

Effects of conventional tobacco smoke and nicotine-free cigarette smoke on airway inflammation, airway remodelling and lung function in a triple allergen model of severe asthma

C. Tilp, H. Bucher, H. Haas, M. J. Duechs, E. Wex and K. J. Erb

Respiratory Diseases Research, Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach a.d. Riss, Germany

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Summary

Background Patients with asthma who smoke have reduced lung function, increased exacerbation rates and increased steroid resistance compared to non-smoking asthmatics. In mice, cigarette smoke has been reported to have both pro- and anti-Th2 response effects.

Objective We hypothesized that combining tobacco cigarette smoke (tCS) with allergen exposure increases inflammation, airway remodelling and lung function in mice. To test this hypothesis, we combined a severe triple allergen model with tCS exposure and investigated whether effects were due to Toll-like receptor 4 signalling and/or nicotine and also observed when nicotine-free cigarettes were used.

Methods Mice were sensitized with ovalbumin, cockroach and house dust mite allergen in alum followed by intratracheal challenges with allergen twice a week for 6 weeks or additionally exposed to tCS during the allergen challenge period. Nicotine or nicotine-free herbal cigarette smoke was also applied to allergen challenged mice.

Results tCS significantly reduced eosinophil numbers, IL-4 and IL-5 concentrations in the lung, total and allergen-specific IgE in serum, improved lung function and reduced collagen I levels. With the exception of collagen I all parameters reduced by tobacco cigarette smoke were also reduced in Toll-like receptor 4-deficient mice. Nicotine-free cigarette smoke also had significant anti-inflammatory effects on eosinophils, IL-4 and IL-5 concentrations in the lung and reduced airway hyperreactivity, albeit weaker than tobacco smoke. Applying nicotine alone also reduced Th2 cytokine levels and eosinophil numbers in the airways.

Conclusion Our experiments show that tCS exposure reduces allergen-induced Th2 response in the lung and associated collagen I production and development of airway hyperreactivity. With the exception on collagen I formation, these effects were not dependent on Toll-like receptor 4. The observed anti-Th2 effects of both nicotine and nicotine-free herbal cigarette smoke together suggests that tCS reduces the Th2 responses through nicotine and other products released by burning tobacco.

Keywords severe asthma, cigarette smoke, anti-inflammatory properties, TLR-4, nicotine

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Correspondence:

Prof. Dr. Klaus Erb, Respiratory Diseases Research, Boehringer-Ingelheim Pharma GmbH & Co. KG, H91-02-01, Birkendorferstr. 65, D-88397 Biberach a.d. Riss, Germany. E-mail: klaus.erb@boehringer-ingelheim.com

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Introduction

Asthma is a heterogeneous chronic disease of the airways consisting of various phenotypes and having numerous risk factors contributing to disease development and severity. Severe asthma can either be of an atopic nature with pronounced Th2 responses or non-atopic with or without an eosinophilic and/or

neutrophilic component [1]. Irrespective of the underlying inflammatory response, all types of severe asthma show persistent inflammation of the airways, airway hyperreactivity (AHR) and pronounced airway remodelling characterized by increased fibrosis and smooth muscle thickening, mucus overproduction and sub-epithelial thickening. Patients with this asthma phenotype often cannot be controlled on standard

therapy (Gold 4–5), often exhibit steroid resistance and are prone to acute exacerbations, leading to the highest mortality rates among all asthma phenotypes [2].

Although a few epidemiological studies suggest that smoking may reduce the development of asthma or some asthma symptoms in specific sub-population of patients [3–5], there is generally no doubt that active or passive tobacco cigarette smoke (tCS) exposure is a major risk factor for developing asthma. Furthermore, smoking asthmatics have worse asthma control and show an excessive decline in forced expiratory volume in 1 s (FEV1) and may have impaired responses to inhaled and oral steroids [6–9] which is especially true for the elderly [10]. In addition, passive smoking may also increase the risk of developing asthma and although smoking cessation helps asthma control and reduces asthma worsening, ex-smoking asthmatics still have a poorer prognosis than never smoking asthmatics [7, 9]. It could be expected that these negative effects of smoking on asthma (together with the well-known tCS associated increased risks of cancer development and developing cardio metabolic diseases and others) would result in less asthmatics smoking. However, the percentage of smokers and ex-smokers is similar in both asthmatic patients and the general population, with about 25–35% of asthmatics being current smokers [11]. Smoking asthmatics can be considered to be an asthma sub-population, which may be in need of therapeutic treatment which differs from non-smoking asthmatics [12, 13]. Overcoming steroid resistance may be of particular importance for these patients, because tCS has been shown to directly cause steroid resistance by impairing histone deacetylase (HDAC) 2 function for example [14].

Although the link between asthma worsening and cigarette smoke exposure has been well established in humans, studies in mice have reported different effects. Recent publications in mice have shown that tCS exposure can on the one hand enhance allergic sensitization and the allergic pulmonary Th2 response [15–19]. However, on the other hand, others have found that tCS attenuates the allergic Th2 response [20–24]. Furthermore, Mortaz *et al.* showed a direct suppressive effect of tCS on mast cell activation *in vitro* [25]. Publications showing strong anti-inflammatory effects of nicotine on Th2 inflammation suggests that nicotine may be at least in part mediating the anti-inflammatory effects of tCS found in mice [26]. Interestingly, in a 4-month mouse OVA/tCS combination experiment, Melgert and Hylkema found no suppressive or enhancing effect of tCS on the allergic Th2 response, AHR or remodelling clearly indicating that generally the effects of tCS on murine Th2 responses depend on the experimental set-up used [24, 27].

We have recently developed a novel triple allergen immunization/challenge model of chronic asthma in mice which displays several hallmarks of human severe asthma including persistent airway inflammation, AHR and remodelling, which is steroid resistant [28]. This triple allergen model, in which mice are immunized with a cocktail of ovalbumin (OVA), extracts of cockroach (CRA) and house dust mite (HDM) followed by twelve challenges (2 per week), alternating between allergens, developed a stronger Th2 type inflammation in the lung, enhanced loss of lung function, higher IgE serum concentrations and collagen I formation in the lung in comparison to only using OVA, CRA or HDM alone in single allergen exposure/challenge models [28]. Furthermore, when mice immunized with all three allergens were challenged with a mixture of all three allergens on the six consecutive weeks following the immunization period, the resulting asthmatic phenotype was much weaker compared to mice where the allergens were alternated during the airway challenges. In the current study, we combined this model with passive tCS exposure (as described previously [29]) by exposing sensitized mice to tCS five times a week during the 6 week allergen challenge period. Our aim was to develop a model mimicking the pathology of asthma worsening observed in smoking asthmatics, including a strong neutrophilia plus eosinophilia, increased AHR, increased remodelling (collagen I formation, mucus production and smooth muscle thickening) and reduced sensitivity to steroid treatment compared to allergen-only exposed mice. We found that the tCS exposure increased the neutrophilia and IL-1 β levels in the lung of allergen treated mice as expected, but reduced all Th2 inflammatory parameters measured, AHR and fibrosis. This clearly shows that in our set-up, tCS is clearly acting as a Th2 response suppressor. We further demonstrated that the suppressive anti-Th2 response effects are not Toll-like receptor 4 (TLR4) dependent but may be mediated by mechanisms involving nicotine and additional substances/particles released by burning tobacco.

