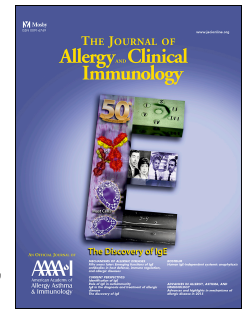


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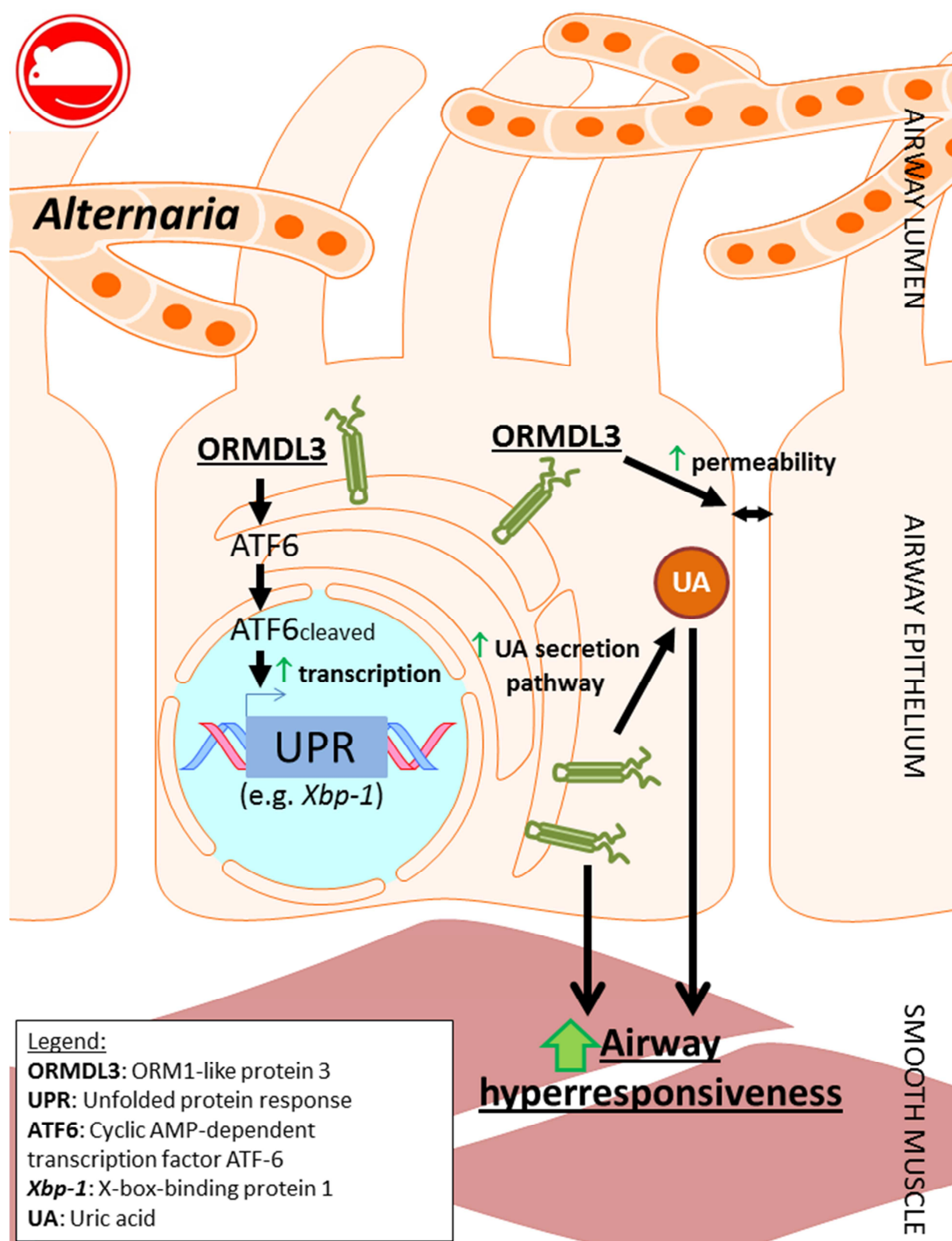
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Pulmonary ORMDL3 is critical for induction of *Alternaria* induced allergic airways disease

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Key Messages:

In response to fungal exposure *Ormdl3* mediates the ATF6 dependent pathway of the unfolded protein response and uric acid release.

The cellular stress promoted by *Ormdl3* orchestrates airway hyperresponsiveness during allergic immune responses.

Epithelial ORMDL3 is crucial for the induction of airway resistance.

Capsule Summary:

ORMDL3, an asthma susceptibility gene identified by GWAS, promotes cellular stress following fungal exposure and orchestrates airway hyperresponsiveness during allergic immune responses. Modulation of cellular stress may be a novel therapeutic avenue for asthma treatment.

Key Words:

ORMDL3, asthma, unfolded protein response, uric acid, *Alternaria*

Abbreviations:

Abbreviations: ORMDL (ORM1-like protein); GWAS (genome wide association study); AHR (airway hyperreactivity); SNP (single nucleotide polymorphism); ER (endoplasmic reticulum); UPR (unfolded protein response); KO (knockout); WT (wildtype); AAD (allergic airways disease); ILC2 (type 2 innate lymphoid cell); AAV (adeno-associated viral vector); EGFP (enhanced green fluorescent protein); BALF (bronchoalveolar lavage fluid). BiP (binding immunoglobulin protein); DAMP (damage associated molecular pattern); lactate dehydrogenase (LDH); ATF (activating transcription factor); IRE (inositol requiring ER-to-nucleus signal

54 kinase); PERK (double-stranded RNA-activated protein kinase (PKR)-like endoplasmic
55 reticulum kinase); *Ddit3* (DNA-damage-inducible transcript 3); *Xbp1* (X-box binding protein 1);
56 *Edem1* (ER degradation enhancer, mannosidase alpha-like 1); CHOP (CCAAT/enhancer-binding
57 protein-homologous protein); cAMP response element (CRE); CREB (cAMP response element
58 binding protein); CDHR3, cadherin regulated family member 3; SERCA2b (sarco-endoplasmic
59 reticulum Ca²⁺ ATPase 2b); ERAD (endoplasmic-reticulum-associated protein degradation).

Abstract:

Background: Genome-wide association studies have identified the *ORMDL3* (ORM (yeast)-like protein isoform 3) gene locus on human chromosome 17q to be a highly significant risk factor for childhood-onset asthma. **Objective:** We sought to investigate *in vivo* the functional role of *ORMDL3* in disease inception. **Methods:** An *Ormdl3* deficient mouse was generated and the role of *ORMDL3* in the generation of allergic airways disease to the fungal aeroallergen *Alternaria alternata* determined. An adeno-associated viral vector was also utilized to reconstitute *ORMDL3* expression in airway epithelial cells of *Ormdl3* KO mice. **Results:** *Ormdl3* knock-out mice were found to be protected from developing allergic airways disease and showed a marked decrease in pathophysiology, including lung function and airway eosinophilia induced by *Alternaria*. *Alternaria* is a potent inducer of cellular stress and the unfolded protein response and *ORMDL3* was found to play a critical role in driving the ATF6 mediated arm of this response through *Xbp1* and downstream activation of the endoplasmic reticulum-associated degradation pathway. Additionally *ORMDL3* mediated uric acid release, another marker of cellular stress. In the knockout mice, reconstitution of *Ormdl3* transcript levels specifically in the bronchial epithelium resulted in reinstatement of susceptibility to fungal allergen-induced allergic airways disease. **Conclusions:** This study demonstrates that *ORMDL3*, an asthma susceptibility gene identified by genome-wide association studies, contributes to key pathways that promote changes in airway physiology during allergic immune responses.

Introduction

Asthma encompasses a range of pulmonary disease phenotypes which are commonly defined by a type 2 inflammatory response to airborne allergen concomitant with airway hyperresponsiveness (AHR).¹ Although the underlying aetiologies for asthma are incompletely understood, it is recognized that the epithelial barrier plays a central role in asthma pathogenesis and that genetic predisposition can be pivotal for asthma development.² A pioneering genome wide association study (GWAS) identified single nucleotide polymorphisms (SNPs) on chromosome 17q21 which were strongly linked to asthma.³ The same SNPs were also found to be associated with increased expression of transcripts of the *ORMDL3* (*ORM-1 like protein 3*) gene.³ However, the functional role of *ORMDL3* in asthma has not as yet been fully elucidated and it is not known how this molecule contributes to disease pathophysiology.

ORMDL3 is expressed in various tissues, including the lung where it is predominantly localised to airway epithelial cells in response to *Alternaria* or ovalbumin challenge.^{4, 5} It is a transmembrane protein located in the endoplasmic reticulum (ER)⁴ where physiologically it acts to negatively regulate sphingolipid synthesis.⁶ Intriguingly, it has been demonstrated that reduction of sphingolipid synthesis with pharmacological compounds increases AHR in mice and human bronchial tissue.⁷ However, in a murine model of house dust mite induced allergic airways disease elevated levels of *ORMDL3* in response to allergen correlate with increased ceramide production.⁸ Prior studies with mice universally overexpressing *ORMDL3* have demonstrated that these mice spontaneously develop increased AHR and airway remodeling which precedes elevated type 2 pulmonary inflammation.⁹

ER stress can be induced upon perturbation of luminal Ca^{2+} levels within the ER consequently decreasing efficiency of correct protein folding.¹⁰ Accumulation of nascent or mis-folded protein

in the ER lumen leads to activation of the unfolded protein response (UPR).^{11, 12} Available data indicate that ORMDL3 modulates the UPR although the pathways involved may be tissue specific. Bone marrow derived macrophages from *Ormdl3* transgenic mice have increased activation of the ATF6 pathway of the UPR,⁹ similar to previous observations utilising lung epithelial A549 cells transfected with *ORMDL3 in vitro*.⁵ In these systems expression of ORMDL3 is positively correlated with expression of SERCA2b (sarco-endoplasmic reticulum Ca^{2+} ATPase 2b), a downstream target of the ATF6 pathway¹³. In HEK293 and Jurkat cells transfected with *Ormdl3* the data illustrate that ORMDL3 binds to and inhibits SERCA thereby impeding entry of cytosolic Ca^{2+} into the ER lumen and initiating the UPR.¹⁴ However, within this experimental setting the main impact of ORMDL3 is on the PERK/eIF2 α arm of the UPR.

