

# Protein Kinase C $\theta$ Is Critical for the Development of In Vivo T Helper (Th)2 Cell But Not Th1 Cell Responses

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## Abstract

The serine/threonine-specific protein kinase C (PKC)- $\theta$  is predominantly expressed in T cells and localizes to the center of the immunological synapse upon T cell receptor (TCR) and CD28 signaling. T cells deficient in PKC- $\theta$  exhibit reduced interleukin (IL)-2 production and proliferative responses in vitro, however, its significance in vivo remains unclear. We found that *pkc- $\theta$ <sup>-/-</sup>* mice were protected from pulmonary allergic hypersensitivity responses such as airway hyperresponsiveness, eosinophilia, and immunoglobulin E production to inhaled allergen. Furthermore, T helper (Th)2 cell immune responses against *Nippostrongylus brasiliensis* were severely impaired in *pkc- $\theta$ <sup>-/-</sup>* mice. In striking contrast, *pkc- $\theta$ <sup>-/-</sup>* mice on both the C57BL/6 background and the normally susceptible BALB/c background mounted protective Th1 immune responses and were resistant against infection with *Leishmania major*. Using in vitro TCR transgenic T cell–dendritic cell coculture systems and antigen concentration-dependent Th polarization, PKC- $\theta$ -deficient T cells were found to differentiate into Th1 cells after activation with high concentrations of specific peptide, but to have compromised Th2 development at low antigen concentration. The addition of IL-2 partially reconstituted Th2 development in *pkc- $\theta$ <sup>-/-</sup>* T cells, consistent with an important role for this cytokine in Th2 polarization. Taken together, our results reveal a central role for PKC- $\theta$  signaling during Th2 responses.

Key words: PKC- $\theta$  • asthma • *Leishmania* • *nippostrongylus* • Th2 cell

## Introduction

Activation of naive T cells in vivo involves a series of interactions between APCs and T cells. Central to these interactions is the presentation of antigen in the context of the MHC to the TCR, and costimulation through nonpolymorphic molecules such as CD28. The integration of these activation signals results in optimal T cell activation and full transcription from the IL-2 promoter. Upon antigen stimulation, many of the molecules involved in T cell activation redistribute to focal points of T cell–APC interaction and form what is commonly referred to as the immunological synapse (1, 2). A key molecule associated with the immunological synapse is protein kinase C (PKC)- $\theta$ , which is a

novel Ca<sup>2+</sup>-independent PKC isoform (3) that unlike the eight other currently identified PKC isoforms (4), is specifically expressed in T cells and skeletal muscle (3). Through a mechanism involving Vav, Rac, and actin cytoskeleton reorganization (5), it localizes into the center of the immunological synapse and to lipid rafts after TCR stimulation and CD28 costimulation (6–8). In addition, upon TCR stimulation, the Src family protein tyrosine kinase, Lck, has been shown to phosphorylate a tyrosine residue in the PKC- $\theta$  regulatory domain, which is required for the translocation of PKC- $\theta$  to lipid rafts (9). Recent studies using T cells from mice genetically deficient in PKC- $\theta$  have shown that its absence leads to reduced IL-2 production and IL-2 receptor expression, with consequent impaired proliferative responses (10, 11). Signaling through PKC- $\theta$  is required for TCR-

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Abbreviations used in this paper: AHR, airway hyperresponsiveness; BAL, bronchoalveolar lavage; i.n., intranasal(ly); MetCh, methacholine chloride; Nb, *Nippostrongylus brasiliensis*; NF, nuclear factor; PKC, protein kinase C.

initiated induction of the transcription factors nuclear factor (NF)- $\kappa$ B and AP1 (10). Activation of NFAT has also been reported to be compromised in one of the PKC- $\theta$ -deficient mouse strains (11). NF- $\kappa$ B-mediated transcription has been linked to the development of Th2 cell immune responses (12–14) as well as IFN- $\gamma$  (15) and IL-12 production (16). NFAT family members have also been shown to have key roles in Th cell differentiation (17, 18), as absence of NFATc1 and NFATc3 results in abnormal Th2 cell polarization.

Although the molecular pathways of PKC- $\theta$ -mediated T cell activation continue to be delineated, the role of PKC- $\theta$  in the development of immune responses in vivo has yet to be established. We have examined Th cell differentiation in response to a variety of stimuli in mice genetically deficient for PKC- $\theta$ , and have found that these animals have a profound defect in Th2 cell responses. PKC- $\theta$  was found to be critical for the development of Th2 cell immune responses after infection by the helminth *Nippostrongylus brasiliensis* (Nb) and for the response to inhaled allergen in a model of atopic asthma. Its absence, however, did not affect the production of IFN- $\gamma$  and Th1 cell-mediated resistance against *Leishmania major* infection by PKC- $\theta$ -deficient mice on a C57BL/6 background. These findings highlight PKC- $\theta$  as a central regulator in the development of Th2 cell immune responses in vivo, and suggest that PKC- $\theta$  could be an important target for immunomodulating drugs.

## Materials and Methods

**Mice and Pathogens.** C57BL/6 and BALB/c wild-type mice were obtained from Charles River Laboratories. PKC- $\theta$ -deficient mice were generated as described previously (10) and backcrossed >15 generations onto the C57BL/6 background and >6 generations onto the BALB/c background. SMARTA-2 (LCMV gp13 TCR transgenic) mice (provided by H. Hengartner, Institute of Experimental Immunology, Zurich, Switzerland) and DO11.10 OVA-TCR transgenic mice (provided by K. Murphy, Washington University School of Medicine, St. Louis, MO) were backcrossed greater than six generations onto the C57BL/6 and BALB/c backgrounds, respectively. Mice were maintained under specific pathogen-free conditions in BioSupport and Skirball Institute animal facilities in isolated, ventilated cages. Animals used in experiments were between 8 and 10 wk of age. Nb was maintained by passage through Lewis rats (provided by N. Harris, Institute of Experimental Immunology, Zurich, Switzerland). Mice were infected subcutaneously with 550 live L3 worms and killed at day 14 after infection. For infection with *L. major*, stationary-phase promastigotes were collected from in vitro culture in biphasic Novy-Nicolle-McNeal blood agar medium, and  $1-2 \times 10^6$  parasites were injected subcutaneously into the right hind footpad. The infection was monitored by measuring footpad swelling with a metric caliper (Kroeplin). Mice were killed and tissues were taken at the indicated time points. All animal experimental procedures were approved by local animal committees (Basel, Zurich and New York University Institutional Animal Care and Use Committee).

