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For Whom the Bell Tolls (and Nods): Spit-acular Saliva

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Abstract Having emerged during the early part of the Cretaceous period, ticks are an ancient group of hematophagous ectoparasites with significant veterinary and public health importance worldwide. The success of their life strategy can be attributed, in part, to saliva. As we enter into a scientific era where the collection of massive data sets and structures for biological application is possible, we suggest that understanding the molecular mechanisms that govern the life cycle of ticks is within grasp. With this in mind, we discuss what is currently known regarding the manipulation of Toll-like (TLR) and Nod-like (NLR) receptor signaling pathways by tick salivary proteins, and how these molecules impact pathogen transmission.

This article is part of the Topical Collection on *Rickettsia*

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Introduction

Ticks are blood-feeding ectoparasites that account for a significant amount of disease worldwide, affecting both humans and livestock [1, 2]. A hallmark feature of these arachnids is the prolonged feeding period on a host, particularly, when compared to other hematophagous arthropods (e.g., mosquitos, tsetse flies, biting flies, fleas, lice, midges, and bedbugs) [1]. The time required for feeding completion varies depending on the species of tick. Soft ticks (Argasidae) will feed repeatedly on one host for minutes to hours [1, 2]. Hard ticks (Ixodidae) will feed for days to weeks and only once per life stage [1, 2]. Nuttalliellidae, the third group, are considered the basal lineage to both Argasidae and Ixodidae and feed rapidly [3]. There have been over 900 species of ticks described to date; approximately 700 belong to Ixodidae and 200 are classified as Argasidae, while Nuttalliellidae contains only one species, *Nuttalliella namaqua* (the South African tick), which is considered a living fossil [3, 4].

For a hard tick to obtain an uninterrupted blood meal, the physical injury caused at the bite site and the prolonged attachment must go unnoticed by the host. This is facilitated, in part, by saliva that is injected into the feeding site [2, 5•]. Tick saliva contains an arsenal of effectors that inhibit multiple modules of the host immune response including inflammation, blood coagulation, wound healing, and vasoconstriction [5•]. A large body of scientific research has been dedicated to uncovering how tick saliva manipulates cellular and humoral responses in mammals [5•]. This work is significant and has greatly contributed to our understanding of the tick's life cycle; however, there is considerably less research investigating the immunomodulatory properties of tick saliva in the context of signaling pathways.

Mammals have several classes of innate immune signaling pathways that are classified by their pattern recognition

receptors: (1) Toll-like (TLR), (2) Nod-like (NLR), (3) C-type lectin (CLR), (4) retinoid acid-inducible gene I-like (RIG I-like) (RLRs), (5) absent in melanoma 2 (AIM2), and (6) cyclic GMP-AMP synthase (cGAS)/STING (stimulator of interferon genes) [6•]. These pathways trigger the immediate immune response to physical injury and/or microbial infection. They recognize pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs), function to combat immune assaults, and facilitate the development of an adaptive immune response [6•]. With this in mind, our review will center on recent advances in the field that examine how tick saliva manipulates innate immune signaling, focusing on TLR and NLR pathways, and the implications this has for pathogen transmission.

TLR Signaling

The TLR family was the first identified group of pattern recognition receptors (PRRs) and is currently the best characterized (Fig. 1a) [6•, 7–9]. This is a large family, consisting of 10 known human TLRs (12 in mice) with varying substrate specificity and cellular localizations. TLRs interact with stimuli at the cell membrane interface, either on the surface or within endosomal compartments [6•]. TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10 are surface-localized, whereas TLR3, TLR7, TLR8, TLR9, TLR11, TLR12, and TLR13 are endosomal [6•]. This compartmentalization controls which PAMPs or DAMPs the TLRs will come into contact with and is, therefore, a means of regulating the inflammatory response. In general, surface TLRs recognize microbial membrane components, such as lipopolysaccharide (LPS), peptidoglycan, flagellin, lipoproteins, lipids, and other proteins. In contrast, endosomal TLRs largely recognize nucleic acids derived from intracellular bacteria and viruses as well as self-derived nucleic acids, which may function as DAMPs [6•, 9, 10].

Upon activation, TLR signaling promotes pro-inflammatory and antimicrobial responses via nuclear factor (NF)-κB and the mitogen-activated protein kinase (MAPK) pathway facilitates the development of adaptive immune responses and can indirectly influence cell death [6•, 10]. TLRs promote these responses by recruiting adaptor molecules with Toll/interleukin-1 receptor (TIR) domains including myeloid differentiation primary response 88 (MyD88), TIR domain-containing adaptor-inducing IFN-β (TRIF), TIR-containing adaptor protein (TIRAP/MAL), or TRIF-related adaptor molecule (TRAM) [6•]. Most TLR signaling can be segregated into either MyD88 or TRIF-dependent pathways [6•, 10]. Excellent reviews within the last few years have comprehensively covered TLR signaling, which the reader is referred to [6•, 9, 10].

