

A Novel Phycobiliprotein Alleviates Allergic Airway Inflammation by Modulating Immune Responses

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Rationale: It has been claimed that phycocyanin exhibits pharmaceutical functions in inhibiting histamine release and leukotriene biosynthesis. In allergic asthma, these inflammatory mediators are crucial for disease progression.

Objectives: The aim of this study was to evaluate the therapeutic potential of R-phycocyanin (R-PC) against allergic airway inflammation.

Methods: Mouse bone marrow–derived dendritic cells (BMDCs) were used to evaluate the immunomodulatory functions of R-PC. In addition, an airway inflammatory model was used to evaluate the therapeutic potential of R-PC.

Measurements and Main Results: R-PC treatment resulted in a decrease of endocytosis, increase of costimulatory molecule expression, and enhancement of interleukin-12 production in mouse BMDCs. Moreover, R-PC–treated cultured dendritic cells were able to promote CD4⁺ T-cell stimulatory capacity and increase interferon- γ expression in CD4⁺ T cells. Intraperitoneal administration of R-PC suppressed ovalbumin (OVA)–induced airway hyperresponsiveness, serum levels of OVA-specific IgE and IgG1, eosinophil infiltration, Th2 cytokine levels, and eotaxin in bronchoalveolar lavage fluid of mice. Antibody against Toll-like receptor-4 was able to inhibit R-PC–induced IL-12 p70 production. Moreover, inhibition of nuclear factor- κ B (NF- κ B) by helenalin and inhibition of the JNK pathway by JNK inhibitor II inhibited R-PC–induced IL-12 p70 production. Western blotting and electrophoretic mobility shift assays showed that R-PC augmented phosphorylation of the inhibitors of NF- κ B and inhibitors of NF- κ B kinase and facilitated NF- κ B activity.

Conclusions: Our data demonstrated that R-PC promoted activation and maturation of cultured dendritic cells and skewed the immunological function toward Th1 activity. Therefore, R-PC may have potential in regulating immune responses and application in reducing allergic asthma.

Keywords: R-phycocyanin; Th1; immunomodulation; allergic asthma

Asthma is a chronic inflammatory disease of the lungs that is increasingly observed in both developed and developing countries (1, 2). Numerous studies performed with asthma animal models have shown increased Th2 cytokine levels and decreased Th1 cytokine levels within the affected airways. Moreover, Th2 cells together with other inflammatory cells, such as eosinophils, mast cells, and B cells, play a major role in the initiation and pathological development of asthma (3, 4).

Dendritic cells (DCs) are professional antigen-presenting cells. In response to various inflammatory stimuli, DCs increase

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Phycocyanin is a phycobiliprotein derived from algae. Pharmaceutical applications of phycocyanin have been claimed, including inhibitory effects on reactive oxygen species, prostaglandin, and leukotriene biosynthesis.

What This Study Adds to the Field

In vitro studies revealed that R-phycocyanin (R-PC) up-regulated IL-12 p70 cytokine production in mouse bone marrow–derived dendritic cells. In an experimental model of airway inflammation R-PC skewed ovalbumin-specific immune responses to Th1, suggesting the therapeutic potential of R-PC in allergic airway inflammation.

expression of costimulatory molecules and produce large amounts of endogenous proinflammatory agents, such as tumor necrosis factor- α , IL-6, IL-12, and leukotriene B4 (5, 6), and antiinflammatory molecules such as prostaglandin E2 and IL-10 (6). These inflammatory mediators promote maturation and recruiting of DCs to the secondary lymphoid organs (7, 8). Functions of DCs can be altered by the cytokines in the environment; therefore, DCs can acquire the ability to skew T-helper responses so that Th1 profiles can be converted to Th2-skewing cells when treated with antiinflammatory cytokines or type two tissue factors, such as IL-10, transforming growth factor- β , prostaglandin E2, or histamine. Airway DCs are crucial immune cells in determining the outcome of inhaled antigens during periods of sensitization and acquire the effector function of Th2 cells in response to allergens leading to allergy. Thus, airway DCs are generally held responsible for initiating and maintaining allergic Th2 cell responses in patients with asthma. Therefore, targeted therapies have been directed toward suppressing the production and action of Th2 cytokines and enhancing Th1 responses (9, 10).

Bangia atropurpurea is a seaweed belonging to Rhodophyta, Bangiales, and Bangiaceae, which are commonly used in many Asian countries as dietary supplements. According to the oldest known Chinese medical text, Bencao Gangmu (51) recorded that seaweed can be used for regulating chronic bronchitis, digesting sputum, and reducing cough. R-phycocyanin (R-PC), derived from *B. atropurpurea*, a water-soluble protein pigment, is linked to bilin chromophores to trimeric α subunits (17.06 kD) and β subunits (26.69 kD) through thioether bonds.

The most common phycocyanin is purified from *Spirulina*, whose nutritional and therapeutic values have been well documented (11). The medicinal and pharmaceutical properties of phycocyanin include antioxidant, antiinflammatory, atherosclerotic, antitumor, and neuroprotective effects (11, 12). To date, few reports have studied the immunological functions of phyco-

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cyanin. Ramirez and colleagues have studied that phycocyanin derived from *Arthrospira maxima* exerts an inhibitory effect on histamine release from mast cells (13). In the present study, we examined the molecular characteristics of R-PC on IL-12 p70 production in mouse BMDCs and explored the regulatory effects on modulating immune functions of inflammatory airway disease.

METHODS

Mice

Female 6- to 8-week-old BALB/c mice were obtained from the Animal Center of the College of Medicine of National Taiwan University and the animal study protocol was approved by the Animal Research Committee of the College of Medicine, National Taiwan University.

Effects of R-PC on DCs

R-PC was kindly provided by Dr. Hung-Non Chou and prepared as described in the online data supplement, treated with Detoxi-Gel (Pierce, Rockford, IL) to remove contaminated endotoxin, and stored at 4°C ready for use. Mouse BMDCs were prepared as previously described (14, 15) ready for further experiments. After R-PC treatment, BMDCs were stained with rat anti-mouse monoclonal antibodies, IA^d (MHC class II), CD80, CD86, CD40, CD205, and CD11c (eBioscience, San Diego, CA) or isotype controls to evaluate surface costimulatory markers expression. Endocytosis was used to evaluate the maturation of BMDCs after R-PC treatment as described previously (16). Stained cells were analyzed by a FACS Calibur (Becton Dickinson). Data were processed with CellQuestPro (Becton Dickinson, Franklin Lakes, NJ) software. Histogram plots were used to show the indicated markers evaluated by flow cytometric analysis after gating the BMDC population expressing CD11c molecule. Cultured supernatants were harvested ready for cytokine analysis by ELISA.

Effects of R-PC on T Cells

Proliferation and cytokine secreting levels were determined by mixed lymphocytes reaction (MLR) as described in the online data supplement to examine the immunomodulatory capability of R-PC.

Measurement of Cytokine Expression

Levels of IL-4, IL-5, IL-10, IL-13, IFN- γ , and eotaxin were measured in bronchoalveolar lavage fluid (BALF) or culture supernatants using commercially available ELISA kits (DuoSet; R&D Systems, Minneapolis, MN). IL-12p70 production was determined using an ELISA kit (BD Pharmingen, San Diego, CA) according to the manufacturer's instructions.

The Effects of R-PC on Ovalbumin-Specific Antibodies

Serum anti-ovalbumin (OVA) IgE, IgG1, and IgG2a antibody titers were determined by ELISA as described by Huang and colleagues (17). Levels of antibodies were compared with standard serum and the concentration of IgG1, IgE, and IgG2a in standard serum was arbitrarily assigned 1 ELISA unit (EU): EU = (a sample – a blank)/(a positive – a blank).

Murine Model of OVA-Induced Allergic Airway Inflammation

A modified mouse model as previously described (18–20) was performed as described in the online data supplement. Briefly, mice were sensitized by six intraperitoneal injections or seven intranasal inhalations of OVA (grade V; Sigma-Aldrich, St. Louis, MO) mixed with R-PC in phosphate-buffered saline (PBS). The disease group of mice was immunized with OVA alone. A naive group of mice was administered PBS. For challenge, all mice were treated intranasally with OVA. Mice in the naive group were administered PBS. Mice were killed at 24 or 36 hours after last immunization.

Histology

Lungs were obtained for histologic analysis and fixed with 10% neutralized buffered formalin. Sections (5 μ m thick) were prepared and subjected to hematoxylin and eosin staining and examined by light microscopy.

Measurement of the Airway Hyperresponsiveness

Lung function was measured by measuring the changes of lung resistance in response to increasing doses of aerosolized methacholine (MCh) (3.125–25 mg/ml) (Sigma-Aldrich) following a modified technique described by Huang and colleagues and Glaab and colleagues (17, 21) using a Buxco Pulmonary Mechanics System (Buxco Electronics, Wilmington, NC). Mechanical ventilation with 150 breaths/min, 0.3 ml of tidal volume, and 4 cm H₂O of positive end-expiratory pressure were provided through the tracheal cannula. Pulmonary parameters were recorded for 3 minutes after 3-minute exposure of aerosol MCh and analyzed by BioSystem XA software. Lung resistance was expressed as RL (cm H₂O $\times$ s/ml).

Preparation of BALF

BALF was collected from each mouse as previously described (19, 20). Supernatant derived from the first lavage was stored at –20°C until measurement. Levels of cytokines were determined by ELISA and the cell pellet was added to two subsequent lavages and resuspended in PBS for cell counting. Based on morphology, a minimum of 200 cells were counted and classified as macrophages, lymphocytes, neutrophils, or eosinophils to analyze the inflammatory cell population in the BALF.

