

Der p2 Activates Airway Smooth Muscle Cells in a TLR2/MyD88-Dependent Manner to Induce an Inflammatory Response

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Der p2 is a major allergen from *Dermatophagoides pteronyssinus*, the main species of house dust mite and a major inducer of asthma, inducing harmful respiratory inflammatory responses by activating cells in the respiratory tract, leading to an unstable status. We hypothesize that Der p2 may induce local inflammatory responses by directly affecting airway smooth muscle (ASM) cells. In this study, we demonstrated that Der p2 raised nuclear factor-kappa B (NF- κ B) and extracellular signal-regulated kinase (ERK) 1/2 activation and induced a high level of proinflammatory cytokines expression in primary cultured ASM cells. Der p2 activated the MyD88 signaling pathway through toll-like receptor (TLR) 2, not through TLR4. Notably, Der p2 stimulated ASM cells to increase phosphorylation of ERK 1/2 and expression of c-Fos, which were also important in the T helper type 2 (Th2) immune response. These results suggest that Der p2 induces asthma through the MyD88 signaling pathway in respiratory tissue.

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Continuous exposure of the respiratory tract of an atopic individual to aeroallergens is an important risk factor for the development of allergic asthma. Exposure to house dust mites (HDMs), one kind of indoor allergen, is positively associated with prevalence of allergic asthma (Rabito et al., 2007), and is a major cause of sensitization with wheezing and airway hyperresponsiveness that is especially problematic in certain countries (Tsai et al., 2000). Der p2, a group II protein antigen from *Dermatophagoides pteronyssinus*, is the major allergen of HDM related to the development of allergic asthma. Der p2-specific immunoglobulin (Ig)E antibodies are positively associated with 87.8% of asthmatic patients (Tsai et al., 2000). Furthermore, Der p2 induces allergic airway inflammation by regulating a specific IgE antibody and interleukin (IL)-4 production in a T helper type 2 (Th2) immune response (Li et al., 2005). Several studies have focused on the stimulation of immune/hematopoietic cells by Der p2, which causes an airway inflammatory response (Tsai et al., 2002; Liu et al., 2003; Li et al., 2005); however, the influence of Der p2 on airway tract tissue is still unknown.

In the development of asthma, not only do immune cells induce airway inflammation, but also airway tract tissue such as airway smooth muscle (ASM) cells may possess proinflammatory and immunomodulatory functions (Sukkar et al., 2006). ASM cells might respond to allergens directly through innate immune receptors and produce various proinflammatory cytokines, causing local inflammatory responses associated with innate immune activation.

Autologous and microbe products could activate toll-like receptors (TLRs) and cause an increase in production of inflammatory mediators, affecting adaptive immunity (Gangloff and Guenounou, 2004; Sukkar et al., 2006). It has been demonstrated that airway infections contribute to the induction of asthma (Micillo et al., 2000), and the immunomodulatory function of ASM cells might occur via TLR activation. ASM cells express functional TLR2, TLR3, and TLR4 (Sukkar et al., 2006), and a variety of allergens may act on ASM

cells and activate TLRs. Activation of ASM cells increases adhesion molecule expression and production of inflammatory mediators such as chemokines and cytokines, which are then activated immune cells, causing airway inflammation. Activation of ASM cells also enhances proliferation and reduces apoptosis, causing airway narrowing and airway hyperresponsiveness in the pathogenesis of asthma (Morris et al., 2005; Shan et al., 2006; Sukkar et al., 2006).

TLRs activate distinct signaling pathways through different adaptor proteins, prominently Myeloid differentiation primary response protein (MyD)88 and Toll/IL-1 receptor-domain-containing adaptor inducing IFN- β (TRIF), leading to the production of inflammatory cytokines and/or type I interferons (IFNs) (Akira et al., 2006; Beutler et al., 2006). MyD88 is a common downstream protein for most TLRs, with the exception of TLR3, and is required for TLR-induced activation of NF- κ B and MAPK kinases (Fitzgerald et al., 2001; Horng et al., 2002; Yamamoto et al., 2002). TLR3, is associated with TRIF rather than MyD88 and induces interferon regulatory factor-3

Abbreviations: ASM, airway smooth muscle; Der p2, group II *Dermatophagoides pteronyssinus*; ERK, extracellular signal-regulated kinase; IRAK, interleukin-1-receptor-associated kinase; MyD88, myeloid differentiation primary response protein 88; NF- κ B, nuclear factor-kappa B; TLR, toll-like receptor.

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(IRF-3) and NF- κ B activation, which is termed the MyD88-independent pathway. TLR4 is associated not only with the MyD88-independent pathway but also the MyD88-dependent pathway for its signaling and induces both proinflammatory cytokine and IFN production (Yamamoto et al., 2003). TLRs regulate particular adaptive immune responses in defense against pathogens and can induce Th1-dominant (autoimmune disease) or Th2-dominant (asthma) diseases (Hoebe et al., 2004). Redecke et al. (2004) reported that the TLR2 agonist promotes Th2 immune responses, causing the development of asthma. The TLR2 agonist promotes the MyD88-dependent signaling pathway to induce phosphorylation of ERK1/2, which increases expression of c-Fos (a suppressor of IL-12) and rarely induces IL-12p70 (Agrawal et al., 2003), and the adaptive immune response tends towards a Th2 response.

