

Mite allergen induces nitric oxide production in alveolar macrophage cell lines via CD14/toll-like receptor 4, and is inhibited by surfactant protein D

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Summary

Background Previously, we have found that dust mite allergens can directly activate alveolar macrophages (AMs), induce inflammatory cytokines, and enhance T-helper type 2 cytokine production. A molecule of innate immunity in the lung, surfactant protein D (SP-D), is able to bind mite allergens and alleviates allergen-induced airway inflammation.

Objectives This study was aimed at investigating the activation pathway of mite allergen (*Dermatophagoides pteronyssinus*, Der p)-induced nitric oxide (NO) production by AMs, and the role of SP-D in the modulation of activated AMs by mite allergens.

Methods Porcine SP-D was purified from bronchoalveolar lavage fluids of Lan-Yu mini-pigs, by affinity chromatography on maltose-sepharose. NO production, inducible expression of lipopolysaccharides (LPS)-related binding and responding surface receptors complex, CD14 and toll-like receptor 4 (TLR4), as well as inducible NO synthase (iNOS) and nuclear factor- κ B activation were studied in two AMs cell lines, MH-S (BALB/c strain), and AMJ2-C11 (C57BL/6 strain), and one peritoneal macrophage cell line (RAW264.7), after stimulation with LPS, or Der p.

Results LPS and Der p elicited different responses of NO production in the different cell lines, and the response might depend upon the expression of the cell surface CD14/TLR4 complex in different genetic backgrounds of macrophage cell lines. Pretreatment of macrophages with SP-D could inhibit NO production from Der p or LPS-stimulated alveolar macrophages.

Conclusion Mite allergen-induced alveolar macrophage activation is mediated by CD14/TLR4 receptors and can be inhibited by SP-D; it further supports the concept that SP-D may be an important modulator of allergen-induced pulmonary inflammation.

Keywords CD14, mite allergen, nitric oxide, surfactant protein-D, toll-like receptor 4

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Introduction

Asthma is characterized by chronic airway inflammation, with an influx of eosinophils and T helper lymphocytes, and associated with bronchial hyper-responsiveness (BHR) [1]. The long-term effects of airway inflammation, involving airway remodelling, are characterized by epithelial damage, microvascular leakage, mucus hypersecretion, and deposition of collagen beneath the basement membrane [2]. Although adaptive antigenic responses are known to play essential roles in allergic asthma, the role of innate immunity is less well characterized, and the pathogenesis and functional consequences of initiating allergic inflammation remain to be clarified. It has been recognized that the lung contains resident components of the innate immune system, such as alveolar macrophages (AMs), that provide a first-line defence

against infectious challenge [3, 4]. In addition, AMs are capable of direct interaction with inhaled allergens, and this interaction causes a rapid up-regulation of inducible NO synthase (iNOS) mRNA and subsequent release of NO and pro-inflammatory cytokines [5, 6]. In a previous study, we found that dust mite allergens from *Dermatophagoides farinae*, Der f, could directly induce AMs activation of inflammatory cytokines and direct naïve T cells to become T-helper type 2 (Th2)-cytokine prone, although the receptors for Der f on AMs remain unidentified [7].

Surfactant-associated protein D (SP-D) is a member of the collectin family of proteins, which has multiple functions in the innate immunity of the lung [8–12]. Previously, we [11, 13] and Madan et al. [14, 15] have demonstrated that SP-A and SP-D could bind to mite (*Dermatophagoides pteronyssinus*, Der p) and fungus (*Aspergillus fumigatus*, Afu) allergens in a specific way, and appeared to inhibit the binding of allergen-specific IgE to these allergens, perhaps through competition or steric hindrance effects. Moreover, both of these surfactant proteins were found to inhibit histamine release from basophils and allergen-induced lymphocyte proliferation,

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two essential steps in the pathogenesis of asthma [13, 16]. Furthermore, both intranasal administration of SP-D and a recombinant fragment of SP-D into allergen-sensitized mice showed beneficial results by decreasing allergen-induced lung inflammation, as displayed by a shift from Th2 to Th1 cytokine profiles in the animals treated [15, 17].

Given the potential roles of surfactant proteins in innate and adaptive immunity in various allergic responses, we hypothesized that SP-D may interact with CD14 and toll-like receptors (TLRs), the pattern recognition receptors of AMs, and inhibit the production of NO and TNF- α from activated AMs after contact with allergens. In this study, we found that lipopolysaccharides (LPS) and Der p elicited different responses, with respect to NO production, in different macrophage cell lines and that this was dependent upon the level of expression of the cell surface CD14/TLR4 complex on these cell lines. Pretreatment with SP-D could inhibit this NO production from Der p or LPS-stimulated AMs, implying that SP-D might play an important role in the modulation of innate immunity in the lung.

Materials and methods

Mice

Specific pathogen-free, female, 6–8-week-old C3H/HeN and C3H/HeJ mice (Laboratory Animal Center, National Cheng Kung University, Tainan, Taiwan) were used. Animals were fed sterile food and water *ad libitum*. All experimental animal care and treatment followed the guidelines set up by the Institutional Animal Care and Use Committee.

Reagents

Der p extract (1 g lyophilized whole-body extract in ether; Allergon, Engelholm, Sweden), or LPS (*Escherichia coli* 055:B5; both from Sigma-Aldrich, St Louis, MO, USA) were dissolved in pyrogenic-free isotonic saline, filtered through a 0.22 μ m filter, and stored at -70°C before use. The LPS concentration of the Der p extract preparation was <0.96 EU/mg of Der p (*Limulus* amoebocyte lysate test; E-Toxate; Sigma-Aldrich). Arg-Gly-Asp-Ser (RGDS) peptide (American Peptide Company, Sunnyvale, CA, USA), mannosyl-bovine serum albumin (BSA)

(EY Laboratories, San Mateo, CA, USA), *N*-monomethyl-L-arginine (L-NMMA), EDTA, polymyxin B, curcumin, and pyrrolidine dithiocarbamate (PDTC, Sigma-Aldrich) were each dissolved in PBS before use.

Purification of surfactant associated protein D from bronchoalveolar lavage fluid of porcine lung

BALF was collected from Lan-Yu mini-pigs. Both lungs were lavaged, total of five times, with 100 mL of PBS containing 5 mM of EDTA. All the BALF collected was pooled and applied to a maltosyl sepharose column (Sigma-Aldrich), as described previously [18, 19]. The amount of endotoxins present in the SP-D preparations was estimated, as described above, and was found to be below 1.0 EU/mg of SP-D.

