

Immunoregulatory role of acid sphingomyelinase in allergic asthma

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Summary

Acid sphingomyelinase (ASM) is one of the enzymes that catalyzes the breakdown of sphingomyelin to ceramide and phosphorylcholine. In this study, we aimed at elucidating the role of ASM in allergic asthma. We used an ovalbumin-induced murine model of asthma where we compared wild-type and ASM-deficient mice. In wild-type mice, secretory ASM activity in the bronchoalveolar lavage fluid was increased in the acute ovalbumin model, but not in a tolerogenic model. Furthermore, in the absence of ASM, the serum IgE level was reduced, compared with wild-type mice, while an accumulation of interstitial macrophages and foreign antigen-induced regulatory T cells along with exhausted CD4⁺ PD1⁺ T cells was observed in the lungs of ASM^{-/-} mice. In conclusion, in the absence of ASM, we observed an accumulation of immunosuppressive antigen-induced regulatory T cells expressing Foxp3 and CTLA4 in the lung as well as multinucleated interstitial macrophages and exhausted CD4⁺ PD1⁺ T cells associated with inhibition of serum IgE in asthma.

Keywords: acid sphingomyelinase; asthma; immunosuppression; macrophages; regulatory T cells.

Introduction

The Global Initiative for Asthma estimates that about 300 million people worldwide suffer from asthma, a chronic inflammatory disease of the airways, characterized by variable obstruction of the airways and airway hyperresponsiveness (AHR) in response to re-exposure to usually innocuous antigens.¹ The immunological mechanisms underlying the pathogenesis are diverse, involving both the innate and adaptive immune systems. Unfortunately, trials that focused on, for example, blocking single

mediators such as interleukin-5 (IL-5), showed limited efficacy only.² Therefore, it is necessary to find new targets to improve asthma therapy and strategies for the prevention of asthma.

The lipid sphingomyelin is an important component of biological membranes. The molecule consists of phosphorylcholine and the bioactive lipid ceramide. Acid sphingomyelinase (ASM) is an enzyme separating those two components of sphingomyelin at the outer leaflet of the cell membrane, thereby releasing ceramide to act as a signal transduction molecule.³ There are two forms of ASM,

Abbreviations: AHR, airway hyperresponsiveness; ASM, acid sphingomyelinase; BALF, bronchoalveolar lavage fluid; Cer, ceramide; CTLA4, cytotoxic T lymphocyte-associated protein 4; FoxP3, forkhead box P3; IL-5, interleukin-5; ILC2, innate lymphoid cell type 2; OVA, ovalbumin; PD-1, programmed cell death 1; PBS, phosphate-buffered saline; SM, sphingomyelin; Treg, regulatory T; WT, wild-type

which derive from the same gene (*SMPD1*), but vary in their glycosylation patterns and are activated differently: lysosomal ASM (L-ASM) can be activated in the absence of zinc ions, whereas secretory ASM (S-ASM) activity is dependent on zinc ions.⁴ Activity of ASM can be further influenced by genetic variations. It has been previously reported that the ASM polymorphism p.G508R was associated with self-reported allergy. As this polymorphism was associated with lower S-ASM activity, there was also an association between S-ASM activity and self-reported allergy. However, when the authors controlled for ASM genotype, there was no difference in S-ASM activity between people with and without self-reported allergy.⁵ Further, in guinea pigs in an ovalbumin (OVA) -induced model of asthma, enhanced ceramide levels have been observed associated with airway epithelial cell apoptosis and inflammation.⁶ Other studies have shown that ceramide in mast cells is able to reduce IL-5, IL-10 and IL-13 production.⁷ ASM-deficient mice have been used mainly to study Niemann–Pick disease, because these patients display mutations in the *SMPD1* gene.⁸ However, they also show resistance to stress-induced apoptosis.⁹

ASM-deficient mice have been shown to develop lung pathology with proceeding age.¹⁰ Furthermore, a recent study reports an amelioration of symptoms and decreased OVA-specific IgE and IL-4 levels in nasal lavage fluid in an OVA-induced model of allergic rhinitis in mice treated with the ASM-inhibitor desipramine.¹¹

By using murine models of allergic asthma, here we aimed to delineate the functional role of ASM in allergic asthma.

Materials and Methods

Mice

All murine experiments were performed with approved licenses (54-2532.1-55/12) from the government of Unterfranken, Bavaria. The mice were bred and kept under specific pathogen-free conditions. For the experiments, wild-type mice on a BALB/c background, C57BL/6 ASM^{-/-} and C57BL/6 wild-type littermates (WT) of mixed gender were used at an age of 6–8 weeks.

OVA-induced model of asthma

The protocol for the OVA-induced model of asthma used for the present studies was previously published.¹² Briefly, mice were sensitized twice with intraperitoneal injections of 100 µg OVA (Calbiochem, Gibbstown, NJ) complexed to the adjuvant alum (10%, Sigma-Aldrich, Steinheim, Germany) on days 0 and 7, intranasal challenge with OVA (50 µg) dissolved in phosphate-buffered saline (PBS) was performed on days 18, 19 and 20, after anesthesia with isoflurane (Abbott, Wiesbaden,

Germany). The experiment ended on day 21, when bronchoalveolar lavage (BAL), serum and lung tissue were collected.

For the tolerogenic model, mice were treated with 50 µg OVA dissolved in PBS intranasally on days 0, 1 and 2. On day 13, all mice (tolerogenic and allergic asthma-OVA) received an intraperitoneal sensitization with 100 µg OVA complexed to alum (10%), followed by intranasal challenge with OVA in PBS (50 µg) on days 20, 21 and 22. The experiment ended on day 23 with the collection of BAL and serum.¹³

Measurement of airway hyperresponsiveness

Airway hyperresponsiveness was determined using a non-invasive, as well as an invasive method, as described previously.¹² Mice were challenged with first PBS, then increasing doses of methacholine (MCh, Sigma Aldrich, Steinheim, Germany). The non-invasive measurement was performed as whole-body plethysmography in a Buxco Electronics device (Data Sciences International, St. Paul, MN). Enhanced pause values (PenH) were recorded. For invasive AHR measurement a FlexiVent FX1 device and FLEXIWARE 7.2.2 software (SCIREQ Scientific Respiratory Equipment, Montreal, Quebec, Canada) were used. Respiratory system resistance (Rrs) was recorded 12 times per dose of PBS/MCh.

