



# Signal Transduction in Immune Cells and Protein Kinases

# 5

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## Abstract

Immune response relies upon several intracellular signaling events. Among the protein kinases involved in these pathways, members of the protein kinase C (PKC) family are prominent molecules because they have the capacity to acutely and reversibly modulate effector protein functions, controlling both spatial distribution and dynamic properties of the signals. Different PKC isoforms are involved in distinct signaling pathways, with selective functions in a cell-specific manner.

In innate system, Toll-like receptor signaling is the main molecular event triggering effector functions. Various isoforms of PKC can be

common to different TLRs, while some of them are specific for a certain type of TLR. Protein kinases involvement in innate immune cells are presented within the chapter emphasizing their coordination in many aspects of immune cell function and, as important players in immune regulation.

In adaptive immunity T-cell receptor and B-cell receptor signaling are the main intracellular pathways involved in seminal immune specific cellular events. Activation through TCR and BCR can have common intracellular pathways while others can be specific for the type of receptor involved or for the specific function triggered. Various PKC isoforms involvement in TCR and BCR Intracellular signaling will be presented as positive and negative regulators of the immune response events triggered in adaptive immunity.

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## 1 Introduction

Cells of the immune system whether appending to the innate or acquired arm undergo complex processes starting from their development

through their maturation and effective action displayed on the aggressor that triggered an immune response (Neagu 2012). Immune cellular responses activated by external and/or internal cues are governed by complex networks of signal transduction pathways. These intracellular pathways have as crucial regulators protein kinases.

Isoforms of Protein kinase C (PKC) span the entire panel of immune cells and are involved in seminal immune processes. PKC families are enzymes involved in signaling pathways. Their action is to specifically phosphorylate substrates at serine/threonine aminoacids. Through this action PKCs regulate important cellular events like cell proliferation, differentiation, immune response and so on. PKCs consist of 10 kinase members. They have a highly catalytic kinase domain that is structurally conserved and a regulatory domain with a less conserved structure (Lim et al. 2015).

In T lymphocyte regulation, there are different PKC isoforms with various physiological roles and non-redundant functions. Just to enumerate some of the PKCs, PKC $\theta$  and PKC $\alpha$  isoforms are involved in antigen-induced T cell activation, inhibition of T cell-mediated responses and in important pathological processes like allograft rejection and autoimmunity. Regulation of T cell proliferation and cell cycle progression have as key molecules distinct PKC isoforms that play positive roles during cell cycle progression, but there are also isoforms like PKC $\delta$  which serves as regulators.

B cells are activated after antigen binding to B cell receptor (BCR) that needs for intracellular signaling CD79 molecules. In B cells pathways involving PKC $\beta$  are triggered. One common intracellular pathway with TCR signaling is the CARD11–BCL10–MALT1 (CBM) signalosome complex activating in the end the nuclear NF $\kappa$ B.

Innate immune system detects pathogens through Toll-like receptors (TLRs), receptors that recognize specific molecular patterns expressed by the aggressors. Different TLRs would lead to various gene expressions, and would recognize different components of microorganisms. In the intracellular signaling events triggered upon TLR

activation various common or less common PKC isoforms are involved (Akira and Takeda 2004).

## 1.1 PKCs Traits

In the signal transduction pathway the extracellular signal that is considered the first messenger will be converted in another intracellular signal that will be the second messenger and this conversion is done at the plasma membrane. Further molecular conversions in the intracellular signaling events will take place in the cytoplasm, at organelle membranes like the nuclear envelope, and finally inside the nucleus. The main protein kinase (PK) in the immune system signaling is PKC because it can be activated by G proteins that are coupled to receptors and tyrosine kinase receptors, so that upon activation it can generate an array of intracellular signals. Activation of various isoforms of phospholipase C (PLC) generates diacylglycerol (DAG) and inositol phosphate 3 (IP3), both of them activating PKC (Nishizuka 1995). IP3 will induce the release of Ca<sup>2+</sup> from intracellular stores that will activate certain PKC sub-families. The “immunological synapse” developed between specific T cell receptors (TCR) and the antigen presenting cell (APC) resides in the activation of PKC triggering the intracellular cascade as a response to a stimulus from the plasma membrane to the nucleus. Interestingly PKCs were found as transducer within the nucleus, pathway different from the cytoplasmic signalling event directly regulating gene expression (Lim et al. 2015). In the journey to develop the nucleus-driven intracellular signaling pathway PKC can trigger MAPK (mitogen-activated protein kinase) cascade by activating Raf (Rapidly Accelerated Fibrosarcoma). Also cytoplasmic PKC, when activated will phosphorylate the inhibitor of transcripion factor nuclear factor (NF) $\kappa$ B (I $\kappa$ B). The phosphorylation will induce the proteolytic degradation of the inhibitor so that NF $\kappa$ B will translocate to the nucleus (Baeuerle and Henkel 1994).

Without being exhaustive, this chapter will highlight some aspects regarding PKC isoforms

involved in intra-cytoplasmic signaling events that are involved in the development of the immune response whether triggered by innate or adaptive immune cells.

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## 2 Toll-Like Receptor Signaling in the Innate System

The innate immune system arm has numerous first line defense functions, one of these functions engages the initial detection of microbes through pattern recognition receptors (PRRs). PRRs will recognize the specific microbe molecular pattern, pathogen-associated molecular patterns (PAMPs). PRRs can recognize also structures that result from damaged cells, damage associated molecules patterns (DAMPs). When activated PRRs will activate intracellular signaling pathways that will further induce complex immune responses by activating the synthesis of inflammatory cytokines, e.g. type I interferon (IFN), and an array of several other immune mediators. This initial activation initiates inflammation, and primes further antigen-specific adaptive immune responses. These orchestrated responses has as final scope the clearance of infecting microbes and the instruction of adaptive immune responses that is antigen-specific (Janeway Jr and Medzhitov 2002). Amidst the signaling pathways, the family of PKCs appending to the larger family of protein serine / threonine kinases keeps the central role in intracellular signal transduction.

