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Acid sphingomyelinase regulates T_H2-cytokine release and bronchial asthma

ASM-a key regulator of asthma

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Abstract

Background: Allergic diseases and especially allergic asthma are widespread diseases with high prevalence in childhood, but also in adults. Acid sphingomyelinase (ASM) is a key regulator of the sphingolipid pathway. Previous studies defined the association of ASM with the pathogenesis of T_H1-directed lung diseases like cystic fibrosis and acute lung injury. Here, we define the role of ASM in T_H2-regulated allergic bronchial asthma.

Methods: To determine the role of Asm under baseline conditions, wildtype (WT) and Asm^{-/-}-mice were ventilated with a flexiVent setup and bronchial hyperresponsiveness was determined using acetylcholine. Flow cytometry and cytokine measurements in bronchoalveolar lavage fluid and lung tissue were followed by *in vitro* T_H2-differentiations with cells from WT and Asm^{-/-} mice and blockade of Asm with amitriptyline. As proof of principle, we conducted an ovalbumin-induced model of asthma in WT- and Asm-KO-mice.

Results: At baseline, Asm-KO-mice showed better lung mechanics, but unaltered bronchial hyperresponsiveness. Higher numbers of Asm^{-/-}-T-cells in bronchoalveolar lavage fluid released lower levels of IL-4 and IL-5 and these results were paralleled by decreased production of typical T_H2-cytokines in Asm^{-/-}-T-lymphocytes *in vitro*. This phenotype could be imitated by incubation of T-cells with amitriptyline. In the ovalbumin asthma model, Asm^{-/-}-animals were protected from high disease activity and showed better lung functions and lower levels of eosinophils and T_H2-cytokines.

Conclusion: Asm-deficiency could induce higher numbers of Th2-cells in the lung, but those cells release decreased T_H2-cytokine levels. Hereby, Asm^{-/-}-animals are protected from bronchial asthma, which possibly offers novel therapeutic strategies, e.g. with ASM-blockade.

Key words:

Acid sphingomyelinase, allergic disease, asthmatic response, T-cells, T_H2

Introduction

Allergic diseases represent significant global health problems with dramatically increasing morbidity during the last decades. Allergies can affect people of all ages, however the prevalence in childhood is up to 39% ^{1,2}. Allergic asthma is characterized by airway inflammation, recurrent bronchospasms, airway hyperresponsiveness (AHR) and mucus hypersecretion ³. Therapies are largely symptomatic and only a small number of medications are available ⁴.

It is well known that sphingolipids, such as sphingosine-1-phosphate, play a crucial role in pathogenesis of bronchial asthma and corresponding airway hyperresponsiveness ^{5,6}. Sphingolipids function as structural elements of the cell membrane and as component of lipoproteins and are increasingly recognized as critical mediators in major pulmonary diseases, such as asthma, acute lung injury, cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD) ⁷⁻¹². Additionally, ORMDL3 (orosomucoid-like 3) is known as protein that regulates sphingolipid synthesis and hereby probably also asthmatic disease and smooth muscle cell contraction ^{13,14}. ASM is encoded by the sphingomyelin phosphodiesterase 1 (SMPD1) gene and localizes predominantly into lysosomes and to the outer membrane leaflet in lipid rafts, where they are involved as key enzymes in signaling processes. The hydrolysis of membrane sphingomyelin to ceramide by ASM leads to the formation of ceramide-rich platforms (CRPs) ¹⁵. Here, ceramide acts as a second messenger and organizes cellular processes and signaling events to allow differentiation, proliferation, apoptosis and inflammation ¹⁵⁻¹⁷.

In asthmatic patients, ceramide and sphingomyelin levels are increased in serum and sputum ¹⁸. Moreover, increased levels of S1P can be found in bronchoalveolar lavage fluid (BALF) of asthmatic patients after allergen provocation ¹⁹. Recently, in a murine 21-day ovalbumin-induced asthma model, Sopel *et al.* observed that Asm-deficiency reduced IgE serum levels and that it increased regulatory T- cells (T_{reg}), but they found no effect on physiological lung functions, such as airway hyperresponsiveness (AHR) and they did not study T_H2 -cells ²⁰. However, ASM interacts with the intracellular domains of CD3 and CD28 and mediates intracellular signals that control CD4⁺ T-cell activation by generating ceramide including those that are involved in CD4⁺ T-cell proliferation and activation. ²¹.

These findings raise the hypothesis that Asm is involved in the regulation of the typical T_H2-phenotype and that this is relevant for allergic lung dysfunctions in a typical 35-day asthma model. In untreated Asm^{-/-}-animals, we observed remarkable changes in the pulmonary leukocyte milieu, including increased amounts of T_H2-, T_H17- and Treg-lymphocytes as well as transforming growth factor (TGF)-β, but decreased levels of IL-4 and IL-5. *In vitro*, Asm-knockout or -blockade by amitriptyline strongly reduced the release of the T_H2-cytokine IL-4 from T-lymphocytes indicating that Asm is required for proper T_H2-responses of these cells. In ovalbumin (OVA)-induced asthma, Asm^{-/-}-animals were protected with a decreased T_H2-response and diminished AHR. Therefore, ASM-blockade may offer novel therapeutic strategies for the very large group of patients with allergic bronchial asthma.

Materials and Methods

For several chapters, additional details are provided in the online supplement.

Animals

Experiments were performed with 7 to 12 weeks old Asm^{-/-} (Smpd1^{-/-}) and WT (Smpd1^{+/+}) littermates (C57BL/6). Smpd1^{-/-} mice have been described previously²². The study was approved by the regional governmental authorities and animal procedures were performed according to the German animal protection law.

Experimental design of ovalbumin-sensitization

On day 0, 14 and 21 mice were injected i.p. with OVA and aluminum hydroxide (Alu). On day 28, 29 and 34, sensitized mice were exposed to nebulized OVA (1 %) for 30 min. Control animals received Alu and were exposed to NaCl (0.9 %). On day 35, mice were mechanically ventilated with the flexiVent ventilator (SCIREQ, Canada).

Cytokine measurements and RT-qPCR analysis

Murine IL-4, IL-5, IL-6, TGF-β and tumor necrosis factor (TNF)-α were analyzed with Multiplex immunoassay (Affymetrix/eBioscience) or ELISA kit (R&D Systems) in supernatants of BALF samples, incubated T-cells and lysed cells of *in vitro* stimulations according to manufacturer's

instructions. Gene expression levels of IL-4, IL-4R, GATA3 and ORMDL3 of PMA/ionomycin stimulated WT and *Asm*^{-/-}-T-cells were quantified by reverse transcription (RT)-qPCR analysis using CFX connect detection system (Bio-Rad, Germany). mRNA levels were normalized to the reference genes ribosomal protein s29 (Rps29) and succinate dehydrogenase complex flavoprotein subunit A (Sdha).

T-cell isolation and T-cell differentiation

Murine T-cells were isolated from splenocytes of untreated *Asm*^{-/-} and WT mice by magnetic activated cell sorting (MACS) separation using the CD4⁺ CD25⁺ Regulatory T-Cell Isolation Kit (Miltenyi, Germany). CD4⁺ CD25⁻ T-cells were incubated with plate coated anti-CD3 and anti-CD28. After 4 days, cell culture supernatants and lysed cells were used for ELISA analysis.

Stimulation and secretion analyses of T-cells

Murine T-cells were isolated from splenocytes of untreated *Asm*^{-/-} and WT-mice by MACS-separation using CD4 (L3T4) MicroBeads (Miltenyi, Germany). CD4⁺ T-cells were then incubated with ionomycin and PMA for 6 hours. Additionally, half of the CD4⁺ T-cells were incubated with GolgiPlug. Lysed cells and cell culture supernatants were used for ELISA measurements of IL-4 and CD4⁺ T-cells were analyzed by flow cytometry. Moreover, WT CD4⁺ T-cells were incubated with ionomycin, PMA and amitriptyline (50 μ M) for 6 hours or incubated with anti-CD3/anti-CD28 and amitriptyline (50 μ M) for 12 hours.

Asm-activity assay (immunofluorescence and radioactivity)

The Asm-activity in BALF, lung tissue and urine was determined using the fluorescent substrate BODIPY-FL-C12 sphingomyelin (N-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoyl) sphingosyl phosphocholine, Invitrogen, Germany) using conditions similar to those described by others^{23,24}. Additionally, Asm-activity was determined using radioactive ¹⁴C-labeled sphingomyelin as previously described in²⁵ using minor modifications.

Statistical analysis

For statistical analysis GraphPad Prism 5.0 (GraphPad Software) was used. For parametric data, we used a one- or two-tailed unpaired Student t test given that data were normally distributed. Nonparametric data were analyzed using Mann-Whitney test. For multiple testing, we conducted One-way ANOVA and p-values were adjusted with the Benjamini-Hochberg false discovery rate (FDR) correction. All data are presented as mean $\pm$ standard error (SEM). P-values < 0.05 were considered to be statistically significant (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$).

Results

Acid sphingomyelinase influences lung mechanics and cell influx in bronchoalveolar lavage

We first studied Asm-activity in lung tissue and in BALF using the non-radioactive, fluorometric Asm-activity assay (Fig. 1A) as well as the C^{14} radioactive activity assay (Fig. 1B). Urine was used as a well-known positive control with relevant levels of Asm-activity. Asm-activity was significantly higher in lung tissue than in BALF (Fig. 1A). As expected, Asm-activity was absent in $Asm^{-/-}$ -animals (Fig. 1B). In addition to well-known ASM-activity in immunological cells, we demonstrated ASM localization at the surface of bronchial epithelium (Supp. Fig. 1). All stained biopsies showed nearly the same result.

To address the pathophysiological significance of these findings for lung mechanics and lung homeostasis, we ventilated untreated WT- and $Asm^{-/-}$ -mice with a flexiVent ventilation setup (Fig. 1C-G). Total resistance of the airway (R_{rs}) and Newtonian resistance (R_n) as resistance of the larger airways tended to be lower in $Asm^{-/-}$ -mice compared to WT. Tissue resistance (G), total elastance (E_{rs}) and tissue elastance (H) were significantly decreased in Asm -KO-mice. Bronchial hyperresponsiveness was not different between WT- and Asm -deficient-animals (Supp. Tab. 1). In conclusion, Asm influences baseline respiratory mechanics, but not bronchial hyperresponsiveness.

