Control of T helper 2 cell function and allergic airway inflammation by PKCζ

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Asthma is a disease of chronic airway inflammation in which T helper (Th) 2 cells play a critical role. The molecular mechanisms controlling Th2 differentiation and function are of paramount importance in biology and immunology. PKCζ has been implicated in the regulation of apoptosis and NF-κB, as well as in the control of T-dependent responses, although no defects were detected in naïve T cells from PKCζ$^{-/-}$ mice. Here, we report that PKCζ is critical for IL-4 signaling and Th2 differentiation. Thus, PKCζ levels are increased during Th2 differentiation, but not Th1 differentiation, of CD4$^+$ T cells, and the loss of PKCζ impairs the secretion of Th2 cytokines *in vitro* and *in vivo*, as well as the nuclear translocation and tyrosine phosphorylation of Stat6 and Jak1 activation, essential downstream targets of IL-4 signaling. Moreover, PKCζ$^{-/-}$ mice display dramatic inhibition of ovalbumin-induced allergic airway disease, strongly suggesting that PKCζ can be a therapeutic target in asthma.

apoptosis | asthma | NF-κB

Asthma is a chronic lung inflammatory disease with increased prevalence in developed countries. The pathology of asthma is associated with aberrant activation of CD4$^+$ lymphocytes differentiated along the T helper (Th) 2 lineage (1). Naïve CD4$^+$ Th cells can differentiate in response to antigen stimulation into two distinct subsets of effector cells, Th1 and Th2, which display distinct cytokine profiles and immune regulatory functions (2). Th1 cells mainly produce IFN-γ and IL-2 and are essential for cell-mediated immune responses against intracellular pathogens. Th2 cells produce a different set of cytokines, including IL-4, IL-5, IL-10, and IL-13, and are important in the control of humoral immunity and allergy (3). The signaling pathways controlling Th2 differentiation and function have been the focus of intense research because they could help to identify therapeutic targets for asthma and other allergic pathologies. IL-4 is important for induction and maintenance of differentiated Th2 cells and for B cell Ig isotype switching to IgE in mice (4). IL-4 and IL-13 share interactions with the IL-4Rα chain and activate the transcription factor Stat6 through a Jak1/Jak3 signaling pathway (3, 5).

The role of the different PKC isoforms in lymphocyte activation and differentiation is a matter of great interest. The recent generation of mutant mice in which different PKC isotypes have been genetically inactivated reveals the selective involvement of each PKC isoform in cell-specific aspects of the immune response (6). The characterization of knockout mice for the diacylglycerol-insensitive atypical PKCζ isoform reveals an important role of this kinase in the immune system (7). Thus, PKCζ$^{-/-}$ adult mice are unable to mount an optimal immune response (8), suggesting alterations in lymphocyte function. Although the humoral response to a T-independent antigen was reduced in the PKCζ$^{-/-}$ mice, the major defects were found in mice challenged with a T-dependent antigen, specifically, in the levels of IgG1, IgG2a, and IgG2b (8). Also, basal IgE levels were dramatically reduced in PKCζ$^{-/-}$ mice compared with WT controls (8), indicating that some kind of T cell alteration, possibly in the Th2 lineage, might be produced by the loss of PKCζ. Surprisingly, although the ability of B cells to proliferate in response to B cell receptor challenge was reproducibly impaired in the PKCζ-deficient mice, no major alterations were observed in the proliferation of naïve T cells (8). However, the potential role of PKCζ in Th2 function and asthma had not been addressed previously.

Materials and Methods

Mice. PKCζ$^{-/-}$ and WT mice (SV129J background) are described in ref. 7. Mice aged between 6 and 8 weeks were used for the *in vitro* experiments. Age- and sex-matched 10- to 12-week-old mice were used for the *in vivo* asthma model.

Antibodies and Reagents. Antibodies to murine CD3ε (145-2C11) and CD28 (37.51) and biotinylated CD8α (53-6.7), CD11b (Mac-1), CD16 (2.4G2), CD19 (1D3), CD24 (M1/69), CD62L (MEL-14), CD117 (2B8), B220 (RA3-6B2), and CD4-FITC (L3T4) as well as IL-4-phycoerythrin (PE) and CD25-PE (PC61) were from Pharmingen. Antibodies to Stat6 (S-20), phospho-extracellular signal-regulated kinase (ERK) (E-4), ERK1 (K-23), phospholipase C-γ (Y783), GATA3 (HG3–31), c-Maf (M-153), actin (I-19), T-bet (4B10), proliferating cell nuclear antigen (FL-261), and nuclear factor of activated T cells (NFAT) c1 (7A6) were from Santa Cruz Biotechnology. Stat5, Jak1, phospho-Stat6 (Tyr-641), phospho-Stat5 (Y694), and phospho-Jak1 (Tyr-1022 and Tyr-1023) antibodies were from Cell Signaling Technology (Beverly, MA). Recombinant murine IL-2, IL-12, and IL-4 as well as anti-IFN-γ, anti-IL-5, anti-IL-4Rα, and anti-IL-4 antibodies were from R & D Systems. IFN-γ, IL-4, IL-5, and IL-10 ELISA kits were from Pharmingen, and the IL-13 ELISA kit was from R & D Systems. Specific polyclonal anti-PKCζ antibody was generated against the sequence encompassing amino acids 185–244 of PKCζ.

