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Resistance to Experimental Autoimmune Encephalomyelitis and Impaired IL-17 Production in Protein Kinase Cζ-Deficient Mice

Seng-Lai Tan, Jinyong Zhao, Chen Bi, XinYi Cynthia Chen, Deena L. Hepburn, Jian Wang, Jonathon D. Sedgwick, Subba R. Chintalacharuvu,1 and Songqing Na1

The protein kinase Cζ (PKCζ) serine/threonine kinase has been implicated in signaling of T cell activation, proliferation, and cytokine production. However, the in vivo consequences of ablation of PKCζ on T cell function in inflammatory autoimmune disease have not been thoroughly examined. In this study we used PKCζ-deficient mice to investigate the potential involvement of PKCζ in the development of experimental autoimmune encephalomyelitis, a prototypic T cell-mediated autoimmune disease model of the CNS. We found that PKCζ−/− mice immunized with the myelin oligodendrocyte glycoprotein (MOG) peptide MOG35–55 were completely resistant to the development of clinical experimental autoimmune encephalomyelitis compared with wild-type control mice. Flow cytometric and histopathological analysis of the CNS revealed profound reduction of both T cell and macrophage infiltration and demyelination. Ex vivo MOG35–55 stimulation of splenic T lymphocytes from immunized PKCζ−/− mice revealed significantly reduced production of the Th1 cytokine IFN-γ as well as the T cell effector cytokine IL-17 despite comparable levels of IL-2 and IL-4 and similar cell proliferative responses. Furthermore, IL-17 expression was dramatically reduced in the CNS of PKCζ−/− mice compared with wild-type mice during the disease course. In addition, PKCζ−/− T cells failed to up-regulate LFA-1 expression in response to TCR activation, and LFA-1 expression was also significantly reduced in the spleens of MOG35–55-immunized PKCζ−/− mice as well as in vitro-stimulated CD4+ T cells compared with wild-type mice. These results underscore the importance of PKCζ in the regulation of multiple T cell functions necessary for the development of autoimmune disease. The Journal of Immunology, 2006, 176: 2872–2879.

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proper activation of T lymphocytes is central to the establishment of adaptive immunity. This process is initiated by stimulation of a TCR by a peptide-MHC complex and co-stimulation of CD28 by ligands on APC, leading to activation of a cascade of intracellular protein phosphorylation events (1). This leads to the formation of a supramolecular complex containing redistributed TCR, adhesion molecules, and signaling components at the interface of the T cell and the APC, known as the immunological synapse. The T lymphocyte–restricted serine/threonine protein kinase protein kinase Cζ (PKCζ)2 is unique among other PKC members because it colocalizes with the TCR in the T cell immunological synapse (2–4). In cultured cell lines, PKCζ-mediated signal transduction has been shown to result in activation of the transcription factors, AP-1 and NF-κB, in response to TCR/CD28 costimulation, ultimately culminating in the induction of expression of genes that are essential for the proliferation and function of activated T cells (5–8). The physiological functions of PKCζ for TCR signaling were subsequently demonstrated by two independent studies with PKCζ-deficient mice (9, 10). Peripheral T cells of PKCζ−/− mice show reduced proliferation and IL-2 production and significant impairment in AP-1 and NF-κB (9, 10) as well as NFAT (10) activation in response to TCR/CD28 stimulation. Although numerous studies have implicated the IκBα kinase IKK complex in linking PKCζ to NF-κB activation (5–8), the pathway responsible for coupling PKCζ to AP-1 appears to be JNK-independent (9). Recently, a study has identified the stress-related STE20/SPO11-like, proline alanine-rich kinase, SPAK, as a substrate and target of PKCζ in a TCR/CD28-induced signaling pathway leading to selective activation of AP-1, but not NF-κB (11).

