

Role of PKC- β in chemical allergen-induced CD86 expression and IL-8 release in THP-1 cells

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Abstract We previously demonstrated an age-related decrease in receptor for activated C-kinase (RACK-1) expression and functional deficit in Langerhans cells' responsiveness. This defect specifically involves the translocation of protein kinase C (PKC)- β . The purpose of this study was to investigate the role of RACK-1 and PKC- β in chemical allergen-induced CD86 expression and IL-8 release in the human promyelocytic cell line THP-1 and primary human dendritic cells (DC). Dinitrochlorobenzene, *p*-phenylenediamine and diethyl maleate were used as contact allergens. The selective cell-permeable inhibitor of PKC- β and the broad PKC inhibitor GF109203X completely prevented chemical allergen- or lipopolysaccharide (LPS)-induced CD86 expression and significantly modulated IL-8 release (50 % reduction). The selective cell-permeable inhibitor of PKC- ε (also known to bind to RACK-1) failed to modulate allergen- or LPS-induced CD86 expression or allergen-induced IL-8 release, while modulating LPS-induced IL-8 release. The use of a RACK-1 pseudosubstrate, which directly activates PKC- β , resulted in dose-related increase in CD86 expression and IL-8 release. Similar results were obtained with human DC, confirming

the relevance of results obtained in THP-1 cells. Overall, our findings demonstrate the role of PKC- β and RACK-1 in allergen-induced CD86 expression and IL-8 production, supporting a central role of PKC- β in the initiation of chemical allergen-induced DC activation.

Keywords PKC · Contact allergy · Chemical allergen

Abbreviations

DAG	Diacylglycerol
DEM	Diethyl maleate
DNCB	2, 4-Dinitrochlorobenzene
PPD	<i>p</i> -Phenylenediamine

Introduction

Chemical allergens are small molecules able to form a sensitizing complex once they bound to proteins (Corsini and Kimber 2007). One of the most frequent manifestations of chemical allergy is contact hypersensitivity, which can have serious impact on the quality of life. A recent general population epidemiological study suggests that the prevalence of contact allergy to at least one sensitizing chemical is ~15–20 % (Peiser et al. 2012; Thyssen et al. 2007), making contact allergy a common and important environmental and occupational health hazard.

The development of allergic contact dermatitis requires the activation of innate immune cells, including keratinocytes necessary for maturation and migration of dendritic cells (DC), and DC required for the activation of specific T cells (Martin 2012). DC are antigen-presenting cells that play a central role in the initiation and regulation of adaptive immune responses. Human DC can arise from a variety of precursors. It has been shown that PKC activation is

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necessary and sufficient to drive human CD34⁺ hematopoietic progenitor cells, peripheral blood monocytes or myeloid leukemic cell lines to DC differentiation (Cejas et al. 2005; Lin et al. 2007).

PKCs play a key regulatory role in a variety of cellular functions, including cell growth and differentiation, gene expression, hormone secretion (Zeng et al. 2012; Cosentino-Gomes et al. 2012). PKCs were originally identified by Nishizuka and colleagues as serine/threonine protein kinases, whose activity was dependent on calcium and phospholipids (Nishizuka 1995; Nakamura and Yamamura 2010). PKC is classified into three subfamilies: PKC classical, novel and atypical. Classical PKC includes α , β I, β II (alternate splicing variants of the PKC- β gene) and γ types, which require diacylglycerol (DAG), calcium and phospholipids for activation. Novel PKC includes δ , ϵ , η and θ isoforms, which require DAG but no Ca²⁺ ion for activation. Atypical PKC includes ζ and ι/λ isoforms, which need neither DAG nor Ca²⁺ ion for activation. Patterns of expression for each PKC isoform differ among tissues (Jaken and Parker 2000; Zeng et al. 2012). It is also clear that different PKCs are not functionally redundant, for example, specific PKCs mediate specific cellular signals required for activation, proliferation, differentiation and survival of immune cells (Leitges et al. 1996; Sun et al. 2000; Miyamoto et al. 2002; Pfeifhofer-Obermair et al. 2012).

Of the classical PKC isoforms, only PKC- β II was consistently activated during DC differentiation-inducing stimuli in normal and leukemic progenitors (Cejas et al. 2005). PKC- β II activation by cytokines (GM-CSF + IL-4 + TNF- α) in DC was demonstrated to be associated with up-regulation of DC surface markers (MHC I and MHC II, CD11c, CD40, CD80, CD86 and CD83), the induction of expression of the NF- κ B family member c-Rel and the ability to stimulate allogeneic T cell proliferation. Lin et al. (2011) found that the cell fate commitment of CD14⁺ monocytes into monocyte-derived macrophages or DC by GM-CSF, or GM-CSF plus IL-4 for 7 days appears to be steered by the selective activation of PKC- α or PKC- β I, respectively. These findings demonstrate the critical role of PKC- β isoforms in cytokine-induced DC differentiation from their progenitor or precursor cells.

As it has been demonstrated, almost 20 years ago by Halliday and Lucas (1993), PKC is of particular interest in the context of skin sensitization as it is required for Langerhans cells' (LCs) migration. Within the epidermis, PKC- β II is exclusively expressed in LCs, and its down-regulation impairs LC function with respect to contact hypersensitivity (Goodell et al. 1996). Decreased level of PKC- β II within LCs by a single application of TPA was associated with a 60 % decrease in contact hypersensitivity to 2,4-dinitrofluorobenzene in TPA-treated mice. Reynolds et al. (1995) demonstrated that down-regulation of

epidermal LCs PKC- β was associated with reduced CD1a⁺ epidermal LCs in allergic and irritant contact dermatitis, suggesting that PKC- β may transduce the signal for migration of LCs from human epidermis. Furthermore, Staquet et al. (2004) demonstrated that chemical-allergen (i.e., Bandrowski's base and 2,4-dinitrobenzenesulfonic acid)-induced migratory capacity of *in vitro*-generated LC was blocked by PKC and matrix metalloproteinase inhibitors.

