SUPPLEMENTARY MATERIAL

Supplement to Materials and Methods.

Plasmid construction

The pJG4-5-GRIP1 NID-RD bait and pGex4T-1-GRIP1 NID-RD were generated by excising the NID-RD (aa 631-1007) fragment from pSG424-GRIP1 NID-RD (Rogatsky et al, 2002) with EcoRI-XbaI/blunt and subcloning it into the EcoRI-XhoI/blunt sites of pJG4-5 and pGex4T-1 (Amersham-Pharmacia), respectively. pcDNA3-GRIP1, pcDNA6(B)-GRIP1 NID (aa 563-765), 2-RD (aa 648-1007) and 3-RD (aa 715-1007) were described previously (Rogatsky et al, 2002; Rogatsky et al, 2001). GRIP1 RD (aa 765-1007) was excised from the pcDNA3-GRIP1 N1007 (Rogatsky et al, 2002) with EcoRI-BspHI/blunt and subcloned into the XhoI-XbaI/blunt sites of modified pcDNA6(A)His. pcDNA6(B)-GRIP1 3-RDmt was generated by replacing the NR box-3 LxxLL motif in GRIP1 3-RD with LxxAA by site-directed mutagenesis (Quickchange, Stratagene) using mutagenic primers F: 5'-GCACTACTGCGCTAT**GC**G**GC**CGACAAAGATGATAC and R: 5'-GTATCATCTTTGTCG**GC**C**GC**ATAGCGCAGTATGC (mutant bases underlined). pET30a-GRIP1 3-RD was generated by subcloning the BamHI-XbaI/blunt 3-RD fragment into the BamHI-SalI/blunt sites of pET30A (Novagen). To generate -533-Luc.mt and -237-Luc.mt, the ISRE was disrupted by site-directed mutagenesis with the primers F: 5'-CTCACGCTTTGGA**C**AGTGA**C**ACCTACCTCACTCG and R: 5'-CGAGTGAGGTAGGT**G**TCACT**G**TCCAAAGCGTGAG (mutant bases underlined). pGex4T1-IRF3 131C was generated by subcloning the IRF3 ScaI-SalI fragment into the SmaI-XhoI sites of pGex4T1 in-frame with GST. pGex4T1-IRF3 N131 was generated by subcloning the IRF3 EcoRI-ScaI fragment into the EcoRI-SmaI sites of pGex4T-1. pGex4T1-IRF3 56-369 was generated by subcloning the EcoRI-XhoI IRF3 library clone from pEG202 into the same sites of pGex4T1. IRF3 188C was PCR-amplified with 2 primers incorporating 5'-BamHI (TGAGGATCCATGTCTGAGAACCCACTGAAGCGGCTG) and 3'-SalI (TAGGTCGACGCTCTCCCCAGGGCCCTGGAAATC) linkers. Following BamHI-SalI digestion, PCR product was ligated into the same sites pGex4T1 generating pGex4T1-IRF3 188C.

***In vitro* binding and competition assays**

For GST pull-down assays, binding reactions containing 20 μl of 50% slurry of the GST-fusion protein on agarose beads and 2 μl of ^{35}S-labeled GRIP1 derivative were incubated in binding buffer (20 mM Tris-HCl, pH 8.0, 0.1 M NaCl, 10% glycerol) supplemented with protease inhibitors (1 mM PMSF and 1 mg/ml each of aprotinin, leupeptin and pepstatin A) and 0.01-0.5% NP-40 for 2 h at 4°C, washed 4 times with binding buffer, boiled in 2xSDS sample buffer and fractionated by SDS-PAGE. The gels were stained with Coomassie blue and autoradiographed.

