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Supplemental Information

Identification of cGAS as an innate immune sensor of extracellular bacterium *Pseudomonas aeruginosa*

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Transparent Methods

Ethics statement

This study followed the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All mice protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of North Dakota (Assurance Number: A3917-01).

Mice

C57BL/6J mice and *STING*$^{-/-}$ mice (8-12 weeks) were obtained from Jackson Laboratory (Bar Harbor, ME). *cGAS*$^{-/-}$ mice (C57BL/6J background) were kindly provided by H. W. Virgin (WASH U) and Charles M Rice (Rockefeller Univ.). Mice were anesthetized using ketamine and xylazine (45 mg/kg and 10 mg/kg). For lung infection model, mice were instilled intranasally with 0.5×10^7 CFU *P. aeruginosa* (six mice/group). Mice were monitored for symptoms every day. Mice were maintained in a specific pathogen free animal facility at the University of North Dakota and Wuhan University.

Primary cells and cell lines

MH-S, RAW264.7, THP-1 cells were kept in our laboratories. BMDMs, AMs and PMs were generated from wild-type C57BL/6 and *cGAS*$^{-/-}$ mice. Macrophages were cultured in RMPI 1640 + 10% FBS + 20 mM HEPES + 2 mM L-glutamine. Primary BMDMs were grown for 10 days stimulated with 25 ng/ml M-CSF. Epithelial cells were cultured in DMEM. The differentiation of THP-1 monocytes into macrophages is conducted at a concentration of 100ng/ml PMA for 48 hours.

Plasmids construction

To construct the mouse *BiP* and *ATF4* promoter luciferase plasmids, the *BiP* (-700 to -400) and *ATF4* (-590 to -290) promoter fragments were amplified from the RAW264.7 cell genome by PCR, then sub-cloned into the *Kpn*I and *Hind*III sites of pGL3-Enhancer Vector (Promega, WI). To construct pET-28a-mcGAS plasmids, cDNA fragments were amplified from BMDMs cDNA with its specific primer and cloned into *Sal*I and *Xho*I sites of the pET-28a vector (Millipore, MA).

Constructed plasmids were transformed into *Escherichia coli* BL21(DE3) (New England Biolabs, MA). PCR primers were shown in Supplemental Table 1.

Generation of gene knockout cell lines

For targeting with CRISPR/Cas9, we used a lentiCRISPR v2 to construct cGAS and STING KO cells. Guide RNA sequences (cGAS: 5′ - CACCG GCTGGAGCCTCCTGCGGCT -3′; STING: 5′ - CACCGCAGGCACTCAGCAGA ACCA -3′). The cGAS or STING lentiCRISPR v2 plasmid and packaging plasmids pVSVg and psPAX2 were co-transfected into HEK293T cells. The cellular supernatants were collected to generate "lentiCRISPR v2" lentivirus. cGAS and STING KO cells were selected with 1 μg/ml puromycin for 7 days followed by 24 h lentivirus treatment.

Bacterial strains

Pseudomonas aeruginosa PAO1, PAO1-GFP, PAO1-mCherry, *K. pneumoniae* and *S. aureus* were cultured in LB broth and agar plates at 37°C. As for invitro infection, bacteria were cultured for 16 h in LB broth medium and then pelleted by centrifugation at 5000g. Cells were then cultured to antibiotic-free medium and infected with bacteria at MOI of 10 for 1 to 4 hours.

RNA isolation and quantitative real-time PCR

Cells were collected in Trizol and RNA was isolated following the manufacturer's instructions. cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Thermo, MA). qPCR was performed using SYBR Green qPCR Master Mix (Thermo) and gene-specific primers (Supplemental Table 1). mRNA levels were normalized to *β-actin*.

Isolation and transfections of *P. aeruginosa* DNA

P. aeruginosa was grown in LB broth with or without 1.2mM EdU. *P. aeruginosa* genomic DNA was isolated using GeneJET Genomic DNA Purification Kit (Thermo). ISD oligos were annealed in 5 mM Tris + 25 mM NaCl buffer at 98°C for 5 min and then cooling to room temperature for 1 h. Cells were transfected with 2 μg of annealed ISD oligos or *P. aeruginosa* DNA using lipofectamine 3000 (Invitrogen). Cells were harvested after 8 h transfection.

