Supplemental Information

The Kinase PKCα Selectively Upregulates Interleukin-17A during Th17 Cell Immune Responses

Marlies Meisel, Natascha Hermann-Kleiter, Reinhard Hinterleitner, Thomas Gruber, Katarzyna Wachowicz, Christa Pfeifhofer-Obermair, Friedrich Fresser, Michael Leitges, Cristiana Soldani, Antonella Viola, Sandra Kaminski, and Gottfried Bader
Supplementary Experimental Procedures

Abbreviations
CNS, central nervous system; EAE, experimental autoimmune encephalomyelitis; EMSA, electrophoretic mobility shift assay; FOXP3, forkhead box p3; i.p., intraperitoneal; IFN-γ, interferon-γ; IL, interleukin; LN, draining lymph nodes; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; PBS, phosphate-buffered saline; Prkca−/−, PKCa-deficient; PMA, phorbol 12-myristate 13-acetate; s.c., subcutaneous; SC, spinal cord; STAT, Signal transducer and activator of transcription; TGFβ, transforming growth factor β; TGFβRI, transforming growth factor β receptor; Th cell, T helper cell; TNF-α, tumor necrosis factor-α.

Antibodies and Reagents
For the stimulations with TGFβ (5ng/ml), the cells were maintained in serum-free X-vivo 20 medium (Lonza). The specific PKCa-β low molecular weight inhibitor G66976 (PKC LMWI; 1µM) (Martiny-Baron et al., 1993) and TGFβRI kinase inhibitor (TGFβRI LMWI; 1µM) were obtained from Calbiochem. [γ-32P]Adenosine 5′-triphosphate (ATP) was purchased from Amersham. Protein lysates were subjected to immunoblotting with antibodies from Cell Signaling against phospho-SMAD2, phospho-SMAD3 (C25A9), SMAD2 (L16D3), and SMAD3 (C67H9), phospho-STAT3, STAT3, phospho-p38, p38, phospho-JNK, JNK, phospho ERK1-2, ERK1-2; from Santa Cruz for TGFβRI (H-100), from Millipore for PKCa (M-4). The antibodies against murine CD4, CD25, IFN-γ, AnnexinV, 7AAD and IL-17A were obtained from BD Pharmingen. CD11c, MHCII, CD40, CD86, Vα2-TCR, CCR6, ROR-γt, T-bet and FOXP3 were obtained from eBioscience. TGFβRII-PE was obtained from R&D Systems. Neutralizing anti-IFN-γ, anti-IL-4 and anti-IL-12 were obtained from BD Pharmingen. The antibodies used for T cell stimulation were monoclonal antibodies (mAbs) against CD28 (clone 28.2) and 2C11 (mouse). The cytokines were obtained from eBioscience. The polyclonal phospho-specific (p)Thr200 TGFβRII antibodies was raised and affinity-purified against the phosphothreonine-containing peptide sequence NH2-LLVQR-T(p)-IARTI-COOH was coupled to KLH.

Antigen Recall Assay
On day 10, splenocyte suspensions were generated from MOG35-55 peptide immunized Prkca+/+ or Prkca−/− mice along with PBS-treated control mice. Splenocytes from individual mice, depleted of red blood cells with lysing buffer (R&D), were plated in duplicates (5x10^5 cells/well) in 200µl IMDM*** medium containing 0, 1, 10, or 100µg/ml MOG35-55 peptide and cultured at 37°C in 5% CO₂. After 72 hours, cytokine production after MOG35-55 peptide restimulation was measured in the cell culture supernatants. Cytokine amounts were analyzed with BioPlex multianalyte technology (BioRad).