When BALF of these animals was analyzed, $Asm^{-/-}$ -animals showed significantly higher numbers of CD45+ cells as unspecific marker of total immune cells (Fig. 2A). Numbers of neutrophils (CD45+CD11b+CD11c-SiglecF-F4/80-Ly6G+), T-lymphocytes (CD45+CD3+), monocytes (CD45+CD11b+CD11c-SiglecF-F4/80-Ly6G-) and dendritic cells (CD45+CD11b+CD11c+SiglecF-F4/80-) were up to 30-fold higher in BALF of Asm -KO-mice compared to WT-mice (Fig. 2B-E). Similar levels were observed in the population of alveolar macrophages (CD45+CD11b+CD11c-

SiglecF+F4/80+) (Fig. 2F). Regarding T-cell subpopulations in homeostasis, we detected significantly higher numbers of CD4⁺ T-cells in Asm-KO- than in WT-mice as well as 10-fold higher numbers of CD4⁺IL17⁺ (T_H17)-cells, more than 3-fold higher levels of CD4⁺GATA3⁺ (T_H2)-cells and around 10-fold higher numbers of CD4⁺CD25⁺FoxP3⁺ (T_{reg})-cells (Fig. 2G-J). We did not detect any CD4⁺IFN- γ cells as typical T_H1-cells in BALF of both groups (data not shown). Additionally, levels of IFN- γ measured by ELISA were below the detection limit (data not shown).

Acid sphingomyelinase-deficiency changes immune cell function *in vivo* and *in vitro*

Having shown that Asm-deficiency dramatically changes cell composition already in homeostasis including T_H2-cells in the lung, we next evaluated immune cell activity and especially T_H2-cell activity in Asm^{-/-} using cytokine ELISAs of BALF. Interestingly, levels of TNF- α were lower in Asm^{-/-} mice whereas IL-6 and TGF- β levels were much higher than in WT-animals correlating with stronger influx of neutrophils, macrophages/monocytes and dendritic cells (Fig. 3A-C). The T_H2-cytokines IL-4 and IL-5 were reduced by approximately 50% in BALF of Asm^{-/-} mice (Fig. 3D-E). Thus, surprisingly, higher numbers of T_H2-cells in BALF of Asm^{-/-} mice correlated inversely with lower levels of typical T_H2-cytokines in homeostasis.

Therefore, we hypothesized that T_H2-cells of Asm^{-/-} mice show a defect in cytokine production. We performed flow cytometry on CD4⁺ T-lymphocytes from spleens of Asm^{-/-} and WT-mice and found significantly higher numbers of CD4⁺GATA3⁺ T_H2-cells (Fig. 4A) in untreated Asm^{-/-}. Next, we isolated CD4⁺CD25⁻ T-lymphocytes and stimulated them with anti-CD3/anti-CD28 for 5 days, which further increased the number of T_H2-cells from Asm^{-/-} animals (Fig. 4B-C). Despite enhanced numbers of CD4⁺GATA3⁺ cells, but concordant with the results *in vivo*, levels of IL-4 were slightly lower in the supernatants of Asm^{-/-}-T-lymphocytes (Fig. 4D). In addition, IL-4 per 1000 T_H2-cells was significantly lower in Asm^{-/-}-T_H2-cells (Fig. 4E). Thus, apparently CD4⁺GATA3⁺ T_H2-cells of Asm^{-/-} animals secrete much lower levels of IL-4 after stimulation. We next stimulated these cells with ionomycin and PMA to trigger cytokine secretion in the presence or absence of GolgiPlug, to inhibit protein transport from Golgi. Asm^{-/-}-cells secreted significantly lower levels of IL-4 than WT-cells; additional incubation with GolgiPlug inhibited IL-4 secretion in both genotypes, however this did not statistically differ in Asm^{-/-}-cells (Fig. 4F-G). Regarding IL-4 levels of lysed cells, we detected lower levels of IL-4 in Asm^{-/-}-cells

compared to WT after stimulation with ionomycin/PMA. Blocking secretion by GolgiPlug led to the expected accumulation of IL-4 in WT-cells. In *Asm*^{-/-}-T-cells, levels of IL-4 were nearly the same in ionomycin/PMA stimulated cells and in those with additional Golgi block (Fig. 4G). Next, we assessed IL-4-gene expression levels in ionomycin/PMA-treated T-cells and we observed, in line with the lower secretion, dramatically reduced IL-4 expression in *Asm*^{-/-} compared to WT (Fig. 4H). In contrast, IL-4R (CD124) was upregulated in ionomycin/PMA stimulated *Asm*^{-/-}-T-cells (Fig. 4I). GATA3 gene expression levels were nearly the same as well as p-STAT6 levels in both groups (Fig. 4J-K). Additionally, we found only a slight difference in ORMDL3 levels (Fig. 4L). Treatment of WT T-cells with ionomycin/PMA or anti-CD3/anti-CD28 and amitriptyline mirrored the phenotype of *Asm*^{-/-}-cells. IL-4 in supernatants of amitriptyline treated probes was reduced in both experimental setups (Fig. 4M-N). From the literature it is known that ionomycin/PMA induces apoptosis, which we could also demonstrate in our experiments (Fig. 4O)^{26,27}. Stimulation with ionomycin/PMA in combination with amitriptyline led to a slightly increased apoptosis of CD4⁺ T-cells compared to exclusive stimulation with ionomycin/PMA. This increased rate of apoptosis was not found in the stimulation with anti-CD3/anti-CD28 and amitriptyline (Fig. 4P), which excludes an apoptotic effect of amitriptyline itself. Therefore, production and secretion of T_H2-cytokines seems to be markedly impaired in *Asm*^{-/-} as well as in amitriptyline treated T-cells independent of apoptosis.

Acid sphingomyelinase-deficiency is protective in a model of bronchial asthma

This strong difference of T_H2-cytokine secretion/production led us hypothesize that *Asm*-deficiency is protective in allergic asthma. Therefore, as proof-of-principle *Asm*^{-/-} and appropriate WT-mice were sensitized with OVA and subsequently lung function measurements were performed on day 35. First, OVA-sensitization itself did not change *Asm*-activity compared to controls (Fig. 5A). Next, we monitored weight of our animals, because ASM inhibitors can cause weight gain; however, WT and *Asm*^{-/-}-animals showed no difference in weight gain over the time (Fig. 5B). We ventilated OVA-sensitized mice with the flexiVent setup. Lung resistance parameters of *Asm*^{-/-}-animals and controls were almost the same, but elastance and tissue elastance differed significantly (data not shown). After Ach-inhalation to provoke bronchial hyperresponsiveness we demonstrated clear differences in resistance parameters but even stronger changes, a reduction by about 50 %, in elastance and tissue

elastance in $Asm^{-/-}$ compared to WT-animals (Fig. 5C-G). Therefore, Asm -deficiency is clearly protective for lung mechanics in this murine model of bronchial asthma.

To confirm the relevance of immune cells, especially T-cells, for the AHR reduction in sensitized $Asm^{-/-}$ -animals, we analyzed airway responses *ex vivo* using PCLS, which lack mobile immune cells (Fig. 5H-I). Airways from sensitized WT- as well as from $Asm^{-/-}$ -animals showed only moderate bronchoconstriction in response to OVA. Additionally, Alu controls exhibited no differences between WT- and $Asm^{-/-}$ -animals in the PCLS samples (data not shown).

Having shown that Asm -deficiency improves AHR in sensitized animals only in the presence of immune cells, we next analyzed the BALF of OVA-treated mice. Cells counts in BALF of sensitized mice showed similar numbers of CD45+ cells as well as neutrophils, monocytes, dendritic cells and T-lymphocytes (Fig. 6A-E); even T-cell subpopulations were similar in both groups (exemplarily T_H17 and T_{reg} cells as Supplemental Fig. 2A-B). Levels of T_H1 cells were very low in both asthma groups (Supp. Fig. 2C). Additionally, levels of IFN- γ were comparable in both groups with low total levels around detection level (Supp. Fig. 2D). In other words, WT-mice exhibited a stronger upregulation of inflammatory cells into the BALF compared to $Asm^{-/-}$ -animals (exemplarily shown for T-lymphocytes, Fig. 6F). Only, the number of eosinophils differed significantly, with lower numbers in sensitized $Asm^{-/-}$ -animals than in WT (Fig. 6G), which corresponded to lower levels of IL-4 and IL-5 in BALF of sensitized $Asm^{-/-}$ (Fig. 6H-I). Both cytokines serve as chemoattractants for eosinophils. IL-6 levels were slightly (but not significantly) higher in $Asm^{-/-}$ BALF and TNF- α levels were nearly the same (Fig. 6J-K). TGF- β levels were clearly increased in $Asm^{-/-}$ -animals in both groups, Alu and OVA (Fig. 6L). Thus, Asm -deficient-mice were protected from upregulation of T_H2 -cytokines upon OVA-stimulation and showed a reduced immune cell influx.

Discussion

Here we provide evidence that ASM acts as modulator of the adaptive immune system and is a critical regulator of asthmatic inflammation by influencing T_H2-responses *in vitro and in vivo*. Normally, during asthmatic reactions, T_H2-cells secrete typical cytokines such as IL-4, IL-5 and IL-13, that then induce the influx of eosinophils and cause bronchial hyperresponsiveness and muscle hypertrophy. In Asm^{-/-}-mice, T-cells differentiate into T_H2-cells in an OVA-sensitization and provocation model, but they do not have the ability to produce and secrete typical T_H2-cytokines. This results in an improved lung function of asthmatic Asm^{-/-}-animals. Of note, even under baseline conditions lungs from Asm-KO-mice lungs are more compliant and airways are more patent; in addition, Asm^{-/-}-T-cells release lower levels of IL-4 and IL-5 *in vivo and in vitro*, also upon stimulation with ionomycin/PMA. Therefore, Asm may offer a novel therapeutic target in bronchial asthma.

Asm is well-investigated in CF and acute lung infections, such as RSV bronchiolitis and bacterial infections^{28,29}, but the role of ASM in T_H2-disease had not yet been studied in detail. CF epithelial cells and macrophages of mice and human CF-patients show an accumulation of ceramide, which leads to activation of the inflammasome³⁰. In CF-mice and human patients, ceramide accumulation in different immune cells and epithelial cells also raises the susceptibility to infection, e.g. *Pseudomonas aeruginosa*. As new therapeutic approach, the ASM-inhibitor amitriptyline was administered in phase II randomised, double-blind, placebo-controlled studies of CF-patients^{31,32}. It significantly increased FEV1 and reduced ceramide in lung cells after treatment with 25 mg amitriptyline twice daily. Therefore, ASM-inhibitors seem to be a new therapeutic strategy in CF and they may as well be in bronchial asthma.