Fig. 1 Overview of Toll-like receptor and Nod-like receptor signaling. **a** ▶ Toll-like receptor signaling. TLR1/TLR2 or TLR2/TLR6 heterodimers and TLR4, TLR5, and TLR10 homodimers signal at the surface of the cell through MyD88 and TIRAP, which recruit IRAK and TRAF6. TRAF6 is an E3 ubiquitin ligase that functions with the E2 ubiquitin-conjugating heterodimer Uev1a:Ubc13 to induce K63 ubiquitination. This ubiquitin chain recruits the TAB1/TAB2/TAB3 and TAK1 signaling complex, which activates either NF-κB or MAPK signaling. Upon stimulation, TLR4 is internalized and signals from the endosome, as do TLR3, TLR7, TLR8, and TLR9. Endocytosed TLR4 signals through TRAM/TRIF and RIPK1, TRADD, and caspase-8 to induce NF-κB activation. This signaling platform also promotes the production of IRF3 through the recruitment of TRAF3 and TANK to TRIF. TLR3 signals through TRIF but does so in a TRAM-independent manner and instead binds TRIF directly. TLR7, TLR8, and TLR9 homodimers signal through TIRAP and MyD88 to activate NF-κB and, in plasmacytoid dendritic cells, the transcription factor IRF7. **b** Nod-like receptor signaling. Nod1 and Nod2 mediate NF-κB and MAPK activation by recruiting RIPK2 to the N-terminal CARD domain. RIPK2 is ubiquitinated in a K63-dependent manner, which recruits the TAB1/TAB2/TAB3 and TAK1 signaling complex. Other NLRs lead to the formation of a multimeric scaffolding platform termed the inflammasome. NLRP molecules require the ASC signaling adaptor molecule for the recruitment of caspase-1 (*green bars*), whereas NLRC molecules possess CARD domains and, therefore, may recruit caspase-1 independent of ASC. Although AIM2 is not an NLR protein, it does form an inflammasome that is also ASC-independent. Activation of the inflammasome causes caspase-1-mediated cleavage of both pro-IL-1β and pro-IL-18 into their mature forms, leading to an inflammatory response

NLR Signaling

In contrast to TLRs, NLRs are cytosolic pattern recognition receptors [6•]. NLRs also sense microbial PAMPs, such as peptidoglycan, and self-derived DAMPs, such as ATP (Fig. 1b) [11•]. Members of this family are intracellular scaffolding proteins, typically structured as follows: (1) an amino-terminal protein-protein interaction domain [caspase activation and recruitment domain (CARD), baculovirus inhibitor of apoptosis protein repeat (BIR), or pyrin domain (PYD)], (2) a central nucleotide-binding oligomerization (Nod) domain (also called NACHT), and (3) carboxy-terminal leucine-rich repeats (LRRs) [11•]. The NLR family is broad with 22 characterized members in humans, 34 in mice, and more recently discovered, over 200 NLR-like coding genes in invertebrates, such as the sea urchin [12•]. Although initially characterized as intracellular receptors that respond to cytosolic pathogens, NLRs also have roles in regulating antigen presentation, embryo development, cell death, adaptive immune response differentiation, and sensing metabolic changes [11•, 12•]. Due to the limited scope of this review, we will be focusing on the role that these receptors have in modulating the innate immune response.

Nod1 and Nod2, the original members of the NLR super family, are both activated by peptidoglycan and are responsible for sensing pathogenic bacteria in the cytosol [11•, 12•, 13]. Nod1 recognizes γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP), which is present in the peptidoglycan of Gram-negative bacteria,

