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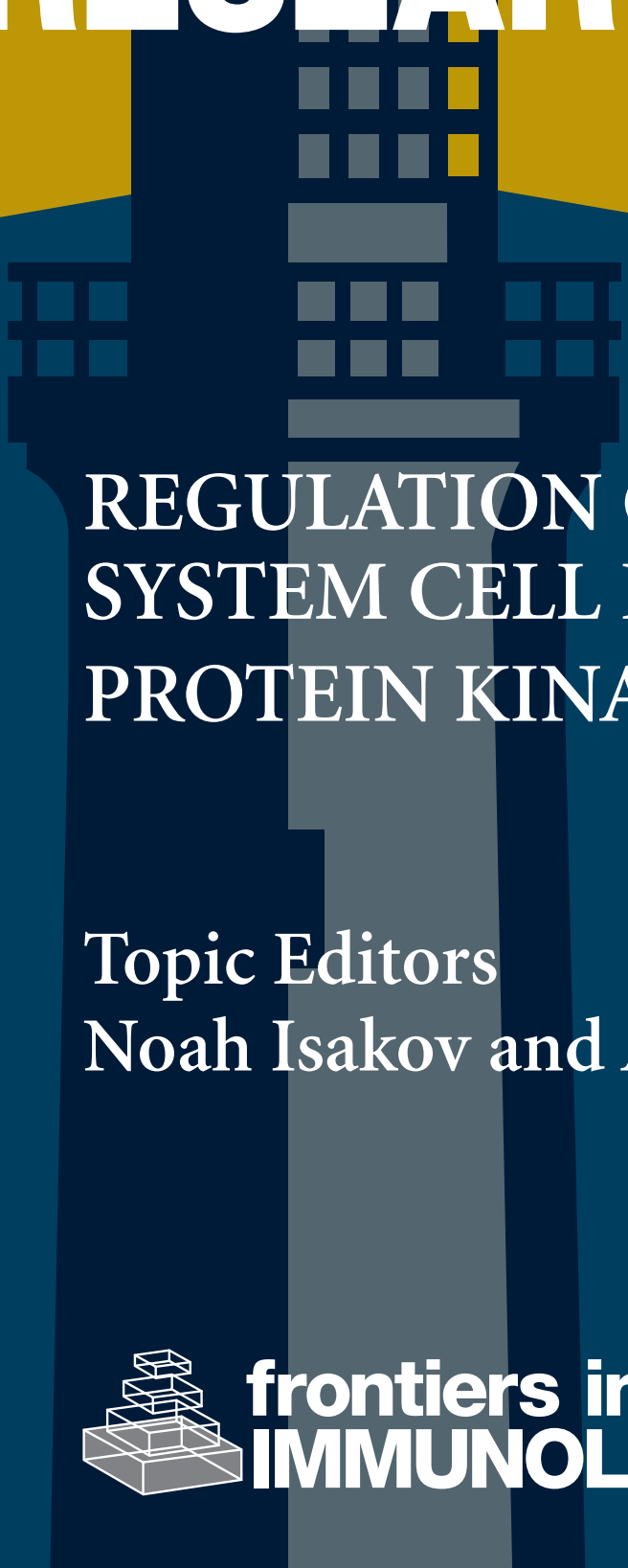

REGULATION OF IMMUNE SYSTEM CELL FUNCTIONS BY PROTEIN KINASE C

Topic Editors
Noah Isakov and Amnon Altman

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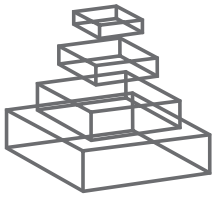

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REGULATION OF IMMUNE SYSTEM CELL FUNCTIONS BY PROTEIN KINASE C

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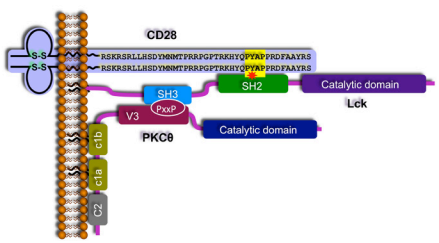

A schematic model of the CD28-Lck-PKCθ tri-partite complex formed in TCR/CD28-stimulated T cells
(Isakov, N. and Altman, A.)

Members of the protein kinase C (PKC) family of Ser/Thr kinases are encoded by nine distinct but closely related genes, which give rise to more than 12 different protein isoforms via a mechanism of alternative RNA splicing. Most PKC proteins are ubiquitously expressed and participate in a plethora of functions in most cell types.

A majority of PKC isoforms is also expressed in cells of the immune system in which they are involved in signal transduction downstream of a range of surface receptors, including the antigen receptors on T and B lymphocytes. PKC proteins are central to signal initiation and propagation, and to the regulation of processes leading to immune cell proliferation, differentiation, homing and survival. As a result, PKC proteins directly impact on the quality and quantity of immune responses and indirectly on the host resistance to pathogens and tendency to develop immune deficiencies and autoimmune diseases.

A significant progress was made in recent years in understanding the regulation of PKC enzymes, their mechanism of action and their role in determining immunocyte behavior.

This volume reviews the most significant contributions made in the field of immune cell regulation by PKC enzymes. Several manuscripts are devoted to the role of distinct PKC isoforms in the regulation of selected immunocyte responses. Additional manuscripts review more general mechanisms of regulation of PKC enzymes, either by post-translational modifications, such as phosphorylation or controlled proteolysis, or by interaction with different binding proteins that may alter the conformation, activity and subcellular location of PKC. Both types of mechanisms can introduce conformational changes in the molecule,

which may affect its ability to interact with cofactors, ATP, or substrates. This topic will be followed by a discussion on the positive and negative impact of individual PKC isoforms on cell cycle regulation.

A second section of this volume concentrates on selected topics relevant to role of the novel PKC isoform, PKC-theta, in T lymphocyte function. PKC-theta plays important and some non-redundant roles in T cell activation and is a key isoform that recruits to the immunological synapse - the surface membrane area in T cells that comes in direct contact with antigen presenting cells. The immunological synapse is formed in T cells within seconds following the engagement of the TCR by a peptide-bound MHC molecule on the surface of antigen-presenting cells. It serves as a platform for receptors, adaptor proteins, and effector molecules, which assemble into multimolecular activation complexes required for signal transduction. The unique ability of PKC-theta to activate the NF-kB, AP-1 and NF-AT transcription factors is well established, and recent studies contributed essential information on the mechanisms involved in the recruitment of PKC-theta to the center of the immunological synapse and the nature of its substrates and the role of their phosphorylated forms in signal transduction. Additional review manuscripts will describe the unique behavior of PKC-theta in regulatory T cells and its role in the regulation of other cell populations, including those of the innate immune response.

This volume brings together leading experts from different disciplines that review the most recent discoveries and offer new perspectives on the contributions of PKC isoforms to biochemical processes and signaling events in different immune cell populations and their impact on the overall host immune response.

Table of Contents

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EDITORIAL
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Regulation of immune system cell functions by protein kinase C

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Keywords: protein kinase C, T cell activation, signal transduction pathways, lymphocyte stimulation, cell growth regulation

Cellular responses to environmental cues are mediated through complex networks of signal transduction pathways. Among the molecules involved in these pathways, members of the protein kinase C (PKC) family stand out because of their ability to acutely and reversibly modulate effector protein functions and control the spatial distribution and dynamic properties of the signals. PKC enzymes also contribute to signaling networks that coordinate many aspects of immune cell function and, therefore, are important players in immune regulation.

Originally discovered in 1977, by Nishizuka and coworkers, PKC was initially identified as a cyclic nucleotide-independent protein kinase that is capable of phosphorylating histone and protamine following Ca^{2+}-dependent limited proteolysis of its proenzyme precursor (1, 2). Subsequently, Nishizuka demonstrated that activation of PKC can occur in the absence of limited proteolysis by a membrane-associated factor and low Ca^{2+} concentration (3). He then characterized the membrane-associated factor as diacylglycerol, which is generated by receptor-stimulated hydrolysis of phosphoinositides, suggesting that this lipid functions as a second messenger (4). These findings and additional data established a new pathway of signal transduction and led to identification of biological roles for PKC in signaling pathways linked to a variety of surface receptors in many different cell types.

The PKC family of serine/threonine kinases consists of 10 distinct isoforms that are differentially expressed in a wide range of cell types and tissues. Despite having a certain degree of redundancy and overlapping substrate specificities, individual PKC isoforms also exhibit non-redundant functions. In addition, activity of distinct isoforms can be deployed in a spatially and temporally dependent manner since most isoforms possess a structurally and differentially activated unique regulatory domain. The activity of PKC can therefore be induced by a large variety of agonists and directed by multiple inputs, including second messengers and a variety of PKC-binding proteins.

This Research Topic focuses on recent developments relevant to the role of PKC in immune cell functions, and includes contributions by many of the leading experts in the field. The first paper by Pfeifhofer-Obermair and colleagues (5) reviews current information related to the physiological role and non-redundant functions of different PKC isoforms in T lymphocytes. The authors emphasize the positive contributions of PKCθ (6) and PKCα (7) to antigen-induced T cell activation and argue that inhibition T cell-mediated responses, including allograft rejection and autoimmunity, requires the inhibition of both PKC isoforms.

Black and Black (8) discuss mechanisms of regulation of T cell proliferation and cell cycle progression that are mediated by distinct PKC isoforms and emphasize their predominant role during the G0/G1 and G2 phases. Although PKC was found to modulate an array of cell cycle regulatory molecules, evidence points to Cdk kinase inhibitors and D-type cyclins as the key mediators of PKC-regulated cell cycle-specific effects. The authors indicate that most PKC isoforms play positive roles during cell cycle progression, except for PKCδ, which serves as a negative regulator.

Another important cellular function that involves PKC is the establishment and maintenance of cell polarity. This mechanism enables, among other things, the formation of a functional immunological synapse (IS) at the T cell-antigen presenting cell (APC) contact area, and the directional release of cytokines and cytolytic factors from cytotoxic T cells toward their specific target cells. The microtubule organizing complex (MTOC) plays an important role in directing this polarity, and the review by M. Huse covers the process through which PKC family members regulate the formation of the MTOC and its link to the T cell IS (9).

The PKCθ isoform, which is selectively enriched in T cells (10), is a key modulator of T cell receptor (TCR) signaling and an essential regulator of T cell activation and survival (11). In antigen-stimulated T cells, PKCθ selectively translocates to the center of the IS, where it mediates critical TCR and CD28 signals leading to the activation of NF-κB, AP-1 and NFAT transcription factors (12).

A review by Wang and coworkers (13) discusses a major mechanism that regulates PKCθ, and is also shared by other kinases. This mechanism involves a post-translational process whereby distinct serine, threonine, and tyrosine residues of PKCθ are autophosphorylated or transphosphorylated, thereby regulating its catalytic activity, as well as its localization within cells and the ability to interact with specific binding partners. The authors focus on the actual PKCθ phosphorylation sites and their potential role in determining PKCθ functions. A short comment on this topic is presented by Freeley and Long (14).

Another manuscript, by Isakov and Altman (15), focuses on the molecular mechanism that regulates the translocation of PKCθ to

the center of the IS. This mechanism was discovered only recently by showing that a proline-rich region within the unique V3 (hinge) domain of PKCθ recruits the enzyme to the cytoplasmic tail of CD28 in a TCR activation-dependent manner (16). The PKCθ-CD28 interaction is mediated via an indirect mechanism involving the Lck protein tyrosine kinase as an intermediate. The authors review progress made in recent years in our understanding of the PKCθ-mediated signal delivery from the TCR/CD28 surface receptors.

Another facet of PKCθ relates to its nuclear function in T cells, where it was found to associate with chromatin in a cell activation-dependent manner. PKCθ interacts with selected nuclear proteins with which it forms active complexes that associate with proximal promoters of inducible T cell genes, including CD69, INF-γ, and TNF-α (17). The nuclear function of PKCθ and its potential involvement in specific transcriptional programs in T cells are discussed by Sutcliffe and colleagues (18).

In addition to the TCR, additional receptors, including OX40, a member of the tumor necrosis factor receptor (TNFR) superfamily, also utilize PKCθ for signal transmission, a topic that is presented in this issue by So and Croft (19). Following activation of T cell-expressed OX40 by its APC-expressed ligand, OX40L, activated OX40 delivers TCR-independent signals that promote optimal activation of NF-κB, using signaling intermediates that are distinct from those utilized by the TCR, e.g., TRAF proteins and RIP1.

PKCθ-deficient mice serve as an important tool for deciphering underlying mechanisms in cellular immune responses. While *in vitro* studies of PKCθ-deficient T cells demonstrated impaired activation responses, *in vivo* studies indicated that the requirement for PKCθ is not universal. Anel and colleagues (20) discuss the involvement of PKCθ in natural killer cell function and anti-tumor immunity, while the importance of PKCθ during the induction of graft-versus-host and graft-versus-leukemia responses, or in antiviral immunity is reviewed by Bronk and colleagues (21).

In another review, Sun (22) elaborates on the potential role of PKCθ in maintaining the normal balance between effector and regulatory T cells and the possibility of targeting PKCθ for intervention in T cell responses and prevention of selected autoimmune diseases and allograft rejection.

Both PKCη and PKCθ are members of the novel PKC subfamily that are highly expressed in T cells. However, in contrast to PKCθ, which is concentrated at the center of the IS in activated T cells, PKCη remains localized in a diffuse pattern over the entire IS, suggesting distinctive roles for these two isoforms in signal relay downstream of the TCR. Fu and Gascoigne (23) discuss the specific roles of PKCη and its functional redundancy with PKCθ in T cell biology.

Members of the atypical PKC subfamily (aPKC), including PKCζ and PKCι/λ, are also expressed in lymphocytes and play important roles in T and B cell differentiation, as well as in the regulation of T cell polarization and survival. A detailed description of the mechanism of action of aPKCs, in conjunction with their adapters, p62 and Par-6, in the PB-1-orchestrated signaling network that regulates lymphocyte behavior, is provide by Martin and Moscat (24).

ACKNOWLEDGMENTS

We would like to thank all these authors for their valuable contribution to this Research Topic, and the referees for their valuable suggestions and critical comments. We hope our readers will find the articles in this Research Topic informative, timely, and enlightening.

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Involvement of distinct PKC gene products in T cell functions

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It is well established that members of the protein kinase C (PKC) family seem to have important roles in T cells. Focusing on the physiological and non-redundant PKC functions established in primary mouse T cells via germline gene-targeting approaches, our current knowledge defines two particularly critical PKC gene products, PKCθ and PKCα, as the "flavor of PKC" in T cells that appear to have a positive role in signaling pathways that are necessary for full antigen receptor-mediated T cell activation *ex vivo* and T cell-mediated immunity *in vivo*. Consistently, in spite of the current dogma that PKCθ inhibition might be sufficient to achieve complete immunosuppressive effects, more recent results have indicated that the pharmacological inhibition of PKCθ, and additionally, at least PKCα, appears to be needed to provide a successful approach for the prevention of allograft rejection and treatment of autoimmune diseases.

Keywords: T cell regulation, protein kinases, PKC isotypes, immune disease therapy

INTRODUCTION

Members of the protein kinase C (PKC) family belong to the serine/threonine protein kinase subfamily, which plays an important role in the regulation of a variety of cell functions (**Figure 2**). The PKC family was originally discovered by Nishizuka and colleagues in 1977 (Takai et al., 1977) and consists of nine isotypes that are expressed in a wide range of cell types and tissues (**Figure 1**). The reasons for the heterogeneity of PKC isotypes are not yet fully understood. T lymphocytes, for example, express up to eight different species of PKC isotypes (**Table 1**), which makes it difficult to determine the specific cellular functions of these individual enzymes. The expression of more than a single PKC isotype in a given cell could suggest functional redundancy and/or specialization. **Table 1** summarizes the overall lymphoid expression patterns and T cell phenotypes of knockout T cells and the different PKC isotypes encoded in the human genome.

ROLE OF PKCθ IN IMMUNE CELL BIOLOGY

The main function of mature T cells is to recognize and respond to foreign antigens. This process involves the activation, adhesion, and differentiation of the resting cell into an effector lymphoblast that actively secretes immunoregulatory lymphokines or displays targeted cytotoxicity, ultimately leading to the recruitment of other cell types and initiation of an effective immune response. T cell activation is triggered by the ability of the T cell receptor (TCR) to recognize a peptide antigen, which is bound to major histocompatibility complex class I (MHCI) or class II (MHCII). T cells then begin to divide and differentiate on the basis of the processed antigen. The effector cell $CD4^+$ T helper cell subset (including T_H1, T_H2, and T_H17 cells) performs effector functions that are necessary to clear the pathogen. T_H1 $CD4^+$ T cells produce IFN-γ and IL-2 and promote cell-mediated immunity. T_H2 $CD4^+$ T cells produce IL-4, IL-5, IL-6, IL-10, and IL-13 and lead to the activation of the humoral immune system. T_H17 $CD4^+$ T cells produce IL-17, IL-21, and IL-22 and play roles in the defense against extracellular bacteria and fungi.

INVOLVEMENT OF PKCθ IN THE IMMUNOLOGICAL SYNAPSE

After cell–cell contact between a T cell and an APC, this contact is stabilized during the initiation of an immune response by interaction of the β2-integrin LFA-1 with its counterligand ICAM-1 (Mazerolles et al., 1988; Dustin and Springer, 1989; Penninger and Crabtree, 1999). LFA-1 avidity is controlled by inside-out signaling via the control of integrin conformation and surface distribution (Lub et al., 1995; Carman and Springer, 2003; Dustin et al., 2004). One important inside-out signaling molecule that controls cell adhesion is the small GTPase Rap1 (Katagiri et al., 2002; Shimonaka et al., 2003). Rap1A-deficient T cells show impaired LFA-1 clustering and adhesion after CD3 stimulation (Duchniewicz et al., 2006). Letschka et al. (2008) found a role of a PKCθ/RapGEF2 complex in regulating LFA-1 avidity in T cells. These authors showed that after T cell activation, PKCθ phosphorylates RapGEF2 at Ser960, which regulates Rap1 activation and LFA-1 adhesiveness to ICAM-1. In agreement, this study showed that in OT-II TCR-transgenic $CD4^+$ T cells, LFA-1 clustering after antigen activation was impaired in PKCθ-deficient $CD4^+$ T cells (Letschka et al., 2008). According to their study, PKCθ seems to positively regulate the adhesive capacity of T lymphocytes.

When a stable contact between a T cell and an APC is formed, the T cell co-stimulatory receptor CD28 is activated by binding to its cell ligands CD80 or CD86. Subsequently, the immunological synapse is generated at the contact area between the T cell and the APC (Rao et al., 1999). Part of the immunological synapse is the supramolecular activation complex (SMAC), which is characterized by different signaling proteins, such as LCK (SRC family tyrosine kinase), LFA-1 (lymphocyte function-associated

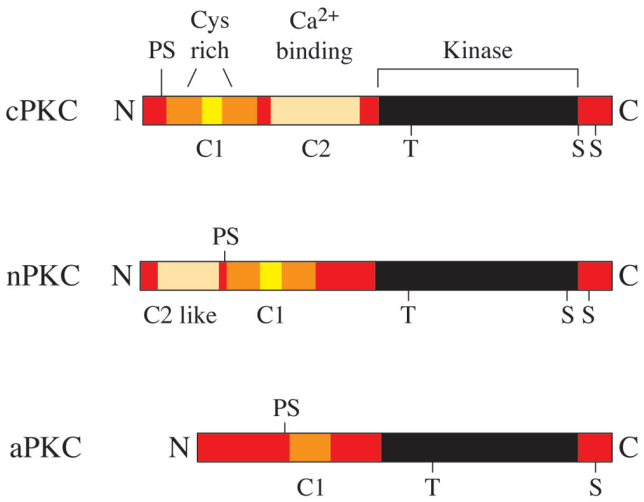

FIGURE 1 | The human PKC gene family. PKC proteins are classified into conventional PKCs (cPKC; α, β, and γ), novel PKCs (nPKC; ε, δ, θ, and η) and atypical PKCs (aPKC; ζ and ι). cPKCs require Ca^{2+} and diacylglycerol (DAG) for activation, nPKCs are Ca^{2+} independent and aPKCs require neither Ca^{2+} nor DAG for activation.

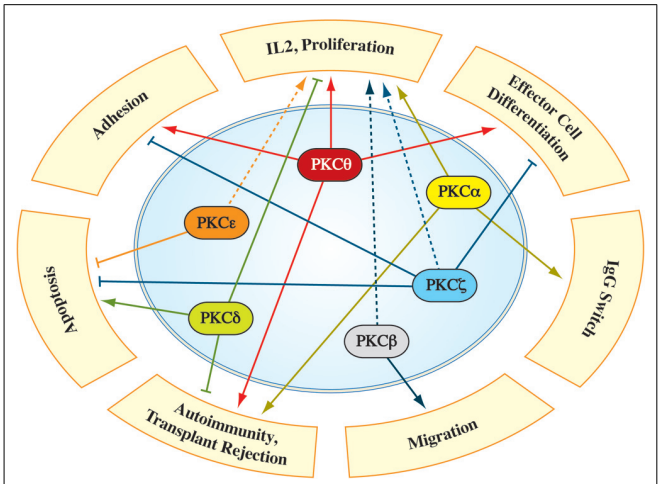

FIGURE 2 | Involvement of individual PKC family members in different aspects of T cell biology. Numerous studies identified PKC isotype-selective functions in signaling pathways, necessary for full T cell activation, differentiation and robust immune responses *in vivo* (for details see text). The dashed line depicts PKC functions which were characterized primarily via overexpression/knockdown studies in immortalized cell lines, while a validation in a more physiological system is pending.

antigen 1), and CD45 (Freiberg et al., 2002). Effective T cell stimulation is characterized by the recruitment of PKCθ to the SMAC (Schaefer et al., 2004), at which it is phosphorylated by LCK at Tyr-90 (Liu et al., 2000). A physical interaction of PKCθ with the cytoplasmatic tail of CD28 has been shown to be essential in this recruitment mechanism (Kong et al., 2011). Subsequently, PKCθ is phosphorylated at different sites (Bauer et al., 2001; Bi et al., 2001; Liu et al., 2002; Freeley et al., 2005; Lee et al., 2005) and autophosphorylated at Thr-219 (Thuille et al., 2005). Recently, Chuang et al. (2011) identified the MAP4K3 GCK-like kinase (GLK) as a kinase that directly phosphorylates PKCθ at Thr-538 which is essential to activation of NF-κB in T cells. Phosphorylation is important to retain PKCθ in the immunological synapse, in which one of its functions seems to be the regulation of the immunological synapse itself. Through the live imaging of components of the immunological synapse, the synapse has been shown to be dynamic in wild-type mice but more stable in PKCθ-knockout mice, which influences the strength, duration and location of signals (Dustin, 2008).

RECRUITMENT AND ACTIVATION OF SIGNALING MOLECULES

Another important role of PKCθ is to recruit and activate signaling molecules, such as phospholipase C (PLC), IL2-inducible T cell kinase (ITK), TEC, phospholipase C γ 1 (PLCγ1), and SPAK (a MAPKKK that ultimately activates AP1) to the immunological synapse. PKCθ was identified to play a critical role in the NF-κB and Ca^{2+}/NFAT pathways to activate the IL-2 promoter. Antigen binding to the TCR leads to an increase in intracellular Ca^{2+}, which activates calcineurin. Calcineurin dephosphorylates NFAT and leads to its nuclear import. Subsequently, NFAT forms complexes with the AP-1 protein transcription factor family and regulates the expression of IL-2 by binding to its promoter. PKCθ-knockout T cells were first described by Sun et al. (2000). They generated PKCθ-knockout mice by replacing the exon encoding the ATP-binding site of the kinase domain with the neomycin resistance gene. In their study they found strongly reduced proliferation of $PKC\theta^{-/-}$ $CD3^+$ T lymphocytes accompanied by a reduced secretion of IL-2. Suitably they could show that TCR-initiated NF-κB activation was absent from $PKC\theta^{-/-}$ $CD3^+$ T lymphocytes but was normal in thymocytes indicating that PKCθ is essential for TCR-mediated T cell activation (Sun et al., 2000).

Pfeifhofer et al. (2003) generated a conditional PKCθ-knockout mouse by using Cre-mediated recombination where the complete coding sequences of exons 3 and 4 are deleted, followed by a frame shift mutation between exons 2 and 5. Additionally to the results Sun et al. (2000) observed, they saw that a deficiency of PKCθ abrogates NFAT transactivation after CD3/CD28 stimulation. In addition, decreased intracellular Ca^{2+} flux was observed (Pfeifhofer et al., 2003).

To induce and maintain the complete IL-2-producing capacity of a T cell after TCR stimulation and activation of CD28, the RING (really interesting new gene)-type E3 ubiquitin ligase Cbl-b must be inhibited. Cbl-b restricts activation of the TCR by inhibiting the activation of PI3K (phosphoinositide-3-kinase; Fang and Liu, 2001) and PLCγ1 (Heissmeyer et al., 2004; Jeon et al., 2004), and it promotes the antigen-induced downregulation of the TCR (Naramura et al., 2002). In response to the stimulation of CD28, Cbl-b is ubiquitinylated and proteasomally degraded. Gruber et al. (2009a) showed that PKCθ directly regulates the ubiquitinylation and degradation of Cbl-b. After co-stimulation of the TCR and CD28, Cbl-b was degraded in wild-type $CD3^+$ T cells but not PKCθ-deficient $CD3^+$ T cells, and the ubiquitinylation of Cbl-b was strongly decreased after treatment with an inhibitor of PKCθ (Gruber et al., 2009a).

Table 1 | Lymphoid expression pattern and immune cell phenotypes of PKC isotype knockout mice.

Gene loci	Tissue expression	Knockout mouse immune phenotype	Reference
Conventional PKCs			
α	Ubiquitous, high in T cells	Reduced proliferation, reduced IFNγ production, defective IgG switching	Pfeifhofer et al. (2006)
β	Ubiquitous, high in B cells	Neutrophil-, B-, mast cell defect	Leitges et al. (1996), Nechushtan et al. (2000)
γ	Brain	ND	
Novel PKCs			
δ	Ubiquitous, high in T cells	Enhanced IL-2 secretion, enhanced proliferation, proapoptotic	Gruber et al. (2005), Lutz-Nicoladoni et al. (2005)
ε	Ubiquitous, high in T cells	Macrophage defect, defective bacterial clearance, influence on the nervous system	Castrillo et al. (2001), Kumar et al. (2002)
η	Ubiquitous, high in T cells	Impairment of epithelial regeneration in wound healing, increased susceptibility to tumor formation in skin carcinogenesis, defective homeostatic proliferation	Chida et al. (2003), Fu et al. (2011)
θ	T cells, platelets, monocytes	Reduced proliferation, reduced IL-2 production, abrogated AP-1, NF-κB, and NFAT transactivation, impaired EAE development, impaired T_H2 immunity against *N. brasiliensis*	Sun et al. (2000), Pfeifhofer et al. (2003), Marsland et al. (2004), Salek-Ardakani et al. (2004, 2005)
Atypical PKCs			
ζ	Ubiquitous	Impaired T_H2 cytokine secretion response	Martin et al. (2005)
ι	Ubiquitous	Lethal phenotype	

IN VIVO IMMUNE RESPONSES

During T cell development, thymocytes undergo a twofold selection process. During positive selection, $CD4^+CD8^+$ double-positive thymocytes bearing TCRs with low or moderate affinity to MHC/antigen complexes expressed on epithelial cells receive a survival signal. During negative selection, the high-affinity interaction of TCRs with self-MHC/self-peptide complexes selects the thymocytes for apoptosis. Selected thymocytes downregulate CD4 or CD8 and leave the thymus as fully mature lymphocytes. To address the question of whether PKCθ is involved in positive selection, Morley et al. (2008) analyzed MHCII-restricted TCR-transgenic and non-transgenic PKCθ-knockout mice. In both mouse models, they found a severe defect in thymocyte positive selection (Morley et al., 2008). In agreement with these results, Gruber et al. (2010) also found a crucial role for PKCθ in the positive selection of thymocytes in a pathway leading to the activation of ERK, NFAT, and NF-κB by analyzing MHCI-restricted TCR-transgenic mice and non-transgenic PKCθ-knockout mice. When a naive $CD4^+$ T cell is activated, it differentiates into the effector subsets T_H1, T_H2, or T_H17. An imbalance of this differentiation leads to autoimmunity and hypersensitivity. Several studies showed that PKCθ is important in the regulation of the T_H2-mediated immune response (Marsland et al., 2004; Salek-Ardakani et al., 2004, 2005; Tan et al., 2006). After infection with *Nippostrongylus brasiliensis*, T_H2 cell immune responses were severely impaired in $PKC\theta^{-/-}$ mice. Consistent with these results, another *in vivo* study showed that PKCθ appears to be involved in lung inflammation responses, which are controlled by T_H2 cells (Marsland et al., 2004; Salek-Ardakani et al., 2004). $PKC\theta^{-/-}$ mice develop drastically reduced pulmonary hypersensitivity responses to inhaled allergens, such as lung inflammation, eosinophil infiltration, and immunoglobulin E production.

To address the question of whether PKCθ is involved in protection against bacterial infections, Sakowicz-Burkiewicz et al. (2008) infected mice with *Listeria monocytogenes* (LM) and found that PKCθ is responsible for normal LM-specific T cell responses. Fauconnier et al. (2011) studied the role of PKCθ after the infection of mice with *Plasmodium falciparum*. They found that PKCθ-deficient mice are resistant to the development of cerebral malaria, and the recruitment and activation of $CD8^+$ T cells in the brains of the resistant mice were reduced. To study the function of PKCθ in a chronic persisting infection model, Nishanth et al. (2010) infected mice with *Toxoplasma gondii*. PKCθ-deficient mice suffered from encephalitis and showed insufficient parasite control. *T. gondii*-specific $CD4^+$ and $CD8^+$ T cells were significantly reduced in the spleens and brains of infected PKCθ-deficient mice, indicating that PKCθ is important for intracerebral parasite control (Nishanth et al., 2010).

Tan et al. (2006) and Salek-Ardakani et al. (2004, 2005) showed that PKCθ is also important for full development of experimental autoimmune encephalomyelitis (EAE), a multiple sclerosis-like autoimmune disease that is T_H17 dependent. $PKC\theta^{-/-}$ mice failed to develop EAE after injection with myelin oligodendrocyte

glycoprotein (MOG). In addition, T_H17 cells produced less IL-17 and failed to infiltrate the CNS.

Recently, Kwon et al. (2012) showed that PKCθ$^{-/-}$ mice had lower levels of Stat3, a transcription factor required for T_H17 differentiation, whereas the activation of Stat4 and Stat6, which are important for T_H1 and T_H2 differentiation was normal. Using a luciferase reporter gene driven by the Stat3 promoter they showed that PKCθ stimulates Stat3 transcription via the NF-κB and AP-1 pathway, resulting in the stimulation of T_H17 differentiation (Kwon et al., 2012).

In striking contrast, PKCθ$^{-/-}$ mice showed normal T_H1 responses after infection with *Leishmania major* (Marsland et al., 2004), suggesting a lineage-specific function of PKCθ.

Garaude et al. (2008) found an impaired anti-leukemic response in PKCθ-deficient mice. These authors induced leukemia with Moloney-murine leukemia virus and found a higher disease incidence and a more rapid disease onset in PKCθ-knockout mice. Additionally, the intravenous injection of EL4 cells induced tumors earlier in PKCθ$^{-/-}$ mice.

To avoid an uncontrolled immune response, the maintenance of the balance between immune tolerance to self-antigens and anti-tumor responses and the regulation of the suppression of effector T cells is mediated by regulatory T cells (T_{reg} cells; Sakaguchi et al., 2008). T_{reg} cells are produced in the thymus (nT_{reg}) or from naive effector T cells (iT_{reg}), and both types of T_{reg} cells express the transcription factor FoxP3, whereas nT_{reg} cells also express Helios (Zheng and Rudensky, 2007; Thornton et al., 2010). T_{reg} cells are able to suppress the function of CD4$^+$ and CD8$^+$ T cells, dendritic cells (DCs), NK cells, and B cells (Gupta et al., 2008b; Shevach, 2009). A deficiency of T_{reg} cells leads to multi-organ inflammatory diseases in mice (Sakaguchi et al., 2008). Gupta et al. (2008a) found a strongly reduced number of T_{reg} cells in PKCθ-knockout mice, but these cells were as potent as wild-type T_{reg} cells in inhibiting effector T cell activation, indicating that PKCθ was not required for T_{reg} cell-mediated inhibitory functions. However, Zanin-Zhorov et al. (2011) found that PKCθ was sequestered away from the T_{reg} immunological synapse with confocal imaging, and using a colitis mouse model and a poorly described PKCθ inhibitor, they postulated a PKCθ-mediated negative feedback loop that enhances the activity of human T_{reg} cells. A very recent publication by Ma et al. (2012) suggested that the differentiation of iT_{reg} cells is inhibited by PKCθ-mediated signals via the AKT-Foxo1/3A pathway.

ROLE OF OTHER PKCs IN IMMUNE CELL BIOLOGY

PKCδ

PKCδ is an isozyme belonging to a novel subclass of the serine/threonine PKC family and is expressed in most tissue and cell types. The kinase catalytic activity of PKCδ is mainly affected by trans- and autophosphorylation at conserved Ser/Thr sites in the catalytic domain (activation loop, turn motif, and hydrophobic motif), by tyrosine phosphorylation (by Src family kinases in the context of oxidative stress and DNA damage; Lu et al., 2007; Lomonaco et al., 2008) and by caspase-mediated proteolysis (during apoptosis; Kikkawa et al., 2002). Generally, upon stimulation, PKCδ translocates from the cytosol or nucleus to membrane/cytoskeletal compartments, enabling the phosphorylation of many target proteins and leading to the activation of several signal transduction pathways. It has also been shown that PKCδ can shuttle to mitochondria (Li et al., 1999; Majumder et al., 2001). PKCδ negatively affects a wide variety of cellular functions by inhibiting cellular growth and proliferation and promoting cell death, but it has also been shown to contribute to mitogenesis (Watanabe et al., 1992; Nakagawa et al., 2005; Santiago-Walker et al., 2005), migration (Jackson et al., 2005), differentiation (Cerda et al., 2001; Yang et al., 2006; Zhang et al., 2008), and tumor progression. Different studies have revealed a role for PKCδ in the initiation, progression, and maintenance of inflammatory processes by affecting NF-κB transactivation (Satoh et al., 2004; Hsieh et al., 2007).

Additionally, a pro-apoptotic role for PKCδ has been described in T cells. The subcellular localization of PKCδ in human T cells during apoptotic induction by cytokine deprivation and Fas ligation and during the prevention of apoptosis by IFNβ addition was analyzed by Scheel-Toellner et al. (1999). The addition of INFβ to T cells in a pro-apoptotic environment led to a rapid re-translocation of PKCδ from the nucleus and inhibited the caspase-3-mediated proteolytic activation of PKCδ (Scheel-Toellner et al., 1999). An essential role for PKCδ in the apoptotic induction of mouse thymocytes was addressed in a study by Lutz-Nicoladoni et al. (2005). Thymocytes from a large panel of PKC-knockout mice were forced to undergo apoptosis *in vitro* via treatment with different apoptotic inducers (PDBu, dexamethasone, FasL, staurosporine, or etoposide), and the selective involvement of PKC isotypes in this process was assessed. PKCδ-deficient primary mouse double-positive thymocytes were protected from apoptotic induction, indicating a clear pro-apoptotic role of PKCδ (Lutz-Nicoladoni et al., 2005). Gruber et al. (2005) investigated the proliferative response and IL-2 cytokine secretion of PKCδ-deficient CD3$^+$ T cells versus control cells *in vitro* via allogenic MHC stimulation and *in vivo* via injection of anti-CD3 antibodies. The significantly enhanced proliferation and IL-2 cytokine production of mature T cells and the increased blood plasma IL-2 levels in PKCδ-null mice led to the assumption that PKCδ acts as a negative regulator of T cell activation responses (Gruber et al., 2005).

An involvement of PKCδ in lytic granule exocytosis of CD8-CTLs (cytotoxic T lymphocytes) was shown by Ma et al. (2007, 2008). The combined use of pharmacological inhibitors and mice with targeted gene deletions allowed these authors to demonstrate that PKCδ is selectively required for lytic granule movement in response to TCR engagement on CD8$^+$ CTLs but is dispensable for activation, cytokine production, and the expression of cytolytic molecules in response to TCR stimulation. In a follow-up study, the authors showed via a time-lapse analysis of living CD8$^+$ CTLs that PKCδ localizes to secretory lysosomes and accumulates at the immunological synapse during target killing (Ma et al., 2007, 2008).

A correlation between impaired PKCδ activation/ phosphorylation and the development of idiopathic and hydralazine-induced lupus was postulated by Gorelik et al. (2007). PMA-stimulated CD4$^+$ T cells from patients with lupus showed an impaired PKCδ activity state compared with CD4$^+$ T cells from healthy donors. This defect was responsible for decreased ERK signaling and led

to increased CD70 expression due to insufficient demethylation of the CD70 promoter (Gorelik et al., 2007).

The expression level and activity state of PKCδ and PKCζ was investigated in amyloid β1–42 (Aβ1–42)-reactive T cell populations in Alzheimer disease (AD) patients in comparison to healthy individuals. This study clearly showed the increased expression and activation of PKCδ in Aβ-stimulated peripheral T cells from early AD patients, whereas the same treatment induced two distinct (p)PKCδ and (p)PKCζ T cell subpopulations in severe AD patients (Miscia et al., 2009).

PKCε

PKCε was first discovered among the novel PKC isotypes and is expressed at high levels in neuronal, hormonal, and immune cells. Essential roles for PKCε have been established in numerous cellular functions, including proliferation, differentiation, gene expression, muscle contraction, transport, tumorigenesis, exocytosis, and endocytosis. In addition to the classical activation by auto- and trans-phosphorylation at conserved sites in the catalytic domain, PKCε is activated by several different second messengers, including diacylglycerol (DAG), phosphatidylinositol-3,4,5-triphosphate, and fatty acids. PKCε is targeted to specific cellular compartments depending on the interaction of second messengers with its C1 domain (DAG and tridecanoic acids evoke a plasma membrane and/or cytoskeleton translocation, whereas arachidonic and linoleic acids lead to recruitment to Golgi networks) and via crosstalk with adaptor proteins (i.e., Rack1 and β-Cop). An association of PKCε (via its actin-binding motif) with actin filaments in response to phosphatidylserine-independent stimulation has been reported (Akita, 2002).

In T cells, numerous studies have directly shown a positive role of PKCε in the regulation of NF-κB/NFAT/AP1 pathways leading to IL-2 upregulation; the activation-dependent translocation of PKCε from the cytosol to the membrane compartment in TCR/CD3- or PMA-stimulated human PBLs has been reported previously (Keenan et al., 1997). The neutralization of PKCε in this cell type via the introduction of antagonistic antibodies led to a downregulation of IL-2 synthesis (Szamel et al., 1998). Jurkat T cells expressing a constitutively active PKCε mutant showed increased AP1 and NFAT1 transactivation (Genot et al., 1995). An inhibitory effect of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in the plasma membrane translocation of PKCε (and PKCα), NF-κB nuclear translocation, and IL-2 transcription in PMA-stimulated Jurkat T cells has been described (Denis et al., 2005). A pivotal role for PKCε in thrombin-mediated ERK1/2 activation in Jurkat cells has been shown by Maulon et al. (2001). The poor ability of neonatal T cells to produce lymphokines was linked to a lower PKCε (and PKCβ, PKCθ, and PKCζ) expression level in this cell type, which is correlated with an activation defect of MAPK pathways (Hii et al., 2003).

Interestingly, Gruber et al. (2005) reported that mice carrying a homozygous disruption of the PKCε locus showed unaltered T cell development and maturation; in addition, mature primary $CD3^+$ T cells isolated from $PKC\varepsilon^{-/-}$ mice showed normal proliferation, IL-2 secretion responses, and NF-κB transactivation upon CD3/CD28 stimulation or allogeneic MHC presentation, suggesting that PKCε loss of function is compensated for by other members of the PKC family. In contrast to the described redundant function of PKCε in mouse T cell proliferation, a role of the PKCε isotype in the regulation of human $CD4^+$ T cell proliferation and sensitivity to TGFβ1 has been shown by Mirandola et al. (2011). PKCε silencing by siRNA led to decreased IL-2 receptor chain expression and proliferation and reduced NF-κB1 and NF-κB2 gene expression upon CD3/CD28 stimulation, whereas the inhibitory effects of TGFβ1 were potentiated by PKCε downregulation. In addition, a possible connection between increased PKCε expression levels in $CD4^+$ T cells from Hashimoto thyroiditis patients and the molecular pathophysiology of this autoimmune disease was postulated (Mirandola et al., 2011).

Some studies have identified an anti-apoptotic role for PKCε: Jurkat T cells were rescued from Fas-mediated apoptosis by PKCε via the p90Rsk-dependent phosphorylation and inactivation of BAD (Bertolotto et al., 2000). The basis for the deletion of autoreactive thymocytes during negative selection was previously addressed (Simon et al., 2000); a lack of the constitutive expression of PKCε in antigen-stimulated $CD4^+/CD8^+$ thymocytes (in comparison to mature T cells) leading to an inhibition of NF-κB activity and increased cell death was postulated as a probable cause.

A positive involvement of PKCε in the recovery of downregulated sphingosine-1-phosphate receptor 1 (S1PR1) in primary mouse $CD4^+$ T cells was investigated (Graeler et al., 2003) in PKCε-null mice and with PKCε-selective inhibitors.

Quann et al. (2011) established a new redundant role for PKCε and PKCη in T cell polarity; the photoactivation of TCR induced a rapid accumulation of both PKC isotypes in a broader domain of the plasma membrane, in which they were required to promote the recruitment of PKCθ to the center of the immunological synapse and subsequent microtubule-organizing center (MTOC) reorientation.

PKCζ

PKCζ is a calcium- and diacylglycerol-independent serine/threonine protein kinase that belongs to the atypical subfamily of PKC isoforms and displays strong homology (more than 70%) to PKCι/λ. It is ubiquitously expressed but is more highly expressed in the lung, brain, and testis. PKCζ contains a PB1 domain in the N-terminus that recognizes OPCA (OPR/PC/AID) motifs of other proteins, such as the scaffold proteins PAR-6 and ZIP/p62 and the kinase MEK5. PKCζ activity is regulated by PDK-1 transphosphorylation of the catalytic domain activation loop, autophosphorylation, and important lipid components, such as phosphatidylinositols, phosphatidic acid, arachidonic acid, PIP3, and ceramide. Prostate apoptosis response-4 (Par-4) and partitioning defective gene-3 (PAR-3) have been reported to inhibit PKCζ activity through a specific protein–protein interaction. PKCζ has been shown to be involved in the regulation of several critical pathways for cell survival, proliferation, differentiation, and cell polarity, thereby affecting the NF-κB and MAPK pathways. A special role in modulating translation via the p70S6 kinase signaling cascade has also been described by numerous studies (Hirai and Chida, 2003). Recently, a link between PKCζ activity

and TGFβ receptor trafficking and degradation has been shown (Gunaratne et al., 2012).

The activation of the PKCζ isotype has been shown to be an important step in the IL-2-mediated proliferation of T cells and in maintaining the integrity of the actin cytoskeletal structure (Gomez et al., 1995). Furthermore, an association between PKCζ and PI3K has been reported to be necessary for the phosphorylation/activation of PI3K in IL-2-stimulated TS1-α/β mouse T cells (Gomez et al., 1996). Through the transient overexpression of wild-type or a dominant-negative mutant of PKCζ in Jurkat T cells, a previous study (San-Antonio et al., 2002) observed that PKCζ can phosphorylate NFAT and regulate its activation status. Additionally, an involvement of both PKCζ and PI3K in NF-κB/c-Rel transactivation regulation in TNFα-stimulated Jurkat T cells was postulated (Martin et al., 2001). A previous study (Sanchez-Valdepenas et al., 2007) addressed the effect of TCR/CD28 co-stimulation on the inducible phosphorylation/transactivation of the NF-κB members p65/RelA and c-Rel. Cot kinase, PKCζ, and NF-κB-inducible kinase (NIK) seemed to be involved in potentiating c-Rel transactivation activity through the phosphorylation of a restricted set of Ser residues, whereas NIK seemed to be unnecessary for the activation of p65. Additionally, Gruber et al. (2008) found a physical and functional interaction between PKCζ and the novel PKCθ isotype in the NF-κB activation of Jurkat T cells. A stimulation-dependent colocalization of the PKCζ/ι–PKCθ complex to lipid rafts was monitored via confocal microscopy. However, peripheral $CD3^+$ T cells isolated from the spleen and lymph nodes of PKCζ-deficient mice showed normal proliferation and IL-2 cytokine responses to CD3/CD28 activation, indicating a possible functional redundancy with PKCι/λ, the closest structural relative (Gruber et al., 2008).

A critical role for PKCζ in IL-4 signaling and T_H2 differentiation *in vitro* and *in vivo* has been reported (Martin et al., 2005). PKCζ-deficient $CD4^+$ T cells showed an impaired secretion of T_H2 cytokines and a defective Stat6/Jak1 pathway. Moreover, $PKC\zeta^{-/-}$ mice were protected from ovalbumin-induced T_H2-driven allergic airway disease in an asthma model.

A protective role for PKCζ against FasL-induced apoptosis was previously described (Leroy et al., 2005); PKCζ interfered with FADD recruitment to the death-inducing signaling complex (DISC) and subsequent caspase-8 processing.

PKCζ has been shown to act in combination with nitric oxide synthase (NOS) in the regulation of thyroid hormone (TH)-mediated T cell proliferation (Barreiro Arcos et al., 2006); TH treatment increased atypical PKCζ expression and NOS activity, whereas PKCζ inhibition abrogated the basal and TH-induced activation of NOS.

A role for PKCζ in the biological processes of adhesion and cell motility has been described by several studies. The mechanism of the CD4-triggered regulation of LFA-1-mediated adhesion was investigated (Trucy et al., 2006). CD4 binding increased the activity of both PDK1 and PKCζ, and both kinases were necessary for the downregulation of LFA-1-dependent adhesion in the A201-$CD4^+$ T cell line in a PI3K-dependent manner. Real et al. (2007) showed that PKCζ and PKCι were both required for T cell motility and the ability to scan DCs downstream of chemokine receptors.

PKCη

PKCη is classified into the novel PKC subfamily and shows a high sequence similarity to PKCε. It was originally isolated from a cDNA library of mouse skin in 1990 (Osada et al., 1990) and is localized on human chromosome 14 (Quan and Fisher, 1999) and mouse chromosome 12 (Chida et al., 1998). It is predominantly expressed in squamous epithelia including skin, tongue, esophagus, and trachea (Koizumi et al., 1993), but at high levels also in T and B cells (Mischak et al., 1991). In addition to phosphatidylserine and diacylglycerol, PKCη can be specifically activated by cholesterol sulfate (Ikuta et al., 1994). An involvement in keratinocyte cell growth, terminal differentiation, and cell cycle arrest has been reported by several studies: PKCη was shown to associate with and to activate Fyn, leading to keratinocyte growth arrest and differentiation (Cabodi et al., 2000); a PKCη induced terminal differentiation through a transcriptional activation of TGas1 and involucrin was described by Ueda et al. (1996) and Efimova and Eckert (2000). In addition, PKCη has been shown to induce G1 arrest in keratinocytes via an inhibition of cyclin-dependent kinase 2 activity (Kashiwagi et al., 2000). An important role in the regulation of cell division and cell death during early B cell development was postulated by the work from Morrow et al. (1999).

The different lipid raft localization pattern of PKCα, PKCη, and PKCθ in cisplatin-induced apoptotic Jurkat T cells was investigated by Solstad et al. (2010). A selective upregulation of PKCα in these microdomains upon apoptosis induction was revealed, whereas the levels of PKCη and PKCθ were significantly reduced.

Recently, Fu et al. (2011) found a pivotal role of PKCη in T cell activation and homeostatic proliferation. Comparing the phenotypes of $PKC\eta^{-/-}$, $PKC\theta^{-/-}$, and mice with a targeted disruption of both PKC isoforms, they were able to show that both isoforms share some redundancy in T cell biology. Both isoforms are recruited to the immunological synapse upon TCR stimulation and double-knockout mice showed a more severe defect in positive selection. Additionally, they found specific non-redundant functions as in self-antigen-dependent homeostatic proliferation. Using a live imaging approach a TCR-induced recruitment of GFP fusion proteins of PKCη and PKCε to the plasma membrane was also described by Quann et al. (2011). The timely well coordinated localized enrichment of these two isoforms served as a prerequisite for the subsequent translocation of PKCθ to the center of the immunological synapse, necessary for the regulation of T cell polarity and T cell effector functions.

PKCβ

The alternative splicing forms PKCβI and PKCβII are members of the calcium-activated, phospholipid- and DAG-dependent classical or conventional PKC subfamily. Numerous studies have shown their role in various cellular processes, such as the regulation of B cell development and activation/proliferation, oxidative stress-induced apoptosis, androgen receptor-dependent transcription regulation, insulin signaling, and endothelial cell proliferation. In B cells, a signaling link between PKCβ and BTK has been described; PKCβ can downregulate BTK function through the direct phosphorylation of BTK at Ser-180, inhibiting its membrane translocation and subsequent activation (Kang et al., 2001). A key

role for PKCβ in BCR-induced NF-κB activation has been shown (Sommer et al., 2005); the direct phosphorylation of CARMA1 at three serines within its linker region induced its translocation into lipid rafts, the recruitment of BCL10/Malt1 and the subsequent activation of signaling molecules downstream of the CBM complex. Furthermore, PKCβ seems to play an important, even dual role in insulin signaling pathways: in muscle cells, PKCβ mediates insulin-dependent DNA synthesis through the RAF1-MAPK/ERK signaling cascade downstream of insulin receptor substrate 1 (IRS1), and in adipocytes, it negatively regulates glucose transport by inhibiting the translocation of the glucose transporters GLUT1 and GLUT4 (Formisano et al., 2000; Bosch et al., 2003; Perrini et al., 2004).

A selective impact of PKCβ on T cell migration has been shown by several studies (Volkov et al., 1998, 2001). LFA-1-triggered T cell locomotion led to the specific recruitment of PKCβ and PKCδ to the MTOC and microtubules. A PKCβ-deficient T cell line was unable to either crawl or develop a polarized microtubule array upon integrin cross-linking, whereas the ability to adhere and form actin-based pseudopodia remained unaffected. The reconstitution of PKCβ(I) in non-motile PKCβ-deficient T cells restored their locomotory behavior in response to an LFA-1 signal.

The possible involvement of PKCβ in IL-2 gene transcription and/or IL-2 protein secretion upon TCR/CD28-induced T cell activation has been addressed by several studies (Long et al., 2001; Dreikhausen et al., 2003). The downregulation of PKCβ synthesis in Jurkat T cells via the addition of antisense oligos resulted in the suppression of the activation of MAPK/NF-κB/NFAT pathways and a complete inhibition of IL-2 transcription and secretion. However, a study performed with a PKCβ-deficient HUT78 T cell clone excluded a possible role for IL-2 transcription and translation but demonstrated an involvement of PKCβ in IL-2 exocytosis. Thuille et al. (2004) investigated the physiological role of PKCβ in primary mouse T cells employing a PKCβ-deficient knockout line and found mostly normal activation-induced proliferation and IL-2 secretion responses. However, it is conceivable that other members of the cPKC family, such PKCα, could compensate for the lack of this redundant PKC isotype in T cells.

In 2010 a re-investigation of IL-2 expression in PKCβ silenced Jurkat T cells via antisense RNA technology revealed a stimulation dependent decreased IL-2 production, whereas the CD25 expression was significantly increased. In addition, PKCβ loss of function affected also CD69 surface levels and IL-8 production (Cervino et al., 2010). In the same year a scientific group investigated the influence of PKCβ on PMA induced apoptosis protection in Jurkat T cells and HL-60 human leukemia cells. The downregulation of PKCβ via shRNA or the specific small inhibitor enzastaurin reversed PMA induced protection of cell death (Meng et al., 2010).

PKCα

Additional to PKCθ also PKCα, a member of the conventional PKCs plays an important role in the induction of a robust immune response. By transfecting fetal thymuses with constitutively active and dominant-negative forms of PKCα, Michie et al. (2001) showed that this isoform plays a specific role in the differentiation and expansion of immature thymocytes. Iwamoto et al. (1992) established a transgenic mouse line carrying rabbit PKCα cDNA under the control of the regulatory element of human CD2. In response to stimulation with anti-CD3, they found that the transgenic thymocytes proliferated extensively and produced IL-2 (Iwamoto et al., 1992). Lallena et al. (1999) and Trushin et al. (2003) showed that PKCα regulates IκB kinase and NF-κB in T cells.

PKCα was shown to be involved in the activation of the PI3K/Akt pathway, which is involved in T cell development, survival, and migration (Jones et al., 2000; Haxhinasto et al., 2008; Sauer et al., 2008). Using PKC inhibitors and *in vitro* kinase assays with recombinant inactive Akt as a substrate, Yang et al. (2006) showed that PKCα could phosphorylate Akt at Ser^{473} dependent on TCR activation. These authors also performed knockdown analysis in Jurkat T cells and found decreased TCR-induced phosphorylation of Akt at Ser^{473}. PKCα and PKCθ are both involved in TCR downregulation (von Essen et al., 2006). von Essen et al. (2006) investigated the role of PKC isotypes in TCR downregulation and found an important role for PKCα in TCR comodulation (downregulation of non-engaged TCRs). Moreover, PKCα seemed to be responsible for the induction of endocytosis of non-engaged TCRs that recycle to the contact zone between the T cell and the APC. PKCθ, however, seemed to be responsible for inducing the endocytosis of directly triggered TCRs at the contact zone. Furthermore, a study showed the involvement of PKCα in allergic processes (Oh et al., 2004).

Our laboratory identified PKCα as a physiological and non-redundant PKC isotype in signaling pathways that are necessary for T cell-dependent IFNγ production and IgG2a/2b antibody responses using PKCα-knockout mice (Pfeifhofer et al., 2006).

PKC LMWI (LOW-MOLECULAR-WEIGHT INHIBITOR) IN THE CLINIC

Studies have shown that $PKC\theta^{-/-}$ mice fail to develop experimental allergic encephalomyelitis (EAE) and display drastically reduced lung inflammation after the induction of allergic asthma and alloreactivity in TX medicine, suggesting that PKCθ by itself is an attractive monotarget for modulation of the immune response. While this published evidence validates PKCθ inhibition being essential, more recent results have indicated that additional PKC isotypes are involved in critical T cell signaling pathways. Because PKCθ and PKCα are both highly expressed in T cells (GNF SymAtlas (http://symatlas.gnf.org/SymAtlas) and have isotype-selective functions in T cells (Sun et al., 2000; Pfeifhofer et al., 2003, 2006), whether PKCθ and PKCα also exert overlapping functions has also been investigated. Gruber et al. (2009b) generated $PKC\alpha^{-/-}\theta^{-/-}$ double-knockout mice and found that the NFAT pathway plays a predominant role in the collaborative action of PKCθ and PKCα. The NFAT kinase GSK3β was hyper-reactive in $PKC\alpha^{-/-}\theta^{-/-}$ double-knockout $CD3^{+}$ T cells. Subsequently, these authors found reduced nuclear translocation and DNA binding of NFAT. In *in vivo* studies, $PKC\alpha^{-/-}\theta^{-/-}$ double-knockout T cells showed strongly reduced IL-2 cytokine secretion after injection of an anti-CD3 monoclonal antibody. Additionally, the mice showed an impaired alloimmune response, leading to significantly prolonged allograft survival in heart transplantation experiments (Gruber et al., 2009b).

To obtain complete immunosuppressive effects, the inhibition of more than PKCθ appears to be needed, and the pharmacologic inhibition of multiple PKC isotypes may provide a successful approach to avert T cell effector functions that are relevant for diseases such as psoriasis, atopic dermatitis, and allergies, as well as other indications, including asthma, rheumatoid arthritis, multiple sclerosis, and transplant rejections.

Sotrastaurin (AEB071) is an immunosuppressive drug that inhibits multiple classical and novel members of the PKC family, resulting in decreased T lymphocyte activation (Evenou et al., 2009). In primary human and mouse T cells, AEB071 abrogated IL-2 secretion and CD25 expression, which are markers of early T cell activation. CD3/CD28-induced T cell proliferation, and LFA-1-mediated T cell adhesion were potently inhibited, and unlike previous PKC inhibitors, the apoptosis of murine T cell blasts was not enhanced (Evenou et al., 2009). These mechanistic studies on NF-κB and NFAT transcription factor transactivation additionally suggest that AEB071 and CsA have a complementary effect, resulting in the combined inhibition of IL-2 secretion. Additionally, other results suggest that AEB071 but not CsA inhibits the adhesive capacities of T lymphocytes.

Skvara et al. (2008) performed a clinical study with patients suffering from psoriasis in which the patients received single and multiple oral doses of AEB071. They found a strong reduction in the clinical severity of psoriasis and a histological improvement in skin lesions, indicating that sotrastaurin may provide a new therapeutic option for psoriasis (Skvara et al., 2008). Even so, we cannot exclude additional PKC isotypes being involved in critical T cell signaling pathways. The effect of AEB071 on PKCθ, including other classical and novel PKC family members expressed in T cells, is the likely mechanism responsible for the strong AEB071 immunosuppressive activity.

NEW CANDIDATE EFFECTOR PATHWAYS MEDIATED BY PKC IN T CELLS

The challenge ahead for immunologists is the further elucidation of the molecular and cellular processes of PKCα and PKCθ that govern the development and function of T cells. PKC-mediated signaling in NFAT/AP-1 transactivation critically involves a pathway of the orphan nuclear receptor NR2F6. There is evidence that PKC-induced signaling involves NR2F6 inactivation, presumably by stimulating the release of NR2F6 from DNA-binding sites. This inactivation facilitates NFAT/AP-1 binding to its enhancers in the IL-2 and IL-17A promoters. In agreement, PKCα$^{-/-}$/θ$^{-/-}$ double-knockout T cells show almost no TCR/NFAT/AP-1 transactivation signaling (Gruber et al., 2009b), whereas NR2F6-knockout T cells show markedly upregulated TCR/NFAT/AP-1 transactivation (Hermann-Kleiter et al., 2008). However, PKCα and PKCθ might have an even broader role in regulating T cell functions than just acting downstream of T cell antigen receptors. Thus, despite the significant progress in assembling the PKC puzzle in T lymphocytes, defining downstream PKC substrates, including their effector functions, triggered by this phosphorylation step remains to be investigated in physiological settings. From these investigations, innovative possibilities are likely to emerge for the manipulation of T cell pathways in treating immunological diseases by suppressing pathophysiological immune responses or augmenting host-protective immunity.

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Protein kinase C signaling and cell cycle regulation

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A link between T cell proliferation and the protein kinase C (PKC) family of serine/threonine kinases has been recognized for about 30 years. However, despite the wealth of information on PKC-mediated control of T cell activation, understanding of the effects of PKCs on the cell cycle machinery in this cell type remains limited. Studies in other systems have revealed important cell cycle-specific effects of PKC signaling that can either positively or negatively impact proliferation. The outcome of PKC activation is highly context-dependent, with the precise cell cycle target(s) and overall effects determined by the specific isozyme involved, the timing of PKC activation, the cell type, and the signaling environment. Although PKCs can regulate all stages of the cell cycle, they appear to predominantly affect G0/G1 and G2. PKCs can modulate multiple cell cycle regulatory molecules, including cyclins, cyclin-dependent kinases (cdks), cdk inhibitors and cdc25 phosphatases; however, evidence points to Cip/Kip cdk inhibitors and D-type cyclins as key mediators of PKC-regulated cell cycle-specific effects. Several PKC isozymes can target Cip/Kip proteins to control G0/G1 → S and/or G2 → M transit, while effects on D-type cyclins regulate entry into and progression through G1. Analysis of PKC signaling in T cells has largely focused on its roles in T cell activation; thus, observed cell cycle effects are mainly positive. A prominent role is emerging for PKCθ, with non-redundant functions of other isozymes also described. Additional evidence points to PKCδ as a negative regulator of the cell cycle in these cells. As in other cell types, context-dependent effects of individual isozymes have been noted in T cells, and Cip/Kip cdk inhibitors and D-type cyclins appear to be major PKC targets. Future studies are anticipated to take advantage of the similarities between these various systems to enhance understanding of PKC-mediated cell cycle regulation in T cells.

Keywords: protein kinase C, signal transduction, T cell activation, cell cycle, cyclin, cyclin-dependent kinase, cyclin-dependent kinase inhibitor

An association between protein kinase C (PKC) signaling and T cell proliferation has been recognized for almost three decades. Over 30 years ago, it was determined that a combination of phorbol esters and elevated intracellular calcium potently induces proliferation of cells of the T cell lineage (e.g., Whitfield et al., 1973; Lyall et al., 1980; Sutherland et al., 1981). Shortly thereafter, it was determined that PKC, then recognized as a calcium-dependent enzyme, represented the major cellular receptor for phorbol esters (Castagna et al., 1982) and it was not long before the connection between these phenomena was made (Isakov et al., 1986; Isakov and Altman, 1987). Since then, a central role for PKC in T cell receptor (TCR) signaling has been firmly established. Despite the long association of PKC with T cell proliferation, details on how PKC signaling interacts with the cell cycle machinery in this cell type are only beginning to emerge. In this regard, our knowledge in T cells lags behind that in other cell types, including other hematopoietic lineages. In this review, we outline our current understanding of the proliferative effects of PKC signaling in T cells within the context of the broader knowledge that has been gained in other systems.

THE PROTEIN KINASE C FAMILY

Protein kinase C represents a family of serine/threonine kinases that belong to the AGC (cAMP-dependent, cGMP-dependent, and protein kinase C) superfamily of protein kinases (Nieto, 2007; Matsuoka et al., 2009; Black, 2010; Rosse et al., 2010; Ryu et al., 2010). PKC isozymes are lipid-dependent kinases (requiring phosphatidylserine binding for activity) and are grouped into three subfamilies based on their structure and requirement for additional co-factors and calcium. Physiological activation of classical PKCs (PKCα, PKCβI and PKCβII, which are splice variants of the *prkcb* gene, and PKCγ) is induced by the lipid second messenger diacylglycerol (DAG) and calcium, while activation of the novel PKCs (PKCδ, PKCε, PKCθ, and PKCη) requires only DAG. In contrast, the atypical PKCs (PKCζ and PKCι/λ) are not dependent on lipid second messengers or calcium for activity. Instead, their function is regulated by protein–protein interactions mediated by a PB1 domain as well as a carboxyl-terminal PDZ ligand motif. Engagement of growth factor or cytokine receptors leads to activation of phospholipase C (PLC) β or PLCγ, which cleave phosphatidylinositol 4,5-bisphosphate to generate DAG and the soluble second messenger inositol trisphosphate (which induces release of calcium from intracellular stores). The production of DAG recruits classical and novel PKCs to the plasma membrane, where they undergo a conformational change resulting in full activation. Unlike other AGC kinases, such as Akt, activation of PKCs does not require acute phosphorylation of the enzyme: phosphorylations necessary for catalytic competence occur shortly after

synthesis and the enzyme is constitutively phosphorylated at these sites (Matsuoka et al., 2009; Rosse et al., 2010). As a result, changes in phosphorylation do not provide an indication of PKC activity; rather signaling-induced translocation of the enzyme to the membrane/particulate fraction represents the most reliable means of monitoring kinase activation. Reversal of signaling can occur by metabolism of DAG by DAG kinase and release of PKCs from the membrane, as well as by agonist-induced enzyme degradation or removal of priming phosphorylation with subsequent rapid degradation (Leontieva and Black, 2004; Newton, 2010). In addition to activation by growth factor signaling, classical and novel PKCs can be stimulated by a number of pharmacological agents that mimic the effects of DAG, such as phorbol esters and macrocyclic lactone bryostatins. However, in contrast to DAG, these agonists, which include phorbol 12-myristate 13-acetate [PMA; also known as 12-*O*-tetradecanoylphorbol-13-acetate (TPA)], phorbol 12,13-dibutyrate (PDBu), and bryostatin 1, are not rapidly metabolized and thus give a more sustained PKC activation.

