

ORIGINAL ARTICLE

Asthma and Lower Airway Disease

Osteopontin protects against pneumococcal infection in a murine model of allergic airway inflammation

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Abstract

Background: In atopic asthma, chronic Th2-biased inflammation is associated with an increased risk of pneumococcal infection. The anionic phosphoglycoprotein osteopontin (OPN) is highly expressed in asthma and has been ascribed several roles during inflammation. This study aimed to investigate whether OPN affects inflammation and vulnerability to pneumococcal infection in atopic asthma.

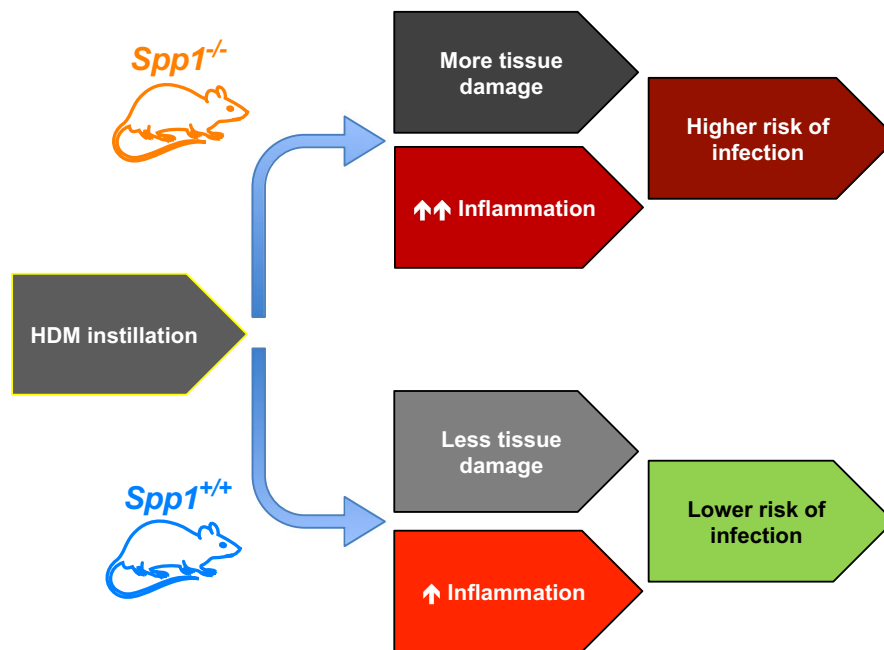
Methods: House dust mite (HDM) extract was used to induce allergic airway inflammation in both wild-type (*Spp1*^{+/+}) and OPN knockout (*Spp1*^{-/-}) C57BL/6J mice, and the airway was then infected with *Streptococcus pneumoniae*. Parameters reflecting inflammation, tissue injury, and bacterial burden were measured. In addition, samples from humans with allergic asthma were analyzed.

Results: Both allergen challenge in individuals with allergic asthma and the intranasal instillation of HDM in mice resulted in increased OPN levels in bronchoalveolar lavage fluid (BALF). More immune cells (including alveolar macrophages, neutrophils, eosinophils, and lymphocytes) and higher levels of proinflammatory cytokines were found in *Spp1*^{-/-} mice than in *Spp1*^{+/+} mice. Moreover, OPN-deficient mice exhibited increased levels of markers reflecting tissue injury. Upon infection with *S. pneumoniae*, *Spp1*^{+/+} mice with allergic airway inflammation had a significantly lower bacterial burden in both BALF and lung tissue than did *Spp1*^{-/-} mice. Furthermore, *Spp1*^{-/-} mice had higher levels of cytokines and immune cells in BALF than did *Spp1*^{+/+} mice.

Conclusion: OPN reduces inflammation, decreases tissue injury, and reduces bacterial loads during concurrent pneumococcal infection and allergic airway inflammation in a murine model. These findings suggest that OPN significantly affects vulnerability to pneumococcal infection in atopic asthma.

KEYWORDS

allergy, asthma, host defense, house dust mite, osteopontin, *Streptococcus pneumoniae*



GRAPHICAL ABSTRACT

HDM-instillation to the airways causes increased inflammation and tissue damage in osteopontin-deficient mice. Osteopontin-deficient mice (*Spp1*^{-/-}) with an ongoing allergic airway inflammation are more prone to pneumococcal infection. The findings suggest that osteopontin plays important roles, modulating inflammation, tissue damage, and host defense against pneumococcal infection during allergic asthma.

1 | INTRODUCTION

Asthma is a prevalent disease affecting approximately 4.3% of all individuals and up to 10%-20% of the population in some countries.¹ Asthma has several phenotypes, but atopic asthma, which features eosinophilia and Th2-mediated airway inflammation with the expression of IL-4, IL-5, and IL-13, is the predominant phenotype.¹ House dust mite (HDM) allergens can trigger Th2 inflammation and are the most common allergens globally associated with asthma and rhinitis.² Atopic asthma is accompanied by a substantially increased risk of *Streptococcus pneumoniae* infection.³⁻⁶ *S. pneumoniae* is a gram-positive bacterium (pneumococcus) commonly found in the upper respiratory tract of asymptomatic carriers; however, it is the leading cause of community-acquired pneumonia.⁷ The factors increasing the risk of pneumococcal infection in atopic asthma remain poorly defined. However, impaired mucociliary clearance, treatment with immunosuppressants, and a dysfunctional host defense resulting from chronic Th2-biased inflammation have been suggested to contribute to this vulnerability.^{4,8} In animal models of asthma, reports conflict regarding whether the allergic inflammation *per se* increases vulnerability to pneumococcal infection or whether the Th2-biased inflammation has a protective effect.⁹⁻¹¹

In addition to the characteristic increase in Th2 cytokines and the influx of eosinophils to the airway during allergic asthma, the level of the anionic phosphoglycoprotein osteopontin (OPN)

increases with disease severity.^{12,13} Several functions of OPN in inflammation have been described, including important roles in cell-mediated immunity, IgE response regulation, inflammatory cell migration, airway fibrosis development, and angiogenesis.^{14,15} Recently, protective roles of OPN during allergic airway inflammation have been described using a murine model of asthma.^{16,17} We recently showed that OPN protects against lung injury through binding and attenuating the effects of cytotoxic damage-associated molecular pattern molecules (DAMPs).¹⁸ However, in mice without prior airway inflammation, OPN has been shown to promote pneumococcal infection.¹⁹ In other models of infection, OPN confers resistance to infection by bacteria such as *Mycobacterium tuberculosis*, *Rickettsia*, and *Listeria monocytogenes*.^{14,20,21} Based on these seemingly contradictory findings, this study aimed to investigate whether OPN plays a role in vulnerability to pneumococcal infection during allergic airway inflammation.

2 | MATERIAL AND METHODS

2.1 | Ethics statement

All human participants gave written informed consent to participate in the study, which was approved by the Regional Ethical Review Board in Lund (LU2012/860). All mouse experiments were conducted according to institutional guidelines and were approved by the Malmö-Lund Animal Care Ethics Committee (M138-13 and M187-15).

