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Defective IgG2a/2b Class Switching in PKC$\alpha^{-/-}$ Mice[1]

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Using model tumor T cell lines, protein kinase C (PKC) α has been implicated in IL-2 cytokine promoter activation in response to Ag receptor stimulation. In this study, for the first time, PKCα null mutant mice are analyzed and display normal T and B lymphocyte development. Peripheral CD3$^+$ PKCα-deficient T cells show unimpaired activation-induced IL-2 cytokine secretion, surface expression of CD25, CD44, and CD69, as well as transactivation of the critical transcription factors NF-AT, NF-κB, AP-1, and STAT5 in vitro. Nevertheless, CD3/CD28 Ab- and MHC alloantigen-induced T cell proliferation and IFN-γ production are severely impaired in PKC$\alpha^{-/-}$ CD3$^+$ T cells. Consistently, PKCα-deficient CD3$^+$ T cells from OVA-immunized PKCα-deficient mice exhibit markedly reduced recall proliferation to OVA in in vitro cultures. In vivo, PKCα-deficient mice give diminished OVA-specific IgG2a and IgG2b responses following OVA immunization experiments. In contrast, OVA-specific IgM and IgG1 responses and splenic PKC$\alpha^{-/-}$ B cell proliferation are unimpaired. Our genetic data, thus, define PKCα as the physiological and nonredundant PKC isotype in signaling pathways that are necessary for T cell-dependent IFN-γ production and IgG2a/2b Ab responses. *The Journal of Immunology,* **2006, 176: 6004–6111.**

Ligand binding to the TCR-CD3 complex, along with accessory molecules, initiates signal transduction that leads to full T cell activation including clonal expansion. Intracellularly, the stimulation of T cells (via elevation of cytosolic calcium concentration and production of diacylglycerols) results in the activation of protein kinase C (PKC)[4], a well-studied family of serine/threonine-specific protein kinases. The physiological role of PKCθ in TCR-induced NF-κB, AP-1 (1), and NF-AT transactivation has been firmly established (2–4). Nevertheless, the physiological and nonredundant functions of all other T cell-expressed PKC isotypes in primary T cells remain mostly unresolved (5, 6).

PKCα is a serine/threonine kinase and an ubiquitously expressed member of the conventional PKCs. Earlier findings (7) using transgenic mice overexpressing rPKCα suggested an important role of this isotype in T cell function. Transfection studies of fetal thymus using either the constitutively active catalytic domain or a dominant negative form of rPKCα showed that this conventional PKC isotype can influence differentiation during thymocyte development. Additionally, thymocytes from PKCα wild-type (wt) overexpressing transgenic mice exhibit increased proliferation and IL-2 production in response to TCR stimulation (8) and a TH1-biased cytokine secretion profile (9). An implication of PKCα in inflammatory responses has also been suggested (10). Overexpression of rPKCα in the epidermis of transgenic mice resulted in striking alterations of PMA-induced edema, infiltration of neutrophils, and expression of genes implicated in inflammation such as cyclooxygenase 2 and TNF-α (10). Conversely, ectopic recombinant expression of mutant PKCα inhibited bacterial LPS-induced cytokine production in macrophages (11). Along this line, and shown by overexpression or inhibition of PKCα in cell lines, PKCα regulates I$\kappa\beta$ kinase and NF-κB also in T cells (12, 13).

Because of these relevant findings plus PKCα's relative high expression in lymphocytes of the T cell lineage (14), we define in this study, for the first time, the in vivo role of PKCα in T cell activation using our recently established PKC$\alpha^{-/-}$ mice (15). Our genetic data now implicate PKCα as a critical factor in Ag receptor signaling leading to T cell proliferation and IFN-γ production in vitro. In vivo, PKCα is selectively required for TH1 T cell-dependent IgG2a/2b Ab responses.

Materials and Methods

Generation and genotyping of PKC$\alpha^{-/-}$ mice

PKC$\alpha^{-/-}$ mice have been recently established (15). DNA was extracted from adult tail tissue, digested with *Bam*HI, and hybridized with an endogenous 3′ probe distinguishing (+/+), heterozygote mutant (+/−), and homozygote mutant (−/−) alleles. Alternatively, mice carrying the PKC$\alpha^{-/-}$ alleles were routinely genotyped by PCR using the primers PKCα3′ E2, 5′-CCT GGT GGC AAT GGG TGA TCT ACA C-3′ and PKCα5′ E2, 5′-GAG CCC TTG GGT TTC AAG TAT AGA-3′. Homozygote PKC$\alpha^{-/-}$ mutant mice have been further backcrossed into the C57BL/6J background ($n = 3$) before functional analysis. To exclude that differences in T cell activation phenotypes between and PKC$\alpha^{-/-}$ T cells are simply due to different genetic backgrounds, littermate controls have been used throughout the study. All studies have been reviewed and approved by the institutional review committee.

Flow cytometry

Single-cell suspensions were prepared and incubated for 30 min on ice in staining buffer (PBS containing 2% FCS and 0.2% NaN_3) with FITC or PE Ab conjugates. Surface marker expression of thymocytes, splenocytes, or lymph node cells were analyzed using a FACScan cytometer (BD Biosciences) and CellQuestPro software (BD Biosciences) according to standard protocols. Abs against murine CD3 (145-2C11), CD4, and CD8 were obtained from Caltag Laboratories and CD28 (37.51), CD69, CD44, and CD25 were obtained from BD Pharmingen, respectively.

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[4] Abbreviations used in this paper: PKC, protein kinase C; PDBu, phorbol 12,13-dibutyrate; wt, wild type.

