PNAS

Supporting Information for

Disulfiram blocks inflammatory TLR4 signaling by targeting MD-2

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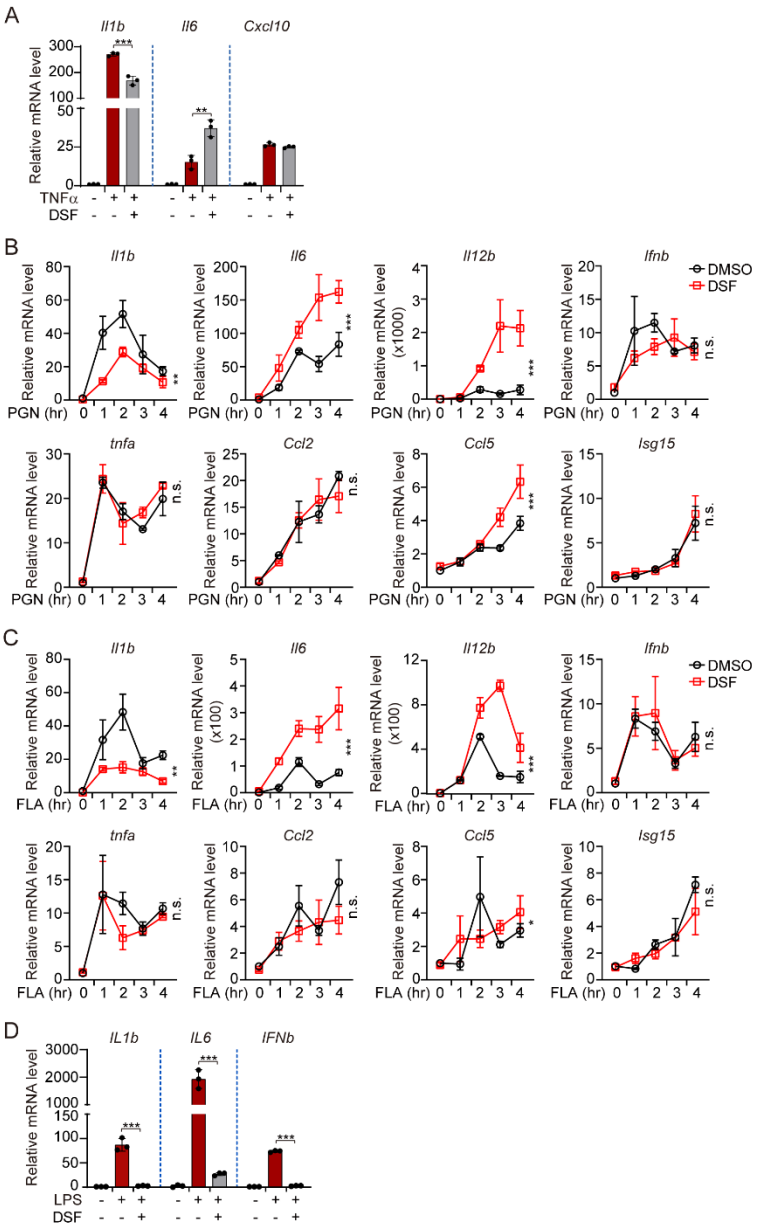

Fig. S1. Disulfiram specifically inhibits LPS-triggered inflammatory response.
(**A**-**C**) Mouse iBMDMs were pretreated or not with disulfiram (5 μM) for 0.5 hr before stimulation with TNFα (10 ng/mL) (**A**), peptidoglycan (PGN) (**B**) or flagellin (FLA) (**C**) for the indicated times. (**D**) Human THP1 cells, pretreated or not with disulfiram for 0.5 hr, were challenged with LPS. mRNA levels of the indicated inflammatory cytokines were assessed by qRT-PCR, normalized to *Gapdh* (**A-C**) or *GAPDH* (**D**), and relative to unstimulated cells. Graphs in **A**-**D** show mean ± s.d.; data are representative of three independent experiments. Data were analyzed using a two-tailed Student's *t*-test (**A** and **D**) or two-way analysis of variance (ANOVA) (**B** and **C**). *P<0.05; **P<0.01; ***P<0.001; n.s., not significant

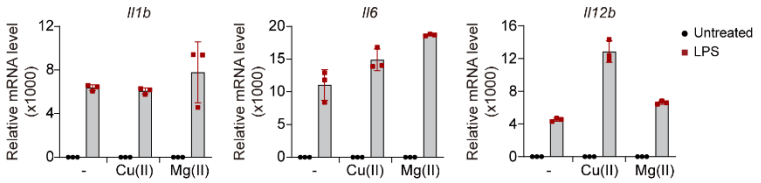

Fig. S2. Cu(II) and Mg(II) do not affect LPS-triggered pro-inflammatory cytokine induction. iBMDMs were pretreated with Cu(II) or Mg(II) (10 μM) for 0.5 hr before stimulation with LPS (1 μg/mL). mRNA levels of the indicated inflammatory cytokines were assessed by qRT-PCR, normalized to *Gapdh* and relative to unstimulated cells. Graphs show mean ± s.d.; data are representative of three independent experiments.

