

Involvement of distal airways in a chronic model of experimental asthma

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Summary

Background Bronchial asthma is characterized by chronic airway inflammation and airway remodelling which occurs in both proximal and distal airways. These changes are associated with development of airway hyper-responsiveness and airflow limitation.

Objective This study was aimed to analyse whether chronic inhalative allergen challenges in mice lead to morphological and physiological changes comparable with this phenotype.

Methods For this purpose, BALB/c mice were systemically sensitized to ovalbumin (OVA) followed by aerosol allergen challenges on 2 consecutive days per week for 12 weeks.

Results In chronically challenged mice, tissue inflammation in proximal as well as distal airways was observed with a predominance of lymphocytes within the cellular infiltrate. In contrast, inflammation in the airway lumen decreased over time. These changes were associated by a shift in bronchoalveolar lavage–cytokine levels from IL-4, IL-5 and TNF- α production (during the acute phase) towards markedly increased levels of TGF- β during the chronic phase. Goblet cell hyperplasia and subepithelial fibrosis occurred throughout the airway tree. In terms of lung function, chronically challenged mice developed persistent bronchial hyper-responsiveness and progressive airflow limitation. Six weeks after OVA aerosol discontinuation, airway inflammation still persisted although lung function was normalized.

Conclusion These data indicate that our model of chronic aerosol allergen challenges leads to a phenotype of experimental asthma with participation of distal airways and persistence of inflammation thereby resembling many morphological and physiological aspects of human bronchial asthma.

Keywords airway inflammation, chronic asthma, distal airways, lymphocytes, remodelling, TGF- β

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Introduction

The increasing prevalence and chronic nature of allergic asthma represent a major health issue and a significant economic burden. Allergic asthma is a chronic disease that is characterized by a significant loss in lung function with bronchial hyper-responsiveness (BHR) and persistent airway obstruction [1]. These clinical manifestations are associated with chronic inflammation of the airways and complex changes in airway structure, including increased smooth muscle mass, goblet cell hyperplasia and subepithelial fibrosis [2, 3]. The pathophysiology of asthma has traditionally been correlated to an inflammatory process and structural changes in large airways [4]. These inflammatory changes are associated with infiltration of the major airways with eosinophils and activated CD4⁺ T lymphocytes [5]. Nonetheless, more recent studies suggest that the inflammatory changes associated with asthma occur throughout the whole

lung, including also distal airways [6–8]. Activated eosinophils and T lymphocytes have also been shown to accumulate in the mucosa of small airways in patients with chronic stable asthma [7]. The pathophysiological consequences of persistent inflammation of small airways are comparable with changes in major airways and include goblet cell hyperplasia with resulting mucus hypersecretion and collagen deposition [4]. A critical role of small airway inflammation and remodelling has been suggested in the development of BHR and airway obstruction in asthmatic patients [9, 10].

Animal models represent an important research tool to examine the complex pathophysiology of allergic asthma. A widely used model is the short-term exposure to aerosolized antigen to induce the development of acute allergic airway inflammation in systemically sensitized mice. However, this approach has significant limitations because of its incomplete presentation of the asthmatic phenotype. Short-term exposure models develop an inflammatory response, which is usually restricted to proximal airways and not associated with airway remodelling.

Here we demonstrate a model which shows involvement of both proximal and distal airways and which exhibits long lasting changes in airway pathology associated with structural

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remodelling. Although several authors have described structural changes in airways of rodents after chronic allergen exposure [11–15], marked inflammatory processes and structural changes in small airways were usually absent in mouse models of experimental asthma. Based on our model of acute allergic inflammation [16, 17], a protocol was developed that demonstrates chronic allergic inflammation of proximal and distal airways mainly with eosinophils and lymphocytes. Moreover airway inflammation was associated with subepithelial fibrosis, goblet cell hyperplasia and increased mass of α -smooth muscle actin (SMA)-positive cells in small airways. Furthermore, the inflammatory and structural changes in this model were associated with stable airflow limitation and increased BHR.

Methods

Animals

Pathogen-free female BALB/c mice (Harlan Winkelmann, Hannover, Germany), weighing 18–22 g and 6–8 weeks of age, were used in all experiments. The animals were maintained under standard housing conditions, fed an ovalbumin (OVA)-free diet and supplied with food and water *ad libitum*.

Sensitization protocol

Mice were sensitized to OVA by three intraperitoneal injections [10 μ g OVA grade VI (Sigma, Deisenhofen, Germany) adsorbed to 1.5 mg Al(OH)₃ (Pierce, Rockford, IL, USA) diluted in 200 μ L phosphate-buffered saline (PBS)] on days 1, 14 and 21. The mice were challenged with OVA (grade V) aerosol (1% wt/vol in PBS) via the airways twice a week on 2 consecutive days over a period of 12 weeks as previously described [16]. Sham sensitization and challenges were carried out with sterile Al(OH)₃ in PBS. Animals were analysed after 1 or 12 weeks of OVA aerosol challenge. To investigate persistence of airway inflammation and lung physiological changes mice were analysed after 6 weeks of OVA aerosol challenge discontinuation following 12 weeks of OVA aerosol challenge.

Differential cell counts in bronchoalveolar lavage fluid

Forty-eight hours after the last allergen challenge bronchoalveolar lavage (BAL) was performed and analysed as previously described [16].

