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PKC-θ inhibits iTreg differentiation via an AKT-FoxO1/3a-dependent pathway

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Abstract

Protein Kinase C-θ (PKC-θ) has been shown to be a critical T cell receptor (TCR) signaling molecule that promotes the activation and differentiation of naïve T cells into inflammatory effector T cells. We demonstrate here that PKC-θ-mediated signals inhibit iTreg differentiation via an AKT-Forkhead Box O1/3a (FoxO1/3A) pathway. Transforming growth factor β-induced iTreg differentiation was enhanced in *PKC-θ*$^{-/-}$ T cells or WT cells treated with a specific PKC-θ inhibitor, but was inhibited by the PKC-θ activator PMA, or by CD28 crosslinking which enhances PKC-θ activation. *PKC-θ*$^{-/-}$ T cells had reduced activity of the AKT kinase, and the expression of a constitutively active form of AKT in *PKC-θ*$^{-/-}$ T cells restored ability to inhibit iTreg differentiation. Furthermore, knockdown or over expression of the AKT downstream targets FoxO1 and FoxO3a was found to inhibit or promote iTreg differentiation in *PKC-θ*$^{-/-}$ T cells accordingly, indicating that the AKT-FoxO1/3A pathway is responsible for the inhibition of iTreg differentiation of iTreg downstream of PKC-θ. We conclude that PKC-θ is able to control T cell-mediated immune responses by shifting the balance between the differentiation of effector T cells and inhibitory Tregs.

Introduction

Naive CD4+ T cells can differentiate into either inflammatory effector T cells or be induced to form regulatory T cells (iTregs) (1, 2), two distinct subsets of T cell helpers with opposite functions. A fine balance between these two opposing T cell types is required for a functional immune system. Understanding the pathways that control the balance between the differentiation of naïve T cells into inflammatory effector T cells and iTregs facilitates the development of novel therapies for treatment of T cell-mediated autoimmunity. Activation of naïve T cells in the presence of TGF-β1 induces expression of Forkhead Box P3 (Foxp3), a master transcription factor instructing iTregs differentiation, and thus a marker for iTreg (3). In contrast to iTregs, natural Tregs (nTregs) are not induced but develop in the thymus. That naive T cells can be differentiated or converted to inhibitory iTregs suggests there is a therapeutic value for such a conversion in the treatment of autoimmunity. However, at present little is known about the mechanisms for regulating this conversion process. One regulatory candidate is AKT, a serine/threonine kinase that is activated following TCR engagement (4). Activation of AKT is significantly reduced in Tregs (5) and studies have shown AKT activation prevents iTreg differentiation by inhibiting the up-regulation of Foxp3 (6, 7). This result was further confirmed by a study showing that Phosphoinositide-3-Kinase (PI3K), an upstream kinase responsible for AKT activation, also inhibited Foxp3 up-regulation (8), supporting that AKT negatively regulates iTreg differentiation. Among the

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downstream targets of AKT, the mammalian target of rapamycin (mTOR) and Forkhead Box O1 and 3a (FoxO1/3a) have been shown to regulate Treg differentiation (9). mTOR signals through two functionally distinct complexes, mTORC1 and mTORC2. AKT acts as an upstream molecule of mTORC1 to regulate the activation of dwonstream p70 ribosomal S6 kinase (S6K). Little is known about both upstream and downstream signaling events involved in mTORC2, although it is clear that mTORC1 and mTORC2 work together but independently to regulate iTreg differentiation (10). Activated AKT also prevents Treg differentiation via the inactivation of FoxO1 and FoxO3a, both of which are thought to promote Treg differentiation through the direct activation of Foxp3 transcription (11, 12). When activated, AKT phosphorylates FoxO1 and FoxO3a, which leads to their exclusion from the nucleus and prevents them from activating transcription of Foxp3. Thus, AKT is an important molecule upstream of FoxO1/3a that regulates Treg differentiation. Little is known however, about the molecules upstream of AKT that are involved in the regulation process.

PKC-θ is a critical TCR signaling molecule required for the activation and differentiation of naïve T cells into inflammatory T effector cells (13–16). Our own studies have contributed to the understanding of PKC-θ function *in vivo* through the creation of a *PKC-θ*$^{-/-}$ mouse knockout strain (13, 17–22). The availability of *PKC-θ*$^{-/-}$ mice has facilitated the study of PKC-θ-regulated T cell function *in vivo*. PKC-θ is essential for the full development of effective Th2 responses to Helminth infection (15) and exposure to model allergens (15, 23). PKC-θ is also required for Th17 responses based on studies using an experimental autoimmune encephalomyelitis (EAE) model (24–26). In a previous study we also demonstrated PKC-θ plays an important role in T cell-mediated cardiac allograft rejection (22). Altogether, previous studies demonstrate a critical role for PKC-θ in the differentiation of naïve T cells into effector T cells that mediate actual immune responses. However, little is known about the function of PKC-θ in the regulation of iTreg differentiation.

In this study, we have shown that PKC-θ-mediated CD28 signaling prevented iTreg differentiation via an AKT-dependent pathway. Deletion of *PKC-θ* or a PKC-θ inhibitor potentiated differentiation of T cells into iTregs, suggesting that PKC-θ negatively regulates iTreg differentiation. We showed that AKT activation was impaired in *PKC-θ*$^{-/-}$ T cells under iTreg priming conditions. As a consequence of impaired AKT activity, phosphorylation of the downstream AKT targets, S6K and FoxO1/3a, was reduced in *PKC-θ*$^{-/-}$ T cells. Forced expression of FoxO1 or FoxO3a, but not active S6K, enhanced iTreg formation in *PKC-θ*$^{-/-}$ T cells, suggesting that PKC-θ inhibits iTreg differentiation via an AKT/FoxO1/3a pathway. Therefore, PKC-θ promotes the differentiation of naïve T cells into inflammatory effectors but prevents inhibitory Treg formation.

Materials and Methods

Mice

PKC-θ$^{-/-}$ mice were described previously (13) and backcrossed with C57BL/6 mice for more than 10 generations. *Foxp3-GFP* mice were obtained from Dr. Vijay Kuchroo (Harvard University) and Dr. Defu Zeng (Beckman Research Institute of City of Hope). B6.SJL (CD45.1) and OTII mice were purchased from The Jackson Laboratory. OTII/*PKC-θ*$^{-/-}$ mice were generated by crossing OTII with *PKC-θ*$^{-/-}$ mice. All mice were housed under specific pathogen-free conditions and experiments were performed in accordance with a protocol approved by the Institutional Animal Care and Use Committee at the Beckman Research Institute of City of Hope (IACUC#07023).

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Plasmids

An empty retroviral expression plasmid MSCV-IRES-Thy1.1 (MIT) and one that encodes constitutively active AKT (MIT-AKT*) were obtained from Dr. Christophe Benoist (Harvard University) (27). LMP vector based retroviral FoxO1 shRNA expressing vectors (LMP-FoxO1 shRNA1 and LMP-FoxO1 shRNA2), MSCV-IRES-GFP (MIG) vector-based retroviral FoxO1, FoxO3a and constitutively active FoxO3a-expressing vectors [MIG-FoxO1, MIG-FoxO3a, and MIG-FoxO3a (3A)] were obtained from Dr. Yun-cai Liu (La Jolla Institute for Allergy and Immunology) (28). MSCV-IRES-human CD4 (MIC) vector based retroviral P70-S6 Kinase 1 (S6K) and constitutively active S6K expressing vectors [MIC-S6K and MIC-S6K (CA)] were obtained from Dr. Craig Walsh (University of California, Irvine).

T-cell isolation and in vitro differentiation of iTreg cells

Naïve ($CD4^+CD25^-CD44^{low}CD62L^{hi}$) T cells were isolated from lymph nodes and spleens of mice by cell sorting using a FACSAria II cell sorter (BD Bioscience). For *in vitro* differentiation of naïve T cells into iTreg cells, 2×10^5 cells were cultured with anti-CD3 (145–2C11, eBioscience), anti-CD28 (37.51, eBioscience), 5 ng/ml TGF-β1 (Peprotech) and 10 ng/ml IL-2 (Peprotech) in goat-anti-hamster IgG (0.2 mg/ml, MP Biomedicals) pre-coated 24-well plates. T cells were cultured for 3 days in RPMI 1640 (Cellgro) containing 2 mM L-glutamine, 50 μM β-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% FBS at 37°C with 5% CO_2. PKC-θ inhibitor was obtained from Rigel, Inc.

In vivo differentiation of iTreg cells

The method was adapted from previous studies (28, 29). In brief, 3×10^6 naïve T cells sorted from OTII or OTII/$PKC\text{-}\theta^{-/-}$ mice were transferred into syngeneic B6.SJL (CD45.1) mice. After 24 h, recipient mice were fed grade VI OVA (20 mg/ml; Sigma-Aldrich) in drinking water for 5 days. Drinking water containing OVA was changed every two days.

