

# Mechanisms Altering Airway Smooth Muscle Cell $\text{Ca}^{2+}$ Homeostasis in Two Asthma Models

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## Key Words

Asthma • Hyperresponsiveness • Calcium • Acetylcholine

## Abstract

**Background:** Asthma is characterized by airway remodeling, altered mucus production and airway smooth muscle cell (ASMC) contraction causing extensive airway narrowing. In particular, alterations of ASMC contractility seem to be of crucial importance. The elevation of the cytoplasmic  $\text{Ca}^{2+}$  concentration is a key event leading to ASMC contraction and changes in the agonist-induced  $\text{Ca}^{2+}$  increase in ASMC have been reported in asthma. **Objective:** The aim of this study was to investigate mechanisms underlying these changes. **Methods:** Murine tracheal smooth muscle cells (MTSMC) from T-bet KO mice and human bronchial smooth muscle cells (HBSMC) incubated with IL-13 and IL-4 served as asthma models. Acetylcholine-induced changes in the cytoplasmic  $\text{Ca}^{2+}$  concentration were recorded using fluorescence microscopy and the expression of  $\text{Ca}^{2+}$  homeostasis regulating proteins was investigated with Western blot analysis. **Results:** Acetylcholine-induced  $\text{Ca}^{2+}$  transients were elevated in both asthma models. This correlated with an increased  $\text{Ca}^{2+}$  content of the sarcoplasmic reticulum (SR). In MTSMC from T-bet KO mice, the expression of the SR  $\text{Ca}^{2+}$  buffers calreticulin and calsequestrin was higher compared

to wild-type mice. In HBSMC incubated with IL-13 or IL-4, the expression of ryanodine receptors, inositol-3-phosphate receptors and sarcoplasmic/endoplasmic reticulum  $\text{Ca}^{2+}$  ATPases 2 was increased compared to HBSMC without incubation with interleukins. The enlarged acetylcholine-induced  $\text{Ca}^{2+}$  transients could be reversed by blocking inositol-3-phosphate receptors. **Conclusions:** We conclude that in the murine asthma model the SR  $\text{Ca}^{2+}$  buffer capacity is increased, while in the human asthma model the expression of SR  $\text{Ca}^{2+}$  channels is altered. The investigation of the  $\text{Ca}^{2+}$  homeostasis of ASMC has the potential to provide new therapeutic options in asthma. Copyright © 2008 S. Karger AG, Basel

## Introduction

In the second half of the 20th century, asthma has become a worldwide epidemic and its prevalence is still increasing [1]. For example, the prevalence of asthma among US children increased from 3.6% in 1980 to 5.8% in 2003, and asthma is the third leading cause of hospitalization among persons under 18 years of age in the US [2]. Al-

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though inhaled corticosteroids have become a standard therapy assuring symptom control in many cases, they represent a broad-spectrum anti-inflammatory approach. Specific therapies targeting pathological alterations particularly found in asthma are still to be developed and a deeper understanding of the pathogenesis of asthma is a prerequisite for such specific therapies.

Asthma is characterized by airway remodeling, altered mucus production and airway smooth muscle cell (ASMC) contraction causing extensive airway narrowing. Alterations of ASMC seem to be of utmost importance in asthma, as its ablation by bronchoscopic thermoplasty led to marked improvement of asthma [3]. However, the question arises if similar effects may be achievable using pharmacological and thereby less invasive interventions. Based on this, the cellular mechanisms leading to excessive ASMC contraction are of particular interest.

The elevation of the cytoplasmic  $\text{Ca}^{2+}$  concentration,  $[\text{Ca}^{2+}]_c$ , is a key event leading to ASMC contraction [4–7]. Consequently, agonist-induced  $\text{Ca}^{2+}$  signaling has been regarded as a possible target in the pathophysiology of asthma [8–11]. In fact, in hyperresponsive inbred rats, enhanced  $\text{Ca}^{2+}$  mobilization was correlated with bronchial hyperresponsiveness [12–14]. Several studies have shown that  $\text{Ca}^{2+}$  responses can be modulated by a multitude of stimuli, including the cytokines IL-1 $\beta$  [15, 16], TNF- $\alpha$  [17–19], IL-4 and IL-13 [20–24]. In addition, CD38/cyclic ADP-ribose-mediated  $\text{Ca}^{2+}$  signaling has been found to contribute to ASMC hyperresponsiveness [25–28].

T-bet is a  $T_H1$ -specific transcription factor, which has the ability to direct  $T_H2$  into  $T_H1$  cells [29]. Naïve mice that have been target deleted of the T-bet gene (T-bet KO mice) spontaneously develop airway remodeling very similar to that seen in asthma and demonstrate multiple functional and inflammatory features characteristic of this disease [30]. Finotto et al. [31] showed that these alterations are mediated by IL-13. Recently, we used lung slices from T-bet KO mice as an asthma model and found both the acetylcholine (ACH)-induced airway contraction and the ACH-induced  $\text{Ca}^{2+}$  increase to be elevated [32]. The question arises as to what mechanisms may mediate the altered  $\text{Ca}^{2+}$  homeostasis in asthma. The exploration of that question was the aim of the present study.

In a former investigation, we used lung slices from T-bet KO mice, but we chose to use cultured ASMC from these mice in the present study. The reason for this was the fact that we aimed at performing Western blot analysis to investigate the protein expression in ASMC. In lung slices, this analysis would have been impaired by the abundant presence of non-smooth muscle cells like fibro-

blasts, epithelial and alveolar cells. In a different study, we recently investigated the effects of IL-4 and IL-13 on the ACH-induced  $\text{Ca}^{2+}$  signaling and receptor expression using primary cultured human bronchial smooth muscle cells (HBSMC). After incubation with 50 ng/ml IL-4 or IL-13 we found the ACH-induced  $\text{Ca}^{2+}$  transient to be elevated [22]. Therefore, HBSMC served as an additional asthma model in this study.

In the present communication, using 2 different asthma models, we showed that ACH-induced  $\text{Ca}^{2+}$  transients in ASMC were increased. This correlated in both models with increased  $\text{Ca}^{2+}$  contents of the sarcoplasmic reticulum (SR). We further showed that in ASMC from T-bet KO mice the expression of the SR  $\text{Ca}^{2+}$  buffers calreticulin and calsequestrin was elevated. In contrast, in human ASMC incubated with IL-13 or IL-4, the expression of ryanodine receptors (RyR), inositol-3-phosphate receptors (IP<sub>3</sub>R) and sarcoplasmic/endoplasmic reticulum  $\text{Ca}^{2+}$  ATPases 2 (SERCA 2) was increased.

## Material 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) and T-bet KO mice on a Balb/C background from Charles River (Needham, Mass., USA). All procedures were approved by the Ethics Committee of the Ludwig Maximilian University Munich.

