The *ORMDL3* Asthma Gene Regulates *ICAM1* and Has Multiple Effects on Cellular Inflammation

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Abstract

Rationale: Polymorphisms on chromosome 17q21 confer the major genetic susceptibility to childhood-onset asthma. Risk alleles positively correlate with *ORMDL3* (orosomucoid-like 3) expression. The locus influences disease severity and the frequency of human rhinovirus (HRV)-initiated exacerbations. *ORMDL3* is known to regulate sphingolipid synthesis by binding serine palmitoyltransferase, but its role in inflammation is incompletely understood.

Objectives: To investigate the role of *ORMDL3* in cellular inflammation.

Methods: We modeled a time series of IL1B-induced inflammation in A549 cells, using cytokine production as outputs and testing effects of *ORMDL3* siRNA knockdown, *ORMDL3* overexpression, and the serine palmitoyltransferase inhibitor myriocin. We replicated selected findings in normal human bronchial epithelial cells. Cytokine and metabolite levels were analyzed by analysis of variance. Transcript abundances were

analyzed by group means parameterization, controlling the false discovery rate below 0.05.

Measurements and Main Results: Silencing *ORMDL3* led to steroid-independent reduction of IL6 and IL8 release and reduced endoplasmic reticulum stress after IL1B stimulation. Overexpression and myriocin conversely augmented cytokine release. Knockdown reduced expression of genes regulating host-pathogen interactions, stress responses, and ubiquitination: in particular, *ORMDL3* knockdown strongly reduced expression of the HRV receptor *ICAM1*. Silencing led to changes in levels of transcripts and metabolites integral to glycolysis. Increased levels of ceramides and the immune mediator sphingosine-1-phosphate were also observed.

Conclusions: The results show *ORMDL3* has pleiotropic effects during cellular inflammation, consistent with its substantial genetic influence on childhood asthma. Actions on *ICAM1* provide a mechanism for the locus to confer susceptibility to HRV-induced asthma.

Keywords: childhood asthma; *ORMDL3*; inflammation; *ICAM1*

Childhood asthma is driven by environmental and genetic influences. Asthma-associated single-nucleotide polymorphisms within the *ORMDL3* (orosomucoid-like 3) asthma locus (1) are

highly positively correlated with the transcript abundance of *ORMDL3* (1, 2). The locus carries a population-attributable fraction for childhood-onset asthma greater than 40% (3). Alleles associated with high

levels of *ORMDL3* transcription confer susceptibility to human rhinovirus (HRV)-induced wheeze, which is the major cause of morbidity in children with asthma (4). Conversely, low-transcription alleles

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At a Glance Commentary

Scientific Knowledge on the

Subject: Polymorphisms within a locus on chromosome 17q21 confer the major genetic susceptibility to childhood-onset asthma. They positively correlate with the transcript abundance of *ORMDL3*. The locus influences disease severity and the frequency of human rhinovirus (HRV)-initiated exacerbations, which are the major causes of morbidity in childhood asthma. *ORMDL3* is known to regulate *de novo* sphingolipid synthesis and stress responses, but systematic knowledge about its actions in inflammation is limited.

What This Study Adds to the

Field: We investigated the role of *ORMDL3* in cellular inflammation, using transcriptomics and metabolomics. Silencing *ORMDL3* led to steroid-independent reduction in inflammatory cytokine release and reduced endoplasmic reticulum stress after proinflammatory stimuli. Transcript abundances of genes regulating host–pathogen interactions, stress responses, and ubiquitination were altered. In particular, *ORMDL3* silencing strongly reduced expression of the HRV receptor *ICAM1*, providing a mechanism for the 17q21 locus to modify the risk of HRV-induced asthma exacerbations. Mediators of glucose metabolism and metabolites integral to glycolysis were strongly affected by knockdown, providing a potential link between metabolic disease and asthma. Knockdown increased levels of ceramides and the immune mediator sphingosine-1-phosphate during inflammation. The results show that *ORMDL3* has a wide range of effects during cellular inflammation, suggesting a range of possible therapeutic benefits from accessing *ORMDL3*-regulated pathways.

predict which children will be protected against wheeze by a rich microbial environment (5). The *ORMDL3* locus also affects susceptibility to type 1 diabetes (6) and may be associated with increased

body mass index in individuals with asthma (7).