The Effects of R-PC on Airway DCs

Mouse peribronchial lymph nodes and cervical lymph nodes were collected and used to analyze the immunomodulatory effects of R-PC on mouse airway DCs as described in the online data supplement. Cultured supernatants were harvested and stored at –20°C until measurement by ELISA.

The Effects of Toll-like Receptor 4 and Cellular Factors on R-PC-Induced IL-12 Production

For Toll-like receptor 4 (TLR4) blocking experiments, mouse BMDCs were pretreated as previously described (16). For cellular factor analysis, BMDCs were pretreated with inhibitors as previously described (16). Some of the BMDCs were left untreated in the presence of medium to serve as controls.

Immunoblots and Electrophoretic Mobility Shift Assay

BMDCs were harvested and stimulated with R-PC for the indicated periods of time. Thereafter, R-PC-stimulated BMDCs were treated with NE-PER nuclear and cytoplasmic extraction reagents (Pierce) according to the manufacturer's instructions to separate nuclear proteins and cytoplasmic proteins from total cellular extracts. The blots were probed with antibodies raised against phosphorylated inhibitors of nuclear factor (NF)- κ B (I κ B) kinase (IKK α / β), phosphorylated I κ B α , total I κ B α (Cell Signaling Technology, Danvers, MA), or actin (Chemicon, Billerica, MA) followed by anti-rabbit or anti-mouse horseradish peroxidase-conjugated antibodies (Cell Signaling Technology). Nuclear extracts were subjected to electrophoretic mobility shift assay (EMSA) according to the gel shift assay system as described previously (16).

Statistical Analysis

One-way analysis of variance comparison tests were used to evaluate the statistical significances of the differences between experimental groups. A *P* value less than 0.05 was considered statistically significant. Results are presented as means $\pm$ SEM.

RESULTS

R-PC Induces Phenotypic Maturation and Th1 Cytokine Production in Mouse BMDCs

Numerous studies have demonstrated that DC activation and maturation induces cytokine production and up-regulation of costimulatory molecules on the cell surface (16, 22, 23). We investigated whether R-PC could induce functional changes in phenotypic maturation of mouse BMDCs. We compared the phenotypes of mouse BMDCs treated with or without R-PC for

48 hours and determined the expression levels of surface markers by measuring mean fluorescence intensity. As shown in Figure 1A, treatment of BMDCs with R-PC resulted in slight increase of CD40, CD80, CD86, CD205, and major histocompatibility complex (MHC) class II molecules on the cell membranes of mouse BMDCs. Furthermore, we investigate whether R-PC could affect the cytokine production in BMDCs. As illustrated in Figure 1B, we found that R-PC significantly enhanced production of IL-12 p70 compared with untreated BMDCs. However, the level of IL-10 was only slightly increased by R-PC treatment. Thus, we examined the potency of R-PC-treated BMDCs to capture fluorescein isothiocyanate (FITC)-labeled dextran by measuring mean fluorescence intensity. Figure 1C illustrates that cytoskeletal rearrangement and cell metabolism were inhibited at 4°C; therefore, BMDCs were incapable of capturing dextran-FITC. However, the untreated BMDCs showed significant uptake of dextran-FITC at 37°C, which was revealed by an increase in green fluorescence. In contrast, lipopolysaccharide (LPS) treatment of BMDCs induced a loss of this capacity. Furthermore, R-PC-treated BMDCs also lost the ability to capture dextran-FITC, which were matured by R-PC as compared with untreated BMDCs. Taken together, these results indicate that R-PC potentially stimulates mouse BMDCs undergoing activation and maturation, and is capable of up-regulating costimulatory markers on the cell surface of BMDCs, reducing the capability of dextran-FITC uptake and elevating the stimulatory effect of R-PC on IL-12p70 production in a dose-dependent manner. Our results suggest that R-PC potentially triggers Th1 polarization through inducing IL-12 p70 production from BMDCs.

BMDCs Cultured with R-PC Elicit High Levels of IFN- γ Production by Allogenic T Cells

To determine whether the R-PC-treated BMDCs were able to promote activation of naive T cells, we examined the efficiency of R-PC-treated BMDCs in the activation of T cells in a MLR. Untreated, LPS-treated, or R-PC-treated BMDCs were irradiated and then used to activate allogenic CD4⁺ T cells derived from C57BL/J mice in a different ratio of DCs to CD4⁺ T cells. Our present data showed that a higher level of T-cell proliferation was obtained in CD4⁺ T cells stimulated by LPS or R-PC-treated BMDCs than that seen in coculture with untreated BMDCs, especially at high DC/T cell ratios (Figure 2A). Furthermore, LPS-treated or R-PC-treated BMDCs increased the IFN- γ production in culture supernatants. In addition, the level of IFN- γ gradually increased with culture time, and was the highest at 96 hours in MLR. Conversely, the levels of IL-4 obtained from these two treatments gradually decreased with time. It was interesting to note that IL-4 was observed to increase with time in allogenic CD4⁺ T cells stimulated with untreated BMDCs, which showed inversion to compare with the level of IL-4 in allogenic CD4⁺ T cells incubated with R-PC-treated BMDCs (Figure 2B). To investigate whether IFN- γ or IL-4 was produced by the activated T cells in this experiment, we performed intracellular cytokine staining to detect IFN- γ or IL-4 in allogenic CD4⁺ T cells (>95% purity by flow), which were cocultured with LPS-treated, R-PC-treated, or untreated BMDCs. Figure 2C shows that LPS-treated or R-PC-treated BMDCs substantially elevated the frequency of IFN- γ -producing CD4⁺ T cells at 96 hours compared with untreated BMDCs. The results showed that the percentages gated of double-positive CD4 and IFN- γ were 55.6% and 58.6% in the group of R-PC-treated BMDCs and LPS-treated DCs, respectively, whereas the percentage gated of double-positive CD4 and IFN- γ was 34.9% in the untreated BMDC group. Conversely, the percentages of IL-4-secreting CD4⁺ T cells in

cultures were 37.3% and 35.4% in the group of R-PC-treated BMDCs and LPS-treated DCs, respectively. Clearly, these results indicate that R-PC-treated BMDCs were potent in inducing T-cell proliferation and augmenting IFN- γ synthesis as well as LPS-treated BMDCs, whereas untreated BMDCs were poor activators.

Inhibitory Effect of R-PC on Airway Inflammation

We next determined whether R-PC could modify an ongoing OVA-induced airway inflammation *in vivo*. Mice were immunized using intraperitoneal injections of OVA and OVA in combination with R-PC (or PBS for the sham group) at Days 0, 5, 10, 15, 20, and 25 and were then subsequently challenged with OVA (or PBS for the sham group) via an intranasal route once a day on Days 35, 36, 37, and 38. We also investigated the eosinophilia in BALF. BALF was collected at 24 hours after the last OVA challenge through intranasal instillation. Total and differential cell counts were performed. As illustrated in Figure 3A, OVA-sensitized and -challenged mice showed significantly increased numbers of total cells in BALF ($P < 0.0001$) at 24 hours after the last OVA challenge as compared with the sham group of mice or mice immunized with OVA in combination with R-PC. In contrast, although intraperitoneal injection of OVA in combination with R-PC resulted in an increased total number of cells ($P < 0.001$) as compared with the PBS controls, the total cell influx was less than for the mice sensitized/challenged with OVA. Furthermore, the numbers of monocytes, neutrophils, and lymphocytes were lower in R-PC admixed with OVA-immunized mice than in OVA-treated group of mice. In particular, the number of eosinophils was significantly reduced in OVA in combination with R-PC-treated mice as compared with OVA-treated mice ($P < 0.0001$). Figure 3B shows that OVA sensitization of mice in combination with or without R-PC induced substantial cytokine release into the BALF as compared with PBS-administered mice. Moreover, OVA-treated mice admixed with R-PC resulted in significant decreases in IL-4, IL-5, IL-10, IL-13, and eotaxin levels in the BALF as compared with OVA-sensitized mice. We also investigated whether R-PC could modulate the immunological functions of DCs *in vivo*. DCs were recovered from peribronchial lymph nodes and cervical lymph nodes derived from OVA-sensitized mice in combination with or without R-PC by CD11c monoclonal antibody-coated microbeads positive selection. Figure 3C illustrates that DCs derived from OVA-sensitized mice in combination with R-PC produced higher amounts of IL-12 p70 as compared with OVA-treated mice. In addition, IL-12p 70 levels were very significantly elevated in DCs cocultured with anti-CD40 antibodies compared with in those obtained from culture with IgG2a serving as isotype control. In contrast, IL-10 levels were unchanged in all analyzed conditions. Thus, R-PC potentially impeded the development of Th2-type airway inflammation. To determine the effect of R-PC on airway function, the mice were exposed to MCh aerosols to measure the airway hyperresponsiveness (AHR). Airway responsiveness was assayed by the invasive technique in anesthetized mice to examine the change of airway resistance in R-PC-treated mice. Figure 3D shows that lung resistance (RL) was slightly affected by high concentrations of MCh in the sham group of mice, but RL was significantly increased when OVA-sensitized/challenged mice inhaled MCh in a dose-dependent manner. In contrast, sensitization with OVA admixed with R-PC resulted in a slight change of RL in mice similar to the sham group of mice. Further, inflammatory cells infiltrated around the airways of OVA-sensitized mice were consistently noted on histopathology analysis. However, there was less inflammatory cell infiltrate in the