2.2 | Animals

Adult female C57BL/6J (wild-type; *Spp1*^{+/+}) and OPN-deficient (*Spp1*^{-/-}) mice (6–7 weeks old) were bred and genotyped at Lund University. The *Spp1*^{-/-} mice have been described elsewhere.²² All animals were housed under specific pathogen-free conditions and had free access to commercial chow and water.

2.3 | Instillation of HDM into mice

For the intranasal (i.n.) instillation of HDM, 6- to 7-week-old *Spp1*^{+/+} and *Spp1*^{-/-} mice were anesthetized using 3%–5% isoflurane. HDM extract (Greer Laboratories, Lenoir, NC) was suspended in saline (0.9% NaCl) at a concentration of 1.0 mg/mL. Mice were exposed to 25 µg of HDM (25 µL, dropwise, alternating between nostrils) on five consecutive days each week for three consecutive weeks. Following the final sensitization with the HDM allergen, mice were allowed to rest for 2 days before being euthanized with isoflurane. The model was adopted from a previously published study, where HDM was instilled intermittently with a two-day rest between week-days.²³

2.4 | Allergen challenge in individuals with atopic asthma

The clinical characteristics included individuals and study protocol are described in Table S1 and in the Data S1.

2.5 | Pneumococcal infection in mice

Following sensitization with the HDM allergen, mice were infected with *S. pneumoniae* (strain A66; ATCC, Rockville, MD) bacteria. Bacteria were grown to mid-logarithmic phase (optical density (OD)₆₂₀ ~ 0.5) in Todd-Hewitt broth supplemented with 0.5% yeast extract (BD, Franklin Lakes, NJ) at 37°C and 5% CO₂. The bacteria were washed and suspended in sterile PBS to a concentration of 2×10^7 colony-forming units (cfu)/mL. Each animal was infected with 50 µL of the bacterial solution by i.n. instillation, whereas uninfected control animals received PBS alone. To maintain a consistent inoculum throughout the experiments, a concentration of bacteria corresponding to a fixed OD was used to infect the animals, and the number of cfu was confirmed by plating in every experiment. The mice were euthanized 48 hours after infection.

2.6 | Bronchoalveolar lavage and cell counts

After the mice were killed, the trachea was cannulated to extract cellular infiltrates from the bronchoalveolar space using a total volume of 1 mL PBS containing 0.1 mmol/L EDTA. When required, red blood cells were removed by suspending the bronchoalveolar lavage fluid (BALF) cells in 100 µL of lysis buffer (150 mmol/L

NH₄Cl, 10 mmol/L KHCO₃, and 0.1 mmol/L EDTA; pH 7.2) for 2 minutes at room temperature (RT) followed by washing in 1 mL PBS. The total number of cells was manually enumerated using a hemocytometer. For differential counts, cytospin preparations of cells were stained with modified Wright-Giemsa stain (Sigma-Aldrich, St. Louis, MO), and a minimum of 300 cells were counted per BALF sample. Cell-free BALF samples were stored at -80°C until further analysis.

The human study subjects and protocols used are described in the Data S1.

2.7 | Measurement of parameters in BALF

To analyze different parameters in the mouse and human BALF samples, commercially available ELISAs were used to measure OPN (R&D Systems, Abingdon, UK), lactate dehydrogenase (LDH) (USCN Life Science, Wuhan, China), and albumin (Bethyl Laboratories, Montgomery, TX). To determine the release of extracellular nucleosomes in BALF, a cell death detection ELISA kit (Roche Applied Science, Penzberg, Germany) was used. To detect multiple mediators, a Bioplex Pro 23-Plex Group I mouse cytokine assay was performed in a Luminex-xMAP/Bioplex-200 system running Bioplex Manager 5.0 software (Bio-Rad, Richmond, CA).

2.8 | Collection of lungs for microscopy

After mice were killed, the lobes of the left lung were perfused with and submerged in Histofix (Histolab, Göteborg, Sweden). After dehydration and paraffin embedding, 4-µm sections were cut from the tissue blocks. Following rehydration and antigen retrieval, the sections were incubated with goat antibodies against murine myeloperoxidase (MPO) or preimmune goat IgG (R&D Systems). Bound antibodies were detected using horseradish peroxidase-conjugated rabbit anti-goat secondary antibodies (diluted 1:1,250; DAKO, Glostrup, Denmark) and visualized using 3,3'-diaminobenzidine as a chromogenic substrate. Some sections were also stained with hematoxylin and eosin (H&E).

2.9 | Statistical analysis

Data are expressed as the means ± standard errors of the mean (SEMs). For the statistical analysis of two experimental groups, the nonparametric Wilcoxon matched-pairs test was used. For the statistical evaluation of more than two experimental groups, either the nonparametric Kruskal-Wallis test with Dunn's *post hoc* test or two-way analysis of variance (ANOVA) with the Tukey *post hoc* test was used. All statistical evaluations were performed using GraphPad Prism software 7.0 (GraphPad Software, La Jolla, CA). *P*-values greater than 0.05 were considered nonsignificant. In the tables and figures, asterisks are used to represent different significance levels as follows: **P* ≤ 0.05, ***P* ≤ 0.01, ****P* ≤ 0.001, and *****P* ≤ 0.0001.