Measurement of cytokines in bronchoalveolar lavage fluid

IL-4, IL-5 and TNF- α were measured in cell-free lavage fluids by cytometric bead array (CBA, BD Biosciences, San Diego, CA, USA). The detection limit for each of the cytokines was 10 pg/mL. Complete BAL TGF- β was measured after acidic activation using chicken anti-human TGF- β (R+D Systems, Minneapolis, MN, USA) in a standard ELISA protocol as described previously [17]. The detection limit for TGF- β was 20 pg/mL.

Lung histology

Lungs were fixed *ex situ* with 4% (wt/vol) paraformaldehyde via the trachea, removed and stored in 4% paraformaldehyde.

Lung tissues were embedded into paraffin, and 3 μ m sections were stained with haematoxylin and eosin or periodic acid-Schiff staining as described previously [18]. For localization of collagen fibrils, Sirius red staining was performed according to Uhal et al. [19].

Immunohistochemistry

Indirect immunohistochemistry was used to stain lung sections for smooth muscle cells and myofibroblasts (mouse monoclonal IgG against α -SMA, clone 1A4, Immunotech, Marseilles, France), and nerve cells (polyclonal rabbit anti-serum against protein gene product 9.5, Biogenesis, Poole, UK) as described earlier [20]. To distinguish smooth muscle cells from myofibroblasts a rat monoclonal IgG against fibroblast-specific peptide (clone ER-TR7; Biogenesis) was utilized.

Quantitative morphology

Paraffin sections stained with Fast Green/Sirius Red and for α -SMA, respectively, were used to quantify changes in airway epithelial cell, collagen and smooth muscle cell layers of distal airways according to standard stereological methods [21]. A distal airway was defined as the segment of a terminal bronchiolus that, starting at the bronchioalveolar duct transition, extended up to five alveoli along the proximal direction. Using a PC-based Olympus light microscope BX 51 equipped with a CAST-Grid System (Visiopharm, Hoersholm, Denmark), all distal airways of a given section were delineated (at a magnification of $\times 426$), and the fields of view to be analysed (at a final magnification of $\times 1700$) were automatically defined according to systematic uniform random sampling. The arithmetic mean thicknesses (τ_{comp}) were determined as the volume of the respective component, determined by counting all points hitting airway epithelium, Sirius Red- and α -SMA-positive components, respectively. Results were referred to the reference surface determined by counting all intersections with the airway epithelial basal membrane [22]. The arithmetic mean thicknesses were calculated according to the formula:

$$\tau_{\text{comp}} = \frac{L(P) \sum P_{\text{comp}}}{2 \sum I_{\text{bl}}}$$

where $L(P)$ is the length per test point, P_{comp} the points hitting the respective component and I_{bl} the intersections of the test line with the epithelial basal lamina. A mean of 559 points hitting airway epithelium, 175 points hitting Sirius Red-stained collagen, 102 points hitting α -SMA-positive smooth muscle cells, and 114 respective 70 intersections with the epithelial basal lamina were counted per lung.

Electron microscopy and definition of proximal and distal airways

For analysis of airway wall ultrastructure, lungs of mice chronically challenged with OVA and control mice challenged with PBS ($n = 3$ per group) were fixed by instillation of 4% (wt/vol) paraformaldehyde via the trachea. Beginning at the lobar bronchus, the airways were microdissected along the axial pathway according to Plopper and co-workers with slight modifications [23, 24]. For histopathological and

ultrastructural analysis, proximal airways were defined as the lobar bronchi and the axial pathway down to the fourth intrapulmonary branch point. Mid-level airways were defined as the axial pathways between the intrapulmonary branch points 6–12. Terminal bronchioles with direct connection to the alveolar ducts were defined as distal airways [25].

Non-invasive measurement of mid-expiratory airflow at baseline and of bronchial responsiveness to β -methacholine

The mid-expiratory airflow (EF₅₀) was measured 24 h after the last OVA aerosol challenge using head-out body plethysmography as described previously [26].

Statistics

Results are presented as mean values $\pm$ SD unless stated otherwise. One-way ANOVA test or Student's unpaired *t*-test were used to determine the significance of differences between animal groups.

Results

Allergic inflammation

Total numbers of eosinophils, neutrophils, lymphocytes and macrophages were assessed in BAL fluid. BAL samples were taken after 1 or 12 weeks of OVA aerosol challenges to differentiate between effects of short-term and chronic exposure. After short-term OVA-exposure, an acute inflammation of the airways with marked increases in eosinophils, lymphocytes and macrophages was detected, in contrast to mice given aerosolized saline only. The leucocyte infiltrate was dominated by eosinophils and to a lesser extent by lymphocytes. After chronic OVA-exposure, numbers of eosinophils, lymphocytes and macrophages significantly declined when compared with the acute phase of airway inflammation, although total leucocyte numbers were still significantly higher than in control mice (Fig. 1a).

Chronic allergen exposure resulted in marked inflammation of the proximal airways which was associated with goblet cell hyperplasia, increased deposition of collagen fibres and an increase in fibroblasts and myo-fibroblasts within the wall of proximal airways.

