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The Critical Role of Protein Kinase C-θ in Fas/Fas Ligand-Mediated Apoptosis[1]

Santhakumar Manicassamy and Zuoming Sun[2]

A functional immune system not only requires rapid expansion of antigenic specific T cells, but also requires efficient deletion of clonally expanded T cells to avoid accumulation of T cells. Fas/Fas ligand (FasL)-mediated apoptosis plays a critical role in the deletion of activated peripheral T cells, which is clearly demonstrated by superantigen-induced expansion and subsequent deletion of T cells. In this study, we show that in the absence of protein kinase C-θ (PKC-θ), superantigen (staphylococcal enterotoxin B)-induced deletion of Vβ8$^+$ CD4$^+$ T cells was defective in *PKC-$\theta^{-/-}$* mice. In response to staphylococcal enterotoxin B challenge, up-regulation of FasL, but not Fas, was significantly reduced in *PKC-$\theta^{-/-}$* mice. PKC-θ is thus required for maximum up-regulation of FasL in vivo. We further show that stimulation of FasL expression depends on PKC-θ-mediated activation of NF-AT pathway. In addition, *PKC-$\theta^{-/-}$* T cells displayed resistance to Fas-mediated apoptosis as well as activation-induced cell death (AICD). In the absence of PKC-θ, Fas-induced activation of apoptotic molecules such as caspase-8, caspase-3, and Bid was not efficient. However, AICD as well as Fas-mediated apoptosis of *PKC-$\theta^{-/-}$* T cells were restored in the presence of high concentration of IL-2, a critical factor required for potentiating T cells for AICD. PKC-θ is thus required for promoting FasL expression and for potentiating Fas-mediated apoptosis. *The Journal of Immunology,* **2007, 178: 312–319.**

To maintain homeostasis, the immune system has developed mechanisms to reduce the significantly expanded T cell population resulting from antigenic stimulation. Activation-induced cell death (AICD)[3] is one of the mechanisms for eliminating clonally expanded T cells (1, 2). Fas (CD95)- and Fas ligand (FasL; CD95L)-mediated apoptosis play an important role in deleting effector T cells, as mice lacking Fas or FasL display defective deletion of peripheral T cells (3, 4), and eventually develop autoimmune disorders (5–7). In addition, FasL-induced apoptosis has been shown to be responsible for protecting immune privileged sites from cellular immune-mediated damage (8, 9). AICD is typically represented by the deletion of Vβ8$^+$ T cells in mice challenged with superantigens such as staphylococcal enterotoxin B (SEB) (10). In response to SEB stimulation, expression of both Fas and FasL is up-regulated. Fas and FasL then interact with each other to induce T cell apoptosis. By binding to the MHC class II molecules of APCs, SEB induces an initial expansion phase of Vβ8$^+$ T cells, followed by a deletion phase. The Vβ8$^+$ T cell deletion phase, but not the expansion phase, is defective in Fas-deficient *lpr* or FasL-deficient *gld* mouse strains (3, 11).

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[3] Abbreviations used in this paper: AICD, activation-induced cell death; FasL, Fas ligand; DAG, diacylglycerol; EAE, experimental allergic encephalomyelitis; IP_3, inositol triphosphate; PKC, protein kinase C; SEB, staphylococcal enterotoxin B; siRNA, small interfering RNA; WT, wild type.

Protein kinase C-θ (PKC-θ), a serine/threonine kinase, plays an important role in T cell activation and proliferation. We and others have shown that PKC-θ is critical for the activation of NF-AT, NF-κB, and AP-1 upon TCR stimulation (12–14). In addition, PKC-θ has been shown to play a role in the development of Th2 and Th1 responses in several different animal models (15–18). Engagement of TCR induces activation of phospholipase Cγ1, which catalyzes the hydrolysis of inositol phospholipids to produce diacylglycerol (DAG) and inositol triphosphate (IP_3). IP_3 induces Ca^{2+} influx, whereas DAG activates PKCs (19). Ionomycin (Ca^{2+} mobilizer) in combination with phorbol esters (PKC activators) mimics the signals required for T cell activation (20), indicating that IP_3-induced Ca^{2+} influx and DAG-mediated PKC activation cooperate with each other to mediate T cell activation. Although phorbol esters (PMA) activate multiple isoforms of PKC, PKC-θ is selectively required for T cell activation (13, 14). T cells obtained from *PKC-$\theta^{-/-}$* mice failed to proliferate and produce IL-2 upon TCR stimulation due to defective activation of NF-κB and AP-1, and these observations are supported by several in vitro studies in Jurkat T cells (21–24). T cells obtained from mice deficient in other isoforms of PKC do not display defects similar to that observed in *PKC-$\theta^{-/-}$* T cells (25), demonstrating the selective requirement of PKC-θ in T cell activation.

Overexpression studies with Jurkat T cells showed that PKC-θ can promote TCR-induced activation of FasL promoter (26, 27). However, most previous experiments examining the role of PKC-θ used either purified T cells from *PKC-$\theta^{-/-}$* mice or transformed Jurkat T cells. Moreover, T cells were usually stimulated with anti-CD3/28 Abs in those studies. Thus, exceptionally little is known about which pathways are necessary for induction of FasL expression in vivo as well as for Fas-mediated apoptosis. Using *PKC-$\theta^{-/-}$* mice, we show in this study that both up-regulation of FasL and Fas-mediated deletion of peripheral T cells require PKC-θ.

Materials and Methods

Mice

PKC-$\theta^{-/-}$ mice on C57BL/6 or 129/Sv or DO11.10-TCR^{Tg} genetic background were used for analyses (28). Mice were housed in the specific

pathogen-free animal facility of the Biological Resource Laboratory of the University of Illinois following the university guidelines. Wild-type (WT) C57BL/6 or 129/Sv or D011.10-Tg mice were purchased from The Jackson Laboratory or Charles River Laboratories.

Plasmids

The WT, constitutively active, and dominant-negative Xpress-tagged human PKC-θ expression vectors were gifts from A. Altman (Division of Cell Biology, La Jolla Institute for Allergy and Immunology, San Diego, CA) and X. Lin (M.D. Anderson Cancer Institute, Houston, TX). Small interfering RNA (siRNA) for human PKC-θ was designed and synthesized, as described in our previous publication (29). The specific cDNA inserts against human PKC-θ were designed and cloned into expression vector system (pSUPERretro-GFP) that directs the synthesis of siRNAs in mammalian cells, under the control of the H1 promoter. These inserts were identical with those described previously (29). A pSUPERretro-GFP construct expressing a scrambled siRNA served as a control.

