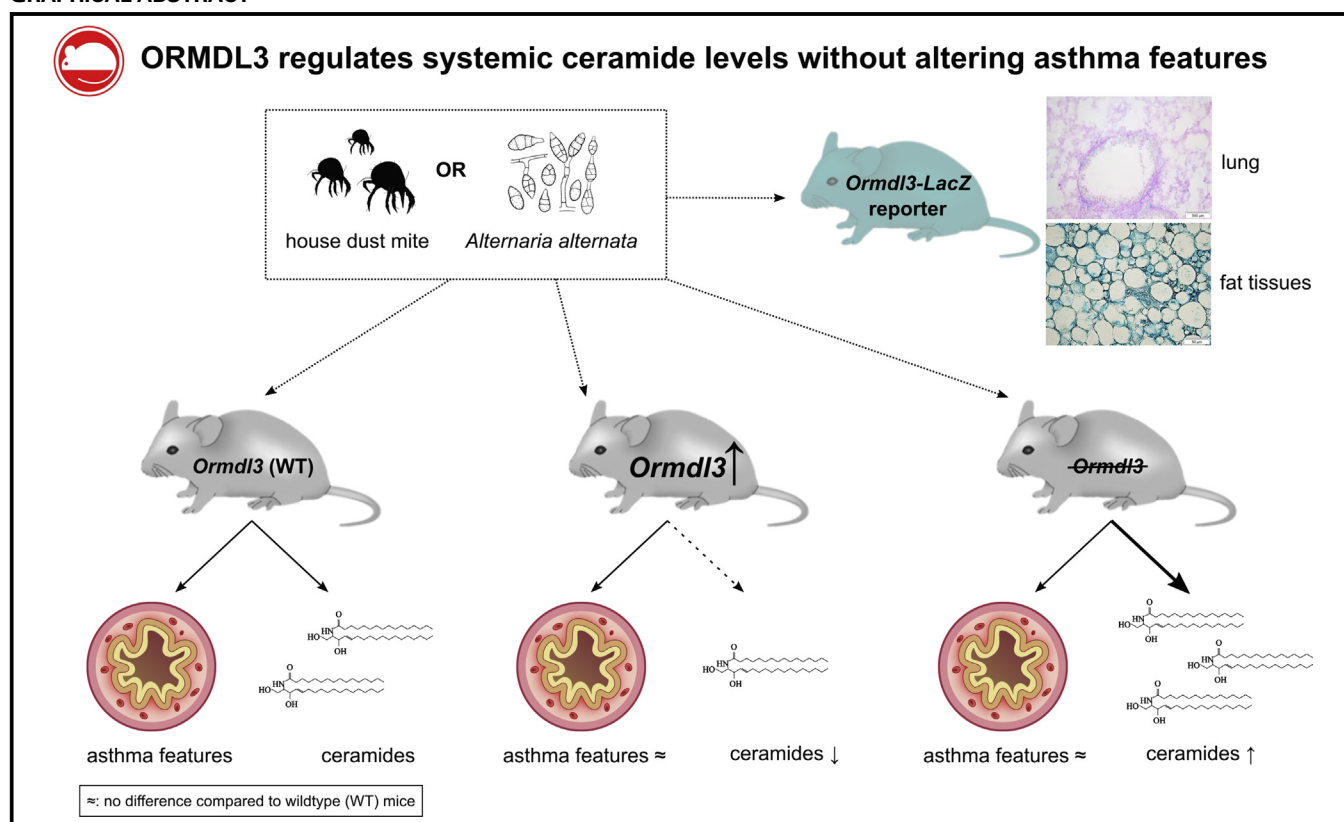


The *ORMDL3* asthma susceptibility gene regulates systemic ceramide levels without altering key asthma features in mice



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GRAPHICAL ABSTRACT



Background: Genome-wide association studies in asthma have repeatedly identified single nucleotide polymorphisms in the ORM (yeast)-like protein isoform 3 (*ORMDL3*) gene across different populations. Although the ORM homologues in yeast are

well-known inhibitors of sphingolipid synthesis, it is still unclear whether and how mammalian *ORMDL3* regulates sphingolipid metabolism and whether altered sphingolipid synthesis would be causally related to asthma risk.

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Objective: We sought to examine the *in vivo* role of ORMDL3 in sphingolipid metabolism and allergic asthma.

Methods: *Ormdl3*-LacZ reporter mice, gene-deficient *Ormdl3*^{-/-} mice, and overexpressing *Ormdl3*^{Tg/wt} mice were exposed to physiologically relevant aeroallergens, such as house dust mite (HDM) or *Alternaria alternata*, to induce experimental asthma. Mass spectrometry-based sphingolipidomics were performed, and airway eosinophilia, T_H2 cytokine production, immunoglobulin synthesis, airway remodeling, and bronchial hyperreactivity were measured.

Results: HDM challenge significantly increased levels of total sphingolipids in the lungs of HDM-sensitized mice compared with those in control mice. In *Ormdl3*^{Tg/wt} mice the allergen-induced increase in lung ceramide levels was significantly reduced, whereas total sphingolipid levels were not affected. Conversely, in liver and serum, levels of total sphingolipids, including ceramides, were increased in *Ormdl3*^{-/-} mice, whereas they were decreased in *Ormdl3*^{Tg/wt} mice. This difference was independent of allergen exposure. Despite these changes, all features of asthma were identical between wild-type, *Ormdl3*^{Tg/wt}, and *Ormdl3*^{-/-} mice across several models of experimental asthma.

Conclusion: ORMDL3 regulates systemic ceramide levels, but genetically interfering with *Ormdl3* expression does not result in altered experimental asthma. (J Allergy Clin Immunol 2019;144:1648-59.)

Key words: Asthma, ORMDL3, house dust mite, *Alternaria alternata*, sphingolipids, ceramides

Allergic asthma results from a complex gene-environment interaction and is currently still on the rise.¹ Single nucleotide polymorphisms (SNPs) on chromosome 17q12-21 confer the most significant and replicated genetic susceptibility to childhood-onset asthma and rhinovirus-induced wheezing illness in childhood.²⁻⁸ Many of these SNPs fall within the promoter region of the ORM (yeast)-like protein isoform 3 (*ORMDL3*) gene and induce slightly increased levels of *ORMDL3*, particularly in T lymphocytes and after stimulation of PBMCs with allergens.^{2,9-11} *Ormdl3* mRNA is also upregulated in murine asthma models, driven by ovalbumin (OVA), house dust mite (HDM), or *Alternaria alternata*.^{12,13} However, studies addressing the functional role of ORMDL3 in asthma generated conflicting conclusions. Both transgenic overexpression and genetic deficiency of *Ormdl3* can enhance key asthma features, whereas one study showed that *Ormdl3* deficiency suppressed only bronchial hyperreactivity (BHR).¹⁴⁻¹⁶ Given the multitude of genetic association studies in human subjects, the currently prevailing hypothesis is still that ORMDL3 overexpression has a causal role in asthma development or progression.

The molecular mechanism by which ORMDL3 contributes to asthma is still a matter of intense debate.^{6,8} ORMDL3 is member of an evolutionarily conserved family of endoplasmic reticulum (ER)-residing proteins and has 2 paralogues in vertebrates, ORMDL1 and ORMDL2, that have not been associated with asthma.¹⁷ In yeast the ORM homologues are described as regulators of *de novo* sphingolipid synthesis by controlling the activity of the rate-limiting enzyme serine palmitoyl transferase (SPT).¹⁸⁻²³ However, in mammals ORMDLs lack the N-terminal phosphorylation site that is crucial for SPT regulation in yeast.

Abbreviations used

BAC:	Bacterial Artificial Chromosome
BAL:	Bronchoalveolar lavage
BHR:	Bronchial hyperreactivity
ER:	Endoplasmic reticulum
HDM:	House dust mite
MLN:	Mediastinal lymph node
ORMDL3:	ORM (yeast)-like protein isoform 3
OVA:	Ovalbumin
PAS:	Periodic acid-Schiff
α-SMA:	α-Smooth muscle actin
SNP:	Single nucleotide polymorphism
S1P:	Sphingosine-1-phosphate
SPT:	Serine palmitoyl transferase
UPR:	Unfolded protein response

Mammalian SPT activity seems to be affected only when all ORMDL paralogues are overexpressed or downregulated simultaneously,^{17,24-26} making it unlikely that SNPs in only *ORMDL3* influence asthma through SPT inhibition. As an ER-resident protein, ORMDL3 has also been described to affect calcium metabolism and the unfolded protein response (UPR), influencing cytokine secretion by structural or immune cells.^{6,12,27-29} However, most molecular studies on ORMDL3 were performed *in vitro* and have led to contradictory results because of the use of different cell lines and distinct approaches to measure total sphingolipid *de novo* synthesis and to control *ORMDL3* expression. Furthermore, many studies were performed on epithelial cells, macrophages, mast cells, and eosinophils,^{6,12,13,29-31} whereas it has been recently demonstrated that chromosome 17q12-21 SNPs affect *ORMDL3* expression most prominently in T cells.⁹

Here we addressed the role of ORMDL3 in sphingolipid metabolism and asthma in newly generated *Ormdl3*-LacZ reporter mice, full *Ormdl3* KO mice (*Ormdl3*^{-/-}), and mice overexpressing murine *Ormdl3* from a Bacterial Artificial Chromosome (BAC) transgene (*Ormdl3*^{Tg/wt}). Using these unique gene-modified mice and their littermate controls, we found that ORMDL3 expression influences ceramide levels in the serum and liver and, to a lesser extent, in lung tissue. At the same time, interfering with *Ormdl3* did not affect key asthma parameters in various allergen-driven asthma models. These data do not support the currently prevailing paradigm that *ORMDL3* drives asthma by interfering with SPT activity or sphingolipid homeostasis.

METHODS

Mice

Ormdl3-LacZ reporter mice were generated by inserting a targeting construct of the European Conditional Mouse Mutagenesis consortium (project 72180) into the first intron of the *Ormdl3* gene (Fig 1, A). This construct contains a sequence that consists of an En2 splice acceptor site, an internal ribosome entry site, a LacZ sequence, a poly-A tail, a loxP site, and a neomycin-coding sequence driven by a human β-actin promoter that is flanked by 2 Flp recombinase target sites. ORMDL3 knockout (*Ormdl3*^{-/-}) mice and ORMDL3 transgenic (*Ormdl3*^{Tg/wt}) mice were previously described.²⁵ All mice were backcrossed for at least 10 crosses onto a C57BL/6 background. 1-DER mice express an MHC class II-restricted T-cell receptor specific for Der p 1 on their CD4 T cells and were generated, as previously described.³²⁻³⁴