Histological analysis of H&E-stained lung sections of chronically exposed mice revealed marked peribronchial inflammation of the airway mucosa that occurred throughout the whole lung, from proximal to distal airways. In contrast, inflammation in acutely exposed mice was almost completely restricted to proximal airways. In distal airways, eosinophils were only rarely seen. To assess leucocyte distribution within

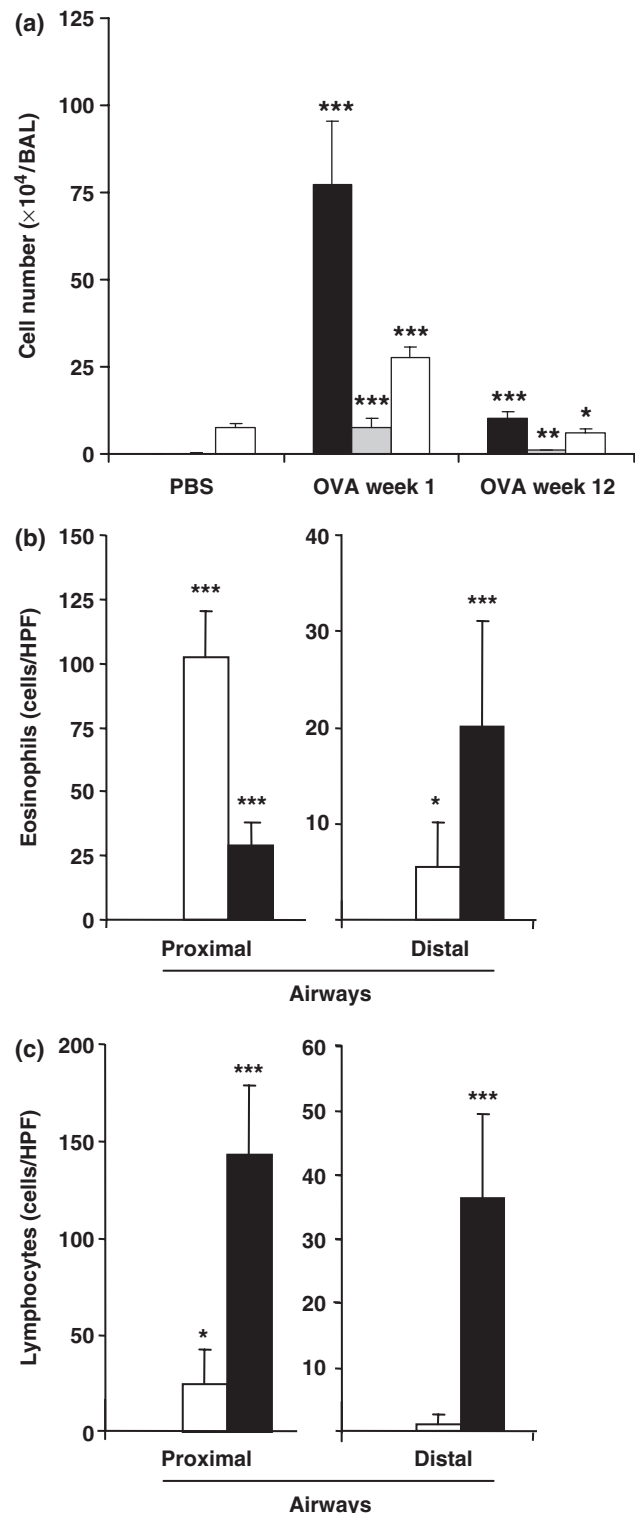


Fig. 1. Acute and chronic airway inflammation (a) Absolute numbers of eosinophils (black), macrophages (white) and lymphocytes (grey) in bronchoalveolar lavage (BAL) samples from ovalbumin (OVA)-sensitized BALB/c mice. Animals were challenged two times (OVA week 1; $n = 8$) or 24 times (OVA week 12; $n = 8$) with aerosolized OVA and compared with sham-sensitized mice challenged with aerosolized saline (phosphate-buffered saline (PBS); $n = 8$). Numbers of eosinophil (b) and lymphocyte (c) cell profiles in mucosa of proximal and distal airways. Determination of leucocyte cell profile counts was carried out in haematoxylin & eosin-stained whole-lung sections from sham-sensitized BALB/c mice challenged with aerosolized saline (grey bars) and OVA-sensitized BALB/c mice challenged two times (white bars) or 24 times (black bars) with aerosolized OVA, using high power field microscopy ($\times 100$ magnification). Leucocyte profile numbers are expressed as cell profile counts per high power field. BAL samples and lung tissues were taken 48 h after the last aerosol challenge. The unpaired one-way ANOVA ($*P < 0.05$; $**P < 0.01$; $***P < 0.001$) was used to compare differences between different OVA groups and PBS control. Data are presented as mean $\pm$ SD.

peribronchial inflammation, cell profiles of infiltrating leucocyte were counted using high power field microscopy. Leucocyte infiltrates in acute inflammation were dominated by eosinophils and to a lesser extend by lymphocytes. A different pattern was observed during the chronic inflammatory response. Lymphocytes dominated in proximal airways, whereas a substantial influx of both, eosinophils and lymphocytes, was detected in distal airways (Figs 1b and 2a–c, g).

The changes in the inflammatory cell pattern were associated with a shift in local cytokine production as measured in BAL fluid. In OVA-sensitized mice challenged twice with OVA, increased levels of IL-4, IL-5 and TNF- α were detectable (Table 1). After chronic allergen challenges, IL-5

levels were significantly decreased as compared with animals with acute inflammation. IL-4 and TNF- α were not detectable or just above the detection limits of the assays. TGF- β was measured in BAL fluids since it has been associated with chronicity and processes of airway remodelling. Although TGF- β was already detectable in BAL samples from acutely challenged mice, a marked increase in TGF- β concentrations was found in chronically challenged animals (Table 1).