Chromatin Immunoprecipitation (ChIP) Assay
The chromatin immunoprecipitation (ChIP) assay was performed with a ChIP assay kit according to the recommendations of the manufacturer (Imagenex) in combination with the Cold Spring Harbor protocol (Carey et al., 2009). Briefly, Prkca+/+ and Prkca−/− naïve T cells were isolated using the CD4+ T cell isolation kit II (Miltenyi Biotech). The polarization of CD4+ T cells was performed using solid-phase anti-CD3 (5µg/ml) and soluble anti-CD28 (1µg/ml) in X-vivo medium supplemented with TGFβ (5ng/ml) and IL-6 (20ng/ml). The cells were harvested after 16 hours, fixed in 1% formaldehyde at 37°C for 10 min, and the cross-linking was quenched by the addition of 1.375 M glycine. The cells were then washed twice with ice-cold PBS and lysed in cold cell lysis buffer for ChIP (5mM PIPES (pH 8.0), 85mM KCl, 0.5% Nonidet P-40 (NP-40)) for 10 min. The cell pellets were lysed following centrifugation in 1 ml nuclei lysis buffer for ChIP (50mM Tris-Cl (pH 8.0), 10mM EDTA, 1% SDS) supplemented with protease inhibitors and were incubated for 10 min on ice. Following sonication with 25-30s pulses using a Bioruptor Next Generation (Diagenode), the samples were centrifuged for 10 min at 12,000 rpm. The sheared chromatin was used to set up immunoprecipitation reactions with 5µg of the indicated Abs (IgG sc-2027 Santa Cruz; STAT3 483 Santa Cruz) at 4°C overnight. Magna ChIP protein G magnetic beads were added for 2 hours, and the samples were sequentially washed once with the buffers provided by the supplier (IMGENEX; high to low salt). The DNA-protein complex was eluted by heating at 65°C overnight, and the DNA was eluted using the IPure kit (Diagenode). Real-time PCR was performed with the following primers and probes using an ABI PRIM 7000 Sequence Detection System (Applied Biosystems): IL17a minimal promoter (-243 to -176) 5’ GAACCTTCTGCCCCCTCCCATCT 3’ and 5’ CAGCACAGAACACCCCTTTT3’ with the following probe 5’ FAM-CCTTGGACAGATGGTGGCGTCA-TAMRA-3‘(Schraml et al., 2009). Unspecific IgG Ab was used as negative control.
Confocal Immunofluorescence

Confocal immunofluorescence staining has been previously described (Nurieva et al., 2009). Briefly, human naïve CD4+ cells were isolated using the human CD4+ CD62L+ T cell isolation kit II (Miltenyi Biotech). The cells were fixed, permeabilized and stained with primary antibodies (anti-PKCα, anti-TGFβRII and anti-gp130). Colocalization was measured on single confocal sections using the colocalization module of Imaris S.0.1, 64-bit version (Bitplane AG). At least 30 individual cells were analyzed for each condition. Colocalization was estimated and expressed with the Mander’s coefficient (range from -1.0 to 1.0, with 1.0 indicating complete colocalization).

Experimental Autoimmune Encephalomyelitis Assay (EAE)

Mice were injected s.c. in the hind flank (100µl) with 200µg MOG35-55 peptide (PolyPeptide group) in complete Freund’s adjuvant (CFA) containing 5mg/ml H37RA (Mycobacterium tuberculosis, Difco Laboratories). In addition, pertussis toxin (Sigma; 200ng/mouse) was administered i.p. on the day of immunization and 48 hours later. Signs of EAE were assigned scores on a scale of 0–4 as follows: 0=none, 0.5=distal weak or spastic tail, 1=complete limp tail, 1.5=limp tail and hind limb weakness (feet slip through cage grill), 2=unilateral partial hind limb paralysis, 2.5=bilateral partial hind limb paralysis, 3=complete bilateral hind limb paralysis, 3.5=complete hind limb and unilateral partial forelimb paralysis, 4=death or total paralysis (Kaminski et al., 2011). The mean EAE disease scores were calculated by comparing the mean values of the WT and Prkca–/– mice at the onset (day 6-13) and at disease progression (day 14-20) using an unpaired Student’s t-test.

