Supplemental Data

Spatiotemporal Regulation of T Cell Costimulation by TCR-CD28 Microclusters and Protein Kinase C θ Translocation

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Supplemental Text

Both molecules might be recruited through lipid raft. It has been reported that CD28 engagement re-distributes lipid raft, and that CD28 and PKCθ associate with downstream molecules in lipid raft (Bi et al., 2001; Viola et al., 1999). However, we failed to observe cluster formation of a raft marker in our systems (data not shown). Alternatively, although PKCθ may be recruited through a DAG gradient generated at the T cell—APC interface (Spitaler et al., 2006), we have not observed any DAG marker gradient (unpublished observation).

Supplemental References

Bi, K., Tanaka, Y., Coudronniere, N., Sugie, K., Hong, S., van Stipdonk, M. J., and Altman, A. (2001). Antigen-induced translocation of PKC-theta to membrane rafts is required for T cell activation. Nat Immunol *2*, 556-563.

Spitaler, M., Emslie, E., Wood, C. D., and Cantrell, D. (2006). Diacylglycerol and protein kinase D localization during T lymphocyte activation. Immunity *24*, 535-546.

Viola, A., Schroeder, S., Sakakibara, Y., and Lanzavecchia, A. (1999). T lymphocyte costimulation mediated by reorganization of membrane microdomains. Science *283*, 680-682.

Supplemental Experimental Procedures

Plasmid construction

Mouse EGFP- or ECFP-CD28, EGFP-CD3ζ, EGFP-CD11a, EGFP- or EYFP-PKCθ, EGFP-PI3K p55α, or EYFP-PI3K p85α was generated by PCR with cDNA from C57/BL6 splenic T cells and subcloned into retroviral vector pMXs (provided by Dr. T. Kitamura, Tokyo University, Japan). A fluorescence tag was added to the C-terminus of each protein except p55α and p85α. Mouse CD28 cDNA was originally provided by Dr. J. P. Allison (University of California, Berkeley, CA). Mutant CD28 was generated by PCR or with the QuickChange II Site-Directed Mutagenesis Kit (Stratagene). All fusion proteins contained an *Xho*I site and a poly-glycine linker (LESGGGGSGGGG, LESGGGG, or SGGGGSGGGGLE).

Preparation of Ig fusion proteins

DNA fragment corresponding to the extracellular domain lacking the signal sequence of mouse WT or YA CTLA-4 was inserted into pME18S expression vector containing a mouse CD150 leader segment at 5' terminus and a human IgG1 Fc segment at 3' terminus. Ig fusion constructs were transfected into HEK293T cells and fusion proteins were purified from the culture supernatant by protein A-affinity chromatography.

Photobleaching analysis

Photobleaching was performed with a Leica DMIRES2 system. AND-Tg T cells expressing EGFP-CD28 or EGFP-PKCθ were imaged for 10 min, photobleached for a specific region of interest (ROI) with a 488-nm laser line, and then imaged every 2.5 s. [Recovery ratio] = ([Average intensity of fluorescence in ROI at indicated time] / [Average intensity of fluorescence in control region at indicated time] – [Average intensity of fluorescence in ROI right after photobleaching] / [Average intensity of fluorescence in control region right after photobleaching]) / ([Average intensity of fluorescence in ROI before photobleaching] / [Average intensity of fluorescence in control region before photobleaching] – [Average intensity of fluorescence in ROI right after photobleaching] / [Average intensity of

fluorescence in control region right after photobleaching]).