Discovered more than 15 years ago PKCs comprises isoforms that are divided in 3 subfamilies: PKC- $\alpha$ ,  $\beta$ I, - $\beta$ II, and - $\gamma$ ; PKC- $\delta$ , - $\epsilon$ , - $\eta$ , and - $\theta$ , and the atypical subfamily, PKC $\zeta$  and  $\lambda$ /i. These 3 sub-families were divided as such due to their characteristics, thus the first mentioned sub-family requires calcium, DAG, and phosphatidylserine (PS); the second sub-family requires DAG and PS in a calcium-independent manner and the last sub-family requires only PS (Newton 2003). As PKC with its various isoforms are involved in cell proliferation, differentiation, apoptosis, and motility these enzymes have been discovered as being involved

in various disease from cancer to cardiovascular disease (Churchill et al. 2008; Ali et al. 2009; Palaniyandi et al. 2009; Gonelli et al. 2009; Bynagari-Settipalli et al. 2010). TLRs are actually the family of receptors that will recognize PRRS and DAMPs to trigger an effective innate immune response (Gay and Gangloff 2007; Kawai and Akira 2010). PKCs are involved in several intracellular signaling events induced by TLRs. Molecular events triggered from various TLRs differ, but the process is generally initiated after recruiting TIR (Toll/IL-1 Receptor homology) containing adaptor proteins [e.g. TIRAP (TIR Adaptor Protein) would recruit MyD88 (myeloid differentiation factor 88), TRAM (TRIF-related adaptor molecule) would recruit TRIF (TIR-domain-containing adapter-inducing interferon- $\beta$ )]. Thereafter MyD88 would recruit IRAK 1,2,4 (IL-1R-associated kinase) and TRAF6 (tumor necrosis factor receptor-associated factor-6). This event would induce finally the pro-inflammatory genes activation. TLR binding to TRIF would recruit TRAF6,  $\beta$ RIP1 (Receptor-interacting serine/threonine-protein kinase 1), and TAK1 (TGF- $\beta$ -activated kinase) and finally induce interferon- $\beta$  (IFN) genes. The activation of TRIF pathway induces the pro-inflammatory cytokines secretion but to a lesser extent than MyD88 pathway. The involvement of various PKC isoforms in TLR signaling was proven when altering PKC enzyme activity within innate immune cells, the process of cytokine secretion was deregulated. Moreover, various TLR ligands activate different PKC isoforms in all innate immune cells, e.g. monocytes, macrophages, dendritic cells and neutrophils (Fronhofer et al. 2006; Zhou et al. 2006; Asehnoune et al. 2005). These PKC isoforms are expressed different in monocytes and macrophages, being involved also in monocyte differentiation. PKC $\alpha$  and  $\beta$  are activated during stimulation and monocyte/macrophage-induced differentiation while PKC $\delta$  expression decreases. Parihar et al. have shown that PKC $\delta$  links and phosphorylates caspase-3, inducing an increase in the apoptotic activity (Parihar et al. 2010). In neutrophils PKC $\delta$  activation downstream of dectin-1 and Mac-1 is

involved in the antifungal action but not the function of macrophages (Li et al. 2016). In dendritic cells (DC) PKC- $\beta$  expression and signaling is important in inducing DC differentiation (Cejas et al. 2005). It was also reported that as DCs are involved in induction of chronic inflammatory diseases (Joffre et al. 2012) PKCs regulate caspase-3-dependent apoptosis (Alcain et al. 2017).

In the signaling pathways of TLRs there are common molecules, but also specific ones. Thus MyD88-dependent intracellular signaling event is used by all TLRs except for TLR3. MyD88-dependent intracellular pathways activates MAPKK (mitogen-activated protein kinase kinase) and IKK (I $\kappa$ B kinase) complex inducing the nuclear translocation of AP-1 (Activator Protein 1) and NF- $\kappa$ B (nuclear factor kappa-light-chain-enhancer of activated B-cells). TRIF is the principal adaptor protein in the MyD88-independent events and can assembly with TRAF6 to activate AP-1 and NF- $\kappa$ B (Patel et al. 2012).

An overview of the major intracellular events where PKCs are involved in TLRs signaling is presented in Fig. 5.1 and some details regarding the involvement of PKC $\alpha$ ,  $\delta$  and  $\epsilon$  in the TLR signaling events are further presented.

## 2.1 PKC Isoforms Involved in TLR Signaling

### 2.1.1 PKC- $\alpha$

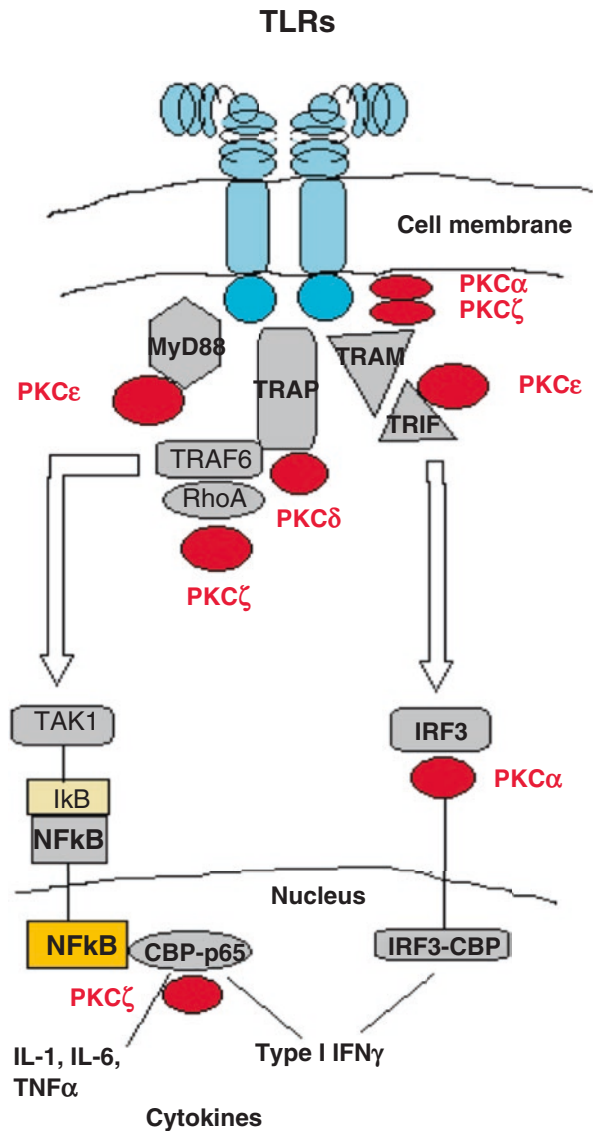
This is one of the conventional isoforms first discovered by using inhibitors that were inhibiting cytokine secretion in macrophages stimulated via TLR (Loegering and Lennartz 2011; Foeyand and Brennan 2004; Catley et al. 2004). Several other studies have shown the link between PKC- $\alpha$  and TLR-related functions. Thus, earlier studies have shown that 264.7 RAW macrophages with low expression of PKC- $\alpha$  have also TNF- $\alpha$ , IL-1 $\beta$ , iNOS, and NF-IL6 (CAAT/enhancer-binding protein  $\beta$ ) low secretion upon LPS-activation (Chano and Descoteaux 2002). Moreover the lack of PKC- $\alpha$  is associated with deregulated phagocytosis, low killing capacity of pathogens, all these functions being non-TLR-related innate

