SPANDIDOS PUBLICATIONS

Therapeutic target validation of protein kinase C (PKC)-ζ for asthma using a mouse model

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Abstract. Protein kinase C (PKC) is a complex family consisting of many types of isoenzymes, of which PKC-ζ, an atypical isoform, has been reportedly implicated in the regulation of apoptosis and NF-κB, as well as control of T-dependent responses. Based on the recent report that PKC-ζ controls TH2 response, the current study was aimed to evaluate PKC-ζ as a potential therapeutic target for asthma using a mouse model. Mouse allergic asthma was induced by repeated sensitization followed by intranasal challenge with OVA and PKC-ζ pseudosubstrate inhibitor (PPI) was intratracheally instilled before each OVA challenge. Airway hyperreactivity (AHR) was measured by ß-methacoline-induced airflow obstruction. Cellular and cytokine profile in bronchoalveolar lavage fluid (BALF) and level of serum IgE as well as cytokine production by draining lymph node cells were compared. AHR and numbers of eosinophils in BALF were significantly lowered by PPI, indicating that blocking of PKC-ζ activation alleviates asthmatic manifestations. Additionally, PPI instillation decreased IL-5 and IL-13 levels in BALF to approximately 20% of controls, but not IFN-γ level. Instillation of PPI also caused a marked fall in the level of TNF-α, another NF-κB-dependent, proinflammatory cytokine. Serum OVA-specific IgE level and *ex vivo* IL-4, IL-5 and IL-13, but not IFN-γ, production by peribronchial lymph node cells was also considerably lower in PPI-treated mice. In conclusion, blockade of PKC-ζ signals by intratracheal instillation of PPI alleviates allergen-specific TH2 response as well as asthmatic manifestations and hence PKC-ζ is a promising target for treatment of asthma.

Introduction

Allergic asthma is a disease initiated by a specific immune response to an allergen and clinically characterized by reversible airway obstruction, airway hyperreactivity (AHR), and lung inflammation (1,2). As more data are accumulated, it becomes increasingly evident that the mechanisms underlying these features are very complex. However, it is widely accepted that type 2 helper T (TH2) cells producing a limited repertoire of cytokines are responsible for inducing those asthmatic manifestations (3,4). $CD4^+$ TH2 cells secreting IL-4, IL-5 and IL-13 are identified in the bronchoalveolar lavage (BAL) and airway biopsy of asthma patients (5,6). The pathogenic role of TH2 cells in asthma has been repeatedly confirmed by adoptive transfer (7,8) or depletion of $CD4^+$ T cells (9) in mouse models. In addition, a critical role of IL-4, IL-5 and IL-13 in the development of asthma has been supported by a number of studies employing cytokine gene targeting (10-12) or neutralizing anti-cytokine antibodies (10,13) and antagonists (14,15).

PKC is a family of serine/threonine kinases which are important in intracellular signal transduction in various cells (16). On the basis of molecular structure and biochemical properties, the PKC family has been divided into three groups; Ca^{2+}-dependent conventional isoforms (cPKC; α, ßI, ßII and γ), Ca^{2+}-independent novel isoforms (nPKC; δ, ε, μ, θ and θ), and diacylglycerol-, and Ca^{2+}-independent atypical isoforms (aPKC; ζ, and λ/ι) (17).

The major activation pathway of ζ isotype of protein kinase C (PKC-ζ) depends on phosphatidylinositol (PI)-3,4,5-trisphosphate (PIP3) (18) and PKC-ζ is important for the regulation of NF-κB transcriptional activity (19). Consequently, a lack of PKC-ζ causes an impairment in B cell receptor signaling and cell proliferation and survival and the transcription of NF-κB-dependent genes (19,20). Recent report showing a dramatic inhibition of allergic airway inflammation in PKC-$\zeta^{-/-}$ mice (21) and availability of a potent PKC-ζ pseudosubstrate inhibitor (PPI) (22) prompted us to evaluate its possible therapeutic potential in a mouse model of asthma.

