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This information is current as of January 26, 2011

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J Immunol; Prepublished online 21 January 2011;
doi:10.4049/jimmunol.0904190
<http://www.jimmunol.org/content/early/2011/01/21/jimmunol.0904190>

Supplementary Data	http://www.jimmunol.org/content/suppl/2011/01/24/jimmunol.0904190.DC1.html
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The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
9650 Rockville Pike, Bethesda, MD 20814-3994.
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.



Lung Effector Memory and Activated CD4⁺ T Cells Display Enhanced Proliferation in Surfactant Protein A-Deficient Mice during Allergen-Mediated Inflammation

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Although many studies have shown that pulmonary surfactant protein (SP)-A functions in innate immunity, fewer studies have addressed its role in adaptive immunity and allergic hypersensitivity. We hypothesized that SP-A modulates the phenotype and prevalence of dendritic cells (DCs) and CD4⁺ T cells to inhibit Th2-associated inflammatory indices associated with allergen-induced inflammation. In an OVA model of allergic hypersensitivity, SP-A^{-/-} mice had greater eosinophilia, Th2-associated cytokine levels, and IgE levels compared with wild-type counterparts. Although both OVA-exposed groups had similar proportions of CD86⁺ DCs and Foxp3⁺ T regulatory cells, the SP-A^{-/-} mice had elevated proportions of CD4⁺ activated and effector memory T cells in their lungs compared with wild-type mice. Ex vivo recall stimulation of CD4⁺ T cell pools demonstrated that cells from the SP-A^{-/-} OVA mice had the greatest proliferative and IL-4-producing capacity, and this capability was attenuated with exogenous SP-A treatment. Additionally, tracking proliferation in vivo demonstrated that CD4⁺ activated and effector memory T cells expanded to the greatest extent in the lungs of SP-A^{-/-} OVA mice. Taken together, our data suggested that SP-A influences the prevalence, types, and functions of CD4⁺ T cells in the lungs during allergic inflammation and that SP deficiency modifies the severity of inflammation in allergic hypersensitivity conditions like asthma. *The Journal of Immunology*, 2011, 186: 000–000.

Lung surfactant is a lipoprotein complex that reduces surface tension at the air–liquid interface and participates in host defense. One of its protein components, surfactant protein (SP)-A, is a member of the collectin family of innate immune molecules, and as such, functions in host defense against a variety of inhaled microbial pathogens by acting as an opsonin (reviewed in Refs. 1–3) and by regulating immune cell function (3–5).

In comparison with our understanding of the role of SP-A in innate immunity and infectious disease models (6–9), relatively little is known about the role of SP-A in allergic hypersensitivity conditions, such as atopic asthma; its increasing prevalence and

related health complications are a top reason for absenteeism from school and work in the United States (10). Mounting evidence suggests that SP-A plays a protective role in allergic hypersensitivity and that this protection is attenuated in conditions in which SP-A levels are decreased or SP-A is inactivated (11–15). We showed in vitro that SP-A modulates the functions of two adaptive immune cells that play critical roles in asthma pathogenesis. Specifically, SP-A inhibits T cell proliferation in an accessory cell-independent manner and inhibits dendritic cell (DC) maturation and their ability to subsequently stimulate T cell proliferation (16, 17). In addition, other reports showed that SP-A inhibits the in vitro proliferation of Ag-stimulated human PBMCs (18), murine splenocytes cocultured with OVA-specific T cell hybridomas (17), and sensitized murine splenocytes to *Aspergillus fumigatus* rechallenge (12). Administration of SP-A in *A. fumigatus*-treated mice was shown to attenuate eosinophilia and cytokine production (19). Taken together, these studies suggested that SP-A functions as a dynamic link between innate and adaptive immunity and that it may be an important regulator of inflammatory consequences associated with allergic lung disease.

DCs and T cells are critical inducers and mediators of adaptive immune responses. Immature DCs are primarily phagocytic, and exposure to inhaled allergens normally results in a state of tolerance by producing anti-inflammatory cytokines, such as IL-10, or by stimulating suppressive activity of T regulatory cells (Tregs) (20, 21). Upon Ag challenge, DCs undergo a maturation process, as evidenced by increased expression of MHC class II (MHCII) and costimulatory molecules CD86 and CD80, which allow for effective Ag presentation to T cells in regional lymph nodes or locally in tissue (22, 23).

CD4⁺ T cells are a heterogeneous population, endowed with different migratory capacities and effector functions. Naive T (T_N) cells are thought to have not yet encountered cognate Ag and to

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Received for publication December 29, 2009. Accepted for publication December 14, 2010.

This work was supported by National Institutes of Health/National Heart, Lung, and Blood Institute Grants 5P50HL084917 and 5R01HL68072. A.M.P. was supported by National Institutes of Health/National Institute of Allergy and Infectious Diseases Grant 1K08AI068822 and by Duke University's Center for Comparative Biology of Vulnerable Populations Grant 5P30-ES-011961-04. G.D.S. is supported by Grant 5R01AG025150 from the National Institute of Aging. The Duke Human Vaccine Institute Flow Cytometry Core Facility is supported by National Institutes of Health Grant AI-051445.

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The online version of this article contains supplemental material.

Abbreviations used in this article: BALF, bronchoalveolar lavage fluid; BMDC, bone marrow-derived dendritic cell; DC, dendritic cell; MHCII, MHC class II; PE-H, allophycocyanin PE; SP, surfactant protein; T_A cell, activated T cell; T_{CM} cell, central memory T cell; T_{EM} cell, effector memory T cell; T_N cell, naive T cell; Treg, regulatory T cell; WT, wild-type.

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have high surface expression of the lymph node-homing receptor L-selectin (CD62L) and low expression of the memory cell marker CD44 (24, 25). Upon recognition of its cognate Ag, the T_N cell acquires an activated phenotype (T_A) with reduced CD62L expression. These cells may further differentiate into memory cells. Memory cells can be divided into central memory T (T_{CM}) cells and effector memory T (T_{EM}) cell subsets. T_{CM} cells express CD62L and lack immediate effector function; however, upon re-stimulation in secondary lymphoid organs, they proliferate and differentiate into effector cells (26). T_{EM} cells lack CD62L and express receptors for migration into inflamed tissue. Upon re-encounter with Ag, these cells have immediate effector function and can rapidly produce inflammatory mediators, such as Th2-associated cytokines IL-4 and IL-5 (27). Naturally occurring Tregs, which constitutively express the α -chain of the IL-2R CD25 and the intracellular transcription factor Foxp3, can inhibit DCs from initiating Th2-driven responses and suppress Th2 effector cell function (28–30). Failure of Tregs to limit the activity of immune cells implicated in asthma may contribute to the development of the disease (31). In the asthmatic condition, effector CD4⁺ T cells accumulate in the lung and perpetuate a Th2 pattern of inflammation. Increased numbers of activated T cells in people with asthma typically correlate with the numbers of activated eosinophils, the levels of cytokines IL-4 and IL-5, the magnitude of decrement in peak expiratory flow rates, and the severity of disease (32, 33).

