

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.

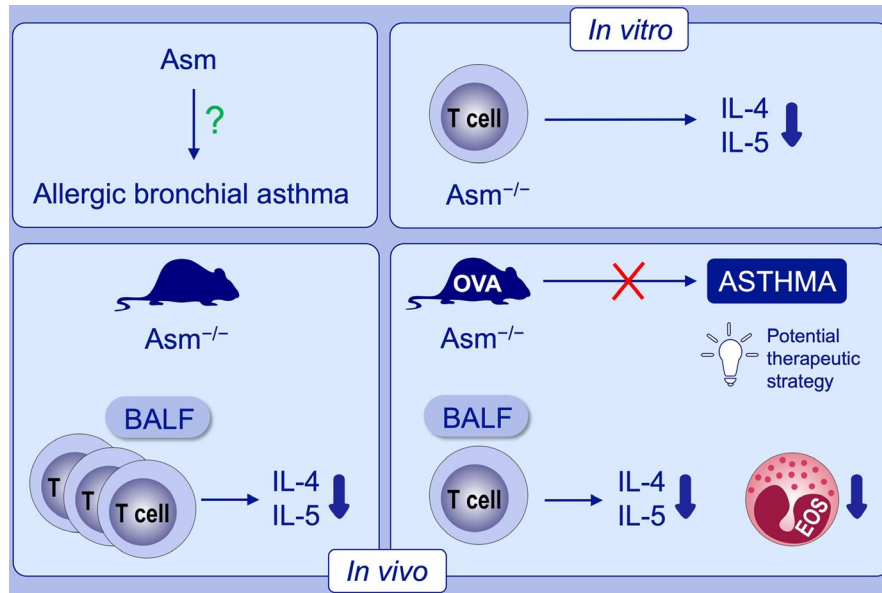
KEYWORDS

acid sphingomyelinase, allergic disease, asthmatic response, T cells, T_H2

*Böll and Ziemann equally contributed to this project and should be considered as co-first authors.

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GRAPHICAL ABSTRACT

This study investigated the role of acid sphingomyelinase (Asm) in allergic bronchial asthma. Asm deficiency resulted in reduced T_H2 cytokine release both in vitro and in vivo. In the ovalbumin model, $Asm^{-/-}$ mice were protected from bronchial asthma, showed decreased production of T_H2 cytokines and lower number of eosinophils.

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%.^{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 (S1P), 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 T_{reg} lymphocytes as well as transforming growth factor (TGF)- β , but decreased levels of IL-4 and IL-5. In vitro, Asm knock-out 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.

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 MACS separation using 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 μ mol/L) for 6 hours or incubated with anti-CD3/anti-CD28 and amitriptyline (50 μ mol/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-dodecano-yl) sphingosyl phosphocholine, Invitrogen) 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.

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 $\pm$ 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 ¹⁴C 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 (*R*_{rs}) and Newtonian resistance (*R*_n) as resistance of the larger airways tended to be lower in *Asm*^{−/−} mice compared with WT. Tissue resistance (*G*), total elastance (*E*_{rs}), 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

