

Allergic Airway Inflammation Initiates Long-Term Vascular Remodeling of the Pulmonary Circulation

Kristina Rydell-Törmänen Lena Uller Jonas S. Erjefält

Division of Vascular and Airway Research, Department of Experimental Medical Science, Lund University, Lund, Sweden

Key Words

Vascular remodeling · Ovalbumin · Smooth muscle · Procollagen I

Abstract

Background: Asthma and allergic airway inflammation are associated with persistent structural alterations in the bronchi, i.e. airway remodeling. Previous studies have shown that during allergic airway inflammation, similar structural alterations may also be evoked in the pulmonary circulation. However, it remained unknown whether remodeling of the pulmonary circulation is as persistent as airway remodeling. The aim of this study is to investigate the reversibility and resolution of vascular remodeling, induced by allergic airway inflammation. **Methods:** A validated mouse model of allergic airway inflammation, utilizing ovalbumin as allergen, was employed. Animals were sacrificed 1 day, 1 week or 1 month after the last allergen exposure, and different parameters of remodeling (smooth muscle mass, proliferation of smooth muscle cells and endothelial cells as well as number of myofibroblasts and procollagen-I-producing cells) were investigated and quantified histologically. **Results:** Allergen exposure resulted in allergic airway inflammation characterized by a transient leukocyte infiltration and in structural alterations in both airway and vascular compartments. The increase in vascular smooth muscle mass and endothelial proliferation persisted at 1 month after the last

allergen exposure. The other parameters and cellular inflammatory response returned to baseline within 1 month after the last allergen challenge. **Conclusions:** Based on the findings in this study, we conclude that acute allergic airway inflammation, although being initiated from the airways, is able to evoke similar long-term structural alterations in pulmonary vessels as described for bronchi.

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Introduction

In allergic diseases, like asthma, the airways are subjected to inflammatory processes and structural alterations in the bronchi, so-called 'airway remodeling' [1–3]. Recent data suggest that, similar to the airways, also the pulmonary circulation is affected and undergoes remodeling as a result of allergic airway inflammation [4–8]. However, this remodeling, which affects the large and mid-sized pulmonary vessels, is far less studied than the structural changes in asthmatic bronchi. Yet, the remodeling of the lung vasculature can be profound and persistent, as exemplified in a variety of non-allergic diseases like pulmonary arterial hypertension (PAH), idiopathic PAH [9, 10], chronic obstructive pulmonary disease [11–13], systemic sclerosis and systemic lupus erythematosus [9, 14, 15]. The changes in these conditions range from increased endothelial proliferation, intimal fibrosis, in-

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Correspondence to: Dr. Kristina Rydell-Törmänen, Division of Vascular and Airway Research, Department of Experimental Medical Science, Lund University, BMC D12, SE-221 84 Lund (Sweden)
Tel. +46 46 222 0570, Fax +46 46 222 4546
E-Mail Kristina.Rydell-Tormanen@med.lu.se

creased medial thickness, luminal occlusion and plexiform lesions [9, 13, 14, 16, 17]. The importance of inflammation and inflammatory processes in the pathogenesis was recently highlighted [18, 19], and previous findings of vascular remodeling induced by allergic airway inflammation [20–22] further support this notion. In contrast to the large and mid-sized pulmonary vessels, remodeling of the bronchial microcirculation in asthma is well described [23, 24] and thought to be persistent. However, data from mice subjected to infectious airway inflammation have revealed a remarkable plasticity of remodeled vessels and a capacity to normalize [25, 26]. Whether the type of remodeling occurring in pulmonary vessels upon allergic inflammation readily resolves has not yet been investigated.

The aim of the present study was to investigate the resolution of vascular remodeling and to determine whether acute allergic airway inflammation is capable of inducing long-lasting vascular remodeling. A mouse model of allergic airway inflammation utilizing ovalbumin (OVA) as allergen was employed [21, 22], where animals were sacrificed at different time points after the last allergen challenge. The time points were selected to represent ongoing airway inflammation, as well as several weeks after the peak of inflammation. Tissue samples were collected in order to assess multiple and previously validated parameters of vascular remodeling [20–22]. Our results demonstrate that the pulmonary vasculature responds to allergic airway inflammation by initiating several features of vascular remodeling that persist after the allergic inflammation has resolved.

