Ismail *et al. Zoonoses* (2022) 2:21
DOI 10.15212/ZOONOSES-2022-0009

Review Article

Protective Immunity and Immunopathology in Ehrlichiosis

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Abstract

Human monocytic ehrlichiosis, a tick transmitted infection, ranges in severity from apparently subclinical to fatal toxic shock-like disease. Models in immunocompetent mice range from abortive to uniformly lethal infection, depending on the *Ehrlichia* species, inoculum dose, and inoculation route. Effective immunity is mediated by $CD4^+$ T lymphocytes and gamma interferon. Lethal infection occurs with early overproduction of proinflammatory cytokines and overproduction of TNF alpha and IL-10 by $CD8^+$ T lymphocytes. Furthermore, fatal ehrlichiosis is associated with TLR 9/MyD88 signaling, upregulation of several inflammasome complexes, and secretion of IL-1 beta, IL-1 alpha, and IL-18 by hepatic mononuclear cells, thus suggesting activation of canonical and noncanonical inflammasome pathways, a deleterious role of IL-18, and a protective role of caspase 1. Autophagy promotes ehrlichial infection, whereas MyD88 signaling hinders ehrlichial infection by inhibiting autophagy induction and flux. During infection of hepatocytes by the lethal ehrlichial species, after interferon alpha receptor signaling, the activation of caspase 11 results in the production of inflammasome-dependent IL-1 beta, extracellular secretion of HMGB1, and pyroptosis. HMGB1 has high levels in lethal ehrlichiosis, thereby suggesting a role in toxic shock. Studies of primary bone marrow-derived macrophages infected by highly avirulent or mildly avirulent ehrlichiae have revealed divergent M1 and M2 macrophage polarization associated with the generation of pathogenic CD8 T cells and neutrophils, and excessive inflammation, or with strong expansion of protective Th1 and NKT cells, resolution of inflammation, and clearance of infection, respectively.

Keywords: Ehrlichiosis, *Ehrlichia*, obligate intracellular bacteria, immunity, inflammasomes, autophagy, type I interferon, pattern recognition receptors, macrophage polarization

Edited by:
Ruifu Yang, Beijing Institute of Microbiology and Epidemiology

Reviewed by:
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Received: March 18 2022
Revised: June 08 2022
Accepted: June 20 2022
Published Online: July 05 2022

INTRODUCTION

Human monocytic ehrlichiosis (HME) is a tick-borne disease caused by the obligately intracellular Gram-negative bacterium *Ehrlichia chaffeensis* [1-4]. Clinical manifestations of HME range from non-specific influenza-like illness to severe and potentially life-threatening disease. The severe form of HME is commonly marked by acute liver damage followed by multi-organ failure and toxic shock-like syndrome [5-9]. Clinical and laboratory diagnosis of HME at early stages of disease is problematic because of non-specific symptoms and challenges regarding the accuracy of the current diagnostic testing. Doxycycline is the drug of choice for the treatment of HME; however, a recent cross-sectional study has determined that late administration of doxycycline is a key factor associated with the development of severe ehrlichiosis

[9-11]. Notably, a history of tick exposure is associated with a decreased rate of admission of patients to intensive care units because effective treatment is given promptly in those cases. Notably, some patients treated in late stages of infection develop a severe disease mimicking hemophagocytic lymphohistiocytosis syndrome, a pathologic hyperactivation of macrophages that occurs in association with infection [12,13]. These findings suggest that multi-system disease and tissue damage in HME are due to immunopathology. This conclusion is further supported by findings in murine models of mild/non-fatal and severe/fatal ehrlichiosis. These findings have suggested that innate and adaptive immune responses against *Ehrlichia* act as a "double-edged sword" in fatal ehrlichiosis [5,14,15], wherein protective immunity is mediated by $CD4^+$ Th1 and NKT cells, and the pathogenic response is attributed to activated neutrophils and TNF-α–producing $CD8^+$ T cells [5,16].

Recent studies have highlighted the essential roles of inflammasomes and autophagy as part of the innate immune responses against several pathogens, thus leading to pathogenic or protective outcomes. Herein, we discuss the cell-specific innate immune responses during ehrlichiosis involving the regulation of autophagy and inflammasomes, the associated signaling pathways, and how these events affect the innate and adaptive immune responses against *Ehrlichia*. Understanding these mechanisms is critical for rational development of novel diagnostic, therapeutic, and preventive countermeasures against ehrlichiosis.

METHODS

Articles on *Ehrlichia* and HME were selected by searching relevant publications from multiple sources. The search was performed via PubMed-Medline. Studies were identified by searching for *Ehrlichia* as well as multiple mechanisms of immunity and pathogenesis during mild and fatal ehrlichiosis. For example, studies on the roles of inflammasomes and autophagy in ehrlichiosis were identified by searching for "*Ehrlichia* and inflammasome," "*Ehrlichia* and autophagy," "*Ehrlichia* and adaptive immunity," and "*Ehrlichia* and immune evasion." We included reports from the past 10 years that are most relevant to the topic of this review.

Ehrlichiosis, a potentially life-threatening infectious disease

HME is a potentially life-threatening tick-borne zoonotic disease increasingly observed in North America. HME is among the most prevalent tick-borne rickettsial diseases, which include spotted fever rickettsiosis and anaplasmosis, caused by spotted fever group *Rickettsia* and *Anaplasma*, respectively [2,4,17,18]. According to the manually completed case report forms (CRFs) and the National Notifiable Diseases Surveillance System, during 2008–2012, people older than 55 years of age had the highest incidence rate among all age groups, although CRFs were highest among children younger than 5 years of age (4%), followed by people older than 70 years of age (3%). Among confirmed cases, CRFs among people older than 70 years of age increased to 53%, whereas CRFs among children younger than 5 years of age increased to 14% [10,19,20]. These findings have been partially due to a significantly higher prevalence of immunosuppressive conditions among older age groups. The median age of patients with immunosuppressive conditions was 60 years. The risk of severe outcomes in immunosuppressive cases was associated with higher rates of hospitalization, presence of life-threatening conditions, and death [21-24]. In contrast, ehrlichiosis cases are believed to be under-reported because patients either are asymptomatic or have mild illnesses not prompting medical consultation, whereas reported cases are believed to over-represent infections with more severe clinical manifestations. Therefore, compared with the younger population, people over 55 years of age are expected to have a higher probability of more severe ehrlichiosis outcomes, owing to age-associated systemic inflammatory responses.

