# Cytoplasmic LPS activates caspase-11: implications in TLR4-independent endotoxic shock

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## Abstract

Inflammatory caspases, such as caspase-1 and -11, mediate innate immune detection of pathogens. Caspase-11 induces pyroptosis, a form of programmed cell death, and specifically defends against bacterial pathogens that invade the cytosol. During endotoxemia, however, excessive caspase-11 activation causes shock. We report that contamination of the cytoplasm by lipopolysaccharide (LPS) is the signal that triggers caspase-11 activation in mice. Specifically, caspase-11 responds to penta- and hexa-acylated lipid A, whereas tetra-acylated lipid A is not detected, providing a mechanism of evasion for cytosol-invasive *Francisella*. Priming the caspase-11 pathway *in vivo* resulted in extreme sensitivity to subsequent LPS challenge in both wild type and *Tlr4*-deficient mice, whereas caspase 11-deficient mice were relatively resistant. Together, our data reveal a new pathway for detecting cytoplasmic LPS.

Caspases are evolutionarily ancient proteases that are integral to basic cellular physiology. Although some caspases mediate apoptosis, the inflammatory caspases-1 and -11 trigger pyroptosis, a distinct form of lytic programmed cell death. In addition, caspase-1 processes IL-1β and IL-18 to their mature secreted forms. Caspase-1 is activated by the canonical inflammasomes, which signal via the adaptor ASC; NLRC4 and NLRP1a/1b can additionally activate caspase-1 directly (1, 2). In contrast to caspase-1, caspase-11 is activated independently of all known canonical inflammasome pathways; the hypothetical caspase-11 activating platform has been termed the non-canonical inflammasome (3). *Casp1*$^{-/-}$ mice generated from 129 background stem cells are also deficient in *Casp11* due to a passenger mutation backcrossed from the 129 background into C57BL/6. Caspase-11 is responsible for certain phenotypes initially attributed to caspase-1, such as shock following endotoxin challenge (3). The physiologic function of caspase-11 is to discriminate cytosolic from vacuolar bacteria (4). In the absence of caspase-11, mice become acutely susceptible to infection by bacteria that escape the phagosome and replicate in the cytosol (4), such as *Burkholderia pseudomallei* and *B. thailandensis*. Caspase-11 also responds to vacuolar Gram-negative bacteria, albeit with delayed kinetics (3, 5–7), which may have relevance to its aberrant activation during sepsis. Although these studies demonstrated both detrimental and protective roles for caspase-11, the precise nature of the caspase-11 activating signal remained unknown.

Because caspase-11 specifically responds to cytosolic bacteria, we hypothesized that detection of a conserved microbial ligand within the cytosol triggers caspase-11. To address

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this hypothesis, we generated lysates of Gram-negative and Gram-positive bacteria and transfected them into LPS primed $Nlrc4^{-/-}Asc^{-/-}Casp11^{+/+}$ or $Casp1^{-/-}Casp11^{-/-}$ bone marrow-derived macrophages (BMMs). By comparing these strains, we can examine caspase-11 activation in the absence of canonical inflammasome detection of flagellin and DNA (fig. S1). Although boiled Gram-negative bacterial lysates were detected through caspase-11 upon transfection into BMMs, Gram-positive lysates were not (Fig. 1A). RNase, DNase, lysozyme, and proteinase K digestion was sufficient to dispose of canonical inflammasome agonists, but failed to eliminate the caspase-11 activating factor(s) (Fig. 1B). We then treated boiled lysates with ammonium hydroxide, which is known to deacylate lipid species (8), and observed that the caspase-11 activating factor was degraded, whereas canonical inflammasome agonists persisted (Fig. 1C).

