Supporting Information

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Materials and Methods

Generation of Mice Lacking *Cdc42* in T Lymphocytes. Conditional targeted $Cdc42^{flox/flox}$ mice were generated as described previously (37). The flox allele contains loxP sites flanking exon 2 of *Cdc42* alleles. To delete *Cdc42* in the T-cell lineage in vivo, $Cdc42^{flox/flox}$ mice were mated with mice expressing Cre recombinase under the control of Lck proximal promoter (Jackson Laboratory). All mice used were 4–8 wk of age. All animals were housed under specific pathogen-free conditions in the Animal Facility at Cincinnati Children's Hospital Research Foundation.

Endogenous Cdc42 Activity Assay. Purified splenic T cells were left unstimulated or were stimulated with 80 ng/mL IL-7 or 10 μg/mL anti-CD3 plus 2 μg/mL anti-CD28 antibodies. Cell-division cycle 42 (Cdc42) activity (GTP-bound Cdc42) was detected by a previously described pull-down assay using the Cdc42 effector probe GST-p21-activated kinase 1 (PAK1) that contains the Cdc42-interactive domain.

Activation-Induced Cell Death. Splenocytes were cultured for 48 h with 20 U/mL IL-2, 1 μg/mL plate-bound anti-CD3, and 1 μg/mL anti-CD28 antibodies. Live cells were purified by a Ficoll density gradient and restimulated with 10 μg/mL plate-bound anti-CD3 antibody for 24 h. Cells then were stained with anti-CD4 and anti-CD8 antibodies followed by Annexin V staining. The percentage of apoptotic cells was analyzed by flow cytometry.

Adoptive Transfer. Purified splenic T cells (2×10^6) were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes) and injected i.v. into $Rag2^{-/-}$ mice (generous gift of Yi Gu, Experimental Hematology, Cincinnati Children's Hospital Medical Center, Cincinnati, OH). Six days later, splenocytes from these mice were isolated and labeled with anti-CD4 and anti-CD8 antibodies. The number of cell divisions was determined by flow cytometry analysis of the CFSE profile in $CD4^+$ or $CD8^+$ T cells.

Memory Cell Proliferation Assay. $Cdc42^{-/-}$ and WT mice were injected i.p. with 2×10^5 pfu Armstrong-3 strain of lymphocytic choriomeningitis virus (LCMV). The mice were killed 50 d later. Splenocytes were isolated and cultured in vitro with or without 10 μg/mL GP61-80 or 2 μg/mL GP33-41 epitopic peptide for 48 h and then analyzed for cell growth rate by a Non-Radioactive Cell Proliferation Assay kit (Promega).

Immunoblotting. Whole-cell lysates were prepared and separated by 10% SDS-PAGE. Expression or activation (phosphorylation) of MAP kinase-ERK kinase (MEK), c-Raf, ERK, JNK, and Cdc42 was probed using the corresponding antibodies. Antibodies against total or phosphorylated MEK, c-Raf, ERK, and JNK were from Cell Signaling Technology; anti-Cdc42 antibody was from Millipore.

Interaction Between Cdc42 Point Mutants and Cdc42 Effectors. NIH 3T3 cells overexpressing the HA_3-tagged Cdc42 mutants were lysed and subjected to the pull-down assay using Cdc42 effector probes containing the Cdc42-interactive domain: GST-PAK1, GST- Wiskott–Aldrich syndrome protein (GST-WASP), GST-IQ motif containing GTPase activating protein 1/2 (GST-IQGAP1), and GST-partitioning-defective protein 6B (PAR6B). The binding activities of GTP-bound Cdc42 mutants to PAK1, WASP, IQGAP1, and PAR6B were detected by Western blotting with anti-HA antibody (Sigma).

Generation of Bone Marrow Chimeric Mice. Low-density bone marrow cells were isolated from WT and $Cdc42^{-/-}$ mice using Histopaque-1083 (Sigma) and then were transduced with one of a number of vectors: mock; Cdc42WT; Cdc42 mutants Cdc42D38A, Cdc42D63H, or Cdc42I173AL174A; or constitutive active mutant of PAK1 (caPAK1) that coexpresses GFP by retroviral infection in the presence of retronectin (Takara). GFP^+-transduced cells were selected by FACS and transplanted into lethally irradiated BoyJ mice. The chimeric mice were killed 8 wk after transplantation, and thymocytes and splenic $CD45.2^+$ T cells derived from donor bone marrow cells were analyzed.

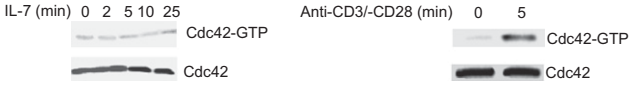

Fig. S1. Cdc42 is not a direct signal transducer of IL-7. Purified splenic T cells from WT mice were stimulated with IL-7 for the indicated time or left unstimulated. As a positive control, splenic T cells from WT mice were stimulated with anti-CD3/-CD28 antibodies for 5 min or left unstimulated. The cells were lysed and subject to GST-PAK1 pull-down assay. The amount of Cdc42-GTP binding to PAK1 was detected by Western blotting of the coprecipitates with anti-Cdc42 antibody. Pooled Cdc42 from total cell lysates also was detected as loading control.

