The Recombinant Profilin from Free-Living Amoebae Induced Allergic Immune Responses via TLR2

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Background: Repeated exposure to recombinant profilin from Acanthamoeba (rAc-PF) induces allergic airway responses in vitro and in vivo. Based on the role of toll-like receptors (TLRs) in allergic airway diseases, TLRs play a central role in innate immune responses and the adaptive immune system and regulate responses against antigens through antigen-specific receptors. In this study, we attempted to determine the molecular mechanisms underlying rAc-PF-induced allergic inflammatory responses.

Methods: We determined the correlation between rAc-PF and TLRs and analyzed changes in allergic immune responses after blocking multiple TLR signaling under rAc-PF treatment conditions in vitro. We also compared allergic inflammatory responses in TLR2 knockout (KO) and wild-type (WT) mice. To investigate the effect of TLR2 on antigen prototyping and T cell activation in the inflammatory response induced by rAc-PF, we assessed maturation of BMDCs and polarization of naïve T cells by rAc-PF stimulation. Additionally, we compared changes in inflammation-related gene expression by rAc-PF treatment in primary lung epithelial cells isolated from TLR2 KO and WT mice.

Results: The rAc-PF treatment increased the expression level of TLR2 and 9 in vitro. But, there were not significantly differ the others TLRs expression by rAc-PF treated group. And then, the mRNA expression levels of inflammation-related genes were reduced in the TLR2 or TLR9 antagonist-treated groups compared to those in the rAc-PF alone, were no difference the treated with the other TLRs (TLR4, 6, and 7/8) antagonist. The difference was higher in the TLR2 antagonist group. Additionally, the levels of airway inflammatory disease indicators were lower in the TLR2 KO group than in the WT group after rAc-PF treatment. Furthermore, the expression of bone marrow-derived dendritic cell (BMDC) surface molecular markers following rAc-PF stimulation was lower in TLR2 KO mice than in WT mice, and TLR2 KO in BMDCs resulted in a remarkable decline in Th2/17-related cytokine production and Th2/17 subset differentiation. In addition, the expression levels of rAc-PF-induced inflammatory genes were reduced in TLR2 KO primary lung cells compared to those in normal primary lung cells.

Conclusion: These results suggest that the rAc-PF-induced airway inflammatory response is regulated by TLR2 signaling.

Keywords: rAc-PF, profilin, toll-like receptors, airway inflammation, T cell activation

Introduction

Toll-like receptors (TLRs) are fundamentals to sensing and responding to inflammation. TLRs are key innate immune sensors that respond to exogenous infectious ligands, pathogen-associated molecular patterns (PAMPs) of infectious ligands and damage-associated molecular patterns (DAMPs) of endogenous molecules.¹² The innate immune response initiated by TLRs is necessary to progress to the most adaptive immune response. TLR signaling is determined by the selective use of signaling adapters and the cells in which they are expressed.³ Dendritic cells (DCs) have a unique ability to sense and present microbial antigens to prime naïve T cells.³⁵ TLR-mediated DC maturation plays an important role in the protective immunity conferred by protein-based vaccines.⁶–⁸ For instance, lipoprotein BP1569, a novel TLR2 agonist, activates DCs via TLR2 and induces Th1/17 and IgG2a antibody responses in mice.⁹ Several immune cell types,
such as DCs, B cells, and CD4+ T cells, express TLRs.9–13 TLR2 influences the initiation of adaptive immunity and regulates DC antigen presentation by altering the expression of surface molecules and cytokine secretion. Alternatively, TLR2 directly affects adaptive immune responses by regulating T cell subset polarization and antibody production.14 TLR2 forms heterodimers with TLR2/1 and TLR2/6. The relationship between TLR2, airway diseases, and allergy prevalence remains uncertain, with recent meta-analyses revealing that rs3804099 may indicate asthma risk or have no association.15,16 However, TLR1 and TLR6 polymorphisms have been reported to be associated with allergic diseases, asthma, and atopy in early life. In addition, TLR2 expression is up-regulated in the nasal mucosa of patients with airway inflammation and is likely a disease-exacerbating factor.15,17