Bronchoalveolar lavage

For the collection of BAL, the lungs were flushed twice with 800 µl of Sterofundin (Braun, Melsungen, Germany) each, as previously described.¹⁴ BAL fluid (BALF) was collected after centrifugation and stored at –20° for further analysis. Cells were counted using a Neubauer chamber (Carl Roth, Karlsruhe, Germany) for total cell count and 5×10^4 cells were used for cytopins. For differential cell counts, May–Grünwald–Giemsa (Carl Roth, Karlsruhe, Germany) staining was performed using an established protocol.

Preparation of lung tissue for histology and lung cell isolation

Lungs were removed from the euthanased mice. Lung tissue for histology was fixed in 10% formalin–PBS solution and embedded in paraffin after dehydration. Sections of 5 µm thickness were prepared and stained with periodic acid–Schiff to determine mucus production.

Total lung cell isolation was performed using an established protocol.¹⁵ Isolated cells were either used for flow cytometry or cell culture (1×10^6 cells per ml RPMI-1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 500 µg/ml OVA) for 24 hr at 37°, 5% CO₂.

Flow cytometry

Freshly isolated lung or spleen cells were incubated with antibodies detecting surface markers for 30 min at 4°, after washing the cells, they were analyzed on a BD FACS Calibur (CELLQUEST PRO Software, BD Biosciences, Heidelberg, Germany) or a BD FACS Canto II (BD FACSDIVA™ software, BD Bioscience). If intracellular markers were stained, after the washing process the cells were first fixed with CytoFix/CytoPerm™ solution (BD Bioscience) for 35 min at 4° and then stained with the respective antibody in Perm/Wash™ solution (BD Bioscience) for an additional 30 min at 4°. Cells were analyzed as mentioned above. The following fluorochrome-labeled antibodies were used: CD4 fluorescein isothiocyanate, CD11b V450, CD25 peridinin chlorophyll protein (PerCP)-Cy5.5, CTLA4 phycoerythrin (PE), PD-1 allophycocyanin, IL-10 PE (BD Biosciences). CD11c, FoxP3 (Miltenyi Biotec, Bergisch Gladbach, Germany). F4/80 PerCP (BioLegend, Fell, Germany). Evaluation of the data was done using FLOWJo software (TreeStar, Ashland, OR).

IL-10 intracellular staining

Total lung murine cells from naive and asthmatic wild-type BALB/c mice were cultured in RPMI-1640 cell culture medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin in pre-coated cell culture wells with 2 µg/ml αCD3 in 0.1 M NaHCO₃-buffer. Cells were cultured with 2 µl anti-CD28/ml for about 20 hr followed by stimulation with 50 ng/ml of phorbol-12-myristate 13-acetate (Sigma-Aldrich), 500 ng/ml ionomycin (Sigma-Aldrich) and using a Golgi-Stop treatment (Golgi-Stop™, BD) for 4 hr at 37°. After cell culture, the cells were harvested, centrifuged (5 min, 423 × g, 4°) and the supernatants were frozen at -80°.

The cell pellet was first stained with cell surface staining, using 50 µl of MasterMix consisting of the selected antibodies, diluted in PBS. For each compensation control 1 µl of the respective antibody (fluorophore) was added to 50 µl PBS. All samples were incubated for 30 min at 4° in darkness.

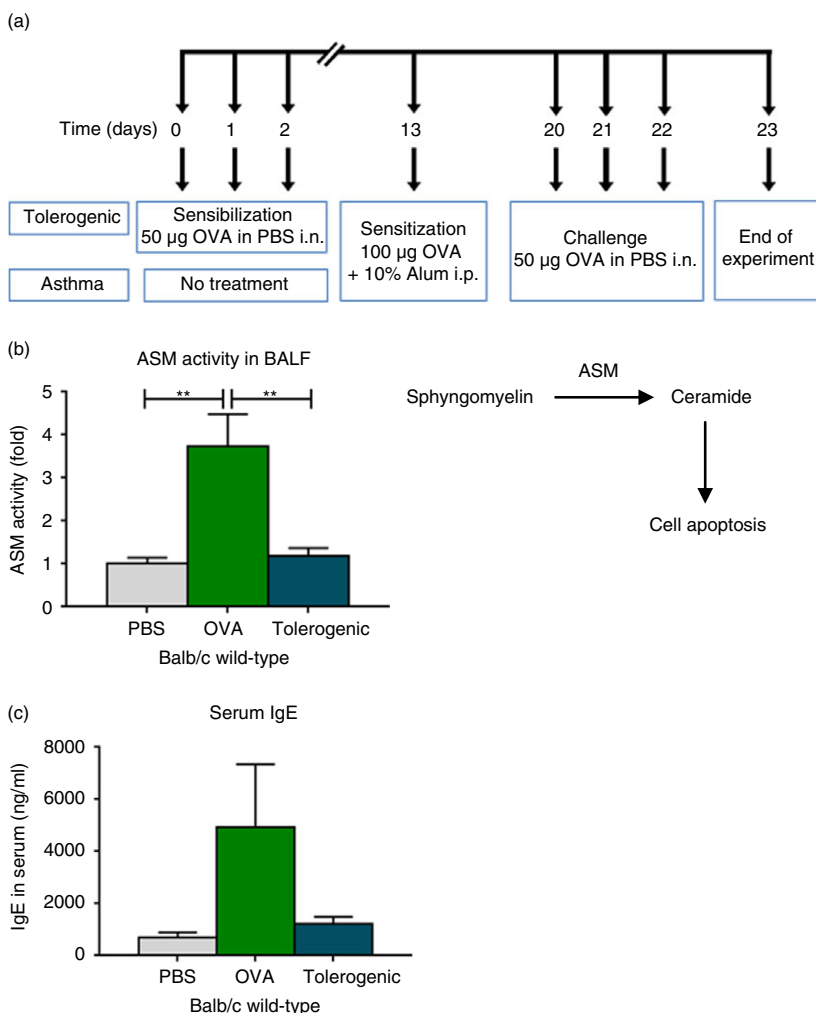


Figure 1. Increased acid sphingomyelinase (ASM) activity in asthma and decreased ASM activity and IgE in an airway tolerogenic model of asthma. (a) Experimental design of the tolerance model of asthma. (b) S-ASM activity in bronchoalveolar lavage fluid (BALF) ($n = 5$ to $n = 7$ /group) from BALB/c wild-type mice subjected to a tolerogenic or asthma model. (c) Serum IgE levels ($n = 4$ or $n = 5$ /group). Data are presented as mean $\pm$ SEM, statistical analysis was performed with two-tailed Student's *t*-test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Data are from representative experiments of at least two independent experiments.

