

Original Paper

IL-13R α 2 Reverses the Effects of IL-13 and IL-4 on Bronchial Reactivity and Acetylcholine-Induced Ca²⁺ Signaling

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^cAnatomical Institute, Ludwig Maximilian University, Munich, Germany**Key Words**Interleukin · IL-13R α 2 · Bronchial reactivity**Abstract**

Background: The interleukins IL-4 and IL-13 play a key role in the pathophysiology of asthma. The interleukin receptor IL-13R α 2 is believed to act as a decoy receptor, but until now, the functional significance of IL-13R α 2 remains vague. **Methods:** Bronchial reactivity was quantified in murine lung slices by digital video microscopy and acetylcholine (ACH)-induced Ca²⁺ signaling was measured in human airway smooth muscle cells (ASMC) using fluorescence microscopy. **Results:** IL-4 or IL-13 up to 50 ng/ml induced bronchial hyperreactivity. But after incubation with 100 ng/ml this effect was lost and bronchial responsiveness was again comparable to the control level. The effects of IL-4 and IL-13 on bronchial reactivity were paralleled by the effects on ASMC proliferation. Fifty nanograms per milliliter of IL-4 and IL-13 increased the Ca²⁺ response of human ASMC to ACH. At 100 ng/ml, however, the effects of the cytokines on the Ca²⁺ response were no longer evident. The expression of IL-13R α 2 increased with increasing concentrations of IL-4 or IL-13, reaching its maximum at 100 ng/ml. Blocking IL-13R α 2, the loss of the effect of IL-4 and IL-13 at 100 ng/ml on human ASMC proliferation and the ACH-induced Ca²⁺ response were no longer present.

Conclusions: IL-4 and IL-13 induce bronchial hyperreactivity by changing the Ca²⁺ homeostasis of ASMC. These effects are counteracted by IL-13R α 2. The biological significance of IL-13R α 2 might be a protective function by regulating IL-13- and IL-4-mediated signal transduction and thereby limiting pathological alterations in Th2-mediated inflammatory diseases.

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Introduction

Asthma is a complex lung disease characterized by bronchial hyperreactivity (BHR) and airway inflammation, which is dominated by Th2 lymphocytes. Interleukin-4 (IL-4) and interleukin-13 (IL-13) are proinflammatory cytokines that are released by Th2 lymphocytes [1, 2] and play a critical role in the development of this airway disease [3, 4]. Both cytokines alter the structural integrity of the airways, leading to an exaggerated response to a variety of stimuli [5]. An increased smooth muscle mass and altered contractile properties of airway smooth muscle cells (ASMC) contribute to the altered responsiveness of the airways [6]. This alteration of the ASMC contractile response can result from a multitude of mechanisms such as changes in cell surface receptors, modifica-

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tion of intracellular signaling molecules or changes in the properties of the contractile apparatus. Cytokines have been found to directly act on ASMC, changing ASMC contractile properties and potentially leading to BHR. Reports using animal models of asthma show the potential role of IL-13 in the development of BHR [7–9]. Venkayya et al. [10] presented data implying that IL-4 and IL-13 can induce BHR in mice even in the absence of mast cells, B or T lymphocytes. In mouse tracheal rings, pretreatment with IL-13 increased carbachol-induced maximum force generation [11] and in rabbit tracheal smooth muscle, IL-13 increased acetylcholine (ACH)-induced contractility [12]. However, the mechanisms by which cytokines promote proasthmatic responses in ASMC have not been clearly established.

Calcium is a common second messenger molecule and has a central role in regulating the contractile function of ASMC. Cytokines such as tumor necrosis factor α or IL-13 have been shown to augment agonist-induced ASMC contractility by enhancing agonist-evoked Ca^{2+} transients to a variety of contractile agonists [13–15]. On the other hand, Madison and Ethier [16] found no effect of 50 ng/ml IL-13 on carbachol-induced Ca^{2+} transients in ASMC and reported that 50 ng/ml IL-4 even diminished these Ca^{2+} transients. Thus, the role of cytokines in the Ca^{2+} homeostasis of ASMC is still controversial.

ASMC express the interleukin-binding proteins IL-4R α , IL-13R α 1 and IL-13R α 2 [17, 18]. IL-4 and IL-13 act through the type II receptor that consists of a dimer of the IL-4R α and the IL-13R α 1 proteins. Upon the binding of IL-4 or IL-13, the type II receptor activates the signal transducer and activator of transcription 6 (STAT-6) signaling cascade [for a review see 19]. IL-13R α 2 is believed to be a decoy receptor that is not capable of initiating signaling pathways. However, in glioblastoma cells, Rahman et al. [20] showed that IL-13R α 2 inhibited IL-4-mediated STAT-6 activation via its cytoplasmic domain, probably by interaction with IL-4R α . Only recently, Syed et al. [21] reported that in cultured human ASMC, IL-13 transcriptionally upregulated IL-13R α 2, but the functional significance of this finding remained unknown. Until now, however, there are no data demonstrating a limitation or even reversal of the effects of Th2 cytokines on BHR, leaving the role of IL-13R α 2 vague.

In our study, we show that IL-4 and IL-13 directly alter ASMC responsiveness by enhancing ACH-induced contractility and Ca^{2+} signaling. Furthermore, we provide evidence that IL-13R α 2 in fact functions to regulate the effects of IL-4 and IL-13, forming a signaling mechanism of protection against the effects of these cytokines.

Materials and Methods

Cell culture reagents were obtained from Life Technologies (Eggenstein, Germany). Other reagents were bought from Sigma-Aldrich (Deisenhofen, Germany) unless stated otherwise. Balb/C mice were purchased from Harlan-Winkelmann (Borchen, Germany). All procedures were approved by the Ethics Committee of the Ludwig Maximilian University in Munich.

Lung Slices

Lung slices were prepared as described before [22]. Briefly, Balb/C mice (42–77 days old) were sacrificed by an intraperitoneal injection of phenobarbital and the chest wall was removed. The trachea was cannulated using an intravenous catheter and the lungs were inflated with 2% agarose-sHBSS at 37°C. Subsequently, 0.1–0.2 ml of air was injected to flush the agarose-sHBSS out of the airways and the agarose was gelled by placing the mouse preparation at 4°C. The lungs were removed, embedded in 2% agarose-sHBSS and slices of ~200 μm thickness were cut with an EMS-4000 tissue slicer (Electron Microscopy Sciences, Fort Washington, Pa., USA). The slices were maintained by floating them in DMEM supplemented with antibiotics and antimycotics at 37°C in 5% CO_2 for up to 5 days. In culture, lung slices maintain their contractile properties for up to 5 days [22]. Experiments were therefore performed between day 2 and 5 of cultivation. The slices were incubated with varying concentrations of recombinant murine IL-13 or IL-4 (R&D Systems, Minneapolis, Minn., USA) for 24 h. Each slice was only used for one experiment. For each group of experiments, slices from at least 4 different mice were used. To measure the airway cross-sectional area, lung slices were placed in culture dishes immersed in sHBSS and held in position by a piece of a nylon mesh. Phase-contrast images were recorded using a digital CCD camera (AxioCam MRm; Carl Zeiss Vision, Munich, Germany). Frames were captured in time lapse (1 frame/s) and the cross-sectional area of the airway was measured by pixel summing using the Scion image analysis software (Scion Corporation, Frederick, Md., USA). Contraction velocity was defined as maximal change in cross-sectional area per second.