HME can present as a mild influenza-like illness or severe disease characterized by initial lymphopenia, thrombocytopenia, and elevated liver enzymes [25]. If untreated, or when treatment with the appropriate antibiotic (doxycycline) is delayed because of misdiagnosis, patients with HME develop complications including meningoencephalitis, adult respiratory distress syndrome, sepsis, and multi-organ failure. HME is an increasingly important public health concern with a high hospitalization rate ranging from 53% to 72%, and a case fatality rate of approximately 1%. The liver is the main site of *Ehrlichia* infection and pathology [26-30]. Most patients with HME have mild-to-moderate increases in serum levels of liver transaminases; some cases show marked cholestasis and progressive hepatosplenomegaly. Histopathologic examination of liver biopsy samples from patients with HME reveals diffuse activation of monocytes and tissue-resident macrophages, as well as lymphocyte infiltration in the hepatic sinusoids; multifocal inflammatory lesions with hepatocellular death that appears to be apoptotic; and nonspecific hepatocyte (HC) swelling and steatosis [29,31,32]. The activation of monocytes and Kupffer cells (liver-resident macrophages) has been observed with and without *E. chaffeensis* infection of host cells, thereby confirming that hepatic injury is not directly associated with ehrlichial burden, but is secondary to the host inflammatory and immune responses.

E. chaffeensis, the causative agent of HME, is an obligately intracellular Gram-negative bacterium that lacks lipopolysaccharide (LPS) and peptidoglycan [8,33]. Other *Ehrlichia* species that cause HME in the United States and worldwide include *E. canis*, *Ixodes ovatus Ehrlichia* (*Ehrlichia* HF strain) often abbreviated as IOE, which has recently been cultivated and named *E. japonica*/IOE, *E. ewingii*, and *E. muris eauclairensis* [34]. IOE/*E. japonica* (the focus of the studies described below) has been detected in *Ixodes ovatus* ticks throughout Japan, *Ixodes apronophorus* ticks in Romania, and *Ixodes ricinus* ticks in France and Serbia [35,36]. Analyses of genome sequences of cultured IOE/*E. japonica* have indicated that this *Ehrlichia* species has a single double-stranded

circular chromosome of 1,148,904 bp, which encodes 866 proteins with similar metabolic functions to those of *E. chaffeensis* [37]. IOE/*E. japonica* encodes homologs of several virulence factors identified in *E. chaffeensis*, such as the type IV secretion system apparatus and effector proteins, the P28/OMP-1 family of outer membrane proteins, tandem repeat proteins, and ankyrin-repeat proteins.

How *E. chaffeensis* or other *Ehrlichia* species cause a spectrum of diseases in humans ranging from mild-to-severe and potentially fatal toxic shock-like syndrome remains elusive. However, as suggested by genome sequence arrangement as well as DNA-DNA hybridization, each of these *Ehrlichia* species has subspecies and strains that vary in virulence [38]. For example, comparative genomic analysis of the Wakulla, Arkansas, and Liberty circulating strains of *E. chaffeensis*, and the disease that they cause in immune-deficient mice, has indicated that those strains have distinct genotypes and phenotypes that define their virulence in immune-deficient mice, in the order of Wakulla, Arkansas, and Liberty from highest to lowest [28,39,40]. The livers of mice infected with the Wakulla and Arkansas strains have more severe diffuse inflammation and granulomatous inflammation than those of mice infected with the Liberty strain.

Animal models of ehrlichiosis

An ideal animal model of human ehrlichiosis should have several criteria that mimic human disease such as: 1) transmission via natural infection, i.e., tick transmission; 2) utilization of major *Ehrlichia* pathogens that cause human ehrlichiosis, such as *E. chaffeensis*, *E. canis*, and *E. ewingii*; 4) a range of disease manifestations and outcomes, varying from mild/non-fatal to severe/fatal in immunocompetent hosts; 5) clinical and pathologic manifestations of mild and severe ehrlichiosis in infected mice similar to those in humans, as well as laboratory findings that recapitulate the findings in HME; 6) infection outcomes dependent on the dose of infectious inoculum, genetic background, and route of transmission, which are key variables affecting the outcomes of infections with most bacterial and viral pathogens; 7) a model that enables mechanistic studies for which reagents and knockout animals lacking specific genes are available. Although several animal models fulfill one or two of the criteria described above, no single model currently fulfills all these criteria. For example, infections with *E. chaffeensis*, the main pathogen causing HME, trigger mild, self-limited infection in immunocompetent hosts, but cause severe and potentially fatal disease in immunocompromised hosts [41,42]. Utilization of this model in the analysis of adaptive immune responses to *Ehrlichia* is limited, and thus may not be optimal for understanding adaptive immunity and pathogenesis during fatal ehrlichiosis. Animal studies of ehrlichial infections in the natural hosts, such as *E. canis* in dogs [43-45], *E. chaffeensis* in white-tailed deer [46-48], and *E. ruminantium* in ruminants [49-51], have revealed a disease that mimics the pathology and defined pathophysiology of the human disease. However, analysis of immunity and pathogenesis through mechanistic approaches is challenging, owing to the outbred nature of the hosts, and the lack of availability of canine or ruminant reagents for examining immune responses. Because of these limitations, an alternative animal model of ehrlichiosis has been developed, which mimics human disease in several aspects. Although data generated from murine models of ehrlichiosis used in analyzing the immunity and pathogenesis of HME, as described below, are intriguing, the ability to translate results from murine experiments to humans and clinical diseases remains limited.

Several mouse strains have been used to examine the immunity, immunopathology, and pathogenesis of ehrlichiosis. These include C57BL/6, C3H/HeJ, C3H/HeN, BALB/c, AKR, C.B 17 SCID, and several knockout mice that lack different aspects of the innate and adaptive immune responses. In immunocompetent C57BL/6 mice, investigators have used several *Ehrlichia* agents to cause a spectrum of disease: *E. chaffeensis* causes self-limited infections, *E. muris* causes mild and persistent infections, and *E. japonica* causes severe and potentially fatal infections [5,52-54]. Notably, the outcomes of infection with *E. japonica*/IOE vary according to the route of infection and the infectious dose. For example, intraperitoneal (i.p.) infection with a high dose of *E. japonica*/IOE is lethal, whereas intradermal infection with the same dose results in sublethal infection and mild disease [55]. Similarly, i.p. or intravenous injection with *E. japonica*/IOE causes dose-dependent lethality (higher dose) or sublethal persistent (lower dose) infection. Interestingly, mice infected i.p. with a high dose of *E. muris* survive and develop protective immunity and long-term memory responses that are protective against not only homologous re-infection but also heterologous re-infection with IOE [56-58]. Recently a tick vector transmission model has been developed, which mimics the natural route of *Ehrlichia* infection as well as the pathology [59-62]. In this model, the *Ixodes scapularis* larvae were fed on mice infected with the human pathogen *E. muris eauclairensis.* After molting, the infected nymphs were placed on naive animals to transmit the pathogen. Mice were infected with *Ehrlichia* when they were infested by 90%–100% of feeding larvae. Many mice fed upon by infected nymphs had sublethal infection, whereas 27% of mice developed lethal disease. Like HME and other needle-transmission models, transmission of *Ehrlichia* via ticks resulted in bacterial dissemination to all tissues, with the highest bacterial burden in the spleen, lungs, liver, kidneys, lymph nodes, bone marrow, and brain. In addition, several foci of cellular infiltration, and death of parenchymal and non-parenchymal cells were observed in the liver.

