

DR. JOSE GARCÍA PICHEL (Orcid ID : 0000-0001-8580-0952)

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Attenuated airway hyperresponsiveness and mucus secretion in HDM exposed *Igf1r*-deficient mice

S. Piñeiro-Hermida¹, J. A. Gregory², I. P. López¹, R. Torrens¹, C. Ruíz-Martínez³, M. Adner^{2#} and J. G. Pichel^{1#*}

¹Centro de Investigación Biomédica de la Rioja (CIBIR), Fundación Rioja Salud, Logroño, Spain.

²Karolinska Institutet, Institute of Environmental Medicine (IMM), Unit of Experimental Asthma and Allergy Research, Stockholm, Sweden.

³Pneumology Service, Hospital San Pedro, Logroño.

These authors share senior authorship.

* José G. Pichel, corresponding author

Correspondence: José G. Pichel, PhD, Lung Cancer Unit, Oncology Area

CIBIR (Centro de Investigación Biomédica de la Rioja)

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Piqueras 98, 2^a Planta

26006 Logroño. La Rioja, SPAIN

e-mail: jgpichel@riojasalud.es

Short title: Igf1r deficiency attenuates HDM allergic response

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Abstract

BACKGROUND: Asthma is a common chronic lung disease characterized by airflow obstruction, airway hyperresponsiveness (AHR) and inflammation. IGFs have been reported to play a role in asthma but little is known about how the Insulin-like Growth Factor 1 Receptor (IGF1R) affects asthma pathobiology.

METHODS: Female Igf1r-deficient and control mice were intranasally challenged with house dust mite (HDM) extract or PBS five days per week for four weeks. Lung function measurements, and bronchioalveolar lavage fluid (BALF), serum and lungs were collected on day 28 for further cellular, histological and molecular analysis.

RESULTS: Following HDM exposure, the control mice responded with a marked AHR and airway inflammation. The Igf1r-deficient mice exhibited an increased expression of the IGF system and surfactant genes, which were decreased in a similar manner for control and Igf1r-deficient mice after HDM exposure. On the other hand, the Igf1r-deficient mice exhibited no AHR, and a selective decrease of blood and BALF eosinophils, lung Il13 levels, collagen and smooth muscle, as well as a significant depletion of goblet cell metaplasia and mucus secretion markers after HDM exposure. The Igf1r-deficient mice displayed a distinctly thinner epithelial layer than control mice, but this was not altered by HDM.

CONCLUSIONS: Herein, we demonstrate by the first time that the Igf1r plays an important role in murine asthma, mediating both AHR and mucus secretion after HDM exposure. Thus, our study identifies IGF1R as a potential therapeutic target, not only for asthma but also for hypersecretory airway diseases.

Introduction

Asthma is a chronic inflammatory disease of conducting airways characterized by airflow obstruction, bronchial hyperresponsiveness and airway inflammation. It is widely considered that the airway epithelium is an essential controller of inflammatory, immune and regenerative responses in asthma. In response to allergen stimulation the airway epithelium secretes fluids,

antimicrobial proteins and mucins which together with club cells represent a major part of the immunomodulatory barrier of the airway epithelium and orchestrate pulmonary innate immunity (1, 2). Geographical variations notwithstanding, the majority of allergic asthmatic individuals are sensitive to house dust mite (HDM). HDM exhibits a complex mixture of molecules including proteases, allergen epitopes and activators of the innate immune system serving as adjuvants to disrupt intercellular tight junctions leading to cytokine, chemokine and growth factor production and cellular influx, as well as airway remodeling and mucus hypersecretion (3-5).

The Insulin-like Growth Factor 1 Receptor (IGF1R) is a ubiquitously expressed membrane-bound tyrosine kinase receptor and a central member of the IGF system. This network comprises two major ligands (IGF1 and IGF2) and different receptors and regulatory proteins, including IGF binding proteins (IGFBPs), acting together to control multiple cellular functions such as growth, proliferation, differentiation, survival, adhesion and migration (6, 7). It has been reported that IGF1 is an important mediator of airway inflammation and remodeling in asthma, and that IGFBP3 blocks the specific physiology of these pathological consequences (8-10). As the mechanisms of the IGF system in asthma are not defined, we aimed to study the implication of Igf1r in asthma. For this purpose we subjected the *Igf1r* conditional mutant mice *UBC-CreERT2; Igf1r^{fl/fl} (CreERT2)* (11) to repeated HDM exposure. We demonstrate that Igf1r play an important role in asthma, with implications in allergic inflammation, airway remodeling, mucus production and in AHR. As such, IGF1R may now be considered as a potential therapeutic target for asthma and other hypersecretory airway diseases.

