SUPPLEMENTAL DATA

Supplemental Materials and Methods

Cell Culture

THP-1 cells (ATCC; Manassas, VA) were cultured in DMEM (Invitrogen-GIBCO, Grand Island, NY), supplemented with 10% FCS (Hyclone, Logan, UT) and penicillin/streptomycin (P/S; Invitrogen-GIBCO, whereas bone marrow macrophages (BMMs) were cultured in RPMI 1640 (Invitrogen-GIBCO) and 20% L929 cell conditioned media, both supplemented with 10% FCS and P/S, as previously reported [1-3].

Additional Treatments and Assays

Some cells were stimulated with nigericin (20 μM; EMD-Millipore), Imject Alum Adjuvant (Alum; 200 μg/ml; Thermo Scientific) and purified *Bacillus anthracis* lethal toxin (LT; lethal factor + protective antigen; a generous gift of J. Mogridge; [4]). Some cells were pretreated with the isopeptidase inhibitor G5 (3,5-bis[(4-Nitrophenyl)methylene]-1,1-dioxide,tetrahydro-4H-thiopyran-4-one; EMD-Millipore, Billerica, MA) that exhibits specificity for BRCC3 [5], caspase 1 inhibitor YVAD (Enzo Life Sciences, Farmingdale, NY) and PKR inhibitor (2-Aminopurine; Sigma-Aldrich; [6]), as indicated.

HMGB1 in culture supernatants was measured by immunoblotting with anti-HMGB1 (Cell Signaling; Beverly, MA) and IL-18 was measured by ELISA (R&D Systems).

Bacterial Infections

Overnight cultures of *S. typhimurium* SL1344 [7] were diluted 1:50 and grown to an OD_{600} = 0.6 in LB. After washing in PBS, bacteria were resuspended in Optimem (10^9 CFU/ml; Invitrogen-GIBCO) and added (MOI = 25) to LPS primed BMMs. One hour later cultures were washed with PBS and grown in Optimem supplemented with gentamycin (100 μg/ml; 5h). *L. pneumophila* (JR32; restriction-defective Philadelphia-1) and flagellin minus (Fla-) strains (JR32 background)

were grown in AYE broth or on CYE plates [1]. Previously characterized strains ectopically over expressing *cdgS3*, *cdgS3B* or *cdgS4* were placed on a flagellin null background [8]. BMMs were infected with post-exponential phase, IPTG (isopropyl-β-D-thiogalactopyranoside; 1 mM, 6h; Sigma Aldrich) treated *L. pneumophila* (MOI = 10), as previously reported [1].

TMRM & mitoSox staining

Mitochondrial reactive oxygen species (mROS) were measured by FACS (LSR Fortessa; BD Biosciences) in mitoSox (Invitrogen; 2.5 μM, 20 min in Optimem) stained BMMs. FACS or fluorescence microscopy (Zeiss Axiovert 200M; Oberkocken, Germany) was used to measure mitochondrial membrane tetramethyl rhodamine methyl ester (TMRM; Anaspec, Fremont, CA; 200 nM, 30 min in Optimem) uptake (i.e., $\Delta\psi_m$). FACS data were analyzed with FlowJo software (Tree Star; Ashland, OR).

Table S1. Primer Sequences

Mu–IFN-β	Forward	5' TCC AAG AAA GGA CGA ACA TTC G 3'
	Reverse	5' TGA GGA CAT CTC CCA CGT CAA 3'
Hu-ASC	Forward	5' CTG CTG GAT GCT CTG TAC 3'
	Reverse	5' GCT GGT GTG AAA CTG AAG 3'
Hu-NLRP3	Forward	5' GTT CAC TGC CTG GTA TCT 3'
	Reverse	5' GTG CTT GCC ATC TTC ATC 3'
Hu-MPYS	Forward	5' ACT TAC AAT CAG CAT TAC AAC A 3'
	Reverse	5' GTC AGC CAT ACT CAG GTT 3'
Mu-MPYS	Forward	5' GCC TGG TCA TAC TAC ATT G 3'
	Reverse	5' GCA GCA TAT CTC GGA ATC 3'
Mu-GAPDH	Forward	5' CAT CAA GAA GGT GGT G 3'
	Reverse	5' CCT GTT GCT GTA GCC 3'
β-actin	Forward	5' GCT CCT CCT GAG CGC AAG T 3'
	Reverse	5' TCG TCA TAC TCC TGC TTG CTG AT 3'

