Guanylate-binding proteins convert cytosolic bacteria into caspase-4 signaling platforms

Michal P. Wandel, Bae-Hoon Kim, Eui-Soon Park, Keith B. Boyle, Komal Nayak, Brice Lagrange, Adrian Herod, Thomas Henry, Matthias Zilbauer, John Rohde, John D. MacMicking and Felix Randow

Bacterial lipopolysaccharide triggers human caspase-4 (murine caspase-11) to cleave gasdermin-D and induce pyroptotic cell death. How lipopolysaccharide sequestered in the membranes of cytosol-invading bacteria activates caspases remains unknown. Here we show that in interferon-γ-stimulated cells guanylate-binding proteins (GBPs) assemble on the surface of Gram-negative bacteria into polyvalent signaling platforms required for activation of caspase-4. Caspase-4 activation is hierarchically controlled by GBPs; GBP1 initiates platform assembly, GBP2 and GBP4 control caspase-4 recruitment, and GBP3 governs caspase-4 activation. In response to cytosol-invading bacteria, activation of caspase-4 through the GBP platform is essential to induce gasdermin-D-dependent pyroptosis and processing of interleukin-18, thereby destroying the replicative niche for intracellular bacteria and alerting neighboring cells, respectively. Caspase-11 and GBPs epistatically protect mice against lethal bacterial challenge. Multiple antagonists of the pathway encoded by *Shigella* flexneri, a cytosol-adapted bacterium, provide compelling evolutionary evidence for the importance of the GBP-caspase-4 pathway in antibacterial defense.

Interferons promote human CASP4 (murine Casp11) responses through upregulation of gene expression, including GTPases of the guanylate-binding protein (GBP) and the immunity-related GTPases (IRGs) families, which protect in a cell-autonomous manner against infection with bacteria, parasites and viruses and promote the activation of human CASP4 (murine Casp11) upon transfection of LPS. GBPs were also reported to foster the rupture of *Salmonella*- (but not *Shigella*-) containing vacuoles, to recruit CASP4 to *Salmonella*-containing vacuoles and to impair the structural integrity of bacteria in murine cells through the downstream action of Irgb10 (ref. 20). However, neither pathogen-specific vacuolar rupture nor Irgb10-dependent bacterial attack sufficiently explains the role of GBPs in pyroptosis, particularly in human cells, which lack genes for most IRGB family members. Here we report that in cells stimulated with IFN-γ, GBPs assemble on the surface of *Salmonella* Typhimurium and *Shigella flexneri* into polyvalent signaling platforms required for the activation of CASP4. Platform assembly, CASP4 recruitment and CASP4 activation are controlled hierarchically by specific GBPs. The GBP-dependent transformation of the bacterial surface into a polyvalent caspase activation platform induces pyroptosis and processing of IL-18, thereby destroying the bacterial niche and alerting neighboring cells.

**Results**

IFN-γ prevents proliferation of cytosol-invading *Salmonella enterica* Typhimurium. The cytosol of mammalian cells is protected against bacterial invasion by antibacterial autophagy, resulting in heightened proliferation of S. Typhimurium in cells lacking essential

---

**Authors**

Michal P. Wandel, Bae-Hoon Kim, Eui-Soon Park, Keith B. Boyle, Komal Nayak, Brice Lagrange, Adrian Herod, Thomas Henry, Matthias Zilbauer, John Rohde, John D. MacMicking and Felix Randow

**Affiliations**

Division of Protein and Nucleic Acid Chemistry, MRC Laboratory of Molecular Biology, Cambridge, UK. Howard Hughes Medical Institute and Systems Biology Institute, Yale University, West Haven, CT, USA. Departments of Immunobiology and Microbial Pathogenesis, Yale University School of Medicine, New Haven, CT, USA. Department of Paediatrics, University of Cambridge, Cambridge, UK. Department of Paediatric Gastroenterology, Hepatology and Nutrition, Cambridge University Hospitals, Addenbrooke’s Hospital, Cambridge, UK. CIRI, Centre International de Recherche en Infectiologie, University of Lyon, Inserm U1111, Université Claude Bernard Lyon 1, CNRS, UMR5308, ENS de Lyon, University of Lyon, Lyon, France. Department of Microbiology and Immunology, Dalhousie University, Halifax, NS, Canada. Department of Medicine, University of Cambridge, Addenbrooke’s Hospital, Cambridge, UK.

**e-mail:** mwan@mlcb.cam.ac.uk; randow@mlcb.cam.ac.uk
Fig. 1 | IFN-γ prevents proliferation of cytosol-invading S. Typhimurium. a, b, Fold replication of S. Typhimurium in HeLa cells. Cells were treated with siRNAs (a) or cytokines (b) as indicated. c, d, Percentage of S. Typhimurium positive for YFP::galectin-8 at 1 h p.i. (c) and endogenous NDP52, ubiquitin (FK2 staining) or GFP::LC3C at 1 and 3 h p.i. in HeLa cells (d). n=100 (for 1 h p.i.), n=200 (for 3 h p.i.) bacteria per coverslip, in triplicate. e, g, Live microscopy of HeLa cells expressing CFP::galectin-8 and infected with S. Typhimurium in medium containing PI. Frames from Supplementary Video 2 (e), time between recruitment of galectin-8 to bacteria and nuclei of host cell becoming PI positive (f) and percentage of PI-positive nuclei among infected cells (g). Cells were imaged every 6 min for 6 h, 12 fields per condition. Blue arrowhead, point of interest. Time p.i. as indicated; scale bar, 10 μm (e). Median in red (f), h, Fold replication of S. Typhimurium in HeLa cells treated with DMSO, 50 μM NEC-1s or 10 μM NSA as indicated. i, Fold replication of S. Typhimurium in HeLa cells expressing FLAG-tagged GFP, Bcl2 or Bcl-xL as indicated. j, Fold replication of S. Typhimurium in HeLa cells expressing flag-tagged GFP or CrmA as indicated. k, Fold replication of S. Typhimurium in HeLa cells treated with DMSO or 50 μM Z-VAD-FMK as indicated. l, Percentage of FAM-VAD-FMK-positive cells among HeLa cells harboring S. Typhimurium positive for endogenous galectin-8 at 90 min p.i.; cells expressing FLAG::CrmA as indicated. n>100 cells with galectin-8-positive bacteria per coverslip, in triplicate. Statistical significance was assessed by two-tailed unpaired Student’s t-test (c), or one-way (g, i) or two-way (a, b, d, h–k) ANOVA with Tukey’s multiple comparisons test; NS, not significant; *P<0.05, **P<0.01 (exact P values are provided in Supplementary Table 1). Data are expressed as the mean±s.e.m. of three (a–d, h–l) or six (g) independent experiments, representative of six (e) independent experiments or pooled from three independent experiments (f). HeLa cells were treated with IFN-γ (e, f) or treated with IFN-γ as indicated (a, c, d, g–l). Bacteria were counted based on their ability to grow on agar plates (a, b, h–k). DMSO, dimethylsulfoxide; gal-8, galectin-8; S. T, S. typhimurium.
components of the pathway, such as the danger receptor galectin-8 (ref. 23), the autophagy cargo receptor NDP52 (refs. 24,25) or FIP200 (ref. 26), a subunit of the autophagy inducing ULK complex (Fig. 1a and Extended Data Fig. 1a). However, antibacterial autophagy provides only partial protection against bacterial proliferation (Fig. 1a and Supplementary Video 1). To investigate whether activated cells mount a stronger response against S. Typhimurium, we treated epithelial cells with interferons and pro-inflammatory cytokines. IFN-γ abrogated replication of S. Typhimurium, even in cells deficient in antibacterial autophagy (Fig. 1a). IFN-γ, TNF, IL-1β and IL-22 had no such effect (Fig. 1b). We conclude that IFN-γ, in an autophagy-independent manner, renders cells nonpermissive for S. Typhimurium proliferation.

Next, we investigated which step in the S. Typhimurium life cycle is antagonized by IFN-γ. IFN-γ-stimulated and unstimulated cells carried similar bacterial loads shortly after infection, suggesting that bacterial invasion of host cells proceeds unimpaired (Extended Data Fig. 1b). Vacular bacteria proliferated normally in IFN-γ-treated cells, as revealed by mutant S. Typhimurium (ΔargH, +inv), which colonize epithelial cells using the inv gene of Yersinia pseudotuberculosis but do not invade the cytosol due to a nonfunctional Spi1 needle apparatus23,24 (Extended Data Fig. 1c). Vacular escape is not controlled by IFN-γ either, as indicated by the unimpaired association of bacteria with proteins that detect bacterial entry into the cytosol (galectin-8, NDP52, ubiquitin, LC3C)24,25,31,32 (Fig. 1c,d and Extended Data Fig. 1d). However, at 3 h post infection (p.i.) the fraction of bacteria associated with NDP52, ubiquitin or LC3C was reduced in IFN-γ-stimulated cells. Taken together, our data reveal that the inhibitory effect of IFN-γ affects bacteria that have reached the host cytosol.

Cytosol-invading bacteria recruit CASP4 for GSDMD-dependent pyroptosis. To investigate the mode of IFN-γ action, we infected cells expressing CFP–galectin-8, a marker of endomembrane damage, in medium containing the membrane-impermeable DNA stain propidium iodide (PI). Indicative of cell death, PI entered cells shortly after the bacterial vacuole was permeabilized, as indicated by the accumulation of galectin-8 (Supplementary Video 2 and Fig. 1e,f). Only IFN-γ-treated cells containing galectin-8-positive bacteria accumulated PI (Fig. 1g). Under similar conditions plasma membranes did not stain with Annexin V (Extended Data Fig. 1e). We conclude that IFN-γ primes cells to execute cell death if S. Typhimurium enters their cytosol. Such heightened alertness towards cytosol-invading bacteria is reminiscent of the interferon-induced antiviral state.