The lungs are continuously exposed to a barrage of environmental irritants that challenge the tight regulation between an active immune defense and tolerance. We hypothesized that SP-A, as part of the local microenvironment of the lung, is critical in modulating the phenotype and prevalence of DCs and CD4⁺ T cells to inhibit characteristic Th2-associated inflammatory indices associated with allergen-induced inflammation. We combined the use of SP-A-deficient mice, the well-characterized OVA-driven model, and in vivo and ex vivo functional assays that used lung-derived cells to examine the dynamic link between innate and adaptive immunity mediated by SP-A.

Materials and Methods

Mice and OVA-alum model of allergic asthma

SP-A^{-/-} mice were backcrossed for 12 generations onto a C57BL/6 background. Age- and sex-matched control C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME) or Charles River Laboratories (Wilmington, MA). Experimental protocols were approved by the Duke University Institutional Animal Care and Use Committee and were carried out in accordance with the standards established by the U.S. Animal Welfare Acts. Mice (6–8 wk) were sensitized on days 0 and 14 by i.p. injections of 0.1 ml saline containing 10 μ g OVA (Grade V, Sigma-Aldrich) complexed with 2.0 mg Inject Alum (Pierce Biotechnology, Rockford, IL). On days 21–23, mice were exposed to 1% aerosolized OVA in sterile saline for 20 min in a 60-l Hinner exposure chamber connected to an ultrasonic nebulizer that delivers aerosol particles with 0.6–0.7 μ m mean diameter (DeVilbiss Ultra-Neb, replaced by Novag Ultrasonic 2000, Susquehanna Micro, PA). Sham mice were given i.p. injections of alum/saline and were aerosolized with saline. Animals were euthanized, and tissue samples were harvested 24 h after the last aerosol challenge. All OVA preparations had an average endotoxin concentration of 70 EU/mg using the *Limulus* ameocyte lysate assay (QCL-1000, BioWhittaker [Lonza], Walkersville, MD).

Lavage and serum protein analysis and histology

Total cell counts, cell differentials, and total protein, as well as SP-D protein analyses in the bronchoalveolar lavage fluid (BALF) and IgE Ab analyses in the serum were performed. Briefly, lungs were lavaged three times with 1 ml PBS/0.1 mM EDTA solution, and collected BALF was centrifuged to pellet the cells; only the supernatant from the first milliliter collected was aliquoted and frozen at -80°C for later use in cytokine, total protein, and Western blot assays. Cell viability was determined via trypan blue exclusion. Cell differentials were determined on ≥ 500 cells using standard

hematological criteria using cytospin preps stained with Wright-Giemsa. Total protein was determined using a Micro BCA[®] Protein Assay Reagent (Pierce, Rockford, IL) using the microtiter plate protocol and a standard curve prepared from assaying known amounts of BSA. Cytokine protein levels were analyzed via ELISA (R&D Systems, Minneapolis, MN). Serum samples were analyzed for total IgE via ELISA (BD Pharmingen, San Diego, CA). To measure SP-D protein levels, equivalent amounts of BALF supernatant (25 μ l/lane) were electrophoresed and transferred to a nitrocellulose membrane. The membrane was immunoblotted with a rabbit anti-mouse Ab directed against SP-D (diluted 1/5,000 in TBS containing 1% Tween 20 and 3% nonfat dry milk) and then incubated with goat anti-rabbit IgG Ab conjugated to HRP (diluted 1/10,000 in TBS containing 1% Tween 20 and 3% nonfat dry milk). Immunoblots were developed by chemiluminescence. To obtain tissue sections for histology, lungs were inflated and fixed with 4% formaldehyde at a pressure of 25 cm of water. Tissues were embedded in paraffin 24 h after fixation. Seven-micron sections were cut and stained with periodic acid-Schiff–diastase.

Isolation of DCs and T cells for flow cytometry

DCs and T cells were isolated by density gradient centrifugation, as previously described (34). Briefly, the lungs were perfused to remove intravascular cells and lavaged to remove cells in the airway lumen. Only lungs that were well perfused, as judged by their degree of whiteness, were excised and used for further analyses. Lung tissues were minced with a razor blade and digested in HBSS with the enzymes collagenase A and DNase I (Roche, Indianapolis, IN) for 1 h with shaking (200 rpm) at 37°C . Single-cell suspensions were obtained by passing the digest through a 40- μ m mesh filter, and erythrocytes were lysed by 1-min incubation in Gey's lysis solution (0.83% NH₄Cl, 0.1% KHCO₃). After lysis and centrifugation, the cell pellet was resuspended in buffer containing HBSS with 5% FCS, 2 mM EDTA, and 100 U/ml penicillin-streptomycin. Cells from the lung digests were layered on top of a 4.0% solution of Optiprep (Axis-Shield PoC AS, Rodeloekka, Norway), placed above a 16% Optiprep solution, and centrifuged at $600 \times g$ for 20 min at room temperature, without applying the brakes at the end. The low-density cells, which included DCs and T cells, were isolated from the 4–16% interface. Splenocytes and lymphocytes from the mediastinal and inguinal lymph nodes were gently teased out from the tissue and passed through a 40- μ m mesh filter, and the erythrocytes were lysed. Thereafter, cells were prepared for flow cytometry.

