Elucidating Human Inflammasome Responses To Legionella Pneumophila And Lipopolysaccharide Variants

Antonia Ronalda Bass
University of Pennsylvania

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Abstract
Host recognition of intracellular bacterial pathogens results in the formation of a multiprotein complex termed the inflammasome, which leads to the recruitment and activation of inflammatory caspases. These caspases promote IL-1 family cytokine release and pyroptosis, a lytic form of cell death, which are critical for anti-bacterial defense. In mice, interferon-gamma (IFN-\(\gamma\)) is a potent inducer of the both the noncanonical and canonical inflammasomes. Specifically, a family of IFN-inducible GTPases known as guanylate binding proteins (GBPs) promote inflammasome responses to a variety of bacteria in mice. The functions of the mouse GBPs include stimulating the rupture of pathogen-containing vacuoles and bacteriolysis of cytosolic bacteria in order to release pathogen-derived products, such lipopolysaccharide (LPS), into the cytosol for downstream host sensing and inflammasome activation. In contrast to mice, which possess 11 GBPs, humans have 7 GBPs and their role in inflammasome activation in human macrophages is poorly understood. Here, we use Legionella pneumophila, a vacuolar intracellular gram-negative bacterium, to elucidate the functions of human GBPs on inflammasome responses in macrophages. We determined that human GBP1 is essential for maximal inflammasome responses to L. pneumophila as well as is important for disrupting the L. pneumophila-containing vacuole (LCV) in order to make this vacuolar bacterium more accessible for cytosolic sensing. In addition, LPS can possess different acylation states within the same or different species of gram-negative bacteria. The noncanonical inflammasome mediates inflammatory responses to intracellular LPS and is comprised of caspase-11 in mice, and the two putative orthologs in humans, caspase-4 and caspase-5. While tetracylated LPS variants evade caspase-11 detection, caspase-4 was found to be activated by a tetracylated LPS variant. However, it is still unclear whether caspase-4 and caspase-5 recognize different LPS variants and whether their activation is promoted by IFN-\(\gamma\). Here, we use primary or THP-1-derived macrophages and CRISPR/Cas9 technology to test human noncanonical inflammasome responses to LPS variants from different bacteria and whether IFN-\(\gamma\) and human GBPs promote these responses in macrophages. Our findings elucidate aspects of human innate immune response to gram-negative bacterial pathogens and may provide insight into developing therapeutics to prevent gram-negative sepsis.

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ELUCIDATING HUMAN INFLAMMASOME RESPONSES TO
LEGIONELLA PNEUMOPHILA AND LIPOPOLYSACCHARIDE VARIANTS

Antonia R. Bass

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Antonia R. Bass

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I dedicate my dissertation work to my beloved family for their continuous love, support, and encouragement throughout my life. To my mother, Angelika, who always reminds me that I can accomplish my dreams as long as I believe in myself and have faith. To my father, Ronald, who taught me the importance of hard work and perseverance. To my sister, Jessica, who is always there to listen and provide continuous support. I am so thankful to you all for helping me get through this process.
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ELUCIDATING HUMAN INFLAMMASOME RESPONSES TO
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CHAPTER 1: INTRODUCTION

Microbial organisms are all around us; some microbes can benefit the host by providing either a commensalism or mutualism relationship, while other microbes, known as pathogens, can be detrimental to the host and cause disease. Mammalian organisms have several lines of defense in order to combat invading pathogens. The initial protection mechanisms are the skin and mucous membranes, which provide physical and chemical barriers by preventing entry of pathogens into the underlying tissues as well as trapping unwanted organisms and destruction of them by antimicrobial enzymes. However, when these primary defenses are damaged the pathogen can now take advantage and enter the body, which is when the next host defense mechanism takes over—nonspecific innate immunity. The innate immune response is the second line of defense against microbial pathogens and is essential for the clearance of these pathogens and host survival. An array of host factors are involved in initial responses to invading bacteria, viruses, parasites and fungi and lead to downstream events including the production of antimicrobial peptides, proinflammatory cytokines, interferon, and/or initiation of cell death pathways. Interplay between these pathways may promote a greater response to combat infection. Additionally, some bacteria are able to evade innate immune pathways by using effector molecules or by altering their morphology to be unrecognizable by host sensors. Here, we highlight the importance of innate immune responses to gram-negative bacterial pathogens and how interferon (IFN) can promote these responses.
1.1. Pattern recognitions receptors and their ligands

In order to detect pathogenic microbes, the host relies on its germline-encoded sensors termed pattern recognition receptors (PRRs) (Janeway, 1989). PRRs function in the surveillance of conserved microbial structures known as pathogen-associated molecular patterns (PAMPs) or endogenous danger signals released from dying cells called damage-associated molecular patterns (DAMPs) (Krakauer, 2019). Recognition of these PAMPs and DAMPs by PRRs leads to downstream signaling events including the release of proinflammatory cytokines and cell death. Upon secretion of cytokines, many different immune responses occur including the recruitment of additional immune cells to the site of infection for host defense and clearance of pathogens as well as the production of host proteins such as interferons and antimicrobial peptides. Furthermore, activation of PRRs can lead to cell death, ultimately eliminating the replicative niche for the pathogen.

There are currently five main classes of PRRs: Toll-like receptors (TLRs), C-type lectin receptors (CLRs), nucleotide-binding oligomerization domain-like receptors (NLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), and absent in melanoma 2 (AIM2)-like receptors (ALRs) (Jang et al., 2015). Within each family of PRRs, there are subfamilies of the receptors that each recognize a specific ligand or signal. This allows the host to initiate an immune response to multiple pathogenic components or danger signals for maximal defense against invading pathogens.
The first family of PRRs that was discovered is the membrane bound TLR family. The Toll pathway was initially identified in a genetic screen for genes that are important for early embryonic development of *Drosophila melanogaster* (Hashimoto, Hudson, & Anderson, 1988). However, it was eventually found that Toll was also essential for immune response in *Drosophila* and that this pathway is conserved in mammals (Wasserman, 1993). There are 10 TLRs in humans (TLR1-10) and 12 TLRs in mice (TLR1-9, TLR11-13), and each TLR recognizes a different stimulus (Broz & Monack, 2013). TLR1-9 are present in both mice and humans, whereas TLR11-13 are only expressed in mice and TLR10 is only expressed in humans since it is a pseudogene in mice. The structure of TLRs consists of an N-terminal leucine-rich repeat (LRR) domain important for ligand sensing, a hydrophobic transmembrane domain, and a cytoplasmic C-terminal Toll IL-1 receptor (TIR) domain essential for signaling (Botos, Segal, & Davies, 2011). TLRs have been shown to localize to two different sites within a cell such as a macrophage. TLR1, TLR2, TLR4, TLR5, and TLR6 mainly localize to the plasma membrane, whereas TLR3, TLR7, TLR8, TLR9, TLR11, TLR12, and TLR13 localize to endosomal compartments (Broz & Monack, 2013). Regardless of their location, TLR activation leads to recruitment of adaptor molecules to the TIR domain for signaling; these adaptor proteins include myeloid differentiation primary response 88 (MyD88) and TIR-domain-containing adaptor protein inducing IFN-β (TRIF) which predominantly signal through NFκB to upregulate proinflammatory cytokine production and through IRF3 to induce IFN production (O’Neill, Bryant, & Doyle, 2009).
As previously mentioned, each TLR recognizes one or more specific microbial ligands. For the TLRs that come into contact with bacterial components in the extracellular milieu, TLR1 recognizes bacterial triacyl lipopeptides, TLR2 recognizes lipoproteins, peptidoglycan, and lipoteichoic acid (LTA), TLR4 recognizes the gram-negative bacterial outer membrane component lipopolysaccharide (LPS), TLR5 recognizes flagellin, and TLR6 recognizes LTA and diacyl lipopeptides (Broz & Monack, 2013). In general for TLRs that recognize their corresponding ligands in endosomal compartments, TLR3, TLR7, TLR8, and TLR9 recognize nucleic acids. Until recently, the location and ligand for human TLR10 was unknown; it was reported that TLR10 localizes to endosomes and its ligand is dsRNA, like TLR3, and that TLR10 negatively regulates TLR3 by competing for dsRNA binding and upregulating the TLR3 signaling inhibitor SARM1 (S. M. Lee et al., 2018). For the remaining mice endosome-localized TLRs, TLR11 recognizes flagellin and profilin, an actin-binding protein found in the parasite Toxoplasma gondii as well as Escherichia coli, TLR12 recognizes profilin, and TLR13 recognizes bacterial ribosomal RNA (Broz & Monack, 2013).

In order for downstream TLR signaling to occur, TLR monomers must first bind to their cognate ligand. This binding results in conformational change and formation of a dimer between two ligand-binding domains of TLRs bringing the transmembrane and cytoplasmic TIR domains of the two TLRs into close proximity, which leads to initiation of the signaling cascade (Botos et al., 2011). TLRs may either homodimerize or heterodimerize, depending on the type of TLR. For instance, TLR1 and TLR2 heterodimerize to recognize triacylated lipopeptides, as
well as TLR2 and TLR6 heterodimerize to recognize diacylated lipopeptides (Oliveira-Nascimento, Massari, & Wetzler, 2012). Once dimerization occurs between two TLR monomers, conformational change allows the TIR domains to recruit TIR-domain-containing adaptors. The five TLR adaptors are MyD88, TRIF, MyD88-adaptor-like (MAL), TRIF-related adaptor molecule (TRAM), and sterile α- and armadillo-motif-containing protein (SARM) (O’Neill et al., 2009). MAL and TRAM are ‘linking adaptors’ where MAL recruits MyD88 and TRAM recruits TRIF to the TLR TIR domains; however, these additional adaptors are not always required for signaling. Specifically, only TLR1, TLR2, TLR4, and TLR6 recruit MAL which further recruits MyD88 for downstream NFκB signaling to produce proinflammatory cytokines (O’Neill et al., 2009). TLR4 also recruits TRAM which recruits TRIF for downstream IRF3 signaling for production of IFN-β or other IFN-inducible genes; nevertheless, it was also found that TLR4-TRIF-dependent signaling also activates NFκB for proinflammatory cytokine production. TLR3 does not signal through MyD88, but it recruits TRIF for IRF3 and NFκB signaling. The remaining TLRs (TLR5, TLR7, TLR8, TLR9, TLR11, TLR12, TLR13) recruit MyD88 for NFκB activation and, in the case of the endosome-localized TLRs, for IRF7 activation to upregulate IFN-α and IFN-inducible genes. More studies need to address the role of TLR10, but it is thought that its function is to negatively regulate MyD88 and TRIF signaling (Jiang, Li, Hess, Guan, & Tapping, 2016). Distinctive from the other four TIR-domain-containing adaptors, SARM is the negative regulator of MyD88- and TRIF-dependent signaling (Carlsson, Ding, & Byrne, 2016; Carty et al., 2006).
CLRs are another family of membrane-bound PRRs that have been implicated in immune responses to microbial pathogens. CLRs are predominantly expressed in myeloid cells and they possess at least one C-type lectin-like domain (CTLD), which is important for binding carbohydrates (mannose, fucose, and glucan) located on bacteria, viruses, parasites, and fungi (Brown, Willment, & Whitehead, 2018). Importantly, CLRs are essential for both innate and adaptive immunity by production of inflammatory mediators and phagocytosis followed by antigen presentation to T lymphocytes, respectively (Chiffoleau, 2018). Examples of CLRs involved in intracellular signaling pathways are Dectin-1, Dectin-2, and macrophage-inducible C-type lectin (Mincle). Dectin-1 directly signals using its immunoreceptor tyrosine-based activation motif (ITAM) found on the cytoplasmic end of the receptor, whereas Dectin-2 and Mincle indirectly signal by associating with an ITAM-containing adaptor (Hoving, Wilson, & Brown, 2014). ITAM further recruits Syk tyrosine kinase for downstream NFκB and mitogen-activated protein kinase (MAPK) signaling. These signaling events lead to cellular responses such as the production of proinflammatory cytokines or activation of the adaptive immune system via cross-presentation.

Some microbial pathogens are uptaken by a host cell through phagocytosis or receptor-mediated endocytosis, resulting in a vacuole-enclosed pathogen. The goal of the host cell is to destroy these phagosomes or endocytosed pathogens by lysosomal degradation. However, pathogens such as bacteria have quickly learned to evade these destructive pathways by using effector molecules either to escape the phagosome and survive in the cytosol or to live within the phagosome
by remodeling their vacuole. In addition, viruses have evolved to hijack the host cell’s endocytic machinery to invade the cell and initiate its replication. In response, host cells developed additional immune defenses within the cytosol that recognize PAMPs and DAMPs, similar to extracellular TLRs and CLRs. These cytosolic PRRs are the NLRs, RLRs, and ALRs and are essential for controlling intracellular infections and promoting host survival.

NLRs are a family of intracellular PRRs that initiate immune responses to pathogenic microbes and cellular stress signals. All NLR proteins have 3 domains: an N-terminal effector domain, a central NACHT nucleotide-binding and oligomerization domain, and a C-terminal LRR domain (Y. K. Kim, Shin, & Nahm, 2016). There are four subfamilies of NLRs based on the structure of the N-terminus, which are the NLRA, NLRB, NLRC, and NLRP subfamilies. The NLRA subfamily has only one member known as MHC-II transactivator (CIITA), which possesses an N-terminal acidic transactivation domain and its function is to regulate the expression of MHC genes. The NLRB subfamily has one member in humans called the NLR Family Apoptosis Inhibitor Protein (NAIP), whereas mice have seven NAIPs (NAIP1-7) and they all contain three N-terminal baculoviral inhibition of apoptosis repeat (BIR)-like domains. The NLRC subfamily has six members, three of which have at least one caspase activation and recruitment domain (CARD) at the amino-terminus (NOD1, NOD2, NLRC4), whereas the remaining three members have an N-terminal domain that is still unknown but are placed in this subfamily due to their homology with the CARD-containing NLRC proteins (NLRC3, NLRC5, NLRX1). The largest NLR subfamily is the NLRP
subfamily containing 14 members which all have an N-terminal pyrin domain (PYD) (NLRP1-14). In general, NLRs are activated upon the binding of cytosolic ligands by the LRR domain, which leads to a conformational change and release of the NLR from its autoinhibitory state. Subsequently, the NACHT domain undergoes oligomerization, which forms an N-terminal scaffold for adaptor proteins followed by effectors to bind. Activation of these NLRs lead to immune responses including inflammasome formation, NFκB and MAPK signaling, and autophagy (Y. K. Kim et al., 2016).

Different NLRs are activated in response to various stimuli such as bacterial flagellin, lethal toxins, and even cell stress responses (i.e. reactive oxygen species (ROS)). These specific ligands lead to the activation of NLRs involved in the formation of inflammasomes, which are cytosolic multimeric protein complexes that promote cell death and proinflammatory cytokine release. Some of the NLRs involved in inflammasome formation include NAIP, NLRC4, NLRP1, and NLRP3 and will be discussed in detail in the ‘canonical inflammasome responses to bacterial components’ section.

RLRs are another family of intracellular PRRs that predominantly recognize viral RNA. There are three members within the RLR family: RIG-I, MDA5 (melanoma differentiation-associated 5), and LGB2 (laboratory of genetics and physiology 2) (Rehwinkel & Gack, 2020). All three members of the RLR family are comprised of a central helical domain followed by a carboxy-terminal domain, both of which are essential for RNA detection (Rehwinkel & Gack, 2020). RIG-I and MDA5 also contain two CARDs at their amino-terminus for signaling transduction,
while LGB2 does not possess these domains and is thought to regulate RIG-I and MDA5. Upon the sensing of viral RNA by RIG-I and MDA5, conformational change results in exposure of their CARDs for interaction with the adaptor mitochondrial antiviral-signaling protein (MAVS) and leads to NFκB activation for type I IFN production. Although RLRs detect nucleic acids derived from viruses, another identified PRR family recognizes nucleic acids from both viruses and bacteria and is known as the ALR family.

The ALR family is the third known family of intracellular PRRs and are important for detecting cytosolic-exposed bacterial and viral DNA. The two members of the ALR family are AIM2 and interferon-inducible protein 16 (IFI16). Their structure is comprised of an N-terminal PYD domain followed by a C-terminal DNA sensing domain known as the 200-amino-acid domain (HIN) (Liao et al., 2011). AIM2 consists of only one HIN-200 domain, while IFI16 contains two domains: HIN-A and HIN-B (Liao et al., 2011). AIM2 interaction with bacterial or viral DNA results in recruitment of the adaptor ASC via PYD-PYD interactions followed by caspase-1 recruitment and activation (Hornung et al., 2009; Rathinam et al., 2010). Caspase-1 activation results in inflammasome responses involving the maturation and release of IL-1 family cytokines and cell death. In contrast, IFI16 recruits the adaptor stimulator of interferon genes (STING) for IFN-β production to combat viral infections (Unterholzner et al., 2010). In addition, studies in murine macrophages showed that IFI204, the murine ortholog of IFI16, mediates IFN-β production in response to DNA from intracellular bacterial infections (Storek, Gertsvolf, Ohlson, & Monack, 2015; Unterholzner et al., 2010). Interestingly, IFI16
is not only important for IFN-β induction, but has also been found to interact with ASC and caspase-1 to initiate inflammasome responses to viral DNA infections as well (Kerur et al., 2011; Monroe et al., 2014). Therefore, AIM2 and IFI16 are considered to be the two inflammasome sensor proteins of the ALR family.

1.2. Canonical inflammasome responses to bacterial components

Upon recognition of intracellular bacterial pathogens, the host initiates the formation of a multimeric protein complex termed the inflammasome. In order for inflammasome activation to occur, a cytosolic sensor protein detects pathogen components or cellular stress signals and recruits an adaptor protein followed by the inactive cysteine protease, caspase-1 (CASP1). CASP1 undergoes oligomerization and autoproteolysis into its mature form which goes on to cleave IL-1β and IL-18 for their release from the cell. CASP1 also cleaves the cytosolic protein gasdermin-D (GSDMD), which is known as the initiator protein for inducing pyroptosis, a lytic form of cell death. GSDMD is made of an N-terminal and C-terminal fragment and upon its cleavage by CASP1, the GSDMD N-terminal fragment translocates to the plasma membrane and oligomerizes to form a pore, allowing release of cellular components and rupture of the cell through osmotic influx. Thus, host cell death and proinflammatory cytokine release are key indicators of inflammasome response to microbial infections.

The CASP1-containing inflammasomes are understood to be known as the canonical inflammasomes. There are six identified canonical inflammasomes: the NLRP1 inflammasome, the NLRP3 inflammasome, the NLRC4 inflammasome, the
AIM2 inflammasome, the IFI16 inflammasome, and the Pyrin inflammasome. Although inflammasome responses can be activated in response to bacterial, viral, parasitic, and fungal pathogens, here only inflammasome responses to bacterial components will be discussed.

The NLRP1 inflammasome is activated by different ligands in humans and mice, although both engage in similar mechanism for downstream activation of the cysteine protease caspase-1. The murine ortholog to human NLRP1 is NLRP1b and it initiates an immune response upon sensing the enzymatic activity of lethal toxin from *Bacillus anthracis* and also recently determined upon the direct N-terminal ubiquitination by E3 ubiquitin ligase IpaH7.8 encoded by *Shigella flexneri* (Sandstrom et al., 2019). The ligand of human NLRP1 is muramyl dipeptide (MDP), a bacterial cell wall component (Faustin et al., 2007). Both NLRP1 and NLRP1b have a C-terminal function-to-find domain (FIIND) followed by CARD. The mechanism of the NLRP1 inflammasome activation involves initial autoproteolysis of the FIIND which results in two fragments that are noncovalently linked and is essential for NLRP1 inflammasome activity (D'Osualdo et al., 2011; Finger et al., 2012; Frew, Joag, & Mogridge, 2012). NLRP1 is subsequently activated by its respective ligands which result in cleavage and ubiquitination of the N-terminal NLRP1 fragment followed by its proteasomal degradation and release of the C-terminal fragment, which contains the FIIND C-terminal portion and the CARD. This leads to the self-assembly of the C-terminal fragment, which recruits caspase-1 to form the inflammasome complex. Some studies showed that the adaptor molecule apoptosis-associated speck-like protein containing a CARD (ASC) is
necessary for NLRP1 inflammasome activation by forming ASC dimers via their PYD-PYD interactions and their free CARD domains mediate CARD-CARD interactions between the NLRP1 CARD and caspase-1 CARD (Finger et al., 2012), while others say that ASC is not required but enhances the NLRP1 inflammasome response (Faustin et al., 2007).

The NLRP3 inflammasome is one of the most studied inflammasomes. In order for this inflammasome activation to occur, two signaling events must happen: a priming signal and activation signal (Kelley, Jeltema, Duan, & He, 2019). A priming signal can include TLR ligands or endogenous cytokines including tumor necrosis factor alpha (TNFα) and interleukin 1 beta (IL-1β). The purpose of priming is to signal through NFκB to upregulate the expression of the NLRP3 protein and pro-IL-1β. Additionally, priming induces post-translational modifications such as phosphorylation and deubiquitylation important for regulating NLRP3 inflammasome activation (Yang, Liu, & Xiao, 2017). The NLRP3 activation signal involves detection of cellular stress that is induced by diverse stimuli, rather than direct binding of ligands as other inflammasomes do. These diverse stimuli include bacterial and fungal toxins, viral DNA and RNA, ATP, nigericin, and alum (Kelley et al., 2019). Upon these stimuli entering the host cell, they can cause different cell stress responses including ion imbalances like potassium (K+) efflux, production of ROS, and lysosomal disruption. All of these cellular events result in activation of the NLRP3 inflammasome, which is then able to oligomerize through its NACHT domain and recruit the adaptor ASC to its N-terminal PYD. Recruitment of ASC leads to the formation of ASC filaments into a single helical structure known as an
ASC speck. Following speck formation, caspase-1 is recruited to ASC through homotypic CARD-CARD interactions, leading to cleavage and maturation of caspase-1 into its active p20 and p10 subunits, which form a heterotetramer that cleaves IL-18 and IL-1β. Although the NLRP3 inflammasome has been studied extensively, there are still many unknowns about how it becomes activated since it doesn’t directly bind to a ligand. Recent studies identified a member of the mammalian NIMA-related kinase family known as NEK7 to be essential for mediating NLRP3 inflammasome assembly and activation (He, Zeng, Yang, Motro, & Nunez, 2016; Schmid-Burgk et al., 2016). It is possible that NEK7 is the upstream sensor of NLRP3 and directly senses the cellular stress responses followed by binding to NLRP3 and facilitating its oligomerization. NEK7 and NLRP3 interaction contributes to promoting pyroptosis in inflammatory bowel disease, thus NEK7 could be a potential therapeutic target for inflammatory disorders or other NLRP3-associated diseases (X. Chen et al., 2019; G. Liu, Chen, Wang, & Yuan, 2020).

The inflammasome containing NLRC4 is unique in that NLRC4 is not the sensor NLR. Instead, NAIP is the sensor NLR and recognizes flagellin as well as the type III secretion system (T3SS) components, the inner rod and needle proteins. Specifically, for the murine NAIPs, NAIP1 recognizes the T3SS needle, NAIP2 recognizes the T3SS inner rod, and NAIP5/6 recognize flagellin through their NACHT domains (Franchi et al., 2006; Kofoed & Vance, 2011; Miao et al., 2006; Yang, Zhao, Shi, & Shao, 2013; Zhao et al., 2011). In contrast, human NAIP acts broadly and recognizes all three of these bacterial ligands, indicating that mice
and human NAIPs function differently (Grandjean et al., 2017; Kortmann, Brubaker, & Monack, 2015; Reyes Ruiz et al., 2017; J. Yang et al., 2013). Based on cryo-EM structures, a single NAIP recruits NLRC4 and promotes the polymerization of NLRC4 to form a ring-like structure (Tenthorey et al., 2017; Zhang et al., 2015). NLRC4 interacts with caspase-1 directly via CARD-CARD interactions; however, it is thought that the adaptor ASC may also be recruited to stabilize the CARD-CARD interactions between NLRC4 and caspase-1, thus enhancing inflammasome activation. The NAIP/NLRC4 inflammasome mounts immune defense against a variety of bacterial pathogens such as *Salmonella enterica* serovar Typhimurium, *Pseudomonas aeruginosa*, and *Legionella pneumophila*. Although *L. pneumophila* does not have a T3SS, it has a type 4 secretion system (T4SS) and flagellin, so it is thought that *L. pneumophila* activates the NAIP/NLRC4 inflammasome by inadvertently translocating flagellin through the T4SS into the cytosol.

It is known that different inflammasomes form their own macromolecular complex in response to a specific ligand or signal. However, during a normal infection, multiple ligands can be released into the cytosol and various endogenous danger signals can be present within a host cell. Therefore, multiple different inflammasomes may be activated within the same cell in order to generate a greater host response to clear pathogens. Interestingly, one study found that, during infection with *S. Typhimurium*, the NLRC4 and NLRP3 inflammasomes are both recruited to the same macromolecular complex which contains ASC, caspase-1, as well as caspase-8 (Man et al., 2014). This shows two novel
mechanisms of inflammasome activation: the first being that two different inflammasome proteins are recruited to the same complex and the second that caspase-8 can also be recruited to the ASC speck. Additional studies determined that caspase-8 can be recruited to the NLRC4 inflammasome independent of caspase-1, but dependent on ASC, thus triggering an alternative pathway when caspase-1 is absent (Kumar, Radha, & Swarup, 2010; B. L. Lee, K. M. Mirrashidi, et al., 2018; Man et al., 2013; Mascarenhas et al., 2017; Rauch et al., 2017). Caspase-8 interaction with ASC PYD is facilitated by its death effector domain (DED) (Vajjhala et al., 2015). Caspase-8 has classically been known to be initiator caspase for apoptosis; however, these findings reveal that caspase-8 has a wide-range of functions in different immune pathways.

The AIM2 inflammasome is activated upon direct binding to double stranded DNA (dsDNA) followed by recruitment of ASC and caspase-1. Studies using macrophages from AIM2-knockout mice or gene silencing of murine macrophages by siRNA revealed that AIM2 is activated by a variety of bacteria including Mycobacterium tuberculosis, Mycobacterium bovis, Francisella tularensis, and Legionella pneumophila (Fernandes-Alnemri et al., 2010; Ge, Gong, Xu, & Shao, 2012; Rathinam et al., 2010; Saiga et al., 2012; Y. Yang et al., 2013). Infection with these bacteria in AIM2-deficient or silenced murine macrophages resulted in decreased caspase-1 activation and IL-1β or IL-18 release compared to wild-type or untreated macrophages, indicating AIM2 is crucial for promoting inflammasome response. Furthermore, there are additional bacterial pathogens that activate both the AIM2 and NLRP3 inflammasomes in parallel to produce robust caspase-1
activation and IL-1β production; these bacteria include *Brucella abortus*, *Listeria monocytogenes*, and *Porphyromonas gingivalis* (Gomes et al., 2013; S. Kim et al., 2010; E. Park et al., 2014). Therefore, it is most likely that multiple inflammasomes are needed to control many other bacterial infections as well due to the presence of various bacterial components that serve as ligands for different inflammasomes and the triggering of cellular stress which typically results in NLRP3 inflammasome activation.

Similar to the AIM2 inflammasome, the IFI16 inflammasome recognizes genomic DNA as it is also within the ALR family. IFI16 was initially found to induce IFN-β in a STING-dependent manner in response to viral DNA (Unterholzner et al., 2010). Later, studies involving gene silencing of IFI16 or its murine ortholog IFI204 in human or murine macrophages, respectively, revealed that they had decreased IFN-β production compared to control-treated macrophages during infection with intracellular bacteria including *L. monocytogenes*, *Francisella novicida*, and *M. bovis* (Chunfa et al., 2017; Hansen et al., 2014; Storek et al., 2015). Thus, IFI16 can recognize both viral and bacterial DNA to induce a type I IFN response. Intriguingly, another study found that IFI204/IFI16 was important for killing of the extracellular bacteria, *Staphylococcus aureus*, by enhancing bactericidal activity which is a novel finding of a DNA sensor (W. Chen et al., 2019). Although there are no reports of IFI16 involvement in caspase-1-dependent inflammasome activation in response to bacteria, there are findings that show IFI16 inflammasome responses to viral infections that are dependent on ASC and caspase-1 (Ansari et al., 2015; Ansari et al., 2013; Kerur et al., 2011). This IFI16
inflammasome is initiated upon the binding of IFI16 to viral DNA in the nucleus, followed by formation of the ASC-caspase-1 inflammasome which is translocated into the cytoplasm for inflammasome activity.

