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*J Immunol* published online 14 May 2012
http://www.jimmunol.org/content/early/2012/05/14/jimmunol.1102941

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Protein Kinase C-0 Promotes Th17 Differentiation via Upregulation of Stat3

Myung-Ja Kwon, Jian Ma, Yan Ding, Ruiqing Wang, and Zuoming Sun

Although protein kinase C-0 (PKC-0)-deficient mice are resistant to the induction of Th17-dependent experimental autoimmune encephalomyelitis, the function of PKC-0 in Th17 differentiation remains unknown. In this article, we show that purified, naive CD4 PKC-0<–/– T cells were defective in Th17 differentiation, whereas Th1 and Th2 differentiation appeared normal. Activation of PKC-0 with PMA promoted Th17 differentiation in wild type (WT) but not PKC-0<–/– T cells. Furthermore, PKC-0<–/– T cells had notably lower levels of Stat3, a transcription factor required for Th17 differentiation, and PMA markedly stimulated the expression of Stat3 in WT but not PKC-0<–/– T cells. In contrast, activation of Stat4 and Stat6, which are critical for Th1 and Th2 differentiation, was normal in PKC-0<–/– T cells. Forced expression of Stat3 significantly increased Th17 differentiation in PKC-0<–/– T cells, suggesting that reduced Stat3 levels were responsible for impaired Th17 differentiation, and that Stat3 lies downstream of PKC-0. Constitutively active PKC-0, or WT PKC-0 activated by either PMA or TCR cross-linking, stimulated expression of a luciferase reporter gene driven by the Stat3 promoter. PKC-0-mediated activation of the Stat3 promoter was inhibited by dominant-negative AP-1 and IkB kinase-β, but stimulated by WT AP-1 and IkB kinase-β, suggesting that PKC-0 stimulates Stat3 transcription via the AP-1 and NF-κB pathways. Lastly, conditions favoring Th17 differentiation induced the highest activation level of PKC-0. Together, the data indicate that PKC-0 integrates the signals from TCR signaling and Th17 priming cytokines to upregulate Stat3 via NF-κB and AP-1, resulting in the stimulation of Th17 differentiation.

The Journal of Immunology, 2012, 188: 000–000.

Diff erentiation of naïve T cells into specific Th lineages is a critical checkpoint for controlling immune responses. Altered regulation of this checkpoint may lead to aggravated autoimmunity by overproduction of Th cells that cause pathogenic inflammation. Historically, Th cells have been classified as either Th1 cells, which produce IFN-γ, or Th2 cells, which produce IL-4 (1). In 2003, however, Cua et al. (2) made the seminal observation that IL-23, rather than the Th1 cytokine IL-12, was critical for the development of experimental autoimmune encephalomyelitis (EAE), a condition long believed to be a Th1-dependent autoimmune disease. Langrish et al. (3) then showed that IL-23 is required for the expansion of a population of IL-17–producing, pathogenic T cells that induce EAE when adoptively transferred to naïve mice. This observation shifted the traditional Th1/Th2 paradigm by addition of a new lineage of Th17 cells to the Th1/Th2 model. Th17 cells produce IL-17, IL-21, IL-22, and GM-CSF cytokines (4–6) that can induce massive tissue inflammation because of the broad distribution of their receptors on both immune and nonimmune cells. It should be noted that IL-17 is not absolutely required for the development of EAE, but it is able to induce and promote EAE development (7). In addition to EAE, Th17 cells are also the pathogenic T cell in animal models of collagen-induced arthritis (8) and inflammatory bowel disease (9). Circumstantial evidence has accumulated to suggest a patho-immunological role for Th17 cells in multiple human autoimmune disorders, including multiple sclerosis (10), rheumatoid arthritis (11), asthma (12), inflammatory bowel disease (13), and psoriasis (14). A greater understanding of the mechanisms responsible for the regulation of Th17 differentiation will facilitate the development of treatments targeting Th17-mediated autoimmunity.

To differentiate into Th17 cells, naïve T cells must be activated via the TCR in the presence of TGF-β and IL-6 (15, 16). TGF-β is required for the expression of retinoic acid-related orphan receptor γt (RORγt), a master transcription factor (17), and IL-6 is needed to activate Stat3 (18, 19). Both factors are critical for Th17 differentiation (20). Upon binding to IL-6, the IL-6R component gp130 dimerizes and activates JAK, which physically associates with gp130 and phosphorylates its cytoplasmic domain. This creates a docking site for the Stat3 Src homology 2 domain, and recruited Stat3 molecules are phosphorylated at Y705. This results in protein dimerization, which is required for translocation of Stat3 into the nucleus and its ability to bind to target DNA. The absolute requirement for Stat3 in Th17 differentiation was demonstrated by the failure to form Th17 cells in the absence of Stat3, as well as the increased production of IL-17 by T cells expressing a constitutively active (CA) form of Stat3 (20, 21). Chen et al. (22) demonstrated that Stat3 directly binds to and activates the IL-17 promoter. Although it is clear that IL-6–induced Stat3 activation is critical for Th17 differentiation, little is known about how the expression of Stat3 is regulated during the differentiation process.

PKC-0 is a critical molecule that mediates TCR signals during T cell activation and differentiation (23). Our studies have con-
truded to understanding the role of PKC-θ in vivo by the creation of PKC-θ−/− mice (23–29). Purified PKC-θ−/− T cells exhibit significantly diminished T cell proliferation and IL-2 production caused by defects in the activation of the AP-1, NF-κB, and NFAT pathways (24, 27, 30), and this result has been confirmed by other investigators using a strain of independently generated PKC-θ−/− mice (31). PKC-θ was initially thought to be required for Th1 responses, based on studies using the EAE model (32). With the recent discovery that Th17 cells are responsible for the EAE phenotype (33), this raises the possibility that PKC-θ is required for the differentiation or function of Th17 cells (34).