The proliferation of S. Typhimurium in IFN-γ-treated cells was not rescued by treatment with the RIPK1 inhibitor NEC-1s or the MLKL inhibitor NSA, or by overexpression of Bcl2 or Bcl-XL, arguing against necroptosis and mitochondria-dependent apoptosis as cause of cell death (Fig. 1h,i and Extended Data Fig. 1f). In contrast, overexpression of the poxvirus caspase inhibitor CrmA or treatment with the pan-caspase inhibitor Z-VAD-FMK enabled bacterial proliferation in IFN-γ-stimulated cells (Fig. 1j,k). Moreover, FAM-VAD-FMK, a fluorescent reporter of caspase activity, accumulated post infection in IFN-γ-stimulated cells containing galectin-8-positive bacteria in a CrmA-inhibitable manner (Fig. II and Extended Data Fig. 1g). Importantly, caspase activity peaked in the proximity of galectin-8-positive bacteria, suggesting that bacteria attract active caspase or initiate caspase activation (Fig. 2a,b). When investigating which caspase accumulates around S. Typhimurium, we found that GFP-tagged CASP4 and, less frequently, CASP5 were recruited in a strictly IFN-γ-dependent manner (Fig. 2c,d and Extended Data Fig. 2a,b). The N-terminal CARD domains of CASP4 and, to a lesser extent, CASP5 were sufficient for recruitment to S. Typhimurium (Fig. 2e), while caspase activity was dispensable (Extended Data Fig. 2c). Endogenous CASP4 accumulated around galectin-8-positive S. Typhimurium in a strictly IFN-γ-dependent fashion (Fig. 2f and Extended Data Fig. 2d). IFN-γ-mediated upregulation of CASP4 is insufficient to cause CASP4 recruitment since even GFP::CASP4, overexpressed beyond levels of IFN-γ-induced endogenous CASP4, was only recruited in IFN-γ-treated cells (Fig. 2d,g), suggesting that an unknown IFN-γ-inducible factor is required for CASP4 recruitment. Depletion of CASP4 but not CASP1 or CASP5 diminished caspase activity in the cytosol of cells containing galectin-8-positive S. Typhimurium and on the bacterial surface (Fig. 2h and Extended Data Fig. 2e,f). CASP4 recruitment preceded cell death (Supplementary Video 3) and deletion or knockout of CASP4, but not other caspases, prevented cell death (Fig. 2i and Extended Data Fig. 2g,h) and rescued the proliferation of S. Typhimurium in IFN-γ-treated cells (Fig. 2j) and Extended Data Fig. 2i). In contrast, S. flexneri, an enterobacterium highly adapted to a cytosolic lifestyle, proliferated unimpaired in cells stimulated with IFN-γ (Fig. 2k). We therefore tested the importance of OspC3, a Shigella-encoded...
inhibitor of CASP4 (ref. 33), and found that within minutes after cytosolic entry bacteria lacking OspC3, but not OspC1, OspC2 or MxiE, a transcriptional regulator of host-induced gene expression, caused host cell death (Fig. 2l,m and Extended Data Fig. 3a,b). *S. flexneri ΔospC3 also triggered cell death in pri-

mmary human enterocytes stimulated with IFN-γ (Extended Data Fig. 3c,d). The proliferation of *S. flexneri ΔospC3 was restricted by IFN-γ (Fig. 2k and Extended Data Fig. 3e,f) in a manner dependent on CASP4 but not CASP1 or CASP5 (Fig. 2n and Extended Data Fig. 4a,b). Since transfected LPS triggers CASP4-mediated
Fig. 3 | GBPs recruit and activate CASP4 at the surface of cytosol-invading bacteria. 

a, Percentage of *S. Typhimurium* positive at 1h p.i. for the indicated GFP::GBP constructs expressed in HeLa cells. *n* > 100 bacteria per coverslip, in triplicate. 

b, Percentage of *S. Typhimurium* positive at 1h p.i. for the indicated GFP::GBP constructs expressed in HeLa cells treated with the indicated siRNAs. *n* > 100 bacteria per coverslip, in triplicate. 

c, Structured illumination micrograph of HeLa cells expressing GFP::GBP1 at 1h p.i. with *S. flexneri* indicated GFP::GBP constructs expressed in HeLa cells treated with the indicated siRNAs. 

d, Percentage of endogenous CASP4-positive bacteria among *S. Typhimurium* for endogenous galectin-8 (d), indicated *S. flexneri* strains (e) or *S. flexneri ΔipaH9.8* (f) at 1h p.i. in HeLa cells. Cells were treated with the indicated siRNAs against GBPs (d, f). *n* > 100 galectin-8-positive bacteria (d) or *n* > 100 bacteria (e, f) per coverslip, in triplicate. 

g-i, Percentages of FAM-VAD-FMK-positive *S. Typhimurium* among bacteria positive for endogenous galectin-8 at 90 min p.i. (g), FAM-VAD-FMK-positive *S. flexneri* of the indicated strains (h) or *S. flexneri ΔospC3 ΔipaH9.8* (i) at 1h p.i. in HeLa cells. Cells were treated with the indicated siRNAs against GBPs (g, h, i). *n* > 100 galectin-8-positive bacteria (g) or *n* > 100 bacteria (h, i) per coverslip, in triplicate. 

j, Lysates of HeLa cells treated with the indicated siRNAs and infected with *S. flexneri ΔospC3* for 1h. Blots were probed with the indicated antibodies; Actin, loading control. 

k, Percentage of PI-positive nuclei in HeLa cells treated with the indicated siRNAs at 2h p.i. with *S. Typhimurium* (k) or *S. flexneri ΔospC3* (l). Statistical significance was assessed by two-tailed unpaired Student’s *t*-test (e), one-way ANOVA with Dunnett’s multiple comparisons test versus siControl (b) or Tukey’s multiple comparisons test (d-i, k, l); NS, not significant; *P* < 0.05, **P* < 0.01 (exact *P* values are provided in Supplementary Table 1). Data are expressed as the mean ± s.e.m. of three (a, b, d-i, four (k) or five (l) independent experiments, or are representative of three (c, j) independent experiments. HeLa cells were treated with IFN-γ (b, c) or treated with IFN-γ as indicated (a, d-i). Uncropped blots (j) are shown in the Source data.
pyroptosis through processing of GSDMD, we tested whether intact bacteria entering the host cytosol trigger the same pathway. *S. flexneri* ΔospC3, but not control strains, caused cleavage of GSDMD (Extended Data Fig. 4c,d) in a CASP4-dependent fashion (Fig. 2o,p) and induced GSDMD-dependent cell death (Fig. 2q and Extended Data Fig. 4q), which resulted in GSDMD-dependent restriction of bacterial proliferation (Extended Data Fig. 4g). GSDMD processing and cell death required the transcription factors STAT1 and IRF-1 (ref. 13), two IFN-γ-induced transcription factors (Fig. 2o,q and Extended Data Fig. 4h). We conclude that upon entry of *S. Typhimurium* or *S. flexneri* ΔospC3 into the cytosol of IFN-γ-treated cells, CASP4 is recruited to the bacterial surface via an unknown factor, where it becomes activated and triggers GSDMD-dependent cell death, thereby destroying the bacterial niche for proliferation. Avoiding the CASP4 pathway via OspC3 is therefore essential for the cytosolic lifestyle of *S. flexneri* in cells exposed to IFN-γ.

Cytosol-invading bacteria recruit and activate CASP4 through GBPs. To identify the genes selectively induced by IFN-γ and required for functionality of the CASP4-GSDMD pathway in response to cytosol-invading bacteria (Fig. 1b), we tested the involvement of GBPs, a family of IFN-γ-induced GTPases, in a hierarchical manner, with GBP1 essential for recruitment of all GBPs (Fig. 3a,b and Extended Data Fig. 5a–c). GBPs are known to coat cytosolic *S. flexneri* to inhibit actin-dependent motility and cell-to-cell spread and have been implicated in cell death.14,15 We confirmed that similar to *S. flexneri*, *S. Typhimurium* also interacted with GBP1, GBP2, GBP3 and GBP4 in a hierarchical manner, with GBP1 essential for recruitment of all GBPs (Fig. 3a,b and Extended Data Fig. 5a–c). GBPs were recruited to cytosol-exposed bacteria, as indicated by staining for NDP52 (Extended Data Fig. 6), where GBP1 accumulated on the bacterial surface and not on damaged vacuoles positive for galectin-8 (Extended Data Fig. 5d). CASP4 was recruited only to bacteria that were also positive for GBPs (Extended Data Fig. 7a), where it formed discontinuous structures on the cytosolic face of

**Fig. 4** Formation of LPS-dependent GBP–CASP4 complexes. a, Pulldown of endogenous CASP4 by FLAG-tagged GBPs from HeLa cell lysates complemented with 10 μg ml⁻¹ *S. Typhimurium* LPS and GDP-AlFx. b, c, Pulldown of endogenous CASP4 by FLAG-tagged GBP1 (b) or GBP3 (c) from HeLa cell lysates complemented with 10 μg ml⁻¹ *S. Typhimurium* LPS and GDP-AlFx, as indicated. d, Pulldown of endogenous CASP4 by FLAG-tagged GBPs from lysates of control or the indicated GBP knockout U937 cells. Lysates were complemented with 10 μg ml⁻¹ *S. Typhimurium* LPS and GDP-AlFx. Data are representative of two independent experiments (a–d). HeLa cells were treated with IFN-γ as indicated (a–d). Flag-tagged proteins were pulled down using magnetic beads and eluted with FLAG peptide. Blots were probed with the indicated antibodies; IP, immunoprecipitation; KO, knockout; PCNA, loading control. Uncropped blots (a–d) are shown in the Source data.
the GBP layer surrounding the bacterium (Fig. 3c), consistent with a role for GBPs in attracting CASP4 to bacteria. Indeed, by infecting cells lacking specific GBPs, we discovered that GBP1, GBP2 and GBP4, but not GBP3 or GBP5, were essential for the recruitment of CASP4 to S. Typhimurium (Fig. 3d and Extended Data Figs. 5a,b and 7b). In contrast, S. flexneri actively avoided CASP4 recruitment to its surface as it was only targeted by CASP4 when lacking either IpaH9.8 (ref. 36), a GBP-specific E3 ubiquitin ligase, or

---

**Fig. 5 | Analysis of LPS-dependent GBP–CASP4 complexes.** a, b, Pulldown of endogenous CASP4 by FLAG-tagged GBP1 from HeLa cell lysates complemented with GDP-AlFx and 10 μg ml⁻¹ LPS from the indicated bacterial species or lipid A (a), with GDP-AlFx and 10 μg ml⁻¹ the indicated agonist (b) or with 10 μg ml⁻¹ S. Typhimurium LPS and the indicated reagents, that is, 200 μM GDP, 300 μM AlCl₃, 10 mM NaF, 200 μM GMP, 0.5 mM GTP-γ-S, 0.5 mM GppNHp or 0.5 mM GppCp (c). c, d, Pulldown of GFP-tagged alleles of CASP4 (c) or endogenous CASP4 (d) by FLAG-tagged WT GBP1 (c) or the indicated GBP1 alleles (d) from HeLa cell lysates. Lysates were complemented with 10 μg ml⁻¹ S. Typhimurium LPS (c, d) and with GDP-AlFx (c) or with GDP-AlFx as indicated (d). Data are representative of two independent experiments (a–e). HeLa cells were treated with IFN-γ as indicated (a–e).