Materials and methods

Animals. Female BALB/c 5- to 6-week-old mice were supplied from Korean branch of Taconics, Samtaco (Osan, Korea). The mice were maintained in an environmentally controlled rearing system and used for experiments when 7- to 8-week-old. Mice were age-matched for each experiment.

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Induction of allergic asthma. Systemic sensitization was performed by two i.p. injections of 50 μg OVA (chicken egg albumin, grade V, Sigma, St. Louis, MO, USA) absorbed to 1 mg alum (aluminum ammonium sulfate, Sigma) in 0.3 ml PBS (phosphate buffer saline, pH. 7.4) on days 0 and 7. On days 15, 16 and 18, mice were anesthetized by i.p. injection of Avertin (2.5% wt/vol in PBS) and intranasally challenged with 2% OVA in PBS (50 μl/mouse).

PKC-ζ pseudosubstrate inhibitor (PPI) and intratracheal instillation. One hour after challenge on days 16 and 18, intratracheal instillation with 50 μM/mouse of PKC-ζ pseudosubstrate attached to cell permeabilization vector peptide (Tocris, Ellisiville, MO, USA) (22) into the immunized mice (50 μl/mouse) was performed non-operatively.

Measurement of airway hyper-reactivity (AHR). At 24 h after the last OVA challenge on day 19, AHR was measured by recording respiratory pressure curves in response to inhaled nebulized methacholine (acetyl-ß-methylcholine chloride; Sigma) using whole-body plethysmography (All-Medicus, Seoul, Korea). AHR was expressed in enhanced pause (Penh), an index of airway obstruction. Penh values were obtained for 5 min and averaged, while mice were exposed to a series of methacholine aerosols (0, 6.25, 12.5, 25 and 50 mg/ml).

Analysis of the BALF (bronchoalveolar lavage fluid). BALF was obtained immediately after bleeding of the mice by lavage of the airways through a tracheal cannula with PBS. For differential BALF cell counts, cytospin preparations were made (1,000 rpm, 5 min) using a cytocentrifuge (Shandon Southern Products, Runcorn, Cheshire, UK) and stained with Diff-Quick (Baxter Healthcare, Miami, FL, USA). Per cytospin preparation, at least 500 cells were counted and differentiated into macrophages, eosinophils, neutrophils and lymphocytes by morphology and staining characteristics.

Collection of serum and lung homogenate. After mice were anesthetized, blood was taken by cardiac puncture. Blood was allowed to clot at room temperature for 30 min and serum recovered by centrifugation (10,000 rpm, 5 min at 4˚C) was stored at 70˚C until use. To collect lung homogenates, lungs were homogenized in PBS using a homogenizer (Janke&Kunkel GmbH, Germany).

Preparation and stimulation of lymph node (LN) cells. The LN cells freshly isolated from the paratracheal and parabronchial regions were made into single cell suspensions and contaminated erythrocyte were lysed by hypotonic shock with sterile distilled water for 9 sec. Cells were stimulated with 0-200 μg/ml OVA in the presence of mitomycin-treated splenic B cells at $1x10^6$ cells/ml for 3 days in IMDM medium (Invitrogen Corp., Carlsbad, CA, USA) supplemented with 10% heat inactivated FBS (Invitrogen), 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B (Sigma), $5x10^{-5}$ M 2-mercaptoethanol (2ME).

Enzyme-linked immunosorbent assay (ELISA). The levels of cytokines in BALF and culture supernatants and serum IgE were determined by sandwich ELISA using antibody pairs against murine IFN-γ, IL-5, IL-13 (Biosource International, Camarillo, CA, USA), IL-4 (BioLegend, San Diego, CA, USA) and TNF-α, and IgE, (BD Pharmingen, San Diego, CA, USA) following manufacturer's instruction.

