

Surfactant protein D deficiency influences allergic immune responses

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Summary

Background The collectin surfactant protein D (SP-D) confers protection against pulmonary infection and inflammation. Recent data suggest a role for SP-D in the modulation of allergic inflammation.

Objective The aim of this study is to characterize the immune responses of SP-D-deficient (SP-D^{-/-}) mice in a kinetic model of allergic inflammation. We determined whether allergic parameters were enhanced in SP-D^{-/-} mice *in vivo*. Further, we examined whether functional immune responses *in vitro* such as lymphocyte proliferation (LP) and cytokine production were modulated in the absence of SP-D.

Methods *In vivo*, wild-type (WT) and SP-D^{-/-} mice were sensitized and challenged with the allergen ovalbumin (OVA) and assessed for allergic parameters (bronchoalveolar lavage (BAL) eosinophils, IL-13 production, pulmonary IFN- γ , IL-10 expression) at early time points (1 and 3 days of challenge) in comparison with late time points (7 days of challenge). *In vitro*, spleen cells from WT and SP-D^{-/-} mice were stimulated with the mitogen concanavalin A (ConA) and lipid A (LpA) and analysed for LP, IL-13 and IFN- γ production. Toll-like receptor 4 (TLR4), ligand for LpA, was assessed by mRNA expression and immunohistochemistry *in vivo*.

Results Following allergen exposure *in vivo*, SP-D^{-/-} mice expressed higher BAL eosinophils and IL-13 concentrations and lower IFN- γ expression at early time points compared with WT mice. IL-10 expression was increased at early time points in SP-D^{-/-} compared with WT mice. Allergen-induced TLR4 expression was increased in WT, but not in SP-D^{-/-} mice. After stimulation with LpA and ConA *in vitro* LP was increased and IFN- γ concentration was decreased in SP-D^{-/-} mice.

Conclusion SP-D may be critical for the modulation of early stages of allergic inflammation *in vivo*.

Keywords allergy, cytokines, inflammation, lung, SP-D, TLR4

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Introduction

The collectin family, which includes surfactant protein D (SP-D) and A (SP-A) as well as mannose-binding proteins, exerts important protective functions in host defence [1–8]. As a member of the collagenous C-type lectin family, SP-D is primarily produced in the lung by alveolar type II cells and by non-ciliated Clara cells, a subset of bronchiolar epithelial cells [9]. Pulmonary collectins may contribute to protection against local pulmonary inflammation including cytokine production [1, 10, 11] elicited by Gram-positive and Gram-negative bacteria [3, 4, 12–18]. More recently, it has been proposed that SP-D plays a role in allergic inflammation by immune modulation via communication between B and T lympho-

cytes [19–21]. SP-D deficiency (SP-D^{-/-}) results in persistent T cell activation in a murine model [21]. *In vitro* human studies reveal that SP-D suppresses allergen-induced lymphocyte proliferation (LP) and IL-2-dependent T cell proliferation [19]. Further, SP-D inhibits allergen-induced histamine release and proliferation of peripheral blood mononuclear cells from asthmatic and non-asthmatic children [22]. Increased SP-D is present in the bronchoalveolar lavage (BAL) fluid of asthmatic adults [23] and we and others have previously demonstrated increased SP-D expression in a murine allergic model [24, 25]. Application of endogenous SP-D can further suppress allergic airway inflammation [26].

To date, no *in vivo* study has examined SP-D^{-/-} mice for the kinetics of allergic inflammation. SP-D^{-/-} mice are described to gradually develop an altered phenotype with impaired alveolar macrophages [27, 28] and alveolar lipoproteinosis [29, 30], increasing up to 7 months of age, when lungs exhibit pulmonary fibrosis and emphysema [29, 30]. We chose to examine 6-week-old SP-D^{-/-} mice, where few baseline

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secondary changes are seen. As an example, SP-D^{-/-} mice in this study exhibited low-grade chronic inflammation characterized by macrophage influx.

Based on work suggesting that SP-D decreases LP [19, 21, 22], we postulated that allergic responses *in vivo* may be increased in SP-D deficiency in an *in vivo* murine model of allergic inflammation. Also, previous data indicate a dependence of SP-A on the innate TLR4 in signalling pathways and cytokine secretion [20]. SP-D and SP-A both have similar effects on T-helper (Th) cell function in allergic immune responses [22, 31]; thus we posited a potential association of SP-D and TLR4, both mediators in the innate immune system. To further characterize the *in vitro* response of SP-D^{-/-} mice, we analysed LP and cytokine responses of spleen cells of wild-type (WT) and SP-D^{-/-} mice *in vitro* following stimulation with the TLR4 agonist lipid A (LpA) as one component of Gram-positive bacteria, and the mitogen concanavalin A (ConA). SP-D deficiency increased parameters of allergic inflammation at early stages in our *in vivo* allergen model and enhanced LP to LpA and ConA *in vitro*.

Materials and methods

Mice

Six-week-old inbred C57BL/6 female and male mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). C57BL/6 mice deficient in SP-D^{-/-} were generated as previously described and backcrossed 10 generations into a C57BL/6 background [29]. The mice were maintained according to the guidelines of the Committee on Animals of the Harvard Medical School and the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources National Research Council.

In vivo study: allergen sensitization and challenge

In this model of allergic inflammation, mice were sensitized with the allergen ovalbumin (OVA) as previously described [24, 32, 33]. In brief, WT and SP-D^{-/-} mice were sensitized by intraperitoneal (i.p.) injection of 10 µg chicken OVA and 1 mg Al(OH)₃ on days 0 and 7 (WT OVA, SP-D^{-/-} OVA). Control mice received i.p. injection of phosphate-buffered saline (PBS) and 1 mg Al(OH)₃ (WT PBS, SP-D^{-/-} PBS). After sensitization, mice were assigned to one of three protocols (Fig. 1). The 15-day protocol comprises challenge with aerosolized 6% OVA dissolved in 0.5 × PBS through an ultrasonic nebulizer (Model 5000, DeVilbiss, Somerset, PA, USA) for 20 min on day 14 and killing 24 h after challenge (day 15). In the 17-day protocol, mice received aerosolized challenge for 3 days (days 14–16) and were killed 24 h after the last challenge (day 17). In the 21-day protocol, mice were challenged with aerosolized OVA for 7 days (days 14–20) and were killed 24 h after the last challenge (day 21). The control groups followed the identical protocols, but were sensitized and challenged with PBS instead of OVA. Two experimental and two control groups were analysed at all three time points. Naïve control mice received no treatment and were kept in the same environment in the laboratory for 21 days (*n* = 4–6 mice/group).

