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Lipid A Variants Activate Human TLR4 and the Noncanonical Inflammasome Differently and Require the Core Oligosaccharide for Inflammasome Activation

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ABSTRACT Detection of Gram-negative bacterial lipid A by the extracellular sensor, myeloid differentiation 2 (MD2)/Toll-like receptor 4 (TLR4), or the intracellular inflammasome sensors, CASP4 and CASP5, induces robust inflammatory responses. The chemical structure of lipid A, specifically its phosphorylation and acylation state, varies across and within bacterial species, potentially allowing pathogens to evade or suppress host immunity. Currently, it is not clear how distinct alterations in the phosphorylation or acylation state of lipid A affect both human TLR4 and CASP4/5 activation. Using a panel of engineered lipooligosaccharides (LOS) derived from *Yersinia pestis* with defined lipid A structures that vary in their acylation or phosphorylation state, we identified that differences in phosphorylation state did not affect TLR4 or CASP4/5 activation. However, the acylation state differentially impacted TLR4 and CASP4/5 activation. Specifically, all tetra-, penta-, and hexa-acylated LOS variants examined activated CASP4/5-dependent responses, whereas TLR4 responded to penta- and hexa-acylated LOS but did not respond to tetra-acylated LOS or penta-acylated LOS lacking the secondary acyl chain at the 3′ position. As expected, lipid A alone was sufficient for TLR4 activation. In contrast, both core oligosaccharide and lipid A were required for robust CASP4/5 inflammasome activation in human macrophages, whereas core oligosaccharide was not required to activate mouse macrophages expressing CASP4. Our findings show that human TLR4 and CASP4/5 detect both shared and nonoverlapping LOS/lipid A structures, which enables the innate immune system to recognize a wider range of bacterial LOS/lipid A and would thereby be expected to constrain the ability of pathogens to evade innate immune detection.

KEYWORDS caspase-4, inflammasome, TLR4, lipid A, lipopolysaccharide

Gram-negative bacteria are responsible for more than 30% of hospital-acquired infections in the United States, making them a costly and deadly public health concern (1). Uncontrolled Gram-negative bacterial infections can lead to detrimental outcomes, including sepsis, which is an overwhelming systemic inflammatory response to an infection. If left untreated, a septic host will succumb to organ failure and, ultimately, death. Preclinical studies in mice successfully treated sepsis using immunomodulators that functioned by neutralizing either host inflammatory mediators or microbial products (2). However, over a hundred clinical trials testing these immunomodulators

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in sepsis patients have failed (2). The reasons for these failures are unclear but may be due to differences between murine and human innate immune responses that play a role in responding to Gram-negative bacterial infections. Understanding further the human innate immune response to Gram-negative bacterial pathogens may aid in identification of potential novel therapeutic targets for the treatment of Gram-negative sepsis.

Gram-negative sepsis is caused by the bacterial endotoxin, lipopolysaccharide (LPS) or lipooligosaccharide (LOS), which is the major lipid component in the outer leaflet of the outer membrane of Gram-negative bacteria (3–5). LPS is composed of a lipid A membrane anchor attached to a core oligosaccharide, which has a variable number of repeating O-antigen carbohydrate units attached to it (6–8). If a particular bacterial species makes lipid A with only the core oligosaccharide attached, it is then named LOS instead of LPS (9, 10). Both LOS and LPS have a lipid A moiety, which acts as a membrane anchor in the outer leaflet of the Gram-negative outer membrane. It is the lipid A subunit that activates both cell surface and cytosolic sensors, which subsequently lead to signaling events resulting in inflammatory cytokine release as well as a form of inflammatory programmed cell death termed pyroptosis (11–13). The Toll-like receptor 4 (TLR4) complex is a plasma membrane-bound receptor that detects lipid A in the extracellular environment or within endosomal compartments. The lipid A/TLR4 signal transduction pathway involves binding of lipid A to the signaling cofactor myeloid differentiation 2 (MD2) (14). Upon lipid A binding, the MD2/TLR4 complex dimerizes, leading to a conformational change in TLR4 and downstream signaling that promotes production of proinflammatory cytokines (15).

In addition to extracellular sensing of lipid A by TLR4, lipid A that enters the cytosol in the context of invasive bacterial pathogens or delivery by bacterium-derived outer membrane vesicles is sensed by the cytosolic receptor caspase-11 (Casp11) in mice or the human orthologs CASP4 and CASP5 (16–18). Binding of lipid A to the caspase activation and recruitment domain (CARD) of Casp11, CASP4, or CASP5 leads to their oligomerization and formation of a noncanonical inflammasome, resulting in their autoproteolytic cleavage and activation (19). Active caspase-11, -4, and -5 subsequently cleave gasdermin-D (GSDMD), the initiator protein of pyroptosis (20–22). Importantly, both TLR4 and the noncanonical inflammasome make independent contributions to host protection in models of systemic Gram-negative infection as well as to immunopathology in models of lethal sepsis.

Lipid A comprises a glucosamine disaccharide linked to hydrophobic acyl chains that vary in number, position, and length depending on the bacterial species (23). Also contingent on the bacterial species, lipid A possesses phosphate groups located on the 1 and/or 4′ positions of the two glucosamine residues (24). The MD2 coreceptor recognizes specific lipid A structures and, upon binding, undergoes a conformational change which initiates the activation of TLR4, whereas intracellularly, the CARD domains of Casp11/4/5 recognize LPS to activate the inflammasome (19, 25).

Intriguingly, pathogenic bacteria modify their acylation and phosphorylation states in response to environmental cues, suggesting that changing these vital features is important for their pathogenesis and potentially allowing these bacteria to evade immune detection or resist innate immune killing mechanisms (26, 27). There are also species-specific differences in lipid A recognition, as evidenced by the observations that murine and human TLR4 can differ in their responses to distinct lipid A variants (28, 29). For example, tetra-acylated *Yersinia pestis* lipid A evades TLR4 detection in humans while maintaining slight agonist activity for murine TLR4, whereas the hexa-acylated form robustly activates TLR4 in both mice and humans (30, 31). Additionally, penta-acylated LPS from *Neisseria meningitidis* LpxL1 potently activates murine TLR4 but not human TLR4 (32). Thus, not only does TLR4 differentially respond to distinct structural lipid A variants, but there are also differences in how human or murine TLR4 respond to a given lipid A structure.

Similar to the murine MD2/TLR4 receptor system, the murine noncanonical Casp11 inflammasome responds poorly to lipid A with a lower number of acyl chains (i.e., tetra-acylated), whereas lipid A containing a higher number of acyl chains (i.e., hexa-acylated) robustly activates Casp11 for downstream pyroptosis and release of inflammatory cytokines (33). Interestingly, some penta-acylated lipid A variants, such as *Francisella novicida lpxF* mutant lipid A, can activate Casp11 (17), while other penta-acylated lipid A variants, such as *Rhizobium galegae* lipid A, fail to activate Casp11 (16). However, it is unknown whether the number of acyl chains is the sole structural feature that dictates noncanonical inflammasome activation or whether other structural features of lipid A also influence Casp11 activation.

In contrast to the murine system, the human noncanonical inflammasome is activated in response to tetra-acylated lipid A, including tetra-acylated *Francisella novicida* lipid A, as well as penta- and hexa-acylated lipid A (18). This indicates that the human noncanonical inflammasome can be activated by lipid A structures with a broader range of acylation states (18). However, whether the position of acyl chains, the number of phosphoryl groups, or other structural modifications play a role in the ability of lipid A to activate the human noncanonical inflammasome has not been studied.

Here, we sought to dissect the relative contribution of lipid A acyl chain number and position, glucosamine phosphorylation, and the core oligosaccharide to the activation of the human lipid A sensing pathways. We used a comprehensive panel of lipid A or LOS structures that vary in acylation and phosphorylation state, and either lack or contain the core oligosaccharide. These structures were isolated from acyltransferase and/or phosphatase bacterial mutants generated in an avirulent strain of *Yersinia pestis*. *Y. pestis* naturally produces LOS due to a mutation that only allows for addition of a single O-antigen unit (34). We found that differences in lipid A phosphorylation state did not affect TLR4 or CASP4/5 activation. However, the acylation state differentially impacted TLR4 and noncanonical inflammasome activation. Specifically, all examined tetra-, penta-, and hexa-acylated LOS variants activated the human noncanonical inflammasome. In contrast, TLR4 responded to penta- and hexa-acylated LOS but did not respond to tetra-acylated LOS or penta-acylated LOS lacking the secondary acyl chain at the 3′ position. Additionally, we found that while lipid A alone was sufficient for TLR4 activation or to activate the noncanonical inflammasome in mouse macrophages, human macrophages required both lipid A and the core oligosaccharide to mount a robust noncanonical inflammasome response. In summary, our findings reveal that the human cytosolic and cell surface lipid A sensing systems respond to both shared and unique structures. Furthermore, our data indicate that the human noncanonical inflammasome can respond to a wider range of lipid A structures than murine Casp11, murine TLR4, and human TLR4. We expect that the collective ability of human TLR4 and human CASP4/5 to recognize a broader range of lipid A structures provides a comprehensive detection strategy that limits Gram-negative pathogen evasion of innate immune sensing.

RESULTS

Structure-activity relationship of LOS with the human TLR4 signaling complex. To determine the effect of acyl chain variation on the activation of the human TLR4 signaling complex, we treated HEK-Blue hTLR4 and THP1-Dual reporter cell lines with a series of LOS variants that were generated in *Y. pestis* using bacterial enzymatic combinatorial chemistry (BECC) (35). We employed a well-characterized set of LOS variants derived from hexa-acylated wild-type (WT) *Y. pestis*, two penta-acylated LOS variants isolated from *Y. pestis* strains lacking the acyltransferase MsbB or LpxP, which add C_{12} and $C_{16:1}$ groups, respectively, to lipid A (*Y. pestis* Δ*msbB* and *Y. pestis* Δ*lpxP*), or the tetra-acylated LOS variant obtained from a *Y. pestis* strain lacking both MsbB and LpxP (*Y. pestis* Δ*msbB* Δ*lpxP*) (36). These molecules differ only in the number and position of acyl chains or phosphates in their lipid A moieties (see Fig. S1 in the supplemental material). HEK-Blue hTLR4, cotransfected with the human TLR4, MD2, and CD14 coreceptor genes, and THP1-Dual reporter cell lines express and secrete alkaline phosphatase downstream of NF-κB activation and were used to measure TLR4 activity via a colorimetric assay.

