

ORIGINAL ARTICLE

Asthma and Lower Airway Disease



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Protein S protects against allergic bronchial asthma by modulating Th1/Th2 balance

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Abstract

Background: Bronchial asthma is a chronic disease characterized by inflammation, obstruction, and hyperresponsiveness of the airways. There is currently no curative therapy for asthma. Type 2 helper T cell response plays a critical role in the pathogenesis of the disease. Protein S is a glycoprotein endowed with anticoagulant, anti-inflammatory, and anti-apoptotic properties. Whether protein S can suppress bronchial asthma and be useful for its therapy is unknown.

Methods: To address this question here we compared the development of allergen-associated bronchial asthma between wild type and protein S-overexpressing transgenic mice. Mice were sensitized and challenged with ovalbumin. We also evaluated the circulating levels of total and active protein S in patients with bronchial asthma and healthy controls.

Results: The circulating level of total protein S and of its active form was significantly decreased in patients with bronchial asthma compared to controls. Allergic protein S transgenic mice showed a significant reduction of airway hyperresponsiveness, lung tissue inflammatory cell infiltration, lung levels of Th2 cytokines and IgE compared to their wild-type counterparts. Administration of exogenous human protein S also decreased airway hyperresponsiveness and Th2-mediated lung inflammation in allergic wild-type mice compared with their untreated mouse counterparts. Human protein S significantly shifted the Th1/Th2 balance to Th1 and promoted the secretion of Th1 cytokines (IL-12, tumor necrosis factor- α) from dendritic cells.

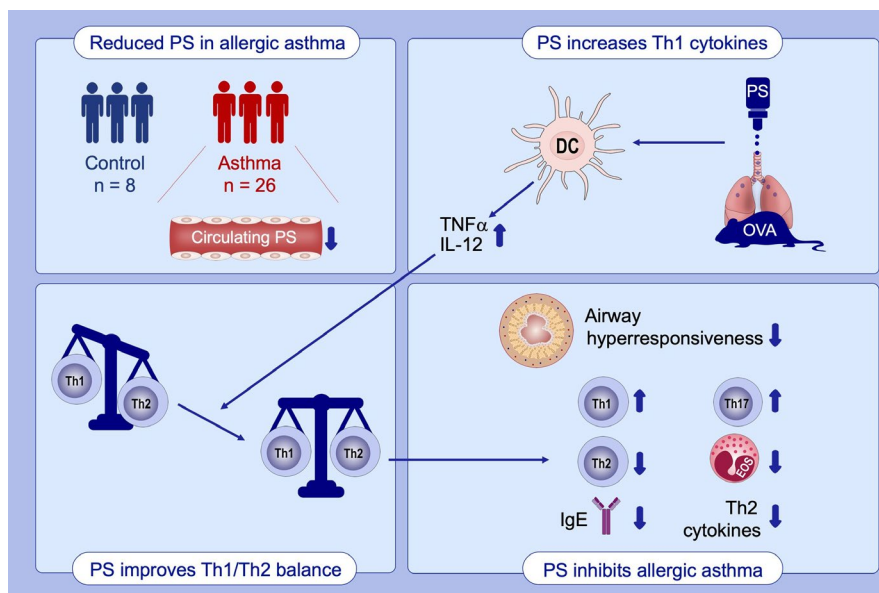
Conclusions: These observations suggest the strong protective activity of protein S against the development of allergic bronchial asthma implicating its potential usefulness for the disease treatment.

Abbreviations: BALF, bronchoalveolar lavage fluid; H&E, hematoxylin & eosin; hPS, human protein S; OVA, ovalbumin; PAS, periodic acid Schiff; PS, protein S; SAL, saline; TG, transgenic; TAM, Tyro, Axl, and Mer; WT, wild type.

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KEYWORDS

asthma, hypersensitivity, Protein S, Th1 cells, Th2 cells



GRAPHICAL ABSTRACT

Patients with allergic bronchial asthma have reduced circulating level of active PS. Administration of exogenous PS attenuates allergic airway inflammation and hyperresponsiveness in mice. In the presence of protein S, allergen presentation by dendritic cells to T-cells shifts the Th1/Th2 immune balance towards Th1 and inhibits allergic response in the airways; therefore, PS has the potential to become a drug for the treatment of allergic bronchial asthma.

1 | INTRODUCTION

Bronchial asthma is a chronic respiratory disease associated with high mortality and morbidity worldwide despite substantial advances in its therapeutic management.¹⁻³ The disease can affect populations of any age and of any geographical region.³⁻⁶ Epidemiological data suggest that there are globally more than 300 millions bronchial asthma patients with an annual number of deaths of more than 200 thousands.^{7,8} It is estimated that by 2025 there will be an additional number of 100 million patients.^{7,8} Rhinitis, rhino sinusitis, and sleep apnea are frequent co-morbidities that worsen the quality of life of many asthma patients.⁹ The disabling effects of bronchial asthma also impose an enormous economic burden due to medications, hospitalizations, and days away from work.¹⁰⁻¹² Apart from underlying genetic abnormalities, intrinsic or environmental factors including allergens play central roles in the pathogenesis of bronchial asthma.¹³ An excessive Th2 response leading to enhanced recruitment of eosinophils and IgE-mediated inflammation is the main driver of the disease.^{13,14}

Protein S (PS) is a vitamin K-dependent glycoprotein with a molecular weight of 69 kDa mainly produced and secreted by liver cells. Structurally, it is composed of an N-terminal 4-carboxyglutamic acid-rich domain, a thrombin-sensitive region, four epidermal growth factor-like domains, and a COOH-terminal region homologous to sex hormone-binding globulin. PS circulates in plasma in a free form

(10 µg/mL) or forming a complex (15 µg/mL) bound to the β chain of C4b-binding protein (C4BP), an inhibitor of the lectin and classical pathways of the complement system.¹⁵ There are two C4BP isoforms. A major isoform having seven α chains and a β chain, and a minor isoform that has no β chain and does not interact with PS.¹⁶ During inflammation, primarily the minor form lacking the β chain increases so that levels of free protein S remain stable.¹⁶ C4BP retains its ability to interact with C4b after binding to PS and thus C4BP can modulate complement activation and inflammation when the C4BP-PS complex is anchored by PS to negatively charged phospholipids at sites of tissue injury.¹⁷ PS plays multiple functions including inhibition of the coagulation system, prolongation of cell survival and regulation of inflammatory and immune responses.¹⁵ PS exerts anticoagulant activity by enhancing the inhibition of blood coagulation by APC, by directly reducing the activity of prothrombinase complex, tenase, or by enhancing the suppressive effect of the tissue factor inhibitory pathway.¹⁵ PS regulates cell survival, inflammation, and immune responses by binding to and activating the Tyro, Axl, and Mer (TAM) tyrosine kinase receptors.¹⁸ Recent experimental studies suggested the potential pharmacological beneficial effects of PS administration in several pathological conditions including acute lung injury, pulmonary fibrosis, diabetes mellitus, and chronic kidney diseases.¹⁹⁻²¹ PS is protective in these pathological conditions because it inhibits apoptosis of key organ parenchymal cells via the activation of the Akt signaling pathway.¹⁹⁻²¹

In the present study, we hypothesized that PS ameliorates allergic bronchial asthma by attenuating Th2 immune responses. To interrogate this hypothesis, we compared the development of allergic bronchial asthma between PS-overexpressing transgenic (TG) mice and wild-type (WT) mice.

2 | METHODS

Additional description of methods is provided in the Appendix S1.

2.1 | Patients

Twenty-six patients with bronchial asthma that received medical attention at the Department of Pulmonary and Critical Care Medicine of Mie University Hospital, and eight healthy volunteers were enrolled in the present investigation. The diagnosis and treatment of bronchial asthma were based on international criteria.²² All patients had stable bronchial asthma under therapy with inhaled corticosteroids. We obtained the characteristics and therapeutic history of the patients from medical records (Table S1). Data obtained from eight healthy male volunteers served as controls (Table S1). The Mie University Committee for Clinical Investigation approved the study protocol (Approval No 2846; February 19, 2015). Written informed consent was obtained from patients and controls before blood sampling.

2.2 | Animals

The TG mice in a C57BL/6 background expressing human (h) PS were characterized in a previous report.²³ The TG mice over-express hPS in the lungs and in many other organs and tissues including liver, brain, kidneys, heart, skin, skeletal muscles, and the mean plasma concentration of hPS is about 85 µg/mL as previously described.²¹ WT mice served as controls. Female mice with body weight between 18 and 22 g and age between 8 and 12 weeks were used in the experiments. All animals were housed in a specific pathogen-free environment with a constant 12:12-h light-dark cycle and under a temperature/humidity control system. Mice were given water and standard mouse food ad libitum. The Committee for Animal Investigation of Mie University approved the experimental protocol (Approval No 25-20/Hen1-Sai; September 12, 2015). All animal procedures were performed in accordance with the institutional guidelines of Mie University and following the internationally approved principles of laboratory animal care published by the National Institute of Health (<https://olaw.nih.gov/>). The research followed the ARRIVE Guidelines for animal investigation. Data collection and measurements were undertaken blindly of the treatment groups.

2.3 | Induction of experimental allergic bronchial asthma

Sensitization of mice was done by intraperitoneal (i.p.) injection of 10 µg ovalbumin (OVA) mixed with 2 mg aluminum hydroxide on day 0. Booster injections were performed on days 7, 14, and 21. Mice receiving intraperitoneal injections of saline (SAL) with the same schedule for OVA injections were used as nonsensitized mouse controls. To induce allergic bronchial asthma, sensitized mice were exposed to aerosolized 2% OVA for 30 min/day on days 29, 30, 32, and 33. Airway hyperresponsiveness was determined by exposing the mice to varying concentrations of aerosolized methacholine on day 33 as enhanced pause (Penh) using a whole-body plethysmograph system (Buxco).²⁴ Two hours after the Penh measurement on day 33, the mice were exposed to aerosolized 2% OVA, and then killed by a pentobarbital overdose on day 34. The UN-511 ultrasonic nebulizer was used for aerosol generation. Control animals were exposed to aerosolized SAL.

2.4 | Design of experiments

The mice were separated in four experimental groups: (a) WT mice (WT)/OVA and (b) human PS TG mice (hPS)/OVA groups received i.p. injection and inhalation challenge of OVA; (c) WT/SAL and (d) hPS/SAL groups received i.p. injection and inhalation challenge of SAL.

2.5 | Stimulation of T cells under neutral or Th2 skewing condition

We prepared suspension of whole splenocytes to evaluate the activation of T cells. Briefly, spleens from OT-II mice were resected under sterile conditions after euthanasia by an overdose of anesthetic (pentobarbital). The spleens were cut into several tissue fragments, incubated in a solution of 0.5 mg/mL of collagenase for 30 minutes at 37°C before filtering through a mesh. The cells were centrifuged and then re-suspended in a solution of 0.8% ammonium chloride to lyse red blood cells.²⁵ After neutralization and washing with RPMI-1640 medium, the whole splenocytes (5×10^5 cells) were cultured in the presence of 20 µg/mL hPS (Enzyme Research Laboratories) for 1 hour. Then, 100 µg/mL of OVA was added and incubated for 7 days under Th2-skewing (10 ng/mL IL-2, 10 ng/mL IL-4, 1 µg/mL anti-IFN γ antibody) or neutral condition (10 ng/mL IL-2).

2.6 | Antigen-presentation assay under neutral or Th2 skewing condition

Bone marrow-derived dendritic cells (BMDCs) (1×10^4 cells) were cultured in the presence of 20 µg/mL hPS for 1 hour, and then 100 µg/

mL of OVA was added and incubated for an additional 1 hour. The cells were then washed with RPMI-1640 medium and co-cultured with OTII spleen-derived naïve CD4⁺ T cells (5×10^5 cells) for 7 days under Th2-skewing (10 ng/mL IL-2, 10 ng/mL IL-4, 1 µg/mL anti-IFN γ antibody) or neutral condition (10 ng/mL IL-2).

2.7 | Flow cytometry

To perform intracellular staining, the cells were stimulated with phorbol 12-myristate 13-acetate (50 ng/mL), ionomycin (500 ng/mL) for 2 hours, and then with brefeldin A for 2 hours. Thereafter, blocking of nonspecific FC receptor bindings was done by incubating with anti-CD16/CD32 for 10 minutes at 4°C, washed once in FACS buffer (phosphate-buffered saline [PBS] containing 1% FCS and 0.1% NaN₃), and then fixed in PBS containing 4% paraformaldehyde. Thereafter, cells were treated with permeabilization buffer (PBS containing 0.5% saponin, 1% FCS, and 2 mM 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid [HEPES]) for 20 minutes and stained using monoclonal anti-mouse interferon (IFN) γ , anti-mouse IL-5, and anti-mouse IL-17, anti-murine Foxp3, anti-murine T-bet, anti-murine GATA-3 or anti-murine ROR γ or their isotype controls. Analysis was performed using a FACScan flow cytometer (BD) for data acquisition, and the Cell-Quest Pro software (BD Biosciences) for data analysis.

2.8 | Statistical analysis

Data were expressed as the mean $\pm$ standard deviation of the mean (SD). For the statistical test, we used analysis of variance along with the Tukey test for the *post hoc* analysis among four variables, and by Mann-Whitney *U* test between two variables. Statistical analyses were done using the Graph Pad Prism package software for Windows (Graph Pad software, Inc). Statistical significance was considered as $P < .05$.

3 | RESULTS

3.1 | Decreased plasma levels of total and free protein S in asthmatic patients

We first measured the circulating level of PS in asthmatic patients to assess its clinical relevance. The plasma concentrations of the total and free forms of PS in patients with asthma were significantly reduced compared to a group of healthy volunteers (Figure 1). Patients with asthma were categorized in three groups according to the daily dose of inhaled fluticasone propionate as follows: high-dose group (≥ 1000 µg/day), moderate-dose group (≥ 500 µg/day - < 1000 µg/day), and low-dose group (< 500 µg/day). Patients of the high-dose group showed significantly increased plasma concentrations of both total PS and free PS compared to patients of the low-dose group (Figure S1). Comparison between male and female patients disclosed no significant difference in the concentrations of total (20.0 ± 7.8 vs 14.6 ± 11.2 µg/

mL; $P = .2$) and free (3.9 ± 1.7 vs 2.9 ± 1.7 µg/mL; $P = .2$) PS. The age of the patients was not significantly correlated with the plasma concentrations of total ($r = .09$, $P = .6$) or free ($r = .1$, $P = .6$) PS. These results suggest that low circulating levels of PS are associated with the asthmatic disease and with a low dose of corticosteroid therapy.

