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J Immunol 2017; 198:3017-3022; Prepublished online 8 March 2017;
doi: 10.4049/jimmunol.1601848
http://www.jimmunol.org/content/198/8/3017

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

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Cutting Edge: Targeting Epithelial ORMDL3 Increases, Rather than Reduces, Airway Responsiveness and Is Associated with Increased Sphingosine-1-Phosphate

Marina Miller,* Arvin B. Tam,† James L. Mueller,*,‡ Peter Rosenthal,* Andrew Beppu,* Ruth Gordillo,§ Matthew D. McGeough,*,‡ Christine Vuong,* Taylor A. Doherty,* Hal M. Hoffman,*,‡ Maho Niwa,† and David H. Broide*

In this study, we used cre-lox techniques to generate mice selectively deficient in ORMDL3 in airway epithelium (*Ormdl3*$^{\Delta 2\text{-}3/\Delta 2\text{-}3}$*/CC10*) to simulate an inhaled therapy that effectively inhibited ORMDL3 expression in the airway. In contrast to the anticipated reduction in airway hyperresponsiveness (AHR), OVA allergen–challenged *Ormdl3*$^{\Delta 2\text{-}3/\Delta 2\text{-}3}$*/CC10* mice had a significant increase in AHR compared with wild-type mice. Levels of airway inflammation, mucus, fibrosis, and airway smooth muscle were no different in *Ormdl3*$^{\Delta 2\text{-}3/\Delta 2\text{-}3}$*/CC10* and wild-type mice. However, levels of sphingosine-1-phosphate (S1P) were significantly increased in *Ormdl3*$^{\Delta 2\text{-}3/\Delta 2\text{-}3}$*/CC10* mice as well as in airway epithelial cells in which ORMDL3 was inhibited with small interfering RNA. Incubation of S1P with airway smooth muscle cells significantly increased contractility. Overall, *Ormdl3*$^{\Delta 2\text{-}3/\Delta 2\text{-}3}$*/CC10* mice exhibit increased allergen-induced AHR independent of inflammation and associated with increased S1P generation. These studies raise concerns for inhaled therapies that selectively and effectively inhibit ORMDL3 in airway epithelium in asthma. *The Journal of Immunology*, 2017, 198: 3017–3022.

A gene, ORMDL3 (orosomucoid like 3), located on chromosome 17q21 (1), has been strongly linked to asthma in genome-wide association studies as well as in candidate gene association studies. ORMDL3 is a member of the ORDML gene family (ORMDL1, 2, and 3), which encodes transmembrane proteins located at the endoplasmic reticulum (ER) (1). ORMDL1 (chromosome 20) (1) and ORMDL2 (chromosome 12) (1) are on different chromosomes from ORMDL3 (chromosome 17q21) (1) and have not been linked to asthma. Both humans and mice express the same three ORMDL family members, with ORMDL3 exhibiting 96% identity between these two species (1). ORMDL3 is a 153-aa ER-localized protein with two predicted transmembrane domains (1). ORMDL3 regulates a number of pathways of potential importance to the pathogenesis of asthma, including ATF6α, sphingolipids, remodeling genes, and chemokines (2–4). We have previously demonstrated that in wild-type (WT) mice inhalation allergen challenge (OVA or *Alternaria*) induces a significant 127-fold increase in ORMDL3 mRNA in bronchial epithelium in vivo (2), suggesting that ORMDL3 in airway epithelium may be a novel therapeutic target in asthma. Additionally, as the single nucleotide polymorphism (SNP) linking chromosome 17q21 to asthma is associated with increased levels of ORMDL3 expression, we generated mice that express increased levels of human (h)ORMDL3 in all cells (termed hORMDL3$^{zp3\text{-}Cre}$) (3) and demonstrated that these mice spontaneously develop increased airway hyperresponsiveness (AHR) characteristic of asthma in the absence of airway inflammation (3).

Identifying pathways that can be targeted to reduce AHR, a cardinal feature of asthma, is a desirable therapeutic goal. Thus, the demonstration that increased ORMDL3 expression in the airway is associated with increased AHR raises the possibility of developing inhalation therapies inhibiting ORMDL3 expression in airway epithelium which could result in reduced AHR. To test this hypothesis we used cre-lox techniques to generate mice selectively deficient in ORMDL3 in airway epithelium (*Ormdl3*$^{\Delta 2\text{-}3/\Delta 2\text{-}3}$*/CC10*) (simulating an effective inhalation therapy that prevented ORMDL3 expression in the airway epithelium) to determine whether this would reduce AHR.

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Received for publication November 10, 2016. Accepted for publication February 13, 2017.

