

β -catenin mediates the inflammatory cytokine expression induced by the Der p 1 house dust mite allergen

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Abstract. The modulations of β -catenin were analyzed during the inflammatory response induced by the Der p 1 house dust mite allergen. Der p 1 induced the dose-dependent expression of inflammatory cytokines, including interleukin (IL)-6, IL-8, monocyte chemoattractant protein-1 (MCP-1) and tumor necrosis factor- α (TNF- α) in THP-1 human monocytic cells. The mRNA expression levels of β -catenin were not altered, however protein levels increased following Der p 1 treatment, demonstrating that β -catenin was modulated by post-transcriptional processes. It was also revealed that nuclear β -catenin levels were significantly increased while cytoplasmic β -catenin levels were reduced, which demonstrated the nuclear translocation of β -catenin by the Der p 1 allergen. Glycogen synthase kinase 3 β (GSK3 β), a regulator of β -catenin stability, was demonstrated to be phosphorylated following Der p 1 treatment. When β -catenin was knocked down by the transfection of its small interfering RNA (siRNA), inflammatory cytokine expression as well as nuclear factor- κ B (NF- κ B) activity, which were induced by Der p 1 treatment, were all significantly reduced. The results demonstrated that Der p 1-induced inflammatory responses were mediated by β -catenin.

Introduction

The house dust mite (HDM), *Dermatophagoides pteronyssinus*, is one of the most common environmental inducers of

allergic diseases, including asthma (1,2). HDM is reported to induce inflammatory responses by modulating various cells, including monocytes (3), macrophages (4), mast cells (5), basophils (6), eosinophils and bronchial epithelial cells (7). These responses induced by HDM have been demonstrated to be caused by inflammatory cytokines, including interleukin (IL)-6, IL-8 and tumor necrosis factor- α (TNF- α) (3,7-9).

Der p 1, one of the major allergens of HDM, is a cysteine protease contained in fecal pellets of mites and is known to be chemically stable for up to 4 years in the environment (10). Inhaled mite fecal-dust sticks to the mucous in the bronchioles, where Der p 1 quickly develops a short-lived inflammatory reaction and repeated inflammatory reactions lead to asthma development (11). The National Academy of Sciences Institute of Medicine (Washington, DC, USA) concluded that there is sufficient evidence of a causal association between Der p 1 allergen exposure and the development and exacerbation of asthma in individuals specifically sensitized to dust mites (12). Therefore, understanding the molecular mechanisms underlying the Der p 1-induced inflammatory response is important for the effective treatment of allergic diseases, including asthma.

Asthma development is well known to be the result of the gene-environment interaction, i.e. environmental allergen stimuli in genetically susceptible individuals (13). In our previous study, β -catenin was suggested to be a candidate gene of asthma as we demonstrated that the promoter SNPs of β -catenin, which affects its mRNA expression, was significantly associated with asthma in human subjects (14). It has been suggested that asthma candidate genes, including β -catenin may modulate the inflammatory response induced by the inhaled environmental allergens, including Der p 1, which has a causal association with asthma development (12,13). In the present study, we investigated whether β -catenin is involved in the mechanisms of the inflammatory response induced by the Der p 1 allergen in THP-1 human monocytic cells, which have been used in studies of HDM-induced inflammatory responses (3,15-17).

Materials and methods

Cell culture. THP-1 human monocytic cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI-1640 medium containing 10%

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Abbreviations: IL, interleukin; GSK, glycogen synthase kinase; HDM, house dust mite; MCP, monocyte chemoattractant protein; PCR, polymerase chain reaction; siRNA, small interfering RNA; SNP, single nucleotide polymorphism; TBP, TATA box binding protein; TNF, tumor necrosis factor

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fetal bovine serum, 100 $\mu\text{g}/\text{ml}$ of streptomycin and 100 U/ml of penicillin. To induce an inflammatory response, the cells were stimulated with a HDM allergen, Der p 1 (GenWay Biotech Inc., San Diego, CA, USA).

