Caspase-11 interaction with NLRP3 potentiates the noncanonical activation of the NLRP3 inflammasome

Julien Moretti1,2, Baosen Jia1,2, Zachary Hutchins1,2,10, Soumit Roy3,11, Hilary Yip1,2, Jiahui Wu4, Meimei Shan1,2,12, Samie R. Jaffrey4, Jörn Coers5,6 and J. Magarian Blander1,2,7,8,9

Caspase-11 detection of intracellular lipopolysaccharide (LPS) from invasive Gram-negative bacteria mediates noncanonical activation of the NLRP3 inflammasome. While avirulent bacteria do not invade the cytosol, their presence in tissues necessitates clearance and immune system mobilization. Despite sharing LPS, only live avirulent Gram-negative bacteria activate the NLRP3 inflammasome. Here, we found that bacterial mRNA, which signals bacterial viability, was required alongside LPS for noncanonical activation of the NLRP3 inflammasome in macrophages. Concurrent detection of bacterial RNA by NLRP3 and binding of LPS by pro-caspase-11 mediated a pro-caspase-11–NLRP3 interaction before caspase-11 activation and inflammasome assembly. LPS binding to pro-caspase-11 augmented bacterial mRNA-dependent assembly of the NLRP3 inflammasome, while bacterial viability and an assembled NLRP3 inflammasome were necessary for activation of LPS-bound pro-caspase-11. Thus, the pro-caspase-11–NLRP3 interaction nucleated a scaffold for their interdependent activation explaining their functional reciprocal exclusivity. Our findings inform new vaccine adjuvant combinations and sepsis therapy.

Murine caspase-11 and its human orthologs, caspases 4 and 5, are cytoplasmic receptors of LPS. Caspase-11 orchestrates defense against Gram-negative bacteria and lethality in sepsis models through cleavage of gasdermin D (GSDMD), which executes pyroptosis of infected cells and damages tissue in endotoxic shock. Caspase-11 also mediates noncanonical (NC)-activation of the highly inflammatory NLRP3 inflammasome to elicit caspase-1 cleavage and interleukin-1β (IL-1β) production. Caspase-11 does not cleave IL-1β directly, while both caspases 1 and 11 cleave GSDMD. Potential mechanisms of NC-activation of the NLRP3 inflammasome include caspase-11 heterodimerization with caspase-1 (ref. 11,13) and caspase-11-mediated GSDMD-dependent cellular perturbations acting through a cell-intrinsic pathway.

Live, but not dead, forms of virulent and avirulent Gram-negative bacteria elicit NC-activation of the NLRP3 inflammasome in macrophages. A specific class of pathogen associated molecular patterns (PAMPs) called vita-PAMPs, such as bacterial messenger RNA (mRNA), and the cyclic dinucleotide c-di-AMP, signals microbial viability and potential infectivity, and augments the immune response. Detection of the vita-PAMP mRNA from live bacteria triggers the activation of the NLRP3 inflammasome in response to Gram-negative, but not Gram-positive bacteria, which do not have LPS, irrespective of bacterial virulence factors that activate inflammasomes.

Here, we report that coincident cytosolic detection of LPS with mRNA was required for NC-activation of the NLRP3 inflammasome in macrophages. Codetection of LPS and bacterial RNA triggered an interaction of pro-caspase-11 with NLRP3 mediated by the NLRP3 LRR and PYD domains and the pro-caspase-11 scaffold domain. Biochemical interaction and interdependent activation of pro-caspase-11 and NLRP3 mechanistically underlie their functional reciprocal exclusivity.

Results
mRNA and LPS activate caspase-11 and the inflammasome. We investigated NC-activation of the NLRP3 inflammasome in bone marrow-derived macrophages (hereafter ‘macrophages’) following phagocytosis of Gram-negative bacteria. We observed similar lysosomal localization of live and dead Escherichia coli (Extended Data Fig. 1a,b). To avoid the compounding effects of replication or virulence factors, we used thymidine auxotrophs of nonpathogenic Escherichia coli K12, strain DH5α. Live E. coli elicited cleavage of caspase-11, caspase-1, GSDMD and IL-1β, and induced pyroptosis as visualized by electron microscopy and measured by lactate dehydrogenase (LDH) release (Fig. 1a,b,c and Extended Data Fig. 1c). These responses, which reflect inflammasome effector functions, were significantly impaired in response to killed E. coli, which have LPS but lack the vita-PAMP mRNA, despite similar inflammasome priming (expression of NLRP3 and proforms of IL-1β, caspase-1 and caspase-11) by either live or killed E. coli (Fig. 1a,c and Extended Data Fig. 1c). Delivery of mRNA or bacterial RNA (RNAvec, which contains 1% mRNAvec) with phagocytosed killed
E. coli, irrespective of RNA_{bac} isolation from either Gram-positive or Gram-negative bacteria, restored caspase-11 and GSDMD cleavage (Fig. 1a,c and Extended Data Fig. 1c) and restored IL-1β secretion and pyroptosis to levels comparable to those elicited by live E. coli (Fig. 1a,c and Extended Data Fig. 1c). Eukaryotic mRNA was not able to do so (Extended Data Fig. 1c). Inflammasome-independent IL-6 and tumor necrosis factor (TNF) production were similar for all stimulation conditions (Fig. 1a,c and Extended Data Fig. 1c). E. coli treatment with Rifampicin, which inhibits RNA_{bac} synthesis, reduced caspase-11, caspase-1 and IL-1β cleavage, while retaining ~70% bacterial viability (Fig. 1d,e).

mRNA_{bac} did not elicit NC-activation of the NLRP3 inflammasome when added to avirulent Gram-positive bacteria (Listeria innocua or Staphylococcus aureusΔSar) that lack LPS, despite similar inflammasome priming (Fig. 1c,f). Live Gram-positive bacteria did not activate the inflammasome either (Fig. 1c,f). Only LPS with mRNA_{bac} from either Gram-positive or Gram-negative bacteria elicited inflammasome activation when delivered to macrophages with

Fig. 1 | Bacterial mRNA and stimulatory LPS are both required for caspase-11 and noncanonical inflammasome activation. a, Immunoblots of macrophage concentrated supernatants (Conc. sup.) (20 h) or whole cell extracts (WCE) (6 h) and cytokine concentrations in culture supernatants (20 h) post stimulation with live (L), heat-killed (HK) or HK E. coli supplemented with 10 μg ml⁻¹ total bacterial RNA (RNA_{tot}) from E. coli or L. innocua. Cl., cleaved. b, Electron microscopy on macrophages stimulated with L or HK E. coli for 8 or 12 h. Scale bar, 2 μm. c, Immunoblots and cytokine concentrations post stimulation with L or HK E. coli, L. innocua or S. aureusΔSar ΔAgr supplemented with their respective 100 ng ml⁻¹ bacterial mRNA. d, Immunoblots as in a post stimulation with L. E. coli grown in 2 μg ml⁻¹ Rifampicin for the times indicated. e, Percentage live E. coli following Rifampicin treatment. f, Immunoblots, cytokine concentrations and LDH release as in c post stimulation with L. E. coli or L, HK or HK L. innocua supplemented with LPS and/or mRNA from E. coli or L. innocua. g, Immunoblots and cytokine concentrations as in a post stimulation with L, HK or HK F. novicida or F. novicida ΔlpxF with 10 μg ml⁻¹ RNA_{bac} from each bacterium. LDH measured by cytotoxicity assay; IL-1β, TNF and IL-6 by ELISA. Error bars, mean ± s.e.m. One-way ANOVA followed by multiple comparisons Sidak tests and FDR correction for multiple comparisons. Scale bar, 2 μm.
killed Gram-positive bacteria (Fig. 1f). Delivery of LPS with dead *L. innocua* without mRNA<sub>bac</sub> did not elicit inflammasome activation (Fig. 1f). Conversely, *L. innocua* mRNA<sub>bac</sub> delivered to cells with killed *E. coli* restored inflammasome activation to levels similar to those elicited by live *E. coli* and killed *E. coli* plus mRNA<sub>bac</sub> (Fig. 1f).

RNA<sub>bac</sub> with LPS from the Gram-negative *Francisella novicida* bacteria, in which lipid A is modified to evade immune stimulation<sup>1</sup>, did not activate the inflammasome (Fig. 1g), indicating that the stimulatory activity of LPS was required for inflammasome effector functions. RNA<sub>bac</sub> with killed mutant bacteria *Francisella tularensis* subsp. *novicida* (F. novicida<sup>ΔlpxF</sup>), which express nonstimulatory tetra-acylated LPS, led to inflammasome effector functions at levels comparable with those elicited by the live *F. novicida<sup>ΔlpxF</sup>* (Fig. 1g). Comparatively, delivery of RNA<sub>bac</sub> with killed *F. novicida*, which express nonstimulatory tetra-acylated LPS, induced significantly less activation (Fig. 1g), as did live *F. novicida*<sup>1</sup> (Fig. 1g). The live or killed forms of all bacteria induced similar inflammasome priming (Fig. 1g). Similar results were observed with *E. coli<sup>LPSmut</sup>* bacteria that express tetra-acylated nonstimulatory LPS<sup>2</sup>. Thus, coincident detection of stimulatory LPS and the vita-PAMP mRNA<sub>bac</sub> significantly augmented NC-activation of the NLRP3 inflammasome in macrophages in response to Gram-negative bacteria.

**mRNA<sub>bac</sub> with low LPS NC-activate the NLRP3 inflammasome.**

Next, we tested whether experimental cytoplasmic delivery of mRNA<sub>bac</sub> with LPS into macrophages augmented NC-activation of the NLRP3 inflammasome. We transfected 2 ng ml<sup>−1</sup> ultrapure LPS and 100 ng ml<sup>−1</sup> of mRNA<sub>bac</sub> with LPS<sub>mut</sub>, *L. innocua* or IVT mRNA<sub>bac</sub> (Fig. 1b). Bacteria:macrophage ratio, 20:1, except 50:1 for *F. novicida*. LDH measured by cytotoxicity assay; IL-1β, TNF and IL-6 by ELISA. Error bars, mean ± s.e.m. One-way ANOVA followed by multiple comparisons Sidak tests. *P* values indicated in bar graphs in a (IL-1β, *n* = 4; LDH, *n* = 3) and b (n = 4). Cleaved caspase-11 corresponds to caspase-11 p30. Results represent at least three independent experiments.