Flow cytometry

Antibodies were purchased from eBioscience, unless otherwise indicated. After blocking the Fc receptor with anti-CD16/32 (clone 93), cells were incubated with anti-mouse CD4 (clone L3T4) and anti-mouse TCR-β (clone H57-597, BD Bioscience) or anti-CD45.2 (clone 104, BioLegend), and fixed/permeabilized in Foxp3 Staining Buffer (eBioscience). Cells were intracellularly stained with anti-mouse Foxp3 (clone FJK-16S), and analyzed using a BD FACSCanto II flow cytometer (BD Biosciences) and FlowJo software (Treestar).

Retroviral Packaging and Transduction

Retroviral expression plasmids were transfected into Phoenix-Eco cells using lipofectamine 2000 (Invitrogen). After 48 hours, 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 (1 μg/ml) and anti-CD28 (1 μg/ml) antibodies in presence of 10 ng/ml IL-2 for 24 hours, then spin infected with viral supernatants (2500 rpm, 30°C for 2 hours) in the presence of 8 μg/ml of polybrene (Sigma-Aldrich). After spin infection, TGF-β1 was added to the culture media to induce iTreg differentiation.

Immunoblot analysis

Naïve T cells (5×10^6) sorted from WT or $PKC\text{-}\theta^{-/-}$ mice were incubated with 1 μg/ml anti-CD3 and anti-CD28 in the presence of 5 ng/ml TGF-β1 for 1 h on ice. Secondary goat-anti-hamster IgG (8 μg/ml) was added and the cells were then incubated at 37°C for variable times. After incubation, cells were lysed in RIPA buffer (Sigma-Aldrich) with phosphatase inhibitor (Sigma-Aldrich) and protease inhibitor (Roche). Soluble proteins were separated

on a NuPAGE 4–12% Bis-Tris gel and transferred to 0.45 μM PVDF membrane. Immunoblot analysis was done with following antibodies (all from Cell Signaling): anti-phospho-AKT (T308, 4056), anti-AKT (4685), anti-phospho-Zap70 (Tyr493, 2704), anti-Zap70 (2705), anti-phospho-S6K (T389, 9234), anti-S6K (2708), anti-phospho-FoxO1 (S256, 9461), anti-FoxO1 (2880), anti-phospho-FoxO3a (S253, 9466), anti-FoxO3a (2497), and anti-β-actin (4970). Band intensity was quantitatively analyzed by ImageJ software (NIH).

Apoptosis assay

WT or *PKC-θ*$^{-/-}$ naïve T Cells were differentiated to iTreg for 3 days, then washed once with ice-cold annexin V binding buffer (10 mM HEPES (pH 7.5), 140 mM NaCl, 5 mM KCl, 1 mM MgCl2, and 1.8 mM CaCl2) and stained with PE-conjugated annexin V and 7-aminoactinomycin D (7-AAD, BD Pharmingen), according to the manufacturer's protocol. Detection of apoptotic cells was performed on a BDCantoII cytometer (BD Biosciences).

Statistical analysis

Statistical analysis was performed using the unpaired, two-tailed Student's *t*-test. P values < 0.05 were considered significant.

Results

PKC-θ inhibitor promotes, whereas PKC-θ activator prevents, iTreg differentiation

Previous studies have demonstrated a role of PKC-θ in the differentiation of naïve T cells into inflammatory T helper cells (15, 26). Here we investigated the function of PKC-θ in the differentiation of inhibitory iTreg cells. A strain of Foxp3-GFP knock-in mice (1) was used in this experiment, so that GFP could be used to monitor live Foxp3 expression as a marker for iTregs. Naïve T cells were first stimulated by 50 ng/ml of anti-CD3 antibody and TGF-β1/IL-2 in combination with increasing amounts of anti-CD28 antibody (0, 50 and 500 ng/ml) which is known to enhance PKC-θ activation significantly (30, 31) (Fig. 1A, top panels). A large proportion of iTregs (92%) were induced by TGF-β1in the absence of anti-CD28 antibody (Fig. 1A-a), indicating the dispensable role of CD28 in iTreg differentiation. However, stimulation of CD28 by 50 ng/ml of anti-CD28 antibody led to a reduction of the iTreg in the differentiated cell populations from 92% (Fig. 1A-a) to 63.1% (Fig. 1A-b). Increasing the anti-CD28 antibody concentration to 500 ng/ml further reduced iTregs to 45.5% (Fig. 1A-c). Similar inhibitory effects of CD28 on iTreg formation were also observed when T cells were stimulated with 500 ng/ml of anti-CD3 antibody. In the absence of anti-CD28 antibody, there were 86.4% iTreg (Fig. 1A-d), which was decreased to 46.8% and 28.6% after treatment with 50 ng/ml and 500 ng/ml anti-CD28 antibody, respectively (Fig. 1A-e and f). Therefore, iTreg formation was inhibited by CD28 co-stimulation in a dose dependent manner. We then tested the effects of a specific PKC-θ inhibitor, REG134, on CD28-mediated inhibition of iTreg differentiation (Fig. 1B). REG134 is 150 and 1200 fold more potent for PKC-θ than for PKCδ and PKCε (data not show), which are the most homologous members of PKC-θ, and belong to the same PKC subfamily (novel PKC). In addition, this inhibitor prevented PKC-θ-mediated function such as CD69 up-regulation and proliferation in WT but not *PKC-θ*$^{-/-}$ T cells (data not shown). Under the same conditions, using 50 ng/ml anti-CD3 antibody (Fig. 1B, top panels), the PKC-θ inhibitor blocked the inhibitory action of 50 ng/ml anti-CD28 antibody and increased the percentage of iTregs from 63.1% (Fig. 1A-b) to 89.6% (Fig. 1B-a), which is almost equal to the percentage of iTregs induced in the absence of CD28 co-stimulation (92%, Fig. 1A-a). The PKC-θ inhibitor therefore abolishes CD28-mediated inhibition under this condition. The use of the PKC-θ inhibitor also reduced the inhibitory effects of 500 ng/ml of anti-CD28 antibody and increased iTreg from 45.5% (Fig. 1A-c) to 67.3 % (Fig. 1B-d) in treated populations.

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Similarly, when T cells were stimulated with 500 ng/ml of anti-CD3 antibody, the PKC-θ inhibitor blocked or prevented the inhibitory action of anti-CD28 and increased iTreg from 46.8% (Fig. 1A-e) to 77.6% in cells treated with 50 ng/ml anti-CD28 antibody (Fig. 1B-c), and from 28.6% (Fig. 1A-f) to 54.7% in cells treated with 500 ng/ml anti-CD28 antibody (Fig. 1B-d). Above results were reproducible as shown in figure 1C which are averaged from three independent experiments. The data therefore suggest that PKC-θ mediates the CD28 signals to inhibit iTreg formation. This hypothesis was further confirmed by an experiment which showed that phorbal ester (PMA), which activates multiple isoforms of PKC including PKC-θ, greatly reduced the percentage of TGF-β1-induced iTregs from 70.4% (Fig. 1D, second panel from left) to 8.49% (2 ng/ml PMA, Fig. 1D, third panel from left) and 3.6% (5 ng/ml PMA, Fig. 1D, fourth panel from left). Due to lack of specificity for PKC-θ, PMA treatment alone is not sufficient to indicate PKC-θ function in iTreg differentiation. However, the fact that PMA and PKC-θ inhibitor have opposite effects on iTreg formation strongly supports that PKC-θ activated by CD28 inhibits iTreg differentiation.