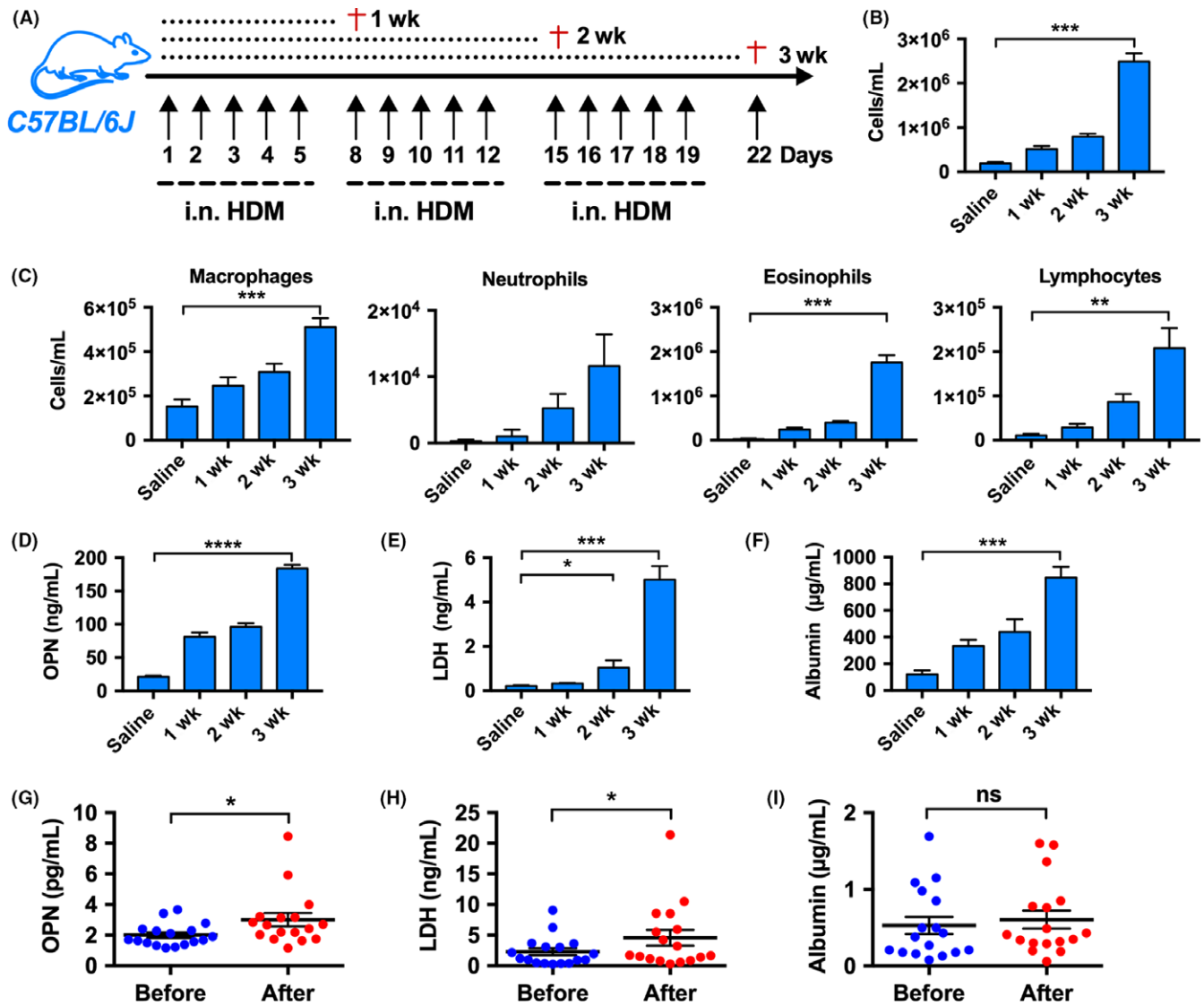
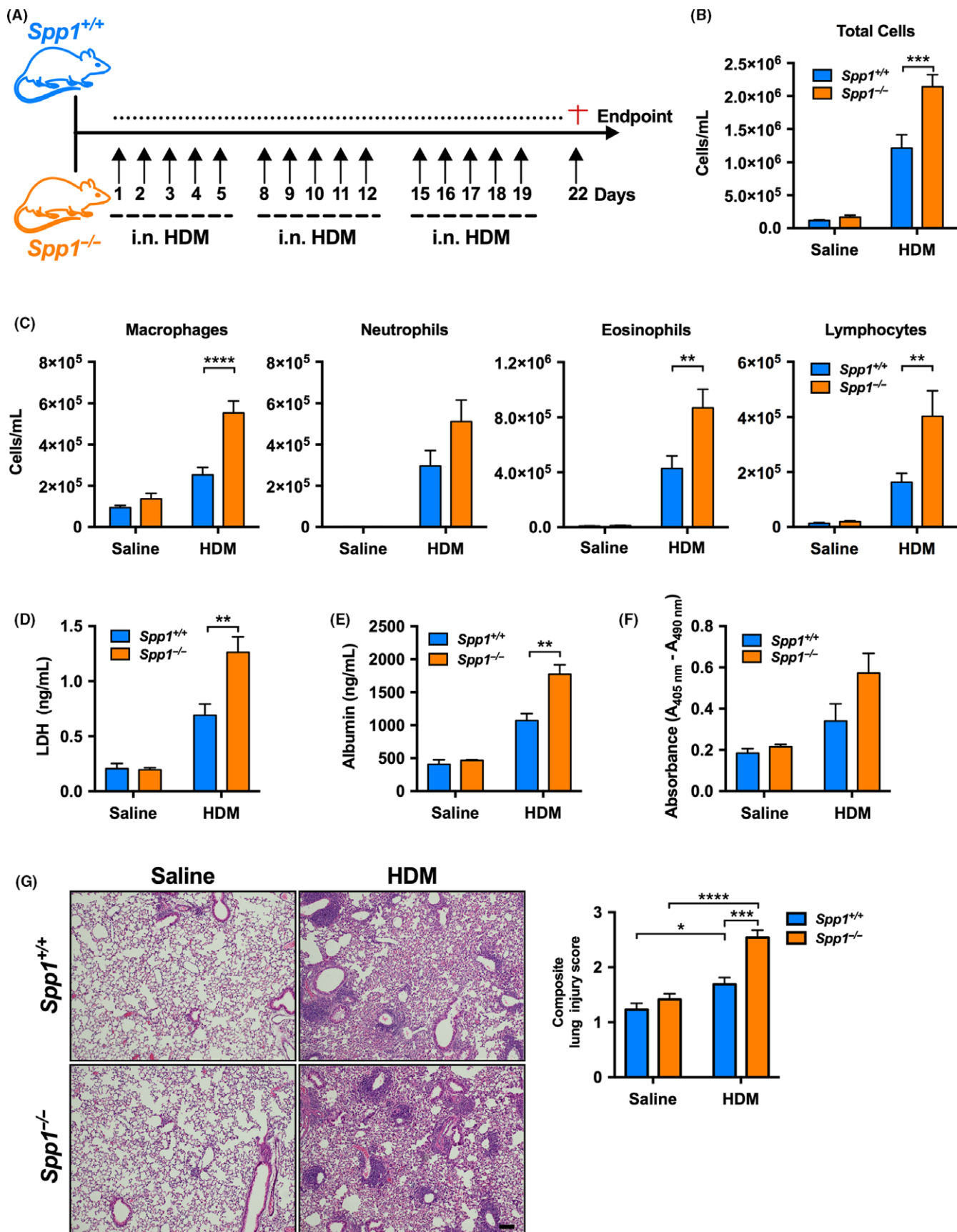


FIGURE 1 Allergic inflammation results in increased osteopontin (OPN) levels and tissue damage in the airway. (A) Schematic presentation of the experimental design and time points of the i.n. instillation of HDM extract (25 µg/dose) into C57BL/6J mice. Two days after the final dose, the animals were sacrificed, and BALF was collected (the red cross indicates the experimental endpoint). The total cell numbers ($***P = 0.0001$) (B), differential cell counts ($***P = 0.0006$ and 0.0001 ; $**P = 0.0013$, respectively) (C), OPN levels ($****P < 0.0001$) (D), LDH levels ($*P = 0.046$ and $***P = 0.0002$) (E), and albumin levels ($***P = 0.0003$) (F) in BALF after 1, 2, and 3 weeks of i.n. instillation with HDM or saline. The data shown are the means and SEMs from 6 to 9 animals in each group. (G) Patients with allergic asthma were subjected to an initial BALF collection (Before) followed by allergen challenge and a subsequent BALF collection 24 h later (After). The OPN levels, as assessed by ELISA, were significantly elevated after allergen challenge ($n = 17$; $*P = 0.027$). (H) LDH levels in BALF were determined to assess lung damage and were significantly higher after allergen exposure ($n = 17$; $**P = 0.0082$). (I) To assess pulmonary microvascular leakage, albumin levels were measured in BALF. No significant differences before and after allergen exposure were found ($n = 17$; $nsP = 0.86$) [Colour figure can be viewed at wileyonlinelibrary.com]