These results suggested lipopolysaccharide (LPS) as the caspase-11 agonist. Consistent with this hypothesis, BMMs underwent caspase-11 dependent pyroptosis following transfection of ultra pure *Salmonella minnesota* RE595 LPS (Fig. 1D). Caspase-11 can promote IL-1β secretion by triggering the canonical NLRP3 pathway (3) (fig. S1). Consistently, IL-1β secretion and caspase-1 processing following transfection of LPS were also caspase-11 dependent (Fig. 1E to G). Moreover, caspase-11 alone promoted pyroptosis (Fig. 1H). In contrast to caspase-1, we were unable to convincingly visualize caspase-11 processing by western blot (Fig. 1F and G; fig. S2A), despite the vast majority of cells exhibiting pyroptotic morphology as seen by phase microscopy. Although these data do not exclude the possibility that processing of a small amount of caspase-11 is required for pyroptosis, they do indicate that processing is not a good proxy measure for rapid caspase-11 activation. This is consistent with direct caspase-1 activation by NLRC4, which is not accompanied by processing (9). These results suggest that the presence of LPS in the cytosol is sufficient to trigger caspase-11; however, we cannot rule out the formal possibility that this signaling arises from a membrane bound compartment such as the ER or golgi. Future identification of the non-canonical inflammasome will permit this determination.

The caspase-11 pathway is not responsive unless macrophages are previously stimulated (primed) with either LPS, poly(I:C), IFN-β, or IFN-γ, which likely induces multiple components of the non-canonical inflammasome pathway including caspase-11 (fig. S2B) (4–7, 10). LPS and poly(I:C) prime via TLR4 and TLR3, respectively, which both stimulate IFN-β production; IFN-β and IFN-γ signaling overlap in their activation of the STAT1 transcription factor, which is critical to caspase-11 activation (5, 7). In order to separate the priming and activation stimuli of caspase-11, we verified that poly(I:C) and IFN-γ could substitute for LPS as priming agents (Fig. 1I).

To corroborate our LPS transfection results, we sought another means to deliver LPS to the cytoplasm. *Listeria monocytogenes* lyses the phagosome via the pore forming toxin LLO, and as a Gram-positive bacterium does not contain LPS. *L. monocytogenes* infection did not activate caspase-11 in BMMs; however, co-phagocytosis of wild type, but not LLO mutant ( hly), *L. monocytogenes* with exogenous LPS triggered pyroptosis, IL-1β secretion, and caspase-1 processing dependent upon caspase-11 (Fig. 2A–F). Despite this genetic evidence of caspase-11 activation, we again did not observe proteolytic processing of caspase-11 (Fig. 2E and F). In conjunction with our previous data indicating that caspase-11 discriminates cytosolic from vacuolar Gram-negative bacteria (4), these results indicate that detection of LPS in the cytoplasm triggers caspase-11 dependent pyroptosis.

Previous studies have shown that another agonist, cholera toxin B (CTB), activates caspase-11. However, LPS was present with CTB for the duration of these experiments (3), and caspase-11 failed to respond to CTB in the absence of LPS (Fig. 2G). The physiological function of CTB is to mediate the translocation of the enzymatically active cholera toxin A

(CTA) into host cells. Therefore, we hypothesized that activation of caspase-11 by CTB results from delivery of co-phagocytosed LPS into the cytosol. Under this hypothesis, CTB should likewise be able to shuttle canonical inflammasome agonists, which are detected in the cytosol. Indeed, when LPS was replaced with PrgJ, an NLRC4 agonist (11), the pyroptotic response switched from caspase-11-dependence to NLRC4-dependence (Fig. 2G). Therefore, in these experiments CTB is not a caspase-11 agonist, but rather an LPS delivery agent. Whether CTB disrupts vacuoles during its use as an adjuvant, or whether complete cholera toxin (CTA/CTB) disrupts vacuoles during infection with *Vibrio cholera* remain to be examined.

We next examined the LPS structural determinants required for detection through caspase-11, and found that the lipid A moiety alone was sufficient for activation (Fig. 3A). It is well established that lipid A modifications enable TLR4 evasion, and we therefore hypothesized that cytosolic pathogens could evade caspase-11 by a similar strategy. Indeed, *Francisella novicida* a Gram-negative cytosolic bacteria, was not detected by caspase-11 (no signal in $Nlrc4^{-/-}Asc^{-/-}$ BMMs; Fig. 3B). *F. novicida* lysates containing DNA activated caspase-1; however, after DNase digestion the remaining LPS failed to activate caspase-11, which was not restored by temperature-dependent alterations in acyl chain length (12) (Fig. 3C). As with *L. monocytogenes* co-phagocytosis of *F. novicida* with exogenous *S. minnesota* LPS resulted in caspase-11 activation (Fig. 3D). Together, these results suggest that *Francisella* species evade caspase-11 by modifying their lipid A. *Francisella* species have peculiar tetra-acylated lipid A unlike the hexa-acylated species of enteric bacteria (13). *F. novicida* initially synthesizes a penta-acylated lipid A structure with two phosphates and then removes the 4' phosphate and 3' acyl chain in reactions that do not occur in *lpxF* mutants (14, 15) (Fig. 3E). Conversion to the penta-acylated structure restored caspase-11 activation, whereas other modifications that maintained the tetra-acylated structures (*flmK* mutant or 18°C growth (12, 16)) did not (Fig. 3F). *lpxF* mutant lipid A is not detected by TLR4 (14), suggesting that the TLR4 and caspase-11 pathways have different structural requirements.