a **Toll-like receptor pathways**

b **Nod-like receptor pathways**
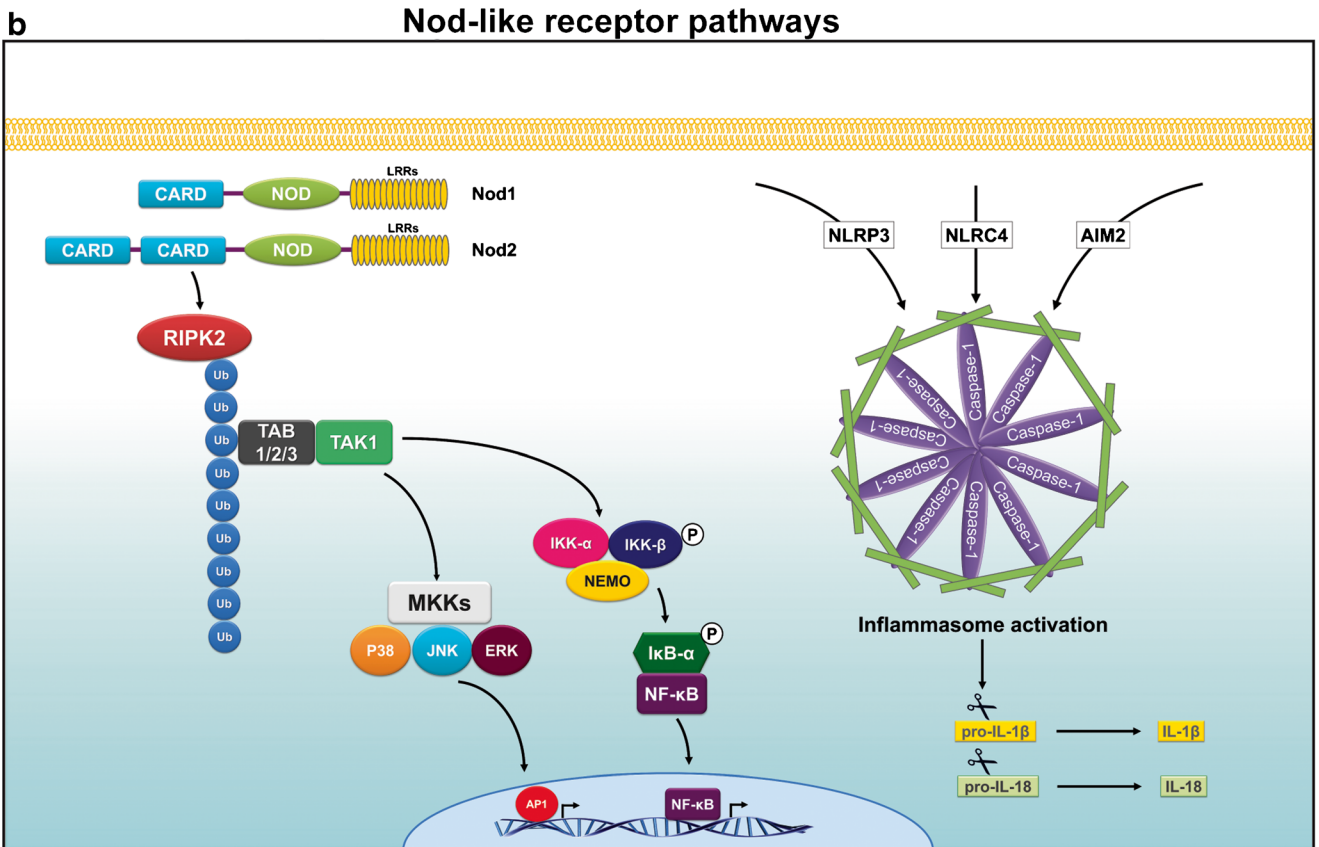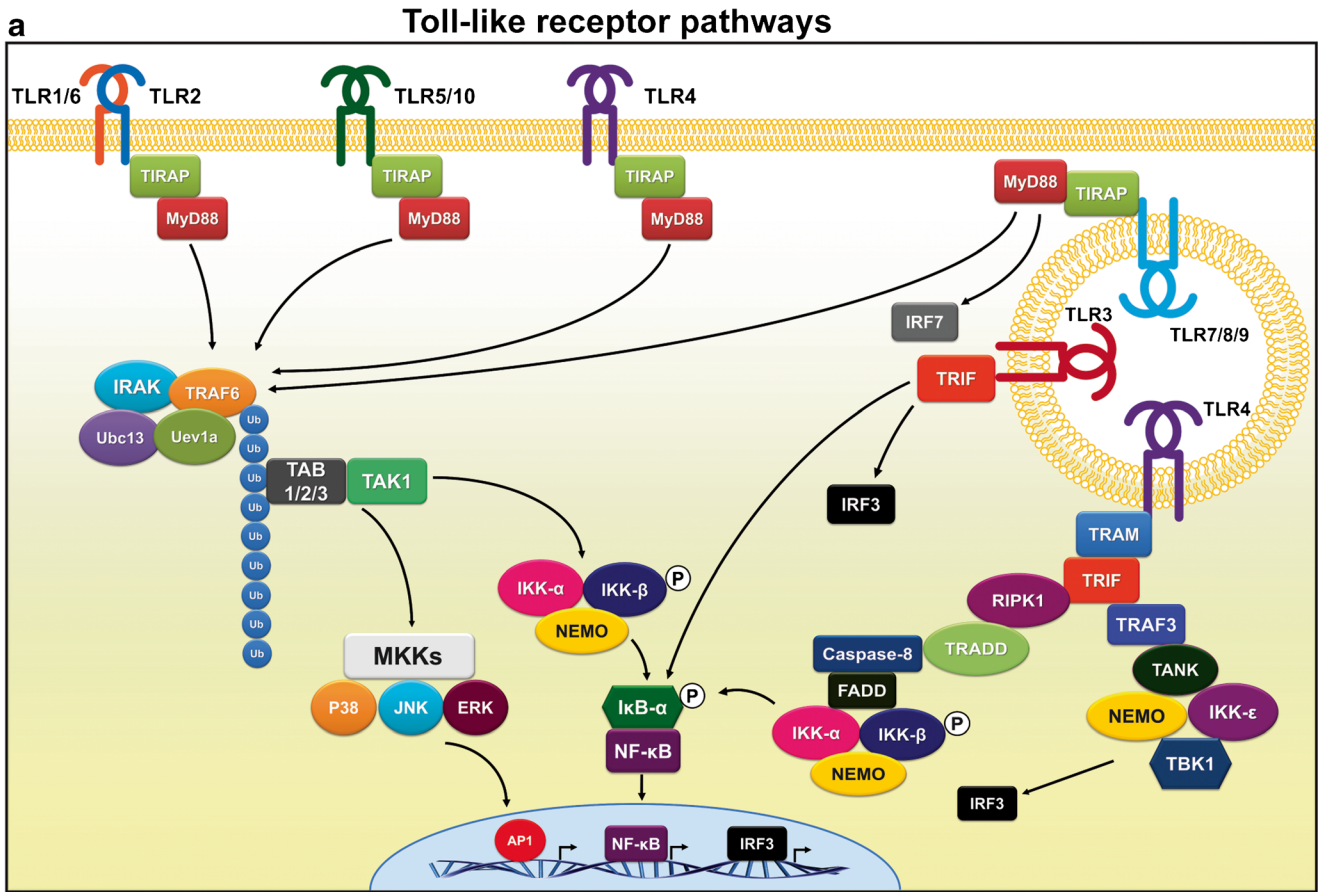
but can also be found in some Gram-positive species [14–16]. Muramyl dipeptide (MDP) which is found in the peptidoglycan of both Gram-positive and Gram-negative bacteria is recognized by Nod2 [13, 17]. Both Nod1 and Nod2 contain an amino-terminal CARD domain and, upon activation, mount a pro-inflammatory immune response by activating the NF-κB pathway [11•, 12•]. This response is dependent on the adaptor serine/threonine kinase, receptor-interacting protein kinase 2 (RIPK2) [18]. Upon activation, NLRs oligomerize via the NACHT domain and serve as a signaling platform for the recruitment of RIPK2 via the CARD-CARD interaction [18] (Fig. 1b). RIPK2 undergoes lysine (K)-63 polyubiquitination, resulting in NF-κB activation [11•, 19]. Alternatively, Nod1 and Nod2 can activate MAPK signaling pathways [11•]. Although this mode of activation is poorly understood, it uses similar signaling molecules to the NF-κB pathway, such as RIPK2 and TAK1, and mounts a pro-inflammatory antimicrobial immune response [20, 21].