CD4$^+$ T Cell Isolation and Differentiation. To obtain naïve CD4$^+$ T cells, single cell suspensions were prepared from spleens and mesenteric lymph nodes of the indicated mice and were incubated with biotinylated antibodies directed at CD8, CD16, CD19, CD24, CD117, major histocompatibility complex class II (I-A^b), and CD11b followed by incubation with anti-biotin-conjugated microbeads. Naïve CD4$^+$ T cells were negatively selected in an autoMACS separator (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. Purified CD4$^+$ T cells were labeled with antibodies specific for CD4, CD25, CD62L, and B220 and analyzed by flow cytometry to

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Abbreviations: Th, T helper; OVA, ovalbumin; BAL, bronchoalveolar lavage; ERK, extracellular signal-regulated kinase; NFAT, nuclear factor of activated T cells; TCR, T cell receptor; H&E, hematoxylin/eosin.

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confirm purity and the naïve status (Fig. 6, which is published as supporting information on the PNAS web site). Naïve $CD4^+$ T cells (10^6 cells per ml) were differentiated in the presence of irradiated antigen-presenting cells (10^6 cells per ml), immobilized anti-CD3 (1 μg/ml), and IL-2 (10 ng/ml). For Th0 cultures, anti IL-4 (4 μg/ml) and anti-IFN-γ (4 μg/ml) were added. For Th1 differentiation, IL-12 (3.5 ng/ml) and anti-IL-4 (4 μg/ml) were added to the culture, whereas IL-4 (4 ng/ml) and anti-IFN-γ (4 μg/ml) were added for Th2 differentiation. After 4 days, the cells were extensively washed, counted, and restimulated as described. No differences in viability were observed in WT and knockout naïve Th0, Th1, or Th2 cells.

FACS Analysis and Intracellular Staining. GATA3 protein levels were assessed by intracellular staining in cells fixed overnight with 70% ethanol at −20°C and stained with anti-GATA3 or control mouse IgG1 followed by FITC-anti-mouse IgG1. To determine intracellular IL-4 levels, brefeldin A (BD GolgiPlug, BD Biosciences) was added 4 h before harvest to Th2 polarized WT and PKC$\zeta^{-/-}$ cells that had been restimulated for 48 h with anti-CD3 in the absence or presence of anti-CD28. Afterward, cells were fixed and permeabilized by using the BD Cytofix/Cytoperm kit (BD Biosciences) and stained with anti-IL-4-phycoerythrin. Analysis was performed in a FACSCalibur cell sorter (BD Biosciences) with CELLQUESTPRO software.

PKCζ Kinase Assay. Cell extracts prepared in lysis buffer (50 mM Tris·HCl, pH 7.5/150 mM NaCl/1 mM EGTA/2 mM EDTA/1% Triton X-100) were immunoprecipitated with the specific anti-PKCζ antibody that does not crossreact with PKCλ/ι for 2 h at 4°C. Immunoprecipitates were captured with protein A and washed extensively in lysis buffer with 0.5 M NaCl. The enzymatic assay was carried out in the immunoprecipitates in assay buffer [35 mM Tris·HCl, pH 7.5/10 mM $MgCl_2$/100 μM $CaCl_2$/0.5 mM EGTA/100 μM ATP/5 μCi (1 Ci = 37 GBq) of [^{32}P]ATP] with 4 μg of myelin basic protein as substrate for 1 h at 30°C.

Cytokine Assays. Naïve $CD4^+$ T cells were stimulated for 72 h with anti-CD3 (10 μg/ml) plus anti-CD28 (5 μg/ml), and Th2-differentiated cells were restimulated for 24 h with anti-CD3 (10 μg/ml), after which, supernatants were collected and cytokine concentrations were measured by ELISA using commercially available kits. ELISA was also used to determine the levels of Th2 cytokines in bronchoalveolar lavage (BAL) fluids.

Immunofluorescent Analysis. Th2 cells (2×10^5) were applied to glass slides by cytocentrifugation. Cells were fixed, permeabilized with 0.1% Triton X-100, and incubated with the different antibodies for 1 h at 37°C and the tetramethylfluorescein tyramide TSA-Direct amplification system (NEN). For the nuclear staining, cells were incubated with TO-PRO. Glass coverslips were mounted on Mowiol and examined with an MRC 1024 confocal system (Bio-Rad) mounted on an Axiovert 135 microscope (Zeiss).

Ovalbumin (OVA)-Induced Allergic Airway Disease. PKC$\zeta^{-/-}$ and WT mice (10–12 weeks old) were immunized as described in ref. 9. Briefly, on day 0, 15 μg of OVA (Sigma) in 200 μl of alum (Pierce) were injected i.p. into sensitized mice. On day 5, the animals received another i.p. injection of 15 μg of OVA in 200 μg of alum and, on day 12, mice were challenged with aerosolized 0.5% OVA in PBS (two challenges of 60 min each that were 4 h apart). Control animals were aerosolized with PBS. On day 14, 40 h after the second OVA challenge, mice were killed for analysis. For histological analysis, lungs from PBS- or OVA-treated mice were inflated through the trachea with 50% Jung tissue-freezing medium (Leica, Vienna) in PBS. The right caudal lobe was trimmed, embedded, frozen, and stored at −80°C. For the adoptive transfer experiments, WT and PKC$\zeta^{-/-}$ mice were i.p. injected with 100 μg of OVA in 2 mg of alum, and, 5 days later, spleens and mesenteric lymph nodes were removed for $CD4^+$ T cell isolation and were cultured for Th2 differentiation for 4 days with irradiated antigen-presenting cells, OVA (50 μg/ml), IL-2 (10 ng/ml), IL-4 (10 ng/ml), and anti-IFN-γ (10 μg/ml). Afterward, Th2-like cells were collected, washed, and i.v. injected into WT and PKC$\zeta^{-/-}$ recipient mice, which, 24 h later, were exposed to inhaled 1% OVA in PBS for a total of 7 days (4 consecutive days exposed, 2 days rested, and 3 consecutive days exposed) for 20 min daily. Mice were killed 24 h after the final exposure to antigen, and histological analysis of lungs was performed. The appropriate institutional animal use and care committees approved all animal experiments.