Culture of MH-S, AMJ2-C11, and RAW264.7 cells

MH-S and AMJ2-C11 cell lines were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). The

RAW264.7 cell line, kindly provided by Dr H. Y. Lei, NCKU, was derived from a tumour obtained from the ascites of male BAB/14 mouse after intraperitoneal injection of Abelson leukaemia virus [20]. These cell lines were maintained regularly in media described in previous reports [20–22].

Stimulation of alveolar macrophages and determination of nitric oxide production

Alveolar macrophages (2×10^5 cells/mL) obtained from the BALF of C3H/HeN or C3H/HeJ mice using the method reported previously [7] were seeded on 96-well culture plates and incubated for 1 h at 37°C and 5% CO_2 . Residual nonadherent cells ($<5\%$) were removed, and adherent BAL macrophages ($>95\%$ viability) were cultured in triplicate in RPMI 1640 supplemented with 10% (v/v) FBS, 20 mM L-glutamine, 10 mM sodium pyruvate, 1% (w/v) non-essential amino acids, 100 U/mL penicillin, 100 g/mL streptomycin, and 50 μM 2-ME. The macrophage cell lines were resuspended at 1×10^6 cells/mL in cultured medium and cultured in 1 mL in six-well plates (Falcon 3072; Becton Dickinson Labware, Franklin Lakes, NJ, USA). A range of different concentrations (0.1, 1.0, 10 $\mu\text{g/mL}$) of LPS, SP-D, or Der p extracts was then added to each well of macrophage cells. Supernatants from these wells were collected and assayed after 0, 12, 24, and 48 h incubation. Aliquots of culture medium from each well were assayed using the Griess reagent (1% (w/v) sulphanilamide, 0.1% (w/v) N-L-naphthylethylenediamine, and 5% (v/v) phosphoric acid) for NO production as described elsewhere [7].

Flow-cytometry analysis

Cultured macrophage cells or cell lines (1×10^6) were incubated with anti-CD16/CD32 mAb (Fc blocker, 2.4G2) followed by FITC-conjugated anti-mouse CD14 (16-10A1) and PE-conjugated anti-TLR4 (AMS-32.1) mAbs (all purchased from BD PharMingen, San Diego, CA, USA) for 30 min on ice. After three washes with PBS, cells were resuspended in 200 μL of PBS and analysed by flow cytometry using a FACScan flow cytometer (Becton Dickinson Immunocytometry, San Jose, CA, USA). For each sample, 15000 events were obtained. Isotype-matched mAb-stained cells were used as a background control in all experiments.

Enzyme-linked immunosorbent assay of cytokine levels

Concentrations of inflammatory cytokines, TNF- α and TGF- β in the supernatants of stimulated macrophages cell were determined using a commercial ELISA assay kit (R&D Diagnostics, MN, USA). The detection limits were 7.5 pg/mL for TNF- α and 15 pg/mL for TGF- β .

Western blotting of inducible nitric oxide synthase production in stimulated alveolar macrophages

AMs (1×10^6 cells) were homogenized in cold lysis buffer (50 mmol/L Tris-HCl, pH 7.5, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 0.1% SDS, 0.1% deoxycholate, 1% IGEPAL [(Octylphenoxy)polyethoxyethanol] CA-630, and a cocktail of protease inhibitors, all from Sigma-Aldrich). Total proteins were extracted by rotating the lysate for 1 h at 4°C . The

sample was then centrifuged for 5 min at 14000g, and the supernatant was harvested. Protein concentrations were determined using an assay kit (Bio-Rad, Hercules, CA, USA). For Western blot analysis, 10 µg of sample protein was separated on a 7.5% denaturing SDS polyacrylamide gel and blotted onto a 0.45 µm pore size polyvinylidene difluoride (PVDF) membrane (Immobilon P, Micropore, Millipore Co., MA, UK). After blocking overnight at 4 °C in block buffer (1% casein in PBS/0.1% Tween) and washing in phosphate-buffered saline (PBS)/0.1% Tween, membranes were incubated with primary monoclonal anti-iNOS antibody (N39120, BD Transduction Laboratories, San Diego, CA, USA) for 1 h. After washing, primary antibodies in the membranes were detected by horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) (BD Transduction Laboratories). Chemiluminescence was used to visualize bands (ECL, Amersham, Arlington Heights, IL, USA). Mouse anti-tubulin- α antibody (BD Transduction Laboratories) was used as the control.

Nuclear extract and electrophoretic mobility shift assay

Nuclear extracts were prepared from cultured AMs cell line (1×10^6) exposed to Der p or LPS for 24 h by the method described previously [7]. Gel shift experiments were performed using a commercial DIG Gel Shift Kit (Cat. No. 3353591, Roche Diagnostics, Mannheim, Germany), which applied the non-isotopic technique for detecting sequence-specific DNA-binding proteins. The labelled DNA fragment containing the sequence of consensus oligonucleotides of nuclear factor- κ B (NF- κ B) (5'-AGTTGAGGGGACTTCCAGGC-3'; Promega, Madison, WI) or AP-1 (5'-CGCTTGATGAGT-CAGCCGGAA-3') was end labelled with digoxigenin-11-ddUTP and incubated with the nuclear extracts to detect sequence-specific DNA-binding protein. The binding reaction consisted of 10 µg of nuclear extracts and 0.5 ng of labelled DNA probe. The mixture was transferred onto a native polyacrylamide gel and submitted to gel electrophoresis. Following the electrophoretic separation, the oligonucleotide-protein complexes were blotted by electroblotting onto BM nylon membranes (Micropore), charged positively. The digoxigenin-labelled probes were subsequently detected by an enzyme immunoassay using anti-digoxigenin-AP, Fab fragments, and the chemiluminescent substrate. The chemiluminescent signals were recorded by exposure to an X-ray film for 16–18 h. Competitive assays with unlabeled oligonucleotides (free probe; F) used 100-fold molar excess relative to the labelled NF- κ B or AP-1 oligonucleotides. The control reaction of this assay was optimized to detect nuclear factor Oct2A by 50% of the DIG-labelled, Oct2 A-binding sequence-specific, control oligonucleotide shifted, under the conditions described in the kit protocol (http://www.rocheappliedscience.com/cfm/proddata.jsp?article_hier=3.6.12.1.11.2).

Statistical analysis

Cytokine contents in AM culture supernatants and nitrite formation values were analysed by a one-way ANOVA test, followed by a *post hoc* comparison between groups. The cellular accumulation and mediator contents in BALF and serum Ab titre were analysed using an unpaired *t* test. The

results are expressed as means $\pm$ SEM. A *P*-value <0.05 was considered significant.