Subsequently the stained cells were washed with 200 μ l PBS, centrifuged (5 min, $423 \times g$, 4°) and the supernatants were discarded. Before intracellular IL-10 staining the cells were incubated in 150 μ l PermFix solution for 35 min at 4° in the dark. After another centrifugation step (5 min, 1500 rpm, 4°), the supernatants were discarded and 50 μ l PermWash mixed with the chosen IL-10 antibody was added to each sample (but not the compensation controls and blank). After another 30 min incubation (4° , darkness), the samples were washed by adding 200 μ l PermWash solution and a centrifugation step (5 min, 1500 rpm, 4°). After discarding the supernatants, 200 μ l PBS was added to each sample and vortexed before FACS analysis.

The following FACS-antibodies were used: PE rat anti-mouse IL-10 (BD; Cat. 554467); PE rat IgG2b, κ isotype control (BD; Cat. 556925); PerCP-Cy5.5 rat anti-mouse CD25 (BD; Cat. 551071); CD4-Vio Blue, mouse (Miltenyi Biotec; Cat. 130-110-411).

Staining solutions: Fixation/Perm Diluent (Invitrogen/eBioscience; Cat. 00-5223-56). Fixation/permeabilization concentrate (Invitrogen/eBioscience; Cat. 00-5123-43). Permeabilization buffer $10\times$ (Invitrogen/eBioscience; Cat. 00-8333-56).

ELISA

For the detection of proteins in serum, BALF or cell culture supernatants, ELISA Kits were used according to the manufacturer's protocol. The following kits were used: OptEIA™ ELISA Kit for the detection of murine IgE, IgG2a and IL-10 (BD Bioscience).

S-ASM activity in BALF

The enzymatic activity of S-ASM in BALF was determined by measuring hydrolysis of fluorescence-labeled SM (BODIPY® FL C12-sphingomyelin; Life Technologies,

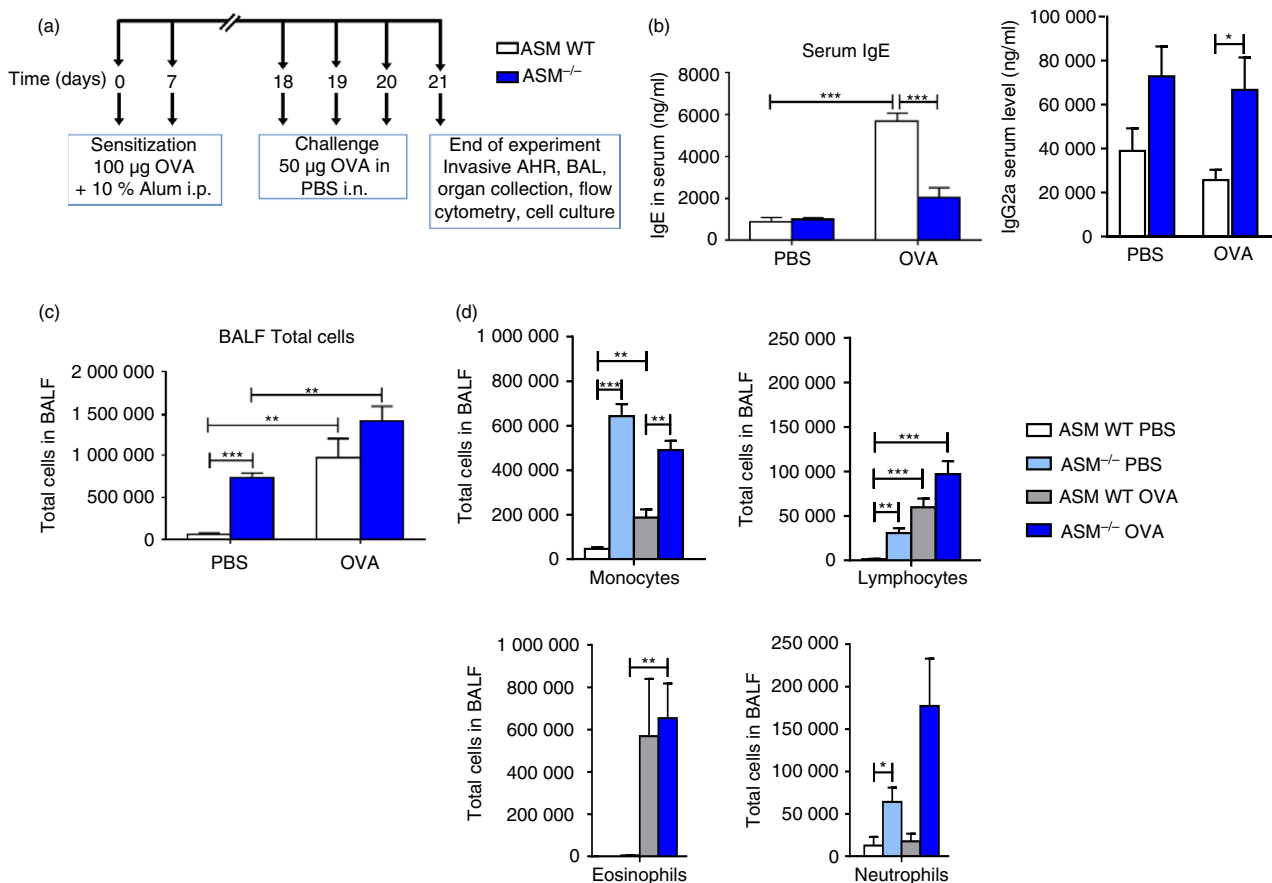


Figure 2. Decreased serum IgE levels and increased serum IgG2a and cell number in the bronchoalveolar lavage fluid (BALF) in acid sphingomyelinase (ASM) -deficient mice. (a) Experimental design of the ovalbumin (OVA) -induced model of asthma. (b) Serum IgE and IgG2a levels measured by ELISA. ($n = 3$ or $n = 4$ /group). (c, d) BALF total (c) and differential cell counts (d) in wild-type and ASM-deficient mice with and without asthma. ($n = 3$ to $n = 5$ /group) Data are presented as mean $\pm$ SEM, representative dot plots are shown. Statistical analysis was performed using a two-tailed Student's *t* test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Data are from representative experiments of two to four independent experiments.