Western blot analysis for PKC-ζ. Whole lung tissues were homogenized in each protein extraction buffer (Cytobuster™ protein extraction reagent or PhosphoSafe™ Extraction Buffer, Novagen) after washing twice in cold PBS. The homogenates were centrifuged at 14,000 rpm at 4˚C for 20 min, and the supernatants were collected. Protein concentration was determined by BCA protein assay. Samples with equal amounts of protein were mixed with 2X SDS sample buffer consisting of 20% glycerol, 4% SDS, 0.16 M Tris-HCl (pH 6.8), 4% 2-ME, and 0.5% bromophenol blue, and then boiled for 5 min. Protein extracts were loaded onto a 10% SDS-PAGE gel and electrophoretically separated. Proteins were transferred onto nitrocellulose membrane (Hybond ECL; Amersham Pharmacia Biotechnology Inc., Piscataway, NJ, USA) and blocked overnight at 4˚C in Tris-buffered saline, pH 7.6, containing 0.1% Tween-20 (TBST) and 5% non-fat dry milk. The membrane was sequentially incubated for 1 h at room temperature with anti-PKC-ζ (Zymed Laboratories, South San Francisco, CA, USA) (0.5 μg/ml) or anti-phospho-PKC-ζ (Cell Signaling Technology Inc., Danvers, MA, USA) (0.2 μg/ ml) and then with an HPR-conjugated goat anti-rabbit (Sigma) diluted to 1:4,000. The membrane was washed repeatedly in TBST between incubations. Visualized with ECL detection reagents (Amersham Biosciences, Buckinghamshire, UK), and finally exposed to photographic film (Hyperfilm ECL; Amersham Biosciences).

Proliferation assay. For the proliferation assays, LN cells were stimulated with 0, 2, 20 or 200 μg/ml OVA in the presence of mitomycin-treated splenic B cells in 96-well plates. Cells were pulsed with 0.5 μCi [^{3}H]-TdR for final 12-16 h and harvested on day 3, and then [^{3}H]-TdR incorporation was counted using a liquid scintillation counter.

Statistical analysis. Statistical analysis and graphical presentation was done using SigmaPlot 5.0 (SPSS Inc., Chicago, IL, USA). Values are given as means ± SE and group means were compared with Student's t-test in which $P<0.05$ was considered significant.

Results

PKC-ζ is activated during OVA sensitization and challenge. At first, we confirmed PKC-ζ activation in our model of asthma, in which mice were sensitized i.p. with 50 μg OVA on days 0 and 7 and intranasally challenged with 2% OVA on days 15, 16 and 18. On day 19, PKC-ζ expression was determined by Western blotting and data showed that sensitization and challenge with allergen substantially induce phosphorylation of PKC-ζ in the lungs (Fig. 1). These results support that PKC-ζ activation is involved in the provocation of asthmatic responses (21).

PPI instillation attenuates AHR and lung inflammation in a mouse model of asthma. AHR is one of the characteristic

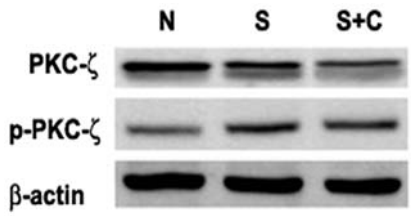

Figure 1. Western blot analysis for PKC-ζ activation. Extracts of lung tissues from sensitized (S) or sensitized and challenged (S+C) or non-immunized (N) mice on day 19 were analyzed by immunoblotting for PKC-ζ or phospho-PKC-ζ. Representative data from 2 independent experiments are shown.