Results

Macrophage cell lines of different genetic backgrounds have different patterns of nitric oxide production when stimulated with lipopolysaccharides, Der p, or surfactant protein D

The mouse alveolar macrophage, MH-S (BALB/c mice), AMJ2-C11 (C57BL/6 mice), and peritoneal macrophage, RAW264.7 cell lines were treated with various concentrations of LPS or Der p extract, respectively, over a number of different incubation periods (Fig. 1). Upon LPS stimulation, there was a dose-dependent increase of NO formation that reached a maximal level after 24 h of stimulation in all three macrophage cell lines, despite their different genetic backgrounds and membrane receptor compositions. However, with Der p stimulation, only in the MH-S cell line was there a detectable, and dose-dependent, nitrite production. MH-S cells produced 30 µM of nitrite after stimulation with 1 µg/mL Der p for 12 h. Nitrite production reached maximum (59.5 µM) after stimulation with 10 µg/mL Der p for 48 h, as compared with 6.5 µM of nitrite produced by unstimulated macrophages ($P < 0.05$). In contrast, in AMJ2-C11 cells treated with the highest concentration of Der p extract (10 µg/mL), there was no detectable level of NO. The nitrite production in Der p-stimulated RAW264.7 cells revealed a pattern similar to that of AMJ2-C11 cells, except that there was some nitrite production (12.5 µM) after 48 h of stimulation with 10 µg/mL Der p extract. There was a similar pattern of nitrite production in the SP-D-stimulated macrophage cell lines, as was observed in the treatment with Der p extract (data not shown).

Previous studies have indicated that there is binding and phagocytosis of aeroallergen such as pollens [6] or mites [7] by AMs through some unidentified C-type lectin and integrin receptors located on the cell membranes of the macrophages. To determine the specific requirements for the mite-induced NO production, the MH-S cells were cultured in the presence of either EDTA, RGDS peptide (inhibit integrins bindings), mannosyl-, or galactosyl-BSA (to compete for lectin-binding receptors), along with either Der p or LPS, respectively (Table 1). We found that there was hardly any change in LPS or Der p-induced NO production with any of the above treatments, except for a reduction in the LPS-induced nitrite release in the presence of RGDS. The results of EDTA incubation are not included, because of the fact that a very small amount of 2 nM EDTA in the culture medium caused apoptosis of the AMs cell line ($>80\%$ for 30 min).

The production of nitric oxide by alveolar macrophages, after stimulation, was accompanied by up-regulation of cellular surface CD14 and toll-like receptor4 receptors.

Studies have shown that LPS-induced activation of macrophages leading to NO production is mainly mediated through the CD14 and TLR4 membrane receptor complex. To determine whether the different response of NO production, observed between different genetic backgrounds of stimulated AM cells, was because of the differential expression of CD14

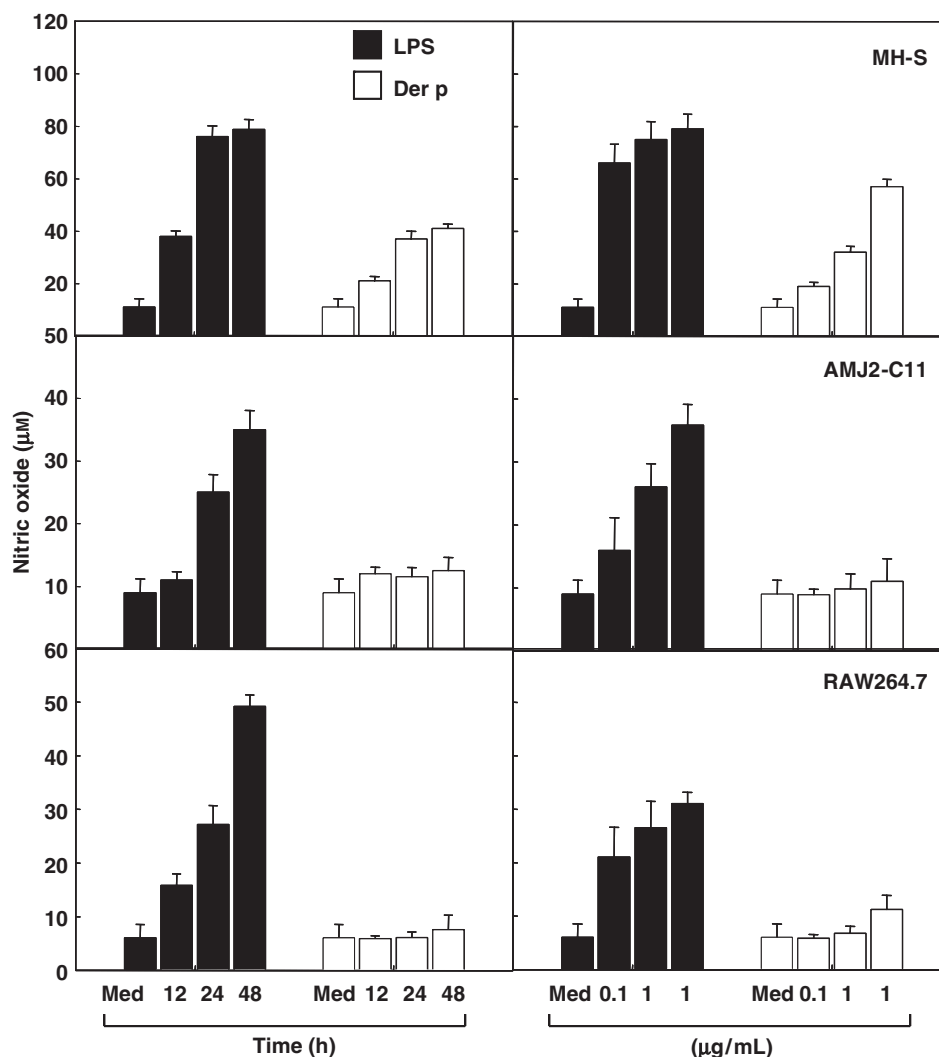


Fig. 1. Time- and dose-dependent nitrite production from lipopolysaccharides (LPS)- and Der p-stimulated macrophage cell lines. The macrophage cell lines, MH-S, AMJ2-C11, and RAW264.7, were re-suspended at 1×10^6 cells/mL in cultured medium in a six-well plate (Falcon 3072; Becton Dickinson). LPS and Der p extracts were then added to each well at different concentrations, 0.1, 1.0, and 10 µg/mL, for 48 h, respectively (right column). Supernatants from these wells were also collected and assayed after 0-, 12-, 24-, and 48-h incubation when cell lines were stimulated with 10 µg/mL of LPS, Der p, and SP-D, respectively (left column). All samples were analysed in triplicate. Each bar represents the mean nitrite levels of mean $\pm$ SE in the culture supernatant from three replicate wells.

