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Deficiency of FHL2 attenuates airway inflammation in mice and genetic variation associates with human bronchial hyperresponsiveness

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Short title: FHL2 deficiency attenuates airway inflammation

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Abstract

Background: Asthma is an inflammatory disease that involves airway hyperresponsiveness and mucus hypersecretion. The LIM-only protein FHL2 is a crucial modulator of multiple signal transduction pathways and functions as a scaffold in specific protein-protein interactions.

Objective: We sought to investigate the role of FHL2 in airway inflammation.

Methods: Allergic airway inflammation was induced in WT and FHL2-knock out (FHL2-KO) mice with ovalbumin (OVA). Lung tissue, bronchoalveolar lavage fluid (BALF) and draining lymphnode cells were analyzed for inflammation. FHL2 loss and gain of function studies were performed in lung epithelial cells.

Results: FHL2-deficient mice challenged with OVA show significantly reduced airway inflammation as evidenced by reduced infiltration of inflammatory cells including eosinophils, dendritic cells, B-cells and T-cells. Furthermore, mucus production was decreased in FHL2-KO mice. In BALF, the levels of IL-5, IL-13, Eotaxin-1, and Eotaxin-2 were significantly lower in FHL2-KO mice. In addition, draining lymph node cells from FHL2-KO mice show reduced levels of IL-5 and IL-13. Consistent with this, OVA-specific serum IgG and IgE levels were reduced in FHL2-KO mice. We also found that phosphorylation of ERK1/2 is markedly attenuated in FHL2-KO lung. Knock-down of FHL2 in human lung epithelial cells resulted in a striking decrease in ERK1/2 phosphorylation and mRNA levels of inflammatory cytokines and MUC5AC, whereas FHL2 overexpression exhibited opposite effects. Finally, the SNP rs4851765 shows an association with the severity of bronchial hyperresponsiveness.

Conclusion: These results highlight functional involvement of FHL2 in airway inflammation and identify *FHL2* as a novel gene associated with asthma severity in human.

Keywords airway inflammation, bronchial hyperresponsiveness, epithelial cells, FHL2, pERK1/2

Introduction

Asthma is a complex inflammatory disorder characterized by airway inflammation, intermittent airway obstruction, eosinophilic infiltration, airway hyperresponsiveness and mucus hypersecretion [1-3]. Asthma involves intricate interaction between the inhaled environment, genetic variation and the structure of the airways [4]. Especially T-helper type 2 (Th2) cells and their cytokine products, including interleukin (IL)-4, IL-5 and IL-13 play a critical role in mediating the inflammatory responses in the diseased lung. Th2 cytokines contribute to the recruitment of eosinophils, activation of mast cells, regulation of IgE synthesis, mucus gland hyperplasia and tissue remodelling[5-8]. The cytokines RANTES and eotaxin1/2 are instrumental in attracting eosinophils to the airways [9-11]. The inflammatory eosinophils, mast cells, neutrophils, dendritic cells and B-cells further modulate the immune response in asthma by promoting Th2 responses [12]. In addition to inflammatory immune cells, airway smooth muscle cells and epithelial cells play a prominent role in the pathogenesis of asthma. Airway smooth muscle cells are major resident cells in airways and their contraction leads to excessive narrowing of the airway at the onset of an asthma attack, and in progressive disease enhanced proliferation of these cells further narrows the airways [13-15].

Excessive production of mucus obstructs mucociliary clearance and airflow in the airways and is a common pathological change observed in asthma [16;17]. *MUC5AC*, among other mucin genes, is the most prominent mucin gene, which is highly expressed in airway inflammation and serves as a major marker of mucus cell hyperplasia [18]. Several studies have shown that inflammatory cytokines, like TNF α , induce *MUC5AC* gene expression in lung epithelial cells [19]. Activation of extracellular signal-

regulated kinase (ERK)-1/2 signalling pathway has been shown to inhibit allergic airway inflammation and asthma through blocking eosinophil infiltration and reducing mucus hypersecretion by inhibiting *MUC5AC* expression [20]. Although several experimental studies in animal models of asthma have led to understanding the nature and mechanisms that drive asthma, the mechanistic processes that underlie the development of asthma are poorly understood. To understand the cellular and molecular mechanisms of asthma, identification of the genes responsible for asthma initiation and progression is crucial.

The LIM-only protein FHL2 contains four LIM domains, which are composed of double zinc finger structures that mediate protein-protein interactions [21-24]. Cumulative evidence shows that FHL2 functions as a scaffold in multiple protein-protein interactions and modulates a number of signal transduction pathways. FHL2 plays a critical role in a range of physiological and pathological processes, among which proliferation, migration, apoptosis and inflammation [22;23;25-27]. FHL2 has been reported to act as a transcriptional cofactor that regulates the activity of the transcription factors such as NFκB, cAMP-responsive element binding protein (CREB), androgen receptor (AR) and Nur77 [22-24;27-30]. The outcome on activity of proteins interacting with FHL2 is highly cell-context dependent. For example, in osteoclast formation FHL2 inhibits TRAF6-induced NFκB activation, whereas FHL2 acts as a positive regulator of NFκB in liver regeneration and carcinogenesis through enhanced protein stability of TRAF6 [31;32]. FHL2 binds and inhibits ERK phosphorylation in cardiomyocytes thereby inhibiting ERK-induced cardiac hypertrophy [33], and we demonstrated that FHL2 regulates vascular smooth muscle cell proliferation and migration through down-regulation of the ERK-CyclinD1 pathway [27]. Furthermore, FHL2 regulates CCL19-induced migration of murine bone marrow-derived dendritic cells [34]. Lung inflammation following bleomycin administration in mice is reduced through the FHL2-mediated increase of tenascin C expression and the reduction of inflammation-resolving macrophage activation [25]. Most recently, FHL2 was shown to protect against chronic inflammatory arthritis [35].

Given that FHL2 is involved in modulation of dendritic cell migration and in inflammatory pathologies, we investigated, to the best of our knowledge for the first time, the function of FHL2 in allergic airway inflammation in mice. We report here that the absence of FHL2 significantly reduces the production of Th2 cytokines, eosinophil infiltrates, mucus production and histopathological changes in the lung. Moreover, we explored the underlying mechanism using both *in vivo* and *in vitro* approaches and we explored whether *FHL2* genetic variation associates with asthma and severity of airway hyperresponsiveness.

Materials and methods

An expanded methods section is available in the online supplementary material.

Animals and care

FHL2-KO mice were generated by R. Bassel-Duby (University of Texas Southwestern Medical Center, Dallas, TX) and were bred onto a C57BL/6 background for >11 generations. All animal experiments were approved by the Committee for Animal Welfare of the Academic Medical Center, University of Amsterdam and were carried out in compliance with guidelines issued by the Dutch government.

Murine model of ovalbumin-induced airway allergic inflammation

Female mice aged 8 weeks were sensitized on days 0 and 7 by intraperitoneal injection of 10 µg OVA (Sigma, grade V) adsorbed in 1 mg aluminium hydroxide (Imject alum, Pierce) (n=8 per group). Control animals received alum only. Subsequently, mice were challenged with aerosolized OVA (Sigma, grade III, 10 mg/ml) or PBS for 30 minutes each day for three consecutive days on days 14, 15, and 16. 48h after the last challenge, mice were sacrificed for harvest of bronchoalveolar lavage fluid (BALF) fluid, lungs and serum.

Lung histology

Sections were stained with haematoxylin/eosin to assess inflammatory infiltrates and with Periodic Acid - Schiff (PAS) to detect mucin in goblet cells. Examining the entire slide surface, the following inflammatory parameters were scored by an experienced histopathologist in a blinded fashion, exactly as described previously [36]: perivascular inflammation, interstitial inflammation, peribronchial inflammation, and edema. Each parameter was graded in a semi-quantitative fashion on a scale of 0 to 4 (0: absent, 1: mild, 2: moderate, 3: severe, 4: very severe). The total pathology score was expressed as the sum of the score for all parameters. In a similar way PAS-staining for polysaccharides in mucus was performed to quantify the amount of mucus. The amount of mucus per lung section was scored semi-quantitatively on a scale of 0-3. Eosinophils were quantified in lung tissue stained by an antibody against major basic protein (MBP; kindly provided by Dr Nancy Lee and Professor James Lee, Mayo Clinic Arizona, Scottsdale, AZ, USA). Phosphorylated ERK1/2 staining was performed using a pERK1/2-specific antibody (Cell Signalling). Morphometric analyses of sections were performed using Leica QWin V3 software.

Assessment of BALF inflammatory cells

BALF was collected and flow cytometric analysis was performed as described before [37].

Measurement of total protein, cytokines and chemokines

Total protein in BALF was measured by standard Bradford's assay as described before [23]. Cytokine levels in BALF were measured by ELISA for IL-5 and IL-13 (Ready-set-go!, eBioscience Inc) and Eotaxin-1 (Qiagen) and Eotaxin-2 (R&D Biosystems). TNF α and IL-6 were measured in BALF and cell culture supernatants using the Cytometric Bead Array Mouse Inflammation Kit (BD Biosciences).

Ex vivo restimulation of lung draining lymph node cells

Lung draining lymph nodes were harvested and cell suspensions were made with cell-strainers. Cells were plated in 96-well round bottom plates at a density of 2×10^5 cells per well and were stimulated for 4 days with 10 μ g/ml OVA (Worthington, Lakewood, NJ) or PBS. Supernatants were analysed for IL-5 and IL-13 production by ELISA.

Serum OVA-specific Immunoglobulins

Serum was analysed to assess the level of total and OVA specific IgE and IgG1 by ELISA (Opteia, BD, San Diego, USA). We used a standard curve of murine IgE and IgG1 respectively, as a quantitative reference, respectively.

Cell culture, Lentiviral transduction, Semi-quantitative RT-PCR, Western blot analysis and PAS staining

Human alveolar epithelial carcinoma (NCI-H292) cells were grown and maintained in RPMI 1640 medium containing 10% FBS and 1% penicillin/streptomycin. Cells were infected with recombinant lentivirus as described previously [23]. Cells were serum-starved for 36 h and stimulated with TNF α (50ng/ml) or EGF (50ng/ml) for indicated time periods. For qRT-PCR experiments, cells were stimulated with TNF α for 6h before harvesting. In some experiments, cells were pre-treated overnight with PD98059 (ERK1/2 inhibitor) at a final concentration of 25 μ M. For PAS staining, the cells were infected with FHL2 lentivirus followed by serum-starvation for 24 h and then cells were stimulated with TNF α (50ng/ml) for 48h. Mucus glycoconjugates were visualized by PAS staining.

Single nucleotide polymorphism (SNP) association with asthma and severity of bronchial hyper-responsiveness (BHR)

For single nucleotide polymorphism (SNP) analyses asthma patients were recruited from the Dutch Asthma GWAS (DAG)-cohort. All available SNPs in *FHL2* in the DAG cohort were tested for an association with asthma and severity of bronchial hyperresponsiveness (BHR).

In the DAG cohort all 920 asthmatics had a physician's diagnosis of asthma, asthma symptoms, and BHR to either histamine or methacholine. The 980 controls had no asthma or chronic obstructive pulmonary disease COPD, nor any evidence of significant airway obstruction. Inclusion criteria for the association with severity of BHR were as follows: cases had a doctor diagnosis of asthma in combination with BHR with data available on smoking and inhaled corticosteroids use at the time of the test. In total 650 asthmatics were included in this analysis. Severity of BHR was measured with a slope. This slope was calculated by dividing the difference between forced expiratory volume in one second (FEV1) at baseline and the dose step at which a $\geq 20\%$ fall in FEV1 was reached. We divided the BHR slopes of the 30-second tidal breathing method by 4 in order to compare the slope of the 30-second tidal breathing method with the 2-minute method. Values were log transformed to reach normal distribution.

