Online Supplemental Material for

Cho et al. http://www.jem.org/cgi/doi/10.1084/jem.20040924

Supplement Materials and Methods

Cell Culture and Antibodies. Jurkat T cells were grown in RPMI medium supplemented with 10% FBS. 293T cells were grown in DME medium supplemented with 10% FBS. Jurkat T cells were electroporated using the Bio-Rad Laboratories electroporator at 250 V and 975 μF in serum-free RPMI medium. $CD2^+$ T cells were extensively purified from PBMCs of healthy volunteers using the CELLection CD2 kit (Dynal Biotech). Flow cytometry showed that >95% of the purified cell population was $CD2^+$ lymphocytes. Anti-ZAP70, anti-Lck, anti-CD3ζ, anti-phospho CD3ζ, anti-PLCγ1, and anti–LFA-1 antibodies were obtained from Santa Cruz Biotechnology, Inc., and anti-phospho ZAP70 (Y_{319}), anti-phospho PLCγ1 (Y_{783}), and anti-phospho LAT (Y_{191}) antibodies were purchased from Cell Signaling Technology Inc. Anti-phospho tyrosine (4G10) antibody was purchased from Upstate Biotechnology. AU1 tag antibody was obtained from BABCO. For stimulation, anti-CD3 antibody (BD Biosciences) and anti-CD3 pan T dynabeads (Dynal ASA) were used.

Preparation of Lentiviral Vector and Infection. Virus stocks were generated by transfecting 293T cells with pHJEF1α-eGFP, pHJEF1α-Tip, or pHJEF1α-TipmLBD, and pHDM-Hgpm2, pRC/CMV-Rev1b, or pHDM.G by calcium phosphate coprecipitation. Medium was changed 16 h after transfection. Supernatants were collected at 48 and 72 h posttransfection and pooled. Virus-containing supernatants were cleared by low speed centrifugation, filtered through a 0.45-μm filter, and concentrated by ultracentrifugation at 25,000 *g* for 90 min. The viral pellets were resuspended in RPMI medium plus 10% FBS and incubated overnight at 4°C. Viral stocks were stored at −80°C. To achieve efficient transduction, viral stock containing 8 μg/ml of Polybrene was mixed with 10^6 cells in 24-well plates, which were then spun at 1,800 rpm for 2 h at 32°C. After 6 h of culture, this procedure was repeated. At 48 h postinfection, cells were analyzed for GFP fluorescence by flow cytometry or immunofluorescence microscopy after staining with anti-Tip antibody. More than 80% of PBMCs and Jurkat T cells were generally transduced with lentivirus using this method.

Flow Cytometry and ELISA. Cells (10^6) were washed with RPMI medium containing 10% FCS and incubated with phycoerythrin-conjugated anti-CD3 or CD69 (BD Biosciences) for 30 min at 4°C. After washing, each sample was fixed with 4% paraformaldehyde solution, and flow cytometry analysis was performed with a FACScan (Becton Dickinson). To measure the production of IL-2, T cells (5×10^4/well) were stimulated with anti-CD3 antibody-coated beads (2×10^5/well; Dynal ASA) in 96-well plates. Culture supernatants were collected after 24 h and assayed for IL-2 by ELISA using OptEIA human IL-2 kit (BD Biosciences).

Calcium Mobilization Analysis. Cells (2×10^6) were loaded with 1 μM indo-1 in 100 μl of RPMI medium containing 10% FBS at 37°C for 30 min, washed once with the medium, resuspended in 1 ml of cold RPMI media containing 10% FBS, and then put on ice until analysis. Baseline calcium levels were established for 1 min before the addition of the antibody. Cells were stimulated with 2 μg of anti–human CD3 antibody. Data were collected and analyzed on a FACS Vantage (Becton Dickinson).

Immunofluorescence. Cells were fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.2% Triton X-100 for 15 min, blocked with 10% goat serum in PBS for 30 min, and reacted with 1:100–2,000 dilutions of primary antibody in PBS for 30 min at room temperature. After incubation, cells were washed extensively with PBS, incubated with 1:2,000 diluted Alexa 488–, Alexa 568–, or Alexa 647–conjugated anti–rabbit or anti–mouse antibody (Molecular Probes) in PBS for 30 min at room temperature, and washed three times with PBS. Confocal microscopy was performed using a Leica TCS SP laser-scanning microscope (Leica Microsystems) fitted with a 40× Leica objective (PL APO, 1.4NA). Images were collected at 512 × 512 pixel resolution using Leica imaging software. The stained cells were optically sectioned in the z axis, and the images in the different channels (photo multiplier tubes) were collected simultaneously. The step size in the z axis varied from 0.2 to 0.5 μm to obtain 8–16 slices/imaged file. The images were transferred to a Macintosh G4 computer (Apple Computer), and NIH Image v1.61 software was used to render the images.

Immunoprecipitation and Immunoblot. For immunoprecipitation, cells were harvested and resuspended in lysis buffer (150 mM NaCl, 0.5% Nonidet P-40, and 50 mM Hepes buffer, pH 8.0) containing protease and phosphatase inhibitors. Immunoprecipitated proteins from precleared cell lysates were used for immunoblot. For protein immunoblots, polypeptides in cell lysates corresponding to 2×10^6 cells were resolved by SDS-PAGE and transferred to nitrocellulose membrane. Immunoblot detection was performed with a 1:1,000 or 1:3,000 dilution of primary antibody with the enhanced chemiluminescence system (Amersham Biosciences).

J. Exp. Med. © The Rockefeller University Press • 0022-1007
Volume 200 September 6, 2004
http://www.jem.org/cgi/doi/10.1084/jem.20040924