

# Aberrant ORM (yeast)-like protein isoform 3 (ORMDL3) expression dysregulates ceramide homeostasis in cells and ceramide exacerbates allergic asthma in mice

Clement Oyeniran, PhD,<sup>a,\*</sup> Jamie L. Sturgill, PhD,<sup>b,c,\*</sup> Nitai C. Hait, PhD,<sup>a</sup> Wei-Ching Huang, PhD,<sup>a</sup> Dorit Avni, PhD,<sup>a</sup> Michael Maceyka, PhD,<sup>a</sup> Jason Newton, PhD,<sup>a</sup> Jeremy C. Allegood, PhD,<sup>a</sup> Alison Montpetit, PhD,<sup>c</sup> Daniel H. Conrad, PhD,<sup>b</sup> Sheldon Milstien, PhD,<sup>a</sup> and Sarah Spiegel, PhD<sup>a</sup> *Richmond, Va*

**Background:** Asthma, a chronic inflammatory condition defined by episodic shortness of breath with expiratory wheezing and cough, is a serious health concern affecting more than 250 million persons. Genome-wide association studies have identified ORM (yeast)-like protein isoform 3 (ORMDL3) as a gene associated with susceptibility to asthma. Although its yeast ortholog is a negative regulator of *de novo* ceramide biosynthesis, how ORMDL3 contributes to asthma pathogenesis is not known. **Objectives:** We sought to decipher the molecular mechanism for the pathologic functions of ORMDL3 in asthma and the relationship to its evolutionarily conserved role in regulation of ceramide homeostasis.

**Methods:** We determined the relationship between expression of ORMDL3 and ceramide in epithelial and inflammatory cells and in asthma pathogenesis in mice.

**Results:** Although small increases in ORMDL3 expression decrease ceramide levels, remarkably, higher expression in lung epithelial cells and macrophages *in vitro* and *in vivo* increased ceramide production, which promoted chronic inflammation, airway hyperresponsiveness, and mucus production during house dust mite-induced allergic asthma. Moreover, nasal administration of the immunosuppressant drug FTY720/fingolimod reduced ORMDL3 expression and ceramide levels and mitigated airway inflammation and hyperreactivity and mucus hypersecretion in house dust mite-challenged mice. **Conclusions:** Our findings demonstrate that overexpression of ORMDL3 regulates ceramide homeostasis in cells in a complex manner and suggest that local FTY720 administration might be a useful therapeutic intervention for the control of allergic asthma. (J Allergy Clin Immunol 2015;■■■:■■■-■■■.)

**Key words:** Asthma, ORMDL3, sphingolipid, ceramide, FTY720, house dust mite

From <sup>a</sup>the Department of Biochemistry and Molecular Biology and the Massey Cancer Center and <sup>b</sup>the Department of Microbiology & Immunology, Virginia Commonwealth University School of Medicine, and <sup>c</sup>the School of Nursing, Virginia Commonwealth University.

\*These authors contributed equally to this work.

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Corresponding author: Sarah Spiegel, PhD, Department of Biochemistry and Molecular Biology and the Massey Cancer Center, Virginia Commonwealth University School of Medicine, PO Box 980614, Richmond, VA 23298. E-mail: [sspiegel@vcu.edu](mailto:sspiegel@vcu.edu).

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## Abbreviations used

|               |                                                                        |
|---------------|------------------------------------------------------------------------|
| AHR:          | Airway hyperresponsiveness                                             |
| BALF:         | Bronchoalveolar lavage fluid                                           |
| CerS:         | Ceramide synthases                                                     |
| FB1:          | Fumonisin B1                                                           |
| GWAS:         | Genome-wide association study                                          |
| HDM:          | House dust mite                                                        |
| H&E:          | Hematoxylin and eosin                                                  |
| LC-ESI-MS/MS: | Liquid chromatography–electrospray ionization–tandem mass spectrometry |
| ORMDL3:       | ORM (yeast)-like protein isoform 3                                     |
| PAS:          | Periodic acid–Schiff                                                   |
| siRNA:        | Small interfering RNA                                                  |
| SPT:          | Serine palmitoyltransferase                                            |
| UPR:          | Unfolded protein response                                              |

Asthma is a common inflammatory disorder characterized by intermittent airflow obstruction, airway hyperreactivity, and increased mucus production. There is a strong genetic component to asthma, and several genome-wide association studies (GWASs) have demonstrated that polymorphisms in a region of chromosome 17q21, which includes the ORM (yeast)-like protein isoform 3 (ORMDL3) gene, contribute to both childhood- and adult-onset asthma in a number of ethnically diverse populations.<sup>1–8</sup> However, the mechanism by which ORMDL3 contributes to asthma pathogenesis is not well understood and is a matter of debate.

ORMDL3 is the only member of the evolutionarily conserved family of 3 endoplasmic reticulum-localized proteins, ORMDL1 to ORMDL3, which has been linked to asthma. In yeast the ORM proteins negatively regulate sphingolipid homeostasis in response to physiologic needs<sup>9–15</sup> by forming a complex with and inhibiting serine palmitoyltransferase (SPT), the first and rate-limiting enzyme in ceramide biosynthesis.<sup>9–12</sup> Recent studies suggest that ORMDL3 also regulates ceramide biosynthesis in mammalian cells.<sup>9,16</sup> Because ORMDL3 polymorphism is associated with its high expression,<sup>4,17</sup> it was suggested that this would correlate with decreased ceramide biosynthesis in asthmatic patients.<sup>18</sup> Inhibiting SPT or genetically reducing its activity in mice decreased lung ceramide levels and increased bronchial reactivity without inflammation, mucus production, or airway remodeling.<sup>18</sup> In sharp contrast, others have reported that ceramide levels are increased in airway epithelia of ovalbumin-sensitized guinea pigs, and inhibition of ceramide biosynthesis with fumonisin B1 (FB1), an inhibitor of ceramide synthases (CerS), before antigen challenge attenuated airway inflammation

and improved abnormal respiratory function and bronchoconstriction.<sup>19</sup> Indeed, increased levels of C16-ceramide observed in CerS2-null mice or its augmentation in the lung by means of intratracheal delivery increased lung inflammation and tissue remodeling and caused airway flow obstruction.<sup>20–22</sup> Ceramide levels are also increased in patients with chronic obstructive pulmonary disease.<sup>21</sup> Although allergen induced ORMDL3 expression in mouse lungs,<sup>23,24</sup> the consequences for lung inflammation are controversial. Although one study in bronchial epithelial cells showed that increased ORMDL3 regulates expression of metalloproteinases, cytokines, and chemokines,<sup>23</sup> ORMDL3 expression did not cause these effects in a later study.<sup>25</sup> Moreover, although it has been suggested that ORMDL3 is an endogenous negative regulator of ceramide biosynthesis with levels that are increased in asthmatic patients, increased rather than decreased ceramide levels appear to contribute to the pathology of asthma, making this a biological paradox. In this study we examined the relationship between expression of ORMDL3 and ceramide levels in epithelial and inflammatory cells and in asthma susceptibility in mice. We provide a mechanism by which high expression of ORMDL3 enhances rather than reduces ceramide levels in cells. We also found that local administration of the immunosuppressive drug FTY720 reduced house dust mite (HDM)–induced increases in ORMDL3 expression and lung ceramide levels and attenuated asthma severity.

## METHODS

### Cell culture

A549 human lung carcinoma cells and RAW264.7 murine macrophages were cultured in high-glucose Dulbecco modified Eagle medium containing 10% FBS and 2 mmol/L L-glutamine. NCI-H292 cells, a human lung mucoepidermoid carcinoma cell line, were cultured in RPMI 1640 medium containing 10% FBS and 25 mmol/L HEPES (pH 7.4). ORMDL3 was downregulated and overexpressed, as previously described.<sup>26</sup>

### Mass spectrometric measurements

Lipids were extracted from cells, lung tissues, and bronchoalveolar lavage fluid (BALF), and sphingolipids were quantified by means of liquid chromatography–electrospray ionization–tandem mass spectrometry (LC-ESI-MS/MS; 4000 QTRAP, AB Sciex, Concord, Ontario, Canada).<sup>26,27</sup> *De novo* ceramide biosynthesis and recycling were analyzed by labeling cells with [<sup>13</sup>C]palmitate (Sigma, St Louis, Mo), as previously described.<sup>28,29</sup>

### HDM model of allergic lung inflammation

Female C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, Me) and kept in the animal care facilities at Virginia Commonwealth University under standard temperature, humidity, and timed light conditions and provided with standard rodent chow and water *ad libitum*. Animal procedures were approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University. Eight-week-old mice were challenged daily by means of intranasal administration with 25  $\mu$ L of saline alone or with 15  $\mu$ g of HDM for 5 consecutive days from days 1 to 5 and from days 8 to 12. Thirty minutes before each challenge, mice were treated intranasally with either vehicle (saline) or FTY720 (0.3 mg/kg). In some experiments mice were injected intraperitoneally with vehicle, myriocin (0.3 mg/kg), or FB1 (0.5 mg/kg) from days 10 to 12. IL-13 knockout mice were kindly provided by Dr McKenzie (MRC, Cambridge, United Kingdom).

## Measurement of airway reactivity

Mice were exposed to aerosol challenges containing increasing doses of acetyl- $\beta$ -methylcholine chloride (methacholine), and resistance, elastance, and tissue damping were measured with FlexiVent software FlexiWare, version 7 (SCIREQ, Montreal, Quebec, Canada).<sup>27</sup>

Data on BALF analysis and lung histology BALF were collected, and cells were quantified, as previously described.<sup>27,30</sup> Mucin secretion was measured by using an ELISA.<sup>27</sup> Paraffin-embedded lung tissues were stained with hematoxylin and eosin (H&E) or periodic acid–Schiff (PAS), as previously described.<sup>27</sup> Expression of ORMDL3 in paraffin-embedded lung tissue sections was evaluated by using rabbit anti-ORMDL3 (0.5  $\mu$ g/mL), as previously described.<sup>23,24</sup>

## Statistical analysis

Statistical analysis was performed with the unpaired 2-tailed Student *t* test for comparison of 2 groups and ANOVA with the Bonferroni *post hoc* comparison for experiments consisting of 3 or more groups (GraphPad Prism; GraphPad Software, La Jolla, Calif). A *P* value of less than .05 was considered significant.

Materials and transfections, quantitative PCR, Western blotting, airway reactivity, lung histology, immunohistochemistry, and BALF analysis are detailed in the [Methods](#) section in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org).

