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Supporting Information for

LPS-aggregating proteins GBP1 and GBP2 are each sufficient to enhance caspase-4 activation both *in cellulo* and *in vitro*

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Other supporting materials for this manuscript include the following:

Movies S1 to S3

Detailed materials and methods

Cell lines and cell culture

HeLa and A549 cells (ATCC) were grown in DMEM (Gibco 11995-065) supplemented with 9% heat inactivated FBS (Omega Scientific) and non-essential amino acids (Gibco 11140-050). Cells were grown at 37°C in 5% CO_2. Cell lines were routinely tested for mycoplasma contamination. Cell lines were authenticated using GenePrint 10 (Promega) performed by the Duke University DNA Analysis Facility.

Knockout cells

For A549 GBP1^{KO}, GBP2^{KO} and GBP4^{KO} cells, single guide RNAs (sgRNAs) sequences to human GBPs were selected using the optimized CRISPR design site crispr.mit.edu. The GBP1 sgRNA sequence was CATTACACAGCCTATGGTGG and the GBP2 sgRNA sequence was CTAGTTCTGCTCGACACTGA. For GBP4 the sgRNA sequences were ATTGTAGGGCTATACCGCACAGG and TATCTCATGAATCGTCTTGCAGG. sgRNAs were cloned into PX459 containing Puromycin resistance ((1); pSpCas9(BB)-2APuro (PX459) was a gift from Feng Zhang (Addgene plasmid #48139) or PX458 containing an eGFP cassette ((1); pSpCas9(BB)-2A-GFP (PX458) was a gift from Feng Zhang (Addgene plasmid #48138) following the Zhang lab, Addgene CRISPR Genome Engineering Toolbox (www.addgene.org/crispr/zhang/). A549 cells were transfected with PX459 or PX458 plasmids containing guide RNAs using FuGENE HD transfection reagent (Promega E2311) following manufacturers guidelines. 48 hours post transfection, PX459 transfected cells were put under Puromycin (Generon 1860-25) selection at 1-2 µg/ml for 48 hours and PX458 transfected cells were sorted one cell per well into a 48 well plate using a FACS Aria Fusion Cell Sorter.

A549 GBP3^{KO}, GBP5^{KO}, and HeLa GBP2^{KO}, GBP3^{KO}, GBP4^{KO}, and GBP5^{KO} cells were generated by the Duke Functional Genomics core as previously described (2). sgRNAs were designed using CHOPCHOP (3) and Cas-OFFinder (4), then cloned into PX459 V2 (Addgene #62988; (1)). For A549 GBP3^{KO} cells the sgRNA sequences were TCGATCTGCCCATTCACCGC and AGAACTTCCGGATACAGAGT. For A549 GBP4^{KO} cells the sgRNA sequences were ATTGTAGGGCTATACCGCACAGG and TATCTCATGAATCGTCTTGCAGG. For A549 GBP5^{KO} cells the sgRNA sequences were GCTCATTAAAGTTCTCGATG and GCAAAGTAACATCCTAGACA. For HeLa GBP2^{KO} cells the sgRNA sequences were AGAGCTGACAGATCGAATCA and TCGTCTACAGAATTGTTACC. For HeLa GBP3^{KO} cells the sgRNA sequence was CCTCATTGAGAACACTAATG. For HeLa GBP4^{KO} cells the sgRNA sequences were CGTCTTGCAGGAAAGCGCAA and ATTGTAGGGCTATACCGCAC. For HeLa GBP5^{KO} cells the sgRNA sequences were GCTCATTAAAGTTCTCGATG and GCAAAGTAACATCCTAGACA. A549 cells were transfected with sgRNAs using Lipofectamine 3000 (ThermoFisher Scientific) according to manufacturer's instructions. 24 hours after transfection, cells were selected with 2 mg/mL puromycin (Sigma) for three days. Cells were diluted to isolate single cells, which were expanded to generate clonal cell lines. Knockouts were validated by western blot or sanger sequencing.

To generate CRISPR Cas-9 mediated knockout of *GBP1* in HeLa cells, we chose an sgRNA sequence targeting exon 2 that would minimize off-target editing of the closely related *GBP3* gene, CATTACACAGCCTATGGTGG. The sgRNA was ordered as modified synthetic sgRNA from Synthego and delivered to HeLa cells by electroporating 200,000 cells with 4 ug TrueCut Cas9 protein (ThermoFisher Scientific) complexed with 180 pmol *GBP1* sgRNA using a Neon system (ThermoFisher Scientific) with the following settings: 1005 V, 35 ms, 2 pulses. Following electroporation, cells were recovered in 6 well dishes for three days before harvesting to check KO efficiency. Cells were diluted to isolate single cells, then expanded to generate a clonal knockout line.

Bacterial strains

Shigella flexneri strain 2457T was used. *S. flexneri* Δ*ipaH9.8*, *S. flexneri* Δ*ospC3*, and *S. flexneri* Δ*ipaH9.8*Δ*ospC3* knockouts were made as previously described (5-7). *S. flexneri* was cultured on tryptic soy broth (TSB, Millipore Sigma) agar plates containing 0.01% Congo Red (Millipore Sigma), then grown in liquid culture in TSB. *Salmonella enterica* Typhimurium strain 14028s was used throughout. *S.* Typhimurium was cultured on lysogeny broth (LB), Miller formulation agar plates, or in liquid LB.

Infections

For *S. flexneri* infections, *S. flexneri* was streaked on an agar plate, then single colonies were picked and grown overnight at 37°C shaking, in 3 ml TSB. The next day 250 µl overnight culture was diluted into 5 ml TSB and grown for approximately 1 hour 45 minutes, until OD600 was between 0.7 and 1. 1 ml bacterial culture was pelleted and resuspended in 1 ml infection media (phenol red free DMEM (Gibco 31053-028), supplemented with 5% FBS and non-essential amino acids). Bacteria was further diluted in infection media to have enough bacteria for a multiplicity of infection (MOI) of 5 in 100 µl per well of 96 well plate, or 1 ml per well of 24 well plate. Media was aspirated from cells and diluted *S. flexneri* was added to each well. Plate was centrifuged for 10 min, 800×g, at room temperature. The end of the centrifugation was considered the start of infection, and plates were then put in 37°C incubator with 5% CO_2. After 30 minutes, cells in 96 well plate were washed once with 200 µl warm HBSS. For microscopy, cells in 24 well plate were washed twice with 1ml warm HBSS. After washing, infection medium containing 50 µg/ml gentamicin was added to wells to kill extracellular bacteria. For *S.* Typhimurium infections, single colonies were grown overnight at 37°C shaking, in 1.5 ml LB. Next day 45 µl overnight culture was diluted into 1.5 ml LB and grown for 2 hours 40 minutes. Bacterial culture was pelleted and resuspended in infection media (described above for *S. flexneri* infections). Bacteria was further diluted in infection media to have enough bacteria for a multiplicity of infection (MOI) of 200 in 100 µl per well of 96 well plate. Media was aspirated from cells and diluted *S.* Typhimurium was added to each well. Plate was centrifuged for 10 min, 800×g, at room temperature. The end of the centrifugation was considered the start of infection, and plates were then put in 37°C incubator with 5% CO_2. After 30 minutes, cells in 96 well plate were washed once with 200 µl warm HBSS. After washing, infection medium containing 50 µg/ml gentamicin was added to wells to kill extracellular bacteria.

Cell death assays, bacterial luminescence and IL-18 ELISA

Cells were plated in 96 well plate with white sides (Corning 3610). *S. flexneri* or *S.* Typhimurium infections were performed as described above, sytox green (Invitrogen) was added at the same time as gentamicin at a final concentration of 1.5-3 nM in 200 µl per well of 96 well plate. For lysed control wells, 1% triton X-100 was added to media containing sytox green and gentamicin. Sytox green fluorescence and bacterial luminescence (*S. flexneri* only) was measured every 30 minutes starting at 1 hpi. Fluorescence and luminescence were measured using Enspire 2300 multilabel plate reader (Perkin Elmer). At 3 hpi, 100 µl supernatant was removed from each well, 30 µl was used for LDH assay and the rest was frozen at -80°C until running IL-18 ELISA.

LDH was measured by CytoTox-ONE homogenous membrane integrity assay (Promega). Briefly, 30 µl supernatant was mixed with 30 µl CytoTox-ONE reagent, incubated for 7 minutes at room temperature, then measured on Enspire 2300 plate reader. IL-18 was measured using IL-18 ELISA matched antibody pair (Thermo Fisher BMS267-2MST) following manufacturer's instructions.

Western blot

Cells were plated in a 12 well plate and primed with IFNγ overnight (if applicable). Next day, cells were washed twice with cold PBS, then lysed in 90 µl RIPA buffer (Millipore Sigma) containing protease inhibitors (Millipore Sigma P8340) on ice for 30 minutes. Lysates were clarified through centrifugation at 20,000×g for 10 minutes at 4°C. Clarified lysate was mixed with Laemmli sample buffer (BioRad) containing beta-mercaptoethanol and boiled for 5 minutes. Samples were run on 4-20% mini-PROTEAN TGX Stain-free gel (BioRad) with all blue protein standards (BioRad) as a ladder. Running buffer contained 25 mM Tris base, 190 mM glycine, 0.1% SDS. Gel was transferred to PVDF using BioRad Trans-blot turbo. Following transfer, membrane was dried then incubated with primary antibody in Tris-buffered saline containing 0.1% tween 20 (TBST) and 5% non-fat milk. Blots were incubated in primary antibody either 1 hour at room temperature, or overnight at 4°C. Following primary antibody, blots were washed three times in TBST, then incubated with secondary antibody in 5% milk in TBST for 45-60 minutes. Blots were then washed five times in TBST then incubated with Clarity ECL substrate (BioRad) or SuperSignal West Femto ECL substrate (Thermo Fisher). Blots were imaged using an Azure 500 imaging system. Primary antibodies were used at the following concentrations: GBP1 (Abcam ab131255, 1:5000), GBP2 (Santa Cruz sc271568, 1:200), GBP4 (Proteintech 17746-1-AP, 1:10,000), GBP5 (Cell Signaling 67798, 1:5,000), GAPDH (Abcam ab9485, 1:10,000), mCherry (Abcam ab183628, 1:10,000), CASP4 (MBL M029-3, 1:1000), GSDMD (Sigma G7422, 1:1000). Secondary antibodies: goat anti-rabbit HRP (BioRad 1706515 or Invitrogen 65-6120, 1:5,000), anti-mouse HRP (Santa Cruz sc-525409, 1:5,000).