FIGURE 2 Osteopontin (OPN) knockout mice display increased inflammatory cell recruitment and tissue damage during allergic airway inflammation. (A) Schematic presentation of the experimental design and time points of HDM instillation in *Spp1*^{+/+} and *Spp1*^{-/-} mice. BALF and lung tissue were harvested 48 h post-HDM instillation (the red cross indicates the experimental endpoint). The total cell numbers ($n = 10$; $**P = 0.0047$) (B), differential cell counts ($n = 10$; $*P = 0.040$ and 0.0001 ; and $*P = 0.0331$, respectively) (C), LDH levels ($n = 6$; $**P = 0.0080$) (D), albumin levels ($n = 6$; $**P = 0.0021$) (E), and free extracellular nucleosomes ($n = 6$; $nsP = 0.16$) (F) in BALF from *Spp1*^{+/+} and *Spp1*^{-/-} mice. (G) H&E staining of lung tissue sections was used to determine lung injury. The left panel shows lung tissue from control animals instilled with either saline or HDM for three weeks. The right panel shows the results of the blinded scoring of lung injury. ($n = 8$; $*P = 0.048$, $***P < 0.0001$, and $****P < 0.001$)



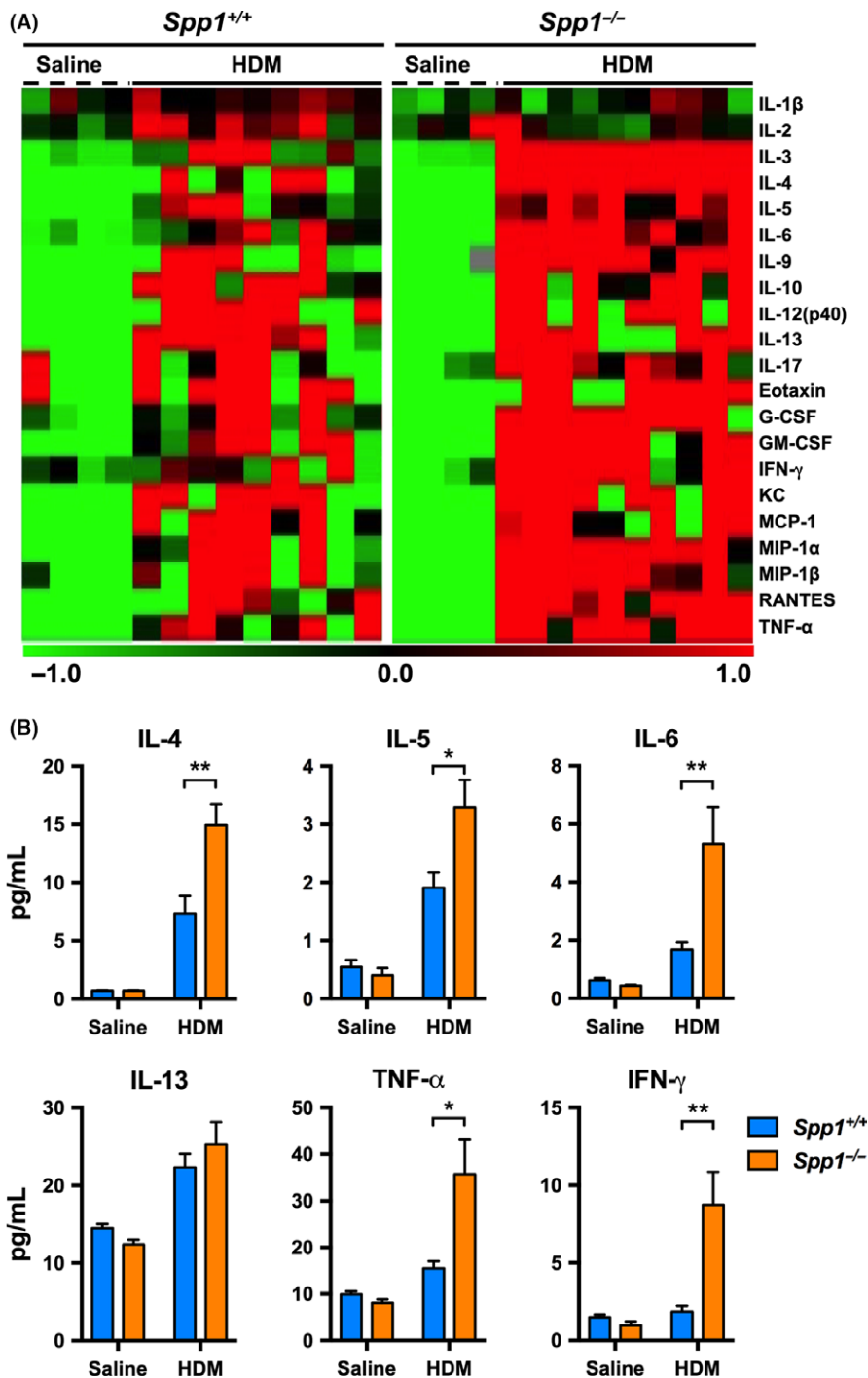


FIGURE 3 Airway inflammation is increased in *Spp1*^{-/-} mice following HDM instillation. (A) Heat map representation of cytokine profiles in BALF from *Spp1*^{+/+} and *Spp1*^{-/-} mice after HDM instillation. Inflammatory mediators were measured using a multiplex bead assay. (B) Graphical presentation of the levels of selected inflammatory cytokines. The data are the means and SEMs; n = 10 per HDM-sensitized group, n = 4 per saline challenged group (IL-4 **P = 0.0023, IL-5 *P = 0.015, IL-6 **P = 0.0065, IL-13 ^{ns}P = 0.56, TNF- α *P = 0.012, and IFN- γ **P = 0.0022)

3 | RESULTS

3.1 | Allergic inflammation increases OPN expression and causes tissue damage

As a model of allergic airway inflammation, mice were repeatedly exposed to i.n. HDM once daily for three consecutive weeks (5 days per week) (Figure 1A). HDM elicited a robust inflammatory response in the airways of mice; this response was measurable in BALF. A time-dependent increase in total cell number, to $\sim 3 \times 10^6$, was seen after

3 weeks of HDM instillation (Figure 1B). The cellular distribution consisted of an increased number of alveolar macrophages (AMs), eosinophils, and lymphocytes but few neutrophils (Figure 1C). The i.n. instillation of saline did not produce an inflammatory response. The OPN levels in BALF increased in parallel with LDH (reflecting cell damage) and albumin (reflecting microvascular leakage) (Figure 1D-F).