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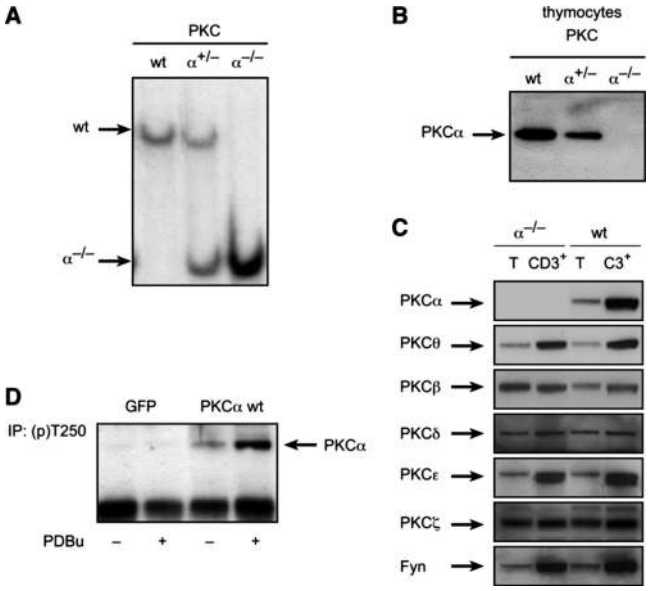

FIGURE 1. Southern and Western blot analysis of PKC$\alpha^{+/-}$, and PKC$\alpha^{-/-}$ T cells. *A*, Genomic DNA was digested with *Bam*HI and hybridized to the 3′ flanking probe; wt and mutant bands are indicated. *B* and *C*, Western blot analysis of 5×10^6/lane thymocytes (Thy) and peripheral purified CD3$^+$ T cells (CD3$^+$). Abs were directed against PKCα, θ, β, δ, ε, ζ, and p59^{fyn}, the latter as loading control, as indicated. *D*, In transfected Jurkat T cells, rPKCα was immunoprecipitated (IP) from resting (−) or 20 min and 100 nM PDBu-stimulated (+) T cells with a (phospho)Thr^{250} specific antiserum and immunoblotted with PKCα-specific mAb. Results shown are representative of at least three independent experiments.

Apoptosis detection

Freshly isolated thymocytes from 6- to 8-wk-old mice were plated in 96-well plates at a density of 2.5×10^5 cells/well in a total volume of 200 μl. Apoptosis induction was performed by addition of either Con A (10 μg ml^{-1}), phorbol 12,13-dibutyrate (PDBu; 1 μg ml^{-1}), ionomycin (1 μg ml^{-1}), camptothecin (1 μM), dexamethasone (10^{-6} M), or staurosporin (100 nM). Percentage of viable cells was determined by propidium iodide staining at time points between 10 and 40 h after apoptosis induction using a FACScan cytometer (BD Biosciences) and CellQuestPro software. Total splenocytes derived from PKCα- proficient or -deficient mice were used to generate activated T cell blasts using Con A (2 μg/ml)/IL-2 stimulation (100 U/ml). After 6 days, activated T cell blasts were washed twice in medium, viable cells were isolated by Lympholyte gradient centrifugation (viability >90%), and incubated in medium to assess apoptosis sensitivity, which was challenged by different concentrations of anti-CD3 cross-linking Abs (clone 2C11) to induce activation-induced cell death.

Analysis of T cell proliferation

Naive CD3$^+$ T cells were purified from pooled spleen and axial lymph nodes via mouse T cell enrichment columns (R&D Systems). T cell populations were typically 95% CD3$^+$, as determined by staining and flow cytometry. Peripheral CD4$^+$ or CD8$^+$ T cells were positively purified on MACS columns with anti-CD4 and anti-CD8 Abs coupled to magnetic beads (Miltenyi Biotec), respectively. For anti-CD3 stimulations, T cells (5×10^5) in 200 μl of medium (RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, and 50 U ml^{-1} penicillin/streptomycin) were added in duplicates to 96-well plates precoated with anti-CD3 Ab (clone 2C11, 10 μg ml^{-1}). Alternatively, PDBu (10 ng ml^{-1}; Sigma-Aldrich) plus Ca^{2+} ionophore (ionomycin, 100 ng ml^{-1}; Sigma-Aldrich) was used. Where indicated, IL-2 (final concentration 40 U ml^{-1}; Roche) or soluble anti-CD28 (1 μg ml^{-1}; BD Biosciences) was also added. Cells were harvested on filters at 64 h after a 16-h pulse with [^{3}H]thymidine (1 μCi/well) and incorporation of [^{3}H]thymidine was measured with a Matrix 96 direct beta counter system.

Analysis of B cell proliferation

Splenic B cells were purified by depletion of non-B cells on MACS columns (Miltenyi Biotec) with anti-CD43 Abs coupled to magnetic beads (Miltenyi Biotec). The purity of B cells was typically 95%, as determined by staining and flow cytometry. B cells were stimulated with 1.2 or 2.4 μg/ml goat anti-mouse IgM F(ab′)$_2$ (Dianova) alone or in combination with 25 U/ml recombinant mouse IL-4 (Roche). The B cells (5×10^5/well) were cultured for 48 h followed by the addition of [^{3}H]thymidine (1 μCi/well) for the next 16 h. Cells were harvested on filters and the incorporation of [^{3}H]thymidine was measured with a Matrix 96 direct beta counter system.

Table I. *T and B lymphocyte development was unaffected by the PKCα mutation*[a]

Hemogram					
RBC (10^6/mm^3)		WBC (10^3/mm^3)		PLT (10^3/mm^3)	
wt	$\alpha^{-/-}$	wt	$\alpha^{-/-}$	wt	$\alpha^{-/-}$
9.99 ± 0.16	9.34 ± 0.16	5.7 ± 1.7	4.5 ± 0.99	240	182 ± 42.43
Whole Cell Number					
Thymus ($\times 10^8$)		Spleen ($\times 10^7$)		Lymph Nodes ($\times 10^6$)	
wt	$\alpha^{-/-}$	wt	$\alpha^{-/-}$	wt	$\alpha^{-/-}$
1.33 ± 0.11	1.63 ± 0.46	6.9 ± 0.85	9.35 ± 0.92	6.4 ± 1.41	9.7 ± 2.12

Subsets						
	Thymus		Spleen		Lymph Nodes	
	wt	$\alpha^{-/-}$	wt	$\alpha^{-/-}$	wt	$\alpha^{-/-}$
CD3$^+$	24.86 ± 14.3	21.82 ± 15.37	22.26 ± 2.62	16.82 ± 7.84	84.58 ± 1.52	79.71 ± 5.69
CD19$^+$/IgM$^+$	ND	ND	45.94 ± 13.01	38.83 ± 10.85	14.33 ± 3.12	13.9 ± 1.15
CD4$^+$	16.26 ± 9.27	15.08 ± 9.47	13.7 ± 1.55	7.93 ± 2.03	49.04 ± 4.36	46.04 ± 8.28
CD8$^+$	3.08 ± 1.25	3.24 ± 2.12	6.87 ± 1.48	4.36 ± 1.6	33.85 ± 2.17	30.97 ± 4.53
Double negative	3.07 ± 2.29	5.43 ± 2.09	79.33 ± 2.55	82.63 ± 12.25	16.66 ± 2.59	22.5 ± 4.07
Double positive	77.6 ± 12.42	76.26 ± 13.5	0.11 ± 0.03	0.09 ± 0.04	0.45 ± 0.4	0.5 ± 0.27
CD4$^+$/CD25$^+$	ND	ND	2.29 ± 0.26	2.11 ± 0.38	4.16 ± 0.46	4.76 ± 1.18

[a] Absolute numbers and cellularity of thymus, spleen, and lymph node: cells were comparable in wt and PKC$\alpha^{-/-}$ mice. Percentages of positive cells ± SEM are indicated ($n = 4$).