Methods

Model

Female BALB/C mice (MoB A/S, Ry, Denmark) were sensitized and challenged with OVA or saline (SAL) as previously described [27]. Animals were sacrificed on day 21, i.e. 1 day after the last challenge (1d; OVA, $n = 8$; SAL, $n = 7$), on day 28, i.e. 1 week after the last challenge (1w; OVA, $n = 8$; SAL, $n = 7$) or on day 51, i.e. 1 month after last challenge (1m; OVA, $n = 4$; SAL, $n = 3$), and lung tissue specimens were obtained for paraffin embedding. All animal protocols were approved by the local ethics committee (Malmö/Lund, Sweden).

Tissue Eosinophilia and Assessment of General Inflammation

Development of an allergic airway inflammation was determined as lung tissue eosinophilia, using enzyme-histochemical staining for eosinophil peroxidase [28]. For a general assessment of the cellular inflammation and to gain an overview, hematoxylin/eosin staining was performed (fig. 1a–c).

Immunohistochemistry

For all immunohistochemistry, a standard protocol was used, as described previously [22]. Briefly, 5- μm paraffin sections were rehydrated and allowed to equilibrate in buffer before a primary antibody was applied onto the section in an appropriate, previously determined dilution, incubated, washed and incubated with secondary antibody before visualization, mounting and analysis. For double labeling, a second primary antibody was applied and the protocol repeated. Details on antibodies and detection of different remodeling parameters are displayed in table 1. Antigen retrieval was performed before procollagen I labeling, using enzymatic retrieval with pepsin/HCl (0.4% pepsin in 0.01 M HCl) for 20 min in 37°C. Hematoxylin was used as background staining.

Quantification and Statistics

The histologic analysis was performed as previously described [22]. Briefly, high-resolution digital images were randomly obtained from tissue areas containing blood vessels. Cross-sectioned blood vessels within the images were divided into subgroups: microvessels (defined as small solitary vessels of 50–249 μm in perimeter; ≥ 150 μm from the closest bronchi), preseptal veins (defined as mid-sized solitary vessels of 250–500 μm in perimeter; ≥ 150 μm from the closest bronchi) and pulmonary arteries (bronchial associated vessels ≥ 250 μm in perimeter). Representative illustrations of the 3 types of vessels investigated are shown in figure 1a–c. Quantification was done as previously described [22]; the length of the basement membrane was assessed by manual cursor tracing using the program ImageJ (ImageJ, Wayne Rasband, NIH, Bethesda, Md., USA). Next, using a fixed threshold, the total area of positive staining directly beneath the luminal perimeter was calculated automatically and correlated with the length of the basement membrane. Positively labeled solitary cells (proliferating cells, procollagen-I-producing cells and myofibroblasts) were analyzed by counting the number of positive cells and correlating the number with the length of the basement membrane. Eosinophilia was calculated by obtaining digital images in a random fashion from slides stained by eosinophil peroxidase; all eosinophils within the image were counted and related to the tissue area. All quantification was made in a blinded fashion, and at least 3 vessels of each type were counted for each parameter and time point. As the SAL-exposed controls only differed slightly between the 3 control groups (a Kruskal-Wallis ANOVA showed no significant differences), they were all pooled into 1 group against which OVA-treated animals were compared. Comparison of allergen-challenged animals with controls was done using the Kruskal-Wallis ANOVA with Bonferroni (Analyze It™, Analyze-it Software Ltd., Leeds, UK). Data are given as the median $\pm$ interquartile range (ICQ, 95% confidence interval), and $p < 0.05$ was considered statistically significant.

