Supplementary information

Protein Kinase C-η Controls CTLA-4-Mediated Regulatory T Cell Function

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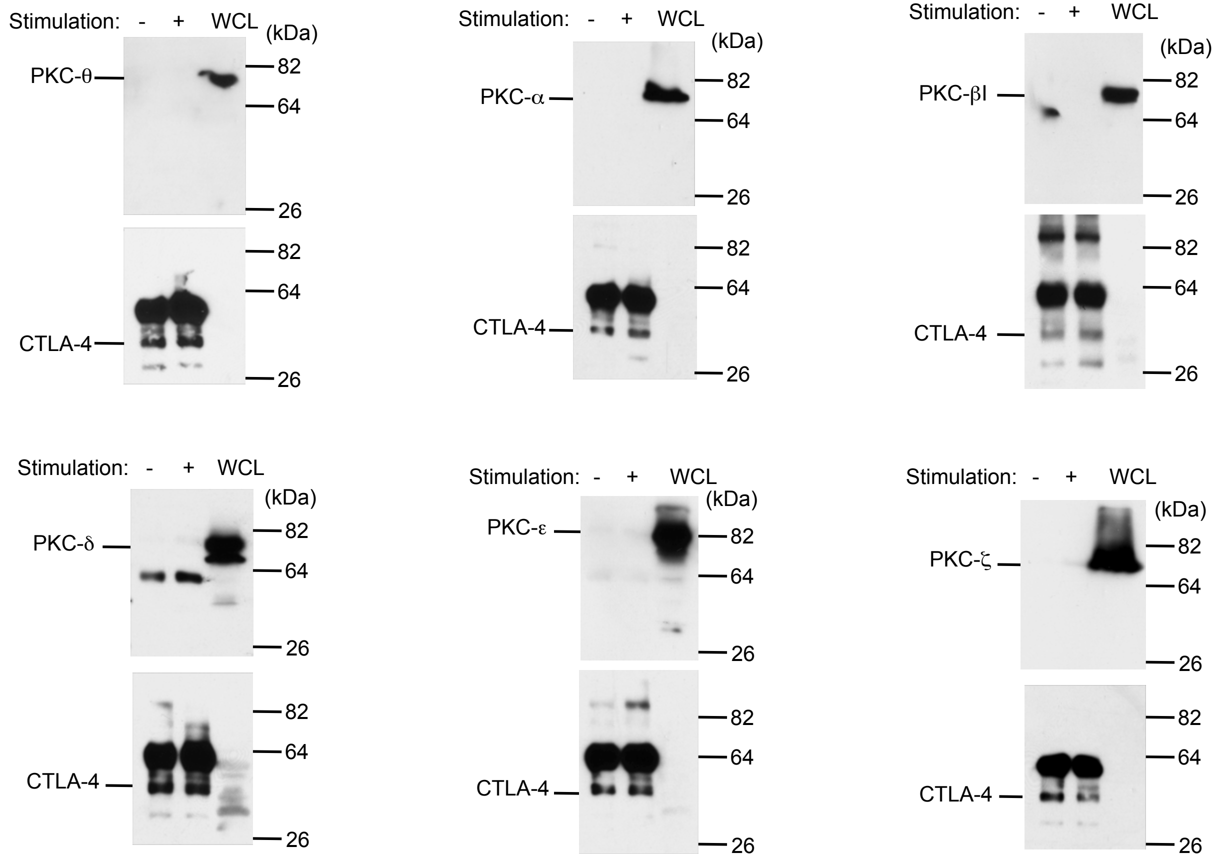
a