Information is limited about the mechanisms through which *ORMDL3* exerts these important effects, beyond that ORM family proteins have known functions as rheostats on *de novo* sphingolipid synthesis (8), inhibiting the *de novo* synthesis of sphingolipids by the human SPT (serine palmitoyltransferase) complex. *ORMDL3* has also been shown to facilitate the unfolded protein response to cellular stress by influencing SERCA (sarcoplasmic/endoplasmic reticulum calcium ATPase) and endoplasmic reticulum (ER)-mediated Ca^{2+} flux (9).

The pulmonary epithelia are highly active immunologically (10), and genes identified by asthma genome-wide association studies often communicate epithelial damage to the adaptive immune system (3). Studies of genetically modified mice showed *Ormdl3* to be induced by allergens and helper T-cell type 2 cytokines and suggested that in the lung *Ormdl3* was expressed predominantly in airway epithelial cells (11, 12). In humans *ORMDL3* is expressed in diverse cell types that include lymphocytes (1, 2) as well as airway epithelial cells (13).

Consequently, we systematically studied the effects of *ORMDL3* on inflammation. We used an established cellular model of lung innate immunity with human A549 cells (14, 15) stimulated by the major proinflammatory cytokine IL1B (15) (Figure 1 shows our study design). In light of previous findings, we concentrated on cytokine (12), transcriptomic (1), and metabolomic (8) consequences of siRNA silencing (knockdown) of *ORMDL3*. Positive findings were explored in normal human bronchial epithelial (NHBE) cells. We contrasted the effects of knockdown by *ORMDL3* upregulation with plasmid transfection. We also investigated the effects of the SPT inhibitor myriocin (16), to explore whether small molecules (drugs) may mimic some of the effects of high *ORMDL3* levels. Enhanced expression of *ORMDL3* is accompanied by poor response to inhaled corticosteroid therapy (17), and so we benchmarked the degree of cytokine reductions against dexamethasone.

Methods

Full details of methods are given in the online supplement.

Epithelial Cell Culture

A549 human lung epithelial cells (American Type Culture Collection) were cultured in Dulbecco's modified Eagle's medium. NHBE cells were obtained from Lonza and were grown in bronchial epithelial medium (BEGM; Lonza).

siRNA-mediated *ORMDL3* Knockdown

ORMDL3 siRNAs were obtained from Dharmacon Research Inc. Nontargeting pool-negative siRNAs were used as controls. siRNA transfections of A549 and NHBE cells were performed with DharmaFECT reagent 1. siRNAs and transfection reagents were mixed according to supplier protocols, and then added to single wells for 48 hours of incubation.

Overexpression of *ORMDL3*

The human *ORMDL3* gene was amplified with template control cDNAs from Clontech. Primer sequences were as follows: F.5'-CACCATGAATGTGGGCACA GCGCACAGCGAG-3'; R.5'-TCAGTAC TTATTGATTCCAAAAATC-3'. The PCR product was cloned into the pcDNA3.1 directional expression vector from Invitrogen. Plasmids were introduced with 1 μl of Lipofectamine 2000 (Invitrogen) per well, and cells were cultured for 48 hours before stimulation.

Epithelial Cell Model of Inflammation

Forty-eight hours after induction of *ORMDL3* and other target gene silencing, cells were starved in serum-free medium for 24 hours before stimulation with IL1B (1 ng/ml; R&D Systems) or TNF- α (tumor necrosis factor- α) (10 ng/ml; R&D Systems). Cells were collected at 0, 2, 4, 6, 8, 10, and 24 hours with supernatants harvested for cytokine measurements as previously established (15), using three replicates for cytokines and four replicates for myriocin studies and other assays as exemplified (14, 18). For metabolite screening, experiments were performed in quadruplicate and cells and supernatants were collected at 0, 2, 4, 8, and 10 hours after stimulation. For dexamethasone pretreatment, dexamethasone was given 1 hour before IL1B stimulation, and cells and supernatants from triplicate samples were collected at 10 hours. The effects of SPT inhibition were tested by

Figure 1. Study design for investigation of *ORMDL3* effects on inflammation. NHBE = normal human bronchial epithelial; *ORMDL3* = orosomucoid-like 3.

the addition of 40 μ M myriocin to quadruplicate samples 2 hours before IL1B stimulation. This is a commonly used protocol to inhibit *de novo* sphingolipid biosynthesis.