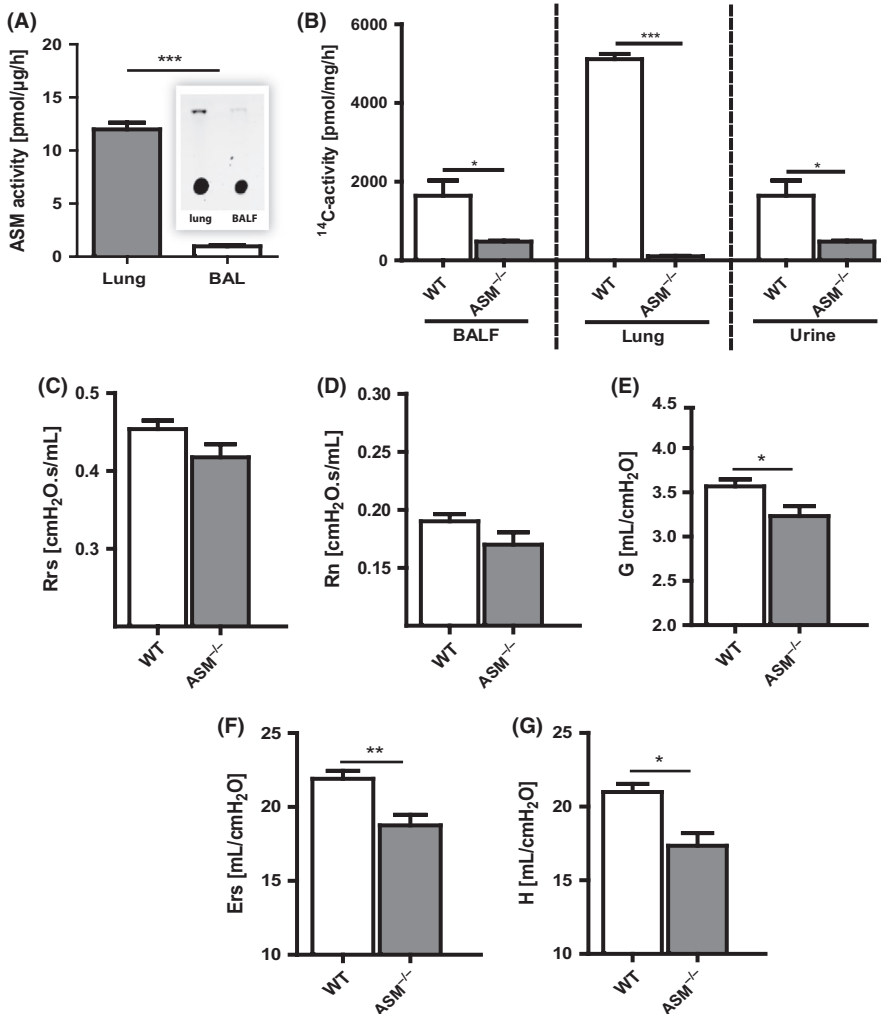


FIGURE 1 Asm activity in different lung compartments in WT and improved lung functions in Asm^{-/-} animals in homeostasis. (A) Asm activity in lung tissue (n = 6) and BALF (n = 5) of WT animals was determined by immunofluorescence assay. (B) Asm activity in BALF, lung, and urine in WT and Asm^{-/-} mice via radioactive ¹⁴C-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 ± SEM; * P < .05, ** P < .01, *** P < .001, (A, C-G) two-sided student's t tests, (B) one-sided student's t test

detected significantly higher numbers of CD4⁺ T cells in Asm^{-/-} 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 (Figure 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).