Isolation of CD4$^+$ T cells

T cells were isolated from spleens and mesenteric lymph nodes of 8- to 12-wk-old mice, as follows. Single-cell suspensions were made by crushing organs. RBCs were lysed with an NH_4Cl buffer. Afterward, CD4$^+$ cells were purified using an autoMACS magnetic cell sorter with a CD4$^+$ isolation kit (Miltenyi Biotec), according to the manufacturer's protocol. Purity of the T cells, determined by flow cytometric analysis, was usually >90%.

Abs and flow cytometric analysis

Cells were stained with indicated Abs in PBS supplemented with 1% FCS for 30 min on ice. Cells were then washed and analyzed on a BD Biosciences FACSCalibur. The following Abs for flow cytometric analyses were obtained from BD Pharmingen: anti-CD4 FITC, anti-CD4 PE, anti-Vβ8.1/8.2 biotin, anti-Vβ6 FITC, anti-Fas FITC, anti-FasL PE, anti-CD69 PE, anti-CD44 PE, anti-CD62L PE, anti-CD25 FITC, streptavidin PE, streptavidin FITC, and anti-Fas. An isotype-matched control Ab was used to evaluate the background staining.

SEB-induced deletion of T cells in vivo

For SEB-mediated deletion experiments, SEB (25 or 50 μg; Sigma-Aldrich) was injected i.p. into 8- to 12-wk-old mice. Four to six mice were sacrificed on days 3, 6, 9, and 12, and spleen and mesenteric lymph nodes were removed. Single-cell suspensions were made by crushing organs. After RBC lysis, cells were stained with anti-Vβ8.1/8.2 FITC or anti-Vβ6 FITC and anti-CD4 PE. A total of 2×10^5 live CD4 cells was collected on a flow cytometer, and the percentage of CD4 cells expressing Vβ8 or Vβ6 was determined.

In vitro activation of DO11.10 CD4$^+$ TCR^{Tg} cells

Spleen and lymph nodes were removed from 8- to 12-wk-old DO11.10^{Tg}/PKC-$\theta^{+/+}$ and DO11.10^{Tg}/PKC-$\theta^{-/-}$ mice. After RBC lysis, single-cell suspensions were made. For in vitro activation, 3×10^6 spleen cells were cultured in 6-well plates with 1 μg/ml OVA peptide. After 24 h, cells were transferred into and expanded in the fresh medium containing 5 ng/ml IL-2 for 5 days.

AICD

Ag-activated DO11.10 TCR^{Tg} T cells were purified using T cell enrichment column. Activated CD4$^+$ T cells were restimulated in the presence of different concentrations of plate-bound anti-CD3 or anti-Fas Ab for different time periods. All cultures were supplemented with 5 ng/ml IL-2 to prevent spontaneous apoptosis. Cells were then harvested and washed once with ice-cold annexin V-binding buffer (10 mM HEPES (pH 7.5), 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 1.8 mM $CaCl_2$). Cell pellets were stained with PE-conjugated annexin V and 7-aminoactinomycin D (BD Biosciences), according to the manufacturer's protocol. Analyses were performed on a FACSCalibur (BD Biosciences) with CellQuest software. For SEB-treated mice, CD4$^+$ T cells were purified from SEB-treated or control mice on day 3 using CD4$^+$ T cell isolation kit (Miltenyi Biotec). A total of 3×10^5 purified CD4$^+$ T cell with 2×10^5 APC from B6 mice was restimulated with 1 μg of SEB for 12 h. To analyze Fas-mediated apoptosis, 3×10^5 purified CD4$^+$ T cells were restimulated with 1 μg of plate-bound anti-Fas Ab. Percentage of apoptotic Vβ8 T cells was determined, as described.

Western blot analysis

Cell lysates were resolved on SDS-PAGE and transferred to nitrocellulose membranes (Hy-bond C Super; Amersham). Membranes were blocked for 1 h in PBS containing 2% BSA and 0.05% Tween 20. Membranes were incubated overnight with rabbit antisera to caspase-8 (sc-7890; Santa Cruz Biotechnology), cleaved caspase-3, and mouse-specific Bid (Cell Signaling Technology), and then washed three times in PBS containing 0.05% Tween 20. Membranes were then incubated with HRP-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology). After three washes in PBS containing 0.05% Tween 20, signals were revealed by ECL Western blotting (Amersham) and visualized by autoradiography. For testing the levels of intracellular active caspase-3, cells were fixed and permeabilized using Cytofix/Cytoperm solution (BD Pharmingen) for 30 min at 4°C. Cells were washed with Perm/wash buffer (BD Pharmingen), and stained for 2 h in the dark at room temperature with 10 μl per sample of anti-active caspase-3-PE-conjugated Ab. Cells were collected, washed once, resuspended in Perm/wash buffer, and analyzed by FACS for PE-positive population.

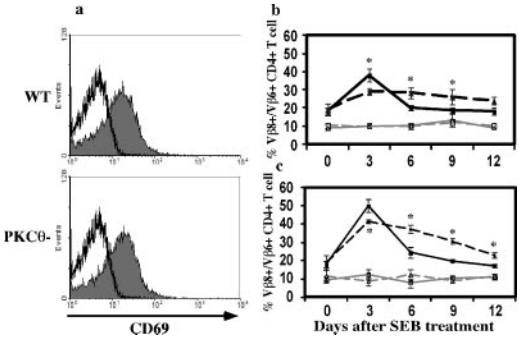

FIGURE 1. PKC-θ is required for SEB-induced deletion of Vβ8$^+$CD4$^+$ T cells. WT and *PKC-$\theta^{-/-}$* mice were injected i.p. with SEB. Vβ8$^+$CD4$^+$ and Vβ6$^+$CD4$^+$ subsets of T cells were then analyzed by a flow cytometer. *a*, CD69 levels on Vβ8$^+$CD4$^+$ T cells were detected before (black line) and 3 days after (shaded area) challenging the mice with SEB. *b*, WT (solid line) and *PKC-$\theta^{-/-}$* (dash line) mice were challenged with 25 μg of SEB. Percentage of Vβ8$^+$CD4$^+$ (black line) and Vβ6$^+$CD4$^+$ (gray line) T cells was determined using a flow cytometer at 0, 3, 6, 9, and 12 days after challenging the mice. $*$, Significantly ($p < 0.05$) differs from WT. *c*, Same analyses as described in *b*, except that the mice were challenged with 50 μg of SEB. Data shown were collected from four to six mice at each of the indicated days after SEB treatment. $*$, Significantly ($p < 0.01$) differs from WT.