In BALF from humans with atopic asthma (described in the Data S1), the levels of OPN and LDH but not albumin increased 24 hours after a single allergen challenge (Figure 1G-I). The cell counts in BALF did not increase significantly (Figure S1). Taken together, these

results indicate that allergic inflammation increases OPN production and inflammation (ie, an influx of immune cells) as well as tissue damage in both mice and humans.

3.2 | Immune cell accumulation and tissue damage increase during allergic inflammation in *Spp1*^{-/-} mice

To investigate whether OPN affects cell death and tissue damage, *Spp1*^{+/+} and *Spp1*^{-/-} mice were subjected to the i.n. instillation of either saline or HDM extract, as described (Figure 2A). Thereafter, the mice were euthanized, and BALF and lung tissue were harvested. HDM extract again induced an influx of immune cells. However, significant increases in the total number of cells as well as in the numbers of AMs and eosinophils were seen in BALF from *Spp1*^{-/-} mice compared with BALF from *Spp1*^{+/+} mice (Figure 2B and C).

To determine the degree of tissue damage and cell death, LDH, albumin, and free nucleosomes (extracellular complexes consisting of DNA and histones resulting from cell death) were measured in BALF from *Spp1*^{+/+} and *Spp1*^{-/-} mice. In mice lacking OPN, significantly higher levels of LDH and albumin were found (Figure 2D and E). The level of free nucleosomes was higher in *Spp1*^{-/-} mice than in *Spp1*^{+/+} mice, but the difference was not significant (Figure 2F). Consistent with these results, blinded lung injury scoring of H&E-stained lung tissue sections confirmed that the tissue damage was more severe in *Spp1*^{-/-} mice subjected to HDM extract instillation for three consecutive weeks than in *Spp1*^{+/+} mice subjected to the same treatment (Figure 2G). In conclusion, *Spp1*^{-/-} mice display increased immune cell influx and tissue damage during HDM-induced allergic inflammation.

3.3 | *Spp1*^{-/-} mice exhibit increased inflammation after HDM instillation

The expression pattern of a broad range of proinflammatory cytokines in BALF from *Spp1*^{+/+} and *Spp1*^{-/-} mice after instillation of HDM extract for 3 weeks was determined using a multiplex assay. A heat map reflecting the degree of differential cytokine expression revealed that OPN-deficient mice have higher levels of inflammation in response to allergen sensitization than do control mice (Figure 3A). On a single-cytokine level, prototypic Th1 (eg, IL-6, TNF- α , and IFN- γ) and Th2 (eg, IL-4 and IL-5) effectors were significantly upregulated in *Spp1*^{-/-} mice (Figure 3B). *Spp1*^{-/-} mice exhibit an increased inflammatory response after HDM instillation, suggesting immunomodulatory roles for OPN in allergic asthma.

3.4 | *Spp1*^{-/-} mice show increased vulnerability to pneumococcal infection after HDM instillation

The vulnerability of *Spp1*^{+/+} and *Spp1*^{-/-} mice to pneumococcal airway infection during HDM-induced allergic inflammation was compared in the described model (Figure 4A). Mice were instilled with HDM extract for 3 weeks, rested for 2 days, instilled intranasally with *S. pneumoniae* (strain A66), and euthanized after 48 hours to evaluate the bacterial load and the levels of inflammatory mediators.

Previously, OPN has been shown to promote pneumococcal infection in mice without prior airway inflammation.¹⁹ A similar decrease in the bacterial load was seen in the current study, confirming that *Spp1*^{-/-} mice are more resistant to pneumococcal infections in the absence of prior inflammation (Figure 4B). In a previous study using a murine asthma model, allergic inflammation promoted resistance to pneumococcal infection.¹¹ A similar effect was observed in the wild-type mice in the current study (Figure 4B). Interestingly, however, *Spp1*^{-/-} mice subjected to HDM instillation followed by pneumococcal infection (HDM + *Bacteria*) had a significantly higher airway bacterial load than did *Spp1*^{+/+} mice, as reflected by the number of cfu in both BALF and lung tissue (Figure 4B).

HDM + *Bacteria* but not pneumococci alone (*Bacteria*) was associated with increased OPN concentrations in BALF (Figure 4C). To determine lung tissue damage, LDH, albumin, and free nucleosomes were measured in BALF (Figure 4D-F). HDM + *Bacteria* increased the release of LDH and the levels of albumin and free nucleosomes in *Spp1*^{-/-} mice to a greater degree than it did in *Spp1*^{+/+} mice (Figure 4D-F). The increased tissue damage in *Spp1*^{-/-} mice was confirmed by blinded lung injury scoring of H&E-stained lung tissue sections (Figure 4G). These findings suggest roles for OPN in protecting against pneumococcal infection during allergic airway inflammation, as shown by the reduced bacterial burdens in *Spp1*^{+/+} mice.

3.5 | HDM instillation into *Spp1*^{-/-} mice promotes inflammation during pneumococcal infection

To compare the inflammatory responses in *Spp1*^{+/+} and *Spp1*^{-/-} mice in response to HDM + *Bacteria*, the cytokine profiles in BALF were evaluated (Figure 5A and B). A heat map revealed that OPN-deficient mice have higher levels of inflammation in response to HDM + *Bacteria* than do control mice (Figure 5A). In mice infected with pneumococci without prior HDM instillation, OPN deficiency was associated with a nonsignificant reduction in several proinflammatory cytokines, including IL-4, IL-6, and TNF- α (Figure 5B). Overall, HDM instillation followed by pneumococcal infection decreased cytokine levels. However, an altered balance was observed; higher levels of cytokines were found in *Spp1*^{-/-} mice than in *Spp1*^{+/+} mice (Figure 5B). Both *Spp1*^{+/+} and *Spp1*^{-/-} mice exposed to HDM prior to bacterial infection had a higher recruitment of inflammatory cells to the airway than did mice exposed to bacterial infection only (Figure 5C). However, in response to HDM + *Bacteria*, mice lacking OPN had a significantly higher influx of immune cells, including neutrophils and eosinophils, than did *Spp1*^{+/+} mice (Figure 5C). This result shows the importance of OPN to the modulation of the host response during concurrent allergic inflammation and pneumococcal infection.

