Supporting Information

Sagi et al. 10.1073/pnas.1121307109

SI Materials and Methods

Antibodies. Anti-human cluster of differentiation 4 (CD4) (clone RM4-5), CD8 (clone HIT8a), CD69 (clone L78), CD25 (clone M-A251), Ki67 (clone B56), CD3 (clone UCHT1), CD28 (clone CD28.2), CD62L (clone Dreg 56), CD45RO (clone UCHL1), phospho-phospholipase γ2 (PLCγ2) (pY759)(clone K86-689.37), phospho- *S*H2 domain-containing Leukocyte Protein of 76 kDa (SLP76)(pY128) (clone H3), phospho-CD3ζ (CD247, pY142) (clone K25-407.69), IL-6 (clone MQ2-13A5), and isotype controls were purchased from BD Pharmingen. Goat $F(ab')_2$ anti-mouse IgG fragments were purchased from Jackson Immunoresearch. Rabbit mAbs specific for phospho-S6 (pS235/236)(clone D57.2.2E) was purchased from Cell Signaling Technology. Anti-actin antibody (clone C4) was purchased from Millipore. Anti CD81 mAbs 5A6 (1) and 1D6 (2) were produced in our laboratory. Anti-CD3 (clone OKT3) was purchased from eBioScience or from Ortho biotech (Muromonoab).

T-Cell Purification. Buffy coat or apheresis Leukoreduction Chamber System (LRS) (3) were obtained from healthy volunteers following informed consent. T cells were purified by negative selection using RosetteSep human T-cell enrichment mixture (STEMCELL, Vancouver, Canada). Human CD4+ T cells were purified by negative selection using RosetteSep human CD4+ T-cell enrichment mixture (STEMCELL). Naïve CD4+ T cells were purified by negative selection using Naïve human CD4+ T-cell isolation kit (Miltenyi Biotec). Memory CD4+ T cells were purified by negative selection using Memory human CD4+ T-cell isolation kit (Miltenyi Biotec). All purifications were performed according to the manufacturers' instructions.

Phospho-Flow Analysis. Purified T cells were rested for 1 h at 37 °C, incubated on ice for 10 min with anti-CD3 (1 μg/mL), either alone or in combination with anti- CD81 (10 μg/mL) or anti-CD28 (10 μg/mL) mAbs, followed by crosslinking with Goat F $(ab')_2$ anti-mouse IgG (4 μg/mL). Cells were stimulated in a 37 °C water bath for 4–20 min, then fixed and permeabilized for 10 min by the addition of 1 mL of "fix/perm" (eBioScience). Fixed cells were washed for 5 min with "perm buffer" (eBioScience), blocked with 50μg of mouse IgG for 20 min on ice and washed twice with "perm buffer". Cells were stained with the following fluorochrome-conjugated antibodies: Pacific Blue-CD4, PE-Cy7-CD45RO, PE-CD3ζ, PE-phospho PLCγ2, 488-phospho SLP76, and 647-phospho S6. The stained samples were acquired in a LSR II flow cytometer (BD Biosciences) and analyzed by Cytobank software (http://cytobank.stanford.edu/).

T-Cell Proliferation. Purified T cells were incubated with 1 μM carboxyfluorescein succinimidyl ester (CFSE; Invitrogen) for 7 min in serum-free RPMI at room temperature. Cells were then washed three times with complete RPMI Medium containing 10% FCS. CFSE-labeled T cells were incubated in sterile tubes at a density of 1×10^6 per ml in the presence of anti- CD3 (0.1 μg/mL), either alone or in combination with anti-CD81 (2.5 μg/mL) or anti-CD28 (2.5 μg/mL). Antibodies were cross-linked with Goat $F(ab')_2$ anti-mouse IgG (4 μg/mL). After 5 d cells were washed once with PBS 1% BSA, blocked with 50μg of mouse IgG for 20 min on ice, washed with PBS containing 1%BSA and stained for the surface markers CD4 and CD45RO. Cell divisions were quantified by CFSE dilution using FlowJo software (http://www.flowjo.com/).

