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The effect of water-soluble chitosan on macrophage activation and the attenuation of mite allergen-induced airway inflammation

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Abstract

Chitin and chitosan have versatile anti-tumor, anti-fungal, and antimicrobial biological properties. Oral intakes and intranasal administration of chitin attenuated allergen-induced airway inflammation in sensitized mice, which may be due to its Th1 adjuvant properties. However, their mechanism of action is not entirely clear. In this report, we demonstrate that water-soluble chitosan (WSC) has specific immunomodulatory effects on dust mite allergen *Dermatophagoides farinae* (Der f)-stimulated, monocyte-derived macrophages (MDM). These effects include polarizing the cytokine balance towards Th1 cytokines, decreasing the production of the inflammatory cytokines IL-6 and TNF-α, down-regulating CD44 and TLR4 receptor expression, and inhibiting T cell proliferation. Scanning electron microscope (SEM) examination found that WSC reduced the rate of pseudopodia formation in Der f-stimulated MDM from allergic asthma patients. The effect of WSC on allergen-stimulated MDM may be mediated via inhibition of PKCζ phosphorylation and NF-κB pathway activation. In a murine model of asthma, we found that intranasal application of WSC attenuates Der f-induced lung inflammation by reducing infiltration of inflammatory cells, epithelial damage, and goblet cell hyperplasia and markedly decreasing production of Arg I, iNOs, and thymic stromal lymphopoietin (TSLP) in the bronchial epithelium. Therefore, we believe that WSC may provide a new therapeutic modality for allergic asthma.
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Keywords: Macrophage; Chitosan; Allergy

1. Introduction

Chitin and chitosan are polymers of β-D-glucosamine and of *N*-acetyl-glucosamine, respectively, and is a key structural component of helminths, arthropods and fungi [1,2]. Chitin is second only to cellulose as the most abundant biological polymer in nature. Chitosan is formed naturally by the action of chitin deacetylases, enzymes involved in either the formation of the cell wall, or in the deacetylation of chitin oligosaccharides, following the action of an endochitinase on cell walls during autolysis [3]. Water-soluble chitosan (WSC) derivatives has been reported to have anti-tumor, anti-fungal, and antimicrobial properties [4,5], to have potential as sustained release carriers for drugs [6], to accelerate wound healing [7], and to enhance the *in vitro* infectivity of adenovirus to mammalian cells [8].

Unlike bacteria and saprophytes which can synthesize and metabolize chitin, mammals are only equipped with a wide range of enzymes, such as acidic mammalian chitinase (AMCase), chitotriosidase (CHIT1), to detect and dispose of chitin and/or chitin-containing organisms [9]. After exposure to chitin by inhalation or ingestion, the hosts respond by increasing the expression of chitinases [9]. The immune response to

Abbreviations: Arg I, arginase I; Der f, *Dermatophagoides farinae*; Der p, *Dermatophagoides pteronyssinus*; iNOs, inducible nitric oxide synthase; HDM, house dust mite; MDM, monocyte-derived macrophages; PBMCs, peripheral blood mononuclear cells; PKCζ, protein kinase Cζ; SEM, scanning electronic microscopy; TSLP, thymic stromal lymphopoietin.

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chitin that develops into T-helper type 1 (Th1) or Th2 response and its role in the allergic asthma remains controversial [10–14]. Oral administration of chitin, for example, has been shown to downmodulate a murine model of allergic airway inflammation [11]. In this study, Shibata et al. [11] not only demonstrate the capacity of chitin to induce the secretion of Th1 cytokines, but also propose that chitin preparations could be an attractive therapy in allergic human disease. Another study by Strong et al. [12] demonstrates that direct application of chitin microparticles (sizes in the 1–20 μm range) to the respiratory tract can alleviate allergic symptoms in a mouse model of ragweed allergy and might be a useful treatment for respiratory allergy and allergic asthma. In contrast, Zhu et al. [13] have shown that during allergic airway inflammation, there is significantly increase of AMCase production in Th2 immune response and IL-13 pathway activation. Further, Reese et al. [14] found direct administration of chitin induced eosinophil and basophil accumulation in helminth-infected murine model of Th2 inflammatory responses. The sensitization role of chitin, they suggested, may through alternatively activated macrophages mediate eosinophil recruitment via leukotriene B4 production [14].

Macrophage plays essential role in allergen-induced reaction such as asthma, atopic dermatitis, and rhinitis [15,16]. Previously, we showed that house dust mites (*Dermatophagoides pteronyssinus*, Der p, and *Dermatophagoides farinae*, Der f) can directly activate innate immune cells such as alveolar macrophages and mast cells without previous *in vitro* or *in vivo* sensitization [17,18]. This activation led to eosinophils infiltration, goblet cell hyperplasia, and hyperplasia of peribronchial smooth muscles in mice repeatedly exposed to Der f. Moreover, we found that Der p can activate and induce nitric oxide (NO) production in the alveolar macrophage cell line (MHS) via CD14/toll-like 4 receptor (TLR4) signaling [19]. Therefore, it was of interest to evaluate the extent of WSC-mediated activation of macrophages during allergen-stimulation. The results reported here demonstrate that water-soluble chitosan attenuates Der f-induced activation of macrophages in allergic asthma patients and reduces allergen-induced airway inflammation in Der f-sensitized mice.

2. Materials and methods

2.1. Reagents

Water-soluble hydroxypropyl chitosan (HPCS) derivatives were synthesized from chitosan (Sigma–Aldrich, St. Louis, MO, USA) by reaction with propylene epoxide under basic conditions. IR, NMR spectroscopy, and elemental analysis were used to characterize these structures and show that the OH groups at C-6 and C-3 and the NH_2 group of chitosan are alkylated. The degree of substitution (DS) of HPCS ranged from 1.5 to 3.1, and the molecular weight (Mw) was between 2.1×10^4 and 9.2×10^4. House dust mite, *Dermatophagoides farinae* (Der f, 1 g of lyophilized whole body extract in ether; Allergon, Engelholm, Sweden), water-soluble chitosan after deacetylation, and hyaluronan (HA) (Mw around 12 kDa, Life Core Biomedical, Chaska, MN, USA) were dissolved in pyogenic-free isotonic saline, filtered through a 0.22-μm filter, and stored at −70 °C before use. LPS concentration of the above preparations of Der f, WSC, and HA was <0.96 EU/mg (*Limulus* amebocyte lysate test; E-Toxate; Sigma–Aldrich).