## RESULTS

### Endogenous ORMDL3 suppresses ceramide biosynthesis in mammalian cells

Although it has been conclusively demonstrated that yeast ORM proteins are negative regulators of SPT,<sup>9–11,13–15</sup> the rate-limiting enzyme in ceramide biosynthesis, only a few studies have addressed their function in regulation of sphingolipid biosynthesis in mammalian cells.<sup>9,16,31</sup> We focused our attention on lung epithelial and macrophage cell lines because ORMDL3 is predominantly induced in airway epithelial cells and macrophages in allergen-challenged mice.<sup>23</sup> Similar to previous results with HeLa cells,<sup>9</sup> simultaneous knockdown of ORMDL1, ORMDL2, and ORMDL3 in lung epithelial cell lines markedly increased levels of all ceramide species (Fig 1, A, and see Fig E1, A, in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). In both A549 and NCI-H292 lung epithelial cells, depletion of ORMDL3 or ORMDL1 greatly increased the major ceramide species, particularly very long-chain C24:0. In contrast, ORMDL2 depletion did not have a significant effect on any of the ceramide species (Fig 1, A, and see Fig E1, A). The same acyl chain species were also increased in dihydroceramides (see Fig E1, B and C), precursors of ceramides, indicating a role of ORMDL proteins in *de novo* ceramide synthesis, as expected. Stronger decreases in ceramide and dihydroceramide levels were observed when all 3 ORMDL proteins were simultaneously downregulated (Fig 1, A, and see Fig E1), suggesting that ORMDL3 and ORMDL1 have redundant or overlapping effects on ceramide synthesis.

Next, we examined the effect of ORMDL3 overexpression because only expression of ORMDL3 and not ORMDL1 has been linked to asthma severity in human subjects<sup>1,3–6,8</sup> and mice.<sup>23,24,32</sup> ORMDL3 overexpression decreased ceramide levels, with the largest effects on C24:0 and C24:1 in A549 cells (Fig 1, B). Similar results were obtained in other cell types, including HEK293 cells (data not shown). Moreover, when all 3 ORMDL proteins were downregulated, overexpression of ORMDL3 alone reduced increased ceramide levels (Fig 1, C), again substantiating

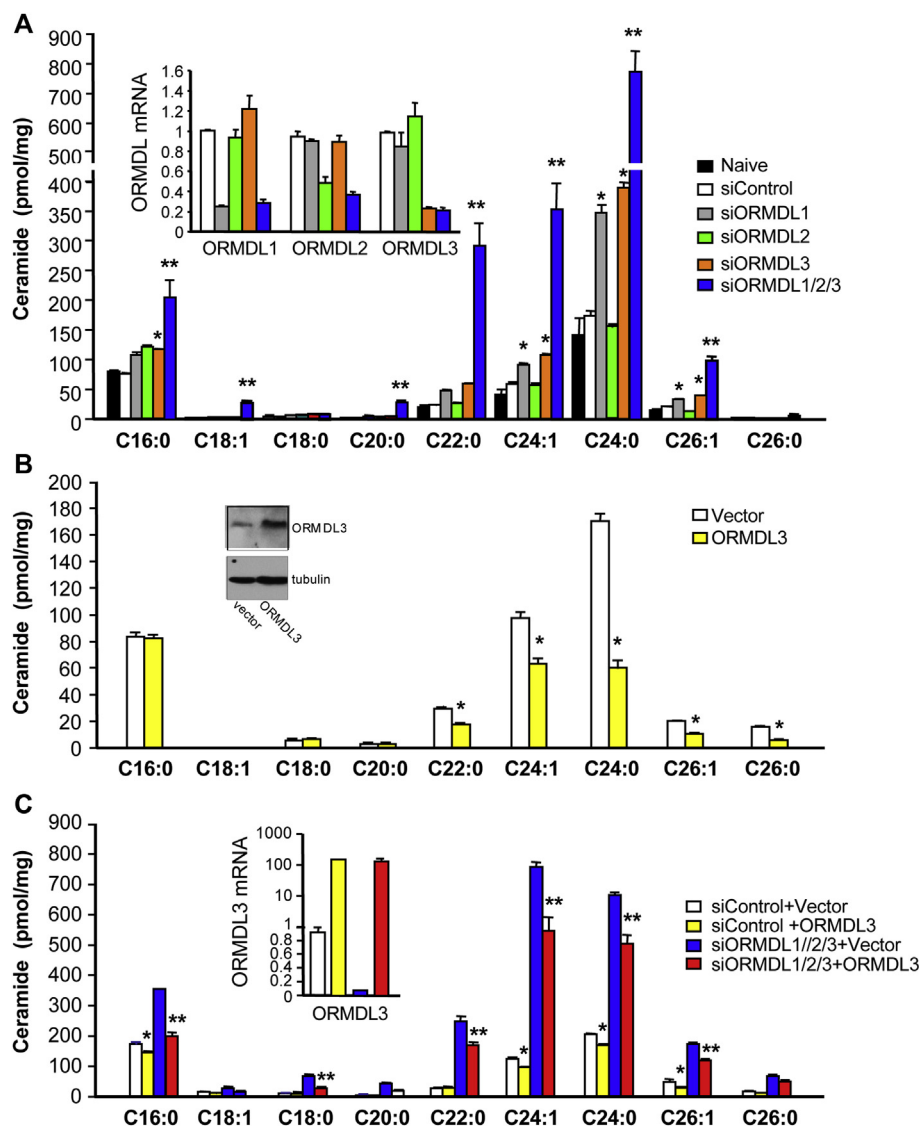


**FIG 1.** Ceramide levels in lung epithelial cells are increased by depletion of ORMDL3 and reduced by its re-expression. **A**, A549 cells were transfected with scrambled siRNA (*siControl*) or specific siRNAs for each ORMDL isoform, as indicated. **B**, Cells were transfected with 1  $\mu$ g of vector or ORMDL3 expression vector. **C**, After downregulation of ORMDL isoforms, cells were transfected with 1  $\mu$ g of vector or ORMDL3. Ceramide species were measured by using LC-ESI-MS/MS. Experiments were performed in biological triplicates. Data are shown as means  $\pm$  SDs and representative of 3 independent experiments. Fig 1, A, \* $P$  < .01 compared with siControl and \*\* $P$  < .001 compared with siORMDL3. Fig 1, B, \* $P$  < .01 compared with vector. Fig 1, C, \* $P$  < .05 compared with vector and \*\* $P$  < .005 compared with siORMDL1/2/3 transfected with vector. Fig 1, A and C, insets, Expression of ORMDL isoforms determined by means of quantitative PCR. Fig 1, B, inset, Cell lysates analyzed by means of immunoblotting with ORMDL3 antibody.

the notion that ORMDL3 functions as a brake on ceramide biosynthesis.

### Higher ORMDL3 expression enhances rather than reduces ceramide levels

Surprisingly, in the course of these experiments, we found that when ORMDL3 was expressed at higher levels in A549 lung epithelial cells, ceramide levels were enhanced rather than reduced (Fig 2, A and B). Similarly, in RAW264.7 macrophages, although low levels of ORMDL3 overexpression (using plasmid concentrations of up to 1  $\mu$ g of plasmid/ $10^6$  cells) also reduced ceramide

levels, higher levels of expression significantly increased ceramide levels (Fig 2, C and D). Taken together, these results suggest that the stoichiometry of ORMDL expression is crucial for its function as a negative regulator of SPT, which is consistent with the demonstration that ORM proteins in yeast form a stoichiometric complex with SPT subunits, inhibiting its activity.<sup>9,10</sup>

It was previously shown that LPS, which elicits strong inflammatory responses, increases ceramide levels in RAW264.7 macrophages.<sup>28</sup> In agreement with this, LPS increased total ceramide levels in these cells from  $1103 \pm 65$  to  $1389 \pm 50$  pmol/mg, which were further enhanced by high expression of ORMDL3 to  $1528 \pm 23$  pmol/mg.

