Protein kinase Cθ: the pleiotropic T-cell signalling intermediate

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Abstract

Activating as well as inhibitory circuits tightly regulate T-cell activation thresholds and effector differentiation processes enabling proper immune response outcomes. Recently, an additional molecular link between T-cell receptor signalling and CD4$^+$ Th17 cell skewing has been reported, namely that protein kinase C (PKC) θ critically regulates Th17/Th1 phenotypic differentiation and plasticity in CD4$^+$ T-cells by selectively acting as a 'reprogramming element' that suppresses Th1-typical genes during Th17-mediated immune activation in order to stabilize a Th17 cell phenotype.

Introduction

Protein kinase C (PKC) θ belongs to the PKC family of serine/threonine kinases (reviewed in [1]). PKCθ expression is restricted to muscle cells, nervous system and, in particular, to the cells of the immune system, with an especially high expression level in T-lymphocytes [2]. The functions of PKCθ have therefore been best investigated in T-lymphocytes. Naïve T-lymphocytes are activated by interaction with a foreign antigen through specific T-cell receptors (TCRs), leading to the formation of a supramolecular complex designated the immunological synapse (IS). Although PKCθ is not the only PKC enzyme recruited to the IS [3], it plays an essential role in signal integration downstream of the TCR and the CD28 co-stimulatory receptor [4–6]. According to Kong et al. [6], an Lck-mediated interaction between PKCθ and the cytoplasmatic domain of CD28 is the molecular mechanism underlying translocation of PKCθ to the IS. Mechanistically, PKCθ promotes transactivation of at least three groups of transcription factors which are essential for T-cell biology: nuclear factor of activated T-cells (NFAT), activator protein 1 (AP-1) and nuclear factor κB (NF-κB) [4,7,8]. Moreover, PKCθ is involved in a T-cell-specific mitogen-activated protein kinase (MAPK) activation pathway mediated by the RasGDP-exchanging factor called RasGRP1 [9]. Other targets of PKCθ enzymatic activity have also been identified. For example, the NFAT competitor NR2F6 [10] and the tolerance-promoting E3 ubiquitin ligase Cbl-b are inactivated by PKCθ-mediated phosphorylation [11]. At a cellular level, PKCθ-deficient CD3$^+$ T-cells are characterized by strongly impaired interleukin 2 (IL-2) production [7,8]. Interestingly, PKCθ-deficiency was found to affect the proliferative potential and survival of CD4$^+$ T-cells more profoundly than of CD8$^+$ T-cells. This might be due to differential regulation of signalling cascade in these two cell populations. PKC$\theta^{-/-}$ CD8$^+$ T-cells, in contrast with PKC$\theta^{-/-}$ CD4$^+$ T-cells, maintain a considerably higher level of NF-κB activation [12]. Deficiency of PKCθ is subset-selective not only in relation to CD8$^+$ T and CD4$^+$ T-cells, but also within the particular CD4$^+$ T helper lineages [13].

PKCθ in regulating T helper effector phenotype

PKCθ not only participates in the proximal TCR signalling during the early stages of a naïve T-cell activation, but also governs differentiation processes of the defined Th1, Th2, Th17 or inducible regulatory T-cell (iT$_{reg}$) helper cell subsets [13]. According to the traditional view, PKCθ contributes to the Th1/Th2 dichotomy [14], since it is essential for the development of Th2-type immune responses but dispensable for Th1-driven immunity. *In vitro* experiments have shown an impaired expression of the Th2 signature transcription factor GATA3 in mouse PKC$\theta^{-/-}$ T-lymphocytes. Thus production of the Th2-specific cytokines IL-4 and IL-5 was negatively affected by PKCθ deficiency whereas the Th1-specific cytokine interferon γ (IFNγ) remained unaltered [15]. *In vivo*, response to infection by *Nippostrongylus brasiliensis*, a parasite inducing Th2-type immunity, was severely impaired in PKC$\theta^{-/-}$ mice, whereas normal immune protection against *Leishmania major*, a pathogen eliciting Th1-type immune reactions was observed in mice deficient in PKCθ [16]. While the absence of PKCθ compromised the protective Th2 immunity against specific external pathogens, it provided protection from Th2-dependent pathological allergic reactions. PKCθ-deficient mice, immunized with OVA peptide and re-challenged intranasally a week thereafter, were protected against adverse hypersensitivity responses to inhaled allergens [16]. Diminished GATA3 expression and down-regulation of

Key words: effector differentiation and plasticity, protein kinase C (PKC) family, protein kinase Cθ (PKCθ), T-cell activation.

