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Expression of IL-4 Receptor alpha on smooth muscle cells is not necessary for development of experimental allergic asthma

Frank Kirstein, PhD^a, William G.C. Horsnell, PhD^a, Douglas A. Kuperman, PhD^b, Xiaozhu Huang, MD^c, David J. Erle, MD^c, Andreas L. Lopata, PhD^{a,d}, and Frank Brombacher, PhD^a

^a Division of Immunology, Institute of Infectious Disease and Molecular Medicine, Health Sciences Faculty, University of Cape Town and International Centre for Genetic Engineering and Biotechnology (ICGEB), 7925 Cape Town, South Africa

^b Department of Medicine, Allergy-Immunology Division, Northwestern University Feinberg School of Medicine, Chicago, USA

^c Lung Biology Center, Department of Medicine, University of California, San Francisco, CA 94158, USA

^d School of Applied Sciences, Food Science-Allergy Research Group, RMIT University, Melbourne, Australia

Abstract

Background—Airflow in the lungs of patients with allergic asthma is impaired by excessive mucus production and airway smooth muscle contractions. Elevated levels of the cytokines IL-4 and IL-13 are associated with this pathology. *In vitro* studies have suggested that IL-4 receptor alpha (IL-4R α) signalling on smooth muscle cells is critical for airway inflammation and airway hyperresponsiveness.

Objective—In order to define the contribution of IL-4 and IL-13 to the onset of asthmatic pathology the role of their key receptor IL-4R α in smooth muscle cells was examined *in vivo*.

Methods—By using transgenic SMC-MHC^{cre}IL-4R α ^{-lox} mice deficient for IL-4R α in smooth muscle cells, *in vivo* effects of impaired IL-4R α signalling in smooth muscle cells on the outcome of asthmatic disease were investigated for the first time. Allergic asthma was introduced in mice by repeated sensitisation with ovalbumin/aluminium hydroxide on days 0, 7 and 14 followed by intranasal allergen challenge on days 21–23. Mice were investigated for the presence of airway hyperresponsiveness, airway inflammation, allergen specific antibody production, Th2 type cytokine responses and lung pathology.

Results—Airway hyperresponsiveness, airway inflammation, mucus production, Th2 cytokine production and specific antibody responses were unaffected in SMC-MHC^{cre}IL-4R α ^{-lox} mice when compared to control animals.

Conclusion—The impairment of IL-4R α on smooth muscle cells had no effect on major aetiological markers of allergic asthma. These findings suggest that IL-4R α responsiveness in

Corresponding Author: Professor Frank Brombacher, Wernher and Beit Building (South), UCT Campus, IIDMM, Anzio Road, Observatory 7925, Cape Town, South Africa, Tel: +27-21-406 6147, Fax: +27-21-406 6029, fbrombac@mweb.co.za.

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airway smooth muscle cells during the early phase of allergic asthma is not, as suggested, necessary for the outcome of the disease.

Clinical Implications—Therapies targeting the IL-4R α might have no direct effect on smooth muscle cells in an allergic asthma response.

Keywords

Smooth muscle cell; Allergy; Asthma; Cytokine Receptors; IL-4; IL-13; gene-deficient mice

Introduction

Allergic asthma is characterised by airway obstruction, inflammation, and airway hyperresponsiveness (AHR). Patients with allergic asthma experience impaired respiratory air flow which is caused by a combination of increased airway mucus secretion and airway smooth muscle cell (ASMC) contraction in response to allergens.

Immunologically it is established that T helper 2 (Th2) type cytokines, particularly IL-4 and IL-13 play an important role in asthma pathogenesis. Indeed, overexpression of these cytokines results in the development of asthmatic symptoms in murine models^{1–4}. Though IL-4 plays an important role in establishing Th2 responses^{5, 6}, IL-13 is the cytokine critical for driving actual asthmatic pathology. In the absence of IL-13, mucus production, AHR and airway inflammation are inhibited. Conversely, administration of recombinant IL-13 will induce these pathological features in non allergic mice^{7, 8}. Such IL-13 driven effects occur irrespective of IL-4, nevertheless induction of a strong Th2 response and immunoglobulin class switching to IgE in mice requires IL-4. These IL-4 and IL-13 mediated effects require signalling through heterodimeric receptors containing IL-4 receptor alpha (IL-4R α). Mice deficient for IL-4R α show protection from experimentally induced allergic asthma⁹ and IL-4R α antagonists abrogate the development of allergic airway symptoms¹⁰.