2.2. Study subjects

The study population was recruited from the Allergy and Asthma Specialty Clinic at the National Cheng Kung University Hospital, and consisted of 20 children with new diagnoses of allergic asthma (mean age, 11.2 years; 12 boys and 8 girls), and 20 age-matched non-atopic controls. The definition of asthma was (1) a history of two or more episodes of wheezing in the last 6 months, during or without concurrent respiratory infection, (2) chronic cough for >1 month and the occurrence of wheezing episode(s) as determined by a physician, and (3) confirmation of a positive response to a bronchodilator (i.e., 15% increase in forced expiratory volume in 1 s). All asthmatic children had a history of atopy and were sensitive to the house dust mite (HDM), *Dermatophagoides farinae* (Der f). Other evaluations included skin-prick tests for responsiveness to six common aeroallergens, a differential blood count with a total eosinophil count, and the measurement of total serum IgE, as well as IgE specific to HDM and mixed pollens using the Unicap system (Pharmacia Diagnostics, Uppsala, Sweden). The positive skin-prick test was defined as the presence of ≧1 reaction with a wheal diameter ≧5 mm. Total serum IgE was measured by a solid-phase immunoassay (Pharmacia IgE EIA; Pharmacia Diagnostics). Allergic status was defined by total IgE levels (≧500 IU/ml) and Der f-specific IgE (i.e., the CAP allergy test). Age-matched controls had no history of any of the above allergic asthmatic symptoms. In all study subjects, no inhaled or oral steroids or bronchodilators were taken for at least 1 week and 6 h, respectively, before blood sampling. This study protocol was approved by the Human Research Committee of the National Cheng Kung University Hospital and informed consent was obtained from all subjects or their guardians.

2.3. Isolation of monocyte-derived macrophages (MDM)

Peripheral blood mononuclear cells (PBMC) were obtained from heparinized blood by Ficoll-Paque PLUS (Amersham, Sweden) density gradient centrifugation. The cells were resuspended in RPMI 1640 containing 10% human serum and seeded onto plastic tissue culture flasks for 1 h to allow monocytes to adhere; the nonadherent cells were then removed by extensive washing. Monocytes were removed from the flask with 530 mM EDTA/PBS and seeded onto 24-well tissue culture dishes. Cells were plated at a density of 4×10^6/well in a six-well plate in Dulbecco's modified Eagle's medium (DMEM) supplemented with glucose, 1 mM sodium pyruvate, 5% fetal bovine serum, macrophage colony-stimulating factor, interleukin-1 (IL-1), IL-6, plasminogen activator, and collagenase (Sigma–Aldrich). Cell viability was assessed by trypan blue exclusion, and always found to be more than 95%. Approximately 80–90% of the MDMs in the differentiated cultures were positive for the macrophage surface marker CD14, with some donor-dependent variability. The culture conditions for Der f (10 μg/ml)-stimulated MDM were at 37 °C under 5% CO_2 incubation for 24 h with or without pretreatment with various dosages of WSC or HA for 10 min.

2.4. Flow cytometric analysis of MDM

Pooled MDM cells (1×10^6) from PBMC were incubated with FITC-conjugated anti-CD44 (IM7, Biolegend, San Diego, CA, USA) or anti-CD14 (MP9, BD PharMingen, San Diego, CA, USA) and PE-conjugated anti-TLR4 (HTA125, Biolegend) or anti-PAR2 (SC-8206, Santa Cruz Biotech, Santa Cruz, CA, USA) mAbs for 30 min on ice. After washing with PBS, stained cells were quantified using FACScan software (BD Immunocytometry Systems, San Jose, CA, USA). All staining steps were performed at 4 °C in RPMI/EDTA/FCS. Isotype-matched, mAb-stained cells were used as a background control in all experiments. At least 10,000 events were analyzed using a BD FACS Caliber Flowcytometer. Monocytes/macrophages, granulocytes, and dead cells were gated out on the basis of forward and right angle light scatter.

2.5. Semi-quantitative RT-PCR assay for mRNA expression in stimulated MDM

RT-PCR was performed to estimate expression of mRNAs for cytokines and chemokines. Total cellular RNA was extracted from pooled monocytes

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(RNeasy Qiagen, Hilden, Germany) and converted to cDNA with SH-reverse transcriptase (Stratagene, La Jolla, CA, USA). The procedures and detailed nucleotide sequences of sense and anti-sense primers for each assessed cytokine were described in our previous report [17].

2.6. MDM cell morphology in scanning electronic microscopy (SEM)

For the morphological study by SEM, cultured MDM cells after various conditions of treatment were fixed with 1.2% glutaraldehyde in 0.1 mol/L phosphate buffer (PB) at pH 7.4 and post-fixed with 1% OsO_4 in 0.1 mol/L PB. The fixed cells were rinsed twice with PBS, subsequently dehydrated in ascending concentrations of ethanol, critical-point dried using carbon dioxide, and coated by vacuum-evaporated carbon and ion-sputtered gold. All samples were observed under JSM-35 scanning electron microscope (LV5610, JEOL, Tokyo) at an accelerated voltage of 10 kV.

2.7. ELISA for the measurement of cytokines

Concentrations of cytokines were measured by a sandwich ELISA technique using commercial matching mAb pairs, one of which was biotinylated (IL-6, MP5-20F3, and MP5-32C11; IL-12, C15.6, and C17.8; IL-4, 11B11, and BVD6-24G2; BD PharMingen). The reaction was developed and measured as in standard procedures. Standards were run in parallel with recombinant cytokines. The detection limits were 7.5 pg/ml for IL-6, IL-4, and 15 pg/ml for IL-12.

2.8. Western blot and EMSA assays for NF-κB and PKCξ

Nuclear extracts were prepared from MDM cells (1×10^6) by the method previously described [18,19]. The PKCξ (PKC0103, Biolegend) was detected using a 1:500 dilution of human monoclonal PKCξ primary antibody, a 1:1000 dilution of HRP-conjugated anti-mouse IgG secondary antibody (Jackson Immunoresearch Laboratories, Inc., West Grove, PA, USA), and the Western blotting luminol-reagent (Santa Cruz Biotech) as detection reagent. The IκBα and p65 NF-κB were assayed using anti-IκBα (44D4, Cell Signaling Technology, Danvers, MA, USA) and NF-κB (p65) antibodies (Santa Cruz Biotech). Electrophoretic mobility shift assay (EMSA) experiments were performed using a commercial DIG Gel Shift Kit (Cat. No. 3353591, Roche Diagnostics, Mannheim, Germany) as previously described [19].