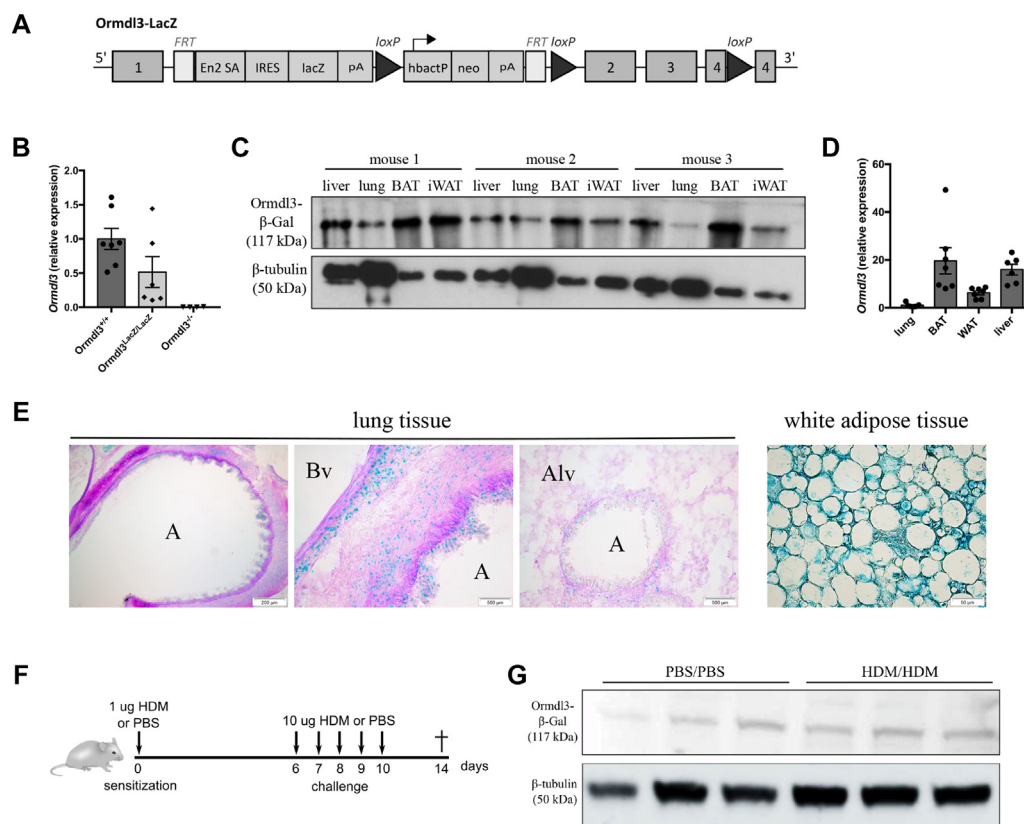


FIG 1. *Ormdl3*-LacZ reporter mice as a useful tool to study ORMDL3 expression. **A**, *Ormdl3*-LacZ reporter mice (*Ormdl3*^{LacZ/LacZ}) were generated by inserting a targeting construct of the EUCOMM consortium into the first intron of *Ormdl3*. Exon 2, exon 3, and part of exon 4 are flanked by 2 loxP sites, which enables generation of conditional knockout mice on crossing with mice expressing Cre-recombinase. **B**, *Ormdl3* mRNA expression levels in lungs from *Ormdl3*^{+/+}, *Ormdl3*^{LacZ/LacZ}, and *Ormdl3*^{-/-} mice. Expression values are shown relative to means of the wild-type group. Data were pooled from 2 experiments (n = 7, 6, and 4, respectively; means ± SEMs). **C**, Western blot showing β-galactosidase expression in liver tissue, lung tissue, brown adipose tissue (BAT), and white adipose tissue (WAT) in 3 individual *Ormdl3*-LacZ reporter mice. β-Tubulin was used as a loading control. **D**, *Ormdl3* transcript levels in lung tissue, BAT, WAT, and liver tissue in wild-type mice. Expression values are shown relative to means of lung samples (means ± SEMs). **E**, Immunohistochemistry analysis of β-galactosidase expression (blue) on lung OCT-inflated cryosections and WAT of *Ormdl3*-LacZ reporter mice. PAS staining was used as counterstaining. A, Airway; Alv, alveoli; Bv, blood vessel. **F**, Scheme representing the acute HDM-dependent asthma model. **G**, Western blot showing β-galactosidase expression in lung tissue from mock- and HDM-challenged *Ormdl3*-LacZ reporter mice.

Mice were bred under specific pathogen-free conditions at the animal house of the VIB/UGhent Center for Inflammation Research. Experiments were carried out with age- and sex-matched groups. All mice were used between 6 and 14 weeks of age. All animal procedures were approved by the Ethical Committee of Ghent University.

Models of allergic asthma

The HDM-induced asthma model was performed, as described previously.³⁵ In brief, mice were sensitized intratracheally on day 0 with 1 μg of HDM extract (Greer Laboratories, Lenoir, NC) or saline, followed by 10 μg of intranasal challenge from days 6 to 10. On day 14, mice were killed by an overdose of pentobarbital. In the chronic HDM-induced asthma model, mice were instilled intranasally with 10 μg of HDM or saline as a control 3 times a week for 5 weeks. Asthma features were determined 3 days after the last challenge. In the *Alternaria* species-induced asthma model, mice were instilled intranasally with 20 μg of *A. alternata* (Greer Laboratories) 3 times a week for 3 weeks. All intratracheal and intranasal treatments were given in 80 and 40 μL of PBS, respectively, and after achievement of light isoflurane anesthesia.

Bronchoalveolar lavage (BAL) was performed with 3 × 1 mL of EDTA-containing PBS (0.5 mmol/L). Blood was obtained from the iliac vein in noncoated Eppendorf tubes to prepare serum. Single-cell suspensions from mediastinal lymph nodes (MLNs) were obtained by homogenizing the organ through a 100-μm cell sieve. Cells were restimulated *ex vivo* with 15 μg/mL HDM for 3 days, and supernatants were collected for ELISA (Ready-SET-Go! kits; eBioscience, San Diego, Calif). The left lung was fixed overnight in 4% paraformaldehyde and used for histology. Right lung tissue was snap-frozen in liquid nitrogen and kept at -80°C until used for RNA extraction.

BHR determination

Mice were anesthetized with urethane, paralyzed with D-tubocurarine, tracheostomized, and intubated with a 20-gauge catheter, followed by mechanical ventilation in a flexiVent apparatus (SCIREQ, Montreal, Quebec, Canada). Respiratory frequency was set at 150 breaths/min with a tidal volume of 10 mL/kg, and a positive end-expiratory pressure of 3 cm H₂O was applied. Increasing concentrations of methacholine were nebulized (0-400 mg/mL) for 12 seconds, and dynamic resistance, elastance, and compliance were recorded.

by 12 repeated measurements for 2 minutes per dose. Baseline resistance was restored between doses.

Histology

After overnight fixation in 4% paraformaldehyde solution, tissues were embedded in paraffin, cut into 5- μ m slices, and stained with either periodic acid–Schiff (PAS) or processed for immunohistochemistry. PAS staining was performed as described by Debeuf et al.³⁵

Lung sections were incubated overnight with an anti- α -smooth muscle actin (α -SMA) primary antibody (A2547; Sigma-Aldrich, St Louis, Mo) to detect peribronchial α -SMA. Species- and isotype-matched antibodies were used as negative controls. Immunoreactivity was detected by means of sequential incubations of lung sections with a biotinylated secondary antibody (E0433; Dako, Heverlee, Belgium) and the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, Calif), followed by 3,3-diaminobenzidine chromogen (Dako). Sections were mounted with Entellan mounting medium (Merck Millipore, Burlington, Mass).

Lipidomics

Individual sphingolipid species were quantified by using a novel liquid chromatography–mass spectrometry method for a comprehensive analysis of typical and atypical sphingolipid species. Lipidomics profiling was done in 20 μ L of mouse serum, or 20 μ g of homogenate from lung tissue was extracted by using the methanol/methyl-tert-butylether/chloroform (MMC) protocol, as described previously.³⁶ d7-Sphinganine, d7-sphingosine, Cer d18:0/12:0, Cer d18:1/12:0, SM d18:1/12:0, GluCer d18:1/18:0, and d7-S1P were used as internal standards. A C18 column–based method for chromatographic separation and mass spectrometry were used, as described previously.³⁶

Statistical analyses

For all experiments, the normality of each group was first checked by using the Shapiro–Wilk statistical test. If data sets were normally distributed, 1-way ANOVA was performed. In case of nonnormally distributed data, a Kruskal–Wallis test was done. All statistical tests were performed in GraphPad Prism software (GraphPad Software, La Jolla, Calif).

RESULTS

Effect of allergen challenge on ORMDL3 expression in asthma

One of the major drawbacks in the ORMDL3 field is the lack of specific antibodies because of the large sequence homology with ORMDL1 and ORMDL2 (84% and 83%, respectively). Therefore many investigators have relied on *ORMDL3* mRNA analysis of isolated tissues or cells without confirming where exactly ORMDL3 is expressed. *Ormdl3*-LacZ reporter mice were generated by inserting a targeting construct into the first intron of *Ormdl3* to be able to better address ORMDL3 expression (Fig 1, A).²⁵ Although these mice still express some basal *Ormdl3* (Fig 1, B), β -galactosidase activity can be used as a readout for *Ormdl3* promoter activation.

We first compared ORMDL3 expression levels in different tissues in steady-state mice. Despite the fact that *ORMDL3* is an asthma susceptibility gene, β -galactosidase expression was relatively low in lung tissue, especially compared with that in liver tissue and white and brown adipose tissues, where ORMDL3 was abundantly present (Fig 1, C, and see Fig E1, A, in this article's Online Repository at www.jacionline.org). Similar results were obtained by using quantitative RT-PCR (Fig 1, D), which is consistent with RNA sequencing data derived from the GENESTIGATOR database (see Fig E1, B).³⁷ Previous studies

suggested that epithelial cells would be the main cell type expressing ORMDL3 in the lung.¹⁴ However, in our hands immunohistochemistry revealed diffuse and low β -galactosidase activity in subepithelial and vascular smooth muscle cells, epithelial cells, and fibroblasts (Fig 1, E). On the contrary, high β -galactosidase activity was seen in white adipose tissue (Fig 1, E), which is consistent with mRNA expression data.

To address whether ORMDL3 expression was induced by allergen challenge *in vivo*, we used a previously established model in which inhaled HDM challenge of HDM-sensitized mice leads to airway eosinophilia (Fig 1, F).³⁵ No increase in lung β -galactosidase activity was seen in allergen-challenged mice compared with that in control PBS-exposed mice (Fig 1, G, and see Fig E1, C). These results showed that ORMDL3 expression in mouse lungs is low and did not increase after allergen challenge using sensitized mice.

Characterization of *Ormdl3*^{Tg/wt} and *Ormdl3*^{-/-} mice

To assess the effects of altered ORMDL3 expression levels *in vivo*, we have recently generated *Ormdl3*-overexpressing mice (*Ormdl3*^{Tg/wt}) and gene-deficient *Ormdl3*^{-/-} mice.²⁵ Before we used these mice in asthma experiments, we wanted to address whether immune cell development was affected by a systemic change in *Ormdl3* expression. Mice from both strains were healthy and did not have a spontaneous phenotype, even with aging (data not shown). We stained cells with a broad panel of antibodies recognizing various lymphocytes and myeloid cells and used the FlowSOM automatic gating and visualization tool to assess the immune cell landscape of *Ormdl3*^{Tg/wt} and *Ormdl3*^{-/-} mice in the most unbiased manner.^{38–40} Although there was a trend toward an increased number of splenic cells in *Ormdl3*^{Tg/wt} mice (Fig 2, A and B), none of the well-known or less defined immune cell clusters were significantly altered compared with those in littermate controls (Fig 2, C and D). Likewise, FlowSOM trees represented a similar immune landscape between *Ormdl3*^{-/-} mice and littermate control mice (Fig 2, E–H). These data suggest that genetic alteration of ORMDL3 does not affect the immunome in a major manner.

Effect of altered ORMDL3 expression on sphingolipid concentration

In yeast it has been conclusively demonstrated that ORM proteins regulate *de novo* sphingolipid synthesis through controlling SPT activity. Therefore dysregulation of sphingolipid *de novo* synthesis was proposed as a pathophysiologic mechanism through which ORMDL3 promotes asthma (for a schematic overview of sphingolipid *de novo* synthesis, see Fig E2, A, in this article's Online Repository at www.jacionline.org).^{8,18–23,41} Therefore we assessed whether ORMDL3 affects sphingolipid metabolism in the context of allergic asthma using our transgenic mice.

Because there are 3 ORM paralogues expressed in mice, it was also important to assess whether deficiency or overexpression of *Ormdl3* causes a compensatory change in *Ormdl1* or *Ormdl2* expression. For lung tissue, *Ormdl3* transcript levels were 6-fold increased in *Ormdl3*^{Tg/wt} mice, and expression levels were comparable for nonasthmatic (PBS/HDM group) and

