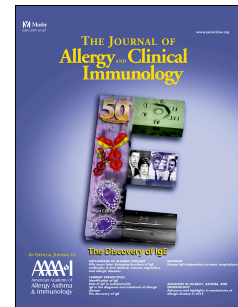


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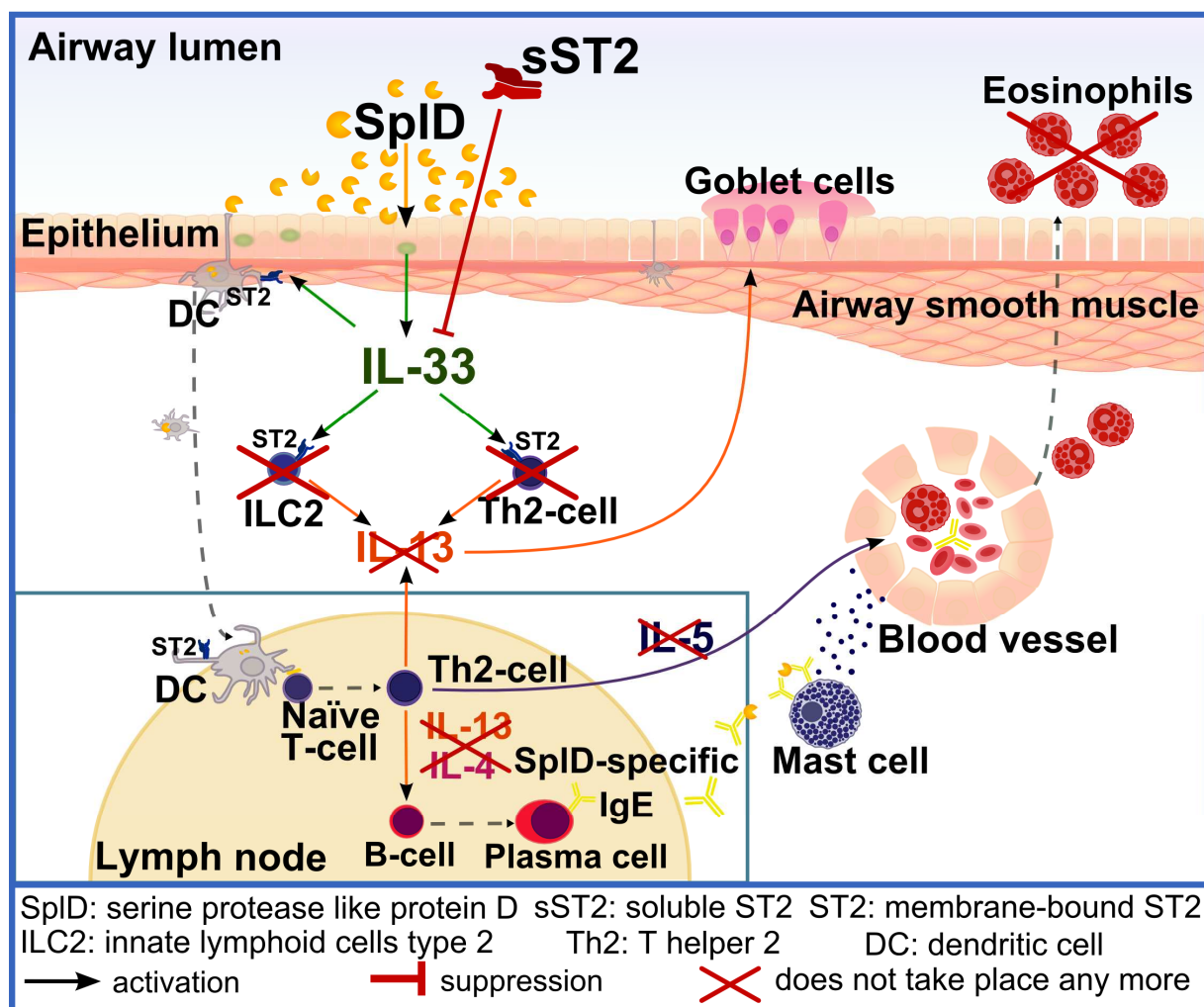
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The IL-33/ST2 axis is crucial in type 2 airway responses induced by the
***Staphylococcus aureus* protease SplD**

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Abstract

Background: Chronic airway inflammatory diseases such as chronic rhinosinusitis with nasal polyps and asthma showed increased nasal *Staphylococcus aureus* (*S. aureus*) colonization. Serine protease like protein D (SplD) and other closely related proteases secreted by *S. aureus* have recently been identified as inducers of allergic asthma in humans and mice but their mechanism of action is largely unknown.

Objective: We investigated the role of recombinant SplD in driving Th2-biased responses and IgE formation in a murine model of allergic asthma.

Methods: Allergic asthma was induced in C57BL/6 J wild type mice, TLR4 knockout mice (TLR4^{-/-}) and Rag2 knockout mice (Rag2^{-/-}) by repeated intratracheal applications of SplD. Inflammatory parameters in the airways were assessed by flow cytometry, ELISA, Luminex and immunohistochemistry. Serum SplD-specific IgE levels were analysed by ELISA.

Results: We observed that repeated intratracheal exposure to SplD led to IL-33 and eotaxin production, eosinophilia, bronchial hyperreactivity and goblet cell hyperplasia in the airways. Blocking the IL-33 activity using a soluble ST2 receptor (sST2) significantly decreased the numbers of eosinophils, IL-13⁺ILC2s, IL-13⁺CD4⁺ T cells, and IL-5 and IL-13 production by lymph node cells but had no effect on IgE production. SplD-induced airway inflammation and IgE production were largely dependent on the presence of the functional adaptive immune system and independent of TLR4 signalling.

Conclusion: The *S. aureus*-derived protein SplD is a potent allergen of *S. aureus* and induces a Th2-biased inflammatory response in the airways in an IL-33-dependent,

but TRL4-independent manner. sST2 could be an efficient strategy to interfere with SplD-induced Th2 inflammation, but does not prevent the allergic sensitization.

Key messages

- sensitization with *S. aureus* serine protease like protein, SplD causes Th2-biased allergic airway inflammation in mice accompanied by the release of the innate cytokine IL-33
- IL-33 targeted treatment could be an efficient therapeutic strategy to counteract the action of SplD on the immune system

Keywords: allergy, asthma, *S. aureus*, sensitization, serine protease

Capsule summary

S.aureus protein SplD acts as an allergen and an adjuvant inducing asthma features via inducing IL-33 production by airway epithelial cells. Soluble IL-33 receptor is a promising treatment option as it interferes with SplD-induced inflammation.

86 **Abbreviations**

87	BALF	bronchoalveolar lavage fluid
88	EDTA	ethylenediaminetetraacetic acid
89	HDM	house dust mite
90	IgE	immunoglobulin E
91	IL	interleukin
92	IL-1RAcP	IL-1R accessory protein
93	ILC2	innate lymphoid cells type 2
94	MHC	major histocompatibility complex
95	MLKL	mixed lineage kinase domain-like protein
96	OVA	ovalbumin
97	PAR	protease-activated receptor
98	PAS	Periodic acid - Schiff
99	PBS	phosphate-buffered saline
100	<i>S. aureus</i>	<i>Staphylococcus aureus</i>
101	SplD	<i>S. aureus</i> -derived serine protease-like protein D
102	sST2	soluble ST2
103	Th2	T helper two
104	TLR	toll-like receptor
105	TSLP	thymic stromal lymphopoietin
106	TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
107	GFP	green fluorescent protein

108

109

Introduction

S. aureus is a versatile germ frequently found colonizing patients with Th2-biased diseases such as atopic dermatitis and chronic rhinosinusitis with nasal polyps¹⁻⁵. It actively manipulates the host immune response by releasing proteins, which facilitate bacterial invasion and colonization^{6,7}. These secreted proteins allow the bacterium to activate virulence and metabolic pathways required for bacterial survival and may exert immune suppressive action on the mucosal environment^{8,9}. Based on *in silico* analyses of the *S. aureus* pangenome it is estimated that the repertoire of secreted proteins comprises more than 1350 proteins, including enterotoxins, toxic shock syndrome toxin-1 and other virulence factors, many of which the function is unknown^{10,11}. It is important to understand the interplay between the immune proteome of *S. aureus* and the immune response of the host and to elucidate its role in the initiation and persistence of chronic airway diseases. Asthmatic patients have increased specific IgE reactivity to various secreted *S. aureus* proteins¹² and several endotypes of chronic rhinosinusitis were proposed based on the presence of *S. aureus*-specific IgE^{13, 5}. Recently, we have observed elevated levels of *S. aureus* serine protease-like protein (Spl)-specific IgE in sera of asthmatic patients, indicating the clinical relevance of these proteases¹⁴. Spls are a group of six *S. aureus* proteases (A-F), which belong to a small subfamily S1B (encompassing staphylococcal V8 protease, epidermolytic toxins, and Spl proteases). 84% of *S. aureus* strains contain at least one Spl-protease-encoding gene¹⁵. Moreover, we could demonstrate that repeated exposure to pure SplD without the addition of any adjuvant results in a Th2 response and SplD-specific IgE production in mice¹⁴. However, the exact mechanisms underlying this SplD-induced Th2 bias are not yet unravelled and are the focus of the current study.

Allergens such as house dust mite (HDM), cockroach or *Alternaria alternata* were shown to act in an important role in allergy development, in part by activation of cell surface protease-activated receptors (PARs)¹⁶ in the airways inducing cytokines and cleave intercellular epithelial tight junctions¹⁷ thereby amplifying the response to allergens. They can also cleave CXCR1 on the surface of neutrophils¹⁸ and CD23 and CD25 receptors on immune cells¹⁹ thereby reinforcing allergy progression.

A key mediator of the type 2 inflammation of the airways is the cytokine IL-33. IL-33 binds to a heterodimeric cell surface receptor consisting of IL-1R accessory protein (IL-1RAcP) and ST2 on immune cells such as Th2 cells, innate lymphoid cells type 2 (ILC2) cells, invariant natural killer T cells, natural killer cells, basophils, eosinophils, mast cells as well as dendritic cells (DCs), eventually activating intracellular signalling pathways and supporting allergic airway inflammation²⁰⁻²². Among the four known isoforms of ST2, two are highly relevant for the regulation of allergic airway inflammation in mice: the soluble ST2 (sST2) and a transmembrane form (ST2L)²³. It has been shown that sST2 antagonizes ST2L, suppressing the IL-33 mediated activation of the NF- κ B pathway as well as downstream effects such as the production of Th2 cytokines, thereby attenuating allergic asthma symptoms in mice^{24,25}. In this respect, sST2 could be a promising therapeutic option for asthma.

In the current work we focused on the molecular mechanisms by which SplD drives allergic asthma in mice. We demonstrated that SplD-induced type 2 cytokine production and eosinophilia in the airways are largely IL-33 dependent, while the production of SplD-specific IgE and goblet cell metaplasia occur independently of this pathway. The above mentioned effects are mediated by the adaptive immune system.

