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Protective Immunity and Immunopathology of Ehrlichiosis

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ABSTRACT

Human monocytic ehrlichiosis, a tick transmitted infection, ranges in severity from apparently subclinical to a fatal toxic shock-like fatal disease. Models in immunocompetent mice range from an abortive infection to uniformly lethal depending on the infecting Ehrlichia species, dose of inoculum, and route of inoculation. 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 signaling via TLR 9/MyD88 with upregulation of several inflammasome complexes and secretion of IL-1 beta, IL-1 alpha, and IL-18 by hepatic mononuclear cells, suggesting activation of canonical and noncanonical inflammasome pathways, a deleterious role for IL-18, and the protective role for caspase 1. Autophagy promotes ehrlichial infection, and MyD88 signaling hinders ehrlichial infection by inhibiting autophagy induction and flux. Activation of caspase 11 during infection of hepatocytes by the lethal ehrlichial species after interferon alpha receptor signaling results in the production of inflammasome-dependent IL-1 beta, extracellular secretion of HMGB1, and pyroptosis. The high level of HMGB1 in lethal ehrlichiosis suggests a role in toxic shock. Studies of primary bone marrow-derived macrophages infected by highly avirulent or mildly avirulent ehrlichiae reveal divergent M1 and M2 macrophage polarization that links with generation of pathogenic CD8 T cells, neutrophils, and excessive inflammation or with strong expansion of protective Th1 and NKT cells, resolution of inflammation and clearance of infection, respectively.

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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 ranges from a non-specific flu-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 the disease is problematic due to non-specific symptoms and challenges in the accuracy of the current diagnostic testing. Doxycycline is the drug of choice for the treatment of HME; however, a recent cross-sectional study determined that late administration of doxycycline as a key factor associated with development of severe ehrlichiosis(9-11). Notably, a history of tick exposure was associated with a decreased rate of admission of patients to intensive care units because the effective treatment was given promptly in those cases. Notably, some patients who are treated at late stage of infection developed a severe disease that mimics hemophagocytic lymphohistiocytosis (HLH) syndrome, a pathologic hyperactivation of macrophages that occurs in association with infection (12, 13). This suggests that multi-system disease and tissue damage in HME are due to immunopathology. This conclusion is further supported by murine models of mild/non-fatal and severe/fatal ehrlichiosis. These murine studies suggested that innate and adaptive immune responses against *Ehrlichia* acts as a "double-edged sword" in fatal ehrlichiosis(5, 14, 15), where protective immunity is mediated by $CD4^+$ Th1 and NKT cells, while pathogenic response is attributed to activated neutrophils and TNF-α-producing $CD8^+$ T cells (5, 16).

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Recent studies highlighted the pivotal role of the inflammasome and autophagy as part of innate immune responses against several pathogens, leading to pathogenic or protective outcomes. In this review, we discuss the cell-specific innate immune responses during ehrlichiosis involving the regulation of autophagy and inflammasome and the associated signaling pathways and how these events impact the innate and adaptive immune responses against *Ehrlichia*. Understanding these mechanisms would be critical for rational development of novel diagnostic, therapeutic, and preventive countermeasures against ehrlichiosis.

Methods. Articles on the *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 role of inflammasome 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 last ten years and included those 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 on the rise in North America. HME is among the most prevalent tick-borne rickettsial diseases that 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 (NNDSS), during 2008-2012, people older than 55 had the highest incidence rate among all the age groups, although CFRs were highest among children younger than 5-years old (4%), followed

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by persons older than 70-years old (3%). Among confirmed cases, CFR among persons older than 70-years old increased to 53%, while CFR among children younger than 5 increased to 14% (10, 19, 20). This is partially due to significantly higher prevalence of immunosuppressive conditions among the older age groups. The median age of patients with immunosuppressive conditions was 60 years. The risk for severe outcomes in immunosuppressive cases were associated with higher rate for hospitalization, presence of life-threatening conditions, and death (21-24). On the other hand, ehrlichiosis cases are believed to be under reported because of patients are either asymptomatic or having mild illnesses not prompting medical consultation, and reported cases over-represent infections with more severe clinical manifestations. Therefore, it is reasonable to infer that compared to the younger population, people over 55-years old have a higher probability of having a more severe outcome when contracting ehrlichiosis, due to age-related 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 due to 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 53-72% and a case fatality rate of approximately 1%. Liver is the main site of *Ehrlichia* infection and pathology (26-30). The majority of HME patients have mild-to-moderate increase in serum level of liver transaminases, in some cases marked cholestasis, and progressive hepatosplenomegaly. Histopathologic examination of HME patients' liver biopsy samples reveals diffuse activation of the monocytes and tissue-resident macrophages as well as lymphocyte infiltration in the hepatic sinusoids, and multifocal inflammatory lesions with hepatocellular death that appears to be apoptotic as well as nonspecific hepatocyte swelling and steatosis (29, 31, 32). The