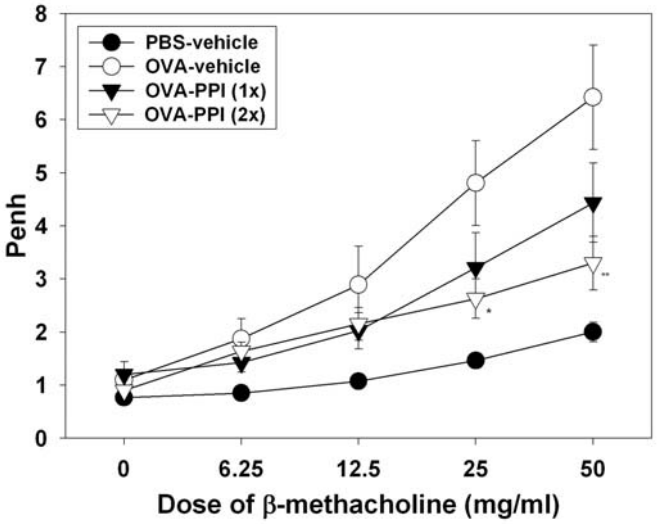

Figure 2. PPI instillation attenuates AHR. Single (1x, on day 16) or double (2x, on days 16 and 18) instillation of PPI or vehicle was performed intratracheally prior to antigen challenge. On day 19, AHR was measured by recording respiratory pressure curves in response to inhaled nebulized methacholine using whole-body plethysmography. Data are shown as the cumulative means ± SE of 13 individual mice from 3 independent experiments. *P<0.05, **P<0.01 vs each control (OVA-vehicle).

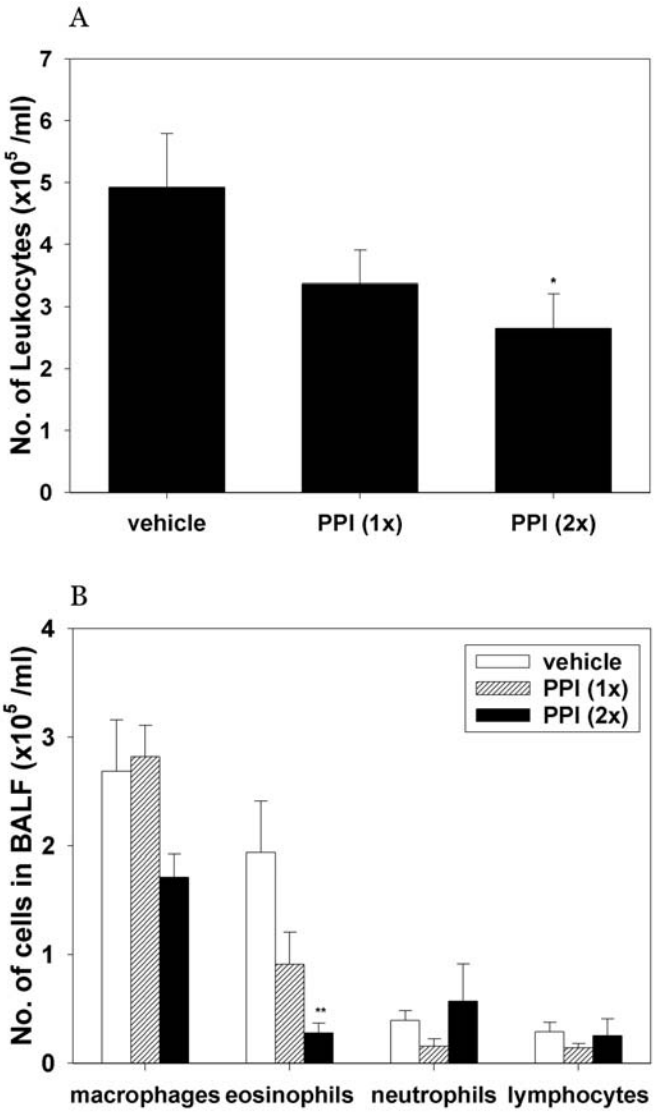

Figure 3. PPI instillation reduces airway inflammation. After antigen challenge and PPI instillation, on day 19, total numbers of leukocytes (A) in recovered BALF were enumerated and their differentials (B) were analyzed with cytosmeared preparations. Data are shown as the cumulative means ± SE of 13 individual mice from 3 independent experiments. *P<0.05, **P<0.01 vs each vehicle control.