3.2 | High circulating level of hPS in transgenic mice

To validate our hPS transgenic mouse model, we measured the plasma concentration of hPS in control and allergic mice. As expected, the circulating level of hPS was significantly higher in both hPS/SAL and hPS/OVA groups compared with their WT counterparts WT/SAL and WT/OVA groups (Figure S2).

3.3 | Reduced airway hyperresponsiveness in hPS TG mice

Airway hyperresponsiveness is a pathognomonic finding of bronchial asthma.¹ In our present mouse model, we used Penh as a marker of airway hyperresponsiveness. The Penh values were significantly increased in the WT/OVA group during inhalation of 20, 30, and 40 mg/mL of methacholine, and in the WT/SAL group during inhalation of 30 and 40 mg/mL of methacholine. The values of Penh remained unchanged in both hPS/OVA and hPS/SAL groups during inhalation of increasing concentrations of methacholine. The Penh values were significantly increased in WT/OVA compared with WT/SAL, hPS/OVA, and hPS/SAL groups. There was also significant difference in Penh values between WT/SAL and hPS/OVA and hPS/SAL groups (Figure 2A). These observations suggest the inhibitory effect of hPS on airway hyperresponsiveness.

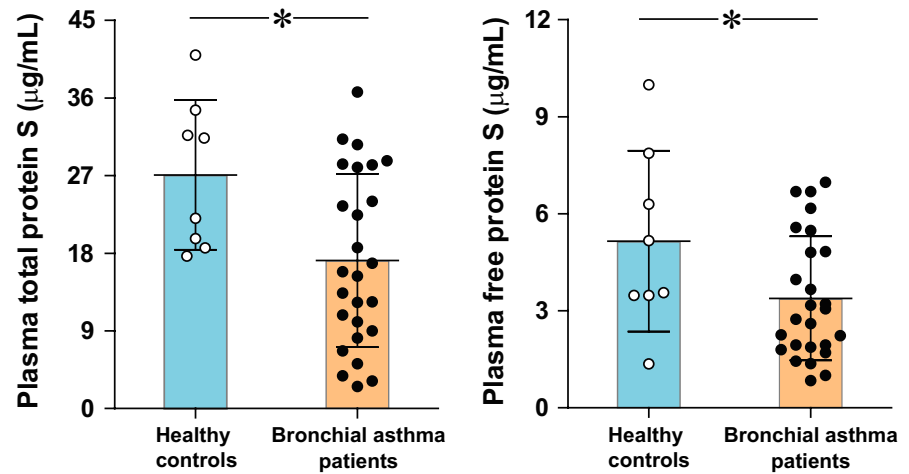
3.4 | Decreased inflammatory cells in the lung from hPS TG mice with allergy

We assessed the effect of hPS overexpression on cell infiltration in the airways and lung tissue. There were increased BALF inflammatory cells with a predominant count of eosinophils in WT/OVA and hPS/OVA mice compared to nonallergic control mice but the number of inflammatory cells and eosinophils were significantly decreased in hPS TG/OVA mice compared to their WT counterparts (Figure 2B). There were no significant changes in the number of lung cells between control saline groups.

3.5 | Reduced infiltration of inflammatory cells in lung tissue from hPS TG mice

Quantification of areas of infiltrating cells in the H&E-stained lung tissues from all mouse groups showed significantly increased infiltration of leukocytes in the intraalveolar spaces and peribronchial and

FIGURE 1 Decreased plasma levels of total and free protein S in asthmatic patients. There were 26 patients with bronchial asthma and eight healthy subjects. The total and active PS were measured by commercial enzyme immunoassay kits. Data are expressed as the mean $\pm$ SD * $P < .05$



peribronchiolar areas of the lungs from WT/OVA mice compared with hPS TG/OVA mice. There was no infiltration of leukocytes in SAL control mice (Figure S3A,B). These observations suggest that hPS overexpression ameliorates allergic inflammation in bronchial asthma.

3.6 | Decreased hyperplasia of goblet cells in hPS-overexpressing TG mice with allergy

Mucin hypersecretion is commonly associated with airway inflammation in allergic asthma.¹ PAS staining showed hyperplasia of goblet cells with increased mucin secretion in the bronchial epithelium of WT/OVA compared with the hPS/OVA group. Control mice revealed normal findings (Figure S4A,B). The results suggest an inhibitory effect of hPS on mucin secretion in allergic asthma.

3.7 | Decreased Th2 response in hPS TG mice

Th2 response is the main driver of allergic diseases.¹ The concentration of markers of Th2 response was measured in plasma samples collected before euthanasia of mice on day 34. The plasma concentrations of IgE and OVA-specific IgE and the BALF concentrations of IL-4, IL-5, IL-13, MCP-1, total IgE, and OVA-specific IgE were significantly increased in the WT/OVA compared with the WT/SAL and hPS/OVA groups. There was no significant difference in the concentrations of IL-4, IL-5, IL-13, MCP-1, and total IgE between hPS/SAL and hPS/OVA groups. There was significant difference in plasma total and OVA-specific IgE and (Figure 3) and in BALF OVA-specific IgE between hPS/SAL and hPS/OVA groups. These observations show that hPS overexpression is associated with a reduced Th2 response.

3.8 | Exogenous hPS inhibits airway hyperresponsiveness, lung eosinophilic inflammation, and Th2 response

To clarify whether hPS exerts its suppressive activity on the effector phase of the immune response, we sensitized WT

female C57BL6 mice with OVA and treated with intranasal SAL or exogenous hPS before challenging them with aerosolized OVA. Nonsensitized mice treated with SAL or hPS in similar fashion served as controls. Sensitized and challenged mice treated with SAL (WT/OVA/SAL) showed significant enhancement of airway hyperresponsiveness, significant lung eosinophilic inflammation, and significant increase in the BALF levels of MCP-1, IL-4, IL-5, and IL-13 compared to sensitized and challenged mice treated with exogenous hPS (WT/OVA/hPS) and nonsensitized mice treated with SAL (WT/SAL/SAL) (Figure 4A,B,C). The area of mucin expression was also significantly increased in the WT/OVA/SAL group compared with the WT/OVA/hPS and WT/SAL/SAL groups (Figure S5). These findings suggest that hPS suppresses allergic asthma by acting in the effector phase.

3.9 | Human PS blocks Th2 response ex vivo

To evaluate whether hPS suppresses the Th2 response ex vivo, we prepared a single cell suspension of whole splenocytes from the spleen of OVA-sensitized mice, cultured in vitro in the presence of hPS or SAL and then with OVA or SAL. The culture supernatant was collected to measure IgE and Th2 cytokines. The culture supernatant from cells treated with OVA and SAL showed significantly increased concentrations of IL-4, IL-5, IL-13, and IgE compared to the culture supernatant from cells treated with OVA and hPS or with SAL alone (Figure S6). These findings corroborate the suppressive activity of hPS on Th2 cells.

3.10 | Decreased number of Th2 cells in the presence of hPS

Next, we evaluated whether hPS affects the differentiation of Th1, Th2, or Th17 cells. To this end, suspensions of whole splenocytes were prepared, pretreated with hPS or SAL before incubating in the presence of OVA under neutral or Th2 condition and the percentage of cells expressing signature cytokines was evaluated. The lymphocyte

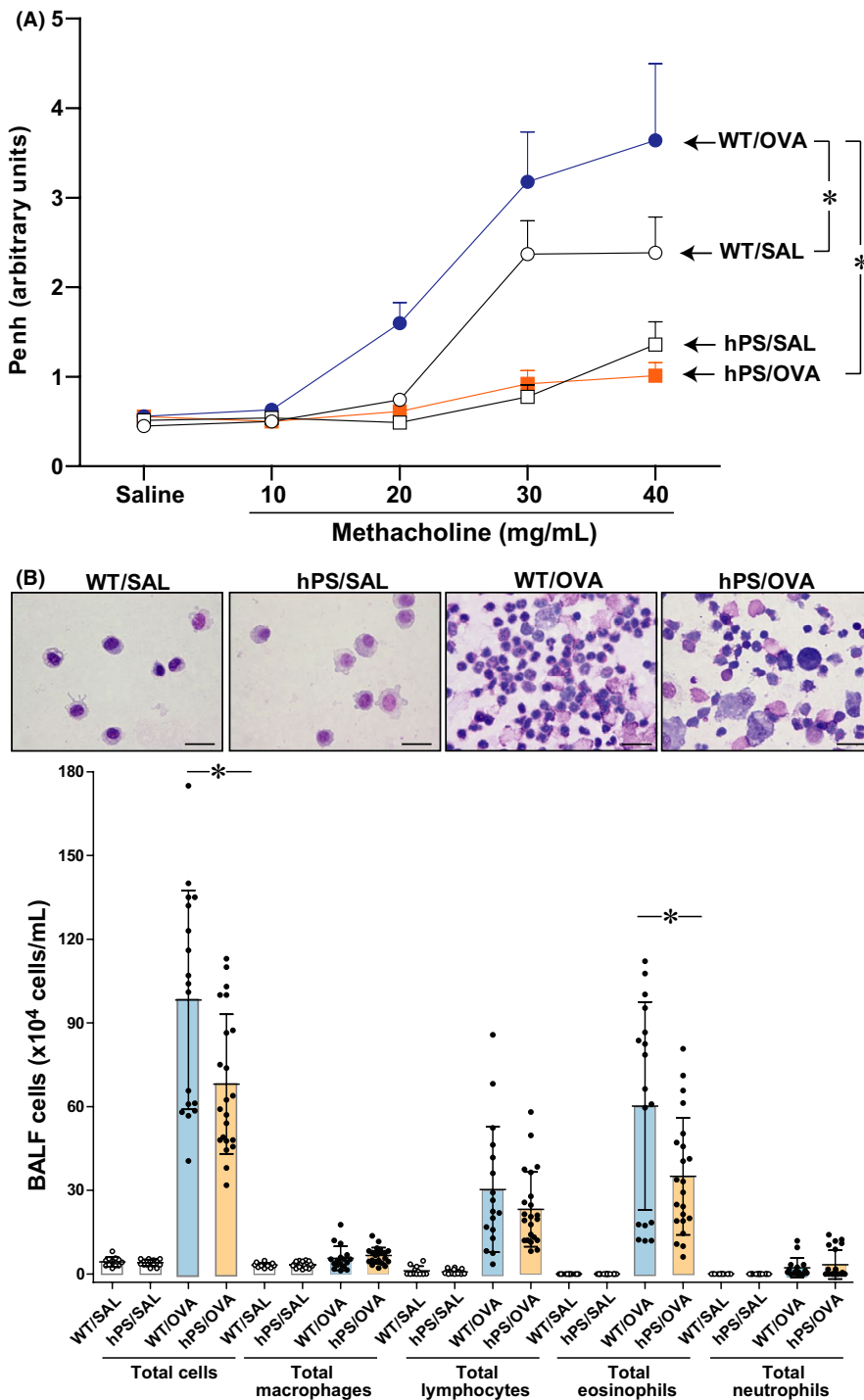


FIGURE 2 Reduced airway hyperresponsiveness and decreased inflammatory cells in bronchoalveolar lavage fluid from human protein S transgenic mice with allergy. Airway hyperresponsiveness to increasing concentrations of aerosolized methacholine was measured by unrestrained plethysmography using a whole-body plethysmograph system (A). A nucleocounter was used to determine the total number of cells in bronchoalveolar lavage fluid (B). Cells were stained with May-Grünwald-Giemsa to determine differential cells. The figure represents the mean of three independent experiments. Scale bars indicate 20 μ m. Data are expressed as the mean $\pm$ SD * P < .05. WT/SAL (n = 12), wild type/saline; WT/OVA (n = 17), wild type/ovalbumin; hPS/SAL (n = 16), human protein S/saline; hPS/OVA (n = 22), human protein S/ovalbumin

population significantly expanded in the presence of OVA but only contaminating cells remained in the absence of OVA after 7 days of culture (Figure S7). Under neutral condition, the number of IL-5 (+) or Foxp3 (+) cells was not affected but the number of IFN γ (+) and IL-17 (+) cells was significantly increased in the presence of hPS compared with the number of cells cultured in the absence of hPS (Figure 5A). On the other hand, under Th2 condition, the number of Foxp3 (+) cells was not affected but the number of IL-5 (+) cells was significantly decreased and that of IFN γ (+) and IL-17 (+) cells was significantly

increased in the presence of hPS compared to cells cultured in the absence of hPS (Figure 5B,C).

We also found that the number of IL-4 (+) was significantly reduced under neutral condition, and the number of both IL-4 (+) and IL-13 (+) was significantly reduced under Th2 condition in the presence of hPS compared with controls (Figure S8A,B,C). hPS showed no toxicity or proliferative activity in vitro. Overall, these findings suggest that hPS increases the population of Th1 or Th17 cells in relation to Th2 cells.