Flow cytometry

Flow cytometry was performed using a BD LSRII (BD Biosciences, San Diego, CA) at the Duke University Human Vaccine Institute Comprehensive Core facilities. Cells were incubated first with anti-mouse CD32/CD16 (Fc γ III/II receptor)-blocking Ab and then incubated with the appropriate staining reagents in PBS and 0.2% BSA buffer for 30 min at 4°C . Stained cells were fixed with 4% formaldehyde. The following mAbs, purchased from BioLegend, eBioScience, or BD Pharmingen (San Diego, CA), were used for various labeling reactions: allophycocyanin/PE (PE-H) anti-CD11c, FITC anti-MHCII (IA/IE), PE-H anti-CD86, FITC/allophycocyanin anti-CD3, PE-Texas red anti-CD4, PE-H anti-CD25 and CD44, and allophycocyanin anti-CD62L. For intracellular staining using FITC-Foxp3 (BD Pharmingen) or anti-BrdU Abs (eBioScience), cells were washed well after surface staining, fixed in 10% neutral buffered formalin, and permeabilized with 0.3% saponin in HBSS + 0.5% FBS for BrdU or Foxp3 staining. Because the anti-BrdU Ab was conjugated to biotin rather than a fluorophore, an additional intracellular staining with streptavidin-AF633 (Molecular Probes) or streptavidin-AF488 (BioLegend) was performed. At least 20,000 events per sample were collected on the FACS instrument. Data were analyzed using FlowJo 8 software (Tree Star, Ashland, OR). Prior to cell marker examination, dead cells, aggregates, and debris were eliminated according to forward scatter height and area.

SP-A preparation. SP-A was purified from the lung lavage fluid of patients with alveolar proteinosis, as described previously (35). Briefly, the lavage fluid was initially treated with butanol to extract SP-A from the lipids. The resulting pellet was then sequentially solubilized with octylglucoside and 5 mM Tris (pH 7.4). Extracted SP-A was treated with polymyxin agarose to reduce endotoxin contamination. SP-A preparations had final endotoxin concentrations <0.01 pg/mg SP-A, as determined by the *Limulus* ameocyte lysate assay, according to manufacturer's instructions (QCL-1000, BioWhittaker [Lonza]).

Ex vivo T cell stimulation assay. Single-cell suspensions from lung digests and spleens were subject to density-gradient centrifugation using Ficoll-Hypaque 1083 (Sigma), and residual RBCs were lysed. Purified CD4⁺ T cells were then obtained by negative selection using paramagnetic microbeads. CD4⁺ T cell purities averaged 97–99%. Bone marrow-derived

dendritic cells (BMDCs) were harvested from the marrow of the tibia and femur, washed, and cultured in complete RPMI 1640 supplemented with 5% GM-CSF conditioned medium for 6 d. Loosely attached cells were harvested and negatively selected with biotinylated Gr-1 Abs (BD Pharmingen) and streptavidin paramagnetic microbeads (Miltenyi Biotec). CD4⁺ T cells (~100,000) were incubated under different experimental conditions in Costar 96-well round-bottom plates in complete RPMI (RPMI 1640 with 5% heat-inactivated FBS [Hyclone]), 25 mM HEPES, 5 μ M 2-ME, 100 U/ml penicillin-streptomycin, and 2 mM L-glutamine (all from Life Technologies, Invitrogen, NY). T cell activation was performed with 2 μ g/ml Con A (Sigma) or indicated ratios of DCs pulsed with OVA. Exogenous SP-A was functionally titrated to saturating levels prior to performing T cell activation assays and was added at 20 μ g/ml at the beginning of the culture. [³H]Thymidine (0.8 μ Ci, 6.7 Ci/mmol; MP Bio) was added to each well for the final 15 h of culture. Incorporated radioactivity (as an indicator of proliferation) was measured by liquid scintillation using CytoScint ES (MP Bio) on a TriCarb 2100TR (Packard Instruments) or MiniBeta counter. IL-4 ELISAs were performed from culture supernatants using eBioScience Ready-Set-Go kits, according to the manufacturer's instructions.

In vivo T cell-proliferation assay. To track the proliferation of T cell subsets in vivo, mice were given i.p. injections of BrdU $\geq$ 2 h before each OVA aerosol treatment and were supplied water supplemented with 0.8 mg/ml BrdU in 4% sucrose ad libitum during the OVA aerosol period (days 21–23). Single-cell preparations from lung digests or spleen were labeled with T cell markers and analyzed by flow cytometry for BrdU incorporation in the nucleus (36).

Statistical analysis

Data were analyzed using SPSS V17.0 (SPSS, Chicago, IL) or Prism 5 (GraphPad Software). Results are reported as group means $\pm$ SEM. Parametric data were analyzed by ANOVA to determine differences among group means and then by the Tukey multigroup comparison to determine which group means differed significantly. Where mentioned, pairwise comparisons using the Mann–Whitney *U* test were performed; *p* values $\leq$ 0.05 were considered statistically significant.

Results

SP-A^{-/-} mice exhibit enhanced Th2-associated responses during allergen-mediated lung inflammation

Hallmark indicators of allergic asthma include airway eosinophilia and the production of mucin, Th2-patterned cytokines, and IgE Abs (37). To assess the influence of SP-A on these parameters, SP-A^{-/-} and wild-type (WT) mice were sensitized and challenged with OVA (SP-A^{-/-} OVA, WT OVA), and the lungs were lavaged, excised, and prepared for analysis. Normal lung histology was observed in the absence of OVA exposure (Fig. 1, WT sham and SP-A^{-/-} sham). WT and SP-A^{-/-} OVA mice exhibited the expected OVA-mediated histological changes, as demonstrated by perivascular and peribronchiolar inflammatory cell infiltration and mucin production (Fig. 1). The SP-A^{-/-} OVA mice exhibited increased total cell and eosinophil counts (Fig. 2A) in the BALF compared with their WT counterparts. SP-A^{-/-} OVA mice had greater BALF IL-4 and IL-5 protein levels (Fig. 2B) and serum IgE levels (Fig. 2C) compared with WT OVA mice, suggesting that SP-A modulates the extent of the Th2 response and subsequent humoral response in allergen-mediated lung inflammation. The levels of Th1-associated cytokines, IFN- γ , IL-1 α , IL-1 β , and IL-12, in the BALF did not differ between the OVA groups (data not shown), suggesting that SP-A preferentially downregulated the Th2-associated response. The levels of total protein, as a measure of epithelial layer integrity, were reported to increase in the BALF of OVA-challenged mice (38, 39). The differences in the levels of inflammation between the WT and SP-A^{-/-} OVA mice, as measured by BALF cell counts and cytokine levels, do not seem to be a consequence of variations in total protein levels (data not shown) or in SP-D levels (data not shown), because these measures increased similarly in the BALF of both OVA groups.