Results

Confirmation of Allergic Inflammation

The presence of allergic airway inflammation was confirmed by tissue eosinophilia. As previously described, the eosinophilia had a foremost peribronchial and perivascular distribution (fig. 1d–f) [22]. At 1d, the

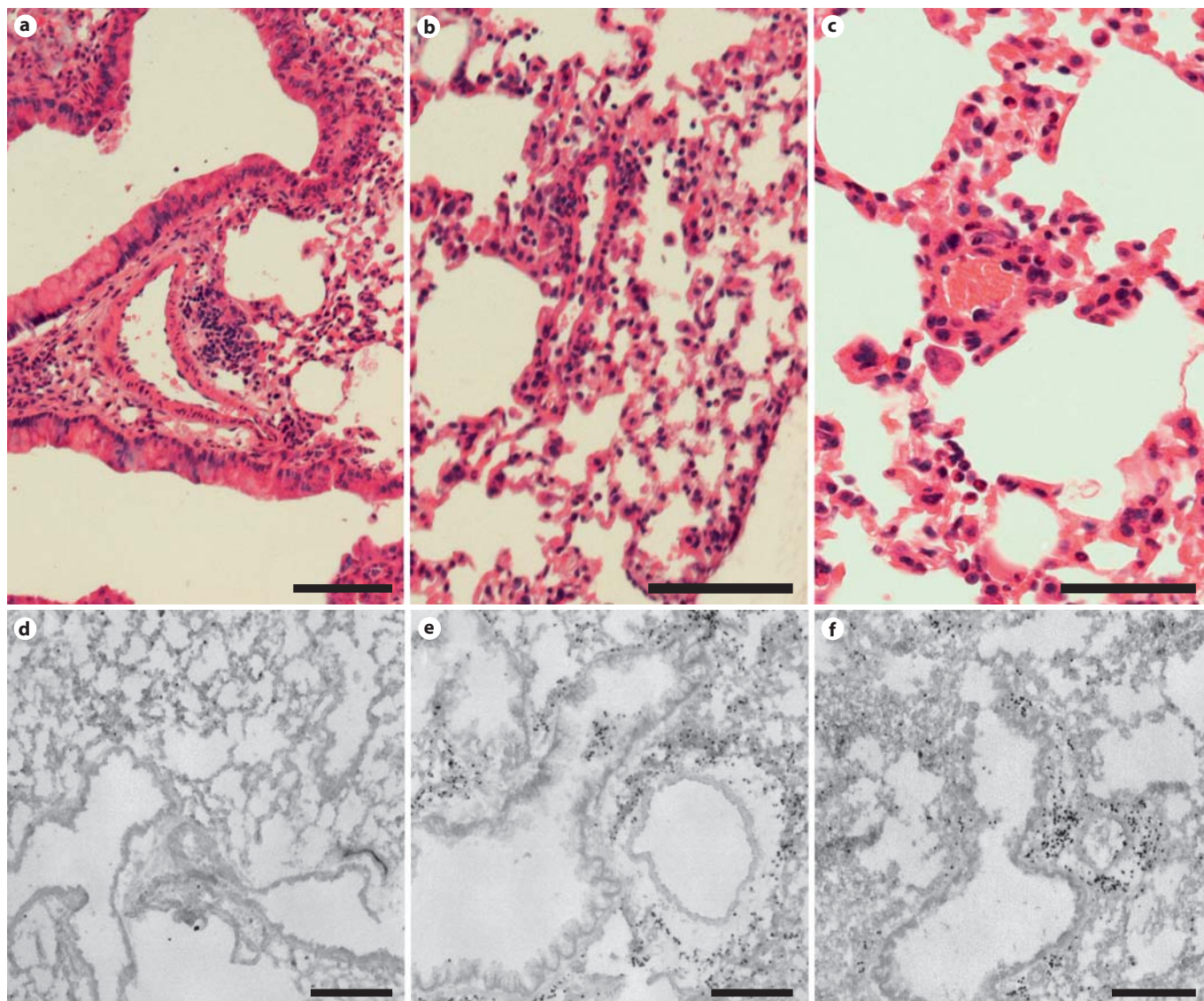


Fig. 1. Representative illustrations of the 3 types of vessels investigated and the distribution of perivascular eosinophilia. Pulmonary arteries (**a**, bronchial associated vessels ≥ 250 μm in perimeter and in close proximity to airways), preseptal veins (**b**, defined as mid-sized solitary vessels of 250–500 μm in perimeter; ≥ 150 μm from the closest bronchi) and microvessels (**c**, defined as small solitary vessels of 50–249 μm in perimeter; ≥ 150 μm from

the closest bronchi). The images are taken at 1 day resolution after 7 daily allergen challenges and stained with hematoxylin/eosin. In comparison with SAL-exposed controls (**d**), the tissue eosinophilia induced by inflammation displayed a perivascular distribution following 1 day of resolution (**e**) and 1 week of resolution (**f**). Eosinophils are visualized by staining for endogenous peroxidase. Scale bars = 100 μm (**a**, **b**, **d**, **e**) and 50 μm (**c**).