It can be appreciated from this that an understanding of the role of IL-4R α in specific cell types would enhance the understanding and treatment of asthma. Key cells involved in the onset and pathogenesis of asthma include T cells, B cells, epithelial and smooth muscle cells. To date the cell specific role of IL-4R α in allergy has only been described *in vivo* for epithelial cells. Here disrupted epithelial IL-4R α signalling results in protection from airway mucus production whereas AHR was not affected¹¹. In addition to immune and epithelial cells it is apparent that ASMC may also play an important role in the onset of asthma. Several *in vitro* studies have shown that IL-4 and IL-13 induce hypercontractility in smooth muscle cells in a STAT6 dependent manner^{12–14}. This IL-4 and IL-13 dependent hypercontractility response of ASMC to carbachol was attenuated in STAT6^{-/-} mice. Use of anti-IL-4R α antibodies also inhibited the contractile responsiveness of IgE sensitised ASMC to acetylcholine while pre-treatment with recombinant IL-13 enhanced contractile ability¹⁵. In addition to effects on ASMC hypercontractility, human airway smooth muscle cells have been shown to release allergy associated chemokines, such as eotaxin^{16–21} in response to IL-4 and/or IL-13 *in vitro*. This IL-4/IL-13 driven eotaxin expression in ASMC can be inhibited by anti-IL4R α antibodies¹⁸ and antisense oligonucleotides to STAT6²².

Owing to this substantial body of *in vitro* data, it was suggested that ASMC responsiveness to IL-4 and IL-13 contributes significantly to asthmatic pathology. To investigate the *in vivo* role of IL-4R α signalling in smooth muscle cells we used a mouse model with smooth muscle cell specific disruption of the *Il4ra* gene (SM-MHC^{cre}IL-4R α ^{-/lox} mice). Recently characterised by our laboratory, SM-MHC^{cre}IL-4R α ^{-/lox} mice showed delayed mucus production, Th2 cytokine production and worm expulsion in a model of nematode infection

¹³. This was the first *in vivo* demonstration of the influence of IL-4R α expression in smooth muscle cells on Th2 type immune responses of the host.

The current study investigated for the first time the *in vivo* effect of IL-4R α signalling in smooth muscle cells in a model of acute allergic asthma. The findings of several *in vitro* studies have suggested a role for IL-4/IL-13 responsiveness in smooth muscle cells on the outcome of allergic airway disease, but our results demonstrated that smooth muscle cell specific disruption of the IL-4R α did not influence the acute phase of the allergic airway response caused by ovalbumin (OVA).

Methods

Mice

Generation and characterization of the SM-MHC^{cre}IL-4R α ^{-lox} (C.Cg-Il4ra^{tm1Fbb}/Il4ra^{tm2Fbb}Tg(Myh11-cre)5013Gko) mice was described previously ¹³. SM-MHC^{cre} mice on a BALB/c background ²³ were crossed with IL-4R α ^{-/-} mice ²⁴. In order to generate SM-MHC^{cre}IL-4R α ^{-lox} mice they were then crossed with IL-4R α ^{-lox} mice ²⁵ as the hemizygous IL-4R α ^{-lox} genotype increases the probability of Cre-mediated *il4ra* gene disruption. All mice were genotyped by PCR before the experiments to confirm smooth muscle cell-specific disruption of the *il4ra* gene. IL-4R α ^{lox/lox} mice produce a functional IL-4R α and hemizygous IL-4R α ^{-lox} littermates were used as control groups. IL-4R α ^{-/-} (Il4ra^{tm1Fbb}/Il4ra^{tm1Fbb}) mice were used as global knock out controls for the IL-4R α . Mice were housed in the animal unit of the University of Cape Town (UCT) under specific pathogen free conditions using individually ventilated cages. All work was approved by the UCT animal ethics committee.

Allergen sensitisation and challenge of mice

Sensitisation and challenge of mice was performed as described previously ¹¹. SM-MHC^{cre}IL-4R α ^{-lox}, IL-4R α ^{-lox} and IL-4R α ^{-/-} mice were sensitised with 50 μ g Ovalbumin (Sigma-Aldrich, grade V) in 200 μ l PBS/1.3% Alum (Sigma-Aldrich) on day 0, day 7 and day 14. On day 21, 22 and day 23, mice were anaesthetised with Ketamine (Centaur Labs, South Africa)/Xylazine (Bayer, South Africa) and challenged with 1mg OVA in 50 μ l PBS by nasal administration. Control groups were treated identically except OVA was missing in the solutions. Mice were killed and studied on day 24.