immunity responses (Ng Yan Hing et al. 2004; Holm et al. 2003). PPAR $\gamma$  regulates phorbol ester-induced NF- $\kappa$ B activation and TNF- $\alpha$  synthesis/secretion by hindering the activation of PKC- $\alpha$  (von Knethen et al. 2007). More recent studies, have shown in human DCs that PKC- $\alpha$  inhibition blocks also the IL-12p40 secretion that was induced by the activation of TLR2/6, TLR 2/1, TLR5, but not through TLR3 (Langlet et al. 2010). DCs from mice with the phenotype PKC- $\alpha$ -/- need PKC- $\alpha$  activation for TLR2/1-activation of MAPK, NF- $\kappa$ B and AP-1; activation that would lead to the secretion of TNF $\alpha$ , IL-6 and IL-10 (Langlet et al. 2010). In an earlier study, Johnson et al. found that a TLR3 ligand can activate PKC- $\alpha$ . Hence, silencing PKC- $\alpha$  with siRNA, TLR3-stimulated IFN- $\beta$  production was blocked in DCs. After silencing the PKC $\alpha$  no hindering of IRF3 activation was recorded but the IRF-3 transcriptional activity was inhibited when induced by TRIF and TBK1 (TANK-binding kinase 1) over-expression. Probably this effect is the consequence of the decreased IRF-3 binding to CBP that needs PKC- $\alpha$  activation (Johnson et al. 2007).

### 2.1.2 PKC- $\delta$

In innate immune cells, TLR-related cytokine secretion involves the activation of PKC- $\delta$ ; this assertion was sustained by the fact that its inhibition decreases the activation of NF- $\kappa$ B, reduces the inflammatory cytokines secretion and the production of nitric oxide (Bhatt et al. 2010; Ikewaki et al. 2007). PKC- $\delta$  interacts with TIRAP via the TIR domain during TLR signaling process (Kubo-Murai et al. 2007). If PKC- $\delta$  is depleted, the kinase activity in the complexed TIRAP is lost proving that PKC- $\delta$  is the major kinase within TIRAP complex. In cecal ligation and puncture (CLP) animal model for sepsis, TLR signaling was studied in vivo. A PKC- $\delta$ -inhibiting peptide was administered intra-tracheally in this model and the administration reduced the lung injury blocking sepsis-induced phosphorylation of this PKC- $\delta$  isoform. In this experimental model reduced levels of various chemokines were obtained, reduced inflammatory cells infiltration in the lungs and lower pul-

**Fig. 5.1** TLR signaling main pathways. PKCs various isoforms can be found at various levels and depending to various TLR types. TLR2 after activation would associate with PKC $\alpha$ . PKC $\alpha$  is also involved in the TLR3 signaling, namely binding to the IRF3 – CBP complex (Interferon Regulatory Factor – CREB Binding Protein) and hence inducing the activation of IFN- $\beta$  gene. PKC $\epsilon$  is needed for TLR2/4 activation, as involved in TRAM phosphorylation. Activation of NF $\kappa$ B needs PKC- $\delta$ . PKC- $\zeta$  is also needed for TLR2/4 activation when it associates to TRAF6 and RhoA (Ras homolog gene family, member A) for transcription of p65 (nuclear factor NF-kappa-B p65 subunit). While both nuclear activation pathways induce Type I IFN $\gamma$ , only CBP-p65 pathway induces IL-1, IL-6, TNF $\alpha$



monary edema. All these tissue effects were attributed to macrophages, endothelial cells, and epithelial cells (Kilpatrick et al. 2011).

In activated macrophages, PKC- $\delta$  is positioned downstream of Sphingosine kinase 1 (SphK1) activation being required for NF- $\kappa$ B activation. In vivo inhibiting SphK1 reduced sepsis-stimulated cytokine secretion and mortality in animal models. This isoform is a link in the activation chain TLR4-SphK1-PKC- $\delta$  -NF- $\kappa$ B-cytokine release (Snider et al. 2010).

### 2.1.3 PKC- $\epsilon$

The first study showing PKC $\epsilon$  involvement in innate immunity-related events was reported almost 20 years ago by Castrillo et al. when PKC- $\epsilon$ -/- mice had spontaneous infections of the uterus and decreased survival rate upon experimental infection (Castrillo et al. 2001). Further studies that have used PKC- $\epsilon$  specific inhibitors, have shown in the same PKC- $\epsilon$ -/- mouse model that PKC- $\epsilon$  is seminal for LPS-stimulated TNF- $\alpha$  and IL-12 secretion performed by DC and macrophages (Comalada et al. 2003;

Koyanagi et al. 2007). This experiment reinforced the involvement of PKC- $\epsilon$  in inflammation and host defense (Aksoy et al. 2004). Actually PKC- $\epsilon$  is phosphorylated by all TLRs that need MyD88 (e.g. TLR1 – 9 except TLR3 in macrophages) (Faisal et al. 2008). If PKC- $\epsilon$  can be phosphorylated also by PKC- $\alpha$ , the effects of PKC- $\alpha$  on cytokine secretion can be shared by PKC- $\epsilon$  (Saurin et al. 2008; Durgan et al. 2008). Moreover, PKC- $\epsilon$  is involved in TLR4 activation but via TRAM pathway that induces IFN- $\beta$  and RANTES (Regulated on Activation Normal T Cell Expressed and Secreted). Recombinant PKC- $\epsilon$ , phosphorylates TRAM, but this phosphorylation does not appear in PKC- $\epsilon$ -/- cells with reduced production of IFN- $\beta$ . Thus, actually PKC- $\epsilon$  is a link in the flow of TLR4- PKC- $\epsilon$  – TRAM- IFN- $\beta$  pathway (McGettrick et al. 2006).

