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Targeting epithelial ORMDL3 increases, rather than reduces, airway responsiveness and is associated with increased sphingosine-1-phosphate

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Abstract

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 responsiveness (AHR), OVA allergen challenged *Ormdl3*$^{\Delta 2\text{-}3/\Delta 2\text{-}3}$*CC10* mice had a significant increase in AHR compared to wild type (WT) mice. Levels of airway inflammation, mucus, fibrosis, and airway smooth muscle (ASM) were no different in *Ormdl3*$^{\Delta 2\text{-}3/\Delta 2\text{-}3}$*CC10* and WT 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 siRNA. Incubation of S1P with ASM 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.

INTRODUCTION

ORMDL3 (orosomucoid like 3), a gene 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 (ORMDL-1,-2,-3) which

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encode transmembrane proteins located at the endoplasmic reticulum (ER)(1). ORMDL-1 (chromosome 20)(1), and ORMDL-2 (chromosome 12)(1) are on different chromosomes from ORMDL-3 (chromosome 17q21)(1) and have not been linked to asthma. Both humans and mice express the same three ORMDL family members with ORMDL-3 exhibiting 96% identity between these two species (1). ORMDL-3 is a 153 amino acid 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, 3, 4). We have previously demonstrated that in 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. In addition, as the SNP linking chromosome 17q21 to asthma is associated with increased levels of ORMDL3 expression, we generated mice that express increased levels of human ORMDL3 in all cells (termed hORMDL3$^{zp3\text{-}Cre}$)(3), and demonstrated that these mice spontaneously develop increased airway responsiveness (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 inhaled 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 inhaled therapy that prevented ORMDL3 expression in the airway epithelium) to determine whether this would reduce AHR.

MATERIALS AND METHODS

Generation of conditional ORMDL3 deficient floxed mouse (*Ormdl3*^{loxP})

Genomic DNA from C57BL/6 mice corresponding to the ORMDL3 genomic locus was PCR amplified with PFU Turbo (Agilent Technologies). PCR products were ligated into the pflox-FRT-Neo vector containing a 5′ MCS (multiple cloning site), a neomycin resistance cassette flanked by FRT sites, another MCS flanked by loxP sequences, and a 3′ MCS. The 5′ homology arm (4511bp) was ligated into the 5′ MCS AscI and FseI restriction sites. A genomic DNA fragment including exons 2–3 (1205bp) 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 DTA (diphtheria toxin) 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 ES cells and ES cell clones were expanded for screening by the UCSD Transgenic Mouse Core. ES cell clones positive for the mutant allele were microinjected into C57BL/6 albino blastocysts followed by implantation into pseudo-pregnant mice by the UCSD Transgenic Core. *Ormdl3*$^{NeoR\text{-}loxP}$ mice were bred to FLPe recombinase transgenic mice (Jackson Labs) to remove the Neomycin cassette in all cells. To delete the *Ormdl3* allele in airway epithelial cells,

Ormdl3^{loxP} mice were crossed with transgenic *CC10-Cre*^{tg} mice (background strain C57/BL; kindly provided by Jeff Whitsett MD, University of Cincinnati, Cincinnati) 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 (non-ciliated, secretory) which represent ≈70% of the adult mouse airway epithelium (5). All experimental mouse protocols were approved by the UCSD 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 wild type or WT mice)(n= 8 mice/group) aged approximately 12 weeks 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 sacrificed and lungs 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/group) (flexiVent ventilator; Scireq) using Scireq software twenty-four hours 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 positive 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)-positive and PAS-negative 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 (S1P)

As ORMDL3 inhibits the enzyme serine palmitoyl transferase the first and rate limiting step in the synthesis of sphingolipids including 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 to WT mice, or in mouse airway epithelial cells in which ORMDL3 was siRNA knocked down, and whether SIP influenced mouse lung smooth muscle contraction.

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

b) 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 200nM thapsigargin (Tg) (Sigma) a known inducer of S1P for 24 h. The supernatants were collected and levels of S1P were quantitated by ELISA (MyBioSource).

c) 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 (SM) cells were used in an *in vitro* SM gel contraction assay, previously described (5).

qRT-PCR

Quantitative RT-PCR (qRT-PCR) was performed as previously described (3) with TaqMan PCR Master Mix and ORMDL1, ORMDL2, ORMDL3, SPTCL1, SPTCL2, and SPTCL3 primers (all from Life Technology).