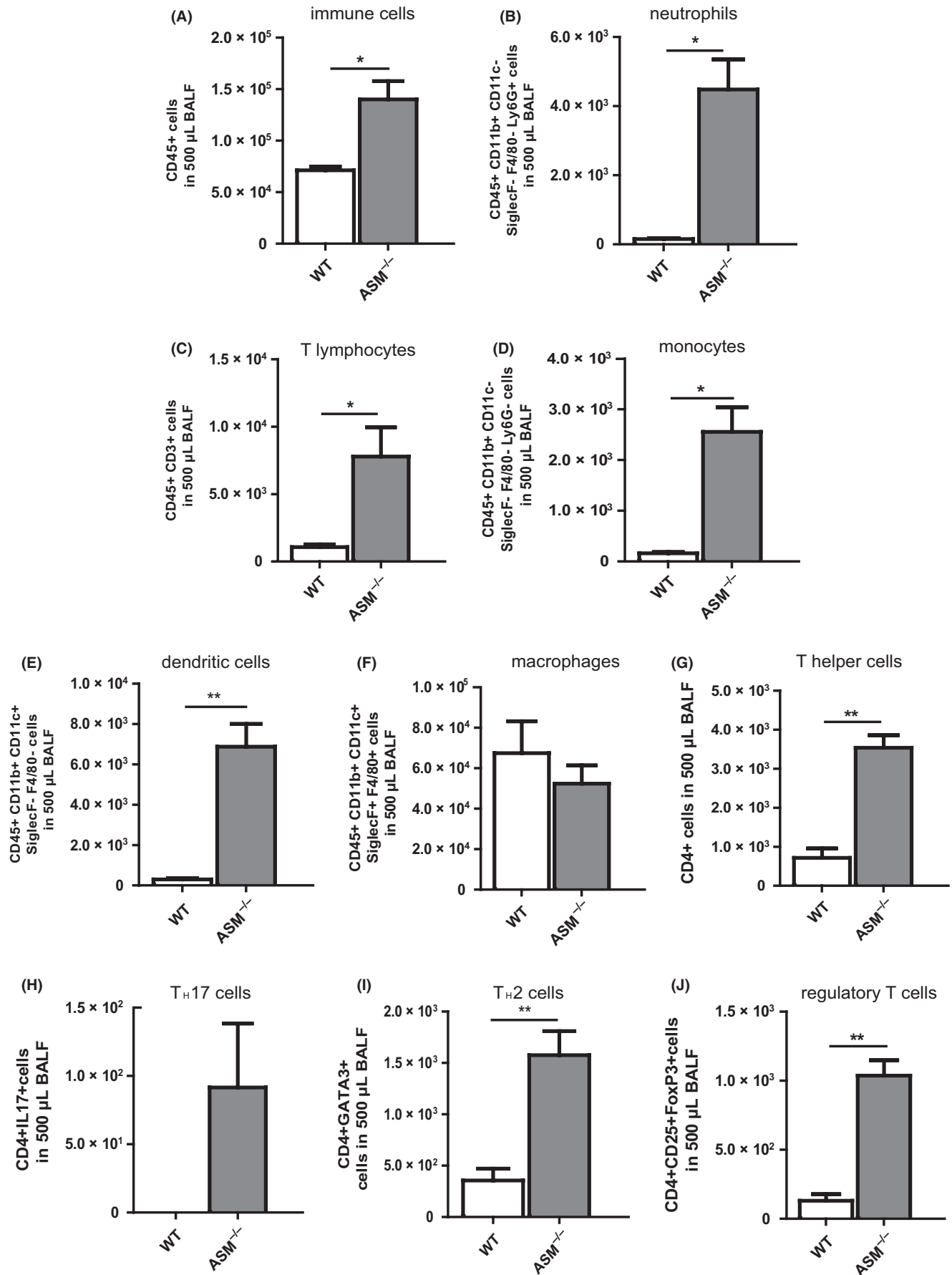
3.2 | Acid sphingomyelinase-deficiency changes immune cell function in vivo and in vitro

Having shown that Asm deficiency dramatically changes cell composition already in homeostasis including 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 (Figure 3A-C). The T_H2 cytokines IL-4 and IL-5 were reduced by approximately 50% in BALF of Asm^{-/-} mice (Figure 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 (Figure 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 (Figure 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 (Figure 4D). In addition, IL-4 per 1000 T_H2 cells was significantly lower in Asm^{-/-} T_H2 cells

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 ± SEM; *P < .05, **P < .01, (A-J) two-sided student's t test or for nonparametric data Mann-Whitney test