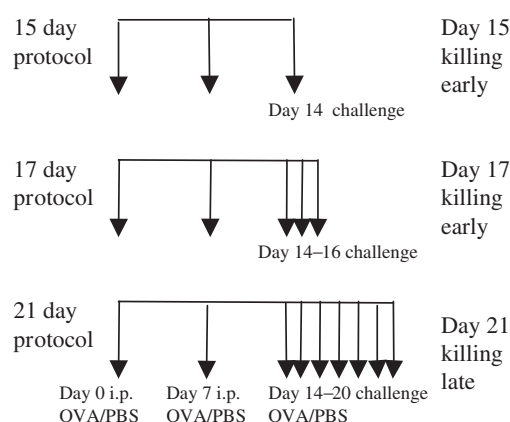


Fig. 1. Murine model of allergic inflammation. In this model of allergic inflammation, wild-type (WT) and surfactant protein D deficient (SP-D^{-/-}) mice received intraperitoneal (i.p.) allergic sensitization on days 0 and 7 or phosphate-buffered saline (PBS) in control mice. The 15-day protocol comprises allergen ovalbumin (OVA) challenge (compared with PBS) on day 14 and killing on day 15. In the 17-day protocol, mice were challenged for 3 days (days 14–16) and killed on day 17. In the 21-day protocol mice were challenged for 7 days (days 14–20) and killed on day 21. Two OVA (WT and SP-D^{-/-}) and two PBS groups were analysed on the 3 time points (*n* = 4–6 mice/group).

Bronchoalveolar lavage, cytokine concentrations and histological analysis

Mice were anaesthetized with ketamine/xylazine i.p. and killed by cardiac puncture 24 h after challenge (days 15, 17, 21). Each mouse underwent BAL, as previously described [24, 32, 34]. BAL cells were pelleted, and following centrifugation the supernatant of BAL cells was stored at -80 °C. Cells were resuspended in RPMI (5 × 10⁵ cells/mL). Slides for differential cell counting were prepared with Cytospin (Shandon Inc., Pittsburgh, PA, USA), fixed and stained with Diff-Quik (Dade Behring, Newark, DE, USA). For each sample, an investigator blinded to the treatment groups performed two counts of 400 cells. Cytokine concentrations were measured with ELISA according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA) and were determined by comparison with known standards. Lower limit of detection for IL-13 was 1.5 pg/mL (*n* = 4–6 mice/group).

For histological analysis, tissue samples from the left lung were removed from the thoracic cavity and snap-frozen in liquid nitrogen; additional lung tissue samples were fixed in 4% paraformaldehyde and routinely processed into paraffin blocks. Frozen sections, 5 µm thick, were cut and the tissues were screened with haematoxylin and eosin to verify the presence of at least three bronchioles per section. For immunostaining, 5 µm thick paraffin-embedded samples were cut. The primary antibodies included polyclonal goat anti-Toll-like receptor 4 (TLR4) (sc-12511, Santa Cruz Biotechnology, Santa Cruz, CA, USA) used at 1:750 dilutions. The identity of cell types staining for TLR4 was assessed by staining serial sections with well-characterized cell markers, such as Mac-3 (purified rat anti-mouse mac-3 mAb, 1:30 dilution, clone M3/84, BD Pharmingen, San Diego, CA, USA) for macrophage staining. Negative controls to verify specificity of immunostaining consisted of pre-absorbing the primary antibody against a 10-fold excess of specific peptide (TLR4) (sc-12511-p, Santa Cruz) and substituting an irrelevant IgG, non-immune goat-IgG (Sigma Aldrich, St Louis, MO, USA),

used at the same concentration as the anti-TLR4 antibody, for the primary antibody. Slides were stained with an avidin–biotin technique with tyramide enhancement [24]. An experienced reader (K. J. H) examined the slides in a blinded fashion using a semi-quantitative (0–4+) method to assess the abundance of immunostaining ($n = 4–6$ mice/group).

Serum IgE

Blood was withdrawn by cardiac puncture and centrifuged at $18\,890 \times g$ at room temperature for 20 min to recover serum. Total serum IgE levels were determined by ELISA as previously described [34]. Total serum IgE concentrations were calculated using a standard curve generated with a commercial IgE standard (BD Pharmingen).

Real-time quantitative polymerase chain reaction

Total RNA was isolated from right lung tissue with TRI Reagent (Sigma-Aldrich). Isolated RNA was reverse transcribed with SuperScript II RNase reverse transcriptase (Life Technologies, Carlsbad, CA, USA). Specific primer pairs for GAPDH (as housekeeping gene: M32599), SP-D, IFN- γ , IL-10 and TLR4 were designed with the Primer Express software (Applied Biosystems, Foster City, CA, USA). The sequences of the forward (FW) and reverse (RE) primer pairs used in the experiments were as follows: SP-D: AGCAGAAATGAAGAGCCTCTCG (FW), AGGGTGCAGGTGTTGGGTAC (RE), IFN- γ : AGGTGTCACAACTGCTGCCA (FW), ACACCCGAATGAGCTGCTCT (RE), IL-10: CCAGTACAGCCGGAAGACA (FW), CTCTGGCCGACTGGGAAGT (RE), TLR4: CCTGGCTGGTTACACGTC (FW), GACATTGCAGAAACATTCGC (RE). Direct detection of the PCR product was monitored by measuring the increase in fluorescence caused by the binding of SYBR Green to dsDNA. Using 5 μ L of cDNA, 5 μ L of primer, and 10 μ L of SYBR Green Master Mix (Applied Biosystems) per well, the gene-specific PCR products were measured continuously by means of GeneAmp 5700 Sequence Detection System (Applied Biosystems) during 40 cycles. All experiments were run in duplicate, and the same thermal cycling parameters were used. Non-template controls and dissociation curves were used to detect primer–dimer conformation and non-specific amplification. The threshold cycle (C_T) of each target product was determined and set in relation to the amplification plot of GAPDH. The C_T is the number of PCR cycles required for the fluorescence signal to exceed the detection threshold value. The detection threshold was set to the log linear range of the amplification curve and kept constant (0.3) for all data analysis. The difference in C_T values of two genes was used to calculate the fold change. The level of mRNA of the individual gene is described as gene expression. The relative quantitation results were used to determine changes in gene expression in WT and SP-D $^{-/-}$ mice compared with naïve mice [35, 36].