Luciferase assays

Jurkat T cells (10^7) were transfected by electroporation with 20 μg of the WT 486-bp CD95 ligand luciferase reporter or reporters containing mutations in the distal (M1) or proximal (M2) or both (M1 plus M2) NF-AT sites together with 15 μg of the PKC-θ siRNA or scrambled siRNA expression plasmids or 15 μg of different indicated expression plasmids. Identical amounts of the corresponding parental vectors were used as control. For normalization, 200 ng of the *Renilla* luciferase reporter vector pTK-*Renilla*-LUC was used. After 36 h, cells were incubated for 8 h with OKT-3 (1 μg/ml) and anti-CD28 (2 μg/ml) Ab, and cross-linked with a secondary goat anti-rat Ig (10 μg/ml). Cells were then lysed and assayed for dual luciferase activity (Promega).

Results

Defective deletion of activated T cells in PKC-$\theta^{-/-}$ *mice*

To determine the function of PKC-θ in the activation-induced deletion of peripheral T cells in vivo, WT and *PKC-$\theta^{-/-}$* mice were challenged with a superantigen SEB, a widely used reagent to study Ag-induced responses in vivo. SEB specifically stimulates T cells with TCRs containing the Vβ8 element. Indeed, CD69, a T cell activation marker, was up-regulated on Vβ8$^+$CD4$^+$ T cells after challenge (Fig. 1*a*), confirming the stimulatory effects of SEB. We then monitored Vβ8$^+$CD4$^+$ T cells in spleens and lymph nodes of four to six WT or *PKC-$\theta^{-/-}$* mice by flow cytometric analysis at days 0, 3, 6, 9, and 12 after SEB treatment (25 μg) (Fig. 1*b*). As previously reported (11, 30), SEB treatment of WT mice (solid black line) resulted in a rapid increase (expansion phase) of Vβ8$^+$CD4$^+$ T cells from 15 to 40% within 3 days. The

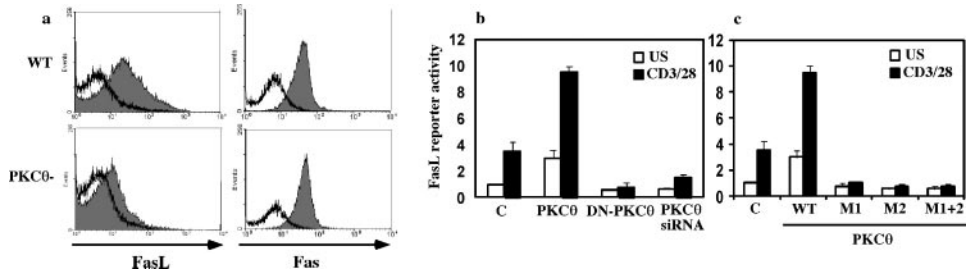

FIGURE 2. PKC-θ stimulates FasL expression. *a*, Surface expression of FasL (*left panels*) and Fas (*right panels*) on V$\beta8^+$CD4$^+$ T cells was detected by flow cytometric analysis before (black line) and 3 days after (shaded area) challenging the mice with SEB. *b*, PKC-θ stimulates FasL reporter activity. A luciferase reporter under the control of a FasL promoter was transfected into Jurkat cells alone (C, control) or together with expression plasmid encoding WT PKC-θ (PKCθ) or inactive PKC-θ (DN-PKCθ) or PKC-θ siRNA. Twenty-four hours after transfection, cells were either left in medium (□) or stimulated with anti-CD3 and CD28 Abs (■) for 8 h. Luciferase activity was then assayed. FasL-luciferase reporter activity is indicated as fold induction relative to the activity obtained from unstimulated cells in control group. *c*, PKC-θ-mediated stimulation of FasL reporter depends on two NF-AT binding sites. PKC-θ expression plasmid was transfected into Jurkat cells together with WT FasL reporter (WT) or reporter having first (M1) or second (M2) or both (M1 + 2) NF-AT binding sites mutated. Data shown are representative of at least three independent experiments.

expansion phase was followed by a slow decrease (deletion phase) in the percentage of V$\beta8^+$CD4$^+$ T cells. The expansion phase was relatively reduced in *PKC-θ*$^{-/-}$ mice (dashed line), which is consistent with the role of PKC-θ in stimulating T cell proliferation reported by previous in vitro studies (13, 14). Because SEB does not bind to TCR Vβ6 element, it had no obvious effects on V$\beta6^+$ T cells either in WT (solid gray line) or *PKC-θ*$^{-/-}$ mice (dashed gray line), indicating the specific stimulatory effects of SEB on V$\beta8^+$ cells. Although both WT and *PKC-θ*$^{-/-}$ mice decreased V$\beta8^+$CD4$^+$ cell population starting at day 3, the percentage of V$\beta8^+$CD4$^+$ cells in *PKC-θ*$^{-/-}$ mice was much higher than the WT mice, suggesting requirement for PKC-θ in the deletion of T cells in addition to expansion. Because PKC-θ is known to regulate the threshold of T cell activation (31), we challenged mice with higher concentrations of SEB (50 μg) (Fig. 1*c*). Expansion phase of V$\beta8^+$CD4$^+$ T cells was enhanced, as expected, by higher concentration of SEB. However, the defective deletion of V$\beta8^+$CD4$^+$ T cells observed in *PKC-θ*$^{-/-}$ mice appeared more obvious compared with WT mice. A total of 50 μg of SEB stimulated an expansion of V$\beta8^+$CD4$^+$ T cells for up to ~40% of total CD4$^+$ T cells in *PKC-θ*$^{-/-}$ mice, which was equivalent to the expansion phase of WT mice challenged with 25 μg of SEB. In contrast to the expansion phase, the deletion phase of the *PKC-θ*$^{-/-}$ mice challenged with 50 μg of SEB was still much reduced compared with WT mice challenged with 25 μg of SEB. These data suggest that defective deletion observed in *PKC-θ*$^{-/-}$ mice is not likely linked to the smaller Vβ8 cell population resulting from reduced expansion phase.