Using murine models of mild and fatal ehrlichiosis caused by systemic infection with mildly and highly virulent *Ehrlichia* species that mimic laboratory findings, as well as clinical and pathologic manifestations in HME, we have shown that the protective immunity during mild ehrlichial infection is due to the generation of both cell-mediated and humoral immunity, mediated by IFN-γ producing $CD4^+$ Th1 cells and *Ehrlichia*-specific IgG antibodies, mainly of the IgG2a isotype [5,63-65]. In contrast, severe and fatal *Ehrlichia*-induced toxic shock-like syndrome is

characterized by the development of initial focal hepatic necrosis and apoptosis, increased serum levels of hepatic enzymes, substantial lymphopenia and leucopenia, apoptosis of myeloid cells and $CD4^+$ T cells, and ultimately multi-organ failure and sepsis. Further analysis has indicated that severe and fatal primary ehrlichiosis in animals is due to a cytokine and chemokine storm characterized by an early overproduction of pro-inflammatory cytokines (including IL-1, TNF-α, IL-18, and IL-12p40) and several chemokines (CCL5/RANTES, CCL3/MIP-1α, CCL4/MIP-1β, CCL2/MCP-1, and IL-8), which is followed by excessive production of anti-inflammatory cytokines (IL-10 and IL-13) during the course of infection [30,52,66]. Our studies have demonstrated that, in contrast to the mild murine ehrlichiosis caused by *E. muris* infection, wherein CD8 T cells play a protective role, $CD8^+$ T cells play a pathogenic role in a murine model of fatal ehrlichiosis [67]. Fatal ehrlichial infections induce substantial expansion of cytotoxic $CD8^+$ T cells producing TNF-α and IL-10. $CD8^+$ T cell deficiency in mice infected with virulent *Ehrlichia* species restores the number of Th1 cells, attenuates the cytokine and chemokine storm, decreases tissue damage, and protects mice against fatal infection. We have also shown that innate cells such as NK cells and neutrophils have deleterious roles in the pathogenesis of ehrlichiosis, because they directly contribute to tissue damage as well as the development of cytokine storms and the expansion of pathogenic $CD8^+$ T cells [66,68]. Although the roles of NK cells and neutrophils during mild ehrlichiosis have not been examined, we have observed differential spatial and temporal changes in NK cells and neutrophils during lethal infection compared with mild infection [66]. NK cells migrate to the liver in fatal ehrlichiosis during lethal infection, whereas they remain in the spleen or peritoneum during mild *Ehrlichia* infection [66]. Although mechanistic studies examining the roles of NK cells and neutrophils in mild ehrlichiosis have not been performed, we believe that NK and neutrophils play a protective role during mild/non-lethal *Ehrlichia* infection, owing to the absence of an excessive inflammatory environment triggering overactivation of these cells and their polarization into a pathogenic phenotype, as seen in fatal ehrlichiosis. Together, these data suggest that NK cells, neutrophils, and $CD8^+$ T cells mediate dysregulated inflammation and tissue injury in fatal HME.

Inflammasomes: cytosolic receptors with key roles in intracellular surveillance

Inflammasomes are key components of the innate immune system and contribute to the initial host defense mechanism against pathogens. Inflammasomes recognize pathogen-derived molecules known as pathogen-associated molecular patterns (PAMPs) [69-71] as well as endogenous host-derived molecules, known as damage-associated molecular patterns (DAMPs), that are released from dying cells during stress or infection [72-75]. Inflammasomes are cytosolic multi-protein complexes that consist of intracellular nucleotide-oligomerization domain (NOD)-like receptor (NLR), nucleotide-binding domain, and leucine-rich repeat (LRR) containing proteins, or the absent in melanoma 2 (AIM2)-like receptors (ALRs), the adaptor protein apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (ASC), and procaspases [76-79]. To date, four inflammasome complexes have been identified and well characterized: the NOD and LRR containing protein (NLR) family members NLRP1, NLRP3, and NLRC4, as well as AIM2. NLRP1 is activated by PAMPs such as muramyl dipeptide. NLRC4 is activated by PAMPs such as flagellin and the type II secretion system, as well as by DAMPs such as neuronal apoptosis inhibitory protein family members. The NLRP3 inflammasome is also activated by several DAMPs including reactive oxygen species (ROS), mitochondrial DAMPs, and adenosine triphosphate, as well as fibrillar proteins (e.g., β-amyloid fibrils). AIM2 is activated by microbial or host double-stranded DNA. AIM2 also binds high mobility group box 1 (HMGB1), which promotes activation during oxidative stress [79].

Two inflammasome pathways are triggered after sensing of a microbial or host ligand: the canonical and non-canonical inflammasome pathways. The canonical inflammasome pathway involves signaling by the NLRP3 complex, after recognition of PAPMs or DAMPs, via the adaptor molecule ASC; consequently, activation of caspase-1 causes cleavage of pro-IL-1β and pro-IL-18, and the release of biologically active IL-1β and IL-18 [80-83]. In the non-canonical inflammasome pathway, cytosolic LPS triggers activation of caspase-11, which in turn activates caspase-1 and promotes secretion of IL-18, IL-1β, and HMGB1, as well as inflammatory cell death, known as pyroptosis [84-88]. The gasdermin D protein is essential for caspase-11-dependent pyroptosis [88-91]. Caspase-11 cleaves gasdermin D, thereby promoting both pyroptosis and NLRP3-dependent activation of caspase-1. Although inflammasomes are critical for defense against pathogens and danger signals, their excessive activation can promote immunopathology and tissue injury. For example, dysregulation of inflammasomes is associated with multiple neurodegenerative diseases, such as Alzheimer disease, Parkinson disease, multiple sclerosis, and amyotrophic lateral sclerosis [92,93]. The ligands causing activation of inflammasomes in these diseases are not completely understood. However, the accumulation of amyloid-beta plaques in the cerebrum in patients with Alzheimer disease has been suggested to be a potential DAMP that triggers NLRP3 inflammasome activation. Activation of NLRP3 has also been widely studied in liver diseases [94-97]. NLRP3-mediated secretion of IL-1β after LPS-TLR signals results in the production of multiple inflammatory cytokines and chemokines, and an excessive inflammatory response. IL-1β in liver diseases recruits inflammatory cells, which in turn activate hepatic stellate cells (HSCs)— key contributors to liver fibrosis [98-101]. IL-1β also triggers triglyceride accumulation in HCs and causes HC cell death mediated by TNF-α.