Figure S1. Additional inflammatory responses in murine BMMs

(**A**) Secreted IL-1β was measured by ELISA in LPS primed BMMs (left panel) treated with ATP (5 mM; 6h) or transfected (X-tremegene HP; Xtrm) with c-diGMP (10 nmol; 6h), c-diAMP (10 nmol; 6h), cGMP (10 nmol; 6h) or GTP (10 nmol; 6h), as in Figure 1A. PMA differentiated THP-1 cells (right panel) were treated with ATP (5 mM; 6h) or transfected (Lipofectamine 2000; lpftm) with c-diAMP (cdA; 5nmol; 6h), c-diGMP (cdG; 5 nmol; 6h) or poly(dA:dT) (dA:dT; 5 μg/ml; 6h), as Figure 1A. Graphs present means ± standard error from three independent experiments. (**B**) Secreted IL-1β was measured by ELISA in LPS primed BMMs treated with KCl, Glibenclamide (Gli), 2-APB or U73122 prior to stimulation with Imject Alum Adjuvant (Alum; 200 μg/ml; Thermo Scientific) or nigericin (20 μM; 1h), as in Figure 2A. Graphs present means ± standard error from two independent experiments.

Figure S2. c-diGMP stimulation of cells deficient of inflammasome components.

(**A**) Secreted IL-1β was measured by ELISA in LPS primed C57Bl/6J wild type (WT), NLRP3[-/-] (NLRP3), or ASC[-/-] (ASC) BMMs treated as in Figure 3. Graphs present means ± standard error from three independent experiments. (**B**) IL-1β was measured by ELISA in LPS primed C57Bl/6J (Bl6) WT and AIM2[-/-] treated as in panel A. Graphs present means ± standard error from two independent experiments. (**C**) IL-1β was measured by ELISA in LPS primed 129 WT and NLRP1b[-/-] BMMs treated with ATP (5 mM; 6h), *B. anthracis* (LT; 1 μg/ml, as indicated), or transfected with c-diGMP (cdG;10 nmol; 6h) and c-diAMP (cdA; 10 nmol; 6h), as in panel A. Graphs present means ± standard error from two independent experiments. (**D**) IL-1β levels were measured by ELISA in LPS primed BMMs, which were pretreated with the PKR inhibitor, 2 amino purine (2-AP; 1 mM, 30 min) prior to stimulation with ATP (5 mM; 30 min) or transfection (X-tremegene HP; Xtrm) with c-diGMP (10 nmol; 6h). Graphs present means ± standard error from three independent experiments. (**E**) IL-18 levels were measured by ELISA on supernatants from panel C. Graphs present means ± standard error from three independent experiments. (**F**)

Expression of ASC (left panel) and NLRP3 (right panel) was evaluated in different THP-1 cell lines by Q-PCR, as indicated. Results were normalized to β-Actin and reported as relative fold change. Graphs present means ± standard error from three independent experiments.

Figure S3. c-diGMP stimulation is not associated with mitochondrial perturbation.

Mitochondrial ROS production (**A**) and mitochondrial membrane potential (**C**) were evaluated by FACS in LPS primed BMMs stimulated with ATP (5 mM, 6h), c-diGMP (cdG; 10 nmol; 6h) or poly(dA:dT) (dA:dT; 5 μM; 6h), after staining with mitoSox (2.5 μM; 20 min) and TMRM (200 nM; 30min), respectively. (**B, D**) Mean fluorescent intensity (MFI) quantification of the data from panels A & C, respectively. Data are representative of 3 independent experiments. (**E**) Mitochondrial membrane potential after TMRM staining was also evaluated by fluorescence microscopy on BMMs cells treated as in panel C.

Figure S4. HMGB1 production and Deubiquitinylation in c-diGMP stimulated BMMs.

(**A**) Secreted HMGB1 was measured by immunoblotting (anti-HMGB1; Cell Signaling) supernatants collected from C57Bl/6J BMMs treated with ATP, c-diGMP and c-diAMP, as in Figure 1A. (**B**) Secreted IL-1β was measured by ELISA in LPS primed C57Bl/6J BMMs treated with YVAD (50 μM; 30 min) or G5 (3,5-bis[(4-Nitrophenyl)methylene]-1,1-dioxide,tetrahydro-4H-thiopyran-4-one; 1 & 2 μM; 30 min) prior to treatment with ATP, c-diGMP and c-diAMP, as in Figure S1. Graphs present means ± standard error from three independent experiments.