Table 1. Inhibitory effect of NO production on LPS- or Der p-stimulated MH-S cell lines by different competitors

Inhibitor	Nitric oxide (μ M)
Medium	2.9 ± 1.45
LPS	62.4 ± 1.91
LPS+Mannosyl-BSA (25 µg/mL)	65.18 ± 3.18
LPS+Galactosyl-BSA (25 µg/mL)	61.77 ± 1.67
LPS+RGDS (25 µg/mL)	$40.03 \pm 0.84^*$
Der-p	41.26 ± 6.11
Der-p+Mannosyl-BSA (25 µg/mL)	39.75 ± 5.50
Der-p+Galactosyl-BSA (25 µg/mL)	38.97 ± 5.26
Der-p+RGDS (25 µg/mL)	38.93 ± 4.46

The MH-S cell line (1×10^6 cells/mL) was incubated with 1 µg/mL of LPS or Der p in the presence or absence of 25 µg/mL mannosyl-BSA, 25 µg/mL galactosyl-BSA, or 25 µg/mL of RGD peptides. After 24 h of incubation, NO production was determined using the Griess reagent as described in Materials and methods. The results were expressed as mean $\pm$ S.E. in the culture supernatant from three replicate wells.

NO, nitric oxide; LPS, lipopolysaccharides; BSA, bovine serum albumin; RGD, Arg-Gly-Asp-Ser.

and TLR4 membranous receptors, the MH-S, AMJ2-C11, and RAW264.7 cell lines were assayed for their spontaneous and inducible expression of CD14 and TLR4 on cell surface membranes (Fig. 2a). The MH-S cell line shows a high expression of these two surface molecules. In contrast, AMJ2-C11 and RAW263.4 cell lines showed relatively low amounts of these two surface molecules. Figure 2b shows the flow cytometry assays for the mean fluorescence intensity (MFI) of CD14 and TLR4 on the surface of LPS- and Der p-stimulated MH-S, and AMJ2-C11 cell lines after 24 h incubation. There was a high expression of both CD14 and TLR4 cell surface molecules in the MH-S cell line, when stimulated with different concentrations of LPS and Der p, respectively. In LPS-stimulated AMJ2-C11 cells, there was a significant, dose-dependent, increase in both CD14- and TLR4-positive macrophages, after 24 h exposure to LPS. In contrast, Der p-stimulated AMJ2-C11 cells did not show an increase of CD14- or TLR4-positive macrophages 24 h post-exposure to increasing concentrations of Der p. This discrepancy in CD14