_Acanthamoeba_ is widely distributed in environments, such as soil, water, and air. They can induce granulomatous amebic encephalitis, _Acanthamoeba_ keratitis and _Acanthamoeba_ pneumonia.18 Although the infection rate is low worldwide, the mortality rate from infection is reported to be very high, especially in immunosuppressed hosts.19 We confirmed that allergic airway disease is induced when repeatedly exposed to the total extracts or excretory/secretory (ES) protein of the free-living amoeba, KA/E2 in vivo. We also published showing that serine protease contained in ES proteins induces allergic respiratory inflammatory disease via protease-activated receptor (PAR)-2 in previous study.20 However, the KA/E2-induced airway inflammatory response was not completed by blocking protease activity. Accordingly, we raised the question whether there is an inflammation-inducing mechanism mediated by receptors other than PAR2 upon KA/E2 exposure, and conducted research on the mechanism mediated by TLR among various antigen receptors.21 In addition, a recombinant profilin was identified from _Acanthamoeba_ (rAc-PF) through clinical research,22 and we suggested that the rAc-PF can induce airway inflammatory disease by increased Th2 immune responses and airway hyper-responsiveness.23 However, the detailed mechanisms underlying the initiation of allergic airway inflammation by rAc-PF remain unclear. In this study, we investigated the correlation between rAc-PF-induced airway inflammatory responses and TLR signaling by analyzing the differences in inflammatory immune responses under normal and TLR depletion conditions following rAc-PF treatment in vitro and in vivo.

**Methods**

**Reagent and Instruments**

Mouse lung epithelial cells (MLE12 cells) were purchased from ATCC (Manassas, VA, USA). Fetal bovine serum (FBS), Roswell Park Memorial Institute (RPMI)-1640 and Dulbecco’s modified Eagle’s Medium (DMEM)/F12 (Gibco, USA), 0.25% Trypsin–EDTA (Gibco, USA), Penicillin-Streptomycin (Gibco, USA), rAc-PF1, antagonists of TLR1/2, TLR4, TLR5, TLR7/8, and TLR9 were also purchased for this study.21

**In vitro Experiment**

To confirm the TLRs gene expression by rAc-PF treatment, after MLE12 cells (4 × 10^5) were stimulated with rAc-PF for 3 h, were synthesized mRNA. For the neutralization of TLRs, each TLR antagonist was treated before rAc-PF stimulation. Total RNA was extracted from the cells using QIAzol (Qiagen, Hilden, Germany) according to the protocol of manufacturer. Quantitative reverse transcription-time PCR was performed using an iCycler™ (Bio-Rad, Hercules, California, USA) in a LightCycler 96 Real-Time PCR System (Roche, Basel, Switzerland). GAPDH was used as a reference gene. The primers used for real-time PCR are listed in Table 1. Amplification conditions: 95°C for 1 min 30 sec, denaturation at 95°C for 25 sec, primer annealing at 55–55°C for 20 sec and elongation at 72°C and 30 sec for 40 cycle.

**Mice and in vivo Experimental Design**

Six-week-old female C57BL/6 mice were purchased from Samtako (Gyeonggi-do, Republic of Korea). TLR2 knock-out (KO) C57BL/6 female mice was supplied by the Pohang University of Science and Technology (Gyeongsangbuk-do, Republic of Korea). The mice were intra-nasally exposed to rAc-PF six times at intervals of 2 days (0, 2, 4, 6, 8, and 10 days) under respiratory anesthesia using isoflurane. All animal experiments were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and protocols approved by the Pusan National
Airway Hyper-Responsiveness (AHR)

After the last administration, airway function was monitored depending on the concentrations (0, 12.5, 25, and 50 mg/mL) of methacholine using whole-body plethysmography (Allmedicus, Korea). After each nebulization, the enhanced pause values, measured every 150 s during the experimental period were averaged and graphs were generated.

Bronchoalveolar Lavage Fluid (BALF)

After inserting the catheter into the trachea, preheated PBS was slowly inserted into the lungs through the catheter and withdrawn to obtain BALF. The samples were centrifuged, and the supernatant was maintained at −70°C until used in enzyme-linked immunosorbent assay (ELISA) detection. Cells were stained with Diff-Quick solution (Dade Diagnostics, Tokyo, Japan) to determine cell differentiation according to established morphological criteria.21

Table 1 Primer Sequences for Real-Time PCR

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<th>Primer</th>
<th>Sequence</th>
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Abbreviations: for, forward; rev, reverse.
Lung Histopathology
Paraffin-embedded sections of the left lobe of the lung tissue were isolated from mice, fixed with formaldehyde and embedded in paraffin. The section of lung tissue was stained with hematoxylin and eosin (H&E) or periodic acid-Schiff (PAS). After staining, we were examined under a microscope for histo-pathological analysis. The disease severity value such as inflammation and mucin production were scored by as previously described.

