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 of cells 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.

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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 Ca2+ and diacylglycerol (DAG); novel PKCs, including PKC-δ, θ, η, and ε, whose activation is dependent on DAG but is independent of Ca2+; and the atypical PKCs, PKC-τ and λ, whose activation occurs independently of both Ca2+ 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 (Bauer et al., 1994; Altman et al., 2000; Bauer et al., 2000).

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 (Moskova 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
PKC-θ as a drug target

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PKC-θ Signaling: A Comprehensive Overview

PKC-θ, a member of the protein kinase C (PKC) family, is widely expressed in various cell types, including T cells, where it plays a crucial role in T cell activation and effector function. PKC-θ signaling is initiated by the activation of TCR in the presence of costimulatory signals. This process leads to the translocation of PKC-θ to the T cell membrane, where it phosphorylates several downstream targets, including AP-1, which is a key transcription factor in T cell activation.

The importance of PKC-θ in T cell activation was first demonstrated by the studies showing that PKC-θ-deficient T cells have impaired activation and proliferation in response to antigen stimulation. This was confirmed by the observation that PKC-θ regulates the expression of several key molecules, such as NFAT, which is essential for the transcription of cytokine genes.

Furthermore, PKC-θ has been shown to be involved in the regulation of T cell proliferation and survival. PKC-θ participates in the feedback inhibition of TCR activation, which is critical for preventing T cell hyperactivation and, consequently, the development of autoimmune diseases.

In conclusion, PKC-θ plays a pivotal role in T cell activation, proliferation, and survival. Its role in T cell function makes it an attractive target for the development of immunomodulatory drugs.
We and others have shown that PKC-θ protein p73. Since NF-κB (Noel et al., 1996; Radvanyi et al., 1996; Van Parijs et al., 1996). The TCR delivers signals that are required not only for stimulating proliferation but also for enhancing survival (Weiss and Littman, 1994; Rose 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 acute 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-xL that intrinsically increases resistance to apoptosis (Bad and thereby inactivating its function (Villalba et al., 2001). 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 IκB 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-deficient 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., 2008), 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 response in PKC-θ−/− mice, it is clear that PKC-θ−/− T cells are 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 are responsible for impaired Th17 differentiation and that Stat3 lies downstream of PKC-θ. Constructively 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 IκB pathways. 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PKC-θ inhibits the differentiation and enhances suppressive function of Treg.

Naive 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 naive 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 iTregs (Curotto de Lafaille and Lafaille, 2009). In contrast to iTregs, natural Tregs (nTregs) are not induced but develop in the thymus. The fact that naive 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 FoxO3α 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 naive 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 iTreg differentiation, a recent study also demonstrated a role for PKC-θ in the regulation of the effecter 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., 1995; Singh et al., 2001), indicating a therapeutic potential for PKC-θ as a drug target 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; Sälek-Ardakani et al., 2004), supporting a requirement for PKC-θ in Th2 type autoimmunity. In contrast, PKC-θ−/− mice played a less critical role in the development of a similar lung inflammatory response mediated by Th1 cells (Sälek-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 (Sälek-Ardakani et al., 2005; Tan et al., 2008), 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

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- Mosyak et al., 2007
- Zanin-Zhorov et al., 2010
- Marsland and Kopf, 2008
- Sälek-Ardakani et al., 2004
- Healy et al., 2006
- Tan et al., 2008
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, which inflammatory cytokines and these cytokines are likely to 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., 2001). Second, autoimmune diseases usually involve more than one type of T helper cell. For example, both Th1 and Th17 responses are likely to 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)

### PKC-θ is a Drug Target for Prevention of 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 NK T cells produce large amounts of cytokines that cause strong inflammation and toxicity to 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 NK T cell development and activation. A dose of ConA (25 mg/kg) 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 INFγ, IL-6, and TNFα, which mediate the inflammatory response 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 PKC-θ in NKT cells is required. 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 the 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; Kasahara et al., 1999; Kusunoki et al., 2008; Kato et al., 2008; 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 allosponses 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 (Manicasamy 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 (Manicasamy 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-xL in PKC-θ−/− T cells was sufficient to restore cardiac allograft rejection (Manicasamy 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 tolerated 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 lifelong 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 FCR-θ−/− mice. FCR-θ−/− mice displayed delayed, but successful cardiac allograft rejection, suggesting there was some compensation for the missing FCR-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 FCR-θ−/− mice. FCR-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 FCR-θ allows long-term survival of cardiac allografts (Manicasamy et al., 2008; Wang et al., 2009).

In addition to heart rejection, the role of FCR-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. Allogeneic 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 GVLR 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, FCR-θ−/− mice have the ability to respond to infection by the listeria bacteria and MCMV virus (Valenzuela et al., 2009). Moreover, FCR-θ−/− mice survived the BMT procedure and did not develop GVHD, whereas majority of WT mice died from GVHD. More importantly, FCR-θ−/− mice retained their ability to induce rejection of tumors. This study demonstrated that FCR-θ inhibitor-based immunosuppressive regimens are able to prevent GVHD but also preserve the protective immune response against infections and tumors.

CONCLUSION

FCR-θ controls fundamental functions of T cells including activation, differentiation, and survival via NFκB, AP-1, and NFAT pathways. FCR-θ also regulates T cell-mediated immune responses 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 FCR-θ inhibitors including how the inhibition of FCR-θ affects the function of other tissues in vivo. It is encouraging to report that many pharmaceutical companies have developed selective FCR-θ inhibitors, and therefore many FCR-θ-regulated functions can be evaluated using these inhibitors instead of FCRθ−/− mice, which have potential developmental caveats. With the availability of FCR-θ inhibitors, it is now possible to test the efficacy in mouse models of human autoimmune diseases including EAE and arthritis, which are likely to lead to clinical trials of FCR-θ-based treatments for human diseases.

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