ORMDL3 and activation of ATF6α

Tracheal airway epithelial cells were grown on 6 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 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 (6)) which allows movement of ATF6 from the ER to the Golgi and the nucleus to be quantitated in epithelial cells co-transfected cells with ATF6-GFP and either no siRNA, ORMDL3 (triple) siRNA, or Scrambled siRNA as a negative control and treated for up to 8 hrs with Tg. For each cell, the location of ATF6 was determined to be either in the ER, in the Golgi (if it co-localized with Golgi marker GM-130), or in the nucleus (if it co-localized 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 6 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 (kindly provided by Dr. Moro (Kyoto University, Japan) (7), or from littermate control mice (n=4/group) using liquid chromatography-tandem mass spectrometry (LC-MS/MS) methodology at the University of Texas South Western Metabolic Phenotyping Core (8).

Statistical Analysis

All results are presented as mean ± SEM. A statistical software package (Graph Pad Prism, San Diego, CA) was used for the two tailed unpaired statistical analysis. P values of < 0.05 were considered statistically significant.

RESULTS

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

To simulate an inhaled therapy which effectively inhibited expression of ORMDL3 in airway epithelium, we generated mice containing the floxed Ormdl3 exon 2 and 3 region (*Ormdl3*^{loxP}) as described in the Methods and as depicted in Figure 1A. These *Ormdl3*^{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*^{loxP} mice with *CC10-Cre*^{tg} mice (5) and demonstrated by genotyping (Figure 1B), and by qPCR (Figure 1C) that the progeny *Ormdl3* $^{\Delta 2\text{-}3/\Delta 2\text{-}3}$*/CC10* mice did not express ORMDL3 mRNA in airway epithelium, but 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 (BAL or bone marrow)(Figure 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 (Mch) (Figure 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 to OVA challenged WT mice ($p<0.05$) (Figure 1E).

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 MBP+ eosinophils (Fig 1F, Supplement Fig 1F), peribronchial CD4+ lymphocytes (Supplement Fig 1A, 1G), or peribronchial F4/80+ macrophages (Supplement Fig 1B, 1H). Similarly there was no difference in PAS+ epithelial mucus cells (Supplement Fig 1C, 1I), the area of peribronchial fibrosis assessed in trichrome stained lung sections (Supplement Fig 1D, 1J). The acute OVA challenge protocol does not induce increased airway smooth muscle hypertrophy (Supplement 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, sphingosine-1-phosphate (S1P), and AHR

ORMDL3 inhibits the enzyme serine palmitoyl transferase (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 assessed. These studies demonstrated that S1P could significantly increase smooth muscle contraction within 15 minutes of exposure of smooth muscle to S1P ($p<0.05$) (Figure 1G–H). 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 to 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 to WT mice ($p<0.0007$)(Figure 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 (SPTCL1, SPTCL2, SPTCL3) 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 the SPTCL1 subunit)($p<0.05$) (Figure 1J), and levels of S1P ($p<0.02$)(Figure 1K) were significantly increased in purified populations of WT mouse primary tracheal epithelial cells in which ORMDL3 was knocked down with siRNA as compared to 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 (Figure 1G–H), 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 endoplasmic reticulum 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 (UPR). Primary airway epithelial cells derived from *Ormdl3* $^{\Delta 2\text{-}3/\Delta 2\text{-}3}$*/CC10* mice activated with Thapsigargin, a UPR inducing agent, had a significantly reduced ability to activate ATF6α at 2 hours as assessed by immunofluorescence detection of nuclear ATF6α (Supplement Figure 2A). However, the reduction of ATF6α activation was diminished at a later time-point (8 hours)(Supplement Figure 2A). Activation of ATF6α is not linked to S1P as levels of S1P are similar in WT mice and ATF6α deficient mice (Supplement Figure 2B). Similarly, in primary airway epithelial cells in which siRNA was used to knockdown ORMDL3, there was significant inhibition of Thapsigargin induced ATF6α activation for up to 4 hrs (Supplement Figure 2C) as assessed by immunofluorescence detection of nuclear ATF6α. However, late activation of ATF6α (8 hours) was not defective (Supplement Figure 2C). siRNA to ORMDL3 significantly inhibited expression of ORMDL3 mRNA (Supplement Figure 2D) in airway epithelial cells as compared to scrambled siRNA. In addition, using an ATF6-GFP reporter (Supplement 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 (Supplement Figure 2E–N). In addition, 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 8hrs (Supplement Figure 2H–M). However, S1P levels are not regulated by ATF6α (Supplement Figure 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 inhaled 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 inhaled 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 which regulates the first step of the sphingolipid pathway which results in the synthesis of S1P (4). There are several studies of sphingolipids which 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), while inhalation of a 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), and 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 have increased in AHR in the absence of inflammation (13). Heterozygous Sptlc2$^{+/-}$ mice have approximately 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). In addition, 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$^{zp3–Cre}$ 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 endoplasmic reticulum (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 hours. 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 upon the cell type in which ORMDL3 is expressed. In addition, the studies of ATF6α activation in epithelial cells deficient in ORMDL3 have the predicted outcome, and 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 human ORMDL3 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 a) differences in hORMDL3$^{zp3\text{-}Cre}$ mice compared to *Ormdl3*$^{\Delta 2\text{-}3/\Delta 2\text{-}3}$CC10 mice in cell types expressing or not expressing ORMDL3 (increased expression in all cells vs selectively deficient in airway epithelium), b) AHR differences detected with no allergen challenge (hORMDL3$^{zp3\text{-}Cre}$mice vs WT mice) vs AHR differences detected with an allergen challenge (OVA + *Ormdl3*$^{\Delta 2\text{-}3/\Delta 2\text{-}3}$CC10 vs OVA + WT mice), and c) 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 vs 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 have not examined whether selective epithelial inactivation of ORMDL3 as in this study effects 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 to 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, while 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 inhaled therapies that selectively and effectively inhibit ORMDL3 in airway epithelium in asthma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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

