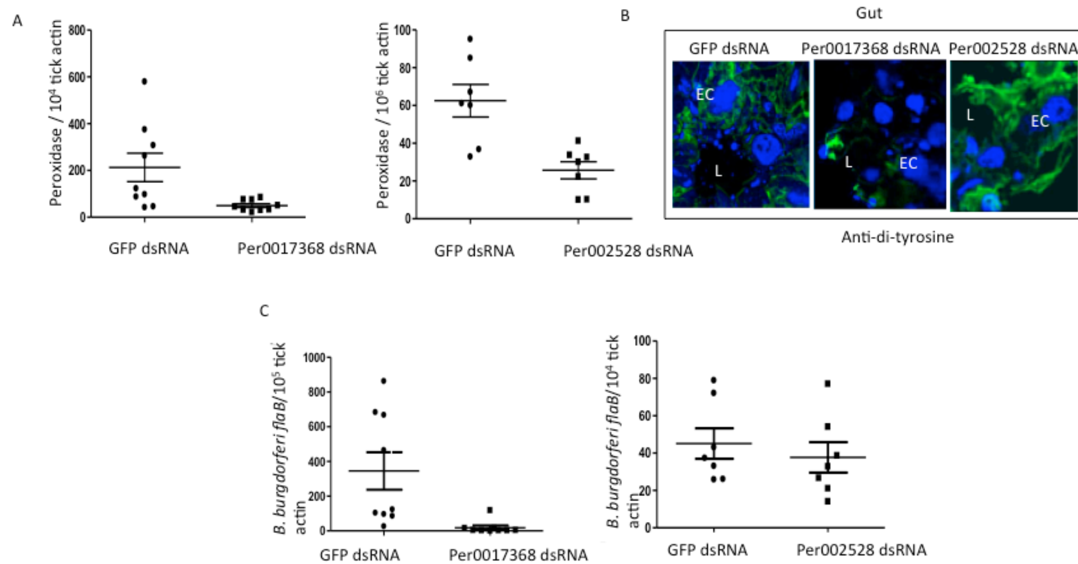

Fig. 5. Knockdown of peroxidase ISCW017368 but not ISCW02528 impairs DTN formation and influences persistence of *B. burgdorferi* in feeding ticks. (**A**) RNA interference-mediated knockdown of peroxidase transcripts in feeding ticks. Nymphal ticks (25/group) were injected with dsRNA from the genes corresponding to ISCW017368 or ISCW002528 (indicated as Per017368 and Per002528, respectively) or control (GFP) and fed on naïve mice, collected at 48h after the onset of feeding, and isolated guts were processed for quantitative RT-PCR using detection primers against target genes and the results normalized against tick *β-actin*. Each circle represents an individual tick that was processed and analyzed separately. Compared to their respective controls (GFP dsRNA), expression of both peroxidases was significantly downregulated ($p < 0.01$). (**B**) Knockdown of peroxidase Per0017368 impaired formation of DTN. The network was detected in dsRNA-microinjected feeding ticks as detailed in panel B using anti-dityrosine (green) and DAPI (blue) for labeling tick cell nuclei. The DTN structures were more obvious in ticks injected with dsRNA for peroxidase ISCW02528 or GFP, compared to those injected with peroxidase ISCW017368 dsRNA. EC, epithelial cell; L, luminal space. (**C**) Knockdown of peroxidase ISCW017368 transcript levels reduces *B. burgdorferi* persistence in feeding ticks. Ticks were microinjected with dsRNA for ISCW017368, ISCW002528, or control (GFP) and allowed to parasitize mice that were infected with *B. burgdorferi*. After feeding, ticks were collected and *B. burgdorferi* burden in ticks was detected by measuring *flaB* transcripts and normalized with tick *β-actin*. Each dot represents an individual tick sample. No significant differences in *B. burgdorferi* burdens between ticks injected with peroxidase ISCW02528 and GFP dsRNA were observed ($p>0.05$) (right panel). However, compared to ticks microinjected with control (GFP dsRNA), those injected with ISCW017368 dsRNA experienced a significant reduction in *B. burgdorferi* burden ($p<0.01$) (left panel).

Induction of nitric oxide synthase potentially contributes to the reduction of B. burgdorferi *in ticks*

Previous results suggested that the RNAi-mediated disruption of the DTN barrier formation could result in a strong pathogen-specific immune response leading to a significant reduction of the pathogen levels. We next examined whether the observed reduction of *B. burgdorferi* levels is due to activation of specific tick innate immune pathway(s) or component(s). The transcript levels of representative potential immune genes from well-known innate immune pathways were compared in *duox*-knockdown and control (GFP dsRNA-injected) ticks. Analysis of a selected set of transcripts grouped under non-self- recognition, signal transduction, and anti-microbial peptide pathways indicated that none of the tested genes showed significant induction in *duox*-knockdown ticks, however, with an exception of the notable induction of a gene encoding nitric oxide synthase (NOS) (**Figure 6A**, $p < 0.05$). In agreement with previous observations showing *B. burgdorferi* responsiveness to nitric oxide (NO)-mediated killing (Ma et al., 1994; Modolell et al., 1994) as well as the anti-parasitic effects of NOS in DTN-silenced mosquitoes (Kumar et al., 2010), the *I. scapularis* NOS gene was also confirmed for its up-regulation in additional groups of *B. burgdorferi*-infected Duox- or peroxidase ISCW017368 knockdown ticks (**Figure 6B**). Enhancement of transcript levels was also complemented by a significant augmentation of total NOS enzymatic activity (**Figure 6C**, $p < 0.05$). Together, these results indicate that the enhancement of potential NO production by NOS could contribute to the destruction of *B. burgdorferi* following impairment of the DTN in ticks.

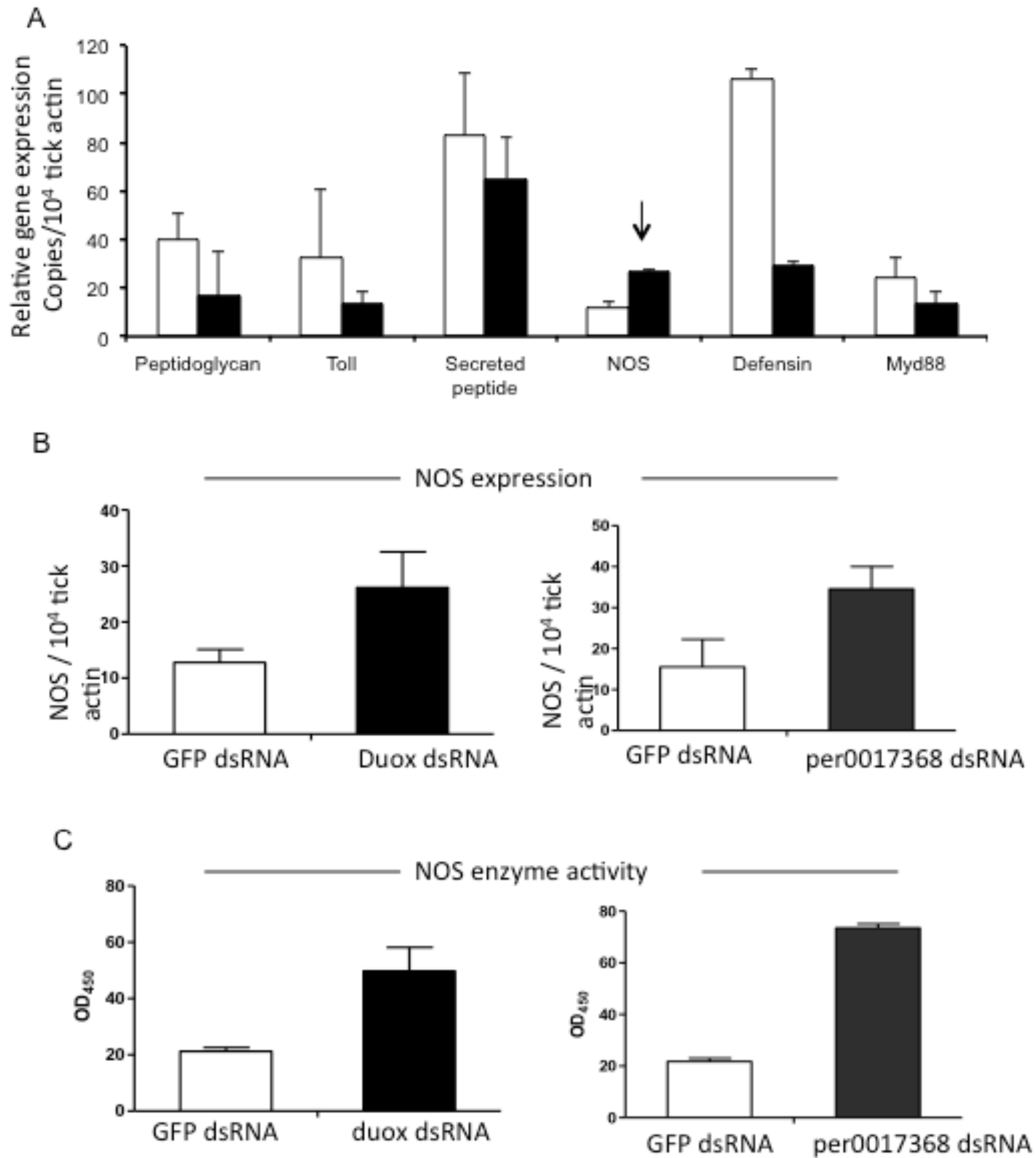

Fig. 6. Knockdown of Duox and peroxidase ISCW017368 induces nitric oxide synthase. (**A**) Expression of representative genes potentially related to innate immune responses in Duox-knockdown ticks. Naïve ticks were microinjected with GFP dsRNA (white bar) or Duox dsRNA (black bar), as detailed in Fig. 2. Transcript levels for selected genes in knockdown and control ticks were measured using qRT-PCR analysis at 48 hours of feeding. While mRNA levels of most genes remained unaltered ($p > 0.05$), expression of the nitric oxide synthase (NOS) gene was significantly upregulated in Duox dsRNA ticks compared to that of corresponding control (GFP dsRNA) ($p < 0.05$). (**B**) Effects of Duox or peroxidase ISCW017368 knockdown on NOS gene expression during tick feeding on *B. burgdorferi*-infected mouse. Knockdown of corresponding Duox or peroxidase ISCW017368 gene expression was performed as detailed in panel A and significantly induced NOS mRNA expression in ticks ($p < 0.05$). **(C)** Enzymatic activity of NOS. Activity of NOS was measured in ticks microinjected with either Duox dsRNA (left panel) or peroxidase ISCW017368 dsRNA (right panel), reflecting significant enhancement of NOS enzyme activity compared to ticks injected with GFP dsRNA ($p < 0.05$).