Requirement of PKC-θ in the up-regulation of FasL in vivo

Fas/FasL-mediated apoptosis plays a critical role in SEB-induced deletion of T cells, as SEB-mediated T cell deletion is defective in Fas- or FasL-deficient mice (3, 11). We therefore first examined surface Fas and FasL expression after SEB treatment by flow cytometric analysis. In agreement with previous reports (32, 33), both FasL and Fas were up-regulated on WT V$\beta8^+$CD4$^+$ T cells after SEB stimulation (Fig. 2*a*, *top two panels*). Fas expression levels on the T cells of SEB-treated WT and *PKC-θ*$^{-/-}$ mice were comparable, suggesting that the observed defective deletion of V$\beta8^+$CD4$^+$ in the absence of PKC-θ is not likely due to differential Fas expression. In contrast, up-regulation of FasL was greatly reduced in *PKC-θ*$^{-/-}$ mice compared with that of the WT mice. Reduced FasL levels on T cells of *PKC-θ*$^{-/-}$ mice were also observed on day 2 after SEB stimulation (data not shown). These results suggest a critical role of PKC-θ in stimulating FasL expression in vivo.

To further dissect the molecular mechanisms responsible for PKC-θ-regulated FasL expression, a FasL reporter described previously (34) was used. Cross-linking CD3/28 moderately stimulated FasL reporter activity in Jurkat cells (Fig. 2*b*). Forced expression of WT PKC-θ activated FasL reporter even in the absence of any stimulation, suggesting that ectopic expression of PKC-θ alone is sufficient to stimulate FasL reporter. The maximum reporter activity was obtained when Jurkat cells were stimulated by cross-linking TCR in the presence of WT PKC-θ, whereas kinase-deficient PKC-θ failed to activate FasL reporter upon TCR stimulation, suggesting that kinase activity of PKC-θ is essential. Furthermore, knockdown of PKC-θ, as we described previously (28, 29), prevented TCR stimulation-mediated activation of FasL reporter. These results clearly suggest that PKC-θ is required for activation of FasL reporter. Two NF-AT binding sites were found to be critical for FasL expression (34). Moreover, we, as well as others, have shown that PKC-θ regulates NF-AT pathway via enhancing Ca^{2+} influx in T cells (13, 29, 35). We therefore determined whether PKC-θ-mediated stimulation of FasL depends on the two NF-AT sites. Indeed, PKC-θ failed to stimulate FasL reporters that had either one or both NF-AT binding sites mutated (Fig. 2*c*). These results suggest that PKC-θ activates FasL expression at least in part via stimulating NF-AT pathway.

Requirement of PKC-θ in Fas-mediated apoptosis

Failed up-regulation of FasL on T cells cannot completely explain the defective depletion of T cells in *PKC-θ*$^{-/-}$ mice, because FasL expression on T cells is not absolutely required for SEB-induced deletion of T cells (33). It is thus possible that there are defects in Fas-mediated apoptosis in the absence of PKC-θ. To test this possibility, WT and *PKC-θ*$^{-/-}$ mice were first challenged with SEB to up-regulate surface Fas. CD4$^+$ T cells obtained from SEB-challenged mice were either left in medium or treated with anti-Fas Ab. Apoptotic V$\beta8^+$CD4$^+$ cells were then detected by annexin V or tetramethylrhodamine ethyl ester staining, as we described previously (28). Without any treatment, no obvious difference in spontaneous apoptosis was observed between WT and *PKC-θ*$^{-/-}$ T cells (Fig. 3*a*). Cross-linking Fas enhanced apoptosis in WT cells, as expected. Fas-induced apoptosis in *PKC-θ*$^{-/-}$ T cells was significantly less than that of WT cells, and was just slightly more than the control spontaneous apoptosis. Such difference in Fas-mediated apoptosis did not result from differential levels of surface Fas, because comparable levels of Fas were detected between WT and *PKC-θ*$^{-/-}$ T cells after SEB challenge (Fig. 2*a*).

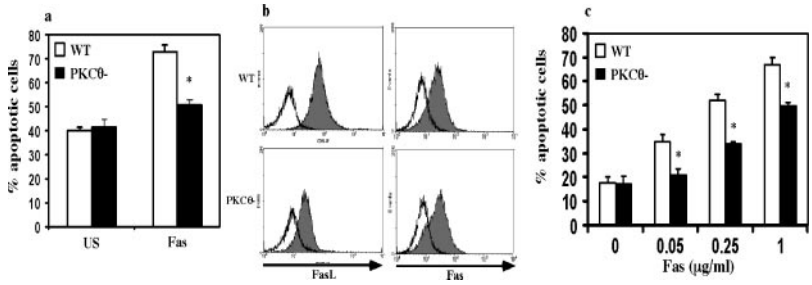

FIGURE 3. PKC-θ plays a role in Fas-mediated apoptosis. *a*, WT (□) and *PKC*-$\theta^{-/-}$ (■) mice were challenged with SEB for 3 days. T cells obtained from SEB-challenged mice were then cultured in medium unstimulated (US) or stimulated with anti-Fas Ab (Fas) for 8 h. Apoptotic cells were then detected with annexin V staining. ∗, Significantly ($p < 0.05$) differs from WT. *b*, Splenocytes obtained from DO11.10 TCR (WT) and *PKC*-$\theta^{-/-}$ DO11.10 TCR (PKC$\theta-$) mice were left in medium (black line) or stimulated with OVA peptide (1 μg/ml, shaded area) in the presence of IL-2 (5 ng/ml) for 5 days. The surface FasL (*left panels*) and Fas (*right panels*) were then analyzed with a flow cytometer. *c*, OVA-stimulated WT (□) and *PKC*-$\theta^{-/-}$ (■) T cells, as described in *b*, were treated with different concentrations of anti-Fas Ab (Fas) in the presence of IL-2 (5 ng/ml) for 8 h. Apoptotic cells were then detected with annexin V staining. ∗, Significantly ($p < 0.01$) differs from WT. Data shown are representative of at least three independent experiments.