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References

1. Hjelmqvist L, Tuson M, Marfany G, Herrero E, Balcells S, Gonzàlez-Duarte R. ORMDL proteins are a conserved new family of endoplasmic reticulum membrane proteins. Genome Biol. 2002; 3:RESEARCH0027. [PubMed: 12093374]
2. Miller M, Tam AB, Cho JY, Doherty TA, Pham A, Khorram N, Rosenthal P, Mueller JL, Hoffman HM, Suzukawa M, Niwa M, Broide DH. ORMDL3 is an inducible lung epithelial gene regulating metalloproteases, chemokines, OAS, and ATF6. Proc Natl Acad Sci USA. 2012; 109:16648–16653. [PubMed: 23011799]
3. Miller M, Rosenthal P, Beppu A, Mueller JL, Hoffman HM, Tam AB, Doherty TA, McGeough MD, Pena CA, Suzukawa M, Niwa M, Broide DH. ORMDL3 transgenic mice have increased airway remodeling and airway responsiveness characteristic of asthma. J Immunol. 2014; 192:3475–3487. [PubMed: 24623133]
4. Breslow DK, Collins SR, Bodenmiller B, Aebersold R, Simons K, Shevchenko A, Ejsing CS, Weissman JS. Orm family proteins mediate sphingolipid homeostasis. Nature. 2010; 463:1048–1053. [PubMed: 20182505]
5. Miller M, Beppu A, Rosenthal P, Pham A, Das S, Karta M, Song DJ, Vuong C, Doherty T, Croft M, Zuraw B, Zhang X, Gao X, Aceves S, Chouiali F, Hamid Q, Broide DH. FSTL1 promotes asthmatic airway remodeling by inducing oncostatin M. J Immunol. 2015; 195:3546–3556. [PubMed: 26355153]
6. Wang Y, Shen J, Arenzana N, Tirasophon W, Kaufman RJ, Prywes R. Activation of ATF6 and an ATF6 DNA binding site by the endoplasmic reticulum stress response. J Biol Chem. 2000; 275:27013–27020. [PubMed: 10856300]
7. Adachi Y, Yamamoto K, Okada T, Yoshida H, Harada A, Mori K. ATF6 is a transcription factor specializing in the regulation of quality control proteins in the endoplasmic reticulum. Cell Struct Funct. 2008; 33:75–89. [PubMed: 18360008]

8. Miller M, Rosenthal P, Beppu A, Gordillo R, Broide DH. Oroscomucoid like protein 3 (ORMDL3) transgenic mice have reduced levels of sphingolipids including sphingosine-1-phosphate and ceramide. J Allergy Clin Immunol. In Press.

9. Roviezzo F, D'Agostino B, Brancaleone V, De Gruttola L, Bucci M, De Dominicis G, Orlotti D, D'Aiuto E, De Palma R, Rossi F, Sorrentino R, Cirino G. Systemic administration of sphingosine-1-phosphate increases bronchial hyperresponsiveness in the mouse. Am J Resp Cell Mol Biol. 2010; 42:572–577.