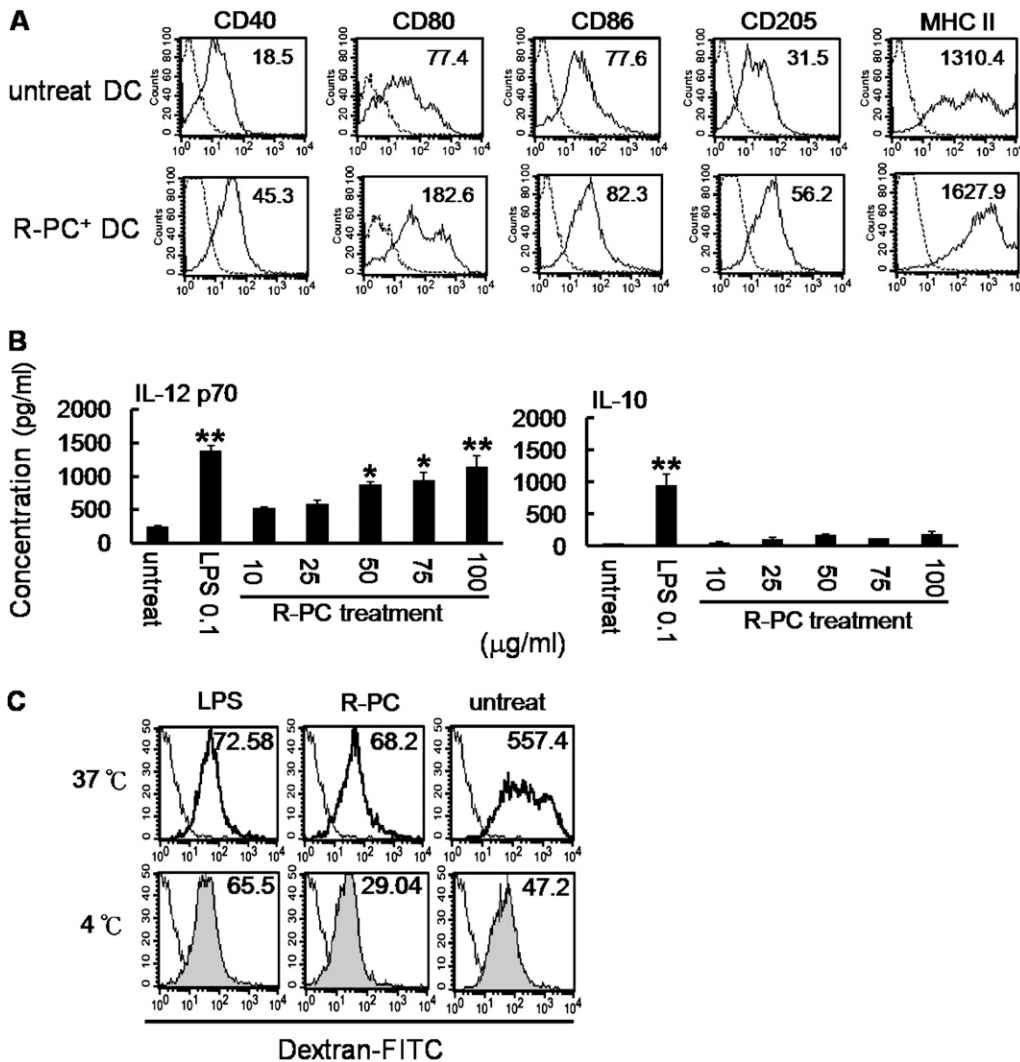


Figure 1. R-phycocyanin (R-PC) induced activation and maturation of bone marrow-derived dendritic cells (BMDCs). (A) R-PC induced up-regulation of costimulatory molecules on BMDC surface. Mouse BMDCs were cultured for 48 hours in the presence of R-PC (75 μg/ml) or medium alone and then stained for the DC-associated activation or maturation markers, CD40, CD80, CD86, CD205, MHC II, plus the CD11c serve as DC marker. The positive cells were first gated by CD11c antibody (Ab) staining and further analyzed for expression of activation- and maturation-related molecules. Dotted lines indicate the cells stained with isotype-matched control Ab; solid lines show the cells stained with specific Ab as indicated. The values shown in the flow cytometry profiles are the mean fluorescence intensity (MFI) indexes. This experiment was repeated three times with similar results. (B) R-PC potentially induces IL-12 p70 cytokine production. Mouse BMDCs were cultured for 48 hours with increasing concentrations of R-PC as indicated. Lipopolysaccharide (LPS, 100 ng/ml)-treated BMDCs were considered as a positive control. At the end of the incubation time, the culture medium was collected for cytokine assay by ELISA. Data represent the mean ± SEM from three independent experiments. Statistical differences are indicated by *P* values (one-way analysis of vari-

ance). **P* < 0.05 and ***P* < 0.001, compared with the levels of cytokine recovered from untreated cells. (C) R-PC reduced the endocytotic capability of mouse BMDCs. On Day 6, mouse BMDCs were harvested and stimulated with or without R-PC (75 μg/ml) for 24 hours. LPS (100 ng/ml) treatment was considered as a positive control. Cells were further incubated with fluorescein isothiocyanate-dextran for 1 hour at 4°C or 37°C. Shaded areas indicate the cells cultured at 4°C; solid lines show the cells cultured at 37°C. The values shown in the flow cytometry profiles are the mean fluorescence intensity indexes. This experiment was repeated three times with similar results.

OVA-treated mice in combination with R-PC (Figure 3E). Collectively, these data indicate that R-PC treatment seems able to block allergic airway inflammation under exposure to allergens.

Regulatory Effect of R-PC on the Th1/Th2 Cell Responses in OVA-Immunized Mice

We examined the regulatory effect of R-PC on Th1/Th2 cell responses in OVA-immunized mice. Mice were immunized with OVA plus R-PC on days 0, 5, 10, 15, 20, and 25. IFN-γ (Th1 cytokine) and IL-4, IL-5, IL-10, IL-13 (Th2 cytokine) production were assayed by splenocytes stimulated with OVA. As illustrated in Figure 4A, compared with OVA-treated mice, IL-4, IL-5, IL-10, and IL-13 cytokine levels were significantly decreased in R-PC admixed with OVA-immunized mice, whereas the production of IFN-γ significantly increased in R-PC admixed with OVA-immunized mice compared with OVA-immunized mice. In addition, OVA in combination with R-PC introduced a higher proliferative activity of OVA-specific T cells in immunized mice as compared with OVA-treated mice (Figure 4B). In addition, we also examined the OVA-specific IgG1,

IgG2a, and IgE immunoglobulin levels in OVA-immunized BALB/c mice. We collected serum from OVA immunized mice in combination with or without R-PC. The sham group of mice was administered PBS and served as normal control animals. The levels of OVA-specific IgE were significantly decreased in OVA admixed with R-PC-treated mice as compared with OVA-sensitized/challenged mice (Figure 4C). Furthermore, R-PC significantly (*P* < 0.05) lowered OVA-specific IgG1 levels. Conversely, OVA-specific IgG2a production from R-PC admixed with OVA-immunized mice was significantly increased at the examined time point after immunization. These results indicated that R-PC potentially inhibits OVA-specific IgG1 and IgE, whereas IgG2a levels were elevated, indicating a specific inhibition on Th2 response by R-PC regulation. These results indicated that R-PC changes the balance of Th1/Th2 cell immune responses from Th2-dominant to Th1-dominant in OVA-immunized mice.

R-PC Induces IL-12 p70 Synthesis through TLR4

We further determined the involvement of these receptors in the interaction of DCs with R-PC by neutralization experi-