Sensing PAMPs and DAMPs by NLRs may also lead to the formation of an oligomeric scaffold termed the "inflammasome." There are several types of inflammasomes, each with fundamental differences that are dependent on their stimuli. Inflammasome activation leads to caspase-1 and caspase-11 (caspase-4 in humans)-dependent cleavage of pro-IL-1β and pro-IL-18 into the active pro-inflammatory forms [22•, 23, 24•, 25]. Although most inflammasomes are dependent on the adaptor molecule ASC for oligomerization, recent advances in inflammasome biology suggest that alternative modes of activation may also exist. For recent reviews on inflammasomes, please refer to Guo et al. [22•] and von Moltke et al. [26].

Recognition of Tick-Borne Pathogens by TLRs and NLRs

The ability of tick-borne pathogens to exploit the immunomodulatory properties of tick saliva upon host colonization may be driven by the co-evolution between ticks and microbes. The large number of pathogens that ticks are capable of transmitting makes them unique among arthropod vectors [2] (Table 1). For the purpose of this review, only bacteria will be discussed, although viruses and other parasites such as protozoa are transmitted by ticks as well. For a more extensive review covering tick-borne pathogens, please refer to *Biology of Ticks*, volume 1, Chapters 6–10 [1].

Table 1 Most prevalent human pathogens transmitted by ticks

Pathogen	Tick host	Disease	Location	Refs
Borrelia burgdorferi sensu stricto	*Ixodes scapularis* *Ixodes pacificus* *Ixodes ricinus*	Lyme disease	North America, Europe	[2, 27]
Borrelia garinii	*Ixodes ricinus* *Ixodes persulcatus*	Lyme disease	Europe, Asia	[2, 27]
Borrelia afzelii	*Ixodes ricinus* *Ixodes persulcatus*	Lyme disease	Europe, Asia	[2, 27]
Borrelia miyamoti	*Ixodes scapularis* *Ixodes persulcatus*	Influenza-like illness, relapsing fever, meningoencephalitis	North America, Europe, Asia	[2, 28–33]
Relapsing fever *Borrelia*	*Ornithodoros spp.*	Relapsing fever	North America, Europe, Asia, Africa	[2, 34–36]
Anaplasma phagocytophilum	*Ixodes scapularis* *Ixodes ricinus* *Ixodes pacificus* *Ixodes persulcatus*	Human granulocytic anaplasmosis	Europe, North America, Asia	[2, 37]
Ehrlichia chaffeensis	*Amblyomma americanum*	Human monocytic ehrlichiosis	North America, Europe, Africa, South and Central America	[2]
Rickettsia rickettsii	*Dermacentor andersoni* *Dermacentor variabilis* *Amblyomma spp.* *Rhipicephalus sanguineus*	Rocky Mountain spotted fever	North, Central and South America	[2]
Rickettsia conorii	*Rhipicephalus sanguineus*	Mediterranean spotted fever	Europe, Africa	[2]
Francisella tularensis	*Amblyomma americanum* *Dermacentor andersoni* *Dermacentor variabilis*	Tularemia	North America, Europe, Asia	[2]
Babesia microti *Babesia divergens*	*Ixodes ricinus* *Ixodes scapularis*	Babesiosis	North America, Europe	[2]
Crimean-Congo hemorrhagic fever virus	*Hyalomma impeltatum* *Hyalomma rufipes*	Crimean-Congo hemorrhagic fever	Europe, Asia, Africa	[2, 38]
Tick-borne encephalitis virus	*Ixodes ricinus* *Ixodes persulcatus*	Tick-borne encephalitis	Europe, Asia	[2, 38]
Powassan virus	*Ixodes cookei*	Encephalitis and neurological disease	North American, Asia	[2, 38]

Interestingly, bacteria vectored by ticks have atypical features when compared to more classically defined pathogens such as *Escherichia coli* or *Salmonella* spp. For example, with the exception of *Rickettsia* spp., most of these microbes either do not have LPS (*Borrelia*, *Anaplasma*, and *Ehrlichia*) or instead have a structurally distinct form of LPS that is not recognized by TLR4 (*Francisella*) [39–43]. Several of these species also do not synthesize canonical peptidoglycans (*Borrelia*) or do not have peptidoglycan altogether (*Anaplasma* and *Ehrlichia*) [39, 41, 42, 44]. How NLR signaling is activated in response to some of these pathogens remains unclear, but these observations suggest that additional PAMPs and/or activation mechanisms may exist.

Borrelia spp.

One of the most important tick-borne pathogens is the causative agent of Lyme disease, *Borrelia burgdorferi* [45]. Infection by this spirochete triggers several TLRs (TLR2, TLR5, TLR7, TLR8, and TLR9) with TLR2 being the most well-characterized [46–52, 53•]. Recent studies have dissected *B. burgdorferi*-mediated TLR2 activation and examined the cross talk with other TLRs. A study published by Petnicki-Ocwieja et al. showed that TLR2-mediated trafficking of *B. burgdorferi* to lysosomal-associated membrane protein-1 (LAMP-1)-tagged compartments is dependent on adaptor protein-3 (AP-3) but did not affect TRAM or MyD88 recruitment to the phagosome. AP-3-deficient mice had increased joint inflammation upon *Borrelia* infection, a phenotype comparable to *tlr2*$^{-/-}$ mice, but counterintuitively did not affect bacterial numbers. The authors note that deficiencies in other TLR2 signaling components (MyD88, CD14, TLR2, and TRIF) also result in increased inflammation, while only CD14 and MyD88 appear to be important for controlling bacterial infection [53•].

Several groups have demonstrated that host infection by this spirochete is enhanced by the presence of tick saliva. Inoculation of *Borrelia* into a mouse in combination with salivary gland extracts increases the level of bacteremia and suppresses pro-inflammatory cytokines in the draining lymph nodes [54–56]. Saliva from *Ixodes scapularis* also reduces adhesion to polymorphonucleocytes (PMNs) by downregulating β2-integrins and facilitating the establishment of *Borrelia* infection [57]. Some specific salivary molecules have been characterized in the context of *B. burgdorferi* infection. These include Salp15 [binds to outer surface protein C (OspC) on the spirochete and protects it from antibody-mediated killing] [58], Salp25D (inhibits the complement system) [59], P8 (inhibits the lectin complement pathway) [60], IRS-2 (prevents Th17 differentiation by inhibiting IL-6/STAT-3 signaling pathway in dendritic cells) [61], and sialostatin L2 (facilitates *B. burgdorferi* population expansion in the skin) [62].

Potential cross talk between TLRs also exists at a tick bite site, as there are bacterial PAMPs and tissue injury DAMPs from skin laceration by the tick's hypostome. Bernard et al. demonstrated

that TLR1/TLR12 inflammatory signaling triggered by the *Borrelia* lipoprotein, L-OspC, was enhanced by the tissue injury signal and the TLR3 agonist, poly (I:C) [63•]. In vitro, tick saliva suppresses both TLR1/TLR2 and TLR3 signaling individually as well as the combined and exacerbated response caused by TLR3-TLR1/TLR2 cross talk (as measured by cytokine secretion) [63•]. Although this is a compelling evidence to suggest that tick saliva directly suppresses TLR signaling induced by *Borrelia* lipoproteins, it remains unclear how this impacts bacteria in vivo.