Western Blotting

Whole-cell protein extracts were prepared with an Active Motif nuclear extract kit (Active Motif Europe). Forty micrograms of protein was separated by electrophoresis on 10% sodium dodecyl sulfate polyacrylamide gels (Invitrogen) and transferred to nitrocellulose membranes. After blocking for 1 hour with 5% milk, the blot was immersed with the first antibody (rabbit anti-human *ORMDL3*, diluted 1:500 [Abgent Cat. No. AP10739c] or mouse anti-human ICAM1, diluted 1:500 [Santa Cruz Biotechnology Cat. No. sc-8439]) and then detected with secondary antibodies conjugated to detection solutions.

ELISAs

Cell-free supernatants were harvested after stimulation and used for cytokine measurements. Human IL-6 and IL-8 were measured with ELISA kits (R&D Systems). Arithmetic means were compared by one-way analysis of variance (ANOVA) (IBM SPSS Statistics 24).

Metabolite Screening

Global biochemical profiling from cells was performed by gas chromatography–mass spectrometry and liquid chromatography–tandem mass spectrometry (MS/MS) platforms (Metabolon Inc.). Active and scrambled siRNA effects on the two treatment groups

were measured in quadruplicate and compared across time points of 0, 2, 4, 8, and 10 hours after stimulation with IL1B. Data were analyzed by two-way ANOVA in R (<https://cran.r-project.org/>). To control for multiple comparisons, results with a *q* value less than 0.01 are reported. Sphingolipid levels were measured by liquid chromatography–electrospray ionization–MS/MS as previously described (19) and compared by one-way ANOVA (IBM SPSS Statistics 24).

Global Gene Expression Profiling and Analysis

RNA samples were hybridized to Affymetrix human gene 1.1 ST arrays on the Affymetrix GeneTitan. Differential expression across 14,488 transcripts and 38 samples was assessed with limma (version 3.22.7). A baseline shift in transcription between *ORMDL3* knockdown and control cells at time point 0 was assessed via group-means parameterization. *P* values were adjusted for multiple testing, controlling the expected false discovery rate below 0.05. The median SD of gene expression for all samples was 0.19380 (first quartile, 0.14950; third quartile, 0.26570), giving 80% power to detect a fold change of 1.59 with a single false positive given four samples per group.

Cell Stress

A549 cells were seeded into 24-well plates and cultured as described above. Thapsigargin (1 μ M; Sigma), which inhibits endoplasmic reticulum calcium ATPase, was added to the cells for 8 hours, and results for triplicate samples were analyzed by ANOVA.

Declarations

This study did not involve human subjects and did not require ethics board approval.

The data sets generated and analyzed during this study are deposited in the GEO database with accession number GSE92484 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE92484>).

Results

ORMDL3 Effects on Cytokine Production

ORMDL3 mRNA was reduced by more than 95% by treating A549 cells with 25 nM siRNA for 48 hours, without affecting viability and with concomitant reduction of the *ORMDL3* protein confirmed by Western blot (Figure 2).

Compared with controls transfected with scrambled siRNA, we observed during the time course after IL1B stimulation a small initial increase in IL8 supernatant after 2 hours, followed later by a greater than 50% reduction in IL8, which was most marked at 10 hours (Figure 2). Similar small initial increases followed by substantial reductions were seen with the production of IL6 (Figure 2) and after TNF- α stimulation (data not shown). In stimulated NHBE cells, *ORMDL3* silencing led to late reduction in IL8 after 16 hours (Figure 2), whereas IL6 levels showed a more marked initial increase before falling significantly below control levels at 16 hours after stimulation (Figure 2). We therefore chose 10 hours after IL1B stimulation in A549 cells as a reference point for downstream experiments.