Real-time polymerase chain reaction (PCR). Cells were cultured in 12-well plates and total RNA was extracted using an RNeasy kit (Qiagen, Hilden, Germany). Total RNA was reverse-transcribed using a cDNA Reverse Transcription kit (Applied Biosystems, Inc., Foster City, CA, USA). Briefly, the reaction was performed in a final volume of 20 μl , which included 100 mM of dNTP, random primers, MultiScribe™ Reverse Transcriptase, RNase inhibitor and 1 μg of total RNA. The reaction mixtures were heated at 25°C for 10 min, 37°C for 120 min and 85°C for 5 sec. Real-time PCR was performed using a StepOne PCR System (Applied Biosystems, Inc.) in triplicate in a final volume of 20 μl , which included TaqMan gene expression master mix, an optimized concentration of each primer, 250 nM of a TaqMan probe and 2 μl of cDNA reaction mixture. The reaction mixtures were preheated at 95°C for 10 min to activate the enzyme and then subjected to 40 cycles of melting at 95°C for 15 sec and annealing/extension at 60°C for 1 min. The efficiency of real-time PCR was ~100%. The assay-on-demand gene expression products (Applied Biosystems, Inc.) were used to evaluate the mRNA expression levels of IL-6 (Hs00174131_m1), IL-8 (Hs99999034_m1), monocyte chemoattractant protein-1 (MCP-1; Hs00234140_m1), TNF- α (Hs01113624_g1), β -catenin (Hs00170025_m1) and 18S rRNA (Hs99999901_s1). The 18S rRNA was used as an internal control. For each sample, the mRNA levels were normalized against 18S rRNA levels and the ratios of normalized mRNA to untreated control sample were determined using the comparative Ct method (18).

Preparation of total cell lysate and western blotting. Cells were lysed with ice-cold RIPA buffer containing 25 mM of Tris-HCl (pH 7.6), 150 mM of NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS and a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). Total cell lysates were obtained following the removal of the insoluble materials by centrifugation at 20,000 \times g for 20 min at 4°C. The protein concentrations were determined using a BCA protein assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA) and 25 μg of proteins were separated by 12% polyacrylamide gel electrophoresis and electrotransferred onto nitrocellulose membranes at 150 mA for 1.5 h. The membranes were then inhibited for 3.5 h at room temperature with phosphate buffered saline containing 5% skimmed milk and 0.1% Tween-20, and incubated with 1:1,000 dilutions of primary antibody overnight at 4°C, and subsequently with a 1:1,000-dilution of horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology, Beverly, MA, USA) for 2 h at room temperature. Peroxidase activity was visualized using an ECL kit (Thermo Fisher Scientific, Rockford, IL, USA). Anti- β -actin antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was used for the loading control of total lysate.

Analysis of nuclear and cytoplasmic β -catenin levels. Cells were harvested and then nuclear and cytoplasmic fractions

were collected using a nuclear extract kit (Active Motif, Carlsbad, CA, USA). Protein concentrations in each fraction were determined using a BCA protein assay kit (Pierce Biotechnology, Inc.). Protein (15 μg) was separated using a 12% polyacrylamide gel electrophoresis and analyzed by western blotting using an anti- β -catenin antibody (BD Biosciences, San Jose, CA, USA) followed by a secondary antibody. Anti-TATA box binding protein (TBP) and anti- β -actin antibodies (Santa Cruz Biotechnology, Inc.) were used for the loading control of nuclear and cytoplasmic extracts, respectively.

Transfection of small interfering RNA (siRNA). Cells were seeded in 12-well plates at a density of 5×10^5 cells/well and then transfected with 50 nM of control siRNA or β -catenin siRNA (Santa Cruz Biotechnology, Inc.) using a lipofectamine RNAiMAX transfection reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). Following 18 h, cells were stimulated with the Der p 1 allergen to induce inflammatory responses.

NF- κ B activity assay. The binding activity of NF- κ B to its target DNA sequence (5'-GGGACTTCC-3') was measured using a TransAM NF- κ B ELISA kit (Active Motif, Carlsbad, CA, USA). Briefly, cells were lysed and 30 μg of proteins in cell lysates were added into the wells coated with oligonucleotide containing the target DNA sequence. Following incubation and washing, the wells were incubated with an anti-NF- κ B antibody followed by a horseradish peroxidase-conjugated secondary antibody.

Statistical analysis. All data are expressed as the mean \pm standard deviation from at least three replicate experiments. Statistically significant differences between treated and untreated samples were detected using unpaired t-tests. $P < 0.05$ was considered to indicate statistically significant differences. All analyses were performed using SPSS ver. 14 (SPSS, Inc., Chicago, IL, USA).

