The *Staphylococcus aureus* serine protease-like protein B is a potent allergen in a murine asthma model

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Conflicts of interests

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Abstract

Background: Asthma is associated with *Staphylococcus aureus* colonization. Two hypotheses were proposed to explain this phenomenon: (1) the allergic environment in asthma favors *S. aureus* colonization and (2) *S. aureus* colonization creates a pro-allergic environment. Since several *S. aureus* virulence factors, such as the serine protease–like protein (Spl) B, elicit a T_H2-biased immune response, we asked whether the pathogen itself can cause asthma.

Objective: Test the ability of recombinant SplB of *S. aureus* to sensitize mice and induce allergic airway inflammation (AAI).

Methods: Mice were treated with repeated intratracheal inoculations of either catalytically active SplB or an inactive mutant. AAI was assessed by evaluating airway hypersensitivity, immune cell infiltration, cytokines, mucus production, fibrosis, and specific serum IgE. We compared the outcome between wild-type and gene-deficient C57BL/6J mice, including recombination-activating gene knockout mice ($Rag2^{-/-}$), interleukin-33 knockout mice ($IL\text{-}33^{-/-}$), and protease-activated receptor 2 knockout mice ($PAR2^{-/-}$).

Results: Intratracheal exposure to SplB sensitized the mice and caused severe eosinophilic airway inflammation and hyperresponsiveness. The development of asthma required both the proteolytic activity of SplB and a functional adaptive immune system. The soluble protease sensor IL-33 was necessary for eosinophil tissue invasion, whereas the membrane-bound protease sensor PAR2 was not.

Conclusion: The serine protease SplB of *S. aureus* is a potent allergen. Based on this finding we propose a third mechanism to explain the relationship between *S. aureus* colonization and

asthma: *S. aureus* can release allergens, such as SplB, that sensitize individuals and lead to the development of asthma.

Key Messages

- The SplB protease, which is secreted by *S. aureus*, is a potent allergen that induces severe eosinophilic airway inflammation.
- SplB's allergenicity depends on its enzymatic activity and on the host's adaptive immune system.
- The IL-33, a soluble protease sensor, is required for eosinophilic airway inflammation but not for the SplB-specific IgE response. The membrane protease sensor PAR2 was only required for lung fibrosis.
- Bacterial proteases may be an underestimated cause of idiopathic asthma.

Capsule summary

The protease SplB, produced by the bacterium *Staphylococcus aureus*, induces allergic airway inflammation in mice, suggesting that *S. aureus* may promote asthma in human patients. These findings imply that bacterial allergens could play a significant role in idiopathic asthma.

Keywords: Allergy, asthma, *Staphylococcus aureus*, sensitization, serine protease, virulence factor, immune escape

Abbreviations

AAI	Allergic airway inflammation
AHR	Airway hyperreactivity
AMC	Amino-methyl-cumarin
APC	Allophycocyanin
BALF	Bronchoalveolar lavage fluid
BCA	Bicinchoninic acid
CBA	Cytometric bead array
CD	Cluster of differentiation
DC	Dendritic cells
ELISA	Enzyme-linked Immunosorbent Assay
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HDM	House dust mite
HE	Hematoxylin Eosin

HpARI2	*Heligmosomoides polygyrus* Alarmin Release Inhibitor 2
HRP	Horseradish peroxidase
Ig	Immunoglobulin
IL	Interleukin
ILC2	Type 2 innate lymphoid cell
i.t.	intratracheally
MHC	Major histocompatibility complex
OD	Optical density
OVA	Ovalbumin
PAR	Protease-activated receptor
PAS	Periodic acid–Schiff
PBS	Phosphate-buffered saline
PE	Phycoerythrin
PerCP	Peridinin-chlorophyll-protein complex
Rag2	Recombination-activating gene
RT	Room temperature
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Spl	Serine protease–like protein
RIPA	Radioimmunoprecipitation Assay
Tfh	T follicular helper
Th2	T helper cell type 2
TSLP	Thymic stromal lymphopoietin
WT	Wild type

Introduction

Asthma is one of the most common chronic inflammatory lung diseases, with over 260 million patients worldwide[1]. Patients suffer from wheezing, cough, chest tightness and shortness of breath. Asthma is a heterogeneous disease, and clinicians differentiate between several asthma phenotypes, of which allergic asthma is the most common[2]. Allergic asthma is characterized by a T helper type 2 cell (Th2) immune response and symptoms are often triggered by the inhalation of airborne allergens. However, the causes of asthma development are debated[3].

Colonization by certain pathogens is suspected to play a role in asthma development and exacerbation, among them *Staphylococcus aureus*[4]. *S. aureus* is a Gram-positive opportunistic pathogen that colonizes the airways in around 30% of the adult human population[5]. Colonization with *S. aureus* is positively correlated with asthma and also associated with other atopic diseases like atopic dermatitis and chronic rhinosinusitis[6,7]. Two mutually non-exclusive scenarios have been proposed to explain the association between *S. aureus* colonization and asthma: (1) the Th2 immune response that characterizes asthma facilitates *S. aureus*

colonization and (2) *S. aureus* colonization creates a Th2 microenvironment that promotes allergic sensitization towards harmless antigens[8].

The immune response towards prominent human asthma allergens like house dust mite (HDM), pollen and cockroach is well-defined[9–11]. Many of these allergens contain proteases, which provoke a Th2 immune response by cleaving tight junction proteins and activating cell-surface protease sensors, such as the protease-activated receptor 2 (PAR2)[12–16]. Moreover, several protease allergens cleave and thereby activate interleukin (IL)-33, an alarmin that is secreted by epithelial cells upon cellular stress and cell death[17].

S. aureus can produce a plethora of virulence factors, some of which have been linked to a Th2 response in humans, such as the serine protease-like proteins (Spls)[8]. The Spls are a group of serine proteases (commonly named SplA-SplF) that are secreted by *S. aureus* and encoded in its genome on the νSaβ genomic island[18–20]. The Spls share amino acid sequence homology between 43,9 and 94,6% and each have distinctive amino acid cleavage motifs[20,21]. In a mouse sepsis model, they act as virulence factors and increase mortality[22]. Moreover, SplD and F cause asthma in an acute mouse asthma model. However, the mechanisms underlying their allergenicity remain poorly defined[23]. In humans who are naturally exposed to *S. aureus*, the Spls elicit a type 2-biased immune response in T cells and B cells[24]. The antibody response directed against the Spls is skewed towards IgE and IgG4 and in asthmatics, the Spl-specific IgE antibody titers are increased[25]. The mechanisms underlying the Spl-specific shift towards type 2 immunity are unknown.

To shed light on the interplay between *S. aureus* and airway allergy, we have studied the *S. aureus* protease SplB. We show that SplB acts as a potent allergen in a mouse model of chronic allergic airway inflammation (AAI). SplB alone – without adjuvant – is sufficient to cause severe eosinophilic inflammation. The development of asthma depends on the adaptive immune system and on SplB's protease activity. IL-33 is necessary for the onset of symptoms, but PAR2 is not. Therefore, we propose a third scenario for the relationship between *S. aureus* and asthma: (3) *S. aureus* secretes allergens that can trigger asthma development and exacerbation.