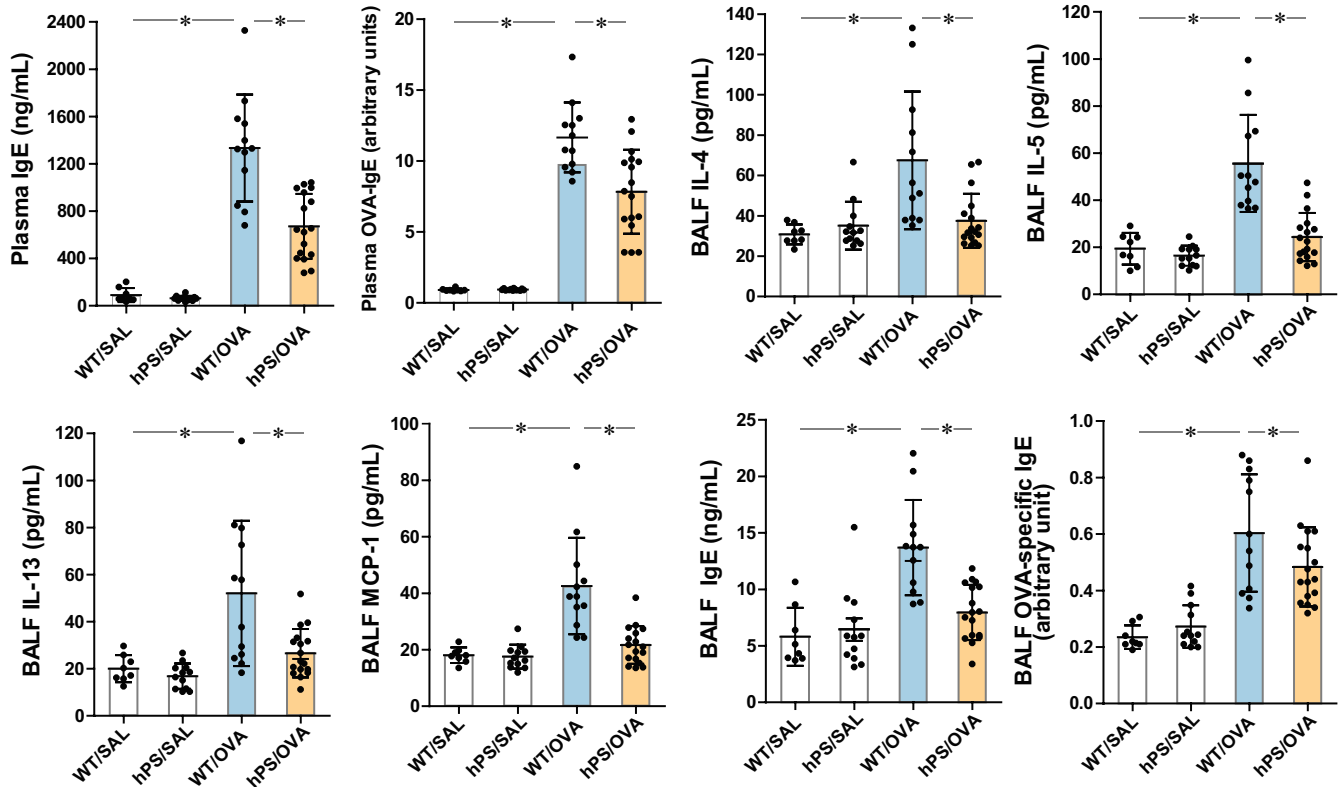


FIGURE 3 Decreased Th2 response in human protein S transgenic mice with allergy. The concentrations of cytokines and IgE were measured by specific enzyme immunoassays using commercial kits. The figure represents the mean of two independent experiments. Data are expressed as the mean $\pm$ SD * $P < .05$. WT/SAL ($n = 8$), wild type/saline; WT/OVA ($n = 12$), wild type/ovalbumin; hPS/SAL ($n = 12$), human protein S/saline; hPS/OVA ($n = 17$), human protein S/ovalbumin

3.11 | Decreased BMDC-mediated differentiation of Th2 cells in the presence of hPS

To evaluate whether hPS affects the BMDC-mediated differentiation of Th1, Th2, or Th17 cells, BMDCs were pretreated with hPS or SAL before sensitizing with OVA and co-cultured with OVA-specific CD4⁺ T cells under neutral or Th2 condition. Under neutral condition, the number of IFN γ (+) and IL-17 (+) cells was significantly increased in the presence of hPS compared with the number of cells cultured in the absence of hPS (Figure 6A). Under Th2 condition, the number of IFN γ (+) was significantly increased and that of IL-5 (+) cells was significantly decreased in the presence of hPS compared to cells cultured in the presence of SAL (Figure 6B,C).

Evaluation of transcription factor activation in T cells showed significantly increased percentage of T cells with activation of T-bet, ROR γ , and Foxp-3 after treatment with hPS under neutral condition compared to controls. The percentage of T cells with GATA-3 activation remained unchanged after treatment with hPS under neutral condition (Figure S9A). Under Th2 condition, the percentage of T cells with activation of T-bet and ROR γ was significantly increased, whereas the percentage of T cells with GATA-3 activation was significantly decreased after treatment with hPS compared to controls. The percentage of T cells with Foxp-3 activation remained unchanged after treatment with hPS under Th2 condition (Figure S9B,C). Overall, these findings indicate that hPS favors the differentiation of Th1 or Th17 cells but not that of Th2 cells.

3.12 | hPS promotes LPS-mediated expression of TNF α

LPS is a well-known Th1 promoting factor.²⁶ We assessed whether hPS may further increase LPS-mediated Th1 response. BMDC was stimulated with LPS in the presence or absence of hPS and the concentrations of cytokines in the cell culture supernatant and their mRNA expression were evaluated. The protein and mRNA expressions of the TNF α , a Th1 cytokine, were significantly increased in cells treated with both LPS and hPS compared to cells treated with LPS alone (Figure S10A,B). There was no significant difference in the protein and/or mRNA expression of IL-12, IL-6, and TGF β 1 between cells treated with both LPS and hPS and cells treated with LPS alone (Figure S10A,B). These findings indicate that hPS further stimulates the LPS-mediated secretion of TNF α from BMDCs.

3.13 | Dendritic cells express Th1 cytokines after stimulation with Der p1 and TSLP in the presence of hPS

To clarify whether hPS can affect Th2 immunity in the context of allergic asthma in humans, we evaluated the effect of hPS on BMDCs stimulated with Der p1 or TSLP. BMDCs were cultured in

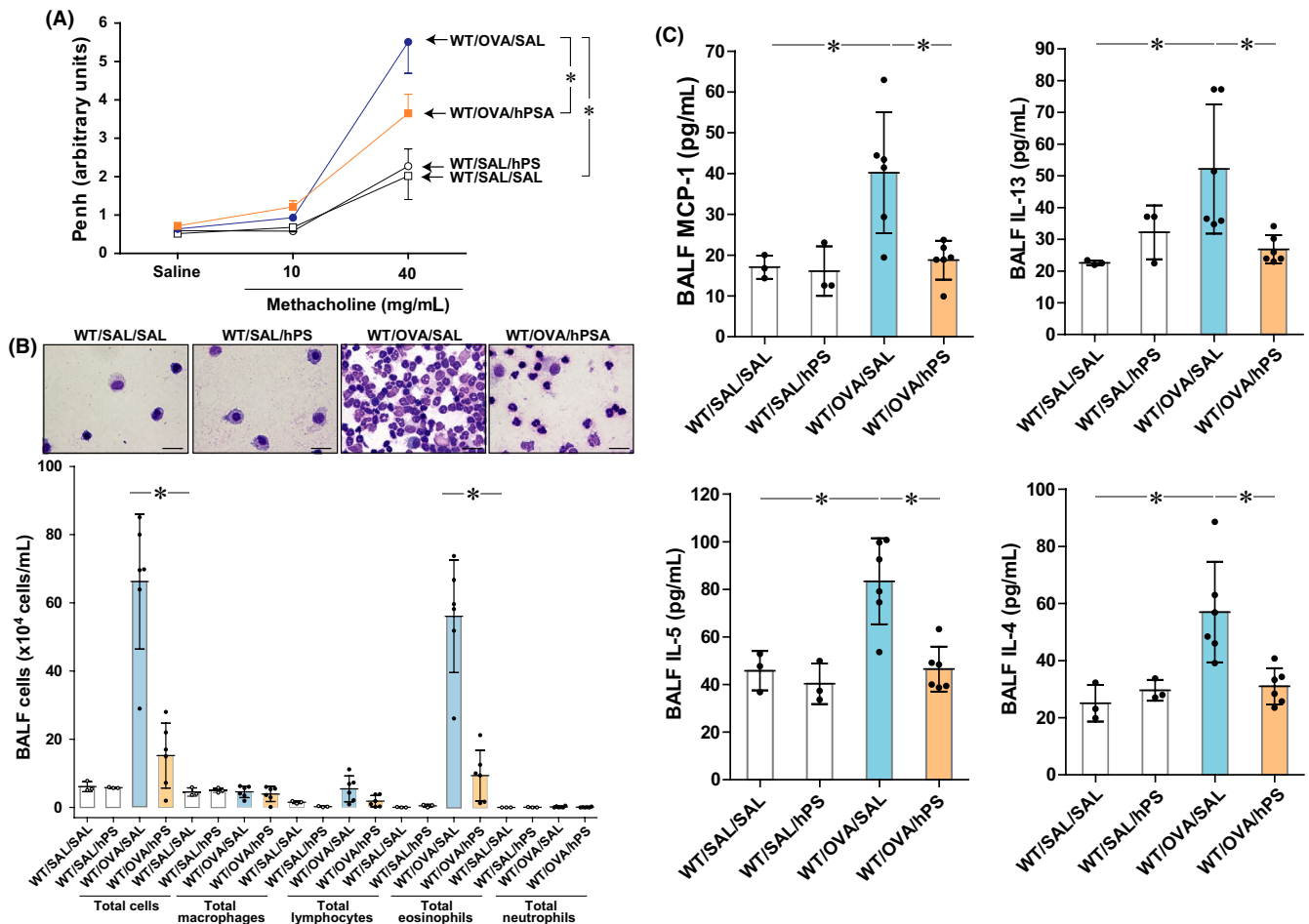


FIGURE 4 Amelioration of airway hyperresponsiveness and Th2 cytokines-mediation inflammation by treatment with exogenous human protein S. Airway hyperresponsiveness to increasing concentrations of aerosolized methacholine was measured by unrestrained plethysmography using a whole-body plethysmograph system (A). A nucleocounter was used to determine the total number of cells in bronchoalveolar lavage fluid (B). Cells were stained with May-Grünwald-Giemsa to determine differential cells. Scale bars indicate 20 μ m. The concentration of cytokines was measured by enzyme immunoassays (C). Data are expressed as the mean $\pm$ SD * P < .05. WT/SAL/SAL (n = 3), wild-type (WT) mice received intraperitoneal saline (SAL), and intranasal saline. WT/SAL/hPS (n = 3), WT mice received intraperitoneal SAL and intranasal human protein S (hPS). WT/OVA/SAL (n = 6), WT mice sensitized by intraperitoneal ovalbumin (OVA) and treated with intranasal SAL. WT/OVA/hPS (n = 6), WT mice sensitized by intraperitoneal OVA and treated with intranasal hPS

the presence of 20 μ g/ml of hPS or SAL for 1h before adding Der p1 or TSLP and continued culturing for an additional 24h. The number of TNF α (+) and IL-12 (+) BMDCs was significantly increased (Figure S11A,B), and the concentrations of IL-12 and TNF α were significantly increased in the culture supernatant of dendritic cells (Figure S12) cultured in the presence of hPS compared with controls. The concentration of IL-6 in the supernatant from BMDCs treated with hPS and SAL (hPS/SAL) was significantly increased compared to cells treated with SAL alone (SAL/SAL) but no difference was observed between other treatment groups (Figure S12). The concentration of TGF β 1 in the supernatant from BMDCs treated with hPS and Der p1 (hPS/Der p1) was significantly increased compared to cells treated with SAL and Der p1 (SAL/Der p1) but no difference was observed between other groups (Figure S12). These findings suggest that hPS may also promote Th1 response in the context of allergic asthma in humans.

4 | DISCUSSION

Bronchial asthma may develop due to genetic abnormalities and/or environmental factors, the most frequent cause being allergens.^{2,13} Allergic bronchial asthma is characterized by lung infiltration of activated Th2 cells that overexpress cytokines including IL-4, IL-5, and IL-13 that, in turn, promote the activation and infiltration of eosinophils and mast cells and B-lymphocytes.^{14,27,28} B cells produce and release allergen-specific IgE,¹⁴ that trigger type I hypersensitivity reactions. In agreement with this pathological picture, here we showed that allergic WT mice have increased lung infiltration of inflammatory cells with predominance of eosinophils, elevated concentrations of Th2 cytokines and IgE in BALF in association with a significant hyperplasia of mucin-producing bronchial cells and significant enhancement of airway hyperresponsiveness compared to nonallergic WT mice. To interrogate the

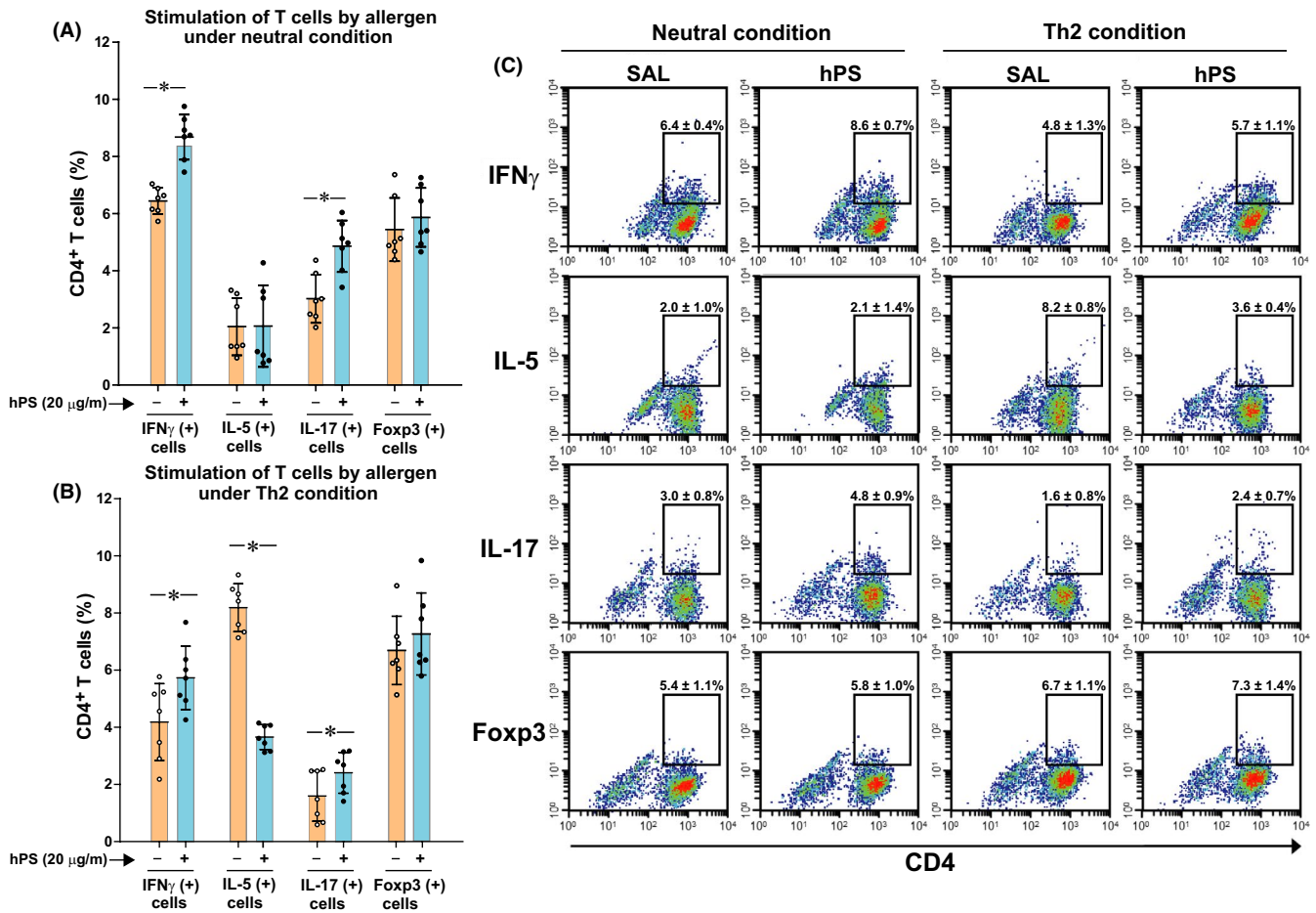


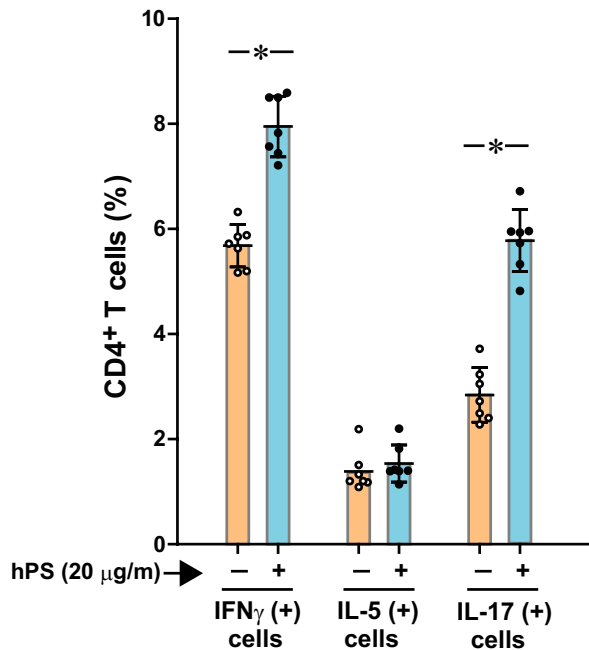
FIGURE 5 Decreased number of Th2 cells in the presence of human protein S. Whole spleen cells were isolated from OTII transgenic, cultured *in vitro* and then pretreated with human protein S (hPS) or saline (SAL) before stimulating with the allergen (ovalbumin) under neutral (A) or Th2 (B, C) conditions and the percentage of cells expressing signature cytokines were evaluated. $N = 7$ in each group. Data are expressed as the mean $\pm$ SD * $P < .05$