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activation of monocytes and Kupffer cells (liver-resident macrophages) has been observed with and without *E. chaffeensis* infection of the host cells, confirming that hepatic injury is not directly related to the 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 is been recently cultivated and named *E. japonica/*IOE, *E. ewingii*, and *E. muris eauclairensis*(34). The IOE/*Ehrlichia japonica* (which will be the focus of studies described below) have been detected in *Ixodes ovatus* ticks throughout Japan, Ixodes apronophorus ticks in Romania, as well as in *Ixodes ricinus* ticks in France and Serbia (35, 36). Analysis of genome sequence of cultured IOE/*Ehrlichia japonica* indicated that this *Ehrlichia* species has a single double-stranded circular chromosome of 1,148,904 bp, which encodes 866 proteins with a similar metabolic function as *E. chaffeensis*(37). IOE/*E. japonica* encodes homologs of several virulence factors identified in *E. chaffeensis*, such as type IV secretion system apparatus and effector proteins, P28/OMP-1 family of outer membrane proteins, tandem repeat proteins and ankyrin-repeat proteins.

How *E. chaffenesis* or other *Ehrlichia* species cause different 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 circulating strains of *E. chaffeensis;* Wakulla, Arkansas, and Liberty, and the disease that they cause in immune-deficient mice indicate that those strains have distinct genotypes and phenotypes that define their virulence for immune-deficient mice with the order of Wakulla,

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Arkansas, followed by Liberty(28, 39, 40). The livers of mice infected with Wakulla and Arkansas strains had severe diffuse inflammation and granulomatous inflammation when compared to the hepatic pathology in mice infected with 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 that varies from mild/non-fatal to severe/fatal in immunocompetent hosts; 5) ehrlichiosis in infected mice should have similar clinical and pathologic manifestations of mild and severe ehrlichiosis as occurs in humans as well as laboratory findings that recapitulate the findings in HME; 6) outcome of infection should be dependent on dose of infectious inoculum, genetic background and route of transmission, which are key variables that affect the outcome of infections with most bacterial and viral pathogens; 7) a model that enables mechanistic studies for which reagents and knockout animals lacking a specific gene (s) are available. Although there are several animal models that fulfill one or two of the criteria mentioned above, there is currently no one model that fulfills all criteria mentioned above. For example, infection with *E. chaffeensis,* the main pathogen causing HME, trigger mild, self-limited infection in immunocompetent hosts, while causing severe and potentially fatal disease in immunocompromised hosts (41, 42). Utilization of this model in 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) as well as *E. ruminantium* in ruminants(49-51), have revealed a disease that mimics the pathology and defined pathophysiology of human disease. However, analysis of immunity and pathogenesis using

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mechanistic approaches is challenging due to the outbred nature of the hosts and lack of availability of canine or ruminant reagents that examine immune responses. Due to the above 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 analyzing immunity and pathogenesis of HME as described below are intriguing, there exists the limitation of translating results from murine experiments to humans and clinical diseases. 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 arms of innate and adaptive immune responses. Employing immunocompetent C57BL/6 mice, investigators have used several *Ehrlichia* agents to cause a different spectrum of disease. *E. chaffeensis* causes self-limited infections, *E. muris* causes mild and persistent infection, and *E. japonica* causes severe and potentially fatal infections (5, 52-54). Notably, the outcome of infection with *E. japonica/*IOE varies based on the route of infection and the infectious dose. For example, intraperitoneal (i.p.) infection with a high dose of *E. japonica/*IOE is lethal, while intradermal (i.e.) infection with the same dose results in sublethal infection and mild disease(55). Similarly, i.p. or intravenous (i.v.) injection with E. japonica/IOE causes dose-dependent lethality (higher dose) or sublethal persistent (lower dose) infection. Interestingly, mice infected via intraperitoneal route with high dose of *E. muris* survive and develop protective immunity and long-term memory responses that are not only protective against homologous re-infection but also against 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.* Following molting, the infected nymphs were placed on naive animals to transmit the pathogen. Mice

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were infected with *Ehrlichia* when they were infested by 90%-100% of feeding larvae, and many mice fed upon by infected nymphs had sublethal infection, while 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 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 liver.

