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Association of *ORMDL3* with rhinovirus-induced endoplasmic reticulum stress and type I Interferon responses in human leukocytes

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Abstract

Background—Children with risk alleles at the 17q21 genetic locus who wheeze during rhinovirus illnesses have a greatly increased likelihood of developing childhood asthma. In mice, overexpression of the 17q21 gene *ORMDL3* leads to airway remodeling and hyper-responsiveness. However, the mechanisms by which *ORMDL3* predisposes to asthma are unclear. Previous studies have suggested that *ORMDL3* induces endoplasmic reticulum (ER) stress and production of the type I interferon (IFN) regulated chemokine CXCL10.

Objective—The purpose of this study was to determine the relationship between *ORMDL3* and rhinovirus-induced ER stress and type I IFN in human leukocytes.

Methods—ER stress was monitored by measuring *HSPA5*, *CHOP* and spliced *XBPI* gene expression, and type I IFN by measuring *IFNB1* (IFN- β) and *CXCL10* expression in human cell lines and primary leukocytes following treatment with rhinovirus. Requirements for cell contact and specific cell type in *ORMDL3* induction were examined by transwell assay and depletion experiments, respectively. Finally, the effects of 17q21 genotype on the expression of *ORMDL3*, *IFNB1*, and ER stress genes were assessed.

Results—THP-1 monocytes overexpressing *ORMDL3* responded to rhinovirus with increased *IFNB1* and *HSPA5*. Rhinovirus-induced *ORMDL3* expression in primary leukocytes required cell-

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cell contact, and induction was abrogated by plasmacytoid dendritic cell depletion. The degree of rhinovirus induced *ORMDL3*, *HSPA5*, and *IFNB1* expression varied by leukocyte type and 17q21 genotype, with the highest expression of these genes in the asthma-associated genotype.

Conclusions & Clinical Relevance—Multiple lines of evidence support an association between higher *ORMDL3* and increased rhinovirus-induced *HSPA5* and type I IFN gene expression. These associations with *ORMDL3* are cell-type specific, with the most significant 17q21 genotype effects on *ORMDL3* expression and *HSPA5* induction evident in B cells. Together, these findings have implications for how the interaction of increased *ORMDL3* and rhinovirus may predispose to asthma.

Introduction

Association with polymorphisms at the 17q21 locus remains one of the strongest and most widely replicated GWAS findings in childhood asthma [1–3]. The 17q21 asthma risk genotype is associated with increased expression of two co-regulated genes at that locus, *GSDMB* (gasdermin B) and *ORMDL3* (orosomucoid like-3) in peripheral blood cells [1, 4, 5]. Furthermore, exposure of PBMC to rhinovirus (RV), a common trigger of wheezing in early childhood and exacerbations in established asthma, upregulates *GSDMB* and *ORMDL3* expression [4]. In the high risk COAST (Childhood Origins of ASThma) birth cohort, children with the TT genotype at rs7216389 who wheezed with RV in the first three years of life had significantly greater prevalence of asthma later in childhood compared to children who did not wheeze, providing evidence for a gene-pathogen interaction [4].

The strong linkage disequilibrium between the nucleotide polymorphisms (SNPs) at 17q21, and the correlation of asthma-associated SNPs with altered expression of both *ORMDL3* and *GSDMB*, have complicated the assignment of causality in asthma risk [4, 6]. Recently, human *ORMDL3* over-expressing transgenic mice have presented compelling evidence for *ORMDL3* as a pathogenic factor. These mice manifest spontaneous airway remodeling, increased fibrosis, mucus production, inflammation, and methacholine-induced hyper-responsiveness [7]. *ORMDL3* is an endoplasmic reticulum (ER) resident protein that appears to have two major functions in cells: 1) inhibition of SPT (serine palmitoyl CoA transferase), reducing *de novo* sphingolipid synthesis and 2) inhibition of the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA2b) pump that maintains the calcium gradient between ER and cytoplasm [8, 9]. Both of these functions could be related to abnormal airway physiology in asthma. By regulating ER calcium stores, *ORMDL3* influences a stress response pathway known as the “Unfolded Protein Response” (UPR).

In immune cells such as macrophages, UPR induction dramatically increases production of type I IFN (with obvious relevance for anti-viral responses), and pro-inflammatory cytokines such as the IL-17-inducing cytokine IL-23 [10–13]. In murine studies, *ORMDL3* has been linked to increased production of the type I IFN regulated chemokine CXCL10 [7, 14]. However, it is unclear how *ORMDL3* alters the function of human leukocytes, particularly vis-a-vis asthma associated infections such as RV. We hypothesize that the increased *ORMDL3* expression associated with the high risk 17q21 genotype may predispose to asthma by regulating RV induced ER stress and type I IFN production.

During a respiratory infection, RV primarily infects and replicates in airway epithelium. However, RV also stimulates responses such as cytokine and chemokine production, proliferation and antigen presentation in different peripheral blood cell types including monocytes, CD4 and CD8 T cells, B cells, dendritic cells and eosinophils [15–21]. In airway tissue from human asthmatic subjects, RV co-localizes with monocytes and macrophages [22]. Furthermore, interactions between RV stimulated monocytes and epithelium dramatically increases CXCL10 production [23]. Therefore, in this study we investigated the relationship between *ORMDL3* expression and RV-induced ER stress and type I IFN production in multiple primary human leukocyte cell types, including cells from individuals with the high risk and low risk genotypes at the 17q21 asthma locus. Together our results revealed associations between elevated *ORMDL3* expression and increased *HSPA5* (BiP) induction and type I IFN responses in response to RV.