#### 2.1.4 PKC- $\zeta$

As a more atypical isoform, component of IL-1R and TNFR intracellular signaling pathways PKC- $\zeta$  was reported more than 15 years ago (Hirai and Chida 2003; Duran et al. 2003). PKC- $\zeta$  was shown to be involved in TLR, IRAK, RhoA, and NF- $\kappa$ B activation (Yang et al. 2007; Huang et al. 2009). Using PKC- $\zeta$  specific inhibitors, ERK activation and TNF- $\alpha$  secretion was blocked upon *Mycobacterium tuberculosis* (MTB) stimulation (Yang et al. 2007). PKC- $\zeta$  was found associated with TLR2 but not TLR4. Thus, in THP-1 cells (a cell line from an acute monocytic leukemia) PKC- $\zeta$  does not associate with TLR2. IRAK phosphorylation is a molecular event in the flow of TLR signaling and if it is degraded is a negative control of the signaling pathway. Using a set of protein kinase inhibitors, it was suggested that IRAK is actually a PKC- $\zeta$  substrate. Several studies have highlighted a role for RhoA in TLR signaling (Chen et al. 2009; Shibolet et al. 2007; Manukyan et al. 2009; Lin et al. 2010). In monocytes it was found that the activation of p65 through TLR2 ligands needs RhoA and PKC- $\zeta$  activation. PKC- $\zeta$  transiently links to RhoA that is activated and such, PKC $\zeta$  aids the observed effect of RhoA on gene transcription. Huang et al. have shown that

PKC- $\zeta$  is associated to RhoA in LPS-stimulated macrophages and by inhibiting RhoA or TRAF6, the PKC- $\zeta$  activation was blocked. Analogous experiments showed that inhibition of PKC- $\zeta$  blocked TAK1 phosphorylation (Huang et al. 2009). Thus actually PKC- $\zeta$  is a link in the flow of TLR2/4-RhoA/TRAF6- PKC $\zeta$  – TAK1 – p65 – cytokine induction.

Various forms of PKCs are involved in the large family of TLRs activation mediating important effector functions in innate immune cells.

### 3 PKCs in T-Cell Receptor Signaling

In contrast to innate immunity, adaptive immunity discriminates minute differences between aggressors. The immune system encounters during its life span a myriad of antigens and hence can specifically raise an immune response towards all the encountered pathogens and antigens. Adaptive immunity would develop a slow but long-lasting specific immune response. T cells and B cells receptors are designed to recognize all potential antigens.

The first stage of recognition in both cell types is the specific linkage with the specific domains in the antigen followed by activation of a single specific cell type. This recognition would trigger cells to proliferate (clonal expansion). Recognition of specific domains on the antigen is done by a specific TCR structurally different from that of BCR. Intracellular signaling triggered by the activation of TCR has both common and different pathways with BCR as further described (Acuto and Cantrell 2000).

#### 3.1 PKCs Families

TCR as being the complex molecular structure delivering the antigen specific recognition is a heterodimer of either  $\alpha\beta$  or  $\gamma\delta$  polypeptides that contain variable regions, responsible for the binding to the complex of antigen peptide-major histocompatibility complex (MHC). In order to

efficiently deliver signals upon activation these chains associate with signal transducing components, CD3 complex, process needed for both cell expression and function accomplishment. The CD3 complex which has 3 parts,  $\zeta$  part being intimately involved in the intracellular signaling pathway (Favero and Lafont 1998). TCR will recognize the antigen presented by the antigen-presenting cells (APCs) and this “immunological synapse” will lead to T-cell differentiation and activation; a multifactorial process including PKC signaling. After TCR-MHC complex formation, PKC localizes to the complex and generates the activation of nuclear transcription factors for further induction of immune effector genes (Isakov and Altman 2002). Activated genes will generate both soluble molecules, like cytokines, chemokines, growth factors and transient cell surface molecules to induce T-cell proliferation and differentiation (Smith-Garvin et al. 2009; Fooksman et al. 2010; Yokosuka and Saito 2010).

There are several PKC isoforms that are involved in the TCR intracellular signaling events.

### 3.1.1 PKC $\theta$

The most predominant isoform used by T cell activation is PKC $\theta$ , while in different T cell subsets other forms can be identified as well (Kong and Altman 2013). This PKC $\theta$  isoform is expressed in T cells within the haematopoietic cell population as shown in the early 90s (Baier et al. 1993). Further studies have shown that after activation, PKC $\theta$  translocates to the plasma membrane associating with the co-stimulatory molecule CD28 through its V3 domain (Kong et al. 2011; Yokosuka et al. 2008). In CD28-deficient mice it was shown that PKC $\theta$  needs the presence of CD28 in activated T cells to develop a mature immunological synapse. PKC $\theta$  is regulated also by the intracellular redox state, and in 2013 the first published study has shown in naive T cells that the oxidized inactive PKC $\theta$  is recruited to the cell membrane and that this process of PKC- $\theta$  activation is redox dependent and needs *de novo* synthesis of glutathione (von Essen et al. 2013). Afterwards, the dependence of

PKC to the oxidative state was proven in muscular disorders as well (Dobrowolny et al. 2018).