Structural changes and airway remodelling

In acute airway inflammation, development of goblet cell hyperplasia associated with increased mucus production was

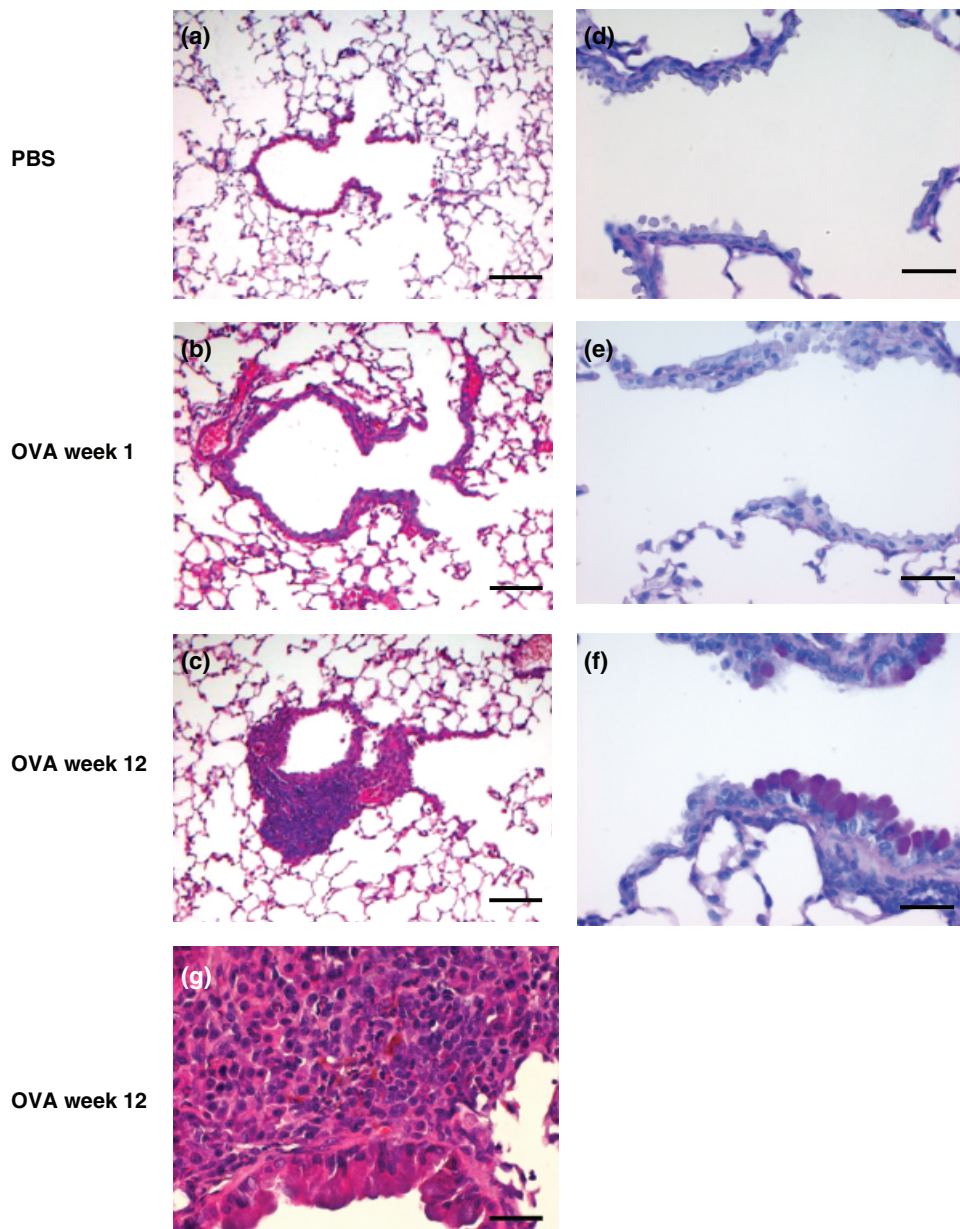


Fig. 2. Progression of airway inflammation in chronic and goblet cell hyperplasia in chronic experimental asthma. Lung histology from ovalbumin (OVA)-sensitized BALB/c mice after two or 24 time challenge with aerosolized OVA. Representative photomicrographs of haematoxylin & eosin (a–c, g) and periodic acid-Schiff (d–f)-stained whole-lung sections showing distal airways from negative control (phosphate-buffered saline (PBS); $n = 8$), acute (OVA week 1; $n = 8$) and chronic (OVA week 12; $n = 8$) allergic inflammation of airways were presented. Scale bar: 100 μ m (a–c); Scale bar: 25 μ m (d–g), E, eosinophils; L, lymphocytes.

restricted to proximal airways, whereas animals following chronic OVA aerosol exposure exhibited progressive hyperplasia of mucus producing cells in proximal as well as in distal airways (Figs 2d–f). Using high power field microscopy a marked increase in goblet cell profile counts was detected in distal airways of chronically exposed compared with acutely exposed mice (Fig 3).