Flow Cytometry

For intracellular cytokine staining, cells were restimulated with phorbol 12-myristate 13-acetate (PMA) (Sigma; 10ng/ml) and ionomycin (Sigma; 125ng/ml) for 4 hours in the presence of Golgi stop (BD Bioscience; 0.65µl/ml). The cell surfaces were stained before the cells were fixed and permeabilized with BD Cytofix-Cytoperm and stained for cytokines. Intracellular staining for FOXP3, ROR-γt or T-BET was done without stimulation, with the eBioscience FOXP3 fixation-permeabilization kit. The affinity-purified (p)Thr180 TGFβRII antibody (Polypeptide group) was labeled using an Alexa Fluor 488 goat-anti-rabbit IgG1 antibody (Molecular Probes).

Preparation of CNS Mononuclear Cells

Mononuclear infiltrating cells were isolated from the CNS (central nervous system) and SC (spinal cord) as described by Korn et al. (Korn et al., 2007). Briefly, mice were perfused through the left cardiac ventricle. Brains and spinal cords were excised and dissociated for 45 min at 37°C by digestion with collagenase D (2.5mg/ml; Roche) and DNaseI (1mg/ml; Sigma) in PBS (containing 2% FCS). Dispersed cells were passed through a 70µm nylon mesh, pelleted by centrifugation, resuspended, layered onto a Percoll density gradient (GE Healthcare) and centrifuged for 20 min at 1200rpm and room temperature without break. CNS mononuclear cells were isolated by collection of the interphase fraction between 30% and 70% Percoll. After intensive washing in PBS, cells were restimulated in vitro with PMA-ionomycin (10ng/ml/125ng/ml) for 4 hours in the presence of Golgi stop (0.65µl/ml; BD Bioscience) and analyzed by flow cytometry.

Protein Kinase Assay

The protein kinase assay has been previously described (Zhou and Littman, 2009). Briefly, the PKCα- and TGFβRII-dependent phosphorylation of the TGFβRII proteins was measured by the incorporation of inorganic phosphate 32P from γ32P-ATP. A TGFβRII LMW (1µM) was used to prevent the auto-phosphorylation of TGFβRII. Peptides of the T200V (YDMTTSGSGGLPLLVRIVARITIV) and T200V mutant (YDMTTSGSGGLPLLVRIVARITIV) motifs were generated. The incorporated radioactivity was measured using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and X-ray autoradiography. For the peptide phosphorylation assays, the level of radioactivity bound to phosphocellulose filters was counted using the Wallac Micro β1450 (Perkin Elmer). The phosphosite prediction was done according to (Fujii et al., 2004).

Reporter Gene Assay

For the reporter gene expression assay Jurkat-TAg cells were transfected with the indicated expression constructs (K368R, kinase-dead PKCα mutant) and measured as previously described (Hermann-Kleiter et al., 2008; Yang et al., 2011).
Transfection of HEK293T Cells

HEK293T cells (Thermo Scientific) were maintained in DMEM medium (Biochrom), supplemented with 10% FCS (Life Technologies) and 1% L-glutamine and antibiotics. Transfection of expression vectors was performed in Opti-MEM medium (Invitrogen) using Metafectene reagent (Biontex) according to the manufacturer’s instructions. In brief, a total of 1x10^6 HEK293T cells were co-transfected (18-24 hours) with expression vectors (5µg) encoding His6-tagged PKCα WT or mutant (A25E, constitutively active mutant; CF, catalytic fragment (Baier, 2003; Yang et al., 2011)) with FLAG-tagged WT TGFβRI as indicated. Using Ni²⁺-NTA Magnetic Agarose beads (Qiagen) PKCα expression constructs were subsequently purified according to the manufacturer’s instructions. In brief, transfected cells were lysed in lysis buffer (0.5% NP-40), incubated with 5% Ni²⁺-NTA beads and His6-tagged proteins bound to magnetic beads were collected in the eluate and resolved by SDS-PAGE.