Consistent with observations made in analyses of mouse TLR4 activation in the companion paper by Harberts et al. (37), activation of human TLR4 in these cell lines

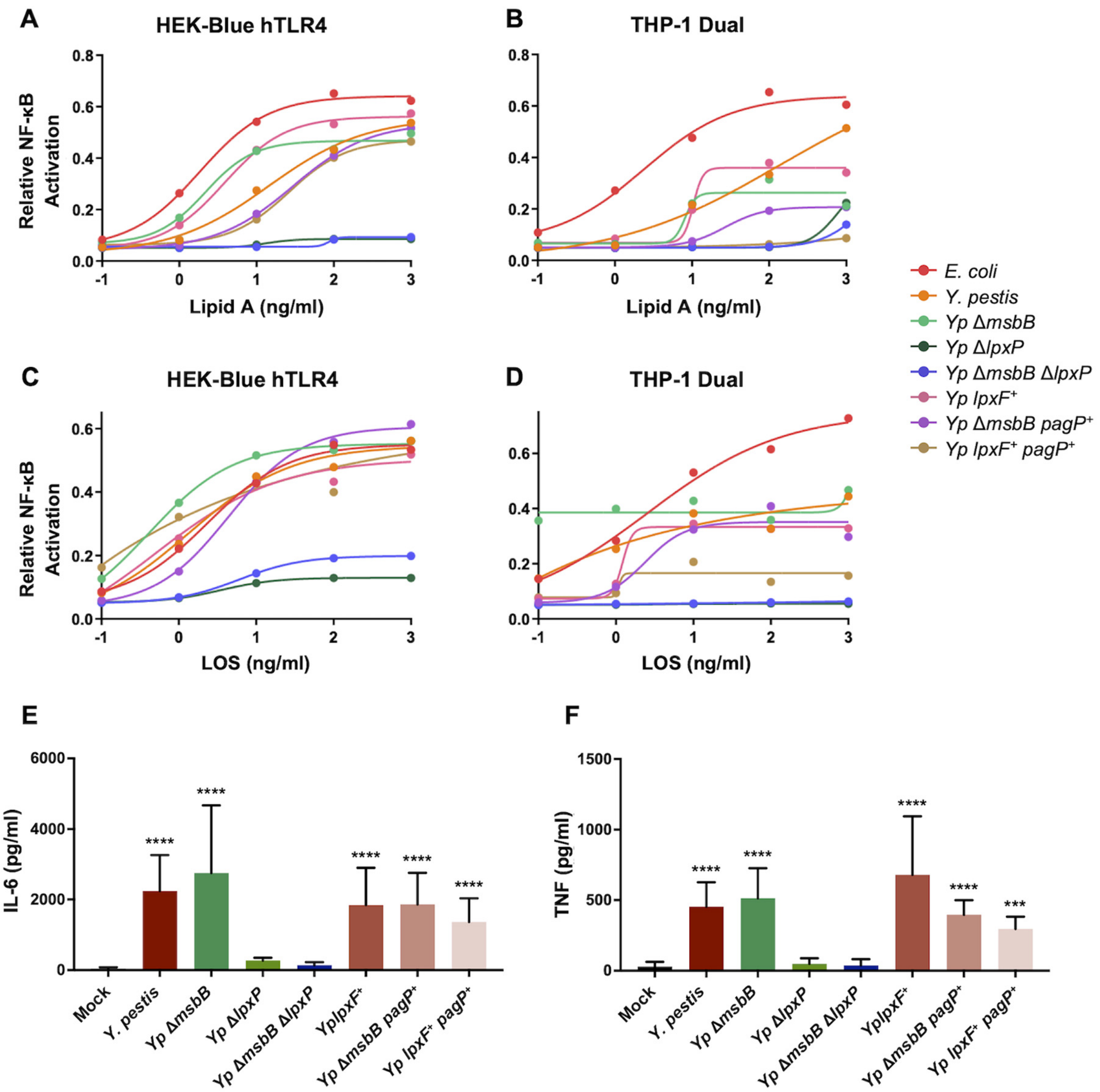

FIG 1 Lipid A structure determines the strength of hTLR4 signaling with the core oligosaccharide minimally contributing. (A to D) LOS (A to B) and lipid A (C to D) structural variants were added at the indicated concentrations to reporter cell lines overexpressing human TLR4/MD2 (HEK-Blue hTLR4) or expressing endogenous levels of TLR4 (THP-1 Dual) for 18 h. Agonists were derived from WT *Escherichia coli* (red), wild type *Yersinia pestis* (orange), *Y. pestis* Δ*msbB* (light green), *Y. pestis* Δ*lpxP* (dark green), *Y. pestis* Δ*msbB* Δ*lpxP* (blue), *Y. pestis lpxF*$^+$(pink), *Y. pestis lpxF*$^+$ *pagP*$^+$ (brown), and *Y. pestis* Δ*msbB pagP*$^+$(purple). Results were graphed using GraphPad Prism v7 with a 4-parameter exponential line of best fit superimposed. Each data point is an average of biological duplicates; representative of three experiments. (E and F) Human monocyte-derived macrophages (hMDMs) were stimulated with 1 μg/mL of purified lipid A from WT *Y. pestis*, *Y. pestis* Δ*msbB*, *Y. pestis* Δ*lpxP*, *Y. pestis* Δ*msbB* Δ*lpxP*, *Y. pestis lpxF*$^+$, *Y. pestis* Δ*msbB pagP*$^+$, or *Y. pestis lpxF*$^+$ *pagP*$^+$. After stimulation with the respective variants for 24 h, TLR4 activation was measured by IL-6 (E) and TNF-α (F) secretion. Data are represented as the mean ± standard deviation (SD) of triplicate wells from 3 to 4 different human donors. Data were analyzed by ANOVA followed by Holm-Šídák's multiple-comparison test; ****, $P < 0.0001$; ***, $P < 0.001$.

was observed following treatment with penta-acylated *Y. pestis* Δ*msbB* LOS lacking the secondary C_{12} acyl chain at the 3′ position, but not penta-acylated *Y. pestis* Δ*lpxP* LOS lacking the secondary $C_{16:1}$ acyl chain at the 2′ position (Fig. 1A and B). As expected, we did not observe TLR4 activation following treatment with tetra-acylated *Y. pestis* Δ*msbB* Δ*lpxP* LOS (Fig. 1A and B). These data support the concept that position-dependent acyl chain additions to the base tetra-acylated bacterial lipid A structure affect human TLR4 activation. We saw results similar to LOS treatment when we instead treated our reporter cell lines with lipid A from each variant, which was derived from the same extraction lots as the LOS (Fig. 1C and D). Variations in response induction kinetics can be observed between lipid A structures, even when the structures have similar maximal signaling capacity. These differences in induction kinetics are likely representative of differential binding affinity between the receptor and ligand (38). These nuanced effects on downstream signaling responses based on initial ligand binding warrant further study.

To confirm our findings that human TLR4 responds to distinct lipid A variants based on the number and position of acyl chains, we also investigated hTLR4 activation by measuring endogenous cytokine production after stimulation with the seven lipid A variants in primary human monocyte-derived macrophages (hMDMs). Like the reporter cell line data, we observed activation of human TLR4 after *Y. pestis* Δ*msbB* treatment but not after *Y. pestis* Δ*lpxP* or *Y. pestis* Δ*msbB* Δ*lpxP* treatment, as determined by interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) cytokine release (Fig. 1E and F). Overall, these data indicate that the TLR4 receptor complex can be activated by lipid A and that MD2/TLR4 is able to discriminate between penta-acylated lipid A containing a secondary acyl chain at the 2′ position but not the 3′ position. Furthermore, our data indicate that lipid A is sufficient to stimulate the TLR4 receptor complex and that the core oligosaccharide moiety is not required for the TLR4-stimulating capacity of lipid A.

The human noncanonical inflammasome is activated by *Y. pestis* LOS variants regardless of acyl chain number. Previous studies indicate that human macrophages can mount noncanonical inflammasome responses to tetra-acylated LPS (37). However, these studies were conducted in macrophages primed with interferon-gamma (IFN-γ), which may induce expression of additional host factors that can promote noncanonical inflammasome responses to LPS. IFN-γ priming mimics conditions following infection when the host already has activated inflammatory pathways leading to IL-12 and IL-18 production and subsequent IFN-γ production by NK and T cells, not the conditions present early during a primary infection. To determine how variation in acyl chain number affects activation of the human noncanonical inflammasome in the absence of IFN-γ priming, we transfected the LOS variants into primary human monocyte-derived macrophages (hMDMs) derived from 3 to 5 healthy human donors. We then assessed inflammasome activation by monitoring cell death via lactate dehydrogenase (LDH) release into the supernatant and IL-1β cytokine secretion. As expected, hexa-acylated LOS derived from WT *Y. pestis* resulted in robust cell death and IL-1β secretion in a dose-dependent manner (Fig. 2A and B). We also found that penta-acylated LOS from either *Y. pestis* Δ*msbB* (Fig. 2C and D) or *Y. pestis* Δ*lpxP* (Fig. 2E and F) both induced robust cell death and IL-1β secretion in hMDMs in a dose-dependent manner, at levels similar to those observed in hMDMs transfected with WT *Y. pestis* LOS (Fig. 2A and B and I and J). Moreover, we observed that although the tetra-acylated *Y. pestis* Δ*msbB* Δ*lpxP* LOS variant induced cell death and IL-1β secretion in a dose-dependent manner (Fig. 2G and H), they were substantially lower than the levels observed in hMDMs transfected with the WT hexa-acylated variant (Fig. 2I and J) indicating decreased inflammasome activation.

These results indicate that in contrast to human TLR4, the human noncanonical inflammasome is robustly activated in response to penta-acylated LOS containing a secondary acyl chain at the 2′ position or at the 3′ position to levels similar to those observed with hexa-acylated LOS. Furthermore, our data indicate that in contrast to the murine noncanonical inflammasome, tetra-acylated lipid A can activate the human noncanonical inflammasome. However, tetra-acylated lipid A is less stimulatory than penta-acylated or hexa-acylated lipid A, suggesting that the presence of either the C_{12} or $C_{16:1}$ secondary acyl chain in *Y. pestis* LOS is required to elicit maximal human noncanonical inflammasome responses.