Deacylation of lipid A is a common strategy employed by pathogenic bacteria. For example, *Yersinia pestis* removes two acyl chains from its lipid A upon transition from growth at 25°C to 37°C (17) (Fig. 3G). Consistent with our structural studies of *F. novicida* lipid A, caspase-11 detected hexa-acylated lipid A from *Y. pestis* grown at 25°C, but not tetra-acylated lipid A from bacteria grown at 37°C (Fig. 3H). Together, these data indicate that caspase-11 responds to distinct lipid A structures, and pathogens appear to exploit these structural requirements in order to evade caspase-11.

In addition to detection of extracellular/vacuolar LPS by TLR4, our data indicate that an additional sensor of cytoplasmic LPS activates caspase-11. These two pathways intersect, however, because TLR4 primes the caspase-11 pathway. However, $Tlr4^{-/-}$ BMMs responded to transfected or CTB-delivered LPS after poly(I:C) priming (Fig. 4A–C). Therefore, caspase-11 can respond to cytoplasmic LPS independently of TLR4.

In established models of endotoxic shock, both $Tlr4^{-/-}$ and $Casp11^{-/-}$ mice are resistant to lethal challenge with 40–54 mg/kg LPS (3, 18, 19), whereas WT mice succumb in 18 to 48 hours (Fig. 4D). We hypothesized that TLR4 detects extracellular LPS and primes the caspase-11 pathway *in vivo*. Then, if high concentrations of LPS persist, aberrant localization of LPS within the cytoplasm could trigger caspase-11, resulting in the generation of shock mediators. We sought to separate these two events by priming and then challenging with otherwise sublethal doses of LPS. C57BL/6 mice primed with LPS rapidly succumbed to secondary LPS challenge in 2 hours (Fig. 4D). TLR4 was required for LPS priming, as LPS primed $Tlr4^{-/-}$ mice survived secondary LPS challenge (Fig. 4E). To

determine whether alternate priming pathways could substitute for TLR4 *in vivo* we primed mice with poly(I:C), and observed that both C57BL/6 and *Tlr4*$^{-/-}$ mice succumbed to secondary LPS challenge (Fig. 4E). This was concomitant with hypothermia (Fig. 4F), seizures, peritoneal fluid accumulation, and occasionally intestinal hemorrhage. In contrast, poly(I:C) primed *Casp11*$^{-/-}$ mice were more resistant to secondary LPS challenge (Fig. 4G), demonstrating the consequences of aberrant caspase-11 activation. Collectively, our data indicate that activation of caspase-11 by LPS *in vivo* can result in rapid onset of endotoxic shock independent of TLR4.

Mice challenged with the canonical NLRC4 agonist flagellin coupled to the cytosolic translocation domain of anthrax lethal toxin also experience a rapid onset of shock (20). In this model, NLRC4-dependent caspase-1 activation triggers lethal eicosanoid production via COX-1 with similar kinetics to our prime-challenge model, suggesting convergent lethal pathways downstream of caspase-1 and caspase-11. Indeed, the COX-1 inhibitor SC-560 rescued poly(I:C) primed mice from LPS lethality (Fig. 4H).