Immunoblotting

Cells were lysed in ice-cold lysis buffer [5mM Na2HPO4, 5mM NaF, 5mM Na3VO4, 5mM EDTA, 150mM NaCl, 50mM tris (pH 7.3), 1% NP-40, aprotinin and leupeptin (50µg/ml each)] and centrifuged at 15000xg for 15 min at 4°C. Cell lysates were electrophoresed on a NuPAGE gel (Invitrogen) and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore) by semidry blotting (90mA, 90 min). The primary antibodies were diluted in tris-buffered saline containing 0.5% Tween-20 and either with 5% nonfat dry milk or with 5% bovine serum albumin (BSA) for phospho antibodies. Peroxidase-conjugated antibodies (Pierce) served as secondary reagents (1:5000). Enhanced chemiluminescence was used for antigen detection (Super Signal, Pierce). As a loading control for whole cell lysates β-Actin or FYN (Santa Cruz) was applied. As a loading control for nuclear fractions, DNA polymerase-δ (Santa Cruz) was applied.

References

Baier, G. (2003). The PKC gene module: molecular biosystematics to resolve its T cell functions. Immunol Rev 192, 62-79.
Carey, M.F., Peterson, C.L., and Smale, S.T. (2009). Chromatin immunoprecipitation (ChIP). Cold Spring Harb Protoc 2009, pdb prot5279.
Fujii, K., Zhu, G., Liu, Y., Hallam, J., Chen, L., Herrero, J., and Shaw, S. (2004). Kinase peptide specificity: improved determination and relevance to protein phosphorylation. Proc Natl Acad Sci U S A 101, 13744-13749.
Kaminski, S., Hermann-Kleiter, N., Meisel, M., Thuille, N., Fresser, F., Penninger, J.M., and Baier, G. (2011). Coronin 1A is an essential regulator of the TGFβ receptor-SMAD3 signaling pathway in Th17 CD4⁺ T cells. J Autoimmun.
Korn, T., Reddy, J., Gao, W., Bettelli, E., Awasthi, A., Petersen, T.R., Backstrom, B.T., Sobel, R.A., Wucherpfennig, K.W., Strom, T.B., et al. (2007). Myelin-specific regulatory T cells accumulate in the CNS but fail to control autoimmune inflammation. Nat Med 13, 423-431.
Martiny-Baron, G., Kazanietz, M.G., Mischak, H., Blumberg, P.M., Kochs, G., Hug, H., Marme, D., and Schachtele, C. (1993). Selective inhibition of protein kinase C isozymes by the indolocarbazole Go 6976. J Biol Chem 268, 9194-9197.
Nurieva, R., Yang, X.O., Chung, Y., and Dong, C. (2009). Cutting edge: in vitro generated Th17 cells maintain their cytokine expression program in normal but not lymphopenic hosts. J Immunol 182, 2565-2568.
Schraml, B.U., Hildner, K., Ise, W., Lee, W.L., Smith, W.A., Solomon, B., Sahota, G., Sim, J., Mukasa, R., Cemerski, S., et al. (2009). The AP-1 transcription factor BATF controls Th17 differentiation. Nature 460, 405-409.
Yang, X.P., Ghoreschi, K., Steward-Tharp, S.M., Rodriguez-Canales, J., Zhu, J., Grainger, J.R., Hirahara, K., Sun, H.W., Wei, L., Vahedi, G., et al. (2011). Opposing regulation of the locus encoding IL-17 through direct, reciprocal actions of STAT3 and STAT5. Nat Immunol 12, 247-254.
Zhou, L., and Littman, D.R. (2009). Transcriptional regulatory networks in Th17 cell differentiation. Curr Opin Immunol 21, 146-152.
Figure S1. Phenotypical Characterization of Prkca<sup>−/−</sup> CD4<sup>+</sup> Th17 Cells (Related to Figure 1)

