Supplemental Material

Type I interferon induction by *Neisseria gonorrhoeae*: Dual requirement of cyclic GMP-AMP synthase and Toll-like receptor 4

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Figure S1

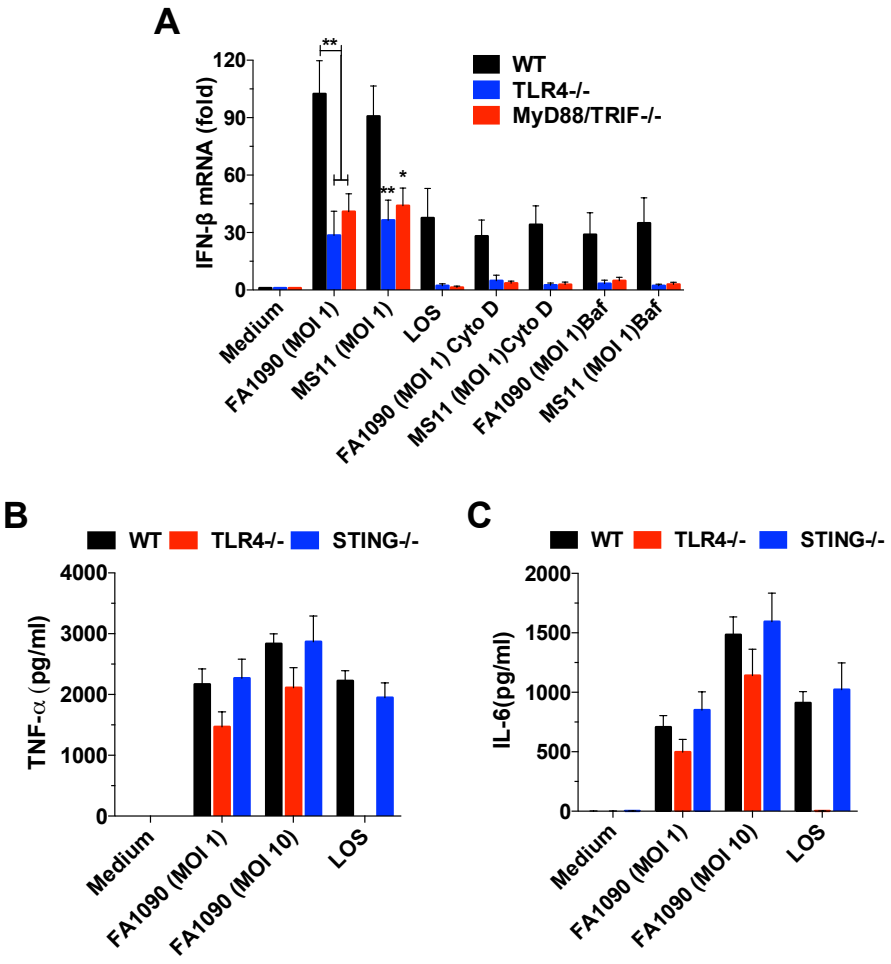

A
IFN-β mRNA (fold)
120
90
60
30
0
WT
TLR4-/-
MyD88/TRIF-/-
**
**
*
Medium
FA1090 (MOI 1)
MS11 (MOI 1)
LOS
FA1090 (MOI 1) Cyto D
MS11 (MOI 1)Cyto D
FA1090 (MOI 1)Baf
MS11 (MOI 1)Baf
B
WT
TLR4-/-
STING-/-
TNF-α (pg/ml)
4000
3000
2000
1000
0
Medium
FA1090 (MOI 1)
FA1090 (MOI 10)
LOS
C
WT
TLR4-/-
STING-/-
IL-6(pg/ml)
2000
1500
1000
500
0
Medium
FA1090 (MOI 1)
FA1090 (MOI 10)
LOS