Darmstadt, Germany), as previously published.¹⁶ Following separation by thin-layer chromatography, ceramide levels were visualized with a Typhoon Trio scanner (GE Healthcare, Munich, Germany) and analyzed using the QUANTITYONE software (Bio-Rad, Munich, Germany).

Statistical analysis

Data are shown as mean values $\pm$ SEM of one representative experiment. All experiments were performed two to four times using three to seven mice per group. Significant differences were evaluated using the Student's two-tailed *t*-test (Windows EXCEL software, Microsoft, Redmond, Washington, USA and GRAPHPAD PRISM Software, San Diego, California, USA). **P* $\leq$ 0.05; ***P* < 0.01; ****P* < 0.001.

Results

Increased ASM in a murine model of allergic asthma was inhibited by airway tolerance

It has been shown previously that respiratory tolerance is highly effective in preventing the development of airway inflammation and hyperreactivity.^{13,17} We therefore asked

if ASM activity was regulated in a model of asthma and airway tolerance, where mice are challenged with OVA intranasally before being sensitized (Fig. 1a). Here, we found that BALF ASM activity was induced in a murine model of asthma and decreased in mice treated in an airway tolerance model compared with the asthma model (Fig. 1b). Hence, airway tolerance suppresses overall ASM activity induced in asthma.

Reduced serum IgE in airway tolerance and in ASM^{-/-} asthmatic mice

In serum, the main antibody class associated with allergy, IgE, was found to be down-regulated in the tolerogenic model compared with asthma (Fig. 1c). Moreover, in a murine model of asthma (Fig. 2a) the serum IgE levels were reduced and IgG2a was induced in ASM-deficient mice (Fig. 2b).

Increased cell number in the BALF of asthmatic ASM^{-/-} mice

Asthma is characterized by airway hyperresponsiveness and induction of inflammatory cells in the airways,

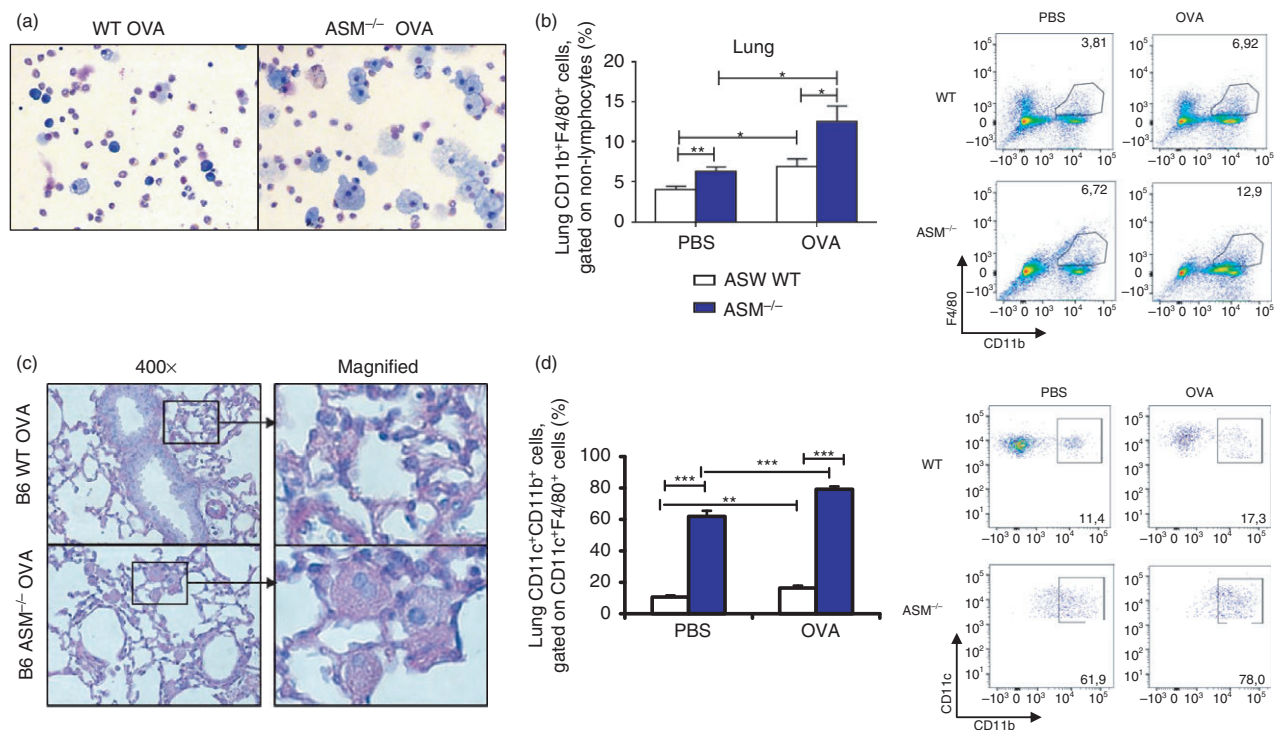


Figure 3. Increased interstitial macrophage survival in acid sphingomyelinase (ASM) -deficient mice in a model of asthma. (a) Representative light microscopic pictures of bronchoalveolar lavage fluid (BALF) cells stained with May-Grünwald-Giemsa. Magnification 400 $\times$ (*n* = 4/group) (b and d). Determination of lung interstitial macrophages (CD11b⁺ F4/80⁺ cells, gated on non-lymphocytes) by flow cytometry (b), and CD11c⁺ CD11b⁺ F4/80⁺ cells, gated on non-lymphocytes by flow cytometry (d). (*n* = 6/group) (c) Representative pictures of lung sections stained with periodic acid-Schiff. (*n* = 4/group). Data are presented as mean $\pm$ SEM. Representative dot plots are shown. Statistical analysis was performed with two-tailed Student's *t*-test. **P* < 0.05; ***P* < 0.01; ****P* < 0.001. Data are from representative experiments of at least two independent experiments.

especially eosinophilic granulocytes.¹⁸ Analysis of WT and ASM-deficient mice in the OVA-induced model of asthma revealed no significant difference between OVA-treated WT and ASM^{-/-} mice in invasively measured AHR. Similarly, by using the non-invasive method, no AHR was observed in WT mice (see Supplementary material, Fig. S1a,b). In accordance with previously published data¹⁰ stating that ASM^{-/-} mice have more cells in the BAL, we were able to confirm this finding in naive and asthmatic ASM^{-/-} mice, as compared with wild-type control mice (Fig. 2c). By looking at the differential cell counts of the cells present in BAL, we noticed, besides the increased presence of lymphocytes and eosinophils, which is typical of allergic asthma, a significant increase in mononuclear cells. Furthermore neutrophils were induced in the absence of ASM, although not statistically significantly in the asthma model (Fig. 2d).