Image processing and data analysis

The area and fluorescence intensity of each region in all images were depicted and measured by ImageJ (National Institutes of Health). Fold intensities in histograms were calculated as described below. [Fold intensity] = ([Intensity of fluorescence of each spot on a diagram] – [Background intensity]) / ([Average intensity of fluorescence on the diagram] – [Background intensity]). $CD28^{hi}$ and $CD3^{hi}$ regions in cSMAC and $CD28^{dim}$ and $CD3^{dim}$ regions in pSMAC were depicted and measured in area and in fluorescence intensity by ImageJ (National Institutes of Health). Fluorescence intensities of the specific regions were calculated as described below. [Intensity] = ([Average intensity of each fluorescence in $CD28^{hi}$ or $CD3^{hi}$ region in cSMAC or in $CD28^{dim}$ or $CD3^{dim}$ region in pSMAC] – [Background intensity]) / ([Maximum intensity of each fluorescence at the entire cell—bilayer interface] – [Background intensity]) (%). Areas and fluorescence intensities of EGFP-p55α clusters were measured by ImageJ. Fluorescence intensities of EGFP-p85α clusters were calculated as described below. [Intensity] = {([Average intensity of EGFP fluorescence at the cluster] – [Background intensity]) x [Area of the cluster]} / {([Average intensity of EGFP fluorescence at the entire cell—bilayer interface] – [Background intensity]) x [Area of the entire cell—bilayer interface]} (%). Significant differences were evaluated by the Student's t test with StatView software. Approximated curves in FRAP studies were depicted by Keleida Graph (Synergy Software, PA, USA).

Co-stimulation analysis

5-10 x 10^4 T cells were incubated with 5 ng/ml PMA with or without 2 μg/ml anti-mouse CD28 (PV-1) in a 96-well round-bottomed dish. Cells were pulsed with 2 Ci/well of ^{3}H-thymidine for 12 h after 48 h culture and measured with the Microbeta scintillation counter.

Western blotting

AND-TCR T cell hybridomas expressing ECFP-CD28 and/or EYFP-PKCθ or

Ag-stimulated $CD4^+$ T cells from AND-Tg *Rag2*$^{-/-}$ mice were lysed with 50 mM Tris-HCl, 50 mM NaCl, and 5 mM EDTA lysis buffer containing 1% digitonin, 1% Nonidet P-40 or 1% Triton X-100, immunoprecipitated with hamster monoclonal anti-CD28 (37.51, eBioscience) or control hamster monoclonal, and blotted with rabbit polyclonal anti-PKCθ (Santa Cruz), goat polyclonal anti-CD28 (Santa Cruz), or HRP-labeled monoclonal anti-GFP (Miltenyi Biotec).

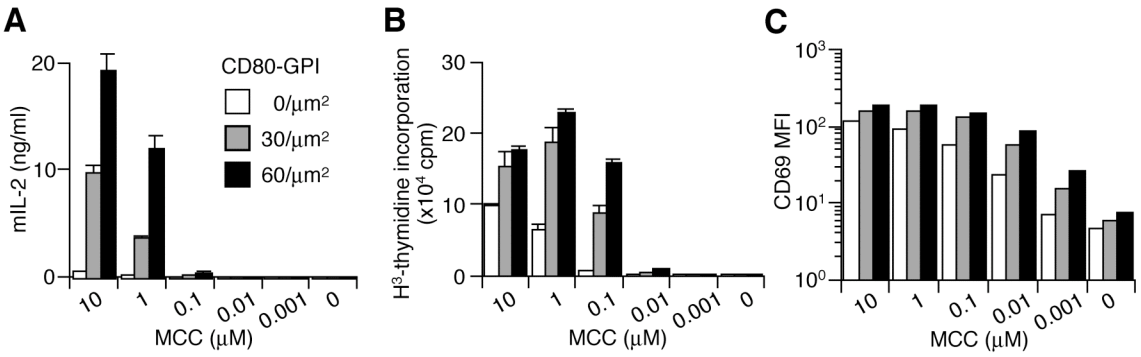

Figure S1. Costimulatory function of CD80-GPI for effector T cell activation

CD4 T cells from AND-Tg mice were stimulated with MCC88-103-pulsed APCs and sequentially cultured with recombinant human IL-2. After one week or later, the effector cells were restimulated with silica beads coated with lipid bilayer containing I-E^k, ICAM-1, and CD80 at various densities. The silica beads were prepulsed with MCC88-103 at the indicated doses.

(**A**) Concentration of IL-2 in the supernatant was measured by ELISA 48 h after stimulation. The graph shows mean +/- SD (n=3). A representative of three independent experiments is shown.

(**B**) Cell proliferation was assessed by measuring ^{3}H-thymidine uptake 60 h after stimulation. The graph shows mean +/- SD (n=3). A representative of three independent experiments is shown.