Roles of canonical and non-canonical inflammasomes in the development and progression of severe ehrlichiosis

Ehrlichia is an obligate intracellular bacterium that resides within specialized membrane-bound inclusions that have early endosome-like characteristics; however, the inclusions lack late endosomal or lysosomal markers [102-104]. Unlike other intracellular bacterial pathogens that access the cytosol, such as *Rickettsia* and *Listeria*, *Ehrlichia* do not escape from phagosomes to the cytosol. However, virulent IOE/*E. japonica* triggers deleterious inflammasome activation. Compared with mild ehrlichiosis in mice, fatal ehrlichiosis is associated with significant upregulation of several inflammasome complexes, including NLRP3, NLRP1, NLRC4, NLRP12, and AIM2; activation of caspase 1 and caspase 11; and secretion of IL-1β, IL-1α, and IL-18 by liver mononuclear cells, including Kupffer cells and infiltrating inflammatory monocytes [63,84,105]. Fatal *Ehrlichia* infection has been found to trigger the activation of canonical and non-canonical inflammasome pathways. Interestingly, our data have demonstrated a deleterious role of IL-18 in the host response to *Ehrlichia*. Mice deficient in the IL-18 receptor (IL-18R$^{-/-}$) are more resistant to fatal ehrlichiosis caused by i.p. infection with *E. japonica*/IOE than wild-type mice. Moreover, infected IL-18R$^{-/-}$ mice have a lower bacterial burden, minimal tissue injury, attenuated inflammation, and greater expansion of protective CD4^{+} Th1 cells [65]. Notably, protective immunity in IL-18R$^{-/-}$ mice results from diminished expansion of pathogenic CD8^{+} T cells, thus suggesting that inflammasome activation leads to induction and an increased number of pathogenic CD8^{+} T cells that cause liver injury [14,65].

Notably, Casp1$^{-/-}$ mice infected with highly virulent IOE are markedly susceptible to fatal ehrlichiosis: they develop overwhelming infection and extensive tissue injury, and succumb to infection at earlier time points after infection than do wild-type controls [14,84]. Therefore, the deleterious inflammasome activation in ehrlichiosis does not appear to be due to the canonical inflammasome pathway. In fact, data suggest that caspase 1 may play a protective role in ehrlichiosis, in agreement with the function of caspase 1 in other infection model systems. Recent studies have suggested that active caspase 1 is hepatoprotective, because deficiency in caspase 1 has been associated with the death of HCs and liver injury in a hemorrhagic shock model [106,107]. Thus, the greater susceptibility of caspase 1$^{-/-}$ mice to fatal ehrlichiosis might be due to altered survival of HCs. Unlike caspase 1$^{-/-}$ mice, mice deficient in NLRP3 (Nlrp3$^{-/-}$ mice) effectively clear ehrlichiae on day 7 post infection; however, these mice still exhibit acute mortality and develop liver injury similarly to wild-type mice [84,85]. Notably, the susceptibility of wild-type, caspase 1$^{-/-}$ and NLRP3$^{-/-}$ mice, and the development of liver damage, are associated with higher expression of active caspase 11 in the liver in these mice than in wild type mice. Therefore, activation of the non-canonical inflammasome pathway may play a key role as a mediator of tissue injury during severe ehrlichiosis.

Regulation of inflammasomes by type I interferon and MyD88 signaling

Type I interferons (IFN-I) include IFN-α and IFN-β cytokines, which are critical components of the innate immune response against viruses [108,109]. However, the role of IFN-I in host responses to bacterial pathogens is dependent on the pathogen. For example, the replication and survival of many cytosolic bacterial pathogens including *Listeria monocytogenes*, *Rickettsia* species, and *Francisella novicida* are restricted by IFN-I signaling [110]. In contrast, we and others have shown that IFN-I contributes to the development of immunopathology during infection with virulent *Ehrlichia* [63,84,85,111]. IFN-I receptor knockout mice (Ifnar1$^{-/-}$) are markedly more resistant to fatal disease than wild-type mice, as evidenced by attenuated liver pathology, lower bacterial burden in the liver and spleen, and prolonged survival. The resistance of Ifnar1$^{-/-}$ mice to fatal ehrlichiosis is associated with the expansion of IFNγ-producing CD4^{+} Th1 cells, which otherwise undergo apoptosis during IOE infection, and a lower number of IL-10 producing T cells with immunosuppressive functions [84]. The protection against lethal infection in Ifnar1$^{-/-}$ mice correlates with attenuated activation of the non-canonical inflammasome pathway, as evidenced by less activation of caspase-11 and lower levels of splenic and hepatic IL-1β than observed in wild type mice. Notably, IFN-I mediated activation of caspase-11 leads to cell death via pyroptosis, a rapid inflammatory cell death that enables exit of intracellular ehrlichiae into the extracellular space, infection of other cells, and bacterial dissemination to peripheral organs (Fig 1).