Methods

Recombinant proteins

Recombinant SplB was produced in the *S. aureus* strain RN4220 using the expression vector pTripleTREP[26]. The sequence of SplB was derived from the *S. aureus* strain USA300 FPR3757 (Fig. E1 in the Online Repository). An amino acid exchange (S193A) was introduced into the catalytic triad of SplB to generate a SplB mutant (SplB mut) without catalytic activity. The recombinant proteins lack the N-terminal signal peptide as this is cleaved off during secretion by the host *S. aureus* strain, and harbor a C-terminal Twin-Strep-tag®.

Mice

Animals were maintained in a 12-hour/12-hour light/dark cycle and had access to water and food *ad libitum*. Animal experiments were approved by the responsible authority, the Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei Mecklenburg-Vorpommern (LALLF MV; AZ 7221.3-1-037/20). All animal experiments were carried out in accordance with the European Union Directive 2010/63/EU, the German Animal Welfare Act (Tierschutzgesetz), and the German Animal Welfare Ordinance on the Protection of Animals Used for Experimental Purposes (TierSchVersV). The following mouse strains were used: C57BL/6J, C57BL/6J-Rag2^{em3Lutzy}/J (RAG2$^{-/-}$), C57BL/6J IL-33$^{Gt/Gt}$ (IL-33$^{-/-}$)[27] and B6.Cg-F2rl1tm1Mslb/J (PAR2$^{-/-}$)[28]. Mice were purchased from Janvier (C57BL/6J), JAX (C57BL/6J RAG2$^{-/-}$ (strain 033526); C57BL/6J PAR2$^{-/-}$ (strain 004993)) or bred in-house (C57BL/6J IL-33$^{-/-}$, originally from Texas A&M Institute for Genomic Medicine (USA)). The mice were certified as *S. aureus*-free. All mice used in animal experiments were female and 8-12 weeks old when treatment started.

Mouse model of chronic allergic airway inflammation (AAI)

Mice were put under anesthesia (75 mg/kg ketamine + 5 mg/kg xylazine) and treated intratracheally (i.t.) with PBS, SplB (10, 25, or 50 µg), SplB mut (25 µg) or OVA (50 µg) on days 0, 7, 14 and 21. Proteins were dissolved in 50 µL PBS. In some experiments, the IL-33 inhibitor HpARI2 (10 µg), the PAR2-blocking monoclonal antibody (mAb) SAM-11 (10 ng; ThermoFisher Scientific, USA; 35-2300) or a monoclonal IgG isotype antibody (10 ng;

ThermoFisher Scientific, USA; 02-6200) were i.t. co-applied with SplB. On days 0, 7 and 14, approximately 100 µL blood was taken from the retroorbital plexus prior to the i.t. application. 72 hours after the fourth treatment, mice were anesthetized and the airway hyperreactivity (AHR) was measured. Afterwards the mice were sacrificed with an overdose of isoflurane.

Statistics

All datasets were tested for normality using the Shapiro–Wilk test. Ordinary one-way ANOVA followed by Tukey's multiple comparisons test was used if all datasets were normally distributed, otherwise the Kruskal–Wallis test followed by Dunn's multiple comparisons test was applied. AHR measurements were analyzed using a two-way ANOVA. A p value < 0.05 was considered significant. Statistical analyses were performed with GraphPad Prism v8.0.1. Data are shown as median with interquartile range if more than one group consisted of less than 4 animals, and as mean ± SD otherwise.

A more detailed description of the materials and methods used in this study can be found in the Methods section in this article's Online Repository.

Results

Catalytically active SplB acts as a potent allergen in murine allergic airway inflammation (AAI)

First, we established a protocol for chronic AAI in female C57BL/6J WT mice. For sensitization we applied SplB i.t. on days 0, 7 and 14. On day 21 the animals were challenged with a fourth i.t. dose of SplB (Fig. 1A). SplB was tested at different doses. Even at the lowest dose (10 µg/application), the protease elicited prominent eosinophilic lung inflammation, accompanied by increased airway hyperreactivity (AHR) (Fig. E6 in the Online Repository). We decided to treat mice with 25 µg/dose in studies prioritizing the detection of small

differences between experimental groups. In studies assessing the effects of an inhibitor, a lower amount of 10 µg/dose was used.

A functional adaptive immune system was required for the development of AAI because Rag$^{-/-}$ mice, who lack T- and B cells, did not develop any signs or symptoms of AAI (Fig. E7 in the Online Repository).

As protease activity is essential for many allergenic proteases to induce symptoms[29–31], we next tested whether SplB's catalytic activity was necessary to elicit AAI. We generated a catalytically inactive SplB mutant by introducing a point mutation (S193A) in its catalytic triad. The resulting SplB mut was completely devoid of protease activity in a specific cleavage assay (Fig. 1B). We then compared the effects of active SplB and SplB mut in our AAI model (Fig. 1A). Ovalbumin (OVA) was used as a specificity control. While the active SplB again induced a strong influx of eosinophils into the BALF and the lung tissue (Fig. 1C, E), OVA and the inactive SplB mutant failed to do so. Remarkably, there was only minimal recruitment of neutrophils to the BALF following sensitization and challenge with SplB (Fig. 1D). Eosinophil counts were almost three orders of magnitude higher than neutrophil counts. The number of neutrophils in the lungs did not change (Fig. 1F). To investigate the systemic immune response, we analyzed the spleen and found a mild but significant eosinophilia in mice treated with the active SplB (Fig. 1G), suggesting that SplB-induced AAI causes systemic immune alterations. Next, we measured type 2 cytokines in the airways. Only active SplB induced elevated IL-4-, IL-5-, and IL-13 concentrations in the BALF (Fig. 1H-J).

To evaluate the impact of SplB's proteolytic activity on the humoral immune response, we measured total and antigen-specific IgE- and IgG levels in the serum (Fig. 2). Catalytic activity of SplB was necessary for production of SplB-specific IgE (Fig. 2A, B). However, SplB mut was able to provoke an antigen-specific IgG response, albeit less effectively than active SplB (Fig. 2E). These results demonstrate that the inactive SplB mutant is immunogenic but not allergenic. OVA-treated mice did not develop OVA-specific antibodies (Fig. 2C, F).

Histological examination of the lungs revealed that, compared to mice exposed to enzymatically active SplB had significantly thicker epithelium in their large airways than those treated with OVA or SplB mut (Fig. 3A). SplB-treated mice also showed increased mucus production (Fig. 3B) and fibrosis (Fig. 3C). Furthermore, treatment with active SplB significantly increased the albumin concentrations in the BALF, indicating impaired epithelial integrity (Fig. 3D). Finally,

IL-33 was increased in the lungs of mice exposed to active SplB (Fig 3E). Concentrations of thymic stromal lymphopoietin (TSLP), granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-1α and IL-25 were below the detection limit.

Taken together, we found that only mice sensitized with enzymatically active SplB developed asthma symptoms and signs of a type 2 immune response, demonstrating that the enzymatic activity of SplB is necessary for the establishment of AAI.

IL-33 is a crucial mediator in SplB-induced AAI

Asthma development often involves the alarmin IL-33[32]. To elucidate its role in SplB-induced asthma, we co-administered the IL-33 inhibitor HpARI2 with SplB. In a parallel approach we tested the effect of SplB on IL-33$^{-/-}$ mice. HpARI2 completely abolished the SplB-induced AHR following methacholine challenge (Fig. 4A) as well as the eosinophilic lung inflammation (Fig. 4B, C). In line with this, only WT mice but not IL-33$^{-/-}$ mice showed a marked accumulation of eosinophils in the lung when challenged with SplB (Fig. 4B, C). Moreover, IL-33 was required for epithelial thickening and increased mucus production (Fig. 4D, E). In contrast, the anti-SplB IgE titers were increased in the sera of all SplB-treated mice, independent of the IL-33 activity (Fig. 4F). Clearly, IL-33 is crucial for recruiting eosinophils to the site of inflammation, but it is not required for the immunoglobulin class switch in SplB-specific B cells.

