

IL-4 and IL-13 Form a Negative Feedback Circuit with Surfactant Protein-D in the Allergic Airway Response¹

Angela Haczku,^{2*} Yang Cao,^{*} Geza Vass,^{*} Sonja Kierstein,^{*} Puneeta Nath,[†] Elena N. Atochina-Vasserman,^{*} Seth T. Scanlon,^{*} Lily Li,[‡] Don E. Griswold,[‡] K. Fan Chung,[†] Francis R. Poulain,[§] Samuel Hawgood,^{||} Michael F. Beers,^{*} and Erika C. Crouch^{||}

The innate immune molecule surfactant protein-D (SP-D) plays an important regulatory role in the allergic airway response. In this study, we demonstrate that mice sensitized and challenged with either *Aspergillus fumigatus* (Af) or OVA have increased SP-D levels in their lung. SP-D mRNA and protein levels in the lung also increased in response to either rIL-4 or rIL-13 treatment. Type II alveolar epithelial cell expression of IL-4Rs in mice sensitized and challenged with Af, and in vitro induction of SP-D mRNA and protein by IL-4 and IL-13, but not IFN- γ , suggested a direct role of IL-4R-mediated events. The regulatory function of IL-4 and IL-13 was further supported in STAT-6-deficient mice as well as in IL-4/IL-13 double knockout mice that failed to increase SP-D production upon allergen challenge. Interestingly, addition of rSP-D significantly inhibited Af-driven Th2 cell activation in vitro whereas mice lacking SP-D had increased numbers of CD4⁺ cells with elevated IL-13 and thymus- and activation-regulated chemokine levels in the lung and showed exaggerated production of IgE and IgG1 following allergic sensitization. We propose that allergen exposure induces elevation in SP-D protein levels in an IL-4/IL-13-dependent manner, which in turn, prevents further activation of sensitized T cells. This negative feedback regulatory circuit could be essential in protecting the airways from inflammatory damage after allergen inhalation. *The Journal of Immunology*, 2006, 176: 3557–3565.

Allergen inhalation elicits characteristic inflammatory symptoms in susceptible patients while normal individuals remain symptom-free. The processes that regulate whether an immune response occurs to inhaled allergens are poorly understood. Increasing evidence supports an immunomodulatory role for the hydrophilic surfactant protein D (SP-D).³ SP-D is a member of the collectin family, composed of an NH₂-terminal collagen and a COOH-terminal lectin domain. This protein is a constitutive mediator of Ag clearance and is capable of interacting with cellular components of both the innate and adaptive immune systems on mucosal surfaces (1, 2). SP-D is a potent inhibitor of T cell function in vitro (3–6) and was shown to protect against various inflammatory responses in the lung in vivo (7–13). SP-D is synthesized and secreted primarily by type II alveolar cells, Clara

cells, and cells of submucosal glands in the lung (14), but this collectin can also be found in epithelial surfaces of many other organs (14–18). Thus, SP-D may have a general immunoregulatory importance associated with protection of epithelial and submucosal tissues.

Recent studies indicated significant changes in levels of SP-D during asthmatic inflammation of the lung in animal models (19–21) as well as in asthmatic patients (22, 23). We previously described that SP-D is selectively increased among the SPs following allergen challenge of sensitized mice (24, 25). In this study, we hypothesize that SP-D production is induced by mechanisms underlying the allergic immune response and that it contributes to regulation of the inflammatory changes. SP-D-deficient mice and the function of rSP-D was studied in models of T cell-driven allergic responses against *Aspergillus fumigatus* (Af) (24, 26, 27), an airborne fungal pathogen, that has been shown to bind to this lung collectin (28, 29).

Materials and Methods

Animals and murine models of allergic sensitization

Changes in SP expression during the allergic response were studied in 8- to 10-wk-old, female normal BALB/c and female STAT-6-deficient BALB/c mice obtained from The Jackson Laboratory, and housed under specific pathogen-free conditions. IL-4/IL-13 double knockout and wild-type (129 $\times$ C57BL/6)F₂ mice were provided by the laboratory of Dr. A. McKenzie (Medical Research Council, Cambridge, U.K.) (30). SP-D-deficient mice were generated from breeding pairs provided to us by the laboratory of Dr. S. Hawgood (University of California, San Francisco, CA). These mice have been backcrossed to C57BL/6 strain for 10 generations (31, 32). For the in vitro type II cell culture studies, postnatal Sprague Dawley rats (Charles River Laboratories) were used as described previously (33). All experimental procedures used in this study were under a protocol approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

Sensitization with Af and with OVA was performed as described previously (24, 27, 34). STAT-6-deficient mice were sensitized and challenged using either Af extract or glycerol (as negative control). IL-4/IL-13 double knockout mice were sensitized and challenged with either OVA or

*Pulmonary, Allergy and Critical Care Division, Department of Medicine, University of Pennsylvania, School of Medicine, Philadelphia, PA 19104; [†]National Heart and Lung Institute, London, United Kingdom; [‡]Centocor, Malvern, PA 19355; [§]Division of Neonatology, University of California, Davis, CA 95817; ^{||}Division of Neonatology, University of California, San Francisco, CA 94143; and ^{||}Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, MO 63110

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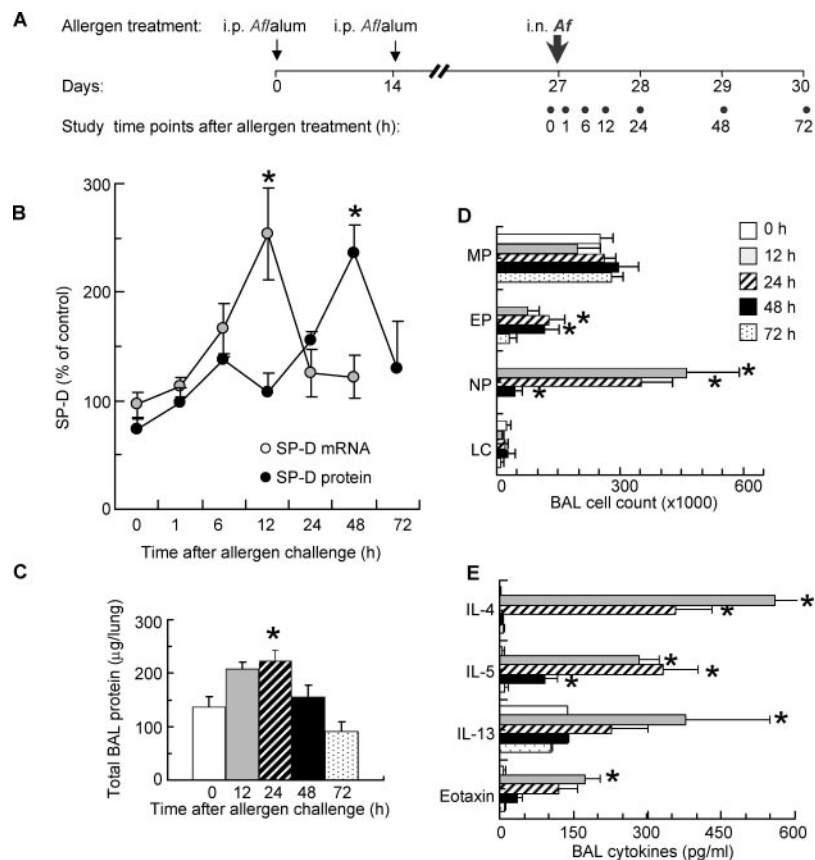
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² Address correspondence and reprint requests to Dr. Angela Haczku, Pulmonary, Allergy and Critical Care Division, Department of Medicine, University of Pennsylvania, 421 Curie Boulevard, Biological Research Building 2-3, Philadelphia, PA 19104-6061. E-mail address: haczku@mail.med.upenn.edu