The human noncanonical inflammasome is activated by *Y. pestis* LOS variants regardless of acyl chain length or phosphorylation state. We next asked whether the secondary acyl chains within LOS can affect noncanonical inflammasome activation. To investigate this, we utilized a hexa-acylated LOS variant containing an additional secondary C_{16} acyl chain that was isolated from *Y. pestis* Δ*msbB* expressing the acyltransferase PagP (*Y. pestis* Δ*msbB pagP*$^{+}$) (39, 40). Transfection of this LOS variant into hMDMs led to similar levels of cell death (Fig. 3A) and IL-1β secretion (Fig. 3B)

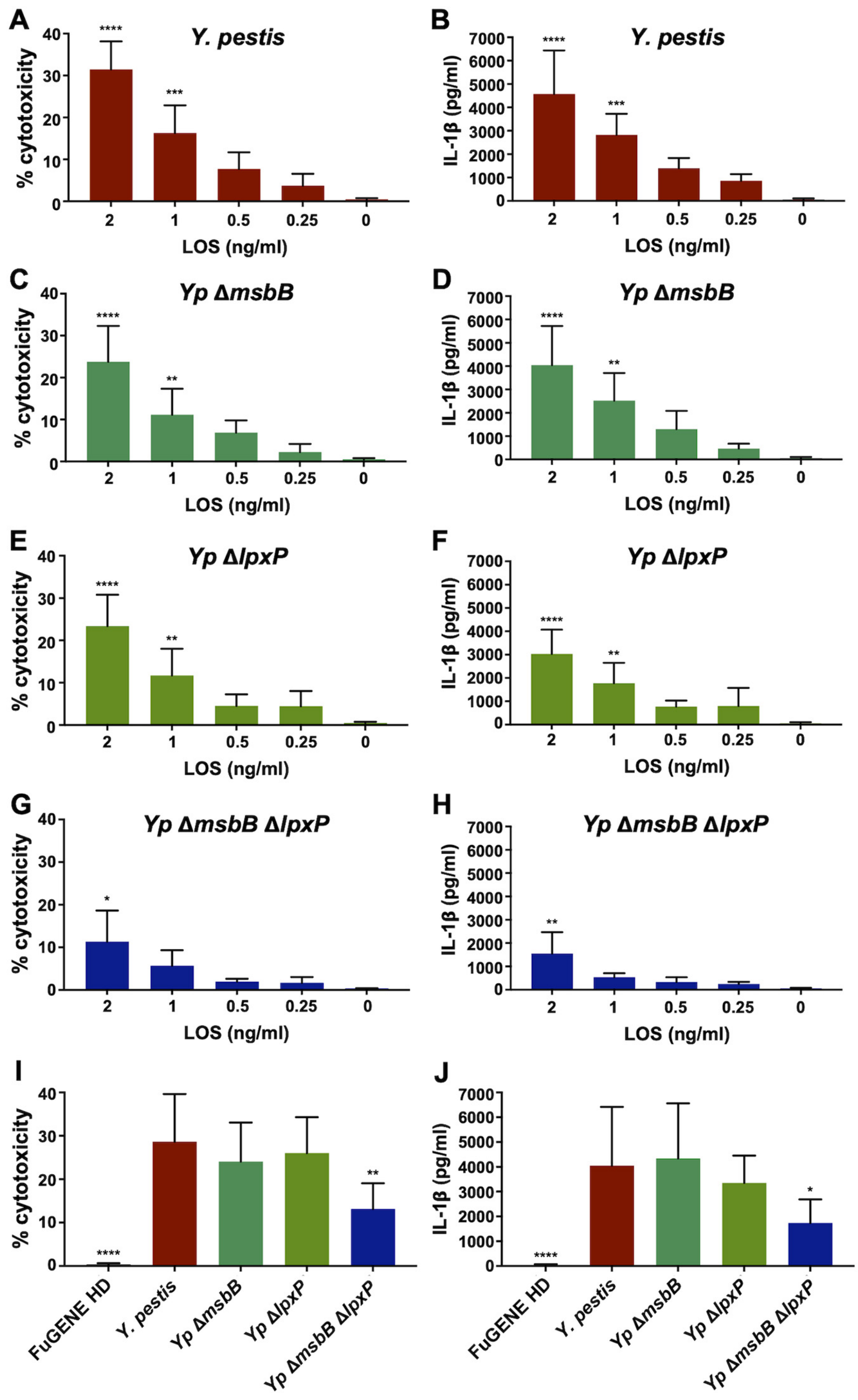

FIG 2 Human noncanonical inflammasome is activated by hexa-, penta-, and tetra-acylated *Yersinia pestis* LOS variants. (A to H) Pam3CSK4-primed human monocyte-derived macrophages (hMDMs) were transfected with the indicated concentration of purified LOS from WT *Y. pestis* (A and B), penta-acylated *Y. pestis* from *Y. pestis* Δ*msbB* (C and D), and *Y. pestis* Δ*lpxP* (E and F) or tetra-acylated *Y. pestis* (*Y. pestis* Δ*msbB* Δ*lpxP*) (G and H). After transfection with the respective LOS variants for 20 h, cell death was assessed by LDH release (A, C, E, and G), and IL-1β secretion was assessed by ELISA (B, D, F, and H). (I and J) Cell death **(I)** and IL-1β release (J) of the indicated LOS variants transfected into hMDMs at 2 μg/mL. Data are represented as the mean ± SD of triplicate wells from 3 to 5 different human donors. Data were analyzed by ANOVA followed by Holm-Šídák's multiple-comparison test; ****, $P < 0.0001$; ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$.

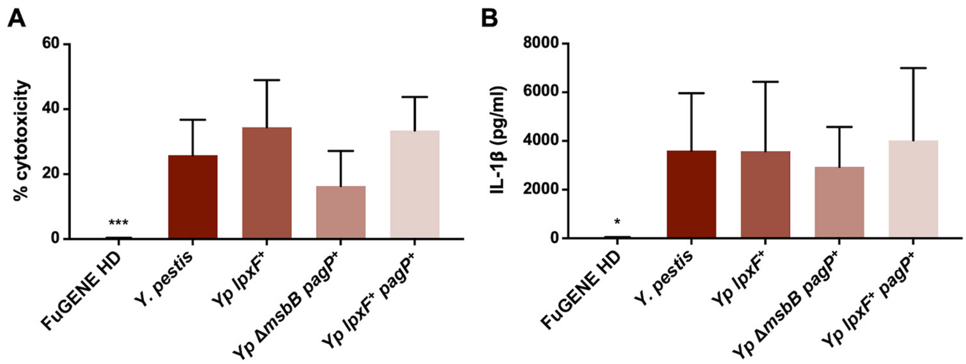

FIG 3 LOS robustly activates the human noncanonical inflammasome regardless of lipid A phosphorylation or acyl chain length. Pam3CSK4-primed hMDMs were transfected with 2 μg/mL of purified LOS from WT *Y. pestis*, *Y. pestis lpxF*$^+$, *Y. pestis* Δ*msbB pagP*$^+$, or *Y. pestis lpxF*$^+$ *pagP*$^+$. (A and B) Then, 20 h post-transfection, cell death was measured by assessing LDH release (A), and IL-1β release was measured by ELISA (B). Data represent the mean ± SD of triplicate wells from seven different healthy human donors. Data were analyzed by ANOVA followed by Holm-Šídák's multiple-comparison test, ***, $P < 0.001$; *, $P < 0.05$.

compared to hMDMs transfected with WT *Y. pestis* LOS. We then tested whether changes in the phosphorylation state of LOS along with changes in acyl chain position can differentially activate the human noncanonical inflammasome. We utilized a hexa-acylated *Y. pestis* LOS variant which is mono-phosphorylated due to expression of the *Francisella novicida* lipid A C-4′ phosphatase LpxF and also has an added secondary C_{16} acyl chain (*Y. pestis lpxF*$^+$ *pagP*$^+$) (41). There were similar levels of cell death and IL-1β secretion after transfection of *Y. pestis lpxF*$^+$ *pagP*$^+$ LOS into hMDMs compared to hMDMs transfected with WT *Y. pestis* LOS (Fig. 3A and B). Furthermore, we observed similar levels of cell death and IL-1β secretion in hMDMs transfected with hexa-acylated *Y. pestis lpxF*$^+$ LOS lacking the 4′ phosphate group compared to WT *Y. pestis* (Fig. 3A and B). Collectively, these data indicate that the human noncanonical inflammasome can be activated by a wide range of tetra-, penta-, and hexa-acylated *Y. pestis* LOS variants regardless of their lipid A phosphorylation or acylation state.

We next asked whether CASP4 and/or CASP5 contribute to detection of these lipid A variants. We used small interfering RNA (siRNA) to silence *CASP4* and/or *CASP5* in primary hMDMs. We observed that knocking down *CASP4* (67% average knockdown), *CASP5* (42% average knockdown), or both (63% and 15% average knockdown for

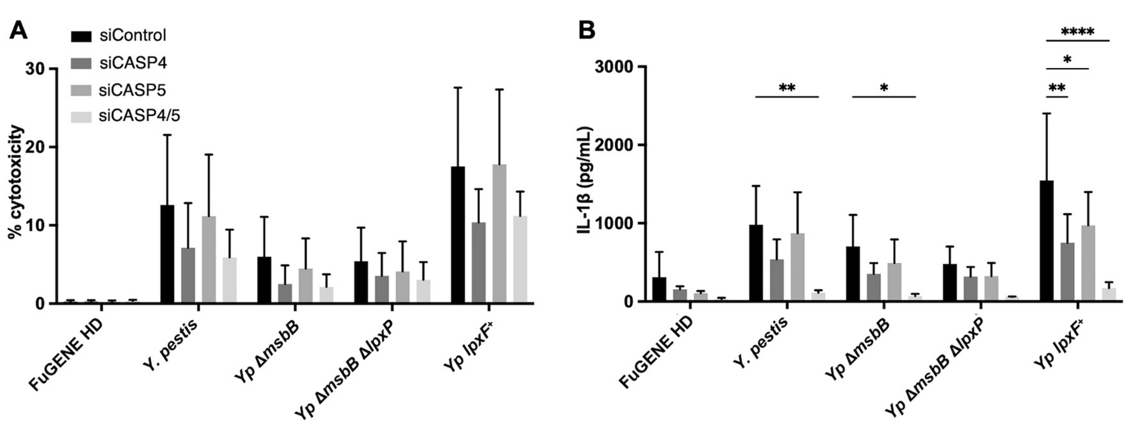

FIG 4 Both caspase-4 and caspase-5 are necessary for maximal inflammasome responses to LOS variants. Pam3CSK4-primed hMDMs were transfected with control siRNA or siRNA against *CASP4* and/or *CASP5*. Then, 24 h after siRNA-mediated knockdown, cells were transfected with 2 μg/mL of purified LOS from WT *Y. pestis*, *Y. pestis* Δ*msbB*, *Y. pestis* Δ*msbB* Δ*lpxP*, or *Y. pestis lpxF*$^+$. (A and B) Then, 24 h post-LOS transfection, cell death was measured by assessing LDH release (A), and IL-1β release was measured by ELISA (B). Data represent the mean ± SD of triplicate wells from four different healthy human donors. Data were analyzed by ANOVA followed by Holm-Šídák's multiple-comparison test; ****, $P < 0.0001$; **, $P < 0.01$; *, $P < 0.05$.