Abbreviations: BMT, bone marrow transplantation; EAE, experimental autoimmune encephalomyelitis; GvHD, graft versus host disease; IFNγ, interferon γ; IL, interleukin; IS, immunological synapse; iT$_{reg}$, inducible regulatory T-cell; NF-κB, nuclear factor κB; NFAT, nuclear factor of activated T-cells; PKC, protein kinase C; STAT, signal transducer and activator of transcription; TCR, T-cell receptor; T$_{reg}$, regulatory T-lymphocyte.

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Th2-specific cytokines was observed in the model of persistent *Toxoplasma gondii* infection in BALBc PKC$\theta^{-/-}$ mice [17]. However, in this chronic inflammatory state mediated by both Th1 and Th2 immune responses, functionality of PKC$\theta^{-/-}$ CD8$^+$ T-lymphocytes appeared also to be affected. Interestingly, the overall effect of PKCθ deficiency was dependent on the genetic background of the infected mice. Pronounced differences (death of PKC$\theta^{-/-}$ mice compared with survival of wild-type controls) were observed only in the BALBc strain. C57BL/6 mice survived infections regardless of their distinct PKCθ genotype [17]. Data from mouse models of influenza and lymphocytic choriomeningitis virus (LCMV) infections provided further evidence that PKCθ is dispensable for induction of effective antiviral Th1 responses *in vivo* [5,18]. Virus clearance, cytolytic functions of CD8$^+$ T-cells and the titre of specific antiviral antibodies were comparable between infected PKCθ-proficient and PKCθ-deficient mice. Notably, isolated PKC$\theta^{-/-}$ CD4$^+$ and CD8$^+$ T-cells required much higher antigen loads than wild-type cells to proliferate in an *in vitro* antigen recall assay, indicating an anergic phenotype. This effect could be reversed by addition of IL-2 [5]. Additional compensatory effects *in vivo* were mediated predominantly by co-stimulatory signals from innate immunity pathways [18].

However, both genetic and/or pharmacological inhibition of PKCθ has been shown to confer significant benefits in controlling an exaggerated immune response. PKCθ-deficient mice infected with *Plasmodium berghei* ANKA were protected from a fatal form of cerebral malaria [19]. This was manifested by the decreased infiltration rate of CD8$^+$ cells into the brain of infected mice. Similarly, pharmacological inhibition of PKCθ prevented tissue damage and decreased pathological T-cell infiltration rates in the cardiac muscle in a mouse model of streptozotocin-induced diabetes. Along this line, intraperitoneal administration of a cell-permeable PKCθ peptide-based inhibitor ameliorated cardiac dysfunction and fibrosis [20]. Genetic ablation of PKCθ was beneficial in the murine model of muscle dystrophy. Generation of dystrophic *mdx* mice deficient in PKCθ resulted in slower disease progression. This might be attributed to alleviated inflammation in muscles that, in turn, might improve tissue regenerative potential. Indeed, bone marrow transplantations (BMTs) demonstrated that the beneficial effect was predominantly dependent on PKC$\theta^{-/-}$ immune cells [21].

Taken together, these data suggest that PKCθ promotes overall stronger immune responses but exerts its threshold regulatory function in a T-cell subset-specific manner. PKCθ is particularly important for effector responses of Th2 cells while Th1 and CD8$^+$ T-cells depend on this function to a lesser extent.