A prominent increase in subepithelial collagen along the bronchial tree including distal airways was revealed by Sirius red staining (Figs 4a, b). This was supported at the ultrastructural level by a considerable increase in collagen micro fibrils and elastin in the subepithelial lamina propria of lungs chronically exposed to OVA aerosol compared with control lungs. The minute epithelial basal membrane, however, showed no ultrastructural alterations (Fig. 5). In addition, an increase in fibroblasts and in α -SMA-positive cell mass was observed by immunohistochemistry (Figs 4c–f). These observations were evidenced by quantitative morpho-

metric analysis which revealed a significant increase in arithmetic mean thicknesses of distal airway collagen and α -SMA-positive cell layer (Table 2).

Progressive airway hyper-responsiveness and airflow limitation

To investigate whether airway inflammation and remodelling associated with a change in lung function, mice were analysed by means of head-out body-plethysmography. Following acute exposure to aerosolized OVA, OVA-sensitized animals developed hyper-responsiveness to β -methacholine (MCh). The concentration that was necessary to induce a 50% decrease in the mid-expiratory airflow (EF₅₀) was significantly

Table 1. Cytokine levels in BAL fluids

	PBS	OVA week 1	OVA week 12
IL-4 (pg/mL)	u.d.	22.8 $\pm$ 2.1***	u.d.
IL-5 (pg/mL)	u.d.	134.5 $\pm$ 21.2***	44.9 $\pm$ 6.3***
TNF- α (pg/mL)	u.d.	34.6 $\pm$ 10.1***	u.d.
TGF- β (pg/mL)	31.9 $\pm$ 8.6	547.8 $\pm$ 149.9*	1502.8 $\pm$ 665.9***

Bronchoalveolar lavage (BAL) levels of IL-4, IL-5, TNF- α and TGF- β in samples from ovalbumin (OVA)-sensitized BALB/c mice. Animals were challenged two times (OVA week 1; $n=8$) or 24 times (OVA week 12; $n=8$) with aerosolized OVA and compared with sham-sensitized mice challenged with aerosolized saline (phosphate-buffered saline, PBS; $n=8$) for 1 or 12 weeks. BAL samples were taken 48 h after the last aerosol challenge. The unpaired student's *t*-test (* $P<0.05$, *** $P<0.001$) was used to compare differences between experimental groups and PBS control. Data are presented as mean $\pm$ SD (u.d. = under detection limit).

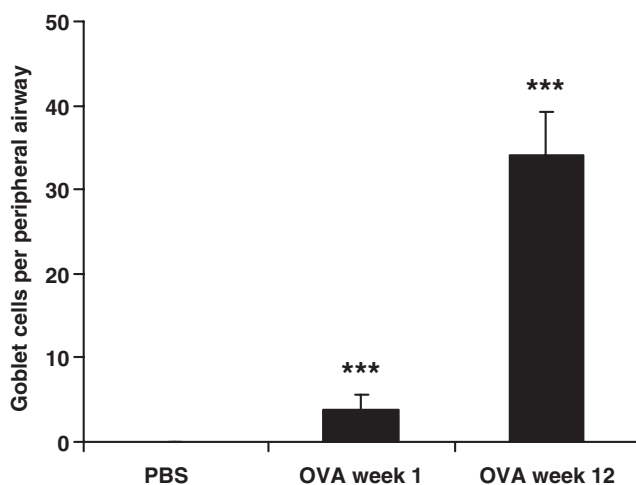


Fig. 3. Progressive goblet cell hyperplasia in chronic experimental asthma. Absolute numbers of goblet cell profiles in mucosa of distal airways of periodic acid-Schiff-stained whole lung sections from negative control (phosphate-buffered saline (PBS); $n=8$), acute (ovalbumin (OVA) week 1; $n=8$) and chronic (OVA week 12; $n=8$) allergic inflammation of airways were counted using high power field microscopy ($\times 100$ magnification). Leucocyte profile numbers are expressed in cell profile counts per cross section of equivalent distal airways. The significance between experimental groups was analysed using one-way ANOVA (*** $P<0.001$). Data are presented as mean $\pm$ SD.

