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Interactions between *Borrelia burgdorferi* and its hosts across the enzootic cycle

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Abstract

The bacterial pathogen *Borrelia burgdorferi* is the causative agent of Lyme disease and is transmitted to humans through an *Ixodes* tick vector. *B. burgdorferi* is able to survive in both mammalian and tick hosts through careful modulation of its gene expression. This allows *B. burgdorferi* to adapt to the environmental and nutritional changes that occur when it is transmitted between the two hosts. Distinct interactions between the spirochete and its host occur at every step of the enzootic cycle and dictate the ability of the spirochete to survive until the next stage of the cycle. Studying the interface between *B. burgdorferi*, the *Ixodes* tick vector, and the natural mammalian reservoirs has been made significantly more feasible through the complete genome sequences of the organisms and the advent of high throughput screening technologies. Ultimately, a thorough investigation of the interplay between the two domains (and two phyla within one domain) are necessary in order to completely understand how the pathogen is transmitted.

1. Introduction

Arthropod borne diseases are becoming increasingly prevalent across the globe, and an understanding of the pathogen-arthropod interface can be an important tool in control of these diseases. *Ixodes* ticks carry many pathogens of human importance, including *Anaplasma, Babesia, Borrelia*, tick-borne encephalitis virus and Powassan virus. The most researched of these pathogens is the spirochete *Borrelia burgdorferi*, the causative agent of Lyme disease and the most common vector-borne disease in the United States (1–3). There are three important components to maintenance of the *B. burgdorferi* life cycle: the spirochete, the invertebrate tick vector, and the vertebrate host (Figure 1).

Ixodes ticks are hematophagous and take one blood meal during the larval, nymph and adult stage of their lifecycle. *B. burgdorferi* is not transmitted from adult ticks to eggs, so larval ticks must acquire the spirochetes from infected animals, such as birds and mice, with the first blood meal. The spirochetes will remain within the tick after feeding and molting into the nymphal stage. A *B. burgdorferi* infected nymph will feed on another reservoir host and transmit the spirochetes, continuing the enzootic cycle. After another molt into the adult stage, the adult tick will typically feed on larger animals (such as deer) that may not be

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competent reservoir hosts for *B. burgdorferi* but are critical to tick mating. Ticks in the nymphal stage are primarily responsible for transmission of *B. burgdorferi* to humans, but humans are not important in the enzootic cycle and are considered dead-end hosts (1).

The tick salivary glands and midgut play important roles in the colonization and transmission of *B. burgdorferi*. During acquisition from an infected host that occurs with the tick blood meal, *B. burgdorferi* enters the tick with blood, and interacts with tick commensal microbes and tick proteins to establish infection and persistence within the vector. Ticks, in response to the bloodmeal and the presence of the pathogen, will modify expression of certain genes. *B. burgdorferi* then has to respond to the vast environmental changes of moving from a vertebrate host to an invertebrate host. These include but are not limited to differences in host immunity, nutrient availability, and temperature. For example, after a blood meal, hard ticks such as *Ixodes* will not feed again for many months. To adapt, the organism needs to slow its growth rate, evade tick immunity and survive a wide range of temperatures. Then, when the tick takes its next blood meal, spirochetes must identify the signals of a new feeding and initiate the changes required for successful migration to the salivary glands in preparation for transmission into a new mammalian host (1,4–8). In this review, we will provide an overview of the enzootic cycle, focusing on the interplay between the mammalian host, the *Ixodes* tick vector, and *B. burgdorferi*, at each step.

2. Tick acquisition and initial *Borrelia* adaptations

During the first stage of the enzootic cycle, *B. burgdorferi* is transmitted into ticks during the larval blood meal. This change in environment requires *B. burgdorferi* to rapidly adapt from the mammal to the tick in order to survive. *B. burgdorferi* must sequester nutrients from the blood meal and evade elimination by components of the tick immune system (Figure 1).

2.1 *Borrelia* transmission into ticks

Ixodes ticks use a feeding apparatus, made of a ventral barbed hypostome, which acts as an anchor and two chelicerae, lined by rows of dentricles, to saw into the host skin (9). In the case of *I. scapularis* but not *I. ricinus*, the tick anchors the mouthparts to the skin via a cement-like material that is created by the salivary glands (10,11). The blood meal lasts for several days, so they have had to develop many strategies to remain attached to the host without triggering key host immune defenses and allowing for adequate blood flow. To accomplish this, tick saliva contains immunosuppressant, vasodilator and anticoagulant molecules that are beneficial to feeding and are utilized by pathogens such as *B. burgdorferi* to assist with transmission (12–14). These will be discussed in detail later in the review. When spirochetes enter the midgut of the larval tick, they utilize the nutrients from the blood-meal and rapid replication occurs (15). Very little is known about spirochete nutrient utilization and metabolism in larval ticks, but nutrient utilization during the nymphal blood meal is better characterized and will be discussed in later sections.

B. burgdorferi's biphasic life cycle requires the spirochete to adapt to the changes in environmental conditions from the vertebrate phase to the arthropod phase, including shifts in pH, temperature, immune defenses, and nutrient availability. *B. burgdorferi* combats these changing conditions by activating transcriptional regulators important in controlling

expression of genes that are involved in attachment to the tick and evading tick immunity (4). A two-component system, consisting of Histidine kinase 1 (Hk1) and response regulatory protein 1 (Rrp1), have been studied in the tick phase and appear to work concurrently during both the larval and nymphal life stages to regulate expression of genes required for survival within the tick. There are still large gaps of knowledge surrounding the regulation and activation of Rrp1 and Hk1 (16–20). Hk1 signaling seems to be induced by stimuli during the process of larval and nymphal feeding but it is unclear if these signals are derived from the vertebrate host at the site of the tick bite or from the tick midgut (21).