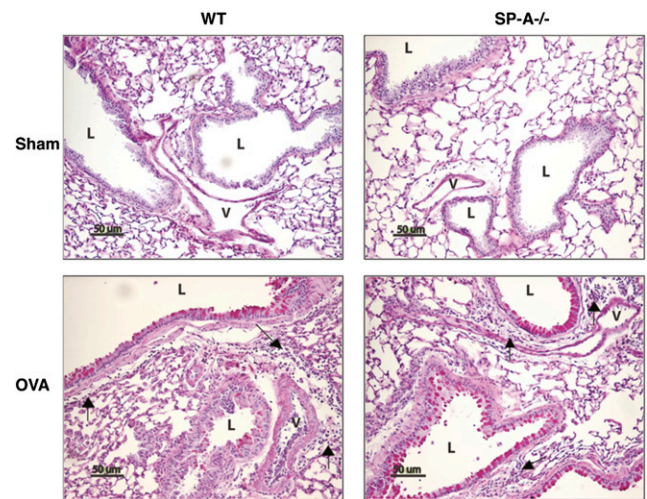


FIGURE 1. Inflammatory cellular infiltrates and mucus production in the lung tissue of OVA mice. Histology was evaluated 24 h after the last aerosol exposure in periodic acid-Schiff–diastase-stained tissue sections. Mucus is stained bright pink. Representative of two mice/group from three separate experiments. L, airway lumen; V, blood vessel lumen; $\rightarrow$, mononuclear inflammatory cell infiltrate.

SP-A^{-/-} and WT mice have similar proportions of mature DCs and Foxp3-expressing CD4⁺/CD25⁺ Tregs in the lungs during allergen-mediated lung inflammation

To determine whether SP-A in the lung microenvironment influences DC phenotype, we analyzed DCs for the expression of MHCII and costimulatory molecules, which are essential for the initiation and amplification of T cell-mediated responses and serve as an index of DC functional maturity (40). CD86 is the predominant costimulatory ligand on DCs responsible for CD28-mediated costimulation that leads to T cell activation (41). In flow cytometric assays, DCs were discriminated from alveolar macrophages by taking advantage of the high autofluorescence of alveolar macrophages and the variation in the levels of MHCII expression (42–45). Thus, we identified DCs as CD11c⁺ and MHCII^{hi} cell surface staining. As expected, the total numbers of low-density cells and the percentage of DCs within that pool of cells increased in response to OVA challenge (Fig. 3A, 3C), and the total number of DCs and CD86⁺ DCs in the lungs increased comparably between WT and SP-A^{-/-} OVA mice in response to OVA challenge (data not shown). In addition, the geometric mean fluorescence intensity of MHCII and CD86 surface expression did not differ among the groups of mice (data not shown). Taken together, these data indicated that SP-A does not specifically alter the phenotype and prevalence of DCs, including those expressing the maturation marker CD86, in response to allergen challenge.

CD25, the high-affinity IL-2R α -chain, contributes to T cell activation by stimulating proliferative pathways in response to autocrine and paracrine IL-2. CD25 is expressed in the global T cell population upon activation. It is also expressed constitutively on Foxp3⁺ Tregs, which inhibit DCs from initiating Th2-driven responses and suppress Th2 effector cell function (28–30). Thus, we determined whether the presence of SP-A in the lung microenvironment influences the proportions of CD25⁺ and Foxp3⁺/CD25⁺ T cells in the lung. Although flow cytometric data revealed that the total numbers of CD3⁺/CD4⁺ cells in the lungs were not statistically different among the groups, the percentage of CD3⁺/CD4⁺ T cells that expressed CD25 tended to be highest in the SP-A^{-/-} OVA group (data not shown). However, there was no difference in the percentage of CD25 cells that concurrently expressed Foxp3 among the groups (data not shown). Taken to-

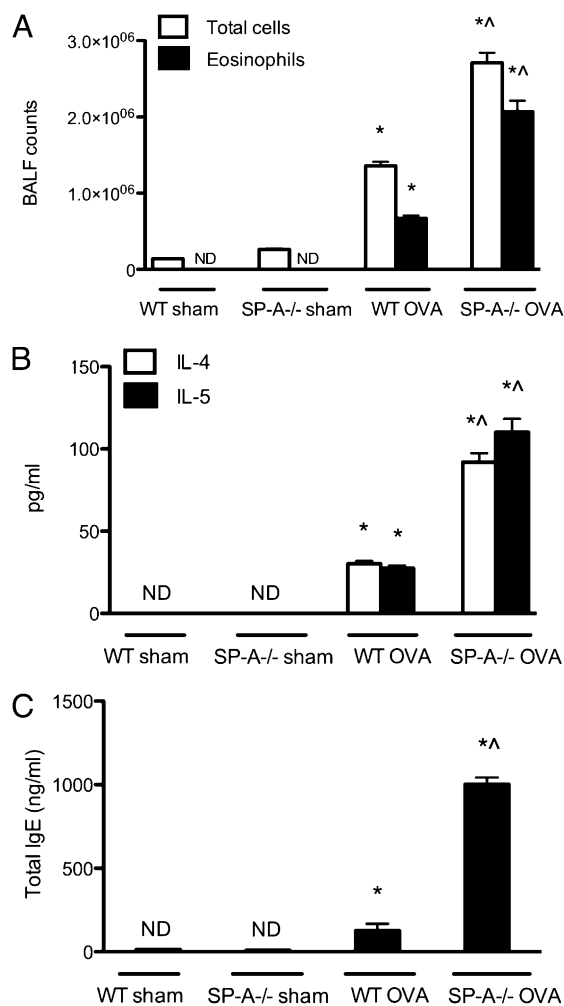


FIGURE 2. SP-A^{-/-} OVA mice have elevated Th2-associated inflammatory indices. **A**, BALF cells were counted, and cell differentials were determined using standard hematological criteria. Eosinophil counts were derived by multiplying the percentage of eosinophils by the total BALF cell counts ($n = 10$ mice/group). * $p < 0.05$, compared with sham-treated controls, which did not differ from each other; $\wedge p < 0.05$, compared with WT OVA. **B**, BALF supernatant was collected and analyzed for IL-4 and IL-5 protein by ELISA ($n = 8$ –10 mice/group). * $p < 0.05$, compared with sham-treated controls, whose levels were not detectable; $\wedge p < 0.05$, compared with WT OVA. Serum samples were monitored for changes in total IgE levels via ELISA, as described in *Materials and Methods* ($n = 10$). * $p < 0.05$ compared with sham-treated controls, whose levels were not detectable; $\wedge p < 0.05$, compared with WT OVA. ND, not detectable.

gether, these results indicated that although SP-A does not modify the prevalence of Foxp3-expressing CD4⁺/CD25⁺ Tregs in lungs in response to allergen challenge, it nominally alters the prevalence of CD4⁺ T cells that express the activation marker CD25. This phenotypic state was further corroborated by functional studies using the pan-T cell mitogen Con A. As seen in Supplemental Fig. 1, the level of proliferation remained inherently higher in the SP-A^{-/-} OVA group. These results directly led us to explore whether the lungs of SP-A^{-/-} mice had a greater proportion of activated or effector T cells.