In the present study *Ormdl3*-deficient mice were generated in order to investigate the role of ORMDL3 in determining the pulmonary response to allergen. In addition, we have reconstituted ORMDL3 specifically in the bronchial epithelium of these knockout (KO) mice to ascertain the specific contribution of epithelial ORMDL3. These mice reveal that ORMDL3 is pivotal in the generation of fungal allergen-induced AHR via modulation of cellular stress pathways. Our data reinforce the findings of asthma GWAS, placing ORMDL3 as an important mediator in the development of allergen induced AHR.

Materials and Methods

Generation of *Ormdl3* KO mice

Two separate *Ormdl3* alleles were utilized in the study. The first was generated by Dr Y Zhang at the Mary Lyon Centre, MRC Harwell and the second was made and kindly donated by Merck Research Laboratories. Both constructs contained LoxP sites flanking Exons 1-4 of *Ormdl3*; the translation initiation site of the gene being contained within Exon 2. Initially, conditional alleles were generated by Flp-mediated removal of selection cassettes. The targeting strategies for both lines were similar however the Merck construct contained dual Neo and Puro selection cassettes, whereas the Harwell allele contained a single Neo selection cassette (Fig. S1). At Merck targeting constructs were micro-injected into ES cells from C57/Bl6NTac mice and complete *Ormdl3* knockouts were subsequently generated by Cre-mediated recombination, using globally expressed Cre-driver mouse lines¹⁵. At Harwell targeting constructs were micro-injected into ES cells from R1 129 ES cells and *Ormdl3* knockouts were subsequently generated by Cre-mediated recombination, using Tg(ACTB-cre)3Mrt mice (Infrafrontier; accession number EM:06107). These *Ormdl3* KO mice were subsequently crossed on to a C57BL/6J background (10 generations). A similar response to allergen was observed in both strains. The MERCK *Ormdl3* KO line (*Ormdl3* KO^{Mer}) was used for experiments shown in figures 1-4, while the experiments in figure 6 and supplementary figure 2, which required in-house breeding at Imperial College, were performed using the *Ormdl3* KO line generated by Dr Y Zhang (*Ormdl3* KO^{Har}).

Construction of *EGFP* and *Ormdl3-EGFP* Adeno-Associated Viruses (AAV)

An *Ormdl3-EGFP* (N-terminal tag) gene fusion was constructed and cloned into plasmid pZac2.1 under control of the ubiquitous cytomegalovirus promoter. AAV vectors were produced by Penn Vector Core (University of Pennsylvania, PA, USA). On day 5 of life neonatal mice

were administered intra nasally 1×10^{11} genome copies of either AAV *EGFP* (control vector) or AAV *Ormdl3-EGFP* (AAV *Ormdl3*) with 50mU neuraminidase (Sigma-Aldrich, Dorset, UK) in PBS. At 8 weeks old, mice received either 10 μ g *Alternaria alternata* extract (Greer Laboratories) or PBS via intranasal instillation, 3 times per week for five weeks. Mice were killed 18 hrs post final *Alternaria* instillation.

Induction of allergic airways disease

Ten to 12 weeks old male WT and *Ormdl3* KO^{Mer} mice were bred at Taconic Biosciences (Germantown, New York, USA). WT and *Ormdl3* KO^{Har} mice (Harwell, UK) were bred at Imperial and neonatal mice used for overexpression studies. All mice were housed in specific pathogen-free conditions and given food and water *ad libitum*. Mice were exposed to 20 μ g of purified *Alternaria alternata* extract (Greer Laboratories, Lenoir, NC, USA) (in 25 μ l PBS) intranasally 3 days/week for 5 weeks. All procedures were conducted in accordance with the Animals (Scientific Procedures) Act 1986.

Assessment of Airway Function

Airway hyper-responsiveness in response to increasing doses of methacholine (10-200 mg/mL; Sigma-Aldrich) was measured as previously described,¹⁶ using the Flexiventtm system (Scireq, Montreal, Canada).

***In vitro* measurement of airway smooth muscle function**

Tracheal tissue was harvested from WT or *Ormdl3* KO^{Mer} mice. Agonist concentration-response curves to methacholine or vehicle were subsequently fitted by least-squares, non-linear regression based upon the Hill equation (Prism 5, GraphPad Software Inc). Mean EC₅₀ values

were calculated by averaging data from interpolation of response curves constructed for each individual tissue within a data set.

Statistical analysis

All data was analysed using Graph Pad Prism 6 (GraphPad Software, La Jolla, USA). Box and whisker plots depict the median and IQR and minimum and maximum values. Line graphs and bar charts are expressed as mean $\pm$ SEM (standard error of the mean) and data was analysed using non-parametric Mann Whitney U tests with significance defined as $*P<0.05$, $**P<0.01$ and $***P<0.001$.

Methods for *in vitro* assessment of airway smooth muscle function, quantitative PCR, RT-PCR, immunofluorescence, isolation and sorting of epithelial and CD45+ cells, flow cytometry, western blotting and mediator analysis are in the Methods section in this article's Online Repository at www.jacionline.org.

Results

***Ormdl3* deficiency protects mice from developing fungal allergen induced AHR**

ORMDL3 is ubiquitously expressed in adult and fetal tissues including the lung^{3, 4} and also in leukocytes.^{5, 18} To assess the impact of ORMDL3 on induction of allergic airways disease (AAD) we generated global *Ormdl3* KO mice. We report that mice lacking *Ormdl3* had no baseline phenotype, appearing healthy and fertile with no alterations in the lung architecture or immune changes at homeostasis compared to wild type (WT) littermate C57bl/6 mice. Despite deletion of *Ormdl3* there was no compensatory up-regulation of the closely related genes *Ormdl1* and *Ormdl2* in the lungs of the KO mice as determined by qPCR (Fig. 1a).

Following a single dose of the fungal allergen *Alternaria*, *Ormdl3* mRNA expression has been shown to be induced in bronchial epithelial cells.⁵ To investigate the functional role of ORMDL3 during the development of asthma we therefore developed a chronic *Alternaria* induced AAD model (Fig. 1b). In WT mice *Alternaria* induced the hallmark features of asthma, including AHR (Figs. 1c-h), lung and airway inflammation, as well as eosinophilia (Figs. 2a-d). IgE concentrations were also augmented following allergen challenge (Fig 2e and f). We also observed an increase in pulmonary IL-13⁺ T cells (Th2) and innate lymphoid cells (ILC2), enumerated by flow cytometry (Figs. 3a and b). Concomitant with the elevated AHR, pulmonary levels of IL-13 were increased (Fig. 3c).

Mice deficient in *Ormdl3* were protected from development of *Alternaria* induced airway resistance and elastance (Figs. 1c-f) and allergen induced decreases in airway compliance (Figs. 1g and h). Changes in airway resistance result from altered contractility of the smooth muscle surrounding the airways. Therefore we chose to investigate whether intrinsic defects in smooth muscle cells could account for differences in lung function between WT and *Ormdl3* KO^{Mer}

mice. Tracheal rings from naïve mice were isolated and the contraction responses towards increasing methacholine concentrations were compared (Fig. 1i). The methacholine EC₅₀ value was significantly reduced in mice lacking ORMDL3 (Fig. 1j) indicating that ORMDL3 plays a role in airway smooth muscle contractility.

To further dissect the mechanisms which may account for the reduced *Alternaria* induced AHR in *Ormdl3* KO^{Mer} mice we profiled the inflammatory cells and mediators present in the lung. *Alternaria* induced cell recruitment to the lung (Figs. 2a and b) and BAL (Fig. 2c) was not affected by pulmonary expression of ORMDL3, although the proportion of eosinophils was minimally but statistically significantly reduced (38% vs 28% SiglecF⁺CD11c⁻CD68⁻ cells of total cells) in allergen exposed *Ormdl3* KO mice^{Mer} (Fig. 2d). Class switching to the allergy associated immunoglobulin IgE was unaffected by the loss of ORMDL3 (Fig. 2e), although there was a modest decrease in the levels of *Alternaria* specific IgE (0.78OD vs 0.61OD) (Fig. 2f) in *Ormdl3* KO mice^{Mer}.

The number of Th2 (CD4⁺IL-13⁺) cells and ILC2s (Lin^{neg}ICOS⁺IL-13⁺) recruited to the lung (Figs. 3a and 3b) and BAL (data not shown) was not different between *Ormdl3* sufficient and deficient mice. Despite the reduction in AHR in mice lacking ORMDL3, these mice responded to *Alternaria* exposure with increased levels of pulmonary Th2 cytokines including IL-13, IL-4 and IL-5 in the lung (Figs. 3c-e). Concentrations of these cytokines were less than that observed in WT mice but the reduction was not statistically significant. A small but significant increase in baseline levels of IL-5 was also noted (204 pg/mL vs 253pg/mL) in the lung tissue (Fig. 3e). Similarly, *Alternaria* induced increases in the levels of the pulmonary alarmin IL-33 were marginally reduced in *Ormdl3* KO^{Mer} mice (Fig. 3f).