Superantigen stimulates T cells differently from a ligand. We thus also examined *fas*-induced apoptosis in T cells stimulated by a ligand. *PKC*-$\theta^{-/-}$ mice were crossed with TCR transgenic mice (DO11.10) that express TCRs with a single specificity for OVA. Splenocytes obtained from DO11.10 and *PKC*-$\theta^{-/-}$ DO11.10 TCR^{Tg} were stimulated with OVA peptide. Previously, we have shown that *PKC*-$\theta^{-/-}$ T cells are susceptible to apoptosis upon TCR stimulation. However, such apoptosis can be prevented by exogenous IL-2 (28). T cells were thus stimulated in the presence of IL-2 (5 ng/ml). After stimulation, surface expression of FasL and Fas was monitored by flow cytometric analysis. In agreement with the results shown in Fig. 2, up-regulation of FasL, but not Fas, was reduced on $CD4^+$ *PKC*-$\theta^{-/-}$ T cells compared with that of the WT cells (Fig. 3*b*). OVA-stimulated WT and *PKC*-$\theta^{-/-}$ $CD4^+$ T cells were then induced to apoptosis by treatment with different concentrations of anti-Fas Ab. *PKC*-$\theta^{-/-}$ T cells were found again to be more resistant to Fas-induced apoptosis compared with WT cells at every concentration of anti-Fas Ab we tested (Fig. 3*c*). These results suggest that maximum apoptosis induced by stimulating Fas requires PKC-θ.

Critical role of PKC-θ in AICD

Activated T cells undergo AICD upon TCR restimulation. Fas/FasL-mediated apoptosis plays an essential role in AICD (2). We therefore compared AICD of T cells obtained from WT and *PKC*-$\theta^{-/-}$ mice. T cells obtained from SEB-challenged mice were either left in medium or stimulated again with SEB. Consistent with previous results (Fig. 3*a*), no obvious difference in spontaneous apoptosis of $V\beta8^+CD4^+$ cells (unstimulated cells) was observed between WT and *PKC*-$\theta^{-/-}$ T cells (Fig. 4*a*). SEB restimulation resulted in a significantly increased apoptosis in WT $V\beta8^+CD4^+$ T cells. However, AICD in the absence of PKC-θ was much reduced compared with the WT, and was only slightly higher than that of unstimulated cells, suggesting a role for PKC-θ in AICD. We also examined AICD using DO11.10 TCR^{Tg} mice. Splenocytes obtained from DO11.10 and *PKC*-$\theta^{-/-}$ DO11.10 mice were stimulated with OVA peptide in the presence of IL-2, as described in Fig. 3*c*. OVA-stimulated CD4 T cells were then induced to undergo AICD by a second stimulation with different concentrations of plate-bound anti-CD3 Ab (Fig. 4*b*). AICD of *PKC*-$\theta^{-/-}$ T cells was much reduced compared with that of WT cells. The differences in AICD observed between WT and *PKC*-$\theta^{-/-}$ T cells were especially obvious when stimulated with physiologically relevant lower concentrations of anti-CD3 Ab. Blocking Fas/FasL pathway with neutralizing anti-FasL Ab significantly reduced AICD both in WT and *PKC*-$\theta^{-/-}$ T cells, confirming the critical role of Fas/FasL in AICD (Fig. 4*c*). In the presence of anti-FasL Ab, there were still differences, although reduced, in the ACID between WT and *PKC*-$\theta^{-/-}$ T cells, suggesting a potential role of PKC-θ in Fas/FasL-independent AICD. These results demonstrated that optimal AICD also requires PKC-θ.

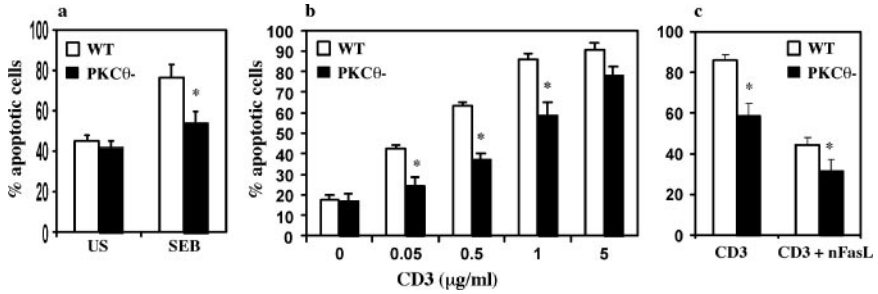

FIGURE 4. PKC-θ is required for AICD. *a*, WT (□) and *PKC*-$\theta^{-/-}$ (■) mice were challenged with SEB for 3 days. T cells obtained from SEB-challenged mice were then cultured in medium unstimulated (US) or stimulated with SEB (1 μg/ml) again for 12 h. Apoptotic cells were then detected with annexin V staining. ∗, Significantly ($p < 0.05$) differs from WT. *b*, Splenocytes obtained from DO11.10 TCR and *PKC*-$\theta^{-/-}$ DO11.10 TCR mice were first stimulated with OVA peptide (1 μg/ml) in the presence of IL-2 (5 ng/ml) for 5 days. OVA-stimulated WT (□) and *PKC*-$\theta^{-/-}$ (■) T cells were induced to undergo AICD by stimulating with different concentrations of plate-bound anti-CD3 Ab (CD3). Apoptotic cells were then detected with annexin V staining. ∗, Significantly ($p < 0.01$) differs from WT. *c*, OVA-stimulated WT (□) and *PKC*-$\theta^{-/-}$ (■) T cells were induced to undergo AICD by stimulating with anti-CD3 Ab (CD3, 1 μg/ml) or in the presence of neutralizing anti-FasL Ab (5 μg/ml). ∗, Significantly ($p < 0.05$) differs from WT. Data shown are representative of at least three independent experiments.