In vitro study: lymphocyte proliferation and cytokine concentrations

Murine spleen cells were isolated [37], washed with RPMI-1640 and suspended in RPMI-1640 with 10% fetal calf serum and antibiotics. For the LP assay, 0.5×10^6 cells/well were cultured in quadruplicates in 96-well round-bottom tissue-

culture plates (Corning, NY, USA) for 48 h, and stimulated with the TLR4 agonist LpA (Salmonella Minnesota Re-595, R mutants, Sigma-Aldrich), the mitogen ConA or kept unstimulated. Cells were pulsed with 1 μ Ci ^{3} H-Thymidine for an additional 8 h. Cultures were performed at 37 °C in a humidified 5% CO $_2$ incubation chamber. Cells were harvested with a Tomcat Mach II harvester (Wallac, Turku, Finland) onto filter plates, which were read using a β -Counter. Proliferation was quantified by stimulation index (SI), which is calculated as the ratio of mean counts per minute (c.p.m.) of stimulated over unstimulated replicates. For analysis of cytokine production *in vitro*, cell culture supernatants were harvested following 48 h of stimulation with LpA, ConA or without stimulation. Cytokine concentrations were measured with ELISA according to the manufacturer's instructions (R&D Systems) and were determined by comparison with known standards ($n = 3–5$ mice/group). Lower limit of detection for IL-13 was 1.5 pg/mL, for IFN- γ 2.0 pg/mL.

Statistical analysis

Data analysis was performed with Sigma Stat software. Statistically significant differences were determined by one-way ANOVA analysis followed by a comparison of groups with the Tukey–Kramer analysis. Data are reported as mean $\pm$ SEM. Statistical significance was defined by $P < 0.05$.

Results

Allergen challenge induced an increase in bronchoalveolar lavage eosinophils and interleukin-13 at early time points in surfactant protein deficient (SP-D $^{-/-}$) compared with wild-type mice

We have previously demonstrated that sensitization and 7 days of challenge were required to induce maximal allergic inflammation in our model [33, 38]. Figure 1 describes the kinetic model of allergic inflammation for this study (see Materials and methods). SP-D gene expression in lung tissue of WT OVA mice was significantly increased at days 15 and 17 as compared with PBS controls (Fig. 2A). We postulated that allergic responses *in vivo* would be enhanced in SP-D $^{-/-}$ mice. The allergic parameters (eosinophils and IL-13) were both significantly increased in WT OVA and SP-D $^{-/-}$ OVA mice as compared with PBS controls on day 17 and 21 for eosinophils or on day 15, 17 and 21 for IL-13, respectively (Figs 2B and C). On days 17 and 21, SP-D $^{-/-}$ OVA mice showed significantly increased eosinophil counts as compared with WT OVA mice ($*P < 0.05$). IL-13 concentration, a representative Th2 cytokine critical in allergic inflammation, was significantly increased in SP-D $^{-/-}$ OVA mice compared with WT OVA mice on days 15 and 17 ($*P < 0.05$) (Fig. 2C). Total serum IgE levels showed a trend to increased levels in SP-D $^{-/-}$ OVA mice as compared with WT OVA mice on all time points, though not significant (Fig. 2D). Together, these results demonstrate, that SP-D $^{-/-}$ mice showed increased allergic parameters compared with WT mice after 1 or 3 days of challenge, respectively, and for eosinophils also after 7 days of challenge.

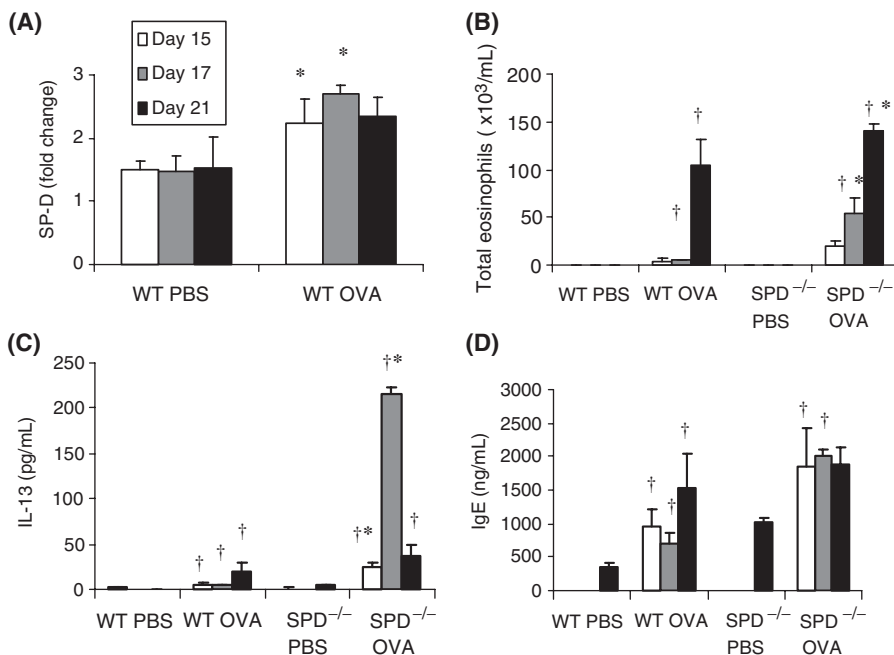


Fig. 2. Increased bronchoalveolar lavage (BAL) eosinophils and IL-13 production in surfactant protein-D (SP-D) deficiency. (A) Pulmonary surfactant protein D (SP-D) gene expression was significantly increased in wild-type (WT) ovalbumin (OVA) as compared with WT PBS mice on days 15 and 17 (* P <0.05). (B) On days 17 and 21, SP-D^{-/-} OVA mice showed increased BAL eosinophils as compared with WT OVA mice (* P <0.05). Each mouse underwent BAL as previously described. Cell counts were determined by differential staining of cells isolated from BAL fluid. (C) Concentration of BAL IL-13 was increased on days 15 and 17 in SP-D^{-/-} OVA compared with WT OVA mice (* P <0.05). (D) Total serum IgE was not significantly increased in SP-D^{-/-} OVA mice as compared with WT OVA mice on all time points. (C, D) Cytokine concentrations in BAL fluid and total serum IgE were measured with ELISA according to the manufacturer's instructions. Data are shown in pg/mL or ng/mL $\pm$ SEM (n = 4–6 mice/group).

Differential interferon- γ and interleukin-10 gene expression in surfactant protein deficient compared with wild-type mice

Pulmonary IFN- γ gene expression, examined as a representative potentially allergy-protective Th1 cytokine, was decreased in SP-D^{-/-} OVA, compared with WT OVA mice on day 15 (* P <0.05) (Fig. 3A). On day 21, IFN- γ gene expression was increased in SP-D^{-/-} OVA, compared with WT OVA mice (* P <0.05). Interestingly, IL-10 as an anti-inflammatory cytokine, showed significantly increased gene expression on day 15 in SP-D^{-/-} OVA mice, compared with WT OVA mice and decreased gene expression on day 21 (* P <0.05) (Fig. 3B).