DNA of subjects was genotyped on the Illumina 317 Chip (Illumina Inc, San Diego, CA) or with the Illumina 370 Duo Chip. Quality control was applied; subjects were removed from analysis if they were not of Caucasian descent, had a low genotyping call rate ($< 95\%$) or were discrepant or ambiguous for genetic sex. SNPs were deleted if the call rates were low ($< 95\%$), not in Hardy-Weinberg Equilibrium ($p < 1E-04$), or if the minor allele frequency was < 0.05 . After quality control 294,932 SNPs were selected for the analysis. All statistical analyses have been performed using PLINK v1.07. Logistic regression analyses in additive models were performed to analyze the associations between *FHL2* SNPs and asthma. Linear regression analyses were performed in additive models to analyze the association of *FHL2* SNPs on slopes of the BHR test with adjustments for smoking and inhaled and/or oral corticosteroid use.

Lung eQTL analysis

SNPs with a significant association with asthma and/or severity of BHR were selected for expression quantitative trait loci (eQTL) analysis. Lung tissues used to identify eQTLs was from 1,111 human subjects who underwent lung surgery at three academic sites, Laval University, University of British Columbia (UBC), and University of Groningen [38]. Genotyping was carried out using the Illumina Human1M-Duo BeadChip. Gene expression profiling was performed using an Affymetrix custom array (see GEO platform GPL10379). In this study, two probe sets testing the mRNA expression levels of the *FHL2* gene were analysed. Adjustments were made for age, gender, smoking and all other lung diseases like cystic fibrosis, chronic obstructive pulmonary disease and idiopathic pulmonary fibrosis. Data of the three centers were analyzed separately and subsequently meta-analyzed. All analyses were performed using R.

Statistical analysis

Statistical analyses for cell and mouse experiments were carried out with GraphPad Prism software (GraphPad Software, San Diego, Calif) and graphing was carried out with Adobe illustrator (Adobe). Comparisons between 2 groups were done with the Student t test for unpaired variables. Data are reported as mean $\pm$ SEM unless otherwise specified. P values < 0.05 were considered as statistically significant.

Results

Deficiency of *FHL2* attenuates infiltration of inflammatory cells in lungs of OVA-challenged mice

To investigate the potential role of *FHL2* in OVA-induced airway allergic inflammation, wild-type (WT) and *FHL2*-KO mice were sensitized and challenged with OVA. BALF was collected 48h after the last OVA aerosol challenge. Assessment of OVA-induced airway inflammation via enumeration of total and differential cell counts in the BALF revealed that the OVA challenge significantly increased total cell, eosinophil, and lymphocyte counts in WT mice as compared to saline control (Fig. 1). Among the inflammatory cell populations, eosinophils were most predominant, followed by macrophages, T-cells, dendritic cells, and B-cells (Fig. 1). The total number of inflammatory cells including the number of eosinophils, dendritic cells, T-cells and B-cells in OVA-challenged *FHL2*-deficient mice were

significantly lower than in OVA-challenged WT mice. There was no difference found in the number of macrophages between WT and FHL2-KO mice after OVA-challenge (Fig. 1E). These results clearly suggest a possible role of FHL2 in eosinophil, dendritic cell and lymphocyte recruitment during allergic inflammation.

FHL2-KO mice show reduced OVA-induced allergic airway inflammation

Inflammation is a major hallmark of allergic airway disease [1-3]. Evaluation of lung histopathology revealed infiltration of inflammatory cells in both WT and FHL2-KO mice following OVA challenge (Fig. 2A). However, OVA-challenged FHL2-KO mice showed a significant decrease in inflammation compared to WT mice (Fig. 2A). Semi-quantitative scoring also revealed that peribronchial inflammation, perivascular inflammation and total inflammation were significantly lowered in FHL2-KO (Fig. 2B-D). Another clinical manifestation of allergic airway disease is the development of mucus cell hyperplasia [16;17]. Assessment of mucus secretion by Periodic Acid Schiff (PAS) staining in the lung sections of WT and FHL2-KO mice challenged with OVA revealed a marked decrease in mucus-producing cells in the proximal airways of FHL2-KO compared to WT mice (Fig. 2E). There were no or very few PAS-positive cells in the proximal airways of saline challenged mice (data not shown). Semi-quantitative scoring also confirmed these results (Fig. 2F), suggesting that FHL2 plays a dominant role in regulation of mucus cell hyperplasia.

Reduced eosinophil accumulation and eotaxin levels in FHL2-KO mice

Eosinophils in the lung tissue were detected by MBP staining, which was quantified by digital imaging and expressed as eosinophils per area (Fig. 3A-B). Consistent with the BALF data, OVA challenge caused an increase in pulmonary eosinophils in WT mice compared to saline control (Fig. 3B). The number of eosinophils in lung tissue of OVA-challenged FHL2-KO mice was substantially decreased compared to WT mice, corroborating the findings in BALF (Fig. 3A-B). Since FHL2-KO mice showed a strong defect in eosinophil recruitment to the lung upon OVA challenge, we determined whether FHL2 deficiency is also associated with impaired production of key chemokines implicated in eosinophil recruitment: Eotaxin-1 and Eotaxin-2. Eotaxin-2 is the major eotaxin isoform recruiting eosinophils into murine airways because this chemokine is highly produced 24 hours after an allergen challenge [9;11;39;40]. In line with the lung histology and BALF data of eosinophils, Eotaxin-1 and Eotaxin-2 protein levels in BALF were significantly increased in OVA-challenged WT mice compared to saline control (Fig. 3C-D). We also observed higher levels of Eotaxin-2 than Eotaxin-1 in BALF of OVA-challenged WT mice (Fig. 3C-D). In contrast to WT mice, FHL2-KO mice failed to produce increased levels of both Eotaxin-1 and Eotaxin-2 after OVA-challenge. Taken together, these data indicate that FHL2 plays an important role in pulmonary chemokine synthesis for recruitment of eosinophils after OVA-challenge.

Effect of FHL2 deficiency on cytokines in BALF

To test the hypothesis that the reduced eosinophilia in the FHL2-KO mice was due to an altered allergic cytokine response, BALF was examined for a variety of potentially relevant cytokines. First, we measured total protein levels showing that FHL2-KO had a significantly lower amount of protein in BALF compared to WT after OVA challenge (Fig. 4A). IL-5, IL-13, TNF α , and IL-6 protein levels in BALF were significantly increased upon OVA challenge in WT mice compared to saline control (Fig. 4B-E). OVA-challenged FHL2-KO mice had a striking reduction in IL-5, IL-13, TNF α , and IL-6 protein levels compared to WT (Fig. 4B-E).

FHL2-KO mice show decreased OVA-specific IgE and IgG1 levels

To investigate whether the inhibition of Th2-mediated allergic airway inflammation in FHL2-KO mice was accompanied by a suppressed humoral immune response, we determined the levels of IgE and IgG1. Total IgE and IgG1 levels in serum were increased after OVA challenge in both WT and FHL2-KO mice (Fig. 5A,C). OVA-specific IgE and IgG1 levels were significantly attenuated in FHL2-KO compared to WT mice after OVA challenge (Fig. 5B,D).

Attenuation of inflammatory cytokine response in the lymph node cells of FHL2-KO

To determine whether attenuated Th2 cell secretion in OVA-challenged mice was reflected at the level of systemic sensitization, we isolated and cultured lung draining lymph node cells from WT and FHL2-KO mice and examined IL-5 and IL-13 levels in the supernatants after *ex-vivo* stimulation with OVA. We noticed that OVA challenge in WT lymph node cells resulted in high levels of IL-5 and IL-13 compared with the saline control (Fig. 6). Consistent with the BALF data, FHL2-KO lymph node cells displayed lower production of IL-5 and IL-13 compared to WT when stimulated *ex vivo* with OVA (Fig. 6A-B). Taken together, the decreased levels of these key cytokines and chemokines may explain, at least partly, the attenuated lung eosinophilia in FHL2-KO mice.

Knock-down of FHL2 reduces inflammation in human lung epithelial cells

To further substantiate our *in vivo* findings, human lung epithelial cells (NCI-H292) were depleted of FHL2 using lentivirus of shRNA targeting FHL2 to investigate cytokine and chemokine release after TNF α stimulation. TNF α stimulation enhanced the expression of several pro-inflammatory cytokines in the control shRNA-infected cells (Fig. 7). In line with our *in vivo* observations, FHL2 knockdown reduced mRNA expression of TNF α and RANTES and protein levels of TNF α and IL-6 (Fig. 7A-D).

FHL2 regulates MUC5AC expression through pERK1/2

Previous reports by others and us revealed that FHL2 binds and regulates ERK1/2 [27;33]. ERK signalling has been proposed to be highly involved in the pathogenesis of asthma [20]. Therefore, we sought to explore the impact of FHL2 on ERK signalling in OVA-challenged WT and FHL2-KO mice. Lung histopathology showed that the number of positive cells of phosphorylated (p)ERK1/2 per area was significantly reduced in OVA-challenged FHL2-KO compared to WT mice (Fig. 8A). To corroborate these *in vivo* findings, we performed Western blotting for pERK1/2 after FHL2 knockdown in NCI-H292 cells. Cells were stimulated with EGF for the indicated time points. Consistent with our *in vivo* observation, knockdown of FHL2 reduced phosphorylation of ERK1/2 compared to control, whereas total ERK1/2 protein levels were not affected (Fig. 8B).

Having established that FHL2 deficiency reduced mucus cell hyperplasia *in vivo*, we sought to examine the effect of FHL2 on expression levels of MUC5AC, which is a key player in mucus production in airway inflammation [41]. Both gain and loss of function experiments were performed in NCI-H292 cells using lentiviruses of FHL2 and shRNA targeting FHL2, respectively. Cells were stimulated with TNF α . qRT-PCR analysis showed that knock-down of FHL2 decreased mRNA expression of MUC5AC in both non-stimulated and stimulated conditions (Fig. 8C). In contrast, over-expression of FHL2 significantly enhanced the mRNA expression of MUC5AC in both non-stimulated and stimulated conditions (Fig. 8D). To substantiate these findings, PAS staining was performed on the lung epithelial cells after over-expression of FHL2. In line with increased MUC5AC gene expression, over-expression of FHL2 strongly enhanced TNF α -induced mucus production (Fig. 8E), further substantiating the role of FHL2 in the regulation of mucus production.

It has been demonstrated that ERK-signalling regulates MUC5AC gene expression in epithelial cells [42]. To determine whether FHL2 modulates MUC5AC gene expression through ERK-signalling, an ERK1/2 inhibitor was employed. Consistent with published reports, mRNA levels of MUC5AC were significantly inhibited upon blocking of ERK-signalling. Furthermore, inhibition of ERK-signalling also significantly

mitigated the FHL2-mediated induction of MUC5AC gene expression, indicating that FHL2 regulates MUC5AC gene expression through ERK-signalling (Fig. 8D). A schematic overview of our data on FHL2 function in airway inflammation is outlined in Fig. 8F.

