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SUPPLEMENTAL MATERIALS AND METHODS

Western blots. Cells were suspended in lysis buffer (1% Triton X-100, 50 mM Hepes, 150 mM NaCl, 5 mM NaF, 1 mM sodium orthovanadate, 1 mM phenylmethanesulphonylfluoride, 1 mM EDTA, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 2 nM okadaic acid) and incubated for 15–30 min on ice. After centrifugation at 15,000 *g* for 15 min at 4°C, supernatants were assayed for total protein (DC protein assay; Bio-Rad Laboratories), and equivalent amounts of protein for each sample were analyzed by SDS-PAGE. For Western blots, we used using specific mAbs and ECL (GE Healthcare). PKB activity was measured using anti–phospho-PKB (Ser473), and p44/42 MAPK activation was measured using anti–phospho-p44/42 MAPK (Thr202/Tyr204; both were obtained from Cell Signaling Technology). As a loading control, we used antibodies to PKB (Millipore) and p42/44 MAPK. We analyzed the profile of Tyr-phosphorylated proteins using anti-PTyr antibody (4G10; Millipore). To measure p110α, p110β, and p110δ levels, we used anti-p110α (a gift from A. Klippel, Merck, Boston, MA), anti-p110β (SC-602), and anti-p110δ (SC-7176; both from Santa Cruz Biotechnology, Inc.).

Immunoprecipitation assays. Total cell lysate was immunoprecipitated with 3 μl anti-p110γ (SC-7177), 5 μl anti-Gq11 (SC-392), or 3 μl anti-Lck (SC-13; all from Santa Cruz Biotechnology, Inc.), or 3 μl anti-Zap70 (Z24820; Transduction Laboratories). In Western blots, we detected p110γ levels using anti-p110γ H1 mAb (1:100; a gift from R. Wetzker, Friedrich-Schiller University, Jena, Germany) and ZAP70 with the mAb described.

Rac activation assay. Small G protein Rac1 activity was assayed with GST protein fused to the Rac1 interactive binding (CRIB) domain from PAK. Cells were stimulated with anti-CD3 or anti-CD3 plus anti-CD28 at 37°C for various time periods, washed rapidly with ice-cold PBS, and lysed in GST-FISH buffer (10% glycerol, 50 mM Tris-HCl [pH 7.4], 100 mM NaCl, 1% NP-40, 2 mM $MgCl_2$, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 0.3 μg/ml benzamide, 1 μg/ml pepstatin A, 1 μg/ml antipain, and 1 mM phenylmethanesulphonylfluoride). The lysate was cleared by centrifugation, and 100 μg of supernatants were incubated for 16 h at 4°C with glutathione-sepharose beads preloaded with a bacterially produced GST-PAK fusion protein. Beads were washed three times in GST-FISH buffer and analyzed by 12% reducing SDS-PAGE, followed by Western blots using anti-Rac1 antibody (BD Biosciences).

FACS-based conjugate formation assay. APCs and T cells were stained with fluorescent cell linker compound PKH26-GL (red) and PKH67-GL (green) kits (Sigma-Aldrich), respectively. For PKH26/PKH67 staining, 5×10^6 cells were washed with serum-free RPMI 1640, resuspended in diluent C at 10^7 cells per milliliter, mixed with an equal volume of 10 μM PKH26/PKH67, and incubated for 5 min at room temperature. The reaction was terminated using 2 vol of FBS, and cells were diluted into 2 vol of medium, washed three times, and rested for 1 h at 37°C.

For conjugation, 10^5 T cells and 2×10^5 APCs were mixed in a final volume of 200 μl in 5-ml round-bottom tubes (model no. 2052; Falcon), centrifuged at 25 *g* (300–350 rpm) for 3 min at 4°C, and incubated in a 37°C water bath for various periods. Cells were resuspended by vortexing for 5–10 s and were fixed by adding 1 ml of ice-cold 0.5% paraformaldehyde in Dulbecco's PBS. The relative proportion of red, green, and red/green events was determined by two-color flow cytometric analysis on a flow cytometer (EPICS XL-MCL; Beckman Coulter). The percentage of conjugation was calculated as the number of two-color events divided by total T cell events (conjugated plus unconjugated).

Actin polymerization assay. Purified T cells or Jurkat T cells were activated with anti-CD3 or anti-CD3 plus anti-CD28, followed by cross-linking with secondary antibody. Cells were washed with ice-cold PBS and fixed with 4% paraformaldehyde, permeabilized, and stained with 1 μg/ml FITC-phalloidin before flow cytometry analysis.

Stimulation with antibody-coated beads and immunofluorescence. Antibodies were adsorbed to microspheres by mixing 0.5 μg of antibody in PBS with 0.5×10^6 microspheres (final volume of 1 ml) and incubating for 1.5 h at room temperature with continuous mixing; 1.5 ml of 1% BSA was added, and mixing continued for 30 min. Microspheres were washed three times and resuspended in PBS. For stimulation, 10^6 Jurkat cells or transfectants were mixed with antibody-coated beads at a 2:1 cell/bead ratio and plated on chambered slides. A time series of images was captured by confocal microscopy.