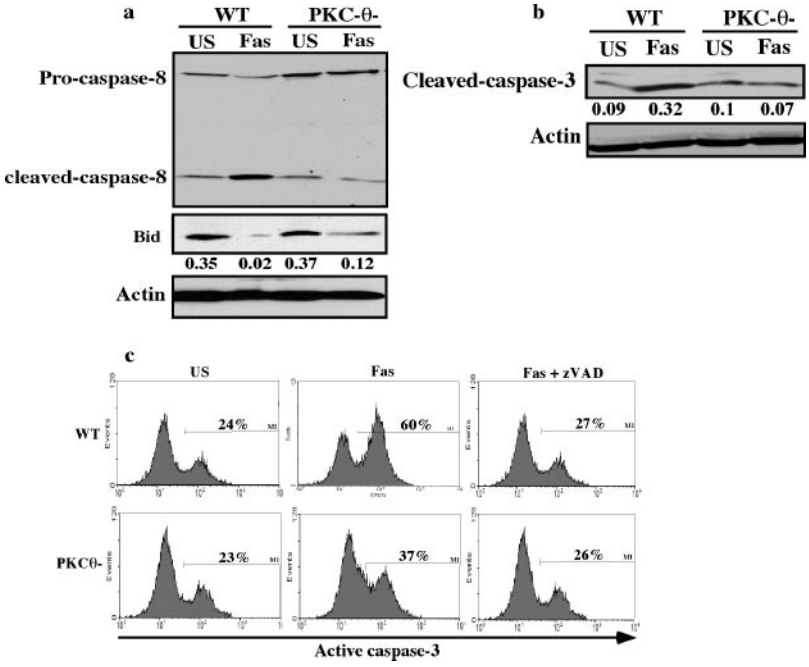

FIGURE 5. PKC-θ is required for Fas-induced activation of apoptotic signaling pathway. *a*, Splenocytes obtained from DO11.10 TCR (WT) and *PKC-θ*$^{-/-}$ DO11.10 TCR (PKC-$^{-}$ mice were first stimulated with OVA peptides (1 μg/ml) in the presence of IL-2 (5 ng/ml) for 5 days. OVA-stimulated WT (□) and *PKC-θ*$^{-/-}$ (■) T cells were left in medium unstimulated (US) or induced to undergo apoptosis by treatment with anti-Fas Ab (Fas). Three hours after Fas treatment, cells were lysed and subjected to Western blot analysis with Abs specifically recognizing caspase-8 (*top panel*) and Bid (*middle panel*). Actin (*bottom panel*) serves as a control for equal loading. Ratio of Bid to actin is indicated. *b*, Same treatment as described in *a*, and cleaved caspase-3 was detected by Western blot analysis. Ratio of cleaved caspase-3 to actin is indicated. *c*, Cells were treated as described in *a*, and intracellular active caspase-3 was detected by flow cytometric analysis with an Ab specifically recognizing only active caspase-3. A broad caspase inhibitor, zVAD, was used to inhibit apoptosis as a control. Data shown are representative of at least three independent experiments.

Requirement of PKC-θ for Fas-initiated apoptotic signaling pathway

Stimulation of Fas results in efficient recruitment of FADD, which then recruits and activates caspase-8 (36). Caspase-8 then cleaves and activates caspase-3 and Bid, resulting in apoptosis (37). We first compared activation of caspase-8 between WT and *PKC-θ*$^{-/-}$ T cells (*top panel*, Fig. 5*a*). OVA-stimulated CD4 T cells obtained from DO11.10 TCR^{Tg} mice were subjected to anti-Fas Ab treatment, as described in Fig. 3*c*. In response to Fas stimulation, procaspase-8 levels were reduced, whereas the cleaved active form of caspase-8 was correspondingly increased in WT cells, as expected. In contrast, there was no obvious decrease in procaspase-8, and increase in cleaved caspase-8 upon Fas stimulation in *PKC-θ*$^{-/-}$ T cells, suggesting a defect in Fas-induced activation of caspase-8. We next examined Bid, a substrate of caspase-8. Because Bid is a small molecule ~22 kDa, the cleaved product of Bid is difficult to detect by regular Western blot analysis. We thus monitored the reduction of full-length Bid resulting from cleavage (*middle panel*, Fig. 5*a*). Before Fas stimulation, levels of Bid between WT and *PKC-θ*$^{-/-}$ T cells were comparable. Stimulation of Fas resulted in greatly reduced full-length Bid in WT cells presumably due to cleavage of Bid, whereas *PKC-θ*$^{-/-}$ T cells maintained relatively higher levels of Bid than that of WT cells after Fas stimulation, which is consistent with the lower levels of active caspase-8 in *PKC-θ*$^{-/-}$ T cells. Lastly, we examined cleaved active form of caspase-3 using both Western blot and flow cytometric analyses. Western blot analysis detected significantly increased levels of cleaved caspase-3 in WT, but not in *PKC-θ*$^{-/-}$ T cells after stimulation of Fas (Fig. 5*b*). This result was confirmed using an Ab that is different from the one used in Western blot analysis, and specifically recognizes active caspase-3 in flow cytometric analysis (Fig. 5*c*). Without stimulation, levels of active caspase-3 were equivalent between WT and *PKC-θ*$^{-/-}$ T cells (~23%). After Fas stimulation, active caspase-3 was detected in 60% of WT cells, but only in 37% of *PKC-θ*$^{-/-}$ T cells. Furthermore, active caspase-3 was inhibited by a pan-caspase inhibitor, zVAD. These results suggest that PKC-θ is required for Fas-induced activation of caspase-8, which relays apoptotic signals to downstream molecules such as Bid and caspase-3.

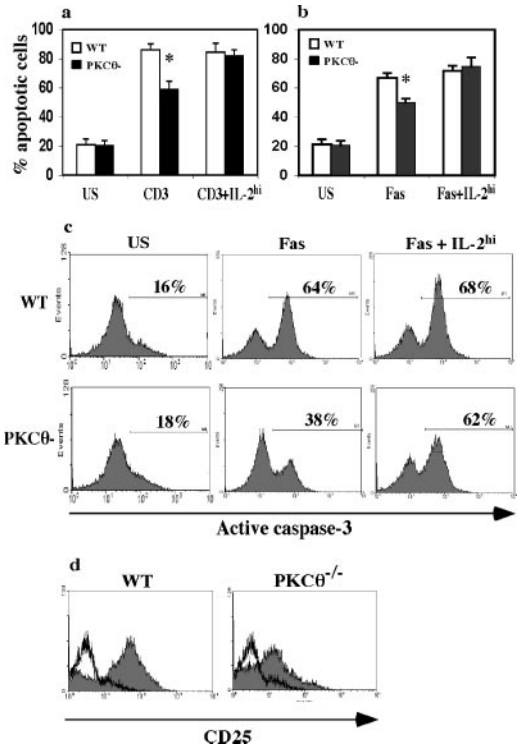

FIGURE 6. IL-2 restored AICD in *PKC-θ*$^{-/-}$ T cells. *a*, Splenocytes obtained from DO11.10 TCR (WT) and *PKC-θ*$^{-/-}$ DO11.10 TCR (PKC^{-} mice were stimulated with OVA, as described in Fig. 3*b*, or in the presence of 50 ng/ml IL-2. OVA-stimulated WT (□) and *PKC-θ*$^{-/-}$ (■) T cells were induced to undergo AICD by stimulating with plate-bound anti-CD3 Ab (CD3). Apoptotic cells were then detected with annexin V staining. *, Significantly ($p < 0.05$) differs from WT. *b*, OVA-stimulated WT (□) and *PKC-θ*$^{-/-}$ (■) T cells, as described in *a*, were treated with anti-Fas Ab (Fas). Apoptotic cells were then detected with annexin V staining. *, Significantly ($p < 0.01$) differs from WT. *c*, Cells were treated, as described in *b*, and intracellular active caspase-3 was detected by flow cytometric analysis with an Ab specifically recognizing only active caspase-3. *d*, CD25 levels on WT and *PKC-θ*$^{-/-}$ T cells before (solid line) and after (shaded area) OVA stimulation. Data shown are representative of at least three independent experiments.