Statistical Analysis. Statistical analyses were performed by using Student's t test. $P < 0.05$ was considered to be significant.

Results

Impaired Th2 Differentiation in PKC$\zeta^{-/-}$ Mice. To determine whether PKCζ may play a role in lineage commitment of $CD4^+$ T helper cells, we initially differentiated $CD4^+$ T cells, either WT or PKC$\zeta^{-/-}$, *in vitro* under Th1 or Th2 polarizing conditions, after which, cells were stimulated with anti-CD3 antibody for 48 h, and the secretion of IFN-γ and IL-4 was determined in the Th1 and Th2 cultures, respectively. Results in Fig. 1*a* show that whereas IFN-γ secretion is not affected, IL-4 is significantly reduced in PKC$\zeta^{-/-}$ cells (Fig. 1*a*). The synthesis of three other Th2 cytokines, IL-5, IL-10, and IL-13, was also dramatically inhibited in PKC$\zeta^{-/-}$ Th2 cells (Fig. 1*a*). When the levels of intracellular IL-4 were determined in the presence of brefeldin A by FACS analysis, it was clear that WT Th2 cells produce significantly more IL-4 than do PKC$\zeta^{-/-}$ Th2 cells (Fig. 1*b*), consistent with the ELISA data in Fig. 1*a*. Together, these results suggest that PKCζ plays a nonredundant role in Th2-polarized $CD4^+$ T cells.

GATA3 expression is a widely established hallmark of the Th2 polarization process (10). Therefore, we initially analyzed by using flow cytometry the levels of this transcription factor in $CD4^+$ T cells incubated under Th0, Th1, and Th2 polarizing conditions. Interestingly, the loss of PKCζ significantly reduces GATA3 expression in Th2 cells (Fig. 2*a*), strongly suggesting that PKCζ plays an important role during Th2 differentiation. Consistent with this notion, the levels of GATA3, as well as the levels of Stat6, c-Maf, RelA, and NFATc1, were dramatically reduced in PKCζ-deficient Th2 cells as determined by immunoblotting (Fig. 2*b*). Interestingly, when Th2-polarized T cells were rechallenged with anti-CD3 plus anti-CD28, the nuclear levels of all of the transcription factors tested were likewise inhibited in the PKC$\zeta^{-/-}$ cells (Fig. 2*c*). Therefore, it seems that PKCζ is required for the Th2 differentiation process to properly occur and for Th2-polarized cells to respond to an anti-CD3 rechallenge.

Signaling Cascades Inhibited in PKC$\zeta^{-/-}$ T Cells Induced to Differentiate Along the Th2 Pathway. Because PKCζ appears to be important for Th2 polarization, we next sought to determine whether this kinase is induced during this important differentiation process. First, we generated a polyclonal antibody that selectively recognizes PKCζ and that does not crossreact with PKCλ/ι. The result in Fig. 3*a* shows the specificity of this antibody in immunoblot analysis of lung extracts, a tissue particularly rich in PKCζ (7), from WT and PKC$\zeta^{-/-}$ mice. Immunoblotting with an anti-PKCλ/ι-specific antibody of the same extracts reveals that the levels of this aPKC isoform were not affected by the loss of PKCζ (Fig. 3*a*). Therefore, we next prepared extracts from $CD4^+$ T cells incubated under Th0, Th1, or Th2 conditions, and the expression of PKCζ was determined by immunoblotting with our selective antibody. The data in Fig. 3*b* demonstrate that PKCζ levels are increased under Th2 polarizing conditions, but not under Th1 polarizing conditions.