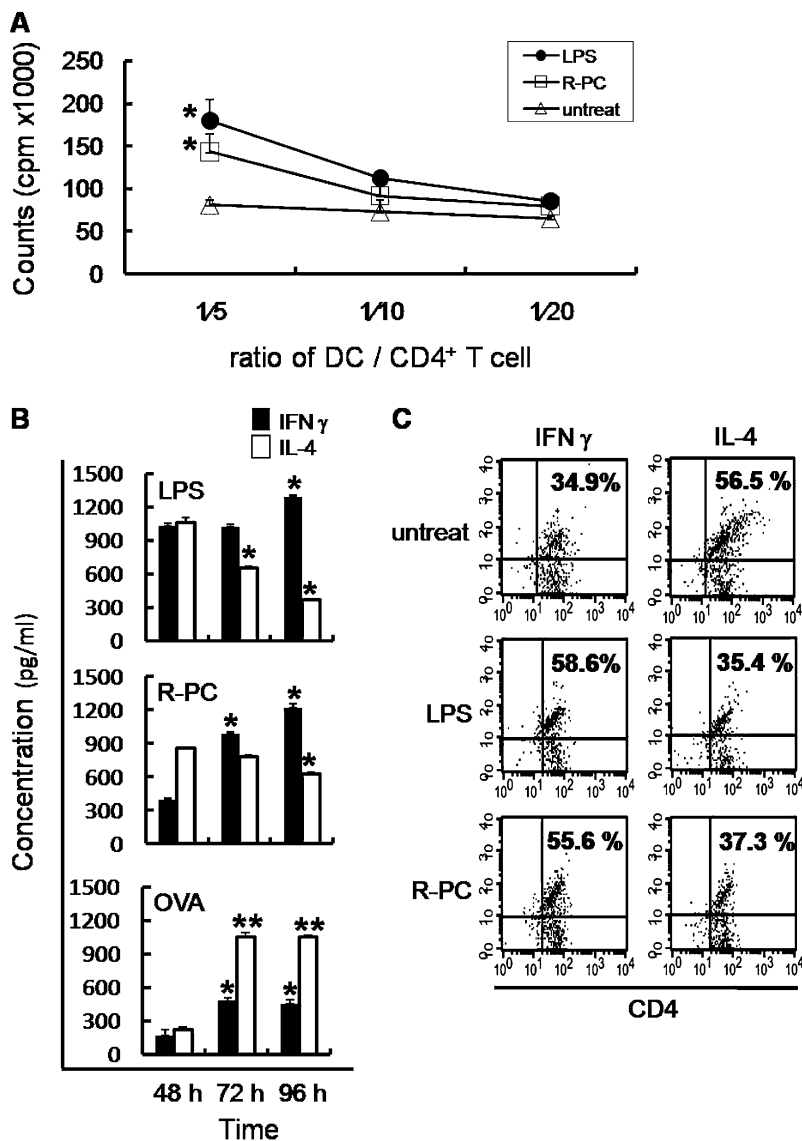


Figure 2. R-phycocyanin (R-PC) augments T-cell proliferation and induces Th1-type immunological responses. On Day 6, mouse bone marrow-derived dendritic cells (BMDCs) were harvested and stimulated with or without R-PC (75 $\mu\text{g/ml}$) for 48 hours. Lipopolysaccharide (LPS, 100 ng/ml) treatment was considered as a positive control. R-PC-treated or LPS-treated BMDCs were further cocultured with allogeneic CD4⁺ T cells for mixed lymphocytes reaction (MLR). (A) R-PC-treated BMDCs potently induced allogeneic CD4⁺ T-cell proliferation. The incorporated radioactivity of T cells was measured by a β -counter at 16 hours after addition of ³H-thymidine. Data represent the mean $\pm$ SEM from three independent experiments. Statistical differences are indicated by *P* values (one-way analysis of variance [ANOVA]). **P* < 0.05 compared with the proliferation activity of T cells cocultured with untreated BMDCs. (B) R-PC-treated BMDCs preferentially induced IFN- γ production over IL-4 production in culture. Culture supernatants were collected from MLR and then both IFN- γ and IL-4 levels were determined by ELISA at the time indicated. Data represent the mean $\pm$ SEM from three independent experiments. Statistical differences are indicated by *P* values (one-way ANOVA). **P* < 0.05 compared the levels of cytokines produced in each group at 48 hours to those at 72 or 96 hours. (C) Both IFN- γ and IL-4 were produced by allogeneic CD4⁺ T cells. Cultured cells were harvested at 96 hours from MLR. Harvested cells were double stained with anti-IFN- γ and anti-CD4 antibodies (Abs) or anti-IL-4 and anti-CD4 Abs. CD4⁺ cells were first gated and further analyzed for cytokine expression by intracellular staining with specific Abs. The values shown in the flow cytometry profiles are the cell percentages of CD4⁺IFN- γ ⁺ or CD4⁺IL-4⁺. This experiment was repeated three times with similar results. cpm = counts per minute.

ments. Mouse BMDCs were pretreated with antibodies raised against TLR4 and further stimulated with increasing concentrations of R-PC. IgG2a-treated BMDCs served as negative controls. As illustrated in Figure 5A, we demonstrated that the addition of an anti-TLR4 monoclonal antibody to BMDC blocked R-PC (5, 10, 25, 50, and 75 $\mu\text{g/ml}$)-induced IL-12 p70 production. The data indicated that R-PC can interact with TLR4 to induce DC activation and IL-12 p70 production in murine BMDCs. Because human monocyte-derived DCs were also activated by R-PC in our study (data not shown), we further demonstrated that R-PC can interact with human TLR4 by the human TLR4-expressed HEK293 cells to show the binding capacity of R-PC with TLR4 (see Figure E1 in the online data supplement). In addition, we further investigated whether the R-PC-induced secretion of IL-12 p70 was affected by the inhibitors of NF- κB , p38 MAPK, p42/44 ERK, or p46/54 JNK. Mouse BMDCs were pretreated with helenalin (an inhibitor of NF- κB), SB203580 (an inhibitor of p38 MAPK), PD98059 (an inhibitor of the ERK pathway), or JNK inhibitor II (an inhibitor of the JNK pathway) for 1 hour and subsequently stimulated with R-PC for 48 hours. The production of IL-12 p70 was quantified by means of ELISA. Figure 5B shows that R-PC-induced IL-12 p70 cytokine production was signif-

icantly reduced by JNK inhibitor II treatment as compared with the levels of cytokines recovered from R-PC-treated BMDCs in culture containing DMSO (0.1%). Moreover, the level of IL-12 p70 was significantly abrogated by helenalin treatment. In contrast, both SB203580 and PD98059 were not affected by R-PC-induced IL-12 p70 production in mouse BMDCs.

R-PC Induces Phosphorylation of IKK α/β , I κ B α , and NF- κB Activation in Mouse BMDCs

As the activation of IKK α/β activity is necessary for I κ B α phosphorylation, the effects of R-PC on IKK α/β activation and I κ B α phosphorylation were likewise studied. Mouse BMDCs were treated with R-PC (75 $\mu\text{g/ml}$) for the indicated periods of time. LPS-treated BMDCs were considered as positive controls. Both phosphorylated IKK α/β and I κ B α proteins were measured by Western blotting with antibodies raised against phosphorylated mouse IKK α/β and I κ B α in cytoplasmic extracts derived from R-PC-treated BMDCs. As illustrated in Figure 6A, the protein levels of phosphorylated IKK α/β and I κ B α were shown as changes in kinetics. After stimulation with R-PC, phosphorylated IKK α/β and I κ B α proteins appeared