Next we overexpressed *ORMDL3* in A549 cells for 48 hours, using a pcDNA3.1 plasmid construct containing the full-length gene. This resulted in significant increases in IL8 and IL6 production after IL1B stimulation (Figure 2). Application of the SPT inhibitor myriocin likewise led to higher IL8 and IL6 release after IL1B stimulation (Figure 2), thus confirming that SPT mediated effects on cytokine production.

Glucocorticoids are a mainstay of therapy for asthma, and enhanced expression of *ORMDL3* is accompanied by a poor response to inhaled corticosteroid therapy (17). We therefore tested whether *ORMDL3* was active in glucocorticoid-regulated

Figure 2. Effects of *ORMDL3* knockdown and overexpression on cytokine production in lung epithelial cells. Shown are levels of supernatant IL8 and IL6 in response to IL1B stimulation. Throughout, purple lines show control levels of IL8 and orange lines show the levels of IL8 with intervention; green lines show control levels of IL6 and coral lines show the levels of IL6 with intervention. (A and B) Decreased IL8 and IL6 production in A549 cells after siRNA knockdown of *ORMDL3*; (C and D) decreased IL8 and IL6 production in NHBE cells after siRNA knockdown of *ORMDL3*; (E and F) IL8 and IL6 production was increased by overexpression of plasmid construct (*pc*) *ORMDL3* and (G and H) by addition of the SPT inhibitor myriocin; (I) Western blot confirming effects of *ORMDL3* siRNA knockdown on *ORMDL3*; (J and K) administration of increasing concentrations of dexamethasone to A549 cells showing that *ORMDL3* knockdown had a marked steroid-sparing effect on IL8 and IL6 production ($t = 10$ h). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Dex = dexamethasone; NHBE = normal human bronchial epithelial; *ORMDL3* = orosomucoid-like 3; SPT = serine palmitoyltransferase.

Table 1. Transcripts Altered by *ORMDL3* Knockdown

Gene	Functions	FC	Average Expression	Adjusted <i>P</i> Value
<i>ORMDL3</i>	Asthma susceptibility gene	−3.46	6.41	1.15×10^{-3}
<i>DIPK2A</i>	Regulates cellular secretory traffic	−1.90	8.21	2.95×10^{-3}
<i>HMOX1</i>	Host defense against physiological insults	−1.84	11.10	6.72×10^{-3}
<i>USP46</i>	Deubiquitinating cysteine protease	−1.72	8.08	2.95×10^{-3}
<i>OSTM1</i>	Ubiquitin-dependent degradation of G proteins	−1.65	7.97	6.72×10^{-3}
<i>ZP3</i>	Structural component of zona pellucida	−1.62	8.82	6.72×10^{-3}
<i>ITGA2</i>	Mediates cellular adhesion	1.64	10.91	6.72×10^{-3}
<i>GALNT5</i>	Catalyzes O-glycosylation of Golgi proteins	1.88	7.53	6.86×10^{-3}
<i>EBI3</i> *	IL27: induction of inflammation	1.89	6.69	2.95×10^{-3}
<i>TRPA1</i>	Receptor for airway oxidative stress	1.94	7.66	7.87×10^{-3}
<i>SNORD38A</i>	Small nucleolar RNA	1.96	4.91	5.46×10^{-3}
<i>GFPT2</i> *	Hyperglycemia and oxidative stress	2.01	9.95	8.54×10^{-3}
<i>BIRC3</i> *	Inhibits apoptosis by binding to TRAF1 and TRAF2	2.09	8.09	2.95×10^{-3}
<i>KLHL4</i>	Kelch-like family member 4	2.09	6.68	1.60×10^{-3}
<i>TMOD1</i>	NF-κB activation and MMP13 induction	2.11	8.49	6.72×10^{-3}
<i>ANKRD1</i>	Inhibitor of ER stress-induced apoptosis	2.25	7.77	2.95×10^{-3}
<i>TNFRSF9</i> *	Enhances development of T cells and monocytes	2.71	6.62	4.25×10^{-3}
<i>ICAM1</i> [†]	Immune synapses, adhesion: HRV receptor	−2.42	10.97	1.01×10^{-4}
<i>NFKB2</i> [†]	Subunit of NF-κB	−1.77	11.27	8.85×10^{-3}