Ki67 Expression. Purified T cells (1×10^6) were incubated with anti-CD3 (0.1 μg/mL), alone or together with anti-CD81 (2.5 μg/mL), anti-CD28 (2.5 μg/mL) or an IgG isotype control (2.5 μg/mL) mAbs. Antibodies were cross-linked with goat $F(ab')_2$ anti-mouse IgG (4 μg/mL). After 96 h, cells were harvested and washed once with PBS, fixed with 1 mL of cold ethanol at −20 °C for 2 h, washed again with PBS and stained with anti-Ki67 mAb. Stained cells were acquired using an LSR II flow cytometer (BD Biosciences) and analyzed by FlowJo software (http://www.flowjo.com/).

Analysis of T-Cell Activation Markers. Purified T lymphocytes (1×10^6) were incubated with anti-CD3 (0.1 μg/mL) either alone or together with anti-CD81 (2.5 μg/mL), anti-CD28 (2.5 μg/mL) or an IgG isotype control (2.5 μg/mL) mAb, followed by cross-linking with goat $F(ab')_2$ anti-mouse IgG (4 μg/mL) for 24 h. Cells were then washed with PBS containing 1% BSA, blocked with 50μg of mouse IgG for 20 min on ice, washed with PBS containing 1% BSA and stained for expression of the surface markers CD4, CD8, CD45RO, CD62L, CD69, and CD25, as indicated. Stained cells were acquired using an LSR II flow cytometer (BD Biosciences) and analyzed by FlowJo software (http://www.flowjo.com/).

Analysis of T-Cell INFγ Production. Purified T lymphocytes (1×10^6) were incubated with anti-CD3 (0.1 μg/mL) either alone or together with anti-CD81 (2.5 μg/mL), anti-CD28 (2.5 μg/mL) or an IgG isotype control (2.5 μg/mL) mAb, followed by crosslinking with goat $F(ab')_2$ anti-mouse IgG (4 μg/mL) for 48 h, GolgiStop (BD Bioscience) was added during the last 6 h. Cells were then washed with PBS containing 1% BSA, blocked with 50μg of mouse IgG for 20 min on ice, washed with PBS containing 1% BSA. Cells were then stained for CD4, followed by fixation and permeabilization with cytofix/cytoperm (BD Bioscience), washed twice with perm buffer (BD Bioscience) and stained with 647-conjugated IFNγ mAb. Cells were acquired using an LSR II flow cytometer (BD Biosciences) and analyzed by FlowJo software (http://www.flowjo.com/).

Analysis of Cytokine and Chemokine Secretion. Purified T lymphocytes (1×10^6) were incubated with anti-CD3 (0.1 μg/mL) either alone or together with anti-CD81 (2.5 μg/mL) for 72 h, followed by centrifugation and collection of the supernatants. Antibodies were removed from the supernatant by incubation with 1.5 mg of protein G Dynabeads (Invitrogen) for 10 min at room temperature. The supernatants were kept at −80 °C until analysis by Luminex Multiplex assay at the core facility at Stanford.

1. Oren R, Takahashi S, Doss C, Levy R, Levy S (1990) TAPA-1, the target of an antiproliferative antibody, defines a new family of transmembrane proteins. *Mol Cell Biol* 10:4007–4015.
2. Schick MR, Levy S (1993) The TAPA-1 molecule is associated on the surface of B cells with HLA-DR molecules. *J Immunol* 151:4090–4097.
3. Néron S, et al. (2007) Characterization of mononuclear cells remaining in the leukoreduction system chambers of apheresis instruments after routine platelet collection: A new source of viable human blood cells. *Transfusion* 47:1042–1049.