This work was supported by National Institutes of Health Grants AI 107779, AI 38425, AI 70535, AI 72115, AI 242236 (to D.H.B.), AI 70535, AI 114585 (to T.A.D.), and GM087415 (to M.N.). A.B.T. is supported by National Institutes of Health Grant T32 AI 07469.

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The online version of this article contains supplemental material.

Abbreviations used in this article: AHR, airway hyperresponsiveness; BAL, bronchoalveolar lavage; ER, endoplasmic reticulum; h, human; MCS, multiple cloning site; PAS, periodic acid–Schiff; qRT-PCR, quantitative RT-PCR; siRNA, small interfering RNA; SNP, single nucleotide polymorphism; S1P, sphingosine-1-phosphate; SPT, serine palmitoyltransferase; WT, wild-type.

Materials and Methods

Generation of conditional ORMDL3-deficient floxed mouse

Genomic DNA from C57BL/6 mice corresponding to the ORMDL3 genomic locus was PCR amplified with PfuTurbo (Agilent Technologies). PCR products were ligated into the pflox-FRT-Neo vector containing a 5′ multiple cloning site (MCS), a neomycin resistance cassette flanked by FRT sites, another MCS flanked by loxP sequences, and a 3′ MCS. The 5′ homology arm (4511 bp) was ligated into the 5′ MCS AscI and FseI restriction sites. A genomic DNA fragment including exons 2–3 (1205 bp) was ligated into BamHI and BglII sites flanked by the loxP sequences. The 3′ homology arm was ligated into the 3′ MCS BamHI restriction site. A diphtheria toxin A negative selection cassette was cloned into the NotI restriction site 5′ to the 5′ homology arm. The finished clone pflox-FRT-Neo_ORMDL3 was sequenced to confirm correct sequence and orientation of inserts. Linearized pflox-FRT-Neo_ORMDL3 was electroporated into C57BL/6 embroyic stem cells and embryonic stem cell clones were expanded for screening by the University of California San Diego Transgenic Mouse Core. Embryonic stem cell clones positive for the mutant allele were microinjected into C57BL/6 albino blastocysts followed by implantation into pseudopregnant mice by the University of California San Diego Transgenic Core. *Ormdl3*$^{\text{NeoR-loxP}}$ mice were bred to FLPe recombinase transgenic mice (The Jackson Laboratory) to remove the neomycin cassette in all cells. To delete the *Ormdl3* allele in airway epithelial cells, *Ormdl3*$^{\text{loxP}}$ mice were crossed with transgenic *CC10-Cre*^{fg} mice (background strain C57/BL; provided by Jeff Whitsett, University of Cincinnati, Cincinnati, OH), which express two transgenes, one an activator that expresses the reverse tetracycline-responsive transactivator (rtTA) in a club cell–specific manner (*CC10-rtTA*), and the second under control of the tet-operator (*tetO*), which controls expression of Cre (*tetO-Cre*), as previously described in this laboratory (5). Club cells are distal airway epithelial cells (nonciliated, secretory) that represent ≈70% of the adult mouse airway epithelium (5). All experimental mouse protocols were approved by the University of California San Diego Institutional Animal Care and Use Committee.

Acute OVA allergen challenge mouse model of asthma

Ormdl3$^{\Delta 2\text{-}3/\Delta 2\text{-}3}$*/CC10* mice and their respective littermate control mice (hereafter referred to as WT mice) (n = 8 mice per group) aged ∼12 wk were sensitized and challenged intranasally with OVA (Worthington, Lakewood, NJ) as previously described (3). Twenty-four hours after the last challenge AHR was measured, mice were sacrificed, and lungs were collected to quantitate levels of airway inflammation and airway remodeling as described (2, 3). AHR to methacholine was assessed in intubated and ventilated mice aged 12 wk (n = 8 mice per group) (flexiVent ventilator; Scireq) using Scireq software 24 h after the last OVA challenge as previously described (3). Lungs were processed for protein and RNA extraction, as well as for immunohistology (paraffin-embedded lung sections) as previously described in this laboratory (3). Numbers of lung eosinophils, CD4$^+$ lymphocytes, and F4/80$^+$ macrophages were quantitated in the peribronchial space in lung sections as previously described (3). To quantitate the level of mucus expression in the airway, the number of periodic acid–Schiff (PAS)$^+$ and PAS$^-$ epithelial cells in individual bronchioles was counted as previously described (3). The area of peribronchial trichrome staining in paraffin-embedded lungs was outlined and quantified under a light microscope (Leica DMLS; Leica Microsystems) attached to an image analysis system (Image-Pro Plus; Media Cybernetics) as previously described (3). The thickness of the airway smooth muscle layer was measured by α-smooth muscle actin immunohistochemistry as previously described (3).