PKCθ in transplantation medicine

The apparent T helper subset selectivity of PKCθ encouraged its clinically oriented investigation in transplantation models. Fully mismatched cardiac allografts were not rejected in Rag1$^{-/-}$ mice reconstituted with PKC$\theta^{-/-}$ T-cells, whereas such allografts in Rag1$^{-/-}$ mice reconstituted with wild-type T-cells were readily rejected. In immune competent PKCθ-deficient but not wild-type recipients, however, rejection could be stopped by a suboptimal treatment dosage of immunosuppressive drugs, suggesting that inhibition of PKCθ may contribute to long-term survival of allografts [22]. Although only a minimal survival benefit was observed in cardiac allograft recipients in another PKCθ knockout strain [23], mice genetically deficient in the two PKC isoenzymes θ and α showed significantly prolonged graft survival. On a molecular level, this protective effect was associated with an almost completely abrogated transactivation of NFAT and additively impaired IL-2 production by PKCθ/α double-deficient T-cells.

Encouraging results have been obtained in BMT experiments. Prior to conducting BMT, Valenzuela et al. [24] confirmed that PKC$\theta^{-/-}$ mice mount sufficient immune responses against *Listeria monocytogenes* infection and responded normally to antigen/adjuvant immunization. In the clinic, allogeneic BMT – mismatched in either MiHA or MHC – is often carried out as immunotherapy for hematopoietic malignances and it is important to prevent devastating graft versus host disease (GvHD) development. In the next experimental step, the authors demonstrated that both sublethally and lethally irradiated mice with MHC- and MiHA-mismatched BMT were protected against GvHD after receiving PKCθ-deficient T-cells or splenocytes. In contrast, mice receiving cells from wild-type donors died due to severe GvHD. Molecular examination indicated an impaired proliferation and survival capability of transplanted PKCθ-deficient T-cells. The authors also investigated the anti-infection activity in the context of GVHD BMT, an issue seldom addressed in studies. They showed that PKC$\theta^{-/-}$ T-cells derived from PKC$\theta^{-/-}$ bone marrow expand *in vivo* in response to murine cytomegalovirus (MCMV) infection, and in these mice as well in those reconstituted with wild-type bone marrow, the infectious agent was cleared in a comparably efficient manner. These findings indicate that targeting PKCθ can prevent GvHD without compromising the ability of BMT recipients to respond to infectious agents. Thus the authors showed that by targeting PKCθ, development of GvHD is prevented while at the same time, anti-infection activity is preserved. This is of enormous benefit for patients receiving allogeneic BMT. Similar results were obtained by Haarberg et al. [25]: Consistent with the data obtained from the cardiac allograft experiments [23], the concomitant deficiency of PKCθ and PKCα allowed protection against GvHD in an additive manner. On the other hand, genetic deficiency of PKCθ rendered mice more prone to a Moloney murine leukaemia virus (M-MuLV)-induced leukaemia [26], suggesting a partially compromised anti-tumour host response in PKCθ-deficient mice.

Taken together, the presented data suggest that elimination of PKCθ function prevents responses to weakly immunogenic antigens, such as alloantigens and self-antigens,

but is less effective in preventing responses to strongly immunogenic antigens, i.e. from exogenous pathogens. Nevertheless, other PKC isoenzymes, such as PKCα, also contribute to alloimmune reactions. Importantly, one recent publication reported unexpected effects of PKCθ genetic ablation and pharmaceutical inhibition. Giambra et al. [27] have elegantly shown that primary PKCθ-deficient mouse T-cells have increased potency to induce acute lymphoblastic leukaemia (T-ALL). Overexpression of PKCθ in leukaemic cells was found to be associated with a reduced pathogenic potential in murine and human cell models. Thus, in this case, inhibition of PKCθ kinase activity might paradoxically promote disease progression.

PKCθ in autoimmunity: new light on established functions

The key role of PKCθ in modulating immune response outcomes raised the question of its function in autoimmune disorders. In general, genetic ablation of PKCθ attenuated the severity of autoimmune diseases in numerous mouse models of colitis and arthritis. Adoptive transfers of PKCθ-deficient or wild-type CD4$^+$ T-cells have shown that PKCθ knockout lymphocytes are unable to induce either Th1- or Th2-mediated colitis [28]. Similarly, disease symptoms in PKCθ-deficient mice were alleviated in diverse arthritis models [29]. Under all these experimental settings, an impaired production of Th1- and Th2-typical cytokines was observed.