**OVA-induced Airway Inflammation.** Mice were immunized by intraperitoneal injection with 100  $\mu$ g OVA (grade V; Sigma-

Aldrich) in 200  $\mu$ l of alum adjuvant (SERVA; Electrophoresis GmbH). 7 d later, mice were challenged by intranasal (i.n.) inoculation with 100  $\mu$ g OVA in 50  $\mu$ l PBS.

**Measurement of Airway Responsiveness.** On day 3 after i.n. challenge with OVA, mice were placed in individual, unrestrained whole body plethysmograph chambers (Buxco Electronics). Airway responsiveness was assessed in mice by inducing airflow obstruction with aerosolized methacholine chloride (MetCh). This procedure estimates total pulmonary airflow in the upper and lower respiratory tracts. The chamber pressure was used as a measure of the difference between thoracic expansion (or contraction) and air volume removed from (or added to) the chamber during inspiration (or expiration). Pulmonary airflow obstruction was assessed by measuring PenH using BioSystem XA software (Buxco Electronics). Measurements of MetCh responsiveness were obtained by exposing mice for 3 min to incremental doses of aerosolized MetCh (Aldrich Chemie) and monitoring the breathing pattern for 5 min after initiation of aerosol dose.

**Collection and Analysis of Bronchoalveolar Lavage (BAL) Cells.** On day 4 after i.n. challenge with OVA, mice were killed by CO<sub>2</sub> inhalation. The trachea was cannulated and BAL was performed by flushing the airways three times with 1 ml PBS. Total BAL cells were counted using a Coulter Counter (IG Instruments) and spun onto glass slides using a Cytospin 2 (Shandon Southern Products, Ltd.). After fixation with methanol for 2.5 min, cells were stained with undiluted May-Grünwald solution (Fluka) for 3 min. An additional staining was performed in a 50% May-Grünwald solution for 3 min. In a last step, cells were stained in 7% Giemsa solution (Fluka) for 12 min. Slides were rinsed with tap water and air-dried overnight. Dried cells were embedded in Eukit solution (O. Kindler GmbH & Co.) under glass coverslips. Percentages of macrophages, lymphocytes, neutrophils, and eosinophils were determined microscopically by counting 200 cells/sample using standard morphological and cytochemical criteria. Total numbers of eosinophils were derived by multiplying the total number of leukocytes by the percentage of eosinophils identified morphologically.

**ELISA Measurement of Antibodies and IFN- $\gamma$ .** At indicated time points, BAL fluid, serum, or culture supernatant was analyzed for either total IgE or IFN- $\gamma$ . 96-well plates (Maxisorp; Nunc) were coated with anti-IgE at a concentration of 5  $\mu$ g/ml, or anti-IFN- $\gamma$  at 5  $\mu$ g/ml in 50  $\mu$ l PBS overnight at 4°C. Between each of the following steps, plates were washed five times with PBS. Coated plates were blocked with PBS/1% BSA for 2 h at room temperature. Samples from individual mice/cultures were serially diluted in PBS/0.1% BSA as indicated, followed by incubation at room temperature for 2 h. Thereafter, alkaline phosphatase-labeled goat anti-mouse antibodies to IgE or IFN- $\gamma$  (Southern Biotechnology Associates, Inc.), respectively, were added at room temperature for 2 h, followed by the addition of the substrate *p*-nitrophenyl phosphate (Sigma-Aldrich). OD was determined at 405 nm.

**Proliferation Assay.** A single cell suspension was made from the indicated lymph nodes by gentle teasing through 70- $\mu$ m nylon cell strainers (Falcon). Cells were washed through the strainers with IMDM into 50-ml (Falcon) tubes. Antigen at the indicated concentrations was diluted in 96-well plates and  $5 \times 10^5$  cells were added per well. Cells were cultured for 72 h at 37°C with [<sup>3</sup>H]thymidine added (1  $\mu$ Ci/well) for the last 12 h. Total [<sup>3</sup>H]thymidine incorporation was measured as an indicator of cell proliferation.

**Intracellular Cytokine Staining and FACS® Analysis.** Approximately  $5 \times 10^5$  cells from either BAL samples or in vitro culture were stimulated with either  $10^{-7}$  M PMA and 1  $\mu$ g/ml ionomy-

cin, 5  $\mu\text{g}/\text{ml}$  plate-bound anti-CD3, or antigen-pulsed DCs for 6 h at 37°C in IMDM medium. For the final 2 h, 10  $\mu\text{g}/\text{ml}$  brefeldin A was added to the cultures to retain cytokines in the cytoplasm. Thereafter, cells were washed with PBS/0.1% BSA and incubated with anti-CD32/CD16 mAb for 30 min at 4°C to block Fc binding. After another washing step, cells were stained with APC-labeled anti-CD4 mAb (BD Biosciences) for 15 min at 4°C. Next, cells were washed with PBS/0.1% BSA, and then again in PBS and fixed with 2% paraformaldehyde for 20 min at room temperature. Fixed cells were then incubated in permeabilization buffer (0.5% saponin/PBS/1% BSA) containing FITC-labeled anti-IFN- $\gamma$ , anti-IL-10, and PE-labeled anti-IL-4 mAb (BD Biosciences) for 30 min at room temperature. Cells were washed twice in permeabilization buffer, and then resuspended in PBS/1% BSA and analyzed by flow cytometry (FACSCalibur™; Becton Dickinson) and FlowJo software (Tree Star, Inc.).

**Determination of Parasite Load.** Infected BALB/c mice were killed at 5 wk before lesions became necrotic. The lesions of three mice were harvested, pooled, and homogenized, debris was removed by brief centrifugation, and parasite numbers were determined in triplicate by limiting dilution (19).