³ Abbreviations used in this paper: SP, surfactant protein; Af, *Aspergillus fumigatus*; i.t., intratracheal; i.n., intranasal; BAL, bronchoalveolar lavage; TARC, thymus- and activation-regulated chemokine.

FIGURE 1. Resolution of *Af*-induced allergic airway inflammation was associated with increased levels of SP-D protein in the BAL fluid of sensitized mice. *A*, Sensitized mice were studied using a separate group of mice before (0 h) and 1, 6, 12, 24, 48, and 72 h following allergen challenge. *B*, SP-D mRNA was analyzed from total RNA extract of the lung tissue. Protein expression was quantified using Western blot of the cell-free supernatant of the BAL fluid. Values are expressed as the percentage of naive control values. *C*, Total protein levels were measured in the BAL supernatant. *D*, Differential cell count of the BAL cell pellet was assessed before (0 h, □) and 12 (▤), 24 (▨), 48 (■), and 72 h (▩) following *Af* challenge of sensitized mice. *E*, IL-4, IL-5, IL-13, and eotaxin levels in the BAL supernatant were measured by ELISA. *, $p < 0.05$ vs the 0 h group; mean $\pm$ SEM of $n = 4-6$ mice.



PBS. Intratracheal (i.t.) injection of recombinant murine IL-4, IL-13, IFN- γ (BD Pharmingen), or BSA of 1.5 μ g in 25 μ l of PBS was conducted as previously described (27). Mice were studied 12, 24, and 48 h later.

Analysis of allergic inflammatory changes

Differential cell count, cytokine, surfactant, and Ig profiles were characterized as previously published (24, 25, 27). For immunohistochemistry, lungs from naive and sensitized mice were snap-frozen and processed as described previously (35) using FITC-conjugated anti-IL-4R and isotype control Abs (BD Pharmingen).

Analysis of SP-D expression

Total RNA was isolated and specific mRNA content was determined by Northern blot analysis (24). Nitrocellulose blots loaded with 10 μ g of total RNA/lane were hybridized under high stringency with [α - ^{32} P]cDNA probes for rat SP-D (33) prepared from purified plasmid inserts by labeling with [α - ^{32} P]dCTP (Ready-to-Go kit; Pharmacia) as previously described. The specific signals were normalized for loading by hybridization of each blot with a ^{32} P end-labeled ([γ - ^{32} P]ATP) 28S rRNA oligonucleotide probe. SP-D protein levels were assessed semiquantitatively, by Western blot as described previously in detail (24). The average density of bands obtained from naive (nonsensitized) mice served as 100% and was used for comparison with every blot in the gels. Western and Northern blot results were expressed as the percent change from the naive animals.

Alveolar type II epithelial cell studies

Alveolar type II cells were isolated as previously described (33, 36). Briefly, after overnight incubation, cells were washed and further incubated in serum-free Weymouth's medium with or without DCI (dexamethasone (10 nM), cAMP (100 μ M), and isobutylmethylxanthine (100 μ M) from Sigma-Aldrich) in the presence or absence of IL-4, IL-13, and IFN- γ (BD Pharmingen) through day 4. The proportion of type II cells on day 4 was ~25%. Western blot analysis and immunocytochemistry was performed as described previously (33). The mAb, 3C9, that recognizes a 180-kDa lamellar body membrane protein ABCA3 of alveolar type II cells (37, 38) was a gift from Dr. H. Shuman (Department of Physiology, University of Pennsylvania, Philadelphia, PA). Phase contrast and fluorescence images were viewed on an Olympus I-70 inverted fluorescence microscope (Chroma Technology).

Recombinant SP-D

Recombinant human SP-D was isolated from the culture medium of stably transfected CHO-K1 cells (39). The secreted protein was then purified by sequential maltosyl-agarose affinity chromatography and gel filtration chromatography as previously described. Endotoxin content of the purified SP-D was measured (*Limulus* Amebocyte Lysate QCL-1000; BioWhittaker) and was found <0.1 pg of LPS/ μ g SP-D. rSP-D was pooled and stored in aliquots at -80°C . The biological activity of the human SP-D on mouse cells was tested using PHA and PMA/ionomycin-stimulated splenic mononuclear cultures (see Fig. 6, A and B).

Cell proliferation and cytokine release assays

Splenic mononuclear cells were isolated from sensitized and challenged mice 24 h after the intranasal (i.n.) *Af* treatment and stimulated with *Af* (0, 1, or 10 μ g/ml), PMA-ionomycin (10 and 500 ng/ml, respectively), or PHA (2.5, 5 or 10 μ g/ml) and treated with SP-D (0.1 or 1 μ g/ml). Seventy-two hours later, the wells were pulsed with [^{3} H]thymidine (4 μ Ci/ml) for an additional 24 h. [^{3} H]Thymidine incorporation of triplicate cultures was determined via liquid scintillation counting and results were presented as proliferation index (ratio over the nonstimulated control values) or percentage of inhibition ($1 - (\text{cpm of SP-D-treated samples} / \text{cpm of no SP-D-containing samples}) \times 100$).

To analyze the released cytokine profile, mononuclear cells were cultured for 48 h. The supernatant was removed and frozen at -80°C until assays were performed. OptEIA mouse IL-4, IL-5, and IFN- γ ELISA kits (BD Pharmingen) were used according to the manufacturer's specifications. To correct for the extent of cell proliferation in the wells, the amount of cytokine detected (picograms per milliliter) was divided by the cpm of the corresponding well. To eliminate individual variability among the different samples, these values were further divided by the nonstimulated (0 μ g/ml *Af*) control samples, the same way as the stimulation index for proliferation is calculated. Results were then expressed as the "cytokine index."