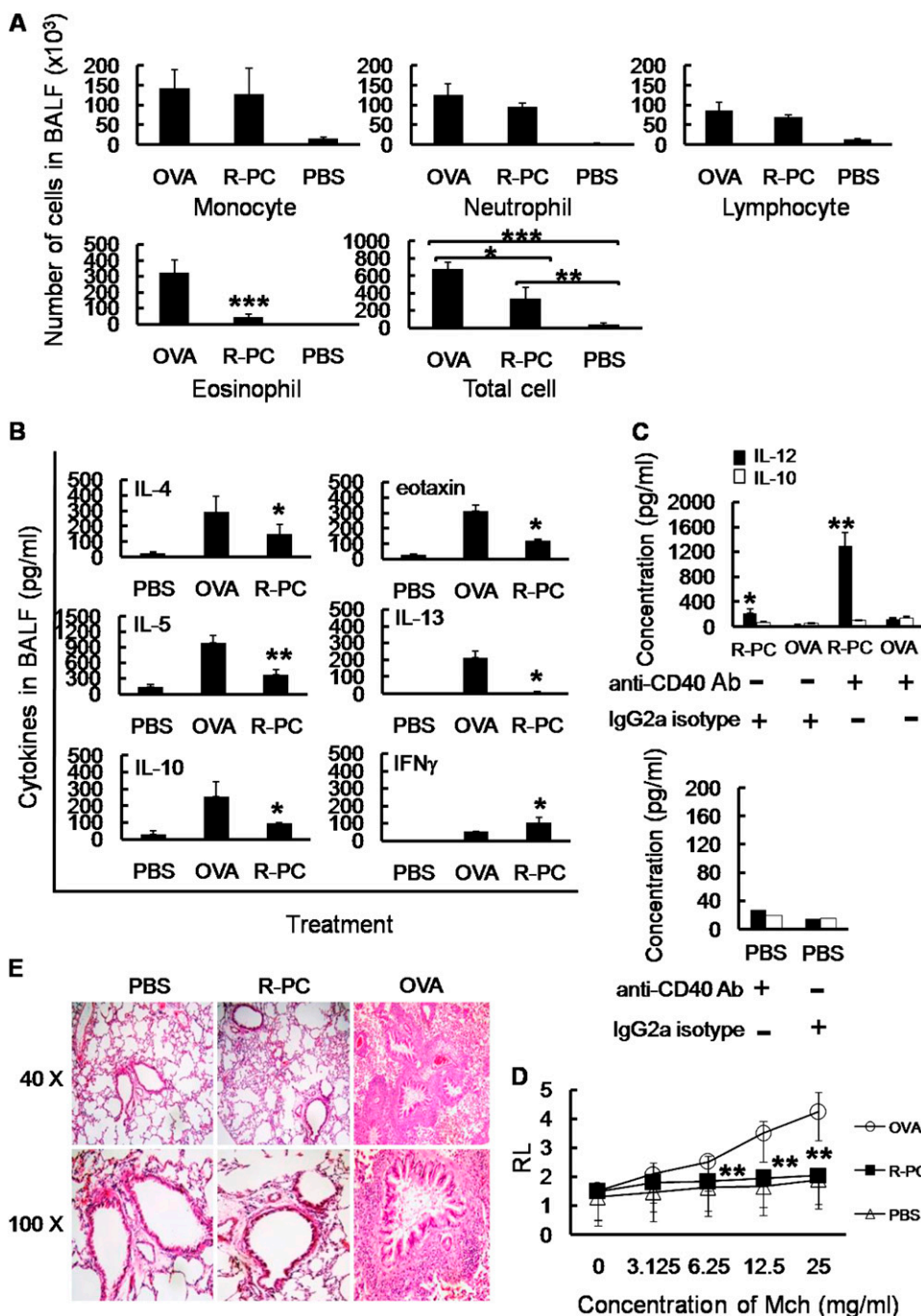


Figure 3. Immunomodulatory effects of R-phycoerythrin (R-PC) administration on airway inflammation in ovalbumin (OVA)-immunized mice. Mice were immunized with OVA, OVA plus R-PC, or phosphate-buffered saline (PBS) and challenged with OVA for four consecutive days. PBS mice were immunized and challenged with PBS as a control of R-PC. R-PC inhibits infiltration of eosinophils. Bronchoalveolar lavage fluid (BALF) was collected at 24 hours after the last OVA challenge. Total and differential cell counts were performed on a minimum of 200 cells to identify Total cell, Eosinophil, Neutrophil, Monocyte, and Lymphocyte counts. Data represent the mean $\pm$ SEM from three independent experiments; $n = 6-8$ per group. Statistical differences are indicated by P values (one-way analysis of variance [ANOVA]). $*P < 0.05$, $**P < 0.001$, and $***P < 0.0001$, compared with the OVA-immunized group. R-PC suppresses Th2 cytokine production. BALF was collected at 24 hours after the last OVA challenge. Levels of IL-4, IL-5, IL-10, IFN- γ , IL-13, and eotaxin were further determined by ELISA. Data represent the mean $\pm$ SEM from three independent experiments; $n = 6-8$ per group. Statistical differences are indicated by P values (one-way ANOVA). $*P < 0.05$ and $**P < 0.001$, compared with the OVA-immunized group. Effects of R-PC on airway dendritic cells (DCs). Airway DCs were collected at 36 hours after the last immunization as described in METHODS. Airway DCs were collected and stimulated with anti-mouse CD40 antibodies or IgG2a isotype control. Levels of IL-12 p70 and IL-10 were estimated by ELISA. Each group consists of 10 mice. Statistical differences are indicated by P values (one-way ANOVA). $*P < 0.05$ and $**P < 0.001$, compared with the OVA-immunized group. (D) R-PC reduced airway hyperresponsiveness in airways of mice. All mice were challenged with

increasing concentrations of aerosolized methacholine (MCh). Results were expressed for each MCh concentration as means $\pm$ SEM of representatives from three independent experiments (PBS, $n = 6$; OVA, $n = 6$; OVA+R-PC, $n = 8$). Statistical differences are indicated by P values (one-way ANOVA). $**P < 0.001$, compared with the OVA-immunized group. (E) R-PC reduced lung tissue allergic inflammation in OVA-induced mice. Histological staining of inflammatory cells in lung tissue. Lung tissues sections were fixed with 10% buffered formalin, sectioned at 5 μ m thickness, and stained with hematoxylin and eosin to analyze the severity of inflammation. The results shown are one of representative from more than five independent sections. (Upper panel, low power field magnification $\times 40$; lower panel, high power field magnification $\times 100$).

from 0.5 to 1 hours and showed slight degradation with time. In addition, the protein level of total I κ B α slightly decreased from 0.5 hours and recovered from 3 hours after R-PC treatment. Interestingly, LPS-induced signaling resulted in degradation of almost all of the I κ B α protein from 0.5 to 2 hours after LPS treatment. These data indicated that although R-PC-induced signaling also activated IKK α/β and I κ B α proteins in mouse BMDCs, the pharmacological mechanism of R-PC seems not totally the same as LPS. It is known that NF- κ B is activated by

LPS derived from gram-negative bacteria or polysaccharides purified from fungus and has been associated with up-regulation of IL-12 p70 in human monocyte-derived DCs (16, 22). To determine whether NF- κ B was also activated in R-PC-induced signaling, we tested the potency of R-PC to activate NF- κ B translocation in the nucleus of R-PC-treated BMDCs. Mouse BMDCs were cultured in the presence of R-PC for the indicated periods of time and nuclear extracts were prepared by NE-PER nuclear and cytoplasmic extraction reagents (Pierce)

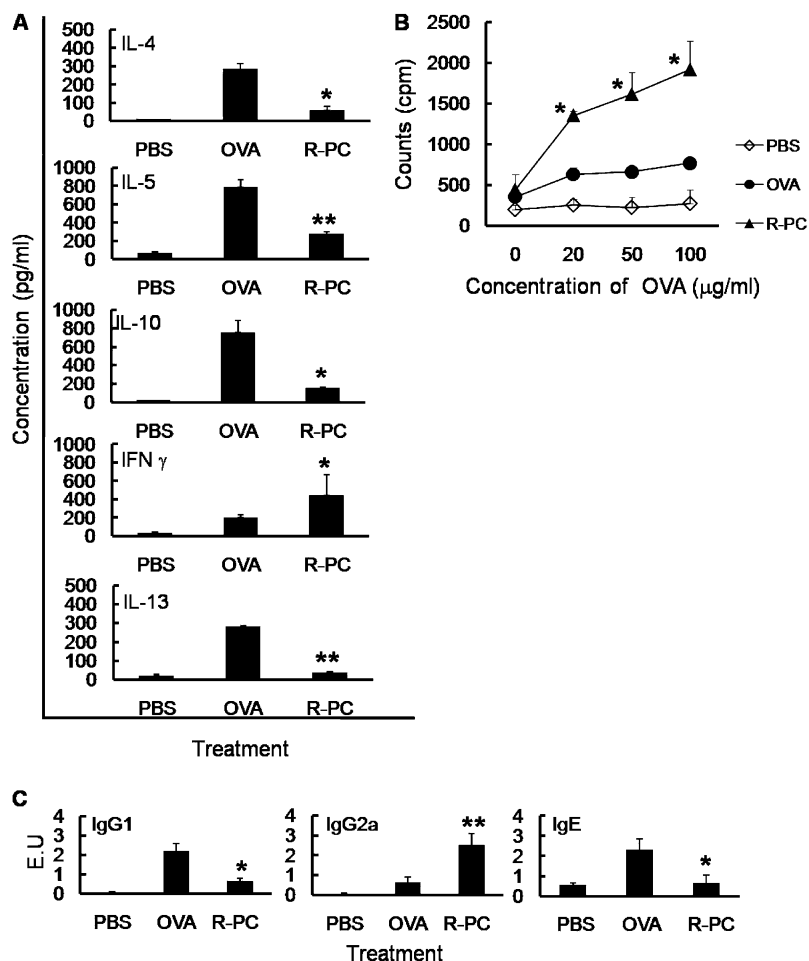


Figure 4. Effects of R-phycocyanin (R-PC) on ovalbumin (OVA)-specific immune responses. Mice were immunized with OVA or OVA plus R-PC. Phosphate-buffered saline (PBS) mice were immunized and challenged with PBS as a naive control. Splenocytes were collected at 24 hours after the last OVA challenge and cocultured with OVA (100 μg/ml) for 5 days. (A) R-PC reduced Th2 cytokine production but elevated Th1 cytokine production. Culture supernatants were collected and levels of IL-4, IL-5, IL-10, IL-13, and IFN-γ were measured by ELISA ($n = 6-8$). Data represent the mean $\pm$ SEM from three independent experiments. Statistical differences are indicated by P values (one-way analysis of variance [ANOVA]). * $P < 0.05$, ** $P < 0.001$, compared with the OVA-immunized group. (B) R-PC enhanced OVA-specific proliferation. Cultured cells were cultured with increasing concentration of OVA for 5 days. ^3H -thymidine was added to cell culture on Day 4. Total cells were harvested on Day 5 and the incorporated radioactivity was measured using a β -counter ($n = 6-8$). Results were shown for each OVA concentration as the mean $\pm$ SEM of representative from three independent experiments. Statistical differences are indicated by P values (one-way ANOVA). * $P < 0.05$, compared with the T-cell proliferation activity derived from the OVA-immunized group. (C) R-PC reduced serum levels of OVA-specific IgG1 and IgE. Serum was collected from mice immunized by OVA, OVA plus R-PC, or PBS at 24 hours after the last OVA challenge. Serum levels of OVA-specific IgG1, OVA-specific IgG2a, and OVA-specific IgE were determined by ELISA ($n = 6-8$ per group). Data represent the mean $\pm$ SEM from three independent experiments. Statistical differences are indicated by P values (one-way ANOVA). * $P < 0.05$, ** $P < 0.001$, compared with the OVA-immunized group.

according to the manufacturer's instruments and further analyzed for NF- κ B binding by EMSA. In agreement with the speculation from previous results, R-PC was able to induce NF- κ B activation and translocation (Figure 6B). Identical results were obtained from LPS-treated BMDCs. The binding of NF- κ B was specific and could be blocked by unlabeled NF- κ B oligonucleotide. These results suggest that R-PC-mediated regulation of cytokine production is potentially dependent on its effects on NF- κ B activation.

DISCUSSION

An increasing prevalence of allergic diseases has been noted in most developed countries, so it is critical to explore and develop possible novel treatment for these health-threatening diseases. Several researchers have demonstrated that polysaccharides derived from *Ganoderma lucidum* display immunomodulatory effects on T lymphocytes and promote the maturation and function of murine DCs (27–29). In our previous study, we cocultured polysaccharides extracted from *G. lucidum* with human monocyte-derived DCs and demonstrated that polysaccharides can effectively boost immunity through inducing activation and maturation of human DCs (16). Therefore, analyzing immunomodulatory actions of interesting molecules can be achieved through evaluating immunological functions of DCs.