The molecular and cellular mechanisms through which IFN-I leads to activation of caspase-11 during *Ehrlichia* infection remain elusive. However, autocrine or paracrine signaling by IFN-I during infection with LPS-containing Gram-negative bacteria leads to upregulation of genes encoding guanylate binding proteins, thereby enabling the release of LPS into the cytosol. Cytosolic LPS acts as a PAMP that triggers cleavage of pro-caspase 11 into active caspase 11 [94,112,113]. Thus, IFNAR signaling during fatal *Ehrlichia* infection might possibly induce the production of guanylate binding proteins, which then disrupt vesicles containing ehrlichiae and enable the escape of PAMPs to the cytosol and the activation of caspase-11. Because *Ehrlichia* lack LPS, the IFN-I-caspase 11 axis is unlikely to be triggered by LPS-like molecules. Our recent studies have suggested that mitochondrial DAMPS might be the ligands that trigger activation of caspase 11 during fatal ehrlichiosis after IFNAR signaling. Infection of macrophages, the main target cells of *Ehrlichia*, with *E. japonica* triggers TLR9/MYD88 signaling, thus leading to activation of the metabolic checkpoint kinase mammalian target of rapamycin complex 1 (mTORC1), a negative regulator of autophagy. MyD88-dependent mTORC1 activation inhibits autophagy induction and flux, and blocks mitophagy (i.e., the elimination of damaged mitochondria via autophagy after binding of mitochondria to autolysosomes) [105]. The MyD88-mediated blocking of of autophagy and mitophagy then leads to

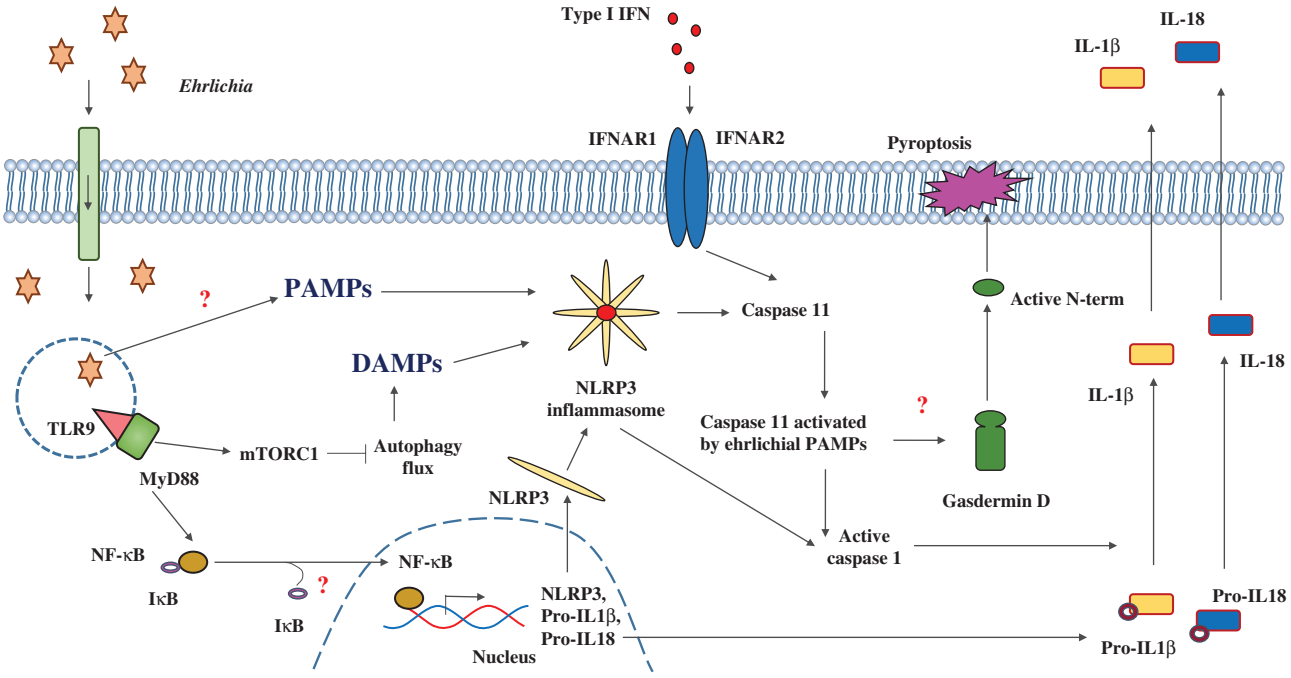

FIGURE 1 | Model of inflammasome activation via canonical and non-canonical pathways. First, *Ehrlichia* invades target cells and induces TLR9/MyD88 signaling, thus upregulating NLRP3 complexes, pro-IL1β, and pro-IL-18 via NF-κB. NRRP3 inflammasome activation occurs after recognition of *ehrlichial* PAMPS and/or mitochondrial DAMPS generated after blockage of autophagy via MyD88/mTORC1 signaling. Activation of canonical inflammasome pathways subsequently results in the cleavage of pro-IL1β and pro-IL-18 into biologically active IL1β and IL-18, and subsequent extracellular secretion. In the second step, binding of type I IFN cytokines to IFNAR triggers cleavage of caspase-11 and the activation of the non-canonical inflammasome pathway, thus leading to secretion of mature IL-1β and IL-18, cleavage of gasdermin D, and pyroptosis.

accumulation of damaged mitochondria, thereby resulting in the release of mitochondrial DNA or other mitochondrial DAMPs (e.g., ROS). Other studies have shown that infection of macrophages with *E. chaffeensis*, the *Ehrlichia* species causing severe and potentially fatal disease in humans, inhibits mitochondrial metabolism [114–116].

Similarly to our studies, studies by Macnamara et al. have shown that IFNα/β promote a lethal, shock-like pathology in mice infected with IOE/*E. japonica* [111]. However, the mechanism through which type I interferon signaling causes fatal ehrlichiosis has been attributed to IFNAR-mediated hemopoietic dysfunction. IFAR signaling triggers severe bone marrow loss, abrogates myelopoiesis during infection, and decreases the number of hematopoietic stem and progenitor cells (HSC/HSPCs) [117]. Deficiency in IFNAR signaling restores the bone marrow and splenic hematopoiesis. Mechanistically, this deleterious effect of type I IFN on HSC/HSPCs is due to caspase 8/RIPK1-mediated inhibition of HSC/HSPC proliferation and increasing HSPC death. Combination RIPK1 antagonist (Necrostatin-1s) and antibiotic therapy in IOE-infected mice has been found to restore HSPC and HSC numbers during infection [117]. Together, these studies suggest that the pathogenic role of type I IFN signaling involves multiple mechanisms that are not restricted to deleterious inflammasome activation (as shown in our study) but also include hematopoietic dysfunction mediated by IFNAR-mediated HSPC cell death as well as HSC quiescence.