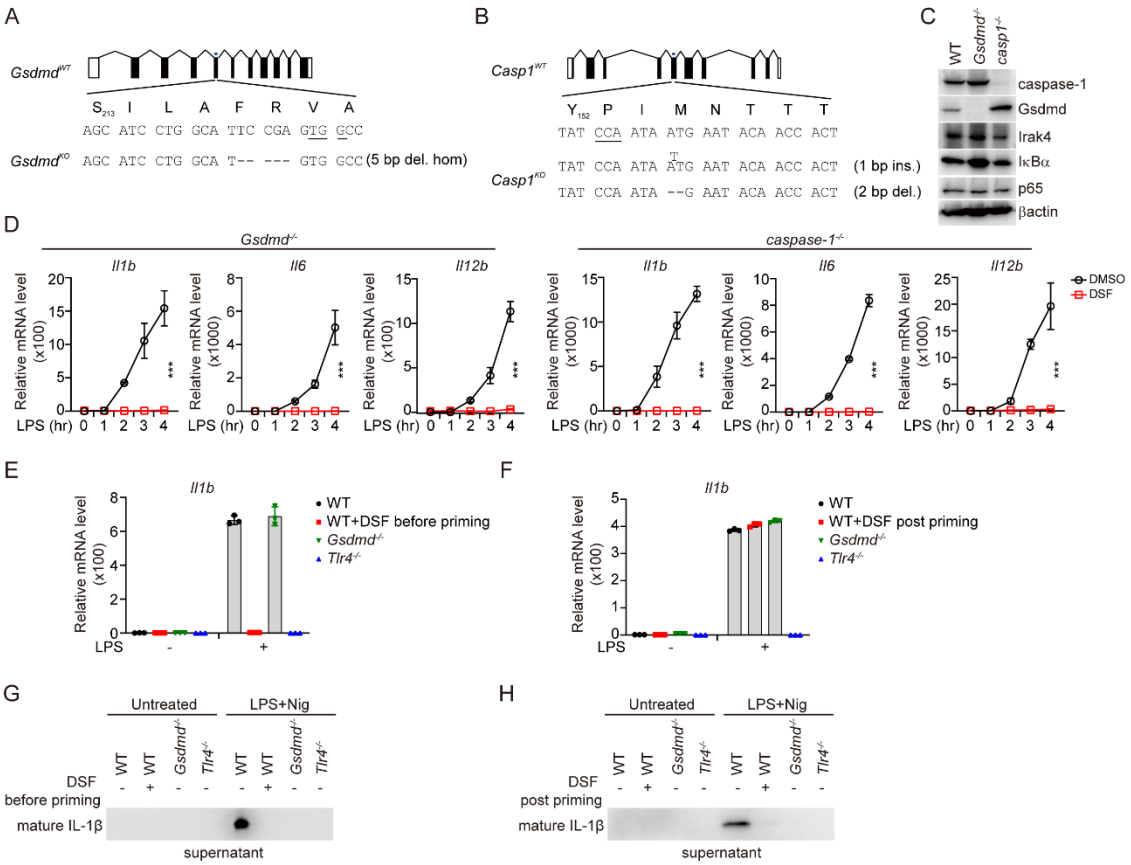

Fig. S3. Disulfiram inhibition of TLR4 signaling is independent of *Gsdmd* and *Caspase-1*. (**A** and **B**) *Gsdmd* (**A**) and *Caspase-1* (**B**) KO iBMDMs used in this study. Black bars and broken-lines represent exons and introns of the target genes, respectively. Location of gRNAs is indicated with blue bar above exon. Shown below is the coding sequence of a wildtype allele with NGG PAM underscored (top) and mutant allele (bottom). (**C**) Wild-type (WT), *Gsdmd* and *Caspase-1* KO iBMDMs analyzed by immunoblot for Caspase-1, Gsdmd, Irak4, IκBα and NF-κB p65. β-actin was a loading control. (**D** *Gsdmd* (left panel) and *Caspase-1* (right panel) KO iBMDMs pretreated or not with disulfiram for 0.5 hr were stimulated with LPS (1 μg/mL) for the indicated times. mRNA levels of the indicated inflammatory cytokines were assessed by qRT-PCR, normalized to *Gapdh* and relative to unstimulated cells. (**E**-**H**) WT, *Gsdmd* and *Tlr4* KO iBMDMs stimulated with LPS (1 μg/mL) for 4 hr. WT iBMDMs were treated or not with disulfiram before (**E** and **G**) or 0.5 hr after (F and H) adding LPS. (**E** and **F**) mRNA levels of *Il1b* were assessed by qRT-PCR, normalized to *Gapdh* and relative to unstimulated cells. (**G** and **H**) Untreated or LPS-primed iBMDMs were treated or not with nigericin for 0.5 hr before cell culture media were collected and analyzed by immunoblot for released mature IL-1β. Graphs in **D**, **E** and **F** show mean ± s.d.; data are representative of three independent experiments. Data were analyzed using two-way analysis of variance (ANOVA). ***P<0.001