Immunohistochemistry

To perform the immunohistochemical analysis, lung slices were cut ~500 μm thick with a scalpel blade and incubated for 24 h at 37°C in 5% CO_2 with varying concentrations of murine IL-4 and IL-13. The fixed tissue samples were dehydrated through a graded series of ethanol and embedded in paraffin. Sections of 4 μm thickness were cut with a Leica SM 2000 R microtome (Leica Instruments, Nussloch, Germany) and mounted on glass slides. After deparaffinization, endogenous peroxidase activity was blocked with 10% H_2O_2 in methanol for 20 min at room temperature. IL-4R α , IL-13R α 1 and IL-13R α 2 immunoreactivity was localized with monoclonal rat anti-mouse IL-4R α , goat anti-human IL-13R α 1 and goat anti-mouse IL-13R α 2 antibodies (dilution 1:100; R&D Systems, Minneapolis, Minn., USA). After the addition of a biotin-conjugated secondary antibody (dilution 1:200; donkey anti-goat IgG or chicken anti-rat IgG), the sections were incubated with an avidin-D-horseradish peroxidase complex (Vectastain ABC kit; Vector Laboratories, Burlingame, Calif., USA) and exposed to 0.1% diaminobenzidine-tetrahydrochloride and 0.05% H_2O_2 as a source of peroxidase substrate. Each section was counterstained with hematoxylin. Incubation with

out the primary antibody served as negative control. The average length of the basement membrane of the airways in the sections was $140 \pm 10 \mu\text{m}$ without significant differences between the experimental groups.

After the incubation with the appropriate antibodies, images of lung sections were taken using a CCD camera. Subsequently, the maximum thickness of the ASMC layer and the number of nuclei in the ASMC layer in each airway of the stained sections were assessed. In representative ASMC, regions of interest (ROI) were set and the labeling was quantified by measuring the mean density of the ROI using the Scion image analysis software. The immunohistochemical analysis was performed in a blinded fashion. All values are expressed as percentage of the control groups (no incubation with interleukins).

Proliferation of Human ASMC

Human ASMC (primary cultures; Cambrex Bio Science, Walkersville, Md., USA) were grown as monolayer cultures and incubated with varying concentrations of recombinant human IL-4 or IL-13 (R&D Systems, Minneapolis, Minn., USA) during the exponential phase of the cell growth. After 24 h of incubation, the cell number of each culture was assessed.

Ca²⁺ Signaling in ASMC

To evaluate the effects of interleukins on ACH-induced Ca²⁺ signaling, human ASMC were incubated for 24 h with IL-4 or IL-13. Subsequently, the ASMC were loaded for 45 min at 37° with the calcium indicator dye Oregon green (10 μM ; Molecular Probes, Eugene, Oreg., USA) in sHBSS containing 0.2% Pluronic F-127 (Calbiochem, La Jolla, Calif., USA). After loading, ASMC were incubated for at least 45 min in sHBSS to allow for complete dye de-esterification and then examined with a fluorescence microscope (Axiovert 200 M; Carl Zeiss, Jena, Germany). Images were recorded in time lapse (1 frame/s) using a digital CCD camera (AxioCam MRm; Carl Zeiss Vision, Munich, Germany). For each image, ROI were defined in single ASMC, and the average fluorescence intensity of each ROI was measured. Final fluorescence values were expressed as a fluorescence ratio (F/F_0) normalized to the initial fluorescence (F_0). Each analysis was performed using custom-written macros in Scion.

Statistics

One-way ANOVA or ANOVA on ranks (combined with pairwise multiple comparisons) were performed using the Sigma Stat software (Jandel Scientific, Chicago, Ill., USA). A p value of less than 0.05 was considered statistically significant.

Results

Influence of IL-4 and IL-13 on Bronchial Reactivity

To evaluate the dependence of bronchial reactivity on varying concentrations of cytokines, lung slices were incubated with increasing concentrations of IL-4 and IL-13 and exposed to 1 μM ACH; the contraction (the maximal decrease in cross-sectional area) as well as the velocity of contraction (the maximal change in cross-sectional area

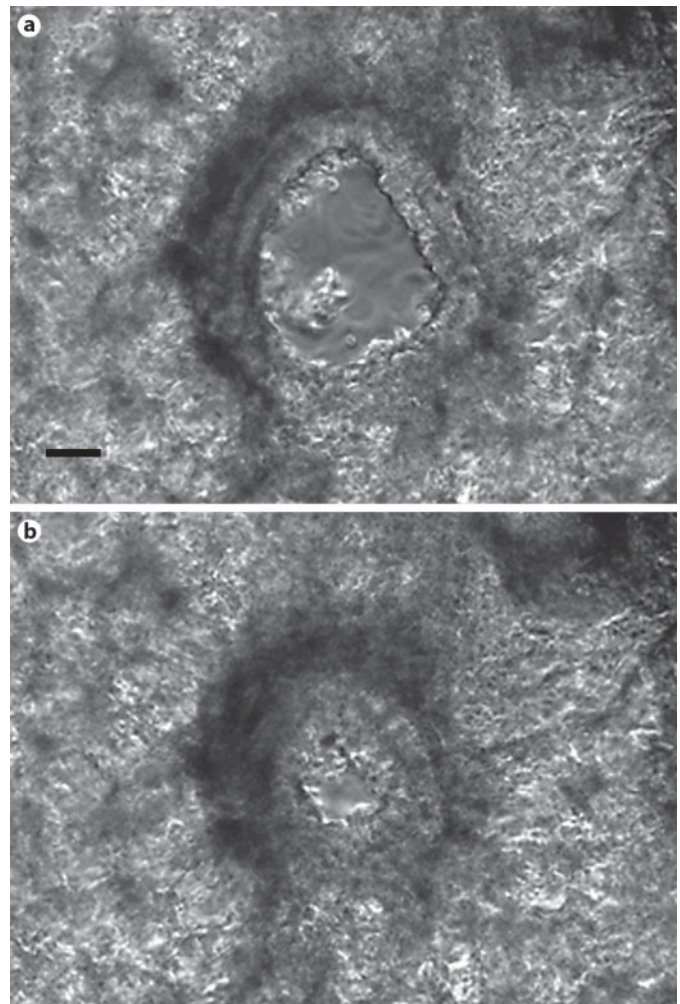


Fig. 1. ACH-induced airway contraction in lung slices. The phase-contrast micrographs show an airway in a lung slice after 24 h of incubation with 50 ng/ml IL-13 immediately before (a) and 150 s after (b) the addition of 1 μM ACH. Scale bar = 10 μm . See online supplement for the corresponding video at www.karger.com/doi/10.1159/000097022.

per second) were quantified using digital video microscopy (fig. 1, see online supplement for the corresponding video at www.karger.com/doi/10.1159/000097022). Before experiments with interleukins were performed, the baseline bronchial reactivity of the airways in the lung slices without IL-4 or IL-13 was evaluated. Without incubation with interleukins, the airways contracted by ~30% of the baseline cross-sectional area in response to 1 μM ACH; the contraction velocity was 3.24 $\mu\text{m}^2/\text{s}$ ($n = 21$). These values served as reference (control) for the subsequent experiments and were set to 100%. Following incubation with IL-13 for 24 h, ACH-induced contraction