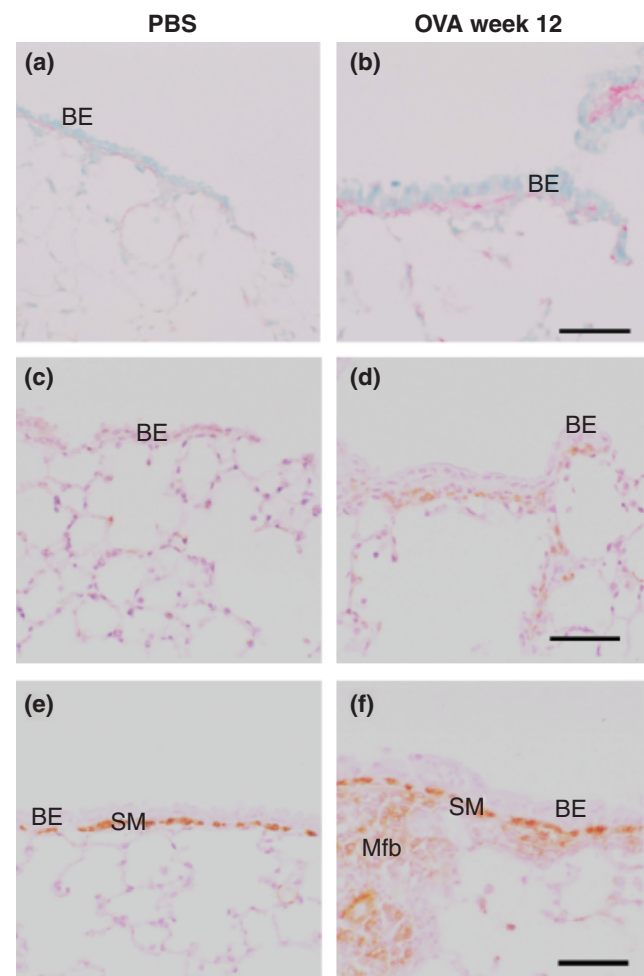


Fig. 4. Airway wall fibrosis in chronic experimental asthma (a, b) Sirius red staining of lung sections of ovalbumin (OVA)-sensitized BALB/c mice demonstrates accumulation of collagen fibrils in the wall of distal airways in chronically exposed (OVA week 12; $n=4$) vs. control (phosphate-buffered saline (PBS); $n=4$) animals. BE, airway epithelium. Scale bar = 50 μ m. (c, d) Immunohistochemical staining of lung sections of OVA-sensitized BALB/c mice for fibroblast-specific peptide demonstrates an increase in adventitial fibroblasts (Fbta) in distal airways of chronically exposed (OVA week 12) vs. control (PBS) animals. Immunoreactivity is also increased in the gas exchange region of chronically exposed mice. AD, alveolar duct; BE, airway epithelium. Scale bar = 50 μ m. (e, f) Immunohistochemical staining for α -smooth muscle actin (α -SMA) of lung sections from chronically OVA-challenged animals (OVA week 12; $n=4$) demonstrate accumulation of α -SMA-positive myofibroblasts in the airway wall as compared with negative controls (PBS; $n=4$). BE, bronchial epithelium; Mfb, myofibroblasts; SM, smooth muscle cell. Scale bar = 50 μ m.

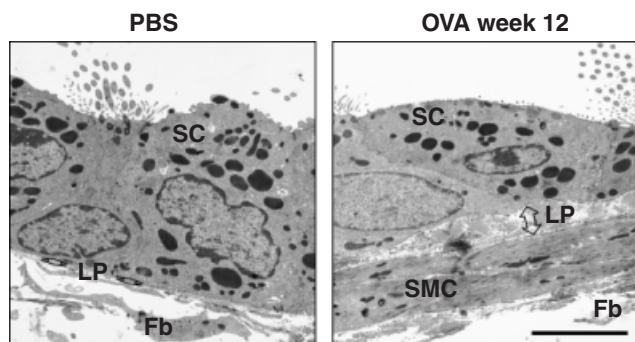


Fig. 5. Thickening of subepithelial lamina propria transmission electron micrographs of airway epithelium of ovalbumin (OVA)-sensitized BALB/c mice demonstrate a marked thickening of the subepithelial lamina propria (LP) in distal airways of chronically exposed (OVA week 12; $n = 4$) vs. control (phosphate-buffered saline (PBS); $n = 4$) animals. Fb, fibroblast; SC, serous cell; SMC, smooth muscle cell. Scale bar = 5 μ m.

Table 2. Quantitative morphological changes in distal airway walls

Arithmetic mean thicknesses of	PBS week 12	OVA week 12
Airway epithelium (μ m)	10.9 $\pm$ 2.6	11.0 $\pm$ 1.4
Airway collagen layer (μ m)	2.8 $\pm$ 0.1	4.5 $\pm$ 0.4***
α -SMA-positive cell layer (μ m)	3.2 $\pm$ 0.5	4.1 $\pm$ 0.7*

Arithmetic mean thicknesses of airway epithelium, airway collagen, and α -smooth muscle actin (α -SMA)-positive smooth muscle cells in paraffin sections from ovalbumin (OVA)-sensitized BALB/c mice. Animals were challenged 24 times (OVA week 12; $n = 5$) with aerosolized OVA and compared with sham-sensitized mice challenged with aerosolized saline (phosphate-buffered saline, PBS; $n = 5$) for 12 weeks. The unpaired student's t -test (* $P < 0.05$, *** $P < 0.001$) was used to compare differences between OVA group and PBS control. Data are presented as mean $\pm$ SD.

lower than in sham-sensitized animals challenged with aerosolized PBS. OVA-sensitized animals challenged chronically with aerosolized OVA revealed an increased BHR as indicated by a lower concentration of β -MCh to induce a 50% decrease in EF_{50} , as compared with animals with acute allergic inflammation (Fig 6a).

Furthermore, baseline levels of lung function were assessed after 1, 4, 6, 8, and 12 weeks, always 24 h after the last OVA challenge. After 8 weeks of challenge to aerosolized OVA, animals developed airflow limitation as indicated by a significant decrease in mid-expiratory airflow (EF_{50}) that persisted during 12 weeks of antigen challenge (Fig 6b).

Persistence of airway inflammation

Six weeks after discontinuation of OVA aerosol challenges lymphocytes remained present in both BAL fluid ($2.4 \pm 0.64 \times 10^4$ cells/BAL as compared with $2.02 \pm 0.30 \times 10^4$ cells/BAL at week 12) and airway walls (data not shown). However, animals did not reveal airflow limitation or airway hyper-responsiveness (AHR) (Table 3).