In 2017, Yoshida *et al.* described the elevation of serum ASM in respiratory tract infections, i.e. RSV bronchiolitis. In that study, Asm was not elevated in the control groups with asthma or adenovirus infections¹¹. This is in line with our results demonstrating that asthmatic sensitization and provocation itself does not alter Asm-activity, but in contrast to the results by Sopel *et al.*²⁰. Yang *et al.* stimulated bone marrow-derived mast cells of WT-mice *in vitro* with antigens, which resulted in an activation of Asm, while Asm-inhibition via amitriptyline decreased antigen induced Ca²⁺-influx and Asm-activity. After induction of systemic anaphylaxis, disease activity was less pronounced in Asm^{-/-}-animals compared to WT-littermates³³. Gupta *et al.* demonstrated higher ASM-levels and stronger activity in erythrocyte membranes of asthmatics compared to healthy controls³⁴. Thus, the expression of Asm in

asthma may be cell- and time-dependent. Importantly, however, our data show that Asm-inhibition is effective regardless of whether Asm is upregulated or not.

This is the first study to focus on the role of Asm in T_H2-directed disease. With high numbers of eosinophils in bronchoalveolar lavage, we show a typical eosinophilic asthma. Although levels of neutrophils were higher in untreated Asm^{-/-}-mice, these animals did not develop neutrophilic asthma. A related study had shown increased numbers of immunosuppressive T_{reg}-cells in a short term (21d) murine asthma model with Asm^{-/-} ²⁰, which we could show only as a tendency. Instead, we showed that ASM-deficiency reduces T_H2-responses such as IL-4 secretion also in T-cells *in vitro* and in the absence of regulatory T-cells, indicating that the role of Asm in T_H2- and T_{reg}-cells requires further study. Clearly, Asm is a key regulator in different immunological cell types.

In our study, levels of IL-4 and IL-5 were lower in Asm^{-/-}-T-cells *in vivo* and *in vitro* and we could mimic this phenotype by amitriptyline blockade. Additionally, absence and presence of GolgiPlug did not alter IL-4 levels in stimulated cells of Asm^{-/-} and WT demonstrating a problem in T_H2-cytokine production. We demonstrated dramatically reduced IL-4 gene expression in Asm^{-/-} after ionomycin/PMA independent of relevant changes in GATA3 and pSTAT6. We speculate that this IL-4-phenotype is primed during development of Asm^{-/-}-animals. IL-4-R may be either upregulated in Asm^{-/-}-T-cells because of ionomycin/PMA stimulation itself or in response to very low IL-4 levels as a means of compensation. Therefore, IL-4-production and subsequent secretion of IL-4 are downregulated in Asm^{-/-}-animals or after amitriptyline treatment and therefore T_H2-response is diminished with lower levels of eosinophils in asthmatic mice and better lung function parameters.

In previous studies it was demonstrated that ASM regulates pathogenic T_H1- and T_H17-differentiation and responses, while it enhances regulatory T-cell development and function ^{35,36}. In line with our data, naïve Asm^{-/-}-mice had higher numbers of systemic T_{reg}-cells. *In vitro* experiments showed that Asm-deficiency in CD4⁺ T-cells correlates with PI3K-Akt signaling, resulting in increased FoxP3 expression and thereby induction of T-cell differentiation ³⁶. Hollmann *et al.* demonstrated that the frequency of T_{regs}, turnover of the effector molecule CTLA-4 and their suppressive activity are upregulated in Asm^{-/-}-animals ³⁷. In our study, T_{regs} were more numerous already in untreated Asm-deficient-animals, while the numbers of T_{regs} were the same in BALF and lung tissue of WT- and Asm^{-/-}-animals after asthma induction. Therefore, altered numbers of T_{reg} alone are not sufficient to explain the observed protective phenotype in asthmatic disease.

ASM is localized in lysosomes but also localizes to the outer leaflet of the plasma membrane and serves to regulate and form ceramide-enriched membrane domains that cluster receptors and other signaling molecules to allow specific cell-stimulation³⁸. Especially, the outer membrane of T-lymphocytes contains high ASM-levels and ASM-activity³⁹. Vice versa, *Asm*^{-/-}-T-cells show 7-10 fold higher levels of sphingomyelin in their surface membrane. In addition, several studies demonstrated, that ASM physically interacts with cell surface receptors⁴⁰, but up to now it has not been clearly shown whether these receptors activate ASM or whether it is the other way around³⁹. We have shown before that, once generated by *Asm*, ceramide will accumulate at the plasma membrane to form ceramide-enriched membrane cluster, in particular within caveolae, leading to initiation of downstream signals^{41,42}. Especially in T-cells it is expected that ASM – amongst others – interacts with cytokine receptors and induces T-cell-differentiation, e.g. T_H2-differentiation⁴³. However, the exact mechanism by which pathways ASM participates in T-cell-receptor (TCR)/CD3 and CD28 signaling remains unknown^{40,44}. Bai *et al.* demonstrated that ASM interacts with the intracellular domains of both, CD3 and CD28²¹. Upon stimulation with antibodies against CD3 and CD28 ASM mediates intracellular downstream signaling pathways of both CD3 and CD28, including CD3-ZAP70-PLC-γ1-MAPK/JNK and CD28-PI3K-Akt-mTOR⁴⁵⁻⁴⁷. Other receptors and signaling pathways regulated by ASM-activity and increased ceramide production include STAT3 and mTOR signaling⁴⁸. First pilot experiments also demonstrated ASM localization at the surface of bronchial epithelium (Supp. Fig. 1). Therefore, in addition to the immunological phenotype ASM seems to regulate function of the epithelium, which clearly interacts with immune cells especially in the lung. Further studies have to uncover mechanisms of ASM-dependent interaction of the local immune system in the lung and lung epithelial cells.

In general, ASM-deficiency leads to decreased levels of ceramide. In line with our results that *Asm*-deficiency impairs asthmatic symptoms in mice, Spiegel *et al.* demonstrated that higher ceramide levels promote stronger chronic airway inflammation, airway hyperresponsiveness and mucus production. The immunosuppressant drug FTY720/fingolimod reduced ORMDL3 expression and hereby ceramide levels and mitigated airway inflammation and hyperreactivity as well as mucus hypersecretion in a house dust mite asthma model⁴⁹. In our model of T cell stimulation ORMDL3 was only slightly different in *Asm*^{-/-} and WT, so that this mechanism seems to be neglectable for our

hypothesis. Nevertheless, ORMDL3 gene expression was higher in WT T cells in line with several studies, which demonstrated a link between pro-inflammatory diseases and ORMDL3 levels⁵⁰

In summary, our findings indicate that ASM plays a crucial role in asthmatic diseases. We believe that these results are relevant for the therapy of different (auto-)immune diseases. We speculate that ASM-inhibitors might be beneficial for asthmatics and allergic patients in general.

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Figure Legends

Figure 1:

Asm-activity in different lung compartments in WT and improved lung functions in $Asm^{-/-}$ animals in homeostasis

(a) Asm-activity in lung tissue (n=6) and BALF (n=5) of WT-animals was determined by immunofluorescence assay. **(b)** Asm-activity in BALF, lung and urine in WT- and $Asm^{-/-}$ -mice via radioactive ^{14}C -activity assay (BALF WT and urine WT n=3, n=8 in all other groups). **(c-g)** Lung function parameters in untreated animals were measured using the flexiVent system (n=5 in both groups). Total lung resistance Rrs **(c)**, central airway resistance Rn (Newtonian resistance) **(d)** and tissue resistance G **(e)**, elastance Ers **(f)** and tissue elastance H **(g)** of untreated $Asm^{-/-}$ and WT-littermates. All results represent means $\pm$ SEM; * p<0.05, ** p<0.01, *** p<0.001, **(a, c-g)** two-sided student's t tests, **(b)** one-sided student's t test.

Figure 2:

Higher levels of different immune cells including T-cell subpopulations in BALF of $Asm^{-/-}$ in homeostasis

Counts of total immune cells **(a)**, neutrophils **(b)**, T-lymphocytes **(c)**, monocytes **(d)**, dendritic cells **(e)** and macrophages **(f)** in untreated $Asm^{-/-}$ and WT-littermates. T-cell subpopulations, including T_H -cells (CD4+) **(g)**, T_H17 -cells (CD4+IL-17+) **(h)**, T_H2 -cells (CD4+GATA3+) **(i)** and regulatory T-cells (CD4+CD25+FoxP3+) **(j)**. **(a-j)** n=5 WT, n=4 $Asm^{-/-}$. All results represent means $\pm$ SEM; * p<0.05, ** p<0.01, **(a-j)** two-sided student's t test or for nonparametric data Mann-Whitney test.

Figure 3:

Cytokine profile in BALF of $Asm^{-/-}$ -mice tends to stronger T_H1 - and attenuated T_H2 -phenotype in homeostasis.

BALF levels of TNF- α **(a)**, IL-6 **(b)**, TGF- β **(c)**, IL-4 **(d)** and IL-5 **(e)** in untreated $Asm^{-/-}$ - and WT-controls. **(a-e)** n=5 in both groups. All results represent means $\pm$ SEM; * p<0.05, ** p<0.01, ***p<0.001, **(a-e)** two-sided student's t test or for nonparametric data Mann-Whitney test.

Figure 4:

$Asm^{-/-}$ and amitriptyline treated T-cells release lower levels of T_H2 -cytokines.

(a) Percentage of T_H2 (CD4+GATA3+)-cells of WT- and $Asm^{-/-}$ -splenocytes in the population of CD4+ cells (n=5 WT, n=4 $Asm^{-/-}$). **(b)** Absolute numbers of T_H2 -cells after 4 d of stimulation with anti-CD3/anti-CD28 of isolated CD4+ T-cells of WT- and $Asm^{-/-}$ -animals (n=5 in both groups). **(c)** Representing dot plots of **(b)** of WT and $Asm^{-/-}$ - T_H2 -cells. **(d-e)** Levels of IL-4 in supernatants of stimulated cells of **(b)** **(d)** and ratio of IL-4/ T_H2 -cells **(e)** (n=5 in both groups). **(f-g)** Isolated CD4+ cells were stimulated with ionomycin/PMA $\pm$ GolgiPlug. IL-4 levels in supernatants of stimulated WT- and $Asm^{-/-}$ -cells **(f)** (n=5 in all groups). IL-4 levels in lysed cells from **(f)** **(g)** (n=5 in all groups). **(h-l)** Gene expression levels of IL-4 **(h)**, IL-4R (CD124) **(i)**, GATA3 **(j)** of ionomycin/PMA stimulated WT- and $Asm^{-/-}$ -cells. **(k-l)** Absolute numbers of p-STAT6+ CD4+ cells **(k)** and gene expression level of ORMDL3 **(l)** of stimulated WT- and $Asm^{-/-}$ -cells. **(h-l)** n=5 in each group. **(m-n)** Isolated CD4+ cells of WT were treated with amitriptyline (50 μ M) and stimulated with ionomycin/PMA **(m)** or anti-CD3/anti-CD28 **(n)**. Levels of IL-4 in supernatants of stimulated cells (n=4 in all groups). **(o-p)** Live cells after stimulation period as percentage of CD4+ cells stimulated with ionomycin/PMA **(o)** and solely anti-CD3/anti-CD28 **(p)** (n=4 in all groups). **(a-p)** All results represent means $\pm$ SEM; * p<0.05, ** p<0.01, *** p<0.001, **(a,b,d,e,h-p)** one-sided student's t test or for nonparametric data Mann-Whitney test, **(f,g)** one-way ANOVA, FDR correction.