4 | DISCUSSION

This study shows that OPN reduces inflammation, decreases tissue injury, and lowers bacterial loads during concurrent pneumococcal

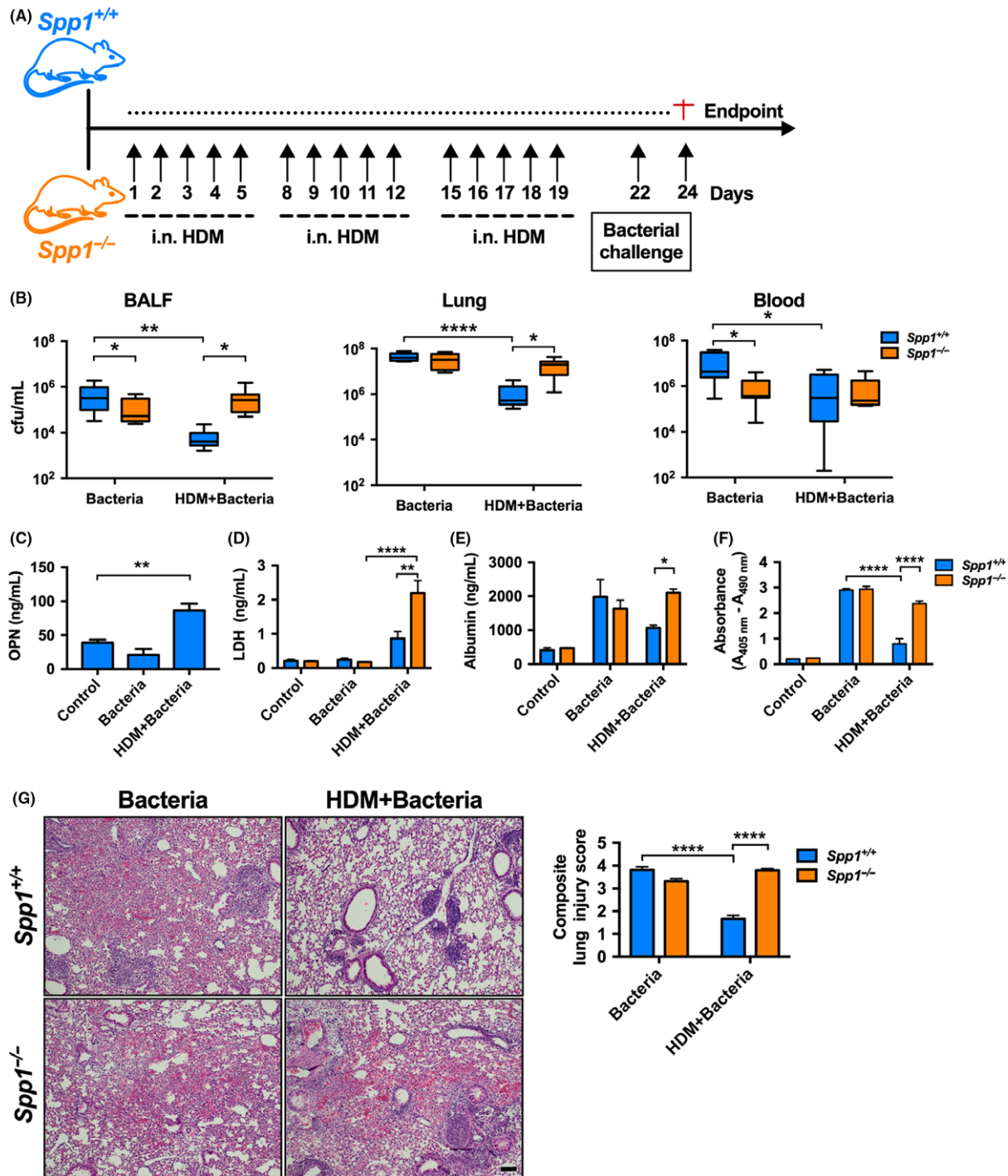


FIGURE 4 *Spp1*^{-/-} mice are more susceptible to pneumococcal infection during allergic airway inflammation. (A) Schematic presentation of the experimental design and time points of HDM instillation and bacterial infection (*S. pneumoniae*, strain A66). Mice were either infected with bacteria alone (*Bacteria*) or treated with HDM instillation followed by bacterial infection (*HDM + Bacteria*) (the red cross indicates the experimental endpoint). (B) BALF, lung homogenates, and blood were collected 48 hours after bacterial infection. The number of cfu was determined after plating on agar. The levels of OPN (***P* = 0.0088) (C), LDH (***P* = 0.0015, *****P* = <0.0001) (D), albumin (**P* = 0.026) (E), and free extracellular nucleosomes (*****P* = <0.0001) (F) were measured in BALF. The data shown are the means and SEMs from 6 to 8 animals in each group. (G) H&E staining of lung tissue sections was used to determine lung injury. The left panel shows lung tissue from animals infected with bacteria alone (*Bacteria*) or treated with HDM instillation followed by bacterial infection (*HDM + Bacteria*). The right panel shows the results of the blinded scoring of lung injury. The data shown are the means and SEMs from *n* = 8 animals in each group. *****P* < 0.0001