**TCR Transgenic T Cell–DC Coculture.** Naive SMARTA-2 PKC- $\theta^{+/+}$  and SMARTA-2 PKC- $\theta^{-/-}$  mice were killed and spleens were removed. CD4 $^{+}$  T cells were isolated by MACS bead separation (Miltenyi Biotec) and were found to be 90–95% CD4 $^{+}$  CD62L $^{\text{high}}$  by subsequent FACS® analysis. DCs were isolated from naive wild-type C57BL/6 spleens as described previously (20). Isolated T cells ( $5 \times 10^4$  cells/well) and DCs ( $10^4$  cells/well) were cultured in 96-well plates in the presence of either 1,000 nM GP13 peptide for Th1 cell differentiation or 0.3 nM GP13 peptide for Th2 cell differentiation. In the indicated cultures, a 1:200 dilution of supernatant from the recombinant human IL-2-producing hybridoma, IL-2-t-6, was also added. On day 3 of culture, cells were activated in the presence of PMA and ionomycin for 4 h. IFN- $\gamma$  and IL-4 production was determined by flow cytometry as described above.

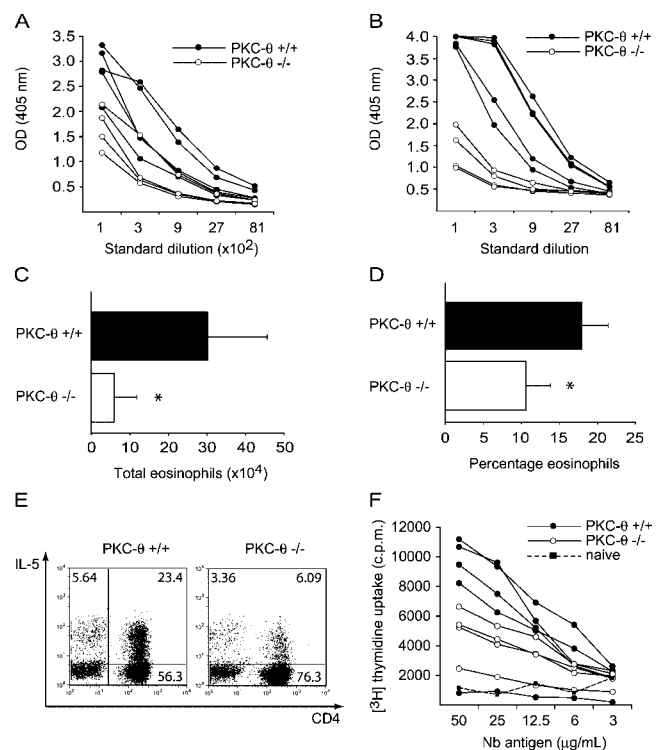
**Statistics.** Statistical significance was analyzed by the Student's *t* test. Unless otherwise indicated, data represents mean  $\pm$  standard deviation with *P* values of  $<0.05$  considered statistically significant.

**Online Supplemental Material.** Fig. S1 shows IL-4 and IFN- $\gamma$  production in a DO11.10 TCR transgenic T cell–DC coculture after activation with high and low doses of OVA peptide (amino acids 323–339; Invitrogen). Fig. S1 is available at <http://www.jem.org/cgi/content/full/jem.20032229/DC1>.

## Results

**PKC- $\theta$ -deficient Mice Have an Impaired Th2 Cell Immune Response against Infection with *Nb*.** It is clear from previous *in vitro* studies that PKC- $\theta$  is important for T cell activation and proliferation (10, 11), but its role in T cell activation during *in vivo* Th1 and Th2 cell immune responses has yet to be investigated. We first sought to establish whether PKC- $\theta$  signaling was required for the development of *in vivo* Th2 cell immune responses. To this end, we examined the immune response mounted against the prototypic Th2 cell-inducing helminth *Nb* by mice genetically deficient in PKC- $\theta$ . Wild-type C57BL/6 control mice and PKC- $\theta$ -deficient mice were infected *s.c.* with *Nb* and 14 d later, serum and BAL fluid were examined for IgE. Total IgE in both serum (Fig. 1 A) and BAL fluid (Fig.

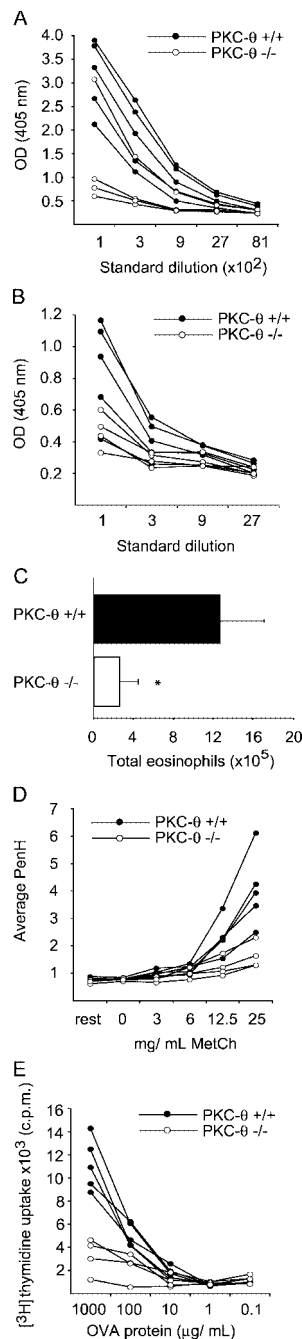
1 B) was dramatically reduced in PKC- $\theta$ -deficient mice compared with similarly infected wild-type controls, indicating that PKC- $\theta$  is required for IL-4-mediated (21) IgE isotype switching. Next, we examined the infiltration of eosinophils into the airways and blood, as this reflects *in vivo* IL-5 cytokine activity (22, 23). As shown in Fig. 1 C, the total number of eosinophils detected in the airways at day 14 after infection was significantly reduced in PKC- $\theta$ -deficient mice compared with wild-type controls. In addition, we examined the percentage of eosinophils circulating in the blood at day 10 after infection and similarly found a significantly reduced proportion of eosinophils (Fig. 1 D). To ascertain whether the reduced eosinophil influx was due to decreased IL-5 production by CD4 $^{+}$  T cells, we stimulated lymphocytes isolated from the airways with PMA and ionomycin and determined the proportion of IL-5-producing CD4 $^{+}$  T cells 6 h later by flow cytometry. In