Flag-tagged proteins were pulled down using magnetic beads and eluted with FLAG peptide. Blots were probed with the indicated antibodies; PCNA, loading control. Uncropped blots (a–e) are shown in the Source Data.
Fig. 6 | Structure–function analysis of the GBP-dependent CASP4 signaling platform. a, d. Percentage of *S. flexneri* positive for the indicated FLAG::GBP1 (a) or FLAG::CASP4 (d) alleles at 1 h p.i. HeLa cells were treated with the GBP1 no. 49 (a) or CASP4 no. 14 (d) siRNA and GBP1 (a) or CASP4 (d) expression was complemented with siRNA-resistant FLAG::GBP1 or FLAG::CASP4 alleles as indicated. *n* > 100 bacteria per coverslip, in triplicate. b. Percentage of endogenous CASP4-positive *S. flexneri ΔipahH9.8* at 1 h p.i. HeLa cells were treated with GBP1 no. 49 siRNA and GBP1 expression was complemented with siRNA-resistant FLAG::GBP1 alleles as indicated. *n* > 100 bacteria per coverslip, in triplicate. c, g. Percentage of PI-positive nuclei in cells infected with *S. flexneri ΔospC3* at 2 h p.i. HeLa cells were treated with the indicated siRNAs and GBP1 (c) or CASP4 (g) expression was complemented with siRNA-resistant FLAG::GBP1 or FLAG::CASP4 alleles as indicated. e. Percentage of GFP::CASP4 CARD domain (aa1–104)–positive *S. flexneri* ospC3 among gal-8-positive (%). Cells were live imaged every 4 min for 2 h, five fields per condition. f. Lysates of cells infected with *S. flexneri ΔospC3* for 1 h. HeLa cells were treated with the indicated siRNAs and CASP4 expression was complemented with siRNA-resistant FLAG::CASP4 alleles as indicated. Blots were probed with the indicated antibodies; Actin, loading control. Samples in f and Fig. 8e were obtained from the same experiment. Statistical significance was assessed by one-way ANOVA with Dunnett's (c) or Tukey's (a, b, d, e, g) multiple comparisons tests; NS, not significant; *P* < 0.05, **P** < 0.01 (exact *P* values are provided in Supplementary Table 1). Data are expressed as the mean ± s.e.m. of three (a, b, d, e), four (c) or five (g) independent experiments, or are representative of two (f) independent experiments. HeLa cells were treated with IFN-γ (a, d) or treated with IFN-γ as indicated (b, c, e–g). Uncropped blots (f) are shown in the Source data.
**Fig. 7 | Gbps and Casp11 protect mice epistatically against bacterial infection.** a, Kaplan–Meier survival plots of WT or the indicated knockout mice infected orogastrically with streptomycin-resistant S. Typhimurium at an MOI of 7×10⁸. Sample sizes, five animals per group. b, Bacterial burden during S. Typhimurium infection in mice. WT or the indicated knockout mice were infected orogastrically with streptomycin-resistant S. Typhimurium at an MOI of 7×10⁸. CFUs of S. Typhimurium in cecum assessed at 96 h p.i. Sample sizes (n=number of animals): n = 3 for WT, n = 2 for Gbp1Δ, n = 4 for Gbp2Δ and Casp11Δ. c, Kaplan–Meier survival plots of WT or the indicated knockout mice infected intraperitoneally with the indicated S. flexneri strains at an MOI of 7.6×10⁴. Sample sizes (n=number of animals) and genotypes (Shigella/mice): n = 8 for WT/WT, WT/Gbp1Δ and WT/Gbp2Δ; n = 6 for WT/Casp11Δ; n = 9 for ∆ospC3/WT and ∆ospC3/Gbp2Δ; n = 10 for ∆ospC3/Gbp1Δ; n = 9 for ∆ospC3/Casp11Δ; n = 8 for ∆ospC3 + ospC3/WT, ∆ospC3 + ospC3/Gbp1Δ and ∆ospC3 + ospC3/Gbp2Δ; n = 6 for ∆ospC3 + ospC3/Casp11Δ. d, Bacterial burden during S. flexneri infection in mice. WT or the indicated knockout mice were infected intraperitoneally with the indicated S. flexneri strains at an MOI of 7.6×10⁴. CFUs of S. flexneri in spleen (left) or liver (right) were determined at 24 h p.i. Sample sizes, five animals per group. Statistical significance was assessed by two-tailed log-rank (Mantel–Cox) test compared with the WT group (a, c) or one-way ANOVA with Dunnett’s multiple comparison test versus the WT mice group (b, d); NS, not significant; *P<0.05; **P<0.01 (exact P values are provided in Supplementary Table 1). Data are shown as the mean±s.d. (b, d). Data are representative of two independent experiments (a–d).

MxiE, a transcriptional regulator of IpaH9.8 expression, or if cells were treated with the proteasome inhibitor Carfilzomib to block IpaH9.8-dependent degradation of GBPs (Fig. 3e and Extended Data Fig. 7c). Recruitment of endogenous GBP1 and endogenous CASP4 to S. flexneri ∆ipaH9.8 was confirmed in primary human enterocytes (Extended Data Fig. 7d). CASP4 recruitment required GBP1, GBP2 and GBP4, but not GBP3 or GBP5 (Fig. 3f), thus mirroring the situation in S. Typhimurium and suggesting that S. flexneri antagonizes CASP4 recruitment by degrading GBPs. To test whether GBPs also affect caspase activation, we infected cells deficient in specific GBPs and used FAM-VAD-FMK to monitor caspase activity. GBP3 was specifically required to induce caspase activity. GBP3 was specifically required to induce caspase activity.
Fig. 8 | Processing and secretion of IL-18 require GBP-dependent CASP4 activity. a–g, Lysates of HeLa cells treated with the indicated siRNAs (a, g), or of the indicated control or knockout HeLa cells (c), collected at 1 h.p.i. with S. flexneri ΔospC3. Samples in c, Fig. 2p and Extended Data Fig. 2g were obtained from the same experiment. b, d, h, Release of IL-18 from HeLa cells treated with the indicated siRNAs (b, h) or from the indicated control or knockout HeLa cells (d) at 1 h.p.i. with S. flexneri ΔospC3. e, i, Lysates of cells at 1 h.p.i. with S. flexneri ΔospC3. HeLa cells were treated with the indicated siRNAs, and GBP1 (i) or CASP4 (e) expression was complemented with FLAG::GBP1 or FLAG::CASP4 alleles as indicated. Samples in e and Fig. 6f were obtained from the same experiment. f, j, Release of IL-18 from cells treated with the indicated siRNAs at 1 h.p.i. with S. flexneri ΔospC3. HeLa cells were treated with the indicated siRNAs, and GBP1 (j) or CASP4 (f) expression was complemented with FLAG::GBP1 or FLAG::CASP4 alleles as indicated. Statistical significance was assessed by one-way ANOVA with Tukey’s multiple comparisons test (b, d, f, h, j); NS, not significant; *P < 0.05, **P < 0.01 (exact P values are provided in Supplementary Table 1). Data are expressed as the mean ± s.e.m. of three (b, d, f, h, j) independent experiments, or are representative of two (c, e, i) or three (a, g) independent experiments. HeLa cells were treated with IFN-γ as indicated (a–f). Blots were probed with the indicated antibodies; Actin, loading control (a, c, e, g, i). Uncropped blots (a, c, e, g, i) are shown in the Source data.
neri rarely stained with FAM-V-AD-FMK (Fig. 3h), indicating lack of caspase activity in its vicinity.

While deficiency in IpaH9.8 was sufficient to cause CASP4 recruitment to S. flexneri (Extended Data Fig. 7c), only bacteria lacking both IpaH9.8 and OspC3 triggered caspase activity (Fig. 3h and Extended Data Fig. 7f,g). We conclude that S. flexneri antagonizes CASP4 at multiple levels. Infection with S. flexneri ΔipaH9.8 ΔospC3 revealed that GBP3 was specifically required to induce CASP4 activity in the bacterial vicinity, in addition to GBP1, GBP2 and GBP4, with established upstream functions (Fig. 3i and Extended Data Fig. 7h–j), again faithfully mirroring the situation in S. Typhimurium. Consistent with the induction of caspase activity in the bacterial vicinity depending on GBP1, GBP2, GBP3 and GBP4, the same set of GBPs was required to induce caspase activity throughout the cell body (Extended Data Fig. 8a,b), to induce processing of GSDMD (Fig. 3j and Extended Data Fig. 8c) and to trigger cell death upon infection with S. Typhimurium or S. flexneri ΔospC3 (Fig. 3k,l and Extended Data Fig. 8d,e), thereby extinguishing the bacterial replicative niche. Overall, we conclude that in cells stimulated with IFN-γ, GBPs associate with Gram-negative bacteria to transform the bacterial surface into a signaling platform required for the recruitment and activation of CASP4, which causes pyroptotic cell death and destroys the bacterial replicative niche. Shigella’s multipronged attempt to prevent formation of the GBP-dependent signaling platform and activation of CASP4 provides compelling evidence for the importance of the pathway in antimicrobial defense.

To investigate how coating of the bacterial surface with GBPs causes CASP4 recruitment, we tested whether GBPs bind CASP4. We discovered that in lysates of epithelial or myeloid cells GBP1 and GBP3, but not GBP2 or GBP4, associate with CASP4 in an LPS-dependent manner (Fig. 4a–d). CASP4 retrieval was enhanced in lysates of IFN-γ-stimulated cells (Fig. 4a–c) and was promoted by LPS from different species, including LPS lacking the O-antigen (Ra LPS) and rough LPS (Rc LPS), but not by lipid A and unrelated bacterial products such as lipoteichoic acid, mycolic acid or Pam,CSK, indicating specificity for LPS and an essential contribution of the LPS core for the association of GBPs with CASP4 (Fig. 5a,b). As predicted, CASP4K19E, an allele deficient in LPS binding12, was inactive (Fig. 5c). Complexes of CASP4 with GBP1 and GBP3 still formed in the absence of GBP3 and GBP1, respectively, indicating that either GBP is sufficient for complex formation (Fig. 4d and Extended Data Fig. 8f,g). CASP4 retrieval required an intact GTPase domain in GBP1 (Fig. 5d) and occurred specifically in the presence of GDP-AlFx (Figs. 4b and 5e), that is, under conditions mimicking the transition state of GTP hydrolysis in oligomeric GBPs49. The LPS-dependent complex formation of CASP4 specifically with oligomeric GBPs suggests that the assembly of GBPs on the bacterial surface into a polyvalent protein array is important for CASP4 activation in response to Gram-negative bacteria.