Measurement of Airway Hyperresponsiveness

Two different methods for measurement of airway resistance were used in this study. First, Measurements of airway resistance in response to intravenously administered acetylcholine were performed as described previously ¹¹. Anaesthetised and paralysed mice were mechanically ventilated and lung resistance was measured at baseline and following increasing intravenous doses of acetylcholine. For the second approach, a flexiVent system (SCIREQ, Canada) was used for airway resistance measurements in response to increasing doses of aerosolised methacholine (0, 5, 10, 20, 40 mg/ml). Mice were anaesthetised with Ketamin and Xylazine, intubated with a 18G cannula and mechanically ventilated at a frequency of 2.5Hz. Mice were exposed for 10s to methacholine aerosol and 15 Single-Frequency Forced Oscillation manoeuvres were performed to determine Dynamic Resistance (R). R is represented as the maximum response for each concentration. In addition to the two invasive methods, Penh values in response to β -methacholine (Sigma) were assessed in conscious unrestrained mice using a whole body plethysmograph (emka Technologies, France). This system measures pressure changes induced by the breathing of the mice and computes enhanced pause (Penh) values, a dimensionless parameter reflecting airway hyperresponsiveness ²⁶. Baseline readings were measured for 5 min before each

exposure to increasing doses of aerosolised β -methacholine in PBS (0, 5, 10, 20 mg/ml). Results are computed as average of first 5 min minus average of baseline reading.

Analysis of airway inflammation

Lungs from sacrificed mice were flushed once with 1ml PBS/1% FCS to obtain bronchoalveolar lavage (BAL) fluid and total cell number was determined using a haemocytometer. A maximum of 2×10^5 cells was centrifuged on a microscope slide and stained with Rapidiff Stain Set (Clinical Diagnostics CC, South Africa). Differential cell counts were made at 400 $\times$ magnification and at least 100 cells were counted per slide. Lung tissue was homogenized in Tris/NP-40 buffer and then centrifuged for 20 minutes at 20000rcf for mouse mast cell protease-1 (mMCP-1) ELISA and eosinophil Peroxidase (EPO) assay. A colorimetric assay for EPO was used to quantify eosinophils in the lung tissue²⁷. 50 μ l of the substrate solution consisting of 16mM o-phenylenediamine (Sigma-Aldrich) in 100mM Tris-HCl buffer pH 8.0 containing 0.1% Triton and 0.01% hydrogen peroxide was added to 50 μ l tissue sample (protein concentration 1mg/ml) and peroxidase standard (Sigma-Aldrich) in 96 well plates. The reaction was stopped after 5 minutes with 50 μ l 2M H₂SO₄ and absorption was measured at 490nm. Mouse mast cell protease concentrations were determined by ELISA (Moredum Scientific, UK) according to the manufacturer's protocol. Serum eotaxin levels were determined by ELISA according to the manufacturer's protocol (R&D Systems, USA).

Histology and Immunohistochemistry

For histological analysis of goblet cell hyperplasia, tissue samples were fixed in 4% phosphate-buffered formalin, cut in 5–7 μ m paraffin sections and stained with Periodic acid-Schiff reagent (PAS). Mucin was stained with anti-Muc5AC antibody (clone 45M-1, Thermo Scientific, 1:100 dilution) and a mouse on mouse staining kit (M.O.M., Vector Laboratories).

Measurement of serum antibodies

Blood samples were collected in plasma separator tubes (BD, USA) by tail vein bleeding and centrifuged for 15 minutes at 6000rcf. OVA-specific antibody isotypes were measured in blood serum by endpoint titration ELISA. Plates were coated with 5 μ g/ml (for IgG) or 1mg/ml (for IgE) OVA. AP-conjugated anti-mouse isotype specific antibodies (Southern Biotechnology, USA) and 4-nitrophenyl phosphate (Fluka, CH) were used for detection. Absorbance was measured at 405nm with 492nm as a reference wavelength.