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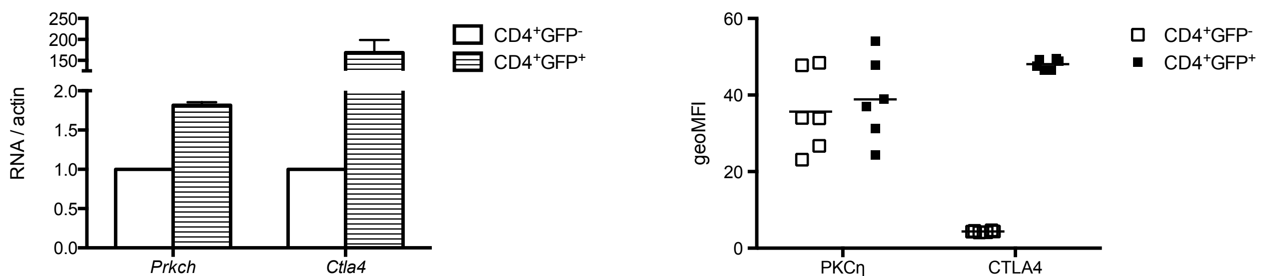
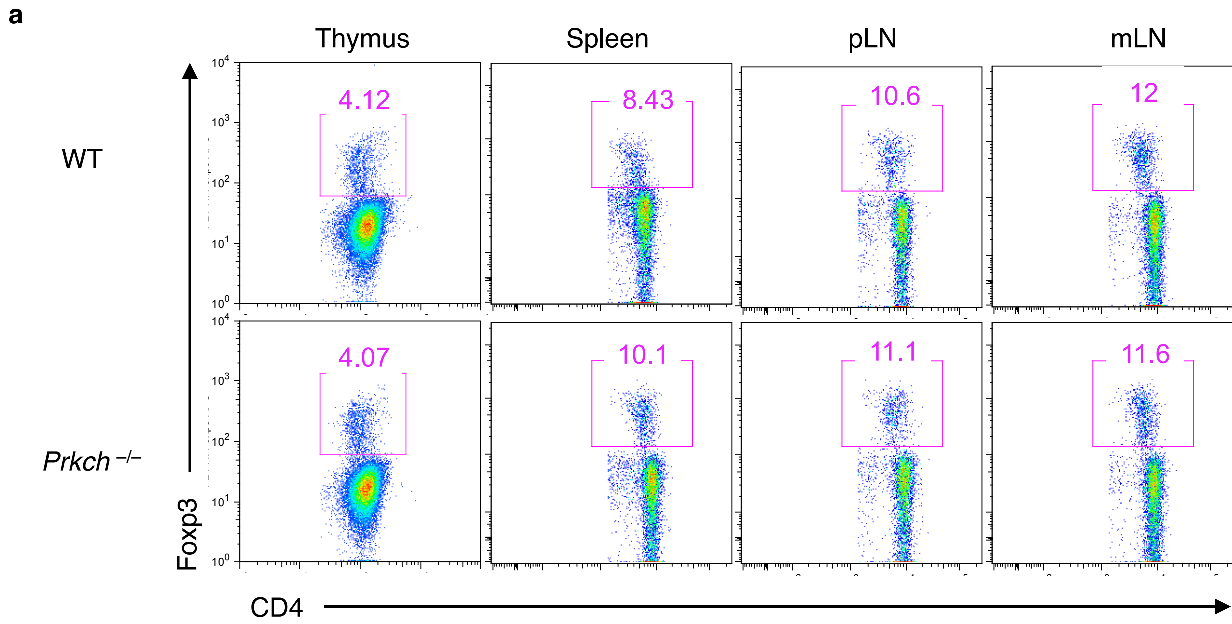
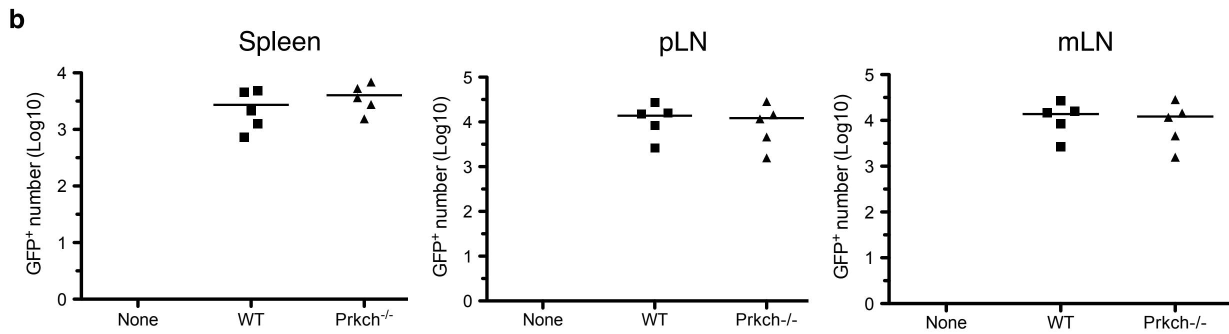
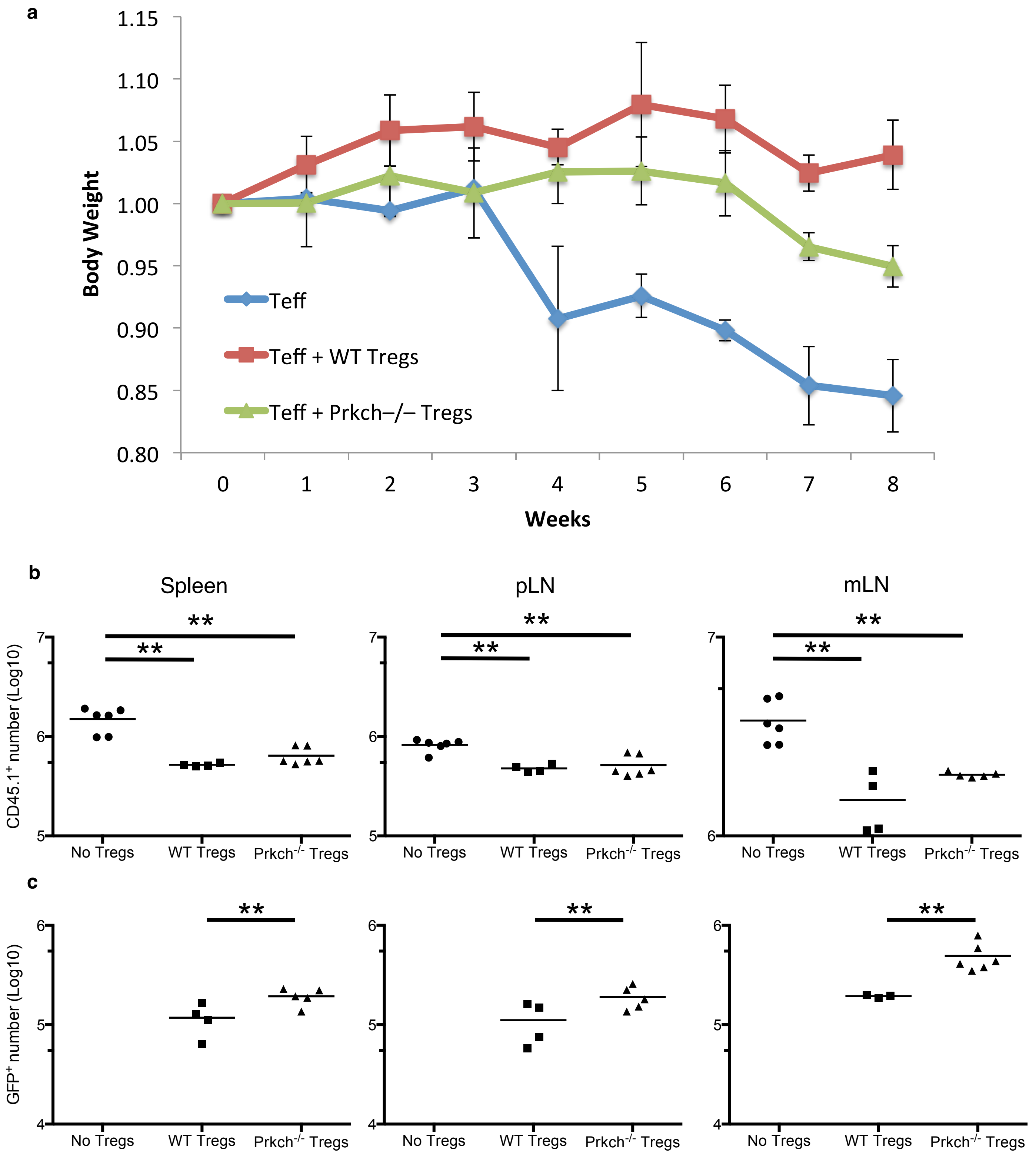