ORMDL3 and sphingosine-1-phosphate

As ORMDL3 inhibits the enzyme serine palmitoyltransferase (SPT), the first and rate-limiting step in the synthesis of sphingolipids, including sphingosine-1-phosphate (S1P) (4), we investigated whether levels of S1P were different in OVA-challenged *Ormdl3*$^{\Delta 2\text{-}3/\Delta 2\text{-}3}$*/CC10* mice compared with WT mice, or in mouse airway epithelial cells in which ORMDL3 was knocked down with small interfering RNA (siRNA) knocked down, and whether S1P influenced mouse lung smooth muscle contraction.

Quantitation of S1P in OVA-challenged Ormdl3$^{\Delta 2\text{-}3/\Delta 2\text{-}3}$/CC10 *mice compared with WT mice.* Levels of S1P level were quantitated in serum by S1P ELISA (MyBioSource).

Quantitation of S1P in airway epithelial cells knocked down with ORMDL3 siRNA. Mouse tracheal epithelial cells were obtained by dissection and culture from C57BL/6 mice as previously described (5). Tracheal epithelial cells from cultures in which ORMDL3 was knocked down with siRNA or scrambled siRNA were plated in 24-well plates in complete epithelial media (Cell Biologics). The cells were stimulated with 200 nM thapsigargin (Sigma-Aldrich), a known inducer of S1P, for 24 h. The supernatants were collected and levels of S1P were quantitated by ELISA (MyBioSource).

Quantitation of S1P-induced smooth muscle contraction. Mouse tracheal smooth muscle cells were obtained by dissection and culture from C57BL/6 mice as previously described (5). These smooth muscle cells were used in an in vitro smooth muscle gel contraction assay, previously described (5).

Quantitative RT-PCR

Quantitative RT-PCR (qRT-PCR) was performed as previously described (3) with TaqMan PCR master mix and ORMDL1, ORMDL2, ORMDL3, SPTLC1, SPTLC2, and SPTLC3 primers (all from Life Technologies).

ORMDL3 and activation of ATF6α

Tracheal airway epithelial cells were grown on six-well plates containing poly-L-lysine–treated cover slips to a confluency of 50% and transfected with siRNA against ORMDL3 (OriGene) using siTran (OriGene), according to the manufacturer's instructions. Immunofluorescence to ATF6α was performed as described previously (2). To further test ATF6 activation in cells knocked down for ORMDL3, we used an ATF6-GFP reporter with GFP attached to the cytosolic side of ATF6 (revised from Ref. 6), which allows movement of ATF6 from the ER to the Golgi and the nucleus to be quantitated in epithelial cells cotransfected cells with ATF6-GFP and either no siRNA, ORMDL3 (triple) siRNA, or scrambled siRNA as a negative control and treated for up to 8 h with thapsigargin. For each cell, the location of ATF6 was determined to be either in the ER, in the Golgi (when it colocalized with Golgi marker GM-130), or in the nucleus (when it colocalized with DAPI). Mouse tracheal epithelial cells were also obtained by dissection and culture from WT and *Ormdl3*$^{\Delta 2\text{-}3/\ \Delta 2\text{-}3}$*/CC10* mice as described (5) and grown on six-well plates containing poly-L-lysine–treated cover slips. Nuclear ATF6 was determined by measuring the fluorescence (in arbitrary units) of ATF6 (red channel) only in the area of the nucleus.

ATF6α and sphingolipid measurements

Levels of S1P were quantitated in serum of ATF6α-deficient mice provided by Dr. K. Mori (Kyoto University, Kyoto, Japan) (7) or from littermate control mice (n = 4 per group) using liquid chromatography–tandem mass spectrometry methodology at the University of Texas Southwestern Metabolic Phenotyping Core (8).

Statistical analysis

All results are presented as mean ± SEM. A statistical software package (GraphPad Prism; GraphPad Software, San Diego, CA) was used for the two-tailed unpaired statistical analysis. A p value <0.05 was considered statistically significant.