Analysis of in vitro cytokine production

For analysis of *in vitro* cytokine production splenocytes and lymphocytes were isolated from pooled spleens or mediastinal lymph nodes of OVA treated and control mice. Cells were incubated for 120h at 2×10^6 cells/ml in 200 μ l IMDM (Gibco, UK)/10% FCS (Delta, South Africa) in 96well plates. Cells were stimulated with OVA (100 μ g/ml) and supernatants were collected after 120 hours. Concentrations of IL-4, IL-5, IL-9, IFN γ (BD Biosciences, USA) and IL-13 (R&D Systems, USA) were measured using sandwich ELISA assays according to the manufacturer's protocol.

Statistics

Values are given as mean $\pm$ SD and significant differences determined using ANOVA (GraphPad Prism 4 software). P values $\leq$ 0.05 were considered significant.

Results

Allergen-induced airway hyperreactivity is not affected in SM-MHC^{cre}IL-4R α ^{-/-} mice

Airway reactivity was measured in sedated and paralyzed, mechanically ventilated SM-MHC^{cre}IL-4R α ^{-/-} and IL-4R α ^{-/-} mice after OVA or saline challenge. Airway resistance was increased in allergen-challenged IL-4R α ^{-/-} and SM-MHC^{cre}IL-4R α ^{-/-} mice, indicating the development of airway hyperreactivity. These increases in airway resistance were not observed in saline challenged mice. Similar increases in airway reactivity were seen in SM-MHC^{cre}IL-4R α ^{-/-} and IL-4R α ^{-/-} mice with no statistically significant differences between both mouse strains (Fig. 1A). The development of airway hyperresponsiveness in both SM-MHC^{cre}IL-4R α ^{-/-} and IL-4R α ^{-/-} mice was confirmed by a second invasive method for determination of airway resistance in response to increasing doses of methacholine aerosol. Airway resistance in both strains treated with OVA was significantly increased when compared to PBS treated IL-4R α ^{-/-} mice with no significant differences between SM-MHC^{cre}IL-4R α ^{-/-} and IL-4R α ^{-/-} mice (Fig. 1B). Similar results were obtained by measuring responses to methacholine by non-invasive whole body plethysmography. Both, OVA treated IL-4R α ^{-/-} mice and SM-MHC^{cre}IL-4R α ^{-/-} mice responded to methacholine challenges with increased Penh values when compared to PBS treated control groups and these increases were not significantly different between both mouse strains (Fig. 1C).

Allergic airway inflammation is not affected in SM-MHC^{cre}IL-4R α ^{-/-} mice

Inflammatory cells are recruited to the lungs as a consequence of allergic sensitisation and challenge with eosinophils being a characteristic cell type for allergic airway inflammation. The total number of cells in the BAL fluid of OVA treated SM-MHC^{cre}IL-4R α ^{-/-} mice did not differ from the cell number recovered from IL-4R α ^{-/-} mice but was significantly higher than the cell number in the BAL fluid of IL-4R α ^{-/-} mice (Fig 2A). Differential cell counts revealed that the main cell type in the BAL fluid in both OVA treated IL-4R α ^{-/-} mice and SM-MHC^{cre}IL-4R α ^{-/-} mice were eosinophils with no significant differences between the strains whereas in IL-4R α ^{-/-} mice it was neutrophils (Fig 2B). To quantify the number of eosinophils in the lung tissue the amount of eosinophil peroxidase (EPO), which is stored in intracellular vesicles in eosinophils until activation, was measured in lung tissue lysate. There was no significant difference in the amount of EPO in the lung tissue between SM-MHC^{cre}IL-4R α ^{-/-} mice and IL-4R α ^{-/-} mice (Fig 2C). In IL-4R α ^{-/-} mice, the concentration of EPO was significantly reduced compared to SM-MHC^{cre}IL-4R α ^{-/-} mice but was higher than in PBS treated control mice. During allergic airway inflammation the lung tissue and airway smooth muscle cells are infiltrated by mast cells. The amount of mMCP-1, a marker for mast cell degranulation, was increased in the lung tissue after challenge with ovalbumin with no significant differences in all mouse strains (Fig. 2D). It has been shown that after *in vitro* stimulation with IL-13, airway smooth muscle cells release eotaxin, a chemoattractant for eosinophils. Levels of this chemokine were measured in the serum of OVA sensitised and challenged SM-MHC^{cre}IL-4R α ^{-/-}, IL-4R α ^{-/-} and IL-4R α ^{-/-} mice. Eotaxin concentrations were similar in both SM-MHC^{cre}IL-4R α ^{-/-} and IL-4R α ^{-/-} mice but significantly lower in IL-4R α ^{-/-} mice (Fig. 2E). These results show that SM-MHC^{cre}IL-4R α ^{-/-} mice are not protected from allergic airway inflammation as is the case with IL-4R α ^{-/-} mice, which showed no signs of eosinophilic cell infiltration in the lungs.