Discussion

This study demonstrates a model of experimental allergic asthma that resembles several features of the human disease.

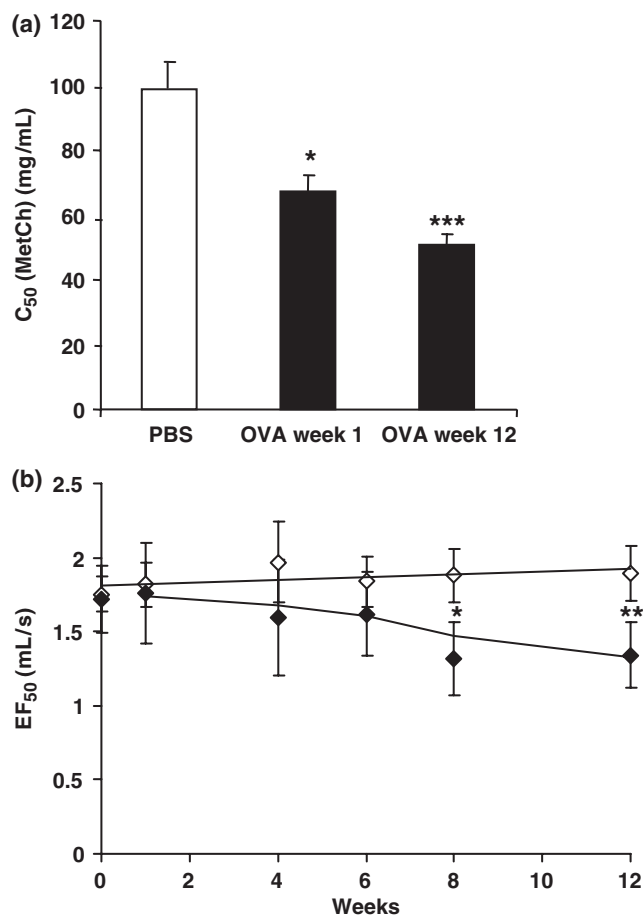


Fig. 6. Lung function in a mouse model of chronic experimental asthma (a) Measurement of airway reactivity to β -methacholine (MCh) in sham-sensitized BALB/c mice challenged with aerosolized saline (phosphate-buffered saline, PBS) and OVA-sensitized and OVA aerosol challenged BALB/c mice. Airway constriction was measured by determining changes in mid-expiratory airflow during consecutive exposure to aerosolized β -MCh in PBS with increasing concentrations and is represented as a concentration of β -MCh (C_{50}) that induces a 50% reduction in mid-expiratory airflow. (b) Measurement of mid-expiratory airflow (EF_{50}) in OVA-sensitized BALB/c mice at indicated time-points of challenge to aerosolized OVA (black diamonds; $n = 8$) or in sham-sensitized animals challenged with aerosolized PBS (white diamonds; $n = 8$). Measurement of EF_{50} was carried out using head-out body plethysmography 24 h after the last challenge. The significance between experimental groups was analysed using one-way ANOVA (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). Data are presented as mean $\pm$ SEM.

The phenotype was achieved following long-term allergen challenges over several months. A hallmark of the inflammatory condition in this model is the development of progressive airway inflammation comprising proximal and distal airways together with an increasing portion of lymphocytes compared with eosinophils in the chronic stage. Furthermore, the cytokine pattern is characterized by a marked increase in TGF- β which is accompanied by significant structural changes in the airways including hyperplasia of epithelial goblet cells, subepithelial fibrosis and increases in airway smooth muscle mass. Furthermore, the mice developed progressive AHR and obstruction.

Our study revealed that allergic inflammation shows a differential pattern of eosinophil/lymphocyte distribution dependent on the stage of inflammation and the type of airways (e.g. proximal vs. distal). Although in chronically

Table 3. Persistence of airway inflammation and lung physiological changes

	PBS	OVA week 12 ¹	OVA week 12+6 ²
Airway inflammation (10 ⁴ cells/BAL)			
Eosinophils	0.09 ± 0.05	10.25 ± 2.0***	0 ± 0
Lymphocytes	0.16 ± 0.06	1.02 ± 0.3***	2.4 ± 0.64
Macrophages	7.56 ± 1.33	5.94 ± 1.24	9.72 ± 0.83
Mucosal airway inflammation (cells profiles/HPF)			
Eosinophils	0 ± 0	20.17 ± 4.45	1.25 ± 0.52
Lymphocytes	0 ± 0	36.33 ± 5.12	9.17 ± 1.25
Macrophages	0 ± 0	6.67 ± 1.43	3.67 ± 0.88
MCh ₅₀ (mg/mL)	98.8 ± 8.37	35.87 ± 4.24	71.76 ± 3.90
EF ₅₀ (mL/s)	1.89 ± 0.20	1.34 ± 0.17	1.92 ± 0.17

BALB/c mice were ovalbumin (OVA)-sensitized and challenged with OVA aerosol for 12 weeks (OVA week 12; *n* = 8). To investigate persistence of airway inflammation BALB/c mice were analysed after 6 weeks of discontinuation of OVA aerosol challenge (OVA week 12+6; *n* = 8). Sham-sensitized animals (phosphate-buffered saline (PBS); *n* = 8) were challenged with aerosolized PBS. For details in methodology, see figure legends for Figs 1 and 6 or Material and Methods section.