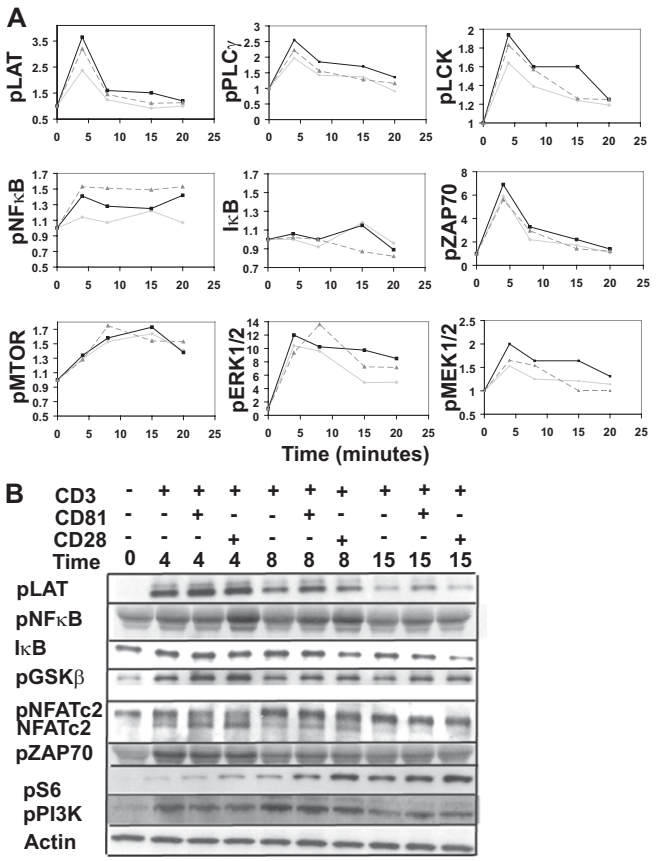

Fig. S1. Signaling pathways induced in response to costimulation by CD28 or by CD81. (*A*) Lysate array assay comparing the response of Jurkat cells to costimulation by 2.5 μg/mL of anti-CD3 mAb or together with 10 μg /mL of either anti-CD28 (dashed line), anti-CD81 (5A6) (black line) or isotype control (gray line) by induction of the indicated phosphorylated proteins. (*B*) Primary isolated T cells were stimulated with the indicated mAbs, cell lysates were separated by SDS/PAGE followed by Western blotting with indicated anti-phosphoprotein antibodies. Data are representative of three independent experiments.

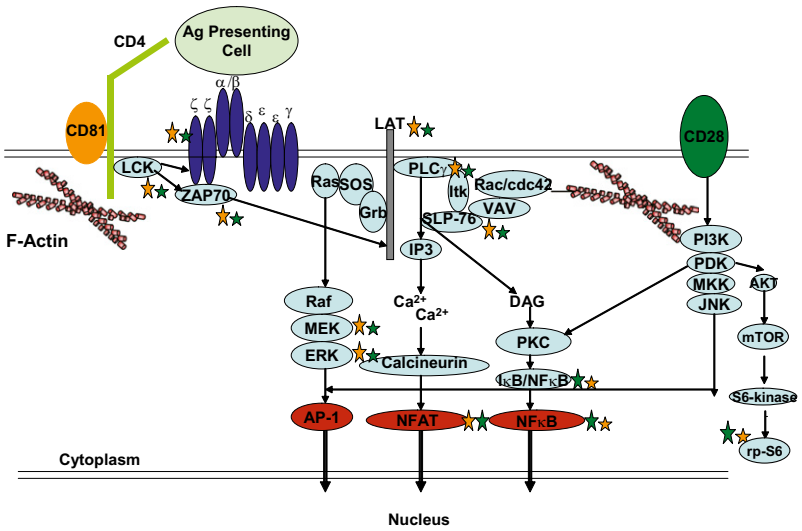

Fig. S2. Effect of costimulation by CD28 and CD81 on T-cell signaling pathways. Signaling molecules (light blue ovals), analyzed by Western blotting, lysate array or phosphoflow cytometry are marked by stars (orange for CD81 and green for CD28). The size of the star represents the relative effect on the activation of the signaling molecule.