Fig. 2 | Low cytosolic concentration of LPS triggers noncanonical activation of the NLRP3 inflammasome when cytotoxic bacterial mRNA is also present. a,b, Immunoblots of macrophage concentrated supernatants (20 h) or WCE (6 h), and cytokine concentrations and LDH release in culture supernatants (20 h) as indicated following transfection of ultrapure LPS (low dose 2 ng ml<sup>−1</sup> or high dose 1 μg ml<sup>−1</sup>) ± mRNA from *E. coli<sup>LPSmut</sup>* or in vitro transcribed (IVT) (100 ng ml<sup>−1</sup>), transfection of LPS (2 ng ml<sup>−1</sup>) ± mRNA from *E. coli* or eukaryotic cells (Euk.) (100 ng ml<sup>−1</sup>) (a) and transfection of WT or *Nlrp3<sup>−/−</sup>* macrophages with indicated doses of ultrapure LPS or mRNA from *E. coli<sup>LPSmut</sup>* or *L. innocua* or IVT (b). Bacteria:macrophage ratio, 20:1, except 50:1 for *F. novicida*. LDH measured by cytotoxicity assay; IL-1β, TNF and IL-6 by ELISA. Error bars, mean ± s.e.m. One-way ANOVA followed by multiple comparisons Sidak tests and *P* values indicated in bar graphs in a (IL-1β, *n* = 4; LDH, *n* = 3) and b (n = 4). Cleaved caspase-11 corresponds to caspase-11 p30. Results represent at least three independent experiments.
Transfection of 2 ng ml⁻¹ ultrapure LPS did not elicit inflammasome effector functions (Fig. 2a,b), despite intact inflammasome priming (Fig. 2a,b), while cotransfection with 100 ng ml⁻¹ mRNAₘₐₙ, irrespective of its source (E. coli, E. coliLPSmut, L. innocua, IVT mRNAₘₐₙ) did elicit inflammasome effector functions (Fig. 2a,b), and in an NLRP3-dependent manner (Fig. 2b); transfected RNAₘₐₙ alone did not (Fig. 2a,b) irrespective of dose (Extended Data Fig. 3a). Cotransfection of eukaryotic mRNA with 2 ng ml⁻¹ ultrapure LPS did not elicit NC-activation of the inflammasome (Fig. 2a). Cotransfection of 100 ng ml⁻¹ IVT mRNAₘₐₙ and 2 ng ml⁻¹ ultrapure LPS into cells also elicited levels of IL-1β production at levels (2–3 ng ml⁻¹) that approximated those elicited by the supplementation of killed bacteria with 100 ng ml⁻¹ bacterial mRNA (Extended Data Fig. 3a). In contrast, transfection of a high concentration of LPS (1 μg ml⁻¹) elicited inflammasome effector functions without mRNAₘₐₙ (Fig. 2a), as previously reported. Caspase-11 cleavage faithfully reflected caspase-11 activation (Extended Data Fig. 3b,c). Notably, cytosolic LPS after transfection of 1 μg ml⁻¹ LPS were higher than after phagocytosis of avirulent E. coli (on average 100-fold higher), virulent E. coli (10- to 11-fold higher) (Extended Data Fig. 2a), and Salmonella typhimurium infection (6-fold higher).

mRNAₘₐₙ (LPS-free RNA from L. innocua or IVT mRNAₘₐₙ, and mRNAₘₐₙ from E. coli or E. coliLPSmut) did not induce expression of NLRP3, pro-caspase-11 and pro-IL-1β protein above that in unstimulated macrophages (Fig. 2a), while ultrapure LPS, at either 2 ng ml⁻¹ or 1 μg ml⁻¹, was sufficient to elicit inflammasome priming (Fig. 2a,b). Transfection of mRNAₘₐₙ alone (either from E. coli, E. coliLPSmut or IVT mRNAₘₙ) did not elicit appreciable IL-6 or TNF production, and did not increase their expression at any dose compared with transfection of LPS alone (Fig. 2a and Extended Data Fig. 3a). Conversely, cotransfection of 2 ng ml⁻¹ LPS with mRNAₘₙ elicited inflammasome-dependent pro-IL-1β cleavage and secretion of IL-1β (Fig. 2a,b), in a manner dependent on the dose of cotransfected mRNAₘₙ (Extended Data Fig. 3a). Thus, the ability of mRNAₘₙ to augment NC-activation of the NLRP3 inflammasome by transfected 2 ng ml⁻¹ LPS was not through inflammasome priming, which was mediated primarily by LPS. At cytosolic concentrations equivalent to those found in macrophages following stimulation with bacteria, cytoplasmic LPS and mRNAₘₙ, which alone did not induce inflammasome activation, synergized in mediating NC-activation of the NLRP3 inflammasome.

LPS augments mRNAₘₙ-driven NLRP3 inflammasome assembly. A prerequisite for NLRP3 activation is the oligomerization of the adapter protein ASC (encoded by Pycard) into prion-like structures, which recruit and activate caspase-1 (refs. 4,26,27). Killed Yersinia pestis, which expresses a stimulatory LPS when grown at 24°C1, and killed E. coli without mRNAₘₙ or RNAₘₙ did not induce detectable ASC oligomerization in macrophages (Fig. 3a–c), although killed and live bacteria similarly induced inflammasome priming (Fig. 3d,e). On the other hand, live bacteria or delivery of mRNAₘₙ or RNAₘₙ isolated from each strain, with killed bacteria, elicited ASC oligomerization (Fig. 3a–c), which was impaired in Nlrp3⁻/⁻ macrophages (Fig. 3c)17,28. ASC oligomerization was triggered by various forms of RNAₘₙ mRNAₘₙ from E. coli or L. innocua, or LPS-free IVT mRNAₘₙ, but not eukaryotic mRNA, if delivered with killed E. coli (Fig. 3f,g) but not when added alone (Fig. 3f). Confocal microscopy showed that live, but not dead, bacteria induced ASC specks29 (Fig. 3b,i and Extended Data Fig. 4a,b). RNAₘₙ delivery with killed E. coli restored ASC speck formation (Fig. 3i and Extended Data Fig. 4a) and was dependent on NLRP3 (Extended Data Fig. 4c), consistent with previous observations17,28.

Live bacteria expressing a nonstimulatory tetra-acetylated LPS (37°C Y. pestis and E. coliLPSmut)17,28 or delivery of RNAₘₙ with killed forms of these bacteria elicited ASC oligomerization and ASC speck formation (Fig. 3a,b,i), indicating that the stimulatory activity of LPS was dispensable for NLRP3 inflammasome assembly, although ASC oligomerization under these conditions was accompanied by inefficient activation of caspase-11 and inflammasome effector functions (Fig. 3d,e). L. innocua, which lacks LPS, had an impaired ability to assemble ASC oligomers, regardless of bacterial viability or the presence of RNAₘₙ from either Gram-positive or Gram-negative bacteria (Fig. 3j). Addition of LPS to killed L. innocua along with RNAₘₙ from either Gram-positive or Gram-negative bacteria augmented ASC oligomerization (Fig. 3j). Supplementation of killed L. innocua with lipid IVa, which binds to but does not activate caspase-11 (ref. 30), also augmented ASC oligomerization when RNAₘₙ was cosupplemented (Fig. 3k). These data showed that viability of Gram-negative bacteria or codetection of mRNAₘₙ with LPS drove efficient ASC oligomerization irrespective of the ability of LPS to stimulate caspase-11, and indicated the uncoupling of inflammasome assembly from inflammasome activation.

NLRP3 and ASC are required for caspase-11 activation. We next tested whether the activation of NLRP3 and the activation of caspase-11 in macrophages were interdependent. Caspase-11 activation and NC-activation of the NLRP3 inflammasome in response to live E. coli were severely impaired in Nlrp3⁻/⁻ and Pycard⁻/⁻ macrophages, despite intact inflammasome priming (Fig. 4a,b), in contrast with the ability of virulent E. coli to elicit NLRP3- and ASC-independent caspase-11 cleavage in macrophages11. Although residual caspase-11 cleavage was detected in Nlrp3⁻/⁻ and Pycard⁻/⁻ macrophages at similar levels in response to either live or killed E. coli, it was not accompanied by the cleavage of GDSDM or release of LDH (as a readout of pyroptosis) observed in wild type macrophages (Fig. 4a,b). A side-by-side comparison showed that, while both virulent and avirulent E. coli triggered inflammasome effector functions, such as caspase-1 cleavage and IL-1β cleavage and secretion, in an NLRP3- and ASC-dependent manner, only virulent
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Shigella caspase-11 in not detected in NLRC4-deficient (-/- or E. coli killed 25%)

710

and virulent Shigella and strains. Results represent at least three independent experiments.

Salmonella E. coli Nlrp3 – Requirement of NLRP3 and aSC for caspase-11 activation in response to live avirulent gram-negative bacteria.

inflammasomes, underwent NC-activation by caspase-11 (ref. 4).

and that the NLRP3 inflammasome, but not the NLRC4 or AIM2 inflammasome) promoted ASC oligomerization similarly of an assembled NLRP3 inflammasome for caspase-11 activation some effector functions (Fig. 4e), indicating a specific requirement E. coli to killed plus RNAbac (Fig. 4d). However, only RNAbac plus AIM2 inflammasome) promoted ASC oligomerization similarly and various canonical inflammasome inducers (flagellin, the NLRC4 inflammasome; poly(dA:dT), the

Virulent bacteria-triggered caspase-1 and pro-IL-β cleavage, which are canonical effector functions of the NLRP3 or NLRC4 inflammasomes, were strongly reduced in Nlrp3–/– and Ipaf–/– macrophages (Fig. 4c), in line with the ability of Salmonella and Shigella to activate the NLRP3 and NLRC4 inflammasomes.

A larger percentage of macrophages showed rapid plasma membrane permeabilization (precedent to pyroptosis) and LDH release in response to virulent compared with avirulent bacteria, and beginning as early as 2 h post stimulation (30% compared with 2.5%) (Extended Data Fig. 5a–d)—a time point when the expression of inflammasome components, including NLRP3, had not yet peaked (Extended Data Fig. 5e)—suggested NLRP3 was not required for GSDMD cleavage and LDH release in response to virulent bacteria. Of note, cleaved caspase-1, caspase-11, GSDMD and IL-1β were detected at 6 h post stimulation with virulent E. coli (Extended Data Fig. 5f). In contrast, a smaller percentage of macrophages (≤25%) were pyroptotic at any given time over the course of 72 h post stimulation with avirulent strains of E. coli, Salmonella and Shigella, and showed delayed kinetics of pyroptosis, which peaked 36 h post stimulation (Extended Data Fig. 5g). Thus, caspase-11 activation, GSDMD cleavage and pyroptosis elicited by avirulent bacteria were dependent on an assembled NLRP3 inflammasome, as they required both NLRP3 and ASC, while these events were independent of NLRP3 in response to virulent bacteria.