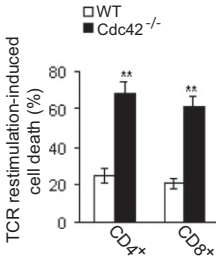

Fig. S2. *Cdc42*-deficient T cells are more susceptible to T-cell receptor (TCR) restimulation-induced apoptosis. Splenocytes were cultured for 48 h with IL-2 and plate-bound anti-CD3 and anti-CD28 antibodies. Live cells were purified by a Ficoll density gradient and restimulated with plate-bound anti-CD3 antibody for 24 h. Cells then were stained with anti-CD4 and anti-CD8 antibodies followed by Annexin V staining. The percentage of apoptotic cells was analyzed by flow cytometry.

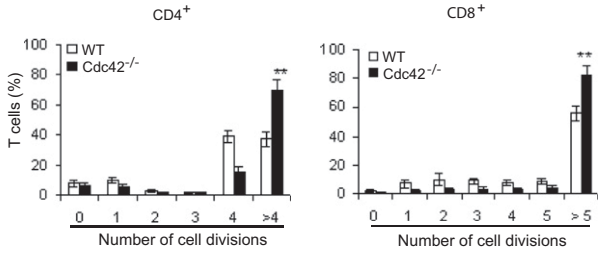

Fig. S3. *Cdc42*-deficient cells undergo homeostatic proliferation. Purified splenic T cells were labeled with CFSE and injected i.v. into recipient *Rag2*$^{-/-}$ mice. After 6 d, the splenocytes from recipient mice were harvested and stained with antibodies against CD4 and CD8. The CFSE profile was analyzed by flow cytometry. Data are represented as means ± SD; $n = 5$. $^{**}P < 0.01$.

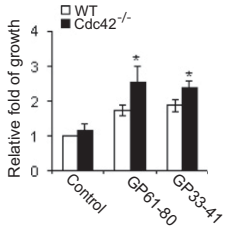

Fig. S4. *Cdc42*-deficient memory T cells are hyperproliferative. *Cdc42*$^{-/-}$ and WT mice were injected i.p. with LCMV. Splenocytes were harvested at day 50 postinjection and cultured with or without (control) epitope GP61-80 or GP33-41 to stimulate LCMV-specific CD4^{+} or CD8^{+} memory T-cell proliferation. Cell grow rate was assessed 48 h later. $n = 6$. $^{*}P < 0.05$.

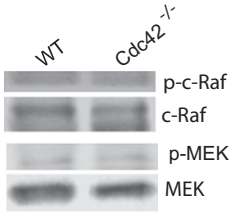

Fig. S5. *Cdc42* deficiency had no effect on c-Raf and MEK activation. Splenic T cells were purified from *Cdc42*$^{-/-}$ and WT mice and immunoblotted with antibodies against phosphorylated (p) or total c-Raf and MEK.

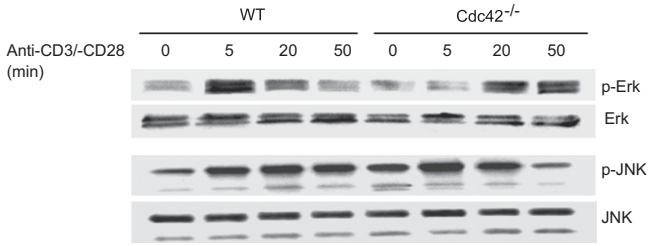

Fig. S6. Effects of *Cdc42* deficiency on TCR-triggered ERK and JNK activation. Splenic T cells were purified from *Cdc42*$^{-/-}$ and WT mice and stimulated with antibodies against CD3 (10 μg/mL) and CD28 (2 μg/mL) for the indicated time periods. Cells then were subjected to Western blotting of phosphorylated (p) or total ERK and JNK.

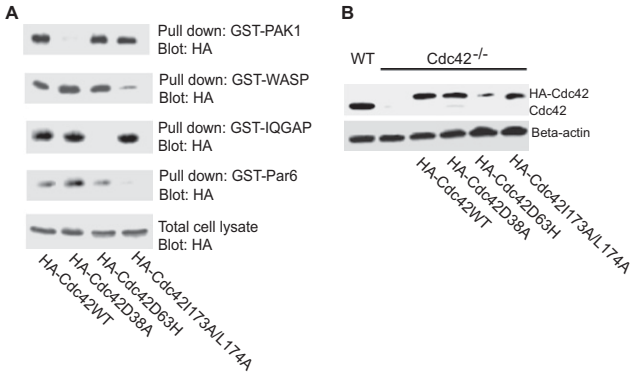

Fig. S7. (*A*) Interaction between Cdc42 mutants and Cdc42 effectors. Cdc42 mutants tagged by HA_3 were transfected into NIH 3T3 cells. The cells were subjected to the pull-down assay using Cdc42 effector probes GST-PAK1, GST-WASP, GST-IQGAP1, and GST-PAR6B. (*A*) The binding activities of GTP-bound Cdc42 mutants to PAK1, WASP, IQGAP1, and PAR6B were detected by Western blotting with anti-HA antibody. (*B*) Bone marrow cells from WT and *Cdc42*-deficient mice were isolated and transduced with Cdc42WT and Cdc42 mutants. The transduced bone marrow cells were sorted and transplanted into lethally irradiated BoyJ mice. Recipient mice were killed 2 mo later. Thymocytes were isolated and subjected to Western blotting with anti-Cdc42 antibody. Cdc42WT and Cdc42 mutants tagged with HA_3 ran 2 K_d higher than the endogenous Cdc42 on SDS/PAGE gel.