Activated PKC translocates from the cytosol to membrane compartments to exert its function (Nishizuka 1995). A family of proteins named receptors for activated C-kinase (RACK) mediates this process (Mochly-Rosen et al. 1991). RACK-1, a 36-kDa protein cloned from rat brain, is the best-characterized member of the RACK family (Ron et al. 1994), shown to preferentially interact with PKC- β and PKC- ϵ . RACK-1 binds to activated PKCs, facilitating their translocation to cellular membranes, stabilizing their active conformation and simultaneously preventing their premature degradation (Ron et al. 1994, 1995). Other proteins such as rat synaptic-vesicle-specific p65 protein, phospholipase C γ , phospholipase A2 and annexin were later discovered to bind to RACK-1 via a sequence similar to the C2 domain of PKC, suggesting that RACK-1 may play a critical role in anchoring a range of proteins to the membrane and may integrate signaling pathways with different physiological functions (McCahill et al. 2002). RACK-1 belongs to the family of WD repeat proteins, carrying seven individual WD repeats. RACK-1 is a scaffolding protein that is involved in the recruitment, assembly and regulation of multiple different signaling molecules (Mochly-Rosen and Gordon 1998; Nilsson et al. 2004). Within the immune system, RACK-1 also functions as an adaptor recruiting the transcription factor STAT1 to the IFN receptor complex and is a scaffold protein for the IFN- α receptor 2/ β -chain of the receptor, Janus kinase 1 and tyrosine kinase 2 (Usacheva et al. 2003). Furthermore, RACK-1 may also serve as a scaffold protein in other cytokine systems such as IL-2, IL-4 and erythropoietin as well as the signaling pathways of TNF-R55 (Tcherkasowa et al. 2002).

We previously demonstrated an age-related decrease in RACK-1 expression, defective PKC- β translocation and reduced cytokine production in rat epidermal cells in response to LPS, phorbol ester and DNCB (Corsini et al. 2009), which provides a new understanding of the consequences of aging on skin immunology. As cytokines and costimulatory molecules are essential for proper antigen presentation and activation of lymphocytes, the purpose of this work was to investigate the role of PKC- β and RACK-1 in chemical allergen-induced CD86 expression and IL-8 release in the human promyelocytic cell line THP-1, employed as surrogate of DC, and in primary human monocyte-derived DC. We focused our attention on CD86 and IL-8 since they have been demonstrated to

be selectively up-regulated by chemical allergens (Mitjans et al. 2008, 2010; Toebak et al. 2006). Here, we demonstrate that PKC- β and RACK-1 play a central role in chemical allergen-induced CD86 up-regulation and IL-8 release.

Materials and methods

Chemicals

Skin sensitizers 2,4-dinitrochlorobenzene (DNCB), *p*-phenylenediamine (PPD), diethyl maleate (DEM) and lipopolysaccharide from *Escherichia coli* serotype 0127:B8 (LPS) were obtained from Sigma (St. Louis, MO, USA), antibodies against PKC- β II (preferentially RACK1-binding isotype) from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and anti-human β -actin from Sigma. Electrophoresis reagents were from Bio-Rad (Richmond, CA, USA). All reagents were purchased at the highest purity available.

Study design

THP-1 cells were diluted to 10^6 cells/mL in RPMI 1640 containing 2 mM L-glutamine, 0.1 mg/mL streptomycin, 100 IU/mL penicillin and 50 μ M 2-mercaptoethanol, supplemented with 10 % heat-inactivated fetal calf serum (media) and cultured at 37 °C in 5 % CO₂ incubator. To investigate the role of PKC in chemical-allergen-induced CD86 expression and IL-8 release, THP-1 cells were treated with the broad inhibitor of protein kinase C GF109203X 5 μ M (Tocris Bioscience, Bristol, UK) for 1 h, and then PPD (60 μ g/ml), DNCB (2 μ g/ml), DEM (100 μ g/ml) or LPS (10 ng/ml) or DMSO as vehicle control were added for 24 h. CD86 expression was evaluated by FACS analysis and IL-8 by ELISA as previously described (Mitjans et al. 2008).

To investigate the specific role of PKC- β and PKC- ϵ in chemical allergen-induced CD86 expression and IL-8 release, THP-1 cells were cultured for 2 h in the presence or absence of a selective cell-permeable inhibitor of PKC- β 5 μ M (PKC- β pseudosubstrate, Tocris Bioscience) or a selective cell-permeable inhibitor of PKC- ϵ (Calbiochem, Billerica, MA, USA). Then, chemical allergens or LPS 10 ng/ml was added for 24 h. The concentration of inhibitor used was not cytotoxic, as assessed by PI staining.

To confirm the role of PKC- β in chemical-allergen-induced CD86 expression and IL-8 release, a pseudo-RACK-1 activator of protein kinase C was used (Tocris). The pseudosubstrate consists of peptide derived from the C2 domain of PKC- β linked by a disulfide bridge to the Antennapedia domain vector peptide. The Antennapedia peptide has been shown to be actively taken up by intact cells, ensuring rapid and effective uptake of the activator

peptide. Once inside the cell, the disulfide bonds were subjected to reduction in the cytoplasm, leading to release of the activator peptide. In this case, THP-1 cells were treated for 24 h with increasing concentration of RACK-1 pseudosubstrate, and then, CD86 expression and IL-8 were evaluated.