Flow cytometric analysis of T cell activation

Cell surface marker staining was performed using PE-conjugated anti-murine CD4, Tricolor-conjugated anti-murine CD3 (Caltag Laboratories),

FITC-conjugated anti-murine CD25 (eBioscience), and allophycocyanin-conjugated anti-mouse CD8 (Caltag Laboratories). Dead cells were excluded by the vital dye TO-PRO-3 (Molecular Probes). The expression of CD25 on CD3⁺CD4⁺ cells was investigated following culture of mononuclear cells with *Af* (0, 1, or 10 μ g/ml) in the presence and absence of SP-D (1 μ g/ml). Flow cytometric analysis was performed after 72, 96, and 120 h of incubation time.

To further investigate the inhibitory effects of SP-D on Ag-stimulated proliferating T cells, we applied a CFSE labeling method (40). Briefly, CFSE (Molecular Probes) was added to the isolated mononuclear cell suspension (10^6 cells/ml) at 1 μ M. Heat-inactivated human serum (Invitrogen Life Technologies) was added to quench the extracellular fluorescence. The cells were then washed and cultured for 7 days. CFSE data files were analyzed using CellQuest acquisition/analysis software (BD Biosciences) and the Proliferation Wizard module in ModFit LT Macintosh software (Verity Software House; Topsham). Fifty-thousand events were collected. All data were acquired on a four-color, dual-laser FACSCalibur (BD Biosciences). Lymphocytes were gated on a side scatter vs forward scatter plot (R1). To identify T cells (CD3⁺ events) and CD4⁺ cells, we used a side scatter vs CD3⁺ plot (R2) and a CD3⁺ vs CD4⁺ (or CD8⁺) plot, respectively.

Data analysis

Data are expressed as mean $\pm$ SEM. Pairwise comparisons were made using the Student *t* test. Multiple comparisons were made using ANOVA followed by the Bonferroni test. Correlations were investigated by regression analysis. Statistical significance was defined as $p < 0.05$. Data were analyzed with the Sigmaplot standard statistical package (Jandel Scientific).

Results

Resolution of inflammatory changes following allergen challenge of sensitized mice was associated with increased SP-D in the airways

To be able to study the onset and resolution of inflammatory changes, we used a previously characterized model of the late phase allergic airway response elicited by a single local challenge. The kinetics of changes in SP-D levels was studied in a different group of sensitized mice before, and 1, 6, 12, 24, 48, and 72 h after i.n. *Af* provocation (Fig. 1A). Because SP-D is constitutively expressed, we quantified the changes by calculating the percent difference from baseline. To determine whether changes in SP-D expression were induced at the pretranslational level, we also studied lung tissue SP-D mRNA expression. Bronchoalveolar lavage (BAL) SP-D protein levels were markedly increased 48 h after the i.n. provocation of sensitized mice with *Af*. This increase was preceded by a peak of SP-D mRNA expression between 6 and 12 h after *Af* (Fig. 1B). SP-D changes were specific because i.n. instillation of the vehicle control, glycerol, in sensitized mice had no effect on SP-D levels. SP-D elevation was also selective because levels of the other SPs (SP-A, SP-B, and SP-C) did not increase upon allergen challenge (data not shown).

The acute inflammatory events of the airways following a single allergen challenge, including increases in total protein (Fig. 1C), IL-4, IL-5, IL-13, and eotaxin (Fig. 1D) culminated 12–24 h later and resolved by 48 h. A marked cellular influx of neutrophils and eosinophils also reached maximum levels at the 12 and 24 h time points, respectively. Although airway eosinophilia persisted over 48 h after *Af* provocation, the numbers of neutrophils returned to normal by 48 h (Fig. 1E). Thus, resolution of the *Af*-induced acute airway changes 48 h after *Af* challenge coincided with a significant rise in BAL SP-D levels.

SP-D protein and mRNA were induced by rIL-4 in the lung of sensitized mice

Regression analysis between IL-4 and SP-D of the BAL fluid obtained from the 0, 1, 6, 12, and 24 h groups ($r = 0.874$, $p < 0.0001$), as well as between BAL IL-4 and SP-D mRNA of the lung tissue obtained from the 0, 1, 6, and 12 h groups ($r = 0.834$,

$p < 0.001$) showed strong positive correlations. However, correlation was lost at the later time points suggesting that IL-4 release was closely associated with initiation of the augmented SP-D expression (Fig. 2A).

To investigate whether the increased SP-D production was causally related to presence of IL-4, mice were sensitized with *Af* (to achieve a “primed,” IL-4 responsive state) and received murine rIL-4, IL-13, or IFN- γ (1.5 μ g/mouse) instead of an *Af* challenge on day 27. SP-D was analyzed in the BAL 12, 24, or 48 h later. The biological activity of the i.t. instilled IL-4 was verified by measuring eotaxin levels (Fig. 2B). Sensitized mice that received IL-4 or IL-13 but not mice that received IFN- γ or vehicle had a significant, time-dependent increase in SP-D in the BAL with the highest values measured 24 h after IL-13 and 48 h after IL-4 treatment. SP-D protein increase was preceded by a transient elevation of SP-D mRNA (Fig. 2, C and D).

IL-4 and IL-13 but not IFN- γ increased intracellular SP-D expression in a dose-dependent manner

The specificity of the IL-4 effect on SP-D induction was studied on isolated type II epithelial cells (33). These studies were conducted in a well-characterized neonatal rat type II cell culture system. Lamellar body expression (37) (Fig. 3A, upper right panel) and the ability to produce SP-D (lower left panel) for up to 4 days after initiating the cell culture was verified by immunochemical staining. Intracellular SP-D expression of type II epithelial cells treated with recombinant rat IL-4, IL-13 or IFN- γ was studied by Western blot analysis. IL-4 and IL-13, but not IFN- γ , increased intracellular SP-D in comparison to the nontreated cells (Fig. 3B). The effects of IL-4 and IL-13 on enhancing intracellular SP-D levels were dose-dependent between 5 and 20 ng/ml.