Increased survival of interstitial macrophages in the absence of ASM in a murine model of asthma

It has been previously shown that with advancing age, more and larger alveolar macrophages with 'foamy' and often multinucleated appearance are present in the airways in the absence of ASM.¹⁰ By looking at the cytopins of the BALF we noticed an increase of big alveolar macrophages in the airways of ASM-deficient mice, compared with the wild-type littermates (Fig. 3a).

In line with previous findings, we also observed large and foamy multinucleated cells in our model of allergic asthma in ASM-deficient mice (Fig. 3a,c). This could be the result of defective apoptosis of these cells, as ASM is an important mediator of this cellular process, especially after cell stress.⁹ Taken together, the increased number of cells in the airways of ASM-deficient naive mice is probably not due to the recruitment of inflammatory cells to the airways, but rather due to a

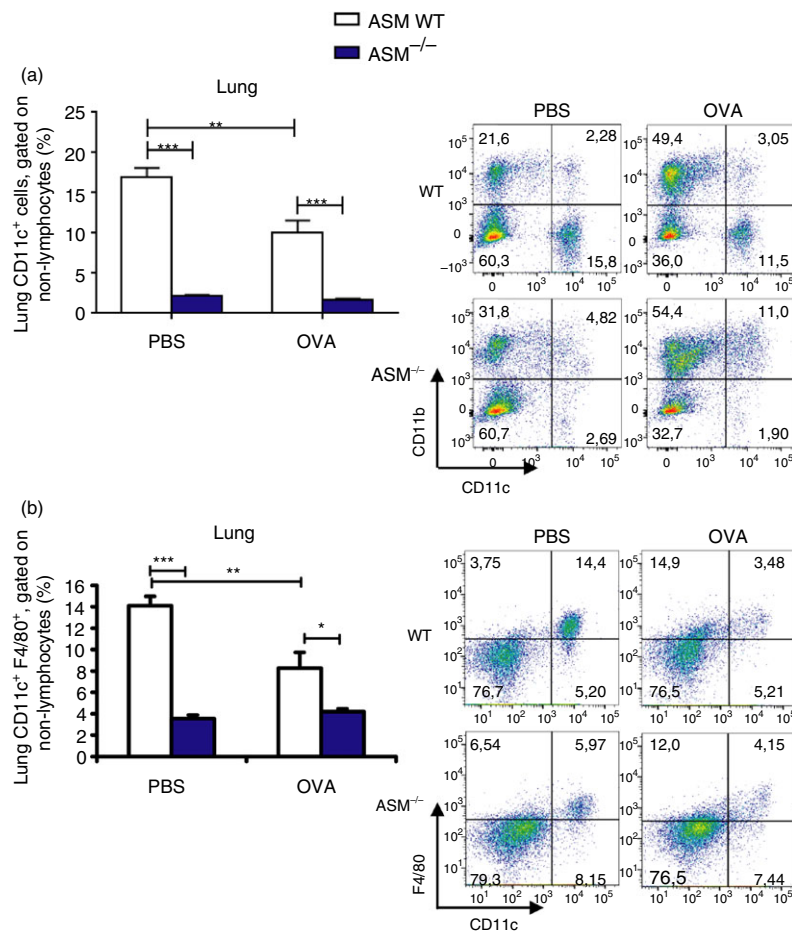


Figure 4. Decreased proportions of CD11c⁺ cells in acid sphingomyelinase (ASM) -deficient asthmatic mice. (a, b) Alveolar macrophages were analyzed as CD11c⁺ (a) and CD11c⁺ F4/80⁺ (b) gated on non-lymphocytes and they were found to be significantly decreased in the lung of ASM^{-/-} mice ($n = 6$ per group). Statistical analysis was performed with two-tailed Student's t test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Data are from representative experiments of at least two independent experiments.

decrease in cell death of alveolar macrophages, which are the predominant cell type in the BAL of naive mice.

By looking at the phenotype of these macrophages in the lung of ASM^{-/-} asthmatic mice, we found that they were of interstitial type, expressing CD11b, CD11c and F4/80 (Fig. 3b,d).¹⁹ Interstitial macrophages promote neutrophil extravasation, so explaining the induction of

neutrophils in the BALF of ASM^{-/-} mice (Fig. 2d). These macrophages were also visible histologically (Fig. 3c).

By contrast, the percentage of CD11c⁺ F4/80⁺ cells among non-lymphocytes, typical markers of alveolar macrophages, was reduced in the absence of ASM both in naive and in asthmatic mice, compared with wild-type control mice (Fig. 4a,b).

