

ORIGINAL ARTICLE

Surfactant protein D inhibits mite-induced alveolar macrophage and dendritic cell activations through TLR signalling and DC-SIGN expression

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Summary

Background Surfactant protein D (SP-D), a secreted pattern recognition molecule associated with pulmonary innate immunity, has been shown to mediate the clearance of pathogens in multiple ways. However, how SP-D interacts with alveolar macrophages (AMs) and dendritic cells (DCs) during allergen exposure remains unclear.

Objective This study was performed to characterize the immunomodulatory effects of SP-D on mite allergen (*Dermatophagoides pteronyssinus*, Der p)-induced inflammatory signalling in AMs and DCs.

Methods Murine AM, alveolar macrophage cell line derived from BALB/c mice (MH-S cells), and human monocyte-derived dendritic cells (MDDC) were used as model systems. The production of nitric oxide (NO) and TNF- α , expression of surface Toll-like receptors (TLRs), and expression of the C-type lectin receptor known as dendritic cell (DC)-specific ICAM-grabbing non-integrin (DC-SIGN) were measured as a function of pretreatment with SP-D and subsequent exposure to Der p. Der p-dependent cellular activations that were modified by SP-D in these model systems were then identified.

Results Pretreatment of MH-S cells with SP-D reduced Der p-dependent production of NO, TNF- α , and the downstream activations of IL-1 receptor-associated kinase, mitogen activated protein kinase (MAPK) kinase, and nuclear factor- κ B. SP-D interacted with CD14 such that CD14 binding to Der p was inhibited and Der p-induced signalling via TLRs was blocked. DC-SIGN expression was suppressed by Der p in MH-S and MDDC; this down-regulation of DC-SIGN expression was prevented by pretreatment with SP-D.

Conclusions These results indicated that the inhibition of Der p-induced activation of MH-S and MDDC by SP-D is mediated through suppression of the CD14/TLR signalling pathway and maintenance of DC-SIGN expression, which may protect allergen-induced airway inflammation.

Keywords allergens, alveolar macrophages, asthma, CD14, DC-SIGN, dendritic cells, surfactant protein D, Toll-like receptor 4

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Components of the innate immune system of the lung, such as mucosal antigen-presenting cells (APCs) and the hydrophilic surfactant proteins (SP), SP-A and SP-D, have long been recognized to provide a first line of defence against infectious challenges [1]. The roles of pulmonary SP-D in defence and maintenance of home-

ostasis have been confirmed by studies on SP-D gene-deficient mice, which exhibit reduced bacterial clearance [2–4] and elevated pro-inflammatory cytokine secretion in response to microbial [5–7] and allergen challenges [8, 9]. Compelling evidence in support of the immunoregulatory role of SP-D in allergen-induced lung inflammation

has been obtained [reviewed in Wang and Reid. [10]]. Previously, this laboratory [11, 12] and that of Madan *et al.* [13] have found that SP-A and SP-D not only interact with house dust mite (*Dermatophagoides pteronyssinus*, Der p) and fungus (*Aspergillus fumigatus*, Afu) allergens in a lectin-binding and calcium-dependent manner but also suppress the binding of allergen-specific IgE to these allergens through competitive inhibitory or steric hindrance mechanisms. Moreover, SP-D was found to inhibit histamine release and allergen-induced lymphocyte proliferation, two essential steps in the pathogenesis of asthma [12]. Administration of recombinant SP-D in murine asthma models results in the down-regulation of eosinophilic inflammation and recruitment, as well as a shift in the cytokine profile towards a T-helper type 1 (Th1) response, subsequently alleviating bronchial hyper-reactivity in treated sensitized mice [13–16]. Whether SP-D performs crucial immunoregulatory function(s) during the period wherein allergens interact with antigen-APCs such as alveolar macrophages (AMs) and dendritic cells (DCs), however, remains to be determined.

Armed with cellular surface receptors, such as Toll-like and C-type lectin receptors, which recognize pathogen-associated molecular patterns (PAMPs), AMs, and DCs play essential roles in the innate immunity of the lung and in allergen-associated disorders such as asthma, atopic dermatitis, and rhinitis [17, 18]. Nonetheless, few studies have been performed that examine the interactions between allergens and APCs, such as AMs and DCs, in the mucosal membranes of the lung [17, 19]. Previously, we demonstrated that dust mite (*Dermatophagoides farinae*, Der f) extracts can directly activate innate immune cells such as AMs [20] and mast cells [21] without previous *in vitro* or *in vivo* sensitization. In mice repeatedly exposed to mite extracts, the activation of AMs and mast cells resulted in eosinophilic infiltration, goblet cell hyperplasia, and hyperplasia of peribronchial smooth muscles [20, 21]. Expression of macrophage Toll-like receptor 4 (TLR4) increases in response to allergens in wild-type but not in SP-D^{-/-} mice [22]. This finding is consistent with the hypothesis that inhibition of allergic airway inflammation by SP-D occurs, at least in part, by modulation of AM function involving PAMP-recognized molecules. We also observed that mite allergens induce nitric oxide (NO) production and NF- κ B activation in AMs via the CD14/TLR4 complex signalling pathway, and that this pro-inflammatory effect is overturned by treatment with SP-D [23]. Both SP-A and SP-D have been shown to interact with cell surface pattern-recognition receptors such as CD14 and TLRs to reduce pro-inflammatory cytokine production in lipopolysaccharide (LPS)- and peptidoglycan-stimulated AMs [24, 25]. Recently, mite allergens were reported to decrease expression of the C-type lectin receptor termed dendritic cell (DC)-specific ICAM-grabbing non-integrin (DC-SIGN)

on the surface of DCs via activation of its inherent protease activity [26]. The decreased expression of DC-SIGN resulted in reduced binding of intracellular adhesion molecule-3 (ICAM-3), an endogenous DC-SIGN ligand expressed on naïve T cells and thought to be involved in Th1 cytokine signalling. Nonetheless, details of the mechanisms whereby SP-D inhibits Der p-dependent AM activation and influences C-type lectin receptor and/or TLR pro-inflammatory signalling pathways remain unknown.

In the present study, we hypothesized that SP-D interacted with CD14, which prevented its binding to Der p allergen and blocked Der p-induced inflammatory signal through the CD14/TLR4/MD2 pathway. Furthermore, Der p-dependent repression of DC-SIGN expression in macrophages and in monocyte-derived dendritic cells (MDDCs) from allergic asthmatics was prevented by pretreatment with SP-D. The results from this study may further clarify the role of SP-D in adaptive immune responses, such as allergen-induced airway inflammation, through interactions with both AMs and DCs.

Methods

Reagents

Methods for expression and purification of recombinant fragments of human SP-D (rfh SP-D) have been described previously in detail [27]. Lyophilized *D. pteronyssinus* was purchased from Allergen, and Der p extracts were prepared as described previously [20]. The endotoxins of *E. coli*-expressed SP-D and Der p extract were removed by an Endotrap[®] column (Cambrex Bio Science Walkersville Inc., Walkersville, MD, USA). Using the *Limulus* amoebocyte lysate test (E-Toxate; Sigma-Aldrich, St Louis, MO, USA), the endotoxin concentration of the Der p preparation was estimated to be <0.96 EU/mg. LPS (*Escherichia coli* 055:B5; Sigma-Aldrich) was dissolved in pyrogenic-free isotonic saline before use. Fluoresceine isothiocyanate (FITC)-conjugated anti-mouse CD14 (16–10A1) and phycoerythrin (PE)-conjugated anti-TLR4 (AMS-32.1) mAbs were obtained from BD PharMingen (San Diego, CA, USA). Cells stained with the appropriate mAbs were analysed for CD14 and TLR4 expression by flow cytometry using a FACScan flow cytometer (Becton Dickinson Immunocytometry, San Jose, CA, USA). For each sample, 15 000 events were obtained. Isotype-matched mAb-stained cells were used as background controls in all experiments.