(A-B; H-I) Naïve CD4<sup>+</sup> CD62L<sup>hi</sup> T cells were cultured under indicated Th cell polarizing conditions for 3 days. (A) Expression analysis of classical PKC<sub>δ</sub>, novel PKC<sub>θ</sub> and PKC<sub>ε</sub>, or atypical PKC<sub>ι</sub> and PKC<sub>ζ</sub> family members revealed no Th17 cell specific upregulation; mRNA expression was determined by quantitative RT-PCR. The data were normalized to GAPDH mRNA and are presented relative to the Th0 levels. Data are from at least three independent experiments. (B) Expression pattern analysis of key cytokines or transcription factors of the Th cell types could confirm specific and efficient Th cell differentiation into the distinct effector cell lineages; mRNA expression was determined by quantitative RT-PCR. One representative experiment out of two is shown. (C-E) Prkca<sup>−/−</sup> CD4<sup>+</sup> Th17 cells display a normal phenotype. The mRNA expression of (C) RUNX1, AHR, IRF4, (D) IL-23R and IL-12Rβ2 was analyzed using qRT-PCR. The results are presented relative to Th0 WT or Th17 WT levels (AHR and IRF4) and are normalized to the expression levels of GAPDH mRNA. The data were derived from two independent experiments of 3 mice per group per experiment. (E) Representative FACS dot plots show the percentages of CD4<sup>+</sup>CCR6<sup>+</sup> gated cells, which are depicted in the upper-right quadrant. One representative experiment out of two is shown. (F-G) Normal IL-6-induced phosphorylation of STAT3 in Prkca<sup>−/−</sup> CD4<sup>+</sup> T cells. (F) Immunoblot analysis of STAT3 phosphorylation in whole-cell lysates of CD4<sup>+</sup> T cells rested for one hour in serum-free medium and stimulated for 30 minutes with IL-6 (40ng/ml). The β-Actin levels control for equal loading. One representative blot out of two independent experiments is shown and quantification of densitometric analysis is shown (below). (G) The mRNA expression of IL-6Rα was analyzed by qRT-PCR. Data are presented relative to Th0 WT levels and are normalized to the expression levels of GAPDH mRNA (n = 3). (H) Cultured under Th17 cell favoring conditions, Prkca<sup>−/−</sup> OTII T cells differentiate into a significantly reduced population of IL-17A<sup>+</sup>IL-17F<sup>−</sup> cells, yet an equal population of IL-17A IL-17F<sup>+</sup> cells, when compared to WT OTII T cells. Splenic DCs were pulsed without (none) or with OVA<sub>323-339</sub> peptide in the presence of lipopolysaccharide (LPS) (100ng/ml) for 24 hours prior to the addition of Prkca<sup>WT</sup> or Prkca<sup>−/−</sup> naïve OTII CD4<sup>+</sup>CD62L<sup>hi</sup> T cells under Th17 cell polarizing conditions (3 days). Cells were re-stimulated (4 hours) with anti-CD3 (5µg/ml; plate coated) plus anti-CD28 (2µg/ml; soluble) in IMDM<sup>+++</sup> medium in the presence of Golgi stop. As determined by flow cytometry, Prkca<sup>−/−</sup> OTII T cells expressed a significantly reduced population of V<sub>α2</sub>TCR<sup>+</sup>IL-17A<sup>+</sup>IL-17F<sup>−</sup> cells, yet an equal population of V<sub>α2</sub>TCR<sup>+</sup>IL-17A<sup>+</sup>IL-17F<sup>+</sup> cells, compared to WT cells. (A; C-D; G-H) The results are indicated as the mean ± SEM; *p<0.05, **p<0.01.
A. 

![Flow cytometry plots showing percentage of Th0 and Th17 cells under different conditions.]