Materials and Methods

Human subjects

Participants were recruited for studies of eosinophil function. All participants provided written informed consent in a University of Wisconsin-Madison Institutional Review Board-approved blood donation protocol (approval #2013-1570). Individuals ranged in age from 19–55 and most had either self-reported allergies (94%), and/or mild asthma (56%). For 17q21 genotyping, whole blood was processed for DNA using the Qiagen Genra Puregene Blood kit according to manufacturer protocols. SNP rs7216389 genotype within the 17q21 locus was assessed using a TaqMan SNP genotyping assay (Applied Biosystems, Foster City, CA, Life Technologies product number: C 29062108 10). Donors with the homozygous TT (high risk) or CC (low risk) genotype were selected for analysis to maximize differences in our samples. Although TT donors had higher eosinophil counts (manuscript submitted), the rest of their cell differentials did not differ from CC donors at the time of blood collection.

Cells, reagents, and stimulations

THP1 (American Type Culture Collection, Rockville, MD) and BJAB (gift of Shannon Kenney, University of Wisconsin-Madison) cell lines were maintained in DMEM/high glucose (Mediatech, Manassas, VA) with 10% FBS (Hyclone, Logan, UT) and antibiotic-antimycotic solution (Mediatech). Comparisons of PBMC, leukocyte fractions and eosinophil subsets, as well as comparisons of cell subsets isolated before or after RV stimulation involved cells from the same donors as cell yields permitted. Percoll enabled separation of eosinophils and PBMC from the same blood donation: For eosinophil isolation, whole blood was centrifuged over Percoll, and eosinophils purified from the granulocyte layer by negative selection using anti-CD16 beads (Miltenyi Biotech, Auburn, CA). Peripheral blood mononuclear cells (PBMC) were isolated from blood by centrifugation over Percoll or Lymphocyte Separation Medium (Fisher, Waltham, MA). CD4, CD8, CD14, CD19 and plasmacytoid dendritic cells (pDC) were separated or depleted from PBMC by positive selection with magnetic microbeads (Miltenyi) according to the manufacturer's instructions. The CD303/BDCA-2 kit was used for pDC depletion. "CD19–CD8–" cells represent column flow through from sequential CD19+ and CD8+ cell

removal. Cell fraction purification studies were carried out on ice with ice-cold solutions to minimize effects on gene expression. For RV stimulations, all primary cells were maintained in DMEM supplemented with 10% FBS (not heat-inactivated; Invitrogen) and antibiotic-antimycotic solution. For plasmid transfection studies, THP1 cells were transiently transfected with plasmids of control-GFP and ORMDL3-GFP (GeneCopoeia, Rockville, MD) using AMAXA electroporation (Lonza, Walkersville, MD) as follows: 4×10^6 cells were combined with 5 μ g DNA in 100 μ L Nucleofector solution (Kit V) and then electroporated using program Y-006 one day prior to stimulation with virus. HRV16A was purified by sucrose gradient and added at the concentration of 2–10 pfu per cell for 24 hrs unless otherwise indicated. For the untreated cells, a vehicle control was added for the same duration. IFN- β protein was detected in supernatant using ProcartaPlex Human IFN-beta Simplex kit (eBioscience, San Diego, CA). The magnetic anti-IFN- β beads were detected on a Luminex 100 machine (Austin, TX). 12-well 0.4 μ M pore size transwell dishes were obtained from Corning (Toledo, OH). These pores are large enough for diffusion of 30 nm rhinovirus. For “co-culture” conditions, CD19+ B cells were positively isolated from PBMC using Miltenyi magnetic beads according to the manufacturer’s directions. 1×10^6 CD19+ cells were placed in the insert with 12×10^6 CD19– PBMC (flow-through) in the well below. The same amount of total RV was used for transwell and whole PBMC stimulations.

Quantitative PCR

Cells were lysed with TRIzol (Invitrogen, Waltham, MA) and processed according to the manufacturer’s instructions. Briefly, RNA was extracted with chloroform and precipitated with isopropanol. RNA quality (260/280 ratio) and quantity was assessed by NanoDrop 2000 (Thermo Scientific, Wilmington, DE). Total RNA was treated with DNase I (Invitrogen) prior to reverse transcription using an Iscript kit (Bio-Rad, Hercules, CA). Gene expression in cDNA was quantitated by SYBR Green (Bio-Rad, Hercules, CA) fluorescence detected on a CFX96 machine (Bio-Rad). Reaction efficiency was assessed using a serially diluted standard curve and product by melting curve. Relative mRNA expression was normalized to the 18S rRNA housekeeping gene using the standard Ct/Ct method. Primers were designed using Beacon design software (Premier Biosoft, Palo Alto, CA): 18S rRNA, F:5’-GGACACGGACAGGATTGACAG3; R:ATCGCTCCACCAACTAAGAACG; *IFNB1*, F: TGGCTAATGTCTATCATCA; R: CTTCAAGTTTCGGAGGTAA; *HSPA5*, F:GTGGAATGACCCGTCTGTG; R:TTGGTTGCTTGGCGTTGG; *CHOP*, F:CTAGTGCCAATGATGTGA; R:ATATACAAGCTGAGACCTT; *CXCL10*, F:GCT GCT ACT ACT CCT GTA; R:GGT CAG AAC ATC CAC TAA G; *ORMDL3*, F:ATCCCGTTTTGTGAGTGTC; R: CTTAGTGTAGAAGCTGGTGAGG; spliced *XBPI*, F: 5’-TGCTGAGTCCGCAGCAGGTG-3’, R:5’-GCTGGCAGGCTCTGGGGAAG-3’.