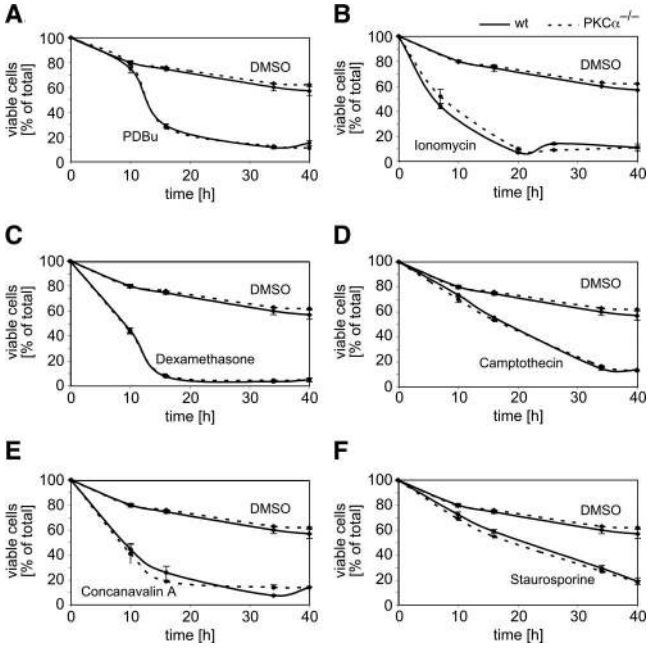

FIGURE 2. Normal ex vivo survival of PKCα-deficient T cells: freshly isolated cells from 6- to 8-wk-old mice were plated in 96-well plates at a density of 2.5×10^5 cells/well. Apoptosis induction was performed by addition of either PDBu (*A*), ionomycin (*B*), dexamethasone (*C*), camptothecin (*D*), Con A (*E*), or staurosporin (*F*) in comparison to the DMSO buffer control, and dead cells were determined by propidium iodide staining. Results shown are the means ± SD of at least three independent experiments.

IL-2 and IFN-γ secretion

T cells were purified from PKCα-deficient and littermate mice and mixed in duplicates at various densities with mitomycin C-treated splenocytes from BALB/c mice. Alternatively, T cells were plated in 96-well plates and incubated with the described CD3 and CD28 Ab stimuli. After 48 h of growth, supernatants were collected and IL-2 and IFN-γ productions were determined by ELISA (Quantikine M; R&D Systems).

Western blot analysis

T cells were stimulated either with medium alone or with solid-phase hamster anti-CD3 (clone 145–2C11), with or without hamster anti-CD28 (clone 37.51; BD Biosciences) at 37°C for various time periods. Cells were lysed in ice-cold lysis buffer (5 mM NaP_2P, 5 mM NaF, 5 mM EDTA, 50 mM NaCl, 50 mM Tris (pH 7.3), 2% Nonidet P-40, and 50 μg/ml each aprotinin and leupeptin) and centrifuged at 15,000 × *g* for 15 min at 4°C. Protein lysates were subjected to Western blotting as previously described (2) using Abs against PKCδ and PKCθ (Transduction Laboratories), PKCα (Upstate Biotechnology), PKCζ, PKCβ, and Fyn (all Santa Cruz Biotechnology), and PKCε (BD Biosciences). To detect the transcription factors, mAb7A6 for NFATc (Affinity BioReagents), mAbG1-D10 for NFATp (Santa Cruz Biotechnology), pAbC-17 for STAT5b (Santa Cruz Biotechnology), and pAb for c-Fos (Geneka) were used.

Gel mobility shift assays

Nuclear extracts were harvested from 2×10^7 cells. Purified $CD3^+$ T cells were washed in PBS and resuspended in 10 mM HEPES (pH7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and protease inhibitors. Cells were incubated on ice for 15 min. Nonidet P-40 was added to a final concentration of 0.6%, and cells were vortex-mixed vigorously, and the mixture was centrifuged for 5 min. The nuclear pellets were washed twice and resuspended in 20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and protease inhibitors, and the tube was rocked for 30 min at 4°C. After centrifugation for 10 min, the supernatant was collected. Extract proteins (2 μg) were incubated in binding buffer with ^{32}P-labeled, double-stranded oligonucleotide probes (NF-κB, 5′-GCC ATG GGG GGA TCC CCG AAG TCC-3′; AP-1, 5′-CGC TTG ATG ACT CAG CCG GAA-3′; NF-AT, 5′-GCC CAA AGA GGA AAA TTT GTT TCA TAC AG-3′; and STAT5, 5′-TGT GGA CTT CTT GGA ATT AAG GGA CTT TTG-3′) (Nushift; Active Motif). In each reaction, 3×10^5 cpm of labeled probe was used, and band shifts were resolved on 5% polyacrylamide gels.

Immunization and recall proliferation

Before an Ag challenge, total serum IgG concentrations were measured by ELISA (Roche). Mice were immunized with protein Ag chicken egg albumin (OVA; Sigma-Aldrich) adsorbed to aluminum hydroxide (Alum; Pierce) (100 μg/mouse i.p.). Serum was collected 14 and 21 days after that challenge. For the recall proliferation assays, T cells were restimulated with soluble OVA using the concentrations indicated and syngenic APCs in vitro. Cells from spleen and axial lymph nodes were incubated in 96-well plates at a concentration of 5×10^5/well. After 3 days, [^{3}H]thymidine (1 μCi/well) was added for an additional 16 h. Cells were harvested on filters and the incorporation of [^{3}H]thymidine was measured with a Matrix 96 direct beta counter system.