PKC-θ negatively regulates iTreg differentiation

To exclude off-target effects of PKC-θ pharmacological regulators, T cells obtained from WT (Fig. 2A, top panels) and $PKC\text{-}\theta^{-/-}$ (Fig. 2A, bottom panels) mice were compared to determine the function of PKC-θ in iTreg differentiation. Although low percentages of iTregs were detected after naïve T cells were stimulated without TGF-β1, more iTregs were detected in the differentiated $PKC\text{-}\theta^{-/-}$ T cell population (3.54%) than WT T cells (1.71%) (Fig. 2A-a and b). Stimulation with anti-CD3 antibody alone in the presence of TGF-β1 induced similar percentages of iTregs in WT (82.2%) and $PKC\text{-}\theta^{-/-}$ T cells (88.5%) (Fig. 2A-c and d), suggesting that PKC-θ is not required for formation of iTregs. Consistent with previous results (Fig. 1A), in the presence of anti-CD28 antibody, the iTreg was significantly reduced to 12.8% in WT T cells (Fig. 2A-e), whereas there were still 69.9% iTregs $PKC\text{-}\theta^{-/-}$ T cells (Fig. 2A-f), revealing the essential function of PKC-θ in CD28-mediated inhibition of iTreg differentiation. Also consistent with previous results (Fig. 1B), the addition of the PKC-θ inhibitor abolished CD28-mediated inhibition and increased the iTregs from 12.8% to 72.6% (100 nM of inhibitor) and 74.1% (200 nM inhibitor) in WT T cells after treatment (Fig. 2A-e, g and i). However, the presence or absence of PKC-θ inhibitor, had no effect on iTreg differentiation in $PKC\text{-}\theta^{-/-}$ T cells; the percentage of iTregs was 69.9% in the absence of PKC-θ inhibitor compared to 67% and 67.4% in those treated with 100 nM and 200 nM PKC-θ inhibitor respectively (Fig. 2A-f, h and j), demonstrating the specificity of the PKC-θ inhibitor and the essential role of PKC-θ in the inhibition of iTreg differentiation. These results were highly reproducible; Figure 2B are the results averaged from four independent experiments that are described in Figure 2A. We also examined the effects of different concentrations of PKC-θ inhibitor on iTreg differentiation (Fig. 2C), and showed that PKC-θ inhibitor could promote iTreg formation in a dose-dependent manner. To exclude the possibility that increased $PKC\text{-}\theta^{-/-}$ iTreg is due to changes in apoptosis, we examined apoptotic cells by annexin V and 7-AAD staining (Fig. 2D). There were very few dead cells, and no obvious difference in apoptosis between WT and $PKC\text{-}\theta^{-/-}$ T cells, which was expected due to presence of IL-2, a critical survival factor for T cells. Apoptosis therefore, is unlikely to be responsible for the huge difference in TGF-β1-induced iTregs observed between WT and $PKC\text{-}\theta^{-/-}$ T cells. Lastly, PKC-θ was introduced back into $PKC\text{-}\theta^{-/-}$ T cells using a retrovirus expressing PKC-θ (MIG-PKC-θ) and compared with an empty virus (MIG) that only expressed GFP. When gated on GFP- cells that were not transduced and therefore did not express viral genes, no significant difference in iTregs was observed between the empty viral control (86.7%) and the PKC-θ expressing virus (87.8%) infected $PKC\text{-}\theta^{-/-}$ T cells (Fig. 2E, two left panels and Fig. 2F). In contrast, when gated on successfully transduced GFP+ cells that expressed viral genes, the

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PKC-θ-expressing virus-infected cells had significantly reduced iTreg (54.6%) compared to those (77.8%) infected by empty virus (Fig. 2E, two right panels and Fig. 2F). Because the restoration of PKC-θ expression restored the ability to inhibit iTreg differentiation in *PKC-θ*$^{-/-}$ T cells, the enhanced iTreg differentiation observed in *PKC-θ*$^{-/-}$ T cells is not due to developmental defects, but due to lack of PKC-θ-mediated inhibition. Taken together, these results confirm the inhibitory role of PKC-θ in iTreg differentiation.

PKC-θ inhibits iTreg formation *in vivo*

In the above studies, purified naïve T cells were cultured in defined medium optimal for iTreg differentiation *in vitro*. To study the role of PKC-θ in iTreg differentiation *in vivo*, iTregs were induced in mice using a model previously described (1, 32). T cells from OT-II TCR transgenic mice with congenic marker CD45.2 were used as donor cells, because these T cells respond to a single ligand, Ova. Sorted naïve OT-II WT and OT-II *PKC-θ*$^{-/-}$ T cells, that lacked Foxp3+ positive cells (Fig. 3A), were adoptively transferred into congenic CD45.1 recipients that then received Ova in the drinking water for five days. Consistent with previous reports (1, 32), Tregs were mostly induced in gut-associated lymphoid tissues such as the lamina propria (LP, 6.14%) and the mesenteric lymph nodes (mLNs, 1.32%) (Fig. 3B, upper two left panels), whereas relatively fewer iTregs were found in other lymphoid tissues such as spleen (SPL, 0.68%) and inguinal lymph nodes (iLNs, 0.54%) (Fig. 3B, upper two right panels). Significantly more iTregs were differentiated from adoptively transferred CD45.1 *PKC-θ*$^{-/-}$ T cells in all the lymphoid tissues examined; 13.4% in LP, 3.23% in mLN, 2.36% in SPL and 1.32% in iLNs (Fig. 3B, lower panels), suggesting that lack of PKC-θ promotes induction of Tregs *in vivo*. These results are reproducible as shown in figure 3C averaged from three independent experiments. Therefore, our data indicate that PKC-θ negatively regulates iTreg formation both *in vitro* and *in vivo*.

Impaired activation of AKT is responsible for enhanced iTreg differentiation in *PKC-θ*$^{-/-}$ T cells

AKT has been shown to negatively regulate iTreg differentiation (6, 7). We therefore examined the phosphorylation of threonine 308 (T308) of AKT, which activates the kinase (33), in WT and *PKC-θ*$^{-/-}$ T cells cultured under iTreg priming conditions (Fig. 4A). In response to stimulation, AKT was strongly phosphorylated in WT cells, however, phosphorylation was greatly impaired in *PKC-θ*$^{-/-}$ T cells (Fig. 4A, left panel). Impaired activation of AKT in *PKC-θ*$^{-/-}$ T cells was confirmed by the quantification of phosphorylated AKT versus total AKT, averaged from several independent assays (Fig. 4A, right panel). In contrast, activation of ZAP70, another critical TCR signaling molecule, used here as a control, was normal in *PKC-θ*$^{-/-}$ T cells (Fig. 4B) which is consistent with our previous results (20). To determine whether impaired AKT activation is responsible for enhanced iTreg differentiation in *PKC-θ*$^{-/-}$ T cells, an active form of AKT was introduced into T cells via retrovirus-mediated transduction. In untreated cells (Fig. 4C, two left panels) and empty virus (MIT)-infected cells (Fig. 4C, two middle panels), *PKC-θ*$^{-/-}$ T cells had significantly higher percentages of iTregs (56.6%–71.5%) (Fig. 4C, two lower left panels) than those of the WT T cells (21%–35.8%) (Fig. 4C, two upper left panels). However, retrovirally expressed active AKT (MIT-AKT*) greatly decreased iTregs to 7.02% in *PKC-θ*$^{-/-}$ T cells (Fig. 4C, lower right panel), although it was still higher than the percentage of similarly treated WT T cells (1.63%) (Fig. 4C, upper right panel). These results suggest that AKT regulate iTreg differentiation even in the absence of PKC-θ. To determine whether endogenous AKT regulates iTreg differentiation, LY294002, an inhibitor for PI3 kinase (PI3K), an upstream kinase required for AKT activation, was tested. LY294002 treatment blocked phosphorylation of T308 in the AKT protein in stimulated T cells (Fig. 4D), suggesting effective inhibition of PI3K-AKT pathway. In WT T cells, treatment with

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LY294002 increased iTreg cells from 31.7% to 65% (Fig. 4E, upper two panels and Fig. 4F), which was close to the percentage of iTregs observed in *PKC-θ*$^{-/-}$ T cells (64.1%), suggesting that endogenous PI3K pathway regulates iTreg differentiation. As expected, LY294002 had minimum effects on iTreg differentiation in *PKC-θ*$^{-/-}$ T cells which already had lower AKT activity; the percentage of iTregs was 64.1% in the absence of LY294002 compared to 70.1% in its presence (Fig. 4D, lower two panels and Fig. 2F). The fact that active AKT inhibits iTreg formation in WT and *PKC-θ*$^{-/-}$ T cells suggests that ATK regulates iTreg differentiation either downstream of PKC-θ, or independent of PKC-θ. However, AKT activity is lower in *PKC-θ*$^{-/-}$ T cells which have enhanced potential to form iTreg, and forced activation of AKT inhibited such potential in *PKC-θ*$^{-/-}$ T cells. Altogether, our data favor the possibility that AKT is downstream of PKC-θ in the regulation of iTreg differentiation.

PKC-θ-regulated iTreg differentiation is independent of S6K, an AKT downstream target

Because S6K is one of the AKT downstream target molecules that may play a role in iTreg differentiation (10, 34), we investigated S6K function in PKC-θ-regulated iTreg differentiation. Upon activation, S6K is phosphorylated at serine 389 (35), which was detected by western blot analysis using a specific antibody that only recognizes this phosphorylated form of S6K (Fig. 5A). Consistent with AKT being the upstream signaling molecule, the LY294002 prevented phosphorylation of S6K in WT T cells. *PKC-θ*$^{-/-}$ T cells also had lower levels of the phosphorylated form of S6K which was expected due to its lower AKT activity. To determine whether lower S6K activation is responsible for the enhanced iTreg differentiation in *PKC-θ*$^{-/-}$ T cells, we evaluated the effects of the WT and the constitutively active form of S6K [S6K(CA)] (36) on iTreg formation using retroviral expression system (Fig. 5B). The percentage of iTregs was 28.9–34% in WT cells (Fig. 5B, top three panels) and 63.2%–67.5% in *PKC-θ*$^{-/-}$ T cells (Fig. 5B, lower three panels) regardless of whether S6K or S6K (CA) was absent or present. Therefore, forced expression of the WT or activated form of S6K did not affect iTreg differentiation in WT cells or *PKC-θ*$^{-/-}$ T cells. This is in striking contrast to the forced expression of AKT, which dramatically affected iTreg differentiation (Fig 4C). The results suggest that S6K is unlikely to be a signaling molecule downstream of PKC-θ-mediated inhibitory pathway of iTreg differentiation.