Results

Der p 1 induces dose- and time-dependent inflammatory responses in THP-1 human monocytic cells. When THP-1 human monocytic cells were treated with various concentrations of the Der p 1 allergen, the expression of inflammatory cytokines, including IL-6, IL-8, TNF- α and MCP-1 were dose-dependently increased (Fig. 1). Among the inflammatory cytokines, the dose response of TNF- α was saturated at the lowest dose of 0.5 $\mu\text{g}/\text{ml}$, followed by that of MCP-1, which was saturated at 1 $\mu\text{g}/\text{ml}$ of the Der p 1 allergen. Dose responses of IL-6 and IL-8 were not saturated up to 3 $\mu\text{g}/\text{ml}$ of Der p 1. Although there were certain differences in the dose responses of cytokines, all cytokines were significantly induced at the concentration of 1 $\mu\text{g}/\text{ml}$ and all the following experiments were conducted at this concentration of the Der p 1 allergen. When cells were treated with 1 $\mu\text{g}/\text{ml}$ of the Der p 1 allergen for various time periods, the cytokine expression was significantly induced following ~1.5 h and was maintained until 6 h following treatment, however MCP-1 was more slowly induced compared with other cytokines (Fig. 2).

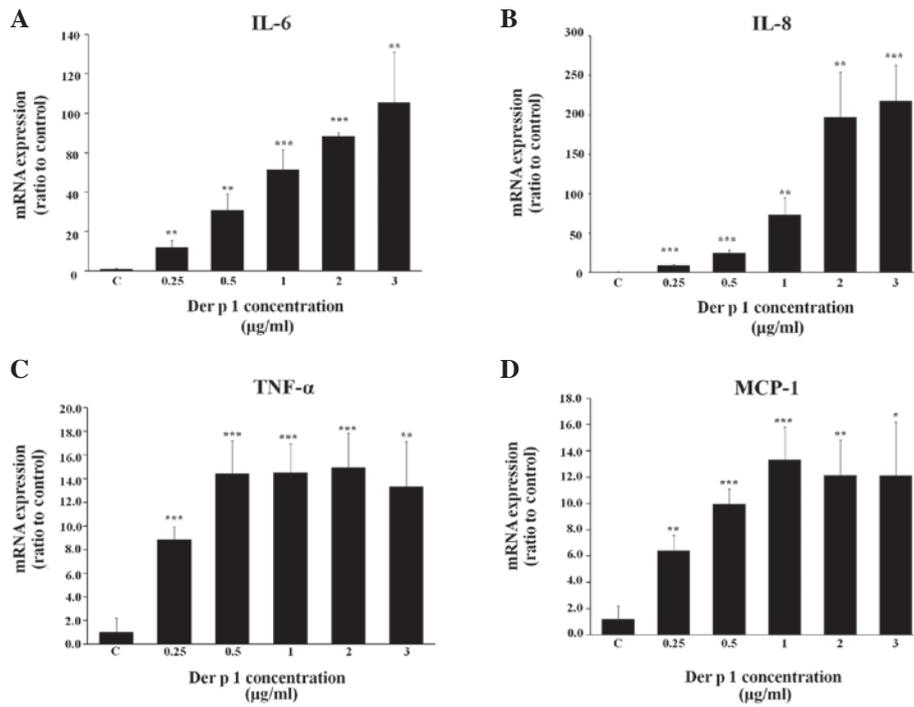


Figure 1. Effects of various concentrations of the Der p 1 allergen on inflammatory cytokine expression. THP-1 human monocytic cells were treated with the Der p 1 allergen for 3 h at various concentrations of 0.25 to 3 $\mu\text{g/ml}$ and total RNA was extracted for the quantification of the mRNA expression levels of inflammatory cytokines, including (A) IL-6, (B) IL-8, (C) TNF- α and (D) MCP-1. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ compared with untreated control marked as C. IL, interleukin; TNF- α , tumor necrosis factor- α ; MCP-1, monocyte chemoattractant protein-1.