We next tested whether NLRP3 and ASC, and assembled inflammasomes, including inflammasomes other than NLRP3, provided a scaffold for caspase-11 activation. Stimulation of macrophages with a combination of killed E. coli and various canonical inflammasome inducers (flagellin, the NLRC4 inflammasome; poly(dA:dT), the AIM2 inflammasome) promoted ASC oligomerization similarly to killed E. coli plus RNAav (Fig. 4d). However, only RNAav, plus killed E. coli induced caspase-11 cleavage and NLRP3 inflammasome effector functions (Fig. 4c), indicating a specific requirement of an assembled NLRP3 inflammasome for caspase-11 activation and that the NLRP3 inflammasome, but not the NLRC4 or AIM2 inflammasomes, underwent NC-activation by caspase-11 (ref. 4). Macrophage stimulation with killed E. coli plus the bacterial toxin nigericin, a canonical NLRP3 inflammasome trigger, also coupled ASC oligomerization to caspase-11 cleavage and NLRP3 inflammasome effector functions (Extended Data Fig. 6a). Direct cytosolic delivery (in contrast to delivery with phagocytosed killed bacteria), of flagellin or poly(dA:dT) into LPS-primed macrophages, or their treatment with nigericin, elicited the cleavage of caspase-1, pro-IL-1β and GSDMD (Extended Data Fig. 6b). Altogether, these results indicated that a scaffold for pro-caspase-11 activation by LPS could be provided only by an assembled NLRP3 inflammasome and not by other assembled inflammasomes.

Caspase-11 is required for NLRP3 inflammasome assembly. We next investigated the requirement of caspase-11 for NC-activation of the NLRP3 inflammasome in response to Gram-negative bacteria. ASC oligomerization and all inflammasome effector functions were impaired in Casp11–/– macrophages following stimulation with live bacteria or killed bacteria plus RNA (compared with wild type macrophages) (Fig. 5a–c), irrespective of whether ASC oligomerization was assessed early (8 h) or late (16 h) post stimulation (Fig. 5b). Nascent ASC oligomers were detected in Casp11–/– macrophages 8 h post stimulation with live E. coli, but not at 16 h post stimulation with either live E. coli or killed E. coli plus RNAav (Fig. 5a,b), indicating an inability to generate stable higher-order ASC clustering in the absence of caspase-1, consistent with prior observations24. ASC specks were also not detected in either Casp11–/– or Casp11–/– macrophages at 16 h post stimulation with live bacteria (Fig. 5d). Pro-IL-1β expression was induced similarly by either live, killed or killed E. coli plus RNAav, in wild type, Casp11–/– or Casp11–/– macrophages (Fig. 5a), although we noted a compensatory increase of pro-caspase-1 expression in Casp11–/– macrophages and pro-caspase-11 expression in Casp11–/– macrophages, while GSDMD and ASC expression were similar in all genotypes (Fig. 5a).

Delivery of poly(dA:dT) with phagocytosed killed E. coli elicited ASC oligomerization in Casp11–/– macrophages (Fig. 5c), indicating that Casp11–/– macrophages did not have a general impairment in ASC oligomerization. Yet, consistent with the specific requirement of an assembled NLRP3 inflammasome for caspase-11 activation, only ASC oligomerization triggered by killed E. coli with RNAav, and not poly(dA:dT), was accompanied by cleavage of caspase-11, caspase-1, pro-IL-1β or GSDMD (Fig. 5c).

Cleaved caspase-11 was detected in concentrated supernatants from Casp11–/– macrophages (Extended Data Fig. 6b), indicating that, unlike the requirement for NLRP3 and ASC, caspase-1 was not required for caspase-11 activation. However, all inflammasome effector functions, including notably GSDMD cleavage and pyroptosis, were impaired in Casp11–/– macrophages compared with wild type macrophages, despite intact caspase-11 activation (Fig. 5a,c), indicating that, like IL-1β, GSDMD was cleaved by caspase-1 in response to RNAav and LPS. Caspase-1, pro-IL-1β and GSDMD cleavage were not detected in Casp11–/– macrophages (Fig. 5a,c). Thus, IL-1β secretion and pyroptosis triggered by RNAav and LPS were direct effector functions of caspase-11-mediated NC-activation of the NLRP3-ASC-pro-caspase-1 inflammasome complex.

Fig. 4 | Requirement of NLRP3 and ASC for caspase-11 activation in response to live avirulent Gram-negative bacteria. a–c, ImmunobLOTS of macrophage concentrated supernatants (20 h) or WCE (6 h), and cytokine concentrations and LDH release in culture supernatants (20 h) post stimulation of WT, Nlrp3–/– or Pycard–/– macrophages with L or HK E. coli (a), WT, Nlrp3–/– or Pycard–/– macrophages with L or HK avirulent or virulent E. coli (b), and WT, Nlrp3–/– or Ipaf–/– macrophages with Live Salmonella ΔSpi1/2 (avirulent, lacking the Salmonella pathogenicity islands 1 and 2 encoded type III secretion system) or WT (virulent) or Shigella BS103 (avirulent virulence plasmid-cured) or WT (virulent) (note the doublet GSDMD bands here likely reflect the nature of the trigger) (c). d, Immunoblot of WT macrophage culture supernatants (16 h) post stimulation with L, HK or HK E. coli supplemented with indicated doses of RNAav, Flagellin or poly(dA:dT). e, ImmunobLOTS of WT macrophage concentrated supernatants (20 h) or WCE (6 h), and cytokine concentrations in culture supernatants (20 h) post stimulation as in d. LDH measured by cytotoxicity assay, IL-1β and IL-6 by ELISA. Error bars, mean ± s.e.m. One-way ANOVA followed by multiple comparisons Sidak tests and P values indicated in bar graphs in a, b, c and e (n = 3). ns, nonsignificant. Bacterial macrophage, 20:1 for E. coli and virulent E. coli; 5:1 for all Salmonella and Shigella strains. Results represent at least three independent experiments.
RNA<sub>tot</sub> and LPS trigger a pro-caspase-11–NLRP3 interaction. We tested whether the functional reciprocal exclusivity of caspase-11 and NLRP3 was due to a physical interaction between the two. NLRP3 stimuli trigger disassembly of the trans-Golgi network (TGN) and recruitment of NLRP3 to the dispersed TGN (dTGN)<sup>32</sup>. Confocal microscopy showed vesicular subcellular colocalization of caspase-11 and NLRP3 in macrophages stimulated with bacteria, irrespective of bacterial viability or virulence, but not in resting bone marrow-derived macrophages (Fig. 6a and Extended Data Fig. 7a,b). We noted TGN dispersion and radiation away from the nucleus after stimulation with either live or dead, virulent or avirulent E. coli (Fig. 6a and Extended Data Fig. 7a), in wild type, Nlrp3<sup>–/–</sup> or Casp11<sup>–/–</sup> macrophages (Extended Data Fig. 7b). NLRP3 was detected on dTGN in wild type macrophages (Fig. 6a and Extended Data Fig. 7a), as reported in Pycard<sup>1/2</sup> macrophages in response to strong triggers such as nigericin, ATP or monosodium urate<sup>15</sup>. Caspase-11 colocalized with dTGN, and specifically to dTGN areas where NLRP3 was also present (Fig. 6a and Extended Data Fig. 7a). Caspase-11 did not localize to dTGN in Nlrp3<sup>–/–</sup> macrophages, while NLRP3 localized to dTGN in Casp11<sup>–/–</sup> macrophages (Extended Data Fig. 7b).

We next tested whether caspase-11 interacted with NLRP3 in macrophages. NLRP3 coimmunoprecipitated with endogenous pro-caspase-11 efficiently following stimulation with live 24°C-grown Y. pestis, F. novicida<sup>poly(dA:dT)</sup> and E. coli or killed forms of these bacteria supplemented with RNA<sub>tot</sub>, and not following stimulation with killed bacteria alone (Fig. 6b,c and Extended Data Fig. 7c). Conversely, the proforms of caspase-11 coimmunoprecipitated with endogenous NLRP3 in response to live E. coli or killed E. coli plus RNA<sub>tot</sub> but not killed E. coli alone (Extended Data Fig. 7c). Transfection of either unprimed or poly(I:C)-primed macrophages with 2 ng ml<sup>–1</sup> ultrapure LPS and/or stimulatory-LPS-free RNA<sub>tot</sub> showed caspase-11 interacted with NLRP3 only when RNA<sub>tot</sub> and LPS were cotransfected (Fig. 6d). Killed E. coli with either RNA<sub>tot</sub> or nigericin, but not poly(dA:dT), promoted the pro-caspase-11–NLRP3 interaction (Fig. 6e and Extended Data Fig. 7d), indicating the interaction was elicited specifically by the combination of LPS and NLRP3 agonists. Pro-caspase-11 did not coimmunoprecipitate.
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with AIM2 in response to either live or killed bacteria, or delivery of RNA<sub>nuc</sub> or poly(dA:dT) with killed bacteria (Fig. 6c). Pro-caspase-11 and NLRP3 did not interact post transfection of high dose (1 μg ml<sup>-1</sup>) LPS (Fig. 6d), but did so post cotransfection of RNA<sub>nuc</sub> with 1 μg ml<sup>-1</sup> LPS (Fig. 6d). NLRP3 coimmunoprecipitated with pro-caspase-11 as early as 3 h post stimulation with bacteria, and irrespective of bacterial virulence (Fig. 6f,g).

NLRP3 did not coimmunoprecipitate with pro-caspase-11, or the opposite, after macrophage stimulation with live Gram-positive <i>L. innocua</i> or killed <i>L. innocua</i> plus either Gram-negative or Gram-positive mRNA<sub>nuc</sub> (Fig. 6h), suggesting that LPS detection was required for pro-caspase-11–NLRP3 interaction. Addition of LPS, in conditions in which RNA<sub>nuc</sub> was also present, to live <i>L. innocua</i> or killed <i>L. innocua</i> restored pro-caspase-11–NLRP3 interaction (Fig. 6i,j).

Stimulation with either <i>E. coli</i><sup>Δ<sub>nuc</sub></sup> (Fig. 6c) or the combination of Lipid IVa with killed <i>L. innocua</i> (Fig. 6f) induced pro-caspase-11–NLRP3 interaction as long as RNA<sub>nuc</sub> was also present, suggesting that LPS binding to caspase-11, but not the stimulatory activity of LPS, was sufficient for mediating the pro-caspase-11–NLRP3 interaction. Thus, LPS binding to caspase-11 concurrent with RNA<sub>nuc</sub> detection by NLRP3 triggers interaction of pro-caspase-11 with NLRP3.

Caspase-11 and NLRP3 interact before their activation. To investigate whether pro-caspase-11 and NLRP3 interacted before caspase-11 activation, we probed the pro-caspase-11–NLRP3 interaction in conditions where the activity of caspase-11 or its products were blocked. GSDMD-dependent cellular perturbations post caspase-11 activation are a prerequisite for NLRP3 inflammasome activation<sup>12,14</sup>. In <i>Gsdmd</i>−/− macrophages, cleaved caspase-11 was detected in the whole cell extracts (WCE), but not in the concentrated supernatants or the WCE (Fig. 7a and Extended Data Fig. 8a,b), while cleaved caspase-11 and IL-1β were not detected in the concentrated supernatants or the WCE (Fig. 7a and Extended Data Fig. 8c). Note, pro-caspase-11 interacted with NLRP3 in <i>Gsdmd</i>−/− macrophages (Fig. 7b). The broad caspase inhibitor zVAD-FMK did not block the pro-caspase-11–NLRP3 interaction (Fig. 7c), although it abrogated inflammasome effector functions (Extended Data Fig. 8d). These observations indicated that the interaction of pro-caspase-11 with NLRP3 was upstream of caspase-11 activation.