Numerous allergens cleave the alarmin IL-33, which increases its biological activity and promotes the activation of innate immune cells and Th2 cells[17]. We therefore asked whether SplB targets IL-33 and incubated full-length murine IL-33 (mIL-33) with active SplB. Immunoblotting revealed that SplB did not cleave mIL-33, although it cleaved the human complement factor C3 under the same conditions (Fig. 4G and published data[33]). Therefore, mIL-33 is not a substrate of SplB.

PAR2 is dispensable for the development of SplB-induced AAI

PAR2 activation by protease allergens promotes Th2-mediated airway inflammation and asthma severity is correlated with increased PAR2 expression[34]. Therefore, we examined the

role of PAR2 in SplB-induced murine AAI. To this end, we treated PAR2$^{-/-}$ mice with SplB or applied a PAR2-blocking monoclonal antibody (SAM-11) to WT mice. PAR2$^{-/-}$ mice exposed to SplB displayed the same number of inflammatory cells in their BALF and lungs as their WT counterparts (Fig. 5A, B). Likewise, WT and PAR2$^{-/-}$ mice had similar titers of anti-SplB IgE in their sera (Fig. 5C). In line with this, PAR2 deficiency had no impact on epithelial thickness or mucus production (Fig. 5D, E). However, remarkably, it significantly reduced lung fibrosis (Fig. 5F). Similarly, blocking of PAR2 with the monoclonal antibody SAM-11 failed to protect mice from AAI, but reduced lung fibrosis (Fig. E8 in the Online Repository).

Discussion

In this study, we show that the protease SplB, which is secreted by *S. aureus*, is a potent allergen that induces type 2 airway inflammation in mice. Four intratracheal applications were sufficient to induce AAI without the need for adjuvants or prior intraperitoneal priming. The AAI phenotype induced by SplB was characterized by increased AHR, a massive influx of eosinophils into the airways and the lung, lung fibrosis, and the production of type 2 cytokines as well as of SplB-specific IgE. Various factors contributed to murine AAI development in distinctive ways: SplB's enzymatic activity was essential for eosinophilic airway inflammation, fibrosis and IgE induction, but not for the production of specific IgG. IL-33 was necessary for eosinophil infiltration and fibrosis, but not for the production of specific IgE or IgG. PAR2 was involved in lung fibrosis but not the other symptoms of AAI.

In our AAI model, the allergenic potential of SplB was strictly dependent on its proteolytic activity, as a catalytically inactive SplB mutant did not elicit AAI nor SplB-specific IgE. Nevertheless, the inactive SplB mutant, but not OVA, induced a specific IgG response. Clearly, enzymatically inactive SplB is immunogenic but not allergenic. The absence of an antibody response to OVA might be attributed to immune tolerance due to OVA's eukaryotic origin and structural similarities with host proteins[35,36]. In contrast, the SplB mutant, which was genetically inactivated by a single point mutation, is structurally probably almost indistinguishable from the active enzyme. Thus, the distinctive immune response to the active and inactive bacterial protease suggests that in its natural form SplB has a dual effect on the immune system, (i) allergenicity, i.e., type 2 polarization requiring protease activity and (ii) immunogenicity

facilitated by structural motifs, possibly conserved in bacteria, that are recognized by innate immune cell receptors[37].

Since catalytically active SplB increased IL-33 concentrations in the lung, we hypothesized that the protease induces AAI via the IL-33 signaling pathway. This was supported by the results of two experimental strategies, IL-33 blockade by HpARI2, a potent anti-IL-33 agent originally derived from helminths[38], and the use of IL-33-deficient animals. The IL-33 function was necessary for the eosinophilic lung inflammation but not for the generation of SplB-specific IgE. We conclude that mediators other than IL-33 must be responsible for the sensitization to SplB, which involves the maturation of immature DCs into cDC2s that drive the polarization of Th2 cells[39,40]. A candidate for this is the alarmin TSLP known to be essential for cDC2 maturation[41]. The concentrations of TSLP, however, remained below the limit of detection in the airways and lungs of the SplB-treated mice. Later during sensitization, T follicular helper (Tfh) cells are critical for B cell activation and the immunoglobulin class switch to IgE in the germinal centers of the secondary lymphoid organs[42]. Tfh cells, however, do not express the IL-33 receptor ST2 and are thus independent of IL-33 signaling[43]. The absence of eosinophilic lung inflammation in mice without active IL-33 could be explained by the role of mast cells. Mast cells express ST2 at high density, and IL-33 lowers their degranulation threshold[44,45]. Degranulating mast cells release large amounts of IL-5, which is critical for eosinophil recruitment[46]. In addition, group 2 innate lymphoid cells (ILC2s) represent a major source of IL-5, and their cytokine production is strongly dependent on IL-33[47]. However, ILC2 activity alone cannot account for the observed phenotype, as IL-33–competent Rag2-deficient mice failed to develop asthmatic features. Thus, catalytically active SplB and IL-33 were both necessary for AAI development, but there is no direct link between the two as the protease SplB does not target murine IL-33.

We reasoned that SplB may cause murine asthma by cleaving another substrate, possibly the membrane-bound protease sensor PAR2, which is targeted by several known protease allergens[15,48]. However, most symptoms in SplB-mediated AAI were independent of PAR2. This is not unprecedented, as other studies have shown that, for example, HDM can also induce AAI independent of PAR2[49,50]. Notably, the absence of PAR2 significantly reduced the extent of lung fibrosis, thereby corroborating previous observations in a murine model of HDM-induced asthma[49,51].

Epithelial barriers are the first line of defense against environmental threats like pathogens and allergens. *S. aureus* needs to overcome host barriers to persist and cause infections. A previous study has shown that Spls play a role in bacterial spread in a rabbit pneumonia model, suggesting that the bacterial proteases damage barriers[52]. Indeed, SplB can disrupt endothelial barriers and cause vascular leakage[53]. In our model of AAI, i.t. application of SplB impaired the integrity of the airway epithelial barrier, resulting in leakage of serum albumin into the airways. The underlying mechanisms, especially the initial molecular target(s) of the bacterial protease SplB in the pathogenesis of murine AAI, remain to be elucidated in future studies. This is the main limitation of our study.

We have identified SplB as a potent bacterial allergen that causes severe eosinophilic lung inflammation on its own without exogenous adjuvants or systemic priming. SplB elicited the production of allergen-specific IgE in both mice and humans[24,25], and promoted airway hyperresponsiveness. Other members of the Spl family, namely SplD and SplF, also induce AAI in mice[23]. These findings suggest that *S. aureus* may play a more active role in asthma than previously thought and they advance our understanding of the role of *S. aureus* airway colonization in the pathogenesis of asthma. Two mutually non-exclusive hypotheses have been proposed: (1) Asthma and the associated type 2 immune response facilitate *S. aureus* colonization and (2) *S. aureus* colonization creates a type 2 microenvironment promoting allergic sensitization towards harmless antigens[8]. Based on our results we propose a third hypothesis to complement these two scenarios: (3) *S. aureus* secretes allergens that can induce asthma development and trigger asthma attacks.

