Supplemental Data

MEKK4 Sequesters RIP2 to Dictate NOD2 Signal Specificity

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Supplemental Material and Methods

Cell Culture, Transfections, Immunoprecipitations, and Western Blotting

HEK-293 cells were grown in DMEM containing 5% FBS. RAW 264.7 cells were grown in DMEM containing 7% FBS. Transfections were performed by calcium phosphate precipitation as previously described [8, 13]. Immunoprecipitations to assay for protein:protein interactions were performed in modified RIPA buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 1% NP-40, 0.25% DOC, 1 mM EDTA, 1 mM EGTA, 1 mM β-glycerophosphate, 1 mM PMSF, 1 mM $NaVO_4$, 10 nM Calyculin A in the presence of protease inhibitor cocktail (Sigma)). After adding Protein G sepharose beads, IPs were washed at least 5 times before Western blotting. For IP-ubiquitination assays, cells were lysed in high-salt modified RIPA buffer containing 1 M NaCl and 0.25% SDS. The last wash of the IPs was performed in 100 mM NaCl. Western blotting was performed on Nitrocellulose membranes (Bio-Rad) as previously described [13].

Antibodies, Plasmids, and Reagents

Myc (9E10), RIP2, and OMNI antibodies were obtained from Santa Cruz Technology. Myc (rabbit), GST (mouse), phospho IKKα/β, phospho-IκBα, IKKβ, IκBα, NEMO, phospho JNK, JNK, phospho p38 and total p38 antibodies were obtained from Cell Signaling Technology. Anti-Flag (M2) and anti-HA (HA-11) were obtained from Covance. FLAG beads (M2) and anti-MEKK4 were obtained from Sigma. OMNI-RIP2, HA-ubiquitin, Myc-NEMO K399R, HA-RIP2, Omni-NOD2, Omni-L1007insC NOD2, Omni-D291N NOD2, FLAG-TAK1 and GST-IKKβ were

used as previously described [8, 13]. The Omni-tagged NOD2 construct was originally subcloned from the mouse EST as described previously [13]. Unlike the human, the insertion of cytosine at L1007 in the mouse allele causes a frameshift leading to a nonsense transcript encoding 41 additional base pairs. MEKK1, 2, 3 and 4 plasmids were provided by Gary Johnson (UNC) (Abell and Johnson, JBC, 280:35793-6 and references therein). Kinase-dead MEKKs 1, 2, and 4 were obtained from Addgene (Cambridge, MA). FLAG-JNK or FLAG-p38 were obtained from the Harvard Institute of Proteomics and were subcloned into FLAG expression vectors. MDP was obtained from Bachem.

ELISAs

An ELISA array was purchased from SuperArray. IL-6 and TNFα ELISAs were purchased from eBioscience. 1 x 10^6 cells were cultured for 24 hours in 0.5% serum. Supernatants were centrifuged to remove cellular debris, and ELISAs were performed according to the manufacturer's protocol. Standard assays were performed using the standards sent with the ELISA array. All experiments were performed in triplicate.

Lentiviral shRNA

Five lentiviral vectors targeting MEKK4 and an empty lentiviral vector were obtained from the Broad Institute (Cambridge, MA). Sequences used for successful mouse MEKK4 knockdown are: CCGGCCTTACGTCATCTGGACTAATCTCGAGATTAGTCC AGATGACGTAA (#3) and CCGGGCTCCTGATGAAGCAGTATTACTCGAGTAATACTGC
TTCATCAGGA (#4). The lentiviral packaging system psPAX2 and pMD2.G was obtained from Addgene, and was used to produce viral particles targeting MEKK4. After transduction of RAW 264.7 cells, the cells were selected in 2 μg/mL Puromycin and clones (>1000) were pooled.