ChIP-PCR

ChIP-PCR was performed using Pierce™ Agarose ChIP Kit (Thermo) following the

manufacturer's instructions. BMDMs were crosslinked with 1% formaldehyde for 10 min at room temperature. Samples were then washed with cold PBS and lysed in the lysis buffer with protease inhibitors. DNA was lysed into the desired fragment length (200 to 600bp) using Micrococcal Nuclease for 15 min at 37°C water bath. Equal amounts of DNA were immunoprecipitated using IgG, IRF3 antibodies. Immunoprecipitated and input DNA was eluted and amplified with primers for UPR molecules (Supplemental Table 1). To relate our studies to the existing data, we downloaded BED files containing the enrichment of IRF3-ChIP binding sites and STAT1-ChIP binding sites from GEO database (GEO Accession number: GSM2698322, GSM925279, GSM1356204, GSM1356222) (Mancino et al., 2015; Purbey et al., 2017). ChIP data were visualized by Integrative Genomics Viewer.

cGAS protein purification

BL21(DE3) transformed with pET-28a-cGAS was cultured in LB medium with kanamycin at 37°C. The cGAS protein was induced with 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) at 16°C for 16 h. The His-tagged protein was purified using Ni-NTA beads. The protein was identified by SDS-PAGE and immunoblotting. hcGAS protein was ordered from NOVUS Biologicals (CO).

Electrophoretic molecular shift assay (EMSA)

Pseudomonas rrs gene probes were amplified by PCR and purified using GeneJet Gel Extraction and DNA Cleanup Micro Kit (Thermo). Briefly, cGAS proteins were mixed with these probes following the EMSA kit (Thermo) manufacturer's instructions and incubated at room temperature for 20 min. The cGAS-probe mixtures were then analyzed by 5% native PAGE gel and electrophoresed in 0.5X TBE buffer at 90V for 90 min. The gels were then stained with SYBR Green EMSA Nucleic Acid Gel Stain.

Co-immunoprecipitation

RAW267.4 cells were infected with *P. aeruginosa*, washed with cold PBS, fixed in 4% PFA and then quenched with 1 M Tris (pH 7.4). Next, cells were lysed using lysis buffer followed by sonication using sonicator in water bath. Samples were then immunoprecipitated with protein A/G

Magnetic Beads pre-cross-linked with cGAS antibody. IP efficiency was detected with Western blot analysis. Samples were de-crosslinked at 65°C for 16 h and then treated with proteinase K. DNA was isolated using Trizol and resuspended in 10 mM Tris. qPCR was then performed to measure the abundance of relevant genes (Watson et al., 2015). All primers were shown in Supplemental Table 1.

Western blotting

Primary antibodies using for immunoblotting: antibodies against cGAS, STING, TBK1, IRF3, p-IRF3, BiP, PERK, ATF4, CHOP, IRE1, XBP1, STAT1, p-STAT1, Histone H3, IRF9 and STAT2 were obtained from Cell Signaling Technology, p-STAT2 (Millipore, MA), p-PERK, ATF6, and β-actin were purchased from Santa Cruz. p-IRE1, mouse and rabbit second antibodies using for immunoblotting were ordered from Invitrogen. Briefly, the cell or tissue samples were lysed in RIPA with protease inhibitor (Roche Diagnostics, IN) for 30 min on ice and then quantitated. SDS Loading Buffer was added into the sample supernatants and then boiled for 10 min at 95°C. 20 μg proteins of each sample were loaded and separated by 10-15% SDS-PAGE gels and transferred onto nitrocellulose and blocked for 1 h at room temperature using 5% nonfat milk. After that, the membranes were incubated using primary Abs overnight at 4°C and second antibody for 2 h. Protein bands were visualized using an enhanced chemiluminescence detection kit and quantified by Quantity One software.

ELISA

IFN-α, IFN-β levels in cell culture supernatants, lung tissues, BALFs, and bloods were detected using mouse IFN-α, IFN-β ELISA Kit (R&D Systems, CO). Briefly, precoated ELISA plates were incubated with 100μL diluted samples, antibody solution, and HRP Solution respectively. PBST was used as wash buffer, each incubation was followed by PBST for 4 times. Lastly, 100 μl of the TMB Substrate Solution was added to each well, incubated 15 min in the dark, and blocked by 100 μl of Stop Solution. Microplate reader was used to determine the absorbance at 450 nm within 5 minutes after the addition of the Stop Solution.

Bacterial burden assay

AMs from BALF, and tissues were homogenized with PBS. CFU were measured using LB agar plates (Guo et al., 2012).

Immunofluorescence

The cells were washed with PBS, fixed in 4% paraformaldehyde for 20 min and then permeabilized with 0.5% Triton X-100 for 20 min. After blocked with 5% non-fat milk for 30 min, the cells were incubated with first antibody and second antibody (diluted by 2% BSA) at 4°C. Nuclear was stained using DAPI. Images were observed under an LSM 510 Meta Confocal Microscope.