The pyrin inflammasome is indirectly activated upon detection of the inactivation of the Rho-GTPase, RhoA. Pyrin is composed of an N-terminal PYD, followed by a B-box and coiled-coil domain (Heilig & Broz, 2018). Human pyrin possesses a C-terminal B30.2 domain, which is absent in mice (Broz & Dixit, 2016). RhoA activates protein kinase 1 (PKN1) and 2 (PKN2) for their phosphorylation of the N-terminal part of pyrin, leading to the binding of 14-3-3 proteins at these phosphorylation sites in order to inhibit pyrin inflammasome activation (Y. H. Park, Wood, Kastner, & Chae, 2016). However, bacterial toxins target and inactivate RhoA, resulting in inactivation of PKN1 and PKN2 and loss of the interaction between pyrin and 14-3-3 proteins (Xu et al., 2014). This releases pyrin from its inhibited state and allows for inflammasome activation, which involves the recruitment of ASC followed by caspase-1 recruitment and activation.

1.3. Noncanonical inflammasome responses to gram-negative bacteria

Recently, an alternative mechanism of inflammasome activation, that does not directly recruit caspase-1, in response to gram-negative bacteria was discovered and is termed the noncanonical inflammasome. The noncanonical inflammasome is comprised of caspase-11 in mice and the two putative orthologous, caspase-4 and caspase-5 in humans (Vigano & Mortellaro, 2013). Caspase-11, caspase-4, and caspase-5 all possess N-terminal CARD domains.
followed by large (p20) and small (p10) catalytic subunits (B. L. Lee, I. B. Stowe, et al., 2018). All of these inflammatory caspases bind to intracellular LPS through their CARD domains, which leads to the oligomerization and activation of caspase-11 and caspase-4 (Shi et al., 2014). Although caspase-5 was shown to bind to LPS, it is unclear whether it oligomerizes and additional studies on the mechanism of caspase-5 function in response to gram-negative bacterial infections are needed. Studies in murine macrophages showed that the caspase-11 noncanonical inflammasome is activated during infection with a number of gram-negative bacteria including *L. pneumophila*, *Escherichia coli*, and *S. Typhimurium* (Aachoui et al., 2013; Broz et al., 2012; Case et al., 2013; Casson et al., 2013; Kayagaki et al., 2011). Human caspase-4 also induces inflammasome activation in response to *L. pneumophila*, *S. Typhimurium*, *Yersinia pseudotuberculosis*, *Francisella novicida*, and *E. coli* in macrophages (Casson et al., 2015; Goddard et al., 2019; Lagrange et al., 2018). In addition, murine and human epithelial cells induce noncanonical inflammasome responses to *Burkholderia pseudomallei*, *Burkholderia thailandensis*, and *S. Typhimurium* (Holly et al., 2020; Knodler et al., 2014; Srisaowakarn, Pudla, Ponpuak, & Utaiisinaroen, 2020; Wang et al., 2018). Thus, the noncanonical inflammasome can be activated in different cell types.

Although there has been very little investigation focused on caspase-5, there are a couple of studies that found interesting results in human monocytes that were treated with LPS or infected with *Pseudomonas aeruginosa* membrane vesicles (Bitto et al., 2018; Vigano et al., 2015). In one study, primary human monocytes were treated with extracellular LPS, which led to the secretion of IL-1α.
and IL-1β in a caspase-4- and caspase-5-dependent manner (Vigano et al., 2015). Intriguingly, only caspase-5 underwent processing in response to LPS internalization, whereas caspase-4 was left uncleaved. These findings may suggest that caspase-5 is differentially expressed in monocytes compared with macrophages and may be a reason of why caspase-5 can stimulate this one-step noncanonical inflammasome response to LPS. The other study found that only caspase-5 is activated in response to P. aeruginosa outer membrane vesicles (OMVs), which enter the monocytes via endocytosis (Bitto et al., 2018). In contrast, caspase-4 was activated upon transfection of P. aeruginosa LPS. These results indicate that caspase-4 and caspase-5 are differentially activated by LPS depending on the form of LPS as well as its mechanism of entry into the cell. Therefore, both of these caspase-5 studies highlight that extracellular treatment of LPS or OMVs result in caspase-5 activation, suggesting that caspase-5 responds to endocytosed vesicles containing LPS, rather than sensing LPS that is forcibly introduced into the cell.

TLR4 was initially believed to be the sole LPS sensor, which recognizes extracellular LPS through a defined mechanism involving an array of proteins that present LPS directly to membrane-bound TLR4 (B. S. Park & Lee, 2013; Poltorak et al., 1998; Takeuchi et al., 1999). Excessive LPS stimulation of cells leads to endotoxic shock or sepsis. Two studies found that intracellular LPS can induce endotoxic shock independent of TLR4 (Hagar, Powell, Aachoui, Ernst, & Miao, 2013; Kayagaki et al., 2013). These studies identified caspase-11 as the noncanonical inflammasome that responds to transfected LPS from different gram-
negative bacteria including *F. novicida, Yersinia pestis*, and *E. coli*. Importantly, the caspase-11 noncanonical inflammasome as well as caspase-1-dependent inflammasomes require two signals for optimal inflammasome response to bacterial pathogens: the priming signal for upregulation of inflammasome proteins and proinflammatory cytokines and an activation signal involving recognition of bacterial components or cell stress by a sensor protein (Kayagaki et al., 2011; Mariathasan, 2007). In agreement with this general rule, these two studies found that LPS-induced lethal sepsis occurred only when wild-type (WT) mice were first primed with a TLR agonist followed by LPS challenge, whereas WT mice treated with either the TLR agonist or LPS alone did not trigger lethality. Furthermore, using these conditions, they showed that caspase-11 knockout (*Casp11<sup>−/−</sup>*)) mice were rescued from LPS lethality, whereas TLR4 knockout (*TLR4<sup>−/−</sup>*)) mice succumbed to the lethal dose of LPS; thus, these data conclude that caspase-11 is the alternative mechanism of LPS sensing within the cytosol (Kayagaki et al., 2013).

In addition to TLR4, it was also previously thought that caspase-1, originally known as the IL-1β converting enzyme (ICE), was important for inducing LPS-induced endotoxic shock in mice (Kuida et al., 1995; Li et al., 1995). However, these published studies generated caspase-1 knockout (*Casp1<sup>−/−</sup>*)) mice using 129 mouse strain embryonic stem cells, which have been found to produce an inactivating passenger mutation of caspase-11 upon the targeting of ICE using homologous recombination (Kenneth et al., 2012). Therefore, due to the close proximity of caspase-1 and caspase-11, these generated *Casp1<sup>−/−</sup>* mice are
actually deficient in both caspase-1 and caspase-11. In order to address this concern, one study generated \( \text{Casp11}^{-/-} \) C57BL/6 mice, caspase-1 and caspase-11 double knockout (\( \text{Casp1/11}^{-/-} \)) C57BL/6 mice, and \( \text{Casp1}^{-/-} \) C57BL/6 mice by injecting the caspase-11 transgene into \( \text{Casp1/11}^{-/-} \) embryos (Kayagaki et al., 2011). These mice helped to distinguish the roles of caspase-1 and caspase-11 during infection of gram-negative bacteria or stimulation with canonical activators such as ATP. This study concluded that canonical stimuli did not activate caspase-11, however non-canonical stimuli such as LPS and gram-negative bacteria led to caspase-11 inflammasome activation as well as caspase-1 inflammasome activation that was dependent on NLRP3 and ASC, but caspase-11 processing and cell death was not dependent on NLRP3 and ASC. Therefore, caspase-11 is upstream of caspase-1. Finally, they also found that caspase-11 deficiency, but not caspase-1 deficiency, protected mice from LPS-induced endotoxic shock.

Since the NLRP3 and ASC proteins were required for inducing a caspase-1-dependent inflammasome response to non-canonical stimuli and that caspase-11 is upstream of caspase-1, it was unclear what the link was connecting caspase-11 activation to downstream NLRP3 inflammasome activation (Kayagaki et al., 2011). Activation of caspase-11 upon infection with various gram-negative bacteria leads to downstream pyroptosis, a lytic form of cell death, and release of IL-1 family cytokines (Hagar et al., 2013; Kayagaki et al., 2011; Kayagaki et al., 2013). However, the mechanism involving pyroptosis initiation was largely unknown. It was discovered that a 53 kDa protein known as gasdermin-D (GSDMD) was essential for inducing noncanonical inflammasome signaling as GSDMD knockout
(GSDMD<sup>−/−</sup>) murine macrophages had decreased cell death and IL-1β release and looked similar to Casp11<sup>−/−</sup> macrophages (Kayagaki et al., 2015). Specifically, this study found that caspase-11 cleaves GSDMD, which results in a p30 pro-pyroptotic N-terminal fragment that also induces NLRP3-dependent caspase-1 activation. Following this breakthrough finding, additional studies determined that the GSDMD N-terminal fragment binds to the plasma membrane to form a circular pore, which results in loss of membrane integrity and osmotic lysis of the cell (Aglietti et al., 2016; Sborgi et al., 2016). The GSDMD pore also disrupts ionic gradients and can lead to potassium (K<sup>+</sup>) efflux, which is a trigger of the NLRP3 inflammasome. This idea was confirmed in a study that showed caspase-11 activated NLRP3 inflammasome by stimulating K<sup>+</sup> efflux (Ruhl & Broz, 2015). Thus, this provides a model in which intracellular LPS activates caspase-11 to cleave GSDMD, resulting in its N-terminal fragment to oligomerize and form a pore in the plasma membrane, which induces pyroptosis as well as K<sup>+</sup> efflux for NLRP3-ASC-caspase-1 inflammasome activation. This process is now well known as the noncanonical NLRP3 inflammasome, which is essential for IL-1 cytokine release due to the cleavage and activation of caspase-1.

Most of these noncanonical inflammasome studies regarding GSDMD and downstream NLRP3 inflammasome have been conducted using mice or murine macrophages, however there are studies that investigated the human noncanonical inflammasome involved in these processes as well. Caspase-4 and caspase-5 mediate downstream NLRP3 inflammasome activation upon the recognition of intracellular LPS (Baker et al., 2015; Schmid-Burgk et al., 2015).
Furthermore, all of the murine and human inflammatory caspases (caspase-1, caspase-11, caspase-4, and caspase-5) were found to cleave GSDMD, which is crucial for induction of pyroptosis (Shi et al., 2015). Therefore, downstream NLRP3 inflammasome activation by caspase-11/4/5 results in caspase-1-mediated maturation of IL-1 family cytokines as well as cleavage of GSDMD, which further promotes pyroptosis of the infected cell.

Intriguingly, the noncanonical inflammasome has been shown to respond to other stimuli in addition to LPS. Caspase-4 is activated by the Shiga toxin 2 (Stx2)/LPS complex derived from pathogenic E. coli and caspase-11 detects lipophosphoglycan (LPG) from Leishmania parasites (de Carvalho et al., 2019; Platnich et al., 2018). In addition, the caspase-4 study also determined that caspase-4-dependent GSDMD cleavage leads to N-terminal GSDMD fragment enriched at the mitochondria, which resulted in mitochondrial ROS production for NLRP3 inflammasome activation (Platnich et al., 2018). These data indicate that GSDMD can activate the NLRP3 inflammasome in multiple ways including induction of K+ efflux and ROS production.

1.4. Type I and II Interferon signaling

Interferons (IFNs) are cytokines that are classically known for their ability to induce an antiviral gene program in response to viral infections. IFNs were named so due to their ability to “interfere” with viral replication, resulting in host defense against viruses (Isaacs & Lindenmann, 1957). There are three main classes within the IFN family: type I, type II, and type III IFNs. Together, they induce the
expression of hundreds of IFN stimulated genes (ISGs) (Platanias, 2005; Stanifer, Pervolaraki, & Boulant, 2019).

Type I IFNs are produced by almost all cell types and are comprised of eight different types: 13 subtypes of IFN-α (alpha), IFN-β (beta), IFN-δ (delta), IFN-ε (epsilon), IFN-κ (kappa), IFN-ω (omega), IFN-ζ (zeta), and IFN-τ (tau) (Platanias, 2005). All of the type I IFNs signal through the type I interferon receptor also known as IFN α/β receptor (IFNAR), which is comprised of two subunits: IFNAR1 and IFNAR2. Upon binding to type I IFNs, the cytoplasmic end of IFNAR1/2 interact with the Janus activated kinase (JAK) family members, JAK1 and TYK2, leading to the phosphorylation of STAT1 and STAT2 for complex formation with IFN-regulatory factor 9 (IRF9). The following STAT1-STAT2-IRF9 complex binds to the IFN-stimulated response element (ISRE) for transcription of hundreds of IFN-stimulated genes. Although type I IFNs were primarily thought to control viral infections, type I IFNs have been shown to promote host defense against bacteria and parasites as well (Boxx & Cheng, 2016; Silva-Barrios & Stager, 2017).

Similar to the type I IFN signaling pathway, type II IFN also signals through the JAK-STAT pathway and is essential for controlling bacterial and parasitic infections in addition to viral infections (Czarniecki & Sonnenfeld, 1993; Lykens et al., 2010; Platanias, 2005). In contrast to type I IFNs, type II IFNs consist of only one type, IFN-γ, which are primarily produced by NK cells, activated T cells, and macrophages. IFN-γ binding to its receptor made up of IFN gamma receptor 1 (IFNGR1) and IFN gamma receptor 2 (IFNGR2) leads to JAK1 and JAK2 association, followed by phosphorylation of STAT1 and production of STAT1-
STAT1 homodimers, which bind to the IFN-γ activated site (GAS) for gene transcription. Type I IFN signaling can also lead to STAT1 homodimers for GAS gene activation. Therefore, type I IFNs can upregulate different genes depending on the combination of STAT complexes for binding either GAS, ISRE, or both.

Type III IFNs are a new class of IFNs and are known as IFN-λs (lamdas) (Stanifer et al., 2019). They have four family members including IFN-λ1, IFN-λ2, IFN-λ3, and IFN-λ4 that signal through a heterodimeric receptor IFNLR1 and IL-10R2 that undergoes JAK-STAT signaling similar to type I IFN signaling through GAS and ISRE elements, however their function is restricted to epithelial cells (Stanifer et al., 2019). These IFNs also protect epithelial barriers from bacterial immunity, further highlighting that type I, II, and III IFNs are essential for host defense against bacterial infections (Odendall, Voak, & Kagan, 2017).

1.5. Interferon-inducible GTPases: Guanylate binding proteins

As it was previously mentioned, type I and II IFNs upregulate a vast repertoire of genes in response to viruses, bacteria, and parasites. Among the most notable family of ISGs are the IFN-inducible GTPase superfamily, which includes the 65-73 kDa guanylate binding proteins (GBPs), 21-47 kDa immunity related GTPases (IRGs), 72-82 kDa myxoma (Mx) resistance proteins, and 200-285 kDa very large inducible GTPases (B. H. Kim, Shenoy, Kumar, Bradfield, & MacMicking, 2012). Specifically, GBPs and IRGs are the two subfamilies involved in cell-autonomous immune responses against intracellular bacterial pathogens.
GBPs are upregulated by type I and II IFNs, however they are more robustly induced by the type II IFN, IFN-γ (Man, Place, Kuriakose, & Kanneganti, 2017). Mice possess 11 GBPs and 2 pseudogenes located on chromosomes 3 and 5. Mouse chromosome 3 contains Gbp1, Gbp2, Gbp3, Gbp5, Gbp7, and one pseudogene, while chromosome 5 has Gbp4, Gbp6, Gbp8, Gbp9, Gbp10, Gbp11, and the second pseudogene (Olszewski, Gray, & Vestal, 2006). In contrast, humans only have seven GBPs located on chromosome 1. Structural and biochemical analysis revealed that GBPs are part of the dynamin superfamily and therefore undergo nucleotide-dependent oligomerization and GTPase activity (Vestal & Jeyaratnam, 2011). Based on the structure of human GBP1, all GBPs are comprised of an N-terminal globular large G domain, which is important for nucleotide binding and GTPase effector activity, followed by an α-helical C-terminal domain (Prakash, Praefcke, Renault, Wittinghofer, & Herrmann, 2000). The N-terminal LG domain is connected by the C-terminal domain by an intermediate region made up of an α-helical domain and two β-sheets.

The C-terminal amphipathic α helices mediate protein-protein and protein-lipid interactions (B. H. Kim et al., 2012). In addition, human and mouse GBP1, GBP2, and GBP5 possess a C-terminal ‘CaaX’ isoprenylation motif important for facilitating protein-protein interactions as well as membrane binding. The ‘C’ signifies a cysteine amino acid residue, ‘aa’ indicates any aliphatic amino acids, and ‘X’ signifies the amino acid that determines which lipid moiety is added, either a leucine or serine. The terminal amino acid for human GBP1 and murine Gbp5 is serine, therefore a C-15 farnesyl group is added at their CaaX motif. In contrast,
human GBP2 and GBP5 as well as murine Gbp1 and Gbp2 have a terminal leucine, which results in addition of a C-20 geranylgeranyl lipid. One study found that membrane binding of human GBP1, GBP2, and GBP5 is dependent not only on prenylation of the CaaX motif, but also dependent on oligomerization of the GBP (Britzen-Laurent et al., 2010). Here, they found that GBP are able to homodimerize or heterodimerize and that prenylated GBP can recruit non-prenylated GBP to their intracellular compartments. Specifically, human GBP1 localizes to the plasma membrane, GBP2 localizes to the perinuclear membrane, and GBP5 to the Golgi. Heterodimerization between prenylated GBP results in a hierarchical positioning effect of GBP; for instance, human GBP1 recruits GBP2 and GBP5 to the plasma membrane, whereas GBP5 repositions GBP2 to the Golgi. These findings suggest that GBP1 is the primary recruiter for the remaining GBP in uninfected IFN-γ-primed cells. However, infection studies have shown to alter the positioning effects of GBP to the pathogen directly, presumably to induce host defense mechanisms.

1.6. GBP role in inflammasome responses to bacteria

Studies in macrophages have proven that GBP employ a variety of functions in a pathogen-specific manner. Initial murine GBP findings showed that GBP can promote rupture of the pathogen-containing vacuole (PCV) or bacteriolysis of cytosolic bacteria (B. C. Liu et al., 2018; Meunier et al., 2014; Meunier et al., 2015). Upon infection with the vacuolar pathogen S. Typhimurium, murine macrophages lacking GBP on chromosome 3 (Gbp<sup>chr3−/−</sup>) showed
decreased cell death and IL-1β release compared to WT macrophages, and more specifically after conducting siRNA-mediated knockdown of individual GBPs, found that Gbp2 is the sole GBP important for mediating this caspase-11-dependent inflammasome response (Meunier et al., 2014). In addition, they determined that Gbp^{chr3/−} macrophages had a decreased amount of galectin-8-positive S. Typhimurium. Galectins are β-galactoside-binding lectins that bind to the carbohydrates found in the inner leaflet of vacuolar membranes and therefore serve as a marker of ruptured vacuoles. As expected due to this finding, they further showed that Gbp^{chr3/−} or Gbp2-deficient (Gbp2^{−/−}) macrophages had decreased presence of cytosolic S. Typhimurium, indicating that GBPs mediate lysis of the PCV of S. Typhimurium. In contrast, two studies revealed that murine GBPs localize to the outer membrane of cytosolic F. novicida and L. pneumophila ΔsdhA mutant. Specifically, murine Gbp2 and Gbp5 individually promote AIM2 inflammasome activation through their direct binding and lysis of F. novicida's outer membrane (Meunier et al., 2015). Additionally, the cytosolic ΔsdhA mutant of L. pneumophila was shown to have lost its rod-like appearance in IFN-β-primed WT murine macrophages, but retained the rod shape in Gbp^{chr3/−} macrophages, indicating that murine GBPs induce bacteriolysis of cytosolic exposed bacteria (B. C. Liu et al., 2018). They further reveal that GBPs on chromosome 3 are essential for mediating the release of DNA from the ΔsdhA mutant. Interestingly, this study also found that IFN-β priming resulted in destabilization of the vacuole of WT L. pneumophila in both WT and Gbp^{chr3/−} macrophages, suggesting that there are additional IFN-induced factors other than GBPs that are rupturing L. pneumophila's
vacuole. It is interesting that murine GBPs rupture the PCV of S. Typhimurium, but not of L. pneumophila; thus, GBPs may take on different roles in a bacterium-specific manner. Nevertheless, all three of these murine GBP studies discussed so far conclude that GBPs control bacterial burden within the host.

In addition to promoting inflammasome responses to bacterial pathogens, GBPs can initiate inflammasome activation in response to bacterial components as well. GBPs promote caspase-11-dependent pyroptosis in response to cytoplasmic LPS from E. coli, Salmonella minnesota, and L. pneumophila, although the specific mechanism of how GBPs do this was unclear (Pilla et al., 2014). It was revealed that murine and human GBP5 can form a tetramer that binds to NLRP3 to promote ASC-caspase-1 inflammasome assembly in response to pathogenic bacteria and bacterial components including LPS and muramyl dipeptide (MDP) (Shenoy et al., 2012). Therefore, GBPs may similarly oligomerize in response to LPS to enhance caspase-11 activation, possibly through binding LPS and transporting it directly to caspase-11, acting in a similar manner as the LPS binding protein (LBP) during TLR4 activation. Furthermore, GBPs on chromosome 3 and more specifically Gbp2 are required for inflammasome activation in response to OMVs derived from E. coli (Finethy et al., 2017). It was later found that GBPs associate with OMVs and transfected LPS, indicating that GBP binding to LPS is the main factor regulating GBP recruitment to OMVs (Santos et al., 2018).

The precise mechanism that governs GBP recruitment to PCVs is poorly understood. We know that GBPs bind to LPS on the outer membrane of bacteria,
but whether there are specific microbial signatures on pathogenic vacuoles is unclear. Two studies determined that murine GBPs are recruited to vacuolar pathogens that contain bacterial secretion systems (E. M. Feeley et al., 2017; Zwack et al., 2017). Specifically, GBPs are delivered to the pathogen-containing vacuoles of *L. pneumophila* and *Yersinia pseudotuberculosis* in a manner dependent on the presence of the Dot/Icm type IV secretion system and hypersecretion of the *Yersinia* type III secretion system, respectively (E. M. Feeley et al., 2017). However, it was determined that the presence of bacterial secretion systems result in vacuolar membrane damage leading to initial recruitment of galectin-3, the damaged vacuole marker, followed by recruitment of GBPs. Galectin-3-deficient cells had a decreased amount of murine Gbp1 and Gbp2 to the *L. pneumophila*-containing vacuoles and *Yersinia*-containing vacuoles, indicating that galectin-3 controls GBP recruitment to PCVs. Furthermore, another study showed that hyperinjection of the *Yersinia* translocon protein, YopD, leads to its association with galectin-3 and Gbp2 and that GBPs on chromosome 3 contribute to *Yersinia* T3SS-induced inflammasome activation (Zwack et al., 2017). Therefore, these findings provide a model wherein *Yersinia* translocon proteins localize to the PCV resulting in galectin-3 recruitment followed by GBP recruitment for promoting noncanonical inflammasome responses to *Yersinia*. Besides the presence of bacterial secretion systems, there may be additional ways in which GBPs are recruited to non-self vacuolar membranes.

The GBP research discussed so far has focused on murine GBPs. Since mice have 11 GBPs and humans have 7 GBPs, there may be differences in the
role that human GBPs play in response to bacterial infections. Recently, investigation on human GBPs have revealed seminal findings on human cell-autonomous immune responses on various bacterial pathogens. Initial studies on the cytosolic bacterium *Shigella flexneri* highlighted that human GBPs are recruited to the outer membrane in order to inhibit *S. flexneri’s* actin motility (Li et al., 2017; Piro et al., 2017; Wandel et al., 2017). Human GBP1 is first recruited to *S. flexneri*, followed by GBP2-4 and GBP6 to form a GBP coat around the bacteria (Piro et al., 2017; Wandel et al., 2017). *S. flexneri* has developed mechanisms to counter host cell defense by using their IpaH family of E3 ubiquitin ligases. Specifically, IpaH9.8 was found to ubiquitylate and degrade GBPs to allow for cell-to-cell spread, while *S. flexneri* lacking IpaH9.8 maintained their GBP coats and were unable to undergo actin-based motility (Li et al., 2017; Wandel et al., 2017). Thus, human GBP1 prevents bacterial spread of *S. flexneri*, which is a novel function of GBPs. Additional findings on this process showed that GBP1 localization with *S. flexneri* is dependent on its CaaX motif, GTPase activity, and triple arginine motif at the C-terminus. Human GBP1 also binds to the *S. flexneri* rough mutant which lacks the O-antigen at a lower affinity compared to the WT *S. flexneri*, indicating that GBP1 binds directly to LPS on the outer membrane of *S. flexneri* (Piro et al., 2017). Moreover, human GBP2 colocalizes with *F. novicida* to promote caspase-4-dependent inflammasome responses (Lagrange et al., 2018). These findings show that human GBPs can take on different roles in a bacterium-specific manner.

Human GBP1 not only is important for inhibiting the bacterial spread of *S. flexneri*, but also plays an essential role in promoting caspase-4 inflammasome
activation during infection with cytosolic exposed S. Typhimurium and S. flexneri mutant that lacks the effectors IpaH9.8 and OspC3 as well (Fisch et al., 2019; Kutsch et al., 2020; Santos et al., 2020; Wandel et al., 2020). GBP1 was initially shown to promote the caspase-4 inflammasome in response to S. Typhimurium in a GTPase and isoprenylation dependent manner (Fisch et al., 2019). In addition, GBP1 localizes to S. Typhimurium and recruits caspase-4. However, this study was not clear on whether GBP1 localizes to the vacuole or outer membrane of S. Typhimurium. A recent study determined that GBP1 is recruited to the outer membrane of cytosolic exposed S. Typhimurium in a CaaX motif and GTPase-dependent manner and does not colocalize with galectin-3+ ruptured vacuolar membranes (Santos et al., 2020). Intriguingly, in contrast to murine GBPs that induce bacteriolysis of cytosolic F. novicida and L. pneumophila, human GBP1 does not induce bacteriolysis of S. Typhimurium but rather controls inflammasome assembly at the bacterial surface. Furthermore, similar to the S. flexneri studies, upon binding LPS on S. Typhimurium, GBP1 recruits GBP2-4 to form a complex, which recruits caspase-4 for its activation. Although it was not clear how caspase-4 is being activated, it could be due to a number of possibilities including a scenario where the GBP complex transfers LPS to caspase-4 or that the GBP complex weakens the bacterial membrane so that caspase-4 can directly target LPS. Two studies investigate how caspase-4 is activated and it seems like latter of these two possibilities is happening (Kutsch et al., 2020; Wandel et al., 2020). While GBP1 is the initiator GBP that recruits GBP2-4, the functions of these recruited GBPs were finally established. GBP2 and GBP4 participate in caspase-4 recruitment,
whereas GBP3 is essential for caspase-4 activation in response to cytosolic S. Typhimurium as well as to S. flexneri that lacks the effectors IpaH9.8 and OspC3, due to their inhibition of the caspase-4 inflammasome (Wandel et al., 2020). Specifically, GBP1-4 assemble into a polyvalent protein array on the outer membrane bacterial surface and leads to an LPS-dependent complex formation with caspase-4. Thus, this investigation provides a model where the GBP complex disrupts the membrane integrity for allowing access of capsase-4 to bind LPS. Furthermore, another study looking at the mechanism of caspase-4 activation showed that GBP1 polymers dock to the outer membrane of S. flexneri through binding to the O-antigen of its LPS, resulting in GBP1 protein coats surrounding the bacteria (Kutsch et al., 2020). They further conclude that GBP1 becomes anchored in the bacterial membrane via their farnesyl tails, which disrupts the LPS barrier and reveals lipid A for caspase-4 detection. Thus, rather than lysing the cytosolic bacteria for release of bacterial components such as LPS for inflammasome activation, human GBPs respond to cytosolic bacteria by orchestrating an organized complex platform for direct access of caspase-4.

Human GBP1 has been involved in microbial restriction of other bacteria and parasites as well. Human GBP1 localizes to the parasite Toxoplasma gondii vacuole to restrict its infection in mesenchymal stromal cells and also mediates restriction of C. trachomatis in THP-1 macrophages (Qin et al., 2017; Xavier, Al-Zeer, Meyer, & Daumke, 2020). Interestingly, GBP1 does not always interact with the pathogen in order to control infection, since it was found that GBP1 does not localize to vacuoles of T. gondii, C. trachomatis, or S. Typhimurium but restricts T.
*gondii* replication in A549 lung epithelial cells (Johnston et al., 2016). Therefore, human GBP s may have different functions in a cell-type-specific manner.