Western blotting

Cells were lysed with RIPA buffer containing protease and phosphatase inhibitor cocktails (Sigma-Aldrich, St. Louis, MO), and whole-cell lysates resolved by 12% SDS-PAGE. Samples were transferred to polyvinylidene difluoride membrane (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) and immunoblotted with primary Abs anti-ORMDL3 Ab (Millipore, Bedford, MA), anti- β -Actin Ab (Santa Cruz, Dallas, TX),

followed by a fluorescence-conjugated secondary Ab (Licor, Lincoln, NE). Proteins were visualized with the Odyssey system (Licor).

Statistical analysis

Gene expression in different cell types and comparisons of genotype differences (CC vs TT at rs7216389) with or without RV treatment were assessed using two way repeated measures ANOVA with treatment, genotype and their interactions as fixed effects. Different PBMC cell types purified before or after simulating with either RV or vehicle control were compared using one way repeated measures ANOVA. All responses were log transformed to better approximate normal distributions. Associations between the expressions of each gene were assessed using Pearson's correlation coefficient in a log transformed scale. $P < 0.05$ was the criterion for statistical significance. Analyses were performed in SAS version 9.4 (SAS Institute, Cary, NC). Error bars in bar graphs denote standard errors of the mean. In all figures, * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$ unless otherwise indicated.

Results

ORMDL3 enables RV upregulation of *HSPA5* and type I IFN gene expression in human monocytes

The UPR encompasses three major signaling cascades initiated by activation of ER resident proteins inositol requiring kinase 1 (IRE1), PKR-like endoplasmic reticulum kinase (PERK) and activating transcription factor (ATF6). IRE1 is both a signaling kinase and an endonuclease that splices the *XBP1* mRNA to generate the active XBP1 transcription factor. PERK phosphorylates eIF2 α , resulting in transient global translational attenuation, apart from specific target genes. Triggering of the UPR also activates the ATF6 transcription factor. Together, these three pathways regulate the transcription of UPR target genes aimed at restoring ER homeostasis [24, 25]. Interestingly, although one study has linked ORMDL3 to eIF2 α phosphorylation (PERK pathway) and BiP induction (ATF6 target), another group has reported unique activation of the ATF6 pathway by ORMDL3 over-expression [7, 9, 14].

The effect of RV stimulation on the UPR was initially assessed in human cell lines: Bjab (non-EBV transformed B cells) and monocytic THP-1 cells were incubated with virus and then activation of the three UPR axes was monitored via the expression of pathway-specific targets: spliced *XBP1* mRNA for the IRE1 pathway, *HSPA5* (encoding BiP/GRP78) for ATF6, and *CHOP* for the PERK pathway [25, 26]. In Bjab B cells RV stimulation induced *IFNB1* and *CXCL10* mRNA, and also upregulated *ORMDL3* and *HSPA5* expression (Fig. 1). Spliced *XBP1* was not significantly up regulated, and *CHOP* expression was unchanged with 2pfu (Fig. 1) and inhibited by 10pfu (Supplemental Fig. S1).

In THP1 monocytes, RV induced expression of spliced *XBP1*, but not *IFNB1* or other UPR target genes (Fig. 2, open bars). As native ORMDL protein was not readily detectable in THP1 monocytes, these cells were transfected with an ORMDL3 expression plasmid. Following ORMDL3 transfection, RV stimulation of THP1 cells increased expression of *HSPA5* and *IFNB1*, but not spliced *XBP1* or *CHOP*. Transfected ORMDL3 also resulted in higher IFN- β production (Supplemental Fig. S2). Together these results suggest that RV

induces cell type specific IFN- β and UPR responses, and that *ORMDL3* expression enhances RV-induced upregulation of *HSPA5* and *IFNB1* expression.

***ORMDL3* expression and *HSPA5* upregulation varies in different primary human leukocyte cell types**

Caliskan et al. described a 2-fold increase in *ORMDL3* expression following RV stimulation of PBMC, a mixed cell population [4]. Recent analysis of *ORMDL3* and *GSDMB* in different unstimulated PBMC cell types revealed elevated gene expression in CD8+ and possibly CD19+ cells, respectively [6]. One study in a mouse model described high *ORMDL3* expression in eosinophils [27]. Therefore, to determine which primary human leukocyte cell types upregulate *ORMDL3* expression after RV stimulation, we incubated eosinophils or PBMC with RV, and then isolated CD19 B cells, CD8 T cells, or CD8-CD19- (mostly monocytes, CD4 T cells, and fewer NK cells and rare dendritic cells) cell fractions. *ORMDL3* expression was highest in eosinophils (Fig. 3) for both untreated and RV stimulated cells, with untreated mean levels 4.7-11 fold higher than other leukocyte populations ($p < 0.001$). Eosinophil *ORMDL3* mRNA was also >18-fold higher than that observed in monocytes (Fig. S3). These differences in mRNA levels correlated with elevated ORMDL protein detected by Western blot. Currently available “ORMDL3” antibodies also recognize ORMDL1 and ORMDL2 related to the high homology between these proteins. However, *ORMDL1* and *ORMDL2* mRNA were not readily detected in freshly isolated human eosinophils (data not shown). A previous report also described greater *ormdl3* expression in murine eosinophils compared to *ormdl1* and *ormdl2*, so the detected band in eosinophils most likely represents primarily ORMDL3 [14]. B cell and CD8 T cells also expressed more *ORMDL3* mRNA than either whole PBMC ($p = 0.02$ to < 0.001) or the CD19-CD8- ($p < 0.001$) populations. RV induced *ORMDL3* expression in all leukocyte cell types (ranging from 1.7-2.0 fold increase ($p = 0.001$)) except eosinophils. Eosinophils failed to upregulate *ORMDL3* expression in response to RV, despite a modest induction of the UPR indicator *HSPA5*, and comparable upregulation of *CHOP* (1.6-2.5 fold increase in all leukocytes, $p = 0.002$). Likewise, eosinophils responded with much less *CXCL10* induction (4.1-fold, $p = 0.01$) compared to other cell types (130-149-fold increase in CD19+ and CD8+ cells and 54-79 fold in PBMC and CD19-CD8- cells respectively, $p < 0.001$). In response to RV, *HSPA5* upregulation was greatest in B-cells (mean 2.9-fold, $p < 0.001$) and greater than in other cell types, particularly PBMC and CD19-CD8- populations ($p < 0.001$), which did not significantly upregulate *HSPA5*. Thus, the cells that increased *ORMDL3* expression in response to RV tended to also upregulate *CXCL10* and *HSPA5*. Furthermore, in PBMC, CD8, and CD19 B cells, RV-induced *HSPA5* gene expression significantly correlated with RV-stimulated *ORMDL3* gene expression (Fig. 4). RV-induced *CXCL10* expression also correlated with *ORMDL3* expression in CD8 T cells and *HSPA5* expression in CD8 T cells, PBMC and CD8-CD19- cells (Fig. S4).