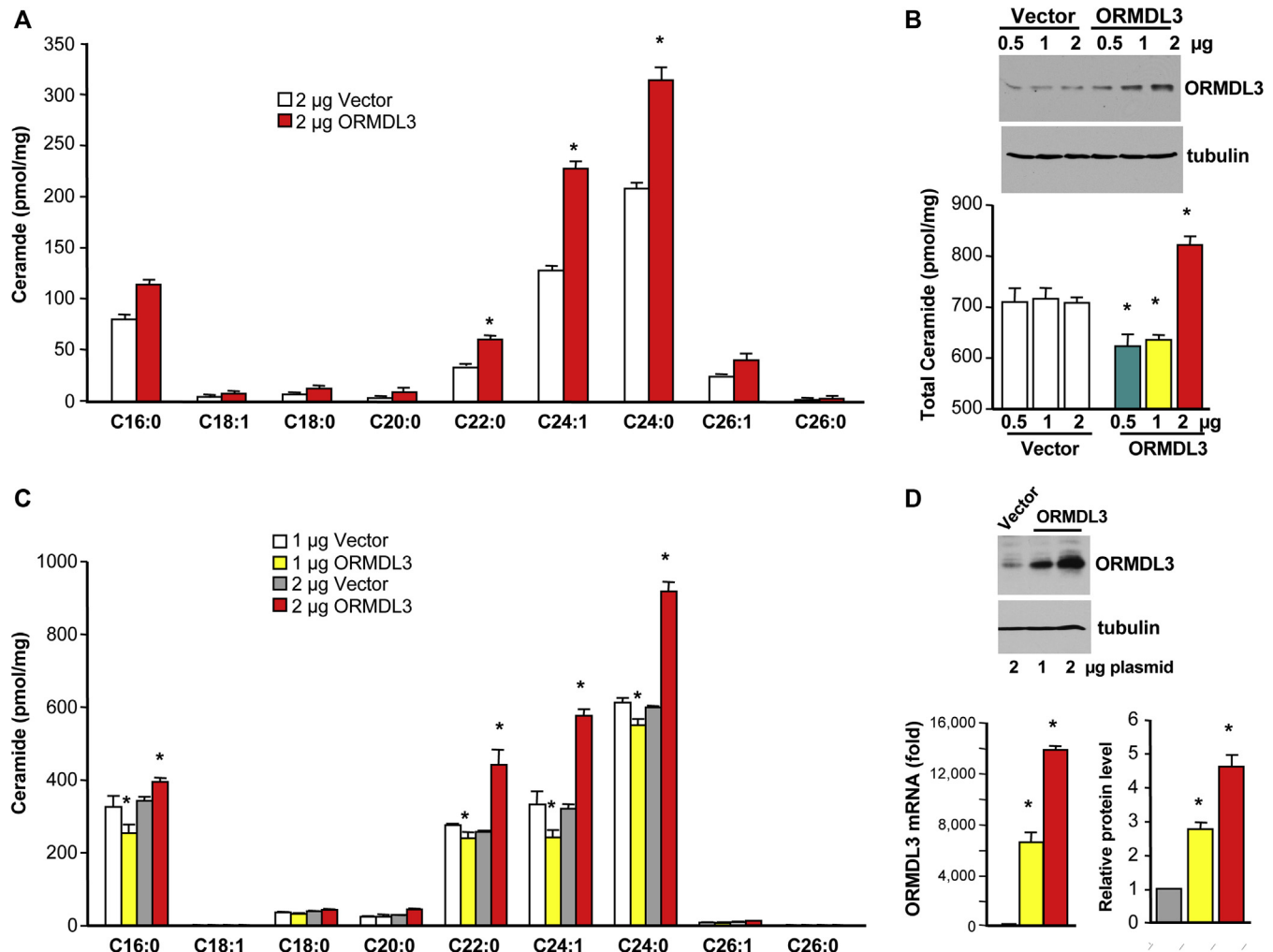


**FIG 2.** High overexpression of ORMDL3 enhances rather than decreases ceramide levels. A549 (**A** and **B**) or RAW264.7 (**C** and **D**) cells were transfected with vector or ORMDL3, as indicated. Ceramide species (Fig 2, **A** and **C**) and total ceramide (Fig 2, **B**) levels were determined by using LC-ESI-MS/MS. Fig 2, **B** and **D**, Cell lysates were analyzed by means of immunoblotting with ORMDL3 antibody and quantified by means of densitometry, and ORMDL3 expression was determined by using quantitative PCR. \* $P < .05$  compared with vector.

Next, we sought to determine the mechanism responsible for increased ceramide levels after high ORMDL3 expression. This could be due to increased *de novo* biosynthesis or the recycling/salvage pathway from degradation of sphingolipids (Fig 3, **A**). Therefore cells overexpressing different levels of ORMDL3 were pulse labeled with [ $^{13}\text{C}$ ]palmitate, which is converted into [ $^{13}\text{C}$ ]palmitoyl-CoA, a substrate for SPT, and also used for N-acylation of sphingosine to ceramide by CerSs. Changes in levels of 4 isotopically distinct ceramide species analyzed by means of mass spectrometry indicate the contribution of the 2 pathways.<sup>28,29</sup> Ceramides labeled in only the sphingoid base backbone (base-labeled ceramides) and ceramides with a labeled sphingoid base plus an amide-linked [ $^{13}\text{C}$ ]palmitate (dual-labeled ceramides) are unambiguously made by using *de novo* biosynthesis. Ceramides labeled in only the amide-linked fatty acid (fatty acid-labeled ceramides) and ceramides with only  $^{12}\text{C}$  (unlabeled ceramides) contain [ $^{12}\text{C}$ ]sphingoid bases that have been released in the recycling/salvage pathway by turnover and then reacylated by CerS using [ $^{13}\text{C}$ ]palmitoyl-CoA or with endogenous

unlabeled fatty acyl CoAs, respectively. As expected from a negative regulator of SPT, ORMDL3 expression reduced levels of base-labeled and dual-labeled ceramides, although low-level ORMDL3 expression had a more pronounced effect on base-labeled ceramides (Fig 3, **B** and **C**). Unlabeled ceramides make up the majority of cellular ceramides. Although no effects on fatty acid-labeled ceramides were observed (Fig 3, **D**), only high expression of ORMDL3 significantly induced increases in several unlabeled ceramide species, particularly C24:0>C24:1>C16:0 (Fig 3, **E**), indicating that increased ceramide levels are not due to effects on the *de novo* pathway but rather on the recycling/salvage pathway.

### ORMDL3 overexpression enhances cytokine and chemokine production induced by allergens

It has been suggested that ceramides are involved in the development of airway inflammation and regulation of proinflammatory cytokine and chemokine production.<sup>19-21</sup> However,

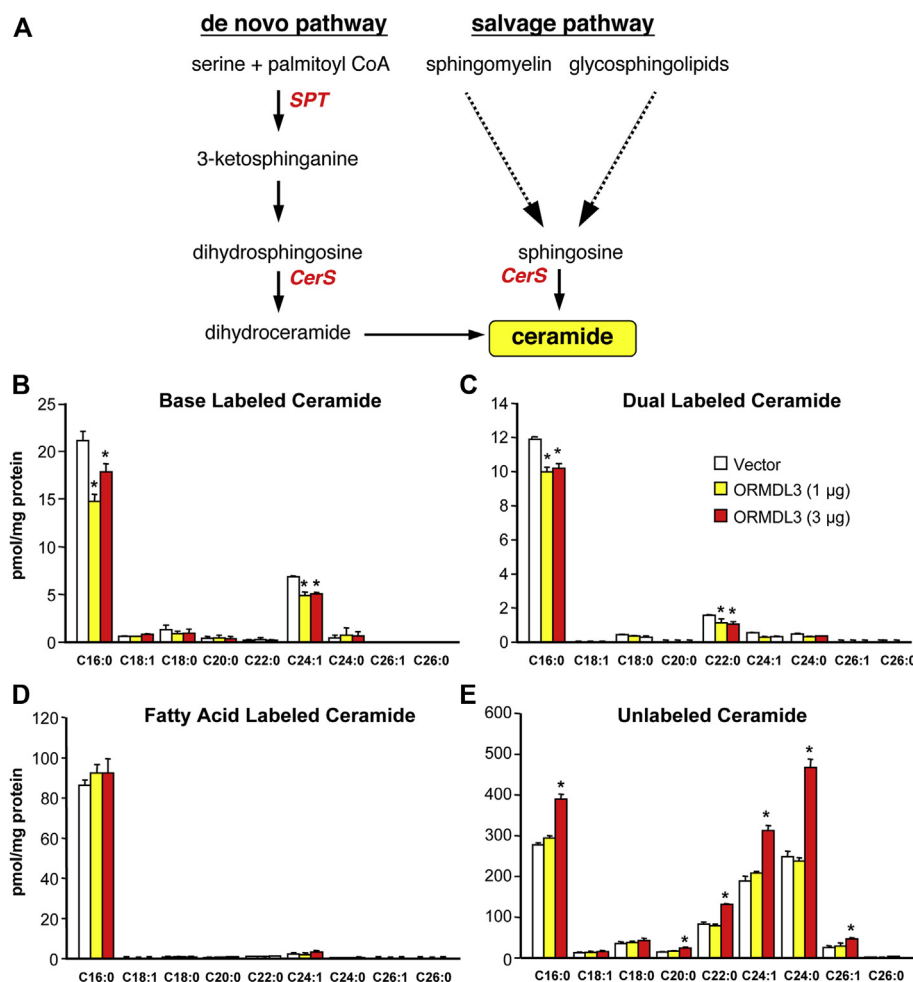


**FIG 3.** Effect of ORMDL3 expression on incorporation of [ $^{13}\text{C}$ ]palmitate into ceramides. **A**, Scheme of ceramide formation by *de novo* biosynthesis and salvage/recycling pathways. **B–E**, A549 cells were transfected with vector or ORMDL3, as indicated, and then incubated for 4 hours with 0.1 mmol/L [ $^{13}\text{C}$ ] palmitic acid. Base-labeled (Fig 3, B), dual-labeled (Fig 3, C), fatty acid-labeled (Fig 3, D), and unlabeled (Fig 3, E) ceramides were determined by using LC-ESI-MS/MS. Data are means  $\pm$  SDs. \* $P < .05$  compared with vector.

whether ORMDL3 regulates inflammatory responses is still an unresolved issue because of conflicting reports.<sup>23,25</sup> Thus it was of interest to examine the effect of ORMDL3 overexpression on cellular responses to common microbial antigens recognized by the innate immune system. The proinflammatory cytokines IL-6, IL-1 $\beta$ , and TNF- $\alpha$ ; the profibrotic cytokine TGF- $\beta$ 1; and the chemokines CCL2 and CXCL10 involved in asthmatic inflammation were markedly increased in RAW264.7 macrophages overexpressing ORMDL3 (Fig 4, A), correlating with enhanced ceramide levels (Fig 2, C). In contrast, lower ORMDL3 overexpression, which did not increase ceramide levels (Fig 2, C), did not enhance the responses to LPS (Fig 4, A). Moreover, in A549 lung epithelial cells overexpression of a high level of ORMDL3 itself, but not a lower level, significantly increased expression of IL-6 and the chemokines CCL2, CCL5, CCL20, CXCL8 and CXCL10 (Fig 4, B), which participate in mucosal innate and adaptive immune responses in airways of asthmatic patients. Similarly, high ORMDL3 expression in lung mucoepidermoid carcinoma NCI-H292 cells that increases ceramide levels (data not shown) significantly enhanced

expression of cytokines and chemokines (see Fig E2 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)) in response to HDM, a ubiquitous indoor allergen contaminated with LPS from colonizing bacteria and one of the most clinically relevant human aeroallergens contributing to allergic asthma worldwide.<sup>33,34</sup> Collectively, these data suggest that when highly overexpressed, ORMDL3 does not decrease ceramide levels but rather increases them, and the excess ceramide enhances production of the same cytokines and chemokines that are increased in human asthmatic patients<sup>35,36</sup> and mouse models of asthma.<sup>34</sup>

### HDM challenge induces expression of ORMDL3 and increases lung ceramide levels

Because ORMDL3 has been linked to asthma susceptibility,<sup>1–8</sup> we next sought to examine its expression in lungs of mice challenged intranasally with HDM in a mouse model of human allergic asthma that recapitulates the cardinal clinical features of human asthma, including airway hyperreactivity, cellular