For competition assays, HIS-tagged GRIP1 3-RD was expressed and purified on cobalt-coated beads (TALON metal affinity resin, Clontech – BD Biosciences) exactly as described for GST-fusion proteins (Krstic *et al*, 1997), rinsed with binding buffer (above) containing 5 mM imidazole and used for binding ^{35}S-labeled GR produced *in vitro* in the presence of 1 μM Dex. Binding reactions were performed as described above in the presence of 0.1% NP-40 and 1 μM Dex. The competitors (GST or GST-IRF3 131C) were eluted of the GST beads with 5 mM reduced glutathione (SIGMA) in 50 mM Tris-HCl pH 8.0, dialyzed in 10 mM Tris-HCl, pH 7.8 – 150 mM NaCl – 10% glycerol overnight at 4°C, and added (2.5-10 μg) to the binding reaction. The gels were stained with Coomassie blue and autoradiographed. In addition, samples were subjected to immunoblotting with IRF3 rabbit polyclonal antibody (Santa Cruz Biotech).

Immunoprecipitation and immunoblotting

Cells were cultured in 6-well plates, collected in PBS, and spun down at 100xg for 5 min. Cell pellets were lysed in RIPA buffer (Rogatsky et al, 2001), supplemented with protease (above) and phosphatase (1 mM NaF, 10 mM β-glycerophosphate, 1 mM Na orthovanadate) inhibitors, and clarified by centrifugation (12,000xg, 15 min, 4°C). One fifth of the lysate was boiled in 2xSDS sample buffer to generate whole cell extracts (WCE), and the rest was incubated with 1.5 μg of rabbit polyclonal antibody to IRF3 (Santa Cruz Biotechnology) for 1 h at 4°C. Immune complexes were collected on protein A/G-PLUS agarose beads (Santa Cruz) for 2 h at 4°C, washed 4 times with ice-cold lysis buffer and boiled in 2xSDS sample buffer. Immunoblotting was performed using standard protocols (Rogatsky et al, 2001) and antibodies to GRIP1 (mouse monoclonal, BD Transduction Laboratories) or IRF3 (goat polyclonal, Santa Cruz). Blots were developed using horseradish peroxidase-conjugated anti-mouse (Promega) and anti-goat (Santa Cruz) secondary antibodies and the enhanced chemiluminescence substrate (Amersham Pharmacia).

siRNA for GRIP1

RAW264.7 cells were scraped off dishes and washed with fresh DMEM. 4 million cells and 4 μg of siGRIP1-1 (F: (CGACAAAGAUGAUACUAAA)dTdT and R: r(UUUAGUAUCAUCUUUGUCG)dAdG), siGRIP1-2 (F: r(GCCAGUAACACAAAGUUAA)dTdT and R: r(UUAACUUUGUGUUACUGGC)dAdG) or scrambled control siRNA were mixed in 100 μl of AMAXA nucleofector solution V and nucleofected as per the manufacturer's instructions. Transfected cells were split into 6-well plates, grown in DMEM-10% FBS for 20 h and treated with pIC as described in Figure Legends. Total protein and RNA were isolated from duplicate wells and analyzed by immunoblotting and qPCR as described in Legend to Figure 3D.

Mouse primary macrophage isolation and culture

Tibia and femurs were isolated from 10-wk old C57BL/6 (wt or MyD88 KO) or SV129 x C57BL/6 (wt or IFNAR KO) mice, and the bone marrow was flushed out with DMEM. Cells were seeded into Petri dishes in RPMI-20% FBS supplemented with 10 ng/ml M-CSF (Peprotech). After 7 days in culture, bone marrow-derived macrophages were scraped off, split into 6-well plates and treated the next day as described in Figure Legends.

To obtain peritoneal macrophages, 10-wk old C57BL/6 or Balb/c mice were injected intraperitoneally with 1 ml/animal of 4% sterile thioglycollate and sacrificed 72 h later. Cells were isolated from peritoneum by lavage using 20 ml RPMI/animal and seeded into 6-well plates using 10^6 cells/well in RPMI-10% FBS. Non-adherent cells were removed 6 h later, whereas adherent cells were refed with RPMI-10% FBS and allowed to recover overnight. The next day, cells were treated as described in Figure Legends for RNA isolation.