Table 11: Oligonucleotide primers used in Dual Oxidase Study

Sequence (5' to 3')	**Purpose**
TGTCCTGGAGGGACAAGTTC	ISCW013159 Forward for RT-PCR and qPCR
TTCAGAACTGCGCAACATTC	ISCW013159 Reverse for RT-PCR and qPCR
GATAAGCGGCAGGATGTGAT	ISCW017070 Forward for RT-PCR and qPCR
TGAAGCTGTGTCCGACTTTG	ISCW017070 Reverse for RT-PCR and qPCR
GGTCAGCTCTAAAGCCATGC	ISCW022537 Forward for RT-PCR and qPCR
AACGGAGTGACCAGTTGACC	ISCW022537 Reverse for RT-PCR and qPCR
TCGGGACATCGATCTCTTTT	ISCW024650 Forward for RT-PCR and qPCR
GCCATCGACGTTTTACGAAT	ISCW024650 Reverse for RT-PCR and qPCR
GTAAAAGCGGAACGCAAGAG	ISCW001759 Forward for RT-PCR and qPCR
TGATTTTCCCCGTTCACTTC	ISCW001759 Reverse for RT-PCR and qPCR
CGAAACCAGACACATCATGG	ISCW002528 Forward for RT-PCR and qPCR
GAGGAGCGTGAGGTTGTAGC	ISCW002528 Reverse for RT-PCR and qPCR
AGCCTCAGAACGTTCGAAAA	ISCW005828 Forward for RT-PCR and qPCR
TCTTCATCCTCCCAATGAGG	ISCW005828 Reverse for RT-PCR and qPCR
ACATCCGTTGGAACTTCGAG	ISCW008495 Forward for RT-PCR and qPCR
GCGTCTTCATCACGACTGAG	ISCW008495 Reverse for RT-PCR and qPCR
GCTGTCGAGCACCTCATACA	ISCW013767 Forward for RT-PCR and qPCR
GCAGGTTTGGAGATCTGAGC	ISCW013767 Reverse for RT-PCR and qPCR
CACCCTAAGGCTCAGACTCG	ISCW017368 Forward for RT-PCR, qPCR,

	and RNAi detection
TGGATGCGTATGAGAATGGA	ISCW017368 Reverse for RT-PCR, qPCR, and RNAi detection
CTCAACGTCAAGTGCCTCAA	ISCW018825 Forward for RT-PCR and qPCR
GACTTGTCCCGCAGAGTAGC	ISCW018825 Reverse for RT-PCR and qPCR
CCAACTTTCCCATGTTCCAG	ISCW020571 Forward for RT-PCR and qPCR
GAAGTTCCAACGGATGTCGT	ISCW020571 Reverse for RT-PCR and qPCR
AGACTACGGCAGCGACAACT	ISCW020569 Forward for RT-PCR and qPCR
ATGTCCTCCGCTATGTCGTC	ISCW020569 Reverse for RT-PCR and qPCR
CTGTTCAACGCCATCAAATG	ISCW015098 Forward for RT-PCR and qPCR
ATGCATGGTTGCGAAATGTA	ISCW015098 Reverse for RT-PCR and qPCR
CCTGACGCAGGAACACTACA	ISCW019584 Forward for RT-PCR and qPCR
ATTTGATGGCGTTGAAGAGG	ISCW019584 Reverse for RT-PCR and qPCR
GCTGCATGAGAAGTTTGCAG	ISCW022517 Forward for RT-PCR and qPCR
GCTGAACATGTCGAACTGGA	ISCW022517 Reverse for RT-PCR and qPCR
CGAGAAGCAGCGCTACGA	ISCW007865 Forward for RT-PCR and qPCR
CATCAACACTTCCGAGGAGA	ISCW007865 Reverse for RT-PCR and qPCR
TCCTACGGGAGGCAGCAGT	16s rRNA Forward for RT-PCR and qPCR
GGACTACCAGGGTATCTAATCCTGTT	16s rRNA Reverse for RT-PCR and qPCR
GGGGTACCCGTCCTCTTCGTCT	Dual Oxidase dsR1 Forward

TCTTCG	
GCGAGCTCGTTCATGATGCCCAGGTAGC	Dual Oxidase dsR1 Reverse
GGGGTACCCTACAGGAGGGACCGGAGTT	Dual Oxidase dsR2 Forward
GCGAGCTCGTCCAGGTTGTCGTGGTACA	Dual Oxidase dsR2 Reverse
GGGGTACCGCTTCTGGTTCGAGAACGTC	Dual Oxidase dsR3 Forward
GCGAGCTCATGTAGGCGACCTCGTTTCC	Dual Oxidase dsR3 Reverse
GGGGTACCGCGCTTCGTGAAAGTGAAG	Dual Oxidase dsR4 Forward
GCGAGCTCTTCCAATTTCCGCTTCTCG	Dual Oxidase dsR4 Reverse
GGGGTACCTCAAGAAGATGATGAAGGAGCA	Dual Oxidase dsR5 Forward
GCGAGCTCATCAGGTTCCGACACATGG	Dual Oxidase dsR5 Reverse
GGTATCACGGGAGTGTGCTT	Dual Oxidase RNAi Detection Forward
CAAGTTGTTGGGGTCGAAAT	Dual Oxidase RNAi Detection Reverse
GATAGCCACGTCCGACTGCG	ISCW002528 dsRNA Forward
GTCCGAGAGGTGTCGATGAG	ISCW002528 dsRNA Reverse

AGATGTGGGACATGTGCCCGG	ISCW002528 RNAi Detection Forward
GTCCGCCGCTTGTCGAGGAC	ISCW002528 RNAi Detection Reverse
CTGCCTACCAAGACGGCATCA	ISCW017368 dsRNA Forward
AGGTCCAGGTACGAGGTCAGA	ISCW017368 dsRNA Detection
AATGAGCTCGAGGTGAAGTTC GAGGGCGA	GFP dsRNA Forward
AATGGTACCTCCATGCCGAGA GTGATCCC	GFP dsRNA Reverse
TTGCTGATCAAGCTCAATATAA CCA	Forward primer for *B. burgdorferi flaB* quantitative RT-PCR (qRT-PCR).
TTGAGACCCTGAAAGTGATGC	Reverse primer for *B. burgdorferi flaB* qRT-PCR.
AGAGGGAAATCGTGCGTGAC	Forward primer for tick *β-actin* qRT-PCR
CAATAGTGATGACCTGGCCGT	Reverse primer for tick *β-actin* qRT-PCR
GATGACCCAGATCACCTG	Forward primer for tick *β-actin* RT-PCR
GCCGATGGTGATCACCTG	Reverse primer for tick *β-actin* RT-PCR
GCTTGGTGGACGAAGAACTG	Toll qPCR Forward
TGAACGTTGGTGACTTTTGG	Toll qPCR Reverse
TGGTCTGACATCGGCTACAG	PGRP qPCR Forward
TTGATACCGCAGGCGATAAG	PGRP qPCR Reverse
CAAGAAGGACTCCTGCCAAG	Secreted peptide qPCR Forward
CTTGAGCCAGTCCAGGTAGC	Secreted peptide qPCR Reverse
ACTTCGGACTCCCAGCTGTA	NOS qPCR Forward

ATGACGTCGCCACAGACATA	NOS qPCR Reverse
TGCAACGGACCCTTCAATA	Defensin qPCR Forward
GGTCGTTTCAGTTGCCTCTC	Defensin qPCR Reverse
TTGAGCAAGCTCAGCAGAAA	Myd88 qPCR Forward

Discussion

B. burgdorferi thrives in nature though an intricate tick-mammal infection cycle (Radolf et al., 2012, Pal and Fikrig, 2003). Even though there has been extensive research on various aspects of Lyme borreliosis, precisely how the pathogen successfully persists in and is transmitted through ticks remains enigmatic, despite a potentially rigorous arthropod innate immune response against invading microorganisms known to exists (Govind and Nehm, 2004, Hoffmann and Reichhart, 2002; Tanji et al., 2007; Tanji and Ip, 2005; Valanne et al., 2011). Here evidence is presented indicating that a molecular barrier, the dityrosine network (DTN), exists in ticks and that could represent a protective strategy to preserve homeostasis of gut commensals that replicate during feeding. Similar to the peritrophic matrix described in the gut of most insects including ticks (Kariu et al., 2013; Zhu et al., 1991), this molecular wall is normally synthesized in response to blood feeding and creates a protective barrier between luminal contents and the underlying gut epithelial cells. In *Drosophila*, the matrix is part of a protective innate immune response since increased susceptibility to bacterial toxins is observed in its absence, (Ha et al., 2005). In contrast, as shown in *Anopheles gambiae* earlier (Kumar et al., 2010), formation of the DTN in ticks also likely decreases the gut permeability to immune elicitors, thereby inadvertently protecting tick-borne pathogens like *B. burgdorferi.* This work shows that DTN formation in *I. scapularis* ticks requires participation of a homologue of NADPH oxidase (NOX), a tick dual oxidase (Duox), along with a specific gut peroxidase. Together, these results highlight a less well-known function of an NOX system in protein cross-linking activity in diverse cellular functions such as cross-linking of the extracellular matrix, cuticular proteins in *Caenorhabditis*

elegans to eggshell hardening, or epithelial barrier (DTN) functions in insects, and as presented here, for the first time, in *Ixodes* ticks as well.

Originally identified in the thyroid, Duox enzymes have been shown to promote formation of dityrosine linkages (Donko et al., 2005). In mammals, formation of these linkages relies upon heme peroxidase activity, such as thyroperoxidase or myeloperoxidase, utilizing hydrogen peroxide often formed from NOX activity. However, Duox contains an extracellular peroxidase domain, which appears to bind heme and serve as a functional peroxidase in *C. elegans* (Edens et al.,2001; Meitzler et al.,2009). Mutations in the peroxidase domain of *C. elegans* Duox lead to a blistering phenotype due to loss of protein crosslinking, without impaired ROS generation and host defense dependent upon Duox (Chavez et al., 2009). The same domain in human Duox, however, does not bind heme nor exert peroxidase or tyrosine crosslinking activity (Meitzler et al., 2009). The sequence analysis data developed here (**Figure 7**) suggests that Duox enzymes in mosquito and tick are also conserved, but lack a key Glu (Glu238) residue that, when mutated in *C. elegans*, impairs heme binding (Meitzler et al., 2010). The mutated protein still has residual peroxidase activity and can mediate tyrosine crosslinking in *C. elegans* (Meitzler et al., 2010). This suggests that arthropod Duox may retain some peroxidase/crosslinking function, and low levels of dityrosine immunoreactivity remains in *ISCW017368* knockdown ticks. Nevertheless, it appears that both Duox and a heme peroxidase are required for proper DTN formation in both mosquito and tick gut.

Fig. 7. Amino acid sequence alignment of potential peroxidase domain of *I. scapularis* Duox with orthologs from other model nematode and arthropod species. Partial amino acid sequences (annotations are shown according to NCBI protein sequences) from *I. scapularis* (EEC10543.1) *C. elegans* (O61213.2), *D. melanogaster* (AAF51201.2), and *A. gambiae* (XP_319115.4) were aligned using Cobalt Constraint-based Multiple Protein Alignment Tool (NCBI). The key residues for heme binding and peroxidase function are identified based upon previous studies on Duox peroxidase domains (PMID: 20947510; PMID: 19460756) and their conservations or deviations are indicated with colored letters and boxes. Alteration of a key glutamic acid residue (Glu238) in *C. elegans* that is important of heme binding (41,43) in *I. scapularis* or other arthropods is indicated in red font.

The *Ixodes* genome is relatively large (approximately 2 Gb) and highly redundant with nearly 70% repetitive DNA. Although it encodes a single Duox, there are at least 16 potential peroxidases, eight of which are annotated as heme peroxidases, four as glutathione peroxidases, three as phospholipid-hydroperoxide glutathione, one as thioredoxin peroxidase, and one as cAMP-regulated phosphoprotein/endosulfine. While each of these enzymes could play individual and potentially important roles in tick physiology and immunity, 11 are dramatically expressed in feeding ticks. Two of these, peroxidase ISCW002528 and peroxidase ISCW017368, are strongly up-regulated in the presence of *B. burgdorferi* suggesting that they could be related to vector immunity. In fact, the data indicate that the latter, which is annotated as a heme peroxidase, is an

indispensible partner of the tick Duox system essential for DTN formation. Although the molecular basis of DTN formation remains unknown, in mosquitoes, the participation of a heme peroxidase is also required (Kumar et al., 2010). Thus, results presented here underscore the importance of this specific class of peroxidases in tyrosine cross-linking critical for DTN formation. The blistering phenotype of peroxidase domain mutants in *C. elegans* Duox suggests the lack of additional peroxidase partner contribution to protein crosslinking in the nematode (Edens et al., 2001; Chavez et al., 2009). However, a peroxidase (MLT-7) has been identified in *C. elegans*, in which loss of expression leads to significant decreases in tyrosine crosslinking of cuticle proteins and a blistering phenotype (Thein et al., 2009; Moribe et al., 2012). In aggregate, the accumulated data suggest that although Duox in higher organisms exhibit absence or loss of tyrosine crosslinking activity, separate heme peroxidases have evolved to complement this function of Duox to mediate or enhance DTN formation, extracellular matrix modification or host defenses from ROS produced by Duox.