It is known that Hk1/Rrp1 play a critical role in promoting *Borrelia* survival within the gut and regulating production of the nucleotide second messenger cyclic-dimeric-GMP (c-di-GMP) (17). Hk1 binding of free amino acids or their derivatives (18) results in a signaling cascade culminating in the phosphorylation of Rrp1 (reviewed in (4)). Rrp1 phosphorylation catalyzes the synthesis of c-di-GMP, which in turn modulates the expression of genes required for survival within ticks including those important for chemotaxis, nucleotide and carbohydrate metabolism (17,20,22). Rrp1-deficient *Borrelia* strains, which are unable to synthesize c-di-GMP, are unable to survive within the tick vector (19), underscoring the importance of both the Hk1/Rrp1 two-component system and c-di-GMP on *Borrelia* survival. Indeed, levels of c-di-GMP help regulate other functions of *Borrelia* survival, including motility and virulence (23,24).

2.2 Tick immune system, detection of *Borrelia*, and *Borrelia* evasion

Ticks take in relatively large blood meals and are therefore vulnerable to the many invading pathogenic species that may be present in the blood. Arthropods defend themselves through many immune signaling pathways like Janus kinase-signaling transducer activator of transcription (JAK-STAT), immune deficiency (IMD), and Toll signaling, but our knowledge of these immune mechanisms in humans and mice cannot necessarily be applied to tick immunity, as ticks are phylogenetically distinct (25,26). *I. scapularis* encodes 33 genes that potentially belong to the Toll pathways, but the roles of these genes and Toll pathways are yet to be elucidated (27). The *I. scapularis* IMD pathway, though significantly different than the traditional insect IMD pathway, produces antimicrobial responses to *B. burgdorferi* and *Anaplasma phagocytophilum* to reduce colonization of these pathogens. During *A. phagocytophilum* infection, this pathway is trigged by infection-derived lipids 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG) and 1-palmitoyl-2-oleoyldiacylglycerol (PODAG), however it is not known whether infection-derived lipids are the trigger for this pathway in *B. burgdorferi* infection (28).

A crucial component of arthropod physiology, the peritrophic membrane (PM) also acts as part of the tick immune defenses. The PM allows metabolites and other materials to be transported between the gut lumen and surrounding tissues, and also acts to prevent abrasive particles from getting through the gut epithelium (29,30). During early stages of feeding, the PM can be detected in *Ixodes* tick midguts, and can act as an immune barrier to pathogens (30,31). Some *Babesia microti* parasites, also transmitted by *Ixodes* ticks, develop an organelle called the arrowhead that passes through the PM and allows parasites to enter the epithelial cells (32). Pathogens that inhabit other arthropods such as *Aedes aegypti*

mosquitoes and sand flies have been known to secrete chitinases that break down the PM barrier and allow for movement through the gut epithelium (33,34). *B. burgdorferi* does not appear to have homologs of this protein. Proteome analyses of PM isolated from fed ticks indicated that there were few unique proteins and the predominant protein was homologous to arthropod chitin deacytlase. Knockdown of this predominant protein, *I. scapularis* CDA-like protein (IsCDA), did not prevent formation of the PM or persistence of *B. burgdorferi* (30). However, passive transfer of antibodies against this protein did increase persistence of *B. burgdorferi* in ticks without impacting the total bacterial population in the gut, perhaps demonstrating a role for the PM in selectively limiting the levels of *B. burgdorferi* in the gut. It is hypothesized from the amino acid sequence that perhaps IsCDA, while containing a conserved enzymatic domain, provides mechanical strength for the PM rather than acting as an active enzyme (30). Yang *et al*. recently discovered that a PM-associated protein, Peritrophic Membrane Chitin Binding Protein (PM_CBP), likely plays a role in the structure and organization of the PM (35). Interference of PM_CBP expression resulted in decreased thickness and increased permeability of the PM, as well as delayed tick feeding (35).

With the blood meal, ticks ingest immune molecules from the vertebrate host that are released in response to the feeding ticks. Vertebrate cytokines and chemokines may impact the tick's own innate immune signaling processes. Smith *et al*. found that vertebrate cytokine IFNγ taken up in the blood meal induces *I. scapularis* Rho-like GTPase (IGTPase) in a STAT-dependent fashion, which then regulates expression of a borreliacidal peptide, domesticated amidase effector 2 (Dae2) (Figure 2). Knockdown of IGTPase in nymphal ticks caused an increase in spirochete burden (36). Dae2, which is expressed during the unfed nymphal and unfed adult stages of *I. scapularis* ticks, is thought to act on the cell wall of *B. burgdorferi* to regulate levels of the spirochete after the tick has acquired the bacteria. It does not, however, limit the amount of bacteria taken in by the tick at the blood, as knockdown in nymphal ticks immediately after engorgement did not result in lower spirochete burden (37).

3. *Borrelia* interactions with the tick gut

Following uptake from the blood meal, *B. burgdorferi* establishes residence within the tick gut. Within this environment, the spirochete interacts with other bacteria within the gut microbiome and tick gut proteins in order to remain viable.

3.1 Tick gut microbiome

The tick gut microbiome may also influence pathogen success and colonization, and manipulation of the microbiome may allow for interruption of this colonization. *B. burgdorferi* may be particularly susceptible to this, as it does not possess the genetic mechanisms for direct interactions with other bacteria, demonstrated by the finding that increases in bacterial genera like *Pseudomonas, Bacillus*, and *Enterobacteriaceae* are associated with decreases in *B. burgdorferi* in the tick midgut (38). However, studies on hard ticks such as *I. scapularis* have observed differing results in terms of the tick microbiome, with some studies finding few bacterial genera in the tick gut and other studies observing 20 or more. The geographical location of field collected ticks, the life stage of the tick, the tick

species, and time from molting and feeding may also play a role in the inconsistencies reported in tick microbiome research, as well as a lack of agreement as to whether the tick microbiome includes environmental microbes and symbionts (39,40).