To further our understanding of how coating of the bacterial surface with GBPs results in CASP4 activation, we deployed mutant GBP1 and CASP4 alleles in complementation experiments12,40. In cells stimulated with IFN-γ and infected with S. flexneri, GBP1 alleles deficient in prenylation (GBP1 S52N), or catalytically inactive due to lack of Mg2⁺ binding (GBP1S152E), GTP binding (GBP1K51A) or GTP hydrolysis (GBP1R84A), did not coat the bacterial surface (Fig. 6a), nor did they recruit CASP4 (Fig. 6b) or trigger cell death (Fig. 6c). Since binding of LPS to the CARD domain of CASP4 has been suggested to activate CASP44,5, we next tested the phenotype of CASP4K19E, a mutant deficient in LPS binding, in a complementation experiment. In cells stimulated with IFN-γ and infected with S. flexneri, wild-type (WT) CASP4 and catalytically inactive CASP4S218A, but not CASP4K19E or its isolated CARD domain (CARDK19E), accumulated on bacteria (Fig. 6d,e). Lack of recruitment in CASP4K19E and lack of catalytic activity in CASP4S218A prevented GSDMD cleavage and cell death in cells stimulated with IFN-γ and infected with S. flexneri (Fig. 6f,g). We conclude that GBP1 and CASP4 do not cause pyroptosis if unable to coat the bacterial surface or if they fail to associate with each other. Lack of CASP4S218A recruitment, considering its reported defect in LPS binding13, is consistent with a direct contribution of LPS to the recruitment of CASP4. However, in the context of an intact bacterial membrane, CASP4 evidently does not bind LPS in the absence of GBPs. The requirement for GBPs in recruiting CASP4 and the accumulation of CASP4 on the cytosolic face of the GBP coat therefore suggest a role for GBPs, possibly assisted by their GTPase activity, in making LPS available for CASP4, possibly by disturbing the integrity of the outer bacterial membrane.

We next tested the in vivo importance of the GBP-CASP4 pathway against Salmonella and Shigella infections. Upon infection with S. Typhimurium, Gbp1−/− mice and Casp11−/− mice suffered a higher bacterial burden in their ceca and succumbed to infection earlier, whereas GBP2 protected mice only during the early stage of infection (Fig. 7a,b). Mice infected with WT S. flexneri had substantially higher bacterial loads in livers and spleens than their littermates exposed to S. flexneri ΔospC3, resulting in significantly better survival of the latter (Fig. 7c,d). Importantly, S. flexneri ΔospC3 was not attenuated in mice lacking Gbp1, Gbp2 or Casp11, the ortholog of human CASP4. Such lack of attenuation for S. flexneri ΔospC3 is best explained by an epistatic interaction of Gbp1 and Gbp2 with Casp11, which provides strong in vivo support for the proposed role of GBPs in converting the surface of cytosol-invading bacteria into a caspase activation platform.

Processing and secretion of IL-18 require GBP-dependent CASP4 activity. In addition to cleaving GSDMD, human CASP4 has been reported to process IL-18 (ref. 41), a pro-inflammatory cytokine, while murine Casp11 appears to be inactive against the latter4. We therefore investigated whether the GBP-dependent conversion of the Shigella surface into a polyvalent caspase signaling platform also controls IL-18 maturation through CASP4. Processing of IL-18, as well as its release from cells infected with S. flexneri, was suppressed by OspC3 and required CASP4 but not CASP1 or CASP5 (Fig. 8a–d and Extended Data Fig. 9a–d). Consistent with species-specific differences, murine Casp11, known not to process IL-18 (ref. 45), failed to complement human cells in which CASP4 had been knocked down (Extended Data Fig. 9e). As expected, GSDMD was required for the release and not the processing of IL-18 (Fig. 8c,d). Complementation of CASP4-depleted cells with catalytically inactive CASP4S218A or recruitment-deficient CASP4K19E failed to rescue the defect in processing and release of IL-18 (Fig. 8e,f). Cells lacking GBP1, GBP2, GBP3 or GBP4, but not GBP5, also failed to process and release IL-18 (Fig. 8g,h and Extended Data Fig. 9g,h). Complementation of GBP1-depleted cells with alleles deficient in prenylation or catalytically inactive GBP1 did not rescue the defect in IL-18 processing or release (Fig. 8i,j). We therefore conclude that the conversion of the bacterial surface by GBPs into a polyvalent signaling platform is required for the CASP4-mediated processing and release of IL-18, suggesting that in addition to inducing pyroptosis and destroying the bacterial replicative niche, human CASP4 also fulfills a pro-inflammatory role by alarming surrounding cells to the presence of cytosol-invading bacteria.

Discussion

Signaling pathways often rely on endomembranes to provide anchoring points and to achieve high local concentrations of signaling components. The outer membrane of Gram-negative bacteria, as shown here, serves a similar purpose through GBP recruitment and the subsequent activation of CASP4 (Extended Data Fig. 10). The establishment of the GBP-derived signaling platform on the bacterial surface, as well as the recruitment and activation of CASP4, are controlled in a hierarchical manner, with upstream GBPs required for all subsequent...
steps. GBP1, the most upstream family member, is specifically needed to recruit GBP2, GBP3 and GBP4, that is, to initiate platform formation. GBP2 and GBP4 are specifically required to recruit CASP4, whereas GBP3 controls its activity.

In contrast to purified LPS, LPS as a constituent of the bacterial outer membrane does not activate CASP4, most likely because the membrane-embedded acyl chains of lipid A remain inaccessible to its ligand-binding CARD domain. GBP3s, besides concentrating CASP4 in a highly relevant location, might also disturb the integrity of the bacterial outer membrane to provide CASP4 with access to otherwise hidden ligands. Further biochemical and structural work will be required to understand the process in more detail, including the possible role of GTP hydrolysis for lipid extraction from the bacterial membrane.

Converting the bacterial surface into a polyvalent signaling platform for caspase activation results in pyroptosis and production of the pro-inflammatory cytokine IL-1β, thereby destroying the replicative niche for intracellular bacteria and alerting neighboring cells to their presence. Bacteria inside pyroptotic cells remain contained in pore-induced intracellular traps until expelled from the tissue with their destroyed host cell or phagocytosed and killed13,14. The dire consequences of pyroptosis for the cell may explain the need for the sequential and regulated nature of the GBP-CASP4 pathway, as it would allow multiple checkpoints before caspase activity is induced.

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/s41590-020-0697-2.

Received: 30 August 2019; Accepted: 29 April 2020; Published online: 15 June 2020

References
1. Poltorak, A. et al. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science 282, 2085–2088 (1998).
2. Takeuchi, O. & Akira, S. Pattern recognition receptors and inflammation. Cell 140, 805–820 (2010).
3. Park, B. S. et al. The structural basis of lipopolysaccharide recognition by the ligand-binding CARD domain. GBPs, besides concentrating CASP4
4. LPS targets host guanylate-binding proteins to the bacterial outer membrane for non-canonical inflammasome activation. EMBO J. 37, e98089 (2018).
5. Meunier, E. et al. Caspase-11 activation requires lysis of pathogen-containing vacuoles by IFN-induced GTPases. Nature 509, 366–370 (2014).
6. Fisch, D. et al. Human GBP1 is a microbe-specific gatekeeper of macrophage apoptosis and pyroptosis. EMBO J. 38, e100926 (2019).
7. Man, S. M. et al. IRG8 liberates bacterial ligands for sensing by the AIM2 and caspases-11–NLPR3 inflammasomes. Cell 167, 382–396.e17 (2016).
8. Beken, C. et al. The interferon-inducible p47 (IRG) GTPases in vertebrates: loss of the cell autonomous resistance mechanism in the human lineage. Genome Biol. 6, R92 (2005).
9. Mitchell, G. & Isberg, R. R. Innate immunity to intracellular pathogens: balancing microbial elimination and inflammation. Cell Host Microbe 22, 166–175 (2017).
10. Matsuzawa-Ishimoto, Y., Hwang, S. & Cadwell, K. Autoptophagy and inflammation. Annu. Rev. Immunol. 36, 73–101 (2018).
11. Thurston, T. L. M., Wandel, M. P., von Muhlinen, N., Foeglein, A. & Randow, F. Gaelecit 8 targets damaged vesicles for autophagy to defend cells against bacterial invasion. Nature 482, 414–418 (2012).
12. Radlke, A. L., Delbridge, L. M., Balachandran, S., Barber, G. N. & O’Riordan, M. X. D. TBK1 protects vacuolar integrity during intracellular bacterial infection. PLoS Pathog. 3, e29 (2007).
13. Isenberg, R. R. & Falkow, S. A single genetic locus encoded by Yersinia pseudotuberculosis permits invasion of cultured animal cells by Escherichia coli K-12. Nature 317, 262–264 (1985).
14. Isenberg, R. R., Voorhis, D. L. & Falkow, S. Identification of invasion: a protein that allows enteric bacteria to penetrate cultured mammalian cells. Cell 50, 769–778 (1987).
15. Perrin, A., Jiang, X., Birmingham, C., So, N. & Brumell, J. Recognition of bacteria in the cytosol of mammalian cells by the ubiquitin system. Curr. Biol. 14, 806–811 (2004).
16. von Muhlinen, N. et al. LC3, Guided selectively by a noncanonical LIR motif in NPD52, is required for antibacterial autophagy. Mol. Cell 48, 329–342 (2012).
17. Kobayashi, T. et al. The Shigella OspC3 effector inhibits caspase-4, antagonizes inflammatory cell death, and promotes epithelial infection. Cell Host Microbe 13, 570–583 (2013).
18. Brien, V. et al. Interferon regulatory factor 1 is required for mouse Gbp gene activation by gamma interferon. Mol. Cell. Biol. 15, 975–982 (1995).
19. Li, P. et al. Ubiquitination and degradation of GBP4 by a Shigella effector to suppress host defence. Nature 511, 378–383 (2013).
20. Wandel, M. P. et al. GBP4 inhibit motility of Shigella flexneri but are targeted for degradation by the bacterial ubiquitin ligase IpA9.H. Cell Host Microbe 22, 507–518.e5 (2017).
21. Piño, A. S. et al. Detection of cytosolic Shigella flexneri via a C-terminal triple-arginine motif of GBP4 inhibits actin-based motility. Molb, 8, e01979-17 (2017).
22. Kane, C. D., Schuch, R., Day, W. A. & Maurelli, A. T. MxiE regulates intracellular expression of factors secreted by the Shigella flexneri 2a type III secretion system. J. Bacterial. 184, 4409–4419 (2002).
23. Ghosh, A., Paecke, G. J. K., Renaud, L., Witthinghofer, A. & Herrman, C. How guanylate-binding proteins achieve assembly-stimulated processive cleavage of GTP to GMP. Nature 440, 101–104 (2006).
24. Prakash, B., Paecke, G. J. K., Renaud, L., Witthinghofer, A. & Herrman, C. Structure of human guanylate-binding protein 1 representing a unique class of GTP-binding proteins. Nature 403, 567–571 (2000).
25. Knodler, L. A. et al. Noncanonical inflammasome activation of caspase-4/caspase-11 mediates epithelial defenses against enteric bacterial pathogens. Cell Host Microbe 16, 249–256 (2014).
26. Ramirez, M. L. G. et al. Extensive peptide and natural protein substrate screens reveal that mouse caspase-11 has much narrower substrate specificity than caspase-1. J. Biol. Chem. 293, 7058–7067 (2018).
27. Jorgensen, I., Zhang, Y., Krantz, B. A. & Miao, E. A. Pyroptosis triggers pore-induced intracellular traps (PITs) that capture bacteria and lead to their clearance by efferocytosis. J. Exp. Med. 213, 2133–2138 (2016).
28. Miao, E. A. et al. Caspase-1-induced pyroptosis is an innate immune effector mechanism against intracellular bacteria. Nat. Immunol. 11, 1136–1142 (2010).