Figure S5. IL-1β is required for c-diGMP mediated neutrophil recruitment in vivo.

(**A**) Peritoneal fluid recovered from mice described in Figure 4 was stained with antibodies to Ly6G and F4/80 and evaluated by FACS. Data are from individual mice, representative of two independent experiments (n=6 mice for c-di-GMP and n=4 mice for PBS treatment). (**B**) Peritoneal fluid was evaluated by FACS analysis 16 h after intraperitoneal injection of PBS or 200

nmol of c-diGMP (cdG) as detailed in Figure 4. In another study 40 µg of anti-IL-1β antibody were injected (IP; 200 µl PBS) 30 min prior to c-diGMP injection (cdG + α-IL-1β). The histogram reports the number of neutrophils recruited to the peritoneum.

References

1. Abdul-Sater AA *et al* (2012) The overlapping host responses to bacterial cyclic dinucleotides. *Microbes and infection / Institut Pasteur* **14:** 188-197

2. Plumlee CR, Lee C, Beg AA, Decker T, Shuman HA, Schindler C (2009) Interferons direct an effective innate response to Legionella pneumophila infection. *J Biol Chem* **284:** 30058-30066

3. Zhao W, Cha EN, Lee C, Park CY, Schindler C (2007) Stat2-Dependent Regulation of MHC Class II Expression. *J Immunol* **179:** 463-471

4. Kassam A, Der SD, Mogridge J (2005) Differentiation of human monocytic cell lines confers susceptibility to Bacillus anthracis lethal toxin. *Cellular microbiology* **7:** 281-292

5. Py BF, Kim MS, Vakifahmetoglu-Norberg H, Yuan J (2013) Deubiquitination of NLRP3 by BRCC3 critically regulates inflammasome activity. *Molecular cell* **49:** 331-338

6. Lu B *et al* (2012) Novel role of PKR in inflammasome activation and HMGB1 release. *Nature* **488:** 670-674

7. Hoiseth SK, Stocker BA (1981) Aromatic-dependent Salmonella typhimurium are non-virulent and effective as live vaccines. *Nature* **291:** 238-239

8. Levi A, Folcher M, Jenal U, Shuman HA (2011) Cyclic diguanylate signaling proteins control intracellular growth of Legionella pneumophila. *mBio* **2:** e00316-00310

Figure S1, Abdul-Sater et al.