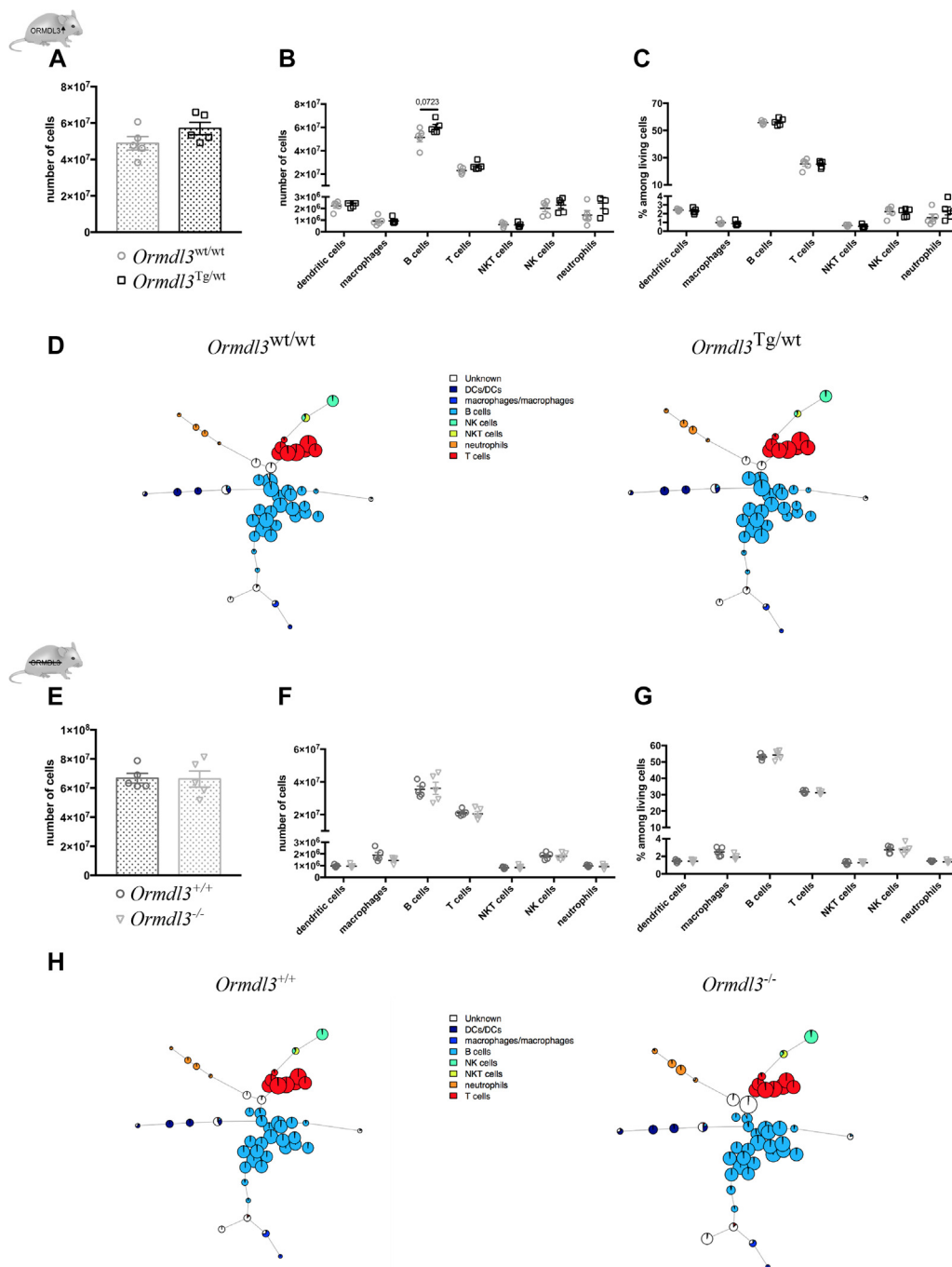


FIG 2. Unbiased analysis of the immune landscape of *Ormdl3*^{Tg/wt} and *Ormdl3*^{-/-} mice. **A**, Splenic cell numbers from 6-week-old *Ormdl3*^{wt/wt} and *Ormdl3*^{Tg/wt} mice (n = 5 and 5, respectively). **B** and **C**, Numbers (Fig 2, B) and percentages (Fig 2, C) of different immune cell types in spleens from *Ormdl3*^{wt/wt} and *Ormdl3*^{Tg/wt} mice (n = 5 and 5, respectively). NK, Natural killer. **D**, FlowSOM trees representing the splenic immune landscape in *Ormdl3*^{wt/wt} and *Ormdl3*^{Tg/wt} mice. **E**, Splenic cell numbers from 6-week-old *Ormdl3*^{+/-} and *Ormdl3*^{-/-} mice (n = 5 and 5, respectively). **F** and **G**, Numbers (Fig 2, F) and percentages (Fig 2, G) of different immune cell types in spleens from *Ormdl3*^{+/-} and *Ormdl3*^{-/-} mice (n = 5 and 5, respectively). **H**, FlowSOM trees representing the splenic immune landscape in *Ormdl3*^{+/-} and *Ormdl3*^{-/-} mice. Data information: All data were analyzed with a Shapiro-Wilk normality test to assess whether the data were normally distributed. Parametric data were analyzed with a *t* test, whereas nonparametric data were analyzed with a Mann-Whitney test. Data are shown as means ± SEMs. **P* < .05. DC, Dendritic cell; NK, natural killer.

asthmatic (HDM/HDM group) conditions. A compensatory change in *Ormdl1* or *Ormdl2* expression was not observed (Fig 3, A). In *Ormdl3*^{-/-} mice transcript levels of *Ormdl3* were absent

without a sign of compensatory upregulation of *Ormdl1* or *Ormdl2* (Fig 3, B). Again, we did not observe any induction of *Ormdl3* mRNA on asthmatic challenge (Fig 3, A and B).

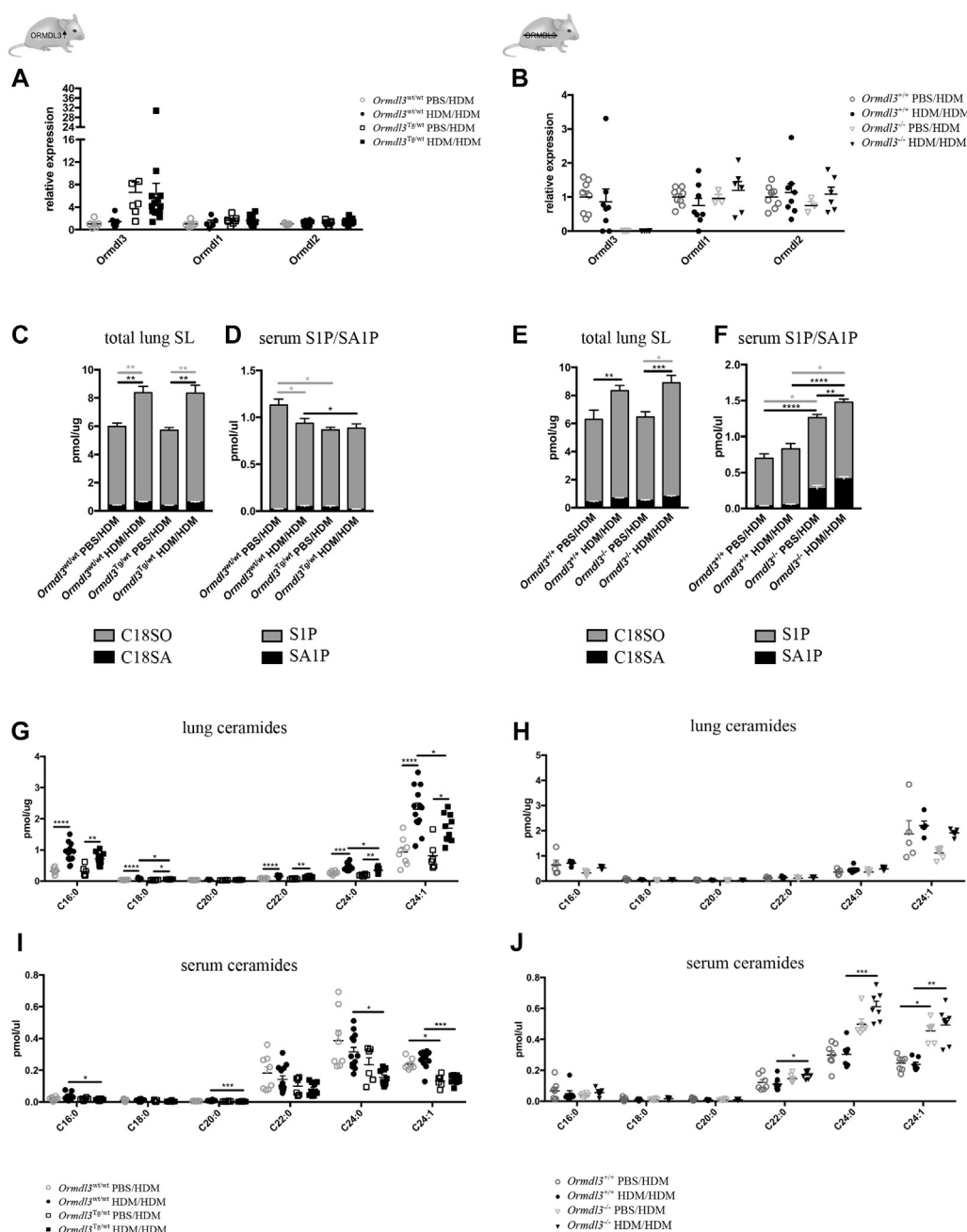


FIG 3. Effect of altered ORMDL3 expression on sphingolipid concentrations. **A** and **B**, *Ormdl3* mRNA expression levels in lung tissue from *Ormdl3*^{wt/wt} and *Ormdl3*^{Tg/wt} mice (Fig 3, **A**) or *Ormdl3*^{+/+} and *Ormdl3*^{-/-} mice (Fig 3, **B**) sensitized with saline (PBS) or HDM and challenged with HDM. Expression values are shown relative to means of the mock-sensitized wild-type group (Fig 3, **A**: n = 5, 10, 7, and 15, respectively; Fig 3, **B**: 8, 8, 3, and 6, respectively). **C** and **E**, C18 sphingoid base levels in lung tissue from (non-)asthmatic *Ormdl3*^{wt/wt} and *Ormdl3*^{Tg/wt} mice (Fig 3, **C**) or *Ormdl3*^{+/+} and *Ormdl3*^{-/-} mice (Fig 3, **E**). Total sphingolipids were extracted, and sphingosine (C18SO, gray) and sphinganine (C18SA, black) levels were analyzed after acid-base hydrolysis by using liquid chromatography–mass spectrometry (Fig 3, **C**: n = 5, 13, 6, and 10, respectively; Fig 3, **E**: n = 5, 5, 7, and 7, respectively). **D** and **F**, S1P (gray) and sphinganine-1-phosphate (SA1P, black) levels in serum from (non-)asthmatic *Ormdl3*^{wt/wt} and *Ormdl3*^{Tg/wt} mice (Fig 3, **D**) or *Ormdl3*^{+/+} and *Ormdl3*^{-/-} mice (Fig 3, **F**; Fig 3, **D**: n = 8, 13, 6, and 10, respectively; Fig 3, **F**: n = 7, 7, 6, and 7, respectively). **G–J**, Ceramide levels in lung tissue (Fig 3, **G** and **H**) or serum (Fig 3, **I** and **J**) from (non-)asthmatic *Ormdl3*^{wt/wt} and *Ormdl3*^{Tg/wt} mice (Fig 3, **G** and **I**) or *Ormdl3*^{+/+} and *Ormdl3*^{-/-} mice (Fig 3, **H** and **J**; Fig 3, **G** and **I**: n = 8, 13, 6, and 10, respectively; Fig 3, **H**: n = 5, 5, 7, and 7, respectively; Fig 3, **J**: 7, 7, 6, and 7, respectively). For Fig 3, **C**, **D**, **G**, and **I**, data were pooled from 3 independent experiments. Data information: All data were analyzed with a Shapiro-Wilk normality test to assess whether the data were normally distributed. Parametric data were analyzed with an ordinary 1-way ANOVA test with multiple comparison correction. Nonparametric data were analyzed with an unpaired Kruskal-Wallis test with multiple correction. Data are shown as means ± SEMs. **P* < .05, ***P* < .01, ****P* < .001, and *****P* < .0001.

Next, we investigated how asthma and ORMDL3 expression influenced the sphingolipid profile in various organs. We observed a mixed picture, depending on the analyzed tissue. In lung tissue total sphingolipid levels were increased in asthmatic mice compared with nonasthmatic mice, confirming previous observations,^{13,42} but this increase was clearly independent of *Ormdl3* (Fig 3, C and E). In line with this, full sphingolipidomics analysis revealed an increase in ceramide levels in the lungs of asthmatic mice (Fig 3, G and H), which appeared unaffected by the absence of ORMDL3 (Fig 3, H). However, on overexpression of ORMDL3, ceramide levels in the lung were decreased slightly, which is consistent with a role for ORMDL3 in blocking ceramide synthesis (Fig 3, G).^{13,43} This was much more pronounced in serum (Fig 3, I and J) and several other tissues, such as liver and fat, in which ORMDL3 is more highly expressed (Fig 1, C, and see Fig E2).

These effects were not limited to ceramides but could also be observed for other sphingolipid species. For instance, sphingosine-1-phosphate (S1P) and sphinganine-1-phosphate were upregulated in sera from *Ormdl3*^{-/-} mice and reduced in sera from *Ormdl3*^{Tg/wt} mice (Fig 3, D and F). A similar pattern was observed for hexosylceramides and sphingomyelins (see Fig E2). In summary, sphingolipid homeostasis seems to be mostly influenced in those tissues in which ORMDL3 is abundantly expressed. For lung tissue, we did not see a major influence of ORMDL3 expression on the asthma-induced change in the sphingolipid profile.

Genetic alteration of ORMDL3 expression levels does not affect key asthma parameters

Having found that genetic interference with expression levels of ORMDL3 led to alterations in ceramide levels, we next addressed whether key asthma parameters would be altered in *Ormdl3*^{Tg/wt} or *Ormdl3*^{-/-} mice, and results of experiments were compared each time with results for proper littermate wild-type control mice. In HDM-sensitized, but not mock-sensitized, *Ormdl3*^{wt/wt} mice, there was eosinophil, T-cell, and B-cell infiltration in BAL fluid; IL-5 and IL-13 cytokine production in the MLNs; HDM-specific IgE production in the serum; and BHR in response to increasing doses of methacholine (Fig 4, A-D, and see Fig E3, A and B, in this article's Online Repository at www.jacionline.org). Strikingly, none of these key HDM-induced asthma parameters were altered in *Ormdl3*^{Tg/wt} mice. A previous study suggested the involvement of ORMDL3 in airway remodeling characterized by goblet cell metaplasia and hypertrophy of α -SMA-expressing myofibroblasts.¹⁴ In *Ormdl3*^{wt/wt} mice HDM challenge of sensitized mice led to an increase in PAS⁺ goblet cell metaplasia and α -SMA staining around the airways compared with that seen in nonsensitized mice (Fig 4, E and F), but again, these features were equally induced in *Ormdl3*^{Tg/wt} mice. Even in a more chronic 5-week model of HDM exposure, overexpression of ORMDL3 did not significantly influence the cardinal features of asthma (see Fig E3, C-G).