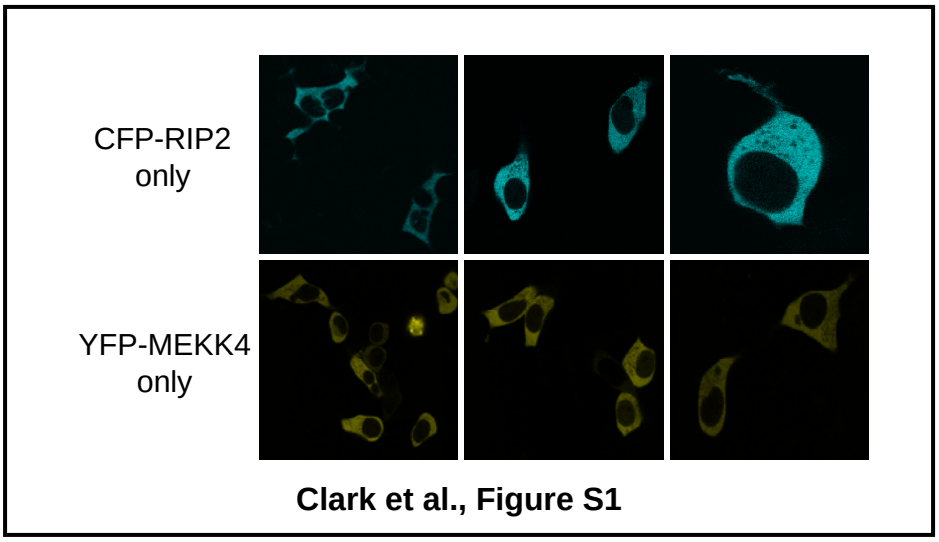


Figure S1. Subcellular Localization of RIP2 and MEKK4

CFP-RIP2 and YFP-MEKK4 were transfected into HEK293 cells. 2 days after transfection, the indicated proteins were visualized using confocal microscopy. Both MEKK4 and RIP2 predominantly showed diffuse cytoplasmic staining with nuclear exclusion.

