Supplemental Material for Methods.

Generation and expression of DNA constructs in cell lines and primary macrophages.

Myc-tagged mouse wild-type and domain deletion mutants of CAPRI in pcDNA3.1/myc vector (Invitrogen) or pMI retrovirus vector[1] were generated by PCR and then cloning using wild-type CAPRI plasmid as template. These constructs were confirmed by sequencing. pMI retrovirus vectors expressing EGFP-tagged constitutively active Cdc42 and Rac1 were generated by inserting active mutant Cdc42EGFP or Rac1EGFP from pEGFP-C3 vector[2] (kindly provided by S. Shaw at NIH) into the pMI vector.

HEK-293 cells were transiently transfected with pcDNA3.1CAPRI myc-tagged constructs by Ca^{2+} precipitation. 48 hrs later, total cell lysates were generated for protein blotting and immunoprecipitation assays. Retroviruses were generated as described previously[1]. BM-derived macrophages were infected with retroviruses by incubating with virus supernatants at 32^{o}C for 8-10 h at day 2 and day 4 of culture. The infection efficiency was ~10% as examined by FACS analysis of $EGFP^{+}$ or $hCD2^{+}$ cells at day 6 of culture.

Microscopy image study.

For visualization of actin polymerization and CAPRI localization, macrophages were incubated with IgG opsonized SRBCs for 30 min at 4 ^{o}C and fixed at RT for 10 min in 3.7% paraformaldehyde. Cells were then permeabilized with 0.2% Triton X-100,

blocked with 0.2% gelatin, stained with 1:50 diluated phallacidin (Molecular Probes) and followed by biotin-anti-CAPRI and Texas-red-avidin. The cells were visualized under a fluorescent microscopy. For observing co-localization of CAPRI and Rac1, wild-type macrophages infected with retroviruses expressing myc-tagged CAPRI were induced to undergo FcγR-mediated phagocytosis as stated above, fixed, permeabilized, stained with TRITC-anti-Rac1 (BD Biosciences), Alexa Fluor 488-anti-myc (clone 9E10) and DAPI. Cells were visualized under a confocal microscopy. Alexa Fluro 488-anti-myc antibody was labeled with a kit from Molecular Probes.

Macrophage activation by FcγR crosslinking and PAMPs.

Elicited macrophages were incubated with an excess amount of anti-FcγRII/III mAb 2.4G2 (eBioscience, San Diego, CA) on ice for 30 min, washed 3 times with cold PBS and crosslinked with anti-rat IgG (Jackson ImmunoResearch Laboratories) at 37 ^{o}C for the indicated time. Cells were lysed for immunoblot or immunoprecipitation. For PAMPs stimulation, macrophages were cultured (1 x 10^{6}/ml, 0.5 ml/well) in 48-well plates for 16-24 hr with zymosan, PGN, LTA and LPS (all from Sigma, St. Louis, MI) and supernatants were collected for the measurement of TNF and IL-6 production using ELISA kits from eBioscience (San Diego, CA). For analysis of cell signaling, whole cell lysates from elicited peritoneal macrophages stimulated with heat-killed *S. pneumoniae* and *S. aureus* (macrophages:bacteria at 1:25) at the indicated time points were blotted with antibodies against phosphorylated ERK1/2, JNK and total ERK2 (Cell Signaling, Beverly, MA).

Macrophage oxidative burst

FcγR-crosslinking induced oxidative burst in macrophages was examined using the Fc OxyBURST reagent (Molecular Probes) according to the manufacturers' instructions. Briefly, elicited macrophages were suspended at $1x10^6$/ml in Krebs' Ringer's PBS buffer (1.0 mM Ca^{2+}, 1.5 mM Mg^{2+} and 5.5 mM glucose, pH7.4) and pre-warmed at 37°C for 15-20 min. BSA-anti-BSA immune complexes labeled with H_2DCF were added to the pre-warmed macrophages at a final concentration of 120 μg/ml and the fluorescence was monitored by FACS immediately for up to 10 min. Plotted are mean fluorescence intensity. Superoxide anion production was measured using a Chemiluminescent Detection Kit (Calbiochem, San Diego, CA). Briefly, macrophages (2 x 10^6/ml) suspended in superoxide anion assay medium were stimulated with PMA (100 ng/ml) for 30 min and assayed for O_2^- activity in a luminometer according to the manufactures' instructions.

Immunoblot and immunoprecipitation

Anti-mouse CAPRI polyclonal antibody was generated by immunizing rabbits with a CAPRI peptide (aa 789-802) conjugated to keyhole limpet hemocyanin and followed by affinity purification on a peptide column. The anti-mouse CAPRI antibody works in immunoblot but not immunoprecipitation assay. For immunoblot, whole cell lysates (10 μg protein/lane) were separated on 10% polyacrylamide gels and transferred to nitrocellulose. The membranes were then probed with the following antibodies: anti-Syk , anti-phospho-Syk, anti-ERK2, anti-phospho-ERK, anti-Cbl, anti-phospho-Cbl, anti-phospho-JNK, anti-phospho-tyrosine (4G10) (all from Cell Signaling) and rabbit anti-

CAPRI polyclonal antibody, followed with HRP-conjugated secondary Abs and detected with enhanced chemiluminescence according to the manufacturer's instructions (Amersham, Arlington Heights, IL). For immunoprecipitation, anti-Rac1, anti-Cdc42 (Upstate, Lake placid, NY), or anti-phospho-Syk bound to protein G beads were incubated with macrophage lysates overnight and blotted for detection. CHO cells expressing wild-type and GRD domain mutated CAPRI and anti-hCAPRI antibody were provided by P. Lockyer (The Babrahm Institute, UK).

Assay of activated Rac1 and Cdc42

Rac1-GTP and Cdc42-GTP were affinity precipitated with GST fusion-protein corresponding to the p21-binding domain of human PAK-1 (Upstate). Fresh macrophage lysates (10-20 x 10^6 cells in 1ml) prepared from elicited peritoneal macrophages with or without FcγR crosslinking were mixed with 5-10 μg of PAK-1 agarose and rotated at 4^oC for 60 min. Precipitates were blotted and detected with anti-Rac1 and Cdc42. To examine CAPRI activity in RAW264.7 macrophage cell line (American Type Culture Collection), a full length cDNA of the *Rasa4* gene was inserted into the *NotI*/*SalI* sites of the retrovirus vector PMI and transduced into RAW264.7 cells by retroviruses as described[1]. Human CD2 positive cells expressing CAPRI or vector only were sorted by FACS and used in Rac1-GTP and Cdc42-GTP assays. CHO cells with or without expression of hCAPRI mutants were directly lysed and subjected to GTP assay as described above.

Reference

1. He, Y.W., Deftos, M.L., Ojala, E.W. & Bevan, M.J. RORgamma t, a novel isoform of an orphan receptor, negatively regulates Fas ligand expression and IL-2 production in T cells. *Immunity* **9**, 797-806 (1998).

2. Salazar-Fontana, L.I., Barr, V., Samelson, L.E. & Bierer, B.E. CD28 engagement promotes actin polymerization through the activation of the small Rho GTPase Cdc42 in human T cells. *J. Immunol*. **171**, 2225-2232 (2003).