RV upregulation of ORMDL3 in primary human leukocytes requires cell-cell contact and the presence of plasmacytoid dendritic cells (pDC)

In contrast to transformed cell lines, or B cells isolated following RV stimulation of whole PBMC, isolated primary human B cells failed to upregulate ORMDL3 in response to RV (Fig. 5A). This suggested that either a soluble factor generated during whole PBMC

stimulation or cell-cell contact between B cells and other cells was required. Previous studies had suggested STAT6 (induced by IL-4 or IL-13 signaling) was sufficient for *ORMDL3* regulation [14, 28]. However, incubation of PBMC with exogenous IL-4 or IL-13 failed to induce *ORMDL3* expression (Fig. S5). Increasing the cell number per well (and thereby cell-cell contact) during RV stimulation enhanced *ORMDL3* upregulation (Fig. 5B). To more directly address the need for cell-cell contact, transwell assays were performed in which the B cells were present in one compartment, PBMC in another, and soluble factors (such as cytokines and RV) could freely diffuse (Fig. 5C). B cells co-cultured with PBMC by transwell did not significantly upregulate *ORMDL3* or *HSPA5* expression. Further, maximal induction of *HSPA5* and *CXCL10* expression required whole PBMC culture. These results indicate a requirement for cell-cell contact.

To determine which cell types in PBMC were critical for *ORMDL3* upregulation by RV, monocytes (Fig. 6A), CD4, CD8 T cells or both T cell subsets (Fig. 6B), or plasmacytoid dendritic cells (pDC) were depleted (Fig. 6C) prior to stimulation with RV. Only depletion of pDC abrogated the otherwise reliable 2-fold increase in RV-induced *ORMDL3* expression. pDC were required but not sufficient for *ORMDL3* upregulation, as isolated B cells incubated with pDC did not increase *ORMDL3* (data not shown), suggesting the need for a more complex cell-cell interaction during the PBMC cultures.

17q21 genotypic differences in RV-induced *ORMDL3* and *HSPA5* are most pronounced in CD19+ B cells

The *ORMDL3* transfection studies, transwell assays and cell depletion studies supported an association between *ORMDL3* upregulation, *HSPA5* and type I IFN gene expression. We next determined whether 17q21 genotype affects *ORMDL3* expression and upregulation of UPR genes and type I IFN in response to RV. Multiple asthma-associated SNPs in 17q21 display significant linkage disequilibrium [4, 29]. As the rs7216389 SNP has been reported to correlate with differential *ORMDL3* expression in whole PBMC, rs7216389 was chosen as a surrogate genotypic marker [4]. The homozygous CC and TT genotypes were chosen to maximize observable differences in a small sample size. Participant characteristics for each genotype are described in Table 1. There was a non-significant trend towards lower age of onset in the TT genotype. *ORMDL3* expression levels were higher in both untreated PBMC and B cells with the asthma risk TT genotype. Following RV-stimulation, the TT genotype was associated with increased *ORMDL3* expression in all PBMC cell fractions (Fig. 7A). The differences in *ORMDL3* expression between CC and TT subjects were most striking and significant ($p = 0.004$) in the CD19+ B cell subset, with or without RV stimulation. Interestingly, only RV-stimulated B cells showed increased *HSPA5* expression in TT cells compared to CC cells ($p = 0.03$, Fig 7B); although there were trends towards increased RV-stimulated *HSPA5* in TT vs. CC PBMCs and CD19-CD8- cells ($p = 0.08$ and 0.09 respectively). *CHOP* and *CXCL10* expression induction did not differ between genotypes (Fig. S6) and PBMCs failed to increase *XBPI* splicing in response to RV (not shown). However, RV-induced *IFNB1* expression in PBMCs was significantly higher in cells with the asthma risk-associated TT genotype (Fig. 7C).

Discussion

Genotype at the 17q21 locus is strongly linked to childhood asthma, especially in children who experience an RV wheezing episode during the first three years of life [4]. The goal of our study was to investigate cell type-specific relationships between RV infection and 17q21 genotype, *ORMDL3* expression, and the UPR. Our results support the hypothesis that *ORMDL3* regulates both ATF6 pathway activity and type I IFN responses to RV in specific blood leukocytes. In monocytic cells, exogenous *ORMDL3* synergized with RV to induce *HSPA5* and *IFNB1* gene expression. Furthermore, RV coordinately induced *ORMDL3* and *HSPA5* expression in PBMC and lymphocytes. Cells with the rs7216389 TT genotype at the 17q21 locus expressed higher levels of *ORMDL3* expression and more RV-induced *HSPA5* and *IFNB1* mRNA. Finally, cell to cell contact appeared to be required for both *ORMDL3* and *HSPA5* upregulation in PBMCs.