To date, only one bacterial allergen is listed in the WHO/IUIS Allergen Nomenclature Sub-Committee's database of allergenic molecules, the serine protease nattokinase from *Bacillus subtilis var. natto* [www.allergen.org][54,55]. Our findings suggest that we may have underestimated the role that bacterial allergens play in disease development, and they encourage the scientific community to increase its efforts to study and clarify this issue.

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Declaration of generative AI and AI-assisted technologies in the manuscript preparation process

During the preparation of this work the authors used deepL write for text revision. After using this tool, the authors reviewed and edited the content as needed and they take full responsibility for the content of the published article.

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Figure legends

Figure 1

Catalytic activity of SplB is essential to cause AAI. Female WT C57BL/6J mice were treated with active SplB or the catalytically inactive SplB mut; OVA-treated mice served as control. **A**, Mouse model of chronic AAI. Mice received weekly intratracheal (i.t.) applications of either PBS or different doses of SplB and were sacrificed 72 hours after the fourth application. **B**, SplB mut is catalytically inactive. The catalytic activity of SplB and SplBmut was determined with the synthetic peptide substrate Ac-VEID-MCA. After substrate addition, fluorescence was measured over 60 min. PBS served as negative control. One out of three similar experiments is depicted. **C** and **D**, Number of inflammatory eosinophils (C) and neutrophils (D) in the BALF measured by flow cytometry. **E** and **F**, Number of inflammatory eosinophils (E) and neutrophils (F) in the lung measured by flow cytometry. **G**, Percentage of eosinophils in the spleen measured by flow cytometry. **H-J**, Concentration of IL-4 (H), IL-5 (I) and IL-13 (J) in the BALF measured by CBA. n = 5-9. Results are presented as mean ±SD. *$p < .05$ and **$p < .01$. Significance was determined using a Kruskal–Wallis test followed by Dunn's multiple comparisons test.

Figure 2

Catalytic activity of SplB is essential for IgE-, but not IgG secretion. Mice were treated with four doses of active SplB, inactive SplB mut or OVA as shown in fig. 1A and serum immunoglobulins were measured by ELISA on days 14 and 21 of the experiment. **A-C**, Serum IgE; total IgE (A), SplB-specific IgE (B) and OVA-specific IgE (C). **D-F**, Serum IgG. Total IgG (D), SplB-specific IgG (E) and OVA-specific IgG (F). n = 5-9. Results are presented as mean ±SD. *$p < .05$, **$p < .01$ and ***$p < .005$. Significance was determined using a Kruskal–Wallis test followed by Dunn's multiple comparisons test.

Figure 3

Only catalytically active SplB induces tissue remodeling in the lungs, and causes epithelial damage. **A**, Hematoxylin and eosin (HE) staining of lung sections and thickness of airway epithelium. Epithelial thickness was determined by measuring the distance from the basement membrane to the apical epithelial surface on five points in three airways per section. **B**, Periodic acid-Schiff (PAS) staining for mucus in lung sections and percentage of PAS-positive airways. The percentage of PAS-positive airways was defined as the ratio of PAS-positive airways to the total number of airways per section. **C**, Sirius red staining for collagen in lung sections and percentage of collagen in the lung area. The percentage of collagen was calculated as the Sirius red-positive area divided by the total lung area. **D**, Serum albumin concentration in the BALF measured by ELISA. **E**, IL-33 concentration was measured by ELISA in 50 µL buffer containing 50 µg of lung protein. n = 5-9. Results are presented as mean ±SD. *$p < .05$, **$p < .01$ and ***$p < .005$. Significance was determined using a one-way ANOVA, except for B, where significance was determined using a Kruskal Wallis test followed by Dunn's multiple comparisons test, depending on normal distribution of data.

Figure 4

SplB induced AAI is IL-33-dependent. WT C57BL/6J mice were treated with SplB or SplB + HpARI2, an IL-33 inhibitor. PBS-treated mice served as control. Additionally, C57BL/6J IL-33$^{-/-}$ mice were treated with either PBS or SplB. The treatment protocol is shown in fig. 1A. **A**, Airway resistance in response to increasing doses of methacholine was measured 72 hours after the last i.t. challenge using the forced oscillation technique. **B** and **C**, Number of inflammatory eosinophils in the BALF (B) and lung tissue (C) measured by flow cytometry. **D**, Epithelial thickness of airway epithelium and **E**, Mucus in lung sections (PAS staining) were determined as described in the legend of fig. 3. **F**, SplB-specific IgE on day 24 measured by ELISA. **G**, Enzymatic cleavage assays. Murine IL-33 or human complement factor C3 were incubated with SplB for 24 h at 37 °C. Cleavage was visualized by Western blotting. n = 11-12. Results are presented as mean ±SD. *$p < .05$, **$p < .01$ and ***$p < .005$. Significance was determined using a Kruskal-Wallis test, except for D, where significance was determined using a one-way ANOVA followed by Tukey's multiple comparisons test as data had normal distribution. AHR measurements were analyzed using a two-way ANOVA followed by Tukey's multiple comparisons test.

Figure 5

SplB-induced AAI is independent of PAR2 activation. WT or PAR2$^{-/-}$ C57BL/6J mice were treated with SplB; PBS-treated PAR2$^{-/-}$ mice served as controls. **A** and **B**, Number of inflammatory eosinophils in the BALF (A) and lung tissue (B) measured by flow cytometry. **C**, SplB-specific IgE measured by ELISA. **D**, Epithelial thickness of airway epithelium, **E**, Mucus in lung sections (PAS staining) and **F**, Collagen quantification (Sirius Red) were determined as described in the legend of fig. 3. n = 4-8. Results are presented as mean ±SD. **p < .01. Significance was determined using a Kruskal-Wallis test followed by Dunn's multiple comparisons test (A-C) or one-way ANOVA followed by Tukey's multiple comparisons test (D-F), depending on normal distribution of data.

Figure E1

Protein sequences of the recombinant preparations of active SplB and catalytically inactive SplB mut. The SplB sequence was derived from the *S. aureus* strain USA300_FPR3757. The expressed SplB and SplB mut proteins contain a signal peptide (grey), which is cleaved off upon secretion by *S. aureus* RN4220, which served as expression system. In the SplB mut protein the catalytic serine was switched to an alanine ((S193A, yellow). A C-terminal Twin-Strep-tag® used for purification is highlighted in blue. The amino acid linker between the SplB protein and the Twin-Strep-tag®is marked in green. Tag and linker were not removed from the mature SplB resp. SplB mut proteins.

Figure E2

Gating strategy for immune cell phenotyping in the BALF. BALF was collected and centrifuged, the supernatant was removed and the cells were stained for flow cytometry analysis. **A**-**C** Gating of single cells, exclusion of debris and duplets. **D**, Gating of living cells. **E**, Distinguishing inflammatory eosinophils (SiglecF^{+}; CD11c^{-}) and alveolar macrophages (SiglecF^{+}; CD11^{+}) from SiglecF^{-} cells. **F**, The SiglecF^{-} cells are further distinguished in neutrophils (SiglecF^{-}; Ly6G^{+}) and CD4^{+} T cells (SiglecF^{-}, CD4^{+}). The following antibodies were used: anti-mouse CD4-VioBlue (130-118-696), CD11c-allophycocyanin (APC) (130-110-839), Ly6-G-fluorescein isothiocyanate (FITC) (130-120-820) and Siglec-F-phycoerythrin (PE)-Vio770 (130-112-334).

