PKC-θ function at the immunological synapse: prospects for therapeutic targeting

Alexandra Zanin-Zhorov1, Michael L. Dustin1* and Bruce R. Blazar2

1Molecular Pathogenesis Program, Helen and Martin Kimmel Center for Biology and Medicine, Skirball Institute of Biomolecular Medicine, Department of Pathology, New York University School of Medicine, New York, NY 10016, USA
2University of Minnesota Cancer Center and Department of Pediatrics, Division of Blood and Marrow Transplantation, Minneapolis, MN 55455, USA

Protein kinase C (PKC)-θ regulates conventional effector T (Teff) cell function. Since this initial finding, it has become clear that the role of PKC-θ in T cells is complex. PKC-θ plays a central role in Teff cell activation and survival, and negatively regulates stability of the immunological synapse (IS). Recent studies demonstrated that PKC-θ is required for the development of novel CD4+Foxp3+ regulatory T (Treg) cells, and mediates negative regulation of Treg cell function. Here, we examine the role of PKC-θ in the IS, evidence for its distinct localization in Treg cells and the therapeutic implications of inhibiting PKC-θ in Teff cells, to reduce effector function, and in Treg cells, to increase suppressor function, for the prevention and treatment of autoimmune and alloimmune disease states.

PKC-θ location in the immunological synapse
The protein kinase C (PKC) family of serine/threonine kinases contributes to signal transduction networks that co-ordinate almost all aspects of immune cell function and thus these kinases play a crucial role in immune regulation. There are three subfamilies of PKC, the conventional, which is activated by Ca2+ and diacylglycerol, the novel, which is activated by diacylglycerol and the atypical, which is insensitive to both Ca2+ and diacylglycerol [1] and all sub-families are represented in T cells. PKC-θ is a novel PKC that is most abundant in hematopoietic cells [2]. PKC-θ gained prominence with the finding that it is the only PKC isoform selectively recruited to the immunological synapse (IS) in conventional effector T (Teff) cells (Figure 1) [3]. The IS is a stable cell–cell junction that forms between a Teff cell and an antigen-presenting cell (APC) during TCR-peptide MHC recognition. The IS is composed of TCR microclusters that form the so-called central supramolecular activation cluster (cSMAC). This is surrounded by the peripheral supramolecular activation cluster (pSMAC), which contains large amounts of LFA-1 and ICAM-1 (Figure 1) [4–7]. Formation of the IS, which is crucial for sustained TCR-induced signaling and full T cell activation, leads to PKC-θ recruitment to the junction between the cSMAC and pSMAC in a CD28 co-stimulatory-dependent manner [3,8–10]. Moreover, both CD28 and PKC-θ are localized to a spatially unique compartment that plays a central role in coordinating the signal transduction pathways downstream of the TCR [10,11]. PKC-θ plays a major role in activation of NF-κB transcription factors and promotes AP-1 and NFAT signals [12,13]. Surprisingly, PKC-θ negatively regulates IS stability by favoring symmetry breaking of the pSMAC in a manner counteracted by expression of the Wiscott Aldrich Syndrome protein (WASP) [14]. In this context, symmetry breaking refers to the conversion of the radially symmetrical pSMAC, which holds the cell in place, into an asymmetric lamella, which drives cell motility. The functional outcome of reduced IS stability might include a greater ability to gather antigen leading to prolonged signaling [15]. In addition, the symmetrical pSMAC ring plays an important role in containing components that are released into the synapse. Consistent with this, inhibition of PKC-θ can enhance killing by CD4+ cytotoxic T cells [16]. The detailed mechanism by which PKC-θ stimulates symmetry breaking in Teff cells is not known, but in other systems symmetry breaking involves cooperative myosin II activation [17]. There are other mechanisms for symmetry breaking: non-responsive anergic T cells, in which PKC-θ is degraded, also break symmetry rapidly [18].