Histological analysis

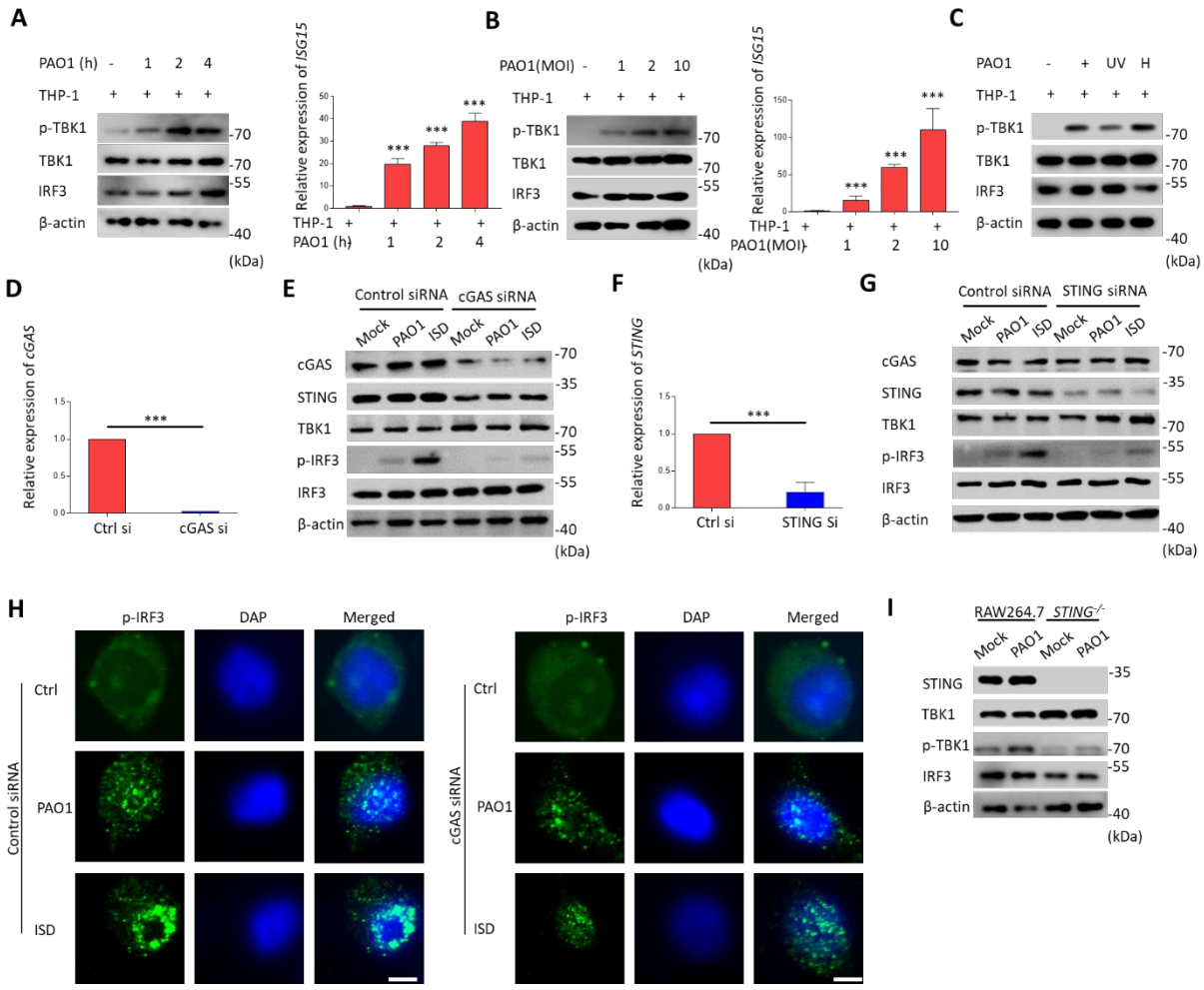
Tissues were fixed in 10% formalin for 24 hours, embedded in paraffin and then processed for H&E staining (AML laboratories, FL).

Statistical analysis

Most experiments were performed for 3 times. One-way ANOVA (Tukey post hoc test) was used for data analysis in GraphPad Prism 7. Besides, survival rates were calculated using Kaplan–Meier curve. ChIP-seq data reported in this paper are from GEO database: IRF3 ChIP (GEO Accession number: GSM2698322, GSM925279), STAT1 ChIP (GEO Accession number: GSM1563689, GSM1563692).

Supplemental Figures and legends

FIGURE S1



S1 Fig. *P. aeruginosa* activated cGAS signaling pathways, related to Figure 1

(A) THP-1 cells were treated with 10 MOI *P. aeruginosa* for 1, 2, or 4 h. Immunoblot analysis of cGAS signaling pathways.

(B) THP-1 cells were treated with 1, 2, or 10 MOI *P. aeruginosa* for 4 h. Immunoblot analysis of cGAS signaling pathways.

(C) THP-1 cells were treated with 10 MOI *P. aeruginosa*, UV-*P. aeruginosa*, or Heated- *P. aeruginosa* for 4 h. Immunoblot analysis of cGAS signaling pathways.

(D) RAW264.7 cells were transfected with control siRNA or cGAS siRNA for 48 h, and qPCR analysis of mRNA levels of *cGAS*.

(E) RAW264.7 cells were transfected with control siRNA or cGAS siRNA and then treated with 10 MOI *P. aeruginosa* or ISD for 4 h. Immunoblot analysis of cGAS signaling pathways.