SNP association with asthma and severity of bronchial hyper-responsiveness (BHR) and lung eQTL analysis

In total of 12 *FHL2* SNPs were available in the DAG cohort and analyzed for association with asthma and severity of BHR. No significant association between SNPs in *FHL2* and asthma was found (Table 1). One significant association was found between SNP rs4851765 and the severity of BHR in asthma. Every added C allele of SNP rs4851765 was associated with less severe BHR (beta (CI95%)= -0.22 (-0.40;-0.03) p=0.02). The SNP rs4851772 in *FHL2* showed a near-significant (p=0.06) association with BHR (Table 2).

Two probe sets were available to test the mRNA expression of FHL2 in the lung eQTL dataset. Rs4851765 was analyzed for association with the expression of both probe sets. No significant association was found as shown in Table 3, suggesting that every added C allele was not associated with a change in gene expression of FHL2 in the lung.

Discussion

Allergic asthma is a complex inflammatory disorder characterized by bronchial hyperresponsiveness, mucus hypersecretion, airway inflammation and eventually airway obstruction[1-3]. The molecular events that contribute to allergic airway inflammation are poorly understood. In this study we utilized FHL2-deficient mice to establish the role of FHL2 in development of allergic airway inflammation using the model of OVA-induced allergic airway inflammation. We demonstrated that FHL2 deficiency significantly attenuates allergic airway inflammation along with reduced infiltration of inflammatory cells, mucus hypersecretion, production of allergen specific IgE and IgG1, Th2 cytokines, eotaxins and phosphorylation of ERK1/2. Furthermore, gain and loss of function studies in cultured human airway epithelial cells demonstrated that FHL2 regulates cyto/chemokine and MUC5AC gene expression, the latter gene through modulation of the ERK1/2 pathway.

OVA-challenged FHL2-KO mice have significantly lower numbers of inflammatory cells, predominantly eosinophils, in BALF compared to WT mice. Furthermore, reduced extravasation of eosinophils was observed in the perivascular, peribronchial, and parenchymal tissues of the lungs of OVA-challenged FHL2-KO mice. Eosinophils play a crucial role in the pathogenesis of allergic inflammation [1;2;4]. The process of eosinophil infiltration into the airways is orchestrated by Th2 cytokines and is coordinated by specific chemokines such as eotaxins [6;8]. Eotaxins are potent chemokines, which induce the accumulation of eosinophils *in vivo* [43]. Th2 cytokine levels in BALF reflect the combined production by Th2 cells and various resident cells such as bronchial epithelial cells and alveolar macrophages, as well as infiltrated eosinophils and lymphocytes. Our present data demonstrate that OVA-challenged FHL2-KO mice display significantly reduced levels of IL-5, IL-6, IL-13, TNF α and eotaxins in BALF compared to WT mice. In addition, expression of IL-5 and IL-13 is decreased in lung draining lymph node cells of OVA-challenged FHL2-KO mice compared to WT cells. FHL2 thus plays a critical role in the regulation of eosinophil migration, however, whether FHL2 affects eosinophils themselves has not been studied so far and requires further research.

Transforming growth factor beta 1 (TGF- β) induces FHL2 expression [44]. TGF- β inhibits the synthesis of GM-CSF and IL-5 by eosinophils and modulates survival and apoptosis of these cells. One may propose that TGF- β -induced FHL2 affects eosinophils, but so far, no direct implication of FHL2 in eosinophils was reported. It will be intriguing to study the role of FHL2 in eosinophils and eosinophilia. Interestingly, FHL2-deficiency results in a trend towards enhanced production of IgE, IgG1, IL-5 and IL-

13 under basal conditions, suggesting that FHL2 is involved in the regulation of expression of these immunoglobulins and cytokines. It will be of interest to study the function of FHL2 in the regulation of IgE, IgG1, IL-5, and IL-13 in further detail.

Disruption of the FHL2 gene also causes reduced influx of dendritic cells and lymphocytes, but not macrophages in BALF, upon OVA challenge. The role for dendritic cells and lymphocytes has been extensively studied in allergic airway inflammation [4;8]. Previously, FHL2-deficient dendritic cells were shown to migrate faster to CCL19 and *in vivo* towards lymphoid organs than WT cells, which was explained by enhanced sphingosine-1-phosphate receptor 1 (S1P-R1) expression [34]. A potential explanation for the discrepancy with our current study may include the fact that the earlier migration studies were performed in healthy WT mice and that only migration of the cells to lymph nodes was monitored, whereas here the migration of dendritic cells towards lungs was determined in diseased FHL2-KO mice. Interestingly, FHL2 is also known to inhibit the activity of sphingosine kinase 1 in cardiomyocytes and endothelial (progenitor) cells [45]. This may implicate that S1P levels are increased in FHL2-KO mice, again not corroborating with, and thus not explaining the observed reduction in cellular influx in the BALF.

Consistent with a reduced Th2 response, FHL2-KO mice showed impaired production of allergen-specific IgG1 and IgE production. Furthermore, we found that knockdown of FHL2 in airway epithelial cells results in reduced expression levels of TNF α , IL-6, and RANTES. FHL2 has been shown to have both pro-and anti-inflammatory effects depending on the cell-type. For example, inflammation in liver regeneration and carcinogenesis is promoted by FHL2 [32], as well as IL-6 expression in skeletal myoblasts. The latter study showed involvement of FHL2 in increased NF κ B and p38 MAPK signaling. Similar as in the current study however, FHL2 inhibits inflammation in lung following bleomycin treatment [25]. Taken together, in airway inflammation FHL2 seems to aggravate disease by enhancing the production of Th2 cytokines and eotaxins.

Previous studies demonstrated that ERK activity is significantly higher in asthmatic mice compared to control mice and that inhibition of ERK signaling markedly diminishes OVA-induced airway inflammation and asthma [20]. ERK activation has been implicated in the induction of cytokine production from a variety of cell types including airway epithelial cells, dendritic cells, smooth muscle cells and T-cells [46]. Moreover, we and others showed that FHL2 binds and regulates ERK [27;33]. As such, the observed reduction of Th2 cytokine levels in BALF and draining lymph nodes from OVA-challenged FHL2-KO mice may be due to the observed inhibition of the ERK signaling pathway in lung.

In this study we demonstrated that FHL2 plays a pivotal role in regulating mucus hypersecretion in airway epithelium, which is an important pathophysiologic feature of allergic airway inflammation [16;17]. OVA-challenged FHL2-KO mice displayed a dramatic reduction in mucus production with less goblet cell hyperplasia compared to WT counterparts. Previous reports suggest that the ERK pathway regulates mucus production through MUC5AC [42]. Given that FHL2 regulates ERK signalling, we hypothesized that FHL2 might modulate mucus secretion through regulation of MUC5AC. Indeed, gain of function studies in epithelial cells showed that FHL2 promotes MUC5AC gene expression through enhanced activation of ERK1/2, whereas knockdown of FHL2 reduced MUC5AC expression. Taken together, the observed reduction in mucus production in FHL2-KO mice may be attributed to the substantial reduction in MUC5AC expression through inhibition of ERK activity.

Based on our GWAS data in the DAG-cohort, no association was observed of FHL2 with asthma; however, severity of the disease does show association with an intronic SNP in the FHL2 gene. We deliberately did not correct for multiple testing given the a prior evidence of the FHL2 gene in BHR. However, since the p-value for the association of the FHL2 SNP with BHR is 0.02, we should be careful concluding that FHL2 is functionally involved in human asthma and further research is required. The

functional effect of this SNP, or SNPs in LD with this SNP is not known, but does not seem to involve an effect on lung gene expression. The FHL2 SNP rs4851765 is located in an intron of the FHL2 gene. Intronic SNPs may have regulatory functions for a specific transcript, however, this regulatory function has not been assigned so far and future work should assess this in more detail.

In conclusion, our study demonstrates for the first time that FHL2 deficiency effectively decreased OVA-induced Th2 cytokine production, pulmonary eosinophilia, serum IgE and IgG1 levels, and mucus hypersecretion in a model of murine airway inflammation. Furthermore, the FHL2 SNP rs4851765 shows an association with BHR in human, but will require replication. Collectively, our data provide compelling evidence that FHL2 exacerbates airway inflammation and may be a novel marker to identify individuals at risk for asthma or alternatively, provide an innovative target to inhibit airway inflammation in patients with asthma.

Author contributions

K. K. and C. J. d. V designed the study; K. K. performed the experiments together with A. L. and L. v. R. M. V. assisted with sensitizing and dissecting the mice and also helped with histological analysis. A. L. helped with ELISAs. J. J. R. helped with histological analysis and pathology scoring. M.A.N, G. H. K, D. S. P, C. A. B, D. D. S, Y. B and D. C. N performed SNP and Lung eQTL analysis. C. J. d. V and L. v. R provided supervision during the study. K. K. analyzed the data and drafted the manuscript with input from C. J. d. V. All authors contributed to and approved the final version of the manuscript.

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Conflict of interest: None declared.

FIGURE LEGENDS

FIGURE 1. Attenuated allergen-driven airway inflammation in OVA-challenged FHL2-KO mice. BALF was collected 48h after the last challenge from OVA- and saline-challenged WT and FHL2-KO mice (n=8 per group). Differential inflammatory cell count analysis for eosinophils (a), dendritic cells (b), B-cells (c), T-cells (d), and macrophages (e) was performed by flow cytometry. There was a progressive increase in the number of inflammatory cells after OVA-challenge in WT mice. The numbers of eosinophils, dendritic cells, B-cells, and T- cells, except macrophages were significantly lower in FHL2-KO mice compared to WT mice. Data are mean±SEM. *, $P \leq 0.05$.

FIGURE 2. FHL2-KO mice show reduced infiltration of inflammatory cells and mucus production after OVA-challenge. a) Histological examination of lung tissue from the OVA-challenged WT and FHL2-KO mice (n = 8) were stained with H&E and examined by light microscopy. b-d) Semi-quantitative pathology scores (described in Materials and methods) for peribronchial inflammation (b), perivascular inflammation (c) and total inflammation (d). e) Representative photomicrographs of PAS-stained lung sections from WT and FHL2-KO mice following OVA-challenge. f) Semi-quantitative mucus score (described in Materials and methods). Data are mean±SEM. *, P≤0.05.

FIGURE 3. FHL2-KO mice demonstrate reduced influx of eosinophils in lung and eotaxin levels in BALF after OVA challenge. a) Representative photomicrographs of MBP-stained lung sections for detection of eosinophils from WT and FHL2-KO mice following saline or OVA-challenge. b) Quantification of MBP-positive cells/area in WT and FHL2-deficient mice. Eotaxin-1 (c) and Eotaxin-2 (d) protein concentrations in BALF were measured by ELISA. Data are mean±SEM (n=8 per group). *, P≤0.05; ns, non-significant.

FIGURE 4. Disruption of FHL2 attenuates cytokines production in BALF. a) FHL2-KO mice have less protein leakage in BALF upon OVA-challenge. The expression of IL-5 (b), IL-13 (c), TNFα (d), and IL-6 (e) was quantified by ELISA. Data are mean±SEM (n=8 per group). *, P≤0.05. ns, non-significant.

FIGURE 5. FHL2 deficiency attenuates production of IgE and IgG1 after OVA-challenge. Total IgE (a), OVA-specific IgE (b), Total IgG1 (c), and OVA-specific IgG1 (d) levels in serum obtained 48h after saline or OVA-challenge. Data are mean±SEM. *, P≤0.05.

FIGURE 6. Attenuation of inflammatory cytokine response in the lymph node cells of FHL2-KO mice. IL-5 (a) and IL-13 (b) secretion by *ex vivo* cultures of draining lymph node cells that had been restimulated with or without OVA was quantified by ELISA. Data are mean±SEM (n=8 per group). *, P≤0.05.