The highly conserved PC protein is present in blue-green and red algae and plays a basic role in connection with boosting immune system or anticancer effects. In the last decade, several approaches have revealed the pharmacological properties attributed to PC, including antioxidant (30), antiinflammatory (31),

hypocholesterolemic (32), neuroprotective, and hepatoprotective activity (33), and anticancer effects. In addition, some studies have mentioned that PC displays immune suppression functions. In a previous study, Kim and colleagues demonstrated an inhibitory effect of PC derived from *Spirulina* on mast cell-mediated immediate-type allergic reactions in rats (34). Furthermore, Ramirez and colleagues also demonstrated that PC derived from the microalgae *Arthrospira maxima* can exert inhibitory effects on the release of histamines from mast cells and block allergic inflammatory responses (13). These results imply that PC potentially suppresses Th2-mediated immune responses. Therefore, in this study, we explored the therapeutic activity of PC in allergic asthma and approached the molecular mechanisms responsible for the regulation of DCs in their activation and maturation. In the present study, we demonstrated that R-PC can induce activation and maturation of mouse BMDCs. Maturation of DCs was characterized by an increase in MHC class II molecule, costimulatory molecules, and activation-associated molecules CD80, CD86, CD205, and CD40 on the cell surface. Moreover, our present data also showed that the uptake of FITC-dextran was observed to be minimal in R-PC-treated BMDCs, similar to that in LPS-treated BMDCs in comparison with untreated BMDCs, and therefore the antigen-processing capacity was also decreased in R-PC-treated DCs. A previous study claimed that DCs from IL-12-deficient mice failed to induce Th1 responses, suggesting the critical role of IL-12 in DC-induced Th1 responses. Therefore, the capacity of DCs to induce efficient Th1 is linked to the synthesis of IL-12 (35). In this study, we found that R-PC preferentially induced the level of IL-12 p70 in mouse BMDCs, but only slightly elicited IL-10 production. Therefore, we suggest

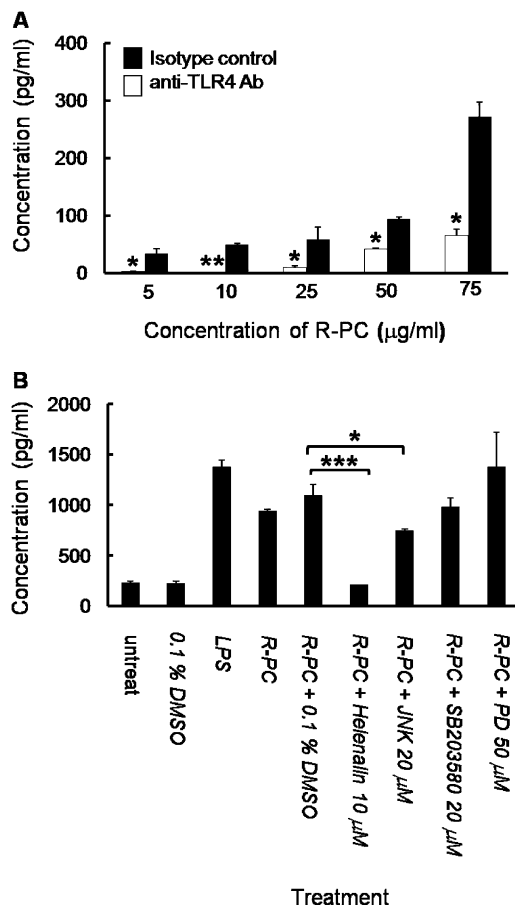


Figure 5. Effects of anti-TLR4 antibody, JNK inhibitor II, helenalin, PD98059, or SB203580 on R-PC-induced up-regulation of IL-12 p70 production in mouse bone marrow-derived dendritic cells (BMDCs). (A) Neutralization with TLR4 monoclonal antibodies (mAb) inhibits the synthesis of IL-12 p70 in R-PC-treated mouse BMDCs. On Day 6, mouse BMDCs were harvested and pretreated for 1 hour with 20 μg/ml of anti-TLR4 Abs or IgG2a served as control. Both anti-TLR4 Abs and IgG2a-treated BMDCs were further incubated with increasing concentrations of R-PC for another 15 hours. The cell culture supernatants were collected for IL-12 p70 analysis by ELISA. Data represent the mean ± SEM from three independent experiments. Statistical differences are indicated by *P* values (one-way analysis of variance [ANOVA]). **P* < 0.05, ***P* < 0.001, compared with the IgG2a-treated mouse BMDCs. (B) JNK pathway and nuclear factor-κB (NF-κB) activation involved R-PC-induced IL-12 p70 production in mouse BMDCs. Mouse BMDCs were pretreated with 0.1% dimethyl sulfoxide (DMSO), 10 μM helenalin (a specific blocker of NF-κB), 20 μM SB203580 (a specific blocker of p38 MAPK), 50 μM PD98059 (an inhibitor of the ERK1/2 pathway), or 20 μM JNK inhibitor II (an inhibitor of the JNK pathway) in culture containing 0.1% of DMSO for 1 hour and further incubated for 24 hours with 75 μg/ml of R-PC. Cells were left untreated in the culture medium alone for 24 hours. At the end of the incubation time, cultured supernatants were collected for IL-12 p70 analysis by ELISA. Data represent the mean ± SEM from three independent experiments. Significant differences are indicated by *P* values (one-way ANOVA). **P* < 0.05, ****P* < 0.0001, compared with the R-PC plus DMSO (0.1%)-treated mouse BMDCs.

that R-PC potentially polarizes the immune system toward a primary Th1 response.

Cytokine analysis in MLR revealed that untreated BMDCs were able to trigger low levels of IFN-γ and a high amount of IL-4 production from CD4⁺ T cells. In contrast, R-PC-treated BMDCs were able to induce allogenic CD4⁺ T cells to secrete

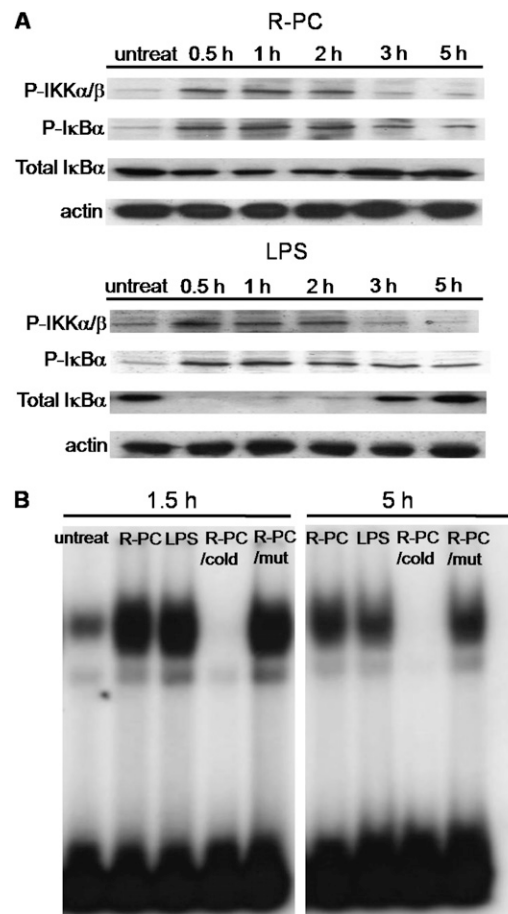


Figure 6. R-phycocyanin (R-PC) induced inhibitor of nuclear factor (NF)-κB (IκB) kinase (IKK)α/β, IκBα phosphorylation, and NF-κB activation in mouse bone marrow-derived dendritic cells (BMDCs). (A) Detection of phosphorylated IKKα/β, IκBα induced by R-PC in mouse BMDCs. Mouse BMDCs were stimulated with R-PC (75 μg/ml) for the indicated periods of time or left untreated. Lipopolysaccharide (LPS, 100 ng/ml)-treated BMDCs served as a positive control. Cytosolic fractions were prepared as described in METHODS and then analyzed by Western blotting that were probed with an antibody against total IκBα, phosphorylated form of IκBα, phosphorylated form of IKKα/β, and incubated with an antibody raised against the actin for loading control. Blots are representative of results obtained in three independent experiments. This experiment was repeated three times with similar results. (B) R-PC induces NF-κB activation. Mouse BMDCs were treated with R-PC (75 μg/ml) for the indicated periods of time or remained unstimulated. LPS (100 ng/ml)-treated BMDCs served as a positive control. Nuclear fractions were prepared as described in METHODS and were assayed for binding to probe containing the NF-κB-binding sites by electrophoretic mobility shift assay. Lanes marked R-PC/cold indicate that the binding reaction was conducted in the presence of a 100-fold excess of unlabeled oligonucleotide probes. To assess the specificity of the binding, mutant probe was added to the R-PC-treated condition, which is marked as R-PC/mu.

high levels of IFN-γ. The levels of IFN-γ gradually increased in culture supernatants, but conversely, the levels of IL-4 production decreased. Therefore, R-PC-treated BMDCs potentially exhibited the capability to skew CD4⁺ T toward Th1 type. Similarly, analysis of the intracellular cytokine production revealed that R-PC-treated BMDCs inhibited IL-4 production while augmenting IFN-γ synthesis in CD4⁺ T cells. Therefore, R-PC-treated BMDCs potentially induce naive T cells to secrete IFN-γ and promote immune responses toward Th1.