We tested whether an assembled NLRP3 inflammasome was required for the pro-caspase-11–NLRP3 interaction. Alongside pro-caspase-11, we detected ASC, pro-caspase-1 and NEK7, which binds NLRP3 and is required for its assembly with ASC and caspase-1 (refs. <sup>33,16</sup>), in the NLRP3 immunoprecipitates after macrophage stimulation with live, but not killed, <i>E. coli</i> (Fig. 7d), suggesting that NLRP3 was ‘licensed’ for assembly<sup>9</sup>. On the other hand, NLRP3, but not ASC or NEK7, were detected in the pro-caspase-11 immunoprecipitates post stimulation with live <i>E. coli</i> (Fig. 7d), indicating that NLRP3, but not NEK7 or ASC, interacted with pro-caspase-11. Pro-caspase-11 and NLRP3 interacted in <i>Pycard</i>−/− or <i>Casp1</i>−/− macrophages following stimulation with live <i>E. coli</i> (Fig. 7e), indicating ASC and caspase-1 were not required and suggesting that the pro-caspase-11–NLRP3 interaction did not reflect caspase-11 recruitment to stable, caspase-1-containing NLRP3-ASC oligomers, following an event such as heterodimerization with caspase-1 (refs. <sup>12,13,14</sup>). These observations suggested that cytosolic detection of RNA<sub>nuc</sub> and LPS from Gram-negative bacteria was necessary and sufficient to mediate the pro-caspase-11–NLRP3 interaction upstream of inflammasome assembly and caspase-11 activation (Extended Data Fig. 9).

Pro-caspase-11 SCAF interacts with NLRP3 LRR and PYD. To identify the domains of NLRP3 and pro-caspase-11 that are important for their interaction, we expressed in 293T cells full-length (NLRP3<sup>FL</sup>) and truncation mutant forms (NLRP3<sup>ΔN</sup>, NLRP3<sup>ΔLRR</sup>, NLRP3<sup>ΔNΔL</sup>, NLRP3<sup>ΔNΔPYD</sup>, NLRP3<sup>ΔLΔR</sup>, NLRP3<sup>ΔLΔPYD</sup>, NLRP3<sup>ΔRΔL</sup>, NLRP3<sup>ΔRΔPYD</sup>, NLRP3<sup>ΔLΔRΔPYD</sup>) of HA-tagged NLRP3, and full-length (casp11<sup>ΔSCAF</sup>) and truncation mutant forms (casp11<sup>ΔSCAF</sup>, casp11<sup>ΔNΔSCAF</sup>, casp11<sup>ΔLΔSCAF</sup>) of FLAG-tagged catalytically inactive caspase-11(C254A)<sup>9</sup> (Extended Data Fig. 10a,b). HA-NLRP3<sup>FL</sup> did not coimmunoprecipitate efficiently with casp11<sup>ΔSCAF</sup>, even when this was loaded at a fourfold higher concentration, compared with casp11<sup>ΔNΔSCAF</sup> and casp11<sup>ΔLΔSCAF</sup> (Extended Data Fig. 10c), indicating that the CARD domain of caspase-11, which binds to LPS, was not required for caspase-11 coimmunoprecipitation with NLRP3<sup>FL</sup>, while the other caspase domain, herein referred to as scaffold (SCAF) domain, was required. Reverse immunoprecipitation (IP) of FLAG-casp11<sup>ΔSCAF</sup> confirmed these results where NLRP3<sup>IE</sup> could not be coimmunoprecipitated with casp11<sup>ΔNΔSCAF</sup> in contrast to its coimmunoprecipitation with casp11<sup>ΔLΔSCAF</sup> (Fig. 8a).

The SCAF domain is common to many caspses, including the inflammatory caspase-1 and caspase-11 and the apoptotic caspses, such as caspase-3, 7 and 9 (Extended Data Fig. 10d), and the SCAF domain of caspase-11 is highly homologous to the SCAF domains of the other caspses (Extended Data Fig. 10e,f). To determine if caspase-11-SCAF was unique in its interaction with NLRP3, we swapped the caspase-11-SCAF with the caspase-9-SCAF (Extended Data Fig. 10g). The FLAG-casp11<sup>ΔNΔSCAF</sup>-casp9<sup>ΔNΔSCAF</sup> chimeric molecule showed impaired coimmunoprecipitation with NLRP3<sup>FL</sup> compared with FLAG-casp11<sup>ΔNΔSCAF</sup> (Fig. 8b). Conversely, replacing caspase-9-SCAF domain with the caspase-11-SCAF (FLAG-casp9<sup>ΔNΔLΔSCAF</sup>-casp11<sup>ΔNΔSCAF</sup> SCAF) allowed coimmunoprecipitation of NLRP3<sup>FL</sup> (Fig. 8b), indicating that the SCAF domain of caspase-11 was required and sufficient for the interaction with NLRP3 in this ligand-independent expression system in 293T cells. On the other hand, NLRP3<sup>ΔPYD</sup> or NLRP3<sup>ΔLRR</sup> showed impaired coimmunoprecipitation with casp11<sup>ΔNΔSCAF</sup> compared with HA-NLRP3<sup>FL</sup> (Fig. 8c). These results indicated that the interaction between NLRP3 and pro-caspase-11 required the PYD and LRR domains of NLRP3 and the SCAF domain of pro-caspase-11.

Discussion

Here, we show that coincident cytosolic detection of LPS with mRNA<sub>nuc</sub> by macrophages simultaneously engaged NLRP3 and caspase-11, and enabled their interaction and interdependent reciprocal
### Table

| Condition | mRNA | LPS (100 ng ml⁻¹) | Poly(dA:dT) (1 µg ml⁻¹) | Lipid IVa (100 ng ml⁻¹) |
|-----------|------|-------------------|------------------------|------------------------|
| Untreated | -    | -                 | -                      | -                      |
| L. innocua | -    | -                 | -                      | -                      |
| Y. pestis | -    | -                 | -                      | -                      |
| E. coli | -    | -                 | -                      | -                      |
| F. novicida | -    | -                 | -                      | -                      |

### Figures

**Figure a**
- Untreated
- L
- HK

**Figure b**
- E. coli
- Y. pestis (24 °C)
- F. novicida
- Pro-caspase-11
- IP
- β-actin

**Figure c**
- E. coli
- E. coli RNA₅ (10 µg ml⁻¹)
- Pro-caspase-11
- IP
- caspase-11

**Figure d**
- No priming
- E. coli
- LPS
- poly(I:C)
- Transfection
- Pro-caspase-11
- IP
- β-actin

**Figure e**
- E. coli
- E. coli RNA₅ (10 µg ml⁻¹)
- Poly(dA:dT) (1 µg ml⁻¹)
- LPS (100 ng ml⁻¹)

**Figure f**
- E. coli
- Virulent E. coli
- L
- HK
- Pro-caspase-11
- IP

**Figure g**
- E. coli
- Virulent E. coli
- L
- HK
- IP

**Figure h**
- E. coli
- L. innocua
- L. innocua RNA₅ (10 µg ml⁻¹)
- Pro-caspase-11
- IP

**Figure i**
- E. coli
- L. innocua
- L. innocua RNA₅ (10 µg ml⁻¹)
- LPS (100 ng ml⁻¹)

**Figure j**
- E. coli
- L. innocua
- L. innocua RNA₅ (10 µg ml⁻¹)
- Lipid IVa (100 ng ml⁻¹)
pro-caspase-11 activation by LPS, indicating the upstream interaction with NLRP3, but not AIM2 inflammasome. Precisely how cytosolic codetection of pro-caspase-11–NLRP3 interaction as early as 3 h. Pro-caspase-11 and NLRP3 may interact directly or indirectly through intermediary protein(s), mRNA, and LPS together, and not each alone, at concentrations equivalent to those detected in the cytosol of macrophages after phagocytosis of bacteria, triggered pro-caspase-11–NLRP3 interaction upstream of the activation of either receptor and before NLRP3-ASC oligomerization. LPS binding to, but not stimulation of, caspase-11 was necessary and sufficient as long as mRNA was also present to engage NLRP3. Although ASC was not required for the pro-caspase-11–NLRP3 interaction, both NLRP3 and ASC (assembled NLRP3 inflammasome) were required for pro-caspase-11 activation by LPS, indicating the upstream interaction with NLRP3 licensed LPS-bound pro-caspase-11 for activation. Reciprocally, caspase-11 protein and not its activity was required for mRNA-driven NLRP3 inflammasome assembly, which also required LPS, a requirement that may have been overlooked due to potential LPS contamination in RNA preparations. Once assembled, the NLRP3 inflammasome, but not the AIM2 or NLR4 inflammasomes, was associated with the activation of LPS-bound caspase-11. Pro-caspase-11 did not interact with AIM2 upon delivery of poly(dA:dT) with phagocytosed killed E. coli. It is possible that NLRP3 and pro-caspase-11 remain in a single complex upon NEK7 and ASC recruitment to NLRP3, which might explain the noted activation of caspase-11 upon assembly of the NLRP3, but not AIM2 inflammasome. Precisely how cytosolic codetection of mRNA, and LPS facilitates caspase-11 activation requires future investigation. Our findings suggest nonmutually exclusive modes of caspase-11 activation in macrophages dependent on the context of cytosolic LPS detection. A fast, NLRP3-dependent mode was triggered by the concurrent expression of bacterial virulence factors during cell invasion by pathogenic Gram-negative bacteria. A slower, NLRP3-dependent mode, was triggered by coincident detection of mRNA from phagocytosed live Gram-negative bacteria and independently of virulence factors.

We propose that the pro-caspase-11 interaction with NLRP3 nucleates a scaffold, the assembled NLRP3 inflammasome, in which NLRP3 and pro-caspase-11 activate one another reciprocally and exclusively. The importance of a platform for caspase signaling emerged from studies showing that caspase-4, -5 or -11 prefer large LPS micelles or LPS aggregates formed upon GBP1 polymerization. LPS micelles occur at higher micromolar range concentrations compared with the LPS monomers at low concentrations, which could explain how microgram levels of cytosolic LPS provide an activation surface for caspase-4, -5 and -11 without the requirement of mRNA. Under conditions when LPS cytotoxic concentrations are lower than the micromolar range,

**Fig. 7 | Pro-caspase-11–NLRP3 interaction is upstream of NLRP3 inflammasome assembly and activation.** a, Immunoblots of macrophage concentrated supernatants (20 h) or WCE (6 h) as indicated post stimulation of WT and Gsdmd−/− macrophages with L or HK E. coli supplemented with E. coli RNA. b–e, IP of endogenous caspase-11 or NLRP3 as indicated, and immunoblotting for coimmunoprecipitating and WCE proteins at 12 h post stimulation of WT and Gsdmd−/− macrophages with L, HK or HK E. coli supplemented with E. coli RNA (E. coli E. coli HK) supernatants (20 h) or WCE (6 h) as indicated post stimulation of WT and Gsdmd−/− macrophages with L, HK or HK E. coli supplemented with E. coli RNA (E. coli E. coli HK). a, All immunoblotted proteins are according to the labels to left of immunoblot panels. Bacteria:macrophage, 20:1. Results represent at least three independent experiments.
such as following phagocytosis of Gram-negative bacteria, the pro-caspase-11-NLRP3 interaction upon codetection of LPS and RNA<sub>bac</sub> from live bacteria, would provide a platform to mediate the activation of pro-caspase-11 and reciprocally, NLRP3.