In PKC $\theta$ -knockout mice, PKC $\theta$  involvement in T-cell signaling was reported more than 15 years ago (Pfeifhofer et al. 2003) and studies that related its dis-functionality in autoimmune diseases followed (Anderson et al. 2006). In mouse experimental models of autoimmune encephalomyelitis it was shown that in PKC $\theta$  deficient mice type Th1 responses are deregulated. Th17 involvement in developing this experimental autoimmune encephalomyelitis needs PKC $\theta$  because it controls Th17 differentiation (Salek-Ardakani et al. 2005; Tan et al. 2006).

PKC $\theta$  controls signal transducer and activator of transcription 3 (STAT3) after Th17 activation by association of activator protein 1 and NF- $\kappa$ B transcription factors to the STAT3 promoter (Kwon et al. 2012). PKC  $\theta$  was reported to suppress Stat4, Tbet (T-box transcription factor) and IFN $\gamma$  upon Th17 activation (Wachowicz et al. 2014).

In immune memory control PKC $\theta$  is also involved. Thus in the antiviral responses performed by CD8+ T-cell, PKC $\theta$  is needed for the antigen immune recall upon re-infection with lymphocytic choriomeningitis virus and influenza virus (Marsland and Kopf 2008) and for developing memory T-cell *per se* (Teixeiro et al. 2009).

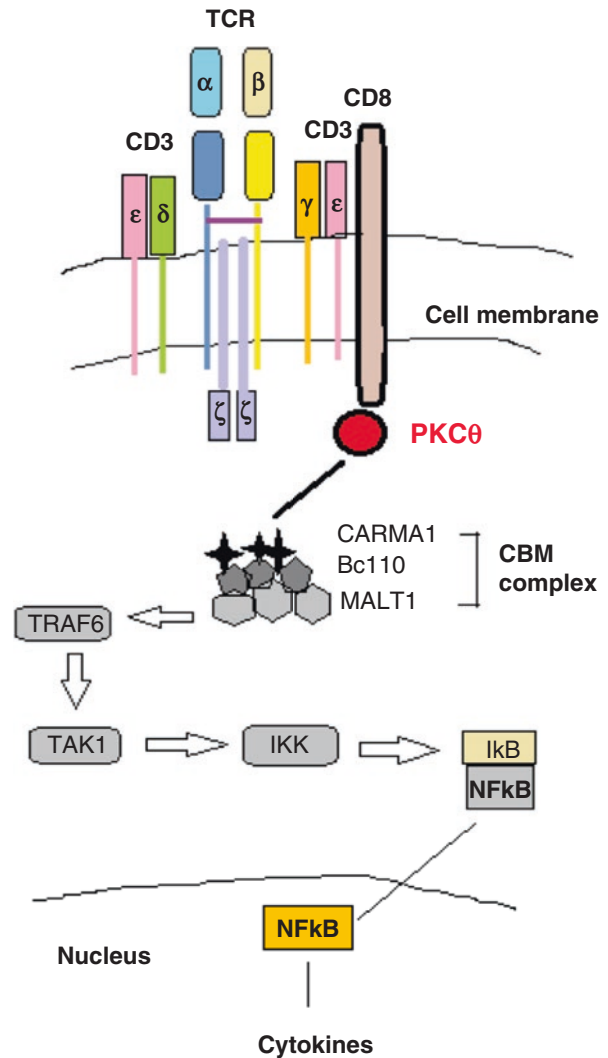
In regulatory T cells (Treg) PKC $\theta$  is localized apart from the immunological synapse, process that leads to a negative regulation of Tregs (Gupta et al. 2008; Zanin-Zhorov et al. 2010). The effect of negative regulation performed by PKC $\theta$  is an inhibited differentiation of Tregs via the AKT/Foxo1/3a pathway (Ma et al. 2012).

PKC $\theta$  has an important role in T-cell immune response regulation and an outline of the main steps in TCR signaling involving PKC $\theta$  is depicted in Fig. 5.2.

### 3.1.2 PKC- $\alpha$

DAG messengers are involved in TCR signaling, but more recently it was demonstrated that it can regulate the duration and amplitude of Ras/ERK signals. Thus, it was shown that PKC $\alpha$  is transiently translocating, process mediated by DAG generation in the contact area of the immune

**Fig. 5.2** TCR signaling pathway involving PKC $\theta$ . Upon TCR stimulation, activation of co-stimulatory CD8 induces the translocation of PKC $\theta$ ; activation induces further the aggregation and phosphorylation of caspase-recruitment domain containing membrane-associated guanylate kinase protein-1 (CARMA1). Aggregated CARMA1 will complex with B cell lymphoma 10 (Bcl-10) and mucosal-associated lymphoid-tissue lymphoma-translocation gene 1 (Malt1). Malt1 in the CBM complex will induce the degradation of the IKK regulatory component, I $\kappa$ B, via activation of TRAF6 and transforming TAK1, the process leading to the activation and nuclear translocation of NF- $\kappa$ B



synapse. It was demonstrated that Diacylglycerol kinase (DGK) $\zeta$  can negatively regulate PKC $\alpha$  translocation kinetics, while PKC $\alpha$  is self-limiting in the immune synapse. These two pillars DGK $\zeta$  and PKC $\alpha$  would regulate the amplitude and duration of Ras/ERK activation after antigen recognition. In animal models characterized by deficient DGK $\zeta$  it was demonstrated that increased DAG signaling induces enhanced PKC $\alpha$ -dependent L-selectin shedding. Thus Gharbi et al. depicted that the early activation of DAG-PKC $\alpha$  axis induces TCR biological responses (Gharbi et al. 2013).

Markers that can detect pathologies associated to T cells were studied by immunohistochemistry

approaches, PKC isoforms included. Thus GADS (GRB2-related adaptor protein 2), DOK2 (Docking protein), SKAP55 (src family associated phosphoprotein), ITK (Interleukin-2 inducible T-cell kinase) markers were associated with lymphoid and myeloid precursor neoplasms. PKC $\alpha$  was found defective in angioimmunoblastic T-cell lymphoma and aberrantly expressed in classical Hodgkin lymphoma, Burkitt lymphoma and plasma cell myeloma. Probably future studies will develop PKC $\alpha$  as a new therapy target (Agostinelli et al. 2014).