OVA-specific serum Abs

OVA-specific serum IgG and IgM concentrations were measured by using ELISA. Immunomax flat-bottom 96-well plates (Techno-Plastic Products) were coated overnight at 4°C with 1 mg ml^{-1} OVA. Wells were blocked with PBS containing 1% BSA at 37°C for 1 h, serum was added (diluted

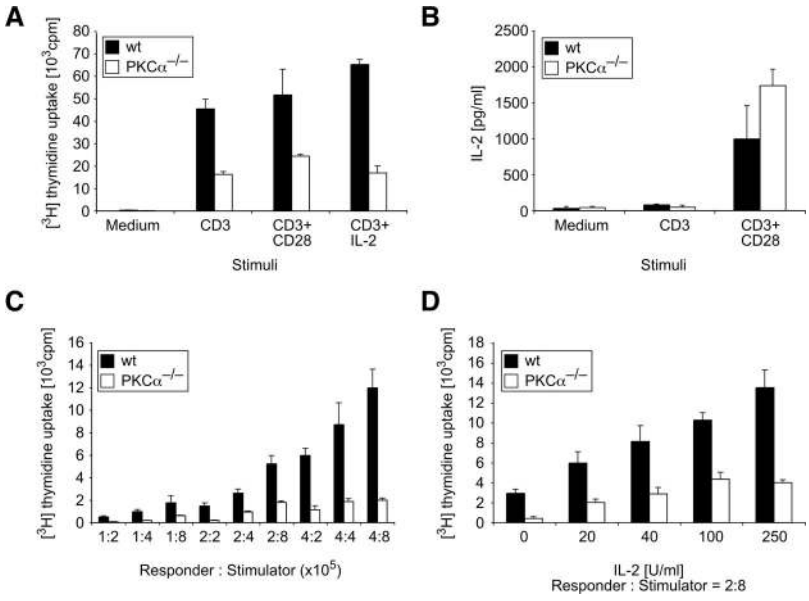

FIGURE 3. Impaired T cell activation in PKCα$^{-/-}$ mice. *A*, Proliferative responses of purified peripheral $CD3^+$ T cells of wt and PKCα$^{-/-}$ mice. After incubation with medium alone or various stimuli such as anti-CD3 (precoated), with or without soluble anti-CD28 or IL-2 (40 U ml^{-1}) for 48 h, cultures were pulsed for 16 h with 1 μCi [^{3}H]thymidine. T cells were subsequently harvested and analyzed using standard procedures. Results shown are the means ± SD of [^{3}H]thymidine incorporation ($n = 7$). *B*, IL-2 cytokine secretion responses of purified peripheral $CD3^+$ T cells treated with various stimuli as indicated. ELISA results shown are the means ± SD ($n = 3$). *C*, For the MLR assays, purified peripheral $CD3^+$ T cells of and PKCα$^{-/-}$ littermates were mixed in duplicates at various densities with mitomycin C-treated splenocytes from BALB/c mice as indicated. After 48 h of growth, the cells were pulsed for 16 h with 1 μCi [^{3}H]thymidine ($n = 11$). *D*, Addition of exogenous IL-2 (from 20 up to 250 U ml^{-1}) does not rescue the proliferative responses to allogenic MHC in PKCα$^{-/-}$ T cells. Results shown are the means ± SD of [^{3}H]thymidine incorporation ($n = 5$)

Table II. *CD25, CD44, and CD69 on wt and PKC$\alpha^{-/-}$ T cells[a]/surface expression*

	CD4$^+$					
	Unstimulated		CD3		CD3 + CD28	
	wt	$\alpha^{-/-}$	wt	$\alpha^{-/-}$	wt	$\alpha^{-/-}$
CD25$^+$	2.37 ± 0.33	3.59 ± 0.64	45.85 ± 1.47	44.94 ± 4.31	48.30 ± 0.45	49.91 ± 5.01
CD44$^+$	23.43 ± 3.62	22.70 ± 1.78	56.37 ± 3.63	51.94 ± 4.41	68.75 ± 2.28	60.61 ± 4.58
CD69$^+$	1.37 ± 0.11	1.28 ± 0.2	42.39 ± 3	43.61 ± 4.76	47.47 ± 0.57	46.75 ± 4.73
	CD8$^+$					
	Unstimulated		CD3		CD3 + CD28	
	wt	$\alpha^{-/-}$	wt	$\alpha^{-/-}$	wt	$\alpha^{-/-}$
CD44$^+$	41.96 ± 2.21	38.18 ± 2.22	82.11 ± 2.02	87.12 ± 4.85	82.40 ± 2.43	87.32 ± 3.75
CD69$^+$	1.08 ± 0.27	0.71 ± 0.12	42.48 ± 4.12	47.65 ± 8.26	45.08 ± 4.43	42.58 ± 6.86

[a] Single-cell suspensions of purified peripheral CD4$^+$ and CD8$^+$ T cells, stimulated or not for 16 h with anti-CD3 and anti-CD3 plus anti-CD28, were stained with anti-CD25, anti-CD69, and anti-CD44, as indicated. Percentages of positive cells ± SEM are indicated (n = 4).

in PBS with 1% BSA), and incubated for 4 h at 37°C. As positive control, anti-OVA (chicken, denatured; AntibodyShop) was used. Secondary Abs peroxidase-monoclonal rat anti-mouse IgG1, peroxidase-rat anti-mouse IgG2a, peroxidase-rat anti-mouse IgG2b, and peroxidase-monoclonal rat anti-mouse IgM (all from Zymed Laboratories) were incubated at 4°C overnight and subsequently peroxidase signaling was detected by the addition of ABTS (Fluka BioChemika).

Results

Generation of PKC$\alpha^{-/-}$ mice

To study the in vivo function of PKCα in mice, a null allele (the PKCα gene was disrupted by insertion of a neomycin resistance gene into exon 2) was generated as described elsewhere (15). Mice have been backcrossed to C57BL/6 to obtain heterozygous PKCα mice (+/−). The intercross of these mice produced homozygous PKCα-deficient (−/−) mutant mice, which were distinguishable by Southern blot (Fig. 1*A*) and/or PCR genotyping. The null mutation of PKCα in thymocytes and peripheral T lymphocytes derived from PKC$\alpha^{-/-}$ mice was confirmed by immunoblotting (Fig. 1, *B* and *C*). As controls, neither of the other CD3$^+$ T cell high-expressed PKC isotypes tested showed any up-regulated expression levels in T cells derived from PKC$\alpha^{-/-}$ mice (Fig. 1*C*).