Thus, analysis of *Ormdl3* KO^{Mer} mice reveal that lack of ORMDL3 protects against development of fungal allergen induced AHR, even though the mice are able to mount a robust Th2 response in the lung and periphery.

Ormdl3* KO mice have reduced cellular stress responses to *Alternaria

In vitro and *in vivo* studies have suggested that ORMDL3 facilitates the UPR.^{5, 9, 14} Therefore to further investigate how *Ormdl3* deficiency, which does not affect type 2 immune responses in response to chronic *Alternaria* exposure, results in protection from allergen induced AHR, the UPR was assessed. We specifically examined whether *Alternaria* promotes ER stress, and consequently UPR signaling in the lung, and more importantly, whether the magnitude of this response is altered in *Ormdl3* KO mice. ER stress leads to detachment of the ER chaperone BiP (binding immunoglobulin protein) from the three key ER stress proteins PERK, ATF6 and IRE-1 α , thus initiating three parallel pathways of the UPR signalling cascade (Fig. 4a).^{11, 12} IRE-1 α and PERK homodimerise and become activated following autophosphorylation of their respective cytoplasmic domains.¹¹ ATF6 is cleaved by proteases in the Golgi complex and subsequently translocates to the nucleus and acts as a transcription factor.¹⁹ Activated PERK phosphorylates eIF2 α resulting in translational arrest of most proteins. Treatment of mice with *Alternaria* resulted in phosphorylation of eIF2 α , however generation of p-eIF2 α was not modulated by ORMDL3 (Fig. 4b). Phosphorylation of eIF2 α also selectively induces the transcription factor ATF4,²⁰ which subsequently increases expression of *Ddit3*, the gene encoding CHOP (CCAAT/enhancer-binding protein-homologous protein).²¹ Baseline expression of *Atf4* mRNA and *Ddit3* were similar between WT and *Ormdl3* KO^{Mer} mice and were not modulated by exposure of mice to *Alternaria* (Figs. 4c and d).

The ATF6 and IRE-1 mediated signaling cascades result in activation of the ERAD (endoplasmic reticulum-associated degradation) pathway. Acting as a transcription factor, cleaved ATF6 increases the expression of a second transcription factor *Xbp1*.²² Interestingly, we observed an up-regulation of total *Xbp1* mRNA expression in WT mice in response to *Alternaria* (Fig. 4e). This was not observed in *Ormdl3* KO^{Mer} mice. IRE-1 α splices *Xbp1* mRNA,²² however there was very little generation of the spliced *Xbp1* gene product in any of the groups (Fig. 4f) indicating that *Alternaria* inhalation does not activate this pathway. Activation of ATF6 induces the transcription of genes that contribute to clearance of unfolded/mis-folded protein from the ER by binding to ER stress response elements. *Edem1* (a protein degradation factor)²³ is an example of an ATF6 inducible gene and its expression was increased in *Alternaria* treated WT mice compared to PBS controls whereas transcription of this gene in response to allergen challenge in *Ormdl3* KO^{Mer} mice was significantly reduced (Fig. 4g). The UPR is also linked to the production of several inflammatory cytokines and ATF6 binds to cAMP response elements promoting IL-6 mRNA transcription.²⁴ Exposure of WT mice to *Alternaria* induced a robust IL-6 response in WT mice and this was significantly blunted in *Ormdl3* KO^{Mer} mice (Fig. 4h). *In vitro* and *in vivo* studies have demonstrated that ORMDL3 overexpression results in increased expression of another ATF6 target gene, *Atap2a2* which encodes SERCA2b.^{5,9} However, utilising *Ormdl3* deficient mice we show that ORMDL3 is not an absolute requirement for expression of SERCA2b since basal and *Alternaria* induced SERCA2b levels were not significantly different between WT and *Ormdl3* KO^{Mer} mice (Fig. 4i). Thus, whilst the UPR is not affected by the absence of ORMDL3 under homeostatic conditions, ATF6 signaling as indicated by increased expression of *Edem1* and IL-6 is significantly impaired in pathological conditions where ER

stress is induced by exposure to *Alternaria*. Thus, the data indicate that ORMDL3 is involved in the regulation of key genes and proteins induced by ATF6 during ER stress. There is also an UPR-independent branch of the ER stress response which regulates activation of the NLRP3 inflammasome.²⁵ *Alternaria* exposure resulted in increased release of the DAMP (damage associated molecular pattern), uric acid. Concomitant with the reduced UPR in *Ormdl3* KO^{Mer} mice, ORMDL3 deficiency also protected against UPR independent cellular stress as evidenced by the significant reduction in uric acid released in these mice compared to WT animals (Fig. 4j). ORMDL3 specifically affects the ER stress pathway since levels of lactate dehydrogenase (LDH) release from cells, a marker of non-specific cellular damage, although modestly increased in response to *Alternaria* was not modulated by lack of ORMDL3 (Fig. 4k). Concurrent with the decrease in allergen induced uric acid levels, the *Ormdl3* KO^{Mer} mice also had significantly lower levels of BALF albumin following *Alternaria* challenge. This is indicative of maintained epithelial integrity and barrier function in *Ormdl3* KO^{Mer} mice compared to WT animals (Fig. 4l). Further studies are needed to determine whether induction of cellular stress is an association or causally related to induction of AHR.

Overexpression of ORMDL3 promotes allergen induced airway hyperresponsiveness

We next investigated the effect of overexpression of ORMDL3 in the lung utilizing an adeno associated viral vector (AAV). Comparative experiments using different AAV serotypes (1, 2, 5, 6, 7, 8, 9 and rh10) encoding the marker gene enhanced green fluorescent protein (EGFP) were conducted. These revealed that intranasal treatment of neonatal mice in the first week of life with AAV9, in combination with neuraminidase to reveal galactose residues on the apical surface of conducting airway epithelial cells to increase AAV9 binding,²⁶ resulted in optimal levels of

EGFP expression in the lung (data not shown). Due to the lack of available ORMDL3 specific antibodies to track expression, an *Ormdl3-EGFP* gene fusion was introduced into the plasmid pZac2.1 to generate an AAV9 *Ormdl3-EGFP* virus (AAV *Ormdl3*). Five day old Balb/c mice were treated with either vehicle (PBS), AAV *EGFP* or AAV *Ormdl3*. At 8 weeks of age (adult) mice were exposed to *Alternaria* for 5 weeks and parameters of AAD were assessed (Fig. 5a). A lower dose of *Alternaria* was specifically chosen in order to determine any potential exacerbation of disease parameters in the mice overexpressing epithelial ORMDL3.

Treatment of neonatal mice with AAV *EGFP* and AAV *Ormdl3* resulted in robust levels of GFP expression primarily in bronchiolar epithelial cells and this expression persisted into adulthood (Fig. 5b). There was no effect of treating neonatal mice with the control AAV *EGFP* vector and these animals were phenotypically indistinguishable from PBS treated littermate controls subsequently exposed to either PBS or *Alternaria* (Figs. 5c-g). At homeostasis overexpression of ORMDL3 did not affect either parameters of lung function or the inflammatory profile of the mice (Figs. 5c-g). However, overexpression of ORMDL3 had a significant effect on *Alternaria* induced AHR (Fig. 5c). At baseline (prior to MCh challenge), *Alternaria* exposed AAV *Ormdl3* mice had significantly higher airway resistance than allergen exposed PBS or AAV *EGFP* treated mice (Fig. 5d). Airway responsiveness to MCh challenge was also increased in the mice overexpressing ORMDL3 (Fig. 5e). In contrast, recruitment of cells to the airways was not affected by ORMDL3 overexpression (Figs. 5f-g). *Alternaria* induced uric acid release was elevated in the ORMDL3 overexpressing mice although the increase was not statistically significant (data not shown).

Epithelial specific ORMDL3 expression restores *Alternaria* induced AAD

The previous series of experiments indicated that overexpression of ORMDL3 regulates lung function. Many of the recently identified asthma susceptibility genes including *ORMDL3* are expressed in epithelial cells.^{3, 5} In WT mice *Ormdl3* is equally expressed by epithelial (EpCam⁺) and hemopoietic (CD45⁺) cells (Fig. 6a). In order to assess the particular contribution of epithelial derived ORMDL3 expression to the generation of *Alternaria* induced pathology, we reconstituted ORMDL3 expression in airway epithelial cells of *Ormdl3* KO^{Har} mice. Intranasal instillation of AAV *Ormdl3* to neonatal mice resulted in expression almost exclusively in bronchial epithelial cells of the conducting airways as shown by immunofluorescence (Fig. 6b) and confirmed by qPCR of sorted epithelial (EpCam⁺) and hemopoietic cells (CD45⁺) (Fig. 6c).

