Published in final edited form as:

Mol Immunol. 2008 December ; 46(2): 213–224. doi:10.1016/j.molimm.2008.08.275.

Differential requirement of PKC- θ in the development and function of Natural Regulatory T cells

Sonal Gupta¹, Santhakumar Manicassamy¹, Chenthamarakshan Vasu², Anvita Kumar³, Weirong Shang⁴, and Zuoming Sun^{1,*}

¹Department of Microbiology & Immunology, School of Medicine, University of Illinois, Chicago, IL 60612, USA

²Department of Surgery, School of Medicine, University of Illinois, Chicago, IL 60612, USA

³Department of Immunology/Microbiology, Rush University Medical Center, Chicago, IL, 60612, USA

⁴Department of Gynecology and Obstetrics, Emory University School of Medicine, Atlanta, GA 30308

Abstract

CD4+CD25+ natural Treg cells, which are developed in the thymus, migrate to the periphery to actively maintain self-tolerance. Similar to conventional T cells, TCR signals are critical for the development and activation of Treg cell inhibitory function. While PKC- θ -mediated TCR signals are required for the activation of peripheral naïve T cells, they are dispensable for their thymic development. Here, we show that mice deficient in *PKC- θ* had a greatly reduced number of CD4+Foxp3+ Treg cells, which was independent of PKC- θ -regulated survival, as transgenic *Bcl-x_L* could not restore the Treg cell population in *PKC- θ ^{-/-}* mice. Active and WT PKC- θ markedly stimulated, whereas inactive PKC- θ and dominant negative NFAT inhibited Foxp3 promoter activity. In addition, mice deficient in calcineurin A β had a decreased Treg cell population, similar to that observed in PKC- θ deficient mice. It is likely that PKC- θ promoted the development of Treg cells by enhancing Foxp3 expression via activation of the calcineurin/NFAT pathway. Finally, Treg cells deficient in PKC- θ were as potent as WT Treg cells in inhibiting T cell activation, indicating that PKC- θ was not required for Treg cell-mediated inhibitory function. Our data highlight the contrasting roles PKC- θ plays in conventional T cell and natural Treg cell function.

Keywords

CD4+CD25+ cells; PKC- θ ; T cell development; survival

Introduction

The maintenance of peripheral tolerance is critical for preventing autoimmunity. It has been demonstrated that Treg cells are important components of the tolerance mechanisms required to prevent activation of autoreactive T cells (Miyara and Sakaguchi, 2007; Sakaguchi et al.,

© 2008 Elsevier Ltd. All rights reserved.

*Corresponding author Mailing address: Division of Immunology, Beckman Research Institute of the City of Hope, 1500 East Duarte Road, Duarte, CA 91010 Phone: 626-256-4673, Ext. 68851, Email: E-mail: zsun@coh.org.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

2006; Shevach, 2006; Shevach et al., 2006). Sakaguchi first identified CD4+CD25+ as Treg cells, as depletion of CD4+CD25+ Treg cells resulted in the development of widespread autoimmune/inflammatory diseases such as autoimmune gastritis, thyroiditis, and type 1 diabetes, as well as inflammatory bowel disease [5]. These autoimmune disorders were prevented by reconstitution of mice with CD4+CD25+ Treg cells (Sakaguchi et al., 1995; Singh et al., 2001). Thornton and Shevach developed a simple and reproducible assay to demonstrate that Treg cells, when co-cultured with responder CD4+CD25- T cells, markedly suppressed proliferation of responders (Thornton and Shevach, 1998). The inhibition of proliferation was dependant upon cell contact between Treg cells and responders, and required the activation of Treg cells via TCR. The observed inhibitory effects were overcome by high concentrations of exogenous IL-2. Cell contact-independent mechanisms were also involved in Treg cell-mediated inhibition. For example, Treg cell-mediated inhibition of autoimmune colitis was dependant upon TGF- β (Izcue et al., 2006). There is data to suggest that in addition to inhibiting T cell activation and dendritic cell maturation (Kriegel et al., 2006), TGF- β could also potentially inhibit immune responses by inducing the production of more Treg cells, as TGF- β can convert CD4+CD25- cells to Treg phenotype by inducing Foxp3 expression (Chen et al., 2003; Fantini et al., 2004). Production of TGF- β by Treg cells also requires stimulation via TCR. *In vitro* and *in vivo* evidence support Treg cell mediation of active maintenance of self-tolerance. Enhancing Treg cell-mediated inhibition of autoreactive T cells is thus considered a novel method to prevent and treat autoimmunity.

Treg cells are developed in the thymus and migrate to peripheral lymphoid tissues (Asano et al., 1996; Itoh et al., 1999). The Foxp3 transcription factor is specifically expressed in natural Treg cells, and is a master gene governing the development of Treg cells. A mutation in Foxp3 gene implicated Foxp3 as a critical component in the development of autoimmune syndrome in mice and humans (Fontenot et al., 2005b; Hori et al., 2003). Forced expression of Foxp3 was sufficient to convert naïve T cells to the Treg cell phenotype (Hori et al., 2003). Additionally, TCR-mediated signals were required to induce the expression of Foxp3, and thus guide the development of Treg cells in the thymus (Cabarrocas et al., 2006; Pacholczyk et al., 2002), making TCR signals critical for both Treg cell development and effector function. However, little is known about the downstream TCR signaling events regulating thymic generation, peripheral activation, and Treg cell function.

While PKC- θ is a critical TCR signaling molecule required for the activation and survival of peripheral naïve T cells, it is dispensable for the development of T cells (Barouch-Bentov et al., 2005; Manicassamy et al., 2006a; Manicassamy and Sun, 2007; Sun et al., 2000). PKC- θ has been shown to play a role in the development of Th2 and Th1 responses in several different animal models (Marsland et al., 2004; Salek-Ardakani et al., 2004; Salek-Ardakani et al., 2005; Tan et al., 2006). T cells obtained from *PKC- θ ^{-/-}* mice failed to proliferate and produce IL-2 upon TCR stimulation due to defective activation of NF- κ B and AP1; these observations were supported by several *in vitro* studies in Jurkat T cells (Baier-Bitterlich et al., 1996; Coudronniere et al., 2000; Li et al., 2004; Lin et al., 2000). PKC- θ also mediates TCR signaling events critical for the activation of NFAT, in addition to NF- κ B and AP1 (Altman et al., 2000; Pfeifhofer et al., 2003; Sun et al., 2000). T cells obtained from mice deficient in other isoforms of PKC do not display defects similar to those observed in *PKC- θ ^{-/-}* T cells (Baier, 2003), demonstrating the selective requirement for PKC- θ in T cell activation. Interestingly, although PKC- θ modulates TCR signals required for peripheral T cell activation, T cell development in the thymus, including positive and negative selection, which are also strictly dependent on TCR-mediated signals, is normal in mice lacking PKC- θ (Sun et al., 2000). Thus, PKC- θ does not mediate signals required for the development of conventional T cells.

As TCR signals are essential for both the development and effector function of Treg cells, we investigated the roles of PKC- θ in both of these processes using *PKC- θ ^{-/-}* mice. We showed

that CD4+Foxp3+ cells were significantly reduced in *PKC-θ*^{-/-} mice. Furthermore, we demonstrated that PKC-θ stimulated Foxp3 promoter activity, likely via the calcineurin/NFAT pathway, implicating the PKC-θ-mediated calcineurin/NFAT pathway in the regulation of Treg cell development through the enhancement of Foxp3 expression. In agreement, development of Treg cells was defective in mice lacking calcineurin Aβ. In spite of the defective development, Treg cells obtained from *PKC-θ*^{-/-} mice inhibited T cell activation as effectively as WT Treg cells. Thus, in contrast to its function in conventional T cells, our data demonstrate that PKC-θ is required for Treg cell development, but is dispensable for Treg cell inhibitory function.

Materials and Methods

Mice

PKC-θ^{-/-} mice on a C57BL/6 (B6) genetic background (Sun et al., 2000) and *Bcl-x_L*^{Tg} mice on a B6 background (Chao et al., 1995) have been described previously. B6 mice served as WT control. *Bcl-x_L*^{Tg} mice were crossed with *PKC-θ*^{-/-} mice, and *Bcl-x_L*^{Tg}/*PKC-θ*^{-/-} mice were screened by genomic Tail PCR (Viagen DirectPCR Cat. 101-T) for the presence of the *Bcl-x_L* transgene. Ova-specific TCR-transgenic OT-2 mice on B6 background were a kind gift from Dr. C Vasu (University of Illinois, Chicago). Mice were housed in a specific pathogen-free animal facility of the University of Illinois, following university guidelines. Animal experiments were done with the permission of national or local authorities. Animal welfare assurance #069R2. Approved RACC#07023.