Changes in CD86 expression are reported as stimulation index (SI) calculated by the following equation:

$$SI = \frac{PCt \times MFI_t}{PCc \times MFI_c}$$

where mean fluorescence intensity is denoted by MFI and percentage of positive cells as PC in chemical-treated cells, whereas PCc and MFIc for relative control cells.

To investigate the role of PKC- β in chemical allergen-induced CD86 expression in primary human immature dendritic cells (iDC), iDC were generated as previously described (Dietz et al. 2010). In brief, monocytes were isolated by CD14⁺ MACS from PBMC isolated by Ficoll gradient centrifugation from fresh blood of human volunteers. These monocytes were differentiated from iDCs by cultivation of up to 10×10^6 cells for 5 days in a 10-cm dish in 10 ml of serum-free medium (Cellgro, obtained from Cell-Genix) supplemented with 500 IU/ml GM-CSF and 50 IU IL-4 (both from Miltenyi Biotech). For stimulation, cells were removed from the dishes and plated at 1×10^6 cells in 1 ml of fresh medium in 24-well plates with or without addition of PKC- β pseudosubstrate (5 μ M) for 2 h. Afterward, cells were either left untreated or stimulated with oxazolone LPS for 24 h and checked by flow cytometry for up-regulation of CD86 expression in live cells (DAPI negative).

PKC- β II translocation assay

THP-1 cells (5×10^6) were incubated overnight and then treated with DNCB 2 μ g/ml for different times. Cells were recovered by centrifugation for 5 min at 1,200 rpm at 4 °C. The pellets were resuspended in 500 μ l of homogenization buffer (50 mM TRIS, 150 mM NaCl, 5 mM EDTA pH 7.5, 0.5 % Triton X-100, 50 μ M PMSF, 2 μ g/ml aprotinin, 1 μ g/ml pepstatin and 1 μ g/ml leupeptin) and homogenized using a Teflon/glass potter. Cytosolic fractions were obtained by centrifugation at 45,000 $\times g$ for 30 min at 4 °C. The pellets were resuspended in 300 μ l of homogenization buffer containing 1.0 % v/v Triton X-100 by sonication 2 $\times$ 15 s; this constituted the membrane fractions. Proteins were denatured for 10 min at 100 °C. The protein content was measured using a commercial kit (Bio-Rad). Equal amounts of protein (10 μ g) per lane were resolved by electrophoresis on 12 % SDS-polyacrylamide gels under reducing conditions (SDS-PAGE). Proteins were then transferred to PVDF membrane (Amersham, Little Chalfont,

UK) and visualized using PCK- β II (1:5,000) and β -actin (1:5,000) as the primary antibodies and developed using enhanced chemiluminescence (ECL, Amersham). Image of the immunoblotting was acquired with a Nikon CCD video camera module. Densitometric analysis was performed with Quantity One v. 4.6 program (Bio-Rad; Hercules, CA, USA). The relative densities of the bands were normalized to data obtained from control sample ran under the same conditions.

Reactive oxygen species

For the determination of intracellular reactive oxygen species stimulated with DNCB, the fluorescent intensity of dichlorofluorescein diacetate (DCFH-DA) (Molecular Probes, Eugene, OR, USA) oxidized product DCF was measured. THP-1 cells were cultured for 2 h in the presence or absence of a selective cell-permeable inhibitor of PKC- β 5 μ M, loaded with DCFH (2 μ M) for other 30 min and then treated in the presence or absence of DNCB (2 μ g/ml) for different times. The fluorescence intensity of fluorophore DCF, which was formed by peroxide oxidation of the nonfluorescent precursor, was measured (in the FL1 channel set for log scale) using FACScan flow cytometry.

Statistical analysis

Data are expressed as mean $\pm$ standard deviation (SD). Statistical analysis was performed using GraphPad InStat version 3.0a for Macintosh (GraphPad Software, San Diego, CA, USA). Tukey's post hoc test was used for multiple-comparison analysis of variance. Between two groups, Student's *t* test was used. Differences were considered significant at $p \leq 0.05$.

Results

As PKC isoforms directly and/or indirectly participate in signal transduction pathways of normal and transformed cells through phosphorylation of target proteins, it is critical to understand the diversity of the intracellular signaling pathways regulated by each PKC isozyme. In order to investigate the role of PKCs in chemical allergen-induced CD86 and IL-8, we used GF109203X, a selective inhibitor of classical PKCs and PKC- ϵ . As shown in Fig. 1a, GF109203X completely inhibited chemical allergen-induced CD86 ($p < 0.01$), suggesting the involvement of classical PKCs and PKC- ϵ , while chemical allergen-induced IL-8 release was only partially modulated (Fig. 1b), suggesting the involvement of other signal transduction pathways. For all allergens tested approximately, a 50 % reduction was obtained ($p < 0.01$). The