Figure E3

Gating strategy for DC subpopulations and eosinophils from lung tissue. Three lobes of the right lung (superior, middle and inferior lobe) were digested and stained for flow cytometry analysis. **A-C**, Gating of single cells, exclusion of debris and duplets. **D**, Gating of living cells. **E**, Distinguishing inflammatory eosinophils (SiglecF^{+}; CD11c^{-}), resident eosinophils (SiglecF^{+} low; CD11c^{+}), alveolar macrophages (SiglecF^{+}; CD11^{+}) from SiglecF^{-} cells. **F**, The SiglecF^{-} cells are further distinguished in dendritic cells (SiglecF^{-}; MHCII^{+}; CD11c^{+}) and MHCII^{-} cells. **G**, MHCII^{-} cells were separated into Ly6G^{-} cells and neutrophils (Ly6G^{+}). **H-I**, DCs subpopulations were further distinguished into type 1 cDCs (SiglecF^{-}; MHCII^{+}; CD11c^{+}, CD11b^{-}, CD103^{+}), type 2 cDCs (SiglecF^{-}; MHCII^{+}; CD11c^{+}, CD11b^{+}, CD103^{-}, CD64^{-}) and moDCs (SiglecF^{-}; MHCII^{+}; CD11c^{+}, CD11b^{+}, CD103^{-}, CD64^{+}). The following antibodies were used: anti-mouse Ly6-G-FITC (130-120-820), MHC Class II-VioGreen (130-112-395), CD103-APC (130-111-686), CD11b-PE-Vio770 (130-113-808), CD64-PE (130-118-684), Siglec-F-PE-Vio615 (130-112-330) and CD11c-VioBlue (130-110-843). The following antibodies were used for dump channel: anti-mouse CD19-APC-Vio770 (130-112-038), CD3-APC-Vio770 (130-119-793) and CD49b-APC-Vio770 (130-116-435).

Figure E4

Gating strategy for T cell subpopulations from lung tissue. Three lobes of the right lung (superior, middle and inferior lobe) were digested and stained for flow cytometry analysis. **A-C**, Gating of single cells, exclusion of debris and duplets. **D**, Gating of living cells. **E**, CD3^{-} cells were separated into NK1.1^{-} cells and NK cells (CD3^{-}, NK1.1^{+}). **F**, CD4^{-} T cells (CD3^{+}, CD4^{-}), CD4^{+} T cells (CD3^{+}, CD4^{+}) were distinguished from CD3^{-} cells. **G**, CD4^{-} T cell subpopulations were distinguished into naïve T cells (CD3^{+}, CD4^{-}, CD62L^{+}, CD44^{-}), effector T cells (CD3^{+}, CD4^{-}, CD62L^{-}, CD44^{+}) and memory T cells (CD3^{+}, CD4^{-}, CD62L^{+}, CD44^{+}). **H**, CD4^{+} T cell subpopulations were distinguished into naïve T cells (CD3^{+}, CD4^{+}, CD62L^{+}, CD44^{-}) and effector T cells (CD3^{+}, CD4^{+}, CD62L^{-}, CD44^{+}). The following antibodies were used: anti-mouse CD3-peridinin-chlorophyll-protein complex (PerCP)-Vio700 (130-120-826), MHC Class II-VioGreen (130-112-395), CD4-Brilliant Violet 605 (BioLegend®, San Diego, USA; 100548), CD11b-PE-Vio770 (130-113-808), CD44-VioBright FITC (130-120287), NK1.1-PE (130-120-506), CD11c-VioBlue (130-110-843) and CD62L-APC (130-112-837).

Figure E5

Gating strategy for immune cells from the spleen. A single cell suspension was prepared rom a part of the spleen and stained for flow cytometry. **A** and **B**, Gating of single cells, exclusion of debris and duplets. **C**, Distinguishing inflammatory eosinophils (SiglecF^{+}, CD11c^{-}) from SiglecF^{-} cells. **D**, CD3^{+} cells were distinguished from CD3^{-} cells. **E**, B-cells (CD3^{-}, CD19^{+}, B220^{+}) were distinguished. **F**, Distinguishing Th cells (CD3^{-}, CD4^{+}, CD8^{-}) from cytotoxic T cells (CD3^{-}, CD4^{-}, CD8^{+}). The following antibodies were used: anti-mouse Siglec-F-PE-Vio770 (130-112-334), CD11c-APC (130-110-839), CD3-PerCP-Vio700 (130-120-826), CD4-VioBlue (130-118-696), CD8-PE (BioLegend®, San Diego, USA; 100708), CD45R-Brilliant Violet 510 (BioLegend®, San Diego, USA; 103248) and CD19-Brilliant Violet 650 (BioLegend®, San Diego, USA; 115541).

Figure E6

SplB induces allergic airway inflammation in mice at different concentrations. **A**, Airway resistance in response to increasing doses of methacholine was measured 72 hours after the last i.t. challenge using a forced oscillation technique. **B** and **C**, Numbers of inflammatory eosinophils in the BALF (B) and lung tissue (C) measured by flow cytometry. **D**, SplB-specific IgE measured by ELISA. **E**, Epithelial thickness of airway epithelium and **F**, Mucus in lung sections (PAS staining) were determined as described in the legend of fig. 3. n = 1-7. Results are presented as median ±interquartile range. *$p < .05$, **$p < .01$ and ***$p < .005$. Significance was determined using a Kruskal–Wallis test followed by Dunn's multiple comparisons test., except for E, where significance was determined using a one-way ANOVA followed by Tukey's multiple comparisons test, depending on normal distribution of data. AHR measurements were analyzed using a two-way ANOVA followed by Tukey's multiple comparisons test.