1.7. Interferon-inducible GTPases: Immunity related GTPases

Another IFN-inducible GTPase family that has been implicated in vacuolar rupture and bacteriolysis are the IRGs. Mice possess 23 IRG proteins, whereas humans possess two IRG proteins, IRGM and IRGC (Bekpen et al., 2005). In contrast to murine IRGs, human IRGs are not IFN-inducible due to the loss of interferon response elements upstream of their transcriptional start site. Therefore, human IRGs would not be involved in IFN-dependent host defense pathways. The murine IRGs are divided into two subfamilies: the GMS and GKS proteins, which are named so because of the presence of either a GMS or GKS amino acid motif on the first nucleotide-binding site (Hunn, Feng, Sher, & Howard, 2011). The GMS proteins include the IRGM subfamily, while the GKS proteins contain the IRGA, IRGB, IRGC, and IRGD subfamilies. The IRGMs are guanine nucleotide dissociation inhibitors that control the localization of other IFN-inducible GTPases including the GKS subfamily of IRGs and GBP s. The presence of IRGMs on membranes prevents the binding of GBP s and GKS IRGs, and therefore the IRGMs serve as regulators for IFN-inducible GTPases involved in vacuolar rupture or bacteriolysis to determine “self” versus “non-self” (Ngo & Man, 2017). Indeed, one study found that Irgm1 and Irgm3 did not localize to “non-self” pathogen-containing vacuoles, but instead localized to “self” organelles, such as lipid droplets (Haldar et al., 2013). They found that when the IRGM proteins were
knocked out, the GKS proteins Irga6 and Irgb6 localized to the lipid droplets, indicating that IRGs and GBPs target membranes that are missing “self” IRGM proteins.

1.8. IRGs role in inflammasome responses to bacteria

IRGs have been shown to destabilize vacuoles of pathogens and in one case have been essential for mediating bacteriolysis of cytoplasmic bacteria. The murine IRGs Irgm1 and Irgm3 are critical for directing Irga6, Irgb6 and Irgb10 to the C. trachomatis inclusions to control bacterial replication (Coers et al., 2008; Haldar et al., 2013). In addition, Irgm1 is important for controlling Mycobacterium tuberculosis infection in the lungs (Tischler, Leistikow, Kirksey, Voskuil, & McKinney, 2013). Although not in the context of bacterial infection, murine IRGs have also been reported to localize to the parasitophorous vacuole of T. gondii in a hierarchal manner in the order of Irgb6, Irgb10, Irga6, Irgm2, and Irgd in order to disrupt the vacuole for killing of the intracellular parasite (Khaminets et al., 2010; Martens et al., 2005).

Irgb10 is the only known murine IRG that has been implicated in inducing bacteriolysis of cytoplasmic bacteria in order to liberate ligands for inflammasome sensing (Man et al., 2016). Specifically, Irgb10 targets the intracellular bacteria F. novicida and E. coli in a GBP dependent manner. Following bacterial targeting, Irgb10 mediates bacteriolysis, which results in the release of bacterial components including DNA for AIM2 inflammasome activation and LPS for noncanonical capsase-11 inflammasome activation. Humans do not possess an ortholog of
mouse Irgb10; however, human IRGM has been shown to be important for controlling *M. tuberculosis* and *S. Typhimurium* infections through autophagy induction (McCarroll et al., 2008; Singh, Davis, Taylor, & Deretic, 2006). While not in the context of inflammasome activation, these findings show that IRGM is important for activating alternative host defense pathways.

1.9. *Legionella pneumophila* infection in macrophages

My primary thesis project focuses on investigating IFN-induced innate immune responses to *Legionella pneumophila* in order to gain an understanding of human GBPs role during infection with this vacuolar pathogen. *Legionella pneumophila* is a gram-negative, opportunistic intracellular bacterial pathogen that is the causative agent for Legionnaires’ disease, a severe pneumonia that primarily affects immunocompromised hosts, or to a lesser extent can cause Pontiac Fever, a flu-like illness (Fields, Benson, & Besser, 2002; Fraser et al., 1977). *L. pneumophila* was first identified during an outbreak that occurred in 1976 at the 56th annual American Legion Convention in Philadelphia (Fraser et al., 1977). There was a total of 182 individuals diagnosed with severe pneumonia and 29 people died from the illness, revealing the detrimental effects of this intracellular pathogen. *L. pneumophila* persists in aquatic environments and replicates within their natural hosts, which are free-living amoebae. However, *L. pneumophila* can also be found as a contaminant in freshwater reservoirs including water towers, hot tubs, and air condition units and, therefore, can become an accidental pathogen to humans upon the inhalation of aerosolized water droplets that are
contaminated with *L. pneumophila*. Upon inhalation, these intracellular bacteria gain access to the lungs where they infect and replicate in alveolar macrophages, although it was determined that *L. pneumophila* can also replicate within neutrophils as well (Copenhaver et al., 2014).

Upon uptake by macrophages, *L. pneumophila* resides within a specialized compartment termed the *L. pneumophila*-containing vacuole (LCV) (Horwitz, 1983). However, in order to survive and replicate within the LCV, *L. pneumophila* relies on its Dot/Icm type IVb secretion system (T4SS) (Berger & Isberg, 1993; Berger, Merriam, & Isberg, 1994; Brand, Sadosky, & Shuman, 1994; Ensminger & Isberg, 2009; Roy & Isberg, 1997). The T4SS translocates over 300 bacterial effectors that are essential for manipulation of host cell processes as well as for vacuolar remodeling (Hubber & Roy, 2010). Specifically, these effectors allow *L. pneumophila* to evade the endocytic pathway by avoiding endosome-lysosome fusion and to remodel the vacuole by recruiting ER-derived vesicles to the LCV membrane (Ensminger & Isberg, 2009; Hubber & Roy, 2010). Modification of the LCV with ER-derived vesicles results the establishment of an ER-like organelle that is maintained by the host cell and, therefore, supports the replication of *L. pneumophila* within this specialized vacuole (Roy, 2002). *L. pneumophila* begin to replicate in the LCV at 4-6 hours upon initial uptake by a host cell. At around 14 hours post-uptake, *L. pneumophila* has replicated to large numbers and at this point escape the LCV and rupture the host cell in order for *L. pneumophila* progeny to infect additional neighboring cells. Although the T4SS is essential for the survival of *L. pneumophila* within the LCV, the T4SS makes *L. pneumophila* susceptible to
host sensing and can activate innate immune pathways in the cytosol, such as the inflammasome (Casson et al., 2013; Casson et al., 2015; Molofsky et al., 2006).

Most of the studies conducted involving *L. pneumophila* use strains that are derived from the serogroup 1 clinical isolate Philadelphia-1, which was collected from the 1976 outbreak. The two strains that were derived from the Philadelphia-1 isolate are the JR32 strain and LP01 strain, both of which are replicative strains (Rao, Benhabib, & Ensminger, 2013). Additional strains were produced downstream from the LP01 strain. LP02 was derived from LP01 and is a thymidine auxotroph; thus, it requires thymidine in order to undergo replication. Using this strain is instrumental in understanding *L. pneumophila*’s influence on the immune response without the confounding variable of replication. Furthermore, LP02 was used to derive LP03, also known as the avirulent *dotA* mutant, which is a thymidine auxotroph that lacks the dot/icm translocation system and is defective for intracellular replication (Berger & Isberg, 1993; Berger et al., 1994). In my studies, I use a flagellin deficient LP02 strain, ΔflaA, in order to investigate inflammasome responses that are NAIP/NLRC4 inflammasome independent (Ren, Zamboni, Roy, Dietrich, & Vance, 2006), as well as use ΔdotA LP03 strain and a dsRED-expressing JR32 strain. *L. pneumophila* has been shown to activate canonical caspase-1 containing inflammasomes as well as noncanonical inflammasome pathways (Case et al., 2013; Casson et al., 2013; Casson et al., 2015; Ge et al., 2012; Miao et al., 2010; Zhao et al., 2011). However, whether IFN-γ or human GBPs promote inflammasome responses to *L. pneumophila* in human macrophages has not been studied.
1.10. Lipopolysaccharide (LPS): immunological consequence of acylation state in macrophages

Lipopolysaccharide (LPS) is the major outer membrane lipid component of gram-negative bacteria. It is composed made up of three components: the lipid A moiety, core oligosaccharide, and O-antigen. LPS is an amphiphilic molecule where the lipid A comprises the hydrophobic part and the polysaccharides within the core and O-antigen make up the hydrophilic portion. The lipid A moiety is the portion of LPS that is directly recognized by the extracellular and intracellular LPS sensors, TLR4 and noncanonical inflammatory caspases 11, 4 and 5, respectively. Specifically, lipid A is has two glucosamine residues that are connected by a β1’-6 linkage that can be phosphorylated at the 1 and 4’ positions and are acylated at the 2,3,2’, and 3’ positions (Bertani & Ruiz, 2018).

Intriguingly, LPS can possess different acylation or phosphorylation states in various bacteria as well as within the same bacterial species. For instance, lipid A can have different numbers and lengths of acyl chains and usually have either one or two phosphate groups. Gram-negative bacteria’s ability to remodel their LPS is important for their pathogenesis as it can allow these particular bacteria to evade immune detection, including TLR4 and the noncanonical inflammatory caspases. In particular for Helicobacter pylori, it has a tetra-acylated LPS and the ability to remove phosphoryl groups from its 1 and 4’ positions through the use of its phosphatases lpxE and lpxF, respectively, which lead to the attenuation of TLR4-MD2 activation (Cullen et al., 2011). This in contrast to E. coli LPS, which is
hexa-acylated and contains two phosphate groups and robustly activated the TLR4-MD2 complex. Another gram-negative bacteria species that has the ability to modify its LPS is Francisella novicida. F. novicida initially synthesizes a penta-acylated LPS with two phosphates and uses its phosphatase lpxF to remove the 4′-phosphate group and a deacylase to remove the 3′-hydroxyacyl chain (Raetz et al., 2009). This process results in a tetra-acylated and monophosphorylated F. novicida LPS, which evades the murine noncanonical inflammasome (Hagar et al., 2013). Additionally, bacteria can switch between acyl states in response to its surrounding environment. Specifically, Yersinia pestis can switch between being tetra-acylated and hexa-acylated. Y. pestis is hexa-acylated when grown at 25°C, the temperature of a flea vector, however it removes two acyl chains to become tetra-acylated upon being grown at 37°C, which is the temperature of a mammalian host (Rebeil, Ernst, Gowen, Miller, & Hinnebusch, 2004). Similar to tetra-acylated F. novicida LPS, it was discovered that tetra-acylated Y. pestis evades murine caspase-11 detection, whereas Y. pestis in its hexa-acylated form activates caspase-11 (Hagar et al., 2013). These results are in agreement with another study that found tetra-acylated H. pylori or lipid IVa, the precursor to E. coli LPS, failed to activate the mouse noncanonical inflammasome as well as TLR4, while hexa-acylated LPS from E. coli or S. Typhimurium robustly activates caspase-11 and TLR4 (Kayagaki et al., 2013). In contrast to what was found in mouse macrophages, a recent study determined that tetra-acylated F. novicida LPS activates capsase-4, suggesting that human noncanonical inflammasome can recognizes under-acylated LPS (Lagrange et al., 2018). This data along with other
human caspase-4 inflammasome studies indicates that caspase-4 can recognize different types of LPS variants with different numbers of acyl chains (Casson et al., 2015; Goddard et al., 2019; Knodler et al., 2014; Lagrange et al., 2018; Srisaowakarn et al., 2020). However, there are no further studies that investigate human noncanonical inflammasome responses to LPS variants that are differentially phosphorylated or acylated.

1.11. Dissertation Aims

Gram-negative bacterial pathogens are responsible for a significant number of hospital-acquired infections, making them a major public health concern. Additionally, antibiotic resistance is on the rise, making it difficult to control many bacterial infections. Inability to control these bacterial infections can lead to sepsis, an overwhelming systemic immune response, and ultimately death if left untreated. Although there are successful treatments for sepsis in mice using immunomodulators, these treatments fail to alleviate sepsis in humans. The reasons for these failures are poorly understood, but one possible reason could be due to the differences between mouse and human innate immune genes that may play a role in the responses to gram-negative bacterial infections. Importantly, mouse studies do not always translate to what happens in humans. Therefore, it is important to understand human innate immune responses to gram-negative bacterial infections in order to gain insight into the cellular components or signaling pathways that can be targeted to enhance or limit the immune response. L. pneumophila is a gram-negative, vacuolar pathogen that replicates in
macrophages and serves as a valuable model pathogen as it robustly activates immune response pathways including the inflammasome. IFN-priming promotes murine inflammasome responses to *L. pneumophila*, but whether and how IFN influences human inflammasome responses to *L. pneumophila* has not been investigated. Additionally, LPS from gram-negative bacteria is a potent activator of the intracellular noncanonical inflammasome; however, it appears that murine caspase-11 is differentially activated depending on the LPS variant, while it is unclear whether human caspase-4 is activated by different LPS variants. To better understand human inflammasome responses to *L. pneumophila* in the context of IFN-γ or to different LPS variants, this dissertation will focus on the following aims (Fig. 1.1):

**Figure 1.1. Human inflammasome responses to *L. pneumophila* and LPS variants.** This dissertation will focus on the role of IFN-γ and GBP s on human inflammasome responses to *L. pneumophila* as well as the roles of caspase-4 and caspase-5 responses to LPS variants.
Aim 1: Determine how IFN-γ promotes human inflammasome responses during *L. pneumophila* infection

IFN-treatment in mice leads to upregulated inflammasome response to a variety of bacteria, including *L. pneumophila*. Specifically, there are unknown IFN-induced factors that promote the rupture of the LCV, while murine GBPs induce bacteriolysis for the release of DNA and LPS into the cytosol for inflammasome sensing and activation. Mice and humans differ in the number of GBPs, and recent findings indicate that human GBP2 promotes caspase-4 inflammasome in response to the cytosolic bacteria, *F. novicida*. However, whether human GBPs contribute to inflammasome responses to vacuolar pathogens is poorly understood. In CHAPTER 2, we investigate whether IFN-γ and human GBPs promote inflammasome response to the vacuolar pathogen, *L. pneumophila*, and found that human GBP1 mediates maximal inflammasome response and promotes the rupture of the pathogen vacuole during *L. pneumophila* infection.

Aim 2: Determine human noncanonical inflammasome responses to different LPS variants

Based on studies testing LPS from different gram-negative bacteria, under-acylated variants evade caspase-11 detection in mice, while one study showed that tetra-acylated LPS activates caspase-4 in humans. Whether the number, position, or length of acyl chains on LPS as well as the number of phosphate groups determines activation of the human noncanonical inflammasome has not been studied. In CHAPTER 3, we investigate the activation of caspase-4 and
caspase-5 in response to different lipooligosaccharide variants derived from Y. *pestis* in order to determine whether there are differences or similarities in their activation. Additionally, we employed CRISPR/Cas9 to knock out caspase-4, caspase-5, GBP1, and GBP2 in THP-1 cell lines. Our preliminary results suggest that caspase-4, but not caspase-5, is activated in response to *E. coli* LPS. Future studies with these cell lines are needed to determine the role caspase-4 and -5 in response to the *Y. pestis* LOS, as well as to determine the role of human GBPs (GBP1 and GBP2) in response to LPS or LOS derived from *L. pneumophila*, *E. coli*, and *Y. pestis*. 
CHAPTER 2

Human GBP1 promotes pathogen vacuole rupture and inflammasome activation during Legionella pneumophila infection

This chapter contains large portions of a submitted manuscript titled “Human GBP1 promotes pathogen vacuole rupture and inflammasome activation during Legionella pneumophila infection” by Antonia. R. Bass and Sunny Shin.
2.1. Abstract

The inflammasome is an essential component of host defense against intracellular bacterial pathogens, such as *Legionella pneumophila*, the causative agent of the severe pneumonia Legionnaires’ disease. Inflammasome activation leads to recruitment and activation of caspases, which promote IL-1 family cytokine release and pyroptosis. In mice, interferon (IFN) signaling promotes inflammasome responses against *L. pneumophila*, in part through the functions of a family of IFN-inducible GTPases known as guanylate binding proteins (GBPs). Within murine macrophages, IFN signaling promotes rupture of the *L. pneumophila*-containing vacuole (LCV), whereas GBPs are dispensable for vacuole rupture. Instead, GBPs facilitate the lysis of cytosol-exposed *L. pneumophila*. In contrast to mouse GBPs, the functions of human GBPs in inflammasome responses to *L. pneumophila* are poorly understood. Here, we show that IFN-γ promotes caspase-1, caspase-4, and caspase-5 inflammasome activation during *L. pneumophila* infection and upregulates GBP expression in primary human macrophages. We find that human GBP1 is important for maximal IFN-γ-driven inflammasome responses to *L. pneumophila*. Furthermore, IFN-γ signaling promotes the rupture of LCVs. Similar to murine GBPs, human GBP1 and GBP2 target LCVs in a T4SS-dependent manner. However, in contrast to murine GBPs, human GBP1 promotes vacuolar lysis of the LCV, resulting in increased bacterial access to the host cell cytosol. Our findings show a key role for human GBP1 in targeting and disrupting pathogen-containing vacuoles and
reveal mechanistic differences in how mouse and human GBPs promote inflammasome responses to *L. pneumophila*.

### 2.2. Significance Statement

The bacterial pathogen *Legionella pneumophila*, the causative agent of the severe pneumonia Legionnaires’ disease, resides within a host-derived vacuole inside macrophages. In response, the macrophage directs assembly of a multiprotein signaling complex termed the inflammasome, which mediates cell death and release of cytokines important for antibacterial defense. The cytokine interferon-gamma (IFN-γ) potentiates inflammasome responses by inducing expression of host factors, including guanylate-binding proteins (GBPs). In mice, IFN signaling promotes rupture of the *L. pneumophila*-containing vacuole (LCV). Mouse GBPs do not mediate LCV disruption, but instead facilitate lysis of cytosol-exposed bacteria. In contrast, the functions of human GBPs are poorly understood. Here, we show that human GBP1 promotes inflammasome responses to *L. pneumophila*. In contrast to mouse GBPs, we find that human GBP1 targets and disrupts the LCV. These findings provide insight into the role of human GBPs in antibacterial defense and reveal mechanistic differences in how mouse and human GBPs function.

### 2.3. Introduction

The innate immune response to bacterial pathogens is essential for mediating host defense and bacterial clearance. This response is initiated through
the recognition of conserved microbial components known as pathogen-associated molecular patterns (PAMPs) by host pattern recognition receptors (PRRs) (Janeway, 1989; Takeuchi & Akira, 2010). In particular for intracellular bacteria, a subset of cytoplasmic PRRs that detect bacterial components contaminating the host cell cytosol and other activities associated with invading pathogens has been implicated in host defense. Upon activation, host sensors such as the nucleotide-binding oligomerization domain-like receptors (NLRs) mediate the formation of a multimeric protein complex termed the inflammasome. Inflammasome activation triggers a cascade of immune responses that culminate in the release of IL-1 family cytokines and an inflammatory form of cell death termed pyroptosis. This response alerts the body of the infection and recruits other innate immune cells to the site of infection, thereby promoting bacterial control and clearance.

The two inflammasomes that have been described are the canonical and noncanonical inflammasomes. In response to a diverse range of ligands, canonical inflammasomes recruit and activate the cysteine protease caspase-1 to promote the processing and secretion of the proinflammatory cytokines IL-1β and IL-18 (Martinon, Burns, & Tschopp, 2002; Ting, Willingham, & Bergstralh, 2008). Additionally, an alternative caspase-1-independent inflammasome termed the noncanonical inflammasome mediates inflammatory responses to gram-negative bacteria (Aachoui et al., 2013; Broz et al., 2012; Case et al., 2013; Casson et al., 2013; Kayagaki et al., 2011; Lamkanfi & Dixit, 2014; Rathinam et al., 2012). The noncanonical inflammasome is formed by caspase-11 in mice and two orthologs
in humans, caspase-4 and caspase-5; these caspases are activated upon binding bacterial lipopolysaccharide (LPS), a potent PAMP and major outer membrane lipid component of gram-negative bacteria (Hagar et al., 2013; Kayagaki et al., 2013; Shi et al., 2014). Following their activation, these inflammatory caspases cleave the substrate gasdermin-D (GSDMD). Upon cleavage, the GSDMD N-terminal fragment translocates to the plasma membrane and oligomerizes to form a pore, leading to pyroptosis (Kayagaki et al., 2015; Shi et al., 2015). Death of the infected cell eliminates the replicative niche for intracellular pathogens and leads to the clearance of bacteria through various mechanisms including the uptake of the bacteria within pore-induced intracellular traps (PITs) by neutrophils and clearance by efferocytosis (Jorgensen, Zhang, Krantz, & Miao, 2016).

Inflammasome responses are potentiated by priming signals recognized by plasma membrane receptors that upregulate the production of inflammatory cytokines and inflammasome components. During an infection, toll-like receptors play a major role in promoting the expression of innate immune genes. Additionally, type I and type II IFNs produced during infection promote inflammasome responses in mice. A subfamily of IFN-upregulated GTPases called GBPs are particularly important in promoting inflammasome responses to gram-negative bacteria in mice (Balakrishnan, Karki, Berwin, Yamamoto, & Kanneganti, 2018; Finethy et al., 2017; B. H. Kim et al., 2016; B. C. Liu et al., 2018; MacMicking, 2004; Meunier et al., 2014; Meunier et al., 2015; Pilla et al., 2014; Shenoy et al., 2012; Zwack et al., 2017). Mouse GBPs can localize to pathogen-containing vacuoles or bacterial membranes of cytosol exposed bacteria (B. H. Kim et al.,
2011; Man et al., 2016; Meunier et al., 2015; Piro et al., 2017; Santos et al., 2018; Wandel et al., 2017). However, the precise steps regulated by GBPs in promoting inflammasome activation are unclear. A study using Salmonella Typhimurium indicated that mouse GBPs promote rupture of pathogen-containing vacuoles (PCVs), whereas other studies with Francisella novicida and L. pneumophila indicate that GBPs function downstream of PCV rupture and facilitate bacteriolysis, resulting in cytosolic release of bacterial components that subsequently trigger inflammasome activation (B. C. Liu et al., 2018; Man et al., 2015; Man et al., 2016; Meunier et al., 2014; Meunier et al., 2015). Additionally, GBPs were found to promote caspase-11 activation in response to transfected LPS and, therefore, revealed that GBPs can operate downstream of vacuolar and outer membrane lysis (Pilla et al., 2014). Mouse GBPs can also promote inflammasome responses in the absence of targeting the PCV, as is the case with the vacuolar pathogen Chlamydia muridarum (Finethy et al., 2015). It is still unclear how mouse GBPs mediate these various functions, although one study showed that GBPs recruit the immunity-related GTPase (IRG) IRGB10 to mediate bacteriolysis (Man et al., 2016).

While studies in mice have linked the functions of IFN signaling and GBPs to inflammasome activation, the degree to which the function of murine GBPs mirror their human counterparts is unknown, as the significant differences in immune genes between mice and humans, including in the GBP superfamily, could translate into differences in immune mechanisms. Notably, mice have 11 GBPs, whereas humans only have seven GBPs (Olszewski et al., 2006). The functions of
human GBP1 colocalizes to the outer membrane of the cytosolic pathogen *Shigella flexneri* and further recruits additional GBPs, specifically GBP2, 3, 4, and 6, to inhibit the actin-based motility of *S. flexneri* (Piro et al., 2017; Wandel et al., 2017). Additionally, human GBP1 and GBP5 promote inflammasome responses to *S. Typhimurium*, which can reside within a specialized vacuole or in the cytosol, while human GBP2 promotes inflammasome responses to the cytosolic pathogen *F. novicida* (Fisch et al., 2019; Lagrange et al., 2018; Shenoy et al., 2012). These findings indicate that different human GBPs function in a bacterium-specific manner. Furthermore, human GBP1 associates with sterilely lysed host vacuoles, and therefore, it may be possible that human GBPs can also associate with bacteria-containing vacuoles that are host derived, similar to mouse GBPs (E. M. Feeley et al., 2017; Piro et al., 2017).

Here, we sought to define the role of IFN-γ signaling and human GBPs in human inflammasome responses to the vacuolar pathogen *L. pneumophila*. *L. pneumophila* is a gram-negative intracellular bacterial pathogen that infects alveolar macrophages and is the causative agent of the severe pneumonia known as Legionnaires’ Disease (Fraser et al., 1977). Upon uptake, *L. pneumophila* resides within a *L. pneumophila*-containing vacuole (LCV) and relies on the Dot/Icm type IV secretion system (T4SS) to survive within the LCV (Berger & Isberg, 1993; Berger et al., 1994; Brand et al., 1994; Horwitz, 1983; X. Liu & Shin, 2019; Roy & Isberg, 1997). The T4SS injects over 300 effector proteins, many of
which enable *L. pneumophila* to evade the endolysosomal pathway and modify its LCV into an ER-derived replicative compartment (Ensminger & Isberg, 2009; Hubber & Roy, 2010; Isaac & Isberg, 2014; Ninio & Roy, 2007; Roy, 2002; Vogel & Isberg, 1999). Despite being essential for *L. pneumophila* virulence, T4SS activity triggers robust canonical and noncanonical inflammasome activation in human macrophages (Casson et al., 2015). The role of IFN signaling and GBPs in promoting human inflammasome responses to *L. pneumophila* is unknown. A recent study found that human GBP1 directly binds to *L. pneumophila* as well as other gram-negative bacterial pathogens (Kutsch et al., 2020); however, this study did not clarify whether GBP1 binds to the LCV or outer membrane.

In this study, we found that IFN-γ promotes inflammasome responses to *L. pneumophila* in a T4SS-dependent manner in both immortalized and primary human macrophages. We further determined that human GBP1 was essential for maximal inflammasome activation and that IFN-γ-primed macrophages had a significant increase in GBP1 and GBP2 localization to the LCV and/or outer membrane of *L. pneumophila* compared to unprimed macrophages. GBP1 and GBP2 were recruited to *L. pneumophila*’s vacuole and/or outer membrane in a T4SS-dependent manner, indicating that human GBPs detect pathogen-containing vacuoles containing virulence-associated bacterial secretion systems. Additionally, IFN-γ treatment led to the increased rupture of LCVs and exposure of *L. pneumophila* to the host cell cytosol, in part through a mechanism involving GBP1. Overall, our findings indicate that IFN-γ-dependent human GBP1 responses promote rupture of the LCV, facilitating bacterial detection in the cytosol.
to enhance inflammasome activation. Furthermore, as human GBP1 facilitates LCV rupture, in contrast to mouse GBPs, which are dispensable for LCV rupture, our findings suggest that mouse and human GBPs have evolved distinct functions.

2.4. Results

2.4.1. IFN-γ promotes inflammasome activation in human macrophages during L. pneumophila infection.

IFN-γ promotes human inflammasome responses to the cytosolic pathogen F. novicida (Lagrange et al., 2018). However, whether IFN-γ upregulates inflammasome responses to a vacuolar pathogen in human macrophages is poorly understood; therefore, we sought to test this with L. pneumophila. To determine whether IFN signaling increases inflammasome activation in response to L. pneumophila, we primed macrophages with IFN-γ prior to infection with L. pneumophila. L. pneumophila requires a T4SS to translocate bacterial products into the host cell cytosol; therefore, we also investigated whether IFN-γ-mediated inflammasome responses to L. pneumophila are dependent on its T4SS. Unprimed or IFN-γ-primed phorbol 12-myristate 13-acetate (PMA)-differentiated THP-1 macrophages were infected with a L. pneumophila dotA mutant lacking a functional T4SS (T4SS-) or a T4SS-sufficient (T4SS+) strain lacking flagellin (ΔflaA) in order to focus on NAIP-independent inflammasome responses. Unprimed THP-1 cells infected with T4SS- Lp or mock infected exhibited little to no cell death, whereas cells infected with T4SS+ Lp underwent increased cell death and IL-1 family cytokine release (Fig. 2.1 A and B), consistent with previous findings showing that
*L. pneumophila* induces T4SS-dependent inflammasome responses in THP-1 cells (Casson et al., 2015). THP-1 macrophages that were primed with IFN-γ and infected with T4SS+ *Lp* had a significant increase in cell death compared to unprimed macrophages (Fig. 2.1 A and 2.2 A). We observed a notable difference in the amount of cell death for THP-1 cells depending on the assay used. Specifically, we found that IFN-γ-primed THP-1 cells infected with T4SS+ *Lp* resulted in around 10% cell death by measurement of LDH at 2 hpi (Fig. 2.1 A), whereas these same cells led to 75% cell death when measuring propidium iodide (PI) uptake (Fig. 2.2 A). This difference may be due to the size of the GSDMD pores in these THP-1 cells, which may be wide enough to allow PI to be easily released due to its smaller size compared to the LDH molecule. IFN-γ-primed macrophages infected with T4SS+ *Lp* also had significantly elevated levels of IL-1β and IL-18 secretion compared to unprimed macrophages (Fig. 2.1 B).