Material and methods

Ethical Statement

All experiments and animal procedures conducted in Spain were carried out in accordance to the guidelines of the European Communities Council Directive (86/609/EEC) and were revised and approved by the CIBIR Bioethics Committee (refs. 03/12 and 13/12). All animal handling, including the experimentation conducted in Sweden was carried out in accordance with ethical permit N152/15 approved by the Regional Committee of Animal Experimentation Ethics (Stockholm, Sweden).

Igf1r-deficient mice and HDM sensitization protocol

UBC-CreERT2; Igf1r^{fl/fl} (CreERT2) mice were used to induce a postnatal *Igf1r* conditional gene deletion using *Igf1r^{fl/fl}* mice as experimental controls, as previously described (11). This transgenic line was in a C57BL/6 enriched (at least six generation backcrosses to C57BL/6 strain) mixed genetic background. 8-10 weeks old female mice were challenged by intranasal administration with 40 µg of HDM extract (Greer Laboratories Inc, Lenoir, NC) in 20 µl of PBS (2 mg/ml) or equal volume of PBS under light isoflurane anesthesia, five days a week for four weeks (Fig. 1). Females were used due to their reported higher susceptibility to allergic airway inflammation (12). In addition, female mice present a less aggressive behavior, reducing casualties during transport between institutions and long experimental protocols implying intensive animal handling. All animals were bred and maintained under specific pathogen-free conditions at CIBIR and Karolinska Institutet animal facilities.

***In vivo* measurement of pulmonary mechanics**

24 hours after the final HDM exposure (day 28) mice were deeply anesthetized with a combination of hypnorm (2.5 µl/g) and midazolam (12.5 µg/g) and lung function following exposure to increasing concentrations of aerosolized methacholine was evaluated using the flexiVent system (Scireq, Montreal, Canada). Newtonian resistance (R_n), tissue damping (G) and tissue elastance (H) were determined by assuming a constant-phase model.

Tissue collection and preparation

Bronchoalveolar lavage was performed immediately after pulmonary mechanics measurements. Lungs were lavaged twice with 0.8 ml cold PBS. Blood was collected by cardiac puncture and serum was obtained by centrifugation and stored at -20°C until further usage. Following lung dissection, the left lung was removed and fixed in 4% formaldehyde and subsequently embedded in paraffin for histopathology and immunohistochemistry. Right lobes were separated and snap frozen in liquid nitrogen for quantitative real-time PCR (qRT-PCR), Western blot (WB) and ELISA analyses.

Quantification of BALF

Total cell number was counted and expressed as cells/ml BALF and differential cell counts were performed on May-Grünwald/Giemsa (Sigma-Aldrich, St. Louis, MO) stained cytopspins, counting a minimum of 300 cells per slide in a blinded fashion. Cells were distinguished into macrophages, lymphocytes, neutrophils, and eosinophils by standard morphology.

Histopathological analysis and immunostaining

Paraffin embedded tissues were cut into 3 µm sections for histopathological evaluation or immunohistochemistry. Hematoxylin and eosin (H&E) staining was performed for quantification of inflamed lung areas and airway thickness. Periodic acid Schiff (PAS) and Masson's trichrome staining protocols served to assess airway epithelial goblet cell abundance and collagen deposition, respectively. Immunostaining was performed using the following antibodies: α-SMA (Clone 1A4 1:100, Sigma-Aldrich, St. Louis, MO), Scgb1a1 (Clone T18 1:400, Santa Cruz Biotech. Inc., Dallas, TX), GluTub or α-tubulin (Clone TU-20 1:300, EMD Millipore, Billerica, MA) and Muc5ac (Clone 45M1 1:50, Thermo Fisher Scientific, Waltham, MA).

Histological quantifications were performed using 6 animals per condition evaluating 4 airways per animal. Quantification of PAS, Scgb1a1, GluTub and Muc5ac positive cells was expressed as the number of positive cells per epithelium length (mm) and both the smooth muscle and airway thickness were assessed by means of 3 different measurements per airway. Fiji Open Source image processing software package (<http://fiji.sc>) was used to quantify the percentage of collagen area as well as for epithelium length measurements.