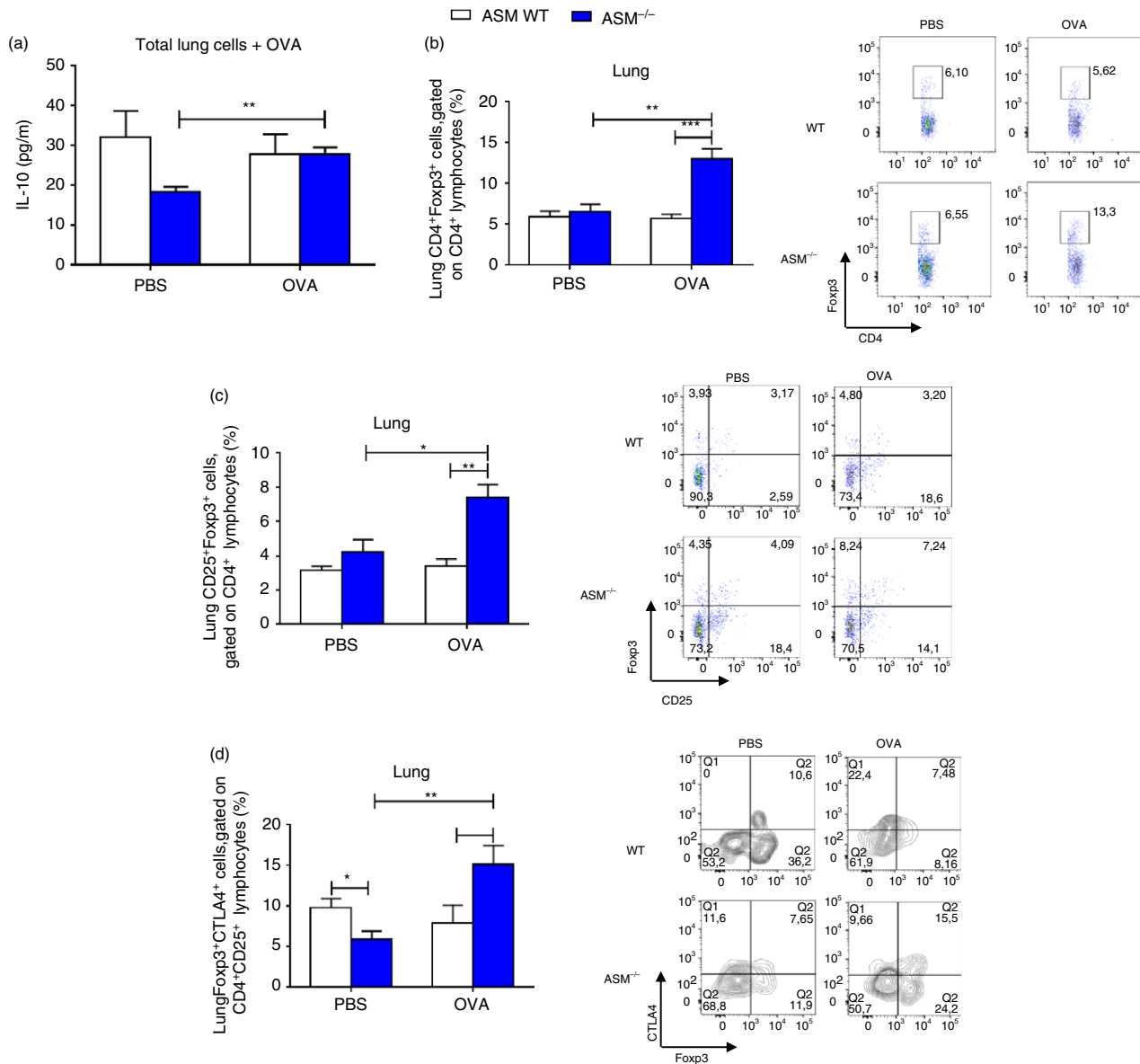


Figure 5. Elevated proportions of induced regulatory T (Treg) cells in acid sphingomyelinase (ASM) -deficient asthmatic mice. (a) Interleukin-10 (IL-10) levels were measured by ELISA in cell culture supernatants of ovalbumin (OVA) -re-stimulated total lung cells isolated from naive or asthmatic B6 wild-type or ASM^{-/-} mice. (b) Flow cytometric analysis of lung CD4⁺ Foxp3⁺ ($n = 4$ /group); (c) CD4⁺ CD25⁺ Foxp3⁺ ($n = 4$ /group), and (d) CD4⁺ CD25⁺ Foxp3⁺ CTLA4⁺ Treg cells ($n = 5$ or $n = 6$ /group) (d). Data are presented as mean $\pm$ SEM and representative dot plots are shown. Statistical analysis was performed using a two-tailed Student's *t*-test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Data are from representative experiments of three to four independent experiments (d).

Elevated immunosuppressive IL-10 levels and proportions of antigen-induced CD4⁺ Foxp3⁺ CD25⁺ and CD4⁺ CD25⁺ CTLA4⁺ regulatory T (Treg) cells in the lungs of asthmatic ASM^{-/-} mice

In addition, the immunosuppressive cytokine IL-10²⁰ was found to be unchanged in the supernatants of *in vitro* OVA-restimulated total lung cells from naive and OVA sensitized and challenged WT mice, but induced by *in vivo* OVA-treatment in ASM-deficient mice (Fig. 5a). These data indicate a more immunosuppressive phenotype of lung cells in the absence of ASM.

In line with the previous findings of a tolerogenic/immunosuppressive phenotype in the absence of ASM, we further observed an increase in the proportion of lung CD4⁺ Foxp3⁺ (Fig. 5b), as well as in CD4⁺ CD25⁺ Foxp3⁺ Treg cells (Fig. 5c) among CD4⁺ T cells in ASM^{-/-} asthmatic mice. These Treg cells have been described to have

a protective role in asthma.²¹ These findings further indicate an immunosuppressive phenotype in the absence of ASM and are in line with a recent discovery of Hollmann *et al.*, describing enhanced Treg cell numbers with a higher suppressive capacity in ASM-deficient mice.²²

We next asked if these Treg cells are induced Treg cells. To answer this question we stained the Treg cells for CTLA4, a marker that indicates that Treg cells are induced by a foreign allergen challenge.²³ We found that these CD4⁺ CD25⁺ Foxp3⁺ CTLA4⁺ T cells were decreased among CD4⁺ CD25⁺ T cells of naive mice and induced in the lung of ASM^{-/-} mice after OVA sensitization and challenge (Fig. 5d). Although additional studies using new identified markers like Helios and Neuropilin-1 for thymus-derived Treg cells are needed,²⁴ these data support the finding that ASM deficiency is associated with the development of allergen-induced Treg cells in the lungs of asthmatic mice.