Recently, it has been shown that T cells are the most prominent immune cells affected by chromosome 17q21 SNPs.⁹ Therefore we wondered whether T-cell function would be intrinsically affected by ORMDL3 overexpression. Although there were no obvious differences in outcomes of the asthma experiments, it was still possible that T-cell function was slightly affected but

that this only became apparent during competition experiments. Therefore we crossed *Ormdl3*^{Tg/wt} mice with 1-DER mice, which contain T cells that specifically react to the Der p 1 peptide of HDM.³²⁻³⁴ *Ormdl3*^{wt/wt} and *Ormdl3*^{Tg/wt} 1-DER T cells were fluorescently labeled and transferred into acceptor mice (see Fig E4, A, in this article's Online Repository at www.jacionline.org), which were subsequently exposed to HDM. Similar proportions of *Ormdl3*^{wt/wt} and *Ormdl3*^{Tg/wt} 1-DER T cells were divided 3 days after transfer, and numbers were equally high (see Fig E4, B and C). Thus, in line with previous results, ORMDL3 did not affect HDM-specific proliferation by T cells intrinsically.

Ormdl3^{Tg/wt} mice were also exposed to a model driven by the fungal extract *A alternata* to exclude allergen-dependent effects. Mice were administered 20 μ g of *A alternata* or PBS 3 times a week for 3 weeks (see Fig E5, A, in this article's Online Repository at www.jacionline.org). *A alternata*-treated mice had increased expression levels of *Ormdl3* mRNA, confirming earlier findings (see Fig E5, B)¹²; however, this could not be confirmed at the protein level with *Ormdl3*-LacZ reporter mice (see Fig E5, C). In *A alternata*-exposed mice there was a marked influx of eosinophils in the BAL fluid, an increase in *A alternata*-specific IgE levels in the serum, T_H2 cytokine production in the MLNs, and development of BHR to methacholine compared with PBS-exposed mice, but none of these features were altered in *Ormdl3*^{Tg/wt} mice (Fig 4, G-I, and see Fig E5, D-F).

It is most likely that the ORMDL3 SNP at 17q12-21 that confers asthma risk leads to overexpression of ORMDL3, as has been demonstrated in T cells.⁹ Because our transgenic mice overexpressing ORMDL3 did not have altered features of asthma, we also decided to perform all key asthma readouts in *Ormdl3*^{-/-} mice in comparison with *Ormdl3*^{+/+} littermates, again sensitizing mice to either HDM or *A alternata*. Whereas all key features of allergic asthma, like eosinophilia, T_H2 cytokine production, HDM-specific serum IgE levels, BHR to methacholine, and airway remodeling, were induced by allergen challenge in *Ormdl3*^{+/+} mice, none of these features were altered in *Ormdl3*^{-/-} mice (Fig 5, A-F, and see Fig E6, A, in this article's Online Repository at www.jacionline.org). There was a small trend toward enhanced IL-17 production by allergen-restimulated MLN T cells, but this was not accompanied by airway neutrophilia (see Fig E6, B). Similar conclusions were reached in the *A alternata* model (Fig 5, G-I, and see Fig E6, C-F). Therefore neither ORMDL3 overexpression nor deletion affected key asthma parameters in various acute and chronic models driven by 2 different allergens.

DISCUSSION

During the last decade, genome-wide association studies have repeatedly and convincingly demonstrated that SNPs in the chromosome 17q12-21 locus are associated with early-life onset of allergic asthma.^{2,3} Genotypes in the core region defined by the first genome-wide association study correlate with expression of 2 genes, ORMDL3 and gasdermin B (*GSDMB*), making these prime candidate asthma genes, although recent studies have implicated gasdermin A (*GSDMA*) distal to and post-glycosylphosphatidylinositol attachment to proteins 3 (*PGAP3*) proximal to the core region as independent loci.⁸ There is much speculation on how ORMDL3 could regulate asthma. In yeast ORM proteins control sphingolipid levels by regulating the enzyme SPT, the rate-limiting enzyme of *de novo* sphingolipid synthesis.¹⁸ A mouse

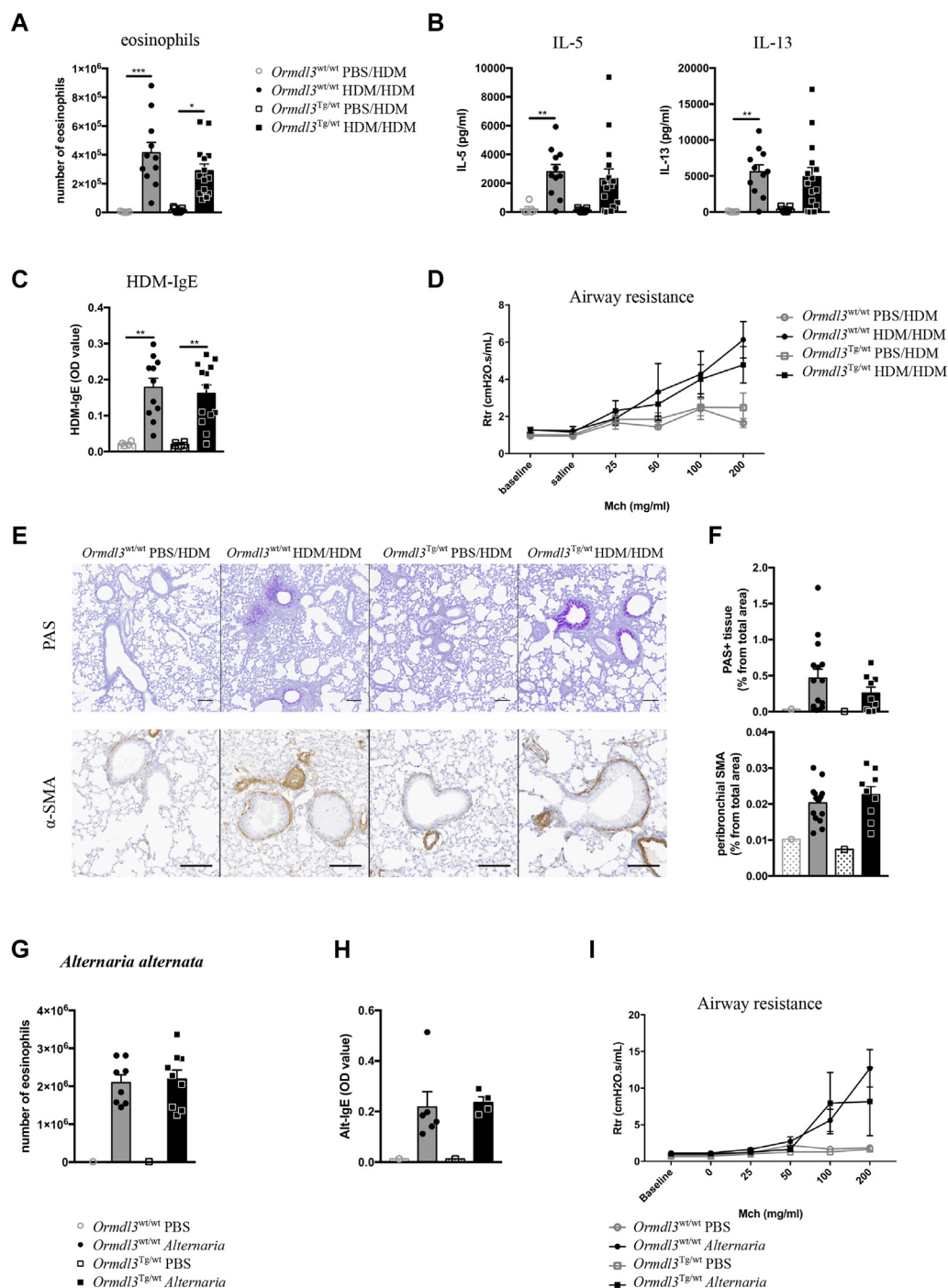


FIG 4. ORMDL3 overexpression does not affect allergic asthma driven by HDM or *A. alternata*. **A**, Eosinophil numbers in BAL fluid from (non-)asthmatic *Ormdl3*^{wt/wt} and *Ormdl3*^{Tg/wt} mice ($n = 5, 11, 7$, and 14 , respectively). **B**, Protein levels of IL-5 and IL-13 secreted by MLN cells during restimulation with HDM ($n = 5, 11, 7$, and 14 , respectively). **C**, Serum HDM-specific IgE levels (OD value; $n = 4, 11, 6$, and 13 , respectively). In Fig 4, A-C, data were pooled from 2 independent experiments. **D**, BHR on exposure to increasing doses of methacholine (*Mch*), as measured with flexiVent ($n = 4, 4, 6$, and 8 , respectively). **E**, Representative images of lung sections stained with either PAS or anti- α -SMA antibody. Scale bar = $100 \mu\text{m}$. **F**, Quantification of PAS⁺ area and peribronchial α -SMA in lung tissue. Data were pooled from 3 independent experiments ($n = 1, 15, 1$, and 9 , respectively). **G**, Eosinophil numbers in BAL fluid on exposure to PBS or *A. alternata* ($n = 1, 8, 1$, and 9 , respectively). **H**, Serum *A. alternata*-specific IgE levels (OD value; $n = 1, 6, 1$, and 4 , respectively). **I**, BHR on exposure to increasing doses of methacholine (*Mch*) as measured with flexiVent (SCIREQ; $n = 1, 7, 1$, and 4 , respectively). Data information: All data were analyzed with a Shapiro-Wilk normality test to assess whether the data were normally distributed. Parametric data were analyzed with an ordinary 1-way ANOVA test with multiple comparison correction. Nonparametric data were analyzed with an unpaired Kruskal-Wallis test with multiple correction. For Fig 4, F-I, a Mann-Whitney statistical test was performed between the HDM-sensitized groups. Data are shown as means $\pm$ SEMs. * $P < .05$, ** $P < .01$, and *** $P < .001$.