The requirement for cell-cell interaction for RV-induced upregulation of *ORMDL3* expression in PBMC contrasts with the sufficiency of STAT6-dependent cytokine stimulation reported in murine and other *in vitro* systems [14]. In particular, the cell type depletion studies suggest that virus-responsive IFN-producing pDC are critical for upregulating *ORMDL3* expression (Figure 6). Moreover, the results of the transwell assays suggest that direct interaction with pDC rather than soluble factors (e.g. IFNs) are necessary to upregulate *ORMDL3* expression upon exposure to RV. The requisite role that pDC are playing in *ORMDL3* regulation will require further study.

Cell-specific patterns of *ORMDL3* expression were highly variable and may provide clues that link this gene to asthma pathogenesis. Our results examining *ORMDL3* expression in different leukocyte populations are consistent with a previous study showing higher expression of this gene in CD8 T cells and B cells and lower expression in monocytes [6]. The strikingly high levels of *ORMDL3* mRNA and *ORMDL* protein in human eosinophils had not been previously reported, and suggest the possibility that *ORMDL3* might affect eosinophil inflammatory functions. In this study, we found that B cells had the greatest genotype-specific differences in *ORMDL3* expression, in both untreated and RV-stimulated cells. B cells have been implicated in asthma exacerbations, asthma severity, and are key effector cells in allergic diseases through the production of allergen specific-IgE [30–32]. Although the 17q21 genotype has not been associated with allergic sensitization, it has recently been associated with allergic rhinitis, suggesting a link between this locus and allergic disease in the presence of asthma [4, 33].

Our studies also demonstrate that RV infection can induce the UPR in a cell-type specific manner. An increasing body of literature has described both induction and inhibition of the UPR by viral infections [13, 34]. Viruses may utilize the pro-survival anti-apoptotic arms of the UPR, as well as increased ER capacity to enhance viral replication in otherwise stressed cells. On the other hand, the transient translational inhibition that decreases protein production during the UPR could hamper viral replication. Results from this study suggest that depending upon cell type (e.g. monocytes vs B-cells or PBMC) RV stimulation may induce all three arms of the UPR, as evident from increases in *HSPA5* (encoding BiP), *CHOP*, and spliced *XBPI* mRNA. In primary human cells, *HSPA5* induction by RV

stimulation was particularly sensitive to cell type, being greatest in CD19 B cells. One possible explanation is that B cells are already “primed” to be ER-stress sensitive, given the central role of the UPR in both the generation and differentiation of B-cells into plasma cells. However, most studies have pointed to XBP1 and the IRE1 pathway in B cell development and function, rather than the ATF6 pathway [35, 36]. As another possibility, one study has suggested that RV replicates and produces infectious virions in B-cells, in contrast to other circulating blood cell types. Viral replication may thus increase ER stress in B cells [19].

To date, the mechanisms by which ORMDL3 influences ER stress are not entirely clear. ORMDL3 has been reported to induce ER calcium depletion and thus ER stress via blockade of the SERCA pump, as seen with the well-known pharmacologic UPR inducer and SERCA inhibitor thapsigargin [9, 37]. Although thapsigargin was first described as an inducer of BiP, one would predict an effect on all three UPR axes [38]. In the Bjab B cells, THP1 monocytes and genotyped primary cells used in this study, *ORMDL3* induction by RV was primarily associated with *HSPA5* expression, and not increases in RV-induced spliced *XBP1* or *CHOP*. These results are consistent with previous studies in the murine system whereby over-expressed ORMDL3 primarily influenced ATF6 activation [7, 14]. The discrepancies with reports supporting a wider effect of ORMDL3 on UPR may reflect differences in experimental system (e.g. stimulus and cell type or species) [9].

It is not clear if the induction of the type I IFN gene *IFNB1* and *HSPA5* are separate or related outcomes of RV stimulation. Our previous studies have described synergistic augmentation of type I IFN genes by the UPR, both through XBP1 binding of an *Ifnb1* enhancer as well as activation of IRF3, a transcription factor essential for IFN- β expression [10, 39]. Further, blockade of ATF6 activation prevented IRF3 phosphorylation by pharmacologic UPR inducers tunicamycin and 2-deoxyglucose [39]. Another study has also implicated ATF6 in the activation of the pro-inflammatory transcription factor NF- κ B, which enhances IFN- β production [40]. Thus, increased ATF6 pathway activation could directly impact RV-induced *IFNB1* expression.

Currently, it is unclear how *increased* IFN- β would contribute to asthma. Indeed, one trial suggested IFN- β may ameliorate virus-induced exacerbations, especially in individuals with more severe asthma [41]. On the other hand, both children and adults who wheeze in response to virus display increased interferon responses, and it is possible that exuberant interferon responses add to airway inflammation and obstruction [42, 43]. Subject phenotype, timing, and magnitude of IFN production in response to viral infections may be critical. Alternatively, ER stress augments the production of other pro-inflammatory cytokines, including TNF- α and IL-23 [11, 12]. These pro-inflammatory cytokines could promote the neutrophilic inflammation seen in acute exacerbations of asthma.