(**C**) CD69 expression was analyzed by FACS 6 h after stimulation. A representative of three independent experiments is shown.

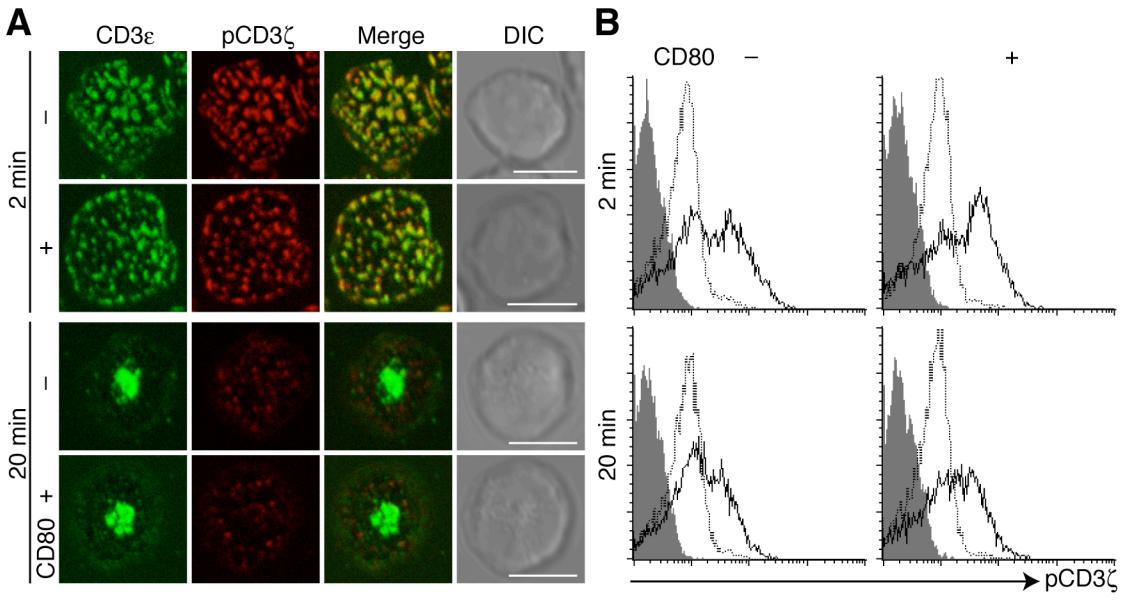

Figure S2. No significant effect on the phosphorylation status of TCR proximal signals by CD28—CD80 binding

(**A**) Freshly isolated T cells from AND-Tg mice were plated on a planar bilayer containing I-E^k and ICAM-1 (row 1 and 3) plus CD80 (row 2 and 4) (prepulsed with MCC88-103), fixed 2 min (top two rows) or 20 min (bottom two rows) after contact, and stained for CD3ε and phospho-CD3ζ. The locations of CD3ε (green) and phospho-CD3ζ (red) were imaged by confocal microscopy. Scale bars represent 5 μm. A representative of three independent experiments is shown.

(**B**) T cells in (**A**) were conjugated with MCC88-103-prepulsed or unpulsed silica beads coated with a lipid bilayer containing I-E^k and ICAM-1 (left) plus CD80 (right). The cells were fixed 2 min (top) or 20 min (bottom) after conjugation, stained for phospho-CD3ζ, and analyzed by FACS. Solid line, Ag-stimulated cells stained by anti-phospho-CD3ζ; dotted line, unstimulated cells stained by anti-phospho-CD3ζ; shadow, Ag-stimulated cells stained by control. A representative of three independent experiments is shown.