Restoration of AICD and Fas-mediated apoptosis in PKC-$\theta^{-/-}$ *T cells by IL-2*

IL-2 is a critical factor required to potentiate T cells for AICD (38, 39). We determined whether the defective AICD of *PKC-*$\theta^{-/-}$ T cells can be reversed in the presence of a high concentration of extracellular IL-2. Splenocytes obtained from DO11.10 and *PKC-*$\theta^{-/-}$ DO11.10 mice were first stimulated with OVA peptide, as described in Fig. 3*b*, or in the presence of 50 ng/ml IL-2. OVA-stimulated CD4 T cells were then induced to undergo AICD by a secondary stimulation with anti-CD3 Ab (Fig. 6*a*). Consistent with previous results, preactivated *PKC-*$\theta^{-/-}$ T cells were relatively resistant to anti-CD3-induced apoptosis. However, IL-2 restored apoptosis of *PKC-*$\theta^{-/-}$ T cells to that of the WT levels. We also examined apoptosis induced by cross-linking Fas (Fig. 6*b*). In contrast to the absence of high concentration of IL-2, there was no significant difference in the Fas-mediated apoptosis between WT and *PKC-*$\theta^{-/-}$ T cells in the presence of 50 ng/ml IL-2. This result was also confirmed by analyzing active form of caspase-3 using a flow cytometer (Fig. 6*c*). As expected, cross-linking Fas triggered rapid apoptosis in WT T cells, reflected by detecting active caspase-3 in 64% of WT T cells after Fas stimulation, whereas only 38% of *PKC-*$\theta^{-/-}$ T cells contained active caspase-3 after Fas-cross-linking. However, IL-2 treatment restored the percentage of T cells containing active caspase-3 to that of the WT T cells (62%) in *PKC-*$\theta^{-/-}$ T cells. These data suggest that T cells are not fully potentiated for AICD in the absence of PKC-θ. We also examined the levels of CD25, a IL-2R. Indeed, CD25 levels were lower in *PKC-*$\theta^{-/-}$ T cells compared with that of the WT T cells. Therefore, PKC-θ promotes AICD at least in part via up-regulating CD25 that mediates the signals required to potentiate T cells for apoptosis.

Discussion

Our previous ex vivo experiments have shown that in contrast to WT cells, naive *PKC-*$\theta^{-/-}$ T cells are susceptible to apoptosis due to lack of TCR-induced up-regulation of Bcl-x_L. However, the observed apoptosis of *PKC-*$\theta^{-/-}$ T cells was prevented by forced expression of Bcl-x_L or IL-2 treatment (28). PKC-θ thus mediates the initial survival signals for T cells by promoting up-regulation of Bcl-x_L. In this study, we examined a different form of apoptosis that is restimulation of previously activated T cells. Fas/FasL-mediated apoptosis or apoptosis of restimulated T cells is closely related to AICD. In contrast to early survival signals that depend on Bcl-x_L, Fas/FasL-mediated apoptosis is independent of Bcl-x_L, as forced expression of Bcl-x_L cannot prevent Fas-mediated apoptosis (40). Using *PKC-*$\theta^{-/-}$ mice, we demonstrated a critical role of PKC-θ in SEB-induced clonal deletion of T cells in vivo. Similarly, AICD of the restimulated *PKC-*$\theta^{-/-}$ T cells is also reduced compared with the WT cells. Furthermore, we also demonstrated that up-regulation of FasL on T cells requires PKC-θ in vivo. PKC-θ thus regulates both up-regulation of FasL and Fas-mediated apoptosis.

We previously reported that purified T cells from *PKC-*$\theta^{-/-}$ mice displayed significant defects in proliferation as well as up-regulation of CD69 upon TCR stimulation (13, 14). In agreement, SEB-induced expansion of V$\beta 8^+$ T cells in PKC-θ null mice is relatively less than that of the WT mice. However, we did observe substantial proliferation of T cells in *PKC-*$\theta^{-/-}$ mice especially when challenged with higher concentration of SEB. In addition, up-regulation of CD69 is normal in *PKC-*$\theta^{-/-}$ mice after SEB challenge. This result suggests that PKC-θ-mediated signals required for T cell proliferation can somehow be compensated in vivo. Lack of NF-κB activation is partially responsible for the defective proliferation of PKC-$\theta^{-/-}$ T cells in vitro (14). Cytokines produced during immune responses may compensate for the requirement of PKC-θ due to their ability to activate NF-κB pathway. We have shown previously that inflammatory cytokines such as IL-1 and TNF-α can induce NF-κB activation in T cells independent of PKC-θ (14, 23).

We observed that FasL levels on the surface of *PKC-*$\theta^{-/-}$ T cells stimulated by SEB in vivo were significantly lower than that of the WT mice. PKC-θ thus is required for optimal stimulation of FasL. PKC-θ modulates T cell activation via regulating NF-κB, AP-1, and NF-AT pathways that also control the expression of FasL (12–14, 35). Villalba et al. (26) showed that forced expression of a constitutively active PKC-θ enhanced FasL expression in Jurkat cells. However, little is known about which pathways are necessary for induction of FasL expression in vivo. To study the role of PKC-θ in vivo in Fas/FasL-mediated apoptosis, we challenged *PKC-*$\theta^{-/-}$ mice with SEB. In addition, *PKC-*$\theta^{-/-}$ mice were crossed to DO11.10 TCR^{Tg} mice. We thus could stimulate CD4 T cells with a ligand. Under such conditions, up-regulation of FasL on T cells was defective in *PKC-*$\theta^{-/-}$ mice. We thus identified PKC-θ as one of the important factors required for stimulating FasL expression in vivo.