To assess whether PKCα is activated by the established autophosphorylation on Thr^{250} (16) in intact T cells, the anti-(phospho)Thr^{250} antiserum reacted strongly with recombinant transfected PKCα in PDBu-stimulated Jurkat T cells but much less in unstimulated cells (Fig. 1*D*). Similar results have been obtained

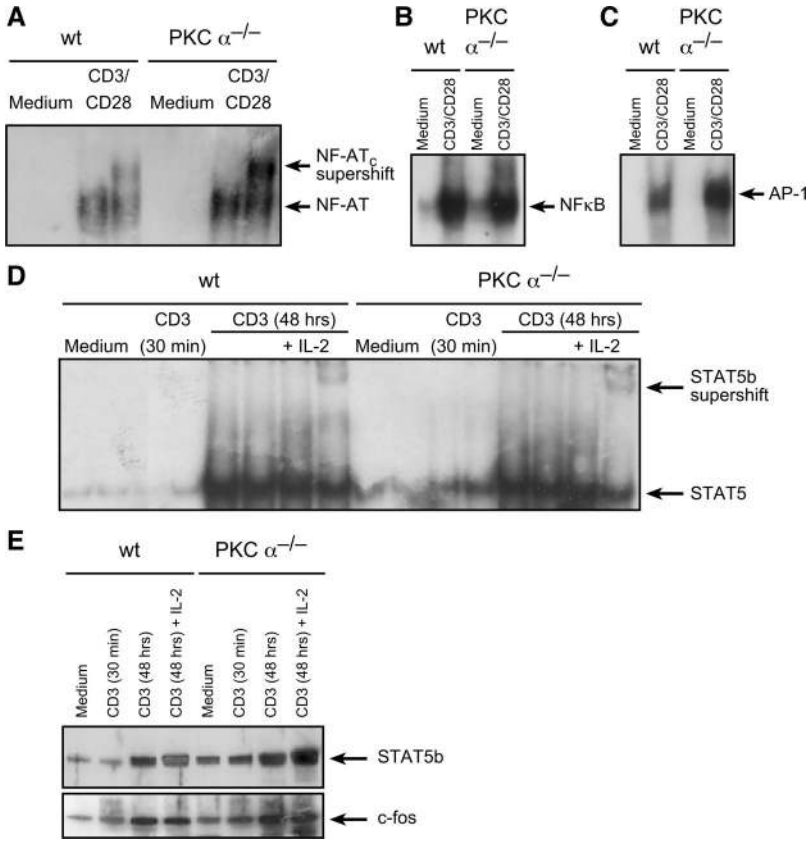

FIGURE 4. Analysis of NF-κB, AP-1, NF-AT, and STAT5 in PKC$\alpha^{-/-}$ T cells: nuclear extracts were prepared from purified peripheral CD3$^+$ T cells, stimulated for 16 h (*A–C*) with medium alone or plate-bound anti-CD3 plus soluble anti-CD28. Gel mobility shift assays were performed using labeled probes containing either NF-AT (*A*), NF-κB (*B*), and AP-1 binding site sequences (*C*). *D*, Normal induction of STAT5b in activated, IL-2-treated T cells. T cells were activated for 48 h with anti-CD3, rinsed, and recultured 30 min with medium alone or supplemented with IL-2 (40 U ml^{-1}). STAT5-binding activity in cells not treated with IL-2 was due to IL-2 release from activated cells, since this band was not present when using extracts from resting or short time (30 min)-stimulated cells. The specificity of STAT5b as well as NF-ATc was confirmed by supershifting the electrophoretic mobility shift with Abs as indicated by the arrow. Experiments were repeated at least three times with similar results. *E*, The normal activation of STAT5b was directly analyzed in nuclear cell extracts of resting cells and cells stimulated for 48 h with anti-CD3. Activated cells were rinsed and recultured for 30 min with medium alone or supplemented with IL-2 (40 U ml^{-1}). Nuclear levels of STAT5b and c-*fos* transcription factors were immunostained with the specific Abs as indicated. Experiments were repeated three times with similar results.

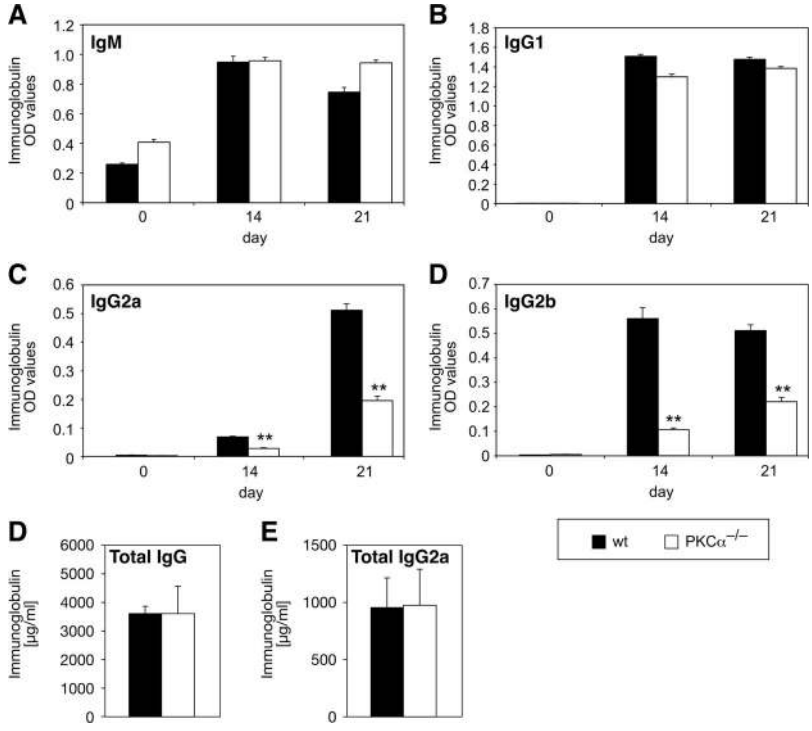

FIGURE 5. Impaired in vivo immune responses in PKC$\alpha^{-/-}$ mice to OVA. The wt and PKC$\alpha^{-/-}$ mice were immunized with OVA and serum anti-OVA IgM (*A*), IgG1 (*B*), and IgG2a (*C*), and IgG2b (*D*) titers were determined at the indicated times by ELISA on OVA-coated plates. Results were averaged by the number of mice in each group. Values are the means ± SD. Asterisks indicate the p value of <0.01 ($n = 9$). *D* and *E*, Normal total serum IgG and IgG2a levels in PKC$\alpha^{-/-}$ mice in vivo were observed ($n = 3$).

upon anti-CD3 ligation (data not shown). Thus, in intact T cells this Thr^{250} autophosphorylation site within the regulatory domain of PKCα was inducibly phosphorylated, indicating a cellular function of the PKCα isotype during T cell activation.