10. Price MM, Oskeritzian CA, Falanga YT, Harikumar KB, Allegood JC, Alvarez SE, Conrad D, Ryan JJ, Milstien S, Spiegel S. A specific sphingosine kinase 1 inhibitor attenuates airway hyperresponsiveness and inflammation in a mast cell-dependent murine model of allergic asthma. J Allergy Clin Immunol. 2013; 131:501–511. [PubMed: 22939756]

11. Ammit AJ, Hastie AT, Edsall LC, Hoffman RK, Amrani Y, Krymskaya VP, Kane SA, Peters SP, Penn RB, Spiegel S, Panettieri RA Jr. Sphingosine 1-phosphate modulates human airway smooth muscle cell functions that promote inflammation and airway remodeling in asthma. FASEB J. 2001; 15:1212–1214. [PubMed: 11344091]

12. Sun X, Ma SF, Wade MS, Flores C, Pino-Yanes M, Moitra J, Ober C, Kittles R, Husain AN, Ford JG, Garcia JG. Functional variants of the sphingosine-1-phosphate receptor 1 gene associate with asthma susceptibility. J Allergy Clin Immunol. 2010; 126:241–249. [PubMed: 20624651]

13. Worgall TS, Veerappan A, Sung B, Kim BI, Weiner E, Bholah R, Silver RB, Jiang XC, Worgall S. Impaired sphingolipid synthesis in the respiratory tract induces airway hyperreactivity. Sci Transl Med. 2013; 5:186ra67. Erratum in Sci Transl Med, 5, 192er7, 2013.

14. Löser S, Gregory LG, Zhang Y, Schaefer K, Walker SA, Buckley J, Denney L, Dean CH, Cookson WO, Moffatt MF, Lloyd CM. Pulmonary ORMDL3 is critical for induction of Alternaria induced allergic airways disease. J Allergy Clin Immunol. 2016 In Press.

15. Greene AL, Lalli MJ, Ji Y, Babu GJ, Grupp I, Sussman M, Periasamy M. Overexpression of SERCA2b in the heart leads to an increase in sarcoplasmic reticulum calcium transport function and increased cardiac contractility. J Biol Chem. 2000; 275:24722–24727. [PubMed: 10816568]

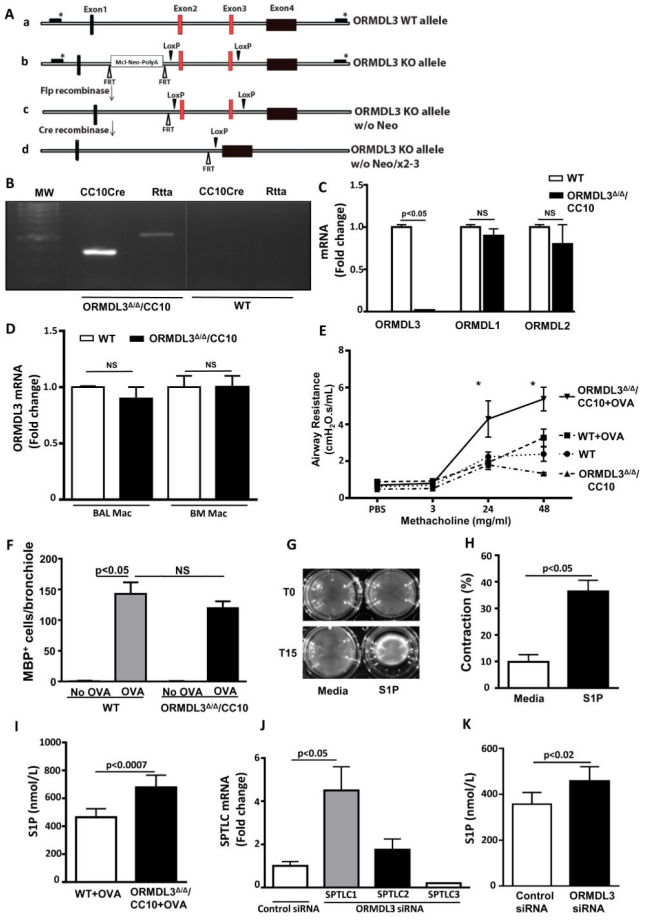

Figure 1. *Ormdl3* $^{\Delta 2\text{-}3/\Delta 2\text{-}3}$*/CC10* mice have increased airway hyperreactivity

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 exon 2 and 3. **B.** Genotyping of *Ormdl3* $^{\Delta 2\text{-}3/\Delta 2\text{-}3}$*/CC10* mice demonstrated presence of CC10 Cre and Rtta (reverse tetracycline-controlled transactivator). **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 to WT mice challenged with OVA as assessed in intubated mice using Scireq software. *p<0.05. **F.** Levels of MBP positive 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–H.** Incubation of lung smooth muscle cells with S1P for 15 min (T15) induced significant contraction compared to 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 qPCR 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.