hypothesis that hPS is protective in bronchial asthma, we induced the disease in mice with systemic overexpression of hPS. We found that hPS with allergic bronchial asthma have significantly decreased lung infiltration of eosinophils, reduced BALF concentrations of IL-4, IL-5, MCP-1, and allergen-specific IgE, reduced hyperplasia of mucin-producing cells and significantly reduced airway hyperresponsiveness compared to their WT counterparts, indicating the protective activity of hPS in allergic bronchial asthma. It is worth noting that there was a complete lack of airway responsiveness to methacholine without complete ablation of inflammatory response in the hPS/OVA group; the differential response to the inhibitory activity of Th1 cytokines between lung compartments reported in previous studies may be the possible explanation for this observation.^{29,30} In addition, the intrapulmonary administration of exogenous hPS before challenging allergen-sensitized mice significantly ameliorated airway hyperresponsiveness, lung eosinophilic inflammation, and the expression of mucin and Th2 cytokines in the lungs, indicating that the beneficial effect of hPS is taking place in the effector phase of the immune response. Overall, to evaluate the clinical relevance of the above findings

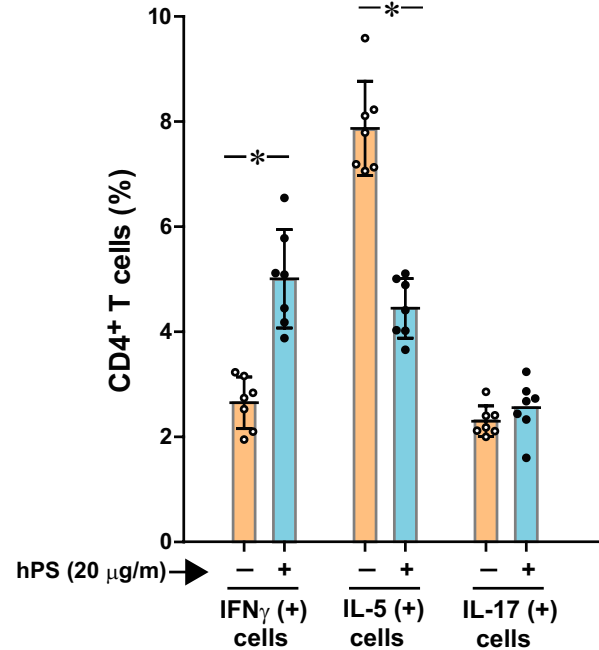
we measured the plasma levels of the total and free forms of PS in patients with bronchial asthma. Patients with bronchial asthma showed significantly reduced circulating levels of both forms of PS compared to healthy subjects. If PS is also protective in humans, we may speculate that the low availability of PS in the systemic circulation might be associated with increased susceptibility to develop allergic bronchial asthma. However, from this clinical finding we cannot infer whether the low plasma concentration of PS is the cause or the consequence of the disease.

The cellular effects of hPS are mediated by the TAM receptors, which are well-recognized inhibitors of the innate and adaptive immune responses.^{18,31} TAM receptors are negative regulators of Th2 responses.³² Here, we hypothesized that hPS inhibits Th2-mediated bronchial asthma by shifting the Th1/Th2 balance toward Th1. To demonstrate this thesis, we pretreated whole splenocytes with hPS before antigen stimulation and evaluated the number of T cells expressing signature cytokines. We found that hPS increases the number of Th1 cytokine-producing cells under neutral condition and decreases that of Th2 cytokine-producing cells under Th2 condition. Interestingly, the number of Th17 cells

(A) Activation of T cells by OVA-pulsed BMDCs under neutral condition



(B) Activation of T cells by OVA-pulsed BMDCs under Th2 condition



Neutral condition

Th2 condition

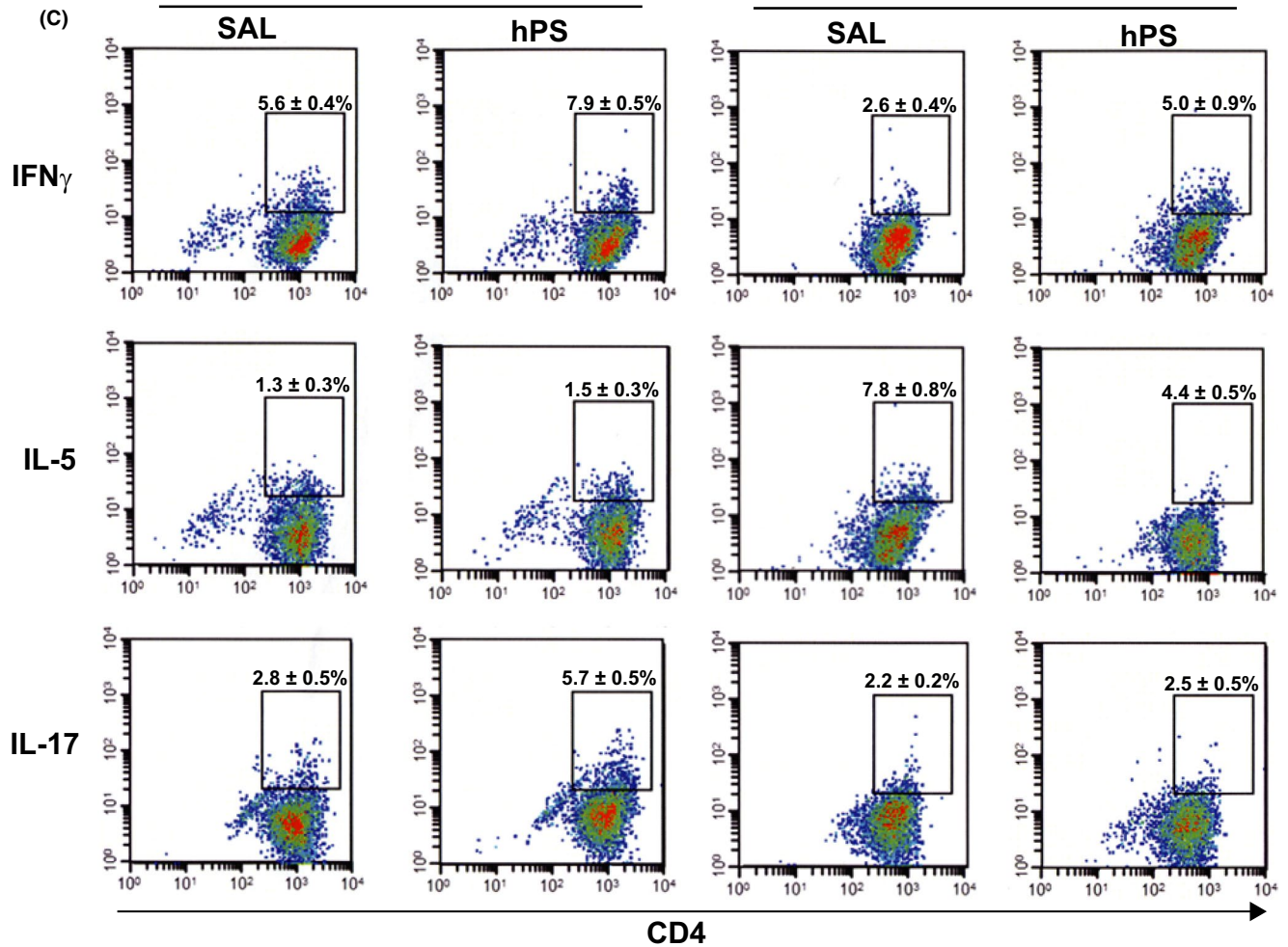


FIGURE 6 Decreased bone marrow-derived dendritic cell-mediated differentiation of Th2 cells in the presence of human protein S. Bone marrow-derived dendritic cells (BMDC) were pretreated with human protein S (hPS) or saline before sensitizing with the allergen (ovalbumin) and co-cultured with ovalbumin-specific CD4⁺ T cells from OTII transgenic mice under neutral (A) or Th2 (B, C) conditions. N = 7 in each group. Data are expressed as the mean $\pm$ SD **P* < .05

also increased in the presence of hPS under both neutral and Th2 conditions. Both IL-6 and TGF β 1 are required for Th17 differentiation.³³ Therefore, stimulation of IL-6 and TGF β 1 secretion from DC by hPS, observed in the present study, may explain the effect of hPS on Th17 cells. In addition, differentiation of CD4⁺ T cells by DCs in the presence of hPS significantly enhanced the number of differentiated Th1 cells compared with Th2 cells. These observations indicate that hPS can tilt the Th1/Th2 balance toward Th1, and this effect may explain the protective activity of hPS in allergic bronchial asthma. To gain further insights into the shifting property of hPS, we evaluated the expression of cytokines from DCs in the presence of hPS. Although there was no significant change in the expression of IL-12, a Th1-polarizing cytokine, we detected a significant increase in the expression of TNF α from DCs stimulated with both LPS and hPS compared to cells treated with LPS alone. TNF α is required for optimal production of IL-12 by DCs.^{34,35} In addition, hPS increased the expression of both IL-12 and TNF α in dendritic cells stimulated with Der p1 or TSLP that are known to induce Th2 immunity in the context of allergic asthma. Overall, these findings suggest that hPS may promote Th1 polarization by increasing the secretion of Th1-promoting cytokines from DCs.

5 | CONCLUSIONS

The present investigation provides strong evidence that hPs is protective in allergic bronchial asthma by shifting the Th1/Th2 balance toward Th1. This novel observation provides a potential alternative approach for the management of allergic bronchial asthma.

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CONFLICT OF INTEREST

None of the authors declared conflict of interest regarding data reported in this work.

AUTHOR CONTRIBUTIONS

YT, CND-G, and KA prepared the allergic asthma mouse models and wrote the first draft of the manuscript. HF RI, HS, and KF performed and analyzed the lung function test. MT, TY, KN, AT, TO, VFD, and TT measured and analyzed several parameters. YY contributed with critical revision of the manuscript and interpretation of the data. TK, ECG, and TK conceived and designed the experiments, analyzed the data and contributed with critical revision and interpretation of the data and revision of the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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Supporting Information

Protein S protects against allergic bronchial asthma by modulating Th1/Th2 balance

Kentaro Asayama, Tetsu Kobayashi, Corina N. D'Alessandro-Gabazza, Masaaki Toda, Taro Yasuma, Hajime Fujimoto, Tomohito Okano, Haruko Saiki, Atsuro Takeshita, Kentaro Fujiwara, Valeria Fidman D'Alessandro, Kota Nishihama, Toshiaki Totoki, Ryo Inoue, Yoshiyuki Takei, and Esteban C. Gabazza.

Appendix S1

Methods

Reagents

The RPMI-1640 medium, chicken egg ovalbumin (OVA) and aluminum hydroxide were from Sigma-Aldrich (Saint Louis, MO) and fetal bovine serum (FBS) from Bio Whittaker (Walkersville, MD). Hamster phycoerythrin/cyanin 5 (PE/Cy5)-labelled anti-mouse CD11c (clone N418; IgG), rat PE-Cy5-labelled anti-mouse CD4 (clone GK1.5; IgG2b κ), rat fluorescein isothiocyanate (FITC)-labelled anti-mouse IFN γ (XMG1.2; IgG1), rat PE-labelled anti-mouse IL-5 (TRFK5; IgG1), rat PE-labelled anti-mouse IL-17A (TC11-18H1; IgG1, κ) and mouse PE-labelled anti-mouse Foxp3 (15OD.1; IgG1, κ) antibody were from BioLegend Inc. (San Diego, CA). Human protein S (hPS) was purchased from Enzyme Research Laboratories (South Bend, IN). Der p1 was purchased from Ray Biotech (Norcross, GA) and thymic stromal lymphopoietin (TSLP) was from R&D Systems (Minneapolis, MN).

Evaluating the effect of exogenous hPS on allergic bronchial asthma

We purchased female wild-type (WT) C54BL6 mice aged 8-week from Nihon SLC (Hamamatsu, Shizuoka, Japan) to use in the experiments. Sensitization of mice to ovalbumin (OVA) was done by intraperitoneal injection of OVA mixed with aluminum hydroxide as described in the main text under the method section. Control mice received intraperitoneal saline (SAL). Thereafter, we separated the mice into four treatment groups. A group of mice (WT/OVA/SAL) sensitized with OVA received intranasal SAL (40 μ l) and another group of OVA-sensitized mice (WT/OVA/hPS) was treated with intranasal hPS (40 ug in 40 μ l saline) 1 h before challenging with aerosolized OVA on days 28, 29, 30, 31 and 32. A group of non-sensitized mice receiving intranasal SAL (WT/SAL//SAL) or intranasal hPS (WT/SAL/hPS) before challenging with OVA, as described above, was used as controls.