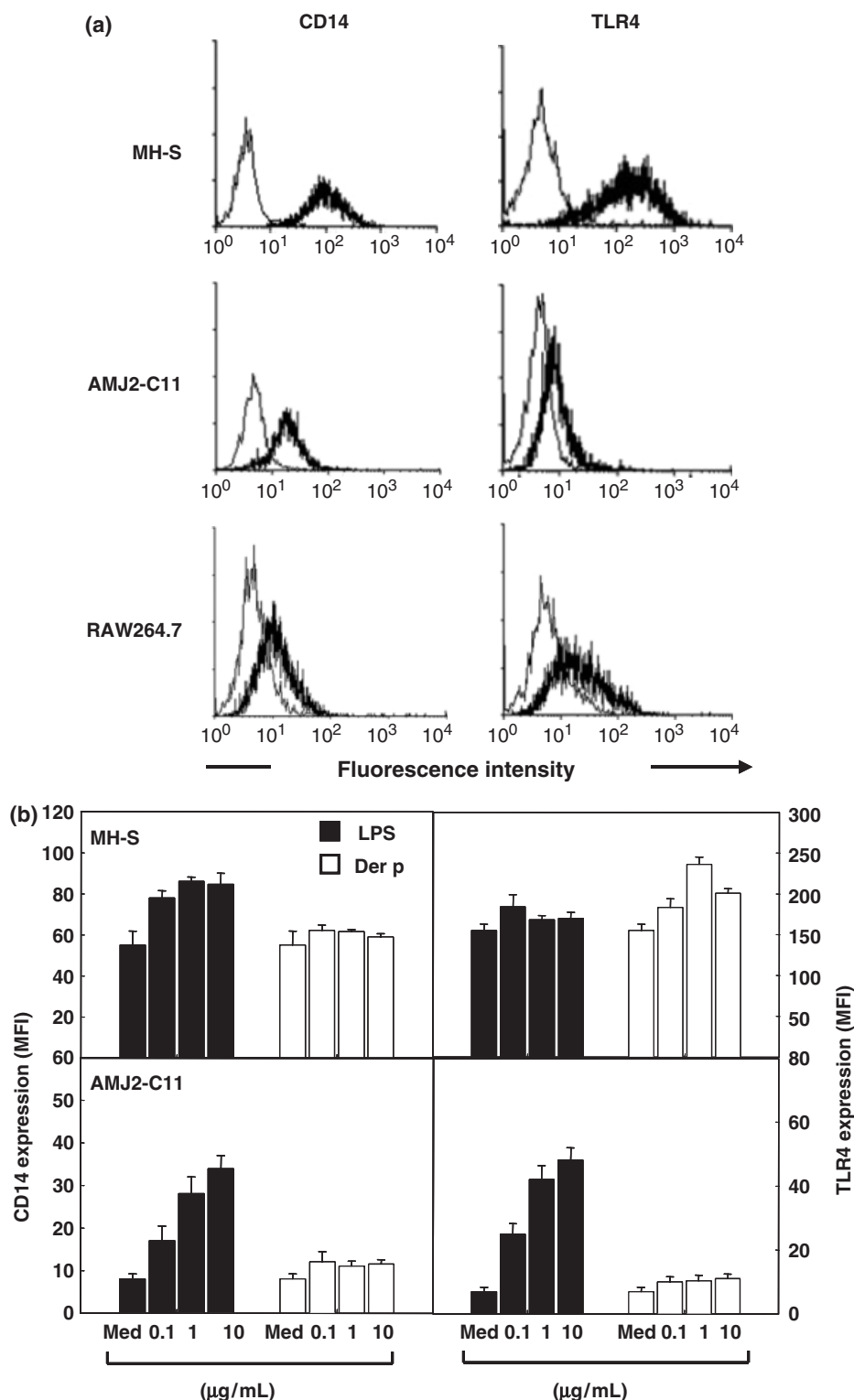


Fig. 2. (a) MH-S, AMJ2-C11, and RAW 264.7 cells were stained with a 1 : 4 dilution of either FITC rat anti-mouse CD14, or PE rat anti-mouse toll-like receptor 4 (TLR4), and analysed by FACScan as described in Materials and methods. The MH-S cell line showed a high expression of these two surface molecules, as shown by the thick lines after labelled monoclonal antibodies were added. In contrast, AMJ2-C11 and RAW 264.7 showed relatively low amounts of these two surface molecules. (b) The flow cytometry assays for the mean fluorescence intensity (MFI) of CD14 and TLR4 on the surface of lipopolysaccharides (LPS), and Der p- stimulated MH-S and AMJ2-C11 cell lines, 24 h after stimulation. 5×10^5 macrophages cells from MH-S and AMJ2-C11 cell lines were stimulated by different concentrations of LPS, and Der p, respectively, for 24 h, and then stained with a 1 : 4 dilution of either FITC rat anti-mouse CD14, or PE rat anti-mouse TLR4, and analysed by FACScan as described in Materials and methods. All samples were analysed in triplicate. Each bar represents the mean nitrite levels of mean $\pm$ S.E. in the culture supernatant from three replicate wells.

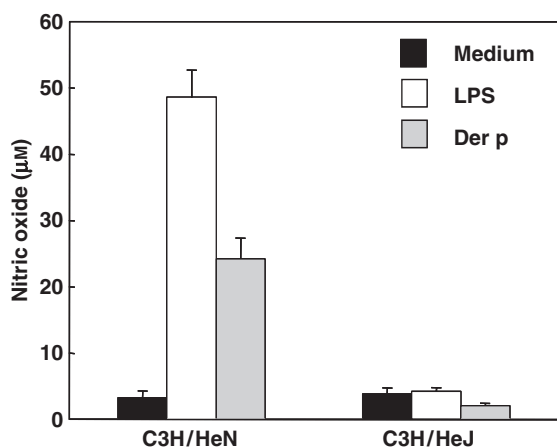


Fig. 3. Production of nitric oxide (NO) in lipopolysaccharides (LPS), and Der p-stimulated alveolar macrophages isolated from bronchoalveolar lavage (BAL) fluids of C3H/HeN and C3H/HeJ mice. BAL cells (2×10^5 /mL), from C3H/HeN and C3H/HeJ mice, were seeded on 96-well culture plates and incubated for 1 h at 37 °C and 5% CO₂. Residual non-adherent cells (<5%) were removed, and adherent BAL macrophages (>95% viability) were cultured in triplicate in culture medium as described in Materials and methods. The cultured alveolar macrophages were treated with 10 µg/mL of LPS and Der p, respectively, for 48 h. Aliquots of culture medium from each well were assayed using the Griess reagent for NO production as described. All samples were analysed in triplicate. Each bar represents the mean nitrite levels of mean $\pm$ SE in the culture supernatants from three replicate wells.

and TLR4 expressions between LPS- and Der p-stimulated AMJ2-C11 cell lines correlated with the results of detectable NO production (>15 µM) as assayed in the culture supernatants under the same treatment conditions (Fig. 1). These findings suggested that the production of NO from Der p- or LPS-stimulated alveolar macrophage cells, as in the case of AMJ2-C11 cell lines, required a certain amount of up-regulation of CD14/TLR4 surface receptors.

In another experiment, AMs isolated from BAL of C3H/HeN and C3H/HeJ mice were stimulated either with LPS or Der p (Fig. 3). AMs from LPS-hyporesponsive C3H/HeJ mice, which have mutations in the *tlr4* gene, showed no nitrite formation on treatment for 48 h with the maximal concentration of LPS as well as Der p. Only AMs from C3H/HeN mice, stimulated by 10 µg/mL of Der p, produced significant amounts of NO as compared with non-detectable NO production in AMs from C3H/HeJ mice.

Production of inducible nitric oxide synthase in alveolar macrophages cell lines

The expression of iNOS is regulated by cytokines and determined primarily by the *de novo* synthesis and stability of iNOS mRNA and protein. To ensure that NO production in LPS- and/or Der p-stimulated AMs was through iNOS expression, cellular extracts from stimulated macrophage cell lines were examined for iNOS protein production by Western immunoblotting. Figure 4 illustrates the results of iNOS production from LPS- or Der p-treated MH-S and AMJ2-C11 cell lines. The iNOS protein was detectable and showed dose and time dependency of production in the MH-S and AMJ2-C11 cell lines at 4–12 h, or 24 h after LPS treatment. In contrast, although iNOS was detected in the Der p-treated

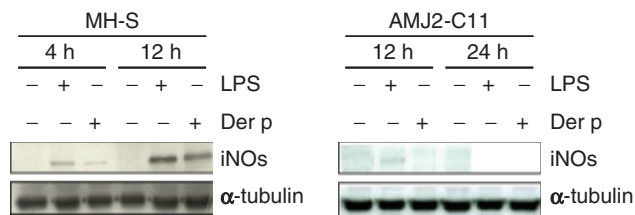


Fig. 4. Western blot analysis of inducible nitric oxide synthases (iNOS) production from stimulated alveolar macrophages cell lines. Cellular extracts from lipopolysaccharides, and Der p-stimulated MH-S and AMJ2-C11 cell lines (1×10^6 cells/mL) for different time periods were evaluated for iNOS protein synthesis, respectively, by Western blot analysis, and α-tubulin was included as the control. A representative blotting, from three independent experiments, for each of stimulated MH-S and AMJ2-C11 cell lines, is shown.

MH-S cells at 4–12 h, there was no iNOS synthesis in the Der p-treated AMJ2-C11 cells.

The production of nitric oxide from alveolar macrophages was through nuclear factor-κB and/or AP-1 pathways

Activation of the iNOS gene promoter is an important mode of iNOS regulation as well as NO production. LPS-induced signal transduction is thought to entail binding to specific cellular receptors, which trigger intracellular signalling cascades leading to the activation of the transcription factors such as NF-κB and AP-1. Nuclear extracts were obtained from LPS- and Der p-stimulated macrophage cell lines. Both NF-κB (Fig. 5a, upper panels) and AP-1 (Fig. 5a, lower panels) activations were assessed by EMSA. Both LPS and Der p were able to activate these two transcription factors in MH-S cells at 30 min after stimulation. When selective inhibitors, PDTC for NF-κB, and curcumin for AP-1, at various concentrations (5, 10, and 50 µM) were pre-incubated with the MH-S cell line, respectively, for 24 h, they could, dose dependently, inhibit subsequent Der p (10 µg/mL)-induced NO production (Fig. 5b). More than 80% inhibition of Der p-stimulated NO production was obtained on pre-incubation with 50 µM of PDTC or 50 µM of curcumin, as compared with non-treated macrophages.