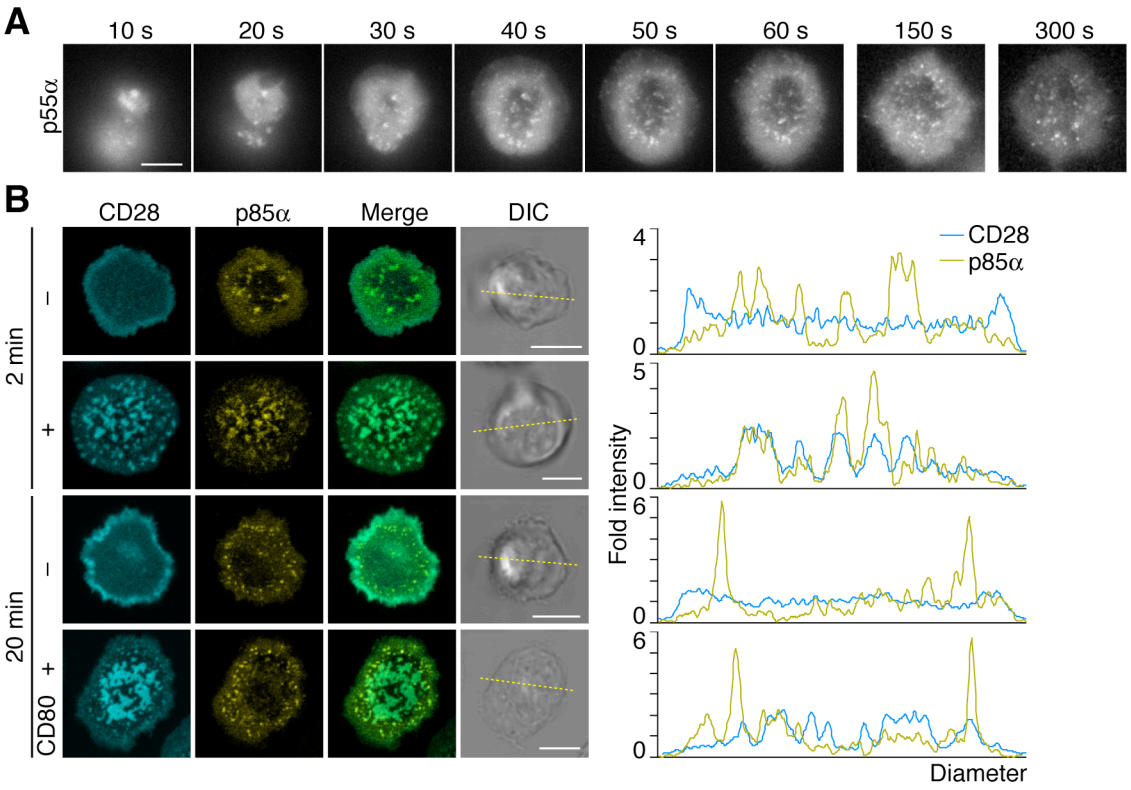

Figure S3. Transient localization of PI3K p55α/p85α at CD28 MCs

(**A**) AND-Tg T cells expressing EGFP-p55α were plated on a planar bilayer containing I-E^k and ICAM-1 (prepulsed with MCC88-103). Images were obtained at video rate (30 frames/s) using TIRFM (time shown above images). A scale bar presents 5 μm. A representative of five independent experiments is shown.

(**B**) AND-TCR T cell hybridomas expressing both ECFP-CD28 and EYFP-p85α were plated on an MCC88-103-prepulsed planar bilayer containing I-E^k and ICAM-1 without (rows 1 and 3) or with CD80 (rows 2 and 4), and real-time imaged 2 and 20 min after contact by confocal microscopy. Histograms on right panels show fold fluorescence intensities of ECFP-CD28 (blue) and p85α (yellow) on the diagonal yellow lines in DIC images. Scale bars represent 5 μm. A representative of two independent experiments is shown.