Employing 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 protective immunity during mild ehrlichial infection is due to generation of both cell-mediated and humoral immunity mediated by IFN-γ producing $CD4^+$ Th1 cells and *Ehrlichia*-specific IgG antibodies, mainly of IgG2a isotype(5, 63-65). In contrast, severe and fatal *Ehrlichia*-induced toxic shock-like syndrome is characterized by development of an initial focal hepatic necrosis and apoptosis, increased serum levels of hepatic enzymes, significant lymphopenia and leucopenia, apoptosis of myeloid cells and $CD4^+$ T cells, followed by multi-organ failure and sepsis. Further analysis indicated that severe and fatal primary ehrlichiosis in animals is due to cytokine and chemokine storm characterized by an early overproduction of pro-inflammatory cytokines (IL-1, TNF-α, IL-18, IL-12p40, etc.), several chemokines (CCL5/RANTES, CCL3/MIP-1α, CCL4/MIP-1β, CCL2/MCP-1, and IL-8) followed by excessive production of anti-inflammatory cytokines (IL-10 and IL-13) during the course of infection(30, 52, 66). Compared to mild murine ehrlichiosis caused by *E. muris* infection, where CD8 T cells play a protective role, our studies have demonstrated that $CD8^+$ T cells play a pathogenic role in murine model of fatal ehrlichiosis(67). Fatal ehrlichial infections induce significant expansion of cytotoxic $CD8^+$ T cells producing TNF-α and IL-10. Deficiency of $CD8^+$ T cells in mice infected

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with virulent *Ehrlichia* species restores the number of Th1 cells, attenuates cytokine and chemokine storm, decreases tissue damage, and protects mice from fatal infection. We also showed that innate cells such as NK cells and neutrophils play deleterious roles in the pathogenesis of ehrlichiosis as they directly contribute to tissue damage as well as the development of cytokine storm and expansion of pathogenic $CD8^+$ T cells (66, 68). Although the role of NK cells and neutrophils during mild ehrlichiosis has not been examined, we have seen a differential spatial and temporal changes in NK and neutrophils during lethal infection compared to mild infection (66). NK cells migrate to the liver in fatal ehrlichiosis during lethal infection, while they remain in spleen or peritoneum, respectively, during mild *Ehrlichia* infection (66). Although mechanistic studies examining the role of NK 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 due to lack of excessive inflammatory environment that trigger overactivation of these cells and their polarization into 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.

Inflammasome: cytosolic receptors that play key roles in intracellular surveillance. Inflammasomes are key component 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 (NBD), and leucine-rich repeat (LRR) containing proteins or the absent in

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melanoma 2 (AIM)-like receptors (ALRs), adaptor protein ASC (apoptosis-associated speck-like protein containing a caspase activation and recruitment domain), and pro-caspases(76-79). To date, four inflammasome complexes have been identified and well characterized including the NOD, LRR containing protein (NLR) family members; NLRP1, NLRP3, NLRC4, as well as the protein Absent in Melanoma 2 (AIM2). NLRP1 is activated by PAMPs, such as muramyl dipeptide. NLRC4 is activated by PAMPs such as flagellin and type II secretion system as well as by DAMPs such as neuronal apoptosis inhibitory protein (NAIP) family members. NLRP3 inflammasome is also activated by several DAMPs including reactive oxygen species (ROS), mitochondrial DAMPs, and adenosine triphosphate (ATP), as well as fibrillar proteins (e.g., β-amyloid fibrils). AIM2 is activated by microbial or host double-stranded DNA (dsDNA). It has been shown that AIM2 also binds to the high mobility group box 1 (HMGB1), which promotes activation during oxidative stress (79).

There are two inflammasome pathways that are triggered upon sensing a microbial or host ligand, the canonical and non-canonical inflammasome pathways. The canonical inflammasome pathway involved signaling by NLRP3 complex, following recognition of PAPMs or DAMPs, via adaptor molecule ASC, which lead to activation of caspase-1, causing cleavage of pro-IL-1β and pro-IL-18 and 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 activates caspase-1 and promotes secretion of IL-18, IL-1β, and HMGB1 as well as inflammatory cell death known as pyroptosis (84-88). Gasdermin D protein is essential for caspase-11-dependent pyroptosis (88-91). Caspase-11 cleaves gasdermin D, and this cleavage promotes both pyroptosis and NLRP3-dependent activation of caspase-1. Although inflammasomes are critical for defense against pathogens and danger signals, excessive activation can promote immunopathology and tissue injury. For example, dysregulation of inflammasomes was associated with multiple neurodegenerative diseases such as Alzheimer disease,

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Parkinson disease, multiple sclerosis, and amyotrophic lateral sclerosis, to name a few(92, 93). The ligand causing activation of inflammasomes in these diseases are not completely understood. However, an accumulation of amyloid-beta plaques in the cerebrum of patients with Alzheimer disease was suggested to be potential DAMPs that trigger NLRP3 inflammasome activation in these patients. Activation of NLRP3 has also been widely studied in liver diseases as well (94-97). NLRP3-mediated secretion of IL-1β following LPS-TLR signals resulted in production of multiple inflammatory cytokines and chemokines and excessive inflammatory response. IL-1β in liver diseases recruits inflammatory cells that in turn activate hepatic stellate cells (HSCs), which are key contributors to liver fibrosis (98-101). IL-1β can also trigger triglyceride accumulation in hepatocyte cells and cause hepatocyte cell death mediated by TNF-α.