Despite limitations related to their lack of specificity for individual PKC isozymes, their ability to promote PKC down-regulation, and the existence of additional targets for these agents (Griner and Kazanietz, 2007), use of pharmacological agonists and membrane permeant DAG analogs has provided significant insight into the downstream effects of PKC activation. However, a complete understanding of PKC signaling will require defining the specific function(s) of individual PKC isozymes, and progress toward this goal has proved technically difficult. Understanding of the functions of atypical PKCs, PKCζ, and PKCι, lags behind that of other members of the PKC family, perhaps largely due to their insensitivity to pharmacological activators (e.g., phorbol esters and bryostatins) and synthetic DAGs. In the absence of isozyme-specific pharmacological PKC agonists and inhibitors, early studies relied on overexpression strategies to decipher the roles of individual isozymes, which can result in non-physiological levels of expression, activity, and regulation. RNA interference technology and genetically altered mice are helping to circumvent these problems, but are not without drawbacks of their own. Potential limitations include the need for a high level of silencing to sufficiently deplete enzyme activity (e.g., >80%, Cameron et al., 2008; M. A. Pysz, A. R. Black, and J. D. Black, unpublished results), and the fact that knockdown of one PKC isozyme can affect accumulation of other members of the family (M. A. Pysz, A. R. Black, and J. D. Black, unpublished data). Overlapping roles of different isozymes means that multiple crosses of transgenic mice may be needed to observe phenotypes.

An additional source of confusion regarding the functions of individual PKC isozymes is the fact that many so-called PKC inhibitors are of questionable specificity (Griner and Kazanietz, 2007; Soltoff, 2007). As an example of particular relevance to T cell activation, special caution is needed when considering studies that have used rottlerin to infer effects of signaling from PKCθ. While this agent was originally considered to be a specific inhibitor of novel PKCs, recent studies have demonstrated that it does not inhibit PKCδ (Soltoff, 2007). In keeping with this finding, the IC_{50} for PKCθ inhibition by rottlerin in the presence of 100 μM ATP is >300 μM (Villalba et al., 1999), a concentration far in excess of that used in studies on its cellular effects. In contrast, rottlerin is a potent inhibitor of other kinases such as PRAK and MAPKAP-K2 (Soltoff, 2007); thus, any effects of this inhibitor cannot be ascribed to direct inhibition of PKCθ.

Despite these limitations, our knowledge of the roles of individual PKCs is emerging. Of note, in addition to the proliferative/cell cycle effects which are the subject of this review, PKC isozymes have been found to regulate multiple cellular processes of direct relevance to T cell development and function, including differentiation, migration, survival, apoptosis, endocytosis, and secretion/exocytosis (Reyland, 2009; Rosse et al., 2010).

THE MAMMALIAN CELL CYCLE

Several excellent reviews have been written on the regulation of the cell cycle (Sherr and Roberts, 2004; Cobrinik, 2005; Malumbres and Barbacid, 2005; Du and Pogoriler, 2006; Satyanarayana and Kaldis, 2009) and only a brief description will be given here. The cell cycle has been classically divided into four phases, G1 (or Gap 1 in which cells prepare for DNA synthesis), S phase (in which DNA is synthesized), G2 (in which cells prepare for division) and mitosis (or M phase, in which sister chromatids are separated and the cell divides; **Figure 1**). Transit through the cell cycle is regulated by four major classes of cyclins whose expression is strictly controlled and limited to particular cell cycle phases. Cyclins are the regulatory subunits for cyclin-dependent kinases (cdks), whose activity is absolutely dependent on association with specific cyclin partners. Entry of quiescent cells into the cell cycle and transit through early G1 is regulated by D-type cyclins, which complex with cdk4 and cdk6 (Musgrove et al., 2011). There are three D-type cyclins, D1, D2, and D3, which are expressed to varying degrees in different tissues; cyclins D2 and D3 appear to be the major players in T cells. Transit through late G1 and progression into S phase is regulated by cyclin E complexed with cdk2 (Hwang and Clurman, 2005; Malumbres and Barbacid, 2005). S phase transit and early G2 are regulated by cyclin A/cdk2 and cyclin A/cdk1 complexes, whereas cyclin B, complexed with cdk1, regulates progression into M phase (Malumbres and Barbacid, 2005; Sanchez and Dynlacht, 2005). In addition to being regulated by cyclin binding, the activity of cdks is under the control of Cip/Kip and Ink4 cdk inhibitor proteins (ckis; Sherr and Roberts, 1995). Members of the Cip/Kip family, including $p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$, have a dual activity in cell cycle regulation. They negatively regulate cell cycle progression by binding to cyclin/cdk2 and cyclin/cdk1 complexes and inhibiting their enzymatic activity. Conversely, these proteins can promote progression by enhancing the association of cyclin D with cdk4 and cdk6 without inhibiting the activity of these complexes (Sherr and Roberts, 1999). The Ink4 ckis, which include $p15^{Ink4b}$, $p16^{Ink4a}$, $p18^{Ink4c}$, and $p19^{Ink4d}$, block the activity of cdk4 and cdk6 by preventing their association with cyclin D. Cdk activity is also regulated by phosphorylation: positive phosphorylation is mediated by cdk activating kinase (CAK or cdk7/cyclin H; Fisher and Morgan, 1994), while negative phosphorylation involves the kinases Wee1 and Myt1. Removal of inhibitory phosphorylation, by e.g., Cdc25 phosphatases, is necessary for full cdk activity.

While there are multiple checkpoints that allow cells to undergo cell cycle arrest in response to various stresses, the most relevant to normal tissue homeostasis and differentiation is that which

directs entry and exit from the cell cycle in G1 (Prasad et al., 1994; Liu et al., 2012). The expression of D-type cyclins is acutely regulated by mitogenic signals. As such, these proteins are the main sensors for the growth environment of the cell, and are intimately involved in regulation of the entry of quiescent cells into the cell cycle. Major targets for cyclin D/cdk complexes include the retinoblastoma protein (pRb) and related pocket proteins, p107 and p130 (Cobrinik, 2005). In the hypophosphorylated state, pocket proteins bind to E2F transcription factors on the promoters of growth-related genes, where they act as transcriptional repressors and actively block expression of genes necessary for DNA replication (Trimarchi and Lees, 2002; Du and Pogoriler, 2006; **Figure 1**). Phosphorylation of pocket proteins relieves this repression, allowing for transcription of E2F-dependent genes, one of which is cyclin E. Cyclin E/cdk2 then completes phosphorylation of pocket proteins, leading to their release from E2F and robust transcription of growth-related genes. At early stages of G1, cells require mitogenic signals to support cyclin expression and cdk activity; however, once sufficient levels of cyclin E have accumulated to maintain its own expression, cells have passed the so-called "restriction point" and are able to proceed through to the next cell cycle without further mitogenic input. In the face of loss of mitogenic signals prior to the restriction point or of negative growth signals, cell cycle progression is halted and cells eventually withdraw into G0 phase and quiescence (Grana et al., 1998; Classon and Dyson, 2001).

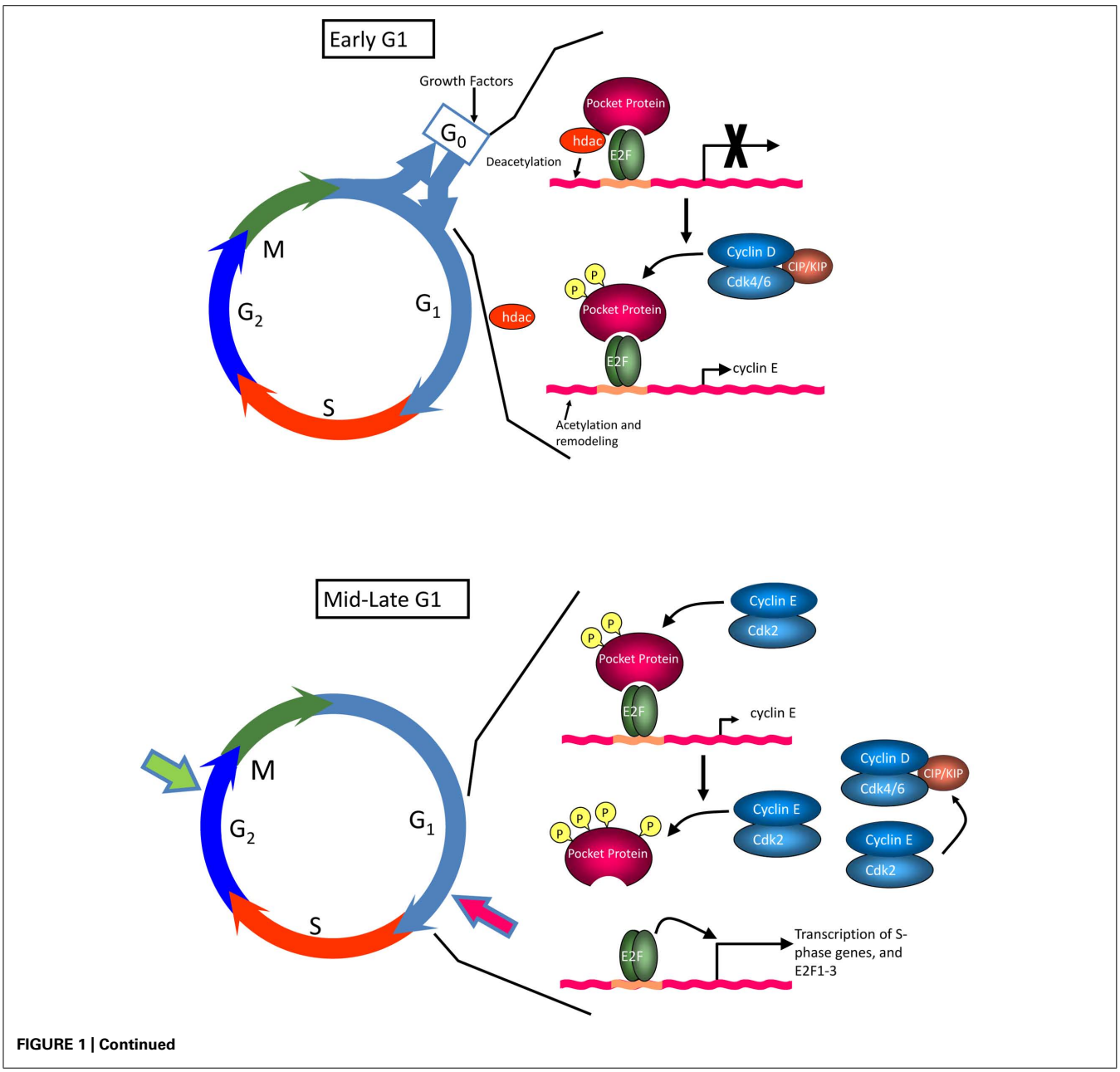

FIGURE 1 | Continued

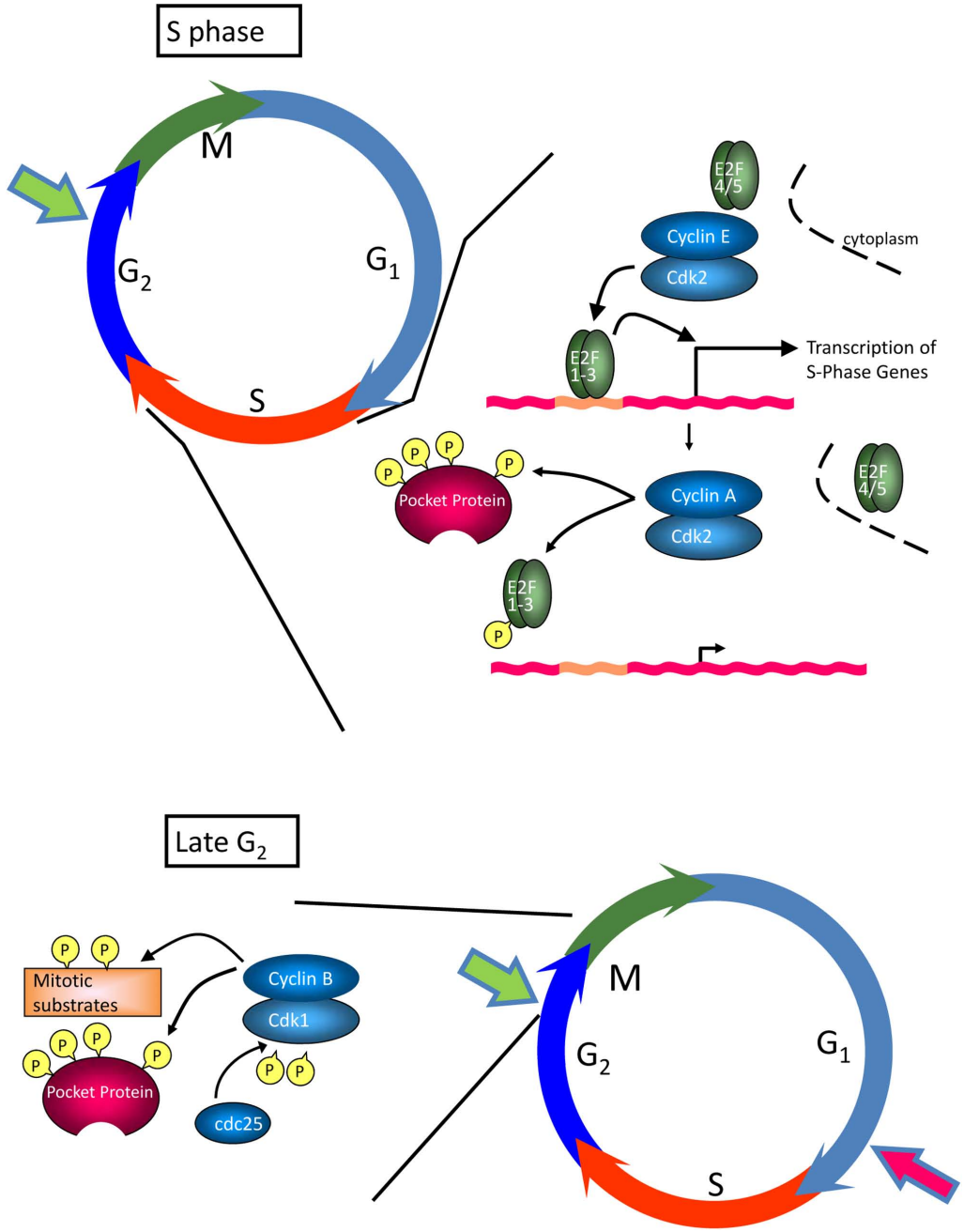

FIGURE 1 | The cell cycle. The cell cycle consists of four phases, G1, S, G2, and M. In early G1, hypophosphorylated pRb binds the E2F transcription factor, and recruits histone deacetylase (HDAC) and other factors to actively repress transcription of E2F-regulated genes important for transition into S phase and DNA replication (e.g., PCNA, topoisomerase I, c-Myc, cyclin E, Cdc25c). Progression through early G1 is dependent on growth factors, which promote expression of D-type cyclins. Formation of cyclin D/cdk4 and cyclin D/cdk6 complexes, which is facilitated by Cip/Kip ckis, leads to phosphorylation of pRb at a subset of available sites and release of HDAC and other inhibitory factors, relieving repression of E2F and promoting upregulation of cyclin E. Cyclin E/cdk2 complexes, relieved from repression by Cip/Kip ckis by sequestration of these inhibitory molecules in cyclin D/cdk complexes, complete pocket protein phosphorylation in mid to late G1, enabling a wave of E2F-dependent transcriptional activity essential for S progression. Together, these events drive cells through the restriction point (large red arrow), which commits cells to the proliferative cycle. If conditions are not optimal to signal this transition, cells exit the cycle and enter G0 or quiescence, a reversible non-replicative state. Once cells enter S phase, cyclin E/cdk2 activity is inhibited by proteasomal degradation of cyclin E in the cytoplasm. Continued inactivation/hyperphosphorylation of pRb allows the transcription of cyclin A and cyclin B, required for subsequent phases of the cell cycle. Cyclin A/cdk2 complexes phosphorylate a number of proteins to facilitate S phase completion and transit into G2/M. Cyclin B is actively synthesized during G2 and associates with cdk1 to trigger mitosis. Cdk1 is maintained in an inactive state by the kinases Wee1 and Myt1. As cells approach M phase, the phosphatase cdc25 is activated to remove inhibitory phosphates on Tyr14 and Thr15, driving the cells into mitosis. A checkpoint in late G2 (large green arrow) prevents cells from entering M phase if the genome is damaged. This DNA damage checkpoint ensures that cells do not initiate mitosis until they have repaired damaged DNA after replication.

PKC SIGNALING AND T CELL PROLIFERATION

T CELL DEVELOPMENT AND TCR SIGNALING

T lymphocytes arise from bone marrow-derived $CD34^+$ stem cells, which seed the thymus and undergo multistage differentiation to become mature circulating cells (for references, see Koch and Radtke, 2011). An early event in this process involves VDJ recombination of the TCR-β chain which then complexes with pre-Tα to form the pre-TCR. Signaling from the pre-TCR leads to proliferation of pre-T cells and rearrangement of the TCR-α chain, which combines with the β chain and CD3 to form the TCR. Further differentiation, accompanied by negative and positive selection, eventually leads to the development of mature naïve T cells, including $CD4^+$ helper T (Th), $CD8^+$ cytotoxic T (Tc), and regulatory T (Treg) cells. These naïve cells exit the thymus and remain dormant as they circulate through secondary lymphoid organs until activated by antigen. These organs, which include the spleen, lymph nodes, and Peyer's patches, transiently house naïve T cells and are the first line of defense against pathogens that traverse the skin or the epithelial lining of the respiratory, gastrointestinal, and urogenital tracts.

Activation of T cells requires interaction of the TCR with major histocompatibility complex (MHC) bound antigen on antigen presenting cells (APCs), such as dendritic cells, macrophages, and B cells (for references, see Marsland and Kopf, 2008; Smith-Garvin et al., 2009; Fooksman et al., 2010; Dustin and Depoil, 2011). The interface between the T cell and APC is marked by the formation of a structure, termed the immune synapse or supramolecular activation cluster (SMAC), which serves to regulate T cell signaling. Productive activation of T cells requires two signals. The first signal is provided by the MHC-bound TCR, while the second signal is provided by co-stimulatory molecules such as CD28 (which binds to B7 proteins on the APC). Additionally, cytokines such as IL-12 and tumor necrosis factor alpha (TNF-α) can provide a third signal that regulates the response to T cell activation. A number of experimental manipulations can activate T cells in the absence of APC interaction; these include crosslinking of the TCR and CD28 with insoluble antibodies and combined treatment of cells with phorbol ester and calcium ionophore.

T cell receptor co-activation leads to the engagement of multiple downstream signaling pathways including those involving phosphatidylinositol 3-kinase (PI-3K), tyrosine kinases such as Lck, and PLCγ (for references, see Altman et al., 2000; Marsland and Kopf, 2008; Smith-Garvin et al., 2009; Fooksman et al., 2010; Dustin and Depoil, 2011). Activation of PLCγ results in production of DAG, which recruits PKCθ to the immune synapse where it interacts indirectly with CD28 through binding to Lck (Kong et al., 2011; Isakov and Altman, 2012). PKCθ then phosphorylates CARMA1, leading to the assembly of the CARMA1–BCL10–MALT1 (CBM) signalosome. PLCγ-generated inositol trisphosphate releases calcium from intracellular stores. The combined action of downstream TCR signaling eventually leads to activation of NF-κB, AP1, and nuclear factor of activated T cells (NFAT) transcription factors (Marsland and Kopf, 2008; Smith-Garvin et al., 2009; Fooksman et al., 2010; Dustin and Depoil, 2011). Together, these events promote functional activation of T cells which is marked by cell proliferation/clonal expansion and cytokine secretion. While the majority of the T cells that arise from activation are eventually cleared from the circulation, a small number develop into memory T cells which are primed for activation upon subsequent antigen exposure.

Antigen-induced proliferation is a key aspect of both T cell differentiation and clonal expansion (Koch and Radtke, 2011). Thus, mechanisms underlying regulation of the T cell cycle machinery are of critical importance to immune function. As the signaling pathways involved in T cell activation are being deciphered, increasing evidence is pointing to the importance of the PKC family in mediating proliferative responses in these cells. The following section outlines our current understanding of the role of individual PKC isozymes in regulating proliferation in T cells within the context of knowledge gained from other systems.

PKCs AND THE CELL CYCLE

As our knowledge of the proliferative roles of the PKC family has developed, it has become increasingly apparent that the effects of these molecules are highly context-dependent. The fact that PKCs are activated by tumor promoting phorbol esters and are downstream of growth factor receptors initially led to the idea that they transduce positive mitogenic signals (Castagna et al., 1982; Kikkawa et al., 1983; Leach et al., 1983). Although a number of early studies supported this idea (Dicker and Rozengurt, 1978; Rozengurt, 1986; Takuwa et al., 1988), it soon became clear that PKCs can negatively and positively regulate cell cycle progression. Indeed, regulation of proliferation by the PKC enzyme system exhibits a high degree of complexity, with effects involving multiple cell cycle regulatory molecules, including cyclins, cdks, and ckis, and impacting various stages of the cell cycle (Black, 2010). Furthermore, individual isozymes can have opposing effects on cell cycle progression in different cell types and even within the same cell type, depending on the signaling environment. A single isozyme can target different cell cycle molecules in different cell types, can have opposite effects on a specific cell cycle target in different systems, and can modulate the same target to produce divergent cell cycle responses (for review, see Black, 2010). Thus, to gain a true understanding of the role of PKCs in regulation of proliferation in any given system, it is important to study the mechanisms by which individual isozymes affect specific cell cycle molecules in that system.

T lymphocytes express all members of the PKC family with the exception of PKCγ (Koretzky et al., 1989; Chen et al., 1994; Thuille et al., 2006). A role for PKC isozymes in cell cycle regulation in $CD3^+$ T lymphocytes was suggested by the early recognition that phorbol esters, in conjunction with calcium ionophore, are potent mitogens for these cells (Altman et al., 1990). While studies have concentrated largely on the role of PKCθ in mediating signaling from the immune synapse, a role for other PKC isozymes is emerging. Notably, different isozymes can have pro-proliferative and/or anti-proliferative functions, arguing that, as in other cell types, PKC signaling can regulate entry into the cell cycle, transit through the various cell cycle phases, as well as cell cycle withdrawal in T cells. The following sections discuss current understanding of the growth regulatory functions of individual PKC family members, followed by a summary of the limited information available on cell cycle-specific effects of these isozymes in T cells.

PROLIFERATIVE EFFECTS OF INDIVIDUAL PKC FAMILY MEMBERS

PKCα

Use of selective pharmacological inhibitors, antisense technology, or siRNA has identified an anti-proliferative and differentiation-inducing role of PKCα in multiple cell types, e.g., intestinal epithelial cells, keratinocytes, mammary epithelial cells, and melanoma cells (Black, 2000, 2010). Anti-proliferative effects of PKCα affecting G1 → S transit include downregulation of cyclin D1 (Detjen et al., 2000; Hizli et al., 2006; Guan et al., 2007), as well as induction of $p21^{Cip1}$ (Frey et al., 1997, 2000; Abraham et al., 1998; Slosberg et al., 1999; Black, 2000; Detjen et al., 2000; Tibudan et al., 2002; Clark et al., 2004; Matsumoto et al., 2006) and $p27^{Kip1}$ (Frey et al., 1997, 2000; Detjen et al., 2000; Tibudan et al., 2002). Induction of $p21^{Cip1}$ is also involved in the ability of this isozyme to delay S phase transit and induce G2/M arrest (Frey et al., 1997; Oliva et al., 2008). Our analysis in intestinal epithelial cells indicated that downregulation of cyclin D1 represents one of the earliest effects of PKCα signaling (Frey et al., 2004; Hizli et al., 2006): PKCα-induced loss of cyclin D1 results from translational and transcriptional inhibition, mediated by activation of the translational repressor 4E-BP1 and downregulation of the Id family of transcription factors, respectively (Clark et al., 2004; Hizli et al., 2006; Guan et al., 2007; Hao et al., 2011). Suppression of cyclin D1 expression by PKCα can involve different intermediate signaling events, including activation of the ERK/MAPK pathway (Clark et al., 2004; Hizli et al., 2006; Guan et al., 2007; Hao et al., 2011) and RORα-mediated suppression of Wnt/β-catenin signaling (Bird et al., 1998). Consistent with a role of PKCα in growth inhibition, activation/membrane association of this isozyme is detected in post-mitotic cells in the intestinal epithelium (Saxon et al., 1994; Frey et al., 2000) and epidermis (Tibudan et al., 2002) *in vivo*. Furthermore, PKCα knockout mice show increased proliferative activity within intestinal crypts, and the tumor suppressive activity of this isozyme in the intestine has been linked directly to its effects on the cell cycle machinery (Oster and Leitges, 2006; Pysz et al., 2009).

Growth-stimulatory effects of PKCα have been reported in glioma cells, osteoblasts, chick embryo hepatocytes, hepatocellular carcinoma cells, and myoblasts, among others (Black, 2000, 2010). Proliferative effects of PKCα on the cell cycle machinery include increased levels of cyclin D1 and cdk4, and enhanced cyclin/cdk2 complex activity (Zhou et al., 2002; Alisi et al., 2004; Wu et al., 2008; Lovatt and Bijlmakers, 2010). PKCα can also elicit a $p21^{Cip1}$-dependent enhancement of proliferation as seen in glioma cells (Besson and Yong, 2000). The ability of PKCα to promote proliferation has been linked to signaling through the ERK/MAPK pathway (Schonwasser et al., 1998; Shatos et al., 2008).

Consistent with the cell cycle effects of PKCα described above, this isozyme is targeted by various physiological stimuli that elicit changes in proliferation (Bird et al., 1998; Black, 2000, 2010). Interestingly, PKCα can mediate opposing cell cycle-specific effects of these agents depending on context. For example, PKCα appears to mediate both proliferative (Buitrago et al., 2003) and growth-inhibitory (Chen et al., 1999; Bikle et al., 2001) effects of vitamin D in different systems. This dichotomy has even been observed in cells of the same tissue origin: decreased PKCα expression mediates all-*trans* retinoic acid (ATRA)-induced inhibition of G1 → S progression in SKRB-3 breast cancer cells (Nakagawa et al., 2003), whereas PKCα is required for ATRA-induced growth arrest in T-47D breast cancer cells (Cho et al., 1997).

A role for PKCα in positive regulation of proliferation in T cells was suggested by the finding that, unlike wild-type cells, T lymphocytes from transgenic mice overexpressing PKCα were able to proliferate in response to soluble anti-CD3 antibody (Iwamoto et al., 1992). This role was confirmed by studies of PKCα knockout mice: while PKCα was not required for differentiation of $CD4^+$ and $CD8^+$ cells or activation-induced IL-2 production, $PKC\alpha^{-/-}$ T cells showed severe defects in TCR-induced proliferation and IFN-γ production (Pfeifhofer et al., 2006). These effects were specific to T cells since B cell proliferation was unaffected (Pfeifhofer et al., 2006; Gruber et al., 2009).

Interestingly, PKCα and PKCθ cooperate in regulation of T cell proliferation: while $PKC\alpha^{-/-}$ and $PKC\theta^{-/-}$ showed only a mild activation defect in a graft-versus-host model, double PKCα/PKCθ knockout mice had a severe defect in alloreactive T cell proliferation (Gruber et al., 2009). This effect is of direct physiological relevance since the double knockout mice had significantly improved transplant survival compared with single knockout and control animals (Gruber et al., 2009). These studies further indicated that the cooperative effects of PKCα and PKCθ are due to a combinatorial effect on NFAT activation. A role for this pathway in effects of PKCα is also supported by the fact that constitutively active PKCα can activate NFAT (and AP1) in T cells (Genot et al., 1995). While these studies indicate that PKCα and PKCθ have overlapping functions in regulation of the alloimmune response and NFAT activation, these isozymes clearly have non-redundant functions in T cells. $PKC\alpha^{-/-}$ mice show a defect in Th1-dependent IgG2a/b switching, indicating that PKCα is particularly important in Th1 cells (Pfeifhofer et al., 2006), a role which contrasts with the more prominent function of PKCθ in Th2 function (Salek-Ardakani et al., 2004). These non-redundant actions of PKCα may reflect its recently identified role in phosphorylation of Akt on serine 473 in T cells (Yang et al., 2010). The relevance of this phosphorylation is supported by the finding that Akt links mTORC2 to Th1 cells whereas PKCθ regulates mTORC2-mediated Th2 differentiation (Lee et al., 2010).

PKCβ

The two major splice variants of the PKCβ gene (*prkcb*), PKCβI and PKCβII, have different functions; however, the fact that early studies did not always differentiate between these forms, and knockdown and knockout strategies can affect both isoforms, has complicated interpretation of their individual roles.

The cell cycle-specific effects of PKCβII, which have been noted in both G1 and G2/M phases, appear to be largely stimulatory (Black, 2010). Effects in G1 have been ascribed to the ability of PKCβII to enhance transcription of cyclin D1 (Li and Weinstein, 2006), promote pRb phosphorylation (Suzuma et al., 2002), or to stimulate CAK activity through direct phosphorylation (Acevedo-Duncan et al., 2002). Studies by Fields and colleagues have established that phosphorylation of lamins contributes to the effects of PKCβII on G2 → M transition (Goss et al., 1994; Walker et al., 1995; Thompson and Fields, 1996; Murray and Fields, 1998), while studies by Newton and colleagues (Chen et al., 2004)

have also determined that PKCβII can affect M phase by regulation of cytokinesis through interaction with pericentrin. However, PKCβII can also inhibit proliferation and induce differentiation in some cell types, with induction of $p21^{Cip1}$ and loss of Cdc25 potentially mediating this activity (Yoshida et al., 2003; Cejas et al., 2005). The PKCβI splice variant has been implicated in positive and negative regulation of proliferation in fibroblasts and colon cancer cells, respectively (Housey et al., 1988; Choi et al., 1990; Sauma et al., 1996); however, these findings relied exclusively on overexpression and further work will be required to determine the specific involvement of the PKCβI isozyme in these effects.

A number of studies indicate that PKCβI and/or PKCβII are involved in regulation of T cell proliferation. For example, antisense-mediated knockdown has implicated PKCβ isozyme(s) in IL-2 signaling (Gomez et al., 1995). Furthermore, PKCβ forms are likely involved in cytoskeletal changes following T cell activation. PKCβII localizes to a cytoskeletal aggregate that forms in close proximity to the microtubule organizing center following T cell activation (Black et al., 1988; Gregorio et al., 1992, 1994) and PKCβI has been shown to associate with microtubules in T cells and to play a role in T cell polarization (Volkov et al., 2001). Since cytoskeletal changes appear to be an important aspect of T cell activation (Repasky and Black, 1996; Martín-Cófreces et al., 2008; Alarcón et al., 2011), these observations are likely to be relevant to T cell signaling. This idea is supported by the finding that antisense-mediated knockdown of PKCβI reduced nuclear translocation of NFAT in TCR/CD28-stimulated Jurkat T lymphoma cells (Dreikhausen et al., 2003). However, PKCβ isozymes do not have an essential role in T cell function since $PKC\beta^{-/-}$ mice have no appreciable T cell-related defects. This contrasts with a critical role for PKCβ in B cell receptor signaling (Thuille et al., 2006) and in dendritic cell differentiation (Farren et al., 2010). Thus, any role of PKCβI/II association with cytoskeletal elements is likely to be redundant. In this regard, it is noteworthy that T cell activation leads to translocation of PKCα and PKCθ to the same PKCβII-associated cytoskeletal aggregate described above (J. D. Black and E. A. Repasky, unpublished data; Wang et al., 1999).

PKCδ

PKCδ broadly inhibits cell cycle progression in G1 in response to pharmacological agonists and physiological activators such as ATRA, inositol hexaphosphate (IP6), interferons, and testosterone (Watanabe et al., 1992; Fukumoto et al., 1997; Ashton et al., 1999; Uddin et al., 2002; Kambhampati et al., 2003; Nakagawa et al., 2005; Vucenik et al., 2005; Cerda et al., 2006; Bowles et al., 2007). Effects on G1 → S phase progression are mediated by direct or indirect targeting of cyclin D1, cyclin E, cyclin A, $p21^{Cip1}$, and/or $p27^{Kip1}$ (Fukumoto et al., 1997; Vrana et al., 1998; Ashton et al., 1999; Nakagawa et al., 2005; Cerda et al., 2006; Afrasiabi et al., 2008). Cyclin D1 expression is downregulated by PKCδ in colon cancer cells (Cerda et al., 2006; Pysz et al., 2009), as well as in PKCδ overexpressing vascular smooth muscle cells (Fukumoto et al., 1997), primary bovine airway smooth muscle cells (Page et al., 2002), and NIH3T3 cells (Soh and Weinstein, 2003). Consistent with these findings, loss of PKCδ activity resulted in increased levels of cyclin D1 in colon cancer cells (Cerda et al., 2006) and bovine airway smooth muscle cells (Page et al., 2002). PKCδ has also been shown to inhibit mitosis in CHO cells and 3Y1 murine fibroblasts (Watanabe et al., 1992; Kitamura et al., 2003).

Although the majority of studies have detected a growth-inhibitory role for PKCδ, it can also act as a positive regulator of the cell cycle (Kitamura et al., 2003; Cho et al., 2004; Jackson and Foster, 2004; Czifra et al., 2006). PKCδ can enhance G1 → S transit through increased expression of cyclin D1, cyclin E, cyclin A, and/or cdk2 (Kitamura et al., 2003; Santiago-Walker et al., 2005; Grossoni et al., 2007), destabilization of $p21^{Cip1}$ (Santiago-Walker et al., 2005; Walker et al., 2006), reduced nuclear localization of $p21^{Cip1}$ (Sipeki et al., 2002; Ranta et al., 2011), and increased E2F promoter activity (Nakaigawa et al., 1996). In many cases, these effects are mediated by the ERK/MAPK pathway (Jackson and Foster, 2004; Grossoni et al., 2007). The opposing effects of PKCδ on cell cycle progression may be regulated by differential phosphorylation on Tyr155 (Acs et al., 2000; Steinberg, 2004).

T cells from PKCδ knockout mice are hyperproliferative and produce more IL-2 cytokine upon stimulation in response to allogeneic MHC. Thus, consistent with a predominant growth-inhibitory role of PKCδ in other systems, this isozyme appears to negatively regulate T cell proliferation, an effect that has been ascribed to attenuation of TCR/CD3-mediated signaling (Gruber et al., 2005a). A similar negative effect of PKCδ on proliferation is also seen in B cells (Miyamoto et al., 2002).

PKCε

PKCε generally mediates pro-proliferative responses, and its effects appear to be predominantly in G1/S rather than G2/M (Graham et al., 2000; Balciunaite and Kazlauskas, 2001). The enzyme has been implicated in mediating PDGF-induced G0/G1 → S progression (Balciunaite and Kazlauskas, 2001). Loss of PKCε activity in NSCLC cells is associated with induction of $p21^{Cip1}$, prolonged G1 → S transition in response to serum, and reduced activation of cdk2 complexes (Bae et al., 2007), indicating that this isozyme suppresses $p21^{Cip1}$ accumulation to facilitate cell cycle progression. PKCε can also induce cyclin D1 transcription and upregulate cyclin D1 and cyclin E protein (Soh and Weinstein, 2003; F. Hao, M. A Pysz, A. R. Black, and J. D. Black, unpublished data). Although PKCε is generally downregulated during differentiation (e.g., Yang et al., 2003), the enzyme promotes adipogenic commitment and is essential for terminal differentiation of 3T3-F442A preadipocytes (Webb et al., 2003). Its expression is also enhanced during myogenic differentiation, resulting in upregulation of cyclin D3 (Gaboardi et al., 2010).

The ability of constitutively active PKCε to activate NFAT and AP1 in Jurkat T lymphoma cells points to a role for this isozyme in T cell activation (Genot et al., 1995). Antisense-mediated knockdown has also implicated this isozyme in IL-2 signaling in T cells (Gomez et al., 1995). Furthermore, siRNA-mediated knockdown of PKCε in $CD4^+$ T cells severely reduced proliferation *in vitro* and enhanced the growth-inhibitory effects of transforming growth factor beta (TGF-β; Mirandola et al., 2011). These findings support a predominantly growth-stimulatory role of PKCε in T cells, as seen in other systems (see above). However, $PKC\varepsilon^{-/-}$ mice show no defects in T cell differentiation, proliferation or activation,

indicating that the functions of this isotype may be in large part redundant, at least in the mouse (Gruber et al., 2005b). In contrast to this finding, analysis of Hashimoto thyroiditis patients points to a potential clinical relevance for proliferative effects of PKCε in T cells. These patients had significantly higher expression of PKCε in their T cells compared with healthy controls (Mirandola et al., 2011). Furthermore, while Hashimoto thyroiditis-derived T cells had diminished TGF-β responses compared with healthy controls, knockdown of PKCε in these cells restored normal responsiveness to TGF-β (Mirandola et al., 2011).

PKCη

PKCη has been associated with post-mitotic cells in a number of tissues including squamous epithelia (Kashiwagi et al., 2002; Breitkreutz et al., 2007), the epidermis (Breitkreutz et al., 2007), and the intestinal epithelium (Osada et al., 1993). Consistent with this localization, PKCη upregulated $p21^{Cip1}$ and $p27^{Kip1}$, decreased cdk2 kinase activity, and induced growth arrest in NIH3T3 cells and keratinocytes (Livneh et al., 1996; Ishino et al., 1998; Cabodi et al., 2000). However, this isozyme can also enhance proliferation as seen in MCF-7 breast cancer cells, where it upregulated cyclin D and cyclin E levels and promoted a redistribution of $p21^{Cip1}$ and $p27^{Kip1}$ from cdk2 to cdk4 complexes (Fima et al., 2001).

PKCη is recruited to the immune synapse, pointing to involvement of this isozyme in T cell activation (Fu and Gascoigne, 2012). This role was confirmed by the finding that $PKC\eta^{-/-}$ T cells have a defective proliferative response to anti-CD3 stimulation *in vitro* (Fu et al., 2011). A somewhat more severe proliferative defect was also observed in response to antigen presentation both *in vitro* and *in vivo* (Fu et al., 2011). Consistent with a role for PKCη in mediating TCR signaling, activated $PKC\eta^{-/-}$ T cells showed a reduction in calcium flux and NF-κB translocation (Fu et al., 2011). While these effects are largely redundant with PKCθ, specific effects of PKCη were seen in T cell homeostatic proliferation, which involves self-antigen recognition and IL-7 and IL-15 signaling (Fu and Gascoigne, 2012). Notably, no defect in homeostatic proliferation was seen in $PKC\theta^{-/-}$ mice, indicating that this effect is largely specific to PKCη, although double knockouts did have a somewhat more severe phenotype.

PKCθ

PKCθ has been implicated as a positive regulator of proliferation in a number of cell types including gastrointestinal stromal tumor cells and breast cancer cells, where it represses expression of $p21^{Cip1}$ and/or $p27^{Kip1}$ (Belguise and Sonenshein, 2007; Ou et al., 2008), and in capillary endothelial cells, where it promotes G2/M progression (Tang et al., 1997).

A large body of evidence has emerged to support a critical role for PKCθ in T cell activation. The functions of this isozyme are the subject of several excellent reviews in this issue (e.g., Freeley and Long, 2012; Isakov and Altman, 2012; Wang et al., 2012) and will only be discussed briefly here. While PKCθ is dispensable for differentiation of $CD4^+$ and $CD8^+$ T cells, it is intimately involved in T cell activation and transduces pro-proliferative signals in multiple pathways, including those triggered by the TCR, CD28, and TNF-α (Altman et al., 2000; So and Croft, 2012). As mentioned above, PKCθ is recruited to the immune synapse early in T cell activation, where it is required for formation of the CBM complex, which plays a central role in mediating downstream signaling during T cell activation (Rawlings et al., 2006). In keeping with this role, PKCθ signaling activates a number of transcription factors that regulate T cell activation and proliferation, including Ap1, NF-κB, and NFAT (Pfeifhofer et al., 2003). Studies using *prkcq* knockout mice have determined that PKCθ plays a central role in mediating proliferative responses during T cell activation. PKCθ-deficient T cells lose the ability to proliferate in response to TCR/CD28 activation *in vitro* (Sun et al., 2000; Pfeifhofer et al., 2003). A role for PKCθ in T cell expansion *in vivo* was also apparent from the defective proliferation seen in $PKC\theta^{-/-}$ mice during allergic asthmatic reactions and in response to bacterial infection (Salek-Ardakani et al., 2004; Sakowicz-Burkiewicz et al., 2008).

As seen with PKC isozymes in other cell types, the action of PKCθ in proliferation appears to be highly context-dependent. For example, while a clear role for this isozyme in regulation of Th2 cell proliferation *in vivo* is seen in the allergic asthmatic response, this was not the case for Th1 cells (Salek-Ardakani et al., 2004). Furthermore, PKCθ-deficiency does not affect T cell proliferation in response to viral infection (Giannoni et al., 2005) and can mediate growth-inhibitory effects of cytokine withdrawal (Li et al., 2006b). Notably, while PKCθ generally plays a positive role in proliferation of effector T cells, it has the opposite effect in Treg cells, where it is sequestered from the immune synapse and promotes growth inhibition (Zanin-Zhorov et al., 2010).

A recent study has given insight into possible explanations for divergent functions of PKCθ (Kong et al., 2011). PKCδ and PKCθ are highly homologous; yet, as noted above, PKCδ is growth inhibitory in T cells. In keeping with these differences, PKCδ is not targeted to the immune synapse, disrupts signalosome assembly and cannot substitute for PKCθ in T cell function. These differences are due to a proline-rich motif in the V3 region of PKCθ that mediates indirect interaction with CD28 through Lck. Mutation of this sequence blocks localization of PKCθ to the immune synapse; conversely, a PKCδ mutant containing this sequence was targeted to the immune synapse and could substitute for PKCθ in T cell signaling (Kong et al., 2011; Isakov and Altman, 2012). These findings point to the importance of alterations in protein–protein interactions and localization in dictating the effects of PKC signaling, and offer a mechanism for the divergent roles of PKC isozymes in different cell types and in different signaling environments.

Atypical PKC isozymes

While analysis of the functions of atypical PKCs is less advanced than that of other PKC isotypes, PKCι and PKCζ generally appear to promote cell cycle progression. Consistent with a cell cycle stimulatory role of PKCζ, keratin-induced blockade of HaCaT cell cycle progression involved inhibition of PKCζ activity, a reduction in cyclin D1 and cyclin E levels, and pRb hypophosphorylation (Paramio et al., 2001). PKCζ can mediate transcriptional activation of cyclin D1 downstream of Ras (Kampfer et al., 2001), and can induce phosphorylation and proteasome-dependent degradation of $p21^{Cip1}$ downstream of PI-3K (Scott et al., 2002). The ability of PKCζ to modulate the subcellular distribution of $p27^{Kip1}$ during

cell cycle reentry of quiescent MCF7 cells is also downstream of PI-3K (Castoria et al., 2004). PKCζ may also enhance cdc25 activity to promote G2/M transit in A549 lung epithelial cells, an effect associated with changes in cdk2 activity (Lee et al., 2011; Kang et al., 2012). Exciting studies by Murray, Fields and colleagues have recently identified PKCι as an oncogene which is required for the transformed growth of various human cancer cell types (Fields and Regala, 2007; Murray et al., 2011). Consistent with these findings, PKCι is upstream of PKCζ in Ras-related upregulation of cyclin D1 (Kampfer et al., 2001). PKCι also phosphorylates and activates CAK in response to PI-3K signaling in glioma and neuroblastoma cells (Acevedo-Duncan et al., 2002; Pillai et al., 2011; Desai et al., 2012) and may target cyclin E in ovarian cancer (Eder et al., 2005).

In contrast to PKCα and PKCε, constitutively active PKCζ had no effect on AP1 and NFAT in Jurkat cells (Genot et al., 1995). However, work of Gruber et al. (2008) points to a role for atypical PKCs in PKCθ-mediated pro-proliferative signaling in T cells. These studies found that PKCζ physically interacts with PKCθ in a yeast two-hybrid screen and that PKCζ is a substrate for PKCθ. This physical interaction likely occurs *in vivo* since PKCζ and PKCι are constitutively localized in lipid rafts to which PKCθ is recruited following activation of primary T cells and Jurkat cells. Use of dominant negative mutant proteins further implicated the atypical isozymes in NF-κB induction by PKCθ. In keeping with their common localization and structure, it appears that PKCι and PKCζ can substitute for each other in most T cell functions. Nonetheless, PKCζ function appears to be particularly important for activation of Th2 cells (Martin et al., 2005): while PKCζ knockout did not result in proliferative or signaling defects in naïve T cells, it dramatically inhibited activation of Th2 cells. This effect was reflected in disruption of STAT6, NFAT, and NF-κB activation following stimulation with anti-CD3. The dramatic upregulation of PKCζ noted during Th2 cell differentiation may account for the inability of PKCι to compensate for loss of PKCζ in these cells (Martin et al., 2005; Gruber et al., 2008). The physiological relevance of PKCζ signaling in Th2 cells is seen in the impaired allergic asthmatic response in PKCζ$^{-/-}$ mice (Martin et al., 2005).

Summary and discussion

From the above discussion, it is apparent that PKC signaling plays an important role in regulation of cell proliferation in a broad spectrum of cell types including T cells. PKC activation can either promote or inhibit transit through multiple stages of the cell cycle. The precise effect of PKCs on the cell cycle is highly context-dependent, and is influenced by the specific isozyme involved, the timing and duration of PKC activation, the cell type, and the signaling environment to which the cell is exposed; however, some themes are beginning to emerge. With regard to individual PKC family members, accumulating evidence indicates that PKCα can exert context-dependent inhibitory or stimulatory effects. While PKCδ can have positive effects on cell cycle progression, its effects are generally inhibitory. On the other hand, effects of PKCβII, PKCε, and atypical PKCs appear to be mainly pro-proliferative, while those of PKCη are generally inhibitory.

In T cells, multiple PKC isozymes mediate proliferative signals associated with TCR/CD28 engagement (**Figure 2**). These effects, which directly impact immune function, involve both redundant and non-redundant functions of individual PKC family members, and a high degree of cooperation between different PKC isozymes is becoming apparent. As in other systems, the effects of PKC signaling are highly context-dependent, with the reliance on individual isozymes differing between T cell subtypes. While the majority of the characterized effects of PKC signaling in T cells have been pro-proliferative, negative effects are also seen: PKCδ appears to play a predominantly inhibitory role and PKCθ can have negative proliferative effects dependent on the signaling environment and cell type.

Although effects of PKC signaling have been noted in all stages of the cell cycle, the predominant actions of PKC isozymes are in G1 and G2 phases. Similarly, while PKCs can modulate the activity of multiple cell cycle regulatory molecules, consistent with effects in G1 and G2, D-type cyclins and Cip/Kip cdk inhibitors ($p21^{Cip1}$ and $p27^{Kip1}$) are emerging as important targets of PKC control. In keeping with the involvement of these proteins in regulation of quiescence, accumulating evidence indicates that controlling cell cycle entry and exit is an important role for PKC signaling. The ability of PKCs to promote $G_0 \rightarrow G_1$ progression has been noted in several cell types (Chiu et al., 2002, 2003; Santiago-Walker et al., 2005). PKC signaling has also been shown to promote cell cycle exit in a number of systems, including intestinal epithelial cells, keratinocytes, PKC-overexpressing fibroblasts, and leukemic cell lines (Black, 2000, 2010). Studies in leukemia cells (Zhang and Chellappan, 1996; Vrana et al., 1998; Wang et al., 1998), non-transformed intestinal epithelial cells (Frey et al., 2000), pancreatic cancer cells (Detjen et al., 2000), and keratinocytes (Tibudan et al., 2002) indicate that PKC family members are capable of activating a complete program of cell cycle withdrawal, which can include downregulation of cyclin D1, upregulation of $p21^{Cip1}$ and $p27^{Kip1}$, alterations in the expression and phosphorylation of the pocket proteins p107, pRb, and p130, and changes in E2F expression and complex formation (Zhang and Chellappan, 1996; Saunders et al., 1998). While the ability of PKC signaling to promote exit from quiescence following TCR/CD28 and pre-TCR engagement is established, further studies are required to define its role in promoting cell cycle exit during T cell development and the establishment of quiescent memory T cells.

SPECIFIC CELL CYCLE TARGETS OF PKC SIGNALING IN T CELLS

Antigen-induced proliferation is a key aspect of both T cell differentiation and clonal expansion (Koch and Radtke, 2011). Thus, the mechanisms underlying PKC isozyme-specific effects on the cell cycle machinery in T cells are of critical importance to immune function. As noted above, the cell cycle is tightly regulated by coordinated actions of cyclins, cdks and ckis, which modulate the activity of the retinoblastoma family and thus expression of E2F-dependent genes (**Figure 1**). Proliferative T cell signaling affects multiple members of this control network. For example, proliferation induced by TCR/CD28 costimulation is associated with increased pRb phosphorylation by cyclin D2/3 and cyclin E, and enhanced transcription of E2F-dependent genes such as cyclins E and A (Colombetti et al., 2006). Analysis of mechanisms underlying these changes has pointed to a particularly important role for cyclin D3, cdk6, and $p27^{Kip1}$ in regulation of T

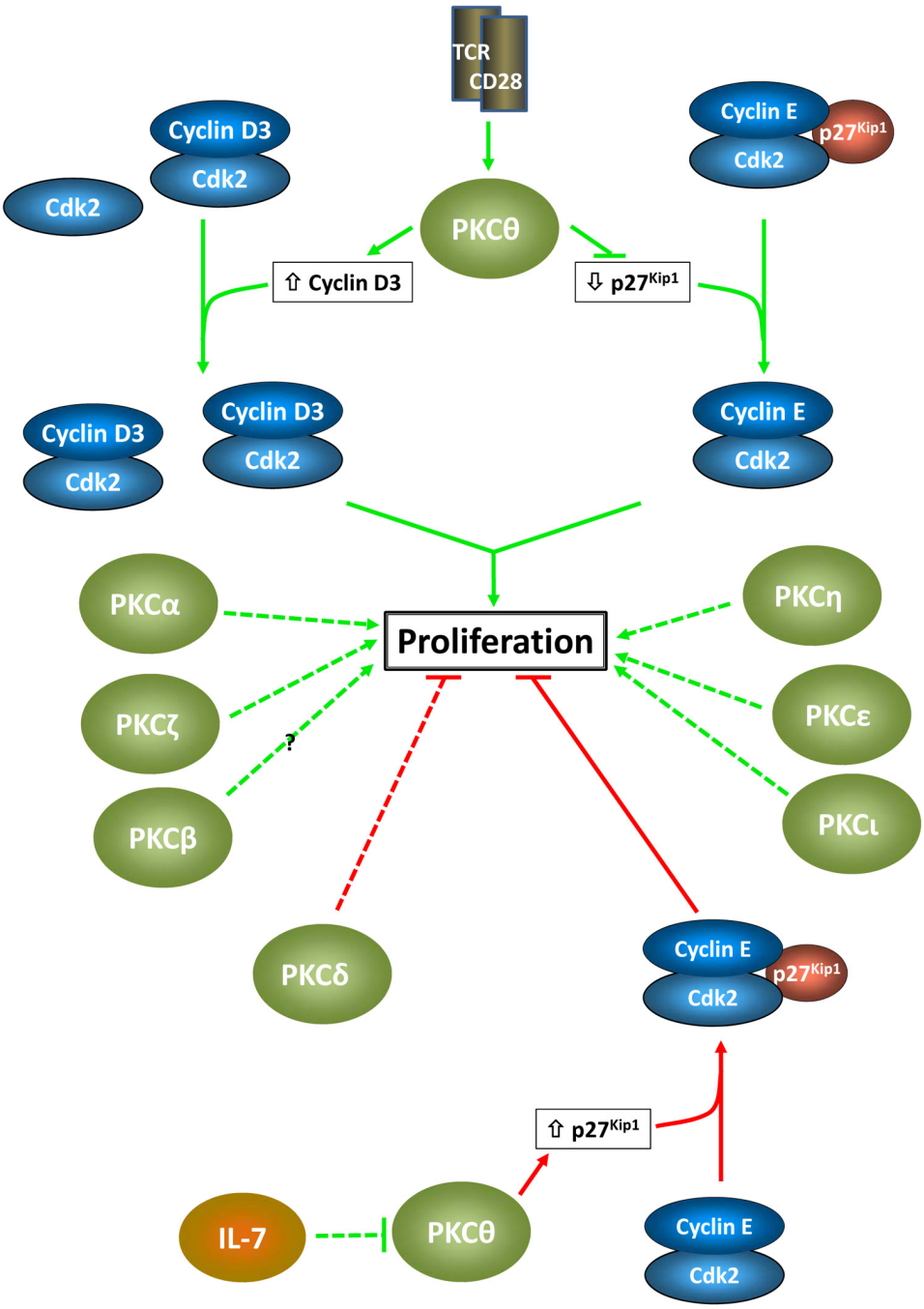

FIGURE 2 | Proliferative effects of PKC isozymes in T cells. Positive and negative proliferative effects of individual PKC isozymes are indicated by green arrows and red barred lines, respectively. Proposed cell cycle targets in the growth-inhibitory and growth-stimulatory effects of PKCθ are shown. These targets reflect the importance of D-type cyclins and Cip/Kip ckis as targets for PKC signaling, as seen in other systems (note that in pre-T cells, cyclin D1 appears to be the target for PKCθ). The dashed lines indicate the lack of knowledge of specific cell cycle targets for other PKC isozymes in T cells.

cell proliferation. For example, cyclin D3 and cdk6 knockout mice show defects in T cell proliferation, whereas cdk4 and cdk2 knockout mice do not (Sicinska et al., 2003; Hu et al., 2009), and $p27^{Kip1}$ null T cells show reduced mitogen requirements and are resistant to anergy (Mohapatra et al., 2001; Rowell et al., 2005; Li et al., 2006a).

While PKC activation mediates TCR signaling to NF-κB, NFAT, and Ap1, transcription factors that have been shown to have a direct role in regulation of the cell cycle machinery in T cells, the function of specific PKCs in these effects remains largely unexplored. However, limited information is emerging to indicate that, as in other cell types, D-type cyclins and Cip/Kip proteins are

important targets of PKC in these cells. In keeping with the greater attention that has been paid to PKCθ, this evidence primarily concerns the effects of this isozyme. For example, saikosaponins inhibit PKCθ translocation and cause a G0/G1 arrest in activated T cells through downregulation of cdk6 and cyclin D3 and upregulation of $p27^{Kip1}$ protein levels (Leung et al., 2005; Sun et al., 2009). A link to $p27^{Kip1}$ is also supported by the finding that PKCθ loss leads to anergy (Deenick et al., 2010), a process that involves upregulation of this cki (Li et al., 2006a; Wells, 2007, 2009). Through its role in assembly of the CBM signalosome, PKCθ has also been implicated in regulation of cyclin E stability in T cells (Srivastava et al., 2012).

Evidence also points to an ability of PKCθ to regulate cyclin D3 and $p27^{Kip1}$ in pre-T cells. These molecules are downstream of the pre-TCR and PKCθ is an important mediator of signaling from this receptor (Felli et al., 2004; Aifantis et al., 2006; Talora et al., 2006). Pre-TCR activation of PKCθ cooperates with Notch3 to induce cyclin D1 in lymphomagenesis, indicating that this cyclin can also be a target for PKCθ in these cells.

Surprisingly, $p27^{Kip1}$ also appears to be involved in PKC-mediated cell cycle arrest following cytokine withdrawal in T cells. IL-7 withdrawal from the D1 thymocyte cell line results in G1 arrest due to upregulation of $p27^{Kip1}$ (Li et al., 2006b). Notably, PKCθ is activated by IL-7 withdrawal in these cells and the upregulation of $p27^{Kip1}$ could be blocked by a general PKC inhibitor. While these studies do not exclude other PKCs, $p27^{Kip1}$ upregulation was not blocked by the classical PKC inhibitor Gö6976, indicating that the effect was mediated by novel or atypical isozyme(s) (Li et al., 2006b).

SIGNALING DOWNSTREAM OF PKC IN REGULATION OF THE CELL CYCLE

While cell cycle-specific effects of PKCs can involve direct phosphorylation of cell cycle regulatory molecules (Goss et al., 1994; Acevedo-Duncan et al., 2002; Scott et al., 2002), the effect of PKCs on the cell cycle is generally indirect and involves downstream signaling cascades. Several signaling pathways, including those involving PI-3K/Akt (e.g., Belguise and Sonenshein, 2007; Bakker et al., 2008; Ou et al., 2008) and Wnt-β-catenin (e.g., Gwak et al., 2009; Murray et al., 2009), have been implicated in PKC proliferative signaling. However, analysis of multiple systems has highlighted the Ras/Raf/MEK/Erk pathway as a particularly important mediator of proliferative effects of PKCs. Most members of the PKC family, including PKCα, PKCβ, PKCλ, PKCδ, PKCε, PKCζ, and PKCθ, can target this pathway in many cell types (Kampfer et al., 2001; Chiles, 2004; Clark et al., 2004; Jackson and Foster, 2004; Koike et al., 2006; Bakker et al., 2008). Activation can occur at multiple steps in the Ras–Raf–MEK–Erk cascade. For example, PKCα can intersect the pathway at the level of Ras (Clark et al., 2004) or downstream of Ras through direct phosphorylation of Raf (Kolch et al., 1993). Erk activation mediates the effects of PKC signaling on several cell cycle regulatory molecules, including D-type cyclins and Cip/Kip ckis (Kampfer et al., 2001; Clark et al., 2004; Koike et al., 2006; Matsumoto et al., 2006; Black, 2010; Ranta et al., 2011). Interestingly, Erk signaling can facilitate both positive and negative effects of PKC on cell cycle targets and cell proliferation, and can mediate divergent effects on individual cell cycle molecules even within a single cell type. For example, our analysis has determined that Erk signaling is required for both PKCα-induced cyclin D1 downregulation and PKCε-induced cyclin D1 upregulation in intestinal epithelial cells (Clark et al., 2004; F. Hao, M. A Pysz, A. R. Black and J. D. Black, unpublished data). Thus, in keeping with the complexity associated with the proliferative consequences of PKC activation in general, the effects mediated by Erk signaling are highly context-dependent. While it has been proposed that the duration of activation dictates the proliferative outcome of Erk signaling (cf. Yasuda and Kurosaki, 2008), the anti-proliferative effects of PKCα and the pro-proliferative effects of PKCε both require prolonged Erk activation, with differences in the localization of activated Erk providing a possible explanation for the divergent effects (Clark et al., 2004).

Erk signaling is important for pre-T cell and T cell proliferation (Yasuda and Kurosaki, 2008), pointing to the possible role of a PKC–Erk signaling axis in these cells. It has been proposed that Sos and RasGRP1 cooperate to regulate the sensitivity, duration, and amplitude of Erk signaling in T cells (Yasuda and Kurosaki, 2008). Although analysis of the roles of PKC isozymes in Erk activation in this system is complicated by the fact that RasGRP1 is also a DAG/phorbol ester activated protein (Yasuda and Kurosaki, 2008), siRNA-based analysis has led to the suggestion that PKC may mediate RasGRP1-independent Erk activation in T lymphocytes (Warnecke et al., 2012). This idea opens the possibility that the proliferative response in T cells may be regulated by the coordinated effects of PKC isozymes, Sos-GRB2 and RasGRP1 on Erk activation.

SUMMARY AND PERSPECTIVES

Although understanding of the impact of PKC signaling on the cell cycle machinery in T cells remains limited, several similarities with other cell types are beginning to emerge (**Figure 2**). As in other cell types, D-type cyclins and Cip/Kip ckis appear to be major targets of PKC signaling in T cells, pointing to effects in G1 and G2. To date, the majority of findings have indicated positive effects of PKCs on cell cycle progression in T cells. However, it should be noted that this may largely reflect a focus on the consequences of T cell activation, which would bias findings in that direction. Evidence for anti-proliferative effects of PKC signaling is indeed accumulating, with PKCδ emerging as a negative regulator. Further analysis is required to identify cell cycle targets which mediate these inhibitory effects. The context-dependence of PKC isozyme-mediated cell cycle regulation observed in other systems has also been noted in T cells, exemplified by the ability of PKCθ to both promote and inhibit T cell proliferation/cell cycle progression. Despite these advances, it is clear that understanding of the cell cycle-specific effects of individual PKC isozymes in T cells is still in its infancy. In addition to delineation of the cell cycle roles of individual PKC isozymes and identification of specific cell cycle targets, issues that remain to be addressed include (a) how the different signaling environments in T cell subsets affect PKC cell cycle signaling, (b) whether PKC signaling plays a role in maintenance of quiescence in T cells and in control of quiescence-related regulators such as FOXO and Krüppel-like transcription factors (Black et al., 2001; Wu and Lingrel, 2004; Vucenik et al., 2005; Hart et al., 2012; Warnecke et al., 2012), and (c) what mechanisms underlie the differential

involvement of individual PKCs in T cell proliferation *in vitro* and *in vivo*. Given the emerging importance of mTOR in immune function (Powell et al., 2012), an area of particular interest is the interplay between PKC and mTOR signaling in control of T cell proliferation under the metabolic conditions in which activation occurs *in vivo*. Other areas that remain to be addressed are the relative contribution of direct activation by TCR/CD28 and of activation by secreted cytokines to PKC-mediated proliferative responses, as well as the role of cell survival in the proliferative effects of PKC manipulation, especially *in vivo*. With increasing knowledge of TCR and cytokine signaling and the availability of mouse models for analysis of PKC isozyme function *in vivo*, it is anticipated that a link between PKC and growth-inhibitory signaling in T cells will be confirmed, and that the molecular details underlying the effects of individual PKC isozymes on the cell cycle in T cell subsets will be elucidated in the near future.

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Microtubule-organizing center polarity and the immunological synapse: protein kinase C and beyond

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Cytoskeletal polarization is crucial for many aspects of immune function, ranging from neutrophil migration to the sampling of gut flora by intestinal dendritic cells. It also plays a key role during lymphocyte cell–cell interactions, the most conspicuous of which is perhaps the immunological synapse (IS) formed between a T cell and an antigen-presenting cell (APC). IS formation is associated with the reorientation of the T cell's microtubule-organizing center (MTOC) to a position just beneath the cell–cell interface. This cytoskeletal remodeling event aligns secretory organelles inside the T cell with the IS, enabling the directional release of cytokines and cytolytic factors toward the APC. MTOC polarization is therefore crucial for maintaining the specificity of a T cell's secretory and cytotoxic responses. It has been known for some time that T cell receptor (TCR) stimulation activates the MTOC polarization response. It has been difficult, however, to identify the machinery that couples early TCR signaling to cytoskeletal remodeling. Over the past few years, considerable progress has been made in this area. This review will present an overview of recent advances, touching on both the mechanisms that drive MTOC polarization and the effector responses that require it. Particular attention will be paid to both novel and atypical members of the protein kinase C family, which are now known to play important roles in both the establishment and the maintenance of the polarized state.