Lymphocytes Stimulation
Lymphocytes were isolated from the lung-draining lymph nodes of each mice to analysis of cytokine expression levels. Cells were stimulated with CD3 pre-coated well for 72 h at 37°C in an atmosphere containing 5% CO2. After incubation, the culture medium was harvested and stored at −70°C for ELISA assay.

ELISA Assay
The levels of IFN-γ, IL-4, IL-5, IL-13, IL-17A, and IL-1β in the lymphocyte culture supernatants and BALF were evaluated using ELISA kits (eBioscience, San Diego, CA, USA), according to the manufacturer’s protocol.

BMDCs Maturation
BMDCs from wild type (WT) and TLR2 KO mice were isolated as described previously. The cells were cultured at 5×10^6 cells/well with rAc-PF for 24 h and co-expressed markers CD40, 80, 86, and MHCII were assessed; anti-MHCII-PE, -CD40-APC, -CD80-FITC and -CD86-BV421 (all from eBioscience). Additionally, cytokines expression level was evaluated in cell supernatants using ELISA assay.

BMDCs and Naïve T Cells Co-Cultivation
Naïve T cells (CD44<sub>low</sub>CD62L<sub>high</sub>CD25<sup>−</sup>) were isolated from WT mice, and sorted naïve T cells were co-cultured with rAc-PF pre-treated BMDCs at naïve T cell:BMDCs ratio of 5:1 for 72 h at 37 °C in presence of an anti-CD3 antibody (eBioscience, San Diego, CA, USA). After co-culture, naïve T cells were stained with anti-CD4-PerCP (eBioscience) according to the protocol of manufacturer, fixed, permeabilized, and stained intracellularly with anti-mouse IL-4-PE-Cy7 and anti-mouse IL-17A-PE. Flow cytometry was performed using a FACS Canto II cytometer (BD Biosciences) equipped with Canto software.

Primary Lung Cell Isolation and Stimulation
As previously described, lung cells were obtained from WT and TLR2 KO mice. Isolated primary lung cells (4 × 10^5 cells/well) were seeded into 24-wells and stimulated with rAc-PF for 3 h and mRNA was extracted from cells for Real-time PCR analysis.

Statistical Analysis
All data were analyzed using Prism 6 (GraphPad Prism, La Jolla, CA, USA). Mean ± standard deviation (SD) was calculated, and significant differences were determined using the Student’s t-test or one-way analysis of variance (ANOVA) with Dunnett’s multiple comparisons post-test comparing all groups with the control group.

Results
TLR2 Influenced on rAc-PF-Induced Airway Inflammation
To measure the functionality of TLRs, we studied the response of MLE12 cells to TLRs characterized by rAc-PF. A check of TLRs gene expression levels after rAc-PF treatment showed that the levels of TLR2 and 9 expression were increased by rAc-PF treatment compared to control. The other TLRs gene expression levels were increased, but the value was not significant. (Figure 1A and Supplementary Figure 1A). Next, we examined the expression levels of inflammation-related genes after rAc-PF stimulation, depending on the presence or absence of TLRs antagonist pretreatment. We confirmed that pro-inflammatory gene expression, IL-25, eotaxin, MDC, and TSLP were decreased in TLR2 or TLR9
antagonist pretreated cells when compared to those treated with rAc-PF alone or other TLRs antagonists (Figure 1B and Supplementary Figure 1B). In particular, the decrease in pro-inflammatory gene expression was most effective when TLR2 function was suppressed (Figure 1B).

The rAc-PF-Induced Airway Inflammatory Response Was Reduced in TLR2 KO Mice

Based on results of In vitro, we determined whether rAc-PF induced airway inflammation through the TLR2 pathway and repeatedly exposed mice to rAc-PF (Figure 2A). Airway hypersensitivity to methacholine decreased to higher levels in the TLR2 KO than in the WT mice (Figure 2B). Additionally, in TLR2 KO mice, the number of inflammatory cells, such as eosinophils and neutrophils, in the BALF was significantly lower than that in WT mice (Figure 2C). Histological analysis of lung tissue showed that immune cell infiltration; especially eosinophils recruitment, mucus production, and hypertrophy of lung epithelium and goblet cells were suppressed in the TLR2 KO compared to WT mice treated rAc-PF (Figure 3A and B). To investigate the immune responses involving TLR2, BALF, and lymphoid cells were obtained from rAc-PF-treated WT and TLR2 KO mice. No difference in T cell proliferation existed between TLR2 KO and WT mice (data not shown). However, TLR2 KO mice showed reduced levels of IL-1β, −4, −5, and −13 in BALF and lymphocytes from lung-draining lymph nodes compared to those in the WT mice (Figure 4A and B). These results indicate that TLR2 affects rAc-PF-induced inflammatory responses both in vitro and in vivo.