Francisella spp.

Francisella tularensis is the causative agent of tularemia, which can manifest as multiple diseases depending on the inoculation route (blood-borne or aerosol). Following escape from the phagolysosome, *F. tularensis* replicates within the cytosol of macrophages where it triggers the inflammasome [64]. This pathogen is Gram-negative but has an atypical form of LPS that is not recognized by TLR4 [43]; instead, the inflammatory response is TLR2-dependent. A 2011 study reported that TLR2, MyD88, and NF-κB were necessary to trigger the *Francisella novicida*-induced inflammasome, as measured by inflammasome assembly, caspase-1 activation, cell death, and IL-18 release [65]. There is no evidence that *Francisella* spp. trigger the NLRP3 or NLRC4 inflammasomes [64]. Instead, it is thought to activate the AIM2 inflammasome with double-stranded DNA, which is exposed when guanylate-binding proteins (GBPs) 2 and 5 lyse the bacteria in the cytosol [66].

Rickettsiales

Several rickettsial pathogens are transmitted by ticks including species of *Anaplasma*, *Ehrlichia*, and *Rickettsia*. All are small, obligate intracellular pathogens that reside either within the host cell cytoplasm or within a membrane-bound, endocytic compartment.

Anaplasma spp.

Anaplasma phagocytophilum causes human granulocytic anaplasmosis (HGA). Other *Anaplasma* species have veterinary importance, including *Anaplasma marginale*, the causative agent of bovine anaplasmosis. In an in vitro infection model, *A. phagocytophilum* induces NF-κB activation through TLR2 [67] as well as NLR signaling [68]. Sukumaran and colleagues found that RIPK2, the signaling molecule for Nod1 and Nod2, was a key regulator in *A. phagocytophilum* infection [69]. *ripk2* transcripts were significantly induced in wild-type *A. phagocytophilum*-infected mice. Moreover, in *ripk2*$^{-/-}$ mice, bacterial burden significantly increased, mice took longer to clear the infection, and pro-inflammatory cytokines such as INF-γ and IL-18 were decreased [69]. Additionally, Chen et al. demonstrated

that both TLR and NLR signaling are dampened when tick saliva is added into the system [68] and that NLR-mediated inflammation is specifically inhibited by the tick saliva molecule, sialostatin L2 (SL2) [70•]. These studies showed that SL2 indirectly blocks caspase-1 enzymatic activity and prevents the secretion of IL-1β and IL-18 by inhibiting reactive oxygen species production by the NADPH oxidase [70•].

Ehrlichia spp.

Ehrlichia chaffeensis is an emerging tick-borne disease that causes human monocytic ehrlichiosis (HME) [71]. *E. chaffeensis* infection induces differential gene regulation of *tlr*, *nod2*, and genes associated with inflammasome formation. Differential expression of the specific genes correlates with distinct disease outcomes. For example, TLR2-dependent responses contributed to protective immunity against *E. chaffeensis*, while Nod2 signaling exacerbates pathogenic immune responses, prompting *Ehrlichia*-induced toxic shock [72•]. In agreement with this, a 2015 study published by Yang et al. suggests that activation of the non-canonical inflammasome, mediated by caspase-11 activation, contributes to fatal ehrlichiosis which is ultimately governed by type I interferon [73]. In this report, *nlrp3*$^{-/-}$ mice cleared the bacteria more efficiently than wild-type mice, although they succumbed to infection at the same rate. Mice deficient for the interferon-α/β receptor, *ifnar*, were highly resistant to fatal ehrlichiosis and had less liver damage, lower bacterial burdens, and prolonged survival. The mechanistic understanding for how IFN-I activates the non-canonical inflammasome remains to be determined, as assays were limited to cytokine measurement [73]. Interestingly, these results imply that there is yet another uncharacterized PAMP that activates the NLR signaling pathway, as *E. chaffeensis* does not have LPS or peptidoglycan [39].

Rickettsia

Rickettsia reside and replicate within the host cell cytoplasm and are responsible for causing Rocky Mountain spotted fever, typhus, rickettsialpox, Boutonneuse fever, and African tick bite fever. The robust inflammatory response caused by infection is characterized by the activation of NF-κB and production of pro-inflammatory cytokines such as IL-1β, IL-6, IL-8, tumor necrosis factor (TNF)-α, and TGF-β [71, 74]. TLR4 recognizes the *Rickettsia* LPS and is a major player in controlling bacterial burden and disease. Activation of this pathway results in significant production of INF-γ. This triggers the expansion of activated NK cells, which also play a key role in controlling microbial numbers [75]. In agreement with this, a typically sub-lethal dose of *Rickettsia* for wild-type mice causes *tlr4*$^{-/-}$ mice

(C3H/HeJ) to succumb to infection. Polymorphisms in TLR4 can also confer disease severity in a *Rickettsia conorii* infection model [76•]. Interestingly, if *tlr4*$^{-/-}$ mice are infected with *R. conorii* alongside feeding *Rhipicephalus sanguineus sensu lato* ticks, levels of IL-1β and NF-κB activation are decreased when compared to *R. conorii* infection alone. Counterintuitively, this did not confer a survival advantage for the bacteria when lung tissue was evaluated [77]. However, this may be due to the distal infection site not being impacted by the localized effects of tick saliva.