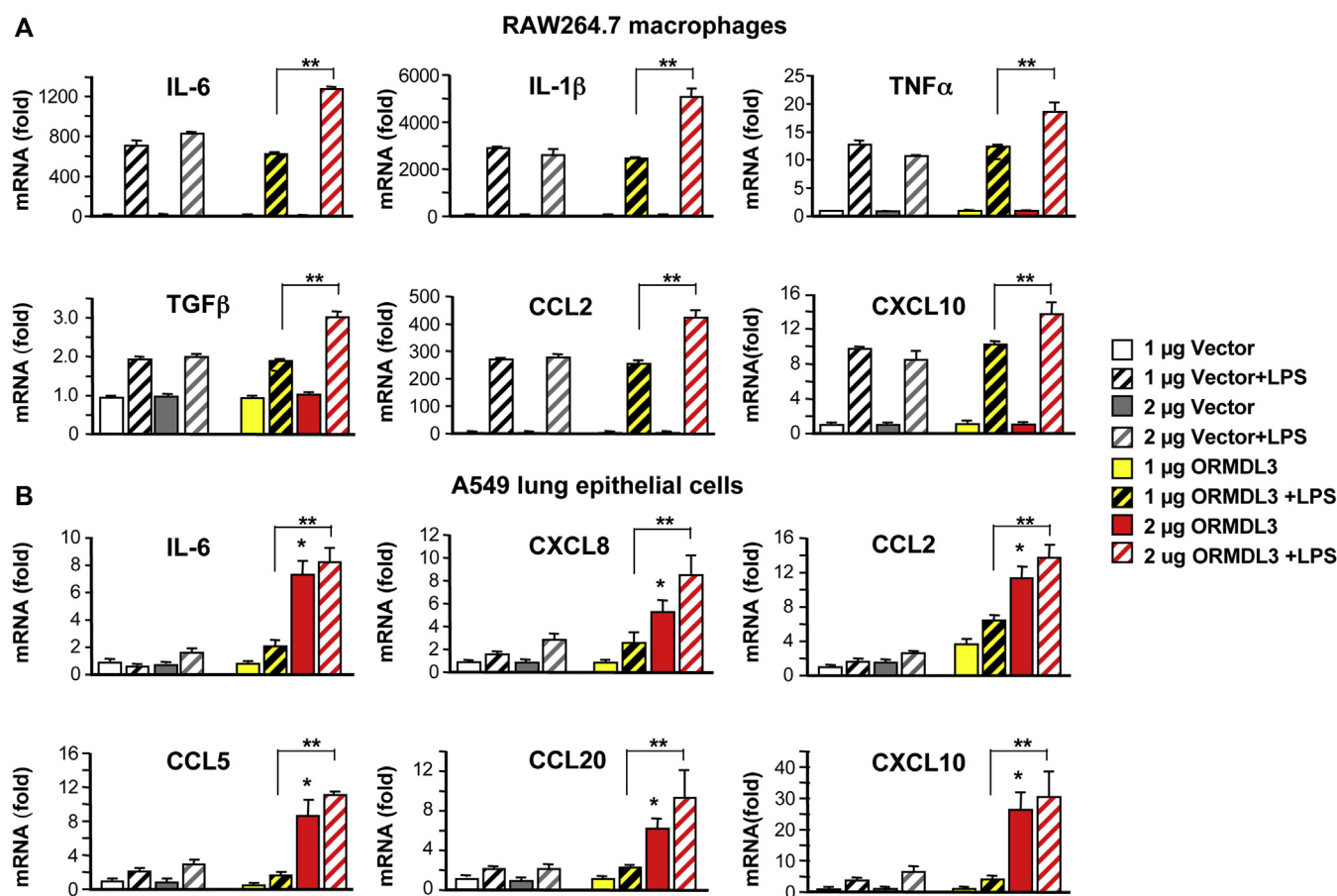


**FIG 4.** High overexpression of ORMDL3 enhances LPS-induced cytokine and chemokine expression. RAW264.7 (A) and A549 lung epithelial (B) cells transfected with vector or 1 or 2  $\mu$ g of ORMDL3 were treated with 10 ng/mL LPS for 4 hours (Fig 4, A) or 1 ng/mL LPS for 24 hours (Fig 4, B). Cytokine and chemokine expression was determined by using quantitative PCR and normalized to glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*). Experiments were performed in biological triplicates. Data are means  $\pm$  SDs and representative of 3 independent experiments. \* $P$  < .05 compared with vector and \*\* $P$  < .05 compared with 1  $\mu$ g of ORMDL3 plus LPS.

infiltration into the lung, inflammation, production of HDM-specific IgE, and mucus hypersecretion.<sup>33,34</sup> Similar to others, we first confirmed that ORMDL3 antibody binds to ORMDL3 using Western blot analysis of lysates from cells overexpressing ORMDL3 (Fig 1, B), which showed a 17-kDa band corresponding to the molecular weight of ORMDL3.<sup>23,24</sup> Because of the high degree of homology of greater than 80% between ORMDL proteins 1, 2, and 3, it is not possible to unequivocally demonstrate that this antibody does not react with ORMDL1 or ORMDL2. However, when expression of ORMDL1, ORMDL2, and ORMDL3 was downregulated by more than 80% in A549 cells, the 17-kDa band was markedly reduced only when ORMDL3 was downregulated (see Fig E3, A and B, in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). Immunohistochemical staining with this antibody was reduced only by siORMDL3 and not by siORMDL2 (see Fig E3, C). Furthermore, there was an increase in the 17-kDa band in RAW264.7 cells treated with LPS, which increased mRNA expression of ORMDL3<sup>23</sup> but not ORMDL1 or ORMDL2 mRNA expression (see Fig E3, D and E).<sup>37</sup>

Similar to previous studies of mice challenged with an extract of the mold *Alternaria alternata*,<sup>23,24</sup> HDM significantly

increased ORMDL3 expression in the lungs detected by means of Western blotting with anti-ORMDL3 antibody (Figs 5, A, and 6, A). Immunohistochemistry of lung sections of unchallenged mice revealed that ORMDL3 was expressed mainly in airway epithelial and endothelial cells (Fig 5, B). In the lungs of mice challenged with HDM, there was a marked increase in ORMDL3 expression not only in airway epithelial and endothelial cells but also in infiltrating immune cells (Fig 5, B). Concomitantly, we observed that HDM challenge increased ceramide levels in the lung, particularly the predominant species there (ie, very long-chain C24:1 and C24:0 ceramides) but not the long-chain ceramides (Figs 5, C, and 6, B). HDM also significantly increased most of the dihydroceramide species (Figs 5, D, and 6, C). In BALF the long-chain C16:0 and dihydro-C16:0 species are the predominant forms of ceramide, probably because of their greater aqueous solubility, and levels of both are also increased after HDM challenge (Fig 5, E and F). Intriguingly, C16:0 ceramide levels were also significantly increased in exhaled breath condensates from seriously ill asthmatic patients compared with those from healthy control subjects (see Fig E4 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)).

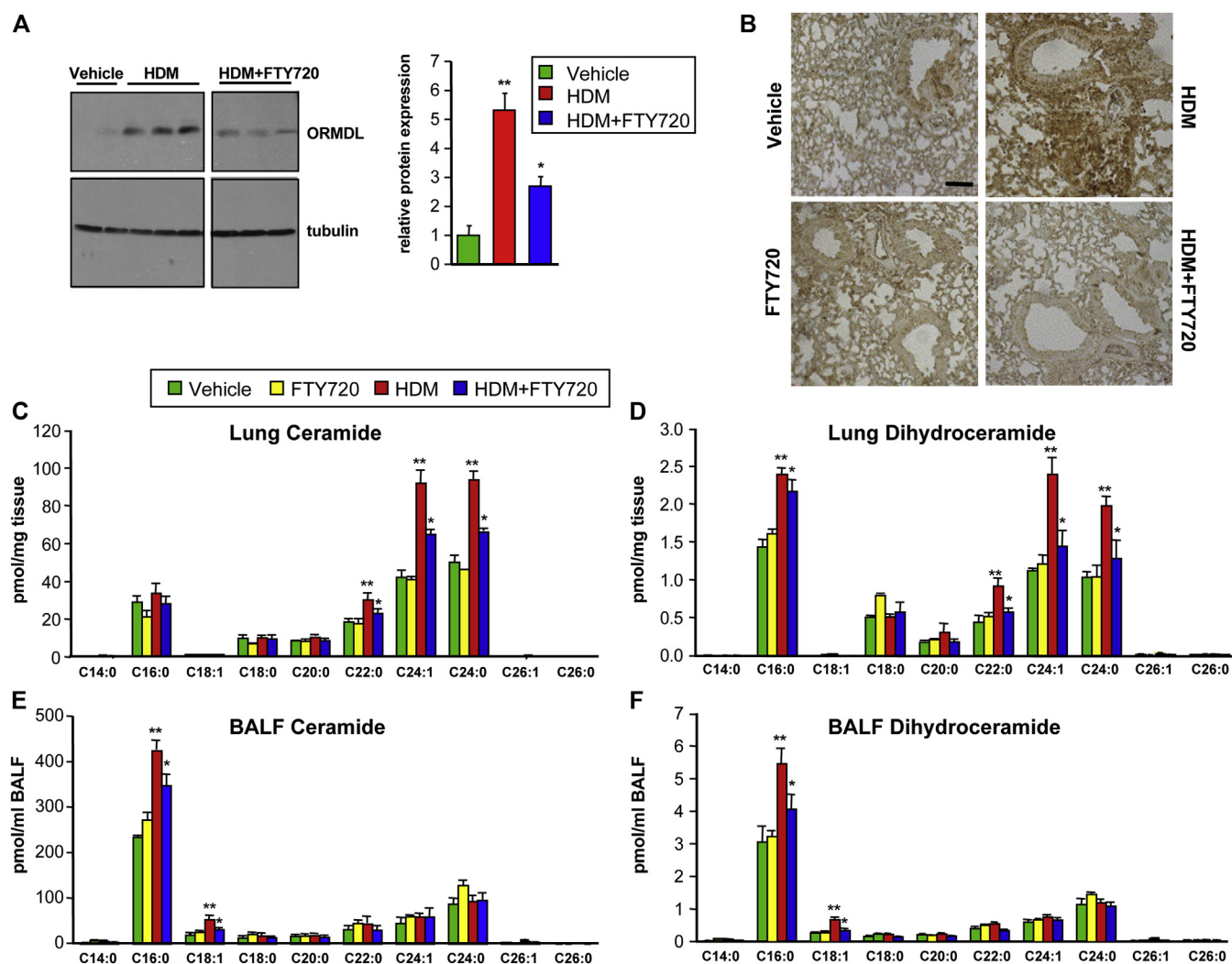


**FIG 5.** HDM increases and FTY720 administration attenuates ORMDL3 expression and increase of lung ceramide and dihydroceramide levels. Mice ( $n = 5$  per group) were challenged intranasally with saline or HDM extract and treated intranasally with vehicle (saline) or FTY720 (0.3 mg/kg) 30 minutes before challenge, and lungs were examined on day 15. **A**, Expression of ORMDL3 in the lungs was determined by means of Western blot analysis with anti-ORMDL3 antibody and quantified by means of densitometry. **B**, Lung sections were immunostained with anti-ORMDL3 antibody. Scale bar = 100  $\mu$ m. **C-F**, On day 15, lung (Fig 5, C and D) and BALF (Fig 5, E and F) ceramide (Fig 5, C and E) and dihydroceramide (Fig 5, D and F) species were analyzed by using LC-ESI-MS/MS. Data are mean  $\pm$  SEMs and representative of 2 independent experiments. \* $P < .05$  compared with HDM and \*\* $P < .05$  compared with vehicle.