There is now clear evidence that Th2 cells play an essential role in the pathogenesis of allergic airway inflammation (36). Our present data showed that R-PC significantly reduced the levels of IL-4, IL-5, IL-10, and IL-13 in BALF. Furthermore, the level of eotaxin was substantially reduced. Th2 cytokines can be produced by various resident cells, such as bronchial epithelial cells, tissue mast cells, and alveolar macrophages, as well as infiltrated inflammatory cells, such as lymphocytes and eosinophils. IL-13 has been considered a potent inducer of eotaxin production by airway epithelial cells. Therefore, reducing the level of eotaxin may be associated with the significant decrease in IL-13 level in BALF. Eosinophils are the principal effector cells for the pathogenesis of allergic inflammation. The transmigration of eosinophils into the airway is a multistep process that is orchestrated by Th2 cytokines, such as IL-4, IL-5, and IL-13, and coordinated by specific chemokines, such as VCAM-1 and VLA-4 (37, 38). Our present findings showed that R-PC prevented eosinophil infiltration into the airways. Similarly, this result was shown by a significant reduction of inflammatory cell infiltration in histological examination. It is believed that inflammatory mediators released during allergic inflammation play a critical role in AHR development (3, 39). Our data showed that R-PC significantly inhibited OVA-induced AHR to inhaled MCh in a dose-dependent manner. It is well known that IL-5 plays a critical role in AHR by mobilizing and activating eosinophils, leading to the release of proinflammatory products, such as major basic protein and cysteinyl-leukotrienes (40, 41). In addition, IL-4 and IL-13 have been shown to induce AHR in mouse asthma models. These findings are consistent with the results of cytokines in BALF. It has been established that IgE-mediated mast cell activation may contribute to AHR by producing inflammatory mediators and cytokines (42). Moreover, IL-4 and IL-13 are important in directing B-cell growth, differentiation, and secretion of IgE and IgG1 (43). Therefore, elevated serum IgE and IgG1 are the hallmarks of a Th2 immune response. We further speculated that the R-PC-induced reduction of AHR, Th2 cytokine production, and tissue eosinophilia may be associated with the reduction in IgE and IgG1 levels in serum. Our data showed that serum levels of OVA-specific IgE and IgG1 were substantially reduced by R-PC. Conversely, the serum levels of OVA-specific IgG2a increased, which is an indicator of Th1 immune response.

Allergic airway inflammation and AHR development involve multiple inflammatory cells and a wide array of mediators. In addition, airway DCs are a crucial factor in determining the outcome of inhaled antigens during sensitization, which acquire Th2-cell effector function in response to encountering antigens and lead to allergy. Our present study revealed that intranasally immunized mice with OVA antigens in combination with R-PC elicited IL-12 p70 production from airway DCs as compared with the OVA-immunized group. Furthermore, the levels of IL-12 p70 more significantly increased with activated airway DCs with anti-CD40 antibody as compared with the isotype control-treated group. In the group of mice bearing intranasal immunization OVA admixed with R-PC, we not only detected high levels of IFN- γ production and OVA-specific CD4⁺ T-cell proliferation, but also observed low levels of OVA-specific IgG1 and IgE in serum and Th2-mediated cytokines in BALF (data not shown). It is likely that R-PC can modulate the immunological capability of airway DCs and potentially skew the OVA-specific immune responses toward Th1.

Little is known about the signal transduction pathways involved in the maturation of mouse BMDCs by R-PC. Toll-like receptors (TLRs) have been identified in humans as an important component of innate immunity against microbial

pathogens or recognize fungal polysaccharide-mediated signaling in DCs and macrophages (24–26). The level of IL-12 p70 production was used to evaluate the binding efficacy of R-PC with TLR4 or TLR2. This experiment further demonstrated that TLR4 plays a critical role in the signal transduction cascade of IL-12 p70 production induced by R-PC. Antibodies against TLR-2 were applied to analyze the binding effect of R-PC, but the results showed that the IL-12 p70 production was unchanged as compared with isotype control antibodies (data not shown). Previous studies have demonstrated that LPS, tumor necrosis factor α , and polysaccharides are able to induce NF- κ B activation and phosphorylation of p38 MAPK, ERK1/2, and p46/54 JNK in monocyte-derived DCs, and can also be used to activate immature human DCs (16, 44). We further investigated whether early phosphorylation of p38 MAPK, ERK1/2, or p46/54 JNK was involved the R-PC-induced intracellular signaling to induce IL-12 p70 production. Cytokine analysis showed that pretreating BMDCs with the inhibitory drugs helenalin or JNK inhibitor II significantly inhibited the production of IL-12 p70 and IL-12 p40 (data not shown) in R-PC-treated BMDCs. Therefore, activation of the NF- κ B or JNK pathway may be involved in R-PC-triggered signaling cascades to elicit IL-12 production. Puig-Kroger and his co-workers reported that by lowering the ratio of ERK to p38 MAPK activation, DCs could be raised with Th1-polarizing capacity, indicating that the ERK signaling pathway in antigen-presenting cells may promote the initial commitment of naive T helper cells toward the Th2 phenotype (45). In our study, PD98059, an ERK1/2 inhibitor II, was observed to slightly increase the level of IL-12 p70 in R-PC-treated BMDCs; therefore, we speculated that R-PC-eliciting signaling might partially activate the ERK 1/2 pathway and facilitate in balancing the immunological shift of cytokine expression in BMDCs. A previous study illustrated that the promoters of human IL-12 p35 and IL-12 p40 genes contain NF- κ B-binding sites, and therefore NF- κ B is involved in IL-12 p35 and IL-12 p40 expression. Furthermore, several reports have also shown that NF- κ B is responsible for LPS-induced DC maturation in an *in vitro* study (46) and cytokine-induced maturation in human monocyte-derived DCs (47). In our study, the EMSA results showed that R-PC induced NF- κ B activation and nuclear translocation. This finding is consistent with the inhibition of IL-12 p70 production obtained from helenalin treatment. In the classic NF- κ B signaling pathway, I κ B α proteins are phosphorylated by an activated IKK complex and trigger polyubiquitination of I κ B α . Therefore, phosphorylated I κ B α would be degraded by 26 S proteasome resulting in the release of free NF- κ B dimers. We further addressed the signaling pathways involving R-PC-induced NF- κ B activation. As shown by immunological blotting analysis, both total I κ B and phosphorylated I κ B α were detected in R-PC-treated BMDCs. The levels of phosphorylated I κ B α were observed from 0.5 hours after R-PC treatment and degraded with time. Interestingly, the levels of total I κ B α were slightly degraded at the indicated time. However, in LPS-treated BMDCs, the levels of total I κ B α were dramatically degraded and regenerated; moreover, the levels of phosphorylated I κ B α were also altered with the indicated time. The present results imply that alternative signaling pathways might be involved in R-PC-induced NF- κ B activation. Because IKK α and IKK β cooperate to induce I κ B phosphorylation, both molecules forming homodimer or heterodimer exist in mediated signaling. Recently, an alternative NF- κ B pathway has been identified, which illustrates upstream of NF- κ B-inducing kinase (NIK) activates an IKK α homodimer, whereas both IKK β and IKK γ are dispensable in the signaling. Our analytic results show that levels of phosphorylated IKK α subunits were apparently

observed, but conversely, only a few phosphorylated IKK β subunits were detected in the R-PC-induced intracellular signaling. This result is distinct from LPS-induced classical NF- κ B pathway activation. Several studies have illustrated that an IKK α -mediated alternative pathway for NF- κ B activation facilitates the promotion of humoral and cell-mediated adoptive immune responses (48, 49). The role of IKK α in DC function and maturation has not been examined. It is only known that IKK α plays a role in the lymphotoxin β -mediated signaling, which is important to maintain DC populations *in vivo* (50). On this basis, R-PC-induced signaling might activate several intracellular molecules, some of which may not have been identified in the study and will be addressed in future work. We speculate that both classic and alternative activation pathways may contribute to R-PC-induced NF- κ B release and nuclear transmigration results in DCs acquiring biological functions in promoting adaptive immunity. Our present study showed that R-PC-treated BMDCs were apparently triggering CD4⁺ T cells to produce high levels of IFN- γ and acquire potent proliferation activity as compared with OVA-treated BMDCs. Furthermore, R-PC also effectively augmented the Th1 type of OVA-specific T cell responses and reduced Th2-mediated inflammation in the airways of OVA-sensitized mice. These results manifestly represent that R-PC potentially establishes a Th1-type of antigen-specific adoptive immune response in mice.

The primary aim of this study was to evaluate the therapeutic potential of R-PC against allergic asthma. Therefore, we evaluated R-PC-mediated immunomodulatory functions on human monocyte-derived DCs in cytokine expression. Our data showed that the levels of IL-12 p70 were elevated in normal human monocyte-derived DCs with R-PC treatment (data not shown). Interestingly, we also observed elevated IL-12 p70 production from R-PC-treated DCs derived from patients with asthma (data not shown). These results imply that R-PC can be potentially applied to modulate immunological functions in humans, which might facilitate reduction of Th2-mediated airway inflammation and improve airway function through modulating immunological functions of airway DCs. Taken together, we conclude that R-PC may skew the immunological characteristics of airway DCs during encounters with antigens, and therefore R-PC can potentially reduce Th2-type inflammation in airways and protect them from allergic disease.