Culture of murine MH-S cells and human MDDCs

Alveolar macrophage cell line derived from BALB/c mice (MH-S cells), which was purchased from the American Type Culture Collection (Rockville, MD, USA), are a

continuous cell line of murine AMs, which were established after transformation of cells obtained by bronchoalveolar lavage from Balb/c mice with simian virus 40 [28]. The cells were cultured in RPMI 1640 medium (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum, 50 U/mL penicillin, 0.05 mg/mL streptomycin, 20 mM L-glutamine, 10 mM HEPES, 10 mM sodium pyruvate, 1% glucose, and 50 μ M 2-mercaptoethanol at 37 °C in 5% CO₂/95% air. Cells were washed with PBS and resuspended in RPMI medium before each experiment. Approval for all experiments with human blood was provided by the Human Ethics Committees of National Cheng Kung University. Peripheral blood mononuclear cells of Der p-sensitive allergic asthmatics and non-allergic subjects were prepared from venous blood as described previously [29]. MDDCs were generated by culture of human monocytes in the presence of 500 U/mL GM-CSF and 500 U/mL IL-4. To induce maturation, LPS (1 mg/mL) was added on day 7, and the mature DCs generated were treated with Der p for another 24 h as detailed in the text and figure legends.

Determinations of nitric oxide and TNF α production

AM cell line, MH-S, were suspended at 1×10^6 /mL in culture medium and maintained in six-well plates (Falcon 3072; Becton Dickinson Labware). Pretreatments with SP-D (10 μ g/mL) were conducted for 4 h. Incubation studies with Der p extract (10 μ g/mL) were conducted as detailed in the text and figure legends. Medium was then collected and analysed for NO content using Griess reagent and for TNF- α production by a commercial ELISA assay kit (R & D systems). The detection limit for TNF- α was 20 pg/mL.

Competitive binding assays

Wells (96-well plates) were coated with the proteins of interest (Der p, CD14, SP-D) overnight at room temperature. After three washes, incubations with various additives (pretreated or non-pretreated under specific conditions) were conducted for 2 h. Samples were washed three times and then treated with specific antibodies for 2 h, followed by three washes. After incubation with streptavidin-HRP (1:200) for 20 min, preparations were washed and incubated with substrate solution [1:1 mixture of colour reagent A (H₂O₂) and colour reagent B (tetramethylbenzidine)] for 20 min. Reactions were terminated with a stop solution (2 N H₂SO₄). The optical density of samples in each well was determined with a microplate reader set to 405 nm.

Assays for expression of 293-hTLR1-TLR9 and 293-hTLR4/MD-2-CD14 proteins by transfected HEK293 cells

TLR activation was determined by measuring the extent of NF- κ B activation in human embryonic kidney (HEK)293

cells expressing each TLR (TLR2, 3, 4, 5, 7, 8, and 9) and secreting alkaline phosphatase under the control of a modified human IL-12p40 promoter inducible by NF- κ B. The amount of secreted alkaline phosphatase was determined using the SEAP Reporter Assay Kit; measurements were performed by the TLR screening service of InvivoGen (Toulouse, France) and findings are presented as arbitrary units. Each test substance (10 μ L) was added to wells (96-well plates, 100 μ L total volume) containing the appropriate cells ($3\text{--}5 \times 10^4$ /well), followed by incubation at 37 °C for 18 h. NF- κ B activation was quantified spectrophotometrically. Initial screenings were performed (in duplicate) by challenge of each of the seven TLR clones with 10 μ g/mL of Der p and comparison of the induced alkaline phosphatase activity with that of negative controls (respective non-induced TLR clones) and that of positive controls stimulated with the following ligands: Pam3CysK4 (10 ng/mL) for TLR2, poly I:C (100 ng/mL) for TLR3, Ec-LPS (100 ng/mL) for TLR4, flagellin (100 ng/mL) for TLR5, R848 (10 μ g/mL) for TLR7 and TLR8, and ODN 2006 (10 μ g/mL) for TLR9 (InvivoGen).

Immunofluorescence assay for NF κ B activation

Cells were fixed with 3.7% paraformaldehyde in PBS for 30 min at room temperature. Washed cells were incubated with rabbit anti-NF- κ B antibodies (Santa Cruz, CA, USA) for 2 h, followed by 2 h of incubation with FITC-conjugated secondary antibodies (Calbiochem, Los Angeles, CA, USA) in PBS. DAPI (4',6-diamidino-2-phenylindole) was used for nuclear staining. Immunofluorescence was observed with a Leica microscope.

Determination of activation of TLR signalling pathways in MH-S cells and measurement of DC-SIGN mRNA expression in MDDCs

Cells were lysed using a Triton X-100-based buffer [1% Triton X-100, 150 mM NaCl, 10 mM Tris (pH 7.5), 5 mM EDTA, 5 mM NaN₃, 10 mM NaF, 10 mM sodium phosphate] in the presence of a protease inhibitor mix and phosphatase inhibitor cocktail I (Sigma-Aldrich). Proteins of whole-cell extracts were resolved by SDS-PAGE, followed by electrotransfer onto nitrocellulose (Bio-Rad) using a semi-dry cell transfer blot (Bio-Rad, Richmond, CA, USA). After blocking, blots were developed with a series of antibodies. Rabbit antibodies specific for IL-1 receptor-associated kinase (IRAK) (#4362), phosphor-IRAK (#4361), mitogen activated protein kinase (MAPK) kinase (MKK)3/6 (#9232), and phospho-MKK3/6 (#9231) were purchased from Cell Signaling (Beverly, MA, USA), and mouse antibodies specific for α -tubulin (#MS-581-P1, MDBio, Taipei, Taiwan) were used as internal controls. Finally, hybridization with horseradish peroxidase-conjugated

goat anti-rabbit or anti-mouse antibodies was performed, and blots were developed using chemiluminescence (ECL, Amersham; Arlington Heights, IL, USA). The Western blotting procedure used to detect transcription factors involved in TLR signalling pathways, and the RT-PCR procedure used to measure human DC-SIGN mRNA expression. The gene-specific primer pairs (sense and antisense, respectively) for human DC-SIGN selected were: hCD209F (5'-ATTTT C CAACT CATT TCAGC C-3') and hCD209R (5'-TCTCA CAGAA AGAGG AGGAC AC-3').

Statistical analysis

Cytokine contents and NO formation values for AM culture media were analysed using a one-way ANOVA test, followed by a *post hoc* comparison between groups. The mean fluorescence intensity (MFI) and percentage of MDDCs positive for DC-SIGN expression for each group of Der p-treated cells, non-allergic controls, and allergic asthmatics were analysed using the paired *t*-test. The results are expressed as means $\pm$ standard error of means (SEM). A *P*-value <0.05 was considered significant.