number of eosinophils was significantly increased (586 ± 47 eosinophils/ mm^2 tissue) compared with controls (29 ± 3 eosinophils/ mm^2 tissue). At 1w, the eosinophilia (243 ± 14 eosinophils/ mm^2 tissue) had decreased somewhat but was still significantly increased compared with controls. At 1m, the eosinophilia had returned to baseline (25 ± 3 eosinophils/ mm^2 tissue).

Endothelial Proliferation

Endothelial proliferation was increased in all types of vessels at 1d and remained significantly increased throughout the study (table 2).

Table 1. Overview of the antibodies and visualization methods used in the study

Remodeling parameter		Antibody	Visualization
Proliferating endothelial cells	Proliferation	PCNA (1:100; Santa Cruz Biotechnology, Santa Cruz, Calif., USA)	HRP-conjugated goat anti-rabbit + DAB (Jackson Immunoresearch, West Grove, Pa., USA)
	Cell nucleus	–	HTX
Proliferating smooth muscle cells	Proliferation	PCNA	HRP-conjugated goat anti-rabbit
	Smooth muscle cells	α -SMA, AP conjugated (1:200; Sigma)	New Fuchsin (DAKO A/S, Glostrup, Denmark)
Smooth muscle mass	Smooth muscle	α -SMA, Cy3 conjugated (1:5,000; Sigma)	–
Procollagen I production	Procollagen-I-producing cells	PINP ¹ (1:100) antigen retrieval: pepsin/HCl	Alexa 488 (goat anti-rabbit; Molecular Probes, Eugene, Oreg., USA)
Actin-procollagen copositivity	Procollagen-I-positive cells	PINP ¹ (1:100)	HRP-conjugated goat anti-rabbit + DAB
	Smooth muscle cells	α -SMA, AP conjugated (1:200)	AP + New Fuchsin (both from DAKO)

PCNA = Proliferating cell nuclear antigen; HRP = horse radish peroxidase; DAB = 3,3-diaminobenzidine tetrahydrochloride; HTX = hematoxylin; α -SMA = α -smooth muscle actin; AP = alkaline phosphatase; PINP = N-terminal of procollagen I.

¹ This antibody was received as a kind gift from Professor Juha Risteli, Oulu University, Oulu, Finland.

Table 2. Proliferation

Treatment	Endothelial cell proliferation			Smooth muscle cell proliferation		
	BA	MSS	SS	BA	MSS	SS
SAL/controls	2.2 $\pm$ 2.3	1.6 $\pm$ 1.6	0.93 $\pm$ 1.4	12 $\pm$ 3.3	8.6 $\pm$ 3.4	6.8 $\pm$ 6.3
OVA/1d	7.4 $\pm$ 3.4**	6.7 $\pm$ 2.3*	8.7 $\pm$ 3.8***	23 $\pm$ 6.2***	22 $\pm$ 8.7***	28.7 $\pm$ 22***
OVA/1w	11.4 $\pm$ 4.8***	7.7 $\pm$ 1.9***	5.1 $\pm$ 4.9*	17 $\pm$ 4.4	14 $\pm$ 5.3**	20.4 $\pm$ 3.8**
OVA/1m	5.3 $\pm$ 3.2	7.9 $\pm$ 1.3*	6.1 $\pm$ 1.7*	8.5 $\pm$ 4.2	9.8 $\pm$ 2.6	14.8 $\pm$ 14.8

BA = Bronchial associated blood vessels; MSS = mid-sized solitary blood vessels; SS = small solitary blood vessels. Cells were labelled with an antibody directed against proliferating cell nuclear antigen and counted manually on digital images, and correlated with the length of the basement membrane. Proliferating smooth muscle cells were detected via double staining with an antibody directed against α -smooth muscle actin. The data represent the number of proliferating cells/mm BM and are given as the median $\pm$ interquartile range. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared with controls.