FIGURE 7. FHL2 knock-down attenuates inflammation in lung epithelial cells. a-b) FHL2 was knocked-down in NCI-H292 lung epithelial cells using lentivirus of shRNA targeting FHL2 and cells were stimulated with or with TNFα. qRT-PCR analysis was performed for TNFα (a) and RANTES (b). c-d) Expression levels of TNFα (c) and IL-6 (d) were quantified in the supernatants of lung epithelial cells after FHL2 knock-down by ELISA. Each experiment (in triplicate) was repeated at least three times. Data are mean±SD. *, P≤0.05.

FIGURE 8. FHL2 regulates MUC5AC expression through phosphorylated (p)ERK1/2. a) Quantification of pERK1/2 positive cells/area of lung tissue sections from the OVA- challenged WT and FHL2-KO mice that were stained for pERK1/2. Data are mean±SEM (n=8 per group). *, P≤0.05. b) Western blot analysis for pERK1/2 in NCI-H292 lung epithelial cells after FHL2 knockdown and stimulation with EGF for the indicated time points. Total ERK1/2 served as a loading control. c) qRT-PCR analysis was performed for MUC5AC after FHL2 knock-down and stimulation with or without TNFα in NCI-H292 cells. d) qRT-PCR analysis was performed for MUC5AC after FHL2 over-expression and treatment with or without TNFα and an ERK1/2 inhibitor (PD98059, 25μM) in NCI-H292 cells. e) PAS staining was performed on lung epithelial cells after over-expression of FHL2 and stimulation with TNFα. Each experiment (in triplicate) was repeated at least two times. Data are mean±SD. *, P≤0.05. f) A schematic summary of FHL2 function in airway inflammation.

Table 1:
Association between genetic variants in the *FHL2* gene and asthma in the DAG cohort

CHR	BP	SNP	Allele	OR (fixed)	OR (random)	P (fixed)	P (random)	Q
2	105345938	rs2278501	T	0.94	0.94	0.31	0.54	0.11
2	105349185	rs2576778	A	1.06	1.06	0.46	0.46	0.93
2	105351660	rs880427	A	0.97	0.97	0.67	0.67	0.35
2	105365441	rs4640402	C	1.00	1.00	0.97	0.97	0.27
2	105379064	rs4851765	C	1.00	1.00	0.98	0.98	0.52
2	105390883	rs4374396	G	1.02	1.02	0.80	0.80	0.35
2	105398723	rs2376740	T	1.00	1.00	0.99	0.99	0.47
2	105402012	rs1914748	T	1.06	1.06	0.40	0.40	0.94
2	105412765	rs4851770	T	0.97	0.97	0.59	0.72	0.16
2	105413221	rs6750100	G	1.06	1.06	0.41	0.59	0.14
2	105418388	rs4851772	G	1.02	1.02	0.85	0.85	0.96
2	105419775	rs7583367	T	1.07	1.07	0.28	0.28	0.36

Table 2:
Association between genetic variants in the *FHL2* gene and severity of BHR in the DAG cohort

CHR	SNP	BP	Allele	Beta	(CI 95%)	P
2	rs2278501	105345938	T	0.13	(-0.04;0.29)	0.14
2	rs2576778	105349185	A	-0.17	(-0.39;0.04)	0.11
2	rs880427	105351660	A	0.07	(-0.11;0.25)	0.43
2	rs4640402	105365441	C	-0.16	(-0.34;0.02)	0.07
2	rs4851765	105379064	C	-0.22	(-0.40;-0.03)	0.02
2	rs4374396	105390883	G	0.00	(-0.17;0.18)	0.98
2	rs2376740	105398723	T	-0.07	(-0.24;0.11)	0.45
2	rs1914748	105402012	T	-0.01	(-0.18;0.16)	0.90
2	rs4851770	105412765	T	-0.01	(-0.18;0.17)	0.94
2	rs6750100	105413221	G	-0.09	(-0.28;0.11)	0.38
2	rs4851772	105418388	G	0.27	(-0.01;0.55)	0.06
2	rs7583367	105419775	T	-0.06	(-0.24;0.11)	0.47

In bold the significant p-values

Table3:

eQTL analyses for BHR-associated SNP rs4851765 with two probe sets testing the mRNA expression levels of *FHL2* in human lung tissues

SNP	Allele	Gene	Chr	Basepair position start	Basepair position end	Cohort	Beta	Se	P-value
rs4851765	C	<i>FHL2</i>	2	105343254	105344297	Groningen	0.03	0.03	0.32
						UBC	0.01	0.03	0.64
						Laval	0.006	0.02	0.23
						<i>meta</i>	0.01	0.01	0.39
rs4851765	C	<i>FHL2</i>	2	105343716	105421392	Groningen	0.03	0.04	0.35
						UBC	-0.003	0.03	0.92
						Laval	0.01	0.03	0.66
						<i>meta</i>	0.009	0.02	0.62

Reference List

- [1] Elias JA, Lee CG, Zheng T, Ma B, Homer RJ, Zhu Z: New insights into the pathogenesis of asthma. J Clin Invest 2003;111:291-297.
- [2] Renaud JC: New insights into the role of cytokines in asthma. J Clin Pathol 2001;54:577-589.
- [3] Wills-Karp M: Immunologic basis of antigen-induced airway hyperresponsiveness. Annu Rev Immunol 1999;17:255-281.
- [4] Holgate ST, Arshad HS, Roberts GC, Howarth PH, Thurner P, Davies DE: A new look at the pathogenesis of asthma. Clin Sci (Lond) 2010;118:439-450.
- [5] Cohn L, Homer RJ, Marinov A, Rankin J, Bottomly K: Induction of airway mucus production By T helper 2 (Th2) cells: a critical role for interleukin 4 in cell recruitment but not mucus production. J Exp Med 11-17-1997;186:1737-1747.
- [6] Herrick CA, Bottomly K: To respond or not to respond: T cells in allergic asthma. Nat Rev Immunol 2003;3:405-412.
- [7] Robinson DS, Hamid Q, Ying S, Tsicopoulos A, Barkans J, Bentley AM, Corrigan C, Durham SR, Kay AB: Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. N Engl J Med 1-30-1992;326:298-304.
- [8] Umetsu DT, DeKruyff RH: TH1 and TH2 CD4+ cells in human allergic diseases. J Allergy Clin Immunol 1997;100:1-6.
- [9] Lloyd C: Chemokines in allergic lung inflammation. Immunology 2002;105:144-154.
- [10] Ono SJ, Nakamura T, Miyazaki D, Ohbayashi M, Dawson M, Toda M: Chemokines: roles in leukocyte development, trafficking, and effector function. J Allergy Clin Immunol 2003;111:1185-1199.

- [11] Pope SM, Zimmermann N, Stringer KF, Karow ML, Rothenberg ME: The eotaxin chemokines and CCR3 are fundamental regulators of allergen-induced pulmonary eosinophilia. *J Immunol* 10-15-2005;175:5341-5350.
- [12] Barry J, Loh Z, Collison A, Mazzone S, Lalwani A, Zhang V, Davidson S, Wybacz E, Garlanda C, Mantovani A, Mattes J, Foster PS, Phipps S: Absence of Toll-IL-1 receptor 8/single immunoglobulin IL-1 receptor-related molecule reduces house dust mite-induced allergic airway inflammation in mice. *Am J Respir Cell Mol Biol* 2013;49:481-490.
- [13] An SS, Bai TR, Bates JH, Black JL, Brown RH, Brusasco V, Chitano P, Deng L, Dowell M, Eidelman DH, Fabry B, Fairbank NJ, Ford LE, Fredberg JJ, Gerthoffer WT, Gilbert SH, Gosens R, Gunst SJ, Halayko AJ, Ingram RH, Irvin CG, James AL, Janssen LJ, King GG, Knight DA, Lauzon AM, Lakser OJ, Ludwig MS, Lutchen KR, Maksym GN, Martin JG, Mauad T, McParland BE, Mijailovich SM, Mitchell HW, Mitchell RW, Mitzner W, Murphy TM, Pare PD, Pellegrino R, Sanderson MJ, Schellenberg RR, Seow CY, Silveira PS, Smith PG, Solway J, Stephens NL, Sterk PJ, Stewart AG, Tang DD, Tepper RS, Tran T, Wang L: Airway smooth muscle dynamics: a common pathway of airway obstruction in asthma. *Eur Respir J* 2007;29:834-860.
- [14] Dowell ML, Lavoie TL, Solway J, Krishnan R: Airway smooth muscle: a potential target for asthma therapy. *Curr Opin Pulm Med* 2014;20:66-72.
- [15] Hershenson MB, Brown M, Camoretti-Mercado B, Solway J: Airway smooth muscle in asthma. *Annu Rev Pathol* 2008;3:523-555.
- [16] Kim WD: Lung mucus: a clinician's view. *Eur Respir J* 1997;10:1914-1917.
- [17] Rose MC, Voynow JA: Respiratory tract mucin genes and mucin glycoproteins in health and disease. *Physiol Rev* 2006;86:245-278.
- [18] Reid CJ, Gould S, Harris A: Developmental expression of mucin genes in the human respiratory tract. *Am J Respir Cell Mol Biol* 1997;17:592-598.
- [19] Thai P, Loukoianov A, Wachi S, Wu R: Regulation of airway mucin gene expression. *Annu Rev Physiol* 2008;70:405-429.
- [20] Duan W, Chan JH, Wong CH, Leung BP, Wong WS: Anti-inflammatory effects of mitogen-activated protein kinase kinase inhibitor U0126 in an asthma mouse model. *J Immunol* 6-1-2004;172:7053-7059.
- [21] El MH, Muller S, Tunggal L, Paulsson M, Aumailley M: Analysis of the adaptor function of the LIM domain-containing protein FHL2 using an affinity chromatography approach. *J Cell Biochem* 6-1-2004;92:612-625.
- [22] Johannessen M, Moller S, Hansen T, Moens U, Van GM: The multifunctional roles of the four-and-a-half-LIM only protein FHL2. *Cell Mol Life Sci* 2006;63:268-284.
- [23] Kurakula K, van der Wal E, Geerts D, van Tiel CM, de Vries CJ: FHL2 protein is a novel co-repressor of nuclear receptor Nur77. *J Biol Chem* 12-30-2011;286:44336-44343.
- [24] Lange S, Auerbach D, McLoughlin P, Perriard E, Schafer BW, Perriard JC, Ehler E: Subcellular targeting of metabolic enzymes to titin in heart muscle may be mediated by DRAL/FHL-2. *J Cell*

Sci 12-15-2002;115:4925-4936.