A greater proportion of preactivated and effector memory CD4⁺ T cells is present in the lungs of SP-A^{-/-} mice during allergen-mediated lung inflammation

Th2-associated cytokine levels and IgE levels were increased in the SP-A^{-/-} OVA mice, suggesting that SP-A directly or in-

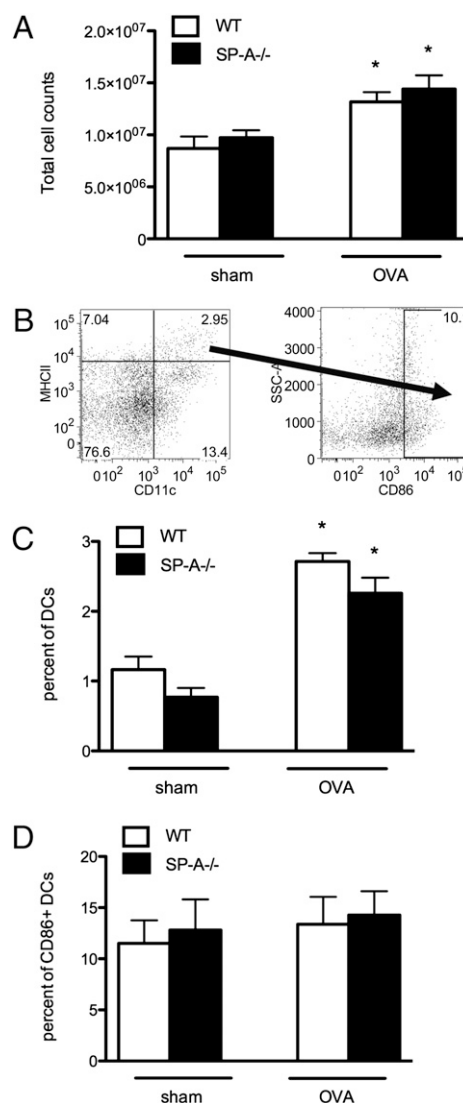


FIGURE 3. WT and SP-A^{-/-} OVA mice have similar proportions of low-density cells and DCs in the lungs. **A**, Representative profile of total lung digest cell counts after density-gradient centrifugation ($n = 3$ –4 lungs pooled/group). **B**, Representative gating strategy shown in SP-A^{-/-} OVA mice to determine the percentage of DCs, identified by CD11c⁺ and MHCII^{hi} cell surface staining, and percentage of DCs that stained CD86⁺ (CD86⁺ DCs) using flow plots. **C**, The percentage of cells expressing CD11c⁺/MHCII^{hi}, as determined by the strategy described in **B**. **D**, The percentage of cells expressing CD86, as determined by the strategy described in **B** ($n = 4$ independent experiments, two mouse samples/group/experiment). * $p < 0.05$, compared with sham-treated controls, which did not differ from each other.

directly modulates CD4⁺ phenotype and Th2 functions. Because SP-A did not alter the phenotype or prevalence of DCs and Tregs, we predicted instead that SP-A influences the immunomodulatory repertoire of CD4⁺ T cells, especially with respect to naive and memory/effector cells, which could account for the differences in inflammatory indices found between the OVA groups.

As described above, the total numbers of CD3⁺/CD4⁺ cells in the lungs did not differ among the groups. However, with regard to the CD3⁺/CD4⁺/CD44^{hi} population, the percentage of T_{CM} cells (CD62L⁺) and T_{EM} cells (CD62L^{neg}) was 24 and 75%, respectively, in the SP-A^{-/-} OVA mice compared with 38 and 61%, respectively, in the WT OVA mice and 50% in both sham mouse groups (Fig. 4B). For the CD3⁺/CD4⁺/CD44^{low} popu-