features in allergic asthma. To examine the symptomatic outcome of the blockade of PKC-ζ activation, we performed single (on day 18) or double (on days 16, and 18) instillation of PPI 1 h after each OVA challenge, while control mice were given vehicle (PBS) instead of OVA. When AHR to increasing concentrations of aerosolized methacholine was measured on day 19, as shown in Fig. 2, vehicle-treated control mice had developed significant AHR. However, mice sensitized and challenged in the same manner with OVA showed ~50% reduced level of Penh following double instillation (2x) of PPI. Significant differences of Penh value were observed with 25 (4.8±0.8 vs 2.6±0.4, P<0.05, n=13) and 50 mg/ml (6.4±0.8 vs 3.3±0.3, P<0.01, n=13) of methacholine. However, mice with single instillation (1x) of PPI failed to show notable effect (Fig. 2).

To validate whether PPI instillation is capable of reducing lung inflammation, airway recruitment of leukocytes were analyzed. Data show that double instillation of PPI considerably reduce total number of leukocytes (4.8±0.9 vs 2.7±0.6x10^5 cells/ml, P<0.05, n=13) (Fig. 3A) and absolute number of eosinophils (1.9±0.5 vs 0.3±0.1x10^5 cells/ml, P<0.01, n=13) (Fig. 3B) in BALF, when compared to those of vehicle-treated mice. However, the number of macrophages was slightly decreased without significance. These results clearly indicate that PPI instillation alleviates allergic responses in asthmatic mice.

PPI instillation suppresses type 2 cytokine levels in BALF. To further define the effectiveness of PPI instillation to inhibit asthmatic manifestation, we evaluated *in vivo* levels of relevant cytokines. Levels of IL-4 (306.1±46.6 pg/ml) and IL-13 (570.5±135.6 pg/ml) in BALF of controls were not significantly different from those in mice with single instillation of PPI. However, those levels were dramatically diminished following double instillation of PPI (IL-4: 80.4±22.0, P<0.01; IL-13: 118.2±24.5 pg/ml, P<0.01, n=12) (Fig. 4A and C). Levels of IL-5 and IFN-γ in BALF were comparable between