Although the role of NLR signaling in *Rickettsia* infection has not been reported, it is reasonable to suspect that this immune pathway could be activated, considering the intracellular nature of this pathogen and the cytosolic localization of NLRs. Examining if and how NLR signaling impacts *Rickettsia* infection will be insightful, given the inflammation and pathology associated with disease.

Tick Saliva Interactions with TLR and NLRs

Due to the role that TLRs and NLRs play as early responders to immune assaults, it is not surprising that hematophagous ectoparasites have evolved mechanisms to dampen or disassemble pro-inflammatory pathways. Components of the saliva from ticks have been experimentally shown to interrupt various TLR pathways, although TLR2 appears to be a prominent target [5•]. This is likely due to the role that TLR2 has in recognizing several tick-transmitted pathogens [78]. Lieskovska and Kopecky demonstrated that when dendritic cells were exposed to tick saliva prior to TLR2 activation with lipoteichoic acid (LTA), NF-κB and ERK1/ERK2 phosphorylation levels were decreased, but not p38 [79]. Tick saliva-primed dendritic cells were subsequently infected with *Borrelia afzelii*, and phosphorylation of NF-κB and ERK1/ERK2 was also decreased, but to a lesser extent than with LTA stimulation [79]. Furthermore, the previously mentioned study by Bernard and colleagues showed that tick saliva from *Ixodes ricinus* decreased TNF-α and IL-8 secretion after stimulation with a TLR1/TLR2 agonist (lipidated OspC from *B. burgdorferi*) and the TLR3 agonist, poly (I:C) [63•].

Identifying specific compounds that act as effector molecules in tick saliva is important for teasing out the molecular mechanisms targeting innate signaling pathways. Oliveira and colleagues demonstrated that pre-treatment of dendritic cells with saliva from *R. sanguineus* inhibited pro-inflammatory cytokine secretion of TNF-α and IL-12p40 after stimulation with TLR2, TLR4, and TLR9 agonists (peptidoglycan, LPS, and CpG, respectively) [80]. This group further examined the active components in the saliva and identified the endogenous purine nucleoside, Ado, in addition to the

well-characterized eicosanoid, prostaglandin E2. Two of the better known molecules in tick saliva, sialostatin L (SL) and SL2, also inhibit TLR signaling [81]. SL inhibits IFN-β production in plasmacytoid dendritic cells when TLR2, TLR7, and TLR9 pathways are stimulated. SL2 inhibits NF-κB activation and components of the MAPK pathway [81]. The precise inhibition mechanisms by SL and SL2 have not been thoroughly characterized. However, a recent study showed that SL inhibits the production of IL-1β and the transcription factor IRF4 in mast cells, which directly binds the promoter of *il-1β* [82•]. This suggests that SL inhibits pro-inflammatory cytokine production at the RNA level by interfering with transcription factors [82•]. These studies are among the few that have parsed out specific effector compounds in tick saliva that affect TLR pathways.

TLR4, the well-known surface receptor for LPS, appears to also be targeted by tick saliva. Carvalho-Costa et al. demonstrated that *Amblyomma cajennense* saliva inhibits LPS-induced secretion of IL-12p40, IL-6, and TNF-α in dendritic cells [83]. Chen et al. also showed that tick saliva inhibits TLR4-mediated cytokine secretion in addition to TLR1, TLR2, and TLR1/TLR2 pathways [68]. This study involved priming bone marrow-derived macrophages (BMDMs) with tick saliva and stimulating with one of the following TLR agonists: LPS (TLR4), Pam3CSK4 (TLR1/TLR2), zymosan (TLR2), and PG-LPS (TLR2) [68]. Under these conditions, tick saliva significantly inhibits secretion of IL-12p40 and IL-6. This same study also showed, for the first time, that tick saliva inhibited NLR-mediated pro-inflammatory signaling. BMDMs were stimulated with the Nod2 agonist, m-DAP, by transfecting it into the cytosol, leading to an increase in IL-6 and IL-12p40 that was inhibited by tick saliva [68]. A subsequent study was published examining the effect of *I. scapularis* SL2 on inflammasome signaling. SL2 inhibited caspase-1 enzymatic activity, which is classically defined as being part of inflammasome activation, downstream from NLR assembly [70•]. When BMDMs were stimulated with *A. phagocytophilum*, caspase-1 was activated and resulted in increased IL-1β and IL-18, but not IL-6; sialostatin L2 mitigated this phenotype. Inhibition of caspase-1 by SL2 appeared to be *A. phagocytophilum*-specific, as SL2 did not suppress caspase-1 in response to canonical NLRP3 and NLRC4 activators [70•]. Interestingly, *A. phagocytophilum* lacks peptidoglycan, the canonical Nod1 and Nod2 agonists, indicating that another unidentified PAMP triggers caspase-1 [39].