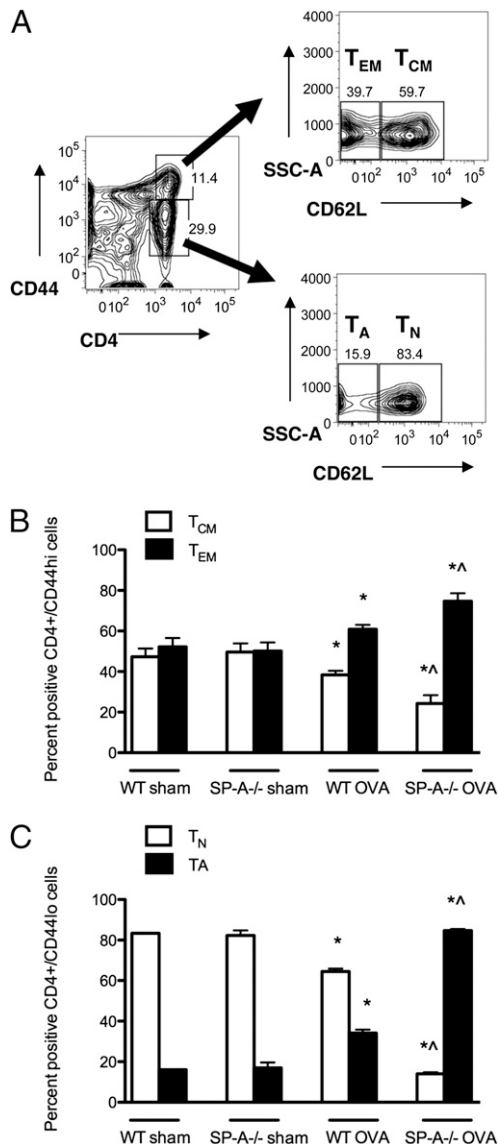


FIGURE 4. SP-A^{-/-} OVA mice have the greatest proportion of T_A and T_{EM} cells in the lungs. *A*, Representative gating strategy used to determine the percentages of T_A, T_N, T_{EM}, and T_{CM} cells in the lungs. CD3⁺/CD4⁺/CD44^{low} T cells that express CD62L^{neg} staining correspond to T_A cells; CD3⁺/CD4⁺/CD44^{low} T cells that express CD62L⁺ staining correspond to T_N cells; CD3⁺/CD4⁺/CD44^{hi} T cells that express CD62L^{neg} staining correspond to T_{EM} cells; and CD3⁺/CD4⁺/CD44⁺ T cells that express CD62L⁺ staining correspond to T_{CM} cells. *B*, Percentage of T_A and T_N cells. *C*, Percentage of T_{EM} and T_{CM} cells ($n = 4$ independent experiments, two mouse samples/group/experiment). * $p < 0.05$, compared with sham-treated controls, which did not differ from each other; $\Delta p < 0.05$, compared with WT OVA.

lation, the percentage of T_N cells (CD62L⁺) and activated T cells (T_A cells) (CD62L^{neg}) was 14 and 85%, respectively, in the SP-A^{-/-} OVA mice. These proportions were dramatically altered in the WT OVA mice to 65 and 34%, respectively. Baseline levels in both sham groups were 83 and 17% (Fig. 4C). To assess whether the effect of SP-A on altering the ratios of T cell subsets was restricted to the lung, T cell subsets in the mediastinal lymph nodes, inguinal lymph nodes, and the spleen were also analyzed. No differences were noted among the groups in the peripheral tissues (Supplemental Fig. 2), indicating that the effects of SP-A on T cell phenotype were localized to the cells found specifically within the lung.

CD4⁺ T cells from the lungs of SP-A^{-/-} OVA mice are hyperresponsive to ex vivo recall stimulation, and this capacity is attenuated with exogenous SP-A treatment

To determine whether the alterations in CD4⁺ T cell phenotypic ratios described above translated into changes in effector function, we isolated CD4⁺ T cells from the spleens and lungs of each mouse group and stimulated the total CD4⁺ T cell population with varying ratios of OVA-primed BMDCs and OVA ex vivo. Cellular proliferation was assayed by [³H]thymidine incorporation, recorded as mean cpm, and representative data are reported as fold differences compared with unstimulated control (T cells + unprimed BMDCs) (Fig. 5A). Lung-derived T cells from the SP-A^{-/-} OVA mice had the greatest proliferative capacity compared with all other groups; SP-A^{-/-} OVA cells had ~2-fold enhancement in proliferation compared with the cells from the sham mice and ~30% enhanced proliferation compared with cells from the WT OVA mice. Supernatant levels of IL-4 were also greatest in the SP-A^{-/-} OVA T cell samples (Fig. 5B). Addition of 20 μ g/ml of exogenous SP-A to the cell culture reduced proliferation by ~40% in the cells from OVA-exposed mice compared with those from the sham mice. Surprisingly, IL-4 secretion was not affected as dramatically, suggesting that SP-A is able to reduce pro-

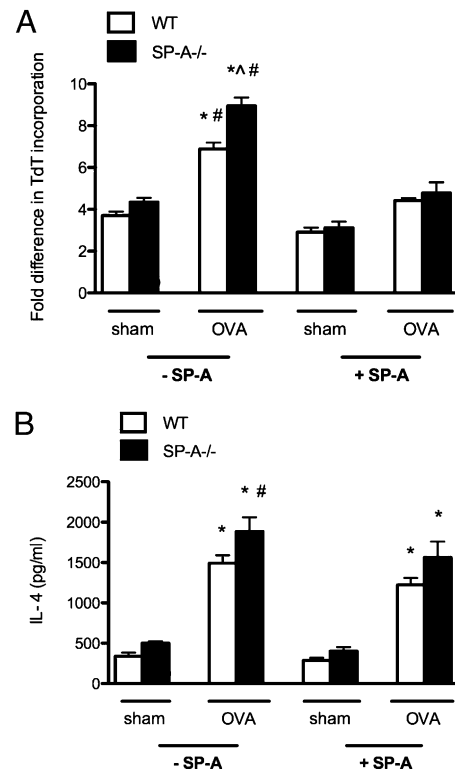


FIGURE 5. CD4⁺ T cells from SP-A^{-/-} OVA mice are hyperresponsive to ex vivo recall stimulation, and this capacity is attenuated with exogenous SP-A treatment. Primary mouse lung-derived T cells were purified and activated ex vivo with 40 μ g/ml of OVA and 1:10 ratio of BMDCs/T cells. Where indicated, cells were treated with exogenous SP-A (20 μ g/ml). *A*, Activation was performed for 50 h with a [³H]thymidine pulse during the last 15 h. Data are represented as fold difference in cellular proliferation normalized to the unstimulated WT cells' condition. *B*, IL-4 was measured from the respective culture supernatants at 30 h post-activation ($n = 3-4$ independent experiments, two mouse samples/group/experiment). Mann-Whitney pairwise comparison. * $p < 0.05$, compared with sham-treated controls that were treated or not with exogenous SP-A, which did not differ from each other; $\Delta p < 0.05$, compared with WT OVA not treated with exogenous SP-A; $\# p < 0.05$, compared with OVA groups treated with exogenous SP-A.

liferation without affecting Th2 cytokine production. These results, combined with the Th2-inflammatory response data (Fig. 1), indicated that the global CD4⁺ T cell population from the SP-A-deficient lungs is hyperresponsive during OVA-stimulatory conditions and provided corroborating evidence that a shift in CD4⁺ T cell profile toward that of an effector phenotype had occurred.