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.
**Methods**

Plasmids, antibodies and reagents. M5P or closely related plasmids were used to produce recombinant MLV for the stable expression of proteins in mammalian cells. Open reading frames encoding full-length caspsases, Bcl2, Bcl-xL, CrmA and GBP1-7 were amplified by PCR. Mutations and protein truncations were generated by PCR and cloned into pEFV5.1.

Antibodies were from Abcam (for western blots: β-actin a82287, Galectin-8 ab18367, NDP52 ab68858), Cell Signaling Technology (Gadamer-D 96548), Clontech (GFP JLB 632831), Invitrogen (Uropornein K2 B1411, M90T 5a) (BMP-3 489 M029-3 18 PM014), Proteintech (FIP200 10043-2-AP, GBPI 15303-1-AP, R&D Systems (for immunofluorescence: Galectin-8 AFI305), Santa Cruz Biotechnology (CASpase-1-20 sc-515, IRF-1 H-8 sc-74530), PCNA PV10 sc-62701, S1AT-111 sc-417), Sigma (for immunofluorescence: FLAG–tag M2 F1804; for western blots: FLAG–tag F7425), Thermo Fisher Scientific (Alexa-conjugated anti-mouse, anti-gout and anti-rabbit antisera) and DABCO (HRP-conjugated reagents). The antiserum against NDP52 used for immunofluorescence was a gift from J. Kendrick-Jones.

Cytokines (all from R&D Systems, apart from IFN-β from PBL) were added for 10–16 h before experiments at the following concentrations: IFN-γ 1 ng ml−1, IFN-β 100 U ml−1, TNFα 10 ng ml−1, IL-1β 10 ng ml−1 and IL-22 10 ng ml−1.

Inhibitors were from Abcam (Nc-1 ab211984), R&D Systems (Necrosisindole 5002, Z-VAD-FMK FKMK001) and Selleck Chemicals (Carfilzomib s2853), FAM-VAD-FMK (92) is from Immunology Chemo Technologies; agonists were from Innaxon (LPS from Salmonella minnesota R60 (Ra) TLpurpere Sterile Solution IAX-100-016, LPS from S. minnesota R5 (Re) TLpurpere Sterile Solution IAX-100-017, Lipid A from S. minnesota R59 (Re) TLpurpere Sterile Solution IAX-100-001, InvivoGen (Pam3CSK4 tlr-3ms), Sigma (LPS S. enterica serotype Typhimurium L5611, LPS Escherichia coli 0127:LB 12755, Lipoteichoic acid from Bacillus subtilis L3265, Mycotic acid from Mycobacterium tuberculosis M4537); and nonhydrolyzable GTP analogs were from Abcam (GTP γ S ab146662, Gpp[Np]Np ab146659, Gpp[c]p ab146600).

**Cell culture.** HeLa cells were obtained from the European Collection of Authenticated Cell Cultures (9302103). HeLa and 293ET cells, as well as all stable cell lines, were grown in IMDM supplemented with 10% FCS at 37 °C and 5% CO2.Authenticated Cell Cultures (9302103). HeLa and 293ET cells, as well as all stable cell lines, were grown in IMDM supplemented with 10% FCS at 37 °C and 5% CO2. L929, B16, NIH3T3 and HEK293T cells (both from The Jackson Laboratory).

**Plasmids, antibodies and reagents.** U937 cells were obtained from the Biological Resource Center CelluloNet and cell lines, were grown in IMDM supplemented with 10% FCS at 37 °C in 5% CO2. M90T 5a (strain M90T 5a) was grown in tryptic soy broth (TSB) or on tryptic soy agar containing 0.003% Congo red. For infection, S. flexneri was grown overnight in TSB and subcultured (1:100) in fresh TSB for 2.5 h. Such cultures were consecutively washed in PBS and resuspended in antibiotic-free IMDM plus 10 FCS immediately before 100 μl was used to infect HeLa cells in 24-well plates (MOI of 1:50). Samples were centrifuged for 10 min at 670 followed by incubation at 37 °C for 20 or 30 min. Following two washes with warm PBS, cells were cultured in 100 μg ml−1 gentamicin for 2 h and 200 μg ml−1 gentamicin thereafter.

To enumerate intracellular bacteria, cells from triplicate wells were lysed in 1 ml of cold PBS containing 0.1% Triton-X-100. Serial dilutions were plated in duplicate on LB agar.

**In vivo Shigella infection.** For in vivo bacterial challenge, S. flexneri WT 5a strain M90T, ΔospC3 mutant and ΔospC3 + complementation lines were grown in TSB at 37 °C overnight and subcultured (1:100) in fresh TSB for 2.5 h before in vivo injection. Age (6–12 weeks old) and sex-matched C57BL/6N WT, Gbp1−/−, Gbp2−/− and Caspase-11–/– mice were intraperitoneally injected with 8×104 colony-forming units (CFUs) of S. flexneri WT or mutant bacterial strains, respectively. Survival rate was monitored every 24 h up to 240 h. To measure bacterial burdens in organs, mice were peritoneally injected with 6×104 CFUs of bacteria and liver tissues were isolated after 24 h p.i. Isolated tissues were washed twice with 1× cold PBS and homogenized. Tissue extracts were diluted with PBS and spread onto TSB agar plates. Bacteria colonies were counted and analyzed by automated colony counting (Scan 300, Interscience).

**In vivo Salmonella infection.** For in vivo bacterial challenge, streptomycin-resistant S. enterica serovar Typhimurium (strain 1344) was grown in LB broth at 37 °C overnight and subcultured (1:100) in fresh LB for 2.5 h before in vivo injection. Age (6–12 weeks old) and sex-matched C57BL/6N WT, Gbp1−/−, Gbp2−/− and Caspase-11–/– mice were intraperitoneally injected with 20 mg per mouse of streptomycin for 24 h at a 7-h fasting period, with 10% sodium bicarbonate for 30 min and with oragotic challenge with 7×108 CFUs of S. Typhimurium. Survival rate was monitored every 24 h up to 24 h. To measure bacterial burdens in organs, mice were injected intragastrically with 7×108 CFUs of bacteria, and cecum tissues were isolated after 96 h p.i. according to Knodler et al.13. Isolated tissues were washed twice with 1× cold PBS and homogenized. Tissue extracts were diluted with PBS and spread onto LB plates in triplicates. Bacteria colonies were counted and analyzed by automated colony counting (Scan 300, Interscience).

**Infection of human intestinal epithelial organoids.** Monolayers of differentiated epithelial organoids were infected with S. flexneri from the basolateral side as described. Briefly, medium in Transwell-containing wells was changed to antibiotic-free differentiation medium the day before infection. Transwells were infected on absorbent paper to remove excess medium and placed upside-down in six-well plates. Then, 15 μl of washed S. flexneri culture was added to the upward-facing side of the Transwell and incubated at 37°C for 1 h. The Transwell was then turned the right way up and both the apical and interior chambers washed three times with warm PBS. Differentiation medium containing 100 μg ml−1 gentamicin was added and incubated further as required.

Cells infected with S. flexneri for 2 h were washed with PBS and either fixed in paraformaldehyde or incubated with Zombie Green (Biolegend; 1:500 in PBS), to label dead cells, at room temperature for 15 min. Cells were washed twice with PBS at 4°C before fixing for 15 min in 4% paraformaldehyde followed by PBS and processing for immunofluorescence with anti-ZO-1 antibody to visualize cell body boundaries (Catalog 40–2200, Thermo Fisher).

**RNA interference.** First, 5×104 cells per well were seeded in 24-well plates. The following day, cells were transfected with 40 pmol of Stealth RNAi siRNA or 6 pmol of Silencer Select siRNA (both Thermo Fisher Scientific) using Lipofectamine RNAiMAX (Thermo Fisher Scientific). Experiments were performed after 3 d. The nontargeting Stealth RNAi negative Control Medium GC or Silencer Select Negative Control No. 1 siRNA was used as control (both Thermo Fisher Scientific).

**Bacterial infections and enumeration of intracellular bacteria.** S. Typhimurium (strain 12023) was grown overnight in Luria broth (LB) and subcultured (1:50) in fresh LB for 2 h before infection. HeLa cells in well plates were infected with 10–20 μl of such cultures for 15 min at 37°C (multiplicity of infection (MOI) of 1:50–1:100). Following two washes with warm PBS and an incubation with 100 μg ml−1 gentamicin for 2 h, cells were cultured in 20 μg ml−1 gentamicin.

**Human intestinal epithelial organoids.** Ethical approval for this project was obtained from the local ethics committee (REC-17/EE/0265) and patients were recruited following informed consent. Intestinal biopsy samples were taken from the distal small bowel (the terminal ileum) from patients undergoing routine endoscopy. All patients included had macroscopically and histologically normal mucosa. Biopsy samples were processed immediately and intestinal epithelial organoids were generated from isolated crypts following an established protocol, as described previously. Organoids were typically grown for 7–9 d before passaging and, once sufficiently expanded, were seeded for growth as a monolayer as described previously. Briefly, 6.5-mm polycarbonate Transwell inserts with 8-μm pore size (Corning) were coated with collagen 2 h before cell seeding. Established intestinal organoids were washed with PBS containing 0.5 mM EDTA and dissociated in 0.5% Trypsin-EDTA. Trypsinization was inactivated by PBS and clumps of cells were removed using a 40-μm cell strainer. Cells were seeded at 104 per Transwell and grown in proliferation medium. After 24 h, cells were maintained in differentiation medium and differentiation was allowed to occur for 5 d before infection.