At 8 weeks of age WT, KO^{Har} and KO^{Har} mice expressing ORMDL3 specifically in the airway epithelium were exposed to *Alternaria* for 5 weeks and parameters of AAD were assessed (Fig. 6d). Expression of epithelial ORMDL3 was associated with a small but statistically significant increase in total cells in the BALF (WT GFP PBS 6.5×10^4 vs KO *Ormdl3* PBS 9.8×10^4) and uric acid (WT *EGFP* PBS 99 μ M and KO *EGFP* PBS 90 μ M vs KO *Ormdl3* PBS 149 μ M) in the airways but there was no other baseline phenotype in the absence of allergen challenge (Figs. 6d and e and S2). Restoration of epithelial ORMDL3 expression in the KO^{Har} mice resulted in enhanced *Alternaria* induced AHR that was equal in magnitude to that recorded in WT mice implying that epithelial derived ORMDL3 governs the deleterious *Alternaria* induced changes in lung function (Figs. 6g-l). The total number of leukocytes recruited to the airway lumen following *Alternaria* challenge was increased in the KO mice expressing epithelial ORMDL3 compared to *Ormdl3* deficient mice (Fig. 6e). Low dose *Alternaria* revealed an effect on Th2 cells, with reduced recruitment in *Ormdl3* KO^{Har} mice (Fig. S2a). Epithelial expression of

ORMDL3 in KO^{Har} mice re-established the allergen induced increase in Th2 cell accumulation (Fig. S2a) There was also a small but significant increase in airway eosinophilia in the epithelial ORMDL3 expressing KO^{Har} mice compared to WT (Fig. S2b). Congruent with the observed changes in type 2 inflammation BALF IL-13 levels were reduced in *Ormdl3* KO^{Har} mice although the reduction was not statistically significant (Fig. S2c). IL-13 concentrations in *Ormdl3* KO^{Har} mice reconstituted with epithelial ORMDL3 were not different from WT. There was no effect of ORMDL3 on total IgE (Fig. S2d) or *Alternaria* specific IgE levels in allergen exposed mice (data not shown). Levels of the cell damage marker LDH, were similar in all groups of mice exposed to allergen (Fig. S2e), congruent with our previous observations. Viral expression of ORMDL3 in KO^{Har} mice restored the uric acid response to *Alternaria* (Fig. 6f). In response to allergen, levels of BALF albumin were decreased in *Ormdl3* KO^{Har} mice compared to WT, indicative of increased epithelial integrity (Fig. 6m). However in the KO^{Har} mice reconstituted with epithelial ORMDL3, albumin levels were equivalent to WT mice (Fig. 6m). These data show that epithelial ORMDL3 governs the cellular stress response and AHR induced by inhaled *Alternaria*.

Discussion

GWAS have established a strong correlation between SNPs at the 17q21 locus and development of asthma and *ORMDL3* has been proposed as an asthma susceptibility gene. However, direct *in vivo* evidence for a pathophysiological function of *ORMDL3* in a long term model of AAD induced by a relevant aeroallergen associated with human disease is lacking.

ORMDL3 expression did not influence the number of Th2 cells or ILC2s recruited to the lung in response to *Alternaria*, however eosinophilia was reduced in mice lacking *ORMDL3*. These data are in agreement with published literature demonstrating that *ORMDL3* regulates eosinophil trafficking, recruitment and degranulation.¹⁸ However, in our lower dose *Alternaria* exposure regime utilised in the epithelial *ORMDL3* reconstitution experiments (Figure 6) we show that the total number of inflammatory cells recruited to the airway lumen are increased in mice expressing epithelial *ORMDL3* compared to the global KO animals. The increased BAL cellularity was due to increased numbers of Th2 cells and eosinophils, indicating that epithelial *ORMDL3* facilitates the trafficking of these cells to the airway lumen. However, the *ORMDL3* dependent element of allergen-induced inflammation is overcome with higher doses of *Alternaria*. Levels of total IgE were unaffected by the loss of *ORMDL3*, indicating that *ORMDL3* primarily affects innate rather than adaptive immunity in the generation of allergic airways disease.

Our data show that *Ormdl3* KO mice are protected from the development of increased airway resistance, a function of smooth muscle contractility, following prolonged *Alternaria* exposure implying that *ORMDL3* is a critical driver of this aspect of AHR. Interestingly, although *ORMDL3* is pivotal for the generation of *Alternaria* induced AHR the same phenotype was not observed in response to the aeroallergen house dust mite (data not shown). *Ormdl3* KO mice

develop AAD when exposed to house dust mite and the magnitude of AHR is equivalent to allergen treated WT mice. This suggests that ORMDL3 mediates an *Alternaria* specific activation of allergic airways disease. Sensitization to *Alternaria* has been implicated in severe asthma risk and fungal exposure is known to be associated with an increase in life-threatening exacerbations of the disease.^{27, 28} Intriguingly, SNPs in the chromosome 17q21 region in addition to being reproducibly associated with asthma have also been linked to asthma exacerbations requiring hospitalisation and/or oral steroids.^{29, 30}

Mice globally overexpressing ORMDL3 have previously been shown to have elevated levels of SERCA2b which transports calcium from the cytosol to the ER.^{5, 9} However, expression of SERCA2b is not solely dependent on the presence of ORMDL3 since levels were not altered in *Ormdl3* deficient mice. ORMDL3 has also been shown to inhibit the activity of SERCA2b and this modulatory effect is suggested to result from direct association of the two proteins.¹⁴ The reduced sensitivity of the smooth muscle from *Ormdl3* KO mice to pharmacologically induced contraction may therefore be due to decreased intracellular calcium concentrations as a result of increased SERCA2b mediated uptake to the ER in the absence of ORMDL3 and in moderate/severe asthmatics reduced activity of SERCA in airway smooth muscle is thought to contribute to airway remodeling.³¹ ORMDL3, which has been shown to be induced by *Alternaria*, also regulates ceramide biosynthesis and high level expression of ORMDL3 increases ceramide production.⁸ Allergen induces elevated ceramide levels with concomitant AHR which is ablated in mice treated with the sphingosine analogue FTY 720 which reduces both ORMDL3 and ceramide levels.⁸ Thus the reduced smooth muscle contractility measured in *Ormdl3* KO mice may also result from dysregulated lipid homeostasis.

We show that ORMDL3 expression specifically in bronchiolar epithelial cells restores the *Alternaria* induced increase in AHR suggesting that although ORMDL3 may have a direct effect on airway smooth muscle it can also act indirectly. Uric acid levels are increased in asthmatic patients following segmental allergen challenge and treatment of HDM challenged mice with uricase ablates allergen induced AHR.³² We have shown that *Alternaria* induced secretion of uric acid into the airways is blunted in *Ormdl3* KO mice. Conversely, viral mediated expression of ORMDL3 in airway epithelial cells of KO mice reinstated the uric acid response to allergen challenge clearly demonstrating the importance of epithelial derived ORMDL3 in mediating *Alternaria* induced release of DAMPs. Interestingly, uric acid is released early in the immune response to protease allergens,³³ and levels can be used as a biomarker for the severity of asthma exacerbations in patients.³⁴ Epithelial cells have an active uric acid transport system and basal secretion of uric acid by human airway epithelial cells has been demonstrated.³⁵ Uric acid is known to have direct effects on smooth muscle cells including stimulating endothelin-1 expression and increased intracellular calcium concentrations, which can induce smooth muscle contraction.^{36, 37} Thus epithelial expressed ORMDL3 can modulate lung function via its effects on uric acid release induced by allergen inhalation.

Many of the recently identified asthma susceptibility genes, including *ORMDL3* are known to be expressed in epithelial cells,^{3, 5} and disrupted airway epithelial barrier function is thought to be a critical controller of disease induction.² Utilizing *Ormdl3* KO mice we can infer that this protein has a vital role in the epithelial response to allergen and dysregulated expression of ORMDL3 likely contributes to a reduction in epithelial integrity as demonstrated by the increase in BAL albumin levels in ORMDL3 sufficient mice compared to KO animals. In KO mice expressing

epithelial ORMDL3, *Alternaria* induced increases in luminal albumin were restored suggesting that epithelial ORMDL3 governs allergen induced loss of epithelial integrity.

Another branch of the ER stress response is the UPR. The protein sensors PERK, IRE-1 α and ATF6 respond to ER stress by increasing expression of proteins such as BiP and CHOP,³⁸ which have been shown to be elevated in patients with asthma and in a murine model of neutrophilic, steroid resistant AAD induced by LPS/OVA. These markers are positively correlated with AHR and therapeutic treatment of mice with 4-phenylbutyric acid, a potent ER stress inhibitor has been shown to reduce AHR implying that activation of the UPR, which is dependent on ORMDL3, is intimately linked with lung function. In the lung ORMDL3 activates the UPR via the ATF6 pathway, increasing transcription of ERAD pathway specific genes (Edem-1) and regulating expression of IL-6 which has been implicated in the pathogenesis of asthma.¹³

It is not known how the levels of ORMDL3 expression achieved by AAV mediated gene transfer to the airway epithelium compare with the *in vivo* levels in WT mice due to the lack of specific reagents to differentiate between ORMDL1, 2 and 3 isoforms because of the high level of homology between these proteins, which we have shown directly using KO mice. Despite this, the data clearly demonstrate that epithelial ORMDL3 is essential for the generation of AAD and GWAS indicate that only the ORMDL3 isoform is associated with asthma susceptibility. ORMDL3 has a cAMP response element (CRE) in the promoter region and protein kinase A dependent CREB (cAMP response element binding protein) - phosphorylation results in binding to the CRE and initiation of *ORMDL3* transcription.³⁹ ORMDL3 activates ATF6, increasing transcription of CRE responsive genes, thus ORMDL3 may mediate its own expression via a positive feedback loop. Additionally, Alternariol, a mycotoxin of *Alternaria*, increases CREB

binding to CREs⁴⁰ and this may be the mechanism by which *Alternaria* increases ORMDL3 expression.