Naive CD4+ T cells can develop into either Th1 or Th2 effector cells as well as a distinct IL-17-secreting T effector cell type (12) upon activation, depending on the cytokines present and the quality and magnitude of both T cell and costimulatory receptor signaling (13–17). Recent studies provide some evidence that PKCζ is involved not only in T cell activation, but also in Th1/Th2 differentiation under different inflammatory conditions in vivo (18, 19). However, little is known about the role of PKCζ in T cell production of IL-17 and Th17 differentiation. In an asthmatic lung inflammation model, Th2 immune responses were preferentially impaired in the absence of PKCζ without a profound defect in Th1 responses in lung, even though PKCζ was required for the initial development of Th1 cells (18). Furthermore, PKCζ−/− mice were able to mount a normal protective Th1 response against Leishmania major infection, but not a Th2 response to Nippostrongylus brasiliensis (19), indicating that PKCζ preferentially modulates Th2 immune responses. However, it is not clear whether PKCζ plays any role in autoimmune disease conditions in which Th1/IL-17 responses predominate. We therefore investigated the effects of ablation of PKCζ in the myelin oligodendrocyte glycoprotein (MOG)35–55-induced mouse experimental autoimmune encephalomyelitis (EAE) model. We found that PKCζ−/− mice were completely resistant to the induction of EAE and exhibited profound
reduction of infiltrating inflammatory cells and demyelination in the CNS compared with wild-type (WT) mice. Consistent with these observations, PKC\textsuperscript{0/−}/CD4\textsuperscript{+} T cells failed to up-regulate surface expression of LFA-1 upon activation in vitro, and LFA-1 expression was also significantly reduced in the spleens of MOG\textsubscript{35-55}-immunized PKC\textsuperscript{0/−} mice compared with WT mice. Furthermore, splenocytes from immunized PKC\textsuperscript{0/−} mice produced significantly lower levels of the Th1 cytokine, IFN-γ, as well as IL-17 upon ex vivo Ag restimulation compared with WT mice. The generation of Ag-specific IL-17-producing cells ex vivo was profoundly impaired in MOG\textsubscript{35-55}-immunized PKC\textsuperscript{0/−} mice. In contrast, production of the Th2 cytokine, IL-4, was marginally affected in PKC\textsuperscript{0/−} under similar conditions. Taken together, these results suggest that PKC\textsuperscript{0} plays an essential role in the regulation of autoimmune effector T cells involved in the immunopathology of EAE and is a potential therapeutic target for treating T cell-mediated autoimmune diseases.

Materials and Methods

Mice

PKC\textsuperscript{0/−}-deficient mice were described previously (9). PKC\textsuperscript{0/−} mice were backcrossed onto C57BL/6 \( \times \) 11 times. Both PKC\textsuperscript{0/−} and WT littermate breeding colonies were established and maintained at Taconic Farms. All animal experimental procedures used in this study were approved by the Eli Lilly animal care and use committee and were conducted in accordance with the guidelines of Lilly.

Induction and clinical evaluation of MOG\textsubscript{35-55}-induced EAE

All mice were sex and age matched (6- to 10wk-old females) at the start of the experiments, and WT littermates were used as controls. PKC\textsuperscript{0/−} and WT mice were immunized s.c. at two sites on the back with 300 \( \mu \)g of MOG\textsubscript{35-55} (MEVGWYRSFSRVRVHLYRNKG; Peptides International), emulsified in a total of 200 \( \mu \)l of CFA (Difco) containing 500 \( \mu \)g of Mycobacterium tuberculosis. Five- to 7-wk-old Macaca nemestrina (Indian rhesus) were infected with 500 \( \mu \)l of tuberculin per mouse. Five- to 7-wk-old BALB/c mice were infected with 100 \( \mu \)l of tuberculin per mouse. The mice were subsequently infected by i.p. injection of 100 \( \mu \)l of CFA containing 500 \( \mu \)g of C. parvum and tuberculin. Mice were monitored daily for clinical signs of EAE.