2.9. T cell proliferation in the co-culture of MDM cells

MDM cells were isolated from Der f-sensitive asthmatic patients and non-atopic controls, and then stimulated with Der f as described above. Autologous T cells were collected and purified by nylon wool column separation. Single-cell suspensions of T cells (2×10^5) were then co-cultured with MDM cells (2×10^4) in the presence of PHA (1 mg/ml; Murex, Dartford, UK). Proliferation rate of T cells in this co-culture condition was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

2.10. Allergen sensitization, challenge, and assessment of lung inflammation in Der f-sensitized mice

Female BALB/c mice aged 6–8 weeks were used for the experiment. Each experiment had the following groups ($n = 6$): mouse controls (neither sensitized nor challenged), and sensitized and allergen challenged mice. The procedures for Der f sensitization and allergen challenge in the murine model of asthma were previously described [20]. The mice in the treatment group received 250 μg/ml of WSC or HA in sterile PBS twice intranasally (i.n.) at 1-week intervals starting on the day of allergen sensitization and ending before intratracheal (i.t.) challenge with allergen. Inflammatory infiltrates and lung architecture in all mice were histopathologically analyzed using light microscopy. For immunohistochemical staining, sections of lung tissues (5-μm) were mounted on sterile glass slides and stained with mouse anti-nitric oxide synthase (NOS) II, anti-arginase I (Arg I; both from Cayman Chemical, Ann Arbor, MI, USA), anti-thymic stromal lymphopoietin (TSLP, Research Diagnostics Inc., Concord, MA, USA) or control IgG for various time intervals.

2.11. Statistics

Data were analyzed by a two-tailed Mann–Whitney *U*-test using GraphPad Prism 4.0 (GraphPad, San Diego, CA, USA). p-Values < 0.05 were regarded as significant.

3. Results

3.1. The effect of WSC on Th2 cytokine production by MDMs

In this study, the purity of MDMs isolated from PBMCs was more than 95% as judged by flow cytometry (data not shown). Stimulation of freshly isolated MDMs with Der f allergen-induced the expression of mRNAs of Th2 cytokines (IL-6 and IL-13), inflammatory cytokines (MIP-1 and TNF-α), and Th2 cytokine transcript factor (GATA3) as detected by semi-quantitative RT-PCR (Fig. 1A). After pretreatment with various dosages of WSC for 6 h, there was a dose-dependent decrease in the mRNA expression of Th2 and inflammatory cytokines as well as GATA3, whereas the mRNA expression of the Th1 cytokine (IL-12) and its transcript factor (T-bet) were increased in WSC-pretreated, Der f-stimulated MDM. Although the mRNA expression of eosinophil chemotactic factor (RANTES) was increased in Der f-stimulated macrophages, pretreatment with WSC did not significantly affect the expression of this chemokine.

To further confirm the effect of WSC on the Der f-stimulated human MDM, the production of IL-1β, IL-6, and IL-12 cytokines in the culture supernatant was assayed with an ELISA kit (Fig. 1B). We found Der f (10 μg/ml)-stimulated MDM released a small amount of IL-12 and large amounts of IL-1β and IL-6 into the culture supernatants. Pretreatment with WSC, but not with HA, reduced IL-1β (138 ± 15 pg/ml *vs.* 58 ± 12 pg/ml) and IL-6 (1180 ± 150 pg/ml *vs.* 650 ± 75 pg/ml) production by more than 58% and 45%, respectively, in Der f-stimulated MDM, but significantly increased IL-12 production ($p < 0.05$) (Fig. 1B).

3.2. The effect of WSC on Der f-induced change in MDM morphology

From light microscopy examination, in contrast to non-stimulated MDM (which had smooth surfaces), Der f-stimulated MDM (white arrows, Fig. 2A) were polymorphic and different sizes. Pretreatment with WSC (250 μg/ml), but not HA, preserved the non-active, smooth surface of the cell despite Der f-stimulation (Fig. 2A). To determine whether the effect of WSC on cell morphology after Der f-stimulation was related to allergy status, human macrophages isolated from PBMCs in non-atopic controls and dust mite (Der f)-sensitive asthmatic patients were examined with SEM (Fig. 2B). Under the high power field (×6000), Der