Definition of abbreviations: *DIPK2A* = divergent protein kinase domain 2A; ER = endoplasmic reticulum; FC = fold change; HRV = human rhinovirus; MMP13 = matrix metalloproteinase 13; NF-κB = nuclear factor-κB; *ORMDL3* = orosomucoid-like 3; TRAF1, TRAF2 = TNF receptor-associated factor 1 and TNF receptor-associated factor 2, respectively.

Shown are transcript clusters that differ with an adjusted $P < 0.01$ between unstimulated A549 cells with *ORMDL3* knockdown and controls. (Results for $P < 0.05$ are given in Table E1.)

*Significant time-course effects of *ORMDL3* knockdown as well as baseline.

[†]Significant changes during time course but not baseline; fold change at 8 hours compared with 6 hours; fold change at 2 hours compared with 0 hours.

pathways by administering increasing concentrations of dexamethasone to A549 cells after IL1B stimulation, with and without *ORMDL3* silencing. Marked effects of silencing on cytokine production persisted in the presence of effective doses of dexamethasone, indicating a steroid-independent effect (Figure 2).

Transcriptomics

To investigate systematically the wider effects of *ORMDL3* silencing, we measured global gene expression with Affymetrix human gene 1.1 ST arrays during the same time course of IL1B-induced inflammation in A549 cells. We found that the transcript abundances of 92 genes were altered at baseline by *ORMDL3* knockdown ($P_{\text{adjusted}} < 0.05$). These involved innate and adaptive immunity (*BIRC3*, *EBI3*, and *TNFRSF9*), stress responses (*HMOX1*, *TRPA1*, *GFPT2*, and *ANKRD1*), and ubiquitination (*USP46* and *OSTM1*) (Table 1 lists the top hit transcripts altered with $P_{\text{adjusted}} < 0.01$; Table E1 in the online supplement includes all results for $P_{\text{adjusted}} < 0.05$).

Transcripts altered in abundance at baseline in general remained at the same levels relative to controls during

the time course after IL1B stimulation (Figure E1). Using limma, we modeled dynamic treatment effects of *ORMDL3* knockdown appearing during the time course. We observed changes in transcript

abundance of 24 genes ($P_{\text{adjusted}} < 0.05$; 9 of the 24 genes were also altered at baseline), with functions associated with apoptosis, inflammation and NF-κB; mitogenesis and cell division;

Figure 3. Downregulation of ICAM1 by *ORMDL3* siRNA knockdown. (A) Dynamic changes in transcript levels of *ICAM1* after IL1B stimulation in A549 cells, comparing *ORMDL3* siRNA silencing (green) and controls (blue) ($P = 0.0001$ for time course, $***P < 0.001$); (B) Western blots confirming downregulation of ICAM1 protein by knockdown. ICAM1 = intercellular adhesion molecule 1; *ORMDL3* = orosomucoid-like 3.

inflammation-related metabolites (NO, Zn^{2+} , Ca^{2+} , dopamine, and ammonia); and lipid and glucose metabolism (Table 1).

The major HRV receptor *ICAM1* (intercellular adhesion molecule 1) (20) exhibited a blunted transcriptional trajectory in *ORMDL3*-depleted A549 cells after IL1B stimulation that was not apparent at baseline (Figure 3). We showed by Western blot that the ICAM1 protein was also downregulated at baseline and at 10 hours after IL1B stimulation (Figure 3). Conversely, administration of myriocin led to

increases in *ICAM1* transcription at similar times after IL1B administration (2.0-fold increase at 6 h, $P = 3.5 \times 10^{-6}$; 2.2-fold increase at 8 h, $P = 3.2 \times 10^{-7}$).

ANKRD1, upregulated 2.2-fold by *ORMDL3* knockdown (Table 1), also has a recognized role in antiviral immunity and viral entry (21); *TNFRSF9* (*CD137*, 2.7-fold increased) moderates antiviral immunity (22); and *EBI3* (a subunit of IL-27 and IL-35, 1.9-fold upregulated) is implicated in immune response to various pathogens (23, 24).