Results

Pretreatment with SP-D inhibits Der p-induced production of nitric oxide and TNF- α in MH-S cells

Der-p allergen was previously found to stimulate the production of NO and TNF- α in a dose-dependent manner in mouse AMs independent of IgE sensitization [23]. In the supplement figure, we found that anti-CD14 monoclonal antibody and anti-TLR4/MD2 monoclonal antibody were able to block Der p-induced TNF- α from THP1 cells (supporting information Fig. S1) or Der p-induced NO from MH-S cells (supporting information Fig. S2), respectively. Pretreatment of Der p-stimulated MH-S cells with rhf SP-D resulted in significant inhibitions of NO (Fig. 1a) and TNF- α (Fig. 1b). However, there were no inhibitions of Der p-induced NO and TNF- α production from MH-S cells when Der p was added simultaneously with SP-D (co-treated; Fig. 1c) or when Der p extracts pre-incubated with SP-D for 4 h at 37 °C were then added simultaneously to MH-S cells (data not shown). These two findings exclude the possibility that inhibition of Der p signalling in MH-S cells by SP-D requires the binding of SP-D to Der p extract.

Der p-induced NF- κ B activation via TLR2 and TLR4 signalling pathways is inhibited by SP-D

Der p-dependent NO and TNF- α production in AMs were previously found to depend on the presence of CD14/TLR cellular receptors [21]. It was therefore of interest to

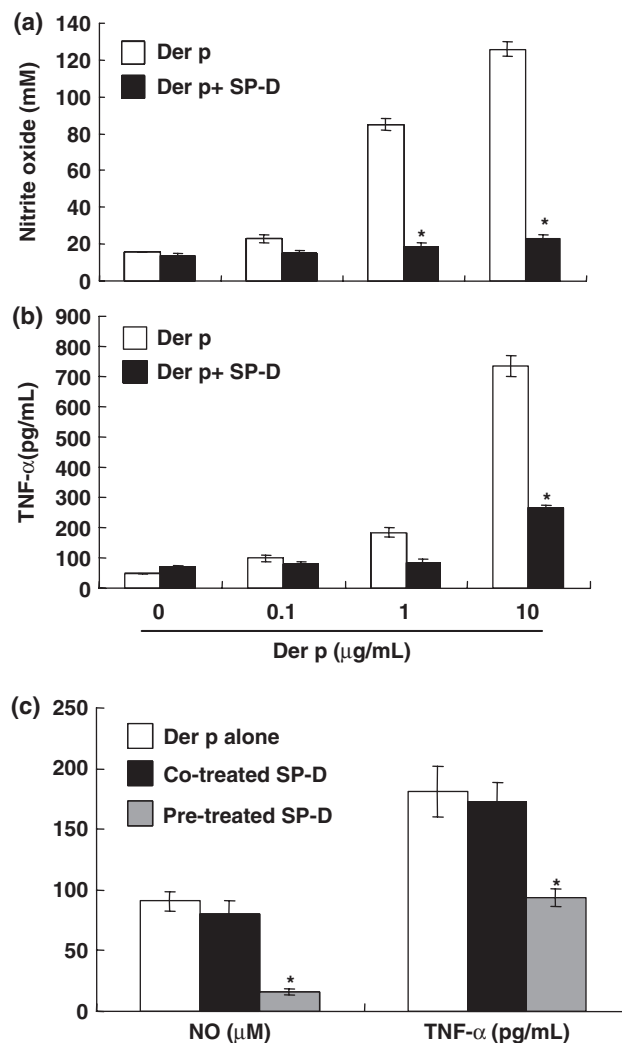


Fig. 1. Pre-treatment with surfactant protein D (SP-D) suppresses production of nitric oxide (NO) (a) and TNF- α (b) by *Dermatophagoides pteronyssinus* (Der p)-stimulated alveolar macrophage cell line derived from BALB/c mice (MH-S cells). MH-S macrophage cells were suspended to 1×10^6 /mL of culture medium for incubations in six-well plates. Cells were pretreated with SP-D (10μ g/mL) for 4 h, followed by addition various concentration of Der p extract. Incubations were continued for 24 h. In the co-treatment study, MH-S cells were treated with SP-D (10μ g/mL) and Der p (10μ g/mL) at the same time (c). Culture medium was then harvested for measurements of NO and TNF- α production. All measurements were performed in triplicate, and results are expressed as means $\pm$ SEM. **P* <0.05 , as compared with the Der p stimulation alone.

identify the TLR(s) susceptible to activation by Der p. In the present study, Der p-induced activation of a reporter gene under the control of an NF- κ B inducible promoter in TLR2, -3, -4, -5, -7, -8, and -9 – transfected HEK 293 cells was monitored. Der p extract activated the NF- κ B-inducible promoter in TLR2 and TLR4-transfected HEK293 cells to degrees comparable with TLR2 and TLR4 agonist stimulation (Fig. 2a). In fluorescence microscopy examination, use of a fluorescent antibody against the

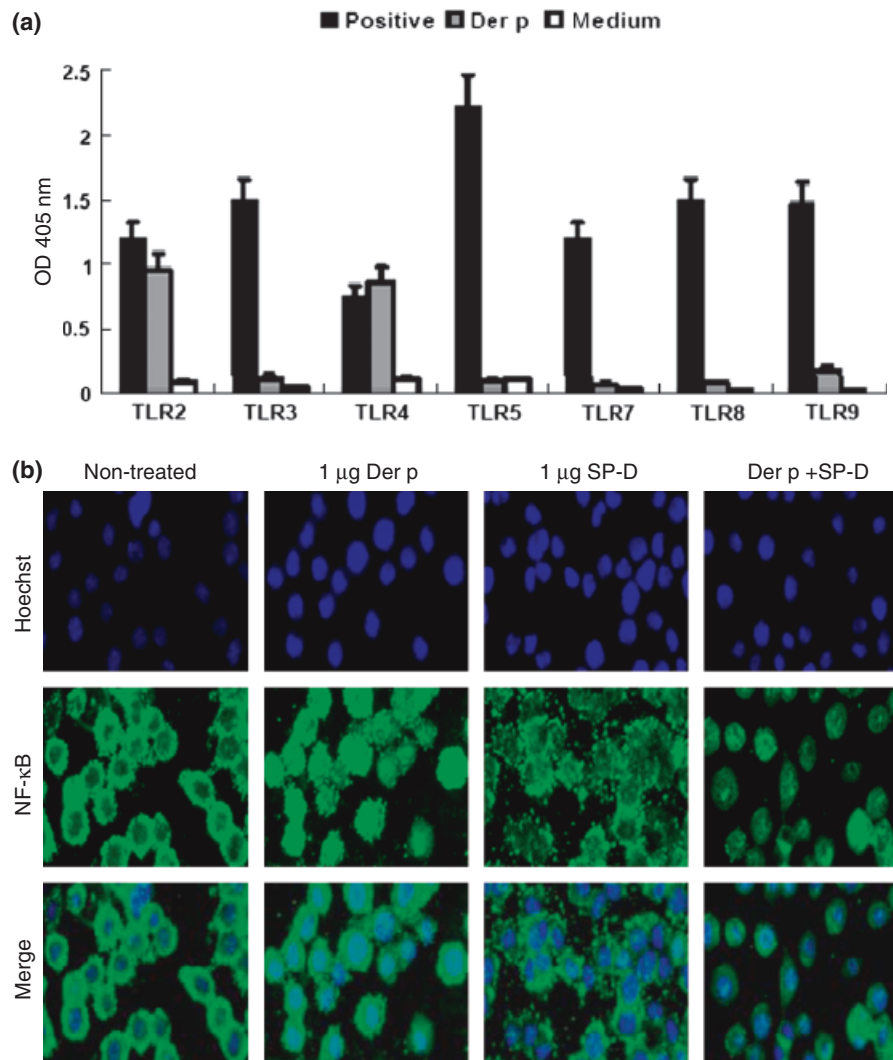


Fig. 2. *Dermatophagoides pteronyssinus* (Der p) interacts with Toll-like receptor 2 (TLR2) and TLR4 and signals translocation of nuclear factor-κB (NF-κB) to the nucleus, which is suppressed by surfactant protein D (SP-D). (a) Interaction of Der p with human embryonic kidney (HEK)293 clones expressing TLR2, -3, -4, -5, -7, -8, or -9 and a reporter gene under the control of an NF-κB inducible promoter were derived. Each TLR-expressing clone was tested for activation by Der p (grey bars) and TLR-specific ligands (black bars). Results are expressed as OD values. White bars indicate background values corresponding to 'activations' detected in the absence of cells. (b) Translocation of activated NF-κB p50/p65 heterodimer (green) from the cytoplasm to the nucleus (blue) in response to Der p (1 µg/mL) and suppression of the translocation by pretreatment with SP-D (10 µg/mL). Co-localization analysis was performed using fluoresceine isothiocyanate-labelled anti-mouse NF-κB p65 antibody and cell nuclei counterstaining with Hoechst dye (original manifestation $\times 400$). Comparable findings were obtained on three separate occasions.