HDM not only regulates immune cells but also airway tissue to cause airway inflammation (Grunstein et al., 2005). Der p2 has enzymatic activity, and this activity could increase bronchial epithelium permeability, may induce epithelial cell detachment, and can generate histological signs of epithelial injury (Herbert et al., 1995). In addition, during acute respiratory syncytial virus (RSV) infection, the airway epithelial barrier is also disrupted and the allergen may pass through the epithelial barrier into the submucosa (Wang et al., 1998). Some studies have recognized that Der p2, which is similar to Der p1, exhibits enzymatic activity (Roche et al., 1997) and pro-asthmatic effects, and the mechanisms of action of Der p1 in ASM have been investigated (Grunstein et al., 2005), but the effect of Der p2 in ASM has not been studied. Der p2 interaction with TLRs in ASM cells remains poorly understood.

Recombinant Der p2 (rDer p2) is distinct from LPS, which can activate MyD88-dependent and -independent pathways (Yamamoto et al., 2004). In this study, we used cultured ASM cells and investigated whether rDer p2 stimulation has a direct effect on the pathway in ASM cells. In addition, the association of MAPKs signaling and TLR activation was also investigated. The aim of the study was to examine whether rDer p2 could induce an inflammatory response in the airway by direct interaction with ASM cells and activate the TLR2 downstream signaling pathway and MAPK that regulate the function of ASM cells.

Materials and Methods

Reagents

All chemicals, antibodies against β -actin and GAPDH were purchased from Sigma (St. Louis, MO). Antibodies to TLR2/4 and monoclonal antibody 2.5 (T2.5) were obtained from Cell Sciences (Canton, MA). Antibodies against phosphorylated ERK1/2, ERK1/2, phosphorylated JNK and JNK were purchased from Cell Signaling Technology (Beverly, MA). rDer p2 was obtained from Indoor Biotechnologies (Manchester, UK). Antibodies against NF- κ B, MyD88, IRAK1, IL-6 and MCP-1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-rabbit IgG and goat anti-mouse IgG antibodies were purchased from Pierce Biotechnology (Rockford, IL).

Isolation and culture of Brown Norway rat airway smooth muscle cells

Adult 6- to 8-week-old male Brown Norway rats were obtained from the National Laboratory Animal Center (Taiwan). Rat trachea tissues were harvested and incubated in Hanks' Balanced Salt Solution (HBSS) with 0.1% collagenase solution at 37°C for 20 min. Loosened connective tissues were scraped and cut into 0.5 mm³ fragments, which were placed in a culture dish with DMEM/F12, 10% FBS, 2 mM glutamine and 100 U/ml penicillin/streptomycin (Gibco, Grand Island, NY). The tissues were then incubated at 37°C. Passages 6–8 were used in all experiments. Cultured

ASM cells expressed α -smooth muscle actins, which were detected immunocytochemically. Confluent ASM cells were growth-arrested by serum deprivation for 48 h, then the cells were treated as indicated and harvested for RNA or protein extraction.

Total cellular RNA extraction and real-time PCR

Cells were stimulated with Der p2 (2 μ g/ml) or MAPK inhibitor for 6 h; the supernatant was then removed and cells were washed in 10 ml 1 $\times$ PBS. Total cellular RNA extraction was performed using a TRI reagent protocol. The cDNA was primed with oligo(dT)_{12–18} and extended with reverse transcriptase (Clontech Laboratories, BD Biosciences, Mountain View, CA). The cDNA fragments of rat TLR2, IL-6, MCP-1, eotaxin and GAPDH were amplified by real-time PCR and IFN- β and RANTES by PCR using specific primer pairs.

Immunoprecipitation and Western blot analysis

Total cellular proteins were extracted by cell lysis buffer (200 mM Tris/HCl, pH 7.4, 5 M NaCl, 200 mM EDTA, 200 mM EGTA, 10% Triton X-100, 200 mM Na₃VO₄, 50 mM NaF, 25 mM PMSF, and 2 mM Leupeptin, pepstatin A). Cytoplasmic proteins and nuclear proteins were obtained using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology). Protein concentration was determined using a Bio-Rad protein assay (Bio-Rad, Hercules, CA). Equal amounts of proteins were subjected to immunoprecipitation and Western blot analysis. Total cell lysates were immunoprecipitated with anti-TLR2 or anti-TLR4 antibody. Lysates and immunoprecipitates were placed in 5 $\times$ sample buffer, boiled for 5 min, and separated via SDS-PAGE. The resulting Western blots were probed using the appropriate antibodies at 4°C overnight then imaged by conjugation with horseradish peroxidase-linked secondary antibody and ECL detection reagent.

Confocal laser scanning microscopy

ASM cells (5 $\times$ 10⁵ cells/well) plated on cover slides. Cells were fixed in 4% paraformaldehyde in PBS for 30 min, permeabilized with 0.2% Triton X-100 in PBS for 10 min with blocking of non-specific binding sites using 1% BSA. Fixed cells were then incubated with primary antibodies to α -smooth muscle actin (red fluorescence) or TLR2 (1:50 dilution) (green fluorescence) overnight and then exposed to secondary antibody (FITC-conjugated goat anti-rabbit IgG or PE-conjugated goat anti-rabbit IgG at 1:100 dilution), followed by DNA staining with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI, blue fluorescence). Photomicrographs were obtained using a Leica TSC SP2 Confocal Spectral Microscope (Hsu et al., 2007).