Although physiological activation of caspase-11 is beneficial in defense against cytosolic bacterial pathogens (4), its aberrant hyperactivation becomes detrimental during endotoxic shock. Our data suggest that when LPS reaches critical concentrations during sepsis, aberrant LPS localization occurs, activating cytosolic surveillance pathways. Clinical sepsis is a more complex pathophysiologic state, where multiple cytokines, eicosanoids, and other inflammatory mediators are likely to be hyperactivated. Eicosanoid mediators and other consequences of pyroptotic cellular lysis (21) should be considered in future therapeutic options designed to treat Gram-negative septic shock. This underscores the concept that Gram-negative and Gram-positive sepsis may cause shock via divergent signaling pathways (22), and that treatment options should consider these as discreet clinical entities.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

## References and Notes

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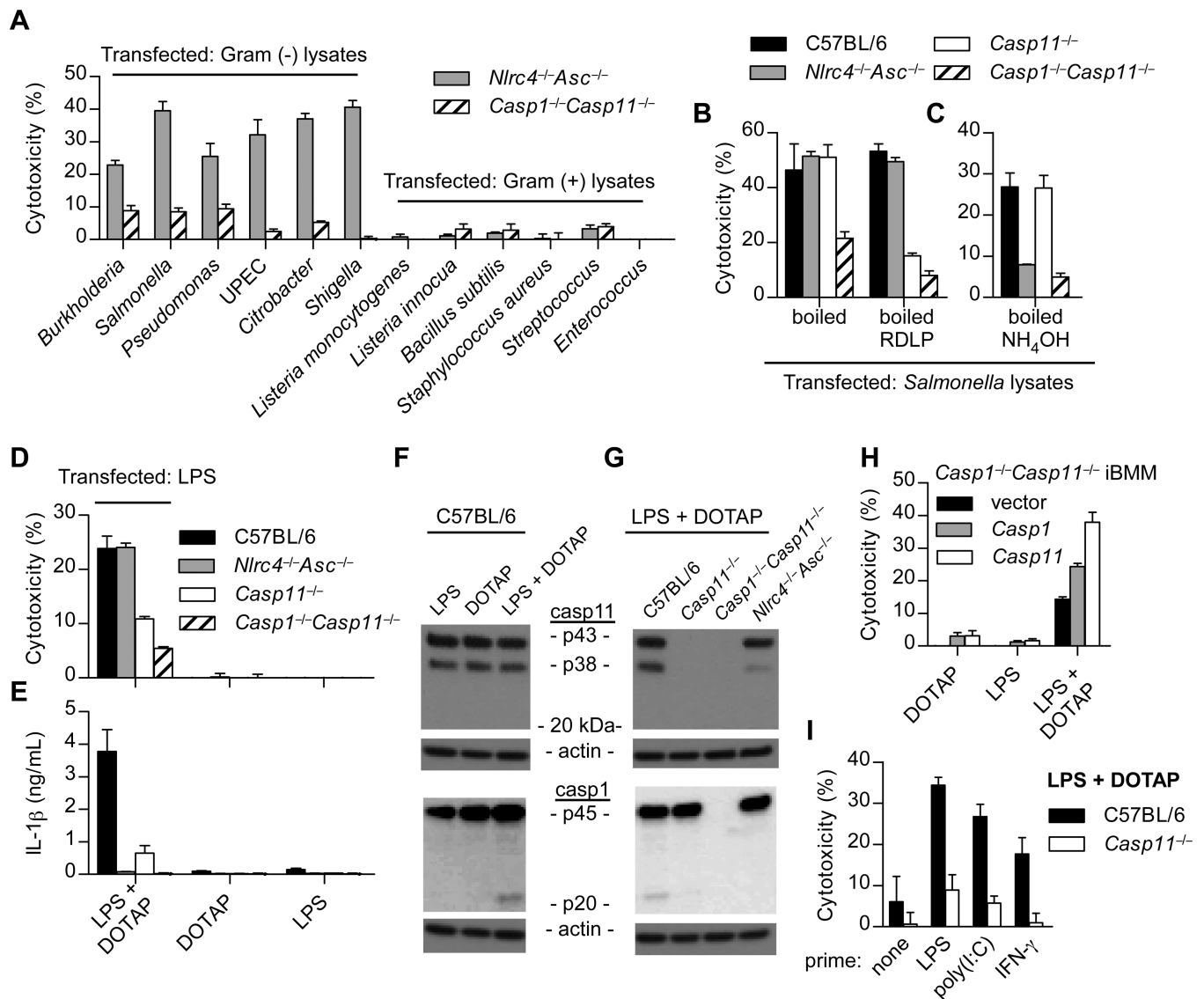
**Fig. 1. Cytoplasmic LPS triggers caspase-11 activation**