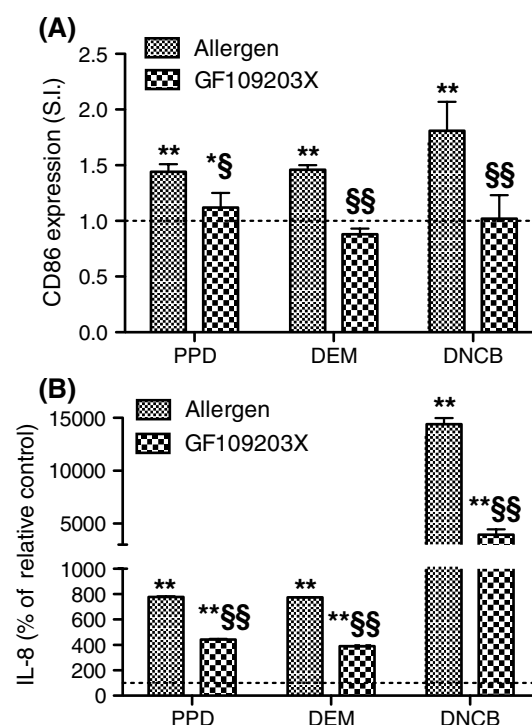


Fig. 1 PKC inhibitor GF109203X blocks allergen-induced CD86 up-regulation and modulates the release of IL-8. **a** Effect of GF109203X on PPD-, DEM- and DNCB-induced CD86 expression after 24 h of treatment. **b** Effect of GF109203X on PPD-, DEM- and DNCB-induced IL-8 release after 24 h of treatment. THP-1 cells were treated for 1 h with GF109203X (5 μ M) and then exposed to PPD (60 μ g/ml), DEM (100 μ g/ml) and DNCB (2 μ g/ml) or DMSO as vehicle control (0.2 % final concentration). CD86 expression was evaluated by flow cytometry analysis and IL-8 by ELISA. Results are expressed as mean $\pm$ SD. Data are representative of three independent experiments. Statistical analysis was performed with Tukey's multiple-comparison test with * $p < 0.01$ versus DMSO-treated cells and ** $p < 0.01$, § $p < 0.05$ versus cells treated with the allergen alone

concentrations of the three allergens used were based on previously published data as the ones resulting in similar CD86 expression (Mitjans et al. 2008, 2010; Galbiati et al. 2012).

As many of the commercially available PKC inhibitors can also inhibit other kinases (Alessi 1997), the use of GF109203X alone is not sufficient to implicate PKC in the regulation of CD86 and IL-8. Therefore, we investigated the role of PKC- β and PKC- ϵ using two PKC isoform-specific inhibitor substrates. As shown in Fig. 2a, the PKC- β pseudosubstrate completely prevented chemical allergen-induced CD86 expression ($p < 0.01$), whereas the PKC- ϵ pseudosubstrate failed to modulate CD86 (Fig. 2c). Similar results were obtained using LPS as stimuli. As shown in Fig. 3a, both GF109203X and the PKC- β pseudosubstrate almost completely abrogated LPS-induced CD86 up-regulation ($p < 0.01$), whereas the PKC- ϵ pseudosubstrate failed to modulate CD86,

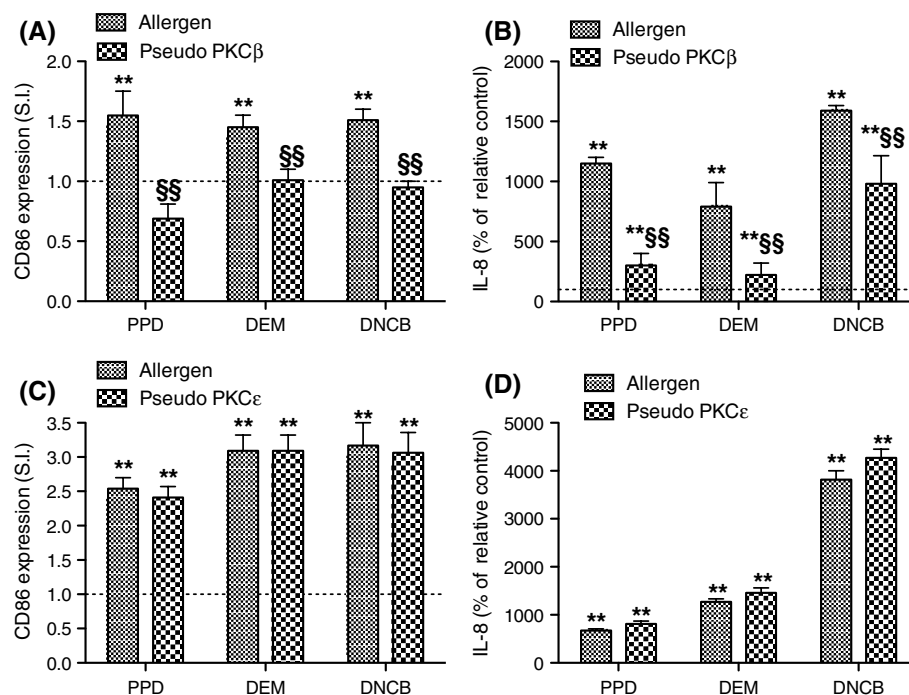


Fig. 2 Effects of specific PKC- β and PKC- ϵ inhibitors on allergen-induced CD86 up-regulation and IL-8 release. **a** Effect of PKC- β pseudosubstrate on PPD-, DEM- and DNCB-induced CD86 expression. **b** Effect of PKC- β pseudosubstrate on PPD-, DEM- and DNCB-induced IL-8 release. **c** Effect of PKC- ϵ pseudosubstrate on PPD-, DEM- and DNCB-induced CD86 expression. **d** Effect of PKC- ϵ pseudosubstrate on PPD-, DEM- and DNCB-induced IL-8 release. THP-1 cells were treated for 2 h with PKC- β and PKC- ϵ