Histology and immunohistochemistry

MOG\textsubscript{35-55}-immunized PKC\textsuperscript{0/−} and WT mice were killed on days 21–25, and spinal cords were removed, fixed overnight in IHC zinc fixative (BD Pharmingen), solution and transferred to 70% ethanol before processing through paraffin. Five- to 7-\( \mu \)m sections were generated by microtome, and sections were placed on positively charged slides. Slides were baked overnight at 60°C, deparaffinized in xylene, and rehydrated through graded alcohols to water. Rehydrated tissue sections were immersed in Luxol Fast Blue (0.1% alcoholic; Poly Scientific R&D) solution at 56°C for 24 h, then washed at 37°C for 2 h, deparaffinized by removing 100 \( \mu \)l of CFA/70% ethanol, and rehydrated in 0.1% Triton X-100, then blocked with 15% normal rabbit serum. Endogenous biotin was blocked with an avidin/biotin blocking kit (Vector Laboratories). Sections were incubated overnight at 4°C with primary Ab at an optimal concentration. After washing, tissue sections were stained with nuclear fast red (Vector Laboratories), dehydrated, and mounted with Clearfilm (Surgipath). For the negative control, equivalent concentrations of rat isotype control IgG2b (Serotec) were used to replace the primary Ab and were processed according to the above-described protocol.

Flow cytometric analysis

CD4\textsuperscript{+} T cells were purified from splenocytes of both WT and PKC\textsuperscript{0/−} mice using magnetic beads according to the manufacturer’s instructions (Miltenyi Biotec). CD4\textsuperscript{+} T cells (1 \( \times \) 10\textsuperscript{6} cells) were activated in 96-well plates that had been precoated with 1 \( \mu \)g/ml anti-CD3 Ab (BD Pharminogen) for the indicated time. For analysis of spinal cord mononuclear cell infiltrates, mononuclear cells from the spinal cords of the mice that were at the peak of disease (day 21) were isolated by Percoll gradient centrifugation. Briefly, pooled spinal cords were dissociated by glass homogenization in ice-cold GNN buffer (8.00 g NaCl, 0.40 g KCl, 3.56 g Na\textsubscript{2}HPO\textsubscript{4}, H\textsubscript{2}O 0.78 g NaH\textsubscript{2}PO\textsubscript{4}, H\textsubscript{2}O 2, and 2 g l- (+) glucose (pH 7.4)) with 0.02% BSA. Dissociated tissues were washed once in GNN/BSA and enzymatically digested for 30 min at 37°C with 250 \( \mu \)g/ml type II collagenase (Sigma-Aldrich). The digested spinal cord preparation was passed through a 40-\( \mu \)m-pore size cell strainer, washed once with GNN/BSA, and resuspended in 30% isotonic Percoll (Pharmacia Biotech). The suspension was fractionated with a 30/37/70% Percoll gradient by centrifugation at 500 \( \times \) g for 30 min at 25°C. Cells were collected from the 37/70% interface and washed with PBS containing 2% FCS and 0.1% BSA.

All flow cytometric data were collected and analyzed with a Cytomix FC500 (Beckman Coulter) using RXP software (Becton Coulter). The following staining Abs were purchased from BD Pharmingen: anti-ICAM-1 (3E2), anti-CD45-CyC (20-F1), anti-CD4-PE (L3T4), and anti-CD8a-FITC (53-6.7), and isotype control Abs (rat IgG-FITC and rat IgG-PE). Anti-ICOS-1 (7E17G9), anti-CD11a-PE (2D7), CD11b-PE (M1/70), and anti-CD49d-PE (PS2) were purchased from Becton. F4/80-FITC (MCA497F) was purchased from Serotec.

Splenocyte cell culture and proliferation assay

Splenocyte cell suspensions were isolated from MOG\textsubscript{35-55}-immunized WT and PKC\textsuperscript{0/−} mice on days 14 and 21, along with naive WT mice, by homogenizing spleens between frosted glass slides (Fisher Scientific) and removing RBC with buffered ammonium chloride lysing buffer (BioWhittaker). Pooled splenocytes of five individual mice from the same group were plated in triplicate in 96-well, round-bottom plates at \( \times \) 10\textsuperscript{5} cells/well in 200 \( \mu \)l of complete RPMI 1640 medium (Invitrogen Life Technologies) supplemented with 2 mM l-glutamine, 25 mM HEPES, 100 \( \mu \)M penicillin, 100 \( \mu \)g/ml streptomycin, 5 \( \times \) 10\textsuperscript{-5} M 2-ME, and 5% FCS (all from Invitrogen Life Technologies) containing 0–50 \( \mu \)g/ml MOG\textsubscript{35-55} (Peptides International) and cultured at 37°C in 5% CO\textsubscript{2}. Proliferation was measured by incorporation of \([\text{H}]\)thymidine (1 \( \mu \)Ci/well; ICN Radiochemicals) during the last 8 h of culture using a Filtermate counter (Pharmacia Biotech).