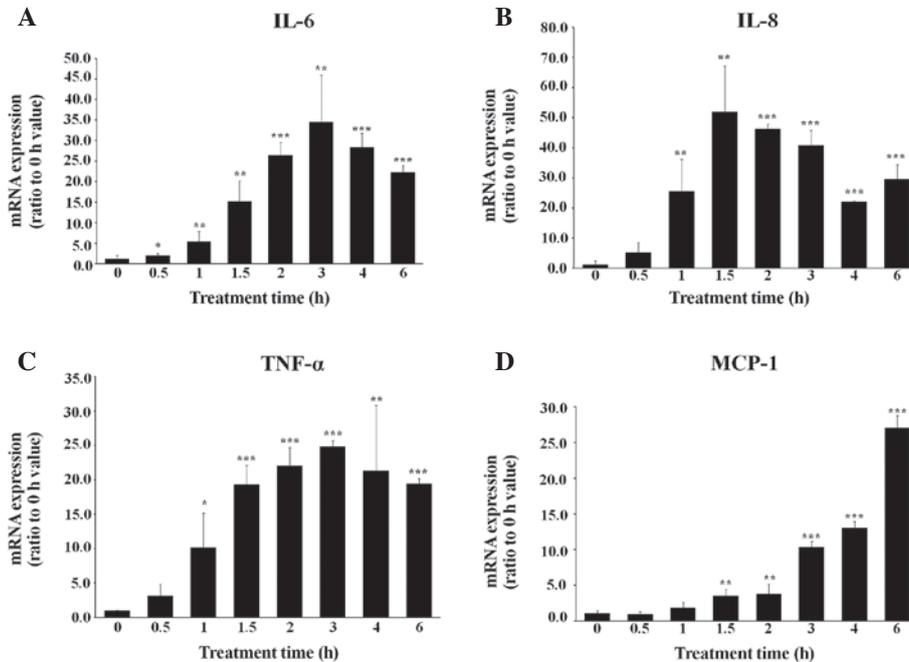


Figure 2. Time courses of Der p 1 allergen-induced inflammatory cytokine expression. THP-1 cells were treated with 1 $\mu\text{g/ml}$ of the Der p 1 allergen for various time periods and total RNAs were extracted for the quantification of mRNA expression levels of inflammatory cytokines, including (A) IL-6, (B) IL-8, (C) TNF- α and (D) MCP-1. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ compared with the untreated control marked as 0. IL, interleukin; TNF- α , tumor necrosis factor- α ; MCP-1, monocyte chemoattractant protein-1.

Der p 1 induces post-transcriptional modulation and nuclear translocation of β -catenin. The modulations of β -catenin were analyzed during the inflammatory response induced by the Der p 1 allergen. The mRNA expression level of β -catenin did not change, however its protein level in total cell lysate

was found to be increased, which demonstrated that β -catenin was modulated by a post-transcriptional process during the Der p 1-induced inflammatory response (Fig. 3A and 3B). When nuclear and cytoplasmic extracts were separated and analyzed for β -catenin protein levels, it was identified that