Understanding of autoimmune pathologies has been profoundly deepened by the discovery of Th17 cells, which participate in acute inflammatory responses and, together with Th1 cells, mediate immune autoreactivity in mouse and man. Initial reports suggested that PKCθ is necessary for development of autoimmune Th17-type responses. Experimental autoimmune encephalomyelitis (EAE), a predominantly Th17-dependent mouse model of multiple sclerosis, was abrogated in PKC$\theta^{-/-}$ mice [30,31]. In a separate study, the observed deficiency of Th17 cell differentiation and function in PKC$\theta^{-/-}$ animals was linked to an impaired signal transducer and activator of transcription 3 (STAT3) expression [32]. However, investigation conducted in our laboratory could not reproduce this finding and shed entirely new light on PKCθ function during pro-inflammatory Th17 immune response [33]. PKCθ-deficient Th17 cells were found to be characterized by enhanced IFNγ production *in vivo* and *in vitro*, allowing immune cells to be generated that produce both IL-17 and IFNγ. As a result, PKC$\theta^{-/-}$ mice were not protected from EAE. In fact, the phase of severe disease symptoms was even prolonged by PKCθ deficiency. The molecular phenotype of PKC$\theta^{-/-}$ Th17 cells was linked to the up-regulation of the STAT4/IFNγ/STAT1/T-bet signalling axis, due to the impaired suppression of *Stat4* transcription in the early effector cell differentiation phase [33]. The new concept of the role of PKCθ in regulating Th17 phenotype stability is schematically depicted in Figure 1.

Apparent discrepancies between the study by Wachowicz et al. [33] and the previously published results [30–32] are likely to be due to the different knockout strategies used to generate the two PKCθ knockout mice model strains [7,8]. A systematic comparison of the phenotypic features of these PKCθ-knockout lines is given in Table 1. Additionally, the different genetic background of animals, namely C57B/6 in the previous [30–32] and 129/Sv in the recent [33] research work, might explain the divergent outcomes of Th17 experiments [34]. A pivotal importance of genetic environment for manifestation of PKCθ-knockout phenotype has also been observed previously in experiments with C57B/6 compared with BALBc mice [17]. These examples suggest that data generated under laboratory conditions with single in-breed mice strains might be potentially misleading. Given the genetic diversity within human populations, this issue is particularly relevant in any future design of small-molecule inhibitors of PKCθ for clinical application.

New aspects of PKCθ functions: implication for therapeutic approaches

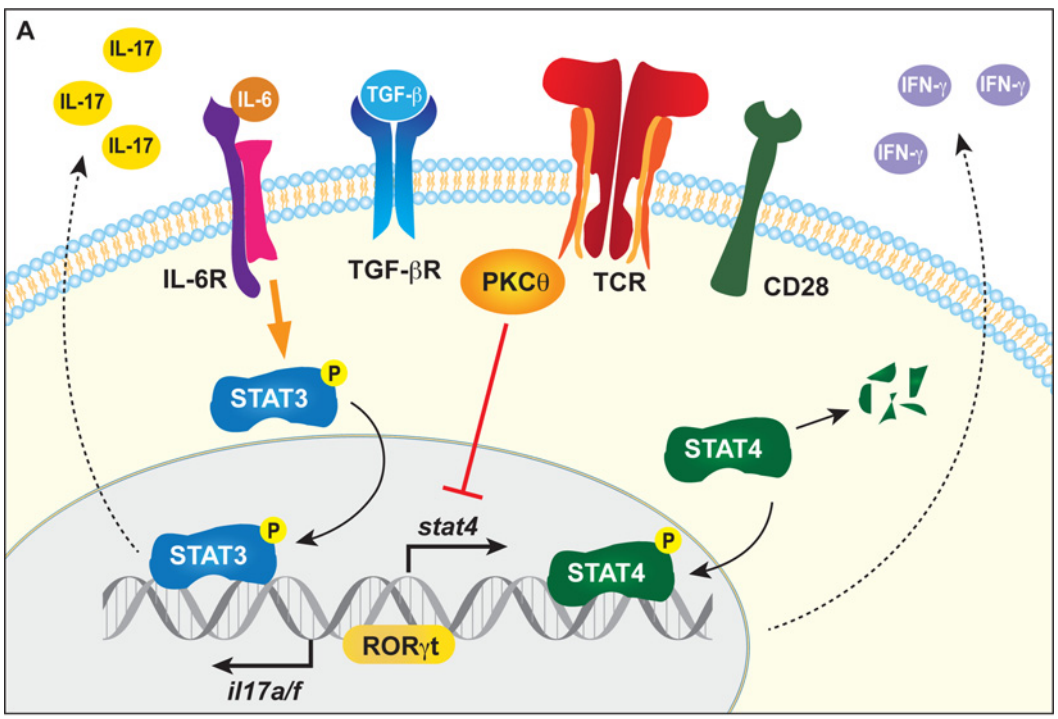
As PKCθ represents a well-validated positive signalling intermediate of the adaptive immune response that acts in a subset-specific manner, PKCθ small-molecule inhibitors are considered as potentially useful immunosuppressive agents in inflammation and autoimmunity [35]. However, in light of the most recent discoveries summarized above, the pros and cons of clinical application of PKCθ inhibitors must be weighed carefully [27,33].