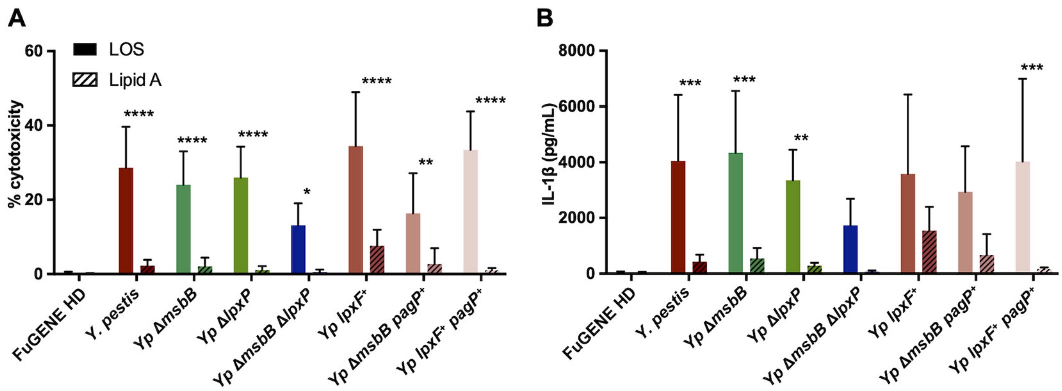

FIG 5 Core oligosaccharide is important for maximal human inflammasome responses to LOS. Pam3CSK4-primed hMDMs were transfected with 2 μg/mL of purified LOS (solid bars) or lipid A (striped bars) from WT *Y. pestis*, *Y. pestis* Δ*msbB*, *Y. pestis* Δ*lpxP*, *Y. pestis* Δ*msbB* Δ*lpxP*, *Y. pestis lpxF*$^+$, *Y. pestis* Δ*msbB pagP*$^+$, or *Y. pestis lpxF*$^+$ *pagP*$^+$. (A and B) After transfection with the indicated variants for 20 h, cell death was measured by assessing LDH release (A) and IL-1β secretion (B). Data represent the mean ± SD of triplicate wells from 5 to 9 different human donors. Data were analyzed by ANOVA followed by Holm-Šídák's multiple-comparison test; ****, $P < 0.0001$; ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$.

CASP4 and *CASP5*, respectively) did not significantly affect cell death after transfecting cells with the LOS variants, compared to control siRNA-treated cells (Fig. 4A). Similarly, we observed that knocking down either *CASP4* or *CASP5* alone did not lead to a significant reduction in IL-1β secretion after transfection with WT *Y. pestis* LOS, penta-acylated *Y. pestis* Δ*msbB* LOS, or tetra-acylated *Y. pestis* Δ*msbB* Δ*lpxP* LOS (Fig. 4B). We did observe that individual knockdown of *CASP4* or *CASP5* led to a significant decrease in IL-1β release in hMDMs transfected with *Y. pestis lpxF*$^+$ LOS, which is the hexa-acylated LOS missing a 4′ phosphate group. However, knocking down both *CASP4* and *CASP5* significantly decreased IL-1β release after transfecting cells with hexa-acylated WT *Y. pestis* LOS or *Y. pestis lpxF*$^+$ LOS, as well as penta-acylated *Y. pestis* Δ*msbB* LOS, compared to control siRNA-treated cells (Fig. 4B). These data suggest that CASP4 and CASP5 both contribute to recognizing LOS containing 3′ and 2′ O-linked acyl chains, which are absent from tetra-acylated *Y. pestis* Δ*msbB* Δ*lpxP* LOS. In addition, these data indicate that the robust IL-1β release seen after transfecting hMDMs with the different LOS variants relies on both CASP4 and CASP5, suggesting that one caspase can compensate for the absence of the other to mediate noncanonical inflammasome activation.

Core oligosaccharide is required for maximum noncanonical inflammasome activation by LOS in human macrophages. Lipid A is sufficient for human TLR4 activation, and absence of the core oligosaccharide does not reduce human or mouse TLR4 activation in response to lipid A compared to LOS (Fig. 1; 37). Previous studies indicate that lipid A is sufficient for noncanonical inflammasome activation in both human and mouse macrophages (16–18), and affinity measurements indicate similar binding affinities of LPS and lipid A for CASP4 (19), suggesting that lipid A and LPS might similarly activate the noncanonical inflammasome. However, it is unknown whether the core oligosaccharide contributes to human noncanonical inflammasome responses to lipid A. To address this question, we compared the noncanonical inflammasome response to purified lipid A lacking the core oligosaccharide or lipooligosaccharide (LOS) derived from each of the seven LOS variants. Surprisingly, and in contrast to the ability of these identical lipid A and LOS preparations to robustly stimulate the murine noncanonical inflammasome to a similar extent (37), transfection of purified lipid A variants resulted in significantly less cell death (Fig. 5A) and IL-1β release (Fig. 5B) compared to their respective LOS variants. Using an alternate route of lipid A delivery via coadministration with the bacterial pathogen *Listeria monocytogenes* (17), which uses the pore-forming toxin listeriolysin O to escape from the phagosome into the host cell cytosol, also resulted in relatively low inflammasome responses to lipid A, similar to the responses observed with transfection (Fig. S2). Taken together, these data indicate that the core oligosaccharide is necessary for maximal human noncanonical inflammasome responses to LOS.

Expression of Caspase-4 in murine macrophages confers responsiveness to hypoacylated LOS and lipid A. Previous studies and our data demonstrate that tetra-acylated lipid A fails to activate murine Casp11 but can activate human CASP4 (16–18, 37). These findings raise the question of whether the ability of human macrophages to respond to tetra-acylated lipid A is due to a property intrinsic to CASP4 itself or another feature unique to human macrophages. To distinguish between these possibilities, we used BMDMs derived from *Casp11*$^{-/-}$ *Casp4*^{Tg} mice, which lack the murine *Casp11* ortholog but express human *CASP4* as a transgene (21). We found that expression of the *CASP4* transgene restored responsiveness of *Casp11*$^{-/-}$ BMDMs to hexa-acylated LOS from WT *Y. pestis*, as well as to both penta-acylated LOS species from *Y. pestis* Δ*msbB* and *Y. pestis* Δ*lpxP* (Fig. S3). *Casp11*$^{-/-}$ *Casp4*^{Tg} BMDMs were also able to respond to tetra-acylated LOS *Y. pestis* Δ*msbB* Δ*lpxP*. This contrasted with WT BMDMs expressing endogenous *Casp11*, as they did not respond to *Y. pestis* Δ*msbB* Δ*lpxP* tetra-acylated LOS or *Y. pestis* Δ*msbB* penta-acylated LOS, or *Casp11*$^{-/-}$ BMDMs, which did not respond to any of the LOS structures (Fig. S3; 37). These data indicate that the ability of the human noncanonical inflammasome to respond to a wide range of *Y. pestis* LOS variants regardless of their acylation state is a property intrinsic to CASP4.

Differences in the ability of the human and mouse noncanonical inflammasomes to respond to lipid A relative to LOS could also be due to an intrinsic property of CASP4 or a caspase-independent difference between human and murine macrophages. To distinguish between these possibilities, we delivered purified LOS or lipid A from the different LOS structural variants into WT, *Casp11*$^{-/-}$, and *Casp11*$^{-/-}$ *Casp4*^{Tg} BMDMs. As expected, there were no differences in cell death or IL-1β release between WT BMDMs transfected with LOS or lipid A (Fig. 6A and B), and cell death and IL-1β release were dependent on Casp11 (Fig. 6C and D; 37). *Casp11*$^{-/-}$ *Casp4*^{Tg} BMDMs showed equivalent levels of cell death and IL-1β release in response to either transfected LOS or lipid A (Fig. 6E and F; Fig. S3). These findings indicate that human macrophages require the core oligosaccharide to respond robustly to cytosolic LOS, whereas mouse macrophages do not and that this differing response is not due to a property inherent to CASP4.

DISCUSSION

In this study, we used the BECC process to create a series of *Y. pestis*-derived LOS with distinct lipid A structures that contain or lack specific acyl chains and/or phosphoryl groups. We used these LOS and lipid A structures to interrogate structure-activity relationships between LOS and TLR4, as well as the CASP4/5 noncanonical inflammasome. Our results show that human TLR4 is activated in response to hexa-acylated LOS and penta-acylated LOS containing the 2′ secondary $C_{16:1}$ acyl chain but not tetra-acylated LOS or penta-acylated LOS missing the 2′ secondary acyl chain (Fig. 1). In contrast, CASP4/5 responded to stimulation by all hexa-, penta-, and tetra-acylated LOS structures tested, with tetra-acylated LOS eliciting a lower response (Fig. 2 and 3). Furthermore, CASP4/5 responded to LOS irrespective of lipid A acyl chain length. These data indicate that although human TLR4 and CASP4/5 respond to overlapping LOS structures, CASP4/5 recognizes a broader range of LOS structures than TLR4.