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Online Data Supplement

Manuscript title: A novel phycobiliprotein alleviates allergic airway inflammation by modulating immune responses

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METHODS

Purification of R-PC from red algae

Filaments of *Bangioatropurpurea* were harvested from an *in vitro* culture system. The filaments were differentiated from releasing spores of thalli in SWM-III cultured medium (Difco, Lawrence, USA). The collected and filtered filaments were dried and suspended with ten times the volume of 10 mM phosphate buffer (pH 6.8). The suspension was centrifuged to remove debris and extraction was precipitated with $(\text{NH}_4)_2\text{SO}_4$ three times. Crude phycocyanin precipitate was subjected to two gel filtration chromatography separations with a Sephadex G200 column and further subjected to hydroxyapatite chromatography purification with a Macro-Prep Ceramic Hydroxyapatite type I gel column (Bio-Rad, Hercules, CA, USA). R-PC was eluted by a linear gradient elution of 10 mM to 200 mM phosphate in 0.1 M NaCl solution. Eluted fractions were monitored by a UV-Visible spectrophotometer, which were characterized by a ratio of $A_{618} / A_{280} > 4$. Finally the purified R-PC was recovered from the eluted fractions and further precipitated with $(\text{NH}_4)_2\text{SO}_4$ and stored at 4 °C ready for use. The level of endotoxin in R-PC for the culture condition was less than 0.01 EU/ml and optimal concentration of LPS for the stimulation of dendritic cells was 100ng/ml (equivalent to 1000EU/ml) in our study.

Effects of R-PC on T cells by allogenic MLR and cytokine production analysis

To examine the stimulating ability of R-PC-treated mouse BMDCs, mixed lymphocytes reaction (MLR) and cytokine secreting levels were determined. CD4^+ T cells were purified from spleens of C57BL/6 mice after incubation with L3T4 (anti-CD4) magnetic beads with MiniMACS (MiltenyiBiotec, BergischGladbach, Germany) according to the manufacturer's instructions. Positively selected cells containing 95-99% CD4^+ cells were collected for cytokine production and proliferation

assay. For the proliferation assay, LPS, R-PC or untreated BMDCs were irradiated at 3000 rads and used as antigen presenting cells (APCs). Freshly isolated C57BL/6 CD4⁺ T cells (3x10⁵ cells/ml) were co-cultured with LPS, R-PC-treated or untreated BMDCs at an indicated ratio of DC/T cells. Cells were cultured in a total volume of 200 μ l in 96-well round-bottom tissue culture plates for 3 days. Cultures were then pulsed with 1 μ l Ci[³H] thymidine for another 17 h of culture and the [³H] thymidine deoxyribose incorporation was measured by a beta scintillation counter (Packard, Meriden). Data were expressed as counts per min (cpm). For the cytokine analysis, LPS, R-PC-treated or untreated BMDCs were co-cultured with C57BL/6 CD4⁺ T cells in the same conditions used for the proliferation assay. After 48 h, 72 h, and 96 h, supernatants were collected and cytokine production was analyzed by ELISA.

Murine model of OVA -induced allergic airway inflammation

The modified mouse model was carried out as described by Hellingset *al.* and Meytset *al.* (E1-E3). Briefly, mice were sensitized with 6 intraperitoneal injections of 50 μ g of ovalbumin (OVA) (Sigma, St Louis, MO, USA) mixed with 50 μ g of R-PC in a total volume of 200 μ l PBS on day 0, day 5, 10, 15, 20 and 25. The disease group of mice was immunized with OVA alone. A naïve group of mice were administered with PBS. For challenge, all mice were treated intranasally with OVA (50 μ g in a total volume of 30 μ l PBS) on day 35, 36, 37, 38. Mice were sacrificed at 24 h after last OVA challenge. On the same day, sera was collected by a retro-orbital puncture and AHR measurements performed, and in addition, BAL fluid, lungs and splenocytes were obtained from each mouse. The lungs, obtained for histologic analysis, were fixed with

10% neutralized buffered formalin. Sections (5 μ m thick) were prepared and subjected to hematoxylin and eosin (H&E) staining and examined by light microscopy. In some experiments, mice were immunized with 7 intranasal inhalations of 50 μ g of OVA (Sigma, St Louis, MO, USA) mixed with or without 50 μ g of R-PC in a total volume of 30 μ l PBS on day 0, 5, 10, 15, 20, 25 and 30. A naïve group of mice were administered with PBS. Mice were sacrificed at 36 h after last immunization. After the mice had been sacrificed, blood, BAL fluid, spleen, peribronchial and cervical lymph nodes were collected.

The effects of R-PC on airway DCs

Mouse peribronchial lymph nodes and cervical lymph nodes were dissected and homogenized using a 40- μ m cell strainer (Falcon, Becton Dickinson, Franklin Lakes, NJ) and suspended in RPMI 1640 supplemented with 2% FCS. The homogenates were washed twice with PBS supplemented with 2% FBS and centrifuged at 1500 r.p.m. for 5 mins. For analysis of *in vivo* primed DC function, freshly isolated CD11c⁺DCs were purified by positive selection using a Macs columns (MiltenyiBiotec, Auburn, CA) from peribronchial lymph nodes and cervical lymph nodes from OVA immunized BALB/c mice in the presence or absence of R-PC through intranasal immunization. 5x10⁵ cells/ well of DCs were cultured for 72 h in the presence of 5 μ g/ml rat anti-mouse CD40 mAbs (clone 3/23; BD PharMingen, San Diego, CA) or control rat IgG2a (BD PharMingen, San Diego, CA). The supernatants were stored at -20 °C until future measurement.

Intracellular cytokine staining

Intracellular cytokine were detected by flow cytometry using the method of Andersson *et al.* with modifications. Briefly, 3×10^5 CD4⁺ T cells and DC were incubated at an indicated DC/T cell ratio (1/5) at 37 °C for 2 days. Monensin was added for the final 6 hours. Then, 10^6 cells were harvested and stained with phycoerythrin-labeled monoclonal antibodies of CD4 (BD PharMingen, San Diego, CA). Subsequently, cells were washed, fixed and permeabilized using eBioscience IC Fixation Buffer / eBioscience Permeabilization Buffer (eBioscience, San Diego, CA) and stained with fluorescein isothiocyanate (FITC) labeled monoclonal antibodies of IFN- γ or IL-4 (BD Biosciences Pharmingen). Staining with isotype control antibodies (BD PharMingen, San Diego, CA) was performed in all experiments. 10^4 of cells were analyzed by flow cytometry. FACS analyses are shown after gating on the CD4⁺ lymphocyte population.

Transfection of TLR in HEK293 cells

For the measurement of the binding capacity of R-PC with TLR4, HEK293 cells (American Type Culture Collection, Manassas, VA, USA) in six-well tissue-culture plates (growing as a monolayer at 70-75 % confluence) were cultured with DMEM supplemented with 10% FBS and transfected using Lipofectamine TM²⁰⁰⁰ (Life Technologies, Carlsbad, CA, USA) with pUNO-hTLR4, pDUO-hTLR4-MD2 and pUNO empty plasmids. Subsequently, transfected HEK293 cells were selected in DMEM containing 10 μ g/ml of blasticidin S-supplemented medium. Transfected cells were cultured in normal DMEM for 36 h, and then were stimulated with R-PC (75 μ g/ml), LPS (50ng/ml) or left as untreated group for additional 24 h. IL-8 protein in the culture supernatant was harvested and measured by ELISA.

RESULTS

R-PC binds TLR4 and induces IL-8 production in TLR4 expressed HEK 293 cells

In order to demonstrate R-PC can binds with human TLR4, therefore, we have followed the methods [4-6] to explore the binding capacity of R-PC with TLR4. Firstly, we have analyzed the TLR4 or TLR4-MD2 expression on cell surface of HEK293 cells by surface markers staining with antibody raised against human TLR4 (Biolegend). As shown in Figure E1-A, The expression of TLR4 was significantly increased in both of pUNO-hTRL4 and pUNO-hTLR4-MD2 transfected HEK293 cells to comparing with pUNO empty plasmids transfected cells. Furthermore, we have analyzed the IL-8 production from hTLR4 or hTLR4-MD2 expressed HEK293 cells following R-PC treatment. LPS was served as positive control to demonstrate the binding effect with TLR4. As illustrated in Figure E1-B and Figure E1-C, a high level of IL-8 was produced from TLR4 or TLR4-MD2 expressed cells following R-PC or LPS treatment. Interestingly, we have found that LPS-mediated TLR4 signaling was MD2 dependent. However, activation signaling of TLR4 with R-PC is not likely to need MD2. Herein, we have provided the evidence to demonstrate R-PC binding with TLR4 through the system composed of TLR4 expressed HEK293 cells and IL-8 production.

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FIGURE LEGENDS

Figure E1. R-PC binds with TLR4 and induces IL-8 production in TLR4 expressed HEK 293 cells. pUNO empty plasmids, pUNO-hTLR4, and pDUO-hTLR4-MD2 were transfected into HEK293 cells and selected with blasticidin S. (A) Three plasmids expressed HEK293 cells were stained with anti-hTLR4 antibody or IgG2a isotype control. Thin lines indicate the cells stained with isotype control Ab; bold lines indicate the cells stained with anti-hTLR4 Abs. Subsequently, three plasmids expressed HEK293 cells were stimulated with LPS (50 ng/ml) (B) or R-PC (75 μ g/ml) (C) or left untreated for 24 h, and the cell culture supernatants were collected and analyzed for IL-8 production by ELISA.

Figure E1

