A Dityrosine Network Mediated by Dual Oxidase and Peroxidase Influences the Persistence of Lyme Disease Pathogens within the Vector*

Background: How tick immune components influence persistence of pathogens remains unknown.

Results: Impairment of dityrosine network formed by a dual oxidase and a peroxidase activates specific immune genes and reduces pathogen survival.

Conclusion: A dityrosine network influences persistence of Lyme disease pathogens.

Significance: Knowledge of a Duox system in protein cross-linking may offer new strategies to disrupt pathogen life cycle.

Ixodes scapularis ticks transmit a wide array of human and animal pathogens including Borrelia burgdorferi; however, how tick immune components influence the persistence of invading pathogens remains unknown. As originally demonstrated in Caenorhabditis elegans and later in Anopheles gambiae, we show here that an acellular gut barrier, resulting from the tyrosine cross-linking of the extracellular matrix, also exists in I. scapularis ticks. This dityrosine network (DTN) is dependent upon a dual oxidase (Duox), which is a member of the NADPH oxidase family. The Ixodes genome encodes for a single Duox and at least 16 potential peroxidase proteins, one of which, annotated as ISCW17368, together with Duox has been found to be indispensable for DTN formation. This barrier influences pathogen survival in the gut, as an impaired DTN in Duox knockdown or in specific peroxidase knockdown ticks, results in reduced levels of B. burgdorferi persistence within ticks. Absence of a complete DTN formation in knockdown ticks leads to the activation of specific tick innate immune pathway genes that potentially resulted in the reduction of spirochete levels. Together, these results highlighted the evolution of the DTN in a diverse set of arthropod vectors, including ticks, and its role in protecting invading pathogens like B. burgdorferi. Further understanding of the molecular basis of tick innate immune responses, vector-pathogen interaction, and their contributions in microbial persistence may help the development of new targets for disrupting the pathogen life cycle.

Lyme disease, the most prevalent tick-borne infection in the United States, is caused by the spirochete bacterium, Borrelia burgdorferi (1, 2). The pathogen thrives in nature through an intricate infectious cycle, including an arthropod vector, Ixodes scapularis, and a vertebrate host, primarily wild rodents (3). 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 (4, 5). 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 (6, 7). 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 (8–11), which pathogens like B. burgdorferi encounters upon its 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 (12, 13). 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 (14–18). How these pathways operate in I. scapularis, especially their activation following B. burgdorferi invasion, however, remains obscure. Nevertheless, ticks likely produce classical antimicrobial peptides in the gut (19), although 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 does not clear the infection (20). Earlier studies also reported the development of annotated catalogs of organ-specific transcripts (21, 22); these studies will have a significant impact on our understanding of tick biology and vector-pathogen interaction. Nonetheless, 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 the 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 (23). The formation of the DTN decreases the gut permeability to immune elictors, 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 (23). Dual oxidase is a prominent member of the NADPH oxidase 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 (24). 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 (25). Similarly, expression of Duox in airway epithelial cells promotes antimicrobial defenses (26).

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, including controlling proliferation of symbiotic or nonsymbiotic bacterial communities in the gut during feeding (23, 25, 27), we sought to investigate how these enzymes affect persistence of a major tick-borne pathogen, *B. burgdorferi*. These studies could 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.

**EXPERIMENTAL PROCEDURES**

*B. burgdorferi, Mice, and Ticks*—A low passage infectious isolate of *B. burgdorferi*, clone B31-A3, was used in this study (28). 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 supplemental Table S1. For analysis of *Duox* and peroxidase expression, the naive 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 (29, 30). The ticks were collected at various time points of feeding or as postfed nymphs. Individual ticks were processed separately. Total RNA was extracted from ticks using TRIzol (Invitrogen), treated with RNase-free DNasel (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) (31). To test the efficiency and exclude nonspecific 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) as detailed. Transcript levels of individual genes were calculated using the 2−ΔΔCT method and further normalized against tick β-actin transcripts (31). For assessment of levels of *B. burgdorferi* or gut bacterial numbers in ticks, the *flaB* or 16S transcript levels, respectively, were quantitated using qPCR as detailed (31).

RNA Interference—RNA interference of target tick genes was accomplished using our published procedures (30, 32). 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 supplemental Table S1. The Duox, peroxidase, and GFP amplimers were separately cloned into the corresponding restriction sites of the pLA4400 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) as described. The injected nymphs were kept overnight in an incubator and used for feeding on mice, and repleted ticks were individually processed for assessment of gene silencing and pathogen levels using qRT-PCR analysis. Primers that bind further upstream and downstream of the target cDNA encompassing the dsRNA sequence were used for the qRT-PCR, as indicated in supplemental Table S1.