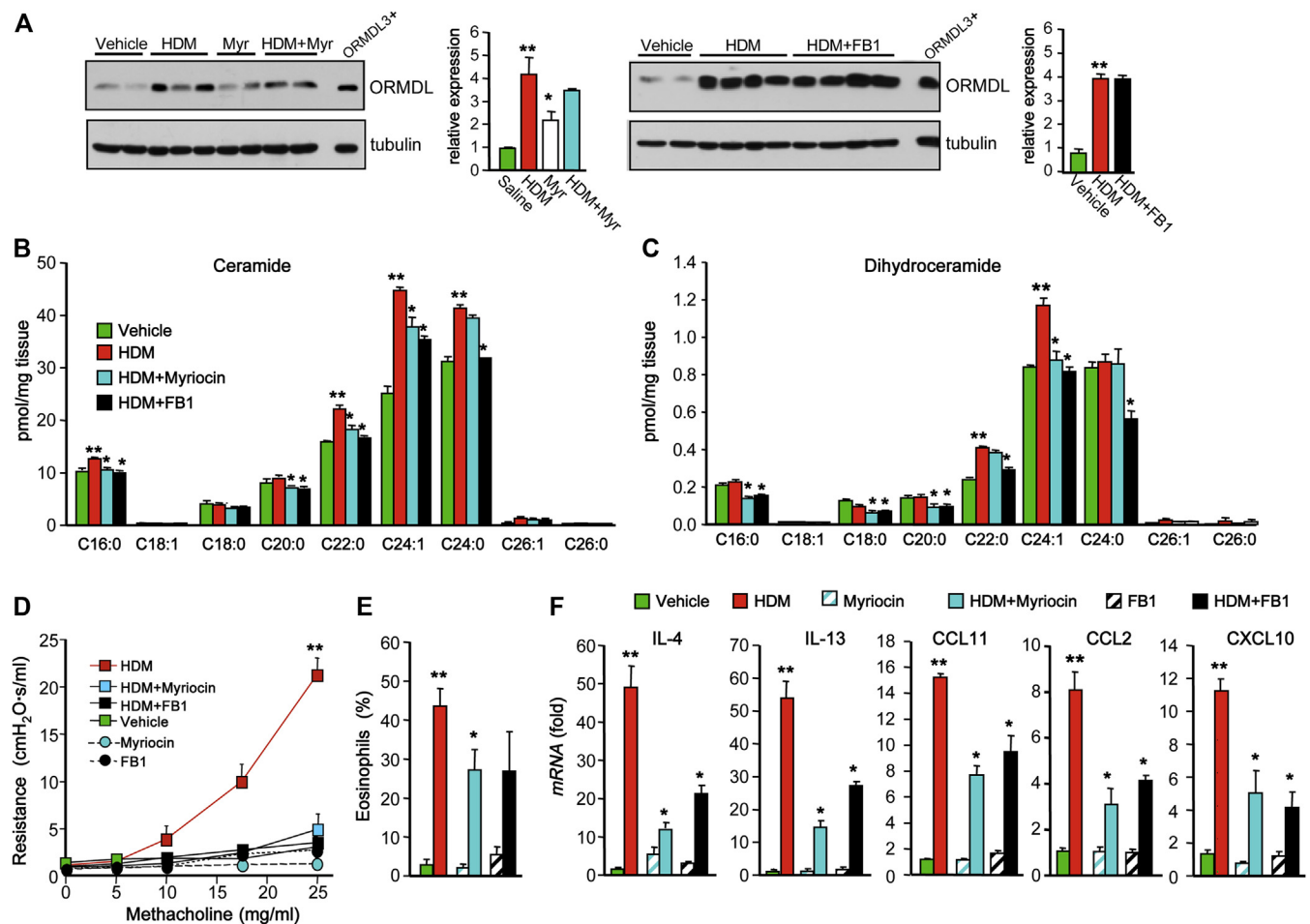
### Prevention of increased lung ceramide level improves respiratory abnormalities and suppresses airway inflammation

HDM-challenged mice were treated with the SPT inhibitor myriocin or the CerS inhibitor FB1 for the last 3 days of allergen challenge to examine the pathologic role of increased ceramide levels in patients with allergic asthma. Although myriocin or FB1 did not significantly affect HDM-induced ORMDL3 expression (Fig 6, A), they markedly suppressed HDM-induced lung ceramide and dihydroceramide level increases (Fig 6, B and C). Remarkably, treatment with myriocin and FB1 completely prevented airway hyperresponsiveness (AHR) to methacholine in HDM-challenged mice (Fig 6, D). These inhibitors also decreased HDM-induced accumulation of eosinophils in BALF (Fig 6, E) and decreased airway expression of the inflammatory cytokines IL-4 and IL-13

(Fig 6, F), which shape the local accumulation and activation of  $T_H2$  responses and have been implicated in the induction of AHR associated with allergic inflammation.<sup>36</sup> Moreover, the HDM-stimulated chemokines CCL11 (eotaxin), CCL2, and CXCL10 (Fig 6, F), which are involved in the recruitment, maturation, and activation of eosinophils and other leukocytes,<sup>34</sup> were also suppressed by myriocin and FB1. These results implicate ceramides in the development of allergic asthmatic response and airway inflammation.

### Administration of FTY720 attenuates upregulation of ORMDL3 and ceramide *in vivo* after HDM challenge

Given that previous studies suggest that ORMDL3 is an allergen- and  $T_H2$  cytokine-inducible gene,<sup>23,24</sup> we sought to



examine the effect of the immunosuppressive drug FTY720, a sphingosine analog that inhibits airway hyperreactivity and inflammation-induced airway remodeling.<sup>38</sup> Intranasal FTY720 drastically reduced HDM-induced ORMDL3 expression (Fig 5, A and B) and increases in ceramide and dihydroceramide levels in the lung (Fig 5, C and D) and BALF (Fig 5, E and F). Consistent with previous studies showing that ORMDL3 mRNA expression is induced in the lungs of mice challenged with IL-13,<sup>23</sup> in IL-13 knockout mice HDM no longer induced expression of ORMDL3 (Fig 7, A), supporting our conclusion that ORMDL3 protein expression is increased in the lungs of HDM-challenged mice. As expected, HDM-induced AHR (Fig 7, B), BALF eosinophil accumulation (Fig 7, C), and Muc5AC expression and mucus secretion (Fig 7, D) were also greatly reduced in IL-13 knockout mice. Importantly, BALF ceramide and dihydroceramide levels were also attenuated compared with those in wild-type littermates challenged with HDM (Fig 7, E and F).

### Intranasal FTY720 treatment mitigates AHR, allergic airway inflammation, and mucus hypersecretion in HDM-challenged mice

In line with previous observations,<sup>38-40</sup> intranasal FTY720 treatment significantly reduced AHR in HDM-challenged mice (Fig 8, A). Lung resistance, elastance, inverse of lung compliance that indicates the rigidity of the lung, and tissue damping were all significantly attenuated compared with those seen in actively sensitized mice (see Fig E5 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). FTY720 also decreased HDM-induced accumulation of eosinophils and neutrophils in BALF (Fig 8, B) and markedly decreased airway expression of the inflammatory cytokines IL-4, IL-13, and IL-33 (Fig 8, C). Likewise, the HDM-stimulated chemokines CCL2, CCL5, CCL11 (eotaxin), and CXCL10 (Fig 8, C) were also suppressed by inhalation of FTY720. Moreover, the increases in mucus production and goblet

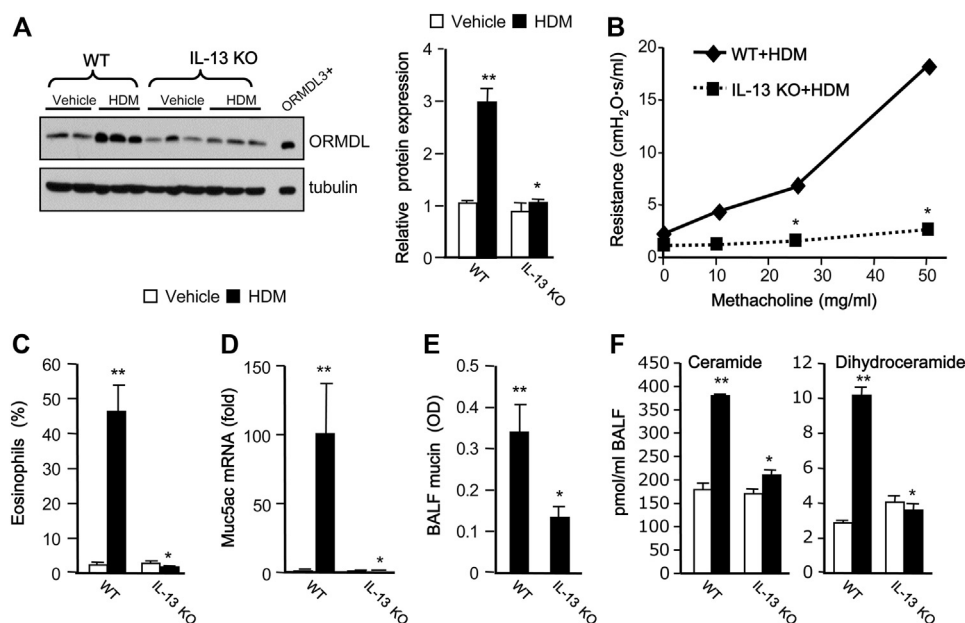


**FIG 7.** HDM-induced ORMDL3 expression, airway resistance, mucus production, and ceramide level increase are suppressed in IL-13 knockout (KO) mice. IL-13<sup>-/-</sup> mice and wild-type (WT) littermates (n = 5 per group) were challenged intranasally with saline or HDM extract, and lungs were examined on day 15. **A**, Expression of ORMDL3 in the lungs was determined by means of Western blot analysis with anti-ORMDL3 antibody and quantified by using densitometry. **B**, Lung resistance in response to inhaled methacholine was measured with the FlexiVent apparatus on day 15. **C**, BALF accumulation of eosinophils determined by using fluorescence-activated cell sorting. **D**, mRNA expression of Muc5AC determined by using quantitative PCR and normalized to glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) and mucin levels in BALF determined by means of ELISA. **E** and **F**, BALF ceramide (Fig 7, **E**) and dihydroceramide (Fig 7, **F**) levels were analyzed by using LC-ESI-MS/MS. Data are means  $\pm$  SEMs. \**P* < .05, IL-13 knockout compared with WT mice challenged with HDM; \*\**P* < .05, WT mice challenged with HDM compared with vehicle.

cell hyperplasia shown by Muc5AC expression (Fig 8, **C**), the BALF content of secreted mucin (Fig 8, **D**), and PAS staining (Fig 8, **E** and **F**) were all greatly reduced by FTY720. These results suggest that suppression of airway inflammation by FTY720 prevents aberrant upregulation of ORMDL3, restores physiologic levels of ceramide, and mitigates airway inflammation, AHR, and mucus production.