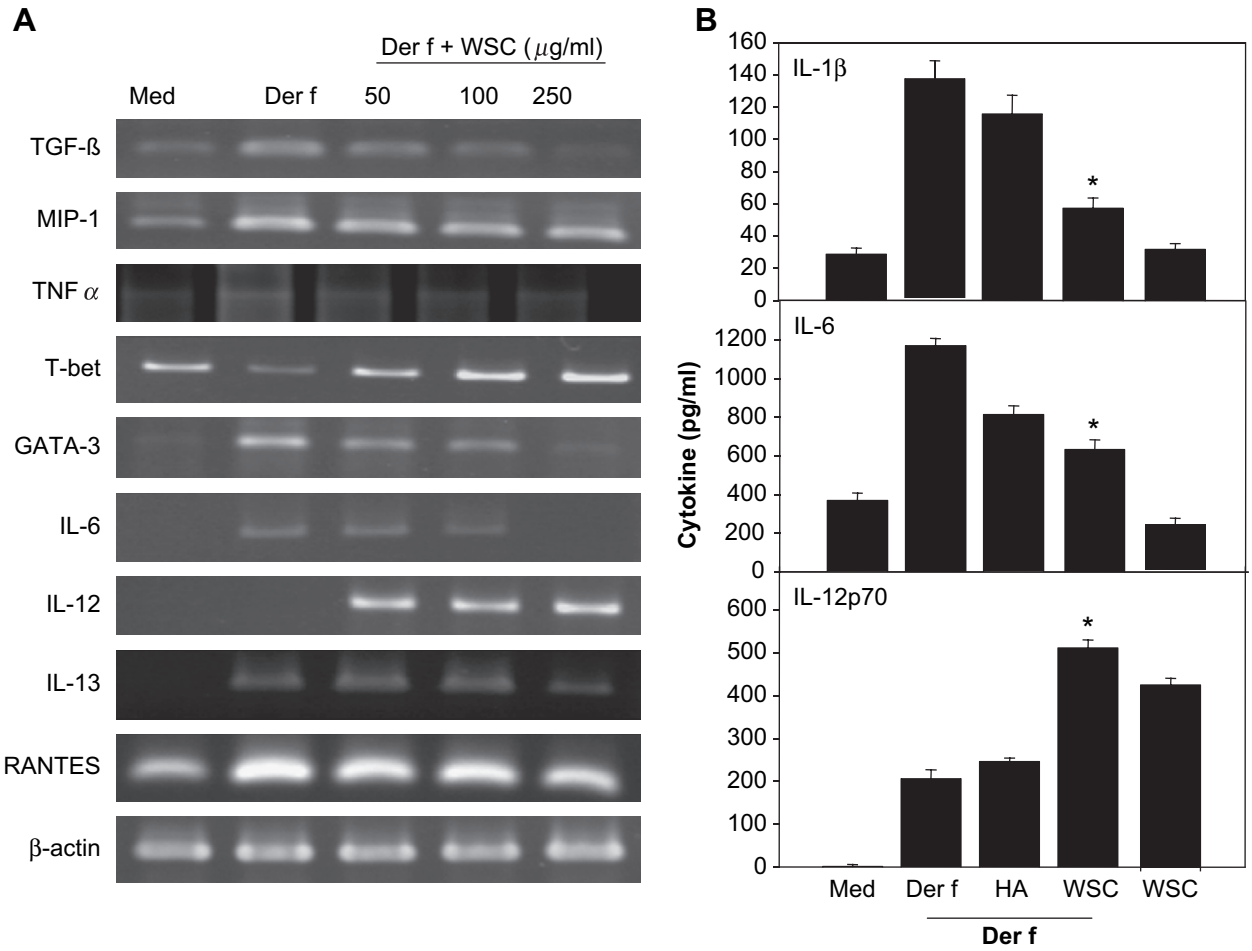

Fig. 1. Effects of WSC on expression of Th1, Th2, and inflammatory cytokine mRNAs and proteins in Der f-stimulated MDM. MDM isolated from human PBMC (2×10^5 cells/ml) were pre-incubated with various dosages of WSC at 37 °C for 6 h prior to the stimulation with 10 μg/ml of Der f. Total RNA was extracted from stimulated cells, and the mRNA expression of each cytokine was evaluated by RT-PCR (A). The effect of WSC on IL-1β, IL-6, and IL-12 cytokines production from Der f (10 μg/ml)-stimulated MDMs. Macrophages (2×10^5 cells/ml) were cultured in the presence of WSC (250 μg/ml) or HA (250 μg/ml) for 24 h. The levels of cytokines in the supernatants were measured by ELISA (B). Data represent four separate experiments performed in triplicate.

f-stimulated MDMs from allergic patients had multiple protuberant pseudopodia and released preformed granules (Fig. 2B, left lower panel), while Der f-stimulated MDMs from non-atopic controls were only polymorphic and had no pseudopodia (Fig. 2B, right upper panel). Furthermore, pretreatment with WSC markedly reduced the number of pseudopodia in Der f-activated MDM from allergic patients and restored the morphology of Der f-activated MDM from non-atopic patients to that of their non-activated MDM counterparts (Fig. 2B, right lower panel).

3.3. The attenuation of CD14, PAR2, CD44 and TLR4 expression by WSC in Der f-stimulated MDMs

To investigate the effect of WSC on the cellular surface proteins of Der f-induced MDM, the CD44 and TLR4 expression on the surface of activated MDMs were studied with flow cytometry and fluorescence microscopy (Fig. 3). Der f-induction significantly increased the amount of TLR4 and CD44 expression. Pretreatment with HA (250 μg/ml) for 6 h significantly decreased TLR4 expression, but not CD44 expression. In contrast, pretreatment with WSC (250 μg/ml) dose-dependently inhibited CD44 and TLR4 expressions in the Der f-stimulated MDM (Fig. 3A). Fluorescence microscopy also found that increasing dosage of WSC caused decreased CD44 expression (green color) on the cell surface of Der f-activated MDM (blue color) (Fig. 3B). Since the level of Der f-induced CD44 and TLR4 expressed by MDM from Der f-sensitive asthmatic patients and MDM from non-atopic controls was similar (data not shown), we decided to assay CD14 and PAR2 on the Der f-activated MDM surface. Levels of expression between these two groups differed significantly (Fig. 3C): CD14 and PAR2 expression was higher on MDMs from allergic asthmatic patients than from controls (59.2 ± 10.3 *vs.* $24.2 \pm 4.5\%$; 46.2 ± 7.8 *vs.* $11.3 \pm 6.6\%$; $p < 0.05$, respectively). Pretreatment with WSC markedly inhibited CD14 and PAR2 overexpression in Der f-stimulated MDM, particularly in atopic subjects (59.2 ± 10.3 *vs.* $28.2 \pm 9.7\%$; 46.2 ± 7.8 *vs.* $18.3 \pm 7.1\%$; $p < 0.05$, respectively).

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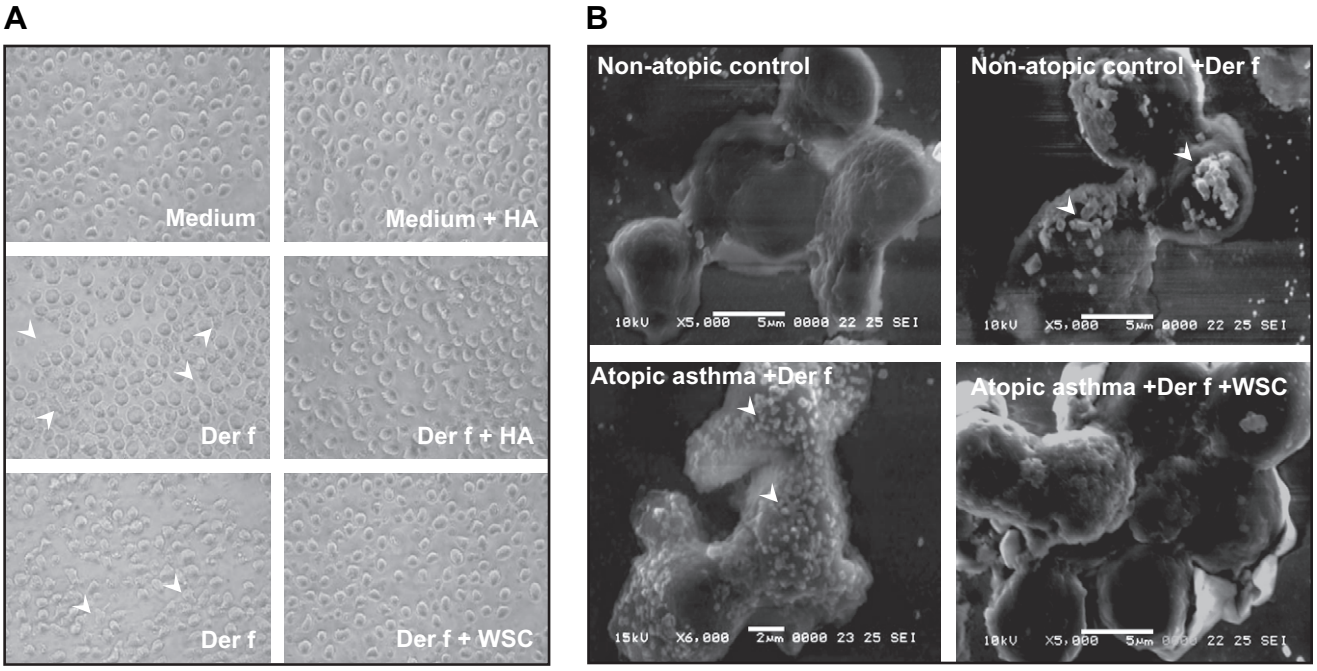

Fig. 2. Effect of WSC on changes in Der f-stimulated MDM morphology. Changes in Der f-stimulated macrophage morphology in response to WSC or HA were observed under a phase contrast light microscope (×100) (A). MDM isolated from non-atopic controls and Der f-sensitive asthmatic patients were pretreated with WSC (250 μg/ml for 6 h) and then stimulated with Der f (10 μg/ml for 24 h). SEM (×6000) revealed Der f-induced many changes in MDM morphology (white arrows) and that pretreatment with WSC prevented these changes. White bar is 2 μm in length (B).

