Original Article

Asthma and Lower Airway Disease

Acid sphingomyelinase regulates $T_{H2}$ cytokine release and bronchial 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, wild-type (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−/− mice.

Results: At baseline, Asm−/− 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 $T_{H2}$ 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, for example, with ASM blockade.

Keywords
acid sphingomyelinase, allergic disease, asthmatic response, T cells, $T_{H2}$
1 | 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%. Allergic asthma is characterized by airway inflammation, recurrent bronchospasms, airway hyperresponsiveness (AHR) and mucus hypersecretion. Therapies are largely symptomatic, and only a small number of medications are available.

It is well known that sphingolipids, such as sphingosine-1-phosphate (S1P), play a crucial role in pathogenesis of bronchial asthma and corresponding airway hyperresponsiveness. 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). Additionally, ORMDL3 (orosomucoid-like 3) is known as protein that regulates sphingolipid synthesis and hereby probably also asthmatic disease and smooth muscle cell contraction. 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). Here, ceramide acts as a second messenger and organizes cellular processes and signaling events to allow differentiation, proliferation, apoptosis, and inflammation.

In asthmatic patients, ceramide and sphingomyelin levels are increased in serum and sputum. Moreover, increased levels of S1P can be found in bronchoalveolar lavage fluid (BALF) of asthmatic patients after allergen provocation. 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 (Treg), but they found no effect on physiological lung functions, such as airway hyperresponsiveness (AHR) and they did not study TH2 cells. 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.

These findings raise the hypothesis that Asm is involved in the regulation of the typical Th2 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 Th2, Th17, 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 Th2 cytokine IL-4 from T lymphocytes indicating that Asm is required for proper Th2 responses of these cells. In ovalbumin (OVA)-induced asthma, Asm−/− animals were protected with a decreased Th2 response and diminished AHR. Therefore, ASM blockade may offer novel therapeutic strategies for the very large group of patients with allergic bronchial asthma.

2 | MATERIALS AND METHODS

For several chapters, additional details are provided in the Data S1.
2.1 | Animals
Experiments were performed with 7-12 weeks old Asm\(^{-/}\) (Smpd1\(^{-/}\)) and WT (Smpd1\(^{+/}\)) littermates (C57BL/6). Smpd1\(^{-/}\) mice have been described previously.\(^{22}\) The study was approved by the regional governmental authorities, and animal procedures were performed according to the German animal protection law.

2.2 | Experimental design of ovalbumin sensitization
On days 0, 14, and 21, mice were injected i.p. with OVA and aluminum hydroxide (Alu). On days 28, 29, and 34, sensitized mice were exposed to nebulized OVA (1%) for 30 minutes. Control animals received Alu and were exposed to NaCl (0.9%). On day 35, mice were mechanically ventilated with the flexiVent ventilator (SCIREQ).

2.3 | 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). mRNA levels were normalized to the reference genes ribosomal protein s29 (Rps29) and succinate dehydrogenase complex flavoprotein subunit A (Sdha).

2.4 | 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). 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.

2.5 | Stimulation and secretion analyses of T cells
Murine T cells were isolated from splenocytes of untreated Asm\(^{-/}\) and WT mice by magnetic-activated cell sorting (MACS) separation using the CD4+ (L3T4) MicroBeads (Miltenyi). 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 µmol/L) for 6 hours or incubated with anti-CD3/anti-CD28 and amitriptyline (50 µmol/L) for 12 hours.

2.6 | 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) using conditions similar to those described by others.\(^{23,24}\) Additionally, Asm activity was determined using radioactive \(^{14}\)C-labeled sphingomyelin as previously described\(^{25}\) using minor modifications.

2.7 | 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’s \(t\) test given that data were normally distributed. Nonparametric data were analyzed using Mann-Whitney test. For multiple testing, \(p\)-values were adjusted with the Benjamini-Hochberg false discovery rate (FDR) correction. All data are presented as mean ± standard error (SEM). \(p\)-values < .05 were considered to be statistically significant (* \(p < .05\), ** \(p < .01\) and *** \(p < .001\)).