Specific clinical asthma phenotypes (recurrent severe exacerbations) have previously been shown to be associated with distinct genotypes (CDHR3, cadherin regulated family member 3, locus).⁴¹ Stratifying patients with severe asthma into two groups based on their response to fungal exposure reveals that the patients that respond to fungal allergens have earlier onset symptoms and require more oral steroid therapy than those who do not and experience a greater number of asthma exacerbations.⁴²⁻⁴⁴ GWAS data predicts that elevated ORMDL3 expression increases the risk of severe asthma and asthma exacerbations, thus given the association between ORMDL3 and *Alternaria* induced AHR revealed in the current study it would be pertinent to determine whether SNPs in the 17q21 locus are associated with severe fungal associated asthma phenotypes. With the current emphasis on personalised medicine, confirmation of the mechanisms and therapeutic targets in these specific patients could offer tailored treatments for this group of asthmatics who frequently present with severe disease and are resistant to current steroid treatments. The ER stress inhibitor sodium phenylbutyrate (4-phenylbutyric acid) is licensed for oral administration and used therapeutically in the management of inherited urea cycle disorders.⁴⁵ Due to its additional chaperone function it would be of interest to determine whether this drug also has efficacy in asthmatic patients carrying genetic variations in the ORMDL3 locus.

In conclusion, ORMDL3 is a crucial regulator of smooth muscle contractility and subsequent AHR acting potentially via cellular stress pathways, the UPR and uric acid release. Modulation of cellular stress may represent a novel therapeutic avenue for the treatment of asthma.

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C.M.L, W.O.C.C and M.F.M conceived the idea and directed the study. S.L, L.G.G and C.M.L designed the experiments. S.L, L.G.G, Y.Z, K.S, S.A.W, J.B and L.D, performed the experiments, analysed the data and generated the figures. C.H.D advised and helped with the generation of the KO mice created by Y.Z at the Sanger Centre and MRC Harwell. S.L, L.G.G, K.S, S.A.W and C.M.L wrote the manuscript.

The authors declare that they have no conflicts of interest.

Figure Legends

Figure 1: *Ormdl3* KO^{Mer} mice are protected from developing *Alternaria* induced AHR.

(a) Expression of *Ormdl1* -3 in lungs of naïve WT and KO mice by qPCR. (b) Experimental plan (c-h) Lung function measured using the FlexiVent system. Dose response curve to methacholine showing (c) airway resistance, (e) airway elastance and (g) airway compliance. (d) Airway resistance (f) airway elastance and (h) airway compliance at 200 mg/mL methacholine. (i) Concentration response curve of tracheas towards increasing concentrations of methacholine. (j) EC₅₀ values of contraction responses. Data was collected from two individually performed experiments. N = 8-12 mice per group. Box and whisker plots depict the median and IQR and minimum and maximum values. **P* < 0.05; ***P* < 0.005 and ****P* < 0.0005.

Figure 2: Analysis of *Alternaria* induced AAD in WT and *Ormdl3* KO^{Mer} mice.

(a) Lung total cell counts. (b) *Alternaria* induced pulmonary inflammation in tissue sections (H and E staining). (c) BALF total cell counts (d) Eosinophils expressed as the percentage SiglecF⁺ CD11c⁻ CD68⁻ lung cells. (e) Total IgE and (f) *Alternaria* specific IgE concentrations. Representative photomicrographs are shown. Original magnification x20. Data was collected from two individually performed experiments. N = 8-16 mice per group. Box and whisker plots depict the median and IQR and minimum and maximum values. **P* < 0.05; ***P* < 0.005 and ****P* < 0.0005.

Figure 3: ORMDL3 does not influence *Alternaria* induced type-2 inflammation or mediators.

(a) Pulmonary Th2 cells (IL-13⁺ CD4⁺ CD3⁺) and (b) ILC2 cells (IL-13⁺ ICOS⁺ CD45⁺ lineage⁺ cells). (c) IL-13, (d) IL-4, (e) IL-5 and (f) IL-33 cytokine levels in lung tissue determined by ELISA. Data was collected from two individually performed experiments. N = 8-16 mice per group. Box and whisker plots depict the median and IQR and minimum and maximum values. **P* < 0.05; ***P* < 0.005 and ****P* < 0.0005.

Figure 4: *Ormdl3* KO^{Mer} mice have diminished cellular stress responses to *Alternaria*.

(a) Schematic showing major components of the unfolded protein response. (b) Western blot showing p-eIF2 α . Specific qPCR analyses were performed for (c) *Atf4* (d) *Ddit3* and (e) total *Xbp1*. (f) PCR of total and spliced *Xbp1*. (g) *Edem1* qPCR. (h) IL-6 determined by ELISA (i) Western blot showing SERCA2b. (j) Uric acid (k) LDH and (l) albumin levels in BALF. Data was collected from two individually performed experiments. N = 8-16 mice per group. Box and whisker plots depict the median and IQR and minimum and maximum values. **P* < 0.05; ***P* < 0.005; ****P* < 0.0005. For analysis of gene expression Mann-Whitney statistical tests were performed between groups where a greater than 2 fold induction of median values was observed.

Figure 5: ORMDL3 overexpression in airway epithelial cells enhances AHR.

(a) Experimental plan. (b) GFP expression (staining - green) in the lungs. (c-e) Lung function measured using the FlexiVent system. (c) Dose response curve to methacholine showing airway resistance. (d) Airway resistance at baseline and (e) 30 mg/mL methacholine. (f) Total cells in the BALF. (g) Eosinophils. Representative photomicrographs are shown. Original magnification x20. Data was collected from three individually performed experiments. N = 8-12 mice per

group. Box and whisker plots depict the median and IQR and minimum and maximum values.

* $P < 0.05$; ** $P < 0.005$ and *** $P < 0.0005$.

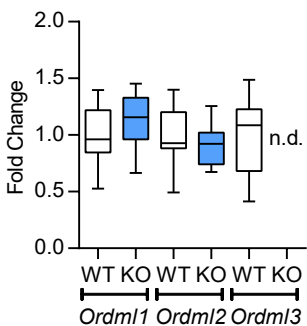
Figure 6: Epithelial ORMDL3 expression drives susceptibility to *Alternaria* induced AHR

(a) *Ormdl3* expression in epithelial (EpCam⁺) cells and leukocytes (CD45⁺) sorted from WT mice. (b) GFP expression (staining - green) in the lungs. (c) *EGFP* expression in sorted epithelial cells and leukocytes from *Ormdl3* KO^{Har} mice following administration of either AAV *EGFP* or AAV *Ormdl3*. (d) Experimental plan. (e) Total cells in the BALF. (f) Uric acid concentration. (g-l) Lung function measured using the FlexiVent system. (g and j) airway resistance, (h and k) airway elastance and (i and l) airway compliance. (m) Albumin levels determined in BALF. Representative photomicrographs are shown. Original magnification x20. Data was collected from two individually performed experiments. N = 7-12 mice per group. Box and whisker plots depict the median and IQR and minimum and maximum values. * $P < 0.05$; ** $P < 0.005$ and *** $P < 0.0005$.

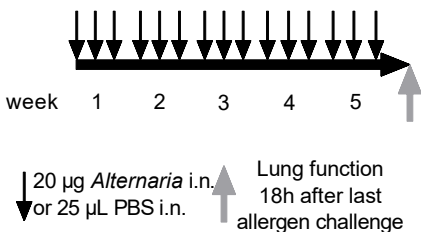
Figure 1

a

Ormdl1-3 expression

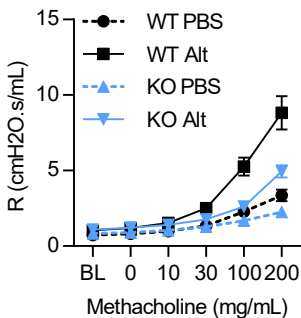


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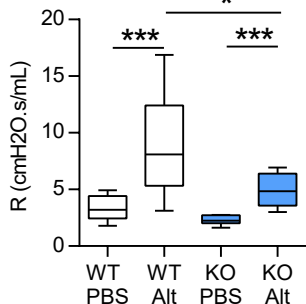
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Resistance



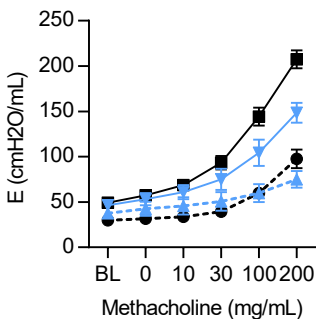
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Resistance at 200 mg/mL



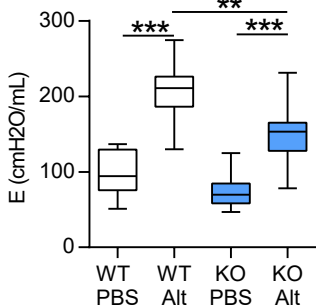
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Elastance



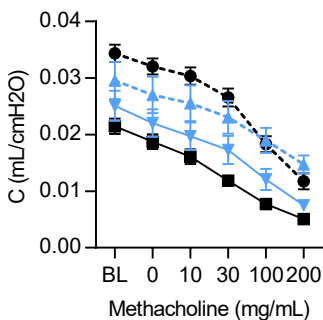
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Elastance at 200 mg/mL



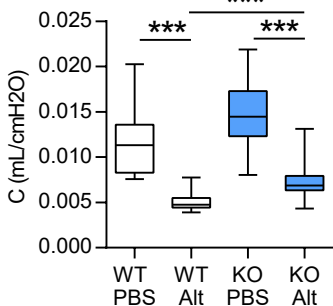
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Compliance



