Supplementary Information

EXPERIMENTAL PROCEDURES

Cell culture - HCT116 and LS 174T cells were maintained in DMEM (Invitrogen, Carlsbad, CA) containing 10% (v/v) heat-inactivated FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin at 37^{o}C under 5% CO_2.

Reagents - Rabbit polyclonal anti-IRAK-1, anti-MyD88, and anti-TRAF6 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal anti-caspase-3, anti-total-ERK, and anti-total-p38 antibodies were from Cell Signaling (Beverly, MA). Siver staining kit was from Thermo Scientific (Rockford, IL).

Zn^{2+}Phos-tag SDS-PAGE – Total cell lysates were subjected to SDS-PAGE with a separating gel (7.5% w/v polyacrylamide and 357 mM Bis-tris-HCl, pH 6.8) containing 25 μM acrylamide-pendant Phos-tag ligand (Wako Pure Chemical, Osaka, Japan) and two equivalents of $Zn(NO_3)_2$. The gel was soaked in a solution containing 1.0 mM EDTA for 10 min, and then soaked in a solution containing 25 mM Tris, 192 mM glycine, and 10% v/v MeOH for 10 min before western blotting in order to remove excess Zn^{2+}.

FIGURE LEGENDS

FIGURE S1. MDP decreases LPS-induced Nod2 and Rip2 expression. BMDM were pretreated with LPS (0.1 ng/ml) for 6 h and then stimulated with MDP for the indicated times. Cell extracts were subjected to Western blot analysis for Nod2, Rip2, and Actin. Data are representative of three independent experiments with similar results.

FIGURE S2. TLR ligands do not induce degradation of Nod2. RAW264.7 cells were pretreated with CpG-B (1 μM) or LPS (0.1 ng/ml or 1 ng/ml) for 6 h and then stimulated with LPS, CpG-B, or MDP for the indicated times. Cell extracts were subjected to Western blot analysis for Nod2, Rip2, and actin. Data are representative of three independent experiments with similar results.

FIGURE S3. Nod2-interacting proteins. HEK293T cells were transiently transfected with empty vector or Flag-Nod2 expression vector. Nod2 was immunoprecipitated with anti-Flag antibody. Nod2-interacting proteins were analyzed by SDS-PAGE and subsequent silver staining.

FIGURE S4. The effect of Hsp90 inhibitors on the expression of intracellular signaling molecules. RAW264.7 cells were pretreated with 17AAG (2 μM) or radicicol (1 μM) for 8 h and then stimulated with MDP (100 μg/ml) for the indicated times. Cell extracts were subjected to Western blot analysis for p-ERK, ERK, p38, TRAF6, MyD88, caspase-3, and actin. Data are representative of three independent experiments with similar results. n.s., non-specific

FIGURE S5. Hsp90 inhibitors decrease MDP-induced NF-κB activity in human intestinal epithelial cell lines. HCT116, LS174T, or SW480 cells were transiently co-transfected with pBVI-Luc reporter and pRLNull plasmids, and cultured for 24 h. (A) HCT116 cells were treated with MDP (100 μg/ml) in the presence or absence of 17AAG (1 μM) or radicicol (2 μM) for 24 h. (B) LS174T cells were co-treated with MDP and 17AAG (1 or 2 μM) or radicicol (2 or 4 μM) for 24 h. (C) SW480 cells were stimulated with MDP in the presence or absence of 17AAG (0.5 or 1 μM) or radicicol (1 or 2 μM) for 24 h. NF-κB activity was measured by dual luciferase assay. Data are representative of three independent experiments with similar results.

FIGURE S6. MDP pretreatment for 16 h does not alter responses to LPS *in vivo*. Wild-type or *Nod2*-deficient mice were injected with 2 ml of autoclaved 4% thioglycollate intraperitoneally. Five days after thioglycollate treatment, MDP (35 mg/kg) was injected intraperitoneally and 16 h later the mice were

challenged with LPS (10mg/kg). Three hours after LPS injection, peritoneal fluid was collected (n=3 per group), and the concentration of IL-6 and TNF-α was measured by ELISA.

FIGURE S7. Rip2 does not phosphorylate Nod2. HEK293T cells were transiently transfected with the indicated HA-tagged expression vectors. Total cell lysates were analyzed by SDS-PAGE in the presence or absence of Phos-tag with Zn2+ and detected by western blotting with anti-HA antibody. Rip2KD; Rip2 kinase dead mutant (Rip2 K38M)

Figure S1

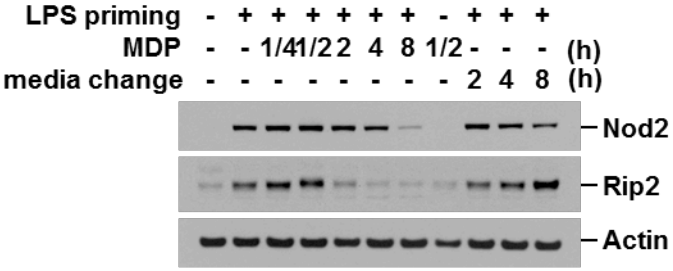

LPS priming - + + + + + + - + + +
MDP - - 1/4 1 1/2 2 4 8 1/2 - - - (h)
media change - - - - - - - - 2 4 8 (h)
Nod2
Rip2
Actin