**Figure 1.** PKC- $\theta$ -deficient mice exhibit an impaired Th2 cell immune response against infection with *Nb*. C57BL/6 and PKC- $\theta$ -deficient mice were infected subcutaneously with *Nb*. On day 14 after infection, total IgE in (A) serum and (B) BAL fluid was determined by ELISA. (C) The total number of eosinophils that had infiltrated into the airways was also determined on day 14. (D) On day 10 after infection, blood was taken and the percentage of circulating eosinophils was determined. On day 14 after infection, (E) lymphocytes isolated from BAL fluid were activated with PMA and ionomycin, and IL-5-producing CD4 $^{+}$  T cells were determined by flow cytometry, (F) mediastinal lymph nodes were removed, and isolated lymphocytes were cultured in the presence of *Nb* antigens for 72 h. [ $^3\text{H}$ ]thymidine incorporation over the last 12 h of culture was measured as an indicator of cell proliferation. \*, significant difference between groups ( $P < 0.05$ ). Data is from representative experiments using four to five mice per group. Similar results were obtained in three independent experiments.

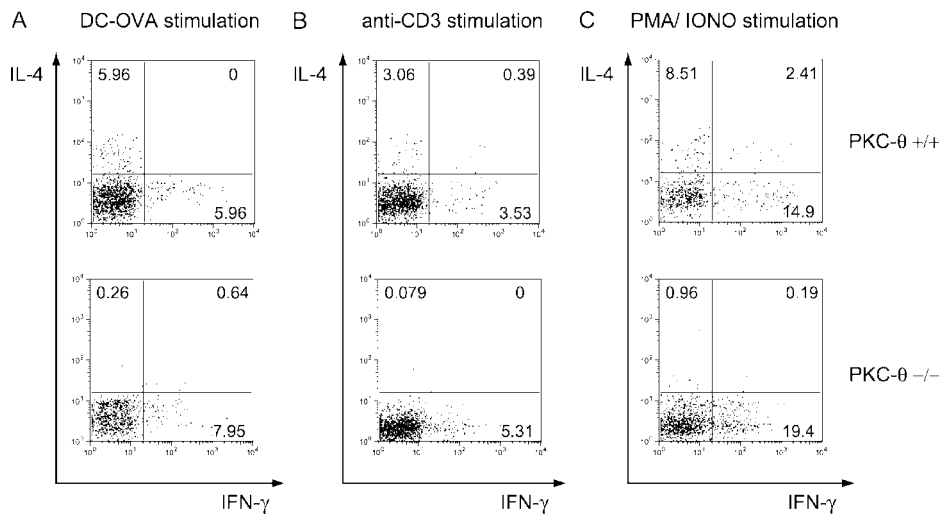
line with the reduced eosinophil infiltration, the proportion of IL-5-producing CD4<sup>+</sup> T cells was reduced in PKC- $\theta$ -deficient mice compared with wild-type controls (Fig. 1 E). This data indicates that the impaired airway eosinophilia is due to a defect in T cell function, rather than a defect in the eosinophils per se. We then examined the proliferative capacity of the cells responding to Nb infection. Lymphocytes were isolated from the draining mediastinal lymph node and cultured in vitro with DCs pulsed with Nb antigens. Lymphocytes from PKC- $\theta$ -deficient mice exhibited greatly impaired antigen-specific proliferation compared with similarly infected wild-type control mice (Fig. 1 F). This finding is consistent with previously published data that show that cells deficient in PKC- $\theta$  have reduced proliferation after stimulation with anti-CD3 (10). Taken together, these data demonstrate that PKC- $\theta$  plays a key role in the induction of Th2 cell immune responses directed against the helminth Nb.

**PKC- $\theta$ -deficient Mice Fail to Develop Th2 Cell-dependent Allergic Airway Inflammation.** To establish whether the defective Th2 cell immune response mounted against Nb infection was also evident in other models of Th2 cell immune responses, we examined the ability of PKC- $\theta$ -deficient mice to develop an allergic airway response against inhaled antigen. Mice were immunized by i.p. injection of OVA adsorbed in alum adjuvant, and then challenged i.n. 7 d later with OVA in PBS. 4 d after the i.n. challenge, serum and BAL fluid were examined for total IgE. In line with the data shown in the previous experiment, both serum (Fig. 2 A) and BAL (Fig. 2 B) IgE levels were dramatically reduced in PKC- $\theta$ -deficient mice compared with wild-type controls. In addition, we quantified the number of eosinophils that had migrated into the airways (Fig. 2 C) and lung parenchyma (not depicted) 4 d after OVA challenge and found that eosinophil migration was similarly reduced in this model. The hallmark feature of allergic asthma is the development of airway hyperresponsiveness (AHR), in which the Th2 cell cytokine IL-13 plays a central role (24). Accordingly, we investigated the development of AHR in PKC- $\theta$ -deficient mice. 3 d after OVA i.n. challenge, mice were exposed to increasing doses of aerosolized MetCh. OVA-sensitized and challenged BALB/c control mice exhibited marked AHR that increased with higher doses of MetCh (Fig. 2 D). However, PKC- $\theta$ -deficient mice only exhibited mild sensitivity to MetCh challenge (Fig. 2 D), indicating that this IL-13-mediated component of the Th2 cell immune response was also impaired. We sought to examine the proliferative capacity of OVA-specific lymphocytes. Lymphocytes were isolated from the draining mediastinal lymph node 4 d after OVA i.n. challenge and cultured in vitro with DCs pulsed with OVA protein. The proliferative response of lymphocytes from PKC- $\theta$ -deficient mice was dramatically reduced compared with wild-type controls (Fig. 2 E). Taken together, these data demonstrate that the Th2 cell effector responses induced in this model of atopic asthma are impaired in the absence of PKC- $\theta$  signaling.