Toll-like receptor 4 gene expression was decreased in surfactant protein deficient ovalbumin mice compared with wild-type ovalbumin mice

TLR4 is a receptor of the innate immune system recently shown to be critical for SP-D-mediated activation. We analysed pulmonary TLR4 gene expression in our *in vivo* model of allergic inflammation in both WT and SP-D^{-/-} mice. TLR4 gene expression was significantly increased in both WT and SP-D^{-/-} OVA mice compared with PBS controls on day 15 ($\dagger P$ <0.05) (Fig. 4A). Interestingly, pulmonary TLR4 gene expression of allergen-challenged SP-D^{-/-} mice was significantly decreased compared with WT mice on day 15 (* P <0.05) and similar on day 21 (Fig. 4A). The total number of BAL macrophages, a major cell type expressing TLR4, was similar in WT OVA and SP-D^{-/-} OVA mice on day 21, and lower in WT OVA as compared with SP-D^{-/-} OVA mice on day 15 (Fig. 4B). Analysing the reagent OVA by Limulus assay and for functional ability to stimulate unprimed splenocytes, we found no evidence for endotoxin contamination (data not shown), which was implicated in other models to modify allergen responses [39]. We confirmed the increase of TLR4 following allergen challenge by immunohistochemical (IH) analysis of TLR4

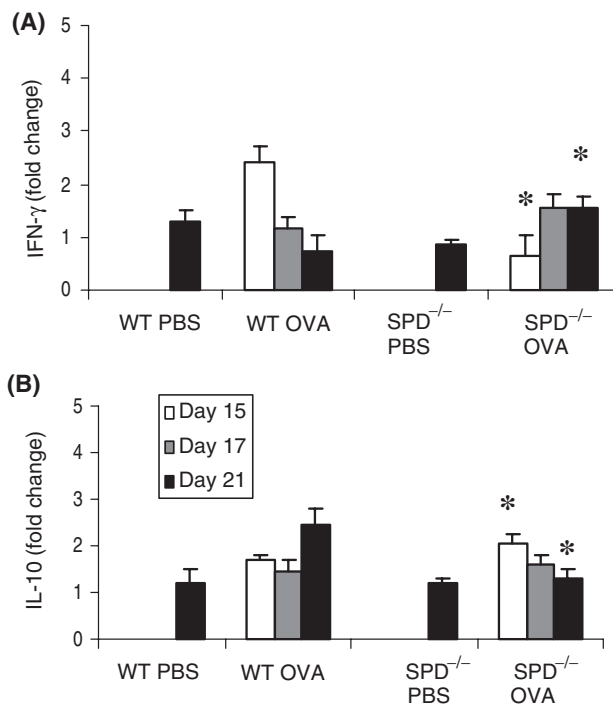
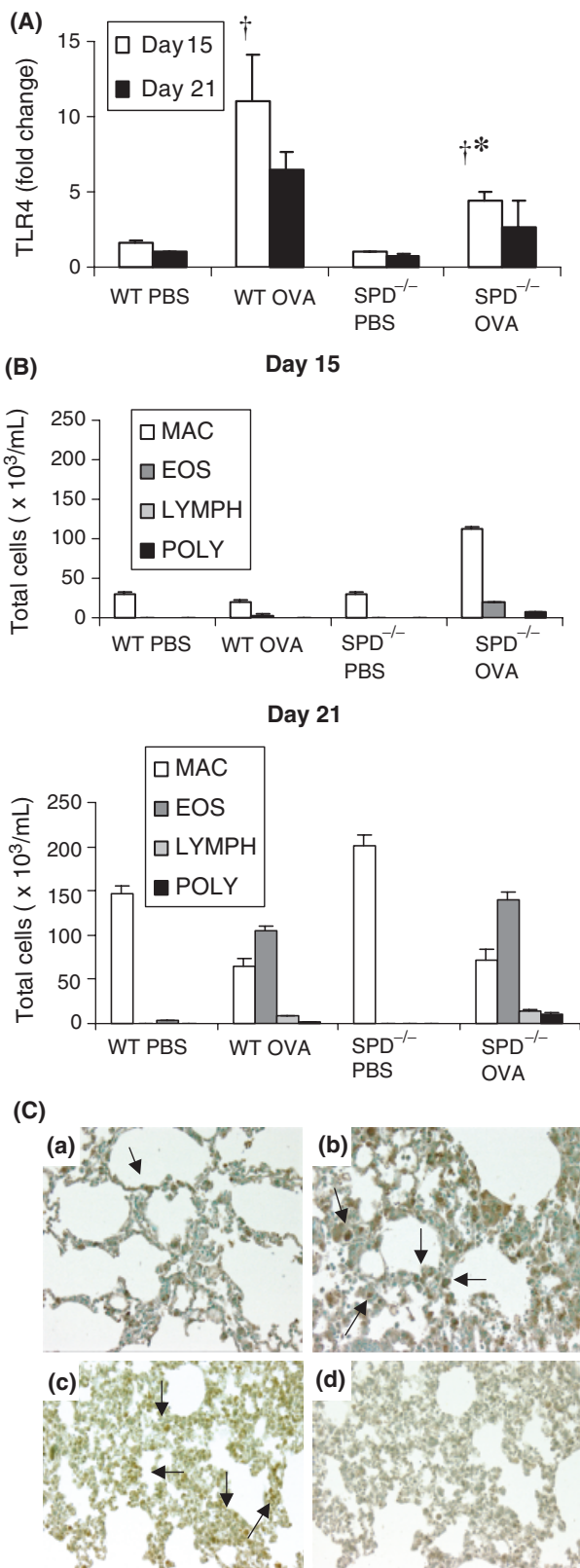


Fig. 3. Effect of surfactant protein D (SP-D) deficiency on pulmonary IFN- γ and IL-10 gene expression. (A) Pulmonary IFN- γ gene expression was significantly decreased on day 15 and increased on day 21 in SP-D^{-/-} OVA mice as compared with WT OVA mice (* P <0.05). (B) In contrast, IL-10 expression was significantly increased on day 15 and decreased on day 21 in SP-D^{-/-} OVA mice as compared with WT OVA mice (* P <0.05). (A, B) Total RNA was isolated from lung tissue and mRNA expression of IFN- γ and IL-10 was measured by real-time PCR as described in Materials and methods. Data are shown as mean fold change $\pm$ SEM (n = 4–6 mice/group).

protein distribution in allergen-induced WT mice (Fig. 4C, b), which revealed increased immunostaining for TLR4 in mononuclear alveolar cells on day 21 compared with WT PBS mice (Fig. 4C, a). Our IH results were consistent with mRNA gene expression (Fig. 4A). TLR4 positive cells

showed increased staining with the macrophage marker Mac-3 (not shown). No significant immunostaining was observed following pre-absorbing the primary antibody with specific peptide for TLR4 (Figs 4C, c and d).