Plasmids

The WT, constitutively active (A148E) and kinase inactive (K409R) human PKC-θ-expression vectors were gifts from A. Altman (Division of Cell Biology, La Jolla Institute for Allergy and Immunology, San Diego, CA). Genomic DNA from tail of C57BL/6 mice was used to PCR-amplify (*PfuTurbo*® DNA Polymerase, Stratagene Cat. 600250) fragments of varying lengths of 5' UTR of mouse Foxp3 gene using specific primers, as shown in the table below, from positions -515, -343, -317, -298 to +180 of transcription start site (TSS). PCR products were cloned into pCR4-TOPO (Invitrogen) after A-tailing and sequenced using M13 sequencing primers at the DNA Sequencing facility of the University of Illinois. Constructs, without base-substitutions, were selected for further cloning into a pGL4.10 vector (Promega) using KpnI and XhoI restriction sites. Desired clones were identified using restriction digestion analysis and sequencing using RVprimer3. The following primers were used to create different lengths of Foxp3 promoter reporters.

Primer	Sequence (5'-3')
Foxp3-515	AAGGTACCTGCTTCCCATTCACATGGC
Foxp3-343	AAGGTACCGTTTAAGAAATTGTGGTTTC
Foxp3-317	AAGGTACCGCCCTGTTATCTCATTGATA
Foxp3-298	AAGGTACCACCTTTTACCTCTGTGGTGA
Foxp3+180	ATTCTCGAGTTCCAAAGTCCTTACCTGGAG
RVprimer3	CTAGCAAAATAGGCTGTCCC

Antibodies

Purified NA/LE Hamster anti-mouse CD3e (145-2C11) (Cat. 553057), NA/LE Hamster IgG1κ isotype control (Cat. 553968), NA/LE Hamster anti-CD28 (37.51) (Cat. 553294), anti-mouse CD4-PE, CD4-FITC, CD8-FITC, CD25-PE, and CTLA-4-PE were purchased from BD

Pharmingen; anti-mouse GITR-APC antibody from BioLegend (San Diego, Ca); Foxp3-APC (Mouse Regulatory T cell staining kit, anti-mouse Foxp3 clone FJK-16s, Cat. 88-8118) and various isotype antibodies from eBioscience (San Diego, Ca); and mouse TGF- β 1 ELISA Ready-SET-Go (Cat. 88-7344) and mouse IL-2 ELISA Ready-SET-Go (Cat. 88-7024) from eBioscience.

Cell Sorting of CD4+CD25⁻ and CD4+CD25⁺ T cells

Spleen and lymph nodes from WT and *PKC- θ ^{-/-}* mice (6–8 week old) were surgically removed, crushed and passed through a nylon mesh (40 mm, BD Falcon, Cat. 352340) to obtain a single cell suspension. CD4⁺ T cell population was isolated on an autoMACS (Miltenyi Biotec) using CD4 T cell Isolation kit (Miltenyi Biotec), according to manufacturer's instructions. Briefly, splenic cell suspension was stained by biotinylated antibody cocktail containing various antibodies, except for CD4, followed by incubation with magnetic beads, and subsequent negative enrichment of CD4⁺ T cells. Enriched CD4⁺ cells were stained with anti-CD4-FITC and anti-CD25-PE or CD25-PE alone, followed by sorting of CD4+CD25⁺ T cells and CD4+CD25⁻ T cells using a high-speed MoFlo sorter (Dako). Purity of sorted populations, as determined by flow cytometric analysis, was $\geq 95\%$.

CFSE staining

CellTrace™ CFSE Cell Proliferation Kit (Cat. C34554) was purchased from Molecular Probes. CFSE (carboxyfluorescein diacetate succinimidyl ester) stock solution (5 mM) was prepared in DMSO, prior to use. Cells were washed twice with cold PBS (Gibco) and resuspended (107/ml) in pre-warmed PBS containing CFSE (5 mM). After incubation (10 min, 37°C, in the dark), staining was stopped by adding equal volume of 20% fetal bovine serum. Cells were pelleted by centrifugation, resuspended in fresh pre-warmed medium, incubated (20 min, 37°C) to ensure complete modification of the probe, washed twice with PBS, counted and used for *in vitro* suppression assays.

Proliferation and *In Vitro* Suppression Assays

After enrichment from the spleen of either WT or *PKC- θ ^{-/-}* mice, CD4+CD25⁻ (5×10^4) and varying amounts of CD4+CD25⁺ T cells were added to U-bottom 96-well plates, containing γ -irradiated (3000 rad) T cell-depleted WT splenocytes (1×10^5) as APCs. Cells were then stimulated with indicated amounts of anti-CD3 or isotype control antibodies. In experiments where cells were stimulated with plate-bound anti-CD3/CD28 antibodies alone, indicated amounts of anti-CD3 and CD28 antibodies were added to 96-well plates and incubated (2 h, 37°C), followed by washing twice with PBS. For proliferation, cells were cultured (72 h, 37°C) and pulsed with 1 mCi of [³H] thymidine for the last 16 h of culture. Cells were then harvested onto a glass-filter using Tomtec 96-well cell harvester and assessed for thymidine incorporation by liquid scintillation in a Wallac MicroBeta counter (Perkin Elmer). Alternatively, CD4+CD25⁻ T cells were loaded with CFSE dye, as described above, before addition to co-culture. After 72 h, cells were collected, surface-stained for CD4 and analyzed by flow cytometry to visualize CFSE dilution. In some experiments, CD4+CD25⁺ T cells from WT and *PKC- θ ^{-/-}* mice were stimulated with indicated amounts of plate-bound anti-CD3/CD28 antibodies plus IL-2 (5U/ml) and cultured (24 h, 37°C) prior to co-culturing with equal numbers of CFSE-stained CD4+CD25⁻ T cells (5×10^4) from ova-transgenic mice (OT-2 mice). γ -irradiated T cell-depleted splenocytes (1×10^5) from OT-2 mice, loaded with soluble ova peptide (1 μ g/ml, Sigma) were used as APCs.

Nucleofection and Luciferase Reporter Assay

Foxp3-promoter-luciferase reporters, along with empty vector or various expression plasmids, were introduced into unstimulated human CD4⁺ T cells (Human T cell Nucleofector kit,

Amaya Biosystems, DPA-1002), according to manufacturer's instructions. In brief, freshly isolated CD4⁺ T cells (5×10^6) from peripheral blood mononuclear cells (PBMCs) of healthy human volunteers were mixed with Foxp3 promoter reporter construct (3 μ g) and maxGFP, various PKC- θ expression plasmids or empty vector to obtain equal amounts of DNA in nucleofector solution (100 μ l). Renilla luciferase vector was added as the internal control for luciferase assays. The V-024 program was used for nucleofection. After pulsing, cells were recovered in pre-equilibrated culture medium, incubated (12 h, 37°C, 5% CO₂), then medium was replaced with fresh equilibrated medium and stimulated with plate-bound anti-CD3 and anti-CD28 antibodies for an additional 24 h. Cells were collected, washed with PBS and lysed using 1 $\times$ passive lysis buffer (Promega), followed by luciferase assay using Dual Luciferase kit (Promega).

Statistical Analysis

Statistical analyses were performed using Unpaired Student's t-Test (Two-Sample Assuming Unequal Variances). Analyses in Figure 2B were performed using ANOVA (Single factor).