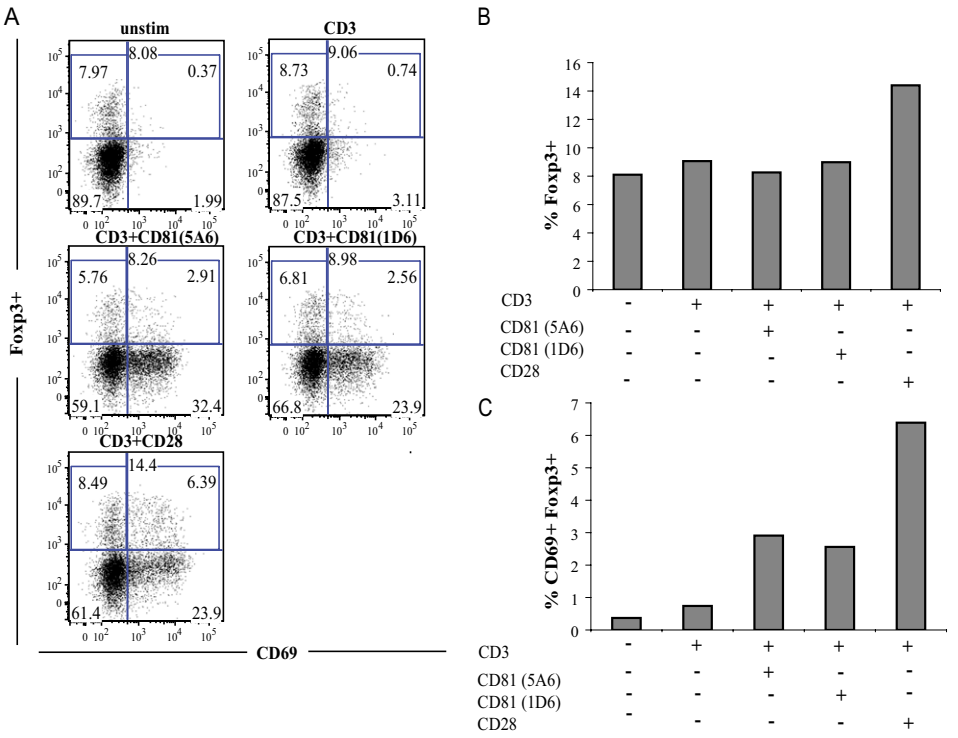

Fig. S3. Costimulation by CD28 and CD81 targets Foxp3 positive cells with different efficiency. Isolated T cells were cultured in the presence of anti CD3 (0.1 μg/mL), anti-CD81 (5A6) and anti-CD28 mAbs, each at a concentration of 2.5 μg/mL The cells were stimulated for 24 h, stained for expression of the surface molecules, CD4 and CD69, followed by an intracellular staining for Foxp3, and then analyzed by flow cytometry. (*A*) Expression of CD69 and Foxp3 on CD4-gated cells. (*B*) Summary of the data showing percentage of CD4+ Foxp3+ positive cells and (*C*) CD4+ Foxp3+ CD69+ positive cells. The data are representative of three independent experiments.