Vascular Smooth Muscle Remodeling

In pulmonary arteries, preseptal veins and microvessels, allergen challenge induced a significant increase in the number of proliferating smooth muscle cells at 1d compared with controls (table 2). The increase remained at 1w in preseptal veins and microvessels, and at 1m, the increase had returned to baseline (table 2). In pulmonary arteries, the smooth muscle mass was significantly increased at 1d and 1m (fig. 2a, 3), although no statistically significant increase was found at 1w. In the preseptal

veins and microvessels, the smooth muscle mass increased significantly at 1d and remained increased throughout the study (fig. 2b, 3). In microvessels, significantly increased smooth muscle mass was present at 1d and 1w and remained increased, although not significantly so, at 1m (fig. 2c, 3).

Total Procollagen I Production and Myofibroblasts

In all 3 types of vessels, pulmonary arteries, preseptal veins and microvessels (fig. 4a–c), the number of myofi-

broblasts increased following allergen exposure, and a significant increase was present at both 1d and 1w, whereas the number of cells had reversed at 1m. In both pulmonary arteries and preseptal veins, the number of procollagen-I-producing cells increased following allergen exposure. A significant increase compared with controls was found at 1d, whereas at 1w and 1m, the number of procollagen-I-producing cells was not significantly elevated compared with controls. Microvessels reacted similarly to the other vessel types and a significant increase in procollagen-I-producing cells was found at 1d but returned towards baseline at later time points.

Discussion

The primary finding of the present study was the induction of long-term structural alterations by a transient acute airway inflammation. The smooth muscle mass and endothelial proliferation increased in response to allergic airway inflammation and remained elevated even at 1m. In contrast, the other parameters investigated increased in response to allergen exposure, but returned to baseline when the inflammation ceased.

In agreement with previous observations in the presently used animal model [20, 22], 7 daily OVA challenges induced a prominent allergic airway inflammation. The number of tissue eosinophils, which was used as indicator of inflammation, revealed that the inflammatory response peaked around 1d and returned to lower but still elevated levels at 1w. After a 1-month resolution period, the cellular inflammatory response had completely ceased. Smooth muscle proliferation, number of myofibroblasts and procollagen I production displayed a similar pattern, suggesting these parameters to be correlated with the inflammatory activity. As procollagen I positivity is an indication of synthesis, rather than of total content, it is not known whether the increased expression is the result of increased collagen deposition or increased turnover, or a combination of both. In contrast to these parameters of remodeling, the smooth muscle mass and endothelial proliferation remained significantly increased even after 1 month of resolution, when the inflammation was completely resolved. These differences suggest that allergic airway inflammation initiates growth and promotes mitotic factors that operate long after the inflammation has ceased. Despite the continuously increased proliferation of endothelial cells, no luminal occlusion or apparent endothelial thickening was present, suggesting increased endothelial cell turnover. This could