In contrast to the deleterious role of IFN-I in fatal ehrlichiosis, recent studies in other infection models, i.e., *Plasmodium yoelii* (causative agent of malaria) and *Rickettsia parkeri* (causative agent of spotted fever rickettsiosis), have shown that the IFN-I response during these infections is protective and negatively regulated by inflammasomes [118,119]. In both infection models, inflammasomes appear to be involved in a non-protective response by inhibiting IFN-β production. Mechanistically, in the malaria model, negative regulation of IFN-β by inflammasomes appears to be mediated by IL-1β-mediated SOCS1 upregulation, which in turn inhibits MyD88-IRF7-mediated-IFN-I signaling and cytokine production in plasmacytoid dendritic cells. However, in the *Rickettsia* model, inhibition of IFN-β by the non-canonical caspase 11-mediated inflammasome pathway is due to inflammatory cell death (pyroptosis), which antagonizes IFN-I. Although we have not examined the cross regulation of type I IFN by inflammasomes in our murine model of fatal ehrlichiosis, temporal and spatial dynamics during infection might potentially contribute to cross-regulation between inflammasomes and IFN-I signaling. In contrast to the protective role of IFN-β in macrophages during infection with *Rickettsia* and *Plasmodium* species, as indicated above, our studies have demonstrated a pathogenic role of IFN-β during fatal ehrlichiosis. This discrepancy might be due to pathogen- or cell-specific differences between these pathogens. Alternatively, low level of IFN-I cytokines might be protective, whereas high levels of IFN-β might be

detrimental, as suggested by other studies. In support of this possibility, we have found that mild ehrlichial infection and protective immunity are associated with low levels of IFN-β.

Toll-like receptors (TLRs) are transmembrane proteins located in both plasma membranes and endosomal membranes, which survey the extracellular and intracellular environment and thus function as pattern recognition receptors (PRRs). TLRs play key roles in the innate immune responses against intracellular pathogens [120]. Surface TLRs such as TLR2 and TLR4 recognize several bacterial ligands. For example, TLR2 recognizes peptidoglycan, lipoteicoic acid, lipopeptides, and lipoprotein, whereas TLR4 recognizes LPS of Gram-negative bacteria [121,122]. Endosomal TLRs include TLR9, which recognizes double-stranded DNA and CpG-containing single-stranded DNA, as well as TLR7, which detects single-stranded RNA. Binding of all TLRs to their respective ligands triggers signals via an adaptor complex consisting of MyD88 [123]. In addition, ligand binding to TLR4 and TLR3 triggers signals via Toll/IL-1R domain-containing adapter-inducing interferon-β (TRIF) [113,124]. Signaling via MyD88 results in transcription and activation of NF-κB- and AP-1-dependent genes, whereas signaling via TRIF results in transcription and activation of not only NF-κB and AP-1-dependent genes, but also induction of IRF3-genes and IFN-I.

We have recently found that virulent *Ehrlichia* triggers activation or signaling via TLR9/MyD88, thus contributing to the activation of both canonical and non-canonical inflammasome pathways and tissue injury during fatal ehrlichiosis [105]. MyD88$^{-/-}$ mice infected with *E. japonica*/IOE show attenuated inflammasome activation and minimal liver injury, and are more resistant to lethal infection than wild type mice. Notably, TLR9$^{-/-}$ and MyD88$^{-/-}$ show ineffective bacterial clearance and protective immunity, as indicated by an elevated bacterial burden in the liver. These data are consistent with earlier findings indicating that *Ehrlichia*-induced liver damage and toxic shock are due not to overwhelming infection but to immunopathology. Although MyD88 deficient mice have diminished inflammasome activation, as evidenced by depressed serum levels of IL-1β and IL-1α, this response is partial, thus suggesting a potential role of MyD88-independent pathways such as TRIF during fatal *E. japonica*/IOE infection. In support of a potential role of TRIF, the lack of TLR9 in macrophages, which signal via both MyD88 and TRIF, completely abrogates secretion of IL-1β and IL-1α, and activation of caspase 1/11, thus suggesting that the TLR9-MyD88-TRIF axis is critical for the activation of both canonical and non-canonical inflammasome pathways. In vivo studies using TLR9 deficient mice have also highlighted a key role of TLR9 as a major endosomal PRR in the development of liver damage and fatal toxic shock after lethal *Ehrlichia* infection [105].

Potential PAMPs that trigger inflammasome activation in *Ehrlichia*-infected cells

Ehrlichia membranes differ from other Gram-negative bacteria in that they lack LPS, including lipid A, peptidoglycan, and cholesterol—major PAMPS that trigger inflammasome activation during infection with these pathogens [125-127]. Although *Ehrlichia* lack genes for cholesterol biosynthesis in their cell walls, *Ehrlichia* hijack host membrane phospholipids from host cells and depend on host-derived cholesterol for survival and infection [128-130]. Genomic analysis has revealed that the *E. Chaffeensis* genome does not encode phosphatidylcholine or cholesterol, but encodes enzymes responsible for phosphatidylethanolamine biosynthesis. Indeed, a recent study has demonstrated that host membrane phospholipids and cholesterol traffic in a unidirectional manner to ehrlichiae inclusions in infected cells [130,131]. This translocation of host-cell membranes and molecules to *Ehrlichia* inclusions is dependent on autophagy as well as host endocytosis, and is mediated by the effector protein *Ehrlichia* translocated factor-1 (Etf-1). This protein translocates from the phagosomal compartment where *Ehrlichia* reside to the host cytoplasm through a type IV secretion system [129,130,132]. Key components of the type IV secretion system include genes encoding VirB and VirD proteins, which are associated with the inner membrane channel and ATPase. Mechanistically, ehrlichial Etf-1 binds RAB5, the autophagy-initiating class III PtdIns3K complex, PIK3C3/VPS34, and BECN1, which are proteins involved in early autophagosome formation [102,103]. Through Etf-1, ehrlichiae induce autophagy to obtain nutrients/amino acids for growth and replication through RAB5 and class III PtdIns3K, while avoiding autolysosomal killing. Whether the ehrlichial membranes containing cholesterol and type IV secreted proteins, such as ETF-1, trigger inflammasome activation in infected cells remains elusive. However, these PAMPS are known inflammasome ligands, thus suggesting that *Ehrlichia* may exploit cholesterol and the type IV secretion system effector to induce inflammasome activation and cell death, thus allowing dissemination to other cells and organs.