B. 

|          | WT  | Prkca<sup>−/−</sup> |
|----------|-----|---------------------|
| Th0      |     |                     |
| Th17     |     |                     |
| AnnV− 7AAD− (%) | 21.9 ± 6.7 | 88.4 ± 2.6 |
| Th0      | 19.0 ± 6.9 |
| Th17     | 88.6 ± 3.5 |

C. 

| Prkca<sup>−/−</sup> OTII | 0µM ova | 1µM ova |
|-------------------------|---------|---------|
| Th17                    |         |         |
| AnnV− 7AAD− (%)         | 15.2 ± 7.7 | 89.1 ± 4.1 |
| Th0                     | 20.6 ± 12.5 |
| Th17                    | 92.2 ± 4.4 |

D. 

![Bars showing absolute number of infiltrating CD4+ cells.]

E. 

![Flow cytometry plots showing CD69 expression on OTII cells.]

F. 

![Bar graph showing relative mRNA expression of FOXP3.]

G. 

![Bar graph showing percentage of brain infiltrating CD4+ cells.]

H. 

![Bar graph showing relative mRNA expression of CD25.]

I. 

![Flow cytometry plots showing CD4+ CD25+ FOXP3+ cells.]

J. 

![Flow cytometry plots showing FOXP3 expression in Thymus and Lymph node.]

---

**Figure Legends:**

- **A:** Flow cytometry plots showing percentage of Th0 and Th17 cells under different conditions.
- **B:** Table showing AnnV− 7AAD− (%) for WT and Prkca<sup>−/−</sup> under Th0 and Th17 conditions.
- **C:** Table showing AnnV− 7AAD− (%) for Prkca<sup>−/−</sup> OTII under 0µM ova and 1µM ova conditions.
- **D:** Bar graph showing absolute number of infiltrating CD4+ cells in brain, SC, and LN.
- **E:** Flow cytometry plots showing CD69 expression on OTII cells.
- **F:** Bar graph showing relative mRNA expression of FOXP3.
- **G:** Bar graph showing percentage of brain infiltrating CD4+ cells.
- **H:** Bar graph showing relative mRNA expression of CD25.
- **I:** Flow cytometry plots showing CD4+ CD25+ FOXP3+ cells.
- **J:** Flow cytometry plots showing FOXP3 expression in Thymus and Lymph node.
Figure S2. No Apparent Th Cell Subset Switch Towards iTreg or Th1 Cells of Prkca−/− Th17 Cells; PKCa-Deficient nTreg and iTreg Cells Display Normal Functions (Related to Figure 2)