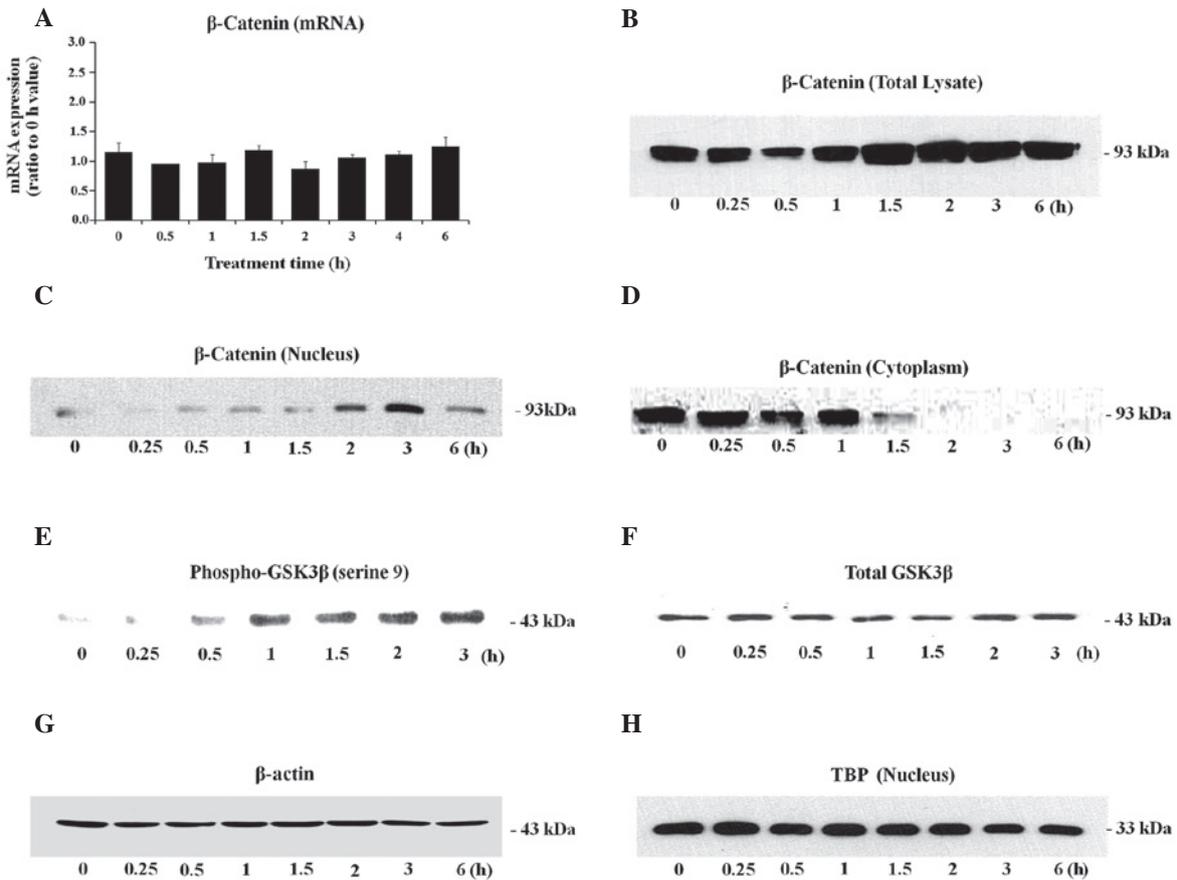


Figure 3. Modulations of β -catenin during Der p 1-induced inflammatory responses. (A) THP-1 cells were treated with 1 μ g/ml of Der p 1 allergen for various time periods and the expression level of β -catenin mRNA was quantified by real-time PCR. In the same experimental condition, protein levels of β -catenin were analyzed by western blotting in (B) the total cell lysate, (C) nuclear extract and (D) cytoplasmic extract. The levels of (E) serine 9-phosphorylated GSK-3 β and (F) total GSK-3 β were analyzed in total cell lysates. (G) β -actin was used as an endogenous control for total cell lysate and cytoplasmic extract and (H) TBP was used as an endogenous control for nuclear extract. PCR, polymerase chain reaction; TBP, TATA box binding protein; GSK-3 β , glycogen synthase kinase-3 β .

nuclear β -catenin levels were increased while its cytoplasmic levels were reduced following 1.5-2 h of Der p 1 treatment (Fig. 3C and 3D), which demonstrated that β -catenin was translocated from the cytoplasm to the nucleus following Der p 1 treatment. When glycogen synthase kinase 3 β (GSK3 β), an upstream regulator of β -catenin stability, was analyzed, the level of the phosphorylated GSK3 β at the serine 9 residue was increased while the total GSK3 β level did not change (Fig. 3E and 3F), demonstrating that GSK3 β was phosphorylated in the inflammatory response induced by Der p 1.

Der p 1-induced inflammatory responses are dependent on β -catenin. To confirm the role of β -catenin in Der p 1-induced inflammatory responses, siRNA-mediated knockdown experiments were conducted. Nuclear β -catenin level, which was increased by Der p 1 treatment, was reduced by β -catenin siRNA transfection, demonstrating a good efficiency of the siRNA-mediated knockdown process (Fig. 4A). In this experimental condition, inflammatory cytokines, including IL-6 (Fig. 4B), IL-8 (Fig. 4C) and MCP-1 (Fig. 4E), which were induced by Der p 1 treatment, were all significantly reduced by β -catenin knockdown. TNF- α expression tends to be reduced by β -catenin knockdown, however statistical significance was not observed (Fig. 4D). The binding activity

of NF- κ B, the major proinflammatory transcription factor, to its target DNA sequence was elevated by Der p 1 treatment and also significantly reduced by β -catenin knockdown (Fig. 4F). These results clearly demonstrated that Der p 1-induced inflammatory responses, including the upregulation of inflammatory cytokine expression and NF κ B activity were mediated by β -catenin, at least partially. It may be suggested that nuclear β -catenin may increase NF- κ B activity and resultant cytokine mRNA expression in Der p 1-induced inflammatory responses.