One limitation to this study was the use of cells from adult subjects with allergies or mild asthma. The enrichment for adults with allergies and asthma facilitated procurement of eosinophils, which are relatively rare cell types in peripheral blood from non-allergic individuals. However, these characteristics of our sample limit the generalizability of our results, which will require validation in healthy subjects and in children. Although our small

sample sizes may have reduced the power to detect some differences, genotype-specific differences in *ORMDL3* expression were highly significant, particularly in CD19+ B cells, attesting to the robust and significant effects of 17q21 genotype on *ORMDL3* expression. Another caveat to interpretation comes from our use of positive selection to obtain multiple cell fractions. Positive selection could inhibit responses to RV. However, untreated populations isolated before (“pre”) or following PBMC culture (“post”) did not display any differences in *ORMDL3* expression (Figure 5A). Also, 17q21 genotype comparisons (Figure 7) and the associations between differing levels of *CXCL10*, *ORMDL3* and *HSPA5* expression (Figure 4 and S4) were all from similarly treated cell populations, so positive selection should not have been an issue for these results.

In summary, our work describes a striking association between elevated *ORMDL3* expression and increased *HSPA5* and type I IFN production in response to RV. Together with other published studies, our results support an association between the risk genotype at the 17q21 asthma locus and elevated *ORMDL3* expression [1, 4, 6, 29, 44]. Our results suggest that immune cells with higher *ORMDL3* (the 17q21 risk genotype) respond to RV with augmented ER stress (at least ATF6 pathway activation) and *IFNB1* expression. The link between ER stress and type I IFN may be direct, for instance through increased activation of the IFN- β regulatory transcription factor IRF3, or indirect. One implication of our results is that increased ER stress could promote increased IFN responses or other pro-inflammatory consequences that predispose to the development of asthma. Alterations in ER stress and type I IFN production may be of particular importance during early childhood, when exposure to viruses is high and anti-viral immunity has not yet matured. Future studies teasing apart the mechanistic relationships between ER stress, *ORMDL3* expression, and type I IFN responses to RV are clearly warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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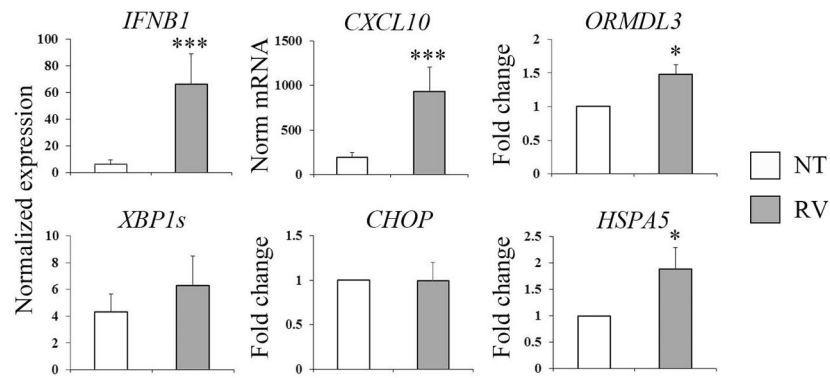


Figure 1. RV induces type I IFN, *HSPA5* and *ORMDL3* gene expression in Bjab cells

B cells were stimulated with RV (gray bars) or untreated (NT, open bars). RNA was reverse transcribed and examined for gene expression by quantitative PCR (qPCR). 18S normalized mRNA or fold changes (NT=1) are shown (note different y-axes). N=7 independent experiments for *IFNB1*, 6 for *HSPA5* and *ORMDL3*, 5 for *CHOP* and 3 for *CXCL10*. *p 0.02 comparing geometric mean expression.

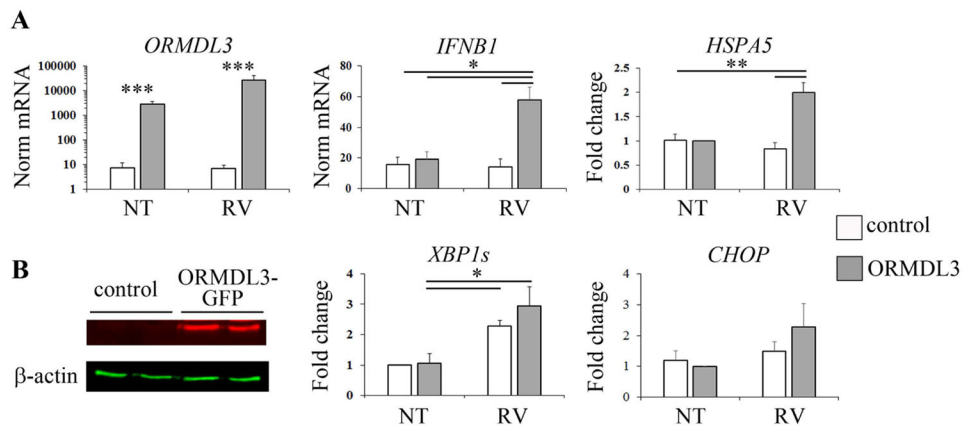


Figure 2. Synergistic *IFNBI* and *HSPA5* expression in ORMDL3 over-expressing monocytes stimulated with RV

THP-1 monocytes were transfected with a control GFP plasmid (control, open bars) or plasmid containing ORMDL3-GFP (gray bars). A) Transfectants were untreated or stimulated with RV as indicated, and then RNA reverse transcribed and analyzed by qPCR for gene expression. Expression was normalized to the 18S housekeeping gene as indicated. Fold change is compared to unstimulated (NT) ORMDL3 transfected cells or control unstimulated cells. N=6 independent experiments for *IFNBI* and *CHOP*, 5 for *ORMDL3*, 4 for *HSPA5* and 3 for *XBP1s*. B) Whole cell lysates (duplicate samples) from control plasmid or ORMDL3-GFP transfectants were resolved by SDS PAGE and blots probed with antibodies specific for actin or ORMDL as indicated.