(F) RAW264.7 cells were transfected with control siRNA or STING siRNA for 48 h, and qPCR analysis of mRNA levels of *STING*.

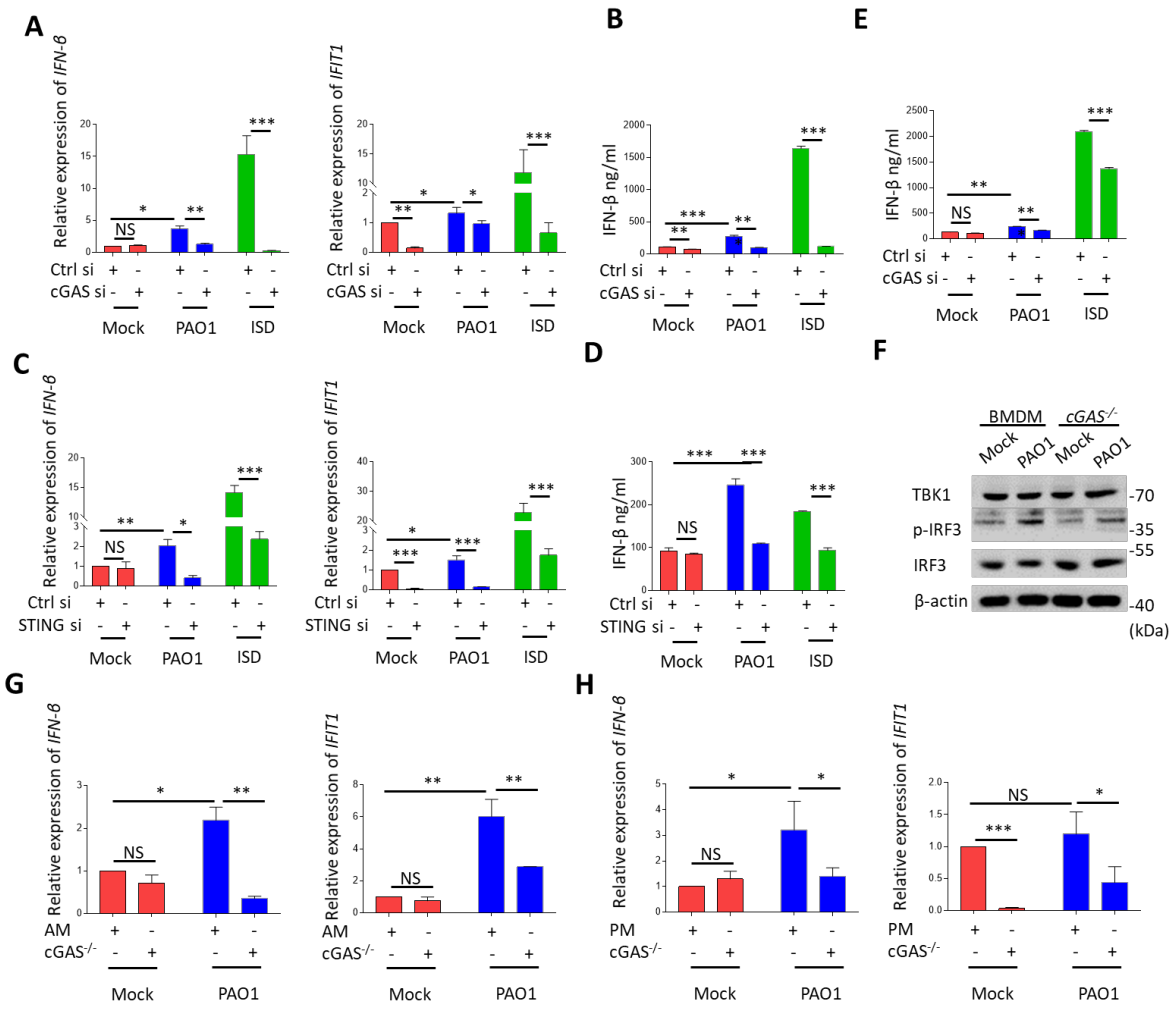
(G) RAW264.7 cells were transfected with control siRNA or STING siRNA and then treated with 10 MOI *P. aeruginosa* or ISD for 4 h. Immunoblot analysis of cGAS signaling pathways.

(H) *STING*$^{-/-}$ RAW264.7 cells were treated with 10 MOI *P. aeruginosa* for 4 h. Immunoblot analysis of cGAS signaling pathways.

(I) RAW264.7 cells were transfected with control siRNA or cGAS siRNA and then treated with 10 MOI *P. aeruginosa* or ISD for 4 h. Immunofluorescence detecting nuclear translocation of IRF3. Scale bar, 5 μm.

qPCR Data (mean ± SEM) are representative of three independent experiments. ($*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$ one-way ANOVA with Tukey post hoc test)

FIGURE S2



S2 Fig. *P. aeruginosa* activated cGAS signaling pathways in macrophages, related to Figure 1

(A) RAW264.7 cells were transfected with control siRNA or cGAS siRNA and then treated with 10 MOI *P. aeruginosa* or ISD for 4 h. qPCR was performed to measure mRNA levels of *IFN-β* or *IFIT1*.