Recent evidence has leaned towards lack of a stable microbiome. Ross *et al.* found through visualization of bacteria and direct measurements of bacterial burden, that *I. scapularis* ticks do not have a stable midgut microbiome (38). A possible explanation for the discrepancies is that many of the initial studies were done by 16s rRNA sequencing and that low biomass has been associated with misinterpretation of sequencing data as well as susceptibility to contamination. Pooling samples when studying the tick microbiome may lead to more consistent results in the future (38,41,42). In a recent study supporting the results from Ross *et al.*, Guizzo *et al.* found that the hard ticks *I. ricinus* and *Rhipicephalus microplus* ticks also have relatively small midgut bacterial communities compared to other blood sucking arthropods, even after the blood meal. Interestingly, the ovaries of both species had much larger microbial communities (43). However, despite a relatively small and inconsistent taxonomic composition, Estrada-Pena *et al.* found that *I. scapularis* midgut microbiomes are functionally redundant. This redundancy implies that the microbiomes have a core functionality that can be accomplished by many taxonomic combinations, and this allows the functional tick microbiome to be quite resistant to perturbation (39).

Narasimhan *et al.* found that the microbiome of the tick may play a key role in the tick's immune defenses against pathogens attempting to colonize the gut (44). They suggest that the expression of STAT, a key component in the JAK/STAT signaling pathway, is modulated by the tick gut microbiome, and that this may facilitate the expression of a core glycoprotein of the tick PM, peritrophin. Increased expression of peritrophin would therefore strengthen the integrity of the PM barrier and they theorize that this may protect the spirochetes from the harsh environment of the gut lumen (44) (Figure 2). Indeed, it has been recently demonstrated that administration of anti-PM_CBP antibodies resulted in impaired spirochete survival in the tick gut (35), again suggesting a potentially critical role of PM integrity on *Borrelia* survival. This research presents a novel and atypical role for the PM, as it is typically thought to be a physical immune barrier that prevents pathogens from breaking through the epithelial layer (29).

Different microorganisms have also been found to manipulate the tick microbiome to facilitate colonization and persistence. A protein upregulated in ticks infected with *B. burgdorferi* and induced by feeding is Protein of *I. scapularis* with a Reeler domain (PIXR). Knockdown of PIXR via RNAi or ticks fed on mice immunized with recombinant PIXR caused a decrease in *B. burgdorferi* colonization in larval and nymphal ticks, and it is hypothesized that PIXR plays a role in inhibiting gram-positive bacterial biofilm formation in the tick gut. This is similar to the role of IAFGP, a protein induced by *Anaplasma phagocytophilum*, which inhibits biofilm formation by binding to the peptidoglycan in the cell walls of gram-positive bacteria (45). Though it is not clear how the induction of PIXR benefits *B. burgdorferi*, it may be elimination of the physical biofilm barrier that allows for easier colonization, or it may be a reduction in some detrimental immune responses provoked by biofilm production and changes in the tick microbiome (46).

3.2 Gut protein-*Borrelia* interactions

The *B. burgdorferi* outer surface proteins OspA and OspB have been shown to be critical for survival within and colonization of the tick gut (47,48). Of the two, much more is known about the importance of OspA for survival within the tick, and the importance of OspA antibodies in preventing binding of *B. burgdorferi* to the tick gut, as this has been the focus of vaccination efforts (49). OspA is selectively expressed when *B. burgdorferi* enters larval ticks, and this allows for attachment and colonization in the gut. During tick feeding, some spirochetes will stop producing OspA, which is thought to allow the spirochetes to detach from the tick gut and travel to the salivary glands for transmission to the host. It is also thought that some adherence to the tick gut occurs via OspA-OspA interactions and that the downregulation of OspA during migration to the salivary glands reduces these interactions and prevents clumping of the spirochetes during transmission to the vertebrate (50). Some spirochetes will continue to produce OspA but these spirochetes disappear during the establishment of mammalian infection (51–53). It is unclear if it is because the spirochetes eventually downregulate OspA or if those that do not are cleared by the host immune system. There is an abundance of research demonstrating that OspA elicits immunity against *B. burgdorferi* and OspA was the antigen for the only approved human vaccine for Lyme disease (54). C3H mice administered recombinant OspA have been shown to be protected against multiple strains of *B. burgdorferi* (54–56). Additionally, antibodies to OspA bind to the spirochetes in the tick gut during feeding, so mice administered OspA antibodies are also protected from challenge with *B. burgdorferi* (57,58).

The related protein, OspB, has also been shown to play a role in tick colonization by the spirochete. Non-borreliacidal antibodies to OspB have been shown to inhibit the interaction between the spirochetes and the tick gut, and some of this inhibition may be due to antibody binding to multiple epitopes, causing steric hinderance (59). It is possible that, due to the genetic and structural similarities of the two outer surface proteins, and the colocalization of these proteins on the surface of the spirochete, antibodies to OspB may impact the binding of OspA to the tick gut (59,60). Neelakanta *et al.* demonstrated in their data that OspB facilitates the survival of spirochetes in the tick, as spirochetes deficient in OspB were not able to colonize, resulting in low numbers of spirochetes in the tick gut despite entering the ticks from infected mice at the same rate as their wild type counterparts (47). The mutant spirochetes that were able to persist may have been able to utilize OspA to adhere to the tick gut, as OspA was not affected in the OspB mutants (47).

At the same time as *B. burgdorferi* is highly expressing OspA, *I. scapularis* is highly expressing the OspA specific ligand, Tick Receptor for OspA (TROSPA). TROSPA expression does appear to be influenced by the presence of spirochetes within the tick, as ticks that do not harbor any *B. burgodorferi* express less TROSPA. This upregulation within the tick would be beneficial to the spirochetes, as the OspA-TROSPA interaction increases spirochete colonization in the tick gut (Figure 2). After tick feeding and engorgement, expression of TROSPA decreases within the tick, in parallel to decreased expression of *B. burgdorferi* OspA (61). Another protein found to impact *B. burgdorferi* colonization in the tick gut is *I. scapularis* protein disulfide isomerase A3 (IsPDIA3). Knockdown of this protein in nymphs resulted in decreased *B. burgdorferi* colonization. Furthermore, larvae fed

on mice that were given anti-recombinant IsPDIA3 sera had decreased *B. burgdorferi* colonization. When IsPDIA3 was knocked down in ticks already infected with *B. burgdorferi*, however, it did not impact the spirochete burden, demonstrating that IsPDIA3 is important for colonization but not viability or persistence of *B. burgdorferi* (62).