(**A–H**) BMM were LPS primed overnight prior to transfection. (**A–C**) BMMs were transfected with the indicated bacterial lysates packaged in Lipofectamine 2000. Cytotoxicity was determined by lactate dehydrogenase release 4 hours later. Where indicated, lysates were treated with RNase, DNase, proteinase K, and lysozyme (RDLP) (**B**) or ammonium hydroxide (**C**). (**D–E**) BMMs were transfected with ultrapure LPS from *S. minnesota* RE595 packaged with DOTAP, a liposomal transfection reagent. Cytotoxicity (**D**) and IL-1β secretion by ELISA (**E**) were determined 4 h post transfection. (**F–G**) BMMs were stimulated as in (**D**) and caspase-1 and -11 processing by western blot were examined 2 h post transfection. (**H**) Immortalized *Casp1⁻/⁻Casp11⁻/⁻* BMMs (iBMMs) complemented by retroviral transduction of *Casp1* or *Casp11* were transfected with LPS from *S. minnesota* RE595. Cytotoxicity was determined after 4 h. (**I**) Macrophages were primed overnight with LPS (50ng/mL), poly(I:C) (1μg/mL), IFN-γ (8ng/mL), or left untreated. Cells were then transfected with LPS from *S. minnesota* RE595 and cytotoxicity was determined 2 h later. Data are representative of at least 3 experiments. Error bars indicate standard deviation of technical replicates.
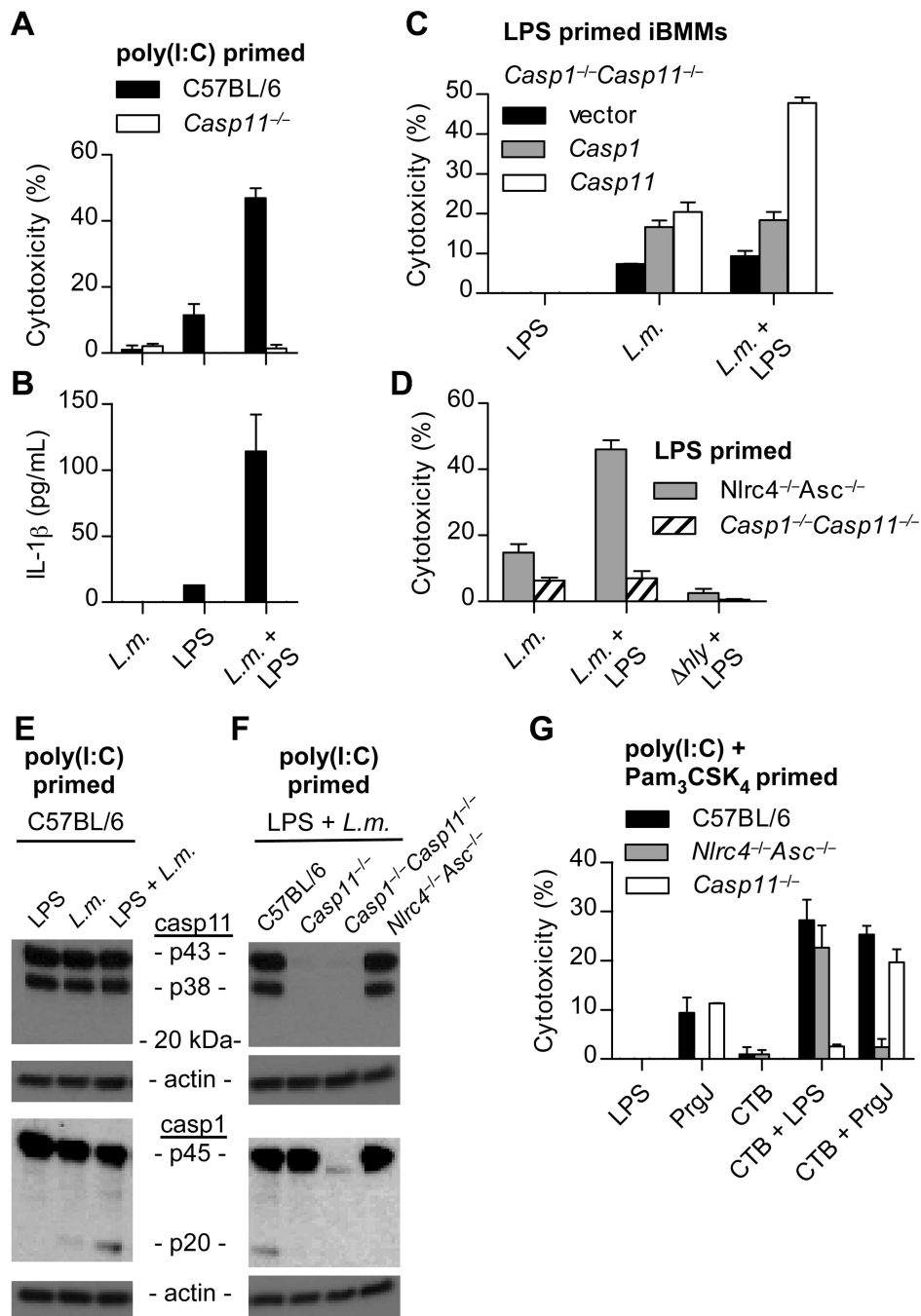
**Fig. 2. *Listeria* and CTB mediate caspase-11 activation by LPS**