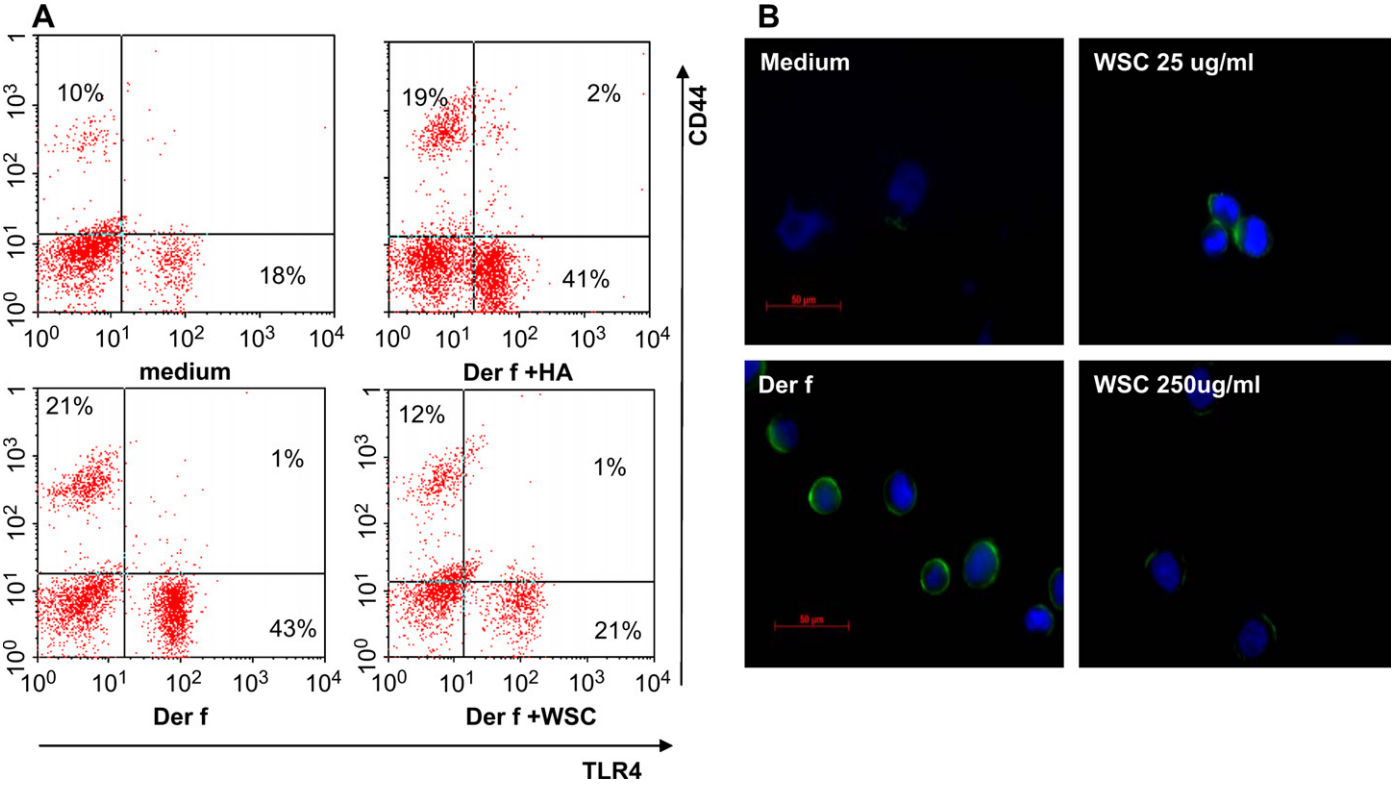

Fig. 3. WSC attenuates expression of CD44 and TLR4 in MDMs. MDMs from PBMCs were incubated alone or stimulated with Der f (10 μg/ml), Der f plus HA (250 μg/ml; pretreatment for 6 h), or Der f plus WSC (250 μg/ml; pretreatment for 24 h) in serum-free RPMI medium and evaluated for expression levels of CD44 and TLR4 by flow cytometry after staining with the corresponding fluorescent antibodies. Percentage values are calculated by Winmidi 2.8 software. The data are representative of three independent experiments (A). Der f-stimulated MDM were treated with different concentrations of WSC for 24 h, collected, fixed, and double-stained for cell nuclei (blue) and CD44 (green), and visualized by fluorescence microscopy (B). MDM from non-atopic controls and Der f-sensitive atopic patients were evaluated for expression levels of CD14 and PAR2 by flow cytometry after staining with the corresponding fluorescent antibodies (C). Comparable results were obtained in three independent experiments.