Methods**Mice and sensitization protocol**

Animals were maintained in a 12/12 h of light/dark cycle and had access to water and food *ad libitum*. Animal experiments were approved by the local ethics committee of Ghent University. Female C57BL/6 J wild type mice (Janvier, Heverlee, Belgium), TLR4 knockout mice (TLR4^{-/-}) and Rag2 knockout mice (Rag2^{-/-}) (both were kindly provided by VIB-IRC, Ghent, Belgium) were treated under light anesthesia with isoflurane/air (Ecuphar, Oostkamp, Belgium) six times every second day by intratracheal applications with either 45µg SplD, 45µg SplF or 100µg ovalbumin (OVA, Worthington Biochemical Corporation, Lakewood, NJ, USA) in 50µL PBS (Gibco Thermo Fisher Scientific, Erembodegem-Aalst, Belgium) and one additional i.t. application of 50µL PBS after each treatment, or 50µL PBS alone. In some experiments a combination of 45µg SplD and 100µg OVA were given six times every second day by intratracheal applications. Soluble ST2 was applied intratracheally at the dose of 25µg/50µL per mouse after each SplD application instead of the second PBS application. 48 hours after the last intratracheal application mice were euthanized by an *i.p.* injection of 100µL Nembutal (Ceva Santé Animale, Libourne, France) and the lungs, serum, bronchoalveolar lavage fluid (BALF) and local draining lymph nodes were collected in each experiment.

Luminex analysis

Concentrations of murine IL-5, IL-13, IL-33 and eotaxin in lung homogenates and lymph node cell supernatants were analysed by Luminex Performance assays (R&D,

Oxon, UK). IL-25 and TSLP were measured by ELISA (R&D Systems, Abington, UK).

Bronchial hyperreactivity test

Bronchial hyperreactivity in response to increasing doses of carbachol (0, 20, 40, 80, 160, 320, 640 $\mu\text{g/kg}$) was measured 48 hours after the last intratracheal application using the forced oscillation technique (Flexivent System, SCIREQ, Montreal, QC, Canada). Neuromuscular blockade was induced by injecting 1mg/kg pancuronium bromide intravenously. A “snapshot perturbation” manoeuvre was imposed to measure the resistance of the whole respiratory system (airways, lung and chest wall).

Flow cytometry

The list of antibodies used in the study and the gating strategy are provided in this article's Online repository.

Statistics

Statistical analysis was performed by using Graph Pad Prism version 6 software and unpaired non-parametric Mann-Whitney test.

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 at www.jacionline.org.

Results

SplD induces key features of asthma and increased expression of IL-33 in mice

We established a sensitization protocol using C57BL/6 J wild type mice that received six intratracheal (i.t.) applications of 45 μg SplD. Mice treated with phosphate

buffered saline (PBS) or the inert antigen ovalbumin (OVA) served as negative control groups. With this model, we previously demonstrated that SplD could attract eosinophils and T cells to the airways, increase Th2 cytokine production in local draining lymph nodes and induce the formation of SplD-specific IgE¹⁴. To study the mechanisms of the type 2-biased asthmatic response to SplD we investigated the IL-33 expression in this mouse model. IL-33 is a crucial cytokine in type 2 allergic inflammation^{20,26}. After SplD exposure, mice expressed higher levels of IL-33 in lung homogenates than controls. Full length IL-33 and, even more distinctly, the cleaved form of IL-33 were increased in SplD-treated mice (Fig.1A). Immunofluorescent co-staining of lung sections demonstrated that IL-33 had a nuclear localization in the airway epithelial cells type II (proSPC⁺) in all treatment groups (Fig.1B), while more IL-33 positive cells were present in the SplD-treated mice. Because IL-33 was shown to be an alarmin that can be passively released during necrosis²⁷, we have analysed cell death in the lungs of SplD-treated mice. Western blotting for phosphorylated mixed lineage kinase domain-like protein (pMLKL), which becomes phosphorylated upon cell necrosis²⁸ was not detectable in the experimental animals irrespective of the treatment (Fig.E1A). In addition, no TUNEL-positive cells in the lung sections were detected either (Fig.E1B), supporting that neither apoptotic nor necrotic cell death has a prominent role in the SplD-induced generation of IL-33 in murine airways. Isolated primary murine airway epithelial cells type II responded to SplD with a increased expression of full length IL-33, which peaked after one hour (Fig.1C). We did not find an increase in TSLP, IL-25, GM-CSF levels in the lungs of mice treated with SplD compared to controls (data not shown).

The IL-33/ST2 axis modulates the type 2 inflammatory responses in a murine model of SplD-induced asthma

To investigate the role of IL-33 in SplD-induced asthma, SplD-sensitized mice were treated with intratracheal applications of recombinant soluble IL-33 receptor, sST2 after each SplD treatment to block IL-33 signalling (Fig.2A). sST2 treatment significantly reduced total cell numbers and eosinophil counts in the bronchoalveolar lavage fluid (BALF) (Fig.2B,C). The low number of neutrophils that were attracted to the BALF upon SplD exposure, was further reduced in sST2 treated mice (Fig.2D). SplD treatment induced an infiltration of CD4⁺ T cells into the BALF (Fig.2E). Furthermore, the activation of Th2 cells and ILC2s, was evident by the increased numbers of IL13⁺ CD4⁺ T and ILC2 cells and IL-5 producing ILC2s in the BALF, which were significantly reduced after sST2 treatment (Fig.2F-H). Eosinophil numbers were reduced in tendency in the lungs of SplD-exposed mice treated with sST2 (Fig.2I). No neutrophil infiltration into the lungs of SplD-exposed mice was observed (Fig.2J). sST2 caused a significant reduction in the number of CD4⁺ T cells in the lungs (Fig.2K). IL-33 and eotaxin levels exhibited a tendency to a decrease when sST2 was given in addition to SplD (Fig.2L,M). In the lung draining lymph nodes, the total cell count was not significantly altered by sST2 treatment (Fig.3A), however, the increase in MHCII⁺CD11c⁺ DCs that was observed after SplD treatment was significantly dampened when sST2 was given in addition (Fig.3B). To analyse the effect of the IL-33 signalling pathway on T cell polarization, the local draining lymph node cells from mice receiving different experimental treatments were re-stimulated with α CD3 and α CD28 antibodies for 5 days. SplD-treated mice showed a significantly higher Th2 cytokine production, i.e. IL-5 and IL-13 levels in the lymph node cell supernatants, compared to PBS-treated groups, corroborating our earlier findings in the same model¹⁴. While Th1 and Th17 differentiation were not affected (data not shown). Remarkably, the sST2 treatment resulted in a strong reduction of

IL-5 and IL-13 levels in the lymph node cell supernatants of SplD-treated mice (Fig.3C,D), supporting the notion of an important role of IL-33 in the SplD-induced T cell activation. Although the inflammatory cell responses were dampened by the sST2 treatment, serum levels of SplD-specific IgE were not significantly reduced by sST2 (Fig.3E). Moreover, SplD-exposure induced bronchial hyperreactivity, which was partially but not significantly lowered by sST2 treatment (Fig.3G). To further characterize our SplD-sensitization model, we investigated lung histology. SplD-treated mice showed marked perivascular and peribronchial infiltration of CD45⁺ leukocytes, which were largely prevented by sST2 (Fig.4A). The key features of asthma, such as the goblet cell hyperplasia in the airways (Fig.4B) and thickening of the peribronchial airway smooth muscles, were present in the SplD-treated mice and indicate incipient airway remodelling (Fig.4C). These were unchanged after the addition of sST2.

In summary, our results underline the crucial role of IL-33 in SplD-induced type 2 inflammation, while airway remodelling features were less affected by intratracheal administration of sST2.

SplD acts as an adjuvant when co-administered with an inert allergen

As we have shown earlier, that SplD administration in the absence of adjuvants results in the production of SplD-specific serum IgE in mice¹⁴ (Fig.3E). In contrast, OVA alone did not induce an OVA-specific IgE response under similar conditions, as it is considered as an inert antigen. Remarkably, intratracheal co-administration of SplD breaks the tolerance to OVA, as shown by significantly higher levels of OVA-specific serum IgE in the mice (Fig.3F).

SplD induces attraction of PAR2 positive cells to the lungs

PAR2 is an important recognition receptor for protease allergens such as HDM or *Alternaria alternata*, and its activation enhances a Th2 response in the lungs^{29,30}. Following SplD treatment, the numbers of PAR2⁺ cells were significantly increased in the lungs, which could be efficiently counteracted by sST2 (Fig.5A). This indicates that the increased PAR2 in the lungs is a consequence of the SplD-induced attraction of inflammatory cells rather than a direct activation of PAR2. Both an *in vitro* assay using an NF- κ B dependent PAR2 reporter HEK-293 cells (Fig.5B) and GFP-tagged PAR2-expressing HeLa cells (Fig.5C) showed an activation with the PAR2 activating peptide SLIGKV-NH₂ and trypsin, by luciferase activity or internalization of PAR2 by endosome formation, but not with SplD.

SplD does not activate TLR2 in vitro

TLR2 is a receptor that detects bacterial cell wall compartments such as peptidoglycan and is thus involved in recognizing *S. aureus*³¹. SplD did not activate TLR2 reporter cells (Hek-Blue hTLR2) *in vitro* at any tested concentration, excluding a direct interaction of SplD with TLR2 (Fig.E2A).

SplD induces asthma features independent from TLR4

Inhalation of allergens with proteolytic capacities, such as HDM, leads to asthma via initiation of the TLR4 signalling pathway³². However, the numbers of eosinophils, neutrophils or T cells in BALF and in lungs were comparable between wild type and TLR4^{-/-} mice when treated with SplD (Fig.E2B-F). In addition, TLR4 deficiency did not affect the levels of SplD-induced IL-33 production (Fig.E2G) and SplD-specific IgE (Fig.E2H) production. This indicated that, in contrast to HDM, SplD-induced asthma is TLR4 independent.

The adaptive immune system is essential for SplD induced asthma

To study the contribution of the adaptive immune system to the SplD-induced allergic airway inflammation we treated Rag2^{-/-} mice, which lack mature T- and B cells but not ILC2s³³, with SplD or PBS. SplD-induced eosinophilia in the BALF was completely abolished in Rag2^{-/-} mice (Fig.6A) and neutrophils were significantly decreased in BALF of SplD-treated Rag2^{-/-} mice compared to the controls (Fig.6B). The levels of IL-33 in the lungs of SplD-treated Rag2^{-/-} mice were comparable to the controls indicating that IL-33 acts upstream of the adaptive immune system (Fig.6C). No production of SplD-specific IgE could be measured in Rag2^{-/-} mice (Fig.6D). Histological analysis of the lungs showed that Rag2^{-/-} mice do not respond to SplD, as they lack inflammatory cell infiltration, airway remodelling or bronchial hyperreactivity (Fig.6E-I) that were observed in the wild type mice. The absence of a functional immune system in Rag2^{-/-} does not allow to analyse the humoral immune response.