Unexpectedly, mice without PKCα, an ubiquitous enzyme with a wide functional spectrum (17), were born at the expected Mendelian frequency, were fertile, appeared healthy and anatomically normal, and lived a normal life span. T and B lymphocyte development was unaffected by the PKCα mutation (Table I): $CD4^+$/$CD8^+$ double-positive thymocytes were able to differentiate into normal total numbers of $CD4^+$ or $CD8^+$ T cells, which express normal levels of CD3. Moreover, the relative and total numbers of the hemogram as well as the peripheral $CD4^+$ and $CD8^+$ T cells in the lymph nodes and spleen were comparable between PKC$\alpha^{-/-}$ and littermates (Table I). Additionally, no significant difference in the susceptibility of thymocytes from adult PKC$\alpha^{-/-}$ mice to several ex vivo apoptotic stimuli was observed (Fig. 2). Consistently, no increased susceptibility to the ex vivo apoptotic stimuli, anti-CD3ε and Fas ligand, were reproducibly obtained in Con A- and IL-2-differentiated PKC$\alpha^{-/-}$ T cell blasts ($n = 8$; data not shown).

Impaired Ag receptor-induced proliferation of PKCα-deficient $CD3^+$ T lymphocytes

To determine whether PKCα influences lymphocyte function, we analyzed T cell responses. As a result, in vitro stimulation with anti-CD3ε mAb alone, or anti-CD3ε plus anti-CD28 mAbs, revealed a significant decrease in the proliferation rate of purified PKC$\alpha^{-/-}$ $CD3^+$ T cells compared with T cell controls (Fig. 3*A*). Externally added IL-2 (IL-2) failed to rescue proliferation in PKC$\alpha^{-/-}$ T cells.

To identify the molecular mechanism accounting for the functional defect in PKC$\alpha^{-/-}$ T cell proliferation, we analyzed signaling pathways downstream of TCR activation. Surprisingly, and despite the strong reduction of TCR-induced proliferation, CD3/CD28 ligation (with or without CD28 costimulation) induced normal IL-2 cytokine production comparable to the littermates (Fig. 3*B*). Cotreatment with PDBu/ionomycin also induced normal IL-2 responses in the PKC$\alpha^{-/-}$ T cells (data not shown). Flow cytometric analysis of the activation markers CD25, CD44, and CD69 on PKCα mutant $CD4^+$ and $CD8^+$ T cells again revealed no significant differences to littermate controls (Table II). This indicates that a signal-transducing pathway involving activation and translocation of other PKC isotype(s) and/or RasGRP family members may compensate for the loss-of-function of PKCα in these activation pathways.

Next, using the MLR assay, we evaluated as well as PKC$\alpha^{-/-}$ T cell proliferation rates induced by allogenic MHC expressed on BALB/c mice-derived splenocytes. As a result, a severe decrease in activation-induced proliferation was observed in PKCα-deficient T cells at all stimulation conditions (Fig. 3*C*). Addition of exogenous IL-2 again, did not efficiently rescue the proliferative defect in PKC$\alpha^{-/-}$ T cells to the allogenic MHC stimulus (Fig. 3*D*). Nevertheless, cotreatment with PDBu/ionomycin restored almost normal proliferative responses of the PKC$\alpha^{-/-}$ T cells, indicating that these cells had no general defect in cell cycle entry (data not shown).

Normal transactivation of the transcription factors NF-AT, NF-κB, AP-1, and STAT5

To further elucidate the molecular basis of the impairment in Ag receptor signaling in the absence of PKCα, we analyzed the NF-κB, AP-1, and NF-AT pathways, known to be PKC dependent (18) and critical in TCR/CD28-induced T cell activation (19, 20). EMSA demonstrated that substantial NF-κB, AP-1, and NF-AT DNA-binding activity was induced in $CD3^+$ peripheral T cells after anti-CD3/anti-CD28 stimulation. Consistent with the normal activation-induced IL-2 cytokine secretion and surface expression of CD25 and CD69 (see Table II), CD3/CD28-mediated transactivation of the transcription factors NF-AT (Fig. 4*A*), NF-κB (Fig. 4*B*), and AP-1 (Fig. *4C*) in PKCα-deficient T cells was not reduced. In contrast, NF-AT and AP-1 transactivation was found to be reproducibly enhanced in the PKC$\alpha^{-/-}$ T cells (Fig. 4, *A* and *C*). Moreover, induction of STAT5 DNA binding as well as

STAT5b nuclear translocation by IL-2 was not affected in cultures of PKC$\alpha^{-/-}$ T cells prestimulated with anti-CD3ε for 48 h (Fig. 4, *D* and *E*). Thus, PKCα is also dispensable in coupling the IL-2 high-affinity cytokine receptor signaling to STAT5 activation in T cells.

PKC$\alpha^{-/-}$ mice show diminished Ag-specific IgG2a/IgG2b responses in vivo

To investigate whether the requirement for PKCα in T cell proliferation in vitro translates into a defective immune response in vivo, we challenged wt and PKC$\alpha^{-/-}$ mice with the protein Ag chicken egg albumin (OVA) adsorbed to aluminum hydroxide. To first examine the humoral immune response, OVA-specific serum IgG concentrations were measured 14 and 21 days after the OVA challenge. Whereas littermates produced high titers of OVA-specific IgM and IgG1, IgG2a, and IgG2b Abs (Fig. 5), the IgG2a and IgG2b response in PKC$\alpha^{-/-}$ mice was selectively impaired (Fig. 5, *C* and *D*). In contrast, OVA-specific IgM and IgG1 responses and total IgG and IgG2a serum levels in PKC$\alpha^{-/-}$ mice were unimpaired (Fig. 5, *A* and *B*, *D* and *E*). This result indicated that PKCα function is selectively required for H chain class switching of Ig IgM to IgG2a and IgG2b.

Responsiveness to an in vivo challenge with the soluble protein Ag OVA in mice was next examined by an in vitro recall proliferation assay of isolated CD3$^+$ T cells. This memory response of splenic T cells to the secondary OVA challenge is shown in Fig. 6*A*. As expected, T cells derived from mice responded to OVA in a dose-dependent manner. However, T cell proliferation in PKC$\alpha^{-/-}$ mutant mice was markedly reduced. These results are consistent with the requirement of PKCα not only in naive but also in memory T cell responses.