(**A–F**) The indicated macrophages were primed with poly(I:C) or LPS and then infected by *L. monocytogenes* (MOI 5) in the presence or absence of LPS from *S. minnesota* RE595 (1μg/mL). Cytotoxicity (**A, C, D**), IL-1β secretion (**B**), or caspase-1 and caspase-11 processing (**E–F**) were examined 4 h post-infection. (**G**) Poly(I:C) and Pam$_3$CSK$_4$ primed macrophages were incubated with the indicated combinations of CTB (20μg/mL), LPS from *E. coli* O111:B4 (1μg/mL), and PrgJ (10μg/mL). Cytotoxicity was determined 16 h later. Data are representative of 3 (**A, D, G**) or 2 (**B, C, E, F**) experiments. Error bars indicate standard deviation of technical replicates.
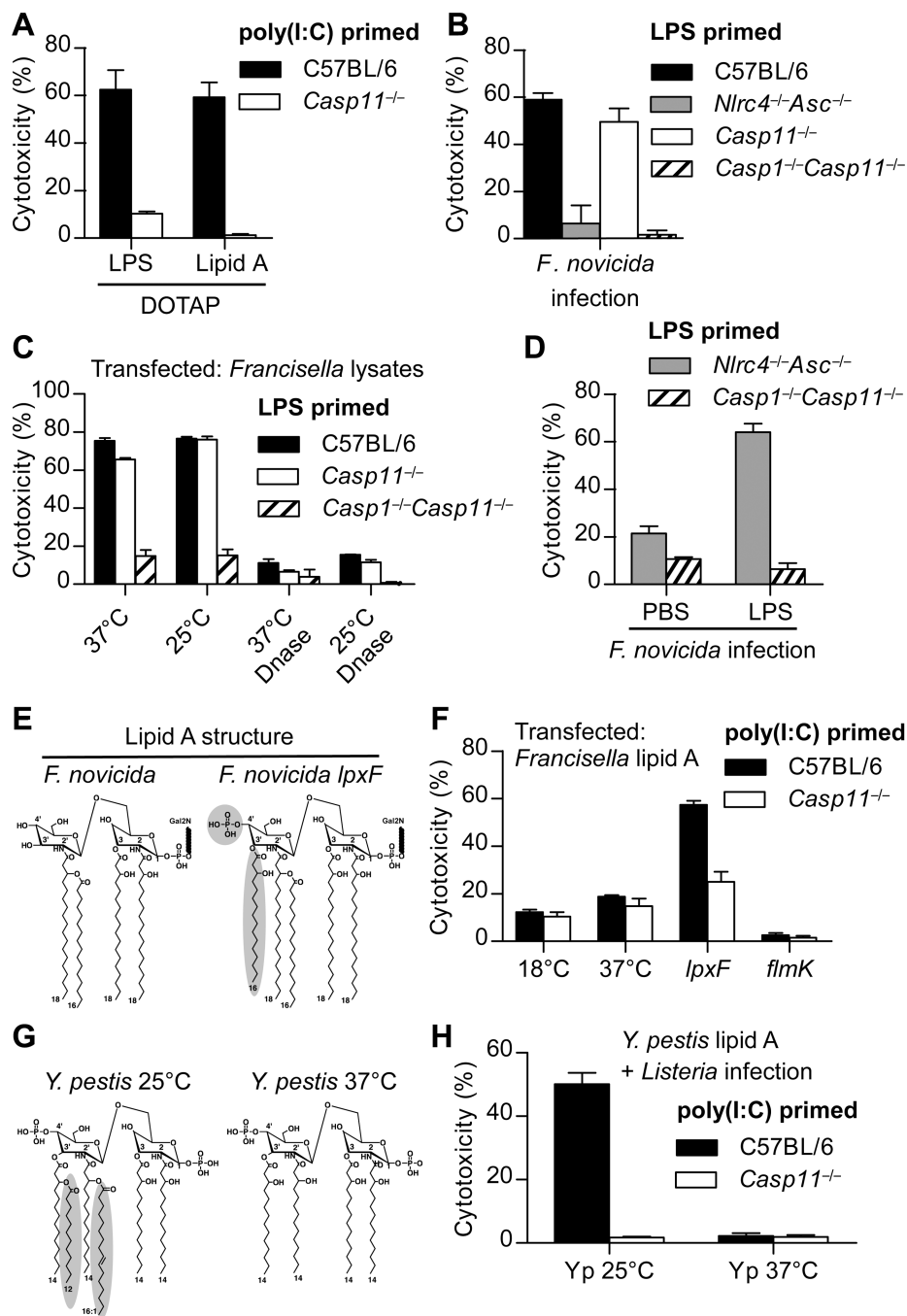
**Fig. 3. Caspase-11 responds to distinct lipid A structures**