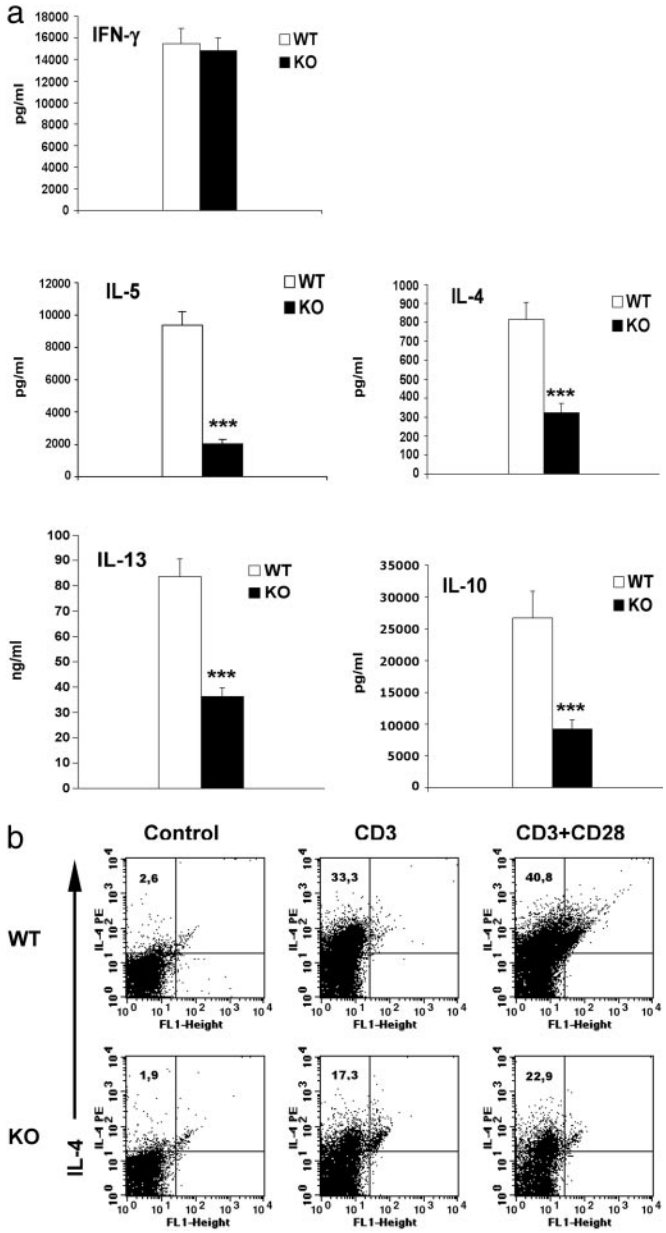

Fig. 1. Role of PKCζ in Th2 differentiation and cytokine synthesis. (*a*) Secretion of IFN-γ in Th1 cells or IL-5, IL-4, IL-13, and IL-10 in Th2 cells in WT or PKC$\zeta^{-/-}$ (KO) mice stimulated with anti-CD3 was determined by ELISA. (*b*) IL-4 intracellular staining of Th2 cells in WT or PKC$\zeta^{-/-}$ (KO) mice either untreated or treated with anti-CD3 alone or in combination with anti-CD28. Inset numbers represent the percentage of IL-4-positive cells. The results in *a* are the mean $\pm$ SD of three independent experiments with incubations in triplicate. The results in *b* are representative of another two experiments. ***, $P < 0.001$.

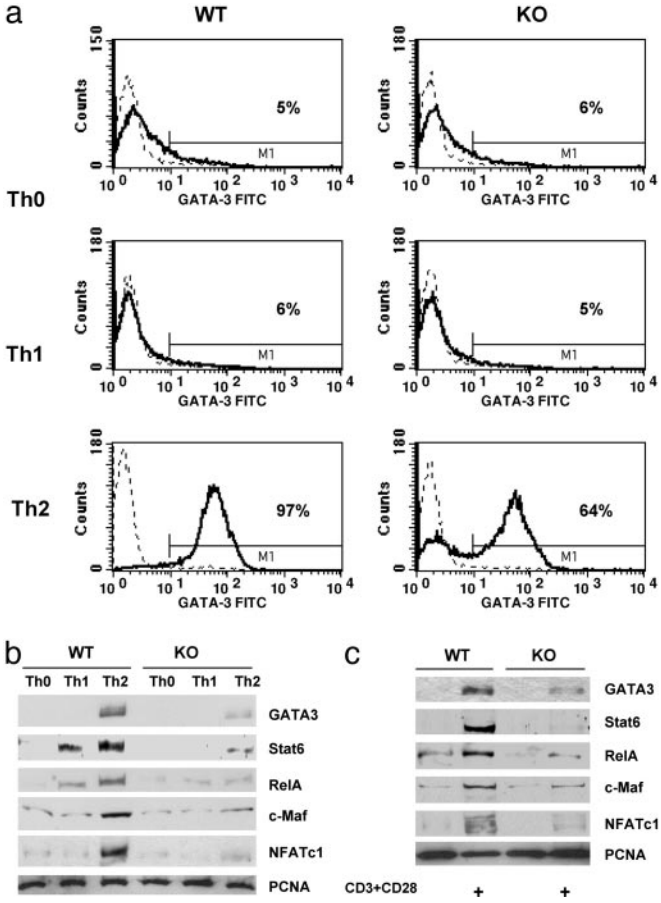

Fig. 2. Th2 transcription factors in PKC$\zeta^{-/-}$ cells. (*a*) Intracellular staining of GATA3 in Th0, Th1, and Th2 cells in WT or PKC$\zeta^{-/-}$ (KO) mice. The solid line represents GATA3 staining; the dashed line corresponds to the mouse IgG1 isotype control. Inset numbers represent the percentage of GATA3-positive cells. (*b* and *c*) Western blot analysis of nuclear levels of GATA3, Stat6, RelA, c-Maf, and NFATc1 in cell cultures as above (*b*) or in Th2 cells stimulated with anti-CD3 plus anti-CD28 (*c*). Anti-proliferating cell nuclear antigen was used as a loading control in *b* and *c*. The results are representative of three independent experiments.

Immunoblotting with anti-GATA3 and anti-T-bet demonstrated that cells were properly polarized to the Th2 and Th1 lineages, respectively (Fig. 3*b*). To determine whether this induced PKCζ is enzymatically active, we immunoprecipitated this kinase from polarized CD4$^+$ T cell extracts, and the enzymatic activity was determined in a standard kinase assay. The data in Fig. 3*b* show that the activity of PKCζ is pronouncedly induced in Th2 cells.

Based on these observations and the impairment of Th2 differentiation and function of PKC$\zeta^{-/-}$ T cells, we considered it important to determine whether PKCζ is activated in Th2 cells upon T cell receptor (TCR) activation. Thus, CD4$^+$ T cells polarized under Th2 conditions were incubated with anti-CD3, and, afterward, PKCζ enzymatic activity was determined as above. The incubation with anti-CD3 triggers a dramatic increase in PKCζ activity (Fig. 3*c*) without appreciable changes in the levels of the enzyme (Fig. 3*c*). Collectively, these results indicate that PKCζ is activated when Th2-polarized CD4$^+$ T cells are rechallenged with anti-CD3. Th differentiation is modulated by signals emanating from the TCR and the cytokines generated during this polarization process, particularly IL-4, which is present in the culture medium of CD4$^+$ T cells induced to differentiate to the Th2 lineage. Importantly, IL-4 is synthesized by Th2 cells when rechallenged with an anti-CD3 antibody in the absence of any exogenous cytokine, which exerts a positive feedback activation loop (11). To determine whether PKCζ activation in CD3-triggered CD4$^+$ T cells polarized under Th2 conditions is a direct effect of TCR signaling or whether it is mediated by the secreted IL-4, WT CD4$^+$ Th2 cells activated with anti-CD3 were incubated in the presence of either neutralizing anti-IL-4 or anti-IL-5 antibodies, after which, PKCζ activity was determined as above. According to the results of Fig. 3*c*, PKCζ activation by anti-CD3 is dramatically inhibited by the presence of anti-IL-4 but not by the presence of anti-IL-5 (Fig. 3*c*). These results indicate that IL-4 is responsible for the activation of PKCζ in CD3-restimulated Th2 cells and that PKCζ could be part of the IL-4