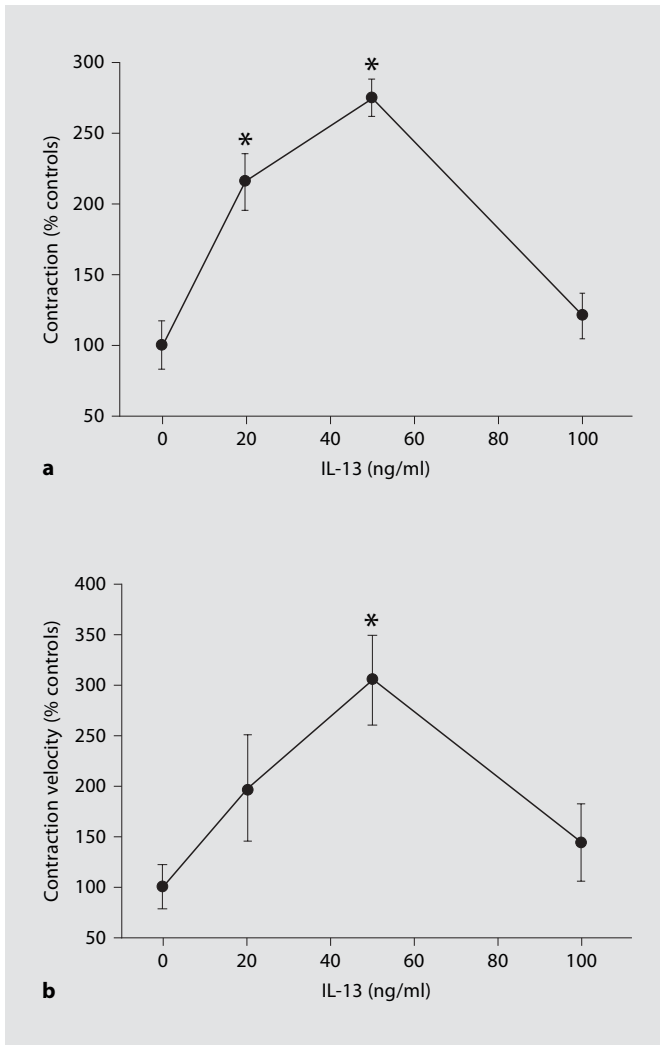


Fig. 2. Effects of IL-13 on bronchial reactivity. After incubation with IL-13 for 24 h, airways in lung slices were exposed to 1 μ M ACH and the maximal decrease in cross-sectional area as well as the velocity of contraction were quantified using digital video microscopy. Up to 50 ng/ml, incubation with IL-13 increased contraction (a) as well as contraction velocity (b). But after incubation with 100 ng/ml IL-13, both values were again comparable to the control group. n = 11–23. * p < 0.01 versus 0 and 100 ng/ml.

increased with increasing concentrations of IL-13 with the maximal contraction (275% of controls, n = 11, p < 0.01; fig. 2a) and contraction velocity (304%, p < 0.01; fig. 2b) being reached after incubation with 50 ng/ml IL-13. However, following incubation with 100 ng/ml IL-13 for 24 h, contraction and contraction velocity were both reduced compared to the lower concentrations of IL-13 (n = 23, p < 0.01 vs. 50 ng/ml IL-13) and showed no sig-

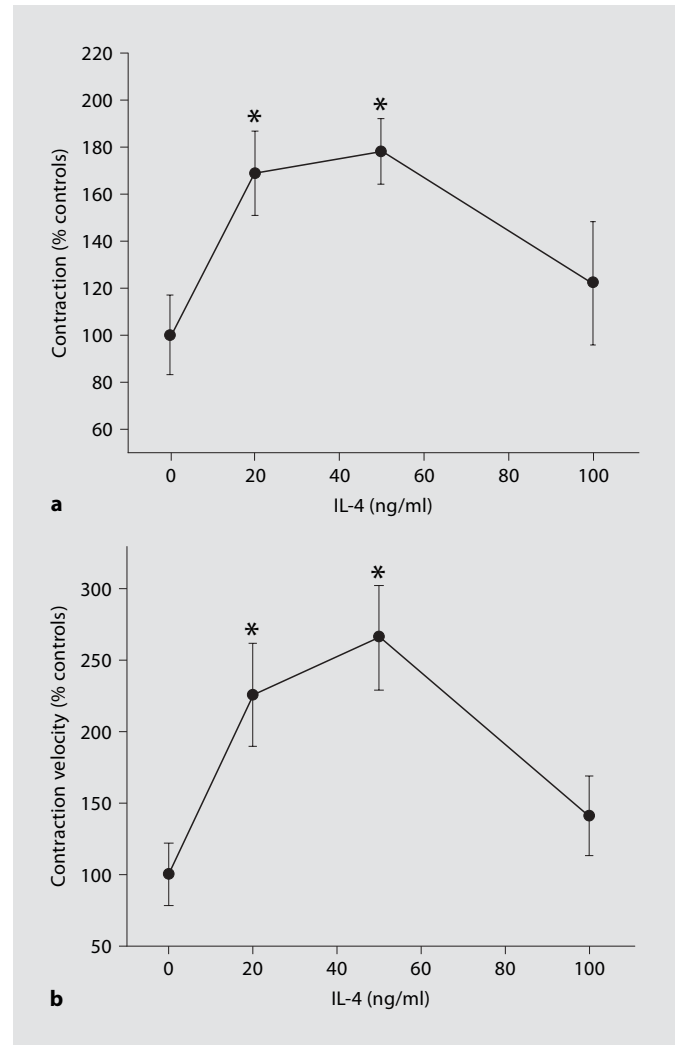


Fig. 3. Effects of IL-4 on bronchial reactivity. After incubation with IL-4 for 24 h, airways in lung slices were exposed to 1 μ M ACH and the maximal decrease in cross-sectional area as well as the velocity of contraction were quantified. Incubation with 50 ng/ml IL-4 increased contraction (a) and contraction velocity (b). Once more, after incubation with 100 ng/ml IL-4, both parameters were again similar to the control group. n = 10–29. * p < 0.01 versus 0 and 100 ng/ml.

nificant differences compared to the control group (contraction: 124% of controls; velocity: 151% of controls).

Similarly, following incubation with 50 ng/ml IL-4 for 24 h, ACH-induced contraction increased to 178% and contraction velocity to 269% (n = 29, p < 0.01; fig. 3). Incubation with 100 ng/ml IL-4 for 24 h resulted in a lower contraction and contraction velocity compared to 50 ng/ml IL-4 and no longer showed significant differences

compared to the control group (contraction: 121% of controls; velocity: 142% of controls; $n = 10$, $p < 0.01$ vs. 50 ng/ml IL-4). Comparing the effects of 50 ng/ml IL-4 with 50 ng/ml IL-13, the contraction induced by IL-13 was significantly higher ($p < 0.01$), while the contraction velocity was not different. Because the maximal contraction and contraction velocity were observed with 50 ng/ml IL-4 or IL-13 and both contraction and contraction velocity decreased with 100 ng/ml of the cytokines, these concentrations were used in the following experiments.