RNA isolation, reverse transcription and qRT-PCR.

Inferior lung lobes were homogenized in Trizol (Invitrogen, Carlsbad, CA) and total RNA was isolated using an RNeasy Mini Kit (Qiagen, Hilden, Germany) and reverse transcribed to cDNA using SuperScript II First-Strand Synthesis System (Invitrogen, Carlsbad, CA) as per manufacturer's specifications. cDNA samples were then amplified by qRT-PCR in triplicate reactions for each primer pair assayed (Table S1) on a 7300 Real Time PCR instrument (Applied

Biosystems, Foster City, CA), using SYBR Premix Ex Taq (Takara Bio Inc., Kusatsu, Japan). Results were normalized using 18S rRNA gene as endogenous control.

ELISAS

Serum total IgE levels were assessed with an IgE mouse ELISA Kit (Abcam, Cambridge, UK) and IL13 levels were determined in homogenized tissue lysates using the middle lung lobes with a mouse IL-13 DuoSet ELISA Kit (R&D Systems, Minneapolis, MN), according to the manufacturer's guidelines.

Western blot analysis

Superior lung lobes were solubilized in a 10 mM Tris/HCl (pH 7.4) buffer containing 0.1% sodium dodecyl sulfate, a protease inhibitor mixture, and DNase (Promega, Fitchburg, WI). Samples were separated on NuPAGE Novex 4%-12% Bis-Tris Gel (Invitrogen, Carlsbad, CA) and transferred to a polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA). Membranes were incubated with primary antibodies for Igf1r (#3027 Cell Signalling Inc., Danvers, MA) and beta-Actin (ab6276 Abcam, Cambridge, UK) at 1:1000 and 1:30000 dilutions respectively, and then incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibodies (DAKO, Agilent technologies, Santa Clara, CA) for Igf1r and beta-Actin respectively, at a 3:10 dilution. Signal was detected using ECL Western Blot Substrate (Thermo Fisher Scientific, Waltham, Ma) and Hyperfilm ECL (GE Healthcare, Little Chalfont, UK). Films were scanned and signals in the linear range were quantified using Image J and normalized to beta-Actin levels.

Statistics

Statistical analyses were performed using SPSS Statistics Software v21 for Windows. Differences between experimental groups were evaluated for significance using Mann-Whitney *U* test and Dunn Sidak multiple comparison test. Results are shown as mean values $\pm$ standard error of the mean (SEM). For all analysis, a *p* value < 0.05 was considered statistically significant.

Results

Depletion of Igf1r in *CreERT2* mutant mice and changes in expression of IGF system genes after the HDM challenge

To verify the Igf1r depletion and investigate the influence of allergic inflammation on the IGF system, qRT-PCR and WB analyses were performed on lung extracts of *CreERT2* mutant mice and their control littermates (*Igf1r^{fl/fl}*) (11) after four weeks of repeated exposure to PBS or HDM (Fig. 1). mRNA expression levels demonstrated an efficient depletion of Igf1r in *CreERT2* PBS-treated mice (90%) and without changes between HDM-treated groups. In addition, HDM treatment decreased Igf1r mRNA levels in *Igf1r^{fl/fl}* mice (81%) (Fig. 2A). WB analysis corroborated these results, showing a significant reduction in Igf1r protein levels in *CreERT2* PBS-treated mice (87%), and no differences between HDM-treated groups (Fig. 2B). Igf1r deficiency caused a significant increase in mRNA expression of *Igf1* and *Insr* expression levels in PBS-treated mice, but not for *Igfbp3* and *Igfbp5*. Even though HDM treatment significantly

reduced the expression levels of *Insr*, *Igfbp3* and *Igf5* in both genotypes without affecting *Igf1*. Igf1r depletion led to a significant increase in the expression of all IGF system genes measured (Fig. 2C).