PKC-θ regulates iTreg differentiation via AKT downstream molecules FoxO1/3a

The other AKT downstream target molecules that may play a role in iTreg differentiation are FoxO1 and FoxO3a (FoxO1/3a) (11, 12). Therefore, we investigated their functions in PKC-θ-enhanced iTreg differentiation. AKT is thought to phosphorylate FoxO1 at serine 256 and FoxO3a at serine 253. This results in their export from the nucleus and prevents activation of their target genes, including Foxp3, in the nucleus (37). We first examined phosphorylation of FoxO1/3a by western blot assays (Fig. 6A-B). In WT cells, stimulation by iTreg priming conditions leads to phosphorylation of FoxO1/3a. Consistent with the lower upstream AKT activity, in *PKC-θ*$^{-/-}$ T cells the phosphorylated forms of FoxO1 (Fig. 6A, upper panel) and FoxO3a (Fig. 6A, third panel) were decreased. This was confirmed by quantification of phosphorylated FoxO1 versus total FoxO1 (Fig. 6B, left panel) and phosphorylated FoxO3a versus total FoxO3a (Fig. 6B, right panel) averaged from three independent assays. The results suggest that *PKC-θ*$^{-/-}$ T cells contain higher levels of the non-phosphorylated forms of FoxO1/3a, which are retained in nucleus and thus capable of activating their target gene Foxp3. This finding is consistent with the increased iTreg ratio in *PKC-θ*$^{-/-}$ T cells after stimulation. Next, we determined whether changes in the levels of FoxO1/3a caused corresponding changes in iTreg differentiation, using knockdown and overexpression approaches. Two different shRNAs that can effectively knockdown FoxO1 (Fig. 6C) were expressed using a retrovirus containing a selectable puromycin resistance

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gene and GFP as the reporter molecule (11). The empty virus (LMP) infected *PKC-θ*$^{-/-}$ T cells had a higher percentage of iTreg cells (41.8%) than the WT cells (24.6%) (Fig. 6D, two left panels), which is consistent with, but not as dramatic as, previous results. This may be due to the different method used in order to accommodate the puromycin selection step. Knockdown by the two shRNAs (LMP-FoxO1 shRNA1 and LMP-FoxO1 shRNA2) led to a decrease in the percentage of iTregs from 24.6% to 16.8% and 17.6% in the respective WT cells (Fig.6D, two upper right panels), suggesting that endogenous FoxO1 stimulates iTreg differentiation. Knockdown of FoxO1 was also able to decrease the percentage of iTregs from 41.8% to 30.3% (shRNA1) and 28.2% (shRNA2) in the *PKC-θ*$^{-/-}$ T cells (Fig. 6D, two lower right panels), suggesting that FoxO1 is likely downstream of PKC-θ, in the regulation of iTreg differentiation. These results were reproducible as shown in figure 6E averaged from three independent experiments. We then determined the effects of overexpression of FoxO1 and FoxO3a on iTreg formation (Fig. 6F). In WT T cells, the percentage of iTregs (8.95%) was significantly increased in cells transduced with virus expressing FoxO1 (32.1%), FoxO3a (27.1%) and FoxO3a (3A) (32.3%) (Fig. 6F, upper panels). The constitutively active form of FoxO3a which has a mutation in the AKT phosphorylation site that prevents its phosphorylation and nuclear export (11). The percentage of iTregs in *PKC-θ*$^{-/-}$ T cells (41%) was also increased by infection and expression of FoxO1 (50.2%), FoxO3a (55.7%) and FoxO3a (3A) (65%) (Fig. 6F, lower panels). These results were reproducible as shown in figure 6G averaged from three independent experiments. In contrast to S6K, FoxO1/3a is able to affect iTreg differentiation in both WT and *PKC-θ*$^{-/-}$ T cells. These results indicate that the decreased phosphorylation of FoxO1/3a, which leads to its nuclear accumulation and availability to stimulate its target gene, Foxp3, is responsible for the enhanced iTreg differentiation in *PKC-θ*$^{-/-}$ T cells.

Discussion

Previous studies of PKC-θ have almost always focused on its role in the stimulation of T cell activation and differentiation (16, 19). *In vitro* assays initially showed that PKC-θ mediates the critical TCR signals required for T cell activation and differentiation into effector T cells (13, 14, 20, 38). PKC-θ's function in T cell activation has also been confirmed *in vivo*. Studies have shown there are defective Th2 and Th17 responses and allograft rejection in *PKC-θ*$^{-/-}$ mice (15, 22–26). PKC-θ, therefore, is known to promote the differentiation of naïve T cells into inflammatory effector T cells that carry out immune responses *in vivo*. In this study, we investigated the role of PKC-θ in Treg differentiation, and our results indicate that PKC-θ-mediated signals inhibited the differentiation of naïve T cells into iTregs. This suggests that activation of PKC-θ is able to boost the immune response by both promoting the differentiation of inflammatory T cells and blocking the differentiation of inhibitory iTregs. In contrast, inhibition of PKC-θ is likely to restrict the immune response by both inhibiting the formation of inflammatory T cells and also promoting iTreg differentiation. PKC-θ inhibitors can also enhance Treg effector function, since PKC-θ inhibits Treg-mediated suppression (39). PKC-θ has long been considered a drug target (16, 19, 40), and highly specific inhibitors similar to the one used in this study are expected to have efficacy in treatment of T cell-mediated autoimmunity and allograft rejection via both the inflammatory and inhibitory arms of the immune system.

Another question addressed in this study was determination of the downstream molecular mechanisms that played a role in PKC-θ-mediated inhibition of iTreg differentiation. Our results support the hypothesis that PKC-θ inhibits iTreg differentiation via an AKT-dependent pathway. The initial evidence for involvement of AKT came from the observation that *PKC-θ*$^{-/-}$ T cells have impaired activation of AKT. This result appears inconsistent with previous results that PKC-θ and AKT works independently to regulate T cell activation (41–43). In contrast to previous studies examining signaling events in T cell activation, here

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we focused on iTreg differentiation. Therefore, T cells were stimulated by iTreg priming conditions to mimic signaling events during iTreg differentiation. Different signaling processes are involved in T cell activation and iTreg differentiation due to presence of specific cytokines required to drive iTreg formation. Crosstalk between PKC and AKT pathway is widely reported in non-hematopoietic cells (44–49) as well as in B (50) and T cells (51, 52). In one study, PKCα in T cells was showed to be able to activate AKT by direct phosphorylation (52). However, PKC pathway can also stimulate AKT function via other mechanisms. For example, Bauer et al showed that PKC-θ and AKT cooperatively stimulated NF-κB (51), which appears to be consistent with the notion that PKC-θ and AKT work independently. However, PKC-θ and AKT were in the same complexes, and dominant negative AKT was found to inhibit PKC-θ-mediated activation of NF-κB, suggesting crosstalk between PKC-θ and AKT. Their research showed that PKC-θ regulated AKT function via promoting its recruitment to the lipid rafts critical for TCR signals. Our results favor that PKC-θ regulates AKT under iTreg priming conditions. The involvement of AKT in PKC-θ-regulated iTreg differentiation was also supported by that LY294002, the PI3K inhibitor, potentiated iTreg formation in WT but not *PKC-θ*$^{-/-}$ T cells. PDK1, downstream of PI3K, is believed to activate PKC-θ by phosphorylation of T538 site (53, 54). In this case, LY294002 is expected to inhibit both AKT and PKC-θ, and therefore, cannot differentiate the effects of AKT and PKC-θ. However, the function of PDK1 in the activation of PKC-θ is questioned by Baier et al (55). To make it more complicated, a recent report suggests that GLK phopshorylates T538 to activate PKC-θ (56). If this is the case, LY294002 does not likely affect PKC-θ activation via inhibition of PI3K-PDK1 pathway. Although these results are not sufficient to determine whether AKT is downstream of PKC-θ or acting independently of PKC-θ to regulate iTreg differentiation, the fact that active AKT inhibits iTreg formation in *PKC-θ*$^{-/-}$ T cells that have lower AKT activity strongly favors the possibility that PKC-θ regulates iTreg differentiation via AKT. Interestingly, the activation of both PKC-θ and AKT are enhanced by CD28 signaling (9, 30, 43, 57), which raises the possibility that stimulation of CD28 inhibits iTreg formation via a PKC-θ-AKT mediated pathway. Indeed, our results demonstrated that crosslinking CD28 inhibited iTreg formation, which is consistent with a previous report (58). Furthermore, crosslinking CD28 failed to inhibit iTreg differentiation in *PKC-θ*$^{-/-}$ T cells or WT cells treated with PKC-θ inhibitor or LY294002 suggesting that a PKC-θ-AKT pathway mediates the CD28 signals essential for inhibiting iTreg formation. Yu's group (59) showed that CD28 is required for iTreg differentiation, which appears to be inconsistent with our results. In contrast to our experiments, iTreg differentiation was performed in the absence of exogenous IL-2 in their report. CD28 stimulation is required for production of IL-2, an essential factor for iTreg formation (60). Indeed, stimulation of CD28 in the presence of exogenous IL-2 inhibited iTreg differentiation in another report from Yu's group (58), which is consistent with our results.