## DISCUSSION

Despite extensive efforts, GWASs have not identified the genetic origins of asthma. Remarkably, however, multiple studies have convincingly and repeatedly identified an association of the 17q21 locus and within it ORMDL3 with asthma in different ethnic populations.<sup>1-8</sup> Yet polymorphisms controlling ORMDL3 expression are not associated with atopy, suggesting that ORMDL3 affects asthma susceptibility independent of atopic or IgE-mediated responses.<sup>5,41,42</sup> Several potential mechanisms of action of ORMDL3 in asthma pathogenesis have been proposed, including (1) inhibition of the sarcoendoplasmic reticulum calcium pump and reduction of endoplasmic reticulum-mediated calcium release and activation of the unfolded protein response (UPR)<sup>43</sup>; (2) inhibition of store-operated calcium entry and lymphocyte activation<sup>44</sup>; (3) activation of the transcription factor ATF6 pathway of the UPR with subsequent upregulation of sarcoendoplasmic reticulum calcium pump 2b and enhanced expression of chemokines and metalloproteinases that contribute to the pathogenesis of asthma<sup>23</sup>; and (4) regulation of eosinophil

trafficking and IL-3–induced expression of CD48 and eosinophil degranulation.<sup>24</sup> However, none have been linked to its evolutionarily conserved role as an endogenous negative regulator of SPT activity and biosynthesis of ceramides that was described in yeast.<sup>9-11</sup> Consistent with this role, we have shown that decreasing ORMDL3 expression in mammalian cells increases ceramide levels and that, conversely, modest increases in ORMDL3 expression decrease ceramide levels by decreasing *de novo* synthesis. Importantly, however, high ORMDL3 levels had the opposite effect and significantly increased ceramide levels. Isotope labeling studies indicated that this was mainly due to increased salvage/recycling of sphingolipids to ceramide. There are several nonmutually exclusive possibilities for this increase in ceramide levels. Previous studies suggested that when *de novo* sphingolipid biosynthesis is inhibited, there is a compensatory increase in the recycling pathway in certain cell types to maintain sphingolipid homeostasis.<sup>45</sup> ORMDL3 overexpression activates the UPR,<sup>23,43</sup> and in yeast, as well as in mammalian cells, induction of the UPR enhances ceramide levels by means of CerS upregulation.<sup>46</sup> In yeast Orm1 forms a complex not only with SPT but also with the CerS homolog Lac1, suggesting an additional role for Orms in regulating ceramide synthesis.<sup>12</sup> Indeed, it was recently shown in yeast that Orm has 2 separate functions in sphingolipid metabolism: inhibition of SPT and activation of complex sphingolipid synthesis.<sup>14</sup>

In agreement with a recent report showing that ORMDL3 expression decreases SPT activity in mammalian cells,<sup>47</sup> we

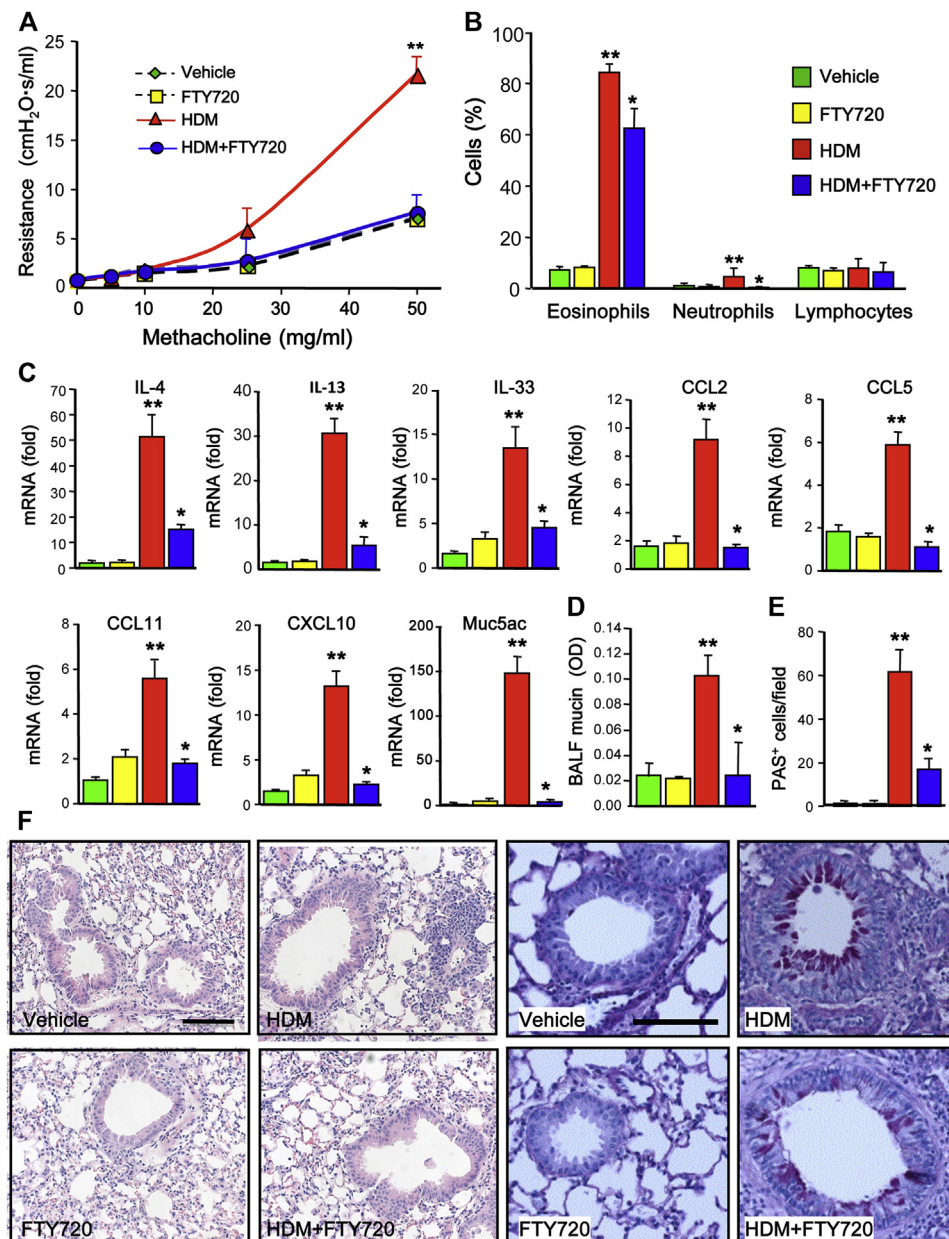


**FIG 8.** FTY720 attenuates HDM-induced airway hyperreactivity and allergic airway inflammation. Mice ( $n = 5$  per group) were challenged intranasally with saline or HDM extract and treated intranasally with vehicle (saline) or FTY720 (0.3 mg/kg) 30 minutes before challenge. **A**, Lung resistance in response to inhaled methacholine was measured with the FlexiVent apparatus on day 15. Note that the responses to methacholine in the unsensitized mice treated with vehicle or FTY720 were indistinguishable. **B**, Eosinophils, neutrophils, and lymphocytes in BALF. **C**, mRNA expression of cytokines and chemokines and Muc5AC in the lung was determined by using quantitative PCR and normalized to glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*). **D**, Mucin levels in BALF were determined by means of ELISA. **E** and **F**, Lung sections were stained with H&E or PAS. Scale bars = 100  $\mu$ m. Fig 8, E, Number of peribronchial, PAS-positive, mucus-producing epithelial cells. Data are means  $\pm$  SEMs and representative of 2 independent experiments. \* $P < .05$  compared with HDM and \*\* $P < .05$  compared with vehicle.

have found that changes in ORMDL3 expression regulate ceramide levels. In contrast, Kiefer et al<sup>31</sup> reported that downregulation or overexpression of ORMDL3 alone did not affect ceramide levels, but rather concurrent changes of all 3 ORMDLs were required. This discrepancy is probably due to the more sensitive mass spectrometric methodology that we used.

Increased ceramide levels have been associated with chronic lung diseases, including emphysema and chronic obstructive pulmonary disease.<sup>21</sup> Similarly, we found that HDM challenge increased ceramide and dihydroceramide levels in lung tissue and BALF, and like ovalbumin- and *Aternaria* species-induced asthma,<sup>23,24</sup> HDM increased ORMDL3 expression in the airways and in infiltrating immune cells. Hence under normal physiologic

conditions, moderate ORMDL3 expression acts as a brake on ceramide synthesis, whereas in pathologic conditions increased ORMDL3 levels lead to increased ceramide production with a concomitant increased inflammatory responses. Although overexpression of ORMDL3 in lung epithelial cells *in vitro* did not affect proinflammatory cytokines,<sup>25</sup> in contrast, we have confirmed and extended a previous report<sup>23</sup> demonstrating that increased ORMDL3 expression increases cytokine and chemokine levels. The biphasic paradigm proposed here might explain these conflicting reports<sup>23,25</sup> because we found that only high ORMDL3 expression, which increases ceramide levels, also enhances inflammatory responses. However, it is not clear whether primary human bronchial epithelial cells function similarly to the cell lines used in our study. Nevertheless, transgenic mice with human ORMDL3 highly expressed in the lung also have increased chemokine expression (CXCL10 and CXCL11), as well as expression of genes associated with airway remodeling (TGF- $\beta$ 1 and ADAM8) and spontaneous airway responsiveness characteristic of asthma.<sup>32</sup> Further studies are needed with ORMDL3 transgenic and knockout mice to investigate whether the ORMDL3-ceramide pathway is causative in patients with allergic asthma and to evaluate the contribution of sphingolipid-independent pathways.