Discussion

The Der p 1 allergen has been reported to induce inflammatory responses by inducing the expression of various cytokines, however its molecular mechanisms were not fully elucidated thus far, although it is extremely important for the efficient treatment of asthma and other allergic diseases. Der p 1 was reported to induce IL-1 β , IL-6 and TNF- α in human eosinophils and co-cultured bronchial epithelial cells through the activation of NF- κ B and p38 MAPK (7). Der p 1, alone or in synergy with the human rhinovirus, induced IL-8 release via NF- κ B nuclear translocation in human bronchial epithelial cells (19). Der p 1 was reported to induce IL-6 and IL-8 release from human respiratory epithelial cells through the activation

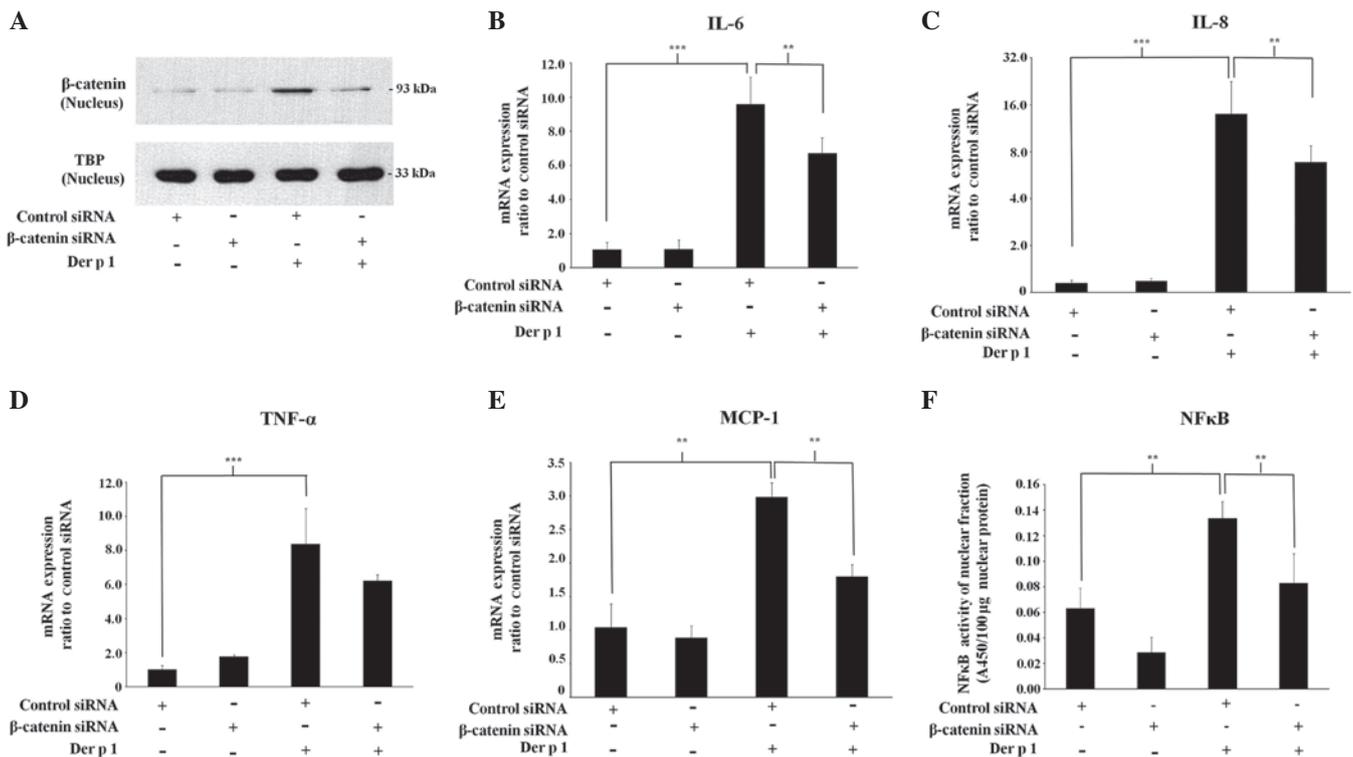


Figure 4. Effects of β -catenin knockdown on Der p 1-induced inflammatory responses. THP-1 cells were transfected with control siRNA or β -catenin siRNA for 18 h and stimulated with 1 μ g/ml of the Der p 1 allergen for 3 h. (A) Nuclear extracts were prepared for the analyses of β -catenin levels and TBP was used as an endogenous control. In the same experimental condition, total RNAs were extracted to measure mRNA expression levels of inflammatory cytokines, including (B) IL-6, (C) IL-8, (D) TNF- α and (E) MCP-1 by real time PCR. (F) Sections of the cells were used for the analyses of NF- κ B activity. ** $P < 0.01$, *** $P < 0.001$. siRNA, small interfering RNA; IL, interleukin; TNF- α , tumor necrosis factor- α ; MCP-1, monocyte chemoattractant protein-1; NF- κ B, nuclear factor- κ B; PCR, polymerase chain reaction.

of protease activated receptor-2 (20). It was also reported that eosinophils released TNF- α following Der p 1 stimulation by a mechanism involving thioredoxin (21). These studies have suggested diverse mechanisms for the inflammatory responses induced by the Der p 1 allergen, however more evidence is needed to confirm that these suggested mechanisms are involved in the allergic diseases of humans.

Since Der p 1 is a well-known environmental allergen of asthma (12) and asthma development is the result of the allergen stimuli in genetically susceptible individuals (13), plausible candidate genes for asthma may also be involved in the Der p 1-induced inflammatory responses. Our interest in β -catenin was prompted by a previous study reporting that the genetic polymorphism of β -catenin was significantly associated with the asthma risk in human subjects, providing evidence that β -catenin is a candidate gene for asthma (14). β -catenin is a member of the WNT/ β -catenin pathway regulating various cellular processes, including proliferation, differentiation and development (22). The levels of β -catenin are regulated by the WNT/ β -catenin pathway. In an inactive state, the β -catenin protein is degraded by a destruction complex composed of AXIN, GSK3 β and adenomatous polyposis coli (APC), and GSK3 β was known to phosphorylate β -catenin at serine 33 and 37 creating a binding site for the E3 ubiquitin for β -catenin ubiquitination and degradation. When the WNT/ β -catenin pathway is activated, the AXIN-GSK3 β -APC complex is disrupted and GSK3 β is inactivated resulting in the protein

stabilization and nuclear translocation of β -catenin (23,24). It was also reported that GSK3 β is inhibited by its phosphorylation of the serine 9 residue (25). Our experimental data demonstrated that GSK3 β was revealed to be phosphorylated at serine 9 by Der p 1 treatment (Fig. 3E). It may be suggested that Der p 1 activates the WNT/ β -catenin pathway leading to GSK3 β phosphorylation followed by the stabilization and nuclear translocation of β -catenin (Fig. 3B and 3C).

Previously, certain studies have reported controversial roles of the WNT/ β -catenin pathway in the modulation of inflammatory responses. Duan *et al* reported that β -catenin negatively regulates the inflammatory response induced by a pathogenic bacterium, *Salmonella typhimurium* (26), while Kim *et al* reported that β -catenin positively regulates the inflammatory response in macrophages stimulated by lipopolysaccharide (27). At present, however, the role of β -catenin has not been elucidated in the Der p 1 HDM allergen-induced inflammatory response. In the present study, we demonstrated that β -catenin is important in the inflammatory response induced by the Der p 1 allergen. By the siRNA-mediated knockdown of β -catenin, we clearly demonstrated that β -catenin, a pivotal molecule in the WNT/ β -catenin pathway, was involved in the induction of cytokine expression as well as NF- κ B activity in the inflammatory response of human monocytic cells stimulated by the Der p 1 allergen (Fig. 4). As the inflammatory cytokine expression is closely associated with asthma development and exacerbation (28), our experimental

data demonstrated that the protein stabilization and nuclear translocation of β -catenin by the Der p 1 allergen contributes to asthma development through the expression of inflammatory cytokines.

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