(B) RAW264.7 cells were transfected with control siRNA or cGAS siRNA or STING siRNA and then treated with 10 MOI *P. aeruginosa* or ISD for 4 h. Cytokine levels of IFN-β in cell culture supernatant were detected using ELISA.

(C) RAW264.7 cells were transfected with control siRNA or STING siRNA and then treated with 10 MOI *P. aeruginosa* or ISD for 4 h. qPCR was performed to measure mRNA levels of *IFN-β* or *IFIT1*.

(D) RAW264.7 cells were transfected with control siRNA or STING siRNA and then treated with 10 MOI *P. aeruginosa* or ISD for 4 h. Cytokine levels of IFN-β in cell culture supernatant were detected using ELISA.

(E) MH-S cells were transfected with control siRNA or cGAS siRNA and then treated with 10 MOI *P. aeruginosa* or ISD for 4 h. Cytokine levels of IFN-β were measured by ELISA.

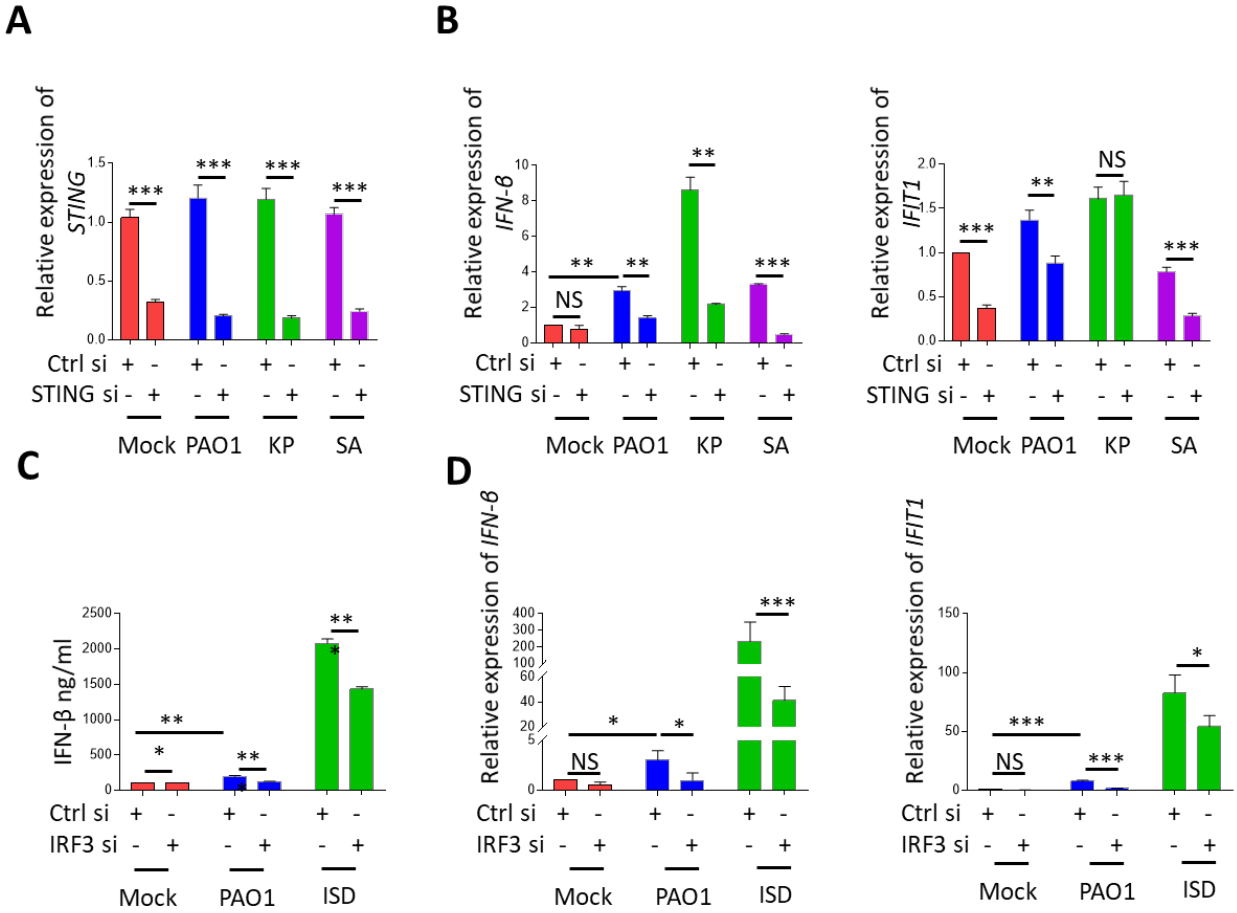
(F) WT and *cGAS*$^{-/-}$ BMDMs were treated with 10 MOI *P. aeruginosa* or ISD for 4 h. Immunoblotting analysis of cGAS signaling pathways.