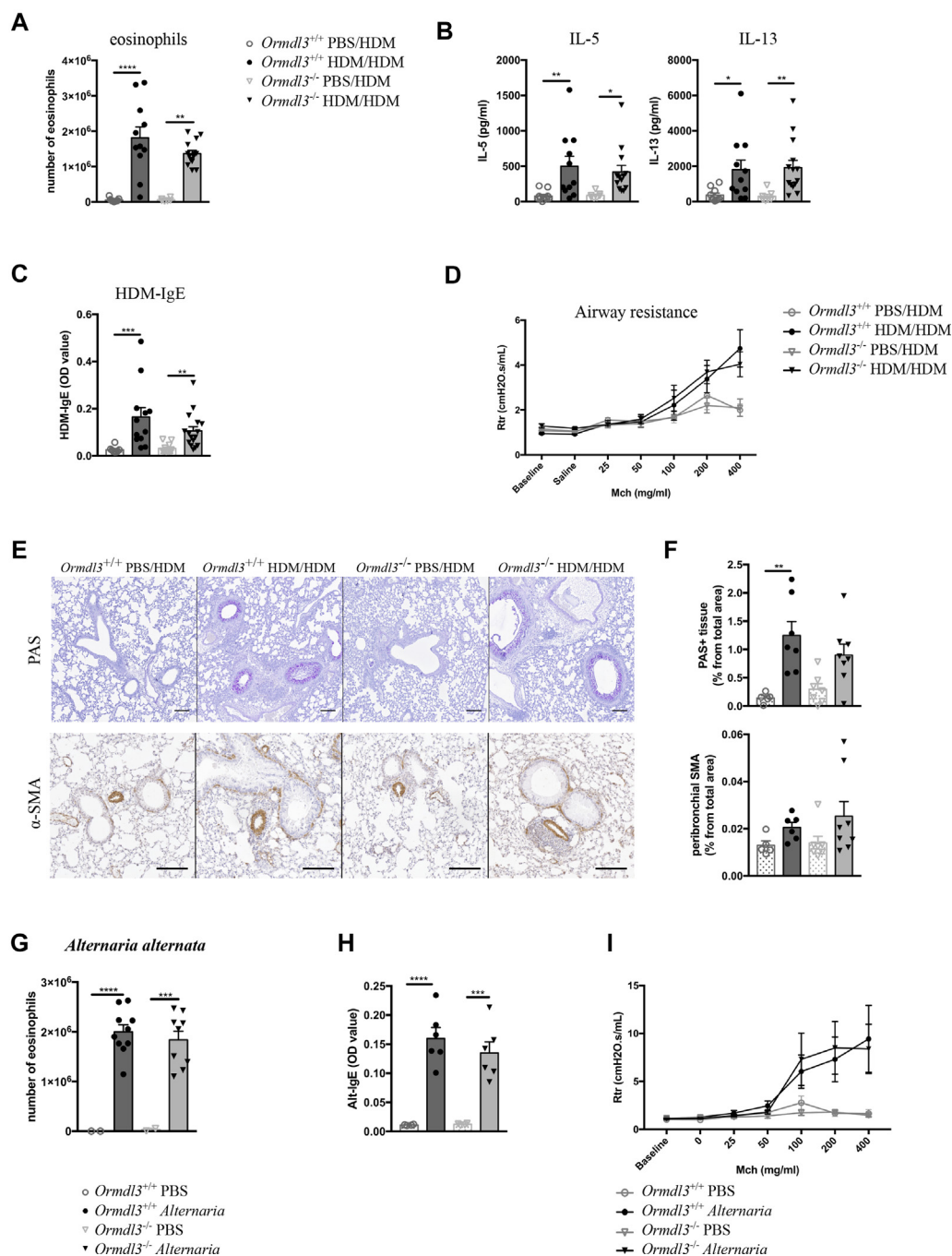


FIG 5. Loss of *Ormdl3* expression does not affect allergic asthma driven by HDM or *A alternata*. **A**, Numbers of eosinophils in BAL fluid from (non-)asthmatic *Ormdl3*^{+/+} and *Ormdl3*^{-/-} mice ($n = 10, 11, 7$, and 14 , respectively). **B**, Protein levels of IL-5 and IL-13 secreted by MLN cells during restimulation with HDM ($n = 10, 11, 7$, and 14 , respectively). **C**, Serum HDM-specific IgG₁, HDM-IgE, and total IgE levels (OD value; $n = 8, 12, 12$, and 16 , respectively). In Fig 5, A-C, data were pooled from 2 experiments. **D**, BHR on exposure to increasing doses of methacholine (*Mch*) as measured with flexiVent ($n = 4, 4, 6$, and 6 , respectively). **E**, Representative images of lung sections stained with either PAS or anti- α -SMA antibody. Scale bar = $100 \mu\text{m}$. **F**, Quantification of PAS⁺ area ($n = 5, 7, 7$, and 8 , respectively) and peribronchial α -SMA ($n = 5, 6, 7$, and 8 , respectively) in lung tissue. **G**, Numbers of eosinophils in BAL fluid from *Ormdl3*^{+/+} and *Ormdl3*^{-/-} mice exposed to PBS or *A alternata* ($n = 2, 10, 2$, and 9 , respectively). **H**, Serum *A alternata*-specific IgE levels (OD value; $n = 4, 6, 4$, and 6 , respectively). **I**, BHR on exposure to increasing doses of methacholine (*Mch*) as measured with flexiVent (SCIREQ; $n = 4, 6, 4$, and 6 , respectively). Data information: All data were analyzed with a Shapiro-Wilk normality test to assess whether the data were normally distributed. Parametric data were analyzed with an ordinary 1-way ANOVA test with multiple comparison correction. Nonparametric data were analyzed with an unpaired Kruskal-Wallis test with multiple correction. Data are shown as means $\pm$ SEMs. * $P < .05$, ** $P < .01$, *** $P < .001$, and **** $P < .0001$.

study subsequently showed that interfering with SPT using the inhibitor myriocin reduced *de novo* sphingolipid synthesis and increased BHR.⁴⁴ This led some to conclude that *ORMDL3* controls asthma through altered sphingolipid metabolism.^{41,45} However, despite extensive efforts, the molecular role of *ORMDL3* in allergic asthma has not been unraveled yet.

Although the studies addressing the effect of inhibition of *de novo* sphingolipid synthesis using myriocin or genetic deficiency of SPT have yielded consistent results,^{13,24,26,44,46-50} mouse studies investigating the role of *ORMDL3* in asthma have been harder to reconcile.¹⁴ It has been reported that C57BL/6 mice expressing high levels of human *ORMDL3* had stronger inflammation and BHR compared with wild-type mice in a mouse model driven by intraperitoneal immunization by OVA in alum, followed by a series of OVA inhalation challenges. However, the same group also reported that BHR (but not inflammation) was increased in C57BL/6 mice lacking *ORMDL3* specifically in epithelial cells,¹⁶ which makes the results hard to interpret. On the contrary, Loser et al¹⁵ reported that full *Ormdl3* gene-deficient C57BL/6 mice were protected from BHR to *A alternata*, whereas inflammation was only slightly affected. From these conflicting studies, the conclusion would be that the role of *ORMDL3* in experimental asthma is highly contextual and might be explained by subtle differences in animal models used by different investigators and whether the model used overexpression of (human) *ORMDL3* or genetic deficiency of endogenous mouse *Ormdl3*.

To address this controversy regarding the models used, we applied multiple models (acute vs chronic) and allergens (HDM, *A alternata*, and OVA; data not shown) in newly generated *Ormdl3* BAC transgenic and *Ormdl3*^{-/-} mice. We observed no differences in the severity of all key asthma parameters when *Ormdl3* was either overexpressed or deleted from the mouse genome. At present, we can only speculate about the differences between our negative results and the positive results reported by others. One major difference is that others studying overexpression have used the human *ORMDL3* gene with a plasmid expression system or adenoviral overexpression of murine *Ormdl3* *in vivo*, which could have led to unphysiologic levels of overexpression or overexpression in cell types that normally do not express *ORMDL3*. In addition, the reported differences in *ORMDL3* expression based on SNP genotype are minor and significantly smaller, as was reported for transgenic mouse models. We have used a BAC transgenic construct in which overexpression of *Ormdl3* is still driven by the endogenous *Ormdl3* promoter. Modeling asthma in the mouse is difficult. It is possible that other environmental triggers that often occur together with allergen exposure are necessary to unravel the link between *ORMDL3* and asthma, such as respiratory viruses or cigarette smoke. In fact, a limitation of this study is the lack of a viral infection model because recent epidemiologic studies suggest that SNPs in the chromosome 17q12-21 locus combined with severe viral respiratory tract infections do have a driving role in causing childhood-onset asthma.^{7,51,52} Interestingly, the same SNPs also lead to protection from asthma on microbial exposure,⁵¹ suggesting that this locus is very environmentally dependent and might be regulated by epigenetic modifications.⁵³ None of the studies addressing the relationship between *ORMDL3* and asthma have thus far studied experimental viral respiratory tract infections. Efforts are underway in our laboratory addressing the role of *ORMDL3* in susceptibility to

respiratory mouse pneumovirus infection and subsequent asthma development.

ORM homologues in yeast are well-known inhibitors of *de novo* sphingolipid synthesis, whereas the role for the mammalian *ORMDLs* is still a matter of debate. Therefore we examined sphingolipid synthesis by using 2 approaches.

First, we analyzed total sphingolipid levels by quantifying the total C18 sphingoid base profile after chemical hydrolysis. If SPT activity would be inhibited by *ORMDL3*, total sphingoid bases would be reduced in *Ormdl3*^{Tg/wt} mice. However, a significant change in total sphingoid base levels could not be detected in lungs of neither *Ormdl3*^{Tg/wt} or *Ormdl3*^{-/-} mice. This corresponds to earlier reports that SPT activity is not affected by changing *ORMDL3* expression levels.²⁵

Next, we performed sphingolipidomic analysis to quantify individual sphingolipid species, including ceramides, sphingomyelins, hexosyl ceramides, and phosphorylated sphingoid bases (S1P and sphinganine-1-phosphate). In particular, S1P has been extensively described as involved in allergic asthma.⁵⁴⁻⁵⁸ Our results demonstrate an increase in serum S1P levels in *Ormdl3*^{-/-} mice (Fig 3, D), which has also been seen in mice lacking *ORMDL3* specifically in epithelial cells.¹⁶ Furthermore, *Ormdl3*^{Tg/wt} mice had reduced levels of S1P (Fig 3, C), which is consistent with previous reports.⁴³ However, the *ORMDL3*-dependent regulation of S1P did not result in an altered asthma phenotype.

Earlier reports have shown that ceramide levels are increased in murine allergic asthma models and asthmatic patients and that inhibition of ceramide synthesis mitigates the allergic response.^{13,42} This was confirmed in our HDM model, which showed a significant increase in lung ceramide levels for asthmatic mice compared with nonasthmatic mice. This asthma-dependent increase in ceramide levels was not influenced in *Ormdl3*^{-/-} mice. On the other hand, it appeared to be hampered in *Ormdl3*^{Tg/wt} mice. Of interest, serum ceramide levels were decreased in *Ormdl3*^{Tg/wt} mice and, conversely, were increased in *Ormdl3*^{-/-} mice. Similar changes were seen in the liver but not in brown or white adipose tissue, which also express significant levels of *ORMDL3* natively (Fig 1, C). A decrease in levels of long-chain ceramides (C:24) has also been observed in mice overexpressing human *ORMDL3*.⁴³ Furthermore, Oyeniran et al¹³ reported that all ceramide species were reduced on overexpression of *ORMDL3* provided that the degree of overexpression is moderate, which is the case in our *Ormdl3*^{Tg/wt} mice. However, although serum (and lung) ceramide levels did change with changing *ORMDL3* expression levels, the allergic response was not affected, suggesting that the association of *ORMDL3* with asthma would be hard to explain through alterations in ceramide levels.

In tissues in which native *ORMDL3* expression was greatest based on the *Ormdl3*-LacZ reporter mouse, the effects on sphingolipid homeostasis were also the most pronounced. This might explain the moderate effects observed in lung tissue. However, it is still unclear at which step *ORMDL3* interferes in the sphingolipid synthesis pathway. In line with our previous data,²⁵ we did not find a consistent change in total sphingolipid levels, which makes a direct effect of *ORMDL3* on SPT activity questionable. Still, especially in liver tissue but also in other tissues, levels of several sphingolipid species were altered in mice with deregulated *Ormdl3*. Interestingly, the largest change was seen for serum sphingomyelins, which is primarily formed in the liver and then

secreted into the blood in the form of lipoproteins. Among the different sphingolipid classes, we observed the most pronounced changes for ceramide and sphingomyelin levels, whereas hexosylceramide levels were not much altered. This shows that mammalian ORMDL3 is somehow involved in the homeostasis of sphingolipids but likely at a level downstream of SPT.

As an ER-residing protein, *in vitro* studies also reported a role for ORMDL3 in calcium homeostasis and the UPR,^{12,27,28} although there are also reports demonstrating no role for ORMDL3 in the UPR.²⁹ We measured UPR target gene expression in different tissues from *Ormdl3*^{-/-} and *Ormdl3*^{Tg/wt} mice but could not find any differences compared with wild-type mice (data not shown), suggesting that the UPR was not affected on changing ORMDL3 expression levels *in vivo*. It should be noted that dramatic overexpression of ER proteins will affect ER homeostasis anyway, and therefore the positive findings described *in vitro* might not be directly linked to ORMDL3.

Thus in this study we found no causal relationship between ORMDL3 expression levels and the severity of key asthma features in a HDM- or *A alternata*-driven allergic asthma model, despite clear alterations in sphingolipid levels in mice overexpressing or lacking ORMDL3. Therefore we do not support the hypothesis that ORMDL3 controls asthma by altering sphingolipid metabolism. More research is needed on the causal relationship between respiratory virus infection, SNPs at 17q21, ORMDL3 levels, and asthma risk before we can definitively exclude a major role for ORMDL3 in asthma.

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Key messages

- *Ormdl3* expression levels did not affect allergic asthma development driven by HDM or *A alternata*.
- ORMDL3 negatively regulated ceramide levels, but this did not result in altered experimental asthma.

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METHODS

Immunoglobulin production

Mice were bled after achievement of terminal anesthesia, and serum was collected by means of centrifugal phase separation to determine IgE and IgG₁ levels by means of ELISA (BD Biosciences, Franklin Lakes, NJ). For HDM-specific IgG₁, ELISA plates were coated with 100 µg/mL HDM (Greer Laboratories). For HDM-specific IgE, ELISA plates were coated with an anti-IgE capture antibody (BD Biosciences), and detection was performed with biotin-labeled HDM (100 µg/mL) diluted in PBS plus 10% FCS.

Flow cytometry

Bronchoalveolar lumen fluid was obtained by flushing the lungs with EDTA-containing PBS (0.5 mmol/L). Cell suspensions were stained with antibody cocktails in PBS for 30 minutes at 4°C and subsequently washed in PBS before readout. Unspecific antibody binding was prevented by adding 2.4G2 (antibody to the Fcγ receptor II/III) during the staining. A fixed amount of counting beads (123count eBeads; Thermo Fisher Scientific, Waltham, Mass) was added to determine absolute cell numbers. Dead cells were excluded by using the Fixable Viability Dye eFluor506 (eBioscience). Data acquisition was performed on a 4-laser Fortessa (BD Biosciences), and data were analyzed with FlowJo software (TreeStar, Ashland, Ore). Eosinophils were gated as CD11c[−]CD3/CD19[−]Ly6G[−]CD11b^{hi}Siglec-F^{hi}SSC-A^{hi} cells, T cells were gated as CD11c[−]CD3/CD19⁺MHCII[−] cells, and B cells were gated as CD11c[−]CD3/CD19⁺MHCII⁺ cells.