Infection Studies—For an assessment of whether the RNA interference-mediated reduction of Duox or peroxidase expression affected *B. burgdorferi* acquisition by nymphal ticks, C3H mice were infected with spirochetes (10^5 spirochetes/mouse). 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 h of feeding, whereas the others were allowed to feed to repletion and then collected. The *flaB* transcripts were detected using qRT-PCR as detailed earlier. 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 incu-

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3 The abbreviations used are: DTN, dityrosine network; Duox, dual oxidase; ROS, reactive oxygen species; dsRNA, double-stranded RNA; qRT-PCR, quantitative RT-PCR; NOS, nitric-oxide synthase.
bated in PBS with 5% goat serum and 0.5% Tween 20, as detailed (30). 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 then washed using PBS with 0.05% Tween 20, stained with DAPI (Invitrogen), and imaged by a LSM 510 laser confocal microscope (Zeiss) as described (30).

Assays for Nitric-oxide Synthase Activity—The NOS activity of the tick tissues was analyzed using a commercial kit (Ultrasmartive colorimetric assay kit; Oxford Biomedical Research) as described (33). Briefly, whole tick samples were homogenized in PBS under the ice and centrifuged briefly, and 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 as detailed, and the OD values at 540 nm were calculated using a standard curve consisting of serial dilutions of nitrite from 0 to 100 μM.

Bioinformatics and Statistical Analysis—Unless indicated otherwise, protein annotation and searches were executed using the VectorBase database. Analyses of protein families and domains were performed using the Pfam and CDD databases. Sequence alignment analysis was performed using Cobalt Constraint-based Multiple Protein Alignment Tool. The results are expressed as the means ± S.E. Statistical significance of differences observed between experimental and control groups were analyzed using GraphPad Prism version 4.0 (GraphPad Software). 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—Ixodid ticks are known to harbor a diverse set of gut bacteria (34, 35). To further study the role of the Duox in the genesis of a DTN and protection of invading pathogens like B. burgdorferi, we first assessed replication of the gut bacterial population during feeding. To accomplish this, naïve mice (3 animals/group) were parasitized with nymphal ticks (15 ticks/mouse), and partially fed ticks were removed at 6–48 h of feeding. Batches of harvested ticks were subjected to total RNA or DNA isolation. Total bacterial numbers were examined by qPCR analyses using tick DNA as a template and generic bacteria-specific 16 S primers, whereas expression of Duox was determined using qRT-PCR. The results showed that bacterial numbers in the feeding tick gut peaks at ~20 h during engorgement (Fig. 1A). Although up-reg-
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FIGURE 2. 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 domains as identified by NCBI conserved domain database search (gray boxes), regions encompassing dsRNA constructs (dsR1–dsR5, red), and detection primers (blue) are shown. Of the dsRNA constructs tested, dsRNA2 (dsR2, spanning nucleotide positions 960–1440, shown in bold type) was most effective and used for subsequent studies. Primers spanning 5′ and 3′ regions of Duox (shown in blue text) were used for detection of transcripts using RT-PCR analysis using agarose gel electrophoresis and quantitative RT-PCR, respectively. B, knockdown of Duox transcripts induced by RNAi interference. Nymphal ticks (25/group) were injected with Duox dsRNA or control GFP dsRNA, fed on naïve mice, and collected at 48 h after onset of feeding; isolated guts were processed for measurement of Duox transcripts. The left panel shows RT-PCR analyses using primers spanning either 5′ FAD domain region of Duox or a loading control gene, tick β-actin, which are expected to amplify same-sized (400 base pairs) products. The right panel shows quantitative RT-PCR analysis using primers targeting 3′ FAD domain region (Fig. 2A), and the results were normalized against corresponding β-actin transcript levels. Each circle represents an individual tick that was processed and analyzed separately. Compared with ticks microinjected with GFP dsRNA, ticks injected with Duox dsRNA experienced significant down-regulation of Duox expression (p < 0.01). C, Duox knockdown interferes with the formation of the DTN. After 60 h of feeding, ticks were collected, and the guts were dissected. DTN formation was detected using anti-dityrosine antibodies (green) 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 control GFP dsRNA, 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 with ticks microinjected with GFP dsRNA, B. burgdorferi burden in ticks was significantly reduced after injection of Duox dsRNA (p < 0.01).

ulation of Duox occurred at multiple time points during feeding, the earliest peak is recorded at 20 h (Fig. 1B), which temporally coincides with the greatest bacterial numbers in feeding ticks. These data suggest that the induction of Duox during the initial hours of feeding potentially in response to gut microbe replication. We then perform a similar tick feeding study to assess whether B. burgdorferi presence within the gut also induces Duox expression. The results show that the ticks that parasitized B. burgdorferi-infected mice contained at least 3-fold higher levels of Duox transcripts in comparison with the ticks that fed on naïve mice (Fig. 1C). We next employed confocal immunofluorescence microscopy 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 h fed ticks, and frozen tissue sections were labeled with a mouse monoclonal antibody specifically against dityrosine or isotype control antibodies. The data indicated specific binding of dityrosine antibodies to the tick gut, indicating the presence of a DTN, whereas no reactivity was recorded for the salivary glands (Fig. 1D) or in gut tissues incubated with isotype control antibodies.