Supplementary Fig. 1. Lack of CTLA-4 interaction with PKC isoforms other than PKC-η, and the expression of PKC-η and CTLA-4 in T_{reg} *vs.* T_{eff} cells. (**a**) MCC-specific T hybridoma cells were left unstimulated (-) or stimulated (+) for 5 min with crosslinked anti-CD3 plus -CTLA-4 mAbs. Cell lysates (WCL) or CTLA-4 immunoprecipitates (2 left lanes in each panel) were resolved by SDS-PAGE, and immunoblotted with the indicated PKC-specific or anti-CTLA-4 Abs. (**b**) $CD4^+$ T cells were purified from spleens of FIG mice, and FACS-sorted into GFP^+ (T_{reg}) and $GFP^–$ (T_{eff}) cells. Equal number of sorted cells were subjected to RNA purification, reverse transcription, and quantitative PCR to determine the mRNA levels of *Prkch* and *Ctla4* (left panel). Intracellular staining of PKC-η and CTLA-4 was performed to determine their respective protein levels (right panel).





Supplementary Fig. 2. Frequency of T_{reg} cell populations. (**a**) The CD4+Foxp3+ cell population from thymi, spleens, peripheral lymph nodes (pLN) and mesenteric lymph nodes (mLN) of 8- to 12-week-old mice were determined by intracellular staining of Foxp3. (**b**) Number of GFP+ T_{reg} cells recovered from mice undergoing homeostatic expansion (see **Fig. 3b-d**). Naïve T cells from allotypically marked CD45.1+ B6.SJL mice were transferred alone (None), or cotransferred with FACS-sorted CD4+GFP+ T_{reg} cells from WT or *Prkch*–/– FIG mice into *Rag1*–/– mice. The numbers of GFP+ cells in spleens, pLN and mLN were determined. Each data point represents a single mouse.



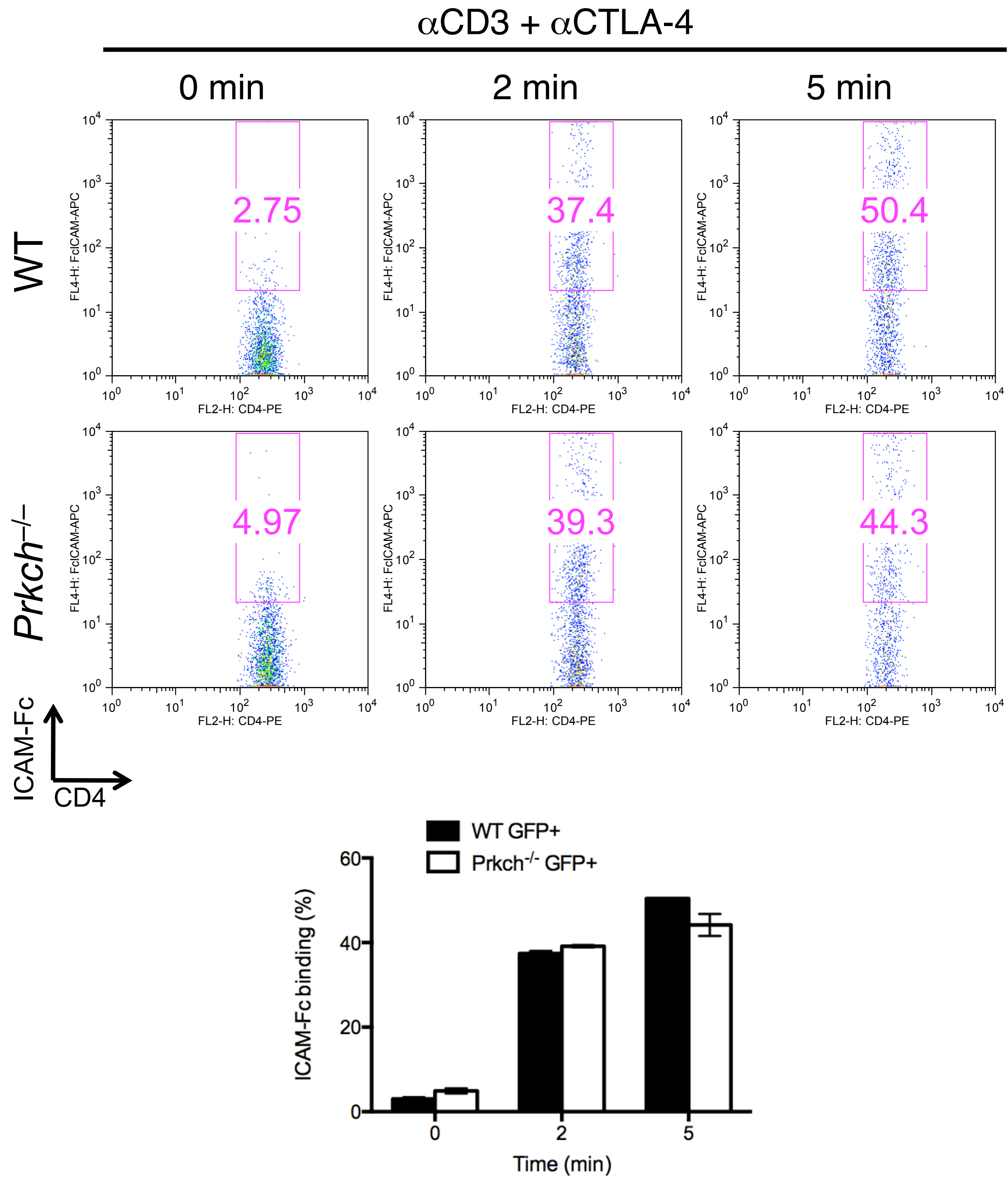
Supplementary Fig. 3. *Prkch*$^{-/-}$ T_{reg} cells protect mice in a T cell transfer model of colitis. (**a**) Sorted CD4^{+}CD62L^{+} naïve T cells (T_{eff}) in the absence or presence of WT or *Prkch*$^{-/-}$ GFP^{+} T_{reg} cells were cotransferred into *Rag1*$^{-/-}$ mice and weight was monitored over time as indicated. Mice were sacrificed 10 weeks post-transfer. (**b**,**c**) The infiltrating T cell populations in spleens, peripheral lymph nodes (pLN) and mesenteric lymph nodes (mLN) were analyzed by flow cytometry and enumerated. $^{**}P < 0.05$