Figure S2

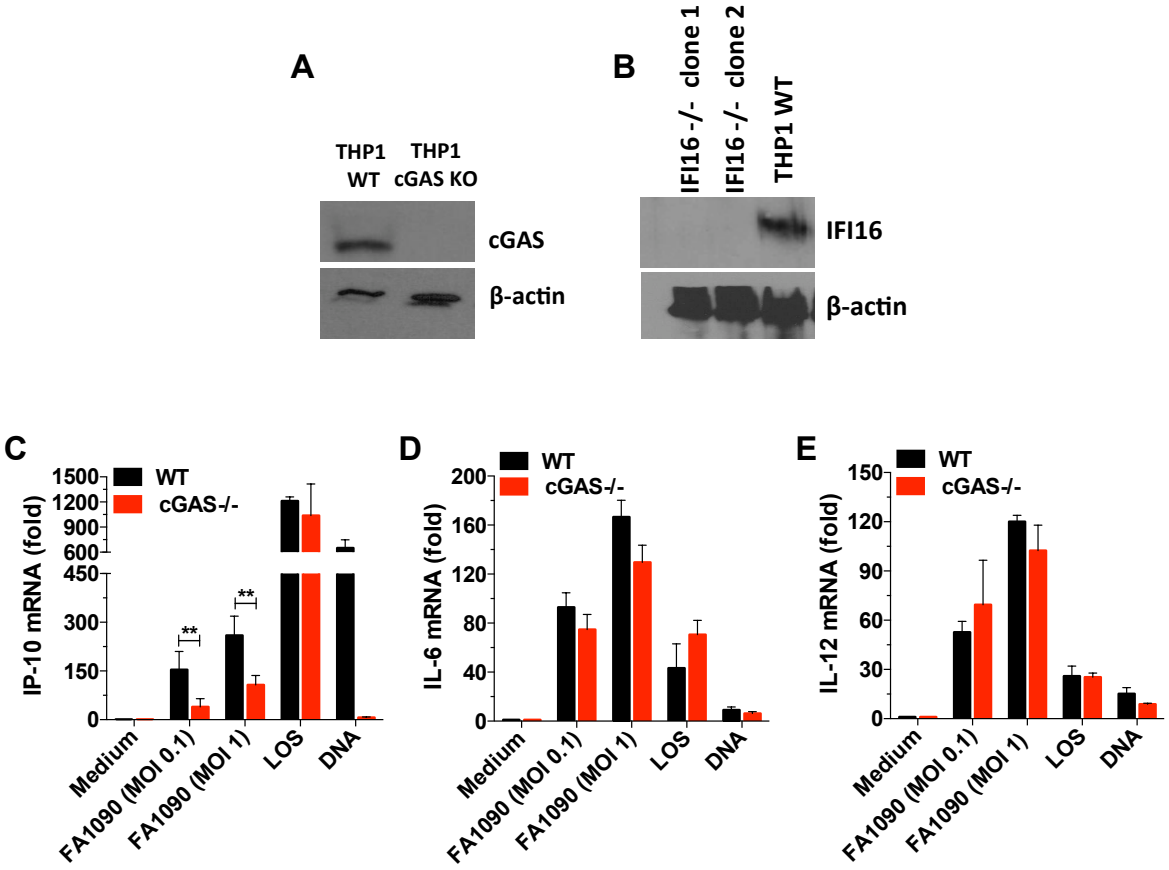
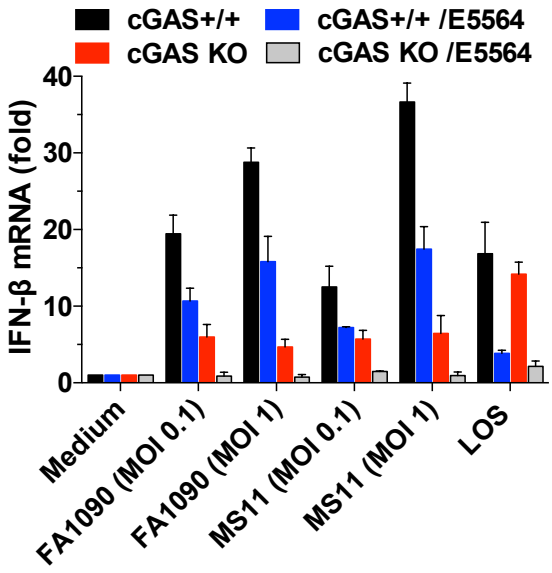