Methods

Animals

Female Balb/cAnCrI mice, C57BL/6J mice, C3H/HeJ mice and C3H/HeOuJ mice, 6 to 10 weeks of age, were purchased from Charles River (Sulzfeld, Germany). The animals were housed under conventional conditions in isolated ventilated cages with free access to water and food. All experiments performed were approved by authorities for the care and use of experimental animals (Regierungspräsidium Tübingen; TVV12-017).

Cigarette smoke exposure

Mice were exposed to CS in a heated (38 °C) whole body exposure chamber for either 6 weeks (5 days per week) in the chronic asthma model, or 4 days in the helminthic model as described previously [29]. Mice were exposed to 4 cigarettes (Roth-Händle without filters, tar 10 mg, nicotine 1 mg, carbon monoxide 6 mg, Imperial Tobacco) per day in the chronic asthma model, or six cigarettes per day in the helminthic infection model. In separate experiments, mice were exposed to the smoke of 4 tobacco- and nicotine-free herbal cigarettes (hCS) (NTB, Kräuterretten, tar 3 mg, nicotine-free, carbon monoxide 3 mg, Arkopharma) per day for a duration of 6 weeks.

Chronic asthma model combined with cigarette smoke exposure

Mice were sensitized and challenged as described previously [28]. Shortly, mice were sensitized by intraperitoneal injection of a combination of 20 µg OVA (salt free albumin egg, Serva, Heidelberg, Germany), 2.5 µg CRA (cockroach, German, *Blattella germanica*, Greer Laboratories, Lenoir, NC, USA) and 2.5 µg HDM (mite, house dust, *Dermatophagoides pteronyssinus*, Greer Laboratories) in a 1 : 2 dilution with aluminium hydroxide (Imject Alum, Rockford, IL, USA) in a total volume of 0.2 mL on day 0, 14 and 21, followed by intratracheal (i.t.) challenges twice a week with alternating allergens for the following 6 weeks (days 26/27, 33/34, 40/41, 47/48, 54/55 and 61/62, respectively). In the first and fourth week, mice received 50 µL of OVA i.t. (20 µg OVA in 50 µL PBS) on two consecutive days; in the second and fifth week i.t., CRA challenge was performed (20 µg CRA in 50 µL PBS); and in the third and sixth week, mice were i.t. challenged with HDM (20 µg HDM in 50 µL PBS).

In a separate experiment, the different groups of mice received a combination of OVA, CRA and HDM (20 µg OVA, 20 µg CRA and 20 µg HDM in 50 µL PBS) during the last two airway challenges in week six. I.t. administration of allergens was performed under short-term isoflourane anaesthesia. All allergens were dissolved in PBS. CS exposure started on day 25, one day before the first i.t. challenge, and ended on day 62. Exposure to the main-stream smoke of four cigarettes was performed 5 days a week, early in the morning and on challenge days 6 h after i.t. application. Mice were exposed to either conventional tCS (as described above) or nicotine-free hCS or received nicotine (nicotine hydrogen tartrate salt, Sigma-Aldrich, Steinheim) by oral gavage (15 mg/kg) on 5 days per week once daily. Mice were sacrificed on day 63 (Fig. 1). In a separate experiment, analysis was performed 7 days after the

last allergen airway challenge on day 70 (mice were exposed to cigarette smoke from day 25 till day 69).

Helminth infection combined with cigarette smoke exposure

Mice were infected intraperitoneally with 750 L₃ *Nippostrongylus brasiliensis* larvae in PBS as described previously [30]. After infection on day 0, mice were exposed to tCS of six cigarettes on days 7, 8, 9 and 10. Mice were sacrificed on day 11 (Fig. 8a).

Airway hyperresponsiveness measurements

AHR as a reaction to methacholine (MCh) challenge was assessed by invasive measurement of resistance and compliance as described before [31]. For the measurements, mice were exposed to MCh in increasing concentrations.

Bronchoalveolar lavage and differentiation of cell types

24 h after the last challenge or CS exposure, mice were euthanized by i.p. application with an overdose of pentobarbital. The trachea was cannulised and a bronchoalveolar lavage (BAL) was performed and the amounts of the different cell types present determined as described previously [28].

Cytokine measurements in BAL fluid and lung homogenate

Cytokines were measured in BAL supernatants using a mouse TH1/TH2 9-Plex cytokine/chemokine multiplex assay (Meso Scale Discovery, Gaithersburg, MD, USA) according to manufacturer's instructions.

Measurement of total and allergen-specific IgE and IgA

Levels of total IgE were measured in serum using a standard enzyme-linked immunosorbent assay (ELISA) Kit from BD Bioscience (BD Biosciences, Erembodegem, Belgium) according to the manufacturer's protocol. Levels of allergen-specific IgE were measured in serial dilutions of serum samples, BAL or lung homogenate with an ELISA technique on an MSD platform using rat anti-mouse IgE (BD Biosciences) and MSD sulfo-tag-labelled goat anti-rat antibody (Meso Scale Discovery) as described before [28]. Allergen-specific IgA measurements were performed in serial dilutions of lung homogenate with an ELISA technique. Plates were coated with 100 µL allergen solution per well (20 µg/mL OVA or HDM or CRA) at 4 °C overnight. The next day, standard and homogenate samples were added and