Isolation of antigen-specific CD4 T-cells

We isolated the whole splenocytes or naive CD4⁺ T-cells from the spleen of OT-II T-cell receptor TG mouse (C57BL/6 background), a generous gift from K. Takahashi, Yokohama City University Graduate School of Medicine. Isolation of naive CD4⁺ T-cells was done using a naïve CD4 T-cell isolation kit II (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) following the manufacturer's instructions. CD4⁺ T-cells of OT-II T-cell receptor TG mouse recognize chicken OVA323-339 in association with I-A^b.

Generation of mouse bone marrow-derived dendritic cells

We extracted bone marrow-derived dendritic cells (BMDCs) from C57BL/6 WT mice and cultured for seven days at 37°C in a humidified atmosphere containing 5% CO₂ using RPMI-1640 medium. The culture medium was supplemented with 10% heat-inactivated fetal bovine serum (FBS; BioWhittaker, Inc., Walkersville, MD), 2mM L-glutamine, 100 U/ml penicillin, 100 µg/mL streptomycin, 50 µM β-mercaptoethanol (Sigma-Aldrich Co, St. Louis, MO) and 200 ng/mL Flt3L (PeproTech, Inc., Rocky Hill, NJ) as previously reported.¹ We then purified CD11c⁺ DCs using mouse CD11c microbeads (Miltenyi Biotec GmbH) following the instructions of the manufacturer.

Sampling of bronchoalveolar lavage fluid and plasma

All animal euthanasia was performed by i.p. injection of high dose pentobarbital to collect samples for biochemical and histological examination. Procedures for plasma and bronchoalveolar lavage fluid (BALF) collection were previously described.² A nucleocounter from ChemoMetec (Allerød, Denmark) was used for counting the total number of cells in bronchoalveolar lavage fluid (BALF). We stained BALF cells with May–Grünwald–Giemsa (Merck, Darmstadt, Germany) to count differential cells.

Histological Examination

The lung circulation was flushed with saline and then the lungs were removed. One of the

lung was perfused with 10% neutral buffered formalin and fixed in formalin. After paraffin embedding, tissue preparations were done to stain with hematoxylin & eosin (H&E) or periodic acid Schiff (PAS), before examining by light microscopy. The positive areas for PAS staining were quantified using an Olympus BX50 microscope combined with an Olympus DP70 digital camera (Tokyo, Japan) and the WinROOF image-processing software (Mitani Corp., Fukui, Japan) for Windows. Ten photos of random areas at high-magnification were taken from the lungs of each mouse and the PAS positive areas were measured.

Biochemical Analysis

The concentrations of IgE, IL-12, tumor necrosis factor (TNF)- α , IL-6 and monocyte chemoattractant protein (MCP)-1 were measured using commercial immunoassay kits from BD Biosciences Pharmingen (San Diego, CA) and IL-5 and IL-13 using kits from R&D Systems (Minneapolis, MN). We measured the total level of PS in plasma by an enzyme-immune assay using anti-human PS antibody (Dako Cytomation, Glostrup, Denmark) as coating antibody, biotin-labeled anti-human PS antibody (Dako Cytomation, Glostrup, Denmark) as detection antibody, horse-radish conjugated streptavidin, and o-phenylenediamine dihydrochloride (Sigma-Aldrich, St. Louis, MO) as substrate following previously described procedures.³ To measure free hPS, we coated a 96-well microplate with C4-binding protein, (ATGen Corp., Sampyeong-dong, Korea), which binds to free PS, washed, blocked, and then added biotin-labelled polyclonal rabbit anti-hPS antibody.⁴ Values were extrapolated from standard curves prepared using PS antigen (Enzyme Research Laboratories).⁴ For measurement of OVA-specific IgE, 96-well plates were coated with 2 μ g/mL OVA by overnight incubation at 4°C. After blocking of nonspecific bindings with 1% bovine serum albumin for 1h at room temperature and subsequent washing, the samples were added and incubated overnight. After appropriate washing, the plates were incubated with biotin-labeled rat anti-mouse IgE for 2h, and then with horseradish

peroxidase-labeled streptavidin for 1h at room temperature. Tetramethylbenzidine (Sigma) was used for development and the reaction was stopped by adding (1M) sulfuric acid solution. Optical density was measured at 450 nm and the values were used as arbitrary units of OVA-specific IgE concentration.

Stimulation of BMDCs with Der p1 and TSLP in the presence of hPS

We cultured BMDCs (4×10^5 cells) in the presence of 20 $\mu\text{g/ml}$ of hPS for 1h before adding Der p1 (1 $\mu\text{g/ml}$) or TSLP (20 ng/ml) and cultured for an additional 24h. The number of TNF α (+) and IL-12 (+) BMDCs and the secretion of cytokines were then evaluated.

Stimulation of OVA-sensitized lymphocytes *ex vivo* in the presence of hPS

After isolation of OVA-sensitized lymphocytes from the spleen of WT/OVA/SAL mice, we cultured the cells in the presence of 20 $\mu\text{g/ml}$ hPS for 1h before adding 100 mg/ml of OVA. The culture supernatant was collected after 48h of incubation to measure the levels of cytokines and IgE.

Analysis of mRNA expression

We used Sepasol RNA I super G (Nacalai) to extract total RNA following the instructions of the manufacturer. The concentration of RNA was measured by using a Nano Drop ND-1000 spectrophotometer (Shimazu). RNA was reverse-transcribed to cDNA using oligo dT primers and ReverTraAce reverse transcriptase (TOYOBO) following instructions of the manufacturer. The sequences of primers used in the study are described in Supplementary

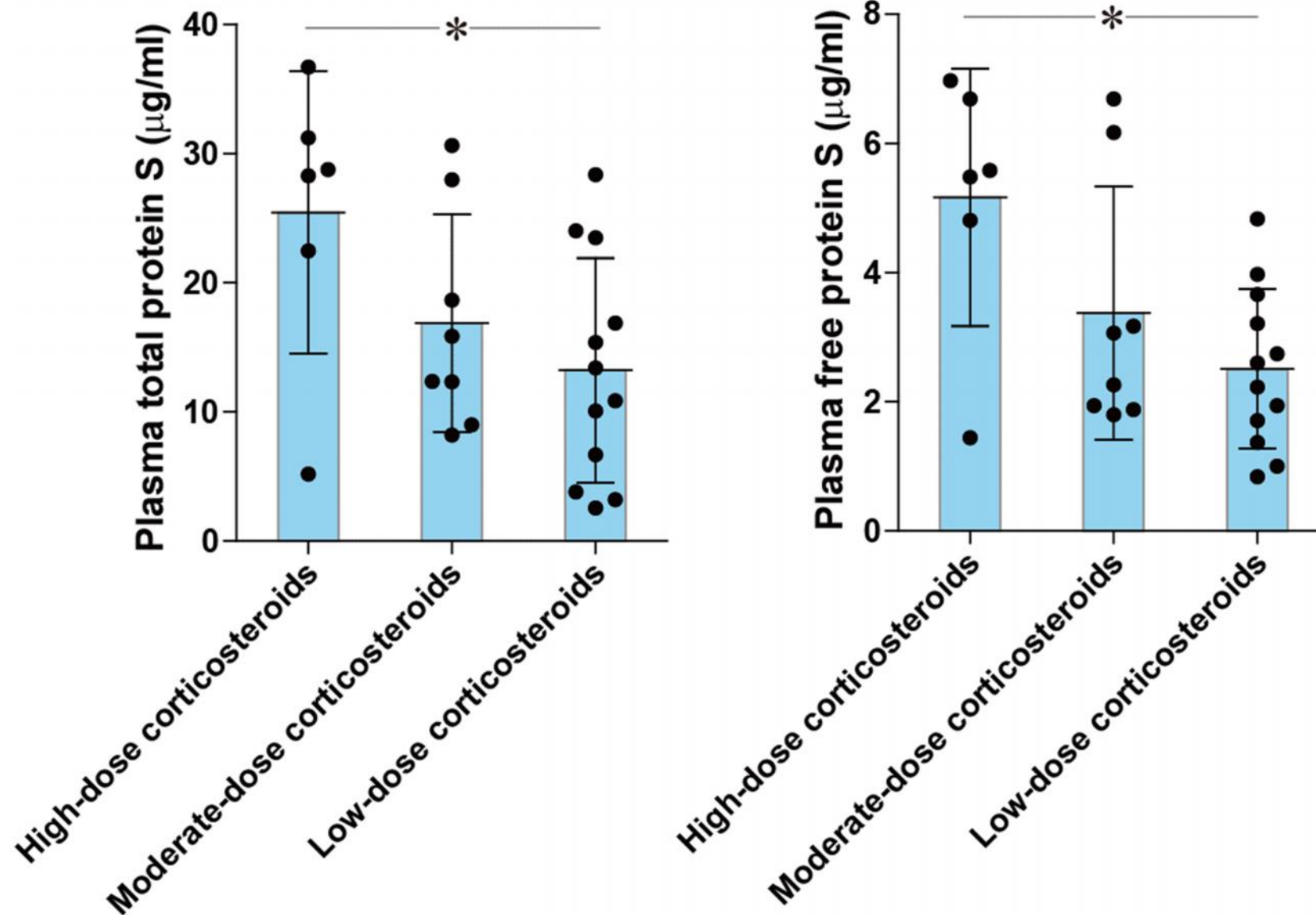
Table 2.

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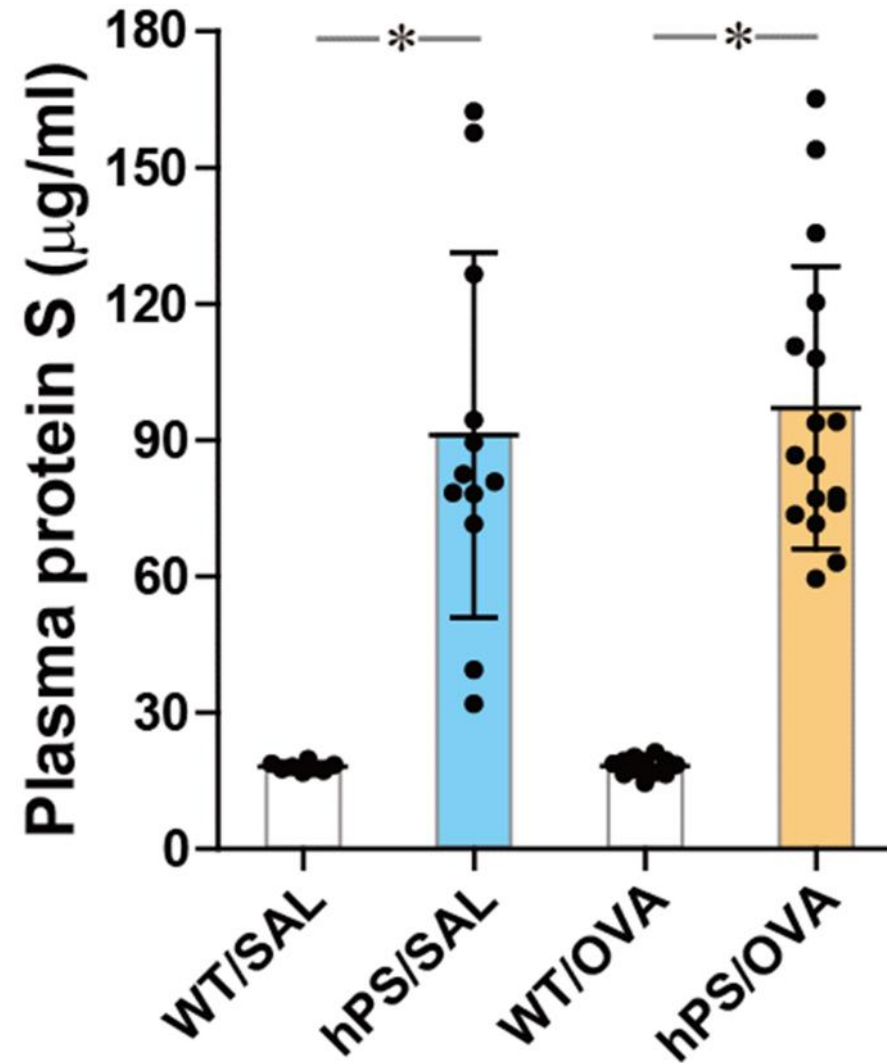
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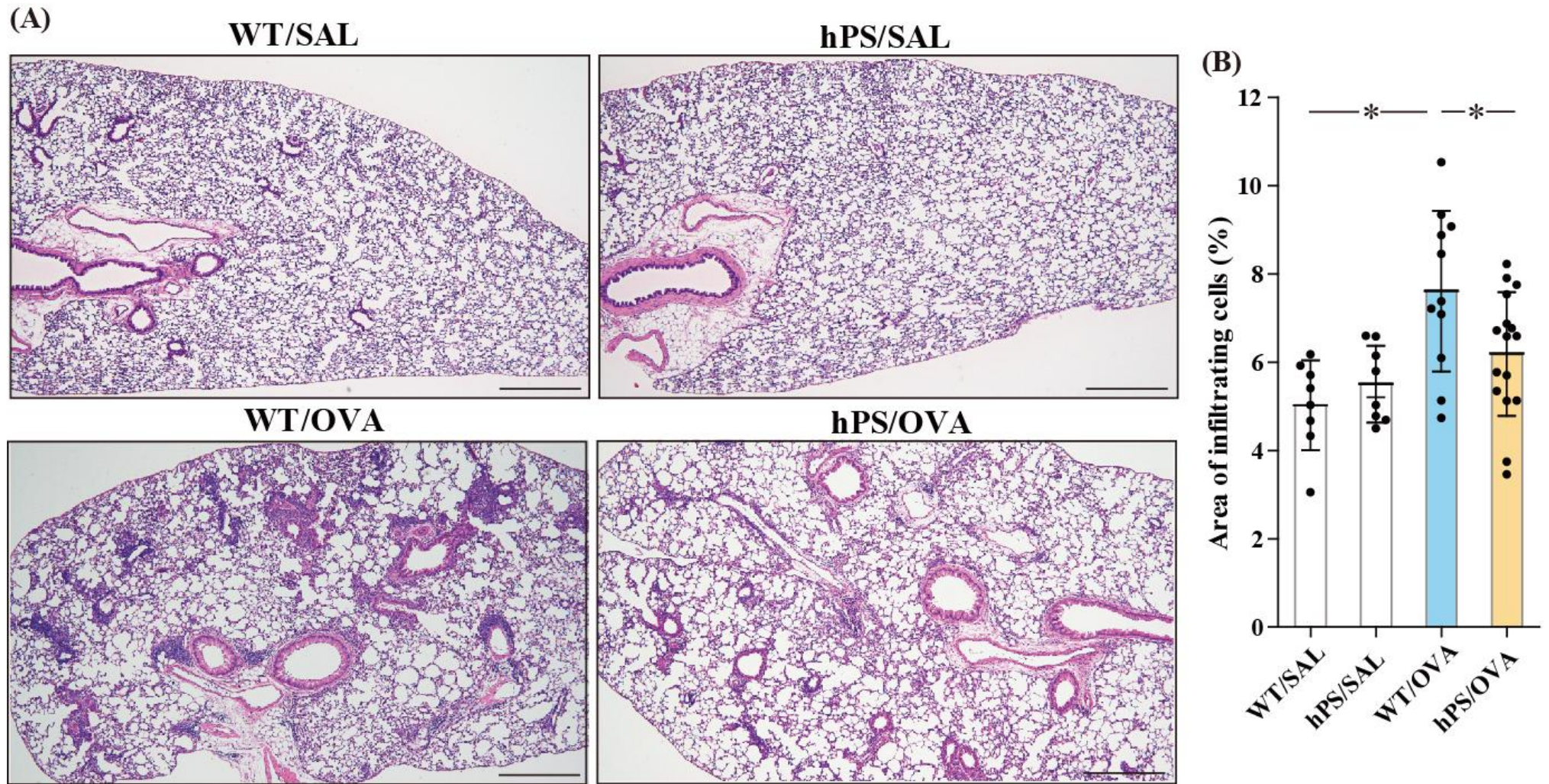
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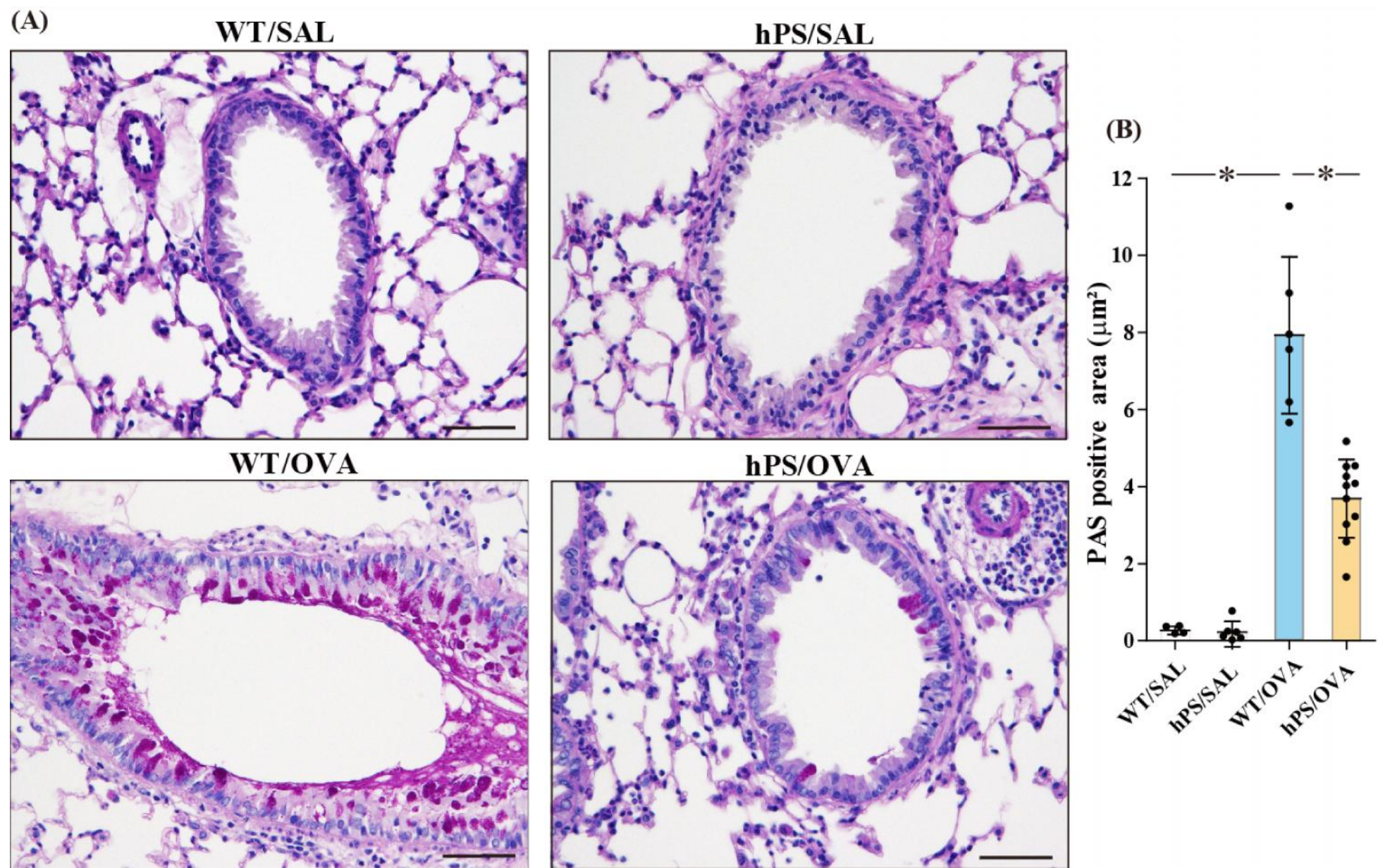
Supplementary Figure 1. Decreased plasma levels of total and free protein S in asthmatic patients receiving low dose of corticosteroids. There were 26 patients with bronchial asthma. The total and active PS were measured by commercial enzyme immunoassay kits. Data are expressed as the mean $\pm$ S.D. * $p < 0.05$.