IL-4R-mediated SP-D accumulation during the asthmatic response

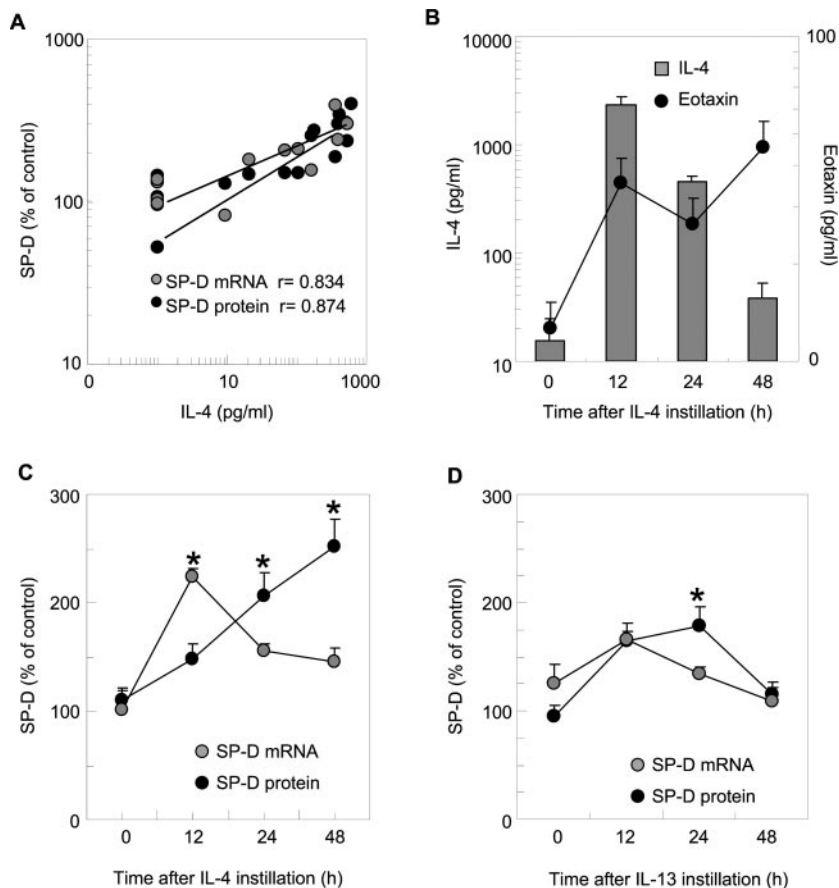
To provide direct evidence for the importance of IL-4 and IL-13 in regulating SP-D levels, IL-4/IL-13 double knockout mice were used in a model of OVA sensitization (34). As we observed following *Af* challenge, wild-type mice produced a significant increase in the BAL SP-D levels in response to OVA challenge. In contrast, mice lacking IL-4 and IL-13 were not able to respond to OVA inhalation by SP-D changes. (Fig. 4A).

Type II alveolar epithelial cells in murine lungs, expressing ABCA3, a lamellar body membrane protein (Fig. 4, upper left panel), had positive immunolabeling for IL-4R in sensitized and *Af*-challenged (upper right panel), but not in vehicle-challenged, mice (lower left panel). To further investigate whether IL-4R signaling plays a role in SP-D up-regulation during allergic changes, we sensitized STAT-6^{-/-} mice and compared their SP-D responses with normal animals 24 h after a single *Af* challenge. Sensitized and challenged wild-type mice had significantly higher SP-D levels in the BAL than vehicle-challenged controls ($p < 0.05$). In contrast, SP-D production after *Af* provocation was absent in mice lacking STAT-6 (Fig. 4B), suggesting an impaired ability to produce SP-D following allergen sensitization and challenge. Baseline SP-D expression of STAT-6^{-/-} mice was equivalent to those of wild-type BALB/c mice (data not shown).

*SP-D^{-/-} mice had increased numbers of CD4⁺ cells, elevated thymus- and activation-regulated chemokine (TARC), and IL-13 expression in the lung and produced exaggerated IgE response upon sensitization with *Af* allergen*

The function of SP-D was first examined in vivo in SP-D-deficient mice. These mice were susceptible to developing secondary lymphoid tissue in the peribronchial areas in their lung in an age and

FIGURE 2. IL-4- and IL-13-induced increased SP-D mRNA and protein production in allergen-sensitized mice in vivo. **A**, Regression analysis between BAL IL-4 and SP-D using samples from sensitized and challenged mice. IL-4 positively correlated with SP-D protein in the first 24 h ($r = 0.87$, $p < 0.0001$, $n = 17$) and with SP-D mRNA in the first 12 h ($r = 0.83$, $p < 0.01$, $n = 13$) after *Af* challenge. y-axis: SP-D (percent of the naive control values), x-axis: IL-4 (pg/ml). **B–D**, Instead of i.n. *Af* challenge, sensitized mice received murine rIL-4 (1.5 μ g) (**B** and **C**) or IL-13 (1.5 μ g) (**D**) i.t., and were studied 12, 24, and 48 h later. **B**, BAL IL-4 and eotaxin were measured by ELISA. **C** and **D**, SP-D mRNA was analyzed using total RNA extract of the lung tissue. Protein expression was quantified by Western blot of the cell-free supernatant of the BAL fluid. Values are expressed as the percentage of naive (nonsensitized) control values. Data represent mean $\pm$ SEM of $n = 6$ mice. *, $p < 0.05$ vs the 0 h group



environment-related manner. Fig. 5A demonstrates a characteristic, dense lymphocytic area in the submucosal tissue of a proximal airway in an 8-wk-old SP-D^{-/-} mouse. Age- and sex-matched wild-type C57BL/6 littermates did not develop any lymphocyte infiltration in their lung and repeated serological and pathological examination excluded possibility of concurrent infection in these mice (data not shown). FACS analysis of mononuclear cells extracted from lung digests (but not from spleen, Fig. 5B, top left panel) showed that the proportion of CD4⁺ T lymphocytes in the SP-D^{-/-} mice was significantly greater than in the wild-type mice (Fig. 5B, top right panel). Increased levels of TARC (CCL-17) and IL-13 in the BAL fluid of SP-D^{-/-} mice indicated a Th2 bias of their immune system (Fig. 5B, bottom panels).

Because CD4⁺ Th2 cells play a central role in allergic airway inflammation, we subjected SP-D^{-/-} mice to allergenic sensitization using two different protocols. In the first, animals received repeated i.n. treatment

with *Af* on days 0, 7, 14, and 21 (Fig. 5C). Although serum IgE gradually increased in both the SP-D^{-/-} and wild-type groups, SP-D^{-/-} mice not only produced higher levels but IgE also appeared in their serum significantly earlier (at day 7, Fig. 5C). The increased susceptibility to produce IgE was confirmed in the SP-D^{-/-} mice using a second protocol in which mice received systemic sensitization with *Af*/alum (Fig. 1A). Serum total and *Af*-specific IgE and IgG1 were markedly elevated while *Af*-specific IgG2a was decreased in the animals lacking SP-D (Fig. 5D). Thus, CD4⁺ T cell accumulation in the lung of SP-D^{-/-} mice was associated with an increased susceptibility to produce IgE following allergic sensitization.