Further work on PKC $\alpha$  involvement in TCR signaling has shown that Ca<sup>2+</sup> elevation is separate from the control of I $\kappa$ B $\alpha$  degradation,



and that the regulatory action via  $\text{Ca}^{2+}$  is dependent on  $\text{PKC}\alpha$ -mediated phosphorylation of p65. Therefore this recent study established a check point controller of  $\text{Ca}^{2+}$  signaling event as being  $\text{PKC}\alpha$  (Liu et al. 2016).

A study published in 2019 by Merida et al. has shown that in T lymphocytes, the interaction between  $\text{DGK}\zeta$  and Sorting Nexin 27 (SNX27) controls  $\text{PKC}\alpha$  activation. When silencing SNX27 in T lymphocytes,  $\text{PKC}\alpha$  activation is enhanced, finding that provides molecular basis for the  $\text{DGK}\zeta$  –  $\text{PKC}\alpha$  deregulations in immune synapse impairment (Mérida et al. 2019).

### 3.1.3 $\text{PKC}\delta$

$\text{PKC}\delta$  isoform was first identified in a mouse model as a  $\text{PKC}$  family member that is required for the control of lytic granule exocytosis by  $\text{CD8}^+$  T cells. Thus  $\text{PKC}\delta$  isoform, although it was shown to regulate this process, was found unessential for activation and cytokine production (Ma et al. 2007). Other studies followed, showing  $\text{PKC}\delta$  involvement in TCR activation. As shown in the previous section, TCR and costimulatory receptors activation have CARMA1 (caspase recruitment domain-containing membrane-associated guanylate kinase 1) as a seminal protein. CARMA1 will recruit B-cell CLL/lymphoma 10 (Bcl10), mucosa-associated lymphoid tissue lymphoma translocation gene 1 (MALT1), and TRAF6 assembling a specific signalosome that would trigger  $\text{NF-}\kappa\text{B}$  activation. In a cellular model with Jurkat T cells,  $\text{PKC}\delta$  was shown to associate with CARMA1 and the over-expression of  $\text{PKC}\delta$  inhibited  $\text{NF-}\kappa\text{B}$  activation through CARMA1. Alternatively, when knocking down  $\text{PKC}\delta$ ,  $\text{NF-}\kappa\text{B}$  activation and IL-2 secretion was noticed. All these results indicated  $\text{PKC}\delta$  as a negative regulator in T cell activation (Liu et al. 2012).

In 2019, it was shown that  $\text{PKC}\delta$  regulates TCR-controlled multivesicular bodies polarization toward the immune synapse and exosome secretion. Moreover at the immune synapse the reorganization of F-actin is inhibited in T lymphocytes were  $\text{PKC}\delta$  is obstructed. Therefore,  $\text{PKC}\delta$  is recently proposed as actin

reorganization regulator in the immune synapse (Herranz et al. 2019).

In TCR signaling there are  $\text{PKCs}$  that up-regulate activation of adaptive immune functions, but also kinases that are negative regulators of this cell type.

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## 4 B-Cell Receptor Signaling

Mature B lymphocytes are a critical component of the adaptive immunity participating to systemic defense by generating highly specific antibodies, regulatory cytokines, chemokines and presenting antigen to T cells to generate an immune response against tumors and pathogens (Harwood and Batista 2008).

B cell activation is initiated upon specific antigen capturing by BCR, this event will trigger various intricate intracellular pathways mediated by Src-family kinases as well as Syk and Bruton's tyrosine kinases (Btk)/Tec that cooperate to uptake the antigen-receptor complex. All this organized assemble of kinases and adaptor proteins form the so called *signalosome* that activates multiple signalling pathways (Packard and Cambier 2013; Lim et al. 2015). Besides antigen capturing, growth and proliferation of B cells are closely regulated through BCR signaling and other membrane receptors that would be activated by different cytokines (Jellusova and Rickert 2016; Taher et al. 2017).

BCR is a heterodimeric transmembrane protein composed of heavy-chain and light chain immunoglobulins,  $\text{CD79A/Ig}\alpha$  and  $\text{CD79B/Ig}\beta$  and located on B cells outer surface (Stephenson and Singh 2017). The engulfed antigen via BCR is processed and presented on B cell surface as specific peptides associated with MHC-II molecules; this process results in the assignation of specific T helper lymphocytes that would provide a second signal for complete B cell activation. Further, activated B cells can differentiate to antibody-secreting plasma cells or enter into germinal centers (GCs) where cells are subjected to BCR affinity maturation and antibody class switch recombination (De Silva and Klein 2015). Subsequently B cells would

leave the GC and differentiate into either high-affinity antibody secreting long-term plasma cells or dormant memory B cells that can reactivate as plasma cells upon re-encountering with the same antigen (Tsui et al. 2018; Kurosaki et al. 2010).

#### 4.1 PKCs Role in BCR Signaling Events

Antigen binding to the BCR and further B cell activation rely on PKC that is translocated from cytosol to the plasma membrane where spatially there are regions comprising the tetraspanin protein CD53 to sustain BCR-dependent PKC stimulation (Zuidscherwoude et al. 2017).

The PKC is a large family of Ser/Thr kinases with pleiotropic expression and controlling many cellular functions like development, differentiation, activation, lymphocytes survival and immune responses development (Tan and Parker 2003; Cooke et al. 2017). From the previously section, the described isoforms of PKC  $\beta$ ,  $\lambda$ , and  $\zeta$  variants prevail in B cells with PKC $\beta$  the most ubiquitously expressed and most important isoform in BCR signaling (Isakov 2018).

The role of PKC $\beta$  in regulating B-cell function was initially revealed through PKC $\beta$  gene knockout mice that exhibited decreased B-cell activation, no proliferation upon BCR activation and altered T-cell-independent immune responses (Leitges et al. 1996).

In establishing the *signalosome*, the isoform  $\beta$  of PKCs mediates phosphorylation of CARMA1 protein leading to a multipart complex comprising CARMA1, Bcl10 and MALT1 linked to membrane lipid rafts (Shinohara et al. 2007). A general outline of the intracellular signaling events triggered by BCR activation regulated by PKC $\beta$  is presented in Fig. 5.3. However, alterations in B cell responses can lead to pathological conditions such as autoimmune diseases when high-affinity autoreactive B lymphocytes develop and produce auto-antibodies leading to tissue damaging.