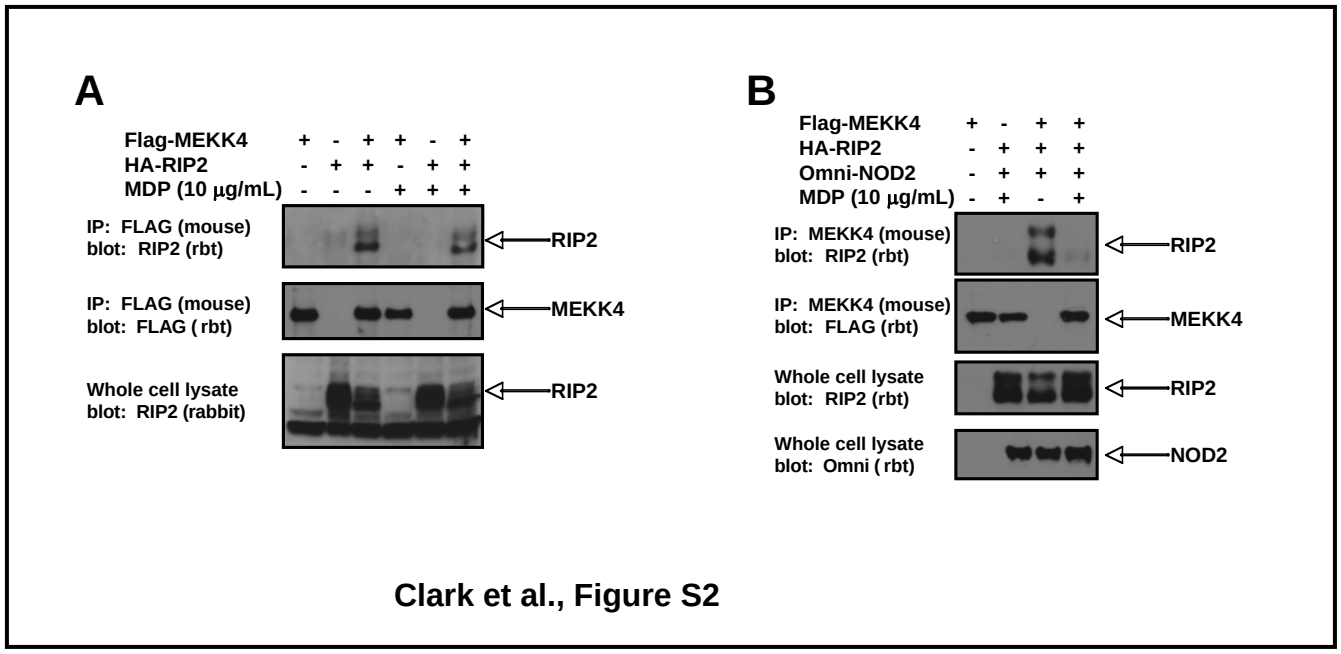
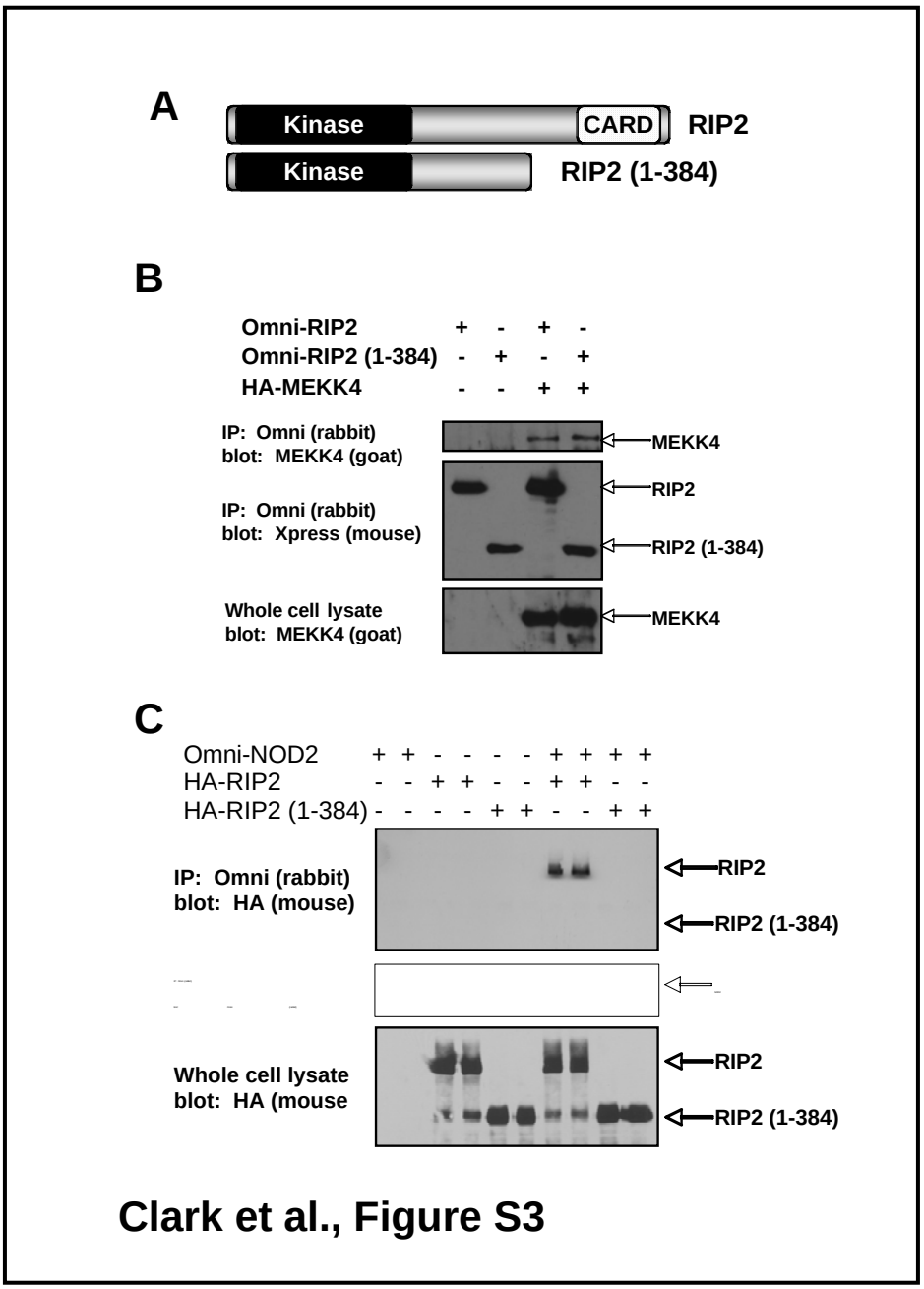