Figure 5:

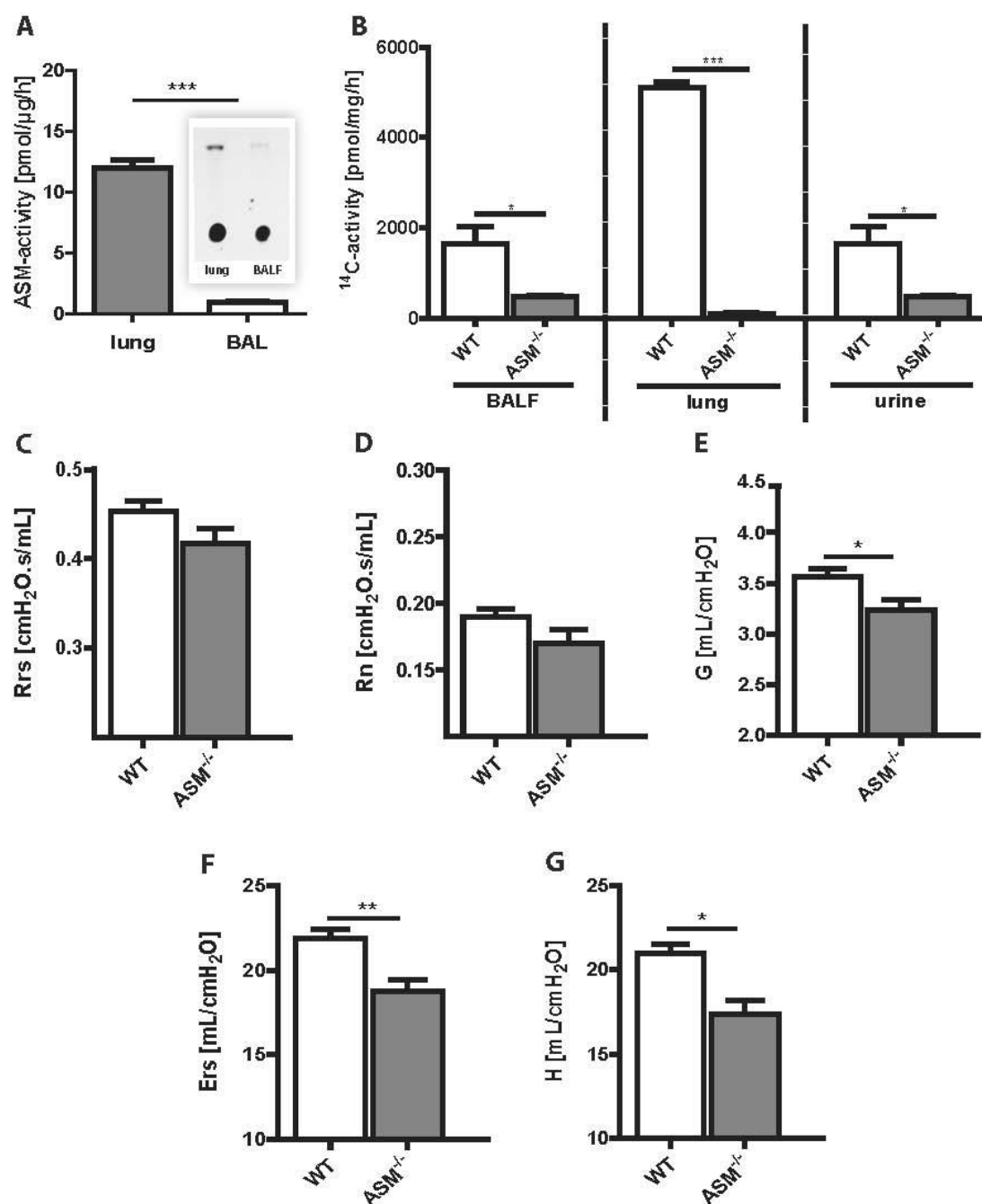
Asm^{-/-} is protective in asthmatic disease: lung mechanics

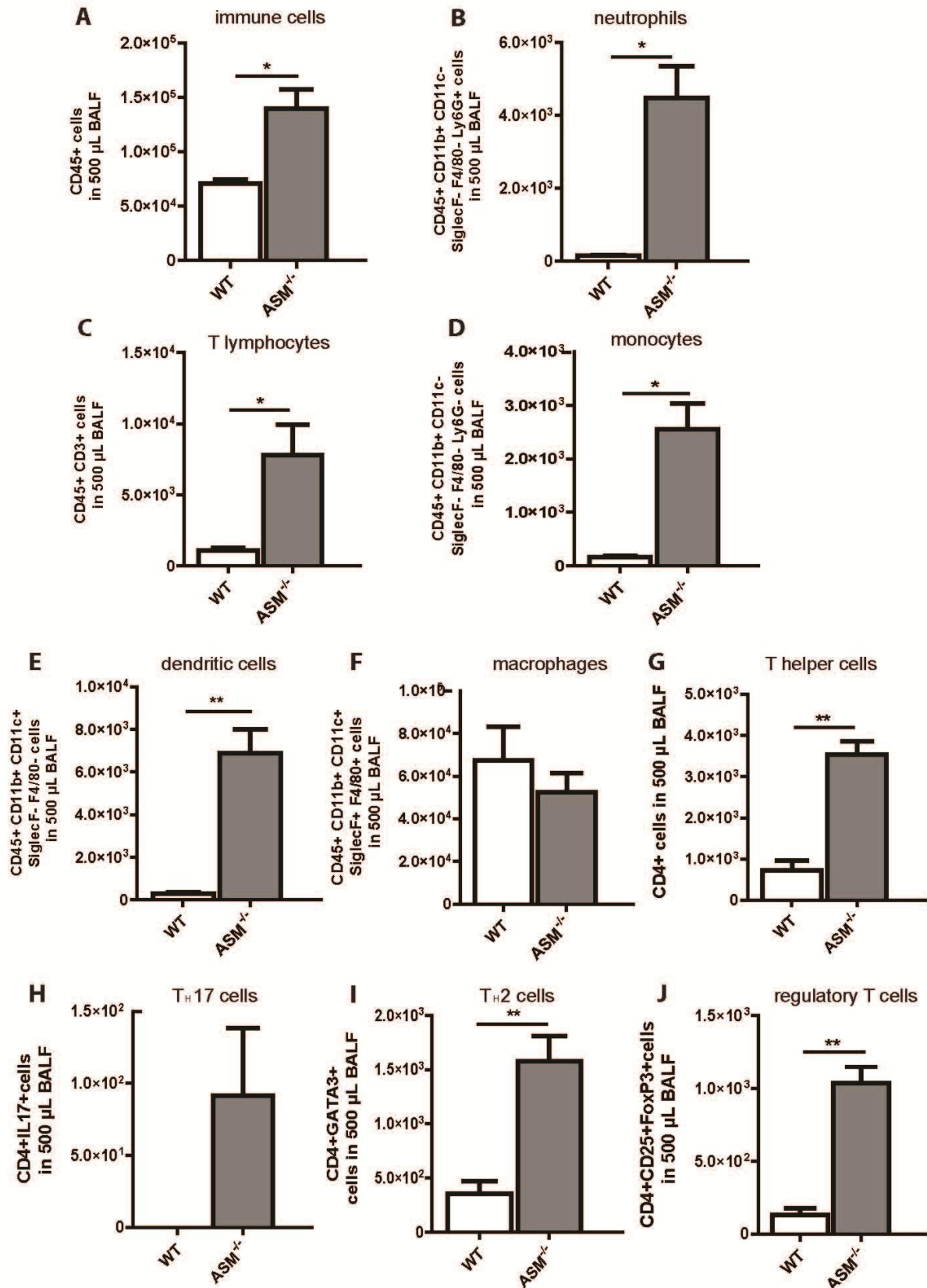
(a) Asm-activity in BALF of sensitized (OVA) (n=5) and non-sensitized (Alu) (n=6) WT-animals via immunofluorescence assay. **(b)** Weight of animals during time of sensitization. Percentage compared to starting weight. **(c-g)** Lung function parameters of sensitized and non-sensitized animals were measured using the flexiVent system. Basal lung function and lung mechanics after application of 3.16 mg/mL Ach. All values were normalized on Alu-controls. (n=7 in WT, n=8 in Asm^{-/-}). Total lung resistance Rrs **(c)**, central airway resistance Rn (Newtonian resistance) **(d)** and tissue resistance G **(e)**, elastance E **(f)** and tissue elastance H **(g)** of Asm^{-/-} and WT-littermates. **(h)** PCLS of sensitized Asm^{-/-} and WT-mice (n=8 in both groups). **(i)** Representative images of PCLS. All results represent means ± SEM; * p<0.05, ** p<0.01, *** p<0.001, **(a-, h)** two-sided student's t, FDR correction.