In this study, we demonstrate that PKC-θ mediates the signals required to stimulate Stat3 expression, which is critical for Th17 differentiation (20, 21). Th17 differentiation was greatly inhibited in PKC-θ−/− T cells, and this was accompanied by reduced levels of Stat3. However, forced expression of Stat3 rescued the Th17 differentiation phenotype in PKC-θ−/− T cells. Reduced Stat3 was observed at both the protein and mRNA levels in PKC-θ−/− T cells, suggesting PKC-θ mediates signals that are required to stimulate Stat3 transcription. Indeed, Stat3 mRNA was upregulated by the PKC-θ activator PMA, to a much greater degree in wild-type (WT) than in PKC-θ−/− T cells. Furthermore, PMA also increased Th17 differentiation in naive WT, but not naive PKC-θ−/− T cells, both results indicating PMA exerts its effects through the activation of PKC-θ. We also showed that PKC-θ−/− mediated activation of the AP-1 and NF-κB pathways stimulated the Stat3 promoter. Taken together, our results indicate that PKC-θ stimulates Th17 differentiation, at least in part, via AP-1 and NF-κB-dependent upregulation of Stat3 expression.

Materials and Methods

Animal care and purification of CD4

WT and PKC-θ−/− C57BL/6 mice have been described previously (29). Mouse care and experimental procedures were performed under pathogen-free conditions following institutional guidance and approved protocols from the Research Animal Care Committee of the City of Hope Medical Center. CD4+ T cells were purified from spleen by negative selection using AutoMax (Miltenyi Biotec, Bergisch-Gladbach, Germany). Purified T cells were cultured in RPMI 1640 media with 10% FBS, 100 μM streptomycin, and 100 U/ml penicillin.

Th cell differentiation

Naïve T cells were stimulated (3 d) with plate-bound anti-CD3, soluble anti-CD28 (BD Pharmingen, San Diego, CA), TGF-β (R&D Systems, Minneapolis, MN), IL-6 (PeproTech, London, U.K.), and IL-23 (R&D Systems, Minneapolis, MN), including anti-mouse IL-4 and anti-mouse IFN-γ (BioLegend, San Diego, CA). For detection of IL-21, IL-22, and GM-CSF, naïve T cells were cultured under Th17 priming conditions for 3 d with anti-CD3 and anti-CD28 in presence of IL-6 (20 ng/ml) and TGF-β1 (1 ng/ml) in IMDM with 10% FBS, 2 mM t-glutamime, 50 μM 2-ME, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C with 5% CO2. Then cells were rested for 2 d with IL-2 (2 ng/ml). Cells were further restimulated with Th17 priming conditions for 3 more days. For Th1 differentiation, naïve T cells were cultured with anti-CD3, anti-CD28, IL-12 (20 ng/ml), and anti-IL-4 (11B11, 10 ng/ml; eBioscience, San Diego, CA) in RPMI 1640 with 10% FBS, 2 mM t-glutamime, 50 μM 2-ME, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C with 5% CO2. For Th2 differentiation, naïve T cells were cultured with anti-CD3, anti-CD28, IL-4 (10 ng/ml), anti–IFN-γ (XMG1.2, 10 ng/ml; eBioscience), and anti–IL-12 (C17.8, 10 ng/ml) in RPMI 1640 for 3 d.

Retrovirus expression of Stat3 to rescue Th17 differentiation

Retroviral murine stem cell virus internal ribosomal entry site GFP (MIG) or Stat3-expressing (MIG-Stat3) plasmids were transfected into Phoenix-Eco cells using Lipofectamine 2000 (Invitrogen). After 48 h, viral supernatants were collected, passed through 0.4-μm filters, and stored at −80°C until use. For transduction, naïve T cells were first activated with anti-CD3 and anti-CD28 Abs for 24 h, then spin infected with viral supernatants (2500 rpm, 30°C for 2 h) in the presence of 8 μg/ml polybrene (Sigma-Aldrich).

After spin infection, IL-6 and TGF-β1 were added to the culture media to induce Th17 differentiation.

Plasmid transfection and luciferase assay

PKC-θ(CA) and dominant-negative (DN) PKC-θ constructs were described previously (23). Anti-Stat3 and phospho-Stat3 (Y705F) Abs were obtained from Cell Signaling (Danvers, MA). The Stat3 promoter reporter and Stat3 promoter reporters with mutant Stat3-binding element (SBE) and mutant cAMP response element (CRE) were obtained from Dr. Toshio Hirano (Osaka University Medical School, Osaka, Japan). Jurkat cells were transfected with plasmid DNA by electroporation as described previously (28) and stimulated 24 h later with PMA or anti-CD3 and anti-CD28 Abs. Luciferase assays were performed using a dual luciferase assay kit (Promega, Milwaukee, WI).

Western blot analysis

Cells were treated with either PMA or Th17 priming cocktails, then lysed. Western blot analyses were performed with Abs recognizing Stat3 (Santa Cruz Biotechnology, Santa Cruz, CA), phospho-STAT3, phospho–PKC-θ and phospho-ERK (Cell Signaling, or β-actin (Sigma, St. Louis, MO).