Keywords: cell polarity, cytoskeleton, lymphocyte, protein kinase C, signal transduction, T cell

Lymphocytes can completely alter their cellular architecture in a matter of minutes in response to cell surface stimulation. This enables them to adapt quickly to multiple disparate tissue environments, which is crucial for effective migration between and within different organ systems. Structural plasticity also plays a key role in promoting and specifying interactions between lymphocytes and other cells. Particularly important among lymphocyte interactions is the immunological synapse (IS), a stereotyped cell–cell contact characterized by the organization of cell surface receptors, adhesion molecules, and signaling proteins into well-defined concentric domains (Dustin et al., 2010). Although the IS was first observed in conjugates between T cells and antigen-presenting cells (APCs), it is clear that natural killer (NK) cells and B cells use similar structures to engage target cells and cells coated with surface-bound antigen, respectively (Dustin and Long, 2010; Harwood and Batista, 2010).

Immunological synapse formation is accompanied by a dramatic change in cell shape. This transformation has been studied most extensively in T cells, which form synapses in response to antigen recognition by the T cell receptor (TCR). T cells search their environment for antigen in a crawling, "hand mirror" configuration that consists of a leading edge followed by a trailing stalk-like projection known as a uropod (Ward and Marelli-Berg, 2009; **Figure 1**). TCR stimulation transforms the leading edge into a radially symmetric lamellipodium that spreads over the surface of the APC, and concomitantly induces collapse of the uropod (Dustin et al., 2010). Within minutes, the extended crawling morphology of the T cell transforms into a more compact shape akin to a sideways cup with its mouth positioned at the IS (Dustin et al., 2010; **Figure 1**).

This structural transformation is associated with and facilitated by extensive remodeling of both the actin and microtubule cytoskeletons (Gomez and Billadeau, 2008; Dustin et al., 2010). Actin polymerization drives the radial growth of the synapse, and plays an important role in stabilizing adhesive contacts and other receptor–ligand interactions. The microtubule cytoskeleton, for its part, rotates so as to position the microtubule-organizing center (MTOC, also called the centrosome) just beneath the IS (**Figure 1**). The MTOC carries along with it the Golgi apparatus and other vesicular compartments. Hence, its polarization to the IS aligns much of the cellular machinery involved in protein trafficking with the APC. This enables the directional secretion of proteins and other cargo toward the APC, which is thought to be crucial for maintaining the specificity of T cell responses (Huse et al., 2008). T cells operate in a dense intercellular milieu packed with healthy bystander cells, and yet paradoxically they use secreted factors for a sizable chunk of their effector function. The importance of directional release into the synapse is most obvious in the case of cytotoxic T cells and NK cells, which kill target cells using soluble, cytolytic factors such as perforin and granzyme (Stinchcombe and Griffiths, 2007). It goes without saying that the effects of these agents must be limited to the target cell alone.

Over the past two decades, our knowledge of the signaling pathways associated with TCR activation has improved dramatically. In contrast, our understanding of the molecular mechanisms that drive concomitant cell shape changes and cytoskeletal

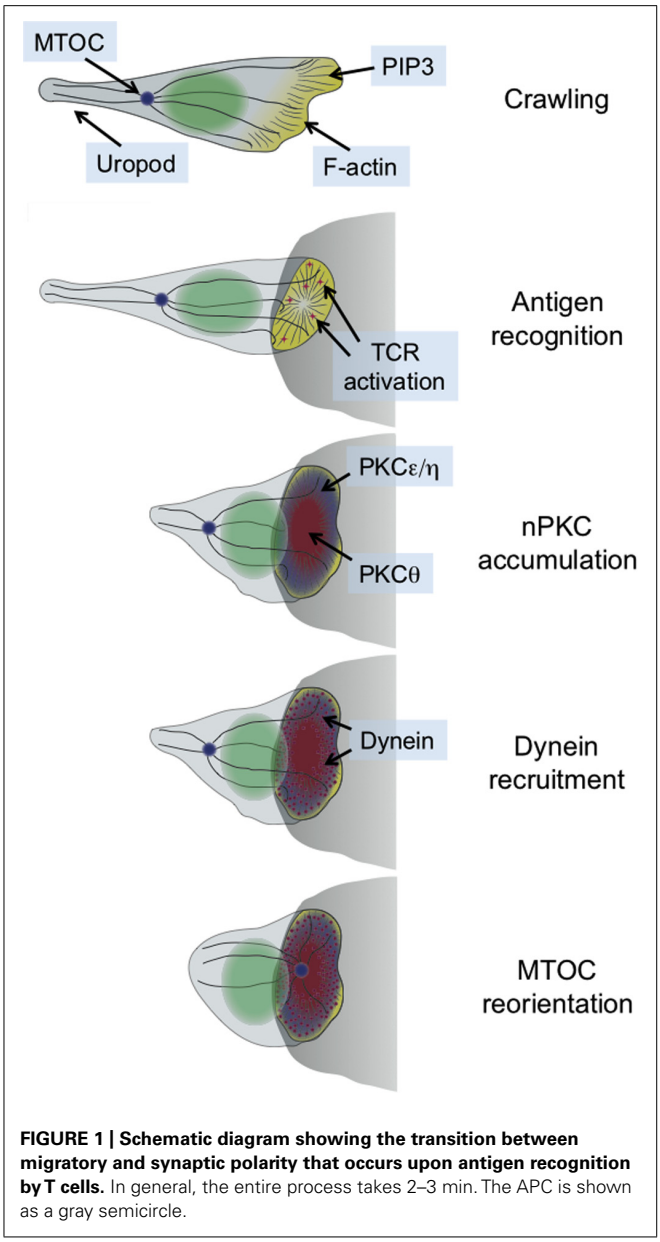

FIGURE 1 | Schematic diagram showing the transition between migratory and synaptic polarity that occurs upon antigen recognition by T cells. In general, the entire process takes 2–3 min. The APC is shown as a gray semicircle.

polarization remains quite poor. In recent years, however, high-resolution imaging approaches have provided investigators with the wherewithal to actually explore the cell biology of T cell activation. This review will focus on what these approaches have taught us about MTOC polarization to the IS, with particular emphasis on the roles played by distinct protein kinase C (PKC) isoforms at various stages during the process.

MTOC POLARIZATION IS DICTATED BY THE TCR

MTOC reorientation to the IS was first documented three decades ago (Geiger et al., 1982; Kupfer et al., 1983). The process was difficult to study, however, largely because it occurs so quickly (typically within 5 min of receptor stimulation) and because T cells are so small. Early efforts focused on defining the basic requirements for the response, and revealed that it was highly dependent on TCR signaling. In a particularly informative set of studies, Burkhardt and colleagues mixed T cells with target cells expressing either the integrin ligand ICAM-1 or agonist peptide-major histocompatibility complex (pMHC; Sedwick et al., 1999). When T cells contacted both kinds of target cell simultaneously, the MTOC polarized toward the cell expressing pMHC. Conversely, actin accumulated at the interface with the cell expressing ICAM-1. In addition to highlighting the importance of TCR signaling, these results provided perhaps the first indication that the molecular pathways involved in integrin-mediated adhesion were separate from those that guided MTOC polarization. Subsequent studies confirmed the dominant role of TCR signaling by demonstrating that several receptor proximal proteins, including the tyrosine kinases Lck and Zap70 and the scaffolding proteins LAT and Slp76, were required for polarization responses (Lowin-Kropf et al., 1998; Kuhne et al., 2003). These proteins, however, play an important role in nearly every aspect of T cell activation. Hence, it remained unclear precisely how early signals emanating from the TCR are coupled specifically to the MTOC.

THE IMPORTANCE OF DIACYLGLYCEROL

As mentioned above, the mechanistic analysis of MTOC reorientation to the IS has long been limited by technical constraints imposed by the dynamics of the response and the size of the cells in question. Beyond this, there were certain complicating issues related to the experimental systems used to quantify polarization. MTOC reorientation was typically assessed by live or fixed imaging of T cell–APC conjugates. In this context, observable polarization could only occur after productive contact formation. Hence, molecules or pathways involved in promoting adhesion with the APC would be implicated in MTOC reorientation, even if their effects on the pathway itself were merely secondary.

To circumvent these issues and improve the spatial and temporal resolution of analysis, we developed a single cell polarization assay in which conjugate formation with an APC was replaced by controlled stimulation of the TCR in a micron-sized region of the T cell membrane (Huse et al., 2007). Our approach is based on a photoactivatable pMHC reagent specific for the 5C.C7 TCR. This reagent bears a large, photocleavable group that blocks TCR binding until it is cleaved off with a pulse of ultraviolet (UV) light. Primary 5C.C7 T cells expressing some sort of fluorescent signaling probe (typically proteins linked to GFP or RFP) are attached to coverslips containing this photoactivatable pMHC and imaged by video microscopy. After a short interval to establish a baseline recording, a micron-sized region beneath each cell is UV irradiated, creating a zone of agonist pMHC that is competent to bind to the 5C.C7 TCR. Subsequent intracellular signaling and cytoskeletal responses are monitored over the next 5–10 min using either epifluorescence or total internal reflection fluorescence (TIRF) illumination.

This protocol typically induces reorientation of the MTOC to the irradiated region in less than 3 min (Quann et al., 2009), and we have been using it as an assay to mechanistically dissect the process. This approach has several advantages over more standard T cell–APC conjugate experiments, the most obvious being a substantial improvement in spatial and temporal resolution.

Indeed, the combination of TIRF microscopy, which provides high-resolution images of the plasma membrane attached to the glass, and the ability to control when and where the T cell is stimulated, has enabled us to resolve events separated by a few as 5 s. In this manner, we have been able to establish a very precise order of operations leading up to MTOC reorientation (Quann et al., 2009, 2011). In addition, because contact with the glass surface is established prior to TCR stimulation (typically using an antibody against a class I MHC protein expressed by the T cells) it is likely that we have isolated the pathways guiding MTOC polarity, which we can study independently of mechanisms controlling adhesion.

Using this assay, we began to explore the relationship between early TCR signaling and MTOC polarization. We were particularly intrigued by the lipid second messenger diacylglycerol (DAG), which is generated by phospholipase C-γ (PLCγ) downstream of TCR activation. DAG transduces signals by recruiting proteins containing DAG-binding C1 domains to the membrane (Colon-Gonzalez and Kazanietz, 2006). It was known that DAG accumulates in a polarized manner at the IS (Spitaler et al., 2006), and we had found that a PLCγ inhibitor completely blocked MTOC polarization (Quann et al., 2009). Using a C1 domain-containing protein biosensor that translocates to membranes containing DAG, we discovered that MTOC reorientation was invariably preceded 10–15 s by the localized accumulation DAG in the region of TCR stimulation (Quann et al., 2009). This close temporal relationship suggested that DAG served to guide the MTOC to the IS. Indeed, using various perturbation approaches, we were able to show that disrupting the ability of the T cell to maintain a localized DAG accumulation or to respond to such an accumulation blocked MTOC polarization. In addition, using a photoactivatable DAG reagent that cannot engage its targets until it is irradiated with UV light, we demonstrated that the localized generation of DAG alone could induce MTOC reorientation, independent of the TCR.

The importance of localized DAG accumulation for the polarization of the MTOC is strikingly reminiscent of the phosphatidylinositol tris-phosphate (PIP3) based direction-sensing mechanism used by *Dictyostelium* and neutrophils to establish migratory polarity (Devreotes and Janetopoulos, 2003; Ward and Marelli-Berg, 2009). In these cell types, the accumulation of PIP3 promotes formation of a leading edge lamellipodium and is important for effective migration within a chemotactic gradient. This system is well suited for rapid and transient direction sensing because PIP3 is continuously metabolized by lipid phosphatases such as PTEN and SHIP, and therefore must be replenished by new PIP3 production in order to maintain directionality. The dynamic balance between production and metabolism enables cells to respond quickly to positional changes in surface receptor stimulation because these changes necessarily lead to positional changes in lipid second messenger production. The same sort of dynamic balance exists for DAG, whose production by PLC isozymes is offset by DAG kinases (DGKs), which convert DAG to phosphatidic acid (PA; Zhong et al., 2008).

The extent to which PIP3-based direction sensing participates in T cell migration is somewhat controversial (Nombela-Arrieta et al., 2004; Reif et al., 2004; Asperti-Boursin et al., 2007; Liu et al., 2007; Smith et al., 2007; Ward and Marelli-Berg, 2009). Nevertheless, it is intriguing to speculate that T cells employ DAG and PIP3 simultaneously as a way to decouple lamellipodial dynamics from MTOC polarization. The ability to control these processes independently would presumably be important for transitioning between synaptic morphology, where the MTOC and leading edge localize to the same interface, and migratory morphology, in which the MTOC localizes to the uropod, distal to the leading edge.

A CASCADE OF NOVEL PKCs

The discovery that DAG plays an important role in T cell MTOC polarization immediately suggested that proteins containing C1 domains were involved in the process. Of these, perhaps the most obvious candidates were the PKCs. It had been known for some time that a combination of phorbol esters (e.g., PMA) and Ca^{2+} ionophores (e.g., ionomycin) can largely recapitulate the effects of T cell activation independent of the TCR (Chatila et al., 1989). These reagents directly activate multiple PKCs, strongly implicating this family of proteins in T cell signaling. Consistent with this notion, PKC inhibitors effectively block many TCR-induced responses, including proliferation and the secretion of inflammatory cytokines (Baier and Wagner, 2009). Although few studies had implicated PKCs in the regulation of lymphocyte architecture, they were known to play an important role in cytoskeletal remodeling in adherent cells such as fibroblasts (Larsson, 2006).

The protein kinase C family is typically divided into three subgroups, which can be distinguished by the structure of their N-terminal regulatory regions (Newton, 2010). Conventional PKCs (cPKCs) contain tandem, DAG-binding C1 domains followed by a C2 domain, which recognizes negatively charged phospholipids in a Ca^{2+}-dependent manner. Novel PKCs (nPKCs), by contrast, contain a C2 domain at their N-termini that cannot bind to phospholipids due to mutations in its Ca^{2+} binding sites. The tandem C1 domains that follow have an unusually high affinity for phorbol esters and DAG. Atypical PKCs (aPKCs) lack C2 domains entirely, and contain only one C1 domain that has lost the ability to bind DAG. These differences in domain structure endow each PKC subfamily with distinct regulatory properties: cPKCs require both Ca^{2+} and DAG for their activation, nPKCs require DAG alone, while aPKCs are largely regulated through protein–protein interactions.

Identifying which of these isoforms contribute to MTOC polarization responses was complicated by the fact that most, if not all, PKCs are expressed in T cells. The importance of localized DAG, however, argued against a role for aPKCs, at least during the early phases of the response. Furthermore, we had shown that Ca^{2+} signaling was not required for polarization (Quann et al., 2009), suggesting that cPKCs were not involved. Hence, we chose to focus first on the nPKC subfamily, comprising PKCδ, PKCε, PKCη, and PKCθ. Of these, probably the best studied was PKCθ, which is highly expressed in both developing and mature T cells. T cells lacking PKCθ display marked deficiencies in antigen-induced proliferation, cytokine secretion, and development into the T_H2 lineage (Sun et al., 2000; Marsland and Kopf, 2008). PKCθ is thought to mediate many of these effects by activating several key

transcription factors, including NF-κB, NFAT, and AP-1, which together account for a significant fraction of TCR-dependent gene expression (Manicassamy et al., 2006).

T cell receptor signaling induces the accumulation of PKCθ at the IS (Monks et al., 1997), where it would presumably be well positioned to promote cytoskeletal polarization. Prior to our work, however, it was unknown whether PKCθ actually contributed to this process. Even less was known about the other nPKCs. Indeed, previous studies implied that PKCε and PKCη were not required for any aspect of T cell activation (Monks et al., 1997; Gruber et al., 2005). Hence, we were quite surprised to find that TCR stimulation in both photoactivation experiments and T cell–APC conjugates induced the robust IS recruitment of not only PKCθ, but also PKCε and PKCη (Quann et al., 2011). Notably, PKCδ was not recruited in this manner, consistent with previous reports indicating that it localizes to intracellular granules instead (Ma et al., 2008).

The synaptic accumulation of PKCε, PKCη, and PKCθ preceded reorientation of the MTOC (**Figure 1**), consistent with a role for all three proteins in the process. Indeed, siRNA-mediated suppression of either PKCθ alone or PKCε and PKCη in combination disrupted polarization responses (Quann et al., 2011). These results indicated that all three proteins participate in MTOC reorientation, but that PKCε and PKCη can functionally compensate for each other. In retrospect, redundancy between PKCε and PKCη should not have been particularly surprising, given the high level of sequence identity (60%) between the two proteins. This may explain why PKCε-deficient T cells display no observable TCR activation phenotype (Gruber et al., 2005). A more concrete answer will await the analysis of PKCε/PKCη double knockout mice.

Interestingly, simultaneous siRNA knockdown of PKCε and PKCη also inhibited the recruitment of PKCθ, while knockdown of PKCθ did not affect PKCη accumulation (Quann et al., 2011). Taken together, these results suggested that PKCε and PKCη operate upstream of PKCθ to promote MTOC polarization. Close examination of the recruitment dynamics of all three proteins was consistent with this hypothesis (Quann et al., 2011). PKCε and PKCη arrived at the region of TCR stimulation first, followed by PKCθ ∼10 s later, and MTOC reorientation 5–10 s after that. PKCε and PKCη had the same accumulation pattern, which covered a broad region of plasma membrane encompassing the entire IS. By contrast, PKCθ occupied a more restricted zone that was entirely contained within the lamellipodial actin ring at the periphery (**Figure 1**). Whether and how these distinct PKC recruitment patterns contribute to polarization responses remains unclear. It is tempting to speculate, however, that the broad accumulation of PKCε and PKCη controls early polarization steps, while the more confined PKCθ distribution contributes to positional refinement of the MTOC at later stages.

We found that the distinct recruitment patterns of PKCη and PKCθ could be largely recapitulated by constructs containing the tandem C1 domains of each protein (Quann et al., 2011). This is remarkable, given that typical C1 domains are all thought to bind to the same ligand, DAG. What then could explain the differences we observed? *In vitro* studies have demonstrated that PKCε binds to bilayers containing DAG with ∼10-fold higher affinity than does PKCθ (Stahelin et al., 2005; Melowic et al., 2007). It is likely that the affinity of PKCη for DAG is similar to that of PKCε, given the close homology between the two proteins. The ability of PKCε and PKCη to bind DAG more tightly than does PKCθ would presumably lead to faster IS recruitment. A higher affinity for DAG could also explain why PKCε and PKCη accumulate in a broader membrane zone than PKCθ, assuming that DAG density declines radially outside of the site of TCR stimulation.

Although differential DAG affinity provides an elegant mechanism for modulating PKC recruitment, other results strongly suggest that there are additional contributing factors. For example, PKCδ recognizes DAG with threefold higher affinity than does PKCθ (Stahelin et al., 2004), and yet PKCδ is not recruited to the IS. This probably has less to do with DAG itself and more to do with the complex protein and lipid environment at the IS in which DAG accumulates. The context within which DAG recognition takes place can have dramatic effects on membrane binding by C1 domains. PKCθ, for example, binds to mixtures of DAG and the charged lipid phosphatidylserine with 28-fold higher affinity than it does to DAG mixtures containing the uncharged phosphatidylglycerol (Melowic et al., 2007). The C1 domains of PKCε have been documented to recognize arachidonic acid and ceramide in addition to DAG (Kashiwagi et al., 2002), and various C1 domains engage in protein–protein interactions in the appropriate environments (Colon-Gonzalez and Kazanietz, 2006).

Hence, it seems reasonable to hypothesize that there are either lipid- or protein-based "contextual factors" that contribute in a combinatorial manner to the accumulation of PKCs within the IS. Although the identity of these molecules remains unknown, the potential to modulate C1 domain localization independent of DAG has important implications for the continued use of C1 domains as DAG "biosensors." Constructs derived from different PKC isoforms as well as protein kinase D are now widely used to monitor *in situ* DAG production in multiple experimental systems. Although we have learned and will continue to learn much from this approach, it is important to keep in mind moving forward that different C1 domains have been evolutionarily tuned to recognize DAG within distinct lipid and protein environments. Therefore, care must be taken when imaging C1 domain constructs, as changes in localization could reflect either a change in DAG density or a change in other contextual factors.

MOVING THE MTOC WITH MOLECULAR MOTORS

Our mechanistic understanding of MTOC polarization in T cells took a significant step forward when it was discovered that the microtubule motor protein dynein is recruited to the IS (Combs et al., 2006). This immediately suggested that dynein, once anchored at the IS, might reorient the MTOC by pulling on the microtubules that radiate from it. This was an attractive hypothesis because microtubules emerge from the MTOC with their minus ends inward and their plus ends outward, effectively matching the polarity of the dynein motor, which moves from plus end to minus end. Indeed, subsequent siRNA experiments demonstrated that knockdown of dynein impaired MTOC reorientation in Jurkat T cells (Martin-Cofreces et al., 2008).

Dynein is a large, multisubunit protein that contains two copies each of a heavy chain and several accessory light chains

(Kardon and Vale, 2009). The heavy chain contains the motor and microtubule binding domains, while the light and intermediate chains provide structural integrity and mediate interactions with accessory factors. The most important of these accessory factors is dynactin, a multisubunit complex roughly equivalent in size to dynein, which enhances dynein processivity and controls its localization to distinct subcellular compartments. Dynein is required for the trafficking of multiple distinct cargos, ranging from proteins such as β-catenin and rhodopsin to organelles like the Golgi apparatus (Vallee et al., 2004; Kardon and Vale, 2009). Although some cargo interactions are mediated by dynein itself, most appear to require dynactin, which plays the role of a large, multifunctional adaptor.

Precisely how the dynein–dynactin complex is recruited to the IS remains unclear. It was initially proposed that dynein associates directly with the TCR signaling machinery by binding to ADAP, a scaffolding protein that interacts with Slp76. In Jurkat cells, ADAP was found to accumulate at the IS, and siRNA-mediated suppression of ADAP inhibited MTOC reorientation (Combs et al., 2006). These results could not be extended to primary T cells, however, raising doubts as to the physiological relevance of this recruitment pathway. TCR photoactivation experiments have revealed that DAG accumulation precedes dynein recruitment by ∼10 s, and that DAG and dynein occupy essentially the same region of membrane prior to MTOC reorientation (Quann et al., 2009). This suggests that DAG might recruit dynein either directly or via some DAG-binding adaptor. Although there is little evidence for this at present, dynein and dynactin are very large, and it is not implausible that one of the less well-studied subunits could indeed be regulated by DAG or a related lipid.

While it is generally thought that dynein–dynactin is necessary for MTOC reorientation in T cells, recent work has cast doubt on the idea that it is entirely sufficient. In primary T cells, polarization responses toward supported lipid bilayers containing cognate pMHC were unaffected by siRNA knockdown of dynein or by the small molecule dynein inhibitor EHNA (Hashimoto-Tane et al., 2011). Although these results imply that dynein-independent pathways exist for MTOC reorientation, the precise identity of these pathways remains to be seen. They are unlikely to involve plus end directed microtubule motors, as overexpression of the dynein recruitment factor RILP (see below), which blocks plus end directed movement, had no effect on MTOC reorientation (Stinchcombe et al., 2006). This leaves the cortical actin cytoskeleton, which interfaces with microtubules just beneath the plasma membrane. In the mature IS, microtubules radiating from the MTOC have been documented to intersect with the actin ring that forms at the periphery of the contact (Kuhn and Poenie, 2002). It has been proposed that the formation of this actin ring applies tension to associated microtubules, thereby dragging the MTOC into close apposition with the synaptic membrane (Stinchcombe et al., 2006). In this manner, the force generated by actin polymerization in the radial lamellipodium could be utilized for MTOC reorientation. Although this hypothesis has not been tested directly, it is interesting to note that several proteins involved in coupling the microtubule cytoskeleton with cortical actin, including the scaffolding molecule IQGAP and the Diaphanous formins, have also been implicated in MTOC reorientation (Gomez et al., 2007). It is possible that these proteins promote polarization responses by establishing and maintaining force-bearing contacts between microtubules and actin.

The collaboration between microtubule- and actin-based remodeling during MTOC polarization is best understood in fibroblasts, which position the MTOC in front of the nucleus during cell migration. In this system, dynein functions to hold the MTOC immobile, while retrograde actin flow drives the nucleus behind it (Gomes et al., 2005). These actin dynamics are, in turn, controlled by myotonic dystrophy kinase-related Cdc42 binding kinase (MRCK) and non-muscle myosin II (Gomes et al., 2005). It is not known whether and to what extent this actin-based pathway contributes to the T cell response, which is much faster and less stable than polarization in fibroblasts. Notably, our photoactivation experiments, which drive MTOC reorientation to a fixed region in space, induce robust MTOC movement, often with minimal net nuclear motion (X. Liu and M. Huse, unpublished results). This is the opposite of what is seen in fibroblasts, suggesting that components of actin-based motility, if they do promote MTOC polarization in T cells, do so via a somewhat distinct mechanism.

STABILIZATION OF THE POLARIZED STATE BY aPKCs

In astrocytes, fibroblasts, and epithelial cells, aPKCs such as PKCζ and PKCι play a central role in the acquisition and maintenance of polarity (Etienne-Manneville and Hall, 2003; Li and Gundersen, 2008). aPKCs operate in this context as part of a complex that also contains the adaptor molecules PAR3 and PAR6 (for partitioning defective). PAR proteins were initially isolated in a screen for factors regulating embryonic polarity, and they are crucial for a variety of processes ranging from asymmetric cell division to the establishment of an apical–basal axis in epithelial cells (Nelson, 2003; Nance and Zallen, 2011). Accumulation of the PAR3–PAR6–aPKC complex in one plasma membrane domain is often accompanied by the accumulation of a distinct complex containing the proteins Scribble and Discs-large (Dlg) in a reciprocal domain. The PAR and Scribble–Dlg complexes mutually antagonize each other's assembly, effectively enhancing and stabilizing the polarized state (Nelson, 2003).

The importance of these complexes for polarity induction in other systems suggested that they might play a similar role in T cells during IS formation. Consistent with this notion, immunocytochemical studies demonstrated that PAR3 accumulates at the T cell–APC interface while Scribble–Dlg localizes to the back of the cell (Ludford-Menting et al., 2005; **Figure 2**). Although the overall distribution of PKCζ was not polarized under these conditions, the phosphorylated and activated form of PKCζ was enriched together with PAR3 at the IS (Bertrand et al., 2010). Blocking this pool of active PKCζ either by application of a small molecule inhibitor or by overexpression of a dominant-negative PKCζ construct disrupted MTOC polarization toward the APC (Bertrand et al., 2010). Polarization responses were also impaired by overexpression of a dominant-negative version of the kinase Par1b (Lin et al., 2009), which is known to antagonize PAR3–PAR6–aPKC in other systems. Although it is somewhat worrying that these perturbation studies exclusively used pharmacological and overexpression approaches,

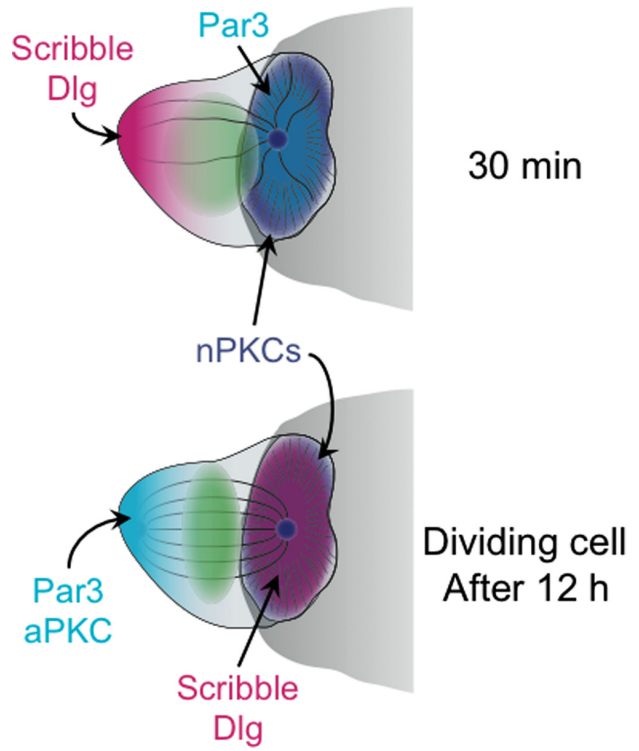

FIGURE 2 | Schematic diagram showing the distribution of polarity complexes in synaptic T cells 30 min (top), and 12 h (bottom) after antigen recognition. The cell at 12 h is depicted as undergoing mitosis. The APC is shown as a gray semicircle.

when taken together the results support a role for the PAR complex and aPKC in synaptic T cell polarity.

Notably, the synaptic accumulation of PAR3 and phospho-PKCζ, which was coupled with Scribble–Dlg localization at the back of the cell, was only observed at relatively late time points, 20–30 min after T cell–APC conjugate formation (Ludford-Menting et al., 2005; Bertrand et al., 2010). Similarly, the perturbation experiments documenting the effects of PKCζ inhibition and overexpression were all scored after 20 min or more (Bertrand et al., 2010). This is well after initial MTOC reorientation, which occurs in less than 5 min. Hence, PAR3–PAR6–aPKC may not be required for the act of polarization, but rather for the subsequent stabilization of the polarized state. This interpretation is consistent with recent live-imaging analysis of B cell polarization (Yuseff et al., 2011). In this study, shRNA-mediated suppression of PKCζ did not block initial MTOC reorientation toward antigen coated beads. It did, however, impair the maintenance of polarization over time.

On balance, the extant data are consistent with a model that divides MTOC polarization into two phases: a fast direction sensing and reorientation phase directed by DAG and nPKCs, followed by a stabilization and consolidation phase that requires the PAR3–PAR6–aPKC and Scribble–Dlg polarity complexes. The separation of polarization responses into two distinct steps would presumably facilitate the modulation of synaptic strength and stability, allowing T cells to tailor their synapses for specific biological functions. Fast, transient MTOC reorientation without subsequent synaptic consolidation would presumably be optimal for cytotoxic T lymphocytes (CTLs), which are most effective when they kill quickly and move on. By contrast, long-lived, stable synapses may be crucial for the directional secretion of cytokines by helper T cells, which occurs hours after initial TCR stimulation. Stabilization may also play a key role in properly aligning naïve T cells to undergo asymmetric cell division on the surface of a dendritic cell (DC; see below). Now that some of the molecules controlling the distinct phases of T cell polarity have come to light, it may be possible to test these predictions in physiologically relevant settings.

Interestingly, PAR3–PAR6–aPKC and Scribble–Dlg also appear to be involved in the maintenance of migratory cell polarity. They display asymmetric localization in migrating T cells, with PAR components in the cell body and Scribble–Dlg in the uropod (Ludford-Menting et al., 2005; Real et al., 2007). Furthermore, overexpression of dominant-negative PKCζ leads to cell rounding, as does siRNA knockdown of Scribble (Ludford-Menting et al., 2005; Real et al., 2007). In light of the model for synaptic polarization described above, it is tempting to speculate that migratory polarity may also be established in two steps. Thus, initial direction sensing and leading edge formation would be driven by PIP3 and regulators of Rho-family GTPases, such as the exchange factor Dock2 (Ward and Marelli-Berg, 2009). This would be followed by the formation of polarity complexes and the stabilization of extended, hand-mirror morphology. Time-resolved studies of polarity induction in response to migratory stimuli, such as chemokines, will be required to examine this hypothesis in more detail.

SYNAPTIC POLARIZATION AND DIRECTIONAL SECRETION

Evidence supporting a role for the IS in directional secretion predates the term "immunological synapse." Pioneering imaging studies of T cell–APC conjugates, performed by Kupfer et al. (1991, 1994), demonstrated that intracellular compartments containing nascent cytokines were tightly apposed to the cell–cell interface, suggestive of targeted release toward the APC. Similarly, by activating T cells that had been forced into membrane pores, Janeway and colleagues provided evidence for preferential cytokine secretion in the direction of TCR stimulation (Poo et al., 1988). It was subsequently found that T cells use at least two directionally distinct pathways for cytokine secretion, one that targets the IS, and another that releases factors in an unpolarized manner (Huse et al., 2006). Although it remains unclear precisely how different cytokines are targeted to different secretory pathways, cell biological analyses have indicated that these pathways are molecularly distinct (Huse et al., 2006). Thus, synaptically secreted cytokines such as IL-2 and IFNγ were observed to traffic in intracellular compartments coated with the GTPases Rab3d and Rab19. Conversely, multidirectionally released factors such as TNF and the chemokine CCL3 occupied vesicles containing the SNARE (soluble *N*-ethylmaleimide-sensitive factor accessory protein receptor) proteins syntaxin-6 and Vti1b. SNAREs and Rabs have been implicated in vesicular trafficking and compartmental specification in numerous systems (Pfeffer, 2001; Jahn et al., 2003), and it is likely they play a central role in the spatial regulation of cytokine secretion from T cells.

The best-studied directional secretion phenomenon in lymphocytes is the synaptic release of cytolytic factors, such as perforin and granzyme, by CTLs and NK cells (Stinchcombe and Griffiths, 2007). Cytotoxic molecules are stored in secretory lysosomes called lytic granules, which are molecularly distinct from compartments involved in cytokine trafficking (Reefman et al., 2010). TCR signaling induces the dynein-dependent trafficking of these granules along microtubules toward the MTOC (Mentlik et al., 2010; Daniele et al., 2011), which concurrently reorients to the IS. In this manner, lytic granules are delivered to the center of the IS, where they fuse with the plasma membrane in a designated "secretory domain" (Stinchcombe et al., 2001; **Figure 3**). Compared to the dense, orthogonal array of actin found in the peripheral lamellipodium, the cortical actin in the secretory domain is sparse and weblike (Brown et al., 2011; Rak et al., 2011). The transient gaps that appear in the actin meshwork in this region provide avenues for the egress of lytic granules and probably also cytokine compartments.

Over the past 10 years, our knowledge of how lytic granules traffic toward the MTOC and the IS has improved substantially. The focusing of lytic granules at the MTOC occurs within minutes of TCR stimulation, and is strongly dependent on Ca^{2+} (Beal et al., 2009). Indeed, the speed of granule trafficking on microtubules scales proportionately with intracellular Ca^{2+} concentration. Notably, both Ca^{2+} influx and DAG accumulation result from the cleavage of PIP2 by PLCγ. Thus, both the reorientation of the MTOC and the focusing of lytic granules respond to the same TCR proximal signaling event, which no doubt facilitates the close temporal coupling of the two responses.

The analysis of proteins associated with lytic granules has revealed other components involved in their trafficking to the MTOC. Particularly relevant to the scope of this review is PKCδ, which binds constitutively to lytic granules and is required for their focusing around the MTOC (Ma et al., 2007, 2008). Importantly, PKCδ deficiency has no effect on MTOC reorientation to the IS, indicating that PKCδ has a specific role in granule trafficking. Recent studies have also implicated the GTPase Rab7 and its associated effector RILP in lytic granule motility (Daniele et al., 2011). The Rab7–RILP complex is involved in coupling multiple distinct organelles to dynein, leading to their accumulation at microtubule minus ends (Wang et al., 2011). In CTLs, both Rab7 and RILP associate with lytic granules, and overexpression of RILP drives copious accumulation of dynein on granule membranes (Daniele et al., 2011). Consistent with a role for this complex in granule trafficking, siRNA knockdown of Rab7 inhibits cytotoxicity. Given the similarities in localization pattern and phenotype, one might imagine that Rab7–RILP and PKCδ cooperate in the same pathway. This remains to be explored, however, as does the relationship between each of these factors and Ca^{2+}.

It is generally thought that synaptic secretion by T cells enables selective communication with or killing of APCs in dense intracellular environments packed with bystander cells (Huse et al., 2008). Consistent with this notion, a considerable amount of *in vitro* and *in vivo* work has established that cytotoxic killing is both contact mediated and antigen specific (Kuppers and Henney, 1977; Kupfer et al., 1986; Lanzavecchia, 1986; Breart et al., 2008; Sanderson et al., 2012). Studies have also documented that APCs in direct contact with $CD4^+$ T cells are preferentially activated. For example, sustained polarization of the T cell MTOC toward antigen bearing DCs is required to induce strong IL-12 production by those DCs (Bertrand et al., 2010; Tourret et al., 2010). Similarly, B cells that are directly conjugated with helper T cells have been observed to divide before other B cells in the culture (Kupfer et al., 1994). It is unclear, however, whether these preferential effects on synaptically engaged APCs depend on directional cytokine secretion *per se*. Indeed, two recent reports have indicated that IFNγ can diffuse away from the IS and stimulate cells at a great distance from the T cell–APC conjugate. In one study (Sanderson et al., 2012), a fluorescently labeled Stat1 reporter was used to monitor the scope of IFNγ secretion from CTLs *in vitro*. In the other study (Muller et al., 2012), IFNγ-activated macrophages were visualized *in vivo* by staining for iNOS. In both of these systems, the most intense responses to IFNγ were observed in the APC or close to it, consistent with the idea that the APC has privileged access to synaptically secreted cytokines. Nevertheless, it is clear that the IS does not act as an impenetrable barrier to diffusion.

In light of this new data, it is worth reevaluating how directional secretion into the IS might maintain the specificity of secretory responses. Because T cell and APC membranes are closely apposed in the IS, it is likely that the APC would have

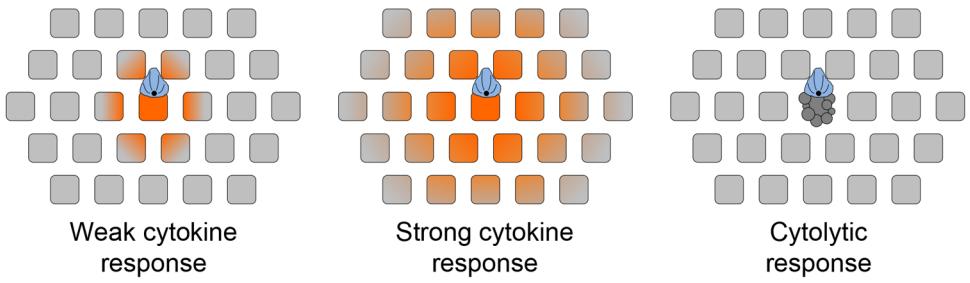

FIGURE 3 | Schematic diagram depicting the effects of synaptic secretory responses. (A) Weak cytokine secretion affects only the APC and cells in its immediate vicinity. **(B)** Strong cytokine secretion affects a larger number of bystander cells. **(C)** Cytolytic killing, mediated by perforin and granzyme, is restricted to the APC. The scope of cytokine diffusion is denoted in orange. T cells are colored blue, with the MTOC and microtubules in black. Bystander cells are depicted as gray squares. Adapted from Sanderson et al. (2012).

the first opportunity to bind synaptically secreted factors. This could substantially affect the scope of the secretory response in circumstances where the number of secreted molecules is comparable to the number of cell surface receptors present in the IS. Of course, stronger responses would overwhelm the available receptors on the APC, leading to diffusion out of the IS and the stimulation of bystander cells (**Figure 3**). A prediction of this "first dibs" mechanism is that small differences in the magnitude of cytokine production could have very substantial effects on the scope of the subsequent response. The extent of bystander effects probably also depends on the physical properties of the secreted factors themselves. Cytokines are small, stable proteins, and they diffuse quickly in tissue environments (G. Altan-Bonnet, personal communication). By contrast, perforins are unstable at neutral pH and readily oligomerize to form pores in cellular membranes (Pipkin and Lieberman, 2007). Indeed, it is likely that secreted perforins have only a short lifetime as soluble factors, associating quickly with the opposing APC. Thus, the specificity of killing would be maintained by the relative instability of perforin, and not by a diffusion barrier imposed by the IS. This model is consistent with the observation that CTLs mediate highly specific killing even while inducing IFNγ signaling responses in distal bystanders (**Figure 3**; Sanderson et al., 2012). Finally, it is possible that specificity is achieved by the combinatorial action of soluble and membrane bound signals. For example, full activation of B cells and DCs requires both cytokine signaling as well as engagement of the cell surface receptor CD40 (Foy et al., 1996; Snijders et al., 1998). CD40 binds to the transmembrane ligand CD154, which accumulates in the T cell IS in response to TCR stimulation (Boisvert et al., 2004; Bertrand et al., 2010; Tourret et al., 2010). The synaptic localization of CD154 provides preferential access to the APC, and would presumably limit the scope of full activation even under conditions where stimulatory cytokine is freely available. An analogous mechanism may also operate during cytotoxic responses, which often require Fas–FasL interactions in addition to perforin- and granzyme-mediated effects (Russell and Ley, 2002).

It is becoming increasingly clear that synaptic secretion, on its own, does not constrain responses to the APC. Identifying the mechanisms or combinations of mechanisms that complement synaptic secretion in order to maintain specificity should provide a more nuanced (and accurate) view of how T cells shape the scope of their effector responses.

POLARITY CUES AND ASYMMETRIC CELL DIVISION

One of the most exciting developments in lymphocyte polarity over the past 5 years is the discovery that antigen-stimulated T cells undergo asymmetric cell division (Chang et al., 2007). Certain proteins, including CD8 and LFA-1, were found to preferentially accumulate on one side of mitotic T cells, implying that they might be inherited unequally by daughter cells. Consistent with this hypothesis, flow cytometric analyses of proliferated T cells revealed a bimodal distribution of these markers. Asymmetric division was only observed under conditions of infection, suggesting that it might play a role in the differentiation of effector cells. Indeed, it was found that daughter cells expressing lower levels of CD8 were more likely to develop into memory cells, while daughters expressing high levels of CD8 became effectors. Hence, asymmetric cell division is likely to play a significant role in the development of distinct T cell subsets after infection. Recently, it was found that activated B cells also undergo asymmetric division (Wang et al., 2011), indicating that this may be a general differentiation mechanism common to all lymphocytes.

The PAR3–PAR6–aPKC and Scribble–Dlg complexes are important for asymmetric division in multiple cell types (Nance and Zallen, 2011). Because previous studies had shown that these proteins accumulate asymmetrically in synaptic T cells (Ludford-Menting et al., 2005), their role in subsequent cell division was investigated. Indeed, perturbation of both complexes inhibited asymmetric division, and disruption of the Par6–PKCζ interaction with a small molecule inhibitor impaired memory cell development *in vitro* (Oliaro et al., 2010). Consistent with these observations, it was found that PAR3, PKCζ, Scribble, and Dlg remained polarized in synaptic T cells at 10–40 h after antigen recognition (Oliaro et al., 2010), the time period during which cell proliferation occurs. However, this polarization pattern was a complete reversal of the one observed at 30 min. After 10 h, Scribble–Dlg became enriched in the IS, while PAR3 and PKCζ localized to the back of the cell (**Figure 2**). When and how this remarkable inversion of polarity occurred is not known. It is also unclear why inverting the localization of polarity complexes should be necessary for promoting asymmetric division. In that regard, it is intriguing to note that PKCθ (and perhaps other nPKCs, as well) remained localized to the IS even at this late stage (Oliaro et al., 2010). Perhaps, the axis of PAR3–PAR6–aPKC/Scribble–Dlg polarity must be reversed relative to the axis of DAG/nPKC polarity in order for productive division to occur? The role of MTOC polarization during asymmetric cell division is also mysterious. One might expect that the placement of the MTOC at the IS could dictate the orientation of spindle assembly. However, imaging of late stage synapses indicated that there is a relaxation of MTOC polarization prior to mitosis (Oliaro et al., 2010). Whether the residual nPKC activity at the IS serves to repolarize the spindle once it forms awaits further studies. There is clearly a need for more mechanistic work in this area.

CONCLUDING REMARKS

Although our understanding of both the mechanisms that drive synaptic polarity in lymphocytes and the effector responses facilitated by that polarization has improved in recent years, many important questions remain. It is now clear that nPKCs and aPKCs play important roles in promoting MTOC polarization. Precisely, how they control dynein and other cytoskeletal components during this process, however, is unknown. To address this question, it will be crucial to identify the PKC substrates and interacting proteins relevant to this system.

It is also unclear how T cells generate and maintain such a stable accumulation of DAG at the IS. The answer likely involves the highly coordinated regulation of DAG production and destruction. In that regard, we have found that pharmacological inhibition of DGKs, which play a major role in the metabolism of DAG in T cells (Zhong et al., 2008), results in profound destabilization of synaptic DAG accumulation and impaired MTOC reorientation (Quann et al., 2009). T cells express multiple DGKs, and

determining which isoform(s) contribute to T cell polarity will be an important challenge.

The concept that MTOC polarization has initiation and stabilization phases is quite intriguing, and requires further investigation. It will be particularly interesting to determine how and when activated T cells transition from one phase to the next. It is conceivable that polarity complexes are recruited to the IS by DAG and nPKC signaling. However, it is also possible that the MTOC itself triggers the requisite signaling events once it is positioned at the IS. A large number of unique signaling proteins are associated with the MTOC, and the close apposition of these proteins with plasma membrane components at the IS could profoundly affect local signaling.

The link between MTOC polarization and directional secretion is well established, and the indications it is important for asymmetric cell division are encouraging. However, it is quite likely that there are other downstream responses requiring polarization of the MTOC to the IS that will remain unknown until we can selectively disrupt the process *in vivo*. Knowledge of the molecular mechanisms controlling cytoskeletal polarization in lymphocytes should, in future years, enable investigators to achieve this overarching goal.

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Regulation of PKC-θ function by phosphorylation in T cell receptor signaling

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Protein kinase C (PKC)-θ is a serine/threonine kinase belonging to the calcium-independent novel PKC subfamily; its expression is restricted to certain tissues and cell types, including T cells. The signals delivered from T cell receptor (TCR) and CD28 costimulatory molecules trigger PKC-θ catalytic activation and membrane translocation to the immunological synapse, leading to activation of NF-κB, AP-1, and NF-AT. These transcription factors are important for T cell survival, activation, and differentiation. Phosphorylation of PKC-θ at multiple Ser/Thr/Tyr residues is induced in T cells during TCR signaling. Some phosphorylation sites play critical roles in the regulation of PKC-θ function and downstream signaling. The regulation mechanisms for PKC-θ phosphorylation sites are now being revealed. In this review, we discuss the current understanding of the regulation of PKC-θ function by phosphorylation during TCR signaling.

Keywords: PKC-θ, phosphorylation, TCR signaling

INTRODUCTION

Protein kinase C (PKC)-θ was originally cloned in 1993 as a novel member of the PKC gene family (Baier et al., 1993; Chang et al., 1993). Because PKC-θ is highly expressed in T cells, immunologists have examined the roles of PKC-θ in T cell activation and immune regulation. Nearly 20 years after its discovery, numerous studies using cell lines and genetic animal models demonstrate that PKC-θ plays essential roles in controlling peripheral T cell activation; preventing T cell anergy; regulating the development of regulatory T cells, T helper (TH)2, and TH17 cells; and modulating autoimmune pathogenesis (Marsland and Kopf, 2008; Zanin-Zhorov et al., 2011). The signals triggered by T cell receptor (TCR) and CD28 costimulatory molecules induce membrane translocation and kinase activation of PKC-θ, leading to subsequent activation of NF-κB and AP-1 (Baier-Bitterlich et al., 1996; Coudronniere et al., 2000; Sun et al., 2000; Bi et al., 2001). These transcription factors induce IL-2 expression and regulate T cell activation and functions. The regulatory mechanisms of the activation and functions of PKC-θ in T cells have been unraveled. Post-translational regulation of PKC-θ by phosphorylation plays important roles in regulating PKC-θ kinase activity and membrane translocation, both of which are essential for PKC-θ function in T cells. Here, we focus our discussion on the function and regulation of critical phosphorylation sites of PKC-θ in T cell activation. Identification of the important regulators of PKC-θ phosphorylation may provide novel therapeutic drug targets for autoimmunity.

STRUCTURE BASIS FOR PKC-θ ACTIVATION

Human PKC-θ is composed of 706 amino acids. The basic structure of PKC-θ is shown in **Figure 1**. Like other PKC family members, PKC-θ consists of an N-terminal regulatory region (amino acids 1–378) and a C-terminal catalytic region (amino acids 379–706; Baier et al., 1993; Chang et al., 1993). The regulatory region displays the domain structure typical of PKC isoforms. The C2-like domain sequence is similar to the Ca^{2+}-binding C2 domain sequences of other PKCs; however, it does not bind to Ca^{2+}. The C2-like domain of PKC-θ contains a phosphorylated Y90 residue, which may mediate the interaction with an SH2 domain-containing protein, as seen in PKC-δ (Benes et al., 2005). In addition, the C2-like domain of PKC-θ may interact with a receptor for activated C kinase (RACK), which may regulate membrane translocation of PKC-θ (Schechtman and Mochly-Rosen, 2001). The interaction of a specific RACK with PKC-θ has not been reported; however, a PKC-δ/θ selective peptide blocking an RACK-binding site on PKC-δ/θ inhibits PKC-θ function (Nagy et al., 2009). Two tandem cysteine-rich C1 domains bind to diacylglycerol; the C1b domain has much higher affinity for diacylglycerol than the C1a domain (Melowic et al., 2007). A pseudosubstrate sequence (RRGAIKQA) within the C1a domain of PKC-θ binds to the substrate-binding region in the catalytic domain, inhibiting the PKC-θ kinase activity in the absence of allosteric effectors. C1 domains are flanked by two variable, or hinge, regions (V1 and V3). A recent study shows that the V3 domain is involved in the indirect association of PKC-θ with CD28, leading to PKC-θ membrane translocation into the immunological synapse; the interaction of lymphocyte-specific protein tyrosine kinase (LCK) with a proline-rich motif-like sequence in the V3 domain may be important for this action (Kong et al., 2011).

The crystal structure of the PKC-θ catalytic domain has been solved (Xu et al., 2004). The three-dimensional structure of PKC-θ is shown in **Figure 2**. The conserved core of the structure includes an N-terminal lobe and a C-terminal lobe, connected by a hinge linker sequence (Xu et al., 2004). The interface of the two lobes constitutes the active site cleft, which is responsible for

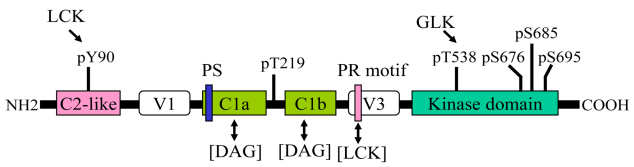

FIGURE 1 | Structural domains and phosphorylation sites of PKC-θ. One-way arrow indicates the phosphorylation of the indicated site by a kinase. Two-way arrow indicates an interaction. PS, pseudosubstrate sequence; PR, proline-rich motif.

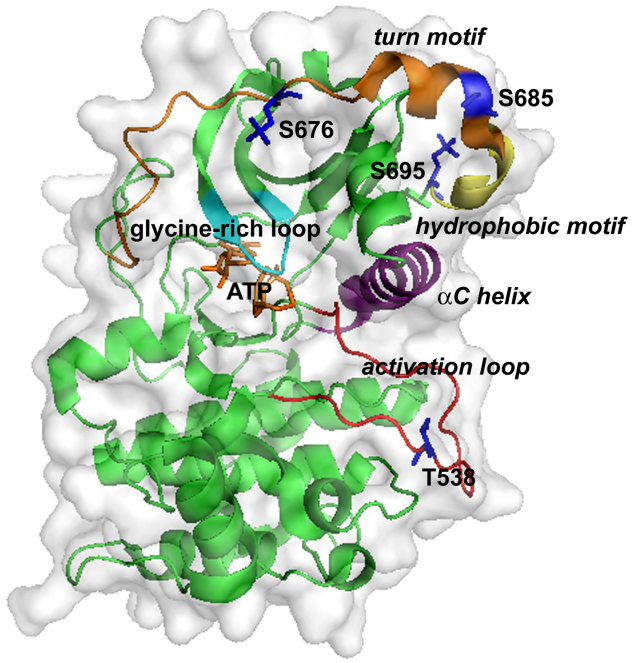

FIGURE 2 | Three-dimensional structure of the kinase domain of PKC-θ. ATP binds at a cleft between the N-lobe (top) and C-lobe (bottom). Key structural elements are highlighted: the glycine-rich loop (cyan), the αC helix (purple), the activation loop (red), the turn motif (orange), and the hydrophobic motif (yellow). Phosphorylation sites and ATP are shown as sticks in blue and yellow-orange, respectively.

the substrate binding and phosphate delivery (Xu et al., 2004). The key and conserved structural elements in the PKC-θ catalytic domain include a glycine-rich loop (GXGXXG) (involved in ATP binding and catalysis), an αC helix (participating in the substrate binding and catalysis), the activation loop bearing the essential phospho-threonine-538 (T538; critical for kinase activation), the hydrophobic motif containing phospho-serine 695 (S695), and the turn motif containing conserved phospho-serine 676 (S676) and phospho-serine 685 (S685) (Xu et al., 2004).

Similar to other protein kinases, PKC-θ displays two main conformational states: "open/active" and "closed/inactive" (Xu et al., 2004; Seco et al., 2012). In the inactive state, the pseudosubstrate sequence in the N-terminal regulatory region of PKC-θ forms intramolecular interaction with the substrate-binding region in the catalytic domain (House and Kemp, 1987). This prevents the catalytic domain from being accessible to substrates (House and Kemp, 1987). The allosteric change of PKC-θ from "closed" to "open" state involves two important mechanisms: diacylglycerol binding to the C1 domains and T538 phosphorylation at the activation loop (Seco et al., 2012). The signal transduction initiated from TCR/CD28 costimulation induces the generation of second messenger diacylglycerol, which binds to C1 domains, resulting in the exposure of the activation loop of PKC-θ (Melowic et al., 2007). The activation loop of PKC-θ is then accessible to phosphorylation by germinal center kinase-like kinase (GLK, also named MAP4K3) and subsequent catalytic activation (Chuang et al., 2011). Phorbol esters such as phorbol-12, 13-dibutyrate (PDBu) and phorbol myristate acetate (PMA) are potent non-physiological PKC-θ agonists that mimic the action of diacylglycerol and are widely used to induce PKC-θ activation (Ron and Kazanietz, 1999). Six phosphorylation sites have been identified on PKC-θ: Y90, T219, T538, S676, S685, and S695. These phosphorylation sites play distinct roles in the regulation of PKC-θ kinase activity or membrane translocation, which will be discussed below.

FUNCTION AND REGULATION OF PKC-θ PHOSPHORYLATION SITES

PKC-θ Y90 PHOSPHORYLATION

Identification and regulation of PKC-θ Y90 phosphorylation

PKC-θ is tyrosine phosphorylated in Jurkat T cells and primary T cells upon anti-CD3 with or without anti-CD28 costimulation (Liu et al., 2000; Bi et al., 2001). Coexpression with LCK, but not other tyrosine kinases including FYN, ZAP-70, SYK, or ITK, induces tyrosine phosphorylation of PKC-θ in COS-1 cells (Liu et al., 2000). Furthermore, tyrosine phosphorylation of PKC-θ is undetectable in LCK-deficient Jurkat T cells upon anti-CD3 stimulation (Liu et al., 2000). Additionally, LCK is constitutively and directly associated with PKC-θ in the transfected HEK293T cells and Jurkat T cells, and PKC-θ can be directly phosphorylated by LCK *in vitro* and *in vivo* (Liu et al., 2000). To identify the LCK-induced tyrosine phosphorylation site of PKC-θ, a series of PKC-θ-derived short peptides containing individual tyrosine residues are used as substrates for LCK in *in vitro* kinase assays (Liu et al., 2000). The peptide containing Y90, but not other tyrosine residues, is significantly phosphorylated by LCK *in vitro* (Liu et al., 2000). Furthermore, substitution of Y90 with phenylalanine abolishes LCK-induced PKC-θ tyrosine phosphorylation in transfected COS-1 cells and anti-CD3-stimulated Jurkat T cells (Liu et al., 2000). These data suggest that LCK directly phosphorylates PKC-θ at Y90. However, *in vivo* Y90 phosphorylation of PKC-θ during TCR signaling remains to be further demonstrated by immunoblotting using a phospho-specific antibody or confirmed by mass spectrometry.

Function of PKC-θ Y90 phosphorylation

Y90 phosphorylation of PKC-θ positively regulates NF-AT and NF-κB activation in T cells. The catalytically active PKC-θ A148E mutant induces NF-AT and NF-κB activation in Jurkat T cells, whereas the Y90F mutation of PKC-θ A148E mutant greatly reduces NF-AT and NF-κB activation (Liu et al., 2000; Bi et al., 2001). This suggests that Y90 phosphorylation regulates PKC-θ function, leading to downstream NF-AT and NF-κB activation.

The evidence of LCK-induced Y90 phosphorylation and membrane translocation of PKC-θ suggests that Y90 phosphorylation may regulate the membrane translocation of PKC-θ (Liu et al., 2000; Bi et al., 2001). However, other studies suggest that LCK regulates membrane translocation via mediating the formation of PKC-θ/LCK/CD28 tri-partite interaction (Tavano et al., 2004; Hofinger and Sticht, 2005; Kong et al., 2011). In these studies, the SH3 domain of LCK interacts with the proline-rich motif in the V3 domain of PKC-θ, whereas the SH2 domain of LCK is bound to the phosphorylated Y207 of the CD28 cytoplasmic tail (Tavano et al., 2004; Hofinger and Sticht, 2005; Kong et al., 2011). PKC-θ mutations at the V3 proline-rich motif suppress membrane translocation of PKC-θ to the immunological synapse and also attenuate T cell activation upon anti-CD3/CD28 costimulation (Kong et al., 2011). It is unknown whether Y90 phosphorylation of PKC-θ is involved in the regulation of PKC-θ/LCK/CD28 tri-partite interaction. The role of Y90 phosphorylation in the regulation of PKC-θ membrane translocation needs to be demonstrated by more direct evidence, such as a study of membrane translocation of PKC-θ Y90F mutant in T cells during TCR signaling. In addition, whether Y90 phosphorylation regulates PKC-θ catalytic activity remains unknown.

PKC-θ T219 PHOSPHORYLATION

Identification and regulation of PKC-θ T219 phosphorylation

T219 was originally identified as a major phosphorylation site of PKC-θ by phosphopeptide mapping (Thuille et al., 2005). The PKC-θ protein used for the phosphopeptide mapping is from purified baculovirus-expressed recombinant PKC-θ after *in vitro* kinase assays in the absence of exogenous substrates, suggesting that T219 is an autophosphorylation site of PKC-θ (Thuille et al., 2005). T219 phosphorylation of recombinant PKC-θ is further demonstrated by immunoblotting using a phospho-specific antibody, which does not recognize PKC-θ mutants with the T219 substitution with either alanine or glutamic acid (Thuille et al., 2005). In Jurkat T cells or primary T cells, PKC-θ T219 phosphorylation is induced by the phorbol ester PDBu, anti-CD3 alone, anti-CD3 plus anti-CD28, or vanadate (a tyrosine phosphatase inhibitor) (Thuille et al., 2005). The mechanism that induces T219 phosphorylation in TCR signaling remains unknown. However, T219 phosphorylation of PKC-θ is undetectable in the kinase-dead PKC-θ K409R mutant in Jurkat T cells upon cellular stimulation, suggesting that T219 is an inducible autophosphorylation site of PKC-θ during T cell activation (Thuille et al., 2005).

Function of PKC-θ T219 phosphorylation

T219 phosphorylation of PKC-θ is important for PKC-θ-mediated T cell activation upon TCR stimulation (Thuille et al., 2005). PKC-θ T219A mutant abrogates TCR-induced activation of NF-κB and NF-AT as well as subsequent IL-2 promoter transactivation in Jurkat T cells (Thuille et al., 2005). Interestingly, although loss of T219 phosphorylation impairs PKC-θ-mediated cellular function, PKC-θ T219A mutant is catalytically intact *in vitro* (Thuille et al., 2005). Similarly, PKC-θ T219A mutant shows slightly increased lipid-binding activity with PDBu (Thuille et al., 2005). These data suggest that T219 phosphorylation regulates PKC-θ-mediated downstream signaling in T cells through a mechanism independent of PKC-θ kinase activity (Thuille et al., 2005). In contrast, PKC-θ T219A mutant is unable to translocate into lipid rafts or the immunological synapse in Jurkat T cells in response to TCR stimulation or in the presence of antigen-presenting cells; forced membrane translocation of PKC-θ T219A mutant by adding a membrane-targeting sequence reconstitutes TCR-induced NF-κB activation in Jurkat T cells (Thuille et al., 2005). Taken together, these data suggest that T219 phosphorylation is required for properly localizing PKC-θ to the cell membrane, allowing PKC-θ to activate downstream effectors in TCR signaling.

PHOSPHORYLATION OF PKC-θ AT T538 IN THE ACTIVATION MOTIF

Identification of PKC-θ autophosphorylation sites at the catalytic domain

Multiple sequence alignment of the catalytic domains of PKC isoforms shows the conserved serine/threonine phosphorylation residues in the activation loop, turn motif, and hydrophobic motif (Liu et al., 2002; Xu et al., 2004). Phosphorylations of T538 at the activation loop, S676 at the turn motif, and S695 at the hydrophobic motif of PKC-θ have been demonstrated using phospho-specific anti-sera and further confirmed by mass spectrometry (Liu et al., 2002; Czerwinski et al., 2005). Phosphorylation of S685 at the turn motif has been demonstrated only by mass spectrometry (Czerwinski et al., 2005). T538, S676, and S695 are constitutively phosphorylated on recombinant PKC-θ isolated from baculovirus, *E. coli*, or HEK293T expression systems (Liu et al., 2002; Czerwinski et al., 2005; Thuille et al., 2005). The PKC-θ kinase-dead mutant (K409W) abolishes phosphorylation of these three sites in the recombinant PKC-θ, suggesting that they are regulated by autophosphorylation (Liu et al., 2002; Czerwinski et al., 2005). Autophosphorylation can be induced in a *cis* or *trans* manner. An intrapeptide reaction in a mixed micelle system shows that PKC-θ autophosphorylation is regulated by intrapeptide phosphorylation (Newton and Koshland, 1987). Catalytic-competent PKC-θ is unable to phosphorylate PKC-θ-derived peptides containing the individual autophosphorylation sites, further supporting that an intramolecular interaction is required for autophosphorylation on these sites (Thuille et al., 2005). Crystal structure of PKC-θ shows that the activation loop, the turn motif, and hydrophobic motif lie close to the active cleft (Xu et al., 2004), supporting that T538, S676, and S695 are autophosphorylated in *cis*. So far, the function and regulation of T538 phosphorylation are the best known and the most intensively studied, whereas the functions and regulations of other three phosphorylation sites in T cells are less clear.