**Figure 2.** PKC- $\theta$ -deficient mice fail to develop Th2 cell-dependent allergic airway inflammation. BALB/c and PKC- $\theta$ -deficient mice were sensitized to OVA by i.p. immunization of OVA protein adsorbed in alum adjuvant and 7 d later, mice were challenged i.n. with OVA protein in PBS. 4 d after challenge, total IgE was determined in (A) serum and (B) BAL fluid by ELISA. (C) The number of eosinophils that had infiltrated into the airways was also determined on day 4 after i.n. challenge. (D) On day 3 after i.n. challenge, mice were exposed to increasing doses of MetCh and AHR was determined in a full body unrestrained plethysmograph. (E) On day 4 after i.n. challenge, mediastinal lymph nodes were removed and isolated lymphocytes were cultured in the presence of OVA protein for 72 h. [<sup>3</sup>H]thymidine incorporation over the last 12 h of culture was measured as an indicator of cell proliferation. \*, significant difference between groups ( $P < 0.05$ ). Data is from representative experiments using four to five mice per group. Similar results were obtained in three independent experiments.

**PKC- $\theta$ -deficient T Cells Exhibit Impaired IL-4 but Normal IFN- $\gamma$  Production.** Next, we examined ex vivo cytokine production of effector CD4<sup>+</sup> T cells isolated from the airways. Mice were sensitized to OVA adsorbed in alum adjuvant and 7 d later were challenged i.n. with OVA in PBS. 4 d after i.n. challenge, lymphocytes from wild-type and PKC- $\theta$ -deficient mice were isolated from the airways and restimulated for 6 h with either OVA protein-pulsed DCs (Fig. 3 A), plate-bound anti-CD3 (Fig. 3 B), or PMA and ionomycin (Fig. 3 C). The proportion of CD4<sup>+</sup> T cells that produced IL-4 and IFN- $\gamma$  was determined by flow cytometry. A comparable proportion of wild-type CD4<sup>+</sup> T cells



**Figure 3.** PKC- $\theta$ -deficient CD4<sup>+</sup> T cells have impaired IL-4 but normal IFN- $\gamma$  production. BALB/c and PKC- $\theta$ -deficient mice were immunized and challenged with OVA as described in Fig. 2. 4 d after challenge, BALB/c was performed and lymphocytes were isolated. Lymphocytes were cultured with (A) DCs pulsed with OVA protein, (B) plate-bound anti-CD3, or (C) PMA and ionomycin for 6 h at 37°C. The proportion of cells producing IL-4 and IFN- $\gamma$  was determined by intracellular cytokine staining and flow cytometry. Dot plots are gated on CD4<sup>+</sup> lymphocytes as determined by forward and side scatter properties and positive staining for CD4. Numbers represent percentage of CD4<sup>+</sup> T cells in each quadrant and are representative of each group. Similar results were obtained in three independent experiments with four to five mice per group.

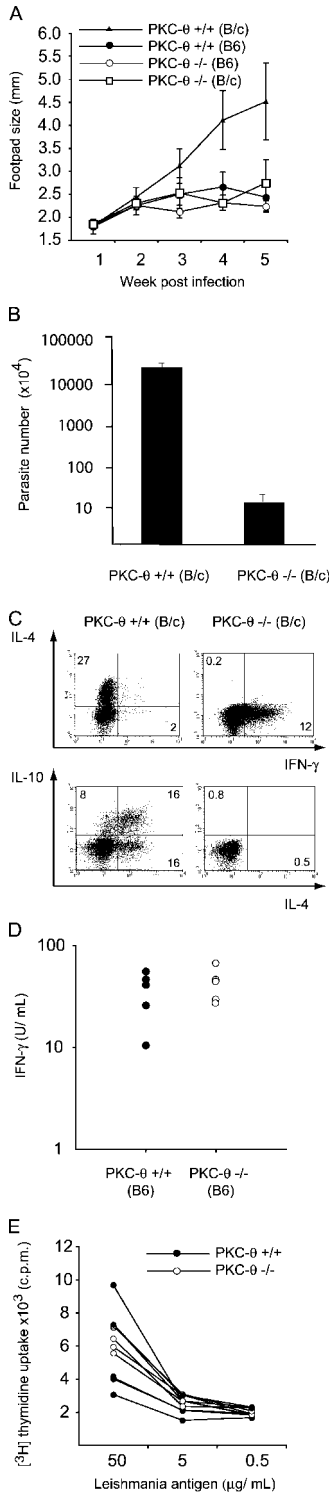
produced either IL-4 or IFN- $\gamma$  after each type of activation stimuli. In contrast, PKC- $\theta$ -deficient T cells failed to produce IL-4 after each of the activation stimuli examined (Fig. 3). This lack of IL-4 production is consistent with the reduced levels of IgE detected in both of the in vivo models tested. Taken together, these data indicate that although PKC- $\theta$  signaling is critically important for IL-4 production and the development of Th2 cell immune responses, it is not necessary for production of the prototypic Th1 cell cytokine, IFN- $\gamma$ .

*PKC- $\theta$ -deficient Mice Develop a Protective Th1 Cell-mediated Immune Response against Infection with *L. major* in Both the Resistant C57BL/6 and Susceptible BALB/c Backgrounds.* We have established that PKC- $\theta$ -deficient mice exhibit impaired Th2 cell immune responses in vivo. To determine whether PKC- $\theta$ -deficient mice exhibit a similar defect in Th1 cell immune responses, we infected PKC- $\theta$ -deficient mice with *L. major*, as resistance to this pathogen has clearly been demonstrated to be dependent upon an effective Th1 cell immune response (25–27). In brief, the C57BL/6 mouse strain mounts a Th1 cell immune response after injection of infectious *L. major* promastigotes and exhibits swelling at the site of infection, which then resolves. In comparison, mice on a BALB/c background mount a predominantly Th2 cell immune response after infection and as a result, fail to control parasite replication and display progressive swelling and necrosis of the lesion (28).