AKT controls S6K, a target of mTOR, and FoxO1/3a, which may regulate iTreg differentiation (9, 11, 12). We asked which of the AKT-regulated pathways are required for PKC-θ-mediated inhibition of iTreg differentiation. mTOR signals through two complexes, mTORC1 and mTORC2, and S6K is an downstream target of mTORC1 (9). Lower S6K activity was detected in *PKC-θ*$^{-/-}$ T cells that had impaired AKT activation, suggesting defective mTORC1 complexes. However, forced expression of either WT or a constitutively active S6K did not affect iTreg differentiation in *PKC-θ*$^{-/-}$ T cells, suggesting that S6K is not involved in the iTreg differentiation pathway. mTORC1 and mTORC2 works together to regulate iTreg differentiation, indicated by defects in both the mTORC1 and mTORC2, but not either one of the two complexes, affected Treg differentiation (10). Therefore, our result that forced activation of kinase S6K alone did not change iTreg differentiation in either WT or *PKC-θ*$^{-/-}$ T cells is consistent with the notion that defects in mTORC1 alone is not sufficient to affect iTreg differentiation. In contrast, iTreg differentiation was changed by

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both over expression and knockdown of FoxO1/3a, which are also regulated by AKT. Mice deficient in both FoxO1 and FoxO3a develop autoimmunity due to a significant decrease in Treg cells (61), suggesting a stimulatory role for FoxO1/3a in Treg differentiation. FoxO1/3a promotes Treg differentiation, most likely via the direct binding and stimulation of Foxp3 transcription (11, 12). Activation of AKT leads to phosphorylation of FoxO1/3a, which results in their export from the nucleus, preventing them from activating Foxp3 transcription (9, 11). Consistent with impaired AKT activity in *PKC-θ*$^{-/-}$ T cells, FoxO1/3a phosphorylation was reduced in these cells, allowing accumulation of more FoxO1/3a in the nucleus to stimulate Foxp3 expression. Knockdown of FoxO1 prevented its accumulation and inhibited formation of Foxp3+ iTregs, whereas overexpression of FoxO1 or FoxO3a increased iTreg differentiation in both *PKC-θ*$^{-/-}$ and WT T cells. Our results suggest that PKC-θ inhibits iTreg differentiation via AKT-mediated phosphorylation of FoxO1/3a, leading to their export from the nucleus and failure to stimulate Foxp3 transcription. PKC-θ is known to stimulate NF-κB via CARM1/BCL10/MALT1 complexes (62). Blockade of NF-κB activation by deletion of CARMA1 or c-Rel prevents iTreg differentiation (63). The reason is that production of IL-2, essential for iTreg differentiation (64, 65), is dependent on NF-κB. Since PKC-θ is required for IL-2 production via activation of NF-κB pathway (66). PKC-θ also likely promotes iTreg differentiation via stimulation of IL-2 production. However, we used exogenous IL-2 in our study. Therefore, the defects we observed are not due to lack of IL-2 production in *PKC-θ*$^{-/-}$ T cells.

We as well as others have shown that *PKC-θ*$^{-/-}$ mice have reduced nTreg development in the thymus (67, 68), suggesting that PKC-θ promotes nTreg development *in vivo*. nTreg development in the thymus depends on positive selection by stronger TCR signals than that required for the development of conventional CD4 and CD8 T cells (69, 70). Therefore, the reduced nTreg development is likely to be due to the overall reduction of TCR signaling strength in the absence of PKC-θ. Development of conventional T cells is normal in *PKC-θ*$^{-/-}$ mice (13), since these cells require weaker TCR signals which do not appear to be affected by the absence of PKC-θ. Conversely, the activation of peripheral T cells, which also requires a strong TCR stimulus, is defective in *PKC-θ*$^{-/-}$ mice (13). Differentiation of naïve T cells to iTreg also requires TCR stimulation. Our results showed that PKC-θ is dispensable for the TCR signaling required for iTreg differentiation, suggesting that iTreg differentiation may require weaker TCR signals. Whereas, stronger TCR signals such as those enhanced by co-stimulation with CD28, actually inhibit iTreg differentiation. Thus, PKC-θ is a critical molecule that controls the threshold of TCR signals which determines the fate of T cell development and differentiation. Manipulation of PKC-θ activity using inhibitors or activators can adjust immune responses by affecting both inflammatory and inhibitory T cell differentiation.

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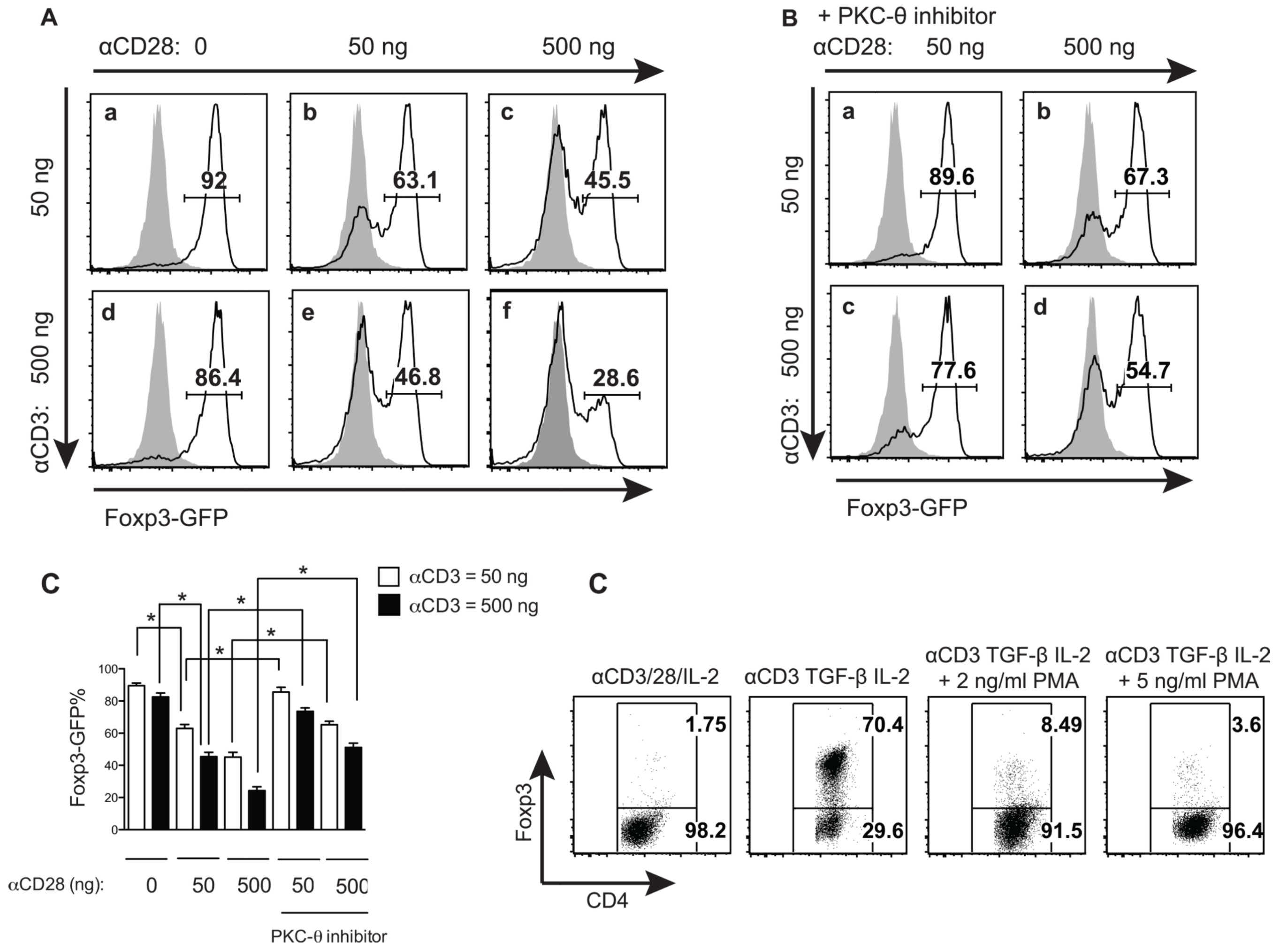

Figure 1.

PKC-θ negatively regulates iTreg differentiation. (A) The effects of TCR signaling strength on iTreg differentiation. Naïve T cells from Foxp3-GFP mice were stimulated with 5 ng/ml TGF-β1, 10 ng/ml IL-2 and the indicated amounts of anti-CD3 and anti-CD28 antibodies for 3 days. Foxp3-GFP expression was detected by flow cytometry. Black lines show the expression of Fox3p-GFP in TGF-β1-induced cells, while the grey shaded areas are GFP in naïve T cells from Foxp3-GFP stimulated with the same anti CD3 and anti-CD28 concentrations but without TGF-β1 and IL2. (B) PKC-θ inhibitor promotes iTreg differentiation. Naïve T cells from Foxp3-GFP mice were differentiated into iTreg cells using the same conditions described in (A), but in the presence of 200 nM of the specific PKC-θ inhibitor REG134. (C) Summary of (A) and (B) averaged from three independent experiments. (D) The PKC-θ activator PMA suppresses iTreg differentiation. Naïve T cells were differentiated into iTreg using the same conditions in (A) plus in the presence of the indicated amount of PMA for 3 days. Tregs were detected by intracellular staining of Foxp3. Plots shown are representative of at least three independent experiments.