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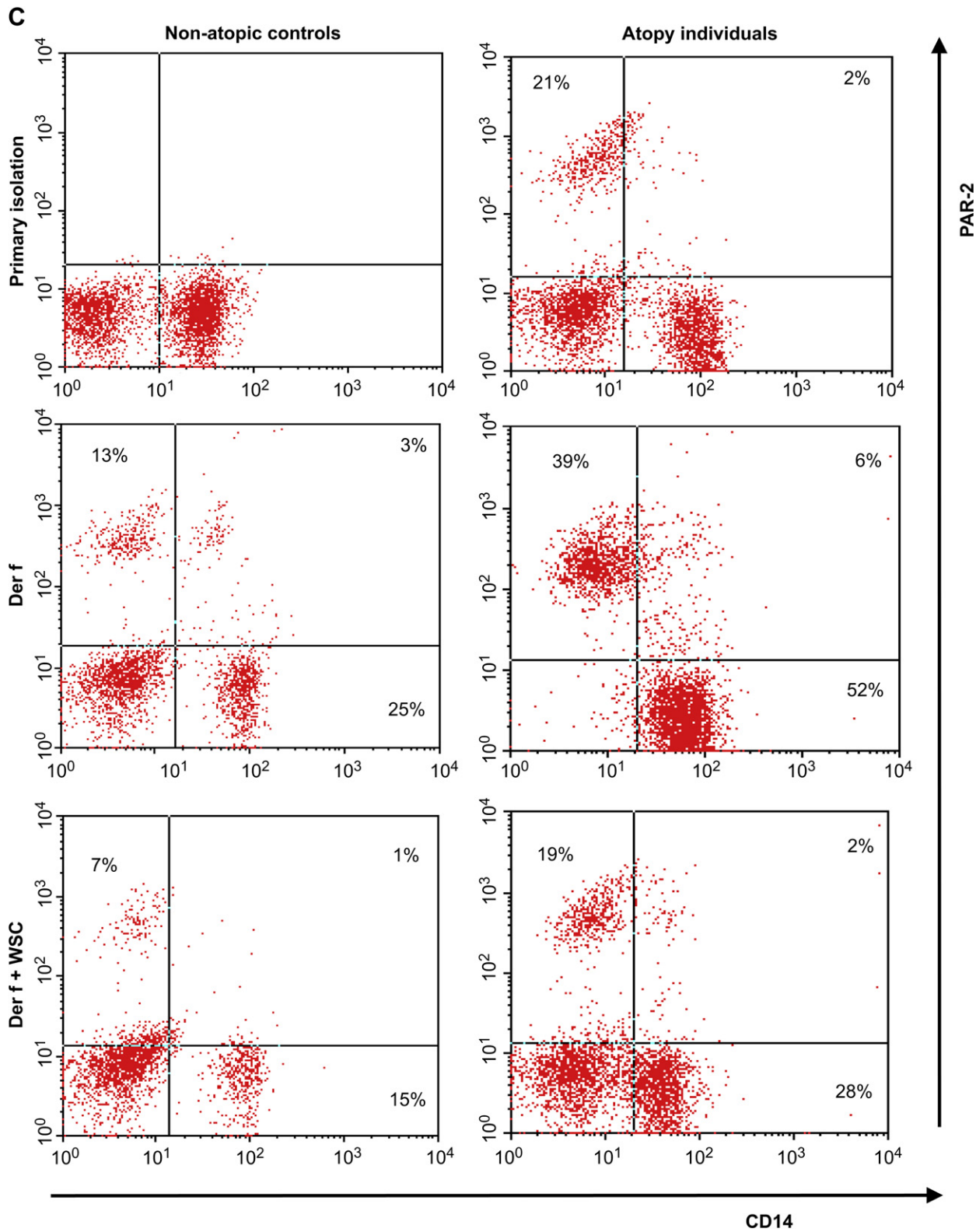

Fig. 3. (*Continued*)

3.4. The involvement of PKCζ and NF-κB pathways in WSC inhibitory actions

The activation signal transduction pathways in the Der f-induced MDM were studied. The effect of signal transduction on phosphorylation of PKCζ was assessed by Western blot analysis of cell extracts for the activation of IκBα in the cytoplasm, and EMSA of nuclear extracts for the translocation of NF-κB. We found WSC (but not anti-CD44 antibody or anti-TLR4 antibody) inhibited PKCζ phosphorylation in

a dose-dependent manner (Fig. 4A). Consequently, pretreatment with WSC (250 μg/ml) but not HA (100 μg/ml) inhibited the Der f-induced activation of the NF-κB activation pathway (Fig. 4B).

3.5. The effect of WSC-pretreated MDMs on T cell proliferation

To further study the effect of WSC on Der f-induced MDM-mediated proliferation of autologous T cells, Der f-treated MDM and autologous naïve CD4 + T lymphocytes were co-incubated for 3 days with or without WSC (Fig. 4C) and cellular proliferation was monitored. The MTT test found that Der f-stimulated MDM dose-dependently enhanced T cell proliferation, but this enhancement was inhibited by co-incubating with increasing dosages of WSC.

3.6. The attenuation of Der f-induced airway inflammation by WSC in a murine model of asthma

Finally, the *in vivo* effect of WSC on the Der f-induced airway inflammation was studied in Der f-sensitized mice. Tracheal instillation of Der f in Der f-sensitized mice induced marked changes in the airways, such as epithelial sloughing, goblet cell hyperplasia (Fig. 5A), and increased Arg I, TSLP, and inducible NOS (iNOS) expression in the subepithelial and peribronchiolar areas (Fig. 5B). These histopathogical findings were similar to those in humans with asthma. Intranasal WSC, but not intranasal HA, significantly reduced the number of inflammatory cells such as eosinophils in the bronchoalveolar lavage (BAL) fluid, airway hypersensitivity (data not shown), epithelial damage, goblet cell hyperplasia (Fig. 5A), and Arg I, TSLP, and iNOs expression in the Der f-treated sensitized mice as compared to non-treated sensitized mice (Fig. 5B).

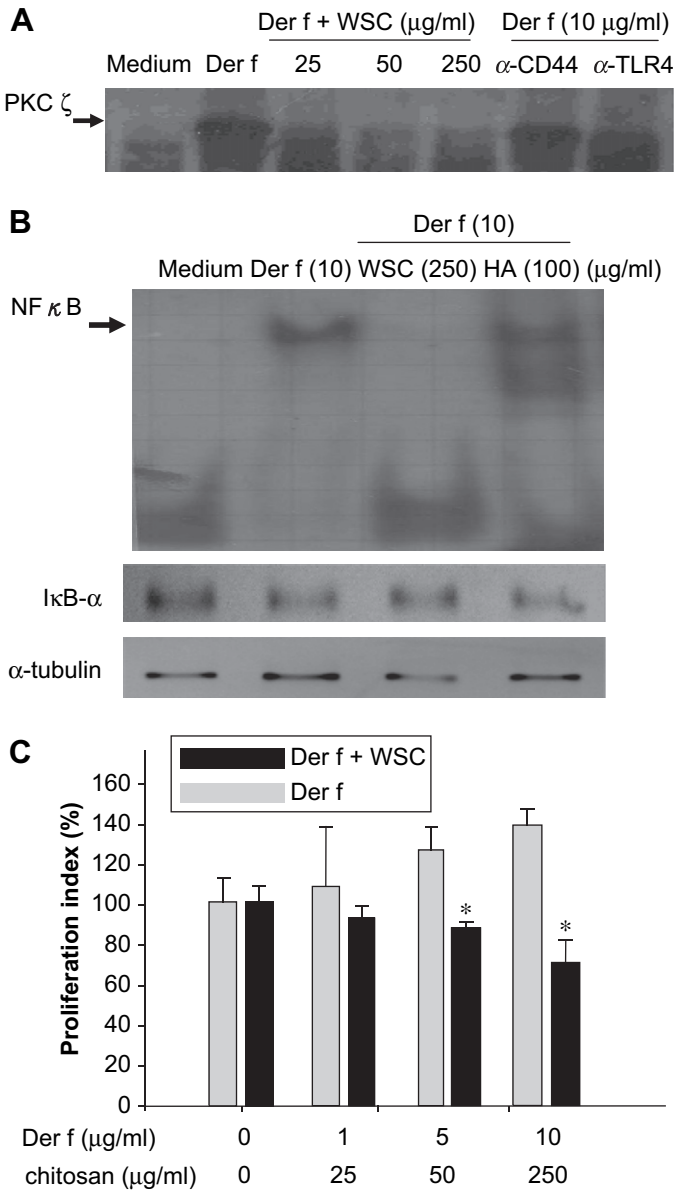

Fig. 4. WSC effects on the activation of PKCζ and NF-κB in Der f-stimulated MDM and on the proliferation of T cells. The effect of WSC, anti-CD44 antibody, and anti-TLR4 antibody on phosphorylation of PKCζ was analyzed in extracts of Der f-stimulated macrophages by Western blot (A). IκBα in extracts of the cytoplasm was analyzed by Western blot, and translocation of NF-κB in extracts of the nucleus was analyzed by EMSA. The data are representative of three independent experiments (B). MDMs were prepared from PBMC after 1 day of treatment with WSC (250 μg/ml) and co-cultured with T cells isolated from PBMC. T cell proliferation rates were determined by the MTT test ($n = 3$) (C).