Two sphingolipid pathway transcripts (the phospholipases *PLA2G15* and *SGPP1*)

were significantly downregulated by *ORMDL3* knockdown, and the abundance of sphingomyelin synthase *SGMS2* was moderately increased (fold change, 1.36; $P_{\text{adjusted}} < 0.05$; Table E1). Examination of other sphingolipid pathway genes (Table E2) showed *ORMDL3* silencing not to induce compensatory increases in transcript abundances of *ORMDL1* and *ORMDL2* and other major sphingolipid modulators.

Metabolomics

Global metabolomic profiling revealed that glucose and glycolytic intermediates

Figure 4. Metabolomic effects of *ORMDL3* knockdown in airway epithelial cells after IL1B stimulation. (A) Changes in glucose metabolites in A549 cells after *ORMDL3* knockdown before and after IL1B stimulation (standard error bars are shown throughout, with a sample size $n = 4$; x-axes: hours after IL1B stimulation); (B) flux of lysophospholipids during the time course of IL1B stimulation, showing differences between *ORMDL3* knockdown and controls not apparent at time 0. *ORMDL3* = orosomucoid-like 3.

including glucose 6-phosphate and lactate were significantly elevated in the *ORMDL3* siRNA-treated samples at baseline and remained higher after IL1B stimulation (Figure 4 and Table 2). IL1B elevated these metabolites in both groups. Sorbitol and pentose phosphate pathway metabolites including ribose were similarly at higher levels in the *ORMDL3* knockdown compared with controls, consistent with an observed increase in NADPH generation (Figure 4; Table 2 for changes with $P_{\text{adjusted}} < 0.01$). The concomitant elevation in transcript abundance of *GFPT2* (glutamine-fructose-6-phosphate transaminase 2) (Table 1) suggests that this gene may contribute to this effect. In addition, we saw levels of lysophospholipids become elevated compared with controls during the time

course after IL1B stimulation (Figure 4 and Table 2), as were the amino acids kynurenine and creatine (Table 2).

Sphingolipid Pathways

Our metabolomic analyses of sphingolipids revealed that *ORMDL3* silencing increased high molecular weight (C24:1, C24:0, and C26:1) ceramide levels in A549 and NHBE cells (Figure 5) at baseline ($t = 0$). IL1B stimulation reduced ceramide levels in both cell types, although the effects were more marked in NHBE cells. The ceramide reduction was substantially opposed by *ORMDL3* knockdown at 10 hours in A549 cells and at 24 hours in NHBE cells (Figure 5).

We did not detect changes in sphinganine, dihydroceramides, sphingosine, or ceramide 1-phosphate. Statistically significant increases were observed in

sphingosine-1-phosphate (S1P) levels (Figure 5), although the concentrations were at the lower limit of sensitivity of the assay.

Silencing of *SPHK1* and *SPHK2*, which synthesize S1P, marginally increased IL6 production, whereas knockdown of *SGPP2* (but not *SGPP1*), which degrade S1P, reduced cytokine production (Figure 6). These findings are consistent with the recognized antiinflammatory signal mediation by S1P (25).

Stress Responses

The previously described facilitation of the unfolded protein response to cellular stress by *ORMDL3* (9) was consistent with our global gene expression results (Table 1). We therefore induced ER stress by adding the SERCA inhibitor thapsigargin to *ORMDL3*-silenced cells. *ORMDL3* knockdown corrected a thapsigargin-induced reduction of ceramide levels (Figure 5). These results confirm that *ORMDL3* is a regulator of ER stress and add weight to the suggestion that ceramide or its metabolites may mediate this effect.