RT-PCR and quantitative PCR

Total RNA was prepared using TRIzol, and cDNA was prepared according to the manufacturers’ instructions (Invitrogen Life Technologies, Carlsbad, CA). Primers for RT-PCR (5′ to 3′) were mouse IL-17A: forward, GATACTCTCTTGATCTTG; reverse, TTCTGACTTTGCTTCGAA-T; mouse Stat3: forward, CTGGAGACA CGTCATCTGAA; reverse, ACTCTTTTGCAAGGAAGCTT; mouse SOCS3: forward, TCTCTAT GTGGG CCGTAGG; reverse, GCACATGTCTCCTGAGTT; mouse HPR: forward, CAA TGGATCCCATCAGAT; reverse, GTGCGGAAATCATCC TGGGAA. Primers used for quantitative RT-PCR (5′ to 3′) were mouse Stat3: forward, CGGAGAAGCTTGTTGAGTTG; reverse, CTTCCGACGACCCAGCTT; GAPDH: forward, ACCACAGTCATGCCATCAT; reverse, TCCACACCCGGTTGCTGA. PCR was performed using an iCycler (Bio-Rad Laboratories, Hercules, CA). All samples were normalized to GAPDH expression.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assays were performed as previously described (35). In brief, chromatin samples were immunoprecipitated with Abs recognizing c-Fos, p65, p50, and acetylated histone H4 (AcH4). Immune complexes were captured using protein A-Sepharose beads and then eluted. After reverse cross-linking and digestion of proteins with proteinase K, DNA was purified by phenol/chloroform extraction. PCR was performed using primers designed to amplify a 368-bp fragment that contained the 𝑔78 –110 region of the Stat3 promoter (5′ to 3′): forward, GTGACACCTGGGGACGCCC; reverse, CGGCGGACCGCTGTACCT; CC. Primers designed to amplify the control β-actin promoter (5′ to 3′) were: forward, CAGGCCAATTTGACGCT; reverse, TTTG GGACACGACCCAGAG.

FACS analysis

Th17 primed cells were activated (4 h) with PMA, ionomycin (Sigma), and GolgiStop (BD Bioscience Pharmingen). Cell surface staining using an FITC-conjugated anti-CD4 Ab and intracellular staining for IL-17 were performed as per manufacturer’s instructions (BD Pharmingen). Allophycocyanin-conjugated Abs specific to IL-17 (eBioscience) were used for FACS analysis using FACS Canto II (BD Bioscience) and FlowJo software.

Results

PKC-θ promotes Th17 differentiation

To investigate the role of PKC-θ in Th17 differentiation, purified naïve CD4 WT and PKC-θ−/− mouse T cells were stimulated with anti-CD3/28 Abs in IMDM containing TGF-β and IL-6, a condition that has been reported to be optimal for Th17 differentiation (36). Under these conditions, WT T cells differentiated into greatly more IL-17–producing cells (44.6%) than those stimulated in IMDM (36). Consistent with the IMDM
FIGURE 1. PKC-θ promotes Th17 differentiation. (A) Impaired Th17 differentiation is observed in PKC-θ<sup>−/−</sup> T cells. Purified naive CD4<sup>+</sup> WT and PKC-θ<sup>−/−</sup> T cells were stimulated by anti-CD3/28 Abs in IMDM containing TGF-β and IL-6 for Th17 differentiation, the Th17 priming conditions. (B) Activation of PKC-θ by PMA stimulates Th17 differentiation in WT, but not PKC-θ<sup>−/−</sup> T cells. WT and PKC-θ<sup>−/−</sup> T cells were differentiated into Th17 in RPMI 1640 medium in the absence (top two panels) or presence of 10 nM PMA (bottom two panels). (C and D) PKC-θ regulates the expression of IL-17 and ROR<sub>γt</sub>. WT T cells (black bars) and PKC-θ<sup>−/−</sup> T cells (open bars) were stimulated as indicated in (B), and expression of IL-17 (C) and ROR<sub>γt</sub> (D) was detected by q-PCR. The expression levels are presented as the fold induction relative to the signals detected in naive T cells. (E) There are no obvious differences in apoptosis between WT and PKC-θ<sup>−/−</sup> T cells. WT and PKC-θ<sup>−/−</sup> T cells were stimulated by Th17-inducing conditions for the indicated times, and apoptotic cells were monitored by Annexin V and 7-aminoactinomycin D. (F–J) Impaired production of Th17 cytokines in PKC-θ<sup>−/−</sup> T cells. WT T cells (open bars) and PKC-θ<sup>−/−</sup> T cells (black bars) were differentiated under Th17-inducing conditions, then expanded in (Figure legend continues)
result, a greater proportion of Th17 cells was detected in the WT T cell (34.2%) than in PKC-θ−/− T cell population (19.8%; Fig. 1B, top two panels). Therefore, the loss of PKC-θ inhibited Th17 differentiation. Conversely, the activation of PKC-θ in WT cells by PMA during differentiation dramatically increased the proportion of Th17 cells from 34.2 to 51.2% (Fig. 1B, left two panels). In contrast, PMA had almost no effect on the differentiation of Th17 cells in PKC-θ−/− T cells (19.8% without PMA versus 23.3% with PMA; Fig. 1B, right two panels), suggesting that PKC-θ plays a specific role in the induction of Th17 differentiation. We used quantitative PCR (q-PCR) to detect the mRNA levels of IL-17 (Fig. 1C) and RORγt (Fig. 1D), which is the master transcription factor instructing Th17 differentiation. Under Th17 priming conditions, the expression of IL-17 and RORγt mRNA was significantly reduced in PKC-θ−/− T cells compared with WT T cells. Furthermore, the addition of PMA dramatically increased IL-17 and RORγt mRNA expression in WT T cells compared with PMA-treated PKC-θ−/− T cells, indicating a specific and positive role for PKC-θ in RORγt-dependent Th17 differentiation. Apoptotic cells were monitored in Th17 differentiation, and no obvious difference in apoptosis was observed between WT and PKC-θ−/− T cells (Fig. 1E). This result excludes the possibility that the reduced numbers of differentiated Th17 cells in PKC-θ−/− T cells is due to apoptosis. Th17 cells also produce IL-21, IL-22, and GM-CSF (4–6), but the differentiation method we used in the earlier studies produces little of these cytokines, so we adopted an alternative differentiation protocol (5) to augment their expression. Initially, differentiated cells, as described in Fig. 1B, were expanded in IL-2, then restimulated in the presence of TGF-β and IL-6 alone or together with either IL-23 or TNF-α. In the absence of TGF-β and IL-6 (condition “ThN”), IL-17A, IL-17F, IL-21, IL-22, and GM-CSF were either undetectable or at very low levels both in WT and PKC-θ−/− T cells (Fig. 1F–J), suggesting absence of Th17 cells. In contrast, the cytokines typically produced by Th17 cells were readily detectable after TGF-β and IL-6 stimulation. The levels of most Th17 cytokines in stimulated WT cells (Fig. 1F–J, open bars) were significantly greater than those in the stimulated PKC-θ−/− T cells (Fig. 1F–J, black bars), except for GM-CSF, which was nearly equivalent (Fig. 1J). These results further confirm an essential role for PKC-θ in the differentiation of Th17 cells but indicate that PKC-θ is dispensable for GM-CSF production. As an additional control, we stimulated naive WT and PKC-θ−/− T cells under conditions that promote Th1 and Th2 differentiation, and did not detect obvious differences between the WT and PKC-θ−/− T cell populations for Th1 and Th2 cells (Fig. 1K, IL), suggesting PKC-θ is not required for Th1 or Th2 differentiation under these conditions. Taken together, our results indicate that PKC-θ is specifically required to promote the differentiation of Th17 cells.