pseudosubstrate inhibitors (5 μ M) and then exposed to PPD (60 μ g/ml), DEM (100 μ g/ml) and DNCB (2 μ g/ml) or DMSO as vehicle control (0.2 % final concentration) for 24 h. Results are expressed as mean $\pm$ SD. Data are representative of three independent experiments. Statistical analysis was performed with Tukey's multiple-comparison test with ** p < 0.01, * p < 0.05 versus DMSO-treated cells and ^{ss}p < 0.01, ^{sp} < 0.05 versus cells treated with the allergen alone

confirming the involvement of PKC- β in the induction of CD86. Concerning the effect on IL-8 release, the PKC- β pseudosubstrate partially prevented chemical allergen-induced IL-8 release (Fig. 2b), while the PKC- ϵ pseudosubstrate had no effect (Fig. 2d). The lack of the effect of PKC- ϵ pseudosubstrate was not due to its ineffectiveness as demonstrated by its ability to modulate LPS-induced IL-8 and TNF- α release (Fig. 3b, c, p < 0.01), indicating that allergens and LPS share common signal transduction pathways but also that LPS triggers different pathways. The inhibitory effects were not due to cytotoxicity as assessed by PI staining: the percentage of viable cells was 90 ± 3 in vehicle-treated cells and 86 ± 1 , 87 ± 1 and 88 ± 1 in GF109203X, pseudo-PKC- β - and pseudo-PKC- ϵ -treated cells, respectively.

Even if it is true that continuous cell lines are not the physiological equivalents of primary cells, they can be valid surrogates. Indeed, similarly, in human primary DC, PKC- β pseudosubstrate prevented LPS- and oxazolone-induced CD86 expression, confirming the relevance of data obtained in THP-1 cells (Fig. 4a, b). In this case, oxazolone was used instead of the other allergens, as at the University Medical Center Freiburg this allergen is commonly used as

positive control with consistent results obtained, while the other allergens have never been tested.

To further support the role of PKC- β activation in CD86 expression and IL-8 production, we used a RACK-1 pseudosubstrate able to directly activate PKC- β . As shown in Fig. 5a, b, the RACK-1 pseudosubstrate was able to induce in a dose-related manner both CD86 and IL-8 (p < 0.01), confirming the critical role of PKC- β .

Finally, we investigated the ability of DNCB to activate PKC by assessing membrane translocation of PKC- β II by Western blot analysis and the induction of ROS. As shown in Fig. 6, DNCB induced a rapid (5 min) and transient PKC- β II translocation from the cytosol (Fig. 6a, b) to the membrane fraction (Fig. 6a, c, p < 0.01 at 5 min); by 30 min, the distribution was back to control levels. Concerning the induction of oxidative stress, as expected, DNCB induced a rapid increase in ROS production as assessed by DCFH-DA and FACS analysis (Fig. 6d, e). The PKC- β pseudosubstrate completely abrogated DNCB-induced ROS production at 5 and 15 min, supporting the notion that as early events allergen induces oxidative/electrophilic stress, PKC- β II activation and ROS production, followed later in time by CD86 expression and IL-8 release.