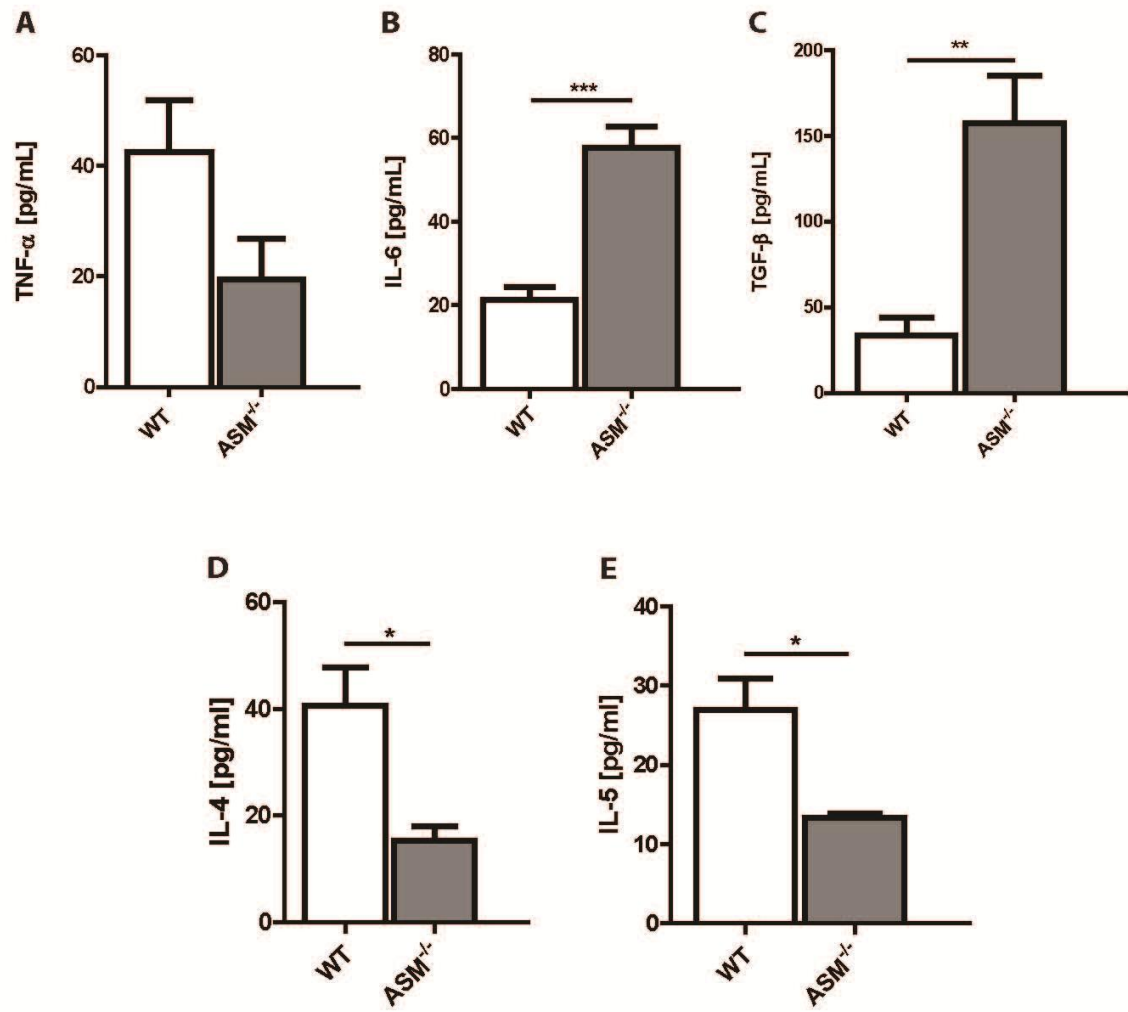
Figure 6:

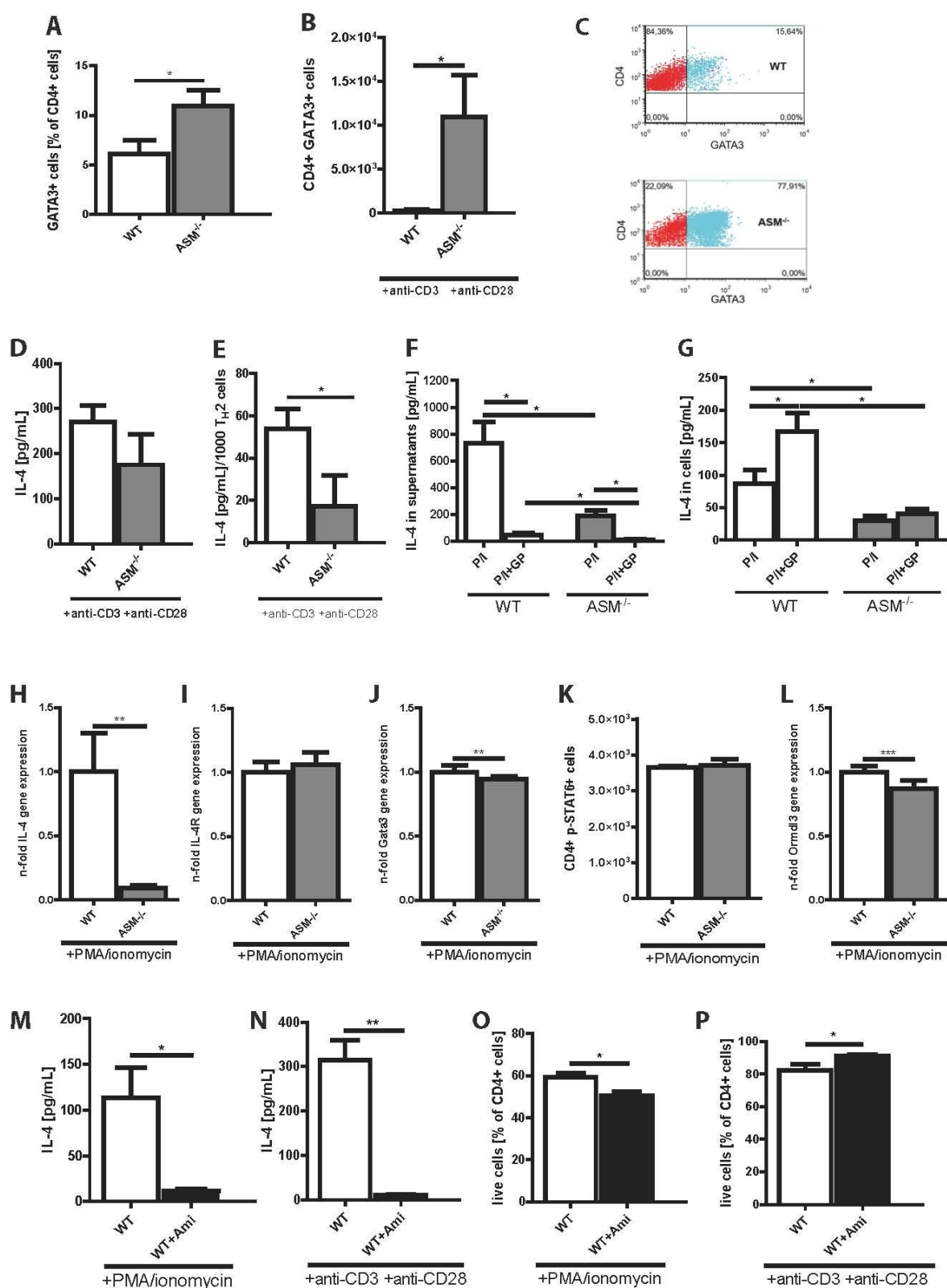
Asm^{-/-} is protective in asthmatic disease: cell composition and cytokine profiles

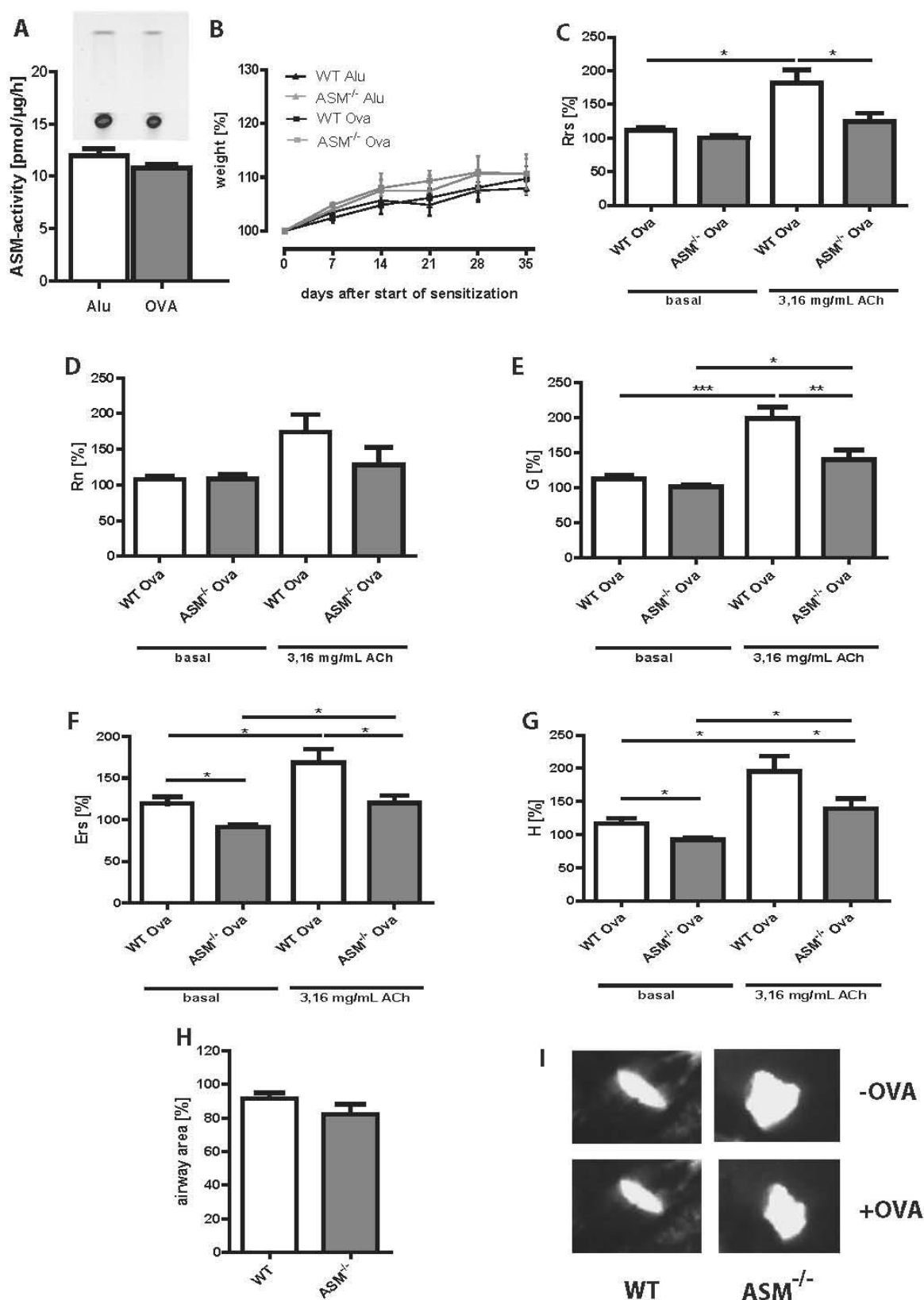
Counts of total immune cells **(a)**, neutrophils **(b)**, monocytes **(c)**, macrophages **(d)** and T-lymphocytes **(e)**. **(f)** Upregulation of CD3+CD4+ cells compared to WT Alu. **(g)** Count of eosinophils in sensitized Asm^{-/-} and WT-littermates. **(a-g)** n=6 Asm^{-/-}, n=7 WT. **(h-l)** BALF levels of IL-4 **(h)**, IL-5 **(i)**, IL-6 **(j)**, TNF-α **(k)** and TGF-β **(l)** in sensitized Asm^{-/-} and WT-mice. **(h-l)** n=4 in both Alu groups, n=6 in both OVA groups. All results represent means ± SEM; * p<0.05, ** p<0.01, *** p<0.001, **(a-l)** two-sided student's t-test, FDR correction.