Duox Knockdown Reduces B. burgdorferi Persistence in Ticks—Because our studies revealed that the replication of gut microbes peaks at 20 h of feeding, which also coincides with Duox expression and formation of the DTN in the gut, we next examined whether 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 (5,418 bp), to identify the region(s) that are most susceptible to RNAi-mediated knockdown, five different dsRNA preparations that encompass various regions of the large gene (5,418 bp), to identify the region(s) that are most susceptible to RNAi-mediated knockdown, five different dsRNA constructs were generated (Fig. 2A) 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 (Fig. 2B, \( p < 0.01 \)) and used in all subsequent studies. Next we examined whether reduction of Duox expression affects formation of the DTN and whether this influences \textit{B. burgdorferi} persistence using dsRNA-injected ticks that fed on \textit{B. burgdorferi}-infected mice. We found that the appearance of the DTN formation was less obvious in Duox knockdown ticks (Fig. 2C), which also retained significantly less \textit{B. burgdorferi} burden when compared with control (GFP dsRNA-injected) ticks (Fig. 2D, \( p < 0.01 \)). Similarly, qPCR analysis using 16 S rRNA levels of total gut bacteria also shows an apparent decrease in Duox knockdown ticks (data not shown). Together, these results suggest that the tick Duox plays a beneficial role for the Lyme disease pathogen while feeding ticks acquire it from infected hosts.

**Identification of a Gut Peroxidase Involved in DTN Formation**

Because previous studies suggested that DTN formation could require participation of a specific gut peroxidase (23), we next labeled the tick gut using an anti-peroxidase antibody. The results show that peroxidase labeling in the gut readily co-localizes with the DTN labeling (Fig. 3A). We further searched for the existence of a gut peroxidase that is potentially involved in DTN formation. Because the \textit{Ixodes} genome encodes for at least 16 potential peroxidase proteins, we studied their expression in our attempt to shortlist potential candidates. RT-PCR analyses indicated that 11 of 16 peroxidases showed dramatic yet variable levels of transcripts in feeding ticks (Fig. 3B). We further assessed whether their expression is modulated during spirochete infection by comparing corresponding mRNA levels in ticks that fed on \textit{B. burgdorferi}-infected mice versus naïve mice. The data show that although expression of a majority of these peroxidases remains unaltered in the presence of \textit{B. burgdorferi} (data not shown), two of them (annotated as ISCW017368 and ISCW002528) displayed dramatic induction when spirochetes are present in feeding ticks (Fig. 3C, \( p < 0.01 \)). Subsequently, a measurement of transcript levels of both of these genes suggested their induction at various different time points of tick feeding (Fig. 3D and E); although mRNA levels of ISCW002528 peak between 6 and 12 h, ISCW017368 is most dramatically expressed between 24 and 36 h.

**Selective Silencing of Peroxidase ISCW017368 Influences DTN Formation and \textit{B. burgdorferi} Acquisition by Ticks**

Because two of the 16 potential peroxidase enzymes, ISCW017368 and ISCW002528, were induced upon spirochete infection of ticks, we used an RNAi-mediated knockdown strategy to assess their rela-
Induction of Nitric-oxide Synthase Potentially Contributes to the Reduction of B. burgdorferi in Ticks—Because disruption of the DTN barrier could result in a strong pathogen-specific immune response, we sought to assess whether the impaired DTN in Duox knockdown ticks and observed reduction of spirochete levels is due to activation of specific tick innate immune pathway(s) or component(s). We compared the transcript levels of representative potential immune genes in Duox knockdown and control (GFP dsRNA-injected) ticks. Analysis of a selected set of putative immune genes grouped under non-self-recognition, signal transduction, and anti-microbial peptides indicated that none of the tested genes showed significant induction in Duox knockdown ticks; however, induction of a gene encoding nitric-oxide synthase (NOS) was observed (Fig. 5A, p < 0.05). Because B. burgdorferi responsiveness to NO-
mediated killing (36, 37) and the anti-parasitic effects of NOS has been documented in DTN-silenced mosquitoes (23), the *I. scapularis* NOS gene was further confirmed for its up-regulation in additional groups of *B. burgdorferi*-infected Duox or peroxidase ISCW017368 knockdown ticks (Fig. 5B), which also reflected an augmentation of total NOS enzymatic activity (Fig. 5C, *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.