(G) WT and *cGAS*$^{-/-}$ alveolar macrophages were treated with 10 MOI *P. aeruginosa* or ISD for 4 h. qPCR was performed to measure mRNA levels of *IFN-β* or *IFIT1*.

(H) WT and *cGAS*$^{-/-}$ peritoneal macrophages were treated with 10 MOI *P. aeruginosa* or ISD for 4 h. qPCR was performed to measure mRNA levels of *IFN-β* or *IFIT1*.

qPCR and ELISA Data (mean ± SEM) are representative of three independent experiments. (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ one-way ANOVA with Tukey post hoc test)

FIGURE S3



S3 Fig. cGAS signaling pathway is responsible for detecting extracellular bacteria, related to Figure 1

(A) BMDMs were transfected with control siRNA or STING siRNA for 48 h and then infected with 10 MOI *P. aeruginosa*, *K. pneumoniae*, and *S. aureus* for 4 h in BMDMs. qPCR analysis of mRNA levels of *STING*.

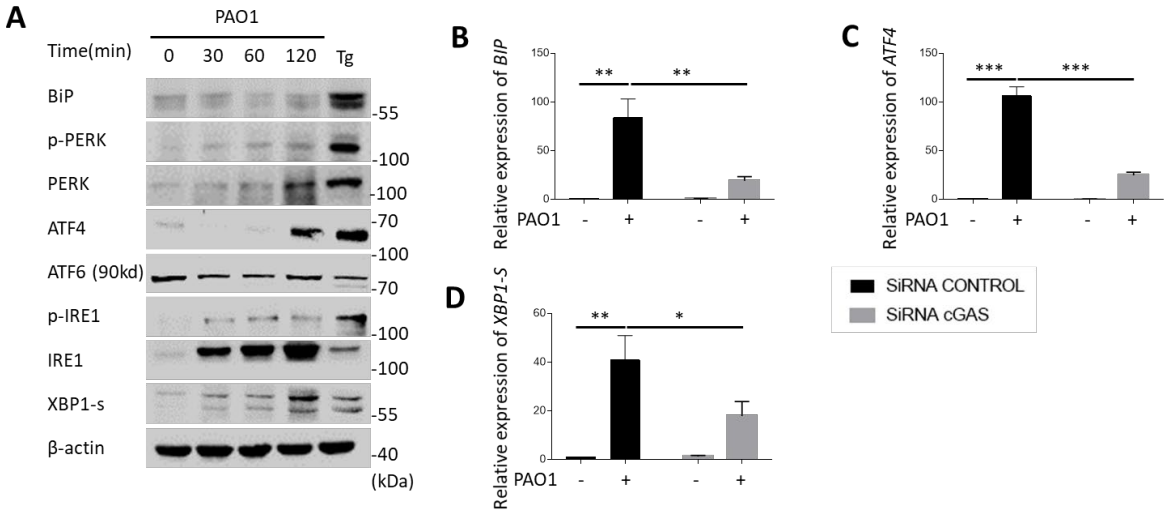
(B) BMDMs were transfected with control siRNA or STING siRNA for 48 h and then infected with 10 MOI *P. aeruginosa*, *K. pneumoniae*, and *S. aureus* for 4 h in BMDMs. qPCR analysis of mRNA levels of *IFN-β* or *IFIT1*.

(C) RAW264.7 cells were transfected with control siRNA or IRF3 siRNA and then treated with 10 MOI *P. aeruginosa* or ISD for 4 h. Cytokine levels of IFN-β in cell culture supernatant were detected using ELISA.

(D) RAW264.7 cells were transfected with control siRNA or IRF3 siRNA and then treated with 10 MOI *P. aeruginosa* or ISD for 4 h. qPCR was performed to measure mRNA levels of *IFN-β* or *IFIT1*.

qPCR and ELISA Data (mean ± SEM) are representative of three independent experiments. (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ one-way ANOVA with Tukey post hoc test)

FIGURE S4



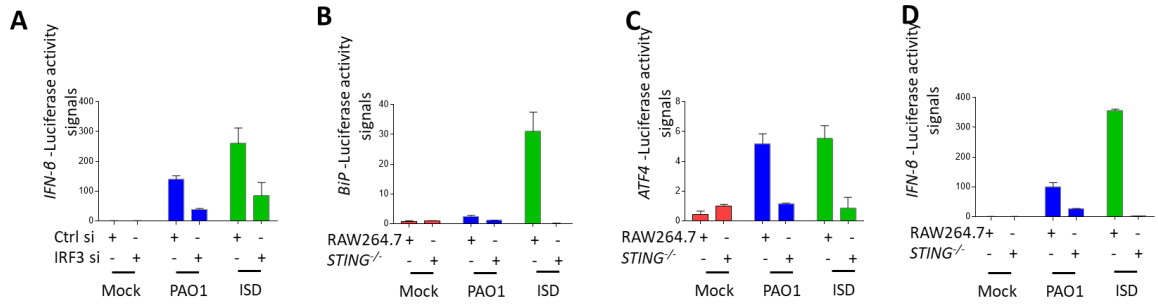
S4 Fig. UPR is activated under *P. aeruginosa* infection, related to Figure 3

(A) WT alveolar macrophages were infected with 20 MOI *P. aeruginosa* for 0.5, 1, or 2h, and Immunoblot analysis of UPR signaling pathways.