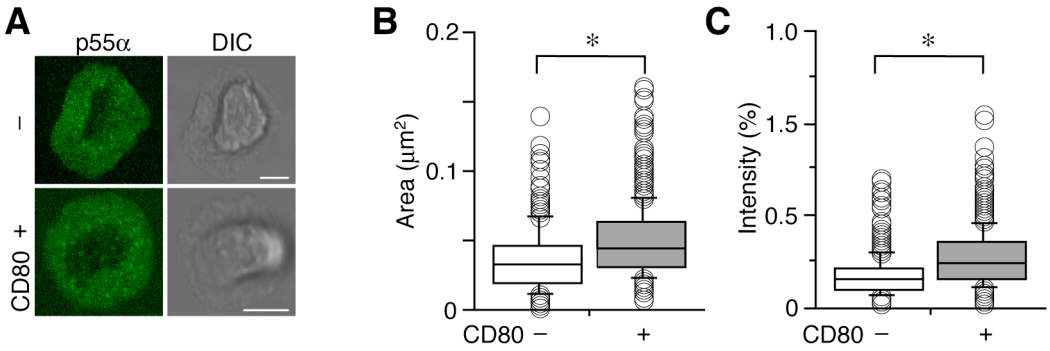

Figure S4. Augmentation of PI3K p55α clusters in size and density by CD28—CD80 binding

(**A**) AND-Tg T cells expressing EGFP-p55α were plated on a planar bilayer containing I-E^k and ICAM-1 without (top) or with CD80 (bottom) (prepulsed with MCC88-103). The cells were real-time imaged from time zero to 5 min by confocal microscopy. The images show T cells 2 min after contact. Scale bars represent 5 μm. A representative of three independent experiments is shown.

(**B** and **C**) EGFP-p55α clusters in 15 cells without CD80-GPI (n=377) and those in 16 cells with CD80-GPI (n=503) were measured in area (**B**) and in fluorescence intensity compared to the total intensity of the entire cell—bilayer interface (**C**). The graphs show mean +/- SD obtained by two independent experiments. *, p value < 0.001 with Student's t test.

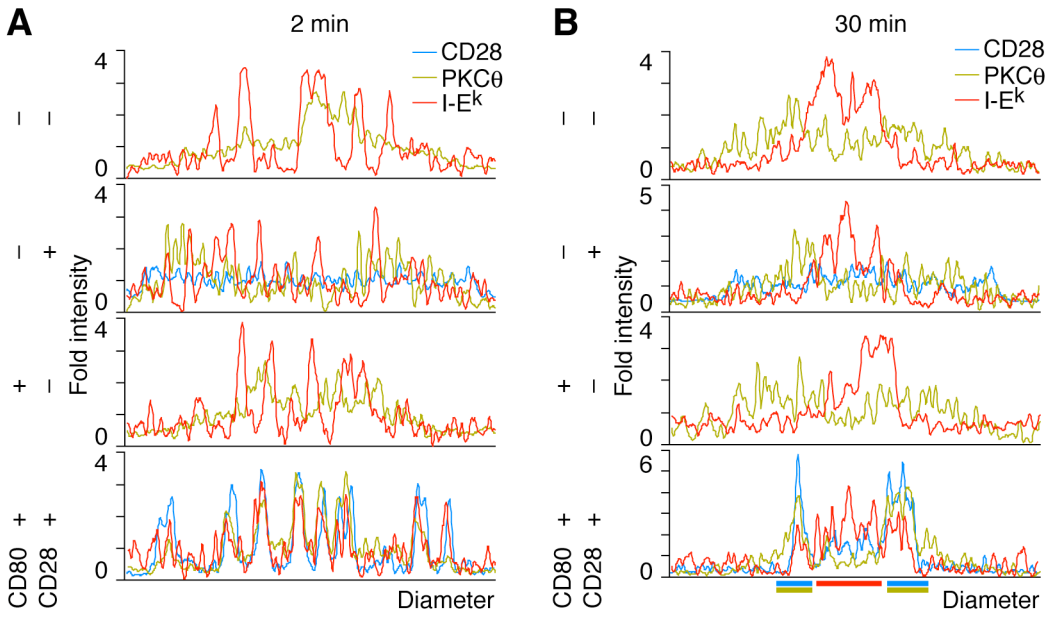

Figure S5. Initial colocalization of PKCθ with CD28 at TCR MCs and annular form of PKCθ clusters with CD28 after cSMAC formation

AND-Tg T cells from *Cd28*$^{-/-}$ mice were transfected with EYFP-PKCθ (**Fig. 5C** and **5E**, rows 1 and 3) plus ECFP-CD28 (**Figure 5C** and **5E**, rows 2 and 4) and were plated on a planar bilayer containing Cy5-labeled I-E^{k} and ICAM-1 (**Figure 5C** and **5E**, top two rows) plus CD80 (**Figure 5C** and **5E**, bottom two rows). Cells were imaged by confocal microscopy 2 min (**Figure 5C**) or 30 min after contact (**Figure 5E**). Fold fluorescence intensities of ECFP-CD28 (blue), EYFP-PKCθ (yellow), and Cy5-I-E^{k} (red) on the diagonal yellow lines in **Figure 5C** and **5E** DIC images were depicted in histograms in (**A**) and (**B**), respectively.