a

Species	Sequence	NCBI Accession
Homo sapiens	AVSLS**K**ML**KKR**SPLTTGVYVKMPPTEPECEKQFQPYFIPIN	NP_005205
Pan troglodytes	AVSLS**K**ML**KKR**SPLTTGVYVKMPPTEPECEKQFQPYFIPIN	XP_001173441
Pongo abelii	AVSLS**K**ML**KKR**SPLTTGVYVKMPPTEPECEKQFQPYFIPIN	XP_002812816
Mus musculus	AVSLS**K**ML**KKR**SPLTTGVYVKMPPTEPECEKQFQPYFIPIN	NP_033973
Rattus norvegicus	AVSLN**R**TL**KKR**SPLTTGVYVKMPPTEPECEKQFQPYFIPIN	NP_113862
Cavia porcellus	AVSLS**K**ML**KKR**SPLTTGVYVKMPPTEPECEKQFQPYFIPIN	XP_003474228
Meleagris gallopavo	AIVVS**K**AIQ**RRRR**LTTGVYVKMP_SEK_LEKKVIPFHITVN	XP_003207551
Gallus gallus	AIVVG**K**AIQ**RR**Q**R**LTTGVYVKMP_SEK_LEKKVIPFHITVN	NP_001035180
Anas platyrhynchos	AVVVG**K**AIQ**RRRR**LTTGVYVKMP_SEK_LEKKVIPFHITVN	ACX71606
Taeniopygia guttata	TVLVG**K**VIQ**KRR**CLTTGVYVKMP_SEK_LEKKVIPFHITVD	XP_002197489
Xenopus tropicalis	AVLLG**K**CQ**RKK**F__TVGNYEKML_ESD_QGNGFSPYYIRVN	XP_002936576
Oncorhynchus mykiss	ALVHQVLQ**RKRR**__FEAIVPMM__S_K_NDGRFD_YGNFQ_	NP_001118005

b

Supplementary Fig. 4. (**a**) Evolutionary conservation of the positively charged proximal motif (underlined, with basic residues in bold) in the cytoplamic tail of CTLA-4. Protein sequence of putative CTLA-4 proteins from the indicated organisms were aligned with human CTLA-4. The consensus sequence was generated using Weblogo (www.weblogo.berkeley.edu). (**b**) . Analysis of “tailess” CTLA-4 mutants for their interaction with PKC-η. WT or truncated CTLA-4 were cotransfected with Xpress-tagged WT PKC-η into JTAg cells. Cells were stimulated with anti-CD3 mAbs + CD86-Fc recombinant protein for 5 min and immunoprecipitated with an anti-CTLA-4 mAb prior to immunoblotting. The right panel shows a schematic representation of mouse CTLA-4 and its cytoplasmic tail. The CTLA-4 tail was partially (Δ192-223) or fully (Δ182-223) truncated.