Figure S2. MDP's Effect on RIP2:MEKK4 Binding in the Absence of NOD2

A. Flag-MEKK4 and HA-RIP2 were transfected into HEK293 cells with or without MDP (10 μg/mL) in the absence of NOD2. 24 hours after transfection, cells were lysed and immunoprecipitations were performed. Western blotting showed similar binding of MEKK4 to RIP2 when MDP was either absent or present in the absence of co-transfected NOD2.

B. To more directly show the effect of MDP treatment (with NOD2 expression) on the MEKK4:RIP2 complex, cells were transfected with MEKK4, RIP2 and NOD2 in either the presence or absence of MDP (10 μg/mL). Immunopreciptations were performed on the lysates. Western blotting showed that the presence of MDP (with NOD2 expression) significantly decreases the binding of MEKK4 to RIP2.



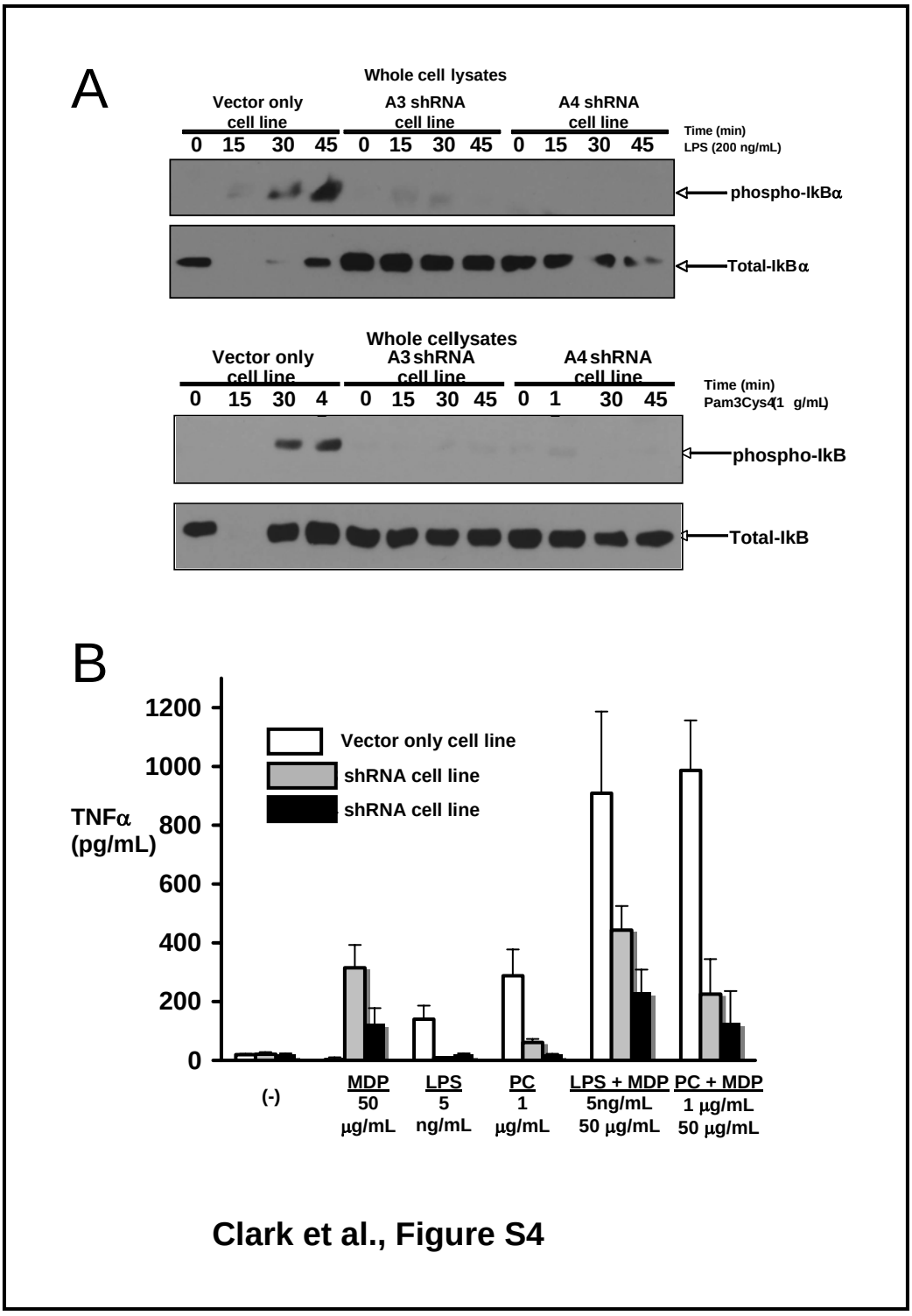
Clark et al., Figure S3

Figure S3. NOD2 and MEKK4 Bind to Distinct Regions of RIP2

A. A deletion mutant of RIP2 that lacks the CARD domain (RIP2 1-384) was generated with both an Omni-tag and a HA-tag. A schematic of both wt RIP2 and this mutant RIP2 is shown.

B. HA-tagged MEKK4 was transfected into 293 cells with either full-length, Omni-tagged RIP2 or with a Omni-tagged RIP2 mutant lacking the RIP2 CARD domain (RIP2 1-384). RIP2 or RIP2 (1-384) was immunoprecipitated via the Omni tag, and Western blots were performed. Antibodies recognizing both full length RIP2 and RIP2 1-384 could immunoprecipitate MEKK4, indicating that RIP2's CARD domain is not essential for the MEKK4 interaction.