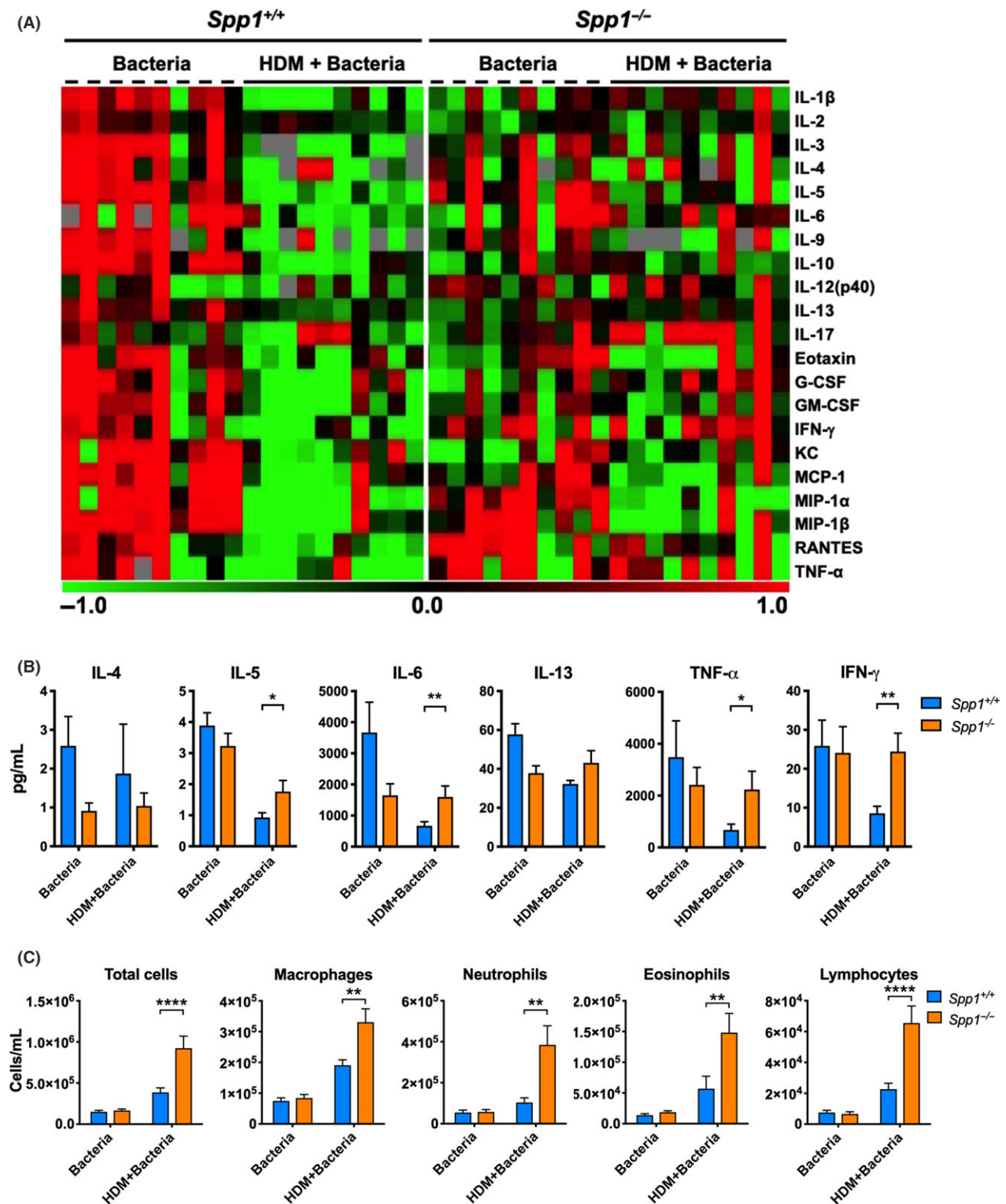


FIGURE 5 *Spp1*^{-/-} mice exhibit increased inflammation in response to allergic airway inflammation followed by pneumococcal infection. (A) Heat map presentation of cytokine profiles in BALF from *Spp1*^{+/+} and *Spp1*^{-/-} mice infected with bacteria (*Bacteria*) or treated with HDM instillation followed by bacterial infection (*HDM + Bacteria*). (B) Graphical presentation of the levels of selected cytokines. (IL-5 *P = 0.049, IL-6 **P = 0.0029, TNF-α *P = 0.036, and IFN-γ **P = 0.023). (C) Total and differential cell counts were determined in BALF. The data are the means and SEMs; n = 10 per group (total cells ****P < 0.0001, macrophages **P = 0.0011, neutrophils **P = 0.0014, eosinophils **P = 0.0051, and lymphocytes ****P < 0.0001)

infection and allergic airway inflammation in a murine model. This study used HDM extract instead of the commonly used ovalbumin to induce allergic airway inflammation; thus, this model more highly resembles human allergic asthma.²⁴ OPN is increased in human asthma, and this study shows that OPN is upregulated in response to sensitization with HDM extract, similar to its response in the ovalbumin model.^{12,13,16} Important roles of OPN during ovalbumin-induced allergic airway inflammation in mice have been identified and include both promoting sensitization and inducing a dampening effect during subsequent allergen sensitization.¹⁶ Indeed, OPN can induce both Th1- and Th17-polarized phenotypes in dendritic cells.^{25,26} The antigenic tolerance conferred by OPN has been attributed to the production of IFN- β by plasmacytoid dendritic cells and the accumulation of regulatory T cells.^{17,27} Accordingly, OPN inhibited the allergic inflammatory response to HDM sensitization in the current study, which compared *Spp1*^{+/+} and *Spp1*^{-/-} mice.

In parallel with the upregulated OPN production, airway tissue damage increased in association with allergen sensitization in both mice and humans. This increase was more pronounced in the mouse model than in the human subjects, probably because the mice were subjected to chronic exposure (3 weeks), while the subjects in the human study were exposed only once. The tissue injury and microvascular leakage in response to allergic inflammation could be a result of the increased influx of immune cells. Cationic granule proteins released from eosinophils and neutrophils, extracellular histones released from damaged cells, proteases, and inflammatory mediators increase microvascular leakage and result in structural damage.²⁸⁻³² In addition, immune cells and epithelial cells succumb to different forms of cell death, such as necrosis, apoptosis, and NETosis, resulting in the release of LDH and free nucleosomes.³³ The tissue damage was significantly more severe in *Spp1*^{-/-} mice, than in *Spp1*^{+/+} mice, suggesting important roles for OPN in tissue protection. Recently, we showed that OPN binds to highly cytotoxic extracellular histones at sites of inflammation.¹⁸ Histones are highly cationic and can damage membranes in the extracellular environment.³⁴ Important detrimental roles for extracellular histones have been demonstrated in pulmonary disease conditions such as acute lung injury and ARDS.³⁵ OPN, however, is anionic, partially due to its high phosphorylation; its anionic nature facilitates electrostatic binding to free histones, resulting in neutralization.¹⁸ The increased tissue damage in *Spp1*^{-/-} mice during allergic airway inflammation modifies the inflammatory response. Released DAMPs, for example histones, can bind Toll-like receptors; this binding may result in a vicious cycle of inflammation and tissue damage.³⁶⁻³⁸ Protective roles of OPN have been shown in other disease models. For example, in models of heart, liver, and kidney reperfusion injury, OPN is essential for decreasing tissue damage.³⁹⁻⁴¹

The increased susceptibility of OPN-deficient mice to pneumococcal infection during allergic airway inflammation has many potential explanations. In a previous study comparing *Spp1*^{+/+} and *Spp1*^{-/-} mice without prior airway inflammation, OPN promoted

pneumococcal infection. OPN possibly protected the bacteria by binding to their surfaces, thereby preventing their eradication by the host immune response.¹⁹ However, during allergic inflammation, the airway microenvironment is profoundly altered. In addition to the tissue damage discussed above, the dysregulated inflammation in *Spp1*^{-/-} mice likely contributes to the increased vulnerability to pneumococcal infection. OPN has several immunoregulatory functions, for example, binding to integrins on leukocytes through an RGD motif in its sequence, and it is also a chemoattractant for both neutrophils and eosinophils.^{42,43} In *Spp1*^{-/-} mice, the absence of OPN may result in an impaired Th1 response, thus preventing the mice from mounting an adequate antibacterial immune response.²⁷

The results from animal models of allergic asthma conflict regarding susceptibility to pneumococcal infection.^{9,10,44,45} Like the current study, another study investigating both ovalbumin- and HDM-induced allergic airway inflammation showed protective effects against pneumococcal infection.¹¹ Neither eosinophils nor neutrophils *per se* were crucial for resistance to pneumococcal infection. Instead, AMs, specifically, a new population of phagocytic SiglecF^{low} AMs, were likely important.¹¹ Importantly, however, the ovalbumin model of allergic airway inflammation is different from the HDM model. Because the HDM extract is crude, even if the eosinophil-rich Th2 inflammatory response predominates, the resulting mixed Th1, Th2, and Th17 inflammatory response may combat bacterial infection more adequately than the response mounted in the ovalbumin model.²⁴ In humans with atopic asthma, other factors can influence susceptibility to pneumococcal infection, for example, treatment with inhaled corticosteroids.⁴⁶

In conclusion, this study shows that OPN could play important protective roles during allergic airway inflammation and could increase resistance to pneumococcal infection. These findings could inform future therapeutic strategies in this context.

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CONFLICT OF INTERESTS

The authors declare no conflict of interests.