The rAc-PF Polarized CD4 T Cells into Th2/Th17 Cells via TLR2

To investigate the interaction between TLR2 and rAc-PF, we measured the levels of co-stimulatory molecules and cytokines in BMDCs from WT and TLR2 KO mice treated with lipopolysaccharide or rAc-PF. The expression of surface markers, CD40, 80, and 86, and the production of cytokines, including IL-4, 5, 13, and 17A were significantly decreased in the rAc-PF-treated TLR2 KO group compared to those in the WT group, and the expression of MHCII on BMDCs in the TLR2 KO group was higher than that in the WT group (Figure 5A and B). These data suggest that rAc-PF interacts with TLR2 to induce DC activation and cytokine production. As TLR2 affects rAc-PF-mediated DC activation, it probably influences Th2 polarization induced by rAc-PF treatment. Co-cultures of BMDCs and naïve T cells were used to verify this hypothesis. The BMDCs from WT and TLR2 KO
Figure 2 Difference of airway hyperresponsiveness and immune cells infiltration depending on the presence or absence of TLR2. Scheme of airway inflammation induced by repeated exposure to rAc-PF (A). Airway hypersensitive response to methacholine (0–50 mg/mL) was compared between WT and TLR2-/- groups (B). Differential immune cells were counted in BAL from WT or TLR2 KO mice using a microscope (C). (n = 5/group, three independent experiments, *; p < 0.05, **; p < 0.001).

Figure 3 Change of the lung histopathological inflammatory manifestations in TLR2 deficiency by rAc-PF. Images of lung sections after H&E or PAS staining show lung tissue inflammation (A). Representative image showing inflammation scoring and PAS-positive cells (mean ± SEM). (The histological grade was described in a previous study21). (n = 5/group, three independent experiments, **; p < 0.01).
mice were treated with rAc-PF for 24 h and then incubated with purified naïve T cells. After 72 h, the T cells population was determined by FACS analysis. As a result, with TLR2 knockout, the Th2-related Th2 and Th17 related cell populations were markedly lower than those in the WT group (Figure 6A and B). These data indicate that TLR2 may play a role in the activation of BMDCs and the production of T cells following rAc-PF treatment in vitro.
TLR2 Deficiency Declines Pro-Inflammation-Related Gene Synthesis in vitro

To investigate the cellular mechanisms of action rAc-PF, we isolated and cultured primary lung cells from WT and TLR2 KO mice treated with rAc-PF in vitro. In response to rAc-PF, cells from WT mice produced significant levels of pro-inflammatory genes, such as IL-25, eotaxin, MDC, and TSLP, but the expression levels of these genes were remarkably decreased in TLR2 KO mice (Figure 7). These results demonstrate that rAc-PF induces inflammatory responses in mouse lung epithelial cells through TLR2 signaling in vitro.

Discussion

Previous studies have shown that repeated exposure to KA/E2, total extracts, and ES proteins induce allergic airway disease, and have also suggested that this immune response is due to the interaction of the PAR2 receptor with the serine protease of KA/E2. Intracellular and extracellular proteases have been reported from numerous other protozoa. Free-living amoeba proteases are important in host tissue invasion, migration, and host pathology. Extracellular proteases from Acanthamoeba caused damage to the collagen barrier in a rat corneal injury model. Other studies showed that extracellular proteases from Acanthamoeba are markers as a distinction between pathogenic and non-pathogenic organisms. To exclude protease activity, boiled ES proteins, protease inhibitors, or PAR2 KO mice were used. These results showed that the inflammatory immune response caused by KA/E2 was reduced by blocking protease activity but was not completely. Based on these results, to determine whether receptors other than protease-activated receptors are
involved, we investigated the relationship between *Acanthamoeba* infection and TLRs. As a result, we observed the role and possible mechanism of action of TLR2 in a mouse model of KA/E2-induced asthma.\textsuperscript{21}