The contribution of phosphate groups to the strength of innate immune response activated by lipid A was also of interest to us, as its potential applicability is highlighted by the use of mono-phosphoryl lipid A (MPL) as an effective vaccine adjuvant that stimulates immune responses without inducing pathogenic inflammation (42–44). Although removal of a phosphate is thought to attenuate signaling through TLR4, recently published studies indicate that with structural changes to lipid A acyl chain length and arrangement, signal strength can be attenuated even if LPS is bisphosphorylated (45–47). Intriguingly, we found that both TLR4 and the noncanonical inflammasome were activated in response to mono- and bis-phosphorylated hexa-acylated LOS that otherwise have identical structures (Fig. 1 and 3), indicating that

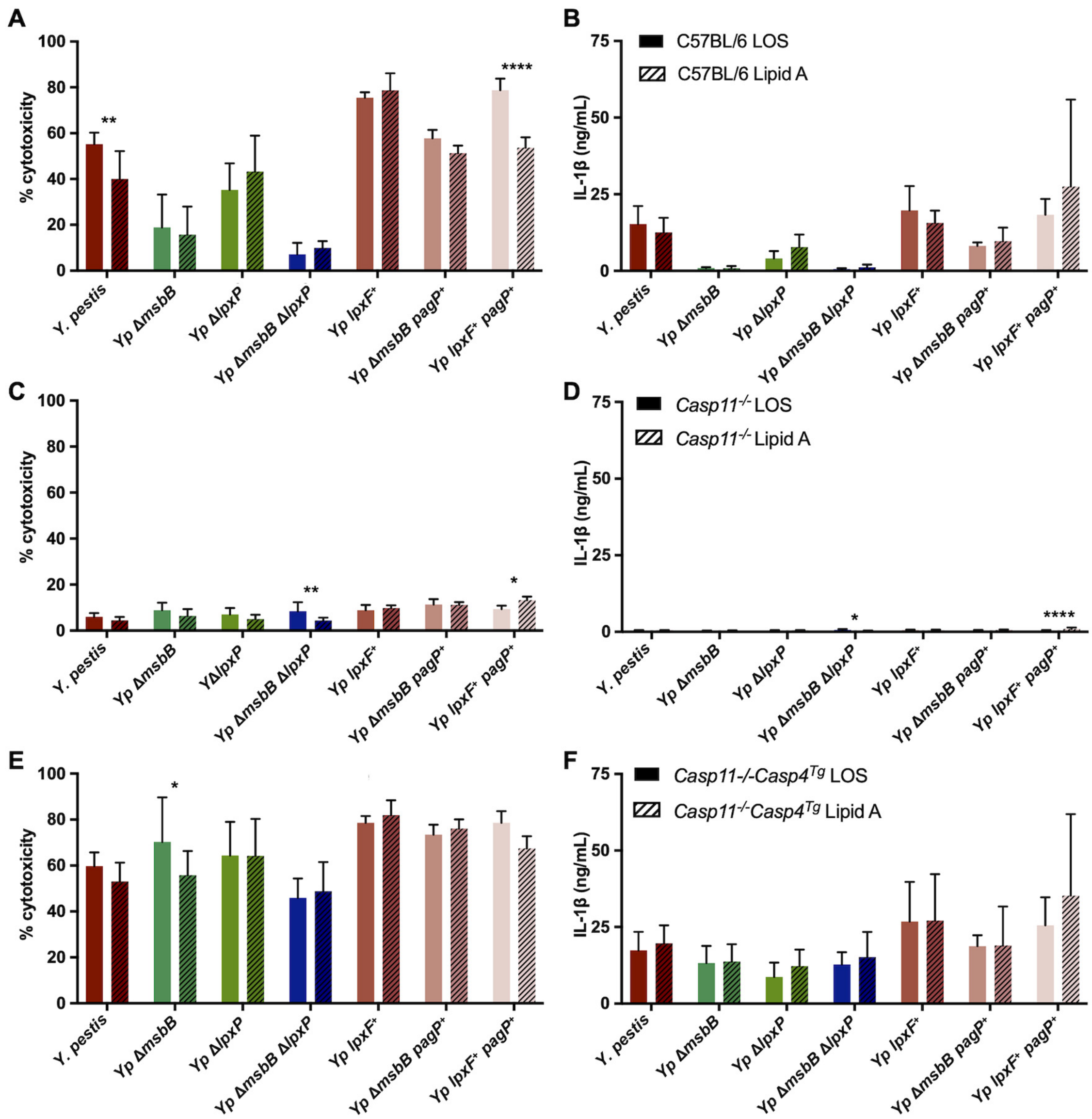

FIG 6 Ectopic expression of caspase-4 confers onto mouse macrophages the ability to respond to all LOS and lipid A variants. (A to F) Pam3CSK4-primed BMDMs from WT (C57BL/6) (A and B), *Casp11*$^{-/-}$ (C and D), and *Casp11*$^{-/-}$ *Casp4*^{Tg} (E and F) mice were transfected with 2 μg/mL of LOS (solid bars) or lipid A (striped bars) from WT *Y. pestis*, penta-acylated *Y. pestis* (*Y. pestis* Δ*msbB*, *Y. pestis* Δ*lpxP*), tetra-acylated *Y. pestis* (*Y. pestis* Δ*msbB* Δ*lpxP*), or hexa-acylated *Y. pestis* (*Y. pestis lpxF*$^{+}$, *Y. pestis* Δ*msbB pagP*$^{+}$, *Y. pestis lpxF*$^{+}$ *pagP*$^{+}$). After transfection with the respective LOS or lipid A variants for 20 h, cell death was measured by LDH release (A, C, and E), and IL-1β secretion was measured by ELISA (B, D, and F). Data represent the mean ± SD of three independent experiments performed in triplicate. Data were analyzed by ANOVA followed by Holm-Šídák's multiple-comparison test; ****, $P < 0.0001$; **, $P < 0.01$; *, $P < 0.05$.

changes in LOS phosphorylation state have no effect on activation of these innate immune pathways.

An unexpected finding from our study is the importance of the LOS core oligosaccharide for maximal noncanonical inflammasome activation in human macrophages (Fig. 5), whereas the core oligosaccharide is not required for noncanonical inflammasome responses in mouse macrophages (Fig. 6; 37). Although previous studies indicated that the presence of the core oligosaccharide allows for maximum TLR4 stimulation (48–50), we found that lipid A lacking the core oligosaccharide induced TLR4 activation to the same extent as LOS. We then investigated whether the ability of human macrophages to broadly recognize LOS variants in a core oligosaccharide-dependent manner was intrinsic to human CASP4 or another feature unique to human macrophages. Using mouse *Casp11*$^{-/-}$ macrophages expressing a human *CASP4* transgene, we found that human CASP4 confers onto mouse BMDMs the ability to recognize different LOS variants regardless of acylation state. These data indicate that the ability of human macrophages to respond broadly to different LOS variants is a property intrinsic to CASP4. Interestingly, we found that in contrast to human macrophages, mouse BMDMs expressing CASP4 responded robustly to both LOS and lipid A (Fig. 6). The ability of mouse BMDMs to respond equally well to both LOS and lipid A, compared to the differential response observed in hMDMs, could be due to the presence of additional mouse-specific host factors in BMDMs that enable inflammasome responses to lipid A and are not present in hMDMs or vice versa.

Guanylate binding proteins (GBPs), which are a subfamily of interferon-inducible GTPases, contribute to intracellular LPS recognition and are involved in cell-autonomous immune responses against intracellular bacterial pathogens (51, 52). It is possible that differences in GBP composition or function are responsible for the differential ability of humans and mice to respond to LOS versus lipid A. Notably, mice have 11 GBPs, whereas humans only have 7 GBPs, and GBPs may also exhibit species-specific functional differences (53). Murine GBPs are thought to promote inflammasome responses to bacterial infection by a variety of mechanisms, including enhancing responses to cytosolic LPS or outer membrane vesicles, mediating lysis of cytosolic bacteria, and promoting rupture of pathogen-containing vacuoles (54–60). Human GBPs mediate immune responses to bacterial infection through several mechanisms, including inhibiting bacterial spread, LPS binding, disrupting the bacterial cell envelope, and promoting inflammasome assembly at the surface of cytosolic bacteria (61–68). Future studies will be needed to investigate the role of GBPs or other host factors in the activation of the murine and human noncanonical inflammasomes in response to LPS, LOS, and lipid A structural variants.

Our data indicate that the human noncanonical inflammasome promiscuously detects a broad range of LOS structures, as it responded to all tetra-, penta-, and hexa-acylated *Y. pestis* LOS variants tested. In contrast, Harberts et al. show in their companion manuscript that the mouse noncanonical inflammasome is more selective, as it failed to respond to tetra-acylated lipid A and only responded to one of the penta-acylated variants, in addition to the hexa-acylated variants (37). Mouse BMDMs expressing human CASP4 also responded to all of the *Y. pestis* LOS variants, indicating that the ability to detect a broad range of LOS structures is intrinsic to CASP4. However, we found that although hMDMs mount inflammasome responses to tetra-acylated LOS, the response was significantly lower than what was observed with penta- or hexa-acylated LOS, indicating that the CASP4/5 inflammasome has some structural preference. Although our studies were conducted in the absence of IFN-γ priming, our findings are largely in agreement with a previous study showing that human CASP4 can be activated by both tetra- and hexa-acylated LPS in the context of IFN-γ-primed human macrophages (18). The mechanism underlying the relatively broad detection of LOS variants by the human noncanonical inflammasome is unclear but could be due to differences in the sequence and resulting structure of human CASP4 and CASP5 compared to mouse Casp11. Intriguingly, both human TLR4 and the human noncanonical inflammasome cannot respond to hexa-acylated LPS structures with C_{16}-length acyl chains derived from deep-sea *Moritella oceanus* strains (69), indicating that there are evolutionary constraints on the types of LPS structures recognized by TLR4 and CASP4/5 toward those found in bacteria able to colonize mammals.

The human noncanonical inflammasome consists of two lipid A sensors, CASP4 and CASP5. Previous studies indicate that CASP4 plays a more dominant role in human inflammasome responses to LPS in macrophages (19, 70–72). We show that both CASP4 and CASP5 contribute to the detection of some LOS variants, as we observed significantly decreased IL-1β release when both CASP4 and CASP5 were knocked down (Fig. 4). Our study begins to address the necessity for CASP4 and CASP5 in the detection of these LOS variants. Additional studies are required to fully parse out whether there are differences in how CASP4 and CASP5 detect and respond to distinct LOS variants. Furthermore, future evaluation of additional LOS or LPS structures from other bacterial species will help broaden our understanding of TLR4 and CASP4/5 recognition and provide insight into host-pathogen interactions. Further defining the rules governing how LPS/LOS/lipid A structural elements activate human TLR4 and CASP4/5 will also enable the development of improved vaccine adjuvants and immunomodulatory therapeutics, as well as more effective treatments for Gram-negative bacterial infections.

Taken together, our findings reveal that human TLR4 and CASP4/5 have differing requirements in their response to LOS structural variants, particularly in terms of LOS acyl chain number and the core oligosaccharide. Many bacterial pathogens have the capability of generating a wide variety of lipid A structures that have the potential to evade either TLR4 or CASP4/5. However, our findings suggest that the ability of TLR4 and CASP4/5 to detect both overlapping and distinct LOS structures enables the innate immune system to sense a wider range of lipid A structures than would be possible with either sensor on its own, thereby imposing a constraint on the ability of pathogens to evolve LPS or LOS structures that can evade immune detection. Furthermore, our data indicate that in contrast to mice, the human noncanonical inflammasome can respond to a broader range of LOS variants (37), indicating that humans may have the ability to mount immune responses against a broader range of Gram-negative bacterial pathogens. Collectively, these findings provide a foundation for further understanding the mechanisms underlying the species-specific differences that enable mice and humans to respond to distinct LPS and LOS structures.