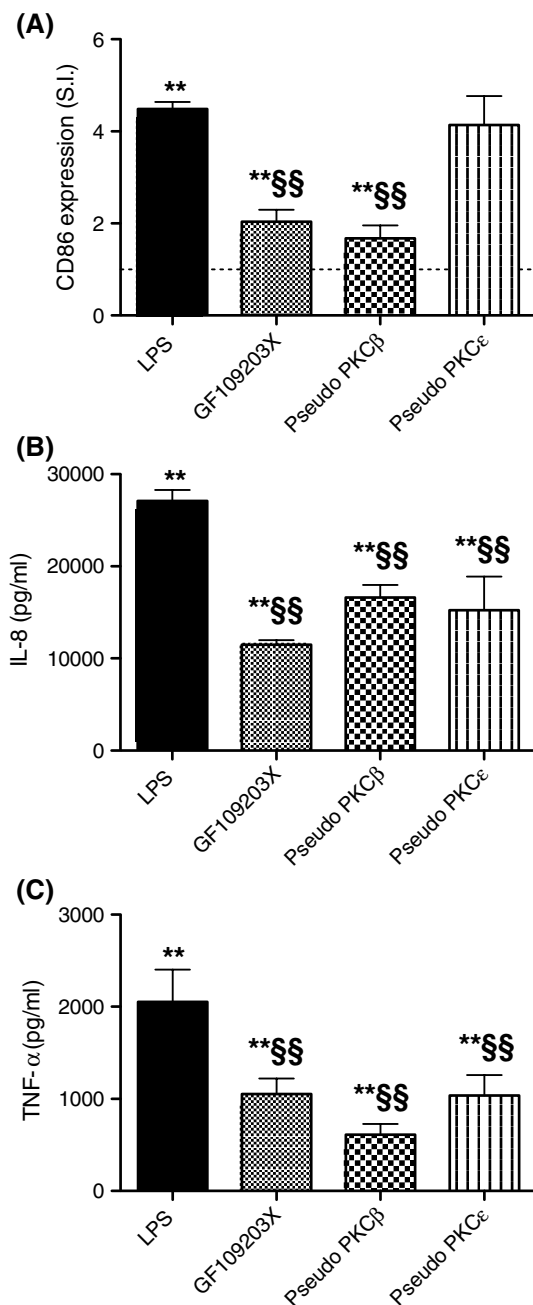


Fig. 3 Effects of PKC inhibitors on LPS-induced CD86 up-regulation and cytokine release. **a** Effect of GF109203X, PKC- β and PKC- ϵ pseudosubstrate inhibitors on LPS-induced CD86 expression. **b** Effect of GF109203X, PKC- β and PKC- ϵ pseudosubstrate inhibitors on LPS-induced IL-8 release. **c** Effect of GF109203X, PKC- β and PKC- ϵ pseudosubstrate inhibitors on LPS-induced TNF- α release. THP-1 cells were treated for 1 h with GF109203X (5 μ M) or for 2 h with PKC- β and PKC- ϵ pseudosubstrate inhibitors (5 μ M) and then exposed to LPS (10 ng/ml) for 24 h. CD86 expression was evaluated by flow cytometry analysis and IL-8 by ELISA. Results are expressed as mean $\pm$ SD. Data are representative of three independent experiments. Statistical analysis was performed with Tukey's multiple-comparison test with ** p < 0.01 versus DMSO-treated cells and §§ p < 0.01, § p < 0.05 versus cells treated with LPS alone

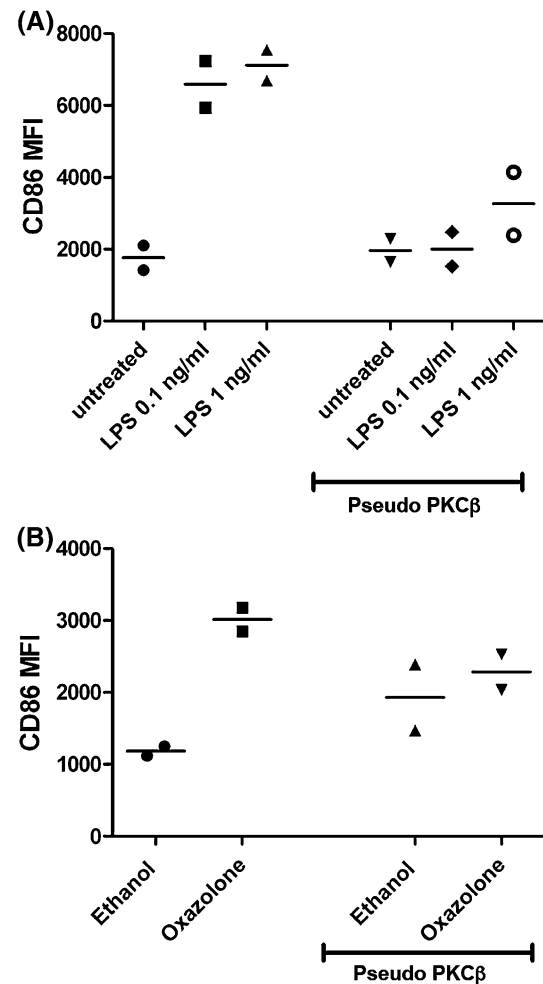


Fig. 4 Effect of specific PKC- β inhibitor on LPS- and oxazolone-induced CD86 up-regulation in primary human DC. iDC were treated for 2 h with PKC- β pseudosubstrate inhibitor (5 μ M) and then exposed to LPS (0.1 and 1 ng/ml) and oxazolone (100 μ M) for 24 h. CD86 expression was evaluated by flow cytometry. Cells were gated on FSC/SSC and live cells by exclusion of DAPI-positive cells. Results shown are from two different donors out of two independent experiments. Results are expressed as CD86 mean fluorescence intensity (MFI)

Discussion

Our findings support a model where PKC- β activation is a key component of the signal transduction pathways that induce DC activation following the exposure to chemical allergens. Specifically, these data indicate the following: (1) PKC is a critical downstream signaling component of chemical allergen-induced CD86 up-regulation and IL-8 production, (2) of the PKC isoforms, PKC- β is most centrally involved in chemical-allergen-induced CD86 up-regulation and IL-8 release and (3) PKC- β is also a critical downstream signaling component of LPS-induced CD86

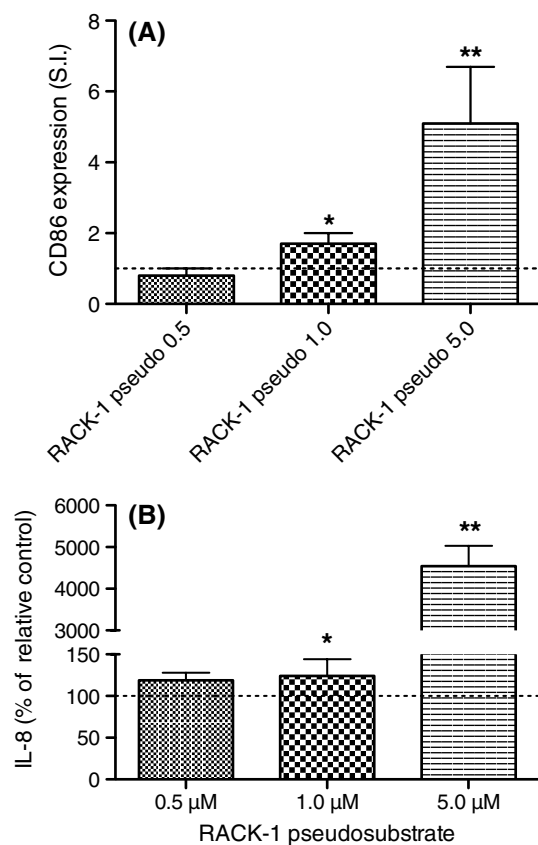


Fig. 5 PKC- β activation by RACK-1 pseudosubstrate results in CD86 up-regulation and IL-8 release. **a** Effect of RACK-1 pseudosubstrate activator on allergen-induced CD86 expression. **b** Effect of RACK-1 pseudosubstrate activator on allergen-induced IL-8 release. To evaluate the effect of a direct activation of PKC- β , a specific RACK-1 pseudosubstrate was used. Cells were exposed to increasing concentration of RACK-1 (0.5–5 μ M) for 24 h. CD86 expression was evaluated by flow cytometry analysis and IL-8 by ELISA. Results are expressed as mean $\pm$ SD. Data are representative of three independent experiments. Statistical analysis was performed with Tukey's multiple-comparison test with ** $p < 0.01$, * $p < 0.05$ versus control cells

up-regulation, but the involvement of PKC- ϵ and possible other pathway in cytokine production was observed as well.