Studies involving mucosal surfaces in mammalian models and Drosophila gut suggest that ROS derived from Duox play an antimicrobial role (Donko et al., 2005). However, data presented here, and that in mosquito (Kumar et al., 2010), suggest that Duox does not exert microbicidal effects in the arthropod gut, since knockdown inhibits bacterial burden. A major fraction of *Plasmodium* parasites has previously been shown to be killed by a vigorous mosquito innate immune response, yet the existence of an analogous phenomenon in ticks, particularly pertaining to the control of *B. burgdorferi* levels in the gut, is unknown. In this work it has been shown that transcript levels of

NOS or their enzymatic activity in Duox-knockdown ticks are significantly enhanced, which may be linked to a potential nitric oxide (NO)-mediated borreliacidal response. Although it has been suggested to play an insignificant role, particularly in murine host immune response against *B. burgdorferi*, NO possesses potent borreliacidal properties against cultured spirochetes and thus could be responsible for the reduction of borrelial numbers in fed ticks. The attempt to study the possible involvement of NOS in the generation of borreliacidal responses in ticks and assess if RNAi or *in vivo* inhibitor-mediated interference of NOS reverses the deleterious effect of Duox silencing on *B. burgdorferi* survival was unsuccessful but could provide clues for the biological significance of NOS up-regulation in ticks with impaired DTNs. Nevertheless, NO production by intestinal epithelial cells has been directly linked to the vector's immune response to other enteric pathogens, including DTN-silenced mosquitoes, and thus likely represents a conserved microbicidal response in diverse arthropod species.

In summary, the evidence presented here that a molecular barrier, recently proposed in studies using malarial vectors and called DTN, catalyzed by a Duox/peroxidase system allows *B. burgdorferi* to proliferate without activating epithelial immunity, such as the NOS enzyme. Arthropod genes that are temporally induced in particular vector tissues could be functionally relevant for *B. burgdorferi* persistence *in vivo.* If any such gene products play beneficial roles in pathogen persistence and/or transmission, this information could be used to develop novel preventive measures to interfere with the spirochete infection cycle. The data show that proteins critical for formation of the DTN, the NADPH oxidase or Duox, as well as a heme peroxidase

protein, are inducible upon *B. burgdorferi* infection and support their persistence in the vector. Gene products involved in innate immunity are conserved across species; however, some are unique to a given species or markedly diversified, which may reflect adaptation to different pathogens, thus presenting an opportunity to assess their roles as immunoprotective antigens.

A portion of this chapter has been published previously:

Yang X*, **Smith AA* (*Co-first authors)**, Williams MS and Pal U. (2014) A Dityrosine Network Mediated by Dual Oxidase and Peroxidase Influences the Persistence of Lyme Disease Pathogens within the Vector. *Journal of Biological Chemistry*. doi:10.1074/jbc.M113.538272

Chapter 4: Dual oxidase immunization and evaluation of protective immunity against *B. burgdorferi* challenge

Introduction

Ixodes ticks are known to transmit a wide variety of pathogens including viruses, bacteria, and protozoan such as tick borne encephalitis, Lyme, and *Babesia* respectively. Since these pathogens are known to infect humans as well as other mammals including those important to agriculture in a global basis, these vectors are of great importance medically. Many recent studies have shown that targeting tick specific proteins can alter the arthropods physiology as well as reduce the pathogen transmission (Merino et al., 2013). Thus, conceptually, by targeting the vector many pathogens that are transmitted by the same arthropod species could be controlled with one vaccine.

In the early 1990's two vaccines were commercialized for the control of cattle ticks. Those two vaccines, TickGARD and Gavac, were recombinant forms of a midgut membrane bound glycoprotein BM86. (Merino et al., 2013) This vaccine reduced the number, weight and reproductive capacity of engorging female ticks. This reduction helped in the controlling of babesiosis in vaccinated cattle. However, BM86 displays remarkable diversity in various tick species and existing of an *I. scapularis* homolog remains obscure. (Merino et al., 2013)Many other *I. scapularis* antigens have been proposed as potential vaccine candidates, including two different salivary proteins, Salp15 and Salp25D (Merino et al., 2013). *B. burgdorferi* is known to exploit the saliva

of the tick to facilitate pathogen transmission. Salp15 is a secreted salivary protein with immunosuppressive properties that inhibits pathways of the innate immune response. Antibodies directed against Salp15 showed to significantly reduce the number of bacteria transmitted to immunized mice (Merino et al., 2013; Anguita et al., 2002). Salp25D, another salivary protein, was shown to be expressed within the gut of the tick as well. Immunization with recombinant Salp25D reduced the number of *Borrelia* that was acquired but did not affect the transmission of the bacteria (Merino et al., 2013; Narasimhan et al., 2007). Neither Salp15 or Salp25D fully protected the host from infection, thus a need to identify a protective antigen still exists.

As shown in chapter 3, Duox is a protein that is important in the formation of the DTN. This network influences bacterial colonization in the gut and plays an important role in transmission. Thus antibodies directed against Duox could potentially interfere with the protein function thus influencing the formation of the network and reduce transmission. The purpose of chapter 4 is to explore this possibility.

Methods

Mice, bacterial strain, and ticks

Five-week-old female C3H/HeN mice were purchased from the National Institutes of Health. All animal experiments were performed in accordance with the guidelines of the Institutional Biosafety Committee and the Institutional Animal Care and Use Committee of the University of Maryland. The *B. burgdorferi* strain B31 A3 is an infectious, clonal derivative of the type strain B31 (Elias et al., 2002). This strain was used throughout this study. *Borrelia* cultures were grown in Barbour-Stoenner-Kelly H (BSK-H) media supplemented with 6% rabbit serum at 34°C. *I. scapularis* ticks used in the present study were reared in the laboratory as described elsewhere.

Phylogenetic tree

Phylogenetic tree was generated using the National Institute of Allergy and Infectious Diseases Bioinformatics Resource Center. Homologous proteins from mice, humans, mosquitos and drosophila were compared using BLASTP software. Next a tree was constructed by viewing the distance tree of results.

Protein expression, purification, polyclonal antibody preparation, and Western blotting

After *in silico* analysis including BLAST search and sequence alignment efforts, two regions within the peroxidase domains of Duox were found to be unique to *I. scapularis*. These regions were named as Peroxidase-1 and Peroxidase-2. Recombinant versions of peroxidase-1 and peroxidase-2 were produced in *E. coli* using the bacteria expression vector pGEX-6P-1 with specific primers listed in Table 12. Expression, purification and enzymatic cleavage of the glutathione transferase (GST) fusion proteins were performed as detailed. *E. coli* clones expressing Peroxidase-1 or Peroxidase-2 were grown overnight in 5 mL of LB medium containing ampicillin at 37° C using a shaking incubator. The next morning, cultures were inoculated into flasks containing 500mL media. These were grown in a shaking incubator to a density where the OD_{650} reached 0.5 at 37° C. Cells were then induced with 0.1M IPTG and left in the incubator for an additional 4 hours prior to centrifuging at 5,000 RPM for 30 minutes. Bacterial pellets were treated with 20 mL of 1% Triton X-100 in PBS, left for 30 minutes on ice, and sonicated under cold conditions. Lysates were centrifuged at 12,000xg for 30 minutes at 4°C to remove the insoluble protein fraction. The supernatant was incubated with Glutathione Sepharose^{TM} 4B(GE Healthcare) for 1 hour. The beads were then washed to eliminate any non-bound proteins. The protein of interest was cleaved overnight using PreScission protease (GE Healthcare)

Polyclonal antibodies against the two *I. scapularis* specific Duox regions were generated in mice as previously described (Kariu et al., 2013). Briefly, ~10 mg/animal was emulsified in complete Freund's adjuvant (Sigma) Animals were boosted at 10 day intervals with the same dose of antigen in incomplete Freund's adjuvant (Sigma). Sera

was collected two weeks following the shown experimentally that this domain is exposed outside the cell second boost. Titers were determined via ELISA as well as antigen specific Western blotting.

Table 12 Oligonucleotide primers used in dual oxidase immunization studies

Sequence (5′ to 3′)	Purpose
CGCGGATCCAGAGCCGTACTCGTCGCA	Peroxidase-1 Forward
CCGGAATTCTTACAGTCCGTCCTCGCCCTT	Peroxidase-1 Reverse
CGCGGATCCCGACGTCAAGACGGGCCA	Peroxidase-2 Forward
CCGGAATTCTTAGGCCGCGATGACGTATCT	Peroxidase-2 Reverse

Localization of dual oxidase via confocal immunofluorescence microscopy

To prepare samples for confocal microscopy, tissues from engorged ticks were fixed for 24 h at 4°C in 4% (w/v) paraformaldehyde in PBS followed by infiltration with 30% sucrose. Cryosectioning was performed at -20°C using a cryotome, and 5 μm thick sections were incubated in PBS with 5% goat serum and 0.5% Tween 20. The sections were labeled with mouse peroxidase-1 or peroxidase-2 polyclonal antibodies, rabbit anti-peroxidase antibodies, or isotype control (mouse or rabbit IgG, Sigma) followed by Alexa-488-labeled goat anti-mouse or Alexa-568-labeled goat anti-rabbit IgG (Invitrogen). Slides were washed using PBS with 0.05% Tween-20, stained with DAPI (Invitrogen), and imaged by a LSM 510 laser confocal microscope (Zeiss).

Immunization of mice with Peroxidase-1 and Peroxidase-2 to assess tick transmission

Groups of mice (4 animals/group) were immunized with adjuvant containing either recombinant peroxidase-1, peroxidase-2 or adjuvant containing the same volume phosphate buffered saline (PBS) as described in the above paragraph. Ten days after the final boost, mice were parasitized with *B. burgdorferi* infected ticks (3 ticks/ mouse), which were allowed to feed to completion. Fully fed ticks were collected and mice were sacrificed 14 days later. Skin, heart, bladder, and joints were collected from each mouse. RNA was isolated from the tissues as well as a pooling of ticks and *B. burgdorferi* burden was measured using quantitative PCR.

Bioinformatics and statistical analysis

Unless indicated otherwise, protein annotation and searches were executed using the VectorBase (www.vectorbase.org) and BLAST (NCBI) databases. Results are expressed as the mean ± standard error (SEM). Statistical significance of differences observed between experimental and control groups were analyzed using GraphPad Prism version 4.0 (GraphPad Software, CA). A two-tailed Student's *t*-test was utilized to compare the mean values, and $p < 0.05$ was considered significant.

Results

Organization and divergence of Dual Oxidase

Results of *in silico* analysis showed that there were two highly specific regions within the peroxidase domain (**Figure 8**). Considering the divergence of *I. scapularis* Duox in comparison to mosquito, *Drosophila*, mice, and human, and existence of tick-specific Duox regions, unique fragment of the protein would be an important target for host vaccination (**Figure 9**)

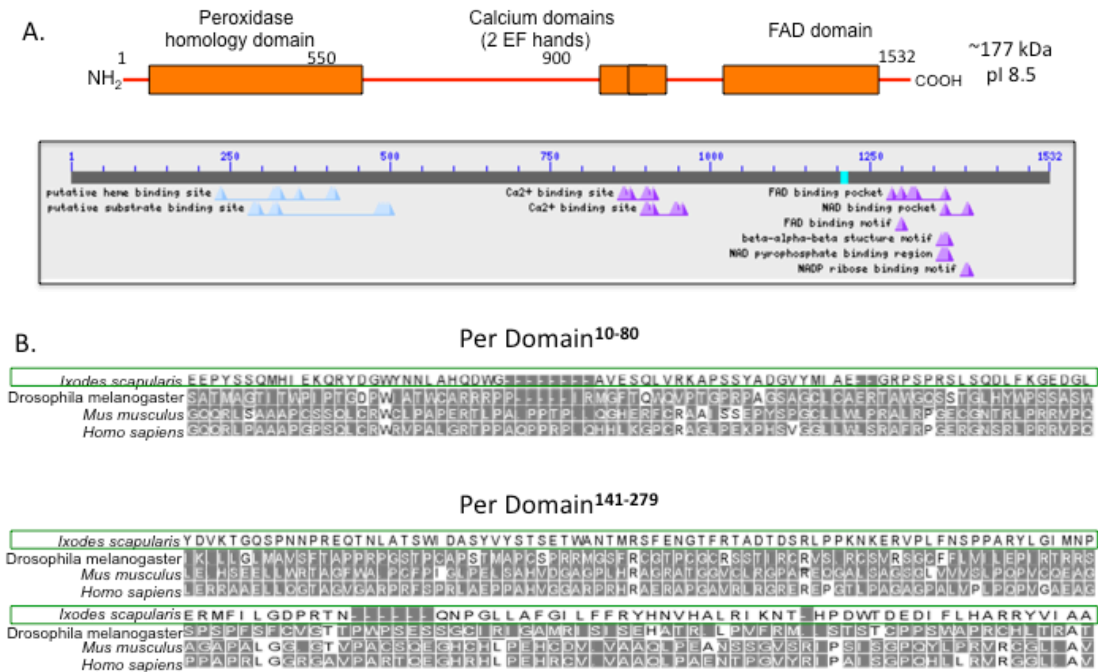

Figure 8 Organization of Dual Oxidase. (**A**) Organization of dual oxidase indicates that there are three major domains present within the protein. The first domain is a peroxidase domain, the second calcium binding domains, and the third being an FAD binding domain. (**B**) Alignment comparisons between *Ixodes, Drosophila melanogaster, Mus musculus, and Homo sapiens* of two tick specific regions within the peroxidase domains.