4. *Borrelia* survival between blood meals

While the blood meal ushers in a large wave of nutrients, spirochetes need to survive within the tick for months after the blood meal nutrients have been depleted until the following blood meal has been taken. During this time, there are many changes in *B. burgdorferi* gene expression, and the spirochete has to modify its utilization of carbon sources in order to survive within the tick until the next blood meal (Figure 1).

Studies have identified several *Borrelia* genes required for persistence within the tick after the blood meal nutrients have waned, although a complete picture of which genes are critical for survival between blood meals remains unknown. BB0690, a Dps protein, homologs of which have been shown to protect other bacteria against DNA damage and oxidative stress (63,64), is highly expressed in ticks and is required for *Borrelia* persistence, as Dps-deficient spirochetes are unable to survive for prolonged periods in unfed ticks (65). While BB0690 does not bind DNA or protect against oxidative stress *in vitro*, it does bind to iron and copper, which in turn helps to protect the spirochete against peroxide stress (65,66). Additionally, the *B. burgdorferi* DnaK suppressor protein (DksA) helps to mediate the response to starvation through downregulating DNA replication machinery proteins, flagellar components, and ribosomal proteins (67,68). The surface lipoprotein of unknown function, *bptA*, has been shown to be a critical regulator of *Borrelia* virulence and persistence within the tick although the mechanism by which it acts is still unknown (69).

Rrp1, the diguanylate cyclase that synthesizes c-di-GMP, is required to control expression of glycerol transport and metabolism in *B. burgdorferi* (19,70). During the initial part of the tick phase of the enzootic cycle, the influx of mammalian blood into the tick gut allows for the utilization of glucose as a carbon source by *B. burgdorferi*. However, in the period of time after the larval blood meal and prior to the nymphal blood meal, glucose and other nutrients provided by the mammalian blood are depleted quickly and *B. burgdorferi* switches its primary carbon source to glycerol, as well as chitobiose and maltose for glycolysis, in order to survive within the tick (20,71–76). Glycerol is readily available in the tick midgut, as it is produced by ticks to serve as “antifreeze” during the winter. Spirochetes that are deficient in either Rrp1 (16,19) or genes encoding the glycerol metabolism operon (71) are unable to survive in ticks. Glycerol utilization and metabolism in *B. burgdorferi* is regulated by the stringent response and Rel_{Bbu} (BB0198) (77,78). Rel_{Bbu} is a homolog of RelA and SpoT (79), enzymes that control the levels of two nucleotide signaling molecules, termed alarmones: guanosine pentaphosphate (pppGpp) and guanosine tetraphosphate (ppGpp). Together, these molecules modulate transcription, translation, and numerous other cellular activities. In *B. burgdorferi*, Rel_{Bbu} is responsible for (p)ppGpp synthesis (77,80,81) and is critical for spirochete survival within the tick in between blood meals, partially due to its regulation of the glycerol metabolism pathway (77). It is possible that the importance of Rel_{Bbu} in spirochete survival is not limited to regulation of glycerol metabolism genes, as

RNA sequencing and microarray analysis has revealed a number of genes that are differentially regulated by Rel_{Bbu} during nutrient stress (77,78). Through identifying specific pathways regulated by Rel_{Bbu}, we can further understand how *B. burgdorferi* survives in the tick between blood meals.

5. *Borrelia* migration from the midgut to the salivary glands to a new host

While *Borrelia* reside in the tick midgut between blood meals, the onset of the blood meal prompts the spirochetes to migrate out of the midgut and into the salivary glands in preparation for transmission into the mammalian host (82). The influx of blood into the tick ushers in further changes to the midgut environment, including temperature and pH, inducing changes to *Borrelia* gene expression necessary for adapting to the new environment (5,83–86). While acquiring the blood meal, ticks remain attached to their host for several days. The tick salivary proteins expressed throughout the feeding period evolve to enhance successful transmission of *Borrelia* to the vertebrate host (14).

5.1 Nutrient uptake during the nymphal blood meal

The onset of the nymphal blood meal ends the period of nutritional deprivation for the spirochete. Immediately following the nymphal blood meal, spirochete numbers in the midgut remain low but increase as the feeding continues in response to the influx of nutrients (15,87,88). Hoxmeier *et al.* used metabolomics to dissect the interaction between *I. scapularis* ticks and *B. burgdorferi* during the blood meal, as *B. burgdorferi* does not possess the pathways for synthesis of nucleotides, amino acids, fatty acids, or enzyme cofactors and therefore it competes with the tick for these nutrients (89). Elucidating these interactions and identifying these metabolites could be important for controlling spirochete growth and persistence within the tick vector.

B. burgdorferi lacks the enzymes for the classical pathway for purine salvage, including hypoxanthine-guanine phophoribosyltransferase (*hpt*), adenylosuccinate synthase (*purA*), and adenylosuccinate lysase (*purB*). It also does not contain the genes that encode the enzymes for de novo synthesis of purines so it sequesters deoxynucleotides and purine bases, the most common purine being hypoxanthine, from the tick blood meal through novel purine salvage pathways (90–92). Similarly, *B. burgdorferi* obtains amino acids from the blood meal, but very little is known about how the spirochetes sequester them from the tick (93). Peptides are a source of amino acids for many bacteria, and this is likely the case for *B. burgdorferi*, as it possesses a peptide transport system similar to oligopeptide permease (Opp) transporters. The genome encodes five peptide-binding proteins, all of which are capable of working with the transporter to facilitate peptide transport (94). The peptide binding proteins show different binding specificity and patterns of expression suggesting that they may play specific roles in particular parts of the lifecycle (94–97). Ablation of this Opp system *in vitro* results in spirochetes that are morphologically abnormal and unable to replicate, further highlighting the critical role of this system for spirochete survival (95). Immediately after the nymphal blood meal, *B. burgdorferi* switches its carbon source to glucose, the most prevalent carbohydrate source in mammalian blood and one of the limited amounts of carbohydrates *B. burgdorferi* can utilize for energy (19,71,98,99). This