The serine protease like protein SplF induces an inflammatory response like SplD

SplF is also encoded in the *Spl*-Operon and shares high sequence similarity with SplD. We tested the response towards SplF *in vivo*, using the same protocol as with SplD. In the BALF of SplF-treated mice, the total cell count (Fig.E3A) as well as the numbers of eosinophils (Fig.E3B), neutrophils (Fig.E3C) and CD4⁺ T cells (Fig.E3D) were significantly higher than in the PBS-treated control mice. The eosinophils are also increased in the lungs (Fig.E3E), while neutrophil numbers remained low (Fig. S3F). A trend towards an increased expression of IL-33 and eotaxin was also observed (Fig.E3G,H). After six applications of SplF, SplF-specific IgE was formed (Fig.E3I). Representative images of CD45⁺ leukocytes attracted to the lungs, goblet

cells, peribronchial smooth muscles and PAR2⁺ cells show comparable results as SplD-treated mice (Fig.E3J).

Discussion

In this study we focus on the mechanisms of the Th2-biased immune response induced by *S. aureus* serine protease like protein, SplD. Our study reveals that sensitization with SplD causes an allergic airway inflammation in mice accompanied by the release of the innate cytokine IL-33, increased airway hyperreactivity, higher mucus production as well as activation of the adaptive immune system with predominant release of Th2 cytokines. Allergen recognition in the airways is characterized by release of IL-1 α , IL-25, IL-33, TSLP, GM-CSF and endogenous danger signals that activate the DCs network and other innate immune cells, such as basophils and type 2 innate lymphoid cells³⁴. In contrast to earlier observations in HDM sensitization models, we did not find an increase in TSLP, IL-25, GM-CSF levels in the lungs of mice treated with SplD.

It became evident that IL-33 and its receptor ST2 are crucial in the regulation of allergic inflammation^{35,36}. IL-33 is expressed by airway smooth muscle cells, endothelial and epithelial cells and it can also be produced by macrophages upon activation in humans³⁷. In murine lungs IL-33 is mostly localized in airway epithelial cells type II³⁸. IL-33 can be released as an alarmin from dying cells²⁷ or upon allergic sensitization^{21,39-41}. In this study we showed that IL-33 expression levels are increased in SplD-treated mice in comparison to the controls. By stimulating isolated primary airway epithelial cells type II with SplD *in vitro*, we demonstrated a fast and direct effect of SplD on IL-33 production that was independent of the presence of inflammatory immune cells. Importantly, the IL-33 upregulation was not

accompanied by cell death as demonstrated by TUNEL staining of lung sections and Western blot for p-MLKL, suggesting that IL-33 is not passively released by dying cells but actively secreted by the airway epithelium. Bacterial and fungal cell wall components were shown to induce IL-33 production via TLR2 activation⁴², however, SplD did not directly activate TLR2 *in vitro*. Moreover, we demonstrated that the facilitating effects of SplD on allergy parameters such as eosinophilia, Th2 cytokine production and SplD-specific IgE levels appeared to be independent of TLR4. This observation is in sharp contrast with the mode of action described for HDM, where the presence of TLR4 on the structural lung cells is required for the development of allergic asthma³². This suggests the possibility that other receptors might be involved, like PAR2, which gets triggered, e.g., by *Alternaria alternata*, to induce an asthmatic response independent of TLR4⁴³. PAR2 activation in the lungs was shown to trigger and enhance a Th2-biased inflammatory response to an inert protein antigen in mice⁴⁴. We observed an increase in PAR2⁺ cells in the lung sections of SplD-treated mice. The higher amount of PAR2 in SplD treated mice can be explained by PAR2 expression on immune cells such as CD4⁺ T cells⁴⁵ or macrophages infiltrating SplD-treated lungs, and by the effects of endogenous proteases that are released, e.g. by mast cells in response to the SplD-induced inflammation⁴⁶. We have demonstrated that no direct protease-dependent activation of PAR2 by SplD occurs.

We have observed an increased production of IL-5 and IL-13 in lymph nodes of the SplD-treated mice. As IL-13 promotes continuous IL-33 production⁴⁷, positive feedback loops could drive allergic airway inflammation in our model. The presence of both uncleaved and cleaved forms of IL-33 in the lungs of mice treated with SplD could be very important, because the mature short form of IL-33 has a 30 fold increased capacity to activate ILC2 cells *ex vivo* via ST2L⁴⁸. We have ruled out the

possibility that SplD could directly cleave IL-33 *in vitro*, as no fragments were generated after co-incubation with of full length IL-33 for up to 24 hours (data not shown). Alternatively, IL-33 activity can be regulated by endogenous proteases released during inflammation. Processing of IL-33 by neutrophil proteases such as neutrophil elastase or cathepsin G may also occur during bacterial, fungal or viral infections⁴⁹. In addition, mast cell tryptase and chymase were shown to generate the shorter mature forms of IL-33⁴⁸. Although the numbers of attracted neutrophils and mast cells are negligibly small in our model (Fig.E5A), they could be a potent source of IL-33 activating proteases. Though, SplD does not cause a direct mast cell degranulation (Fig.E5B). However, so far, the mechanism of IL-33 cleavage in SplD-treated mice remains a matter of speculation.

To block the IL-33 pathway we treated mice with intratracheal applications of sST2. In contrast to former studies involving sST2, we applied sST2 intratracheally to mimic the non-invasive administration route of therapeutic inhalers. The sST2 treatment during SplD sensitization resulted in a diminished eosinophilic attraction to the airways, lower IL-33- and eotaxin levels, lower numbers of IL-13⁺ ILC2s and CD4⁺ T cells in the BALF and less IL-5 and IL-13 production in local draining lymph nodes. In contrast, intratracheal application of sST2 did not reduce SplD-specific IgE levels. This is possibly due to the intratracheal administration route, as previously the IL-33 pathway was shown to interfere with IgE production in ST2^{-/-} mice using HDM⁵⁰ or a mix of allergens from *Alternaria alternata*, *Aspergillus* or HDM^{51,52}. In a study by Willart and colleagues, mice injected i.p. with sST2 had reduced levels of HDM-specific IgE, while no effect on Th2 cytokine levels in the lymph nodes could be seen²⁵. SplD-induced airway resistance was non-significantly lowered and airway remodelling was unaffected by sST2 treatment.

DCs are known to be the primary sensors of antigens and infectious agents in the airways and they initiate appropriate adaptive immune responses⁵³. During allergic sensitization IL-33 can activate DC maturation, resulting in the expression of co-stimulatory molecules, the priming of T cells and ultimately the production of Th2 cytokines^{54,55}. IL-33 treatment was shown to increase DC numbers in the lungs while local T and B cell populations were not affected⁵⁵. We have demonstrated that SplD treatment attracts DCs to the lung as well as the local lymph nodes, where they probably prime naïve T cells for differentiation into Th2 cells. We observe that SplD induced the migration of DCs to the local lymph nodes. As expected, DC activation was IL-33 dependent as it was reduced in mice treated with sST2.

These results prompted us to analyse the role of the adaptive immune system in our experimental model of SpD-induced asthma using Rag2^{-/-} mice. Importantly, eosinophilia, SplD-specific IgE production and goblet cell hyperplasia were completely abolished in Rag2^{-/-} mice that received SplD. This demonstrates an essential function of the adaptive immune system in SplD-induced allergic airway inflammation. Since IL-33 levels were unaltered in SplD-treated Rag2^{-/-} mice, IL33 must act upstream of T cell sensitization, probably by tuning DCs. Moreover, the data clearly shows that the pronounced eosinophilia in the inflamed airways and lungs was not elicited by IL33 directly, but mediated by adaptive immune cells. In agreement with this, Rag2^{-/-} mice treated with HDM had very few eosinophils in the airways and reduced goblet cell metaplasia³³.

At the first glance, our findings appear to contradict reports by Halim et al. who showed that ILC2s rather than T cells are important at the initiation stage of papain-induced allergic inflammation (days 2-3), promoting type 2 cytokine production, eosinophilia and mucus hyperproduction^{56,57}. However, at later stages (days 14-21),

CD4⁺ T cells were the main producers of Th2 cytokines⁵⁸. After two weeks of SplD-treatment, we found, besides infiltration of CD4⁺ T cells, a significant expansion of ILC2s in the airways of SplD-treated wild type mice. It appears plausible that also in our model, the initial response to SplD is carried out mainly by resident ILC2s. However, the lack of inflammation in Rag2^{-/-} mice after two weeks of SplD-exposure shows, that an amplification of this initial ILC2-mediated response by T cells is required for the development of the observed eosinophilic type 2 airway inflammation in wild type mice.

We have previously shown that *S. aureus* enterotoxin B (SEB), a potent T cell activating agent, can break immune tolerance in the airways. In the presence of this toxin intratracheal application of OVA, otherwise an inert protein, primes a Th2 response characterized by bronchial hyperreactivity and the production of specific IgE⁵⁹. This is regulated by the activation of inflammatory DCs and polyclonal T cells independent of the IL-1R pathway⁶⁰. Similarly, SplD promoted the production of OVA-specific IgE antibodies when given in combination with OVA. Importantly, in our experimental model intratracheal application of OVA alone did not elicit OVA-specific IgE production, which indicates that it functions as a model of airway tolerance. To render OVA allergenic, prior priming by systemic application of OVA together with an adjuvant is usually required⁶¹. Hence, our data indicates that SplD is capable of breaking tolerance in the airways.

Next to SplD, we showed that SplF can also induce an eosinophilic inflammatory response and allergic sensitization in mice. SplF shares 94.6% sequence homology with SplD⁶². Considering its high prevalence of 71%⁶³ in *S. aureus* strains, our findings make SplF another relevant *S. aureus*-derived allergen.

Our observations support the hypothesis that *S. aureus* is persistently colonizing the nasal mucosa of patients, protected by a biofilm or inside of immune cells ⁴ and constantly producing a panel of factors that could initiate and/or aggravate a Th2-biased immune response. The chronic exposure to *S. aureus* secreted proteins, like Spls, would also hinder resolution of the inflammation, fostering chronification.

In summary, we have demonstrated that SplD causes a Th2 response in an IL-33 dependent manner, because Th2 cytokine production and eosinophilic airway inflammation could be controlled by sST2 treatment. This identifies IL-33 as a potential therapeutic target in chronic rhinosinusitis and asthma patients who are carriers of *S. aureus*.

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Author contributions

A.R.T. conducted experiments, acquired data, analysed data and wrote manuscript. M.N., S.P., K.D., C.O. performed experiments, acquired data, analysed data, revised the manuscript. G.H., H.B. and A.G. performed experiments. D.V.K., W.D., P.V., C.B., B.M.B., R.B and C.B. revised the manuscript. H.B., R.B., H.H. and T.M. provided reagents, revised manuscript. O.K. performed study design, conducted experiments, acquired data, analysed data, wrote and revised manuscript.

Figure legends

Figure 1: IL-33 upregulation upon SplD exposure. A, detection and semi-quantitative analysis of full length- and short form-IL-33 in lung homogenates of PBS, OVA or SplD-treated mice by Western blot. Data is presented as means $\pm$ SEMs for four mice per group. B, co-staining of IL-33 (green) and airway epithelial cells type II (proSPC, red) and DAPI (blue). Scale bars represent 25 μ m. C, Western blot detection and semi-quantitative analysis of IL-33 in isolated murine primary airway epithelial cells type II (AECIIs) that were stimulated with 25 μ g/mL SplD for 0, 1 and 5h. n=3-5. Results are presented as means $\pm$ SEMs, *P<0.05 are determined with Mann-Whitney U test.