Therefore, KA/E2 could be a new allergen for respiratory diseases of unknown etiology. In clinical trials, patients who tested positive in a skin prick test using *Acanthamoeba* antigen showed higher levels of *Acanthamoeba*-specific IgE compared to patients who did not and normal patients.\textsuperscript{22} Additionally, the patient’s serum contained a common antibody that reacted with an unknown KA/E2-derived protein of 13–15 kDa size.\textsuperscript{20,22} Two types of profilin from, (rAc-PF), profilinI and -II have been reported in *Acanthamoeba*.\textsuperscript{28} Several recent studies have shown that profilin in certain environments, acts as a marker of severity and an allergen capable of inducing airway symptom.\textsuperscript{29,30} Additionally, in its natural form, it is a potential candidate for the treatment of allergic diseases. Under specific conditions, profilin induces symptoms at all levels of the respiratory system, with uncertainty about whether airway symptoms are triggered and to what extent these symptoms contribute. Several studies have shown that sensitization to profilin is significantly associated with a more severe presentation of allergic inflammation.\textsuperscript{31} We examined that rAc-PF may be an allergen in *Acanthamoeba*, but further studies needed to identify the mechanisms of allergenic reactions induced by Ac-PF\textsuperscript{1}.

Pattern recognition receptors, including TLRs, retinoic acid-inducible gene-I-like receptors, nucleotide-binding oligomerization domain-like receptors, C-type lectin receptors, protease activated receptors, and purinergic receptors are present in airway epithelial cells.\textsuperscript{32,33} TLR2 is the most widely expressed member of the TLR family on the surface of immune cells, macrophages, neutrophils, monocytes, and dendritic cells.\textsuperscript{34} The occurrence or acute exacerbation of asthma is reportedly associated with infection by a variety of pathogens. Therefore, the occurrence of acute exacerbation of asthma after pathogen infection is speculated to be closely related to a TLR2-mediated mechanism.\textsuperscript{32,33,35} Upon recognition of PAMPs or DAMPs, pattern recognition receptors activate downstream signaling products of pro-inflammatory cytokines and chemokines.\textsuperscript{21,34,35} TLRs detect invading pathogens and play an essential role in the development of allergic airway diseases such as asthma.\textsuperscript{36} TLR2 is a major receptor that recognizes-glucans in fungi and peptidoglycans in Gram-positive bacteria.\textsuperscript{37} Previous studies have reported that TLR2 agonists ameliorate allergic airway inflammation by promoting Th1 response. However, an earlier study showed that the activation of TLR2 induces a Th2 cytokine response and promotes experimental airway inflammatory responses.\textsuperscript{38–41} Although various studies have indicated that TLR2 is associated with airway disease induction, the role of TLR2 and possible downstream signaling pathways in allergic airway inflammation induced by rAc-PF in mouse models remain to be elucidated.\textsuperscript{20,21}

In this study, to investigate the correlation between KA/E2-derived rAc-PF, which lacks protease activity and TLRs, we examined the mechanisms underlying the airway inflammatory response induced by rAc-PF in WT and TLR2 KO mice. A TLR2 antagonist significantly decreased rAc-PF-induced inflammation-related gene expression. In TLR2\textsuperscript{−/−} mice, rAc-PF exposure reversed airway inflammation phenotypes, such as airway hypersensitivity, immune cell infiltration around the airway, lung histology, and pro-inflammatory cytokine production. In addition, TLR2 likely modulates the production of DC-derived Th2/17 cell-polarizing Th2/17-related cytokines by stimulating rAc-PF.

**Conclusion**

In this study, our results indicate that TLR2 influences the activation of rAc-PF-induced innate and adaptive airway inflammatory responses in vitro and in vivo. In particular, TLR2 expressed on DCs can influence adaptive T-cell immunity against rAc-PF stimulation by regulating cytokine production and T-cell polarization. Although TLR2 signaling is essential for the occurrence and development of rAc-PF-induced airway inflammation, the detailed mechanism of the correlation rAc-PF exposure and TLR9 will be needed in further studies. Taken together, TLR2 antagonists may be a good treatment option for allergens-triggered airway hyperresponsiveness, thus preventing the development of asthma. These findings have potential applications for the design and development of novel allergenic therapies.

**Acknowledgments**

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT) (NRF-2020R1A2C1014361).
Disclosure

The authors report no conflicts of interest in this work.

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