**DISCUSSION**

*B. burgdorferi* thrives in nature though an intricate tick-mammal infection cycle (3, 38). Notwithstanding an intense research focus on Lyme borreliosis, precisely how the pathogen successfully persists in and is transmitted through ticks, despite a potentially rigorous arthropod innate immune response against invading microorganisms (14–18), remains enigmatic. Here, we present evidence that a molecular barrier, termed dityrosine network (DTN) exists in ticks that could represent a protective strategy to preserve homeostasis of gut commensals that replicate during feeding. Homologous to peritrophic matrix described in the gut of most insects including ticks (30, 39), this molecular wall is normally synthesized in response to blood feeding and creates a protective barrier between luminal contents and underlying gut epithelial cells. In *Drosophila*, the matrix is part of a protective innate immune response because in its absence, increased susceptibility to bacterial toxins is observed (25). In contrast, as shown in *A. gambiae* earlier (23), formation of the DTN in ticks also likely decreases the gut permeability to immune elicitors, thereby inadvertently also protecting pathogens like *B. burgdorferi*. We show that DTN formation in *I. scapularis* ticks required participation of an NADPH oxidase, a tick Duox, along with a specific gut peroxidase. Together, our results highlight the paradigm of a less well known yet critical function of a NADPH oxidase system in protein cross-linking activity related to diverse cellular functions that specifically range from cross-linking of the extracellular...
matrix or cuticular proteins in 

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The *Ixodes* genome is relatively large and highly redundant; although it encodes a single Duox, there are at least 16 potential peroxidases, 8 of which are annotated as heme peroxidases, 4 as glutathione peroxidases, 3 as phospholipid-hydroperoxide glutathione, 1 as thioredoxin peroxidase, and 1 as cAMP-regulated thioredoxin peroxidase. Although each of these enzymes could play individual and potentially important roles in tick physiology and immunity, 11 are dramatically expressed in feeding ticks. Considering further up-regulation of two of these in the presence of *B. burgdorferi*, the functions of peroxidase ISCW002528 and peroxidase ISCW017368 could certainly be related to vector immunity. In fact, our data indicate that the latter, which is annotated as a heme peroxidase, is an indispensable partner of the tick Duox system essential for DTN formation. Interestingly, although the molecular basis of DTN formation remains unknown, in mosquitoes, the participation of a heme peroxidase is also required (23). Thus, our results 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* that is important of heme binding (41, 43) in *I. scapularis* or other arthropods is indicated in red text.

matrix or cuticular proteins in Caenorhabditis elegans to egg-shell hardening or epithelial barrier (DTN) functions in insects. Originally identified in the thyroid, Duox enzymes have been shown to promote formation of dityrosine linkages (24). In mammals, formation of these bonds relies upon heme peroxidase activity, such as thyroperoxidase or myeloperoxidase, utilizing hydrogen peroxide often formed from NADPH oxidase activity, such as thioredoxin peroxidase. Although each of these enzymes plays a role in tick physiology and immunity, 11 are dramatically expressed in feeding ticks. Considering further up-regulation of two of these in the presence of *B. burgdorferi*, the functions of peroxidase ISCW002528 and peroxidase ISCW017368 could certainly be related to vector immunity. In fact, our data indicate that the latter, which is annotated as a heme peroxidase, is an indispensable partner of the tick Duox system essential for DTN formation. Interestingly, although the molecular basis of DTN formation remains unknown, in mosquitoes, the participation of a heme peroxidase is also required (23). Thus, our results 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* that is important of heme binding (41, 43) in *I. scapularis* or other arthropods is indicated in red text.

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4. Piesman, J., Oliver, J. R., and Sinsky, R. J. (1990) Growth kinetics of the burden. A major fraction of Plasmodium parasites were 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. We show that transcript levels of NOS or their enzymatic activity in Duox knockdown ticks are significantly enhanced, which may be linked to a potential 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. Our attempts to study the possible involvement of NOS in the generation of borreliacidal responses in ticks and assess whether RNAi or in vivo inhibitor-mediated interference of NOS reverses the deleterious effect of Duox silencing on B. burgdorferi survival remained 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 immune response of the vector to other enteric pathogens, including DTN-silenced mosquitoes, and thus likely represents a conserved microbialidal response in diverse arthropod species.

In summary, here we present evidence that a molecular barrier, recently proposed in studies using malarial vectors, termed DTN, and catalyzed by a Duox/peroxidase system, allows B. burgdorferi potentially 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. Our data show that proteins critical for formation of the DTN, the NADPH oxidase, or Duox, as well as a heme peroxidase protein, are inductive 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. Together, this information will help clarify the biology of tick immune responses, will provide insights about adaptive strategies of a bacterium that persists in a diverse array of host and vector tissues, and may contribute to new targets for the disruption of the spirochete life cycle.

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