Airway mucus production is not affected in SM-MHC^{cre}IL-4R α ^{-/-} mice

Hyperplasia of mucus-producing goblet cells in the lungs is a characteristic of allergic asthma. The predominant mucin glycoprotein secreted in the respiratory tract of healthy and asthmatic individuals is MUC5AC and its expression in goblet cells is upregulated by

IL-4R α signals in murin models of asthma¹¹. Histological examination of PAS and anti-MUC5AC stained lungs of OVA sensitised and challenged IL-4R $\alpha^{-/lox}$ mice (Fig. 3A) and SM-MHC^{cre}IL-4R $\alpha^{-/lox}$ mice (Fig. 3B) showed similar severity of goblet cell hyperplasia and mucin production, whereas IL-4R $\alpha^{-/-}$ mice (Fig. 3C) and the alum treated control group showed no signs of increased mucus production (Fig. 3D).

Antigen-specific Th2 type cytokine and antibody responses in IL-4R $\alpha^{-/lox}$ SM-MHC^{cre}IL-4R $\alpha^{-/lox}$ mice

To determine whether T cell dependent immune responses were affected by disrupted smooth muscle cell IL-4R α , lymphocytes from lung draining mediastinal lymph nodes (Fig. 4A) and splenocytes (Fig. 4B) were stimulated *in vitro* with OVA. Sensitised and challenged SM-MHC^{cre}IL-4R $\alpha^{-/lox}$ and IL-4R $\alpha^{-/lox}$ mice showed antigen-specific Th2 type cytokine responses characterised by production of IL-4, IL-5, and IL-13 in lymphocytes with production of IL-5 and IL-13 being significantly higher in SM-MHC^{cre}IL-4R $\alpha^{-/lox}$ mice (Fig. 4A). When compared to cytokine production by lymphocytes, splenocytes produced similar levels of IL-4 but only low levels of IL-5 and IL-13 (Fig. 4B). IL-4R $\alpha^{-/-}$ mice developed a Th1 type cytokine response to OVA characterised by production of IFN- γ by lymphocytes but not splenocytes. Antigen specific cytokine responses were not detected in PBS control groups. To investigate whether antibody responses to allergens are affected by IL-4R α signalling on smooth muscle cells, specific antibody levels in SM-MHC^{cre}IL-4R $\alpha^{-/lox}$ mice were compared to those of IL-4R $\alpha^{-/lox}$ and IL-4R $\alpha^{-/-}$ mice after sensitisation and challenge with OVA. Compared to alum treated control groups, serum levels of OVA-specific IgE (Fig. 5A) and IgG1 (Fig. 5B), were increased in IL-4R $\alpha^{-/lox}$ and in SM-MHC^{cre}IL-4R $\alpha^{-/lox}$ mice with no significant differences between the two mouse strains. As expected IL-4R $\alpha^{-/-}$ mice showed significantly increased levels of specific IgG2a and IgG2b (Fig. 5C, D) but impaired OVA specific IgE and IgG1 antibody titres.¹⁰

Discussion

To investigate the contribution of smooth muscle cell IL-4 and IL-13 signalling in the onset of allergic asthma *in vivo*, SM-MHC^{cre}IL-4R $\alpha^{-/lox}$ mice were used, which have a cell specific disruption of IL-4R α on smooth muscle cells.¹³ Comparison of these mice to IL-4R $\alpha^{-/lox}$ and IL-4R $\alpha^{-/-}$ mice showed that impaired IL-4R α signalling in smooth muscle cells had no effect on the major aetiological markers of allergic asthma. In a recently published study, we demonstrated delayed mucus production, Th2 cytokine production and worm expulsion in SM-MHC^{cre}IL-4R $\alpha^{-/lox}$ mice in a model of gut infection with the nematode *Nippostrongylus brasiliensis*¹³. This disparity between our two studies might be explained by intrinsic differences between the two disease models and different roles for IL-4R α in SMC of the gut and the lung in clearance of intestinal worm infections or induction of allergic airway disease. Airway hyperresponsiveness is a characteristic feature of allergic asthma. As the best method for the determination of AHR in mice is under debate²⁸ two invasive and one non-invasive method were used in this study. The results obtained from each method were comparable and both SM-MHC^{cre}IL-4R $\alpha^{-/lox}$ and IL-4R $\alpha^{-/lox}$ mice developed AHR after sensitisation and challenge with OVA but not PBS. Furthermore, equivalent responses to acetylcholine and methacholine were seen in both groups. In agreement with our results a study using a different mouse model with smooth muscle cell specific IL-4R α disruption (SMP8^{cre} IL-4R $\alpha^{-/lox}$ mice) found development of AHR in response to house dust mite allergens or recombinant IL-13 in the absence of smooth muscle IL-4R α (C. Perkins, F. Finkelman, personal communication). These results indicate that IL-4R α expression on smooth muscle cell is not required for the development of AHR in mice. This does not exclude the possibility that IL-4R α signalling on smooth muscle cells contributes to the onset of AHR in wild type animals, as the airway response observed in our