Table 2. Metabolites Altered by *ORMDL3* Knockdown over the Time Course of IL1B-induced Inflammation in Airway Epithelial Cells

Pathway	Biochemical Name	P Value	q Value	Ratio 8 h
Carbohydrate	Fructose	<0.001	0.0002	2.30
	Mannose 6-phosphate	<0.001	0.0008	1.76
	Sorbitol	<0.001	0.0000	3.67
	Glucose 6-phosphate (G6P)	<0.001	0.0007	2.07
	Glucose	<0.001	0.0025	2.12
	Fructose 1,6-diphosphate, glucose 1,6-diphosphate	<0.001	0.0003	1.79
	Lactate	<0.001	0.0001	1.39
	Ribitol	<0.001	0.0000	1.67
	Ribose	<0.001	0.0013	1.56
	Ribose 5-phosphate	<0.001	0.0027	1.55
	Cytidine 5'-diphosphocholine	<0.001	0.0014	1.41
Lipid	2-Palmitoylglycerophosphoethanolamine	<0.001	0.0027	1.36
	2-Palmitoleoylglycerophosphoethanolamine	<0.001	0.0018	1.34
	2-Linoleoylglycerophosphoethanolamine	<0.001	0.0022	1.29
Nucleotide	Adenosine 2'-monophosphate (2'-AMP)	<0.001	0.0001	1.46
	Guanosine	<0.001	0.0002	1.37
	Uridine	<0.001	0.0014	1.32
Amino acid	Kynurenine	<0.001	0.0016	1.33
	Creatine	<0.001	0.0019	1.22
Peptide	Aspartylphenylalanine	0.0029	0.0099	1.41
	γ -Glutamylvaline	<0.001	0.0025	0.76
	γ -Glutamylleucine	<0.001	0.0025	0.87
	γ -Glutamylisoleucine	<0.001	0.0025	0.90
	γ -Glutamylmethionine	<0.001	0.0013	0.82
	γ -Glutamylphenylalanine	<0.001	0.0001	0.86
	γ -Glutamyltyrosine	<0.001	0.0016	0.82
	γ -Glutamylthreonine	<0.001	0.0019	0.80
	γ -Glutamyltryptophan	0.0019	0.0067	0.91
Cofactor	Nicotinamide adenine dinucleotide reduced (NADH)	0.0018	0.0067	1.65*
Xenobiotic	Erythritol	<0.001	0.0000	1.56

Definition of abbreviations: *ORMDL3* = orosomucoid-like 3; Ratio 8 h = ratio of *ORMDL3* knockdown to scrambled siRNA controls 8 hours after IL1B administration.

*Ratio at 10 hours (ratio = 1.07 at 8 h).

Discussion

The results implicate manifold actions of *ORMDL3* on pathways for epithelial-microbial interactions, inflammation, and glucose metabolism that are in addition to its recognized inhibition of *de novo* ceramide synthesis. ORM proteins are highly conserved in eukaryotes, which taken with our findings might suggest an ancient role for them in coordinating metabolism under conditions of stress.

Early HRV infection is a marker of subsequent asthma, and significant increases in the number of childhood HRV wheezing illnesses are observed in children with enhanced transcription genotypes at the 17q21 *ORMDL3* locus (4). Our finding that *ORMDL3* knockdown decreased the transcript and protein expression of *ICAM1*, the major HRV receptor (20), suggests a mechanism for the association between *ORMDL3*, HRV infections, and asthma. In addition, *ICAM1* is a receptor for the bacterial airway pathogen *Haemophilus influenzae* (26), which is increased in abundance in asthmatic airways between exacerbations (27), potentially providing positive feedback between chronic bacterial colonization and acute viral infections.

Figure 5. Effects of *ORMDL3* knockdown on sphingolipid pathways in airway epithelial cells after IL1B stimulation. (A and B) Abundance of ceramide species in A549 and primary NHBE cells with and without *ORMDL3* silencing and before and after 10 hours of IL1B stimulation; (C) abundance of sphingosine-1-phosphate (S1P) and dihydrosphingosine-1-phosphate (DHS1P) in the same experiment; (D) changes in ceramide species in A549 cells after 8 hours of thapsigargin-induced ER stress, with and without *ORMDL3* knockdown. ER = endoplasmic reticulum; NHBE = normal human bronchial epithelial; *ORMDL3* = orosomucoid-like 3.