The observation that NLRP3-ASC oligomerization was not sufficient for inflammasome activation is in contrast to the existing view that NLRP3-ASC oligomers are automatically activated, leading to the cleavage of caspase-1 and IL-1β. Our findings do not necessarily imply that the NLRP3-ASC oligomers elicited by LPS plus mRNA<sub>bac</sub> are different from the oligomers formed through the canonical pathway of inflammasome activation, either qualitatively or quantitatively. Future studies are necessary to decipher whether this is the case. Rather, the use of nonstimulatory LPS with mRNA<sub>bac</sub>, ‘freeze-framed’ each step of the process, and revealed an additional regulatory layer to allow activation after NLRP3-ASC oligomerization. Our data indicated that the NLRP3-ASC oligomers did not activate caspase-1 for the subsequent cleavage of GSDMD unless caspase-11 was activated beforehand. Therefore, while the LPS-bound proform of caspase-11 was important for mRNA<sub>bac</sub>-driven NLRP3 inflammasome assembly, the active form of caspase-11 allowed assembled NLRP3-ASC oligomers to proceed to full activation manifesting in cleavage of caspase-1 and leading to IL-1β secretion and pyroptosis. The requirement for GSDMD in the process may reflect a positive feedback role to maintain NLRP3 inflammasome assembly and activity.

mRNA<sub>bac</sub> and LPS synergistically coordinated NC-activation of the NLRP3 inflammasome. mRNA<sub>bac</sub> was the NLRP3 trigger, similar to nigericin, and its requirement alongside LPS was not a function of inflammasome ‘priming’, which can be mediated by various PAMPs during macrophage phagocytosis of bacteria. However, LPS binding to pro-caspase-11 and the interaction of LPS-bound pro-caspase-11 with NLRP3 in the presence of RNA<sub>bac</sub> ultimately set LPS apart from other PAMPs. Codetection of LPS with eukaryotic mRNA did not elicit NC-activation of the NLRP3 inflammasome, likely due to differences between eukaryotic versus bacterial mRNAs at 3′ ends<sup>43</sup>, which may be important for NLRP3 inflammasome activation<sup>12,45,46</sup>. Future investigations are necessary to elucidate how mRNA<sub>bac</sub> stimulate NLRP3.

Detection of mRNA<sub>bac</sub> in the context of a prototype vaccine comprised of live or dead Gram-negative bacteria elicited follicular CD4<sup>+</sup> T helper (T<sub>H</sub>) cell and amplified antibody responses<sup>16,20,47</sup>. Although Casp11<sup>−/−</sup> Casp1<sup>−/−</sup> mice were used in those studies<sup>16</sup>, the present work indicated that these triggers did not activate caspase-1 in the absence of caspase-11, pointing to a role for NC-activation of the NLRP3 inflammasome in mediated the T<sub>H</sub> cell and antibody responses in vivo. Our findings provide a rationale for creating combination adjuvants based on LPS and mRNA<sub>bac</sub> to synergistically enhance immune responses that benefit from inflammasome activation<sup>12,20,47,48</sup>. While more work is necessary to extend our findings to human caspases 4 and -5, the establishment of mRNA<sub>bac</sub> and LPS as a combinatorial trigger for caspase-11 activation should inform much needed therapies for Gram-negative sepsis<sup>49</sup>.

**Online content**

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41590-022-01192-4.
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Methods

Mice. C57BL/6j mice were from The Jackson Laboratory and bred inhouse. Nlpφ3- and PyCARD- mice were from R. Flavell, Casp1- and Casp11- mice from T.D. Kanneganti (MTA with Genentech), and females from Gsmd mice from K. Fitzgerald for 12-week-old male and female mice were used randomly. Animal procedures were approved by the Icahn School of Medicine at Mount Sinai (ISMSM) and Weill Cornell Medicine (WCM) IACUC in accordance with the ‘Guide for the Care and Use of Laboratory Animals’ (NIH publication 86–23, revised 1985).

Bone marrow-derived macrophages. Bone marrow progenitors harvested from femurs of mice were differentiated into macrophages over 7–10 days in RPMI 1640 with M-CSF (conditioned medium from I.292 cell line) and 10% fetal bovine serum, plus 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM l-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 1% MEM nonessential amino acids, and 55 μM β-mercaptoethanol (Sigma).

Bacterial strains. E. coli K12, DH5α was from Invitrogen. Naturally occurring thyminidine auxotrophs (thyA-) were selected and E. coli RFP thyA- were generated as described. E. coli (Migula) Castellani and Chalmers strain (pathogenic E. coli serovar O1:H7), ATCC 17775. E. coli CM300 (ref. 21) (E. coli mcr3579) was provided by V. Dixit. All E. coli strains were grown in Luria-Bertani broth (LB), supplemented with 500 μg/ml trimethoprim and 50 μg/ml trimethoprim (Sigma) when thyB-.

Y. pestis KIM derivative 19 (D19), BEI Resources (NR-6681) was grown in Tryptic Soy Broth at 24 or 37°C. Y. pestis KIM D19 and E. coli ST19 and Y. pestis KIM D19 or E. coli ST19 were imported to the Blander laboratory with permits from the US Department of Agriculture. I.M. was tested for circulating iron levels before work with Y. pestis KIM D19, L. innocua Selieger (ATCC 33091)22 was grown in BH broth (Gibco). S. aureusOkla25, lacking global regulators Sar (Staphylococcal accessory regulator) and Agr (Accessory gene regulator) were from L. Stuart and grown in BH broth (Gibco). Salmonella enterica serovar typhimurium SL1344 WT and Salmonella enterica serovar typhimurium Δspir2, lacking T3SS, ATCC, were grown in LB broth. Shigella flexneri WT and BS103 (virulence plasmid-cured strain) were from M. Goldberg and grown in Terrific Broth (TB). Work with all strains of bacteria was approved by the Institutional Biosafety and EHS of ISMSM and WCM.

E. coli cultured in 2 μg/ml rifampicin (Sigma) were washed three times in PBS, serial dilutions plated on LB agar containing 500 μg/ml trimethoprim and 50 μg/ml trimethoprim (Sigma), and colony counts determined. For heat-killing, bacteria were grown to mid-log phase, washed, resuspended in PBS and incubated at 60°C for 2h. E. coli RFP (E. coli mCherry) were killed by treatment with 50 μg/ml Gentamicin sulfate at 37°C shaking for 12–16h. Killing confirmed by plating on LB agar.

Stimulation of macrophages. At 12–16h before stimulation with bacteria, bone marrow-derived macrophages (hereafter ‘macrophages’) were replated as follows: 5×10^4 cytokines or LPS or LPS, 5×10^4 antibiotics (cocktail, 1 μg/ml each), and RNA expression vectors (2 μg/ml each). Macrophages were transfected with 1 μg/ml plasmid DNA (Complete Protein Transfection Solution, Promega). ASC oligomers were detected at the expected molecular weight (monomer (~22 kDa), dimer (~45 kDa) and polymers (above ~55 kDa)) and impaired in ASC-deficient macrophages validating assay specificity.

For priming before stimulation or transfection, macrophages were treated with Lipofectamine 2000 (ThermoFisher). For 1 well of a 24-well plate, 1.5 μl Lipofectamine per well was diluted in 50 μl opti-MEM medium (Gibco) to transfect indicated concentrations of LPS (E. coli 055:BS, Sigma), ultrapure LPS (E. coli 0111:B4, Invivogen) or RNA Bac. For indication, when transfected with 2 ng/ml LPS, 1 well of a 24-well plate containing 500,000 macrophages plated at 250,000 macrophages 200 μl−1 cm2 received 0.8 ng LPS, while 1 well of a 6-well plate received 4 ng LPS. When transfected with 1 μg/ml LPS, the same number of cells received respectively 400 ng and 2 μg LPS.

The 293T cells were transfected using JetPrime (Polyplus Transfection). Transfections were according to manufacturer’s instructions. For a 1 cm² plate, 10 μg DNA was diluted in 500 μl JetPrime buffer before adding 20 μl JetPrime Reagent.

Preparation of RNA Bac. RNA Bac was isolated from mid-log phase growing bacteria using RNAeasy prep kits (Qiagen) following the manufacturer’s instructions. To enrich mRNA Bac, ribosomal 16S and 23S RNA (rRNA) were removed by magnetic bead-based capture hybridization using MICROBEx kit (Ambion/Applied Biosystems). Remaining mRNA was purified using RNeasy miniprep (Qiagen) following the RNA cleanup protocol. RNA concentration and purity were determined by nanodrop measuring 260/280 nm and 260/230 nm absorbance ratios, and RNA integrity by 1% agarose gel electrophoresis.

Immunoblot. Macrophages were washed in cold PBS, then lysed in 50 mM Tris-HCl (pH 7.9), 300 mM NaCl, 1% Triton X-100, supplemented with protease and phosphatase inhibitor cocktails (Complete Protein Transfection Solution, Promega). WCE were centrifuged at 3,640 g, 10 min at 4°C. Protein concentrations were determined using the Bradford method. Samples were denatured in Laemmli sample buffer for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) resolution. Proteins were transferred onto a polyvinylidenedifluoride membrane (Millipore). All immunoblots represent at least three independent experiments. All blots were probed for β-actin as a loading control.

Concentration of supernatants using TCA precipitation. Proteins of macrophage supernatants (prepared in serum-free Opti-MEM) were concentrated using trichloroacetic acid (TCA) precipitation and washed in cold acetone. Precipitated proteins were resuspended and denatured in 50 μl 2x Laemmli buffer for SDS-PAGE resolution on 12% polyacrylamide gels. All immunoblots represent at least three independent experiments. All blots were probed for β-actin as a loading control.

Concentration of supernatants using TCA precipitation. Proteins of macrophage supernatants (prepared in serum-free Opti-MEM) were concentrated using trichloroacetic acid (TCA) precipitation and washed in cold acetone. Precipitated proteins were resuspended and denatured in 50 μl 2x Laemmli buffer for SDS-PAGE resolution on 12% polyacrylamide gels. All immunoblots represent at least three independent experiments. All blots were probed for β-actin as a loading control.