Function of PKC-θ T538 phosphorylation

The activation loop is a short critical polypeptide that lies outside the active site cleft of the kinase domain (Nolen et al., 2004). The activation loop contributes to binding surfaces for substrates and co-factors (Nolen et al., 2004). The crystal structure of PKC-θ shows that the phosphate of T538 in the activation loop forms hydrogen-bonding interaction with the αC helix, which helps to stabilize the correct orientation of the αC helix and to maintain

active conformation of this kinase (Xu et al., 2004). Loss of T538 phosphorylation in PKC-θ T538A mutant abolishes PKC-θ kinase activity, indicating that T538 is a critical activation site regulating PKC-θ kinase activity (Liu et al., 2002; Czerwinski et al., 2005). Therefore, T538 phosphorylation is widely used as a surrogate marker for PKC-θ kinase activation. T538 phosphorylation does not seem to regulate PKC-θ membrane translocation, because PKC-θ T538A mutant still translocates into lipid rafts in T cells upon anti-CD3/CD28 stimulation (Thuille et al., 2005). Consistent with loss of kinase activation of PKC-θ T538A mutant, the downstream NF-κB and NF-AT activations are abrogated in PKC-θ T538A-transfected Jurkat T cells upon anti-CD3/CD28 costimulation (Liu et al., 2002; Thuille et al., 2005), indicating that T538 is a critical phosphorylation site required for PKC-θ function and T cell activation.

Inducible PKC-θ T538 phosphorylation in T cell signaling

While it is undoubtedly true that T538 is constitutively autophosphorylated in the recombinant PKC-θ proteins isolated from different expression systems (Liu et al., 2002; Xu et al., 2004; Czerwinski et al., 2005; Thuille et al., 2005), whether PKC-θ T538 phosphorylation is constitutive or inducible during T cell activation has been controversial. A constitutive T538 phosphorylation of endogenous PKC-θ has been detected in Jurkat and primary T cells using the phospho-specific antibodies from different sources, and T538 phosphorylation is not further enhanced by TCR or PMA stimulation (Freeley et al., 2005; Thuille et al., 2005; Gruber et al., 2006). The observation of constitutive PKC-θ T538 phosphorylation in T cells reported in some studies maybe due to high basal PKC-θ activity; for example, Jurkat T cells can be easily stimulated with serum (Lee et al., 2006). Earlier studies of PKC-θ phosphorylation in T cells used an electrophoretic mobility shift of PKC-θ in T cells upon anti-CD3/CD28 or PMA/ionomycin stimulation; these studies show that PKC-θ migrates more slowly in activated T cells than in unstimulated T cells (Thebault and Ochoa-Garay, 2004; Puente et al., 2005). Although the phosphorylation of PKC-θ at T538 is not directly examined in these studies due to the lack of use of anti-phospho-PKC-θ (T538) antibody, PKC-θ T538A mutation abolishes the mobility shift of PKC-θ induced by PMA (Thebault and Ochoa-Garay, 2004; Puente et al., 2005), suggesting that T538 phosphorylation is induced by T cell signaling. Using the phospho-specific PKC-θ T538 antibodies from different sources, several groups show that T538 phosphorylation is induced during T cell activation upon anti-CD3 or interferon stimulation (Srivastava et al., 2004; Lee et al., 2005; Liu and Lai, 2005; Park et al., 2009; Chuang et al., 2011). The inducible T538 phosphorylation of PKC-θ is consistent with the inducible PKC-θ kinase activation in T cells upon TCR stimulation (Monks et al., 1997; Coudronniere et al., 2000; Puente et al., 2005). Furthermore, our group has identified GLK as the direct upstream kinase that phosphorylates PKC-θ T538 upon TCR stimulation (Chuang et al., 2011). Therefore, PKC-θ-dependent downstream signaling such as IKK-NF-κB activation may need to be examined as controls for the basal PKC-θ activity in unstimulated T cells (Lee et al., 2005, 2006). Taken together, PKC-θ T538 phosphorylation and its kinase activation are induced in T cells upon TCR stimulation.

Regulation of PKC-θ T538 phosphorylation and activation by GLK in TCR signaling

The kinase that directly phosphorylates PKC-θ was a mystery before the discovery of the role of GLK in TCR signaling. Initially, 3-phosphoinositide-dependent kinase-1 (PDK1) was proposed to be the kinase directly phosphorylating PKC-θ based on the following evidence: (i) PKC-θ T538 phosphorylation is impaired in PDK1-knockdown Jurkat T cells and PDK1-deficient primary T cells upon anti-CD3/CD28 stimulation (Lee et al., 2005; Park et al., 2009); (ii) PDK1 is associated with PKC-θ in the lipid rafts of T cells during anti-CD3/CD28 stimulation (Lee et al., 2005); (iii) PDK1 directly phosphorylates the conserved phosphorylation site at the activation loop of the other PKC isoforms (Le Good et al., 1998; Balendran et al., 2000). However, several observations indicate that PDK1 may not be the direct upstream kinase for PKC-θ: (i) anti-CD3 stimulation alone without anti-CD28 costimulation is sufficient to induce phosphorylation and activation of PKC-θ but not PDK1 (Liu and Lai, 2005; Park et al., 2009); (ii) PDK1-deficient T cells still show residual PKC-θ T538 phosphorylation (Park et al., 2009); and (iii) there is lack of *in vitro* phosphorylation of PKC-θ by PDK1 to demonstrate that PKC-θ is a direct substrate of PDK1. A recent study suggests that AMP-activated protein kinase (AMPK) may be involved in PKC-θ T538 phosphorylation in Jurkat T cells upon PMA/ionomycin stimulation (Lee et al., 2012). In addition, APMK is also implicated in phosphorylation of PKC-ζ on its activation loop in the cells under the hypoxia (Gusarova et al., 2009). However, there is no direct evidence that AMPK directly phosphorylates PKC-θ at T538 in TCR signaling (Lee et al., 2012).

GLK is a Ste20-like serine/threonine kinase that activates the JNK pathway in response to stress stimulation (Diener et al., 1997). Our recent study of the GLK function in T cells revealed an important role of GLK in controlling TCR signaling, T cell activation, immune responses, and autoimmunity via activating PKC-θ (Chuang et al., 2011). GLK-deficient T cells show abolished phosphorylation of PKC-θ T538 and IKK, as well as reduced activation of NF-κB, upon anti-CD3 stimulation; this effect is unlikely mediated by PDK1 because PDK1 is not activated by anti-CD3 stimulation alone (Park et al., 2009; Chuang et al., 2011). Furthermore, PDK1 activation is unaffected in GLK-deficient T cell upon anti-CD3/CD28 costimulation, even though phosphorylations of PKC-θ and IKK are abolished in these cells (Chuang et al., 2011). These data indicate that GLK activates PKC-θ independent or downstream of PDK1. GLK is inducibly and directly associated with PKC-θ in T cells upon anti-CD3 stimulation (Chuang et al., 2011). GLK directly phosphorylates PKC-θ at T538 but not at S695 or S676 residue *in vitro* (Chuang et al., 2011). These data unequivocally demonstrate that GLK is the kinase that directly phosphorylates and activates PKC-θ during TCR signaling. Our unpublished data show that PKC-θ is unable to translocate to cell membrane in GLK-deficient T cells upon anti-CD3 stimulation. This effect is not likely mediated by a loss of PKC-θ T538 phosphorylation in GLK-deficient T cells, because T538A mutation does not affect PKC-θ membrane translocation (Thuille et al., 2005). How GLK regulates PKC-θ membrane translocation remains unknown. One potential mechanism is that GLK may directly or indirectly induce another phosphorylation site

(e.g., T219 or a novel S/T residue) that regulates PKC-θ membrane translocation. Further characterization of GLK-mediated PKC-θ phosphorylation may reveal a novel mechanism that regulates PKC-θ membrane translocation.

PHOSPHORYLATION OF PKC-θ AT S676 AND S685 IN THE TURN MOTIF

S676 is constitutively autophosphorylated in the turn motif of recombinant PKC-θ isolated from HEK293T cells and *E. coli* expression system (Liu et al., 2002; Czerwinski et al., 2005). Basal S676 phosphorylation of PKC-θ has also been observed in Jurkat T cells, CTL clone AB.1 cells, and primary $CD4^+$ T cells (Freeley et al., 2005; Puente et al., 2005; Lee et al., 2010). The basal level of PKC-θ S676 phosphorylation is moderately increased in mouse primary $CD4^+$ T cells upon anti-CD3/CD28 costimulation (Lee et al., 2010). However, some controversy remains about the effects of S676 phosphorylation on the PKC-θ kinase activity and downstream NF-κB activation (Liu et al., 2002; Czerwinski et al., 2005; Thuille et al., 2005). PKC-θ S676A mutant isolated from Jurkat T cells or *E. coli* expression system retains only 30–40% *in vitro* kinase activity compared to WT (Czerwinski et al., 2005; Thuille et al., 2005); furthermore, PKC-θ S676A mutant dramatically suppresses NF-κB activity in Jurkat T cells stimulated by phorbol ester or anti-CD3 (Thuille et al., 2005). In contrast, PKC-θ S676A mutant isolated from HEK293T cells displays intact kinase activity and does not affect TCR-induced NF-κB activity in Jurkat T cells (Liu et al., 2002). These different results could be due to using experimental systems or conditions. It has been observed in PKC-βII that a single mutation of a conserved serine/threonine residue in the turn motif does not affect PKC-βII function due to compensatory phosphorylation at the neighboring residues (Edwards et al., 1999). In a similar note, combinational mutations of S676 and other neighboring serine residues (S662 and S685) in the turn motif of PKC-θ result in a greater loss of its kinase activity (Liu et al., 2002).

Mass spectrometry studies using PKC-θ isolated from *E. coli* expression system reveal that S685 is an autophosphorylation site in the turn motif of PKC-θ (Czerwinski et al., 2005). Another mass spectrometry study using PKC-θ isolated from Jurkat T cells shows that S685 phosphorylation is induced in Jurkat T cells upon anti-CD3 stimulation (Mayya et al., 2009). Although S685 phosphorylation of PKC-θ remains to be further verified using phospho-specific antibody, PKC-θ S685A mutant shows significantly reduced kinase activity (Czerwinski et al., 2005). The effect of S685 phosphorylation on the downstream NF-κB activation has not been reported; but it would be expected to positively regulate PKC-θ function and T cell activation during TCR signaling. How the phosphorylation at the turn motif affects PKC-θ catalytic activity is not clear. The electron density maps in crystallization studies of PKC-θ do not define the turn motif (Xu et al., 2004); however, the crystallization studies of the kinase domains of PKC-βII and PKC-ι show that the phosphate on the turn motif forms intramolecular ionic contacts to help stabilize the active conformation of these enzymes (Messerschmidt et al., 2005; Grodsky et al., 2006). Therefore, the phosphorylation at the turn motif of PKC-θ may contribute to the regulation of its catalytic activity.

PHOSPHORYLATION OF PKC-θ AT S695 IN THE HYDROPHOBIC MOTIF

S695 is a constitutive autophosphorylation site in the C-terminal hydrophobic motif of PKC-θ when isolated from *E. coli*/baculovirus/HEK293T expression systems (Liu et al., 2002; Xu et al., 2004; Czerwinski et al., 2005; Thuille et al., 2005). In Jurkat T cells, human primary T cells, and mouse primary T cells, S695 phosphorylation of PKC-θ is induced upon anti-CD3 with or without CD28 costimulation (Villalba et al., 2002; Freeley et al., 2005; Lee et al., 2010). A deficiency of an essential mTOR complex 2 (mTORC2) subunit RICTOR suppresses PKC-θ phosphorylation at S696 but not at S676 in mouse primary $CD4^+$ T cells upon anti-CD3/CD28 stimulation. Thus, mTORC2 regulates S696 phosphorylation of PKC-θ during TCR signaling. S696 phosphorylation may contribute to catalytic activity of PKC-θ, which is important for mTORC2-mediated Th2 differentiation (Lee et al., 2010). PKC-θ S695A mutant diminishes PKC-θ *in vitro* kinase activity, supporting that S695 phosphorylation is required for optimal PKC-θ kinase activation (Liu et al., 2002; Czerwinski et al., 2005; Thuille et al., 2005). Crystallographic data of PKC-θ catalytic domain further support that S695 phosphorylation contributes to an active conformation of PKC-θ by tightening intramolecular interaction between the hydrophobic motif and the N-lobe to align the αC helix (Xu et al., 2004). Phosphorylation of PKC-βII at the hydrophobic motif regulates subcellular localization in addition to catalytic activity of this kinase (Edwards et al., 1999). Membrane-associated PKC-θ, but not cytosolic PKC-θ, is phosphorylated at S695, raising the possibility that S695 phosphorylation may regulate membrane translocation of PKC-θ (Villalba et al., 2002; Freeley et al., 2005). Two lines of evidence suggest that S695 phosphorylation may not be involved in membrane translocation of PKC-θ: (i) the conventional PKC inhibitor Gö6976 abrogates S695 phosphorylation without affecting membrane translocation of PKC-θ in Jurkat T cells upon anti-CD3/CD28 stimulation (Freeley et al., 2005); and (ii) the N-terminal regulatory region but not the C-terminal kinase domain regulates membrane translocation of PKC-θ during T cell activation (Bi et al., 2001; Thuille et al., 2005). Future studies of PKC-θ membrane translocation in T cells using S695A mutant will provide a conclusive answer. The effect of S695 phosphorylation of PKC-θ on T cell activation varies in different studies. Catalytically active PKC-θ A148E mutant induces NF-κB and NF-AT activation in Jurkat T cells upon anti-CD3/CD28 costimulation, which is greatly inhibited by S695A mutation in PKC-θ A148E mutant (Thuille et al., 2005). In contrast, another study shows that PKC-θ S695A mutant does not affect NF-κB activation in the Jurkat T cells upon anti-CD3/CD28 costimulation; paradoxically, the kinase activity of PKC-θ S695A mutant is impaired in the same study (Liu et al., 2002). However, the regulation of PKC-θ kinase activation by S695 phosphorylation supports that S695 phosphorylation of PKC-θ positively regulates T cell activation during TCR signaling.

Interdependency of different PKC-θ autophosphorylation sites

Previous studies suggest interdependency of different PKC-θ autophosphorylation sites. Loss of T538 phosphorylation in PKC-θ T538A mutant leads to reduced or abolished S676 and S695 phosphorylation in HEK293 cells (Liu et al., 2002). This is likely the consequence of low catalytic activity of T538A mutant, since

S676 and S695 are autophosphorylated on the recombinant PKC-θ protein *in vitro* (Liu et al., 2002). In addition, treatment with a serine/threonine phosphatase inhibitor okadaic acid restores the S676 and S695 phosphorylation but not the catalytic activity of PKC-θ T538A mutant (Liu et al., 2002), supporting an additional or alternative explanation that mutation of one phosphorylation site may affect the phosphorylation status at another site by increasing the susceptibility of this mutant to dephosphorylation by phosphatases. On the other hand, PKC-θ S695A mutant results in a great loss of T538 phosphorylation in HEK293 cells and reduced *in vitro* kinase activity (Liu et al., 2000; Czerwinski et al., 2005); this effect may be attributed to a secondary effect of reduced catalytic activity of S695A mutant. However, this does not seem to apply to PKC-θ S676A mutant, which shows reduced catalytic activity but appears to have intact T538 phosphorylation in HEK293 cells (Liu et al., 2002; Czerwinski et al., 2005). The unphosphorylated turn motif in PKC-βII is bound to HSP70, which stabilizes PKC-βII, allowing re-phosphorylation of this enzyme (Gao and Newton, 2002); this study suggests that PKC-θ S676A may be more stable and ready for autophosphorylation at T538 due to a loss of phosphorylation in the turn motif of this mutant. Another possibility is that PKC-θ T538 can be phosphorylated by other kinases. The relationship of S676 and S695 phosphorylation has not been reported. Phosphorylations of T219 and T538 in PKC-θ are independent of each other, as mutation of either site does not affect phosphorylation on the other site on baculovirus-expressed PKC-θ proteins (Thuille et al., 2005). This finding supports that T219 phosphorylation does not regulate the catalytic activity of PKC-θ (Thuille et al., 2005); however, it is not clear why T219 autophosphorylation is not affected by T538A mutation. One possibility is that T219 of PKC-θ could also be regulated by other kinases. While multiple residues of PKC-θ are constitutively autophosphorylated *in vitro*, the relationship of these phosphorylation residues during TCR signaling has not been studied. Is there sequential phosphorylation of different sites on PKC-θ upon TCR stimulation? Is there spatial regulation of PKC-θ phosphorylation during T cell activation? Future studies of these questions may reveal a novel regulation of PKC-θ activation and function by phosphorylation.

CONCLUDING REMARKS

In conclusion, phosphorylations play critical roles in regulating PKC-θ catalytic activity and membrane translocation, both of which are required for the proper function of PKC-θ in T cell activation. The regulation of PKC-θ phosphorylation is much more complex than we initially thought. The individual PKC-θ phosphorylation sites can be induced by autophosphorylation or upstream kinases depending on different cell types or cell stimuli. In TCR signaling, T538 and Y90 are phosphorylated on PKC-θ by GLK and LCK, respectively. Although phosphorylations of T219, S676, S685, and S695 are all induced during TCR signaling, the direct kinases for the phosphorylation of these sites on PKC-θ remain unclear. T538 phosphorylation is most critical for PKC-θ catalytic activation, whereas S676, S685, and S695 may be required for optimal catalytic activation of PKC-θ during TCR signaling. Phosphorylations of Y90 and T219 regulate PKC-θ membrane translocation and T-cell activation. PKC-θ is recruited to different locations in effector T cells and regulatory T cells and performs opposite functions in these cells (Zanin-Zhorov et al., 2010). For example, PKC-θ localizes to the immunological synapse and positively regulates cellular activation in effector T cells, while PKC-θ is sequestered away from the immunological synapse in stimulated regulatory T cells and negatively regulates cell function (Zanin-Zhorov et al., 2010). On a similar note, a substantial amount of PKC-θ is constitutively located in the nucleus of T cells, where it regulates gene expression (Sutcliffe et al., 2011). It might therefore be appropriate to speculate that different phosphorylation sites (e.g., Y90, T219, or other sites) of PKC-θ may influence its distribution to different subcellular locations. The model of regulation of PKC-θ phosphorylation in TCR signaling is shown (**Figure 3**).

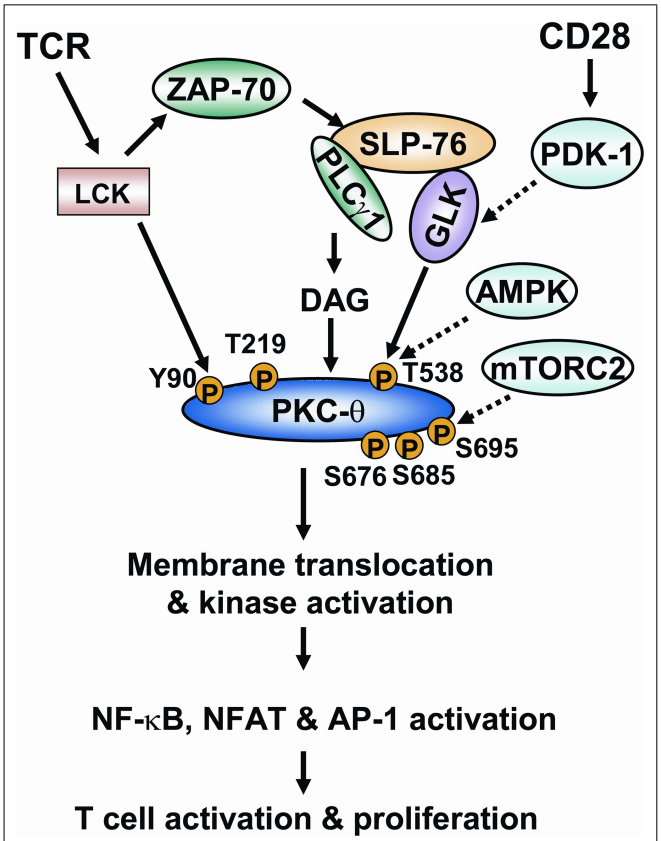

FIGURE 3 | The model of regulation of PKC-θ phosphorylation during TCR signaling. Ligation of TCR induces the activation of tyrosine kinase LCK. LCK activates ZAP-70, which in turn induces tyrosine phosphorylation of the adaptor SLP-76. SLP-76 directly interacts with and activates PLCγ1 and GLK. PLCγ1 cleaves a phospholipid, generating the second messenger diacylglycerol. The binding of diacylglycerol with PKC-θ induces the conformational change of PKC-θ; T538 of PKC-θ is then phosphorylated by GLK, leading to catalytic activation of PKC-θ. The CD28 costimulatory activates PDK-1, which facilitates PKC-θ T538 phosphorylation possibly via activating GLK. AMPK is also implicated in PKC-θ T538 phosphorylation in T cells. LCK directly interacts with and phosphorylates PKC-θ at Y90. The phosphorylation of Y90 and another residue (T219) induces membrane translocation of PKC-θ. S676, S685, and S695 residues are also phosphorylated on PKC-θ upon TCR stimulation. mTORC2 activity is required for PKC-θ S695 phosphorylation in T cells. Catalytic activation and membrane translocation of PKC-θ lead to the activation of transcription factors NF-κB, NF-AT, and AP-1, and to subsequent T cell activation.

How such a complex regulation of PKC-θ phosphorylation is integrated with TCR/CD28 signaling is the key for the future understanding of PKC-θ function and T cell activation. Specifically, what kinases or phosphatases regulate the important PKC-θ phosphorylation sites in T-cell signaling? Does the phosphorylation of PKC-θ at different residues occur in a sequential and spatial manner during T cell signaling? How does PKC-θ phosphorylation at a specific site regulate its catalytic activity and membrane translocation? Future studies of the regulatory mechanisms of PKC-θ phosphorylation are important not only for the understanding of the underlying mechanism of PKC-θ-mediated cell activation, but also for the design of novel therapeutic targets for PKC-θ-mediated inflammatory diseases.

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Regulating the regulator: phosphorylation of PKC θ in T cells

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A commentary on

Regulation of PKC-θ function by phosphorylation in T cell receptor signaling
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Protein kinase C θ (PKC θ) is a serine/threonine kinase that is now firmly established as a central component in T cell activation, proliferation, differentiation, and apoptosis (Hayashi and Altman, 2007). Since it was first discovered that PKC θ re-localizes to the immunological synapse (IS) in conventional effector T cells following T cell stimulation, many roles have now been defined for this kinase in these cells such as (a) activation of NF-κB, AP-1, and NFAT transcription factors that control the synthesis of pro-inflammatory cytokines and the anti-apoptotic molecule Bcl-x_L (Hayashi and Altman, 2007), (b) regulation of IS dynamics (Sims et al., 2007), (c) up-regulation and clustering of the integrin LFA-1 on the T cell surface (Tan et al., 2006; Letschka et al., 2008) – thus facilitating stable adhesion between T cells and antigen-presenting cells (APC) and/or migration into inflamed tissues, (d) re-orientation of the microtubule-organizing center toward the APC (Quann et al., 2011), and (e) fine tuning of T cell activation by regulating the intracellular localization, degradation, and internalization of key signaling molecules (Nika et al., 2006; von Essen et al., 2006; Gruber et al., 2009). A new function for PKC θ has also recently been revealed with the finding that this kinase regulates an inducible gene expression program in T cells by associating with chromatin in the nucleus (Sutcliffe et al., 2011).

A host of studies have now convincingly demonstrated that targeting PKC θ could be a viable therapeutic strategy to block the T cell inflammatory response in autoimmunity, allergy, and allograft rejection (Marsland and Kopf, 2008; Zanin-Zhorov et al., 2011; Altman and Kong, 2012). For example, PKC θ-deficient mice (PKC $\theta^{-/-}$) have reduced incidence and severity of Th2 and Th17-mediated inflammatory disorders, including asthma, inflammatory bowel disease, multiple sclerosis, arthritis, and allograft rejection in comparison to their wild-type littermates (PKC $\theta^{+/+}$; Marsland and Kopf, 2008; Zanin-Zhorov et al., 2011; Altman and Kong, 2012). Intriguingly, PKC$\theta^{-/-}$ mice are still capable of mounting relatively normal Th1 and $CD8^+$ T cell-mediated immune responses to infectious viruses (Marsland and Kopf, 2008; Zanin-Zhorov et al., 2011; Altman and Kong, 2012). Secondly, the recent finding that inhibition of PKC θ increases the suppressive activity of regulatory T cells (Zanin-Zhorov et al., 2010) suggests that therapeutic strategies designed to inhibit this kinase may hold great promise in diverting the pro/anti-inflammatory balance toward a reduction in inflammation in T cell autoimmunity and allergy, whilst at the same time maintaining immunity to viral pathogens. Lastly, that PKC θ has a restricted tissue expression profile and is highly expressed in T cells suggests that targeting this molecule with specific inhibitors should have minimal effects in other cells and tissues (Hayashi and Altman, 2007; Altman and Kong, 2012). In spite of all this promising data however, a number of studies have demonstrated that targeting PKC θ could potentially have some undesired effects. For example, it has been reported that $CD8^+$ T cells from PKC $\theta^{-/-}$ mice have a survival defect following activation (Barouch-Bentov et al., 2005; Saibil et al., 2007; Kingeter and Schaefer, 2008). In addition, it has been reported that PKC $\theta^{-/-}$ mice have an impaired anti-leukemic response (Garaude et al., 2008), which likely results from reduced tumor surveillance *in vivo*. It is important therefore that these issues are addressed in respect of any PKC θ-targeting strategies that are developed in the future.

Although much has been learned about PKC θ in T cells, considerable gaps still exist in our knowledge as to how this kinase is regulated, including the upstream signals and interacting partners that control its intracellular localization and catalytic activation at various locations in the cell. Furthermore, although a plethora of substrates that are phosphorylated by PKC θ *in vitro* have now been characterized (Nika et al., 2006; Hayashi and Altman, 2007; Letschka et al., 2008), whether any of these are *bona fide* substrates *in vivo* remains to be addressed. Like many other kinases, PKC θ is also regulated by phosphorylation on a host of serine, threonine, and tyrosine residues that influence its activity and intracellular localization. Six phosphorylation sites have been mapped on PKC θ in T cells to date. Some of these sites appear to be phosphorylated by unrelated upstream kinases, while other sites are regulated via auto-phosphorylation. Three of these phosphorylation sites are highly conserved on most other PKC isoforms, which suggests that they may regulate aspects that are central to all isoforms, such as stability. In contrast, PKC θ contains three phosphorylation sites that appear to be unique to this isoform.[1] Therefore PKC θ may execute distinct functions and/or be regulated differently in T cells (Freeley et al., 2011). In this issue of *Frontiers in T Cell Biology*, Wang et al. (2012) summarize the regulation of PKC θ by phosphorylation during T cell signaling. Understanding the pathways

[1]Some of these three residues on PKC θ may also be found in other PKC isoforms, but their phosphorylation on other PKCs has not been described.

that regulate PKC θ in T cells may provide additional therapeutic targets for the treatment of inflammatory diseases.

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PKC-theta-mediated signal delivery from the TCR/CD28 surface receptors

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Protein kinase C-theta (PKCθ) is a key enzyme in T lymphocytes, where it plays an important role in signal transduction downstream of the activated T cell antigen receptor (TCR) and the CD28 costimulatory receptor. Interest in PKCθ as a potential drug target has increased following recent findings that PKCθ is essential for harmful inflammatory responses mediated by Th2 (allergies) and Th17 (autoimmunity) cells as well as for graft-versus-host disease (GvHD) and allograft rejection, but is dispensable for beneficial responses such as antiviral immunity and graft-versus-leukemia (GvL) response. TCR/CD28 engagement triggers the translocation of the cytosolic PKCθ to the plasma membrane (PM), where it localizes at the center of the immunological synapse (IS), which forms at the contact site between an antigen-specific T cell and antigen-presenting cells (APC). However, the molecular basis for this unique localization, and whether it is required for its proper function have remained unresolved issues until recently. Our recent study resolved these questions by demonstrating that the unique V3 (hinge) domain of PKCθ and, more specifically, a proline-rich motif within this domain, is essential and sufficient for its localization at the IS, where it is anchored to the cytoplasmic tail of CD28 via an indirect mechanism involving Lck protein tyrosine kinase (PTK) as an intermediate. Importantly, the association of PKCθ with CD28 is essential not only for IS localization, but also for PKCθ-mediated activation of downstream signaling pathways, including the transcription factors NF-κB and NF-AT, which are essential for productive T cell activation. Hence, interference with formation of the PKCθ-Lck-CD28 complex provides a promising basis for the design of novel, clinically useful allosteric PKCθ inhibitors. An additional recent study demonstrated that TCR triggering activates the germinal center kinase (GSK)-like kinase (GLK) and induces its association with the SLP-76 adaptor at the IS, where GLK phosphorylates the activation loop of PKCθ, converting it into an active enzyme. This recent progress, coupled with the need to study the biology of PKCθ in human T cells, is likely to facilitate the development of PKCθ-based therapeutic modalities for T cell-mediated diseases.

Keywords: protein kinase C-theta, PKCθ, CD28, Lck, signal transduction, costimulation

INTRODUCTION

Protein kinase C-theta (PKCθ) is a key regulator of signal transduction in activated T cells that is linked to multiple pathways downstream of the T cell antigen receptor (TCR; Isakov and Altman, 2002). Engagement of the TCR and the resulting formation of diacylglycerol (DAG) are sufficient for promoting PKCθ recruitment to cell membranes (Monks et al., 1997, 1998). However, localization of PKCθ to the immunological synapse (IS) is entirely dependent on the concomitant ligation of the CD28 coreceptor (Huang et al., 2002). Localization of PKCθ at the center of the IS is essential for activation of signaling pathways that promote T cell-dependent immune responses against distinct antigens and pathogens. While the recruitment of PKCθ to the IS of TCR/CD28 engaged T cells has been extensively studied, information on the molecular basis for this highly selective process has been relatively scarce until recently. The present manuscript provides background information on the molecules involved in this process and describes in more detail the studies that clarified a new mechanism by which PKCθ is being recruited to the center of the IS and is essential for the induction of PKCθ-dependent activation signals.

THE PKC FAMILY

Protein kinase C was discovered by Nishizuka and colleagues, who demonstrated a new kinase that undergoes activation by limited proteolysis (Inoue et al., 1977), or by translocation to the plasma membrane (PM), where it associates with specific cofactors (Takai et al., 1979). The membrane-associated PKC-activating factor turned to be DAG (Kishimoto et al., 1980). DAG, together with inositol 1,4,5-trisphophate (IP_3), are products of phospholipase C-mediated hydrolysis of the membrane phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP_2; Berridge and Irvine, 1984; Nishizuka, 1984). These two second messengers transduce signals from a plethora of activated receptors: the hydrophobic

DAG remains bound to the cell membrane where, in addition to PKC, it activates effector molecules such as RasGRP, a guanine nucleotide exchange factor (GEF) for Ras (Lorenzo et al., 2000), while the hydrophilic IP_3 diffuses through the cytosol and binds IP_3-receptors, which function as ligand-gated Ca^{2+} channels in the endoplasmic reticulum (ER), thereby triggering the release of free Ca^{2+} ions into the cytoplasm (Takai et al., 1979; Khan et al., 1992; Bourguignon et al., 1994). The utilization of phorbol esters, which mimic the activity of DAG, together with Ca^{2+} ionophores, demonstrated that PKC also plays an essential role in the induction of T lymphocyte proliferation (Truneh et al., 1985; Isakov and Altman, 1987) and reactivation of effector cytotoxic T cells (Isakov and Altman, 1985; Isakov et al., 1987).

Protein kinase C enzymes transduce a myriad of signals from a large number of cell surface receptors that are coupled to phospholipase C and phospholipid hydrolysis. They regulate the function of effector molecules by phosphorylating specific serine and threonine residues. The PKC family includes 10 structurally and functionally related isoforms (for more details, see the first review by Pfeifhofer-Obermair et al., 2012), grouped into three subfamilies based on the composition of their regulatory domains and their respective cofactor requirements (Newton, 1995; Mellor and Parker, 1998). The first subfamily includes conventional PKCs (cPKC; α, βI, βII, γ) that are regulated via two DAG-binding C1 domains organized in tandem near the cPKC amino terminus (Hurley et al., 1997; Johnson et al., 2000; Ho et al., 2001) and an adjacent Ca^{2+} and phospholipid-binding C2 domain (Nalefski and Falke, 1996; Johnson et al., 2000). The second group includes novel PKCs (nPKC; δ, ε, η, θ) that are DAG-dependent, but Ca^{2+} and phospholipid independent for their activity. The third group includes atypical PKCs (aPKC: ζ, λ/ι) that are DAG-, Ca^{2+}-, and phospholipid-independent. While PKC enzymes are involved in metabolic processes in different cell types, many studies implicate PKC enzymes in signal transduction networks that convert environmental cues into cellular actions (Rosse et al., 2010). Six of the PKC isoforms, including PKCα, δ, ε, η, θ, and ζ are expressed at varying amounts in T cells (Meller et al., 1999). Immunological studies using different genetic models and pharmacological drugs indicated that distinct PKC isoforms are required for different aspects of the activation and effector functions of T cells. The results suggest that distinct PKC isoforms may serve as drug targets for different T cell mediated adaptive immune responses (Baier and Wagner, 2009).

PROTEIN KINASE C-THETA

Protein kinase C-theta is a Ca^{2+}-independent nPKC isoform exhibiting a relatively selective pattern of tissue distribution, with predominant expression in T lymphocytes (Baier et al., 1993; Meller et al., 1999), platelets (Chang et al., 1993; Meller et al., 1998; Cohen et al., 2009), and skeletal muscle (Osada et al., 1992; Chang et al., 1993). It has a unique ability to translocate to the center of the IS of activated T cells (Monks et al., 1997, 1998) where its full activation requires the integration of TCR and CD28 costimulatory signals (Huang et al., 2002; Tseng et al., 2008; Yokosuka et al., 2008). Engagement of the TCR and the CD28 coreceptor initiates a series of PKCθ-dependent signaling events leading to activation of transcription factors, including NF-κB, AP-1, and NF-AT, which are critical for T cell activation, proliferation and differentiation (Baier-Bitterlich et al., 1996; Coudronniere et al., 2000; Dienz et al., 2000; Lin et al., 2000; Sun et al., 2000; Pfeifhofer et al., 2003). Under certain activation conditions, PKCθ can translocate to the nucleus where it directly associates with chromatin and is involved in the regulation of microRNAs and T cell-specific inducible gene expression program (Sutcliffe et al., 2011). The exact mechanism by which the membrane-bound PKCθ delivers signals to the nucleus has not been fully resolved but studies provided information on a number of effector molecules that operate along this pathway in activated T cells. These studies demonstrated that PKCθ-mediated regulation of NF-κB activity involves the multisubunit inhibitor of κB (IκB) kinase (IKK) complex (Coudronniere et al., 2000; Dienz et al., 2000; Khoshnan et al., 2000; Lin et al., 2000; Bauer et al., 2001).

An important upstream effector in the NF-κB signaling pathway is IκBα, which binds NF-κB in the cytoplasm of resting T cells and mask its nuclear localization signal (NLS), thereby preventing NF-κB translocation to the nucleus (Mercurio et al., 1997; Regnier et al., 1997; Jacobs and Harrison, 1998). IKK-mediated phosphorylation of IκBα signals the protein for degradation (Karin, 1999), exposes the NF-κB NLS and promotes NF-κB translocation to the nucleus and the induction of NF-κB-mediated gene transcription. T cells from PKCθ-deficient ($Prkcq^{-/-}$) mice fail to respond to TCR stimulation with degradation of IκBα (Sun et al., 2000), supporting the model whereby PKCθ regulates NF-κB activity through its effect on IKK-IκBα. Some of the effector molecules that link PKCθ to IKK have been identified and include the PKCθ substrate protein, caspase activation and recruitment domain (CARD) and membrane-associated guanylate kinase (MAGUK) domain-containing protein-1 (CARMA1). This scaffold protein is primarily expressed in lymphocytes (Bertin et al., 2001; Hara et al., 2003), where it links PKCθ to NF-κB activation in T cells (Ruland et al., 2001, 2003; Ruefli-Brasse et al., 2003; Xue et al., 2003). Phosphorylation of CARMA1 by PKCθ in TCR/CD28-stimulated T cells, promotes CARMA1 association with the B-cell lymphoma/leukemia 10 (Bcl10) and mucosa-associated lymphoid tissue 1 (MALT1) proteins (Matsumoto et al., 2005; Sommer et al., 2005) leading to recruitment of the trimolecular complex to the IS (Gaide et al., 2002; Che et al., 2004; Hara et al., 2004) and activation of the IKK complex (McAllister-Lucas et al., 2001). Furthermore, overexpression of CARMA1, Bcl10, and MALT1 in T cells, followed by TCR/CD28 stimulation, resulted in the formation of a CARMA1-Bcl10-MALT1 trimolecular complex, where all three proteins were required for maximal activation of NF-κB (McAllister-Lucas et al., 2001; Ruland et al., 2001). It should be noted that in some studies (Khoshnan et al., 2000), but not others (Lin et al., 2000), PKCθ was found to directly associate with members of the IKK complex, particularly IKKβ, suggesting the potential existence of an additional linear route from PKCθ to NF-κB. The transcription factor AP-1, similar to NF-κB, is a primary physiological target of PKCθ (Baier-Bitterlich et al., 1996; Li et al., 2004), while regulation of the NF-AT transcription factor requires cooperation between PKCθ and calcineurin, a Ca^{2+}-dependent serine/threonine phosphatase (Pfeifhofer et al., 2003).

All three PKCθ-regulated transcription factors have corresponding binding sites on the IL-2 gene promoter, and their binding to the IL-2 gene is essential for optimal IL-2 response (Isakov and Altman, 2002).

While PKCθ-mediated regulation of NF-κB activity in TCR/CD28-stimulated T cells has been studied in great detail, PKCθ is also involved in the regulation of additional cellular functions, and physically associates with additional binding partners. Besides CARMA1, PKCθ physically associate with 14-3-3τ (Meller et al., 1996), Cbl (Liu et al., 1999), Fyn (Ron et al., 1999), Lck (Liu et al., 2000), AKT (Bauer et al., 2001), moesin (Pietromonaco et al., 1998), PICOT (Witte et al., 2000), and the HIV nef protein (Smith et al., 1996). Some of these molecules (i.e., Lck) phosphorylate PKCθ and may affect its activity and/or subcellular distribution, while others, which serve as substrates for PKCθ (i.e., Cbl, 14-3-3τ and moesin) may regulate cellular functions, such as cytoskeletal reorganization.

DIFFERENTIAL REQUIREMENTS FOR PKCθ BY DISTINCT T CELL SUBPOPULATIONS

Initial characterization of PKCθ-deficient T cells suggested the involvement of PKCθ in cellular responses leading to T cell activation, proliferation, and cytokine production (Sun et al., 2000; Pfeifhofer et al., 2003; Anderson et al., 2006). Subsequent *in vitro* and *in vivo* investigations and the analysis of *Prkcq*$^{-/-}$ mice in different disease models demonstrated differential requirements for PKCθ by distinct T cell subpopulations and during the induction of selected types of immune responses. Thus, PKCθ was found to be essential for the induction of Th2-type immune responses to allergens or helminth infection (Marsland et al., 2004; Salek-Ardakani et al., 2004) and the induction of Th17-mediated experimental autoimmune encephalomyelitis (EAE) that serves as a model of multiple sclerosis (Salek-Ardakani et al., 2005; Anderson et al., 2006; Tan et al., 2006; Marsland et al., 2007; Kwon et al., 2012), and other experimental autoimmune diseases (Anderson et al., 2006; Healy et al., 2006; Marsland et al., 2007; Chuang et al., 2011). In contrast, Th1-dependent mouse resistance to *Leishmania major* infection was intact in *Prkcq*$^{-/-}$ mice (Marsland et al., 2004; Ohayon et al., 2007), and PKCθ was dispensable for CTL-mediated protective antiviral responses, most likely reflecting compensation by innate immunity signals (Berg-Brown et al., 2004; Giannoni et al., 2005; Marsland et al., 2005, 2007; Valenzuela et al., 2009). Consistent with the *in vivo* findings, *in vitro* induction of CD4+ T cell polarization by optimal T cell-antigen-presenting cell (APC) coculture conditions, demonstrated a requirement for PKCθ during Th2 and Th17 cell development, and only moderate effect of PKCθ on Th1 cell development (Marsland et al., 2004; Salek-Ardakani et al., 2004, 2005). Additional studies performed in *Prkcq*$^{-/-}$ mice demonstrated the requirement for PKCθ in the induction of graft-versus-host (GvH) and alloreactive T cell-mediated immune responses (Valenzuela et al., 2009). In contrast, PKCθ-deficient T cells retained the ability to induce graft-versus-leukemia (GvL) responses in allogeneic bone marrow (BM) transplanted mice (Valenzuela et al., 2009).

Protein kinase C-theta also contributes to allograft rejection, as shown by Manicassamy et al. (2008) using an adoptive transfer model. In these studies, *Rag*$^{-/-}$ mice reconstituted with *Prkcq*$^{-/-}$ T cells were unable to reject cardiac allografts, in contrast to the acute allograft rejection observed in the wild-type T cell reconstituted *Rag*$^{-/-}$ mice. However, this was due to lack of PKCθ-regulated expression of anti-apoptotic molecules, such as Bcl-x_L, which led to apoptosis of the effector T cells; transgenic expression of Bcl-x_L in *Prkcq*$^{-/-}$ T cells restored their ability to reject the cardiac allografts. The rejection of cardiac allograft by *Prkcq*$^{-/-}$ mice was only slightly delayed (Manicassamy et al., 2008; Gruber et al., 2009), suggesting compensation by other PKC isoforms. Indeed, mice lacking both PKCθ and PKCα, demonstrated a significantly delayed rejection of cardiac allografts (Gruber et al., 2009).

The overall positive role of PKCθ in the activation of effector T cells (T_{eff}) and the promotion of adaptive immune responses raise questions about the nature of its function in regulatory T cells (T_{reg}) that suppress T_{eff} functions. This issue has recently been partially resolved by Zanin-Zhorov et al. (2010) who found that PKCθ mediates negative feedback on T_{reg} functions. Furthermore, activation of T_{reg} resulted in sequestration of PKCθ away from the IS, and inhibition of PKCθ activity (using the C20 compound) increased the suppressive activity of T_{reg} (Zanin-Zhorov et al., 2010, 2011). *In vivo* studies demonstrated that T_{reg} development in the thymus of *Prkcq*$^{-/-}$ mice is impaired leading to reduced numbers of T_{reg} cells in the periphery (Schmidt-Supprian et al., 2004; Zanin-Zhorov et al., 2010, 2011), although activity of these mature PKCθ-deficient T_{reg} cells was intact (Gupta et al., 2008).

THE IMMUNOLOGICAL SYNAPSE

Adaptive immune responses are dependent on the effective communication between antigen-specific T cells and APCs. At the very early phase of the activation response, T cells interact via their TCR with cognate peptide-MHC complexes on the surface of APCs and both cell types respond by redistributing their receptors/ligands to the contact area that rearranges as a platform for effective signaling (Dustin and Zhu, 2006). The IS, representing the interface between a T cell and an APC, is formed by specific protein microclustering (Yokosuka et al., 2005) and their segregation into one of two separate regions: a central core [central supramolecular activation clusters (cSMAC)], which contains the TCR and costimulatory receptors, and a peripheral region [peripheral supramolecular activation clusters (pSMAC)], which contains adhesion molecules, such as LFA-1 (Dustin, 2009). T cell surface receptor engagement triggers signaling cascades that result in the recruitment of multiple membrane-anchored and cytoplasmic effector molecules, including kinases, adaptor proteins, and cytoskeletal components, to the IS (Dustin et al., 2010). One of the most prominent proteins to be recruited to the IS of antigen-responding T cells is PKCθ, which localizes at the cSMAC (Monks et al., 1997, 1998). Additional high-resolution imaging analysis by TIRF microscopy demonstrated that PKCθ colocalizes with CD28, and demonstrated that the cSMAC is divided into two structurally and functionally distinct compartments: a central TCR^{high} compartment, where signaling is terminated (Vardhana et al., 2010) and TCR-associated signaling complexes are internalized and degraded, and an outer TCR^{low} "ring" where PKCθ and CD28 colocalize (Yokosuka et al., 2008).

CD28

CD28 is a type 1 transmembrane glycoprotein that is constitutively expressed as a disulfide-linked homodimer on all CD4+ and CD8+ murine T cells and majority of CD4+ and CD8+ human peripheral blood T cells (Gross et al., 1990; Vallejo, 2005). The human CD28 precursor protein is 220 amino acids long (218 in mouse) and the mature protein possesses 202 amino acids (218 in mouse) due to cleavage of an amino-terminal leader sequence (18 and 19 amino acids in the human and mouse CD28, respectively). In addition, CD28 possesses a cytoplasmic tail of 41 amino acids (38 in mouse) that is critical for signal transduction and coreceptor-induced cell stimulation. Physiological activation of CD28 is mediated by one of two natural ligands expressed on the surface of APCs, CD80, and CD86, which directly associate with a conserved motif [MYPPPY (single amino-acid letter code)] in the extracellular region of CD28 (Kariv et al., 1996; Truneh et al., 1996). Engagement of CD28 provides costimulatory signals that complement or synergize with those provided by the TCR, leading to optimal activation of T cells (Thompson et al., 1989; Harding et al., 1992). CD28 engagement increases IL-2 production (Thompson et al., 1989; Jain et al., 1995; Reichert et al., 2001) and IL-2 receptor expression (Shahinian et al., 1993), and provides survival signals by upregulating the anti-apoptotic protein, Bcl-X_L (Boise et al., 1993). In addition, CD28 synergizes with the TCR in providing potent signals for activation of c-Jun kinase (JNK), p38 MAP kinase, and IKK pathways (Su et al., 1994; Harhaj and Sun, 1998), and activation of the NF-κB (Michel et al., 2000; Diehn et al., 2002) AP-1 (Rincon and Flavell, 1994) and NF-AT transcription factors (Michel et al., 2000; Diehn et al., 2002).

The positive role of CD28 in T cell activation was demonstrated in CD28-deficient (*Cd28*$^{-/-}$) T cells, in which TCR engagement in the absence of CD28 costimulation resulted in anergy and/or tolerance induction upon rechallenge with the same antigen (Appleman and Boussiotis, 2003). T cell proliferation and Th2-type cytokine secretion were also severely impaired in *Cd28*$^{-/-}$ mice or wild-type mice treated with CD28 antagonists (Green et al., 1994; Lucas et al., 1995; Rulifson et al., 1997; Schweitzer et al., 1997; Gudmundsdottir et al., 1999). Furthermore, lack of CD28-mediated costimulation led to reduced immune responses against infectious pathogens (Shahinian et al., 1993; King et al., 1996; Mittrucker et al., 2001; Compton and Farrell, 2002) and allografts (Salomon and Bluestone, 2001) and impaired GvH disease (Via et al., 1996), contact hypersensitivity (Kondo et al., 1996), and asthma (Krinzman et al., 1996).

T cell receptor engagement in the absence of CD28 costimulation induces an unbalanced signaling response in which TCR-mediated Ca^{2+} influx predominates. This leads to activation of calcineurin which dephosphorylates NF-AT leading to its nuclear translocation and induction of a limited set of anergy-associated genes resulting in T cell anergy (Macian et al., 2004). CD28, in contrast to the TCR, does not induce a Ca^{2+} response (Lyakh et al., 1997). Instead, CD28-coupled costimulatory signals induce the activation of NF-κB and AP-1, and concomitant AP-1 association with NF-AT, conditions that promote IL-2 prod uction and rescue of the T cells from a state of anergy (Macian et al., 2004).

SIGNALING DOWNSTREAM OF CD28

CD28 delivers signals in activated T cells via its cytoplasmic tail, which has no intrinsic catalytic activity, but possesses several protein–protein interaction motifs that enable it to associate with enzymes and other effector molecules (Boise et al., 1993; see **Figure 1**). In resting T cells, non-phosphorylated CD28 associates with the serine/threonine protein phosphatase protein 2A (PP2A), which dissociates from CD28 upon activation induced-phosphorylation of CD28 (Chuang et al., 2000). CD28 triggering by its ligands leads to phosphorylation of tyrosine residues (Raab et al., 1995; Teng et al., 1996; King et al., 1997) in the cytoplasmic tail of CD28, creating new docking sites for different effector molecules that initiate the activation of signaling cascades, and define the costimulatory functions of CD28 (Raab et al., 1995; Andres et al., 2004; Dodson et al., 2009).

The first motif in the human CD28 cytoplasmic tail, juxtaposed to the PM, contains a Y^{173}MNM sequence that undergoes tyrosine phosphorylation following the engagement of CD28 and serves as a binding site for the SH2 domain of p85, the regulatory subunit of the lipid kinase, phosphatidylinositol 3-kinase (PI3K; August and Dupont, 1994; Pages et al., 1994; Prasad et al., 1994; Truitt et al., 1994). The methionine residue at the +3 position confers specificity for p85 binding (Takeda et al., 2008), while the asparagine at the +2 position confers additional specificity for the SH2 domain of Grb2 and GADS (Songyang et al., 1993; Raab et al., 1995; Sanchez-Lockhart et al., 2004; Schneider et al., 1995; Harada et al., 2001). The relative concentration of PI3K, Grb2, and GADS at the vicinity of CD28 cytoplasmic tail, and the relative affinity of their SH2 domain for the phospho-Tyr^{173}-containing

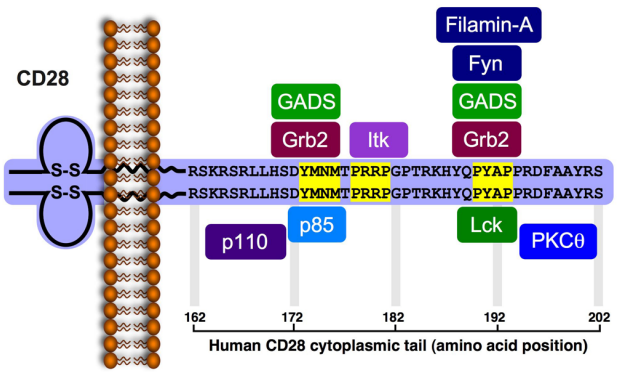


FIGURE 1 | Signaling motifs in the cytoplasmic tail of the human CD28 and binding partners. The human *CD28* encodes a 220 amino acid-long protein (218 in the mouse) that includes a leader sequence of 18 residues (19 residues in the mouse). The mature protein (202 residues) possesses a 41 amino acid-long cytoplasmic tail that includes three potential protein-protein interaction motifs (highlighted in yellow). The phospho-Tyr^{173} within the YMNM motif serves as a docking site for the SH2-containg proteins, p85, Grb2 and GADS. The P^{178}RRP motif can interact with the SH3 domain of Itk. The P^{190}YAP motif can interacts with the SH3 domain of Grb2, GADS and Lck, as well as with filamin-A. Phosphorylation of Tyr^{191} within the PYAP motif creates a docking site for the Lck SH2 domain and enables PKCθ to interact via its V3 domain with the Lck SH3. Studies indicate that Tyr^{191} is important for CD28 and PKCθ localization to the cSMAC, and that the PYAP motif contributes to T cell activation and cytokine expression.

motif likely determine which of the three potential binding partners interacts with the activated CD28 and, hence, the resulting functional outcome. A second, nearby motif possesses the P^{178}RRP sequence, and serves as a binding site for the SH3 domain of IL-2-inducible T cell kinase (Itk; Marengere et al., 1997; Garcon et al., 2004). CD28-mediated activation of Itk is dependent on Lck (Gibson et al., 1996), but the actual role of Itk in CD28-induced costimulation is still controversial (Liao et al., 1997; Gibson et al., 1998; Yang and Olive, 1999; Li and Berg, 2005). A third, more distal, P^{190}YAP motif serves as a potential docking site for several different effector molecules. These include filamin-A, an actin binding protein and a scaffold for lipid raft formation, which utilizes repeat 10 (amino acids 1158–1246) for interaction with CD28 (Tavano et al., 2006), Grb2 and GADS adaptor proteins, which bind the P^{190}YAP motif via their SH3 domain (Okkenhaug and Rottapel, 1998; Ellis et al., 2000), and the Lck and Fyn protein tyrosine kinases (PTKs; Hutchcroft and Bierer, 1994; zur Hausen et al., 1997; Holdorf et al., 1999; Tavano et al., 2004). Both Lck and Fyn were implicated in the early phase of the CD28 signaling pathway (August et al., 1994) and coexpression studies demonstrated that the two PTKs could phosphorylate CD28, primarily on Tyr^{173} at the Y^{173}MNM motif, thereby increasing the binding of p85- and Grb2-SH2 to CD28 (Raab et al., 1995). Lck and Fyn were also found to coimmunoprecipitate with CD28 from activated T cells (Hutchcroft and Bierer, 1994), where Lck interacted with the P^{190}YAP motif via its SH3 domain (Holdorf et al., 1999; Tavano et al., 2004), and Fyn interacted with the same motif using its SH2 domain (zur Hausen et al., 1997), although other studies indicated no interaction between CD28 and Fyn (Marengere et al., 1997). While presence of the two proline residues in the P^{190}YAP motif predicts interaction with SH3-containg proteins, binding studies demonstrated that the Lck-SH3 domain interacts with relatively low affinity ($K_d > 1$ μM) with peptides that contain the P^{190}YAP motif and correspond to residues 188–202 of human CD28, or 186–196 of murine CD28, respectively (Hofinger and Sticht, 2005).

Other studies demonstrated that Tyr^{191} within the P^{190}YAP motif is one of two major phosphorylation sites in CD28-stimulated Jurkat T cells, and the only tyrosine residue within the CD28 cytoplasmic tail that is essential for delivery of costimulatory signals leading to CD69 expression and synthesis and secretion of IL-2 (Sadra et al., 1999). The latter findings raise the possibility that CD28 engagement-induced phosphorylation of Tyr^{191} creates a new and transient binding site for SH2-containing proteins, possibly Lck, since CD28 and Lck were shown to colocalize at the cSMAC (Tavano et al., 2004; Kong et al., 2011). Binding studies provided further support for this hypothesis by showing that a CD28-derived peptide that possesses phospho-Tyr^{191} interacts with the Lck-SH2 domain with a relatively high affinity ($K_d = 2.13$ μM; Hofinger and Sticht, 2005), at the range of other SH2-ligand interactions (Bauer et al., 2004). This binding affinity is about three orders of magnitude stronger than that for the Lck-SH3 domain. High affinity binding of Lck-SH2 to P^{190}pYAP occurs despite the difference between this sequence and the phospho-YEEI sequence predicted to be the preferred binding site of the Lck-SH2 domain (Songyang et al., 1993). More recent studies indicated that PKCθ can also interact with the cytoplasmic tail of CD28, and that this interaction involves Lck as an intermediate molecule, as discussed below.

CD28 AND THE IS

Upon binding of its ligand, B7, CD28, similar to the engaged TCR, accumulates at the cSMAC of the IS although the two receptors initiates distinct but complementary signaling pathways. The transient recruitment of CD28 to the immature IS of TCR engaged T cells is very rapid and occurs within seconds of the onset of the calcium signal (Andres et al., 2004). Engagement of the TCR in *Cd28*$^{-/-}$ T cells results in altered, diffuse pattern of distribution of PKCθ and LFA-1 at the IS, suggesting an essential role for CD28 in the initiation and stabilization of the mature IS (Huang et al., 2002; Sanchez-Lockhart et al., 2004). Furthermore, *in vivo* blocking of CD28 impairs the activity of effector molecules, including PKCθ (Jang et al., 2008), and inhibits T cell-dependent immune responses (Linsley and Nadler, 2009). CD28 engagement promotes a cytoskeleton-dependent recruitment of cell surface receptors (Wulfing and Davis, 1998) and signaling molecules-containing lipid rafts that support building the IS and contribute to signal transduction from IS-residing receptors (Dustin and Shaw, 1999; Viola et al., 1999).

More recent studies demonstrated that in activated T cells, CD28 is recruited coordinately with the TCR to form microclusters at the cSMAC (Yokosuka et al., 2008). Upon progression of this initial step, the CD28 and TCR segregate to two spatially distinct subregions within the cSMAC, a central TCR^{high} subregion, where signaling is terminated and TCR-associated signaling complexes are internalized and degraded, and an outer TCR^{low} annular form that contain CD28 clusters, as well as PKCθ. CD28 and PKCθ were physically associated, as shown by PKCθ coimmunoprecipitation with CD28 from a lysate of PMA-stimulated T cells (Yokosuka et al., 2008).

PKCθ–CD28 INTERACTION AND RECRUITMENT OF PKCθ TO THE IS

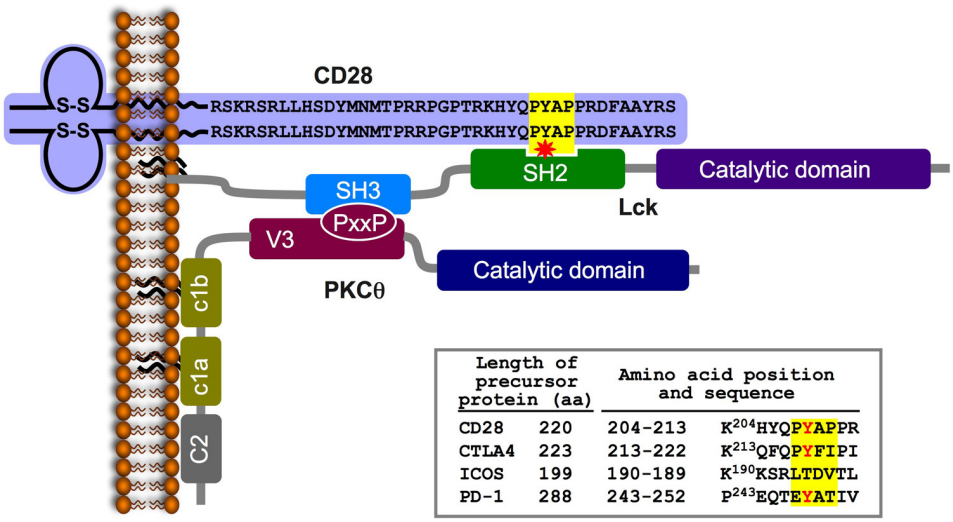
T cell receptor engagement polarizes PKCθ and induce its recruitment to the IS, a response that is greatly augmented by CD28 ligation (Huang et al., 2002; Tseng et al., 2008; Yokosuka et al., 2008). Although the recruitment of PKCθ to the center of the IS (cSMAC) of is well documented, information on the molecular basis for this highly selective localization has been relatively scarce. Early studies have shown that PKCθ recruitment to the IS is indirectly dependent on the PI3K interaction motif within the CD28 cytosolic tail (Harada et al., 2001). Thus, mutation of Met^{173} within the mouse YMNM motif, which binds PI3K upon its tyrosine phosphorylation, resulted in decreased ability of CD28 to direct PKCθ recruitment to the cSMAC, and inhibited PKCθ-dependent activation of NF-κB to and the *Il2* gene (Sanchez-Lockhart et al., 2004).

Following the recently reported PKCθ–CD28 association in PMA-stimulated T cells (Yokosuka et al., 2008), we conducted a detailed structure-function analysis of this association in TCR-stimulated T cells (Kong et al., 2011). We demonstrated that PKCθ physically associated with the cytoplasmic tail of CD28

following TCR/CD28 costimulation. Taking advantage of the fact that PKCδ, the closest relative of PKCθ, does not translocate to the IS after T cell-APC interaction (Monks et al., 1997), we compared the amino acid sequence analysis of PKCθ and PKCδ and found that they diverged significantly only in their V3 (hinge) domain, corresponding to amino acids ~291–378 of human PKCθ, suggesting a potential role for this region in targeting PKCθ to the IS. Indeed, a V3-deletion mutant of PKCθ (PKCθ-ΔV3) or an exchange mutant of PKCθ, in which the native V3 domain was replaced by the PKCδ V3 domain, did not coimmunoprecipitate with CD28, and failed to translocate to the IS (Kong et al., 2011) and to activate PKCθ-dependent reporter genes such as the CD28 response element (RE/AP). Conversely, the isolated V3 domain of PKCθ localized in the center of the IS and associated with CD28. Moreover, T cells recovered from mouse BM chimeras on a *Prkcq*$^{-/-}$ background reconstituted with the same PKCθ mutants failed to proliferate and produce IL-2 in response to CD3/CD28 costimulation, and their ability to upregulate CD69 or CD25 expression was reduced. Given the critical role of the V3 domain in directing the CD28 association and IS localization of PKCθ, we argued that this domain will function as a dominant negative mutant by disrupting the activation-dependent association between endogenous CD28 and PKCθ. As expected, ectopic expression of the isolated PKCθ V3 domain blocked the recruitment of endogenous PKCθ to CD28 and the IS, and severely inhibited PKCθ-dependent functions, including CD25 and CD69 upregulation, T cell proliferation and IL-2 production, and Th2 and Th17 (but not Th1) differentiation and inflammation.

Fine mapping of the PKCθ V3 domain identified an evolutionarily conserved proline-rich (PR) motif (ARPPCLPTP; corresponding to amino acid residues 328–336 of human PKCθ) within the PKCθ-V3 domain, which was required for PKCθ–CD28 association, PKCθ localization to the IS, and induction of PKCθ-mediated functions. Insertion of this motif into the V3 domain of PKCδ enabled this altered PKCδ form to translocate to the IS and activate PKCθ-dependent signal. The two internal proline residues in this motif (Pro-331 and -334) were particularly critical in this regard (Kong et al., 2011).

In trying to more precisely define the nature of the inducible PKCθ-Lck complex, we focused on the potential contribution of Lck kinase. This possibility was considered in view of previous studies demonstrating a functional relationship between CD28, PKCθ, and Lck. First, in stimulated T cells, Lck can be recruited to the tyrosine-phosphorylated distal PR motif (P^{190}Y*AP) in the cytoplasmic tail of CD28 via its SH2 and SH3 domains, respectively (Miller et al., 2009; see **Figure 2**), This motif directs the colocalization of PKCθ and CD28 to the cSMAC (Yokosuka et al., 2008) and is apparently involved in additional biological functions, including the stabilization of IL-2 mRNA, reorganization of lipid rafts, and sustained autophosphorylation and activation of Lck at the IS (Holdorf et al., 2002; Sanchez-Lockhart et al., 2004; Dodson et al., 2009). Second, Lck phosphorylates and associates with PKCθ, and mutation of the major Lck phosphorylation site on PKCθ (Tyr^{90})



Length of precursor protein (aa)		Amino acid position and sequence	
CD28	220	204-213	K^{204}HYQPYAPPR
CTLA4	223	213-222	K^{213}QFQPYFIPI
ICOS	199	190-189	K^{190}KSRLTDVTL
PD-1	288	243-252	P^{243}EQTEYATIV

FIGURE 2 | A schematic model of the CD28-Lck-PKCθ tri-partite complex formed in TCR/CD28-stimulated T cells. TCR/CD28 engagement triggers the activation of tyrosine kinases and phosphorylation of multiple substrates, including the cytoplasmic tail of the CD28. Phosphorylation of a tyrosine within the PYAP motif (Y^{191} in the mature human CD28) forms a high affinity-binding site for the SH2 domain of the Lck PTK. Lck is an IS-residing molecule in activated T cells; it is tethered to the plasma membrane via its N-terminal palmitic and myristic fatty acids (Paige et al., 1993), and is constitutively associated with the cytoplasmic tail of the IS-residing accessory molecules, CD4 or CD8 (Rudd et al., 1988, 1989; Veillette et al., 1988; Barber et al., 1989; Paige et al., 1993). Simultaneous activation of phospholipase C and hydrolysis of the membrane phospholipid (PIP_2) forms DAG, which enables PKCθ anchoring to the plasma membrane. Colocalization of PKCθ and CD28 is regulated by an interaction between the PKCθ PXXP motif and the Lck-SH3 domain, which results in the formation of a trimolecular complex comprising CD28-Lck-PKCθ. The inset table shows the amino acid sequence of a region within the cytoplasmic tail of the immature CD28 that includes the PYAP motif (on a yellow background) compared to homologous sequences of three additional members of the CD28 coreceptor family (obtained using the ClustalW multiple sequence alignment program). A partially conserved tyrosine is marked in red.

inhibited PKCθ-dependent activation events in stimulated T cells (Liu et al., 2000).