3 | RESULTS

3.1 | Acid sphingomyelinase influences lung mechanics and cell influx in bronchoalveolar lavage
We first studied Asm activity in lung tissue and in BALF using the nonradioactive, fluorometric Asm activity assay (Figure 1A) as well as the \(^{14}\)C radioactive activity assay (Figure 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 (Figure 1A). As expected, Asm activity was absent in Asm\(^{-/}\) animals (Figure 1B). In addition to well-known Asm activity in immunological cells, we demonstrated ASM localization at the surface of bronchial epithelium (Figure S1). 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 (Figure 1C-G). Total resistance of the airway (Rrs) and Newtonian resistance (Rn) as resistance of the larger airways tended to be lower in Asm\(^{-/}\) mice compared with WT. Tissue resistance (G), total elastance (Ers), and tissue elastance (H) were significantly decreased in Asm\(^{-/}\) mice. Bronchial hyperresponsiveness was not different between WT and Asm deficient animals (Table S1). 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 (Figure 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\(^{-/}\) mice compared with WT mice (Figure 2B-E). Similar levels were observed in the population of alveolar macrophages (CD45+CD11b+CD11c-SiglecF-F4/80+) (Figure 2F). Regarding T cell subpopulations in homeostasis, we
detected significantly higher numbers of CD4+ T cells in Asm−/− than in WT mice as well as 10-fold higher numbers of CD4+IL17+ (Th17) cells, more than 3-fold higher levels of CD4+GATA3+ (Th2) cells and around 10-fold higher numbers of CD4+CD25+FoxP3+ (Treg) 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).

3.2 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 Treg cells in the lung, we next evaluated immune cell activity and especially TH2 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 (Figure 3A–C). The Th17 cytokines IL-4 and IL-5 were reduced by approximately 50% in BALF of Asm−/− mice (Figure 3D–E). Thus, surprisingly, higher numbers of Th17 cells in BALF of Asm−/− mice correlated inversely with lower levels of typical Th1 cytokines in homeostasis.

Therefore, we hypothesized that Th1 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+ Treg cells (Figure 4A–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 (Figure 4D). In addition, IL-4 per 1000 Treg cells was significantly lower in Asm−/− Treg cells.
immune cells

neutrophils

T lymphocytes

monocytes

dendritic cells

macrophages

T helper cells

T_\text{H}17 cells

T_\text{H}2 cells

regulatory T cells

\text{CD}45^+ \text{CD}11b^- \text{CD}11c^- \text{SiglecF}^- \text{F}4/80^- \text{Ly6G}^- \text{cells in 500 µL BALF}
Acid sphingomyelinase deficiency is protective in a model of bronchial asthma

This strong difference of T\textsubscript{H}2 cytokine secretion/production led us to hypothesize that Asm deficiency is protective in allergic asthma.
Therefore, as proof-of-principle Asm<sup>−/−</sup> 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 with controls (Figure 5A). Next, we monitored weight of our animals, because ASM inhibitors can cause weight gain; however, WT and Asm<sup>−/−</sup> animals showed no difference in weight gain over the time (Figure 5B). We ventilated OVA-sensitized mice with the flexiVent setup. Lung resistance parameters of Asm<sup>−/−</sup> 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 with WT animals (Figure 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 (Figure 5H-I). Airways from sensitized WT and 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 (Figure 6A-E); even T cell subpopulations were similar in both groups (exemplarily Treg cells as Figure 5A-B). Levels of Treg cells were very low in both asthma groups (Figure 5C). Additionally, levels of IFN-γ were comparable in both groups with low total levels around detection level (Figure 5D). In other words, WT mice exhibited a stronger upregulation of inflammatory cells into the BALF compared with Asm−/− animals (exemplarily shown for T lymphocytes, Figure 6F).

Only, the number of eosinophils differed significantly, with lower numbers in sensitized Asm−/− animals than in WT (Figure 6G), which corresponded to lower levels of IL-4 and IL-5 in BALF of sensitized Asm−/− (Figure 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 (Figure 6J-K). TGF-β levels were clearly increased in Asm−/− animals in both groups, Alu and OVA (Figure 6L). Thus, Asm deficient mice were protected from upregulation of Treg cytokines upon OVA stimulation and showed a reduced immune cell influx.