The pattern of inflammatory cytokines, tumour necrosis factor-α and transforming growth factor-β, production is different from nitric oxide production in AMJ2-C11 cell lines

Figure 6 shows the results of the production of TNF-α and TGF-β, from stimulated alveolar macrophage cell lines. There were similar patterns of dose- and time-dependent production of TNF-α from MH-S, as well as AMJ2-C11 cell lines, when stimulated with LPS and Der p, respectively. The productions of TNF-α and TGF-β in MH-S cell line were detectable within 6 h and reached a plateau within 24 and 48 h, when stimulated with 1 µg/mL LPS or 10 µg/mL of Der p (data not shown). However, the production of TGF-β in the stimulated AMJ2-C11 cells was shown to be very different from that in MH-S cells. While there was a dose- and time-dependent TGF-β production in AMJ2-C11 cells starting from 0.1 to 10 µg/mL of LPS on 6–48 h stimulation, Der p could only stimulate TGF-β production from MH-S cells, but

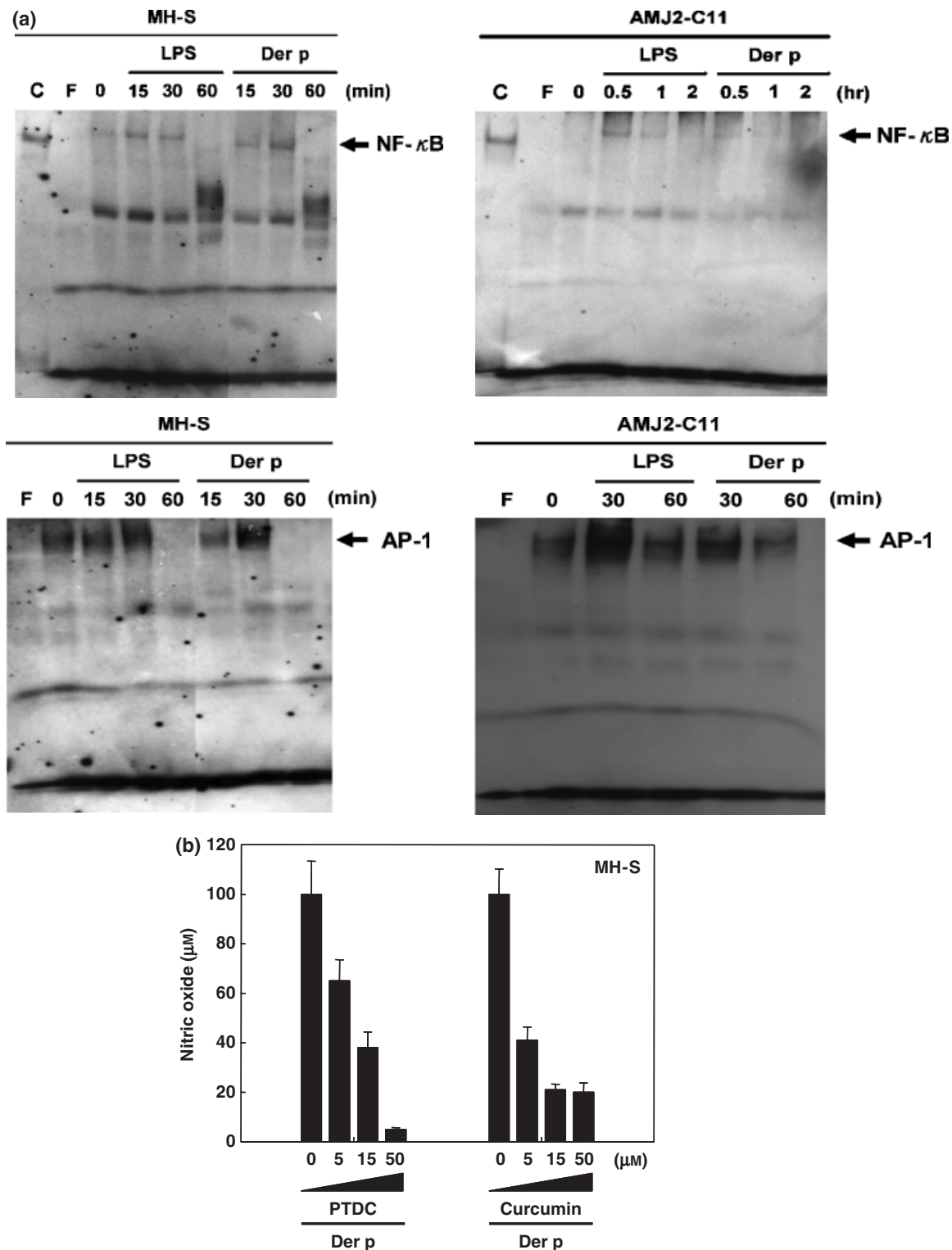


Fig. 5. (a) Nuclear proteins were extracted from MH-S (left) and AMJ2-C11 (right) cell lines after incubation with 10 $\mu\text{g}/\text{mL}$ of lipopolysaccharides and Der p, respectively, at different time periods. The activation of nuclear factor- κB (NF- κB) (upper panel) and AP-1 (lower panel) was evaluated by the DIG Gel shift assay kit as described in the Materials and methods. The gel shift experiment was performed using labelled DNA probes containing a consensus sequence of NF- κB or AP-1, respectively. Unlabelled oligonucleotide (F)-containing binding site for NF- κB or AP-1 was used for competition. The control reaction (C) of this assay was optimized to detect nuclear factor Oct2A by 50% of the control oligonucleotide shifted, which contained the binding site for Oct2A, under the conditions provided in the Kit protocol. Figures shown are representative of three independent experiments. (b) MH-S cell line (1×10^6 cells/mL) incubated with 10 $\mu\text{g}/\text{mL}$ Der p alone or with different concentrations of NF- κB inhibitor, PTDC, and AP-1 promoter inhibitor, curcumin, respectively. After 24 h of incubation, aliquots of culture medium from each well were assayed using the Griess reagent for NO production as described in the Materials and methods. All samples were analysed in triplicate. Each bar represents the mean nitrite levels as the mean $\pm$ SE in the culture supernatant, from three replicate wells.