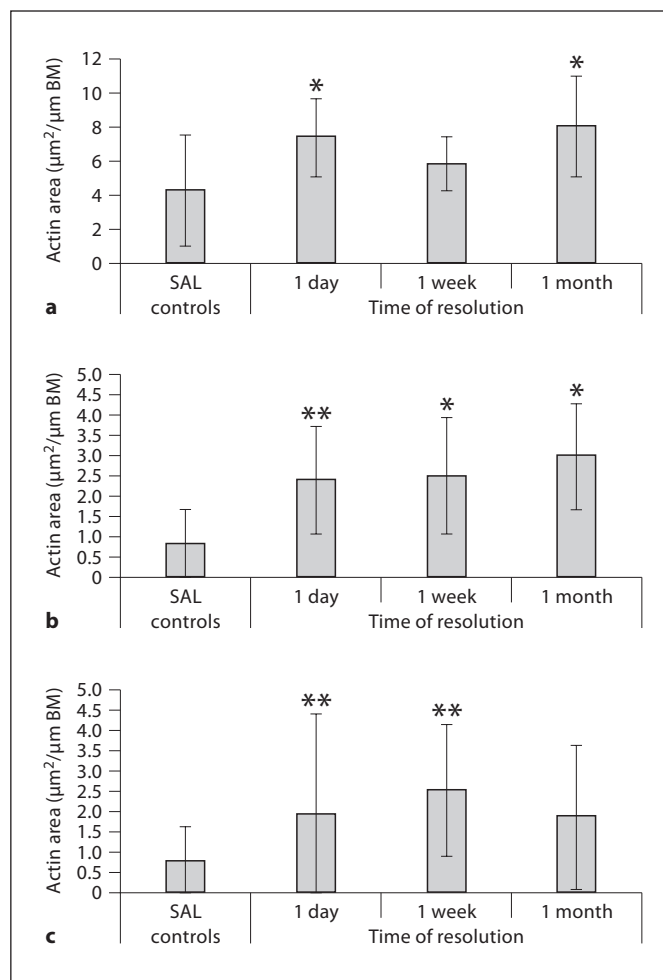


Fig. 2. Effects of allergen exposure on smooth muscle. Smooth muscle mass increased significantly in pulmonary arteries (a), preseptal veins (b) and microvessels (c) at 1d compared with controls (SAL). Following 1 month of resolution, the smooth muscle mass was still increased in bronchial associated vessels and mid-sized vessels. BM = Basement membrane. * $p < 0.05$, compared with control; ** $p < 0.01$. Data are given as the median $\pm$ interquartile range (ICQ, 95% confidence interval), and statistical analyses were made by Kruskal-Wallis ANOVA (Analyze It, Analyze-it Software Ltd.).

theoretically be accomplished by increased apoptosis; however, a previous study investigating cell turnover found no increased apoptosis [22]. In relation to the present increase in smooth muscle mass, one underlying mechanism, apart from the obvious proliferation of smooth muscle cells, could be endothelial mesenchymal transition [29] or transdifferentiation [30] where the endothelial cells could be differentiating into smooth muscle cells and thereby contribute to the increased smooth