Figure S2

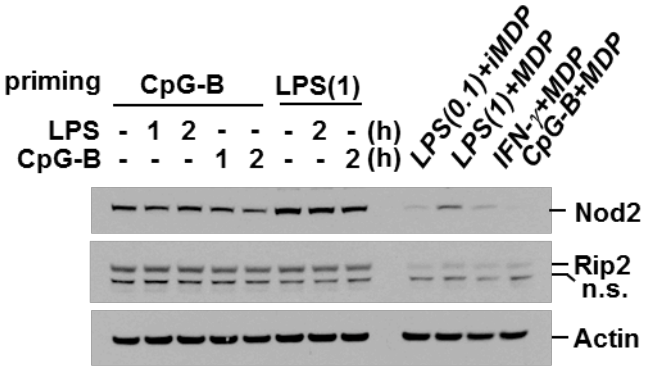

priming
CpG-B
LPS(1)
LPS - 1 2 - - - 2 - (h)
CpG-B - - - 1 2 - - 2 (h)
LPS(0.1)+iMDP
LPS(1)+MDP
IFN-γ+MDP
CpG-B+MDP
Nod2
Rip2
n.s.
Actin

Figure S3

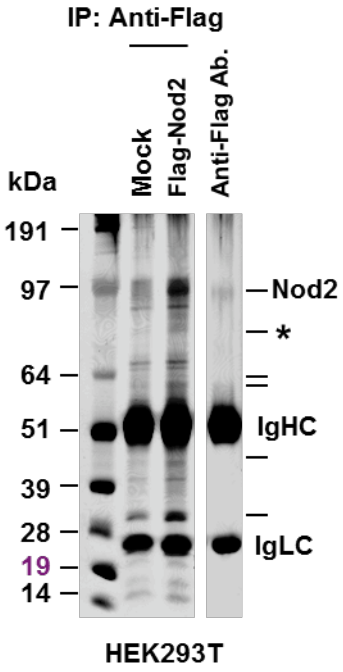

IP: Anti-Flag
Mock
Flag-Nod2
Anti-Flag Ab.
kDa
191
97
64
51
39
28
19
14
Nod2
*
IgHC
IgLC
HEK293T

Figure S4

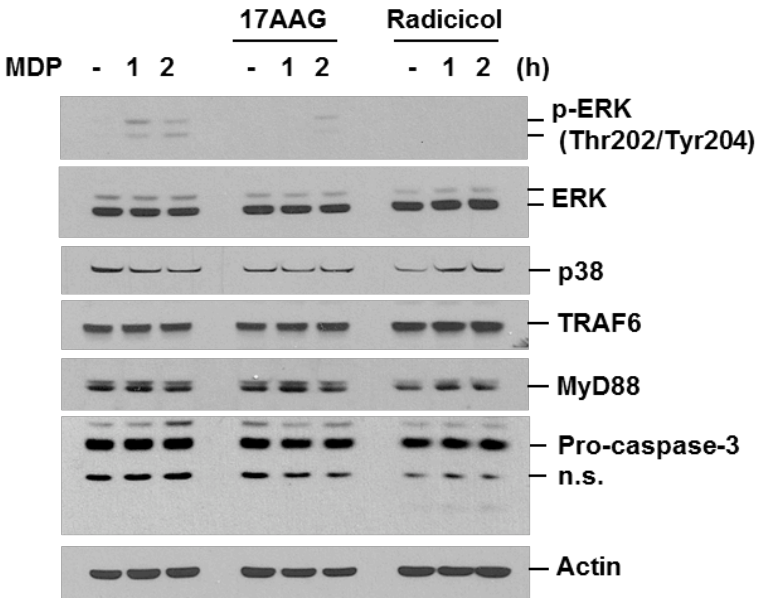

17AAG
Radicicol
MDP - 1 2 - 1 2 - 1 2 (h)
p-ERK
(Thr202/Tyr204)
ERK
p38
TRAF6
MyD88
Pro-caspase-3
n.s.
Actin