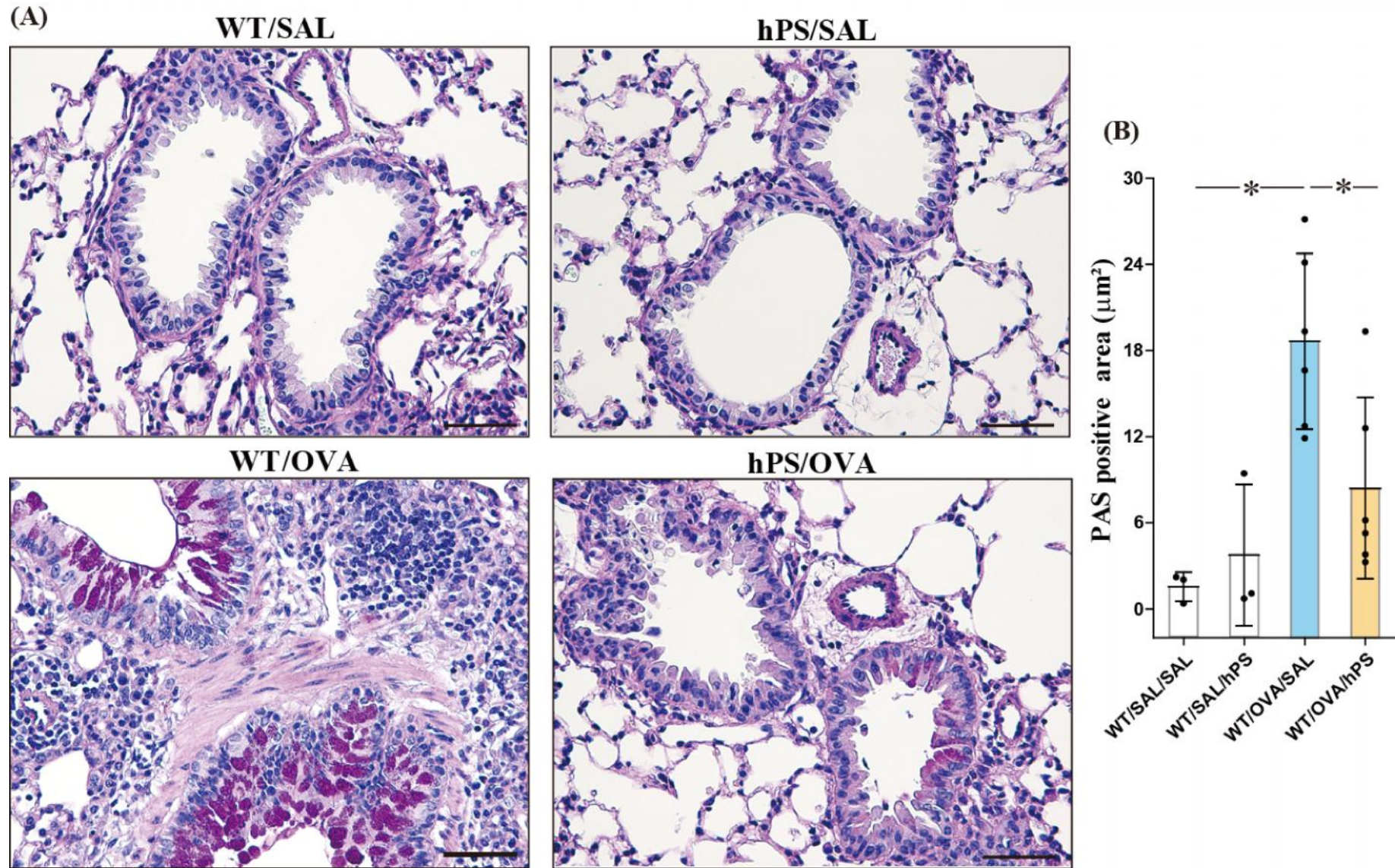
Supplementary Figure 2. Increased circulating level of human protein S in transgenic mice. Human protein S (hPS) was measured by enzyme immunoassays. Data are expressed as the mean $\pm$ S.D. * $p < 0.05$. WT/SAL (n=8), wild type/saline; WT/OVA (n=12), wild type/ovalbumin; hPS/SAL (n=8), human protein S/saline; hPS/OVA (n=17).



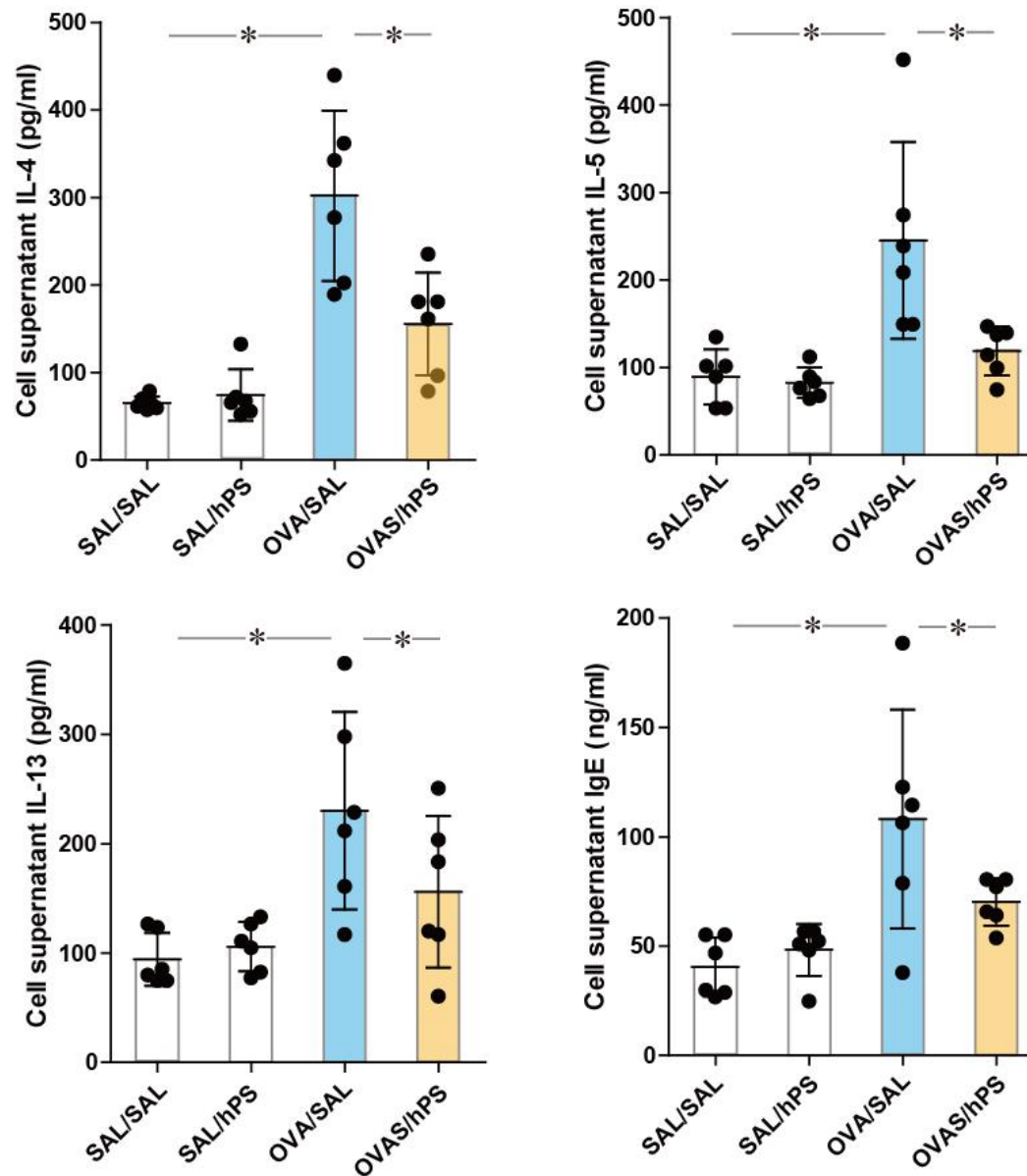
Supplementary Figure 3. Reduced infiltration of inflammatory cells in lung tissue from human protein S transgenic mice with allergy. Lung tissue was stained with hematoxylin & eosin (A) and the grade of inflammatory cell infiltration was quantified using the WindROOF imaging software (B). The figure represents the mean of two independent experiments. Scale bars indicate 500 μ m. Data are expressed as the mean $\pm$ S.D. * p <0.05. WT/SAL (n=8), wild type/saline; WT/OVA (n=11), wild type/ovalbumin; hPS/SAL (n=8), human protein S/saline; hPS/OVA (n=16), human protein S/ovalbumin.