Figure E7

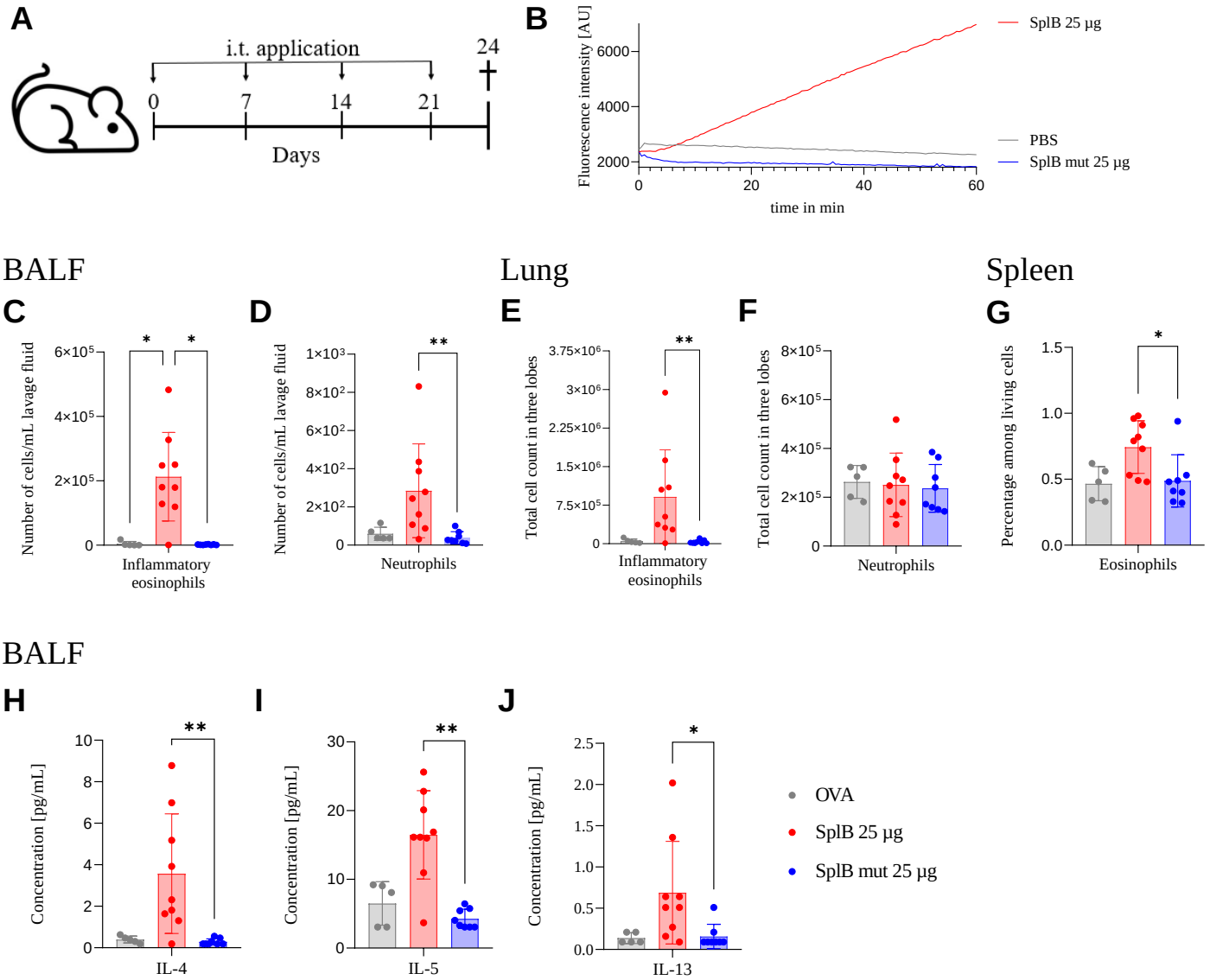
AAI induction by SplB requires an intact adaptive immune system. WT or Rag2$^{-/-}$ C57BL/6J mice were treated with SplB as shown in fig. 1 in the main paper, PBS-treated Rag2$^{-/-}$ mice served as control. **A** and **B**, Number of inflammatory eosinophils in the BALF (A) and lung tissue (B) measured by flow cytometry. **C**, Epithelial thickness of airway epithelium and **D**, Mucus in lung sections (PAS staining) were determined as described in the legend of fig. 3. n = 3-6. *$p < .05$, **$p < .01$ and ***$p < .005$. Results are presented as mean ±SD. Significance was determined using a Kruskal–Wallis test followed by Dunn's multiple comparisons test. (A-

B) or a one-way ANOVA followed by Tukey's multiple comparisons test (C-D), depending on normal distribution of data.

Figure E8

SplB-induced AAI is independent of PAR2 function. WT C57BL/6J mice were treated with SplB or SplB + SAM-11, a PAR2-inhibiting monoclonal antibody. Mice treated with SplB + IgG2a served as isotype control. **A** and **B**, Numbers of inflammatory eosinophils in the BALF (A) and lung tissue (B) measured by flow cytometry. **C**, SplB-specific IgE concentrations measured by ELISA. **D**, Epithelial thickness of airway epithelium, **E**, Mucus in lung sections (PAS staining) and **F**, Collagen quantification (Sirius Red) were determined as described in the legend of fig. 3. n = 5. **p < .01. Results are presented as mean ±SD. Significance was determined using a Kruskal–Wallis test followed by Dunn's multiple comparisons test.

Figure 1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7
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24
Days
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time in min
SplB 25 µg
PBS
SplB mut 25 µg
BALF
Lung
Spleen
C
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6×10⁵
4×10⁵
2×10⁵
0
Inflammatory eosinophils
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6×10²
4×10²
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Neutrophils
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2.25×10⁶
1.5×10⁶
7.5×10⁵
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Inflammatory eosinophils
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Total cell count in three lobes
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6×10⁵
4×10⁵
2×10⁵
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G
Percentage among living cells
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6
4
2
0
IL-4
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Concentration [pg/mL]
30
20
10
0
IL-5
J
Concentration [pg/mL]
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2.0
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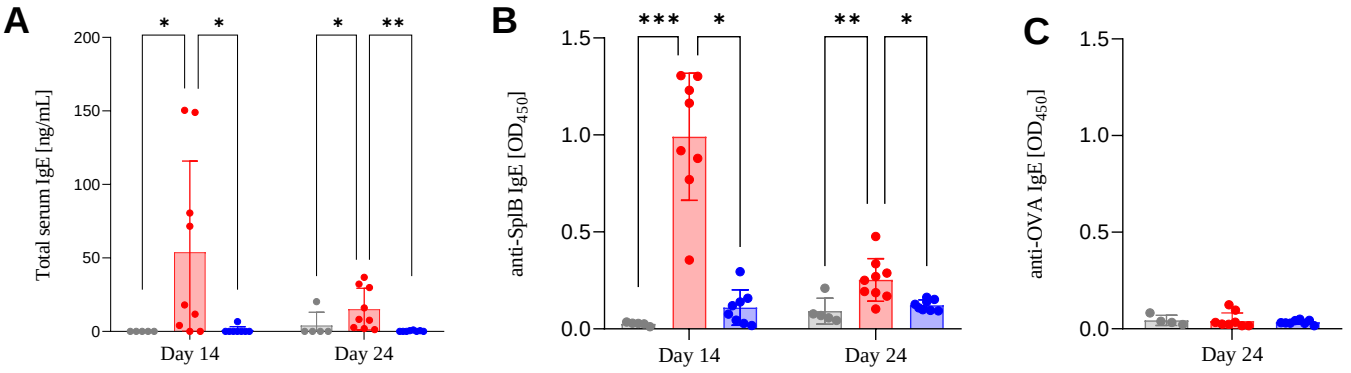
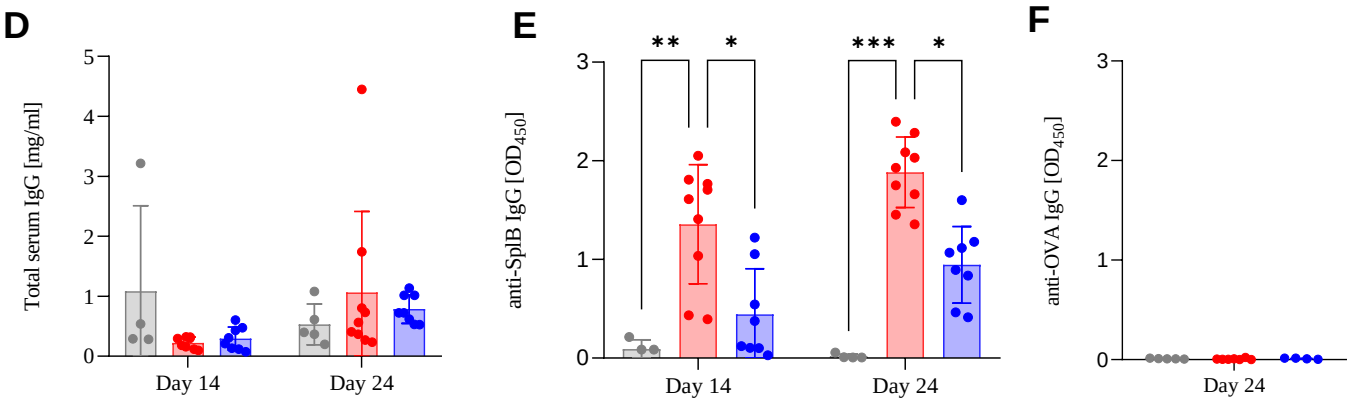
1.0
0.5
0.0
IL-13
OVA
SplB 25 µg
SplB mut 25 µg