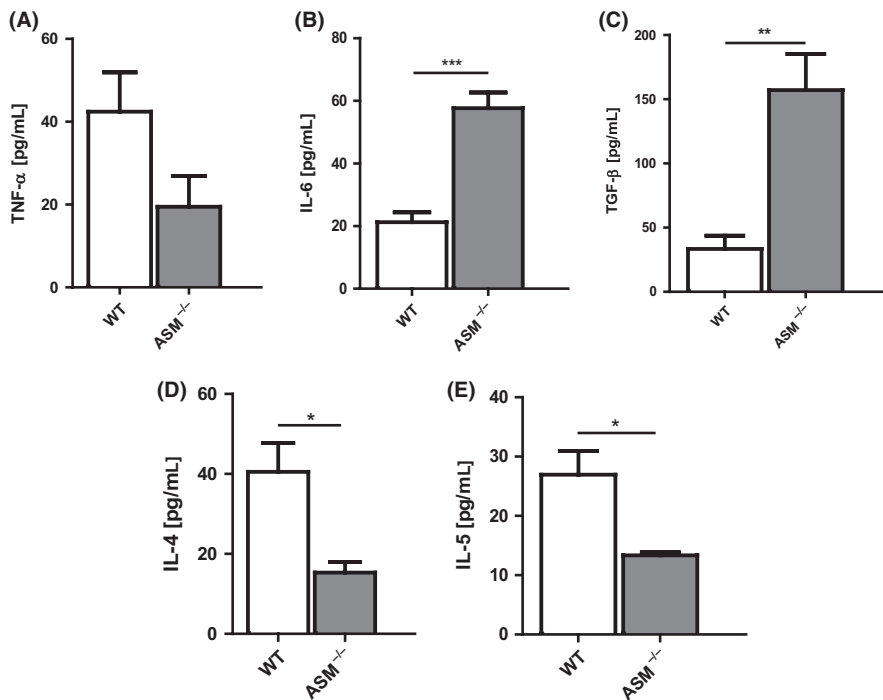


FIGURE 3 Cytokine profile in BALF of Asm^{-/-} mice tends to stronger T_H1- and attenuated T_H2-phenotype in homeostasis. BALF levels of TNF-α (A), IL-6 (B), TGF-β (C), IL-4 (D), and IL-5 (E) in untreated Asm^{-/-} and WT controls. A-E, n = 5 in both groups. All results represent means ± SEM; * *P* < .05, ** *P* < .01, *** *P* < .001, (A-E) two-sided student's *t* test or for nonparametric data Mann-Whitney test

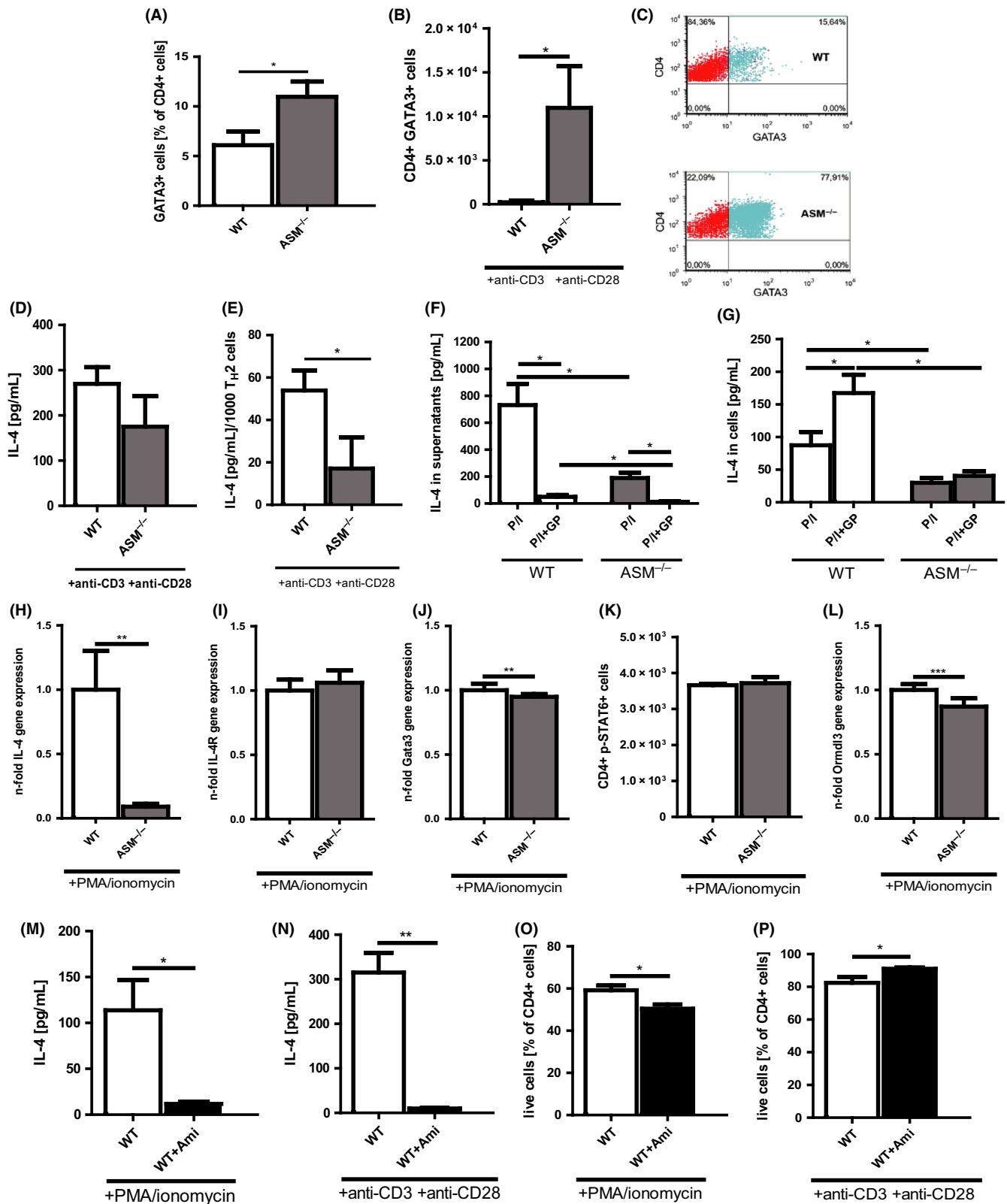
(Figure 4E). Thus, apparently CD4⁺GATA3⁺ T_H2 cells of Asm^{-/-} animals secrete much lower levels of IL-4 after stimulation. We next stimulated these cells with ionomycin and PMA to trigger cytokine secretion in the presence or absence of GolgiPlug, to inhibit protein transport from Golgi. Asm^{-/-} 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 (Figure 4F-G). Regarding IL-4 levels of lysed cells, we detected lower levels of IL-4 in Asm^{-/-} cells compared with 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 (Figure 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 with WT (Figure 4H). In contrast, IL-4R (CD124) was upregulated in ionomycin/PMA stimulated Asm^{-/-} T cells (Figure 4I). GATA3 gene expression levels were nearly the same as well as p-STAT6 levels in both groups (Figure 4J-K). Additionally, we found only a slight difference