C. Omni-tagged NOD2 was transfected into 293 cells with either HA-tagged full length RIP2 or HA-tagged RIP2 1-384 (in duplicate). NOD2 was immunoprecipitated by virtue of its Omni tag and Western blotting was performed. The Xpress antibody recognizes a 9 amino acid epitope within the 22 amino acid Omni tag, and thus recognizes Omni-tagged proteins. Unlike MEKK4, RIP2's CARD domain was required for the NOD2 interaction, showing that MEKK4 and NOD2 bind to different regions of RIP2.



Clark et al., Figure S4

Figure S4. Synergy between NOD2 and TLR2/4 Is Decreased in the MEKK4 Knockdown Cells

A. MEKK4 knockdown cells were treated with the indicated agonist (upper blots: Pam3Cys4 – TLR2 agonist; lower blots: limiting dose of Highly purified LPS-TLR4 agonist) for the indicated times. Lysates were generated, and protein concentration was standardized via the Bio-Rad Protein Assay. Western blots were performed using the indicated antibodies. In both TLR2 and TLR4 stimulation, the activation of the NFκB pathway was blunted relative to the vector-only control cell line.

B. Cells were treated with the indicated agent for 16 hours. Media was collected, and a TNF ELISA was performed. MDP stimulated TNF production in both the MEKK4 knockdown cell lines while the production was decreased upon treatment with LPS and Pam_3Cys_4. The synergy between MDP and these agents was also decreased in the MEKK4 knockdown cell lines.