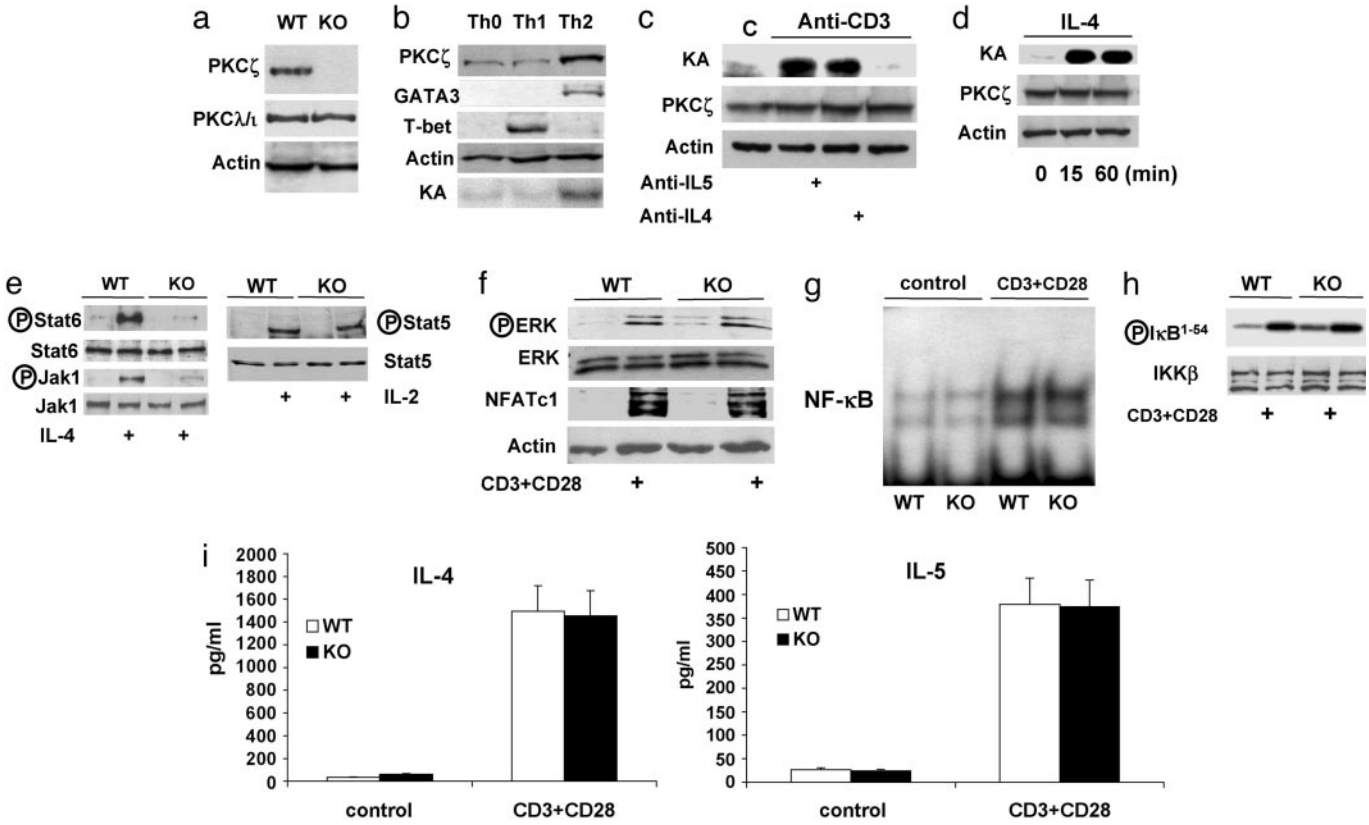

Fig. 3. Biochemical signaling pathways in PKC$\zeta^{-/-}$ cells. (*a*) Western blot analysis of PKCζ and PKCλ/ι in lung from WT and PKC$\zeta^{-/-}$ (KO) mice. (*b*) Extracts from CD4$^+$ T cells incubated under Th0, Th1, or Th2 polarizing conditions were analyzed by immunoblotting for PKCζ, GATA3, and T-bet expression and by PKCζ kinase activity (KA). (*c*) WT Th2-polarized T cells were incubated with or without anti-CD3 in the presence or absence of neutralizing anti-IL-4 or anti-IL-5 antibodies, and PKCζ kinase activity (KA) and PKCζ levels were determined. (*d*) PKCζ kinase activity (KA) and PKCζ levels were measured in Th2-polarized cells stimulated with IL-4 for different times. (*e*) Naïve CD4$^+$ T cells were stimulated or not stimulated with IL-4 or IL-2 for 10 min; afterward, phospho-Stat6, phospho-Jak1, and phospho-Stat5 levels, as well as levels of Stat6, Jak1, and Stat5, were determined by immunoblotting. (*f*) Western blot analysis of phospho-ERK, ERK, and NFATc1 in naïve CD4$^+$ T cells stimulated with anti-CD3 plus anti-CD28. (*g*) NF-κB activation was analyzed by EMSA in nuclear extracts prepared from naïve CD4$^+$ T cells stimulated with anti-CD3 plus anti-CD28. (*h*) IκB kinase activity was determined in the above extracts. The results are representative of three independent experiments. (*i*) Cytokine production of IL-4 and IL-5 analyzed by ELISA in naïve CD4$^+$ T cells, either WT or PKC$\zeta^{-/-}$ (KO), stimulated with anti-CD3 plus anti-CD28. The results are the mean ± SD of three independent experiments with incubations in triplicate.