Flow cytometric analysis

Flow cytometric analyses were performed using a FACSCalibur cytometer (BD Biosciences). PBMC were isolated from heparinized blood by Isopaque-Ficoll by Lymphoprep (Nycomed Pharma AS, Oslo, Norway) through gradient centrifugation, resuspended in 10% FCS/RPMI-1640 (2.5 $\times$ 10⁶ cells/ml). Cells were stained with anti-TLR2 Ab (1:250) or control IgG at 4°C for 30 min, followed by fluorescein isothiocyanate (FITC)-conjugated secondary Ab (1:1,000) (BioSource International, Camarillo, CA) at 4°C for an additional 30 min.

Statistical analysis

Statistical analyses were carried out using SPSS/Windows software. The statistical significance was evaluated by one-way analysis of variance (ANOVA).

Results

TLR1 through TLR10 mRNA expression under basal condition

To characterize ASM cells, we used monoclonal anti- α -smooth muscle actin to qualify the ASM cells. This antibody could specifically recognize the α -smooth muscle actin isoform of actin. It does not react with the other major actin isoforms presenting in fibroblasts or epithelial cells. We demonstrated ASM cells which were stained with anti- α -smooth muscle actin monoclonal antibody by using confocal laser scanning microscopy (Fig. 1a).

To determine expression of TLR2 on ASM cells, we assessed the expression of TLR2 by using Immunofluorescence staining assay and flow cytometry. The results showed that ASM cells were positive stained with anti-TLR2 monoclonal antibody by using confocal laser scanning microscopy (Fig. 1b). In Figure 1c, the middle gray area indicated that ASM cells were stained with TLR2 antibody and the light gray area indicated that PBMCs were stained with TLR2 antibody by using flow cytometric analysis. The expression intensity of TLR2 on ASM

cells (9.7%) was lower than PBMCs (62.3%) (Fig. 1c). These results indicated constitutional expression of TLR2 in ASM cells.

We also demonstrated that rat ASM cells which were not stimulated with serum and cytokines could express TLR1 through TLR10 mRNA by using real-time PCR. Threshold cycle numbers for TLR2 and TLR4 were less than 25, whereas those for TLR1, TLR3, TLR7, TLR8, TLR9, and TLR10 were equal to or higher than 30 (Fig. 1d).

Der p2 induced production of proinflammatory cytokines

We first determined whether rDer p2 could, without the presence of immune cells, stimulate ASM cells and induce the cells to produce proinflammatory cytokines directly. After in vitro activation with rDer p2, the expression of proinflammatory cytokines of ASM cells was measured. It has been shown that human ASM cells express TLR2 and TLR4, which are the potent receptors involved in the innate immunity