Results

PKC- $\theta^{-/-}$ mice have significantly reduced CD4⁺Foxp3⁺ Treg cells

Foxp3, specifically expressed in natural Treg cells, is critical for Treg cell development and its forced expression is sufficient to convert naïve T cells to the Treg cell phenotype (Hori et al., 2003). We thus examined Treg cells in PKC- $\theta^{-/-}$ mice by first identifying CD4⁺Foxp3⁺ positive cells in the thymus utilizing a monoclonal antibody that recognizes intracellular Foxp3, (Fig. 1A, left panel). The total number of CD4 single-positive thymocytes was equivalent in WT and PKC- $\theta^{-/-}$ mice (data not shown), in accordance with the normal thymic development of CD4 T cells in PKC- $\theta^{-/-}$ mice (Sun et al., 2000). Consistent with previous studies (Hori et al., 2003), Foxp3⁺ cells represented about 5% of the CD4 single positive thymocytes in WT mice. In contrast, PKC- $\theta^{-/-}$ mice had markedly reduced CD4⁺Foxp3⁺ cells, usually less than 1% (Fig. 1A, right panel) (Schmidt-Suppran et al., 2004). Furthermore, the mean fluorescent intensity (MFI) of Foxp3 expression was greatly reduced in PKC- $\theta^{-/-}$ thymocytes (MFI=45) compared to WT T cells (MFI=104), suggesting that not only the number of CD4⁺Foxp3⁺ cells, but also the levels of Foxp3 protein in each cell were reduced in thymocytes from PKC- $\theta^{-/-}$ mice.

We next analyzed Treg cells in peripheral lymphoid tissues (Fig. 1B). Consistent with the phenotype in thymus, PKC- $\theta^{-/-}$ mice had a lower percentage of CD4⁺Foxp3⁺ cells in both the spleen and lymph node, compared to WT mice. Interestingly, there was no obvious difference in the MFI of Foxp3 in peripheral T cells between WT and PKC- $\theta^{-/-}$ mice. We also investigated the surface expression of glucocorticoid-induced tumor necrosis factor receptor family-related gene (GITR), considered another Treg cell marker given its predominant expression in these cells (Shimizu et al., 2002). Indeed, the number of CD4⁺GITR⁺ cells was greatly reduced in the spleen and lymph nodes of PKC- $\theta^{-/-}$ mice compared to WT (Fig. 1c), confirming the defective development of Treg cells in PKC- $\theta^{-/-}$ mice. These data confirmed PKC- θ was a critical molecule required for the normal development of Treg cells.

PKC- θ -regulated Treg development is independent of PKC- θ -mediated survival

Previous studies, by our laboratory and others, have shown that PKC- θ regulates T cell survival by up-regulating Bcl-x_L, a critical survival factor (Barouch-Bentov et al., 2005; Manicassamy et al., 2006a; Saibil et al., 2007). This raises the possibility that PKC- θ may enhance Treg cell survival by a similar mechanism, and that the reduced Treg cell population observed in PKC- $\theta^{-/-}$ mice resulted from apoptosis. To determine whether PKC- θ -regulated Bcl-x_L plays a role in Treg cell development, PKC- $\theta^{-/-}$ mice were crossed to Bcl-x_L transgenic mice (Bcl-x_L^{Tg}).

The *Bcl-x_L* transgene is specifically targeted to T cell compartments using a Lck proximal promoter (Chao et al., 1995). To ensure that *Bcl-x_L^{Tg}* would prevent T cell apoptosis resulting from the lack of PKC- θ , apoptosis was compared between T cells obtained from spleens of WT, *PKC- $\theta^{-/-}$* , *Bcl-x_L^{Tg}*, and *PKC- $\theta^{-/-}$ /*Bcl-x_L^{Tg}* mice (Fig. 2A). Purified CD4⁺ T cells from different genotypes of mice were cultured and stimulated (or not) with anti-CD3/28 antibodies; apoptotic cells were then detected by annexin V (Manicassamy et al., 2006a; Xie et al., 2005a; Xie et al., 2005b). Consistent with our previously published data (Manicassamy et al., 2006a), in the absence of stimulation, *PKC- $\theta^{-/-}$* CD4⁺ T cells displayed slightly more apoptosis than their WT counterparts. However, CD3 and CD28 stimulation resulted in *PKC- $\theta^{-/-}$* CD4⁺ T cells undergoing accelerated apoptosis (~65% apoptotic cells) compared to the WT cells (~30% apoptotic cells). *Bcl-x_L^{Tg}* restored the survival of *PKC- $\theta^{-/-}$* T cells to levels similar to those observed in WT cells (~30%), suggesting that *Bcl-x_L^{Tg}* was sufficient to prevent apoptosis resulting from the lack of PKC- θ . We then examined CD4⁺Foxp3⁺ cells in the thymus of different mouse genotypes (Fig. 2B) and found that they represented 5% of the CD4 single-positive thymocytes in WT and *Bcl-x_L^{Tg}* mice, suggesting that *Bcl-x_L^{Tg}* did not significantly affect the development of Treg cells. Although, the total number of CD4 single-positive thymocytes were increased in *Bcl-x_L^{Tg}* mice, it did not affect the overall percentage of Foxp3⁺ Tregs among this subset. Consistent with previous results (Fig. 1A), *PKC- $\theta^{-/-}$* mice had greatly reduced levels of CD4⁺Foxp3⁺ cells. While *Bcl-x_L^{Tg}* prevented apoptosis of *PKC- $\theta^{-/-}$* T cells, it failed to restore the Treg cell population in *PKC- $\theta^{-/-}$ /*Bcl-x_L^{Tg}* mice. Furthermore, MFI data suggested Foxp3 levels were kept at lower levels in *PKC- $\theta^{-/-}$ /*Bcl-x_L^{Tg}* mice, similar to that observed in the *PKC- $\theta^{-/-}$* mice, suggesting that forced expression of *Bcl-x_L* had no effects on the defective development of Treg cells. These results indicated that the reduced number of thymic Treg cells in *PKC- $\theta^{-/-}$* mice was not due to defective survival in the absence of PKC- θ .***

To investigate whether there was an intrinsic requirement for PKC- θ in Treg development, we adoptively transferred bone marrows from WT and *PKC- $\theta^{-/-}$* mice to *Rag1^{-/-}* mice and analyzed Tregs in the thymus (Fig. 2C) six weeks after adoptive transfer. Similar to what was observed in *PKC- $\theta^{-/-}$* mice, *Rag1^{-/-}* mice adoptively transferred with *PKC- $\theta^{-/-}$* bone marrow contained a lower percentage of CD4⁺Foxp3⁺ cells in the thymus compared to *Rag1^{-/-}* mice reconstituted with WT bone marrow. This result suggested that defective Treg development in *PKC- $\theta^{-/-}$* mice was likely due to lack of PKC- θ in T cells or progenitors, but not in other tissues.

PKC- θ stimulates Foxp3 promoter activity in a NFAT-dependent manner

PKC- $\theta^{-/-}$ mice not only had reduced CD4⁺Foxp3⁺ cells, but also had displayed lower MFI for Foxp3 compared to WT cells (Fig. 1A), suggesting that PKC- θ was essential for maintaining high levels of Foxp3 expression. We then analyzed the mouse Foxp3 promoter region and identified three potential NFAT-binding sites (Fig. 3A), which have also been shown to be conserved on the human Foxp3 promoter (Mantel et al., 2006). Previous studies showed that PKC- θ was able to stimulate the Ca²⁺/calcineurin/NFAT pathway in T cells (Altman et al., 2004; Manicassamy et al., 2006b; Pfeifhofer et al., 2003). We first investigated whether PKC- θ stimulated Foxp3 promoter activity. Several different DNA fragments of the Foxp3 promoter were cloned into pGL4, a luciferase reporter. The general scheme of the mouse Foxp3 promoter and positions of various promoter reporter constructs are depicted in Figure 3A. Construct p515 contained all three potential NFAT-binding sites, while construct p343 contained only one and constructs p317 and p298 had all three NFAT-binding sites deleted. pGL4 and four Foxp3 reporters were transfected into CD4⁺ T cells utilizing Amaxa system. Twelve hours after transfection, cells were stimulated (24 hr), or not, with anti-CD3 and CD28 antibodies (Fig. 3B). Our data showed minimal reporter activity associated with p317 and p298, similar to the activity obtained from pGL4 (promoterless parental plasmid), while p343 displayed much

higher activity. The highest reporter activity was obtained from p515. These results suggested that NFAT-binding sites were potentially important for regulating Foxp3 promoter activity. Furthermore, TCR stimulation, which is known to activate the calcineurin/NFAT pathway, greatly stimulated the activity of reporters p343 and p515, which contained NFAT-binding sites, but not reporters p317 and p298 that did not, suggesting TCR-mediated signals were able to stimulate Foxp3 promoter activity.