Our further analysis confirmed the physical and functional CD28-Lck-PKCθ link by demonstrating that Lck function as an intermediate to recruit PKCθ to CD28 upon T cell stimulation. The Lck-SH3 domain interacted with the PR motif in the PKCθ V3 domain, while the Lck SH2 domain interacted with phospho-Tyr^{191} in the P^{190}YAP motif in the CD28 cytoplasmic tail. Taken together, the above findings demonstrate a unique signaling mode of CD28 and establish the molecular basis for the specialized localization and function of PKCθ in antigen-stimulated T cells.

THE GLK-PKCθ LINK

Recent studies demonstrated that recruitment of PKCθ to the cSMAC in activated T cells is essential but not sufficient for the full activation of PKCθ and its downstream target molecules. These studies further showed that the germinal center kinase (GSK)-like kinase (GLK) also translocates to the IS of TCR-engaged T cells where it phosphorylates the activation loop of PKCθ, converting it into an active enzyme (Chuang et al., 2011). Of interest, however, despite the importance of PKCθ in the thymic development of natural regulatory T cells (nTregs; Schmidt-Supprian et al., 2004), GLK-deficient mice displayed normal nTreg development (Chuang et al., 2011). These results emphasize the important role of post-transcriptional regulation of PKCθ that occurs at several steps and involve different checkpoints at distinct sites within the activated T cell.

CONCLUSIONS AND FUTURE PERSPECTIVES

Identification and characterization of the molecular mechanism by which PKCθ associates with CD28 and colocalizes with it at the cSMAC has provided important information relevant to the mechanism by which CD28 and PKCθ contribute to signal transduction in TCR/CD28-engaged T cells. These findings also raise new questions relevant to the mechanism of interaction of CD28 and PKCθ and their specific role in the induction of distinct T cell-mediated immune responses. One obvious question relates to the mechanism by which PKCθ is sequestered away from the IS of activated T_{reg} cells. It would be interesting to determine whether a CD28-Lck-PKCθ tri-partite complex (Kong et al., 2011) occurs in T_{reg} cells, and determine the mechanism that enables PKCθ recruitment away from the T_{reg}-APC contact area. A possible explanation for this process was provided by Yokosuka et al. (2010) showing that CTLA-4 competes with CD28 in recruitment to the cSMAC. In addition, it is not known whether PKCθ is involved in a second signal delivery during the costimulation of γδ T cells (Ribot et al., 2011).

Despite the extensive amount of studies on the biology of PKCθ in mouse T cells, very little is known about its regulation and function in human T cells. This is a substantial gap that would need to be filled if PKCθ is destined to fulfill its promise as a clinically relevant drug target (Altman and Kong, 2012). As discussed earlier, the dependence of T cell-mediated deleterious autoimmune/inflammatory responses, including GvHD, on PKCθ, but its dispensable role in beneficial responses (antiviral immunity and GvL response) make it an attractive clinical drug target with potentially advantage over global immunosuppressive drugs such as calcineurin inhibitors (e.g., cyclosporine A), which have pronounced toxic side effects. Indeed, there has been considerable interest among pharmaceutical companies in developing small molecule selective PKCθ catalytic activity inhibitors, and AEB071,the most advanced of these compounds, which inhibits other PKC family members in addition to PKCθ, is currently in early clinical trials (Evenou et al., 2009).

Nevertheless, small molecule inhibitors of protein kinases often have toxic side effects because of their lack of absolute specificity, which reflects the relatively high conservation of catalytic domains within the protein kinase family, and even more so within the PKC family. Furthermore, since catalytic kinase inhibitors in current clinical use are ATP competitors, they need to be used at relatively high and potentially toxic concentrations in order to effectively compete with ATP, whose intracellular concentration is ∼1 mM. As a result, there has recently been considerable interest and progress in developing allosteric kinase inhibitors, which bind to sites other than the catalytic site in kinases and, thus, are likely to be much more selective and less toxic (Lamba and Ghosh, 2012). Our recent study (Kong et al., 2011) demonstrates a new potential approach for attenuating PKCθ-dependent functions utilizing allosteric compounds based on the critical PR motif in the V3 domain of PKCθ that will block its Lck-mediated association with CD28 and recruitment to the IS, which is obligatory for its downstream signaling functions. This new approach could serve as a basis for the development of new therapeutic agents that would selectively suppress undesired T cell-mediated inflammation and autoimmunity or prevent graft rejection, while preserving desired immunity, such as antiviral responses.

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Chromatinized protein kinase C-θ: can it escape the clutches of NF-κB?

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We recently provided the first description of a nuclear mechanism used by Protein Kinase C-theta (PKC-θ) to mediate T cell gene expression. In this mode, PKC-θ tethers to chromatin to form an active nuclear complex by interacting with proteins including RNA polymerase II, the histone kinase MSK-1, the demethylase LSD1, and the adaptor molecule 14-3-3ζ at regulatory regions of inducible immune response genes. Moreover, our genome-wide analysis identified many novel PKC-θ target genes and microRNAs implicated in T cell development, differentiation, apoptosis, and proliferation. We have expanded our ChIP-on-chip analysis and have now identified a transcription factor motif containing NF-κB binding sites that may facilitate recruitment of PKC-θ to chromatin at coding genes. Furthermore, NF-κB association with chromatin appears to be a prerequisite for the assembly of the PKC-θ active complex. In contrast, a distinct NF-κB-containing module appears to operate at PKC-θ targeted microRNA genes, and here NF-κB negatively regulates microRNA gene transcription. Our efforts are also focusing on distinguishing between the nuclear and cytoplasmic functions of PKCs to ascertain how these kinases may synergize their roles as both cytoplasmic signaling proteins and their functions on the chromatin template, together enabling rapid induction of eukaryotic genes. We have identified an alternative sequence within PKC-θ that appears to be important for nuclear translocation of this kinase. Understanding the molecular mechanisms used by signal transduction kinases to elicit specific and distinct transcriptional programs in T cells will enable scientists to refine current therapeutic strategies for autoimmune diseases and cancer.

Keywords: PKC-theta, microRNA, chromatin, T cells, signaling kinase, immune response gene, NF-κB, nuclear PKC-theta

INTRODUCTION

In the past, the nuclear role of Protein Kinase C (PKC) has been dominated by the cytoplasmic signaling role of this kinase family. A complex series of molecular events is initiated upon stimulation of the TCR and CD28 co-receptor. This results in the selective activation of the novel PKC family member, PKC-θ, which activates transcription factors such as NF-κB, ultimately leading to induction of a distinct cohort of immune response genes (Sun et al., 2000; Isakov and Altman, 2002). However, recent studies have demonstrated that signaling molecules and kinases play a key role in the nucleus under activating conditions (Martelli et al., 1999; Passalacqua et al., 1999).

An emerging concept is that "chromatin structure" forms a novel platform for signal transduction. One elegant example of this is the activation of the stress-induced MAP kinase Hog1 that involves recruitment of this protein to the chromatin of most osmo-inducible genes in yeast (Pascual-Ahuir et al., 2006; Pokholok et al., 2006; Proft et al., 2006). Genome-wide analysis showed the binding pattern of Hog1 was not only within the promoter but also the transcribed regions of osmotic stress responsive genes (Proft et al., 2006). Several kinases have now been shown to occupy both the promoters and active transcribed regions of genes they activate (Pokholok et al., 2006), and this has helped to revolutionize our view of how signaling pathways regulate gene expression.

Recently, PKC-β_1 was shown to associate with chromatin, phosphorylate threonine-6 on histone H3, and influence the action of the histone demethylase, LSD1 (Metzger et al., 2010). Analogous to these studies, we found that the PKC-θ enzyme tethers to chromatin and appears to be an integral component of transcription complexes, where it functions as a structural adaptor or locally phosphorylates other chromatin-associated proteins (Sutcliffe et al., 2011). In certain cohorts of inducible genes in T cells, nuclear PKC-θ intimately interacts with chromatin by forming an active transcription complex with RNA polymerase II, MSK-1, LSD1, and 14-3-3ζ (Sutcliffe et al., 2011). Specifically, we showed that nuclear PKC-θ is recruited in an activation-dependent manner to the proximal promoters of inducible T cell genes, such as *CD69*, *TNF-α*, *IFN-γ*, and *heparanase*, as well as to microRNA genes (Sutcliffe et al., 2011). One possible consequence of the association of activated signal transduction kinases with the genes they regulate is that it could provide a more efficient mechanism

of controlling gene expression. Our findings revealed a model in which chromatin-anchored PKC-θ could regulate inducible T cell gene transcription by two opposing mechanisms. Firstly, via the direct tethering of PKC-θ to inducible genes to form an active transcription complex. Secondly, indirectly by docking onto the chromatin of microRNA genes that modulate key repressor proteins that regulate cytokines. Ultimately, the balance of both these processes allows appropriate levels of inducible gene transcript to be maintained in activated T cells.

Here, we extend upon our recent observations describing PKC-θ as a novel chromatin-associated enzyme, by identifying two distinct NF-κB-containing motifs. The first may facilitate the assembly of the PKC-θ active complex at protein coding genes and the second enables negative regulation of microRNA genes. Finally, we have identified a new nuclear localization sequence for PKC-θ that may help to delineate the nuclear and cytoplasmic functions of this important kinase.

RESULTS AND DISCUSSION

IDENTIFICATION OF A NOVEL NUCLEAR LOCALIZATION SIGNAL FOR PKC-θ

Many proteins that are targeted to the nucleus contain a canonical nuclear localization signal (NLS) and indeed a putative NLS domain has been described for PKC-θ (DeVries et al., 2002). Alternatively, proteins can bind to NLS-containing proteins in order to enter the nucleus. Preliminary computational analysis using publically available yeast two-hybrid screens revealed candidate proteins that could facilitate the transport of PKC-θ into the nucleus, namely AKT1, HABP4, CHD3, and TCLA1. Based on our preliminary studies, these two mechanisms are unlikely to solely account for the existence of nuclear PKC-θ.

Chuderland et al. (2008) have demonstrated that the Serine-Proline-Serine (SPS) sequence in ERK2 is phosphorylated following cellular stimulation and that this is essential for nuclear translocation. Other signaling molecules, such as SMADS, MEK, and p53 use the same mechanism. Indeed, mutation of SPS to APA (which cannot undergo phosphorylation), greatly reduced nuclear translocation of ERK2, whereas mutation to EPE (which mimics a phosphorylated SPS sequence), resulted in highly upregulated nuclear translocation of ERK2 (Chuderland et al., 2008). SPS phosphorylation was required for the interaction of ERK2 with the nuclear import protein, Importin 7 (Chuderland et al., 2008). Although the specific kinases responsible for SPS phosphorylation were not identified, S/T-P-S/T was suggested to be a general nuclear translocation signal for NLS-independent translocation of signaling molecules into the nucleus.

Thus, in our experiments we searched the sequences of the human PKC family members for S/T-P-S/T (SPT-like) motifs and corresponding NLS sequences previously described (DeVries et al., 2002). **Figure 1A** showed that out of all the possible residue combinations for this motif, the sequence "SPT" was the most common. Additionally, all nPKC and cPKC isoforms contain at least one SPT-like motif (**Figure 1A**). In contrast, aPKC isoforms do not contain any SPT-like motifs. Interestingly, there is an SPT-like motif in the second C1 domain, C1b, in all cPKC isoforms, and nPKC δ and θ (**Figure 1A**). The nPKC isoforms ε and η do not contain this conserved motif, instead they contain the sequence VPT at this location in C1b. However, both these isoforms have an additional SPT-like motif in between C1b and the catalytic domain (**Figure 1A**). This could indicate that there has been a compensatory mutation in these two isoforms. Two additional SPT motifs were found in PKC-γ, one of which is repeated (SPSPSPT). Additionally, there is a C-terminal TPT motif present in PKC-βI that is not in βII (**Figure 1A**). Certain SPT-like motifs in PKC-η, α, and βI, have been experimentally shown phosphorylation sites, and others have been predicted to be phosphorylated (see "Table in Materials and Methods").

We designed two mutants of the SPT motif within the full length PKC-θ, expressed them as HA-tagged fusion proteins, and assessed the distribution of each PKC-θ mutant construct following transfection into Cos-7 cells. Our results show that mutation of the SPT motif in PKC-θ to APA, which cannot be phosphorylated, resulted in a significant decrease in the nuclear localization of this kinase (**Figures 1B,C**). Mutation of this motif to EPE, a change that mimics constitutionally phosphorylated PKC-θ, led to an increase in nuclear localization (**Figures 1B,C**). Interestingly, mutation of the putative NLS sequence (pNLS-mut) did not significantly alter cytoplasmic-nuclear distribution of this kinase in Cos-7 cells (**Figures 1B,C**). These mutants will be a valuable tool to determine how essential nuclear localization of PKC-θ is to inducible gene regulation.

Taken together, our findings suggest that PKCs belong to the class of signaling kinases whose nuclear transportation and role may be accomplished through this alternative NLS motif. Further studies will be required to determine which kinases mediate the phosphorylation of this motif in T cells and to what extent the NLS and SPT sequences of PKC-θ participate in mediating nuclear shuttling of this kinase in different cell types and activation states.

PKC-θ IS ACTIVE IN BOTH CYTOPLASMIC AND NUCLEAR FRACTIONS

Protein Kinase C-theta clearly has distinct functions in both the cytoplasm and nucleus (Sutcliffe et al., 2011). However, the relative activity of this isoform in these two compartments is unknown. Our PKC-θ isoform specific kinase assay revealed that this kinase is active in both the cellular and nuclear compartments in resting and activated T cells (**Figure 2**). Interestingly, cytoplasmic PKC-θ activity increased following T cell activation, whereas there was no significant induction in the nuclear fraction (**Figure 2**). Although there is a lack of induction in nuclear PKC-θ activity following T cell stimulation, it remains to be determined how activity may influence the function of this kinase when it is tethered to chromatin. Indeed, our preliminary results indicate that the active form of this kinase (phosphorylation of serine-695) is much more transient on chromatin than the occupancy of PKC-θ in the non-phosphorylated form (Sutcliffe et al., 2011). Ultimately, the structure and function of chromatin-tethered PKC-θ needs to be elucidated to further understand how this kinase mediates gene regulation.

AN NF-κB TRANSCRIPTION FACTOR MODULE EXISTS WITHIN PKC-θ BOUND GENES IN HUMAN T CELLS

The mechanisms that control the recruitment of signaling kinases to particular chromatin regions remain elusive, although the binding location of PKC-θ at specific regions of DNA and on selected

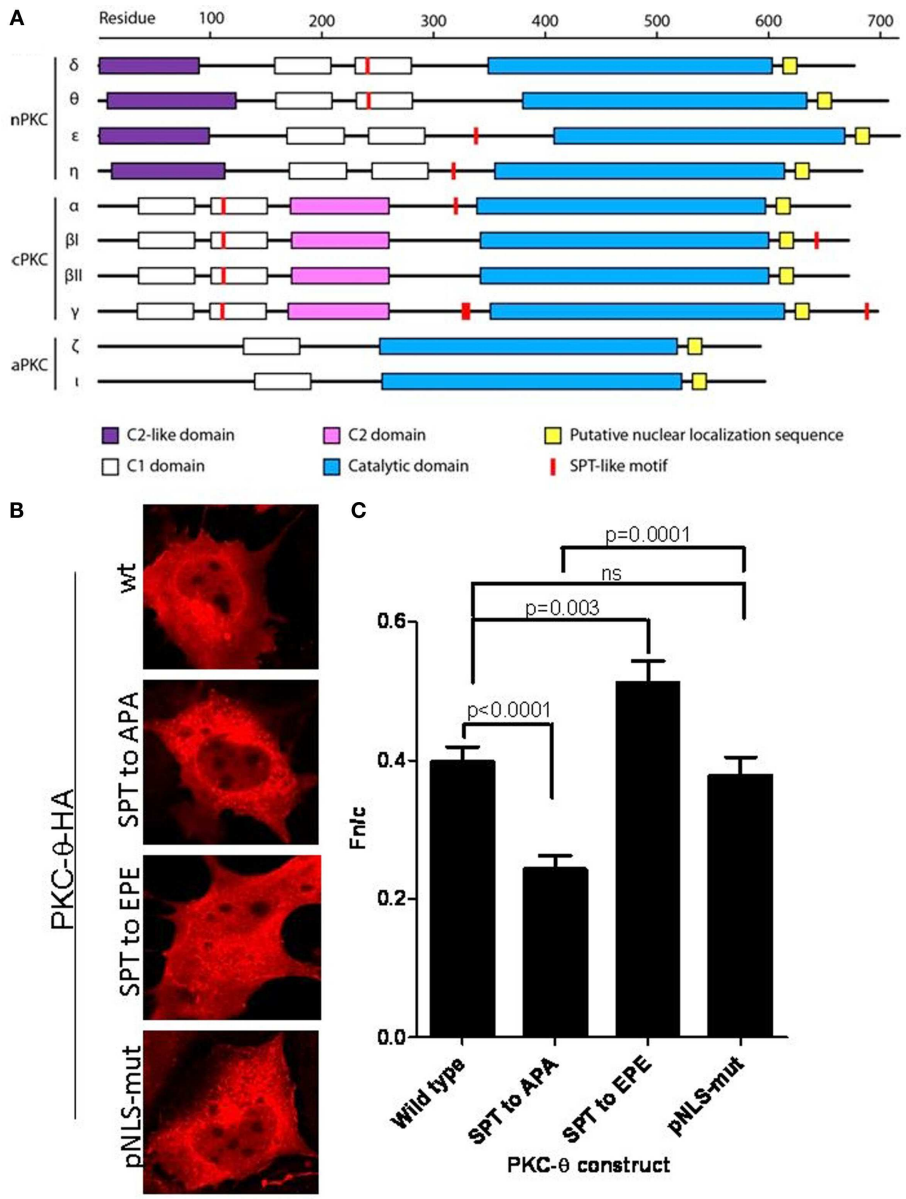

FIGURE 1 | SPT^{243} is the minimum motif for nuclear localization of PKC-θ regulated by phosphorylation at S^{241} and T^{243}. (A) SPT-like motifs and nuclear localization sequences (NLS) were displayed relative to the domain organization of PKC members (nPKC: δ, θ, η, ε; cPKC: α, βI, βII, γ; and aPKC: ζ, ι, or λ). SPT-like motifs are depicted in red and the locations of NLS are shown in yellow. The C2-like domain (purple) is characteristic of nPKCs, while cPKCs share the calcium binding C2 domain (pink). Both nPKC and cPKC isoforms possess the C1 domain (white), that is composed of C1a and C1b. In comparison, aPKC isoforms only contain C1a of the C1 domain. All PKC family members contain a C-terminal catalytic domain (blue). **(B)** The full length PKC-θ wild type gene sequence and its derivatives wherein putative phosphorylation sites at S^{241} and T^{243} were mutated to either the non-phosphorylatable alanine (SPT to APA) or the phosphomimetic glutamine (SPT to EPE), were cloned into the pTracer-CMV vector in frame with a C-terminal HA-tag. The vector also codes for GFP, which is translated independent of the insert and serves as an internal marker for transfected cells. Subconfluent cultures of Cos-7 cells were transfected and subsequently the fixed cells were probed with rabbit antibody to HA-tag, followed by secondary antibody to rabbit immunoglobulins conjugated to Alexa-Fluor 568. Localization of expressed PKC-θ was studied with confocal laser scanning microscopy as detailed in the methods. Representative images for each construct are shown. **(C)** $F_{n/c}$ values for each construct are shown in **(C)**, with significant differences between datasets indicated. Data shown are mean ± SEM, $n > 15$ for each dataset.

genes may provide a clue to both its function and mode of recruitment (Edmunds and Mahadevan, 2006). Based on various occupancy patterns across genes, Pokholok et al. (2006) present several models of how signaling kinases can be recruited and associate with the genome. Kinases could be recruited to regulatory regions of inducible genes initially by specific marks such as PTMs on histones or by specific transcription factors in response to T cell activation. Once recruited, these kinases could

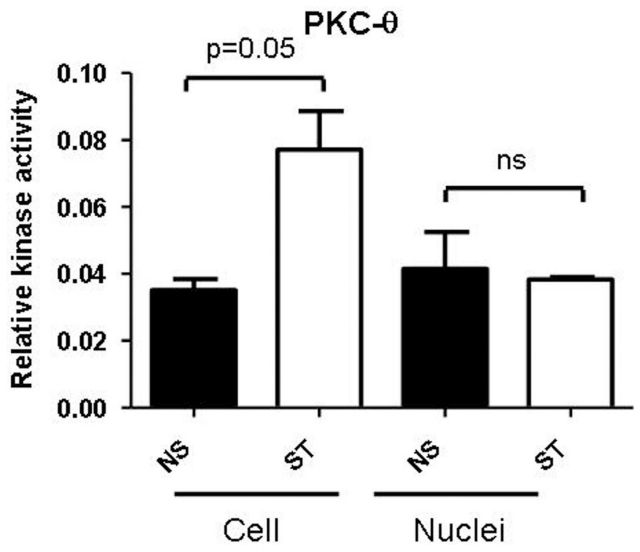

FIGURE 2 | PKC-θ activity in T lymphocytes. Jurkat T cells were either non-stimulated (NS) or stimulated (ST) with PMA/Cal for 4 h. The PKC-θ specific antibody was used to immunoprecipitate PKC-θ from either whole cells or nuclei from both NS and ST treated Jurkat T cells. PKC ELISA-based kinase assays were performed on these PKC-θ fractions and absorbance was measured at 450 nm. Data is plotted as relative kinase activity compared to the negative control that did not have any antibody. Data is representative of the mean ± SE of three independent experiments and statistical significance was determined by a two-tailed paired *t*-test using GraphPad Prism 5.03.

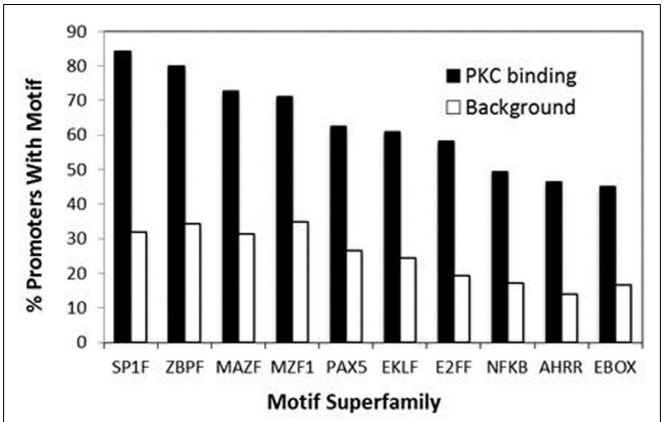

FIGURE 3 | Motif superfamilies identified within promoters of PKC-θ bound genes. A greater proportion of the promoters of the PKC-bound genes had motifs for members of the SP1F, ZBPF, MAZF, MZF1, PAX5, EKLF, E2FF, NFKB, AHRR, and EBOX motif superfamilies, when compared to a background set of promoters. The representative members of the superfamilies shown are SP1_01 (SP1F), ZNF219_01 (ZBPF), MAZR_01 (MAZR), MZF1_01 (MZF1), PAX5_01 (PAX5), EKLF_01 (EKLF), NFKAPPAB_01 (NFKB), E2F_03 (E2FF), AHRARNT_02 (AHRR), and MYCMAX_03 (EBOX).

associate with the transcriptional machinery or with remodeling complexes.

Our recent genome-wide analysis identified many novel PKC-θ targeted genes that might be implicated in Th1 cell development, differentiation, apoptosis, and proliferation (Sutcliffe et al., 2011). To examine the transcription factor candidates that may be responsible for recruiting PKC-θ to target genes, we undertook bioinformatic analysis of our ChIP-on-Chip data. Forty-nine over-represented transcription factor binding motifs were identified in the promoters of the PKC-θ binding genes. These motifs belonged to 10 superfamilies ZBPF, MAZF, MZF1, SP1F, EBOX, E2FF, EKLF, PAX5, NFKB, and AHRR (**Figure 3**). The promoters of *IL-2*, *CD69*, *TNF-α*, and *IFN-γ* (all strongly transcriptionally regulated during T cell activation) were examined for these motifs (**Figure 4A**). NF-κB motifs were located near E2FF and EBOX family members in all four genes (**Figure 4A**).

In other PKC-θ binding genes, the area around the PKC-θ binding site was analyzed for commonly occurring combinations (co-regulatory motifs, CRM) of three motifs, where the most frequently occurring superfamily member was used and the frequency of three motifs occurring within 300 bp of each other was calculated. MYCMAX_03 (EBOX) and AHRARNT_02 (AHRR) commonly occurred together and this was attributed to the similarity of their matrices. The combination NFKB_SP1F_E2FF occurs in these promoter regions significantly more than expected by chance (**Figure 4B**), whereas the NFKB_E2FF_AHRR and NFKB_AHRR_PAX5 combinations are significantly over-represented in the within-gene PKC binding regions (**Figure 4C**). The PAX5 DNA binding motif is bound by both Pax5 and Pax-9 of which only Pax-9 is expressed in Jurkat cells. ChIP-seq data for GM12878 and K562 cells (Lee et al., 2012) available in the UCSC Hg18 genome browser suggests that the AHRR sites may actually be bound by c-Myc and/or Max. This study also showed that NF-κB can bind near many of the predicted sites (Lee et al., 2012).

Currently it is unknown what targets chromatin-remodeling factors and signaling kinases, such as PKC-θ, to specific chromatin regions. This data provides a clue that a novel NF-κB transcription factor module may be involved in tethering PKC-θ to chromatin in T cells.

NF-κB IS A PREREQUISITE FOR THE TETHERING OF THE PKC-θ ACTIVE TRANSCRIPTION COMPLEX

The NF-κB family of transcription factors encompasses a heterogenous set of inducible proteins, including NF-κB1 (p50 and precursor p105), NF-κB2 (p52 and precursor p100), RelA (p65), RelB, and c-Rel (May and Ghosh, 1998; Caamano and Hunter, 2002; Kane et al., 2002). The phosphorylated NF-κB dimer translocates into the nucleus where it binds to DNA at cognate κB sites in promoters and enhancers to control transcription of various NF-κB dependent genes (Donovan et al., 1999; Rayet and Gelinas, 1999; Seetharaman et al., 1999; Das et al., 2001). These genes are expressed in a cell- and tissue-specific manner and this provides an additional level of regulation. Generation of mice deficient in, or having mutated forms of, the different members of the NF-κB family has helped to delineate characteristics of each member in various immune responses and their role in disease (Kontgen et al., 1995; Ghosh et al., 1998; Rao et al., 2003).

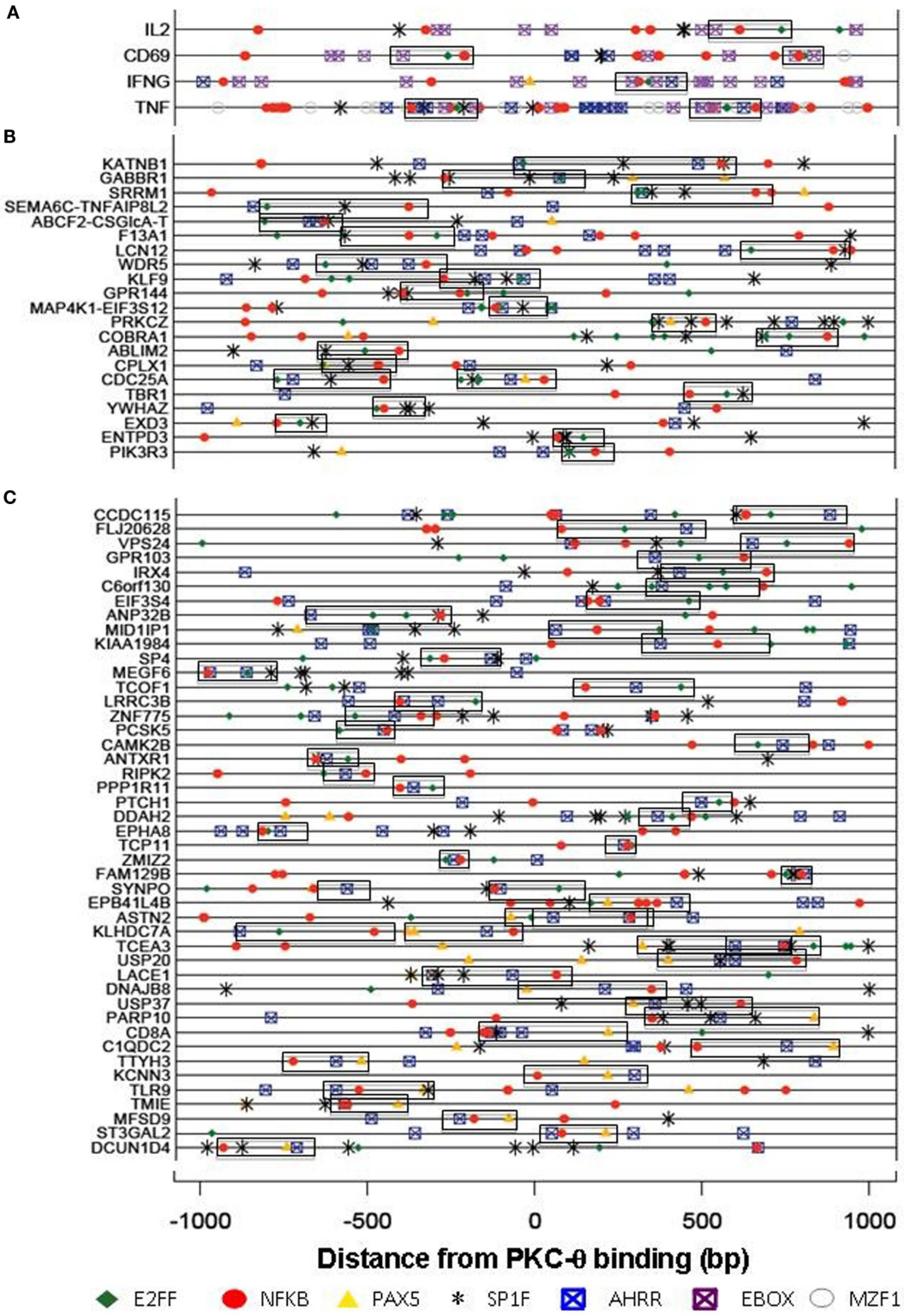

FIGURE 4 | Transcription factor binding motifs in the PKC-bound sequences. Motifs for members of the NFKB (red), E2FF (green), PAX5 (orange), AHRR (blue), EBOX (purple), MZF (gray), and SP1F (black) superfamilies were identified by their Position Weight Matrices. PKC binds four inducible genes [+150 bp downstream from the transcription start site **(A)**], in promoter sequences **(B)** or within genes **(C)**. All motifs within a superfamily are shown for **(A)** while only NFKAPPAB_01 (NFKB), E2F_03 (E2FF), AHRARNT_02 (AHRR), PAX5_01 (PAX5), and SP1_01 (SP1F) are shown for **(B)** and **(C)**. Commonly co-occurring motifs are boxed together. NFKB_SP1F_E2FF commonly co-occur in the promoter regions **(B)**, while the NFKB_E2FF_AHRR and NFKB_AHRR_PAX5 combinations are significantly over-represented in the within-gene PKC binding regions **(C)**.

To functionally validate that NF-κB may be involved in the direct tethering of PKC-θ to chromatin, we utilized two well established NF-κB inhibitors. Pentoxifylline directly inhibits c-Rel activity, preventing its nuclear recruitment in $CD4^+$ T cells (Wang et al., 1997), whilst Bay disrupts the phosphorylation of IκB, resulting in the inappropriate cytoplasmic retention of NF-κB (Garcia et al., 2005). Consistent with previous findings, pretreatment of Jurkat T cells with either of these NF-κB inhibitors significantly abolished inducible gene transcription of two key immune response genes, *IL-2* and *CD69* (**Figure 5A**).

Next, we examined whether recruitment of PKC-θ, Pol II, or LSD1 were impaired in the inhibitor treated cells. Our chromatin immunoprecipitation (ChIP) results clearly show that the lack of NF-κB severely reduced assembly of the PKC-θ-containing active transcription complex (**Figure 5B**). These results further support our previous findings of the interdependency of PKC-θ and LSD1 (Sutcliffe et al., 2011), where both proteins are essential for the active complex to remain associated with chromatin. Ultimately to truly ascertain the specificity of the NFkB-PKC-θ axis at the level of chromatin structure, further experiments will be required with inhibitors that target other T cell transcription factors such as NFAT.

To examine whether PKC-θ and LSD1 have the potential to directly interact with each other, we modeled the interaction between PKC-θ and LSD1 using the ClusPro server (Kozakov et al., 2010) with the LSD1-CoREST crystal structure (pdb code 2IW5) and chain A from the crystal structure of the catalytic domain of PKC-θ (pdb code 2JED). This computational modeling revealed a high degree of complementarity between the LSD1 and PKC-θ surfaces at the putative interaction interface (**Figure 5C**). Interestingly, LSD1 is bound where the peptide substrate would bind PKC-θ. Functional studies are required to confirm there is a direct interaction between these two proteins.

NF-κB RELIEVES TRANSCRIPTIONAL REPRESSION OF PKC-θ TARGETED microRNA, miR-200C, IN HUMAN T CELLS

Our recent ChIP-on-Chip data revealed that PKC-θ binds to both promoters and transcribed regions of genes, as well as to microRNA promoters that are crucial for cytokine regulation (Sutcliffe et al., 2011). Since PKC-θ regulates microRNAs in human T cells, we wanted to determine whether, like coding genes, an NF-κB-containing motif exists on these genes. To address this, a cohort of PKC-θ bound microRNAs were interrogated for the presence of the superfamily motifs identified in **Figure 3**. Interestingly, in these sequences a distinct combination of NFKB_PAX5_E2FF was commonly found (**Figure 6A**).

To functionally validate the importance of NF-κB in the transcription of microRNAs, we specifically investigated the behavior of miR-200c, which is dependent on PKC-θ recruitment (Sutcliffe et al., 2011). In addition, we again utilized the pentoxifylline and Bay inhibitors. As shown in **Figure 6B**, treatment with both these compounds resulted in increased transcription of miR-200c following T cell stimulation.

In parallel at the chromatin level, pentoxifylline and Bay increased recruitment of PKC-θ, Pol II, and LSD1 at the miR-200c promoter following T cell activation (**Figures 6C–E**). These ChIP data further support the interdependence of these proteins and implicate NF-κB in the regulation of miR-200c. However, in contrast to the coding genes so far examined, the PKC-θ active transcription complex appears to be recruited to microRNAs independently of NF-κB to relieve transcriptional repression.

CONCLUSION

The findings presented here provide a new dimension to the chromatin-associated role we previously described for PKC-θ (Sutcliffe et al., 2011). In this present study, we have shown that PKC-θ may be recruited onto the chromatin template via distinct NF-κB transcription factor modules depending on the gene context. Our results reveal the existence of an NF-κB dependent mechanism for the tethering of the active transcription complex comprising of at least PKC-θ, Pol II, and LSD1 on coding genes (**Figure 7**). We propose this mechanism involves PKC-θ mediated cytoplasmic activation of c-Rel leading to binding of this transcription factor on target genes (**Figure 7**). In the genes we have surveyed, these promoter complexes also comprise nuclear PKC-θ.

We have established that a novel SPT sequence must be phosphorylated in order for PKC-θ to be translocated into the nucleus (**Figure 7**). This event appears to be independent of its intrinsic kinase activity. The nuclear and cytoplasmic contributions of PKC-θ remain to be delineated, to unravel how they are coordinated and integrated to achieve the appropriate physiological response. Our findings of the existence of the novel SPT motif in novel and conventional PKC isoforms is exciting and will have a tremendous impact by allowing us to manipulate the nuclear role of PKC-θ. It will also be important to ascertain whether this nuclear phenomenon is intrinsic to all PKC family members depending on the cell type, activating signal, and differentiation state of the cell.

Several lines of evidence support our model of the involvement of c-Rel in mediating PKC-θ recruitment over other NF-κB family members: (1) the delayed kinetics of nuclear c-Rel translocation and activation following T cell stimulation closely correlates with the formation of the active transcription complex; (2) pentoxifylline is a c-Rel specific inhibitor that successfully abolishes the assembly of the PKC-θ complex; (3) our preliminary c-Rel knockout studies suggest that c-Rel is essential for anchoring PKC-θ to chromatin (data not shown); and (4) both c-Rel and PKC-θ are essential for chromatin accessibility necessary for transcription of immune response genes (Rao et al., 2003; Sutcliffe et al., 2011). The notion that nuclear kinases work synergistically with NF-κB is not without precedence. Saccani et al. (2002) found that p38-dependent H3 phosphorylation marked selected promoters for increased recruitment of NF-κB leading to increased transcriptional activity. It remains to be determined whether PKC-θ is linked to the recruitment of histone modifying complexes or chromatin remodelers, such as SWI/SNF, as was previously demonstrated for p38α (Simone et al., 2004). In addition, other molecules of the NF-κB pathway such as IKK-α, NIK, and IKK1 have also been shown to have a nuclear role (Carlotti et al., 2000; Birbach et al., 2002; Anest et al., 2003; Yamamoto et al., 2003).

Our results also imply that NF-κB forms a repressive complex on microRNA genes by impeding the formation of the PKC-θ active transcription complex (**Figure 7**). Indeed we have identified that a unique NF-κB transcription factor module exists on microRNA genes which is distinct to that within PKC-θ bound

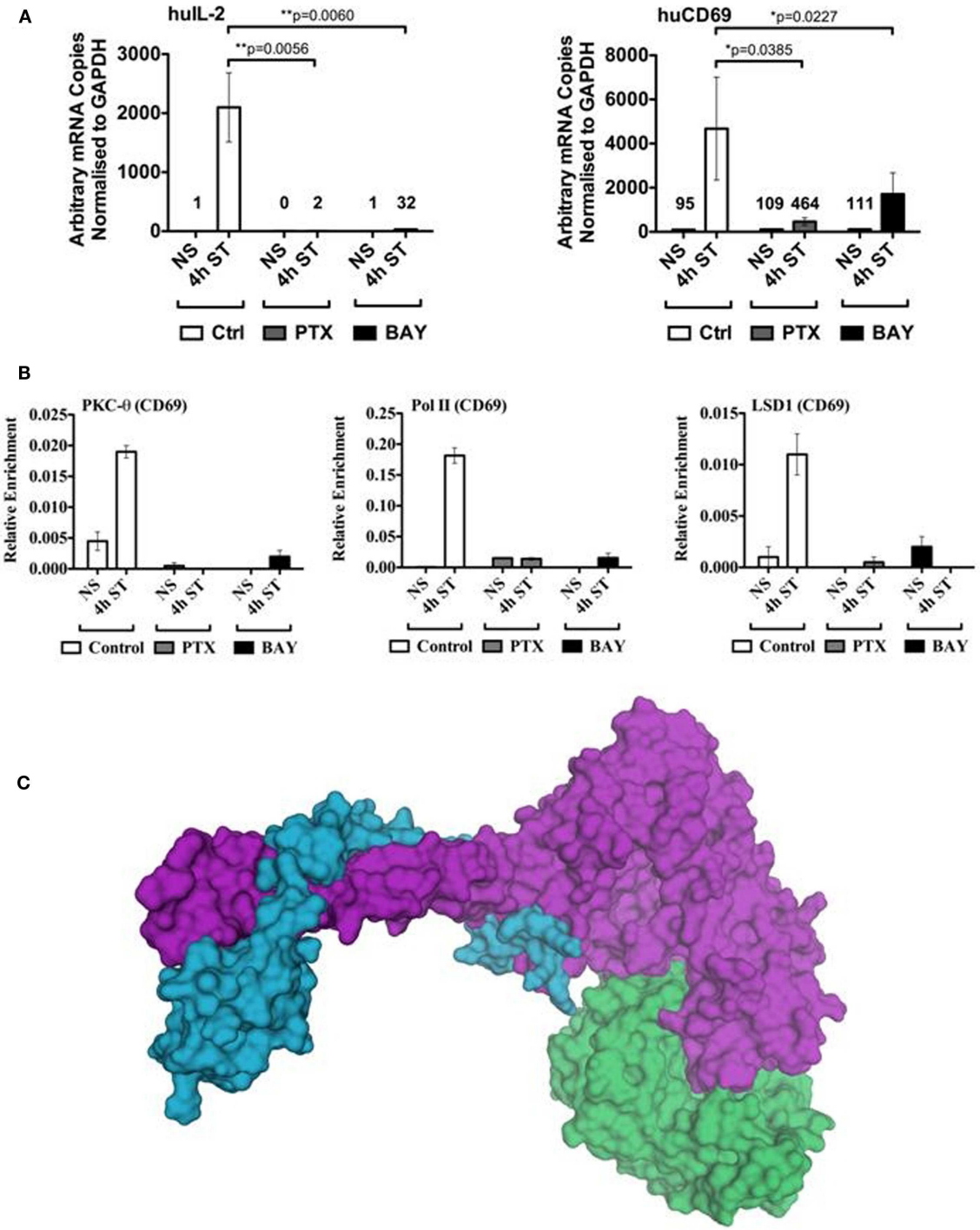

FIGURE 5 | Binding of the PKC-θ active transcription complex to the *CD69* promoter appears dependent on NF-κB. (A) Resting Jurkat T cells were pre-incubated with either Pentoxifylline (PTX), Bay, or left untreated (control) for 1 h. Cells were then left non-stimulated (NS) or activated with PMA/CaI (ST) for 4 h. Total mRNA was isolated for cDNA reverse transcription. Gene specific Taqman® expression assay was used to detect the transcript levels of human *IL-2* and *CD69*. Data is expressed as arbitrary mRNA copies normalized to *GAPDH*. Data represent the mean ± SD of four independent experiments. Statistical significance between the activated control and inhibited samples was determined by a two-tailed paired *t*-test using GraphPad Prism 5.03. PKC-θ, Pol II, and LSD1 ChIP experiments were performed on the samples described in **(A)**. **(B)** Sybr-green real-time PCR was used to detect the relative enrichment of these proteins across the *CD69* promoter (−214 to −52 relative to the transcription start site). **(C)** The catalytic domain of PKC-θ (green, pdb code 2JED) is modeled interacting with the LSD1-CoREST complex (purple and blue, respectively, pdb code 2IW5). In this model the peptide-binding groove of PKC-θ contacts the SWIRM and amine oxidase domains of LSD1.

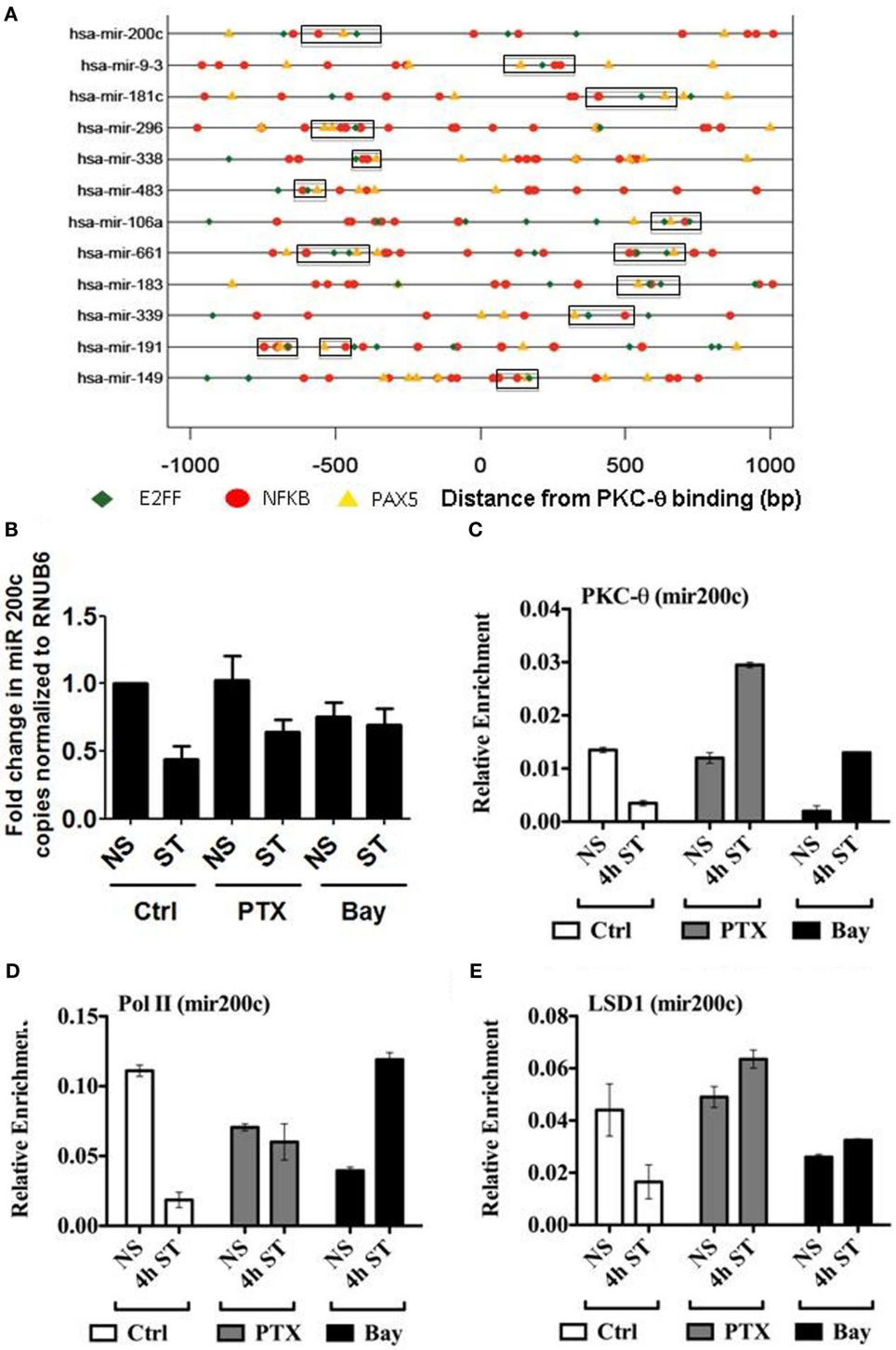

FIGURE 6 | PKC-θ binding to the miR-200c promoter is not impaired by the absence of NF-κB in T cells. (A) Transcription factor binding motifs in the microRNA PKC-θ bound sequences. Motifs for NFKAPPAB_01 (NFKB, red), E2F_03 (E2FF, green), and PAX5_01 (PAX5, orange) were identified by their Position Weight Matrices. The three motifs commonly occur within 300 bp of each other near the PKC binding sites in microRNA genes. **(B)** TaqMan miR-200c microRNA cDNA was isolated from resting (NS) and 4 h PMA/CaI stimulated (ST) Jurkat T cells in the presence or absence of Pentoxifylline (PTX) or Bay. Data expressed as fold change in miR-200c relative to NS set to 1 and normalized to RNU6B. Data representative of the mean ± SE of three independent experiments. **(C)** PKC-θ, **(D)** Pol II, and **(E)** LSD1 ChIP were carried out on samples as described for **(B)**. Sybr-green real-time PCR was used to detect the relative enrichment of these proteins across the *miR-200c* promoter. ChIP data are expressed as relative enrichment and plotted as the mean ± SE of two independent experiments performed in duplicate.

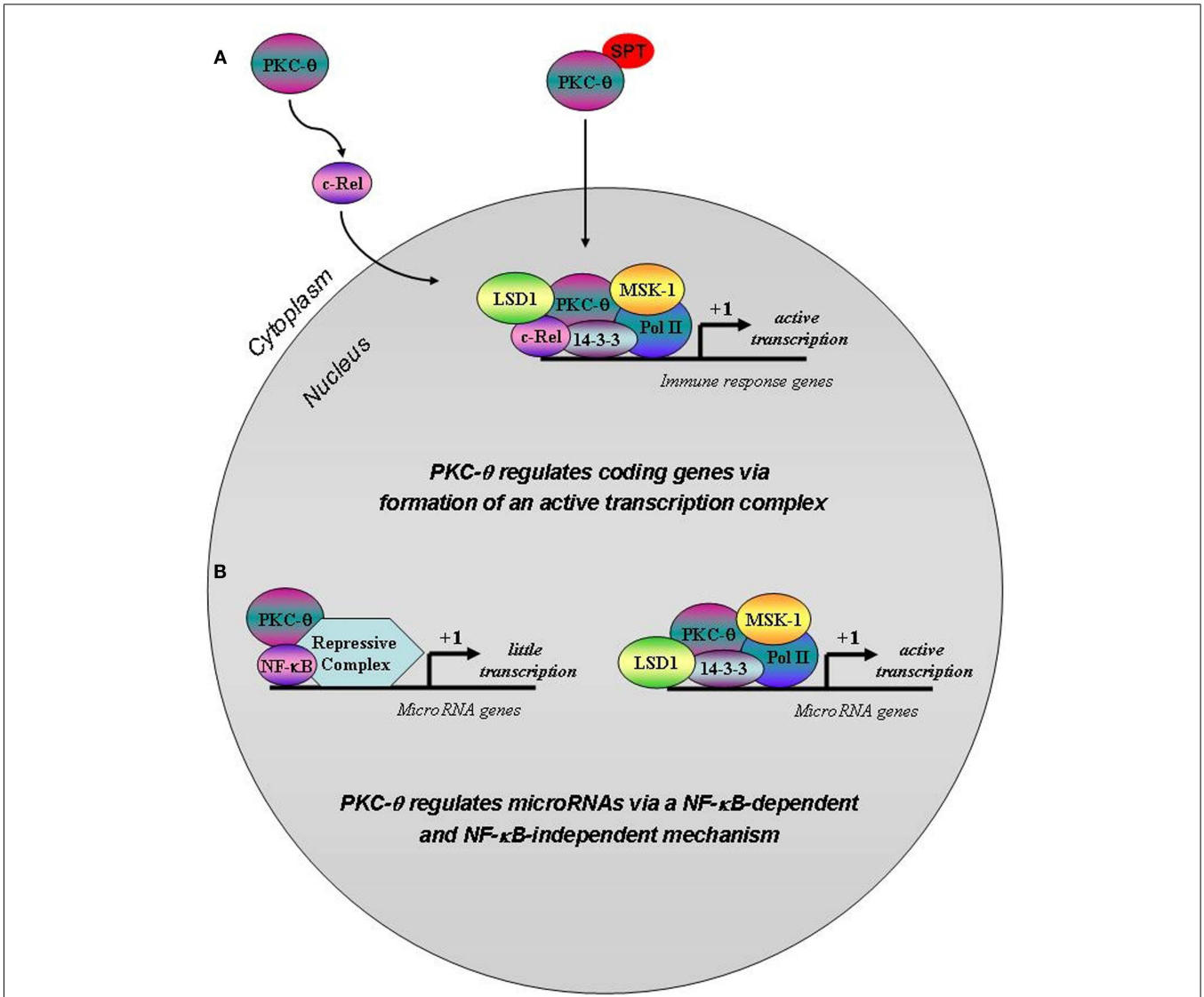

FIGURE 7 | Putative model for PKC-θ tethering to coding vs. microRNA genes in T cells. (A) T cell activation induces a complex signaling cascade that involves PKC-θ, which provokes the activation and nuclear translocation of the transcription factor c-Rel. This event ultimately results in the binding of c-Rel to a unique transcription factor module at gene regulatory regions. We propose that c-Rel then initiates the recruitment of PKC-θ that may enter the nucleus via a phosphorylatable SPT sequence and bind to chromatin, thereby allowing the formation of the active transcription complex on coding genes to enable chromatin accessibility and gene transcription. **(B)** Following T cell stimulation, there is a reduction in PKC-θ binding to chromatin at microRNA genes compared to resting T cells. Instead, NF-κB is recruited via a distinct transcription factor motif and may form part of a repressive complex on these genes to dampen gene transcription. When NF-κB is removed from this system, more of the active PKC-θ complex assembles on microRNA gene promoters thereby overcoming transcriptional repression.

coding genes. In this scenario when NF-κB is inhibited, the PKC-θ active transcription complex binds to target microRNAs relieving transcriptional repression. Future studies are required to decipher the make-up of this repressive complex, determine the order of recruitment during complex assembly and assess the phosphorylation status of chromatinized PKC-θ.

A fundamental question remains as to how PKC-θ tethers to chromatin at microRNA genes in the absence of NF-κB. One possibility being that PKC-θ is recruited via an epigenetic signature on histone tails or chromatin-associated proteins. For example, the 14-3-3ζ adaptor protein which we have shown to be part of the PKC-θ active complex (Sutcliffe et al., 2011) may act as an anchoring target for PKC-θ. Our findings provide a foundation for investigating the contribution of individual NF-κB family members in their ability to modulate nuclear PKC-θ mediated transcriptional events, both at coding genes and microRNA genes.

Our discovery of a molecular function of nuclear PKC-θ has challenged the previous concept that this kinase's sole purpose is signal transduction in the cytoplasm following T cell activation. Given that PKC-θ has also been shown to play a pivotal role in the generation of auto-reactive effector T cells in autoimmune conditions and immunological memory (Marsland et al., 2005; Healy

et al., 2006; Tan et al., 2006), understanding the molecular mechanisms used by PKC-θ to elicit specific and distinct transcriptional programs in T cells may provide new avenues for therapeutic drug design.

MATERIALS AND METHODS

CELL CULTURE

Human Jurkat T cells and mouse EL-4 T cells were cultured and stimulated with 24 ng/mL of PMA (Sigma) and 1 μM of CaI (Sigma) as previously described (Chen et al., 2005; Sutcliffe et al., 2009). For inhibitor studies, cells were pre-treated with 6 mg/mL pentoxifylline (Sigma), 15 μM Rottlerin (Calbiochem), 15 μM H89 (Sigma), or 5 μM Bay (Calbiochem) as specified for 1 h prior to stimulation. Cos-7 cells were cultured in DMEM high glucose medium with 10% heat inactivated FCS and antibiotics.

PROTEIN EXTRACT PREPARATION

Both whole cell and nuclear extracts were prepared for PKC kinase assays. For whole cell extracts, cells were collected and washed once in 1 X PBS then resuspended in RIPA buffer [20 mM Tris pH 8.0, 15 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.2 mM Pefabloc (Roche), 1:200 Protease Inhibitor Cocktail Set III, EDTA-Free (Cat. No: 539134, Merck), 1:500 Phosphatase Inhibitor Cocktail Set V (Cat. No: 524629, Merck)] and incubated on ice for 15 min. Samples were centrifuged at 13,000 rpm for 15 min at 4˚C and the supernatant was collected as whole cell extracts. For nuclear extracts, cells were collected and washed once in 1× PBS then resuspended in Nuclei Lysis Buffer A+ [10 mM Tris pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.1 mM EDTA, 0.5% NP-40, 0.2 mM Pefabloc (Roche), 1:200 Protease Inhibitor Cocktail Set III, EDTA-Free (Cat. No: 539134, Merck), 1:500 Phosphatase Inhibitor Cocktail Set V (Cat. No: 524629, Merck)] and incubated on ice for 5 min. Samples were centrifuged at 3000 rpm for 3 min at 4˚C then the supernatant was removed. The remaining pellet was resuspended in Nuclei Lysis Buffer A− (as in Nuclei Lysis Buffer A but without NP-40) and centrifuged for 3000 rpm for 3 min at 4˚C. The supernatant was removed and the nuclei were resuspended in SDS Lysis Buffer (1% SDS, 10 mM EDTA, 50 mM Tris, pH 8.1).

PKC-θ KINASE ASSAY

Protein Kinase C-theta enzymatic activity was assayed using a modification of a chromatin immunoprecipitation method previously described (Sutcliffe et al., 2009). Briefly, whole cell and nuclear extracts were prepared and sonicated for 7 min. The sonicated samples were centrifuged at 14,000 rpm for 5 min to remove debris and the supernatant was collected. Samples were diluted with ChIP dilution buffer (Millipore) and incubated overnight at 4˚C with 5 μg of anti-PKC-θ (sc212, Santa Cruz) and goat anti-rabbit IgG conjugated to agarose beads, affinity isolated antibody (Cat. No: A8914, Sigma-Aldrich). Samples were then processed with ChIP wash buffers (Millipore) and the Kinase Wash Buffer (Enzo Life Sciences). The beads were then resuspended in Kinase Assay Dilution Buffer (Enzo Life Sciences). The samples were loaded in duplicate wells on the PKC kinase activity plate and the assay was performed as per manufacturer's guidelines (PKC kinase activity kit, Cat. No: ADI-EKS-420A, Enzo Life Sciences). The PKC kinase activity was measured at an absorbance of 450 nm on the Benchmark Plus™ Microplate Spectrophotometer (Bio-Rad). PKC kinase activity was analyzed by firstly, subtracting the blank readings from the average of duplicate sample wells to correct for background absorbance. Then, the no antibody control well readings were subtracted from the corrected sample readings to give the relative kinase activity.

TRANSFECTIONS AND CONFOCAL MICROSCOPY

Within the full length PKC-θ wild type gene sequence the putative phosphorylation sites S^{241} and T^{243} were mutated to either the non-phosphorylatable alanine (SPT to APA) or the phosphomimetic glutamine (SPT to EPE) and were cloned into the pTracer-CMV vector in frame with a C-terminal HA-tag. This vector also codes for GFP which is translated independently of the insert and serves as an internal marker for transfected cells. Subconfluent cultures of Cos-7 cells grown on coverslips were transfected with purified plasmids using lipofectamine 2000 (Ghildyal et al., 2005) and fixed 24 h later with 4% paraformaldehyde in PBS, followed by permeabilization with Triton X-100 as described previously (Li et al., 2008). Fixed cells were probed with rabbit antibody to HA-tag (Sigma), followed by secondary antibody to rabbit immunoglobulins conjugated to Alexa-Fluor 568 (Life Technologies); coverslips were mounted on glass microscope slides with ProLong Gold antifade (Life Technologies). Localization of expressed PKC-θ was studied with confocal laser scanning microscopy as described previously (Ghildyal et al., 2009a). Single sections of 0.5 μM were obtained with Nikon x60 oil immersion lens using a Nikon C1 plus confocal system and NIS-Elements AR 3.2 software; the final image was obtained by averaging four sequential images of the same section. Digital confocal images were analyzed with ImageJ public domain software to determine the nuclear/cytoplasmic fluorescence ratio ($F_{n/c}$), determined by using the equation: $F_{n/c} = (F_n - F_b)/(F_c - F_b)$, where F_n is the nuclear fluorescence, F_c is the cytoplasmic fluorescence, and F_b is the background fluorescence (autofluorescence; Ghildyal et al., 2005, 2009a,b). Mann–Whitney non-parametric test (GraphPad Prism) was used to determine significant differences between datasets.

TOTAL RNA ISOLATION AND QUANTITATIVE REAL-TIME PCR

Total RNA was extracted, converted to cDNA, and real-time PCR was performed as previously described (Sutcliffe et al., 2009). Human arbitrary *IL-2* and *CD69* transcript levels were detected using gene specific Taqman assays [*IL-2* (Hs00174114) and *CD69* (Hs00934033) Applied Biosystems]. Inducible gene transcript levels were normalized to the housekeeping gene, *GAPDH* (Hs99999905). MiR-200c and RNU6B microRNA assays were performed using the TaqMan® MicroRNA Reverse Transcription Kit (ABI 4366596) and probes previously described (Sutcliffe et al., 2011).

CHROMATIN IMMUNOPRECIPITATION ASSAYS

ChIPs were performed following the protocol supplied by Upstate Biotechnology, as previously detailed (Sutcliffe et al., 2009, 2011). Five micrograms of the following antibodies were used for ChIP: anti-PKC-θ (Santa Cruz), anti-Pol II (Abcam), and anti-LSD1

(Millipore). Immuno-complexes were enriched with Magna ChIP Protein A Magnetic beads (16-661, Millipore) and washed with ChIP wash buffers prior to DNA elution. Immuno-precipitated DNA was quantified using SYBR-green real-time PCR and normalized to total genomic input ($2^{-\Delta Ct}$). Primers for human CD69 were:−241 to −52 relative to the TSS (Fwd: CCCACTTTCCTCCTGCTACA and Rev: GCCGCCTACTTGCTTGACTA) and +137 to +377 relative to the TSS (Fwd: CCGGAGAGTGGACAAGAAAG and Rev: GGGGTTTACCTCTTCCCTGA); and for the human miR200c promoter: (Fwd: CCACTGCCTTAACCCCTTC and Rev: AGGGGTGAGACTAGGCAGGT) previously described in reference (Wiklund et al., 2010).

BIOINFORMATICS

To help determine which transcription factors may be aiding the recruitment of PKC to DNA, we used the Genomatix program to examine the promoters of 69 of the top PKC binding genes. Our strategy was to look for transcription factor binding sites as defined by their TRANSFAC position weight matrices. The sequences of the PKC binding regions (±1 kb; Sutcliffe et al., 2011) were obtained from UCSC (Hg18). Match (Kel et al., 2003) was used to find where the over-represented motifs in the sequences occur with the core similarity score cut-off set to minimize the false negative rate and the matrix similarity score set to 0.9 for ZBPF, MAZF, MZF1, and SP1F members, 0.85 for EBOX and PAX5 members and 0.8 for E2FF, EKLF, and NFKB and 0.7 for AHRR members. The over-represented motifs belong to several superfamilies so to remove the redundancy when searching for co-occurring motifs, we reduced the set to NFKAPPAB_01 (NFKB), MYCMAX_03 (EBOX), E2F_03 (E2FF), MAZR_01 (MAZR), AHRARNT_02 (AHRR), PAX5_01 (PAX5), MZF1_01 (MZF1), and SP1_01 (SP1F). We calculated how frequently these motifs occurred together in groups of three within 300 bp. To determine if this was higher than by chance we also calculated the average number and SD of sequences that would have the three motifs within 300 bp if the motifs were distributed randomly among a similar number of sequences 1000 times. A co-regulatory motif was considered significant if it occurred more frequently than 2 SD above the mean of the 1000 random permutations. The calculations and visualization were performed in R.

COMPUTATIONAL MODELING

The interaction between PKC-θ and the LSD1-CoREST complex was modeled using the ClusPro server (Kozakov et al., 2010), with the LSD1-CoREST crystal structure (pdb code 2IW5) and chain A from the crystal structure of the catalytic domain of PKC-θ (pdb code 2JED). The model shown in **Figure 5C** is the top ranked model.

PROTEIN KINASE C SEQUENCE ALIGNMENTS

Protein sequences were retrieved from Uniprot for 11 of the PKC members (see Table in Materials and Methods for identification). By using Jalview (Waterhouse et al., 2009), combinations of nuclear localization motifs SPT, SPS, TPS, and TPT (termed SPT-like motifs) were identified. These motifs were displayed relative to the domains in PKC isoforms using CLC Main workbench 6 (CLC Bio). References demonstrating or predicting phosphorylation at the SPT-like sites are listed below.