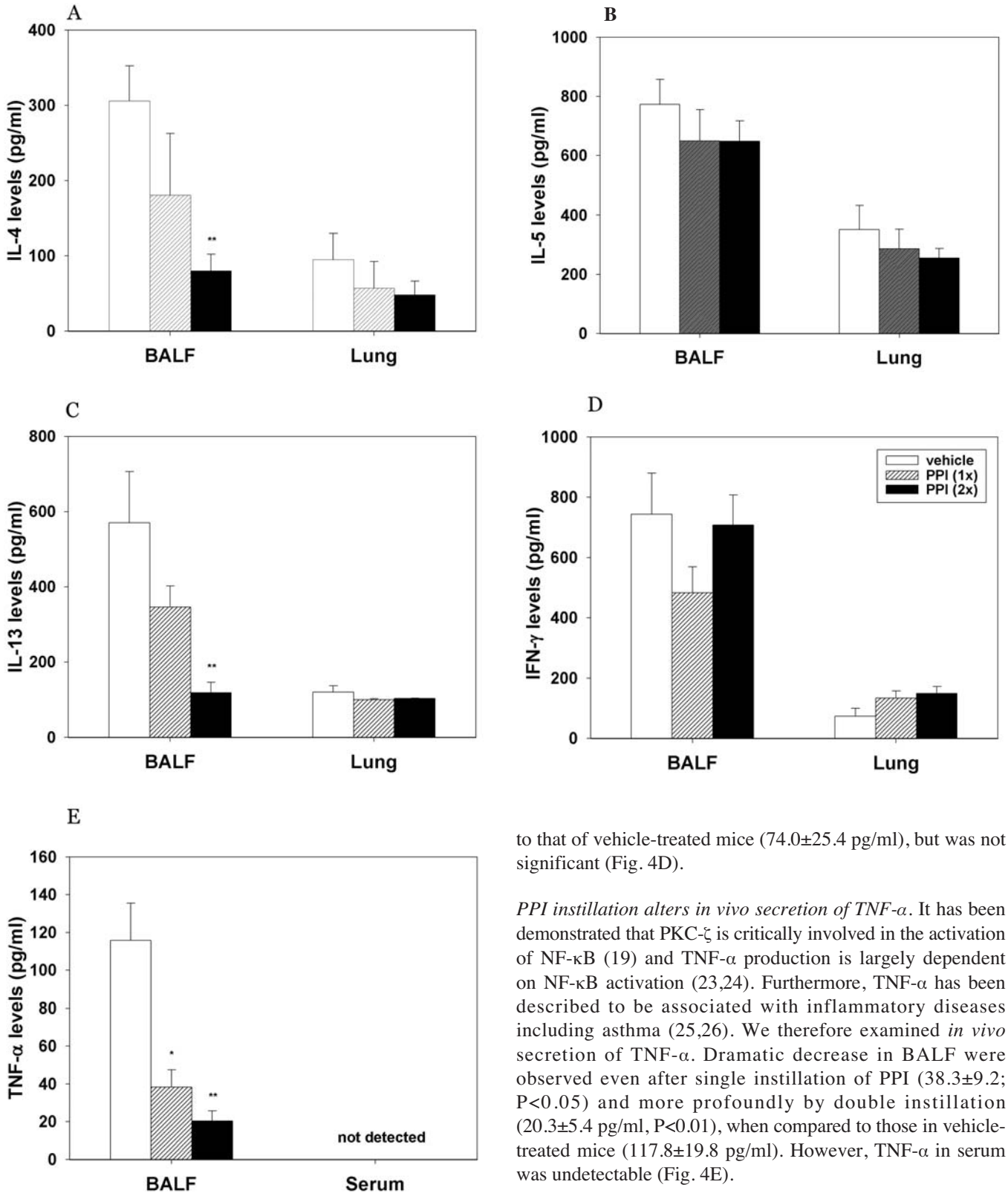

Figure 4. PPI instillation inhibits *in vivo* secretion of TH2 cytokines. BALF and lung homogenate were recovered on day 19 and levels of IL-4 (A), IL-5 (B), IL-13 (C), IFN-γ (D) and TNF-α (E) were determined by ELISA. Data are shown as the cumulative means ± SE of 9-13 individual mice from 2 to 3 independent experiments. *P<0.05, **P<0.01 vs each vehicle control.

all groups. Levels of type 2 cytokines in lung tissues showed no significant difference, although IFN-γ level in PPI (2x) group (149.2±23.2 pg/ml) increased by 2-fold when compared to that of vehicle-treated mice (74.0±25.4 pg/ml), but was not significant (Fig. 4D).

PPI instillation alters in vivo secretion of TNF-α. It has been demonstrated that PKC-ζ is critically involved in the activation of NF-κB (19) and TNF-α production is largely dependent on NF-κB activation (23,24). Furthermore, TNF-α has been described to be associated with inflammatory diseases including asthma (25,26). We therefore examined *in vivo* secretion of TNF-α. Dramatic decrease in BALF were observed even after single instillation of PPI (38.3±9.2; P<0.05) and more profoundly by double instillation (20.3±5.4 pg/ml, P<0.01), when compared to those in vehicle-treated mice (117.8±19.8 pg/ml). However, TNF-α in serum was undetectable (Fig. 4E).

PPI instillation decreases Ag-specific IgE levels in sera. Based on a previous study showing that PKC deficiency dramatically reduced serum IgE level (20), we evaluated whether PPI displays similar effect *in vivo* by measurement of OVA-specific IgE levels in serum. As shown in Fig. 5, markedly lower levels of OVA-specific IgE was detected in serum (P<0.01) in PPI-instilled mice.