in ORMDL3 levels (Figure 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 (Figure 4M-N). From the literature, it is known that ionomycin/PMA induces apoptosis, which we could also demonstrate in our experiments (Figure 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 (Figure 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.

3.3 | Acid sphingomyelinase deficiency is protective in a model of bronchial asthma

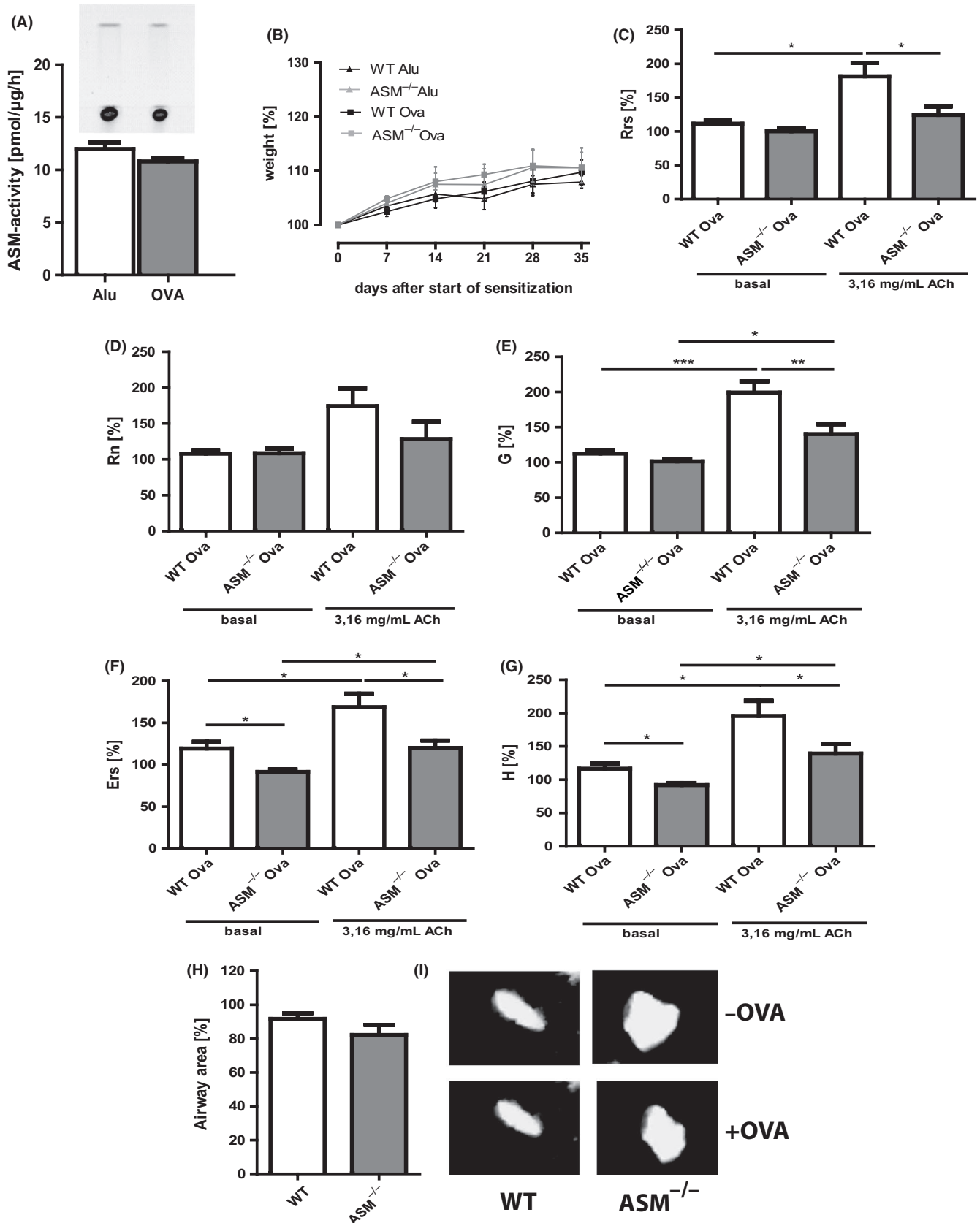
This strong difference of T_H2 cytokine secretion/production led us hypothesize that Asm deficiency is protective in allergic asthma.