To determine whether PKC- θ was critical for TCR-mediated Foxp3 reporter stimulation, WT, constitutively active, and inactive PKC- θ were used in transient transfection assays, together with pGL4 or p515 reporters (Fig. 3C). As expected, the various forms of PKC- θ had no obvious effects on pGL4 with or without TCR stimulation, suggesting that the stimulated reporter activity was due to the presence of the Foxp3 promoter. Even in the absence of TCR stimulation, WT PKC- θ slightly stimulated, whereas constitutively active PKC- θ greatly stimulated p515 activity, suggesting that forced expression of PKC- θ alone was sufficient to activate the Foxp3 reporter. TCR stimulation further potentiated PKC- θ -mediated Foxp3 reporter activation. More importantly, inactive PKC- θ abrogated TCR activation-induced Foxp3 reporter activity, clearly demonstrating the critical role of PKC- θ in the stimulation of Foxp3 reporter. To determine whether NFAT played a role in PKC- θ -mediated activation of Foxp3 reporter, a dominant-negative isoform of NFAT (dnNFAT) (Chow et al., 1999) was introduced into cells, together with active PKC- θ . Indeed, dnNFAT abrogated PKC- θ -mediated activation of Foxp3 reporter activity in the presence and absence of TCR stimulation, implicating NFAT as a critical factor, downstream of PKC- θ , in the stimulation of Foxp3 promoter activity.

Mice-deficient in calcineurin A β display defective development of CD4+Foxp3+ cells

Calcineurin (Cn) is a Ca²⁺/calmodulin-dependent phosphatase that dephosphorylates and activates NFAT (Manicassamy et al., 2008). There are three different isoforms of Cn transcribed from distinct genes (Crabtree, 1999). T cells predominantly expressed the CnA β isoform, and mice-deficient in CnA β , but not CnA α , displayed significant defects in T cell activation (Bueno et al., 2002; Zhang et al., 1996). Since NFAT was associated with PKC- θ -regulated Foxp3 reporter activity, we analyzed the development of CD4+Foxp3+ Treg cells in the thymus of CnA β ^{-/-} mice (Fig. 4). Similar to PKC- θ ^{-/-} mice, CnA β ^{-/-} mice also had reduced CD4+Foxp3+ cells compared to WT. Furthermore, Foxp3 levels in CnA β ^{-/-} thymocytes (MFI=154) were also lower than those displayed by WT mice (MFI=195). These results suggested that development of Treg cells and maintenance of high Foxp3 levels was dependent on CnA β as well as PKC- θ .

PKC- θ is dispensable for Treg cell-mediated inhibition of T cell activation

Activation of Treg cell inhibitory function requires stimulation via TCR. Since PKC- θ mediates TCR signals essential for conventional T cell activation, we investigated whether PKC- θ also mediated TCR signals required for Treg cell activation. CD4+CD25+ Treg cells (Fig. 5A) were sorted from spleens of WT and PKC- θ ^{-/-} mice. Over 90% of the sorted CD4+CD25+ cells expressed Foxp3 (Fig. 5B), confirming they were Treg cells. Treg cells were then mixed with WT CD4+CD25- responder cells at different ratios. Proliferation of the responders was monitored by [³H] thymidine incorporation (Fig. 5C). In response to stimulation with anti-CD3/28 antibodies, responders, but not Treg cells from WT and PKC- θ ^{-/-} mice, proliferated as expected. Consistent with previous reports (Sakaguchi, 2004; Thornton and Shevach, 1998), the proliferation of responders was inhibited by WT Treg cells in a concentration-dependent manner. Mixing WT Treg cells with responders at a 1:1 ratio almost completely inhibited responder proliferation, which was overcome by exogenous high concentrations of IL-2. More importantly, there were no obvious differences between WT and PKC- θ ^{-/-} Treg cell-mediated inhibition. Given that inhibition of T cell activation also resulted in the inhibition of IL-2 production by responders, we measured IL-2 concentration in the supernatant (Fig.

5D). Indeed, both WT and *PKC-θ*^{-/-} Treg cells inhibited IL-2 production in a concentration-dependent manner, confirming that *PKC-θ*^{-/-} Treg cells were as potent as WT Treg cells in the inhibition of T cell activation.

Cell proliferation was also monitored by labeling responders with carboxyfluorescein succinimidyl ester (CFSE), a fluorescent dye that permits direct detection of cell division as measured by the decreasing intensity of CFSE with each division (Fig. 5E). In the absence of stimulation, CD4+CD25⁻ responders displayed a major single CFSE peak (top left panel), confirming the lack of proliferation. As expected, in response to TCR stimulation, responders proliferated vigorously, as indicated by the increased population (80%) of CFSE-positive cells in the gated area (top right panel). In contrast, both CFSE-labeled WT (left panel, second row) and *PKC-θ*^{-/-} Treg cells (right panel, second row) did not proliferate in response to TCR stimulation. Responder proliferation was almost completely inhibited by mixing with either WT (left panel, third row) or *PKC-θ*^{-/-} (right panel, third row) Treg cells at a 1:1 ratio. As expected, reduction of Treg cells by mixing cells at a 5:1 (responders:Treg) ratio (bottom panels), resulting in a significant reduction of inhibitory effects. However, even at this ratio, *PKC-θ*^{-/-} Treg cells were able to inhibit responders at a potency similar to that observed in WT Treg cells. These results confirmed that *PKC-θ*^{-/-} Treg cells inhibited T cell activation as efficiently as WT Treg cells.

To determine whether the strength of TCR stimulation affected *PKC-θ*^{-/-} Treg cell-mediated inhibition, CD4+CD25⁻ responders were obtained from TCR transgenic mice (OT-2) expressing TCRs with a single specificity for Ova peptide 323–339 (Fig. 5F). While unstimulated CFSE-labeled responders displayed a single peak (Fig. 5F, top left panel), responders displayed multiple peaks in response to Ova stimulation, suggestive of proliferation (Fig. 5F, top right panel). Prior to mixing with responders, CD4+CD25⁺ Treg cells were first activated with 0.5 mg/ml (Fig. 5F, middle panels) or 5 mg/ml (Fig. 5F, bottom panels) anti-CD3 antibody. Treg cells were then mixed with responders at a 1:1 ratio. Similar to previous results, WT and *PKC-θ*^{-/-} Treg cells almost completely inhibited the proliferation of responders stimulated by Ova, confirming that PKC-θ was not essential for Treg cell-mediated inhibition even at the relatively lower strength of TCR stimulation.

PKC-θ is not required for IL-2-induced expansion of Treg cells, or for production of TGF-β by Treg cells

Treg cells proliferate in the presence of high concentrations of IL-2, which is critical for Treg cell homeostasis and function (Fontenot et al., 2005a; Furtado et al., 2002). We therefore investigated the role of PKC-θ in Treg cell expansion *in vitro* (Fig. 6A). WT and *PKC-θ*^{-/-} CD4+CD25⁺ cells were stimulated with anti-CD3/28 antibodies in the presence of IL-2. Viable cells were then counted at different days following IL-2 treatment. As expected, IL-2 treatment resulted in a significantly increased number of WT Treg cells. An equivalent number of WT and *PKC-θ*^{-/-} Treg cells were observed even 21 days after IL-2 treatment, suggesting PKC-θ was not required for IL-2-induced Treg cell proliferation.

In response to TCR activation, Treg cells produce TGF-β which is a potential mediator of T cell function inhibition. We consequently determined whether the TCR signals required for Treg cells to produce TGF-β were mediated by PKC-θ (Fig. 6B). As expected, with or without stimulation, CD4+CD25⁻ cells did not produce TGF-β. Additionally, in the absence of stimulation, CD4+CD25⁺ Treg cells did not secrete TGF-β. However, in response to TCR stimulation, WT and *PKC-θ*^{-/-} CD4+CD25⁺ cells produced equivalent amounts of TGF-β, suggesting that PKC-θ was not required for TGF-β production upon TCR stimulation.