Igf1r deficiency improves lung function and counteracts allergic airway inflammation and airway remodeling in HDM-treated mice

To assess whether Igf1r deficiency has an effect on lung mechanics, we measured airway responsiveness to inhaled methacholine (Fig. 3). Compared with PBS, HDM-challenged *Igf1r^{fl/fl}* mice displayed AHR with an increased response for the proximal, *Rn*, and distal parameters, *G* and *H*. The AHR seen in both the proximal and distal parts of the lung were, however, not present in *CreERT2* HDM-treated mice, which showed similar functional responses as PBS-treated mice. These findings show that *Igf1r* deficiency improves mouse lung function at both central and peripheral levels after HDM-mediated asthma.

Although we did not find differences counting circulating macrophages, lymphocytes and neutrophils, HDM-treated *Igf1r^{fl/fl}* mice showed increased eosinophil numbers in blood (4-fold), when compared to PBS-treated. The *CreERT2* HDM-challenged mice did not show such an increase when compared to PBS-treated (Fig. 4A). Allergen-treated *Igf1r^{fl/fl}* mice demonstrated a significant increase in total cells in BALF (8-fold) compared to PBS-treated control mice. HDM-challenged *CreERT2* mice also displayed an increase in total cell numbers (6-fold), although significantly lower than that observed in *Igf1r^{fl/fl}* mice (Fig. 4B). Differential cell counts in BALF from *Igf1r^{fl/fl}* and *CreERT2* mice exhibited a marked increase in lymphocyte (49- and 25-fold, respectively) and neutrophil (45- and 40-fold, respectively) numbers in HDM-challenged mice, without significant differences between genotypes. The eosinophils were also increased after HDM in *Igf1r^{fl/fl}* mice (40-fold), however this phenomenon was not observed in *CreERT2* mice, which roughly showed basal eosinophil counts (Fig. 4B).

We found that in HDM-exposed mice the total IgE concentration in serum was highly increased in both genotypes after HDM exposure when compared to PBS-exposed mice (> 90%) (Fig. 4C). In addition, we found that the level of Il13 in lung homogenates was significantly higher in HDM-treated *Igf1r^{fl/fl}* mice compared to PBS-exposed mice (27%) whereas HDM-challenged *CreERT2* lungs displayed normal values (Fig. 4D).

In an attempt to determine how *Igf1r* deficiency protects against HDM-mediated allergic airway inflammation at the histopathological level, we measured several asthma-related features in airways: inflamed lung area, number of PAS⁺ cells, collagen staining and smooth muscle thickness. Assessment of all of these features revealed increased values in HDM-challenged lungs of both genotypes compared to their PBS-treated controls. Measurements that were markedly increased in case of *Igf1r^{fl/fl}* mice displayed a milder increase in allergen-treated *Cre-ERT2* lungs (Fig. 5).

Igf1r depletion attenuates airway hyperreactivity and enhances surfactant expression

Since we have previously reported that *Igf1r* deletion alters airway epithelium morphology and cellular composition (11, 13), we next measured airway wall thickness in the four experimental groups. Accordingly, *CreERT2* mice showed a significantly thinner airway after both treatments. However, whereas in *Igf1r^{fl/fl}* mice HDM challenge induced airway thickening (19%), HDM-exposed *CreERT2* mutant lungs did not show such thickness (Fig 6A). In

order to validate the increase in PAS⁺ cell numbers we observed, mRNA levels of *Muc5ac* and the master regulator *Spdef* were measured. As expected, HDM-challenged *Igf1r^{fl/fl}* lungs showed significantly increased *Muc5ac* levels (16-fold) respect to PBS-exposed controls. HDM-challenged *CreERT2* mice also demonstrated increased *Muc5ac* levels respect PBS-treated mice, however, this increase was significantly lower than that observed in *Igf1r^{fl/fl}* mice (7-fold) (Fig. 6B, left). Correspondingly, mRNA levels of the master regulator *Spdef*, were also reduced in HDM-challenged *CreERT2* mice (42%) (Fig 6B, right). Immunohistochemical analyses of the airway epithelium cellular composition in HDM-exposed lungs revealed a significant decrease in Scg1a1⁺ club cells (35%) and a concomitant increase in GluTub⁺ cells in *CreERT2* mice (17%). Furthermore, the proportion in Muc5ac⁺ cells was also diminished in *Igf1r*-deficient mice (50%) (Fig. 6C).