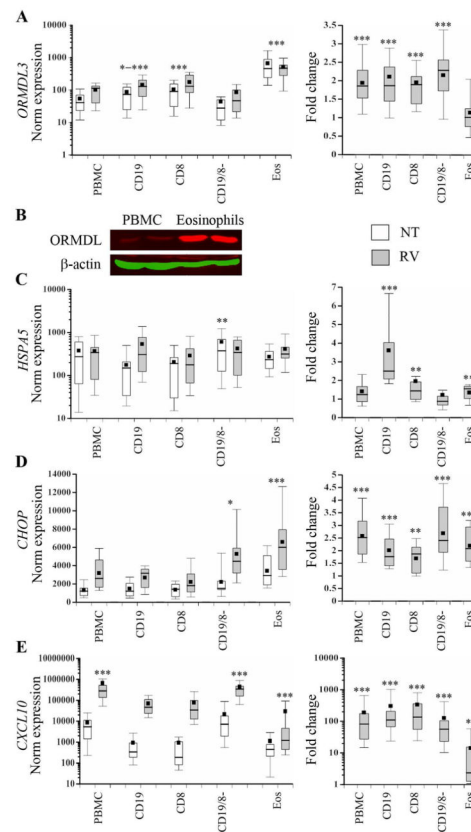


Figure 3. *ORMDL3*, *HSPA5*, *CHOP* and *CXCL10* gene expression in primary leukocytes
 A, C–E) Eosinophils or PBMC were unstimulated (NT, open bars) or stimulated with RV (gray bars). After PBMC stimulation, CD19+ (B cells), CD8+ (T cells) or CD19–CD8– (CD19/8–) fractions were isolated prior to RNA extraction. Gene expression was analyzed by qPCR and normalized to 18S (left graphs) and further normalized to NT for ratio of stimulation (Fold change, right graphs). Standard box plots denote 25th percentile, median and 75th percentiles with whiskers at 10th and 90th percentiles and black boxes indicating means. For *ORMDL3*, *HSPA5* and *CXCL10*, data are from 19 donors (PBMC, CD19, CD8), 17 donors (CD19–CD8–) or 12 donors (eosinophils). For *CHOP*, data are from 11 donors. B) Western blots of whole cell lysates from PBMC or eosinophils (duplicate samples) probed with fluorescence-tagged antibodies specific for β –actin or *ORMDL* as indicated. Results are representative of 3 experiments showing elevated *ORMDL* protein in eosinophils. A) *ORMDL3* expression: CD19 vs. PBMC *p 0.02, CD19 vs. CD19–CD8– ***p<0.001. CD8s vs both PBMC and CD19–CD8– ***p<0.001. Eosinophils vs. all others, ***p<0.001. C) *HSPA5* expression: untreated CD19–CD8– vs. other cell types, **p 0.003. D) *CHOP* expression: *p<0.03 comparing CD19–CD8– vs. PBMC, CD19 and CD8. ***P<0.001 for eosinophils vs PBMC, CD19 and CD8. E) *CXCL10* expression: ***p<0.001 for PBMC and CD19–CD8– vs. CD19, CD8 and Eos. Eosinophil expression lower than other cell types ***p<0.001. Fold change asterisks refer to comparisons of RV stimulated and untreated samples for those cell fractions.

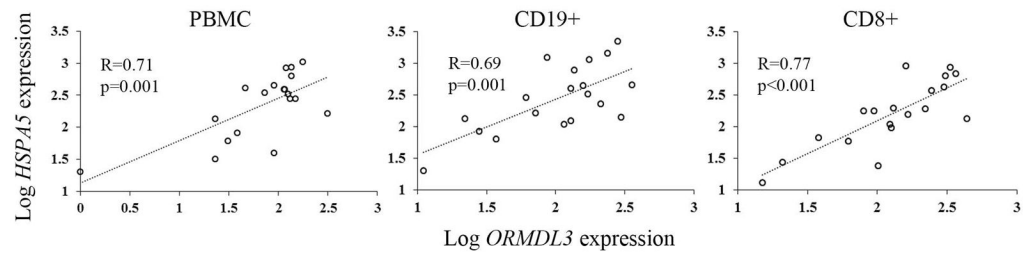


Figure 4. Correlation of RV-induced *ORMDL3* with BiP gene expression

CD19+ and CD8+ cells were isolated from PBMC following stimulation with RV. Gene expression was determined by qPCR. Statistical evaluation of association was performed on log transformed data as shown. Data are from 19 donors.