Interestingly, we noticed significantly increased secretion of IL-1β and IL-18 levels in T4SS- *Lp*-infected THP-1 cells primed with IFN-γ compared to unprimed cells. However, unprimed and IFN-γ-primed THP-1 cells infected with T4SS- *Lp* showed significantly lower levels of cytokine release compared to their T4SS+ *Lp* infected counterparts. This indicates that there is a T4SS-dependent cytokine release in both unprimed and IFN-γ-primed THP-1 cells, where the latter shows a more dominant phenotype. Furthermore, we observed processing of IL-1β into its mature p17 form in the supernatant of both T4SS- and T4SS+ *Lp*-infected THP-1 cells primed with IFN-γ (Fig. 2.1 C). These data indicate that IFN-γ priming promotes inflammasome responses to both T4SS- and T4SS+ *L. pneumophila* in THP-1 cells.
cells, although maximal inflammasome activation occurs in primed cells infected with bacteria that harbor a functional T4SS.

We next asked whether IFN-γ also enhances inflammasome responses to *L. pneumophila* in primary human monocyte-derived macrophages (hMDMs) derived from healthy human donors. IFN-γ-primed hMDMs infected with T4SS+ *Lp* also exhibited significantly increased levels of cell death (Fig. 2.1 D), as well as IL-1β and IL-18 release (Fig. 2.1 E), compared to unprimed or IFN-γ-primed hMDMs that were uninfected or infected with T4SS- *Lp*. Overall, our data indicate that IFN-γ promotes inflammasome responses and IL-1 family cytokine release in response to *L. pneumophila* infection in both PMA-differentiated THP-1 cells and primary hMDMs.
PBS for two or four hours, respectively. (A and D) Cell death was measured using

\[ (100 \text{ U/ml}) \]

overnight and infected with T4SS

\[ \text{macrophages (hMDMs)} \]

differentiated THP

\[ \text{pneumophila} \]

Figure 2.1. IFN-\( \gamma \) promotes inflammasome activation in response to \( \text{L. pneumophila} \) in human macrophages. Phorbol 12-myristate 13-acetate (PMA)-differentiated THP-1 cells (A, B, C) or primary human monocyte-derived macrophages (hMDMs) (D, E, F) were either left unprimed or primed with IFN-\( \gamma \) (100 U/ml) overnight and infected with T4SS- \( \text{Lp} \), T4SS+ \( \text{Lp} \), or mock-infected with PBS for two or four hours, respectively. (A and D) Cell death was measured using

\[ \text{IL-18 (pg/mL)} \]

and

\[ \text{IL-18 2hpi} \]

were either left unprimed or primed with IFN-\( \gamma \) (100 U/ml) overnight and infected with T4SS- \( \text{Lp} \), T4SS+ \( \text{Lp} \), or mock-infected with PBS for two or four hours, respectively. (A and D) Cell death was measured using

\[ \text{IL-18 (pg/mL)} \]

and

\[ \text{IL-18 2hpi} \]

were either left unprimed or primed with IFN-\( \gamma \) (100 U/ml) overnight and infected with T4SS- \( \text{Lp} \), T4SS+ \( \text{Lp} \), or mock-infected with PBS for two or four hours, respectively. (A and D) Cell death was measured using

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and

\[ \text{IL-18 2hpi} \]

were either left unprimed or primed with IFN-\( \gamma \) (100 U/ml) overnight and infected with T4SS- \( \text{Lp} \), T4SS+ \( \text{Lp} \), or mock-infected with PBS for two or four hours, respectively. (A and D) Cell death was measured using

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and

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and

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\[ \text{IL-18 (pg/mL)} \]

and

\[ \text{IL-18 2hpi} \]

were either left unprimed or primed with IFN-\( \gamma \) (100 U/ml) overnight and infected with T4SS- \( \text{Lp} \), T4SS+ \( \text{Lp} \), or mock-infected with PBS for two or four hours, respectively. (A and D) Cell death was measured using

\[ \text{IL-18 (pg/mL)} \]

and

\[ \text{IL-18 2hpi} \]

were either left unprimed or primed with IFN-\( \gamma \) (100 U/ml) overnight and infected with T4SS- \( \text{Lp} \), T4SS+ \( \text{Lp} \), or mock-infected with PBS for two or four hours, respectively. (A and D) Cell death was measured using

\[ \text{IL-18 (pg/mL)} \]

and

\[ \text{IL-18 2hpi} \]

were either left unprimed or primed with IFN-\( \gamma \) (100 U/ml) overnight and infected with T4SS- \( \text{Lp} \), T4SS+ \( \text{Lp} \), or mock-infected with PBS for two or four hours, respectively. (A and D) Cell death was measured using
lactate dehydrogenase release assay and normalized to mock-infected cells. (B and E) IL-1β and IL-18 levels in the supernatant were measured by ELISA. (C, F) Immunoblot analysis was conducted on supernatants (sup) and lysates from THP-1 cells (C) or hMDMs (F) for full-length IL-1β (pro-IL-1β), cleaved IL-1β (mature IL-1β), full length caspase-1 (pro-casp1), cleaved casp1 (casp1 p20), pro-casp4, cleaved caspase-4 (casp4 p32), pro-casp5, casp5 p35, full-length Gasdermin-D (GSDMD), intermediate and cleaved GSDMD (GSDMD int. and GSDMD p30), and β-actin. Western blots are representative of three independent experiments. (A and B) Shown are the results representative of three independent experiments. *P<0.05, **P<0.01, and ***P<0.001 by unpaired t-test. (D and E) Shown are the pooled results of six independent experiments using hMDMs from different healthy human donors. Each data point represents the mean of triplicate infected wells from an individual donor. *P< 0.05, **P<0.01, and ***P< 0.001 by paired t-test.

2.4.2. Caspase-1, caspase-8, and additional caspases promote inflammasome activation in response to L. pneumophila.

We next investigated which caspases are involved in promoting inflammasome activation in response to L. pneumophila following IFN-γ priming. L. pneumophila activates the human noncanonical caspase-4 inflammasome in macrophages that were not initially primed (Casson et al., 2015), but whether IFN-γ priming affects canonical or noncanonical inflammasome activation in L. pneumophila-infected human macrophages has not been studied. We observed caspase-1 processing into its mature p20 form in T4SS+ Lp-infected hMDMs primed with IFN-γ (Fig. 2.1 F). Both caspase-4 and caspase-5 were upregulated at the RNA and protein level following IFN-γ priming of THP-1 cells and hMDMs (Fig. 2.1 F and 2.2 B-D). Additionally, we observed release of full-length and processed forms of caspase-4 and caspase-5, as well as GSDMD processing and release, into the supernatants of IFN-γ-primed hMDMs infected with T4SS+ Lp (Fig. 2.1 F). Intriguingly, we noticed that the lysates of unprimed primary hMDMs either mock-
infected or infected with T4SS- *Lp* showed caspase-5 expression, suggesting that it may be constitutively expressed in these cells (Fig. 2.1 F). Additionally, IFN-γ treatment resulted in the generation of processed caspase-5 p35 subunit in primary hMDMs and THP-1 cells and indicates that caspase-5 is undergoing autoprocessing in the presence or absence of infection. The upregulation of caspase-4 and caspase-5 by IFN-γ treatment in THP-1 cells and primary hMDMs as well as the released processed forms of these caspases in the supernatant of IFN-γ-primed hMDMs may account for the observed increase in cell death and cytokine release during *L. pneumophila* infection. Together, these data demonstrate that caspase-1, -4, and -5 are processed into their mature forms upon IFN-γ priming and infection with T4SS+ *Lp* (Fig. 2.1 F).
Figure 2.2. IFN-γ promotes inflammasome activation in response to L. pneumophila in human macrophages and upregulates caspase-4 and caspase-5. (A) PI uptake time course of PMA-differentiated THP-1 cells that were either left unprimed or primed with IFN-γ (100 U/ml) for 24 h and infected with T4SS+ Lp MOI=50. Data are representative of three independent experiments with each data point representing the mean of triplicate infected wells. *P< 0.05, **P< 0.01, ***P< 0.001 by unpaired t-test. PMA-differentiated THP-1 cells (B) or primary hMDMs (D) were either left unprimed or primed with IFN-γ (100 U/ml) for 18 or 20 hours, respectively. Transcrit levels of caspase-4 (CASP4) and caspase-5 (CASP5) were determined by quantitative RT-PCR. Fold change was calculated by normalizing to the housekeeping gene HPRT for each sample and then to the unprimed sample. Relative expression of each gene was calculated by normalizing to the housekeeping gene HPRT. Shown are pooled results of three independent experiments (B) or six independent experiments using hMDMs from different healthy human donors (D), with each data point representing the value for each experiment (B) or an individual donor (D). *P< 0.05, **P< 0.01, and ****P< 0.0001 by paired t-test. (C) PMA-differentiated THP-1 cells were either left unprimed or primed with IFN-γ (100 U/ml) overnight and infected with T4SS- Lp, T4SS+ Lp, or mock-infected with PBS for two hours. Immunoblot analysis was performed on
PMA-differentiated THP-1 lysates for full-length caspase-4 (pro-casp4), full-length caspase-5 (pro-casp5), caspase-5 intermediates (casp5 p44 and casp5 p35), and \( \beta \)-actin (same \( \beta \)-actin blot as shown in Fig. 2.1C since from same experiment). Western blots are representative of three independent experiments.

We next tested whether caspase activity is required for inflammasome responses to *L. pneumophila* in the presence or absence of IFN-\( \gamma \). Our previous data indicated that caspase-1 is cleaved and activated upon IFN-\( \gamma \) priming and infection with T4SS+ *Lp* (Fig. 2.1 F). Therefore, we wanted to confirm that caspase-1 is involved in inflammasome responses to *L. pneumophila* through inhibitor treatment. Additionally, recent studies have found novel functions of caspase-8 being implicated in inflammasome responses to a variety of bacteria (Antonopoulos et al., 2015; Man et al., 2014; Man et al., 2013; Sarhan et al., 2018); thus, we wanted to determine whether caspase-8 is also promoting inflammasome responses to *L. pneumophila*. Cell death was significantly decreased in either unprimed or IFN-\( \gamma \)-primed hMDMs that were treated with the pan-caspase inhibitor ZVAD prior to infection with T4SS+ *Lp*, compared to the levels of cell death observed in vehicle control-treated cells (Fig. 2.3 A). Intriguingly, we observed higher cell death in unprimed and IFN-\( \gamma \)-primed hMDMs that were treated with DMSO and infected with T4SS+ *Lp* compared to the unprimed and IFN-\( \gamma \)-primed hMDMs that were only infected with T4SS+ *Lp* in Fig. 2.1 D. This may suggest that DMSO propagates cell death in the presence of T4SS+ *Lp* through additional activation of cell stress pathways or inflammasomes, such as the NLRP3 inflammasome. IL-1\( \beta \) and IL-18 secretion was also significantly decreased in IFN-\( \gamma \)-primed hMDMs treated with ZVAD, compared to DMSO-treated hMDMs (Fig. 2.3
B and C). In addition, IL-18 secretion was significantly decreased in unprimed hMDMs treated with ZVAD. Importantly, treatment with the caspase-1-specific inhibitor YVAD or the caspase-8-specific inhibitor IETD significantly reduced cell death and IL-1\(\beta\) and IL-18 secretion in IFN-\(\gamma\)-primed hMDMs, compared to DMSO-treated hMDMs. Similar to ZVAD treated cells, YVAD and IETD also significantly reduced cell death and IL-18 release in unprimed hMDMs. Interestingly, we observed lower amounts of cell death and IL-1 family cytokine release in hMDMs treated with the broader-spectrum inhibitor ZVAD compared to treatment with the caspase-1 or caspase-8 selective inhibitors. These data indicate that caspase-1, caspase-8, and likely additional caspases are involved in promoting inflammasome responses to \textit{L. pneumophila}. Furthermore, we noticed that unprimed and IFN-\(\gamma\)-primed hMDMs infected with T4SS+ \textit{Lp} had comparable levels of IL-1\(\beta\), which may be due to donor variability as well as to the low levels of IL-1\(\beta\) production from lack of TLR priming. As both caspase-4 and caspase-5 are processed in IFN-\(\gamma\)-primed T4SS+ \textit{Lp}-infected hMDMs (Fig. 2.1 F), these noncanonical inflammatory caspases may play a role together with caspase-1 and caspase-8 to promote IFN-\(\gamma\)-mediated inflammasome responses.
Figure 2.3. Caspase-1, caspase-8, and additional caspases promote inflammasome activation in response to *L. pneumophila*. (A, B, C) Primary hMDMs were left unprimed or primed with IFN-γ (100 U/mL) overnight and treated with the inhibitors ZVAD, YVAD, IETD, MCC950, or DMSO control for one hour followed by infection with T4SS+ *Lp* for four hours. (A) Cell death was measured using lactate dehydrogenase release assay and normalized to mock-infected cells. (B and C) IL-1β and IL-18 levels in the supernatant were measured by ELISA. Shown are the pooled results of four to six independent experiments using hMDMs from different healthy human donors. Each data point represents the mean of triplicate infected wells from an individual donor. *P< 0.05, **P< 0.01, and ***P< 0.001 by paired t-test.
We also wanted to determine whether the NLRP3 inflammasome plays a role in response to *L. pneumophila* during IFN-γ treatment, since it has been shown that the NLRP3 inflammasome can be activated downstream of caspase-11 during *L. pneumophila* infection (Case et al., 2013; Casson et al., 2013). While the NLRP3 inhibitor MCC950 did suppress inflammasome responses upon LPS and nigericin treatment, the inhibitor had no effect on cell death or IL-1β and IL-18 levels in both unprimed and IFN-γ-primed hMDMs infected with *L. pneumophila* (Fig. 2.4). In addition, ZVAD, YVAD, and IETD treatments significantly decreased IL-18 levels in hMDMs treated with LPS and nigericin. These results suggest that the NLRP3 inflammasome does not play a role in response to *L. pneumophila* in the context of IFN-γ priming. However, it is still possible that the NLRP3 inflammasome contributes to controlling *L. pneumophila* infection in human macrophages at a later timepoint, since we only looked four hours after infection. Collectively, our data show that caspase-1, caspase-8, and likely caspase-4 and caspase-5 participate in inflammasome responses to *L. pneumophila* infection in IFN-γ-primed hMDMs.
Figure 2.4. NLRP3 inflammasome activation is inhibited by MCC950, YVAD, ZVAD, and IETD. (A) Primary hMDMs were primed with *E. coli* LPS (0.5 μg/mL) for four hours and treated with nigericin (10 μM) for four hours. Inhibitors were added one hour before addition of nigericin. IL-1β and IL-18 levels in the supernatant were measured by ELISA. Shown are the results representative of three independent experiments. *P< 0.05, ***P< 0.001, ****P< 0.0001 by unpaired t-test.

2.4.3. IFN-γ upregulates human GBPs.

IFN-γ induces expression of a large number of genes that contribute to antimicrobial defense. In mice, two IFN-inducible gene families that promote inflammasome activation in macrophages are the GBPs and IRGs. Their assigned functions include binding and rupturing the phagosome of vacuolar pathogens, as well as directly lysing bacteria that escape the phagosome and enter the cytosol (B. C. Liu et al., 2018; Man et al., 2016; Meunier et al., 2014; Meunier et al., 2015). These activities lead to release of pathogen-derived products such as lipopolysaccharide (LPS) and DNA into the cytosol, resulting in downstream inflammasome activation. Mice have 11 GBPs and 23 IRGs, whereas humans have seven GBPs and two IRG genes (Bekpen et al., 2005). Human GBPs, like
their murine counterparts, are IFN-inducible, whereas human IRGs are not induced by IFN stimulation (Bekpen et al., 2005; Lagrange et al., 2018; Qin et al., 2017).

Thus, we chose to test whether human GBPs might play a role in the enhanced inflammasome responses of IFN-γ-primed cells to *L. pneumophila*. We first asked whether GBP expression is upregulated by IFN-γ in THP-1-derived macrophages and hMDMs. In THP-1 cells, we found that expression of all GBPs was induced in response to IFN-γ, and GBP1-5 mRNA levels were significantly upregulated in hMDMs following IFN-γ treatment (Fig. 2.5 A and B). Following IFN-γ-priming, we observed high relative expression of *GBP1, GBP2, GBP3, GBP4,* and *GBP5*, whereas there was very low relative expression of *GBP6* and *GBP7* in THP-1 cells (Fig. 2.6 A) and hMDMs (Fig. 2.6 B), in agreement with previous findings (Lagrange et al., 2018). Furthermore, priming hMDMs with increasing amounts of IFN-γ led to a dose-dependent increase in GBP mRNA levels (Fig. 2.5 C and 2.6 C). Protein levels of GBP1, GBP2, GBP4, and GBP5 were also increased in a dose-dependent manner in response to IFN-γ (Fig. 2.5 D). Thus, human GBPs are transcriptionally and translationally induced by IFN-γ in macrophages, in agreement with previous findings (Lagrange et al., 2018; Qin et al., 2017).
Figure 2.5. Human GBP s are transcriptionally and translationally upregulated in response to IFN-γ. PMA-differentiated THP-1 cells (A) or primary hMDMs (B) were either left unprimed or primed with IFN-γ (100 U/mL) for 18 or 20 hours, respectively. (C and D) hMDMs were left unprimed or primed with IFN-γ at the indicated concentrations for 20 hours. (A, B, C) Transcript levels of GBP1-7 were determined by quantitative RT-PCR and fold change was calculated by normalizing to the housekeeping gene HPRT for each sample and then to the unprimed sample. Shown are the pooled results of three independent experiments (A) or six independent experiments using hMDMs different healthy human donors.
(B), with each data point representing the value for each experiment (A) or an individual donor (B). *P< 0.05, **P< 0.01, and ***P< 0.001 by paired t-test. (C) Shown are the pooled results of four independent experiments using hMDMs from different healthy human donors and each data point represents the value of an individual donor. (D) Immunoblot analysis was conducted on lysates for GBP1, GBP2, GBP4, GBP5, and β-actin. Western blot is representative of four independent experiments using hMDMs from different healthy human donors.

Figure 2.6. Human GBPs are transcriptionally upregulated by IFN-γ in macrophages. PMA-differentiated THP-1 cells (A) or primary hMDMs (B) were either left unprimed or primed with IFN-γ (100 U/mL) for 18 or 20 hours, respectively. (C) hMDMs were left unprimed or primed with IFN-γ at the indicated concentrations for 20 hours. (A, B, C) Transcript levels of GBP1-7 were determined by quantitative RT-PCR and relative expression of each gene was calculated by
normalizing to the housekeeping gene HPRT. Shown are the pooled results of three independent experiments (A) or six independent experiments using hMDMs from different healthy human donors (B), with each data point representing the value for each experiment (A) or an individual donor (B). *P< 0.05 and **P< 0.01 by paired t-test. (C) Shown are the pooled results of four independent experiments using hMDMs from different healthy human donors and each data point represents the value of an individual donor.

2.4.4. Human GBP1 contributes to maximal IFN-γ-dependent inflammasome responses to L. pneumophila.

Since GBP1-5 were significantly upregulated in hMDMs, we next wanted to test whether these GBPs play a role in human inflammasome responses to L. pneumophila. We therefore individually silenced expression of GBP1-5 prior to IFN-γ treatment and T4SS+ Lp infection in hMDMs. Notably, specific knockdown of GBP1 significantly decreased cell death and IL-1β and IL-18 secretion following L. pneumophila infection in IFN-γ-primed hMDMs, indicating that GBP1 plays a non-redundant role in inflammasome responses against L. pneumophila infection (Fig. 2.7 A and B). Importantly, we do not observe complete decrease of cell death and cytokine release upon knockdown of human GBP1. This indicates that human GBP1 plays a partial role in promoting inflammasome responses during L. pneumophila infection, possibly through rupture of the LCV or bacteriolysis of the outer membrane in order for release of L. pneumophila components into the cytosol for inflammasome sensing. Additionally, we notice that the cell death is around 20% upon GBP1 knockdown of IFN-γ-primed hMDMs (Fig. 2.7 A), which is comparable to the amount of cell death we observe in unprimed hMDMs (Fig. 2.1 D). Thus, the remaining cell death observed is most likely due to inflammasome
activation by secreted T4SS effector molecules as well as cell stress signals that are typically activated upon detection of \textit{L. pneumophila}. GBP3 knockdown resulted in significantly decreased IL-1\(\beta\) release but did not affect cell death or IL-18 release. In contrast, knockdown with siRNAs against \textit{GBP2}, \textit{4}, and \textit{5} did not decrease cell death or cytokine secretion. Furthermore, we examined the knockdown efficiencies for hMDMs treated with siRNA for each GBP and found that siRNA knockdown was specific for each GBP and did not affect the expression levels of the remaining GBPs (Fig. 2.7 C). Collectively, these data indicate that human GBP1 is important for promoting maximal cell death and IL-1 family cytokine release.
Figure 2.7. GBP1 is required for maximal inflammasome activation. Primary hMDMs were transfected with 30 nM siRNA specific for individual GBP or scrambled control siRNA (siControl), primed with IFN-γ (100 U/mL) overnight, and infected with T4SS+ Lp for four hours. (A) Cell death was measured using lactate dehydrogenase release assay and normalized to mock infected cells. (B) IL-1β and IL-18 levels in the supernatant were measured by ELISA. (C) Transcript levels of GBP1-5 in ‘mock’ samples were determined by quantitative RT-PCR and fold change was calculated by normalizing to the housekeeping gene HPRT for each sample and then to the siControl sample. (A, B, C) Shown are the pooled results.
of three independent experiments using hMDMs from different healthy human donors. (A and B) Each data point represents the mean of triplicate infected wells from an individual donor. *P< 0.05, **P< 0.01, and ***P< 0.001 by paired t-test. (C) Each data point represents the value of an individual donor.

2.4.5. IFN-γ promotes GBP localization to *L. pneumophila* in a T4SS-dependent manner.

Since our data indicated that human GBP1 is required for maximal inflammasome activation during infection with *L. pneumophila*, we next wanted to elucidate how GBP1 could be promoting this response. Mouse Gbp2 colocalizes with *S. Typhimurium* and promotes the rupture of the *Salmonella*-containing vacuole (SCV), while its predicted human ortholog, GBP1, was recently shown to target and bind the outer membrane of *S. Typhimurium* to form a GBP complex and disrupt the membrane integrity for recruitment and activation of caspase-4 (Fisch et al., 2019; Fisch et al., 2020; Kutsch et al., 2020; Meunier et al., 2014; Santos et al., 2020; Wandel et al., 2020). In addition, recent findings showed that human GBP1 directly binds to gram-negative bacteria including *L. pneumophila*, although they did not determine whether GBP1 binds to the LCV or outer membrane (Kutsch et al., 2020). We hypothesized that human GBP1 might play a similar role as mouse Gbp2 during *S. Typhimurium* infection and would be predicted to colocalize with the LCV for vacuolar rupture in IFN-γ-primed macrophages. To test this hypothesis, we infected IFN-γ-primed and unprimed hMDMs with dsRED-expressing T4SS+ *Lp* and stained for GBP1. While there was little to no GBP1 expression or colocalization with *L. pneumophila* in unprimed cells, there was a significant increase in the percentage of infected cells containing
GBP1-positive *L. pneumophila* following IFN-γ priming (Fig. 2.8 A and B). Approximately 60% of infected cells contained *L. pneumophila* that colocalized with GBP1. In contrast, GBP1 was distributed throughout the cytoplasm in uninfected IFN-γ-primed hMDMs (Fig. 2.9 A). The secondary antibodies used for anti-GBP1 staining did not associate with *L. pneumophila* when used alone and only stained cells when primary anti-GBP1 antibodies were used (Fig. 2.9 B). These data indicate that GBP1 is recruited to *L. pneumophila* and/or the LCV within IFN-γ-primed hMDMs.

While it is unclear whether GBP1 binds to the LCV or the bacterial outer membrane, human GBP1 colocalizes with the outer membrane of the cytosolic bacterium, *S. flexneri*, and additional GBPs are also recruited to inhibit its actin motility (Piro et al., 2017; Wandel et al., 2017). Thus, we tested whether GBP2 also localized to *L. pneumophila*. We also observed a significantly increased percentage of hMDMs harboring GBP2+ *L. pneumophila* following IFN-γ priming compared to unprimed cells (Fig. 2.8 C and D), although to a lower extent compared to GBP1+ *L. pneumophila*. Furthermore, secondary antibodies used for anti-GBP2 staining did not stain when used alone and only colocalized with *L. pneumophila* when primary anti-GBP2 antibodies were used (Fig. 2.9 C). Collectively, these findings show that both GBP1 and GBP2 are recruited to *L. pneumophila* and/or the LCV in IFN-γ-primed hMDMs.

In mouse macrophages, colocalization of GBPs with *L. pneumophila* is dependent on the T4SS, while GBP colocalization with *Yersinia pseudotuberculosis* requires the presence of type III secretion system translocon...
components (E. M. Feeley et al., 2017; Zwack et al., 2017). These findings indicate that murine GBPs respond to secretion systems that are key signatures of bacterial virulence. It is also possible that GBPs associate with the virulence factors secreted by these pathogens to aid in the process of PCV rupture and bacterial access to the cytosol. However, whether human GBPs also detect PCVs that contain bacteria expressing virulence-associated secretion systems is unclear. Notably, only T4SS+ Lp, but not T4SS- Lp, exhibited robust colocalization with GBP1 and GBP2 in IFN-γ-primed hMDMs (Fig. 2.8 E-H). Collectively, these data suggest that GBP1 and GBP2 are upregulated in response to IFN-γ priming and following infection, are recruited to L. pneumophila in a T4SS-dependent manner.
Figure 2.8. IFN-γ promotes the colocalization of GBP1 and GBP2 with *L. pneumophila* in a T4SS-dependent manner. (A-D) Primary hMDMs were either left unprimed or primed with IFN-γ (100 U/mL) overnight and infected with dsRED-
expressing T4SS+ Lp for two hours. Representative fluorescence micrographs of anti-GBP1 (A) or anti-GBP2 (C) antibody staining in dsRED-T4SS+ Lp-infected hMDMs and quantification of the percentage of hMDMs containing GBP1+ Lp (B) or GBP2+ Lp (D) out of total infected hMDMs. Graphs show the mean and s.d. of technical triplicates and data are representative of three independent experiments using hMDMs from different healthy human donors. **P< 0.01 by unpaired t-test. (E-H) Primary hMDMs were primed with IFN-γ (100 U/mL) overnight and infected with dsRED-expressing T4SS- Lp or T4SS+ Lp for two hours. Representative fluorescence micrographs of anti-GBP1 (E) or anti-GBP2 (G) antibody staining in dsRED-T4SS- or dsRED-T4SS+ Lp-infected hMDMs and quantification of the percentage of hMDMs containing GBP1+ Lp (F) or GBP2+ Lp (H) out of total infected hMDMs. Graphs show the mean and s.d. of technical triplicates and data are representative of two independent experiments using hMDMs from different healthy human donors. *P< 0.05 and ****P<0.0001 by unpaired t-test.

**Figure 2.9. GBP1 is distributed throughout the cytoplasm in uninfected hMDMs.** Primary hMDMs were either left unprimed or primed with IFN-γ (100 U/mL) overnight and infected with dsRED-expressing T4SS+ Lp or left uninfected for two hours. (A) Representative fluorescence micrographs of anti-GBP1 staining in uninfected hMDMs. (B and C) Representative fluorescence micrographs of dsRED-T4SS+ Lp-infected hMDMs stained with only secondary-antibody anti-rabbit (B) or anti-mouse (C) Alexa Fluor 488. (A, B, C) Images are representative of three independent experiments using hMDMs from different healthy human donors.
2.4.6. IFN-γ and GBP1 promote the rupture of LCVs.