- [25] Alnajar A, Nordhoff C, Schied T, Chiquet-Ehrismann R, Loser K, Vogl T, Ludwig S, Wixler V: The LIM-only protein FHL2 attenuates lung inflammation during bleomycin-induced fibrosis. *PLoS One* 2013;8:e81356.
- [26] Huang PH, Chen CY, Lin CP, Wang CH, Tsai HY, Lo WY, Leu HB, Chen JW, Lin SJ, Chu PH: Deletion of FHL2 gene impaired ischemia-induced blood flow recovery by modulating circulating proangiogenic cells. *Arterioscler Thromb Vasc Biol* 2013;33:709-717.
- [27] Kurakula K, Vos M, Otermin R, I, Marinkovic G, Buettner R, Heukamp LC, Stap J, de W, V, van Tiel CM, de Vries CJ: The LIM-Only Protein FHL2 Reduces Vascular Lesion Formation Involving Inhibition of Proliferation and Migration of Smooth Muscle Cells. *PLoS One* 2014;9:e94931.
- [28] Muller JM, Isele U, Metzger E, Rempel A, Moser M, Pscherer A, Breyer T, Holubarsch C, Buettner R, Schule R: FHL2, a novel tissue-specific coactivator of the androgen receptor. *EMBO J* 2-1-2000;19:359-369.
- [29] Xiong Z, Ding L, Sun J, Cao J, Lin J, Lu Z, Liu Y, Huang C, Ye Q: Synergistic repression of estrogen receptor transcriptional activity by FHL2 and Smad4 in breast cancer cells. *IUBMB Life* 2010;62:669-676.
- [30] Kurakula K, Sommer D, Sokolovic M, Moerland PD, Scheij S, van Loenen PB, Koenis DS, Zelcer N, van Tiel CM, de Vries CJ: LIM-only protein FHL2 is a positive regulator of liver X receptors in smooth muscle cells involved in lipid homeostasis. *Mol Cell Biol* 2015;35:52-62.
- [31] Bai S, Kitaura H, Zhao H, Chen J, Muller JM, Schule R, Darnay B, Novack DV, Ross FP, Teitelbaum SL: FHL2 inhibits the activated osteoclast in a TRAF6-dependent manner. *J Clin Invest* 2005;115:2742-2751.
- [32] Dahan J, Nouet Y, Jouvion G, Levillayer F, Adib-Conquy M, Cassard-Doulcier AM, Tebbi A, Blanc F, Remy L, Chen J, Cairo S, Werts C, Si-Tahar M, Tordjmann T, Buendia MA, Wei Y: LIM-only protein FHL2 activates NF-kappaB signaling in the control of liver regeneration and hepatocarcinogenesis. *Mol Cell Biol* 2013;33:3299-3308.
- [33] Purcell NH, Darwis D, Bueno OF, Muller JM, Schule R, Molkentin JD: Extracellular signal-regulated kinase 2 interacts with and is negatively regulated by the LIM-only protein FHL2 in cardiomyocytes. *Mol Cell Biol* 2004;24:1081-1095.
- [34] Konig K, Diehl L, Rommerscheidt-Fuss U, Golletz C, Quast T, Kahl P, Kolanus W, Knolle P, Buettner R, Heukamp LC: Four-and-a-half LIM domain protein 2 is a novel regulator of sphingosine 1-phosphate receptor 1 in CCL19-induced dendritic cell migration. *J Immunol* 8-1-2010;185:1466-1475.
- [35] Wixler V, Cromme C, Retser E, Meyer LH, Smyth N, Muhlenberg K, Korb-Pap A, Koers-Wunrau C, Sotsios Y, Bassel-Duby R, Baeten D, Tak PP, Niederreiter B, Redlich K, Bertrand J, Skryabin BV, Ludwig S, Pap T: FHL2 regulates the resolution of tissue damage in chronic inflammatory arthritis. *Ann Rheum Dis* 8-14-2014.
- [36] Daan de BJ, Roelofs JJ, de Vos AF, de BR, Schouten M, Hommes TJ, Hoogendijk AJ, de Boer OJ, Stroo I, van der Zee JS, Veer CV, van der Poll T: Lipopolysaccharide inhibits Th2 lung

inflammation induced by house dust mite allergens in mice. *Am J Respir Cell Mol Biol* 2013;48:382-389.

- [37] van Rijt LS, Kuipers H, Vos N, Hijdra D, Hoogsteden HC, Lambrecht BN: A rapid flow cytometric method for determining the cellular composition of bronchoalveolar lavage fluid cells in mouse models of asthma. *J Immunol Methods* 2004;288:111-121.
- [38] Hao K, Bosse Y, Nickle DC, Pare PD, Postma DS, Laviolette M, Sandford A, Hackett TL, Daley D, Hogg JC, Elliott WM, Couture C, Lamontagne M, Brandsma CA, van den Berge M, Koppelman G, Reicin AS, Nicholson DW, Malkov V, Derry JM, Suver C, Tsou JA, Kulkarni A, Zhang C, Vessey R, Opiteck GJ, Curtis SP, Timens W, Sin DD: Lung eQTLs to help reveal the molecular underpinnings of asthma. *PLoS Genet* 2012;8:e1003029.
- [39] Ben-Yehuda C, Bader R, Puxeddu I, Levi-Schaffer F, Breuer R, Berkman N: Airway eosinophil accumulation and eotaxin-2/CCL24 expression following allergen challenge in BALB/c mice. *Exp Lung Res* 2008;34:467-479.
- [40] Ying S, Robinson DS, Meng Q, Barata LT, McEuen AR, Buckley MG, Walls AF, Askenase PW, Kay AB: C-C chemokines in allergen-induced late-phase cutaneous responses in atopic subjects: association of eotaxin with early 6-hour eosinophils, and of eotaxin-2 and monocyte chemoattractant protein-4 with the later 24-hour tissue eosinophilia, and relationship to basophils and other C-C chemokines (monocyte chemoattractant protein-3 and RANTES). *J Immunol* 10-1-1999;163:3976-3984.
- [41] Evans CM, Kim K, Tuvim MJ, Dickey BF: Mucus hypersecretion in asthma: causes and effects. *Curr Opin Pulm Med* 2009;15:4-11.
- [42] Perrais M, Pigny P, Copin MC, Aubert JP, Van S, I: Induction of MUC2 and MUC5AC mucins by factors of the epidermal growth factor (EGF) family is mediated by EGF receptor/Ras/Raf/extracellular signal-regulated kinase cascade and Sp1. *J Biol Chem* 8-30-2002;277:32258-32267.
- [43] Griffiths-Johnson DA, Collins PD, Rossi AG, Jose PJ, Williams TJ: The chemokine, eotaxin, activates guinea-pig eosinophils in vitro and causes their accumulation into the lung in vivo. *Biochem Biophys Res Commun* 12-30-1993;197:1167-1172.
- [44] Zhang W, Jiang B, Guo Z, Sardet C, Zou B, Lam CS, Li J, He M, Lan HY, Pang R, Hung IF, Tan VP, Wang J, Wong BC: Four-and-a-half LIM protein 2 promotes invasive potential and epithelial-mesenchymal transition in colon cancer. *Carcinogenesis* 2010;31:1220-9.
- [45] Ebrahimian T, Arfa O, Simeone S, Lemarie CA, Lehoux S, Wassmann S: Inhibition of four-and-a-half LIM domain protein-2 increases survival, migratory capacity, and paracrine function of human early outgrowth cells through activation of the sphingosine kinase-1 pathway: implications for endothelial regeneration. *Circ Res* 1-3-2014;114:114-123.
- [46] Cui CH, Adachi T, Oyamada H, Kamada Y, Kuwasaki T, Yamada Y, Saito N, Kayaba H, Chihara J: The role of mitogen-activated protein kinases in eotaxin-induced cytokine production from bronchial epithelial cells. *Am J Respir Cell Mol Biol* 2002;27:329-335.