Role of canonical and non-canonical inflammasome 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, but the inclusions lack late endosomal or lysosomal markers (102-104). Unlike other intracellular bacterial pathogens that access cytosol such as Rickettsia and Listeria, *Ehrlichia* do not escape from phagosome to cytosol. However, virulent IOE/*E. japonica* trigger deleterious inflammasome activation. Compared to 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, as well as secretion of IL-1β, IL-1α, and IL-18 by liver mononuclear cells including Kupffer cells and infiltrating inflammatory monocytes (63, 84, 105). These data indicate that fatal *Ehrlichia* infection triggers activation of canonical and non-canonical inflammasome pathways. Interestingly, our data demonstrated a deleterious role of IL-18 in the host response to

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Ehrlichia. Mice deficient in IL-18 receptor (IL-18R$^{-/-}$) are more resistant to fatal ehrlichiosis caused by i.p. infection with E. japonica/IOE when compared to wild-type mice. 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 is a result of reduced expansion of pathogenic CD8^{+} T cells, suggesting that inflammasome activation leads to induction and increased number of pathogenic CD8^{+} T cells that cause liver injury (14, 65).

Notably, Casp1$^{-/-}$ mice infected with highly virulent IOE are highly susceptible to fatal ehrlichiosis as they develop an overwhelming infection, extensive tissue injury and succumb to infection at earlier time points after infection compared to wild-type controls(14, 84). These data suggest that the deleterious inflammasome activation in ehrlichiosis is not due to the canonical inflammasome pathway. In fact, these data suggest that caspase 1 may play a protective role in ehrlichiosis, which is consistent with caspase 1 function in other infection model systems. Recent studies suggest that active caspase 1 is hepatoprotective as deficiency of caspase 1 is associated with death of hepatocytes and liver injury in a hemorrhagic shock model (106, 107). Thus, it is possible that greater susceptibility of caspase 1$^{-/-}$ mice to fatal ehrlichiosis could be due to altered survival of hepatocytes. Unlike caspase 1$^{-/-}$, mice deficient of NLRP3 (Nlrp3$^{-/-}$ mice) effectively cleared ehrlichiae on day 7 post infection; however, these mice still exhibited acute mortality and developed liver injury like wild-type mice (84, 85). Notably, susceptibility of wild-type, caspase 1$^{-/-}$ and NLRP3$^{-/-}$ mice and their development of liver damage were associated with increased expression of active caspase 11 in the liver compared to wild type mice. This suggested that activation of non-canonical inflammasome pathway may play a key role as mediator of tissue injury during severe ehrlichiosis.

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Regulation of inflammasome by Type I interferon and MyD88 signaling. Type I interferons (IFN-I) include IFN-α and -β cytokines, which are critical components of innate immunity 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). On the other hand, 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 highly resistant to fatal disease compared with wild-type mice, as they have attenuated liver pathology, lower bacterial burden in liver and spleen, as well as prolonged survival. Resistance of Ifnar1$^{-/-}$ mice to fatal ehrlichiosis was associated with expansion of IFNγ-producing CD4^{+} Th1 cells, which otherwise undergo apoptosis during IOE infection, and lower number of IL-10 producing T cells that have immunosuppressive function (84). The protection conferred to Ifnar1$^{-/-}$ mice against lethal infection correlated with attenuated activation of non-canonical inflammasome pathway as evidenced by decreased activation of caspase-11 and decreased levels of splenic and hepatic IL-1β, compared with wild type mice. Notably, IFN-I mediated activation of caspase-11 leads to cell death via pyroptosis, i.e., a rapid inflammatory cell death that enabled exit of intracellular ehrlichiae to the extracellular space infecting other cells, as well as caused bacterial dissemination to peripheral organs (**Figure 1**).

The molecular and cellular mechanisms by which IFN-I leads to activation of caspase-11 during *Ehrlichia* infection remains elusive. However, it has been shown that autocrine or paracrine signaling by IFN-I during infection with LPS-containing Gram-negative bacteria leads to upregulation of genes encoding guanylate binding proteins (GBPs), which enables release of LPS into cytosol. Cytosolic LPS acts as a PAMP that triggers cleavage of pro-caspase 11 into active caspase 11(94, 112, 113).