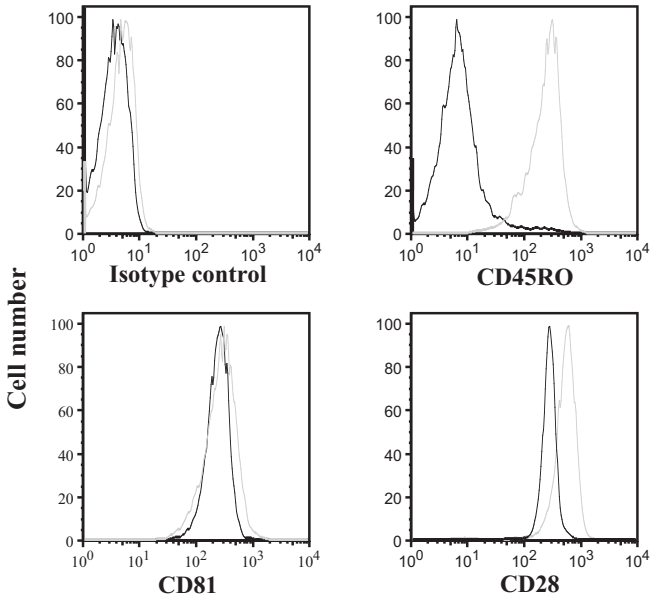

Fig. S4. Expression of CD45RO, CD28 and CD81 on isolated naïve and memory subsets. Isolated naïve (black-line histograms) and memory (gray-line histograms) CD4 T cells were stained for the surface markers CD45RO, CD28, and CD81 and by an isotype control mAb, followed by flow cytometry. The data represent results of three experiments analyzing expression on both isolated and nonisolated CD4 T-cell subsets.

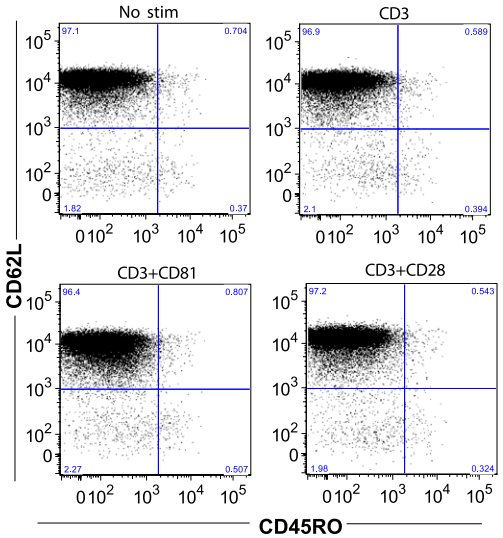

Fig. S5. Isolated naïve cells do not convert to memory cells after a short stimulation. Naïve and memory CD4 T cells were purified by negative isolation kits, costimulated separately with the indicated mAbs and analyzed for CD45RO and CD62L expression after 22 h. Data are representative of three independent experiments.