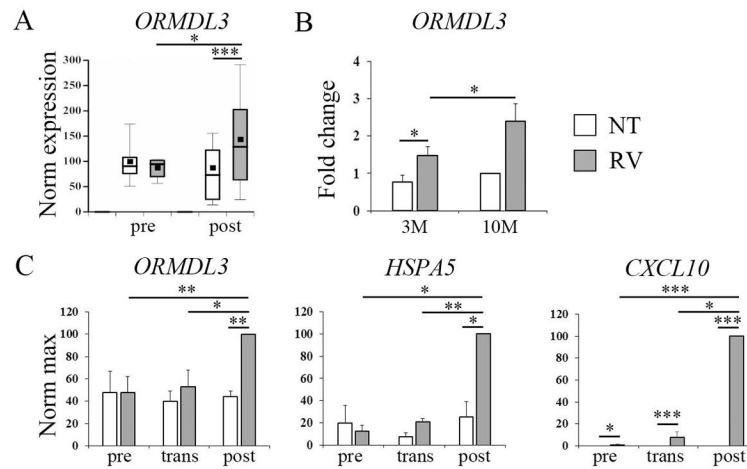


Figure 5. Upregulation of *ORMDL3* by RV in B cells requires cell-cell contact

A) B cells were isolated prior to stimulation (pre), or after PBMC stimulation (post) with media (NT, open bars) or RV (gray bars) for 24h. Gene expression was examined by qPCR with normalization to 18S. Data is from 6 pre donors and 19 post donors. * $p=0.02$, *** $p<0.001$. B) PBMC were incubated with 2 pfu/cell RV for 24h at a concentration of 3×10^6 cells per well (3M) or 10×10^6 cells per well (10M). Fold change *ORMDL3* (vs. 10M non-treated) expression was determined by qPCR. $N=5$ independent experiments (5 donors). * $p=0.04$. C) B cells were isolated prior to stimulation (pre), incubated in a transwell with PBMC (trans), or isolated following stimulation with RV (post). RNA expression was normalized to post stimulation (=100). Data are from 4–5 independent experiments. *ORMDL3* * $p=0.01$, ** $p=0.005$; *HSPA5* * $p=0.04$, ** $p=0.005$; *CXCL10* * $p=0.02$, *** $p<0.001$.

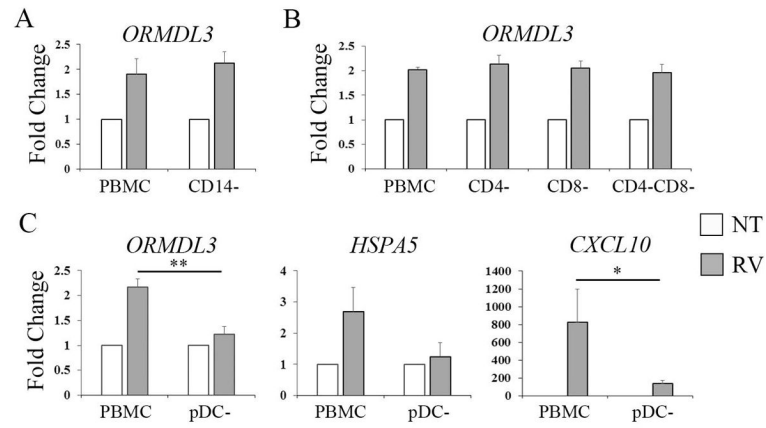


Figure 6. RV-induced *ORMDL3* in PBMC requires pDC

Individual populations of cells including CD14 monocytes (A), T cells (B) and pDC (C) were depleted from PBMC prior to stimulation with RV. Gene expression was determined by qPCR with normalization to unstimulated (NT). A) N=4 experiments. B) N=3 experiments. C) N= 6 experiments for *ORMDL3* and *HSPA5* and 4 for *CXCL10*. *p=0.03, **p=0.003.

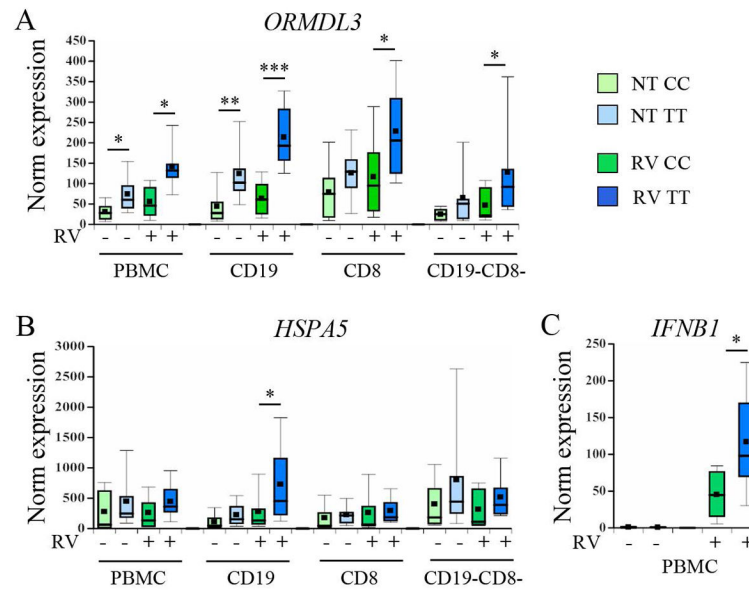


Figure 7. 17q21 genotypic differences in *ORMDL3*, *HSPA5* and *IFNB1* gene expression
 PBMC from donors with CC (low risk) or TT (high risk) genotypes at the 17q21 locus were stimulated with media (pale boxes) or RV (dark boxes) prior to isolation of different cell subsets. Gene expression was determined by qPCR with normalization to 18S. CC genotypes are green and TT are in blue. For *ORMDL3* and *HSPA5* expression, PBMC, CD19 and CD8 cells were obtained from 9 CC and 10 TT donors. CD19-CD8- data are from 9 CC and 8 TT donors. For *IFNB1*, PBMC data were from 9 CC and 8 TT donors. A) *ORMDL3* *p 0.03, **p=0.004, ***p<0.001. B) *HSPA5* and C) IFN- β *p=0.03.

Table 1
Demographics and clinical characteristics of genotyped study participants

CC and TT represent genotypes at the childhood asthma-associated SNP rs7216389. SD=standard deviation.

Characteristics	CC (n=9)	TT (n=10)	p-value
Sex (% male)	33	30	0.80
Race (% White, Non-Hispanic)	89	100	0.47
Asthma (%)	67	90	0.30
Age (mean years (SD))	43.9 (10.3)	43.9 (10.7)	1.00
Age asthma onset (mean years (SD))	25.3 (16.8)	14.1 (10.6)	0.15

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