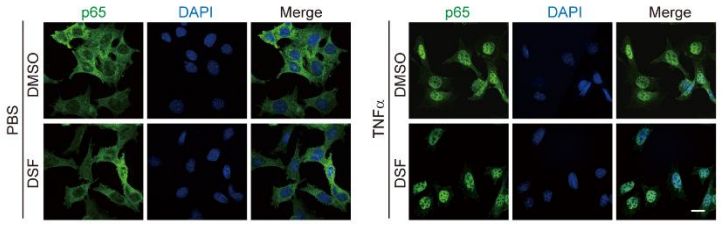

Fig. S4. Disulfiram does not inhibit TNFα-induced nuclear translocation of p65.
MEF cells, pretreated or not with disulfiram (10 μM) for 0.5 hr, were stimulated with TNFα (10 ng/mL) for another 0.5 hr and then analyzed by immunostaining for NF-κB p65. Representative immunofluorescence images of NF-κB p65 subcellular localization are shown.

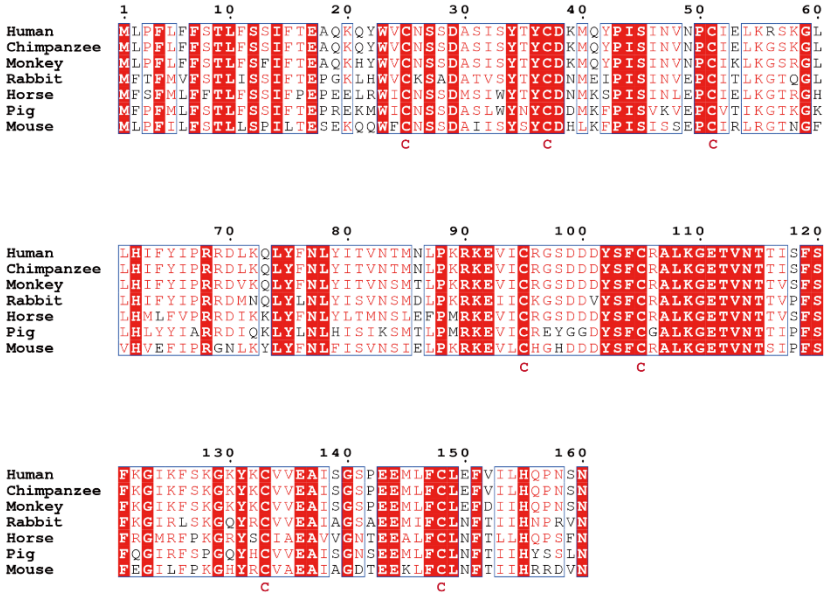

Fig. S5. Sequence alignment of MD-2 protein from multiple species.
Sequence alignment of human, chimpanzee, monkey, rabbit, horse, pig and mouse MD-2 was performed using the ClustalW2 algorithm and plotted by ESPript program. Identical residues are highlighted by red background, and conserved residues are colored in red. Cysteine residues are marked with red “C” underneath the sequences.

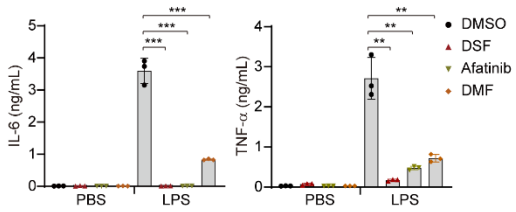

Fig. S6. Disulfiram (DSF), afatinib and DMF inhibit LPS-induced pro-inflammatory cytokine secretion.

iBMDMs were pretreated with afatinib (10 μM), DMF (20 μM) or DSF (10 μM) for 0.5 hr before stimulation with LPS (1 μg/mL) for 2 hr. The amount of indicated inflammatory cytokines secreted into the culture medium was determined by ELISA. Graphs show mean ± s.d. Data were analyzed using a two-tailed Student's *t*-test. **P<0.01; ***P<0.001