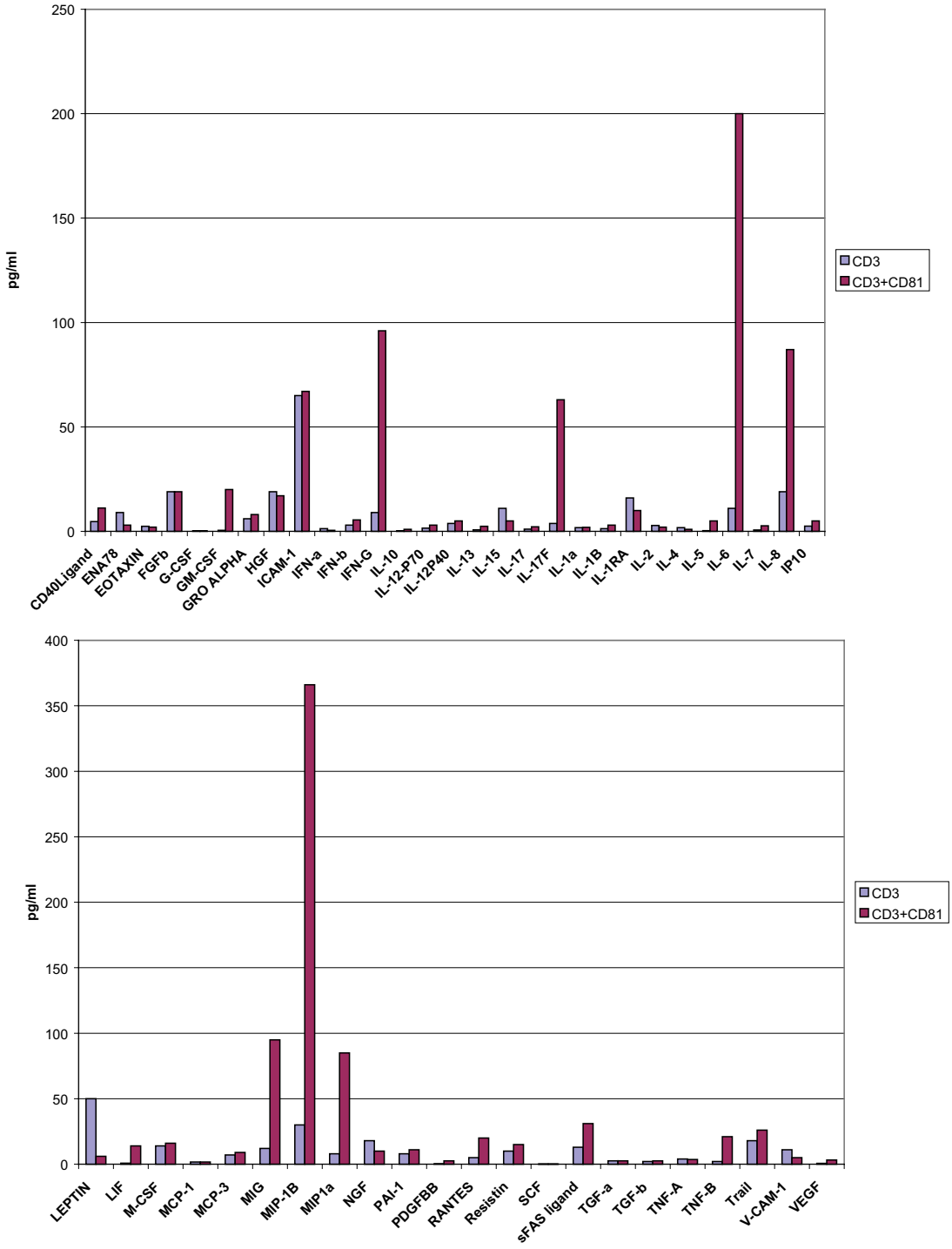

Fig. S6. Cytokine and chemokine production following CD81 costimulation. Isolated CD4 T cells were stimulated by the indicated antibodies for 72 h. Following the stimulation, supernatants were collected and analyzed by the multiplex Luminex assay.

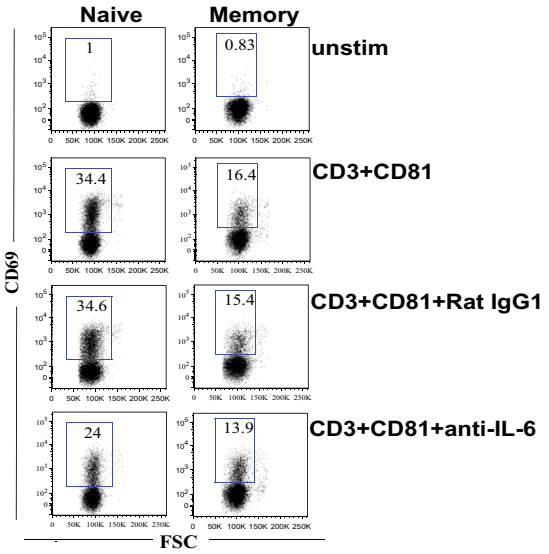

Fig. S7. IL-6 affects the extent of naïve T-cell costimulation by CD81. Total CD4 T cells were incubated in the presence of anti CD3 (0.1 μg/mL) plus 2.5 μg/mL of anti-CD81 (5A6) and in the presence of anti-IL-6 or the rat IgG1 isotype control (each at the concentration of 10 μg/mL), as indicated. The cells were stained for expression of CD45RO, CD62L, CD4, and CD69 after 22 h and analyzed by flow cytometry. Shown is CD69 expression on gated naïve and memory subsets. Data are representative of three independent experiments.