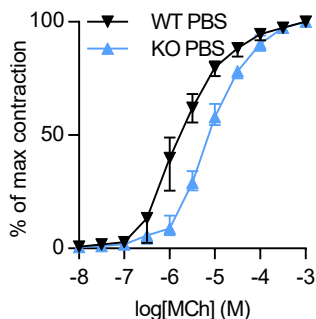
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Compliance at 200 mg/mL



i

Tracheal contraction



j

Methacholine EC₅₀ values

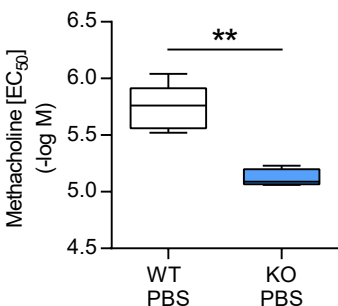


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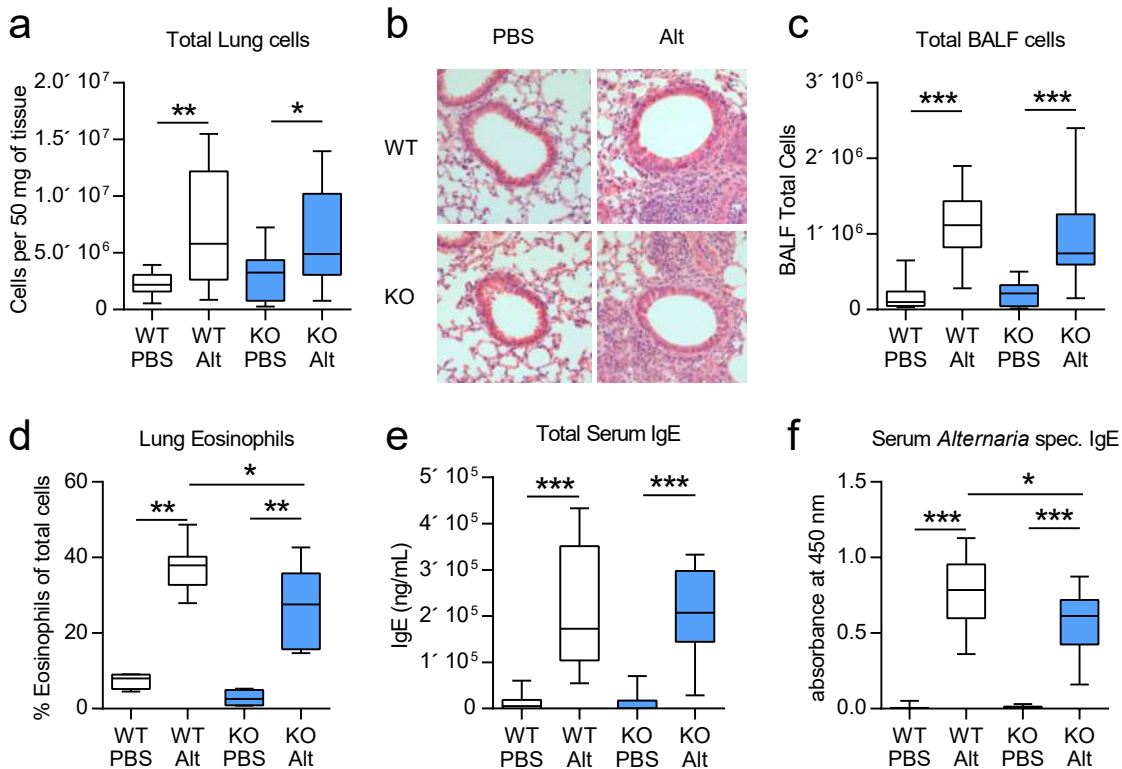


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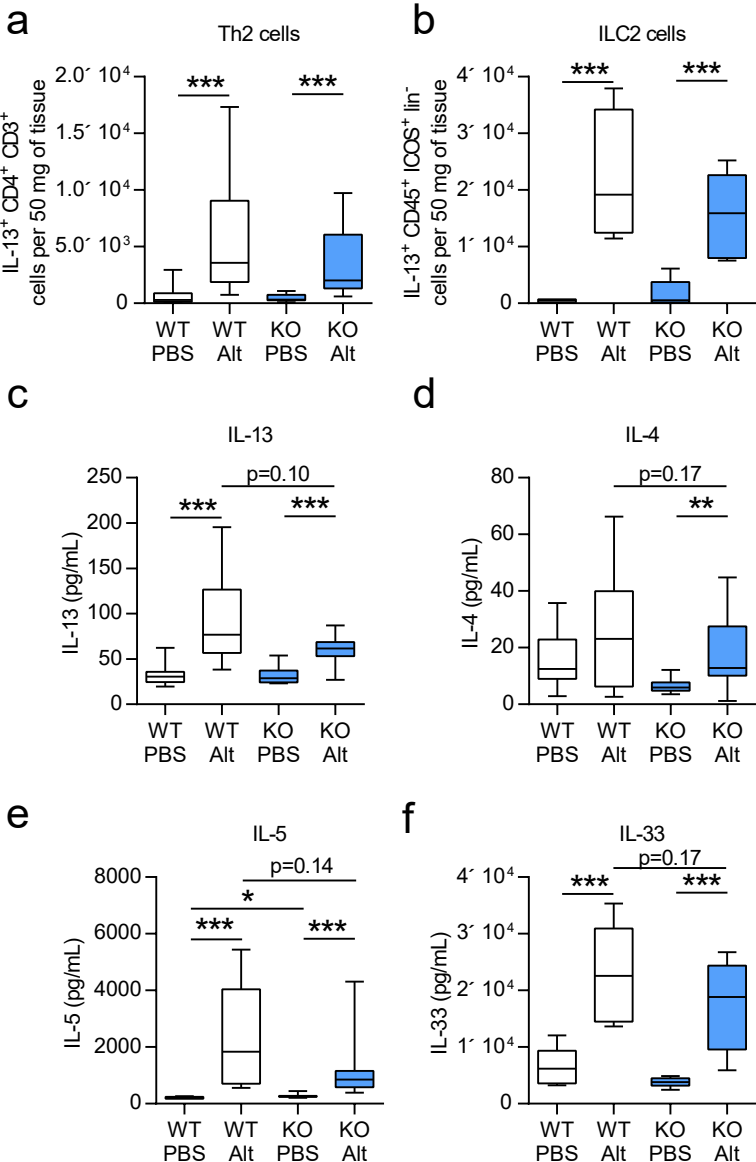


Figure 4

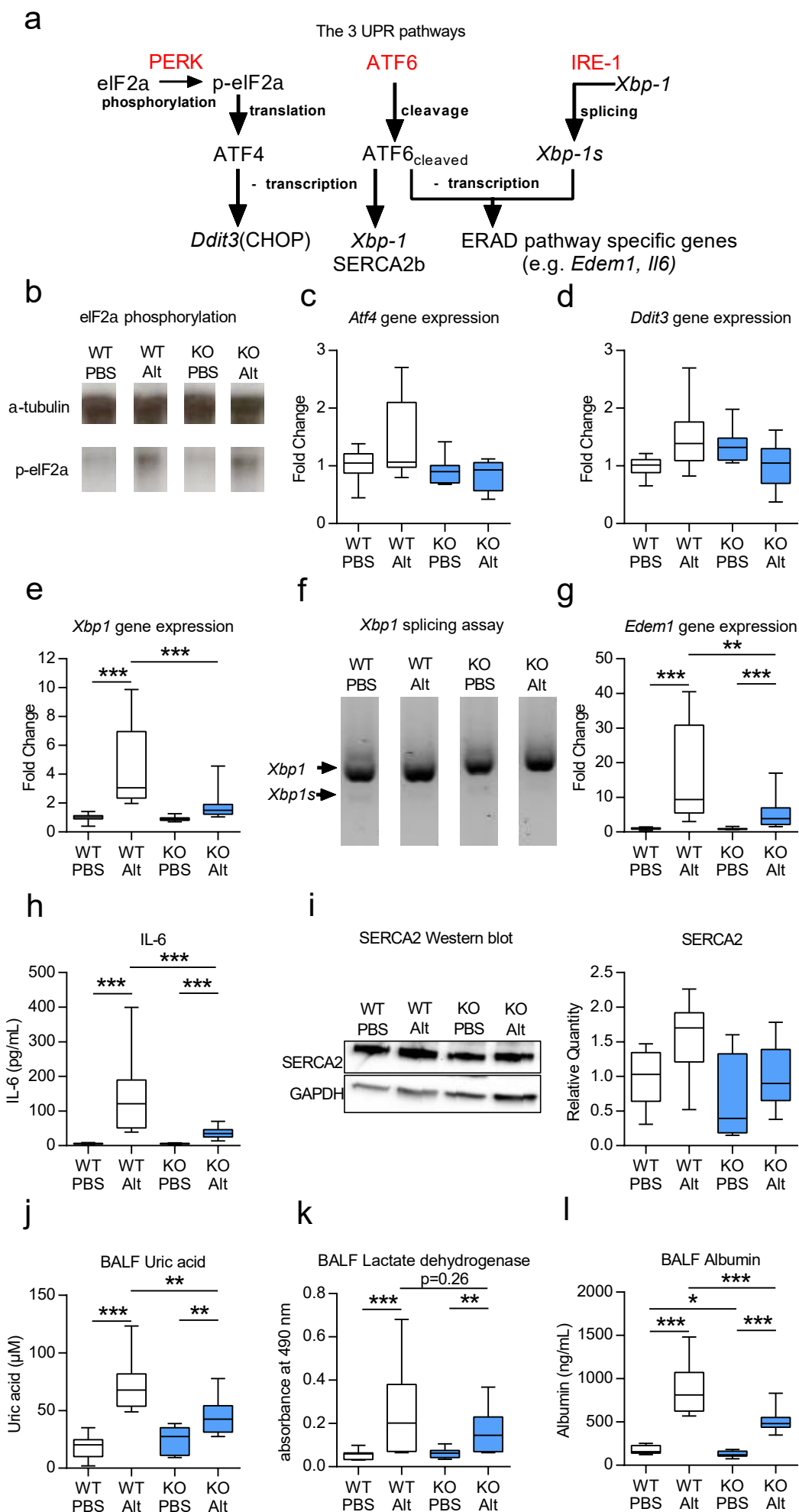
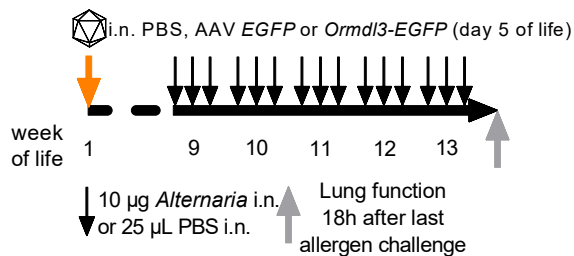
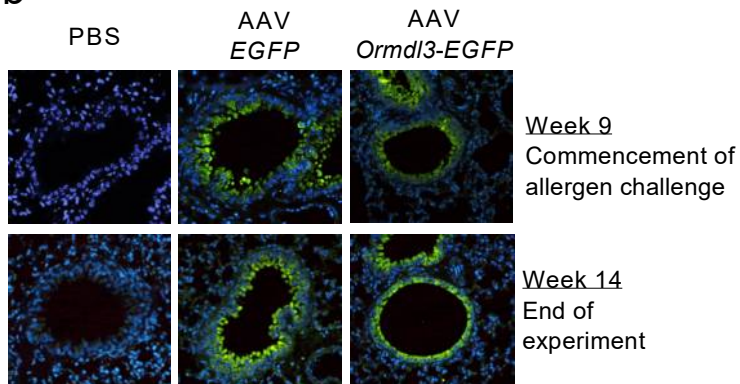