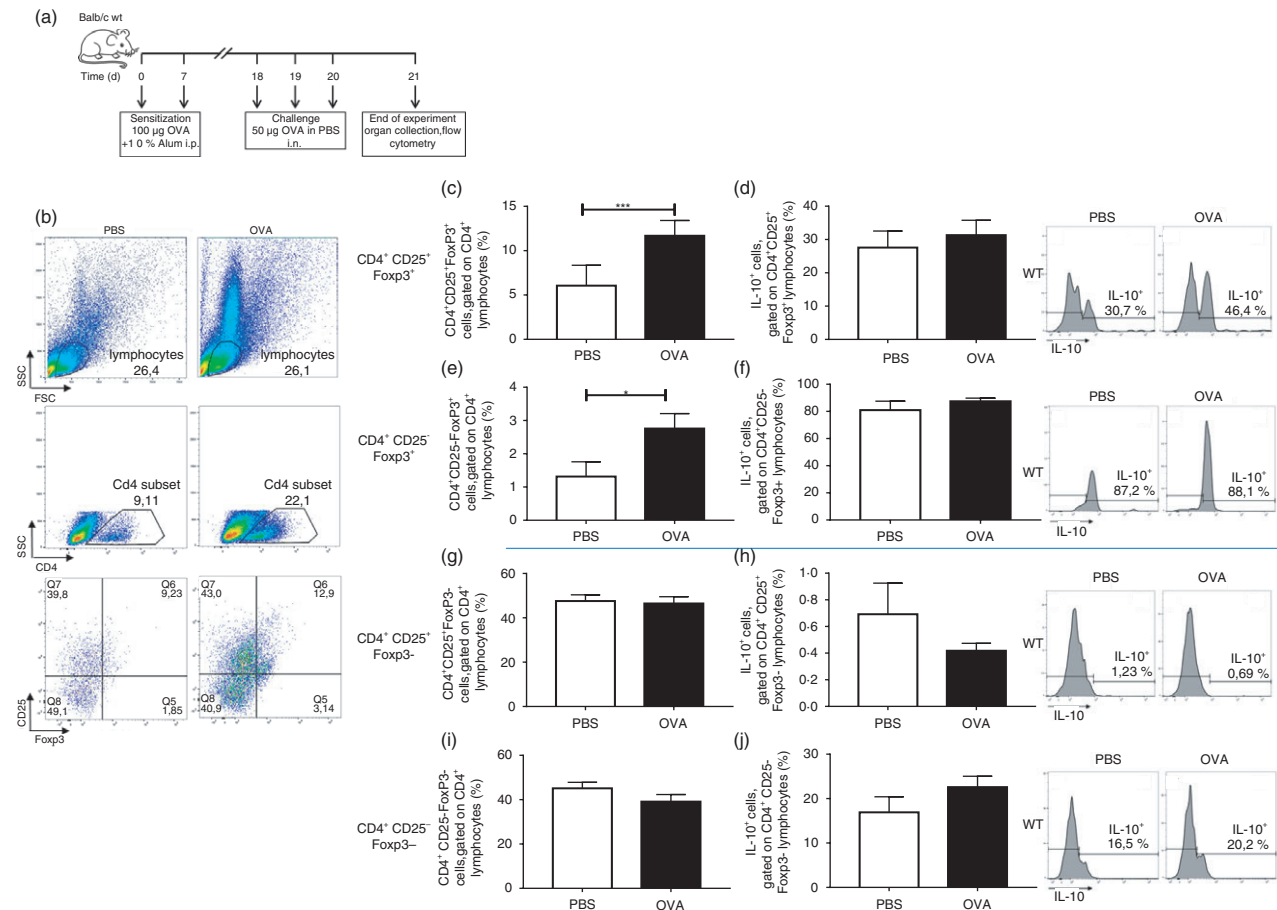


Figure 6. Proportions of subsets of IL-10⁺ regulatory T (Treg) cells and conventional CD4⁺ T cells in asthmatic mice. (a) Experimental design of the ovalbumin (OVA) -induced model of asthma. (b, c, e, g, i) Flow cytometric analysis of lung CD4⁺ T cells isolated from naive or asthmatic BALB/c mice: representative dot plot (b), CD4⁺ CD25⁺ Foxp3⁺ (c), CD4⁺ CD25⁺ Foxp3⁺ (e), CD4⁺ CD25⁺ Foxp3⁺ (g) and CD4⁺ CD25⁺ Foxp3⁺ (i) (*n* = 6/group). (d, f, h, j) Flow cytometric analysis of IL-10, gated on lung CD4⁺ CD25⁺ Foxp3⁺ (d), CD4⁺ CD25⁺ Foxp3⁺ (f), CD4⁺ CD25⁺ Foxp3⁺ (h) and CD4⁺ CD25⁺ Foxp3⁺ (j). Data are presented as mean ± SEM and representative dot plots are shown. Statistical analysis was performed using a two-tailed Student's *t*-test. **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

Distribution of IL-10 producing CD4⁺ T cells in the lungs of mice with and without asthma

We next asked what the cellular CD4⁺ T-cell source of IL-10 production in our model is, whether they are conventional T cells or Treg cells. To this aim we performed intracellular FACS staining for IL-10 in Treg cells or conventional T cells.²⁵ As we reported in Fig. 5, as well as in BALB/c in Fig. 6(a–d), CD4⁺ CD25⁺ Foxp3⁺ T cells (Treg cells) represent around 5% of the CD4⁺ T cells in the lung, and CD4⁺ CD25[−] Foxp3⁺ cells represent only 1% of the CD4⁺ T-cell population (Fig. 6c,e). Both populations were found induced by the allergen in a BALB/c background. By analyzing IL-10, using FACS, we found that around 30% of the CD4⁺ CD25⁺ Foxp3⁺ T cells express IL-10 (Fig. 6d). Moreover, 80% of the CD4⁺ CD25[−] Foxp3⁺ cells express IL-10 intracellularly (Fig. 6f). The majority of the CD4⁺ T cells in the lung in our model are Foxp3 negative, both CD4⁺ CD25⁺ and CD4⁺ CD25[−] account for around 40% each (Fig. 6g,i respectively). Further, few CD4⁺ Foxp3[−] CD25⁺ T cells express IL-10 whereas around 20% of the CD4⁺ Foxp3[−] CD25[−] cells express it in the lung (Fig. 6h,j). Hence, these latter cells represent the predominant source of IL-10 in the CD4⁺ T-cell

compartment, along with the conventional Treg CD4⁺ CD25⁺ Foxp3⁺ cells, and could be classified as Tr1 cells. Further studies are necessary to better define this population by using specific markers.

The only cell population induced by allergen in our model of asthma was the CD4⁺ CD25[−] Foxp3[−] T-cell population (Fig. 6g).

Increased proportion of PD1⁺ and CTLA4⁺ CD4⁺ T cells in the absence of ASM in the lung of asthmatic mice

Moreover, the expression of the inhibitory molecule PD-1 was enhanced in the lungs of ASM^{−/−} mice, in an OVA-treated setting (Fig. 7a). In addition, CTLA4 was found induced on lung CD4⁺ T cells from naive ASM-deficient mice compared with wild-type littermates, consistent with a tolerogenic role of ASM-deficiency in asthma (Fig. 7b).