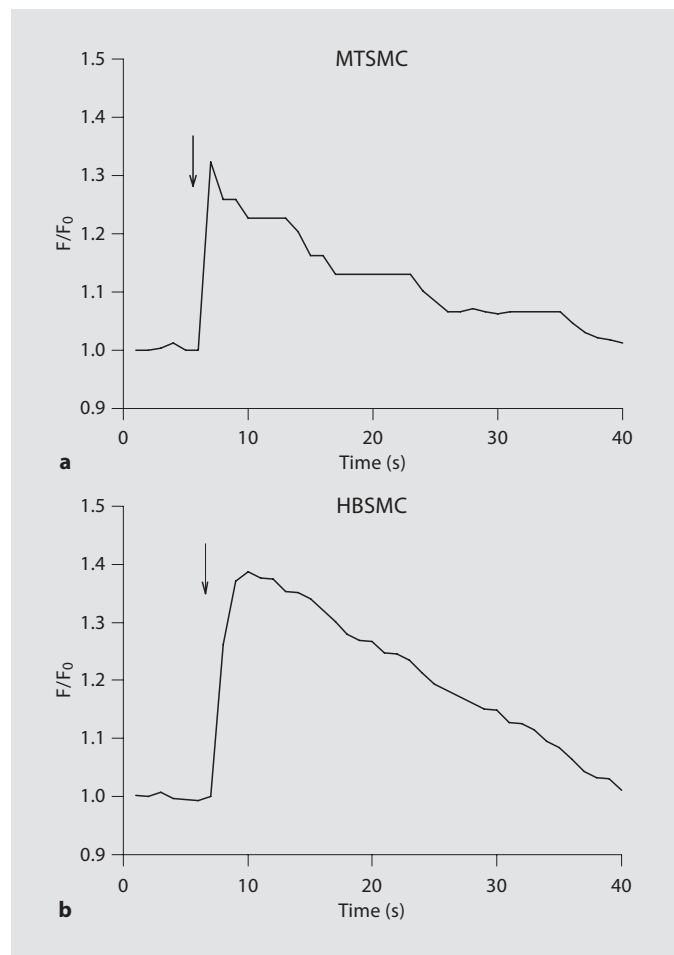
To grow murine tracheal smooth muscle cells (MTSMC), mice aged 42–77 days were sacrificed by intraperitoneal injection of phenobarbital. The trachea was excised, the connective tissue and the epithelium removed, and the trachea was placed in 3 mg/ml (720 U/ml) collagenase for 1 h. The harvested cells were washed and seeded on rat collagen-covered tissue plates to prevent growth of fibroblasts. MTSMC were identified by anti- $\alpha$ -actin staining and contractile responses to ACH. Primary cultured HBSMC were purchased from Cambrex Bio Science (Charles City, Iowa, USA), grown as monolayer and incubated for 24 h with recombinant human IL-4 or IL-13 (R&D Systems, Minneapolis, Minn., USA). In a previous study, dose-response curves for both interleukins were performed with 50 ng/ml, providing maximum effects in terms of contractile responses to ACH and morphological changes [22]. Therefore, this concentration was used in the present study. HBSMC without incubation with interleukins served as controls.

To quantify the ACH-induced  $\text{Ca}^{2+}$  increase, MTSMC (1 mM ACH) and HBSMC (1  $\mu\text{M}$  ACH) were loaded for 30 min at 37°C with the calcium indicator dye Oregon Green (10  $\mu\text{M}$ ; Molecular Probes, Eugene, Oreg., USA) in supplemented Hanks balanced salt solution containing 0.2% Pluronic (Pluronic F-127; Calbiochem, La Jolla, Calif., USA). After loading, the cells were incubated for at least 30 min in supplemented Hanks balanced salt

solution to allow for complete dye de-esterification and examined with a fluorescence microscope (Axiovert 200 M; Carl Zeiss, Jena, Germany). Images were recorded in time lapse (0.5 or 1 frame/s) using a digital CCD camera (AxioCam MRm; Carl Zeiss Vision, Munich, Germany). For each image, regions of interest were defined in single cells, and the average fluorescence intensity of each region of interest 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 the image analysis software Scion.

To perform Western blot analysis with MTSMC, the cells were washed twice with ice-cold phosphate-buffered saline (10 mM, pH 7.4) and trypsinized or scraped in 0.02% EDTA solution for intracellular or transmembrane proteins, respectively. The cell suspensions were treated according to user protocols using the Mammalian Protein Preparation Kit (Qiagen GmbH, Hilden, Germany) for intracellular proteins and Native Membrane Protein Extraction Kit (Merck Biosciences, Darmstadt, Germany) for transmembrane proteins. The extracts were collected, aliquoted and then stored at  $-20^\circ\text{C}$  until use with Western blot analysis. The extracts were treated with Laemmli sample buffer (at a final concentration of 32.5 mM Tris, 2.5%  $\beta$ -mercaptoethanol, 1% SDS and 12.5% glycerol) at  $85^\circ\text{C}$  for 10 min and separated by SDS-PAGE on 4–12% Bis-Tris (for calreticulin, calsequestrin and SERCA 2) or 3–8% Tris-acetate mini gels (for  $\text{IP}_3\text{R}$  and RyR) depending on the size of the target protein. Staining was performed using specific antibodies [rabbit anti-calreticulin, dilution 1:5,000; rabbit anti-calsequestrin, dilution 1:5,000; goat anti-SERCA2 (Santa Cruz Biotechnology), dilution 1:2,000; goat anti-RyR (Santa Cruz Biotechnology), dilution 1:1,000; rabbit anti- $\text{IP}_3\text{R}$  (Santa Cruz Biotechnology), dilution 1:1,500] and secondary antibodies [horseradish peroxidase-linked goat anti-rabbit-IgG or donkey anti-goat-IgG (Santa Cruz Biotechnology), dilution 1:10,000]. The protein concentrations of the extracts were determined with the Non-Interfering Protein Assay Kit according to the manufacturer's protocol (Merck Biosciences).  $\beta$ -Actin served as a loading control.

To perform Western blot analysis with HBSMC, the cells were incubated for 24 h with 50 ng/ml IL-4 and IL-13. Protein expression was determined by immunoblotting with protein extracts prepared with the Compartmental Protein Extraction Kit (Chemicon International, Hampshire, UK) according to the manufacturer's instructions. Protein concentrations of the extracts were quantified using the Bradford protein assay kit (Bio-Rad, Munich, Germany). Proteins were separated by SDS-PAGE on a 6% separating and 4% stacking gel (for SERCA 2), a 4% separating and 3% stacking gel (for  $\text{IP}_3\text{R}$  and RyR) and a 10% separating and 4% stacking gel (for calreticulin and calsequestrin), and transferred to nitrocellulose membranes (Hybond ECL Membrane; Amersham Biosciences, Little Chalfont, UK). After blocking for 2 h in a 5% solution of nonfat dried milk/TBS with 0.05% Tween 20, the membranes were incubated overnight at  $4^\circ\text{C}$  with specific antibodies [mouse anti-SERCA 2-antibody (2A7-A1; Abcam Inc., Cambridge, Mass., USA); mouse anti- $\text{IP}_3\text{R}$ -antibody (Chemicon International); mouse anti-RyR-antibody (34C; Abcam Inc.), dilution 1:1,000; rabbit anti-calreticulin-antibody (H-170; Santa Cruz Biotechnology), dilution 1:1,000]. Sheep anti-mouse or donkey anti-rabbit IgG horseradish peroxidase-linked whole antibodies (Amersham Biosciences; dilution 1:1,500) were used as secondary antibodies.  $\beta$ -Actin served as a loading control.