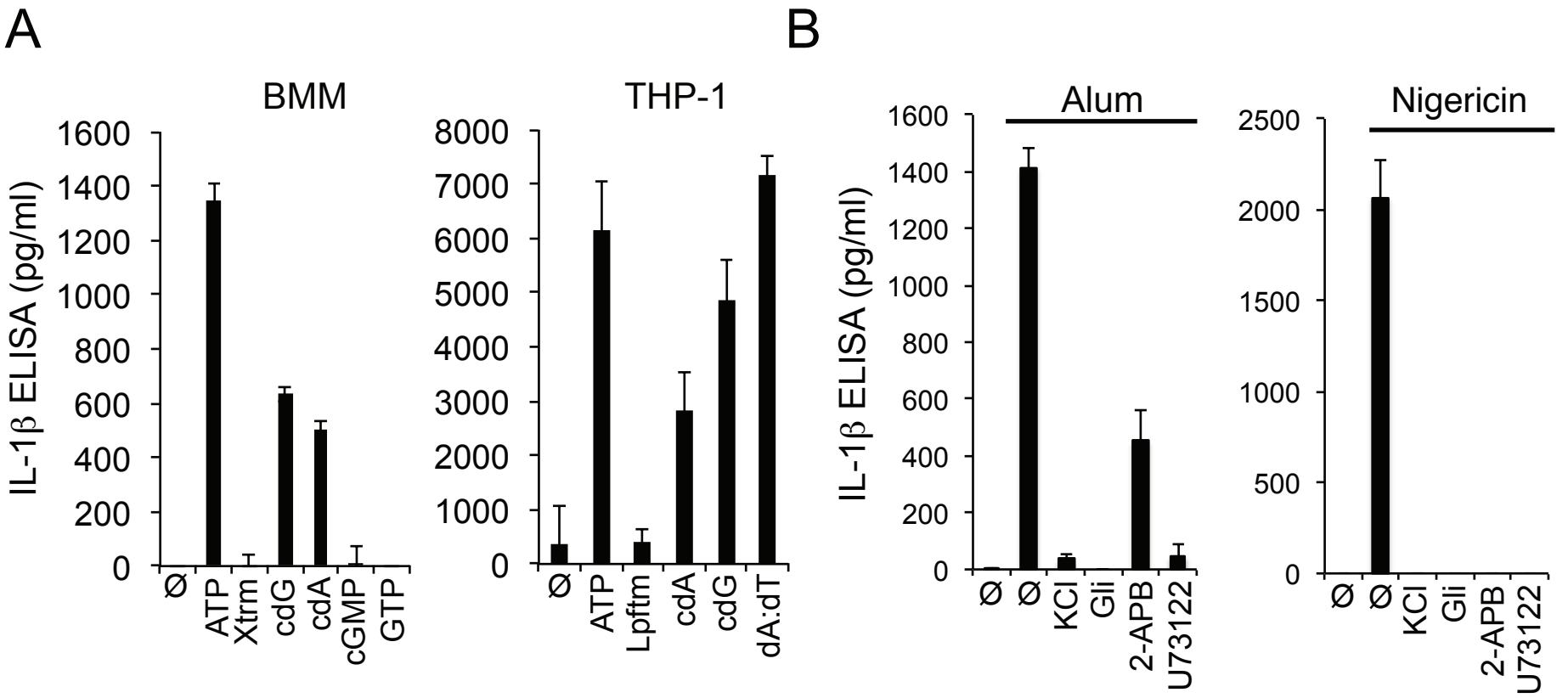

Figure S2, Abdul-Sater et al.