Figure 9 Divergence of Dual Oxidase. Phylogenetic tree comparison of dual oxidase between *Ixodes, Drosophila melanogaster, Mus musculus, and Homo sapiens.*

Duox is predicted to be a transmembrane protein and polyclonal antibodies against Duox indeed indicated that Duox is surface exposed. This protein was found to be produced only on the lumen of the gut in the same location as the DTN, agreeing with the previously discussed data in chapter 3. (**Figure 10**)

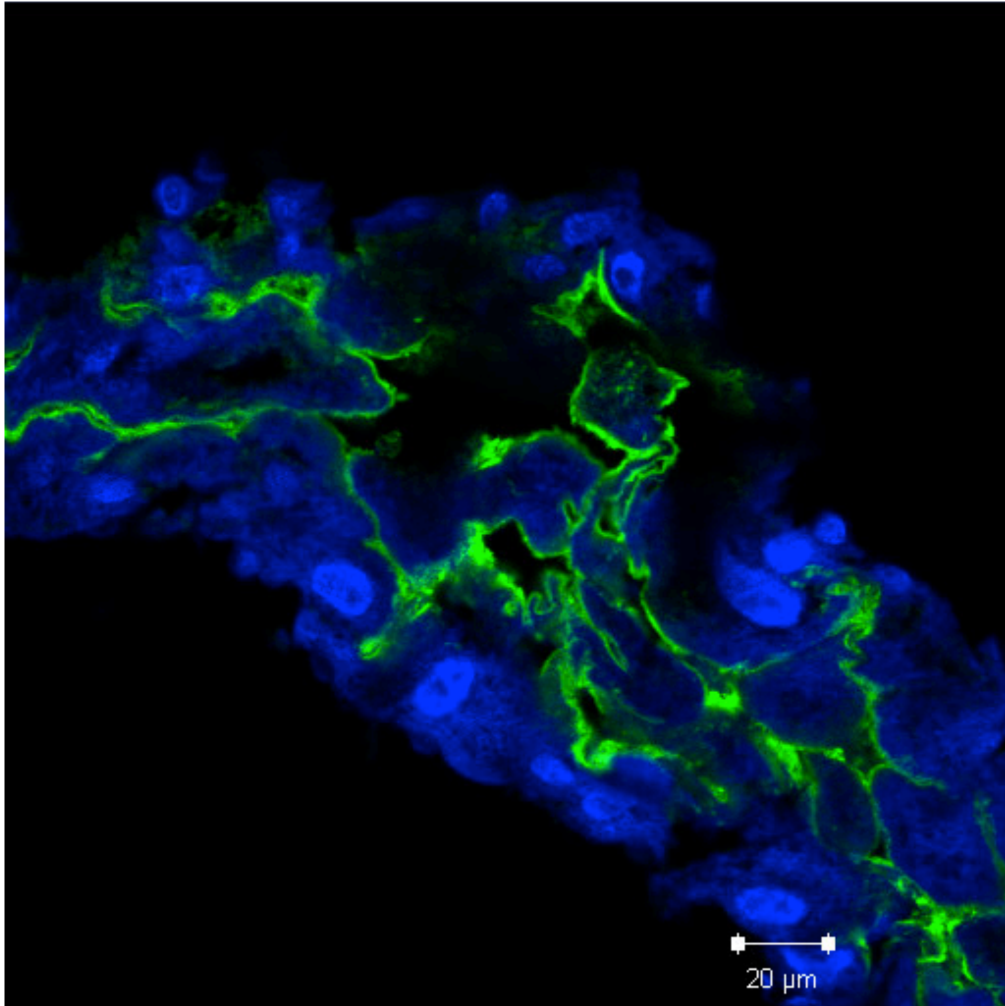

Figure 10 Dual Oxidase is localized to the lumen of the *I. scapularis* gut. Confocal immunofluorescence staining of tick gut at 60 hours of feeding was performed using anti-duox antibodies (green) and DAPI (blue) to stain the nuclear.

Duox immunization could interfere with transmission of *Borrelia* or the tick feeding given that Duox was found to be exposed on the luminal surface of the *I. scapularis* gut and that it was also shown in Chapter 3 to be essential in the formation of the DTN. This possibility was explored as follows. To mimic the natural transmission of *B. burgdorferi,* infected nymphs were generated by allowing larva to acquire spirochetes from infected mice and then molt into nymphs. These infected ticks were then placed on mice immunized with PBS, Peroxidase-1, or Peroxidase-2. Mice were sacrificed and the bacteria burden was analyzed via qRT-PCR. The ticks that fed on these immunize mice

were also analyzed for bacteria burden after feeding. When analyzing the bacteria burden in the sacrificed mice there was a significant decrease in *flab* expression levels within the skin indicating that both fragments gave some protection. This experiment was repeated independently with different protein preparations for immunization. The first experiment showed that there was not a significant decrease in bacteria levels of mice immunized with Peroxidase-1. This data set contained an outlier and the repeat experiment showed a 90% reduction in transmission (**Figure 11**). Peroxidase-2 showed a 99% and 88% reduction in bacterial levels in experiment 1 and 2 respectively. Neither experiment resulted in any decrease in the bacteria levels within the fed immunized ticks.

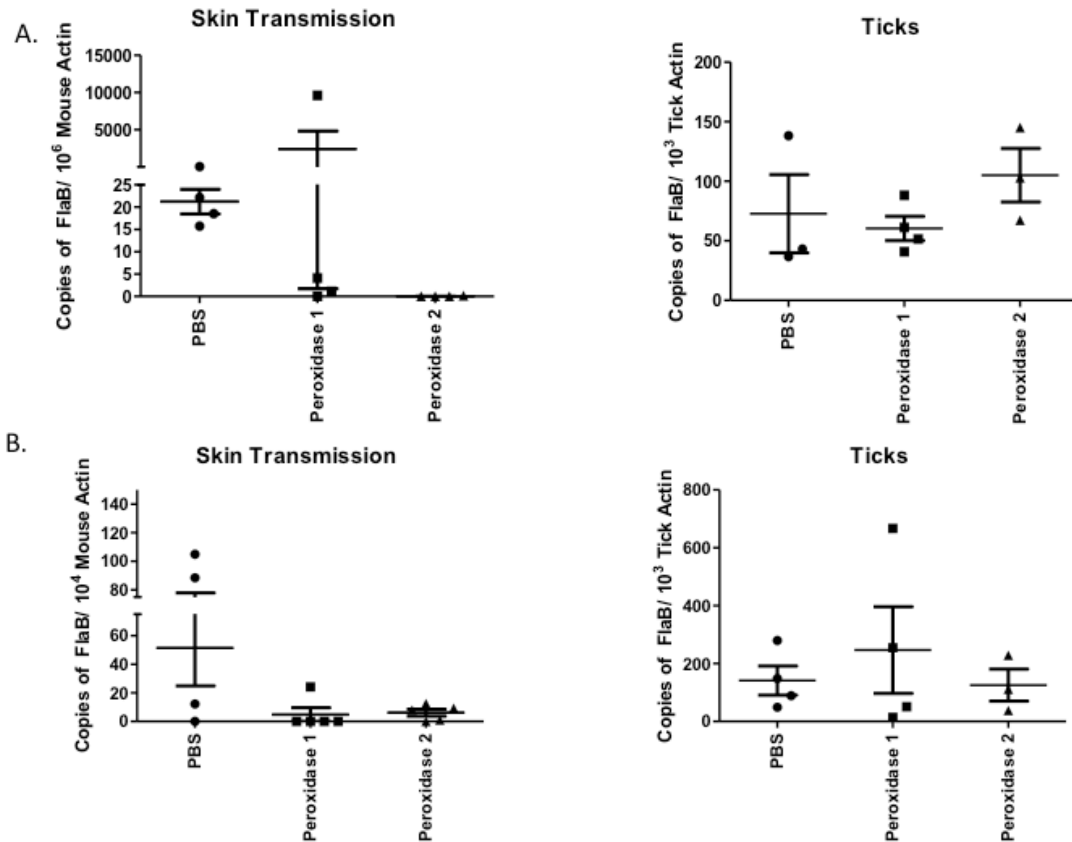

Figure 11 Immunization with *I. scapularis* specific proteins elicited significant protection. (**A**) The first experiment immunizing mice with duox fragments resulted in a significant decrease in *Borrelia* levels in mice immunized with Peroxidase-2. (p<0.05) There was a nonsignificant reduction in bacterial levels within the fed ticks. (**B**) The second experiment immunizing mice with duox fragments resulted in a significant decrease in *Borrelia* levels in mice immunized with either Peroxidase-1 or Peroxidase-2. (p<0.05)

Discussion

Since ticks are successful ectoparasites that are known to transmit a wide variety of pathogens to various different hosts, the development of a tick vaccine is important. Duox, a protein previously described in chapter 3, was further explored as a potential vaccine target. Since Duox is a highly conserved protein among many different eukaryotes, the first aim is to determine which regions of the protein are specific to *I. scapularis*. Regions that are non-specific would not be good targets since they would not be able to elicit an immune response but they could influence physiology of non-intended species.

Since *B. burgdorferi* has existed in nature in a complex tick-mammal life cycle for thousands of years, the bacteria have developed many mechanisms to evade the vector's immune responses and thrive in nature. A great amount of research has been directed toward discovery of a Lyme disease vaccine against a bacterial antigen that is important to the infection cycle (Kung et al., 2013). However, relatively limited research has been done on which vector proteins influence the pathogen life cycle, providing a strong rationale for the work presented within this chapter. The work presented in Chapter 3 shows that dual oxidase along with a specific peroxidase enzyme catalyze the formation of the DTN, a network that is essential in maintaining gut bacteria as well as transmitted pathogens like *B. burgdorferi.* A drastic decrease in the transmission of *B. burgdorferi* into the host is seen if the network fails to form. Taken together, above sets of data suggested Duox is a potential target to develop a tick vaccine.

As indicated in the earlier section, since Duox is a highly conserved protein amongst all eukaryotes, regions of the protein that are unique to *I. scapularis* were identified prior to conducting further experiments. It is important to determine if there are any conserved regions of the gene that are present in the host that is being immunized prior to immunization. *In silico* analysis identified two regions within the peroxidase domain of Duox. It was predicted and shown experimentally above that this domain is exposed outside the cell, making these regions ideal vaccine targets.

Production of antibodies against these regions, called Peroxidase-1 and Peroxidase-2, revealed that Duox is a membrane protein that is expressed on the luminal side of the gut. This discovery correlates with formation of the DTN along the lumen that was discussed in Chapter 3. Consequently, experiments were conducted to test if antibodies against either of these regions are protective against either tick engorgement process or pathogen transmission. In two independent experiments, Peroxidase-2 was found to be highly protective against *B. burgdorferi* transmission. However, the protein did not exhibit any effect on the duration of *I. scapularis* feeding or engorged tick weights. Thus, this protein would not be a good target for an anti-tick vaccine but it does show promise as a *B. burgdorferi* transmission-blocking agent. In one of the two experiments Peroxidase-1 was also found to be highly protective. The experiment where Peroxidase-1 was not found to be protective included one mouse out of the group of 4 that was highly infected, skewing the data. This could be due to minor differences in the immune systems of individual mice or other unknown factors. Future experiments should focus on discovering which epitopes from each region mount a protective immunity.