Finally, to elucidate how *Igf1r* depletion improves peripheral lung function, we evaluated mRNA expression levels of the surfactant markers *Sftpa1*, *Sftpb*, *Sftpc* and *Sftpd* (Fig. 6D). *Igf1r* deficiency caused a generalized increase in all markers measured after both PBS and HDM treatment, although *Sftpc* changes were not significant in PBS-exposed mice. In addition, whereas *Sftpa1* and *Sftpd* levels showed similar changes in expression levels in PBS- and HDM-challenged mice, *Sftpc* levels were significantly reduced in HDM-challenged mice of both genotypes, and *Sftpb* was only found to be reduced in *Igf1r^{fl/fl}* animals.

Discussion

The present study shows that the marked AHR and airway inflammation induced by HDM in control mice was absent or attenuated in the *Igf1r*-deficient mice. This is the first study to report that *Igf1r* can be important for both AHR and airway inflammation in asthma.

Igf1r-deficient mice are bred from a C57BL/6 genetic background, which usually do not display strong airway inflammation or AHR in response to allergen challenge (14, 15). In order to overcome this constraint a specific protocol using repetitive exposures to HDM during four weeks was used to induce AHR. HDM exposure decreased the *Igf1r* levels in *Igf1r^{fl/fl}* mice similarly to those observed in the *CreERT2* animals. Although the mechanism behind this unexpected reduction is unknown, it could be a long term effect occurring after tissue remodeling has been established. We also observed an increased expression of additional IGF system genes in *CreERT2* mutant lungs. The up-regulation of *Igf1*, *Insr*, *Igfbp3* and *Igfbp5* in PBS exposed mice are possibly compensatory effects in response to *Igf1r* deficiency, as we have previously reported in lungs from mice with compromised *Igf1r* signaling in the respiratory epithelium (13). Accordingly, it has been previously reported that exogenous *Igfbp3* and *Igfbp5* administration blocks physiological consequences of asthma in OVA challenged mice, and enhances epithelial cell adhesion to maintain the epithelial-mesenchymal boundary (8, 9, 16). Thus, *Igfbp3* and *Igfbp5* could mediate protective properties against HDM-mediated allergic inflammation.

A marked increase of AHR was found in both the proximal and distal parts of *Igf1r^{fl/fl}* mice after HDM exposure, as has been reported in mice subjected to a similar allergenic challenge (17, 18). The repeated exposure of mice to HDM also increased total cell number in BALF as well as airway remodeling parameters such as cell hyperplasia, collagen deposition,

smooth muscle cells and mucus production, all findings previously reported (5). Furthermore, increases in airway thickness, serum total IgE and lung Il13 levels similar to those observed herein have also been reported elsewhere (17, 19, 20). All of these features were reflected in *Igf1r^{fl/fl}* HDM-challenged lungs and support the effectiveness of our HDM sensitization protocol.

Interestingly, the HDM-induced effects for several of our measured parameters were decreased in the *CreERT2* mice. In particular, AHR was not found in the Igf1r-deficient mice. AHR is considered to be dependent on several features, such as remodeling and mucus production (21, 22). These features were all found decreased, albeit not totally normalized in the *CreERT2* Igf1r-deficient mice. On this basis, it was recently reported that Igf1r plays an important role in the initiation of the inflammatory process (23) and that Igf1r signaling promotes activation of the NF- κ B pathway, a critical regulator of immune responses orchestrating HDM-induced airway inflammation, AHR and fibrotic remodeling (24, 25). In a lung fibrosis model, it was reported that Igf1r blockade decreased α -SMA protein expression and collagen content in bleomycin injured mice (26, 27). Blockade of Il13 activity after chronic HDM sensitization has been shown to reduce eosinophilia in BALF, peribronchial collagen and goblet cell hyperplasia, findings that are accordant with our observation that Il13 levels were not altered by HDM exposure in lungs from *CreERT2* mutant mice (20).

Whereas HDM caused a clear increase of eosinophils in blood and BALF of *Igf1r^{fl/fl}* mice, their levels remained close to basal in *CreERT2* Igf1r-deficient mice. It has been previously shown that Igf1r deregulation decreases the number of peripheral white blood cells, thus providing evidence implicating Igf1r in promoting bone marrow myeloid cell generation (28, 29). The reduced eosinophil counts observed in blood and BALF from *CreERT2* mutant mice, led us to speculate that Igf1r could have an important role in proliferation and/or differentiation of myeloid progenitors and their recruitment to the lung, especially with respect to eosinophils. Further studies are needed to confirm this hypothesis.