Mice were infected with  $2 \times 10^6$  infectious *L. major* promastigotes in the hind footpad. Footpad swelling was measured in infected C57BL/6, BALB/c wild-type control, and PKC- $\theta$ -deficient mice. As expected, wild-type BALB/c mice exhibited progressive footpad swelling (Fig. 4 A) and high parasite titre (Fig. 4 B). However, wild-type C57BL/6 and PKC- $\theta$ -deficient mice on both C57BL/6 and BALB/c backgrounds were capable of mounting an effective Th1 cell immune response, as indicated by transient footpad swelling (Fig. 4 A) and very low parasitemia (Fig. 4

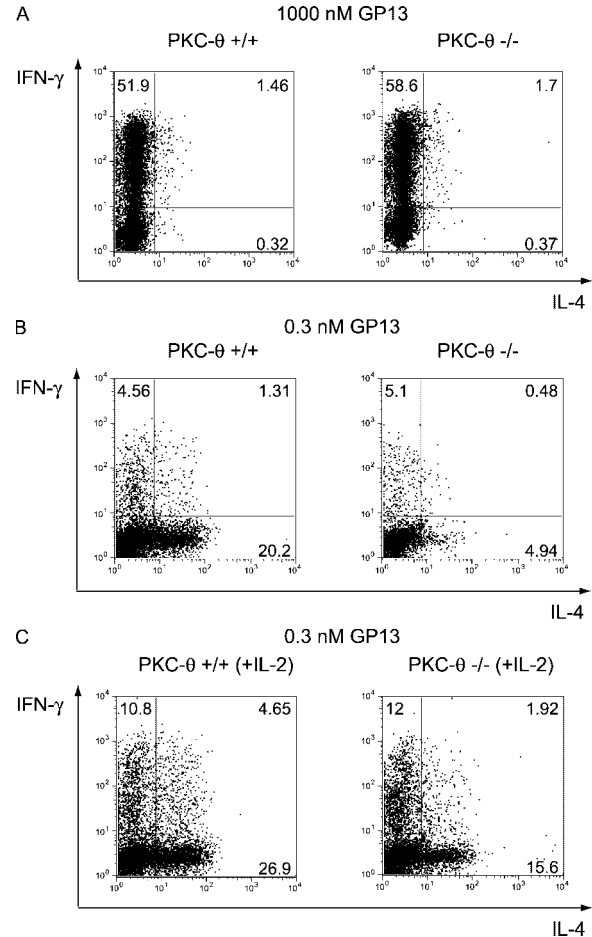
B and not depicted). We continued to monitor PKC- $\theta$ -deficient mice for as long as 9 mo after infection, and no disease development was observed (unpublished data). Next, we examined cytokine production by wild-type and PKC- $\theta$ -deficient BALB/c mice after *L. major* infection. As expected, a large proportion of CD4<sup>+</sup> T cells from wild-type mice produced IL-4 and IL-10, but very little IFN- $\gamma$ , consistent with failure to clear the infection (Fig. 4 C). In contrast, PKC- $\theta$ -deficient mice on a BALB/c background produced no IL-4 or IL-10, but did produce IFN- $\gamma$  (Fig. 4 C). We also measured the total amount of IFN- $\gamma$  produced by wild-type and PKC- $\theta$ -deficient mice on C57BL/6 backgrounds by ELISA after 48 h of culture with *L. major* antigens. In line with their similar resistance to *L. major* infection, we found that comparable amounts of IFN- $\gamma$  were produced in both wild-type and PKC- $\theta$ -deficient mice (Fig. 4 D). Next, we examined antigen-specific proliferation of lymphocytes isolated from the popliteal lymph node, and similarly found no difference between wild-type and PKC- $\theta$ -deficient mice (Fig. 4 E). Thus, in striking contrast to its requirement for development of in vivo Th2 cell responses, PKC- $\theta$  signaling does not appear to be critical for the development of Th1 cell immune responses.

*IL-2 Partially Restores Th2 Cell Differentiation of PKC- $\theta$ -deficient T Cells in an In Vitro TCR Transgenic T Cell-DC Coculture System.* To ascertain whether PKC- $\theta$ -deficient T cells were capable of differentiating into Th2 cells, we used an in vitro TCR transgenic T cell-DC coculture system. CD4<sup>+</sup> T cells were isolated from the spleens of naive SMARTA2 TCR transgenic mice on a C57BL/6 wild-type background or a PKC- $\theta$ -deficient background, and cultured in the presence of primary DCs with either a high (1,000 nM) or low (0.3 nM) concentration of GP13 peptide. We have recently shown that high antigen concentration promotes differentiation of IFN- $\gamma$ -producing Th1 cells through up-regulation of CD40L on naive CD4<sup>+</sup> T cells and CD40-mediated IL-12 production by DCs,



**Figure 4.** PKC- $\theta$ -deficient mice exhibit a Th1 cell immune response after infection with *L. major*. C57BL/6, BALB/c, and PKC- $\theta$ -deficient mice on a C57BL/6 or BALB/c background were infected with  $2 \times 10^6$  promastigotes of *L. major* in the hind footpad. (A) At the indicated time points, the course of disease was monitored using metric callipers to determine footpad swelling. (B) 5 wk after infection, lesions from infected footpads were removed and parasite load was determined. (C) Lymphocytes were isolated from popliteal lymph nodes draining the infected footpads and cultured in the presence of soluble *L. major* antigens for 72 h. (C) Cultured lymphocytes from BALB/c wild-type and PKC- $\theta$ -deficient mice were restimulated with PMA and ionomycin for 4 h and the proportion of CD4<sup>+</sup> T cells producing IFN- $\gamma$ , IL-10, and IL-4 was determined by flow cytometry. Numbers represent percentage of cytokine<sup>+</sup> cells in each quadrant. (D) IFN- $\gamma$  levels in the supernatant of cultured lymphocytes from C57BL/6 wild-type and PKC- $\theta$ -deficient mice were determined by ELISA after 48 h of stimulation with soluble *L. major* antigens. (E) Lymphocytes isolated from the popliteal lymph node were cultured in the presence of soluble *L. major* antigens for 72 h. [<sup>3</sup>H]thymidine incorporation over the last 12 h of culture was measured as an indicator of cell proliferation. Data is from representative experiments using three to five mice per group. Similar data was obtained in two independent experiments.