Nevertheless, these data suggest that a vaccine could be designed based on knowledge of these epitopes.

Chapter 5: A specific *Ixodes scapularis* small GTPase influences *B. burgdorferi* survival in the vector

Introduction

B. burgdorferi sensu lato, the causative agent of Lyme disease, is the most prevalent tick-borne bacteria in the United States, Europe, and many parts of Asia. (Piesman and Eisen, 2008; Piesman and Gern, 2004; Steere, 2001; Barbour and Hayes, 1986; Burgdorfer et al., 1982) This group of spirochete is maintained in a complex enzootic life cycle relying on the transmission as well as acquisition of the vector tick, *I. scapularis* complex to the mammalian host. *I. scapularis*, commonly known as the black-legged tick, has previously been shown to be the vector host of various different pathogens. Our understanding of the tick innate immune response to these invading pathogens is not well investigated despite the many recent advances involving model arthropods. However, it is established that ticks respond to invading pathogens with both a cellular and humoral response during feeding, thus it is assumed that during acquisition *B. burgdorferi* likely encounters a hostile environment.

During acquisition of blood meal, endocytosis is rapidly occurring allowing tick gut cells to capture many different necessary nutrients present within the blood meal. During this process involving infected blood meal, it is likely that innate immune components of the vector, including Toll and IMD pathway, phagocytosis, and antimicrobial peptides responses could also be activated, due to the presence of molecular patterns present on pathogens. The IMD and toll pathways, once induced, are responsible

for the activation of many different immune effector genes including antimicrobial peptides. Both pathways have been heavily studied in regards to insect biology, especially in model insects although their role in *B. burgdorferi* survival within the ticks remains obscure. Phagocytosis is also a key innate immune response that is known play important role in host defense. This event can be initiated by binding of various pathogens to a cell surface receptor initiating an intracellular signaling response. (Walters and Papadimitriou, 1972) To date *B. burgdorferi* ingestion via phagocytosis is rarely observed within the arthropod vector; however, the mechanism in which spirochetes evade this mechanism is unknown. (Mattilaet al., 2007)

In order to gain additional insight into which genes are playing important rolls in the immune response of *I. scapularis* to the infectious agent *B. burgdorferi*, genes that have been previously studied in other arthropods were screened for differential regulation in engorged ticks that parasitized *B. burgdorferi*-infected mice and uninfected mice during acquisition of a blood meal. This screening is described earlier in Chapter 1, which indicated a gene was identified to be significantly up regulated, which is termed herein as *I. scapularis Rho GTPase* (*IRGTPase*). The experiments presented in this chapter seek to further explore the role of IRGTPase in *I. scapularis* innate immune response.

Methods

Bacteria, Mice, and Ticks

The infectious isolate of *B. burgdorferi*, clone B31-A3 (Elias et al., 2002), was used throughout this study. Mice were four- to six-week-old C3H/HeN females that were purchased from the National Institutes of Health. For some experiments, *B. burgdorferi* clone B31-A3 was transfected with a plasmid expressing green fluorescence protein (GFP). All ticks that were used were reared in the laboratory as described in previous sections. All animal experiments were performed in accordance with the guidelines of the Institutional Biosafety Committee and the Institutional Animal Care and Use Committee.

RNA interference

RNA interference of target tick genes was accomplished using published procedures (Kariu et al., 2013; Pal et al., 2004). Tick cDNA was prepared as described above and used as a template to amplify various fragments of the open reading frames of the corresponding target gene *IRGTPase* (accession number *ISCW004348*). A fragment of the *gfp* gene was also amplified as a control. The primers used to amplify the specific DNA fragments are listed below. The *IRGTPase* and *gfp* amplicons were separately cloned into the corresponding restriction sites of the L4440 double T7 script vector, and the dsRNA was synthesized and purified using a commercial kit (MEGAscript RNAi kit, Ambion, Inc.). Five µl of the dsRNA (2 µg/µl) was loaded into capillary tubes and microinjected into the gut of unfed nymphs (25 ticks/group). The injected nymphs were

kept overnight in an incubator and used for feeding on mice. Repleted ticks were individually processed for assessment of RNA silencing as well as pathogen levels using qRT-PCR analysis. Primers that bind upstream and downstream of the target cDNA encompassing the dsRNA sequence were used for the qRT-PCR. These primers can be found in Table 13.

Infection studies

C3H mice were infected with spirochetes (10^5 spirochetes/mouse) in order to assess whether the RNA interference-mediated reduction of *IRGTPase* expression affected *B. burgdorferi* acquisition by nymphal ticks. IRGTPase or GFP dsRNA preparations were administrated to nymphs, and the microinjected ticks were placed on *B. burgdorferi*-infected mice (25 ticks/mouse) that were infected for 14 days. Groups of ticks were forcibly detached from the mice following 48 hours of feeding, while the others were allowed to feed to repletion and were collected. The *flaB* transcripts were detected using qRT-PCR. At least three independent experiments were performed and 6-10 ticks were recovered from each mouse. Each tick was analyzed individually for measurement of target transcripts or pathogen burden using qRT-PCR. All primers used in these experiments can be found in **Table 13**.

Table 13 Oligonucleotide primers used in IRGTPase Studies.

Sequence (5′ to 3′)	**Purpose**
CCGAGCTCGATCAGCCCGTGGACCGGAAG	IRGTPase dsRNA Forward
GGGGTACCGCCGGACCTCGGGCACCATGT	IRGTPase dsRNA Reverse
ATACGGGCCTCGGCTTAC	IRGTPase RNAi Detection Forward
AGTGCACCGCAAAGTCTTCT	IRGTPase RNAi Detection Reverse
AATGAGCTCGAGGTGAAGTTCGAGGGCGA	GFP dsRNA Forward
AATGGTACCTCCATGCCGAGAGTGATCCC	GFP dsRNA Reverse
TTGCTGATCAAGCTCAATATAACCA	Forward primer for *B. burgdorferi flaB* quantitative RT-PCR (qRT-PCR).
TTGAGACCCTGAAAGTGATGC	Reverse primer for *B. burgdorferi flaB* qRT-PCR.
AGAGGGAAATCGTGCGTGAC	Forward primer for tick *β-actin* qRT-PCR
CAATAGTGATGACCTGGCCGT	Reverse primer for tick *β-actin* qRT-PCR

Pharmacological inhibition of Rho GTPase

Naïve nymphal ticks were microinjected with either 10 ng/ml *Clostridium difficile* Toxin-B (Sigma-Aldrich, MO) or PBS (Ami et al.,1998). C3H mice were infected with spirochetes (10^5 spirochetes/mouse) in order to assess inhibition of protein activity. Following 14 days of infection, Toxin B or PBS was administered to the nymphs and placed on infected mice (25 ticks/mouse). Ticks were allowed to feed on *B. burgdorferi* infected mice for 48 hours before being forcefully removed. RNA was extracted from a pooling of three ticks and converted to cDNA. Bacteria load was then quantified via qRT-PCR.

Levels of *B. burgdorferi* were also analyzed using confocal microscopy as described previously above. Briefly, guts were dissected from 48 hour fed ticks and placed on poly-L-lysine coated slides. Tissues were acetone fixed for 10 minutes. Samples were than blocked for 1 hr with 5% goat serum and washed with PBS-T. Next the gut samples were incubated with anti-*B. burgdorferi* labeled with FITC. As a counter stain, cells were also incubated with DAPI (4′,6-diamidino-2-phenylindole) and then visualized using the LSM510 scanning laser confocal. (Zeiss Microimaging Inc, Germany)

Cytokine Assay

Naïve nymph ticks were microinjected with various concentrations of individual cytokines according to **Table 14**. Microinjected ticks were allowed to recover for 3 hours before being allowed to feed on naïve mice. Ticks were collected 48 hours into feeding and analyzed for *Rho GTPase* expression via qRT-PCR.

Table 14: Cytokine Concentrations

Cytokine	Concentration
TNF-α	0.3 ng/mL
IFN-γ	5 ng/mL
IL-6	6000u/mL
IL-1β	0.3 ng/mL
IL-12	50 ng/mL

Infection studies using IFN-γ Knock-out Mice

Mice deficient for IFN- γ (B6.129S7-*IfngtmTs/J*) or IFN-γ receptor (B6129S7-*Ifngr1tmAgt/*J) were commercially obtained from Jackson laboratories. Mice were needle infected with $1x10^5$ *B. burgdorferi*/animal. Following 14 days of infection, mice were parasitized with naïve *I. scapularis.* Ticks were collected 48 hours into feeding and total RNA was extracted and converted to cDNA. Expression of *IRGTPase* as well as bacterial burden was assessed by qRT-PCR.

Tick Cell Culture

The *I. scapularis* ISE6 cell line was grown containing 5 mL of L15B supplemented with 5% fetal bovine serum, 5% tryptose phosphate broth, and 0.1% cholesterol concentrate. Cultures were maintained at 34°C and media was changed weekly. For assays, tick cells were re-suspended in fresh culture medium, counted with a hemocytomer, and adjusted to a density of $1x10^5$ cells/mL. 0.5 mL cell suspension was seeded into an 8 well tissue culture plate and was given 1 day to adhere prior to further assays.

Phagocytosis assays

ISE6 cells were incubated with 0.1 ug IFN-γ or IL-12 for 6 hours prior to challenge with GFP-producing *B. burgdorferi* overnight at 34°C. Culture medium was removed and cells were washed with cold PBS to remove any unbound bacteria. Cells were then fixed with acetone for 10 min and incubated with Texas Red®-X Phalloidin

(Invitrogen) and 4′,6-diamidino-2-phenylindole (Invitrogen). The cells were visualized using the LSM510 scanning laser confocal. (Zeiss Microimaging Inc, Germany) Counting the number of cells where the bacteria was found to be inside the cells and dividing by the total number of cells determined the percent of phagocytic cells.

Bioinformatics and statistical analysis

Unless indicated otherwise, protein annotation and searches were executed using the VectorBase (www.vectorbase.org) database. Results are expressed as the mean ± standard error (SEM). Statistical significance of differences observed between experimental and control groups were analyzed using GraphPad Prism version 4.0 (GraphPad Software, CA). A two-tailed Student's *t*-test was utilized to compare the mean values, and $p < 0.05$ was considered significant.

Results

Identification of I. scapularis phagocytic genes that are up regulated in the presence of B. burgdorferi.

The immune response of *I. scapularis* ticks to *B. burgdorferi* infection currently understudied. Lacking an adaptive immune response, ticks likely react with cellular and humoral responses when encountering a pathogen, which include recognized innate immune pathways such as agglutination, antimicrobial peptide production and phagocytosis. Since phagocytosis, in particular, plays a key role in the innate immune system of other hematophagous insects, such as mosquitos, we sought to characterize phagocytic pathways within the tick. Due to the completion of the *I. scapularis* genome as well as the availability of comparative genomic analysis tools, we were able to identify several genes that are likely to be involved in *I. scapularis* phagocytic pathways (**Table 8**). In order to determine which of these genes are differentially regulated in the presence of *B. burgdorferi*, expression levels of target immune genes were compared between infected and uninfected ticks. Genes that displayed a differential regulation of greater than two-fold alterations (increase or decrease) were considered to be significant.