(B-D) RAW264.7 cells were transfected with control siRNA or cGAS siRNA for 48 h, and then treated with 10 MOI *P. aeruginosa* or ISD for 4 h. qPCR measuring mRNA levels of *BiP*, *ATF4*, and *XBP1-s*.

qPCR Data (mean ± SEM) are representative of three independent experiments. (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ one-way ANOVA with Tukey post hoc test)

FIGURE S5

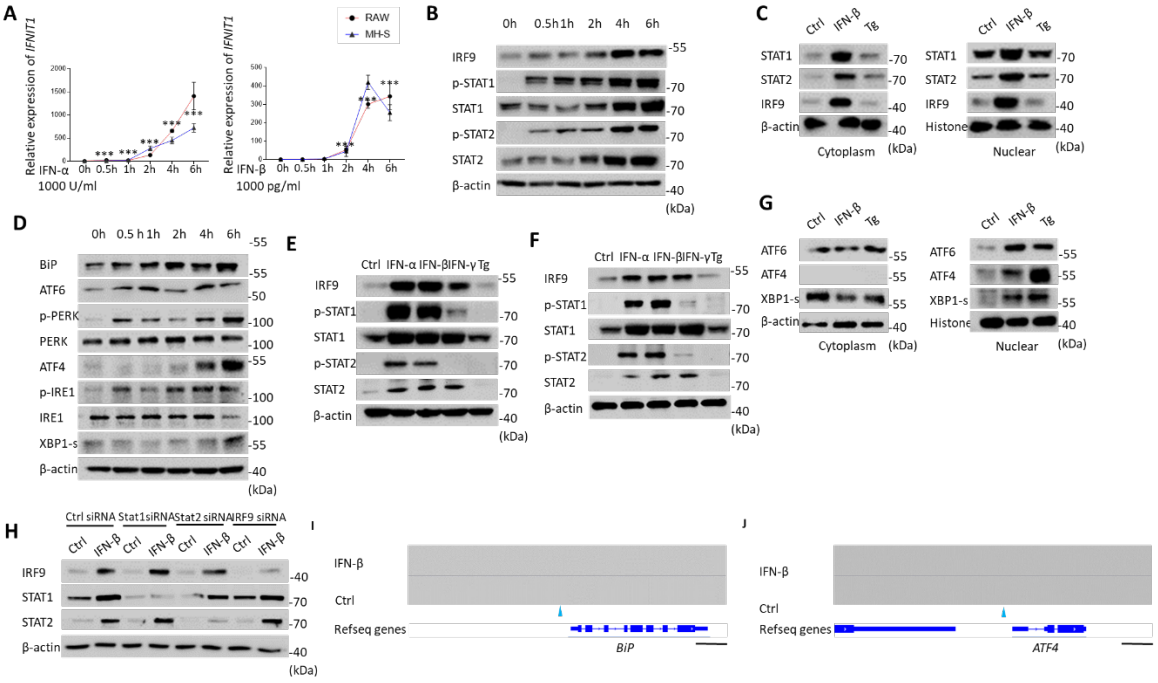


S5 Fig. *P. aeruginosa* activated IFN-dependent UPR, related to Figure 4

(A) Control siRNA or IRF3 siRNA-treated RAW264.7 cells were pre-transfected with *IFN-β* luciferase vectors for 24 h and then treated with 10 MOI *P. aeruginosa* or ISD for 4 h. Luciferase activity were detected using Dual-Luciferase Reporter Assay System.

(B) *STING*$^{-/-}$ RAW264.7 cells were were pre-transfected with *BiP*, *ATF4* and *IFN-β* luciferase vectors for 24 h and then treated with 10 MOI *P. aeruginosa* for 4 or ISD for 4 h. Luciferase activity were detected using Dual-Luciferase Reporter Assay System.

FIGURE S6



S6 Fig. *P. aeruginosa* activated IFN-dependent UPR, related to Figure 4

(A) RAW264.7 and MH-S cells were treated with IFN-α or IFN-β at different time points and then detected mRNA levels of *IFIT1*.

(B) RAW264.7 cells were treated with IFN-β at different time points. Immunoblotting analysis of JAK-STAT signaling pathways.