**Bacterial infections and enumeration of intracellular bacteria.** S. Typhimurium (strain 12023) was grown overnight in Luria broth (LB) and subcultured (1:50) in fresh LB for 2 h before infection. HeLa cells in well plates were infected with 10–20 μl of such cultures for 15 min at 37°C (multiplicity of infection (MOI) of 1:50–1:100). Following two washes with warm PBS and an incubation with 100 μg ml−1 gentamicin for 2 h, cells were cultured in 20 μg ml−1 gentamicin.
CRISP–Cas9–mediated gene knockout. For HeLa knockouts, oligonucleotides (Sigma) for the sgRNA were phosphorylated with T4 PNK (NEB), annealed by heating to 95 °C and subsequently cooled down slowly to room temperature. Hybridized oligonucleotides were cloned into the lentiviral sgRNA expression vector pKLV-U6gRNA(BbsI)-PGKpupe2ABFP (Addgene 50946). HeLa cells were constructed to stably express Cas9 through lentiviral transduction of vector pHRSIN-Psfv-Cas9-Pppg-Hygro and hygromycin selection. Subsequently, sgRNA-containing vector was introduced by transduction and puromycin selection. The resultant transduced knockout populations were used to generate single-cell clones by limiting dilution. Gene disruption was validated by immunoblotting using corresponding antibodies. sgRNA: CASP1 (GTTCATGTCTCATGTTATTC), CASP4 (GAGAAAAACCGCGCCAGC), GSDMD (GCATGTTGGCTGCCCTTTTGA), GBP1 (GAACACTAATGGCGGACGTAA), GBP3 (GTTTACGTGATTTCACCG), GBP2 (GGGCCCCGAAGATTGCTTCT), GBP2 (GTCTCATGCTATTGTACACGA), GBP1 (TTTAGTGTGAGACTGCACCG), GBP2_S1 (TCCTATGCTATTGTACACGA), GBP3_S1 (ACTCTGCTGTAACATTACAGT), GBP2_S2 (CTTCCCTGAAAGTCTAGCCA), GBP3_S2 (TAATCCCTGAAAGTCTAGCCA).

Microscopy. HeLa cells were grown on glass coverslips before infection. After infection, cells were washed twice with warm PBS and fixed in 4% paraformaldehyde for 20 min. Cells were washed twice with PBS and then simultaneously permeabilized and blocked in PFB (PBS, 0.1% saponin, 2% BSA). Coverslips were incubated with primary followed by secondary antibodies for 1 h in PBB. Samples were mounted either in mounting medium with 4',6-diamidino-2-phenylindole (DAPI) or in Prolong Antifade mounting medium for confocal imaging and super-resolution microscopy, respectively. Marker-positive bacteria were scored by eye amongst at least 100 bacteria per coverslip. Confocal images were taken with a x63/1.4 numerical aperture (NA) oil immersion lens on a Zeiss 780 microscope.

Live imaging was performed on a Nikon Eclipse Ti equipped with an Andor Revolution XD system and a Yokogawa CSU-X1 spinning disk unit using a x63/1.4 NA water immersion lens.

Super-resolution images were acquired using an Elvira S1 structured illumination microscope (SIM; Carl Zeiss). The system has four laser excitation sources (405 nm, 488 nm, 561 nm and 640 nm) with fluorescence emission filter sets matched to these wavelengths. SIM images were acquired with a x63/1.4 NA oil immersion lens with grating projections at five rotations and five phases in accordance with the manufacturer’s instructions. The number of z planes varied with sample thickness. Super-resolution images were calculated from the raw data using Zeiss Zen software.

High-content analysis (HCA) microscopy was performed on a Nikon Eclipse Ti equipped with an Andor Neo sCMOS camera. HCA wide-field images were taken with a x20/0.75 NA air lens on an inverted microscope using µ-Plate 24-well ibiTreat (Ibidi). PI-positive nuclei were identified using the General Analysis module in Nikon NIS-Elements HC Software.

In vitro pulldown. First, 1.5 × 10^6 HeLa cells expressing FLAG-tagged proteins were scraped, washed with PBS and lysed at 4 °C in the lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM MgCl₂, 100 μM digoxin (Sigma, D141), complete Mini EDTA-free protease inhibitor (Roche)). Then, 1 × 10^7 U937 cells expressing FLAG-tagged proteins were pelleted, washed twice with PBS and lysed at 4 °C in the lysis buffer. Post-nuclear supernatants were obtained by centrifugation at 6,000g for 10 min at 4 °C. Cell lysate was incubated with 10 μg/ml LPS or other agonist, 200 mM GDP (Sigma, G7127), 300 μM AICl, and 10 mM NaF for 2 h at room temperature with rotation. Flag-tagged proteins were pull down using Anti-DYKDDDDK Magnetic Agarose (Pierce, A36797) for 1 h at 4 °C. Following four washes with wash buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM MgCl₂, 100 μM digoxin, 200 μM GDP, 300 μM AICl, 10 mM NaF), Flag-tagged proteins were eluted with 3x DYKDDDDK Peptide (Pierce, A36805). After addition of SDS, loading buffer samples were separated on 4–12% denaturing Bis-Tris gels (Thermo Fisher Scientific).

Statistical analysis. All data were tested for statistical significance with Prism software (GraphPad Prism 7 and 8). The unpaired two-tailed Student’s t-test was used to test whether two samples originated from the same population. Differences between more than two samples were tested using a one-way (for one variable) or a two-way (for two variables) analysis of variance (ANOVA). Either Dunnett’s multiple comparison test (to compare all samples against a control) or Tukey’s multiple comparison test (to compare all samples against each other) was applied. Unless otherwise stated, all experiments were performed at least three times and the data were combined for presentation as a mean ± s.e.m. All differences do not specifically indicated as significant were not significant (P > 0.05). Significant values are indicated as *P < 0.05 and **P < 0.01. Statistical details, including sample size (n), are reported in the figures and figure legends. Exact P values are provided in Supplementary Table 1.

Microscopy: for scoring marker positive bacteria, at least three independent experiments with three replicates (that is, triplicate coverslips) each were performed. Bacteria were scored by visual enumeration as ≥ n/100 (for 1 h p.i.) or ≥ n/200 (for ≥2 h p.i.) bacteria per replicate. Graphs show mean ± s.e.m.

Cell death (PI nuclear stain): for scoring cell death, at least three independent experiments were performed. PI-positive nuclei were identified using the General Analysis module in Nikon NIS-Elements HC Software and an average from 16 images per sample was calculated. Graphs show mean ± s.e.m.

Scoring intracellular bacteria: to score bacterial burdens, cells from triplicate wells were lysed and bacteria were plated in duplicate on LB agar. Each experiment was performed at least three times. Bacterial colonies were counted using the aColyte3 system (Synbiosys). Graphs show mean ± s.e.m. Significance is shown for the latest time point.

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

Data availability. All data generated or analyzed during this study are included in this published article (and its supplementary information files). The source data that support the findings of this study are available from the corresponding author upon reasonable request.

References
45. Randow, F. & Sale, J. E. Retroviral transduction of DT40. Subcell. Biochem. 40, 383–386 (2006).
46. Nowarski, R. et al. Epithelial IL-18 equilibrium controls barrier function in colitis. Cell 163, 1444–1456 (2015).
47. Krcza, J. et al. DNA methylation defines regional identity of human intestinal epithelial organs and undergoes dynamic changes during development. Gut 68, 49–61 (2019).
48. Ettayebi, K. et al. Replication of human noroviruses in stem cell-derived human enteroids. Science 353, 1387–1393 (2016).
49. Koestler, B. J., Ward, C. M. & Payne, S. M. Shigellosis pathogenesis modeling with tissue culture assays. Curr. Protoc. Microbiol. 50, e57 (2018).
50. Brinkman, E. K., Chen, T., Amendola, M. & van Steensel, B. Easy quantitative assessment of genome editing by sequence trace decomposition. Nucleic Acids Res. 42, e168 (2014).
51. Sidik, S. et al. A Shigella flexneri virulence plasmid encoded factor controls production of outer membrane vesicles. *G3 (Bethesda)* 4, 2493–2503 (2014).

**Acknowledgements**

We thank J. Kendrick-Jones (MRC Laboratory of Molecular Biology, Cambridge) for providing antiserum against NDP52. This work was supported by the MRC (grant no. U1051708648) and the Wellcome Trust (grant no. WT104752MA) to F.R., and by the NIH National Institutes of Allergy and Infectious Diseases (grant nos. R01AI068041-13 and R01AI108834-05) to J.D.M. J.D.M. is an investigator of the Howard Hughes Medical Institute.

**Author contributions**

M.P.W. performed and analyzed all experiments with the following exceptions: K.B.B. performed and analyzed experiments in enterocytes and bacterial proliferation assays in knockout cells; B.-H.K., E.-S.P. and J.D.M. designed, performed and analyzed Salmonella and Shigella infections in mice; A.H. and J.R. generated Shigella mutants; K.N. and M.Z. generated human enteroids; and B.L. and T.H. generated U937 knockouts. M.P.W. and F.R. designed the study and wrote the manuscript.

**Competing interests**

The authors declare no competing interests.

**Additional information**

Extended data is available for this paper at https://doi.org/10.1038/s41590-020-0697-2.

Supplementary information is available for this paper at https://doi.org/10.1038/s41590-020-0697-2.

Correspondence and requests for materials should be addressed to M.P.W. or F.R.

**Peer review information** Zoltan Fehervari was the primary editor on this article and managed its editorial process and peer review in collaboration with the rest of the editorial team.