CD4+ Foxp3+ regulatory T (Treg) cells suppress inflammatory responses in a TCR-dependent manner [19] and are central in maintaining a balance between immune tolerance to self-antigens and anti-tumor responses [20–22]. There is evidence that stable Treg cell–dendritic cell (DC) interactions are important for Treg cell function in vivo [20–22], but the organization of the IS in Treg cells is not well studied. Recent data showed that during IS formation in Treg cells, PKC-θ is sequestered away from the IS to the distal pole complex (Figure 1) [23]. Thus, PKC-θ is differentially localized in Teff cells and Treg cells during IS formation. Furthermore, through use of supported planar bilayers to present anti-CD3 antibodies and ICAM-1 to T cells to mimic IS formation in vitro, Treg cells were found to form a more stable IS than Teff cells. Here, we examine the function of PKC-θ in Teff cells and Treg cells with emphasis on the role of PKC-θ at the IS. Also discussed is the therapeutic potential for targeting PKC-θ as an anti-inflammatory approach.
PKC-θ signaling pathways

Signal transduction downstream of TCR is initiated in microclusters within nascent IS. PKC-θ mediates activation of transcription factors important for T cell function, such as nuclear factor κB (NF-κB), activation protein-1 (AP-1) and nuclear factor of T cells (NFAT) (Figure 2). PKC-θ activates NF-κB through phosphorylation of the membrane-associated guanylate kinase (MAGUK) Carma1 (also called Card11) [24]. The N-terminus of Carma1 contains a caspase recruitment domain (CARD) and a coiled-coil domain [25]. Phosphorylation of Carma1 by PKC-θ initiates assembly of an oligomeric complex with the CARD domain containing adapter Bcl10 and the death domain (DD) containing paracaspase Malt1, which mediates activation of NF-κB transcription factors [24]. PKC-θ also phosphorylates the ste20-family kinase SPAK, which is important for AP-1 transcription factor activation [26].

One group found that Ca2+ and NFAT activation was relatively normal in PKC-θ-deficient T cells in vitro [13], whereas another group found that the Ca2+ and NFAT pathway was defective [12]. The role of PKC-θ in NF-κB signaling is selective for the TCR–CD28 signaling pathways [27,28] because inflammatory cytokine-mediated activation of NF-κB is independent of PKC-θ [13]. PKC-θ is an essential TCR-induced signal that activates IL2 gene expression [12] and it appears to mediate enhanced IL-2 production in response to CD28 co-stimulation [8,10].

Moreover, recent study has demonstrated that PKC-θ might regulate gene expression outside the IS in T cells by association with chromatin in the nucleus [29]. Thus, PKC-θ plays a crucial role in integration of TCR–CD28 co-stimulatory signals required for optimal NF-κB activation and this pathway could be considered the canonical pathway for function of PKC-θ in T cell activation.

PKC-θ and T cell function

Treg cells

Treg cells are central in maintaining a balance between immune tolerance to self-antigens and anti-tumor responses [19]. There are two developmentally distinct populations of CD4+ Treg cells, both of which depend upon the transcription factor Foxp3 [37–39]. Natural Tregs (nTreg) are produced in the thymus and express both Foxp3 and the transcription factor Helios [40]. Induced Treg (iTreg) cells are generated from naive Th cells in the periphery and do not express Helios [40]. Foxp3 deficiency leads to defects in Treg cell function, which manifest as a multi-organ fatal inflammatory disease in mice [41,42]. A unique feature of Treg cells is their ability to suppress a range of target cell types that includes CD4+ and CD8+ subsets of T cells, B cells, NK cells and DCs [19,43]. The proposed mechanisms of Treg-mediated suppression include suppressor cytokine secretion, IL-2 consumption, alteration of APC function, target cell cytolyis and cell surface molecules [19,43].

In mice lacking PKC-θ, Treg development in the thymus is impaired leading to reduced numbers of Treg cells in the periphery, although the per cell function of peripheral Treg cells remains intact [44,45]. It has been suggested that during T cell development TCR ligands that induce strong signals drive differentiation of Treg cells as opposed to Th cells. The requirement for PKC-θ in Treg cell development might relate to the ability of PKC-θ to augment signal transduction in TCR ligated cells. For example, cRel, a downstream target of PKC-θ, is crucial for Treg cell development and expansion [46]. Furthermore, as discussed, PKC-θ deficiency impairs IL-2 production by Th cells, and Treg cells need high levels of IL-2 for function, survival and proliferation [19,42,47]. Therefore, reduced IL-2 in PKC-θ-deficient mice might also contribute to Treg cell defects [44,46].