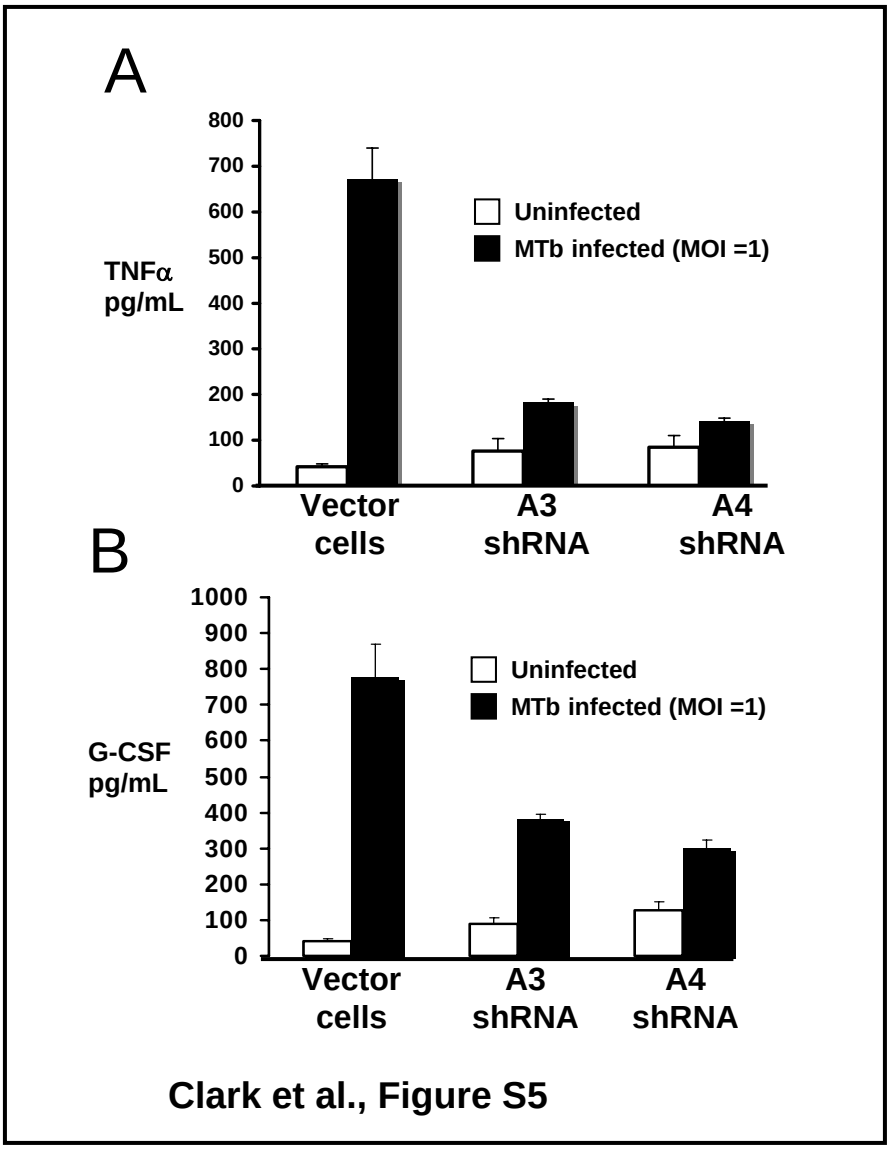


Figure S5. TNF and G-CSF Production Are Decreased in MEKK4 Knockdown Cells Infected with M. Tuberculosis

A, B. The indicated cell lines were infected at an MOI of 1:1 with M. Tuberculosis. 16 hours post-infection, media was collected and assayed for either TNF or G-CSF. In both the MEKK4 knockdown cell lines, the M.Tb-induced cytokine release was decreased.