Figure 2

Serum IgE

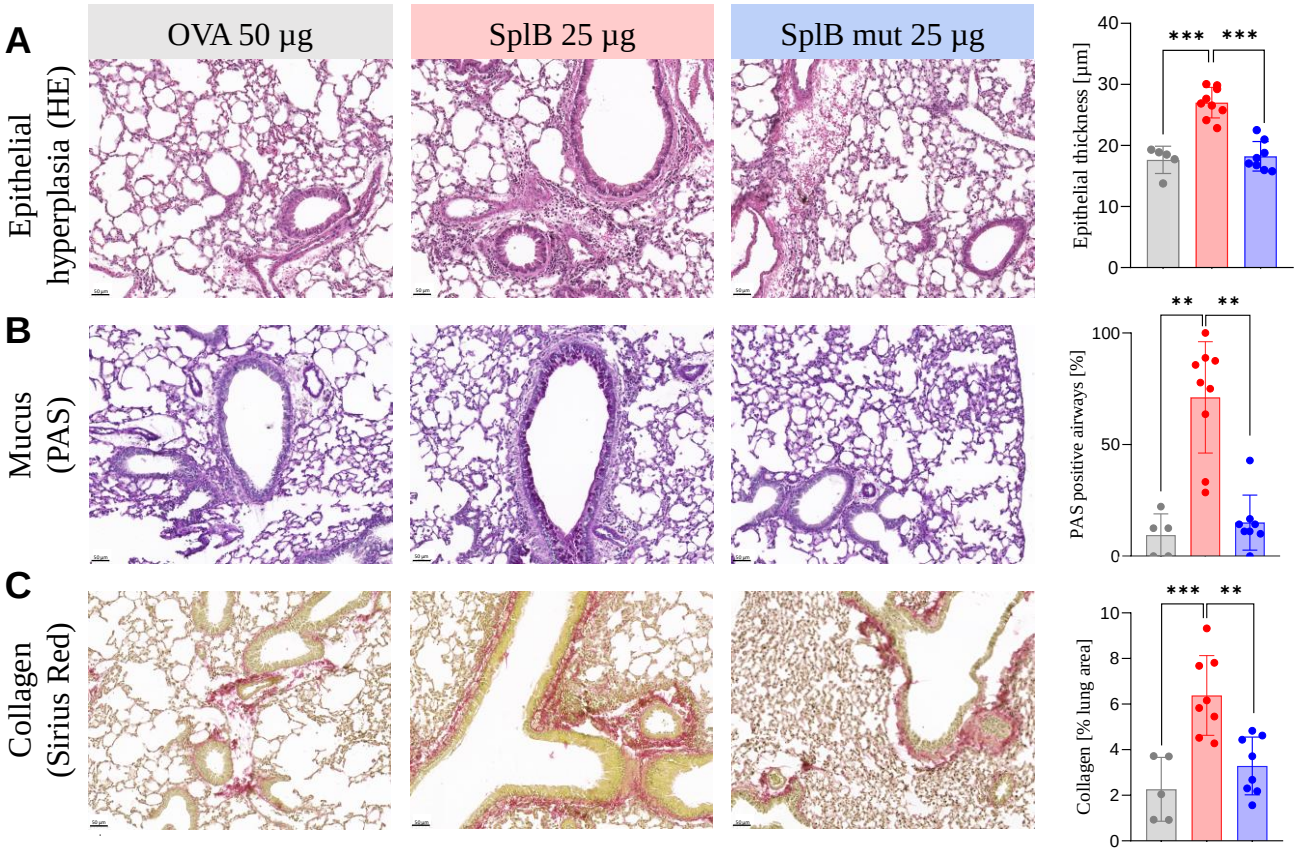


Serum IgG



OVA 50 µg SplB 25 µg SplB mut 25 µg

Figure 3



BALF

Lung

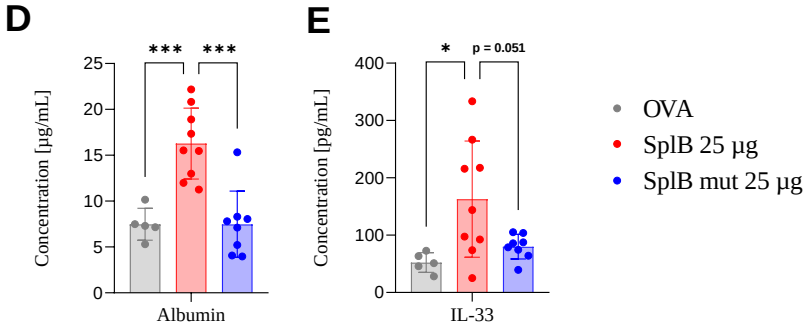
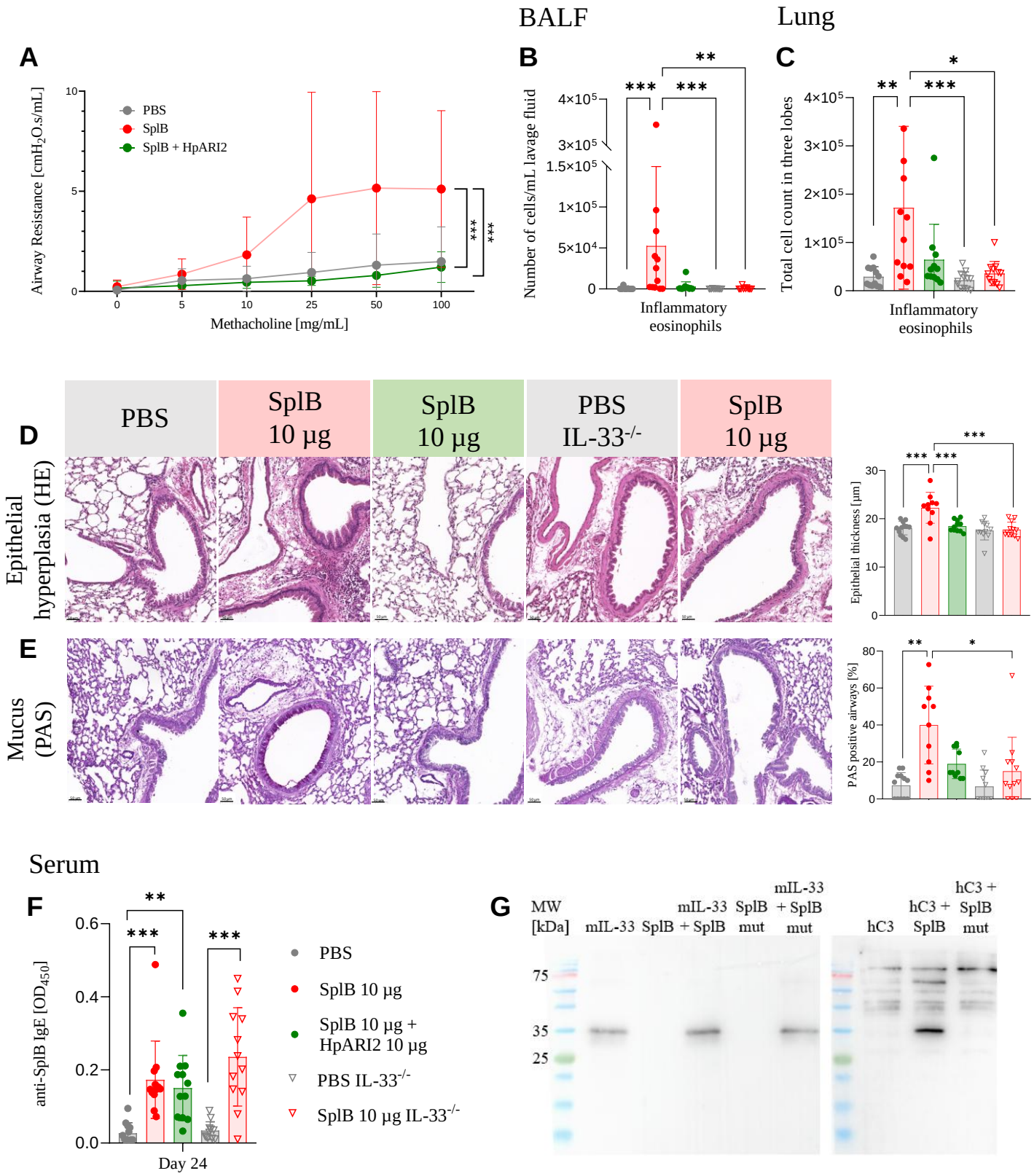