A
THP1 WT
THP1 cGAS KO
cGAS
β-actin
B
IFI16 -/- clone 1
IFI16 -/- clone 2
THP1 WT
IFI16
β-actin
C
IP-10 mRNA (fold)
WT
cGAS-/-
1500
1200
900
600
450
300
150
0
**
**
Medium
FA1090 (MOI 0.1)
FA1090 (MOI 1)
LOS
DNA
D
IL-6 mRNA (fold)
WT
cGAS-/-
200
160
120
80
40
0
Medium
FA1090 (MOI 0.1)
FA1090 (MOI 1)
LOS
DNA
E
IL-12 mRNA (fold)
WT
cGAS-/-
150
120
90
60
30
0
Medium
FA1090 (MOI 0.1)
FA1090 (MOI 1)
LOS
DNA

Figure S3



Supplemental Figure Legends

Figure S1. Induction of type I IFN is partially dependent on TLR4 and MyD88/TRIF pathways. Related to Figure 1.
(A) WT, TLR4$^{-/-}$, and MyD88/TRIF$^{-/-}$ immortalized macrophages were infected with GC (FA1090 and MS11 strains) at the indicated MOI or stimulated with LOS (100ng/ml) for 6 hrs. Cells were pretreated with cytochalasin D (Cyto D) or bafilomycin A (Baf) for 1 hr. (B-C) WT, TLR4$^{-/-}$ and STING$^{-/-}$ BMDMs were infected with GC strain FA1090 or stimulated with LOS (100ng/ml). Levels of IFN-β were determined by qPCR and normalized to GAPDH levels. Levels of TNF-α and IL-6 were measured by ELISA after 18 hr. Data are represented as mean ± SD of one experiment of two (A) and one of three (B-C) experiments (each performed in triplicate) that yielded similar results. Asterisks indicate that differences are statistically significant (*, 0.05 > p <> 0.05 and **, 0.01 > *p* > < 0.001).

Figure S2. IP-10, but not IL-6 and IL-12 induction by GC is dependent on cGAS. Related to Figure 2.
A) Whole lysates from WT and cGAS KO THP-1 cells, and (B) WT and IFI16 KO THP-1 cells, were subjected to western blotting using antibodies against cGAS, IFI16, and β-actin.
(C - E) Undifferentiated cGAS$^{+/+}$ and cGAS KO THP-1 cells were infected with GC, transfected with GC DNA (3μg/ml), or stimulated with LOS (100ng/ml). Levels of IP-10 (C), IL-6 (D) and IL-12 (E) were measured by qPCR and normalized by GAPDH levels. Data are represented as mean ± SD of one experiment of three experiments (each performed in triplicate) that yielded similar results. Asterisks indicate that differences are statistically significant (**$p < 0.01$).

Figure S3. Induction of type I IFN is completely dependent on cGAS and TLR4. Related to Figure 3.
cGAS$^{+/+}$ and cGAS KO THP-1 cells were infected with GC or stimulated with LOS (100ng/ml) with or without the TLR4/MD-2 inhibitor E5564 (10μg/ml) for 6 hr. Levels of IFN-β were measured by qPCR and normalized by GAPDH levels. Data are represented as mean ± SD of one experiment of two that yielded similar results.