utilization of glucose is supported by the presence of multiple phosphotranferase-type glucose transporter genes in the *B. burgdorferi* genome (4,72,73,100). *B. burgdorferi* is also dependent on the blood meal in the tick for fatty acids and cholesterol as it cannot synthesize them, elongate fatty acid chains or oxidize exogenous fatty acids on its own (101–103). The spirochete's outer membrane is composed of cholesterol and fatty acids and free cholesterol appears to be crucial to the interactions with the tick through lipid rafts (104).

5.2 Changes in *Borrelia* and tick gene expression occurring during the blood meal(s)

As previously discussed, Rrp1-Hk1 mediates the initial transition of *B. burgdorferi* to the tick host during the blood meal. The environment during the blood meal is harsh, with activation of reactive oxygen and nitrogen species as well as activation of the tick immune system (7,27,28,105–107). By studying a transposon library of *Borrelia* mutants (108), Phelan *et al.* were able to identify 46 genes that were essential to *Borrelia* survival in ticks after the blood meal (109). Most of these genes were of unknown function, and likely have a variety of functions critical for *Borrelia* survival and migration out of the gut. However, several of the genes appeared to be involved in protection against reactive oxygen species. BB0017, a protein known to protect *B. burgdorferi* against reactive oxygen species, is required for tick survival through control of expression of other genes (109).

After establishing infection in the tick midgut, the next major event in the lifecycle is the nymphal blood meal. Spirochete gene expression changes in response to nymphal blood uptake are critical for *B. burgdorferi* survival and transmission to a new host. During uptake of the nymphal blood meal, the Hk2/Rrp2 two-component system becomes active, although the specific signal that leads to this activation is unknown. Phosphorylation of Rrp2 by Hk2 results in Rrp2 activation (110–112). In turn, Rrp2 acts as a transcriptional activator for RpoN, which controls expression of RpoS, both of which are alternate sigma factors (112–117). While many components of this pathway have yet to be elucidated, the Rrp2-RpoN-RpoS pathway is essential for successful spirochete transmission into mice. Genes in this regulon, including *ospc* (important in early mammalian infection) and decorin binding protein A (*dbpa*, important in binding to mammalian extracellular matrix proteins), are involved in the tick-mammal transmission cycle (116,118–120).

Spirochete motility is also critical for survival in the tick following the blood meal (88). Spirochetes deficient in *flaB* (121), the periplasmic flagellar filament, or *cheY3* (122), a chemotaxis response regulator, survive in ticks to a lower extent than wild-type *Borrelia*. While it is unknown when during the tick phase of the enzootic cycle these genes are required for survival, it is possible that motility is upregulated during feeding when the spirochetes need to migrate from the midgut into the salivary glands. In addition to motility, other genes have been documented to change expression in feeding ticks or are important for spirochete transmission from ticks to mammals, including *bba52* (123), *bba03* (124), *bba07* (125), and *bba66* (126). In addition to identifying *Borrelia* proteins that are critical for spirochete transmission from ticks, researchers have also begun to identify tick proteins that impact spirochete transmission. For example, the tick gut protein ISDLP (*I. scapularis* dystroglycan-like protein) is upregulated during tick feeding and is important for *B. burgdorferi* transmission (127). In order to understand the specific impact of each of these

proteins on spirochete survival and successful transmission from tick to mammal, the specific functions and interacting partners of these *B. burgdorferi* and tick proteins need to be elucidated.

Interactions between spirochete proteins and tick proteins are critically important to both establishment of the spirochete in the midgut and migration out of the midgut and into the salivary glands. While the interaction between OspA and TROSPA is critical for spirochete maintenance in the midgut, downregulation of OspA and TROSPA during the blood meal help promote spirochete egress from the midgut (50,61). To identify additional interacting proteins, Narasimhan *et al.* screened a yeast display library of tick gut proteins against total *Borrelia* membrane extracts (128). From this screen, the tick gut protein Ixofin3D was found to help congregate spirochetes into clusters in the midgut epithelium, which was required for transmission to the salivary glands and egress from the midgut (128). While the *Borrelia* protein that specifically interacts with Ixofin3D is unknown, other *Borrelia* genes have been shown to be important for interacting with tick proteins and inducing migration to the salivary glands. Interactions between the *Borrelia* surface protein BBE31, which is upregulated during nymph feeding, and the secreted gut protein TRE31 results in spirochete migration through the hemolymph to the salivary glands (129).

5.3 Salivary gland composition

Upon egress from the tick midgut, *Borrelia* enter the salivary glands prior to transmission into the vertebrate host. The tick salivary gland is a complex environment, composed of several hundred known proteins (130). While many of these proteins are critical for *Borrelia* transmission into the mammalian host (see “Immunomodulatory effects of tick saliva”), there have been recent studies that have emphasized the diversity of the salivary gland proteins at different stages of feeding.

Acquiring a blood meal from a mammal is a lengthy process, with the tick remaining attached to the mammal for a few days to up to one week (14). During this time, the proteins secreted from the salivary glands change rapidly. Using proteomics, researchers have determined that ticks secrete functionally similar but distinct proteins every 24 hours in order to evade detection by the mammalian immune system (131). Other groups have used sequencing technologies, including cDNA libraries (130,132,133), phage display (134), and next generation sequencing (135,136), to characterize salivary gland proteins and have found that the complexity and diversity observed in tick salivary gland proteins is likely due to the long host attachment time *Ixodes* ticks require in order to acquire a blood meal. Many of the proteins identified in these studies have known functions, including proteases, cell adhesion proteins, cytoskeletal proteins, antimicrobial proteins, and others (132). However, a substantial portion of the salivary gland proteins are of unknown function. Determining whether these proteins interact with *Borrelia* surface proteins or mammalian proteins can help identify their role and function in the tick salivary gland.