p65 subunit of the NF-κB p50/p65 heterodimer revealed that NF-κB was mainly localized to the cytoplasm (inactive form linked to IκB) in non-activated MH-S cells treated with or without SP-D (Fig. 2b). In contrast, MH-S cells activated by Der p display a uniform distribution of NF-κB p65 fluorescent labelling (Fig. 2b). This homogeneous distribution is the result of translocation of the activated NF-κB p50/p65 heterodimer from the cytoplasm to the nucleus and its subsequent binding to nuclear DNA. Pretreatment with SP-D suppressed Der

p-inducible translocation of NF-κB to the nucleus of MH-S cells (Fig. 2b).

SP-D suppresses Der p-dependent nitric oxide production in MH-S cells through IKK and ERK signalling pathways

The effects of SP-D on activations of IRAK and MKK, both of which are involved in the TLR MyD88-dependent signalling pathway shared by TLR2 and TLR4 receptors, were examined. MH-S cells displayed significant

phosphorylation of IRAK within 15 min of exposure to Der p, with maximum phosphorylation observed at 60 min of exposure. Activation of MKK phosphorylation in response to Der p stimulation was somewhat slower, with 60 min of treatment required for detection (Fig. 3a). Der p-dependent NO production by MH-S cells was also inhibited by UO126 (a selective inhibitor of upstream MKK-1 and MKK-2) and by SB203580 (a highly selective and cell-permeable inhibitor of p38 MAPK) (Fig. 3b). In a dose-dependent manner, SP-D was found to block the phosphorylations of IRAK and MKK in response to Der p (Fig. 3c).

SP-D binds CD14 and thereby reduces its availability for the binding to Der p allergen

Regardless of dosage, treatments with SP-D produced no significant changes in CD14 expression at 12 h (Fig. 4a) or in TLR4 expression at 24 h (Fig. 4b) in MH-S cells. Fluorescence microscopy revealed that FITC-labelled SP-D antibody and fluorescent antibody against CD14 localized to identical sites on the plasmalemma of non-activated, SP-D-treated MH-S cells (Fig. 4c). This finding was further confirmed using a solid-phase ELISA binding assay, which showed that SP-D interacted with CD14 in culture plates in a dose-dependent manner whether surfaces were coated with CD14 (Fig. 4d) or SP-D (Fig. 4e). This interaction was calcium-dependent and inhibited by maltose (data not shown). Soluble CD14 molecules bind Der p extracts selectively in our previous report [30]; the effects of SP-D on this binding were therefore of interest. Experiments were performed to determine whether SP-D interferes with binding of CD14 to Der p extracts. Plates coated with Der p extract were incubated with soluble CD14 and/or SP-D, with the order of addition of additives varied. Pre-addition of SP-D to coated plates did not inhibit binding of CD14 to Der p. By contrast, when SP-D was incubated with CD14 before addition to plates or when SP-D and CD14 were added simultaneously to plates, the binding of CD14 to Der p was decreased by more than 80% (Fig. 4f). These findings imply that the interaction binding between CD14 and SP-D, which results in CD14 becoming unavailable to Der p binding, inhibits the binding of CD14 to Der p.

SP-D inhibits Der p-induced down-regulation of DC-SIGN expression in MH-S cells

In order to determine how SP-D modulates the cell surface molecules in the MH-S cells that render it non-responsive to Der p allergen stimulation, studies were performed to examine the effects of Der p and SP-D on the cellular receptors that induce inhibitory signals in MH-S cells. *In vitro*, the C-type lectin receptor, DC-SIGN, was found

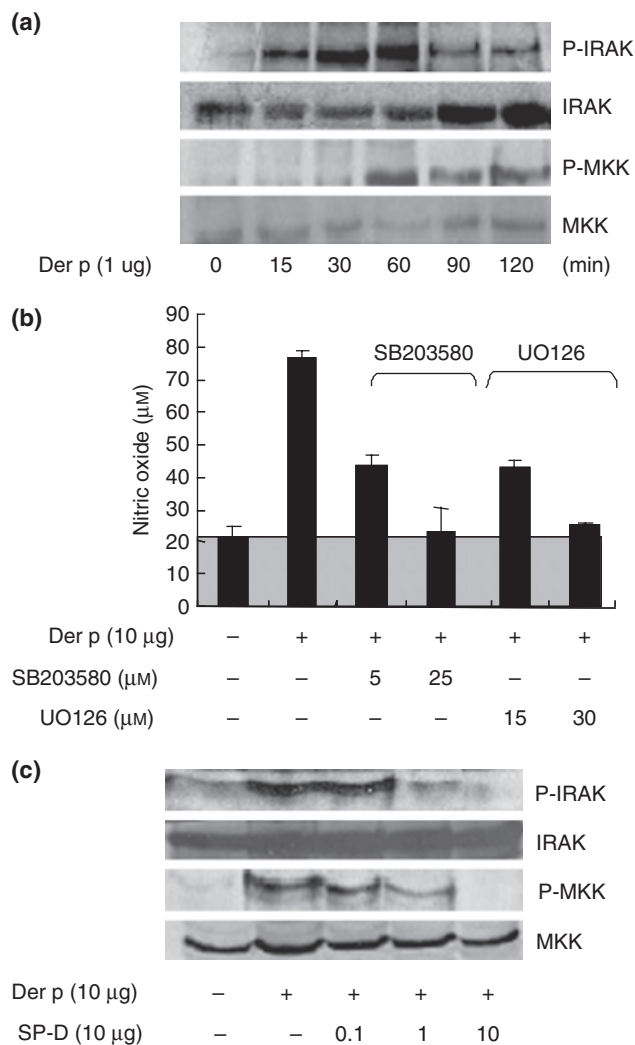


Fig. 3. Pretreatment of alveolar macrophage cell line derived from BALB/c mice (MH-S cells) with surfactant protein D (SP-D) suppresses phosphorylation of IL-1 receptor-associated kinase (IRAK) and mitogen activated protein kinase (MAPK) kinase (MKK) in response to *Dermaphagoides pteronyssinus* (Der p). MH-S cells (6×10^6 /mL) were treated with Der p (10 µg/mL) for 24 h (a and b), or pretreated for 4 h of SP-D at the indicated concentrations and then treated for 24 h with Der p (10 µg/mL) as indicated (c). Extracts were prepared for measurements of p-IRAK and p-MKK protein expression by western blotting. Comparable findings were obtained on three separate occasions.

to interact with Der p extracts in a dose-dependent manner (Fig. 5a). Moreover, DC-SIGN expression in MH-S cells was decreased by increasing doses of Der-p stimulation, but not by SP-D (Fig. 5b). When MH-S cells were pretreated with SP-D, however, the decreased expression of DC-SIGN caused by Der p stimulation was inhibited (Fig. 5c).