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Thus, it is possible that IFNAR signaling during fatal *Ehrlichia* infection induces production of GBPs, which then disrupt vesicles containing ehrlichiae, allowing the escape of PAMPs to the cytosol and activation of caspase-11. Since *Ehrlichia* lacks LPS, the IFN-I-caspase 11 axis is less likely triggered by LPS-like molecules. Our recent studies suggest that mitochondrial DAMPS might be the ligands that trigger activation of caspase 11 during fatal ehrlichiosis upon IFNAR signaling. Infection of macrophages, the main target cells of *Ehrlichia*, with *E. japonica* trigger TLR9/MYD88 signaling. The latter leads to activation of the metabolic checkpoint kinase, mammalian target of rapamycin complex 1 (mTORC1), the negative regulator of autophagy. MyD88-dependent mTORC1 activation leads to inhibition of autophagy induction and flux as well as block of mitophagy (i.e., elimination of damaged mitochondrea via autophagy following binding of mitochondrea to autolysosome) (105). MyD88-mediated block of autophagy and mitophagy then leads to accumulation of damaged mitochondria, which in turn results in release of mitochondrial DNA (mtDNA) or other mitochondrial DAMPs (e.g., ROS). Other studies showed that infection of macrophages with *E. chaffeensis*, the *Ehrlichia* species that cause severe and potentially fatal disease in humans, inhibits mitochondrial metabolism (114-116).

Similar to our studies, studies by Macnamara et al. have shown that IFNα/β promote lethal, shock-like pathology in mice infected with IOE/*E. japonica* (111). However, the mechanism by which type I interferon signaling causes fatal ehrlichiosis was attributed to IFNAR-mediated hemopoietic dysfunction. IFAR signaling triggered severe bone marrow (BM) loss, abrogated myelopoiesis during infection, and reduced the number of hematopoietic stem and progenitor cells (HSC/HSPCs) (117). Deficiency of IFNAR signaling restored the BM and splenic hematopoiesis. Mechanistically, this deleterious effect of type I IFN on HSC/HSPCs was due to caspase 8/RIPK1-mediated inhibition of HSC/HSPCs proliferation and increasing HSPC death. Combination therapy of IOE-infected mice

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with RIPK1 antagonist (Necrostatin-1s) together with antibiotic rescued 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 only restricted to deleterious inflammasome activation (as shown in our study), but also hematopoietic dysfunction mediated by IFNAR-mediated HSPCs cell death as well as HSC quiescence.

Unlike the deleterious role of IFN-I in fatal ehrlichiosis, recent studies in other infection models; *Plasmodium yoelii* (causative agent of malaria) and *Rickettsia parkeri* (causative agent of spotted fever rickettsiosis) have shown that IFN-I response during these infection is protective and negatively regulated by inflammasome(118, 119). In both infection models, inflammasome appears to play a non-protective response by inhibiting IFN-β production. Mechanistically, in the malaria model, negative regulation of IFN-β by inflammasome seems 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. On the other hand, in the Rickettsia model, inhibition of IFN-β by the non-canonical, caspase 11-mediated inflammasome pathway was due to inflammatory cell death (pyroptosis) that antagonizes IFN-I. Although we have not examined the cross regulation of type I IFN by inflammasome in our murine model of fatal ehrlichiosis, but it is possible that temporal and spatial dynamics during infection may contribute to cross regulation between inflammasome and IFN-I signaling. On the other hand, unlike the protective role of IFN-β in macrophages during infection with *Rickettsia* and *Plasmodium* species as indicated above, our studies demonstrated a pathogenic role of IFN-β during fatal ehrlichiosis. This discrepancy could be due to pathogen- or cell-specific differences between these pathogens. Alternatively, while low level of IFN-I cytokines could be protective, a substantially high level of IFN-β could be detrimental as suggested by other

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studies. In support of this argument, we have found that mild ehrlichial infection and protective immunity are associated with low level of IFN-β.

Toll-like receptors (TLRs) are transmembrane proteins located in both plasma and endosomal membranes that survey the extracellular and intracellular environment and thus function as pattern recognition receptors (PRRs). TLRs play a key role in the innate immune responses against intracellular pathogens(120). Surface TLRs such as TLR2 and TLR4 recognize several bacterial ligands. For example, TLR2 recognize peptidoglycan, lipoteicoic acid, lipopeptides, and lipoprotein, while TLR4 recognize LPS of Gram-negative bacteria (121, 122). Endosomal TLRs include TLR9 that recognizes double-stranded DNA and CpG-containing single-stranded DNA as well as TLR7 that detects single-stranded RNA. Ligation of all TLRs with their respective ligand(s) trigger signals via an adaptor complex consisting of MyD88(123). In addition, ligation of 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 recently found that virulent *Ehrlichia* trigger activation or signaling via TLR9/MyD88, which contributes to activation of both canonical and non-canonical inflammasome pathways and tissue injury during fatal ehrlichiosis (105). MyD88$^{-/-}$ mice infected with *E. japonica*/IOE have attenuated inflammasome activation, minimal liver injury, and were more resistant to lethal infection compared to wild type mice. Notably, these mice had ineffective bacterial clearance and protective immunity as marked by increased bacterial burden in the liver. These data are consistent with earlier findings indicating that *Ehrlichia*-induced liver damage and toxic shock is not due to overwhelming infection but rather due to immunopathology. Although MyD88 deficient mice had decreased