Figure 2: The SplD-induced IL-33 dependent Th2 inflammation. A, application

scheme, the different groups got six i.t. applications every 48 h of either PBS, OVA, SplD or SplD in combination with soluble ST2 (sST2) and were euthanized 48 h after the last application. B, total cell count and flow cytometric analysis of C, eosinophils ($CD11c^{-}SiglecF^{+}CD11b^{+}$), D, neutrophils ($GR1^{+}CD11b^{+}F4/80^{-}$) and E, $CD3^{+}CD4^{+}$ T cells in BALF. F, IL-13 producing $CD4^{+}$ T cells G, IL-5 and H, IL-13 producing ILC2s in the BALF. I, eosinophils, J, neutrophils and K, $CD4^{+}$ T cells in lungs, analysed by flow cytometry. L, IL-33 and M, eotaxin measurement of lung homogenates by Luminex. Mean values $\pm$ SEM are presented. $n=7-13$, $*P<0.05$; $**P<0.01$; $***P<0.001$; $****P<0.0001$; are determined with Mann-Whitney U test.

Figure 3: IL-33 dependent T memory cell formation in local lymph nodes, specific IgE formation and airway hyperreactivity assay. A, total cell count of lymph node cells B, flow cytometric analysis of DCs in lymph nodes and C, D, cytokine release by lymph node cells after stimulation for 5 days with anti-CD3 and anti-CD28 antibodies or medium (RPMI, controls). E, SplD-specific IgE and F, OVA-specific serum IgE concentrations of mice treated with either PBS, OVA, SplD or a combination of SplD and sST2 or SplD and OVA were measured by ELISA. G, airway resistance in response to increased doses of carbachol administered 48 h after the last intratracheal challenge of PBS, OVA, SplD or SplD and sST2 was measured by forced oscillation technique. $n=7-13$, results are presented as means $\pm$ SEMs, $*P<0.05$; $**P<0.01$, $***P<0.001$ are determined with Mann-Whitney U test.

Figure 4: SplD-induced leukocyte infiltration and tissue remodelling in the lungs. A, anti-CD45-staining in the lungs, B, Periodic acid-Schiff (PAS) staining of lung sections and goblet cell quantification in the airways (800-2000 μ m perimeter) and C,

anti-smooth muscle actin (SMA) staining with measurement of the peribronchial airway smooth muscle thickness in the lungs of PBS, ovalbumin (OVA), SplD and SplD with sST2-treated mice. Scale bars represent 200 μ m. n=5, results are presented as means $\pm$ SEMs, *P<0.05; **P<0.01 are determined with Mann-Whitney U test.

Figure 5: PAR2 is indirectly increased in SplD treated mice. A, anti-PAR2 stained lung sections and quantification of PAR2 positive area. n= 5, results are presented as means $\pm$ SEMs, *P<0.05; **P<0.01 are determined with Mann-Whitney U test. B, luciferase activity based activation assay of Hek-293 cells transfected with human PAR2 (hPAR2) or an empty vector control. Cells were either untreated or treated with SplD, PAR2 activating peptide SLIGKV-NH₂ or trypsin for 10 hours. Luciferase values are presented as fold activation relative to untreated cells. Graphs represent mean values of quadruplicates $\pm$ SD. C, visualization of PAR2 translocation in HeLa cells transfected with a human PAR2-EGFP fusion construct. The cells were either untreated, or stimulated with SplD, SLIGKV-NH₂ or trypsin and recorded for 60 min.

Figure 6: The role of the adaptive immune system in SplD-induced asthma using Rag2^{-/-} mice. Flow cytometric analysis of A, eosinophils and B, neutrophils in the BALF, C, IL-33 measurement of lung homogenates by Luminex and D, SplD-specific IgE measurement in serum by ELISA of PBS or SplD-treated Rag2^{-/-} mice in comparison to wild type mice. E, anti-CD45 staining and quantification F, PAS-staining and goblet cell count, G, anti-smooth muscle actin (SMA) with measurement of the peribronchial airway smooth muscle thickness and H, anti-PAR2 staining and quantification on lung sections. I, airway resistance in response to increased dosages of carbachol administered 48 h after the last intratracheal challenge of PBS- or SplD-

treated Rag2^{-/-} mice was determined by the forced oscillation technique. Scale bar represents 200 μ m, n=5-8. Mean values $\pm$ SEM are presented. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001; are determined with Mann-Whitney U test.