Pre-treating mouse Treg cells with a PKC-θ inhibitor increases engraftment at one week after injection into TCRδ−/− mice together with a CD4+CD25+CD45RBhigh subset of Teff cells [23]. As Treg cells divide after introduction into lymphopenic mice [48], we can conclude that murine Treg cells with inhibited PKC-θ show no major defect in proliferation in vivo, and might even proliferate more rapidly than Treg cells with fully active PKC-θ. By
contrast, inhibition of PKC-θ abolishes the ability of human Treg cells to proliferate in vitro upon anti-CD3 and CD28 stimulation in the presence of high levels of IL-2 [23]. There could be several explanations for the discrepancy between the results of in vivo and in vitro studies regarding the role of PKC-θ in Treg cell expansion, including the presence of additional factors in vivo that contribute to IL-2 production, other growth factors that might be present in vivo, or species-specific differences. Thus, in Treg cells, PKC-θ is required for thymic development, but is dispensable for in vivo homeostatic expansion and suppressive function.

TCR signals are required for Treg cell-suppressive function and these signals can be received through a stable IS. Human Treg cells form a more stable IS compared to Teff cells [23], which could be related to reduced PKC-θ signaling at the IS in Treg cells [14]. Consistent with this idea, markers of signaling downstream of TCR, including phosphorylated Src family kinases, PKC-θ and CARMA-1, are reduced at the Treg cell IS (Figure 2). Furthermore, inactivation of PKC-θ in Treg cells with an irreversible small molecule inhibitor resulted in ~4-fold increase in Treg cell function [23]. Knock-down of PKC-θ by siRNA generates a similar increase in Treg cell function. The precise mechanism by which PKC-θ signaling mediates negative feedback in Treg cell is not known, but it might involve inhibition of NF-κB-mediated transcription in the presence of FoxP3 or other Treg cell-associated transcription factors. Many questions remain about which signaling molecules interact with PKC-θ at the distal pole complex and how the role of PKC-θ and NF-κB in Treg cell development in the thymus is related to the role of those molecules in maintenance of the Treg cell phenotype and function.

**Targeting PKC-θ in disease**

Defects in or insufficiency of Treg cells has been linked to autoimmune disease in human and animal models [49–51]. For example, Treg cells isolated from peripheral blood of patients with rheumatoid arthritis (RA) have reduced suppressive function due to negative regulation by tumor necrosis factor-α (TNF-α) [23,51]. Interestingly, conventional pathways for TNF-α signaling are not PKCθ dependent [13] but treatment with TNFα results in relocation of PKC-θ in Treg cells to a more Teff cell-like pattern [23]. Moreover, PKC-θ inhibition protects Treg cells from TNF-α-mediated suppression and partially restores the diminished suppressive activity of Treg cells purified from RA patients [23,51]. Finally, PKC-θ inhibition in Treg significantly increases the potential to protect mice against inflammatory colitis in vivo [23]. It quite remarkable that pre-treating Treg cells with a small molecule inhibitor for only 30 min, followed by washing away the free drug and infusing the treated Treg cells into a lymphopenic host would have a sufficiently durable effect to change the course of a disease that develops over a period of weeks. Because Treg cells expand rapidly in lymphopenic hosts and PKC-θ inhibition does not block this expansion, the drug is almost certainly diluted out. Thus, these effects could take place early and then set the stage for the development of disease, perhaps by determining the final ratio of Treg cells to Teff cells in the host after reconstitution. PKC-θ-suppressed, more potent Treg cells could change this ratio both by proliferating faster and by inhibiting Teff cell proliferation.