Immunofluorescence microscopy

Cells were plated on glass coverslips in 24 well plates and primed overnight with IFNγ. The next day cells were infected with *Shigella* at an MOI of 5. At the indicated time points after infection, media was aspirated from wells and cells were fixed in 4% paraformaldehyde in PBS for 15 minutes at room temperature. Cells were washed three times with PBS, then permeabilized with 0.1% triton X-100 in PBS for 15 minutes. Cells were blocked for 30 minutes in PBS containing 5% BSA and 2.2% glycine. Anti-GBP1 (Abcam ab131255, 1:150 dilution) was added in blocking buffer for 1 hour at room temperature. Cells were washed three times with PBS containing 0.05% triton X-100, then incubated with donkey anti-rabbit IgG Alexa Fluor 568 (Thermo Fisher A10042, 1:1000 dilution) and Hoechst 33258 (1µg/mL) for 45 minutes in blocking buffer. Cells were then washed three times with PBS containing 0.05% triton X-100 and mounted on glass slides. For experiments without any antibody staining, following fixation, cells were washed three times in PBS then incubated with 1 µg/mL Hoechst for 20 minutes in PBS, washed twice with PBS and mounted on glass slides. Coverslips were mounted with mounting media containing 9 parts Mowiol solution (100 mM Tris-HCl, pH 8.5, 25% glycerol, 125 µg/mL Mowiol 4-88) and 1 part PPD solution (0.1 mg/mL 1,4-Phenylenediamine dihydrochloride in water). Mounting media was allowed to harden overnight at room temperature. Images were acquired using a Zeiss Axio Observer Z1 microscope using Zeiss Plan-Apochromat 63×/1.4 oil objective, or a DeltaVision Elite Deconvolution microscope with UPLSAPO 100×/1.40 oil objective. Images were processed with DeltaVision software for deconvolution, or with Fiji.

Time-lapse microscopy of infected cells

700,000 A459 GBP1-KO pInducer-mCherry, -GBP1, or GBP1^{3R} cells were plated on glass bottom 10 mm microwell dishes and treated with 2 µg/ml aTc and 100 U/ml IFNγ for 20-22 h to stimulate mCherry fusion protein expression. Cells were infected with GFP-expressing *S. flexneri* Δ*ipaH9.8*Δ*ospC3* as described above with the following alterations. After incubating cells at 37 °C and 5% CO_2 following centrifugation for 10 min at 700 x g at room temperature, cells were washed three times with 2 ml HBSS. After washing,

2 ml infection media supplemented with 150 nM sytox blue (Invitrogen) was added to each dish to stain cells undergoing pyroptosis. Time-lapse images of infected cells were acquired every 3 min with a Zeiss 880 AiryScan Fast Inverted Confocal on AxioObserver Z1 microscope using a Zeiss Plan-Apochromat 63×/1.4 oil objective, with stage incubator set to 37°C and 5% CO_2 buffering. All images were processed with Fiji.

LPS electroporation

Electroporation was done using the Neon Transfection System (Thermo Fisher). Cells were plated in a 6 well plate and were either left untreated or were treated with 100 U/ml IFNγ overnight. The next day, cells were trypsinized, washed twice with PBS and the cell number was determined. Cells were pelleted and diluted in Neon buffer R to 5,000 cells/µl. *E. coli* O55:B5 LPS (Invivogen tlrl-pb5lps) was diluted in Neon R buffer to 500 µg/ml, 100 µg/ml and 50 µg/ml. Cell and LPS dilutions were gently mixed and 500,000 cells were electroporated with either 2.5 µg, 0.5 µg or 0.25 µg LPS in a 100 µl Neon pipette tip using electrolytic buffer E2 and 2 pulses of 1005 V for 35 ms. After electroporation prewarmed infection media supplemented with 3 µg/ml propidium iodide was quickly added to cells and 50,000 cells were plated per well in black tissue culture treated 96-well plates in triplicates. Non-electroporated cells left untreated or treated with 1% triton served as live and dead controls. Fluorescence was measured using an Enspire 2300 multilabel plate reader (Perkin Elmer) at 1, 2, and 4 hours post electroporation. After measuring fluorescence, 100 µl supernatant was removed from each well and was frozen at -80°C until running IL-18 ELISA. IL-18 was measured using IL-18 ELISA matched antibody pair (Thermo Fisher BMS267-2MST) following manufacturer's instructions.

LPS transfection

Transfection protocol was modified from Santos et al. (8) A549 cells were seeded in a 96 well plate, 2.5×10^4 cells per well. The next day, LPS transfection mixture was prepared- for each well 75 µl optimem (31985-062), 1 µl lipofectamine 2000 (Invitrogen), and either 1 µg or 0.1 µg *E. coli* O55:B5 LPS (Invivogen tlrl-pb5lps). LPS and lipofectamine mixture was incubated for 20 minutes before adding to cells. During incubation, media in 96 well plate was aspirated and 75 µl optimem containing 0.5 µM sytox green (Invitrogen) was added to each well. Lysed control wells contained 75 µl optimem, 0.5 µM sytox green, and 1% triton X-100. 75 µl lipofectamine and LPS mixture was added on top of media containing sytox green.

Plasmids

All GBP expression plasmids were in the lentiviral pInducer20 backbone (9) containing a C-terminal mCherry (GBP4) or N-terminal mCherry (all other constructs). Full plasmid sequences are provided as a supplemental file. For experiments using these plasmids, expression was induced by adding 1 µg/ml anhydrotetracycline (Takara) overnight in cell culture media, or for titrations at concentration indicated in figure. When applicable, anhydrotetracycline was added at the same time as IFNγ.

For *S. flexneri* luminescence experiments, strains were transformed with ilux pGEX(-), which was a gift from Stefan Hell (Addgene plasmid #107879; http://n2t.net/addgene:107879 ; RRID:Addgene_107879) *S. flexneri* was transformed with pGFPmut2 (10) for microscopy experiments.

For knockdown of CASP4, lentiviral vectors from the Mission shRNA collection (Sigma). Plasmids expressed shRNAs targeting GFP or CASP4 (shRNA TRCN0000003512) in the pLKO.1 backbone.

Lentivirus production and cell line complementation

293T cells were plated in a 6 well plate with 1×10^6 cells per well in 2ml. The next day cells were transfected using TransIT 293 transfection reagent (Mirus), following manufacturer's instructions. Each

well was transfected with 1 µg pInducer plasmid, 750 ng pSPAX2 (Addgene), 250 ng VSVG. At 24 hours post transfection, media was removed, and 3 ml fresh media added. Supernatant containing virus was collected at 48 and 72 hours post infection and filtered through 0.45 µm nylon filters (corning). Virus was frozen at -80°C until use.

For transduction, A549 cells were trypsinized and resuspended in media containing 10 µg/mL polybrene (Millipore Sigma) to a concentration of 3.33×10^4 cells/ml. In 6 well plate, 250 µl lentivirus supernatant (described above) and 1.5 ml diluted cells were added to each well. Cells were incubated for 48-72 hours, then 2 mg/ml geneticin was added for approximately 10 days to select for cells containing the pInducer plasmid. Cells transduced with pLKO.1 shRNA plasmids were selected with 1 µg/ml puromycin for 5 days.

Expression, purification, and prenylation of recombinant protein

Recombinant GBP1, GBP5, and FTase were expressed and purified as described previously(11). N-terminally His_6-tagged GBP1 and GBP2 were expressed in *E. coli* strain BL21 CodonPlus (DE3) RIL from bacterial vector pQE-80L. N-terminally His_{10}-tagged GBP5 was expressed in *E. coli* strain Rosetta (DE3) pLysS from bacterial vector pQE-80L. N-terminal His_6-tagged farnesyltransferase (FTase) and geranylgeranyltransferase (GGTase) were expressed in *E. coli* strains Rosetta (DE3) pLysS and BL21 CodonPlus (DE3) RIL from pRSF-Duet1 vector. Bacteria were cultivated in terrific broth media (GBPs), or terrific broth media supplemented with 60 µM $ZnCl_2$ (Ftase, GGTase) and grown at 37 °C and 90 rpm to an OD_{600} of 0.4–0.8. After decreasing the temperature to 20 °C, protein expression was induced with 100 µM IPTG. For FTase and GGTase expression, an additional 0.5 mM $ZnCl_2$ was added to the culture. Bacteria were harvested after 16–18 h at 3000 x g for 15 min at 4°C (Sorvall LYNX 6000 centrifuge, F9-6x1000 LEX rotor, Thermo Fisher Scientific).

Buffer compositions for the purification of recombinant GBPs and FTase or GGTase differed in the use of 50 mM HEPES, pH 7.8 for FTase and GGTase instead of 50 mM Tris-HCl, pH 7.9 for GBPs. Harvested bacteria were resuspended in buffer A (50 mM Tris-HCl, pH 7.9, 500 mM NaCl, 5 mM $MgCl_2$) supplemented with 1 mM phenylmethylsulfonyl fluoride to inhibit proteases and sonicated on ice at 30% amplitude pulsing at 1 sec on/ 1 sec off for a total of 10 min (Ultrasonic homogenizer Sonoplus HD 2200, Bandelin) with the temperature of the resuspension kept below 8 °C. Cell debris was removed from lysate containing soluble protein by centrifugation at 35,000 x g and 4 °C for 45 min (Sorvall LYNX 6000 centrifuge, F21-8x50y rotor, Thermo Fisher Scientific). Proteins were further purified by immobilized metal affinity chromatography (IMAC) followed by size-exclusion chromatography (SEC). All chromatography columns were connected to ÄKTA Purifier or Prime systems (GE Healthcare Life Sciences). After loading of soluble proteins, the IMAC column (30 ml HisPur Cobalt Resin, 30 ml) was sequentially washed with 5-10 column volumes (CVs) buffer A and 3-4 CVs buffer B_{10} (50 mM Tris-HCl, pH 7.9, 150 mM NaCl, 5 mM $MgCl_2$, 10 mM imidazole). His-tagged protein was eluted with 2 CVs buffer B_{150} (50 mM Tris-HCl, pH 7.9, 150 mM NaCl, 5 mM $MgCl_2$, 150 mM imidazole). GBP containing fractions from IMAC were pooled and precipitated by slowly adding 3 M $(NH4)_2SO_4$. $(NH4)_2SO_4$ protein precipitates were dissolved in buffer C (50 mM Tris-HCl, pH 7.9, 150 mM NaCl, 5 mM $MgCl_2$) and loaded on a with buffer C equilibrated SEC column (Superdex 200 26/ 60, 320 ml) to remove $(NH_4)_2SO_4$ and aggregated protein from monomeric protein. FTase and GGTase containing fractions from IMAC were pooled, concentrated via ultra-filtration using Vivaspin 20 centrifugal columns (10 kDa cut-off, Sartorius), and loaded on the SEC column (Superdex 200 26/ 60, 320 ml) to isolate monomeric protein. Fractions containing monomeric GBPs, FTase, and GGTase were pooled, concentrated via ultra-filtration using 10 kDa cut-off Vivaspin 20 centrifugal columns, frozen in liquid nitrogen, and stored at -80°C.