PPI instillation selectively suppresses ex vivo TH2 response of LN cells. Our results suggest that PPI instillation selectively

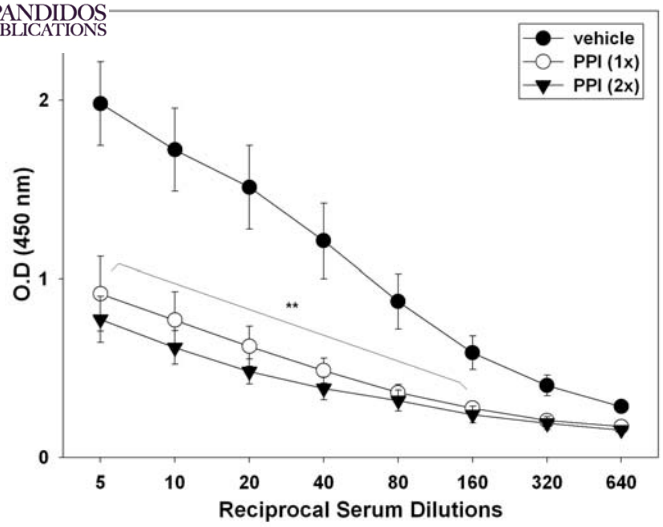

Figure 5. PPI instillation inhibits *in vivo* secretion of Ag-specific IgE. After harvesting airway lavage, blood was taken by cardiac puncture and serum was recovered. Ag-specific serum IgE level was measured by ELISA. Data are shown as the cumulative means ± SE of 10-13 individual mice from 3 independent experiments. **P<0.01 vs each vehicle control.

down-regulates *in vivo* TH2 response. For further insight into this conclusion drawn from the above experiments, we analyzed *ex vivo* Ag-specific responses of draining LN cells from OVA-immunized mice. As shown in Fig. 6A, PPI instillation does not affect Ag-specific proliferative response of LN cells. However, production of TH2 cytokines, IL-4 (688.8±44.7 vs 404.4±59.1 pg/ml; P<0.01, n=10), IL-5 (4,705.1±1207.5 vs 1,797.5±376.2 pg/ml; P<0.05, n=10) and IL-13 by OVA-stimulated LN cells was markedly diminished by double instillation of PPI, whereas there was no significant change in IFN-γ production (Fig. 6B).

Discussion

Although great advancement has been recently achieved in understanding the immunopathogenesis of bronchial asthma, the development and regulation of allergic response remains still incompletely understood. Accumulated data strongly support that TH2 cells play a pivotal role in orchestrating airway inflammation, and unraveling of TH2 signaling pathways has thus been an area of intensive study for identifying therapeutic target in allergic diseases including asthma.

PKC-ζ plays an essential role in the regulation of NF-κB transcriptional activity (19). Given that members of the NF-κB family are important in the pathogenesis of allergic airway inflammation in murine models of asthma (27,28), it is not surprising that PKC-ζ deficiency inhibits allergic airway disease (21). Nonetheless, *in vivo* employment of PKC-ζ inhibitor to control allergic asthma has not been tried so far. In the present study, we took advantage of the ability of synthetic pseudosubstrate peptides, with sequences based on the endogenous PKC-ζ pseudosubstrate region, to inhibit PKC-ζ kinase activity in a mouse model of allergic asthma.

As observed by other investigators in eosinophils after allergen challenge (29) and in the lungs of asthmatic mice (21), PKC-ζ activation was prominent in our mouse model of