Discussion

Extensive studies have established Treg cells as a subset of T cells critical for maintaining self-tolerance (Sakaguchi et al., 2006; Shevach, 2006). A breakthrough in defining Treg cells was the identification of Foxp3 as a master transcription factor for controlling Treg cell development and function (Hori et al., 2003). However, less progress has been made in elucidating the molecular mechanisms responsible for the regulation of Foxp3-mediated Treg cell development. In this study, we established a link between the PKC- θ -regulated calcineurin/NFAT pathway and Foxp3-regulated Treg cell development. A recent report demonstrated that NFAT together with Smad3 regulates Foxp3 expression (Tone et al., 2008). PKC- θ is thus likely a critical upstream signaling molecule regulating Foxp3 expression via NFAT pathway. Previous research has suggested a potential link between PKC- θ -regulated NF- κ B and Treg cell development (Schmidt-Supprian et al., 2004). These data, and ours, suggest PKC- θ regulates Treg cell development via multiple signaling pathways. PKC- θ has been shown to regulate TCR-mediated activation of NF- κ B, NFAT and AP1 activity in peripheral T cells (Altman et al., 2000; Baier-Bitterlich et al., 1996; Coudronniere et al., 2000; Li et al., 2004; Lin et al., 2000; Pfeifhofer et al., 2003; Sun et al., 2000). However, it remains to be determined whether PKC- θ -regulated AP1 plays a role in Treg cells. Recent studies have clearly demonstrated a requirement for PKC- θ in the activation of peripheral T cells, and that PKC- θ is dispensable for the development of T cells in thymus (Pfeifhofer et al., 2003; Sun et al., 2000). In contrast to its role in conventional T cells, our data showed that while PKC- θ was required for Treg cell development, it was dispensable for its inhibitory function, confirming PKC- θ plays different roles in different subsets of T cells.

We observed reduced numbers of Foxp3-positive thymocytes in *PKC- θ ^{-/-}* mice compared to WT mice. As expected, most of the reduction was in the CD4 single positive thymus subset (data not shown). We did not observe obvious differences between WT and *PKC- θ ^{-/-}* mice in terms of CD4+CD25+ thymocytes (data not shown), suggesting a normal development of thymocytes until the CD4+CD25+ stage, and a selective defect of Foxp3 upregulation in thymocytes from *PKC- θ ^{-/-}* mice. The remaining Foxp3+ thymocytes in *PKC- θ ^{-/-}* mice expressed lower levels of Foxp3 protein in individual cells. Since PKC- θ is required to activate T cells to produce IL-2, which is believed to regulate Treg homeostasis (Fehervari et al., 2006; Setoguchi et al., 2005), one may argue that the defective development of Treg cells in *PKC- θ ^{-/-}* mice resulted from the lack of IL-2. However, IL-2 is not essential for thymic production of Tregs in terms of frequency, cell number and suppressive function (D'Cruz and Klein, 2005; Fontenot et al., 2005a). Also, Foxp3 mRNA in Tregs was comparable in *IL-2^{-/-}* and *IL-2^{+/-}* mice. Similarly, there was no defect in the thymic generation of Foxp3+ Tregs in *IL-2R α ^{-/-}* mice, suggesting the CD25 (IL-2 receptor) has no function in thymic development of Tregs (D'Cruz and Klein, 2005; Fontenot et al., 2005a; Soper et al., 2007). It is therefore unlikely that the defective development of Treg in *PKC- θ ^{-/-}* mice was due to the lack of IL-2. However, Treg frequency was reduced in the thymus and periphery of *IL-2R β ^{-/-}* mice (Soper et al., 2007). This defect in functional CD4+CD25+ Treg development was restored by thymic expression of IL-2R β in *IL-2R β ^{-/-}* mice (Malek et al., 2002). Interestingly, Foxp3+ Tregs were not present in the thymus or periphery of *IL-2R γ ^{-/-}* or *γ c^{-/-}* mice (Fontenot et al., 2005a). IL-2R β is the common subunit between IL-2 and IL-15 cytokine receptors, while γ c is the common subunit among IL-2, IL-15 and IL-7 receptors. It is thus possible that IL-15, IL-7 and IL-2 have redundant functions in the thymic development of Foxp3+ Treg cells.

To explore whether PKC- θ directly affected Foxp3 gene expression, we cloned Foxp3 promoter region and tested the effect of PKC- θ on their activation. In isolated mouse CD4 T cells, TCR stimulation does not upregulate Foxp3 expression, as also observed by other investigators (Hori et al., 2003). Therefore, we created an artificial system using human CD4 T cells, in

which Foxp3 expression is up-regulated in response to TCR stimulation (Walker et al., 2003). This system was used to demonstrate as 'proof-of-principle' that mouse Foxp3 promoter can be stimulated by TCR signaling and PKC- θ activation. Previous studies, including ours, have shown that PKC- θ -mediated TCR signals were able to stimulate the calcineurin/NFAT pathway, most likely via the enhancement of Ca^{2+} influx (Altman et al., 2004; Manicassamy et al., 2006b). Our results support PKC- θ regulation of Foxp3, at least in part via the calcineurin/NFAT pathway. First, three potential NFAT binding sites were identified in the Foxp3 promoter region, and the deletion of these three sites resulted in significantly reduced reporter activity. These three conserved functional NFAT-binding sites were also previously identified on the human Foxp3 promoter (Mantel et al., 2006), further confirming the role of NFAT in the regulation of Foxp3 expression. Second, PKC- θ -mediated activation of Foxp3 reporter was inhibited by dominant negative NFAT. Lastly, defective development of Treg cells was also observed in *CnA β ^{-/-}* mice. Therefore, it is possible that PKC- θ regulated the development of Treg cells via calcineurin/NFAT, as well as other pathways such as NF- κ B (Schmidt-Supprian et al., 2004). Interestingly, Foxp3 has been shown to interact with NFAT and inhibit NFAT activity (Wu et al., 2006). It thus raises the possibility that once Foxp3 is produced, it can inhibit NFAT, an activator of Foxp3 expression. This feedback mechanism could be important for maintaining Foxp3 expression levels in Treg cells.

One of the functions of PKC- θ is to enhance peripheral T cell survival by upregulating Bcl- x_L (Barouch-Bentov et al., 2005; Manicassamy et al., 2006a; Saibil et al., 2007). Additionally, PKC- θ stimulates Bcl- x_L expression by activating the NF- κ B pathway. Considering the requirement of both PKC- θ and NF- κ B for the development of Treg cells (Schmidt-Supprian et al., 2004), we initially hypothesized that the significantly reduced number of Treg cells observed in *PKC- θ ^{-/-}* mice resulted from apoptosis due to lack of PKC- θ -mediated survival. While the *Bcl-x_L* transgene rescued peripheral T cells from apoptosis, it did not rescue Treg cell development. Thus, in contrast to its survival function in peripheral T cells, PKC- θ -regulated Treg cell development is not likely dependent on PKC- θ -mediated survival, implicating PKC- θ in controlling different functions by regulating target genes in distinct T cell subsets.

In contrast to its essential role in the development of Treg cells, we found that PKC- θ is not required for Treg cell-mediated inhibition of T cell activation. Thornton and Shevach have long noted that in order for Treg cells to inhibit T cell activation *in vitro*, they need to be activated via stimulation of TCR (Thornton and Shevach, 1998). Here we report that *PKC- θ ^{-/-}* Treg cells inhibited T cell activation almost as efficiently as WT Treg cells. We have previously demonstrated that PKC- θ was required for NFAT activation when T cells were activated with relatively low concentrations of anti-CD3 antibody (0.5 and 1 $\mu\text{g/ml}$) (Manicassamy et al., 2006b). However, at higher antibody concentrations (5 $\mu\text{g/ml}$), NFAT activation was normal, even in the absence of PKC- θ . These results suggested that PKC- θ function might be compensated by strong TCR stimulation. We thus activated Treg cells with different concentrations of anti-CD3 antibody, and found that *PKC- θ ^{-/-}* Treg cells were efficiently activated, even with 10-fold lower concentrations of anti-CD3 antibody. Thus, it is unlikely that PKC- θ function was compensated in Treg cells by overwhelming TCR stimulation. We also found that PKC- θ -deficient Treg cells were able to respond to exogenous IL-2 and expand during *in vitro* culture, to an extent similar to what was observed in WT control Tregs. One of the effector mechanisms for Treg cell-mediated inhibition is the production of cytokine TGF- β . We did not observe differences in the production of TGF- β by Treg cells compared to WT and *PKC- θ ^{-/-}* mice. These data confirmed PKC- θ was required for Treg cell development, as well as for the activation of conventional T cells, but was not required for Treg cell-mediated inhibition.