Because of the established role of T cell-derived IFN-γ in IgG2a class switching (21), we compared IFN-γ production of CD3$^+$ T cells in in vitro cultures. Despite the normal IL-2 responses, CD3 ligation (with or without CD28 costimulation) of PKC$\alpha^{-/-}$ T cells resulted in reduced IFN-γ production in in vitro culture, when compared with controls (Fig. 6*B*). Next, using the MLR assay, we evaluated as well as PKC$\alpha^{-/-}$ T cytokine production induced by allogenic MHC expressed on BALB/c mice-derived splenocytes. Consistent with the results above, PKC$\alpha^{-/-}$ T cells again demonstrated a severe decrease in allogenic-MHC-induced IFN-γ production in in vitro culture, when compared with controls (Fig. 6*C*). Because of the cytokine-selective defect observed for IFN-γ but not for IL-2 secretion, simple inhibitory effects on the translation and/or secretion machineries in PKCα-deficient T cells are unlikely. Consistent with a transcriptional defect, mRNA levels of IFN-γ transcripts were reduced in stimulated PKC$\alpha^{-/-}$ T cells, when compared with controls (data not shown).

In contrast to the CD3$^+$ T cells (Fig. 3*A* above), PKCα-deficient splenic B cells responded efficiently to an anti-IgM and IL-4 stimulation (Fig. 6*D*). This normal in vitro B cell proliferation is paralleled by normal total serum IgG in PKC$\alpha^{-/-}$ mice in vivo (Fig. 5, *D* and *E* above).

To test whether PKC$\alpha^{-/-}$ CD4$^+$ and CD8$^+$ subsets are differently affected in T cell activation, purified peripheral CD4$^+$ or CD8$^+$ T cells of and PKC$\alpha^{-/-}$ mice were analyzed for proliferation, IFN-γ, and IL-2 cytokine secretion responses to allogenic MHC, respectively (Fig. 7). Loss of PKCα selectively impaired IFN-γ (but not IL-2) production in both subsets (Fig. 7, *C–F*), whereas proliferation appeared more profoundly inhibited in the CD8$^+$ T cells due to significantly stronger stimulation indices.

Discussion

The physiological PKC isotype functions are a field of intensive studies, although several critical issues in T lymphocytes remain unanswered or ill-defined. Although PKCθ is firmly established to play a key role in TCR signaling leading to IL-2 production but also to immune response controlled by TH2 cells (5), other PKC isotype-specific roles of T cell-expressed PKC family members have not been resolved in a physiological setting. In this study, using for the first time PKCα KO mice, we investigated the potential of PKCα in primary T cell activation.

As result, T cell proliferative responses were strongly reduced in PKC$\alpha^{-/-}$ CD3$^+$ T cells (Fig. 3, *A* and *C*). Nevertheless, the marked decrease in proliferation, observed in PKCα-deficient T cells is neither accompanied by a significant reduction in the level of secreted IL-2 (Fig. 3*B*) nor surface expression of CD25, CD44, and CD69 activation markers (Table II). PKC$\alpha^{-/-}$ thymocytes and CD3$^+$ T cells are only defective in cell cycle progression but do not have an enhanced cell death (Fig. 2 and data not shown).

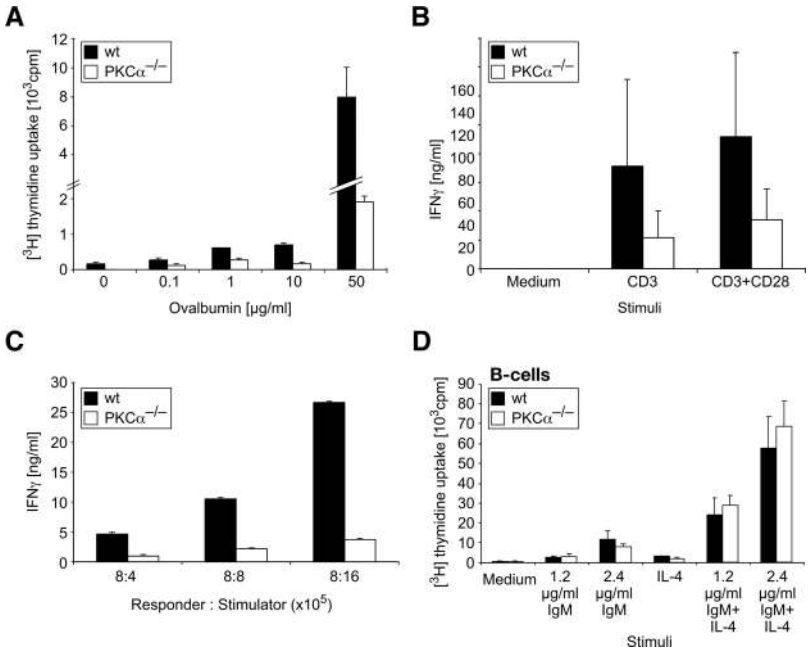

FIGURE 6. PKCα is critical for T cell activation in vivo. *A*, Impaired T cell-dependent response to Ag in PKC$\alpha^{-/-}$ mice. OVA was injected i.p. After 3 wk, splenocytes were restimulated with OVA in vitro and T cell proliferative responses were measured. Results were averaged for four mice in each group ($n = 5$). *B* and *C*, Selective requirement of PKCα for IFN-γ production. CD3$^+$ T cells were stimulated for 48 h with CD3 and CD3/CD28 cross-linking or allogenic MHC, derived from BALB/c splenocytes, as indicated. IFN-γ was determined from collected supernatants by ELISA. *D*, PKCα is not essential for purified splenic B cell proliferation. The wt and PKC$\alpha^{-/-}$ B cells were activated with the indicated stimuli and proliferation was determined 48 h later by [^{3}H]thymidine incorporation ($n = 4$).