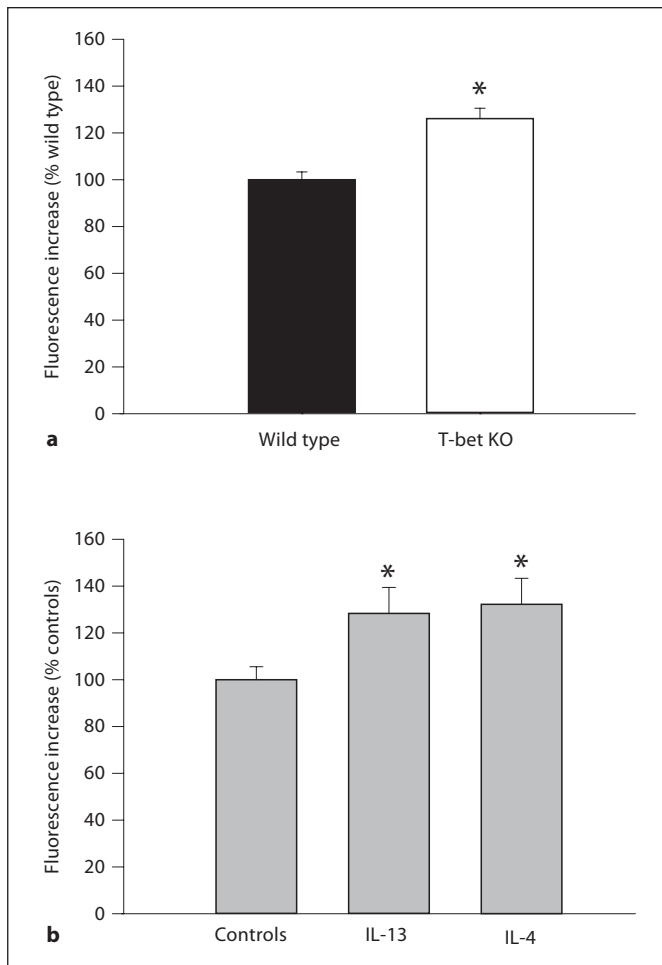
**Fig. 1.** MTSMC and HBSMC were loaded with the  $\text{Ca}^{2+}$  indicator Oregon Green, monitored using fluorescence microscopy and exposed to ACH. ACH ( $1\ \mu\text{M}$ , arrow) induced a transient increase in  $[\text{Ca}^{2+}]_c$  as shown by the representative curve of MTSMC (a) and HBSMC (b).

For MTSMC and HBSMC, antibody complexes were visualized using Hyperfilm ECL chemiluminescence (Amersham Biosciences) and evaluated using the ImageJ analysis software.

Statistical analysis was performed using the Sigma Stat software (Jandel Scientific, Chicago, Ill., USA).  $p < 0.05$  was considered statistically significant.

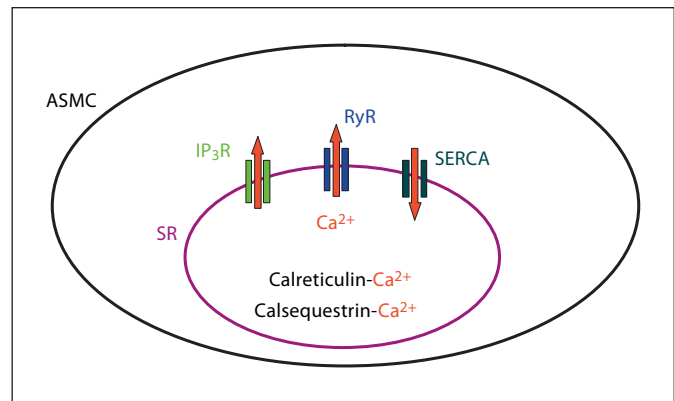
## Results

MTSMC and HBSMC were loaded with the  $\text{Ca}^{2+}$  indicator Oregon Green and recorded using fluorescence microscopy. Exposure to ACH ( $1\ \text{mM}$  for MTSMC,  $1\ \mu\text{M}$  for HBSMC) induced a transient increase in  $[\text{Ca}^{2+}]_c$  (fig. 1). To assess the effects of an asthmatic phenotype on the



**Fig. 2. a** To assess the effects of an asthmatic phenotype on the ACH-induced  $\text{Ca}^{2+}$  response, MTSMC from T-bet KO and wild-type mice were compared. The ACH-induced  $\text{Ca}^{2+}$  transients were higher in MTSMC from T-bet KO mice ( $n = 151$ ). \*  $p < 0.001$ . **b** HBSMC were incubated with 50 ng/ml IL-13 or IL-4 for 24 h. After incubation with the interleukins, HBSMC showed increased ACH-induced  $\text{Ca}^{2+}$  transients ( $n = 57$ ). \*  $p < 0.05$  versus controls.

$\text{Ca}^{2+}$  response, 2 asthma models were used. In the murine model, MTSMC from T-bet KO mice served as asthmatic ASMC, while MTSMC from wild-type mice served as control [30, 32]. In this setting, the ACH-induced  $\text{Ca}^{2+}$  transients were significantly higher in MTSMC from T-bet KO mice (126% of MTSMC from wild-type mice,  $n = 151$ ,  $p < 0.001$ ; fig. 2a). In the human model, HBSMC were incubated for 24 h with 50 ng/ml IL-13 or IL-4. In a previous study, dose-response curves for both interleukins were performed. Because 50 ng/ml provided maximum effects in terms of contractile responses to ACH and mor-