Increased lymphoproliferative responses, decreased interferon- γ (IFN- γ) and sustained IL-13 concentrations in SP-D^{-/-} mice following *in vitro* stimulation with Lipid A

To characterize the lymphoproliferative responses of SP-D^{-/-} mice, we stimulated spleen cells with the TLR4 agonist LpA, a major component of lipopolysaccharide (LPS), at different doses. The lymphoproliferative response of SP-D^{-/-} mice was increased compared with WT mice in doses ranging from 0.1 to 100 μ g/mL LpA ($*P < 0.05$) (Fig. 5A). To determine the capacity of SP-D^{-/-} mice to produce cytokines following innate stimulation, we examined IFN- γ and IL-13 concentrations as cytokines relevant to allergic inflammation after stimulation with different doses of LpA (Figs 5B and C). IFN- γ concentration was significantly diminished in SP-D^{-/-} compared with WT mice following stimulation with LpA at any dose ($*P < 0.05$) (Fig. 5B). In contrast, IL-13 concentration was similar compared with WT mice following stimulation with LpA (Fig. 5C).

Mitogen stimulation increased lymphocyte proliferation, and decreased interferon- γ and IL-13 concentrations in surfactant protein deficient mice

To contrast the phenotypic immune responses in SP-D^{-/-} following innate stimulation, we investigated whether the proliferative response to a T cell mitogen (ConA) was also increased in SP-D^{-/-} mice *in vitro*. Mitogen-induced proliferation was significantly enhanced in SP-D^{-/-} mice as compared with WT mice ($*P < 0.05$) (Fig. 5D). Mitogen-induced IFN- γ and IL-13 concentrations were significantly reduced in SP-D^{-/-} mice as compared with WT controls ($*P < 0.05$) (Figs 5E and F). These results support the concept that stimulation of TLR4 with LpA and ConA increases LP in the absence of SP-D and may influence the immune system to inhibit IFN- γ secretion.

Discussion

Our results indicate that SP-D influences allergic immune responses, which are characterized as predominant Th2 cytokine responses. We present a temporal profile of allergic

Fig. 4. Toll-like receptor 4 (TLR4) expression in allergen-challenged SP-D^{-/-} and wild-type (WT) mice. (A) TLR4 gene expression was comparable in WT PBS and SP-D^{-/-} PBS mice on days 15 and day 21. TLR4 gene expression was significantly increased in WT and SP-D^{-/-} OVA mice compared with both PBS controls on day 15 ($P < 0.05$). Gene expression of TLR4 in SP-D^{-/-} OVA was significantly decreased as compared with WT OVA mice on day 15 ($*P < 0.05$). Total RNA was isolated and real-time PCR performed as described in Materials and methods. Data are shown as mean fold change $\pm$ SEM. (B) Total bronchoalveolar lavage (BAL) macrophage counts were similar in SP-D^{-/-} as compared with WT mice before and after allergen challenge on day 21, and higher on day 15. BAL cell counts were determined as described in Material and methods. Data are shown as total cell counts $\pm$ SEM. (C) Immunohistochemistry showed increased TLR4 expression after allergen exposure on day 21 in WT OVA (b) as compared with PBS mice (a). Arrows designate alveolar cells immunopositive for TLR4. Co-localized Mac-3 staining for macrophages showed the identity of TLR4-positive cells. (c) Immunostaining with TLR4. (d) Negative control demonstrating no immunostaining on a serial section, which was stained using TLR4 antibody pre-absorbed against specific peptide. Immunohistochemistry was performed as described in Material and methods. The staining of one representative mouse is shown. (A–C) $n = 4$ –6 mice/group.

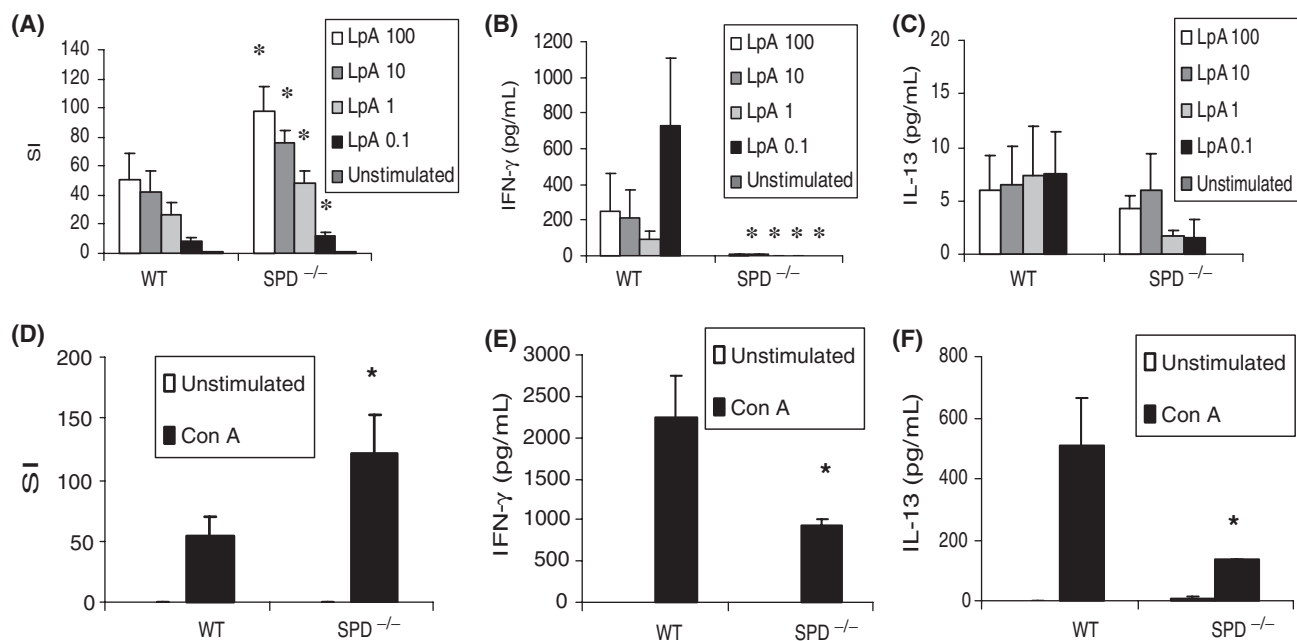


Fig. 5. Altered immune responses: increased proliferative responses and decreased IFN- γ production in SP-D^{-/-} following Lipid A (LpA) and concanavalin A (ConA) stimulation. (A) Lymphocyte proliferation (LP) was increased in SP-D^{-/-} mice following stimulation with LpA at multiple doses (0.1–100 µg/mL) as compared with WT controls (* P < 0.05). (B) IFN- γ production was absent in SP-D^{-/-} mice after low doses of LpA stimulation (0.1 and 1 µg/mL, * P < 0.05) and decreased compared with wild-type (WT) mice after higher doses of LpA (10 and 100 µg/mL, * P < 0.05). (C) IL-13 production was dose independent and similar in SP-D^{-/-} and WT controls. (D) LP was increased in SP-D deficiency following stimulation with the mitogen ConA as compared with WT controls (* P < 0.05). (E, F) IFN- γ and IL-13 production was decreased in SP-D^{-/-} mice as compared with WT controls after ConA stimulation (* P < 0.05). (A–F). LP was determined following stimulation with the indicated doses of LpA or ConA for 48 h by ³H-Thymidine uptake as described in Materials and methods. Supernatants of spleen cells were harvested 48 h after LpA or ConA stimulation and cytokine concentrations were measured with ELISA as described in methods (n = 3–5 mice/group).