4 | 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 Treg responses in vitro and in vivo. Normally, during asthmatic reactions, Treg 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 Treg cells in an OVA sensitization and provocation model, but they do not have the ability to produce and secrete typical Treg cytokines. This results in an improved lung function of asthmatic Asm−/− animals. Of note, even under baseline conditions lungs from Asm−/− 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, but the role of ASM in Treg2 disease had not yet been not 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 inflammasome. In CF mice and human patients, ceramide accumulation in different immune cells and epithelial cells also raises the susceptibility to infection, for example Pseudomonas aeruginosa. As new therapeutic approach, the ASM inhibitor amitriptyline was administered in phase II randomised, double-blind, and placebo-controlled studies of CF patients. 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, that is RSV bronchiolitis. In that study, Asm was not elevated in the control groups with asthma or adenovirus infections. 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. 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 with WT littermates. Gupta et al demonstrated higher ASM levels and stronger activity in erythrocyte membranes of asthmatics compared with healthy controls. 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 Treg2-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 Treg cells in a short term (21d) murine asthma model with Asm−/−, which we could show only as a tendency. Instead, we showed that ASM deficiency reduces Treg 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 Treg2
Immune cells

- Neutrophils
- T lymphocytes
- Dendritic cells
- Eosinophils

WT Al

Alu
–/–

AS
M

WT Ova

Ova
–/–

ASM

0

5.0 × 10⁵

1.0 × 10⁶

1.5 × 10⁶

***

CD45+ cells in 500 µL BAL

CD45+ CD11b+ CD11c− SiglecF− F4/80− Ly6G+ cells in 500 µL BAL

WT Al

Alu
–/–

AS
M

WT Ova

Ova
–/–

ASM

0

1.0 × 10⁴

2.0 × 10⁴

3.0 × 10⁴

4.0 × 10⁴

5.0 × 10⁴

**

CD45+ CD11b+ CD11c− SiglecF− F4/80− Ly6G− cells in 500 µL BAL

Monocytes

WT Al

Alu
–/–

AS
M

WT Ova

Ova
–/–

ASM

0

5.0 × 10³

1.0 × 10⁴

1.5 × 10⁴

2.0 × 10⁴

**

CD45+ CD11b+ CD11c+ SiglecF− F4/80− cells in 500 µL BAL

CD45+ CD3+ cells in 500 µL BAL

WT Al

Alu
–/–

AS
M

WT Ova

Ova
–/–

ASM

0

5.0 × 10⁵

1.0 × 10⁶

1.5 × 10⁶

***

CD45+ CD11b+ CD11c− SiglecF+ F4/80+ cells in 500 µL BAL

IL-4 [pg/mL]

WT Al

Alu
–/–

AS
M

WT Ova

Ova
–/–

ASM

0

100

200

300

400

**

IL-5 [pg/mL]

WT Al

Alu
–/–

AS
M

WT Ova

Ova
–/–

ASM

0

100

200

300

***

IL-6 [pg/mL]

WT Al

Alu
–/–

AS
M

WT Ova

Ova
–/–

ASM

0

50

100

150

* **

TNF-α [pg/mL]

WT Al

Alu
–/–

AS
M

WT Ova

Ova
–/–

ASM

0

100

200

300

400

500

**

TGF-β [pg/mL]

WT Ova

Ova
–/–

ASM

0

10

20

30

40

* **

Upregulation of CD3+ cells (n-fold)

(A) Immune cells

(B) Neutrophils

(C) Monocytes

(D) Dendritic cells

(E) T lymphocytes

(F) Eosinophils

(G) IL-4 [pg/mL]

(H) IL-5 [pg/mL]

(I) IL-6 [pg/mL]

(J) TNF-α [pg/mL]