Confirmation of this finding was sought. Expression of DC-SIGN by MDDCs from Der p-sensitized allergic asthmatic patients was significantly lower than that from

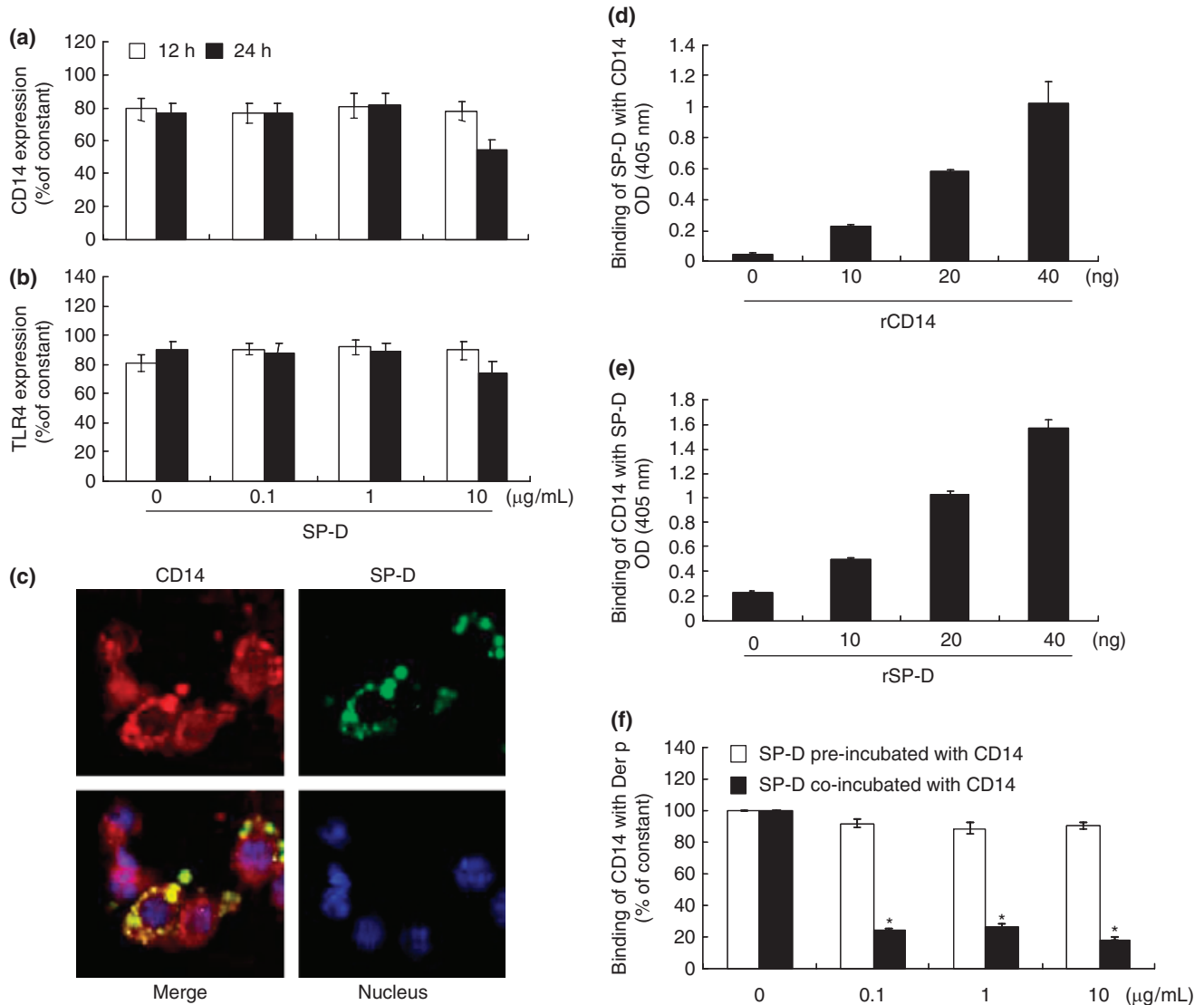


Fig. 4. Surfactant protein D (SP-D) associates with CD14 rendering it unavailable for interaction with *Dermatophagoides pteronyssinus* (Der p). The stimulation of SP-D does not influence the expression of CD14 (a) and Toll-like receptor 4 (TLR4) (b) on the alveolar macrophage cell line derived from BALB/c mice (MH-S cells). Fluorescence microscopy revealing co-localization of CD14 and SP-D on the plasmalemma of MH-S cells (original magnification $\times 400$) (c). There were dose-dependent bindings of recombinant CD14 to solid-phased SP-D (d) and SP-D to coated recombinant CD14 (e) in the ELISA assay. Incubation of CD14 with SP-D prevents CD14 from binding to Der p (f). Plates coated with Der p extract were incubated with soluble CD14 and/or SP-D at the concentrations indicated, followed by measurements of binding of CD14 to Der p by ELISA assay. Open bars indicate addition of SP-D to plates before addition of CD14 (pre-incubated). Filled bars indicate incubation of SP-D with CD14 before addition of the mixture to plates (co-incubated). Each bar represents the mean value of measurements conducted in triplicate $\pm$ SEM. * $P < 0.05$.

non-allergic controls ($P < 0.05$, Fig. 6a). When in contact with Der p extracts for 24 h, MDDCs from both allergic asthmatics (AS) and controls (NC) displayed significant reductions in the overall DC-SIGN expression and in the percentage of DC-SIGN-positive cells ($P < 0.001$; Figs 6a and b). The mRNA expression of DC-SIGN in MDDCs was not changed by Der p stimulation both in allergic asthmatics and in control subjects (Fig. 6c). However, pretreatment of MDCCs from either group of subjects with SP-D served to block

the Der p-dependent down-regulation of DC-SIGN protein expression ($P < 0.05$; Fig. 6d). These findings are comparable with those obtained with MH-S cells (Fig. 5c).

Discussion

Previously, we have shown that an endogenous protein, surfactant protein D (SP-D), which belongs to the innate