model could have been induced by other cell types, like epithelial cells. It has been demonstrated that IL-13 signals on airway epithelial cells alone are sufficient to induce AHR in mice ²⁹ despite the fact that disruption of IL-4R α on epithelial cells did not affect development of AHR.¹¹

Allergic asthma is characterised by an inflammatory response in the lungs. Comparable levels of airway inflammation were observed in SM-MHC^{Cre}IL-4R $\alpha^{-/lox}$ and IL-4R $\alpha^{-/lox}$ mice following treatment with allergen. No difference in concentration of eotaxin in blood serum or was seen between SM-MHC^{Cre}IL-4R $\alpha^{-/lox}$ and IL-4R $\alpha^{-/lox}$ mice. As expected, IL-4R $\alpha^{-/-}$ mice failed to generate a hyperplastic goblet cell mucus response, however both SM-MHC^{Cre}IL-4R $\alpha^{-/lox}$ and IL-4R $\alpha^{-/lox}$ mice showed hyperplastic goblet cell driven mucus responses. Finally lymphocytes from SM-MHC^{Cre}IL-4R $\alpha^{-/lox}$ mice produced similar amounts of IL-4 and increased amounts of IL-5, and IL-13 in after *in vitro* stimulation with OVA compared to IL-4R $\alpha^{-/lox}$ mice. The differences were only observed after antigen specific stimulation but not after stimulation with anti-CD3 antibodies (data not shown) and increased *in vitro* IL-5 and IL-13 production did not result in different outcomes of AHR, mucus production, BAL eosinophils and Th2 type antibody response typical of allergic reactions.

Major symptoms of allergic asthma are mediated by IL-4 and IL-13 and a recent study demonstrated that IL-4R α signalling in epithelial cells is required for enhanced mucus production in response to allergens. Using mice with disrupted expression of IL-4R α on clara cells (goblet cell precursors), it was possible to demonstrate a cell specific requirement for IL-4R α for allergen induced mucus production in a non-immune cell type ¹¹. Although epithelial cell IL-4R α signalling was required for mucus production, it was not required for development of AHR suggesting that IL-4R α signals on other cell types must contribute to induction of AHR.

The finding that loss of IL-4R α on smooth muscle cells had no effect on any of the major aetiological markers of allergic asthma is surprising. A large body of *in vitro* data indicated a contribution of smooth muscle cell IL-4R α in allergic asthma. Several studies showed enhanced responses of cultured murine smooth muscle cells to contractile agonists after treatment with IL-4 and IL-13 ^{12, 14, 30}. In human smooth muscle cells, IL-13 increased intracellular Ca²⁺ responses induced by contractile agonists ¹⁴ and decreased responsiveness to bronchodilators ³¹. Based on these results it was believed that narrowing of the airways in allergic asthma was influenced by direct effects of IL-4 and IL-13 on ASMC. However, it was shown here that AHR in this model was independent of IL-4R α signals in ASMC, indicating that enhanced responses to contractile agonists after treatment with IL-4 or IL-13 observed *in vitro* are not necessary for AHR development *in vivo*. Furthermore, it was shown that IL-4R α signalling on smooth muscle cells is not essential for inflammatory responses and eotaxin release during acute phase allergic asthma. As lung endothelial cells express eotaxin in response to IL-13 ³² in a STAT6 dependent manner ³³ these cells may be responsible for the IL-4R α dependent increases in serum eotaxin observed here contributing to the airway inflammation. On the other hand we have previously shown that direct effects of IL-13 on airway epithelial cells did not induce airway inflammation *in vivo* ²⁹.