**Reduced expression of Stat3 is responsible for impaired Th17 differentiation in PKC-θ−/− T cells**

To identify molecules downstream of PKC-θ in the Th17 differentiation pathway, we looked in PKC-θ−/− T cells for altered expression of proteins that were known to regulate Th17 differentiation. We found that levels of Stat3, a transcription factor essential for Th17 differentiation (20, 21), were significantly lower in PKC-θ−/− T cells on Western blots (Fig. 2A, top panel). The lower levels of Stat3 were observed even in untreated naive PKC-θ−/− T cells (Fig. 2A, top panel, 0 time point), suggesting that PKC-θ is required for maintaining Stat3 expression in naive peripheral T cells. In response to TCR stimulation, which greatly stimulates PKC-θ activity (26, 37), Stat3 levels increased with the stimulation time (10 h, 30 h) to a much greater degree in WT T cells than in PKC-θ−/− T cells, confirming the requirement of PKC-θ for Stat3 expression. Phosphorylation of Y705 is essential for activation of Stat3 (19); therefore, an Ab specifically recognizing the Y705-phosphorylated form of Stat3 (p-Stat3) was used to detect activated Stat3 on Western blots (Fig. 2A, second panel). Phospho-Stat3 was not detected in untreated naive T cells, and TCR stimulation led to phosphorylation of Stat3 as expected. Although phospho-Stat3 was readily detected in WT T cells after stimulation, phospho-Stat3 levels were markedly lower in PKC-θ−/− T cells, probably reflecting the overall lower levels of Stat3 in these cells (Fig. 2A). Shorter stimulation times (10–60 min) were also tested (Fig. 2B), and Stat3 levels were consistently lower in PKC-θ−/− T cells compared with WT T cells (Fig. 2B, top panel). This was also reflected by lower levels of phospho-Stat3 in the PKC-θ−/− T cells (Fig. 2B, middle panel). However, in contrast with longer stimulation times (Fig. 2A), no obvious increase in Stat3 protein levels was observed after stimulation of either cell type (Fig. 2B), possibly because longer time periods are needed for transcription and protein synthesis.

Because Th17 priming conditions increased Stat3 expression (Fig. 2A), we examined whether TCR signaling or Th17 differentiation cytokines were responsible for the stimulation of Stat3 expression (Fig. 2C). TCR stimulation only slightly increased Stat3 levels, whereas Th17 priming conditions, including both TCR and TGF-β/IL-6 stimulation, increased Stat3 expression to maximum levels, suggesting that TCR and cytokine signals cooperate to stimulate Stat3 expression. Furthermore, we showed that IL-6, but not TGF-β, treatment increased Stat3 expression (Fig. 2C), suggesting that IL-6-mediated signals cooperate with TCR signals to maximally stimulate Stat3 expression. In addition, PMA, the PKC-θ activator, greatly increased Stat3 levels (Fig. 2C). To determine the role of PKC-θ in the observed upregulation of Stat3, both WT and PKC-θ−/− T cells were stimulated by TCR or Th17 cytokines (Fig. 2D). Stat3 messengers were much lower in PKC-θ−/− T cells compared with WT T cells, indicating that PKC-θ is a critical regulator for Stat3 expression. As controls, we examined the expression of Stat4 (Fig. 2E), which is critical for Th1 differentiation, and Stat6 (Fig. 2F), which is critical for Th2 differentiation. No obvious difference in the levels of the phosphorylated active form or total Stat4 were detected between WT and PKC-θ−/− T cells during Th1 differentiation (Fig. 2E, left panel), which was also confirmed by the ratio of phospho-Stat4 to the β-actin loading control (Fig. 2E, middle panel) and total Stat4 to β-actin (Fig. 2E, right panel). Similarly, no differences were detected in the phosphorylated active form of Stat6 or total Stat6 between WT and PKC-θ−/− T cells during Th2 differentiation (Fig. 2F). These results are consistent with our observation that the differentiation of PKC-θ-deficient cells into Th1 and Th2 is not affected in vitro (Fig. 1K, IL), and suggest the selective requirement for PKC-θ in the expression of Stat3 is essential for Th17 differentiation. To determine whether the lower Stat3 levels in PKC-θ−/− T cells begin early during T cell development, we...
FIGURE 2. PKC-θ is required to upregulate Stat3. (A and B) Reduced Stat3 levels in PKC-θ−/− T cells. Purified WT and PKC-θ−/− T cells were stimulated with Th17 conditions for the indicated times in (A) and (B). Cells were then lysed for Western blot analysis that used Abs specific for Stat3, p-Stat3, and PKC-θ. β-actin served as the loading control. The number above each Stat3 and p-Stat3 band is the ratio of intensity between the band and the corresponding load control. (C) Th17 priming cytokines and PMA stimulate Stat3 expression. T cells were stimulated by indicated conditions, and Stat3 expression was analyzed by Western blot. The number above each band is the ratio of intensity between the band and the corresponding load control. (D) Reduced Stat3 messengers in the absence of PKC-θ. WT and PKC-θ−/− T cells were stimulated as indicated, and Stat3 messengers were detected by q-PCR. (E) Normal activation of Stat4 in PKC-θ−/− T cells. WT and PKC-θ−/− T cells were differentiated under neutral or Th1 conditions, and the p-Stat4 (left top panel) and total Stat4 (left middle panel) were analyzed by Western blots. Ratios of p-Stat4 to loading control β-actin (middle panel) and Stat4 to β-actin (right panel) were averaged from three independent experiments. (F) Normal activation of Stat6 in PKC-θ−/− T cells. WT and PKC-θ−/− T cells were differentiated under neutral or Th2 conditions, and the p-Stat6 (left top panel) and total Stat6 (left middle panel) were analyzed by Western (Figure legend continues)
PKC-θ activates by PMA stimulation increased Stat3 protein to much higher levels in WT cells than in PKC-θ−/− T cells. WT and PKC-θ−/− T cells were either untreated (−) or stimulated with increasing amounts of PMA. Total Stat3 protein was detected on Western blots using β-actin as a loading control. The number above each band is the ratio of intensity between the band and the corresponding load control. (*p < 0.05, **p < 0.01).