Figure 1

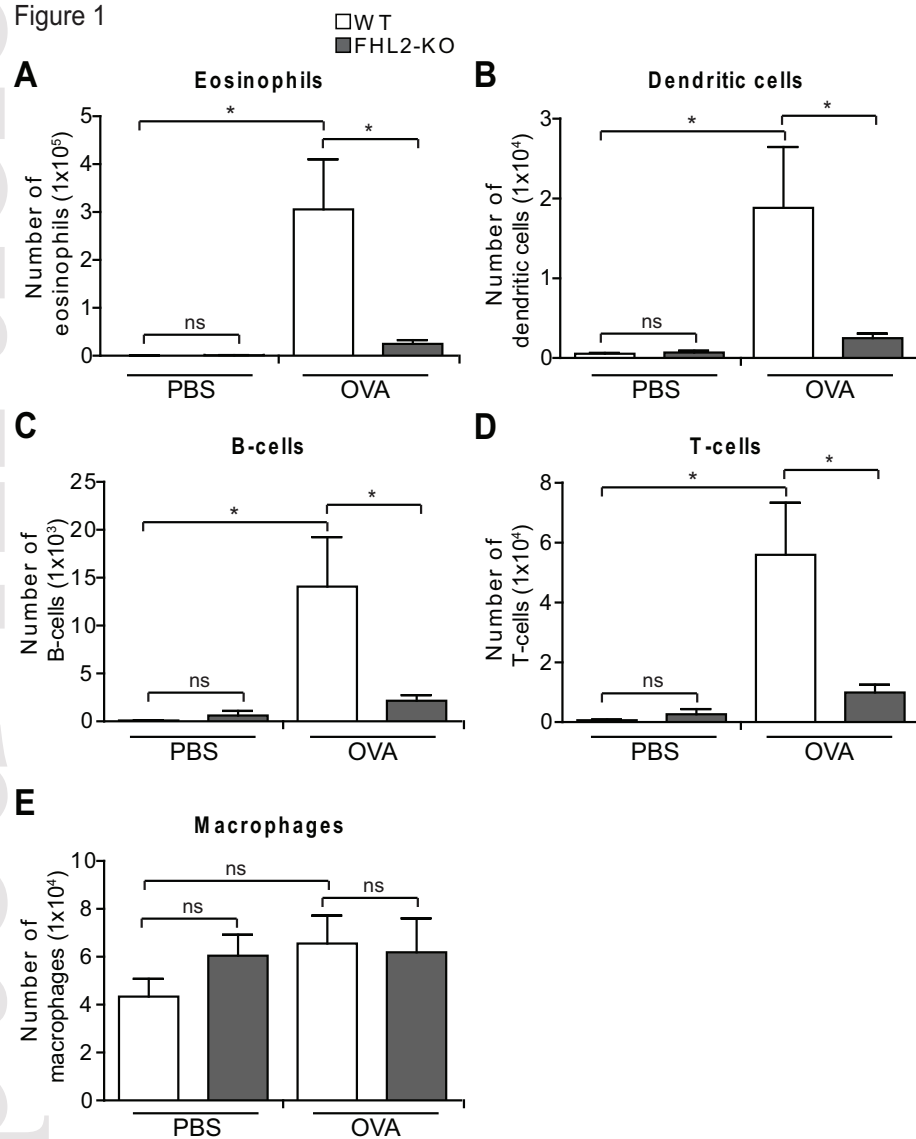


Figure 2

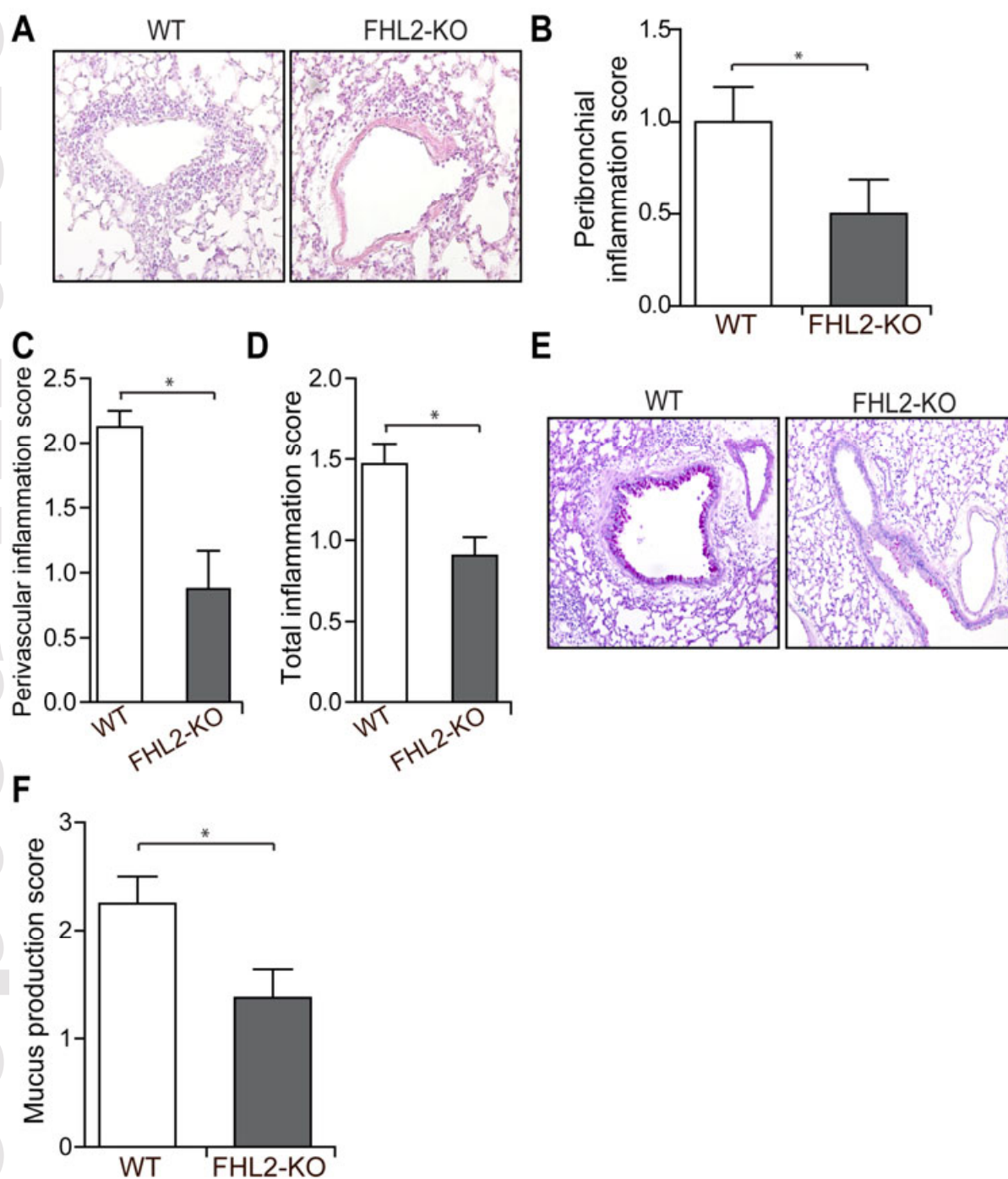


Figure 3

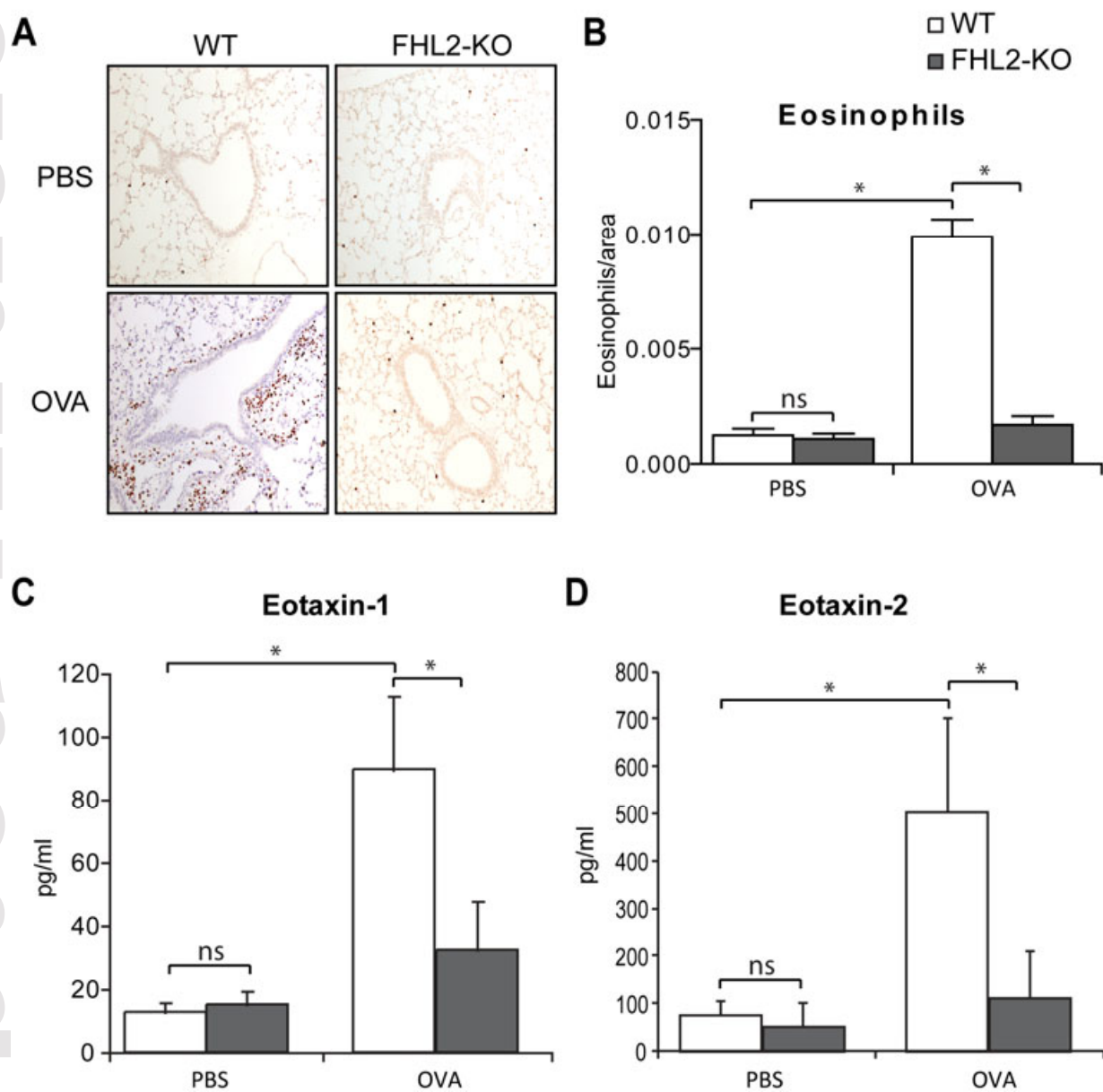


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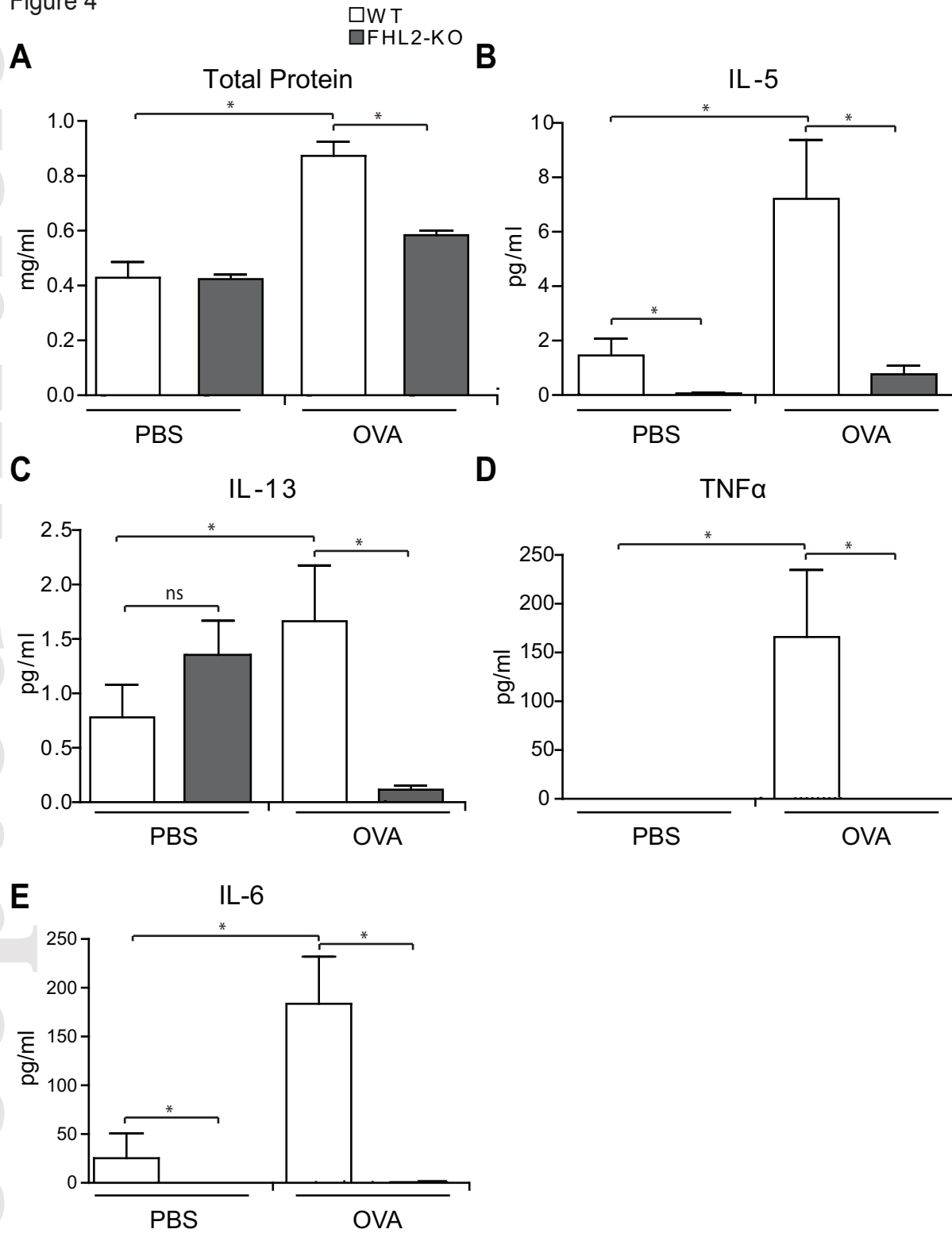