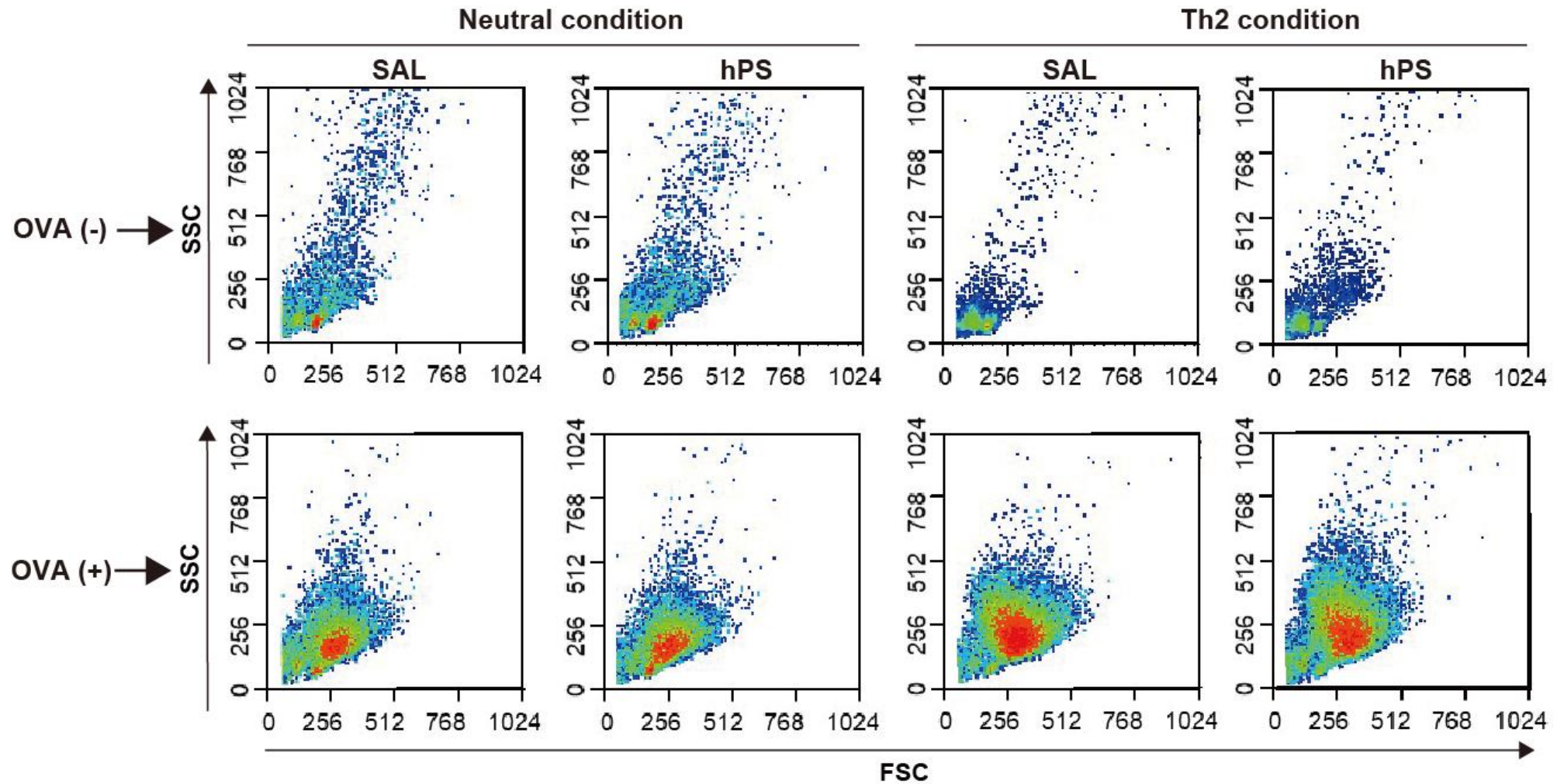
Supplementary Figure 4. Reduced number of mucin-secreting cells in human protein S transgenic mice with allergy. Lung tissue was stained with periodic acid Schiff (A) and quantified using the WindRoof imaging software (B). The figure represents the data of one independent experiment. Scale bars indicate 50 μm . Data are expressed as the mean $\pm$ S.D. * $p < 0.05$. WT/SAL (n=4), wild type/saline; WT/OVA (n=4), wild type/ovalbumin; hPS/SAL (n=6), human protein S/saline; hPS/OVA (n=11), human protein S/ovalbumin.



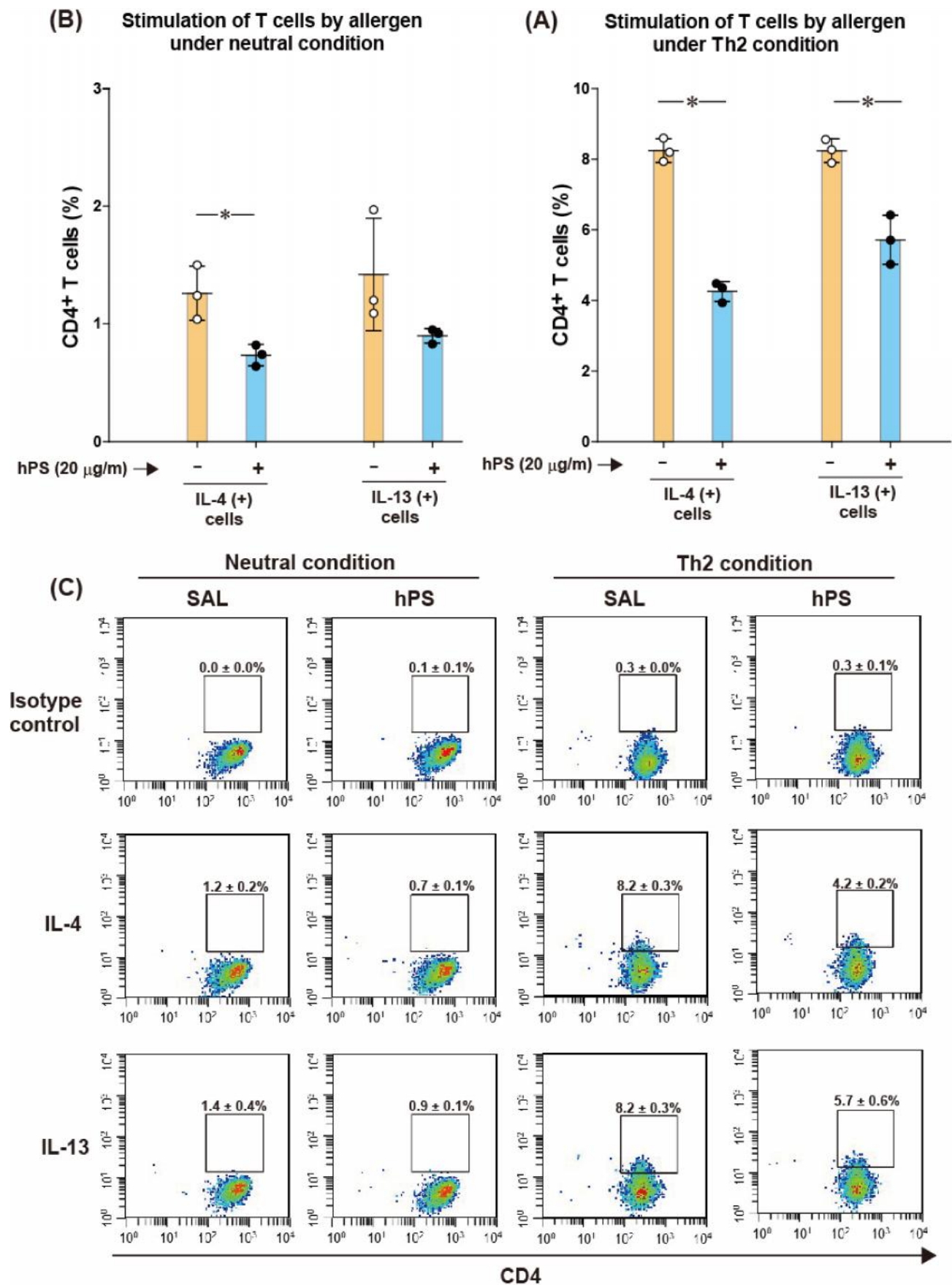
Supplementary Figure 5. Reduced secretion of mucin in allergen-sensitized mice treated with exogenous human protein S. Lung tissue was stained with periodic acid Schiff (A) and quantified using the WindRoof imaging software (B). Scale bars indicate 50 μm . Data are expressed as the mean $\pm$ S.D. WT/SAL/hPS (n=3), WT mice received intraperitoneal SAL and intranasal human protein S (hPS). WT/OVA/SAL (n=6), WT mice sensitized by intraperitoneal ovalbumin (OVA) and treated with intranasal SAL. *p<0.05.



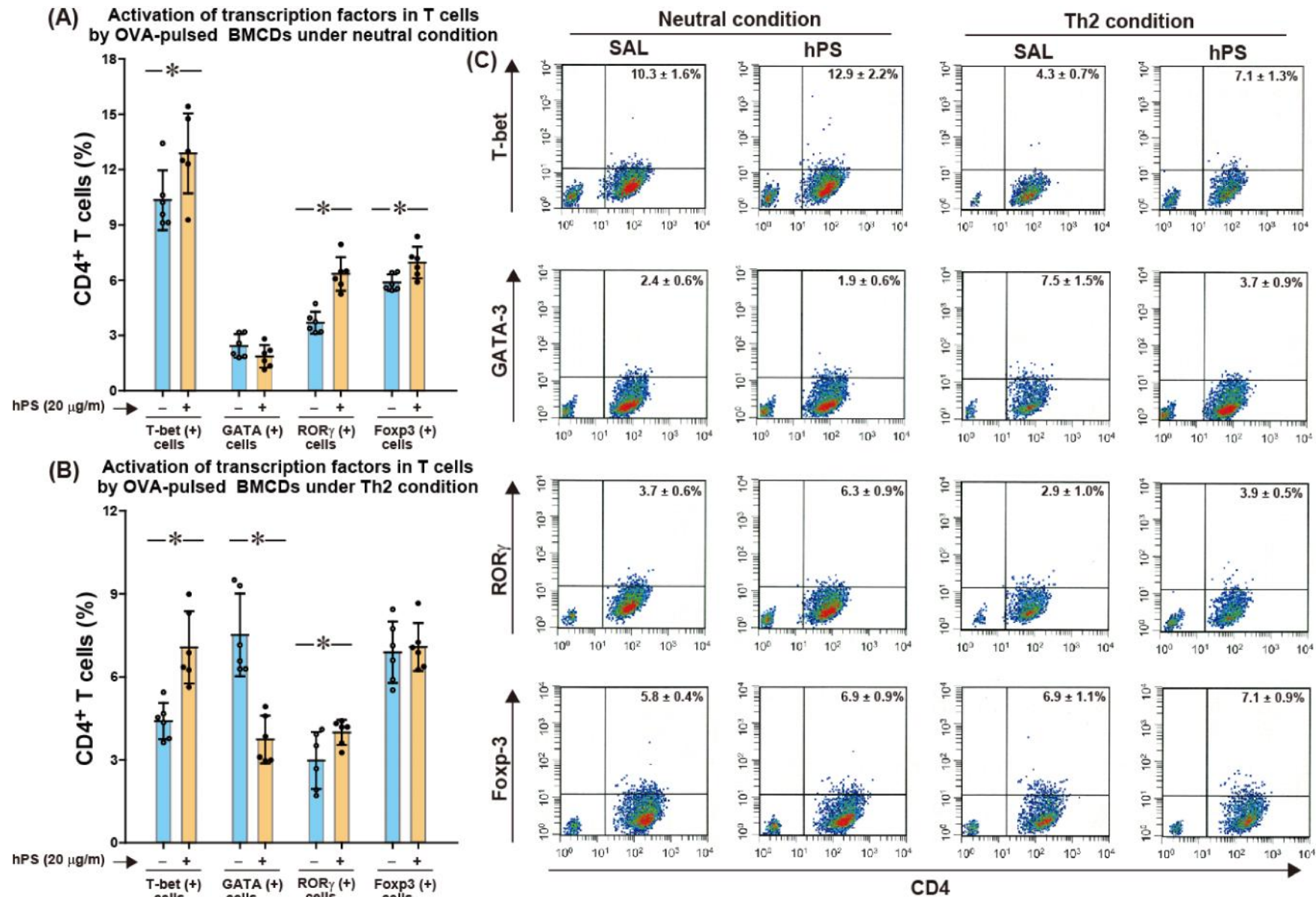
Supplementary Figure 6. Human PS inhibits Th2 response *ex vivo*. Lymphocytes from the spleen of OVA-sensitized mice, were cultured in the presence of hPS or saline and treated with OVA or saline. Cytokines and IgE were measured by enzyme immunoassays. Data are expressed as the mean ± S.D. WT/SAL/hPS (n=3), WT mice received intraperitoneal SAL and intranasal human protein S (hPS). WT/OVA/SAL (n=6), WT mice sensitized by intraperitoneal ovalbumin (OVA) and treated with intranasal SAL. *p<0.05.



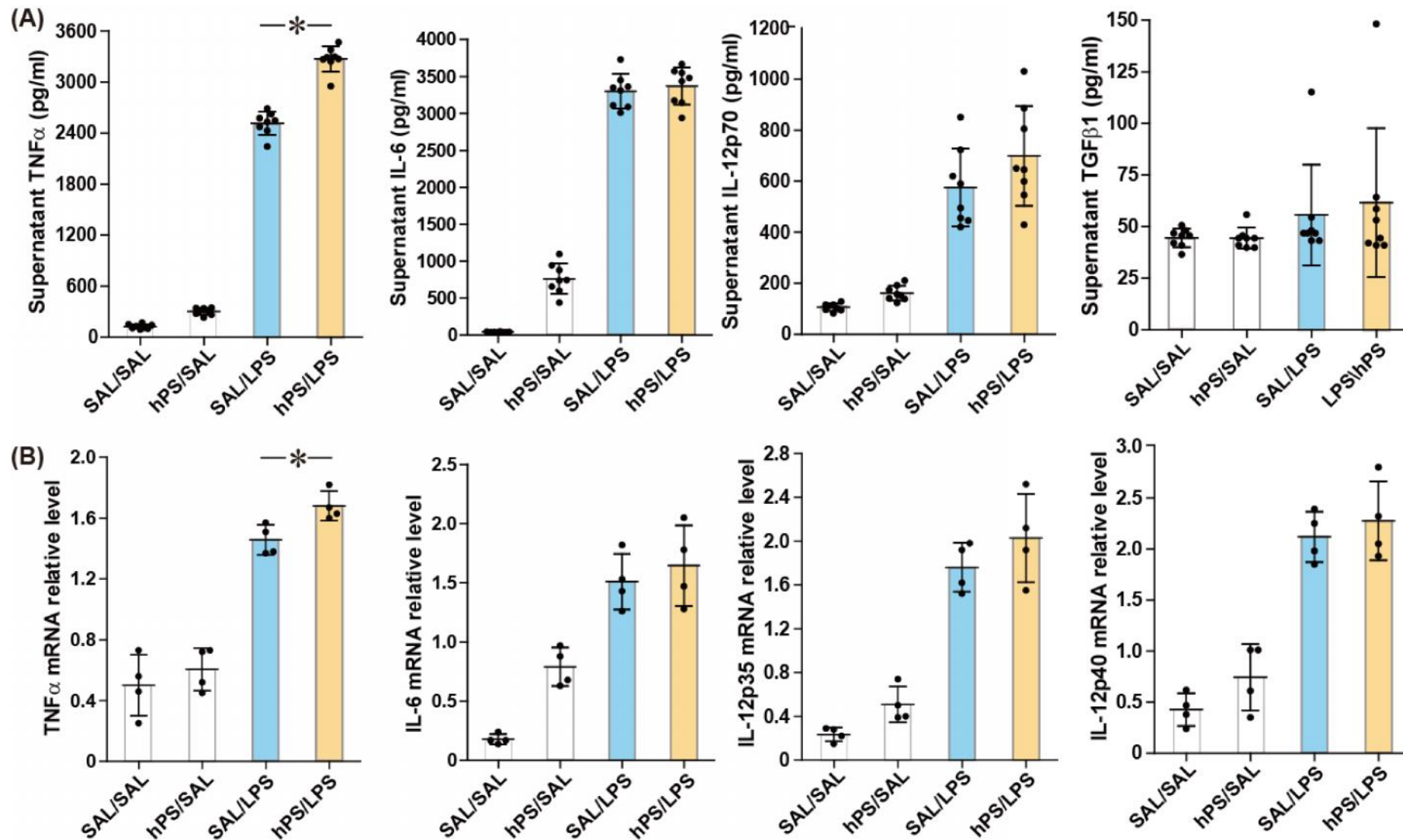
Supplementary Figure 7. The response of lymphocytes under different treatment conditions and conditions is specific to ovalbumin. After isolation from OT-II T-cell receptor TG mouse, whole spleen cells were cultured in the presence of hPS for 1h, and then ovalbumin [OVA (+)] or saline [(OVA (-))] was added and incubated for 7 days under Th2-skewing or neutral condition. SAL, saline; hPS, human protein S; OVA, ovalbumin; SSC, side scatter; FSC, forward scatter.