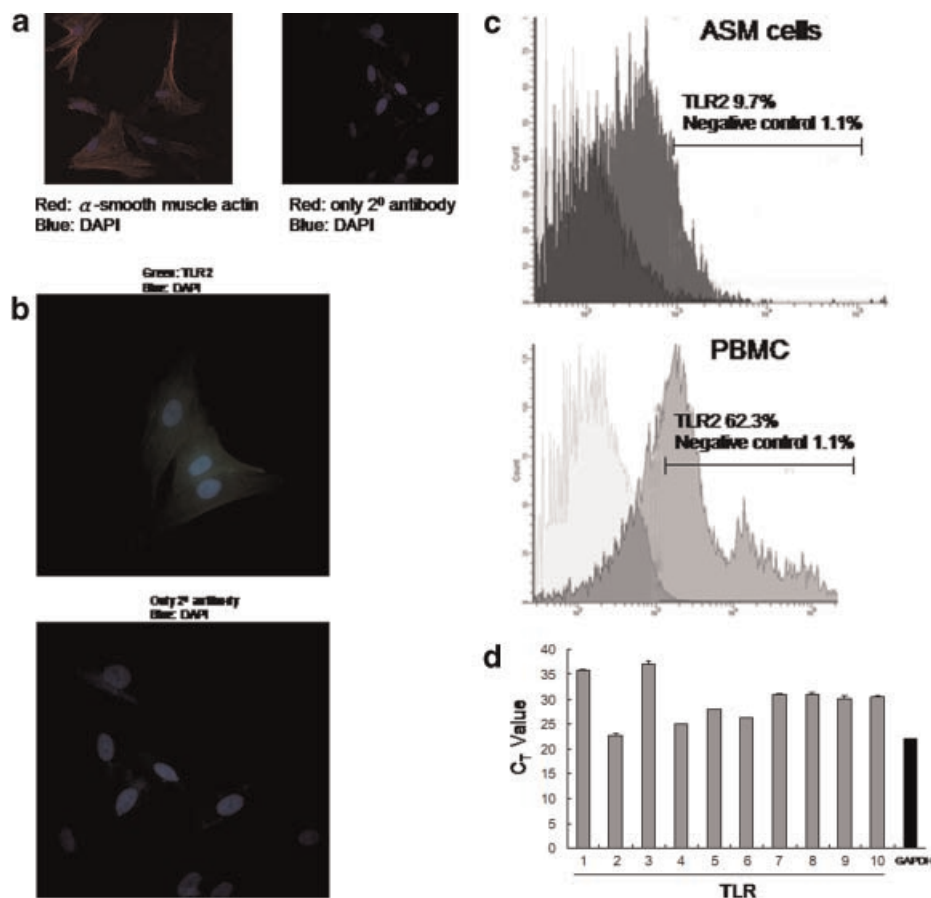


Fig. 1. The pattern of TLRs expressed in rat ASM cells under basal condition. **a:** 10^5 cells/well were incubated with FBS-free medium for 48 h. Cells were stained with anti- α -smooth muscle actin antibodies (left graph) or anti-IgG antibodies (right graph). Then, PE-labeled secondary antibodies (1:100) were used to stain with cells. The nuclei were stained with DAPI (blue fluorescence). The results were detected by using confocal laser microscopic system. **b:** ASM cells were stained with anti-TLR2 antibody (1:50) or anti-IgG antibodies. Then, FITC-labeled secondary antibodies (1:100) were used to stain with cells (green fluorescence). The results were detected by using confocal laser microscopic system. **c:** ASM cells were stained with anti-TLR2 antibody (1:250) (middle gray area) or anti-IgG antibodies (dark gray area). PBMCs also were stained with TLR2 (1:250) (light gray area) or control IgG (grayline). Then, FITC-labeled secondary antibodies were used to stain with ASM cells and PBMCs. The results were detected by using flow cytometry. **d:** TLR1 through TLR10 mRNA expression. Specific TLRs primers were used to detect various TLRs mRNA expression by using real-time PCR. Note that the scale of threshold cycle (C_T), which lower histogram bars represented higher levels of mRNA expression. Bars represented means $\pm$ SD for triple times. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

of ASM cells to proinflammatory response induction (Sukkar et al., 2006). In order to test the function of these two receptors in the ASM cells of our system, we activated cells with LPS and Pam₃CSK₄, agonists for TLR4 and TLR2, respectively. Cultured ASM cells were stimulated with LPS (10 μ g/ml), rDer p2 (2 μ g/ml) or Pam₃CSK₄ (100 ng/ml) for 4 h, and the results showed higher MCP-1 and IL-6 mRNA expression in cells stimulated with Pam₃CSK₄ and rDer p2 as compared with those stimulated by LPS (Fig. 2a). The cytokine level of MCP-1 and IL-6 were confirmed after ASM cells were stimulated with LPS, rDer p2, or Pam₃CSK₄ for 24 h (Fig. 2b).

LPS is a potent immune stimulator and can induce strong cytokine expression in most cell types. Interestingly, mildly increased eotaxin mRNA expression in ASM cells was observed upon LPS stimulation, but not rDer p2 or Pam₃CSK₄ stimulation (Fig. 2c). These data indicate that rDer p2 can be recognized by ASM cells and can induce a proinflammatory response directly.

Der p2 activated JNK and ERK of the MAPK pathway

The production of several chemokines has been shown to be regulated by MAPKs signaling pathways (Nakamichi et al., 2005). In order to find out which signaling pathways are involved in the activation of rDer p2 in ASM cells, phosphorylation of MAPKs in ASM cells was examined by Western blotting. ASM cells were stimulated with rDer p2 for either 15 or 30 min, and phospho-MAPKs and MAPKs levels were measured in the total cell lysates. We found that rDer p2 could enhance the phosphorylation of ERK and JNK in ASM cells (Fig. 3a), but p38 could not (data not shown). To evaluate the role of MAPKs in the production of inflammatory mediators, a blocking study using MAPKs inhibitors was performed, the results of which showed that Der p2-induced MCP-1 and IL-6 expression was significantly inhibited by MAPKs inhibitors (Fig. 3b,c), and rDer p2 also induced expression of c-Fos (Fig. 3d).

Der p2 activated the MyD88-dependent signaling pathway only

NF- κ B is the key signaling pathway in the immune system for the initiation of an inflammatory response in both hematopoietic

and non-hematopoietic cells. We studied the translocation of NF- κ B by Western blot, and the results showed that rDer p2 promoted translocation of NF- κ B, as shown in Figure 4a.

To assess whether rDer p2 activates the MyD88-dependent pathway or the MyD88-independent pathway, we used LPS and rDer p2 to stimulate ASM cells. We found that rDer p2 induced expression of MyD88 and degradation of IRAK1 as with LPS stimulation in ASM cells (Fig. 4b,c); moreover, LPS, which is recognized by TLR4, has been shown to use the MyD88-independent pathway to induce the production of type I IFN. Instead of the MyD88-dependent pathway, TLR3 and TLR4 might therefore use the MyD88-independent/TRIF-dependent pathway. Activation of the TRIF-dependent pathway will induce NF- κ B and IRF activation, leading to type I IFN-like cytokine expression, such as expression of IFN- β and RANTES. IFN- β and RANTES production in ASM cells was observed after LPS stimulation; however, rDer p2 did not affect the production of these mediators (Fig. 4d).

Thus, these findings indicate that rDer p2 stimulation up-regulated ASM cells in a different manner to LPS stimulation, which is consistent with the cytokine expression patterns observed (Fig. 2). rDer p2 activated ASM cells to generate inflammatory cytokines via the MyD88-dependent pathway only.