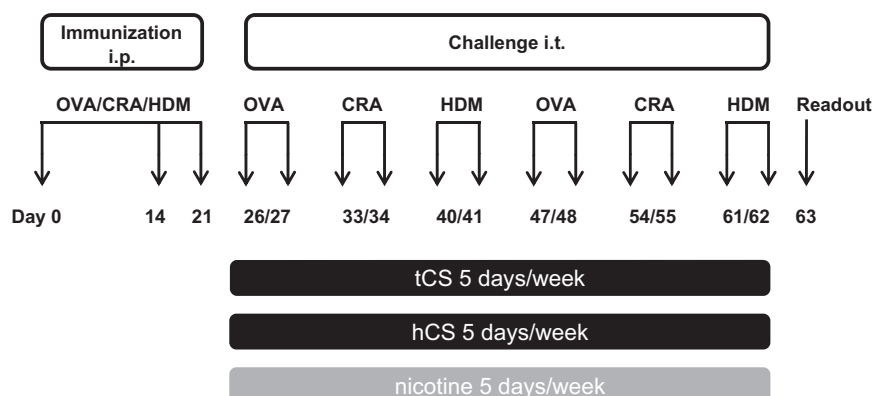


Fig. 1. Treatment protocol. Mice were sensitized intraperitoneally on days 0, 14 and 21 with a mixture of ovalbumin (OVA), cockroach antigen (CRA), house dust mite antigen (HDM) and ALUM. Mice were then challenged i.t. over 6 weeks with one allergen on two consecutive days per week alternating the allergens. The negative controls were sham treated with PBS. Separate groups of mice were simultaneously exposed to conventional tobacco cigarette smoke (tCS), nicotine-free herbal cigarette smoke (hCS) or nicotine for 5 days per week in addition to allergen exposure.

incubated at 4 °C overnight. 24 h later, 50 µL biotinylated goat anti-mouse IgA (Bio-Rad AbD Serotec GmbH, Puchheim, Germany) was added and incubated for 2 h at room temperature. Afterwards, 50 µL streptavidin-peroxidase was added per well and incubated for 30 min in the dark. In a last step, 50 µL OPD was added per well. After 10 min, 50 µL stopping solution was added and plates were measured at 450 nm.

Collagen I measurements

Collagen levels were assessed in supernatant of lung homogenate using the mouse type I collagen detection kit (Chondrex, Redmond, WA, USA) according to manufacturer's instructions and as described previously [28].

Antigen re-stimulation from spleen cells and Th2 cytokine measurement

Spleen cells were isolated by squeezing the organs through a 70-µm cell strainer. Remaining erythrocytes were removed using ACK lysing buffer (Lonza, Basel, Switzerland), and tissue debris was removed by filtering samples through a 40-µm cell strainer. After centrifugation for 5 min at 1000 rpm, the cell pellet was re-suspended in RPMI containing 10% FCS and 1% Pen/Strep (culture medium). Subsequently, 1 million cells/well were seeded in 96-well format.

Cells were cultured in 200 µL culture medium containing 0.02 mg OVA, HDM and CRA allergen. After 48 h, culture plates were centrifuged for 5 min at 1000 rpm. Supernatant was collected and stored at –80 °C for analysis of cytokine release. IL-13 was measured using a mouse IL-13 ultra-sensitive kit (Meso Scale Discovery) according to manufacturer's instructions, while IL-4 and

IL-5 were measured using a V-PLEX pro-inflammatory panel (Meso Scale Discovery).

Histological analysis

Left lungs were fixed in 4% formalin for a minimum of 24 h and embedded in paraffin wax. Slices were taken at the position where the main bronchus enters the lung. Lung sections (2–3 µm) were stained with haematoxylin and eosin (H&E) reagent (Merck, Darmstadt, Germany). To measure smooth muscle thickness, sections were immunostained with an α -smooth muscle actin antibody (Clone 1A4, Dako, Glostrup, Denmark). To detect collagen deposition, a picro-sirius red staining with picric acid (Sigma-Aldrich, Steinheim, Germany) was performed. Mucus production and goblet cell metaplasia was visualized by a periodic acid-Schiff (PAS) staining. Transverse sections of individual mice showing the main bronchus of the left lung lobe were analysed to quantify smooth muscle thickening, epithelial layer thickening and goblet cell metaplasia using Halcon software (MVTec Software GmbH, Munich, Germany) as described previously [28].

Statistical significance

Data were analysed by using the Prism 6 software package (GraphPad Software, San Diego, CA, USA). All data were expressed as mean and standard error of the mean (SEM) and was analysed using one-way ANOVA with a Dunnett post-test. A *P* value of < 0.05 was considered statistically significant. Normalization of allergen-specific IgE levels was calculated as follows: % Change = $(Y/K1) \times 100$. With K1 being the mean value of the allergen group and Y being the value of interest.

Results

Tobacco cigarette smoke exposure reduces Th2 responses in a chronic asthma model in Balb/c mice

We combined a chronic asthma model with daily smoke treatment (see Fig. 1 and methods) in Balb/c mice and investigated the effects of conventional tCS on pulmonary inflammation, AHR and airway remodelling. Figure 2a shows total and differential cell counts in the BAL fluid of the different groups. The highest total cell count, eosinophil, macrophage and lymphocyte numbers were detected in the allergen group. In the group treated with allergen + tCS additionally, total cell, eosinophil, lymphocyte and macrophage numbers were significantly lower compared to the allergen-only treated group. Neutrophil numbers were highest in the allergen + tCS group. Figure 2b and c shows the concentrations of the Th2 cytokines IL-4 and IL-5 measured in BAL. Consistent with eosinophil counts, IL-4 and IL-5 concentrations were most prominently increased in the allergen group. Significantly, lower values for both cytokines were detected in the allergen + tCS group. PBS and tCS group samples were below the detection limit for both cytokines. Similar results were found for IL-10 (Table 1). IL-12 was highest in the tCS group, while IL-1 β , KC and TNF- α were highest in the allergen + tCS group (Table 1). Furthermore, tCS also strongly reduced all measured Th2 response parameters, AHR and collagen I deposition when the mice were analysed 1 week after the last HDM challenge on day 70 and exposed to conventional tCS up to day 69 (Figure S1). We also repeated the experiment where the last two challenges were performed with a mixture of all three allergens instead of HDM only. We found similar suppressive effects of tCS on the asthmatic phenotype irrespective if the mice were challenged with HDM or all three allergens (Figure S2).

Airway hyperreactivity and allergen-induced IgE is suppressed by tobacco cigarette smoke

Figure 2d shows resistance (RI) values [cm H₂O/mL/s] and Fig. 2e compliance (Cdyn) values [mL/cm H₂O] after PBS administration followed by 0.625, 2.5, 5.0 and 12.5 mg/mL doses of MCh in the different treatment groups. Baseline RI values and values after MCh challenge were highest in the allergen group. PBS, tCS and allergen + tCS groups were all significantly lower than the allergen group, with the tCS group being least responsive to rising MCh doses. The allergen-only treated group also showed a significant reduction in compliance compared to the PBS and tCS only treated animals. Additional tCS exposure attenuated the reduction in compliance observed in the allergen group. Total

IgE levels were significantly elevated in the serum of mice treated with allergen compared to PBS or tCS only treated animals and tCS exposure significantly reduced total and allergen-specific IgE levels in the allergen treated animals in serum samples (Fig. 2f–i), and also in BAL fluid and lung homogenate (Table 2).