FlowSOM

Automated analysis is done by using the FlowSOM algorithm.^{E1,E2} FlowSOM uses a self-organizing map to cluster cells in different nodes based on expression of the distinct markers used in a given flow cytometric data set and subsequently structures the nodes in a minimally spanning tree. We first concatenated live cells and used FlowSOM to generate a single FlowSOM tree.

Image quantification

A Fiji-based software script was applied to quantify PAS⁺ cells and α-SMA in the lung to perform objective image quantification. RGB images were converted to 8-bit images, and binary masks were created by using the “autothreshold mean” method to detect the whole tissue.^{E3} Filtering by size allowed us to distinguish lung tissue from air space in the lung. Then, depending on the staining method, color deconvolution was applied, as described in Ruifrok and Johnston.^{E4} Positively stained cells were detected by using the Renyi entropy thresholding method,^{E5} and binary particle analysis was performed to filter particles by size. The positively stained area was then normalized to the total area of the lung.

Immunoblotting

Lung tissue was snap-frozen in liquid nitrogen during dissection. Lungs were homogenized with TissueLyser II (Qiagen, Hilden, Germany) in 400 µL of E1A buffer (1% NP-40, 20 mmol/L HEPES [pH 7.9], 250 mmol/L NaCl, and 1 mmol/L EDTA) complemented with cOmplete-ULTRA (Roche, Mannheim, Germany) protease inhibitors. Before SDS-PAGE, samples were spun at 12,000g to remove insoluble material. Protein concentrations were determined by using the Bradford assay. Samples were boiled for 5 minutes at 95°C before loading. After wet transfer to polyvinylidene difluoride membrane (Immobilon; Millipore), proteins were analyzed by means of immunoblotting and visualized by means of chemiluminescence (NEL 104001EA; PerkinElmer, Waltham, Mass). Primary antibodies used recognize β-galactosidase (ab4761, 1:1000; Abcam, Cambridge, Mass) and β-tubulin (ab21058, 1:5000; Abcam). Goat anti-rabbit-horseradish peroxidase (P0448, 1:2000; Dako) was used as a secondary antibody.

1-DER T-cell adoptive transfer

1-DER T cells were isolated out of spleens and lymph nodes from *Ormdl3*^{wt/wt} and *Ormdl3*^{Tg/wt} 1-DER mice. T cells were purified by making use of the MagniSort Mouse CD4 T cell Enrichment Kit (Thermo Fisher) and were labeled with Cell Trace Violet (Invitrogen, Carlsbad, Calif) during 10 minutes at 37°C. 1-DER cells (1 × 10⁶) of each genotype were intravenously injected into CD45.1 B6 acceptor mice, followed by intratracheal installation of 10 µg of HDM 2 hours after intravenous injection. Acceptor mice were killed 3 days later, and MLNs, lungs, and spleens were dissected for analysis.

Quantitative real time-PCR

Total RNA was isolated from mouse lung tissues by using TriPure Isolation Reagent (Roche) and isolated according to the manufacturer's instructions. One microgram of RNA was converted to cDNA by using an iScript advanced reverse transcriptase (Bio-Rad Laboratories, Hercules, Calif), according to the manufacturer's instructions. The target cDNA was amplified by 30 cycles of PCR with LightCycler 480 Probe Master Mix (Roche) and the following primers: *Ormdl3* (forward, CCCTCACCAACCTTATCCAC; reverse, GGACCCCGTAGTCCATCTG) and universal probe #109 (Universal Probe Library; Roche, Indianapolis, Ind). Expression levels were calculated by using qBase+ software (Biogazelle, Ghent, Belgium) and normalized to the 2 most stable reference genes among *Hmbs*, *Sdha*, *Hprt*, *Rpl13a*, and *Gapdh*. To amplify reference genes, SensiFAST SYBR No-ROX Kit (GC Biotech, Wadinxveen, the Netherlands) was used, and primers were as follows: *Hmbs* (forward, GAAACTCTGCTTCGTCGATT; reverse, TGCCCATCTTTCA TCACTGTATG), *Sdha* (forward, TTTCAGAGACGGCCATGATCT; reverse, TGGGAATCCCACCCATGTT), *Hprt* (forward, TGAAGAGCTACTGTAAT GATCAGTCAAC; reverse, AGCAAGCTTGCAACCTTAACCA), *Rpl13a* (forward, CCTGCTGCTCTCAAGGTTGTT; reverse, TGGTTGTC ACTGCCTGGTACTT), and *Gapdh* (forward, GCATGGCCTTCCGTGTTTC; reverse, TGTCATCATACTTGGCAGGTTTCT).

Tissue homogenization preceding lipid extraction

Tissues were homogenized in lysis buffer (25 mmol/L HEPES [pH 8] and 0.2% Triton X-100 [vol/vol]) by using a Precellys 24 tissue homogenizer (Bertin Technologies, Montigny-le Bretonneux, France). A volume that contained 100 mg of protein (as determined by using the Bradford assay) was filled up to 100 mL with saline for sphingolipid extraction.

Lipid extraction and sphingoid base analysis

Methanol (500 mL; Honeywell, Morris Plains, NJ) containing 200 pmol internal standards (d7-sphinganine and d7-sphingosine; Avanti Polar Lipids, Alabaster, Ala) was added to tissue homogenate containing 100 mg of protein or 100 µL of serum, followed by gentle rotation at 1400 rpm and 37°C for 1 hour (Thermomixer comfort; Eppendorf, Hamburg, Germany). Samples were then centrifuged at 16,000g for 5 minutes to pellet precipitated proteins, and the supernatant was transferred to a new 2-mL Eppendorf tube. HCl (≥32%; Sigma-Aldrich) was added, and lipids were hydrolyzed for 16 hours at 65°C and then neutralized with 10 mol/L KOH. Free sphingoid bases were extracted by adding chloroform (99.8%; Sigma-Aldrich) in basic conditions that contained 2N ammoniac (Sigma-Aldrich). Sphingoid bases were analyzed by using TSQ Quantum Ultra and Q Exactive liquid chromatography–mass spectrometry, as previously described.^{E6,E7}

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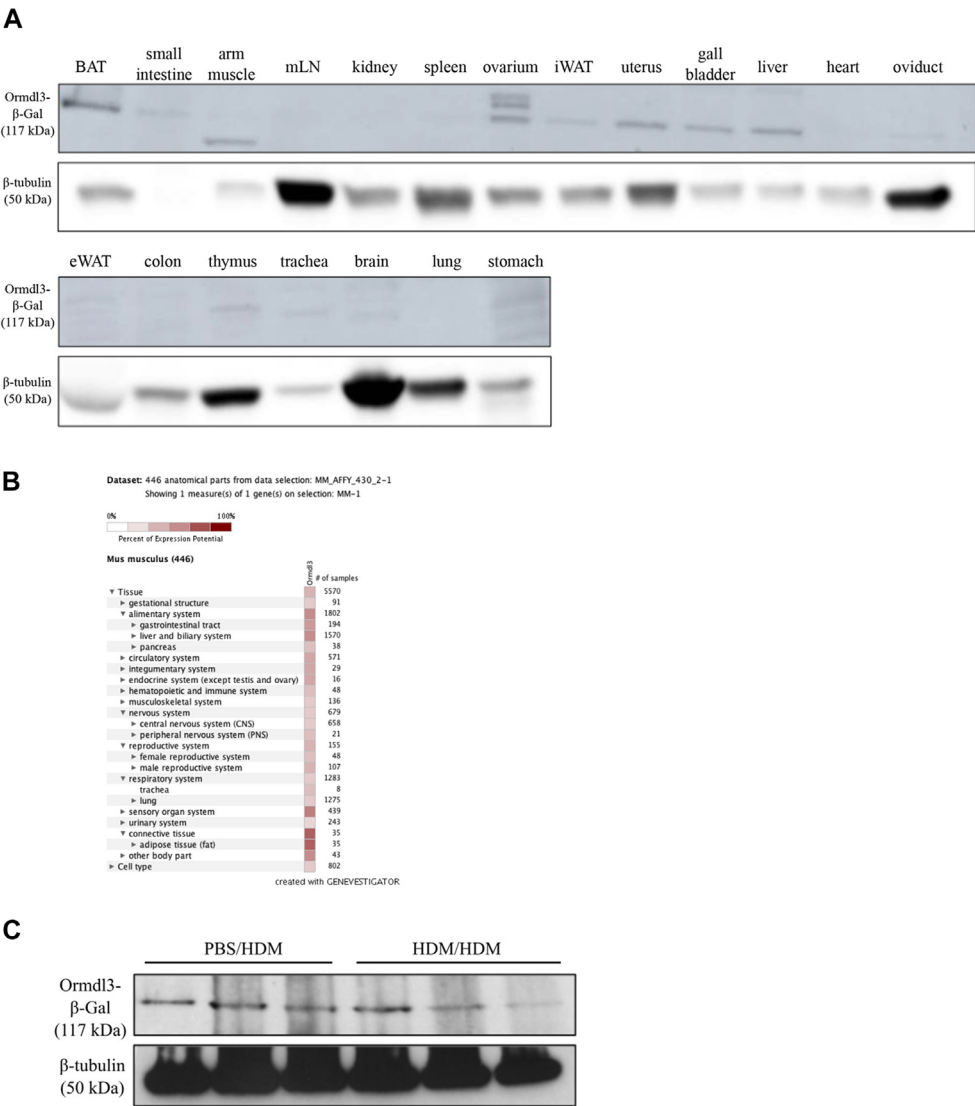


FIG E1. *Ormdl3*-LacZ reporter mice as a useful tool to study ORMDL3 expression. **A**, Western blot showing β -galactosidase expression in different tissues from an *Ormdl3*-LacZ reporter mouse. For each tissue, 50 μ g of protein (determined by using the Bradford assay) was loaded. β -Tubulin was used as a loading control. *eWAT*, Epididymal white adipose tissue; *iWAT*, inguinal white adipose tissue. **B**, Heat map showing *Ormdl3* mRNA expression in different murine tissues. The heat map demonstrates publicly available RNA sequencing data from the GENEVESTIGATOR database. **C**, Western blot showing β -galactosidase expression in lung tissue from PBS-sensitized (nonasthmatic) mice and HDM-sensitized (asthmatic) *Ormdl3*-LacZ reporter mice. β -Tubulin was used as a loading control.