Figure 5

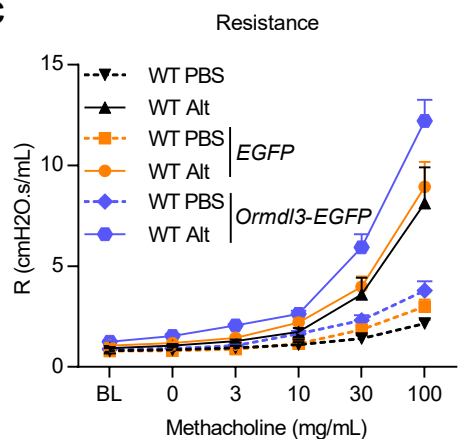
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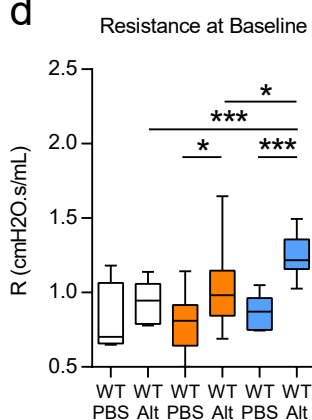
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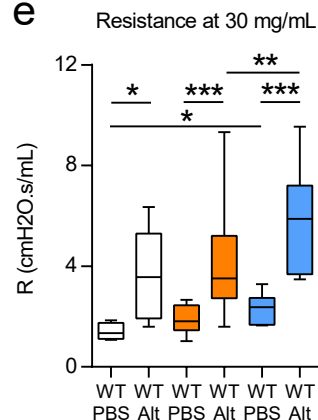
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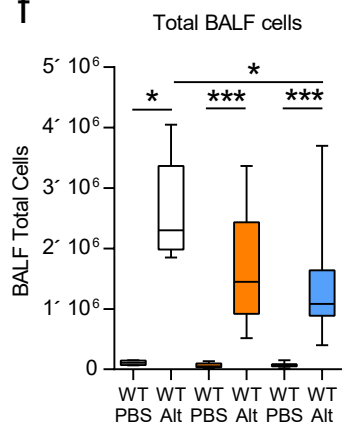
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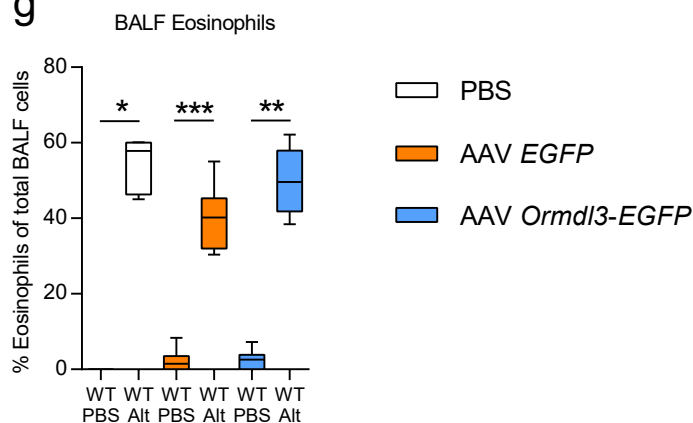
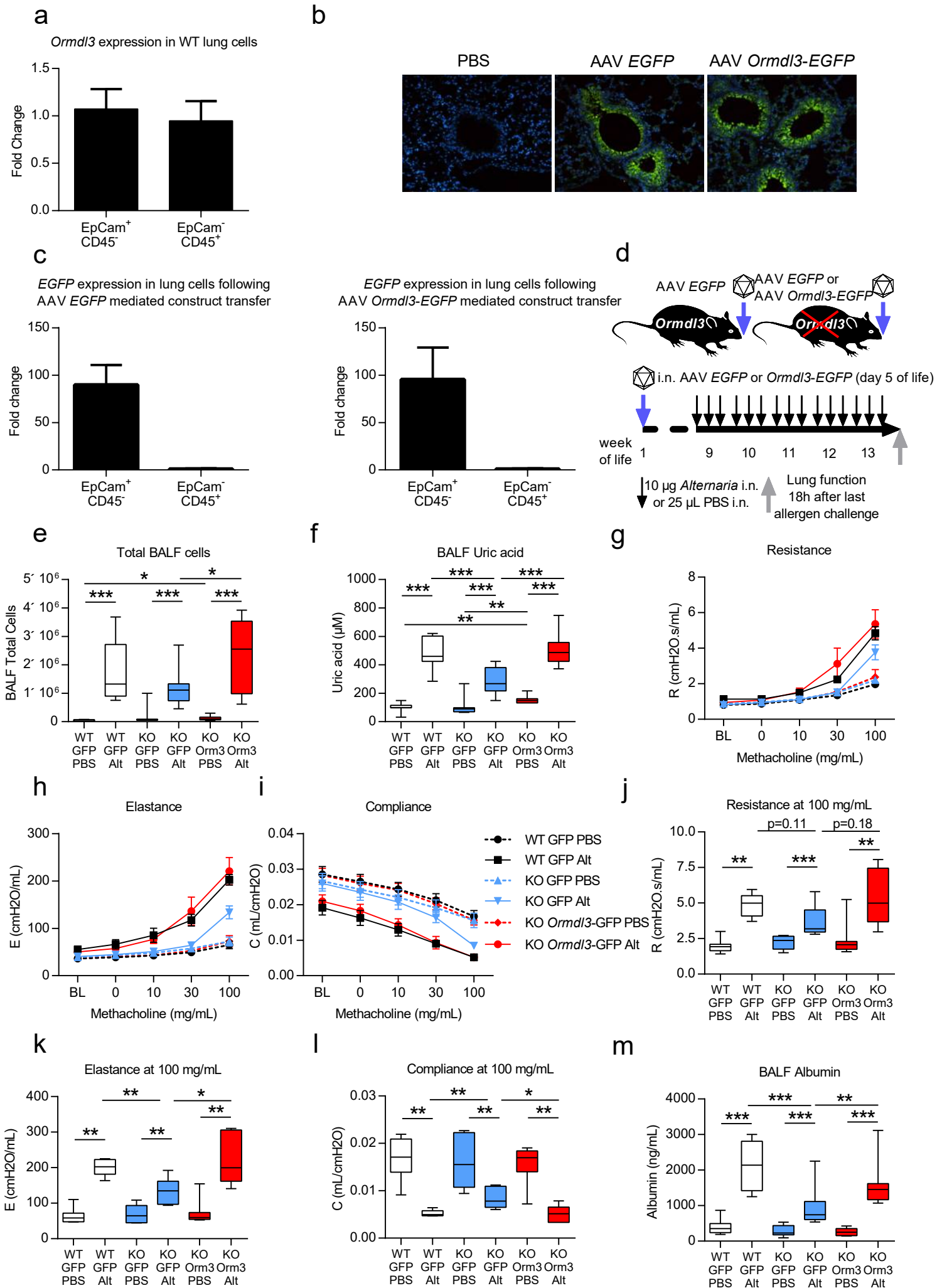


Figure 6



Supplementary Data

Materials and Methods

RT-PCR

Lung tissue samples were homogenized in TRIzol® solution (Life Technologies) followed by addition of chloroform to extract nucleic acids. RNA was isolated using the RNeasy® Mini Kit (QIAGEN), including an on-column DNase digest with an RNase-free DNase set (QIAGEN) as per manufacturer's instructions. cDNA was amplified with random primers and the High-Capacity cDNA Reverse Transcription Kit (Life Technologies) according to the manufacturer's instructions.