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inflammasome activation as evidenced by decreased serum levels of IL-1β and IL-1α, such decrease was partial, suggesting a potential role of MyD88-independent pathways such as TRIF during fatal *E. japonica/*IOE infection. In support of potential role of TRIF, the lack of TLR9 in macrophages, which signal via both MyD88 and TRIF, completely abrogated secretion of IL-1β and IL-1α, and activation of caspase 1/11, suggesting that TLR9-MyD88-TRIF axis is critical for activation of both canonical and non-canonical inflammasome pathways. In vivo studies using TLR9 deficient mice also highlighted a key role of TLR9, as a major endosomal PRR, in the development of liver damage, and fatal toxic shock following lethal *Ehrlichia* infection (105).

Potential PAMPs that trigger inflammasome activation in *Ehrlichia*-infected cells. *Ehrlichia* membranes are different than other Gram-negative bacteria as they lack LPS, including lipid A, peptidoglycan, and cholesterol, major PAMPS that trigger inflammasome activation during infection with these pathogens (125-127). Although *Ehrlichia* lacks genes for biosynthesis of cholesterol in their cell wall, it was found that *Ehrlichia* hijack host membrane phospholipids from the host cell and are dependent on host-derived cholesterol for survival and infection (128-130). Genomic analysis revealed that the *E. chaffeensis* genome do not express phosphatidylcholine or cholesterol but encodes enzymes for phosphatidylethanolamine (PE) biosynthesis. Indeed, recent study demonstrated that host membrane phospholipids and cholesterol traffic in unidirectional manner to ehrlichiae inclusion in the infected cells (130, 131). This translocation of host-cell membranes and molecules to *Ehrlichia* inclusions was found to be dependent on both autophagy as well as host endocytosis and 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 Type IV secretion system include genes coding

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for 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 early proteins involved in 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 membrane containing cholesterol and type IV secreted proteins such as ETF-1 triggers inflammasome activation in infected cells remains elusive. However, the fact that these PAMPS are known inflammasome ligands suggests that *Ehrlichia* may exploit cholesterol and type IV secretion system effector to induce inflammasome activation and cell death, which enables them to disseminate to other cells and organs.

The *E. chaffeensis* genome contains several other genes involved in host-pathogen interactions. Of particular interest the genes that encode tandem repeat proteins (TRPs) and ankyrin repeat containing proteins (133, 134) as these proteins could be secreted into cytosol and access inflammasome complexes. *E. chaffeensis* TRPs are secreted via type 1 secretion system, which is commonly utilized by Gram-negative bacteria and is employed to secrete various exotoxins, adhesins and enzymes. TRPs interact with various proteins, DNA. RNA, and small molecules to 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 as they induce strong host antibody responses and recognized by polyclonal antibodies exist in the sera from patients with HME. Examples of TRPs are TRP32, TRP47, TRP75 and TRP120(138-142). Many *E. chaffeensis* TRPs and Ankyrin (Anks) repeats such as TRP32, TRP47, TRP120, and Ank200 are considered nucleomodulins due to their translocation and localization in the nucleus and ability to

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alter or modify gene expression using various mechanisms, including interaction with host proteins, upregulation of genes associated with host cell survival or death as well as direct binding to the protein–DNA complexes(143, 144). Although the role of these TRPs and Anks in activation of inflammasome and autophagy regulation during fatal ehrlichiosis is not yet examined, their function and cellular location suggest that they may act as potential PAMPs for inflammasomes.

Role of autophagy in regulation of inflammasome and host responses to *Ehrlichia*. Autophagy is a host homeostatic mechanism that is pivotal for innate host defense against several intracellular pathogens (14, 145-148). Recent studies have shown that autophagy induction enhances survival and/or replication of *Ehrlichia*. Pharmacologic blocking autophagy induction using 3-MA treatment or knockdown of autophagy genes such as *atg5* or *beclin-1* also impair bacterial survival and replication (102, 103, 149, 150). Interestingly, as a host defense mechanism, MyD88 signaling following infection of mice with *E. japonica*/IOE impaired bacterial replication by inhibiting autophagy induction (105). Inhibition of autophagy induction by MyD88 signaling occur via activation of mTORC1. Notably, although MyD88 deficiency enhanced autophagy flux (i.e., autophagsome-lysosomal fusion), this process did not result in effective elimination of virulent *E. japonica/*IOE as the bacteria did not colocalize with the LC3II/autophagosomes and lysosomes.