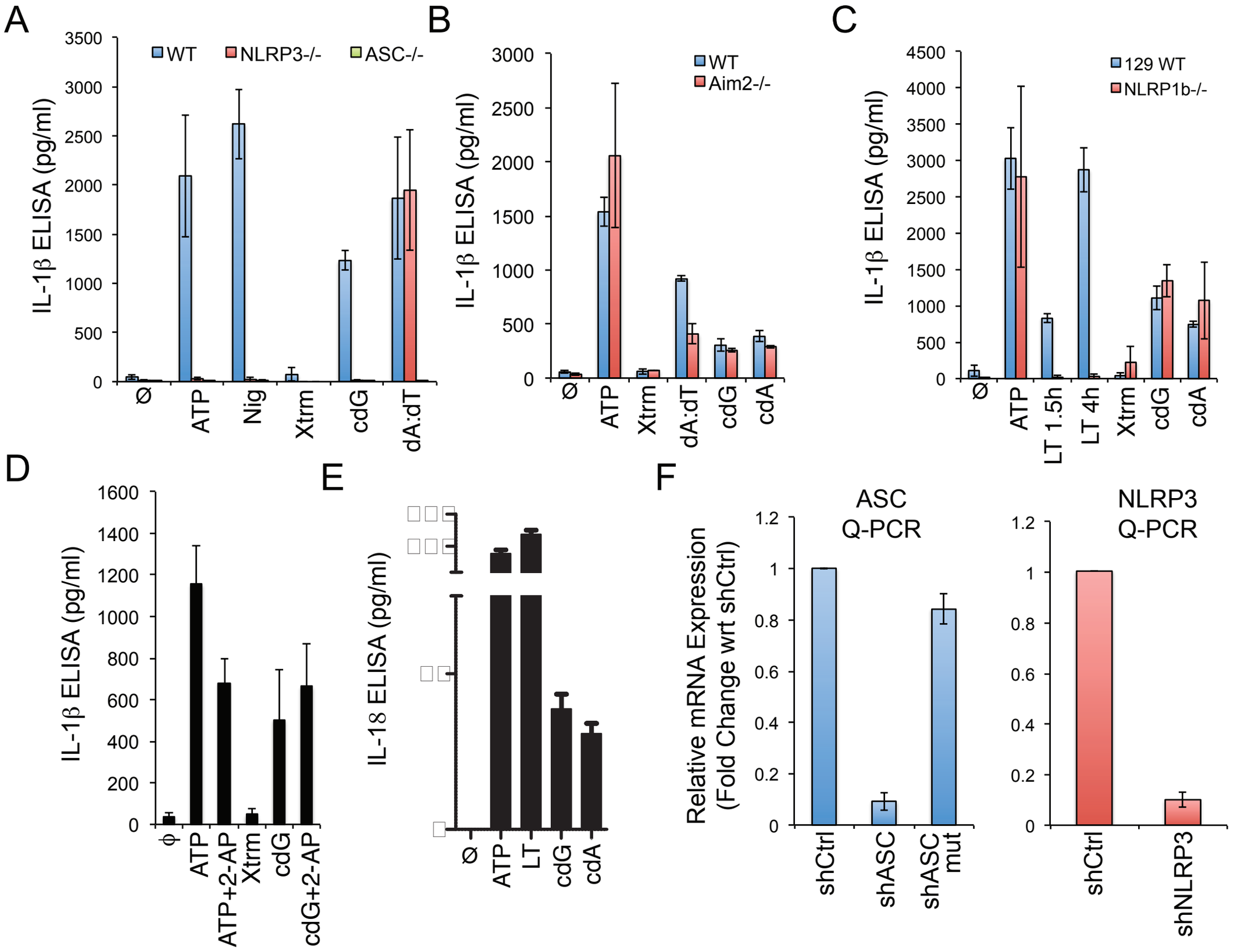

Figure S3, Abdul-Sater et al.