Figure 3. PKC-θ regulates Stat3 transcription. (A) PKC-θ activated by PMA stimulation increased Stat3 protein to much higher levels in WT cells than in PKC-θ−/− T cells. WT and PKC-θ−/− T cells were either untreated (−) or stimulated with increasing amounts of PMA. Total Stat3 protein was detected on Western blots using β-actin as a loading control. The number above each band is the ratio of intensity between the band and the corresponding load control. (*p < 0.05, **p < 0.01).

Results were averaged from three independent experiments described in (A). 

**Figure 3. PKC-θ regulates Stat3 transcription. (A)** PKC-θ activated by PMA stimulation increased Stat3 protein to much higher levels in WT cells than in PKC-θ−/− T cells. WT and PKC-θ−/− T cells were either untreated (−) or stimulated with increasing amounts of PMA. Total Stat3 protein was detected on Western blots using β-actin as a loading control. The number above each band is the ratio of intensity between the band and the corresponding load control. (*p < 0.05, **p < 0.01).

**Figure 3. PKC-θ regulates Stat3 transcription. (B)** PKC-θ activated by PMA or Th17 differentiation conditions increased Stat3 mRNA to much higher levels in WT cells than that of PKC-θ−/− T cells. WT and PKC-θ−/− T cells were either untreated (−), stimulated with increasing amounts of PMA, or stimulated using Th17 priming conditions. Stat3 and SOCS3 mRNA was detected by RT-PCR (B) or q-PCR (C, D). (E) Loss of PKC-θ does not affect Stat4 and Stat6 levels. WT and PKC-θ−/− T cells were either untreated (−) or stimulated with increasing amounts of PMA or Th17 priming conditions, and Stat4 and Stat6 mappers were then detected by q-PCR. Data shown are representative of at least three independent experiments. *p < 0.05, **p < 0.01.

**Figure 3. PKC-θ regulates Stat3 transcription. (C)** PKC-θ activated by PMA or Th17 differentiation conditions increased Stat3 mRNA to much higher levels in WT cells than that of PKC-θ−/− T cells. WT and PKC-θ−/− T cells were either untreated (−), stimulated with increasing amounts of PMA, or stimulated using Th17 priming conditions. Stat3 and SOCS3 mRNA was detected by RT-PCR (B) or q-PCR (C, D). (E) Loss of PKC-θ does not affect Stat4 and Stat6 levels. WT and PKC-θ−/− T cells were either untreated (−) or stimulated with increasing amounts of PMA or Th17 priming conditions, and Stat4 and Stat6 mappers were then detected by q-PCR. Data shown are representative of at least three independent experiments. *p < 0.05, **p < 0.01.

**Figure 3. PKC-θ regulates Stat3 transcription. (D)** PKC-θ activated by PMA or Th17 differentiation conditions increased Stat3 mRNA to much higher levels in WT cells than that of PKC-θ−/− T cells. WT and PKC-θ−/− T cells were either untreated (−), stimulated with increasing amounts of PMA, or stimulated using Th17 priming conditions. Stat3 and SOCS3 mRNA was detected by RT-PCR (B) or q-PCR (C, D). (E) Loss of PKC-θ does not affect Stat4 and Stat6 levels. WT and PKC-θ−/− T cells were either untreated (−) or stimulated with increasing amounts of PMA or Th17 priming conditions, and Stat4 and Stat6 mappers were then detected by q-PCR. Data shown are representative of at least three independent experiments. *p < 0.05, **p < 0.01.

**Figure 3. PKC-θ regulates Stat3 transcription. (E)** PKC-θ activated by PMA or Th17 differentiation conditions increased Stat3 mRNA to much higher levels in WT cells than that of PKC-θ−/− T cells. WT and PKC-θ−/− T cells were either untreated (−), stimulated with increasing amounts of PMA, or stimulated using Th17 priming conditions. Stat3 and SOCS3 mRNA was detected by RT-PCR (B) or q-PCR (C, D). (E) Loss of PKC-θ does not affect Stat4 and Stat6 levels. WT and PKC-θ−/− T cells were either untreated (−) or stimulated with increasing amounts of PMA or Th17 priming conditions, and Stat4 and Stat6 mappers were then detected by q-PCR. Data shown are representative of at least three independent experiments. *p < 0.05, **p < 0.01.