Supplemental Experimental Procedures

Reagents

All cell culture reagents were obtained from Mediatech. LPS derived from *Escherichia coli* strain 0111:B4 was purchased from Sigma and re-extracted by phenol chloroform to remove lipopeptides as described (Hirschfeld et al., 2000). LOS (strain FA1090) was kindly provided by Peter Rice (UMass Medical School). Lipofectamine 2000 was purchased from Invitrogen, and digitonin from Sigma. Recombinant mouse IFN-β and human type I IFN-α were purchased from PBL Assay Science. Sendai virus (SeV, Cantrell strain) was obtained from Charles River Laboratories (Wilmington, MA). Anti-cGAS was purchased from Sigma, and anti-IFI16 from Abcam.

Cell culture, stimulation, ELISA and NO.

Cells including WT, TLR4$^{-/-}$ and MyD88/TRIF$^{-/-}$ immortalized macrophages, and primary BMDMs from WT, cGAS$^{-/-}$, TLR4$^{-/-}$, TLR4/cGAS$^{-/-}$ and STING$^{-/-}$ mice (C57BL/6 background housed at UMass Medical School animal facility) were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL) containing 4 mM glutamine and 10% fetal bovine serum (FBS). THP-1 (human promonomyelocytic cell line) cells (ATCC), cGAS KO and IFI16 KO generated by CRISPR/CAS9 system were grown in RPMI 1640/glutamine supplemented with 10% FBS. Transient transfections with GC DNA were performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. WT, TLR4$^{-/-}$, cGAS$^{-/-}$, TLR4/cGAS$^{-/-}$ and STING$^{-/-}$ BMDMs and undifferentiated THP-1 cells were infected or stimulated and supernatant collected after 16 hr. Where indicated, cells were pretreated with Bafilomycin A (200nM) or Cytochalasin D (2μM) (Sigma) for 1 hr.

CRISPR/Cas9-Mediated Knockout Cell Line Generation

THP-1 cells were coelectroporated with a gRNA- and a mCherry-Cas9-expression plasmid as described (Schmid-Burgk et al., 2014). The gRNA target sequences used were 5'-GAA CTT TCC CGC CTT AGG CAG GG-3' for cGAS (Wassermann et al., 2015) and 5'-CGG ACA CCT TAC TCC CTT TG-3' for IFI16. After FACS enrichment of mCherry-Cas9-positive cells, monoclones were grown for 2 weeks and were genotyped using deep sequencing. For each target gene, clones bearing all-allelic frameshift mutations were expanded for further study.

Neisseria gonorrhoeae strains

Strains used in this study were *Neisseria gonorrhoeae* FA1090, MS11, ND500, HH549 -traH (T4SS in-frame deletion) and HH557 -traH/+traH (T4SS complemented). The two clinical gonococcal isolates (#39 and #61) were chosen from women identified as contacts of men with gonorrhea. These strains were collected as part of an NIH grant (AI084048) at the STD Clinic located adjacent to the Institute of Dermatology, Chinese Academy of Medical Sciences in Nanjing, China. All Gonococci strains were grown overnight on chocolate agar plates and re-passaged onto fresh chocolate agar and allowed to grow for 5 hr to log phase at 37°C in an atmosphere containing 5% CO_2. These strains were resuspended in DMEM + 10% FBS and the optical density adjusted to give 1 x 10^7 number of organisms/mL.

Quantitative real-time PCR

Total RNA was extracted using Trizol (Invitrogen) according to the manufacturer's instructions. One microgram of total RNA was used for cDNA synthesis using iScript Select cDNA synthesis kit (Bio-Rad), and quantitative PCR using iQ SYBR Green Supermix was performed. The following primers were used: human IFN-β forward: 5'-AGG ACA GGA TGA ACT TTG AC-3'; human IFN-β reverse: 5'-TGA TAG ACA TTA GCC AGG AG-3'; human cGAS forward: 5'-GGG AGC CCT GCT GTA ACA CTT CTT AT-3'; human cGAS reverse: 5'-CCT TTG CAT GCT TGG GTA CAA GGT-3'; human TNFα forward: 5'-TGC TTG TTC CTC AGC CTC TT-3'; human TNFα reverse: 5'-GGT TTG CTA CAA CAT GGG CT-3'; human IL-6 Forward: 5'-ACT CAC CTC TTC AGA ACG AAT TG-3'; human IL-6 reverse: 5'-CCA TCT TTG GAA GGT TCA GGT TG-3'; human IL-12 forward: 5'-CCT TGC ACT TCT GAA GAG ATT GA -3'; human IL-12 reverse: 5'-ACA GGG CCA TCA TAA AAG AGG T-3'; human IP10 forward: 5'-GTG