Figure 5

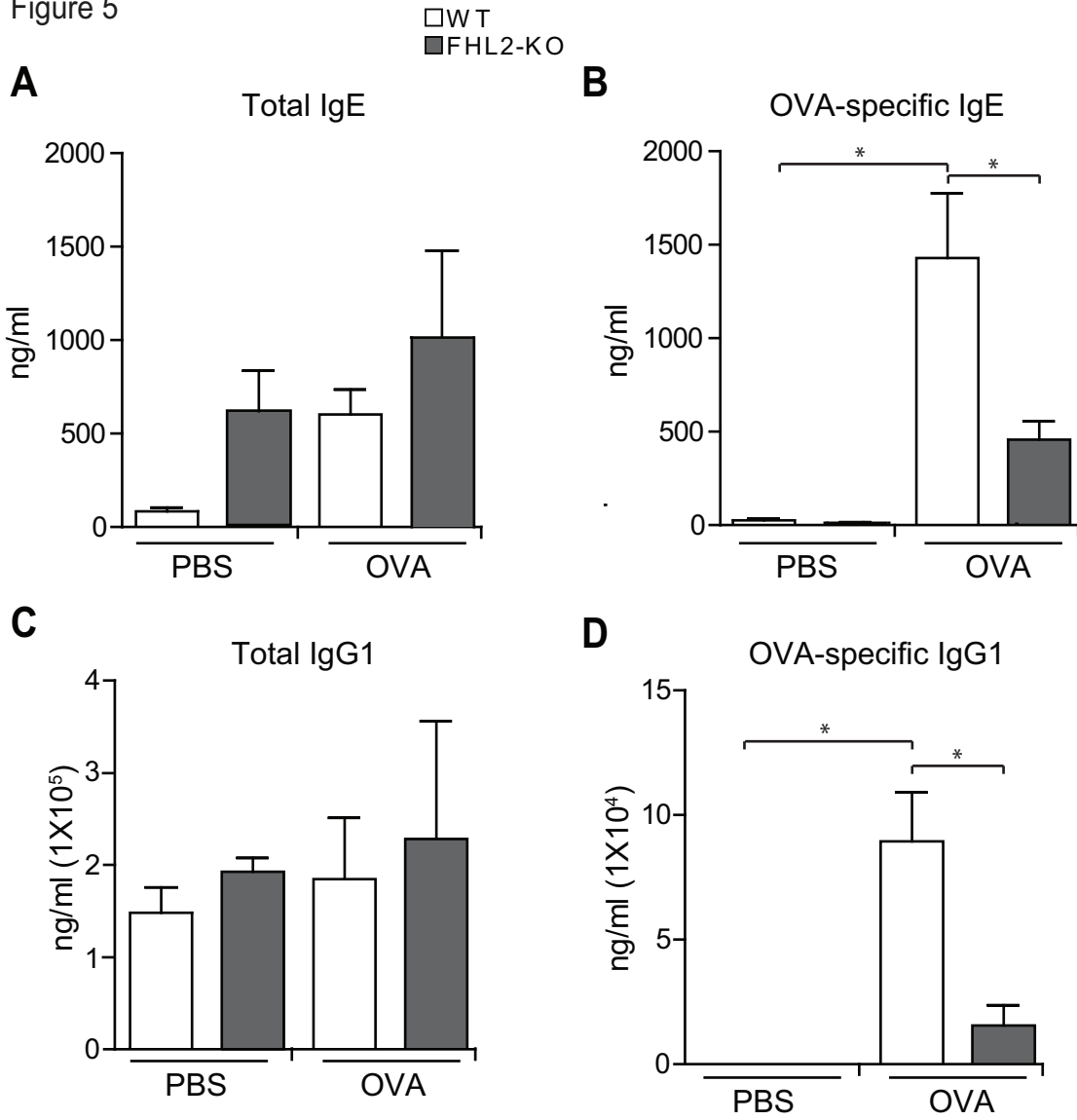


Figure 6

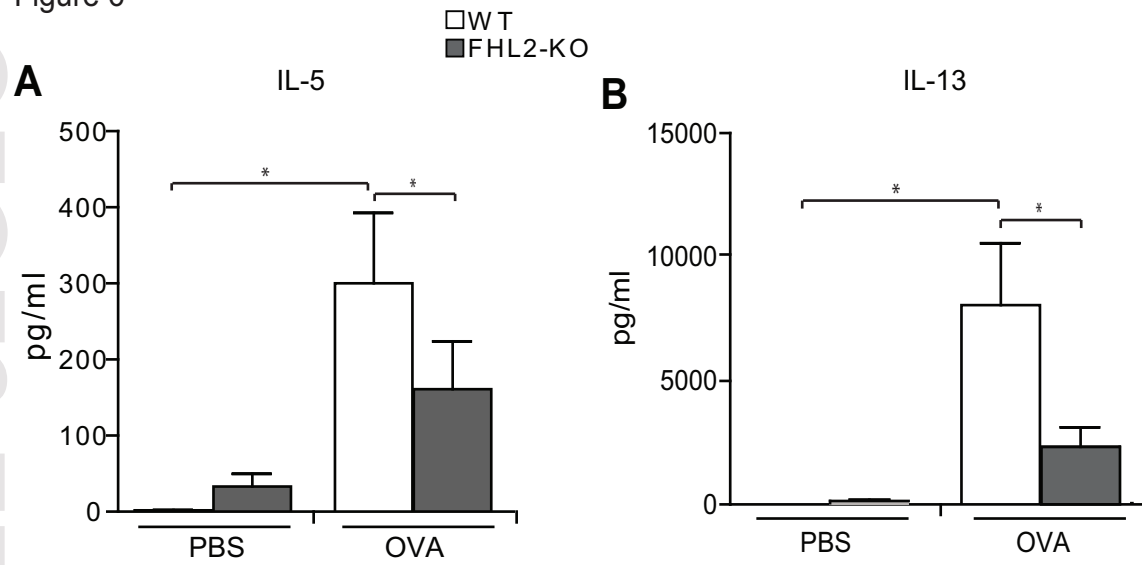


Figure 7

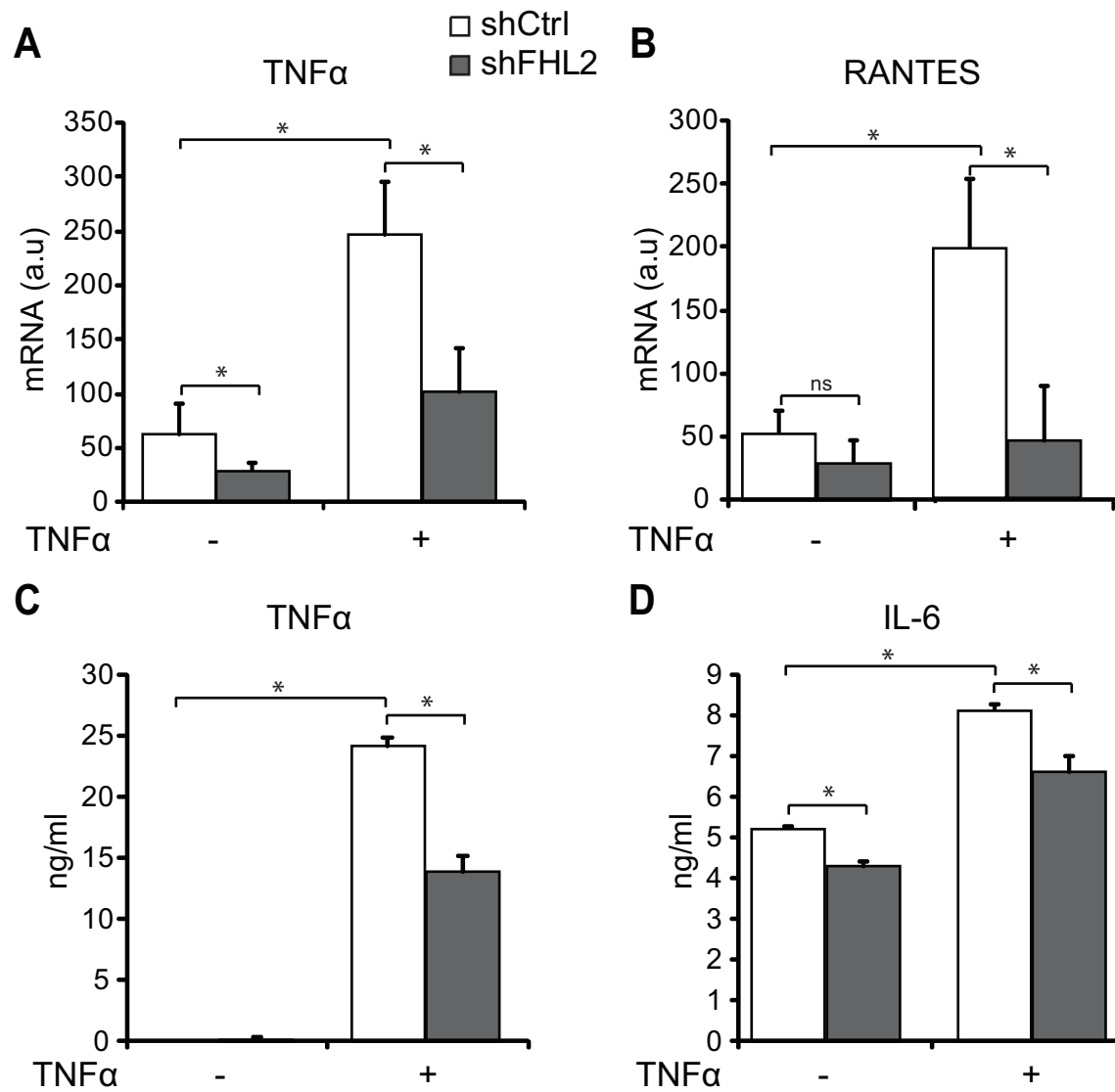
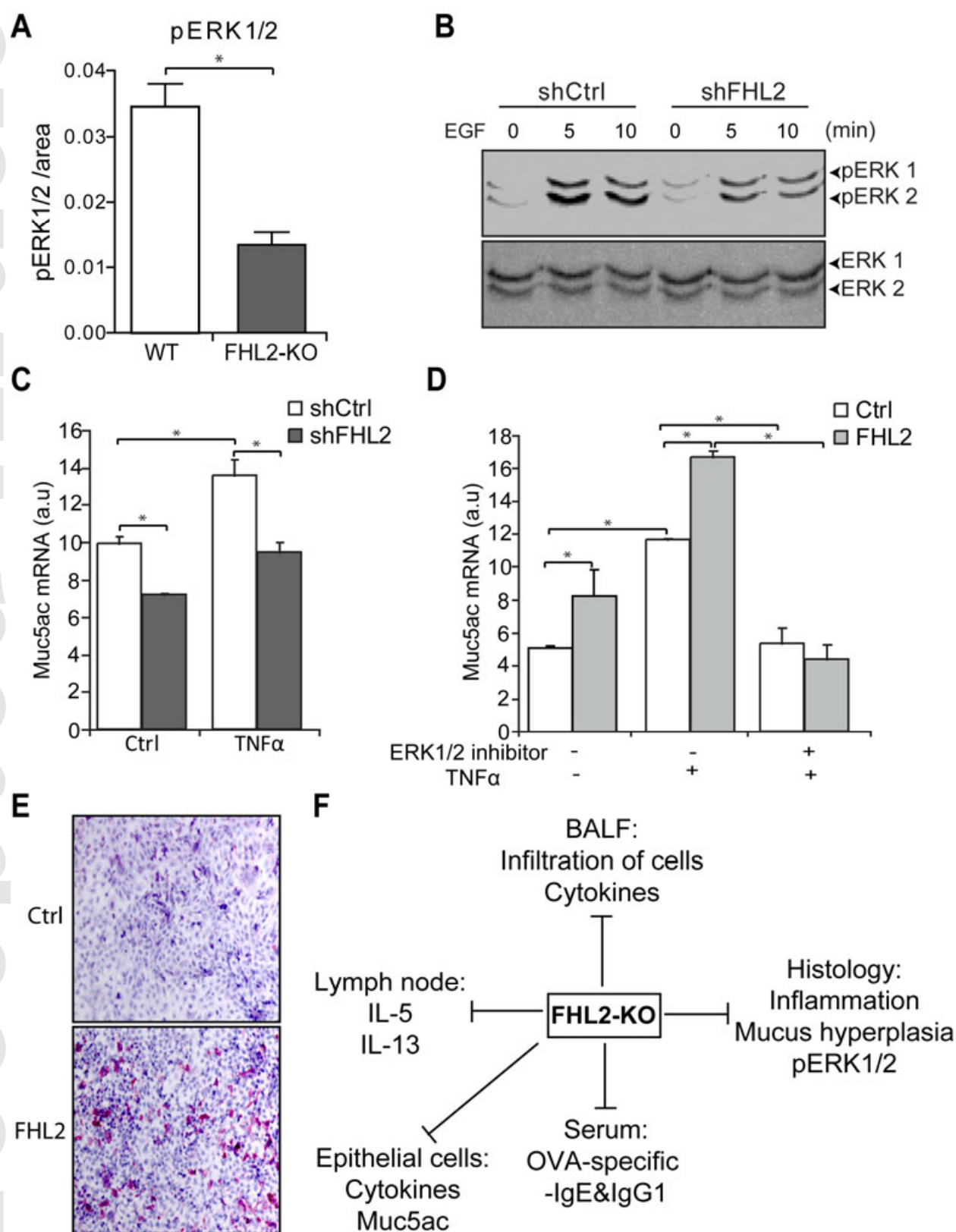


Figure 8



Supplementary material

Deficiency of FHL2 attenuates airway inflammation in mice and genetic variation associates with human bronchial hyperresponsiveness

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Materials and methods

Animals and care

FHL2-KO mice were generated by R. Bassel-Duby (University of Texas Southwestern Medical Center, Dallas, TX) and were bred onto a C57BL/6 background for >11 generations. All animal experiments were approved by the Committee for Animal Welfare of the Academic Medical Center, University of Amsterdam and were carried out in compliance with guidelines issued by the Dutch government.