GBP1 was farnesylated *in vitro* as described previously(12). Monomeric GBP1, GBP2, and GBP5 were incubated for 16 h at 4°C in glass vials with farnesyl pyrophosphate (FPP) or geranylgeranyl

pyrophosphate (GGPP) and FTase or GGTase in buffer D (50 mM Tris-HCl, pH 7.9, 5 mM $MgCl_2$, 150 mM NaCl, 10 µM $ZnCl_2$). For farnesylation, 60 µM GBP1 was supplemented with 150 µM FPP and 1.25 µM FTase in a total volume of 4 ml. For geranylgeranylation, 60 µM GBP2 or GBP5 were supplemented with 150 µM GGPP and 5 µM GGTase in a total volume of 4 ml. Reaction mixtures were supplemented with $(NH_4)_2SO_4$ (3 M stock solution) to a final concentration of 1.25 M and loaded on a hydrophobic interaction chromatography (HIC) column (Butyl FF 16/10, 20 ml), equilibrated with buffer E (50 mM Tris-HCl, pH 7.9, 5 mM $MgCl_2$, 1.2 M $(NH_4)_2SO_4$). For GBP1, after loading, the HIC column was sequentially washed with 2 CVs buffer E and with 2 CVs of buffer E with its initial $(NH_4)_2SO_4$ concentration decreased to 60%. Farnesylated GBP1 was separated from non-farnesylated GBP1 by decreasing the $(NH_4)_2SO_4$ concentration further in a continuous gradient over 3 CVs from 60% to 45% $(NH_4)_2SO_4$ (elution of farnesylated GBP1) followed by a continuous gradient over 3.75 CVs from 45% to 25% $(NH_4)_2SO_4$ (elution of non-farnesylated GBP1). For GBP2 and GBP5, after loading, the HIC column was sequentially washed with 2 CVs buffer E and with 2 CVs of buffer E with its initial $(NH_4)_2SO_4$ concentration decreased to 80%. Geranylgeranylated GBP2 and GBP5 were eluted from the HIC by decreasing the $(NH_4)_2SO_4$ concentration from 80% to 0% in a continuous gradient over 20 CVs. Fractions with prenylated GBPs were pooled, concentrated via ultra-filtration using 10 kDa cut-off Vivaspin 20 centrifugal columns and further purified by SEC to isolate monomeric protein. Following SEC, fractions with monomeric prenylated GBPs were pooled, concentrated via ultra-filtration, frozen in liquid nitrogen, and stored at -80°C. Concentrations of proteins were calculated according to Lambert–Beer law, using absorption at 280 nm in buffer F (6 M guanidine hydrochloride, 20 mM potassium phosphate, pH 6.5) and respective molar absorption coefficients (GBP1 43,240 $M^{-1}cm^{-1}$, GBP2 52,050 $M^{-1}cm^{-1}$, GBP5 46,005 $M^{-1}cm^{-1}$, FTase 158,235 $M^{-1}cm^{-1}$, GGTase 138,170 $M^{-1}cm^{-1}$).

Labeling of recombinant proteins with fluorescent dyes

After exchanging buffer C to buffer G (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 5 mM $MgCl_2$), recombinant GBPs were incubated with Alexa Fluor 488 C_5 maleimide dye or Alexa Fluor 647 C_2 maleimide dye (Invitrogen) in a ratio of 1:1 or 1:2 on ice for 10–20 min. Labeling reactions were stopped by changing buffer G to buffer C supplemented with 2 mM DTT via ultra-filtration using 10 kDa cut-off Vivaspin Turbo 4 centrifugal columns. Concentrations of proteins and labeling efficiencies were calculated according to Lambert-Beer law, using absorptions at 280 nm, 491 nm, and 651 nm in buffer C, respective molar absorption coefficients (GBP1 45,840 $M^{-1}cm^{-1}$, GBP2 53,860$M^{-1}cm^{-1}$, GBP5 45,380 $M^{-1}cm^{-1}$, Alexa Fluor 488 71,000 $M^{-1}cm^{-1}$, Alexa Fluor 647 268,000 $M^{-1}cm^{-1}$), and correction factors for fluorescent dyes (Alexa Fluor 488 0.11, Alexa-Fluor647 0.03). Labeling efficiencies for Alexa Fluor 488-labeled proteins ranged from 44% to 120%. Labeling efficiencies for Alexa Fluor 647-labeled proteins ranged from 13% to 49%.

Binding of protein to bacteria

S. flexneri expressing RFP was streaked on an agar plate, then single colonies were picked and grown overnight at 37 °C shaking, in 5 ml TSB. The next day 175 µl overnight culture was diluted into 5 ml TSB and grown for 1 hour 20 minutes. 2.5 ml bacterial culture was pelleted, washed with 1 ml PBS, and resuspended in 1 ml 4% formaldehyde in PBS, pH 7.4 for 20 min to fix. Formaldehyde-fixed bacteria were washed twice with PBS and resuspended in PBS supplemented with 0.03% NaN3. Final concentrations for *in vitro* binding experiments were 10^5-3 x 10^6 bacteria/ml, 5 µM GBP, and 2 mM GTP. Bacteria were diluted in buffer C, supplemented with 50 µM BSA, and the dilution was applied to the cover slide of a glass bottom 10 mm microwell dish. Following centrifugation for 1 min at 3,000 x g bacteria were incubated for 5 min at 25 °C on the temperature-controlled microscope stage. Alexa Fluor-labeled GBPs were diluted in buffer C supplemented with 50 µM BSA, mixed with GTP, and the mixture was

added to bacteria at t = 0 min. The samples were gently mixed, and images were collected every 1.5 min. After recording time-lapse images for 60 min different field of views were imaged for quantification. Imaging was performed on a Zeiss 880 Airyscan Fast Inverted Confocal on Axio Observer Z1 microscopes using Zeiss Plan-Apochromat 63×/ 1.4 oil objectives. All images were processed with Fiji.

LPS aggregation

Final concentrations for LPS aggregation microscopy experiments were 5 µM recombinant GBP, 50 µg/ml LPS, and 2 mM GTP. Alexa Fluor 568-labeled *E. coli* O55:B5 LPS (Invitrogen) was diluted in buffer C, supplemented with 50 µM BSA, and the dilution was applied to the cover slide of a glass bottom 10 mm microwell dish. Following centrifugation for 1 min at 3,000 x g bacteria were incubated for 5 min at 25 °C on the temperature-controlled microscope stage. GBPs were diluted in buffer C supplemented with 50 µM BSA, mixed with GTP, and the mixture was added to LPS at t = 0 min. The samples were gently mixed, and after 20 min, different field of views were imaged for quantification. Imaging was performed on a Zeiss 880 Airyscan Fast Inverted Confocal on Axio Observer Z1 microscopes using Zeiss Plan-Apochromat 63×/ 1.4 oil objectives. All images were processed with Fiji and LPS areas and numbers were quantified with the integrated analyze particle tool.

Dynamic light scattering

Dynamic light scattering (DLS) experiments of recombinant GBPs and LPS was performed with Delsa Max Pro instrument (Beckman Coulter). GBPs were diluted in buffer C or buffer C (apo) supplemented with 300 µM AlF_X, 10 mM NaF, and 250 µM GDP (GDP·AlF_X) to a final concentration of 5 µM in the presence or absence of 0.05 mg/ml *E. coli* O55:B5 LPS. Samples were incubated at room temperature (RT) for 1 hour prior starting DLS measurements in a temperature-controlled cuvette set to RT. The particle size was measured over three measurements, each consisting of ten runs. The number-weighted radius (R_n) of the particles was determined with the manufacturer's software. A series of controls were also measured to establish the underlying particle sizes of the filtered aqueous buffers and the protein solutions. These were buffer C, buffer C supplemented with GDP·AlF_X, and 5 µM bovine serum albumin (BSA) or 0.05 mg/ml LPS in the respective buffers.

Absorbance-based light scattering

Absorbance-based light scattering experiments were performed with a Specord200 UV/Vis spectrophotometer (Analytik Jena) as described previously(13). GBP1, GBP2, and GBP5 were diluted in buffer C supplemented with 50 µM BSA to a final concentration of 5 µM. Protein dilutions were incubated in a temperature-controlled cuvette at 25°C for 5 min. GTP was added to protein dilutions at a final concentration of 2 mM to start GBP self-assembly. Polymerization of GBPs was followed as absorbance signal at 350 nm over time. To determine formation of mixed polymers, absorbance of 5 µM GBP1 in the presence of 5 µM GBP1, GBP2, or GBP5 following GTP addition was monitored (final GBP concentration 10 µM, polymerization of 5 µM GBP1 is shown as control).

Native polyacrylamide gel electrophoresis

Native GBP-LPS complexes were analyzed with native polyacrylamide gel electrophoresis (NPAGE). Recombinant GBP1, GBP2, GBP5, BSA (control), and *E. coli* O55:B5 LPS were diluted in buffer C or buffer C supplemented with 300 µM AlF_X and 10 mM NaF. Protein and LPS dilutions were mixed, and GDP was added to induce complex formation. Final concentrations were 5 µM protein, 250 µM GDP, 1 mg/ml, 0.1 mg/l, or 0.01 mg/ml LPS (Fig. 7B), and 2 mg/ml to 0.008 mg/ml (1:1 dilution, Fig. 7C and Fig. S6C). After incubation at RT for 30 min, samples were mixed 1:1 with 2 x native sample buffer (BioRad) and loaded on a 4-20% precast protein gel (BioRad) for electrophoresis in Tris/Glycine running buffer

(BioRad) at 80 V for 15 min followed by 180 V for 1 h. After electrophoresis, gels were fixed overnight in 60% methanol and 10% acetic acid. The next day, gels were rehydrated in 3% acetic acid and successively stained using Pro-Q Emerald 300 Lipopolysaccharide Gel Stain Kit (Invitrogen) and Coomassie Brilliant Blue R-250. LPS staining and Coomassie staining were visualized with an Azure 500 Biosystem using excitation at 365 nm and emission at 595 nm or the UV320 Coomassie detection program (utilizing the orange tray), respectively. All gel images were processed with Fiji. Gel bands were analyzed with the built-in Fiji tool.