Our data substantiate the link between ceramide level increases and acute asthmatic responses.<sup>19</sup> Increased formation of ceramides after HDM challenge promotes AHR and recruitment of eosinophils and enhances allergic inflammation, and inhibitors that block the ceramide level increase mitigate these responses. Moreover, we found that levels of the C16:0 ceramide are also significantly increased in exhaled breath condensates from asthmatic patients. Taken together, these data suggest that strategies aimed at reducing increased ceramide levels might be a promising therapeutic approach.

FTY720/fingolimod is an anti-inflammatory prodrug approved for the treatment of multiple sclerosis that sequesters lymphocytes into lymph nodes by modulating S1PR1 after phosphorylation to a S1P mimetic.<sup>48</sup> Although there are 2 recent case reports that prolonged systemic treatment of patients with multiple sclerosis worsened their asthma,<sup>49,50</sup> local administration of FTY720 abrogates allergen-induced asthma in animals without causing systemic lymphopenia.<sup>38,40</sup> In agreement with this, we found that intranasal administration of FTY720 to mice exposed to HDM significantly reduced ORMDL3 expression, ceramide level increase, AHR, airway inflammation, and mucus hypersecretion, substantiating the correlation between asthma severity, ORMDL3, and ceramide levels. The effect of FTY720 administration on ORMDL3 levels is likely indirect and due to decreased T<sub>H</sub>2 cytokines that regulate its expression,<sup>23,24</sup> which is consistent with the lack of increased ORMDL3 expression by HDM in IL-13 knockout mice. In addition, inhibition of protein phosphatase 2A<sup>51</sup> and cytosolic phospholipase A<sub>2</sub><sup>52</sup> by FTY720 might also contribute to its suppressive actions in experimental asthma. Our data show that AHR, inflammation, and asthma severity correlated with the extent of ORMDL3 expression and support the notion that local administration of FTY720 might be a new therapeutic weapon in the fight against asthma.

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## Key messages

- Overexpression of ORMDL3 in cells antithetically increases ceramide levels and enhances cytokine and chemokine production.
- During HDM-induced allergic asthma in mice, lung ceramide levels are increased, and their inhibition suppressed AHR and cytokine production.
- Local administration of FTY720 attenuates HDM-induced ORMDL3 expression and airway inflammation and hyper-reactivity and might be a useful therapeutic intervention for the control of allergic asthma.

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## METHODS

### Materials

ON TARGETplus SMARTpool small interfering RNA (siRNA) oligonucleotides for human ORMDL1 (catalog no. L-018403-01), ORMDL2 (catalog no. L-017035-02), and ORMDL3 (catalog no. L-017002-02) were from Dharmacon (Lafayette, Colo). Lipofectamine RNAiMAX, Lipofectamine and Plus reagent, Lipofectamine transfection reagents, and TRIzol were from Life Technologies (Grand Island, NY). All tissue culture cell lines (A549, NCI-H292, HEK293, and RAW264.7) were purchased from the American Type Culture Collection. Cell-culture media and supplies were from Life Technologies. FBS was from Atlanta Biologicals (Flowery Branch, Ga), HDM extract was from Greer Laboratories (Lenoir, NC; lot no. 228282), *Escherichia coli* LPS was from Sigma (catalog no. L4516), and FTY720 was from Cayman Chemicals (Ann Arbor, Mich).

### Downregulation and overexpression of ORMDL3

A549 and NCI-H292 cells were transfected with ON TARGETplus SMARTpool siRNA oligonucleotides (Dharmacon) for human ORMDL1, ORMDL2, and ORMDL3 or scrambled siRNA by using Lipofectamine RNAiMAX transfection reagent. For overexpression, A549 and RAW264.7 cells were transfected with ORMDL3 plasmid by using Lipofectamine or Lipofectamine LTX and Plus reagents, respectively.

### Quantitative real-time PCR

Total RNA was isolated with TRIzol and reverse transcribed with High Capacity cDNA Reverse Transcription Kit and premixed primer probe sets from Applied Biosystems (Foster City, Calif), and cDNA was amplified with the ABI 7900HT. Relative amounts of transcripts were normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) mRNA and compared between the different genes by using the  $\Delta\Delta$  cycle threshold method, as previously described.<sup>E1</sup>

### Western blot analysis

Lung tissue samples or cell pellets were homogenized in buffer containing 50 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1% NP-40, 2 mmol/L sodium orthovanadate, 4 mmol/L sodium pyrophosphate, 100 mmol/L NaF, and 1:500 protease inhibitor mixture (Sigma). Immunoblotting was carried out essentially as previously described.<sup>E2</sup> Equal amounts of protein were separated by using SDS-PAGE, transblotted to nitrocellulose, and incubated with primary antibodies against ORMDL3 (1:3000; Abgent, San Diego, Calif) and tubulin (1:8000; Cell Signaling, Danvers, Mass). Immunopositive bands were visualized by using enhanced chemiluminescence with secondary antibodies conjugated with horseradish peroxidase (goat anti-rabbit; 1:5000; Jackson ImmunoResearch, West Grove, Pa) and Super-Signal West Pico chemiluminescent substrate (Pierce, Rockford, Ill), as previously described.<sup>E1</sup> ODs of bands associated with proteins of interest were quantified with AlphaEaseFC software (Alpha Innotech, San Leandro, Calif) and normalized to the ODs of their respective tubulin bands.

### Measurement of airway reactivity

On day 15, mice were anesthetized by means of intraperitoneal administration of ketamine/xylazine and mechanically ventilated after tracheotomy. Measurements of baseline lung function were made with the FlexiVent apparatus (SCIREQ), as previously described.<sup>E3</sup> Mice were then exposed to aerosol challenges containing increasing doses of acetyl- $\beta$ -methylcholine

chloride (methacholine). Resistance, elastance, and tissue damping were measured with the FlexiVent software FlexiWare, version 7.0.

### BALF collection and analysis

BALF was collected by lavage of the lungs with 1 mL of PBS containing 1% BSA. Cells and supernatant were collected by means of centrifugation, and cells were resuspended in 100  $\mu$ L of PBS. Cytospin specimens were prepared and stained with Diff-Quik (Siemens Healthcare Diagnostics, Malvern, Pa), and proportions of different cell types were quantified by counting at least 200 cells per cytospin preparation.<sup>E3</sup> In some experiments cellular composition of BALF was also determined by using fluorescence-activated cell sorting.<sup>E4</sup>

### Lung histology

After lavage, lungs were inflated through the trachea, removed, and fixed in 10% neutral buffered formalin. The tissues were then embedded in paraffin, and 5- $\mu$ m sections were stained with H&E or PAS, as previously described.<sup>E3</sup> A Zeiss Axioimager A1 equipped with an AxioCam MRC camera was used to photograph the sections. For enumeration of mucus-producing airway epithelial cells, PAS-positive epithelial cells were counted in at least 10 bronchioles in each slide in a blinded manner.

### Immunohistochemistry

Expression of ORMDL3 in paraffin-embedded lung tissue sections was evaluated by using rabbit anti-ORMDL3 (0.5  $\mu$ g/mL), as previously described.<sup>E2,E5</sup> Rabbit IgG (R&D Systems, Minneapolis, Minn) was used as the negative control. Biotinylated secondary anti-rabbit antibody was added and incubated at room temperature for 20 minutes. After 5 minutes with streptavidin-horseradish peroxidase, sections were stained with 3,3'-diaminobenzidine substrate and counterstained with hematoxylin. Slides were examined with a Zeiss Axioimager A1 (Zeiss, Oberkochen, Germany) at  $\times 200$  magnification, and images were captured with an AxioCam MRC camera at  $1 \times 1$  binning.

### Mucin secretion

Mucin levels in BALF were measured by using an ELISA with a murine Muc5AC-specific antibody (Pierce).<sup>E3</sup>

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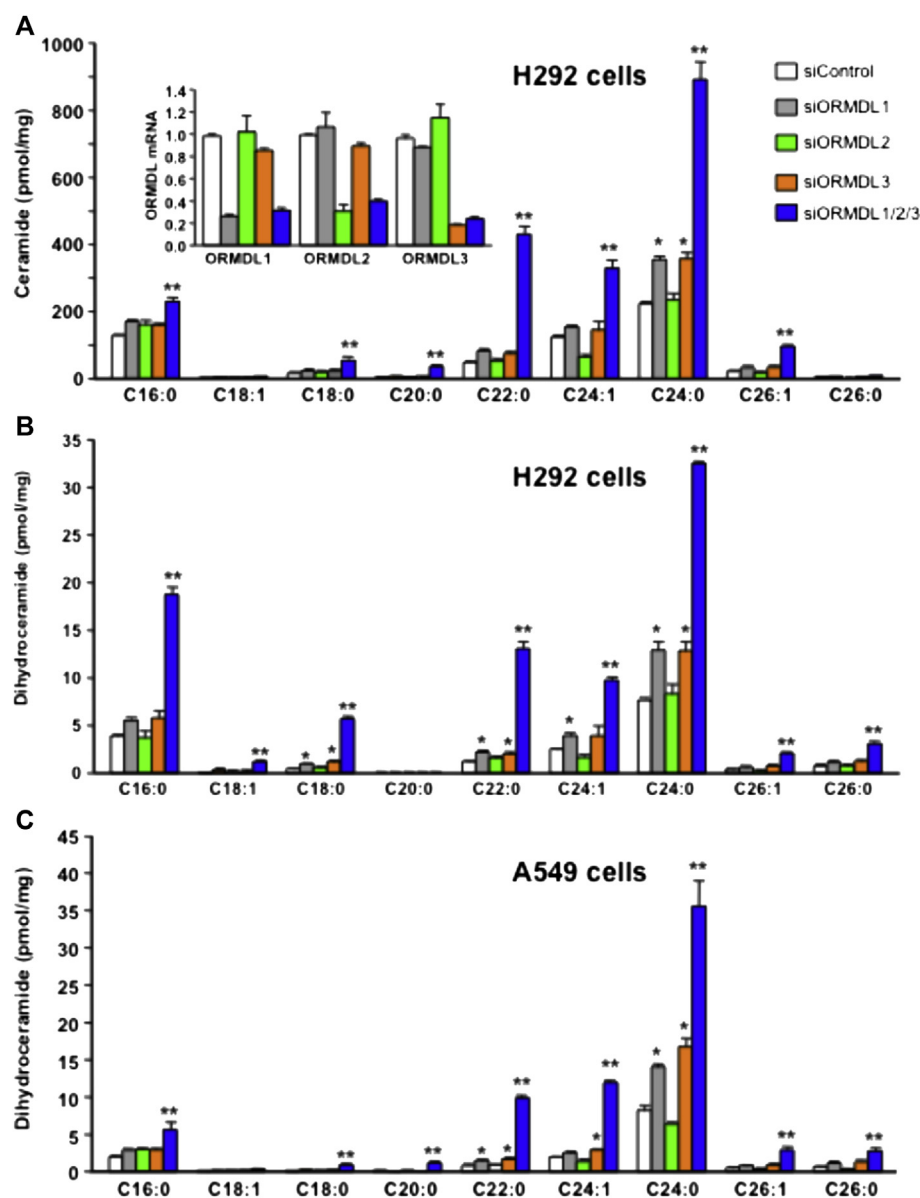