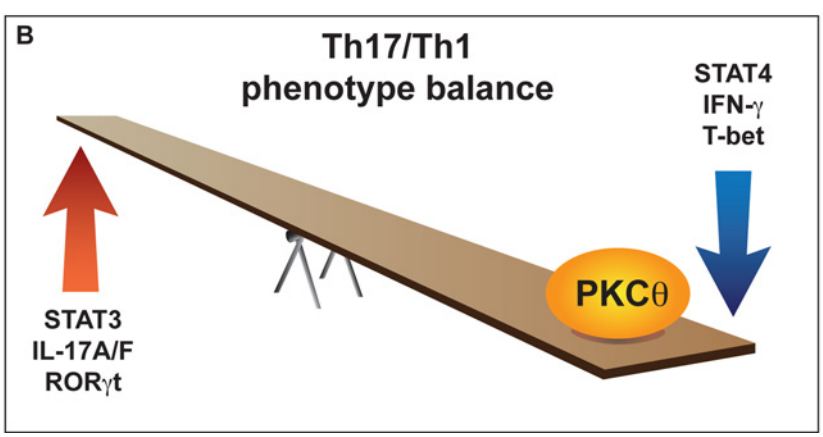
First, EAE is a good example of an autoimmune condition regulated differently by Th17 and Th1 cytokines [36]. The role of IFNγ in the regulation of inflammatory responses is especially complex, as this cytokine exerts both host protective and detrimental effects. In general, IFNγ usually alleviates acute Th17 inflammation, while mediating a chronic inflammatory state [37,38]. Th17 cells are characterized by highly plastic effector phenotype and, after re-differentiation, can serve as a main IFNγ source at an infection site [39,40]. Identification of PKCθ as an important regulator of Th17 phenotype stability and Th17/Th1 balance is crucial for the understanding of its role in autoimmunity and inflammation. An underlying molecular mechanism might be related to the strength of TCR stimulation, which is known to determine a T-cell differentiation outcome [41]. Once an effective activation threshold of T-cell stimulation has been reached, PKCθ may determine both the quality of the signal and direction of T helper differentiation. In fact, decreased strength of CD28 co-stimulation, a characteristic feature of PKCθ-deficient CD4$^+$ T-cells, was shown to promote Th17 effector fate [42,43]. The strength of TCR stimulation also governs cell fate decisions in Th1/Th2 dichotomy [15,44].