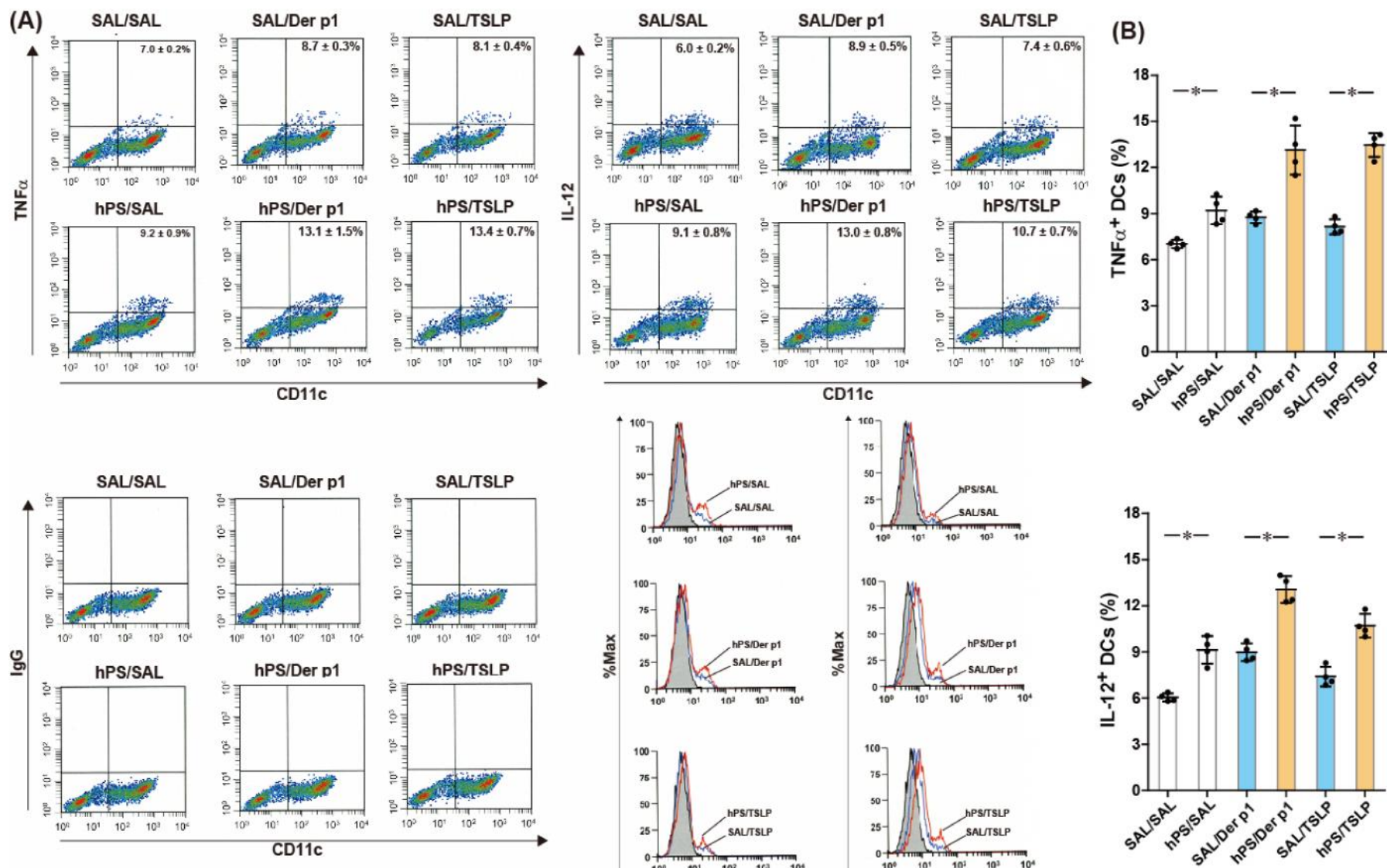
Supplementary Figure 8. Decreased number of IL-4 (+) and IL-13 (+) cells in the presence of human protein S. Whole spleen cells isolated from OT-II transgenic mice were pre-treated with human protein S (hPS) or saline (SAL) for 1h before stimulating with the allergen (ovalbumin) under neutral (A) or Th2 (B, C) condition. The percentage of cells expressing signature cytokines were evaluated by flow cytometry. N=3 in each group. Data are expressed as the mean $\pm$ S.D. * $p < 0.05$.



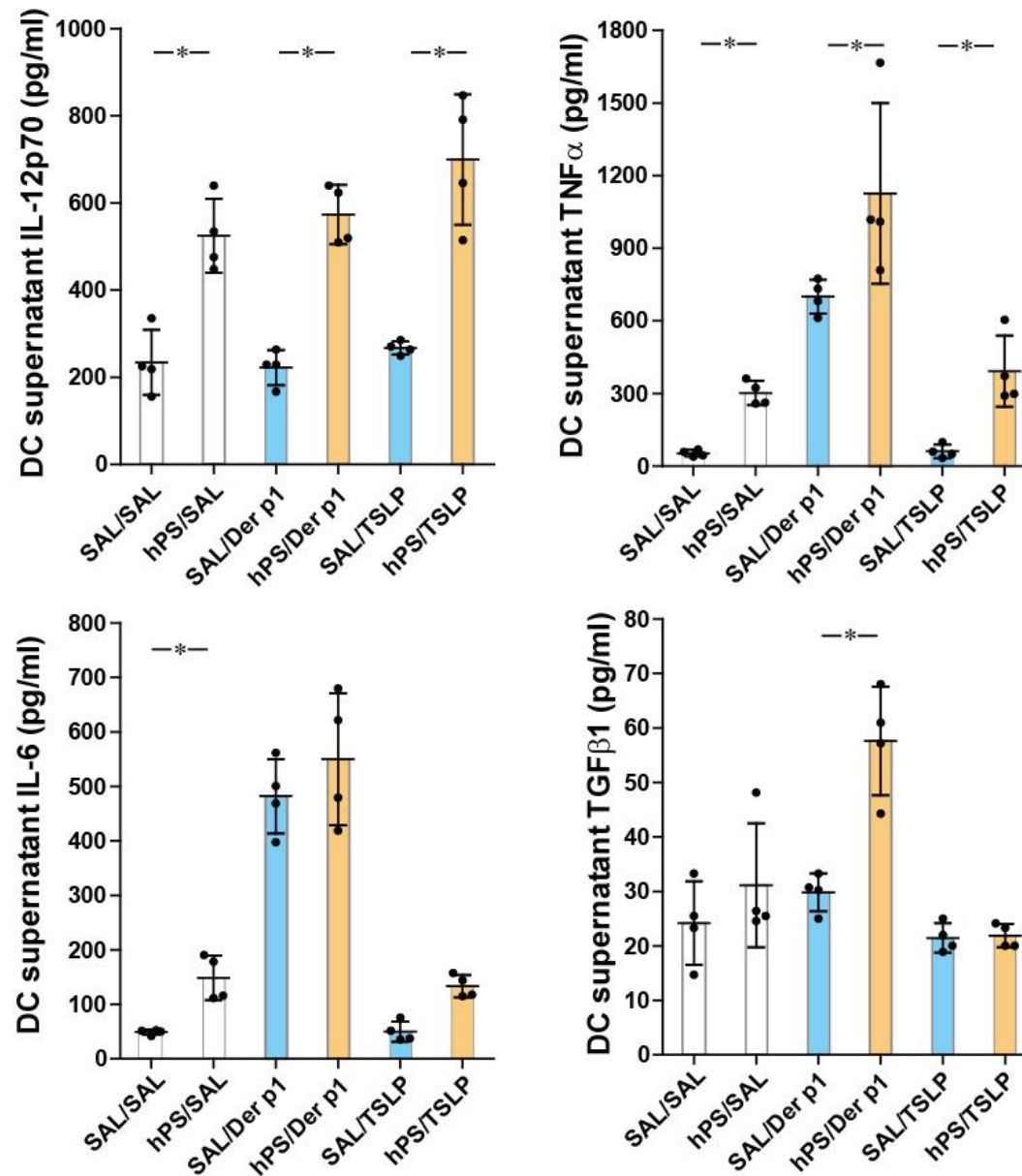
Supplementary Figure 9. Decreased activation of transcription factors in T cells by ovalbumin-pulsed dendritic cells in the presence of human protein S. Bone marrow-derived dendritic cells (BMDC) were pre-treated with human protein S (hPS) or saline before sensitizing with the allergen (ovalbumin) and co-cultured with ovalbumin-specific CD4⁺ T cells from OTII transgenic mice under neutral (A) or Th2 (B, C) conditions. N=6 in each group. Data are expressed as the mean ± S.D. *p<0.05.



Supplementary Figure 10. Human protein S (hPS) promotes lipopolysaccharide-mediated expression of TNF α . Bone marrow-derived dendritic cells were stimulated with lipopolysaccharide (LPS) in the presence or absence of hPS and the secretion of protein (A, n=8) and the mRNA expression (B, n=4) of cytokines were evaluated. Data are expressed as the mean $\pm$ S.D. *p<0.05



Supplementary Figure 11. Dendritic cells express Th1 cytokines after stimulation with Der p1 and TSLP in the presence of human protein S. Bone marrow-derived dendritic cells (BMDCs) were generated from C57BL/6 WT mice after culturing for seven days in the presence of Flt3L followed by purification of CD11c⁺ DCs as described under methods in the Online Supporting Information. BMDCs were washed, seeded in 24-well plates and cultured using RPMI-1640 medium in the presence of 20 µg/ml human protein S (hPS) for 1h before adding Der p1 (1 µg/ml) or thymic stromal lymphopoietin (TSLP) (20 ng/ml) and cultured for an additional 24h. IL12 (+) or TNFα (+) BMDCs were gated by flow cytometry and the percentage of cells in each treatment group was calculated. Data are expressed as the mean ± S.D. *p<0.05.



Supplementary Figure 12. Human protein S increases the expression of Th1 cytokines from dendritic cells. Bone marrow-derived dendritic cells were cultured in the presence of human protein S (hPS) before stimulating with Der p1 (1 μ g/ml) or thymic stromal lymphopoietin (TSLP) (20 ng/ml) and continued culturing for 24h. The concentration of tumor necrosis factor α and interleukin (IL)-12 were measured by enzyme immunoassays. Data are expressed as the mean $\pm$ S.D. SAL, saline; * p <0.05.

Supplementary Table 1. Characteristics of the patients

Clinical parameters	Patients	Controls
No of subjects	26	8
Sex		
Male/female	12/14	8/0
Age (years-old)	61.6 ± 17.4	47.4 ± 12.4
Lung function test		NA
VC (L)	3.0 ± 0.9	
VC (% predicted)	97.7 ± 17.5	
FVC (L)	2.9 ± 1.0	
FVC (% predicted)	96.1 ± 19.9	
FEV1 (L)	2.2 ± 1.0	
FEV1/FVC (%)	72.5 ± 12.4	
Blood eosinophils (cells/ μ L)	327.3 ± 268.9	NA
Blood eosinophils (%)	5.3 ± 4.1	NA
Serum IgE (U/ml)	426.1 ± 713.9	NA
Severity of BA (GINA)		
2/3/4	4/16/6	

Data are the mean $\pm$ standard deviation. FEV1, forced expiratory volume in one second; FVC, forced volume vital capacity; BA, bronchial asthma. GINA, Global Initiative for Asthma. NA, not available.

Supplementary Table 2. Primers for RT-PCR

Sequence (5' -> 3')		Tm	Reference	Location	Product size
GAPDH					
Sense	TGGCCTTCCGTGTTTCCTAC	65.0	NM_008084	686-704	178 bp
Antisense	GAGTTGCTGTTGAAGTCGCA	64.2		863-844	
IL-6					
Sense	CTGGTCTTCTGGAGTACCATAG	62.3	NM_031168	375-396	374 bp
Antisense	AAGTCAGATACCTGACAACAGG	62.7		748-727	
TNF α					
Sense	ACGTGGAACCTGGCAGAAGAG	64.4	NM_013693	182-201	284 bp
Antisense	CTCCTCCACTTGGTGGTTTG	64.5		465-446	
IL-12p35					
Sense	TCCTGCACTGCTGAAGACATC	65.6	NM_001159424	238-258	340 bp
Antisense	TCTCGCCATTATGATTCAGAGAC	64.1		577-555	
IL-12p40					
Sense	CCCTGACTCTCGGGCAGTGAC	70.6	NM_008352	477-497	400 bp
Antisense	ACCTTTCTGGTTACACCCCTC	63.0		876-856	

GAPDH: glyceraldehyde-3-phosphate dehydrogenase; IL: interleukin; TNF α , tumor necrosis factor α .