We next wanted to determine how IFN-γ and GBP1 promote increased inflammasome activation during *L. pneumophila* infection. We first tested whether IFN-γ treatment results in an increase of ruptured LCVs, which would allow *L. pneumophila* to become more accessible for recognition by cytosolic inflammasome sensors. We utilized a differential permeabilization assay to distinguish between vacuolar and cytosolic *L. pneumophila* in the presence and absence of IFN-γ priming (Meunier & Broz, 2015). We compared unprimed and IFN-γ-primed hMDMs that were infected with dsRED-expressing T4SS+ *Lp* and then treated with the detergent digitonin, which selectively permeabilizes the plasma membrane while leaving intracellular membranes intact. The cells were then immunostained with an antibody for *L. pneumophila*, followed by staining with an Alexa 488-labeled secondary antibody that fluoresces green. Thus, dsRED-expressing *L. pneumophila* contained within an intact vacuole only fluoresce red, while dsRED-expressing *L. pneumophila* within a ruptured vacuole will fluoresce both green and red (Fig. 2.10 A). We found that a significantly increased percentage of hMDMs primed with IFN-γ contained *L. pneumophila* that stained with anti-*L. pneumophila* antibody and fluoresced green compared to unprimed cells (Fig. 2.10 B and C). We observed around 25% of unprimed hMDMs contained cytosolic *L. pneumophila*, which may be due to additional IFN-independent host factors that contribute to the targeting and destabilization of the LCV. Treatment with the detergent saponin, which permeabilizes all cell membranes, resulted in
similar percentages of unprimed and IFN-γ-primed hMDMs containing bacteria that were stained by anti-\textit{L. pneumophila} antibody (Fig. 2.11 A and B). The secondary antibody stained only in the presence of anti-\textit{L. pneumophila} antibody (Fig. 2.11 C), indicating that the secondary antibody does not bind to \textit{L. pneumophila} directly. These results indicate that IFN-inducible host factors promote rupture of the LCV, resulting in increased \textit{L. pneumophila} exposure to the host cell cytosol.

Since GBP1 colocalizes with \textit{L. pneumophila} (Fig. 2.8 A and B) and GBP1 is important for maximal inflammasome responses to \textit{L. pneumophila} in IFN-γ-primed hMDMs (Fig. 2.7), we hypothesized that GBP1 might contribute to the disruption of LCV integrity. Therefore, we conducted the phagosome integrity assay in GBP1-silenced IFN-γ-primed hMDMs. We confirmed efficient and specific \textit{GBP1} knockdown at the mRNA and protein levels compared to control siRNA treatment (Fig. 2.10 D and E). Additionally, a significantly lower percentage of infected hMDMs treated with \textit{GBP1} siRNA contained GBP1+ \textit{L. pneumophila} compared to control siRNA-treated hMDMs (Fig. 2.11 D and E). Interestingly, a significantly decreased percentage of \textit{GBP1} siRNA-treated hMDMs contained bacteria that were stained by anti-\textit{L. pneumophila} antibody following digitonin permeabilization compared to hMDMs treated with control siRNA (Fig. 2.10 F and G), indicating that there is a significant decrease in the percentage of cells containing ruptured LCVs following \textit{GBP1} knockdown. In contrast, following saponin permeabilization of all cellular membranes, a similar percentage of hMDMs contained bacteria that stained positive for anti-\textit{L. pneumophila} antibody following control or GBP1 siRNA treatment (Fig. 2.11 G and H), whereas staining
with secondary antibody alone revealed negligible background staining (Fig. 2.11 F and I). Collectively, these findings indicate that GBP1 plays a key role in IFN-γ-dependent disruption of the LCV in primary human macrophages, thus allowing for increased access of *L. pneumophila* to the host cell cytosol.

**Figure 2.10. IFN-γ and GBP1 promote the rupture of LCVs in hMDMs.** (A) Schematic of vacuolar *Lp*, which fluoresces red, and cytosolic *Lp*, which is stained green and fluoresces red. (B and C) Primary hMDMs were either left unprimed or primed with IFN-γ (100 U/mL) overnight and infected with dsRED-expressing T4SS+ *Lp* for two hours. (B) Representative fluorescence micrographs of anti-*Lp* antibody staining followed by Alexa 488-conjugated secondary antibody staining in digitonin-permeabilized dsRED-T4SS+ *Lp*-infected hMDMs. (C) Quantification
of the percentage of hMDMs harboring cytosolic Lp out of total infected hMDMs. (D-G) Primary hMDMs were transfected with 5 pmol siRNA specific for GBP1 (siGBP1) or scrambled control siRNA (siControl) for at least 48 h, primed with IFN-γ (100 U/mL) overnight, and infected with dsRED-expressing T4SS+ Lp for two hours. (D) GBP1 transcript levels in ‘mock’ samples were determined by quantitative RT-PCR. Fold change was calculated by normalizing to the housekeeping gene HPRT and then to the siControl sample. (E) Immunoblot analysis was conducted on ‘mock’ lysates for GBP1, GBP2, and β-actin. (F) Representative fluorescence micrographs of anti-Lp antibody staining followed by Alexa 488-conjugated secondary antibody staining in digitonin-permeabilized dsRED-T4SS+ Lp-infected hMDMs. (G) Quantification of the percentage of hMDMs harboring cytosolic Lp out of total infected hMDMs. Graphs show the mean and s.d. of technical triplicates and data are representative of three independent experiments using hMDMs from different healthy human donors. *P<0.05 and **P<0.01 by unpaired t-test. (D and E) Data are representative of three independent experiments using hMDMs from different healthy human donors.
Figure 2.11. Controls for phagosome integrity assay and GBP1 immunostaining assay. (A-C) Primary hMDMs were either left unprimed or primed with IFN-γ (100 U/mL) overnight and infected with dsRED-expressing T4SS+ Lp for two hours. (A) Representative fluorescence micrographs of anti-Lp primary antibody and Alexa Fluor 488-conjugated anti-rabbit secondary antibody staining in saponin-permeabilized unprimed and IFN-γ-primed dsRED-T4SS+ Lp-infected hMDMs and (B) quantification of the percentage of anti-Lp antibody (Ab) positive hMDMs out of total infected hMDMs. (C) Representative fluorescence micrographs of unprimed and IFN-γ-primed dsRED-T4SS+ Lp-infected hMDMs stained with only secondary-antibody anti-rabbit Alexa Fluor 488 as a control for digitonin phagosome integrity assay. (D-I) Primary hMDMs were transfected with 5 pmol siRNA specific for GBP1 (siGBP1) or scrambled control siRNA (siControl)
for at least 48 h, primed with IFN-γ (100 U/mL) overnight, and infected with dsRED-expressing T4SS+ Lp for two hours. (D) Representative fluorescence micrographs of anti-GBP1 antibody staining in dsRED-T4SS+ Lp-infected hMDMs. (E) Quantification of the percentage of hMDMs containing GBP1+ Lp out of total infected hMDMs. (F) Representative fluorescence micrographs of IFN-γ-primed siControl and siGBP1 dsRED-T4SS+ Lp-infected hMDMs stained with only Alexa Fluor 488-conjugated anti-rabbit secondary antibody as a control for GBP1 immunostaining assay. (G) Representative fluorescence micrographs of anti-Lp primary antibody and Alexa Fluor 488-conjugated anti-rabbit secondary antibody staining in saponin-permeabilized IFN-γ-primed siControl and siGBP1 dsRED-T4SS+ Lp-infected hMDMs and (H) quantification of the percentage of anti-Lp Ab positive hMDMs out of total infected hMDMs. (I) Representative fluorescence micrographs of IFN-γ-primed siControl and siGBP1 dsRED-T4SS+ Lp-infected hMDMs stained with only Alexa Fluor 488-conjugated anti-rabbit secondary antibody as a control for digitonin phagosome integrity assay. (A-I) Data and images are representative of three independent experiments using hMDMs from different healthy human donors. ****P<0.0001 by unpaired t-test.

2.5. Discussion

Our data reveal that human GBP1 is crucial for robust inflammasome activation in response to *L. pneumophila* infection in IFN-γ-primed primary human macrophages. These findings are the first to report the role of human GBPs in inflammasome activation in response to *L. pneumophila* infection. We show that IFN-γ leads to enhanced cell death and proinflammatory cytokine release during *L. pneumophila* infection and that this inflammasome response involves caspase-1, caspase-4, caspase-5, and GSDMD processing. Although we conclude that IFN-γ-primed cells lead to a T4SS-dependent inflammasome response to *L. pneumophila*, we did observe a small but significant amount of cytokine release in IFN-γ-primed THP-1 cells that were infected with T4SS- Lp compared to unprimed cells. However, IFN-γ-primed THP-1 cells infected with T4SS- Lp showed significantly decreased cell death and cytokine release compared to cells infected
with T4SS+ *Lp*, indicating that there is a T4SS-dependent inflammasome response. The reason for release of cytokines upon infection with T4SS- *Lp* may be due to an alternative IFN-dependent innate immune pathway that is independent of GBPs, since we did not observe GBP localization to T4SS- *Lp*, and would be of interest to investigate in the future. We also find that GBP1 colocalizes with *L. pneumophila* in a T4SS-dependent manner and promotes increased access of *L. pneumophila* to the host cell cytosol, indicating that GBP1 facilitates disruption of the LCV. Our findings suggest a model in which human GBP1 promotes the liberation of *L. pneumophila* components into the host cell cytosol to allow for increased inflammasome sensing and activation. Intriguingly, murine GBPs do not disrupt the LCV, but rather promote outer membrane disruption of cytosolic *L. pneumophila* (B. C. Liu et al., 2018). Together, these findings suggest that human and murine GBPs play distinct roles in mediating inflammasome responses against *L. pneumophila*.

Although mice encode 11 GBPs and humans encode seven GBPs, there are some GBPs shared between mice and humans, with mouse Gbp2 and Gbp5 thought to be the orthologs of human GBP1 and GBP5, respectively (Olszewski et al., 2006). These murine orthologs may provide insight into the functions of human GBPs, since most experimental studies aimed at elucidating GBP functions have been conducted in mice. Mouse GBPs colocalize with pathogens that harbor bacterial secretion systems or bacterial translocon components (E. M. Feeley et al., 2017; Zwack et al., 2017). Mouse Gbp2 promotes lysis of the SCV and activation of the noncanonical inflammasome, while its ortholog human GBP1
colocalizes with S. Typhimurium and promotes caspase-4-mediated pyroptosis (Fisch et al., 2019; Meunier et al., 2014). Mouse GBPs do not mediate vacuole disruption for other bacterial pathogens, but instead facilitate lysis of cytosolic bacteria (B. C. Liu et al., 2018; Meunier et al., 2014; Meunier et al., 2015). Whether human GBP1 is recruited to pathogen-containing vacuoles and whether it promotes lysis of pathogen-containing vacuoles or bacteria was unknown. Importantly, our findings reveal that GBP1 targets the LCV in a T4SS-dependent manner and furthermore, that GBP1 promotes vacuolar disruption and increased exposure of *L. pneumophila* to the host cell cytosol. Thus, human and mouse orthologs may have both distinct and overlapping functions. Additional studies will further elucidate the roles of human GBPs in response to other bacterial infections.

Our data show that human GBP1 and GBP2 colocalize with *L. pneumophila* in a T4SS-dependent manner, but whether and how these GBPs are recruited and bound to the LCV and/or bacterial outer membrane still remains to be determined. Mouse Gbp2 colocalizes with bacterial pathogens containing bacterial secretion systems in a galectin-3-dependent manner (E. M. Feeley et al., 2017). Whether galectins facilitate human GBP1 recruitment to pathogen-containing vacuoles is unknown. Furthermore, human and mouse GBP1, GBP2, and GBP5 have a C-terminal CaaX prenylation motif that facilitates membrane binding and oligomerization with other GBPs (Vestal & Jeyaratnam, 2011). Human GBP1 colocalizes to the outer membrane of *S. flexneri* and colocalizes with *S. Typhimurium* in a manner dependent on its isoprenylation and GTPase activity (Fisch et al., 2019; Piro et al., 2017; Wandel et al., 2017). In addition, human GBP1
colocalizes with a *S. flexneri* mutant lacking the O-antigen less frequently than with the wild-type strain, indicating that host recognition of O-antigen enables GBP1 targeting to *S. flexneri* (Piro et al., 2017). It would be of interest to determine whether the CaaX motif in human GBP1 and GBP2 are necessary for colocalization with *L. pneumophila* and what bacterial or vacuolar components they are binding to. Although we found that GBP2 colocalized with *L. pneumophila*, siRNA-mediated silencing of GBP2 did not have an effect on inflammasome activation. It is possible that GBP2 is not required for inflammasome responses to *L. pneumophila* or that siRNA-mediated knockdown in primary hMDMs was not efficient enough to reveal a role for GBP2. Further studies will discern between these possibilities. Since we found that GBP1 promotes inflammasome activation, it would also be of interest to determine whether GBP1 may act as an initiator GBP that recruits additional GBPs, similar to what has been observed with *S. flexneri* (Li et al., 2017; Piro et al., 2017; Wandel et al., 2017), and whether there is a synergistic role for human GBPs.

Inflammasome activation is triggered in response to sensing of bacterial products within the cytosol. Vacuolar localization of *L. pneumophila* within its ER-derived vacuole would presumably limit the ability of host cells to recognize *L. pneumophila* components. However, when the integrity of the LCV is compromised, either by host factors or in the case of bacterial mutants that cannot maintain vacuolar integrity *L. pneumophila* becomes more accessible for recognition by host cytosolic sensors (B. C. Liu et al., 2018). We show that IFN-γ priming in primary human macrophages results in an increased frequency of
ruptured LCVs, indicating that IFN-inducible host cell factors promote disruption of the LCV. Our data indicate that GBP1 is one such factor. While we cannot formally conclude that GBP1-mediated rupture of the LCV is the proximal cause of downstream inflammasome activation, this rupture likely results in increased exposure of *L. pneumophila* products to the host cell cytosol, thus making the bacteria vulnerable to inflammasome sensing. Human GBPs may also target and promote destabilization of the outer membrane of *L. pneumophila* to enable the release of bacterial components, including LPS and DNA, for inflammasome sensing. Murine GBPs encoded on chromosome 3 promote the disruption of the outer membrane of the cytosolic *L. pneumophila* mutant lacking the effector SdhA, which is important for maintaining the vacuole integrity of the LCV (B. C. Liu et al., 2018). Mouse macrophages lacking chromosome 3 GBPs that were infected with the ∆sdhA mutant showed a decrease in pyroptosis and release of DNA into the cytosol, indicating that one or more chromosome 3 GBPs contribute to inflammasome activation in response to cytosolic bacteria. Since mouse GBPs mediate the disruption of cytosolic *L. pneumophila*, it is possible that human GBP1 or other GBPs may also enable disruption of the *L. pneumophila* outer membrane to release bacterial components that subsequently lead to inflammasome activation.

Overall, our findings reveal a critical role for IFN-γ and human GBP1 in promoting human inflammasome responses against *L. pneumophila*. In particular, our study illuminates a key function for human GBP1 in disrupting the pathogen-containing vacuole. These findings indicate that human GBPs have distinct roles...
compared to mouse GBPs in promoting inflammasome responses to *L. pneumophila* and provide insight into human cell-autonomous responses to a vacuolar bacterial pathogen.

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### 2.7. Materials and Methods

#### 2.7.1. Primary Human Samples
All studies on primary human monocyte-derived macrophages (hMDMs) were performed in compliance with the requirements of the US Department of Health and Human Services and the principles expressed in the Declaration of Helsinki. Samples obtained from the University of Pennsylvania Human Immunology Core are considered to be a secondary use of deidentified human specimens and are exempt via Title 55 Part 46, Subpart A of 46.101 (b) of the Code of Federal Regulations.

2.7.2. Cell Culture

THP-1 cells (TIB-202; American Type Culture Collection) were maintained in RPMI supplemented with 10\% (vol/vol) heat-inactivated FBS, 0.05 nM \( \beta \)-mercaptoethanol, 100 IU/mL penicillin, and 100 \( \mu \)g/mL streptomycin at 37°C in a humidified incubator. The day before stimulation, cells were replated in media without antibiotics in a 48-well plate at a concentration of \( 2 \times 10^5 \) cells per well or in a 96-well plate at a concentration of \( 1 \times 10^5 \) cells per well and incubated with phorbol 12-myristate 13-acetate (PMA) for 24 hours to allow differentiation into macrophages. Media was replaced with RPMI without serum for infections in 48-well plate.

Primary human monocytes from deidentified healthy human donors were obtained from the University of Pennsylvania Human Immunology Core. Monocytes were cultured in RPMI supplemented with 10\% (vol/vol) heat-inactivated FBS, 2 mM L-glutamine, 100 IU/mL penicillin, 100 \( \mu \)g/mL streptomycin, and 50 ng/mL recombinant human M-CSF (Gemini Bio Products). Cells were
cultured for 4 days in 10 mL of media in 10 cm-dishes at 4-5 × 10^5 cells/mL, followed by addition of 10 mL of fresh growth media for an additional 2 days for complete differentiation into macrophages. The day before macrophage stimulation, cells were rinsed with cold PBS, gently detached with trypsin-EDTA (0.05%) and replated in media without antibiotics and with 25 ng/mL M-CSF in a 48-well plate at a concentration of 1 × 10^5 cells per well or in a 24-well plate at a concentration of 2 × 10^5 cells per well.

2.7.3. Macrophage Stimulation

In infection experiments, PMA-differentiated THP-1 cells and primary human monocyte-derived macrophages (hMDMs) were either left unprimed or were primed overnight with recombinant human IFN-γ (R&D Systems) at a concentration of 100 U/mL. In dose-response experiments, hMDMs were either left unprimed or primed with 0.1, 1, 10, or 100 U/mL of IFN-γ for 20 hours.

2.7.4. Bacterial Strains and Macrophage Infection

All *Legionella pneumophila* infections used strains derived from the serogroup 1 clinical isolate Philadelphia-1. Where indicated, strains utilized were derived from the Lp02 strain (*rpsL, hsdR, thyA*), which is a thymidine auxotroph. The isogenic Lp02 (*rpsL, hsdR, thyA*) flagellin mutant, ΔflaA (T4SS+ *Lp*), and avirulent dotA mutant, Lp03 (T4SS− *Lp*), which are both thymidine auxotrophs, were used to infect PMA-differentiated THP-1 cells and primary hMDMs (Berger & Isberg, 1993; Berger et al., 1994; Ren et al., 2006). ΔflaA (T4SS+) or ΔdotA (T4SS−) *L.*
*pneumophila* strains on the JR32 background (*rpsL, hsdR*) carrying pSW001, which allows for constitutive dsRED expression, were used in immunofluorescence experiments (Mampel et al., 2006; Marra & Shuman, 1989). All *L. pneumophila* strains were grown as a stationary patch for 48 hours on charcoal yeast extract agar plates at 37°C (J. C. Feeley et al., 1979). Bacteria were resuspended in PBS and added to the cells at a multiplicity of infection (MOI) of 10 in 48-well and 24-well plate experiments. Infected cells were then centrifuged at 290 × g for 10 min and incubated at 37°C. For immunofluorescence experiments, primary hMDMs were infected for 2 hours. For infection experiments involving THP-1-derived macrophages, cells were infected for 2 hours. For additional infection experiments involving primary hMDMs, cells were infected for 4 hours. For all experiments, mock-infected cells were treated with PBS.

### 2.7.5. Inhibitor Treatments

25 µM of caspase-1 inhibitor Ac-YVAD-cmk (Sigma-Aldrich SML0429), 20 µM of caspase-8 inhibitor Z-IETD-FMK (SM Biochemicals SMFMK004), 20 µM of pan-caspase inhibitor Z-VAD(Gomes et al.)-FMK (SM Biochemicals SMFMK001), and 1 µM of NLRP3 inhibitor MCC950 (Sigma-Aldrich PZ0280) were added to primary hMDMs 1 hour before infection.

### 2.7.6. NLRP3 Inflammasome Assay
The NLRP3 inflammasome was activated by priming primary hMDMs with 0.5 µg/mL *E. coli* LPS (055:B5;Sigma) for 4 hours followed by 10 µM nigericin treatment for 4 hours.

### 2.7.7. siRNA-Mediated Knockdown

All of the Silencer Select siRNA oligos targeting human GBP mRNA were purchased from Thermo Fisher Scientific. Individual siRNA targeting GBP1 (s5620), GBP2 (s5623), GBP3 (5628), GBP4 (s41805), and GBP5 (s41810) were used. The two Silencer Select negative control siRNAs (Silencer Select Negative Control No. 1 siRNA and Silencer Select Negative Control No. 2 siRNA) were purchased from Life Technologies (Ambion). In experiments where GBP1-5 were individually knocked down, primary hMDMs were replated in media without antibiotics in a 48-well plate, as described above, three days before infection. Two days before infection, 30 nM of total siRNA were transfected into macrophages using HiPerFect transfection reagent (Qiagen) following the manufacturer’s protocol. 16 hours before infection, media was replaced with fresh antibiotic-free media containing 100 U/mL IFN-γ. In immunofluorescence experiments where GBP1 was knocked down, primary hMDMs were replated in media without antibiotics on glass coverslips in a 24-well plate as described above four days before infection. Three days before infection, 5 pmol of total siRNA were transfected into macrophages using Lipofectamine RNAiMAX transfection reagent (Thermo Fisher Scientific) following the manufacturer’s protocol. 16 hours before
infection, media was replaced with fresh antibiotic-free media containing 100 U/mL IFN-γ.

2.7.8. Quantitative RT-PCR Analysis

RNA was isolated using the Rneasy Plus Mini Kit (Qiagen) following the manufacturer’s protocol. Cells were lysed in 350 µL RLT buffer with β-mercaptoethanol and centrifuged through a QiAshredder spin column (Qiagen). cDNA was synthesized from isolated RNA using SuperScript II Reverse Transcriptase (Invitrogen) following the manufacturer's protocol. Quantitative PCR was conducted with the CFX96 real-time system from Bio-Rad using the SsoFast EvaGreen Supermix with Low ROX (Bio-Rad). Transcript levels for each gene were normalized to the housekeeping gene HPRT for each sample, and samples were normalized to unprimed sample or to control siRNA-treated sample using the $2^{-\Delta\Delta Ct}$ (cycle threshold) method to calculate fold change. Relative expression was calculated by normalizing gene-specific transcript levels to HPRT transcript levels for each sample using the $2^{-\Delta Ct}$ method. Primer sequences from primer bank used for HPRT1, GBP1-6, CASP4, and CASP5 or from Lagrange, et al. for GBP7 are the following (all 5’ → 3’):

HPRT1 forward: CCTGGCGTCGTGATTAGTGAT
HPRT1 reverse: AGACGTTCAGTCCTGTCCATAA
GBP1 forward: AGGAGTTCCTTCAAAGATGTGGA
GBP1 reverse: GCAACTGGACCCTGTCGTT
GBP2 forward: CTATCTGCAATTACGCAGCCT
GBP2 reverse: TGTTCCTGCTTCTTGGGATGA
GBP3 forward: ATCCCTGAAGCTAAGCAAG
GBP3 reverse: GGGCAGATCGAAGCATAACT
GBP4 forward: ATGGGTGAGAGAACTCTTCACG
GBP4 reverse: TGGCGGTATAGCCCTACAATTG
GBP5 forward: CCATGTGCCTCATCGAGAACT
GBP5 reverse: ACAGGTTTCGTAATGGCCAC
GBP6 forward: ATGGAAATCTGGACCCAAAATGT
GBP6 reverse: GCTGGTTCACCAATAAGCTGCT
GBP7 forward: TGCCTTCTTACCAAGTCCAGA
GBP7 reverse: TCTCTGATGCCATGTTCAGG
CASP4 forward: TCTCGGAACTGTGCATGATG
CASP4 reverse: TGTGTGATGAAGATAGAGCCCAT
CASP5 forward: TCACCTGCCTGCAAGGAATG
CASP5 reverse: TCTTTTCGTCAACCACAGTGTAG

2.7.9. LDH Cytotoxicity Assay

Macrophages were infected in a 48-well plate as described above and harvested supernatants were assayed for cell death by measuring loss of cellular membrane integrity via lactate dehydrogenase (LDH) activity. LDH release was quantified using an LDH Cytotoxicity Detection Kit (Clontech) according to the manufacturer's instructions and normalized to mock-infected cells.
2.7.10. Real-Time Propidium Iodide Uptake Assay

To measure live kinetics of cell membrane permeability, THP-1 cells were plated as described above in a black, flat-bottom 96-well plate (Cellstar), primed with 100 U/mL IFN-γ for 24 hours, and infected with T4SS+ Lp at an MOI of 50 in media containing 1X HBSS without phenol red, 20 mM HEPES, and 10% (vol/vol) heat-inactivated FBS. Infected cells were centrifuged at 290 × g for 10 min. The cells were supplemented with 5 μM propidium iodide (PI, P3566, Invitrogen) and incubated for 10 min at 37°C to allow the cells to equilibrate. Then, the plate was sealed with adhesive optical plate sealing film (Microseal, Bio-Rad) and placed in a Synergy H1 microplate reader (BioTek) pre-heated to 37°C. PI fluorescence was measured every hour for 4 hours.

2.7.11. ELISA

Macrophages were infected in a 48-well plate as described above and harvested supernatants were assayed for cytokine levels using ELISA kits for human IL-1β (BD Biosciences) and IL-18 (R&D Systems).

2.7.12. Immunoblot Analysis

In experiments where macrophages were plated in a 48-well plate, cells were lysed in 1X SDS/PAGE sample buffer, and low-volume supernatants (90 μL media per well of a 48-well plate) were mixed 1:1 with 2× SDS/PAGE sample buffer containing Complete Mini EDTA-free Protease Inhibitor Mixture (Roche). In experiments where primary hMDMs were plated in a 24-well plate and infected
with T4SS- Lp, T4SS+ Lp, or mock infected with PBS, cells were lysed in 1X SDS/PAGE sample buffer, and supernatants were treated with trichloroacetic acid (TCA) overnight at 4°C and centrifuged at maximum speed for 15 min. Precipitated supernatant pellets were washed with ice-cold acetone, centrifuged at maximum speed for 10 min, and resuspended in 1X SDS/PAGE sample buffer. Protein samples were boiled for 5 min, separated by SDS/PAGE on a 12% (vol/vol) acrylamide gel, and transferred to PVDF Immobilon-P membranes (Millipore). Primary antibodies specific for human IL-1β (clone 8516; R&D Systems), caspase-1 (2225S; Cell Signaling), caspase-4 (4450S; Cell Signaling), caspase-5 (D3G4W; 46680S; Cell Signaling), Gasdermin-D (126-138; G7422; Sigma-Aldrich), GBP1 (ab131255, Abcam), GBP2 (sc-271568, Santa Cruz), GBP4 (17746-1-AP, Proteintech), GBP5 (D3A5O, 67798S; Cell Signaling) and β-actin (4967L; Cell Signaling) were used. HRP-conjugated secondary antibodies anti-rabbit IgG (7074S; Cell Signaling) and anti-mouse IgG (7076S; Cell Signaling) were used. For detection, ECL Western Blotting Substrate or SuperSignal West Femto (both from Pierce Thermo Scientific) were used as the HRP substrate.

2.7.13. Immunofluorescence Microscopy

Primary hMDMs were plated on glass coverslips in a 24-well plate as described above. After 2 hours of infection with dsRED-Lp, cells were washed 2 times with PBS and fixed with 4% paraformaldehyde for 10 min at 37°C. Following fixation, cells were washed and permeabilized with 0.2% Triton X-100 for 10 min. Cells were washed, blocked with 10% BSA for 1 hour, and stained with primary
antibodies (identified below) for 1 hour. Cells were washed with PBS and incubated with the appropriate Alexa-Fluor-conjugated secondary antibodies (identified below) for 1 hour, followed by washes and mounted on glass slides with DAPI mounting medium (Sigma Fluoroshield). Primary antibodies used were rabbit anti-GBP1 (1:100 dilution; Abcam) and mouse anti-GBP2 (1:50 dilution; Santa Cruz). Secondary antibodies used at a dilution of 1:4000 were goat anti-rabbit conjugated to Alexa Fluor 488 (4412S; Cell Signaling) and goat anti-mouse conjugated to Alexa Fluor 488 (A11029; Life Technologies). Coverslips were imaged on a Leica SP5 FLIM confocal microscope at a magnification of 63× and the percentage of infected cells containing GBP1+ or GBP2+ intracellular bacteria out of the total number of infected cells were quantified.