The reduced proportion of club cells found in distal airways of *CreERT2* mutant lungs is in accordance with recent data published by our group where a lung epithelial-specific Igf1r-deficiency in mice caused delayed differentiation of club cells in terminal bronchioles (13). In response to allergen stimulation, club cells differentiate into goblet cells through transcriptional activation of the master regulator *Spdef*. Subsequently, the increase in *Muc5ac* expression through transcriptional activation of *Spdef* in goblet cells, contributes to both goblet cell hyperplasia and mucus hyperproduction (30, 31). In this context, delayed club cell differentiation in *CreERT2* airways may result in decreased transdifferentiation into goblet cells, with a concomitant reduction in goblet cell hyperplasia and mucus production. In accordance with depleted *Spdef* and *Muc5ac* expression in *CreERT2* mice, *Spdef*-deficient mice showed an improved lung function as well as a reduced number of BALF eosinophils, and targeted silencing of SPDEF in airway epithelial cells *in vitro* reduces MUC5AC expression (32, 33). Furthermore, *Muc5ac* deficiency in mice abolishes AHR and inflammation (22, 34). As such, a reduction in mucus production may contribute to the improved lung function in *CreERT2* mutant mice observed in our study. It has also been demonstrated that Il13 stimulation of human airway epithelial cells induced MUC5AC expression (35). Together, these findings support a role for Igf1r in lung airway epithelium transdifferentiation to the mucosecretory cell fate during chronic allergic airway inflammation.

Pulmonary surfactant proteins are essential for lung function and homeostasis after birth (36). While surfactant proteins B and C showed a critical role in the preservation of lung function, the immunomodulatory proteins A and D were reported to play an important role during allergic airway inflammation (37-40). Here we show that Igf1r deficiency causes a general increase in surfactant expression basally and after the HDM challenge. On this basis, we conclude that increased expression of surfactant markers improves lung function of *CreERT2* mice and also is important for maintenance of homeostasis in the lung.

Although little is known about the role of IGFs in patients with asthma, previous reports in humans support data found in mice. Thus, corticosteroid treatment in asthma can reduce the lamina reticular thickness by reduction of IGF1 expression with consequent inhibition of the airway infiltration by inflammatory cells, and therefore help to prevent remodeling of the airways (41). Moreover, IGFBP3 secreted by IGF1-stimulated airway epithelial cells during allergic inflammation was suggested to be involved in allergic airway remodeling (42). Considering that there are no effective therapies available to prevent asthma, targeting IGF signaling can be an attractive therapeutic strategy. Actually, treatment to inhibit the IGF1 action in cancer has been performed but did not show a uniform benefit among patients (43). Conversely, targeting IGF1R, which currently is exhaustively evaluated in clinical trials for oncologic patients (44), could be emphasized as an approach for the treatment of asthma.

In conclusion, our results demonstrate that Igf1r plays a key role in murine asthma with important implications regarding allergic inflammation, airway remodeling, mucus production and ultimately in AHR. Based on this putative prophylactic effect of Igf1r deficiency in counteracting asthma, it will be interesting to develop a therapeutic model to test if targeting Igf1r signaling resolves established allergic airway inflammation. At present, there are no effective therapies to treat mucus hyperproduction associated to goblet cell hyperplasia. Thus, our study identifies IGF1R as a potential target for therapeutic development, not only for the treatment of asthma but also for hypersecretory airway diseases.

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Author Contributions

S.P.-H., J.A.G., M.A. and J.G.P. proposed and conceived the project and designed the experiments. S.P.-H., J.A.G., I.P.L., R.T., M.A. and J.G.P. performed the experiments. S.P.-H., J.A.G., I.P.L., C.R.-M, M.A. and J.G.P. analyzed the data, interpreted the experiments and contributed with reagents, materials and analytical tools. S.P.-H. and J.G.P. drafted and wrote the manuscript. All authors read, revised and approved the manuscript before submission.

Conflict of interest

The authors declare no conflict of interest.

Figure legends

Figure 1. Protocol for intranasal exposure to house dust mite. 8-10 weeks old female *UBC-CreERT2*; *Igf1^{fl/fl}* mice (*CreERT2*) and their controls (*Igf1^{fl/fl}*) (11) were challenged by intranasal administration of 40 µg of house dust mite (HDM) extract in 20 µl of phosphate buffered saline (PBS) (2 mg/ml) or equal volume of PBS under inhaled isoflurane anesthesia, five days a week during four weeks. Lung function assessment and BALF, serum and lungs were collected 24 hours after the last exposure on day (D) 28.