Using the SM-MHC^{Cre}IL-4R $\alpha^{-/lox}$ mice enabled us, for the first time, to test *in vivo* the assumptions about the role of IL-4R α on ASMC derived from *in vitro* studies. No evidence was found to support an important role for smooth muscle cell IL-4R α in allergic asthma. The presence of IL-4R α on ASMC in allergic asthma was not, as previously suggested, critical for the disease outcome, highlighting the importance of *in vivo* studies of complex diseases such as allergic asthma which are characterised by complicated interactions of different cell populations. However, despite not being necessary for the development of

allergic airway disease in this model, our results do not rule out a contribution of IL-4R α on ASMC to allergic asthma. ASMC do have the ability to produce a wide range of cytokines and chemokines upon stimulation³⁴ which are likely to play an important immunoregulatory role in allergic asthma independent of direct responsiveness to IL-4R α . Such candidates are likely to define the role of ASMCs in allergic asthma.

The results presented here were derived from a mouse model of allergic asthma and the situation might be similar in the human disease. As such the data presented here could have important consequences for the development of new treatments of allergic asthma and therapies targeting IL-4R α in the lung might have no direct effect on ASMC.

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Abbreviations

AHR	Airway hyperreactivity
OVA	Ovalbumin
IL-4R α	IL-4 receptor alpha
SM-MHC	smooth muscle myosin heavy chain
mMCP-1	mouse mast cell protease-1
ASMC	airway smooth muscle cell
PAS	periodic acid Schiff

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Figure 1. Airway resistance is not affected by loss of the IL-4R α on smooth muscle cells
 Airway resistance to increasing concentrations of intravenously administered acetylcholine (A), aerosolised methacholine (B) or Penh values in response to methacholine (B) were measured in IL-4R $\alpha^{-/-}$ and SM-MHC^{cre} IL-4R $\alpha^{-/-}$ mice after OVA or PBS sensitization and challenge. Values represent pooled data with SD from two experiments with 4–8 mice per group. (* $p < 0.05$; *** $p < 0.001$ vs. PBS control)

Figure 2. Markers of lung inflammation are not affected by loss of the IL-4Ra on smooth muscle cells after sensitisation and challenge with OVA

Total cell numbers (A) and numbers of eosinophils (EOS), neutrophils (Neu), lymphocytes (Lym) and macrophages (Mac) were counted in the BAL fluid. Eosinophil peroxidase (EPO) (B) and mouse mast cell protease (mMCP-1) (C) levels were measured in lung tissue lysate. Eotaxin concentration was measured in the blood serum by ELISA (D). Values represent pooled data with SD from two experiments with 4–6 mice per group. (*, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.001$ vs. PBS control)

Figure 3. Goblet cell hyperplasia and mucin production after sensitisation and challenge with OVA is not affected by loss of the IL-4R α on smooth muscle cells

Mucus producing goblet cells were stained with PAS or anti-MUC5AC antibody in the airways of OVA sensitised and challenged IL-4R $\alpha^{-/-lox}$ (A), SM-MHC^{cre} IL-4R $\alpha^{-/-lox}$ (B) and IL-4R $\alpha^{-/-}$ mice (C) and PBS sensitised and challenged IL-4R $\alpha^{-/-lox}$ mice (D). Pictures were taken with a magnification of $\times 400$ and representative examples of three experiments are shown here. Arrows: mucus producing goblet cells; Aw: airways

Figure 4. Sensitisation and challenge with OVA induces a Th2 type immune response in IL-4Rα^{-/-/lo x} and SM-MHC^{cre}IL-4Rα^{-/-/lo x} mice

Lymphocytes (A) from mediastinal lymph nodes and splenocytes (B) were restimulated *in vitro* with OVA (100μg/ml) and cytokine concentrations were measured in the supernatant by ELISA. (ND: not detected, * p< 0.05; ** p< 0.005; *** p< 0.001 vs. IL-4Rα^{-/-/lo x} OVA)

Figure 5. The allergen specific antibody response of SM-MHC^{cre}IL-4R $\alpha^{-/-}$ mice resembles the antibody response of IL-4R $\alpha^{-/-}$ mice

OVA specific antibodies were measured by endpoint titration ELISA. Tested subclasses were IgE (A), IgG1 (B), IgG2a (C) and IgG2b (D). Data represent pooled values with SD from two experiments with 4–6 mice per group.