2.7.14. Phagosome Integrity Assay

The phagosome integrity assay was performed as previously published (Meunier & Broz, 2015), with some modifications. To distinguish between cytosolic and vacuolar bacteria, primary hMDMs were plated on glass coverslips in a 24-well plate as described above and infected with dsRED-Lp. After 2 hours of infection, cells were washed 3 times with KHM buffer (110 mM potassium acetate, 20 mM HEPES, and 2 mM MgCl₂, pH 7.3) and incubated for 1 min in KHM buffer with 50 µg/mL digitonin (Sigma-Aldrich). Cells were washed 3 times with KHM buffer and stained for 15 min at 37°C with primary antibody to L. pneumophila (1:1000 dilution; gift from Craig Roy) in KHM buffer with 3% BSA. Cells were washed with PBS, fixed, and quenched with 0.1 M glycine for 10 min. Cells were
washed and incubated with secondary antibody anti-rabbit Alexa Fluor 488 for 1 hour, followed by washes and mounted on glass slides with DAPI mounting medium. Cells were analyzed by microscopy. 0.1% saponin in KHM buffer was used as a positive control for this assay. The percentage of infected cells harboring cytosolic bacteria out of the total number of infected cells were quantified.

2.7.15. Statistical Analysis

GraphPad Prism software was used for graphing of data and all statistical analyses. Statistical significance for experiments with THP-1 cells was determined using the unpaired two-way Student’s t test. Statistical significance for hMDMs was determined using the paired two-way t test in experiments comparing multiple donors and the unpaired two-way t test in experiments involving infections with dsRED-expressing Lp for immunofluorescence assay. In hMDM experiments that compare cells from multiple donors, data are graphed so that each data point represents the mean of triplicate wells for each donor, and all statistical analysis was conducted comparing the means of each experiment. Differences were considered statistically significant if the P value was <0.05.
CHAPTER 3

IFN-\(\gamma\) and GBP5s promote human noncanonical inflammasome responses to LPS variants derived from different bacteria

This chapter contains unpublished data generated by Antonia R. Bass, Stephanie Shreiner, and Brian Yan and portions of the introduction are part of a manuscript by Erin Harberts*, Jasmine Alexander-Floyd*, Antonia R. Bass*, Robert K. Ernst, and Sunny Shin. *indicates co-first authorship
3.1. Abstract

Cytosolic detection of lipopolysaccharide (LPS), the major outer membrane lipid component of gram-negative bacteria, is accomplished by the noncanonical inflammasome and leads to gasdermin-D (GSDMD)-mediated inflammatory cell death known as pyroptosis as well as downstream IL-1 family cytokine release. The noncanonical inflammasome is comprised of the cysteine protease caspase-11 in mice, while humans possess the two putative orthologs caspase-4 and caspase-5. Intriguingly, LPS can vary in its acylation and phosphorylation state contingent on the bacterial species and its environment. Gram-negative bacteria containing under-acylated LPS has been shown to evade murine caspase-11 detection in macrophages, while hexa-acylated LPS variants robustly activate the noncanonical inflammasome. In contrast to mice, a recent study found that LPS with a lower acylation state can activate the human caspase-4 noncanonical inflammasome. However, the role of caspase-5 still remains unclear and whether caspase-4 or caspase-5 can be activated in response to LPS variants with differential acylation and phosphorylation states has not been investigated. Furthermore, IFN-inducible GBPs promote noncanonical inflammasome responses in murine macrophages and also recently found in human macrophages. Whether IFN-γ and human GBPs promote caspase-4 and caspase-5 activation in response to LPS from different bacteria is unknown. Here, we test the human noncanonical inflammasome response to LPS variants isolated from Yersinia pestis that vary in the number and position of acyl chains as well as the number of phosphate groups in primary human macrophages. We also use THP-
1-derived macrophages and CRISPR/Cas9 technology to delineate the roles of caspase-4, caspase-5, GBP1, and GBP2 in inflammasome responses to *Legionella pneumophila* or *Escherichia coli* LPS. We determine that caspase-4 plays a major role in detecting *E. coli* LPS, whereas caspase-5 does not and that human GBP1 may be important for cell death in response to *L. pneumophila* and *E. coli* LPS. This study provides a better understanding of the distinct roles that caspase-4 and caspase-5 have upon sensing different LPS variants. Additionally, the CRISPR/Cas9 knockout clones developed in this study offer useful tools in order to further investigate the functions of the human noncanonical inflammasome and GBPs in the context of other LPS variants or bacterial infections.

### 3.2. Significance Statement

Gram-negative bacterial LPS is recognized by two innate immune sensors: the extracellular sensor, TLR4, and the intracellular sensor, the noncanonical inflammasome. Detection of LPS by these sensors is crucial for combating host defense against gram-negative bacterial pathogens. However, overactivation of these pathways can lead to detrimental outcomes including sepsis, an overwhelming inflammatory response that can result in organ failure and ultimately death. As there are treatments for sepsis in mice, there are no successful approved treatments for sepsis in humans. In addition, the mouse noncanonical inflammasome is comprised of only one inflammatory caspase, caspase-11, whereas the human noncanonical inflammasome is made up of two inflammatory caspases, caspase-4 and caspase-5, and their functions are relatively poorly
understood. Our study focuses on determining the specific roles of caspase-4 and caspase-5 in response to LPS variants from different bacteria, as well as examining whether IFN-inducible GBPs play a role in noncanonical inflammasome responses. This study elucidates aspects of human innate immune responses to gram-negative bacterial pathogens and provides insight into identifying potential therapeutic targets for treating gram-negative sepsis.

3.3. Introduction

Gram-negative bacteria cause more than 30% of hospital-acquired infections in the US, making them a major public health concern (Peleg & Hooper, 2010). Additionally, antibiotic-resistance is on the rise among gram-negative bacterial pathogens, further highlighting the need for more therapeutic approaches to controlling these infections (Exner et al., 2017). Uncontrolled gram-negative bacterial infections can lead to detrimental outcomes including sepsis, which is an overwhelming systemic inflammatory immune response to an infection. If left untreated, the host will succumb to organ failure and ultimately death. Preclinical studies in mice showed successful treatments for sepsis using immunomodulators that functioned by neutralizing either host inflammatory mediators or microbial products (Marshall, 2014). However, over 100 clinical trials testing these immunomodulators in sepsis patients have failed. The reasons for these clinical trial failures are unclear but may be due to differences between murine and human innate immune genes that play a role in responses to gram-negative bacterial infections. Therefore, it is important to understand human innate immune
responses to gram-negative bacterial pathogens in order to identify potential novel therapeutic targets for the treatment of gram-negative sepsis.

Gram-negative sepsis is caused by the bacterial endotoxin, lipopolysaccharide (LPS), which is the major lipid component in the outer membrane of gram-negative bacteria. LPS activates innate immune sensors and subsequently leads to host defense signaling events including inflammatory cytokine release as well as cell death of the infected cell. The first identified LPS sensor is toll-like receptor 4 (TLR4), a membrane-bound receptor located on the plasma membrane or endosomal compartment membrane and recognizes extracellular LPS (Takeuchi et al., 1999). Although TLR4 is essential for regulating responses to gram-negative bacteria, its dysregulation can lead to sepsis. Studies in mice identified an alternative LPS sensor that leads to TLR4-independent endotoxic shock (Hagar et al., 2013; Kayagaki et al., 2013). This second LPS sensor is known as the noncanonical inflammasome and recognizes LPS within the cytosol.

The noncanonical inflammasome is formed by the cysteine protease caspase-11 in mice and the two putative orthologs, caspase-4 and caspase-5 in humans (Kayagaki et al., 2011; Shi et al., 2014). These inflammatory caspases recognize and bind LPS through their caspase activation and recruitment domain (CARD) resulting in their oligomerization and activation (Shi et al., 2014). Active caspase-11, -4, and -5 mediate cleavage of gasdermin-D (GSDMD), the initiator protein of pyroptosis. The N-terminal fragment of GSDMD translocates to the plasma membrane and oligomerizes to form a pore resulting in osmotic influx and
cell lysis. This GSDMD pore also leads to potassium (K+) efflux which triggers activation of the canonical NLRP3 inflammasome. The NLRP3 inflammasome involves activation of caspase-1, which also cleaves GSDMD as well as cleaves IL-1 family cytokines into their mature forms for their release to signal bystander cells, which are then activated and recruited to the site of infection. This noncanonical inflammasome response is crucial for controlling intracellular bacterial infections, but similar to TLR4, its overactivation can result in LPS-induced septic shock. Intriguingly, humans are among the most sensitive to endotoxins, whereas mice are more resistant (Kajiwara et al., 2014). One possibility as to why humans are more sensitive to the endotoxin LPS may be due to the presence of two intracellular LPS sensors compared to mice that contain only one intracellular LPS sensor. Humans evolved to develop caspase-4 and caspase-5 most likely due to a gene duplication of the ancestral caspase-11 gene in order to recognize and respond to a variety of gram-negative bacterial pathogens they come into contact with. The functions of caspase-4 and caspase-5 in response to LPS are relatively poorly understood. Therefore, we aim to determine how these inflammatory caspases contribute to the human noncanonical inflammasome response to LPS.

LPS is made up of three distinct components: the lipid A moiety, core oligosaccharide, and O-antigen polysaccharide. Specifically, the lipid A moiety is the component that is directly recognized and bound to by the CARD domain. Lipid A is comprised of two glucosamine residues that contain hydrophobic acyl chains that vary in number, position and length depending on the bacterial species. Also
contingent on the bacterial species, lipid A possesses either one or two phosphate groups located on the 1 and/or 4' positions of the two glucosamine residues, respectively, and these phosphate groups provide a negative charge that help to facilitate binding to the positively charged amino acid residues in the CARD domains of caspase-11, -4, and -5. Interestingly, some bacteria within the same species are able to modify their acylation and phosphorylation states, suggesting that changing these vital features is important for their pathogenesis and may allow these bacteria to evade immune detection.

The murine noncanonical inflammasome varies in response to different LPS variants. It has been determined that LPS with a lower quantity of acyl chains (i.e. tetra-acylated) evade caspase-11 immune detection, whereas LPS containing higher number of acyl chains (i.e. hexa-acylated) activate caspase-11 for downstream pyroptosis and release of inflammatory cytokines (Hagar et al., 2013; Kayagaki et al., 2013). Interestingly, some penta-acylated LPS variants, such as *Francisella novicida lpxF* mutant, can activate caspase-11 (Hagar et al., 2013), while other penta-acylated LPS variants, like *Rhizobium galegae*, evade caspase-11 detection (Kayagaki et al., 2013). This brings up the question of whether the number of acyl chains is the only factor involved in promoting noncanonical inflammasome responses. It was newly discovered that the human noncanonical inflammasome functions differently than the murine system. The human noncanonical inflammasome is activated in response to tetra-acylated LPS variants, including the tetra-acylated *F. novicida* LPS, as well as penta- and hexa-acylated LPS variants, which indicates that the human noncanonical
inflammasome is activated by LPS with different acylation states (Lagrange et al., 2018). Surprisingly, there are no other studies that further explore the human noncanonical inflammasome response to different LPS variants. Whether the position of acyl chains or the number of phosphoryl groups on lipid A play a role in human noncanonical inflammasome response has not been studied.

Furthermore, IFN promotes inflammasome activation in murine macrophages in response to gram-negative bacteria as well as to bacterial components including LPS and outer membrane vesicles (OMVs) (Finethy et al., 2017; B. C. Liu et al., 2018; Meunier et al., 2014; Meunier et al., 2015; Pilla et al., 2014; Santos et al., 2018). Specifically, the IFN-inducible family of GTPases known as guanylate bindings proteins (GBPs) facilitate these enhanced inflammasome responses by different functions, including the rupture of pathogen-containing vacuoles and disruption of the outer membrane of cytosol exposed bacteria. It was recently determined that human GBP2 promotes noncanonical inflammasome responses to the tetra-acylated LPS derived from F. novicida as well (Lagrange et al., 2018). However, there are no additional studies that investigate whether IFN and GBPs promote human noncanonical inflammasome responses to other LPS variants from different bacteria.

Here, we investigate the activation of the human noncanonical inflammasome in response to seven lipooligosaccharide variants, which lack the O-antigen, that were derived from Y. pestis in order to determine whether its activation differs in response to each LOS variant. Specifically, we explore whether the number of acyl chains, number of phosphoryl groups, or position of acyl chains
can influence human noncanonical inflammasome activation and if IFN-γ can promote this response. Additionally, we utilize CRISPR/Cas9 technology to knockout either caspase-4 (CASP4), caspase-5 (CASP5), human GBP1, or human GBP2 in the immortalized monocytic cell line, THP-1, in order to determine the roles of these host proteins in response to LPS variants derived from *L. pneumophila* and *E. coli*. Overall, this study provides a better understanding of caspase-4 and caspase-5 and how IFN-γ and GBPs contribute to human noncanonical inflammasome activation in response to LPS variants from different bacteria.

3.4. Results

3.4.1. The human noncanonical inflammasome is activated by different LOS variants.

Caspase-4 was shown to be activated in response to tetra-acylated LPS derived from *F. novicida*, as well as to penta- and hexa-acylated LPS variants from other bacteria (Lagrange et al., 2018). However, whether the human noncanonical inflammasome can be activated in response to additional LPS variants that have differential acylation and phosphorylation states has not been studied. To determine whether the human noncanonical inflammasome responds to other LPS variants, we tested seven different LOS variants, which lack the O-antigen, that were isolated from wild-type (WT) *Y. pestis* or different mutants of *Y. pestis* that were grown at 26°C. The variants tested include four hexa-acylated LOS, two penta-acylated LOS, and one tetra-acylated LOS. Specifically, the hexa-acylated
LOS include LOS from WT *Y. pestis* (358@26), a mutant that lacks one phosphate group at the 4’ position (468@26), a mutant that is missing an acyl chain at the 3’ position but has an added acyl chain at the 2 position (438@26), and a mutant that lacks a phosphate group at the 4’ position as well as has a missing acyl chain at the 2’ position and an added acyl chain at the 2 position (470@26) (Fig. 3.1 A). The penta-acylated LOS variants are missing an acyl chain at either the 2’ position (47@26) or at the 3’ position (46@26). Finally, the tetra-acylated LOS variant lacks the two acyl chains at the 2’ and 3’ positions (48@26). Upon transfection with these seven LOS variants in primary human monocyte-derived macrophages (hMDMs), we determined that all of the variants promote cell death and IL-1β release; however, it does appear that transfection of the tetra-acylated LOS, 48@26, shows a lower inflammasome response compared to all of the other variants (Fig. 3.1 B and C). This may indicate that the presence of either the 2’ or 3’ acyl chains that are missing in this LOS variant help to promote maximal noncanonical inflammasome responses in human macrophages. Overall, these data suggest that *Y. pestis* LOS variants containing different number and position of acyl chains as well as different number of phosphate groups all activate the human noncanonical inflammasome.
Figure 3.1. *Yersinia pestis* LOS variants of different acylation and phosphorylation states activate the human noncanonical inflammasome. (A) Schematic representations of the seven *Y. pestis* LOS variants. The two glucosamine residues, which are represented by hexagons, can have one or two phosphate groups (orange circles) and have either four, five, or six acyl chains attached that can vary in position. LOS mutants that have missing phosphate groups or acyl chains or have added acyl chains in a different position compared to the WT *Y. pestis* LOS (358@26) are outlined in red circles. (B and C) Primary hMDMs were primed with Pam3CSK4 (1 µg/mL) for four h and either mock transfected with FuGENE HD alone or transfected with FuGENE HD and 2 µg/mL LPS for 20 h. (B) Cell death was measured using lactate dehydrogenase release assay and normalized to mock-infected cells. (C) IL-1β levels in the supernatant
were measured by ELISA. (B and C) Shown are pooled results of two to three independent experiments using hMDMs from different healthy human donors. Each data point represents the mean of duplicate wells from an individual donor. Bar graphs are color coated based on the number of acyl chains (Green=Hexa-acylated, Blue=Penta-acylated, Pink=Tetra-acylated).

3.4.2. IFN-γ upregulates human noncanonical inflammasome responses to *E. coli* and *Y. pestis* LOS variants.

IFN has been shown to promote inflammasome responses in both mouse and human macrophages infected with a variety of bacteria including *S. Typhimurium, L. pneumophila*, and *F. novicida* (Lagrange et al., 2018; B. C. Liu et al., 2018; Meunier et al., 2014; Meunier et al., 2015). In particular, the family of IFN-inducible GTPases known as GBPs are upregulated by both type I and II IFNs, but more robustly upregulated by the type II IFN, IFN-γ, and are the key players in promoting these inflammasome responses. Here, we investigated whether IFN-γ promotes human noncanonical inflammasome responses to a hexa-acylated or tetra-acylated LOS derived from *Y. pestis*. The 470@26 LOS variant produced a robust inflammasome response in primary human macrophages (Fig. 3.1 B and C); therefore, we used this hexa-acylated LOS variant to determine how IFN-γ affects this response along with the tetra-acylated variant that did not produce as strong of a response. As a control, we looked at human noncanonical inflammasome activation in response to the hexa-acylated LOS derived from the W3110 *E. coli* strain in unprimed and IFN-γ-primed THP-1-derived macrophages. Upon transfection of these three LOS variants in THP-1-derived macrophages, we observed that IFN-γ priming led to a significant increase in IL-1β release compared
to unprimed cells (Fig. 3.2). Moreover, all of the IFN-γ-primed cells have similar amount of IL-1β release in response to the two hexa-acylated LOS variants and the tetra-acylated variant. This indicates that IFN-γ enhances human noncanonical inflammasome activation in response to differentially acylated LOS.

![IL-1β release](image)

**Figure 3.2. IFN-γ promotes human noncanonical inflammasome responses to *E. coli* and *Y. pestis* LOS variants.** Phorbol 12-myristate 13-acetate (PMA)-differentiated THP-1 cells were either primed overnight with IFN-γ (100 U/mL) or left unprimed, then primed with Pam3CSK4 (1 µg/mL) for 4 h, and either mock transfected with FuGENE HD alone or transfected with FuGENE HD and 2 µg/mL LPS for 20 h. IL-1β levels in the supernatant were measured by ELISA. **P<0.01 and ***P<0.001 by unpaired t-test.

3.4.3. Development of CASP4 or CASP5 knockout THP-1 clones using CRISPR/Cas9 technology.

Caspase-4 and caspase-5 comprise the human noncanonical inflammasome. While most studies of the noncanonical inflammasome have been conducted using mice and murine macrophages, there are a few studies that investigated caspase-4 inflammasome activation in response to different gram-negative bacteria including *L. pneumophila, E. coli*, and *F. novicida* (Casson et al.,
Very little research has looked into the role of caspase-5 in macrophages, which may be due to its low relative expression level in macrophages. However, one study using primary human macrophages determined that caspase-5 did not undergo processing in response to transfected *E. coli* LPS or infection with *L. pneumophila*, *S. Typhimurium*, or *Y. pseudotuberculosis*, but did undergo proteolytic cleavage in response to treatment with extracellular *E. coli* LPS (Casson et al., 2015). In contrast, this study showed that caspase-4 undergoes processing in response to infection with *L. pneumophila* and *S. Typhimurium*. These results suggest that caspase-4 and caspase-5 may be activated through different pathways in response to gram-negative bacteria or LPS. Interestingly, additional studies using human monocytes found that caspase-5 undergoes processing in response to extracellular LPS and activation in response to treatment with *Pseudomonas aeruginosa* outer membrane vesicles (OMVs) (Bitto et al., 2018; Vigano et al., 2015). Thus, these results in addition to the primary human macrophage results indicate that caspase-5 activation may be initiated through an external pathway, but this has not been thoroughly investigated. Since caspase-5 has been shown to directly bind LPS via its CARD domain (Shi et al., 2014), similar to caspase-11 and caspase-4, it is likely that caspase-5 can be activated in response to intracellular LPS. Therefore, in order to investigate the distinct roles of caspase-4 (CASP4) and caspase-5 (CASP5) in response to intracellular LPS variants, we used the Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR) system together with the RNA-
guided exonuclease Cas9 to disrupt the *CASP4* and *CASP5* genes in the immortalized monocytic THP-1 cell line.

We generated lentivirus using a lentiCRISPR version 2 plasmid, which contains the Cas9 protein and the guide RNA (gRNA) target sequence for either *CASP4* or *CASP5*, VSV-G envelope plasmid, and psPAX2 packaging plasmid. The gRNA sequences for *CASP4* or *CASP5* are located within exonic regions of the gene in order to promote a double-stranded break (DSB) at the target DNA site within the protein coding area. Specifically, the *CASP4* gRNA targets exon 3 and the *CASP5* gRNA targets exon 5, both of which targeted sequences are highlighted in Fig. 3.3 A and B. This DSB results in either non-homologous end joining (NHEJ) or homology directed repair (HDR), which can cause base-pair insertions, deletions, or frameshift mutations and lead to a premature stop codon and nonfunctional gene. After infecting the THP-1 monocytic cell lines with the generated lentivirus containing either *CASP4* or *CASP5* gRNA and Cas9 protein, followed by puromycin selection and clonal selection, we expanded 12 single cell clones to determine whether their RNA and protein levels of *CASP4* or *CASP5* were absent. Based on the decreased gene and protein expression we chose three clones to validate and ensure that they were clones that originated from a single cell. THP-1 cells are a human monocytic cell line that are derived from an acute monocytic leukemia patient; however, they possess a diploid karyotype, unlike other leukemia cell lines (Fleit & Kobasiuk, 1991). Therefore, a single cell clone should therefore contain only two allelic mutations at the target sequence. Of the three clones chosen for the *CASP5* gene, we validated two clones for *CASP5*,
clone 1 and clone 8, both of which possess two distinct allelic mutations (Fig. 3.3 C and D). The sequencing electropherogram for the DNA target sequence and surrounding regions for both CASP5 alleles of each clone are shown. The sequence alignment comparing WT THP-1 and CASP5\textsuperscript{-/-} THP-1 clones are also shown, and the nucleotide deletions within the CASP5 target sequence are indicated by red boxes. The CASP5 alleles in clone 1 have either 10 or 13 nucleotides deleted, which result in premature stop codons (Fig. 3.3 C). The CASP5 alleles in clone 8 have either two or four nucleotide deletions and also result in premature stop codons and therefore nonfunctional CASP5 protein (Fig. 3.3 D). qPCR analyses and western blots revealed decreased RNA and absent protein expression levels of CASP4\textsuperscript{-/-} clones 2 and 6 (Fig. 3.4 A-B and E). Future experiments are required to validate these as single cell clones with only two allelic mutations.
Figure 3.3. Validation of CASP4 and CASP5 knockout THP-1 clones generated using CRISPR/Cas9 technology. Schematic representations of the CASP4 gene (A) or the CASP5 gene (B) with exons (filled boxes) and introns (lines). The respective guide RNA target sequences for CASP4 and CASP5 are highlighted in red. Shown are the mutations of the two alleles for CASP5-/- clone 1 (C) or CASP5-/- clone 8 (D) THP-1 genomic DNA by electropherogram and sequence alignment with WT THP-1 genomic DNA. The CASP5 target sequence is underlined and nucleotide deletions are indicated by the red boxes. The mutation starting point of missing nucleotide is indicated in the electropherogram by a black line.
is underlined and nucleotide deletions are indicated by the red boxes. The mutation starting point of missing nucleotide is indicated in the electropherogram by a black line.

Besides one study that showed caspase-4-dependent inflammasome activation in response to *F. novicida* in IFN-γ-primed human macrophages (Lagrange et al., 2018), no other studies have investigated the roles of caspase-4 or caspase-5 in IFN-γ-primed human macrophages in response to LPS variants from different bacteria. We compared caspase-4 and caspase-5 RNA and protein expression levels in WT THP-1 cells as well as the *CASP4*−/− and *CASP5*−/− THP-1 clones (Fig. 3.4). This is to confirm that knockout of either gene did not cross react and lead to unintentional knockout of the other gene. For instance, *CASP4*−/− THP-1 clones 2 and 6 present a decreased fold change and relative expression of *CASP4*, but *CASP5*−/− THP-1 clones 1, 6, and 8 have normal *CASP4* gene expression similar to WT THP-1 cells (Fig 3.4 A and B). *CASP5*−/− THP-1 clone 6 was examined because it initially showed decrease *CASP5* expression and absent protein expression when comparing the original 12 expanded clones; however, after conducting validation experiments it was revealed that it was not a single cell clone and had more than two allelic mutations. Additionally, *CASP5*−/− THP-1 clones 1, 6, and 8 have decreased RNA fold change and relative expression levels of *CASP5*, while *CASP4*−/− THP-1 clones 2 and 6 have higher *CASP5* RNA expression, although *CASP4*−/− clone 6 show slightly lower *CASP5* RNA expression compared to WT THP-1 cells (Fig. 3.4 C and D). Finally, we compared the CASP4 and CASP5 protein levels in unprimed and IFN-γ-primed WT THP-1 cells and the
CASP4 and CASP5 knockout clones. Unprimed or IFN-γ-primed CASP4/− THP-1 clones 2 and 6 show no CASP4 protein expression, whereas WT THP-1 and CASP5/− THP-1 clones 1 and 8 have CASP4 expressed (Fig. 3.4 E). Notably, IFN-γ-primed WT THP-1 and CASP5/− THP-1 clones have increased CASP4 protein expression compared to unprimed cells. Furthermore, unprimed WT THP-1 cells as well as the CASP4 and CASP5 knockout clones do not show any CASP5 protein expression (Fig. 3.4 F). CASP5 is not constitutively expressed, unlike CASP4, and requires a priming signal for its upregulation. In addition, CASP5 has a very low relative expression compared to CASP4 (Fig. 3.4 B and D). IFN-γ-primed WT THP-1 and CASP4/− THP-1 clones 2 and 6 present CASP5 protein expression, while CASP5/− THP-1 clones 1 and 8 do not express CASP5 (Fig. 3.4 F). These results indicate that disruption of the CASP4 gene does not affect CASP5 expression levels and that mutation of the CASP5 gene does not abrogate CASP4 expression. Thus, these THP-1 knockout clones can be used to distinguish the functions of caspase-4 and caspase-5 in response to intracellular LPS variants.
Figure 3.4. Expression of CASP4 and CASP5 in CASP4 or CASP5 knockout THP-1 clones. (A-D) PMA-differentiated WT THP-1 cells, CASP4\(^{-}\) THP-1 clones, and CASP5\(^{-}\) THP-1 clones were primed with IFN-\(\gamma\) (100 U/mL) for 16 h. CASP4 and CASP5 transcript levels were determined by quantitative RT-PCR and relative expression was calculated by normalizing to the housekeeping gene HPRT. Fold change was further calculated by normalizing to the WT THP-1 sample. (E-F) PMA-differentiated WT THP-1 cells, CASP4\(^{-}\) THP-1 clones, and CASP5\(^{-}\) THP-1 clones were either left unprimed or primed with IFN-\(\gamma\) (100 U/mL) for 18 h. Immunoblot analysis was conducted on lysates for full length caspase-4 (pro-casp4), full length caspase-5 (pro-casp5), and caspase-5 intermediates (casp5 p44 and casp5 p35). Blots were stripped and reprobed for \(\beta\)-actin.
3.4.4. CASP4 is activated in response to *E. coli* LPS, while CASP5 may not play a role in response to *E. coli* or *L. pneumophila* LPS.

The role of caspase-4 and caspase-5 in response to different LPS variants is not well defined. In addition, whether IFN-γ promotes human caspase-4 or caspase-5 responses to different hexa-acylated LPS variants, including LPS derived from *L. pneumophila* or *E. coli* has not been studied. Here, we use WT, CASP4−/−, and CASP5−/− THP-1-derived macrophages to define the distinct roles of caspase-4 and caspase-5. Inflammasomes require an initial priming signal to upregulate sensor proteins, such as caspase-4 and caspase-5. First, we either left the THP-1 cells unprimed or primed them with IFN-γ in order to compare the roles of caspase-4 and caspase-5 in these distinguished cell types. Next, we primed all of the THP-1 cells with the TLR1/2 ligand, Pam3CSK4, which not only promotes the expression of sensor proteins but also of IL-1 family cytokines including IL-1β. The noncanonical inflammasome is characterized by cleaving GSDMD, which is the initiator of pyroptosis, followed by K+ efflux, which activates the NLRP3 capsase-1-containing inflammasome for IL-1 processing and release.