responses in a murine model *in vivo* and the impact of SP-D deficiency. Early in allergen challenge, lack of SP-D skews allergic responses in favour of a greater Th2-like response, suggesting that SP-D may participate in the early immune response by dampening the subsequent allergic reactivity. However, later in the allergic response, potentially other mediators predominate, leading to a late rise in IFN- γ and a decrease in IL-10 expression. Further, we provide novel data demonstrating that allergen-induced TLR4 expression is increased in WT, but decreased in SP-D-deficient mice. These data suggest additional pathways by which SP-D and TLR4 may influence allergic responses.

This study suggests that SP-D contributes to decrease pulmonary allergic inflammation at early time points in allergic challenge. BAL eosinophils and IL-13 production were increased in SP-D^{-/-} mice at early time points. These data complement prior studies, which demonstrate that SP-D administration suppresses LP and the development of allergy in sensitized mice [21, 22, 31, 40–42]. SP-D may influence allergic immune responses in several ways. One potential explanation is that mannoseylated glycoprotein OVA may be bound and cleared by SP-D, since recombinant SP-D was shown to bind mannose-glucose [43]. The multifaceted immunoregulatory potential of SP-D is also shown by direct binding to allergens. SP-D can bind allergens from *Aspergillus fumigatus* [44, 45] and glycoprotein allergens of house dust mite [40, 46] via interaction with carbohydrate components. SP-D further inhibits the binding of specific IgE to these allergens, possibly through direct, CRD-dependent binding to lymphocytes [46]. SP-D may modulate T cell responses [19, 22, 31, 47] and enhance antigen presentation by dendritic cells

[48]. Further, SP-D coupled to OVA may increase the effective activation of macrophages and also contribute to the clearance of apoptotic cells by macrophages [30, 49]. At early time points in our study, WT OVA mice express a lower degree of allergic inflammation than allergen-challenged SP-D^{-/-} mice. However, at later time points, the immunomodulatory effects of SP-D, whether by direct binding or cytokine production, may not be sufficient to dampen a maximal allergic response.

Production of cytokines is one potential mechanism contributing to a modulatory effect of SP-D. Decreased IFN- γ production in SP-D deficiency at early time points in our study may indicate that IFN- γ is an allergy-protective marker in the presence of SP-D. Consistent with our data, Takeda et al. [50] showed increased IFN- γ secretion following application of SP-D with allergen, although in an isolated population of alveolar macrophages. We analysed an additional cytokine, IL-10, with known anti-inflammatory properties and secreted by T cells, non-T cells, epithelial cells and dendritic cells as well as macrophages [51, 52]. Prior work indicates that culture of allergen sensitized macrophages with SP-D results in increased production of IL-10 [50]. These data suggest a potential negative regulatory property of SP-D in allergic inflammation in part by modulating the function of alveolar macrophages [50]. Our data, showing that allergen-induced IL-10 levels are increased over time in WT OVA mice as allergic responses increase, support the concept that IL-10 may be anti-inflammatory [53, 54]. The decrease in IL-10 over time in SP-D deficiency may be due to dysfunctional alveolar macrophages in SP-D deficiency.

The *in vitro* analyses in SP-D deficiency suggest a role for SP-D in suppressing LP following mitogen (ConA) as well as innate (LpA, a TLR4 agonist) stimulation. Our work complements studies indicating that administration of SP-D can dampen lymphocyte responses [55, 56] and that lymphoid aggregates are seen in SP-D-deficient mice [21].

We postulate that factors in addition to SP-D may mediate an allergy-protective mechanism. Previous reports indicate that an intact TLR4 complex is necessary for SP-A-induced activation of the transcription factor nuclear factor κ B and of several cytokines such as TNF- α and IL-10 [20]. In this study, we describe for the first time, that TLR4 expression is increased in WT mice *in vivo* after one allergen challenge. TLR4 expression was diminished at early stages of allergen challenge in the absence of SP-D. Vandivier et al. [49, 30] showed that SP-D enhanced apoptotic cell ingestion *in vitro* and apoptotic cell clearance *in vivo*. Clark confirmed that SP-D^{-/-} mice have increased alveolar macrophages, which are known to express TLR4 and an increased number of apoptotic and necrotic alveolar macrophages. In our model, spleen cells of SP-D^{-/-} mice showed intact *in vitro* responses after TLR4 stimulation as exemplified by increased lymphoproliferation and sustained IL-13 production. In other studies, SP-D was shown to alter interactions between LPS and the cofactor CD-14, associated with TLR4, which are critical for innate immune responses [57]. Whether TLR4 expression may be SP-D dependent or co-acting in allergen-induced immune responses requires further investigation.

In summary, our data add to the emerging role of SP-D in modulating cellular immune responses after allergen challenge [19, 22–24, 31, 50]. SP-D influences allergic responses, potentially by suppressing T cell activation and influencing a distinct cytokine pattern. At maximal allergen exposure, SP-D may not be sufficient to reduce allergic inflammation. Administration of SP-D to SP-D^{-/-} mice is a potential means to assess SP-D effects, taking into consideration the type of SP-D, dose, time and site of application and distribution. Interestingly, several studies have highlighted the potential of collectins and their recombinant fragments as well as protein-free synthetic phospholipid-based surfactant as therapeutic molecules [11, 26, 43, 58–60]. Taken together, we suggest that the pulmonary collectin SP-D participates in the modulation of allergic inflammation. Whether SP-D will be shown protective in allergic inflammation in humans has yet to be elucidated.