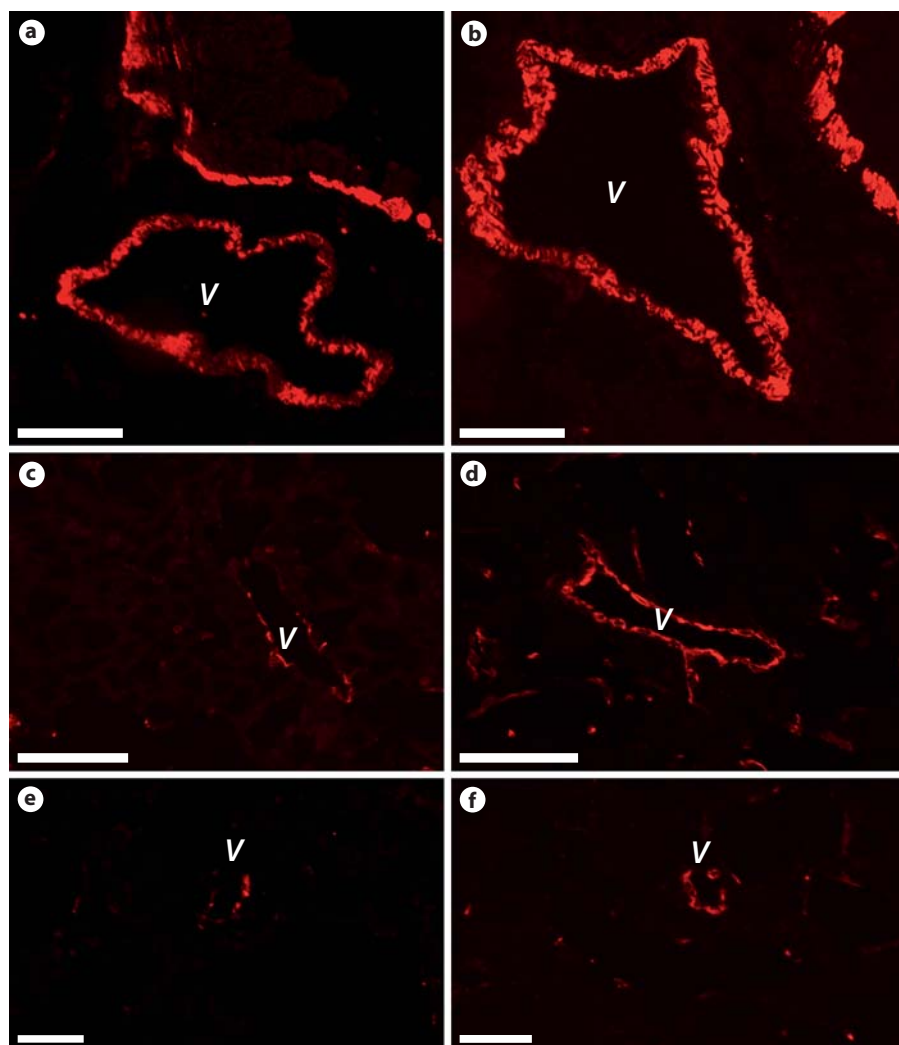


Fig. 3. Representative illustrations of the differences in smooth muscle mass following allergen challenge. The smooth muscle mass, measured as area of actin labeling, increased in response to allergen exposure (**b, d, f**, OVA, 1 day resolution after 7 daily allergen challenges) compared with controls (**a, c, e**). The increase displayed a similar pattern in all 3 types of vessels (**v**) investigated in this study: pulmonary arteries (**a, b**, bronchial associated vessels ≥ 250 μm in perimeter and in close proximity to airways), preseptal veins (**c, d**, defined as mid-sized solitary vessels of 250–500 μm in perimeter; ≥ 150 μm from the closest bronchi) and microvessels (**e, f**, defined as small solitary vessels of 50–249 μm in perimeter; ≥ 150 μm from the closest bronchi). Actin labeling was achieved by staining with an antibody directed against α -smooth muscle actin (Cy3 conjugated, 1:5,000; Sigma, St. Louis, Mo., USA). Scale bars = 100 μm (**a–d**) and 50 μm (**e, f**).

muscle mass. Although we cannot exclude these latter possibilities, proliferation and/or growth of existing smooth muscle cells are likely responsible for the present sustained smooth muscle enlargement in pulmonary vessels.

The vessels investigated within this study were divided into groups based on size and location, representing the distinct vessel types. However, interestingly, similar structural alterations were present among all vessels, regardless of size or location. The present findings of vascular remodeling of pulmonary arteries, preseptal veins and microvessels at 1d are in accordance with previous findings in this model [21, 22] and more chronic allergen administration models [4, 20]. However, the sustained features of vascular remodeling even after 1 month of resolution, represents a novel observation.

Irreversible bronchial [31] and vascular [20] remodeling has been reported in a chronic model of allergic airway inflammation, which differs from the model used in this study on 2 important aspects; first, the house dust mite model utilizes no adjuvant [32], and secondly, the house dust model extract is administered for a prolonged period of time [20, 31, 33]. These 2 aspects may suggest the model to imitate the human situation more closely. However, the present model is mimicking a more acute situation, and initially, it seemed unlikely that an acute inflammation could result in similar alterations as a chronic inflammation. Therefore, the most intriguing finding of this study was that acute inflammation could result in similar remodeling as chronic inflammation. Furthermore, it appears that the type of allergen and the method of sensitization/exposure are of less importance,