Effects of IL-4 and IL-13 on ASMC Proliferation

To investigate the effects of IL-4 and IL-13 on the ASMC mass, the thickness of the ASMC layer and the number of ASMC nuclei in lung sections were quantified (fig. 4). To compensate for the different airway sizes, both parameters were divided by the length of the basement membrane of the respective airway. Fifty nanograms per milliliter IL-4 and 50 ng/ml IL-13 caused a significant thickening of the ASMC layer (IL-4: 214% of controls; IL-13: 207% of controls; $n = 8-13$, $p < 0.01$; fig. 5a). After incubation with 100 ng/ml IL-4 or 100 ng/ml IL-13, the thickness of the ASMC layer was found to be comparable to the control group (100 ng/ml IL-4: 124% of controls; 100 ng/ml IL-13: 121% of controls; $p < 0.01$ vs. 50 ng/ml). The number of ASMC nuclei showed a similar pattern, increasing after incubation with 50 ng/ml (IL-4: 211% of controls; IL-13: 245% of controls; $n = 8-13$, $p < 0.001$; fig. 5b) and decreasing to starting level after 100 ng/ml (IL-4: 154% of controls; IL-13: 153% of controls; $p < 0.001$ vs. 50 ng/ml).

In human primary ASMC cultured as monolayer, incubation with 50 ng/ml IL-4 or IL-13 for 24 h also induced an increase in cell number (IL-4: 168% of controls; IL-13: 165% of controls; $n = 6$, $p < 0.05$; fig. 6). Similarly to murine ASMC in lung slices, the cell numbers returned to starting levels after incubation with 100 ng/ml of each interleukin (IL-4: 106% of controls; IL-13: 95% of controls; $p < 0.05$ vs. 50 ng/ml).

Effects of IL-4 and IL-13 on ACH-Induced Ca^{2+} Signaling in ASMC

The rise in intracellular calcium is an essential event in the signaling cascade leading to ASMC contraction. We therefore investigated the effects of IL-4 and IL-13 on ACH-induced Ca^{2+} signaling. Human ASMC were loaded with the Ca^{2+} indicator dye Oregon green and examined with fluorescence microscopy. The Ca^{2+} response to ACH consisted of an initial Ca^{2+} transient followed by a Ca^{2+} plateau (fig. 7a). Without incubation

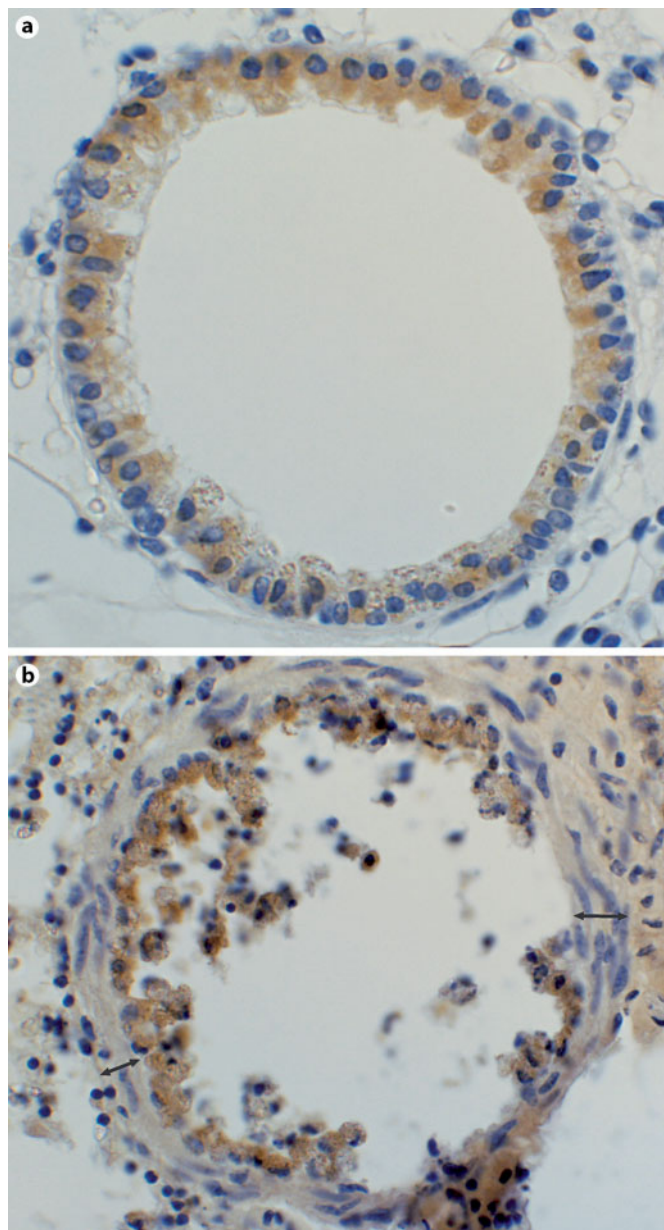
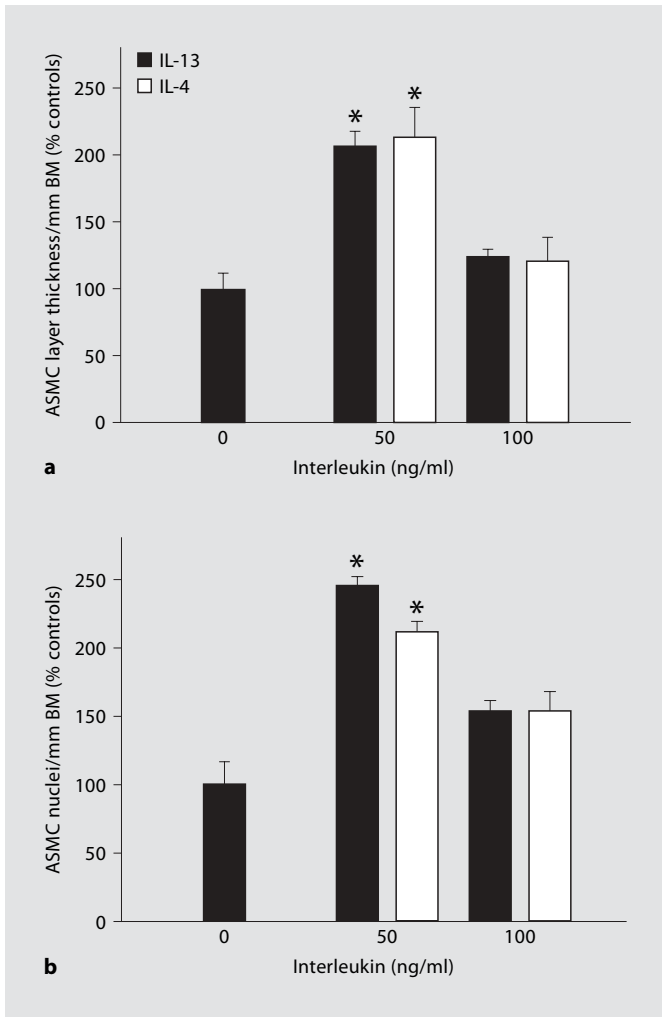
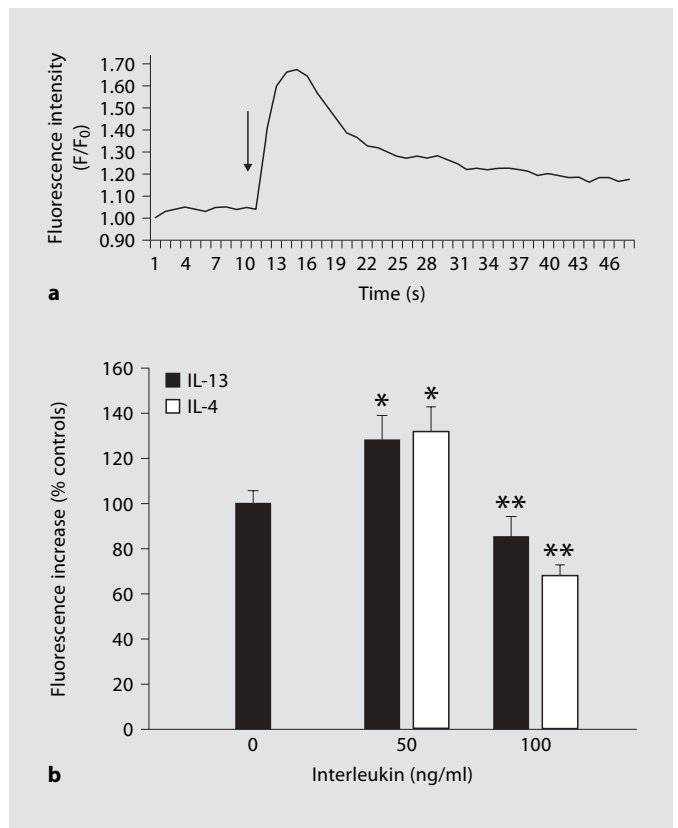
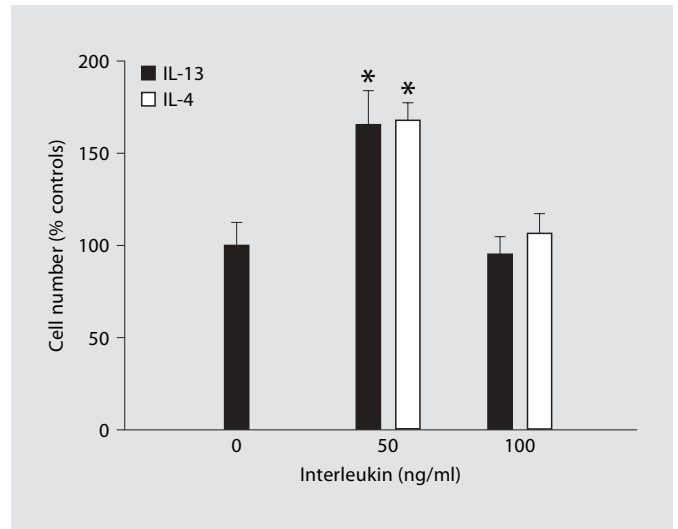