Upon transfection with *E. coli* LPS and not *L. pneumophila* LPS, both CASP4−/− and CASP5−/− THP-1 cells show a significant decrease in cell death compared to WT THP-1 cells in the Pam3CSK4-primed only condition (Fig. 3.5 A). Notably, CASP4−/− THP-1 cells show a much greater decrease compared to when CASP5 is knocked out. This indicates that caspase-4 plays an essential role in cell death in response to *E. coli* LPS, while caspase-5 may play a small role. Furthermore, when these cells are additionally primed with IFN-γ and transfected with *E. coli* LPS,
CASP4−/− THP-1 cells have significantly reduced cell death, while CASP5−/− THP-1 cells do not (Fig. 3.5 B). This could be due to an upregulated expression of caspase-4 in the CASP5−/− THP-1 cells upon the treatment of IFN-γ. Similar to the Pam3CSK4-primed cells, additional treatment of IFN-γ does not result in a caspase-4- or caspase-5-dependent cell death in response to L. pneumophila LPS (Fig. 3.5 B). Interestingly, both CASP4−/− THP-1 clones either primed with Pam3CSK4 alone or primed with IFN-γ and Pam3CSK4, showed significantly decreased IL-1β release in response to E. coli, while the CASP5−/− THP-1 clones do not have a reduction (Fig. 3.5 C and D). In addition, for both primed conditions, only CASP4−/− clone 6 THP-1 cells have a significant reduction in IL-1β release in response to L. pneumophila LPS. This may suggest that caspase-4 is important for downstream IL-1β secretion in response to L. pneumophila LPS, but not cell death. These results are perplexing since cell death is upstream of IL-1 cytokine release for noncanonical inflammasome activation. Since these experiments were only conducted two times, repeated experiments are necessary and additional future experiments involving different timepoints and LPS treatment conditions are crucial to determine the role of caspase-4 and caspase-5 responses to L. pneumophila and E. coli LPS. It would be interesting to observe the inflammasome responses of caspase-4 and caspase-5 to treatment of extracellular LPS. Based on these results, we conclude that caspase-4 promotes noncanonical inflammasome responses to E. coli LPS and that caspase-5 may not play a role in response to E. coli or L. pneumophila LPS.
Figure 3.5. CASP4 promotes noncanonical inflammasome responses to *E. coli* LPS, while CASP5 may not play a role in detecting *E. coli* or *L. pneumophila* LPS. PMA-differentiated WT THP-1 cells, CASP4−/− THP-1 clones, and CASP5−/− THP-1 clones were either left unprimed (A and C) or primed overnight with IFN-γ (100 U/mL) (B and D), then primed with Pam3CSK4 (1 µg/mL) for 4 h and either mock transfected with FuGENE HD alone or transfected with FuGENE HD and 10 µg/mL *L. pneumophila* LPS or 2 µg/mL *E. coli* LPS for 4 h. (A and B) Cell death was measured using lactate dehydrogenase release assay and normalized to mock-infected cells. (C and D) IL-1β levels in the supernatant were measured by ELISA. Shown are results representative of two experiments. *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001 by unpaired t-test.

3.4.5. Development of GBP1 or GBP2 knockout THP-1 clones using CRISPR/Cas9 technology.

The IFN-inducible family of GTPases known as GBPs promote inflammasome responses to a variety of gram-negative bacteria as well as to bacterial components including LPS and OMVs (Finethy et al., 2017; Fisch et al., 2019; Lagrange et al., 2018; Meunier et al., 2014; Meunier et al., 2015; Pilla et al., 2014; Santos et al., 2020; Santos et al., 2018). Recent findings for human GBPs
revealed that human GBP1 promotes caspase-4-dependent inflammasome responses to S. Typhimurium and E. coli LPS, while human GBP2 promotes noncanonical inflammasome responses to the under-acylated LPS derived from F. novicida (Fisch et al., 2019; Lagrange et al., 2018; Santos et al., 2020). In addition, our lab discovered that human GBP1 is essential for maximal inflammasome responses to L. pneumophila (Chapter 2); however, we used RNAi-mediated knockdown techniques rather than a complete cell knockout of GBP1. Although we show that siRNA specific for GBP1 led to efficient knockdown, a GBP1 knockout cell line would eliminate the remaining residual GBP1 and, therefore, would abolish any inflammasome response due to the remaining GBP1 left. Here, we generated human GBP1 as well as GBP2 knockout THP-1 cells using the lentivirus-mediated CRISPR-Cas9 genome editing technology. The lentiCRISPR plasmids used contained either GBP1 gRNA that targets exon 8 or GBP2 gRNA that targets exon 6, both of which these sequences are highlighted in Fig. 3.6 A and B. Targeting these exonic regions will lead to nucleotide mutations within the protein coding region and, therefore, will result in a premature stop codon and nonfunctional protein. Applying the same process we used to develop the CASP4−/− and CASP5−/− THP-1 single cell clones, after infecting THP-1 cells with the lentivirus containing the gRNA target sequence for GBP1 or GBP2 as well as the Cas9-exonuclease protein, we treated the cells with puromycin to maintain the cells that contained the lentivirus. We next expanded 12 single cell clones and checked for decrease in RNA expression or absence of protein for either GBP1 or GBP2 in order to select the three best clones for validating that they originated
from a single cell. We validated two GBP1- THP-1 clones that each possess two allelic mutations. The sequencing electropherogram shows the DNA target sequence and adjacent nucleotide sequence of both GBP1 alleles for each clone (Fig. 3.6 C and D). In addition, we show the sequence alignment between the WT THP-1 and GBP1- THP-1 clones, and the nucleotide changes within the GBP1 target sequence are specified by red boxes or outlines. The GBP1 alleles in clone 1 have either five nucleotides deleted or have one nucleotide deleted and a nucleotide switch (Fig. 3.6 C). Both of these mutations revealed that they lead to premature stop codons. Additionally, the GBP1 alleles for clone 6 show either two nucleotides deleted or a nucleotide insertion, which result in premature stop codons (Fig. 3.6 D). Due to technical complications, we were unable to validate the GBP2- THP-1 clones, although we did choose two clones that showed the best RNA decrease as well as absence of GBP2 protein to use in experiments. Future experiments are needed in order to validate and ensure that these GBP2- THP-1 clones are derived from a single cell.
Figure 3.6. Validation of GBP1 and GBP2 knockout THP-1 clones generated using CRISPR/Cas9 technology. Schematic representations of the GBP1 gene (A) or the GBP2 gene (B) with exons (filled boxes) and introns (lines). The respective guide RNA target sequences for GBP1 and GBP2 are highlighted in red. Shown are the mutations of the two alleles for GBP1\(^{-/-}\) clone 1 (C) or GBP1\(^{-/-}\) clone 6 (D) THP-1 genomic DNA by electropherogram and sequence alignment with WT THP-1 genomic DNA. The CASP5 target sequence is underlined and nucleotide deletions are indicated by the red filled-in boxes. Nucleotide insertion or switch is marked by a red outline. The mutation starting point of missing nucleotide or inserted nucleotide is indicated in the electropherogram by a black line.
or switch is marked by a red outline. The mutation starting point of missing or inserted nucleotide is indicated in the electropherogram by a black line.

After choosing two GBP2−/− THP-1 clones for experimental use, in addition to the validated GBP1−/− THP-1 clones, we compared the protein expression levels of GBP1 and GBP2 in these clones as well as in WT THP-1 cells. The purpose of this is to confirm that the GBP1 gRNA did not affect GBP2 expression and vice versa. We initially primed the WT THP-1 cells and GBP knockout clones with IFN-γ to upregulate GBP expression, since GBPs are interferon-inducible and will not be expressed unless stimulated with IFN. We found that GBP1−/− THP-1 clones 1 and 6 have no GBP1 protein expression, while WT THP-1 and GBP2−/− THP-1 clones 1 and 7 have normal GBP1 levels (Fig. 3.7 A). Moreover, GBP2−/− THP-1 clones 1 and 7 show absent GBP2 protein expression, whereas WT THP-1 and GBP1−/− THP-1 clone 1 have normal GBP2 expression (Fig. 3.7 B). Interestingly, GBP1−/− THP-1 clone 6 shows lower GBP2 protein levels, although this observed decrease may be due to lower amount of lysate added since β-actin levels are lower in this sample compared to the other samples. A repeat of this western blot is needed to confirm whether GBP1−/− THP-1 clone 6 has its GBP2 protein levels affected by the GBP1 gRNA. Since we see that GBP1 is knocked out of the GBP1−/− THP-1 clones and that GBP2 is knocked out of the GBP2−/− THP-1 clones, we felt that we could continue and conduct experiments with these clones in order to determine their role in response to intracellular LPS derived from L. pneumophila and confirm their role in response to E. coli LPS.
3.4.6. GBP1 and GBP2 are important for cell death in response to *L. pneumophila*, while GBP1 plays a role in cell death in response to LPS derived from *L. pneumophila* and *E. coli*.

We recently found that human GBP1 promotes inflammasome responses to *L. pneumophila* and that these inflammasome responses led to caspase-1, -4, and -5 activation (Chapter 2). In addition to knocking down human GBP1 using siRNA, we also individually knockdown GBP2, 3, 4, and 5, but we did not see a decrease in inflammasome activation upon knockdown of these GBPs. We sought to use the newly developed GBP1−/− and GBP2−/− THP-1 clones in order to confirm the role of human GBP1 in inflammasome responses to *L. pneumophila* and whether the complete absence of GBP2 influences inflammasome responses as well. Since THP-1 cells have different kinetics of inflammatory responses to pathogens compared to primary human macrophages, we conducted a time course experiment using IFN-γ-primed WT THP-1 cells, GBP1−/− THP-1 clones, and

![Image](image-url)
GBP2\(^{-/-}\) THP-1 clones that were infected with *L. pneumophila*. We determined at each time point (1, 2, or 4 hpi) that knockout of GBP1 and GBP2 in THP-1 cells results in a significant decrease of cell death but not IL-1\(\beta\) secretion (Fig. 3.8 A and B). These results are in partial agreement with our previous findings that knockdown of GBP1 leads to a decrease in cell death. However, IL-1\(\beta\) is not decreased upon knockout of GBP1, contrary to what we found with siRNA knockdown of GBP1. Additionally, our results reveal a new finding that human GBP2 may also be important for promoting cell death during *L. pneumophila* infection.

Previous studies showed that GBPs are important for promoting noncanonical inflammasome responses to LPS. Specifically, it was found that human GBP1 promotes caspase-4 activation in response to *E. coli* LPS, while human GBP2 promotes caspase-4 activation in response to *F. novicida* LPS and not *E. coli* LPS (Lagrange et al., 2018; Santos et al., 2020). In addition, mouse GBPs located on chromosome 3 promote caspase-11 activation in response to LPS derived from *L. pneumophila* (Pilla et al., 2014). Therefore, we wanted to investigate whether human GBP1 or GBP2 promoted noncanonical inflammasome responses to *L. pneumophila* LPS as well as to confirm that human GBP1 and not GBP2 promotes inflammasome activation in response to *E. coli* LPS. Using the *GBP1\(^{-/-}\)* and *GBP2\(^{-/-}\)* THP-1 clones, we found that knockout of only GBP1 resulted in decreased cell death in response to *L. pneumophila* LPS and *E. coli* LPS, while IL-1\(\beta\) release was not affected (Fig. 3.8 C and D). These results are in agreement with the previous findings that human GBP1, and not GBP2, is important for
stimulating inflammasome response to *E. coli* LPS. It is also interesting that the absence of GBP2 does not lead to decrease in cell death in response to *L. pneumophila* LPS as it does in response to *L. pneumophila* bacteria. This may suggest that GBP2 is activated in response to a different bacterial component of *L. pneumophila* other than LPS. Overall, these data indicate that human GBP1 and GBP2 promote cell death during infection with *L. pneumophila*, while GBP1 stimulates cell death in response to LPS derived from *L. pneumophila* and *E. coli*. 
GBP1 and GBP2 play a role in cell death but not IL-1β release in response to *L. pneumophila*, while GBP1 plays a role in cell death in response to LPS derived from *L. pneumophila* or *E. coli* in THP-1 cells. (A and B) PMA-differentiated WT THP-1 cells, *GBP1*<sup>−/−</sup> THP-1 clones, and *GBP2*<sup>−/−</sup> THP-1 clones were primed with IFN-γ (100 U/mL) overnight and either mock infected with PBS or infected with T4SS+ Lp for the timepoints indicated. (C and D) PMA-differentiated WT THP-1 cells, *GBP1*<sup>−/−</sup> THP-1 clones, and *GBP2*<sup>−/−</sup> THP-1 clones were primed with IFN-γ (100 U/mL) overnight and either mock transfected with FuGENE HD alone or transfected with FuGENE HD and 10 µg/mL *L. pneumophila* LPS or 2 µg/mL *E. coli* LPS for 2.5 h. (A and C) Cell death was measured using lactate dehydrogenase release assay and normalized to mock-infected cells. (B and D) IL-1β levels in the supernatant were measured by ELISA. Shown are the results representative of one experiment.
measured using lactate dehydrogenase release assay and normalized to mock-infected cells. (B and D) IL-1β levels in the supernatant were measured by ELISA.

3.5. Discussion

Our data suggests that the human noncanonical inflammasome is activated in response to differentially acylated and phosphorylated LOS derived from Y. pestis, and that IFN-γ promotes these inflammatory responses. One possibility of why the human noncanonical inflammasome is activated by these various LOS structures, in contrast to the murine noncanonical inflammasome that is not activated by under-acylated LPS, is that humans possess two putative orthologs of murine caspase-11, which are caspase-4 and caspase-5. Thus, these two inflammatory caspases may be working together in order to recognize different types of LPS structures. The CASP4−/− and CASP5−/− THP-1 clones we developed using CRISPR/Cas9 genome editing technology are useful tools that can help to distinguish the functions of caspase-4 and caspase-5 to differentially phosphorylated and acylated LPS variants. Future studies testing the inflammasome responses to the seven LOS variants from Y. pestis in these THP-1 clones are needed to fully examine the distinct roles of caspase-4 and caspase-5. Additionally, previous work found that caspase-5 may be activated by a different pathway compared to caspase-4. Specifically, caspase-5 is cleaved and activated in response to treatment with extracellular E. coli LPS in monocytes and macrophages (Casson et al., 2015; Vigano et al., 2015). No additional work has been done to investigate this alternative LPS activation process. Thus, additional experiments testing extracellular LPS treatment in these CASP4−/− and CASP5−/−
THP-1 clones either left as monocytes or in differentiated macrophages to determine whether there is a difference in caspase-5 activation. It would be of interest to also determine whether TLR4 is important for the activation of caspase-5, as TLR4 is the extracellular LPS sensor and may somehow internalize LPS after binding and transport LPS to caspase-5 directly. Therefore, caspase-4 and caspase-5 may be activated by different LPS structures but may also be involved in different mechanisms of activation depending on the route of LPS entry into the cytosol.

Our data indicate that caspase-4 promotes noncanonical inflammasome responses to \textit{E. coli} LPS in both Pam3CSK4-primed macrophages as well as in Pam3CSK4 and IFN-\(\gamma\)-primed macrophages, while we do not see a role for caspase-5 in response to transfected \textit{L. pneumophila} LPS or \textit{E. coli} LPS. However, we did see that \textit{CASP4}^{-/-} THP-1 clone 6 transfected with \textit{L. pneumophila} LPS resulted in significantly decreased IL-1\(\beta\) release, indicating that caspase-4 is important for promoting inflammatory cytokine release. It is intriguing, however, that we see decreased IL-1\(\beta\) release but no decrease in cell death, since cell death is initiated before cytokine release during noncanonical inflammasome activation. Therefore, an alternative and more sensitive cell death assay, such as propidium iodide (PI) uptake, is needed in order to determine whether the cell death phenotype of the \textit{CASP4}^{-/-} THP-1 clones in response to \textit{L. pneumophila} LPS is accurate. PI is a DNA-binding dye that is significantly smaller in size compared to the LDH enzyme. Thus, PI is able to enter cells more easily through smaller pores, while LDH may not be released from these pores.
We also developed GBP1−/− and GBP2−/− THP-1 clones to determine the roles of human GBP1 and GBP2 in response to intracellular *L. pneumophila* LPS and to confirm the role of human GBP1 in response to *L. pneumophila* bacteria. Our data suggest that GBP1 and GBP2 were necessary for cell death in response to *L. pneumophila*, highlighting a potential new role for GBP2 that we did not observe in previous experiments using siRNA-mediated knockdown. It is possible that siRNA treatment results in residual amounts of GBP2 protein and that this remaining presence of GBP2 may account for the observed cell death. Therefore, this may be why we detect a decrease in cell death only in the complete absence of GBP2. The mechanism of how GBP2 is promoting cell death during *L. pneumophila* infection is unclear, but future experiments that test whether GBP2 is important for vacuolar or outer membrane rupture would be of interest and would reveal another human GBP that facilitates the exposure of *L. pneumophila* into the cytosol, as we previously saw with human GBP1 (Chapter 2).

Additionally, we observed that GBP1 may be important for promoting cell death in response to *L. pneumophila* LPS, whereas GBP2 does not. However, we only observed this phenotype with GBP1−/− THP-1 clone 1. Thus, repeated experiments are required to confirm whether human GBP1 is an essential mediator of cell death in response to *L. pneumophila* LPS and whether the second GBP1−/− THP-1 clone reveals the same phenotype. Additionally, we noticed that there was no change in IL-1β release between WT THP-1 cells and the GBP1−/− and GBP2−/− THP-1 clones. One reason as to why there is no difference in IL-1β secretion between WT and GBP knockout cells is that these THP-1 cells are differentiated
into macrophages using PMA, which robustly promotes IL-1β production. Consequently, there may be such a high background of IL-1β being produced that knockout of one gene that is partially responsible for IL-1β release is not sufficient enough to see a difference. Therefore, we may need to change the differentiation conditions to generate THP-1-derived macrophages that have normal IL-1β production as well as may need to conduct a time course since we may be looking too early for IL-1β release. Different differentiation conditions, such as with Vitamin D3, may generate THP-1-derived macrophages that produce normal amounts of IL-1β in order to examine the role of human GBPs in inflammatory cytokine release in response to LPS.

Our study focuses on the individual roles of caspase-4 and caspase-5. Whether knockout of both caspase-4 and caspase-5 would produce a synergistic effect and greater decrease in inflammasome responses to LPS is unclear. The development of a double CASP4/5−/− THP-1 cell line is needed to address this question. Since caspase-4 and caspase-5 may be activated through different mechanisms, it would be helpful to compare the inflammasome response to the treatment of extracellular LPS or transfection of LPS into the cytosol using these CASP4/5−/− cells in addition to the CASP4−/− and CASP5−/− THP-1 clones.

Furthermore, the GBP knockout experiments in this study suggest that human GBP1 plays a role in response to *L. pneumophila* LPS and *E. coli* LPS. It would be of interest to determine how GBP1 is promoting this cell death response in THP-1-derived macrophages. A couple of experiments that could be done are to conduct confocal microscopy after transfection of biotinylated LPS and staining
of GBP1 or perform an LPS pull-down assay and determine whether these LPS variants are binding to GBP1, which may be transferring the LPS directly to these inflammatory caspases. Thus, GBP1 may be acting as an LPS binding protein, similar to the TLR4 pathway.

This study provides a better understanding of the roles of caspase-4 and caspase-5 to LPS variants and how IFN-inducible GBPs contribute to these inflammasome responses. Our findings provide insight into potential therapeutic targets for gram-negative sepsis.

3.6. Acknowledgements

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3.7. Materials and Methods

3.7.1. Primary Human Samples

All studies on primary human monocyte-derived macrophages (hMDMs) were performed in compliance with the requirements of the US Department of Health and Human Services and the principles expressed in the Declaration of Helsinki. Samples obtained from the University of Pennsylvania Human Immunology Core are considered to be a secondary use of deidentified human specimens and are exempt via Title 55 Part 46, Subpart A of 46.101 (b) of the Code of Federal Regulations.

3.7.2. Cell Culture

Primary human monocytes from deidentified healthy human donors were obtained from the University of Pennsylvania Human Immunology Core. Monocytes were cultured in RPMI supplemented with 10% (vol/vol) heat-inactivated FBS, 2 mM L-glutamine, 100 IU/mL penicillin, 100 µg/mL streptomycin, and 50 ng/mL recombinant human M-CSF (Gemini Bio Products). Cells were cultured for 4 days in 10 mL of media in 10 cm-dishes at 4-5 × 10^5 cells/mL, followed by addition of 10 mL of fresh growth media for an additional 2 days for complete differentiation into macrophages. The day before macrophage stimulation, cells were rinsed with cold PBS, gently detached with trypsin-EDTA (0.05%) and replated in media without antibiotics and with 25 ng/mL M-CSF in a 48-well plate at a concentration of 1 × 10^5 cells per well.
THP-1 cells (TIB-202; American Type Culture Collection) were maintained in RPMI supplemented with 10% (vol/vol) heat-inactivated FBS, 0.05 nM β-mercaptoethanol, 100 IU/mL penicillin, and 100 µg/mL streptomycin at 37°C in a humidified incubator. One or two days before stimulation with IFN-γ or Pam3CSK4, respectively, cells were replated in media without antibiotics in a 48-well plate at a concentration of 2 × 10⁵ cells per well and incubated with phorbol 12-myristate 13-acetate (PMA) for 24 hours to allow differentiation into macrophages. Media was replaced with RPMI without serum for *L. pneumophila* infections or replaced with Opti-MEM I Reduced Serum Media for LPS transfection experiments.

### 3.7.3. Macrophage Stimulation

For the experiment using primary human macrophages, cells were primed with Pam3CSK4 (1 µg/mL) for four hours before LPS transfection.

For the LPS transfection experiment using only PMA-differentiated WT THP-1 or the LPS transfection experiment using PMA-differentiation WT THP-1 cells, *CASP4*⁻/⁻ THP-1 clones, and *CASP5*⁻/⁻ THP-1 clones, cells were either left unprimed or were primed overnight with recombinant human IFN-γ (R&D Systems) at a concentration of 100 U/mL, followed by priming with Pam3CSK4 (1 µg/mL) for four hours before LPS transfection.

For the *L. pneumophila* infection and LPS transfection experiment using PMA-differentiated WT THP-1 cells, or *GBP1*⁻/⁻ THP-1 clones, and *GBP2*⁻/⁻ THP-1 clones, cells were primed overnight with recombinant human IFN-γ at a concentration of 100 U/mL before infection or transfection.
For experiments involving harvesting lysates for RNA and protein analyses from PMA-differentiated WT THP-1, \textit{CASP4}^-/\textit{CASP5}^- THP-1 clones, and \textit{GBP1}^-/\textit{GBP2}^- THP-1 clones, cells were either left unprimed or primed with recombinant human IFN-\textgreek{y} at a concentration of 100 U/mL for 16 or 18 hours.

3.7.4. Bacterial Strain and Macrophage Infection

The \textit{Legionella pneumophila} infection experiment comparing WT THP-1 cells to \textit{GBP1}^- and \textit{GBP2}^- THP-1 clones used the strain derived from the serogroup 1 clinical isolate Philadelphia-1. The strain utilized was a flagellin mutant, \textit{\textit{\textDelta}flaA}, derived from the Lp02 strain (\textit{rpsL}, \textit{hsdR}, \textit{thyA}), which is a thymidine auxotroph (Ren et al., 2006). \textit{L. pneumophila} \textit{\textDelta}flaA was grown as a stationary patch for 48 hours on charcoal yeast extract agar plates at 37°C (J. C. Feeley et al., 1979). Bacteria were resuspended in PBS and added to the cells at a multiplicity of infection (MOI) of 10 in a 48-well plate. Infected cells were then centrifuged at 290 × g for 10 min and incubated at 37°C for 1, 2, or 4 hours. Mock-infected cells were treated with PBS.

3.7.5. Lipopolysaccharide (LPS) or Lipooligosaccharide (LOS) Variants and Macrophage Transfection

All LPS and LOS used in this study were isolated from whole bacteria in the laboratory of Robert Ernst. \textit{L. pneumophila} LPS was isolated from \textit{L. pneumophila} LP02 \textit{\textDelta}flaA strain. \textit{E. coli} LPS was isolated from \textit{E. coli} K-12 W3110 strain. \textit{Y. pestis} LOS was isolated from \textit{Y. pestis} grown at 26°C, including the WT strain as
well as the mutants generated through the addition of specific enzymes to the WT strain. After stimulation of primary human macrophages or THP-1-derived macrophages with IFN-γ and/or Pam3CSK4 as described above, the media was replaced with 300 µL of Opti-MEM I Reduced Serum Media per well. Cells were either mock-transfected with FuGENE HD (Promega) alone or transfected with a mixture of 0.75 µL FuGENE HD [0.25%(vol/vol)] plus LPS or LOS (2 µg/mL of Y. pestis LOS or E. coli LPS and 10 µg/mL L. pneumophila LPS). Plates were then centrifuged at 805 × g for 5 min and incubated at 37°C for 4 or 20 hours.

3.7.6. LDH Cytotoxicity Assay

Macrophages were infected or transfected in a 48-well plate as described above and harvested supernatants were assayed for cell death by measuring loss of cellular membrane integrity through lactate dehydrogenase (LDH) release from cells. LDH release was quantified using an LDH Cytotoxicity Detection Kit (Clontech) according to the manufacturer’s instructions and normalized to mock-infected cells.

3.7.7. ELISA

Macrophages were infected or transfected in a 48-well plate as described above and harvested supernatants were assayed for cytokine levels using ELISA kits for human IL-1β (BD Biosciences).

3.7.8. Quantitative RT-PCR Analysis
RNA was isolated using the RNeasy Plus Mini Kit (Qiagen) following the manufacturer's protocol. Cells were lysed in 350 µL RLT buffer with β-mercaptoethanol and centrifuged through a QIAshredder spin column (Qiagen). cDNA was synthesized from isolated RNA using SuperScript II Reverse Transcriptase (Invitrogen) following the manufacturer's protocol. Quantitative PCR was conducted with the CFX96 real-time system from Bio-Rad using the SsoFast EvaGreen Supermix with Low ROX (Bio-Rad). Transcript levels for each gene were normalized to the housekeeping gene HPRT for each sample, and samples were normalized to unprimed sample or to control siRNA-treated sample using the 2−ΔΔCt (cycle threshold) method to calculate fold change. Relative expression was calculated by normalizing gene-specific transcript levels to HPRT transcript levels for each sample using the 2−ΔΔCt method. Primer sequences from primer bank used for HPRT1, CASP4, CASP5, GBP1, and GBP2 are the following (all 5' → 3'):

**HPRT1** forward: CCTGGCGTCGTGATTAGTGAT
HPRT1 reverse: AGACGTTCAGTCCTGTCCATAA

**CASP4** forward: TCTGCGGAACTGTGCATGATG
CASP4 reverse: TGTGTGATGAAGATAGAGCCCAT

**CASP5** forward: TCACCTGCCTGCAAGGAATG
CASP5 reverse: TCTTTTCGTCAACCACAGTGTAG

**GBP1** forward: AGGAGTTCCCTTCAAAGATGTGGA
GBP1 reverse: GCAACTGGACCCTGTCGTT

**GBP2** forward: CTATCTGCAATTACGCAGCCT
GBP2 reverse: TGTTCTGGCTTCTTGGGATGA
3.7.9. Immunoblot Analysis

In experiments where THP-1-derived macrophages were used, cells were lysed in 1X SDS/PAGE sample buffer. Protein samples were boiled for 5 min, separated by SDS/PAGE on a 12% (vol/vol) acrylamide gel, and transferred to PVDF Immobilon-P membranes (Millipore). Primary antibodies specific for caspase-4 (4450S; Cell Signaling), caspase-5 (D3G4W; 46680S; Cell Signaling), GBP1 (ab131255, Abcam), GBP2 (sc-271568, Santa Cruz), and β-actin (4967L; Cell Signaling) were used. HRP-conjugated secondary antibodies anti-rabbit IgG (7074S; Cell Signaling) and anti-mouse IgG (7076S; Cell Signaling) were used. For detection, ECL Western Blotting Substrate or SuperSignal West Femto (both from Pierce Thermo Scientific) were used as the HRP substrate.