(A-B; H-I) Naïve CD4+ CD62Lhi T cells were cultured under indicated Th cell polarizing conditions for 3 days. (A) Under Th17 cell polarizing conditions, no apparent Th cell subset shift into either iTreg or Th1 cells was detected as analyzed by the expression (flow cytometry) of their key transcription factors FOXP3 and T-BET (ROR-γt-gated events), respectively. One representative experiment out of two is shown. (B-C) Prkca−/− OTII and Prkca−/− OTII Th17 cells and Prkca+/− and WT Th17 cells display equal survival rates, respectively. (B) Naïve CD4+ Prkca−/− and WT cells were polarized under Th17 cell conditions for 3 days. (C) Naïve CD4+ Prkca+/− OTII and Prkca−/− OTII T cells were polarized under Th17 cell skewing conditions with LPS-activated OVA323-339-primed DCs for 3 days. The percent of non-apoptotic and non-necrotic annexin V (AnnV)- and 7-amino-actinomycin D (7AAD)-gated cells are shown (cells in (B) are gated on CD4+ events; cells in (C) gated on CD4+ Vα2TCR+ events). The data were obtained from at least three independent experiments. (D and G) On day 14 post-immunization (peak), mononuclear cell infiltrates were isolated from the brain (CNS), spinal cord (SC) and draining lymph nodes (LN) and stimulated for 4 hours with PMA plus ionomycin in the presence of Golgi stop. The cells were fixed, permeabilized, stained and analyzed via flow cytometry. Comparable mean absolute CD4+ cell numbers ± SEM in the brain (left), spinal cord (middle) and draining lymph nodes (right) between genotypes are shown in (D). (E-F) Prkca−/− OTII Th17 cells produce only a marginal amount of IFN-γ. Splenic DCs were not pulsed (control) or pulsed with 1μM OVA323-339 peptide in the presence of LPS (100ng/ml) for 24 hours prior to the addition of Prkca+/− or Prkca−/− naïve OTII CD4+ CD62Lhi T cells under Th17 cell polarizing conditions for 3 days. Cells were restimulated (4 hours) with anti-CD3 (5µg/ml; plate coated) plus anti-CD28 (2µg/ml; soluble) in IMDM+/+ medium in the presence of Golgi stop. Cells were stained for CD4 (surface) and intracellular IL-17A and IFN-γ. As determined by flow cytometry, Prkca−/− OTII Th17 cells expressed a significantly reduced population of IL-17A IFN-γ cells, yet an equal and marginal population of IL-17A IFN-γ and IL-17A IFN-γ+ cells, compared to WT OTII Th17 cells. The data are derived from 3 mice per group per experiment from 2 independent experiments. (G) Prkca−/− mice display reduced FOXP3+ infiltrates during acute EAE. The cells expressing FOXP3 in the brain (left), spinal cord (middle) and in lymph nodes (right) are shown. The data are derived from three mice per group per experiment from two independent experiments. (H-J) The absence of PKCa does not affect the development of nTreg and in vitro differentiated iTreg cells. (H) The mRNA expression of FOXP3 was analyzed using qRT-PCR. The results are presented relative to WT Th0 cell levels and are normalized to the expression levels of GAPDH mRNA. One representative experiment out of two is shown. (I) The percentage of the CD4+CD25+FOXP3+ population, analyzed by flow cytometry in iTreg differentiated cells (3 days), was comparable between the genotypes under all investigated doses of TGFβ. (J) Representative FACS plots display CD25+FOXP3+ (CD4+-gated events) nTreg cells in the thymus (left panels) or lymph nodes (right panels). (I-J) The data are derived from two independent experiments of three separate mice per group. (B-C; F-G; I) The results are indicated as the mean ± SEM; *p<0.05, **p<0.01.
A. IMMATURE DCs

|          | CD11c⁺ | CD11c⁺MHCII⁺ | CD11c⁺CD40⁺ | CD11c⁺CD86⁺ |
|----------|--------|--------------|-------------|-------------|
| WT       | 65.7 ± 4.1 | 77.8 ± 2.0  | 29.3 ± 4.0  | 20.7 ± 1.8  |
| Prkca⁻⁻⁻ | 57.7 ± 5.0 | 74.0 ± 3.2  | 29.6 ± 3.5  | 24.2 ± 3.9  |

MATURE DCs

|          | CD11c⁺ | CD11c⁺MHCII⁺ | CD11c⁺CD40⁺ | CD11c⁺CD86⁺ |
|----------|--------|--------------|-------------|-------------|
| WT       | 72.6 ± 1.9 | 74.8 ± 2.4  | 42.6 ± 4.5  | 27.0 ± 2.4  |
| Prkca⁻⁻⁻ | 61.3 ± 4.3 | 72.1 ± 2.6  | 42.8 ± 5.3  | 29.6 ± 4.2  |

B. 