(**A**) Poly(I:C) primed BMMs were transfected with LPS from *S. minnesota* RE595 or *S. typhimurium* lipid A. Cytotoxicity was determined after 2 h. (**B**) Cytotoxicity in LPS primed BMMs was determined 4 hours after infection with *F. novicida* (MOI 200). (**C**) LPS primed BMMs were transfected with mock or DNase treated *F. novicida* lysates. Cytotoxicity was determined 4 hours later. (**D**) Macrophages were infected as in (**B**) in the presence or absence of LPS from *S. minnesota* RE595. (**E**) Structural comparison of lipid A from wild type *F. novicida* or the *lpxF* mutant. Structural changes are indicated. (**F**) Poly(I:C) primed macrophages were transfected with lipid A from *F. novicida* grown at 18°C or 37°C, or the

indicated *F. novicida* mutants grown at 37°C. Cytotoxicity was determined after 2 h. (**G**) Structural comparison of lipid A from *Y. pestis* grown at 25°C or 37°C. (**H**) Poly(I:C) primed BMMs were infected with *L. monocytogenes* in the presence of lipid A from *Y. pestis* grown at 25°C or 37°C. Cytotoxicity was determined after 4 h. Data are representative of at least 3 (**A, F, G**) or 2 (**B, C, D**) experiments. Error bars indicate standard deviation of technical replicates.
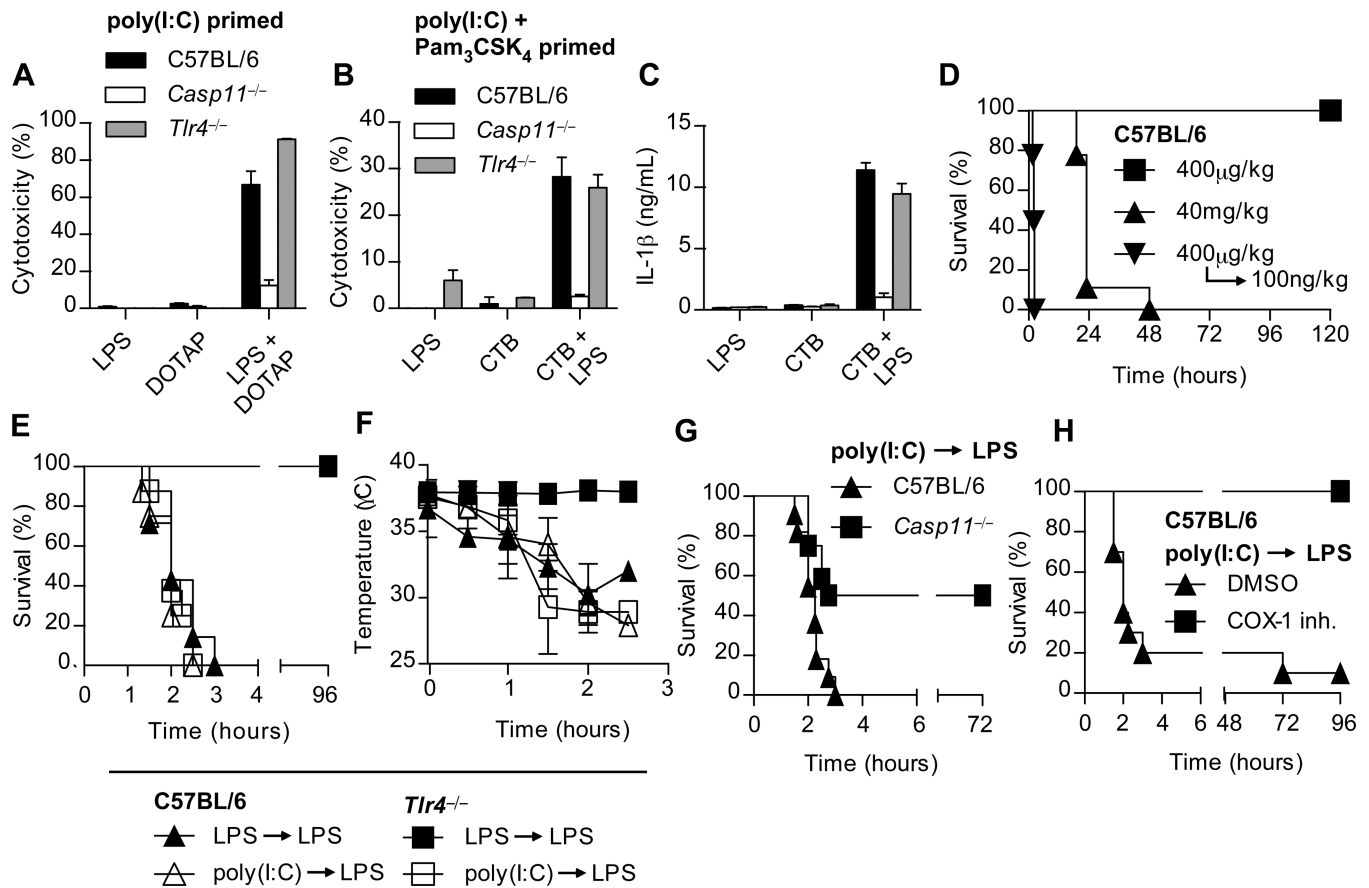