Der p2 activated TLR2 signal transduction in ASM cells

rDer p2 signaling is MyD88-dependent, and we hypothesize that recognition of rDer p2 by ASM cells might occur through MyD88-dependent TLRs. ASM cells express mainly TLR2, 3, and 4 (Sukkar et al., 2006), in which TLR2 and TLR4 are MyD88-dependent, and both receptors are not only expressed but also have biological functions in ASM cells (Fig. 2). To investigate whether Der p2 is recognized by TLR2 or TLR4, we measured MyD88/TLR association by immunoprecipitation after exposure to rDer p2. We proved the specific recognition of Der p2 by TLR2, and we also tried to use a TLR2 antagonistic antibody (T2.5) to block the rDer p2-activated TLR2 downstream signal in ASM cells. Cells were pretreated with or without T2.5 antibody for 1 h before exposure to rDer p2 or TLR2 agonist Pam₃CSK₄. MyD88 and TLR2 expression was detected by Western blot (Fig. 5a). We also confirmed MyD88/TLR association by immunoprecipitation using MyD88 antibody

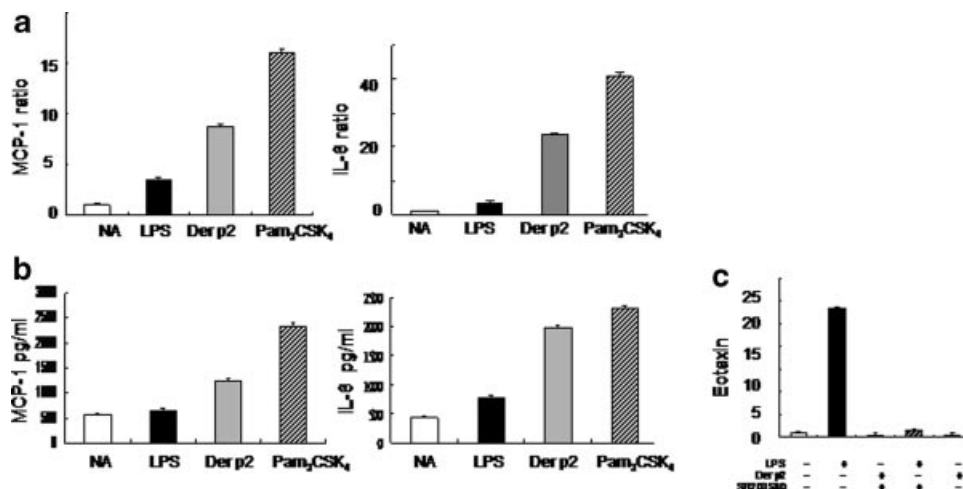


Fig. 2. rDer p2 induced proinflammatory production of cytokines. Growth-arrested cells were treated with LPS (10 μ g/ml), rDer p2 (2 μ g/ml), or Pam₃CSK₄ (100 ng/ml) for 4 or 24 h. a,c: Specific MCP-1, IL-6 and eotaxin primers were subjected to real-time PCR and (b) determined protein levels of MCP-1 and IL-6 by ELISA. Experimental results are presented as means $\pm$ SD from three experiments.

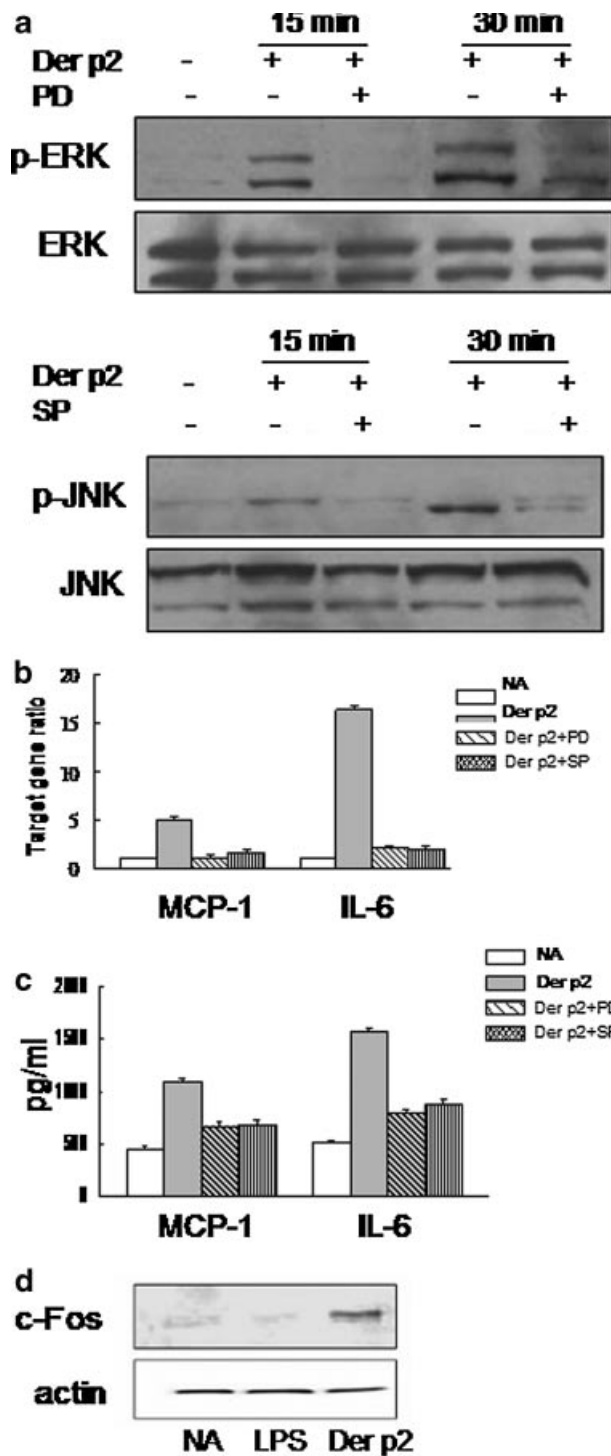


Fig. 3. rDer p2 activated JNK and ERK of the MAPK pathway. Growth-arrested cells were treated with rDer p2 (2 μ g/ml) in addition to various MAPK inhibitors (PD98059, 10 μ M; SP600125, 10 μ M) (a) for 15 or 30 min, and Western blotting was performed using the antibody. Growth-arrested cells were treated with rDer p2 in addition to MAPK inhibitors for 2 or 4 h, and (b) specific MCP-1 and IL-6 primers were subjected to real-time PCR. c: Determined protein levels of MCP-1 and IL-6 by ELISA. d: Western blotting using the c-Fos antibody. Experimental results are presented as means $\pm$ SD from three experiments.