Production of IL-4, IL-5 and IL-13 by spleen cells from mice treated with allergen or allergen and tCS after in vitro re-stimulation with allergen

Figure 5j shows that spleen cells from mice of the allergen group re-stimulated with a mixture of OVA/CRA/HDM secreted IL-4, IL-5 and IL-13 *in vitro*. Spleen cells from mice of the allergen + tCS group produced significantly lower amounts of IL-5 and lower amounts of IL-4 and IL-13 in comparison with cultures obtained from the allergen group after allergen-re-stimulation. No IL-4, IL-5 or IL-13 could be detected in the spleen cell cultures from control mice after allergen re-stimulation. When the spleen cells from the different groups of mice were only cultured in medium, without allergens, no IL-4, IL-5 or IL-13 could be detected (data not shown).

Effects of cigarette smoke on airway remodelling

Staining of lungs sections from mice treated with allergen + tCS revealed a much weaker cell infiltration and inflammation compared to the allergen-only treated group (Fig. 3a). However, additional tCS exposure did not reduce the significant increase in smooth muscle and epithelial layer thickness and goblet cell metaplasia observed around the main bronchus of the allergen-only treated animals compared to the PBS controls (Fig. 3a–c and data not shown). However, tCS treatment significantly reduced the collagen I levels detected in lung homogenates (Fig. 3d). Lower collagen deposition could also be observed in sections of the lung stained with Picro Sirius Red (Fig. 3a).

Cigarette smoke mediated suppressive effects on allergic Th2 responses are not dependent on TLR4 signalling

To investigate whether the observed suppressive effects of tCS on Th2 responses were dependent on TLR4, we repeated the above-described experiments in C3H/HeJ mice which lack TLR4 signalling (Fig. 4) and in wild-type control animals (C3H/HeOuJ mice) without TLR4 deficiency (Figure S3). In C3H/HeJ mice, the suppressive effects of tCS on allergen-induced readouts, including eosinophil levels in the lung (Fig. 4a), IL-4, IL-5 (Fig. 4b and c), AHR (Fig. 4d and e), IL-10 and IL-12 concentrations in BAL (Table S1) as well as total and allergen-specific serum IgE levels (Fig. 4f–i), were

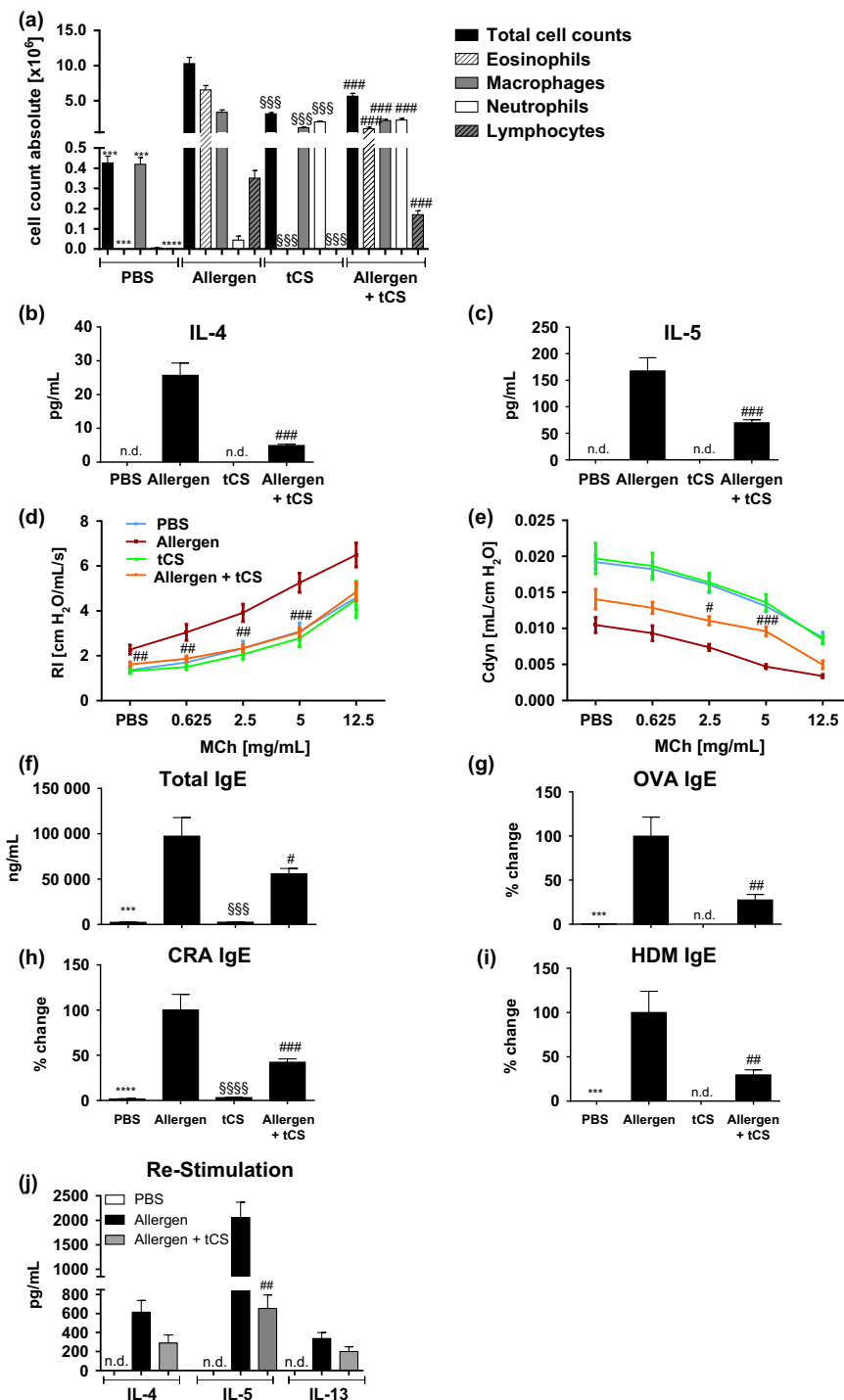


Fig. 2. Tobacco cigarette exposure reduces allergen-induced Th2 responses and airway hyperreactivity. Balb/c mice were treated with tCS and allergen as described in Fig. 1. (a) Total cell, eosinophil, macrophage neutrophil and lymphocyte numbers in the BAL fluid of the different groups of mice. IL-4 (b) and IL-5 (c) levels in the BAL fluid. (d and e) The different groups of mice were challenged with increasing doses of methacholine and resistance and compliance measurements are shown. Levels of total IgE (f) and specific for the allergens OVA (g), CRA (h) and HDM (i) in the serum of the different groups of mice. (j) Spleen cells of the different groups of mice were re-stimulated with OVA, CRA and HDM *in-vitro* and the cytokines IL-4, IL-5 and IL-13 were measured in the supernatant. Results represent mean $\pm$ SEM ($n = 8-12$ per group). Significant changes between PBS group and allergen group: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$; between tCS group and allergen group: § $P < 0.05$, §§ $P < 0.01$, §§§ $P < 0.001$, §§§§ $P < 0.0001$; between allergen + tCS group and allergen group: # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$, #### $P < 0.0001$. Allergen-specific IgE data is normalized to the mean of the allergen group. The experiment was repeated once with similar results.