Caspase-4 activation

Caspase-4 activity was determined as described previously (14) with the following modifications. Recombinant GBP1, GBP1^{R48A}, GBP1^{3R}, and GBP2, were diluted in buffer H (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 5 mM $MgCl_2$, 2 mM EDTA, 10 mM DTT) supplemented with *E. coli* O55:B5 LPS, and GTP (or buffer as control) was added to induce aggregate formation. After incubation at 37 °C for 30 min, reactions were mixed 1:1 with recombinant caspase-4 diluted in buffer H in a black 96 well plate with non-binding surface (Corning), and Z-VAD-AMC (Cayman Chemical) diluted in buffer H was added to each sample. Fluorescence was measured with an Enspire 2300 multilabel plate reader (Perkin Elmer) using excitation at 365 nm and emission at 450 nm. Final concentrations were 5 µM GBP, 0.05 mg/ml LPS, 1 mM GTP, 0.05 µM caspase-4, and 75 µM Z-VAD-AMC. N-terminal GST tagged, human full-length caspase-4 expressed in and purified from wheat germ (Novus Biologicals, H00000837-P01) was used to avoid LPS contamination. Fluorescence intensities were normalized to basal caspase-4 activities (Fig. 7D and E, Fig. S9D lower panel). Free AMC amounts upon Z-VAD-AMC cleavage were determined by correlating fluorescence intensities to fluorescence intensities measured for defined AMC concentrations in calibration experiments following established protocols (15) (Fig. S9D upper panel).

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Supplementary Figures

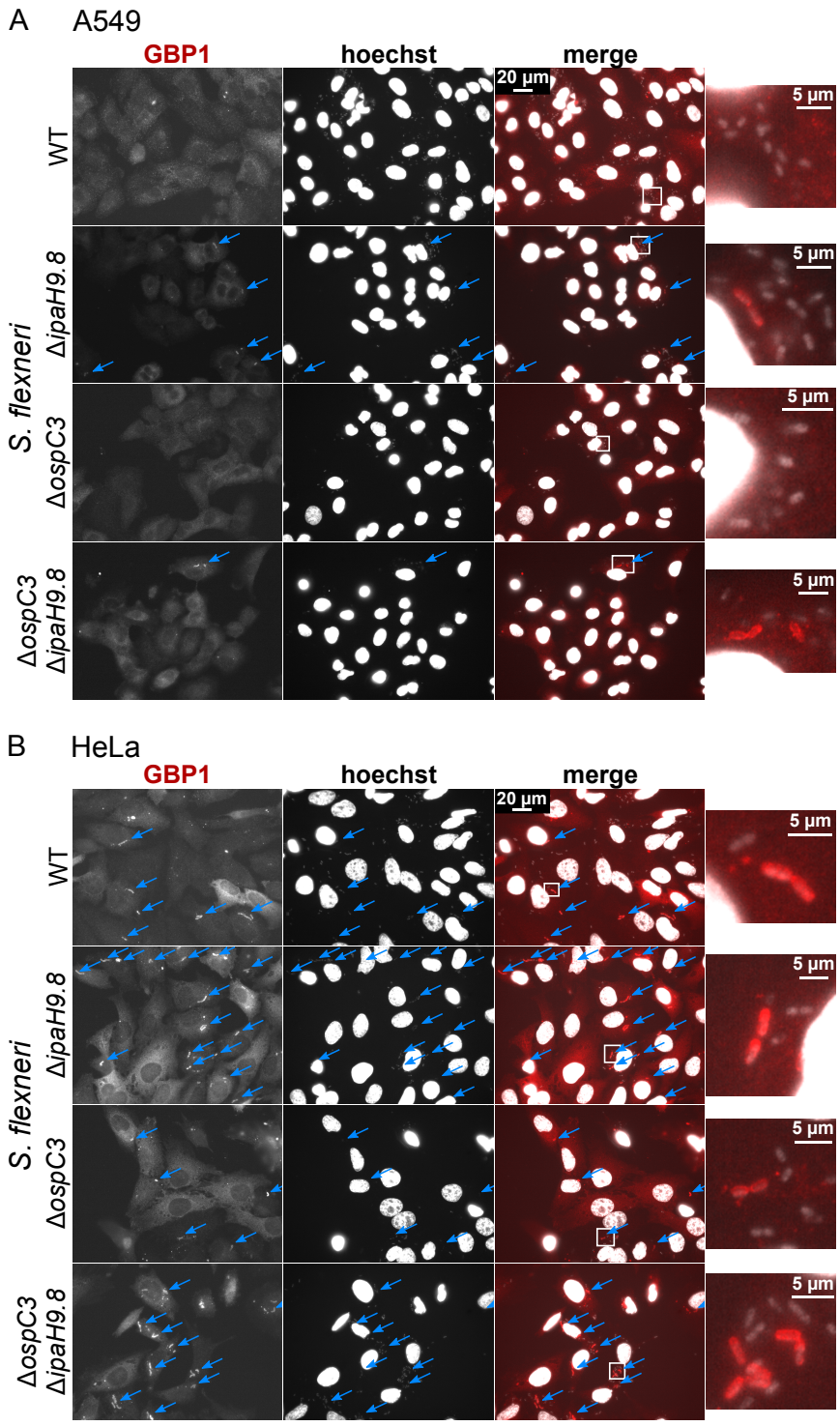


Figure S1. A549 cells have less frequent GBP1 binding to *S. flexneri*, compared to HeLa cells. A549 (A) and HeLa (B) cells were infected with the indicated strains, fixed at 1.5 hours post infection and immunostained for GBP1. Bacteria were visualized with DNA stain Hoechst. *S. flexneri* with GBP1 surrounding >50% of the bacterial surface were counted as GBP1 positive. Blue arrows indicate clusters of *S. flexneri* that are coated with GBP1. Images were taken at 63× magnification and are representative of three independent experiments.

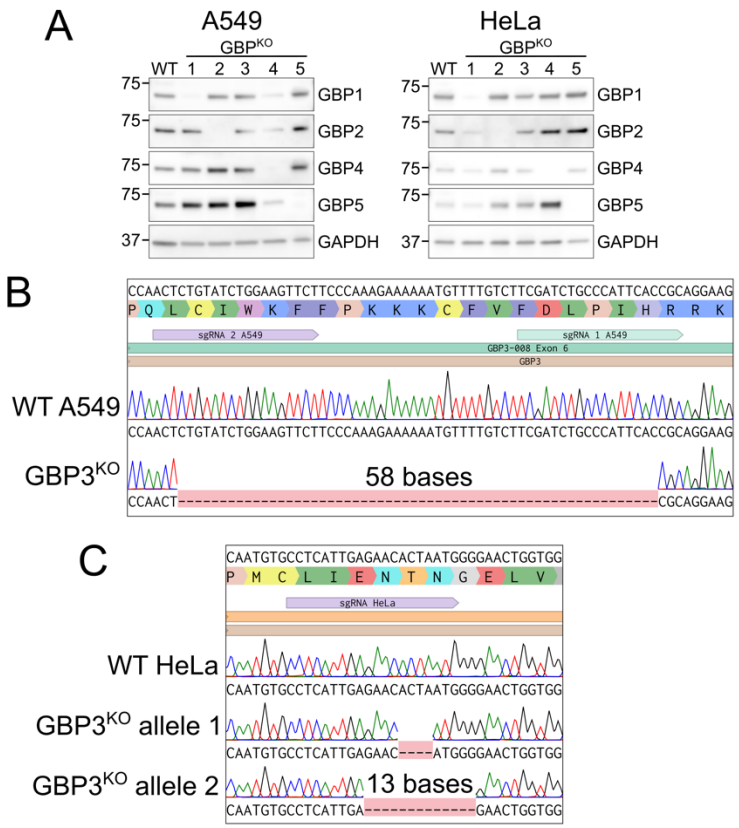


Figure S2. GBP1-5 were individually knocked out in A549 and HeLa cells. A549 and HeLa wildtype or CRISPR knockout clones were plated and primed with 100 U/ml IFNγ overnight. Cells were then lysed and GBP or GAPDH expression was analyzed by western blot. (B) Genomic DNA was extracted from wildtype and GBP3^{KO} A549 cells, PCR was used to amplify the edited region of the GBP3 gene, then PCR product was sequenced using sanger sequencing. Alignments were made using Benchling. (C) Genomic DNA was extracted from wildtype and GBP3^{KO} HeLa cells, PCR was used to amplify the edited region of the GBP3 gene, then PCR cloning was used to separate different alleles. Colony PCR and sanger sequencing were used to determine the sequence of each allele. Alignments were made using Benchling.