Transcriptional regulation of FasL expression is a critical mechanism for controlling Fas/FasL-mediated apoptosis. A variety of transcription factors has been shown to regulate FasL expression. NF-AT is a key regulator of FasL expression in response to T cell activation (34, 41). Two NF-AT binding sites, proximal and distal, were identified on FasL promoter region by footprint analysis. Previous studies, including ours, have shown that PKC-θ regulates NF-AT activation by enhancing Ca^{2+} influx in T cells (13, 29, 35). In this study, we show that PKC-θ regulates FasL expression at least in part via these two NF-AT binding sites on the promoter region, as mutation of the NF-AT sites resulted in abolished PKC-θ-mediated activation of FasL promoter activity. It has also been shown that AP-1 is required to cooperate with NF-AT to activate FasL expression (42). In addition to direct stimulation of FasL promoter, NF-AT is also likely to enhance FasL expression by inducing Egr family of transcription factors, Egr 2 and Egr3 (43), both of which are potent activators of FasL gene (44, 45). NF-κB is another positive regulator of FasL expression, as inhibitory nondegradable IκBαM prevents up-regulation of FasL (46). By deletion analyses, Villalba et al. (26) have shown that binding sites for NF-κB, AP-1, and NF-AT are all required for optimal FasL promoter activity, and deletion of NF-AT sites has most dramatic effects on FasL promoter activity. It thus suggests that NF-AT is the key regulator for FasL up-regulation, which is consistent with our in vitro analysis in Jurkat cells with M1 and M2 mutants (Fig. 2*c*). In this study, we demonstrated at the first time that PKC-θ is required for stimulating endogenous FasL gene expression using ex vivo and in vivo stimulation of T cells with SEB or a ligand, OVA. Based on these results, we believe that PKC-θ regulates FasL expression in vivo also via multiple signaling pathways, including NF-κB and AP-1, in addition to NF-AT.

Previously, we have shown that naive *PKC-*$\theta^{-/-}$ T cells are susceptible to apoptosis, especially in response to TCR stimulation (28). To our surprise, *PKC-*$\theta^{-/-}$ T cells are relatively resistant to Fas-mediated apoptosis compared with the WT cells. Because surface Fas levels are equivalent between WT and *PKC-*$\theta^{-/-}$ T cells after SEB or OVA stimulation, the observed resistance to Fas-induced apoptosis is not due to differential Fas expression. It is thus possible that PKC-θ plays a role in Fas-mediated death signals. Indeed, in the absence of PKC-θ, T cells are less sensitive to Fas-induced apoptosis. Fas/FasL-mediated apoptosis contributes significantly to AICD in vitro and in vivo (3, 47, 48). In agreement

with this notion, we showed that blocking FasL resulted in reduced AICD. Compared with WT cells, *PKC-θ*$^{-/-}$ T cells are less sensitive to AICD, confirming the potential role of PKC-θ in Fas/FasL-mediated AICD. The differences in AICD between WT and *PKC-θ*$^{-/-}$ T cells are reduced in the presence of FasL blocking Ab. PKC-θ-regulated Fas/FasL apoptosis is thus mostly responsible for the observed differences in AICD between WT and *PKC-θ*$^{-/-}$ T cells. Moreover, stimulation of Fas failed to fully activate caspase-8, a critical caspase required to initiate the apoptotic process. This observation was further confirmed by lack of activation of downstream molecules of caspase-8, caspase-3, and Bid. Therefore, PKC-θ is required for activating Fas-mediated apoptotic pathway. The exact molecular mechanisms responsible for the function of PKC-θ in Fas-mediated apoptosis are currently under investigation. One of the possibilities is that PKC-θ directly mediates Fas signalings. However, knockdown of PKC-θ had no effects on apoptosis induced by cross-linking Fas in Jurkat T cells as well as in several other transformed human T cells (data not shown). In agreement with our results, Villalba et al. (26) also reported that a pharmacological inhibitor of novel PKCs, including PKC-θ, did not affect Fas-mediated apoptosis in Jurkat cells. Thus, the available data do not favor the possibility that PKC-θ directly regulates Fas-mediated death signals. However, because these results were obtained using human transformed T cell lines, we cannot exclude the possibility that PKC-θ regulates Fas-mediated signals in mouse primary T cells. The other possibility is that PKC-θ is required for maximally priming naive T cells for Fas-mediated apoptosis. Naive T cells are resistant to Fas-mediated apoptosis unless being activated. T cell activation process primes naive T cells for apoptosis not only by up-regulation of apoptotic molecules such as FasL and Fas, but also by down-regulation of antiapoptotic molecules such as c-FLIP (39). Our results indicate that T cells are not fully potentiated for AICD- and Fas-mediated apoptosis in the absence of PKC-θ, because high concentration of IL-2 can restore AICD in *PKC-θ*$^{-/-}$ T cells. Up-regulation of Fas is normal in *PKC-θ*$^{-/-}$ mice. Moreover, the antiapoptotic molecule c-FLIP is kept at low levels in *PKC-θ*$^{-/-}$ mice (data not shown). However, we did find that CD25 levels are lower in *PKC-θ*$^{-/-}$ T cells. Because IL-2 is a critical factor for potentiating T cells for AICD (38), PKC-θ most likely promotes AICD at least in part by up-regulating CD25 that mediates IL-2 signals required to potentiate T cells for Fas-mediated apoptosis.

Fas/FasL-mediated apoptosis contributes to the tissue damage of CNS associated with experimental allergic encephalomyelitis (EAE), as *lpr* and *gld* mice are relatively resistant to the development of clinical EAE (49, 50). Similarly, PKC-θ null mice were also found to be resistant to EAE partly due to impaired Th1 responses (16, 18). Interestingly, PKC-θ was shown to be critical for Th2, but not Th1, responses in several other disease models (15, 17). The question is why *PKC-θ*$^{-/-}$ mice are able to develop efficient Th1 immunity in other models, whereas they fail to induce Th1-dependent tissue damage in EAE. It is possible that impaired FasL/Fas pathway may also contribute to the resistance to EAE in *PKC-θ*$^{-/-}$ mice in addition to impaired Th1 responses. Besides T cell activation and survival, PKC-θ-regulated FasL/Fas pathway is thus another layer of control in PKC-θ-regulated immune responses. Altogether, PKC-θ is a critical molecule that regulates T cell function at multiple stages in T cell-mediated immune responses.

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Disclosures

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