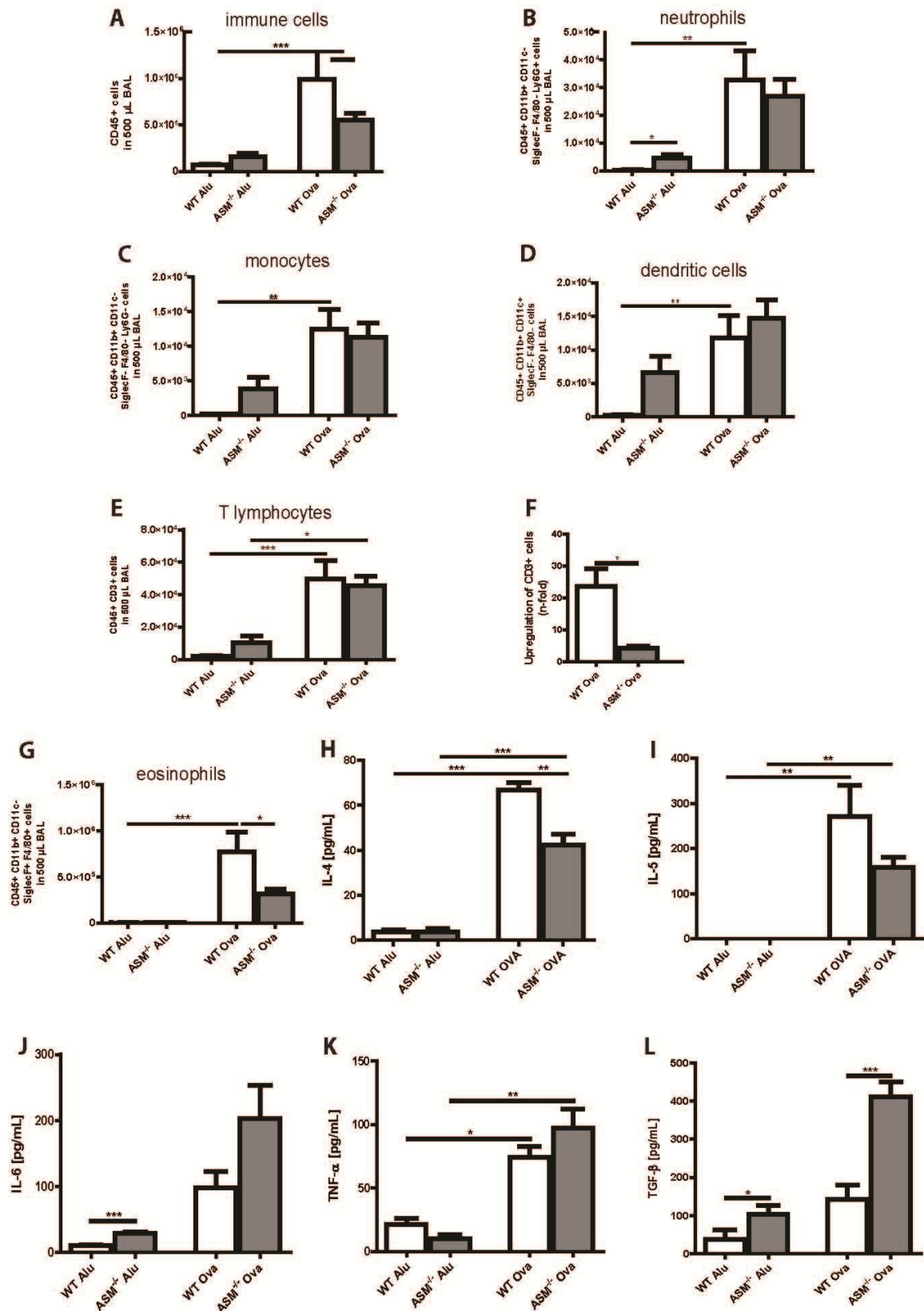




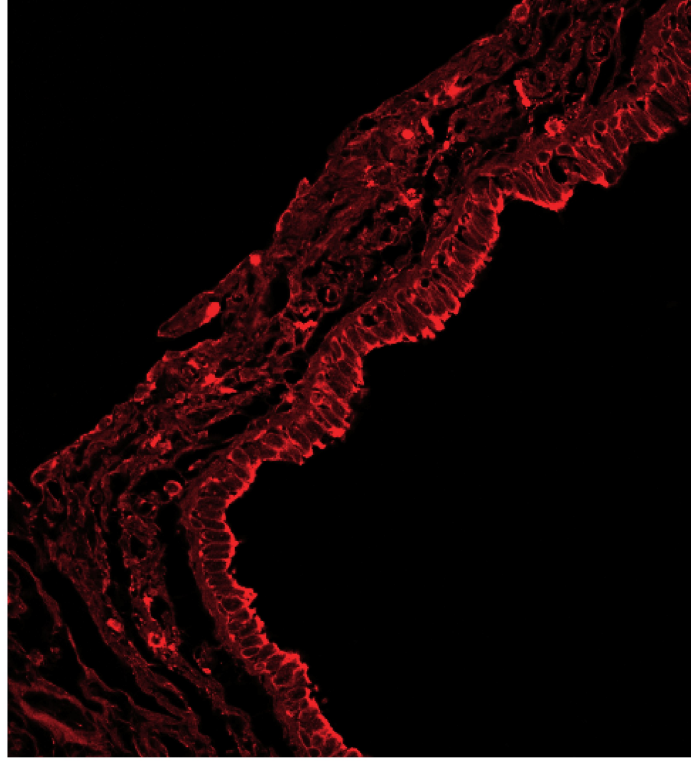




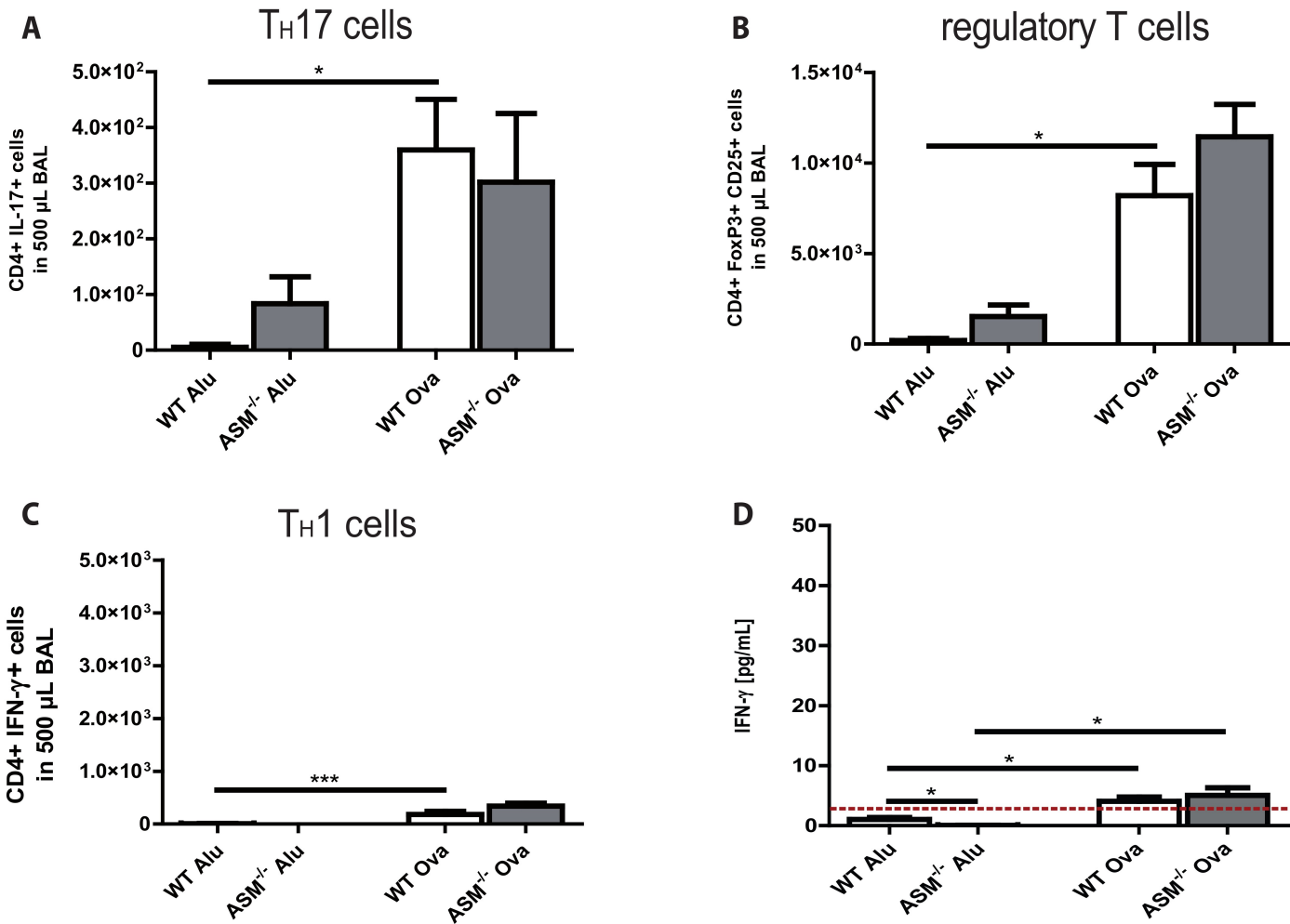




Supp. Fig. 1:



Supp. Fig. 2:



Supp. Tab. 1:

	WT	Asm^{-/-}
Total Resistance (Rrs)	0.69 ± 0.03	0.64 ± 0.04
Newtonian Resistance (Rn)	0.35 ± 0.06	0.31 ± 0.03
Tissue Resistance (G)	5.25 ± 0.26	4.93 ± 0.45
Elastance (Ers)	27.99 ± 0.69	27.04 ± 1.87
Tissue Elastance (H)	25.19 ± 0.68	27.27 ± 3.92

Supplemental Methods

Animals

Experiments were performed with 7 to 12 weeks old $Asm^{-/-}$ ($Smpd1^{-/-}$) and WT- ($Smpd1^{+/+}$) littermates (C57BL/6). $Smpd1^{-/-}$ mice have been described previously¹. All experiments were performed according to the rules of the Directive 2010/63/EU of the European Parliament and were approved by the Landesamt für Natur, Umwelt und Verbraucherschutz (LANUV) Nordrhein-Westfalen (84-02.04.2013.A124, 81-02.04.2018.A095) or by the Institute of Laboratory Animal Science RWTH Aachen (50.025A4). All mice were bred in the animal facility of RWTH Aachen or UK Essen and were kept under specific pathogen-free conditions. WT and transgenic mice were age-matched for all experiments.