**FIG E1.** Downregulation of ORMDL1 and ORMDL3 increases ceramide and dihydroceramide levels in lung epithelial cells. H292 (**A** and **B**) and A549 (**C**) lung epithelial cells were transfected with scrambled siRNA (*si-Control*) or specific siRNAs for each ORMDL isoform, as indicated. Lipids were extracted, and ceramide and dihydroceramide species were analyzed by using LC-ESI-MS/MS. Experiments were performed in biological triplicates. Data are means  $\pm$  SDs and representative of 3 independent experiments. \* $P < .01$  compared with siControl and \*\* $P < .001$  compared with siORMDL3. Fig E1, *A*, inset, ORMDL1/2/3 mRNA levels were determined in duplicate cultures by using quantitative PCR and normalized to expression of glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*).

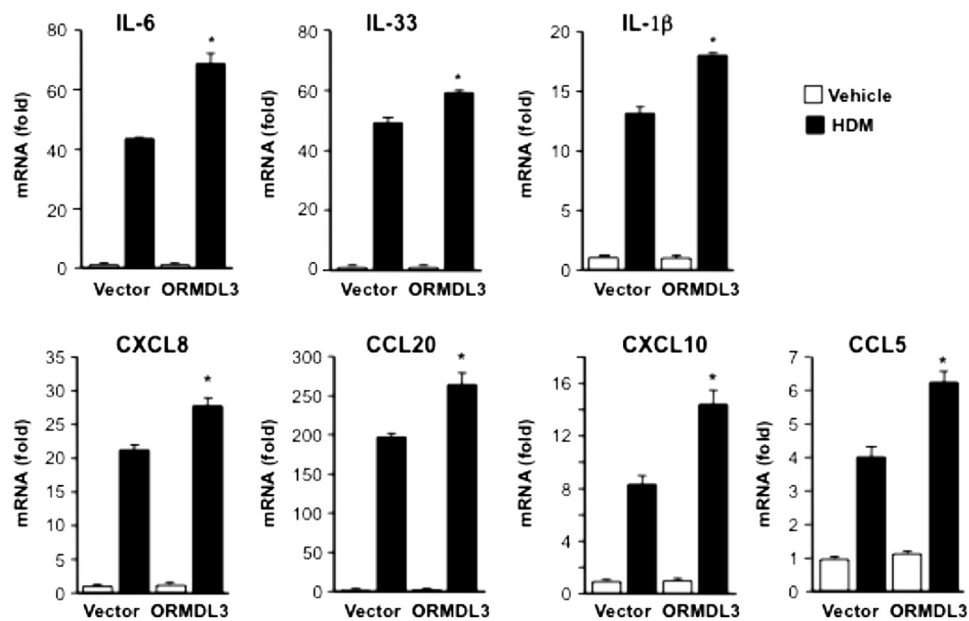


**FIG E2.** High expression of ORMDL3 enhances HDM-induced cytokine and chemokine expression in lung epithelial cells. NCI-H292 lung mucoepidermoid carcinoma cells transfected with vector or 2  $\mu$ g of ORMDL3 were treated with 50  $\mu$ g/mL HDM extract for 24 hours. Expression of the indicated cytokines and chemokines was determined by using quantitative PCR and normalized to glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*). Experiments were performed in biological triplicates. Data are means  $\pm$  SDs and representative of 3 independent experiments. \* $P < .05$  compared with vector treated with HDM.

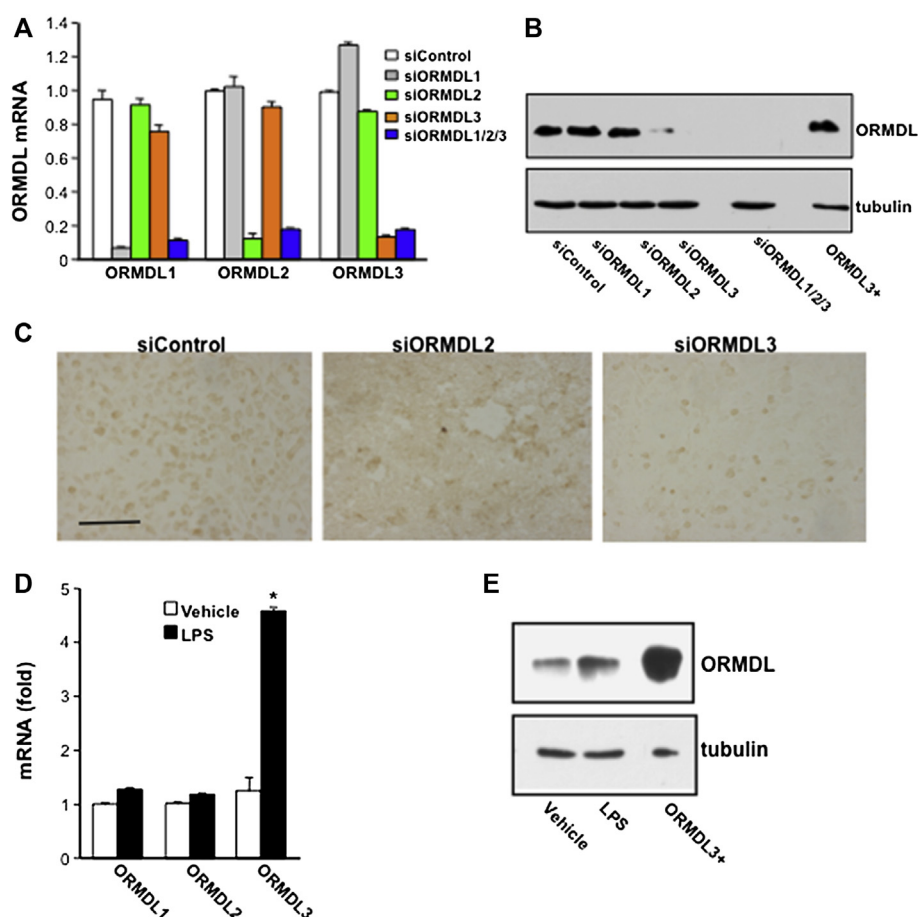


**FIG E3.** ORM DL3 antibody validation. **A-C**, A549 cells were transfected with siControl or specific siRNAs for each ORM DL isoform, as indicated. **D** and **E**, RAW264.7 cells were treated with 0.1  $\mu$ g of LPS for 24 hours. Fig E3, **A** and **D**, Expression of ORM DL isoforms, as determined by using quantitative PCR. Fig E3, **B** and **E**, Cell lysates were analyzed by means of immunoblotting with ORM DL3 antibody. Tubulin was used as a loading control. Fig E3, **C**, Immunohistochemical staining with anti-ORDL3 antibody. Size bar = 50  $\mu$ m. \* $P$  < .05 compared with vehicle.

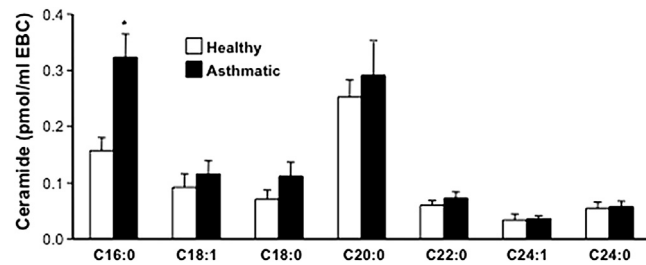


**FIG E4.** Ceramide is increased in exhaled breath condensates (EBC) from asthmatic patients. Exhaled breath condensate collection was approved by the Institutional Review Board of Virginia Commonwealth University. EBCs were collected from healthy control subjects, according to recommended procedures,<sup>E6</sup> by using an RTube and cooling sleeve (Respiratory Research, Austin, Tex) and from patients admitted to the intensive care unit with a history of asthma by using an RTube VENT and cooling sleeve connected to the endotracheal tube and expiratory limb of a mechanical ventilator. The RTube VENT was left inline for 10 minutes, which was sufficient to collect 1 to 2 mL of EBCs. EBCs were stored at  $-80^{\circ}\text{C}$  before analysis. Lipids were extracted from 1 mL of EBCs, and ceramide species were analyzed by using LC-ESI-MS/MS. Data are means  $\pm$  SEMs. \* $P < .01$  compared with controls.

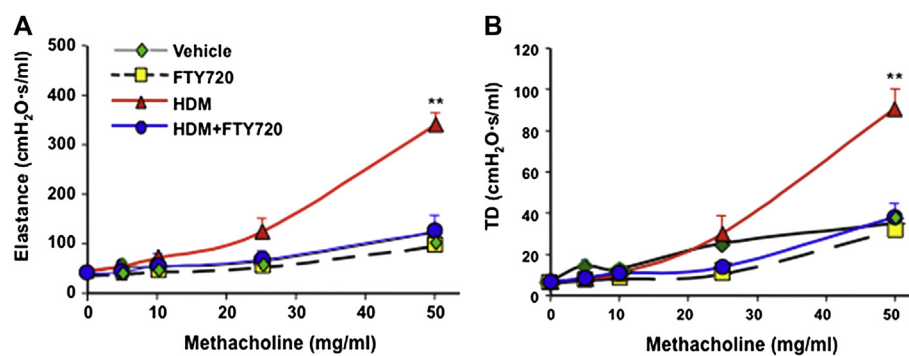


**FIG E5.** Effect of FTY720 on development of HDM-induced AHR. Mice ( $n = 5$  per group) were sensitized, challenged, and treated as described in Fig 3. Airway responses to methacholine were measured with the FlexiVent apparatus on day 15. **A**, Elastance. **B**, Tissue damping. Data are means  $\pm$  SEMs and representative of 2 independent experiments. \*\* $P < .05$  compared with vehicle.