signaling machinery but that it is not a direct step in the TCR signal transduction cascade. To address this point, naïve CD4$^+$ T cells were incubated with IL-4 for different times, and PKCζ enzymatic activity was determined as above. Interestingly, the addition of IL-4 provokes a reproducible and robust activation of PKCζ in these cells (Fig. 3*d*), demonstrating that PKCζ constitutes a potentially important step in IL-4 signaling. If this model is correct, that would imply that the activation of signaling cascades of the TCR should not be impaired in the PKC$\zeta^{-/-}$ naïve CD4$^+$ T cells but that the stimulation of the IL-4 pathway should be inhibited. To address this point, we incubated naïve WT and PKC$\zeta^{-/-}$ CD4$^+$ T cells with or without IL-4, after which, tyrosine phosphorylation of Stat6 and Jak1 was determined by immunoblotting with phospho-site-specific antibodies. Results in Fig. 3*e Left* demonstrate that the loss of PKCζ dramatically impairs the activation of Stat6 and Jak1 in naïve CD4$^+$ T cells. However, the loss of PKCζ does not affect the activation of ERK or NFATc1 (Fig. 3*f*) or that of NF-κB (Fig. 3*g*) or IκB kinase activity (Fig. 3*h*) in these cells when stimulated with anti-CD3 plus anti-CD28. The secretion of IL-4 and IL-5 in naïve CD4$^+$ T cells activated with anti-CD3 is normal in the PKC$\zeta^{-/-}$ cells (Fig. 3*i*), reinforcing the notion that PKCζ is not a direct target of TCR signaling. Likewise, the loss of PKCζ did not affect IL-2-induced Stat5 activation in this system (Fig. 3*e Right*). Taken together, these results suggest that the defect in Th2-polarized cells is accounted for by the important role played by PKCζ in IL-4 signaling but not in TCR downstream events. This model is consistent with our previously published results that demonstrated the lack of defects in the proliferation of naïve T cells from PKC$\zeta^{-/-}$ mice, a phenomenon that is clearly dependent on IL-2 production and signaling (8). In contrast, because the common γ-chain receptor is essential for IL-2 and IL-4 function, the fact that IL-2 signaling is intact in the PKC$\zeta^{-/-}$ mice (Fig. 3*e Right*) indicates that the defects observed in the IL-4 pathway cannot be explained by potential alterations in the levels of this receptor subunit. In addition, the data in Fig. 7, which is published as supporting information on the PNAS web site, demonstrate that the levels of the α-chain of the IL-4 receptor are intact in naïve and Th2-polarized T cells as demonstrated by FACS analysis to detect surface expression of the receptor (Fig. 1*a*) and immunoblotting (Fig. 1*b*). Interestingly, the nuclear translocation of activated Stat6 is dramatically inhibited in the PKC$\zeta^{-/-}$ Th2 cells (Fig. 4*a*), and the exogenous addition of IL-4 does not bypass this blockade (Fig. 4*b*). These results are consistent with the notion that PKCζ is required for the efficient activation of Jak1 and the subsequent phosphorylation and nuclear translocation of Stat6.

Loss of PKCζ Inhibits Allergic Airway Disease. It is well established that the Th2 immune response is responsible of the development of allergic airway inflammation (12–15). Because the loss of PKCζ results in impaired Th2 activation, we reasoned that PKC$\zeta^{-/-}$ mice would display reduced inflammatory response in a model of

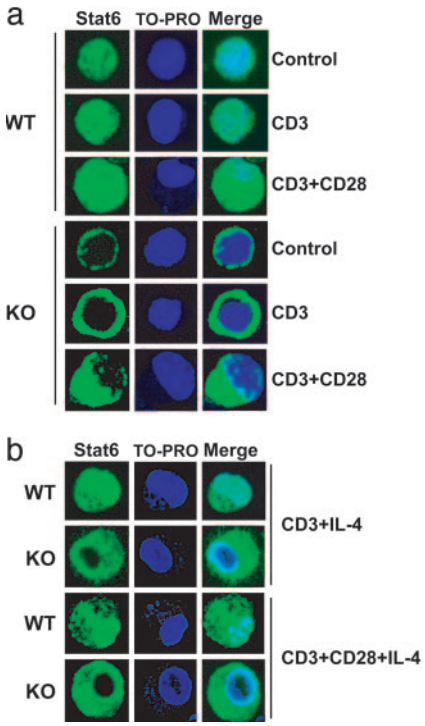

Fig. 4. PKCζ is required for Stat6 nuclear translocation. Th2 polarized WT or PKC$\zeta^{-/-}$ (KO) cells were restimulated or left unstimulated with anti-CD3 or anti-CD3 plus anti-CD28 in the absence (*a*) or presence (*b*) of IL-4, and nuclear translocation of Stat6 was determined by confocal analysis. Stat6 (green) or TO-PRO nuclear (blue) staining was visualized. The results are representative of three independent experiments.