Table 1. Cytokine levels in the lung of Balb/c mice treated with allergen, tCS and allergen + tCS. Different groups of mice were treated as described in Fig. 1. Cytokines were measured in the BAL fluid using mouse TH1/TH2 9-Plex cytokine/chemokine multiplex assay. All data are presented as mean $\pm$ SEM ($n = 8-12$ /group)

Cytokines (BAL) pg/mL	PBS	Allergen	tCS	Allergen + tCS
IL-10	1.91 $\pm$ 0.81***	93.41 $\pm$ 11.36	3.24 $\pm$ 1.26***	12.83 $\pm$ 1.77***
IL-12	25.34 $\pm$ 2.86***	296.24 $\pm$ 37.08	364.78 $\pm$ 12.46 n.s.	211.45 $\pm$ 13.18*
IL-1 β	2.18 $\pm$ 0.38 n.s.	6.55 $\pm$ 1.35	5.36 $\pm$ 0.69 n.s.	19.67 $\pm$ 1.68***
KC	44.64 $\pm$ 4.25***	164.09 $\pm$ 16.48	127.43 $\pm$ 11.91 n.s.	191.05 $\pm$ 9.53 n.s.

Significant changes compared to the allergen group: * $P < 0.05$, *** $P < 0.001$. The experiment was repeated once with similar results.

Table 2. Allergen-specific IgE levels in BAL supernatant and lung homogenate of Balb/c mice treated with allergen, tCS and allergen + tCS. Different groups of mice were treated as described in Fig. 1. IgE concentrations were measured via ELISA technique on a MSD platform as described in material and methods. Results represent mean $\pm$ SEM ($n = 8-10$ per group)

IgE % change		PBS	Allergen	tCS	Allergen + tCS
BAL	OVA	0.58 $\pm$ 0.26**	100.00 $\pm$ 31.51	1.0 $\pm$ 0.26***	15.7 $\pm$ 4.18**
	CRA	0.12 $\pm$ 0.02***	100.00 $\pm$ 36.97	0.05 $\pm$ 0.01***	9.39 $\pm$ 2.37***
	HDM	0.43 $\pm$ 0.09*	100.00 $\pm$ 40.77	0.48 $\pm$ 0.08**	14.21 $\pm$ 6.27*
Homogenate	OVA	15.95 $\pm$ 0.75****	100.00 $\pm$ 12.68	16.16 $\pm$ 0.44****	35.45 $\pm$ 3.95****
	CRA	4.62 $\pm$ 0.22****	100.00 $\pm$ 12.47	4.97 $\pm$ 0.17****	35.31 $\pm$ 6.37****
	HDM	4.45 $\pm$ 0.20***	100.00 $\pm$ 26.44	6.26 $\pm$ 0.29***	36.07 $\pm$ 13.10*

Significant changes compared to the allergen group: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

found to be equivalent to the results obtained in Balb/c mice (see Figs 2 and 3) and C3H/HeOuJ mice (Figure S3). However, surprisingly and in contrast to Balb/c mice and wild-type control mice (C3H/HeOuJ), no significant difference between the allergen and the allergen + tCS group was observed with respect to collagen I levels, suggesting that this effect was dependent on TLR4 (Figure S4).

Effects of nicotine-free herbal cigarette smoke and oral nicotine administration on allergen-induced inflammation in mice chronically treated with allergens

Publications have shown, that nicotine, an active ingredient of tobacco smoke, has anti-inflammatory properties both *in vivo* and *in vitro* [32–34]. To investigate whether the tCS mediated suppression of allergen-induced Th2 responses is solely mediated by nicotine, we performed the following experiment. Balb/c mice subjected to allergen sensitization and challenge protocol were additionally exposed to nicotine-free herbal smoke (hCS) or 15 mg/kg nicotine hydrogen tartrate salt orally 5 days a week, a dose shown to have anti-inflammatory properties and to be tolerated *in vivo* [35, 36]. Similar to the results obtained in the experiments performed with conventional tCS in Balb/c mice (see Figs 2 and 3), total eosinophils and macrophage numbers as well as IL-4 and IL-5 concentrations were significantly lower in the allergen + hCS group compared to the allergen-only treated group (Fig. 5a–f). In contrast to the data obtained with tCS, neutrophil

numbers were also reduced in the allergen + hCS treated group compared to the allergen treated group (see Fig. 2). A significantly lower cell influx of eosinophils and lower IL-4 and IL-5 concentrations in the BAL was also seen in the allergen + nicotine group compared to the allergen group (Fig. 5a–f). Allergen-specific IgE was reduced by hCS but not by nicotine with the exception of CRA-specific IgE levels (Fig. 5g–j). The development of AHR was also significantly reduced by hCS but not nicotine in the allergen-treated mice (Fig. 6a and b). Nicotine or hCS had no effect on the increase in goblet cell numbers (data not shown), epithelial layer or collagen I formation observed in the allergen treated mice (Fig. 7b and c). Nicotine but not hCS significantly reduced the increase in smooth muscle thickness (Fig. 7a).

Interestingly, KC levels in the allergen + hCS group were significantly lower compared to the allergen + tCS group (36.3 ± 1.09 vs. 191 ± 9.53 pg/mL **** (mean of 8–12 mice/group, **** $P < 0.0001$). IL-1 β could not be detected in the BAL in any of the different groups of mice. However, we detected IL-1 β in lung homogenate and found in the allergen + hCS group significantly tenfold lower amounts of IL-1 β compared to the allergen + tCS group (181 ± 27.74 pg/mL vs. 1922 ± 228 **** (mean of 8–12 mice/group, **** $P < 0.0001$). These much lower KC and IL-1 β values correlate with much lower numbers of neutrophils in BAL fluid of both hCS-only treated mice and allergen + hCS-treated mice in comparison with tCS-treated mice and allergen + tCS-treated animals (Figs 2a and 5c).