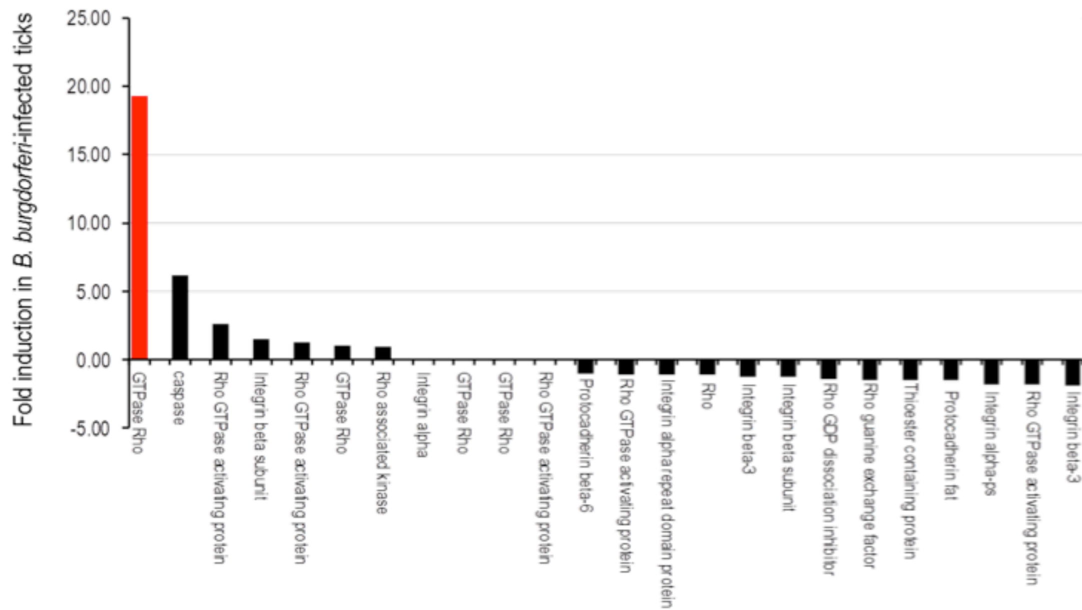

Figure 12 Relative fold induction of phagocytic pathway genes in presence of *Borrelia*. Total RNA was isolated from a pooling of 48 hour fed ticks that were either infected with *Borrelia* or naïve. Total RNA was converted to cDNA for measuring gene specific transcripts by quantitative real time PCR (qRT-PCR). Fold increase of expression levels of individual genes was calculated based on the threshold cycles (Ct) values. All genes were normalized against *B-actin* Ct values. Infected samples were thean compare to the naïve sample values.

Of the 24 different genes, a sole member of *I. scapularis Rho GTPases*, termed *IRGTPase*, is dramatically induced during acquisition of spirochetes from infected mice (**Figure 12**). However, notably, *IRGTPase* was not induced during transmission of *B. burgdorferi* from infected ticks to a naïve mouse (**Figure 17A)**. IRGTPase was readily detectable within the gut, but not found to be localized to the salivary glands, as shown by confocal microscopy, suggesting IRGTPase is specific to the gut. To determine the expression of *IRGTPase* during feeding, a time course analysis was performed where ticks were forcibly detached from mice at various times of tick engorgement and processed for quantitation of transcripts. The result shows that levels of *IRGTPase* gradually increase during tick feeding process (**Figure 13 B**). Next, we assessed whether the up regulation of this gene was specific to infection with *B. burgdorferi* or other tick-

borne pathogens. To examine this, we compared expression levels of IRGTPase in ticks that were infected with *Anaplasma phagocytophilum*. Interestingly, *Anaplasma* –infected ticks did not display a similar up-regulation of IRGTPase, suggesting its modulation is specific to *B. burgdorferi* infection of ticks (**Figure 13**).

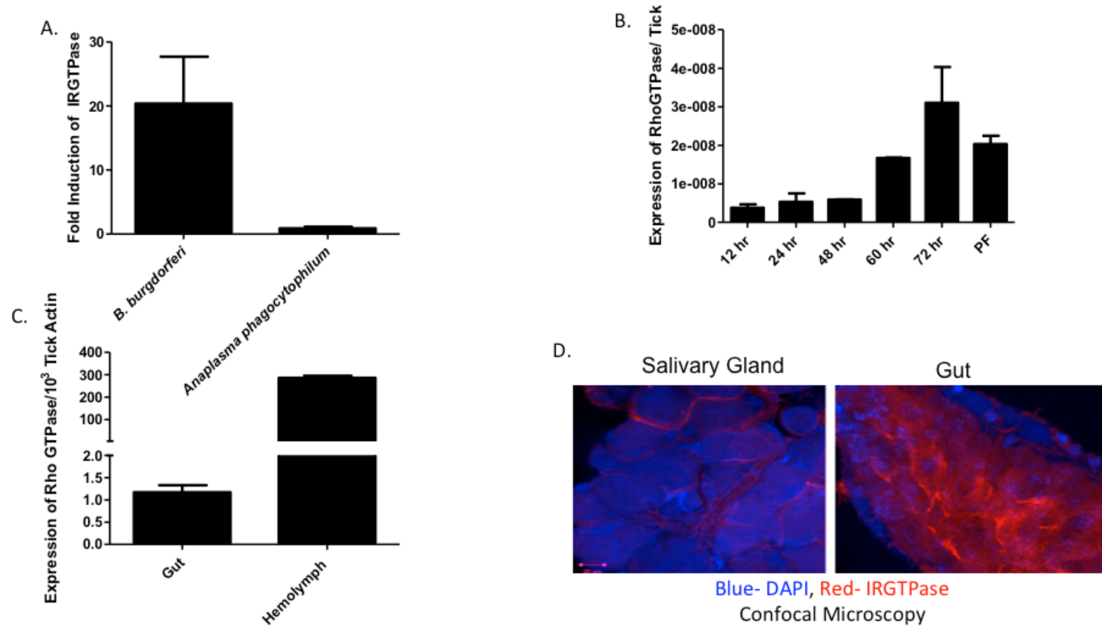

Figure 13 ***IRGTPase expression is specifically induced in the presence of Borrelia.*** (**A**) Expression of *IRGTPase* is specifically induced in the presence of *Borrelia* in comparison to *Anaplasma phagocytophilum*. (**B**) Expression of *IRGTPases* was assessed throughout feeding of *Ixodes*. There is a peak in expression around 72hr into feeding. (**C**) *IRGTPase* is expressed highly in the hemolymph of the tick when assessed by qPCR, while *IRGTPase* is also expressed to a lesser degree within the gut. (**D**) Confocal microscopy indicates that IRGTPase is not expressed in the salivary glands of the tick, while it is expressed in the gut. Red color stains IRGTPase protein and blue stains the nuclear of the cell.

IRGTPase knockdown increases B. burgdorferi persistence in ticks

Silencing of *IRGTPase*, via RNAi-mediated silencing in nymphal ticks fed on *B. burgdorferi* infected mice, reflected a slight increase in bacterial burden at 48 hours into feeding, however, with a significant increase in fully engorged repleted ticks (**Figure 14**). These results were acquired from mice confirmed by Western blotting to be infected with *B. burgdorferi*.

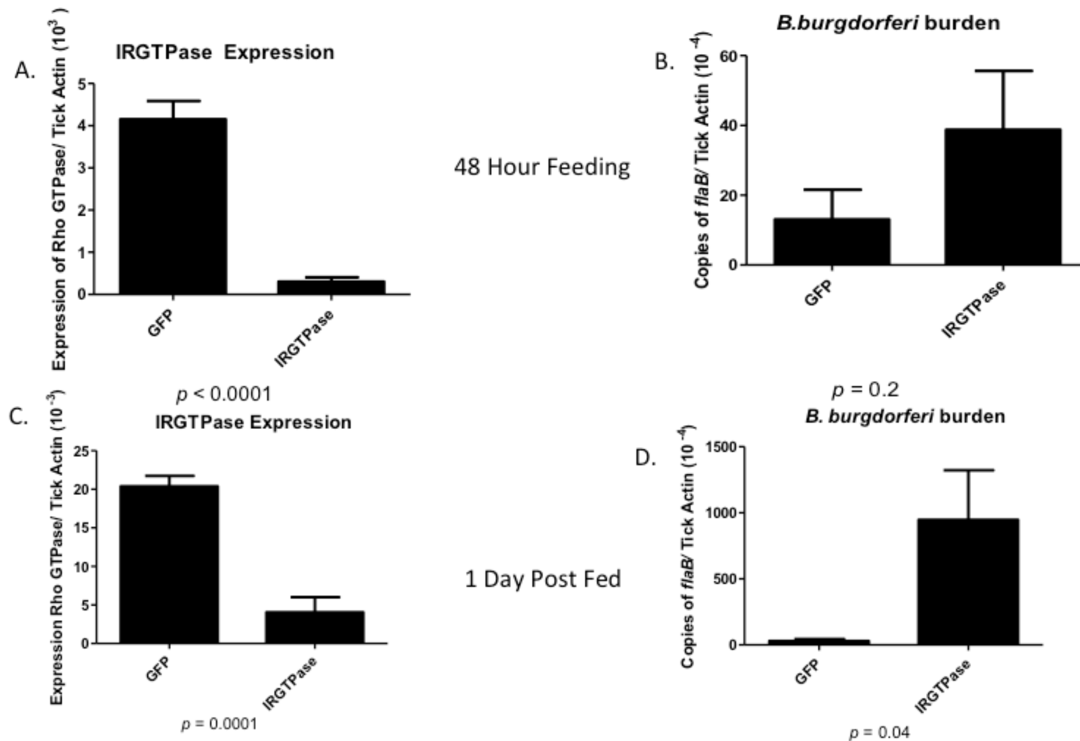

Figure 14 *IRGTPase* knockdown increases *B. burgdorferi* persistence in ticks. (**A**) RNA interference-mediated knockdown of peroxidase transcripts in feeding ticks. Nymphal ticks (25/group) were injected with dsRNA of IRGTPase or control (GFP) and fed on *Borrelia* infected mice, collected at 48h after the onset of feeding, and ticks were processed for quantitative RT-PCR using detection primers against target genes and the results normalized against tick *β-actin*. Compared to their respective controls (GFP dsRNA), expression *IRGTPase* was significantly downregulated ($p < 0.01$) (**B**) Knockdown of *IRGTPase* expression reduces *B. burgdorferi* persistence in feeding. Ticks were microinjected with IRGTPase dsRNA or GFP dsRNA and allowed to parasitize mice that were infected with *B. burgdorferi*. 48 hours into feeding, ticks were collected and *B. burgdorferi* burden in ticks was detected by measuring *flaB* transcripts and normalized with tick *β-actin*. Compared to ticks microinjected with GFP dsRNA, *B. burgdorferi* burden in ticks was higher but the value was not significant. (**C**) (A) RNA interference-mediated knockdown of peroxidase transcripts in feeding ticks. Nymphal ticks (25/group) were injected with dsRNA of IRGTPase or control (GFP) and fed on *Borrelia* infected mice, fully engorged ticks were collected, and were processed for quantitative RT-PCR using detection primers against target genes and the results normalized against tick *β-actin*. Compared to their respective controls (GFP dsRNA), expression *IRGTPase* was significantly downregulated ($p < 0.01$) (**D**) Knockdown of *IRGTPase* expression reduces *B. burgdorferi* persistence in feeding. Ticks were microinjected with IRGTPase dsRNA or GFP dsRNA and allowed to parasitize mice that were infected with *B. burgdorferi*. 48 hours into feeding, ticks were collected and *B. burgdorferi* burden in ticks was detected by measuring *flaB* transcripts and normalized with tick *β-actin*. Compared to ticks microinjected with GFP dsRNA, *B. burgdorferi* burden in ticks was significantly higher in ticks with *IRGTPase* knocked down. ($p<0.05$)

In order to further study role of IRGTPase, we proceeded to use a well known inhibitor of Rho GTPase called Toxin B which is produced by *Clostridium difficile*, a mono-glucosyltransferase, which utilizes UDP-glucose residues to transfer glucose to a critical motif rending Rho GTPase in a non-active state. In correlation with our previous finding, ticks that were microinjected with the protein inhibitor exhibited higher levels of bacteria in comparison to PBS control ticks. (**Figure 15a**). To determine whether Toxin B exert any affect on the general morphology of the gut, particularly within 48 hours of treatment, confocal microscopic analysis of gut tissues was performed, which indicated a negligible effect of the inhibitor on gut appearances (**Figure 15b**).