In addition to differential expression over the course of feeding, it has become evident that proteins in the salivary gland, including lipocalins, Kunitz-domain containing proteins, and metalloproteases have molt stage-specific expression, suggesting that the interactions between the mammal and the tick are likely to differ between stages (136). Likewise, tick

salivary protein expression differs depending on the mammalian host, as the tick transcriptome and proteome composition is distinct when fed on mice or guinea pigs (137). These adaptions by the tick that are dependent on the molt stage or mammalian host help to ensure adequate blood meal uptake and evasion of mammalian immune detection.

5.4 Immunomodulatory effects of tick saliva

Proteins in tick saliva have immunomodulatory properties that impact multiple aspects of mammalian host defense. While their primary purpose is to ensure that the tick can feed to repletion, *B. burgdorferi* take advantage of some of these salivary proteins or their functions to facilitate movement from the tick to the mammalian host (138–140).

One salivary gland protein, Salp15, is particularly influential in impacting the mammalian $CD4^+$ T cell response to *Borrelia*. Salp15 has been documented to bind to the CD4 co-receptor on $CD4^+$ T cells, preventing T cell receptor signaling through the inhibition of Lck and Zap70 phosphorylation (138,141) (Figure 2). Together, this results in a decrease in IL-2 production, a cytokine critical for T cell expansion (138). The impact on the $CD4^+$ T cell response is not limited to Salp15, as other unknown tick salivary gland proteins have been shown to bind IL-2 directly, preventing T cell proliferation (142). Other salivary proteins, such as Iris and Iristatin, inhibit production of several proinflammatory cytokines, including IL-6, TNFα, and IFNγ (143,144). Tick saliva has also been documented to impact more upstream pathways, specifically impairment of dendritic cell maturation, antigen presentation, and cytokine production (139,145,146), all of which can inhibit the downstream $CD4^+$ T cell response.

The impact of tick saliva on the mammalian host response is not limited to T cells. In addition to its influence in inhibiting cytokine production, Salp15 has also been documented to bind to OspC on *Borrelia*, protecting the bacterium from antibody mediated killing in mice (147). Antibody targeting of Salp15 resulted in enhanced phagocytosis of *Borrelia* and targeting of OspC antibodies to the *Borrelia* surface (148). Other salivary gland proteins, such as the B cell inhibitory protein (BIP), inhibit B cell proliferation and activation (140,149). Salp15 and other salivary gland proteins, including Isac, Salp20, TSLPI and IRAC, have also been reported to interfere with the complement cascade, preventing both neutrophil phagocytosis and complement-induced lysis of *Borrelia* (150–154) (Figure 2). Furthermore, tick saliva can reduce integrin expression on neutrophils, reducing chemotaxis and inhibiting neutrophil mediated killing of *Borrelia* (155,156). Through inhibition of multiple aspects of the mammalian immune response, tick salivary proteins help ensure adequate transmission of *Borrelia* from the tick to the mammal and completion of the enzootic cycle.

6. Utilizing insights from the tick-pathogen interface

Repeat exposure to tick salivary proteins can induce tick immunity in hosts such as rabbits and guinea pigs, but not in the natural *Peromyscus leucopus* reservoir (157). Tick immunity can result in a decrease in tick feeding and longer time to engorgement, among other detriments to tick health. Immunity to tick proteins can be driven by several immune components, including eosinophils, basophils, mast cells, and antibodies against the salivary

proteins (158,159). Interestingly, although repeat tick bites do not appear to cause tick immunity in mice, when infected ticks are fed on mice that had been repeatedly infested with ticks, the immune responses against tick proteins did reduce transmission of *B. burgdorferi* (160). Furthering this, when serum from rabbits with tick immunity was passively transferred to mice, *B. burgdorferi* transmission was reduced upon challenge with infected ticks, demonstrating the importance of tick salivary proteins to the transmission of *B. burgdorferi* (161). This effect can also be seen in humans, as individuals living in endemic Lyme disease areas who have been repeatedly exposed to tick bites have lower rates of Lyme disease (162). However, it is unclear if this is driven entirely by immunity to tick saliva, or if the reaction to the tick bite simply allows for a more rapid identification and removal of the tick.

Interfering with tick salivary proteins that impede mammalian immunity against *Borrelia* is an attractive option to combatting long-term *Borrelia* infections in mammals. There have been numerous studies that have utilized gene silencing technologies in ticks to determine the effects of salivary proteins on *Borrelia* transmission and survival within the tick, and are promising targets for therapeutics aimed to decrease *Borrelia* transmission. RNA-interference (RNAi) mediated silencing of the salivary protein tick histamine release factor (tHRF), which binds to basophils and stimulates histamine release, has been shown to reduce *B. burgdorferi* burden in mice and reduce tick feeding efficiency (163). Similarly, silencing of Isac reduced tick weight and *Borrelia* burdens in ticks (164). RNAi silencing of the salivary protein sialostatin L, which has a predominately anti-inflammatory role in mammals through targeted inhibition of several proteases (165), reduced tick weight and survival following attachment (166).