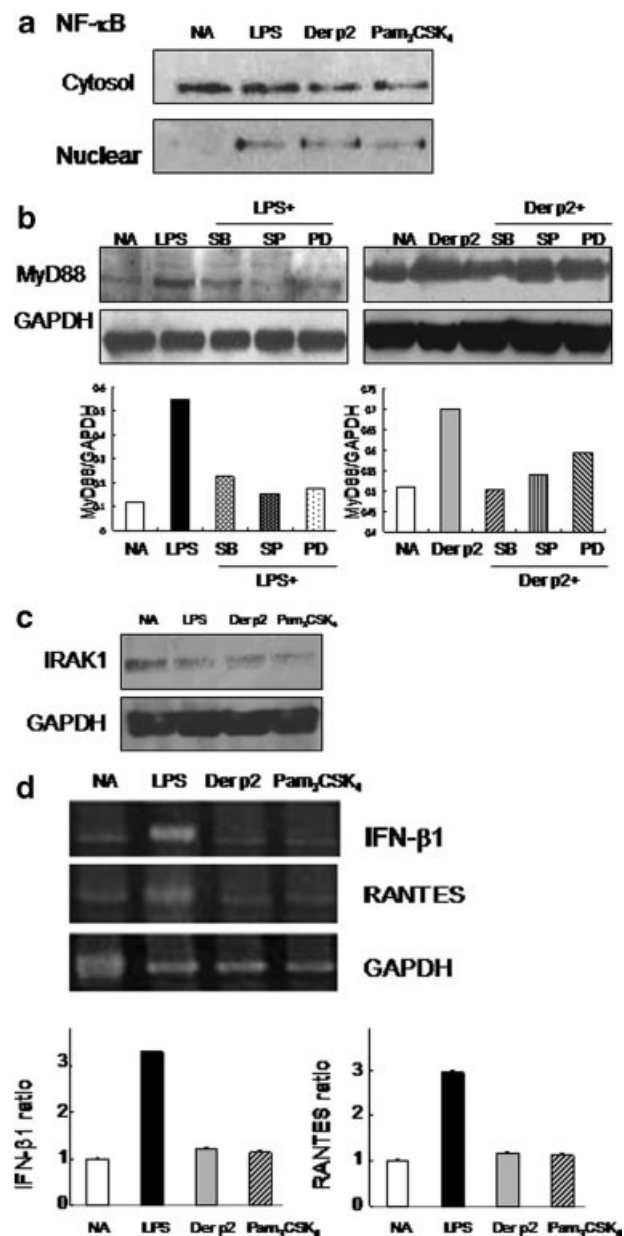


Fig. 4. Der p2 only activated the MyD88-dependent signaling pathway in ASM cells. a: Cells were treated with LPS (10 μ g/ml), rDer p2 (2 μ g/ml), or Pam₃CSK₄ (100 ng/ml) for 15 min. Western blotting was performed using anti-NF- κ B antibody in cytosol and nuclear protein. b, c: Growth-arrested cells were treated with LPS (10 μ g/ml) or rDer p2 (2 μ g/ml) in addition to various MAPK inhibitors (PD98059 10 μ M, SP600125 10 μ M and SB 203580 10 μ M) for 15 min. Western blotting was performed using anti-MyD88, anti-IRAK1 and anti-GAPDH antibodies. d: Specific IFN- β and RANTES primers were subjected to RT-PCR. All data represent two independent experiments. Experimental results are presented as means $\pm$ SD from three experiments.

(Fig. 5b). The results showed significantly increased association between MyD88 and TLR2 in ASM cells after exposure to rDer p2 or TLR2 agonist Pam₃CSK₄ and decreased association between MyD88 and TLR2 in ASM cells pretreated with T2.5 antibody.

However, no time- or dose-independent increases in MyD88/TLR4 complexes were seen (Fig. 5c). We determined