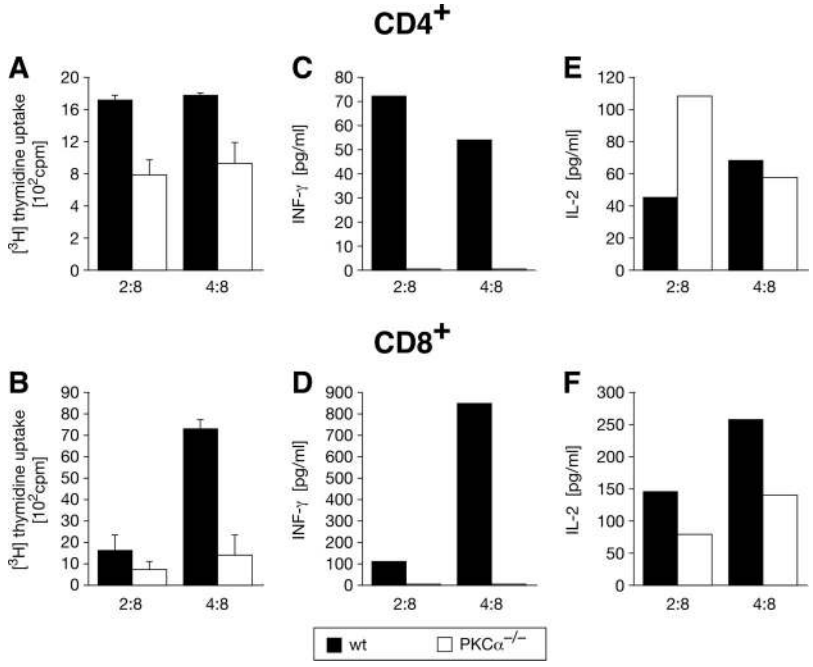

FIGURE 7. Impaired T cell activation to allogenic MHC in PKC$\alpha^{-/-}$-derived CD4$^+$ and CD8$^+$ subsets. Purified peripheral CD4$^+$ or CD8$^+$ T cells of wt and PKC$\alpha^{-/-}$ mice were mixed in duplicates at 2:8 and 4:8 densities with mitomycin C-treated splenocytes from BALB/c mice as indicated. After incubation for 48 h, cultures were analyzed for [^{3}H]thymidine incorporation (*A* and *B*), IFN-γ (*C* and *D*), and IL-2 cytokine secretion (*E* and *F*) responses, respectively. Results shown are the means ± SD of two independent experiments.

Consistently, treatment with CD3/CD28 demonstrated substantial NF-κB, AP-1, and NF-AT DNA-binding activity in purified peripheral PKC$\alpha^{-/-}$ CD3$^+$ T cells, which was comparable to the situation for NF-κB and reproducibly enhanced for NF-AT and AP-1 (Fig. 4, *A–C*). The addition of exogenous IL-2 to the medium (even up to a concentration of 250 U ml^{-1}, known to activate also the low-affinity IL-2R) does not restore the proliferative response of the mutant T cells (Fig. 3*D*). Inhibition of IL-2-dependent proliferation in T cells suggests a potential interference with cytokine receptor signaling. Nevertheless, STAT5 activation downstream of the IL-2 high-affinity receptor is unimpaired in PKC$\alpha^{-/-}$ T cells, as determined by EMSA as well as nuclear translocation experiments of STAT5b in CD3$^+$ T cells (Fig. 4, *D* and *E*).

In contrast to the PKC$\alpha^{-/-}$ T cells, splenic PKC$\alpha^{-/-}$ B cell proliferation in vitro was found to be comparable to controls (Fig. 6*D*). Thus, PKC$\alpha^{-/-}$ cells appear to have a T cell lineage-specific defect in cell cycle progression downstream of IL-2 high-affinity receptor function. Selective knockdown studies of PKCα (via antisense oligonucleotides, RNA interference as well as pharmacological inhibitors), and biochemical studies using PKC isotype-specific cDNA (wt, constitutively active, and dominant negative), already strongly implicated a role of PKCα in the control of cell cycle machinery. Importantly, the biological responses obtained by the manipulation of PKCα were found to be cell-type specific (17); it promoted proliferation in some types of cells (human breast cancer cells, neonatal cardiomyocytes (22), and mediated cell cycle arrest in other cell types (epithelial cells) by inducing cyclin-dependent kinase inhibitors, p21$^{Waf1/Cip1}$ (23) and p27^{Kip1} (24). The distinct responses are modulated by dynamic interactions with cell-type-specific anchoring proteins such as the receptor for activated PKC, vinculin, talin, β_1 integrin, and caveolin. Thus, the observed T but not B cell lineage-specific cell cycle progression defect in the PKC$\alpha^{-/-}$ T cells can be explained along this line of published findings. Consistently, loss of PKCα resulted in a severe block of memory recall proliferation in in vitro cultures, when restimulated with soluble Ag and syngenic APCs (Fig. 6*A*).

When studying the role of PKCα in the priming of T cells in vivo using the T cell-dependent Ag OVA, a significant suppression of OVA-specific IgG2a and IgG2b production, the two complement-fixing IgG subclasses, was observed (Fig. 5, *C* and *D*). Two pathways for the genetic switch from IgM to IgG production are known (21). One pathway requires the TH1-type cytokine IFN-γ to stimulate IgM-secreting cells to switch to IgG2a-secreting cells. Another pathway requires the TH2-type cytokines IL-4 and IL-6 to stimulate IgM-secreting cells to switch to IgG1-secreting cells. When the IgG1 and IgG2a levels were measured, these two pathways were differentially affected in the PKC$\alpha^{-/-}$ mice (Fig. 5, *B* and *C*). The IgG1 responses were normal and only the IgG2a plus IgG2b levels were decreased following OVA administration in PKC$\alpha^{-/-}$ mice. Furthermore, this decrease in IgG2a was accompanied by decreased IFN-γ production of isolated CD3$^+$ T cells in vitro (Fig. 6, *B* and *C*). Consistently, in parallel assays, IL-4 production was not significantly affected in PKC$\alpha^{-/-}$ T cells (data not shown). Thus, these data indicate that loss of PKCα alters the ability to switch from IgM to the IgG2a and IgG2b production, possibly by reducing IFN-γ secretion of CD^{3+} T cells.

Along this line, splenic PKC$\alpha^{-/-}$ B cell proliferation in vitro was found to be comparable to that of controls (Fig. 6*D*). Comparable total serum IgG levels (Fig. 5, *D* and *E*) and, more importantly, normal OVA-specific IgM and IgG1 responses (Fig. 5, *A* and *B*) in PKC$\alpha^{-/-}$ mice were detected. Thus, PKC$\alpha^{-/-}$ mice appear not to alter the initial generation of the Ab response but rather to inhibit Ab class switching from IgM to the Ig isotypes, IgG2a and IgG2b. Because this appears to reflect a mostly T cell-intrinsic defect, PKCα, both in vitro and in vivo, is a critical and T lineage-specific positive regulator and the first PKC isotype to be identified that couples the T cell Ag receptor signaling to lymphocyte activation and function downstream of IL-2 cytokine/IL-2 receptor function.

Taken together, loss of PKCα has no apparent effect on T cell development and selection in the thymus. Instead, PKCα is required for the activation and in vivo function of mature CD3$^+$ T cells, such as recall Ag-induced T cell growth, IFN-γ production, and support for class switching to IgG2a and IgG2b to OVA Ag challenge in vivo. Functionally, these data indicate that, next to PKCθ, also PKCα is part of a signaling pathway that is necessary for full Ag receptor-mediated T cell activation and T lymphocyte

immunity. How the loss of PKCα mechanistically results in defective cell cycle progression and IFN-γ production, however, remains to be resolved.

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Disclosures

The authors have no financial conflict of interest.

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