Recently it was shown that *PKC-θ*^{-/-} mice were resistant to experimental autoimmune encephalomyelitis (EAE) due to impaired IL-17 production (Tan et al., 2006), suggesting PKC-θ was also required for Th17 differentiation. Interestingly, differentiation of both Tregs and Th17 requires TGFβ (Zhou et al., 2008). It is thus possible that lack of TGFβ was responsible for the reduction of Tregs and Th17 cells in *PKC-θ*^{-/-} mice. Tregs are capable of producing TGFβ. Although our data showed that TGFβ production from *PKC-θ*^{-/-} Tregs was comparable to WT Tregs, overall TGFβ production by Tregs was likely significantly reduced due to the reduced number of Tregs in *PKC-θ*^{-/-} mice. It was proposed that TGFβ produced by Tregs promoted Th17 differentiation (Weaver et al., 2007). It is not clear, however, whether reduced Th17 in EAE was related to TGFβ produced by Tregs in *PKC-θ*^{-/-} mice. It is also possible that TGFβ produced by other cells, such as dendritic cells, may require PKC-θ. Further studies are needed to further elucidate the roles of PKC-θ in Th17 and its potential relationship with Tregs in normal and disease conditions.

Conclusions

In summary, we found how a single protein, like PKC-θ can highlight the biological differences between two subsets of CD4 T cells, namely, Treg cells and conventional CD4 T cells. Absence of Foxp3⁺ Tregs in mice leads to a severe phenotype characterized by massive infiltration of activated T cells and inflammation of various organs. In contrast, *PKC-θ*^{-/-} mice used in this study has normal phenotype, even after severe reduction of Foxp3⁺ Tregs in thymus, thus representing a good model system to study Treg cell development in thymus. This phenotype is explained by the reported impairment of PKC-θ-deficient CD4 T cells to divide rapidly and secrete various cytokines involved in inflammation.

Acknowledgement

We thank Dr. Lena Al-Harhi for providing human CD4 T cells and nucleofection system, Drs. Xin Lin and Amnon Altman for sharing expression plasmids encoding active and inactive isoforms of PKC-θ, Drs. Roger Davis and Chi-Wing Chow for sharing expression plasmid encoding dn-NFAT, and Dr. Jeffery D. Molkentin for calcineurin Aβ knockout mice. This work was supported by grants from American Cancer Society of Illinois Division, Schweppe Foundation, UIC Cancer center and UIC IRB and NIH R01-AI053147.

References

- Altman A, Isakov N, Baier G. Protein kinase Cθeta: a new essential superstar on the T-cell stage. *Immunol Today* 2000;21:567–573. [PubMed: 11094261]
- Altman A, Kaminski S, Busuttil V, Droin N, Hu J, Tadevosyan Y, Hipskind RA, Villalba M. Positive feedback regulation of PLCγ1/Ca(2+) signaling by PKCθeta in restimulated T cells via a Tec kinase-dependent pathway. *Eur J Immunol* 2004;34:2001–2011. [PubMed: 15214048]
- Asano M, Toda M, Sakaguchi N, Sakaguchi S. Autoimmune disease as a consequence of developmental abnormality of a T cell subpopulation. *J Exp Med* 1996;184:387–396. [PubMed: 8760792]
- Baier G. The PKC gene module: molecular biosystematics to resolve its T cell functions. *Immunol Rev* 2003;192:64–79. [PubMed: 12670396]
- Baier-Bitterlich G, Uberall F, Bauer B, Fresser F, Wachter H, Grunicke H, Utermann G, Altman A, Baier G. Protein kinase C-θeta isoenzyme selective stimulation of the transcription factor complex AP-1 in T lymphocytes. *Mol Cell Biol* 1996;16:1842–1850. [PubMed: 8657160]
- Barouch-Bentov R, Lemmens EE, Hu J, Janssen EM, Droin NM, Song J, Schoenberger SP, Altman A. Protein kinase C-θeta is an early survival factor required for differentiation of effector CD8⁺ T cells. *J Immunol* 2005;175:5126–5134. [PubMed: 16210616]
- Bueno OF, Brandt EB, Rothenberg ME, Molkentin JD. Defective T cell development and function in calcineurin A beta -deficient mice. *Proc Natl Acad Sci U S A* 2002;99:9398–9403. [PubMed: 12091710]

- Cabarrocas J, Cassan C, Magnusson F, Piaggio E, Mars L, Derbinski J, Kyewski B, Gross DA, Salomon BL, Khazaie K, et al. Foxp3+ CD25+ regulatory T cells specific for a neo-self-antigen develop at the double-positive thymic stage. *Proc Natl Acad Sci U S A* 2006;103:8453–8458. [PubMed: 16709665]
- Chao DT, Linette GP, Boise LH, White LS, Thompson CB, Korsmeyer SJ. Bcl-x_L and Bcl-2 repress a common pathway of cell death. *J Exp Med* 1995;182:821–828. [PubMed: 7650488]
- Chen W, Jin W, Hardegen N, Lei KJ, Li L, Marinos N, McGrady G, Wahl SM. Conversion of peripheral CD4+CD25– naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med* 2003;198:1875–1886. [PubMed: 14676299]
- Chow CW, Rincon M, Davis RJ. Requirement for transcription factor NFAT in interleukin-2 expression. *Mol Cell Biol* 1999;19:2300–2307. [PubMed: 10022916]
- Coudronniere N, Villalba M, Englund N, Altman A. NF-kappa B activation induced by T cell receptor/CD28 costimulation is mediated by protein kinase C-theta. *Proc Natl Acad Sci U S A* 2000;97:3394–3399. [PubMed: 10716728]
- Crabtree GR. Generic signals and specific outcomes: signaling through Ca²⁺, calcineurin, and NF-AT. *Cell* 1999;96:611–614. [PubMed: 10089876]
- D'Cruz LM, Klein L. Development and function of agonist-induced CD25+Foxp3+ regulatory T cells in the absence of interleukin 2 signaling. *Nat Immunol* 2005;6:1152–1159. [PubMed: 16227983]
- Fantini MC, Becker C, Monteleone G, Pallone F, Galle PR, Neurath MF. Cutting edge: TGF-beta induces a regulatory phenotype in CD4+CD25– T cells through Foxp3 induction and down-regulation of Smad7. *J Immunol* 2004;172:5149–5153. [PubMed: 15100250]
- Fehervari Z, Yamaguchi T, Sakaguchi S. The dichotomous role of IL-2: tolerance versus immunity. *Trends Immunol* 2006;27:109–111. [PubMed: 16459146]
- Fontenot JD, Rasmussen JP, Gavin MA, Rudensky AY. A function for interleukin 2 in Foxp3-expressing regulatory T cells. *Nat Immunol* 2005a;6:1142–1151. [PubMed: 16227984]
- Fontenot JD, Rasmussen JP, Williams LM, Dooley JL, Farr AG, Rudensky AY. Regulatory T cell lineage specification by the forkhead transcription factor foxp3. *Immunity* 2005b;22:329–341. [PubMed: 15780990]
- Furtado GC, Curotto de Lafaille MA, Kutchukhidze N, Lafaille JJ. Interleukin 2 signaling is required for CD4(+) regulatory T cell function. *J Exp Med* 2002;196:851–857. [PubMed: 12235217]
- Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 2003;299:1057–1061. [PubMed: 12522256]
- Itoh M, Takahashi T, Sakaguchi N, Kuniyasu Y, Shimizu J, Otsuka F, Sakaguchi S. Thymus and autoimmunity: production of CD25+CD4+ naturally anergic and suppressive T cells as a key function of the thymus in maintaining immunologic self-tolerance. *J Immunol* 1999;162:5317–5326. [PubMed: 10228007]
- Izcue A, Coombes JL, Powrie F. Regulatory T cells suppress systemic and mucosal immune activation to control intestinal inflammation. *Immunol Rev* 2006;212:256–271. [PubMed: 16903919]
- Kriegel MA, Li MO, Sanjabi S, Wan YY, Flavell RA. Transforming growth factor-beta: recent advances on its role in immune tolerance. *Curr Rheumatol Rep* 2006;8:138–144. [PubMed: 16569373]
- Li Y, Hu J, Vita R, Sun B, Tabata H, Altman A. SPAK kinase is a substrate and target of PKCtheta in T-cell receptor-induced AP-1 activation pathway. *Embo J* 2004;23:1112–1122. [PubMed: 14988727]
- Lin X, O'Mahony A, Mu Y, Geleziunas R, Greene WC. Protein kinase C-theta participates in NF-kappaB activation induced by CD3-CD28 costimulation through selective activation of IkappaB kinase beta. *Mol Cell Biol* 2000;20:2933–2940. [PubMed: 10733597]
- Malek TR, Yu A, Vincek V, Scibelli P, Kong L. CD4 regulatory T cells prevent lethal autoimmunity in IL-2Rbeta-deficient mice. Implications for the nonredundant function of IL-2. *Immunity* 2002;17:167–178. [PubMed: 12196288]
- Manicassamy S, Gupta S, Huang Z, Molkentin JD, Shang W, Sun Z. Requirement of calcineurin a beta for the survival of naive T cells. *J Immunol* 2008;180:106–112. [PubMed: 18097009]
- Manicassamy S, Gupta S, Huang Z, Sun Z. Protein Kinase C-{theta}-Mediated Signals Enhance CD4+ T Cell Survival by Up-Regulating Bcl-x_L. *J Immunol* 2006a;176:6709–6716. [PubMed: 16709830]
- Manicassamy S, Sadim M, Ye RD, Sun Z. Differential roles of PKC-theta in the regulation of intracellular calcium concentration in primary T cells. *J Mol Biol* 2006b;355:347–359. [PubMed: 16309697]