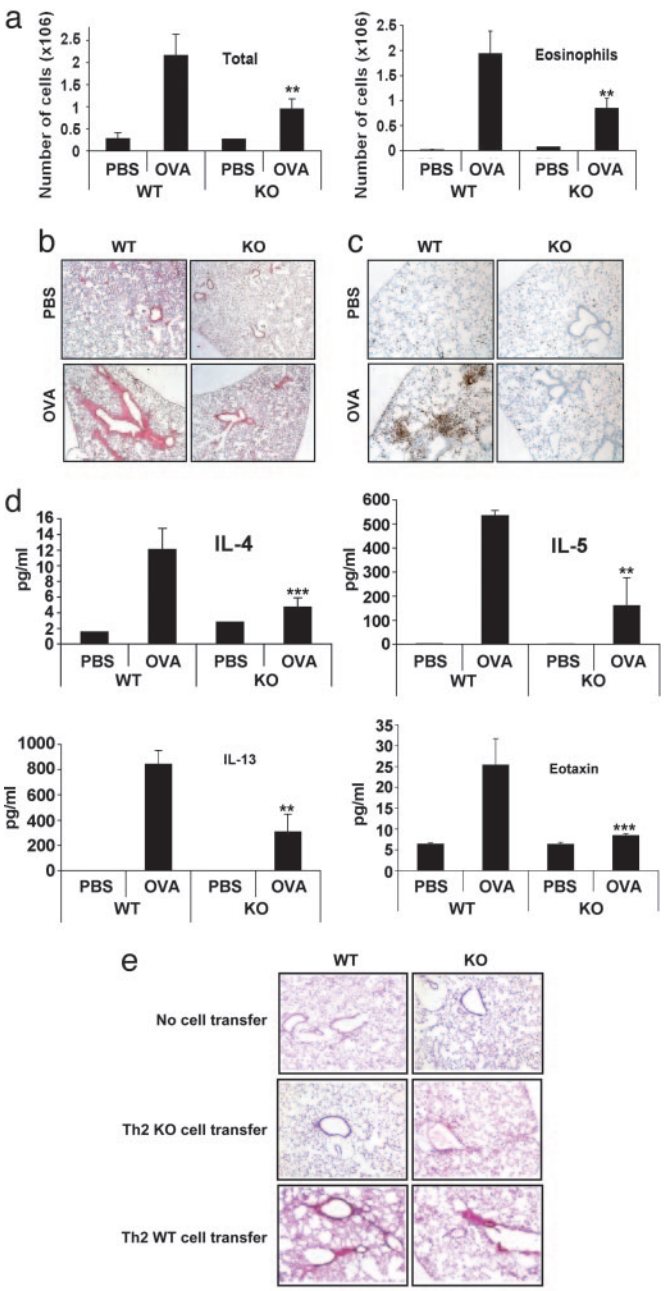

Fig. 5. Role of PKCζ in OVA-induced allergic airway disease. (*a–d*) OVA-induced allergic airway inflammation is impaired in PKC$\zeta^{-/-}$ mice. Total cell numbers and eosinophils in BAL fluids were determined (*a*). Lung sections were prepared and stained with H&E (*b*) or with anti-CD11b (*c*). Levels of IL-4, IL-5, IL-13, and eotaxin in the BAL fluids were measured by ELISA (*d*). (*e*) Adoptively transferred Th2 WT into PKC$\zeta^{-/-}$ (KO) cells can generate airway inflammation. Th2 WT and PKC$\zeta^{-/-}$ (KO) cells were generated by using mice sensitized with OVA and alum. Th2 cells were collected and injected into either WT or PKC$\zeta^{-/-}$ (KO) recipient mice. The mice were exposed to aerosolized OVA, and lungs were prepared for histology and stained with H&E. Data shown are representative of two experiments that each time involved six WT and six PKC$\zeta^{-/-}$ (KO) mice. *a* and *d* are the mean ± SD; $n = 6$ for each genotype. **, $P < 0.01$; ***, $P < 0.001$.

OVA-induced allergic airway disease that predominantly generates a Th2 response. Therefore, mice that are either WT or PKC$\zeta^{-/-}$ were sensitized to OVA and then challenged twice with aerosolized antigen or PBS on the same day. Forty-eight hours after the aerosol challenge, mice were killed, the lungs were examined histologically by hematoxylin/eosin (H&E) staining for eosinophilic infiltration, and BAL was performed to determine inflammatory cell recruitment. There was a robust increase in total BAL cell numbers in WT mice that were OVA sensitized and challenged with aerosolized antigen compared with PBS-challenged mice (Fig. 5*a Left*), due especially to eosinophils (Fig. 5*a Right*). However, this increase was dramatically reduced in the PKC$\zeta^{-/-}$ mice (Fig. 5*a*), indicating that the impairment in the Th2 response observed in these mutant mice inhibits airway inflammation. Consistent with this notion, H&E histological analysis of lung sections from this experiment shows that whereas the challenged WT mice display a prominent inflammatory response with massive perivascular and peribronchial infiltration with abundant eosinophils, PKC$\zeta^{-/-}$ mice display a much more attenuated response (Fig. 5*b*). CD11b staining confirms a dramatic reduction in granulocyte infiltration in the OVA-challenged PKC$\zeta^{-/-}$ mice (Fig. 5*c*). The production of mucus in asthmatic patients is severely debilitating and may lead to death. Periodic acid Schiff's base staining of lung sections consistently showed mucus production in the airway epithelium and alveoli of WT mice. In contrast, and concurring with the milder allergic response to the OVA challenge observed in the PKC$\zeta^{-/-}$ mice, mucus production was not observed in lung sections from these animals (data not shown). In addition, BAL IL-4, IL-5, IL-13, and eotaxin (Fig. 5*d*) supernatant levels, which were dramatically increased in OVA-challenged WT mice, were severely reduced in similarly treated PKC$\zeta^{-/-}$ mice.