Results

Ormdl3$^{\Delta 2\text{-}3/\Delta 2\text{-}3}$/CC10 *mice have increased AHR*

To simulate an inhalation therapy that effectively inhibited expression of ORMDL3 in airway epithelium, we generated mice containing the floxed Ormdl3 exon 2 and 3 region (*Ormdl3*$^{\text{loxP}}$) as described in *Materials and Methods* and as depicted in Fig. 1A. These *Ormdl3*$^{\text{loxP}}$ mice displayed no apparent phenotypic abnormalities and their lungs were normal at birth. To study mice deficient in airway epithelial expression of ORMDL3, we crossed *Ormdl3*$^{\text{loxP}}$ mice with *CC10-Cre*^{fg} mice (5) and demonstrated by genotyping (Fig. 1B) and by quantitative PCR (Fig. 1C) that the progeny *Ormdl3*$^{\Delta 2\text{-}3/\Delta 2\text{-}3}$*/CC10* mice did not express ORMDL3 mRNA in airway epithelium, but they did express ORMDL1 and ORMDL2 mRNA in airway epithelium. The selective deletion of ORMDL3 in airway epithelium in *Ormdl3*$^{\Delta 2\text{-}3/\Delta 2\text{-}3}$*/CC10* mice was evident from the demonstration that ORMDL3 was not deleted in macrophages (bronchoalveolar lavage [BAL] or bone marrow) (Fig. 1D), a cell type we have previously shown to highly express ORMDL3 (2). These *Ormdl3*$^{\Delta 2\text{-}3/\Delta 2\text{-}3}$*/CC10* mice also displayed no phenotypic abnormalities and their lungs were normal at birth. *Ormdl3*$^{\Delta 2\text{-}3/\Delta 2\text{-}3}$*/CC10* mice and WT mice had similar baseline levels of AHR to methacholine (Fig. 1E). However, OVA allergen–challenged *Ormdl3*$^{\Delta 2\text{-}3/\Delta 2\text{-}3}$*/CC10* mice had a significantly greater increase in AHR as compared with OVA-challenged WT mice ($p < 0.05$) (Fig. 1E).