Fig. 4. Effects of IL-13 on ASMC and IL-13R α 2 expression. Lung slices were incubated with 50 ng/ml IL-13 for 24 h, embedded in paraffin and exposed to an IL-13R α 2 antibody followed by a biotin-conjugated secondary antibody. Counterstaining was performed with hematoxylin. **a** Control sections without incubation with IL-13 showed weak staining for the IL-13R α 2 receptor. **b** After incubation with 50 ng/ml IL-13, the thickness of the ASMC layer (double-head arrows) as well as the number of ASMC nuclei were increased and the staining for IL-13R α 2 was intensified. $\times 40$.



5 **Fig. 5.** Effects of IL-4 and IL-13 on ASMC in lung slices. Lung slices were incubated with IL-4 or IL-13 for 24 h, embedded in paraffin, stained with hematoxylin and eosin, and the thickness of the ASMC layer, the number of ASMC nuclei and the length of the basement membrane were quantified for each airway. To compensate for the different airway sizes, the thickness of the ASMC layer and the number of ASMC nuclei were divided by the length of the basement membrane of the respective airway. BM = Basement membrane. **a** Incubation with 50 ng/ml IL-4 or 50 ng/ml IL-13 caused a considerable thickening of the ASMC layer. But after incubation with 100 ng/ml, the thickness of the ASMC layer was again similar to the control group. $n = 8-13$. * $p < 0.01$. **b** The number of ASMC nuclei showed a similar pattern with an increase after 50 ng/ml and a decrease after 100 ng/ml. $n = 8-13$. * $p < 0.001$.



7 Oregon Green and examined with fluorescence microscopy. **a** The Ca^{2+} response to ACH (arrow) consisted of an initial Ca^{2+} transient followed by a Ca^{2+} plateau as shown by the representative trace. **b** After incubation with 50 ng/ml IL-4 or IL-13 for 24 h, the magnitude of the initial Ca^{2+} transient was increased compared to the control group. When incubating with 100 ng/ml IL-4 or IL-13, the magnitude of the initial Ca^{2+} transient was reduced and no longer showed significant differences compared to the control group. $n = 34-43$ cells. * $p < 0.05$ versus 0 ng/ml; ** $p < 0.01$ versus 50 ng/ml.

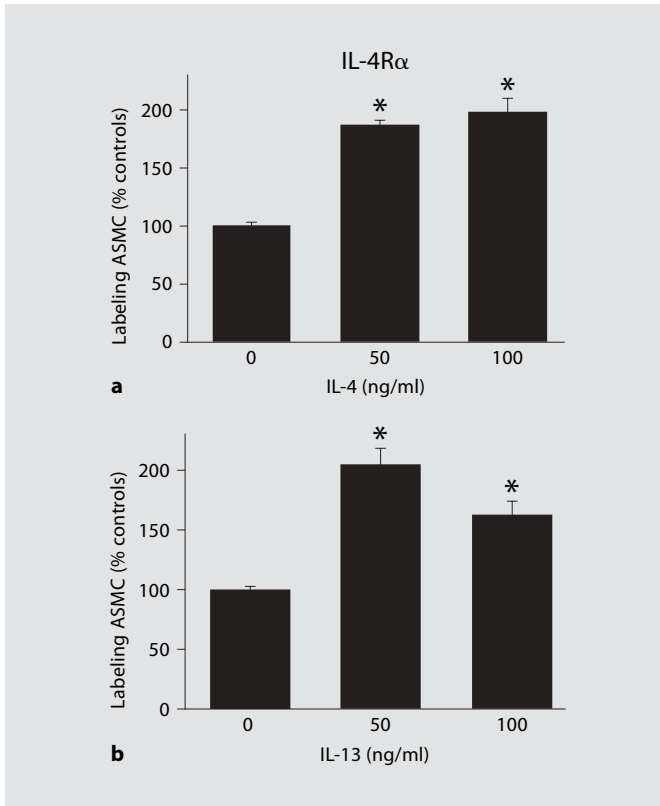


Fig. 8. Effects of IL-4 and IL-13 on the expression of IL-4R α . Lung slices were incubated for 24 h with IL-4 or IL-13, embedded in paraffin and labeled with an IL-4R α antibody. Staining of ASMC was quantified using the Scion image analysis software. Incubation with IL-4 (**a**) or IL-13 (**b**) increased the expression of IL-4R α in ASMC. No concentration dependence could be observed. n = 8–13. * p < 0.01 versus 0 ng/ml.