The recent success of small molecule inhibitors of PKC-θ in phase I clinical trials for psoriasis raises the possibility of therapeutically targeting PKC-θ to interfere with the balance between Teff cells and Treg cells in patients [52]. It is therefore useful to consider the role of PKC-θ in both Teff and Treg cell. PKC-θ-deficient Teff cells are more prone to apoptosis, a finding associated with reduced levels of BclXL expression (Figure 2). However, forced expression of BclXL in Teff cells does not improve their survival [44]. The absence of PKC-θ results in a reduced survival of CD4+, and to a greater extent, CD8+ T cells [53–55]. These data predict that PKC-θ inhibition could be a useful approach to inhibit Teff cell responses, such as those that would occur during allogeneic transplantation of solid or hematopoietic organs or autoimmunity (reviewed in [56]). Inhibiting PKC-θ would function either to reduce the number of

**Figure 2.** The role of PKC-θ in TCR-CD28-induced signaling in T cells. In response to TCR-CD28 stimulation, PKC-θ is recruited to the IS and activated in a Lck and PI3K-dependent manner. Then, PKC-θ recruits a MAGUK protein, Carma-1, that enables the assembly of a Carma-1-Bcl10-Malt1 complex necessary for NFκB activation. PKC-θ also activates the AP-1 transcription factor by interaction with SPAK kinase. The PKC-θ-mediated activation of NFAT is controversial, but some have reported that the Ca2+-NFAT pathway is defective in PKC-θ-deficient T cells. Taken together, PKC-θ plays the central role in the TCR-CD28-mediated induction of gene transcription leading to proliferation and cytokine secretion in T cells.
activated Teff cells, by failing to protect Teff cells from apoptosis and activation-induced cell death, or to minimize the contribution of signals downstream of CD28, predisposing T cells to become anergic. Conversely, blocking CD28–B7 ligand interactions using an anti-CD28 monoclonal antibody results in PKC-θ and Jnk suppression, along with long-term cardiac allograft survival [57].

Regulation of IL-2 production and alloreactivity in vivo is influenced by PKC-α and PKC-θ, which both affect NFAT translocation [58]. Whereas PKC-α or PKC-θ-deficient recipients have only minimal prolongation of cardiac allograft survival, there is an additive effect on allograft survival in double-deficient recipients, indicating that PKC-α and PKC-θ are non-redundant in controlling alloreactivity in vivo. PKC-θ deficiency is partially compensated because cardiac allograft rejection in PKC-θ deficient recipients is delayed although not eliminated. The strength of this partial compensation is modest, however, because cardiac allografts in PKC-θ-deficient recipients are hypersusceptible to blockade with anti-CD154 antibody or CTLA4-Ig [54]. In T cell adoptive transfer experiments, PKC-θ-deficient T cells fail to reject cardiac allografts unless Bclxl is expressed by transgenesis [54]. Thus, inhibition of PKC-θ with small molecule inhibitors or drugs might be highly useful in eliminating detrimental Teff cell responses or reducing their frequency below the necessary threshold for mediating auto- or allo-aggressive T cell-mediated tissue injury.