GCA TTC AAG GAG TAC CTC-3'; human IP10 reverse: 5'-TGA TGG CCT TCG ATT CTG GAT T-3'; human GAPDH forward: 5'- GAG TCA ACG GAT TTG GTC GT-3'; human GAPDH reverse: 5'- TTG ATT TTG GAG GGA TCT CG-3'; mouse IFN-β forward: 5'-TCC GAG CAG AGA TCT TCA GGA A-3'; mouse IFN-β reverse: 5'-TGC AAC CAC CAC TCA TTC TGA G-3'; mouse Hamp1 forward: 5'-AGA AAG CAG GGC AGA CAT TG-3'; mouse Hamp1 reverse: 5'-CAC TGG GAA TTG TTA CAG CAT T-3'; mouse Ferroportin forward: 5'-TGG AAC TCT ATG GAA ACA GCC T-3'; mouse Ferroportin reverse: 5'-GGC ATT CTT ATC CAC CCA GT-3'; mouse GAPDH forward: 5'-CGA CTT CAA CAG CAA CTC CCA CTC TTC C-3'; mouse GAPDH reverse: 5'-TGG GTG GTC CAG GGT TTC TTA CTC CTT-3'. To evaluate expression of iron-responsive genes in intracellular GC strain FA1090 after BMDM infection, total RNA was extracted using RNeasy columns (Qiagen) and genomic DNA was removed by DNase treatment and gDNA Wipeout (Qiagen) following the manufacturer's instructions. The extracted bacterial RNA was then reverse transcribed to cDNA using the QuantiTect Reverse Transcriptase kit (Qiagen) following the manufacturer's instructions. Quantitative PCR using iQ SYBR Green Supermix was performed and results were normalized to 16S rRNA expression for each individual sample. The expression of iron-responsive genes *fetA*, *tbpB*, *mpeR*, and non-iron-responsive gene *serC* in macrophages-associated GC FA1090 after 6 hr of infection was compared to that after 1 hr of infection. The following primers were used: tbpB forward: 5'-AGG GCA AGG CGA CAA ATA CA-3', and reverse: 5'-CGA ATC AGT TTG CCC GTC AA-3'; fetA forward: 5'-AGA GTT TGC CGT CAG CGA AA-3', and reverse: 5'-TAG GCG TTG GCA TCC AGT TT-3'; mpeR forward: 5'-AAA CAG CCC GGT TTG CAT CT-3', and reverse: 5'-GCG CAG TTG TGG CTG AAA TT-3', serC forward: 5'-TGT TGC CTG AAG CTG TGT TG-3', and reverse: 5'-TGT TCC GCA TGA TGC AGG AT-3'; 16S forward: 5'-CCA TCG GTA TTC CTC CAC ATC TCT-3', and reverse: 5'-CGT AGG GTG CGA GCG TTA ATC-3'.

References

Hirschfeld, M., Ma, Y., Weis, J.H., Vogel, S.N., and Weis, J.J. (2000). Cutting edge: repurification of lipopolysaccharide eliminates signaling through both human and murine toll-like receptor 2. J Immunol *165*, 618-622.

Schmid-Burgk, J.L., Schmidt, T., Gaidt, M.M., Pelka, K., Latz, E., Ebert, T.S., and Hornung, V. (2014). OutKnocker: a web tool for rapid and simple genotyping of designer nuclease edited cell lines. Genome Res 24, 1719-1723.