**Figure 3. PKC-θ regulates Stat3 transcription. (F)** PKC-θ activated by PMA or Th17 differentiation conditions increased Stat3 mRNA to much higher levels in WT cells than that of PKC-θ−/− T cells. WT and PKC-θ−/− T cells were either untreated (−), stimulated with increasing amounts of PMA, or stimulated using Th17 priming conditions. Stat3 and SOCS3 mRNA was detected by RT-PCR (B) or q-PCR (C, D). (E) Loss of PKC-θ does not affect Stat4 and Stat6 levels. WT and PKC-θ−/− T cells were either untreated (−) or stimulated with increasing amounts of PMA or Th17 priming conditions, and Stat4 and Stat6 mappers were then detected by q-PCR. Data shown are representative of at least three independent experiments. *p < 0.05, **p < 0.01.

**Figure 3. PKC-θ regulates Stat3 transcription. (G)** PKC-θ activated by PMA stimulation increased Stat3 protein to much higher levels in WT cells than in PKC-θ−/− T cells. WT and PKC-θ−/− T cells were either untreated (−) or stimulated with increasing amounts of PMA. Total Stat3 protein was detected on Western blots using β-actin as a loading control. The number above each band is the ratio of intensity between the band and the corresponding load control. (*p < 0.05, **p < 0.01).

**Figure 3. PKC-θ regulates Stat3 transcription. (H)** PKC-θ activated by PMA stimulation increased Stat3 protein to much higher levels in WT cells than in PKC-θ−/− T cells. WT and PKC-θ−/− T cells were either untreated (−) or stimulated with increasing amounts of PMA. Total Stat3 protein was detected on Western blots using β-actin as a loading control. The number above each band is the ratio of intensity between the band and the corresponding load control. (*p < 0.05, **p < 0.01).

**Figure 3. PKC-θ regulates Stat3 transcription. (I)** PKC-θ activated by PMA stimulation increased Stat3 protein to much higher levels in WT cells than in PKC-θ−/− T cells. WT and PKC-θ−/− T cells were either untreated (−) or stimulated with increasing amounts of PMA. Total Stat3 protein was detected on Western blots using β-actin as a loading control. The number above each band is the ratio of intensity between the band and the corresponding load control. (*p < 0.05, **p < 0.01).
ences in the percentages of IL-17-producing cells (Fig. 2J), suggesting that rescued Th17 is proportional to increased RORγt levels. Therefore, it appears that Stat3 rescued Th17 cells via an RORγt-dependent pathway, and overall, our data indicate that PKC-θ promotes Th17 differentiation via stimulation of Stat3 expression.

Critical role of PKC-θ in the regulation of Stat3 expression

Studies showed that PMA treatment stimulated Stat3 protein levels in T cells (Fig. 2C). However, because PKA activates multiple isoforms of PKC including PKC-θ, we therefore determined whether PKC-θ is responsible for PMA-stimulated Stat3 levels using the PKC-θ−/− T cells (Fig. 3A). PMA treatment resulted in the upregulation of Stat3 protein detected by Western blot in WT T cells, suggesting that activation of the PKC pathway alone is sufficient to stimulate Stat3 expression. In response to PMA stimulation, PKC-θ−/− T cells failed to increase Stat3 levels similar to those observed in WT T cells, suggesting it is likely that PKC-θ mediated the signals responsible for Stat3 upregulation. To determine whether increased Stat3 protein levels are due to increased transcription, we analyzed Stat3 mRNA by semiquantitative RT-PCR (Fig. 3B, top panel) and q-PCR (Fig. 2C). Consistent with the Stat3 protein data, PMA stimulated the expression of Stat3 mRNA to much greater levels in WT T cells than in PKC-θ−/− T cells, suggesting that PKC-θ mediates Stat3 expression via regulation of transcription. As a control, we determined that PMA did not affect the mRNA levels of SOCS, a critical regulator of the Stat3 pathway, in either cell type using RT-PCR (Fig. 3B, middle panel) or q-PCR (Fig. 3D). In addition, we detected similar levels of Stat4 and Stat6 in WT and PKC-θ−/− T cells stimulated with PMA or Th17 priming conditions (Fig. 3E), suggesting the requirement for PKC-θ in the regulation of Stat3 expression is specific. Taken together, our results indicate that activation of PKC-θ by PMA specifically promotes Th17 differentiation (Fig. 1B) and leads to increased Stat3 transcription.