PKC isoforms	Uniprot name	Location	Sequence	Ref to show phosphorylation
PKC-δ	KPCD_human	240–242	SPT	
PKC-θ	KPCT_human	241–243	SPT	
PKC-ε	KPCE_human	337–339	SPS	
PKC-η	KPCL_human	317–319	SPT	Gauci et al. (2009), Mayya et al. (2009), and Oppermann et al. (2009)
PKC-α	KPCA_human	111–113	SPT	
		319–321	SPS	Beausoleil et al. (2006), Daub et al. (2008), Olsen et al. (2006), and Zahedi et al. (2008)
PKC-β (I)	KPCB1_human	111–113	SPT	
		642–644	TPT	Grodsky et al. (2006) and Zahedi et al. (2008)
PKC-β (II)	KPCB2_human	111–113	SPT	
PKC-γ	KCPG_human	110–112	SPT	
		326–332	SPSPSPT	
		326–332	SPT	
PKC-ζ	KCPZ_human	–	–	
PKC-λ/ι	KCPI_human	–	–	

NLS

PKC isoforms	Uniprot Name	Location	Sequence
PKC-δ	KPCD_human	613–625	KRRLEPPFRPKVK
PKC-θ	KPCT_human	644–656	RKEIDPPFRPKVK
PKC-ε	KPCE_human	678–690	QKKIKPPFKPRIK
PKC-η	KPCL_human	624–636	HRQIEPPFRPRIK
PKC-α	KPCA_human	607–619	NREIQPPFKPKVC
PKC-β (I)	KPCB_human P05771-1	610–622	RKEIQPPYKPKAR
PKC-β (II)	KPCB_human P05771-2	610–622	KEIQPPYKPKAR
PKC-γ	KCPG_human	624–636	RLEIPPPFRPRPC
PKC-ζ	KCPZ_human	528–540	KKQALPPFQPQIT
PKC-λ/ι	KCPI_human	552–544	QKQVVPPFKPNIS

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Regulation of the PKCθ-NF-κB axis in T lymphocytes by the tumor necrosis factor receptor family member OX40

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Antigen primed T lymphocytes need to expand and persist to promote adaptive immunity. The growth and survival signals that control this are in large part provided by the NF-κB pathway in activated or effector/memory T cells. Although several membrane receptors impact NF-κB activation, signaling from OX40 (CD134, TNFRSF4), a member of the tumor necrosis factor receptor (TNFR) superfamily, has proven to be important for T cell immunity and a strong contributor to NF-κB activity. PKCθ directs the T cell receptor (TCR) and CD28-dependent assembly of a CBM complex (CARMA1, BCL10, and MALT1) for efficient activation of NF-κB, raising the question of whether other membrane bound receptors that activate NF-κB also require this PKCθ-CBM axis to control TCR-independent T cell activity. We discuss here our recent data demonstrating that after ligation by OX40L (CD252, TNFSF4) expressed on antigen-presenting cells, OX40 translocates into detergent-insoluble membrane lipid microdomains (DIM or lipid rafts) in T cells irrespective of TCR signals, and assembles into a signaling complex containing PKCθ, together with TRAF2, RIP1, the CBM complex, and the IKKα/β/γ complex. PKCθ is required for optimal NF-κB activation mediated by OX40 and thus works as an essential component of this OX40 signalosome. We also discuss the likelihood that other TNFR superfamily molecules might complex with PKCθ in T cells, and whether PKC isoforms may be critical to the function of TNFR molecules in general.

Keywords: OX40, PKCθ, TRAF, NF-κB, IKK, CBM, TNFSF, TNFRSF

INTRODUCTION

Interactions between several members of the tumor necrosis factor (TNF) superfamily and the TNF receptor (TNFR) superfamily are vital for regulation of T cell activation, differentiation, and survival (Croft, 2003, 2009). Many TNFR molecules, such as TNFR2 (TNFRSF1B), OX40 (TNFRSF4), CD27 (TNFRSF7), CD30 (TNFRSF8), 4-1BB (TNFRSF9), HVEM (TNFRSF14), GITR (TNFRSF18), and DR3 (TNFRSF25) are constitutively or inducibly expressed on T cells. They can be viewed as major sources of nuclear factor κB (NF-κB) signals, through TNF ligand-dependent recruitment of adaptors (TNF receptor-associated factors or TRAFs), making them important components of the T cell signaling machinery (Croft, 2003, 2009, 2010; Sugamura et al., 2004; Watts, 2005; So et al., 2006; Nocentini et al., 2007; Nolte et al., 2009; Faustman and Davis, 2010). Although the molecular machinery through which TNFR1 (TNFRSF1A) regulates signaling has been intensively studied in non-T cells and has become a framework to understand the classical or canonical NF-κB (NF-κB1) pathway as well as induction of apoptosis, it is unclear how other members of the TNFR superfamily organize their signaling complexes on the membrane, especially in T cells, and whether the respective complexes are comparable to that recruited by TNFR1.

Activation of NF-κB1 is initiated by signal-dependent phosphorylation, ubiquitination, and subsequent degradation of inhibitory IκB. This allows cytoplasmic NF-κB1/RelA to stably translocate to the nucleus and activate gene transcription. IκB phosphorylation is catalyzed by the IκB kinase (IKK) complex that contains two homologous catalytic subunits, IKKα and IKKβ, and the regulatory subunit IKKγ. Activation of IKKβ is essential for NF-κB1 in response to all pro-inflammatory stimuli (Hayden and Ghosh, 2008; Vallabhapurapu and Karin, 2009). Although all stimuli leading to NF-κB1 activation appear to converge on IKKβ-mediated IκB phosphorylation, the upstream events controlling activation of the IKK complex are possibly distinct and specific to the individual type of NF-κB-activating stimulus. In T cells, the T cell receptor (TCR) and the Ig superfamily costimulatory molecule CD28 are capable of synergizing together and activating NF-κB1. CARMA1 [caspase-recruitment domain (CARD)-membrane-associated guanylate kinase (MAGUK) protein 1] has been shown to control this NF-κB1 activation by forming a complex with B cell lymphoma 10 (BCL10), and mucosa-associated-lymphoid-tissue lymphoma-translocation gene 1 (MALT1), termed CBM (Gaide et al., 2002; Wang et al., 2002; Thome, 2004). Importantly, PKCθ (protein kinase C θ) is also recruited after cross-linking the TCR and CD28 (Bi et al., 2001). Phosphorylation of CARMA1 by PKCθ induces the assembly of the CBM complex that then activates IKKβ (Matsumoto et al., 2005; Sommer et al., 2005).

The question was then raised as to whether this PKCθ-CBM module to activate IKKβ was specific to cooperation between the TCR and CD28 or might be a shared pathway with other molecules,

either in T cells or non-T cells. Initial studies of CARMA1 suggested the former. TNF binding with TNFR, largely on non-T cells such as embryonic fibroblasts, has been extensively characterized, and shown to recruit TRAF2 that links the serine/threonine kinase RIP1 (receptor interacting protein kinase-1) to activation of IKKβ (Chen and Goeddel, 2002; Wajant et al., 2003; Muppidi et al., 2004; Kovalenko and Wallach, 2006). In contrast, TNF was found to induce NF-κB activation equivalently in CARMA1-deficient T cells (Gaide et al., 2002; Wang et al., 2002), implying that TNFR superfamily members may not use or require the PKCθ-CBM pathway for their activities. We have now recently defined a novel signaling complex of OX40, which does contains PKCθ and the CBM complex, as well as TRAF2, RIP1, and the IKK complex (IKKα, IKKβ, and IKKγ), but not TCR, CD28, or other TCR-proximal signaling kinases (So et al., 2011b). Upon OX40L (TNFSF4) stimulation, this signaling module is organized in detergent-insoluble membrane lipid microdomains (DIM or lipid rafts) and regulates TCR-independent NF-κB1 activation. This review focuses on these new findings regarding the OX40 complex and discusses its relevance to other TNFR members in terms of regulation of PKCθ and other PKC isoforms.

NF-κB1 SIGNALING THROUGH OX40 IS ESSENTIAL FOR ACTIVATED/EFFECTOR T CELL RESPONSES

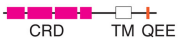
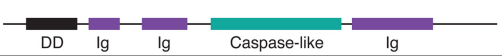
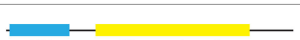
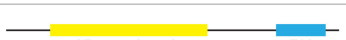
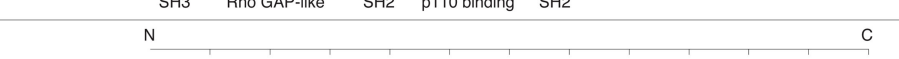
The TNF receptor OX40 is induced on activated $CD4^+$ and $CD8^+$ T cells and the TNF ligand OX40L is induced on activated antigen-presenting cells (APCs; Croft, 2010). Signaling through OX40 dominantly regulates T cell turnover at the peak of the expansion phase of many immune responses and the subsequent survival of activated/effector T cells when antigen becomes limiting (Gramaglia et al., 2000; Rogers et al., 2001; Bansal-Pakala et al., 2004). OX40-deficient T cells cannot persist well and exhibit decreased survival rates, resulting in reduced accumulation of memory cells with time (Gramaglia et al., 2000; Murata et al., 2000; Humphreys et al., 2007; Soroosh et al., 2007; Mousavi et al., 2008). The signaling mechanisms by which OX40 contributes to T cell survival are reasonably well defined in $CD4^+$ T cells. Little has been done in terms of signaling in CD8 T cells but the targets and molecules involved are likely similar. One critical pathway that regulates $CD4^+$ T cell survival mediated by OX40 is NF-κB1 (Song et al., 2008). Phosphorylation of IκB, nuclear translocation of NF-κB1/RelA, and NF-κB1 activities, are impaired in antigen-responding $CD4^+$ T cells which lack OX40. In accordance with this, OX40-deficient $CD4^+$ T cells cannot maintain high levels of several anti-apoptotic Bcl-2 family members that are under the control of NF-κB1. Correspondingly, retroviral transduction of a constitutively active form of IKKβ into OX40-deficient $CD4^+$ T cells rescues the poor survival phenotype and increases the expression of Bcl-2 family members (Song et al., 2008).

The TNF ligand OX40L is a type II transmembrane and homotrimeric protein composed of three TNF homology domains, whereas OX40 is a type I transmembrane protein monomer and is trimerized through binding with OX40L, resulting in formation of a quaternary organized hexamer complex. OX40 has four cysteine-rich domains (CRDs) and the first three CRDs from the N-terminus interact with OX40L in the extracellular space (Compaan and Hymowitz, 2006). OX40 has the potential to recruit TRAF2, TRAF3, and TRAF5 to a QEE motif existing in its ∼40 amino acid cytoplasmic tail (Arch and Thompson, 1998; Kawamata et al., 1998; **Table 1**). However, whether all TRAFs are recruited *in vivo* is not clear and the downstream signaling that is controlled by these TRAFs has not been investigated in detail. To easily visualize and uncover the signaling modules induced by OX40 ligation, we established an MCC-specific T cell hybridoma cell from OX40-deficient and TCR transgenic mice, and introduced cMyc-tagged-OX40 into this T cell (So et al., 2011b). Although the cMyc-tag is attached to the N-terminus of OX40, this cMyc-OX40 can interact normally with OX40L and induce strong NF-κB1 activity in the T cell. Furthermore, the cMyc-tagged OX40 can be efficiently precipitated from this cell (So et al., 2011b). After triggering OX40 with membrane bound OX40L expressed on a fibroblast cell (Gramaglia et al., 1998), we observed recruitment of the canonical TRAF2, RIP1, and IKK complex, and also PKCθ and the CBM complex (**Table 1**). Importantly, this signalosome did not require TCR signals, and was formed without antigen recognition and in the complete absence of a TCR. Moreover, an anti-OX40 agonist antibody immobilized on a plate induced the same signaling complex (So et al., 2011b).

A MOLECULAR FRAMEWORK FOR THE OX40 SIGNALOSOME

An important issue is how OX40 builds the functional signaling complex for NF-κB1 in the absence of TCR signals. In the TNFR1-NF-κB1 pathway, a pro-survival complex I is formed by recruitment of TNF receptor-associated death domain (TRADD), RIP1, TRAF2, cellular inhibitor of apoptosis protein 1 and 2 (cIAP and cIAP2), and the linear ubiquitin chain assembly complex (LUBAC; Chen and Goeddel, 2002; Wajant et al., 2003; Muppidi et al., 2004; Kovalenko and Wallach, 2006; Iwai and Tokunaga, 2009; Vallabhapurapu and Karin, 2009; Walczak, 2011). RIP1 and TRAF2 are conjugated with non-degradative Lys-63 (K63)-linked polyubiquitin chains, which are thought to be critical to recruit a transforming-growth-factor-β-activated kinase-1 (TAK1)/TAK1-binding protein (TAB) 2/TAB3 complex and the IKK complex, leading to IKK activation (Wertz and Dixit, 2008; Li et al., 2009; Skaug et al., 2009; Napolitano and Karin, 2010). TRAF2 acts as an adaptor and it may function as part of the E3 ubiquitin ligase for RIP1 in concert with cIAP1/2 (Ea et al., 2006). In contrast, OX40 does not have a death domain (DD) to recruit TRADD but may simply rely on its QEE motif to recruit TRAFs (**Table 1**; **Figure 1**). Short hairpin RNA mediated silencing of TRAF2 significantly decreased the association between OX40 and the IKK complex and blocked NF-κB1 activation (So et al., 2011b), showing that TRAF2 is as an essential keystone for the OX40-NF-κB1 axis. RIP1 was ubiquitinated following OX40 triggering, but the deficiency in TRAF2 did not change the level of ubiquitination and did not affect recruitment of RIP1 to OX40 (So et al., 2011b). Although RIP1 is thought to play a role in TNFR1 driven NF-κB signaling as described above, it has been reported that TNF-induced NF-κB1 activation is normal in some RIP1-deficient cells, suggesting that the requirement for RIP1 is cell-type specific (Bertrand and Vandenabeele, 2010). The functional significance of RIP1 in the OX40 complex has yet to be determined, but it is possible that it is not sufficient for recruitment of the IKK complex or IKK phosphorylation. This may

Table 1 | Proteins involved in the OX40 signalosome[a, b].

Protein	Structure	Interactors
OX40		OX40L, TRAF2
TRAF2		OX40, IKKα, IKKβ, TAK1/TAB?, GLK?
IKKα		IKKβ, IKKγ, PKB, TRAF2
IKKβ		IKKα, IKKγ, PKB, TRAF2
IKKγ (NEMO)		IKKα, IKKβ, CBM, RIP1, TAK1/TAB
RIP1		TRAF2, TAK1/TAB, IKKγ
CARMA1		BCL10, MALT1, PKCθ, PDK1, PKB, IKKγ
BCL10		CARMA1, MALT1, IKKγ
MALT1		BCL10, IKKγ
PKCθ		PDK1, CARMA1, PKB, GLK
PKB		PKCθ, IKKα, IKKβ, CARMA1
PDK1		PKCθ
p85 (PI3K)		p110 (PI3K)
		

[a] Protein name abbreviations: TRAF, tumor necrosis factor receptor-associated factor; IKK, inhibitor of NF-κB (IκB) kinase; NEMO, NF-κB essential modulator; RIP, receptor interacting protein kinase; CARMA, caspase-recruitment domain (CARD)-membrane-associated guanylate kinase (MAGUK) protein 1; BCL, B cell lymphoma; MALT, mucosa-associated-lymphoid-tissue lymphoma-translocation gene; PKC, protein kinase C; PKB, protein kinase B; PDK, 3-phosphoinositide-dependent protein kinase; PI3K, phosphoinositide 3-kinase; TAK, transforming-growth-factor-β-activated kinase; TAB, TAK1-binding protein; GLK, germinal center kinase-like kinase; CBM, CARMA1, BCL10, and MALT1. [b] Domain name abbreviations: CRD, cysteine-rich domain; TM, transmembrane; QEE, a motif containing three amino acids, glutamine-glutamic acid-glutamic acid; RING, really interesting new gene; Zn, zinc finger; CC, coiled coil; TRAF-C, C-terminal TRAF domain; ULD, ubiquitin-like domain; SDD, scaffolding and dimerization domain; NBD, NEMO-binding domain; LZ, leucine zipper; RHIM, RIP homotypic interaction motif; DD, death domain; CARD, caspase-recruitment domain; PDZ, PSD95, DLGA and ZO1 homology; SH3, src homology 3; GUK, guanylate kinase; Ig, immunoglobulin-like domain; C2, calcium binding domain in PKC; C1, phosphatidylserine- and diacylglycerol-binding domain in PKC; PH, pleckstrin homology; SH2, src homology 2; Rho GAP, Rho GTPase-ativating protein.

explain our finding that PKCθ and the CBM complex associate with OX40.

PKCθ is highly expressed in T cells and the importance for mature T cell activation is well recognized (Sun et al., 2000; Isakov and Altman, 2002). We had previously observed in one *in vivo* system that OX40 signaling could not compensate for defective activation of PKCθ-deficient $CD4^+$ T cells even though OX40 was expressed (Salek-Ardakani et al., 2005). This implied that PKCθ was a possible mediator of OX40 signals. Although TRAF2, RIP1, CARMA1, and the IKK complex were pulled down with OX40 under conditions of immunoprecipitation with a stringent buffer (RIPA), the PKCθ-CBM complex was only pulled down using a milder buffer containing n-dodecyl-β-maltoside, a detergent that preserves membrane protein structure. This shows that the PKCθ-CBM compartment of the OX40 complex is weaker in association and may require additional intermediates, and that the membrane environment is required to organize the compartment.

It has been demonstrated that PKCθ specifically interacts with lipids or protein components in DIM (Bi et al., 2001; Melowic et al., 2007; Kong et al., 2011). Phosphoinositide 3-kinase (PI3K) participates in the selective membrane recruitment of PKCθ (Villalba et al., 2002). Protein kinase B (PKB or Akt; Bauer et al., 2001) and 3-phosphoinositide-dependent protein kinase-1 (PDK1; Park et al., 2009) interact with PKCθ, and can also control NF-κB1 activity. The interaction between PKB and CARMA1 additionally may play an important role for NF-κB1 (Narayan et al., 2006). In our experiments, OX40 translocated into DIM after interaction with OX40L and although we found that the interaction

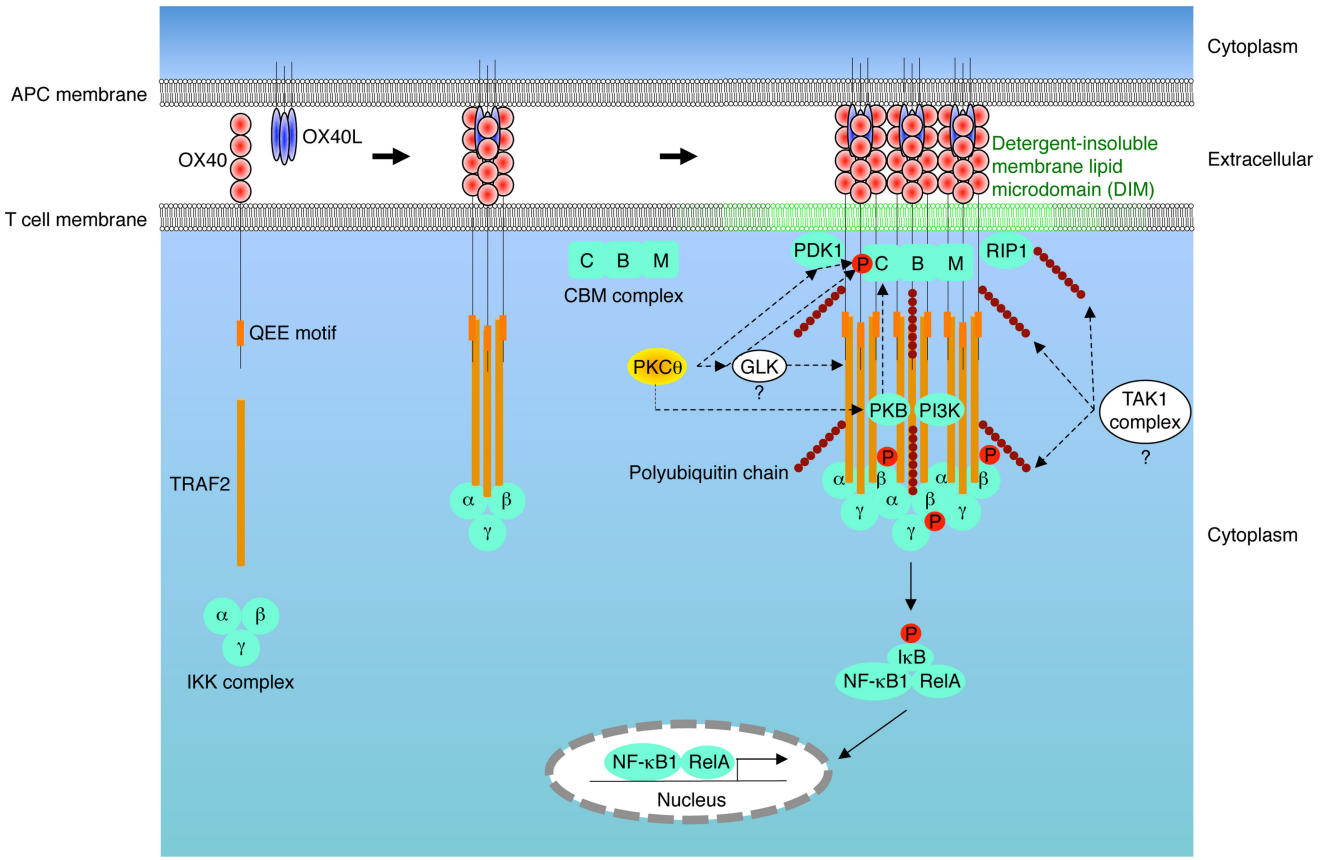


FIGURE 1 | A model of the OX40 complex cascade leading to IKK activation. OX40L binding to OX40 on T cells results in trimerization of OX40 monomers and recruitment of TNFR-associated factor 2 (TRAF2) to the cytoplasmic QEE motif in OX40. The IκB kinase (IKK) complex (IKKα, IKKβ, and IKKγ) is then recruited to the OX40-TRAF2 module, but this event is not sufficient for IKK activation. The OX40-OX40L hexamer complex translocates into detergent-insoluble membrane lipid microdomains (DIM). This organizes the higher ordered multimolecular receptor-ligand architecture that is required for IKK activation in a T cell receptor (TCR)-independent manner. Phosphoinositide 3-kinase (PI3K), 3-phosphoinositide-dependent protein kinase-1 (PDK1), and protein kinase B (PKB) are recruited to OX40 in the DIM. Although not yet determined, germinal center kinase-like kinase (GLK) is possibly incorporated into the OX40 signalosome through TRAF2. Protein kinase C θ (PKCθ) translocates to the DIM resident OX40, likely through the activities of PDK1, PKB, or GLK, and may be activated by either PDK1 or GLK. Caspase-recruitment domain (CARD)-membrane-associated guanylate kinase (MAGUK) protein 1 (CARMA1) also associates with OX40 in DIM, possibly through PKB. Activated PKCθ phosphorylates CARMA1, and this subsequently induces the CBM complex [CARMA1, B cell lymphoma 10 (BCL10), and mucosa-associated-lymphoid-tissue lymphoma-translocation gene 1 (MALT1)]. Receptor interacting protein 1 (RIP1) is also incorporated into the OX40 complex in DIM in a TRAF2-independent manner. OX40, TRAF2, and RIP1 are highly polyubiquitinated in the OX40 complex, and thus the transforming-growth-factor-β-activated kinase-1 (TAK1) complex might be recruited through these polyubiquitin chains. The IKK complex activated in the OX40 complex phosphorylates and degrades IκB, and then this facilitates entry of NF-κB1/RelA into the nucleus.

between OX40 and the TRAF2-IKK compartment was independent of DIM, depletion of cholesterol or suppression of synthesis of sphingolipid/cholesterol strongly inhibited OX40-dependent NF-κB1 activation (So et al., 2011b). This showed that additional molecular events in the DIM are required for activation of the IKK complex by OX40. In accordance, we observed that PKCθ associated with OX40 in DIM and this association was dependent on TRAF2 (So et al., 2011b). PI3K and PKB, and to a minor extent PDK1, were also inducibly recruited into the OX40 complex (So et al., 2011a). PI3K was phosphorylated in this complex (So et al., 2011a) and thus is probably important for conversion of phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2) into phosphatidylinositol-3,4,5-triphosphate (PtdIns(3,4,5)P_3) in the neighboring membrane where OX40 translocates in the immune synapse. The localization of PtdIns(3,4,5)P_3 at the inner leaflet of the plasma membrane is known to recruit pleckstrin homology (PH) domain containing signaling proteins, such as PDK1 and PKB. Activated PDK1 can phosphorylate PKCθ (Park et al., 2009) and PKB may link PKCθ and CARMA1 (Bauer et al., 2001; Narayan et al., 2006), which in turn could lead to activation of CARMA1 and induction of the CBM complex (Matsumoto et al., 2005; Sommer et al., 2005). Furthermore, PKB can directly or indirectly lead to phosphorylation of IKKα and IKKβ (Ozes et al., 1999; Romashkova and Makarov, 1999). Therefore, it is likely that PKCθ may be recruited to OX40 through PDK1 and/or PKB allowing PKCθ to phosphorylate CARMA1 and providing the maximal stimuli necessary to phosphorylate the IKK complex (**Table 1**; **Figure 1**). Consistent with this, PKCθ- or CARMA1-deficient

primary $CD4^+$ T cells displayed severely reduced activation of NF-κB1 when stimulated by OX40L in spite of normal expression of OX40 (So et al., 2011b).

It is also possible that the cross-talk between OX40 and PKCθ is mediated through the germinal center kinases (GCKs). Four of the mammalian group I GCKs, GCK, GCK-related (GCKR), GCK-like kinase (GLK), and hematopoietic progenitor kinase-1 (HPK1), have a conserved carboxyl terminal regulatory domain that was suggested to target TRAF proteins (Kyriakis, 1999) and thus these four kinases may be recruited to members of the TNFR superfamily. Both GCK and GCKR can physically associate with TRAF2 (Yuasa et al., 1998; Chin et al., 1999; Shi et al., 1999; Shi and Kehrl, 2003) although the stimuli that may induce this are unclear. Most interestingly, GLK was recently found to directly phosphorylate and activate PKCθ in T cells (Chuang et al., 2011). These data then suggest that OX40 might activate PKCθ through the TRAF2-mediated recruitment of GLK (**Figure 1**). Whether GCKs are recruited to OX40 and function to control PKCθ activity needs to be addressed in the future.

The OX40 complex is likely to be tightly controlled by polyubiquitin chains. A polyubiquitin chain is formed when one of the eight amino groups within ubiquitin (seven ε-amino groups of internal lysines and one α-amino group of N-terminal methionine) is linked to the C-terminal glycine of another ubiquitin. The best characterized linkages utilize ubiquitin K48 and K63. K48-linked polyubiquitination usually targets substrates for proteosomal degradation, whereas K63-linked polyubiquitin chains can function as scaffolds to assemble signaling complexes, such as the TAK1/TAB2/TAB3 and the IKK complexes (Wertz and Dixit, 2008; Skaug et al., 2009). The cytoplasmic tail of OX40 contains three lysine residues, which might be targets for ubiquitination. Indeed, upon triggering with OX40L, OX40 is highly ubiquitinated and the protein level of OX40 is transiently decreased (So et al., 2011a,b). Disruption of DIM decreases the level of polyubiquitin chains, correlating with reduced complex formation and weak NF-κB1 activity induced by OX40 (So et al., 2011a,b). This suggests that DIM works as a platform to attach polyubiquitin chains to OX40 and that this event plays an essential role for IKK activation. At the present, we do not know how many K48- and K63-linked polyubiquitin chains are conjugated to OX40, but we think that both types of polyubiquitin chains should be critical for regulation of the OX40-NF-κB1 axis. Whether ubiquitination of OX40 will affect recruitment of PKCθ remains to be seen.

Blocking interactions between OX40L and OX40 concomitantly block survival of pathogenic effector T cells and promote clonal expansion and suppressive function of $Foxp3^+$ regulatory T cells. OX40 is therefore a promising drug target for T cell-mediated inflammatory diseases. Mice treated with anti-OX40L blocking mAb or OX40- and OX40L-deficient mice have revealed significantly attenuated inflammation in murine models of colitis, asthma, diabetes, multiple sclerosis, rheumatoid arthritis, atherosclerosis, graft-versus-host disease, sepsis, and uveitis (Croft, 2009, 2010). PKCθ also has a similar dual role in effector and regulatory T cells, i.e., inhibition of PKCθ decreases inflammation mediated by effector T cells, whereas it promotes suppressive functions of regulatory T cells (Zanin-Zhorov et al., 2011). This suggests that inhibitors that target the molecular machinery of OX40 (**Table 1**; **Figure 1**) also have a great therapeutic potential with inflammatory and autoimmune diseases.

REGULATION OF PKC ISOFORMS BY OTHER MEMBERS OF THE TNFR SUPERFAMILY

Of the TNFR family members most closely related to OX40 (TNFR2, HVEM, 4-1BB, CD30, GITR, CD27, DR3), only 4-1BB has been assessed in terms of potentially modulating or requiring PKCθ. Ligation of 4-1BB in activated $CD8^+$ T cells was found to induce translocation of PKCθ into lipid raft domains augmenting PKCθ accumulation in the contact area between a T cell and an APC (Nam et al., 2005). The signaling complex of 4-1BB has not been visualized, but this data implies 4-1BB may recruit a signalosome that is closely related to that recruited by OX40. 4-1BB also binds TRAF2, and given our finding that TRAF2 knockdown inhibited PKCθ association with OX40, it is likely that any TNFR molecule that binds TRAF2 might have the capacity to engage PKCθ. TRAF2 can bind TNFR2, HVEM, CD30, GITR, CD27, and DR3 in transient transfection systems, implying this molecule may be central to the activities of all of these molecules. This remains to be determined, but in this regard, induction of a monocyte inflammatory mediator, TGF-β-inducible gene h3 (βig-h3), by cross-linking DR3 was blocked by several PKC inhibitors that might target PKCθ, although no direct data was provided (Lee et al., 2010).

As discussed above, current ideas suggest that PKCθ is not required for the activity of TNF through TNFR1, however other PKC isoforms may be involved in TNFR family signaling in some situations. It is well known that activation of PKC by phorbol ester can antagonize death (apoptosis) induced by DD containing TNFR members, such as TNFR1, FAS (TNFRSF6), and TRAIL-R1/2 (TNFRSF10A/B; Ruiz-Ruiz et al., 1997; Gomez-Angelats et al., 2000; Meng et al., 2002; Harper et al., 2003). Pretreatment of HeLa cells with phorbol ester inhibits recruitment of key obligatory DD-containing adaptor proteins to the death-inducing signaling complex (DISC) organized by TRAIL-R and TNFR1 (Harper et al., 2003). In the TNFR1 complex, RIP1 may recruit atypical PKCs (PKCζ and λ or ι) through p62 (Sanz et al., 1999). In human neutrophils, TNFR1 was found to recruit PKCδ to the complex and this counteracted apoptotic signaling mediated by the DISC through activation of NF-κB1 (Kilpatrick et al., 2004). Furthermore, in the TNFR1 complex of mouse embryonic fibroblast, PKCδ and PKCε were recently shown to be responsible for phosphorylation of TRAF2, controlling the introduction of K63-linked polyubiquitin chains into TRAF2, and recruitment of the TAK1/TAB2/TAB3 complex and activation of the IKK complex (Li et al., 2009). In another example, in human peripheral blood lymphocytes and leukemic T cell lines, FAS upon stimulation with FASL (TNFSF6) induced rapid localization of stromal interaction molecule 1 (STIM1) and Orai1 into the membrane receptor cluster and this led to Ca^{2+} entry and recruitment of PKCβ2 into the DISC. PKCβ2 in turn also delayed DISC formation and prevented induction of the apoptotic pathway (Khadra et al., 2011). Thus, in the apoptosis-inducing members of the TNFR superfamily, PKC recruitment may primarily limit cell death, or function to help molecules like TNFR1 to switch their signaling toward the pro-inflammatory NF-κB pathway (**Figure 2**).

In other TNFR members that do not contain DD, such as CD40 (TNFRSF5), BAFF-R (TNFRSF13C), RANK (TNFRSF11A), NGFR (TNFRSF16), and GITR, alternate PKC isoforms also appear to play roles in cellular functions. In peritoneal macrophages, strong or weak engagement of CD40 reciprocally regulated PKC isoforms, resulting in differential cellular responsiveness to *Leishmania major* infection. A higher concentration of anti-CD40 induced phosphorylation and membrane translocation of PKCα, β1, β2, and ε, which favored Th1-type protective immunity effective for the parasite elimination, whereas a lower concentration induced phosphorylation and membrane translocation of PKCδ and ζ/λ, which favored Th2-type immunity and thus permitted parasite growth (Sudan et al., 2012). In mature B cells, triggering of BAFF-R with BAFF (TNFSF13B) also induced membrane translocation of PKCβ which controlled B cell survival through PKB activation (Patke et al., 2006). Stimulation of RANK with RANKL (TNFSF11), in a pre-osteoclast cell line RAW264.7 and in primary bone marrow-derived macrophages, led to recruitment of atypical PKCs through a RANK-TRAF6-p62-PKC linkage. This activated NF-κB1/NFATc1 and played a critical role for osteoclastogenesis (Duran et al., 2004). Moreover, in P12 cells, a rat pheochromocytoma cell line, NGFR stimulation with NGF induced a receptor complex that contained K63-polyubiquitinated TRAF6, p62, IKKβ, and PKCι, which induced NF-κB1 and was involved in neuronal survival (Wooten et al., 2005). Lastly, stimulation of a macrophage cell line with soluble GITR induced recruitment of PKCδ to the cell membrane fraction (Lee et al., 2004). These data then collectively imply that overall usage of PKC isoforms by members of the TNFR superfamily is likely to be widespread. It is tempting to speculate that the TNFR-PKC axis may be critical for life and death decisions in many different types of cells by inducing NF-κB1 activation or activities of other signaling pathways (**Figure 2**).

CONCLUSIONS

Based on results obtained in our biochemical studies, we present an original model that can explain how PKCθ contributes to the NF-κB1 pathway mediated by the OX40 stimulatory receptor in T cells (**Figure 1**). Upon interaction with membrane OX40L, OX40 moves into the DIM of T cells and builds a multimolecular complex irrespective of antigen/TCR engagement. This complex provides the molecular machinery that controls IKKβ through PKCθ. PKCθ is recruited to the OX40-TRAF2 compartment, activates CARMA1, and then induces the CBM complex to augment IKK activities. This OX40 complex, which contains several upstream kinases for IKK, is an important source of NF-κB1 in T cells and controls longevity of T cells through induction of pro-survival genes. Although OX40 can provide classical costimulatory signals to T cells in concert with those from antigen and CD28, OX40 also sustains signals initiated by the TCR and CD28 while functioning as an independent signaling unit. PKC is central to signal transduction pathways involved in T cell activation, differentiation, and survival. Our data suggests that PKCθ is an integral component of the complex that allows OX40 to function in this regard, and we speculate that an equivalent signaling complex containing PKCθ is likely to be found in complexes formed by other members of the

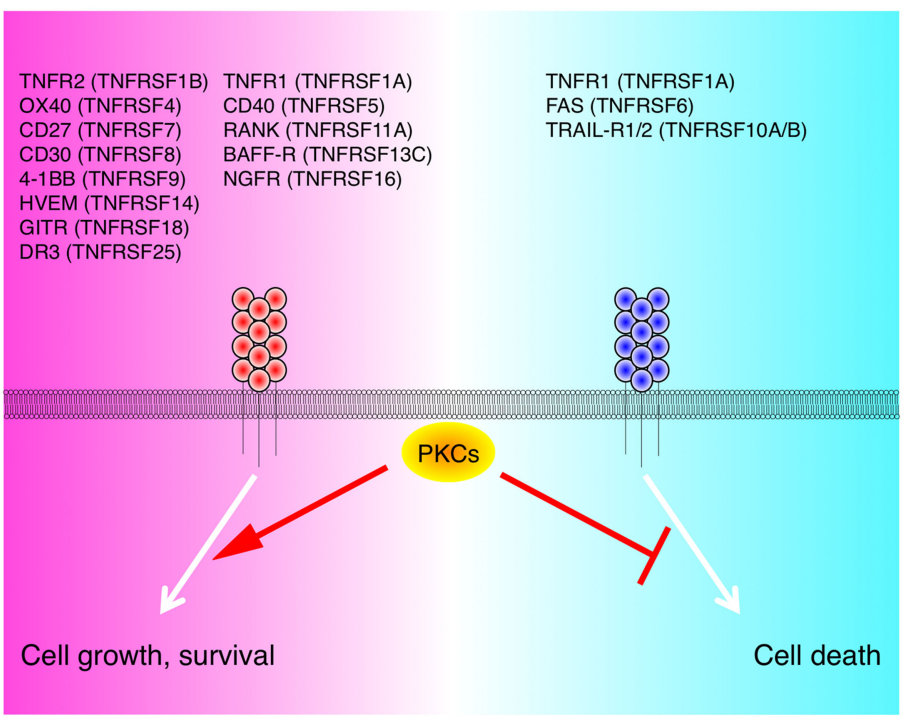


FIGURE 2 | PKC isoforms control life and death induced by TNFRSF. The TNFR-PKC axis activates NF-κB1 or other signaling pathways, which concomitantly promotes cell growth/survival and inhibits cell death.

TNFR superfamily. This may explain the global requirement for many of these molecules in driving T cell responses. It will be very important to compare the molecular mechanisms by which TNFR members control T cell activity in the future and to determine if one or several PKC isoforms are central regulators of TNFR family molecule action.

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Protein kinase C-θ (PKC-θ) in natural killer cell function and anti-tumor immunity

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The protein kinase C-θ (PKCθ), which is essential for T cell function and survival, is also required for efficient anti-tumor immune surveillance. Natural killer (NK) cells, which express PKCθ, play a prominent role in this process, mainly by elimination of tumor cells with reduced or absent major histocompatibility complex class-I (MHC-I) expression. This justifies the increased interest of the use of activated NK cells in anti-tumor immunotherapy in the clinic. The *in vivo* development of MHC-I-deficient tumors is much favored in *PKC*θ$^{-/-}$ mice compared with wild-type mice. Recent data offer some clues on the mechanism that could explain the important role of PKCθ in NK cell-mediated anti-tumor immune surveillance: some studies show that PKCθ is implicated in signal transduction and anti-tumoral activity of NK cells elicited by interleukin (IL)-12 or IL-15, while others show that it is implicated in NK cell functional activation mediated by certain killer-activating receptors. Alternatively, the possibility that PKCθ is involved in NK cell degranulation is discussed, since recent data indicate that it is implicated in microtubule-organizing center polarization to the immune synapse in CD4$^+$ T cells. The implication of PKC isoforms in degranulation has been more extensively studied in cytotoxic T lymphocyte, and these studies will be also summarized.

Keywords: PKC-θ, NK cells, anti-tumor immunity, CTL

PKCθ IN T CELLS

The protein kinase C-θ (PKC-θ) was initially isolated as a PKC isoform expressed in T cells (Baier et al., 1993), although it was demonstrated afterward that its expression was not restricted to them (Isakov and Altman, 2002). Structurally, PKCθ is a member of the novel, Ca^{2+}-independent, PKC subfamily (which also includes PKCδ, ε, and η). PKCθ rapidly translocated to the immunological synapse (IS), suggesting a central role in T lymphocyte signal transduction (Monks et al., 1997). Seminal discoveries by the group of Amnon Altman demonstrated that PKCθ was indeed essential in T cell activation through the triggering of key transcription factors such as AP-1 (activating protein-1; Baier-Bitterlich et al., 1996), nuclear factor-κB (NF-κB; Coudronniere et al., 2000), and nuclear factor of activated T cell (NF-AT; Altman et al., 2004). This was confirmed by two independent groups who developed two different strategies to knockout PKCθ mice (Sun et al., 2000; Pfeifhofer et al., 2003). These transcription factors control the expression of target genes implicated in proliferation, survival, and/or cytotoxicity. One of them, Fas Ligand (FasL), is also implicated in immune homeostasis maintenance through activation-induced cell death (AICD). PKCθ deficient mice show normal lymphocyte development but display a selective phenotype in their mature T cell compartment, characterized by impaired proliferation and interleukin (IL)-2 production in response to T cell receptor (TCR)/CD28 co-stimulation (Sun et al., 2000; Pfeifhofer et al., 2003). This phenotype is also linked to the regulation of genes essential for survival, such as B-cell lymphoma (Bcl)-2 family members (Bertolotto et al., 2000; Villalba et al., 2001b; Barouch-Bentov et al., 2005). The mechanism by which PKCθ contributes to the first steps of T cell activation has recently been uncovered. A motif in the V3 domain determines its localization to the IS through the direct interaction with the co-stimulation molecule CD28 (Kong et al., 2011). Other experts in this issue extensively describe the biochemical and functional aspects of PKCθ regulation in T cells.

NATURAL KILLER CELLS

The appearance of clinically detectable tumors may be the result of the proliferation of cells that have developed sophisticated strategies to escape the immune response. Arguably, the most relevant is the total or selective loss of expression of the major histocompatibility complex class-I (MHC-I). MHC-I, known as human leukocyte antigen (HLA) in humans, mediates self-recognition and thus present endogenously synthesized antigens to CD8$^+$ cytotoxic T lymphocytes (CTLs). Changes in MHC-I allow tumor cells to avoid CTLs and thereby the adaptive immune response (Aptsiauri et al., 2007). However, a small amount of surface MHC-I must be maintained, because its absence would make tumor cells targets of natural killer (NK) cells.

Natural killer cells are members of the innate immune system with natural cytotoxicity against tumor cells. This lymphocyte lineage produces cytokines and shows cytotoxicity and effector

functions (Lanier, 2008; Velardi, 2008). NK cells predominantly target cells lacking MHC-I, which include transformed or virus-infected cells, which down-regulate MHC-I expression to avoid recognition by CTLs. Therefore the "missing self" hypothesis proposes that NK cells discriminate target cells from other healthy "self" cells based on MHC-I expression. However, it is now clear that NK cell activation depends on a complex signaling process mediated by activating and inhibitory receptors, being the functional outcome the final result of the different activating and inhibitory signals received (see the schematic representation shown in **Figure 1**).

One of the most potent activating NK cell receptor (killer-activating receptor, KAR) is CD16, a receptor for the Fc domain of IgG, also termed FcγRIII, which is responsible for antibody-dependent cell-mediated cytotoxicity (ADCC), one of the main physiological functions of NK cells. CD16 signals through its association with the immunoreceptor tyrosine-based activation motif (ITAM)-containing adaptors FcεR1γ and CD3ζ, which finally activate ZAP-70 and Syk protein tyrosine kinases (López-Botet et al., 2000; Lanier, 2008; KEGGpathway, 2011). Other human KARs that share a similar signal transduction machinery are NKp46 and NKp30. Influenza virus hemagglutinin (HA) and B7-H6 expressed on the surface of tumors, respectively, have been identified as ligands for these KAR, but it is also possible that other unidentified endogenous ligands exist.

NKp44, which ligand is also unknown, completes, together with NKp30 and NKp46, the so-called natural cytotoxicity receptors (NCR) family. However, NKp44 signals through a different ITAM-containing adaptor, DAP12, that also results in the activation of ZAP-70 and Syk. This signaling adaptor is shared by several activating receptors belonging to the killer cell immunoglobulin-like receptors (KIR) family: KIR2DS1 and KIR2DS4, which ligands are specific haplotypes of HLA-C, and KIR2DS2 and KIR3DS1, which ligands are still unknown. Finally, the DAP12 adaptor is also used by NKG2C, a member of a different family of NK cell receptors, the C-lectin type family. This activating receptor is expressed as a heterodimer with CD94 and its ligand is HLA-E.

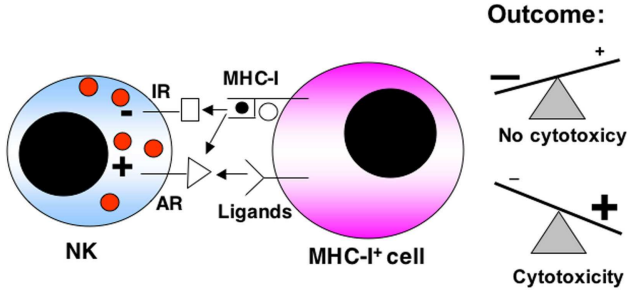

FIGURE 1 | Schematic representation of the balance between activating (AR) and inhibitory receptors (IR) in NK cell anti-tumoral function. Receptors for MHC-I can be either inhibitory or activating. The expression levels of MHC-I or of the different ligands for the activating receptors on the target cell is of paramount importance in the final response. The final outcome between both types of signaling will determine if there is a cytotoxic response or not.

Another activating receptor of the C-lectin type is NKG2D. This protein is expressed as a homodimer, and uses a different adaptor for signaling, DAP10. This adaptor does not contain ITAM domains in its cytoplasmic tail, but it contains one YINM motif, that, upon tyrosine phosphorylation recruits both PI3K and the Grb2-Vav1-Sos1 complex. The downstream signaling of this receptor does not require ZAP-70 and/or Syk activation, but results also in ERK and PKC activation. NKG2D ligands in humans are members of the MIC and ULBP families of proteins, which are expressed by virus-infected or tumoral cells.

Another family of activating receptors is the SLAM family, which includes CD244 (also known as 2B4), CRACC (CD319), and NTB-A. CD244 ligand is CD48, while CRACC and NTB-A promote homophilic interactions. These receptors posses TIYXX motifs in their cytoplasmic tails (also called immunoreceptor tyrosine-based switch motif, ITSM), which, upon tyrosine phosphorylation recruit the adaptors SAP or EAT2. If SAP is recruited, then the signals generated, including the activation of the protein tyrosine kinase Fyn, result in activation. However, if EAT2 is recruited, then signals are rather inhibitory.

Finally, two other activating receptors are expressed in human NK cells, DNAM-1, also known as CD226, and NKp80, also known as CLEC5C. It seems that DNAM-1 signaling is dependent on specific domains present in its own cytoplasmic tail (Shibuya et al., 1998). On the other hand, it has been recently demonstrated that NKp80 transmits signals through a semi-ITAM domain present in its cytoplasmic tail, activating directly Syk (Dennehy et al., 2011). The ligands for DNAM-1 are CD112 and CD155, molecules implicated in leukocyte adhesion, and the ligand for NKp80 is AICL, a very close homolog to NKp80 expressed on the surface of solid tumors.

In mice, NK cell activating receptors belong rather to the C-lectin family, normally forming homodimers on the surface of NK cells, and transmitting signals through the DAP12 adaptor. These are Ly49D, Ly49H, and Ly49P, which ligands are, respectively, H-$2D^d$ and murine cytomegalovirus antigens. Two other members of this family, NK1.1 and NKR-P1F signals instead through FcεR1γ and recognize members of a new C-type lectin-related (Clr) family.

PILRβ has been identified as an activating NK cell receptor in mice, being its signaling dependent also on DAP12 and its ligand CD99.

Activating NK cell receptors used by mice and humans include CD16, CD244, the heterodimer CD94/NKG2C, the homodimer NKG2D, and DNAM-1.

Inhibitory NK cell receptors posses ITIM domains in their cytoplasmic tails, and its ligation results in activation of protein tyrosine phosphatases, mainly SHP-1 and SHP-2, that counteract the signals given by activating receptors, initiated by tyrosine phosphorylation.

Most of the killer cell immunoglobulin-like receptors (KIR) have inhibitory activity, being their ligands specific or broad HLA haplotypes. Among them, KIR2DL1 recognizes HLA-Cw4 and related, "group2" alleles. KIR2DL2 and KIR2DL3 recognize HLA-Cw3 and related, "group1" alleles. KIR3DL1 is the receptor for HLA-B allotypes with Bw4 motifs. Finally, KIR3DL2 is the receptor for HLA-A3/11 and KIR2DL5 ligands are unknown.

Some members of the C-lectin type family of NK cell receptors are inhibitory. These are the heterodimer CD94/NKG2A, which ligand is HLA-E, and the homodimers Mafa and NKR-P1 (CD161), which ligands, are, respectively, cadherins and lectin-like transcript 1 (LLT1), respectively.

Other human NK cell inhibitory receptors are immunoglobulin-like transcript 2 (ILT2), which binds to most HLA-I haplotypes; LAIR-1, which binds to collagen; CEACAM-1, that binds to the different CD66 variants; and SIGLEC7, that binds to sialic acids.

Finally, KIR2DL4 (CD158d) is a member of the KIR family, which ligand is the non-classical HLA protein HLA-G, that has both activating and inhibitory capacities, similar to the described situation with CD244 (2B4).

Again, most of the inhibitory receptors in mouse NK cells belong to the C-type lectin family, Ly49a, c, e, f, g, and i which ligands are different MHC-I haplotypes (see **Table 1** for the specific ligands of each receptor). Other inhibitory receptors in mouse NK cells are gp49b1, that binds to integrins, and PILRα, which ligand is CD99. Inhibitory receptors shared by mouse and human NK cells are Mafa, CD94/NKG2A, and LAIR-1. See **Table 1** for a summary of all receptors, ligands, and adaptors.

NK CELLS IN TUMOR IMMUNOTHERAPY

A large interest in NK cells is currently coming from the field of cancer immunotherapy, which tries to increase the anti-tumoral immune response of cancer patients. Data from several laboratories suggest that exploiting NK cell alloreactivity could be largely beneficial independently of the NK cell source for the treatment of blood-borne cancers (Terme et al., 2008; Velardi, 2008; Cho and Campana, 2009). Donor-versus-recipient NK cell reactivity is mediated by KIRs, which sense missing expression of donor KIR-ligand(s) in the recipient and mediate alloreactions. KIR-ligand mismatching is a prerequisite for NK cell alloreactivity because in 20 donor–recipient pairs that were not KIR-ligand mismatched in the graft-versus-host direction, no donor alloreactive NK clones were found (Ruggeri et al., 2007b).

The most advanced clinical use of NK cells is related to hematological cancers in which current clinical protocols fail inducing

Table 1 | Natural killer cell activating and inhibitory receptors in mice and humans and their ligands.

Adaptor	Activating receptor	Ligand	Inhibitory receptor	Ligand
HUMAN AND MOUSE				
FcεRIγ, CD3ζ	CD16	IgG	CD94-NKG2A	HLA-E ($Qa1^b$)
	NKp46	HA, ?	Mafa	Cadherins
DAP12	CD94-NKG2C	HLA-E ($Qa1^b$)	LAIR-1	Collagen
DAP10	NKG2D	MIC, ULBP (Rae-1, H60)		
SAP/EAT2	CD244 (2B4)*	CD48		
–	DNAM-1	CD112, CD155		
HUMAN				
FcεRIγ, CD3ζ	NKp30	B7-H6, ?	KIR2DL1	HLA-Cw4
			KIR2DL2	HLA-Cw3
			KIR2DL3	HLA-Cw3
DAP12	NKp44	?	KIR3DL1	HLA-Bw4
	KIR2DS1	HLA-Cw3	KIR3DL2	HLA-A3/11
	KIR2DS2	?	KIR2DL5	?
	KIR2DS4	HLA-Cw4	NKR-P1 (CD161)	LLT1
	KIR3DS1	?	ILT2	HLA-I
FcεRIγ	KIR2DL4*	HLA-G	CEACAM-1	CD66
SAP/EAT2	CRACC*	CRACC	SIGLEC7	Sialic acids
	NTB-A*	NTB-A		
–	NKp80	AICL		
MOUSE				
DAP12	Ly49D	$H\text{-}2D^d$	Ly49a	$H\text{-}2D^d$, $\text{-}D^k$
	Ly49H	MCMVm157	Ly49c	$H\text{-}2K^b$, $\text{-}K^d$, $\text{-}D^d$, $\text{-}D^k$
	Ly49P	MCMV?		
	PILRβ	CD99	Ly49e	?
FcεRIγ	NK1.1	Clr?	Ly49f	$H\text{-}2^d$
	NKR-P1F	Clrg	Ly49g	$H\text{-}2D^d$
			Ly49i	$H\text{-}2D^k$
			gp49b1	Integrins
			PILRα	CD99

*The adaptors used by each family of activating receptors are also indicated. In the mouse and human activating receptor ligand row, parenthesis indicates the mouse ligand. *Indicates receptors with both activating and inhibitory capabilities. ?, Unknown ligand.*

long-term survival in a significant number of patients. Those refractory to standard treatment, i.e., radio- and/or chemotherapy are often subjected to a myeloablative regimen followed by allogenic hematopoietic stem cell transplantation (HSCT). However, the mortality linked to this treatment approaches to 20%. In addition, Graft-versus-Host (GvH) and HvG diseases and opportunistic infections hamper this procedure. Moreover, relapse has become the leading cause of death following allogenic HSCT: the relapse rate has not decreased over the past 20 years (Kroger, 2011). In general, prognosis is poor for patients who relapsed to an allograft since effective treatment options are limited. These include donor lymphocyte infusions, withdrawal of immunosuppressive medication and second allogeneic HSCT. However, new specific cellular approaches are under investigation. In particular, the use of alloreactive NK cells after umbilical cord blood transplantation (UCBT) seems promising. KIR-ligand incompatibility in the GvH direction improves outcomes after UCBT in the clinic (Stern et al., 2008; Willemze et al., 2009). Moreover, NK cells: (1) are not responsible of GvHD; (2) can be injected as "differentiated" cells without the need of long time survival on patients body; (3) protect from opportunist infections (Willemze et al., 2009), probably through their immunoregulatory effects on B cells, T cells and macrophages, and more importantly on polymorphonuclear cells (PMNs; Bhatnagar et al., 2010). Finally, NK cell-mediated therapy after hematopoietic cell transplantation seems safe (Miller et al., 2005; Ruggeri et al., 2007a; Rubnitz et al., 2010). Immunotherapy, in particular NK cell-mediated, is probably the only approach to eliminate these highly resistant tumor cells.

In vitro studies on primary lympho-hematopoietic lineage tumor cells showed that alloreactive NK cells kill acute and chronic myeloid leukemia, as well as T cell acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia, non-Hodgkin's lymphoma, and multiple myeloma. The only non-susceptible target was common ALL, however, KIR-ligand incompatibility in the GvH direction improves outcomes after UCBT in ALL patients. Despite their source, alloreactive NK cells home to lympho-hematopoietic sites and ablate recipient lympho-hematopoietic cells, including leukemic cells, while sparing other healthy organs. This explains why NK cells immunotherapy would be mainly useful in blood-borne cancers (Stern et al., 2008; Willemze et al., 2009).

New strategies are being developed to use NK cells in the treatment of solid tumors in the clinic. Several tumor-targeted monoclonal antibodies (mAbs) are included in the clinical care for certain tumors. Besides inducing antibody-dependent cell-mediated cytotoxicity (ADCC), these mAbs can kill their targets through activation of the complement, which in certain cases could be associated to clinical toxicity, i.e., anti-GD2 therapy (Sorkin et al., 2010). Nowadays the clinical use of mAbs tries to mainly exploit ADCC, which induces a more satisfactory clinical response and is mainly mediated by NK cells *in vivo* (Alderson and Sondel, 2011). Between the several activating or inhibitory Fc receptors for IgG (FcγR), NK cells express almost exclusively the activating FcγRIIIa (CD16). The importance of this receptor in the clinic is highlighted by the fact that patients with a valine at position 158 of FcγRIIIa (FcγRIIIa^{158v}) respond better to mAbs-mediated therapy. This is linked to the higher affinity for IgG of FcγRIIIa^{158v} NK cells, leading to a more sensitivity activating receptor and increase ADCC (Alderson and Sondel, 2011).

PKCθ IN NK CELLS

Compared to T cells, much less is known about the role of PKCθ in NK cells. PKCθ is expressed in NK cells (Balogh et al., 1999; Vyas et al., 2002b) and it has been demonstrated that it mediates the phosphorylation of WASP-interacting protein (WIP) during NK cell activation (Krzewski et al., 2006). In addition, it has been shown that PKCθ translocates to the IS during NK recognition of target cells (Davis et al., 1999).

It was shown that NK cells from PKCθ deficient mice had impaired IL-12-stimulated interferon (IFN)-γ production, without affecting their cytotoxic potential on YAC-1 cells (Page et al., 2008), which are extremely sensitive to NK cells, including naïve, *ex vivo* obtained, NK cells. However, in a different study (Tassi et al., 2008), no effect of PKCθ deficiency was observed on IFN-γ secretion induced by IL-12, IL-18, or a combination of both cytokines. In this study no effect of PKCθ deficiency was either observed on cytotoxicity against YAC-1 cells or against tumoral cells over-expressing KAR ligands (Tassi et al., 2008). It is also possible that the protocol used to generate NK cells, *ex vivo* culture in the presence of IL-2, may have masked the effect of PKCθ during the cellular cytotoxicity assays, since the tumor cells used in that study are especially sensitive to NK cells (Van den Broek et al., 1995; Screpanti et al., 2001; Pardo et al., 2002). Nevertheless, PKCθ was required to induce IFN-γ and TNF-α secretion by KAR that contain ITAMs such as NK1.1 and Ly49D. Finally, the early control of murine cytomegalovirus infection, that is dependent on NK cell activity, was not affected by the absence of PKCθ (Tassi et al., 2008).

In human γδ T cells the co-stimulation mediated by NKG2D was dependent on PKCθ (Nedellec et al., 2010). However, this study mostly relies on the use of the PKCθ inhibitor rottlerin, which has been shown to also inhibit other cellular kinases (Davies et al., 2000).

In addition, our group has demonstrated that PKCθ plays a prominent role in tumor immune surveillance mediated by NK cells (see below, Aguiló et al., 2009).

PKCθ IN ANTI-TUMOR IMMUNE SURVEILLANCE

The cancer immuno-surveillance hypothesis proposes that the immune system detects and eliminates cells undergoing tumor transformation. Immuno-deficient mice develop more tumors than immuno-competent mice and clinical data support the notion that cancer immuno-surveillance also occurs in humans (Dunn et al., 2002, 2006; Aptsiauri et al., 2007). In addition, the adaptive immune system is thought to maintain small cancer lesions in an equilibrium state (Koebel et al., 2007; Melief, 2007). Therefore, the relevant cellular effectors of immuno-surveillance must perform two critical tasks to eradicate developing tumors: directly kill tumor cells and produce cytokines such as IFN-γ to stimulate the host immune response (Dunn et al., 2006).

We tested the role of PKCθ in T cell leukemia progression by inducing the disease in wild-type (*wt*) and PKCθ-deficient mice with moloney murine leukemia virus (M-MuLV). In line with the above-mentioned studies, we found that disease incidence

and onset were enhanced in *PKC*θ$^{-/-}$ mice. Transfer of leukemic T cells from *wt* donors into PKCθ-deficient and *wt* recipients induced leukemia in 100 and 40% of the mice, respectively. Interestingly, leukemic cells from *PKC*θ$^{-/-}$ donors were less efficient at forming tumor since only 50% of the PKCθ-deficient and 10% of the *wt* recipients developed the disease. Consistent with these observations, intravenous injection of low numbers of the murine lymphoma T cell line EL4 induced tumors more rapidly in *PKC*θ$^{-/-}$ mice compared to their wt counterpart. These results showed that PKCθ was essential for the immune response to leukemia in mice. This response probably involved CTL function, since both M-MuLV-induced tumors and EL4 cells expressed MHC-I (Garaude et al., 2008). These results also suggest a role of PKCθ in cancer immune surveillance, since tumors generated in the absence of this protein were less aggressive than those generated in the presence of PKCθ. In this connection, it has been also described that PKCθ, by mediating the activation of NF-κB by pre-TCR in immature thymocytes, contributes to the development of Notch3-dependent T cell lymphoma (Felli et al., 2005). This indicates that PKCθ could be somehow required for lymphoma cell "fitness," and the results obtained in *PKC*θ$^{-/-}$ mice could reflect both effects.

Cells of the innate immune system (γδ T, NK, and NK T cells) can also mediate anti-tumor responses. Specifically, NK cells play an important role in tumor immune surveillance (Ljunggren and Karre, 1985; Terme et al., 2008; Vivier et al., 2008), as they control progression of MHC-I-deficient tumors using perforin/granzyme- and FasL-mediated cytotoxicity (Van den Broek et al., 1995; Screpanti et al., 2001; Pardo et al., 2002). Moreover, NK cells also function as mediators between innate and adaptive immunity in anti-tumor responses (Moretta et al., 2008).

Since most tumors cells down-regulate MHC-I expression to escape the CTL-mediated response (Garrido et al., 2010), it is important to study the role of NK cells in this context.

The *in vivo* development of a MHC-I-deficient tumor (RMA-S) is favored in *PKC*θ$^{-/-}$ mice compared with wild-type mice (Aguiló et al., 2009). Previous studies clearly demonstrated that the *in vivo* development of this tumor is not dependent on T cells and that it is controlled by NK cell activity (Kärre et al., 1986; Smyth et al., 1998, 2000; Kelly et al., 2002; Vosshenrich et al., 2005). The enhanced tumor growth in *PKC*θ$^{-/-}$ mice was associated with a deficient recruitment of NK cells to the site of tumor development and with a decreased activation status of recruited NK cells. This correlated with a reduced *ex vivo* cytotoxic potential of NK cells isolated from *PKC*θ$^{-/-}$ mice on RMA-S cells after poly I:C treatment (Aguiló et al., 2009). Interestingly, and confirming previous data (Page et al., 2008; Tassi et al., 2008), no difference was observed on the killing of YAC-1 cells suggesting that in addition to the absence of MHC-I, other regulatory events might be required for the tight control of NK cytotoxicity. YAC-1 cells are sensitive to naïve NK cells and are not able to induce tumors in syngeneic mice because of their extreme sensitivity to NK cell-mediated lysis, mediated by both perforin/granzymes and FasL (Aguiló et al., 2009). However, NK cells do not target RMA-S cells unless they are previously activated, i.e., by *in vivo* injection of poly I:C. Interestingly, only perforin/granzymes, but not FasL, are responsible for the elimination of RMA-S cells mediated by activated NK cells (Pardo et al., 2002; Aguiló et al., 2009). Adoptive transfer of naïve NK cells from wt or *PKC*θ$^{-/-}$ mice to *PKC*θ$^{-/-}$ mice was also performed to demonstrate that the defect in activation was intrinsic to NK cells, and not due to any other cellular component. Hence, PKCθ seems to be implicated in NK cell-mediated anti-tumor immunity at least by acting on the cytolytic potential of activated NK cells.

Poly I:C has been extensively used as an indirect *in vivo* NK cell activator, through the secretion of cytokines by macrophages and/or dendritic cells (Djeu et al., 1979). Poly I:C, mimics viral double-stranded RNA, and is recognized by the member of the Toll-like receptor family TLR3, which is expressed by antigen-presenting cells (Alexopoulou et al., 2001), and also by cytosolic receptors such as MDA5 and RIG-I (Kato et al., 2006). Poly I:C treatment increased the level of expression and the activation status of PKCθ in NK cells, both *in vivo* and *in vitro*. In the latter case, the presence of the whole splenocyte population was needed, being the effect presumably mediated by macrophages and/or dendritic cells (Aguiló et al., 2009). This was in agreement with previous studies demonstrating the increase in anti-tumor activity of NK cells activated by poly I:C (Akazawa et al., 2007). The increase in PKCθ expression depended on cell-to-cell contact, while its activation was mediated by a soluble factor. This soluble factor should be one of the cytokines secreted by macrophages and/or DCs that are known to activate NK cells. Since IL-12 signal transduction was reported to be affected in NK cells from *PKC*θ$^{-/-}$ mice (Page et al., 2008), this cytokine was tested first. IL-15 was also included in those studies, since it is important in regulating NK cell function and survival (Carson et al., 1994; Cooper et al., 2002), and for efficient anti-tumoral NK cell activity (Liu et al., 2012). Indeed, both, IL-12 and IL-15, activated PKCθ in NK cells, with IL-15 being more potent at inducing PKCθ phosphorylation. More importantly, neutralizing antibodies to IL-15, but not those blocking IL-12, reduced substantially NK cell PKCθ phosphorylation induced *in vitro* by poly I:C treatment of a mixed splenocyte population (Aguiló et al., 2009). How could IL-15 be coupled to PKCθ activation? Interestingly, PKCθ is the only T cell expressed PKC isoform that is activated through a PI3K-dependent pathway, which is also activated by TCR ligation (Villalba et al., 2002). Although the main signaling pathway elicited by cytokine receptors is mediated by JAK and STATs, cytokines such as IL-2 are also able to activate the PI3K pathway (Brennan et al., 1997). It is interesting to note that IL-2 and IL-15 share the same signaling receptors suggesting that IL-15 could also trigger the PI3K pathway, as has been suggested in some studies in T cells (Ben Ahmed et al., 2009).