Experimental design of ovalbumin-sensitization

On day 0, 14 and 21 mice were injected i.p. with 200 μ l OVA and Alu (OVA:10 μ g/Alu:1.5mg, Sigma, Germany). Control animals received only Alu. On day 28, 29 and 34 sensitized animals were exposed to nebulized OVA (1 %) for 30 min each day. Controls were exposed to NaCl (0.9 %). On day 35, mice were ventilated with the FlexiVent setup (SCIREQ, Germany).

Ventilation

On the day of ventilation, mice were anesthetized with pentobarbital (70-80mg/kg) and fentanyl (0,15mg/kg) and tracheotomized with a 20-gauge cannula and directly connected to a ventilator device. All mice were mechanically ventilated with a tidal volume (V_t) of 10 mL/kg and a positive end-expiratory pressure (PEEP) of 2 cm H_2O using the flexiVent (SCIREQ, Canada) ventilation setup. Breath rate was set as 150/min. Inspiratory fraction of oxygen was 0.21. Dynamic lung mechanics was measured by applying a sinusoidal standardized breath and analyzed with forced oscillation technique (FOT). We used a 1.2 s, 2.5 Hz single-

frequency forced oscillation manoeuvre (SnapShot perturbation) and a 3 s, broadband low frequency forced oscillation manoeuvre, containing 13 mutually prime frequencies between 1 and 20.5 Hz (Quick Prime perturbation). Total lung resistance (R_{rs}) and elastance (E_{rs}) were calculated using *flexiVent* software (flexiWare 7.0.1, SCIREQ, Canada) by fitting measured SnapShot values to the linear single compartment model applying multiple linear regressions. Respiratory system input impedance was calculated from the QuickPrime data and Newtonian (central airway) resistance (R_n), tissue resistance (tissue damping) (G), and tissue elastance (H) were assessed by iteratively fitting the constant-phase model to input impedance. During the first 30 min of ventilation time, baseline values were recorded using a standardized script with continuous measurements every 30 s. Every 5 min short volume-controlled recruitment maneuvers (3 s) were used to avoid atelectasis. AHR was provoked with increasing concentrations of nebulized Ach.

Body temperature was rectally controlled and adjusted to 37 ± 0.5 °C during the entire ventilation period. Continuous data recording of heart rate and ECG was performed to monitor the function of the cardiovascular system.

After the period of ventilation, the anesthetized animals were euthanized by bleeding of both carotid arteries.

Precision-cut lung slices

Precision-cut lung slices (PCLS) were prepared according to Martin *et al.* [24] in order to investigate airway reactivity upon acetylcholine (Ach)-provocation. Airway area was calculated with Optimas 6.5 (Media Cybernetics, USA) to evaluate bronchoconstriction.

Bronchoalveolar lavage fluid (BALF)

The whole lung was used for BALF by instilling 1000 μ L ice-cold phosphate buffered saline (PBS).

T-cell isolation and T-cell differentiation

Murine T-cells were isolated from splenocytes of untreated *Asm*^{-/-} and WT-mice by magnetic activated cell sorting (MACS) separation using the CD4+CD25+ Regulatory T Cell Isolation Kit (Miltenyi, Germany). CD4+CD25- T-cells were then incubated with plate coated anti-CD3 (10 µg/mL) and anti-CD28 (1 µg/mL). For T_H0-differentiation cells were incubated with 1 µg/mL ionomycin (Sigma-Aldrich, Germany) and 50 ng/mL PMA (Sigma Aldrich, Germany) in the presence of 1 µg/mL GolgiPlug (BD Biosciences, Germany) without any additional cytokines at 37°C. GolgiPlug (Brefeldin A) inhibits intracellular transport of proteins, so cytokines produced during activation will be retained inside the cell^{5,6}. After 4 days, cell culture supernatants were used for ELISA measurements of IL-4 and cells were lysed by ultrasonic treatment and intracellular cytokines were washed out and collected for ELISA analysis. CD4+ T-cells (after T_H0-differentiation) and splenocytes (before MACS-separation) were also analyzed by flow cytometry. For surface staining, cells were stained with anti-CD3 (eBioscience, Germany), anti-CD4 (BD Pharmingen, eBioscience, Germany), anti-CD11b (eBioscience, Germany), anti-Gr1 (eBioscience, Germany), anti-F4/80 (eBioscience, Germany) and anti-CD25 (eBioscience, Germany). For intracellular staining, cells were fixed and permeabilized with FoxP3 staining buffer set (eBioscience, Germany) following manufacturer's instructions and stained with anti-FoxP3 (eBioscience, Germany), anti-Gata3 (eBioscience, Germany), anti-IFN-γ (eBioscience, Germany) and anti-IL-17 (BD Pharmingen, Germany). For a complete list of all antibodies used, see Supp. **Table 1**. Flow cytometry was carried out using FACS-LSR-Fortessa (BD Biosciences, Germany) and data analysis was performed using FCS Express Software (DeNovo Software).

Stimulation and secretion analyses of T-cells

Murine T-cells were isolated from splenocytes of untreated *Asm*^{-/-} and WT-mice by MACS-separation using CD4 (L3T4) MicroBeads (Miltenyi, Germany). CD4+ T-cells were then

incubated with ionomycin and PMA for 6 hours. Additionally, half of the CD4⁺ T-cells were incubated with GolgiPlug. CD4⁺ T-cells were stimulated with 1 µg/mL ionomycin (Sigma Aldrich, Germany) and 50 ng/mL PMA (Sigma Aldrich, Germany) and as appropriate with 1 µg/mL GolgiPlug (BD Biosciences, Germany) for 6 hours. After incubation time, lysed cells and cell culture supernatants were used for ELISA measurements of IL-4. Moreover, WT CD4⁺ T-cells were incubated with 1 µg/mL ionomycin (Sigma Aldrich, Germany), 50 ng/mL PMA (Sigma Aldrich, Germany) and amitriptyline (50 µM) for 6 hours or incubated with 10 ng/mL anti-CD3 (eBioscience, Germany) and 1 µg/mL anti-CD28 (eBioscience, Germany) and amitriptyline (50 µM) for 12 hours. Cell culture supernatants were used for ELISA measurements of IL-4 and CD4⁺ T-cells were analyzed by flow cytometry. For apoptosis rate, CD4⁺ T-cells were stained with anti-CD4 (BD Pharmingen, eBioscience, Germany), Annexin V Apoptosis Detection Set (ThermoFisher, Germany) and 7-AAD (BioLegend, Germany) following manufacturer's instructions. For intracellular staining, cells were fixed and permeabilized with FoxP3 staining buffer set (eBioscience, Germany) following manufacturer's instructions and stained with anti-CD4 (BD Pharmingen, eBioscience, Germany), anti-Gata3 (eBioscience, Germany), anti-CD124 (BD Biosciences, Germany) and anti-p-STAT6 (eBioscience, Germany). For a complete list of all antibodies used, see Supp. Table 2. Flow cytometry was carried out using BD LSRFortessa (BD Biosciences, Germany) and data analysis were performed using FCS Express Software (DeNovo Software, USA).

Asm-activity assay (immunofluorescence)

The Asm-activity in BALF and lung tissue was determined using the fluorescent substrate BODIPY-FL-C12-sphingomyelin (N-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-dodecano-1-yl) sphingosyl phosphocholine, Invitrogen, Germany) using conditions similar to those described by others²⁻⁴. Depending on the sample type, different amounts of protein (BALF 1 µg, lung 3 µg) are used and diluted with lysis buffer (250 mM sodium acetate, 100 µM ZnCl₂, 1 % NP40, pH 5) to a final volume of 20 µL. After incubation for 24 h

(BALF) or 1 h (lung) at 37°C with 100 pmol substrate in 180 µL assay buffer (250 mM sodium acetate, 100 µM ZnCl₂, 0.1 % NP40, pH 5), lipids were extracted with 250 µL chloroform:methanol (2:1, v/v). The lower organic phase was then concentrated in nitrogen flow, dissolved in 20 µL chloroform:methanol (2:1, v/v) and spotted on silica gel 60 thin layer chromatography plates (Macherey-Nagel). Ceramide and uncleaved sphingomyelin were separated using chloroform:methanol (80:20, v/v) as solvent and were quantified on a Typhoon 9400 scanner (GE Healthcare) and ImageJ. Asm-activity is calculated in hydrolysis rate of sphingomyelin (pmol/µg) per time (h).

Asm-activity assay (radioactivity)

Asm-activity in BALF, lung tissue and urine was determined using ¹⁴C-labeled sphingomyelin as previously described using minor modifications (Gulbins E. et al., 2000). 10 µg protein of the samples were diluted with H₂O to a final volume of 10 µL and incubated for 2 h at 37°C with 40 µL ¹⁴C-labeled sphingomyelin mixture (73 nmol ¹⁴C-labelled sphingomyelin, 400 nmol unlabeled sphingomyelin). Radioactive phosphocholine produced from the ¹⁴C-labeled sphingomyelin was extracted with 750 µL chloroform:methanol (2:1, v/v) and 250 µL H₂O. The release of ¹⁴C-labeled phosphorylcholine was measured in 250 µL of the upper phase after adding 4 mL scintillation liquid by liquid scintillation counting with Scintillation Counter LS 6000SC. Asm-activity is calculated in hydrolysis rate of sphingomyelin (pmol/mg) per time (h).

Bronchial epithelial cell staining

Lung biopsies were obtained from remaining healthy donor lungs (n=5) that were used to transplant CF patients. Biopsies were fixed in 4% PFA for 48 h. We used samples from female and male patients. The identity of the patients and donors, respectively, was blinded

to the investigators. The studies were approved by the local ethics committees Essen, all patients gave informed consent. The permission number was 17-7326-BO.