ASC oligomerization. Following a published protocol, macrophages were resuspended in hypotonic buffer (20 mM HEPES, 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, with protease inhibitor cocktail) and lysed mechanically by passing through a 27G needle 20 times. Intact cells, debris and nuclei were removed by centrifugation at 340g for 8min. Supernatant containing soluble and insoluble proteins was collected and mixed 1:1 (volume) with CHAPS buffer (20 mM HEPES, 5 mM MgCl2, 0.5 mM EGTA 0.1% CHAPS, protease inhibitors). ASC oligomers were pelleted by centrifugation at 2,650g for 8 min and cross-linked in CHAPS buffer containing 2x SDS (ThermoScientific) for 30 min at room temperature before centrifugation at 2,650g for 8 min. Pellets were resuspended in 2x Laemmli buffer and denatured 5 min at 95°C. Cross-linked fractions were resolved by SDS-PAGE on 12% polyacrylamide gels. Detection of ASC oligomers was performed initially using the anti-ASC-N-15 antibody (sc-22514-R, Santa Cruz technology), which was obtained via a collaborative effort. Later experiments used anti-ASC AL177 (AG-25B-0006-C100, Adipogen). ASC oligomers were detected at the expected molecular weight (monomer (~22 kDa), dimer (~45 kDa) and polymers (above ~45 kDa)) and impaired in ASC-deficient macrophages validating assay specificity.

Immunoprecipitation. For IP of endogenous NLPR3 and AIM2, macrophages were harvested, washed in cold PBS, lysed in 50 mM Tris-HCl (pH 7.9), 130 mM NaCl, 0.25% Triton X-100 supplemented with protease and phosphatase inhibitor cocktails (Complete Protease and Phosstop, Roche), and IP performed in the same
buffer. Alternatively, for IP of endogenous caspase-11, lysis and IP were performed in 50 mM Tris-HCl (pH 7.9), 300 mM NaCl and 0.5% Triton X-100 supplemented with protease inhibitor and phosphatase inhibitor cocktails.

For immunoprecipitation of overexpressed NLPR3 and Caspase-11, 24-h-transfected 293T cells were lysed in 50 mM Tris-HCl (pH 7.9), 300 mM NaCl, 1% Triton X-100 supplemented with protease and phosphatase inhibitor cocktails. For all IP conditions, portions of cleared WCE were preadsorbed on Protein G-agarose beads (Sigma) for 30 min before incubation with anti-caspase-11, anti-NLPR3, anti-AIM2, anti-HA or anti-FLAG M2 antibodies for 2 h at 4 °C in the respective lysis/IP buffer, and final binding on Protein G-agarose beads for 30 min. After three washes in the corresponding lysis/IP buffer, beads pellets were resuspended and denatured in 40 µL 2x Laemmli buffer before SDS-PAGE of both immunoprecipitates and WCE. Alternatively, for anti-FLAG M2 immunoprecipitates, elution was performed using 100 µg·mL⁻¹ 3x FLAG peptide (Sigma, catalog no. F4799) in lysis/IP buffer for 30 min before denaturation in Laemmli buffer.

**Pulldown of active caspases.** Biotinylated zV-AMF (5 µg, Cayman Chemical, catalog no. 1135608-15-1) was applied to macrophages 2 h before bacterial stimulation or transfection. For zV-AMF FMK pulldown of entire macrophage output (WCE plus supernatant), cells were lysed directly by adding 1% IGEPAL CA630 to the cell medium. Mixed lysates were incubated on ice for 5 min in the presence of protease and phosphatase inhibitors. Pulldown was performed using Neutravidin agaroze beads (ThermoScientific, catalog no. 29200). Beads were washed five times in 50 mM Tris-HCl (pH 7.9), 300 mM NaCl, 1% IGEPAL CA630 buffer before resuspension in 2xLaemmli 2X and denaturation 10 min at 95 °C.

**In vitro caspase activity assay.** Immunoprecipitation of endogenous caspase-11 was performed as described in Immunoprecipitation. Hydrolysis of fluorogenic substrate zV-AMC by immunoprecipitates was performed by mixing 50 µL of immunoprecipitates with 50 µl assay buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 3 mM EDTA, 0.05% Tween-20 and 10 mM DTT). zV-AMC was added to the reaction at a final concentration of 75 nM and hydrolysis was calculated by measuring fluorescence intensity of free AMC hydrolyzed from zV-AMC (excitation 450 nm, emission 365 nm) on a SpectraMax DX3 fluorimeter.

**Measurement of cytosolic LPS.** Macrophages were washed several times in cold PBS with gentle tration wash PBS, followed by stimulation with E. coli to co-localize extracellular LPS or bacteria. WCE were prepared and LPS levels were determined using a Limulus Amebocyte Lysate (LAL) pyrochrome detection kit (Associates of Cape Cod Incorporated), using Glucashield reconstitution buffer to overcome interference from (1-3)-β-glucan.

**Preparation of cytosolic and residual fractions.** Subcellular fractionation of macrophages was conducted by a digestion-based method as described, with modifications. 2 h after stimulation or transfection, macrophages were washed five times for 5 min each in cold PBS with gentle shaking at 4 °C to remove extracellular bacteria and LPS. Plated cells were treated with 0.05% digitonin buffer for 10 min and supernatant containing of cytosol was collected. The residual cell fraction (containing cell membrane, organelles and nucleus) was collected in 0.1% CHAPS buffer. Cytosol and residual fractions were used for LPS quantification via LAL assay or for reverse transcription–quantitative PCR (RT–qPCR) quantification of RNAbac. Fractions were additionally immunoblotted for organelle and cytosolic markers to validate purity of cytosolic fractions.

**Detection of RNAbac using Pepper RNA-Ideg system.** We used Pepper RNA-Ideg for imaging RNAbac in cells. Pepper RNA binds to and prevents RNA synthesis. We targeted the 3′ end of cytoplasmic RNAs encoding for ectopic mCherry or endogenous GroEL, both labeled with Alexa Fluor 647. Additional staining for LAMP1 protein or LPS was performed before the probe hybridization according to the kit manual. Fluorescence was analyzed by confocal microscopy.

**Quantification of RNAbac by RT–qPCR.** At 2 h after macrophage stimulation, cytosolic fractions were prepared and RNAs extracted using RNeasy mini kit. RNAs were treated with TURBO DNase (ThermoFisher), and ‘no RT’ qPCR controls were run together with RNAase-free conditions. Reverse transcription was performed using SuperScript III Reverse Transcriptase (ThermoFisher) and qPCR using SYBR Green PCR Master Mix (ThermoFisher) for bacterial genes: groES (forward (F) primer: ACTAATCTGTGGCAGGCGAT; reverse (R) primer: AAATCTTCATACCCAGGCGG), groEL (F primer: CGTGGGTC ATGCTTGGCCAC; R primer: AAGGCCGTCACTCATCTCCCCG), R primer: ATCTCCGGCTCAGGAGTA) and dnaE (F primer: CGCTTGGGACAATTCGGT; reverse primer: ACCGTCTCCG AACAATGG). For gapdh for gpdh (F primer: AGGTGCTGTGTTACGACGGTT; R primer: TGTTAACCAGATGTTGAGCTGA) was used as eukaryotic control and amplification of bacterial genes was reported to gdh.

**Cytokine enzyme-linked immunosorbent assay.** Supernatants from cultured macrophages were collected 24 h poststimulation or at indicated times. Enzyme-linked immunosorbent assay (ELISA) capture/detection antibodies used were: IL-6, MP-20-3F3/MP-32C11 (BDPharmingen); IL-1β, B2/ rabbit polyclonal antibody (e Bioscence); TNFα 14-7423-85 (Bioscence)/13-7341-85 (Invitrogen). All antibodies were 2 µg·mL⁻¹ capture and 0.5 µg·mL⁻¹ detection, except IL-6 capture, which was at 1 µg·mL⁻¹. Detection antibodies were biotinylated and labeled by streptavdin-conjugated horseradish peroxidase (HRP), and visualized by adding 3, 5, 5′-tetramethylbenzidine (TMB, KPL). Color development was stopped with TMB-Stop Solution (KPL). Recombinant cytokines served as standards (Peprotech). Absorvances at 450 nm were measured on a microplate reader (Molecular Devices). Cytokine supernatants were calculated by extrapolating absorbance values from standard curves where known concentrations were plotted against absorbance using SoftMax Pro 5 software.

**Measurement of inflammatory cell death.** Cell death of macrophages was measured using the Cytox96 cytotoxicity assay (Promega) following the manufacturer’s instructions. The assay measures the release of lactate dehydrogenase (LDH) into the supernatant calculated as the percentage of maximum LDH content, measured from total cellular lysates (100%).

**Measurement of cell death by SYTOX incorporation.** Macrophages were plated in 96-well plates and stimulated with bacteria as indicated in triplicates. At 1 h poststimulation or transfection, SYTOX red dead cell stain (Invitrogen) was added to the cell medium at a final concentration of 60 nM. Kinetics of SYTOX incorporation into macrophages was determined using Incucyte (Sartorius) for 74 h. Percentages of SYTOX− macrophages at each time point were calculated by reporting the amount of SYTOX− macrophages to the number of plated macrophages. Representative images were selected at different time points.

**Confocal microscopy.** Macrophages were plated on glass coverslips. After bacterial stimulation, coverslips were washed with PBS, fixed in 2% paraformaldehyde and quenched in 50 mM NH₄Cl. Cells were then permeabilized with 0.1% Triton X-100 in PBS before blocking with 10% FBS PBS and incubation with appropriate antibodies prepared in 10% FBS PBS. When indicated, Phalloidin Alexa 647 was used together with secondary fluorescent antibodies to stain Actin and delineate the cell area. Finally, cells were stained with 4,6-diamidino-2-phenylindole (DAPI). Coverslips mounted with ProLong Diamond Antifade Mountant (Invitrogen). Images acquired on a Leica SP5 DM microscope with a x63/1.4 numerical aperture oil immersion objective. The partial nuclear staining pattern noted in Extended Data Fig. 4 has been reported and is ASC-specific given its absence in PyCARD− macrophages.