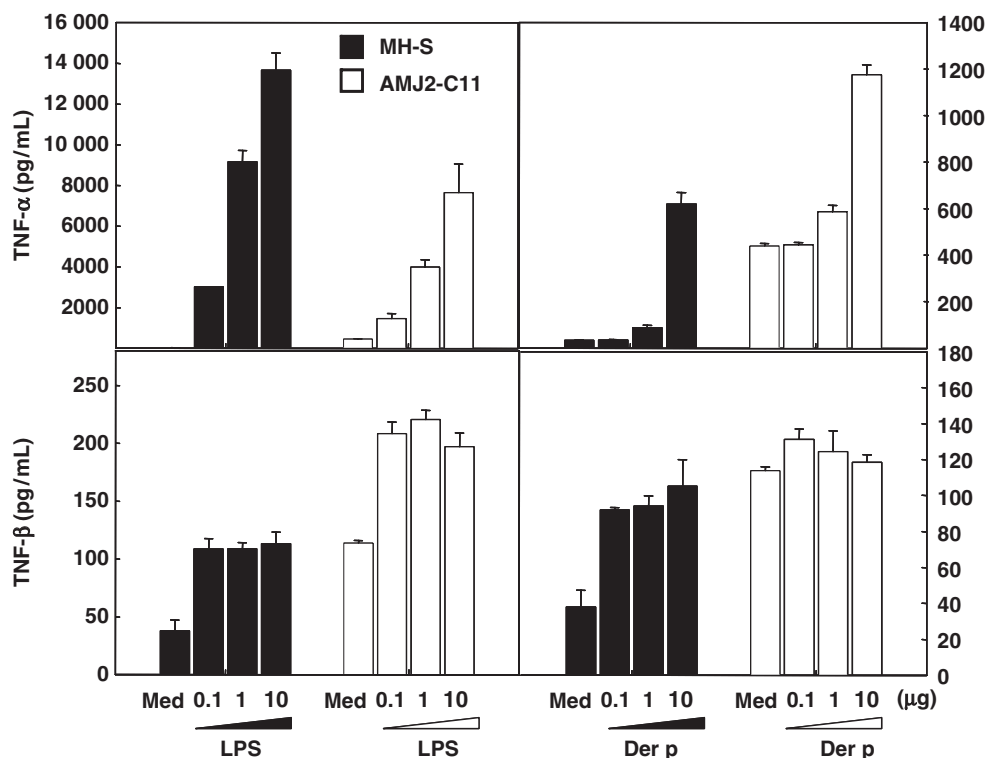


Fig. 6. Production of inflammatory cytokines from lipopolysaccharides (LPS), and Der p-stimulated alveolar macrophages cell lines. MH-S and AMJ2-C11 cell lines (1×10^6 cells/mL) were stimulated for 24 h with various doses of LPS, or Der p. The accumulation of pro-inflammatory mediators of TNF- α and TGF- β in the supernatants was evaluated using ELISA, as described in the Materials and methods. The results shown are the means $\pm$ SEM of three to six separate experiments performed in triplicate.

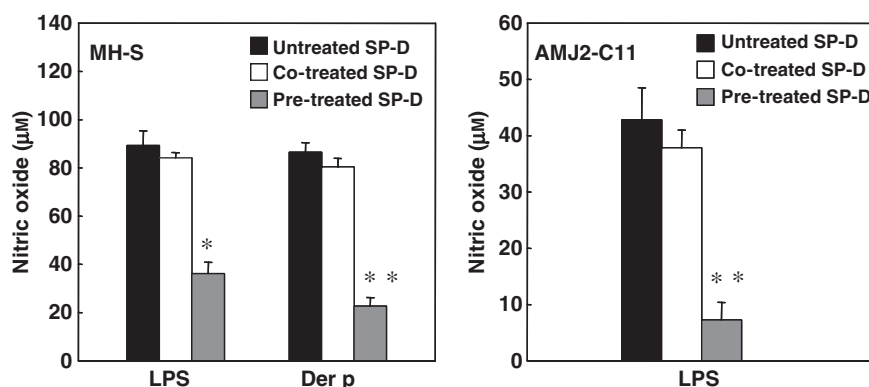


Fig. 7. Inhibitory effect of nitric oxide (NO) production on lipopolysaccharides (LPS)-, or Der p-stimulated alveolar macrophages cells by SP-D. MH-S and AMJ2-C11 cell lines (1×10^6 cells/mL) were stimulated with $10 \mu\text{g/mL}$ of Der p or LPS for 24 h (untreated); or stimulated with $10 \mu\text{g/mL}$ of Der p, or LPS, combined with $10 \mu\text{g/mL}$ of SP-D (co-treated) for 24 h; or first incubated with SP-D ($10 \mu\text{g/mL}$) for 24 h, and then stimulated with $10 \mu\text{g/mL}$ of Der p, or LPS for another 24 h (pre-treated). At the end of incubation, aliquots of culture medium from each well were assayed using the Griess reagent for NO production as described in Materials and methods. All samples were analysed in triplicate. Each bar represents the nitrite levels expressed as mean $\pm$ SE in the culture supernatant, from three replicate wells.

not from AMJ2-C11 cells even after the maximal dosage ($10 \mu\text{g/mL}$) stimulation for 48 h (Fig. 6).

Pre-treatment with surfactant-associated protein D inhibits lipopolysaccharides- and Der p-induced nitric oxide production in MH-S and AMJ2-C11 cell lines

Although SP-D in itself can induce NO production in AMs, the potential of SP-D to inhibit production of NO in LPS- or Der p-stimulated macrophage cell lines is shown in Fig. 7.

Pre-treatment of the MH-S cells with various concentrations (0.1, 1.0, and $10 \mu\text{g/mL}$) of SP-D 24 h before stimulation with LPS or Der p (pre-treated) led to a dose-dependent reduction of NO production. The inhibitory effect of NO synthesis was detected starting from the pre-treatment of $0.1 \mu\text{g/mL}$ of SP-D (1 : 10 of LPS or Der p), and reached maximal inhibition when $10 \mu\text{g/mL}$ of SP-D was added as shown in Fig. 7. A similar effect of SP-D inhibition on NO production was observed on the LPS-stimulated AMJ2-C11 cell lines. When SP-D was added simultaneously (co-treated) with LPS or Der

p to the MH-S cell culture or with LPS to the AMJ-C11 cells, this inhibition effect on NO production was not observed.