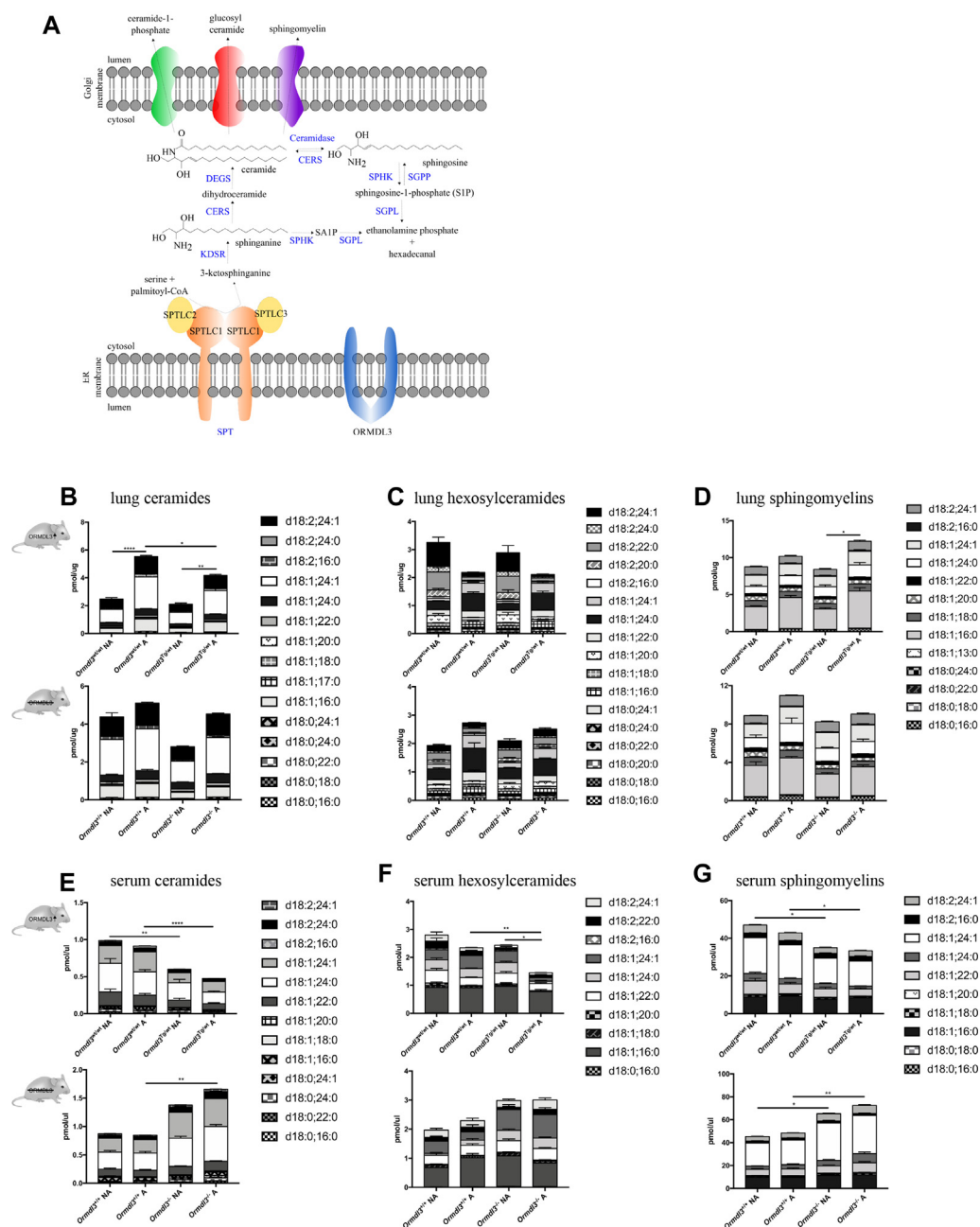


FIG E2. Effect of altered ORMDL3 expression on sphingolipid concentration. **A**, Schematic overview of sphingolipid synthesis. *De novo* sphingolipid synthesis starts with the action of SPT, which mediates the condensation of serine and palmitoyl-CoA into long-chain bases, such as sphinganine. Sphinganine is subsequently acylated to form (dihydro)ceramides by using the action of ceramide synthases (CERS). In the Golgi, ceramides can be converted to more complex sphingolipids, such as sphingomyelin, or can be broken down by ceramidases to form sphingosine. S1P, the phosphorylated form of sphingosine, is the substrate of S1P lyase (SGPL), which is the only enzyme converting sphingolipids into nonsphingolipid molecules. DEGS, sphingolipid delta(4)-desaturase; KDSR, 3-ketodihydrosphingosine reductase; SGPP, S1P phosphatase 1; SPHK, sphingosine kinase. **B–P**, Ceramide, hexosylceramide, and sphingomyelin levels in different tissues from (non-)asthmatic *Ormdl3*^{wt/wt} and *Ormdl3*^{Tg/wt} mice (upper part) and *Ormdl3*^{+/-} and *Ormdl3*^{-/-} mice (lower part). Fig E2, B–D, Lung; Fig E2, E–G, serum; Fig E2, H–J, liver; Fig E2, K–M, brown adipose tissue (BAT); Fig E2, N–P, white adipose tissue (WAT). Data information: All data were analyzed with a Shapiro-Wilk normality test to assess whether the data were normally distributed. Parametric data were analyzed with an ordinary 1-way ANOVA test with multiple comparison correction. Nonparametric data were analyzed with an unpaired Kruskal-Wallis test with multiple correction. Data are shown as means $\pm$ SEMs. **P* < .05, ***P* < .01, ****P* < .001, and *****P* < .0001. Upper panels (*Ormdl3*^{wt/wt} and *Ormdl3*^{Tg/wt} mice): *n* = 8, 13, 6, and 10, respectively; lower panels (*Ormdl3*^{+/-} and *Ormdl3*^{-/-} mice): *n* = 5, 5, 7, and 7, respectively.

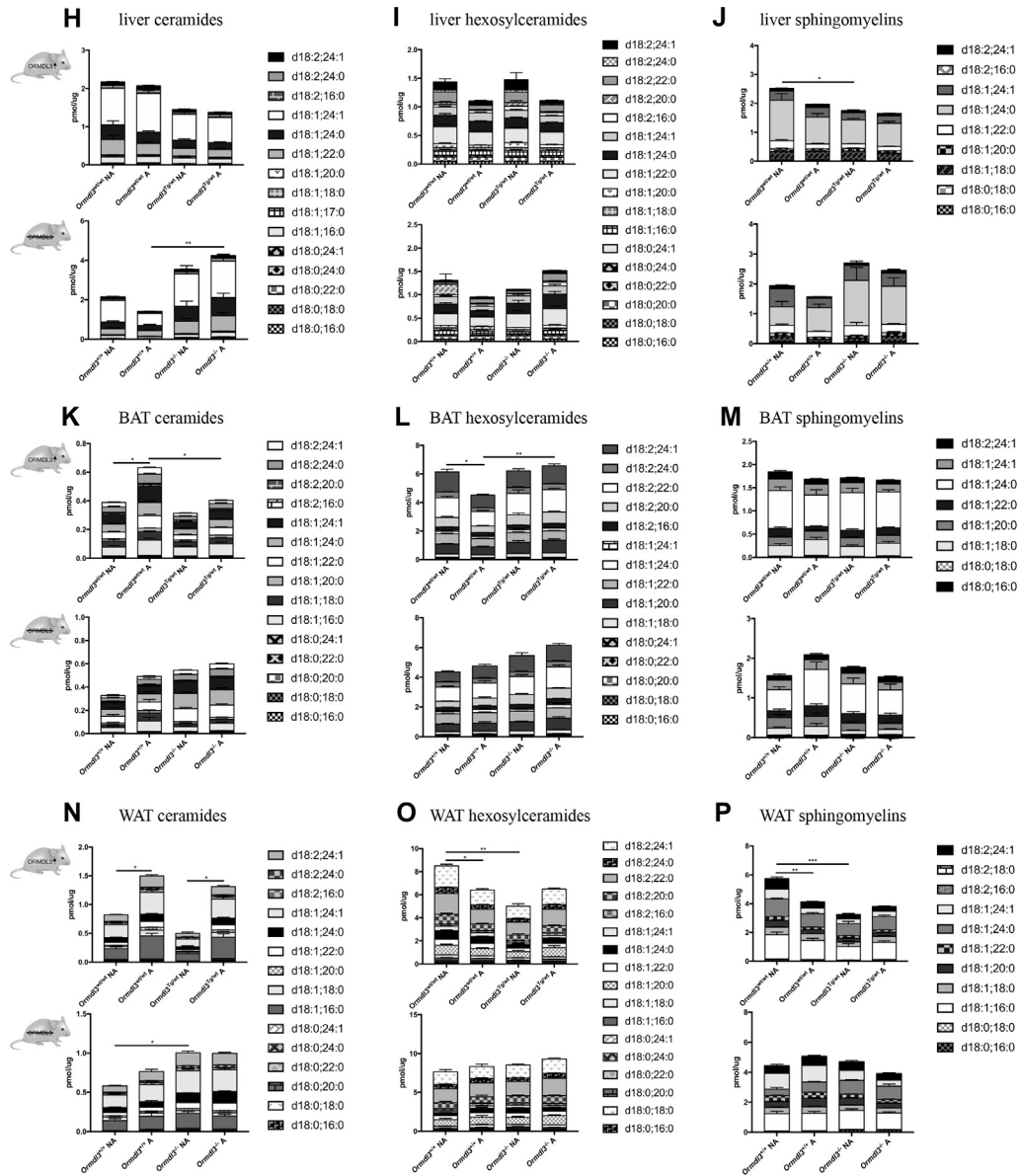


FIG E2. (Continued).

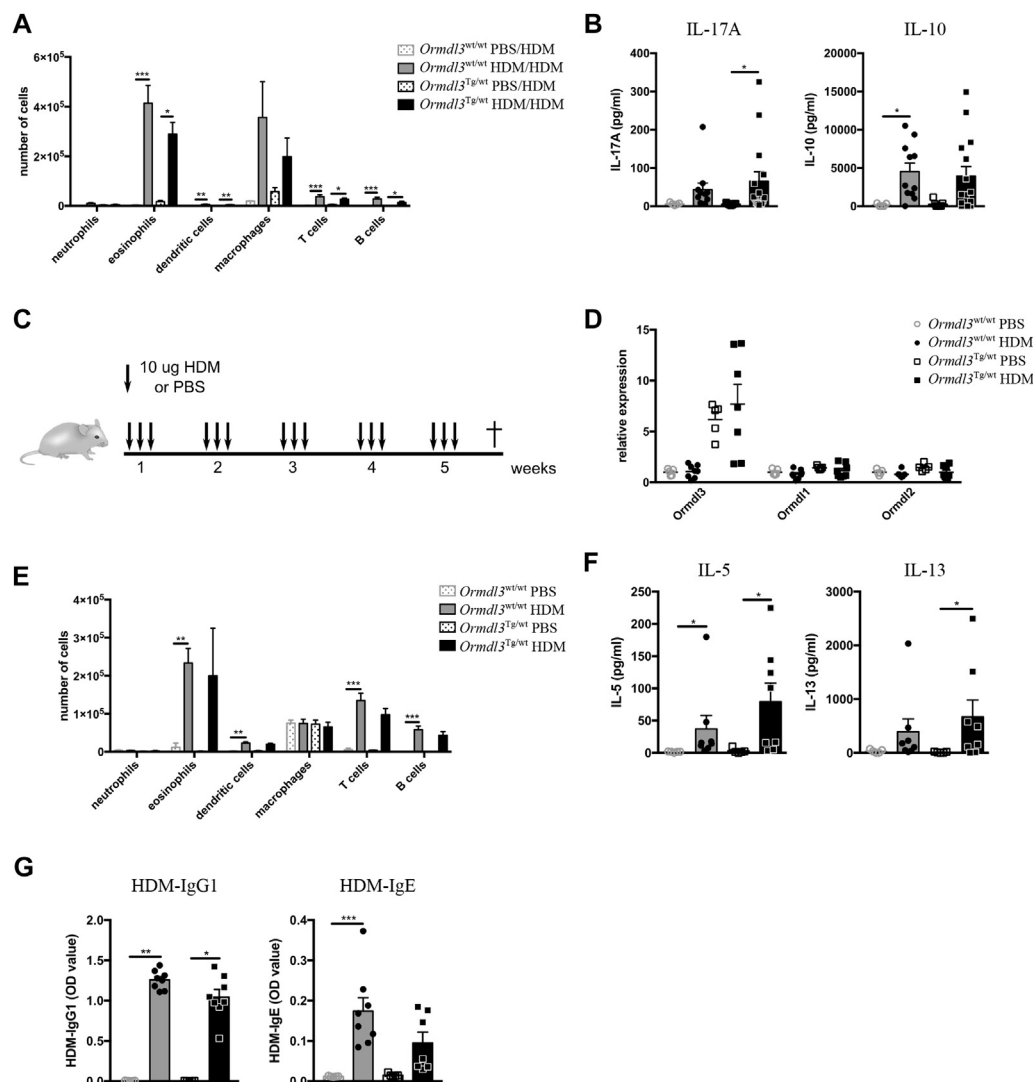


FIG E3. ORMDL3 overexpression does not affect allergic asthma driven by acute or chronic HDM exposures. **A**, Numbers of inflammatory cells in BAL fluid from mock- and HDM-sensitized *Ormdl3^{wt/wt}* and *Ormdl3^{Tg/wt}* mice ($n = 5, 11, 7$, and 15 , respectively). **B**, Protein levels of IL-10 and IL-17A secreted by MLN cells during restimulation with HDM for 3 days ($n = 5, 11, 7$, and 15 , respectively). In Fig E3, **A** and **B**, data were pooled from 2 independent experiments. **C**, Scheme representing the chronic HDM-dependent asthma model. **D**, *Ormdl* mRNA expression levels in lung tissue from PBS and HDM-treated *Ormdl3^{wt/wt}* and *Ormdl3^{Tg/wt}* mice. Expression values were calculated by using qBase+ and were normalized to the reference genes *Hmbs* and *Hprt*. Expression values are shown relative to means of the PBS-treated *Ormdl3^{wt/wt}* group ($n = 5, 8, 5$, and 7 , respectively). **E**, Numbers of inflammatory cells in BAL fluid from PBS- and HDM-treated *Ormdl3^{wt/wt}* and *Ormdl3^{Tg/wt}* mice ($n = 5, 8, 5$, and 8 , respectively). **F**, Protein levels of IL-5 and IL-13 secreted by MLN cells during restimulation with HDM for 3 days ($n = 5, 8, 5$, and 8 , respectively). **G**, Serum HDM-specific IgG₁ and HDM-IgE levels, as measured by using ELISA ($n = 5, 8, 5$, and 8 , respectively). Data information: All data were analyzed with a Shapiro-Wilk normality test to assess whether the data were normally distributed. Parametric data were analyzed with an ordinary 1-way ANOVA test with multiple comparison correction. Nonparametric data were analyzed with an unpaired Kruskal-Wallis test with multiple comparison correction. Data are shown as means $\pm$ SEMs. * $P < .05$, ** $P < .01$, *** $P < .001$, and **** $P < .0001$.