MCh, β -methacholine; EF₅₀, mid-expiratory airflow; BAL, bronchoalveolar lavage; HPF, high power field.

OVA-exposed mice the inflammatory response within the airway lumen was less prominent as compared with the acute situation, tissue inflammation was markedly increased. This increase in tissue inflammation was not only because of the quantitatively elevated amount of infiltrating leucocytes, but also to the qualitative expansion of airway inflammation from proximal throughout intermediate down to distal airways. Compared with acute allergic inflammation, the chronic inflammation was dominated by lymphocytes. Thus, we observed not only a progression of airway inflammation from proximal to distal airways, but also a shift in the lymphocyte/eosinophil ratio within the infiltrate. These lymphocytes may be characteristic for a chronic inflammatory response induced by repeated allergen challenge and remained still in alveolar lumina and in the airway walls even after 6 weeks of aerosol OVA challenge discontinuation. The numbers of lymphocytes and macrophages revealed a moderate but not significant increase as compared with the situation before aerosol OVA challenge discontinuation. At the same time-point eosinophils are completely absent in airway tissue as well as in BAL fluid. The mechanism for this observation is still speculative and remains to be investigated in more detail. Eosinophils may have disappeared because of the absence of specific survival factors and/or chemotactic factors. In contrast, lymphocytes and macrophages seemed to be further attracted into the affected tissue either as a consequence of continued expression of specific adhesion molecules or chemotactic attraction. In addition, these cell populations are characterized by a prolonged survival when compared with eosinophils.

However, the role of these lymphocytes remains to be investigated in more detail. The decrease in BAL leucocytes after prolonged antigen exposure is consistent with previously reported results [14, 15]. However, a progression to distal airways and an increase of the inflammatory response to chronic allergen exposure has not been described in murine models of experimental asthma so far.

The decreased numbers of leucocytes in BAL fluid and the extensive collagen deposition in the airway wall in our model

may be in part linked to the markedly increased TGF- β levels in BAL fluids. Since TGF- β levels increased dramatically over time, we investigated whether chronic exposure to aerosolized OVA leads to structural changes in the airways. Indeed, an increased deposition of elastin and collagen fibres was observed by histochemistry and electron microscopy in the epithelial lamina propria and in the tunica muscularis of proximal and distal airways. This was accompanied by increased mass of α -SMA-positive cells.

The bronchial epithelium is suspected to be the major source of TGF- β [27] in asthmatic patients, but infiltrating inflammatory cells, such as eosinophils and lymphocytes have been reported to also express TGF- β [27, 28]. Furthermore, higher TGF- β levels were detected in BAL fluids of stable asthmatic patients and it is reported that TGF- β is synthesized during the late phase response to allergens [29]. Dependent on the situation and site of production, TGF- β exhibits anti-inflammatory and pro-fibrotic activities [30]. Within the airways, TGF- β has various effects and seems to play a major role in matrix regulation. For example, it stimulates lung fibroblast proliferation [31], promotes matrix protein synthesis [32] and enhances anti-protease activity [33]. On the other hand, TGF- β can inhibit T and B lymphocyte proliferation [34], and suppresses T helper type 2 cell function and cytokine production [35]. Both observations, decreased inflammation of the airway lumen and increased collagen deposition in the airway wall, are in accordance with both, the anti-inflammatory and pro-fibrotic effect of TGF- β .

We finally investigated the association of inflammatory and structural changes in the airways with changes in lung function by using head-out body-plethysmography. Mice that were chronically exposed to OVA developed stable airflow limitation as indicated by a significant decrease in mid-expiratory airflow and chronically exposed mice were more sensitive to MCh as acutely challenged animals. In our model, the persistence of airway hyper-reactivity and particularly the progression of airflow limitation were associated with a progression of airway inflammation. These changes occurred in parallel to the development of signs of airway remodelling. It still remains to be elucidated how airway hyper-reactivity is regulated in this model, however, both immunological and neuronal dysfunction seem to play a critical role [36].

Wagner et al. [37] provided evidence that hyper-reactivity is a feature of distal lung elements as well, though the underlying mechanisms seem to differ from those of proximal airways. Distal airways may play a critical role in the development of stable airflow limitation in patients suffering from chronic asthma. In distal airways any decrement of the luminal diameter results in significantly increased airway resistance that leads to airflow limitation. Several pathophysiological events might be intimately involved in limiting the diameter of distal airways in a direct or indirect fashion. These include structural changes in distal airways such as airway wall thickening because of a deposition of elastin and collagen fibres, increased mass of α -SMA-positive cells, mucus plugging because of mucus hyper-secretion and accumulation of inflammatory cells accompanied by tissue oedema [37]. The decrease in mid-expiratory airflow as observed in our model could be consistent with such structural changes and tissue inflammation in distal airways.

In conclusion, this novel model of experimental asthma reflects several structural and physiological changes similar to human bronchial asthma. They include progressive airway inflammation in association with epithelial goblet cell hyperplasia and subepithelial fibrosis in proximal as well as in distal airways. There was an association with a progression of airflow limitation and persistent airway hyper-reactivity. Inflammation in chronic experimental asthma was associated with a significant shift in cytokine production with a down-regulation of inflammatory cytokines accompanied by a marked increase in TGF- β . This model may now also open the opportunity to further investigate the contribution of small airways to the pathophysiology of allergic asthma.

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