Murine model of ovalbumin-induced airway allergic inflammation

Female mice aged 8 weeks were sensitized on days 0 and 7 by intraperitoneal injection of 10 µg OVA (Sigma, grade V) adsorbed in 1 mg aluminium hydroxide (Imject alum, Pierce) (n=8 per group). Control animals received alum only. Subsequently, mice were challenged with aerosolized OVA (Sigma, grade III, 10 mg/ml) or PBS for 30 minutes each day for three consecutive days on days 14, 15, and 16. 48h after the last challenge, mice were sacrificed for harvest of bronchoalveolar lavage (BAL) fluid, lungs and serum.

Lung histology

Lungs were inflated and fixed with 4% formaldehyde via a tracheal cannula and stored in 4% formaldehyde for fixation before histochemical processing. The whole lung was embedded in paraffin, sectioned at a 4-µm thickness, deparaffinized and rehydrated. Sections were stained with haematoxylin/eosin to assess inflammatory infiltrates and with Periodic Acid - Schiff (PAS) to detect mucin in goblet cells. Examining the entire slide surface, the following inflammatory parameters were scored by an experienced histopathologist in a blinded fashion, exactly as described previously (1): perivascular inflammation, interstitial inflammation, peribronchial inflammation, and edema. Each parameter was graded in a semi-quantitative fashion on a scale of 0 to 4 (0: absent, 1: mild, 2: moderate, 3: severe, 4: very severe). The total pathology score was expressed as the sum of the score for all parameters. In a similar way PAS-staining for polysaccharides in mucus was performed to quantify the amount of mucus. The amount of mucus per lung section was scored semi-quantitatively on a scale of 0-3. Eosinophils were quantified in lung tissue stained by an antibody against major basic protein (MBP; kindly provided by Dr Nancy Lee and Professor James Lee, Mayo Clinic Arizona, Scottsdale, AZ, USA). γ -phosphorylated ERK1/2 staining was performed using a pERK1/2-specific antibody (Cell Signalling). Morphometric analyses of sections were performed using Leica QWin V3 software.

Assessment of BAL inflammatory cells

The lungs were lavaged with 3x 1ml PBS containing 0.1mM EDTA by intratracheal cannulation. BALF inflammatory cells were collected by centrifugation at 400xg for 7 min at 4°C. The cell pellet was resuspended and flow cytometric analysis was performed as described before to assess the cellular composition of the BALF [34].

Measurement of total protein, cytokines and chemokines

Total protein in BALF was measured by standard Bradford's assay as described before (2). Cytokine levels in BALF were measured by ELISA for IL-5 and IL-13 (Ready-set-go!, eBioscience Inc) and Eotaxin-1 (Qiagen) and Eotaxin-2 (R&D Biosystems) according to the manufacturer's directions. TNF α and IL-6 were measured in BALF and cell culture supernatants using the Cytometric Bead Array Mouse Inflammation Kit (BD Biosciences).

Ex vivo restimulation of lung draining lymph node cells

Lung draining lymph nodes were harvested and cell suspensions were made with cell-strainers. Cells were plated in 96-well round bottom plates at a density of 2×10^5 cells per well and were stimulated for 4 days with 10 μ g/ml OVA (Worthington, Lakewood, NJ) or PBS. Supernatants were analysed for IL-5 and IL-13 production by ELISA.

Serum OVA-specific Immunoglobulins

Serum was analysed to assess the level of total and OVA specific IgE and IgG1 by ELISA (Opteia, BD, San Diego, USA). Nunc maxisorp plates were coated with 100 μ g OVA (grade V, Sigma)/ml and were incubated overnight at 4°C. After blocking with 10%FCS/PBS, plates were incubated with serum samples for 2h and the OVA specific IgE and OVA specific IgG1 were detected following the manufacturer's instructions. We used a standard curve of murine IgE and IgG1 respectively, as a quantitative reference.

Cell culture, lentiviral transduction, semi-quantitative RT-PCR, Western blot analysis and PAS staining

Human alveolar epithelial carcinoma (NCI-H292) cells were grown and maintained in RPMI 1640 medium containing 10% FBS and 1% penicillin/streptomycin. Recombinant lentiviral particles encoding FHL2, shRNAs targeting FHL2 were produced, concentrated and titrated as described previously (2). Cells were seeded at 2.4×10^4 cells/ml and were infected with recombinant lentivirus for two times with an interval of 12h and incubated for 24h. After 24h, the medium was refreshed and the cells were cultured for another 24h. Cells were serum-starved for 36h and stimulated with TNF α (50ng/ml) or EGF (50ng/ml) for indicated time periods. For qRT-PCR experiments, cells were stimulated with TNF α for 6h before harvesting. In some experiments, cells were pre-treated overnight with PD98059 (ERK1/2 inhibitor) at a final concentration of 25 μ M. Transduction efficiency was determined by immunofluorescence and semi-quantitative RT-PCR (qRT-PCR). qRT-PCR and Western blot analysis were performed as described previously (3). The following primers were used for qRT-PCR: TNF α forw: 5'-AGGACACCATGAGCACTGAAAG-3', TNF α rev: 5'-AGGAGAGGCTGAGGAACAAG-3'; RANTES forw: 5'-CGCTGTCATCCTCATTGC-3', RANTES rev: 5'-CCACTGGTGTAGAAATACTCC-3'; Muc5ac forw: 5'-GGAAGTGTGGGGACAGCTCTT-3', Muc5ac rev: 5'-GTCACATTCTCAGCGAGGTC-3'. Antibodies applied in Western blot analysis were anti-ERK1/2 (Cell signalling), and anti-pERK1/2 (Santa Cruz). For PAS staining on NCI-H292 cells, the cells were infected with FHL2 lentivirus followed by serum-starvation for 24 h and then cells were stimulated with TNF α (50ng/ml) for 48h.

After the incubation, cells were fixed with formaldehyde for 30 min and mucus glycoconjugates were visualized by PAS staining.

Single nucleotide polymorphism (SNP) association with asthma and severity of bronchial hyper-responsiveness (BHR)

For single nucleotide polymorphism (SNP) analyses asthma patients were recruited from the Dutch Asthma GWAS (DAG)-cohort. All available SNPs in *FHL2* in the DAG cohort were tested for an association with asthma and severity of bronchial hyperresponsiveness (BHR).

In the DAG cohort all 920 asthmatics had a physician's diagnosis of asthma, asthma symptoms, and BHR to either histamine or methacholine. The 980 controls had no asthma or chronic obstructive pulmonary disease COPD, nor any evidence of significant airway obstruction. Inclusion criteria for the association with severity of BHR were as follows: cases had a doctor diagnosis of asthma in combination with BHR with data available on smoking and inhaled corticosteroids use at the time of the test. In total 650 asthmatics were included in this analysis. Severity of BHR was measured with a slope. This slope was calculated by dividing the difference between forced expiratory volume in one second (FEV1) at baseline and the dose step at which a $\geq 20\%$ fall in FEV1 was reached. We divided the BHR slopes of the 30-second tidal breathing method by 4 in order to compare the slope of the 30-second tidal breathing method with the 2-minute method. Values were log transformed to reach normal distribution.

DNA of subjects was genotyped on the Illumina 317 Chip (IlluminaInc, San Diego, CA) or with the Illumina 370 Duo Chip. Quality control was applied; subjects were removed from analysis if they were not of Caucasian descent, had a low genotyping call rate ($< 95\%$) or were discrepant or ambiguous for genetic sex. SNPs were deleted if the call rates were low ($< 95\%$), not in Hardy-Weinberg Equilibrium ($p < 1E-04$), or if the minor allele frequency was < 0.05 . After quality control 294,932 SNPs were selected for the analysis. All statistical analyses have been performed using PLINK v1.07. Logistic regression analyses in additive models were performed to analyze the associations between *FHL2* SNPs and asthma. Linear regression analyses were performed in additive models to analyze the association of *FHL2* SNPs on slopes of the BHR test with adjustments for smoking and inhaled and/or oral corticosteroid use.

eQTL analysis

SNPs with a significant association with asthma and/or severity of BHR were selected for eQTL analysis with probes located in *FHL2* in a lung tissue database (4). Adjustments were made for age, gender, smoking and all other lung diseases like cystic fibrosis, chronic obstructive pulmonary disease and idiopathic pulmonary fibrosis. Data of the three centers were analyzed separately and consequently meta-analyzed. All analyses were performed using R.

Statistical analysis

Statistical analyses for cell and mouse experiments were carried out with GraphPad Prism software (GraphPad Software, San Diego, Calif) and graphing was carried out with Adobe illustrator (Adobe). Comparisons between 2 groups were done with the Student t test for unpaired variables. Data are reported as mean $\pm$ SEM unless otherwise specified. P values < 0.05 were considered as statistically significant.

References

1. Daan de BJ, Roelofs JJ, de Vos AF, de BR, Schouten M, Hommes TJ, Hoogendijk AJ, de Boer OJ, Stroo I, van der Zee JS, Veer CV, van der Poll T. Lipopolysaccharide inhibits Th2 lung inflammation induced by house dust mite allergens in mice. *Am J Respir Cell Mol Biol* 2013;**48**:382-389.
2. Kurakula K, van der Wal E, Geerts D, van Tiel CM, de Vries CJ. FHL2 protein is a novel co-repressor of nuclear receptor Nur77. *J Biol Chem* 2011;**286**:44336-44343.
3. Kurakula K, Vos M, Otermin R, I, Marinkovic G, Buettner R, Heukamp LC, Stap J, de W, V, van Tiel CM, de Vries CJ. The LIM-Only Protein FHL2 Reduces Vascular Lesion Formation Involving Inhibition of Proliferation and Migration of Smooth Muscle Cells. *PLoS One* 2014;**9**:e94931.
4. Hao K, Bosse Y, Nickle DC, Pare PD, Postma DS, Laviolette M, Sandford A, Hackett TL, Daley D, Hogg JC, Elliott WM, Couture C, Lamontagne M, Brandsma CA, van den Berge M, Koppelman G, Reicin AS, Nicholson DW, Malkov V, Derry JM, Suver C, Tsou JA, Kulkarni A, Zhang C, Vessey R, Opiteck GJ, Curtis SP, Timens W, Sin DD. Lung eQTLs to help reveal the molecular underpinnings of asthma. *PLoS Genet* 2012;**8**:e1003029.