Discussion

The lipid sphingomyelin is an important element of biological membranes involved in cell survival and death, as well as in exocytosis. Previous studies have shown that

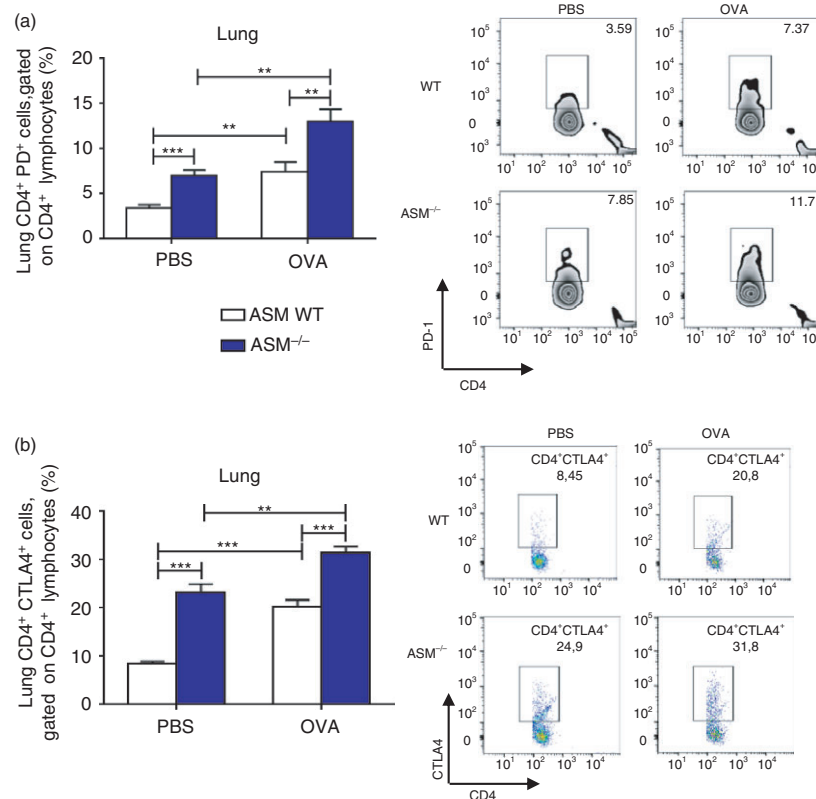


Figure 7. Increased expression of different exhaustion markers in acid sphingomyelinase (ASM) -deficient mice. (a, b) Flow cytometric analysis of lung CD4⁺ PD-1⁺ T cells (a) and CTLA4 expression on CD4⁺ T cells (b) ($n = 4$ to $n = 6$ /group). Data are presented as mean $\pm$ SEM, representative dot plots are shown. Statistical analysis was performed using a two-tailed Student's *t*-test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Data are from representative experiments of two to four independent experiments.

ASM is released from lysosomes into the extracellular medium during cell injury.⁴ Here, we have determined the functional role of ASM in allergic asthma.

In this study, we describe an increase in S-ASM activity in the airways after allergen-induced experimental asthma. Further, we found that ASM^{-/-} mice have decreased serum IgE levels and increased proportions of foreign antigen-induced Treg cells in the lung.²⁶ Consistent with this concept, we observed that airway tolerance reduced ASM activity in the airways and IgE in serum.²²

As mentioned above and consistent with previous reports,^{11,22} we observed an increase in the proportion of allergen-induced lung CD4⁺ FoxP3⁺ Treg cells in the absence of ASM, which have been described to have a protective role in asthma.²¹ Other T-cell subsets, such as T helper type 1 and type 2 cells, were not altered by ASM deficiency (data not shown) whereas inhibitory markers such as PD-1 and CTLA4 were found induced on lung CD4⁺ T cells. This could also be the reason for the down-regulation of the IgE production in ASM-deficient mice.

Previous studies on the role of ASM in T cells have revealed a defective activation and differentiation of T helper cell subsets in the absence of ASM and its downstream metabolite ceramide,¹⁰ therefore further experiments are needed to clarify the underlying mechanisms linking the ASM–ceramide-pathway to T-cell differentiation. We hypothesize that the increased Treg cell fraction, especially in naive and in asthmatic ASM-deficient mice, could be responsible for the decreased recruitment of inflammatory cells to the lungs of asthmatic ASM^{-/-} mice and probably also for the dysregulated secretory function of several relevant immunological cells including B cells, which regulate IgE production in asthma. In conclusion, the increase of Treg cells could be responsible for the resolution of allergic asthma in ASM-deficient mice.

It has been previously shown in ASM deficiency that more abundant and bigger alveolar macrophages with a ‘foamy’ and multinucleated characteristic accumulate in the airways with proceeding age, which we were able to confirm in our studies and which is probably a result of defective apoptosis in the absence of ASM.^{9,10} The precise phenotype of these cells, probably macrophages, remains to be elucidated. In conclusion, we assume that the overall increased number of cells in the airways of ASM-deficient mice is not caused by a recruitment of inflammatory cells to the site of inflammation, as usually observed in asthma, but rather by an accumulation of interstitial macrophages, unable to undergo cell death due to ASM-deficiency.¹¹

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Disclosures

The authors declare no conflict of interest.

Author contribution

NS performed all the experiments described in this manuscript, wrote the manuscript and prepared the manuscript text and the figures. MR determined the S-ASM activity, SK contributed with the murine models. CR. took care of the mice and contributed to the S-ASM activity, JK provided the ASM knockout mice and contributed to the scientific background of the hypothesis and discussion and SF supervised the entire work and contributed to figure generation. Susetta Finotto with Julia Koelle and Sonja Dumendiak did the manuscript revisions and entirely generated Fig. 6.

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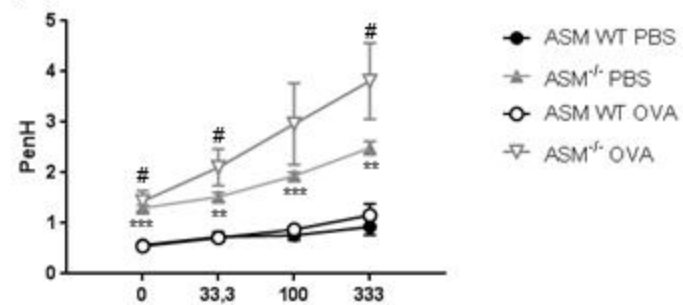
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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. A–B, Airway hyperresponsiveness determined by non-invasive whole-body plethysmography (PenH, A) and an invasive method (Rrs, B) in naïve and OVA-treated WT and ASM-/- mice.

(a)



(b)