(C) RAW264.7 cells were treated with IFN-β or Tg for 6h. Immunoblotting analysis of STAT1, STAT2 and IRF9 in cytoplasmic and nuclear fractions of RAW264.7 cells.

(D) RAW264.7 cells were treated with IFN-β at different time points. Immunoblotting analysis of UPR signaling pathways.

(E) RAW264.7 cells were treated with IFN-α, IFN-β, IFN-γ and Tg for 6h. Immunoblotting analysis of JAK-STAT signaling pathways.

(F) BMDMs were treated with IFN-α, IFN-β, IFN-γ and Tg for 6h. Immunoblotting analysis of JAK-STAT signaling pathways.

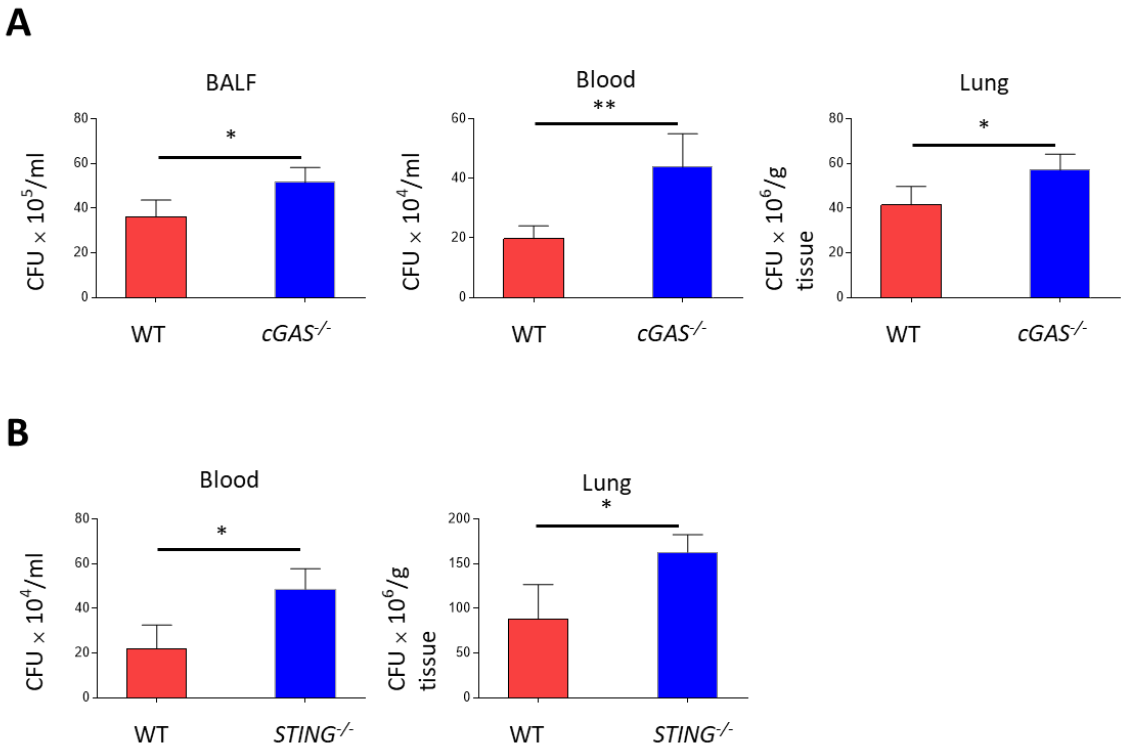
(G) RAW264.7 cells were treated with IFN-β or Tg for 6h. Immunoblotting analysis of ATF6, ATF4 and XBP1 in cytoplasmic and nuclear fractions of RAW264.7 cells.

(H) RAW264.7 cells were transfected with control siRNA, STAT1 siRNA, STAT2 siRNA and IRF9 siRNA IFN-β for 48h and then treated with IFN-β for 6h. Immunoblotting analysis of JAK-STAT signaling pathways.

(I, J) Chromatin immunoprecipitation sequencing (ChIP-seq) for STAT1 binding in BiP, ATF4 normalized by input. Blue arrows indicate transcription start site (TSS) of *BiP* or *ATF4*. Scale bar 1000bp.

qPCR Data (mean ± SEM) are representative of three independent experiments. (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ one-way ANOVA with Tukey post hoc test)

FIGURE S7



S7 Fig. The cGAS signaling pathway modulates bacterial burdens under *P. aeruginosa* infection, related to Figure 5

(A) Bacterial burdens of *P. aeruginosa* in BALF, blood and lung were detected after 24 h post *P. aeruginosa* infection in *cGAS*$^{-/-}$ mice compared with WT mice.

(**B**) Bacterial burdens of *P. aeruginosa* in blood and lung were detected after 24 h post *P. aeruginosa* infection in *cGAS*$^{-/-}$ mice compared with WT mice.

CFU (mean ± SEM) are representative of three independent experiments. (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ one-way ANOVA with Tukey post hoc test)

Supplemental References

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