Discussion

In this study, we have demonstrated that the extract of house dust mite, Der p, was able to induce NO production in AM cell lines in a dose-dependent and time-dependent manner. However, this effect of NO production depended upon the genetic background of the cell lines used. While LPS significantly stimulated NO production in the MH-S (expressing a high amount of surface CD14/TLR4), AMJ-2 (expressing a low amount of surface CD14/TLR4), and RAW 264.7 (which expresses only TLR4) cell lines, it was found that Der p could only stimulate NO production in the MH-S, but not in AMJ-2 or RAW 264.7 cell lines. The CD14/TLR4 surface complex-dependent NO production stimulated by Der p was also confirmed in LPS non-responsive AMs purified from C3H/HeJ mice, which had both mutations in the CD14 and *tlr4* genes. Der p and SP-D could induce NO production in AMs from C3H/HeN, but not from C3H/HeJ mice.

Although innate immunity in the lung is recognized to play important roles in allergic development [23], there are only a few reports regarding the detailed molecular interaction between allergens and AMs. Currie et al. [6] found that pollen starch granules (PSG), which are aeroallergens in hayfever, were rapidly bound and phagocytosed by AMs. This phagocytosis of PSG was dependent on Mg^{2+} and Ca^{2+} and was inhibited by neo-glycoproteins such as galactose BSA and *N*-acetylgalactose BSA, and there was partial inhibition with RGDS peptides. A role was suggested for both C-type lectins and β_2 integrins in the binding and internalization of PSG, but the exact receptor(s) on the surface of AMs, which may have mediated recognition of inhaled allergenic particles, were not identified. Also, it has been reported by Peake et al. [24] that house dust mite fecal materials (HDMFP, containing Der p 1 and Der p 2) stimulated the production of NO from rat AM cell lines, and this HDMFP-mediated NO release was down-modulated after treatment with the NO inhibitor, *N*(G)-nitro-L-arginine methyl ester (L-NAME), dexamethasone, EDTA, or cysteine, but not by heat treatment of the allergens. Although we have found the same type of inhibition with respect to Der f-induced NO production in the purified AMs from BALB/c mice [7], the inducible NO production in Der p stimulated in AM cell lines, in this study, was not inhibited by maltosyl-, or galactosyl-BSA and RGDS peptides, which suggested that this effect of NO production did not involve C-type lectin or integrin receptors on the AMs cell lines. The mechanism of this discrepancy is not yet clear, but may be because of different characteristics of the freshly isolated cells and transformed AMs cell lines that we used.

Previously, we have reported that HDM, Der f, is a potent stimulator of AMs isolated from sensitized as well as from naïve mice. HDM-stimulated NO production via iNOS expression, activated NF- κ B, mobilized a variety of proinflammatory cytokines, and up-regulated B7 expression [7]. In this study, we have noted the same effects on NO production, i.e. increased iNOS expression and NF- κ B and AP-1 activation, stimulated by Der p in the alveolar macrophage

cell lines. However, an unexpected finding is that this activation pathway of NO production was correlated with the amounts of CD14/TLR4 molecules expressed on the targeted cell surfaces, as seen from the results of dose-dependent increase of CD14 and TLR4 signals in LPS-stimulated AMJ2-C11 cells, while the Der p treatment did not raise the level of synthesis of these two molecules, to the required threshold concentration for NO production (Fig. 2). To our knowledge, there is no report regarding the interaction of CD14 with HDM allergens, although from a clinical point of view, it has been suggested that the level of soluble CD14 in serum is related to allergic diseases and asthma, and single-nucleotide polymorphisms (SNPs) sites on the promoter regions of CD14 gene have been reported to have an association with asthma susceptibility [25, 26]. Although the direct evidence of allergens binding to CD14 is lacking, our study does reveal the significance of CD14 in the allergen-induced AM activation, such as NO formation and inflammatory cytokine production.

In the present study, we found that TNF- α and TGF- β , the two macrophage-related inflammatory cytokines were produced from LPS- or Der p-stimulated MH-S cell line. However, Der p-induced TNF- α , but not TGF- β synthesis in stimulated AMJ2-C11 cells. These results indicated that the synthesis of Der p-induced TNF- α and nitrite formation, although it is through the NF- κ B activation pathway, might use different activation surface molecules, i.e. CD14/TLR4 receptor independent, to synthesize TNF- α as in the AMJ2-C11 cell line. Therefore, the mechanisms behind these different pathways between TNF- α and NO synthesis require further investigation.

Previously, we have demonstrated that SP-A and SP-D could bind to mite (*D. pteronyssinus*, Der p) allergens in a specific manner [11], and could also inhibit the binding of allergen-specific IgE to these allergens, through competition or steric hindrance effects [13]. Moreover, both of these surfactant proteins were found to inhibit histamine release and allergen-induced lymphocyte proliferation, two essential steps in the pathogenesis of asthma [13]. It is suggested that surfactant proteins may be involved in allergen sensitization, or may participate in the allergic inflammatory processes that are frequently seen in asthmatic patients. In the present study, we found that pre-treatment of SP-D induced a significant inhibition of NO production in Der p- or LPS-stimulated MH-S cells as well in LPS-stimulated AMJ2-C11 cells. This inhibition was lost when SP-D was added simultaneously with LPS, or Der p, to the cultured cells. Previously, Sano et al. [27, 28] found that SP-A and D, although through different binding moieties, could bind CD14 and modulate the interaction between LPS and CD14. Murakami et al. [29] further reported that SP-A binds to both CD14 and TLR2 and that this interaction likely contributes to the ability of SP-A to affect LPS- and peptidoglycan-mediated cell responses, respectively. Although the detailed mechanisms of how SP-D exerts an inhibitory effect on NO production by AMs are not clear, we speculated that SP-D might first block the CD14/TLR4 receptors on the cell surface and thus prevent further activation stimulated by LPS, or Der p. Alternatively, as suggested by Gardai et al. [30], it might have been because of some unidentified inhibitory mechanisms exerted by SP-D on AMs, that resulted in the cells becoming unresponsive to LPS

or Der p. However, our results do demonstrate the important role of SP-D in the immunomodulation of Ams, and suggest that SP-D may prevent the development of inflammatory events seen in asthma or infectious lung diseases.

In summary, the data presented in this report demonstrate that mite allergen-induced alveolar macrophage activation is mediated by CD14/TLR4 receptors and can be inhibited by SP-D; it further supports the concept that SP-D may be an important modulator of allergen-induced pulmonary inflammation.

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