Furthermore, vaccinating mice and other mammals against tick salivary proteins is a potential way to combat *Borrelia* transmission from ticks to mammals and vice versa. There have been several promising studies indicating that vaccination against tick salivary proteins could be a successful avenue to developing an effective anti-tick vaccine. Vaccination of mice with recombinant Salp15 has been shown to protect mice from *B. burgdorferi* colonization (148), evidence that tick salivary proteins can be immunogenic in mammals. Similarly, mice vaccinated with recombinant TSLPI reduced *B. burgdorferi* burdens, while RNAi silencing of TLSPI in ticks reduced *Borrelia* survival in ticks following the blood meal (151). Oral vaccination of mice using recombinant vaccinia virus expressing the tick gene subolesin resulted in significantly lower tick infestation on vaccinated mice and substantially reduced uptake of *B. burgdorferi* by ticks that fed to repletion (167). Larval ticks fed on mice administered antiserum against the secreted salivary gland protein IsPDIA3 showed a reduction in *B. burgdorferi* colonization both in the fed larvae and once the ticks had molted into nymphs (62), suggesting that reducing acquisition by ticks from mice could further limit the spread of the spirochete from ticks to other mammals. These vaccination studies are not limited to efficacy in mice – vaccination of tick sialostatin L2 in guinea pigs lead to decreased feeding ability of ticks, including longer feeding time, increased tick rejection rate, and enhanced inflammation (168). Although guinea pigs differ from the natural mouse hosts, these studies are encouraging when thinking about potential tick vaccination strategies.

Proteomic studies have also aided in the discovery of potential salivary protein vaccine candidates. Comparing the tick salivary proteome at 24 hours and 66 hours post-attachment, Narasimhan *et al.* discovered that salivary gland proteins expressed in the first 24 hours following attachment are sufficient to induce mammalian directed anti-tick immunity and can prevent tick feeding and *Borrelia* transmission (161). Yeast display libraries have been used to identify salivary gland proteins that impair mammalian immune function (169). These data have been useful to further immunization studies, as vaccination of rabbits with several newly discovered tick salivary proteins led to a reduction in nymph feeding (169). Together, these studies suggest that targeted interference with tick salivary proteins, whether through RNAi silencing or vaccination with recombinant protein, is a promising method for an anti-tick vaccine that can ultimately reduce *Borrelia* transmission from ticks to mammals.

While vaccinating humans against one or several of these tick proteins is a potential preventative approach, it does not address the geographic spread of the disease or the increasing prevalence of infection among ticks. Reservoir targeted vaccine approaches targeting *B. burgdorferi* infection in ticks and wild mice have the potential to greatly reduce the prevalence of disease. Vaccination of wild *P. leucopus* mice could help reduce total spirochete burdens in the wild, thereby lessening the chances of human *B. burgdorferi* infection. Success for this approach was shown in a field study in southern Connecticut, where mice were hand captured and injected with OspA. This resulted in a decreased prevalence of infected ticks in the subsequent season (170). Another method of reservoir targeted vaccines involves oral vaccination of animals. The benefit of oral vaccination is that delivery of an anti-tick or anti-*B. burgdorferi* vaccine could occur through placed baits. Successful oral strategies of vaccination have utilized recombinant *B. burgdorferi* proteins or viral vectors encoding either spirochete or tick genes (171–174). Targeting tick antigens in a reservoir targeted vaccine approach also has added benefits of reducing transmission rates of other tick borne pathogens, including *B. microti* and *A. phagocytophilum* (167).

7. Conclusions and Perspectives

The stages of the enzootic cycle result in several distinct environments to which *B. burgdorferi* must adapt in order to survive (Figure 1). Spirochete gene regulation is a critical component of this adaptation, as it is necessary for preventing elimination by host defenses and altering metabolism according to nutrient availability. Entry into the tick via a blood meal from a mammal requires *B. burgdorferi* to change its gene expression from evading mammalian host detection to evading the tick immune pathways. The nutrient availability from the larval blood meal wanes quickly, and *B. burgdorferi* must alter its carbon source in order to survive until the nymphal blood meal. Upon tick uptake of the nymphal blood meal, *B. burgdorferi* must again change anatomical locations within the tick by migrating from the gut to the salivary glands, which contain an entirely new set of defenses that the spirochete must overcome in order to be successfully transmitted into the mammalian host. Ultimately, when *B. burgdorferi* returns to the mammal, it must alter its gene expression yet again in order to evade mammalian immune detection. Studying the interface between *B. burgdorferi* and its tick and mammalian hosts is now more accessible than in the past, as the genomes of all three species have been sequenced and are readily available (101,175,176). High throughput screens that utilize sequencing technologies have emerged as critical methods to

identify points of intersection and interaction between the spirochete and its hosts. There still remains much to be known about specific protein-protein interactions and signaling pathways between the hosts and *B. burgdorferi*, and how these interactions change according to tick molt stage and the different mammalian species. While there is no approved vaccine to prevent transmission of the spirochete from ticks to mammals, unique perspectives on the enzootic cycle and interactions between *B. burgdorferi* and the *Ixodes* tick vector can help identify potential targets for blocking mammal to tick transmission, tick to mammal transmission, or spirochete survival in the tick vector. It is our hope that this review provides adequate context for future research designed to identify interactions between *I. scapularis* and *B. burgdorferi*.