The ability of the PKC inhibitor GF109203X to inhibit chemical allergen-induced CD86 expression and modulate IL-8 production indicates that PKC is an upstream component of these signaling pathways. Among the different PKC isoforms, the critical role of PKC- β II in DC maturation has been demonstrated (Cejas et al. 2005; Lin et al. 2007). To address the involvement of PKC- β in chemical allergen-induced CD86 expression and IL-8 production, we asked this seemingly straightforward question by using a selective cell-permeable PKC- β inhibitor and by assessing PKC- β II translocation following DNCB treatment. We preferred not to use the genetic silencing of the PKC- β isoform to avoid any possible compensatory and confounding

mechanisms of other PKC isoforms. Furthermore, we have previously demonstrated in leukocytes, by using RACK-1 antisense oligonucleotides, the role of PKC- β signaling pathway in LPS-induced cytokine production (Corsini et al. 1999; Corsini et al. 2009). We used three contact allergens, namely DNCB, DEM and PPD, at concentrations resulting in similar CD86 expression, and the classical activator of natural immunity LPS. The ability of the PKC- β pseudo-substrate to completely prevent chemical allergen-induced CD86 expression and significantly reduce IL-8 release, together with the rapid activation of PKC- β II (5 min), indicates that it is involved in the primary signaling rather than a secondary response. These data suggest that chemical allergen-induced CD86 expression is specifically signaled through PKC- β , while other signal transduction pathways are involved in IL-8 production. To strengthen the role of PKC- β in CD86 expression and IL-8 release, we used a RACK-1 pseudosubstrate to specifically activate PKC- β . A dose-related induction of CD86 expression and IL-8 release was observed, further demonstrating the selective involvement of PKC- β .

We focused on PKC- β II as the available evidence indicates that PKC- β I and PKC- β II have different functional roles in monocytes: PKC- β I has been associated with the induction of apoptosis (Pongracz et al. 1996), while PKC- β II with proliferation and differentiation (Cejas et al. 2005; Lin et al. 2007). We could not rule out the involvement of other PKC isoforms or degree of PKC signal strength or other signal transduction pathways. As shown in LPS-treated cells, also the selective inhibitor of PKC- ϵ can significantly reduce LPS-induced IL-8 and TNF- α release. However, there is reasonably strong evidence that PKC- β is playing a major role. It would not be surprising if there were some degree of functional redundancy between PKC isoforms.

Mizuashi et al. (2005) showed that all the sensitizers tested (Ni, formaldehyde, DNCB, MnCl₂ and thimerosal) and none of the nonsensitizers reduced the GSH/GSSG ratio, which was accompanied by p38 MAPK activation in DC. The antioxidant *N*-acetyl-L-cysteine, which prevented the reduction in the GSH/GSSG ratio, abrogated p38 MAPK activation and CD86 expression, a common marker of DC maturation induced by chemical sensitizers. Chemical-induced oxidation of the cell-surface thiols appears to be one of the triggers of DC maturation, resulting in intracellular redox imbalance (Suzuki et al. 2009; Kagatani et al. 2010; Corsini et al. 2013). Proteins that are reversibly modulated by redox imbalance are of high interest. In this context, protein kinases and phosphatases have been described to be the key elements in oxidative-stress-mediated signaling events. PKC isoforms have been shown to contain a unique structural feature that is susceptible to oxidative modification (Cosentino-Gomes et al. 2012). In