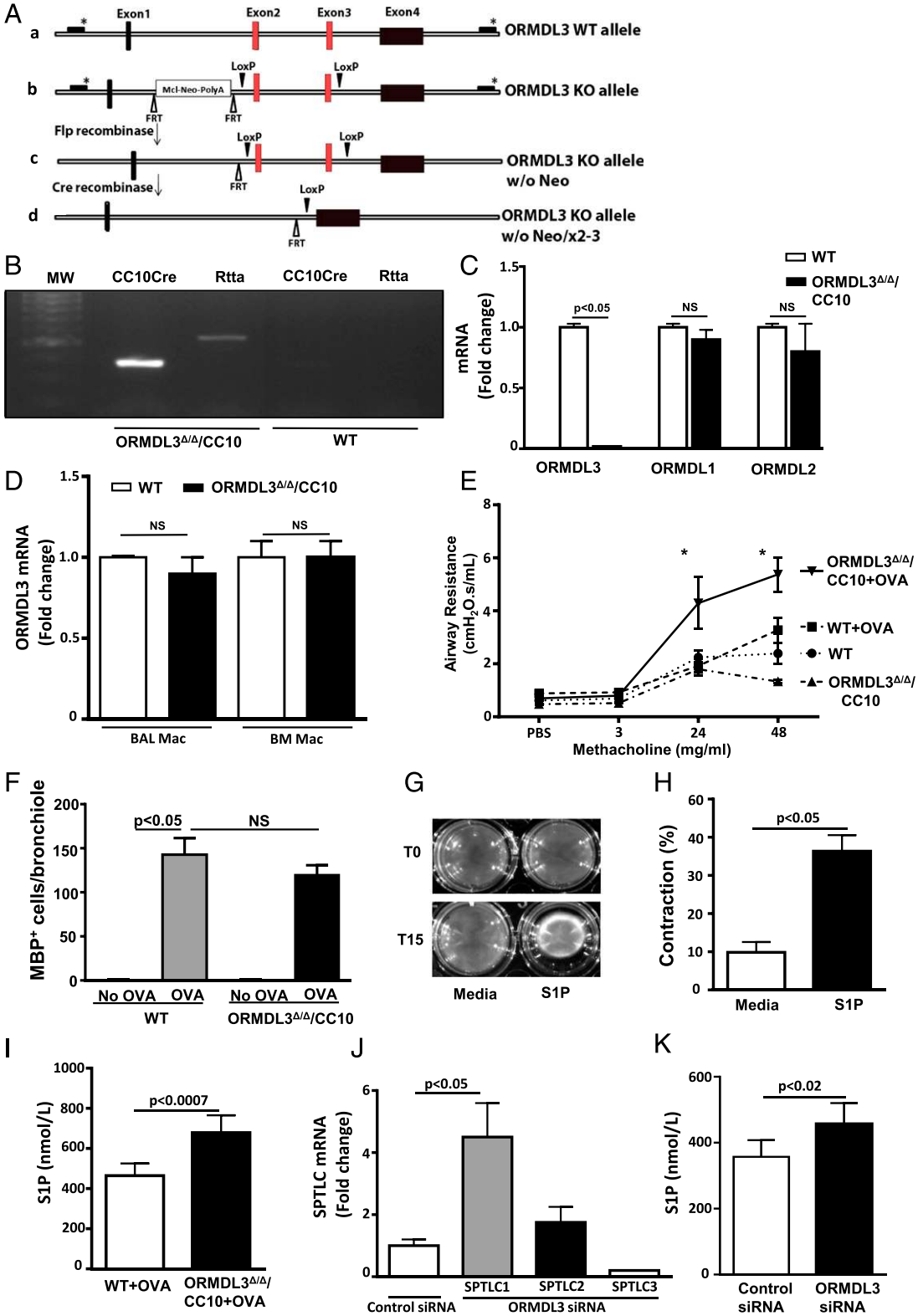

FIGURE 1. *Ormdl3*$^{\Delta 2\text{-}3/\Delta 2\text{-}3}$*/CC10* mice have increased AHR. (**A**) *Ormdl3*$^{\Delta 2\text{-}3/\Delta 2\text{-}3}$*/CC10* mice were generated by crossing ORMDL3 floxed mice with CC10 cre mice to delete ORMDL3 exons 2 and 3. The asterisks mark the 5′ and 3′ probes (represented as black rectangles) used for Southern blot analysis of embryonic stem cell clones. (**B**) Genotyping of *Ormdl3*$^{\Delta 2\text{-}3/\Delta 2\text{-}3}$*/CC10* mice demonstrated the presence of CC10 Cre and reverse tetracycline-controlled transactivator (Rtta). (**C**) Airway epithelial cells from *Ormdl3*$^{\Delta 2\text{-}3/\Delta 2\text{-}3}$*/CC10* mice expressed ORMDL1 and ORMDL2 but not ORMDL3 mRNA as assessed by qRT-PCR. (**D**) Macrophages (BAL or bone marrow derived) from *Ormdl3*$^{\Delta 2\text{-}3/\Delta 2\text{-}3}$*/CC10* and WT mice expressed similar levels of ORMDL3 mRNA as assessed by qRT-PCR. (**E**) *Ormdl3*$^{\Delta 2\text{-}3/\Delta 2\text{-}3}$*/CC10* mice challenged with OVA had significantly increased AHR to methacholine compared with WT mice challenged with OVA as assessed in intubated mice using Scireq software. $^{*}p < 0.05$. (**F**) Levels of major basic protein (MBP)$^{+}$ eosinophils were quantitated in the peribronchial space by immunohistochemistry in *Ormdl3*$^{\Delta 2\text{-}3/\Delta 2\text{-}3}$*/CC10* mice and control WT mice before and after OVA challenge. (**G** and **H**) Incubation of lung smooth muscle cells with S1P for 15 min (T15) induced significant contraction compared with media. (**I**) Levels of S1P (ELISA) were increased in serum from *Ormdl3*$^{\Delta 2\text{-}3/\Delta 2\text{-}3}$*/CC10* mice. (**J**) Levels of SPT isoforms SPTCL1, SPTCL2, and SPTCL3 mRNA were quantitated by quantitative PCR in epithelial cells transfected with ORMDL3 or control siRNA. (**K**) Levels of S1P (ELISA) were quantitated in epithelial cells transfected with ORMDL3 or control siRNA.

Ormdl3$^{\Delta 2\text{-}3/\Delta 2\text{-}3}$/CC10 *mice have no increased airway inflammation to explain increased AHR*

There was no difference in either baseline or acute OVA allergen–induced levels of peribronchal major basic protein$^+$ eosinophils (Fig. 1F, Supplemental Fig. 1F), peribronchial CD4$^+$ lymphocytes (Supplemental Fig. 1A, 1G), or peribronchial F4/80$^+$ macrophages (Supplemental Fig. 1B, 1H). Similarly, there was no difference in PAS$^+$ epithelial mucus cells (Supplemental Fig. 1C, 1I), the area of peribronchial fibrosis assessed in trichrome-stained lung sections (Supplemental Fig. 1D, 1J). The acute OVA challenge protocol does not induce increased airway smooth muscle hypertrophy (Supplemental Fig. 1E, 1K), and thus changes in levels of airway smooth muscle cannot explain the increased AHR in *Ormdl3$^{\Delta 2\text{-}3/\Delta 2\text{-}3}$/CC10* mice.