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ACCEPTED MANUSCRIPT

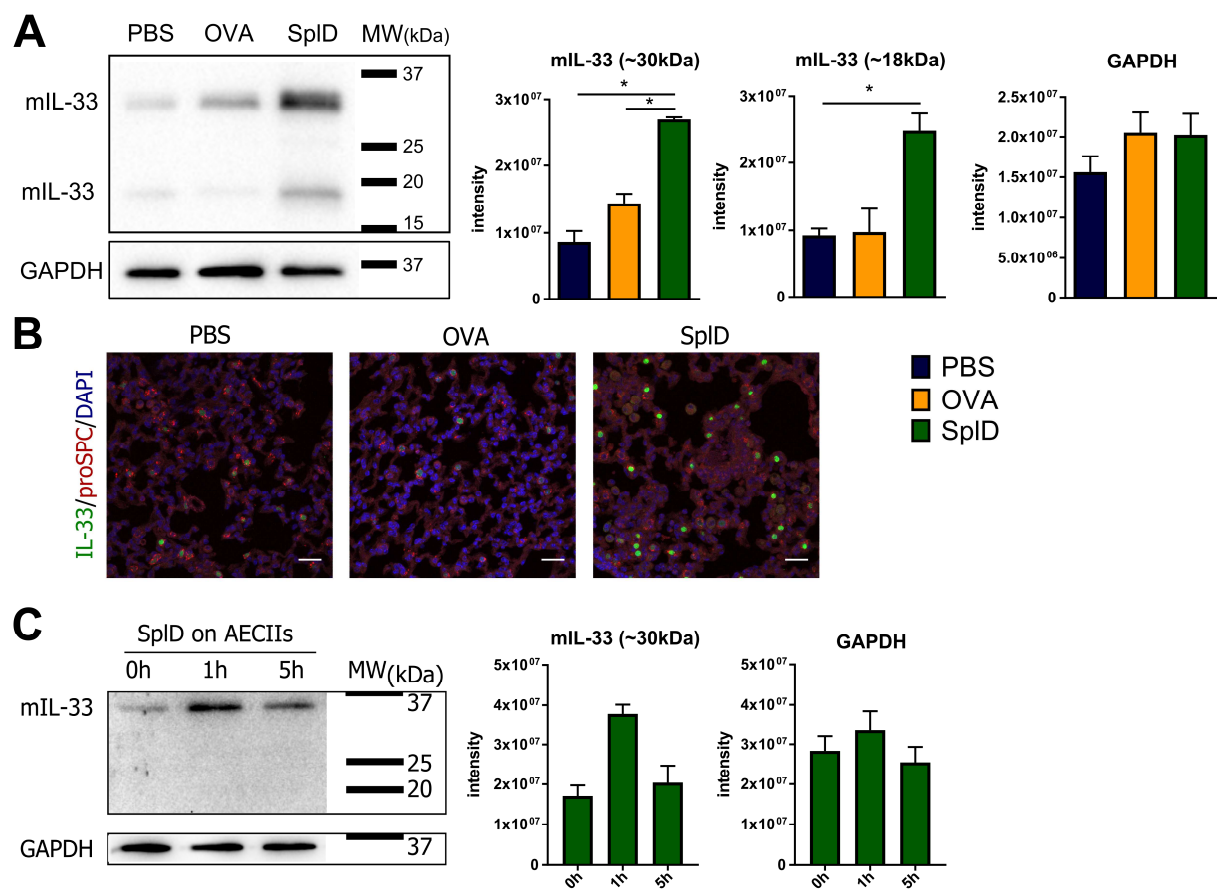


Figure1

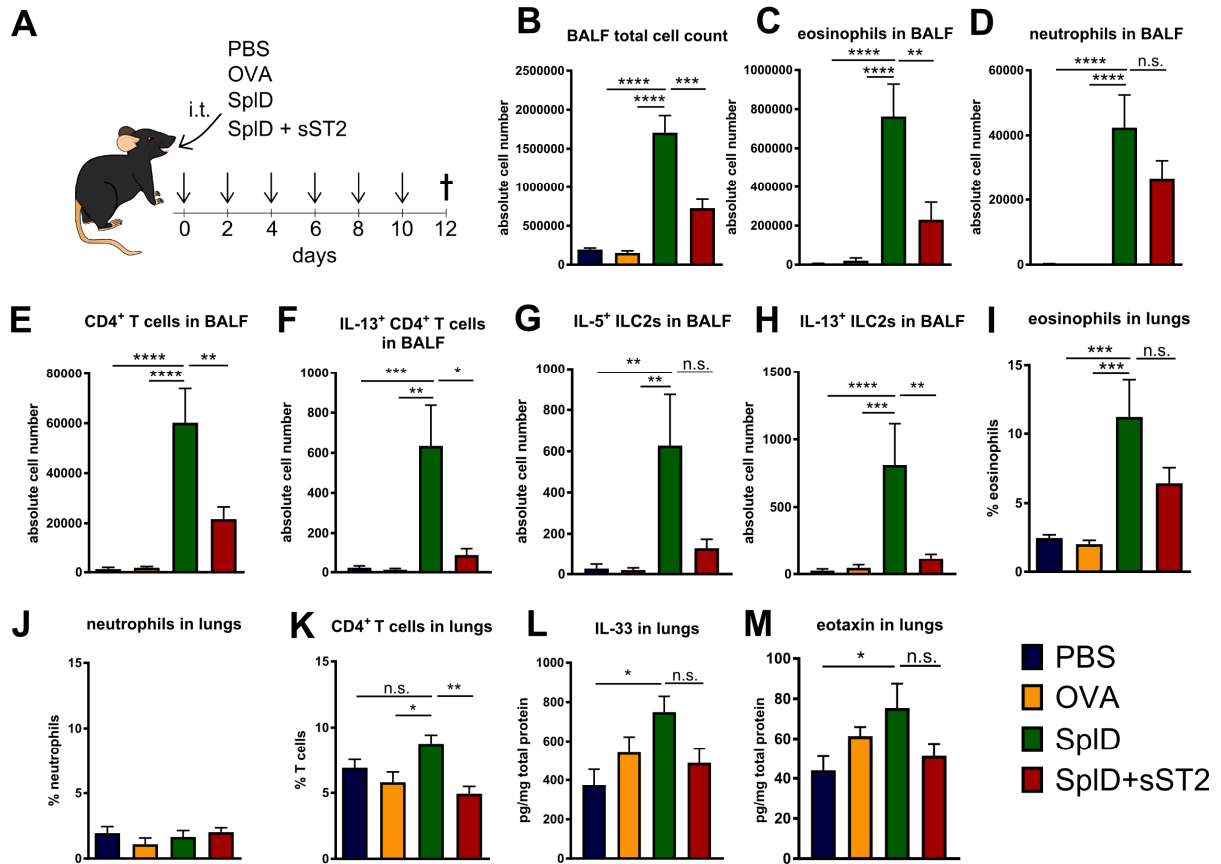


Figure 2

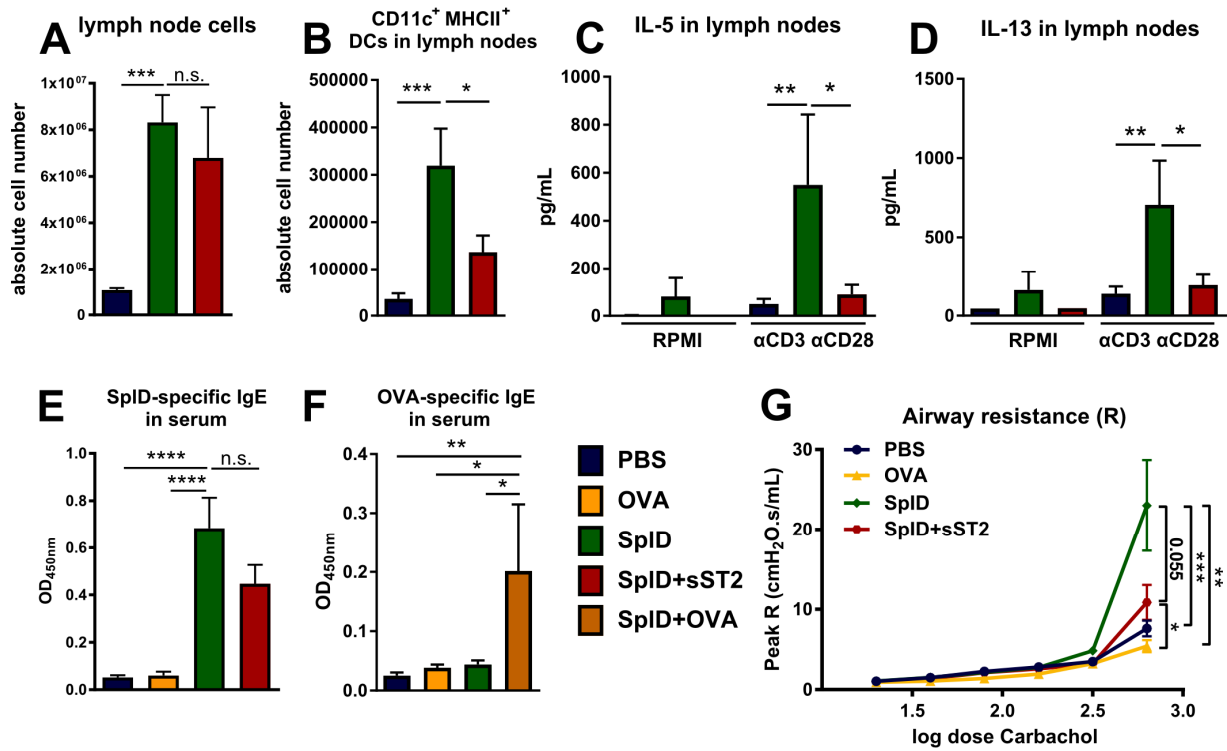


Figure 3

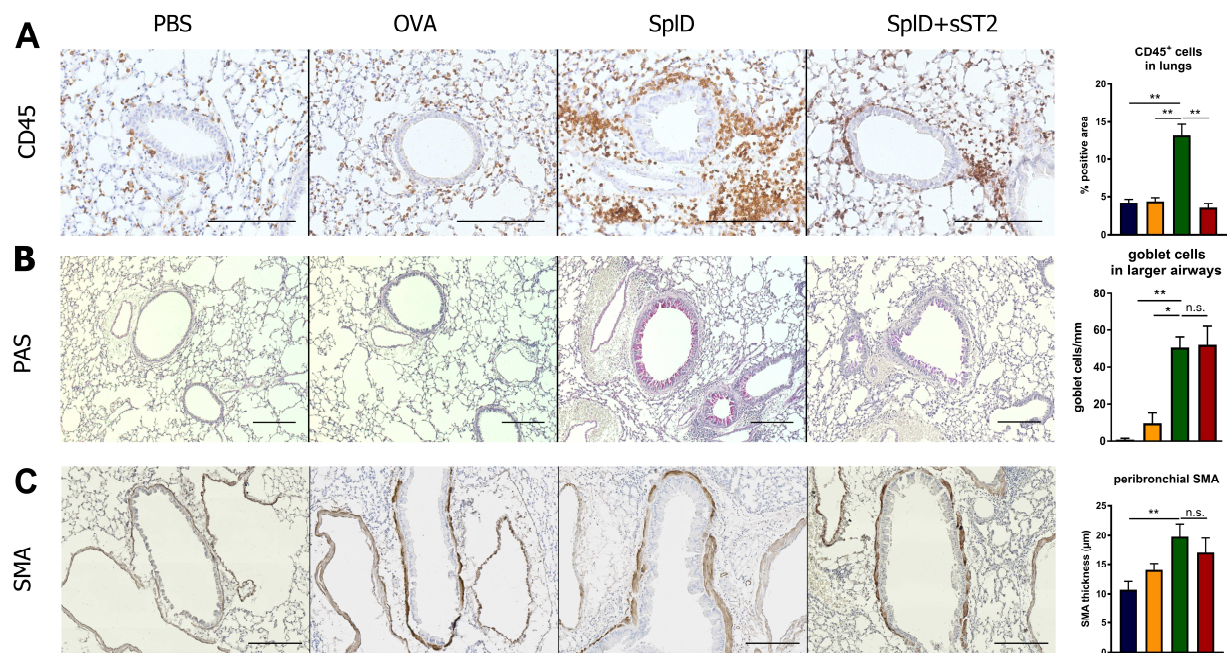


Figure 4

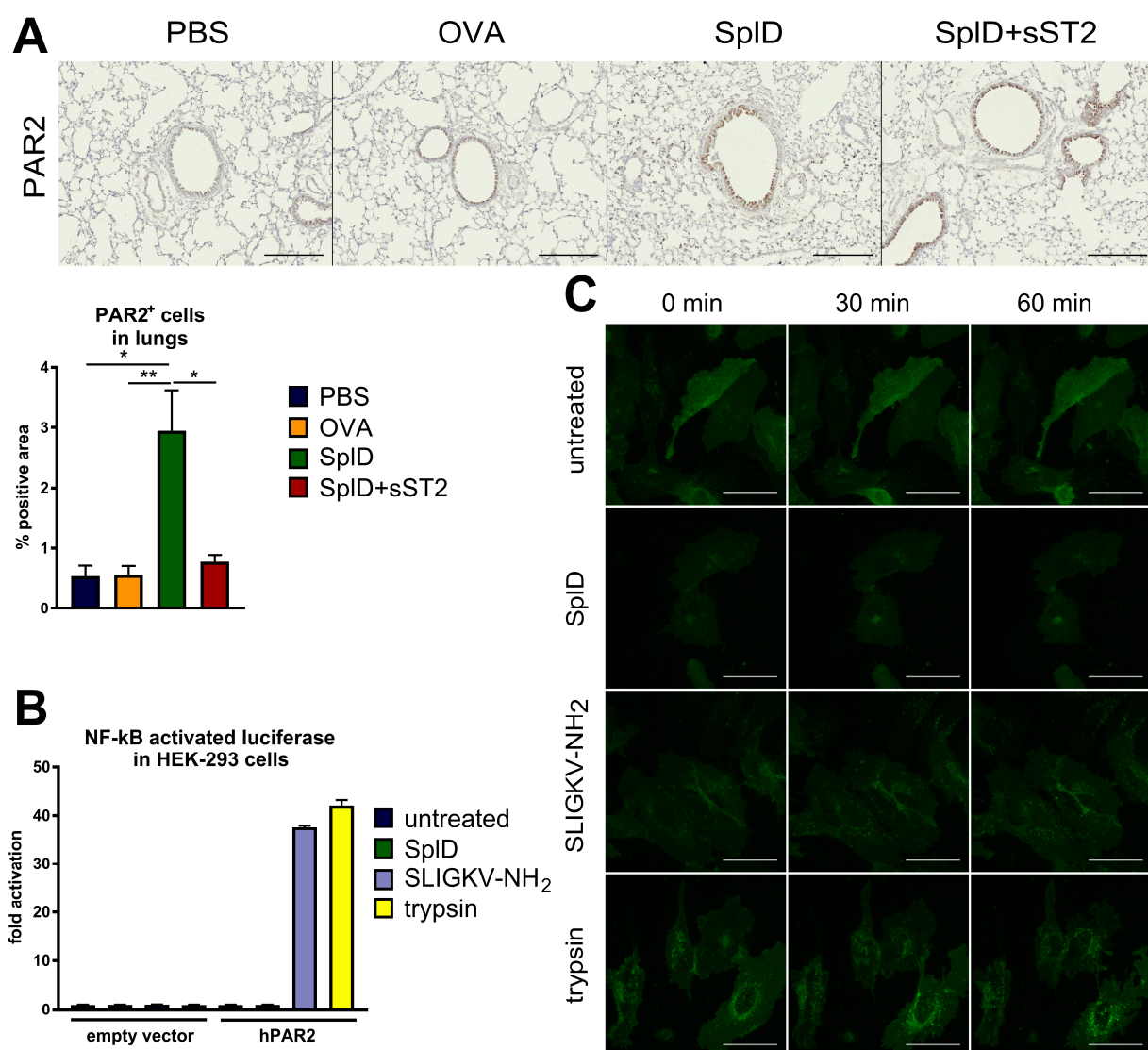


Figure 5

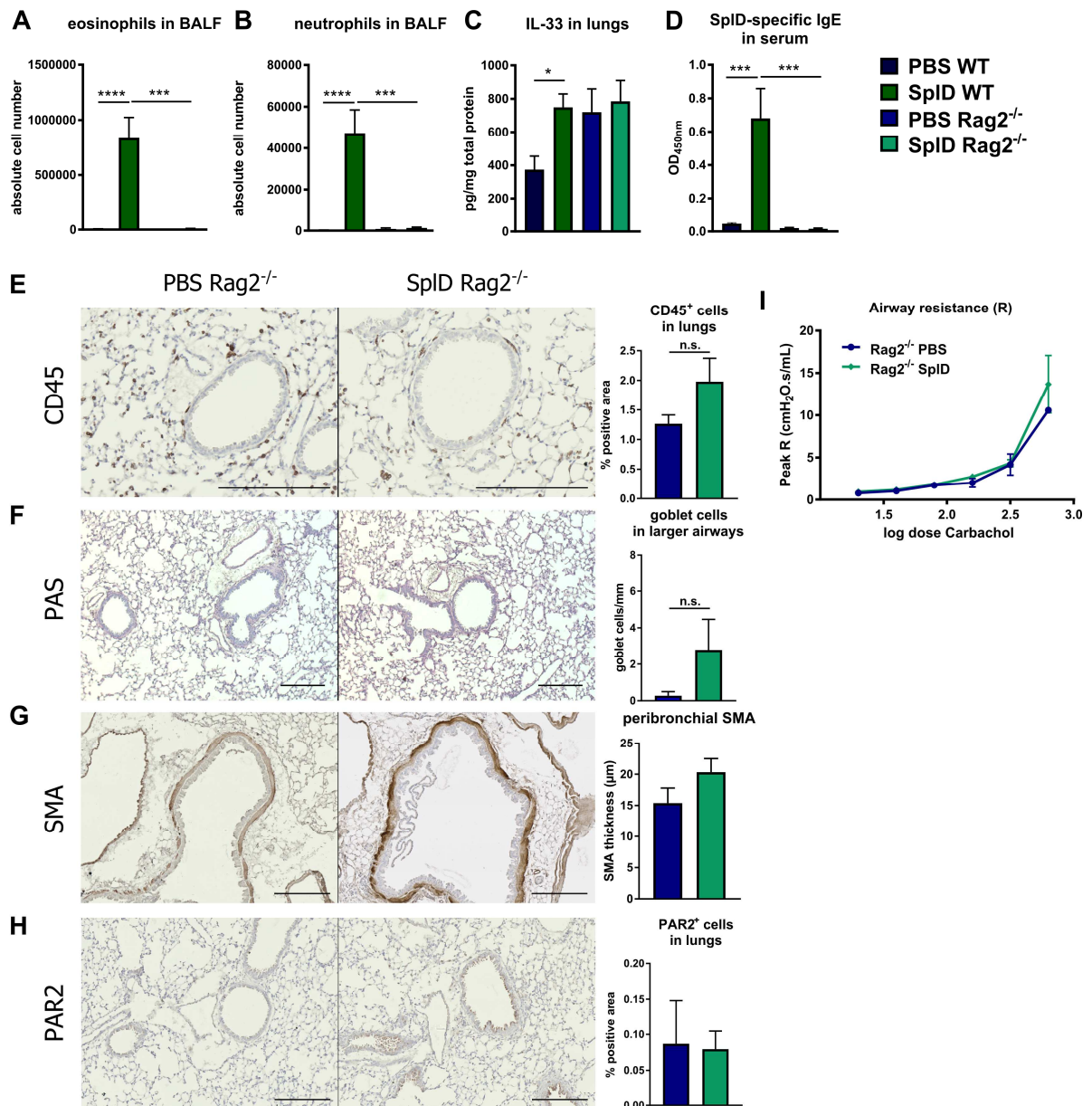


Figure 6

Repository methods**Recombinant protein production**

The recombinant SplD was produced in the *B. subtilis* strain 6051HGW LS8P-D, which lacks the proprietary proteases WprA, Epr, Bpr, NprB, NprE, Vpr, Mpr, AprE, as described in details before¹⁴. Shortly, cell-free supernatants were collected and subjected to tangential flow filtration to exchange the buffer with 20mM Tris/HCl (pH 7.5). SplD was purified by ion-exchange chromatography on a SP Sepharose Fast Flow column (GE Healthcare), followed by a two-step purification using centrifugal filter units (Amicon Ultra 30K/10K; Merck Millipore). Thereby the buffer was exchanged with PBS. The quality of the native SplD preparation was verified by SDS-PAGE. The recombinant murine sST2 was generated and purified with standard chromatographic procedures from sST2-His transfected HEK- 293 cells at the VIB Protein Service Facility, VIB Center for Inflammation Research (Ghent, Belgium).

Organ processing

Blood was collected in EDTA microvettes 200Z (Sarstedt, Nümbrecht, Germany), centrifuged for 10 min at 3000 rpm, serum was collected and stored at -20°C before further analysis. BALF was collected by rinsing the airways 3 times with 0.3 mL of 5% BSA (Sigma-Aldrich, Diegem, Belgium) in PBS with complete protease inhibitor cocktail (Roche Diagnostics, Anderlecht, Belgium) and 2 times with 1 mL of 0.2% EDTA (Sigma-Aldrich) in PBS and kept at 4°C. Red blood cells were lysed for 2 min with VersaLyse buffer (Beckman-Coulter). Cells were used for FACS analysis. Lungs were perfused with 10 mL of 0.9% NaCl (Braun, Meslungen, Germany). The left lobe of the lung was fixed with 10% formalin (Sigma-Aldrich) for paraffin embedding, whereas the right lobes were either snap frozen or minced and digested with 1%

collagenase type II (Worthington) for FACS analysis. Snap frozen lungs were homogenized for 2 min at 50 oscillations per seconds with 10 times more w/v T-Per Tissue Protein Extraction reagent (Thermo Fisher Scientific, Waltham, MA, USA) with 1x HALT protease inhibitor cocktail kit (Thermo Fisher Scientific) using the TissueLyser LT (Qiagen, Antwerp, Belgium). After 10 min of centrifugation at 3000 rpm, supernatants were collected and repeatedly centrifuged at 15000 rpm for 3 min. Supernatants were collected as lung homogenates and protein concentration was measured by the Bio-Rad Protein assay (Bio-Rad, Temse, Belgium).

AECII sorting an in vitro stimulation with SplD

AECIIs were sorted from 3 naïve C57BL/6 WT mice as previously described⁶⁴. After sorting, cells were stimulated for 0, 1 and 5 h with 25µg/mL SplD in serum free IMDM medium (Gibco) and lysed with T-Per buffer (Thermo Fisher Scientific) containing 1x HALT protease inhibitor cocktail kit (Thermo Fisher Scientific). Protein concentration was measured by the Bio-Rad Protein assay (Bio-Rad).

Lymph node re-stimulation assay

Peribronchial lymph nodes were collected and put through a 70µm mesh. Red blood cells were lysed with VersaLyse buffer (Beckman-Coulter, Suarlée, Belgium) and lymph node cells were seeded in triplicates per condition at 2×10^5 cells/well in RPMI medium (Gibco) with 10% FBS (Gibco) and Pen/Strep (Gibco) in a 96 well plate. The wells were either uncoated or coated with 2 µg/mL purified anti-mouse CD3 (eBioscience) for 2 h prior to cell incubation. Purified anti-mouse CD28 (eBioscience) was added to the CD3 stimulated cells to have a final concentration of 2 µg/mL and unstimulated controls were filled up with RPMI medium to a final volume of 200

μL/well. Cells were incubated for 5 days at 37°C, 5% CO₂ and supernatants were used for IL-5 and IL-13 analysis.

Western blotting