ELISA and ELISPOT analysis

Cytokine levels produced by cultured splenocytes from MOG\textsubscript{35-55}-immunized PKC\textsuperscript{0/−} and WT mice along with naive WT spleens were analyzed by removing 100 \( \mu \)l of cell culture supernatant/well after 72 h of culture as described above. Supernatants were filtered using Millipore plates (Mab-nob) and stored at \( \sim \) 80°C. Cytokines were analyzed with an R&D Systems MAP Kit (LUM000) with the following beads: IL-2 (CLUM402), IL-4 (LUM404), IFN-γ (LUM485), IL-4 (LUM404), and IL-17 (M1700).

The quantitative determination of the frequency of cells releasing mouse IL-17 was performed using R&D Systems mouse IL-17 ELISPOT (EL421). Briefly, freshly isolated spleen cells from immunized WT and PKC\textsuperscript{0/−} mice were plated in HL-11/1% glutamine-supplemented medium at \( \times \) 10\textsuperscript{5} cells/well (100 \( \mu \)l/well) with or without MOG\textsubscript{35-55} at 0, 5, and 50 \( \mu \)g/ml. The plates were incubated at 37°C in a 5% CO\textsubscript{2} incubator for 3 days. Proliferation was measured by incorporation of \([\text{H}]\)thymidine (1 \( \mu \)Ci/well; ICN Radiochemicals) during the last 8 h of culture using a Filtermate harvester (Packard Instruments) and a 1450 Microbeta liquid scintillation counter (Pharmacia Biotech).

TagMan quantitative RT-PCR analysis

Total cellular RNA pooled from spinal cords of WT or PKC\textsuperscript{0/−} mice on day 21 was prepared using a RNeasy Mini Kit (Qiagen); DNase treated, and quantified by spectrophotometric analysis. A two-step quantitative RT-PCR was performed to determine IL-17 and LFA-1 expression using the relative standard curve method, with the cellular housekeeping enzyme GAPDH as the normalization control. cDNA was synthesized with a High
Results
PKCθ-deficient mice are resistant to induction of MOG35–55-induced EAE

To investigate the role of PKCθ in the development of autoimmune T cell responses and disease induction, we immunized WT and PKCθ−/− mice with MOG35–55 peptide to induce EAE. As depicted in Fig. 1, the disease onset in WT mice occurred around days 13–14 and peaked between days 21 and 30, with a mean maximum disease score of 2.2. In contrast, PKCθ−/− mice were completely resistant to EAE induction. WT or PKCθ−/− mice immunized with CFA alone did not display EAE signs (data not shown). These results show that PKCθ is necessary for the induction of MOG35–55-induced EAE in mice.

Reduced inflammation of CNS in PKCθ-deficient mice after EAE induction

EAE is characterized by inflammatory cell infiltration into tissues of the CNS (20). To determine whether resistance to EAE induction in PKCθ−/− mice was associated with a reduction of CNS inflammation, we next examined leukocyte infiltration and demyelination in the spinal cords of WT and PKCθ−/− mice by both immunohistochemical staining and flow cytometric analysis of spinal cords at the peak of disease (on day 21). As shown in Fig. 2, spinal cords from PKCθ−/− mice did not show visible signs of demyelinating lesions, as revealed by Luxol Fast Blue staining (Fig. 2B), or cellular infiltrates by CD45-positive staining (Fig. 2D), consistent with the clinical EAE scores. In contrast, WT mice showed extensive demyelination (Fig. 2A), closely associated with heavy leukocyte infiltration (Fig. 2C) into the spinal cord parenchyma. These results suggest that the resistance of PKCθ−/− mice to MOG35–55-induced EAE is primarily due to the reduction of mononuclear cell infiltration and the absence of demyelination in CNS during EAE induction.