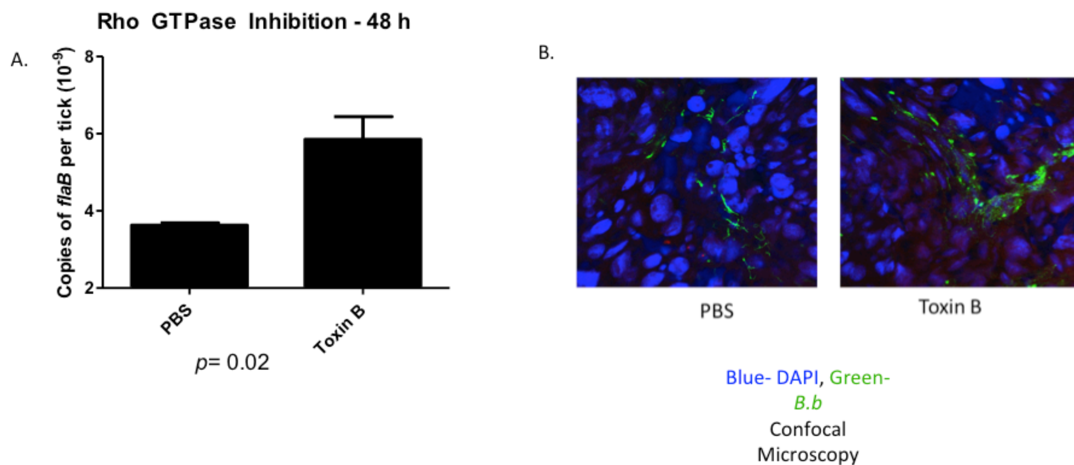

Figure 15 IRGTPase protein inhibition increases *B. burgdorferi* persistence in ticks. (**A**) Toxin B mediated protein inhibition in feeding ticks. Ticks were microinjected with Toxin B or PBS and allowed to parasitize mice that were infected with *B. burgdorferi*. 48 hours into feeding, ticks were collected and *B. burgdorferi* burden in ticks was detected by measuring *flaB* transcripts and normalized with tick *β-actin*. Compared to ticks microinjected with PBS, *B. burgdorferi* burden in ticks injected with Toxin B was significantly higher. ($p<0.05$) (**B**) Toxin B did not affect the morphology of the tick gut. Given that Toxin B can be harmful confocal microscopy was used to assess the morphology of the gut. No apparent effect was seen.

How an intracellular molecule (IRGTPase) is induced by the presence of a extracellular bacteria (B. burgdorferi)? - Possible involvement of a mammalian factor present during an infected blood meal

Since *B. burgdorferi* is an extracellular bacterium, we sought to examine how an intracellular protein, IRGTPase, is being activated in feeding tick gut. As Rho GTPases are known to be activated by intracellular signaling through various cell surface molecules (Jaffe and Hall, 2005), we began to search for a specific ligand that could induce IRGTPase. As previous studies have shown that integrin binding can lead to Rho GTPase activation (Schwartz and Shattil, 2000), and that *B. burgdorferi* produce surface exposed integrin binding ligands, such as P66 (LaFrance et. al, 2011), so initially we compared ticks that were infected with wild type *B. burgdorferi* to a well characterized integrin P66 deletion mutant. However, no significant difference in expression of IRGTPase was observed when ticks encountered wild type spirochetes or P66-deficient mutants (**Figure 16**).

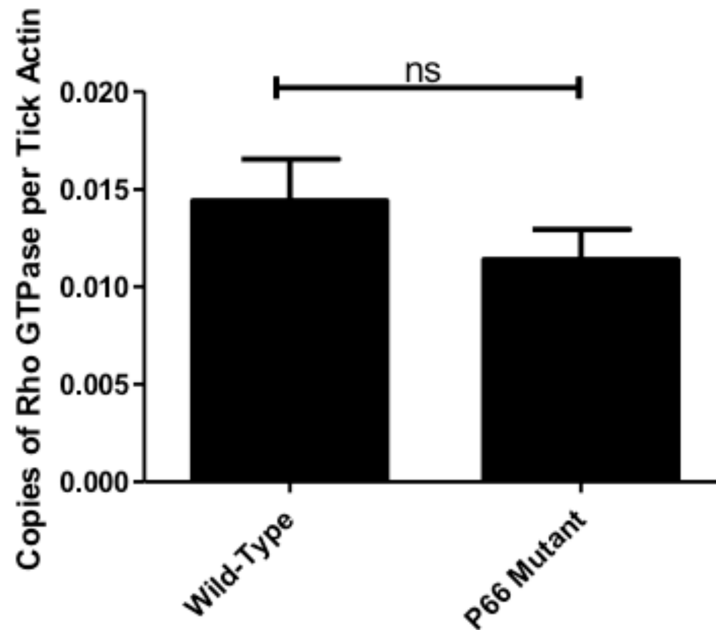

Figure 16 P66 ligand does not induce IRGTPase. Ticks were microinjected with a P66⁻ *Borrelia* or wild-type *Borrelia* and allowed to parasitize naïve mice. 48 hours into feeding, ticks were collected and IRGTPase transcripts were measured and normalized with tick *β-actin*. No significant difference in IRGTPase was detected.

Certain cytokines are amongst other known activators of Rho GTPases. Since ticks do not produce cytokines, at least one is not yet identified; we explore the possibility that any cytokines could be acquired from infected hosts and present in the blood meal. During mammalian infection with *B. burgdorferi* there are five systemic cytokines that are known to be present. These include IFN- γ, TNF-α, IL-1β, IL-6, and IL-12 (Zeidner et al., 1997). When active and recombinant forms of these cytokines were directly transferred to ticks prior to engorgement, only one of them, IFN- γ, was able to significantly induce transcript levels of *IRGTPase* (**Figure 17**). In order to mimic a natural method of transferring IFN- γ during tick engorgement process groups of nymphs were allowed fed upon infect IFN- γ knock out mice. As IFN- γ influence *B. burgdorferi*

levels in mice, as a more appropriate control, a second group of ticks were fed on IFN- γ receptor knock out mice to insure similar levels of pathogens but with the presence of IFN- γ. In mice that were able to still produce IFN- γ (i.e. IFN- γ receptor knock out mice), higher levels of *IRGTPase* transcripts were observed which in turn led to lower levels of *B. burgdorferi* in feeding vector (**Figure 18**).

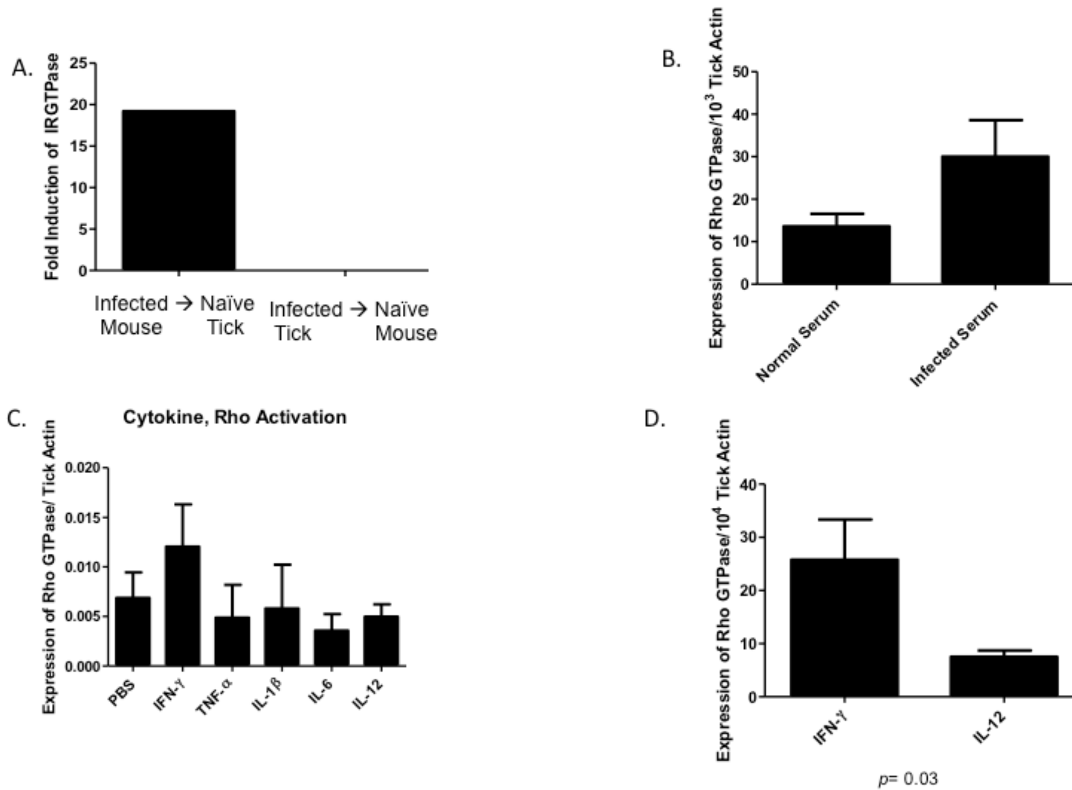

Figure 17 Identification of IRGTPase activator in feeding ticks. (**A**) *IRGTPase* is induced only during acquisition of the pathogen from an infected host. Fold induction of *IRGTPase* during both acquisition of *Borrelia* from an infected host to a naïve tick as well as during transmission of *Borrelia* from an infected tick to a naïve host was assessed by qPCR. (**B**) Expression of *IRGTPase* is increased in a non-significant amount in the presence of infected serum. Ticks were microinjected with fresh normal mouse sera or infected mouse sera and allowed to parasitize naïve mice. 48 hours into feeding, ticks were collected and *IRGTPase* transcripts were measured and normalized against tick *β-actin*. No significant difference in *IRGTPase* was detected. (**C**) Expression of *IRGTPase* is induced in the presence of IFN-γ. Ticks were microinjected with PBS, IFN-γ, TNF-α, IL-1β, IL-6, or IL-12 and allowed to parasitize naïve mice. 48 hours into feeding, ticks were collected and *IRGTPase* transcripts were measured and normalized against tick *β-actin*. (**D**) Expression of *IRGTPase* is significantly induced in the presence of IFN-γ. Nymphal ticks (25/group) were injected with IFN-γ or TNF-α, and allowed to parasitize naïve mice. 48 hours into feeding, ticks were collected and *IRGTPase* transcripts were measured and normalized against tick *β-actin*. A significant increase in *IRGTPase* expression was observed in ticks that were introduced to IFN-γ. ($p<0.05$)

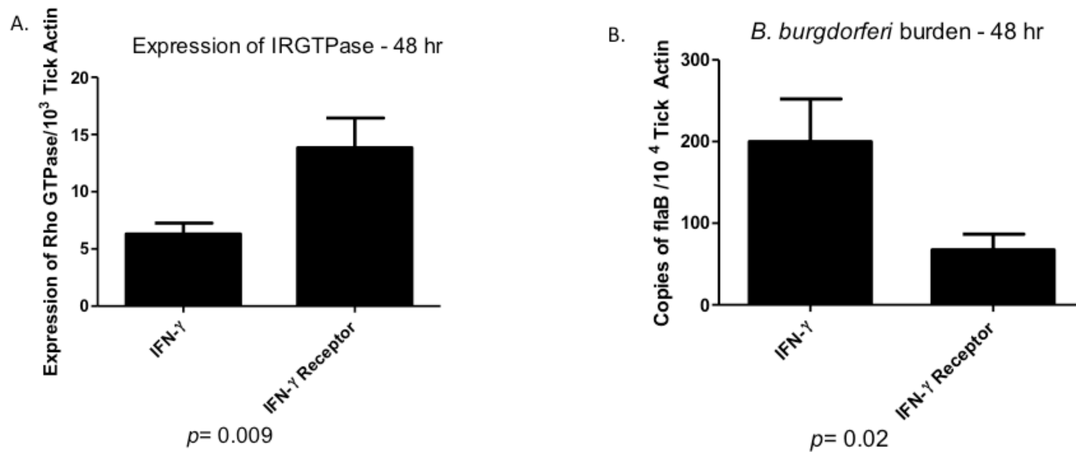

Figure 18 *IRGTPase* is induced in mice producing IFN-γ, which causes a decrease in pathogen persistence. (**A**) Expression of *IRGTPase* is significantly induced in the presence of IFN-γ. Nymphal ticks (25/group) were allowed to parasitize on either IFN-γ knockout mice or IFN-γ receptor knockout mice that were infected with *Borrelia.* 48 hours into feeding, ticks were collected and *IRGTPase* transcripts were measured and normalized against tick *β-actin*. When IFN-γ is being produced we observed a significant increase in *IRGTPase* expression. ($p<0.01$) (**B**) Bacterial burden decreased in the presence of IFN-γ. Nymphal ticks (25/group) were allowed to parasitize on either IFN-γ knockout mice or IFN-γ receptor knockout mice that were infected with *Borrelia.* 48 hours into feeding, ticks were collected and *IRGTPase* transcripts were measured and normalized against tick *β-actin*. When IFN-γ is being produced we observed a significant increase in bacterial burdens in ticks. ($p<0.05$)

In order to determine if INF-γ is involved at increasing the frequency of phagocytosis events we proceeded with further *in vitro* studies. Previous works have shown that ISE6 *I. scapularis* cells are poorly able to phagocytize *B. burgdorferi* (Mittila et al., 2007). Since these cells were found to produce *IRGTPase* transcripts (**Figure 19**) a phagocytosis assay where cells were exposed to wither IFN-γ or IL-12 and *B. burgdorferi* was adopted. It was observed that cells that were pre-incubated with IFN-γ had a significant increase in phagocytosis (**Figure 19**).