The *E. chaffeensis* genome contains several other genes involved in host-pathogen interactions. Of particular interest are the genes that encode tandem repeat proteins (TRPs) and ankyrin (Ank) repeat containing proteins [133,134], because these proteins are secreted into the cytosol and can interact with inflammasome complexes. *E. chaffeensis* TRPs are secreted via a type 1 secretion system, which is commonly used by Gram-negative bacteria to secrete various exotoxins, adhesins, and enzymes. TRPs interact with various proteins, DNAs, RNAs, and small molecules, and consequently mediate several processes important for cell survival and function, such as cell adhesion, signal transduction, protein folding, immune responses, RNA processing, transcription regulation, intracellular transport, and cell death [135-137]. *E. chaffeensis* TRPs are immunogenic as well as immunoreactive, because they induce strong host antibody responses and are recognized by polyclonal antibodies in the sera of patients with HME. Examples of TRPs are TRP32, TRP47, TRP75, and TRP120 [138-142]. Many *E. chaffeensis* TRPs and Ank repeats, such as TRP32, TRP47, TRP120, and Ank200, are considered nucleomodulins,

owing to their translocation and localization in the nucleus, and their ability to alter or modify gene expression through various mechanisms, including interaction with host proteins, upregulation of genes associated with host cell survival or death, and direct binding to protein–DNA complexes [143,144]. Although the roles of these TRPs and Anks in inflammasome activation and autophagy regulation during fatal ehrlichiosis remain to be examined, their functions and cellular locations suggest that they may act as potential PAMPs for inflammasomes.

Roles of autophagy in the regulation of inflammasomes and host responses to *Ehrlichia*

Autophagy is a host homeostatic mechanism that is essential for innate host defense against several intracellular pathogens [14,145-148]. Recent studies have shown that autophagy induction enhances the survival and/or replication of *Ehrlichia*. Pharmacologic blocking of autophagy induction with 3-MA treatment or knockdown of autophagy genes, such as *atg5* or *beclin-1*, also impairs bacterial survival and replication [102,103,149,150]. Interestingly, as a host defense mechanism, MyD88 signaling after infection of mice with *E. japonica*/IOE impairs bacterial replication by inhibiting autophagy induction [105]. Inhibition of autophagy induction by MyD88 signaling occurs via activation of mTORC1. Notably, although MyD88 deficiency enhances autophagic flux (i.e., autophagosome-lysosomal fusion), this process does not effectively eliminate virulent *E. japonica*/IOE, because the bacteria do not colocalize with the LC3II/autophagosomes and lysosomes.

Several human and murine studies have reported negative regulation of inflammasomes by autophagy [151-154]. Inhibition of autophagy in macrophages leads to the accumulation of DAMPs, such as damaged mitochondria, host DNA, ROS, mitochondrial DNA, and oxidized mitochondrial cardiolipin [154-156]. Generation of mitochondrial ROS or mitochondrial DNA, and their release into the cytosol cause activation of the cytosolic NLRP3 inflammasome pathway. Mechanistically, MyD88-mediated mTORC1 activation triggers inflammasome activation in macrophages after IOE/*E. japonica* infection via inhibition of autophagy and mitophagy [105], thus resulting in the accumulation of NLRP3 ligands such as mitochondrial DAMPs, as described above. Blocking mTORC1 signaling *in vivo* in infected WT mice, and *in vitro* in primary macrophages, enhances autophagy and attenuates inflammasome activation [105].

Roles of hepatocytes and macrophages in *Ehrlichia*-induced immunopathology

The liver is a major site of pathology and infection in patients with *Ehrlichia*-induced sepsis [29]. The precise effects of inflammation caused by dysregulated inflammasomes and autophagy in different cell types remain unexplored in infections with *Ehrlichia*. Macrophages and monocytes are the major target cells for *Ehrlichia*; however, this bacterium can infect other cell types, such as HCs and endothelial cells, in mice and humans. Whether *Ehrlichia* infect other liver parenchymal cells, such as HSCs or bile duct cells, remains elusive and is an area of future research. However, we and other investigators have recently examined the effects of deleterious type I IFNs during fatal ehrlichial infection on hematopoietic and nonhematopoietic cells by using bone marrow chimeric mice. These studies have demonstrated that IFN-α receptor signaling in nonhematopoietic cells is important for the pathogenesis of *Ehrlichia*-induced sepsis. We have recently demonstrated that virulent *E. japonica*/IOE infects and replicates in primary murine HCs *in vitro*, and that IFNAR signaling in HCs promotes bacterial replication and inflammation via the induction of autophagy and activation of the non-canonical inflammasome pathway, respectively [85,157]. The activation of caspase-11 (non-canonical inflammasome pathway) in *E. japonica*/IOE infected HCs after paracrine IFNAR signaling results in three key events; 1) production of the inflammasome-dependent cytokine IL-1β; 2) cytosolic translocation of HMGB1 and extracellular secretion; and 3) pyroptotic cell death. Notably, fatal murine ehrlichiosis is associated with high serum levels of HMGB1, thus suggesting that HMGB1 may contribute to *Ehrlichia*-induced liver injury and sepsis. HMGB1 is a nuclear protein that acts as a DAMP when translocated to the cytosol and is secreted actively or passively after cell death during many infectious and inflammatory diseases. The mechanism through which HMGB1 contributes to *Ehrlichia*-induced liver injury and sepsis remains elusive. However, as suggested by other studies, extracellular hepatic HMGB1 triggers caspase 1 activation and cell death via binding the receptor for advanced glycation end products (RAGE) on adjacent uninfected macrophages or HCs [158-161]. In contrast, intracellular HMGB1 has been found to induce autophagy through direct interaction with beclin-1 (a key protein that initiates autophagosome formation) [159,162,163]. This HMGB1–beclin1 complex is positively regulated by unc-51-like autophagy activating kinase 1 (Ulk1) and mitogen-activated protein kinase (MAPK). As described above, *Ehrlichia* acquires amino acids, iron, and other essential nutrients through fusion of *Ehrlichia*-containing inclusions with autophagosome and endosome pathways. Because ehrlichial replication is dependent on autophagy induction involving beclin-1, intracellular HMGB1-induced autophagy might possibly enhance bacterial survival and replication in target cells.

Unlike HCs, macrophages are innate immune cells that play key roles in the regulation of innate and adaptive immunity against several pathogens [164,165]. Macrophages are not only the initial immune cells that respond to infections with various pathogens but also function as antigen-presenting cells that prime the adaptive immune response, drive inflammation and host defense against infections, and mediate tissue repair after the resolution of infection and inflammation. Two major lineages are currently known: the first lineage is cells that are derived from myeloid progenitor cells in the bone marrow and give rise to blood-circulating monocytes, and the second lineage is tissue-resident macrophages such as alveolar macrophages in the lung and

Kupffer cells in the liver [157,166,167]. After stimulation, macrophages differentiate or polarize into either classically activated macrophages (M1) or alternatively activated macrophages. M1 macrophages exhibit strong microbicidal function and thus contribute to host defense against several viral, bacterial, and protozoal pathogens; they also play roles in antitumor immunity via several mechanisms [168,169]. M1 cells are characterized by high expression of MHC class II and costimulatory molecules such as CD86 and CD40, but low expression or downregulation of mannose receptor (CD206). At the functional level, M1 cells can phagocytose microorganisms; secrete pro-inflammatory and Th1-promoting cytokines (e.g., IL-12, IL-1β, IL-6, and TNF-α) and chemokines; and produce multiple microbicidal molecules such as nitric oxide and ROS. In contrast, M2 cells are marked by upregulation of CD206, arginase-1, IL-10, and TGF-β. M2 cells exhibit anti-inflammatory or immunosuppressive phenotypes, and thus play roles in tissue repair and wound healing, as they phagocytose apoptotic bodies and cellular debris. These M2 macrophages are strong inducers of T helper 2 (Th2) cells and/or regulatory T cells, where these cells are commonly associated with suppression of tumor microenvironment and thus tumor growth. M1 polarization is enhanced by both IFN-γ and LPS, whereas M2 polarization is promoted via IL-4, IL-10, and IL-13.