**Fig. 4. LPS detection and rapid induction of shock in primed mice occur independently of TLR4**
(**A**) BMMs were primed overnight with poly(I:C) and then transfected with *S. minnesota* RE595 LPS. Cytotoxicity was determined 2 hours later. (**B–C**) Poly(I:C) and Pam₃CSK₄ primed macrophages were incubated with the indicated combinations of CTB (20μg/mL) and LPS from *E. coli* O111:B4 (1μg/mL). Cytotoxicity (**B**) and IL-1β secretion (**C**) were determined 16 hours later. Data are representative of at least 3 experiments; error bars indicate standard deviation of technical replicates (A–C). (**D**) Survival of mice challenged with the indicated doses of *Escherichia coli* LPS, or primed with LPS and then re-challenged 7 hours later. Data are pooled from three experiments; n = 9 per condition. (**E**) Survival of mice primed with LPS (400μg/kg) or poly(I:C) (10μg/kg) and then challenged 7 hours later with LPS (100ng/kg). Data are pooled from three experiments; n = 7 per LPS prime group and n = 8 for poly(I:C) prime group. (**F**) Rectal temperatures of mice in panel (**E**) after LPS challenge. Data are representative of 3 experiments; n = 4 per condition. (**G**) Survival of poly(I:C) primed mice challenged 6 hours later with LPS (10ng/kg). Data are pooled from 3 experiments, n = 11 (C57BL/6) or 12 (*Casp11⁻/⁻*). (**H**) Mice were primed with poly(I:C) and then challenged 6 hours later with LPS (100ng/kg) and monitored for survival. 1 h before LPS challenge, mice were given 5mg/kg of COX-1 inhibitor or DMSO control. Data are pooled from 2 experiments; n = 11 per condition.