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References

- Crouch E, Wright JR. Surfactant proteins a and d and pulmonary host defense. *Annu Rev Physiol* 2001; 63:521–54.
- Kuan SF, Persson A, Parghi D, Crouch E. Lectin-mediated interactions of surfactant protein D with alveolar macrophages. *Am J Respir Cell Mol Biol* 1994; 10:430–6.
- van de Wetering JK, van Eijk M, van Golde LM, Hartung T, van Strijp JA, Batenburg JJ. Characteristics of surfactant protein A and D binding to lipoteichoic acid and peptidoglycan, 2 major cell wall components of gram-positive bacteria. *J Infect Dis* 2001; 184: 1143–51.
- Palaniyar N, Zhang L, Kuzmenko A et al. The role of pulmonary collectin N-terminal domains in surfactant structure, function, and homeostasis *in vivo*. *J Biol Chem* 2002; 277:26971–9.
- Shrive AK, Tharia HA, Strong P et al. High-resolution structural insights into ligand binding and immune cell recognition by human lung surfactant protein D. *J Mol Biol* 2003; 331:509–23.
- Holmskov U. Lung surfactant proteins (SP-A and SP-D) in non-adaptive host responses to infection. *J Leukoc Biol* 1999; 66: 747–52.
- van Golde LM. Potential role of surfactant proteins A and D in innate lung defense against pathogens. *Biol Neonate* 1995; 67 (Suppl. 1):2–17.
- Vaandrager AB, van Golde LM. Lung surfactant proteins A and D in innate immune defense. *Biol Neonate* 2000; 77 (Suppl. 1):9–13.
- Crouch E, Parghi D, Kuan SF, Persson A. Surfactant protein D: subcellular localization in nonciliated bronchiolar epithelial cells. *Am J Physiol* 1992; 263 (1 Part 1):L60–6.
- LeVine AM, Whitsett JA. Pulmonary collectins and innate host defense of the lung. *Microbes Infect* 2001; 3:161–6.
- Kishor U, Madan T, Sarma PU, Singh M, Urban BC, Reid KB. Protective roles of pulmonary surfactant proteins, SP-A and SP-D, against lung allergy and infection caused by *Aspergillus fumigatus*. *Immunobiology* 2002; 205:610–8.
- Kuan SF, Rust K, Crouch E. Interactions of surfactant protein D with bacterial lipopolysaccharides. Surfactant protein D is an *Escherichia coli*-binding protein in bronchoalveolar lavage. *J Clin Invest* 1992; 90:97–106.
- McIntosh JC, Swyers AH, Fisher JH, Wright JR. Surfactant proteins A and D increase in response to intratracheal lipopolysaccharide. *Am J Respir Cell Mol Biol* 1996; 15:509–19.
- Van Rosendaal BA, Van de Lest CH, Van Eijk M, Van Helden HP, Haagsman HP. Pulmonary surfactant proteins A and D are involved in the early response to intratracheally aerosolized lipopolysaccharide. *Biochem Soc Trans* 1997; 25:S656.
- Sahly H, Ofek I, Podschun R et al. Surfactant protein D binds selectively to *Klebsiella pneumoniae* lipopolysaccharides containing mannose-rich O-antigens. *J Immunol* 2002; 169:3267–74.
- Wu H, Kuzmenko A, Wan S et al. Surfactant proteins A and D inhibit the growth of Gram-negative bacteria by increasing membrane permeability. *J Clin Invest* 2003; 111:1589–602.
- Bufler P, Schmidt B, Schikor D, Bauernfeind A, Crouch EC, Griesse M. Surfactant protein A and D differently regulate the immune response to nonmucoid *Pseudomonas aeruginosa* and its lipopolysaccharide. *Am J Respir Cell Mol Biol* 2003; 28:249–56.
- Noah TL, Murphy PC, Alink JJ et al. Bronchoalveolar lavage fluid SP-A and SP-D are inversely related to inflammation in early CF. *Am J Respir Crit Care Med* 2003; 168:685–91.
- Borron PJ, Crouch EC, Lewis JF, Wright JR, Possmayer F, Fraher LJ. Recombinant rat surfactant-associated protein D inhibits human T lymphocyte proliferation and IL-2 production. *J Immunol* 1998; 161:4599–603.
- Guillot L, Balloy V, McCormack FX, Golenbock DT, Chignard M, Si-Tahar M. Cutting edge: the immunostimulatory activity of the lung surfactant protein-A involves Toll-like receptor 4. *J Immunol* 2002; 168:5989–92.
- Fisher JH, Larson J, Cool C, Dow SW. Lymphocyte activation in the lungs of SP-D null mice. *Am J Respir Cell Mol Biol* 2002; 27:24–33.
- Wang JY, Shieh CC, You PF, Lei HY, Reid KB. Inhibitory effect of pulmonary surfactant proteins A and D on allergen-induced lymphocyte proliferation and histamine release in children with asthma. *Am J Respir Crit Care Med* 1998; 158:510–8.