MATERIALS AND METHODS

Ethics statement. All studies on primary human monocyte-derived macrophages (hMDMs) were performed in compliance with the requirements of the U.S. 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. All animal studies were performed in compliance with the federal regulations set forth in the Animal Welfare Act (31), the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, and the guidelines of the University of Pennsylvania Institutional Animal Use and Care Committee. All protocols used in this study were approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania (protocols no. 804523 and no. 804928).

Mice. Bone marrow-derived macrophages from WT C57BL/6 mice (Jackson Laboratory), *Casp11*$^{-/-}$ mice (Jackson Laboratory) (73), and *Casp11*$^{-/-}$ *Casp4*^{Tg} mice (from Joseph Buxbaum's laboratory) (21) were used in this study.

Lipooligosaccharide (LOS) and lipid A variants. LOS structural variants were created using bacterial enzyme combinatorial chemistry in the *Yersinia pestis* KIM6+ strain, an avirulent, nonselect agent variant (74). Due to a mutation that only allows for the addition of one O-antigen unit, *Y. pestis* makes only LOS, not LPS. Lipid A structural variants include *Y. pestis* Δ*msbB*, *Y. pestis* Δ*lpxP*, *Y. pestis* Δ*msbB* Δ*lpxP*, *Y. pestis lpxF*$^+$, *Y. pestis lpxF*$^+$ *pagP*$^+$, and *Y. pestis* Δ*msbB pagP*$^+$ (35, 36, 40) (Fig. S1). Briefly, to the tetra-acylated lipid A structure, *msbB* adds a 3′ O-linked C_{12} acyl chain, *lpxP* adds a 2′ O-linked $C_{16:1}$ acyl chain, and *lpxF* removes the 4′ phosphate. Bacteria were cultured and harvested, and LOS was extracted as described in reference 37. Lipid A was collected from the extracted LOS using a mild acid hydrolysis as previously described (35).

Cell culture. Reporter cell lines with a secreted alkaline phosphatase (SEAP) gene under the control of the NF-κB promoter were used to determine TLR4 activation levels in response to various LOS structures. HEK-Blue hTLR4 cells (InvivoGen) were cultured in Dulbecco's modified Eagle's medium (DMEM), and THP-1 Dual cells (InvivoGen) were cultured in RPMI; both media were supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 IU/mL penicillin, and 100 μg/mL streptomycin. Cells were plated at a density of 100,000 cells/well in a 96-well flat-bottom plate and cultured in a 5% CO_2 humidified incubator. THP-1 Dual cells were cultured with 100 ng/mL vitamin D_3 to promote adherence and surface expression of CD14, a TLR4 coreceptor. Cells were stimulated for 18 h with a 5-log dose range of agonist as previously described (35). SEAP amounts were then measured in the cell culture supernatants using Quanti-Blue detection medium (InvivoGen) according to the manufacturer's instructions. The optical density at 620 nm (OD_{620}) represents the activation level of NF-κB in each well.

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 medium in 10-cm- dishes at 4–5 $\times$ 10^5 cells/mL, followed by addition of 10 mL of fresh growth medium for an additional 2 days for complete differentiation into macrophages. The day before macrophage stimulation, cells were rinsed with cold phosphate-buffered saline (PBS), gently detached with trypsin-EDTA (0.05%), and replated in medium without antibiotics and with 25 ng/mL macrophage colony-stimulating factor (M-CSF) in a 48-well plate at a concentration of 1×10^5 cells per well.

Mouse bone marrow-derived macrophages (BMDMs) were cultured in DMEM supplemented with 10% FBS, 10 mM HEPES, 1 mM sodium pyruvate, and 30% L929 cell-conditioned medium. Cells were grown for 6 to 7 days in non-tissue culture-treated plates before being reseeded into 48-well tissue culture plates at a density of 1×10^5 cells per well for 20 h before LOS and lipid A transfection.

LOS and lipid A delivery. For TLR4 stimulation, primary human monocyte-derived macrophages (hMDMs) were either mock treated with Opti-MEM reduced serum medium (Thermo Fisher Scientific) alone or with 2 μg/mL of lipid A for 24 h. For intracellular delivery, hMDMs or murine bone marrow-derived macrophages (BMDMs) were primed with 1 μg/mL or 400 ng/mL Pam3CSK4 (InvivoGen), respectively, for 4 h. The medium was then replaced with 300 μL of Opti-MEM per well, and cells were either mock-transfected with FuGENE HD (Promega) alone or treated with a mixture of 0.75 μL FuGENE HD (0.25% [vol/vol]) plus LOS or lipid A (2 μg/mL or the indicated concentrations). Plates were then centrifuged at $805 \times g$ for 5 min before culturing at 37°C for 20 h. *Listeria monocytogenes* coinfection of lipid A into hMDMs was performed as described in reference 17. Briefly, primary hMDMs were primed with Pam3CSK4 for 4 h and then infected with *L. monocytogenes* strain 10403S at an MOI of 5 in the presence of 2 μg/mL lipid A. After 1 h of infection, 20 μg/mL gentamicin was added to kill extracellular bacteria. Then, 4 h after infection, cell supernatants were harvested to assess cell death and cytokine secretion.

LDH cytotoxicity assay. Primary hMDMs and BMDMs were transfected in a 48-well plate as described above, and harvested supernatants were assayed for cell death by measuring the loss of cellular membrane integrity via lactate dehydrogenase (LDH) activity. LDH release was quantified using an LDH cytotoxicity detection kit (TaKaRa BioProducts) according to the manufacturer's instructions and normalized to mock-infected cells.

ELISA. To measure human IL-6, TNF-α, and IL-1β secretion, primary hMDMs were stimulated or transfected in a 48-well plate as described above, and harvested supernatants were assayed for cytokine levels using enzyme-linked immunosorbent assay (ELISA) kits for human IL-6 (BioLegend), TNF-α (R&D Systems), and IL-1β (BD Biosciences) according to the manufacturer's instructions.

To measure mouse IL-1β secretion, supernatants and recombinant cytokine standards were applied to anti-IL-1β antibody-coated (eBioscience) Immulon ELISA plates (ImmunoChemistry Technologies). IL-1β was detected using biotinylated anti-IL-1β (eBioscience) and streptavidin conjugated to horseradish peroxidase (BD Biosciences). Peroxidase activity was detected using an o-phenylenediamine hydrochloride (Sigma-Aldrich) solution in citrate buffer. Reactions were stopped with 3 M H_2SO_4, and absorbance at 490 nm was read with a spectrophotometer.

siRNA knockdown. All Silencer Select siRNA oligonucleotides were purchased from Ambion (Life Technologies). For CASP4, the siRNA used was siRNA identification no. s2414, and s2417 was used for CASP5. To knock down CASP4 or CASP5 alone, 30 nM the appropriate oligonucleotide was used per well. To knock down both CASP4 and CASP5, 15 nM each oligonucleotide was used per well. As a control, Silencer select negative-control siRNAs (Silencer Select negative-control no. 1 siRNA 4390843 and Silencer Select negative-control no. 2 siRNA 4390846) were used at 15 nM each per well. Transfection of the pooled siRNAs into macrophages was performed using Lipofectamine RNAiMAX transfection reagent (Thermo Fisher Scientific) following the manufacturer's protocol. Treatment with the appropriate siRNAs was performed for 24 h.

Reverse transcription-quantitative PCR (qRT-PCR) analysis. Cells were lysed, and RNA was isolated using the RNeasy Plus kit (Qiagen). Synthesis of the first-strand cDNA was performed using SuperScript II reverse transcriptase and oligo(dT) primer (Invitrogen). Quantitative PCR (qPCR) was performed with the CFX96 real-time system (Bio-Rad) using the SsoFast EvaGreen supermix with the Low-ROX kit (Bio-Rad). The following primers from PrimerBank (75–77) were used. The PrimerBank identifications are *CASP4* (73622124c2), *CASP5* (209870072c1), and *HPRT* (164518913c1; all 5′ to 3′): *CASP4* forward: TCTGCGGAACTGTGCATGATG, *CASP4* reverse: TGTGTGATGAAGATAGAGCCCAT, *CASP5* forward: TCACCTGCCTGCAAGGAATG, *CASP5* reverse: TCTTTTCGTCAACCACAGTGTAG, *HPRT* forward: CCTGGCGTCGTGATTAGTGAT, and *HPRT* reverse: AGACGTTCAGTCCTGTCCATAA.

For analysis, mRNA levels of siRNA-treated cells were normalized to control siRNA-treated cells using the $2^{-\Delta\Delta CT}$ (cycle threshold) (78) method to calculate fold induction.

Statistical analyses. All graphed data and analysis of variance (ANOVA) analyses were carried out in GraphPad Prism (San Diego, CA). ANOVA was followed by multiple comparison with the Holm-Šídák posttest. The resulting significance levels are indicated in the figures. All *P* values and significance levels are indicated in the figures and figure legends.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1 MB.