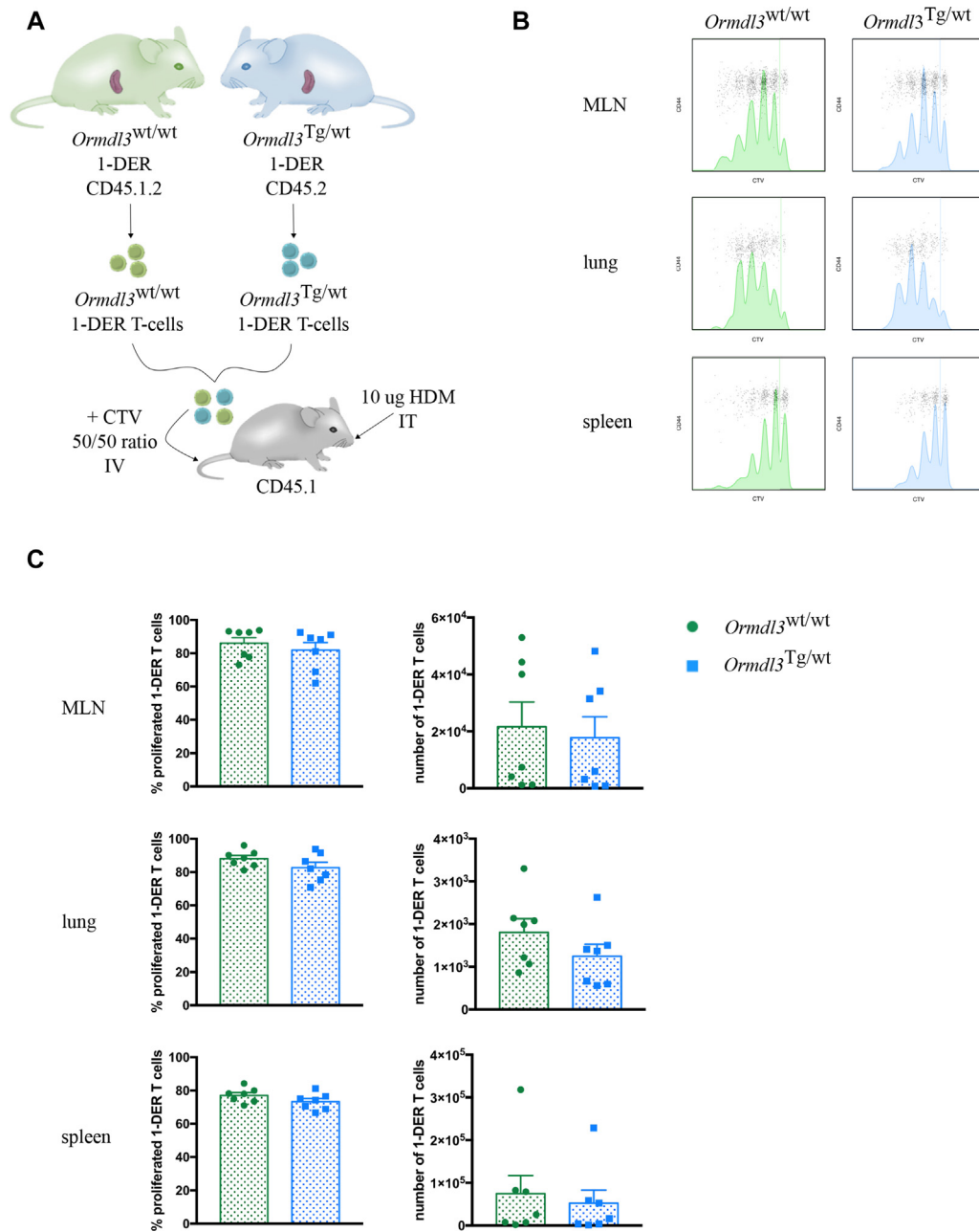


FIG E4. ORMDL3 does not affect T-cell function intrinsically. **A**, Scheme representing adoptive transfer of *Ormdl3*^{wt/wt} and *Ormdl3*^{Tg/wt} 1-DER T cells into CD45.1 B6 acceptor mice, which were consequently exposed to HDM. *IT*, Intratracheal; *IV*, intravenous. **B**, Proliferation profile of adoptively transferred 1-DER T cells labeled with CellTrace Violet (CTV) 3 days after transfer. **C**, Proliferation and numbers of adoptively transferred 1-DER T cells 3 days after transfer ($n = 7$ and 7 , respectively).

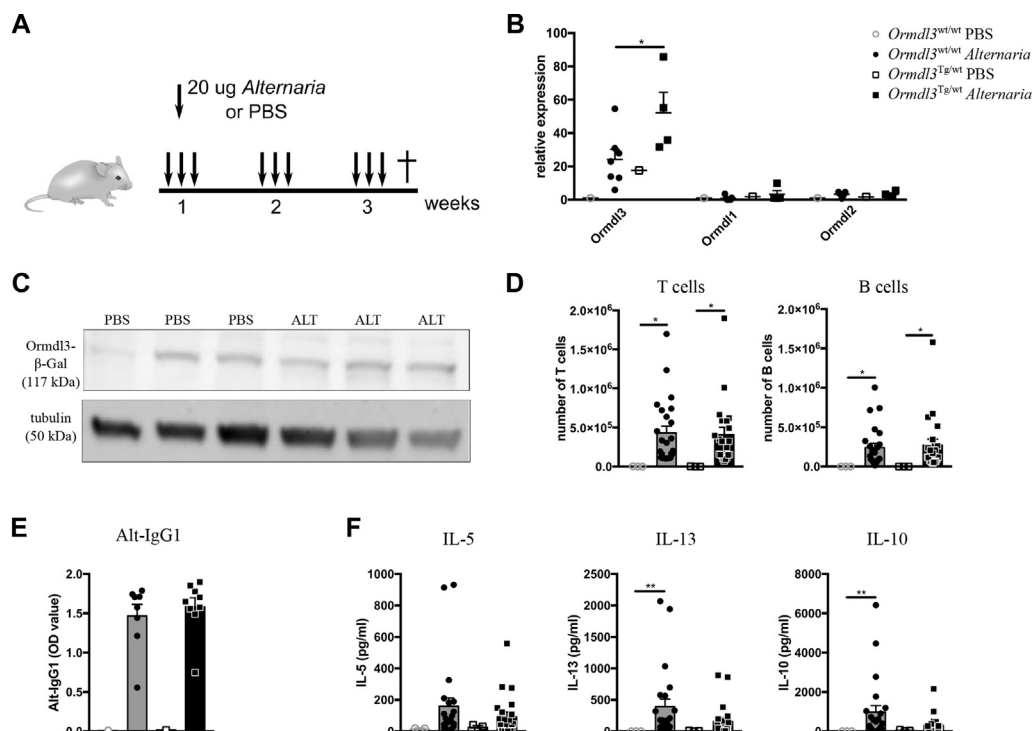


FIG E5. ORMDL3 overexpression does not affect allergic asthma driven by *Alternaria alternata* (ALT). **A**, Scheme representing the *A. alternata*-dependent asthma model. **B**, *Ormdl* mRNA expression levels in lung tissue from *Ormdl3*^{wt/wt} and *Ormdl3*^{Tg/wt} mice exposed to either PBS or *A. alternata*. Expression values were calculated by using qBase+ and were normalized to the reference genes *Hprt* and *Sdha*. Expression values are shown relative to means of the PBS-treated *Ormdl3*^{wt/wt} group (n = 1, 7, 1, and 4, respectively). **C**, Western blot showing β-galactosidase expression in lung tissue from nonasthmatic (PBS-treated) and asthmatic (*A. alternata*-treated) *Ormdl3*-LacZ reporter mice. β-Tubulin was used as a loading control. **D**, Numbers of B and T lymphocytes in BAL fluid from *Ormdl3*^{wt/wt} and *Ormdl3*^{Tg/wt} mice treated with PBS or *A. alternata* (n = 3, 24, 3, and 22, respectively). **E**, Serum *A. alternata*-specific IgG₁ levels (OD value), as measured by using ELISA (n = 1, 8, 1, and 9, respectively). **F**, Protein levels of IL-5, IL-13, and IL-10 secreted by MLN cells during restimulation with *A. alternata* for 3 days (n = 3, 24, 3, and 22, respectively). In Fig E5, **D** and **F**, data were pooled from 3 independent experiments. Data information: All data were analyzed with a Shapiro-Wilk normality test to assess whether the data were normally distributed. Parametric data were analyzed with an ordinary 1-way ANOVA test with multiple comparison correction. Nonparametric data were analyzed with an unpaired Kruskal-Wallis test with multiple comparison correction. Data are shown as means ± SEMs. **P* < .05, ***P* < .01, ****P* < .001, and *****P* < .0001.

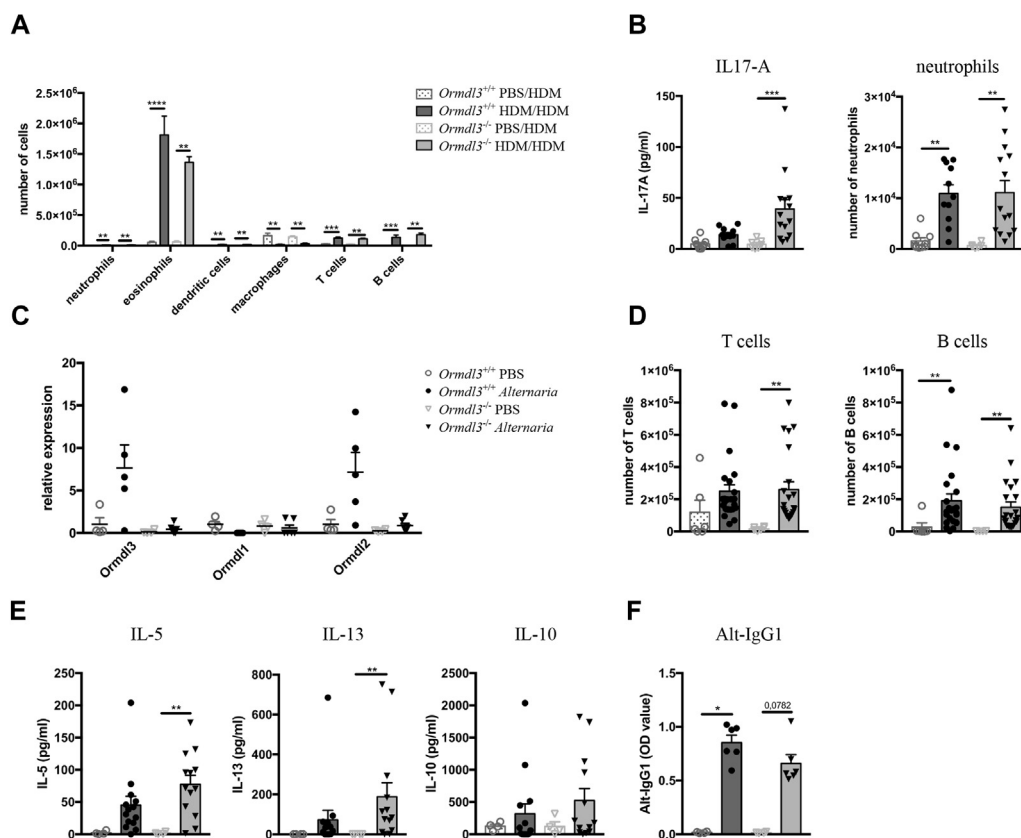


FIG E6. Loss of *Ormdl3* expression does not affect allergic asthma driven by HDM or *A alternata*. **A**, Numbers of inflammatory cells in BAL fluid from mock- and HDM-sensitized *Ormdl3*^{+/+} and *Ormdl3*^{-/-} mice ($n = 10, 11, 7$, and 14 , respectively). **B**, Numbers of neutrophils in BAL fluid and protein levels of IL-17A secreted by MLN cells during restimulation with HDM for 3 days ($n = 10, 11, 7$, and 14 , respectively). **C**, *Ormdl* mRNA expression levels in lung tissue from *Ormdl3*^{+/+} and *Ormdl3*^{-/-} mice exposed to either PBS or *A alternata*. Expression values were calculated by using qBase+ and normalized to the reference genes *Hprt* and *Rpl13a*. Expression values are shown relative to means of the PBS-treated *Ormdl3*^{+/+} group ($n = 4, 5, 4$, and 6 , respectively). **D**, Numbers of B and T lymphocytes in BAL fluid from *Ormdl3*^{+/+} and *Ormdl3*^{-/-} mice treated with PBS or *A alternata* ($n = 6, 23, 2$, and 22 , respectively). Data were pooled from 3 independent experiments. **E**, Protein levels of IL-5, IL-13, and IL-10 secreted by MLN cells during restimulation with *A alternata* for 3 days ($n = 4, 14, 4$, and 13 , respectively). **F**, Serum *A alternata*-specific IgG₁ levels (OD value), as measured by using ELISA ($n = 4, 6, 4$, and 6 , respectively). In Fig E6, **A**, **B**, and **E**, data were pooled from 2 independent experiments. Data information: All data were analyzed with a Shapiro-Wilk normality test to assess whether the data were normally distributed. Parametric data were analyzed with an ordinary 1-way ANOVA test with multiple comparison correction. Nonparametric data were analyzed with an unpaired Kruskal-Wallis test with multiple comparison correction. Data are shown as means $\pm$ SEMs. * $P < .05$, ** $P < .01$, *** $P < .001$, and **** $P < .0001$.