4. Discussion

The mechanisms of WSC-mediated immunostimulation are thought to involve macrophage activation [21,22]. Many reports suggest that chitosan can upregulate TNF-α, IL-1β, NO, and colony-stimulating factor (CSF) production in macrophages [23–26]. However, the mechanism by which oligochitosan interacts with and activates macrophages that leads into different immunological response is not clear. Several factors, such as the administration route and particle size, might account for the Th1 *vs*. Th2 response to chitin [10–14]. A Th1 response is elicited when macrophages phagocytose microparticles of chitin (sizes in the 1–20 μm range), but not when soluble chitin or particles too large to engulf are used [27]. Further evidences show that administration of unphagocytosable Sephadex (cross-linked dextran) beads can also induce a local eosinophilia in the lung and peritoneal cavity that shows a similar dependence on leukotriene B4 [28,29].

In this study, we demonstrated that water-soluble, low molecular weight chitosan had specific immunomodulatory effects on Der f-stimulated human MDM including the shifting of Th2 cytokine polarization, decreasing the production of the inflammatory cytokines IL-6 and TNF-α, down-regulating CD44, CD14, TLR14, and PAR2 receptor expressions, and inhibiting T cell proliferation in the presence of allergen-stimulated MDM. These results further emphasize that the anti-inflammatory and anti-allergic effects of WSC are related to the macrophage activation. The inhibitory effect of WSC on Der f-stimulated MDM seemed to depend on inhibition of inflammatory cytokines by Der f rather than interference with

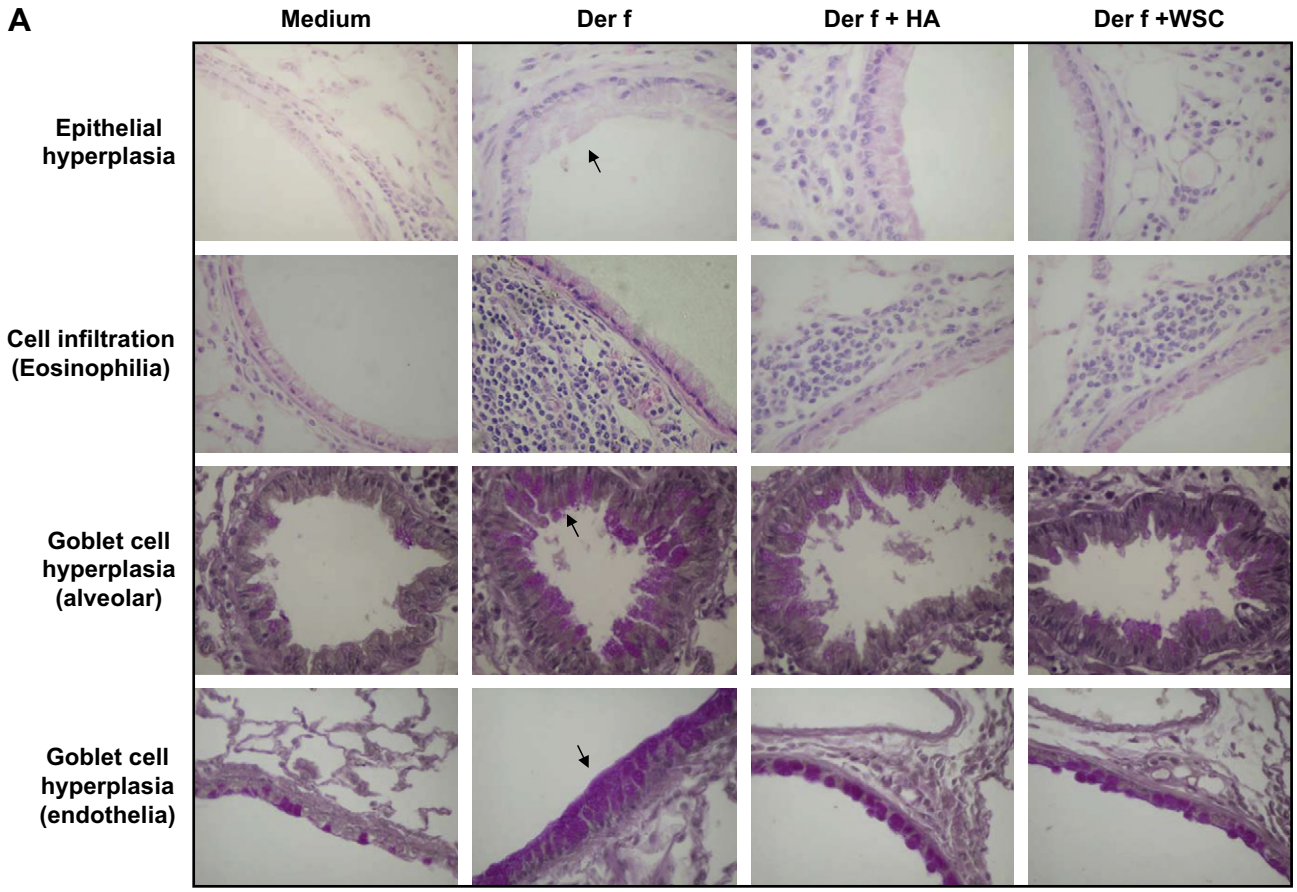

Fig. 5. WSC attenuates allergen-induced airway inflammation in Der f-sensitized mice. Histological examination (hematoxylin and eosin stain, ×100) of representative lungs taken from groups of mice ($n = 6$ in each group) on the third day after allergen challenge. Arrows denote the areas of marked epithelial hyperplasia, inflammatory cell infiltrates, goblet cell hyperplasia in the alveoli and bronchial respiratory epithelium under light microscopy (A). Immunohistochemical analysis (×100) of the production of Arg I, TSLP, and iNOs in representative lungs taken from groups of mice on the third day after allergen challenge. Lung tissue sections were stained with antibodies specific for iNOS, Arg I, and TSLP as described in Section 2 (B).