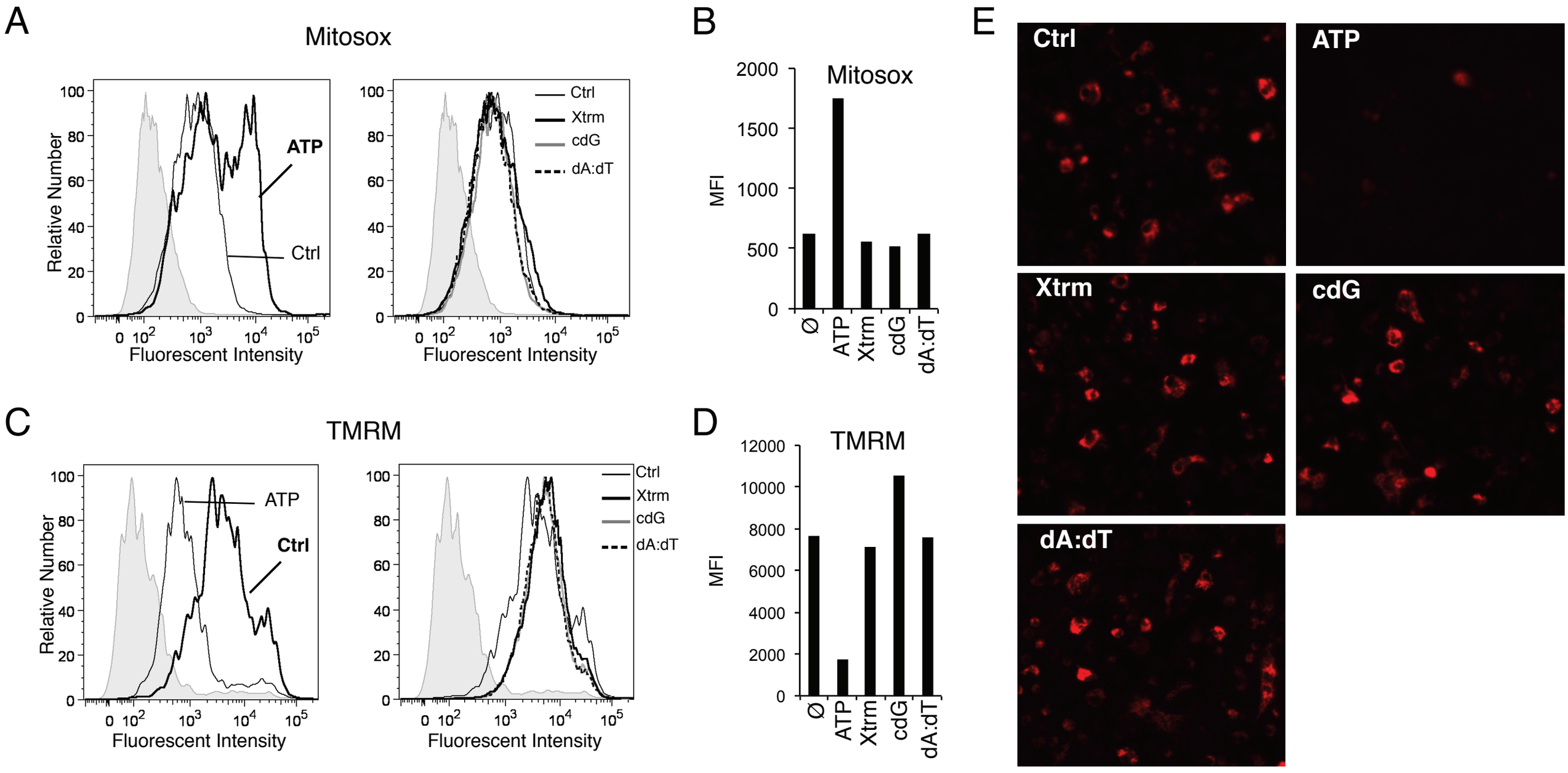

Figure S4, Abdul-Sater et al.

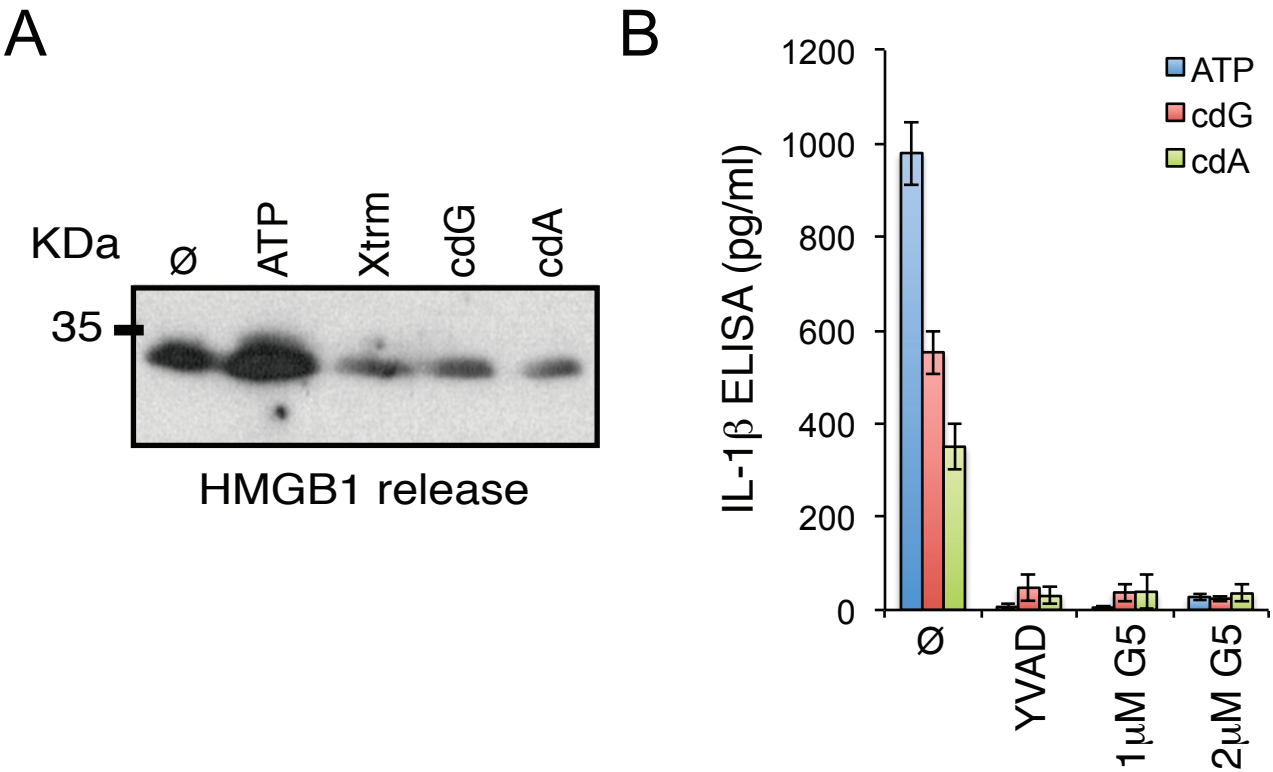

Figure S5, Abdul-Sater et al.