Conclusion

The influence that tick saliva has in facilitating tick-borne pathogen transmission is well-documented; however, the mechanistic details are only beginning to emerge. Currently, the vast majority of knowledge is limited to downstream cytokine phenotypes. We know very little about individual salivary molecules and how they interfere with immune signaling cascades. Given the role that TLR and NLR signaling has in controlling tick-borne diseases and the reported effect that tick saliva has on these pathways, it is reasonable to speculate that pathogen transmission is inadvertently promoted by the immunomodulatory properties of salivary components. Both TLR and NLR signaling cascades converge on NF-κB and MAPK activation and play complementary roles in controlling microbial infections. It is, therefore, possible that components from tick saliva act on one or both pathways in a redundant manner to maximize host immune suppression, which is then exploited by tick-borne pathogens.

The main focus of research on the immunomodulatory properties of tick saliva has been on how molecules manipulate the host immune system. An alternative consideration is how the saliva influences the contents of a blood meal ingested by the tick. As previously discussed, multiple studies have indicated that tick saliva alters cytokine secretion in the host. Cross talk between host signaling molecules and tick cells/tissues is a possibility with unknown consequences. Moreover, what effect ingested tick saliva (mixed with the blood meal) may have on the tick's immune system is poorly characterized. For example, after a *B. burgdorferi*-infected tick molts, bacteria remain dormant in the midgut prior to the next blood meal [27, 84]. Upon feeding, the spirochete population expands rapidly [85, 86]. While much of this is likely caused by the influx of nutrients and the temperature increase that comes with a blood meal, the ingested saliva may also suppress the tick immune system, which would benefit the expansion of a microbial population.

Anti-inflammatory properties of tick saliva present a unique opportunity to discover novel therapeutics. These could be applied in a variety of clinical scenarios caused by inappropriate inflammation, as is seen with some microbial infections. The pathology associated with certain diseases can be directly attributed to the host immune response, rather than the pathogen itself. For example, it is well-known that some Lyme disease patients can have autoimmune symptoms, such as treatment-resistant Lyme arthritis [87, 88], and as discussed earlier in this review, inflammation resulting from Nod2 signaling in ehrlichiosis can cause toxic shock syndrome (while TLR2 responses are protective) [73]. Potential to combat these diseases with salivary molecules exists if they can skew an immune reaction from a pathological outcome to a protective response.

Inappropriate inflammation is also the cause for several sterile diseases including asthma, inflammatory bowel and autoimmune diseases, chronic inflammation resulting from long-term exposure to irritants, and cancer growth encouraged by immune cell infiltrates [82•, 89–94]. Furthermore,

detrimental outcomes can result from an inflammatory response to an injury such as atherosclerosis or a brain aneurysm [95]. The pathology from these types of inflammation results in a significant amount of morbidity, loss in the quality of life, decreased physiological function, and/or death. If individual salivary molecules are characterized, potential exists to harness the anti-inflammatory properties as therapeutics to prevent pathology associated with these sterile diseases. Although this area is relatively unexplored, some studies are beginning to emerge [91, 92, 96].

Apart from clinical applications, tick salivary components may also be developed into research tools. For example, a salivary molecule could be used for imaging techniques if it targets and binds components of an immune signaling pathway. This could be useful with either total cell populations or with single cells to monitor immune pathway activation or differentiation under physiological and/or pathological conditions. Importantly, these salivary molecules can be used to study the immune response itself. The innate immune field is rapidly expanding, indicating that there is much to discover. The adaptation of ticks to their mammalian hosts has resulted in elegantly tailored immune manipulation strategies, which presents a unique opportunity for scientists to exploit this relationship as a learning opportunity.

Historically, experimental manipulation of ticks has been challenging, given their exclusively hematophagous diet and relatively slow growth rate/life cycle when compared to other arthropods. Moreover, these arachnids are not easily amenable to genetic manipulation as their insect counterparts are (*Drosophila*, mosquitos, etc.), which have presented hurdles and slowed the progress of discovery. However, with the advent of new molecular tools such as genome engineering technologies and the increasingly common use of high-throughput methods, there is potential for these impediments to be overcome. Having a precise, molecular understanding on how saliva manipulates immune pathways will pave the way for novel tick control strategies and will lay the foundation for vaccine development. Importantly, this knowledge will provide insights into the basic biology of ticks, which is imperative for comprehending why and how these arthropods are exceptionally competent vectors for disease.

Compliance with Ethical Standards

Conflict of Interest Dana K. Shaw, Michail Kotsyfakis, and Joao H.F. Pedra declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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Papers of particular interest, published recently have been highlighted as:
• Of importance

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