whereas low antigen concentration leads to the differentiation of IL-4-producing Th2 cells (20). After 3 d of culture, cells were activated with PMA and ionomycin for 4 h and the proportion of CD4<sup>+</sup> T cells producing IFN- $\gamma$  and IL-4 was determined by flow cytometry. When cells were activated with 1,000 nM GP13 peptide, wild-type cells differentiated with high frequency selectively into Th1 cells



**Figure 5.** IL-2 partially restores Th2 cell differentiation in an in vitro TCR transgenic T cell-DC coculture system. PKC- $\theta$ <sup>+/+</sup> and PKC- $\theta$ <sup>-/-</sup> SMARTA-2 TCR transgenic CD4<sup>+</sup> T cells were cultured with C57BL/6 primary DCs in the presence of GP13 peptide for 3 d as described in Materials and Methods. After a 4-h restimulation with PMA and ionomycin, IFN- $\gamma$  and IL-4-producing TCR transgenic T cells were identified by flow cytometry. (A) Cells were activated with 1,000 nM GP13 peptide for Th1 cell differentiation. (B) Cells were activated with 0.3 nM GP13 peptide for Th2 cell differentiation. (C) On day 0, IL-2 was added to cultures stimulated with 0.3 nM GP13 peptide. Numbers represent percentage of CD4<sup>+</sup> T cells in each quadrant. Data is from one representative experiment and similar data was obtained in two independent experiments.

(IFN- $\gamma$ <sup>+</sup> IL-4<sup>-</sup>), which was comparable in PKC- $\theta$ -deficient cells (Fig. 5 A). However, although wild-type SMARTA2 T cells differentiated into predominantly Th2 (IL-4<sup>+</sup> IFN- $\gamma$ <sup>-</sup>) cells after activation with 0.3 nM GP13 peptide, PKC- $\theta$ -deficient SMARTA2 T cells failed to differentiate into IL-4-producing cells and remained mainly undifferentiated (IL-4<sup>low</sup> IFN- $\gamma$ <sup>low</sup>) cells (Fig. 5 B). IL-2 is important for T cell proliferation and also plays a role in Th2 cell differentiation (29). Considering that PKC- $\theta$ -deficient mice exhibit impaired IL-2 production and up-regulation of CD25 (10), one likely mechanism underlying the impaired Th2 cell differentiation could be the reduced level of IL-2. Accordingly, we investigated whether the addition of IL-2 into the coculture system could restore Th2

cell differentiation in PKC- $\theta$ -deficient mice. Fig. 5 C shows that indeed the addition of IL-2 resulted in increased production of IL-4 by PKC- $\theta$ -deficient T cells, although not to the level of wild-type cells. In a BALB/c DO11.10 transgenic T cell-DC coculture system, we similarly found that PKC- $\theta$ -deficient mice exhibited impaired Th2 cell differentiation, and this was partially restored by the addition of exogenous IL-2 (Fig. S1, available at <http://www.jem.org/cgi/content/full/jem.20032229/DC1>). These data further highlight the specific impairment of Th2 cell immune responses in the absence of PKC- $\theta$ .

## Discussion

In this work we have demonstrated that PKC- $\theta$  is required for in vivo development of Th2 cell- but not Th1 cell-dependent immune responses. PKC- $\theta$ -deficient mice exhibited a highly impaired Th2 cell immune response against the helminth Nb, as shown by reduced IL-4-dependent IgE antibody production and IL-5-dependent blood and airway eosinophilia. In addition, and as expected from published in vitro observations, antigen-specific T cell proliferation was also significantly impaired after ex vivo restimulation. In support of these data, PKC- $\theta$ -deficient mice failed to develop allergic airway inflammation, IgE antibody responses, and AHR in a model of atopic asthma. A key finding was that although IL-4 production was selectively impaired in PKC- $\theta$ -deficient mice, a comparable number of antigen-specific CD4<sup>+</sup> T cells from PKC- $\theta$ -deficient mice and wild-type controls produced IFN- $\gamma$ . This finding was supported by the observation that the Th1 cell response against *L. major* infection was not affected by the absence of PKC- $\theta$ , although the Th2 cell response was compromised. Resolution of footpad swelling, parasite titer, IFN- $\gamma$  production, and proliferation in response to *L. major* antigen were similar in PKC- $\theta$ -deficient and control C57BL/6 mice. Moreover, in BALB/c mice, which are normally susceptible to infection due to an early IL-4 response, resolution of footpad swelling was observed in animals lacking PKC- $\theta$ , further demonstrating the failure of a Th2 cell response to the parasite.

PKC- $\theta$  is required for activation of the transcription factors NF- $\kappa$ B and AP-1 after TCR ligation (10). Both transcription factors have been implicated in Th cell development. NF- $\kappa$ B family members regulate IFN- $\gamma$  and IL-12 as well as Th2 cell cytokine expression (12–16), whereas AP-1, specifically Jun B, activates IL-4 expression in conjunction with c-MAF (30). However, both transcription factors are also required for IL-2 expression, which is severely compromised in PKC- $\theta$ -deficient T cells (10). Extensive evidence points to a role for IL-2 in regulation of Th2 cell cytokine responses. Le Gros et al. (29) originally showed that IL-2 production enhances the development of Th2 cells. This result was consistent with the finding that in vivo blockade of IL-2 resulted in resistance to *L. major* infection in normally susceptible BALB/c mice (31). IL-2 is required for IL-4 expression in vitro (29, 32, 33) and has recently

been demonstrated to stabilize chromatin accessibility for STAT5 binding to the *il4* gene, suggesting that it functions to maintain transcriptional activity at the *il4* locus (34). Interestingly, IFN- $\gamma$  expression appears to be less sensitive to the levels of IL-2, as mice treated with anti-IL-2 antibodies or lacking PKC- $\theta$  express sufficient levels of IFN- $\gamma$  to combat *L. major* infection (Fig. 4 and reference 31). Anti-IL-2 treatment also inhibited IL-4 expression in DO11.10 TCR transgenic T cells stimulated in vivo (34). Our in vitro T cell stimulation experiments demonstrate that IL-4 expression is partially rescued by the addition of exogenous IL-2 (Fig. 5). Therefore, failure to produce IL-2 might be a central mechanism underlying the impaired Th2 cell immune responses found in PKC- $\theta$ -deficient mice.