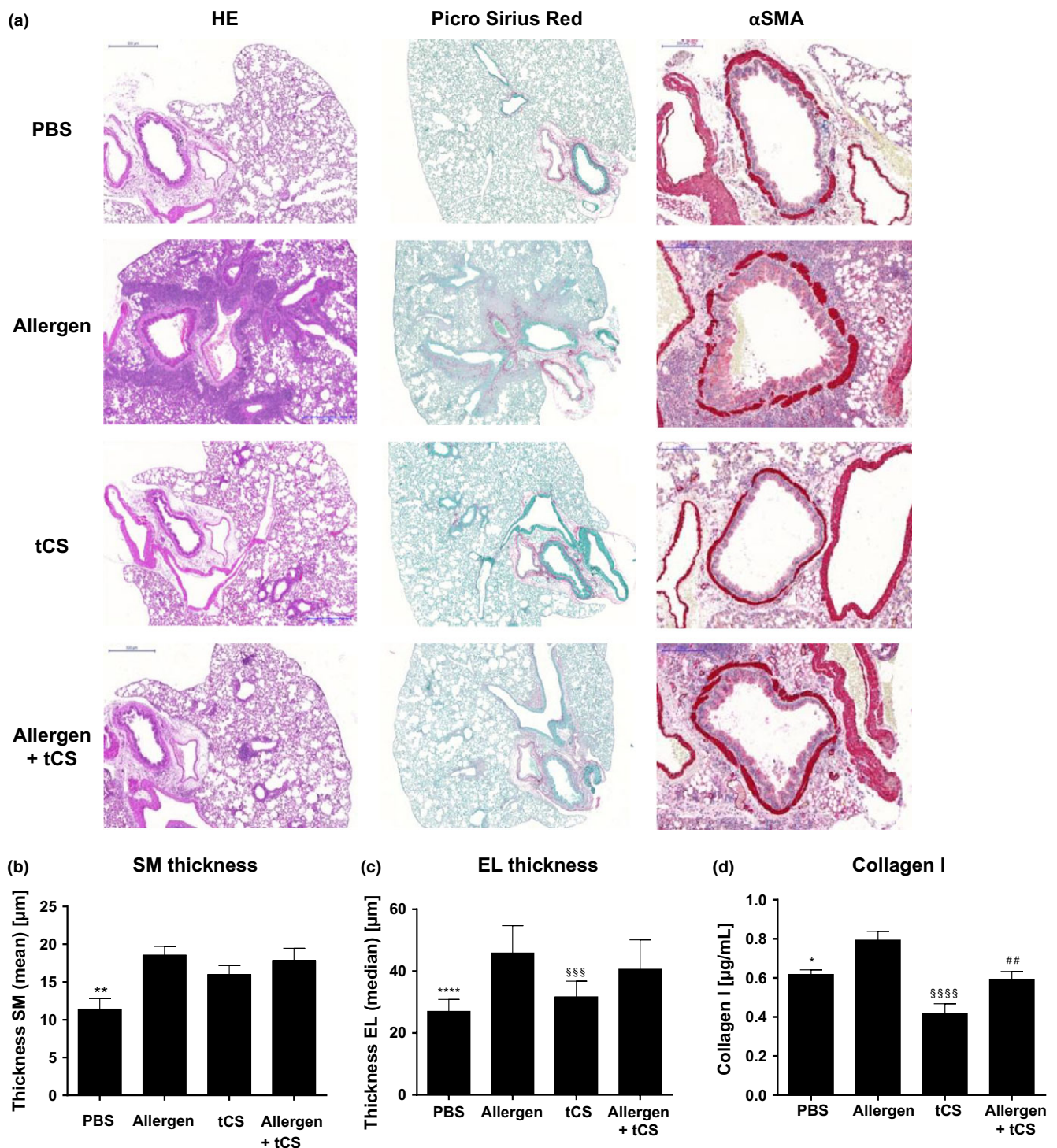


Fig. 3. Effects of tobacco cigarette smoke on allergen-induced remodelling: Balb/c mice were treated with tCS and allergen as described in Fig. 1. (a) H&E, Picro Sirius red and α -smooth muscle actin staining (SMA) stain of sections from the lung of PBS, allergen, tCS and allergen + tCS-treated mice. Shown are representative sections of 8–12 mice/group. Smooth muscle thickness (b) epithelial layer thickness (c) and collagen I in lung homogenates (d) are shown. Results represent mean $\pm$ SEM (8–12 mice/group). Significant changes between PBS group and allergen group: * P < 0.05, ** P < 0.01, **** P < 0.0001; between tCS group and allergen group: \$\$\$ P < 0.01, SSSS P < 0.0001; between the allergen + tCS group compared to allergen group: ## P < 0.01. The experiment was repeated once with similar results.