Another level of complexity that has to be taken into consideration when interpreting pharmacological experiments with PKCθ inhibitors is that these compounds

Figure 1 | **Th17/Th1 CD4$^+$ T-cell effector differentiation and plasticity regulation model**

(**A**) Under pro-inflammatory conditions, PKCθ is dispensable for activation of STAT3 and subsequent transcription of the Th17 marker genes *Rorγt* and *Il17a/f*. However, PKCθ critically stabilizes the Th17 phenotype by transcriptional suppression of the *Stat4* gene promoter. STAT4 protein undergoes a constant turnover, and concomitant stimulation by IL-6 and transforming growth factor β (TGF-β) initiates *Stat4* transcriptional suppression in a PKCθ-dependent manner, ultimately terminating IFNγ production in Th17 cells. (**B**) Thus (genetic) inhibition of PKCθ leads to enhanced STAT4 and STAT1 activities, elevated expression of T-bet and continued and profound IFNγ production by these 'would-be Th17 cells'.





might also block other PKC isoenzymes. For example, the most commonly used low-molecular-mass PKC inhibitor AEB071/sotrastaurin targets both novel (PKCθ) and classical (PKCα) PKC isoenzymes [35]. This fact is of particular importance in the light of the recent finding that PKCα is a signalling intermediate specific to the Th17 cell subset and PKCα-deficient mice are resistant to induction of EAE [45]. Therefore caution must be exercised when interpreting pharmacological experiments with 'PKCθ inhibitors' that may in fact block more than one PKC isoenzyme.

Open questions

Finally, in order to understand the general impact of PKCθ on adaptive immune response, contradictory results concerning the role of PKCθ in regulatory T-lymphocytes (T_{reg}) need

Table 1 | Direct comparison of the two PKCθ knockout mouse strains

Bold text indicates phenotypic differences between the two mouse strains. AP-1, activator protein 1.

	Sun et al. [7]	Pfeifhofer et al. [8]
Targeting strategy:	Exon 11 replaced by PKG-neo	Cre/loxP null allele (exon 3/4 deletion)
Residual open reading frame	365 aa	9 aa
Molecular and phenotypic characterization of peripheral T-cells ([7] and [8])		
Proliferation after CD3/CD28 stimulation	Impaired	Impaired
Proliferation after PMA/ionomycin stimulation	Impaired	**Normal**
IL-2 production after CD3/CD28 stimulation	Impaired	Impaired
IL-2 production after PMA/ionomycin stimulation	Impaired	**Normal**
NF-κB CD3/CD28 transactivation	Impaired	**Partially impaired**
AP-1 CD3/CD28 transactivation	Impaired	**Partially impaired**
NFAT CD3/CD28 transactivation	Normal	**Partially impaired**
Th17 CD4$^+$ phenotype characterization ([32] and [33])		
Genetic mouse background	C57B/6	129/Sv
Production of IL17	Impaired	**Normal**
STAT3 expression level	Impaired	**Normal**
IL-6 induced STAT3 phosphorylation	Impaired	**Normal**
IFNγ production in Th17 cells	N.D.	**Strongly enhanced**
STAT4 activation in Th17 cells	N.D.	**Strongly enhanced**
IFNγ production in Th1 cells	Normal	Normal
STAT4 activation in Th1 cells	Normal	Normal

to be resolved. Based on experiments with PKCθ knockout mice, Gupta et al. [46] suggested that PKCθ influences thymic development but not the functionality of mature T_{reg} cells. In utter contrast, an inhibitory role of PKCθ in differentiation of iT_{reg}s was proposed [47]. Yet another group reported normal T_{reg} differentiation *ex vivo*, but increased suppressive potential of PKCθ-inhibited T_{reg} cells [48]. It should be noted that the data presented in the latter paper were generated by partial siRNA knockdown and pharmacological inhibition of PKCθ, and not by employing a PKCθ germline knockout strategy. Finally, PKCθ is expressed not only in T-lymphocytes but also in other cells of the immune system [49,50], so that inhibition of this enzyme in the whole organism might influence immune response in manifold ways. Therefore these issues have to be carefully revisited. Our ongoing investigations with both CD4$^+$ T-cell and Foxp3$^+$ T_{reg} lineage-specific conditional PKCθ knockout mouse strains can be expected to help resolve the apparent discrepancies.

Conclusion

The immediate and membrane-proximal signalling function of PKCθ that regulates the strength of the TCR and CD28 co-stimulatory signals has been known for years, but the recent discovery of additional functions of PKCθ can be expected to shed more light on its complex role in T-cell biology. For a fuller understanding of the role of PKCθ in immune responses, studies elucidating the molecular mode of its action and validation in human T-cells are needed.

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