Figure 4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Lung
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B
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PBS
SplB
SplB + HpARI2
Airway Resistance [cmH2O.s/mL]
Methacholine [mg/mL]
Number of cells/mL lavage fluid
Total cell count in three lobes
Inflammatory eosinophils
D
E
PBS
SplB 10 µg
SplB 10 µg
PBS IL-33-/-
SplB 10 µg
Epithelial hyperplasia (HE)
Mucus (PAS)
Epithelial thickness [µm]
PAS positive airways [%]
Serum
F
G
anti-SplB IgE [OD450]
Day 24
PBS
SplB 10 µg
SplB 10 µg + HpARI2 10 µg
PBS IL-33-/-
SplB 10 µg IL-33-/-
MW [kDa]
mIL-33
SplB
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mIL-33 + SplB mut
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hC3 + SplB
hC3 + SplB mut
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35
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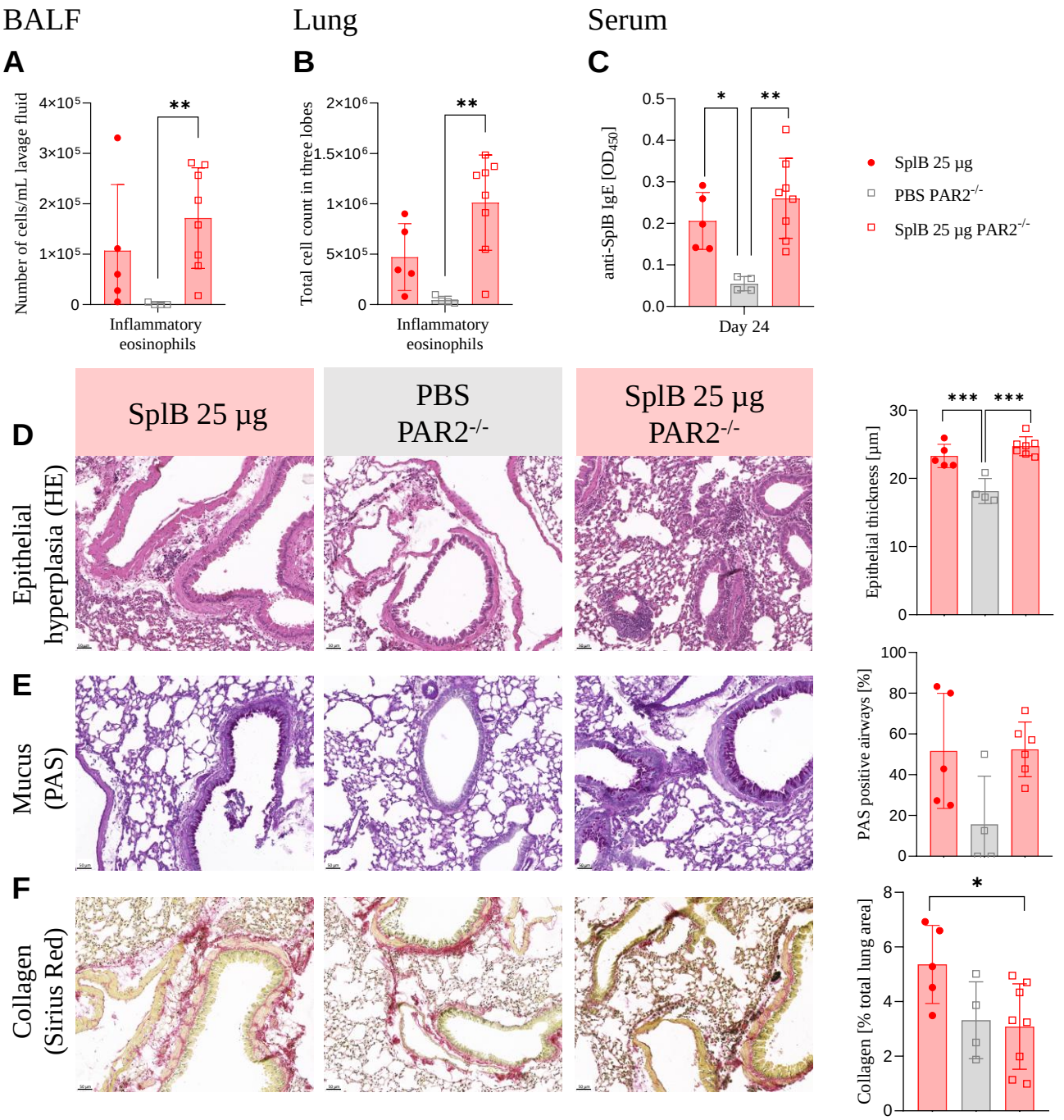
Figure 5


BALF
Lung
Serum
A
B
C
Number of cells/mL lavage fluid
4×10^5
3×10^5
2×10^5
1×10^5
0
**
Inflammatory eosinophils
Total cell count in three lobes
2×10^6
1.5×10^6
1×10^6
5×10^5
0
**
Inflammatory eosinophils
anti-SplB IgE [OD450]
0.5
0.4
0.3
0.2
0.1
0.0
*
**
Day 24
SplB 25 µg
PBS PAR2-/-
SplB 25 µg PAR2-/-
D
SplB 25 µg
PBS PAR2-/-
SplB 25 µg PAR2-/-
Epithelial hyperplasia (HE)
Epithelial thickness [µm]
30
20
10
0
***
***
E
Mucus (PAS)
PAS positive airways [%]
100
80
60
40
20
0
F
Collagen (Sirius Red)
Collagen [% total lung area]
8
6
4
2
0
*