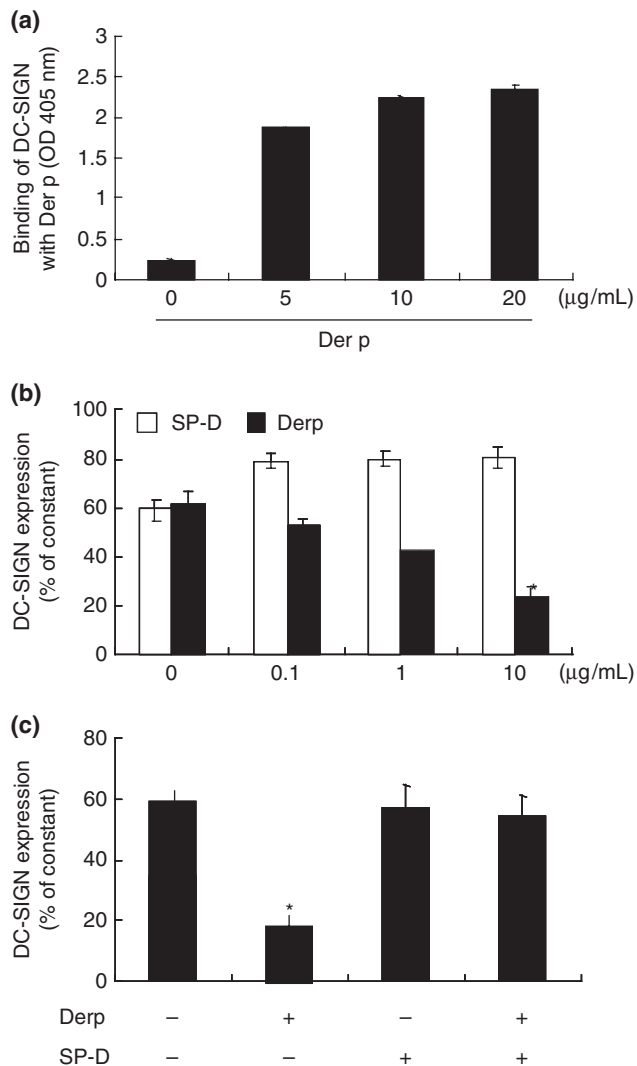


Fig. 5. *Dermatophagoides pteronyssinus* (Der p) interacts with dendritic cell (DC)-specific ICAM-grabbing non-integrin (DC-SIGN) and down-regulates its expression in alveolar macrophage cell line derived from BALB/c mice (MH-S); surfactant protein D (SP-D) antagonizes the down-regulation. (a) In solid phase ELISA binding assay, Der p interacts with DC-SIGN in a dose-dependent manner. (b) Expression of DC-SIGN is decreased by Der p but not by SP-D stimulation. Cells were treated with either Der p or SP-D for 24 h at the concentrations indicated. Extracts were then prepared for measurements of DC-SIGN mRNA by RT-PCR. (c) SP-D antagonizes the down-regulation of DC-SIGN in response to Der p. Preparations were pretreated with SP-D (10 μg/mL) for 4 h and then treated with Der p (10 μg/mL) as indicated, followed by measurements of DC-SIGN mRNA by RT-PCR. Each bar represents the mean value ± SEM of measurements performed in triplicate. * $P < 0.05$, as compared with Der p and SP-D stimulation.

immunity of the lung, can inhibit airborne allergen-induced NO and TNF- α production in AM cell lines [23]. In this study, we found that the inhibitory effects of SP-D on Der p-induced AMs and DC activation may be exerted through two different mechanisms. Firstly, although SP-D can directly bind to allergen and LPS-

containing pathogens, this binding cannot prevent NO and TNF- α production from AMs activation, as suggested by the lack of inhibition observed when SP-D and Der p were added simultaneously to the MH-S cells (Fig. 1c), while treatment of MH-S cells with SP-D before the addition of Der p clearly resulted in a significant decrease of NO and TNF- α production (Figs 1a and b). Therefore, in order to determine whether the inhibitory effect of SP-D is associated with the interaction with PAMP recognition receptors, we found that the binding of SP-D to the CD14 molecule inhibited its binding to Der p, which directly interferes with the Der p-induced NF- κ B activation via CD14/TLR4 receptors. These results imply that SP-D plays a role in the maintenance of the immune tolerance status of the lung during routine exposure to airborne allergens.

The modulation of allergen-induced AMs and DCs activation that attenuates airway inflammation may also result from the interaction of SPs with TLRs [31, 32]. Recently, it was also found that SP-D bound to the extracellular domains of TLR2 and TLR4 through its carbohydrate recognition domain (CRD) [32]. Whether SP-D modulates PAMP-induced TLR2- or TLR4-dependent signalling through similar mechanisms as in the interference of CD14-Der p bindings has not yet been definitely assessed. In the present study, pretreatment of AMs with SP-D was found to inhibit the phosphorylations of IRAK and MKK and to block NF- κ B activation in response to Der p through modulation of TLR2 or TLR4 signalling pathways (Figs 2 and 3). There is a possibility that SP-D may act on as yet unidentified cellular receptors in AMs that induce inhibitor signals on the TLR inflammatory pathways and become non-responsive to allergen-stimulation. Further investigation is required to determine whether this anti-inflammatory effect of SP-D is mediated through its direct interaction with TLRs or that occurs indirectly through its interactions with other surface receptors.

DC-SIGN (also known as CD209), a receptor present in the plasma membranes of AMs and DCs, binds schistosome glycoproteins as well as a number of pathogens associated with suppression of Th1 and/or enhancement of Th2 immunity [33, 34]. DC-SIGN was recently shown to recognize peanut allergen (Ara h 1), with internalization of the allergen enhanced as a function of receptor expression [35]. Moreover, it was found that DC-SIGN activation modulated TLR signalling at the level of the transcription factor NF- κ B [36]. A recent study also demonstrated that treatment of DC-SIGN-expressing cells with the cysteine protease Der p 1 led to loss of detectable surface DC-SIGN and DC-SIGNR [26]. Thus, we hypothesized that SP-D may exert its inhibitory function directly or indirectly through the up-regulation of the expression of inhibitory signal pathway-associated receptors, such as macrophage mannose receptor (MMR) or DC-SIGN, which may regulate Der