- Manicassamy S, Sun Z. The critical role of protein kinase C-theta in Fas/Fas ligand-mediated apoptosis. *J Immunol* 2007;178:312–319. [PubMed: 17182568]
- Mantel PY, Ouaked N, Ruckert B, Karagiannidis C, Welz R, Blaser K, Schmidt-Weber CB. Molecular mechanisms underlying FOXP3 induction in human T cells. *J Immunol* 2006;176:3593–3602. [PubMed: 16517728]
- Marsland BJ, Soos TJ, Spath G, Littman DR, Kopf M. Protein kinase C theta is critical for the development of in vivo T helper (Th)2 cell but not Th1 cell responses. *J Exp Med* 2004;200:181–189. [PubMed: 15263025]
- Miyara M, Sakaguchi S. Natural regulatory T cells: mechanisms of suppression. *Trends Mol Med* 2007;13:108–116. [PubMed: 17257897]
- Pacholczyk R, Kern J, Singh N, Iwashima M, Kraj P, Ignatowicz L. Nonself-antigens are the cognate specificities of Foxp3+ regulatory T cells. *Immunity* 2007;27:493–504. [PubMed: 17869133]
- Pacholczyk R, Kraj P, Ignatowicz L. Peptide specificity of thymic selection of CD4+CD25+ T cells. *J Immunol* 2002;168:613–620. [PubMed: 11777953]
- Pfeifhofer C, Kofler K, Gruber T, Tabrizi NG, Lutz C, Maly K, Leitges M, Baier G. Protein kinase C theta affects Ca2+ mobilization and NFAT cell activation in primary mouse T cells. *J Exp Med* 2003;197:1525–1535. [PubMed: 12782715]
- Saibil SD, Jones RG, Deenick EK, Liadis N, Elford AR, Vainberg MG, Baerg H, Woodgett JR, Gerondakis S, Ohashi PS. CD4+ and CD8+ T cell survival is regulated differentially by protein kinase Ctheta, c-Rel, and protein kinase B. *J Immunol* 2007;178:2932–2939. [PubMed: 17312138]
- Sakaguchi S. Naturally arising CD4+ regulatory t cells for immunologic self-tolerance and negative control of immune responses. *Annu Rev Immunol* 2004;22:531–562. [PubMed: 15032588]
- Sakaguchi S, Ono M, Setoguchi R, Yagi H, Hori S, Fehervari Z, Shimizu J, Takahashi T, Nomura T. Foxp3+ CD25+ CD4+ natural regulatory T cells in dominant self-tolerance and autoimmune disease. *Immunol Rev* 2006;212:8–27. [PubMed: 16903903]
- Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* 1995;155:1151–1164. [PubMed: 7636184]
- Salek-Ardakani S, So T, Halteman BS, Altman A, Croft M. Differential regulation of Th2 and Th1 lung inflammatory responses by protein kinase C theta. *J Immunol* 2004;173:6440–6447. [PubMed: 15528385]
- Salek-Ardakani S, So T, Halteman BS, Altman A, Croft M. Protein kinase Ctheta controls Th1 cells in experimental autoimmune encephalomyelitis. *J Immunol* 2005;175:7635–7641. [PubMed: 16301673]
- Schmidt-Suprian M, Tian J, Grant EP, Pasparakis M, Maehr R, Ovaa H, Ploegh HL, Coyle AJ, Rajewsky K. Differential dependence of CD4+CD25+ regulatory and natural killer-like T cells on signals leading to NF-kappaB activation. *Proc Natl Acad Sci U S A* 2004;101:4566–4571. [PubMed: 15070758]
- Setoguchi R, Hori S, Takahashi T, Sakaguchi S. Homeostatic maintenance of natural Foxp3(+) CD25(+) CD4(+) regulatory T cells by interleukin (IL)-2 and induction of autoimmune disease by IL-2 neutralization. *J Exp Med* 2005;201:723–735. [PubMed: 15753206]
- Shevach EM. From vanilla to 28 flavors: multiple varieties of T regulatory cells. *Immunity* 2006;25:195–201. [PubMed: 16920638]
- Shevach EM, DiPaolo RA, Andersson J, Zhao DM, Stephens GL, Thornton AM. The lifestyle of naturally occurring CD4+ CD25+ Foxp3+ regulatory T cells. *Immunol Rev* 2006;212:60–73. [PubMed: 16903906]
- Shimizu J, Yamazaki S, Takahashi T, Ishida Y, Sakaguchi S. Stimulation of CD25(+)CD4(+) regulatory T cells through GITR breaks immunological self-tolerance. *Nat Immunol* 2002;3:135–142. [PubMed: 11812990]
- Singh B, Read S, Asseman C, Malmstrom V, Mottet C, Stephens LA, Stepankova R, Tlaskalova H, Powrie F. Control of intestinal inflammation by regulatory T cells. *Immunol Rev* 2001;182:190–200. [PubMed: 11722634]

- Soper DM, Kasproicz DJ, Ziegler SF. IL-2Rbeta links IL-2R signaling with Foxp3 expression. *Eur J Immunol* 2007;37:1817–1826. [PubMed: 17559173]
- Sun Z, Arendt CW, Ellmeier W, Schaeffer EM, Sunshine MJ, Gandhi L, Annes J, Petrzilka D, Kupfer A, Schwartzberg PL, Littman DR. PKC-theta is required for TCR-induced NF-kappaB activation in mature but not immature T lymphocytes. *Nature* 2000;404:402–407. [PubMed: 10746729]
- Tan SL, Zhao J, Bi C, Chen XC, Hepburn DL, Wang J, Sedgwick JD, Chintalacharuvu SR, Na S. Resistance to experimental autoimmune encephalomyelitis and impaired IL-17 production in protein kinase C theta-deficient mice. *J Immunol* 2006;176:2872–2879. [PubMed: 16493044]
- Thornton AM, Shevach EM. CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *J Exp Med* 1998;188:287–296. [PubMed: 9670041]
- Tone Y, Furuuchi K, Kojima Y, Tykocinski ML, Greene MI, Tone M. Smad3 and NFAT cooperate to induce Foxp3 expression through its enhancer. *Nat Immunol* 2008;9:194–202. [PubMed: 18157133]
- Walker MR, Kasproicz DJ, Gersuk VH, Benard A, Van Landeghen M, Buckner JH, Ziegler SF. Induction of FoxP3 and acquisition of T regulatory activity by stimulated human CD4+CD25– T cells. *J Clin Invest* 2003;112:1437–1443. [PubMed: 14597769]
- Weaver CT, Hatton RD, Mangan PR, Harrington LE. IL-17 family cytokines and the expanding diversity of effector T cell lineages. *Annu Rev Immunol* 2007;25:821–852. [PubMed: 17201677]
- Wu Y, Borde M, Heissmeyer V, Feuerer M, Lapan AD, Stroud JC, Bates DL, Guo L, Han A, Ziegler SF, et al. FOXP3 controls regulatory T cell function through cooperation with NFAT. *Cell* 2006;126:375–387. [PubMed: 16873067]
- Xie H, Huang Z, Sadim MS, Sun Z. Stabilized beta-catenin extends thymocyte survival by up-regulating Bcl-xL. *J Immunol* 2005a;175:7981–7988. [PubMed: 16339534]
- Xie H, Sadim MS, Sun Z. RORgammat recruits steroid receptor coactivators to ensure thymocyte survival. *J Immunol* 2005b;175:3800–3809. [PubMed: 16148126]
- Zhang BW, Zimmer G, Chen J, Ladd D, Li E, Alt FW, Wiederrecht G, Cryan J, O'Neill EA, Seidman CE, et al. T cell responses in calcineurin A alpha-deficient mice. *J Exp Med* 1996;183:413–420. [PubMed: 8627154]
- Zhou L, Lopes JE, Chong MM, Ivanov II, Min R, Victora GD, Shen Y, Du J, Rubtsov YP, Rudensky AY, et al. TGF-beta-induced Foxp3 inhibits T(H)17 cell differentiation by antagonizing RORgammat function. *Nature*. 2008