Therefore, IL-15 looked as a very feasible candidate to be a mediator in PKCθ-dependent anti-tumoral NK cell activation. We have performed additional experiments, analyzing the effect of IL-15 on different NK cell functions. We have found that, although IL-15 is able to signal through PKCθ, this signaling is not needed for the main functional effects of IL-15 on NK cells (Aguiló et al., in preparation). Hence, IL-15 probably is not the main cytokine implicated in PKCθ-dependent NK cell anti-tumoral activity. Additional experiments will be required to uncover cytokines that mediate dendritic cell-mediated NK cell activation.

As a summary (**Figure 2**), we propose that poly I:C, through TLR3 activation on macrophages or dendritic cells, elicits the secretion of cytokines that activate NK cells, including IL-15. During tumor development, danger signals are generated that activate antigen-presenting cells, which in turn ensure tumor antigen presentation and secrete a set of cytokines that regulate NK cells. Some of these cytokines may signal through PKCθ to control NK cell activation. This activated state is needed to counteract the growing of MHC-I negative tumors such as RMA-S, while other tumors, extremely sensitive to NK cell-mediated cytotoxicity, such as YAC-1, do not grow in syngenic mice. Once KARs are activated through the ligands expressed on tumoral cells, activated NK cells would be able to eliminate the tumor in a defined cytokine environment. On the other hand, KAR ligation induces the NK cell secretion of TNF-α and IFN-γ from activated NK cells, a process in which PKCθ is implicated (Tassi et al., 2008). TNF-α is important for the recruitment of more NK cells to the tumor environment (Smyth et al., 1998), which could explain the reduced recruitment of NK cells observed in *PKC*θ$^{-/-}$ mice (Aguiló et al., 2009).

A ROLE FOR PKCθ IN NK CELL DEGRANULATION?

Natural killer cells from *PKC*θ$^{-/-}$ mice had a reduced capacity to degranulate against RMA-S cells, which correlated with the impairment in their lytic ability (Aguiló et al., 2009). It has been recently shown that PKC-θ, together with other members of the novel PKC family, is implicated in the formation of the IS in CD4$^+$ T cells, probably through a function in microtubule-organizing center (MTOC) polarization (Quann et al., 2011). However, this study did not address the possible involvement of this mechanism in lytic granule secretion and on cytotoxicity.

IMMUNOLOGICAL SYNAPSE FORMATION

Most of the studies performed to determine the relevant molecular components in lytic granule secretion have been realized on CTL. The IS of CTL is quite distinct from that of CD4$^+$ T cells since it should allow both signaling and degranulation. The description of the CTL immune synapse showed the presence of a "cleft" that allowed the secretion of lytic granules components (Stinchcombe et al., 2001b). Hence, the signaling regulating synapse formation could be different in CD4$^+$ and CD8$^+$ T cells, and even if this signaling is shared, the regulation of lytic granule secretion should be particular to cytotoxic cells (CTL and NK cells). At the functional level, not all cytokines are secreted into the synapse of CD4$^+$ T cells: IL-2 and IFN-γ follow a synapse-mediated secretion, while TNF-α and some chemokines do not follow a directional type of secretion (Huse et al., 2006). On the other hand, cytokine release and degranulation proceed by different pathways also in NK cells (Reefman et al., 2010).

The IS of NK cells is similar to the one described for CTLs although it has some particularities (Davis et al., 1999; Krzewski and Strominger, 2008). Two different IS have been named, the activating IS and the inhibitory IS. Activating IS is formed by a

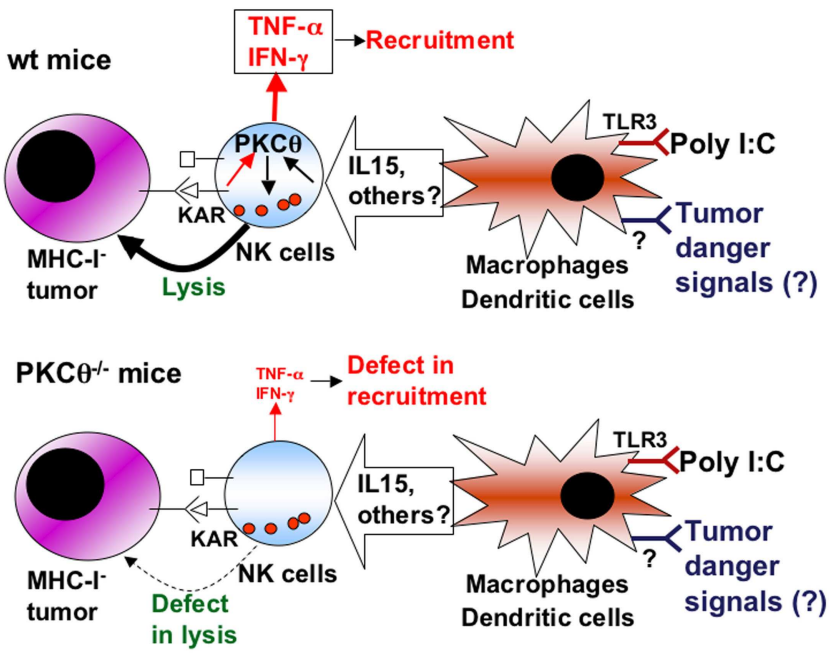

FIGURE 2 | Schematic showing the role of PKCθ in anti-tumoral NK cell activation. Upper panel, situation in NK cells from wild-type (wt) mice: cytokines produced by macrophages and/or dendritic cells upon poly I:C activation, or upon the sensing of tumor "danger" signals, including IL-15, induce the activation of NK cells through signaling dependent on PKC-θ (black arrows). This activation increases the cytotoxic potential of NK cells that can lyse tumoral target cells, especially those that are negative for MHC-I expression, and prevent tumor development. In addition, the production of TNF-α and IFN-γ by NK cells induced by certain killer-activating receptors (KAR) is also dependent on PKC-θ (red arrows), allowing the recruitment of more immune cells and sustaining immune activation. Lower panel, situation in NK cells from PKC-θ$^{-/-}$ mice: as a consequence of the absence of PKC-θ, cytokines produced by macrophages and/or dendritic cells do not activate properly NK cells, which then show a defect in lysis of tumoral cells, allowing tumoral development. In addition, the production of TNF-α and IFN-γ by NK cells is decreased, causing defects in recruitment.

central supramolecular activating cluster (SMAC) where all receptors involved in signal transduction accumulate and a peripheral SMAC where the adhesion molecule LFA-1 is placed. Similarly, inhibitory receptors accumulate in the cSMIC and LFA-1 in the pSMIC (Carlin et al., 2001). However, given the different number of activating and inhibitory receptors (see Natural Killer Cells), the molecular mechanisms regulating signaling from the synapse is more complex than in the case of other lymphocytes subsets like CTLs (Vyas et al., 2002a; Krzewski and Strominger, 2008). Specifically, formation and signaling through IS in NK cells is dynamically regulated by activation thresholds dictated by the balance between activating and inhibitory receptors. Most of the studies have characterized the IS formed after engagement of target cells by the activating NKG2D receptor (Krzewski and Strominger, 2008). However, as explained above, signaling from NKG2D receptors is regulated differentially from other receptors. Formation of the NK cell IS is regulated at different stages (Orange et al., 2003). The first stage implies an activation process after target cell engagement, followed by signal amplification and the rearrangement of the actin cytoskeleton in the pSMAC. This process is regulated by the WASP protein. Subsequently, MTOC is polarized and granule content is released through the cSMAC, as in the case of CTLs. All these activation steps are negatively regulated by signaling transduced by SHP phosphatases from inhibitory receptors (Krzewski and Strominger, 2008).

MOLECULES REGULATING CTL AND NK CELL DEGRANULATION

The molecular defects responsible of several diseases have allowed the identification of important molecules implicated in CTL degranulation (Clark and Griffiths, 2003; Stinchcombe and Griffiths, 2007; de Saint Basille et al., 2010). Granule movement through tubulin filaments is regulated by the adaptor protein-4 (AP-3; Clark et al., 2003), docking onto the plasma membrane is regulated by Rab27a (Haddad et al., 2001; Stinchcombe et al., 2001a), and priming or fusion with the membrane by Munc-13-4 (Feldmann et al., 2003). Finally, ASMAse is involved in the proper shrinkage of secretory granules after docking and priming steps to efficiently release granule content into target cells (Herz et al., 2009).

Regarding NK cells, it has been demonstrated that myosin IIA and WIP are required for granule polarization and NK cell-mediated cytotoxicity (Krzewski et al., 2006). Moreover, it could be suggested that Munc-13-4 is also involved in NK cell granule exocytosis since patients deficient in this protein present deficient NK cell-mediated cytotoxicity (Marcenaro et al., 2006; Bryceson et al., 2007). Syntaxin-11 also participates in the fusion of granules with the membrane of human NK cells (Arneson et al., 2007; Bryceson et al., 2007).

TCR EARLY SIGNALING DURING CTL DEGRANULATION. IMPLICATION OF PKC ISOFORMS

Although the most important regulators of lytic granule exocytosis are well described, at least in CTL, the connection between activating receptor-triggered signal transduction and lytic granule secretion is not completely understood. In the case of CTL, TCR signal transduction is initiated through protein tyrosine kinases of the src family that, together with the action of recruited ZAP-70 results in the phosphorylation and activation of phospholipase C-γ1 (Mustelin et al., 1990; Chan et al., 1992). CTL degranulation depends on these tyrosine kinase-mediated signaling pathways (Secrist et al., 1991; O'Rourke and Mescher, 1992; Anel and Kleinfeld, 1993). PLC-γ activation produces diacyl glycerol (DAG), which activates PKCs, and inositol-trisphosphate (IP_3), which increases the intracellular Ca^{++} concentration. A combination of the phorbol ester phorbol myristate-acetate (PMA) and the calcium ionophore ionomycin induces CTL degranulation. Granule movement was dependent on Ca^{++} and CTL shape change was dependent on PKC activity (Takayama and Sitkovsky, 1987; Haverstick et al., 1991). Depletion of PMA-sensitive PKC isoforms by prolonged PMA exposure prevented TCR-induced degranulation in CTL clones (Nishimura et al., 1987; Anel et al., 1994). In addition, PKC inhibition prevented MTOC polarization in CTL (Nesic et al., 1998).

A closer look at the role of individual PKC isoforms in CTL function revealed that PKCθ was implicated in the induction of FasL expression at a transcriptional level (Villalba et al., 1999; Villunger et al., 1999; Pardo et al., 2003). This is probably due to the role of PKCθ in NF-AT and NF-κB activation (Sun et al., 2000; Pfeifhofer et al., 2003), transcription factors which are involved in the control of FasL gene transcription (Latinis et al., 1997; Kasibhlatla et al., 1999). As already commented above, PKCθ is the only PKC isoform that is activated through a PI3K-dependent pathway that is triggered by TCR ligation (Villalba et al., 2002). This is in agreement with the fact that functional FasL expression is also prevented by PI3K inhibitors in long-term CTL clones (Anel et al., 1995; Pardo et al., 2003). Perforin/granzyme mediated lysis of Fas-negative target cells and CTL degranulation is sensitive to broad-spectrum PKC inhibitors, and also to Gö-6976, a specific inhibitor of the classical PKC isoforms (α, β, γ), but not to low doses of rottlerin, an inhibitor that prevents PKCθ activation (Villalba et al., 1999; Pardo et al., 2003). In addition, transfection with constitutively active PKCα, but not with PKCθ, cooperated with ionomycin to promote degranulation in murine CTL clones (Pardo et al., 2003). Later works demonstrated that both constitutively active PKCα and PKCθ cooperated with thapsigargin to induce degranulation in a human CTL tumoral cell line, although PKCα seemed more efficient (Grybko et al., 2007).

DOWNSTREAM SIGNALING DURING CTL DEGRANULATION

Cytotoxic T lymphocyte and NK cell degranulation is dependent on both actin cytoskeleton and on tubulin microtubules (Gomez and Billadeau, 2008). The proximal signaling described above connects initially with remodeling of the actin cytoskeleton mainly through the adaptors Vav1 and SLP76 (Villalba et al., 2001a; Zeng et al., 2003). These actin cytoskeleton remodeling events are needed for immune synapse formation in $CD4^+$ T cells, in CTL and also in NK cells. Once the immune synapse is formed, the next step for degranulation to occur is MTOC polarization (Kuhn and Poenie, 2002). Once MTOC is polarized, granules should move on tubulin rails, dock to the plasma membrane, and fuse to release their content in the IS. For these steps to occur, the granule and membrane proteins described above are needed, but signaling events are not completely elucidated. A role for ERK activation in CTL degranulation was clearly established

(Berg et al., 1998). This activation was dependent on PI3K, and paxillin has been identified as the ERK substrate that mediates MTOC polarization to the IS (Robertson et al., 2005; Robertson and Ostergaard, 2011). However, a role for PKCθ in ERK activation was not clearly demonstrated, and primary CTL from *PKCθ*$^{-/-}$ mice degranulated normally (Puente et al., 2006).

EARLY SIGNALING DURING NK CELL DEGRANULATION

Regarding NK cell degranulation elicited by KARs, signal transduction pathways are similar to those described in CTL in the case of those receptors that use FcεR1γ, CD3ζ, or DAP12 adaptors, but different in receptors that use DAP10 or SAP adaptors, such as NKG2D and CD244, respectively. However, by activating the PI3K pathway and the PLCγ-mediated increase in intracellular calcium concentration in the case of NKG2D and by the SAP-mediated activation of the protein tyrosine kinase Fyn in the case of CD244, these KAR arrive to generate similar downstream signaling that leads finally to degranulation (Cerwenka and Lanier, 2001; Lanier, 2008). In fact, it has been demonstrated that mouse NK cells do not require Syk and ZAP-70 kinases to kill different types of target cells, even those that do not express ligands for NKG2D, and that only abrogating at the same time the activity of src kinases and PI3K, degranulation was prevented (Colucci et al., 2002). In addition, the consequences of signaling transduced by the different receptors do not seem to be redundant. It has been previously shown that not a single activating receptor is sufficient to induce NK cell-mediated cytotoxicity against target cells (Bryceson et al., 2006). Only certain synergistic combinations of receptors are able to trigger this process. For example, it has been shown that engagement of CD16 induces degranulation in a non-polarized manner, meanwhile engagement of LFA-1 was able to polarize granules toward the IS. However, only after simultaneous engagement of both molecules NK cells were able to kill target cells (Bryceson et al., 2005), indicating also the importance of adhesión receptors such as LFA-1.

DOWNSTREAM SIGNALING DURING NK CELL DEGRANULATION. IMPLICATION OF PKC

Regarding downstream signaling in NK cell degranulation, it has been clearly demonstrated that the PI3K-mediated activation of ERK is involved in NK cell degranulation elicited by CD16 (Bonnema et al., 1994), by KARs that use DAP12 (Jiang et al., 2000, 2002) and also by those using DAP10 as adaptor (Billadeau et al., 2003; Upshaw et al., 2006). ERK activation results finally in MTOC polarization, similarly to what was observed in CTL degranulation (Chen et al., 2006). In the case of NKG2D, the PI3K pathway also results in the recruitment of the adaptor CrkL, needed for efficient MTOC polarization (Segovis et al., 2009). A role for JNK activation has been also demonstrated in NKG2D-mediated cytotoxicity (Li et al., 2008).

The role of PKC in NK cell degranulation was initially demonstrated since a combination of PMA and ionomycin is able to induce degranulation in these cells (Bonnema et al., 1994). It was also demonstrated that DNAM-1 signal transduction is dependent on PKC expression (Shibuya et al., 1998). Less is known about the role of different PKC isoforms in NK cell degranulation. As mentioned above, PKCθ is expressed by NK cells. During activation of the human NK cell line YTS with target cells with no HLA-I expression, a PKCθ pseudo-substrate inhibitor prevented WIP phosphorylation (Krzewski et al., 2006). WIP forms a complex with WASP and myosin IIA that regulates actin cytoskeleton dynamics during NK cell activation, and WIP knockdown prevents NK cell degranulation (Krzewski et al., 2008). However, it was not demonstrated that the formation of this complex was dependent on WIP phosphorylation by PKCθ (Krzewski et al., 2006). Interestingly, it has been recently published the kinome of NK cells activated by CD16 or simultaneous CD244 and DNAM-1 ligation, being PKC-θ identified as the only PKC isoform that increase phosphorylation upon receptor ligation (König et al., 2012).

The results described above, most of them obtained in CTL, suggest that PKCθ is not mandatory for granule exocytosis. However, it has not been studied yet in detail its possible involvement in NK cell immune synapse signaling and in NK cell granule polarization and/or secretion.

CONCLUSION

PKCθ plays an important role in tumor immune surveillance *in vivo* (Garaude et al., 2008; Aguiló et al., 2009). In the case of CTL-mediated tumor control, this can be explained by its role as a CTL survival factor (Barouch-Bentov et al., 2005), the impaired cytokine response observed in PKCθ deficient animals (Sun et al., 2000) and its implication in FasL expression (Villalba et al., 1999; Villunger et al., 1999; Pardo et al., 2003).

In the case of NK cell-mediated tumor control, several functional consequences of PKCθ deficiency can also contribute to

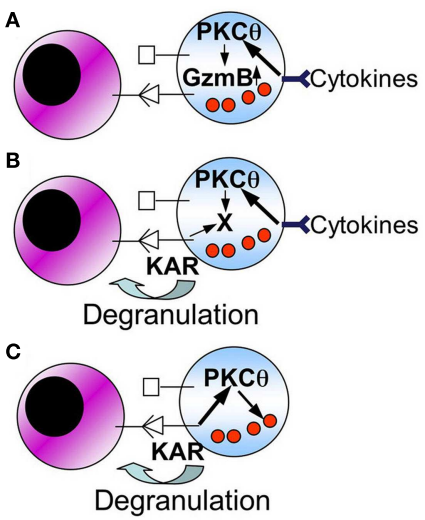

FIGURE 3 | Schematic showing possible mechanisms for the role of PKCθ in NK cell-mediated anti-tumor immunity. The following working hypothesis are formulated (see the text for details): **(A)** cytokines produced by macrophages and/or dendritic cells, by signaling through PKC-θ, increase granzyme B expression. An increase in granzyme B expression is directly associated with the augmentation of the cytolytic potential of NK cells; **(B)** cytokines produced by macrophages and/or dendritic cells, by signaling through PKC-θ, induce or increase the expression of a protein (X) implicated in degranulation of NK cells; **(C)** PKC-θ could be directly implicated in degranulation of NK cells through specific KARs.

the final outcome. As depicted in the schematic **Figure 2**, KAR-induced TNF-α and IFN-γ secretion is defective in *PKC*θ$^{-/-}$ mice (Tassi et al., 2008), and this can contribute to a defective recruitment of effector cells to the site of tumor development (Aguiló et al., 2009). Also, PKCθ could be implicated in the induction of FasL expression (Pardo et al., 2003), although this has not been demonstrated in NK cells. In addition, the possibilities shown in **Figure 3** may be considered, although they should be taken as working hypothesis that need to be better characterized:

a) Macrophage or dendritic cell derived cytokines, through a pathway that implicates PKCθ, induce an increase in granzyme B expression.
b) Macrophage or dendritic cell derived cytokines, through a pathway that implicates PKCθ, induces or increases the expression of a cellular component (X) that is needed to increase the degranulation potential of NK cells.
c) PKCθ is directly implicated in NK cell degranulation elicited by specific KARs.

These three possibilities are not mutually exclusive, and all of them can contribute to the regulation of NK cell anti-tumoral potential. In addition, different signaling and/or functional responses can be activated by different KARs, and PKC-θ could be relevant for signaling elicited by some KARs but not by others. Undoubtedly, uncovering the precise molecular mechanisms that direct these processes will allow a rational improvement of NK cell-mediated anti-tumor immunotherapy protocols.

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Targeting PKCθ in alloreactivity and graft-versus-host-disease: unanswered questions and therapeutic potential

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Protein kinase C isoform θ (PKCθ) is a key modulator of TCR signaling and mediates activation of NF-κB, NF-AT, and AP-1 transcription factors. Although *in vitro* studies of PKCθ$^{-/-}$ T cells have shown impaired activation responses, *in vivo* studies indicate that PKCθ requirement is not universal. While PKCθ is important in induction of experimentally induced autoimmune diseases in mice and generation of Th2 responses, it is not essential for induction of T cell proliferative and cytotoxic responses against influenza virus, LCMV, and vaccinia virus. The context-specific involvement of PKCθ in T cell responses suggests that inhibition of PKCθ may be beneficial in some but not all situations. In the bone marrow transplantation (BMT) setting, we have shown that graft-versus-host-disease (GVHD) cannot be induced in the absence of PKCθ. However, graft-versus-leukemia effects and T cell ability to clear virus infection remains intact. Therefore, PKCθ is a potential therapeutic target in BMT, inhibition of which may prevent GVHD while retaining anti-tumor and anti-infection responses.

Keywords: alloreactivity, graft-versus-host-disease, protein kinase C, T cell activation, anti-tumor effect

Studies of PKCθ$^{-/-}$ mice have shown normal T cell development but greatly impaired *in vitro* proliferative responses (Sun et al., 2000; Pfeifhofer et al., 2003; Grumont et al., 2004). Several studies have shown that PKCθ exerts functions leading to survival of T cells and activation versus tolerance outcomes *in vivo* (Berg-Brown et al., 2004; Barouch-Bentov et al., 2005). In addition, PKCθ is crucial for induction of experimentally induced autoimmune diseases in mice, including encephalomyelitis, arthritis, and myocarditis (Salek-Ardakani et al., 2005; Anderson et al., 2006; Healy et al., 2006; Marsland et al., 2007). It has also been shown that T cells cannot mount Th2 responses in the absence of PKCθ, while induction of Th1 responses against *Leishmania major* are not substantially impacted (Marsland et al., 2004). PKCθ$^{-/-}$ mice can also induce robust infection clearing CD8 T cell responses against multiple viruses including influenza virus, LCMV, and vaccinia virus (Marsland et al., 2004). Although *in vitro* proliferation of PKCθ$^{-/-}$ T cells is typically impaired, why *in vivo* responses to infectious agents remain intact is not well understood. It is thought, however, that stimulation of innate immunity by these infectious agents bypasses the requirement for PKCθ *in vivo* (Marsland et al., 2005, 2007; Marsland and Kopf, 2008). Precisely how this is accomplished and which molecular pathways are involved in PKCθ-independent T cell activation is not clear. The context-specific requirement of PKCθ in T cell activation suggests that inhibition of PKCθ may be beneficial in some but not all situations. Thus, inhibiting PKCθ may be therapeutically beneficial, but these specific circumstances need to be identified.

ESSENTIAL ROLE OF PKCθ IN ALLOREACTIVITY AND GRAFT-VERSUS-HOST-DISEASE

Alloreactivity is initiated by T cells that specifically recognize mismatched (non-self) MHC/peptide complexes. Graft-versus-host-disease (GVHD) is a potentially lethal complication of allogeneic bone marrow transplantation (BMT) in which alloreactive T cells from the donor are activated by mismatched major and/or minor histocompatibility complex antigens of the recipient. Although side-effects of BMT are severe, for many cancer patients this represents a last line of hope to remove residual tumor cells, as the alloantigen response mediating GVHD can also promote donor T cells to exert graft-versus-leukemia (GVL) effects (Shlomchik, 2007; Welniak et al., 2007). In this therapeutic BMT procedure, GVHD is the major complication as it leads to high morbidity and mortality of patients (Appelbaum, 2001; Shlomchik, 2007). To date, no clinical strategy has been established that can selectively prevent GVHD while preserving the GVL effect.

Given the differential requirement for PKCθ in distinct T cell-mediated responses, we investigated a potential role for PKCθ in the alloreactive responses of GVHD and GVL (Valenzuela et al., 2009). To investigate the necessity of PKCθ, we used an acute model of GVHD with the donor and recipient mismatched for both major and minor histocompatibility complex antigens (Liang et al., 2007). CD4 and CD8 T cells from wild-type (WT), PKCθ$^{-/-}$ or NF-κB p50$^{-/-}$cRel$^{-/-}$ mice were transferred together with WT T cell-depleted (TCD) BM cells into lethally irradiated MHC mismatched recipients. p50$^{-/-}$cRel$^{-/-}$ T cells (Zheng et al., 2003)

were used to investigate a potential requirement for NF-κB in this setting. As expected, recipients of WT T cells showed typical signs of GVHD with more than 70% mortality within 60 days after BMT. In a poignant divergence, the mice receiving PKCθ$^{-/-}$ or p50$^{-/-}$cRel$^{-/-}$ T cells showed little evidence of GVHD and survived through the duration of the experiment. Additionally, histologic analysis of the small intestine (a major site of GVHD-induced tissue destruction) was normal in mice receiving PKCθ$^{-/-}$ T cells, while their WT counterparts showed glandular destruction, lymphocytic infiltrate, and loss of mucosa. Although a clear mechanism remains elusive, additional studies indicated that GVHD does not occur in the absence of PKCθ because these T cells have impaired proliferation and increased apoptosis.

PRESERVATION OF ANTI-VIRUS AND GVL RESPONSES BY T CELLS LACKING PKCθ

Allogeneic BMT results in increased risk of life threatening infections due to conditioning regimens these patients must undergo. Previous studies showed that responses to bacterial and viral agents remain intact in the absence of PKCθ (Marsland et al., 2005; Marsland and Kopf, 2008); yet, such studies have not been performed in a post-BMT setting. Because CMV is one of the most prevalent viruses carried by humans, it represents a major threat to BMT patients (Meyers et al., 1986). To recapitulate this disease, we used an MCMV infection model (Hossain et al., 2007, 2008). Importantly, both anti-MCMV T cell response and virus clearance was comparable between WT and PKCθ$^{-/-}$ T cell transplanted mice (Valenzuela et al., 2009). Therefore, PKCθ is dispensable for a successful anti-MCMV response post-BMT.

The purpose of BMT in the cancer treatment settings is for donor T cells to be able to target malignant cells. Therefore, it is imperative that PKCθ$^{-/-}$ T cells retain this GVL activity. To this end, we performed studies (Valenzuela et al., 2009) using the A20 B cell lymphoma line (Liang et al., 2007). Different numbers of WT and PKCθ$^{-/-}$ T cells were transplanted, and evaluated for their ability to induce GVHD and mediate GVL activity. In the absence of T cells, all recipients died from lymphoma growth. All doses of WT T cells induced moderate to severe GVHD but little or no lymphoma growth. In contrast, all recipients of PKCθ$^{-/-}$ T cells survived through the duration of the experiment with mild body weight loss and low GVHD. Furthermore, all the recipients of high PKCθ$^{-/-}$ T cell numbers and most recipients of low or intermediate T cell numbers remained largely free of tumor. This result led us to the conclusion that PKCθ plays a substantial role in the induction of GVHD, but is not essential for GVL (Valenzuela et al., 2009). Importantly, these results indicate that pharmacological PKCθ targeting may impair GVHD without significantly impacting GVL responses.

KEY UNRESOLVED ISSUES IN PKCθ FUNCTION

Previous studies and our findings indicate relatively normal responsiveness of PKCθ$^{-/-}$ cells to infectious agents as well as high affinity antigenic stimulation (e.g., OVA) *in vivo* (Valenzuela et al., 2009). However, PKCθ$^{-/-}$ T cell alloreactivity and GVHD-inducing ability is severely impaired, likely due to reduced proliferation and survival in recipient mice (Valenzuela et al., 2009). This fundamental difference in the requirement of PKCθ in various settings is a key unanswered question, and is central for understanding how detrimental and beneficial functions of T cells in BMT can be separated. The specific inability of PKCθ$^{-/-}$ T cells to induce GVHD can be due to several mutually non-exclusive reasons. First, the conditioning regimen used for BMT may play an important role. Thus, lethal irradiation prior to BMT severely depletes recipient APC required for donor T cell activation. It is possible that reduction in APC impacts PKCθ$^{-/-}$ T cell responses more severely than WT T cells. Interestingly, allograft survival in heart transplantation models showed a relatively small requirement for PKCθ in transplant rejection (Manicassamy et al., 2008; Gruber et al., 2009), likely due to presence of compensatory functions of PKCα (Gruber et al., 2009). Therefore, it is possible that impaired alloreactivity in the absence of PKCθ is more pronounced in the BMT setting. Second, it is possible that defects in CD4 and CD8 T cell migration (Letschka et al., 2008) contribute to lack of GVHD induction in the absence of PKCθ. Thus, impaired migration of PKCθ$^{-/-}$ T cells to GVHD target organs such as the gut, lungs, and skin may be responsible for reduced GVHD. Third, the function of PKCθ in alloreactivity may not be limited to effector T cell responses. Previous studies investigating a role for PKCθ in Tregs suggest that PKCθ function in Treg may also be important in alloreactivity (Zanin-Zhorov et al., 2010). While PKCθ localizes to the immune synapse (IS) in effector T cell, PKCθ is sequestered in a distal complex away from the IS in Treg (Zanin-Zhorov et al., 2010). As such, PKCθ is responsible for mediating a negative effect on the suppressive function of Treg. Consequently, PKCθ inhibition enhances Treg function leading to protection from inflammatory colitis in mice (Zanin-Zhorov et al., 2010). A caveat worth mentioning here is that while PKCθ inhibition leads to enhanced Treg function, PKCθ absence does not have the same effect (Gupta et al., 2008). The underlying reason for this is not completely clear (Zanin-Zhorov et al., 2011).

The easiest albeit simplistic way to understand why PKCθ absence does not impact anti-infection and anti-tumor responses is to consider a role for functionally redundant pathways. As mentioned above, PKCθ is involved in regulating activation of NF-κB, AP-1, and NF-AT. Studies by Marsland and colleagues have shown that microbial stimulation through pattern recognition receptors (PRR) can induce NF-κB activation in PKCθ$^{-/-}$ T cells (Marsland et al., 2005, 2007; Marsland and Kopf, 2008). Thus, PRR may play a key functionally redundant role with PKCθ during infection with microbial agents. In contrast, why anti-tumor responses are only slightly reduced in the absence of PKCθ is more difficult to understand. BMT is primarily used for leukemia treatment. Leukemic cells, in particular B lymphocytes, have naturally high expression of MHC and co-stimulatory molecules, reflecting the natural function of these lineages in antigen presentation. In the above-mentioned study (Valenzuela et al., 2009), A20 B cell lymphoma cells were used as tumor targets. Whether PKCθ$^{-/-}$ T cells are specifically (or only) able to eradicate leukemic tumors can be directly tested by determining PKCθ requirement in eradication of non-leukemic tumors. Mechanistically, one possibility is that functionally redundant pathways are strongly activated in PKCθ$^{-/-}$ T cells by A20 and potentially other leukemic tumors. Furthermore, leukemic tumors may represent better targets for

PKCθ$^{-/-}$ T cells than epithelial cells targeted during GVHD. Regardless of precise mechanisms, it is likely that both responses to infectious agents and leukemic tumors are maintained in the absence of PKCθ through functionally redundant pathways. A recent study identified a novel role for PKCθ as a transcriptional co-activator capable of interacting with promoters of several immune function genes (Sutcliffe et al., 2011). How this function impacts alloreactivity and other known functions for PKCθ remains to be determined.

INVESTIGATING THE EFFECT OF PKCθ INHIBITION IN GVHD AND GVL

The above-mentioned studies provide strong rationale for targeting PKCθ in a BMT settings (Valenzuela et al., 2009). The PKC family has been implicated in tumor cell proliferation, survival, invasion, metastasis, and tumor angiogenesis. Therefore, targeting PKC isotypes may present an attractive target for novel anticancer therapies. Consequently, several inhibitors of PKC isoforms have been developed by different pharmaceutical companies. Although some selective PKCθ inhibitors have been reported (Cywin et al., 2007; Cole et al., 2008), their *in vivo* toxicity or efficacy remains to be determined. A major advance was achieved recently with AEB071 (developed by Novartis), a very potent and selective inhibitor of both novel and classical PKCs. Skvara et al. (2008) reported that treatment with AEB071 significantly improved symptoms of patients with severe psoriasis. Another potentially important PKC inhibitor is enzastaurin (Ly317615, from Eli Lilly and Company; Baier and Wagner, 2009). Enzastaurin was identified as an inhibitor of the PKCβ isoform, but it also impacts the AKT pathway (Graff et al., 2005); importantly, enzastaurin inhibits the PKCθ isoform approximately fivefold more potently than the beta isoform (Graff et al., 2005). *In vivo* studies indicate that enzastaurin is very well tolerated with a favorable safety profile, allowing it to be dosed for extended durations (Herbst et al., 2007). Furthermore, enzastaurin has anti-proliferative and pro-apoptotic activity in solid tumors as well as hematological malignancies, including leukemia and lymphoma (Herbst et al., 2007). Thus, while not exclusively specific for PKCθ, enzastaurin has the major advantage of being well tolerated *in vivo* and the added benefit of anti-tumor effects. Hence, PKCθ inhibition by enzastaurin may prevent GVHD while preserving GVL responses, which will act cooperatively with the direct anti-tumor effects of enzastaurin. We expect future studies to define more potent and specific inhibitors of PKCθ and consequently help move this important therapeutic target to the clinical arena.

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Intervention of PKC-θ as an immunosuppressive regimen

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PKC-θ is selectively enriched in T cells and specifically translocates to immunological synapse where it mediates critical T cell receptor signals required for T cell activation, differentiation, and survival. T cells deficient in PKC-θ are defective in their ability to differentiate into inflammatory effector cells that mediate actual immune responses whereas, their differentiation into regulatory T cells (Treg) that inhibits the inflammatory T cells is enhanced. Therefore, the manipulation of PKC-θ activity can shift the ratio between inflammatory effector T cells and inhibitory Tregs, to control T cell-mediated immune responses that are responsible for autoimmunity and allograft rejection. Indeed, PKC-θ-deficient mice are resistant to the development of several Th2 and Th17-dependent autoimmune diseases and are defective in mounting alloimmune responses required for rejection of transplanted allografts and graft-versus-host disease. Selective inhibition of PKC-θ is therefore considered as a potential treatment for prevention of autoimmune diseases and allograft rejection.

Keywords: PKC-θ, T cell activation, T cell differentiation, autoimmunity, allograft rejection

INTRODUCTION

T cells that are newly migrated out of thymus are naïve T cells incapable of mediating immune responses. Engagement of antigens with their T cell receptors (TCR) initiates the activation and differentiation programs that transform naïve T cells into inflammatory Th1, Th2, and Th17 effector cells or regulatory T cells (Treg), both of which participate in actual immune responses. The interference with molecules involved in T cell activation and differentiation is therefore considered an effective strategy to control the overwhelming immune responses that mediate autoimmunity and allograft rejection. However, most molecules involved in the processes of T cell activation and differentiation are not likely to be good targets for interference or inhibition because they also play essential roles in other cell types and signaling pathways. The ideal targets should meet at least the following criteria: (1) they selectively regulate T cell function; (2) small molecule inhibitors of them are easy to isolate or develop; (3) rather than inhibit, they actually promote Treg function. Ideally, inhibition of the target molecule should inhibit inflammatory T cells, and at the same time, enhance the suppressive function of Tregs. In this review, we summarize the evidence that supports PKC-θ as an excellent target for development of immunosuppressive agents.

PKC-θ IS SELECTIVELY ENRICHED IN T CELLS

PKC-θ belongs to a family of serine/threonine kinases that consists of 12 different isoforms, each with distinct roles in the regulation of cellular functions (Newton, 1997). Members of this family can be divided into three subfamilies (Newton, 1997): conventional PKCs, including PKC-α, β, and γ, which are activated by Ca^{2+} and diacylglycerol (DAG); novel PKCs, including PKC-δ, θ, η, and ε, whose activation is dependent on DAG but is independent of Ca^{2+}; and the atypical PKCs, PKC-ζ and λ, whose activation occurs independently of both Ca^{2+} and DAG. PKC-θ was first cloned as a novel PKC predominantly expressed in skeleton muscle (Osada et al., 1992; Chang et al., 1993) and found to be significantly enriched in hematopoietic compartments and skeleton muscle (Baier et al., 1994; Altman et al., 2000; Bauer et al., 2000). In hematopoietic compartments, PKC-θ is primarily expressed in T cells, but not B cells, and this has also been confirmed by *in situ* hybridization to mouse whole body sections (Bauer et al., 2000). The selective expression pattern of PKC-θ strongly suggests it plays a unique function in T cell compartments, and therefore may be a good immunosuppressive target for controlling T cell-mediated immunity. However, it is important to point out that T cells also express other isoforms of PKC (Bauer et al., 2000). Furthermore, although most studies have so far focused on PKC-θ function in T cells, there is evidence supporting that PKC-θ is also expressed and play a role in other tissues including muscle (Kim et al., 2004; Benoit et al., 2009; Paoletti et al., 2010), platelets (Nagy et al., 2009; Harper and Poole, 2010; Cohen et al., 2011), natural killer (NK) cells (Aguilo et al., 2009), and likely mast cells (Kempuraj et al., 2005). Therefore, inhibition or targeting of PKC-θ for immunotherapeutic treatments may also affect other tissues in addition to T cells.

PKC-θ SELECTIVELY TRANSLOCATES TO IMMUNOLOGICAL SYNAPSE

PKC-θ attracted significant attention when it was shown among all the isoforms of PKC expressed in T cells, PKC-θ selectively translocates to the immunological synapse (IS), the stable cell–cell junction formed between T cells and antigen-presenting cells (Monks et al., 1997, 1998). The IS is a cluster of specialized membrane microdomains where TCR signaling molecules, including the TCR itself, are assembled (Grakoui et al., 1999). Formation of the IS is an active process that requires Lck-mediated signals to initiate re-organization of cytoskeleton (Morgan et al., 2001). Although there is still some controversy (Lee et al., 2002), it is

generally believed that the IS serves as the platform that delivers integrated signals essential for T cell activation (Moran and Miceli, 1998). The IS consists of three major compartments: the central supramolecular activation cluster (cSMAC), the peripheral SMAC (pSMAC), and the distal SMAC (dSMAC; Barouch-Bentov et al., 2005; Dustin, 2009). The cSMAC was initially referred to as a signaling structure (Monks et al., 1998; Freiberg et al., 2002) and is located at the center of the IS. Around this center is the pSMAC, a ring of LFA-1/ICAM-1 co-localized with the cytoskeletal integrin linker talin (Monks et al., 1998). The outermost ring is the dSMAC, a zone enriched in the CD45 tyrosine phosphatase (Freiberg et al., 2002). Recently, it was demonstrated that the TCRs initially microcluster in the dSMAC, and then move through the pSMAC into the cSMAC, and is believed to be critical for the generation of continuous TCR signals that are required for T cell activation (Varma et al., 2006). PKC-θ is recruited to the junction between the cSMAC and pSMAC and co-localizes with TCRs in a CD28 co-stimulatory-dependent manner (Monks et al., 1997, 1998; Somersalo et al., 2004). Microscopic studies of IS have shown that T cells expressing PKC-θ periodically break open the pSMAC to create an asymmetric focal zone accumulation pattern that relocates to nearby areas where the pSMAC reformed (Sims et al., 2007). This periodic breaking of the symmetric pSMAC to form a polarized focal zone allows short bursts of migration, facilitating T cell interaction with multiple antigen presenting cells (Lindquist et al., 2004). This observation is also consistent with the asymmetric cell division theory that suggests that the IS leads to asymmetric cell division, a feature that is important for memory/effector differentiation of lymphocytes (Chang et al., 2007). A recent study has identified a unique region of PKC-θ, called the V3 domain, that is responsible for the selective translocation of PKC-θ to the IS (Kong et al., 2011). V3 was found to interact with the SH3 domain of Lck which is in turn, tethered to the phosphorylated tail of CD28 via its SH2 domain. The PKC-θ–Lck–CD28 interaction explains why PKC-θ recruitment to the IS depends on CD28 co-stimulation. However, in a different study the active kinase domain of PKC-θ was reported to be essential for PKC-θ translocation into the IS (Cartwright et al., 2011) and is not clear why there is a discrepancy. One possibility is that the two studies used different sources of T cells: primary T cells transduced with retrovirus and a D10 cell line. In contrast to conventional T cells, PKC-θ does not translocate to IS of Tregs. In fact it is actually sequestered away from the IS (Zanin-Zhorov et al., 2010), suggesting that the function of PKC-θ in Tregs is likely to be different from its functions in conventional T cells. Altogether, the fact that selective translocation of PKC-θ (but not other isoforms of PKC) to the IS is critical for T cell activation, strongly suggests it has unique functions in mediating TCR signals, and that selective inhibition of PKC-θ could specifically interfere with T cell function.

PKC-θ MEDIATES TCR SIGNALS ESSENTIAL FOR T CELL ACTIVATION

TCR signals are initiated by activation of the Src family protein tyrosine kinase (PTK) Lck (Weiss and Littman, 1994), which leads first to recruitment of ZAP 70 and then the subsequent recruitment of the adaptor proteins LAT, SLP76, and VAV. LAT then recruits PLCγ1 (Clements, 2003; Berg et al., 2005) which catalyzes phosphatidylinositol 4,5-biphosphate into inositol triphosphate (IP3), a Ca^{2+} mobilizer and DAG, the PKC-θ activator (Weiss and Littman, 1994). In addition to DAG, PKC-θ activation seems to also require phosphorylation of threonine 538 (T538) in its activation loop (Liu et al., 2002; Lee et al., 2005). Previously, PDK1 was believed to phosphorylate T538 (Liu et al., 2002). However, a recent study indicates GLK is the upstream kinase responsible for T538 phosphorylation (Chuang et al., 2011). Upon activation, PKC-θ mediates the activation of NF-κB, AP-1, and nuclear factor of activated T cells (NFAT), critical transcription factors that are required for activation of the IL-2 gene (Sun et al., 2000; Pfeifhofer et al., 2003). Several adaptor proteins play a critical role in mediating PKC-θ-induced NF-κB activation including membrane-associated guanylate kinase (MAGUK), CARMA1, B-cell lymphoma 10 (Bcl 10), and mucosa-associated lymphoid tissue 1 (MALT1). Together with PKC-θ, these adaptors facilitate the activation of IKK, leading to the phosphorylation, ubiquitination, and degradation of IκB. Degradation of IκB releases NF-κB to the nucleus, where it participates in the activation of target genes essential for T cell activation (Weil et al., 2003; Lin and Wang, 2004; Weil and Israel, 2004). Studies using PKC-θ-deficient T cells or cells overexpressing the constitutively active PKC-θ or the catalytically inactive form of PKC-θ have demonstrated that PKC-θ also selectively activates the AP-1 signaling pathway in T cells (Sun et al., 2000). AP-1 is composed of c-Jun and c-Fos, which regulate many cellular events (Baier-Bitterlich et al., 1996). Although the exact mechanism responsible for PKC-θ-mediated activation of AP-1 is still unclear, several studies have provided some insight into this process. Ras and MAP kinases: JNK, ERK, and P38 are all involved in PKC-θ-mediated AP-1 activation (Baier-Bitterlich et al., 1996; Shaulian and Karin, 2002). Li et al. (2004) isolated a PKC-θ-interacting upstream MAP kinase, originally termed Ste20/SPS1-related proline and alanine-rich kinase (SPAK or PASK), and demonstrated that SPAK selectively interacts with PKC-θ and participates in PKC-θ-mediated activation of AP-1, but not NF-κB. Activation of T cells through the TCR also leads to IP3-mediated elevation of cytosolic $[Ca^{2+}]_i$ by inducing Ca^{2+} influx. Elevated intracellular Ca^{2+} ultimately leads to activation of the calcineurin phosphatase, which dephosphorylates NFAT, resulting in its translocation to the nucleus. Translocated NFAT cooperates with AP-1 to activate IL-2 expression and it has been shown that, in the absence of AP-1, NFAT activation can lead to T cell anergy (Macian et al., 2002). Several studies have shown that PKC-θ enhances the activation of NFAT by stimulating Ca^{2+} influx; TCR-induced Ca^{2+} influx, and NFAT activation is defective in T cells from *PKCθ*$^{-/-}$ mice (Pfeifhofer et al., 2003; Manicassamy et al., 2006a). Although it is clear that PKC-θ regulates Ca^{2+} signals via stimulation of PLCγ1, it is not known how PKC-θ stimulates PLCγ1. The Tek kinase family member Itk may be the missing link. Itk-deficient T cells display defective Ca^{2+} influx and PLCγ1 activation (Liu et al., 1998), whereas overexpression of Itk leads to stimulation of PLCγ1 activity (Tomlinson et al., 2004). Therefore, it is possible that PKC-θ regulates PLCγ1 activation via Itk. Altogether, PKC-θ-mediated TCR signaling regulates multiple signaling pathways including NF-κB, AP-1, and NFAT that are all critical for T cell activation, which were clearly

summarized in our previously published review articles (Manicassamy et al., 2006b; Kwon et al., 2010). Inhibition of PKC-θ is therefore expected to prevent T cell activation by blocking these pathways.

PKC-θ ENHANCES T CELL SURVIVAL

Productive engagement of the TCR leads to T cell activation, resulting in cell proliferation and production of IL-2. Proliferating T cells especially during S phase of the cell cycle are susceptible to apoptosis (Boehme and Lenardo, 1993; Radvanyi et al., 1996). The TCR delivers signals that are required not only for stimulating proliferation but also for enhancing survival (Weiss and Littman, 1994; Boise et al., 1995). Such survival signals ensure the completion of the T cell activation process that is essential for differentiating naïve T cells into effectors that can mediate actual immune responses (Radvanyi et al., 1996). During T cell activation, survival of the T cells is enhanced by IL-2, which acts as an extrinsic survival factor. In addition, activated T cells substantially up-regulate Bcl-x_L that intrinsically increases resistance to apoptosis (Noel et al., 1996; Radvanyi et al., 1996; Van Parijs et al., 1996). We and others have shown that PKC-θ is required for the survival of activated T cells (Barouch-Bentov et al., 2005; Manicassamy et al., 2006a). *PKC-θ*$^{-/-}$ T cells undergo apoptosis in response to TCR stimulation, which correlates with the reduced expression of NF-κB-dependent Bcl-x_L. Forced expression of Bcl-x_L and Bcl-2 restores the survival of *PKC-θ*$^{-/-}$ T cells and exogenous IL-2 can partially overcome the defective survival and proliferation of *PKC-θ*$^{-/-}$ T cells. Similar to primary T cells, PKC-θ is also required for the survival of T cell lines such as Jurkat. It has been shown that PKC-θ promotes Jurkat survival by phosphorylating Bad and thereby inactivating its function (Villalba et al., 2001). However, primary T cells deficient in PKC-θ showed comparable levels of Bad phosphorylation to that of wild-type (WT) T cells (Barouch-Bentov et al., 2005), suggesting that the observed apoptosis of *PKC-θ*$^{-/-}$ T cells is unlikely to be due to lack of Bad phosphorylation. Other pathways may also be involved in the apoptosis of *PKC-θ*$^{-/-}$ T cells. For example, up-regulation of pro-apoptotic molecule Bim might be partially responsible for the observed apoptosis in *PKC-θ*$^{-/-}$ T cells (Barouch-Bentov et al., 2005). Wan and DeGregori (2003) reported that inhibition of NF-κB leads to an increase in the expression of pro-apoptotic protein p73 . Since *PKC-θ*$^{-/-}$ T cells are defective in NF-κB activation, it is possible that down-regulated p73 may contribute to the apoptosis of *PKC-θ*$^{-/-}$ T cells. Because T cell survival ensures T cell-mediated immune responses, one of the mechanisms for PKC-θ to regulate immune responses is to enhance T cell survival and this is confirmed by our allograft rejection study discussed later in the review.

PKC-θ PROMOTES THE DIFFERENTIATION OF NAÏVE T CELLS TO INFLAMMATORY Th17 CELLS

Differentiation of naïve T cells into specific T helper lineages is a critical checkpoint for controlling immune responses. Altered regulation of this checkpoint can lead to aggravated autoimmunity by overproduction of T helper cells that cause pathogenic inflammation. Similarly, allograft rejection also depends on the effectors that differentiate from allo-specific naïve T cells. *In vitro* stimulation of naïve CD4 T cells in the presence of appropriate cytokines up-regulates master transcription factors that instruct their differentiation into Th1, Th2, or Th17 inflammatory T helper cells (Dong, 2010). When stimulated with IL-12 or interferon-γ (INF-γ), naïve T cells express the T-box transcription factor T-bet as their lineage-specific transcription factor and differentiate into Th1 cells that secrete IFN-γ as their signature cytokine. In the presence of IL-4, naïve T cells express GATA3 as their lineage-specific transcription factor and differentiate into Th2 cells that secrete the signature cytokines IL-4, IL-5, and IL-13 (Murphy and Reiner, 2002). TCR stimulation in the presence of IL-6 and TGF-β leads to up-regulation of RORγt and differentiation of naïve T cells into Th17 cells that produce IL-17, IL-21, IL-22, and GM-CSF. We compared the differentiation of WT and *PKC-θ*$^{-/-}$ T cell under Th1, Th2, and Th17 priming conditions *in vitro* to determine the function of PKC-θ in these processes and found that PKC-θ preferentially involved in the regulation of Th17 formation (Kwon et al., 2012). We showed that purified naive *PKC-θ*$^{-/-}$ T cells were defective in Th17 differentiation, whereas Th1 and Th2 differentiation appeared normal. Activation of PKC-θ with PMA promoted Th17 differentiation in WT but not *PKC-θ*$^{-/-}$ T cells. Furthermore, *PKC-θ*$^{-/-}$ T cells had notably lower levels of Stat3, a transcription factor required for Th17 differentiation, and PMA markedly stimulated the expression of Stat3 in WT but not *PKC-θ*$^{-/-}$ T cells. In contrast, activation of Stat4 and Stat6, which are critical for Th1 and Th2 differentiation, was normal in *PKC-θ*$^{-/-}$ T cells. Forced expression of Stat3 significantly increased Th17 differentiation in *PKC-θ*$^{-/-}$ T cells, indicating that reduced Stat3 levels were responsible for impaired Th17 differentiation and that Stat3 lies downstream of PKC-θ. Constitutively active PKC-θ or WT PKC-θ activated by either PMA or TCR cross-linking, stimulated the expression of a luciferase reporter gene driven by the Stat3 promoter. PKC-θ-mediated activation of the Stat3 promoter was inhibited by dominant negative AP-1 and IκB kinase-β, but stimulated by WT AP-1 and IκB kinase-β, suggesting that PKC-θ stimulates Stat3 transcription via the AP-1 and NF-κB pathways. Finally, conditions favoring Th17 differentiation induced the highest activation level of PKC-θ. Altogether, the data indicate that PKC-θ integrates the signals from the TCR activated with Th17 priming cytokines to up-regulate Stat3 via NF-κB and AP-1, which stimulate Th17 differentiation. The results are also consistent with the observation that PKC-θ-deficient mice are resistant to the induction of experimental autoimmune encephalomyelitis (EAE; Salek-Ardakani et al., 2005; Tan et al., 2006), which is a Th17-associated autoimmune disease. In contrast, and although *PKC-θ*$^{-/-}$ mice have been reported to be defective in development of Th1 and Th2 immune responses, depending on the mouse model used (Marsland et al., 2004; Salek-Ardakani et al., 2004; Healy et al., 2006), our *in vitro* assays have shown that Th1 and Th2 differentiation is normal in the absence of PKC-θ. In addition to the apparently different priming conditions *in vitro* and *in vivo*, defects in other PKC-θ-regulated functions such as survival are likely to contribute to the overall defective Th1 and Th2 immune responses observed in *PKC-θ*$^{-/-}$ mice *in vivo*. Although it is difficult to define which PKC-θ-regulated functions are responsible for the defective immune

responses *in vivo*, we excluded the possibility that PKC-θ-regulated survival affected our differentiation assays *in vitro* by adding exogenous IL-2 which inhibits *PKC-θ*$^{-/-}$ T cell apoptosis (Manicassamy et al., 2006a). Altogether, both *in vitro* and *in vivo* studies indicate that PKC-θ promotes the differentiation of Th17 cells that are associated with multiple autoimmune disorders (Huang et al., 2007).

PKC-θ INHIBITS THE DIFFERENTIATION AND ENHANCES SUPPRESSIVE FUNCTION OF Tregs

Naïve CD4+ T cells can differentiate into either inflammatory effector T cells or Tregs (iTregs; Bettelli et al., 2006; Zhou et al., 2008), 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. The activation of naïve T cells in the presence of TGFβ induces expression of Forkhead Box P3 (Foxp3), a master transcription factor instructing iTreg differentiation, and is also a marker for iTreg (Curotto de Lafaille and Lafaille, 2009). In contrast to iTregs, natural Tregs (nTregs) are not induced but develop in the thymus. The fact that naïve T cells can be differentiated into inhibitory iTregs suggests there is a therapeutic value for such a conversion in the treatment of autoimmunity. Our data has demonstrated that PKC-θ-mediated signals inhibit iTreg differentiation (Ma et al., 2012). We found that TGFβ-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 cross-linking which enhances PKC-θ activation. Further, we showed that *PKC-θ*$^{-/-}$ T cells had reduced activity of the AKT kinase, and that the expression of a constitutively active form of AKT in *PKC-θ*$^{-/-}$ T cells restored their ability to inhibit iTreg differentiation. In addition, 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 respectively, indicating that the AKT-FoxO1/3A pathway is responsible for the inhibition of iTreg differentiation downstream of PKC-θ. Considering the positive role played by PKC-θ in the activation and differentiation of naïve T cells into inflammatory T effector cells (Altman et al., 2000; Sun et al., 2000; Pfeifhofer et al., 2003; Marsland et al., 2004), together the data indicate 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.

In addition to Treg differentiation, a recent study also demonstrated a role for PKC-θ in the regulation of the effector function of Tregs (Zanin-Zhorov et al., 2010). Tregs inhibit inflammatory effector T cell function via cell contact-dependent and independent mechanisms (Sakaguchi et al., 2008). Such inhibitory functions of Tregs are important for establishing tolerance mechanisms required to prevent activation of autoreactive T cells that lead to autoimmunity (Sakaguchi et al., 2006; Shevach, 2006; Shevach et al., 2006; Miyara and Sakaguchi, 2007). The depletion of CD4+CD25+ Tregs from mice results in the development of widespread autoimmune/inflammatory diseases such as autoimmune gastritis, thyroiditis, type 1 diabetes, and inflammatory bowel disease. These autoimmune disorders are prevented by reconstituting the mice with CD4+CD25+ Treg cells (Sakaguchi et al., 1995; Singh et al., 2001), indicating a therapeutic potential for Tregs for the treatment of autoimmunity. To increase the efficacy of Treg-mediated inhibition, it is important to enhance the suppressive function of Treg. Inhibition of PKC-θ either by knockdown or a specific PKC-θ inhibitor has been shown to significantly boost the potential of Tregs to inhibit T cell activation (Zanin-Zhorov et al., 2010). However, in the presence of TGFβ neutralizing antibody, the PKC-θ inhibitor fails to enhance the suppressive function of Tregs, suggesting that inhibition of PKC-θ stimulates Tregs to produce the TGFβ that is responsible for inhibition of T cell activation. Interestingly, the suppressive function of Treg was also enhanced by inhibiting the activation of NF-κB, a critical downstream target of PKC-θ (Sun et al., 2000), indicating the possibility that PKC-θ inhibitor enhances Treg function by blocking activation of the NF-κB pathway. Furthermore, PKC-θ inhibitor-treated Tregs were more potent than untreated Tregs in preventing inflammatory colitis *in vivo* (Zanin-Zhorov et al., 2010), supporting the potential clinical application of PKC-θ inhibitors for Treg-mediated treatment of autoimmunity. Taken together, inhibition of PKC-θ can interfere with T cell-mediated immunity by inhibiting inflammatory T cell differentiation, by promoting Treg differentiation and by enhancing the suppressive function of Tregs.

PKC-θ PLAYS A CRITICAL ROLE IN T CELL-DEPENDENT AUTOIMMUNITY

Due to the unique roles played by PKC-θ in the regulation of T cell activation and differentiation, PKC-θ is believed to be a potential drug target and pharmaceutical companies have developed highly specific PKC-θ inhibitors (Cywin et al., 2007; Mosyak et al., 2007). Mouse models of autoimmune diseases have been used to define PKC-θ function in T cell-dependent autoimmunity (Marsland and Kopf, 2008). Two independent studies have shown that *PKC-θ*$^{-/-}$ mice were resistant to the induction of Th2-dependent lung inflammation in airway hyper responsiveness (AHR; Marsland et al., 2004; Salek-Ardakani et al., 2004), supporting a requirement for PKC-θ in Th2 type autoimmunity. In contrast, PKC-θ played a lesser role in the development of a similar lung inflammatory response mediated by Th1 cells (Salek-Ardakani et al., 2004), suggesting different functions of PKC-θ in Th1 and Th2 responses. However, PKC-θ was found to be essential for both methylated BSA and type II collagen-induced arthritis, a Th1-mediated autoimmunity disease (Healy et al., 2006). The results suggest that PKC-θ function is dependent on the model used. *PKC-θ*$^{-/-}$ mice were reported by two different groups to be resistant to the development of Th17-mediated EAE, the mouse model for multiple sclerosis (Salek-Ardakani et al., 2005; Tan et al., 2006), indicating a requirement for PKC-θ in Th17-dependent autoimmunity.

The evaluation of PKC-θ function *in vivo* is complicated by several factors. First, PKC-θ function may be compensated for *in vivo*. Most *in vitro* assays clearly indicated essential role of PKC-θ in T cell functions (Manicassamy et al., 2006b). However, we found that the requirement for PKC-θ may be bypassed if T cells are stimulated by overwhelmingly strong TCR signals such as high concentrations of phorbol ester and ionomycin or anti-CD3/28 antibodies (unpublished data). It is therefore possible that PKC-θ

function *in vivo* may also depend on the strength of TCR stimulation. In contrast to *in vitro* assays using purified T cells, *in vivo* immune responses involve many different types of cells that can produce factors to compensate for PKC-θ function. For example, one of the major signaling pathways that PKC-θ regulates is NF-κB. NF-κB can also be activated by PKC-θ-independent pathways such as TNFα and IL-1 (Sun et al., 2000). Many *in vivo* inflammation conditions produce TNFα and IL-1 inflammatory cytokines and these cytokines are likely compensate for PKC-θ function at least for the activation of NF-κB in T cells. Toll like receptors (TLR) can also activate the NF-κB pathway and it was indeed found that TLR-mediated signals can overcome the requirement for PKC-θ in T cell activation and the development of autoimmune myocarditis (Marsland et al., 2005). Second, autoimmune diseases usually involve more than one type of T helper cell. For example, both Th1 and Th17 responses are likely contribute to the development of EAE (Salek-Ardakani et al., 2005). In humans, it is even more difficult to specifically define the types of T helper cells involved in autoimmunity. Therefore, inhibition of PKC-θ may inhibit one type of T helper, but not other types of T helpers that can induce autoimmunity. Conversely, PKC-θ-mediated T cell differentiation is not the only PKC-θ function essential for the development of autoimmunity; PKC-θ also regulates other T cell functions including activation and survival. Therefore, the observed defects in the development of autoimmunity in *PKC-θ*$^{-/-}$ mice are likely to be due to the disruption of several PKC-θ-regulated functions including those that have not yet been identified. However, despite the possible complications, the potential of PKC-θ as a drug target has been indicated by a trial for the treatment of psoriasis (Skvara et al., 2008). In this study, the clinical severity of psoriasis was reduced up to 69% after 2 weeks of treatment with a PKC inhibitor, AEB071 which inhibits multiple isoforms of PKC with strong specificity for PKC-θ, PKC-α, and PKC-β and lesser specificity for PKC-δ, PKC-ε, and PKC-η. Other clinical trials are expected because many companies have developed PKC-θ inhibitors. One of potential trials will be systemic lupus erythematosus, because patients with this disease show considerably enhanced activation of both PKC-θ and its potential upstream kinase GLK (Chuang et al., 2011).

PKC-θ IS REQUIRED FOR NKT-MEDIATED AUTOIMMUNE HEPATITIS

Autoimmune hepatitis (AIH) results from the mistaken attack on healthy liver cells by an individual's own immune system (McFarlane, 1999). In mice, acute hepatitis can be induced by treatment with concanavalin A (ConA), which causes rapid activation of CD1d-positive NK T cells. These activated NKT cells produce large amounts of cytokines that cause strong inflammation responsible for damaging liver tissues. Our research has shown that *PKC-θ*$^{-/-}$ mice were resistant to ConA-induced hepatitis due to an essential requirement for PKC-θ during NKT cell development and activation. A dose of ConA (25 mg/kg) that was lethal to WT mice failed to cause death due to liver injury in *PKC-θ*$^{-/-}$ mice (Fang et al., 2012). Correspondingly, the ConA-induced production of cytokines such as IFNγ, IL-6, and TNFα, which mediate the inflammation responsible for liver injury, were significantly lower in *PKC-θ*$^{-/-}$ mice. In addition, upon stimulation with an NKT cell-specific lipid ligand, peripheral *PKC-θ*$^{-/-}$ NKT cells produced lower levels of inflammatory cytokines than that of WT NKT cells, suggesting that activation of NKT cells requires PKC-θ. Our results suggest PKC-θ is an essential molecule required for activation of NKT cells to induce hepatitis (Fang et al., 2012), and thus, is a potential drug target for prevention of autoimmune hepatitis. NKT cells are also thought to be involved in liver injury induced by LPS, α-galactosylceramide (α-GalCer), *Salmonella* infection, chronic hepatitis C infection, and primary biliary cirrhosis (Ishigami et al., 1999; Kawano et al., 1999; Kaneko et al., 2000; Kato et al., 2000; Kim et al., 2002). Inhibition of PKC-θ is also likely to have therapeutic value in the treatment of liver injury in patients with these conditions.