SP-D inhibited mitogen- and Ag-induced CD3⁺CD4⁺ T cell activation in vitro

To test the hypothesis that SP-D is directly capable of affecting T cell function, recombinant human SP-D dodecamers (purified from

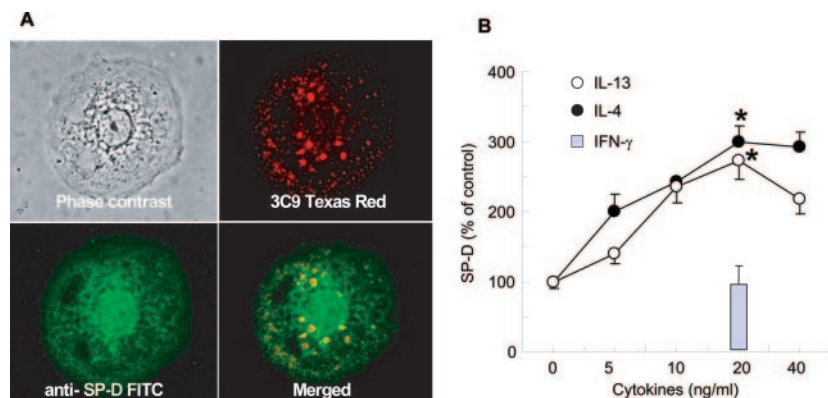
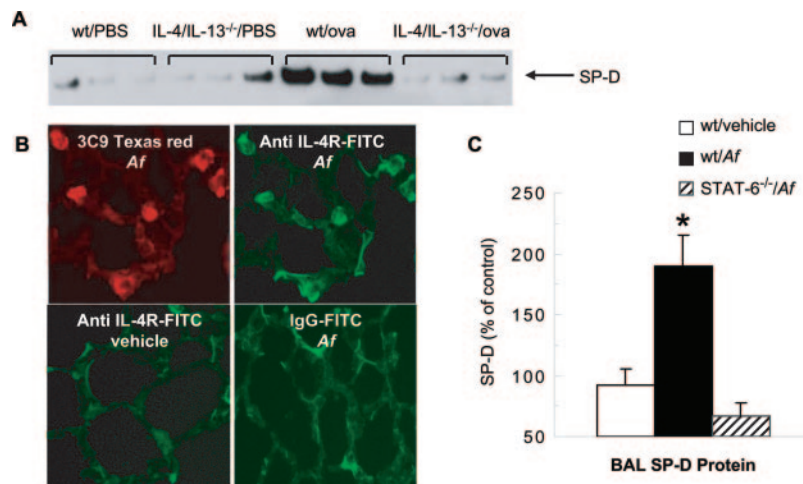


FIGURE 3. IL-4 and IL-13, but not IFN- γ , up-regulated SP-D production in type II alveolar epithelial cells. **A**, A single type II cell (phase contrast, upper left) shows double labeling with anti-SP-D (green, lower left) and 3C9 (anti-ABCA3, red, upper right) by immunofluorescence. Magnification, $\times 100$. **B**, Cells were treated with IL-4, IL-13, or IFN- γ (20 ng/ml) from days 1 to 4 and harvested on day 4 for Western blot analysis. The effect of cytokines on SP-D production is expressed as the percentage of the nontreated controls. Mean $\pm$ SEM of $n = 4$ separate experiments. *, $p < 0.05$ vs no cytokine

FIGURE 4. Allergen-induced SP-D production in the airways was associated with IL-4R-mediated pathways. *A*, Western blot analysis of BAL fluid SP-D expression in wild-type (wt) and IL-4/IL-13^{-/-} mice sensitized with OVA and challenged with PBS or OVA as described previously (34). Each band represents a separate mouse. One of two gels is shown. *B*, Lung sections of mice sensitized and challenged with Af or vehicle and sacrificed on day 28 (as shown in Fig. 1A) were immunolabeled with 3C9 (anti-ABCA3, red) and anti-IL-4R (green). Positive staining for IL-4R of type II alveolar epithelial cells in Af-challenged (upper right) but not in vehicle-challenged mice (lower left). Anti-IL-4R isotype control labeling of an Af-challenged lung (lower right). Magnification, $\times 40$. *C*, Sensitized wild-type (wt) and STAT-6^{-/-} mice were challenged with vehicle or Af (as shown in Fig. 1A) 24 h before study. SP-D production was measured by Western blot in the BAL fluid. Sensitized wild-type mice (■) had increased SP-D in comparison with naive controls (□) and with mice lacking STAT-6 (▨). *, $p < 0.05$ vs wild-type naive. Mean $\pm$ SEM of $n = 6$.



the conditioned medium of stably transfected CHO-K1 cells) were administered to splenic mononuclear cells at 0.1–1.0 $\mu\text{g/ml}$ concentrations. We chose these concentrations because SP-D was previously measured to be 552 ± 174 ng/ml in BAL of female C57BL/10 mice 6–8 wk of age (41). First, we tested whether SP-D would inhibit thymidine incorporation in PMA-ionomycin-stimu-

lated cells (Fig. 6A) and cells induced by PHA (Fig. 6B). We observed significant inhibitory effects that were partially abolished when addition of SP-D was delayed by 24 h (Fig. 6C). IL-2 (10 ng/ml) also partially reversed SP-D inhibition of proliferation (Fig. 6D). These results suggested that SP-D acts at the early phase of T cell activation and, at least partly, via inhibition of IL-2. Baseline