Major effector mechanisms of autoimmune demyelination in the CNS are mediated, at least in part, by effector CD4+ encephalitogenic T cells, followed by phagocytosis of myelin debris by local infiltrated macrophages. To confirm the reduced mononuclear cell infiltration and differentiate the infiltrated cell population in the CNS, we next analyzed the nature of the infiltrating leukocytes in the CNS of WT and PKCθ−/− mice. Mononuclear cells isolated from the spinal cords of PKCθ−/− and WT mice harvested on day 21 were stained for CD45 and CD4 or CD11b and analyzed by flow cytometry. As shown in Fig. 3a, there was a profound reduction in the percentage of infiltration of T cells (CD45+CD4+) as well as macrophages (CD45+CD11b+) in the spinal cords of PKCθ−/− mice (right panels) compared with WT mice. The diminished infiltration of CD4+ T cells and CD11b+ macrophages was confirmed by immunohistochemical staining of the spinal cords of PKCθ−/− mice (Fig. 3B). These results support the idea that the lack of PKCθ leads to reduction of mononuclear cell infiltration into the CNS and spinal cord demyelination during EAE progression.

Reduced IFN-γ production in PKCθ−/− splenocyte cultures

To determine whether resistance of EAE induction in PKCθ−/− mice was simply due to a reduced response of T cells to immunized Ag or to an impairment in T cell effector and proliferative functions, we examined cytokine production and T cell proliferation from MOG35–55-immunized PKCθ−/− or WT splenocytes on day 14 (onset of the disease) or 21 (peak of the disease) upon ex vivo stimulation in the presence of different concentrations of MOG35–55 peptide. Compared with WT animals, splenocytes from PKCθ−/− mice produced significantly less IFN-γ in response to MOG35–55 stimulation at all MOG35–55 concentrations tested at both stages of the disease (Fig. 4A). In contrast, IL-2 production by PKCθ−/− cells was only slightly decreased in comparison with WT cells on day 14 and was not different from WT on day 21 (Fig. 4B). Similar to IL-2 production, the Th2 cytokine, IL-4, was slightly lower in PKCθ−/− splenocytes compared with WT on day 14, but levels were comparable or slightly higher on day 21 (Fig. 4C). Collectively, these data suggest that PKCθ was important for optimal T cell responses, including Th2 cytokine production at initial Ag encounter. For Th1 IFN-γ production, PKCθ remained an essential signaling factor at both early and late time points.

Ex vivo MOG35–55 concentration-dependent T cell proliferative responses of PKCθ−/− splenocytes were slightly lower than WT responses on day 14 (Fig. 4D), which was consistent with lowered IL-2 secretion compared with WT (Fig. 4B). These effects were significant. However, there was no significant difference in MOG35–55-stimulated proliferation between WT and PKCθ−/− splenocytes on day 21 after immunization (Fig. 4D), which is in agreement with the equivalent levels of IL-2 secretion (Fig. 4B). Thus, PKCθ−/− T cells were efficiently primed with MOG35–55 immunization.

PKCθ is necessary for IL-17 production in spleen and CNS

We next examined the levels of the proinflammatory cytokine, IL-17, which is produced by a subset of IL-23-driven Th cells and is important for the development of EAE (12). As shown in Fig. 5A, IL-17 was reduced >50% in splenocyte culture from
PKCθ−/− mice compared with WT mice on days 14 and 21, suggesting that PKCθ is important for the generation of this pathogenic cytokine.

To gain insight into the possible mechanisms by which PKCθ−/− may regulate IL-17 production, we determined the number of IL-17-secreting cells from MOG35–55-immunized PKCθ−/− or WT splenocytes on day 21 upon ex vivo stimulation in the presence of the indicated concentrations of MOG35–55 peptide (Fig. 5B). The frequency of Ag-specific IL-17-producing cells in the spleen after MOG restimulation was significantly reduced in PKCθ−/− splenocytes compared with WT spleen. In contrast, there was no significant difference in IL-17 synthesis upon OVA

FIGURE 2. Prevention of CNS inflammation and demyelination in PKCθ−/− mice. Spinal cords were isolated from EAE mice randomly selected from PKCθ−/− and WT mice on day 21 for histopathological analysis. A representative section from each group is shown. A and B, Demyelination (stained by Luxol Fast Blue); C and D, leukocyte infiltration (stained by anti-CD45 Ab). Note the reduced intensity of myelin staining in areas associated with inflammation.