with interleukins, the magnitude of the initial Ca²⁺ transient (the difference in percent between F/F₀ measured immediately before and at the maximum value of the Ca²⁺ transient) was 43% above baseline values (n = 57 cells). These values served as reference (control) for the subsequent experiments and were set to 100%. After incubation with 50 ng/ml IL-13 for 24 h, the magnitude of the initial Ca²⁺ transient was increased compared to the control group (128% of controls; p < 0.05; fig. 7b). When incubated with 100 ng/ml IL-13, the magnitude of the initial Ca²⁺ transient was reduced and no longer different from the of the control group (85% of controls; p < 0.01 vs. 50 ng/ml IL-13). Incubation with 50 ng/ml IL-4 for 24 h also augmented the ACH-induced Ca²⁺ response (132% of controls; p < 0.05; fig. 7b). Again, incubation with 100 ng/ml IL-4 resulted in a lower magni-

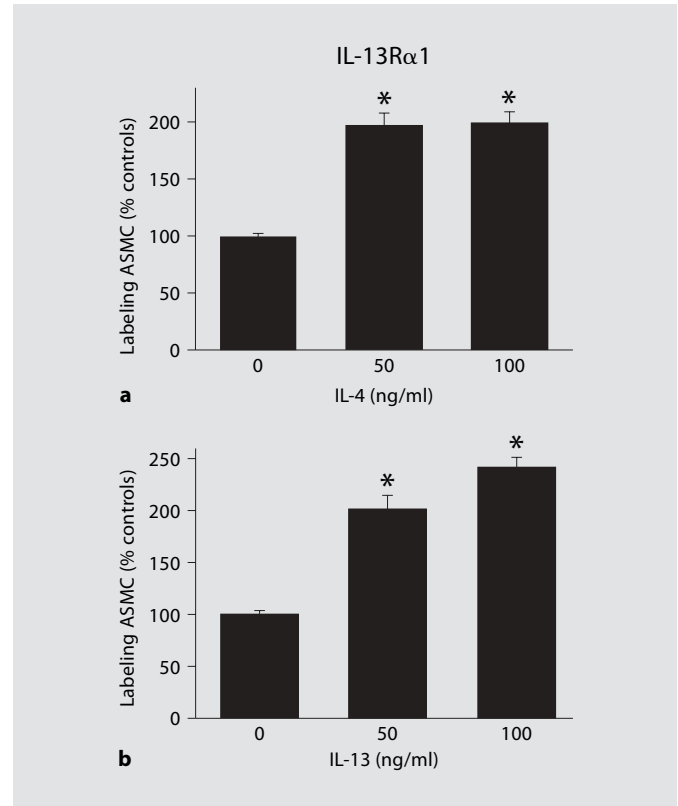


Fig. 9. Effects of IL-4 and IL-13 on the expression of IL-13R α 1. Incubation with IL-4 (**a**) or IL-13 (**b**) for 24 h increased the expression of IL-13R α 1 in ASMC. These effects were not concentration dependent. n = 8–13. * p < 0.01 versus 0 ng/ml.

tude of the initial Ca²⁺ transient (68% of controls; p < 0.01 vs. 50 ng/ml IL-4).

Effects of IL-4 and IL-13 on Interleukin Receptor Expression

To test if changes in the interleukin receptor expression caused the lack of effectiveness of IL-4 and IL-13 at 100 ng/ml, we performed immunohistochemistry with antibodies specific for the IL-4R α , IL-13R α 1 and IL-13R α 2 proteins in lung slices. Subsequently, the staining of the ASMC was quantified using the Scion image analysis software.

Incubation with 50 ng/ml IL-4 or IL-13 increased the expression of IL-4R α (IL-4: 186% of controls; IL-13: 203% of controls; n = 8–13, p < 0.01; fig. 8). Incubation with 100 ng/ml IL-4 or IL-13 had no further effect.

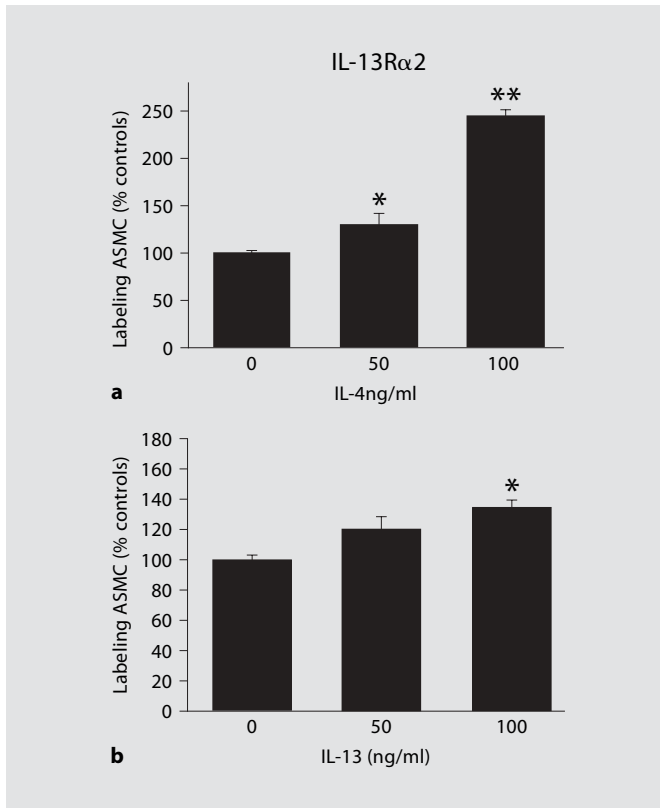


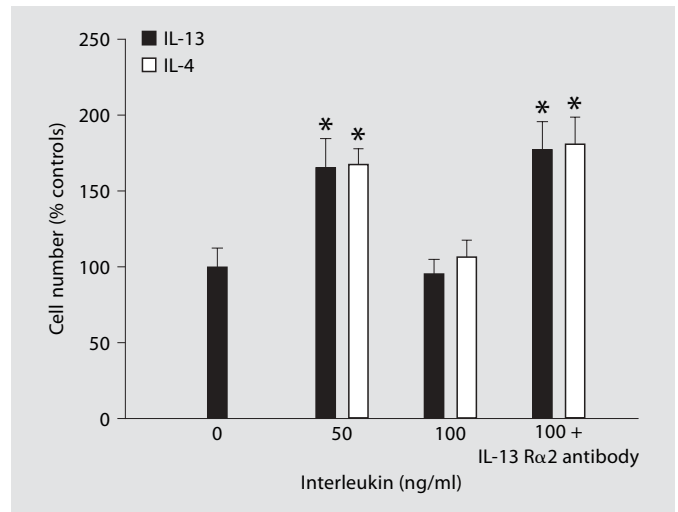
Fig. 10. Effects of IL-4 and IL-13 on the expression of IL-13R α 2. Incubation with IL-4 (a) or IL-13 (b) increased the expression of IL-13R α 2 in ASMC. In contrast to IL-4R α and IL-13R α 1, the expression of IL-13R α 2 increased in a concentration-dependent manner. n = 8–13. * p < 0.01 versus 0 ng/ml; ** p < 0.01 versus 0 and 50 ng/ml.