- 23 Cheng G, Ueda T, Numao T et al. Increased levels of surfactant protein A and D in bronchoalveolar lavage fluids in patients with bronchial asthma. *Eur Respir J* 2000; 16:831–5.
- 24 Haley KJ, Ciota A, Contreras JP, Boothby MR, Perkins DL, Finn PW. Alterations in lung collectins in an adaptive allergic immune response. *Am J Physiol Lung Cell Mol Physiol* 2002; 282:L573–84.
- 25 Kasper M, Sims G, Koslowski R et al. Increased surfactant protein D in rat airway goblet and Clara cells during ovalbumin-induced allergic airway inflammation. *Clin Exp Allergy* 2002; 32:1251–8.
- 26 Hickling TP, Bright H, Wing K et al. A recombinant trimeric surfactant protein D carbohydrate recognition domain inhibits respiratory syncytial virus infection *in vitro* and *in vivo*. *Eur J Immunol* 1999; 29:3478–84.
- 27 Hawgood S, Ochs M, Jung A et al. Sequential targeted deficiency of SP-A and -D leads to progressive alveolar lipoproteinosis and emphysema. *Am J Physiol Lung Cell Mol Physiol* 2002; 283:L1002–10.
- 28 Zhang L, Ikegami M, Dey CR, Korfhagen TR, Whitsett JA. Reversibility of pulmonary abnormalities by conditional replacement of surfactant protein D (SP-D) *in vivo*. *J Biol Chem* 2002; 277:38709–13.
- 29 Botas C, Poullain F, Akiyama J et al. Altered surfactant homeostasis and alveolar type II cell morphology in mice lacking surfactant protein D. *Proc Natl Acad Sci USA* 1998; 95:11869–74.
- 30 Clark H, Palaniyar N, Strong P, Edmondson J, Hawgood S, Reid KB. Surfactant protein D reduces alveolar macrophage apoptosis *in vivo*. *J Immunol* 2002; 169:2892–9.
- 31 Wang JY, Shieh CC, Yu CK, Lei HY. Allergen-induced bronchial inflammation is associated with decreased levels of surfactant proteins A and D in a murine model of asthma. *Clin Exp Allergy* 2001; 31:652–62.
- 32 Mark DA, Donovan CE, De Sanctis GT et al. Both CD80 and CD86 co-stimulatory molecules regulate allergic pulmonary inflammation. *Int Immunol* 1998; 10:1647–55.
- 33 Arestides RS, He H, Westlake RM et al. Costimulatory molecule OX40L is critical for both Th1 and Th2 responses in allergic inflammation. *Eur J Immunol* 2002; 32:2874–80.
- 34 Donovan CE, Mark DA, He HZ et al. NF-kappa B/Rel transcription factors: c-Rel promotes airway hyperresponsiveness and allergic pulmonary inflammation. *J Immunol* 1999; 163: 6827–33.
- 35 Gibson UE, Heid CA, Williams PM. A novel method for real time quantitative RT-PCR. *Genome Res* 1996; 6:995–1001.
- 36 Heid CA, Stevens J, Livak KJ, Williams PM. Real time quantitative PCR. *Genome Res* 1996; 6:986–94.
- 37 Fleming CM, He H, Ciota A, Perkins D, Finn PW. Administration of pentoxifylline during allergen sensitization dissociates pulmonary allergic inflammation from airway hyperresponsiveness. *J Immunol* 2001; 167:1703–11.
- 38 Krinzman SJ, De Sanctis GT, Cernadas M et al. T cell activation in a murine model of asthma. *Am J Physiol* 1996; 271 (3 Part 1): L476–83.
- 39 Eisenbarth SC, Piggott DA, Huleatt JW, Visintin I, Herrick CA, Bottomly K. Lipopolysaccharide-enhanced, toll-like receptor 4-dependent T helper cell type 2 responses to inhaled antigen. *J Exp Med* 2002; 196:1645–51.
- 40 Madan T, Kishore U, Shah A et al. Lung surfactant proteins A and D can inhibit specific IgE binding to the allergens of *Aspergillus fumigatus* and block allergen-induced histamine release from human basophils. *Clin Exp Immunol* 1997; 110:241–9.
- 41 Strong P, Reid KB, Clark H. Intranasal delivery of a truncated recombinant human SP-D is effective at down-regulating allergic hypersensitivity in mice sensitized to allergens of *Aspergillus fumigatus*. *Clin Exp Immunol* 2002; 130:19–24.
- 42 Madan T, Kishore U, Singh M et al. Protective role of lung surfactant protein D in a murine model of invasive pulmonary aspergillosis. *Infect Immun* 2001; 69:2728–31.
- 43 Ogasawara Y, Voelker DR. Altered carbohydrate recognition specificity engineered into surfactant protein D reveals different binding mechanisms for phosphatidylinositol and glucosylceramide. *J Biol Chem* 1995; 270:14725–32.
- 44 Madan T, Eggleton P, Kishore U et al. Binding of pulmonary surfactant proteins A and D to *Aspergillus fumigatus* conidia enhances phagocytosis and killing by human neutrophils and alveolar macrophages. *Infect Immun* 1997; 65:3171–9.
- 45 Allen MJ, Harbeck R, Smith B, Voelker DR, Mason RJ. Binding of rat and human surfactant proteins A and D to *Aspergillus fumigatus* conidia. *Infect Immun* 1999; 67:4563–9.
- 46 Wang JY, Kishore U, Lim BL, Strong P, Reid KB. Interaction of human lung surfactant proteins A and D with mite (*Dermatophagoides pteronyssinus*) allergens. *Clin Exp Immunol* 1996; 106: 367–73.
- 47 Borron PJ, Mostaghel EA, Doyle C, Walsh ES, McHeyzer-Williams MG, Wright JR. Pulmonary surfactant proteins A and D directly suppress CD3+/CD4+ cell function: evidence for two shared mechanisms. *J Immunol* 2002; 169:5844–50.
- 48 Brinker KG, Martin E, Borron P et al. Surfactant protein D enhances bacterial antigen presentation by bone marrow-derived dendritic cells. *Am J Physiol Lung Cell Mol Physiol* 2001; 281:L453–63.
- 49 Vandivier RW, Ogden CA, Fadok VA et al. Role of surfactant proteins A, D, and Clq in the clearance of apoptotic cells *in vivo* and *in vitro*: calreticulin and CD91 as a common collectin receptor complex. *J Immunol* 2002; 169:3978–86.
- 50 Takeda K, Miyahara N, Rha YH et al. SP-D regulates airway function and allergic inflammation through regulation of macrophage function. *Am J Respir Crit Care Med* 2003; 168:783–9.
- 51 Romani L, Mencacci A, Cenci E et al. Neutrophil production of IL-12 and IL-10 in candidiasis and efficacy of IL-12 therapy in neutropenic mice. *J Immunol* 1997; 158:5349–56.
- 52 Keisari Y, Wang H, Mesika A et al. Surfactant protein D-coated *Klebsiella pneumoniae* stimulates cytokine production in mononuclear phagocytes. *J Leukoc Biol* 2001; 70:135–41.
- 53 Zuany-Amorim C, Haile S, Leduc D et al. Interleukin-10 inhibits antigen-induced cellular recruitment into the airways of sensitized mice. *J Clin Invest* 1995; 95:2644–51.
- 54 Colavita AM, Hastie AT, Musani AI et al. Kinetics of IL-10 production after segmental antigen challenge of atopic asthmatic subjects. *J Allergy Clin Immunol* 2000; 106:880–6.
- 55 Borron P, McCormack FX, Elhalwagi BM et al. Surfactant protein A inhibits T cell proliferation via its collagen-like tail and a 210-kDa receptor. *Am J Physiol* 1998; 275 (4 Part 1):L679–86.
- 56 Crouch E, Chang D, Rust K, Persson A, Heuser J. Recombinant pulmonary surfactant protein D. Post-translational modification and molecular assembly. *J Biol Chem* 1994; 269:15808–13.
- 57 Beutler B. Toll-like receptors: how they work and what they do. *Curr Opin Hematol* 2002; 9:2–10.
- 58 Hartshorn KL, White MR, Voelker DR, Coburn J, Zaner K, Crouch EC. Mechanism of binding of surfactant protein D to influenza A viruses: importance of binding to haemagglutinin to antiviral activity. *Biochem J* 2000; 351 (Part 2):449–58.
- 59 Madan T, Kishore U, Singh M et al. Surfactant proteins A and D protect mice against pulmonary hypersensitivity induced by *Aspergillus fumigatus* antigens and allergens. *J Clin Invest* 2001; 107:467–75.
- 60 Babu KS, Woodcock DA, Smith SE, Staniforth JN, Holgate ST, Conway JH. Inhaled synthetic surfactant abolishes the early allergen-induced response in asthma. *Eur Respir J* 2003; 21:1046–9.