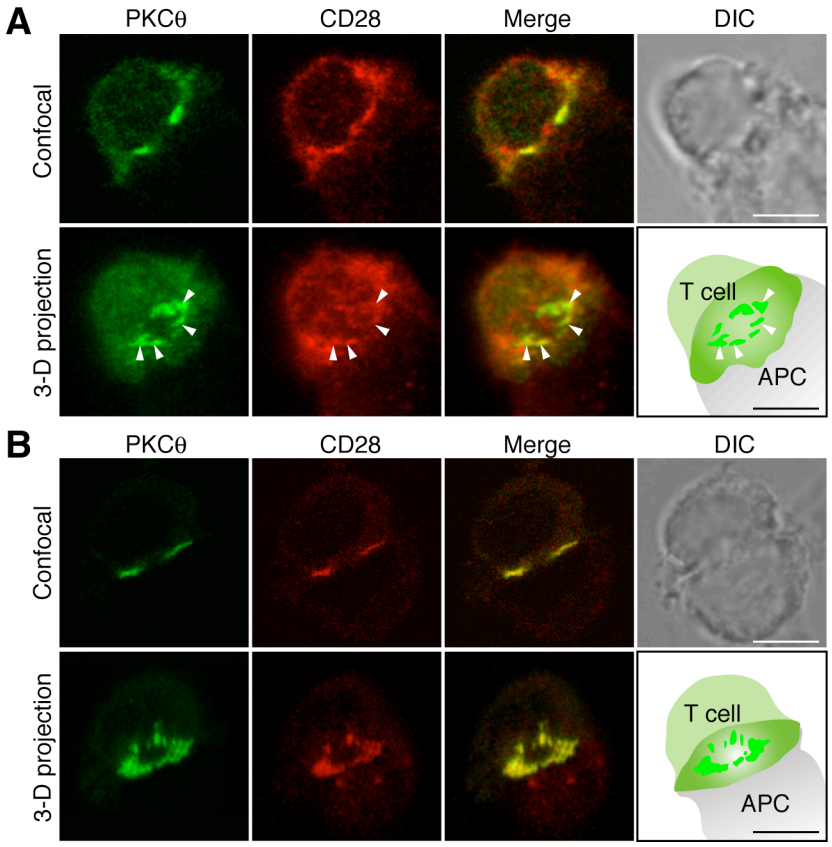

Figure S6. An annular form of PKCθ clusters at the T cell—APC interface

5C.C7-Tg T cells expressing EGFP-PKCθ were conjugated with MCC88-103-pulsed DC-1 expressing CD80, fixed 30 min after conjugation, stained with anti-mouse CD28 (PV-1) and Alexa Fluor 568 anti-hamster IgG, and imaged by confocal microscopy (top row). Images were obtained every 0.25 μm along the z-axis and reconstructed to 3-D projection (bottom row). Both (**A**) and (**B**) are representative images in three independent experiments. Arrowheads represent PKCθ and CD28 clusters. Scale bars represent 5 μm. The 3-D reconstructed images are available in **Movie S9**.