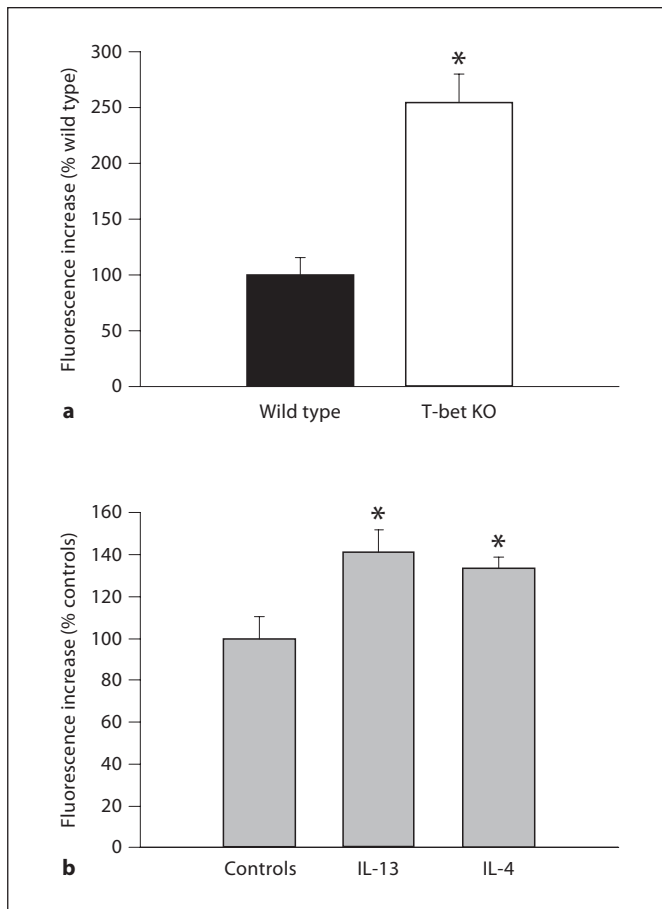


**Fig. 3.** In ASMC, the equilibrium of the  $\text{Ca}^{2+}$  content of the SR is maintained by SERCA pumping calcium into the SR and  $\text{IP}_3\text{R}$  and RyR releasing calcium out of the SR. Within the SR, calcium is buffered by calreticulin and calsequestrin.

phological changes [22], this concentration was used in the human asthma model in this study. After incubation with IL-13 or IL-4, the ACH-induced  $\text{Ca}^{2+}$  transient was increased (IL-13: 128% of controls; IL-4: 132% of controls;  $n = 57$ ,  $p < 0.05$  versus controls; fig. 2b).

The equilibrium of the  $\text{Ca}^{2+}$  content of the SR is maintained by SERCA pumping calcium into the SR, while  $\text{IP}_3\text{R}$  and RyR release calcium out of the SR (fig. 3) [33]. Within the SR, calcium is buffered by the  $\text{Ca}^{2+}$  chelators calreticulin and calsequestrin. To test the hypothesis that an increased  $\text{Ca}^{2+}$  content of the SR may have caused the increased  $\text{Ca}^{2+}$  transients in the asthmatic models, SERCA were inhibited, leading to a net  $\text{Ca}^{2+}$  efflux out of the SR. The resulting increase in  $[\text{Ca}^{2+}]_c$  was used as an estimate of the  $\text{Ca}^{2+}$  content of the SR. In the murine model, SERCA were inhibited using 10  $\mu\text{M}$  thapsigargin. The following increase in  $[\text{Ca}^{2+}]_c$  was markedly higher in MTSMC from T-bet KO mice (255% of MTSMC from wild-type mice,  $n = 5-30$ ,  $p < 0.05$ ; fig. 4a). In HBSMC, 1  $\mu\text{M}$  cyclopiazonic acid (CPA) was used to block SERCA. The resulting increase in  $[\text{Ca}^{2+}]_c$  was significantly elevated after incubation of HBSMC with 50 ng/ml IL-13 or IL-4 for 24 h, indicating a higher  $\text{Ca}^{2+}$  content of the SR in the asthma model (IL-13: 141% of controls; IL-4: 134% of controls;  $n = 21-92$ ,  $p < 0.05$  versus controls; fig. 4b). Thapsigargin and CPA were used because these compounds caused maximal increase in  $[\text{Ca}^{2+}]_c$  in the respective cell type.

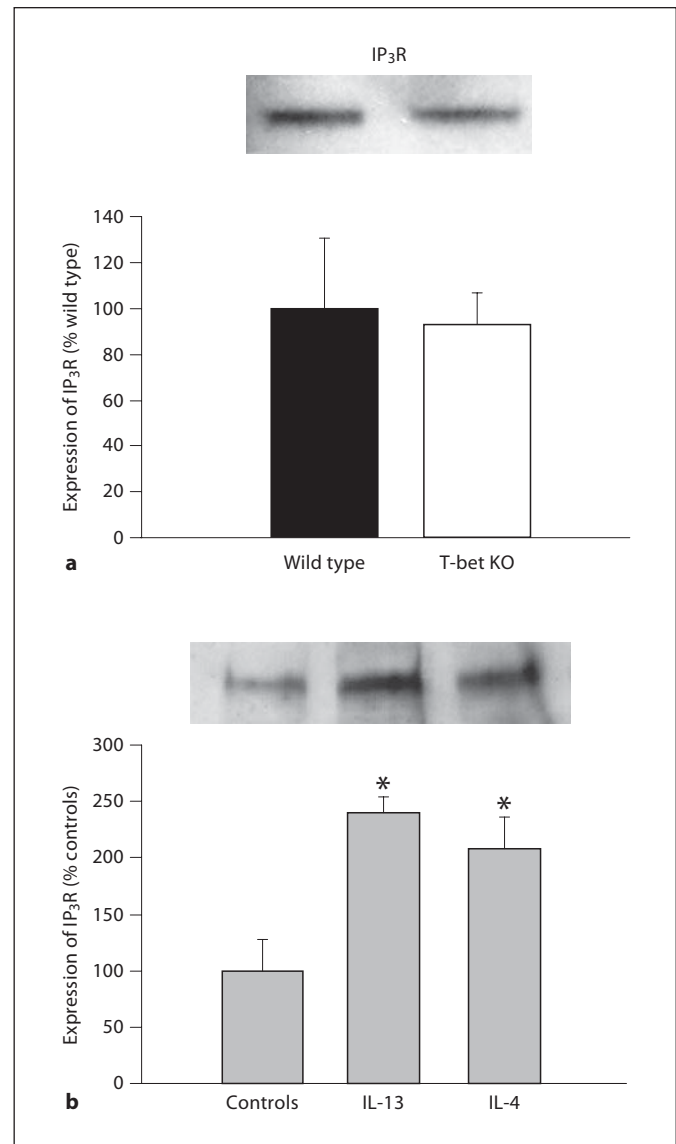
To further identify the mechanisms responsible for the altered  $\text{Ca}^{2+}$  homeostasis in asthmatic ASMC, the expression of proteins regulating the  $\text{Ca}^{2+}$  content of the SR