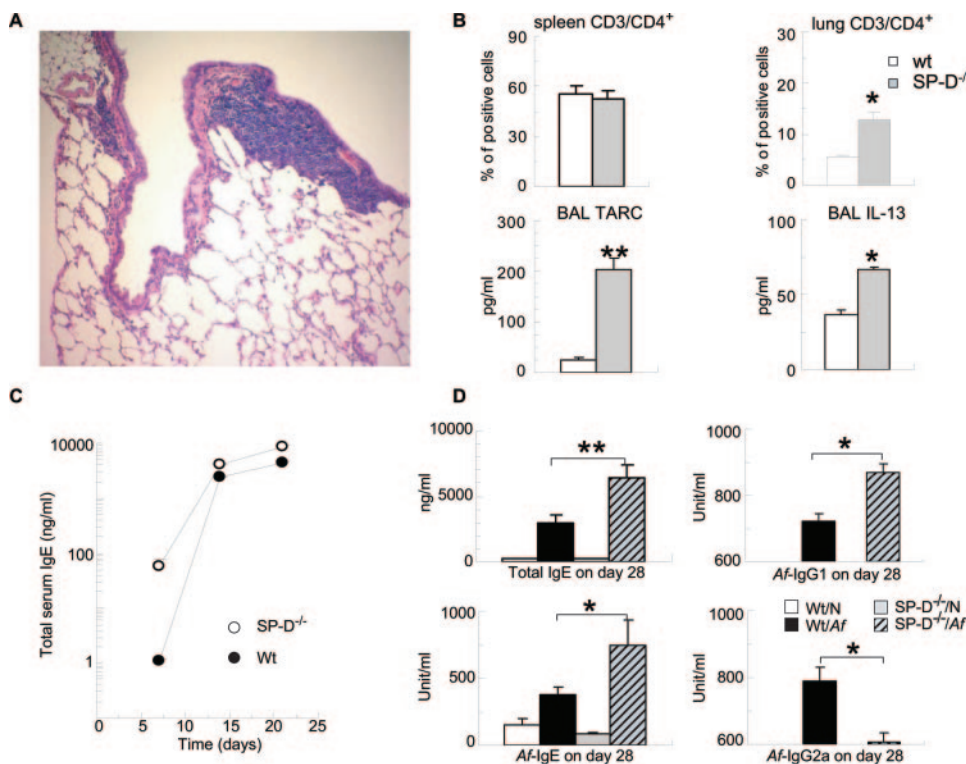
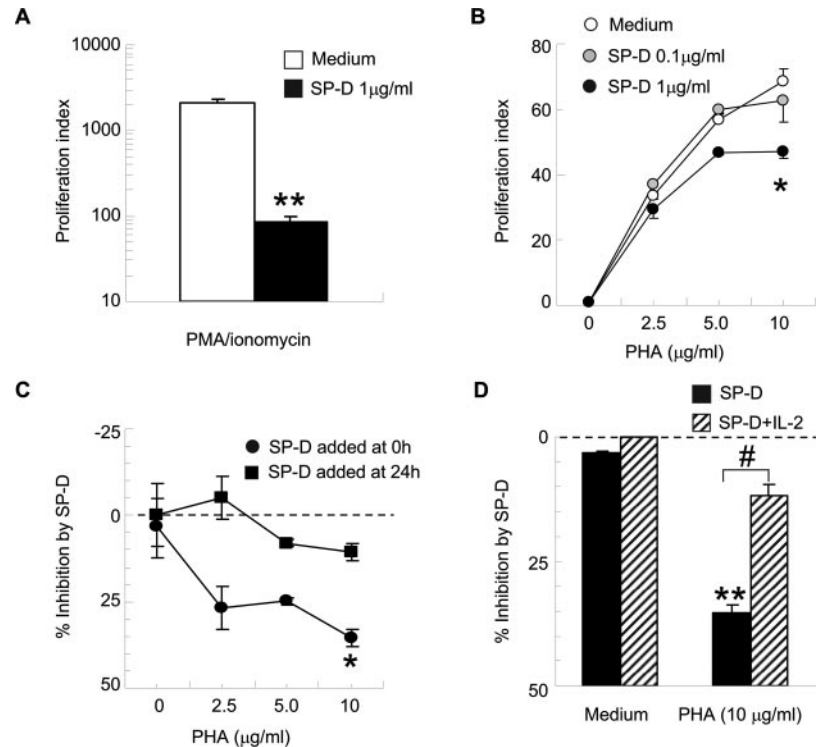


FIGURE 5. SP-D deficiency resulted in accumulation of lymphocytes in the lung and enhanced IgE responses to allergenic sensitization in vivo. *A*, Lymphocyte infiltration of the submucosal tissue of a proximal airway in an 8-wk-old SP-D^{-/-} mouse. *B*, Comparison of the number of CD4⁺ lymphocytes in the lung and the spleen and the TARC and IL-13 levels of BAL between wild-type (□) and SP-D^{-/-} C57BL/6 mice (▨) by FACS analysis of purified mononuclear cells gated on the CD3⁺ population. *, $p < 0.05$. Mean $\pm$ SEM of $n = 6$. *C*, Wild-type (●) and SP-D^{-/-} (○) mice received sensitization exclusively via the airways by i.n. Af treatment on days 0, 7, 14, and 21. Serum total IgE levels were analyzed on days 7, 14, and 21. Mean $\pm$ SEM of $n = 8$. *D*, Serum total IgE, Af-specific IgE, IgG1, and IgG2a levels were measured on day 28 in mice sensitized and challenged with Af (as shown in Fig. 1A). SP-D^{-/-} mice had significantly higher serum IgE and IgG1 levels (▨) than wild-type mice (■), **, $p < 0.01$. Nonsensitized wild-type (□) and SP-D^{-/-} mice (▨) were used as controls. For quantitation of Af-specific Abs (Af-IgE, Af-IgG1, Af-IgG2a), the OD of an in-house positive control serum (1000 U/ml) was used mean $\pm$ SEM of $n = 6$.

FIGURE 6. SP-D suppressed lymphocyte proliferation in vitro in a dose- and time-dependent manner. *A* and *B*, SP-D inhibited mitogen-induced proliferation of splenic mononuclear cells. Lymphocytes isolated from spleens of naive mice were cultured (*A*): with PMA (10 ng/ml) and ionomycin (500 ng/ml), or (*B*): with PHA (0, 2.5, 5, and 10 μ g/ml), in the absence or presence of SP-D (0.1 or 1 μ g/ml). [^{3} H]Thymidine uptake was assessed 72 h after initiation of the culture. Results are expressed as proliferation index. *C* and *D*, SP-D lost its suppressive activity on preactivated cells and in the presence of exogenously added IL-2. Percent inhibition by SP-D (1 μ g/ml, $\bullet$ and $\blacksquare$) compared with “no SP-D” control (dotted lines). *C*, SP-D was given at (0 h) or 24 h after (24 h) the initiation of the cell culture. *D*, Cells were cultured in the presence of SP-D (1 μ g/ml, $\blacksquare$). Mouse rIL-2 (10 ng/ml) was added at the initiation of the culture ($\boxtimes$). Mean $\pm$ SEM of $n = 4$ separate experiments (each performed in triplicates). *, $p < 0.05$ vs medium control; #, $p < 0.05$ SP-D alone vs SP-D + IL-2.



(unstimulated) cpm of naive lymphocytes was not affected by SP-D indicating that it has no toxic effects on these cells and that an activated state of the lymphocytes is a prerequisite for inhibition by this lung collectin.

Because SP-D^{-/-} mice had increased levels of CD4⁺ T cells in the lung, we wanted to investigate whether presence of SP-D would inhibit the function of this population of lymphocytes. Expression of the activation marker CD25, [^{3} H]thymidine uptake, and the generational progression of T cells were investigated in allergen (Af)-stimulated splenic mononuclear cells harvested 24 h after Af challenge of sensitized mice. CD25 was increased on CD3⁺CD4⁺ cells 48 h after Af stimulation of the sensitized, but not naive, lymphocytes in vitro. Enhanced CD25 expression persisted over 120 h of culture (data not shown). This increase was significantly inhibited in the presence of 1 μ g/ml SP-D ($p < 0.05$; Fig. 7A).

CFSE-labeled cell cultures were assessed by FACS 7 days after stimulation with Af. The amount of this intracellular dye is halved as the lymphocytes divide, allowing identification of individual generations. Mononuclear cells were gated for positive CD3/CD4 or CD3/CD8 expression and marked as parental, first, second, and third generation according to their CFSE content. Cells incubated in the presence of 1 μ g/ml SP-D were compared with equivalent populations of cells stimulated in the absence of SP-D and the results are expressed as a percentage of the SP-D-free control. Fig. 7B shows that SP-D increased the number of cells that remained in the parental population following Af stimulation in CD3⁺/CD4⁺ lymphocytes and decreased the number of cells in the second and third generations, in comparison with medium treatment (the middle line). Importantly, no inhibitory effects by SP-D were observed in CD3⁺/CD8⁺ cells (data not shown).