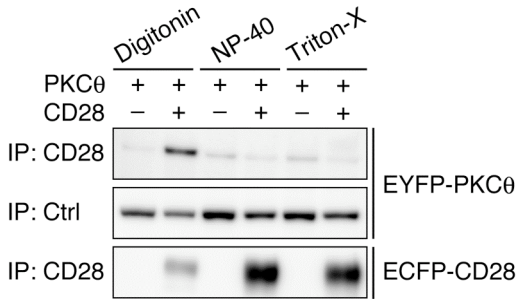

Figure S7. Weak assembly of CD28 with PKCθ detected upon PMA stimulation

AND-TCR T cell hybridomas expressing EYFP-PKCθ and/or ECFP-CD28 in **Figure 4A** and **4B** were stimulated for 2 min with 50 nM PMA. Cells were lysed with 1% digitonin, 1% NP-40, or 1% Triton-X lysis buffer as depicted in the figure, precipitated with anti-CD28 (top and bottom) or control (middle), and blotted for PKCθ (top and middle) or CD28 (bottom). A representative of three independent experiments is shown.

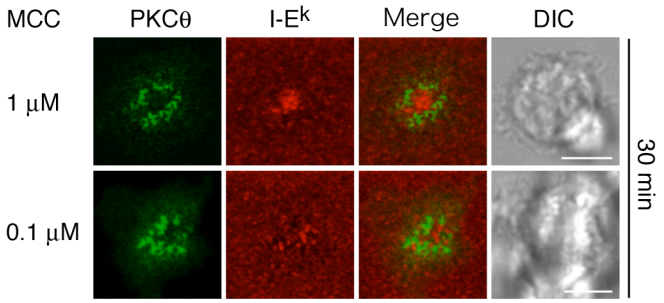

Figure S8. Generation of the annular form of PKCθ clusters without cSMAC formation

5C.C7 Tg T cells expressing EGFP-PKCθ (green) were plated on a planar bilayer containing Cy5-I-E^k (red) and ICAM-1 prepulsed with 1 μM (top) or 0.1 μM MCC88-103 (bottom) and imaged by confocal microscopy 30 min after contact. Scale bars represent 5 μm. A representative of two independent experiments is shown.

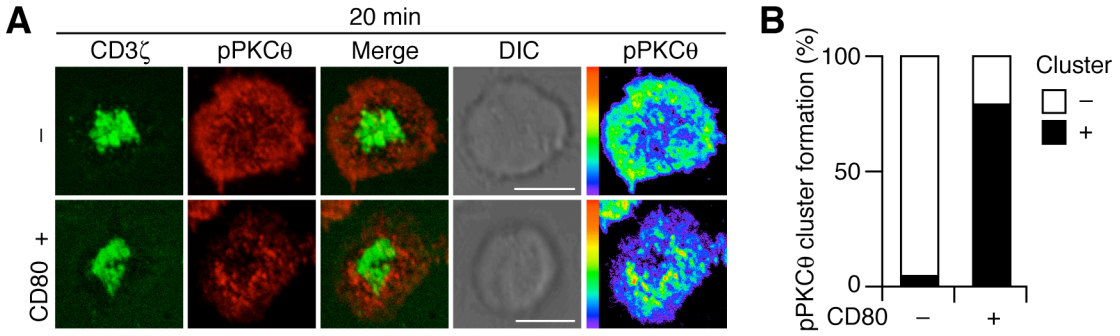

Figure S9. Accumulation of phospho-PKCθ at the outer region of cSMAC

(**A**) AND-Tg T cells expressing EYFP-CD3ζ (green) were plated on a planar bilayer containing I-E^k and ICAM-1 (top) plus CD80 (bottom) (prepulsed with MCC88-103). Cells were fixed 20 min after contact, stained for phospho-PKCθ (red), and imaged by confocal microscopy. Scale bars represent 5 μm. A representative of two independent experiments is shown.

(**B**) Formation of phospho-PKCθ clusters in (**A**) was evaluated by pattern recognition in the presence or absence of CD80-GPI. n=131 and 88 for $CD80^-$ and $CD80^+$, respectively. A representative of two independent experiments is shown.