Der f–MDM interaction, since co-incubation of Der f with WSC, or pretreatment with WSC (less than 6 h) did not inhibit the inflammatory cytokine production of Der f-stimulated MDM (data not shown).

Furthermore, we found that Der f-stimulated MDM from allergic asthmatics (but not from non-atopic subjects) had multiple micropseudopodia (Fig. 2). This morphological difference in allergen-stimulated MDM between atopic and non-atopic subjects has not been reported before, and its significance in the pathogenesis of allergen-induced inflammation is not understood. We suspect that WSC prevents allergen-induced degranulation and perturbation in the MDM membrane events that trigger pathways leading to inflammatory activation. This membrane-stabilizing activity of WSC may act through ligand and receptor interaction of macrophage [30,31]. Our findings that the inhibitory effect of WSC on Der f-stimulated MDM involves inhibition of intracellular signal transduction cascades, such as the deactivation of PKCξ, reduction in the degradation of IκBα, and nuclear translocation of p65 NF-κB (Fig. 4) support this hypothesis. These intracellular effects eventually decrease production of inflammatory cytokines and expression of activated molecules on the cell surface.

Notably, HA, which has a similar structure to that of WSC with a linear repeating polymer of *N*-acetylglucosamine linked β1,4 to glucuronic acid, did not have any significant effect on the allergen-induced Th2 and inflammatory cytokine production, as well as on Der f-induced changes in MDM morphology. Moreover, Der f-induced significant amounts of the HA-binding receptor, CD44, and the protease-activated receptor (PAR2) in MDM (Fig. 3), which play important roles in the allergen-induced inflammation [32,33]. In contrast to WSC, pretreatment with HA in Der f-stimulated MDM did not suppress CD44, PAR2, CD14, and TLR4 receptor expression (Fig. 3).

Finally, using the Der f-sensitized mouse model of asthma, we found WSC had the same effect as chitin microparticles on the prevention and treatment of allergen-induced airway inflammation [11,12]. Moreover, Der f-induced inflammatory mediators important in airway remodeling in the bronchial subepithelial tissue such as Arg I, nitric oxide [34], and TSLP [35] were significantly reduced by the intratracheal administration of WSC (Fig. 5). A signature gene induced in alternatively activated macrophages is Arg I [36,37]. Arg I cleaves arginine to generate L-ornithine, a precursor for polyamines and praline, which have been

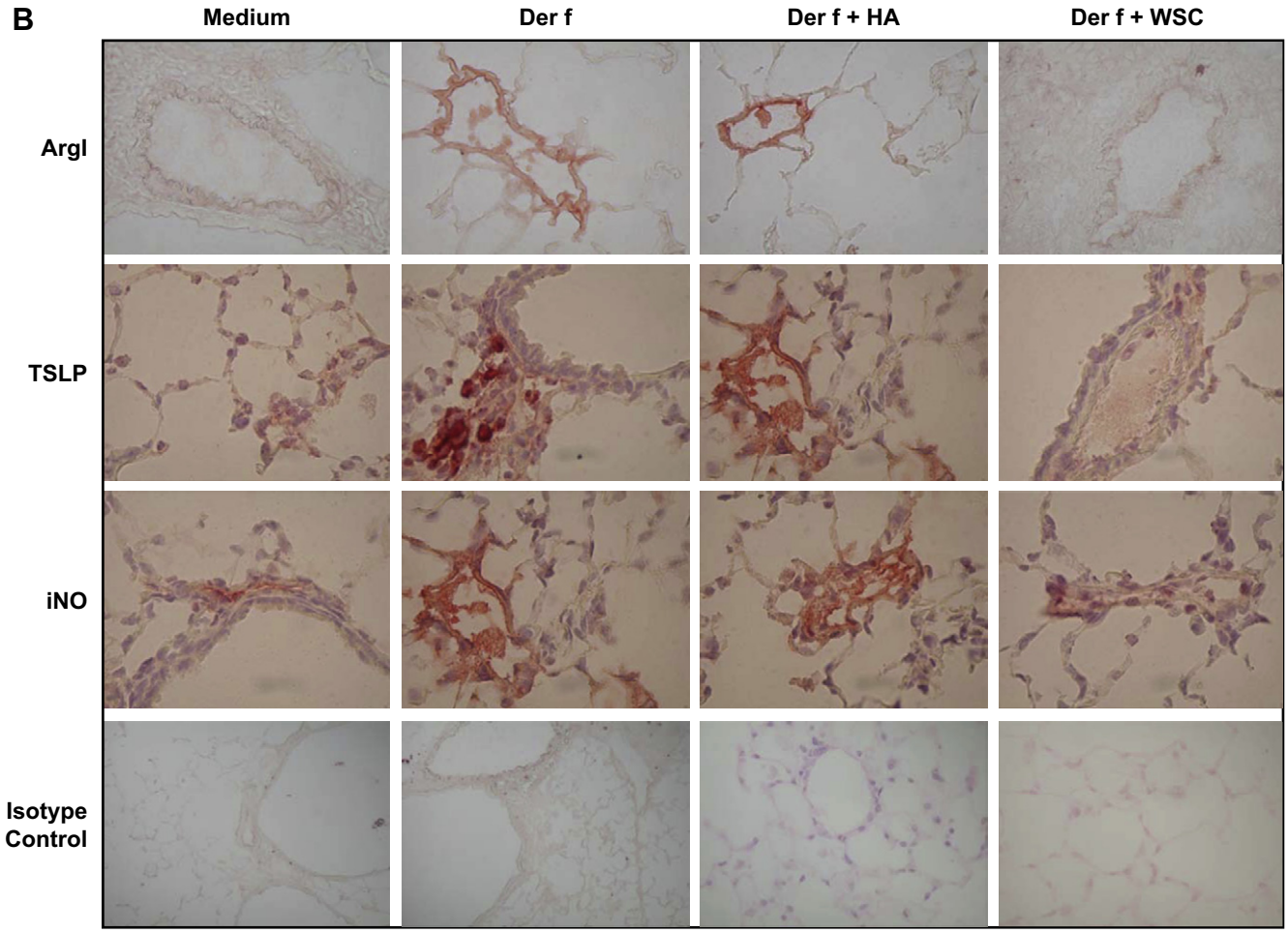

Fig. 5. (*Continued*)

implicated in cell proliferation and collagen production, respectively.

5. Conclusions

Our results showed that in addition to its Th1 adjuvanticity, water-soluble WSC is a strong immunomodulator of the alternative activation of macrophages to allergen-stimulation, i.e., it attenuates inflammatory processes frequently accompanying chronic asthma-induced airway remodeling. Such properties of WSC might be useful in combination, or as an alternative to treatment with current anti-asthma medications.

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