**Fig. 4.** To assess the  $\text{Ca}^{2+}$  content of the SR, SERCA were inhibited and the resulting increase in  $[\text{Ca}^{2+}]_c$  was quantified. **a** Using  $10 \mu\text{M}$  thapsigargin to block SERCA in MTSMC, the resulting increase in  $[\text{Ca}^{2+}]_c$  was markedly higher in MTSMC from T-bet KO compared to wild-type mice ( $n = 5-30$ ). \*  $p < 0.05$ . **b** Blocking SERCA in HBSMC with  $1 \mu\text{M}$  CPA, the resulting increase in  $[\text{Ca}^{2+}]_c$  was significantly elevated after incubation with  $50 \text{ ng/ml}$  IL-13 or IL-4 ( $n = 21-92$ ). \*  $p < 0.05$  versus controls. Thapsigargin and CPA were used because these compounds were found to cause maximal increase in  $[\text{Ca}^{2+}]_c$  in the respective cell type.

were investigated by Western blot analysis.  $\text{IP}_3\text{R}$  is an SR  $\text{Ca}^{2+}$  release channel that opens upon the binding of  $\text{IP}_3$ . In MTSMC, we found no difference between the expression of this receptor in T-bet KO and wild-type mice (T-bet KO mice 93% of wild-type mice,  $n = 13$ ; fig. 5a). In contrast, incubation of HBSMC with IL-13 or IL-4 considerably increased the expression of  $\text{IP}_3\text{R}$  (IL-13: 241% of controls; IL-4: 209% of controls;  $n = 5$ ,  $p < 0.001$  versus controls; fig. 5b).

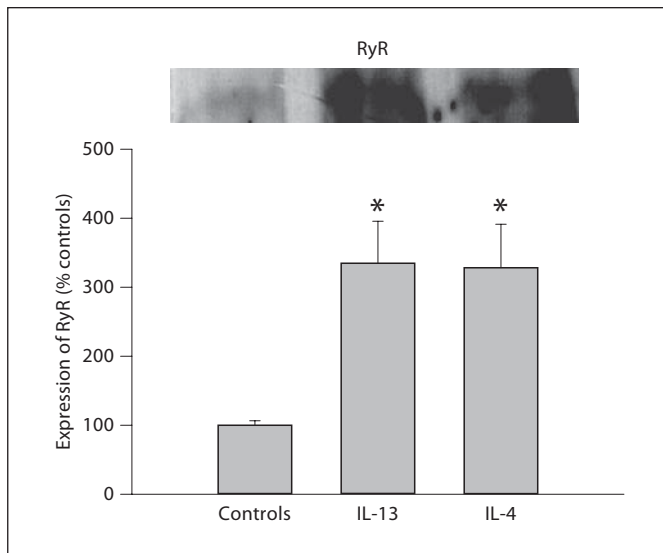
The other receptor releasing calcium from the SR is RyR. In MTSMC, the expression of RyR was very weak,



**Fig. 5.** The expression of  $\text{IP}_3\text{R}$  was investigated using Western blot analysis. **a** In MTSMC, no difference between cells from T-bet KO and wild-type mice could be observed ( $n = 13$ ). **b** In contrast, incubation of HBSMC with IL-13 or IL-4 considerably increased the expression of  $\text{IP}_3\text{R}$  ( $n = 5$ ). \*  $p < 0.001$ .

prohibiting quantitative analysis. However, no apparent differences between T-bet KO and wild-type mice could be observed (data not shown). In HBSMC, the expression of RyR was stronger and incubation with IL-13 or IL-4 noticeably increased it (IL-13: 335% of controls; IL-4: 327% of controls;  $n = 5$ ,  $p < 0.001$  versus controls; fig. 6).

SERCA pump calcium against a concentration gradient from the cytoplasm into the SR. So far, 3 SERCA iso-

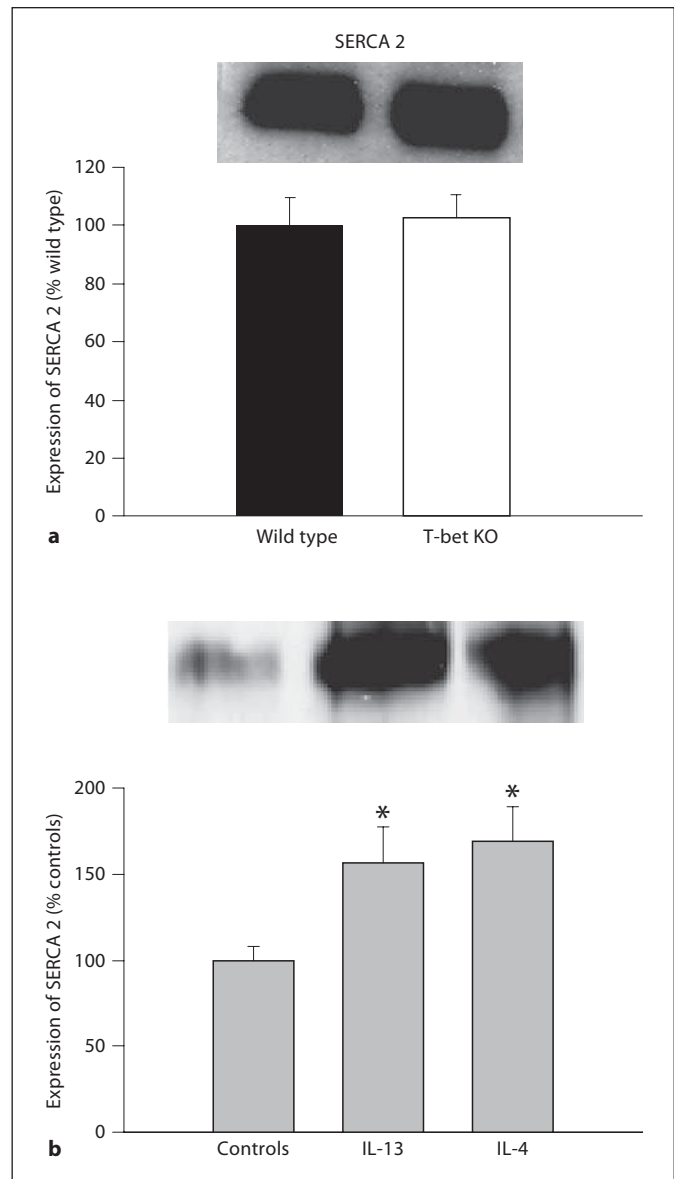


**Fig. 6.** Investigating the expression of RyR in MTSMC, it was too weak to allow quantitative analysis. However, no apparent differences between T-bet KO and wild-type mice could be observed (data not shown). In HBSMC, the expression of RyR was stronger and noticeably further increased after incubation with IL-13 or IL-4 (n = 5). \* p < 0.001 versus controls.

forms have been identified, with SERCA 2 being the isoform that is predominant in smooth muscle [33]. Analyzing the expression of SERCA 2, we found no difference between MTSMC from T-bet KO and wild-type mice (T-bet KO mice 102% of wild-type mice, n = 7; fig. 7a). In contrast, HBSMC incubated with IL-13 or IL-4 showed an increased expression of SERCA 2 (IL-13: 157% of controls; IL-4: 169% of controls; n = 6, p < 0.01 versus controls; fig. 7b).