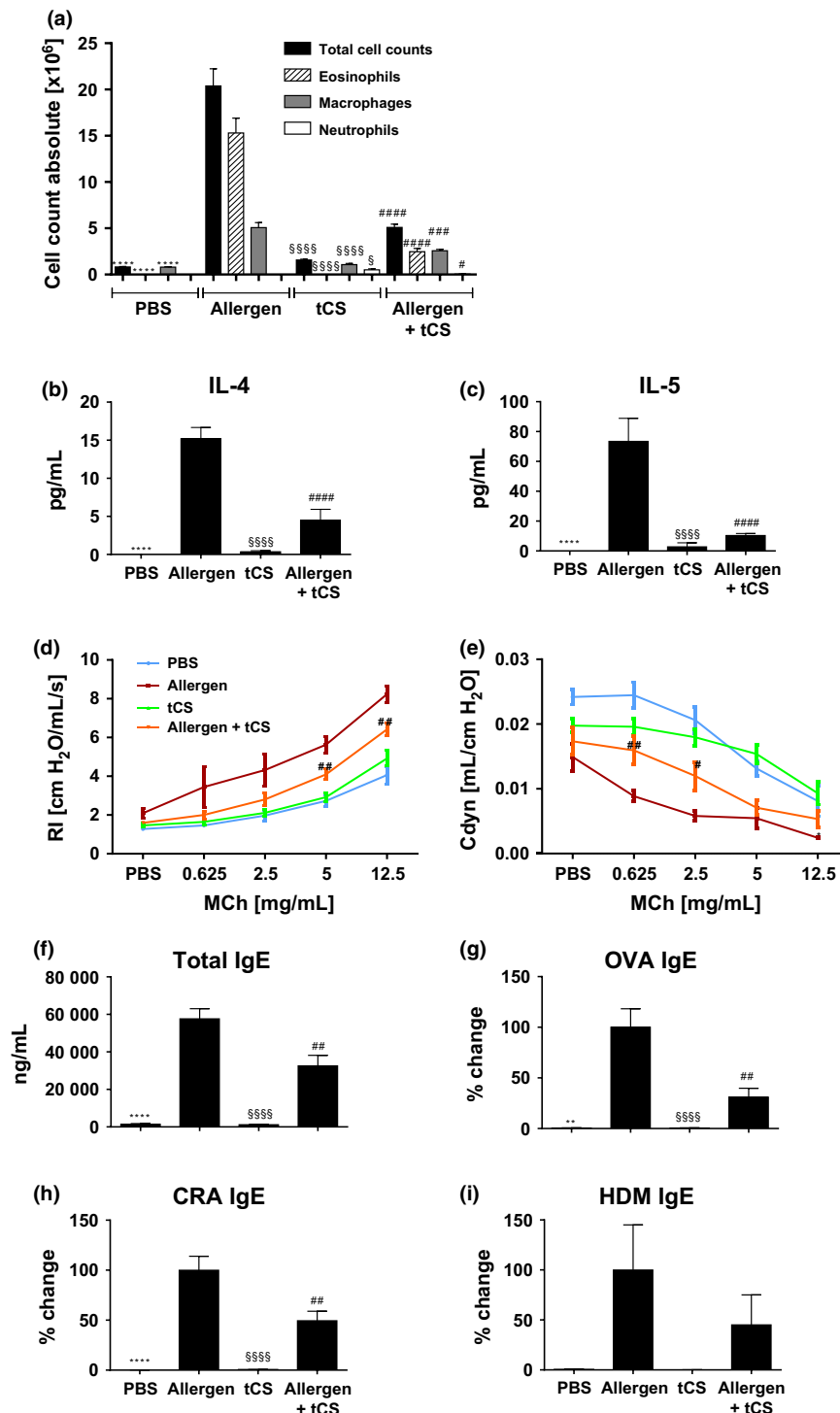


Fig. 4. Reduction of Th2 responses by tobacco cigarette smoke is not dependent on TLR4: C3H/HeJ mice (TLR4 deficient) were treated with tCS and allergen as described in Fig. 1. (a) Total cell, eosinophil, macrophage and neutrophil numbers in the BAL fluid of the different groups of mice. IL-4 (b) and IL5 (c) levels in the BAL fluid. (d and e) The different groups of mice were challenged with increasing doses of methacholine and resistance and compliance measurements are shown. Total IgE (f) and specific for the allergens OVA (g), CRA (h) and HDM (i) in the serum of the different groups of mice. All graphs are presented as mean $\pm$ SEM ($n = 8-12$ mice/group). Significant changes between PBS group and allergen group: $**P < 0.01$, $****P < 0.0001$; between the tCS group and the allergen group: $\$P < 0.05$, $****P < 0.0001$; between the allergen + tCS group and allergen group: $\#P < 0.05$, $\#P < 0.01$, $###P < 0.001$, $####P < 0.0001$. Allergen-specific IgE data are normalized to the mean of the allergen group. The experiment was repeated in wild-type control mice (C3H/HeOuJ) with the same results.

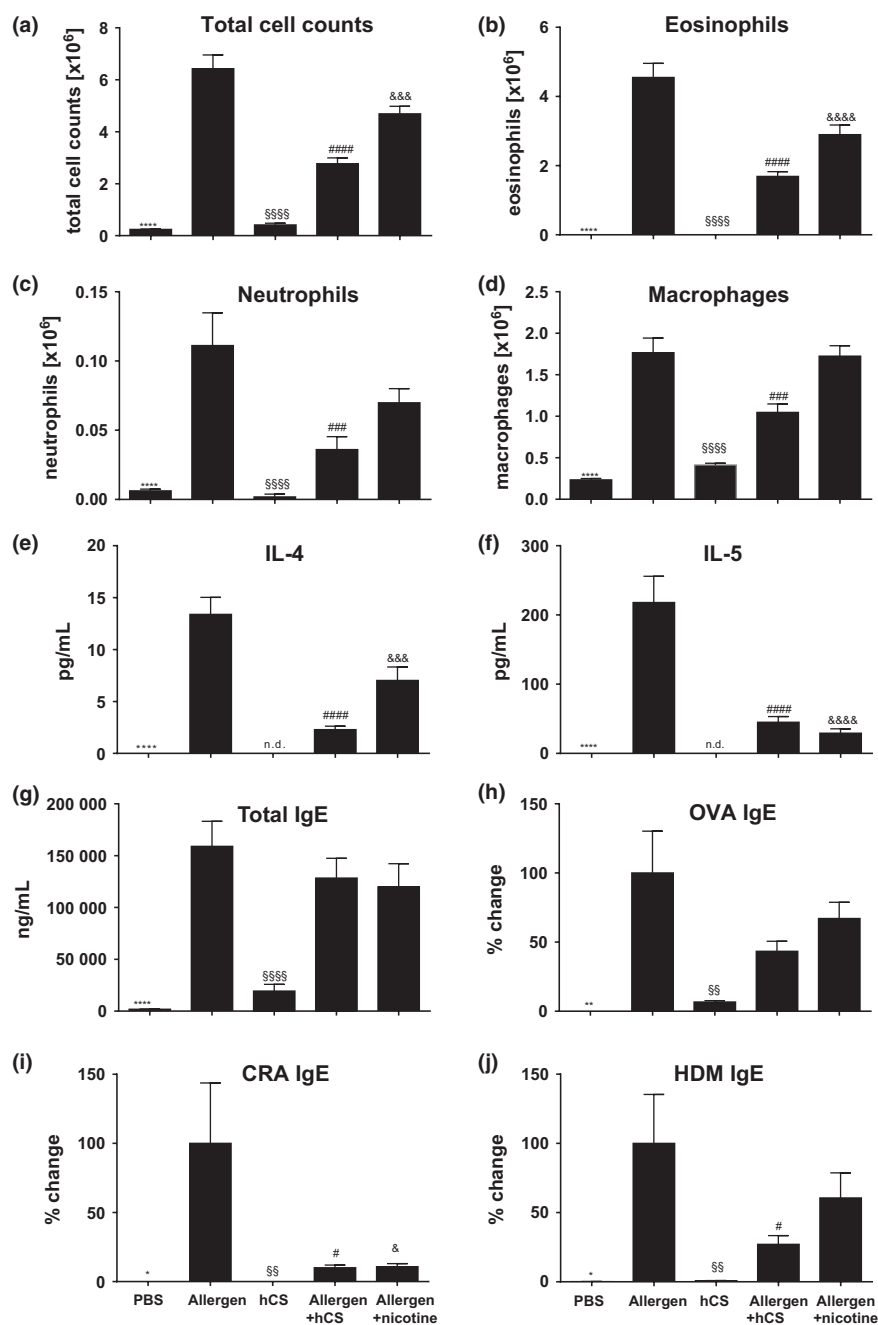


Fig. 5. Nicotine-free herbal cigarette smoke (hCS) and nicotine reduce allergen-induced Th2 responses in the lung: Balb/c mice were treated with hCS or nicotine and allergen as described in Fig. 1. (a) Total cell, (b) eosinophil, (c) neutrophil and (d) macrophage numbers in the BAL fluid of the different groups of mice are shown. IL-4 (e) and IL5 (f) levels in the BAL fluid and total IgE (g) and specific for OVA (h), CRA (i), and HDM (j) in the serum of the different groups of mice. All graphs are presented as mean $\pm$ SEM ($n = 8$ –12 mice/group). Significant changes between PBS group and allergen group: * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$; between the hCS group and the allergen group: §§ $P < 0.01$, §§§ $P < 0.0001$; between the allergen + hCS group and the allergen group: # $P < 0.05$, ### $P < 0.001$, #### $P < 0.0001$; between the allergen + nicotine group and the allergen group: & $P < 0.05$, && $P < 0.001$, &&& $P < 0.0001$. Allergen-specific IgE data are normalized to the mean of the allergen group.