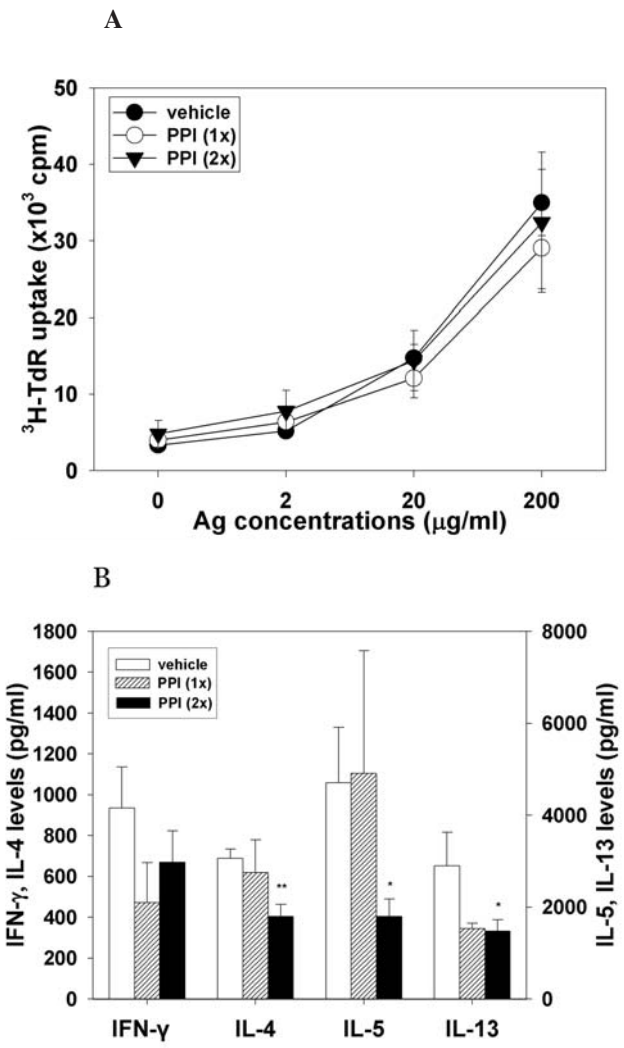

Figure 6. PPI instillation suppresses *ex vivo* production of TH2 cytokines. Draining LN cells were recovered on day 19 and stimulated with 0-200 μg/ml OVA for proliferation assay (A) or 200 μg/ml OVA for cytokine induction (B) in the presence of mitomycin-treated splenic B cells for 3 days. Proliferation was evaluated by 3H-TdR incorporation assay and levels of cytokines in culture supernatants were determined by ELISA. Data are shown as the cumulative means ± SE of 9-10 individual mice from 2 independent experiments. *P<0.05, **P<0.01 vs each vehicle control.

asthma after antigen sensitization and challenge (Fig. 1) and which provides a rationale for the present study. Our data showed that PPI instillation can attenuate asthmatic manifestation including airway hyper-reactivity (Fig. 2) and lung inflammation (Fig. 3) and inhibit *in vivo* secretion of TH2 cytokines (Fig. 4) and allergen-specific IgE (Fig. 5). Although IL-5 level in BALF was not significant between control and PPI-treated groups, the hypothesis that PPI down-regulates *in vivo* TH2 response was supported also by additional data showing that *ex vivo* production of IL-4, IL-5 and IL-13 by draining LN cells were significantly suppressed by PPI (Fig. 6).

Consistent with a previous report, PPI instillation failed to reduce IFN-γ secretion as well as LN cell proliferation, indicating that PPI selectively controls TH2 response without alteration of TH1 response and T cell proliferation. Data from Martin *et al* (21) have suggested a mechanism underlying this effect of PPI. They described that PKC-ζ is required for

the activation of Jak1 and the subsequent phosphorylation and nuclear translocation of Stat6, which are essential downstream targets of IL-4 signaling, but not in TCR downstream events.

TNF-α is a cytokine associated with inflammatory diseases including asthma (25,26). TNF-α secretion is likely to be the most sensitive to blocking of PKC-ζ because TNF-α is the sole cytokine defectively secreted even with single instillation of PPI (Fig. 4E). It thus may reinforce that PKC-ζ is critically involved in the activation of NF-κB (19) on which TNF-α production is largely dependent (23,24). However, PKC-ζ is not a direct target of TCR signaling so that lack of PKC-ζ does not affect naïve T cell proliferation and NF-κB activation (21). These findings also provide a promise that PPI may be applied without risk of suppression in normal immune response. Additionally, it is notable that local administration of PPI can reduce antigen-specific TH2 response, as it suggests that PPI can be easily applied for clinical use.

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