In the SR, calcium binds to specific high-capacity, low-affinity Ca<sup>2+</sup> buffers to ensure high-capacity Ca<sup>2+</sup> storage as well as rapid Ca<sup>2+</sup> release [33]. The most important SR Ca<sup>2+</sup> buffer is believed to be calreticulin. Analyzing the expression of calreticulin, we found it slightly but significantly elevated in MTSMC from T-bet KO mice (107% of MTSMC from wild-type mice, n = 6, p < 0.05; fig. 8a). In HBSMC, the expression of calreticulin was modestly elevated after incubation with IL-13, but not after incubation with IL-4 (IL-13: 115% of controls; IL-4: 103% of controls; n = 7, p < 0.05 versus controls and IL-4; fig. 8b).

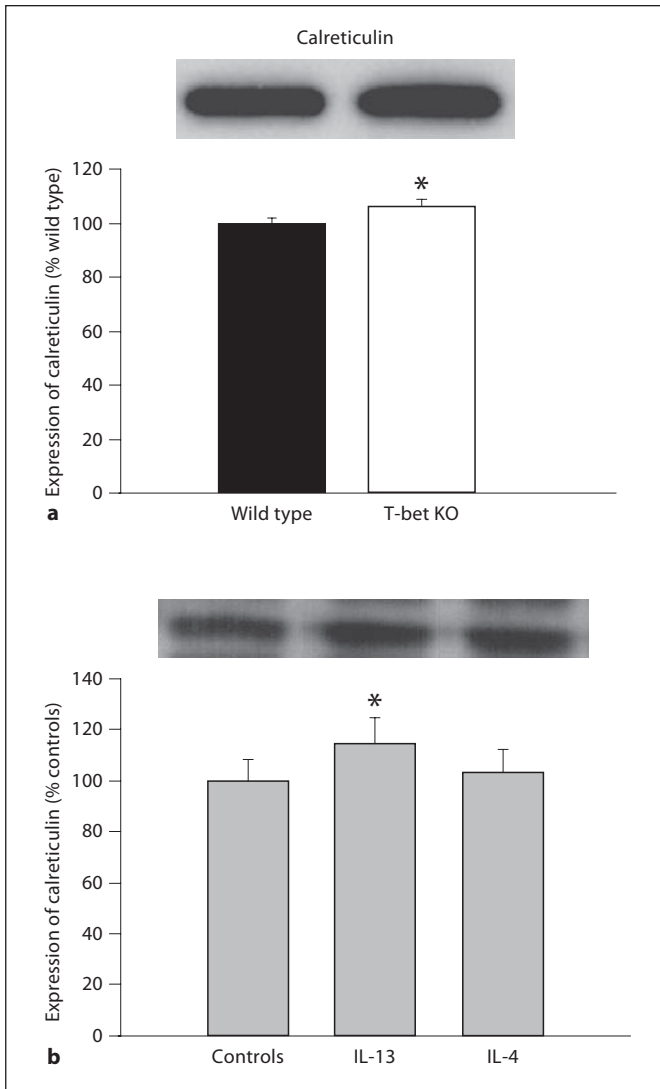
Another SR Ca<sup>2+</sup> buffer is calsequestrin. Analyzing its expression in MTSMC, it was found to be weak in cells from wild-type mice but stronger in cells from T-bet KO mice (189% of MTSMC from wild-type mice, n = 5, p < 0.05; fig. 9). In HBSMC, the expression of calsequestrin



**Fig. 7. a** In MTSMC, no differences between the expression of SERCA 2 in cells from T-bet KO and wild-type mice could be found (n = 7). **b** Conversely, in HBSMC, incubation with IL-13 or IL-4 increased the expression of SERCA 2 (n = 6). \* p < 0.01 versus controls.

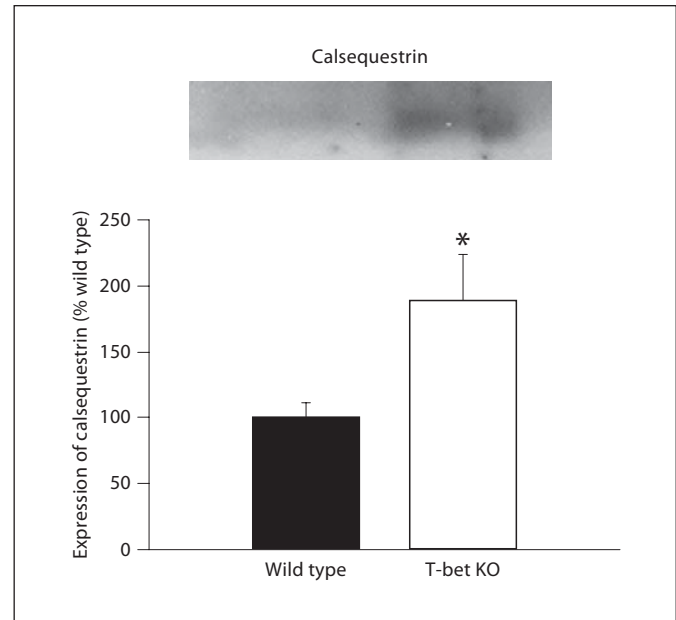
was too weak to allow quantitative analysis. However, no influence of incubation with IL-13 or IL-4 could be observed (data not shown). Table 1 gives an overview of the findings on the expression of the Ca<sup>2+</sup>-regulating proteins that were investigated.

The agonist-induced elevation in [Ca<sup>2+</sup>]<sub>c</sub> is a key event leading to ASMC contraction, airway narrowing and fi-



**Fig. 8. a** Analyzing the expression of the SR  $\text{Ca}^{2+}$  buffer calreticulin, it was slightly, but significantly elevated in MTSMC from T-bet KO compared to wild-type mice ( $n = 6$ ). \*  $p < 0.05$ . **b** In HBSMC, the expression of calreticulin was modestly elevated after incubation with IL-13 but not after incubation with IL-4 ( $n = 7$ ). \*  $p < 0.05$  versus IL-4 and controls.

nally dyspnea in patients with asthma. Based on the findings of this study, the question arises whether the altered  $\text{Ca}^{2+}$  signaling cascade in asthma can be influenced using specific inhibitors. As an example of such an intervention, we used xestospongins in the HBSMC asthma model. Xestospongins are a family of potent inhibitors of  $\text{IP}_3\text{R}$ , whose expression we found to be increased after incubation with interleukins. Xestospongins C is believed to be the most potent one, with xestospongins D being less