(K) TGF-β [pg/mL]
and T<sub>reg</sub> 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<sup>−/−</sup> 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<sup>−/−</sup> and WT demonstrating a problem in T<sub>H</sub>2 cytokine production. We demonstrated dramatically reduced IL-4 gene expression in Asm<sup>−/−</sup> after ionomycin/PMA independent of relevant changes in GATA3 and p-STAT6. We speculate that this IL-4 phenotype is primed during development of Asm<sup>−/−</sup> animals. IL-4-R may be either upregulated in Asm<sup>−/−</sup> 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<sup>−/−</sup> animals or after amitriptyline treatment and therefore T<sub>H</sub>2 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<sub>H</sub>1- and T<sub>H</sub>17-differentiation and responses, while it enhances regulatory T cell development and function. In line with our data, naïve Asm<sup>−/−</sup> mice had higher numbers of systemic T<sub>reg</sub> cells. In vitro experiments showed that Asm deficiency in CD4<sup>+</sup> T cells correlates with PI3K-Akt signaling, resulting in increased FoxP3 expression and thereby induction of T cell differentiation. Hollmann et al demonstrated that the frequency of T<sub>reg</sub> turnover of the effector molecule CTLA-4 and their suppressive activity are upregulated in Asm<sup>−/−</sup> animals. In our study, T<sub>reg</sub> were more numerous already in untreated Asm deficient animals, while the numbers of T<sub>reg</sub> were the same in BALF and lung tissue of WT and Asm<sup>−/−</sup> animals after asthma induction. Therefore, altered numbers of T<sub>reg</sub> alone are not sufficient to explain the observed protective phenotype in asthmatic disease.

Acid sphingomyelinase 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 stimulation. Especially the outer membrane of T lymphocytes contains high ASM levels and ASM activity. Vice versa, Asm<sup>−/−</sup> 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 receptors, but up to now it has not been clearly shown whether these receptors activate ASM or whether it is the other way around. 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. Especially in T cells, it is expected that ASM—among others—interacts with cytokine receptors and induces T-cell differentiation, for example T<sub>H</sub>2 differentiation. However, the exact mechanism by which pathways ASM participates in T cell receptor (TCR)/CD3 and CD28 signaling remains unknown. Bai et al demonstrated that ASM interacts with the intracellular domains of both, CD3 and CD28. 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-mTOR. Other receptors and signaling pathways regulated by ASM activity and increased ceramide production include STAT3 and mTOR signaling. First pilot experiments also demonstrated ASM localization at the surface of bronchial epithelium (Figure S1). 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 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 model. In our model of T-cell stimulation, ORMDL3 was only slightly different in Asm<sup>−/−</sup> 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 levels.

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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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

SB, EV, NW, CM, and KT involved in the conception and design of the study. SB, SZ, EV, KO, and AR performed the research. SB, SZ, PK, EV, KO, SU, KT, and CM involved in data analysis and interpretation. EV, SU, KT, CM, MK, KB, and EG contributed reagents/materials/tissue specimens. SB, EV, SU, SZ, CM, and KT wrote the paper.