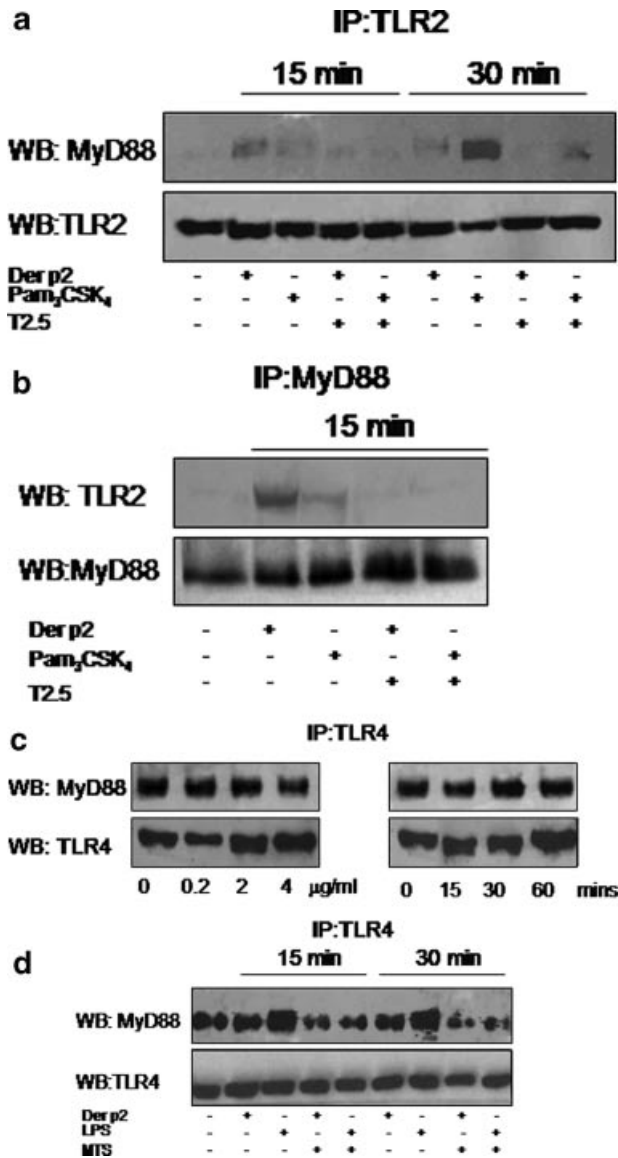


Fig. 5. rDer p2 activated TLR2 signal transduction in ASM cells. Antagonist TLR2 antibody T2.5 inhibits rDer p2-induced events in ASM cells. **a:** ASM cells were cultured with the addition of either no antibody or T2.5 antagonistic antibody for 1 h before exposure to rDer p2 (2 μg/ml) or Pam₃CSK₄ (100 ng/ml) for 15 and 30 min. Protein was normalized and subjected to immunoprecipitation (IP) with anti-TLR2 or (b) anti-MyD88 antibody. **c:** Growth-arrested cells were stimulated with different doses of rDer p2 (0.2, 2, and 4 μg/ml) for three lengths of time (15, 30, and 60 min). Total protein was harvested at each dose and time point, and protein was normalized and subjected to immunoprecipitation (IP) with anti-TLR4 monoclonal antibody. **d:** ASM cells were treated with MTS510 (5 μg/ml) antibody, an antagonist TLR4 antibody, or without MTS510 antibody or for 1 h before exposure to rDer p2 (2 μg/ml) or LPS (10 μg/ml) for 15 and 30 min. Protein was normalized and subjected to immunoprecipitation (IP) with anti-TLR4 monoclonal antibody. The subsequent precipitates were separated by SDS-PAGE and Western blotting was performed using the indicated antibodies.

whether rDer p2 activates the TLR4 downstream signal in ASM cells using TLR4 antagonistic antibody (MTS510). Cells were pretreated with or without MTS510 antibody for 1 h before exposure to rDer p2 or TLR4 agonist LPS. The result showed that rDer p2 was not recognized by TLR4 in ASM cells, as shown

in Figure 5d, and that Der p2 only activated TLR2 pathways in ASM cells.

rDer p2 induced expression of TLR2 mRNA

In order to investigate rDer p2-regulated expression of TLR2 mRNA in ASM cells, cultured ASM cells were stimulated with rDer p2, TLR4 agonist LPS or TLR2 agonist Pam₃CSK₄ for 4 h. Cells treated with LPS exhibited increased expression of TLR2 mRNA. As shown in Figure 6a, expression of TLR2 mRNA was up-regulated by rDer p2, and the stimulation results exhibited statistical differences. Higher expression of TLR2 mRNA was observed in ASM cells treated with rDer p2 and Pam₃CSK₄.

However, different MAPKs inhibitors resulted in different expression levels of TLR2 after rDer p2 stimulation of ASM cells. Regarding expression of TLR2 mRNA, MAPK inhibitors down-regulated the expression of TLR2 by rDer p2 (Fig. 6b).

Discussion

We have demonstrated in this study that ASM cells can recognize and produce inflammatory cytokines in vitro when directly stimulated by Der p2. The biochemical data obtained showed that Der p2 can activate ERK, JNK, and NF-κB pathways in a MyD88-dependent manner. Finally, using an antibody blocking assay, we have shown that recognition of Der p2 in ASM cells is through TLR2. These data indicated that the ASM cells, instead of being affected by the inflammatory environment and presenting asthmatic clinical features, are able to sense the allergen directly and initiate the local inflammatory response.

According to the pathogenesis of asthma, inflammation of ASM cells is a risk factor for airway remodeling (Oliver and Black, 2006; Odaka et al., 2007). ASM cells secrete RANTES, MCP-1, IL-6, IL-8 and eotaxin following stimulation with IL-1β, TNF-α, and IFN-γ (Pype et al., 1999; Hallsworth et al., 2001; Doganci et al., 2005). After activation, ASM cells secrete various chemokines/cytokines, leading to the recruitment and activation of hematopoietic cells including mast cells, lymphocytes and eosinophils during airway inflammation (Rose et al., 2003; Doganci et al., 2005; Lee et al., 2008). Traditionally, it is thought that pathogen invasion is detected by immune/hematopoietic cells, and that the production of proinflammatory cytokines by those cells initiates a local to systematic inflammatory response. However, it has been shown that some recently identified pathogen receptors that can recognize the pathogen product are not only expressed in hematopoietic cells but also in various other mammalian cells (Becker et al., 2000; Muzio et al., 2000; Visintin et al., 2001; Shan et al., 2006). It is possible that a local inflammatory response is