Incubation with 50 ng/ml IL-4 or IL-13 increased the expression of IL-13R α 1 (IL-4: 198% of controls; IL-13: 202% of controls; n = 8–13, p < 0.01; fig. 9). Again, incubation with 100 ng/ml IL-4 or IL-13 had no additional effect.

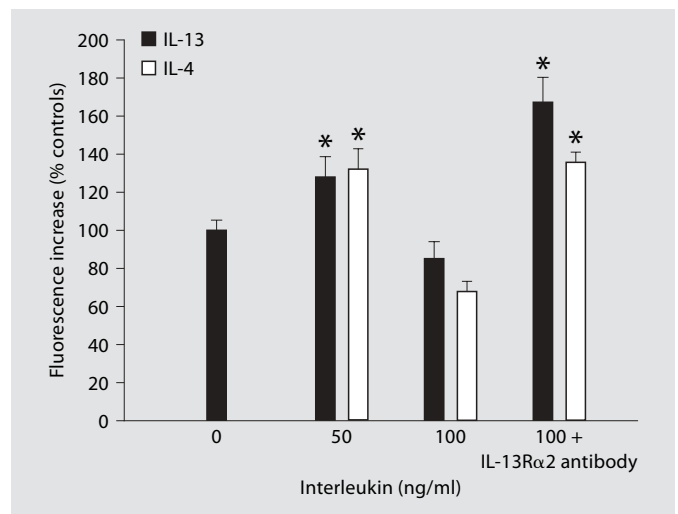
In contrast, the expression of IL-13R α 2 increased in a concentration-dependent manner after incubation with IL-4 or IL-13 (100 ng/ml IL-4: 244% of controls; 100 ng/ml IL-13: 134% of controls; n = 8–13, p < 0.01; fig. 10).

Effects of IL-13R α 2 Inhibition

Syed et al. [21] reported a 1.6-fold upregulation of IL-13R α 2 in response to 100 ng/ml IL-13 and our data on the expression of IL-13R α 2 suggested an involvement in the lack of effectiveness of IL-4 and IL-13 at 100 ng/ml. To further test this hypothesis, we used an anti-IL-13R α 2 antibody to block IL-13R α 2. Because of the specificity of this antibody for the human IL-13R α 2, only its effect on



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Fig. 11. Effect of IL-13R α 2 inhibition on the proliferation of IL-4- and IL-13-treated ASMC. In human primary cultured ASMC, the decrease in proliferation after incubation with 100 ng/ml IL-4 or IL-13 compared to 50 ng/ml was no longer evident when treated with a human anti-IL-13R α 2 antibody. n = 6. * p < 0.05 versus 0 and 100 ng/ml.

Fig. 12. Effect of IL-13R α 2 inhibition on the ACH-induced Ca²⁺ signaling of IL-4- and IL-13-treated human ASMC. In human primary cultured ASMC, the decrease in the ACH-induced Ca²⁺ transient after incubation with 100 ng/ml IL-4 or IL-13 compared to 50 ng/ml was reversed when treated with a human anti-IL-13R α 2 antibody. n = 34–43 cells. * p < 0.05 versus 0 and 100 ng/ml.

human ASMC could be assessed. After incubation with 4 μ g/ml of the IL-13R α 2 antibody, the decrease in ASMC proliferation after 100 ng/ml IL-4 or IL-13 was no longer evident (100 ng/ml IL-4 + IL-13R α 2 antibody: 181% of controls; 100 ng/ml IL-13 + IL-13R α 2 antibody: 177% of

controls; $n = 6$, $p < 0.05$ vs. 0 and 100 ng/ml; fig. 11). Regarding the ACH-induced Ca^{2+} mobilization, incubation with the IL-13R α 2 antibody completely inhibited the decrease observed after the incubation with 100 ng/ml IL-4 or IL-13 (100 ng/ml IL-4 + IL-13R α 2 antibody: 136% of controls; 100 ng/ml IL-13 + IL-13R α 2 antibody: 167% of controls; $n = 34$ – 43 cells, $p < 0.05$ vs. 0 and 100 ng/ml; fig. 12).

Discussion

In this study, we showed that IL-4 or IL-13 in concentrations up to 50 ng/ml induced BHR in murine lung slices in terms of both increased contraction and contraction velocity. But after incubation with 100 ng/ml this effect was lost and bronchial responsiveness was again comparable to the control level. The effects of IL-4 and IL-13 on bronchial reactivity were paralleled by the effects on ASMC proliferation. Furthermore, IL-4 and IL-13 increased the Ca^{2+} response of human ASMC to ACH. Again, at 100 ng/ml the effects of the cytokines on the Ca^{2+} response were no longer evident. IL-4 and IL-13 increased the expression of IL-4R α and IL-13R α 1 in ASMC with the expression being comparable after incubation with 50 or 100 ng/ml. In contrast, the expression of IL-13R α 2 increased with increasing concentrations of IL-4 or IL-13, reaching its maximum at 100 ng/ml. When blocking IL-13R α 2, the loss of effect of IL-4 and IL-13 at 100 ng/ml on the ASMC proliferation and the ACH-induced Ca^{2+} response was no longer evident. This pattern of findings suggests that IL-4 and IL-13 induce BHR by changing the Ca^{2+} homeostasis of ASMC and that these effects are regulated by IL-13R α 2, constituting a protective mechanism against the effects of the proinflammatory cytokines known to be involved in asthma.

In bronchoalveolar lavage samples from patients with atopic asthma, the number of activated Th2 lymphocytes [23], derived from CD4+ T lymphocytes [24], is increased and Th2 lymphocytes appear to be critically important for the pathophysiological events leading to asthma [25, 26]. Furthermore, IL-13 released by Th2 lymphocytes [1, 2] was identified to be a crucial participant in the allergic responses of the respiratory tract [3, 4]. Although numerous studies also noticed a role for IL-4 in asthma [27, 28], other reports suggested that IL-4 was not obligatory for this airway disease [29, 30]. In the present study, we aimed at examining the effects of both IL-4 and IL-13 on bronchial reactivity as well as on ASMC Ca^{2+} signaling and at investigating how these effects might be regulated.

We used murine lung slices to investigate the agonist-induced contractility of ASMC. Lung slices have previously been used by us and others to study a variety of responses including bronchial contractility [22, 31–35], vascular responses [36] and mucociliary function [37]. The major advantage of this approach is that the in situ organization of the lung tissue and the contractility of the ASMC are maintained for several days. Thus, murine lung slices in combination with video microscopy appear to be particularly suited to study bronchial reactivity in vitro. The culture of human lung slices was established by Wohlsen et al. [38], using material obtained after lung or lobe resections. Unfortunately, patients undergoing lung resections due to malignant diseases, i.e. the majority of these patients, for the most part suffer from chronic obstructive airway disease. Under these conditions, the investigation of BHR would have been impaired. To circumvent this drawback, we investigated the influence of IL-4 and IL-13 on ACH-induced Ca^{2+} signaling using primary cultured human ASMC that were obtained post mortem from donors without lung diseases. With this approach, we were able to combine information obtained with murine ASMC in lung slices with data acquired with primary cultured human ASMC.