Figure 1. *PKC-θ*^{-/-} mice have reduced Treg cells

A). Thymocytes from WT (*PKCθ*⁺) and *PKC-θ*^{-/-} (*PKCθ*⁻) mice were prepared from freshly isolated thymus, and analyzed for surface CD4 and intracellular Foxp3 expression by flow cytometry. Numbers indicate the percentage of cells among CD4 single positive cells in the gated area. MFI (mean fluorescent intensity) of Foxp3 is also indicated. Average percentage of Foxp3⁺ among CD4 single positive cells (Right panel, $\pm$ s.d., n=10 mice). **B).** Similar to **A**, however with cells obtained from spleens and lymph nodes (LN) of WT and *PKC-θ*^{-/-} mice. Average percentage of Foxp3⁺ cells among CD4⁺ cells (Right panel, $\pm$ s.d., n=10 mice). **C).** Surface CD4 and GITR expression were analyzed on cells obtained from spleens

or LN of WT and *PKC- $\theta^{-/-}$* mice. Average percentage of GITR+ cells among CD4+ cells (Right panel, $\pm$ s.d., n= 6 mice, $P<0.0001$).

Figure 2. Transgenic *Bcl-x_L* failed to rescue the defective development of Treg cells in *PKC-θ*^{-/-} mice

A). *Bcl-x_L*^{Tg} prevented the apoptosis of *PKC-θ*^{-/-} T cells. T cells purified from spleens of WT, *PKC-θ*^{-/-}, *Bcl-x_L*^{Tg}, and *PKC-θ*^{-/-}/*Bcl-x_L*^{Tg} mice stimulated (36 h), or not (None), with anti-CD3 (1 mg/ml) and CD28 (2 mg/ml) antibodies. Apoptotic cells were detected by flow cytometry analysis of annexin V staining in cells. (n= at least 3 independent experiments).

B). Thymocytes of WT, *PKC-θ*^{-/-}, *Bcl-x_L*^{Tg}, and *PKC-θ*^{-/-}/*Bcl-x_L*^{Tg} mice were prepared from freshly isolated thymus, and analyzed for surface CD4 and intracellular Foxp3 expression by flow cytometry. Numbers indicate the percentage of cells in the gated area. MFI (mean fluorescent intensity) of Foxp3 is also indicated. Average percentage of CD4+Foxp3+ cells

among CD4 single positive cells (Right panel, $\pm$ s.d., n= 9 mice. C). Bone marrows obtained from WT or *PKC- θ ^{-/-}* mice were adoptively transferred to *Rag-I^{-/-}* mice. Development of Tregs in *Rag-I^{-/-}* mice was analyzed six weeks after adoptive transfer. Percentage of CD4 +Foxp3 is indicated. (*P<0.0001).

Figure 3. PKC-θ stimulates Foxp3 promoter activity in a NFAT-dependent manner

A). Schematic representation of the mouse Foxp3 promoter region. Three potential NFAT-binding sites are indicated (black squares). Arrows: positions of the four oligonucleotide primers used to generate the deletion of the Foxp3 promoter-luciferase reporters. Numbers on the arrows: nucleotide positions upstream of the transcriptional initiation site. **B).** Different lengths of Foxp3 reporters were transfected into CD4⁺ T cells (human); 12 hrs after transfection, cells were stimulated overnight with anti-CD3/28 antibodies (black bars), or not (open bar). Luciferase activity was then measured. **C).** pGL4 or p515 Foxp3-promoter reporter was transfected into CD4⁺ T cells alone or with the expression plasmid encoding WT PKC-θ, inactive PKC-θ, constitutively active PKC-θ, or dominant negative NFAT (DN-NFAT) as

indicated; 24 h after transfection, cells were stimulated overnight with anti-CD3/28 antibodies (black bars), or not (open bars). Luciferase reporter activity is indicated as the fold induction relative to the activity obtained from unstimulated cells in the control group. Error bars: $\pm$ s.d., n=3 independent experiments.

Figure 4. *CnAβ*^{-/-} mice have reduced Treg cells

Thymocytes of WT (*CnAβ*⁺) and *CnAβ*^{-/-} (*CnAβ*⁻) mice were prepared from freshly isolated thymus, and analyzed for surface CD4 and intracellular Foxp3 expression by flow cytometry. Numbers indicate the percentage of cells in the gated area among CD4⁺ cells. MFI (mean fluorescent intensity) of Foxp3 is also indicated. Average percentage of Foxp3⁺ cells among CD4 single positive cells (Right panel, $\pm$ s.d., $n = 5$ mice, $*P < 0.0001$).

Figure 5. PKC- θ is dispensable for Treg cell-mediated inhibition of T cell activation

A). Isolation of Treg cells. CD4+CD25+ cells in the gated area represent cells that were isolated from the spleens of both WT and *PKC- θ ^{-/-}* mice. **B).** Flow cytometry analysis of Foxp3 expression in isolated CD4+CD25+ cells. Percentage of Foxp3 positive cells is also indicated. **C).** WT CD4+CD25⁻ responders mixed with WT (open bars) or *PKC- θ ^{-/-}* (black bars) Treg cells at different ratios, as indicated. Mixed cells were stimulated with anti-CD3/28 alone or in the presence of IL-2. Proliferation of responders was measured by [³H] thymidine incorporation. **D).** IL-2 in the supernatant of cells described in C was measured by ELISA. **E).** Top panels: CFSE-labeled responders were either unstimulated (top left panel) or stimulated with anti-CD3/28 antibodies (TCR-stimulated, top right panel). Responders (not labeled with

CFSE) mixed with CFSE-labeled WT Treg cells (top left panel, second row) or *PKC-θ*^{-/-} Treg cells (right panel, second row) at a 1:1 ratio, and stimulated with anti-CD3/28 antibodies. CFSE-labeled responders mixed with WT Treg cells or *PKC-θ*^{-/-} Treg cells (not labeled with CFSE) at a 1:1 ratio (third row panels) or 5:1 (bottom panels), and stimulated with anti-CD3/28 antibodies. Marked area indicates the percentage of proliferated cells. **F**). CFSE-labeled responders obtained from OT-2 TCR transgenic mice were either unstimulated (top left panel) or stimulated with peptide ligand, Ova (TCR-stimulated, top right panel). Bottom two panels: CFSE-labeled OT-2 responders mixed at a 1:1 ratio with WT (left panels) or *PKC-θ*^{-/-} (right panels) Treg cells (not labeled with CFSE) that were previously activated with 0.5 mg/ml (middle panels) or 5 mg/ml anti-CD3 antibody (bottom panels). Marked area indicates the percentage of proliferated cells. (n= at least 3 independent experiments).

Figure 6.

A). PKC- θ is not required for IL-2-induced *in vitro* Treg cell expansion. WT or PKC- $\theta^{-/-}$ CD4+CD25+ cells (5×10^5) were cultured in medium supplemented with 100 U/ml IL-2. Total numbers of viable cells were counted at different days as indicated. **B).** PKC- θ is not required for the production of TGF- β by Treg cells. Production of TGF- β by Treg cells. CD4+CD25- responders, WT (open bar) or PKC- $\theta^{-/-}$ (black bars) Treg cells were stimulated with anti-CD3/28 antibodies or not (None); TGF- β in the supernatant was measured by ELISA 48 h after stimulation. (n=at least 3 independent experiments).