Adoptive Transfer Experiments Support a Critical Role of PKCζ in Th2 Function. The results from the asthma model experiments are consistent with a critical role of PKCζ in Th2 function. However, it would be of great interest to determine whether this phenotype is mostly due to defects in Th2 cells or whether the loss of PKCζ in

the lung resident cells might contribute to the inhibition in allergic airway inflammation observed in the PKCζ mutant mice. To address this question, we transferred *in vitro*-generated WT and PKC$\zeta^{-/-}$ Th2 cells into PKC$\zeta^{-/-}$ mice and determined the sensitivity of these mice to the asthma model. Following a previously established method (16), we induced Th2 cells by i.p. injections of WT and PKC$\zeta^{-/-}$ mice with OVA, and, after 5 days of immunization, CD4$^+$ T cells from spleens and lymph nodes were isolated and subsequently cultured with antigen-presenting cells, OVA, and IL-4 for 4 days. Afterward, Th2-polarized cells were i.v. injected in equal numbers into WT and PKC$\zeta^{-/-}$ mice, whereas some control WT and PKC$\zeta^{-/-}$ mice did not receive cells. Mice were then challenged with inhaled OVA as described in *Materials and Methods*. Twenty-four hours after the final challenge, mice were killed, and lung inflammation was analyzed histologically by H&E staining as above. Importantly, H&E histological analysis of lung sections from this experiment shows that whereas the challenged PKC$\zeta^{-/-}$ mice that have been injected with PKC$\zeta^{-/-}$ Th2 cells showed little or no inflammation, the mutant mice injected with WT Th2 cells displayed a prominent inflammatory response with massive perivascular and peribronchial infiltration (Fig. 5*e*). In contrast, the WT control mice that were not challenged showed no inflammation at all (Fig. 5*e*). Also, the injection of PKC$\zeta^{-/-}$ Th2 cells into WT mice did not produce significant inflammation (Fig. 5*e*). These results suggest that the loss of PKCζ in the lung resident cells does not contribute in a significant manner to the OVA-induced airway inflammatory response and demonstrate the critical role of PKCζ in Th2 function *in vivo*.

Discussion

The understanding of the signaling cascades that regulate asthma, and Th2 polarization in particular, is an important issue in immunology. Because Th2 cells are critical players in the orchestration of the networks activated during allergic airway inflammation, these signaling pathways are a rich source of therapeutic targets in asthma and possibly other allergic diseases. The fact that PKCζ is a critical modulator of the Th2 response strongly suggests that it is a potentially relevant target for these pathological alterations of the immune system. Our data indicate that the loss of PKCζ leads to a clear impairment in the secretion of Th2 cytokines in *ex vivo* and *in vivo* experiments due to the inability of the PKC$\zeta^{-/-}$ CD4$^+$ T cells to differentiate adequately along the Th2 lineage. Thus, the loss of PKCζ results in the generation of Th2 cells in *ex vivo* cultures that poorly activate GATA3, c-Maf, Stat6, and NFATc1 during the Th2 differentiation program. Also, RelA activation is impaired in the PKC$\zeta^{-/-}$ cells. Although PKCζ has been shown to be involved in NF-κB nuclear translocation and IκB kinase activation in lung (7) and liver (17), we think that the defect observed in RelA activation, and in the other transcription factors, in Th2-polarized cells is secondary to an impaired differentiation program in the mutant cells due to the essential role played by PKCζ in IL-4 signaling. In this regard, Th2 differentiation *in vitro* is triggered by TCR activation and the IL-4 present in the culture medium. Notably, the activation of PKCζ in anti-CD3-rechallenged Th2 cells requires the autocrinely secreted IL-4, suggesting that PKCζ is not a direct downstream target of the TCR pathway but is a critical mediator of IL-4 signal transduction. Consistent with this model, IL-4 is sufficient to activate PKCζ in naïve T cells, and the loss of this kinase impairs Jak1/Stat6 activation by IL-4 in these cells, whereas it is dispensable for the activation of TCR proximal signals such as ERK and NF-κB activation. Therefore, the results presented here establish PKCζ as a critical player in the Th2 differentiation programs downstream the IL-4 receptor and independently from TCR-activated signals. The data are consistent with our previously published results that demonstrated that PKCζ is not involved in TCR-driven activation of naïve T cell proliferation and IL-2 production (8). Interestingly, the loss of PKCζ leads to impaired Stat6 tyrosine phosphorylation and nuclear translocation due to the fact that PKCζ is required for the proper stimulation of Jak1. Our previous results indicated that PKCζ interacts with and phosphorylates Jak1 *in vitro* and in IL-4 activated cells (17), which offers a mechanistic explanation to the Th2 inflammatory phenotype of the PKCζ mutant mice reported here.

Particularly relevant from the point of view of lung inflammatory pathologies are our results from the OVA-induced allergic airway disease model. These data validate PKCζ in asthma, because the mutant mice show a dramatically reduced response to OVA-induced airway inflammation. This response is the consequence of a complex set of cellular interactions involving the recruited Th2 lymphocytes and lung resident cells (1, 11). Of note, adoptive transfer experiments reported in this study demonstrate that the loss of PKCζ in lung resident cells does not contribute significantly to the impairment of the inflammatory response in this system, whereas the loss of PKCζ in Th2 cells is of great importance. These data reinforce the notion that PKCζ is required for Th2 function due to its critical role in IL-4 signaling. Our results also explain previous observations that PKC$\zeta^{-/-}$ mice have defects in mounting an optimal adaptive immune response to a T-dependent antigen without a defect in naïve T cell activation (8). Thus, our system is different from the PKC$\theta^{-/-}$ mice. In contrast to the PKC$\zeta^{-/-}$ mice, which have a defect in Th2 cells but not in naïve T cell proliferation (8), in the case of the PKC$\theta^{-/-}$ mice, the alterations were not restricted to the Th2 differentiation program (18–20); defects were also found in NF-κB activation and proliferation of naïve T cells (21, 22), suggesting that the role of PKCζ is more constrained to the Th2 polarization mechanism and that the role of PKCθ has a broader impact in T cell function.

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