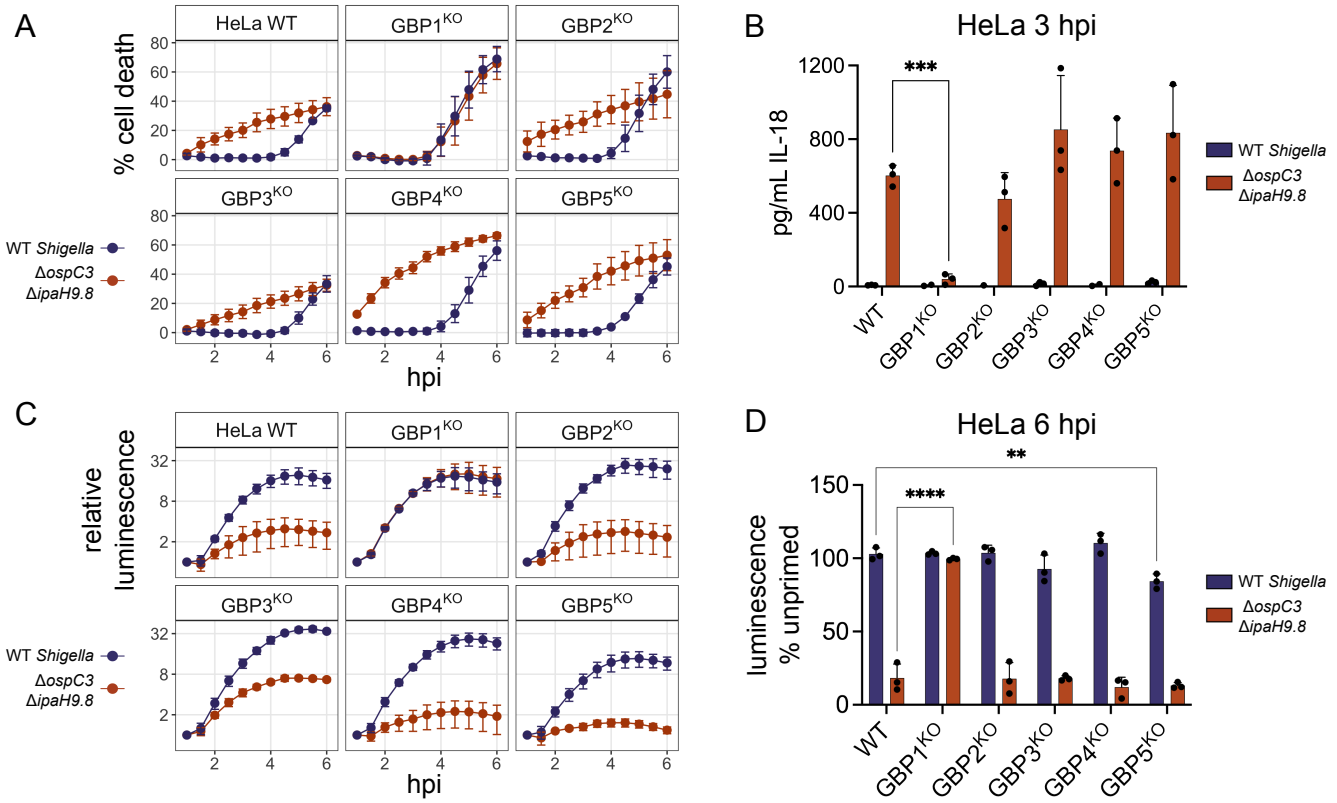


Fig. S3. HeLa cells require endogenous GBP1 for pyroptosis and restriction of *S. flexneri* growth. HeLa cells were primed overnight with 100 U/mL IFNγ, then infected with wild-type *S. flexneri* or *S. flexneri* Δ*ipaH9.8*Δ*ospC3* expressing a bioluminescent reporter plasmid. Cell death was measured using sytox green (A). Supernatant was taken at 3 hpi to measure IL-18 secretion (B). Bacterial growth was monitored by luminescence (C). Luminescence measurements from the 6 h timepoint were used to calculate the growth of each strain in primed cells relative to unprimed cells (D). Data are averages from three independent experiments and are represented by mean ± SD. Two-way ANOVA with Dunnett's multiple comparisons test was used; for each bacterial strain, values for each knockout cell line were compared to the wild-type cells. All statistically significant comparisons are shown. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

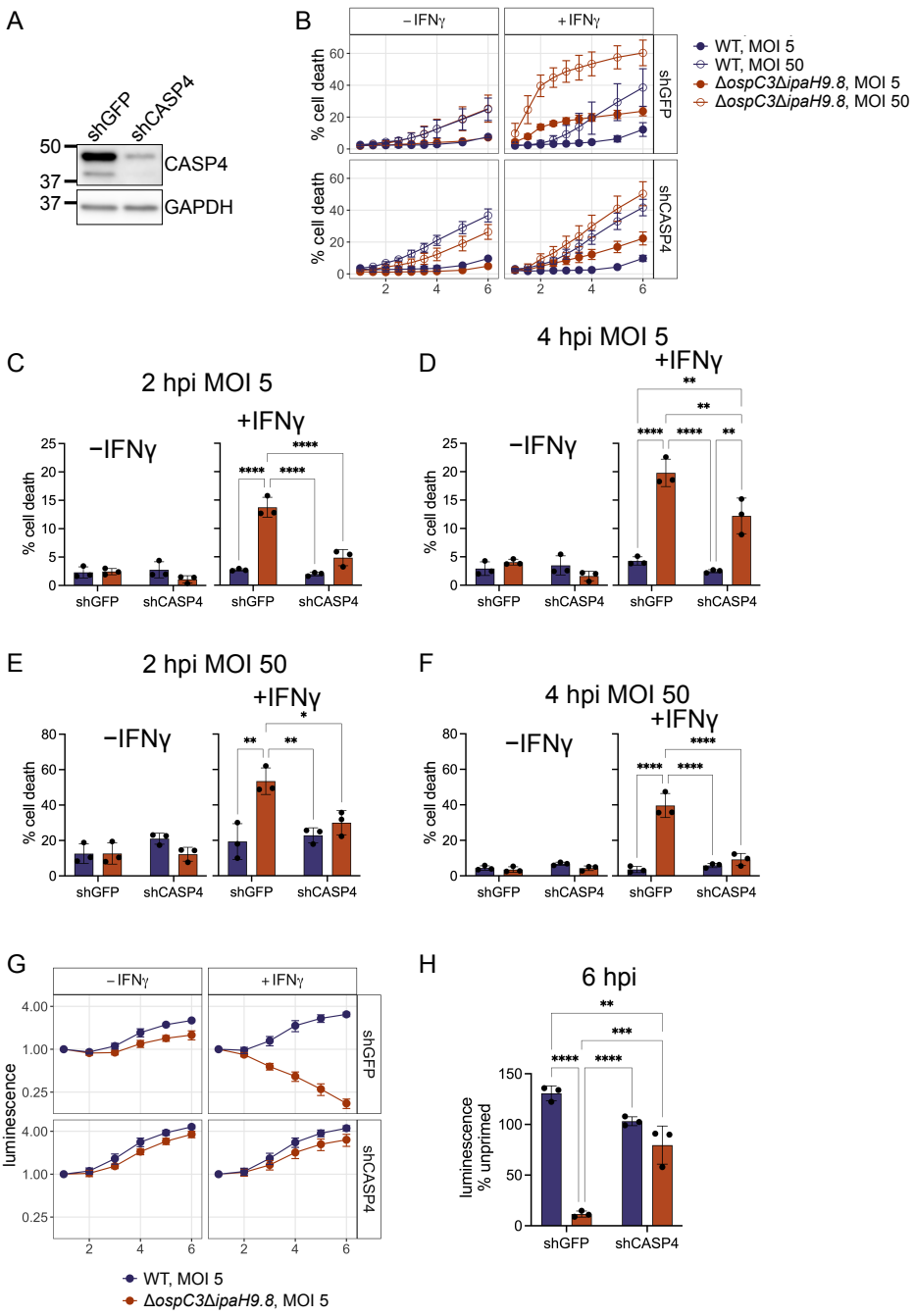


Figure S4. *S. flexneri* Δ*ospC3*Δ*ipaH9.8*-induced pyroptosis requires CASP4. A549 cells were transduced with lentiviral vectors expressing a control shRNA targeting GFP or an shRNA targeting caspase-4. (A) Western blot was used to determine knockdown efficiency. (B) Cells were infected with indicated strains of *S. flexneri* at a multiplicity of infection (MOI) of 5 or 50, and cell death was measured over time using sytox green. Percent cell death at 2 hpi (C and E) or 4 hpi (D and F) was used for statistical analysis. Luminescence was used to measure bacterial growth over time (G). *S. flexneri* replication in IFNγ primed cells divided by luminescence signal in unprimed cells at 6 hours post infection (H). Data in graphs show averages of three independent experiment and error bars show standard deviation. In C, D, E, F, H statistical significance was determined using two-way ANOVA with Tukey's multiple comparisons test. All significant comparisons are shown, * = P < 0.05, ** = P < 0.01, *** = P < 0.001, **** = P < 0.0001.

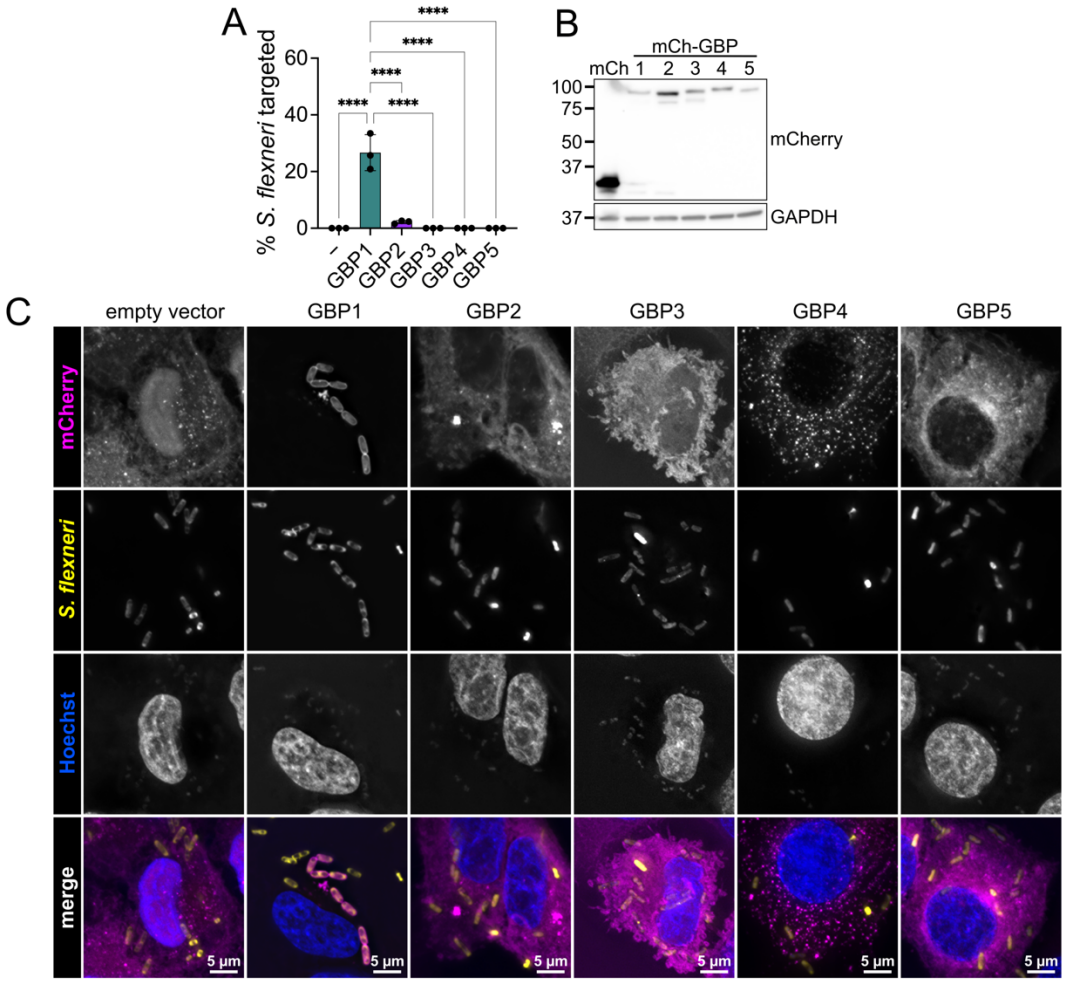


Figure S5. GBP1, but not GBP2-5, can bind bacteria in cells. (A) A549 GBP1^{KO} cells overexpressing mCherry or mCherry-GBPs were primed with 100 U/ml IFNγ overnight, then infected with *S. flexneri* Δ*ipaH9.8* expressing GFP. Cells were fixed at 2 hpi, images were taken at 63x by widefield microscopy, then total number of *S. flexneri* as well as mCherry positive *S. flexneri* were counted using ImageJ. *S. flexneri* with mCherry signal around at least 50% of the bacterial membrane were counted as targeted. (B) Expression levels of each mCherry constructs were tested using western blot. (C) A549 GBP1^{KO} cells overexpressing mCherry or mCherry-GBPs were primed with 100 U/ml IFNγ overnight, then infected with *S. flexneri* Δ*ipaH9.8* expressing GFP. Cells were fixed at 2 hpi and images were taken at 100x magnification by widefield microscopy. Images were deconvolved and z-projections are shown. (A) Graph shows averages from three independent experiments and is represented by mean ± SD. One-way ANOVA with Tukey's multiple comparisons test was used. All significant comparisons are shown. **** = P < 0.0001. (B and C) Images are representative of three independent experiments.