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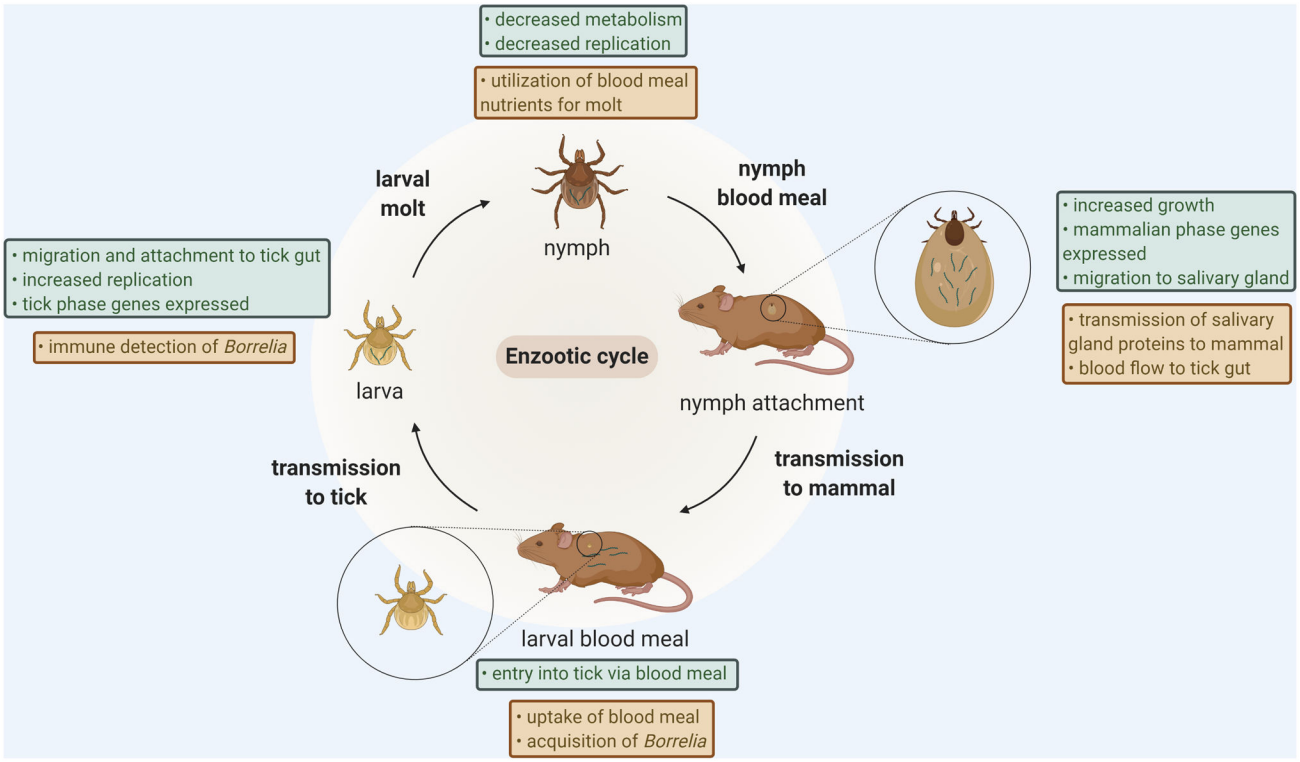

Figure 1: The enzootic cycle of *Borrelia burgdorferi*.

Throughout the enzootic cycle, *B. burgdorferi* must adapt to new challenges within the tick. Upon entering the tick, *B. burgdorferi* must rapidly adapt to changes in environment and upregulate tick-phase genes, while at the same time evade detection by the tick immune system. The influx of blood into the tick gut during the nymphal blood meal promotes the migration of *B. burgdorferi* to the salivary glands and eventual entry into the host, where it must again adjust to environmental changes. *B. burgdorferi* associated changes are indicated in green boxes, changes in the tick are indicated in brown boxes. Created with BioRender.com.

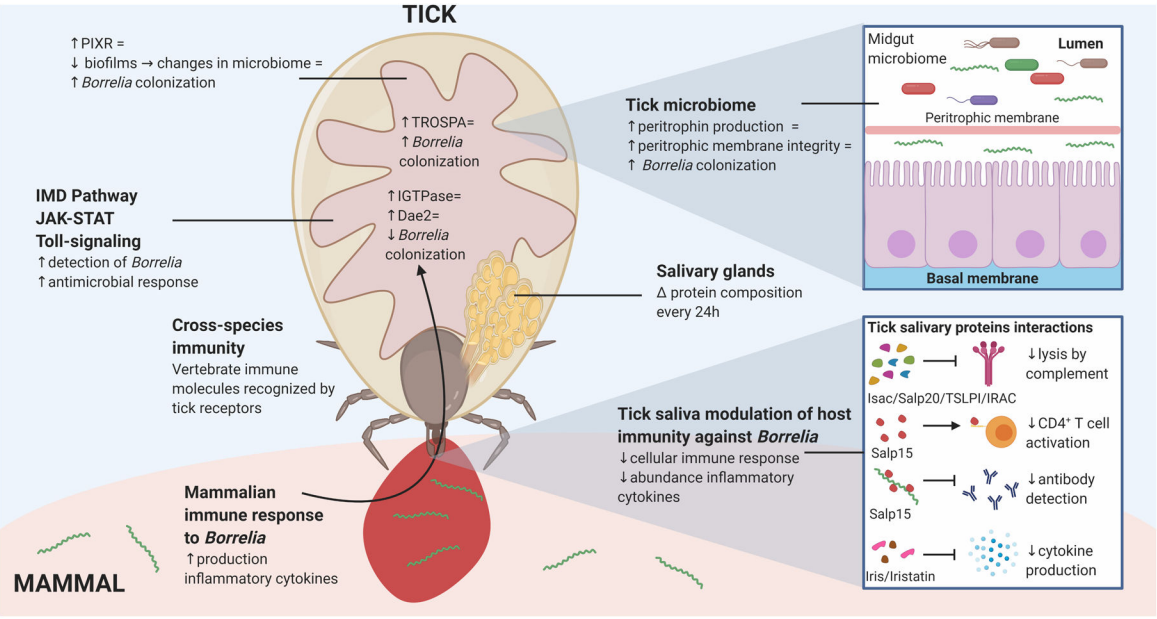

Figure 2: Interactions between *B. burgdorferi* and the *Ixodes* tick vector.

Interactions between *B. burgdorferi* and tick proteins occur at multiple locations within the tick. The tick midgut provides an opportunity to influence *B. burgdorferi* colonization through the peritrophic membrane, tick gut microbiome, and other tick gut proteins, including TROSPA and PIXR. Tick salivary gland proteins are able to impair the mammalian immune response and aid *B. burgdorferi* survival within the mammal. Some of these interactions include Isac, Salp20, TSLPI and IRAC prevention of complement mediated lysis of *B. burgdorferi*, Salp15 binding to $CD4^+$ T cells and inhibiting their activation, while simultaneously binding to *B. burgdorferi* and preventing antibody mediated detection of the spirochete, and Iris and Iristatin mediated reduction of proinflammatory cytokine production. The equal sign denotes “leads to.” Created with BioRender.com.