FIGURE 3. Population analysis of infiltrating leukocytes in the CNS of PKCθ−/− and WT mice with EAE. A, Mononuclear cells isolated from the spinal cord of PKCθ−/− and WT mice on day 21 were stained for CD45 and CD4 (top panels) or for CD45 and CD11b (bottom panels) and analyzed by flow cytometry. The percentage of CD4+ T cells or CD11b+ macrophages was counted on gated CD45+ cells. The data represent one of two independent experiments. B, Spinal cords from PKCθ−/− and WT mice were analyzed by immunohistochemistry for CD4, CD11b, and MHC class II staining.
stimulation, suggesting that the reduction in IL-17 production may be Ag specific.

In addition, spleen cell cultures from PKC0−/− mice produced significantly less IL-17 in response to IL-23 stimulation in vitro (Fig. 5C), indicating that PKC0 may play a role in IL-23-induced Th17 polarization. To put the relevance of these observations within the context of EAE resistance, we examined potential differences in IL-17 expression in the CNS of PKC0−/− and WT mice. Consistent with the pathogenic role of IL-17 in EAE, quantitative TaqMan RT-PCR analysis revealed a 6-fold reduction in IL-17 expression in the spinal cords of PKC0−/− mice compared with WT mice at the peak of the disease (Fig. 5D).

Reduced LFA-1 expression in PKC0−/− CD4+ T cells and spleens

As shown in Figs. 2 and 3, there was strikingly less mononuclear cell infiltration in the CNS of PKC0−/− mice compared with WT mice. We therefore measured the surface expression of the major integrins known to be involved in activated pathogenic T cell migration into the CNS during EAE development. Purified CD4+ T cells of naive PKC0−/− and WT mice were stained for various surface markers before (Fig. 6, A and B) and after (Fig. 6, C and D) stimulation with anti-CD3 and were subsequently analyzed by flow cytometry (Fig. 6). Anti-CD3 stimulation of WT cells resulted in an
increased number of cells with high surface expression of LFA-1 (Fig. 6, A and C, LFA-1, dashed line histograms). In contrast, PKCθ−/− T cell cultures failed to up-regulate LFA-1 surface expression after treatment with anti-CD3 (solid line histograms). Importantly, the reduced LFA-1 surface expression on PKCθ−/− CD4+ T cells appeared to be specific, because the surface expression of ICOS-1 (21) before or after T cell activation did not change appreciably under the same conditions (Fig. 6, B and D). The dependence of LFA-1 expression on PKCθ−/− mice was further confirmed by quantitative TaqMan RT-PCR analysis (Fig. 6, E). Spleens were isolated from EAE mice randomly selected from PKCθ−/− and WT mice on day 21 for quantitative TaqMan RT-PCR analysis. * p < 0.01 compared with WT.

**FIGURE 5.** PKCθ is necessary for optimal IL-17 production. Splenocytes were isolated from five each of PKCθ−/− and WT mice on days 14 and 21 after immunization with MOG35-55 in CFA. The MOG35-55-primed cells were cultured in the presence of the indicated concentration of MOG35-55, and culture supernatants were collected at 72 h. A, ELISA was used to measure the Ag-stimulated production of IL-17 in the cultures. B, Spleen cells from MOG35-55-immunized animals were collected for ELISPOT analysis to enumerate the frequency of IL-17-secreting cells. C, Mouse spleen mononuclear cells purified from PKCθ−/− or WT mice were cultured in 96-well tissue culture plates in the presence of 100 ng/ml mouse IL-2 and the indicated concentrations of human rIL-23 for 6 days. IL-17 in cell supernatants was measured using the mouse IL-17 ELISA kit. D, Spinal cords were isolated from EAE mice randomly selected from PKCθ−/− and WT mice on day 21 for quantitative TaqMan RT-PCR analysis. * p < 0.01 compared with WT.