ORMDL3, S1P, and AHR

ORMDL3 inhibits the enzyme SPT (4), the first and rate-limiting step in the synthesis of sphingolipids (including S1P, which has been implicated in asthma) (9–12). We therefore investigated whether S1P could contribute to the increased AHR we observed in these mice by performing an in vitro airway smooth muscle contraction assay (5) in which S1P was incubated with airway smooth muscle, and smooth muscle contraction was assessed. These studies demonstrated that S1P could significantly increase smooth muscle contraction within 15 min of exposure of smooth muscle to S1P ($p < 0.05$) (Fig. 1G, 1H). As S1P could directly induce smooth muscle contraction independent of inflammation, we examined whether levels of S1P were different in OVA allergen–challenged *Ormdl3$^{\Delta 2\text{-}3/\Delta 2\text{-}3}$/CC10* mice, compared with WT mice. These studies demonstrated that levels of S1P were increased in OVA allergen–challenged *Ormdl3$^{\Delta 2\text{-}3/\Delta 2\text{-}3}$/CC10* mice compared with WT mice ($p < 0.0007$) (Fig. 1I), suggesting a sphingolipid pathway through which inactivation of ORMDL3 selectively in airway epithelium could increase AHR by direct effects on airway smooth muscle. In addition to demonstrating that in vivo a deficiency of ORMDL3 in epithelial cells resulted in increased levels of S1P, we also quantitated in vitro levels of the three subunits (SPTLC1, SPTLC2, SPTLC3) of the enzyme SPT (regulated by ORMDL3), as well as S1P levels, in WT bronchial epithelial cells in which ORMDL3 was knocked down with siRNA. These studies demonstrated that levels of the SPT enzyme (in particular SPTCL1) ($p < 0.05$) (Fig. 1J) and levels of S1P ($p < 0.02$) (Fig. 1K) were significantly increased in purified populations of WT mouse primary tracheal epithelial cells in which ORMDL3 was knocked down with siRNA as compared with control siRNA. Thus, taken together, our studies demonstrate that OVA allergen–challenged *Ormdl3$^{\Delta 2\text{-}3/\Delta 2\text{-}3}$/CC10* mice have increased S1P due to removal of the inhibitory effect of ORMDL3 on SPT, the enzyme mediating the first step in S1P synthesis. The local increase in S1P in airway epithelium in close proximity to airway smooth muscle can directly induce increased smooth muscle contractility (Fig. 1G, 1H), and thus potentially contribute to increased AHR detected in acute OVA allergen–challenged *Ormdl3$^{\Delta 2\text{-}3/\Delta 2\text{-}3}$/CC10* mice.

A deficiency in epithelial ORMDL3 delays ATF6α and BiP activation, but does not regulate S1P

We have previously demonstrated that increased expression of ORMDL3 (an ER-localized protein) in vitro in airway epithelium transfected with ORMDL3 (2), and in vivo in the lung of ORMDL3 transgenic mice (3), upregulates the ATF6α branch of the unfolded protein response. Primary airway epithelial cells derived from *Ormdl3$^{\Delta 2\text{-}3/\Delta 2\text{-}3}$/CC10* mice activated with thapsigargin, an unfolded protein response–inducing agent, had a significantly reduced ability to activate ATF6α at 2 h as assessed by immunofluorescence detection of nuclear ATF6α (Supplemental Fig. 2A). However, the reduction of ATF6α activation was diminished at a later time point (8 h) (Supplemental Fig. 2A). Activation of ATF6α is not linked to S1P, as levels of S1P are similar in WT mice and ATF6α-deficient mice (Supplemental Fig. 2B). Similarly, in primary airway epithelial cells in which siRNA was used to knock down ORMDL3, there was significant inhibition of thapsigargin-induced ATF6α activation for up to 4 h (Supplemental Fig. 2C) as assessed by immunofluorescence detection of nuclear ATF6α. However, late activation of ATF6α (8 h) was not defective (Supplemental Fig. 2C). siRNA to ORMDL3 significantly inhibited expression of ORMDL3 mRNA (Supplemental Fig. 2D) in airway epithelial cells as compared with scrambled siRNA. Additionally, using an ATF6-GFP reporter (Supplemental Fig. 2E–N) there is a significant delay in the movement of ATF6 from the ER to the Golgi in epithelial cells with ORMDL3 knocked down (Supplemental Fig. 2E–N). Additionally, the ORMDL3-induced delay in movement of ATF6 from the ER to the nucleus has functional consequences, as it reduces levels of activation of downstream ATF6α target genes such as BiP even at 8 h (Supplemental Fig. 2H–M). However, S1P levels are not regulated by ATF6α (Supplemental Fig. 2B).