Activation of Stat3 promoter by PKC-θ–mediated signals

Reduced levels of Stat3 mRNA in PKC-θ–deficient cells suggest the possibility that PKC-θ–mediated signals activate Stat3 transcription via stimulation of its promoter. To test this possibility, we looked for binding of AcH4, an epigenetic marker for active promoters, to the Stat3 promoter region in vivo using ChIP (Fig. 4A) (38). No signal was detected with an isotype control Ab (C), but the anti-AcH4 immunoprecipitated DNA contained the Stat3 promoter from WT T cells, suggesting that AcH4 was associated with the Stat3 promoter region. A weaker signal was detected in PKC-θ−/− T cells, suggesting there is much lower Stat3 promoter activity in the absence of PKC-θ. No differences were observed between association of AcH4 with the β-actin control promoters from WT and PKC-θ−/− T cells, indicating specificity of PKC-θ in the regulation of endogenous Stat3 promoter activity. We next investigated PKC-θ–mediated regulation of Stat3 promoter activity by transfecting Jurkat cells with a luciferase reporter gene driven by a 498-bp region that contains the Stat3 proximal promoter (39). Stat3-promoter reporter activity (Fig. 4B, black bars) was determined in Jurkat cells stimulated with increasing concentrations of PMA, as well as CD3/28 TCR stimulation, both of which are known to activate PKC-θ (24, 26). To determine the contribution of PKC-θ to Stat3 reporter activity, a PKC-θ(DN) was also introduced (open bars). Stat3-promoter activity was greatly increased by PMA and CD3/28 stimulation, but almost completely inhibited by the PKC-θ(DN), strongly suggesting that PKC-θ is responsible for the stimulation of the Stat3 promoter. Finally, we tested effects of a PKC-θ(CA) on Stat3 promoter ac-
tivity. The Stat3-promoter reporter was activated by PKC-θ(CA), but not by PKC-θ(DN) (Fig. 4C), indicating that activation of PKC-θ alone is sufficient to stimulate the Stat3 promoter, which is consistent with our observation that activation of PKC-θ by PMA alone is sufficient to stimulate Stat3 expression. To determine the role of endogenous PKC-θ, we transfected the Stat3-promoter reporter construct into primary WT and PKC-θ−/− T cells that were then stimulated by PMA or TCR cross-linking (Fig. 4D). Stat3 promoter activity was consistently lower in the absence of PKC-θ. Altogether, these results indicate that PKC-θ plays a critical role in the stimulation of the Stat3 promoter both in vitro and in vivo.

PKC-θ-mediated activation of AP-1 and NF-κB for stimulation of the Stat3 promoter

To determine the mechanisms responsible for PKC-θ-mediated activation of Stat3, we examined two DNA elements known to work cooperatively to regulate Stat3 expression: SBE and CRE (40). The WT Stat3-promoter reporter or Stat3-promoter reporters containing mutations in the SBE binding site (Stat3[mSBE] reporter) or the CRE binding site (Stat3[mCRE] reporter; Fig. 5A) were introduced to Jurkat cells alone or together with the activated form of PKC-θ (PKC-θ(CA); Fig. 4A). There was a 40-fold stimulation of both mutant reporters by PKC-θ(CA) (Fig. 5C, 5D) that was equivalent to the fold stimulation observed for the WT Stat3 reporter (Fig. 5B), strongly suggesting that PKC-θ-mediated stimulation occurs independently of SBE and CRE. Because previous studies have demonstrated that PKC-θ activates the NF-κB, AP-1, and NFAT pathways in T cells (24, 26, 27, 41), we determined whether the Stat3 reporter gene was regulated by these pathways in Jurkat cells using WT and DN constructs of critical proteins that either activate or block each pathway. The Stat3-promoter reporter gene was stimulated by WT IκB kinase-β,
but not by a IKKβ(DN) (Fig. 5E), suggesting the NF-κB pathway was able to activate the Stat3 reporter. More importantly, the DN IKKβ inhibited stimulation of the Stat3 reporter by PKC-θ(CA).

Similarly, WT AP-1 stimulated Stat3 reporter activity, whereas the AP-1(DN) inhibited PKC-θ(CA) stimulation of the Stat3 reporter (Fig. 5F). However, DN NFAT failed to inhibit PKC-θ(CA)–mediated Stat3 reporter activity (data not shown). These results suggest that PKC-θ–mediated activation of the Stat3 promoter is dependent on the NF-κB and AP-1 pathways, but not the NFAT pathway.

ChIP assays were performed to determine whether there were any direct interactions between the Stat3 promoter region and AP-1 (Fig. 5G, top left panel). An anti–c-Fos Ab was used because c-Fos is an essential component of AP-1 (24). Strong signals were detected in WT cells after PMA treatment (Fig. 5G, top left panel), suggesting PMAPMA promotes binding of c-Fos to the Stat3 promoter in vivo. In contrast, the signals in PKC-θ–/– T cells were extremely weak (Fig. 5G, top right panel), indicating that it is PKC-θ that promotes the binding of c-Fos to the Stat3 promoter after PMAPMA stimulation. We also investigated the interaction between Stat3 promoter and NF-κB, which consists of p50 and p65 subunits. Although no signals were detected with an anti-p50 Ab (data not shown), very strong signals were detected with an anti-p65 Ab in WT T cells, after PMA stimulation (Fig. 5G, middle left panel). The signals were weaker in PKC-θ–/– T cells (Fig. 5G, middle right panel). Furthermore, the signal strength increased with increasing PMAPMA stimulation time. As an input control, DNA templates that were not subjected to immunoprecipitation (input) were used in the PCR, and a band of expected size was amplified (Fig. 5G, bottom panels). These results suggested that AP-1 and NF-κB directly interact with the Stat3 promoter in vivo, and that activated PKC-θ regulates or promotes these interactions.

Enhancement of PKC-θ activation by Th17 priming conditions

Our data indicate that the Th17 priming cytokines TGF-β and IL-6, combined with TCR stimulation, induced maximal Stat3 expression (Fig. 2C). We therefore monitored the activation of PKC-θ using an Ab that recognized T538 phospho–PKC-θ (Fig. 6), because the phosphorylation of this site is thought to be critical for PKC-θ activation (42). Purified naive WT T cells were stimulated by TCR cross-linking (TCR) or Th17 priming conditions (TCR stimulation + TGF-β + IL-6) (Th17). Consistent with our previous results (43), phosphorylation of PKC-θ at T538 increased with increasing TCR stimulation time, suggesting PKC-θ is activated by TCR signaling. Phosphorylation of PKC-θ was greatly enhanced by the Th17 priming conditions compared with TCR cross-linking alone, indicating that the TGF-β and IL-6 cytokines enhance the activation of PKC-θ. Therefore, Th17 priming conditions that induced maximum levels of Stat3 expression were correlated with the highest PKC-θ activation status.

