Supplemental Data

Itk Functions to Control Actin Polymerization at the Immune Synapse through Localized Activation of Cdc42 and WASP

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Supplemental Experimental Procedures

Mice

The generation of *Itk−/−, Rlk−/−*, and *Rlk−/−* × *Itk−/−* mice and their backcrossing to the AND TCR Tg background have been described previously [S1, S2]. AND Tg control mice were obtained from Jackson Laboratory, and C3H mice were from Frederick.

Antibodies and Reagents

PV1 anti-CD28 antibody was a gift from Dr. Anne Sperling (University of Chicago). Anti-TCRβ (H57) was from BD-Pharmingen. Rhodamine phalloidin, Alexa 488-phalloidin, and rabbit anti-GFP pAb were purchased from Molecular Probes. Rabbit anti-Arp3 antibody was a gift from Dr. Matt Welch (UC Berkeley, Berkeley, CA; [S3]). Rabbit anti-Vav antibody was purchased from BD Biosciences. Mouse anti-WASP monoclonals were developed by immunizing mice with peptides containing WASP residues 248–310 and 461–485 linked with a GGSGGS linker and were screened for conformation-specific reactivity by ELISA. These antibodies will be described in detail elsewhere (D.W.L., C.M. Labno., D.Y., J.K.B., and M.K.R., unpublished data). The GFP-WASP-GBD reagent has been previously described [S4]. All secondary antibodies were obtained from Jackson Immunoresearch. DASP peptide (residues 86–90; 94–103 of moth cytochrome c) was made by the University of Chicago Peptide Facility.

Cell Culture

The I-E^k-positive B cell line CH12 [S5] was cultured in DMEM (GIBCO-BRL) with 10% FCS (BioWhittaker). Lymph node T cells were expanded on irradiated C3H splenocytes pulsed with 30 μg/ml DASP peptide and grown in DMEM with 10% FCS, 45 U/ml IL-2, and 1 μg/ml anti-CD28 in 10% CO_2 at 37°C. At 7–10 days after activation, live T cell blasts were isolated by passage over a Histopaque (Sigma) gradient and were rested at least 1 hr prior to use.

Analysis of T Cell Conjugates

The FACs-based conjugation assay was done as previously described [S6]. Immunofluorescence microscopy and digital deconvolution were performed as described previously [S4]. For studies of interactions with anti-TCR beads, polystyrene latex microspheres (5.2 μm, Interfacial Dynamics) were coated with 1 μg/ml anti-TCRβ in PBS, washed with 3% BSA/PBS, and resuspended in RPMI/10% FCS at 10^8 beads/ml. Peripheral T cells from spleen were isolated by negative selection on T cell enrichment columns (R&D Systems) and were incubated with an equal number of beads at 4°C for 5 min, then at 37°C for 20 min. The mixture was then fixed with 4% paraformaldehyde and was stained with 10 μg/ml Alexa 594-phalloidin for 40–60 min at room temperature. Cells were washed with 0.5% BSA in PBS, spotted onto slides, and imaged on a Zeiss LSM 510 Confocal microscope. A total of 50–100 bead conjugates per sample were scored for increased F-actin adjacent to the bead.

Flow Cytometry

Murine T cell blasts were prepared as described above for conjugate experiments, and expression levels of the AND Tg TCR were evaluated by flow cytometry after labeling with FITC-anti-Vα11 (Pharmingen). For analysis of F-actin content, splenic T cells were isolated by negative selection, stimulated for 5 min with anti-CD3-biotin (15 μg/ml) plus streptavidin (8 μg/ml), fixed with 4% parformaldehyde, and stained with Alexa 488-phalloidin. Cells were analyzed on a FACsCalibur (Becton Dickinson), and data analyses were performed by using FlowJo (Treestar) or CellQuest (Becton Dickinson) software.

Supplemental References

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