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REFERENCES

1. Bateman ED, Hurd SS, Barnes PJ, et al. Global strategy for asthma management and prevention: GINA executive summary. Eur Respir J. 2008;31:143–178. The European Respiratory Journal. 2018;51(2).
2. Bush A, Fleming L. Diagnosis and management of asthma in children. BMJ. 2015;350:h996.
3. Buc M, Dzurilla M, Vrljak M, Bucova M. Immunopathogenesis of bronchial asthma. Arch Immunol Ther Exp. 2009;57(5):331–344.
4. Tese R, Borrelli G, Mongelli G, Mastrorilli V, Cardinale F. Treating pediatric asthma according guidelines. Front Pediatr. 2018;6:234.
5. Liu L, Zhai C, Pan Y, et al. Sphingosine-1-phosphate induces airway smooth muscle cell proliferation, migration, and contraction by modulating Hippo signaling effector YAP. Am J Physiol. 2018;315(4):L609-L621.
6. Kawa Y, Nagano T, Yoshizaki A, et al. Role of S1P/S1PR3 axis in release of CCL20 from human bronchial epithelial cells. PLoS One. 2018;13(9):e0203211.
7. Becker KA, Riethmuller J, Zhang Y, Gubins B. The role of sphingolipids and ceramide in pulmonary inflammation in cystic fibrosis. Open Respir Med J. 2010;4:39–47.
8. Aureli M, Schiumarini D, Loberto N, et al. Unravelling the role of sphingolipids in cystic fibrosis lung disease. Chem Phys Lipid. 2016;200:94-103.
9. Ghidoni R, Caretti A, Signorelli P. Role of sphingolipids in the pathobiology of lung inflammation. Mediators Inflamm. 2015;2015:487508.
10. Sturgill JL. Sphingolipids and their enigmatic role in asthma. Adv Biol Regul. 2018;70:74-84.
11. Yoshida S, Naguchi A, Kikuchi W, Fukaya H, Igarashi K, Takahashi T. Elevation of serum acid sphingomyelinase activity in children with acute respiratory syncytial virus bronchiolitis. Tohoku J Exp Med. 2017;243(4):275-281.
12. Jenkins RW, Canals D, Hannun YA. Roles and regulation of secretory and lysosomal acid sphingomyelinase. Cell Signal. 2009;21(6):836–846.
13. Zhang Y, Willis-Owen S, Spiegel S, Lloyd CM, Moffatt MF, Cookson W. The ORMDL3 asthma gene regulates ICAM1 and has multiple effects on cellular inflammation. Am J Respir Crit Care Med. 2019;199(4):478-488.
14. Chen J, Miller M, Unno H, Rosenthal P, Sanderson MJ, Broide DH. Orosomucoid-like 3 (ORMDL3) upregulates airway smooth muscle proliferation, contraction, and Ca(2+) oscillations in asthma. J Allergy Clin Immunol. 2018;142(1):207–218.
15. Stancevic B, Kolesnicky R. Ceramide-rich platforms in transmembrane signaling. FEBS Lett. 2010;584(9):1728-1740.
involves the activation of acid sphingomyelinase. J Immunol. 2006;176(4):2397-2406.

39. Schneider-Schaulies J, Beyersdorf N. CD4+ Foxp3+ regulatory T cell-mediated immunomodulation by anti-depressants inhibiting acid sphingomyelinase. Biol Chem. 2018;399(10):1175-1182.

40. Bai A, Guo Y. Acid sphingomyelinase mediates human CD4(+) T-cell signaling: potential roles in T-cell responses and diseases. Cell Death Dis. 2017;8(7).

41. Collenburg L, Walter T, Burgert A, et al. A functionalized sphingolipid analogue for studying redistribution during activation in living T cells. J Immunol. 2016;196(9):3951-3962.

42. Grassme H, Schwarz H, Gubins E. Molecular mechanisms of ceramide-mediated CD95 clustering. Biochem Biophys Res Comm. 2001;284(4):1016-1030.

43. Schuchman EH. Acid sphingomyelinase, cell membranes and human disease: lessons from Niemann-Pick disease. FEBS Lett. 2010;584(9):1895-1900.

44. Boucher LM, Wiegmann K, Futterer A, et al. CD28 signals through acidic sphingomyelinase. J Exp Med. 1995;181(6):2059-2068.

45. Razzaq TM, Ozegbe P, Jury EC, Semb P, Blackwell NM, Kabouridis PS. Regulation of T-cell receptor signalling by membrane micro-domains. Immunology. 2004;113(4):413-426.

46. Kane LP, Lin J, Weiss A. It's all relative: NF-kappaB and CD28 costimulation of T-cell activation. Trends Immunol. 2002;23(8):413-420.

47. Cheng J, Montecalvo A, Kane LP. Regulation of NF-kappaB induction by TCR/CD28. Immunol Res. 2011;50(2-3):113-117.

48. Bai A, Moss A, Kokkotou E, et al. CD39 and CD161 modulate Th17 responses in Crohn's disease. J Immunol. 2014;193(7):3366-3377.

49. Oyeniran C, Sturgill JL, Hait NC, et al. Aberrant ORM (yeast)-like protein isoform 3 (ORMDL3) expression dysregulates ceramide homeostasis in cells and ceramide exacerbates allergic asthma in mice. J Allergy Clin Immunol. 2015;136(4):1035-1046.

50. Kiefer K, Casas J, Garcia-Lopez R, Vicente R. Ceramide imbalance and impaired TLR4-mediated autophagy in BMDM of an ORMDL3-overexpressing mouse model. Int J Mol Sci 2019;20(6).

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

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