Discussion

These studies of *Ormdl3$^{\Delta 2\text{-}3/\Delta 2\text{-}3}$/CC10* mice demonstrate that selectively inhibiting ORMDL3 in airway epithelial cells plays a significant role in induction of allergen-induced increases in AHR in vivo through a pathway independent of inflammation and associated with a pathway of increased S1P generation, which can induce increased smooth muscle contraction. These results further suggest that utilizing an inhalation therapeutic approach in asthma targeting a selective significant reduction in ORMDL3 expression in airway epithelium may paradoxically result in increased AHR as a consequence of increased epithelial-derived S1P increasing airway smooth muscle contractility. Whether an inhalation therapy targeting a less than complete inhibition of ORMDL3 expression by airway epithelium would also result in increased AHR is at present unknown. However, a therapy inducing only partial inhibition of ORMDL3 expression in the airway may also not be an effective therapy in asthma.

In this study, we demonstrated that inhibition of ORMDL3 in primary lung epithelial cells using either cre-lox strategies or siRNA knockdown results in increased levels of S1P as well as increased levels of SPTCL1, a subunit of the SPT enzyme that regulates the first step of the sphingolipid pathway, which results in the synthesis of S1P (4). There are several studies of sphingolipids that suggest that increased levels of S1P are likely to contribute to increased AHR in asthma. For example, administration of S1P to WT mice significantly increases AHR (9), whereas inhalation of an Sphk1 inhibitor inhibits asthma outcomes in mouse models (10). The importance of S1P to asthma is also suggested from studies demonstrating increased BAL levels of S1P in human allergic asthmatics following endobronchial allergen challenge (11), as well as genetic linkage studies showing an association between functional SNPs in the S1P receptor 1 and asthma (12). In contrast to these studies demonstrating that increased S1P plays a role in increasing AHR (9, 10), Sptlc2$^{+/-}$ mice, which have a heterozygous deficiency in SPT, the enzyme regulating the first step of sphingolipid synthesis, have reduced synthesis of sphinganine and ceramide (but not reduced S1P), and they have increased AHR in the absence of inflammation (13). Heterozygous Sptlc2$^{+/-}$ mice have ~60% decreased hepatic SPT activity (13). Although ORMDL3 inhibits SPT (4), studies of mice deficient in ORMDL3 selectively in airway epithelium, which have increased expression of SPT and increased S1P, are not directly comparable to studies of mice with a heterozygous deficiency of SPT in all cell types in which levels of S1P are not altered (13). Additionally,

hORMDL3$^{zp3-Cre}$ mice, which universally express increased levels of ORMDL3, have reduced levels of S1P (8), suggesting that pathways other than S1P may be contributing to AHR in these mice. As S1P can induce increased smooth muscle contractility, further studies are needed to determine which ORMDL3-regulated pathway (e.g., inhibition of sphingolipid synthesis, inhibition of ATF6α and SERCA2b, inhibition of remodeling genes, or other as yet unidentified pathways) could contribute to increased AHR observed in hORMDL3$^{zp3\text{-}Cre}$ mice (3).

As ORMDL3 not only regulates S1P, we also examined whether inactivation of ORMDL3 in airway epithelial cells also influenced another downstream pathway of ORMDL3, namely ATF6α (2, 3). ORMDL3 and ATF6α are both localized to the ER (2, 3). We demonstrated that reductions in ORMDL3 in primary epithelial cells delayed transport of ATF6α from ER to Golgi to nucleus and delayed ATF6α activation, which has prolonged functional consequences, as it reduces levels of activation of downstream ATF6α target genes such as BiP even at 8 h. However, studies of ATF6α-deficient mice demonstrated that ATF6α did not regulate levels of S1P. Thus, ORMDL3 regulates several downstream pathways, including sphingolipids and ATF6α, which are separate pathways with separate downstream effects on cellular function likely dependent on the cell type in which ORMDL3 is expressed. Additionally, the studies of ATF6α activation in epithelial cells deficient in ORMDL3 have the predicted outcome, and they underscore that not all outcomes such as increased AHR are unanticipated when ORMDL3 is inhibited in epithelium.

We have previously demonstrated that hORMDL3$^{zp3\text{-}Cre}$ mice expressing increased levels of hORMDL3 universally in all cell types have increased airway responsiveness in the absence of associated airway inflammation (3). As the SNP linking chromosome 17q21 is associated with increased levels of expression of ORMDL3, these studies of hORMDL3$^{zp3\text{-}Cre}$ mice provide insight into how ORMDL3 may contribute to the pathogenesis of asthma in those asthmatics having the SNP associated with increased ORMDL3 expression. As the studies of ORMDL3 transgenic mice were performed in mice expressing increased ORMDL3 in all cell types, we do not yet know which cell type expressing increased levels of ORMDL3 contributes to increased AHR.