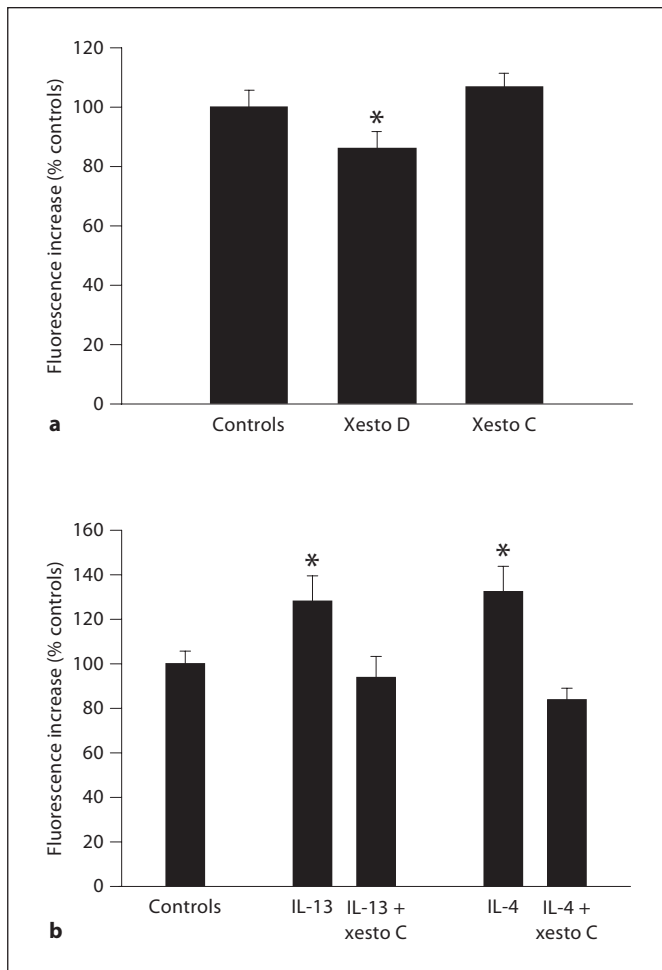


**Fig. 9.** In MTSMC, the expression of the SR  $\text{Ca}^{2+}$  buffer calsequestrin was higher in cells from T-bet KO compared to wild-type mice, which showed only weak expression ( $n = 5$ ). \*  $p < 0.05$ . In HBSMC, the expression of calsequestrin was too weak to allow quantitative analysis. However, no influence of incubation with IL-13 or IL-4 could be observed (data not shown).

**Table 1.** Expression of SR  $\text{Ca}^{2+}$  homeostasis-regulating proteins

	RyR	$\text{IP}_3\text{R}$	SERCA	Calreticulin	Calsequestrin
T-bet KO mice (MTSMC)	→	→	→	↑	↑
IL-13/IL-4 (HBSMC)	↑	↑	↑	(↑)	→

effective [34]. In HBSMC without interleukin incubation, preincubation with  $10 \mu\text{M}$  xestospongins D for 30 min significantly decreased ACH-induced  $\text{Ca}^{2+}$  transients, while incubation with  $10 \mu\text{M}$  xestospongins C had no effect (xestospongins D: 86% of controls; xestospongins C: 107% of controls;  $n = 33-57$ ,  $p < 0.05$ ; fig. 10a). But, interestingly, xestospongins C reversed the increase in the ACH-induced  $\text{Ca}^{2+}$  transients after incubation with IL-13 or IL-4 (IL-13: 128% of controls without and 94% of controls with



**Fig. 10.** HBSMC were loaded with the  $\text{Ca}^{2+}$  indicator Oregon Green, monitored with fluorescence microscopy and exposed to  $1 \mu\text{M}$  ACH. **a** Blocking  $\text{IP}_3\text{R}$  using  $10 \mu\text{M}$  xestospongins D, the ACH-induced  $\text{Ca}^{2+}$  transients were reduced. Using  $10 \mu\text{M}$  xestospongins C, no effect could be observed ( $n = 33\text{--}57$ ). \*  $p < 0.05$  versus controls and xestospongins C. **b** But,  $10 \mu\text{M}$  xestospongins C reversed the increased ACH-induced  $\text{Ca}^{2+}$  transients after incubation with IL-13 or IL-4 ( $n = 20\text{--}57$ ). \*  $p < 0.05$  versus controls and interleukin/xestospongins C. Xesto = Xestospongins.

xestospongins C; IL-4: 132% of controls without and 84% of controls with xestospongins C;  $p < 0.05$ ,  $n = 20\text{--}57$ ; fig. 10b).

## Discussion

Using 2 different asthma models, we have shown that ACH-induced  $\text{Ca}^{2+}$  transients in asthmatic ASMC were increased. This correlated in both models with increased

$\text{Ca}^{2+}$  contents of the SR. We have further shown that in ASMC from T-bet KO mice the expression of the SR  $\text{Ca}^{2+}$  buffers calreticulin and calsequestrin was elevated. In contrast, in human ASMC incubated with IL-13 or IL-4, the expression of RyR,  $\text{IP}_3\text{R}$  and SERCA 2 was increased.

In this study, we used 2 asthma models: MTSMC from T-bet KO mice and HBSMC incubated with interleukins. While HBSMC were commercially purchased, MTSMC were isolated from the murine trachea and identified by anti- $\alpha$ -actin staining and contractile responses to ACH. HBSMC were incubated for 24 h with  $50 \text{ ng/ml}$  IL-13 or IL-4. In a previous study, dose-response curves for both interleukins were performed. Because  $50 \text{ ng/ml}$  provided maximum effects in terms of contractile responses to ACH and morphological changes [22], this concentration was used in the human asthma model.

The 2 models were different in 3 aspects. Firstly, the cells originated from different species, namely mice and humans. Secondly, cells from the trachea on one hand and from bronchi on the other hand were used. Thirdly, asthma was mimicked using a genetically engineered mouse model or by incubation of cells with IL-13 or IL-4. In both models, the ACH-induced  $\text{Ca}^{2+}$  transients were increased most probably by elevated  $\text{Ca}^{2+}$  contents of the SR. Yet, the mechanisms changing the  $\text{Ca}^{2+}$  homeostasis of the SR were different in the 2 experimental settings. While in MTSMC the SR  $\text{Ca}^{2+}$  buffers were upregulated, the expression of  $\text{Ca}^{2+}$  channels and a  $\text{Ca}^{2+}$  pump was increased in HBSMC. Which or how many of the 3 different aspects of the 2 models were responsible for the differences remains speculatively, but needs to be addressed in future studies. However, our results remind us of the need to be careful when generalizing results obtained in a single experimental model.

Elevation of the  $[\text{Ca}^{2+}]_c$  can be caused by  $\text{Ca}^{2+}$  release from the intracellular  $\text{Ca}^{2+}$  stores or by  $\text{Ca}^{2+}$  influx from the extracellular space. In a previous study, we could show that in ASMC the latter plays a minor role and probably only serves to compensate for  $\text{Ca}^{2+}$  losses to the extracellular space [4]. But, we explicitly do not exclude the possibility that  $\text{Ca}^{2+}$  influx may play a role in the changes in the  $\text{Ca}^{2+}$  homeostasis seen in asthma. However, in the present study, we concentrated on changes in the regulation of the SR  $\text{Ca}^{2+}$  content.