**Plasmids and constructs.** The plasmid encoding Casp11 (NM_007609.3) was purchased from E. Shah, Nipr3 cDNA clone (NM_145827.3) was purchased from OriGene. Full-length and truncated Casp11 or Nlrp3 were PCR-amplified from the corresponding plasmids and subcloned into vector pCDH-CMV-MCS-EF1-Puro between XbaI and BamHI sites for Casp11, or BstBI and NotI sites for Nlrp3. A 3xFLAG Tag and HA tag were introduced into the N-terminus of Casp11 and Nlrp3, respectively, by PCR. The Casp11 (C254A) mutation was generated using Agilent mutagenesis kit (catalog no. 200521)

**Antibodies.** The following antibodies were used for immunoblots: anti-β-Actin (Cell Signaling Technology, catalog no. 3700), anti-ERP72 (Cell Signaling Technology, catalog no. 5033), anti-S6K (Cell Signaling Technology, catalog no. 2441), anti-β-Tubulin (Cell Signaling Technology, catalog no. 2141), anti-FLAG (Cell Signaling Technology, catalog no. 14793), anti-Caspase-1 p20 (e Bioscence, catalog no. 14-9382-82), anti-ALM2 (1e Biosciences, catalog no. 4-6008-93), Santa Cruz Technologies anti-ASC (Santa Cruz Technologies, catalog no. sc-22514-R), anti-TGN38 (Santa Cruz Technologies, catalog no.sc-166594), anti-HistoneH1 (Santa Cruz Technologies, catalog no.s.c993338), anti-Caspase-11 (Sigma, catalog no. C1354), anti-AspergimmD (Sigma, catalog no. G7422, anti-FlagM2 (Sigma, catalog no. F3165), anti-NLRP3 (Adipogen, catalog no. AG-20B-0014-C100), anti-ASC (Adipogen, catalog no. AG-25B-0006-C100), anti-Phospho-p62 S403 (Millipore,
catalog no. MABC186), anti-IL-1β (Research & Development, catalog AF-401-NA), anti-HA (Roche, catalog no. 1185016001), anti-GasderminD (Abcam, catalog no. ab219880), anti-NK7 (Abcam, catalog no. ab133514), anti-Calreticulin (Abcam, catalog no. ab92516), anti-GM130 (Abcam, catalog no. ab52649), anti-CathepsinS (Abcam, catalog no. ab232740), anti-LPS (Abcam, catalog no. ab35654) and anti-LAMP1 (Biolegend, catalog no. 121602). For immunoprecipitation of HA-NLRP3, we used anti-HA (Cell Signaling, catalog no. 3724).

Statistical analysis. For statistical analyses of more than three group experiments, one-way analysis of variance (ANOVA) was performed followed by multiple comparisons Sidak tests to allow two-by-two comparisons. For experiments with two groups (Extended Data Figs. 1b and 4b), Student’s t-test was performed. Error bars were mean ± s.e.m.; ns indicated nonsignificant; P values and group sizes are indicated in the corresponding figures and legends. Analyses were conducted on biological replicates.

**Reporting Summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

Datasets generated or analyzed during this study are available on reasonable request. Source data are provided with this paper.

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**Author contributions**

J.M. and J.M.B. directed the study, designed experiments and wrote the manuscript. J.M. performed most experiments, data and statistical analyses and all macrophage stimulations. B.J. performed molecular biology, cloning, transfection and ViewRNA ISH related experiments. Z.H. conducted the experiments related to measuring RNA_m in cytosolic extracts and kinetics of macrophage cell death. S.R. performed experiments related to Fig. 1cf and the dual LPS and RNA_m requirement for noncanonical NLRP3 inflammasome activation during early stages of the work. M.S. conducted contolocal microscopy lysosomal localization of bacteria and quantification. H.Y. performed experiments related to Extended Data Fig. 8c. J.W. and S.R.J. provided conceptual advice.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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**Correspondence and requests for materials** should be addressed to J. Magarian Blander.

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Extended Data Fig. 1 | Bacterial mRNA and stimulatory LPS are both required for caspase-11 and noncanonical inflammasome activation.

**a**, Immunofluorescence confocal microscopy on bone marrow derived macrophages (hereafter ‘macrophages’) 2 h post stimulation with L or HK E. coli or untreated. **b**, Bar graph, % cells with LAMP1-LPS colocalization. **c**, Immunoblots of macrophage concentrated supernatants (20 h) or WCE (6 h), and cytokine concentrations and LDH release in culture supernatants (20 h) post stimulation with L, HK or HK E. coli supplemented with 100 ng ml–1 mRNA isolated from E. coli or eukaryotic cells. LDH measured by cytotoxicity assay; IL-1β, TNF and IL-6 by ELISA. Error bars, mean ± s.e.m. t-test was performed in **b** (n = 3). ns: nonsignificant. Bacteria:macrophage=20:1. Results represent at least three independent experiments.
Extended Data Fig. 2 | See next page for caption.
Extended Data Fig. 2 | Cytosolic levels of LPS and bacterial mRNA after macrophage stimulation with live or heat-killed bacteria. a, Measurements of LPS in the cytosolic (C) or residual (R) fractions prepared from macrophages 2 hr post-transfection of indicated doses of LPS alone, co-transfection of 2 ng ml⁻¹ LPS with 100 ng ml⁻¹ E. coli<sup>LPSmut</sup> mRNA, or stimulation with L or HK E. coli or virulent E. coli as indicated. LPS was measured via Limulus Amebocyte Lysate method. b, Immunoblots of cytosolic (C) and residual (R) fractions from macrophages 2 h post stimulation with L E. coli, HK E. coli or L virulent E. coli, and probed with marker antibodies for indicated subcellular compartments. c, RT-qPCR for the bacterial gene groES, groEL, era and dnaE on cytosolic fractions prepared 2 h post macrophage transfection with indicated doses of ultrapure LPS and mRNA prepared from E. coli, or stimulation with L, HK or HK E. coli or virulent E. coli supplemented with mRNA prepared from each bacterium. d,e, mNeonGreen fluorescence measurements on total extracts (d) or cytosolic versus residual fractions (e) of macrophages stably expressing tDeg-tagged mNeonGreen 2 h post stimulation with E. coli expressing or not Pepper RNA, or after treatment with proteasome inhibitor MG132 as indicated. By expressing a Pepper RNA-regulated fluorogenic protein (mNeonGreen-tDeg) in the cytosol of macrophages, we noted 1.5-2-fold increase in mNeonGreen fluorescence following phagocytosis of recombinant Pepper RNA-expressing E. coli compared to the 2-2.5-fold increase with the proteasomal inhibitor MG132, indicating cytosolic access of Pepper RNA derived from phagocytosed E. coli bound to and stabilized the fluorescent protein in the cytosol of macrophages. f,h, Confocal microscopy of direct fluorescence RNA in situ hybridization (ViewRNA ISH) to detect two RNA<sub>out</sub> transcripts encoding for either mCherry (f) or endogenous GroES (h) from recombinant mCherry E. coli showed significantly more probe signal in macrophages at 6 h post-phagocytosis of live (L) compared with killed (K) bacteria. Killed bacteria were visualized by anti-LPS staining due to loss of mCherry fluorescence. RNA probe signal localized with live bacteria in lysosomes labeled with LAMP-1 as expected, but almost half of this signal did not colocalize to these bacteria suggesting cytosolic access. Inserts show magnification of indicated area. g,i, Bar graphs show quantification of probe signal in (f) and (h), respectively. Bacteria:macrophage=20:1. Error bars, mean ± s.e.m. One-way ANOVA followed by multiple comparisons Sidak tests and p values are indicated in bar graphs in g (Total number, Untreated: n = 13, L: n = 19, K: n = 15, K(LPS): n = 17 - Not colocalized, Untreated: n = 11, L, K: n = 14) and i (Total number, Untreated: n = 11, L: n = 14, K, K(LPS): n = 10 - Not colocalized, n = 10). Results represent at least three independent experiments.
Extended Data Fig. 3 | Bacterial mRNA and LPS are both required for IL-1β secretion and generation of the active form of caspase-11. a, Cytokine concentrations and LDH release in culture supernatants (20 h) post-transfection of WT or Nlrp3−/− macrophages as indicated with 2 ng ml⁻¹ ultrapure LPS and/or 10, 30 and 100 ng ml⁻¹ of mRNA prepared from E. coli LPSmut, L. innocua, or in vitro transcribed (IVT). b, Immunoblots of macrophage concentrated supernatants, WCE, mixed concentrated supernatants and WCE, or pulldown of caspases with biotinylated zVAD-FMK from WT, Nlrp3−/− or Casp11−/− macrophages 20 h post stimulation with L, HK or HK E. coli supplemented with E. coli total RNA (RNAtot), or transfection with indicated doses of ultrapure LPS and E. coli RNARNAtot. c, In vitro zVAD-AMC fluorescence post-incubation with immunoprecipitates of endogenous caspase-11 from macrophages stimulated for 6 h or 12 h as indicated with L, HK, or HK E. coli supplemented with E. coli RNARNAtot (10 µg ml⁻¹). No IgG served as a control for Protein G-bound proteins alone. Bacteria:macrophage=20:1. Error bars, mean ± s.e.m. Results represent at least three independent experiments.
Extended Data Fig. 4 | LPS is required alongside bacterial mRNA for assembly of the NLRP3 inflammasome and regardless of LPS-stimulatory activity.

a–c, Immunofluorescence confocal microscopy on macrophages 16 h post stimulation of indicated macrophage genotypes with L, gentamicin-killed (K) or K red fluorescent protein (RFP) expressing recombinant E. coli supplemented with E. coli RNA

Notes, the partial nuclear staining pattern has previously been observed (see Methods) and appears to be a specific signal given its absence in PyCARD

In a,b, insets show magnification of indicated areas. White arrowheads point to ASC specks. Phalloidin delineates the macrophage actin cytoskeleton. Scale bar = 10 µm. Bar graphs, % cells exhibiting ASC specks. Error bars, mean ± s.e.m. t-test was performed in b (n = 5). One-way ANOVA followed by multiple comparisons Sidak tests were performed in a (n = 5) and c (n = 6). P values are indicated in bar graphs. Bacteria:macrophage = 20:1. Results represent at least three independent experiments.
Extended Data Fig. 5 | See next page for caption.
Extended Data Fig. 5 | Kinetics of noncanonical activation of the NLPR3 inflammasome in macrophages in response to virulent and avirulent bacteria.