Reprints and permissions information is available at www.nature.com/reprints.
Extended Data Fig. 1 | IFN-γ prevents proliferation of cytosol-invading S. Typhimurium. a, Lysates of HeLa cells treated with the indicated siRNAs. Blots were probed with the indicated antibodies. PCNA – loading control. b, Colony-forming units (CFU) of S. Typhimurium in HeLa cells at 1 h p.i. c, Fold replication of S. Typhimurium ΔprgH + inv in HeLa cells. d, Confocal micrographs of HeLa cells infected with S. Typhimurium taken at 1 h p.i. stained with DAPI and antibodies against Galectin-8 and ubiquitin (FK2 antibody) (top panel) or over-expressing GFP::LC3C and stained with DAPI and antibody against NDP52 (bottom panel). e, Percentage of Annexin V positive HeLa cells expressing CFP::Galectin-8 amongst cells harbouring intracellular S. Typhimurium. Negative or positive – none or at least one bacterium per cell positive for CFP::Galectin-8. Live imaged every 6 min for 6 h, 12 fields per condition. f, Percentage of PI positive nuclei in HeLa cells infected with S. Typhimurium at 2 h p.i. Cells were treated with DMSO, 50 μM NEC-1s, 10 μM NSA or 50 μM Z-VAD-FMK as indicated. g, Confocal micrograph of HeLa cells infected with S. Typhimurium in the presence of FAM-VAD-FMK and stained with DAPI and antibody against Galectin-8. Image taken at 90 min p.i. Statistical significance was assessed by two-tailed unpaired Student’s t-test (b), one-way (e,f) or two-way (c) analysis of variance (ANOVA) with Tukey’s multiple comparisons test; ns, not significant, **P < 0.01 (exact p values are provided in Supplementary Table 1). Data are expressed as the Mean ± SEM of three (c, e, f) or five (b) independent experiments, or representative of two (a) or three (d, g) independent experiments. HeLa cells were treated with IFN-γ (g) or treated with IFN-γ as indicated (a–c, e, f). Bacteria were counted based on their ability to grow on agar plates (b, c). Scale bar, 10 μm (d, g). Uncropped blots (a) are shown in the Source Data. PI - propidium iodide, p.i. - post-infection, S.T. - S. Typhimurium.
Extended Data Fig. 2 | Cytosol-invading bacteria recruit caspase-4. a, Confocal micrographs of HeLa cells over-expressing GFP::Caspase-4 or -5 at 1 h p.i. with S. Typhimurium and stained with DAPI. Scale bar, 10 μm. b, f, g, Lysates of HeLa cells expressing the indicated GFP::Caspase constructs (b), of cells treated with the indicated siRNAs (f), or of the indicated control or knock-out cells (g). Blots were probed with indicated antibodies, PCNA (b, f), Actin (g) – loading control. Samples in Extended Data Fig. 2g, Fig. 2p and Fig. 8c were obtained from the same experiment. c, d, Percentage of S. Typhimurium positive for the indicated GFP::Caspase constructs (c) or staining positive for endogenous Galectin-8 and/or Caspase-4 (d) in HeLa cells at 1 h p.i.: n > 100 bacteria per coverslip, in triplicate. e, Percentage of FAM-VAD-FMK positive S. Typhimurium amongst bacteria staining positive for endogenous Galectin-8 at 90 min p.i. in HeLa cells treated with siRNAs against caspases as indicated. n > 100 Galectin-8 +ve bacteria per coverslip, in triplicate. h, Percentage of PI positive nuclei in the indicated control or knock-out HeLa cells uninfected or infected with S. Typhimurium at 2 h p.i. i, Fold replication of S. Typhimurium in HeLa cells treated with the indicated siRNAs against caspases. Bacteria were counted based on their ability to grow on agar plates. Statistical significance was assessed by one-way (e, h) or two-way (i) analysis of variance (ANOVA) with Tukey’s multiple comparisons test; ns, not significant, **P < 0.01 (exact p values are provided in Supplementary Table 1). Data are expressed as the Mean ± SEM of three (c, d, e, i) independent experiments, or representative of two (b, f, g) or three (a) independent experiments. HeLa cells were treated with IFN-γ (c) or treated with IFN-γ as indicated (a, d-i). Uncropped blots (b, f, g) are shown in the Source Data. PI - propidium iodide, p.i. - post-infection, +ve - positive.
Extended Data Fig. 3 | The S. flexneri effector OspC3 inhibits interferon-induced pyroptosis. a, PI positive nuclei in HeLa cells infected with the indicated S. flexneri strains at 2 h p.i. b, Percentage of PI positive nuclei in CFP::Galectin-8 expressing HeLa cells infected with the indicated S. flexneri strains in the presence of PI. n = three (WT) and four (ΔospC3) independent repeats. Live imaged every 5 min for 5 h, 10 fields per condition. c, Percentage of Zombie Green positive (that is dead) cells in monolayers of differentiated human epithelial organoids at 2 h p.i. with the indicated S. flexneri strains. n > 50 infected cells per coverslip. d, Confocal micrograph of a monolayer of differentiated human epithelial organoids stained with Zombie Green and antibody against ZO-1 at 1 h p.i. with the indicated S. flexneri strains. Scale bar, 10 μm. e, f, Fold replication of the indicated S. flexneri strains in HeLa cells. Bacteria were counted based on their ability to grow on agar plates. Statistical significance was assessed by one-way (a, b) or two-way (e, f) analysis of variance (ANOVA) with Tukey’s multiple comparisons test; ns, not significant, **P < 0.01 (exact p values are provided in Supplementary Table 1). Data are expressed as the Mean ± SEM of three (a, b, e, f) or four (b) independent experiments, or representative of two (d) independent experiments. HeLa cells were treated with IFN-γ as indicated (a-f). PI - propidium iodide, p.i. - post-infection.
Extended Data Fig. 4 | Cytosol-invading bacteria trigger caspase-4 and gasdermin-D dependent pyroptosis. a, b, g. Fold replication of *S. flexneri* ΔospC3 in HeLa cells treated with the indicated siRNAs against caspases (a) and in control or knock-out HeLa cells (b, g). Bacteria were counted based on their ability to grow on agar plates. c, d. Lysates of HeLa cells at 1 h p.i. with the indicated *S. flexneri* strains. Pro - full length pro-form of GSDMD (shorter exposure), NT - N-terminal domain of GSDMD (longer exposure). Samples in Extended Data Figs. 4c,d and 9a, c were obtained from the same experiment. e, h. Lysates of HeLa cells treated with the indicated siRNAs. * unspecific band. f. PI positive nuclei in the indicated control or knock-out HeLa cells at 2 h p.i. with *S. flexneri* ΔospC3. Statistical significance was assessed by one-way (f) or two-way (a, b, g) analysis of variance (ANOVA) with Tukey's multiple comparisons test; ns, not significant, **P < 0.01 (exact p values are provided in Supplementary Table 1). Data are expressed as the Mean ± SEM of three (b, g, f) or four (a) independent experiments, or representative of two (e, h) or three (c, d) independent experiments. HeLa cells were treated with IFN-γ as indicated (a-h). Blots were probed with indicated antibodies, PCNA (e, h), Actin (c, d) - loading control. Uncropped blots (c-e, h) are shown in the Source Data. PI - propidium iodide, p.i. - post-infection.
Extended Data Fig. 5 | GBP1 recruits GBP2-4 to S. Typhimurium. a, b, Lysates of HeLa cells treated with the indicated siRNAs (a), or from the indicated control or knock-out cells (b). Blots were probed with indicated antibodies, PCNA (a), Actin (b) - loading control. Samples in Extended Data Fig. 5b, 8c and 9f were obtained from the same experiment. c, Percentage of S. Typhimurium positive for the indicated GFP::GBP constructs at 1 h p.i. in the indicated control or knock-out HeLa cells. n > 100 bacteria per coverslip, in triplicate. d, Structured illumination micrograph of HeLa cells expressing GFP::GBP1 and antibody-stained for Galactin-8 at 1 h p.i. with S. Typhimurium. Scale bar, 1μm. Statistical significance was assessed by one-way analysis of variance (ANOVA) with Tukey’s multiple comparisons test (c); ns, not significant, **P < 0.01 (exact p values are provided in Supplementary Table 1). Data are expressed as the Mean ± SEM of three (c) independent experiments, or representative of two (a, b) or three (d) independent experiments. HeLa cells were treated with IFN-γ (c, d) or treated with IFN-γ as indicated (b). Uncropped blots (a, b) are shown in the Source Data. p.i. - post-infection.
Extended Data Fig. 6 | GBPs target cytosol-invading S. Typhimurium. Confocal micrographs of HeLa cells over-expressing GFP::GBP1-7 and stained with DAPI and antibody against NDP52 at 1 h p.i. with S. Typhimurium. Representative of three independent experiments. Scale bar, 10 μm.
Extended Data Fig. 7 | GBP5 recruit and activate caspase-4. a, Percentage of S. Typhimurium positive for GFP:GBP1-4 and/or staining positive for endogenous Caspase-4 in HeLa cells at 1 h p.i. n > 100 bacteria per coverslip, in triplicate. b, Percentage of endogenous Caspase-4 (b) or FAM-VAD-FMK (e) positive S. Typhimurium amongst bacteria staining positive for endogenous Galectin-8 in the indicated control or knock-out HeLa cells at 1 h (b) or 90 min (e) p.i. n > 100 Galectin-8 positive bacteria per coverslip, in triplicate. c, Percentage of endogenous Caspase-4 (c) or FAM-VAD-FMK (f) positive bacteria of the indicated S. flexneri strains in HeLa cells at 1 h p.i. n > 100 bacteria per coverslip, in triplicate. d, Confocal micrograph of a monolayer of differentiated human epithelial organoids antibody-stained for GBP1 and Caspase-4 with S. flexneri ΔipaH9.8. g, Confocal micrographs of HeLa cells treated with DMSO or Carfilzomib as indicated at 1 h p.i. with S. flexneri ΔospC3 in the presence of FAM-VAD-FMK and stained with DAPI. h-j, Percentage of FAM-VAD-FMK positive S. flexneri ΔospC3 ΔipaH9.8 (h, j) or S. flexneri ΔospC3 (i) at 1 h p.i. in the indicated control or knock-out HeLa cells (h,i) or in HeLa cells treated with the indicated siRNAs against caspases and 1 μM Carfilzomib (j). n > 100 bacteria per coverslip, in triplicate. Statistical significance was assessed by one-way analysis of variance (ANOVA) with Tukey’s multiple comparisons test (b, c, e, f, h-j); ns, not significant, **P < 0.01 (exact p values are provided in Supplementary Table 1). Data are expressed as the Mean ± SEM of three (a-c, e, f, h-j) independent experiments, or representative of two (d, g) independent experiments. HeLa cells were treated with IFN-γ (a, b, d, e, g, h) or treated with IFN-γ as indicated (c, f, i, j). Cells were treated with DMSO or 1 μM Carfilzomib as indicated (c, f, g). Scale bars, 10 μm (d, g). p.i. - post-infection, +ve - positive.
Extended Data Fig. 8 | GBPs govern gasdermin-D dependent pyroptosis. a, b, Percentage of FAM-VAD-FMK positive cells among HeLa cells containing S. Typhimurium positive for endogenous Galectin-8 at 90 min p.i. (a) or containing S. flexneri ΔospC3 at 1 h p.i. (b); cells treated with siRNAs against GBPs as indicated. n > 100 cells with Galectin-8 +ve bacteria (a) or n > 100 infected cells (b) per coverslip, in triplicate. c, Lysates of the indicated control or knock-out HeLa cells infected with S. flexneri ΔospC3 for 1h. Pro - full length pro-form of GSDMD (shorter exposure), NT - N-terminal domain of GSDMD (longer exposure). Samples in Extended Data Fig. 8c, Extended Data Fig. 5b and Extended Data Fig. 9f were obtained from the same experiment.