- **IL-6** [pg/ml]
  - WT: none, LPS
  - Prkca⁻⁻⁻: none, LPS

- **IFN-γ** [pg/ml]
  - WT: none, LPS
  - Prkca⁻⁻⁻: none, LPS

- **TNF-α** [pg/ml]
  - WT: none, LPS
  - Prkca⁻⁻⁻: none, LPS
Figure S3. PKCα-Deficient DCs Display an Unimpaired Phenotype (Related to Figure 3)

(A-B) WT and Prkca⁻/⁻ DCs were not pulsed (none; immature DCs) or pulsed with 1µM OVA₃₂₃₋₃₃₉ peptide (LPS; mature DCs) in the presence of LPS (100ng/ml) for 24 hours. (A) The critical DC markers CD86 and CD40 are appropriately expressed on the surface of Prkca⁻/⁻ DCs compared to WT DCs. Cells were stained for CD11c, MHCII, CD40 and CD86 and analyzed by flow cytometry. The data were obtained from two independent experiments using two separate mice per genotype per experiment. Data represent percentage of positive gated cells. (B) LPS stimulated Prkca⁻/⁻ DCs hyper-produce TNF-α, IL-6 and IFN-γ. The amount of IL-6 (left panel), IFN-γ (middle panel) and TNF-α (right panel) production in WT or Prkca⁻/⁻ DCs in the supernatant was assessed by BioPlex technology. The data are derived from two independent experiments of four separate mice. Error bars, mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.
| A. | TGFβRI 

| PKCα |
|------|
| Ni²⁺-NTA |
| control | wt | A25E | CF |
| TGFβRI |
| PKCα FL |
| PKCα CF |
| INPUT |
| TGFβ |

B. | GST-TGFβRI |
|-----|
| GST T |
| (p)Thr²⁰⁰ TGFβRI |
| Coomassie |
Figure S4. TGFβRI Physically Interacts with PKCα (Related to Figure 4)

(A) HEK293T cells were co-transfected with expression vectors encoding HIS6-tagged PKCα WT or mutant (A25E, constitutively active mutant; CF, catalytic fragment; described elsewhere (Baier, 2003; Yang et al., 2011) and FLAG-tagged TGFβRI. Post transfection, cells were stimulated with TGFβ (5ng/ml; 15min) as indicated. HIS6-tagged PKCα mutant proteins were precipitated with Ni²⁺-NTA-beads. Precipitates and whole cell lysates (INPUT) were analyzed by immunoblotting with respective anti-FLAG and anti-PKCα antibodies. FL, full length. Representative result out of three independent experiments is shown. (B) The immunoreactivity of the (p)Thr²⁰⁰ antibody is Thr²⁰⁰ phosphosite-specific. The specificity of the antibody raised against phosphorylated Thr²⁰⁰ on TGFβRI peptide was assessed by immunoblot analysis. No immunoreactivity was detected when the T200V mutant protein was used at the same levels (Coomassie band). Representative result out of two independent experiments is shown.
A. 

|        | WT | Prkca<sup>−/−</sup> |
|--------|----|---------------------|
| TGFβ 0' |    |                      |
| 30'    |    |                      |
| 60'    |    |                      |

B. 

C. 

D. 

E. 

F. 

G. 

H. 

I. 

EOMES
Figure S5. PKCα Is Dispensable in the Noncanonical TGFβR-Mediated Signaling Pathways
(Related to Figure 5)

Naive CD3⁺ T cells were stimulated in serum free X-vivo 20 with TGFβ (5ng/ml) as indicated. Cells were lysed and levels of (A) phospho-JNK (D) phospho-p38 and (F) phospho-ERK1-2 were determined by immunoblot. Levels of total proteins confirmed equal loading and respective quantification of densitometric analysis is shown in (B-C; E-G). (H) The phosphorylation level of AKT (T308) between WT and Prkca⁻/⁻ CD3⁺ T cells was determined by flow cytometry. (I) Naïve CD4⁺ CD62L⁺ T cells were cultured under indicated Th cell polarizing conditions for 3 days and mRNA expression of Eomesodermin (EOMES) was determined by quantitative RT-PCR. The data were normalized to GAPDH mRNA and are presented relative to the Th0 levels (n = 3). (A-H) The data are representative of at least two independent experiments. (B-C; E; H-I) Error bars, mean ± SEM.