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±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), and 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 μmol/L) 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 ± SEM; * *P* < .05, ** *P* < .01, *** *P* < .001, (A,B,D,E,H-P) one-sided student's *t* test or for nonparametric data Mann-Whitney test, (F,G) two-sided student's *t* test, FDR correction



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 with controls (Figure 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 (Figure 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 with WT animals (Figure 5C-G). Therefore, *Asm* deficiency is clearly protective for lung mechanics in this murine model of bronchial asthma.

FIGURE 5 $Asm^{-/-}$ is protective in asthmatic disease: lung mechanics. A, Asm activity in BALF of sensitized (OVA) ($n = 5$) and nonsensitized (Alu) ($n = 6$) WT animals via immunofluorescence assay. B, Weight of animals during time of sensitization. Percentage compared with starting weight. C–G, Lung function parameters of sensitized and nonsensitized 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 $\pm$ SEM; * $P < .05$, ** $P < .01$, *** $P < .001$, (A–H) two-sided student's t test, FDR correction

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 T_H17 and T_{reg} cells as Figure S2A–B). Levels of T_H1 cells were very low in both asthma groups (Figure S2C). Additionally, levels of IFN- γ were comparable in both groups with low total levels around detection level (Figure S2D). 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 T_H2 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 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 $Asm^{-/-}$ 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 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.³⁰ 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.^{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, that is RSV bronchiolitis. In that study, Asm was not elevated in the control groups with asthma or adenovirus infections.¹¹ 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 Ca^{2+} influx and Asm activity. After induction of systemic anaphylaxis, disease activity was less pronounced in $Asm^{-/-}$ animals compared with WT littermates.³³ Gupta et al demonstrated higher ASM levels and stronger activity in erythrocyte membranes of asthmatics compared with healthy controls.³⁴ 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