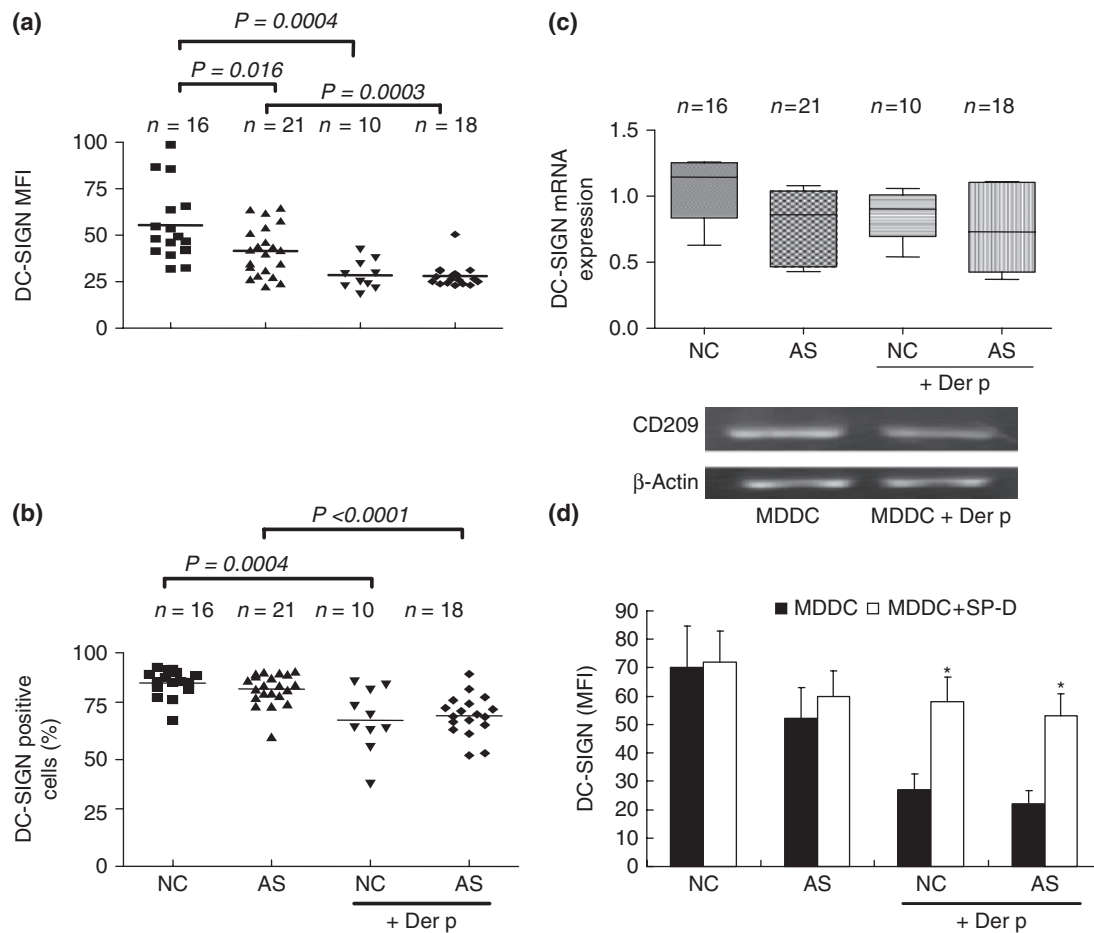


Fig. 6. Expression of dendritic cell (DC)-specific ICAM-grabbing non-integrin (DC-SIGN) by monocyte-derived dendritic cells (MDDCs) from *Dermatophagoides pteronyssinus* (Der p)-sensitized allergic asthmatic patients (AS) and non-allergic controls (NC), and effects of Der p and surfactant protein D (SP-D) on expression. (a) mean fluorescence intensities for DC-SIGN; (b) percentages of DC-SIGN-positive cells. Each bar represents the mean value $\pm$ SEM of measurements performed in triplicate. * $P < 0.01$.

p-induced Th2 immune response and cellular activation. However, we did not find any difference in MMR expression in the mouse macrophage upon SP-D stimulation (data not shown). Accordingly, we observed a significant reduction in the amount of DC-SIGN on the surface of human MDDC, obtained either from allergic asthmatics or control subjects, after treatment with Der p (Figs 6a and b). The Der p-induced down-regulated DC-SIGN expression was not due to DC-SIGN mRNA reduction (Fig. 6c). The possibility of proteolytic activity of Der p that cleaved surface DC-SIGN on MH-S cells cannot be confirmed at this stage. However, we found that, although SP-D cannot modulate DC-SIGN expression, pre-treatment of both allergic patients's and controls's MDDCs with SP-D obviously prevented the Der p-induced reduction of DC-SIGN (Fig. 6d). These findings provide a novel mechanism of the inhibitory effect of SP-D on the allergen-induced AMs and DCs activations.

In summary, we found that the inhibitory effects of SP-D on Der p-stimulated AMs might be exerted through both

direct and indirect mechanisms. We have demonstrated that SP-D can directly bind to the CD14 molecule and inhibit its binding to Der p, which may block Der p-induced NF- κ B activation via CD14/TLR4 receptors (Fig. 7, pathway 1). Besides, Der p can also bind to DC-SIGN, and decrease DC-SIGN cell surface expression. Also, SP-D can maintain the level of DC-SIGN expression probably by preventing its enzymatic digestion and/or degradation by Der p (Fig. 7, pathway 2). Finally, the third possibility is that SP-D may act on as yet unidentified cellular receptors in AMs that induce inhibitor signals on the TLR inflammatory pathways and become non-responsive to allergen stimulation (Fig. 7, pathway 3). Therefore, AMs and DCs, which act as scavengers and professional APCs to activated naïve T cells, are tightly regulated by SP-D to maintain homeostasis through multiple cellular mechanisms as proposed in this study. These results also demonstrate the potential role of SPs in regulating adaptive immune responses through interactions with AMs and DCs.

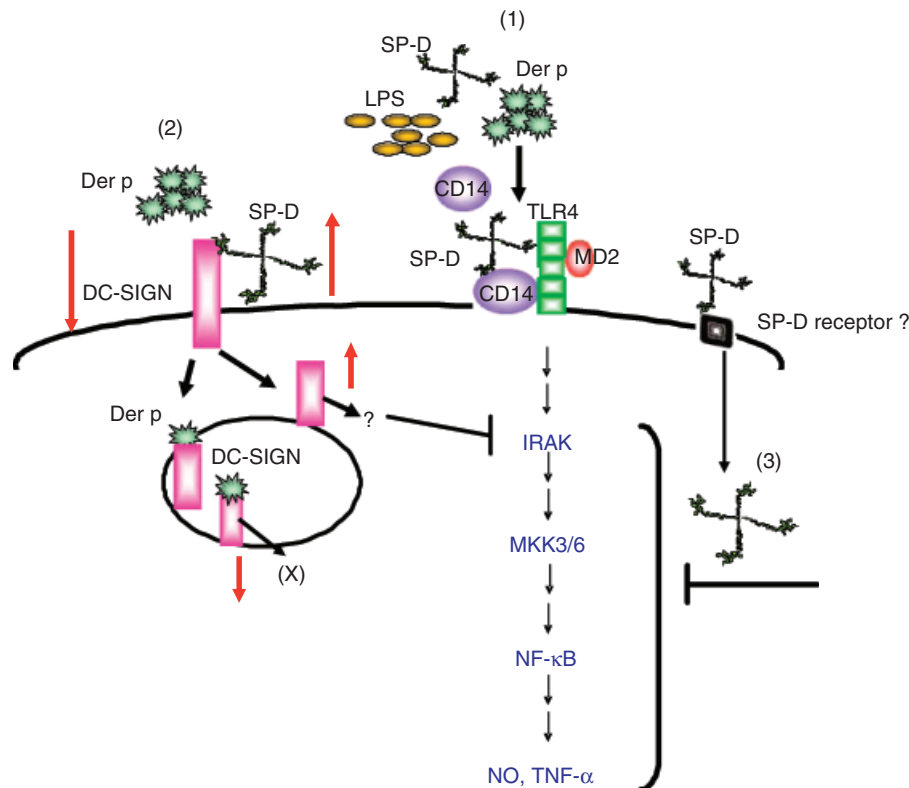


Fig. 7. Schematic representation of three possible mechanisms whereby surfactant protein D (SP-D) treatment may suppress inflammatory signalling by *Dermatophagoides pteronyssinus* (Der p). SP-D can directly bind to CD14 molecule and inhibit its binding to Der p which may block Der p-induced nuclear factor-κB (NF-κB) activation via CD14/Toll-like receptor 4 (TLR4) receptors (pathway 1). Also, SP-D can maintain the level of dendritic cell (DC)-specific ICAM-grabbing non-integrin (DC-SIGN) expression probably by preventing its enzymatic digestion and/or degradation by Der p (pathway 2). Finally, the third possibility is that SP-D may act on now still unidentified cellular receptors in alveolar macrophages or DCs that evoke inhibitor signals on the TLR inflammatory pathways and render non-responsive to allergen-stimulation (pathway 3).

Acknowledgement

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Anti-CD14 monoclonal antibody MEM-18 blocks Der p-induced TNF α production in THP1 cells. Human mononuclear cell lines, THP1 cells, were dispensed in 1 mL at 2×10^6 /mL into each well of a 24-well culture plate. The cells were pre-incubated with various concentrations of anti-CD14 monoclonal blocking antibody MEM-18 (gifted from Dr. Yoshio Kuroki, Department of Biochemistry, Sapporo Medical University School of Medicine) or isotype control antibody for 1 h at 37 °C. Der p extracts (1 μ g/mL and 10 μ g/mL) were added in cultured cells for another 24 h at 37 °C, 5% CO₂ in a humidified incubator. The culture supernatants were collected for TNF α assay, as described under Materials and methods. The data shown are mean $\pm$ SEM of three experiments. * P < 0.01; ** P < 0.001, compared with the culture supernatant collected from the cells pre-incubated with control IgG.