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REFERENCES

1. Peleg AY, Hooper DC. 2010. Hospital-acquired infections due to Gram-negative bacteria. N Engl J Med 362:1804–1813. https://doi.org/10.1056/NEJMra0904124.
2. Marshall JC. 2014. Why have clinical trials in sepsis failed? Trends Mol Med 20:195–203. https://doi.org/10.1016/j.molmed.2014.01.007.
3. Hotchkiss RS, Moldawer LL, Opal SM, Reinhart K, Turnbull IR, Vincent J-L. 2016. Sepsis and septic shock. Nat Rev Dis Primers 2:16045. https://doi.org/10.1038/nrdp.2016.45.
4. Vincent JL, Mongkolpun W. 2018. Current management of Gram-negative septic shock. Curr Opin Infect Dis 31:600–605. https://doi.org/10.1097/QCO.0000000000000492.
5. Munford RS. 2006. Severe sepsis and septic shock: the role of Gram-negative bacteremia. Annu Rev Pathol 1:467–496. https://doi.org/10.1146/annurev.pathol.1.110304.100200.
6. Valvano MA. 2022. Remodelling of the Gram-negative bacterial Kdo2-lipid A and its functional implications. Microbiology (Reading 168). https://doi.org/10.1099/mic.0.001159.
7. Lüderitz O, Galanos C, Lehmann V, Rietschel ET, Rosenfelder G, Simon M, Westphal O. 1973. Lipid A: chemical structure and biological activity. J Infect Dis 128:17–29.
8. Hartley JL, Adams GA, Tornabene TG. 1974. Chemical and physical properties of lipopolysaccharide of Yersinia pestis. J Bacteriol 118:848–854. https://doi.org/10.1128/jb.118.3.848-854.1974.
9. Hase S, Rietschel ET. 1976. Isolation and analysis of the lipid A backbone. Lipid A structure of lipopolysaccharides from various bacterial groups. Eur J Biochem 63:101–107. https://doi.org/10.1111/j.1432-1033.1976.tb10212.x.
10. Preston A, Mandrell RE, Gibson BW, Apicella MA. 1996. The lipooligosaccharides of pathogenic Gram-negative bacteria. Crit Rev Microbiol 22:139–180. https://doi.org/10.3109/10408419609106458.
11. Takeuchi O, Akira S. 2001. Toll-like receptors; their physiological role and signal transduction system. Int Immunopharmacol 1:625–635. https://doi.org/10.1016/S1567-5769(01)00010-8.
12. Fink SL, Cookson BT. 2005. Apoptosis, pyroptosis, and necrosis: mechanistic description of dead and dying eukaryotic cells. Infect Immun 73:1907–1916. https://doi.org/10.1128/IAI.73.4.1907-1916.2005.
13. Franchi L, Eigenbrod T, Muñoz-Planillo R, Nuñez G. 2009. The inflammasome: a caspase-1-activation platform that regulates immune responses and disease pathogenesis. Nat Immunol 10:241–247. https://doi.org/10.1038/ni.1703.
14. Shimazu R, Akashi S, Ogata H, Nagai Y, Fukudome K, Miyake K, Kimoto M. 1999. MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. J Exp Med 189:1777–1782. https://doi.org/10.1084/jem.189.11.1777.
15. Means TK, Golenbock DT, Fenton MJ. 2000. The biology of Toll-like receptors. Cytokine Growth Factor Rev 11:219–232. https://doi.org/10.1016/S1359-6101(00)00006-X.
16. Kayagaki N, Wong MT, Stowe IB, Ramani SR, Gonzalez LC, Akashi-Takamura S, Miyake K, Zhang J, Lee WP, Muszyński A, Forsberg LS, Carlson RW, Dixit VM. 2013. Noncanonical inflammasome activation by intracellular LPS independent of TLR4. Science 341:1246–1249. https://doi.org/10.1126/science.1240248.
17. Hagar JA, Powell DA, Aachoui Y, Ernst RK, Miao EA. 2013. Cytoplasmic LPS activates caspase-11: implications in TLR4-independent endotoxic shock. Science 341:1250–1253. https://doi.org/10.1126/science.1240988.
18. Lagrange B, Benaoudia S, Wallet P, Magnotti F, Provost A, Michal F, Martin A, Di Lorenzo F, Py BF, Molinaro A, Henry T. 2018. Human caspase-4 detects tetra-acylated LPS and cytosolic Francisella and functions differently from murine caspase-11. Nat Commun 9:242. https://doi.org/10.1038/s41467-017-02682-y.
19. Shi J, Zhao Y, Wang Y, Gao W, Ding J, Li P, Hu L, Shao F. 2014. Inflammatory caspases are innate immune receptors for intracellular LPS. Nature 514:187–192. https://doi.org/10.1038/nature13683.
20. Lin XY, Choi MS, Porter AG. 2000. Expression analysis of the human caspase-1 subfamily reveals specific regulation of the CASP5 gene by lipopolysaccharide and interferon-gamma. J Biol Chem 275:39920–39926. https://doi.org/10.1074/jbc.M007255200.
21. Kajiwara Y, Schiff T, Voloudakis G, Gama Sosa MA, Elder G, Bozdagi O, Buxbaum JD. 2014. A critical role for human caspase-4 in endotoxin sensitivity. J Immunol 193:335–343. https://doi.org/10.4049/jimmunol.1303424.
22. Agnew A, Nulty C, Creagh EM. 2021. Regulation, activation and function of caspase-11 during health and disease. Int J Mol Sci 22:1506. https://doi.org/10.3390/ijms22041506.
23. Raetz CRH, Reynolds CM, Trent MS, Bishop RE. 2007. Lipid A modification systems in gram-negative bacteria. Annu Rev Biochem 76:295–329. https://doi.org/10.1146/annurev.biochem.76.010307.145803.
24. Bertani B, Ruiz N. 2018. Function and biogenesis of lipopolysaccharides. EcoSal Plus https://doi.org/10.1128/ecosalplus.ESP-0001-2018.
25. Maeshima N, Fernandez RC. 2013. Recognition of lipid A variants by the TLR4-MD-2 receptor complex. Front Cell Infect Microbiol 3:3.
26. Needham BD, Trent MS. 2013. Fortifying the barrier: the impact of lipid A remodelling on bacterial pathogenesis. Nat Rev Microbiol 11:467–481. https://doi.org/10.1038/nrmicro3047.
27. Teghanemt A, Zhang D, Levis EN, Weiss JP, Gioannini TL. 2005. Molecular basis of reduced potency of underacylated endotoxins. J Immunol 175:4669–4676. https://doi.org/10.4049/jimmunol.175.7.4669.
28. Hajjar AM, Ernst RK, Tsai JH, Wilson CB, Miller SI. 2002. Human Toll-like receptor 4 recognizes host-specific LPS modifications. Nat Immunol 3:354–359. https://doi.org/10.1038/ni777.
29. Akira S. 2000. Toll-like receptors: lessons from knockout mice. Biochem Soc Trans 28:551–556. https://doi.org/10.1042/bst0280551.
30. Montminy SW, Khan N, McGrath S, Walkowicz MJ, Sharp F, Conlon JE, Fukase K, Kusumoto S, Sweet C, Miyake K, Akira S, Cotter RJ, Goguen JD, Lien E. 2006. Virulence factors of Yersinia pestis are overcome by a strong lipopolysaccharide response. Nat Immunol 7:1066–1073. https://doi.org/10.1038/ni1386.
31. Matsuura M, Takahashi H, Watanabe H, Saito S, Kawahara K. 2010. Immunomodulatory effects of Yersinia pestis lipopolysaccharides on human macrophages. Clin Vaccine Immunol 17:49–55. https://doi.org/10.1128/CVI.00336-09.
32. Steeghs L, Keestra AM, van Mourik A, Uronen-Hansson H, van der Ley P, Callard R, Klein N, van Putten JPM. 2008. Differential activation of human and mouse Toll-like receptor 4 by the adjuvant candidate LpxL1 of Neisseria meningitidis. Infect Immun 76:3801–3807. https://doi.org/10.1128/IAI.00005-08.
33. Zamyatina A, Heine H. 2020. Lipopolysaccharide recognition in the crossroads of TLR4 and caspase-4/11 mediated inflammatory pathways. Front Immunol 11:585146. https://doi.org/10.3389/fimmu.2020.585146.
34. Reeves PR, Pacinelli E, Wang L. 2003. O antigen gene clusters of Yersinia pseudotuberculosis. Adv Exp Med Biol 529:199–206. https://doi.org/10.1007/0-306-48416-1_39.
35. Gregg KA, Harberts E, Gardner FM, Pelletier MR, Cayatte C, Yu L, McCarthy MP, Marshall JD, Ernst RK. 2017. Rationally designed TLR4 ligands for vaccine adjuvant discovery. mBio 8:e00492-17. https://doi.org/10.1128/mBio.00492-17.
36. Rebeil R, Ernst RK, Jarrett CO, Adams KN, Miller SI, Hinnebusch BJ. 2006. Characterization of late acyltransferase genes of Yersinia pestis and their role in temperature-dependent lipid A variation. J Bacteriol 188:1381–1388. https://doi.org/10.1128/JB.188.4.1381-1388.2006.