**FIGURE 6.** Reduced LFA-1 expression in PKCθ−/− spleens and CD4+ T cells. A, Purified CD4+ T cells from WT and PKCθ−/− spleens were activated in 96-well tissue culture plates that were precoated with anti-CD3 mAb for 0 h (A and B) or 24 h (C and D). Cells were recovered and stained for LFA-1 or ICOS-1. Dashed and solid line histograms represent WT and PKCθ−/− mice, respectively. The y-axis represents relative cell number, and the x-axis represents log10 fluorescence intensity. Percentages are the number of cells with high surface expression of LFA-1 or ICOS-1 as defined by the indicated bar. Isotype control mAb staining is shown as a shaded histogram. The data represent one of two independent experiments. E, Spleens were isolated from EAE mice randomly selected from PKCθ−/− and WT mice on day 21 for quantitative TaqMan RT-PCR analysis. * p < 0.05 compared with WT.
up-regulation on PKCθ was substantiated by quantitative TaqMan RT-PCR analysis of the spleens from MOG_{35-55}-immunized PKCθ^−/− mice and WT mice (Fig. 6E). Thus, the decreased expression of LFA-1 may at least partly account for the markedly reduced leukocyte infiltration into the CNS of PKCθ^−/− mice during EAE development.

**Discussion**

Previous in vitro and in vivo studies have suggested that PKCθ is crucial for signaling events leading to activation, proliferation, and cytokine production of mature T cells (9, 10). The role of PKCθ in T cell differentiation and function in inflammatory diseases is less clear. For example, the induction of an immune response to viral infections is not impaired in the absence of PKCθ (22–24). Interestingly, PKCθ has been shown to be critical for Th2 inflammatory responses in vivo after respiratory challenge with OVA (18) and N. brasiliensis infection (19), but is dispensable for Th1 responses. Therefore, the role of PKCθ in T cell differentiation may depend on the specific Ag(s) and organs. In this study we examined the role of PKCθ in the Th1/IL-17 effector T cell-dependent MOG_{35-55}-induced EAE model using PKCθ^−/− mice. We found that PKCθ^−/− mice were markedly resistant to the induction of EAE immunized by MOG_{35-55}. Consistent with this observation, there were profound reductions in leukocyte infiltration into the CNS, including CD4^+ T cells and macrophages, in PKCθ^−/− mice compared with WT (Figs. 2 and 3). Furthermore, the Th1 cytokine, IFN-γ (Fig. 4A), and the proinflammatory/pathogenic cytokine, IL-17 (Fig. 5), were diminished significantly upon Ag stimulation ex vivo in PKCθ^−/− cells from postimmunized mice on both days 14 and 21.

In contrast, the levels of the Th2 cytokine, IL-4, were not affected or were higher in PKCθ^−/− splenocyte culture compared with WT, with slightly lower levels in the PKCθ^−/− splenocyte culture on day 14 after immunization, which could be due to the reduced cell proliferation (Fig. 4D). Our data suggest that PKCθ plays an essential role in the development of autoimmune Th1 (IFN-γ) responses as well as in the production of the key pathogenic cytokine, IL-17, in vivo.

Previous studies have demonstrated that T cell proliferation is impaired in the absence of PKCθ upon anti-CD3 stimulation in vitro (9, 10). We also observed a small, but significant, impairment of T cell proliferative responses upon MOG_{35-55} stimulation on day 14, but not day 21, after immunization (Fig. 4D). In our study, splenocytes from both WT and PKCθ were able to produce comparable levels of IL-2 in response to MOG_{35-55}, especially on day 21 (Fig. 4B). This is also different from previous results that showed a marked decrease in anti-CD3-stimulated IL-2 production in the absence of PKCθ (9, 10). Addition of exogenous rIL-2 can rescue anti-CD3-dependent T cell proliferation, suggesting that diminished IL-2 production is primarily responsible for the defect in T cell proliferation (9). The different outcomes of MOG_{35-55} and anti-CD3 stimulation of T cells could be due to the fact that anti-CD3 stimulation acts mainly on naive T cells, whereas MOG_{35-55} stimulation operates through previously Ag-primed T cells.