Recently, we have shown that infection of unprimed primary bone marrow-derived macrophages with highly virulent *E. japonica*/IOE (which causes fatal ehrlichiosis in mice) or mildly virulent *E. muris* (which causes mild and self-limited ehrlichiosis in mice) induces polarization of macrophages into M1 and M2 phenotypes, respectively [157,170]. IOE-induced polarization of macrophages into the M1 phenotype is interesting, because *Ehrlichia* species do not express LPS, an important M1 stimulus. Similarly, the polarization of M1 and M2 macrophages after *E. japonica*/IOE and *E. muris* infection, respectively, is not associated with substantial production of M1- or M2-promoting cytokines, such as IFN-γ, IL-4, and IL-10. Using murine models of mild and fatal ehrlichiosis, we have further shown that *Ehrlichia*-induced liver damage and sepsis are associated with the accumulation of infiltrating pro-inflammatory M1 macrophages/monocytes in the liver. This M1 expansion in the liver correlates with the generation of pathogenic $CD8^+$ T cells and neutrophils, excessive inflammation, liver injury, and high bacterial burden. In contrast, expansion of M2 in the liver in *E. muris*-infected mice is associated with strong expansion of protective Th1 cells and NKT cells, resolution of inflammation, and clearance of infection. Mechanistically, polarization of M2 macrophages in *E. muris*-infected mice is due to enhanced autophagy induction, whereas blocking autophagy induction in mice infected with IOE/*E. japonica* induces polarization of M1 macrophages [157,170]. Blocking mTORC1 signaling in *E. japonica*/IOE-infected macrophages enhances autophagy and decreases polarization of M1 macrophages, thus suggesting that mTORC1 is a key regulator of M1 polarization during *Ehrlichia*-induced liver injury and toxic shock. The finding that mTORC1, metabolic sensor, is a key factor in macrophage polarization in ehrlichiosis suggests metabolic regulation of macrophage polarization. Studies have shown that the activation of M1 macrophages correlates with aerobic glycolysis and the induction of a pentose phosphate pathway that provides NADPH to produce ROS, whereas M2 macrophages use fatty acid oxidation [171-175]. However, whether M1/M2 polarization occurs in ehrlichiosis after infection with different *Ehrlichia* strains in humans remains elusive and is a topic of future investigation.

CONCLUSION AND FUTURE PERSPECTIVES

Although much progress has been made in recent years in the understanding of immunity and immunopathogenesis of *Ehrlichia* spp. infection, better understanding of

TABLE 1 | Proposed uses of inhibitors of specific detrimental pathways in fatal ehrlichiosis as potential immunotherapies.

Targets	Function in *Ehrlichia* pathogenesis	Inhibitors used
HMGB1	Fatal ehrlichiosis in mice is associated with high levels of HMGB1. HMGB1 has emerging roles in liver disease [157]. Studies have indicated that HMGB1 contributes to *Ehrlichia*-induced liver injury [153-158].	HMGB1-specific polyclonal and monoclonal antibodies, and glycyrrhizin can be used as inhibitors of HMGB1 [176].
IL-1β	IL-1β has proinflammatory effects in ehrlichiosis [28,50,64] and has been shown to contribute to liver fibrosis [95-98].	Anakinra, rilonacept, and canakinumab are the target drugs developed by different pharmaceutical companies for blocking IL-1β [177]. These agents are known to target inflammation in a broad spectrum of disease.
Caspase 11	*Ehrlichia* infection has been shown to upregulate canonical and non-canonical inflammasome pathways [61,81,102].	Scutellarian inhibits caspase 11 in macrophages [178]. Wedelolactone [179] inhibits caspase-11, and prevents IL-1β maturation and apoptosis.
Type I interferon	Type I interferon mediates inflammasome activation and HMGB-1 translocation [82,109], and also has important roles in other infectious diseases [108].	Anifrolimab, a human monoclonal antibody to type I interferon receptor subunit 1, suppresses interferon gene expression and is used in treatment of systemic lupus erythematosus [180].

intracellular bacterial infections and immunology is greatly needed in many important areas of research. The critical gaps in knowledge include: 1) defining the roles of inflammasome activation and autophagy in ehrlichiosis by using the natural model of infection (tick-transmitted infection); 2) defining the PAMPs and DAMPs activating inflammasomes during *Ehrlichia* infection; 3) defining cell-specific responses to *Ehrlichia* infection, for not only immune cells but also parenchymal cells such as endothelial cells, HCs, and HSCs (given that HSCs have been shown to act as antigen-presenting cells expressing PRR and thus modulate immune responses against pathogens); 4) defining crosstalk between macrophages and parenchymal cells in the pathogenesis of severe ehrlichiosis; 5) examining the early events that occur at points of entry in the skin and how they lead to bacterial dissemination in intradermal and tick-transmission models as well as *in vitro* models, such as liver and skin organoid and organ-on-a-chip models; 6) analyzing the potential of multiple inhibitors of specific inflammasome genes or type I IFN signaling used to treat other diseases (Table 1) as immune based strategies in the treatment of severe ehrlichiosis. The results of these investigations will establish concepts that should also apply to other infectious diseases.

ACKNOWLEDGEMENT

This work was partially supported by NIH grants AI097679 and GM089641 to NI; AI156536 and AI132674 to LS; AI021242 to DHW; and startup funds from the Pathology Department, University of Illinois Hospitals & Health Science System to NI.

CONFLICTS OF INTEREST

Lynn Soong is Editor-in-Chief of *Zoonoses*. He was not involved in the peer-review or handling of the manuscript. The other authors have no other competing interests to disclose.

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