Because GVHD is iatrogenic, in that donor anti-host reactive allogeneic T cells are purposefully infused at the time of transplant, we know the onset of disease generation. GVHD has a relatively narrow time window for induction and targeting PKC-θ during a well defined and relatively short time frame might present new opportunities for clinical testing. Because GVHD is inhibited by Treg cells and worsened by high numbers of Teff cells, manipulating Treg cell and Teff cell numbers or function for a transient period of time post-transplant (within the first 3 months) might result in an avoidance of this potentially lethal complication of hematopoietic cell transplantation. Consistent with the finding that PKC-θ regulates CD4+ and CD8+ T cell survival, alloreactivity and IL-2 production, GVHD lethality in irradiated recipients of allogeneic PKC-θ-deficient CD8+ or -CD4+ and -CD8+ T cells is reduced [35]. Although GVHD causes a high degree of systemic inflammation and tissue destruction, PKC-θ-deficient T cells were hypoproliferative and had a higher frequency of annexin V staining after adoptive transfer in irradiated allogeneic recipients and gave rise to significantly fewer splenic CD4+ and CD8+ T cells early post-transplant. Interestingly, neither TLR4 nor TLR9 signals could overcome the defect of PKC-θ-deficient T cells in GVHD induction. This contrasts with the situation in control of bacterial and viral infection, in which TLR stimulation could compensate for PKC-θ deficiency. This is important because it suggests that PKC-θ inhibition might effectively block GVHD without eliminating antimicrobial defenses. Moreover, the presence of strong proinflammatory signals, such as those derived from a mitogenic anti-CD40 antibody or lipopolysaccharide, enhanced, but did not fully restore, the in vivo expansion and cytotoxicity of PKC-θ-deficient TCR transgenic CD8+ T cells administered to recipients expressing the relevant high-affinity ligand. Skewing of cytokine responses away from a Th2 response [30], which have been associated with decreased GVHD in some models, was not evident. Intriguingly, despite these profound defects in CD8+ T cell proliferation and cytotoxicity, clearance of a viral pathogen and leukemia cell line that are controlled by CD8+ T cells was intact. Reduced PKC-θ expression might lead to lower levels of FasL expression [59] that could also contribute to the lower GVHD lethality seen in recipients given PKC-θ-deficient versus PKC-θ-sufficient T cells. Taken together, these results suggest that the sterile inflammatory signaling driving GVHD might be an insufficient activator of NF-κB expression in donor T cells early post transplant in the absence of PKC-θ. By contrast, the innate sensing mechanisms in graft-versus-leukemia that further contribute to the inflammatory signals present in GVHD might be better activators of NF-κB, resulting in compensation of the PKC-θ defect in TCR signaling early post-transplant, an important time for graft-versus-leukemia effector mechanisms. Alternatively, the reduced T cell expansion and cytotoxicity of PKC-θ-deficient T cells might be insufficient to cause GVHD lethality owing to too few donor anti-host allogeneic T cells to exceed a threshold required for mediating GVHD. However, failure of donor T cells to migrate to GVHD target organs, or persistence of donor T cells, might be sufficient to reduce viral load and tumor burden but insufficient to cause GVHD lethality. Regardless of the mechanism, pharmacological targeting of PKC-θ could be an ideal approach to decrease GVHD lethality while preserving anti-infection and anti-tumor T cell responses post-transplant.

With respect to autoimmune diseases, PKC-θ-deficient recipients are resistant to the development of experimental autoimmune encephalomyelitis [60–62] and myosin-induced autoimmune myocarditis associated with reduced IL-17 secretion [63]. Under these conditions, TLR9 signaling can act directly on PKC-θ-deficient T cells to activate NF-κB, resulting in near-normal T cell expansion under Th1 polarizing conditions, restoring BclXL expression and promoting the generation of autoimmune myocarditis [63].

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Figure 3. The stability of the IS and localization of the NF-κB activating complex in Teff and Treg cells. The NF-κB activating complex consisting of PKC-ii, Carma-1 and other components is localized to the center of the IS in Teff cells and the distal pole complex in Treg cells. The IS is destabilized by PKC-ii in Teff cells leading to more broken IS patterns, whereas exclusion of PKC-ii from the IS of Treg cells stabilizes the IS. Red, ICAM-1; green, TCR; white, actin.
However, PKC-θ inhibition might prove to be useful in preventing autoimmune colitis, as discussed above [23,32]. Thus, PKC-θ inhibition could be highly useful in preventing and treating autoimmune diseases via targeting T effector cells and Treg cells to reduce and conversely increase their in vivo survival and function.

**Concluding remarks**

Our attention was initially called to PKC-θ by its unique localization in the T effector cell IS [3,4]. A dozen years later, application of a second-generation technology (for example supported lipid bilayers) to study the IS revealed that PKC-θ is excluded from the Treg cell synapse (Figure 3) [23]. These studies led to our current understanding that PKC-θ has dual roles in T effector and Treg cells that can be targeted to reduce inflammation in the context of autoimmune and alloreactivity. In T effector cells, PKC-θ promotes inflammation by increasing activation of the NF-κB transcription factors at the IS to promote growth and survival [13,56]. In Treg cells, PKC-θ is sequestered to a distal complex away from the IS and mediates a negative feedback on the Treg suppressive function [23]. The dual role of PKC-θ in controlling T cell function represents a therapeutic potential of PKC-θ inhibition as a powerful tool to block undesirable inflammatory responses.

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