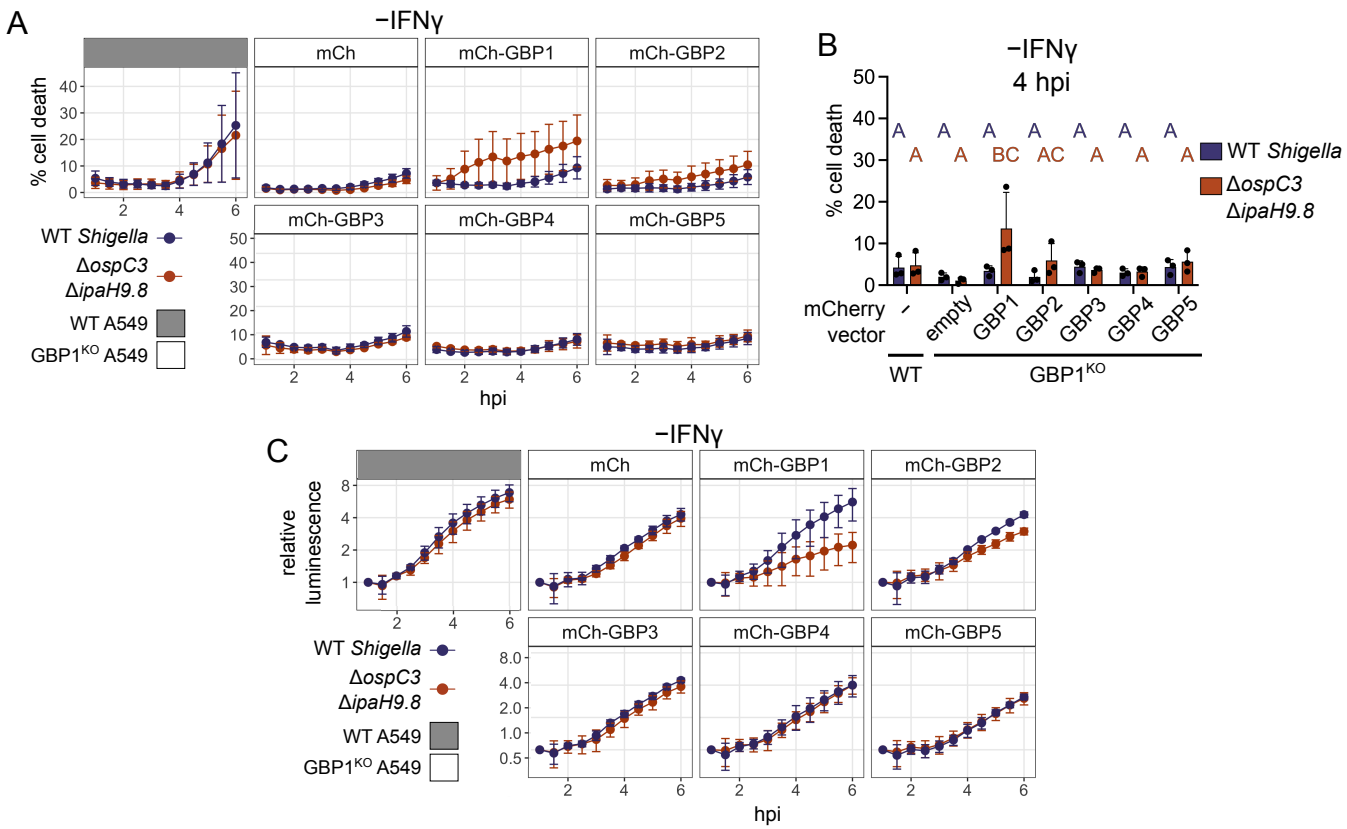


Fig. S6. Overexpression of GBP1 or GBP2 in unprimed cells has little effect on pyroptosis and bacterial restriction. A549 cells were stably transduced to express mCherry or mCherry-GBPs. Cells were then infected with bioluminescent *S. flexneri* and cell death was measured over time using sytox green fluorescence in unprimed cells (A). The sytox green signal at 4 h was used to determine statistical significance (B). Bacterial luminescence was measured over time (C). Data are averages from three independent experiments and are represented by mean ± SD. Two-way ANOVA with Tukey's multiple comparisons test was used. Statistical comparisons are shown by letters, with bars sharing no matching letters being significantly different. Purple letters correspond to statistical comparisons for wild-type *S. flexneri*, and orange letters correspond to *S. flexneri* Δ*ipaH9.8*Δ*ospC3*.

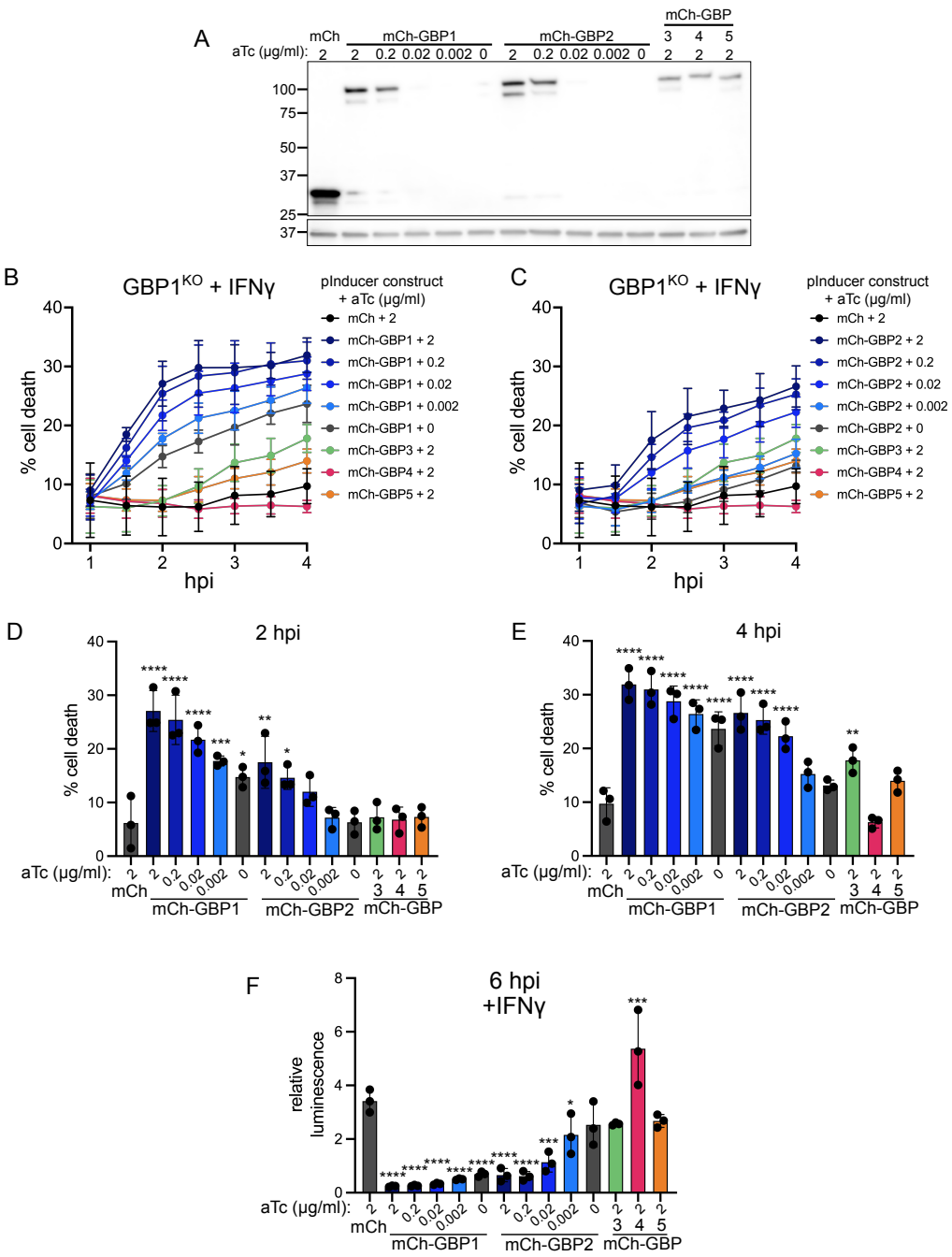
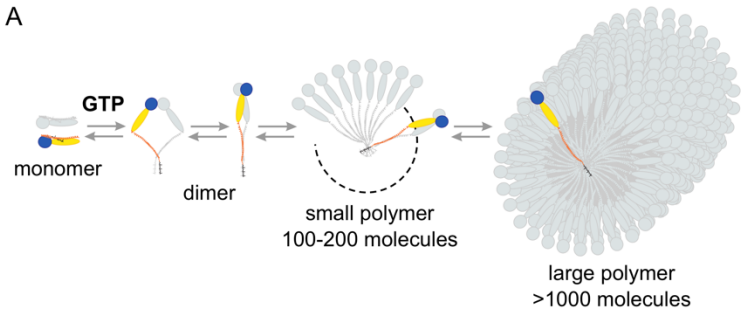
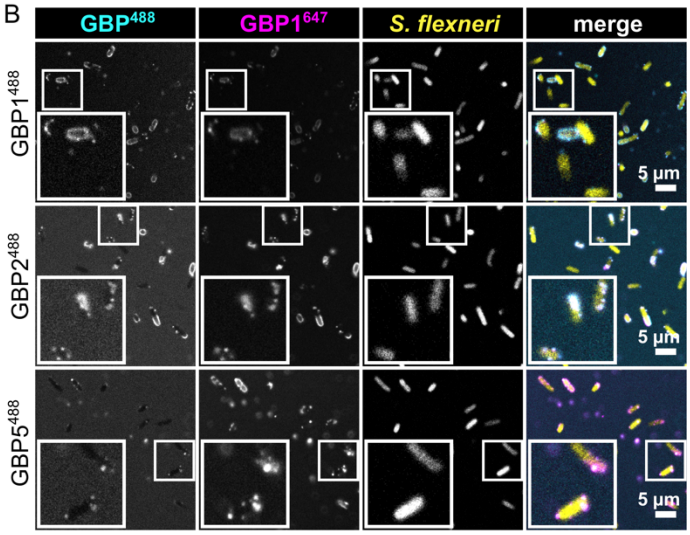