Autoreactive IL-17-producing Th cells are highly potent at inducing CNS immune pathology, and adoptive transfer of IL-17-producing CD4^+ T cells into recipient mice efficiently induces EAE (12). Such IL-17-producing CD4^+ T cells appear to emerge from previously activated Th cells in the presence of IL-23 (12). Furthermore, administration of neutralizing IL-17 mAb results in partial protection of EAE induction, suggesting that IL-17 is one of the key cytokines in the pathogenesis of EAE (12). Consistent with its critical role in EAE pathogenesis, we found that IL-17 production from MOG_{35-55}-activated primed splenocytes from PKCθ^−/− mice was significantly reduced compared with that in WT mice (Fig. 5A). PKCθ^−/− mice showed a profound reduction of IL-17 synthesis per cell upon MOG_{35-55} stimulation ex vivo, but no difference in IL-17 synthesis upon OVA stimulation (Fig. 5B), suggesting that the defect in IL-17 production may be Ag specific. Indeed, IL-17 expression was markedly reduced in the CNS of immunized PKCθ^−/− mice (Fig. 5D). This could be due to a reduction in the number of differentiated and/or survival of IL-17-producing Th cells as well as IL-17 expression at the mRNA level.

With respect to the latter, a crucial role for NFAT, a downstream effector of PKCθ (10), in TCR-mediated regulation of IL-17 gene transcription has been demonstrated (25). However, we have not ruled out the possible role of PKCθ in IL-23-mediated IL-17 synthesis in the development of EAE, and our data support this concept (Fig. 5C).

The reduced IL-17 production by PKCθ-deficient T cells may only partially account for the resistance to EAE induction, because administration of neutralizing IL-17 mAb produced only partial inhibition of EAE in mice, suggesting that other factors contribute to EAE in mice (12). Cell traffic may also be a key factor. T cell transendothelial adhesion is mediated by two major integrins, LFA-1 (CD11a), interacting with ligands ICAM-1/ICAM-2/ICAM-3, and VLA-4, interacting principally with VCAM-1 (26). LFA-1-mediated transendothelial migration of encephalitogenic T cells has been suggested to be involved in the pathogenesis of EAE (27). Furthermore, leukocytes from patients with multiple sclerosis were found to display significantly higher adhesion capacity and expressed higher levels of LFA-1, suggesting that LFA-1/ICAM interactions may promote the adherence of leukocytes to brain microvascular endothelial cells, contributing to the disease pathology (28, 29). Thus, our findings that PKCθ^−/− mice had reduced ability to up-regulate LFA-1 cell surface expression on CD4^+ T cells upon activation (Fig. 6A) and decreased expression of LFA-1 in their spleens during the disease course (Fig. 6B) may explain, at least in part, the markedly reduced leukocyte infiltration into the CNS. In support of this view, anti-LFA-1 Abs have been shown to block completely the induction of EAE (30, 31). Down-modulation of LFA-1 (and VLA-4) was found recently to correlate with clinical responses to IFN-β treatment in patients with relapsing-remitting multiple sclerosis (32), and similar mechanisms were demonstrated in rat EAE upon treatment with lovastatin (33), which apparently is an inhibitor of the LFA-1/ICAM-1 interaction (34). Surface expression of VLA-4 was not differentially regulated between WT and PKCθ^−/− T cells (data not shown), suggesting that LFA-1 and VLA-4 may have nonredundant roles in mediating pathogenic T cells infiltrating the CNS during EAE development.

It is possible that PKCθ deficiency may also affect the expression of chemokines and/or chemokine receptors, which may contribute to the lack of infiltrating T cells in the CNS. Furthermore, we have not ruled out the possibility that PKCθ deficiency may render T cells more prone to apoptosis in vivo, given that PKCθ has been reported to protect T cells from Fas-induced apoptosis via phosphorylation and inactivation of pro-apoptotic protein BAD (35, 36). It would not be surprising if the cumulative impact of multiple defects in T cell function were ultimately responsible for the profound resistance of PKCθ^−/− mice to EAE. Although future studies will be necessary to test these hypotheses to determine the exact mechanism(s) by which PKCθ regulates the development of EAE, our data clearly show that PKCθ modulates both Th1 and Th17 cytokine profiles, affects processes necessary for leukocyte infiltration into the CNS, and collectively plays an important role in the development of EAE. Thus, PKCθ may serve as an attractive therapeutic target for treating autoimmune diseases such as multiple sclerosis.
Note added in proof. While this paper was under review, similar findings were reported by Salek-Ardakani et al. (37).

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Disclosures

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