Figure 6. Sphingolipid pathways and knockdown of genes regulating S1P. (A) Schematic of sphingolipid synthetic pathways with main metabolites and enzymes; (B) knockdown of S1P-modifying genes. IL6 and IL8 levels in supernatants after 8 hours of IL1B stimulation are shown as a fraction of levels in controls. CERK = ceramide kinase; ER = endoplasmic reticulum; KDSR = 3-ketodihydrosphingosine reductase; ORMDL3 = orosomucoid-like 3; S1P = sphingosine-1-phosphate; SGPP1, SGPP2 = sphingosine-1-phosphate phosphatase 1 and 2, respectively; SM = sphingomyelin; SMPD1 to SMPD4 = sphingomyelin phosphodiesterase 1–4, respectively; SPHK1, SPHK2 = sphingosine kinase 1 and 2, respectively; SPT = serine palmitoyltransferase; SPTLC = serine palmitoyltransferase long chain base subunit.

ORMDL3 silencing affected gluconeogenesis during inflammation. We observed concomitant upregulation in the transcription of *GFPT2* (Table 1), which is associated in diabetics with insulin resistance, hyperglycemia, and oxidative stress (28). Obesity and asthma form a complex multifactorial syndrome with

poor responses to therapy (29, 30). Our results may suggest a mechanism for a reported genetic component to obese asthma from the *ORMDL3* locus (7).

Taken in the context of the reduced airway responsiveness observed in *Ormdl3* knockout mice (12) and the increased responsiveness of *Spt*^{+/-} knockout (31)

and *Ormdl3* transgenic mice (12, 32), our study identified candidates that may regulate the nonspecific airway hyperresponsiveness that accompanies human asthma (33). *TRPA1* is a receptor that recognizes oxidative stress and modulates airway hyperresponsiveness (34), and *ATP2B4* varies the production of

the bronchodilator nitric oxide (35). The elevated metabolite kynurenine is a vasodilator and immune signaling molecule (36) that by inference may also alter bronchial smooth muscle tone.

ORMDL3 is known to inhibit *de novo* synthesis of sphingolipids by SPT (8). We found that *ORMDL3* knockdown increased intracellular levels of ceramide, some lysophospholipids, and S1P. We were not able to distinguish completely between direct effects of these sphingolipids on the various changes we have observed and as yet unknown consequences of *ORMDL3* activation, but our administration of myriocin and knockdowns of other pathway enzymes support central roles for sphingolipids in the observed effects of *ORMDL3* on inflammation and metabolism.

The results of our study should be considered in light of several limitations. Ceramide synthesis is known to be multifaceted, and ceramide effects vary in different cellular compartments (25) that we have not tested independently in our experiments. We have studied mostly

A549 cells, because the scope of our experiments required cell lines that grow robustly and at a reasonable cost, and because there is a substantial literature underpinning A549 cell use in inflammation research (14, 18). A549 cells are, however, derived from an alveolar malignancy, so in selected experiments we investigated NHBE cells to represent bronchial epithelium. Primary NHBE cells grow much more slowly than malignant A549 cells, complicating direct comparisons. The results from both cell types nevertheless show that *ORMDL3* knockdown reduced late cytokine responses to IL1 β , although an early increase in supernatant cytokine release was more marked after knockdown in NHBE cells. NHBE cells also showed larger increases than A549 cells in C24 ceramides after knockdown, but concomitant downstream increases in S1P were qualitatively similar. Fully differentiated NHBE cells, which best reflect the airway epithelium, may show further differences. Further caution should

be applied to extrapolating the results to the complex mixed cell interactions that are present in asthmatic airway inflammation.

Polymorphisms within the *ORMDL3* locus carry the highest known risk for childhood asthma (37). Genetic associations between disease and a drug target more than double the chance of success in phase 2 trials (38, 39). Our results suggest a range of possible therapeutic benefits from accessing *ORMDL3*-regulated pathways on airway viral infection, inflammation, and bronchial responsiveness. S1P is a recognized target for therapy of inflammatory disorders (40, 41) and manipulation of other steps in sphingolipid assembly may be of therapeutic benefit (42). Investigation of upstream regulatory phosphorylation of *ORMDL3* (8) and its interactions with SPT may also provide novel opportunities. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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