Stainings of biopsies were performed as previously reported (Grassmé *et al.* 2001, 2003; Teichgräber *et al.*, 2008; Pewzner-Jung *et al.*, 2014). The tissue was serially dehydrated with an ethanol to xylol gradient and then embedded in paraffin. Samples were sectioned at 7 µm, dewaxed, rehydrated, and treated with pepsin (Digest All; #003009, Invitrogen) for 30 min at 37°C. They were then washed with water and PBS and blocked for 10 min at room temperature with PBS, 0.05% Tween 20 (Sigma), and 5% fetal calf serum (FCS). The samples were stained with a goat anti-human acid sphingomyelinase antibody (kindly provided by Dr. K. Sandhoff, University of Bonn, Germany) in H/S (132 mM NaCl, 20 mM HEPES [pH 7.4], 5 mM KCl, 1 mM CaCl₂, 0.7 mM MgCl₂, 0.8 mM MgSO₄) plus 1% FCS at room temperature for 45 min. Samples were washed three times with PBS plus 0.05% Tween 20 and once with PBS. The tissue was secondarily labeled with Cy3-coupled anti-goat F(ab)₂ fragments (Jackson ImmunoResearch) in H/S plus 1% FCS for 30 min. Tissue was again washed three times with PBS plus 0.05% Tween 20 and once with PBS; finally, they were embedded in Mowiol.

The immunostainings were controlled with control serum (Dako). These stainings showed no or very weak staining. We also included controls with secondary Cy3-coupled antibodies only.

Samples were examined with a Leica TCS-SP5 confocal microscope equipped with a 100× lens, and images were analyzed with Leica LCS software version 2.61 (Leica Microsystems, Mannheim, Germany). All comparative samples were measured at identical settings.

Flow cytometry

Absolute numbers of BALF-cells and antibody-stained populations were measured by FACS analysis with the FACS-Canto II device (BD Bioscience, Germany). A minimum of 10,000 events were collected for evaluation. For surface staining, BALF-cells were stained with anti-CD11b (eBioscience, Germany), anti-CD11c (eBioscience, Germany), anti-Ly6G (Miltenyi Biotec, Germany), anti-F4/80 (Bio-Rad, Germany), anti-SiglecF (eBioscience, Germany), anti-CD45 (eBioscience, Miltenyi Biotec, Germany), anti-CD-3 (eBioscience, Germany), anti-CD4 (eBioscience, Germany) and anti-CD25 (eBioscience, Germany). For intracellular staining, cells were fixed and permeabilized with FoxP3 staining buffer set (eBioscience, Germany) following manufacturer's instructions and stained with anti-FoxP3 (eBioscience, Germany), anti-IFN- γ (eBioscience, Germany) and anti-IL-17 (BD Biosciences, Germany). For a complete list of all antibodies used, see Supp. **Tab. 2**. Flow cytometry was carried out using BD LSRFortessa (BD Biosciences, Germany) and data analysis were performed using FCS Express Software (DeNovo Software, USA). To calculate absolute cell numbers, BALF-cells were analyzed without staining.

Supp. Tab. 2: Flow cytometry antibodies

Protein Target	Conjugate	Species Target	Company
Annexin V	PE-Cyanine7	Mouse	ThermoFisher
CD3	APC	Mouse	eBioscience
CD3	PE	Mouse	eBioscience
CD3	PE-Cyanine5	Mouse	eBioscience
CD4	APC	Mouse	eBioscience
CD4	PB	Mouse	BD Pharmingen
CD4	PE-Cyanine7	Mouse	eBioscience
CD11b	eFluor® 450	Mouse	eBioscience

CD11b	PB	Mouse	eBioscience
CD11b	PE-Cyanine7	Mouse	BD Bioscience
CD11c	PE	Mouse	eBioscience
CD25	APC	Mouse	eBioscience
CD25	PE	Mouse	eBioscience
CD45	APC-Cyanine7	Mouse	eBioscience
CD45	PB	Mouse	Miltenyi Biotec
CD124	PE	Mouse	BD Biosciences
FoxP3	FITC	Mouse	eBioscience
F4/80	FITC	Mouse	Bio-Rad
F4/80	PE-Cyanine7	Mouse	eBioscience
Gata3	PerCP-Cyanine5.5	Mouse	eBioscience
Gr1	PE	Mouse	eBioscience
IFN- γ	APC	Mouse	eBioscience
IFN- γ	PB	Mouse	eBioscience
IL-17	FITC	Mouse	BD Pharmingen
IL-17	PE	Mouse	BD Bioscience
Ly6G	APC	Mouse	Miltenyi Biotec
p-STAT6	APC	Mouse	eBioscience
Siglec F	PE-Cyanine5.5	Mouse	eBioscience
Siglec F	PerCP-eFluor® 710	Mouse	eBioscience
7-AAD	-	Mouse	BioLegend

RNA isolation and quality control

RNA of stimulated WT and Asm^{-/-}-T-cells (stored at -80°C) was extracted using RNeasy Mini Kit (QIAGEN, Germany) according manufacturer's instructions. RNA concentration and quality ($A_{260}/A_{280} \sim 2$) were measured by NanoDrop (ThermoFisher, Germany).

cDNA synthesis

Reverse transcription of 1 µg of total RNA in a total volume of 12.5 µL was performed using Maxima Reverse Transcriptase (ThermoFisher, Germany), RiboLock RNase Inhibitor (ThermoFisher, Germany), Oligo(dt) 18 Primer (ThermoFisher, Germany) and dNTP Mix (ThermoFisher) according manufacturer's instructions. cDNA was stored at 4°C for up to 2 weeks. A negative control without reverse transcriptase (NRT) and a negative control without template (NTC) were included.

qPCR

Real-time qPCR was performed using CFX Connect Detection System (Bio-Rad, Germany). The reaction takes place in a total volume of 10 µL with 1 µL cDNA, 5 µL iTag Universal SYBR Green Supermix (Bio-Rad, Germany) and final concentrations of 500 nM forward and 500 nM reverse primer. Following thermal cycling parameters were used: DNA polymerase activation at 95°C for 300 s, then 45 cycles consisting of denaturation at 95°C for 10 s, annealing at 58°C (IL-4R) or 60°C (IL-4) or 62°C (GATA3) or 64°C (ORMDL3) for 10 s and extension at 72°C for 15 s. Cycling was followed by melt curve analysis from 65°C to 97°C. Data analysis was performed using CFX Maestro Software (Bio-Rad, Germany). Automatic baseline settings (Baseline Subtracted Curve Fit) were used. Standards and samples were analyzed in duplicate. The following quality control parameters were ensured: NTCs and NRTs Cq (quantification cycle) greater than 38 or a Cq which is at least 5 greater than the Cq of the samples, efficiency between 90 and 110, R^2 of the standard curve greater than 0,980, replicate group Cq standard deviation less than 0,2. To exclude genomic DNA contamination NRT controls of each sample were analyzed and only exon spanning primers were used. Relative RNA expression levels were calculated using Single threshold Cq determination and were normalized to the reference genes Rps29 and Sdha. The forward and reverse primer sequences and annealing temperatures of the reference and target genes are given in Supp. Tab. 3.

Reference gene validation

A panel of 10 candidate reference gene primer were evaluated in order to establish the genes with the most stable levels of expression in stimulated WT and Asm^{-/-}-T-cells. qPCR was used to quantify the RNA expression levels of Glyceraldehyde-3-Phosphate Dehydrogenase (Gapdh), Actin Beta (Actb), Eukaryotic Translation Elongation Factor 2 (Eef2), Ribosomal Protein s29 (Rps29), Beta-2-Microglobulin (B2m), Hypoxanthine Phosphoribosyltransferase 1 (Hprt1), Ribosomal Protein L13 (Rpl13), Phospholipase A2 (Pla2), Succinate Dehydrogenase Complex Flavoprotein Subunit A (Sdha), TATA-Box Binding Protein (Tbp) in 3 stimulated WT and 3 stimulated Asm^{-/-}-T-cell samples in duplicate. The evaluation was conducted using qBase+ software, which utilizes the geNorm selection algorithm. LinReg was used to calculate the efficiency and its standard deviation. The forward and reverse primer sequences and annealing temperatures of the reference and target genes are given in Supp. **Tab. 3**.

Supp. Tab. 3: Reference and target genes

Primer	Forward sequence	Reverse sequence	Annealing temperature
Gapdh	CATGGCCTTCCGTGTTCTTA	ACTTGGCAGGTTTCTCCAGG	60°C
Actb	CACTGTCGAGTCGCGTCC	TCATCCATGGCGAACTGGTG	60°C
Eef2	TCACAATCAAATCCACCGCC	ATGGCCTGGAGAGTCGATGA	60°C
Rps29	CCTTTCTCCTCGTTGGGCG	GAGCAGACGCGGCAAGAG	61°C
B2m	TTCTGGTGCTTGTCTCACTGA	CAGTATGTTTCGGCTTCCCATTC	61°C
Hprt1	TCAGTCAACGGGGGACATAAA	GGGGCTGTACTGCTTAACCAG	61°C
Rpl13	GCGGATGAATACCAACCCCT	CCACCATCCGCTTTTTCTTGT	61°C
Pla2	GAAAAGTTCTTGATCCCCAATGC	TGTGACTGGTCCACAATTCCTT	60°C
Sdha	GGAACACTCTCCAAAAACAGACCT	CCACCACTGGGTATTGAGTAGAA	60°C
Tbp	ATATACCGTGAATCTTGGCTGT	GATTGTTCTTCACTCTTGGCTC	61°C

IL-4	GTCATCCTGCTCTTCTTTCTC	TCTCTGTGGTGTTCTTCGT	60°C
IL-4R	AGAGGGACCTGGCTTCTGAT	CAAAAGGTGCCTGCACAAGG	58°C
GATA3	AGAACCGGCCCCTTATCAA	AGTTCGCGCAGGATGTCC	62°C
ORMDL3	GGGGGTGGTCAGGAAAGAGGCT	GGGTTGCCAGGAAGCCCACAAA	64°C

1 Supplement Table and Figure Legends

2 Supp. Tab. 1:

3 **No differences in bronchial hyperresponsiveness between $Asm^{-/-}$ and WT-animals**

4 Lung function parameters in untreated $Asm^{-/-}$ and WT-animals were measured using the flexiVent
5 system after application of 3.16 mg/mL Ach (n=5 in both groups).

7 Supp. Fig. 1:

8 **ASM is located at the surface of human bronchial epithelial cells**

9 Confocal microscopy of stainings of human bronchial epithelial cells with ASM-antibody. The
10 immunostainings were controlled with control serum (Dako) and with secondary Cy3-coupled
11 antibodies only. Both showed no staining.

13 Supp. Fig. 2:

14 **No differences in T-cell subpopulations and IFN- γ in BALF of sensitized $Asm^{-/-}$ and WT-animals**

15 Counts of T_H17 - **(a)**, T_{reg} - **(b)** and T_H1 -cells **(c)** in sensitized $Asm^{-/-}$ and WT-littermates. **(d)** BALF levels
16 of IFN- γ in sensitized $Asm^{-/-}$ and WT-mice. **(a-d)** n=4 in both Alu groups, n=6 in both OVA groups. All
17 results represent means $\pm$ SEM; * p<0.05, *** p<0.001, **(a-d)** two-sided student's t test, FDR
18 correction.