Because exogenous IL-2 failed to fully restore differentiation of PKC- $\theta$ -deficient Th2 cells, other pathways involved in Th1 cell differentiation may additionally be compromised in the mutant mice. In mice lacking the guanine nucleotide exchange factor Vav, which has been reported to interact with PKC- $\theta$ , there was also reduced IL-4 mRNA, but normal IFN- $\gamma$  mRNA, in draining lymph nodes after immunization (35). A similar phenotype to the one described in this work was found for *Itk*-deficient mice (36), in which development of the Th2 cell immune responses against Nb and *L. major* were impaired, whereas Th1 cell responses were normal. Although no direct association between *Itk* and PKC- $\theta$  has thus far been described, the strikingly similar phenotype of these mice suggests that these pathways of Th2 cell induction intersect. Moreover, Btk was reported to be associated with PKC- $\beta$  in B cells, suggesting that Tec family kinases and PKCs may function in the same signaling pathway (37). Therefore, the defect in the PKC- $\theta$ -deficient mice might be due to interruption of signals mediated through *Itk* and Vav after TCR and/or CD28 ligation. It is possible, however, that the impaired in vivo and in vitro Th2 cell responses of *Itk*-deficient mice may also be due to reduced levels of IL-2 (36). In these mice, the reduced expression of IL-2 was shown to be due, at least in part, to defective activation of NFAT. Thus, the impaired Th2 cell development observed in both *ITK* and PKC- $\theta$ -deficient mice may derive from reduced IL-2 expression rather than from defects in PKC- $\theta$ -derived signals to activate the *il4* locus.

PKC- $\theta$  and the CD28 costimulatory pathway closely interact in the activation of T cells. PKC- $\theta$  requires CD28 signaling to localize into the center of the mature immunological synapse (7) and is thought to integrate TCR and CD28 signals, leading to complete T cell activation (38). Ectopic expression of the serine-threonine kinase Akt in CD28-deficient mice has been shown to compensate for some of the costimulatory signal that is provided by CD28. Specifically, Kane et al. (39) were able to reconstitute IFN- $\gamma$  production, but not IL-4 or IL-5, by ectopic expression of Akt in CD28-deficient mice. These data are similar to the specific impairment of IL-4 and IL-5 production that we describe in this work, raising the possibility that although either Akt-mediated signaling or PKC- $\theta$ -mediated signaling

might be sufficient to induce Th1 cell immune responses, Th2 cell immune responses require a productive interaction between the two molecules. Such ectopic expression of Akt may not have been adequate for the integration of PKC- $\theta$  into the immunological synapse, or its Lck-mediated activation, and consequently resulted in impaired production of Th2 cell cytokines. Indeed, PKC- $\theta$  and Akt have been reported to physically associate upon T cell activation and to activate the NF- $\kappa$ B signaling pathway (40).

Whether loss of PKC- $\theta$  may directly affect Th2 cell cytokine gene expression remains unclear. Earlier studies have implicated members of the NF- $\kappa$ B and NFAT families of transcription factors in regulation of Th1 and Th2 cell differentiation. Although NF- $\kappa$ B family members have been reported to regulate the expression of both Th1 (15, 16) and Th2 cell cytokines (12–14), IL-4 production and Th2 cell-dependent airway eosinophil responses were compromised in mice lacking expression of the p50 subunit of NF- $\kappa$ B (12, 14). In these mice, IFN- $\gamma$  production was comparable to levels in wild-type animals (14). Because PKC- $\theta$  is required for NF- $\kappa$ B activation after TCR ligation, impairment of this pathway in mutant mice may explain, at least in part, the defect in Th2 cell cytokine regulation. The role of NFAT signaling in the observed Th2 cell defect in the PKC- $\theta$ -deficient mice is more difficult to interpret. Although we did not observe a defect in NFAT activation in T cells from mutant mice, another group reported impaired nuclear NFAT DNA binding activity in a different strain of *pkc $\theta$ <sup>-/-</sup>* mice. Even if NFAT signaling were defective, the observed reduction in IL-4 levels cannot be readily explained by current literature, which implicates members of the NFAT family in both positive and negative regulation of expression of the cytokine. Moreover, NFAT proteins often associate with AP-1 heterodimers to bind to composite sites in regulatory regions of cytokine genes, and the defective AP-1 activation in PKC- $\theta$ -deficient mice may therefore contribute to the impaired Th2 cell polarization.

Although PKC- $\theta$  may play a synergistic role in the transduction of both Th1 and Th2 cell activation signals, our data indicate that it might be a central regulator of Th2 cell immune responses, whereas Th1 cell responses are maintained in its absence through alternate pathways. In purified PKC- $\theta$ -deficient T cells, induction of IFN- $\gamma$  was enhanced even in the absence of proliferation, and after 2 h of anti-TCR stimulation we observed higher levels of IFN- $\gamma$  mRNA in CD4<sup>+</sup> cells from mutant versus control mice (unpublished data). These results suggest that the mutant T cells have an intrinsic propensity to express this Th1 cell cytokine. However, we did not observe enhanced Th1 cell responses *in vivo* in the mutant animals, and we cannot rule out that other factors, such as proinflammatory cytokines, sustain normal Th1 cell responses in the absence of PKC- $\theta$ . In an *in vivo* setting, the inflammatory cytokine milieu, strong costimulatory signals and activation of Toll-like receptor pathways may bypass the requirement for PKC- $\theta$  in T cell activation and differentiation during Th1 cell-inducing in-

fections, whereas signals induced during helminth infection or allergic asthma might not be sufficient to bypass the requirement for PKC- $\theta$  in Th2 cell immune responses.

In summary, the experiments detailed in this work show that PKC- $\theta$  is critical for the development of *in vivo* Th2 cell immune responses. These data highlight PKC- $\theta$  as a potentially important target in therapy against Th2 cell-related diseases.

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