25 μg protein of lung homogenates or lysed AECII cells per slot were loaded on a 15% TrisHCl gel, separated by SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad). For Western blot, mouse IL-33 antigen affinity-purified polyclonal goat IgG (dilution 1:300) (R&D Systems, Abingdon, UK) antibody and donkey anti-goat HRP (dilution 1:5000) (Santa Cruz, Heidelberg, Germany), as well as the rabbit anti-GAPDH antibody (dilution 1:10000) (Sigma-Aldrich) in combination with the donkey anti-rabbit HRP antibody (dilution 1:2000) (GE healthcare, Little Chalfont, UK) were used. For p-MLKL and MLKL Western blots, lungs were homogenized with addition of PhosStop (Roche Diagnostics) in the above mentioned lysis buffer. A cell lysate of necrotic TNFα-treated CT26 cells was used as a positive control. Anti-MLKL (phosphoS358) (dilution 1:2000) (Abcam) in combination with the donkey anti-rabbit HRP antibody (dilution 1:2000) (GE healthcare) and anti-MLKL clone 3H1 (dilution 1:1000) (Merck Millipore, Billerica, MA, USA) in a combination with the anti-rat IgG, HRP linked antibody (dilution 1:2000) (Cell Signaling Technology, Leiden, The Netherlands) were used. Bands were visualized using the Immobilon Western Chemiluminescence HRP substrate (Merck Millipore). 4 mice were tested per group for the lung homogenates and 3 for AECIIs, one representative series is shown. Semi quantitative analysis of band intensities was performed by measuring the area under the peak of plotted lanes with the ImageJ software.

Immunofluorescence staining

Formalin fixed and paraffin embedded lung tissues were cut at 5 μ m sections. Sections were deparaffinised, non-specific binding sites were blocked with 3% normal horse serum (Vector Laboratories, Peterborough, UK) in PBS. The sections were incubated over night at 4°C with the following primary antibodies: mouse IL-33, (dilution 1:50) (R&D Systems) in combination with anti-prosurfactant protein C (proSP-C) antibody (1:1000) (Merck Millipore). As secondary antibodies, donkey anti-goat Alexa 488 (1:750) antibody (Invitrogen, Thermo Fisher Scientific) was used in combination with donkey anti-rabbit Alexa 594 (1:750) (Invitrogen). Slides were mounted with Vectashield Mounting medium for Fluorescence with DAPI (Vector Laboratories). Images were obtained with a Zeiss Axioplan 2 confocal microscope.

Immunohistochemistry

Formalin fixed and paraffin embedded lung tissues were cut at 5 μ m sections. Sections were deparaffinised and rehydrated and antigen retrieval with citrate buffer (2 mM citric acid and 10 mM sodium citrate, Sigma-Aldrich), peroxidase blockage with 3% H₂O₂ (Sigma-Aldrich) and blockage of non-specific binding sites with 3% goat serum (Sigma-Aldrich) in PBS with 0.4% Triton-X 100 (Sigma-Aldrich) was performed. Slides were either stained with anti-PAR2 antibody (dilution 1:100) (Abcam) in combination with goat anti-rabbit HRP (1:100), with anti-smooth muscle actin (1:3000 dilution) (Abcam) in combination with goat anti-rabbit HRP (1:200) or with purified rat anti-mouse CD45 (dilution 1:100) (BD Biosciences) in combination with biotin goat anti-rat Ig (dilution 1:500) (BD Bioscience). The CD45 staining signal was amplified using the Vectastain elite ABC kit (Vector Laboratories). All sections were developed with DAB+ Substrate chromogen system (Dako, Glostrup, Denmark) and counterstained with hematoxylin (Sigma-Aldrich), dehydrated and mounted with Pertex (Histolab, Gothenburg, Sweden). Images were taken with an

AxioScanZ.1 (Zeiss, Germany) using a 20X Plan-Apochromat 0.8 NA dry objective, captured by a Hitachi HV-F202SCL camera. The percentage of CD45 and PAR2 positive area of lung sections were quantified using the trainable WEKA segmentation tool of the Fiji software. Airway smooth muscle thickness was calculated as the radius of the SMA⁺ stained peribronchial smooth muscle area.

Toluidine blue staining

Lung sections were deparaffinised, rehydrated and stained for 15 min with 0.1 % Toluidine Blue (Sigma-Aldrich) in 7% ethanol and 1% NaCl, pH 2.3, followed by dehydration with 100% ethanol and mounting with Pertex (Histolab, Gothenburg, Sweden). Mast cell numbers were quantified in 5 non-adjacent lung sections per mouse (n=7). Images were acquired with an AxioScanZ.1 (Zeiss, Germany) using a 20X Plan-Apochromat 0.8 NA dry objective, captured by a Hitachi HV-F202SCL camera.

Periodic acid-Schiff staining

Deparaffinised and rehydrated lung sections were stained for mucus producing goblet cells, using the periodic acid-Schiff (PAS) kit (Sigma-Aldrich). The magenta stained goblet cells were counted in airways of a perimeter of 800-2000 μ m, using the Aviso software.

TUNEL staining

Deparaffinised and rehydrated lung sections were stained using the TUNEL Apoptosis Detection Kit (Merck Millipore) according to the manufacturer's manual.

As a positive control, 200 µg/mL DNase I (Qiagen) in 1 mg/mL BSA, TrisHCl pH 7.5 was applied for 10 min after the endogenous peroxidase-quenching step.