Proliferation of cells isolated from sensitized but not naive mice was significantly increased by Af in vitro, but presence of SP-D abolished the Af-induced changes ($p < 0.01$; Fig. 7C). Similarly to CD25 expression (Fig. 7A) and CFSE distribution (Fig. 7B), no effects of SP-D were seen in [^{3} H]thymidine uptake of nonstimulated cells (Fig. 7C). Indeed, greater antigenic stimulation resulted

in greater suppression of T cell function confirming a lack of toxic effects and a requirement of an activated state for effective suppression by SP-D.

SP-D inhibited Af-induced Th2 cell function

To study whether Ag-stimulated CD4⁺ lymphocytes are also inhibited in their cytokine production, we analyzed the release of IL-4, IL-5, and IFN- γ into the culture supernatant 48 h after initiation of the cell cultures. Although there was a significant, dose-dependent IL-4 and IL-5 release by sensitized cells upon Af stimulation (Fig. 7, D and E), no significant elevations were observed in the levels of IFN- γ (Fig. 7F). Addition of SP-D (1 μ g/ml) to sensitized lymphocytes abolished the Af-induced dose-response curve in IL-4 (Fig. 7D) and significantly inhibited IL-5 (Fig. 7E). However, levels of IFN- γ were increased in comparison with the nontreated samples ($p < 0.05$). Thus, in addition to inhibiting cell proliferation, SP-D also suppressed allergen-induced Th2 cytokine production by individual cells. To rule out the effect of LPS on proliferating lymphocytes, purified SP-D was assayed for its endotoxin content and was found to have <0.1 pg of LPS/ μ g SP-D. This level of endotoxin has no effect on lymphocyte function.

Discussion

The lung is a hyporesponsive immunological environment and failure to restrain immune reactions can precipitate lung injury. This study demonstrates that allergen exposure increased production of the immunosuppressive innate immune molecule SP-D. The rise in the BAL SP-D levels coincided with resolution of the inflammatory changes 48 h after a single allergen challenge. Enhancement in SP-D production was reproduced in vivo and in vitro by administration of IL-4 and IL-13, but not IFN- γ . In addition, IL-4/IL-13 double knockout and STAT-6-deficient mice failed to increase SP-D upon allergen challenge indicating a regulatory role of Th2 cytokines acting on the IL-4R. Mice lacking SP-D developed a greater IgE response upon allergic airway sensitization than wild-type animals, while addition of SP-D inhibited allergen-induced T cell activation and Th2 cytokine

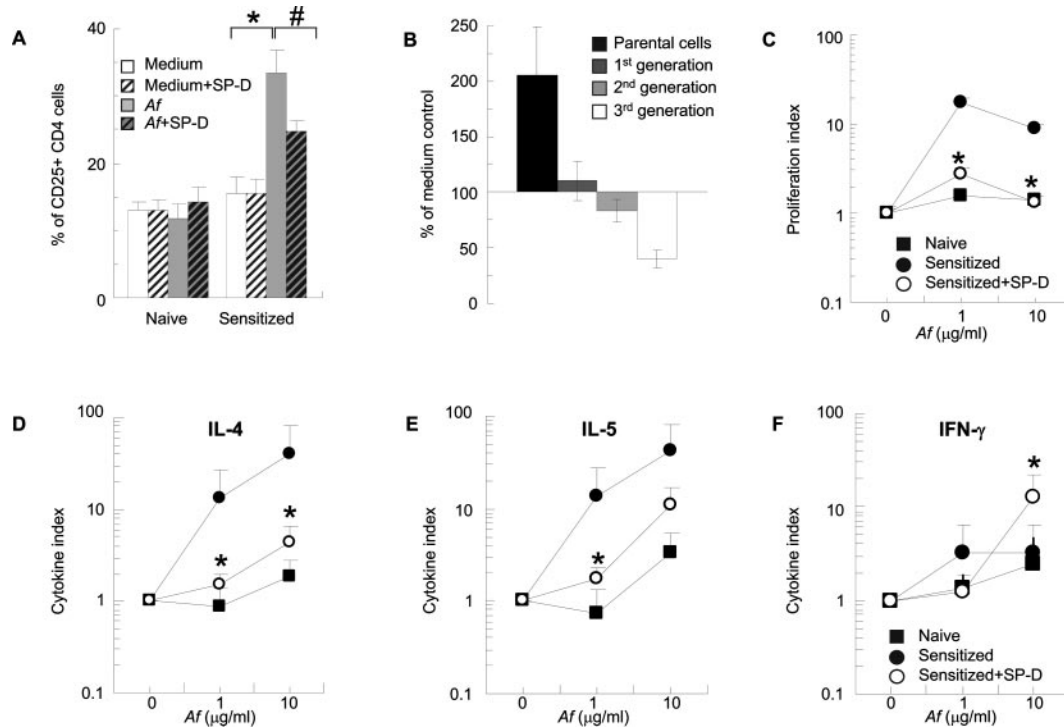


FIGURE 7. SP-D inhibited allergen-driven Th2 cell responses in vitro. Splenic mononuclear cells (2×10^6 /ml) of either naive or sensitized mice were cultured with Af in the absence or presence of rSP-D (1 μ g/ml). **A**, Expression of CD25 on the CD3⁺CD4⁺ T cells was assessed by FACS analysis 48 h after initiation of the cell culture. (Naive cells, *left group of bars*; sensitized cells: *right group of bars*) □: medium; ▨: Af (1 μ g/ml). ▩: SP-D (1 μ g/ml). Mean $\pm$ SEM of $n = 4$ separate experiments; *, $p < 0.05$ vs medium; #, $p < 0.05$ vs sensitized cells without SP-D. **B**, Proliferation was assessed by [³H]thymidine uptake 96 h after initiation of the culture and expressed as proliferation index. **C**, Distribution of CFSE between the parental (■) and subsequent generations (gray, light gray, and white bars) was assessed by FACS analysis of the CD3⁺CD4⁺ cells 96 h after Af stimulation. The effects of SP-D treatment were compared with and expressed as the percentage of the nontreated controls. **D–F**, Af rechallenge of sensitized (●) and naive cells (■) and the effects of addition of SP-D (1 μ g/ml, ○) were studied in vitro for IL-4 (**D**), IL-5 (**E**), and IFN- γ (**F**). Mean $\pm$ SEM of $n = 4$ separate experiments *, $p < 0.05$; **, $p < 0.01$ vs sensitized cells without SP-D.

release in vitro. Thus, the presence of SP-D in the distal air spaces is very likely to be important for maintaining a normal level of suppression of adaptive immunity.