Outlining biomarkers that designate an abnormal B cell response can assist in elucidating dis-

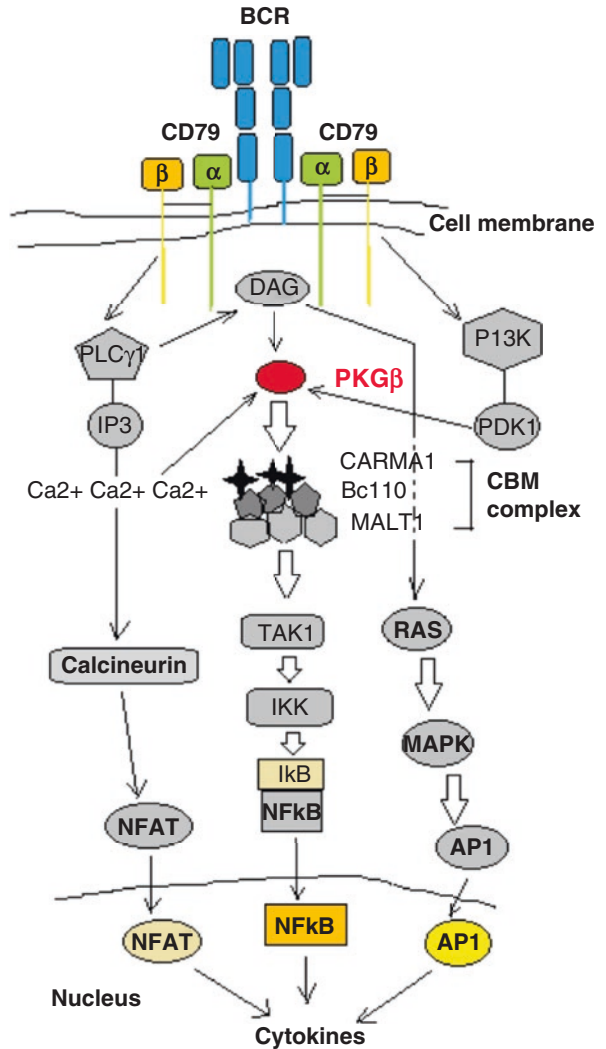
ease mechanisms and provide new therapeutic avenues for precision medicine (Taher et al. 2017).

Another B cells subclass essential in immune homeostasis maintenance is represented by regulatory B lymphocytes (Bregs) that modulate immune responses mostly *via* anti-inflammatory cytokines (e.g. IL-10) enabling Tregs commencement, and thus hindering anti-tumor responses. Aberrations in Breg numbers and function have been observed in immune-related pathologies, including cancer. However, despite a pivotal role for Bregs in upholding inflammation and carcinogenesis, the phenotypic diversity of the Bregs remains unclear (Mauri and Menon 2017; Sarvaria et al. 2017). The PKC $\beta$  is actively involved in NF- $\kappa$ B signaling cascade upon BCR engagement that initiate an influx of Ca<sup>2+</sup> into the cytoplasm (Saijo et al. 2002). Secondary mediators such as DAG are generated as part of the BCR activation cascade to initiate Ca<sup>2+</sup> influx and PKCs signaling events, leading to activation of several transcription factors (Myc, NF- $\kappa$ B, activator protein 1 etc.) essential for B-cell life program (Packard and Cambier 2013).

The mandatory role of PKC isoforms and their involvement in NF- $\kappa$ B pathway modulation in lymphocytes is well known (Guo et al. 2004). The membrane localization of PKC $\beta$  is favorable for I $\kappa$ B kinase (IKK) complex activation that is tightly regulated by Ca<sup>2+</sup> levels (Guo et al. 2004; Berry et al. 2018). The enzyme complex IKK is located in the upstream of the NF- $\kappa$ B signaling cascade playing an essential role for B cells development and function (Kaisho et al. 2001). PKC $\beta$  has a significant role in IKK cascade as IKK becomes activated upon phosphorylation of the adaptor protein CARMA1 by PKC $\beta$ ; this will fit together IKK and TAK1 allowing TAK1 to phosphorylate and activate IKK. As a consequence, I $\kappa$ B is further phosphorylated by IKK leading to NF- $\kappa$ B activation, highlighting the role of PKC $\beta$  in BCR-triggered NF- $\kappa$ B pathway (Shinohara et al. 2005; Thome et al. 2010).

The central mechanism that regulates intracellular Ca<sup>2+</sup> levels in lymphocytes is represented by store-operated calcium entry (SOCE) through calcium release-activated calcium (CRAC) comprising *Stromal Interaction Molecule 1 and 2*

**Fig. 5.3** BCR signaling pathway involving PKCβ. B cells are activated after antigen binding to BCR that needs for intracellular signaling CD79 molecules. In B cells pathways involving PKCβ are triggered. One common intracellular pathway with TCR signaling is the CBM complex activating finally the nuclear NFκB. Secondary messengers (DAG) are generated upon PLCγ1 activation that due to generated calcium (Ca<sup>2+</sup>) would activate PKCβ. 3-phosphoinositide dependent protein kinase-1 (PDK1) activated through phosphoinositide 3-kinase (PI3K) would activate PKCβ. The signaling pathways lead to the activation of nuclear transcription factors: NFκB, activator protein 1 (AP1) and nuclear factor of activated T cells (NFAT) that will induce cytokines' synthesis



proteins (STIM) and Orai protein channels (Feske 2007). STIM and Orai channels discovery, almost a decade ago, has advanced knowledge regarding Ca<sup>2+</sup> multiple actions such as gene expression activation program. Mostly, Ca<sup>2+</sup> dynamics is induced by activation of antigen receptor that would trigger distinct patterns of gene expression responsible for a distinct transcriptionally fate in lymphocytes' life. Interestingly, a clear connection between strength of antigen binding to BCR, pro-inflammatory transcription factors NF-κB and NFAT and Ca<sup>2+</sup> levels effects was reported. Thus, a high affinity antigen interaction with BCR will cause STIM/Orai-mediated Ca<sup>2+</sup> entrance and activation of