Activated and effector memory CD4⁺ T cells proliferate to a greater extent in the lungs of SP-A^{-/-} mice during allergen-mediated lung inflammation

Finally, to investigate the mechanism for the increased prevalence of T_A and T_{EM} cells in the SP-A-deficient condition, BrdU incorporation was analyzed in the subpopulations of lung CD4⁺ T cells in vivo. Data were normalized to the levels of BrdU incorporation in the control group, which was composed of splenocytes from saline-treated WT mice. Results showed that T_{EM} and T_A cells from SP-A^{-/-} OVA mice have a greater proliferation rate compared with WT OVA mice (Fig. 6). Interestingly, T_{EM} cells from sham-treated SP-A^{-/-} mice had reduced levels of BrdU incorporation compared with cells from sham-treated WT mice. The largest fold change in percentage and numbers was observed in the T_A cells, which are the predominant responder population and have the potential to convert to memory cells under conditions of OVA stimulation. Thus, lung effector memory and activated CD4⁺ T cells displayed enhanced proliferation in SP-A^{-/-} mice during allergen-mediated inflammation.

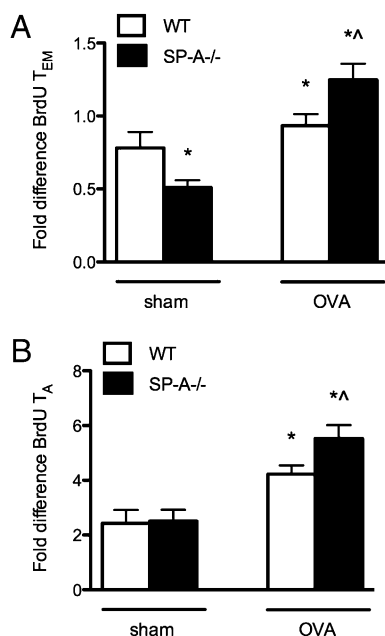


FIGURE 6. T_{EM} and T_A cells proliferate to the greatest extent in the lungs of SP-A^{-/-} OVA mice. Mice were given i.p. injections of BrdU before each OVA aerosol treatment, and the drinking water was supplemented with BrdU during the OVA aerosol period. Single-cell preparations from lung digests or spleen were labeled with T cell phenotypic markers (CD3, CD4, CD44, CD62L) and simultaneously analyzed by flow cytometry for BrdU incorporation in the nucleus. Data are represented as mean fold difference normalized to the sham-treated WT CD4⁺ splenic T cell population. **A**, Fold differences in BrdU⁺ T_{EM} cells ($n = 3$ independent experiments, three mouse samples/group/experiment). Mann-Whitney pairwise comparison. * $p < 0.05$, compared with WT sham; ^ $p < 0.05$, compared with WT OVA. **B**, Fold differences in BrdU⁺ T_A cells ($n = 3$ independent experiments, three mouse samples/group/experiment). Mann-Whitney pairwise comparison. * $p < 0.05$, compared with sham-treated controls, which did not differ from each other; ^ $p < 0.05$, compared with WT OVA.

Discussion

To our knowledge, our study is the first to investigate the phenotype and prevalence of lung-derived adaptive immune cells during allergen-induced inflammation in an SP-A-deficient condition. We demonstrated that T_A and T_{EM} cells from OVA-exposed SP-A-deficient mice exhibited the greatest extent of in vivo proliferation, correlating with striking phenotypic data showing that these mice had the greatest proportions of CD4⁺ T_A and T_{EM} cells and the lowest proportions of T_N and T_{CM} cells in their lungs. This phenotypic shift correlated with an increased proliferative and IL-4-producing capacity ex vivo and with a greater extent of eosinophilia and IL-4 and IL-5 cytokine levels in the BALF and IgE levels in the serum. Taken together, we identified an important role for SP-A in inhibiting the proliferation of CD4⁺ T_A and T_{EM} subpopulations and inhibiting Th2-associated inflammatory indices.

SP-A levels were reported to increase or decrease (14, 46, 47) in allergic hypersensitivity conditions. For instance, Haley et al. (48), who used a shorter priming period but a longer and more concentrated OVA aerosol protocol than we did, found increased expression of SP-A in lavage fluid and in nonciliated epithelial cells of noncartilaginous airways. However, a specific decrease in SP-A levels during allergic inflammation was observed in mouse studies that used *A. fumigatus*, a ubiquitous airborne saprophytic fungi, or dust mite allergen (11, 49). A recently published, well-controlled human study by Erpenbeck et al. (14) showed that segmental allergen challenge in subjects with asthma resulted in massive eosinophil influx, with specific increases in SP-B, SP-C, and SP-D and a decrease in SP-A BALF levels. Importantly, in this study, SP levels were compared with baseline and saline control challenge in the same subjects. These alterations occurred in the absence of a concomitant change in total phospholipid levels in the cell-free BALF, suggesting that SP levels are regulated independently from surfactant phospholipid synthesis and secretion. Increasing evidence also shows that polymorphisms in the SP-A genes (*SP-A1* and *SP-A2*) affect the protein expression of functional SP-A (reviewed in Ref. 50). Our study provides further evidence that SP deficiency or inactivation has the potential to modify the severity of inflammation in allergic lung diseases like asthma.

Under steady-state conditions, lung DCs undergo slow but constitutive migration to draining lymph nodes and can remain there for several days to confer Ag-specific tolerance. In response to allergen exposure in asthma, the numbers of DCs increase (51), particularly in the lower airways as a result of recruited myeloid precursors (52), and then migrate with an increased rate and magnitude to the lymph nodes (53, 54). The concept that SP-A can affect the phenotype and chemotactic responses of DCs was shown in an in vitro study conducted in our laboratory by Brinker et al. (16). Incubation with SP-A decreased the numbers of BMDCs that migrated toward the secondary lymphoid tissue chemokine/CCL21-induced chemokine gradient (16) and decreased the extent of DC maturation. In contrast, we did not find an increase in the prevalence of matured DCs in the absence of SP-A in the sham or the OVA condition. Brinker et al. (16) used a relatively short-term acute LPS exposure to stimulate the maturation of the BMDCs. Thus, the lack of differences in MHCII and CD86 mean fluorescence intensity in our current study may be a consequence of the different agonists used (LPS versus OVA) or the time of analysis. Another possibility is that the previous study was conducted using BMDCs, which may respond differently to SPs than do lung-derived DCs (34, 55, 56). In addition, we examined the later phase of the secondary challenge (i.e., after the

third day of OVA aerosol challenge), but it is possible that SP-A interacts differentially with DCs to alter the maturation and trafficking processes during the early phases of the secondary challenge.