Figure 2. Efficient depletion of Igf1r and changes in expression of the IGF system genes in lungs of *CreERT2* mice after HDM induced allergic airway inflammation. A) *Igf1r* mRNA expression levels normalized to 18S expression; (B) representative Western blots for Igf1r and graphical representations of densitometric measurements of band intensities normalized to beta-actin levels; and (C) lung tissue mRNA expression of IGF system related genes in PBS- and HDM-exposed *Igf1^{fl/fl}* and *CreERT2* mice. Numbers within graphic bars indicate the number of

mice analyzed and data are expressed as mean $\pm$ SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (Mann-Whitney U test and Dunn Sidak multiple comparison test). HDM, house dust mite; PBS, phosphate buffered saline.

Figure 3. Igf1r deficiency decreases AHR after house dust mite challenge. Resistance of conducting airways (Rn), tissue damping (G) and tissue elastance (H) to MCh evaluated with the flexiVent system. Numbers in graphic legends indicate the number of mice analyzed and data are expressed as mean $\pm$ SEM. * *Igf1r^{fl/fl}* + HDM vs. *CreERT2* + HDM; # *Igf1r^{fl/fl}* + HDM vs. *Igf1r^{fl/fl}* + PBS. One symbol, $p < 0.05$; two symbols, $p < 0.01$; three symbols, $p < 0.001$ (Dunn Sidak multiple comparison test). MCh, methacholine; HDM, house dust mite; PBS, phosphate buffered saline.

Figure 4. Igf1r depletion attenuates the increase of blood and BALF eosinophils, and Il13 levels in lung homogenate after exposure to house dust mite. A) Peripheral white blood cell counts on blood smear preparations; (B) total and differential cell counts performed on cytopsin preparations of BALF; and C) serum total IgE and Il13 levels in lung homogenate quantified by ELISA, determined in *CreERT2* mice. Numbers within graphic bars indicate the number of mice analyzed and data are expressed as mean $\pm$ SEM. ** $p < 0.01$; *** $p < 0.001$ (Dunn Sidak multiple comparison test). HDM, house dust mite; PBS, phosphate buffered saline.

Figure 5. Reduced allergic airway inflammation, goblet cell hyperplasia, collagen content and smooth muscle thickness in Igf1r-deficient mice after HDM exposure. Representative images of proximal airways showing inflamed lung areas (left) (scale bar: 0.5 mm); mucus producing cells per epithelium length (red arrowheads in insets) (center left) and airway collagen content (center right) (scale bars: 50 μ m); and smooth muscle thickness (right) (scale bar: 20 μ m). Bottom graphs represent quantification of the abovementioned parameters. Numbers within graphic bars indicate the number of mice analyzed and data are expressed as mean $\pm$ SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (Dunn Sidak multiple comparison test). H&E, hematoxylin and eosin; PAS, periodic acid schiff; α -SMA, alpha-Smooth Muscle Actin; AW, airway; HDM, house dust mite; PBS, phosphate buffered saline; SM, smooth muscle.

Figure 6. Attenuation of airway hyperreactivity and mucus secretion and increased expression of surfactant markers in HDM-exposed Igf1r-deficient mice. (A) Proximal airway wall thickness; (B) lung mRNA expression of the mucus secretion markers *Muc5ac* and *Spdef* normalized to 18S expression; (C) proportion of club (in red) and ciliated (in green) cells and quantification of *Muc5ac* (in red) positive cells in terminal and proximal bronchioles, respectively; and (D) changes in mRNA expression of surfactant (*Sftp*) markers *a1*, *b*, *c* and *d* in *CreERT2* mice. Scale bars: 20 μ m. Numbers within graphic bars indicate the number of mice analyzed and data are expressed as mean $\pm$ SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (Mann-Whitney U test and Dunn Sidak multiple comparison test). HDM, house dust mite; PBS, phosphate buffered saline; tb, terminal bronchioles; pb, proximal bronchioles; Scgb1a1, Secretoglobin 1A1; GluTub, Glu Tubulin; *Muc5ac*, Mucin 5ac.