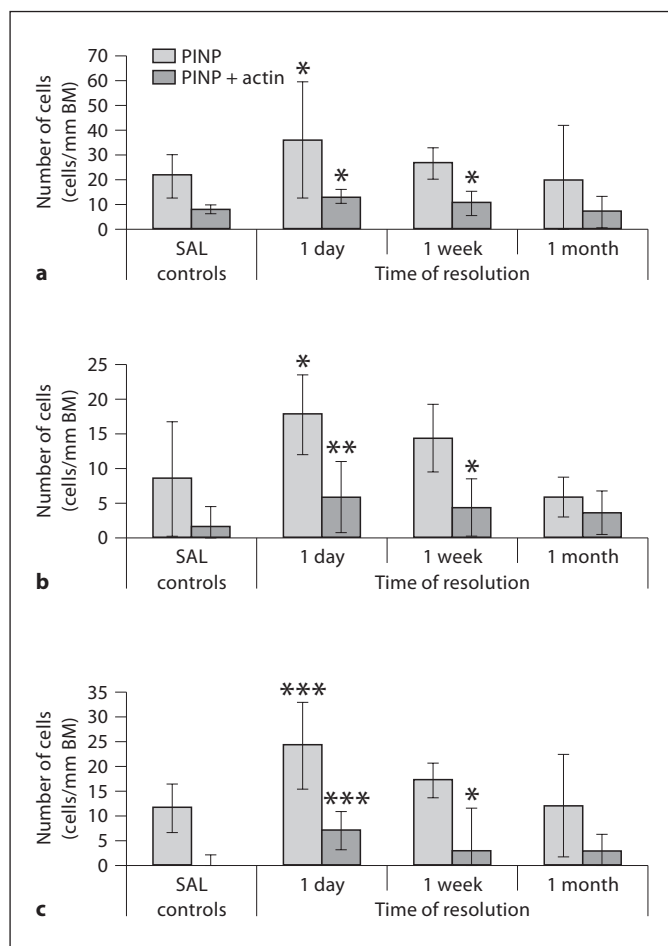


Fig. 4. Effects of OVA challenge on the number of procollagen-I-producing cells (PINP) and myofibroblasts. Both the number of procollagen-I-producing cells and myofibroblasts (cells co-positive for α -smooth muscle actin and procollagen-I-producing cells) increased after allergen (OVA) challenge and displayed a similar reaction in all 3 vessel types: pulmonary arteries (a), pre-septal veins (b) and microvessels (c). BM = Basement membrane. * $p < 0.05$, compared with control; ** $p < 0.01$; *** $p < 0.001$. Data are given as the median $\pm$ interquartile range (ICQ, 95% confidence interval), and statistical analyses were made by Kruskal-Wallis ANOVA (Analyze It, Analyze-it Software Ltd.).

as similar alterations are present in both models [20–22], as well as in other models of allergic airway inflammation [4].

Although the present study did not investigate any physiological effects, a recent study by Daley and colleagues [4] showed that pulmonary vascular remodeling, induced by allergic airway inflammation, does not result in pulmonary hypertension but rather in a hyper-reactive response to e.g. hypoxia. This is in accordance with the

findings by Witzernath et al. [34] who found the pulmonary vasculature of animals subjected to allergic airway inflammation to be hyper-reactive to endothelin-1 and serotonin. Notably, both these physiological studies also applied a limited histology, and their findings are in accordance with the remodeling found in our present and previous studies [20–22]. The mechanism behind the hyper-reactivity of remodeled vessels is yet unknown. Tentatively, for a given stimulus, a greater contraction force may be evoked simply due to the increased numbers and size of smooth muscle cells. Other possibilities include altered receptor expression or threshold for intracellular signaling.

Although this and most other experimental studies on allergen-induced vascular remodeling were performed in mice, several indications suggest that the revealed features are applicable to human disease. Allergic airway inflammation in man induces vascular involvement of both the bronchial and the pulmonary arteries [5, 8], and remodeling of the pulmonary circulation occurs in diseases such as systemic lupus erythematosus, systemic sclerosis, chronic obstructive pulmonary disease and idiopathic PAH [9–15], where recent observations suggest an inflammatory component as contributing factor [18, 19, 35]. Indeed, corticosteroids and immunosuppressant drugs may improve PAH [35]. Details on the inflammatory processes underlying PAH are not known. However, the results presented in this paper suggest that the inflammatory process does not have to be located within the vessel itself, nor does it have to be chronic in nature.

In conclusion, the present study suggests that an acute airway inflammation is able to initiate long-term vascular remodeling of the pulmonary circulation. This observation highlights the ability of the vascular system to react to an allergic inflammation, even if not directly exposed to the inflammatory stimulus, and increases the rationale for anti-remodeling strategies in asthma.

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