The increased  $\text{Ca}^{2+}$  transients were correlated with an increased  $\text{Ca}^{2+}$  content of the SR. The  $\text{Ca}^{2+}$  content was estimated by blocking SERCA and quantifying the increase in  $[\text{Ca}^{2+}]_c$  caused by calcium leakage out of the SR. To block SERCA, thapsigargin was used in MTSMC and CPA in HBSMC. The different drugs were used because



each of them gave a maximal response in the respective cells. Speculatively, the reason for this difference may be different SERCA subtypes, as SERCA 2 exists in the isoforms 2A and 2B, although the investigation of this question was beyond the scope of this study. In cardiac smooth muscle cells, a decreased  $\text{Ca}^{2+}$  content of the SR has been shown in cardiac hypertrophy [35] and has been found to be responsible for defective excitation-contraction coupling in heart failure [36]. On the other hand, Asano and Nomura [37] found the  $\text{Ca}^{2+}$  content of the SR in arterial smooth muscle cells to be increased in spontaneously hypertensive rats. In a previous study, we could show that increased bronchial reactivity in lung slices of T-bet KO mice correlated with increased  $\text{Ca}^{2+}$  content of the SR in bronchial ASMC from these mice [32]. We now confirm these data in MTSMC from T-bet KO mice and in interleukin-treated HBSMC. We believe that in the future an altered regulation of the  $\text{Ca}^{2+}$  content of the SR may prove to be involved in a number of diseases with altered muscle cell function.

In the SR, calcium is buffered by  $\text{Ca}^{2+}$  chelators, which are high-capacity/low-affinity buffers combining the storage of large quantities of calcium with its rapid release. The storage proteins that are believed to be expressed in smooth muscle at the highest levels are calreticulin and calsequestrin [33]. In MTSMC from T-bet KO mice, we found these 2 buffers to be upregulated. Although the increase in expression of calreticulin was statistically significant, it appeared low given the strong baseline expression of calreticulin, rendering the meaning of this finding somewhat unclear. In contrast, the expression of calsequestrin was considerably higher in MTSMC from T-bet KO mice. In HBSMC, we found a statistical increase in the expression of calreticulin after incubation with IL-13, but not with IL-4. Given the strong upregulation of  $\text{IP}_3\text{R}$ , RyR and SERCA 2, this finding appears to be of minor importance. However, this is – to our knowledge – the first report of an upregulation of SR  $\text{Ca}^{2+}$  buffers in ASMC in an asthma model.

ATP-dependent SERCA pumps force calcium out of the cytoplasm and into the SR. Three SERCA isoforms have been identified, with SERCA 2 being the isoform that is predominant in smooth muscle [33]. In HBSMC, the expression of SERCA 2 was increased after incubation with IL-13 or IL-4. A higher capacity to pump calcium against the concentration gradient into the SR could lead to a higher SR  $\text{Ca}^{2+}$  content. However, the question arises how the calcium is stored in the SR, as the 2  $\text{Ca}^{2+}$  buffers investigated were not upregulated accordingly. Speculatively, so far undescribed  $\text{Ca}^{2+}$  buffers may contribute to

the overall buffer capacity of the SR and may have been upregulated by the incubation with the interleukins. Alternatively, the capacity to buffer calcium was sufficient to store the increased amount of calcium without the need to increase protein expression. In heart failure, the activity of SERCA was found to be decreased [38] and these pumps are currently being investigated as a possible target for heart failure therapy [39]. Thus, decreased contractility of cardiac myocytes leads to heart failure, while increased contractility of ASMC may lead to asthma. The investigation of SERCA therefore seems promising in diseases connected with altered muscle contractility.

After the binding of ACH to its receptor, activation of phospholipase C produces  $\text{IP}_3$ , which releases calcium from the SR via the  $\text{IP}_3\text{R}$  channel. As an example for a pathophysiological role for  $\text{IP}_3\text{R}$ , it has been shown to support proarrhythmic  $\text{Ca}^{2+}$  signals in cardiac myocytes [40]. We found the expression of  $\text{IP}_3\text{R}$  to be increased after incubation of HBSMC with IL-13 or IL-4. A cytoplasmic  $\text{Ca}^{2+}$  increase via  $\text{IP}_3\text{R}$  triggers calcium-induced calcium release from the SR via RyR [41]. RyR dysfunction has been detected in heart failure and ventricular tachycardia as well as in striated muscle diseases, and RyR-stabilizing drugs have been demonstrated to be effective in the corresponding disease models [42]. In MTSMC, only weak expression of RyR could be detected, with no apparent difference between T-bet KO and wild-type mice. But, in HBSMC, incubation with IL-13 or IL-4 noticeably increased the expression of RyR. The upregulation of  $\text{IP}_3\text{R}$  and RyR in HBSMC obviously did not directly lead to an increased SR  $\text{Ca}^{2+}$  content, as these receptors are SR  $\text{Ca}^{2+}$  release channels. However, these receptors contributed to the release of a greater amount of calcium out of the SR, as seen in response to ACH. To take advantage of this finding, we used xestospongins to investigate if inhibition of  $\text{IP}_3\text{R}$  could be used to reduce the elevated ACH-induced  $\text{Ca}^{2+}$  signaling in HBSMC after incubation with IL-13 or IL-4. Xestospongins are a family of potent inhibitors of  $\text{IP}_3\text{R}$ , of which xestospongins C is believed to be the most potent one and xestospongins D to be less effective [34]. Without interleukins, xestospongins D decreased ACH-induced  $\text{Ca}^{2+}$  transients, while incubation with xestospongins C had no effect. But, xestospongins C reversed the IL-13- and IL-4-mediated increase in ACH-induced  $\text{Ca}^{2+}$  transients. The different effects of xestospongins C and D may be due to different affinities to  $\text{IP}_3\text{R}$ . In turn, differences in the affinities may be dependent on the isoform of the receptor [34]. So far, 3 isoforms of  $\text{IP}_3\text{R}$  have been isolated and all 3 are expressed in smooth muscle [33]. But, to our knowledge, the affinities

of the xestospongins family members to the different isoforms have not been investigated systematically. However, xestospongins C is an example of a drug that reverses the pathological  $Ca^{2+}$  signaling without affecting the normal  $Ca^{2+}$  homeostasis.

Using 2 different asthma models, we showed that ACh-induced  $Ca^{2+}$  transients in ASMC were increased. This correlated in both models with increased  $Ca^{2+}$  contents of the SR. We further showed that in the murine asthma model the SR  $Ca^{2+}$  buffer capacity is increased,

while in the human asthma model the expression of SR  $Ca^{2+}$  channels is altered. We conclude that the  $Ca^{2+}$  homeostasis of ASMC is a field with the potential to provide new therapeutical options in asthma.

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