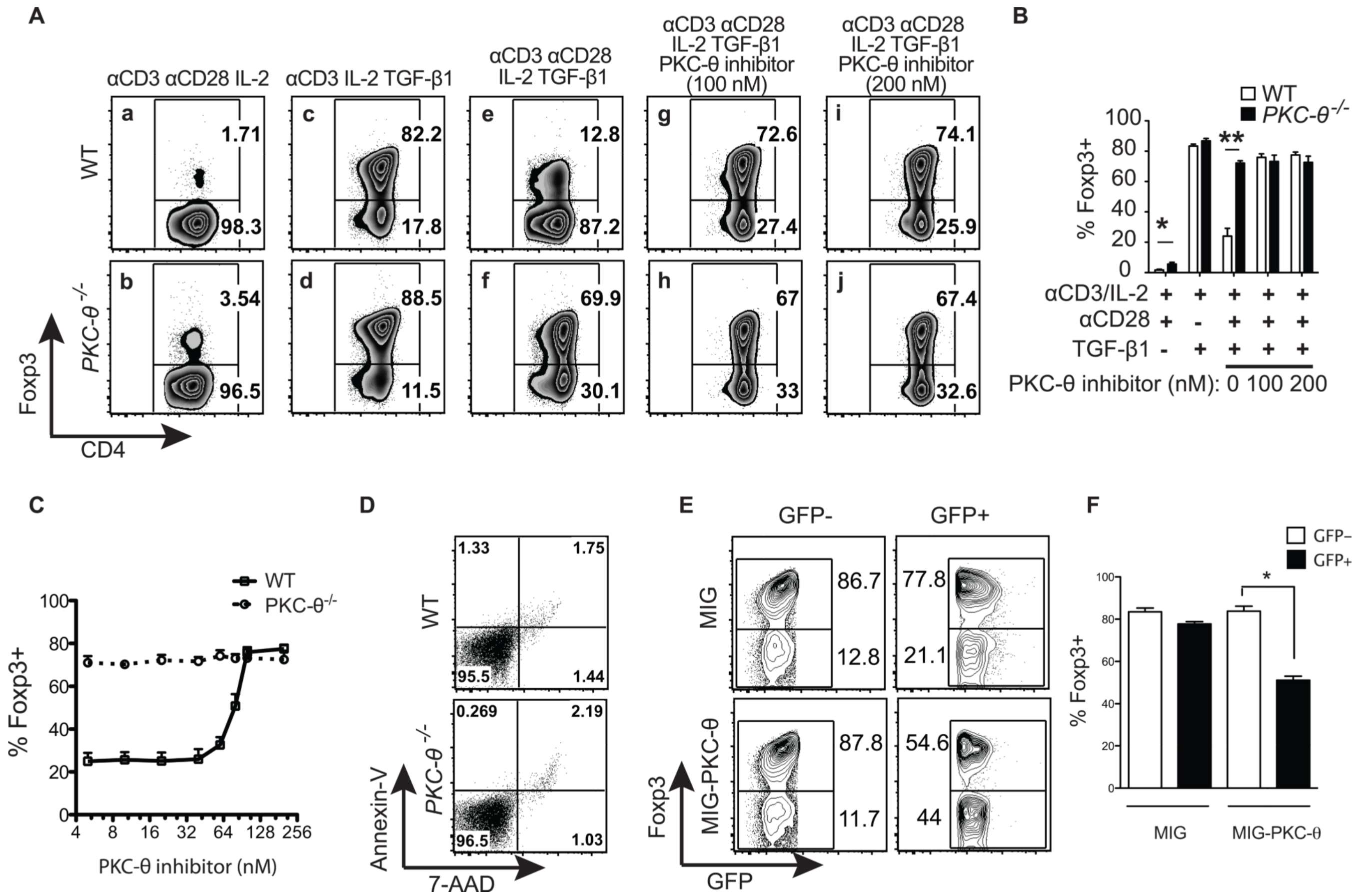

Figure 2.

PKC-θ$^{-/-}$ T cells have enhanced potential to differentiate into iTregs. (A) Isolated naïve CD4^{+} T cells from WT or *PKC-θ*$^{-/-}$ mice were differentiated into iTregs for 3 days under the conditions indicated. Intracellular Foxp3 detected with a specific antibody was then analyzed by flow cytometry. (B) The percentage of Foxp3+ cells were averaged (mean ± SEM) from four independent experiments described in (A), *, $p<0.05$; **, $p<0.01$; error bars indicate ±SD. (C) PKC-θ inhibitor promotes iTreg formation in a dose dependent manner. CD4^{+} T cells from WT (solid line) or *PKC-θ*$^{-/-}$ (dotted line) mice were differentiated into iTregs for 3 days in the presence different concentrations of PKC-θ inhibitor (X-axies), and percentage of iTreg induced is indicated (Y-axies). (D) No obvious difference in apoptosis was observed between WT and *PKC-θ*$^{-/-}$ T cells. Naïve CD4^{+} T cells from WT control or *PKC-θ*$^{-/-}$ mice were differentiated into iTreg cells with 5 ng/ml TGF-β1 and 10 ng/ml IL-2 for 3 days. Apoptotic cells were then detected by Annexin-V and 7-AAD staining. (E) Restoration of PKC-θ in *PKC-θ*$^{-/-}$ T cells restored the inhibition of iTreg differentiation. Naïve *PKC-θ*$^{-/-}$ T cells infected with MIG-PKC-θ or an empty virus control were differentiated into iTreg with anti-CD3/28, TGF-β1 and IL-2. Percentage of Foxp3- or Foxp3+ cells in GFP- and GFP+ cell populations are indicated. The plots shown are representative of at least three independent experiments. (F) Summary of (E) averaged from three independent experiments.

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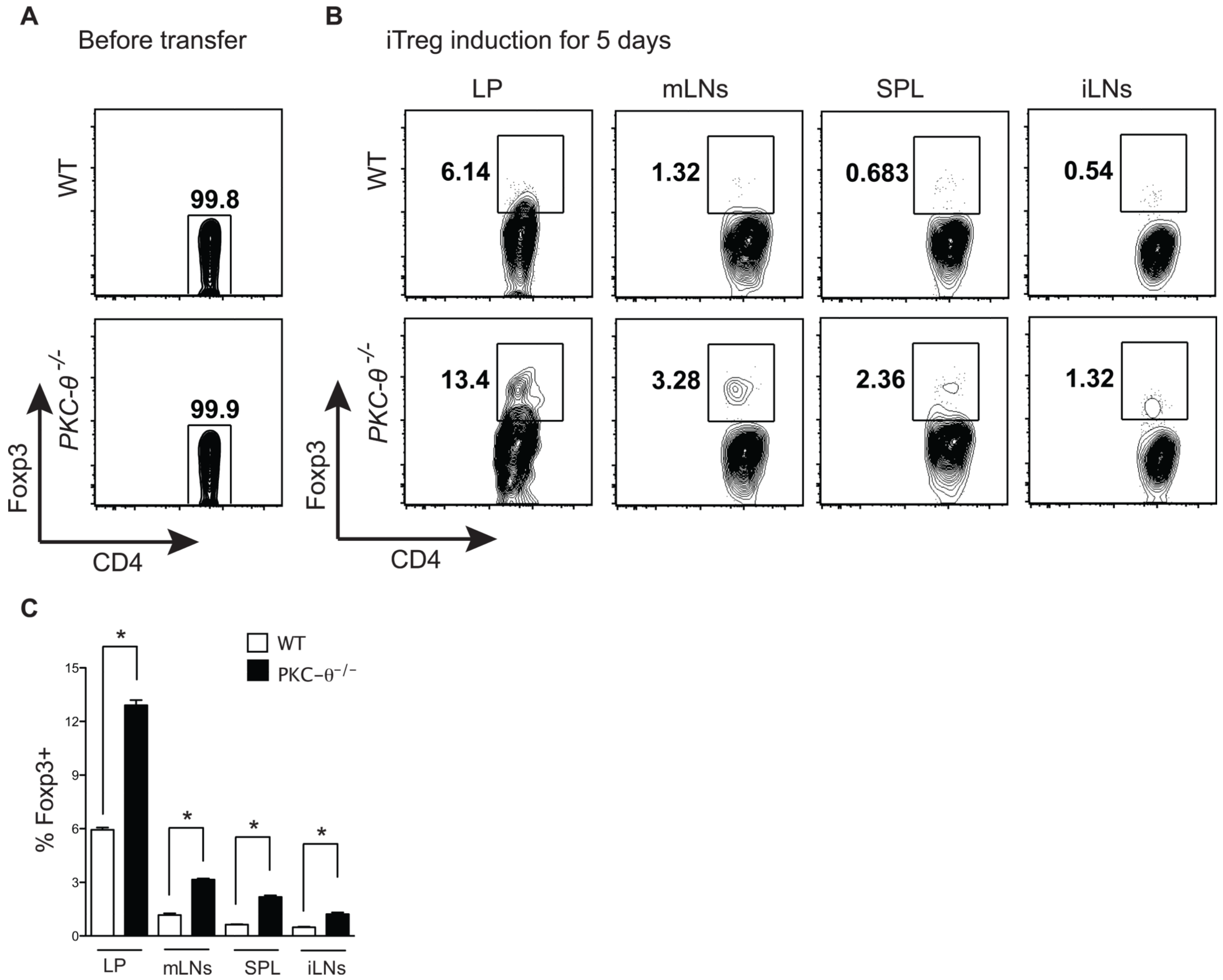

Figure 3.