AUTHOR CONTRIBUTIONS

GK, HH, and AE designed the experiments; GK, RKVB, PP, and ET performed the experiments and analyzed the data; ET, HS, and LB provided the patient samples; AHN provided the OPN knockout mice; and GK and AE wrote the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Supplementary data

Methods

Study subjects

Seventeen individuals with allergic asthma according to GINA guidelines were included (clinical characteristics are described in Supplementary Table 1) (GINA)¹. All subjects had clinically stable asthma. None of the subjects were treated with oral corticosteroids, anti-IgE, allergen-specific immunotherapy, phosphodiesterase inhibitors, muscarinic or leukotriene receptor antagonists for at least six months before inclusion. None were previous or current smokers. All subjects signed a written informed consent, and the study was approved by the Regional Ethics Review Board in Lund, Sweden (2012/860).

Study design

The study consisted of: 1. screening visit, 2. baseline bronchoscopy, 3. inhaled allergen challenge and 4. second bronchoscopy 24 h after the allergen challenge. The baseline bronchoscopy and the second bronchoscopy were performed within 3–14 weeks [median 4 (IQR 3–4) weeks]. All subjects sensitized to any pollen were tested outside of the relevant pollen season. At screening, subjects completed an Asthma Control Test (ACT) questionnaire² and a methacholine inhalation challenge, a skin prick test (ALK-Abello, Hørsholm, Denmark) was performed and serum IgE levels (RAST) were analyzed to determine sensitizations to 10 allergens, including house dust mite (*Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*), cat, horse, dog, *Alternaria alternata*, *Cladosporium herbarum*, grass, birch and ragweed pollen. All subjects had a positive skin prick test (wheal

diameter ≥ 3 mm)³, a positive methacholine challenge, and confirmed lower airway symptoms to the specific allergen used for the inhaled allergen challenge.

Spirometry

Spirometry (Jaeger MasterScope, Erich Jaeger GmbH, Würzburg, Germany) was measured according to the American Thoracic Society (ATS)/European Respiratory Society (ERS) guidelines. Per cent of predicted values (%p) were calculated from reference spirometric values by Crapo *et al*⁴.

Methacholine inhalation challenge

The methacholine challenge was carried out to screen for airway hyper-responsiveness, using five inhalations with increasing methacholine doses (50, 150, 300, 600 and 900 μ g, maximal cumulative dose 2000 μ g) as previously described⁵. The test was considered positive if FEV₁ dropped $\geq 20\%$ from baseline below maximum cumulative dose.

Inhaled allergen challenge

A single diluted allergen extract (cat, horse, HDM, birch or grass pollen; ALK-Abelló, Hørsholm, Denmark, chosen based on maximal response according primarily to anamnesis and self-reported subject's history, secondarily to skin prick test and/or serum IgE levels) was administered by counted deep breaths over several steps with gradually increasing doses of 1.2-20 000 Standardized Quality units (SQ-U) as described previously⁵. Whenever FEV₁

dropped $\geq 20\%$ from baseline, the challenge was considered completed and no further allergen was inhaled.

Bronchoscopy with bronchoalveolar lavage

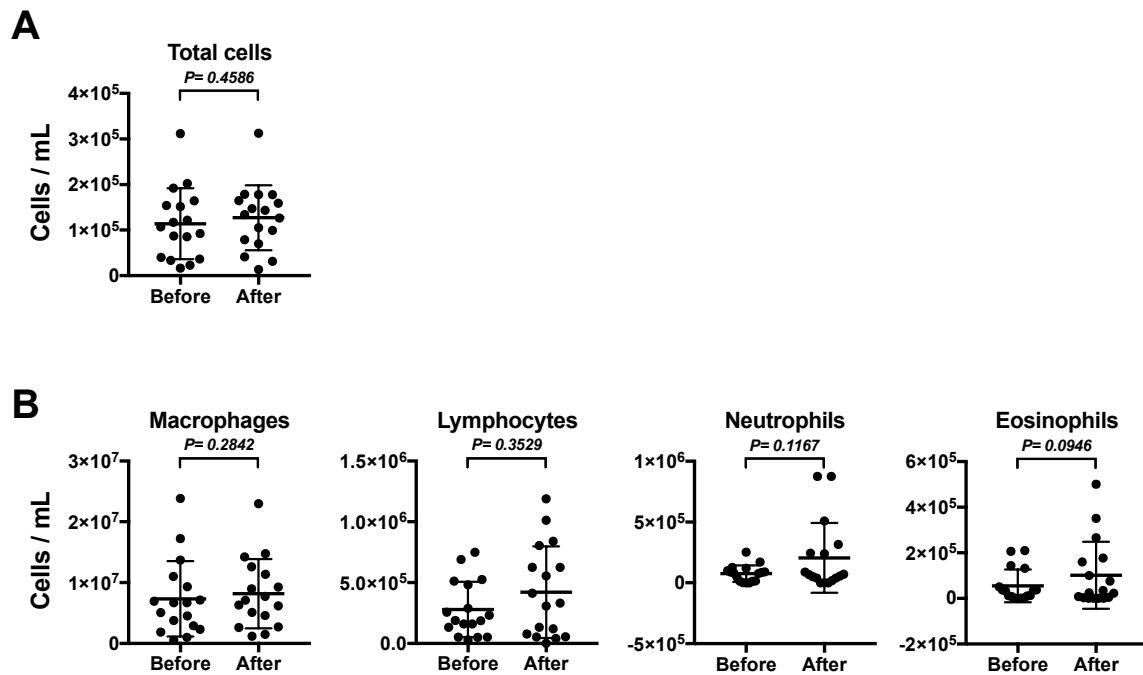
Bronchoscopies were performed according to clinical routine. A flexible bronchoscope (Olympus IT60, Tokyo, Japan) was inserted into the trachea and the airways were systematically examined. BAL was sampled by infusion of 150 mL (3x50 mL) of 0.9% phosphate buffered saline (PBS) at room temperature, re-aspirated by gentle suction. The first 50 ml sample was defined as the bronchial fraction while subsequent samples were pooled and defined as the alveolar fraction, as previously described ⁶. The BAL was filtered through a 100 μm filter and centrifuged at 200 x g for 5 min (+4° C), and the BAL supernatant was analyzed for osteopontin as described in Material and Methods.

Supplementary Table 1. Characteristics of the individuals enrolled in the study

	median
Sex, male /female (n)	9/8
ACT-score	22 (21-24)
FEV1 (L)	3,9 (3.5-4.1)
FEV1 (% of predicted)	95 (89-103)
Budesonide (µg/day)	200 (0-400)
Age (y)	27 (25-40)
Specific IgE (kU/L)	3.3 (1.9-10.7)
Total IgE (kU/L)	81 (44-430)
Number of sensitizations	3 (3-5)
Dose of allergen administered (SQ-U)	373 (175-1604)
Methacholine PD20 (µg)	137 (131-514)

Data are shown as median (and interquartile range); ACT = Asthma Control Test; FEV1 = forced expiratory volume in 1 second; Methacholine PD20 is the cumulative dose of methacholine causing a fall in FEV1 of 20%. ACT = Asthma Control Test; FEV1 = forced expiratory volume in 1 second

Supplementary Figure 1



Supplementary Figure 1. Influx of immune cells before and after allergen challenge in allergic asthmatics.

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