3.7.10. Development of CRISPR/Cas9 Knockout THP-1 cells

To generate CASP4, CASP5, GBP1, or GBP2 knockout in THP-1 cells, pLentiCRISPR v2 plasmids encoding the specific gRNA and Cas9 were purchased from GenScript. The following target sequences were used:

CASP4 gRNA 1: TCCTGCAGCTCATCCGAATA
CASP5 gRNA 2: CGTCAACCACAGTGTAGCCC
GBP1 gRNA 3: ACAAAAGAGACGATAGCCCC
GBP2 gRNA 2: AACTTTCGGATGCACAACCG

Initial production of lentiviral particles were made using the pCMV-VSV-G and psPAX2 plasmids that were generously provided by Paul Bates at the
University of Pennsylvania. HEK293T cells were plated at $2.0 \times 10^6$ cells in a 10 cm-dish in 10 mL of DMEM supplemented with 10% (vol/vol) heat-inactivated FBS, 2 mM L-glutamine, 100 IU/mL penicillin, 100 µg/mL streptomycin. 24 hours after plating HEK293T cells, the plasmids were transfected using the Lipofectamine 2000 protocol. A DNA master mix of the plasmids contained 1 µg of pCMV-VSV-G, 2.5 µg of psPAX2, and 5 µg of pLentiCRISPR v2 with the specific gRNA. 50 µL of Lipofectamine 2000 transfection reagent was used per 10 cm-dish. Transfected HEK293T cells were incubated at 37°C for 18 hours, followed by careful removal of the media and replacement with 6 mL of fresh HEK293T cell growth media per dish. 24 hours later, the supernatant containing the lentiviral particles was harvested and filtered using a 0.22 µM filter. $5 \times 10^5$ THP-1 cells were infected with 1 mL of lentiviral particles treated with 8 µg/mL of polybrene and plated in a TC-treated 12-well plate. Infected THP-1 cells were then centrifuged at $1250 \times g$ for 90 min at 25°C. Cells were then carefully pipetted out of the wells and added to a conical tube respective for each gRNA condition. Cells were centrifuged at $805 \times g$ for 3 min at 25°C. Media was aspirated and cells were resuspended in fresh THP-1 growth media and 2 mL of resuspended cells were added per well of a 12-well plate. Cells were incubated at 37°C for 48 hours. After 48 hours, puromycin was added at a final concentration of 1 µg/mL. Cells were maintained in puromycin for 2-3 weeks and then harvested for western blot analysis and clonal selection. For clonal selection, cells were plated at a concentration of 0.5 cell per 200 µL of THP-1 growth media in flat-bottom 96-well plates and incubated at 37°C for 4-6 weeks until single clones were noticeable at the bottom of the well. 12 single cell
clones for each gene were expanded from the 96-well plate to a 48-well plate, 24-well plate, 12-well plate, 6-well plate, and finally 10 cm-dish. The cells from each single cell clone were plated in a 48-well plate at a concentration of $2 \times 10^5$ cells per well in 500 µL of media treated with PMA for differentiation into macrophages, the next day either stimulated with IFN-γ overnight or left unstimulated, and harvested for DNA, RNA, and western blot analyses.

3.7.11. Validation of CRISPR/Cas9 Knockout THP-1 Single Cell Clones for CASP5 and GBP1

After choosing three single cell clones for CASP5 or GBP1 based on RNA knocked down and protein knocked out, DNA from the selected single cell clones was purified using the DNeasy Blood and Tissue Kit (Qiagen). The genomic region comprising the gRNA target sequence for each gene was amplified by PCR using the following primers (all 5' → 3'):

**CASP5** forward: GGTAGGGGAAGGTGGCAGC
**CASP5** reverse: GGGGCTACATCCCAATCACC
**GBP1** forward: GGTGAGGAGGCTGTCAGTTTC
**GBP1** reverse: ACTCTCTTTTGATGAGCACCTAGGAC

The PCR product was purified using the QIAquick PCR purification Kit (Qiagen). The purified PCR product was then ligated into the pGEM-T vector (Promega) and transformed into DH5α high efficiency competent cells using the protocol from the pGEM-T Vector Systems Protocol. Following blue-white screening, colony PCR was conducted on 10-15 white colonies using the primer
sequences shown above and PCR product was run on an agarose gel. Positive colonies showing the correct bp amount were sequenced using the M13/pUC primer: 5’ CCCAGTCACGACGTTGTAACG 3’.

3.7.12. Statistical Analysis

GraphPad Prism software was used for graphing of data and all statistical analyses. Statistical significance for experiments with THP-1 cells was determined using the unpaired two-way Student’s t test. Statistical significance for hMDMs was determined using the paired two-way t test in experiments comparing multiple donors. In hMDM experiments that compare cells from multiple donors, data are graphed so that each data point represents the mean of duplicate wells for each donor, and all statistical analysis was conducted comparing the means of each experiment. Differences were considered statistically significant if the P value was <0.05.
A. Data Summary

The innate immune response to bacterial pathogens is crucial for pathogen clearance and host survival. Specifically, the inflammasome, which is a cytosolic multimeric protein complex activated in response to bacterial components or danger signals, is important for initiating an inflammatory form of cell death known as pyroptosis and IL-1 family cytokine release to signal to additional immune cells for their recruitment and/or activation. IL-1 family cytokines can act in an autocrine or paracrine manner to upregulate a family of cytokines termed interferons (IFNs). For instance, IL-18 is a cytokine produced and released by infected cells that can then stimulate other immune cells, such as NK or T cells, to produce the cytokine IFN-γ. IFN-γ has been shown to promote inflammasome responses to a variety of gram-negative bacterial pathogens as well as to bacterial components. Specifically, a family of IFN-induced GTPases known as guanylate binding proteins (GBPs) are the key factors responsible for promoting inflammasome responses through a number of functions, including the rupture of pathogen-containing vacuoles or bacteriolysis of bacterial outer membranes. Although most studies focused on the role of GBPs during inflammasome activation have been conducted using murine models, recent findings using human cells have identified key human GBPs for promoting inflammasome responses to certain bacteria. However, the role of human GBPs in inflammasome responses during infection with the vacuolar bacterium Legionella pneumophila has not been investigated.
Additionally, the noncanonical inflammasome, which is activated by intracellular LPS from gram-negative bacteria, is comprised of caspase-11 in mice and the two orthologs, caspase-4 and caspase-5, in humans. LPS can vary in its acylation and phosphorylation state depending on the bacterial species and environmental conditions. Recent findings revealed key differences between murine caspase-11 and human caspase-4 activation. Specifically, tetra-acylated LPS was found to evade caspase-11 detection, while it activates caspase-4 in human macrophages. Moreover, the role of caspase-5 in response to intracellular LPS is unclear and whether IFN or human GBPs promote inflammasome responses to LPS from *L. pneumophila* has not been studied.

Mice and humans possess differences in their innate immune genes and these differences may play a role in how they respond to invading pathogens. Therefore, it is essential to gain a better understanding of human inflammasome responses to the vacuolar intracellular bacterial pathogen, *L. pneumophila*, and its corresponding LPS and whether IFN or human GBPs play a role in these responses.

In Chapter 2, we determined that IFN-γ promotes caspase-1, caspase-4, and caspase-5 inflammasome responses to *L. pneumophila* in human macrophages. By utilizing siRNA-mediated knockdown of the individual human GBPs, we found that human GBP1 is essential for this maximal inflammasome response. Furthermore, we revealed that IFN-γ treatment leads to the rupture of *L. pneumophila*-containing vacuoles (LCVs) and that GBP1 contributes to this rupture. In contrast to the murine model which shows that GBPs do not rupture the
LCV but instead rupture the outer membrane of *L. pneumophila*, our findings indicate that human GBP1 does rupture the LCV. It remains to be determined whether additional human GBPs, along with human GBP1, help promote the rupture of the LCV or whether human GBPs also induce bacteriolysis of the outer membrane of *L. pneumophila*.

In Chapter 3, we found that the human noncanonical inflammasome is activated by a variety of differential acylated and phosphorylated LPS variants isolated from *Y. pestis* in primary human macrophages, and that IFN-γ promotes this response to both tetra-acylated and hexa-acylated *Y. pestis* LPS. We also utilized CRISPR/Cas9 gene editing technology to knockout caspase-4, caspase-5, GBP1, and GBP2 in the THP-1 monocytic cell line. We used these generated knockout cell lines to elucidate the role of these genes in response to LPS derived from *L. pneumophila* and *E. coli*. We found that caspase-4 is essential for promoting noncanonical inflammasome responses to *E. coli* LPS, while caspase-5 may not play a role in detecting intracellular *E. coli* or *L. pneumophila* LPS. Furthermore, we found that human GBP1 may play a role in promoting cell death to *L. pneumophila* LPS and *E. coli* LPS, while human GBP2 may not. While these experiments need to be repeated to confirm these findings, they suggest that caspase-4 and caspase-5 have distinct roles in response to different LPS variants, and that human GBP2 does not promote inflammasome responses to *L. pneumophila* LPS but may promote cell death during *L. pneumophila* infection.

Overall, our studies bring insight into human inflammasome responses to the vacuolar pathogen, *L. pneumophila*, as well as to LPS variants from different
bacteria. Moreover, our studies reveal key findings on how IFN-γ and, more specifically, IFN-inducible GBPs contribute to the activation of the human inflammasome. However, there are additional questions pertaining human GBPs and the human noncanonical inflammasome that are still left unanswered. In this chapter, I will discuss these questions and propose future experiments in order to gain a better understanding of human inflammasome responses to gram-negative bacterial pathogens (Fig. 4.1 and 4.2).

B. Future Directions

Figure 4.1. Model and future directions for human inflammasome responses to *L. pneumophila*. Our data reveal that human GBP1 is important for maximal inflammasome responses during *L. pneumophila* infection and indicate that human GBP1 mediates the rupture of the LCV. Whether and how additional human GBPs or host factors are involved in inflammasome responses to *L. pneumophila* are unknown. Future directions for these studies are discussed in this chapter.

Does human GBP1 have a synergistic role with additional human GBPs to promote inflammasome responses to *L. pneumophila*?
We find that upon individual knockdown of human GBP1-5 using siRNA, only knockdown of GBP1 resulted in significantly decreased cell death and IL-1 family cytokine release, indicating that human GBP1 is important for maximal inflammasome response to *L. pneumophila*. One possible reason as to why only GBP1 was shown to be essential for inflammasome response compared to the remaining GBPs tested is that human GBP1 may be the initiator GBP that responds during *L. pneumophila* infection and additional GBPs follow suit. This assumption is based on human GBP findings during *Shigella flexneri* infection. Specifically, it was revealed that human GBP1 is recruited to the outer membrane of *S. flexneri*, followed by human GBP2, 3, 4, and 6 to inhibit the actin motility of *S. flexneri* (Piro et al., 2017; Wandel et al., 2017). In addition to *S. flexneri*, human GBP1 initiates the assembly of a GBP complex, including GBP1-4, on the surface of *S. Typhimurium* outer membrane in order to recruit caspase-4 for inflammasome activation (Santos et al., 2020; Wandel et al., 2020). Therefore, human GBP1 may also initiate binding to the LCV as well as the outer membrane of *L. pneumophila* in order to recruit additional GBPs for their disruption of the LCV and outer membrane to release *L. pneumophila* components into the cytosol for downstream inflammasome sensing. In fact, we found that GBP2, in addition to GBP1, is also recruited to *L. pneumophila*. However, we did not test whether GBP1 knockdown resulted in decreased GBP2 binding.

Since GBPs can homo- or hetero-dimerize (Britzen-Laurent et al., 2010), we want to investigate whether knockdown of other human GBPs, in addition to human GBP1, leads to a further decrease in inflammasome response during *L.
*pneumophila* infection compared to knockdown of human GBP1 alone. This experiment can be accomplished by conducting double knockdowns of human GBP1 with either human GBP2, 3, 4, or 5, followed by IFN-γ priming and *L. pneumophila* infection. In addition, we can conduct siRNA-mediated knockdown of human GBP2, 3, 4, or 5 in the GBP1/− THP-1 clones. These experiments will determine whether human GBPs play a synergistic role with human GBP1 to stimulate inflammasome responses during *L. pneumophila* infection.

**Does human GBP1 directly interact with and rupture the LCV?**

Our study in Chapter 2 indicates that human GBP1 ruptures the LCV of *L. pneumophila*, however the precise mechanism of vacuolar rupture as well as the vacuolar components that GBP1 may be binding to are unknown. Experiments to address these questions are technically challenging because *L. pneumophila* derives its vacuole from the ER and, thus, isolation of the LCV following infection with *L. pneumophila* in human macrophages may include ER components as well as the LCV. However, purification of LCVs from amoebae and phagocytes has been conducted (Hoffmann, Finsel, & Hilbi, 2012). Therefore, we can purify the LCV from *L. pneumophila* infected human macrophages using this specific optimized protocol followed by proteomic approaches to identify any interacting proteins. An additional step that would be needed to specifically determine what vacuolar components are interacting with human GBP1, would be to conduct a pull-down of GBP1 following the LCV purification and then use mass spectrometry to identify the interacting protein partners of GBP1. Furthermore, super-resolution
microscopy may also be used to stain for any identified interacting LCV proteins with GBP1 to visualize these interactions. Although technically challenging, if these proposed methods are successful, then this study would reveal novel human GBP1 interactions with bacterial vacuolar components.

In Chapter 2, the phagosome integrity assay we conducted in control siRNA-treated and GBP1 siRNA-treated primary human macrophages suggests that GBP1 is essential for LCV rupture, since we saw decreased anti-L. pneumophila antibody staining in macrophages with GBP1 knocked down. However, these results may also indicate that GBP1 simply promotes anti-L. pneumophila antibody binding to the bacterial surface. Thus, additional experiments are needed to address whether GBP1 actually promotes rupture of the LCV. An alternative experiment to assess the vacuolar integrity of the LCV would be to stain for galectin proteins. Galectins are a family of β-galactoside-binding proteins that target the inner leaflet of ruptured vacuoles and are therefore characterized as being markers for vacuolar rupture (E. M. Feeley et al., 2017). To determine if human GBP1 induces rupture of the LCV, we can stain for galectin-3 or galectin-8 in IFN-γ-primed primary human macrophages that are treated with control siRNA or GBP1 siRNA and infected with dsRED-expressing L. pneumophila. If we observe a decreased presence of galectin staining with L. pneumophila upon GBP1 knockdown, then these results indicate that there are fewer disrupted vacuoles in the absence of GBP1 and would reveal that human GBP1 contributes to the rupture of the LCV. Additionally, another approach of quantifying the amount of cytosolic L. pneumophila or L. pneumophila with
ruptured vacuoles would be to conduct a flow cytometry-based assay in digitonin and saponin permeabilized primary human macrophages in the presence and absence of GBP1. These results would reveal whether or not human GBP1 contributes to the presence of cytosolic *L. pneumophila*, as we see using microscopy. Flow-cytometry can also be used to stain galectin as previously mentioned and can therefore serve as a confirmatory assay for our microscopy-based experiments. These proposed experiments may provide insight on whether human GBP1 contributes to the vacuolar rupture of *L. pneumophila* or just allows the anti-*L. pneumophila* antibody to target the bacteria more efficiently.

**Does human GBP1, as well as other human GBPs, disrupt the outer membrane of *L. pneumophila*?**

Mouse GBPs on chromosome 3 do not rupture the LCV, but they induce bacteriolysis on the outer membrane of *L. pneumophila* (B. C. Liu et al., 2018). In contrast, our study in Chapter 2 indicates that human GBP1 ruptures the LCV, although it is possible that GBP1 can disrupt the outer membrane as well. Recent studies focused on human GBPs revealed that human GBP1-4 bind to the outer membrane of cytosol exposed S. Typhimurium and potentially result in the loss of membrane integrity, thus making LPS more accessible for caspase-4 detection (Santos et al., 2020; Wandel et al., 2020). Therefore, in order to determine whether human GBP1 facilitates the disruption of the *L. pneumophila* outer membrane, we need to utilize the cytosol-exposed *L. pneumophila ΔsdhA* mutant, which is not able to maintain the vacuole integrity of the LCV. We can conduct a bacterial
morphology assay coupled with super-resolution microscopy as a way to qualitatively determine whether the *L. pneumophila ΔsdhA* mutant either sustains its rod-shape, indicating that its outer membrane is intact, or assumes a swollen and truncated morphology, signifying that its outer membrane was disrupted. This assay would be conducted in IFN-γ-primed WT and GBP1−/− THP-1-derived macrophages infected with a dsRED-expressing *L. pneumophila ΔsdhA* mutant. Thus, if human GBP1 facilitates the disruption of the *L. pneumophila* outer membrane, WT macrophages would show a perturbed *L. pneumophila* shape, while GBP1−/− THP-1-derived macrophages would reveal an undamaged *L. pneumophila*. Furthermore, we can also test whether additional human GBPs disrupt the *L. pneumophila* outer membrane by applying RNAi-mediated knockdown of an individual GBP compared to control-knockdown in either primary human macrophages or in THP-1-derived macrophages, followed by IFN-γ treatment and infection with the dsRED-expressing *L. pneumophila ΔsdhA* mutant.

Another method to determine whether the *L. pneumophila* outer membrane is disrupted is by measuring the amount of *L. pneumophila* DNA released into the cytosol comparing WT macrophages or macrophages with individual human GBPs knocked down. This experiment requires a *L. pneumophila ΔsdhA* mutant that harbors a non-transferable pJB908 plasmid that is only released into the cytosol if the outer membrane is disturbed (B. C. Liu et al., 2018). Altogether, this proposed experiment along with the bacterial morphology assay will reveal whether human GBPs promote bacteriolysis of *L. pneumophila*, ultimately leading to its increased access in the host cytosol for inflammasome sensing.
Are additional host factors involved in promoting inflammasome responses during *L. pneumophila* infection?

In addition to GBPαs, another IFN-inducible family of GTPases was discovered to promote inflammasome responses to bacterial pathogens in mice—the immunity related GTPases (IRGs). Specifically, IRGB10 is recruited to *F. novicida* in a GBP-dependent manner and is essential for mediating bacterial killing for downstream AIM2 inflammasome activation in mice (Man et al., 2016). In contrast to mice, which have 23 IRG proteins, humans only possess two IRG proteins, IRGM and IRGC, both of which are not interferon inducible due to the loss of the interferon response element ahead of their transcription start site (Bekpen et al., 2005). Additionally, there is no human ortholog of mouse IRGB10 and the only function that human IRGM has been shown to have during bacterial infections is to limit *M. tuberculosis* infection through an autophagy-mediated pathway (Singh et al., 2006). It is unknown whether human IRG proteins are involved in inflammasome responses during bacterial infections. We have previously conducted knockdown experiments of IRGM and IRGC in primary human macrophages, followed by infection with *L. pneumophila*, and found that the relative expression levels of these genes are very low and their knockdown does not lead to decreased inflammasome response. Therefore, IRGM and IRGC may not play a role in inflammasome responses during *L. pneumophila* infection in human macrophages.
There are most likely additional undiscovered host factors that are important in promoting inflammasome responses to *L. pneumophila* in human macrophages. A genome-wide RNAi screen followed by *L. pneumophila* infection and analysis of cell death and IL-18 or IL-1β release is one way to discover novel host genes that are important for contributing to the inflammasome response. An alternative method would be to conduct a pull-down followed by mass spectrometry. In Chapter 2, we show that *L. pneumophila* activates both canonical and noncanonical inflammasomes and that human GBP1 plays a large role in these inflammasome responses. To identify additional host factors that may play a role with human GBP1, we can conduct a pull-down assay of GST-tagged GBP1 using glutathione agarose beads. This experiment would involve initial transfection of GST-tagged recombinant GBP1 into human macrophages, then infection with *L. pneumophila*. The cell lysate from these infected macrophages would be incubated with glutathione agarose beads and, after washing steps, would elute the GST-tagged GBP1. Any bound proteins to human GBP1 can be analyzed by mass spectrometry to identify these unknown interactors. Therefore, this experiment may bring insight into host proteins that interact with human GBP1 to help facilitate the rupture of the LCV and/or promote inflammasome responses to *L. pneumophila*. 
Figure 4.2. Future directions for human inflammasome responses to LPS variants. Our data suggest that the human noncanonical inflammasome is activated by different LPS variants and that IFN-γ promotes these responses. However, the specific roles that caspase-4 and caspase-5 have in response to different LPS variants are unclear and whether human GBP1 is directly involved in recognizing *L. pneumophila* LPS is unknown. Future directions for these studies are discussed in this chapter.

Do caspase-4 and caspase-5 have a synergistic function or play distinct roles in response to intracellular LPS from different gram-negative bacteria?

It is intriguing that humans possess two inflammatory intracellular caspases that bind to LPS, whereas mice only have one caspase that recognizes LPS. Human caspase-4 and caspase-5 are thought to have resulted from a gene duplication event from the ancestral gene, caspase-11. It is also known that humans are more sensitive to LPS compared to mice. Since human caspase-4 was found be activated in response to tetra-acylated LPS, which is known to evade murine caspase-11 detection in addition to other under-acylated LPS, it is possible that humans need two LPS-sensing caspases in order to identify diverse LPS from different gram-negative bacteria to initiate inflammatory responses to eliminate the
these invading pathogens. Thus, it would be of interest to determine whether caspase-4 and caspase-5 work together to respond to different gram-negative bacterial infections, either through synergistic functions or distinct roles. Using $\text{CASP4}^{-/-}$ and $\text{CASP5}^{-/-}$ THP-1 cells, we observed in Chapter 3 that caspase-4 is important for mounting an inflammasome response to $\text{E. coli}$ LPS as well as possibly to $\text{L. pneumophila}$ LPS, while caspase-5 did not seem to play a strong role in response to either LPS. This indicates that caspase-4 and caspase-5 may respond to LPS variants from different bacteria. Moreover, we can use these $\text{CASP4}^{-/-}$ and $\text{CASP5}^{-/-}$ THP-1 cells to test the roles of caspase-4 and caspase-5 to other LPS variants, including the seven differential acylated and phosphorylated LOS variants derived from $\text{Y. pestis}$ that we determined all activate the human noncanonical inflammasome. This investigation will be a starting point to determine whether caspase-4 and caspase-5 identify and respond to distinct LPS variants.

However, it may be also possible that caspase-4 and caspase-5 are activated by the same LPS variants from specific gram-negative bacteria. Therefore, comparing the single knockout clones of $\text{CASP4}^{-/-}$ and $\text{CASP5}^{-/-}$ THP-1 cells to double $\text{CASP4/5}^{-/-}$ THP-1 cells will be necessary to determine whether caspase-4 and caspase-5 stimulate a synergistic response to LPS. If the $\text{CASP4/5}^{-/-}$ THP-1 cells show a decreased inflammasome response compared to the $\text{CASP4}^{-/-}$ and $\text{CASP5}^{-/-}$ THP-1 cells, then capase-4 and caspase-5 carry out a synergistic role. Following LPS binding on the CARD domains of caspase-4 and caspase-5, these caspases oligomerize before activation (Shi et al., 2014). In addition, the caspase-4 and caspase-5 CARD domains share 56% protein identity, suggesting
that they are capable of interacting with each other through their CARD domains. Therefore, it may be possible that caspase-4 and caspase-5 hetero-oligomerize to generate a stronger inflammasome response. To test whether caspase-4 and caspase-5 form a hetero-dimer, we can conduct a bimolecular fluorescence complementation (BiFC) assay to visualize the interactions of caspase-4 and caspase-5. First, we will fuse the N- and C-terminal non-fluorescent fragments of Venus fluorescent protein, a variant of yellow fluorescent protein (YFP), to the amino-terminus of caspase-4 and caspase-5, respectively. Then, we will clone these fused proteins into a mammalian expression vector, followed by transfection of these caspase-4 and caspase-5 vectors into HEK293T cells, which do not possess inflammasome components, and subsequent transfection of LPS. If caspase-4 and caspase-5 hetero-dimerize, then the N- and C-terminal non-fluorescent fragments would come together to produce a fluorescent signal, which we would visualize by microscopy. This study would provide molecular insight into caspase-4 and caspase-5 interactions that may contribute to noncanonical inflammasome activation.

**Do caspase-4 and caspase-5 engage in different pathways of activation depending on the mode of entry of LPS?**

Caspase-4 and caspase-5 were previously shown to have different mechanisms of activation in response to *E. coli* LPS. Specifically, caspase-4 was found to be cleaved and activated in response to transfected LPS, whereas caspase-5 is cleaved in response to extracellular LPS treatment in primary human
macrophages (Casson et al., 2015). These results suggest that caspase-4 and caspase-5 may engage in distinct pathways of activation based on the mode of entry of LPS. Additional experiments in monocytes revealed that caspase-5 is activated upon extracellular treatment with *E. coli* LPS or *P. aeruginosa* OMVs, both of which enter the monocytes via endocytosis (Bitto et al., 2018; Vigano et al., 2015). These combined findings indicate that caspase-5 activation may involve an alternative extracellular pathway through endocytosis. TLR4 is present on the plasma membrane and is known as the extracellular LPS sensor that is endocytosed upon LPS detection. Whether TLR4 and its cofactors are involved in caspase-5 activation through this extracellular LPS sensing mechanism has not been studied. Since we have only looked at the role of caspase-4 and caspase-5 in THP-1-derived macrophages that are transfected with LPS derived from *L. pneumophila* or *E. coli*, we additionally want to investigate the role of caspase-4 and caspase-5 in human macrophages that are extracellularly treated with these LPS variants. Thus, we would conduct an experiment in which either WT, CASP4−/−, and CASP5−/− THP-1-derived macrophages are either extracellularly treated or transfected with LPS from *L. pneumophila* or *E. coli* in order to distinguish the conditions in which caspase-4 and caspase-5 are activated. Additionally, to determine whether TLR4 is involved in the activation of caspase-4 or caspase-5 during treatment or transfection with these LPS variants, we can treat the three different THP-1 cells with the TLR4 pharmacological inhibitor, TAK-242, before introduction of LPS. These findings would bring insight into the mechanism of
human noncanonical inflammasome activation by extracellular LPS or intracellular LPS and how TLR4 may be involved.

**Does human GBP1 directly bind to *L. pneumophila* LPS?**

Human GBPs localize to the outer membrane of different gram-negative bacteria including *S. flexneri*, *S. Typhimurium*, and *F. novicida* (Kutsch et al., 2020; Lagrange et al., 2018; Piro et al., 2017; Santos et al., 2020; Wandel et al., 2020; Wandel et al., 2017). Human GBP1 was found to target the *S. flexneri* rough mutant, which lacks the O-antigen on its LPS, less efficiently compared to *S. flexneri* that possesses its O-antigen (Piro et al., 2017). More specifically, the O-antigen is important for allowing the transition of surface-docked GBP1 polymers into GBP1 protein sheets that coat the entire surface of *S. flexneri* outer membrane (Kutsch et al., 2020). These results combined with recent findings that human GBP1 directly binds to LPS from *E. coli* and *S. Typhimurium* indicate that human GBP1 initially binds to LPS on the outer membrane of these bacteria followed by recruitment of addition GBPs to control host response against these pathogens (Santos et al., 2020). However, whether human GBP1 or other GBPs can bind to *L. pneumophila* LPS has not been investigated. Our results in Chapter 3 showed that human GBP1 may be promoting cell death in response to *L. pneumophila* LPS, therefore, GBP1 may be directly binding to LPS in this setting. A pull-down assay would be one way to determine whether this human GBP1-*L. pneumophila* LPS interaction is occurring. To test this, we would transflect biotynlated *L. pneumophila* LPS in human macrophages, followed by a pull-down using
streptavidin magnetic beads, washes, and immunoblot for human GBP1. These results may bring a better understanding of human GBP1 interactions and may reveal that human GBP1 also binds to the outer membrane in addition to the LCV.

**Concluding Remarks**

Our results bring insight into human inflammasome responses to *L. pneumophila* and LPS variants from different bacteria. We found that human GBP1 is important for promoting inflammasome responses and vacuolar disruption during *L. pneumophila* infection, which is in contrast to mouse GBPs that rupture the outer membrane of *L. pneumophila*. Additional experiments must be conducted to understand the mechanism of GBP1 rupture of the LCV and whether human GBPs contribute to bacteriolysis as well. We also show that the human noncanonical inflammasome is activated by LPS variants that differ in their acylation and phosphorylation state and that caspase-4 plays a significant role in response transfected LPS. Future studies must be conducted to establish the role of caspase-5 and human GBPs in response to LPS.

Overall, these studies provide insight into the role that IFN-γ plays during infection with gram-negative bacteria in human macrophages. There are currently no approved treatments for sepsis in humans and studies in mice do not always correlate to what happens in humans. Therefore, it is important to understand human innate immune responses involved in combatting bacterial infections, which, if left unregulated, can ultimately lead to septic shock and death. These studies investigate host-pathogen interactions in human macrophages, which are
one of the first responders to invading pathogens, and reveal potential therapeutic host targets that can be used to treat gram-negative sepsis in humans.


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