37. Harberts E, Grubaugh D, Akuma DC, Shin S, Ernst RK, Brodsky EI. 2022. Position-specific secondary acylation determines detection of lipid A by murine TLR4 and caspase-11. Infect Immun https://doi.org/10.1128/iai.00201-22.
38. Shin HJ, Lee H, Park JD, Hyun HC, Sohn HO, Lee DW, Kim YS. 2007. Kinetics of binding of LPS to recombinant CD14, TLR4, and MD-2 proteins. Mol Cells 24:119–124.
39. Chandler CE, Harberts EM, Pelletier MR, Ernst RK. . 2020. Correction for Chandler et al., Early evolutionary loss of the lipid A modifying enzyme PagP resulting in innate immune evasion in Yersinia pestis. Proc Natl Acad Sci U S A 117:32817. https://doi.org/10.1073/pnas.2019986117.
40. Chandler CE, Harberts EM, Pelletier MR, Thaipisuttikul I, Jones JW, Hajjar AM, Sahl JW, Goodlett DR, Pride AC, Rasko DA, Trent MS, Bishop RE, Ernst RK. 2020. Early evolutionary loss of the lipid A modifying enzyme PagP resulting in innate immune evasion in Yersinia pestis. Proc Natl Acad Sci U S A 117:22984–22991. https://doi.org/10.1073/pnas.1917504117.
41. Jones JW, Cohen IE, Tureček F, Goodlett DR, Ernst RK. 2010. Comprehensive structure characterization of lipid A extracted from Yersinia pestis for determination of its phosphorylation configuration. J Am Soc Mass Spectrom 21:785–799. https://doi.org/10.1016/j.jasms.2010.01.008.
42. Qureshi N, Takayama K, Ribi E. 1982. Purification and structural determination of nontoxic lipid A obtained from the lipopolysaccharide of Salmonella typhimurium. J Biol Chem 257:11808–11815. https://doi.org/10.1016/S0021-9258(18)33836-5.
43. Ribi E, Cantrell JL, Takayama K, Qureshi N, Peterson J, Ribi HO. 1984. Lipid A and immunotherapy. Rev Infect Dis 6:567–572. https://doi.org/10.1093/clinids/6.4.567.
44. Tomai MA, Solem LE, Johnson AG, Ribi E. 1987. The adjuvant properties of a nontoxic monophosphoryl lipid A in hyporesponsive and aging mice. J Biol Response Mod 6:99–107.
45. Gregg KA, Harberts E, Gardner FM, Pelletier MR, Cayatte C, Yu L, McCarthy MP, Marshall JD, Ernst RK. 2018. A lipid A-based TLR4 mimetic effectively adjuvants a Yersinia pestis rF-V1 subunit vaccine in a murine challenge model. Vaccine 36:4023–4031. https://doi.org/10.1016/j.vaccine.2018.05.101.
46. Harberts E, Varisco D, Haupt R, Jain A, Middaugh CR, Ernst RK. 2020. Novel lipid A mimetics, BECC438 and BECC470, act as potent adjuvants in bacterial and viral subunit vaccines. J Immunol 204:166.14.
47. Zacharia A, Harberts E, Valencia SM, Myers B, Sanders C, Jain A, Larson NR, Middaugh CR, Picking WD, Difilippantonio S, Kirnbauer R, Roden RB, Pinto LA, Shoemaker RH, Ernst RK, Marshall JD. 2021. Optimization of RG1-VLP vaccine performance in mice with novel TLR4 agonists. Vaccine 39:292–302. https://doi.org/10.1016/j.vaccine.2020.11.066.
48. Zughaier S, Agrawal S, Stephens DS, Pulendran B. 2006. Hexa-acylation and KDO(2)-glycosylation determine the specific immunostimulatory activity of Neisseria meningitidis lipid A for human monocyte derived dendritic cells. Vaccine 24:1291–1297. https://doi.org/10.1016/j.vaccine.2005.09.039.
49. Gaekwad J, Zhang Y, Zhang W, Reeves J, Wolfert MA, Boons G-J. 2010. Differential induction of innate immune responses by synthetic lipid a derivatives. J Biol Chem 285:29375–29386. https://doi.org/10.1074/jbc.M110.115204.
50. Muroi M, Tanamoto K. 2002. The polysaccharide portion plays an indispensable role in Salmonella lipopolysaccharide-induced activation of NF-kappaB through human toll-like receptor 4. Infect Immun 70:6043–6047. https://doi.org/10.1128/IAI.70.11.6043-6047.2002.
51. Vestal DJ. 2005. The guanylate-binding proteins (GBPs): proinflammatory cytokine-induced members of the dynamin superfamily with unique GTPase activity. J Interferon Cytokine Res 25:435–443. https://doi.org/10.1089/jir.2005.25.435.
52. Kim B-H, Shenoy AR, Kumar P, Bradfield CJ, MacMicking JD. 2012. IFN-inducible GTPases in host cell defense. Cell Host Microbe 12:432–444. https://doi.org/10.1016/j.chom.2012.09.007.
53. Olszewski MA, Gray J, Vestal DJ. 2006. In silico genomic analysis of the human and murine guanylate-binding protein (GBP) gene clusters. J Interferon Cytokine Res 26:328–352. https://doi.org/10.1089/jir.2006.26.328.
54. Liu BC, Sarhan J, Panda A, Muendlein HI, Ilyukha V, Coers J, Yamamoto M, Isberg RR, Poltorak A. 2018. Constitutive interferon maintains GBP expression required for release of bacterial components upstream of pyroptosis and anti-DNA responses. Cell Rep 24:155–168.e5. https://doi.org/10.1016/j.celrep.2018.06.012.
55. Meunier E, Dick MS, Dreier RF, Schürmann N, Kenzelmann Broz D, Warming S, Roose-Girma M, Bumann D, Kayagaki N, Takeda K, Yamamoto M, Broz P. 2014. Caspase-11 activation requires lysis of pathogen-containing vacuoles by IFN-induced GTPases. Nature 509:366–370. https://doi.org/10.1038/nature13157.
56. Meunier E, Wallet P, Dreier RF, Costanzo S, Anton L, Rühl S, Dussurgey S, Dick MS, Kistner A, Rigard M, Degrandi D, Pfeffer K, Yamamoto M, Henry T, Broz P. 2015. Guanylate-binding proteins promote activation of the AIM2 inflammasome during infection with Francisella novicida. Nat Immunol 16:476–484. https://doi.org/10.1038/ni.3119.
57. Santos JC, Dick MS, Lagrange B, Degrandi D, Pfeffer K, Yamamoto M, Meunier E, Pelczar P, Henry T, Broz P. 2018. LPS targets host guanylate-binding proteins to the bacterial outer membrane for non-canonical inflammasome activation. EMBO J 37:e98089. https://doi.org/10.15252/embj.201798089.
58. Finethy R, Luoma S, Orench-Rivera N, Feeley EM, Haldar AK, Yamamoto M, Kanneganti T-D, Kuehn MJ, Coers J. 2017. Inflammasome activation by bacterial outer membrane vesicles requires guanylate binding proteins. mBio 8:e01188-17. https://doi.org/10.1128/mBio.01188-17.
59. Pilla DM, Hagar JA, Haldar AK, Mason AK, Degrandi D, Pfeffer K, Ernst RK, Yamamoto M, Miao EA, Coers J. 2014. Guanylate binding proteins promote caspase-11-dependent pyroptosis in response to cytoplasmic LPS. Proc Natl Acad Sci U S A 111:6046–6051. https://doi.org/10.1073/pnas.1321700111.
60. Shenoy AR, Wellington DA, Kumar P, Kassa H, Booth CJ, Cresswell P, MacMicking JD. 2012. GBP5 promotes NLRP3 inflammasome assembly and immunity in mammals. Science 336:481–485. https://doi.org/10.1126/science.1217141.
61. Li P, Jiang W, Yu Q, Liu W, Zhou P, Li J, Xu J, Xu B, Wang F, Shao F. 2017. Ubiquitination and degradation of GBPs by a Shigella effector to suppress host defence. Nature 551:378–383. https://doi.org/10.1038/nature24467.
62. Piro AS, Hernandez D, Luoma S, Feeley EM, Finethy R, Yirga A, Frickel EM, Lesser CF, Coers J. 2017. Detection of cytosolic Shigella flexneri via a C-terminal triple-arginine motif of GBP1 inhibits actin-based motility. mBio 8:e01979-17. https://doi.org/10.1128/mBio.01979-17.
63. Wandel MP, Pathe C, Werner EI, Ellison CJ, Boyle KB, von der Malsburg A, Rohde J, Randow F. 2017. GBPs Inhibit Motility of Shigella flexneri but Are Targeted for Degradation by the Bacterial Ubiquitin Ligase IpaH9.8. Cell Host Microbe 22:507–518.e5. https://doi.org/10.1016/j.chom.2017.09.007.
64. Kutsch M, Sistemich L, Lesser CF, Goldberg MB, Herrmann C, Coers J. 2020. Direct binding of polymeric GBP1 to LPS disrupts bacterial cell envelope functions. EMBO J 39:e104926. https://doi.org/10.15252/embj.2020104926.
65. Santos JC, Boucher D, Schneider LK, Demarco B, Dilucca M, Shkarina K, Heilig R, Chen KW, Lim RYH, Broz P. 2020. Human GBP1 binds LPS to initiate assembly of a caspase-4 activating platform on cytosolic bacteria. Nat Commun 11:3276. https://doi.org/10.1038/s41467-020-16889-z.
66. Wandel MP, Kim B-H, Park E-S, Boyle KB, Nayak K, Lagrange B, Herod A, Henry T, Zilbauer M, Rohde J, MacMicking JD, Randow F. 2020. Guanylate-binding proteins convert cytosolic bacteria into caspase-4 signaling platforms. Nat Immunol 21:880–891. https://doi.org/10.1038/s41590-020-0697-2.
67. Fisch D, Bando H, Clough B, Hornung V, Yamamoto M, Shenoy AR, Frickel E-M. 2019. Human GBP1 is a microbe-specific gatekeeper of macrophage apoptosis and pyroptosis. EMBO J 38:e100926. https://doi.org/10.15252/embj.2018100926.
68. Fisch D, Clough B, Domart M-C, Encheva V, Bando H, Snijders AP, Collinson LM, Yamamoto M, Shenoy AR, Frickel E-M. 2020. Human GBP1 differentially targets Salmonella and Toxoplasma to license recognition of microbial ligands and caspase-mediated death. Cell Rep 32:108008. https://doi.org/10.1016/j.celrep.2020.108008.
69. Gauthier AE, Chandler CE, Poli V, Gardner FM, Tekiau A, Smith R, Bonham KS, Cordes EE, Shank TM, Zanoni I, Goodlett DR, Biller SJ, Ernst RK, Rotjan RD, Kagan JC. 2021. Deep-sea microbes as tools to refine the rules of innate immune pattern recognition. Sci Immunol 6:eabe0531. https://doi.org/10.1126/sciimmunol.abe0531.
70. Casson CN, Yu J, Reyes VM, Taschuk FO, Yadav A, Copenhaver AM, Nguyen HT, Collman RG, Shin S. 2015. Human caspase-4 mediates noncanonical inflammasome activation against Gram-negative bacterial pathogens. Proc Natl Acad Sci U S A 112:6688–6693. https://doi.org/10.1073/pnas.1421699112.
71. Baker PJ, Boucher D, Bierschenk D, Tebartz C, Whitney PG, D'Silva DB, Tanzer MC, Monteleone M, Robertson AAB, Cooper MA, Alvarez-Diaz S, Herold MJ, Bedoui S, Schroder K, Masters SL. 2015. NLRP3 inflammasome activation downstream of cytoplasmic LPS recognition by both caspase-4 and caspase-5. Eur J Immunol 45:2918–2926. https://doi.org/10.1002/eji.201545655.
72. Schmid-Burgk JL, Gaidt MM, Schmidt T, Ebert TS, Bartok E, Hornung V. 2015. Caspase-4 mediates non-canonical activation of the NLRP3 inflammasome in human myeloid cells. Eur J Immunol 45:2911–2917. https://doi.org/10.1002/eji.201545523.
73. Wang S, Miura M, Jung YK, Zhu H, Li E, Yuan J. 1998. Murine caspase-11, an ICE-interacting protease, is essential for the activation of ICE. Cell 92:501–509. https://doi.org/10.1016/s0092-8674(00)80943-5.

74. Sun W, Six D, Kuang X, Roland KL, Raetz CRH, Curtiss R. 2011. A live attenuated strain of Yersinia pestis KIM as a vaccine against plague. Vaccine 29:2986–2998. https://doi.org/10.1016/j.vaccine.2011.01.099.
75. Spandidos A, Wang X, Wang H, Dragnev S, Thurber T, Seed B. 2008. A comprehensive collection of experimentally validated primers for Polymerase Chain Reaction quantitation of murine transcript abundance. BMC Genomics 9:633. https://doi.org/10.1186/1471-2164-9-633.
76. Spandidos A, Wang X, Wang H, Seed B. 2010. PrimerBank: a resource of human and mouse PCR primer pairs for gene expression detection and quantification. Nucleic Acids Res 38:D792–9. https://doi.org/10.1093/nar/gkp1005.
77. Wang X, Seed B. 2003. A PCR primer bank for quantitative gene expression analysis. Nucleic Acids Res 31:e154. https://doi.org/10.1093/nar/gng154.
78. Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25:402–408. https://doi.org/10.1006/meth.2001.1262.