Several human and murine studies reported a negative regulation of inflammasome by autophagy (151-154). Inhibition of autophagy in macrophages leads to accumulation of DAMPs such as damaged mitochondria, host DNA, reactive oxygen species (ROS), mitochondrial DNA, and oxidized mitochondrial cardiolipin (154-156). Generation of mt ROS or mtDNA and their release into cytosol causes activation of cytosolic NLRP3 inflammasome pathway. Mechanistically, we found that MyD88-mediated mTORC1 activation triggers inflammasome activation in macrophages

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following IOE/*E. japonica* infection via inhibition of autophagy and mitophagy(105), resulting in 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 resulted in enhanced autophagy and attenuated inflammasome activation (105).

Role 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 hepatocytes (HCs) and endothelial cells in mice and humans. Whether *Ehrlichia* infect other liver parenchymal cells such as hepatic stellate cells (HSCs) or bile duct cells remains elusive and is an area of future research. However, we and other investigators recently examined the effect of deleterious type I IFNs during fatal ehrlichial infection on hematopoietic and nonhematopoietic cells using bone marrow chimeric mice. These studies demonstrated that IFN-α receptor signaling in nonhematopoietic cells is important for pathogenesis of *Ehrlichia*-induced sepsis. We 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 induction of autophagy and activation of non-canonical inflammasome pathway, respectively(85, 157). The activation of caspase-11 (non-canonical inflammasome pathway) in *E. japonica*/IOE infected HCs following paracrine IFNAR signaling resulted in three key events; 1) production of 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 a high serum level of

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HMGB1, 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 following cell death during many infectious and inflammatory diseases. The mechanism by which HMGB1 contributes to *Ehrlichia*-induced liver injury and sepsis remains elusive. However, as suggested by other studies, extracellular hepatic HMGB1 could trigger caspase 1 activation and cell death via binding to the receptor for advanced glycation end products (RAGE) on adjacent uninfected macrophages or HCs, (158-161). On the other hand, intracellular HMGB1 was found to induce autophagy by 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 by fusion of *Ehrlichia*-containing inclusions with autophagosome and endosome pathways. Since ehrlichial replication is dependent on autophagy induction involving beclin-1, it is possible that intracellular HMGB1-induced autophagy enhances bacterial survival and replication in their target cells.

Unlike hepatocytes, macrophages are innate immune cells that play a key role in regulation of innate and adaptive immunity against several pathogens (164, 165). Macrophages are not only the initial immune cells that respond to infections with variable pathogens, but they also function as antigen-presenting cells priming the adaptive immune response, driving inflammation, and host defense against infections as well as mediating tissue repair following resolution of infection and inflammation. Two major lineages are currently known: cells that are derived from myeloid progenitor cells in the bone marrow and give rise to blood-circulating monocytes and tissue-resident macrophages such as alveolar macrophages in the lung and Kupffer cells in the liver (157,

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166, 167). Upon stimulation, macrophages differentiate or polarize into either the 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, and they also play a role in antitumor immunity via several mechanisms (168, 169). Markers of M1 cells include high expression of MHC class II and costimulatory molecules such as CD86 and CD40, but low expression or downregulation of mannose receptor (CD206). At function level, the M1 cells can phagocytose microbes, secrete pro-inflammatory and Th1-promoting cytokines (e.g., IL-12, IL-1β, IL-6, TNF-α) and chemokines, and produce multiple microbicidal molecules such as nitric oxide (NO) and reactive oxygen species (ROS). On the other hand, M2 are marked by upregulation of CD206, arginase-1, IL-10, and TGF-β. M2 cells exhibit anti-inflammatory or immunosuppressive phenotype and thus play a role 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, and thus are commonly associated with suppressive tumor microenvironment and tumor growth. M1- Mφ polarization is enhanced by both IFN-γ and LPS, while 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 (that causes fatal ehrlichiosis in mice), or mildly virulent *E. muris* (that causes mild and self-limited ehrlichiosis in mice) induce polarization of macrophages into M1 and M2 phenotypes, respectively (157, 170). IOE-induced polarization of macrophages into M1 phenotype is rather interesting since *Ehrlichia* species do not express LPS, which is an important M1 stimulus. Similarly, the polarization of M1 and M2 macrophages following *E. japonica/*IOE and *E. muris* infection, respectively, was not associated with significant production of M1- or M2-promoting cytokines such as IFN-γ, IL-4, and IL-10. Using murine