SplD-, SplF- and OVA-specific IgE analysis

Sera of mice were taken 48 hours after the last SplD application and the titers of SplD specific IgE were performed using an ELISA as described before¹⁴. For OVA or SplF-specific IgE measurements, 5 µg/mL OVA or SplF were used to coat the plates, otherwise the assay was performed in the same way as for SplD-specific IgE.

Hek-Blue hTLR2 cell stimulation assay

Hek-Blue hTLR2 (InvivoGen, San Diego, CA, USA) are TLR2 reporter cells that produce secreted embryonic alkaline phosphatase (SEAP) upon TLR2 activation via NF-κB. Hek-Blue hTLR2 cells were stimulated with 50 µg/mL, 25 µg/mL, or 5 µg/mL SplD at 37°C, 5% CO₂, overnight. As a positive control, peptidoglycan (PGN) was added at 1 µg/mL. Cell supernatants were diluted 1 in 10 with Quanti Blue (Invivogen, San Diego, CA, USA) according to the supplier's manual and the SEAP activity was measured at 620 nm after 24 h. Untreated cell supernatants were used as negative control. All conditions were measured in duplicates and the experiment was repeated three times.

Generation of PAR2-reporter HEK293T cells

Cells were seeded one day before transfection at a density of 4×10^4 cells/well in 1 mL DMEM, 10% FCS medium in 24 well plates. Cells were transfected by calcium-phosphate precipitation with an NF-κB inducible luciferase reporter plasmid, a β-galactosidase reporter for normalization of the luciferase values and a human PAR2 expression vector. For control transfections without PAR2 the total amount of DNA

was equalized with empty vector DNA. After 4h of incubation at 37°C and 5% CO₂, the medium was replaced by fresh DMEM with 10%FCS. 24h after the transfection, cells were used for the PAR2 activation assay.

PAR2 activation assay using HEK293 PAR2 reporter cells

The medium was replaced to serum free DMEM and cells were stimulated for 10 h in quadruplicates with 25µg/mL SplD, 50µM SLIGKV-NH₂ (Sigma-Aldrich), 1µM trypsin (Gibco) or were left untreated. After lysis of the cells in 200 µL luciferase lysis buffer (25 mM Tris-phosphate pH 7.8, 2 mM dithiothreitol, 2 mM 1,2-cyclohexanediaminetetraacetic acid, 10% glycerol, 1% Triton X-100), luciferase and β-galactosidase activities were measured. To correct for differences in transfection efficiencies, the obtained luciferase values were normalized with the corresponding β-galactosidase values.

Generation of PAR2-EGFP expressing HeLa cells and PAR2 receptor translocation assay

HeLa cells were transfected with a human PAR2-EGFP fusion construct and stimulated with 25µg/mL SplD, 100µM SLIGKV-NH₂ (Sigma-Aldrich), 1µM trypsin or were left untreated. The simulated cells were imaged every 2.5 minutes with the Zeiss Spinning Disk microscope for 1 h. The experiment was repeated three times. Representative images at 0, 30, 60 min of each experimental condition are shown.

Flow cytometry

Anti-mouse CD3-PE, CD4-FITC, CD11b-PerCP-Cy5.5, purified CD16/32, CD25-APC, Gr-1-FITC, MHCII-FITC, F4/80-APC, CD25-PECy7, CD90.2-APC, CD5-PerCP Cy5, NK1.1-PerCP Cy5, TCRβ-PerCP Cy5, CD11c-APCeFluo780, CD11b-

APCeFluo780, CD45R-APCeFluo780, CD3 AF700, CD45-BV421, IL-13-PE and IL-5-PE were purchased from (eBioscience) and CD11c-PE-Cy7 (BD Biosciences), Siglec F-PE (Miltenyi Biosciences) was used to stain mouse BAL and lungs. Secondary antibody HRP-APC-Cy7 was purchased from (eBioscience). Cells were stained for 30 min at room temperature, washed with PBS, and LIVE/DEAD[®] Fixable Aqua dead cell stain, LIVE/DEAD[®] Fixable Near-IR dead cell stain (Invitrogen) or 7-AAD Viability Staining Solution (eBioscience) were used for 10 min to exclude dead cells. Cells were washed and analysed by flow cytometry using FACS Canto II (BD Biosciences). For the intracellular IL-5 and IL-13 staining, BALF cells were stimulated with the cell stimulation cocktail (plus protein transport inhibitors) (eBioscience) for 4 h at 37°C, 5% CO₂, fixed and permeabilized with the intracellular fixation and permeabilization buffer set (eBioscience).

Mast cell degranulation assay

Bone marrow derived murine mast cells were generated from C57Bl/6 J mice, n=4, according to the protocol of Vukman et al. The mast cell growth medium was enhanced by 1 ng/mL recombinant IL-3 (PeproTech, London, UK). Mast cells were grown for 4 weeks and analyzed for c-kit and FcεRI and expression by flow cytometry (c-kit-PE, FcεRI-PE-Cy7, both from eBioscience). Cells were used for the experiments at a purity of >90 % c-kit⁺ and FcεRI⁺ cells. Experiments were repeated three times. Mast cell degranulation was tested by a beta-hexosaminidase assay using the protocol of Ali et al. with some modifications: 2.4x10⁵ cells/well were plated in 96-well plates and pre-incubated over night with 1 µg/mL anti-DNP IgE (Sigma-Aldrich) and stimulated for 1 h with 200 ng/mL DNP-HSA (Sigma-Aldrich). Cell pellets were lysed in 1% hexadecyltrimethylammonium bromide (Sigma-Aldrich) with 20 mM EDTA for 10 min at RT, incubated with p-nitrophenyl-n-acetyl-β-d-

glucosaminid (Sigma-Aldrich) for 1.5 h at RT. The reaction was quenched with 100 μ L 2M NaOH and the absorbance was measured at 405 nm.

Repository figures

Figure E1: IL-33 upregulation upon SplD exposure is cell death independent. **A**, detection of phosphorylated MLKL (p-MLKL) and unphosphorylated MLKL (MLKL) by Western blot. Necrotic TNF α -treated CT26 cells lysate served as positive control. **B**, TUNEL stained lung sections of SplD-treated mice. The DNase I treated lung section on the right served as positive control. Scale bar represents 200 μ m.

Figure E2: Possible interplay of SplD with the surface receptors TLR2 or TLR4. **A**, Secreted embryonic alkaline phosphatase (SEAP) activity of the TLR2 reporter cell line (Hek-Blue hTLR2) after overnight stimulation with different concentrations of SplD or peptidoglycan (PGN). Values were measured in duplicates and the experiment was repeated three times. **B**, total cell count and **C-E**, flow cytometric analysis of eosinophils, neutrophils and CD4⁺ T cells in the BALF and **F**, eosinophils in the lungs. **G**, IL-33 measurement in lung homogenates by Luminex. **H**, SplD-specific IgE in serum, measured by ELISA of SplD-treated TLR4^{-/-} mice in comparison to the results of SplD-treated wild type mice. Results are presented as means $\pm$ SEMs. n=4-11. *P<0.05 are determined with Mann-Whitney U test.

Figure E3: SplF induced airway inflammation. **A**, total cell count, **B**, eosinophils, **C**, neutrophils and **D**, CD4⁺ T cells in the BALF. **E**, eosinophils (CD11c⁻ SiglecF⁺CD11b⁺) and **F**, neutrophils (GR1⁺ CD11b⁺F4/80⁺) in the lungs, measured by flow cytometry. **G**, IL-33 and **H**, eotaxin measured in lung homogenates by Luminex. **I**, SplF-specific IgE in serum of PBS or SplF-treated mice. **J**, representative images of anti-CD45, Periodic acid-Schiff (PAS), anti-smooth muscle actin (SMA) and anti-PAR2 stainings of lung sections of SplF treated mice.

Figure E4: Representative dot plots and gating strategy of ILCs positive for intracellular IL-5 or IL-13 in the BALF of wild type C57BL/6 J mice repeatedly exposed to 45µg SplD. ILC2s were identified as Lin⁻ (CD3⁻, CD5⁻, NK1.1⁻, TCRβ⁻, CD11c⁻, CD11b⁻, and CD45R⁻), CD90⁺ cells. FSC, Forward scatter; SSC, side scatter; IL, Interleukin-5 or 13; Iso, isotype control.

Figure E5: A, mast cell quantification of toluidine blue stained lungs. n=5-13. Mast cell numbers are expressed per area of the section. B, beta-hexosaminidase assay of bone marrow derived mast cells from C57BL/6 J mice to test the mast cell degranulation. For B, results are presented as means ±SEMs. n=4-11. *P<0.05 are determined with Mann-Whitney U test.