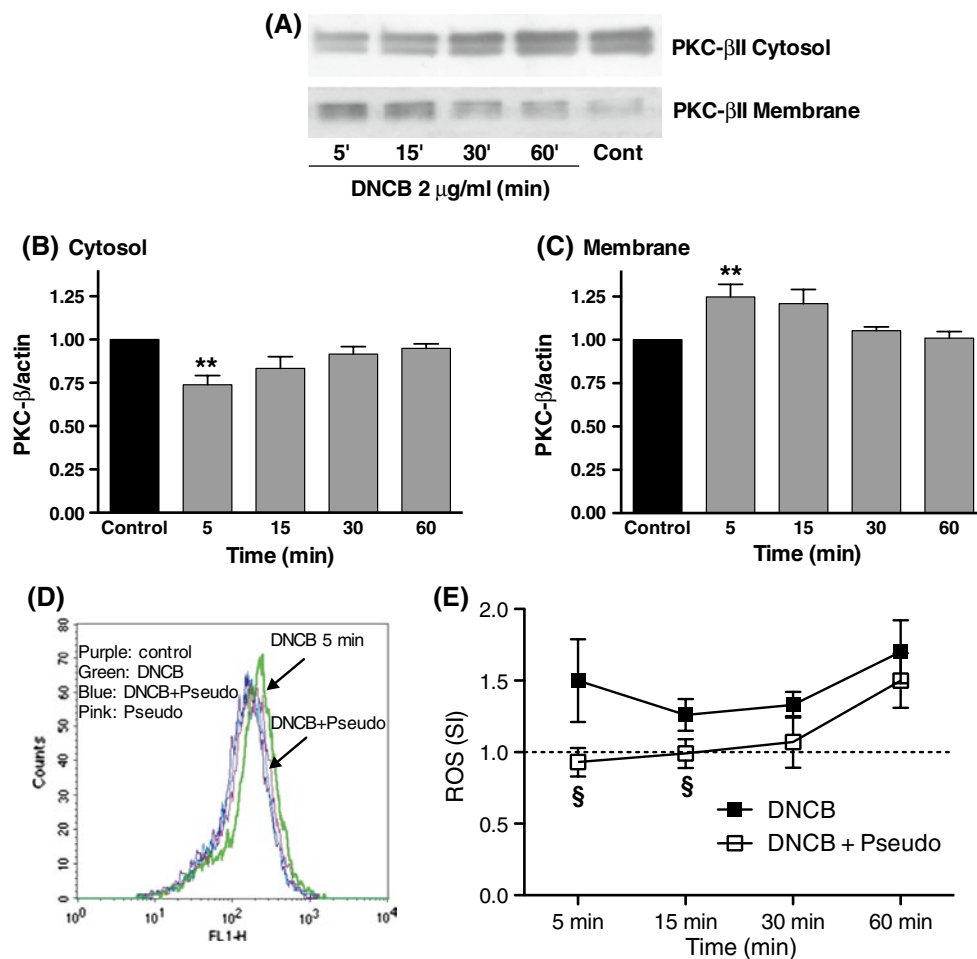


Fig. 6 DNCB induces a rapid PKC-βII translocation and ROS production. **a** Representative Western blot analyses of PKC-βII immunoreactivities in THP-1 cells after DNCB treatment. Proteins from soluble (20 μg) and membrane (10 μg) fractions were loaded and electrophoresed on the gels (12 % SDS-PAGE). Quantification by densitometric analysis of PKC-βII immunoreactivities normalized for β-actin (not shown) content in cytosol (**b**) and membrane fractions (**c**) after DNCB (2 μg/ml). Values are expressed as optical density (PKC-βII/actin) relative to control values (time 0, control). Results are expressed as mean ± SD. Data are representative of three independent experiments. **d** and **e** to detect ROS and to investigate the role of PKC-β, THP-1 cells were treated for 1.5 h with PKC-β

pseudosubstrate inhibitor (5 μM) and loaded for 30 min with DCFH-DA (2 mM), followed by DNCB (2 μg/ml) or DMSO as vehicle control for additional 1 h total. The relative fluorescence intensity of fluorophore DCF was then detected by flow cytometric analysis. In **d**, representative histograms are reported after 5 min of treatment with DNCB, while in **e**, ROS production over time is reported. ROS production is expressed in SI unit, calculated by dividing MFI of treated cells for MFI in relative control cells. Each value represents the mean ± SD of three samples. Statistical analysis was performed with Dunnett's multiple-comparison test, with $p < 0.05$ versus cells treated with DNCB alone

particular, two pairs of zinc fingers are found within the regulatory domain (sites of diacylglycerol and phorbol ester binding). Each zinc finger is composed of six cysteine residues and two zinc atoms. The high levels of cysteine residues render the regulatory domain susceptible to redox regulation (Giorgi et al. 2010; Gopalakrishna and Jaken 2000). Oxidative stress destroys the zinc finger conformation, and the autoinhibition is relieved, resulting in a PKC form that is catalytically active in the absence of Ca^{2+} or phospholipids. Currently, evidence supports the direct activation of different PKC isoforms by oxidative stress and ROS generation (Gopalakrishna and Jaken 2000; Del Carlo and Loeser

2006). Furthermore, not only can redox imbalance activate PKC, but PKC also has been shown to promote the production of endogenous ROS. The β-isoform of PKC induces ROS generation through mitochondrial damage (Pinton et al. 2007). PKC-β is responsible for the activation/phosphorylation of the mitochondrial p66Shc protein, resulting in ROS generation (Migliaccio et al. 1999; Lin et al. 2011). In myeloid leukemia cells, ROS are not necessary for the translocation of PKC-βII from the cytosol to the cell membrane. PKCβII was shown to be essential for ROS production induced by the PKC activator TPA (Datta et al. 2000), indicating that TPA-induced ROS production is dispensable

for the translocation of PKC β II from the cytosol to the cell membrane. Our findings support as early events an allergen-induced oxidation of cell-surface thiols $\rightarrow$ oxidative/electrophilic stress $\rightarrow$ GSH depletion $\rightarrow$ PKC- β II activation $\rightarrow$ ROS $\rightarrow$ $\rightarrow$ CD86 expression and IL-8 release.

Other PKC isoforms, including PKC- α , PKC- ϵ and PKC- ζ , have also been involved in mitochondrial ROS production in different experimental models (Wang et al. 2006; Agudo-López et al. 2011). Further studies are necessary to characterize the specificity by which chemical-allergen-induced ROS may activate different PKC isoforms. Our results, however, contribute to the explanation of the role of PKC- β in CD86 up-regulation and IL-8 production as hallmarks of contact allergy.

Overall, our findings contribute to the understanding of the mechanism responsible for the initiation of chemical-allergen-induced DC activation. The selective activation of specific PKC isoforms may also provide novel therapeutic strategies to manipulate DC activation and contact allergy.

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Conflict of interest For the past 3 years since the beginning of this work, all authors declare of not having any financial or personal association with any individuals or organizations that could have inappropriately influenced the submitted work.

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