both NF-κB and NFAT factors. An intermediary antigen affinity binding to BCR causes possibly Ca<sup>2+</sup> fluctuations that preferentially activate NF-κB. Faint interactions in antigen-BCR complex will generate a transitory raise in cytosolic Ca<sup>2+</sup> leading to the selective activation of NF-κB dependent gene expression. Thus Ca<sup>2+</sup> levels dictated by bonding strength in antigen-receptor complex are able to regulate important checkpoints in canonical NF-κB signaling in both T and B lymphocytes. More accurately, in resting lymphocytes, homo- and heterodimers of NF-κB p50, p65, and c-Rel proteins are sequestered in the cytosol by IκB proteins including IκBα. BCR engagement activates a series of PTKs, β iso-

forms in case of BCR, that subsequently activate PLC $\gamma$  isoforms, cleaving PI(4,5)P<sub>2</sub> to IP<sub>3</sub> and DAG. Further, IP<sub>3</sub> binds to IP<sub>3</sub> receptors channels from endoplasmic reticulum (ER) allowing release of Ca<sup>2+</sup> in cytosol. The resulting collapse of Ca<sup>2+</sup> level in ER induce STIM1 proteins oligomerization and stimulation of plasma membrane Orai1 channels enabling extracellular Ca<sup>2+</sup> admission and a significant elevation in cytoplasmic Ca<sup>2+</sup> level. STIM1 plays a dominant role in BCR-induced Orai1 activation while STIM2 regulates cytosolic Ca<sup>2+</sup> levels in quiescent conditions (Roos et al. 2005; Liou et al. 2005; Berry et al. 2018). Additional roles for STIM/Orai in B cell life were revealed in Bregs where expression of immunosuppressive cytokines IL-10 and TGF- $\beta$  are deficient in the absence of STIM/Orai mediated Ca<sup>2+</sup> channels (Berry et al. 2018).

Recent data designate a critical role of PKC $\beta$  in antigen polarization which is supposed to enable the effective antigen processed peptides linking to MHC-II molecules. Mice with PKC $\beta$ -deficient B cells would exhibit impaired antigen polarization and presentation that could hinder GC development, thus highlighting the significant role of PKC $\beta$  in B cells fate determination and metabolic program (Tsui et al. 2018).

In the last years there were several studies revealing that PKC could intercede as a possible target in B cell diseases taking into account that deregulation in BCR signaling represents one of the mark for *activated B-cell diffuse large B-cell lymphoma* (ABC-DLBCL); a number of BCR targeted therapies are current in progress, including kinase inhibitors against PKC (Stephenson and Singh 2017; Kuppers 2005).

PKC $\beta$ II is overexpressed in chronic lymphocytic leukemia (CLL) where it's associated with poor prognosis and survival as well as with anti-cancer drug resistance development (Kabir et al. 2013; Kazi et al. 2013). PKC $\beta$ II is viewed as a potential therapeutic target for CLL treatment but inhibition of PKC $\beta$ II with specific inhibitors (e.g., LY379196). This inhibitor had a minor effect on the cellular viability and failed to induce

a decrease of mRNA levels for PKC $\beta$ II in CLL cells. Certain growth factors like VEGF seem to sustain PKC $\beta$ II activity. In a milieu deprived of VEGF there is a clear inhibition of PKC $\beta$ II by LY379196 (Abrams et al. 2010). The role of PKCs in CLL cell survival is to inhibit pro-apoptotic signals and stimulate pro-survival ones (PKC $\beta$ II) and activate anti-apoptotic effectors like NOTCH2 (PKC $\delta$ ) (Hubmann et al. 2010; Kang 2014). Furthermore, PKC $\delta$  regulates peripheral B-cell development and immune homeostasis (Salzer et al. 2016) and mediates mitochondrial-dependent apoptosis in an epigenetic manner like phosphorylating histones at specific residues such as H3S10 or H3T45 known to be linked to apoptosis (Hurd et al. 2009; Park and Kim 2012).

PKC $\delta$  is recognized to be involved in autoimmunity as recently, autosomal recessive mutations in gene encoding PKC $\delta$  have been identified in patients with systemic lupus erythematosus. Moreover *Prkcd*<sup>-/-</sup> knockout mice models revealed a distinctive role of PKC $\delta$  in controlling B-cell tolerance as immune complex-mediated glomerulonephritis, lymphadenopathy, splenomegaly, and multi-organ infiltrations of B cells were observed in this animal model (Miyamoto et al. 2002). Therefore the mechanisms of B-cell tolerance could be elucidated studying PKC $\delta$  related deficiencies (Salzer et al. 2016) as well as its immune therapy targeting (Lim et al. 2015).

Nevertheless, after almost three decades of cancer clinical trials with targeting PKC inhibitors, the results are still controversial for patient outcome. Increasing evidence from cancer genomic and proteomic outline provide a novel research line where PKCs isoenzymes largely act as tumor suppressors and therefore their activity should be restored instead of inhibited in antitumor approaches (Newton 2018). However the clear immunologic role of PKC in lymphocytes biology is still to be portrayed and progresses in this area are still to come (Harton 2019).

## 5 Conclusion

PKCs in the immune system have become a check –point for seminal functions and control of intracellular signaling events. Nevertheless, after almost three decades of fundamental studies and cancer clinical trials targeting PKC with inhibitors, there is still information lacking. For example in the clinical trials results there are still controversies and sometimes even the clinical outcome of the patient subjected to this therapy worsed. Increasing evidence from cancer genomic and proteomic outline provide a novel approaches namely PKCs isoenzyme largely act as tumor suppressors and therefore their activity should be restored instead of inhibited in antitumor approaches (Newton 2018). However the clear immunologic role of PKC in lymphocytes biology is still to be portrayed. Apart from its potential role in antigen presentation and TCR/BCR triggered events immune cell activation/differentiation and transcriptomic pattern related events, still little is known about the contribution of PKC to cell's immunobiology.

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