Gaining increasing attention is the notion that DCs may not actually be a separate cell type with unique functions but rather part of the mononuclear phagocyte system because they are derived from a common precursor, respond to the same growth factors (including CSF-1), express the same surface markers (including CD11c), and have no unique adaptation for Ag presentation that is not shared by other macrophages (57). Guth et al. (58) recently demonstrated that the airway environment with locally high concentrations of GM-CSF and, to a lesser extent, SP-D, promotes the development of macrophages with unique DC-like characteristics, illustrating that the phenotype was not predetermined but was, instead, a product of the environment. Thus, the distinction between macrophages and DCs is not always clear, particularly in the lung.

Previously published data from our laboratory and other investigators showed that T cells from the alveolar airspace are functionally different from T cells from the circulation and that surfactant lipids and proteins alter T cell functions in vitro (17, 59–62). The results presented in this study clearly showed that T cells isolated from lung digests of SP-A^{-/-} mice challenged with OVA differed from those isolated from the WT mice challenged with OVA. Therefore, SP-A must be acting on T cells locally in the airspace, making its way from the airspace to the parenchyma to affect T cells there or acting indirectly by mediating the functions of APCs or other cells that, in turn, modulate T cells. Determining whether SP-A can act directly on T cells while they are in the airspace or parenchyma and/or whether SP-A is influencing the activity of other cells that, in turn, modulate T cells in these different locations are important questions to be addressed in future studies.

The proliferative capacity of T cells from the lungs of the SP-A^{-/-} OVA mice was greater in ex vivo assays using Con A or OVA-recall stimulation compared with T cells from the WT OVA mice. However, the proliferative capacity of T cells from the WT and SP-A^{-/-} sham mice did not differ, and addition of exogenous SP-A to cultures of WT OVA or SP-A^{-/-} OVA T cells resulted in inhibition of OVA-induced proliferation, both of which suggested that there are no differences in intrinsic proliferative capacity or responsiveness to SP-A between T cells from WT and SP-A^{-/-} T mice. We hypothesized that the greater proliferative capacity in the SP-A^{-/-} OVA cells can be attributed to the larger proportion of T cells that are primed to respond to stimuli in these mice (i.e., larger proportion of CD25-expressing cells and T_H cells in the case of the Con A assays and larger proportion of T_{EM} cells in case of the OVA-recall assays).

The presence or absence of proinflammatory subsets of T cells has major effects on the course and outcome of inflammatory reactions in the lungs secondary to the distinct cytokines secreted (63–67). Th2 T_{EM} cells play an important role in the pathogenesis of asthma because they are known to accumulate in the lung and produce cytokines rapidly. Although it is debatable whether there is a true shift toward a Th2 bias or whether there is a concomitant diminishment of Th1 activity, increased levels of Th2 cell-secreted IL-4 and IL-5 have been repeatedly observed in the BALF of subjects with asthma (32). The increased Th2 cytokine profile, IgE production, and eosinophilia, as well as hyperresponsivity of the isolated CD4⁺ T cells, we observed are consistent with a predominant prevalence of an effector CD4⁺ T cell phenotype in the SP-A-deficient condition.

Based on our findings, we propose the following model. The presence of adequate amounts of functional SP-A in the lung milieu

negatively regulates the prevalence of effector memory CD4⁺ T cells in the lung tissues during allergen-induced inflammation. Because of a decreased pool of effector memory CD4⁺ T cells, there is a concomitant decrease in the production of Th2-associated cytokines, which, in turn, affects the recruitment or survival of eosinophils and diminishes the IgE-mediated humoral response. Thus, the overall effect of the presence of SP-A in the lung is a reduction in several inflammatory indices associated with allergen-mediated lung inflammation. Understanding the mechanisms by which SP-A influences the phenotype and homing specificity of cells important in asthma pathogenesis could provide new targets for immunotherapy, perhaps unique SP-A-based therapies, for chronic inflammatory lung diseases.

Acknowledgments

We thank Dr. William M. Foster and Erin Potts for assistance with the OVA exposures and Dr. Julia K. Walker for critical review of the manuscript. We also thank Dr. John F. Whitesides and Patrice McDermott in the Duke Human Vaccine Institute Research Flow Cytometry Shared Resource Facility for assistance with flow cytometry procedures.

Disclosures

The authors have no financial conflicts of interest.

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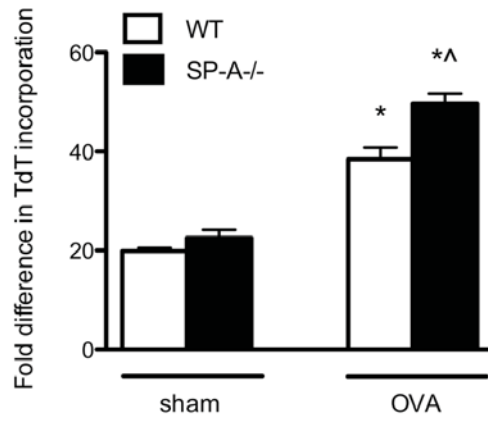
SUPPLEMENTAL FIGURE LEGENDS

Supplement 1. T cells from SP-A^{-/-} OVA mice show the greatest proliferative response to Con A stimulation.

Primary mouse lung-derived T cells were purified and activated *ex vivo* as described in Methods with 2 µg/ml of Con A. Activation was performed for 65 h with a [³H]-thymidine pulse during the last 15 h. Data is represented as fold difference in proliferation from replicate wells, normalized to the unstimulated WT cells alone condition. (*n* = 3 independent experiments, replicate wells from 2 - 3 mouse samples/group/experiment; Mann-Whitney pairwise comparison, *p* < 0.05; *, as compared to sham-treated controls, which did not differ from each other, ^ as compared to WT OVA. T cells from SP-A^{-/-} mice treated with OVA typically showed ~30% greater proliferation in response to the pan-T cell mitogen, Con A.

Supplement 2. WT and SP-A^{-/-} OVA mice have similar proportions of CD4⁺ T subsets in the MLN, spleen, and ILN. *CD4/CD44^{low}* column, left side gate corresponds to previously activated cells (T_A) and right side gate corresponds to naïve cells (T_N). *CD4/CD44^{hi}* column, left side gate corresponds to effector memory cells (T_{EM}) and right side gate corresponds to central memory cells (T_{CM}). (Representative flow plots from 2 independent experiments with 2 samples of individual mice pooled/group). MLN = mediastinal lymph node, ILN = inguinal lymph nodes.

Supplemental 1.



Supplemental 2.