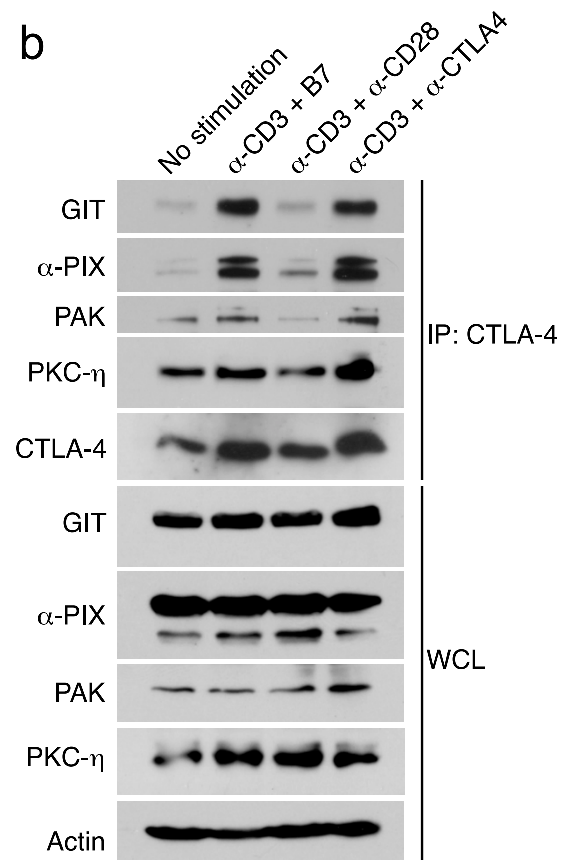


Supplementary Fig. 5. Effect of *Prkch* deletion on LFA-1 function. Purified CD4+ cells from WT or *Prkch*-/- FIG mice were stimulated with anti-CD3 plus anti-CTLA-4 Abs for the indicated times. The function of LFA-1 was measured by its ability to bind to ICAM1-Fc. Cells were stained with fluorophore-conjugated anti-CD4 and anti-Fc antibodies. Shown are representative data gated on GFP+ cells (top panel) and cumulative data of 2 independent experiments (bottom panel).

a

Protein	Putative function	Fold change
PAK2	GTPase kinase	2.28
Arfgap	GTPase	2.13
Mtap4	Cytoskeleton	1.92
Gm12250	GTPase	1.91
Lap3	Prot turnover	1.80
Git2	GTPase	1.76
Slc1a5	Transporter	1.73
Tcp1	Chaperon	1.68
Dock11	GEF	1.60
Prkcb	Signaling	1.58
Ubr4	E3 ubiquitin ligase	1.58
Fam65B	Cytoskeleton	1.55
Phc3	Chromatin modifier	1.52

b



Supplementary Fig. 6. (**a**) Phosphoproteome analysis of CD3- plus CTLA-4-costimulated *in vitro*-induced T_{reg} cells. Purified $CD4^+$ T cells from WT or *Prkch*$^{-/-}$ FIG mice were differentiated for 6 days into iTregs in the presence of TGF-β and IL-2 in standard SILAC media. The cells were stimulated with anti-CD3 plus anti-CTLA-4 mAbs for 5 min before cell lysis and sample preparation for phosphoproteomic analysis. Shown are representative hypophosphorylated proteins in *Prkch*$^{-/-}$ Tregs as compared to WT Tregs with a fold-change of >1.5. (**b**) Recruitment of GIT-PIX-PAK complex to CTLA-4-PKC-η complex is dependent on CTLA-4, but not CD28. MCC-specific T hybridoma cells were left unstimulated or stimulated with anti-CD3 and anti-B7, anti-CD3 and anti-CD28, or anti-CD3 and anti-CTLA-4 for 5 min prior to CTLA-4 immunoprecipitation. Immunoblotting was carried out with indicated antibodies.