**a.** LDH released in culture supernatants of macrophages at indicated time points post-stimulation with Live *E. coli* (avirulent), virulent *E. coli* or virulent *Salmonella*. LDH measured by cytotoxicity assay. **b,c.** Kinetics of SYTOX Red incorporation over 72 h in macrophages stimulated with Live *E. coli* or virulent *E. coli*, *Salmonella ΔSpi1/2* (avirulent) or wild type (WT) (virulent), or *Shigella BS103* (avirulent) or WT (virulent) (**b**), and representative images of SYTOX Red incorporation at selected time points (**c**). Peaks of SYTOX incorporation occurred faster (blue boxes) in response to virulent bacteria, before decreasing likely due to destruction of cell structure, while macrophages stimulated with avirulent bacteria were still incorporating SYTOX and peaked later (orange boxes). Scale bar=150 μm. **d.** Counting of live macrophages at the indicated time points post stimulation with Live *E. coli* or virulent *E. coli*. Counts were normalized to the unstimulated macrophages for each time point. **e.** Immunoblots of macrophage WCE at the indicated time points post stimulation with Live *E. coli*, virulent *E. coli* or virulent *Salmonella*. **f.** Immunoblots of macrophage concentrated supernatants 6 h post stimulation with Live *E. coli* or virulent *E. coli*. Error bars, mean ± s.e.m. Bacteria:macrophage =20:1 for *E. coli* and virulent *E. coli*, 5:1 for *Salmonella* and *Shigella* strains. Results represent at least three independent experiments.
Extended Data Fig. 6 | NLRP3 stimuli specifically couple with LPS in mediating noncanonical activation of the NLRP3 inflammasome. **a,b,** Immunoblots of macrophage concentrated supernatants (20 h), WCE (6 h), or cross-linked fractions (16 h), and cytokine concentrations in culture supernatants (20 h) as indicated post stimulation with L, HK or HK E. coli supplemented with indicated doses of RNA_{tot} or Nigericin (**a**), and post-treatment with Nigericin or transfection of indicated doses of poly(dA:dT) or Flagellin with or without prior priming with 100 ng ml⁻¹ LPS for 12-16 h (**b**). IL-1β and IL-6 by ELISA. Error bars, mean ± s.e.m.
Extended Data Fig. 7 | dTGN colocalization and biochemical pro-caspase-11–NLRP3 interaction. a,b, Immunofluorescence confocal microscopy 16 h post stimulation of WT macrophages with L or HK virulent E. coli (a), and WT, Casp11–/– and Nlrp3–/– macrophages as indicated with L E coli (b). Scale bar=10 µm. In (a), side micrograph insets in the triple merges show magnification of the indicated areas. c,d, Immunoprecipitation (IP) of endogenous caspase-11 or NLRP3 as indicated, and immunoblotting for co-immunoprecipitating proteins and WCE proteins (labels to left of immunoblot panels) from macrophages stimulated 12 h with L, HK or HK E. coli supplemented with indicated dose of E. coli RNA_{tot} (c) and L, HK or HK E. coli supplemented with indicated dose of E. coli RNA_{tot} or Nigericin, E. coli RNA_{tot} alone or Nigericin alone (d). Bacteria:macrophage=20:1. Results represent at least three independent experiments.
Extended Data Fig. 8 | GSDMD is important for noncanonical activation of the NLRP3 inflammasome in response to Gram-negative bacteria. a–c, Immunoblots of macrophage concentrated supernatants (20 h) or WCE at 6 h (a,b) and 20 h (c) post stimulation of WT and Gsdmd−/− macrophages with L or HK E. coli (avirulent) or virulent E. coli as indicated. (d) Immunoblots of macrophage concentrated supernatants (20 h) or WCE (6 h) post stimulation of macrophages with L or HK E. coli with or without zVAD-FMK treatment.
Extended Data Fig. 9 | See next page for caption.
Extended Data Fig. 9 | Model for noncanonical activation of the NLRP3 inflammasome by live Gram-negative bacteria. Following phagocytosis of live Gram-negative bacteria, two classes of PAMPs are exposed to cytoplasmic pattern recognition receptors: the classical PAMP LPS, shared by live and killed bacteria, and the vita-PAMP bacterial mRNA (mRNA$_{bac}$), present only in live bacteria. Coincident detection of mRNA$_{bac}$ and LPS from virulent or avirulent Gram-negative bacteria alike promotes a physical and mutually exclusive interaction between NLRP3 and the intracellular LPS receptor pro-caspase-11. This interaction localizes to the dispersed Trans-Golgi Network (TGN) and is mediated through the pro-caspase-11 SCAF domain and the LRR and PYD domains of NLRP3. The interaction of NLRP3 and pro-caspase-11 is upstream of their activation: It does not require the ability of LPS to activate caspase-11 and can still occur in the absence of GSDMD. It also does not require ASC and caspase-1 which are important for NLRP3 activation. Besides their interaction, NLRP3 and pro-caspase-11 are reciprocally required for their function: LPS binding to but not activation of pro-caspase-11, is necessary for mRNA$_{bac}$-mediated NLRP3 inflammasome assembly. Reciprocally, NLRP3 and ASC but not caspase-1 are required for pro-caspase-11 activation, indicating the necessity for ‘nascent’ NLRP3 inflammasome assembly upon sensing the viability of Gram-negative bacteria (detection of mRNA$_{bac}$) and irrespective of bacterial virulence factor expression. Although NLRP3-ASC oligomers can form in the absence of pro-caspase-1, these oligomers are unstable indicating stabilization of the ‘nascent’ assembled NLRP3 inflammasome upon pro-caspase-1 recruitment. Furthermore, higher concentrations of intracellular LPS, likely due to virulence factor activity during infection with virulent cell-invasive Gram-negative bacteria, trigger faster kinetics of plasma membrane permeabilization/pyroptosis compared with avirulent bacteria, and independently of NLRP3 and ASC$^{9}$. Collectively, the model that emerges demonstrates two modes of pro-caspase-11 activation by LPS, a fast NLRP3-independent mode triggered by the concurrent expression of bacterial virulence factors, and a slower NLRP3-dependent mode triggered by coincident detection of the vita-PAMP mRNA$_{bac}$ that signifies bacterial viability. (SCAF, scaffold; PYD, Pyrin; LRR, leucine rich repeat).
Extended Data Fig. 10 | See next page for caption.
Extended Data Fig. 10 | Characterization and mapping of the pro-caspase-11 and NLRP3 interaction. 

a, Schematic indicating the different truncated mutants of NLRP3 used for co-immunoprecipitation experiments in 293 T cells. All forms were fused to 3xHA tag in N-Terminus.

b, Schematic indicating the different truncated mutants of casp11C254A used for co-immunoprecipitation experiments in 293 T cells. All forms were fused to 3xFLAG tag in N-Terminus.

c, Immunoprecipitation and immunoblotting for coimmunoprecipitating and WCE proteins of overexpressed FLAG-caspase-11 either casp11C254A FL, Casp-11 C254A ΔCARD or Casp-11 C254A ΔSCAF with or without HA-NLRP3FL in 293 T cells 24 h post-transfection. To equilibrate the levels of FLAG-caspase-11 mutants in the immunoprecipitates, 4 times more protein extracts were submitted to anti-FLAG immunoprecipitation when FLAG-casp11C254A ΔSCAF was expressed (labelled 4X) compared to when either FLAG-casp11C254A FL or FLAG-casp11C254A ΔCARD were expressed (labelled 1X). Therefore, note that all proteins from FLAG-casp11C254A ΔSCAF samples (including HA-NLRP3FL) were 4 times more abundant during anti-FLAG immunoprecipitation, yet HA-NLRP3FL was still much less co-immunoprecipitated with FLAG-casp11C254A ΔSCAF compared to FLAG-casp11C254A FL. Immunoblotted proteins are indicated to left of each immunoblot panel.

d, Schematic representation of CARD, DED, CASPASE p20 and CASPASE p10 domains of murine inflammatory and apoptotic caspases. SCAF domain is composed of CASPASE p20 and CASPASE p10 domains. All caspases, with the exception of the short forms of mouse and human caspase-12, have SCAF domains. The alignment E values were calculated using the NCBI alignment tool.

e, Similarity coefficients between caspase-11 and other murine caspases. The alignment diagram was generated using the alignment module of SnapGene software.

f, Protein sequence alignment for murine caspases. The alignment diagram was generated using the alignment module of SnapGene software.

g, Schematic indicating the different caspase-11C254A and caspase-9 chimeras used for co-immunoprecipitation experiments in 293 T cells. All forms were fused to 3xFLAG tag in N-terminus.
Reporting Summary

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- [ ] For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- [ ] For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

- [ ] Data collection: SoftMaxPro 5 was used to collect ELISA/LDH data.
- [ ] Data analysis: For statistical data analysis, GraphPad Prism 9 was used.

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A data availability statement is included. Accession codes and clinical data sets are not applicable. Datasets generated during this study are available upon request.
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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

- Sample size: No sample sizing was performed for experiments using primary murine bonne marrow-derived macrophages.
- Data exclusions: No data was excluded.
- Replication: Experiments/experimental conditions have been replicated at least 3 times to confirm the biological conclusions.
- Randomization: No randomization was used.
- Blinding: Most experiments on primary murine bone marrow-derived macrophages or cell lines could not be blinded as the same experimenter performed all experimental steps, data collection and analysis.

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We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

| Materials & experimental systems | Methods |
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| n/a | n/a |
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| [ ] Antibodies | [ ] Flow cytometry |
| [ ] Eukaryotic cell lines | | |
| [ ] Palaeontology and archaeology | [ ] MRI-based neuroimaging |
| [ ] Animals and other organisms | |
| [ ] Human research participants | |
| [ ] Clinical data | |
| [ ] Dual use research of concern | |

Antibodies

Antibodies used

Antibodies for Western Blot were obtained from: 1) Cell Signaling Technology: anti-beta-Actin (3700), anti-ERF2 (5033), anti-S6K (2708), anti-alpha-Tubulin (3873), anti-FLAG [14793]. 2) From eBioscience: anti-Caspase-1 p20 (14-9832-82), anti-AIM2 (14-6008-93). 3) From Santa Cruz Technologies: anti-ASC (sc-22514-R), anti-TGN38 (sc-166594), anti-HistoneH2 (sc393358). 5) From Sigma Aldrich: anti-Caspase-11 (C1354), anti-GasderminD (G7422), anti-FlagM2 (F3165). 6) From Adipogen: anti-NLRP3 (AG-20B-0014-C100), anti-ASC (AG-25B-0006-C100). 7) From Millipore: anti-Phospho-p62 S403 (MABC186). 8) From Research & Development: anti-IL-1beta(AF-401-NA). 9) From Roche: anti-HA (11815016001). 10) From Abcam: anti-GasderminD (ab219800), anti-NEK7 (ab133514), anti-Calreticulin (ab92516), anti-GM130 (ab52649), anti-CathepsinS (ab232740), anti-LPS (ab35654). 11) From Biolegend: anti-LAM1 (121602). For immunoprecipitation of HA-NLRP3, anti-HA was obtained from Cell Signaling (3724). For more information and details, please refer to Online Methods.

Validation

All antibodies were validated based on the manufacturer information available for each reference cited above. Several antibodies were also validated on cells from mice deficient [knock-out] for the molecule the antibody is against.

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)

293T cells

Authentication

Purchased from ATCC and multiple aliquots labeled with date and name, and cryo-preserved. Upon thawing, cells were passed or fed at least twice a week and maintained in a 37°C/5% CO2 incubator. Cells were maintained between 10% and 90% confluency. Cells were not allowed to reach confluency.
Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals
CS7BL/6j mice were purchased from The Jackson Laboratory and bred in-house. Nlrp3 \(-/-\) and Pycard (ASC)-/- mice were obtained from Richard Flavell (Yale University). Caspase1 \(-/-\) and Caspase11 \(-/-\) mice were obtained from Thirumala D. Kanneganti (St Jude Hospital) under an MTA with Genentech. Femurs from GSDMD \(-/-\) mice were obtained from Kate Fitzgerald (UMass). For generation of bone-marrow derived macrophages, 6 to 12 weeks old male and female mice were randomly used. Animals were maintained in the Weill Cornell Medicine Research Animal Resource Center in standard housing SPF cages kept at ambient temperatures with 12 hr dark/light cycles.

Wild animals
No wild animals used

Field-collected samples
No field-collected samples

Ethics oversight
All experiments with mice were approved by the institutional animal care and use committees of both the Icahn School of Medicine at Mount Sinai and Weill Cornell Medicine, and carried out in accordance with the 'Guide for the Care and Use of Laboratory Animals' (NIH publication 86–23, revised 1985).

Note that full information on the approval of the study protocol must also be provided in the manuscript.