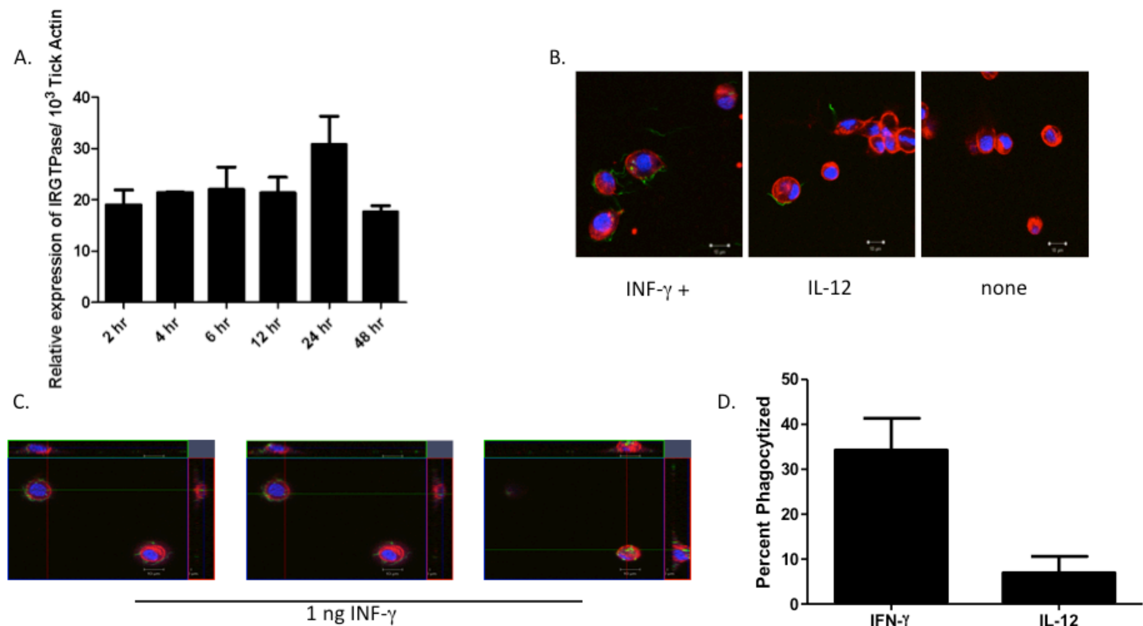

Figure 19 Role of Rho GTPase in phagocytosis of *B. burgdorferi in vitro.* (**A**) ISE6 cells produce IRGTPase. About $1x10^5$ ISE6 cells were incubated with 0.1 ug IFN-γ. Cells were harvested at 2 hours, 4 hours, 6 hours, 12 hours, 24 hours, and 48 hours. RNA was extracted and converted to cDNA. Expression of *IRGTPase* transcripts were measured by qPCR. No significant increase in expression was observed. (**B**) ISE6 cells produce IRGTPase. $1x10^5$ ISE6 cells were incubated with 0.1 ug IFN-γ for 6 hours prior to challenge with GFP-producing *B. burgdorferi* overnight at 34^oC. Culture medium was removed and cells were washed with cold PBS to remove any unbound bacteria. Cells were then fixed with acetone for 10 min and incubated with Texas Red®-X Phalloidin (red) and DAPI (4′,6-diamidino-2-phenylindole) (blue). (**C**) Orthogonal view of a representative Z-stack image of cells incubated with IFN-g show that *Borrelia* is being phagocytized. (**D**) Counting the number of cells where the bacteria was found to be inside the cells and dividing by the total number of cells observed determined the percent of phagocytic cells. Cells were either incubated with either IFN-γ or IL-12.

Discussion

The innate immune response of *I. scapularis* to the presence of *B. burgdorferi* is not well characterized and remains as understudied area of investigation. There is gap in knowledge in the field pertaining to whether most ingested bacteria in the tick blood meal are killed during engorgement process or if only a few bacteria are initially acquired which rapidly replicate following detachment. If bacteria within the gut were initially killed upon acquisition, it would be assumed that the innate immune responses would have been activated leading to differential regulation of innate immune genes. However, which genes are important to this response are unknown.

Since the innate immune response is characterized by several different well-studied pathways that can control microbial persistence, we sought to focus on one specific pathway, phagocytosis. Phagocytosis is the pathway that is responsible for the ingestion of foreign particles including bacteria (Walters et al., 1978). After phagocytosis, these foreign particles can further be broken down that help reduce the infection. 24 potential genes that could play a role in phagocytosis events linked to *B. burgdorferi* persistence in ticks were initially identified through literature searches. Quantitative RT-PCR was able to identify one specific Rho GTPase that was differentially regulated during *B. burgdorferi* infection. Although Rho GTPase is a family of proteins with diverse functions and the tick genome encodes for at least five different Rho GTPases, only one specific member was up-regulated during spirochete infection. It was, therefore, assumed that this protein could play an important role in infection and *B.*

burgdorferi persistence in the vector. These efforts are fundamental to understanding of innate immune responses that are specific to Lyme disease pathogens.

Rho GTPases are monomeric intracellular proteins roughly 20-30 kDa GTP binding proteins known to be important for many cellular functions in a wide range of organisms that includes actin modulation, activation of NADPH oxidase, stress fiber dynamics, cell cycle progression and cell adhesion (Jaffe and Hall, 2005). When Rho GTPases are bound to GTP they are in an active state. It is during this active state that these proteins are able to interact with effector or target molecules for further downstream signaling (Jaffe and Hall, 2005). Of the five different tick Rho GTPases, only one was found to be up regulated (ISCW004348), which we termed IRGTPase. This particular Rho GTPase bears closest similarity to a specific class of Rho GTPase in mouse, termed as RhoA. To define the role of IRGTPase in acquisition and persistence of the bacteria within the tick gut, we used two key approaches: RNAi-mediated knock down of *IRGTPase* transcripts and inhibition protein function using well-characterized inhibitors, such as Toxin B.

When inhibiting Rho GTPase function, via knock down or chemical inhibition, the bacterial load within the tick significantly increases indicating that IRGTPase likely plays a beneficial role in maintaining low bacteria burden within the tick gut. How this intracellular molecule was activated was further investigated, since *B. burgdorferi* is a known extracellular pathogen. As Rho GTPases can be regulated by several different factors including bacterial ligands that interact with cellular receptors like integrin, or

host cytokines, we further explored these possibilities in the blood (Schwarts and Shattil, 2000; Jaffe and Hall, 2005).

The results show that the up regulation of *IRGTPase* might not be due to the presence of a factor(s) on bacterial surface including a prominent integrin binding ligand, P66. However, *B. burgdorferi* is known to produce a large number of surface proteins and particularly, there are additional integrin binding proteins that are produced by spirochetes. So there still remains a possibility that an unknown spirochete integrin binding ligand could be involved in the activation of IRGTPase in feeding ticks.

Asides ligands present on the bacteria surface, host-derived factors including certain cytokines that are present in the tick blood meal acquired from infected mice could lead to the differential regulation of Rho GTPases. Therefore tested the different cytokines that are systemically produced during a mammalian infection with *B. burgdorferi* were tested. Only one cytokine, IFN- γ, was found to induce a significant up regulation of *IRGTPase*. This observation was further confirmed in studies involving mice that were lacking IFN- γ.

IFN- γ, has previously been shown in other studies to play an important role in the up regulation of Rho GTPases leading to endocytosis (Utech et al., 2005). These observations also supports our speculation that a host-derived cytokine induces a specific Rho GTPase, which in turn could mediate phagocytosis or other innate immune

responses that ultimately help controlling the levels of ingested pathogens like *B. burgdorferi* in the feeding vector.

Chapter 6: Conclusion

I. scapularis ticks transmit a wide array of human and animal pathogens including *B. burgdorferi*. Due to the evolutionary divergence from other bacteria, and the possession of a unique cellular structure, *B. burgdorferi* cannot be classified as a conventional Gram-positive or Gram-negative bacterial species. Additionally, key pattern recognition molecules or PAMPs, such as Lipopolysaccharides and peptidoglycans, are absent or structurally distinct, respectively, in *B. burgdorferi* (Schleifer and Kandler, 1972; Takayame et al., 1987; Fraser et al., 1997). Thus, the wealth of knowledge generated in other model arthropods, regarding the genesis of host immune responses against classical bacterial pathogens, might not be readily applicable for *B. burgdorferi*. Primary goal for this study was to characterize components of the tick immune responses that modulate *B. burgdorferi* infection and use this information to better understand specific aspects of tick immunity as well as to contribute to the development of new strategies that interfere with pathogen persistence and transmission.

The first goal of this work was to assess the expression profile of *I. scapularis* innate immune transcriptome to identify genes that are induced in the *B. burgdorferi*-infected vector. These pathogen-induced gene-products were than further studied for their possible beneficial or harmful roles in microbial persistence within the vector. All subsequent studies presented in the thesis focused on two specific gene-products that are involved in the maintenance of gut microbe homeostasis (Duox and peroxidase) and phagocytosis (Rho GTPase).

In chapter 2, it has been shown that there are 20 up-regulated genes, 53 down-regulated genes, and 194 non-regulated genes in the presence of *B. burgdorferi*. Of these 20 up-regulated genes, functions of two particular gene-products were investigated in chapters 3, 4, and 5. Chapter 3 shows that dual oxidase (along with a specific peroxidase) work together to crosslink tyrosine to for the DTN. This network is essential for the survival of gut microbiota. If the network fails to fully form, nitric oxide is induced and bacteria are killed. It was then shown that immunization of hosts with two unique portions of the peroxidase domain of dual oxidase were able to interfere with *Borrelia* transmission, and significantly reduce levels of spirochete infection in mice. One of the future directions for this project is to determine which epitopes of these proteins provide the protection. If these neutralizing epitopes could be identified, more efficient transmission-blocking vaccine against *B. burgdorferi* could be developed.

It was shown in chapter 5 that Rho GTPase, another gene-product that is up-regulated in the presence of *B. burgdorferi***,** is induced by a host-derived cytokine, IFN-γ. This induction than causes an increase in potential phagocytosis events in ticks which results in a decrease in *B. burgdorferi* survival in the vector. The future directions for this project are to identify the tick INF-γ receptor, which is critical in our understanding of how intricate host-vector-microbial interaction influence persistence of tick-borne in nature.

Appendices

REVIEW

1) **Smith AA** and Pal U. Immunity-related genes in *Ixodes scapularis* – perspectives from genome information in "Lyme Disease: Recent Advances and Perspectives" hosted by Dr(s) Catherine Ayn Brissette, Tanja Petnicki-Ocwieja in Frontiers in Microbiology, (In press) 2014.

RESEARCH ARTICLES

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