To assess BHR, we used two parameters: maximal contraction and contraction velocity. Duguet et al. [39] analyzed bronchial reactivity among inbred mouse strains in vivo and in vitro and found that the contraction velocity of lung slices correlated with the bronchial reactivity in vivo, whereas contraction did not. In a previous paper, we also analyzed contraction and contraction velocity in the mouse strains that Duguet et al. [39] used and found both measures to be closely related and to give identical readings of BHR [32]. In this study, IL-4 and IL-13 also showed comparable effects on contraction and contraction velocity. A possible explanation for these differences might be the thickness of the slices used, which was 0.5–1 mm in the study by Duguet et al. [39] compared to 200 μ m in our studies. Differences in smooth muscle mass and its surface-to-volume ratio might have led to the differences in the mechanical contraction properties.

After incubation with IL-4 and IL-13, we found the thickness of the ASMC layer to be increased. An increased ASMC mass alone could have accounted for the elevated maximal airway contraction. However, the contraction velocity was also increased after incubation with the interleukins and this finding pointed towards intrinsic alterations in the contractile properties of the ASMC. An increased ASMC mass could have resulted from an increase

in cell size, leaving the number of ASMC unchanged. Alternatively, cell proliferation with increased cell numbers could have been the cause of the thickening of the ASMC layer. Evaluating the number of nuclei in the ASMC layers in lung slices and the cell numbers of cultured human ASMC under the influence of IL-4 and IL-13, we found that indeed ASMC proliferation rather than an increase in cell size accounted for the increase in ASMC mass.

Calcium is a ubiquitous signaling molecule that is involved in the regulation of a broad variety of cellular events in almost all mammalian cell types [40–42]. In ASMC, the elevation of $[Ca^{2+}]_c$ leads to contraction. In our study, we found an enhanced Ca^{2+} response to ACH in human ASMC treated with 50 ng/ml IL-13 or IL-4. But incubation with 100 ng/ml IL-13 or IL-4 attenuated the ACH-induced Ca^{2+} signaling and this pattern of response paralleled the effects of IL-4 and IL-13 on ASMC contraction in murine lung slices. Furthermore, this concentration dependence was also found for the effects on the proliferation of both murine and human ASMC. Therefore, the attenuation of the effects of IL-4 and IL-13 at high concentrations does not appear to depend on the culture form nor to be species specific.

Deshpande et al. [14] reported enhanced intracellular Ca^{2+} responses to bradykinin, thrombin and histamin in IL-13-treated human ASMC. Tliba et al. [11] showed an increase in force generation induced by both carbachol and KCl in murine cultured tracheal rings pretreated with 100 ng/ml IL-13. The same group noticed that in cultured human ASMC, 50 ng/ml IL-13 significantly enhanced the calcium signals induced by bradykinin, histamine and carbachol. These findings are in agreement with our results. In contrast, Madison and Ethier [16] noticed no effect of 50 ng/ml IL-13 and even an inhibitory effect of 50 ng/ml IL-4 on calcium transients in response to carbachol in bovine ASMC. The most obvious methodological difference is the use of bovine ASMC by Madison and Ethier [16] compared to murine and/or human ASMC used by our group, Deshpande et al. [14] and Tliba et al. [11]. Although our data suggest comparable patterns of response to IL-4 and IL-13 in murine and human ASMC, the effects of these cytokines may be different in bovine tissue.

The data reported in this paper demonstrate that both cytokines, IL-13 and IL-4, possess the potential to increase the contractile responses of ASMC to ACH and to augment ACH-induced Ca^{2+} signaling. These effects occurred at cytokine concentrations up to 50 ng/ml. When incubating lung slices or human ASMC with 100 ng/ml IL-13 or IL-4, these effects were no longer evident, indi-

cating some kind of negative feedback mechanism. One possible mechanism would have been the up- or downregulation of interleukin receptors. IL-4 and IL-13 bind to the type II receptor consisting of a dimer of the IL-4R α and the IL-13R α 1 proteins. Upon binding, both interleukins affect gene transcription via the STAT-6 pathway. Myrtek et al. [43] reported that in peripheral blood eosinophils, IL-13 and IL-4 downregulate IL-13R α 1 expression. The downregulation of IL-4R α and/or IL-13R α 1 would have been one possible mechanism underlying the negative feedback on BHR and Ca^{2+} signaling observed in our study. However, there was no decrease in the labeling for IL-4R α or IL-13R α 1 with increasing concentrations of IL-4 or IL-13, rendering this possibility unlikely. In contrast, the expression of IL-13R α 2, which is believed to act as a decoy receptor, increased in a concentration-dependent manner after incubation with IL-4 and IL-13. Zheng et al. [44] demonstrated that IL-4 and IL-13 can upregulate IL-13R α 2 expression in murine epithelial cells. In ASMC, the regulation of genes by interleukins has been studied using microarray techniques [45, 46] and Syed et al. [21] only recently found that IL-13 increases IL-13R α 2 gene expression although the physiological significance of this finding remained unknown. In agreement with these data, our results show that IL-13 and IL-4 have the potential to upregulate IL-13R α 2. Because of the correlation between the decrease of BHR and Ca^{2+} signaling at 100 ng/ml IL-4 or IL-13 and the high expression of IL-13R α 2 at this cytokine concentration, IL-13R α 2 appears to negatively control the effects of IL-13 and IL-4. To further test the hypothesis that IL-13R α 2 is involved in the limitation or even reversal of the effects of Th2 cytokines, IL-13R α 2 was blocked in IL-4- and IL-13-treated human ASMC with a human anti-IL-13R α 2 antibody. Because, to our knowledge, there is no murine antibody, IL-13R α 2 could not be blocked in the experiments on BHR in lung slices. However, blocking IL-13R α 2 in human ASMC reversed the decrease in ASMC proliferation and ACH-induced Ca^{2+} signaling observed at 100 ng/ml IL-4 and IL-13. Our data therefore demonstrate for the first time that IL-13R α 2 acts as a negative regulator in IL-4- and IL-13-mediated signal transduction.

Debinski et al. [47] and Murata et al. [48] reported that IL-4 does not bind to IL-13R α 2. But our data suggest that IL-13R α 2 also functions as a negative controller for the IL-4 signaling pathway. Whether IL-4 directly binds to IL-13R α 2 or forms an additional signaling complex using a second molecule yet has to be studied.

Taken together, we have shown that IL-13 and IL-4 induce BHR and that both interleukins elevate ACH-in-

duced intracellular Ca²⁺ mobilization. We have – to our knowledge for the first time – demonstrated that the effects of IL-4 and IL-13 underlie a control mechanism at high concentrations and that this mechanism is mediated by IL-13Rα2. We believe that our findings contribute to a better understanding of the relevance of IL-13Rα2, which might be a protective function by regulating IL-13- and IL-4-mediated signal transduction and thereby limiting pathological alterations in Th2-mediated inflammatory diseases.

Acknowledgements

Supported by the Deutsche Forschungsgemeinschaft grants BE 2356/2–1 and BE 2356/2–3 to A.B.

Data presented in this paper are part of the doctoral thesis of J.K.

The technical assistance provided by A. Sulz and U. Fazekas is gratefully acknowledged.

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