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models of mild and fatal ehrlichiosis, we further showed that *Ehrlichia*-induced liver damage and sepsis are associated with accumulation of infiltrating pro-inflammatory M1 macrophages/monocytes in the liver. This M1 expansion in the liver correlated with generation of pathogenic $CD8^+$ T cells and neutrophils excessive inflammation, liver injury and high bacterial burden. In contrast, expansion of M2 in the liver of *E. muris*-infected mice was associated with strong expansion of protective Th1 cells and NKT cells, resolution of inflammation, and clearance of infection. Mechanistically, we found that polarization of M2 macrophages in *E. muris*-infected mice is due to enhanced autophagy induction, while blocking autophagy induction in mice infected with IOE/*E. japonica* induced polarization of M1 macrophages (157, 170). Blocking mTORC1 signaling in *E. japonica*/IOE-infected macrophages enhanced autophagy and decreased polarization of M1 macrophages, 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 Mφ polarization in ehrlichiosis suggests metabolic regulation of macrophage polarization. Studies have shown that activation of M1 macrophages correlated with aerobic glycolysis, and induction of a pentose phosphate pathway that provides NADPH to produce ROS, while M2 macrophages utilize fatty acid oxidation (171-175). However, whether M1/M2 polarization occurs in ehrlichiosis following infection with different *Ehrlichia* strains in humans remains elusive and is a topic of future investigation.

Conclusion and Future Perspectives. While much progress has been made in the recent years for our understanding of immunity and immunopathogenesis of *Ehrlichia* spp. infection, many important areas of research are in great need for a better understanding of intracellular bacterial infections and immunology. The critical gaps in knowledge include: 1) defining the role of inflammasome

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activation and autophagy in ehrlichiosis 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, not only immune cells but also parenchymal cells such as endothelial cells, hepatocytes, and hepatic stellate cells. The latter has been shown to act as antigen-presenting cells expressing PRR and thus can 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 the portal of entry in skin and how this leads to bacterial dissemination using the intradermal and tick-transmission models as well as *in vitro* models such as liver and skin Organoid and Organ-on-a-chip; 6) analyzing the potential of multiple inhibitors of specific inflammasome gene(s) or type I IFN signaling used in other diseases (Table 1) as an immune based strategies in treatment of severe ehrlichiosis. The results of these investigations will establish concepts that will also apply to other infectious diseases.

Conflict of Interest: The authors have declared that no competing interests exist.

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Targets	Function in *Ehrlichia* Pathogenesis	Inhibitors used
HMGB1	Fatal ehrlichiosis in mice is associated with high level of HMGB1. HMGB1 has emerging role in liver disease (157). There are studies available that showed HMGB1 contributes to *Ehrlichia*-induced liver injury (153-158).	HMGB1-specific polyclonal and monoclonal antibodies, glycyrrhizin (GR) can be used as inhibitor of HMGB1 (176).
IL-1β	IL-1β has proinflammatory effect in ehrlichiosis (28, 50, 64). It has been shown to contribute to liver fibrosis (95-98).	Anakinra, Rilonacept, Canakinumab are the target drug for blocking IL-1β develop by different Pharma companies(177). These agents are known to target inflammation in broad spectrum of disease.
Caspase 11	*Ehrlichia* infection shown to upregulate canonical and non-canonical inflammasome pathway (61, 81,102).	Scutellarian acts as an inhibitor for caspase 11 in macrophage(178).
		Wedelolactone (179) acts inhibitor for caspase-11. It prevents IL-1β maturation and apoptosis.

TABLE 1: Proposed utilization of inhibitors of specific detrimental pathways in fatal ehrlichiosis as potential immunotherapy.

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Type I interferon	It is shown to mediate Inflammasome activation and HMGB-1 Translocation (82, 109). It also has important role in other infectious disease (108).	Anifrolimab, human monoclonal antibody to type I interferon receptor subunit 1 suppressed interferon gene expression. It is used in treatment of System lupus erythematosus (SLE)(180)

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Figure 1: Model of inflammasome activation via canonical and non-canonical fashion. Firstly, *Ehrlichia* invades the target cell and induces TLR9/MyD88 signaling to upregulate NLRP3 complexes, pro-IL1β and pro-IL-18 via NF-κB. Then, activation of NRRP3 inflammasome following recognition of Ehrlichial PAMPS and/or mitochondrial DAMPS generated upon block 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 trigger cleavage of caspase-11 and activation of non-canonical inflammasome pathway leading to secretion of mature IL-1β and IL-18, cleavage of gasdermin D, and pyroptosis.

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