PKC-θ PLAYS A CRITICAL ROLE IN ALLOIMMUNE RESPONSES ESSENTIAL FOR TRANSPLANT REJECTION

Solid organ transplants that benefit end-stage organ failure patients are severely limited by the occurrence of rejection. Alloreactive T cells are critical targets for tolerance induction since they mediate the immune responses required for rejection. The alloreactive T cell pool is very large (Suchin et al., 2001), which explains why immune responses against allografts are at least two orders of magnitude stronger than immune responses against a specific antigen. Therefore, long-term tolerance to allografts is extremely difficult to establish. The initial evidence for requirement of PKC-θ in alloresponses came from the impaired *in vitro* mixed lymphocyte reaction of *PKC-θ*$^{-/-}$ T cells (Sun et al., 2000). Injection of allogeneic cells into the footpad of PKC-θ-deficient mice provoked a significantly diminished local T cell response compared to WT mice similarly challenged, suggesting an essential role for PKC-θ in the allo-reaction *in vivo* (Anderson et al., 2006). We tested PKC-θ function in transplant rejection using a cardiac allograft model (Manicassamy et al., 2008). *Rag1*$^{-/-}$ mice reconstituted with WT T cells readily rejected fully mismatched cardiac allografts, whereas, *Rag1*$^{-/-}$ mice reconstituted with *PKC-θ*$^{-/-}$ T cells failed to promote rejection, suggesting that PKC-θ is required for T cell-mediated allograft rejection. One of the important mechanisms responsible for establishing tolerance to allografts is to reduce the number of alloreactive T cells by inducing apoptosis (Wells et al., 1999). Since PKC-θ is required for survival of activated T cells (Manicassamy et al., 2006a), we therefore tested the role of PKC-θ-regulated survival in cardiac allograft rejection and demonstrated that the transgenic expression of Bcl-x_L in *PKC-θ*$^{-/-}$ T cells was sufficient to restore cardiac allograft rejection (Manicassamy et al., 2008). This result suggests that apoptosis of alloreactive T cells in the absence of PKC-θ is responsible for the observed tolerance to cardiac allografts. Alloreactive T cells can be tolerized through anergy, suppression and deletion. Tolerizing mechanisms through anergy and Treg-mediated suppression are unlikely change the size of alloreactive T cell pool which is ready to destroy allografts. This is the problem for cyclosporin A (CsA), the most successful immunosuppressive drug used clinically so far. CsA prevents apoptosis of alloreactive T cells by inhibition of T cell activation (Li et al., 1999), resulting in accumulation of large amounts of alloreactive T cells that destroy allografts once the immunosuppressive drugs are discontinued (Li et al., 2001).

Therefore, prevention of allograft rejection usually requires transplant recipients to take life-long immunosuppressive drugs, which can result in complications including infections and malignancy. Whereas, deletion induces tolerance by decreasing the number of alloreactive T cells via apoptosis, and thus avoids the potential risk of accumulating alloreactive T cells. In addition to an adoptive transfer model, we also tested cardiac rejection using intact *PKC-θ*$^{-/-}$ mice. *PKC-θ*$^{-/-}$ mice displayed delayed, but successful cardiac allograft rejection, suggesting there was some compensation for the missing PKC-θ function. Finally, a sub-therapeutic dose of anti-CD154 antibody or CTLA4-Ig, which was not sufficient to prevent cardiac allograft rejection in WT mice, prevented heart rejection in *PKC-θ*$^{-/-}$ mice. *PKC-θ*$^{-/-}$ mice treated with sub-therapeutic doses of Anti-CD154 or CTLA4-Ig also accepted donor-type second cardiac allografts but rejected third-party allografts (Wang et al., 2009). Thus, in combination with other treatments, the inhibition of PKC-θ allows long-term survival of cardiac allografts (Manicassamy et al., 2008; Wang et al., 2009).

In addition to heart rejection, the role of PKC-θ was also examined in a bone marrow transplantation (BMT) model (Valenzuela et al., 2009). BMT is used to replace damaged bone marrow with healthy stem cells or used as therapy for hematopoietic malignancies. In the latter case, allogeneic BMT boosts the patient's immune system to aid in fighting against the cancer, which is called the graft-versus-leukemia (GVL) effect. However, graft-versus-host disease (GVHD), a potentially lethal consequence of BMT, limits the clinical application of this very effective treatment. Alloreactive donor T cells recognize the mismatched MHC of the recipient and undergo robust activation, expansion, and differentiation, resulting in GVHD, which causes severe damages to multiple tissues including gut, liver, skin, and kidney (Shlomchik, 2007). Immunosuppressive drugs are therefore needed clinically to prevent GVHD-induced damage. However, commonly used immunosuppressive drugs such as CsA and FK506 also inhibit the immune response against pathogens as well as tumors (GVL), and consequently limit the effects of GVL on the elimination of residual tumor cells (Reddy et al., 2005). The optimal immunosuppressive regimens are able to prevent GVHD, but also preserve the immune responses against infectious pathogens. Similar to WT mice, *PKC-θ*$^{-/-}$ mice have the ability to respond to infection by the listeria bacteria and MCMV virus (Valenzuela et al., 2009). Moreover, *PKC-θ*$^{-/-}$ mice survived the BMT procedure and did not develop GVHD, whereas majority of WT mice died from GVHD. More importantly, *PKC-θ*$^{-/-}$ mice retained their ability to induce rejection of tumors. This study demonstrated that PKC-θ inhibitor-based immunosuppressive regimens are able to prevent GVHD but also preserve the protective immune response against infections and tumors.

CONCLUSION

PKC-θ controls fundamental functions of T cells including activation, differentiation, and survival via NF-κB, AP-1, and NFAT pathways. PKC-θ also regulates T cell-mediated immune responses *in vivo* and selective PKC-θ inhibitors are believed to have the potential for clinical application in the treatment of autoimmunity and prevention of allograft rejection. However, PKC-θ function in T cell-mediated immune responses is dependent on the mouse models used. Therefore, the mechanisms involved in each of the diseases should be carefully examined. More questions need to be addressed prior to the clinical application of PKC-θ inhibitors including how the inhibition of PKC-θ affects the function of other tissues *in vivo*. It is encouraging to report that many pharmaceutical companies have developed selective PKC-θ inhibitors, and therefore many PKC-θ-regulated functions can be evaluated using these inhibitors instead of *PKCθ*$^{-/-}$ mice, which have potential developmental caveat. With the availability of PKC-θ inhibitors, it is now possible to test their efficacy in mouse models of human autoimmune diseases including EAE and arthritis, which are likely to lead to clinical trials of PKC-θ-based treatments for human diseases.

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The role of protein kinase Cη in T cell biology

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Protein kinase Cη (PKCη) is a member of the novel PKC subfamily, which also includes δ, ε, and θ isoforms. Compared to the other novel PKCs, the function of PKCη in the immune system is largely unknown. Several studies have started to reveal the role of PKCη, particularly in T cells. PKCη is highly expressed in T cells, and is upregulated during thymocyte positive selection. Interestingly, like the θ isoform, PKCη is also recruited to the immunological synapse that is formed between a T cell and an antigen-presenting cell. However, unlike PKCθ, which becomes concentrated to the central region of the synapse, PKCη remains in a diffuse pattern over the whole area of the synapse, suggesting distinctive roles of these two isoforms in signal transduction. Although PKCη is dispensable for thymocyte development, further analysis of PKCη- or PKCθ-deficient and double-knockout mice revealed the redundancy of these two isoforms in thymocyte development. In contrast, PKCη rather than PKCθ, plays an important role for T cell homeostatic proliferation, which requires recognition of self-antigen. Another piece of evidence demonstrating that PKCη and PKCθ have isoform-specific as well as redundant roles come from the analysis of CD4 to CD8 T cell ratios in the periphery of these knockout mice. Deficiency in PKCη or PKCθ had opposing effects as PKCη knockout mice had a higher ratio of CD4 to CD8 T cells compared to that of wild-type mice, whereas PKCθ-deficient mice had a lower ratio. Biochemical studies showed that calcium flux and NFκB translocation is impaired in PKCη-deficient T cells upon TCR crosslinking stimulation, a character shared with PKCθ-deficient T cells. However, unlike the case with PKCθ, the mechanistic study of PKCη is at early stage and the signaling pathways involving PKCη, at least in T cells, are essentially unknown. In this review, we will cover the topics mentioned above as well as provide some perspectives for further investigations regarding PKCη.

Keywords: development, homeostatic proliferation, immune synapse, immunological synapse, protein kinase C, signaling, T cell, T cell activation

INTRODUCTION

Protein kinase C (PKC) is a large family of serine/threonine kinases that can be divided into three subfamilies based on their structural homology and requirement of cofactors for activation (Baier, 2003). The conventional PKC subfamily contains α, βI, βII, and γ, requiring calcium and diacylglycerol (DAG) for activation. The novel PKC subfamily contains δ, ε, θ, and η, and requires DAG but not calcium for activation. In contrast, the atypical PKC subfamily (i.e., ζ and λ/ι) requires neither DAG nor calcium for their activation (Pfeifhofer et al., 2003). Studies using PKC isoform-specific knockout mice have shown differential roles of each isoform in T cell development and function (Sun et al., 2000; Thuille et al., 2004; Gruber et al., 2005a,b; Pfeifhofer et al., 2006). For example, PKCα-deficient mice have a normal T cell development phenotype. In peripheral T cells, PKCα is dispensable for normal T cell activation and IL2 production, but it is required for proliferation and IFN-γ production (Pfeifhofer et al., 2006). PKCβ is dispensable for normal T cell development and function (Thuille et al., 2004), although it was found to be important for LFA-1-mediated T cell locomotion in a PKCβ-deficient cell line (Volkov et al., 2001). PKCδ is a negative regulator of T cell activation, as PKCδ-deficient T cells are hyperproliferative and produce more IL2 cytokine upon stimulation (Gruber et al., 2005a). This negative regulatory role is also reflected in PKCδ-deficient B cells (Mecklenbrauker et al., 2002; Miyamoto et al., 2002). In striking contrast, PKCθ-deficient T cells completely lose the ability to proliferate or to produce IL2 after stimulation through the T cell receptor (TCR) in *in vitro* assays (Sun et al., 2000; Pfeifhofer et al., 2003), even though both δ and θ have the closest identity (60%) within the novel PKC subfamily (Kong et al., 2011; Quann et al., 2011). PKCε was dispensable for T cell development and activation (Gruber et al., 2005b). In PKCζ-deficient mice, there is no overt defect in T cell development (Leitges et al., 2001), but these mice showed impaired Th2 cell differentiation (Martin et al., 2005). Interestingly, although discovered more than two decades ago (Osada et al., 1990), and like PKCθ, highly expressed in T cells (Baier, 2003; **Figure 1**: data from www.biogps.org (Su et al., 2004; Wu et al., 2009) the role of PKCη had never been thoroughly examined in T cells until the recent study from our group (Fu et al., 2011). This despite the fact that PKCη-deficient mice have existed for almost 10 years (Chida et al., 2003). Meanwhile, although discovered only a little later than PKCη, PKCθ is considered paramount in T cell function. Our recent work on PKCη has significantly filled this gap by showing both isoform-specific and redundant (with PKCθ) roles of PKCη

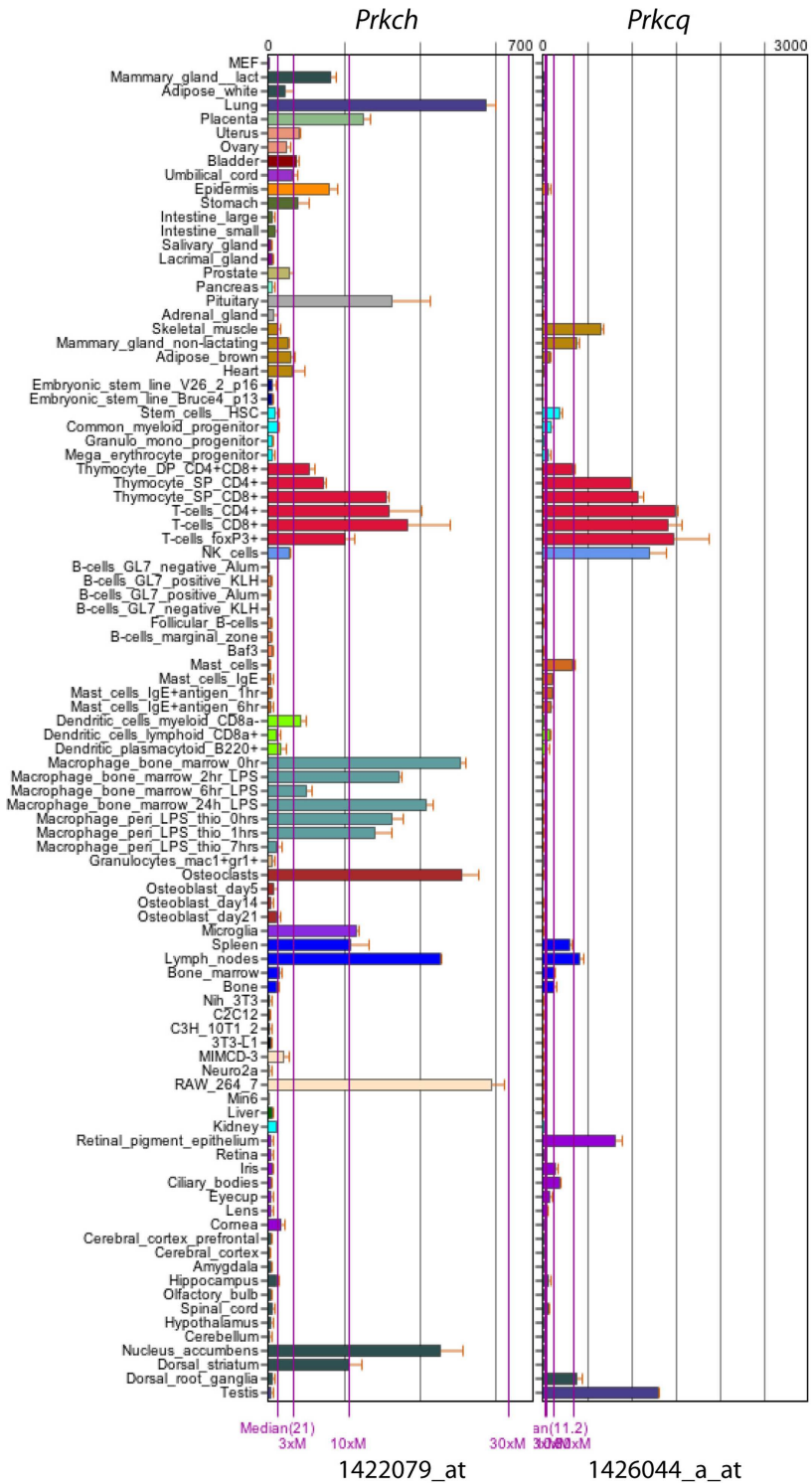

FIGURE 1 | mRNA expression profile of mouse PKCη (*Prkch*) and PKCθ (*Prkcq*). Data were obtained from www.biogps.org using GeneAtlas MOE430, gcrma (Su et al., 2004; Wu et al., 2009), with probes 1422079_at (*Prkch*) and 1426044_a_at (*Prkcq*).

in T cell development and function (Fu et al., 2011; Fu and Gascoigne, 2012). In this article, we will first briefly review some earlier studies on PKCη, then mainly focus on four subjects currently under study: (1) the recruitment of PKCη to the immunological synapse; (2) its role in T cell development; (3) its role in T cell function; (4) its role in TCR signaling. Finally, we would like to share

some of our thoughts with the readers about future investigations regarding PKCη.

COMPARISON OF PKCη AND PKCθ MOLECULES

In the novel PKC subfamily, PKCδ and PKCθ are closely related (60% identity), as are PKCε and PKCη (also 60% identity; Baier, 2003; Quann et al., 2011). A cross comparison between PKCη and PKCθ reveals that these two isoforms bear 42% identity (**Figure 2A**). The overall domain structure of PKCη and PKCθ proteins shows a high degree of similarity. This domain architecture is shown in **Figure 2B**. In both isoforms, there is a "C2-like" domain near the amino-terminal of the protein, which cannot bind calcium, unlike the C2 domains in conventional PKC isoforms (Baier, 2003). Following the C2-like domain, there are tandem repeats of two DAG binding C1 domains and the V3 hinge region. This is the most different region between PKCη and PKCθ (**Figure 2C**). In PKCθ, V3 is important in association of the kinase with CD28 and as a result is required to mediate PKCθ's localization in the central synapse (Kong et al., 2011). The motif within PKCθV3 domain that is required for CD28 interaction, including the conserved PXXP sequence (Kong et al., 2011), is missing in PKCη (**Figure 2C**). The C2-like, C1, and V3 domains together form a regulatory region, which likely performs the isoform-specific functions, as the carboxyl-terminal serine/threonine kinase domain is rather conserved across all PKC isoforms. The difference between the V3 domains of PKCθ and PKCη suggests that this may be responsible for their different localization in the immunological synapse.

A BRIEF HISTORY OF PKCη STUDIES

PKCη was originally identified from a mouse epidermis cDNA library and found to be highly expressed in mouse tissues such as skin, lung, and heart (Osada et al., 1990). Because of this tissue-specific expression pattern, most studies regarding PKCη were historically focused on keratinocyte proliferation and differentiation (Ohba et al., 1998; Cabodi et al., 2000). However, development of skin was normal in PKCη-deficient mice in steady state (Chida et al., 2003). In contrast, under challenging conditions, these PKCη-deficient mice were susceptible to skin tumor induction and showed impaired wound healing (Chida et al., 2003). In immune cells, PKCη is highly expressed in mouse macrophages and T cells, but not B cells (**Figure 1**). However, interestingly, potential roles of PKCη in B cells were suggested in a number of studies (Morrow et al., 1999; Oda et al., 2008). For example, PKCη was shown to be specifically transcribed in pro-B but not pre-B cells, and a pro-apoptotic role of PKCη in B cells was suggested (Morrow et al., 1999). In another study, PKCη was shown to direct IRF4 expression and Igκ gene rearrangement in pre-BCR signaling (Oda et al., 2008). Surprisingly, nothing was known about the specific role of PKCη in T cells until quite recent work from our group and others (Singleton et al., 2009; Fu et al., 2011; Quann et al., 2011; Sewald et al., 2011), which is the topic we address below.

RECRUITMENT OF PKCη TO THE IMMUNOLOGICAL SYNAPSE

The immunological synapse or supramolecular activation cluster (SMAC) forms at the interface between a T cell and an

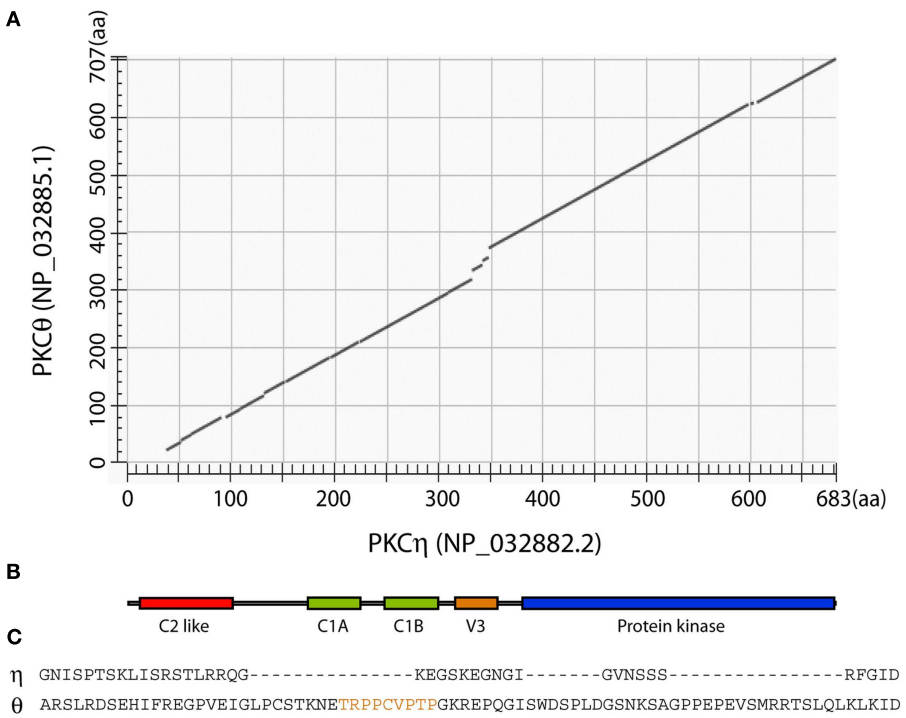

FIGURE 2 | Comparison of mouse PKCη and PKCθ proteins. (A) Alignment of mouse PKCη and PKCθ was performed using NCBI BLAST program. **(B)** Cartoon showing the arrangement of known conserved domains that applies to both PKCη and PKCθ proteins. **(C)** Alignment of PKCη and PKCθ V3 domains. The region identified as important for PKCθ interaction with CD28 (Kong et al., 2011) is highlighted in orange.

antigen-presenting cell (APC; or a surrogate), and is the site at which early signaling events occur (Grakoui et al., 1999). The widely accepted importance of PKCθ in T cells is largely due to its identification as the only PKC isoform recruited to the immunological synapse (Monks et al., 1997), and particularly to the central synapse region (cSMAC), along with TCR and other molecules (Monks et al., 1998). Since then, PKCθ has served as a landmark for defining the immunological synapse. However, studies from our group and others challenged the view that only PKCθ is recruited to the synapse (Singleton et al., 2009; Fu et al., 2011; Quann et al., 2011). PKCη is recruited to the immunological synapse upon T cell recognition of its cognate antigenic peptide-MHC (pMHC), but not non-stimulatory pMHC, presented by APCs (**Figure 3**; Fu et al., 2011). More interestingly, PKCη and PKCθ showed different recruitment patterns, as PKCη forms a diffuse pattern at the immunological synapse, whereas PKCθ concentrates into the central region (**Figure 3**; Singleton et al., 2009; Fu et al., 2011), suggesting different functions in time and space of these two PKC isoforms. In addition to PKCη and PKCθ, PKCε is also recruited to the immunological synapse (Quann et al., 2011). In this study, polarization of the T cell microtubule-organizing center (MTOC) is directed by diacylglycerol (DAG) at the immunological synapse via three PKC isoforms, in two sequential steps. Initially, PKCε and PKCη accumulate in a broad region of the interface between T cell and APC, followed by PKCθ concentrating in a smaller, central, zone (Quann et al., 2011). It seems that in different cell types, recruitment of PKC isoforms could also be different. For example, it has been shown that, in contrast to the immunological synapse-localization in effector T cells, PKCθ is sequestered away from the immunological synapse in regulatory T cells (Treg), and thus mediates negative feedback on Treg cell function (Zanin-Zhorov et al., 2010). This intriguing observation may be also worth examination for PKCε and PKCη.

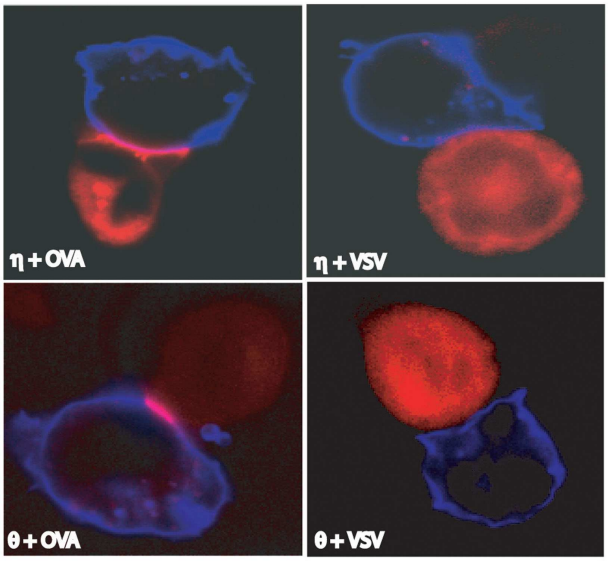

FIGURE 3 | Recruitment of PKCη and PKCθ to the immunological synapse. Both PKCη and PKCθ were recruited to the immunological synapse by antigenic stimulation (i.e., with stimulatory peptide OVA) but not by non-antigenic stimulation (i.e., with non-stimulatory peptide VSV). Blue are EL4 cells used as antigen-presenting-cells. Red are OT-I T hybridoma cells transfected with PKCη- or PKCθ-RFP as indicated. Adapted from Fu et al. (2011).

PKCη IN T CELL DEVELOPMENT

Our initial speculation that PKCη may play a role in T cell development was based on the finding that PKCη mRNA expression was upregulated during thymocyte positive selection (Mick et al., 2004; Niederberger et al., 2005). These observations were surprising given the established important role of PKCθ in T cell biology, but intriguing because PKCθ-deficient mice have only a very minor defect in thymocyte development. Initial phenotyping of PKCθ-deficient mice did not identify any defects in thymocyte development (Sun et al., 2000; Pfeifhofer et al., 2003), although later studies did find a mild thymocyte development defect in such mice (Morley et al., 2008; Fu et al., 2011). However, phenotyping of PKCη-deficient mice showed rather normal thymocyte development. This was not completely unexpected given the multiple novel PKC isoforms co-expressed in T cells, and redundancy could play a role to compensate for the absence of any particular isoform. We also noted that induction of PKCη mRNA is much higher and earlier in PKCθ-deficient mice than in wild-type mice (i.e., induction during positive selection in wild-type mice, but induction before positive selection in PKCθ-deficient mice), suggesting a compensatory effect due to redundancy of function between PKCη and PKCθ (Fu et al., 2011). In accord with this notion, PKCη is recruited to the immunological synapse in immature $CD4^+CD8^+$ (DP) thymocytes in the $PKC\theta^{-/-}$ mice, as is PKCθ in the PKCθ-sufficient DP cells. In PKCθ-sufficient cells, PKCη is only recruited to the synapse in mature $CD4^+$ or $CD8^+$ (SP) thymocytes. These results are only suggestive of redundant function, but clear redundancy between PKCη and PKCθ in thymocyte development was confirmed when we phenotyped $PKC\eta^{-/-}\theta^{-/-}$ mice. Positive selection of thymocytes in these double-knockout mice was more severely impaired than either single PKC-knockout mice. However, the blockade of thymocyte development in $PKC\eta^{-/-}\theta^{-/-}$ mice was not complete, as SP cell numbers were only reduced by about 50% (**Figure 4A**; Fu et al., 2011). Therefore, it is possible that other PKC isoforms than PKCη and θ can still compensate for their deficiency, perhaps most likely those members within the same subfamily (e.g., PKCε).

It is natural to speculate that in a multimember protein family, there are some overlapping functions between individual members (i.e., redundancy), as well as isoform-specific functions. In our study, we found that PKCη and PKCθ had opposite effects on the CD4 to CD8 T cell ratios in the secondary lymphoid organs (**Figure 4B**). PKCη-deficient mice had a higher CD4/CD8 ratio than wild-type mice, whereas PKCθ-deficient mice had a lower ratio, indicating an isoform-specific role of these PKCs in balancing CD4 and CD8 T cell homeostasis. Interestingly, these effects are "neutralized" by each other in that $PKC\eta^{-/-}\theta^{-/-}$ mice exhibited normal CD4/CD8 ratios. Multiple factors can affect the CD4/CD8 T cell ratio during thymocyte development (Corbella et al., 1994; Suzuki et al., 1995;Sim et al., 1998a,b). The

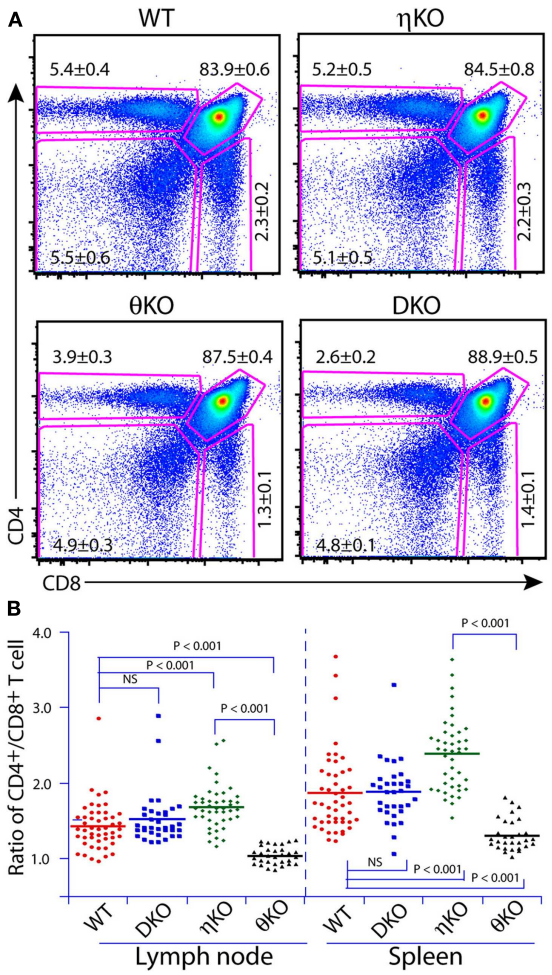

FIGURE 4 | Redundancy and isoform-specific role of PKCη and PKCθ in T cell development. (A) Redundant role of PKCη and PKCθ is revealed by phenotyping T cell development in the thymus of double-knockout mice. **(B)** Isoform-specific role of PKCη and PKCθ is revealed by the CD4/CD8 T cell ratio in the periphery. Adapted from Fu et al. (2011).

SP thymocytes from these knockout mice did not show altered CD4/CD8 ratios, indicating that the effects on the CD4/CD8 ratio occur post-thymically (Fu et al., 2011). Another intriguing observation is that PKCη-deficient mice have an irregular distribution of T cells between spleen and peripheral lymph nodes. The total T cell numbers are increased in the lymph nodes of PKCη-deficient mice, which mirrored the phenomenon that the lymph nodes are much larger in size in PKCη-deficient mice compared to wild-type mice. In contrast, the total T cell numbers are reduced in the spleen in PKCη-deficient mice compared to wild-type mice. Enlarged lymph nodes (i.e., lymphadenopathy) were also observed in PKCδ-deficient mice, which was mainly attributed to the increased B cell numbers (Mecklenbrauker et al., 2002). Currently, it is not clear what causes this biased T cell distribution in PKCη-deficient mice. We speculate that altered lymphocyte homing and/or homeostasis could be one of the reasons.

PKCη IN PERIPHERAL T CELL HOMEOSTASIS AND RESPONSE TO ANTIGEN

For the sake of simplicity, we focused on CD8 T cells for most functional studies on PKCη$^{-/-}$ mice (Fu et al., 2011). PKCη-deficient CD8 T cells showed a mild proliferation defect compared to wild-type T cells upon anti-CD3 antibody stimulation. In contrast, under the same conditions, PKCθ-deficient CD8 T cells were completely non-proliferative, as previously reported (Sun et al., 2000; Pfeifhofer et al., 2003). However, this striking difference between PKCη$^{-/-}$ and PKCθ$^{-/-}$ CD8 T cells was blurred under more physiological conditions. For example, when we used APCs pulsed with antigenic peptide to stimulate these PKC-deficient CD8 T cells, both PKCη$^{-/-}$ and PKCθ$^{-/-}$ CD8 T cells still proliferated less well than wild-type cells, but the relative difference between PKCη$^{-/-}$ and PKCθ$^{-/-}$ CD8 T cells is much more subtle than with anti-CD3 crosslinking (Fu et al., 2011). In general, we observed that antigen-specific proliferation of PKCη-deficient T cells was more severely reduced compared to wild-type cells than was anti-CD3 antibody induced proliferation. It may be that this is because the anti-CD3 stimulation does not involve the formation of the immunological synapse, whereas the synapse is important in the antigen-specific responses. The proliferation defect of PKCη$^{-/-}$ CD8 T cells was also confirmed in *in vivo* experiments, where wild-type and PKCη$^{-/-}$ CD8 T cells were co-transferred into recipient mice and stimulated by antigenic peptide (**Figure 5A**; Fu et al., 2011).

Therefore the proliferation defect of PKCη$^{-/-}$ CD8 T cells is consistent both *in vitro* and *in vivo*. However, in the case of PKCθ$^{-/-}$ CD8 T cells, *in vivo* reductions in responses were much less severe than those observed *in vitro*. For instance, the absence of PKCθ does not impair antigen-specific proliferation (Barouch-Bentov et al., 2005) or antiviral immune responses, in which PKCθ$^{-/-}$ CD8 T cells were found to proliferate normally (Berg-Brown et al., 2004; Marsland et al., 2005). The role of PKCθ in the *Listeria* infection model is controversial, with one group showing PKCθ is not important (Valenzuela et al., 2009) and another group claiming the opposite (Sakowicz-Burkiewicz et al., 2008). These conflicting results may be due to the different bacterial infection doses used between these two groups. One common explanation of PKCθ's dispensable role in these infection models is that *in vivo* innate signals can compensate for the absence of PKCθ (Marsland et al., 2005; Valenzuela et al., 2009), however it is also possible that PKCη functions in place of PKCθ in these cases.

In stark contrast, in an experiment to measure T cell homeostatic proliferation, we found that PKCη, but not PKCθ, is required (**Figure 5B**; Fu et al., 2011). In these experiments, no matter whether we used polyclonal T cells or monoclonal TCR transgenic T cells as donor cells, only PKCη$^{-/-}$ CD8 T cells showed impaired proliferation in lymphopenic animals, whereas PKCθ$^{-/-}$ CD8 T cells showed normal homeostatic proliferation. The non-essential role of PKCθ in T cell homeostatic proliferation was also independently reported by others (Valenzuela et al., 2009). This was indeed an unexpected result: one would have assumed that defective homeostatic proliferation might occur in PKCθ$^{-/-}$ T cells, at least as a reflection of strong deficiency in *in vitro* proliferation. Both TCR mediated signaling and the cytokines IL7 and IL15 are required to support normal homeostatic proliferation (Jameson, 2002; Surh and Sprent, 2005). However, we think

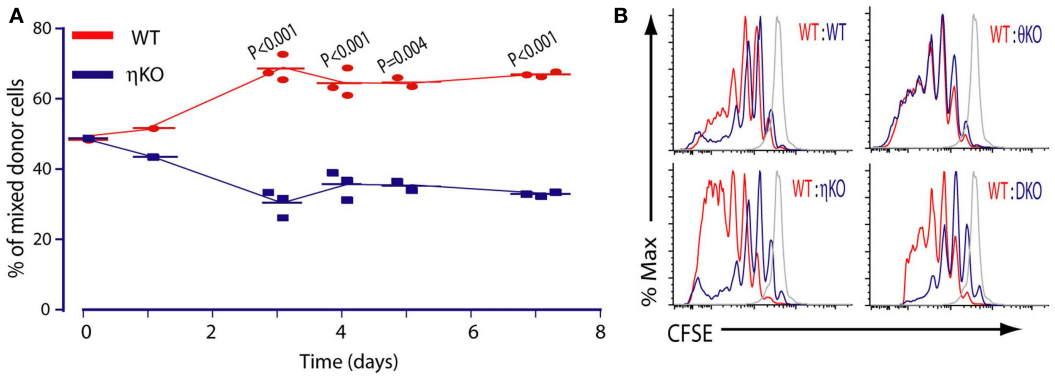

FIGURE 5 | Requirement of PKCη in T cell proliferation. (A) PKCη is required for efficient antigen-specific T cell proliferation *in vivo*. **(B)** PKCη but not PKCθ is required for T cell homeostatic proliferation *in vivo*. Adapted from Fu et al. (2011).

altered responsiveness to these cytokines is unlikely to contribute to the defective homeostatic proliferation in PKCη-deficient T cells, because the amounts of IL7Rα (CD127) and IL15R (CD122) on the PKCη-deficient T cells were the same as those of wild-type T cells (Fu et al., 2011). We were also unable to find any difference in the numbers of apoptotic cells between PKCη-deficient and -sufficient mice, suggesting that the requirement for PKCη for homeostatic proliferation is not due to differential cell survival. PKCθ has been found to be a survival factor for CD8 T cells. In contrast to antigen-specific T cell proliferation, which is the clonal expansion of particular T cells recognizing their cognate antigen, homeostatic proliferation is the response of T cells to self-MHCp complexes for survival. Therefore the strength of TCR signaling is different in these two scenarios. It is possible that PKCη and PKCθ play dominant roles in homeostatic and antigen-specific proliferation respectively. PKCθ may be more important in antigen-specific activation because of its reported role in breaking the "symmetry" of the synapse (Sims et al., 2007). This is required for T cell movement, such as during scanning over the surface of an APC.

PKCη IN T CELL RECEPTOR SIGNALING

Compared to the very well characterized mechanisms regarding PKCθ in the molecular signaling machinery in T cells (Egawa et al., 2003; Wang et al., 2004; Roose et al., 2005; Manicassamy et al., 2006), similar studies of PKCη are at a very early stage. In our study, we showed that Ca^{2+} flux and NFκB nuclear translocation were impaired in PKCη$^{-/-}$ T cells, but that TCR-proximal signaling pathways were intact. These signaling defects are similar to those defects reported in PKCθ$^{-/-}$ T cells (Sun et al., 2000; Pfeifhofer et al., 2003). Thus two questions remain: First, if the signaling defects are the same in PKCη- and PKCθ-deficient T cells, why are the defects in PKCη-deficient T cells not as strong as PKCθ-deficient T cells, at least *in vitro*? One possibility is that more signaling pathways are interrupted by PKCθ-deficiency compared to PKCη-deficiency, in addition to NFκB (Sun et al., 2000) and NFAT (i.e., Ca^{2+} signaling-related) defects (Pfeifhofer et al., 2003). For example, it was recently shown that PKCθ can bind to CD28 and thus mediates a co-stimulation-driven signaling pathway from the immunological synapse (Yokosuka et al., 2008; Kong et al., 2011). More importantly, are there non-overlapping or distinct pathways between PKCη and PKCθ? The answer is likely yes. First of all, as shown in our study, PKCη and PKCθ have distinct roles in homeostatic proliferation, with η being required but θ being dispensable (Fu et al., 2011). Second, the different spatio-temporal localization of PKCη and

Table 1 | Comparison of PKCη and PKCθ in T cell biology.

	PKCθ	PKCη
T cell development in KO mice	Mildly impaired[1]	Normal[2]
MATURE T CELLS IN KO MICE		
CD4/CD8 ratio	Lower than WT[2]	Higher than WT[2]
Proliferation		
to αCD3 *in vitro*	Severely impaired[3,4]	Mildly impaired[2]
to PMA/ionomycin	Normal[4] or Impaired[3]	Normal[2]
to antigen *in vivo*	Normal[5–8] or Impaired[9]	Impaired[2]
to antigen *in vitro*	Impaired[1,3,4]	Impaired[2]
Homeostatic proliferation		
Non-tg CD8 T cells	Normal[2,7]	Impaired[2]
OT-I tg CD8 T cells	Normal[2]	Impaired[2]
SIGNALING EVENTS IN KO CELLS		
Calcium flux	Impaired[4]	Impaired[2]
NFκB	Impaired[3,4]	Impaired[2]
NFAT	Normal[3] or Impaired[4]	Not available
AP-1	Impaired[3,4]	Not available
IMMUNOLOGICAL SYNAPSE (IS)		
In effector T cells	Recruited to IS[10,12,15]	Recruited to IS[2,12]
Spatial pattern	Central region[11,12]	Diffuse pattern[2,12]
Temporal kinetic	Late, after η[13]	Early, before θ[13]
Domain(s) required	V3 domain[14]	Not available
In regulatory T cells	Not recruited to IS[15]	Not available

[1] Morley et al. (2008), [2] Fu et al. (2011), [3] Sun et al. (2000), [4] Pfeifhofer et al. (2003), [5] Berg-Brown et al. (2004), [6] Barouch-Bentov et al. (2005), [7] Valenzuela et al. (2009), [8] Marsland et al. (2005), [9] Marsland et al. (2004), [10] Monks et al. (1997), [11] Monks et al. (1998), [12] Singleton et al. (2009), [13] Quann et al. (2011), [14] Kong et al. (2011), [15] Zanin-Zhorov et al. (2010).

PKCθ in the immunological synapse, with η showing an earlier and more diffuse pattern and θ showing a later and more concentrated pattern in the central region of the synapse (Singleton et al., 2009; Fu et al., 2011; Quann et al., 2011). Finally, there is a study showing PKCη and PKCθ having differential downstream functions in EL4 thymoma cells (Resnick et al., 1998). Collectively, these results strongly indicate the existence of an at least partially independent signaling pathway involving PKCη.

FUTURE DIRECTIONS

As mentioned earlier, the study of PKCη in T cell biology and the immune system in general, is far behind the state of knowledge we have on its cousin PKCθ (Fu and Gascoigne, 2012). Several recent studies have finally brought PKCη under the spotlight (Singleton et al., 2009; Suzuki et al., 2009; Fu et al., 2011; Quann et al., 2011; Sewald et al., 2011). In **Table 1**, we summarize the available results regarding PKCη in comparison with PKCθ. However, much more work needs to be done before we have a comprehensive understanding of the role of PKCη. First, what molecular machinery is involved in PKCη signaling? Does PKCη share the same signaling complex with PKCθ, such as the CAMA1/MALT1/Bcl10 complex? Second, what drives PKCη to the immunological synapse and what is the importance of differential localization of PKCη compared to PKCθ in the synapse? A recent study shows that the V3 domain is required for PKCθ recruitment to the immunological synapse (Kong et al., 2011). Is this also true for PKCη, considering their generally similar structures, or is the diffuse synapse-localization of PKCη due to the lack of the relevant motif in the V3 domain? Since PKCθ interacts with CD28 through a V3 motif, does PKCη also interact with CD28, or if not, is it due to the different V3 sequences? Does PKCη interact with other co-stimulatory molecules? Third, what roles does PKCη have in other T cell subsets or in other immune cells? In mice, it has been shown that PKCθ-deficiency impairs regulatory T cell development (Schmidt-Supprian et al., 2004), and in humans it has been shown that PKCθ plays a negative feedback role in regulatory T cell function, which is in contrast to its positive feedback role in naïve conventional T cells (Zanin-Zhorov et al., 2010). Thus it may be informative to check the role of PKCη in Treg cell development and function. PKCθ-deficiency has been shown to specifically impair Th2 cell responses but not Th1 responses, and thus has various effects in anti-pathogen immune responses (Berg-Brown et al., 2004;Marsland et al., 2004, 2005; Sakowicz-Burkiewicz et al., 2008). Could PKCη play an opposing role in these cases or a redundant role? What effects may PKCη have on CD4 T-helper cell subset differentiation? The role of PKCη in infection models and autoimmune diseases is another area that clearly needs attention. A simple but very informative study would be to directly compare the immune responses in η-, θ-, or ηθ-double deficient mice to the same viral and bacterial pathogens to get a full picture of the role of these two PKC isoforms in immunity. All these questions deserve more systematic studies in the future.

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Th1/Th2 differentiation and B cell function by the atypical PKCs and their regulators

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The members of the atypical Protein Kinase Cs (aPKC) kinase subfamily, PKCζ and PKCλ/ι, as well as their adapters, p62 and Par-6, form part of the PB1-domain-containing group of signaling regulators. Both adapters serve to locate through heterotypic interactions the aPKCs into the NF-κB and cell polarity pathways, respectively. Both signaling cascades have been critically implicated in T cell function *in vitro* and *in vivo*. The analysis of gene-knockout (KO) mice deficient in the different PB1 molecules is providing more definitive information on the actual role that the aPKCs and other PB1-containing molecules play in B cell biology and T cell polarity, survival, and differentiation toward the different effector lineages *in vivo* and at the cellular *ex vivo* level. Here we discuss recent data generated from the analysis of KO mice linking the control of cell polarity by PKCλ/ι and PKCζ, their adapter p62, and the Par-4 inhibitor, in the control of B and T cell signaling and differentiation. Altogether, these genetic and biochemical evidences reveal the existence of a PB1-orchestrated signaling network that acts to control Th2 differentiation *in vitro* and *in vivo*, and the gene transcriptional programs that are essential during the B cell maturation and function and Th2 differentiation.

Keywords: atypical PKCs, B cells, T cells, Th1, Th2, adaptive immune responses, asthma

INTRODUCTION

It is well known that cells from the adaptive immune system need proper activation of the nuclear factor κB (NF-κB) for their function and development. NF-κB provides necessary mediators for the survival of immature T and B cells in antigen receptor-mediated negative and positive selection of non-reactive clones in thymus and bone marrow, respectively. NF-κB activation is also necessary for mature lymphocyte differentiation and function (Hayden et al., 2006; Schulze-Luehrmann and Ghosh, 2006). However, uncontrolled activation of NF-κB can induce cancer development, autoimmune, and chronic inflammatory diseases as a result of exacerbated lymphocyte function (Karin and Greten, 2005). The Protein Kinase Cs (PKCs) family is an important piece in the puzzle of NF-κB-mediated cell activation and several studies on recent years have demonstrated that proteins of this family are potent mediators of antigen receptor downstream signaling in B and T cells. Hence, these proteins are key in both, the development and the control of innate and adaptive immune responses. Since there are three different families of PKCs; classical PKCs (PKCα, βI, βII, γ), novel PKCs (PKCδ, ε, η, θ), and atypical PKCs (aPKCs; PKCζ, λ/ι), some of the lymphocyte signaling pathways controlled by these kinases can be redundant although they have distinct *in vivo* functions due to their broad substrate specificity. For example, in addition to NF-κB they are also involved in AP-1 or NFAT transcription factors activation in T cells (Tan and Parker, 2003). In this review we discuss the mechanisms by which aPKCs regulate T and B cell signaling after antigen stimulation and the role of the aPKCs-associated molecules Par-4, a potent inhibitor of aPKCs, and the scaffolding protein p62 in lymphocyte activation and differentiation.

ATYPICAL PKCs IN B CELL BIOLOGY

B cells are lymphocytes originated from lymphoid precursors in the bone marrow after rearrangement of the immunoglobulin genes to provide the immune system with the specific repertoire of B cells to protect the body against pathogens (Harwood and Batista, 2010). B cells generation from hematopoietic precursors needs four different steps to take place: early pro-B cells, late pro-B cells, pre-B cells, and immature B cells formation. Before immunoglobulin gene rearrangement starts, the early pro-B cell subset emerges from the precursors giving rise to the following subset of late pro-B cells. In this step begins the rearrangement of D and J gene segments to generate pre-B cells with intact immunoglobulin heavy chains. When the rearrangement of the light-chain genes is completed the immature B cell subset is formed, expressing IgM on cell surface ($B220^{low}$ $MHC\text{-}II^{high}$ IgM^{high} IgD^{low}). At this step, B cells undergo a selection process to eliminate self-reactive cells to avoid autoimmunity before going out to periphery. Once positioned in secondary lymphoid organs, immature B cells become transitional B cells that are ready to maturate within the follicles in lymph nodes or in the marginal zone of the spleen (marginal zone B cells). After antigen challenge, mature B cells ($B220^{high}$ $MHC\text{-}II^{high}$ IgM^{low} IgD^{high}) become antibody-secreting plasma cells in germinal centers or memory B cells distributed elsewhere (Hardy and Hayakawa, 2001). The role of PKCs in these processes as well as in the activation and

function of mature B cells is from diverse nature. Is important to remark that, unlike what is described for other PKCs, PKCδ is involved in the generation of B cell tolerance and anergy (Mecklenbrauker et al., 2002; Miyamoto et al., 2002) whereas PKCβ and PKCζ have a different role in B cell biology. Mice deficient for PKCβ has defective B cell signaling (Leitges et al., 1996) and PKCζ deficiency is associated to defects in B cell development, proliferation, and survival (Leitges et al., 2001; Martin et al., 2002; Moscat et al., 2003).

Role of PKCζ in secondary lymphoid organ maturation and B cell differentiation

Although the lack of PKCζ in mice generates no apparent abnormality, the formation, and maturation of secondary lymphoid organs is altered during the first weeks after birth. In this regard, 2–4 weeks old mice fail to develop an appropriate number of Peyer's patches (PP) with an important reduction in follicles size and total cell number within each PP. Moreover, internal structure of PPs is also impaired as B and T cell zones are disrupted, this is due in part to the almost total lack of follicular dendritic cells (FDCs) that organized follicles structure. B cell zones are reduced and there is an imbalance between $B220^{high}$ MHC-II^{high} IgM^{low} IgD^{high} mature and $B220^{low}$ MHC-II^{high} IgM^{high} IgD^{low} immature B cells that is also observed in adult mice (Leitges et al., 2001). Regarding the spleen of these mice, although grossly normal, they present a defect in the formation of B cell follicles in the white pulp. The deficiency of FDCs in this organ induces disruption of the marginal zone architecture. Accordingly there is a decrease in the percentage of mature BCs in lymph nodes. However most of these defects are overcome in adult animals indicating that PKCζ could have an important role in B cell maturation in young animals that can be compensated by other molecules in adult mice (Leitges et al., 2001). In this regard it is important to note that B cell subpopulations in spleens and lymph nodes are normal and also microarchitecture of these organs has no apparent defects in adult animals.

B cell activation and survival is dependent of PKCζ signaling

In depth study of the role of PKCζ in B cell signaling indicates that this aPKC is required for an optimal survival rate, cell cycle entry, and proliferation after B cell receptor (BCR) stimulation in adult animals (Martin et al., 2002). As stated before, B cell subpopulation and lymphoid organ architecture of 4–6 weeks old animals is quite normal. In contrast to that observed in embryonic fibroblasts (EFs; Leitges et al., 2001), studies with PKCζ-deficient mice indicate that the defects in B cell activation and proliferation correspond to impaired activation of extracellular signal-regulated kinase (ERK), whereas the activation of other mitogen-activated protein kinases (MAPK) is not altered. Regarding transcription factors activation through PKCζ, a small decrease in activating protein-1 (AP-1) transcription factor activation is observed along with an inhibition of c-Fos induction in PKCζ-deficient mice (Martin et al., 2002; **Figure 1A**). Although PKCζ is an important mediator of the NF-κB pathway (Leitges et al., 2001), the activation and nuclear translocation of this transcription factor is not inhibited in BCs from PKCζ-deficient mice. However, activation of I*k*B transcription is severely impaired in PKCζ-deficient B cells after BCR stimulation and more importantly, transcription of NF-κB-dependent genes such as IL-6 or Bcl-X_L (important for B cell survival) is also inhibited (**Figure 1A**). These data demonstrate that although PKCζ is important for the transcription of NF-κB-dependent genes associated with proliferation and survival, and consistent with previous data of EF from PKCζ-deficient mice (Leitges et al., 2001), NF-κB nuclear translocation is not affected in the absence of this molecule in B cells (Martin et al., 2002).

Adaptive immune responses are altered in PKCζ-deficient mice

PKCζ is an essential kinase for B cell survival and function through the activation of I*k*B and Bcl-X_L transcription (Martin et al., 2002). These data agree with the notion that Rel, RelA, and NF-κB1 transcription factors are essential in B cell development, survival, proliferation, and immunoglobulin expression (Grossmann et al., 2000; Gugasyan et al., 2000). The lack of Rel/NF-κB genes or the individual subunits of the IκB kinase, IKKα and IKKβ, alters the development of adaptive immune response (Gerondakis et al., 1999). Accordingly, although PKCζ-deficient mice develop immune responses against T-independent and -dependent antigens and are able to induce the formation of germinal centers in the spleen, the T-dependent humoral immune responses are very faint. The secretion of IgG_1, IgG_{2a}, and IgG_{2b} immunoglobulins, specific for dinitrophenyl-ovalbumin (DNP-OVA) T-dependent antigen, is almost abrogated in PKCζ-deficient mice, indicating that these mice are not able to mount a proper humoral response against T-dependent antigens (Martin et al., 2002; **Figure 1A**).

Even nowadays there is little information on the role of the other family member of aPKCs, the PKCλ/ι, in secondary lymphoid organ development or signaling downstream BCR in mature B cells. This is in part due to the lack of viable homozygous deficient mice for PKCλ/ι as these mice die before birth (Soloff et al., 2004; Yang et al., 2009), which is incompatible with the study of mature B cells. While more effort needs to be done to clarify this issue, PKCλ/ι seems not to be activated after BCR stimulation or after incubation with anti-CD40 indicating that this aPKC could not be playing any role in B cell biology (Martin et al., 2002). In summary, PKCζ plays a major role in lymphoid organ formation and B cell differentiation and function that could be non-redundant with the other aPKCs, the PKCλ/ι.

Role of PKCλ in B cell development

The role of PKCλ/ι in BC development or function has not been studied in such detail so as PKCζ. However it is known that PKCλ is involved in pre-BCR-induced activation of NF-κB in B cells undergoing maturation process (Saijo et al., 2003). Src-family protein tyrosine kinases (SFKs) have an important role in the first steps during B cell development, controlling the generation, and survival of pre-B cells (Wechsler and Monroe, 1995). These effects are mediated by NF-κB thus in the absence of SFKs, IKK phosphorylation is inhibited and the nuclear translocation of NF-κB is impaired inducing defects in the formation of pre-B cells. It has been proposed that the atypical PKCλ is a mediator of SFK induced activation of NF-κB in immature B cells, since PKCλ activation in pro-B cells deficient for SFK restores IKK activation and NF-κB-mediated signaling (Saijo et al., 2003). The mechanisms of

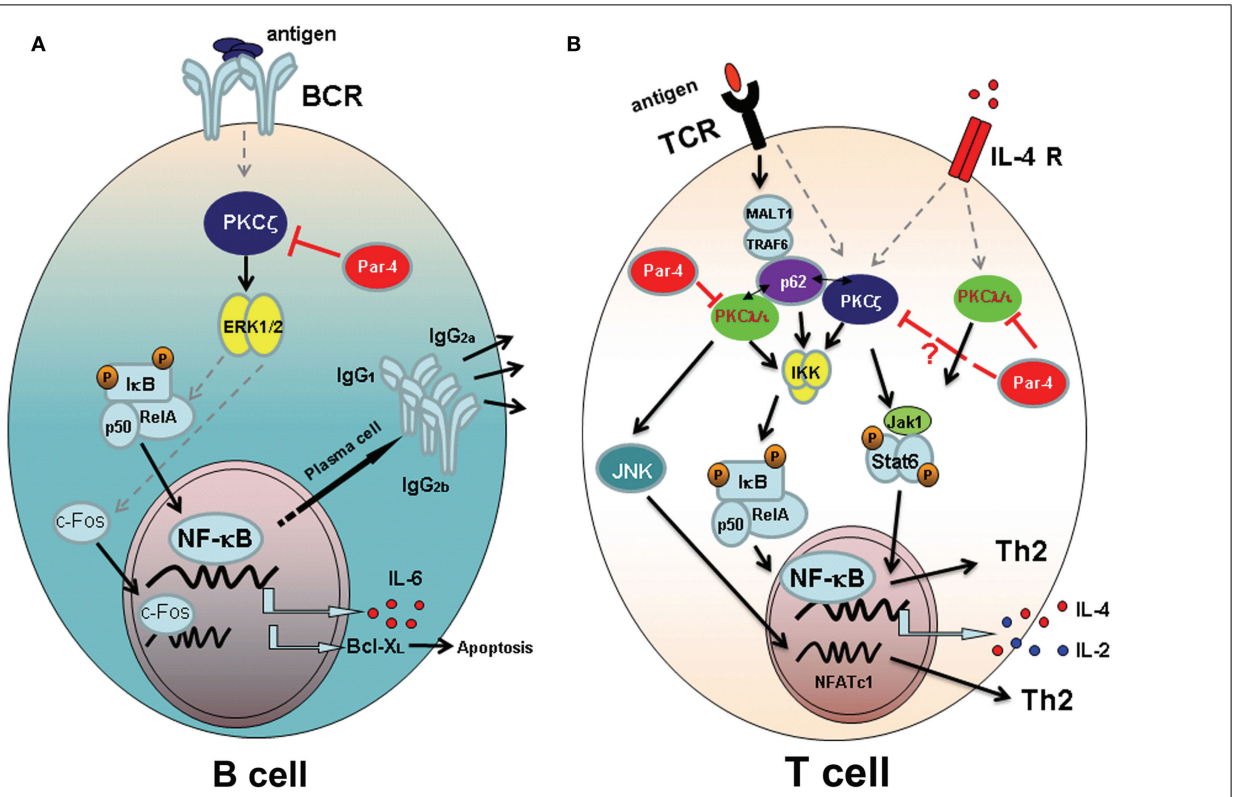

FIGURE 1 | Atypical PKC signaling pathways leading to lymphocyte activation and/or differentiation. (A) Signaling through B cell antigen receptor (BCR) in B-lymphocytes. PKCζ is required for the activation of ERK after antigen challenge and necessary for IκB transcription and for the activation and nuclear translocation of c-Fos. The transcription of NF-κB-dependent genes IL-6 and Bcl-X_L, and the secretion of T-dependent immunoglobulins depend on PKCζ-mediated signaling. The aPKC inhibitor Par-4 blocks PKCζ activation and B cell proliferation. **(B)** Signaling pathways in T lymphocytes. After antigen presentation, downstream signaling through T cell receptor (TCR) takes place. Atypical PKCζ and PKCλ/ι are important for IKK activation and NF-κB nuclear translocation, together with p62 scaffold protein that links the aPKCs with TCR activation through MALT-1 and TRAF-6 proteins. Moreover PKCλ/ι activates JNK and NFATc1 nuclear translocation, required for Th2 differentiation of T cells. Signaling through IL-4 receptor (IL-4R) requires both atypical PKCs to fully activate Jak1/Stat6 pathway and therefore to Th2 differentiation. Par 4 inhibits TCR and IL-4R signaling pathways blocking the activation of aPKCs.

IKK activation by PKCλ are not addressed, however it could be a direct association by the consensus phosphorylation site Ser177 (Lallena et al., 1999), or by an indirect interaction with Bcl10 and Carma1 (Gaide et al., 2002).

T CELL SIGNALING AND ATYPICAL PKCs

The nature of effector T lymphocytes and their functional properties into inflammatory tissues has been a matter of study during decades. However, in the last few years the paradigms of effector cell populations at the site of inflammation has changed considerably with the emergence of new players. After the infection of invading pathogens or during inflammation, T cell activation, and subsequent differentiation of T helper lymphocyte subsets are crucial for the development of immune responses. Once the antigen presenting cell presents the antigen to naïve CD4 T cells, these cells experienced differentiation toward Th1, Th2, or Th17 T helper cell subsets in the lymph nodes. Afterward, T helper cells migrate to the inflamed tissue and develop an adaptive immune response. Th1 cells are characterized by the expression of T-bet and the release of the Th1-related cytokine IFN-γ. Th1 cells are involved in the generation of autoimmunity and inflammatory disorders mediated by cellular immune responses such as the clearance of pathogens. The effector subset Th2 is essential for the development of inflammatory conditions mediated by antibodies and in the generation of allergic responses such as asthma. Th2 cells produce mainly IL-4, IL-5, and IL-13 among other cytokines and express the transcription factors GATA-3, Stat6, and c-Maf. And the more recently discovered T helper subset, the Th17 cells are characterized by the synthesis of IL-17A, IL-17F, and IL-22. Th17 cell emerged as an independent differentiation pathway as the express a different panel of transcription factors such as Stat3 and RORγt (Harrington et al., 2005; Bettelli et al., 2007; Nakayamada et al., 2012). The role of classical and novel PKCs in T cell receptor (TCR) signaling and T cell differentiation into helper T cells have been addressed extensively. PKCθ is recruited to the T cell synapse after TCR stimulation, what makes this PKC essential for the downstream signaling through NF-κB (Liu et al., 2000). The function of this PKC in T cell signaling is shared with PKCα, since both PKCs mediate T lymphocyte activation (Gruber et al., 2009). However PKCθ have different roles in Th1, Th2, or Th17 differentiation. PKCθ-deficient mice develop normal Th1 responses toward intracellular pathogens whereas Th2 asthmatic response against

that this protein acts at the level of antigen receptor signaling. Interestingly Par-4 deficient T cells secrete high amounts of IL-2 *in vitro* after stimulation. As Par-4 is a pro-apoptotic protein, Par-4 deficient T cells are more resistant to apoptosis induced cell death than their WT T cell control (Lafuente et al., 2003). As it was previously mentioned, aPKCs downstream signaling cascade controls NF-κB activation (Leitges et al., 2001) and, in turn, Par-4 inhibits this pathway and proliferation of T cells through the inhibition of aPKCs phosphorylation. Then, it seems that Par-4-mediated inhibition of T cell proliferation is not controlled through PKCζ since the proliferation of T cells from PKCζ-deficient mice is normal (Martin et al., 2002). Hence Par-4 should exert its effect through the other aPKC, the PKCλ/ι. Moreover, primary EFs from Par-4-deficient mice have decreased JNK activation that is observed also in T cells from these mice (Garcia-Cao et al., 2003; Lafuente et al., 2003). JNK is a potent inhibitor of NFATc1 transcription factor essential for T cell differentiation and function and accordingly Par-4 deficient T cells have an increase in NFATc1 activation, which may explain the high levels of IL-2 secretion by these cells that could directly influence the high rate of proliferation of Par-4-deficient T cells (Chow et al., 2000; Crabtree and Olson, 2002). A well as PKCζ and PKCλ/ι play an important role in T cell differentiation toward helper subsets, Par-4 is important for the control of Th2 responses. Par-4 deficiency leads to aPKC hyperactivation and subsequent inhibition of JNK, signaling pathway required for the control of IL-4 secretion, and Th2 differentiation (Dong et al., 1998; **Figure 1B**). Moreover, *in vivo* experiments in a model of T cell-dependent hepatitis have demonstrated that Par-4 deficiency leads to an increase in IL-4 signaling pathway, increasing the levels of Th2-cytokines, eotaxin, apoptosis, and liver injury after treatment with Con-A. These results are consistent with an enhanced signaling downstream IL-4 in EFs from Par-4 deficient mice, where Jak1 and Stat6 phosphorylation is increased demonstrating a connection with an enhanced signaling through aPKC (Duran et al., 2004a; Martin et al., 2005; Yang et al., 2009).

aPKC scaffold protein p62 controls T helper differentiation

The adapter protein p62 interacts with aPKCs through their PB1 protein interaction domain and is essential in the maintenance of NF-κB activity during osteoclastogenesis (Duran et al., 2004b). Similarly is a key protein for the long-term NF-κB activation after TCR stimulation of naïve $CD4^+$ T cells (Martin et al., 2006). Unlike PKCζ and PKCλ/ι do, p62 controls Th2 differentiation and function by an IL-4 independent mechanism. Th2 cells deficient for p62 have impaired activation and nuclear translocation of GATA-3 and RelA, playing an important role in NF-κB activation, whereas Stat6 signaling through IL-4 receptor is not affected (Das et al., 2001; Martin et al., 2006). In this regard, proximal TCR downstream signaling pathway is not altered in the absence of p62 as Zap-70, ERK, or AKT activation and Ca^{2+} flux are normal after antigen receptor triggering. Degradation of IκBα, an early indicator of NF-κB activation, is also intact in p62-deficient cells while IκBα activation is inhibited several hours after TCR triggering along with NF-κB inhibition. Interestingly, p62 expression is induced at later time points after TCR stimulation and particularly under Th2 differentiation conditions. This is possible because p62 establish a connection with Malt-1 and TRAF6, both proteins involved in IKK ubiquitination (Wooten et al., 2005), favoring NF-κB activation after long-term TCR stimulation probably through PKCλ/ι (**Figure 1B**). Accordingly with impaired Th2 differentiation *in vitro*, p62-deficient mice also show a dramatically reduction of lung inflammation toward OVA-induced allergic airway disease demonstrating a key role of p62 in Th2 function *in vivo* (Martin et al., 2006).

CONCLUDING REMARKS

It is clear that over the recent years the aPKCs have emerged as critical players in the control of T and B cell function, which has important implications as potential therapeutic targets in inflammation and immunity. Importantly due to their role also in cancer, they can also be considered critical players in the ability of the tumor cell to grow and proliferate but also as key players in the tumor microenvironment whereby they can be central in orchestration signals controlling the impact that inflammation has in tumor progression and initiation. Future studies using the animal models described here will be instrumental in addressing these important questions.

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