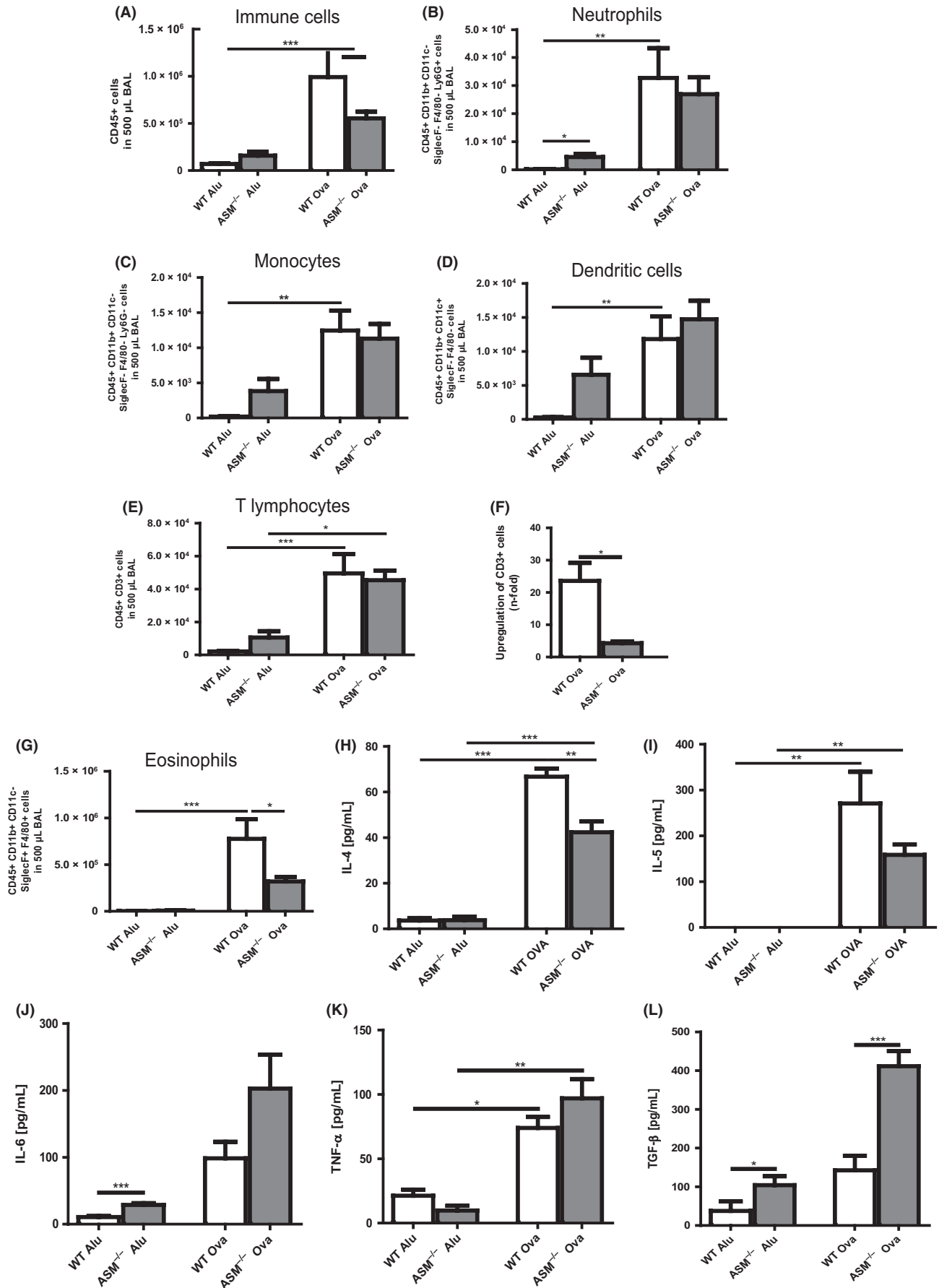


FIGURE 6 $Asm^{-/-}$ is protective in asthmatic disease: cell composition and cytokine profiles. Counts of total immune cells (A), neutrophils (B), monocytes (C), macrophages (D), and T lymphocytes (E). F, Upregulation of CD3+CD4+ cells compared with WT Alu. G, Count of eosinophils in sensitized $Asm^{-/-}$ 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 $\pm$ SEM; * $P < .05$, ** $P < .01$, *** $P < .001$, (A–L) two-sided student's t test, FDR correction

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 p-STAT6. 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.

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.³⁸ Especially, the outer membrane of T lymphocytes contains high ASM levels and ASM activity.³⁹ 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 receptors,⁴⁰ but up to now it has not been clearly shown whether these receptors activate ASM or whether it is the other way around.³⁹ 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—among others—interacts with cytokine receptors and induces T-cell differentiation, for example T_H2 differentiation.⁴³ However, the exact mechanism by which pathways ASM participates in T cell receptor (TCR)/CD3 and CD28 signaling remains unknown.^{40,44} Bai et al²¹ 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- γ 1-MAPK/JNK and CD28-PI3K-Akt-mTOR.^{45–47} Other receptors and signaling pathways regulated by ASM activity and increased ceramide production include STAT3 and mTOR signaling.⁴⁸ 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 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 model.⁴⁹ 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 levels.⁵⁰

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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SUPPORTING INFORMATION

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