Figure S2. Anti-TLR4/MD2 monoclonal antibody, MTS510, blocks Der p-induced NO production in MH-S cells. Murine alveolar macrophage cell lines, MH-S cells, were dispensed in 1 mL at 2×10^6 /mL into each well of a 24-well culture plate. The cells were pre-incubated with various concentrations of anti-TLR4/MD2 monoclonal blocking antibody, clone MTS510 (Cat. No. 16–9924,

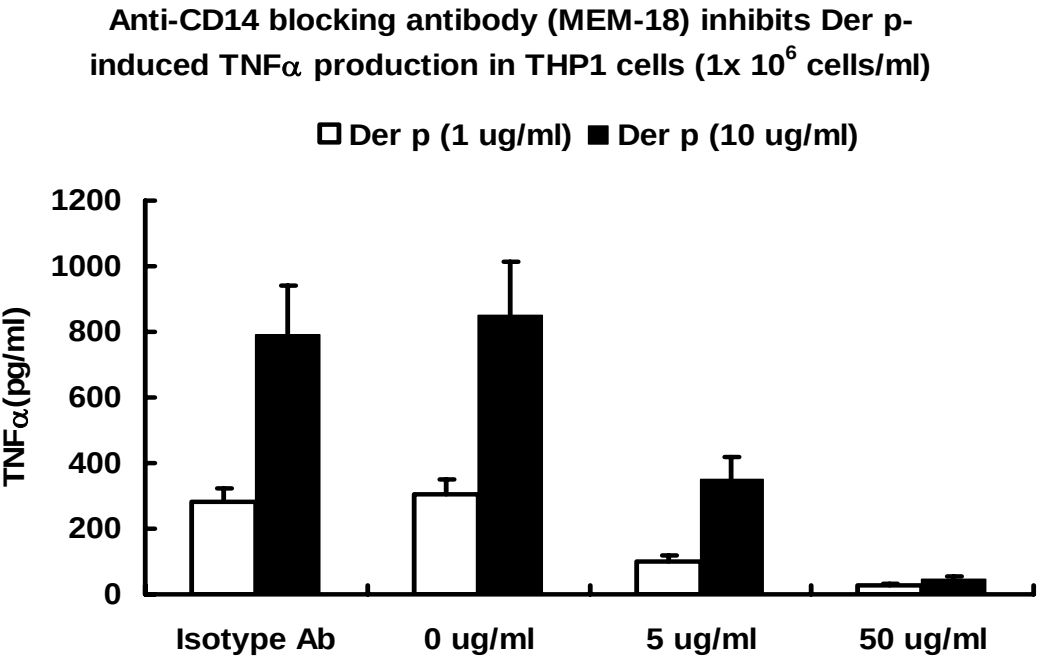
eBioscience Inc., San Diego, CA, USA) or isotype control antibody for 1 h at 37 °C. Der p extracts (1 µg/mL and 10 µg/mL) were added in cultured cells for another 24 h at 37 °C, 5% CO₂ in a humidified incubator. The culture supernatants were collected for NO assay, as described under Materials and methods. The data shown are mean ± SEM of three experiments. * $P < 0.01$; ** $P < 0.001$,

compared with the culture supernatant collected from the cells pre-incubated with control IgG.

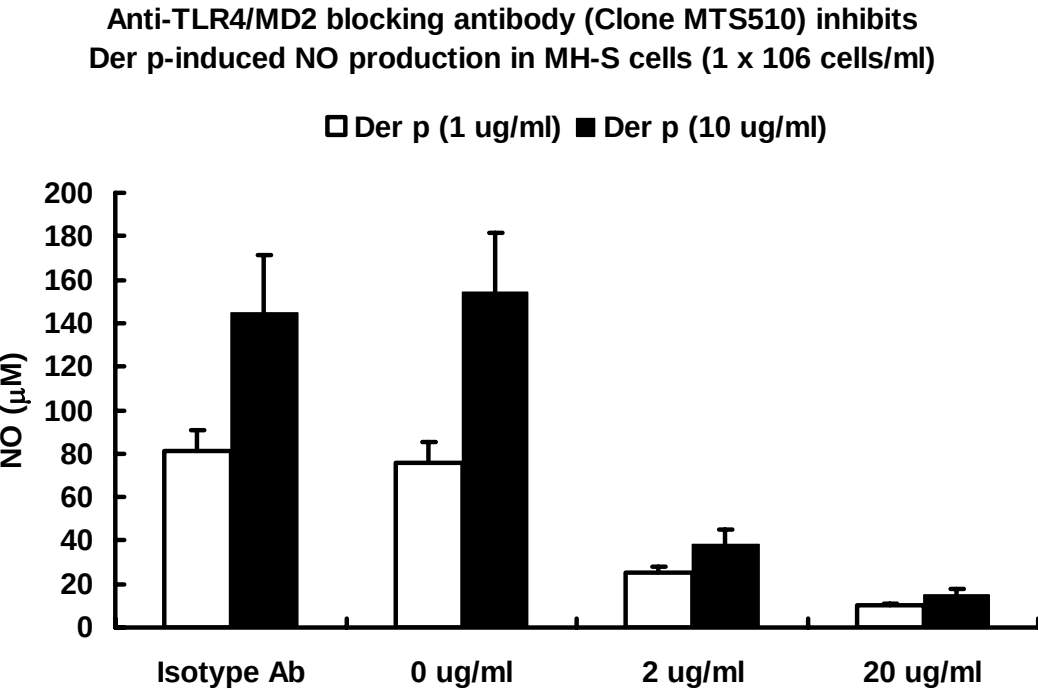
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Supplement figure:

(A).



(B).



Supplement figure legend:

(A). Anti-CD14 monoclonal antibody MEM-18 blocks Der p-induced TNF α production in THP1 cells. Human mononuclear cell lines, THP1 cells, were dispensed in 1 ml at 2×10^6 /ml into each well of a 24-well culture plate. The cells were pre-incubated with various concentrations of anti-CD14 monoclonal blocking antibody MEM-18 (gifted from Dr. Yoshio Kuroki, Department of Biochemistry, Sapporo Medical University School of Medicine) or isotype control antibody for 1 hour at 37°C. Der p extracts (1 ug/ml and 10 ug/ml) were added in cultured cells for another 24 hours at 37°C, 5% CO₂ in a humidified incubator. The culture supernatants were collected for TNF α assay, as described under Materials and methods. The data shown are means $\pm$ SEM of three experiments. *p < 0.01; **p < 0.001, compared with the culture supernatant collected from the cells pre-incubated with control IgG.

(B). Anti-TLR4/MD2 monoclonal antibody, MTS510, blocks Der p-induced NO production in MH-S cells. Murine alveolar macrophage cell lines, MH-S cells, were dispensed in 1 ml at 2×10^6 /ml into each well of a 24-well culture plate. The cells were pre-incubated with various concentrations of anti-TLR4/MD2 monoclonal blocking antibody, clone MTS510 (Cat. No. 16-9924, eBioscience Inc., San Diego, CA, USA) or isotype control antibody for 1 hour at 37°C. Der p extracts (1 ug/ml and 10 ug/ml) were added in cultured cells for another 24 hours at 37°C, 5% CO₂ in a humidified incubator. The culture supernatants were collected for NO assay, as described under Materials and methods. The data shown are means $\pm$ SEM of three experiments. *p < 0.01; **p < 0.001, compared with the culture supernatant collected from the cells pre-incubated with control IgG.