Although inflammatory changes of the lung generally induce enhanced expression of both collectins (SP-A and SP-D) (42, 43), allergic inflammation appeared to result in an intriguing selective increase of SP-D protein levels in our previous studies (24, 25). SP-D expression is known to be induced by hormones and second messengers including dexamethasone, insulin, cAMP, and phorbol ester (44, 45) but cytokines that are central for development of a Th2-type inflammatory response have also been implicated. Transgenic mice overexpressing IL-4 (46, 47), and IL-13 (48) showed markedly elevated SP-D levels in the lung. To better understand the mechanisms that regulate SP-D expression, in this study we used two different models of the allergic airway response. In the first one, we used a ubiquitous airborne allergen, shown to bind both SP-A and SP-D (49). To verify that the increase in SP-D was not due specifically to treatment with Af but was the result of the elicited allergic inflammation, we applied a second model based on sensitization and repeated challenges with OVA, the most commonly used Ag to induce allergic airway inflammation in mice (34). Allergen challenge in both models induced increased SP-D expression. Our time-course studies revealed that SP-D expression followed a characteristic kinetics with a late onset after allergen provocation that coincided with resolution of most of the proinflammatory changes. A strong positive correlation between IL-4 and SP-D early after Af challenge suggested the possibility of a regulatory relationship between these molecules.

Because IL-4 and IL-13 have well-established effects on altering epithelial cell function in allergic inflammation, we hypothesized that they may also directly affect SP-D production. Recombinant IL-4 and IL-13 (but not IFN- γ) significantly enhanced SP-D mRNA and protein levels in isolated rat type II epithelial cells and in vivo, in mouse lungs confirming in two species, that SP-D up-regulation during the allergic changes was specific to the action of these Th2 cytokines. Our results also showed that allergic sensitization and challenge of BALB/c mice induced the expression of IL-4Rs on type II alveolar epithelial cells and that the SP-D production elicited after Af challenge in sensitized wild-type BALB/c mice was absent in mice lacking IL-4 and IL-13 or STAT-6. Thus, allergen exposure increased SP-D mRNA and protein levels in the lung that was mediated by IL-4 and IL-13. However, the role of such enhancement in production of this lung collectin needed to be clarified.

A protective immunoregulatory function of SP-D has recently been suggested by studies in gene knockout mice. Animals lacking this protein have shown an enhanced susceptibility to inflammation, particularly following microbial challenge (7, 31, 32, 50, 51). Consistent with a protective role of this molecule, the lung of SP-D^{-/-} mice showed significant modifications (in comparison with wild-type mice). As previously described, a constitutive presence of activated macrophages, accumulation of lipoproteins in the distal air spaces, and a consequent emphysematous tissue morphology (31, 32, 51) is associated with physiological alterations such as increased tidal volume (52). We also observed airway submucosal accumulation of lymphocytes of the CD3/CD4 subclass

with increased expression of IL-13 and TARC in the BAL fluid and enhanced IgE and IgG1 response to allergic sensitization.

The SP-D^{-/-} mice have been studied recently by Schaub et al. (53). As suggested by their studies, we found that SP-D^{-/-} mice have a Th2-biased immune system in the lung with increased IL-13 expression. However, while Schaub et al. (53) saw no increased IgE, we found enhanced serum total and Af-specific IgE and IgG1 in the SP-D^{-/-} mice. In contrast, they described an increased tissue eosinophilia in these animals that we did not observe in our model. The increased eosinophil number was only present at the early time points after sensitization (53). The discrepant findings are probably due to the different allergens (Af in our study and OVA in theirs) and different sensitization protocols used in the two studies. The discrepancies between the different studies in the future maybe resolved by aligning the allergic sensitization protocols, modifying the genetic background of the SP-D^{-/-} mice (currently these mice are on a C57BL/6 background), and by eliminating the constitutively activated macrophages by the use of conditional SP-D knockouts (54).

Because of development of a secondary lymphoid tissue in the peribronchial areas with enhanced IgE and IgG1 responses, it is possible that SP-D directly affects B cells without T cell involvement. However, the increased number of CD4⁺ cells in SP-D^{-/-} mice together with earlier studies by Fisher et al. (55) who demonstrated accumulation and activation of T lymphocytes in these mice, suggest a suppressive SP-D effect on T lymphocyte function. In addition, direct T cell suppression has been shown (3, 4, 6), and it is now established that SP-D treatment can alleviate Th2-mediated allergic inflammatory changes in mice (reviewed in Ref. 56). In this study, we further investigated the mechanisms of the SP-D effects on T cells.

Binding of allergens, such as particles of Af (28, 29) and the house-dust mite (57), could provide one mechanism by which SP-D interferes with allergen-induced T cell activation. Indeed, SP-D can block IgE immune complex-mediated histamine release (58) and enhance Ag presentation by dendritic cells (59). However, our data suggest that the inhibitory effects of this lung collectin did not depend exclusively on interference with the Ag-TCR interaction because cells stimulated with PMA and ionomycin or by PHA were also inhibited by SP-D, indicating that SP-D directly suppresses T cell activation. Inhibition of lymphocyte proliferation by SP-D was dose-dependent and was not due to toxic effects because baseline (nonstimulated) proliferation of the lymphocytes was not affected. This is in agreement with Borron et al. (6) who showed that SP-D inhibited lymphocyte proliferation but it did not affect cell viability or apoptosis of the cells. In our Ag-stimulated cultures, cells exposed to greater Ag concentrations appeared more susceptible to inhibition by SP-D. Indeed, at higher Af concentrations CD3⁺CD4⁺ (but not CD8⁺) lymphocytes had a significantly greater proportion of their cells in the parental population, and reduced expression of CD25, the α -chain of the IL-2R, in the presence of SP-D.

SP-D can inhibit T cell proliferation through both an IL-2-independent action that involves attenuation of cytosolic free calcium, and through an IL-2 and TCR-dependent mechanism (3, 6). Interestingly, we found that the effects of SP-D were partially diminished when IL-2 was added to PHA-stimulated cultures. Furthermore, when the addition of SP-D was delayed by 24 h, its inhibitory effects were abolished suggesting that SP-D acts at an early phase of T cell activation. The inhibitory effects of SP-D treatment on Th2 cytokine expression were previously suggested by ex vivo studies (5) showing that there was less IL-4 and IL-5 in the splenic supernatant of SP-D-treated mice than in nontreated controls. We hypothesized here that SP-D exerts a direct inhibitory

effect on Ag-induced Th2 processes and found that Af-stimulated IL-4 and IL-5 production was abolished by SP-D, strongly suggesting that allergen-stimulated CD4⁺ T lymphocytes are inhibited by this lung collectin.

In summary, resolution of the allergic response to inhaled Af is associated with an IL-4/IL-13-mediated increase of SP-D. Lack of this molecule results in heightened CD4 activation with increased IgE to allergenic sensitization in vivo while addition of SP-D to Af-stimulated lymphocyte cultures directly inhibits Th2 cell activation in vitro. This study suggests the existence of a negative feedback regulatory circuit with the function of maintaining hyporesponsiveness to inhaled allergens.

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