Figure S7. GBP1 and GBP2 promote pyroptosis even with low levels of expression. A549 GBP1^{KO} cells were transduced with pInducer-mCherry constructs and anhydrotetracycline (aTc) was used to induce mCherry-GBP expression. Expression of mCherry-GBP1 and mCherry-GBP2 was titrated using different concentrations of aTc. mCherry control vector, mCherry-GBP3, mCherry-GBP4, and mCherry-GBP5 were expressed at maximal levels. Western blot using an antibody against mCherry was used to assess expression levels (A). These cells were infected with *S. flexneri* Δ*ospC3*Δ*ipaH9.8* and sytox green fluorescence was used to determine cell death promoted by each construct. Cell death data showing GBP1 titration (A) and GBP2 titration (B) is from same experiments, split into two graphs to make it easier to read. Data for mCherry, mCherry-GBP3, mCherry-GBP4, and mCherry-GBP5 is identical in both graphs. 2 hpi and 4hpi from B and C were used to compare cell death levels between cells expressing different constructs (D and E). Luminescence was used to measure bacterial growth over time, and luminescence at 6 hpi relative to 1hpi was used to assess growth differences in the presence of indicated constructs (F). Data in graphs contains mean ± SD from three independent experiments. Significance in D, E, F was determined using one-way ANOVA with Dunnett's multiple comparisons test, comparing to mCherry. * = P < 0.05, ** = P < 0.01, *** = P < 0.001, **** = P < 0.0001.





C

	R_n (nm)			
	apo		GDP·AlFx	
GBP1	3.3 ± 0.1	m	13.0 ± 0.2	p
GBP1^{nf}	3.7 ± 0.1	m	5.2 ± 0.1	d
GBP2	3.3 ± 0.1	m	14.7 ± 0.5	p
GBP2^{ngg}	3.3 ± 0.2	m	4.8 ± 0.0	d
GBP5	3.8 ± 0.3	m	4.4 ± 0.9	d
GBP5^{ngg}	4.0 ± 0.2	m	4.8 ± 0.1	d

Figure S8. Mixed GBP1-GBP2 and GBP1-GBP5 polymers attach to the bacterial surface. (A) Mechanism of GBP1 polymerization. GBP1 exit its closed monomeric state, in which the farnesyl moiety is buried inside a hydrophobic pocket, and forms dimers in a GTP hydrolysis-dependent manner. GBP1 dimers can assemble into small polymers consisting of 100-200 molecules and continue their growth to form large polymers holding over 1000 molecules. (B) Confocal microscopy time frames of recombinant Alexa Fluor 488-labeled GBP1, GBP2, or GBP5 mixed with Alexa-647-labeled GBP1 supplemented with GTP at 5 min after addition to formaldehyde-fixed RFP-expressing *S. flexneri*. (C) Number-weighted mean radius (R_n) of nucleotide-free (apo) or GDP·AlF_X-bound nonisoprenylated (non-farnesylated = nf, non-geranylgeranylated = ngg) and isoprenylated GBPs determined in DLS experiments (values for data shown in Fig. 4G). monomer – m; dimer – d; polymer – p. (B) Representative images of three independent experiments. (C) Mean ± SD from three independent experiments.

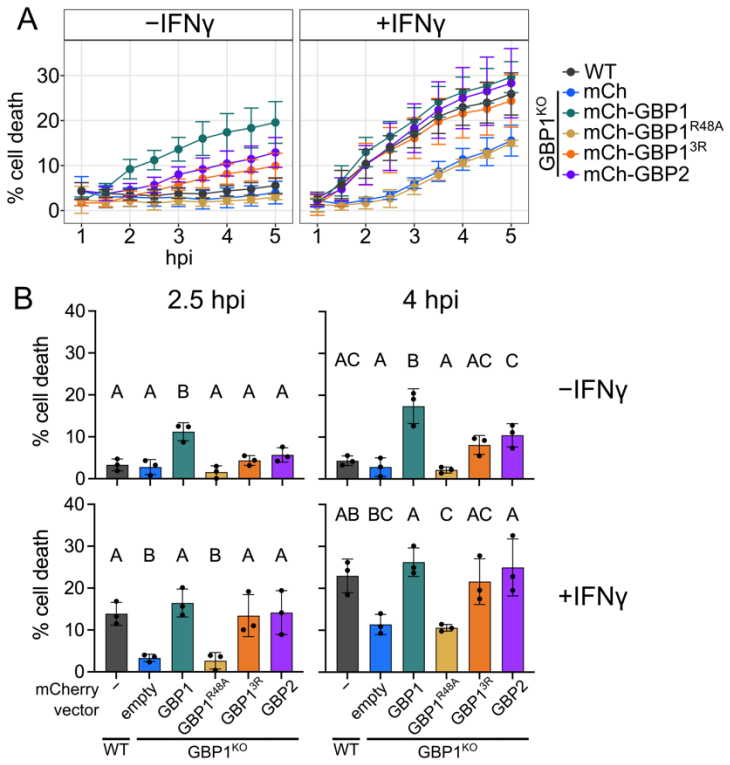


Figure S9. GBP1, GBP1^{3R}, and GBP2 can rescue pyroptosis in GBP1^{KO} cells during *S.* Typhimurium infection. Wildtype A549 or GBP1^{KO} A549 cells overexpressing mCherry or mCherry-GBPs were plated and cells were unprimed or primed with 100 U/ml IFNγ overnight, then infected with *S.* Typhimurium. Cell death was monitored over time using sytox green fluorescence (A). For statistical analysis, the sytox green signal at 2.5 hours and 4 hours post infection was used (B). All graphs show averages from three independent experiments and are represented by mean ± SD. (B) One-way ANOVA with Tukey's multiple comparisons test was used. Statistical comparisons are shown by letters, with bars sharing no matching letters being significantly different. * = P < 0.05, ** = P < 0.01, *** = P < 0.001, **** = P < 0.0001.

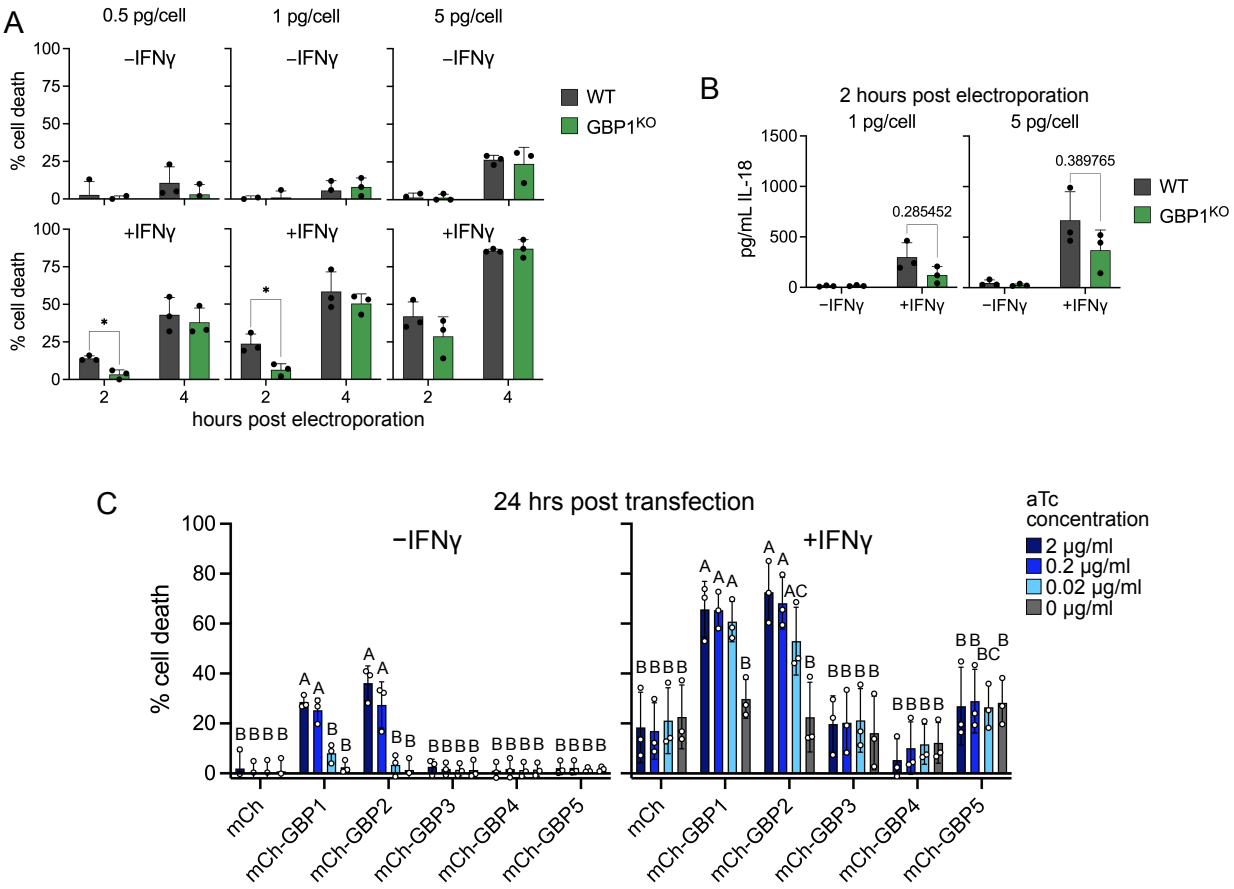


Figure S10. GBP1 is not required for pyroptosis in response to electroporated LPS.
(A, B) Wildtype and GBP1^{KO} A549 cells unprimed or primed with 100 U/ml IFNγ overnight were electroporated with 0.5 pg/cell, 1 pg/cell, or 5 pg/cell *E. coli* O55:B5 LPS. Cell death was measured using sytox green fluorescence at 2 and 4 hours post electroporation (A). (B) IL-18 secretion was measured in supernatants taken at 2 hours post electroporation. (C) GBP1^{KO} A549 cells were transduced with tet-inducible expression vectors and expression of each construct was titrated with different concentrations of anhydrotetracycline (aTc). Cells were unprimed or primed with 100 U/ml IFNγ overnight, then transfected with 1 µg *E. coli* O55:B5 LPS per well. Cell death was measured using sytox green fluorescence at 24 hours post transfection. All graphs show averages from three independent experiments and are represented by mean ± SD. (A and B) Statistical significance was determined using multiple unpaired T tests with Welch correction, with multiple comparisons corrected with Holm-Šídák method. All significant comparisons are shown. * = P < 0.05. Exact P values are shown in (B) for IFNγ primed cells. (C) Statistical significance was determined using two-way ANOVA Tukey's multiple comparisons test. Significance is represented by letters, with bars sharing no matching letters being significantly different.