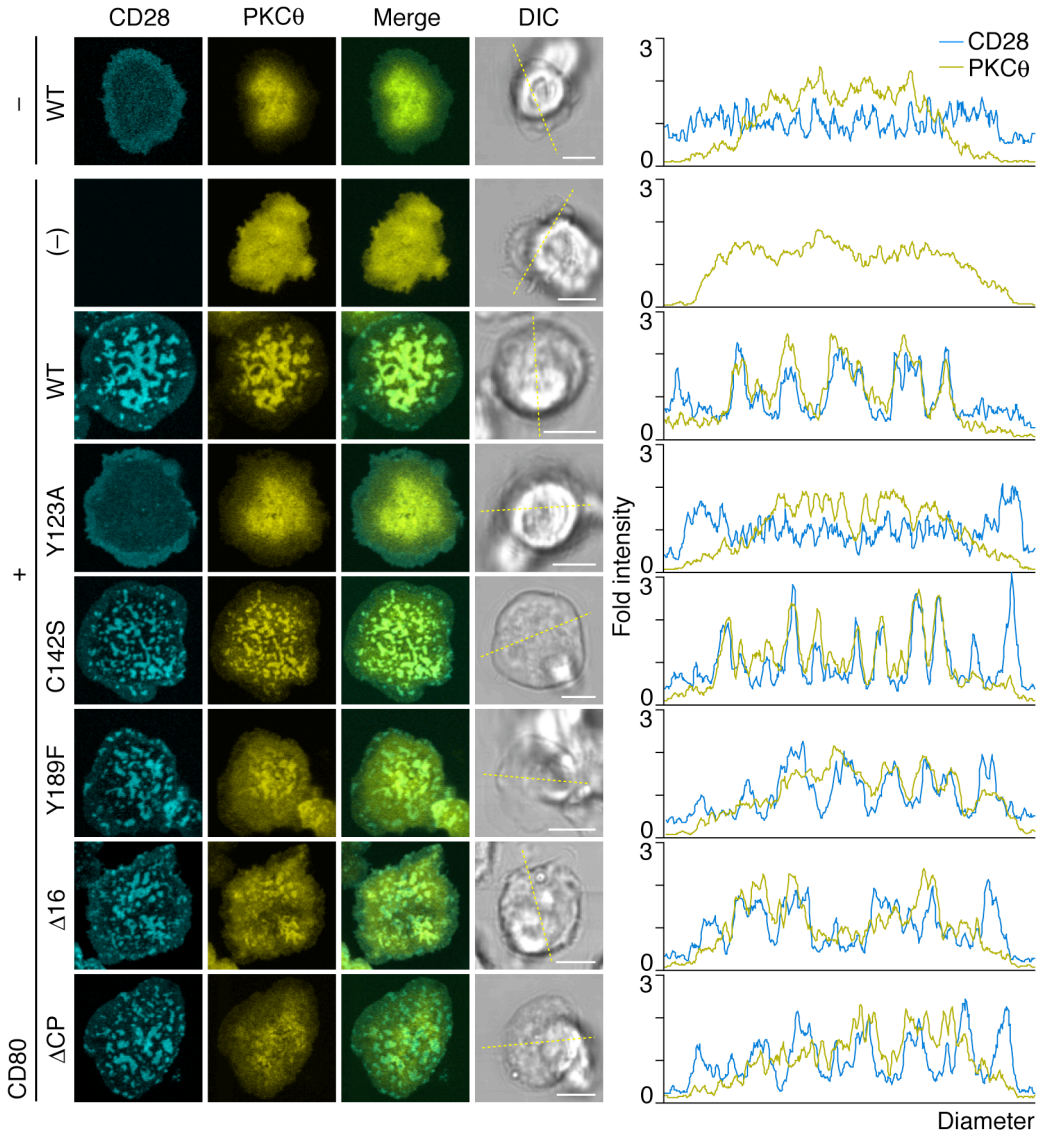

Figure S10. Function of CD28 cytoplasmic tail in the translocation of PKCθ to TCR-CD28 MCs

AND-TCR T cell hybridomas expressing EYFP-PKCθ (yellow) and ECFP-WT or mutant CD28 (cyan) were plated on a planar bilayer containing I-E^k and ICAM-1 (top) plus CD80 (prepulsed with MCC88-103) (bottom seven rows), and real-time imaged by confocal microcopy 2 min after cell—bilayer contact. Histograms show the fold fluorescence intensities of ECFP-CD28 (blue) and EYFP-PKCθ (yellow) on the diagonal yellow lines in

DIC images. Scale bars represent 5 μm. A representative of three independent experiments is shown.