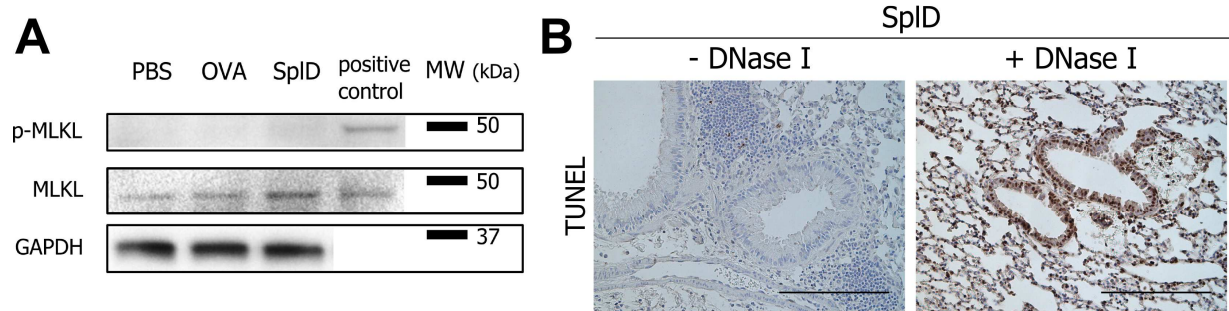


Figure E1

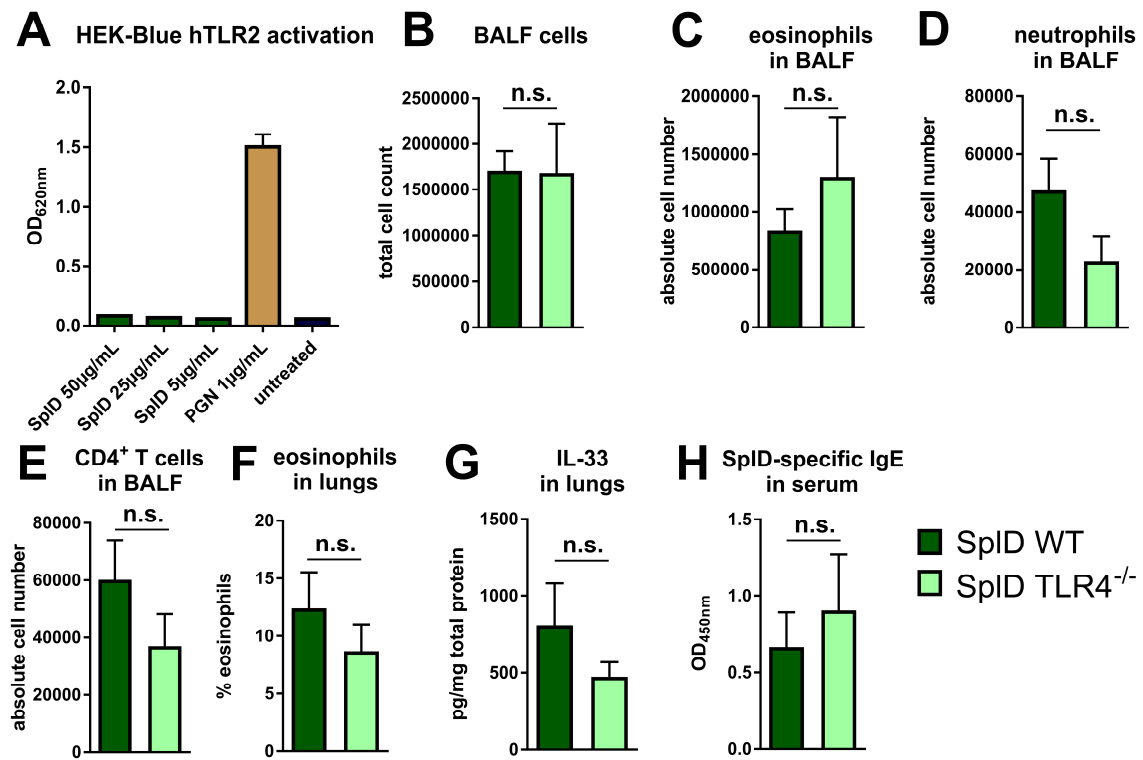


Figure E2

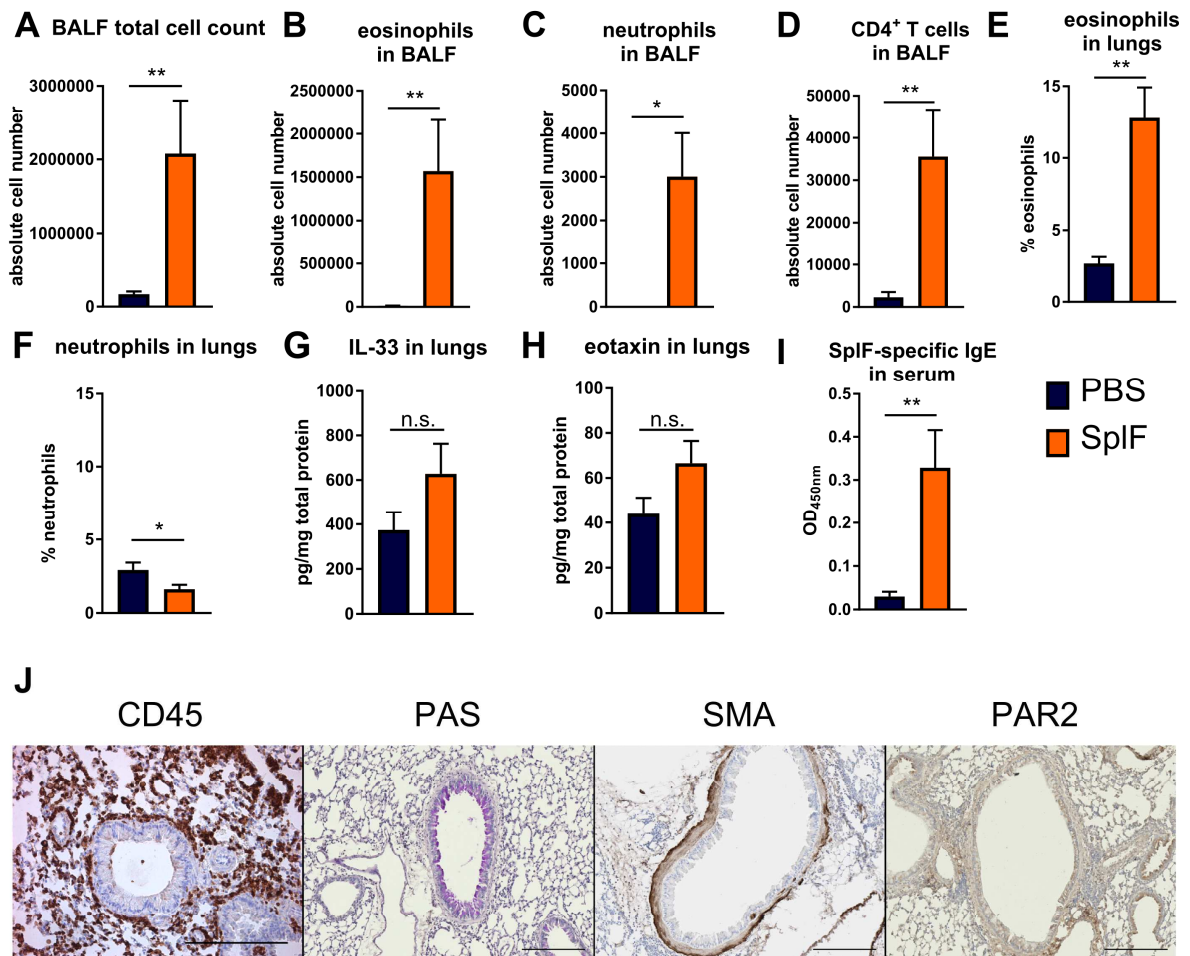


Figure E3

gating strategy for ILCs in the BALF

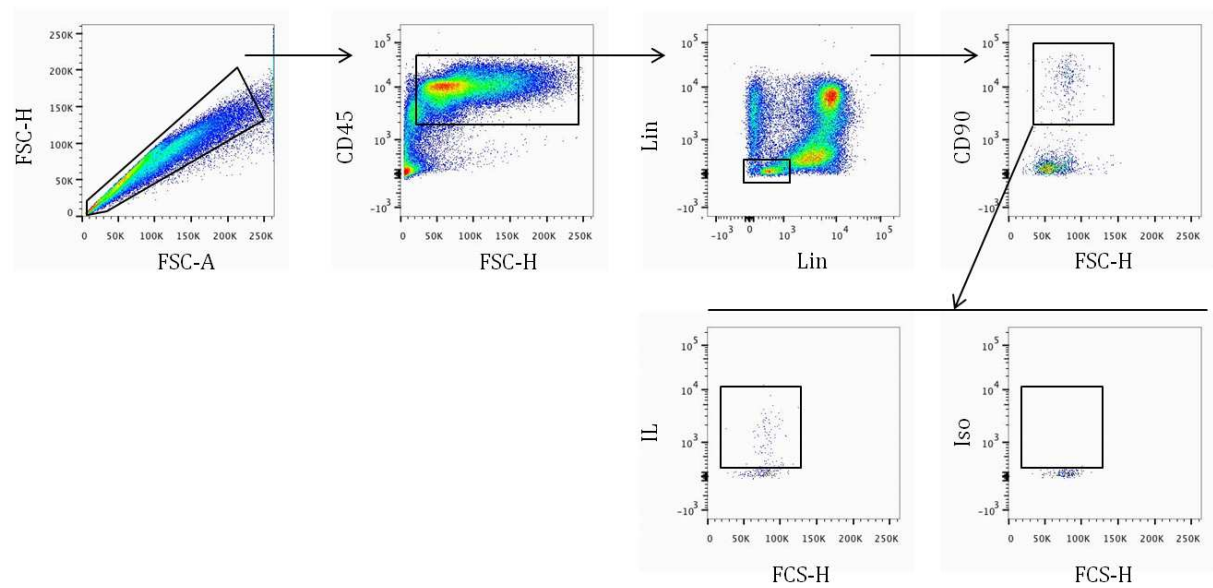


Figure E4

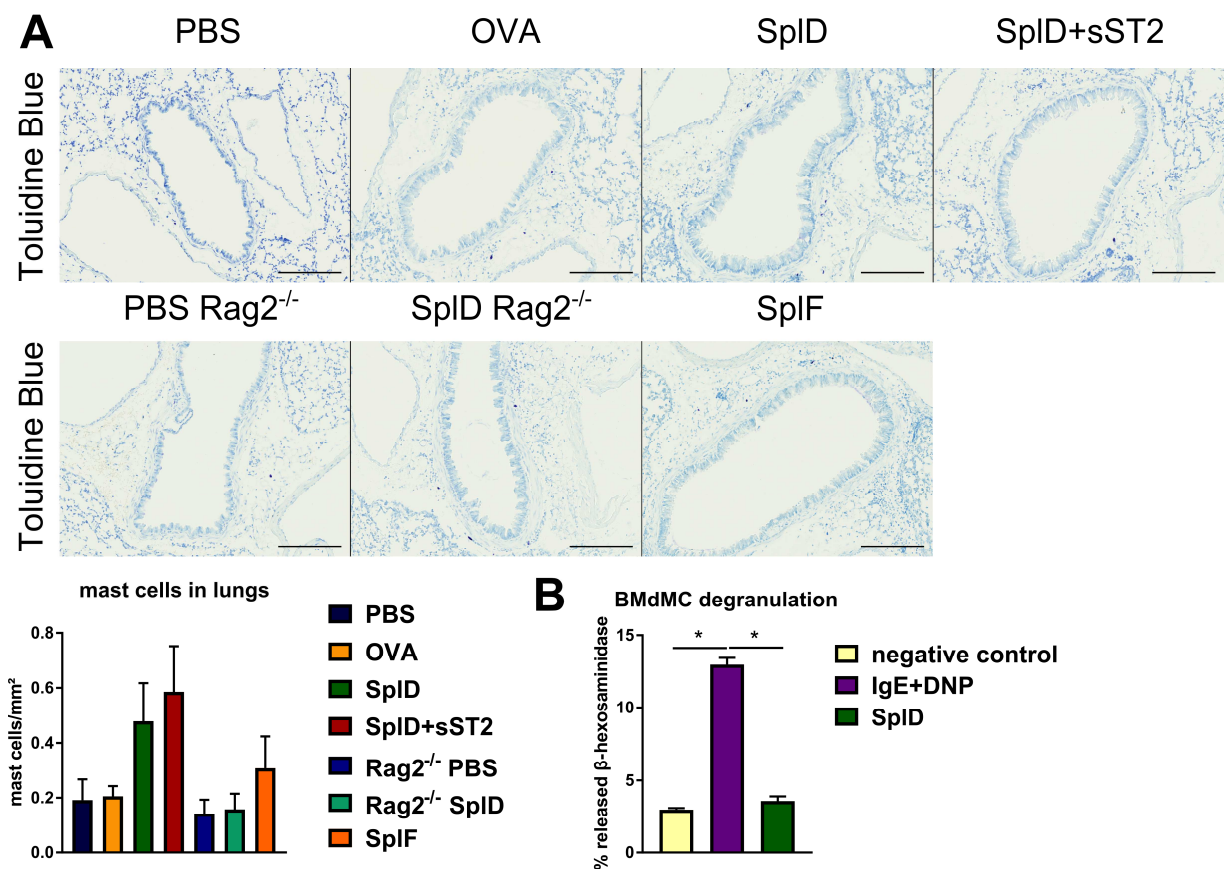


Figure E5