PKC-θ$^{-/-}$ T cells form more iTreg cells *in vivo*. (A) Lack of Foxp3 expression in sorted naïve T cells prior to adoptive transfer. Foxp3 expression in freshly sorted $CD4^{+}CD25^{-}$ $CD44^{lo}CD62L^{hi}$ cells from OTII or OTII/*PKC-θ*$^{-/-}$ mice was detected by flow cytometry. (B) Sorted donor cells (CD45.2+) as indicated in (A), were transferred into CD45.1 syngeneic hosts that were then fed OVA in the drinking water for 5 days. Intracellular Foxp3 in $CD45.2^{+}CD4^{+}TCR\text{-}\beta^{+}$ cells in the LP, mLNs, iLNs and SPL were detected by flow cytometric analysis. Plots shown are representative of three independent experiments. (C) Summary of (B) averaged from three independent experiments.

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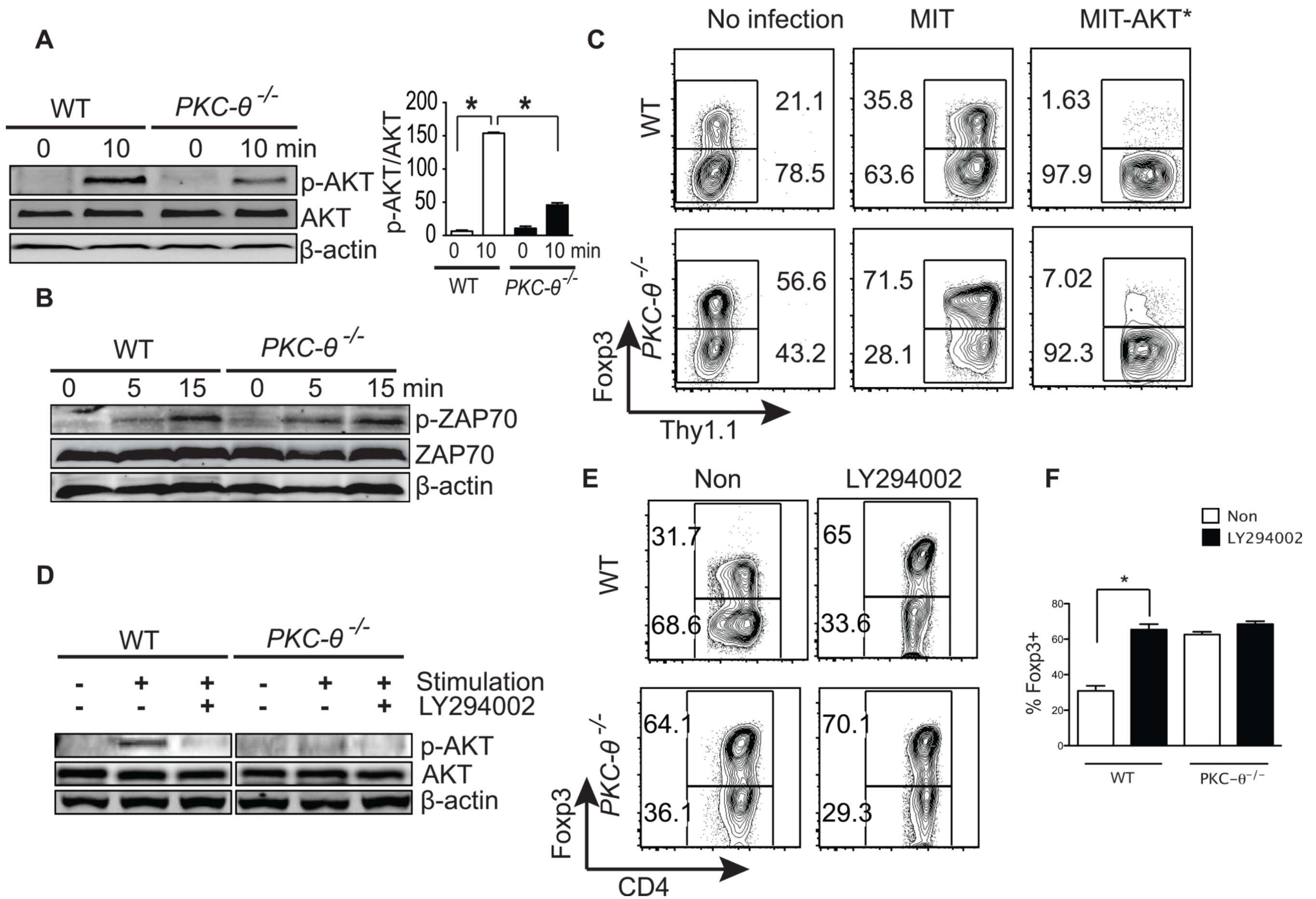

Figure 4.

Impaired activation of AKT in *PKC-θ*$^{-/-}$ T cells is responsible for enhanced iTreg differentiation. (A) Impaired AKT activation in *PKC-θ*$^{-/-}$. Naïve WT and *PKC-θ*$^{-/-}$ T cells were either untreated or stimulated with 1 μg/ml anti-CD3, 1 μg/ml anti-CD28 and 5 ng/ml TGF-β1. Phosphorylation of AKT (T308), expression of total AKT, and the β-actin control were detected by Western blot analysis. The ratio between phosphorylated-AKT and total AKT bands were averaged (mean ± SEM) from three experiments; *, $p<0.01$; error bars indicate ±SD. (B) Normal activation of ZAP70 in *PKC-θ*$^{-/-}$ T cells. WT and *PKC-θ*$^{-/-}$ T cells were either untreated or stimulated with similar conditions as in (A) and phosphorylation of ZAP70 (Tyr493), expression of total ZAP70, and the β-actin control were detected by Western blot analysis. (C) Forced expression of constitutively active AKT (AKT*) inhibits iTreg differentiation. WT and *PKC-θ*$^{-/-}$ T cells were either untreated or infected with control virus (MIT) or virus expressing activated AKT (MIT-AKT*). T cells were then differentiated into iTreg cells with anti-CD3/28, TGF-β1 and IL-2. Thy1.1 was a marker for successful retroviral transduction, and thus expression of the protein of interest. Percentages of Foxp3 negative or positive cells are indicated. (D) LY294002 prevents AKT phosphorylation. WT and *PKC-θ*$^{-/-}$ T cells were either untreated or stimulated with the conditions described in (A) in the presence or absence of 10 μM LY294002. Phosphorylation of AKT (T308), expression of total AKT and the β-actin control were detected by Western blot analysis. (E) LY294002 potentiates iTreg differentiation. Naïve WT and *PKC-θ*$^{-/-}$ T cells were differentiated into iTreg cells with or without 10 μM LY294002. Tregs were detected by flow cytometric analysis of intracellular Foxp3 detected

with a specific antibody. Results shown are representative of at least three independent experiments. (F) Summary of (E) averaged from three independent experiments.

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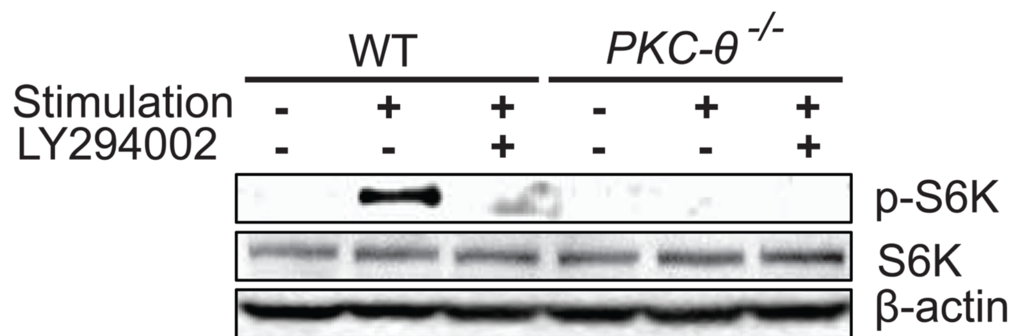