We had anticipated based on the studies demonstrating increased AHR in hORMDL3$^{zp3\text{-}Cre}$ mice (3) that studies in *Ormdl3*$^{\Delta 2\text{-}3/\Delta 2\text{-}3}$/*CC10* mice would demonstrate the reverse phenotype (i.e., reduced AHR). There are several potential reasons why this was not the case, including 1) differences in hORMDL3$^{zp3\text{-}Cre}$ mice compared with *Ormdl3*$^{\Delta 2\text{-}3/\Delta 2\text{-}3}$/*CC10* mice in cell types expressing or not expressing ORMDL3 (increased expression in all cells versus selectively deficient in airway epithelium), 2) AHR differences detected with no allergen challenge (hORMDL3$^{zp3\text{-}Cre}$ mice versus WT mice) versus AHR differences detected with an allergen challenge (OVA plus *Ormdl3*$^{\Delta 2\text{-}3/\Delta 2\text{-}3}$/*CC10* versus OVA plus WT mice), and 3) potential different ORMDL3 downstream pathways mediating increased AHR (S1P derived from airway epithelium in *Ormdl3*$^{\Delta 2\text{-}3/\Delta 2\text{-}3}$/*CC10* mice versus SERCA2b in airway smooth muscle in hORMDL3$^{zp3\text{-}Cre}$ mice). Studies have also demonstrated that universal ORMDL3-deficient mice have reduced AHR when challenged with *Alternaria*, but they have not examined whether selective epithelial inactivation of ORMDL3, as in this study, influences AHR (14). Thus, further studies are needed to determine which cell types expressing ORMDL3 contribute to increased AHR and through which of several downstream pathways regulated by ORMDL3 (ATF6α, SERCA2b, S1P, chemokines, remodeling genes, or other pathways) (2, 3) this may be mediated. Although ORMDL3 regulates several downstream pathways (2, 3), it is likely that in different cell types some downstream pathways may be more important than in others. For example, SERCA2b is downstream of ORMDL3, and in smooth muscle SERCA2b induces contraction (15). The ability of ORMDL3 to induce SERCA2b in airway epithelium may not be as important as induction of SERCA2b in smooth muscle, as contraction is a less important function of airway epithelium as opposed to airway smooth muscle. At present, it is not known which upregulated and/or downregulated ORMDL3 pathway (including the generation of S1P) in epithelium in *Ormdl3*$^{\Delta 2\text{-}3/\Delta 2\text{-}3}$/*CC10* mice results in increased AHR. Further studies in which the generation of S1P was inhibited in epithelium in *Ormdl3*$^{\Delta 2\text{-}3/\Delta 2\text{-}3}$/*CC10* mice would help to determine whether S1P, or an alternative ORMDL3-regulated pathway, contributed to increased AHR in these mice.

In summary, in this study we used cre-lox techniques to generate mice selectively deficient in ORMDL3 in airway epithelium (*Ormdl3*$^{\Delta 2\text{-}3/\Delta 2\text{-}3}$/*CC10*) to simulate an inhaled therapy that effectively inhibited ORMDL3 expression in the airway. In contrast to the anticipated reduction in AHR, OVA allergen–challenged *Ormdl3*$^{\Delta 2\text{-}3/\Delta 2\text{-}3}$/*CC10* mice had a significant increase in AHR compared with WT mice, which would raise concerns about targeting epithelial ORMDL3 in asthma. Levels of S1P were significantly increased in *Ormdl3*$^{\Delta 2\text{-}3/\Delta 2\text{-}3}$/*CC10* mice as well as in airway epithelial cells in which ORMDL3 was inhibited with siRNA, whereas incubation of S1P with airway smooth muscle cells significantly increased contractility. Overall, these studies demonstrate that *Ormdl3*$^{\Delta 2\text{-}3/\Delta 2\text{-}3}$/*CC10* mice exhibit increased allergen-induced airway responsiveness independent of inflammation and associated with increased S1P generation, which induces airway smooth muscle contraction. These studies raise concerns for inhalation therapies that selectively and effectively inhibit ORMDL3 in airway epithelium in asthma.

Acknowledgments

ATF6α-deficient mice were provided by Dr. K. Mori (Kyoto University), and *CC10-Cre*^{tg} mice were provided by Dr. Jeff Whitsett (University of Cincinnati).

Disclosures

The authors have no financial conflicts of interest.

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