Real-time RT-PCR reactions were performed on a ViiA™ 7 Real-time PCR System (Life Technologies) in conjunction with TaqMan® Fast Advanced Master Mix (Life Technologies) and following TaqMan® Gene Expression Assays (Life Technologies): *Atf4* (Mm00515325_g1), *Ddit3* (Mm01135937_g1), *Edem1* (Mm00551797_m1), *Xbp1* (Mm00457357_m1), *Gapdh* (Mm99999915_g1) and *Hprt* (Mm01545399_m1). Expression of each target gene was normalized to the arithmetic mean of the two housekeeping genes *Gapdh* and *Hprt*. Data were then expressed as fold-change of relative mRNA expression in experimental groups versus naïve wild-type animals.

Xbp-1 and spliced *Xbp-1* (*Xbp-1s*) transcripts were amplified by PCR (FWD primer: ACACGCTTGGGAATGGACAC; REV primer: CCATGGGAAGATGTTCTGGG). The products were separated using a 4% agarose gel (Ultrapure agarose; Invitrogen).

Isolation and sorting of CD45⁺ Epcam⁻ and CD45⁻ EpCam⁺ cells

Airway epithelial cells were sorted as previously reported,¹ with minor amendments. Lungs were perfused with ice cold PBS through the right cardiac ventricle. 1.5 mL of dissociation

solution (DMEM/F12 (no phenol red; Life Technologies) substituted with 25 mM HEPES (Sigma Aldrich), penicillin/streptomycin and 5 mg/mL dispase II (Sigma Aldrich) was injected into the lungs through the trachea utilizing a 1 mL syringe, followed by 0.5 mL of 1% low gelling temperature agarose (Sigma Aldrich). The trachea was consequently closed using a hemostat and the chest was cooled down with ice to facilitate agarose solidification. The lung lobes were then harvested and incubated at 37°C for 1h in 5 mL dissociation solution under slight agitation. The digested tissue was passed through a 100 µm cell strainer (BD), cells were precipitated by centrifugation, washed through a 100 µm cell strainer for a second time to remove any remaining, solidified agarose and incubated at room temperature for 10 min in DMEM/F12/25 mM HEPES in the presence of 50 µg/mL DNase I (Roche). Erythrocytes were lysed and the cell suspension was extracellularly stained in sorting buffer (1% BSA in PBS) with PE conjugated EpCam (eBioscience) and Brilliant Violet 421 conjugated CD45 (Biolegend) anti-mouse antibodies and sorted using a BD FACS Aria III cell sorter. QIAshredder and RNeasy micro kits (Qiagen) were used in combination to isolate RNA. cDNA was prepared using the SuperScript VILO Master Mix (ThermoFisher). RNA/cDNA preparation steps were performed according to manufacturers' instructions. Transcript levels of *EGFP* were assessed by qRT-PCR utilizing *EGFP* targeting Taqman gene expression assays (Mr04329676_mr; Thermo Fisher). *Gapdh* served as reference gene.

Immunofluorescence

Paraffin sections were stained sequentially with rabbit anti-GFP antibody (Abcam), biotinylated goat anti-rabbit (Jackson) and streptavidin AlexaFluor488 (Molecular probes, Invitrogen). Tissue sections were visualised and images overlaid using a LeicaDM2500 microscope and QWin software (Leica Microsystems (UK) Ltd., Milton Keynes, UK).

49

50 Western blot

51 Lung tissue was homogenised at 50 mg/ml tissue in RIPA buffer (Sigma) followed by
52 centrifugation at 13,000 rpm for 10 mins at 4°C. The resultant supernatant was used for
53 Western blot. Proteins were separated by SDS-PAGE and transferred to PVDF membranes.
54 Membranes were blocked with 5% (w/v) non-fat milk in TBS with 0.05% tween-20. The
55 following primary antibodies were used; rabbit monoclonal anti-SERCA2 (abcam), rabbit
56 polyclonal anti-GAPDH (Millipore), rabbit polyclonal anti-GAPDH Phospho-eIF2 α (Ser51)
57 (Cell Signaling Technology) and rabbit monoclonal Anti-alpha tubulin antibody (Abcam).

58 Flow Cytometry

59 BAL fluid recovery, lung tissue digest, cell counting and differential cell counting was
60 performed as described previously.²
61 Single cell suspensions from lung tissue and BAL were stimulated with 40 ng/mL PMA
62 (Sigma-Aldrich) and 3 μ g/mL ionomycin (MERCK Millipore, Watford, UK) in
63 RPMI/10%FCS containing 10 μ g/mL Brefeldin A (Sigma-Aldrich) at 37°C, 0.5% CO₂ for
64 3h. Cells were then washed and incubated for 20min at room temperature with 10 μ l normal
65 rabbit serum (Sigma-Aldrich). After washing, cells were stained for extracellular antigens in
66 5%FCS/1%BSA in PBS for 30min at 4°C. Cells were then washed, fixed and permeabilised
67 using Fix/Perm kit (eBioscience, Hatfield, United Kingdom) before being stained for
68 intracellular antigens. All antibodies were purchased from eBioscience except anti-ICOS
69 (Biolegend, London, UK). Acquisition was performed using Fortessa™ (BD) and analysis
70 with FlowJo™ software (Flowjo, Ashland, Alabama, USA).

71 Mediator Analysis.

Lung tissue was homogenized in HBSS (Life technologies) containing protease inhibitor cocktail (Roche). Lung tissue supernatant, BAL and serum were analysed by ELISA; IL-4 and IL-5 (BD PharMingen, Oxford, UK), IL-13 (Mouse Quantikine Kit (R&D systems, Abingdon, UK)), IL-33 (Mouse IL-33 DuoSet ELISA, R&D systems) and albumin (Bethyl Laboratories Inc, Montgomery, TX, USA). Paired antibodies for IgE (R&D systems) were used to measure serum Ig levels. For determination of *Alternaria* specific IgE levels high binding ELISA plates were coated with 50 µg/ml *Alternaria* extract. Uric acid levels were determined by Amplex Red uric acid assay (Life Technologies) and LDH levels by a lactate dehydrogenase based, In vitro toxicology assay kit (Sigma-Aldrich), according to the manufacturers' instructions.

***In vitro* measurement of airway smooth muscle function**

Tracheal tissue was harvested from WT or *Ormdl3* KO^{Mer} mice and bathed in Kreb's Henseleit solution (KHS) warmed to 37°C and aerated with 95% O₂/5% CO₂. The tracheas were then cleared of extraneous tissue and split into two rings by cutting along the transverse plane. Tissues were then sutured to force-displacement transducers and placed in tissue baths. Following time to stabilize, tissues were challenged with acetylcholine (1 mM) three times to confirm viability and to standardize responses. Cumulative concentration-response curves (10 nM – 1 mM) were then generated to methacholine or vehicle (KHS). Agonist concentration-response curves were subsequently fitted by least-squares, non-linear regression based upon the Hill equation (Prism 5, GraphPad Software Inc). Mean EC₅₀ values were calculated by averaging data from interpolation of response curves constructed for each individual tissue within a data set.

Supplementary References

1. Juncadella IJ, Kadl A, Sharma AK, Shim YM, Hochreiter-Hufford A, Borish L, et al. Apoptotic cell clearance by bronchial epithelial cells critically influences airway inflammation. *Nature* 2012; advance online publication.
2. Snelgrove RJ, Gregory LG, Peiro T, Akthar S, Campbell GA, Walker SA, et al. *Alternaria*-derived serine protease activity drives IL-33-mediated asthma exacerbations. *J Allergy Clin Immunol* 2014; 134:583-92.e6.

Supplementary Figure legends

Figure S1: Comparative schematic of strategies used to generate *Ormdl3*^{MER} KO and *Ormdl3*^{HAR} KO mice.

(a) Strategy used by MERCK. The gene *Ormdl3* contains 4 exons of which exons 2-4 are encoding the ORMDL3 protein. Exons 2-4 were consequently flanked by 2 antibiotic selection cassettes (Puro: puromycin; Neo: neomycin) and loxP recombination sites were inserted upstream of the Puro cassette and downstream of the Neo cassette. Following homologous recombination, Flp recombination and Cre recombination steps, the resulting *Ormdl3*^{MER} KO strain retains a loxP recombination site downstream of exon 1. (b) The approach used by MRC Harwell slightly differed from the MERCK strategy, as a single neomycin selection cassette was included in the targeting vector. LoxP recombination sites were located between exons 1 and 2 and between exon 4 and the FRT flanked Neo cassette. The resulting *Ormdl3*^{HAR} KO mice strain therefore retains a loxP, as well as a FRT recombination site after homologous, Flp and Cre recombination. *Abbreviations: CDS: coding sequence; UTR: untranslated region*

Figure S2: Effect of epithelial ORMDL3 expression on type 2 inflammation.

(a) Th2 cells (IL-13⁺ CD4⁺ CD3⁺) in the BALF. (b) Eosinophils expressed as the percentage SiglecF⁺ CD11c⁻ CD68⁻ in the BALF. (c) IL-13 levels in the BALF. (d) Serum IgE levels (e) Lactate dehydrogenase levels in the BALF. Data was combined from 2 individually

123 performed experiments. N = 7-12 mice per group. Box and whisker plots depict the median
124 and IQR and minimum and maximum values. * $P < 0.05$; ** $P < 0.005$ and *** $P < 0.0005$.

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126

Supplementary Figure 1

a

MERCK *Ormdl3* KO strategy

Mouse Genomic Locus:



Targeting Vector:



Allele after homologous recombination



Conditional KO allele after Flp recombination



Constitutive KO allele after Cre recombination



b

Harwell *Ormdl3* KO strategy

Mouse Genomic Locus:



Targeting Vector:



Allele after homologous recombination



Conditional KO allele after Flp recombination



Constitutive KO allele after Cre recombination



CDS

UTR

loxP

F3

FRT

Supplementary Figure 2