Figure S5

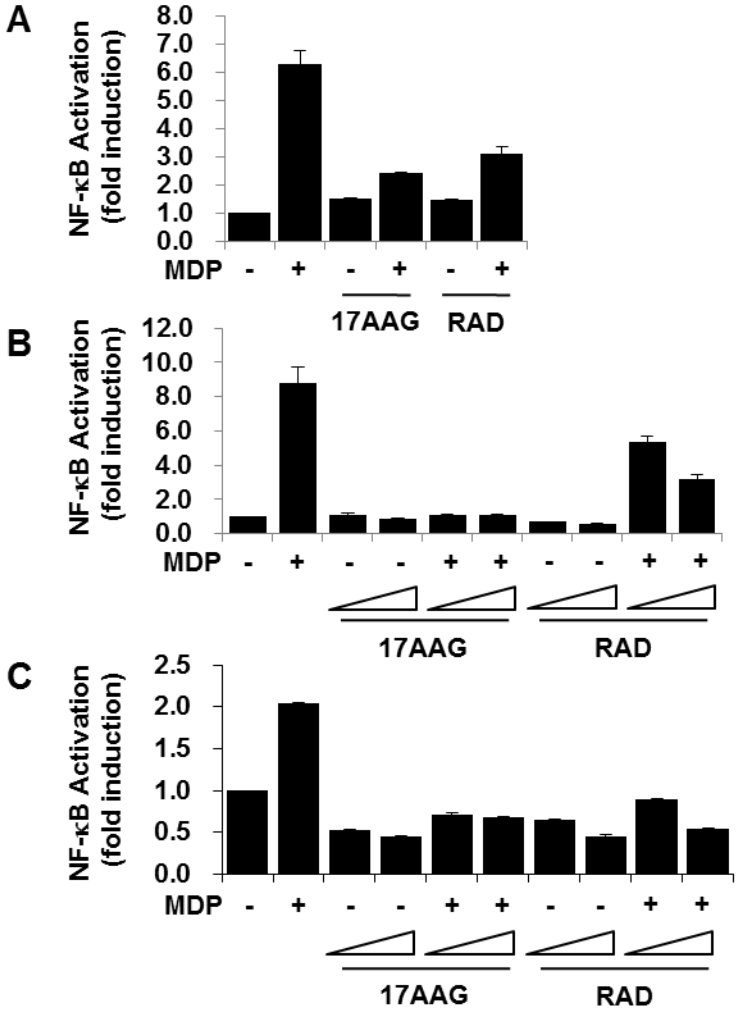

A
NF-κB Activation (fold induction)
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
MDP - + - + - +
17AAG
RAD
B
NF-κB Activation (fold induction)
12.0
10.0
8.0
6.0
4.0
2.0
0.0
MDP - + - - + + - - + +
17AAG
RAD
C
NF-κB Activation (fold induction)
2.5
2.0
1.5
1.0
0.5
0.0
MDP - + - - + + - - + +
17AAG
RAD

Figure S6

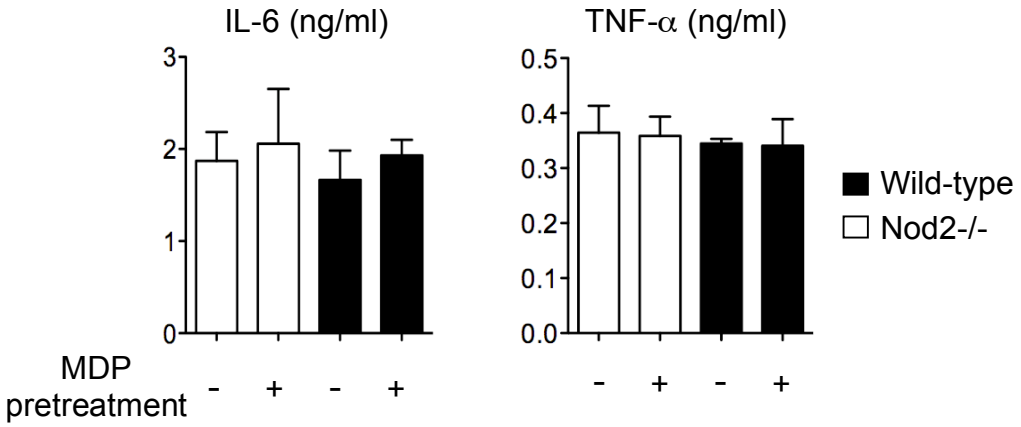
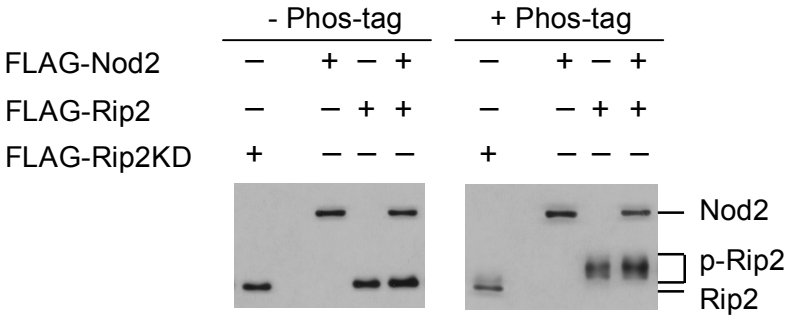

IL-6 (ng/ml)
TNF-α (ng/ml)
3
2
1
0
0.5
0.4
0.3
0.2
0.1
0.0
MDP
pretreatment
- + - +
- + - +
Wild-type
Nod2-/-

Figure S7


- Phos-tag
+ Phos-tag
FLAG-Nod2
FLAG-Rip2
FLAG-Rip2KD
Nod2
p-Rip2
Rip2