d, e, Percentage of PI positive nuclei in the indicated control or knock-out HeLa cells at 2 h p.i. with S. Typhimurium (e) or S. flexneri ΔospC3 (d). f, Lysates of the indicated control or knock-out U937 cells. g, Sanger sequencing chromatogram of control and GBP3 knock-out U937 cells. Statistical significance was assessed by one-way analysis of variance (ANOVA) with Tukey’s multiple comparisons test (a, b, d, e); ns, not significant, **P < 0.01 (exact p values are provided in Supplementary Table 1). Data are expressed as the Mean ± SEM of three (b, d, e) or four (a) independent experiments, or representative of two (c, f) independent experiments. HeLa cells were treated with IFN-γ as indicated (a-f). Blots were probed with indicated antibodies, Actin – loading control (c, f). Uncropped blots (c, f) are shown in the Source Data. PI - propidium iodide, p.i. - post-infection, +ve - positive. S. T - S. Typhimurium.
Extended Data Fig. 9 | Processing and secretion of IL-18 during S. flexneri infection. a, c. Lysates of HeLa cells prepared at 1 h p.i. with the indicated S. flexneri strains. Samples in Extended Data Fig. 9a, c and Extended Data Fig. 4c, d were obtained from the same experiment. b, d. Release of IL-18 from HeLa cells infected with the indicated S. flexneri strains for 1 h. e. Lysates of HeLa cells expressing the indicated FLAG-tagged caspase alleles and treated with the indicated siRNAs prepared at 1 h p.i. with S. flexneri ΔospC3. f. Lysates of the indicated control or knock-out HeLa cells prepared at 1 h p.i. with S. flexneri ΔospC3. Samples in Extended Data Fig. 9f, Extended Data Fig. 5b and Extended Data Fig. 8c and were obtained from the same experiment. g. Release of IL-18 from the indicated control or knock-out HeLa cells infected with S. flexneri ΔospC3 for 1 h. Statistical significance was assessed by one-way analysis of variance (ANOVA) with Tukey’s multiple comparisons test (b, d, g); ns, not significant, *P < 0.05, **P < 0.01 (exact p values are provided in Supplementary Table 1). Data are expressed as the Mean ± SEM of three (b, d, g) independent experiments, or representative of two (e, f) or three (a, c) independent experiments. HeLa cells were treated with IFN-γ as indicated (a-g). Blots were probed with indicated antibodies, Actin – loading control (a, c, e, f). Uncropped blots (a, c, e, f) are shown in the Source Data.
Extended Data Fig. 10 | Schematic illustration of the GBP-CASP4 pathway. Interferon-induced guanylate-binding proteins (GBPs) transform Gram-negative bacteria into a caspase activation platform by coating their surface with a polyvalent protein array. The bacterial GBP coat may serve to foster contacts between CASP4 and its microbial ligand, the hydrophobic lipid A moiety of LPS, an integral and otherwise inaccessible component of the bacterial outer membrane. GBPs control CASP4 activation in a hierarchical manner; GBP1 initiates platform assembly, GBP2 and GBP4 control CASP4 recruitment, whereas GBP3 governs CASP4 activation. Once activated CASP4 cleaves GSDMD and IL-18 to cause pyroptotic cell death and cytokine release, thereby destroying the bacterial niche and alerting neighbouring cells of imminent danger. The cytosol-adapted bacterium *Shigella flexneri* antagonizes the pathway through secretion of the CASP4 inhibitor OspC3.
Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Research policies, see Authors & Referees and the Editorial Policy Checklist.

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

- [x] The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- [ ] A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- [x] The statistical test(s) used AND whether they are one- or two-sided
  - *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
- [x] A description of all covariates tested
- [ ] A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- [ ] A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- [ ] For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted
  - *Give P values as exact values whenever suitable.*
- [ ] 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
- [x] Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

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

- Confocal images were taken on a Zeiss 780 microscope using ZEN (Black) software version 2010 and 2012.
- Live imaging was performed on a Nikon Eclipse Ti equipped with an Andor Revolution XD system and a Yokogawa CSU-X1 spinning disk unit using Andor software version IQ2 and IQ3.
- Super resolution images were acquired using an Elyra S1 structured illumination microscope (Carl Zeiss Microscopy Ltd, Cambridge, UK) using ZEN (Black) software version 2010 and 2012.
- High Content Analysis microscopy was performed on a Nikon Eclipse Ti equipped with Andor Neo sCMOS camera using Nikon NIS-Elements HC Software version 4.4.

Data analysis

- High Content images were analysed using General Analysis module in Nikon NIS-Elements HC Software version 4.4.
- Imaris versions 7.4-8.0 [Bitplane] and/or Adobe Photoshop CS6 version 13.0.6 was used to analyze immunofluorescence images.
- Microsoft Excel version 16.16.09 was used for data analysis.
- All data were tested for statistical significance with Prism software version 7 and 8 [GraphPad Software, La Jolla, CA].
- Adobe Illustrator CS6 version 16.0.4 was used for creating figures.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.
Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

All data supporting the findings of this study are included in the paper and the Supplementary Information. The source data that support the findings of this study are available from the corresponding author upon reasonable request.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

- [x] Life sciences
- [ ] Behavioural & social sciences
- [ ] Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/mr-reporting-summary-list.pdf

Life sciences study design

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

**Sample size**
No statistical methods were used to predetermine sample size for experimentation. Rather, sample size was chosen following standard practice in the field. Given the minimal experimental variation in clonal cell lines, a maximum of three technical replicates were used per sample, and experiments were repeated multiple times independently. Each data set was compiled from at least 3 (or at least 2 for in vivo) separate experiments. Statistical significance was determined across all samples. The number of independent experiments is mentioned in the respective figure legend.

**Data exclusions**
No technical replicates were excluded.

**Replication**
Experimental results shown in the manuscript were reliably reproducible. For mouse studies, at least two biologically independent experiments were repeated for all results in the manuscript. If group size was small (e.g., due to limited availability of mouse strains), data from replicate experiments were pooled for graphical representation. All results are biological replicates obtained in independent experiments.

**Randomization**
No randomization; all experiments were started from common pools of cells and bacteria. In vivo experiments used both male and female mice at 6-8 weeks of age.

**Blinding**
No blinding was applied. Blinding was not necessary for experiments, where data were generated by a digital reading or by quantitative measurement.

Reporting for specific materials, systems and methods

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**

| n/a | Involved in the study |
|-----|-----------------------|
|     | Antibodies            |
|     | Eukaryotic cell lines |
|     | Palaeontology         |
|     | Animals and other organisms |
|     | Human research participants |
|     | Clinical data         |

**Methods**

| n/a | Involved in the study |
|-----|-----------------------|
|     | ChiP-seq              |
|     | Flow cytometry        |
|     | MRI-based neuroimaging |

**Antibodies**

Antibodies used:
- Antibody: clone (if applicable), catalog number, manufacturer, dilution(s)
  - β-actin: ab8227, Abcam, 1:5000 WB
  - Galectin-3: ab183637, Abcam, 1:1000 WB
  - NDRG2: ab68888, Abcam, 1:10000 WB
  - GBP1: EPR8285, ab131255, Abcam, 1:20000 WB
Validation

All common antibodies used consistent with the literature and data sheets.

- B-actin: ab8227, loading control, https://www.abcam.com/beta-actin-antibody-ab8227.html
- Galectin-8: ab183637, WB - siRNA knock-down
- NQ52: ab66586, WB - siRNA knock-down
- GBP2: ab132355, WB - CRISP/Cas9 knock-out
- Gasdermin-D: 96458, WB - siRNA knock-down & CRISP/Cas9 knock-out
- GFP: 632381, WB - siRNA knock-down
- Ubiquitin: BML-PW8810, https://www.enzolifesciences.com/BML-PW8810/mono-and-polyubiquitylated-conjugates-monoclonal-antibody-rk2/
- Caspase-4: MO29-3, WB & IF - siRNA knock-down & CRISP/Cas9 knock-out
- IL-18: PM014, https://www.mibio.bio.com/bio/g/dl/A/index.html?pcd=PM014
- Cas9: MAC133, https://www.merckmillipore.com/G8/en/product/Anti-Cas9-Antibody-clone-7A9,MM_NF-MAC133
- GBP2: NBP1-77478, WB - CRISP/Cas9 knock-out
- FIP200: 10047-2-AP, WB - siRNA knock-down
- GBP1: 15303-1-AP, WB & IF - siRNA knock-down & CRISP/Cas9 knock-out
- Galectin-8: A1305, IF & qPCR - siRNA knock-down
- Caspase-1: sc-515, WB - siRNA knock-down & CRISP/Cas9 knock-out
- IRF-1: sc-74530, WB - siRNA knock-down
- PCNA: sc-56, loading control, https://www.scbt.com/p/pcna-antibody-pc10
- STAT1: sc-417, WB - siRNA knock-down
- GBP2: sc-10588, WB & qPCR - siRNA knock-down
- FLAG-tag: F1804, WB - siRNA knock-down
- FLAG-tag: F7425, WB - siRNA knock-down
- Actin: A3853, loading control, https://www.sigmaaldrich.com/catalog/product/sigma/sa3853?lang=en&region=GB
- The antisemir against NQ52, WB & IF - siRNA knock-down
- IF - immunofluorescence, WB - western blot

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s) - HeLa cells were obtained from the European Collection of Cell Culture ECACC No: 93021013.
- U937 cells were obtained from the Biological Resource Centre "Cellulonet".

Authentication - Cell lines obtained directly from ECACC and Cellulonet. Cell morphology was assessed for authentication.

Mycoplasma contamination - All cells tested negative for mycoplasma.

Commonly misidentified lines (See ITCC register) - No commonly misidentified cell line was used.

Animals and other organisms

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

Laboratory animals - Six to eight week-old male and female C57BL/6N, Gbp1-/- (B6N), Gbp2-/- (B6N) and Casp11-/- (B6N) mice originated from The Jackson Laboratory or were generated in-house as published or reported in the Methods.

Wild animals - No wild animals were used in this study.
| Field-collected samples | No field collected samples were used in this study. |
|-------------------------|-----------------------------------------------------|
| Ethics oversight         | All procedures used in this study complied with federal guidelines and institutional policies by the Yale animal care and use committee (protocol 2017-11647). |

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