Discussion

Studies using PKC-θ–/– mice have demonstrated that PKC-θ is required for the development of Th17–dependent EAE (32, 34). Consistent with this, we have shown that naïve PKC-θ–/– T cells were defective in their differentiation into Th17 cells in vitro. Given that PKC-θ–deficient cells showed impaired Th17 differentiation and reduced levels of Stat3, activation of PKC-θ by PMA significantly promoted Th17 differentiation and dramatically increased Stat3 levels in WT T cells compared with PKC-θ–/– T cells, and forced expression of Stat3 increased Th17 differentiation in PKC-θ–/– T cells, our data support a critical role for PKC-θ in the regulation of Th17 differentiation, at least in part, via the upregulation of Stat3 transcription. In addition, we have shown that conditions that induced Th17 differentiation also greatly facilitated the activation of PKC-θ, implicating PKC-θ as a critical checkpoint for integrating signals from the TCR, TGF-β, and IL-6 for Th17 differentiation. Previously, we and others have shown that PKC-θ regulates the survival of activated T cells (23, 44), which raised the possibility that defective Th17 differentiation was due to apoptosis of PKC-θ–/– T cells. However, we did not observe any obvious difference in apoptosis between WT and PKC-θ–/– T cells during Th17 differentiation, which is likely to be due to presence of Th17 priming cytokines. Furthermore, exogenous IL-2, which inhibits the apoptosis of PKC-θ–/– T cells (23, 44), did not affect Th17 differentiation (data not shown). In contrast with impaired differentiation of Th17 cells, we did not observe any obvious defects in Th1 and Th2 cell differentiation in PKC-θ–/– T cells in vitro (Fig. 1K), and we observed normal activation of Stat4 and Stat6, which are critical for Th1 and Th2 differentiation, in both WT and PKC-θ–/– T cells (Fig. 2E, 2F). However, PKC-θ was previously shown to be essential for the development of effective Th2 responses induced by helminth infection (45) and model allergens, but was dispensable for Th1 responses against viruses (45, 46). This discrepancy with our results may reflect the differences between in vitro differentiation and in vivo immune responses. Multiple PKC-θ–regulated functions may contribute to the defects observed in PKC-θ–/– mice including T cell activation (24), survival (23), activation-induced cell death (28), and T cell differentiation. Therefore, the defective Th2 responses in PKC-θ–/– mice could result from one or more other defects, rather than defective Th2 differentiation. Our in vitro differentiation experiments using purified T cells suggest a selective function for PKC-θ in Th17, but not Th1 or Th2 differentiation, and this is supported by a specific defect in Stat3 activation, but not Stat4 or Stat6 activation, observed in PKC-θ–/– T cells.

In this study, we also addressed the mechanisms responsible for PKC-θ–regulated Stat3 expression. PMA induced stimulation of Stat3 transcription to greater levels in WT compared with PKC-θ–/– T cells, strongly suggesting that PKC-θ activation is necessary and sufficient to activate the Stat3 gene. Indeed, expression of a Stat3 promoter reporter gene construct was stimulated by coexpression of a PKC-θ(CA), and completely suppressed by coexpression of a DN PKC-θ. When we asked how PKC-θ activates the Stat3 promoter, we found that PKC-θ–mediated stimulation of the Stat3 promoter was inhibited by DN IKKβ and AP-1, indicating that AP-1 and NF-κB are required. The direct interaction between c-Fos (AP-1) or p65 (NF-κB) and the Stat3 promoter was then confirmed by ChIP analysis. Although both anti–c-Jun and anti–NF-κB p50 Abs worked well in our electromobility shift
assay, the fact that we could not detect Stat3 promoter binding to c-Jun or NF-κB p50 is probably due to these Abs not working well in ChIP assays (24). We showed that Stat3 levels were much lower in naive PKC-θ−/− T cells than naive WT cells, suggesting that PKC-θ is required to maintain a high basal level of Stat3 in naive T cells. The fact that reduced Stat3 was detected only in naive T cells but not in immature thymocytes in the absence of PKC-θ does not support an argument that lower Stat3 expression results from defective T cell development. TCR signals, which are significantly weaker than the signals mediating T cell activation, are required to maintain the homeostasis of T cells (47). It seems likely that PKC-θ mediates weak TCR signals to maintain Stat3 levels in naive cells. Although our results do not exclude other pathways except NFAT, they do suggest that PKC-θ-regulated signals upregulate Stat3 transcription via the AP-1 and NF-κB pathways. The fact that Stat3 could be upregulated by PMA or Th17 priming conditions, rather than TCR signals, was much lower in naive T cells than naive WT cells, suggesting that TGF-β reduces Stat3 levels in naive T cells such as cell activation and survival. It worth mentioning that GM-CSF is unlikely to affect Th17 differentiation directly because T cells do not express GM-CSF receptors (5, 6), but it may indirectly affect Th17 differentiation via stimulation of other cells such as dendritic cells and macrophages. Given the potential involvement of Th17 cells in multiple autoimmune disorders, including multiple sclerosis, rheumatoid arthritis, and inflammatory bowel disease (4), our research identifies PKC-θ as a potential therapeutic target for treatment of Th17-dependent autoimmunity.

Acknowledgments
We thank Drs. Amnon Altman and Xin Lin for providing the PKC-θ expression plasmids, Dr. Yanfen Hu for the AP-1 expression plasmids, Dr. Richard Ye for the IKKβ expression plasmids, Dr. Toshio Hirano for providing the STAT3 promoter-driven luciferase reporters, and Dr. Margaret Morgan for expert editorial assistance.

Disclosures
The authors have no financial conflicts of interest.

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