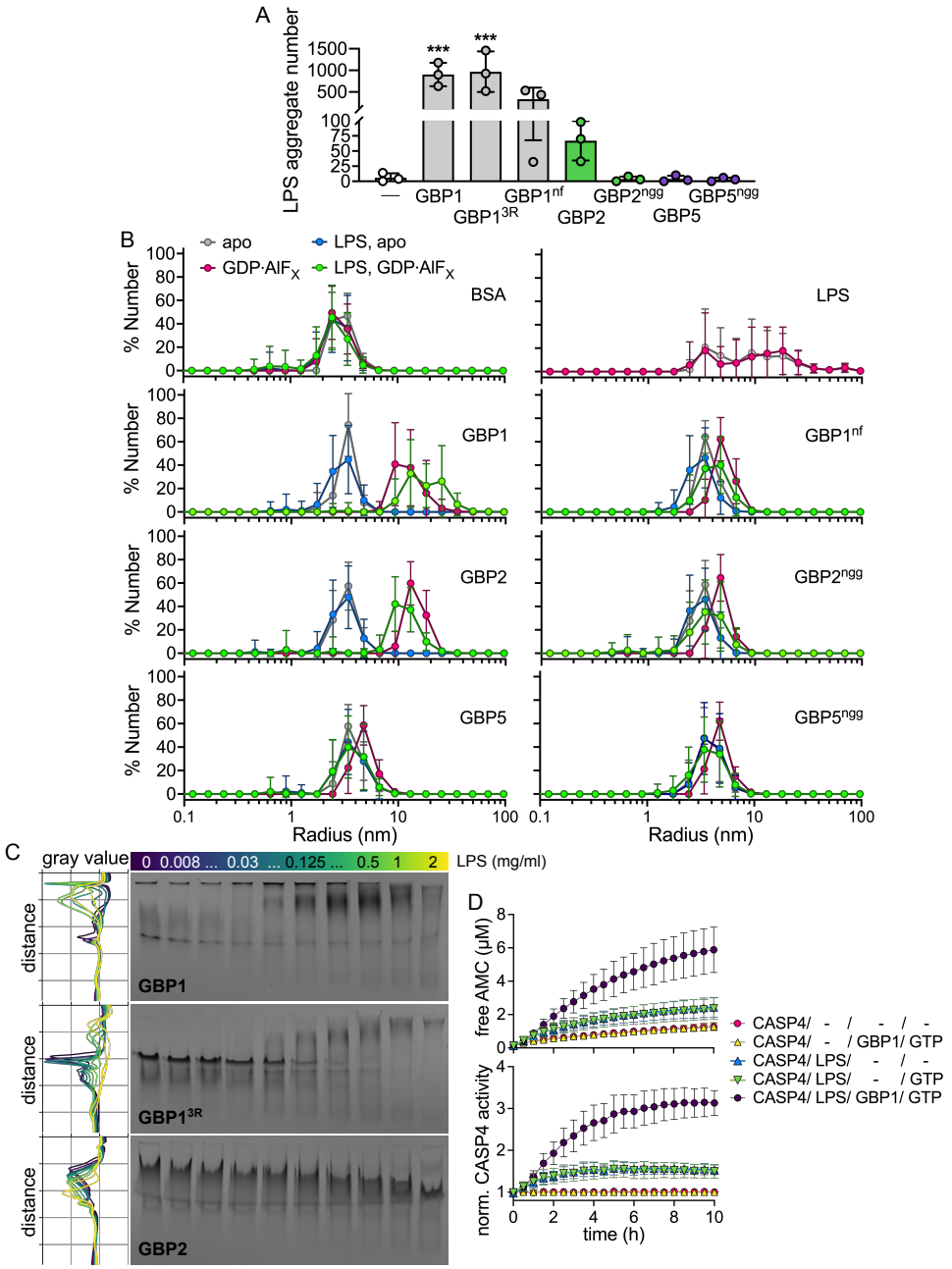


Figure S11. GBP2 binds directly to LPS and polymerizing GBPs promote caspase-4 activation. (A) Recombinant isoprenylated and nonisoprenylated GBPs were supplemented with GTP and added to Alexa Fluor 568-labeled *E. coli* O55:B5 LPS. LPS particles were analyzed from different fields of views taken after 20 min with Fiji, and the number of LPS aggregates was plotted. (B) Number-weighted mean radius (R_n) of nucleotide-free (apo) or GDP·AlF_X-bound nonisoprenylated (non-farnesylated - nf, non-geranylgeranylated - ngg) and isoprenylated GBPs in the presence and absence of LPS were determined in DLS experiments. (C) NPAGE of GBP1, GBP1^{3R}, and GBP2 titrated with LPS (final concentrations 2 mg/ml to 0.008 mg/ml) supplemented with GDP·AlF_X. Grey values for each gel lane were plotted with Fiji (GBP1 gel from Fig. 7C). (D) Following mixing of recombinant proteins with *E. coli* O55:B5 LPS in the absence or presence of GTP, protein-LPS complexes were added to recombinant caspase-4, and caspase-4 activity was determined by monitoring release of free 7-Amino-4-methylcoumarin (AMC) upon cleavage of fluorogenic caspase substrate Z-VAD-AMC over time. Fluorescence intensities were either correlated with fluorescence intensities measured for defined AMC concentrations to determine free AMC amounts upon Z-VAD-AMC cleavage (upper panel), or normalized to basal caspase-4 activities (lower panel). Caspase-4 – CASP4. All graphs show averages from three independent experiments and are represented by mean ± SD. (A) One-way ANOVA with Dunnett's multiple comparisons test comparing to control (no GBP addition) was used. All significant comparisons are shown. *** = P < 0.001. (C) Representative NPAGEs from three independent experiments are shown.

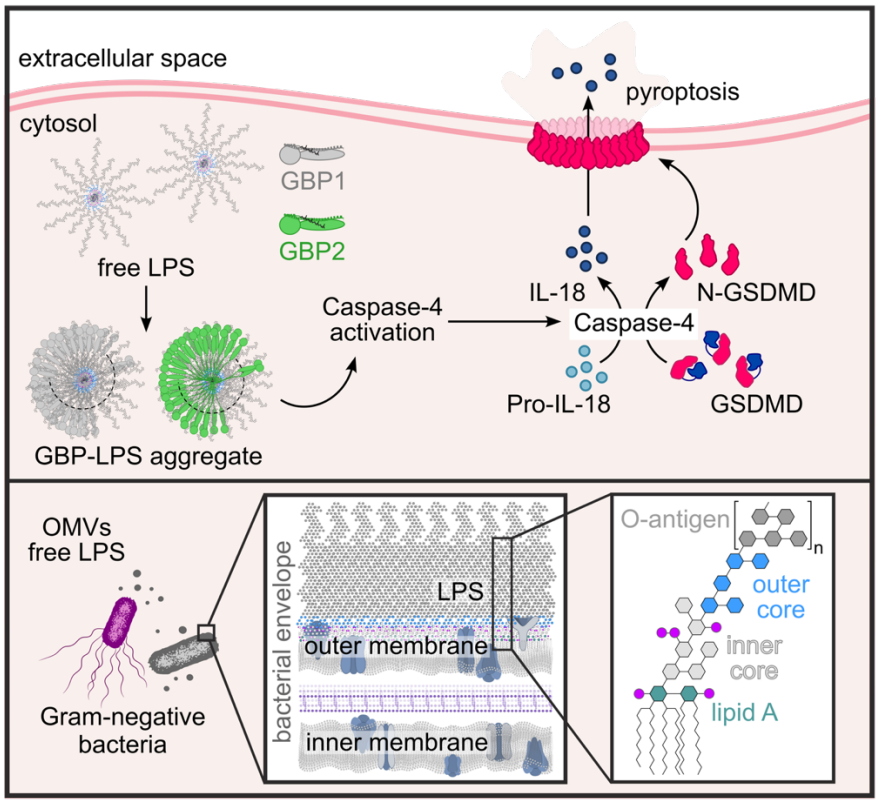


Figure S12. LPS surfactants GBP1 and GBP2 activate the non-canonical inflammasome in the cytosol without binding to the bacterial surface. Polymerizing GBP1 and GBP2 aggregate free LPS in the host cell cytosol. GBP1-LPS and GBP2-LPS complexes both enhance caspase-4 activation resulting in IL-18 release and GSDMD-induced pyroptotic cell death, ultimately depleting cytosolic Gram-negative bacteria of their replicative niche.

Movie S1 (separate file). GBP2 and GBP5 fail to encapsulate bacteria on their own. Recombinant Alexa Fluor 488-labeled GBP1, GBP2, or GBP5 were supplemented with GTP and added to formaldehyde-fixed RFP-expressing *S. flexneri*.

Movie S2 (separate file). Both GBP2 and GBP5 associate with bacteria GBP1-dependently but only GBP2 incorporates in the microcapsule. Recombinant Alexa Fluor 488-labeled GBP1, GBP2, or GBP5 mixed with Alexa-647-labeled GBP1 were supplemented with GTP and added to formaldehyde-fixed RFP-expressing *S. flexneri*.

Movie S3 (separate file). Pyroptosis of *S. flexneri* does not require GBP1 binding to bacteria. GBP1^{KO} cells primed with 100 U/ml IFNγ overnight expressing mCherry (control), mCherry-GBP1, or mCherry-GBP1^{3R} (magenta) infected with GFP-expressing *S. flexneri* Δ*ospC3* Δ*ipaH9.8* (yellow). Dying cells are shown in blue (sytox blue).

Plasmid sequences shown below for GBP overexpression constructs. mCherry shown in red, GBPs in blue, point mutations in green.

>pInducer-mCherry

gcacctttttgaaatgtaatcatttgggtcaatatgtaatttttcagtgttagactagtaaattgtccgctaaattctggccgtttttggcttttttgttagacgaagcttggtacc
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