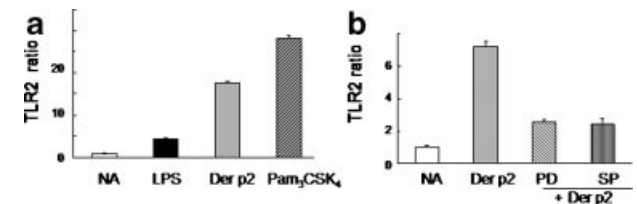


Fig. 6. rDer p2 induced expression of TLR2 mRNA. **a:** Growth-arrested cells were treated with LPS (10 μg/ml), rDer p2 (2 μg/ml) or Pam₃CSK₄ (100 ng/ml) for 4 h. Specific TLR2 primers were subjected to real-time PCR. **b:** Growth-arrested cells were treated with rDer p2 (2 μg/ml) in addition to various MAPK inhibitors (PD 98059, 10 μM; SP600125, 10 μM) for 2 h. Experimental results are presented as means ± SD from three experiments.

initiated by the recognition of the pathogen/allergen by non-hematopoietic cells. ASM cells affected by a chronic allergen-induced inflammatory response could process a pathological remodeling in asthmatic individuals. In this study, we have shown that, rather than being affected by immune cells, ASM cells alone could directly react with allergens through innate receptors and produce the proinflammatory response. This discovery might change our understanding of the process of asthma and provide new ideas for the treatment of asthma.

The important relationships between allergens from HDMs, cockroaches, fungi and pollens and asthma development have been examined in numerous clinical studies (Dahl et al., 2005; Denning et al., 2006). In this study, we demonstrated that the rDer p2-induced inflammation of ASM cells up-regulates the expression of TLR2. Other studies have demonstrated that proinflammatory cytokines such as TNF- α up-regulate TLR2 via the NF- κ B pathway (Syed et al., 2007). It has also been verified that proinflammatory cytokines up-regulate expression of TLR2, thereby promoting inflammatory responses (Parker et al., 2004). We also determined in this study that the rDer p2-induced MAPK signaling cascade in ASM cells plays a crucial role in cytokines synthesis and increased TLR2 expression by using MAPK inhibitors. The inhibition of TLRs expression by MAPK inhibitors might directly affect the transcriptional level by inactivation of MAPK-dependent transcriptional factors; or, these inhibitors could block the production of proinflammatory cytokines and indirectly diminish the inflammation induced up-regulation of TLRs expression. These results also confirm those of *in vivo* animal studies that have demonstrated airway hyperresponsiveness and elevated intrapulmonary response and cytokine levels in rDer p2-sensitized *C56BL/6* and *Balb/c* mice (Tsai et al., 2002; Qiu et al., 2006).

Pathogens or allergens contact TLRs to activate target cells, producing cytokines that influence adaptive immune responses. In general, the TLR signaling pathway of immune cells leads to the production of IFN- α , TNF- α , IL-1, and IL-6. These cytokines up-regulate the production of IL-12 and IL-18, promoting Th1 differentiation (Wagner, 2001). Because TLRs induce a strong Th1 immune response, many studies have used various types of TLR ligands to treat the Th2 immune response in allergic diseases; however, these reports suggest that whether Th1 or Th2 differentiation occurs depends on the dosage of the ligand (Agrawal et al., 2003; Didierlaurent et al., 2004; Velasco et al., 2005)—for example, a low concentration of LPS may induce Th2 differentiation, whereas a higher LPS concentration induces Th1 differentiation (Eisenbarth et al., 2002; Kaisho et al., 2002). Additionally, using different TLRs to induce the signaling pathway could cause different adaptive immune responses: for example, the TLR2 signaling pathway causes Th2 differentiation in asthma pathogenesis. The TLR2 agonist Pam₃CSK₄ induces IL-13, IL-1 β , and GM-CSF in support of Th2 differentiation (Ritz et al., 2002; Herrick and Bottomly, 2003; Wynn, 2003). The results of another study have already proven that agonists of TLR2 (MyD88-dependent pathway) stimulate phosphorylation of ERK1/2, thereby increasing expression of c-Fos and enhancing Th2 responses (Agrawal et al., 2003).

Der p2 is considered an allergen in HDMs that induces asthma (Tsai et al., 2000). When HDMs are inhaled into the airway, epithelia cells are the first cells to interact with the allergen. Some antigens from HDMs contain active proteolytic enzymes that break down tight epithelial cell junctions (Gershwin, 2007). Moreover, Der p2 enhances NO production of alveolar macrophages via a non-enzymatic pathway (Peake et al., 2003), and this NO production causes lung inflammation and epithelial injury (Li et al., 1996). The local inflammatory response and its associated tissue damage might assist other allergens in penetrating to the tissue underneath, such as ASM cells. These mechanisms indicate that Der p2 might therefore make contact with ASM cells. Furthermore, the use of a mouse

animal model might be required in order to demonstrate this interaction *in vivo*.

In conclusion, we have demonstrated that the aeroallergen rDer p2 could act directly on ASM cells. rDer p2 activated the TLR2 signaling pathway in ASM cells, but not TLR4. rDer p2 is likely to be essential for the promotion of the MyD88/IRAK1/NF- κ B pathway to induce an inflammatory response and bronchial hyperresponsiveness, not through a TRIF pathway, and we determined the mechanism by which Der p2 affected the immune response in the context of asthma should provide us with invaluable insight into the processes that lead to the development of the disease.

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