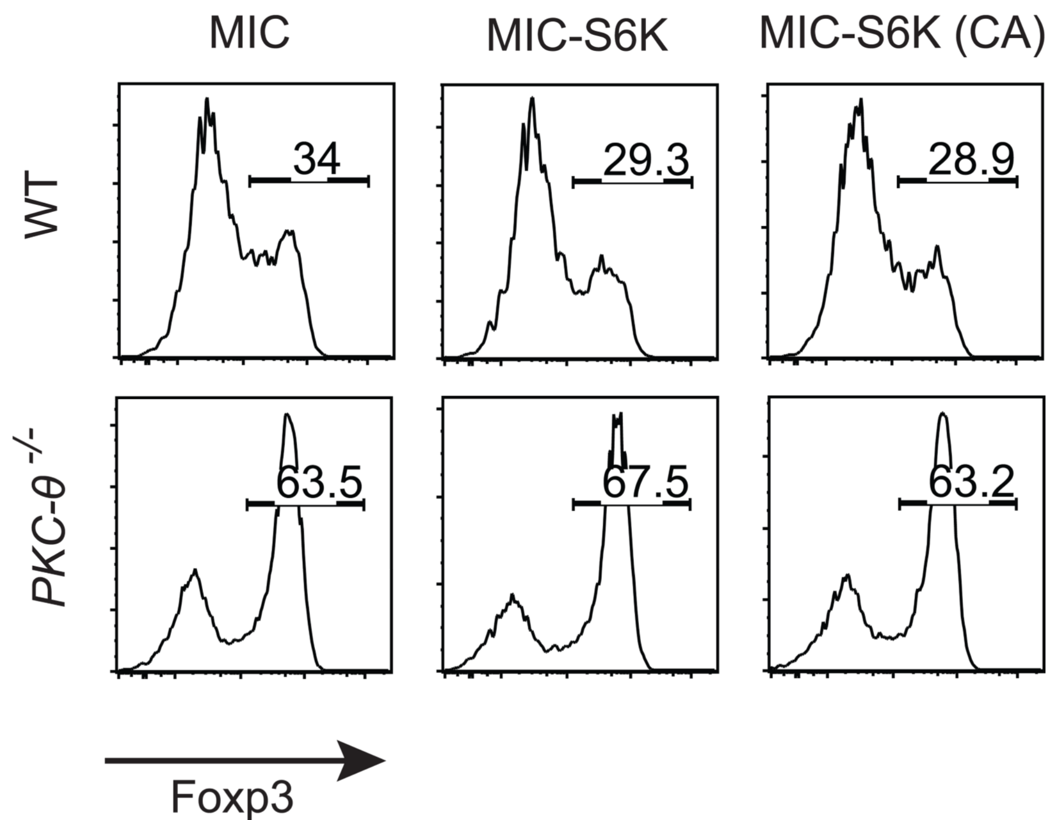

Figure 5.

S6K is not involved in PKC-θ-mediated iTreg differentiation. (A) Activation of S6K is impaired in *PKC-θ*$^{-/-}$ T cells. T cells were either untreated or stimulated with 1 μg/ml anti-CD3, 1 μg/ml anti-CD28, 5 ng/ml TGF-β1 in the presence or absence of LY294002 (10 μM). Phosphorylation of S6K (T389), expression of total S6K and the β-actin control were detected by Western blot analysis. (B) Forced expression of WT S6K or active S6K [S6K (CA)] does not affect iTreg differentiation. T cells infected with MIC-S6K, MIC-S6K (CA) or control virus were differentiated into iTreg cells for 3 days with anti-CD3/28, TGF-β1 and IL-2. Tregs were detected by flow cytometric analysis of intracellular Foxp3. Results shown are representative of at least three independent experiments.

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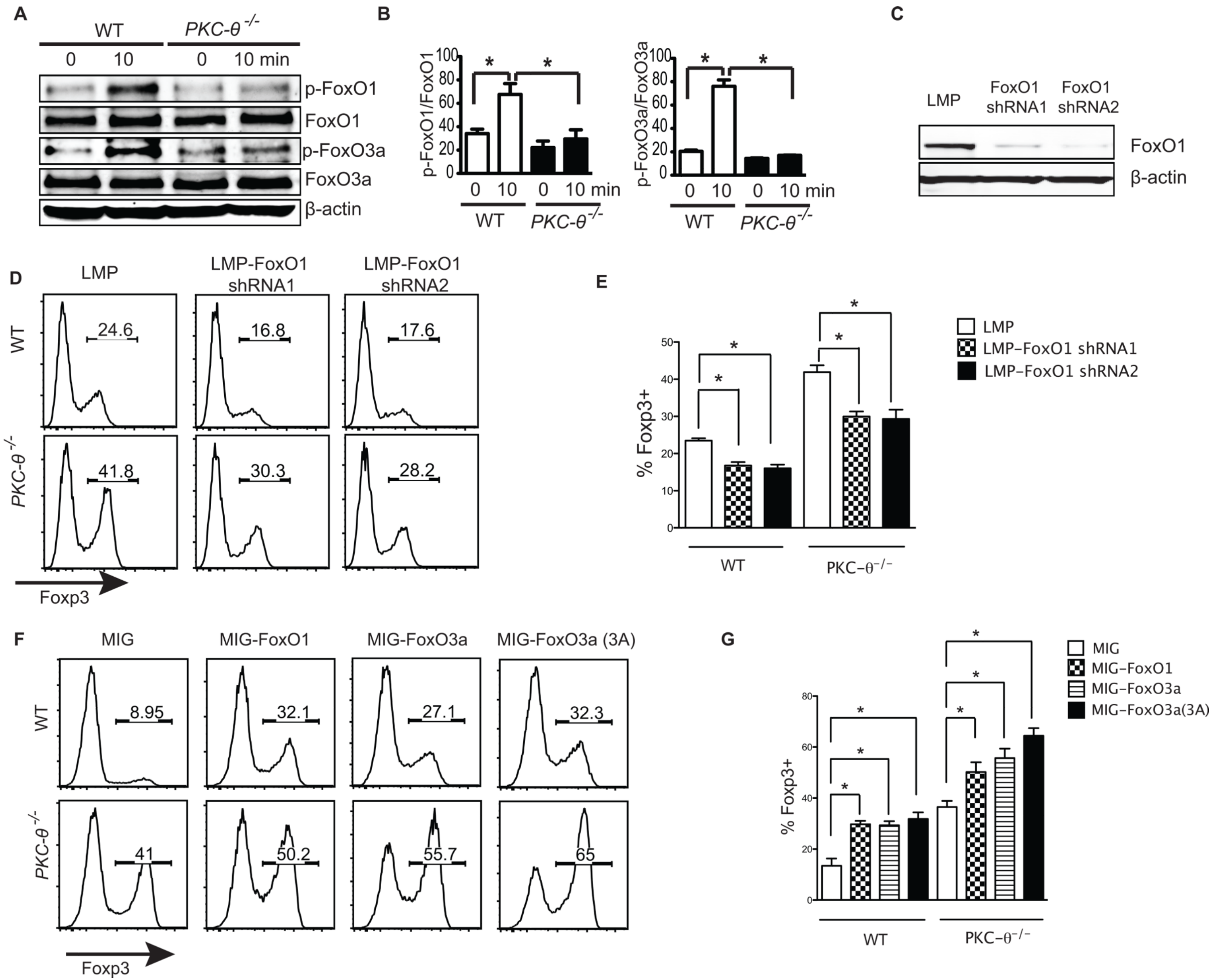

Figure 6.

FoxO1 and FoxO3a mediate PKC-θ-enhanced iTreg differentiation. (A) Reduced phosphorylation of FoxO1 and FoxO3a in *PKC-θ*$^{-/-}$ T cells. Naïve WT and *PKC-θ*$^{-/-}$ T cells were either untreated or stimulated with 1 μg/ml anti-CD3, 1 μg/ml anti-CD28 and 5 ng/ml TGF-β1. Phosphorylation of FoxO1 (S-256) and FoxO3a (S-253), expression of total FoxO1, FoxO3a and the β-actin control were detected by Western blot analysis. (B) Ratio of bands representing phosphorylated-FoxO1 and total FoxO1, phosphorylated-FoxO3a and total FoxO3a. Bands were averaged (mean ± SEM) from three experiments; *, $p<0.01$; error bars indicate ±SD. (C) Knockdown of FoxO1 by shRNA. T cells were transduced with LMP expression shRNA1 and shRNA 2 targeting FoxO1, and FoxO1 expression was detected by westernblot analysis. (D) Knock down of FoxO1 impairs iTreg differentiation. T cells infected with two different LMP-FoxO1 shRNAs or a control virus were differentiated into iTreg cells. Expression of Foxp3 was detected by intracellular staining. (E) Summary of (D) averaged from three independent experiments. (F) Forced expression of FoxO1, FoxO3a or constitutively active FoxO3a (FoxO3a [3A]) enhances iTreg differentiation. Naïve WT and *PKC-θ*$^{-/-}$ T cells infected with MIG-FoxO1, MIG-FoxO3a, MIG-FoxO3a (3A), or a control virus were differentiated into iTreg cells. Tregs were detected by flow cytometric analysis of

intracellular Foxp3. Results shown are representative of at least three independent experiments. (G) Summary of (F) averaged from three independent experiments.

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