Reduction of Th2 responses by cigarette smoke in C57BL/6 mice infected with the helminth *Nippostrongylus brasiliensis*

To determine whether the strong suppressive effects of tCS on Th2 responses can also be found in other

Th2-driven models, we investigated whether tCS exposure also reduced the development of a Th2 response in the lung induced by an infection with *N. brasiliensis*. C57BL/6 mice were infected on day 0 and were exposed to tCS on days 7–10 with the readout on day 11 (Fig. 8a). Eosinophil and macrophage numbers in the

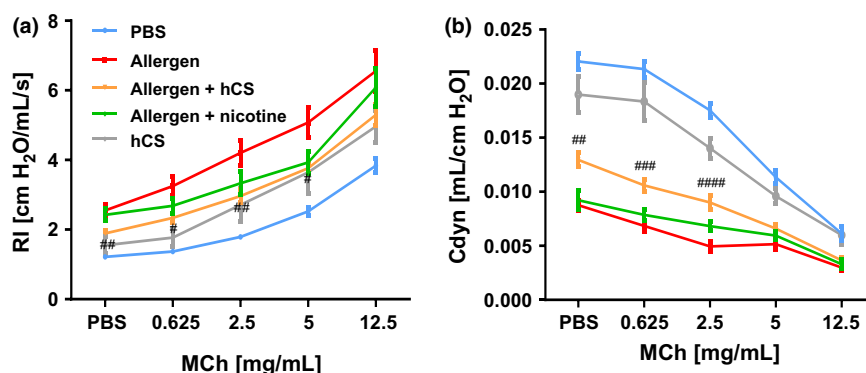


Fig. 6. Effects of nicotine-free herbal cigarette smoke and nicotine on allergen-induced airway hyperreactivity. The different groups of Balb/c mice were challenged with increasing doses of methacholine and resistance (a) and compliance (b) measurements are shown. Results represent mean $\pm$ SEM (8–10 mice/group). Significant changes between the allergen + hCS group compared to allergen group: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, $^{\#\#\#\#}P < 0.0001$.

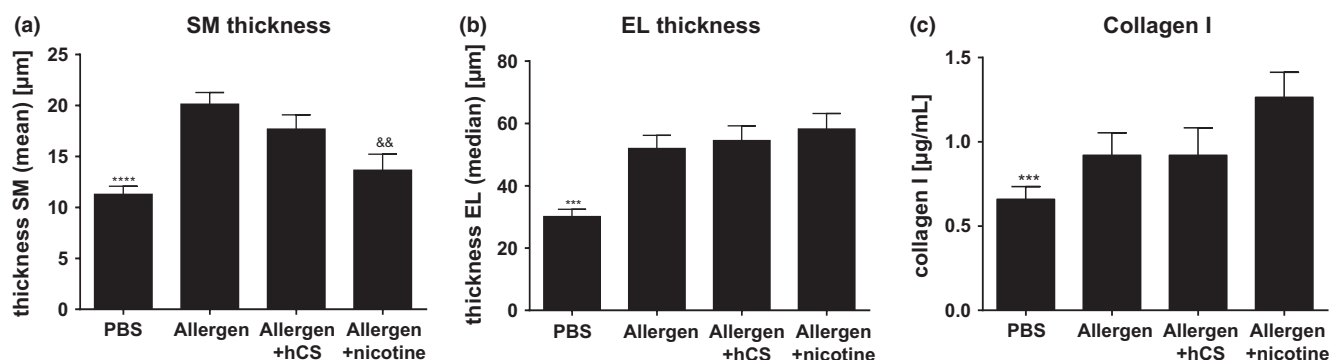


Fig. 7. Effects of nicotine-free herbal cigarette smoke (hCS) and nicotine on allergen-induced increase in smooth muscle and epithelial layer thickness and collagen I levels in the lung. Animals were treated with allergen, hCS and nicotine as described in Fig. 1. Smooth muscle (SM) thickness (a) epithelial layer (EL), thickness (b) and collagen I in lung homogenates (c) are shown. Results represent mean $\pm$ SEM (8–10 mice/group). Significant changes between PBS group and allergen group: $^{***}P < 0.001$, $^{****}P < 0.0001$; between allergen + nicotine and the allergen group: $^{\&\&}P < 0.01$.

BAL were highest in the helminths infected mice compared to PBS group and tCS-only treated animals. Mice infected with helminths and exposed to tCS had significantly lower numbers of eosinophils in the BAL compared to the helminths only infected mice. Neutrophils were highest in the tCS group and again significantly lower in the helminths + tCS group (Fig. 8b). IL-4 and IL-5 levels in BAL supernatant were also highest in the helminths group, and levels were significantly lower in the helminths + tCS group (Fig. 8d and e).

Discussion

The vast majority of available clinical data investigating the association of tCS on asthma suggest that active or passive tCS exposure is a major risk factor for developing asthma and worsens asthma symptoms [6–8, 10, 37–40]. Furthermore, there is no doubt that smoking asthmatics dramatically increase their risk of developing

other serious health conditions compared to non-smoking asthmatics. In mice, some studies show that tCS exposure increases allergic responses [15–19, 41] but other studies demonstrate anti-asthmatic effects [20–24]. However, little is known about tCS effects in severe murine models reflecting hallmarks of severe asthma. Thus, the aim of our study was to investigate the effects of tCS on the development of a severe asthma phenotype in mice using a recently developed novel model. In this developed novel animal model, mice are immunized with a mixture of OVA, CRA and HDM and challenged over 6 weeks with the allergens, alternating between them, starting with an OVA provocation in week one and ending with a HDM provocation in week six. This allergen-combination protocol induces a stronger asthma phenotype compared to a similar protocol where only one or the other allergen is used [28]. Furthermore, the asthmatic phenotype was less severe when a combination of all three allergens was used during the

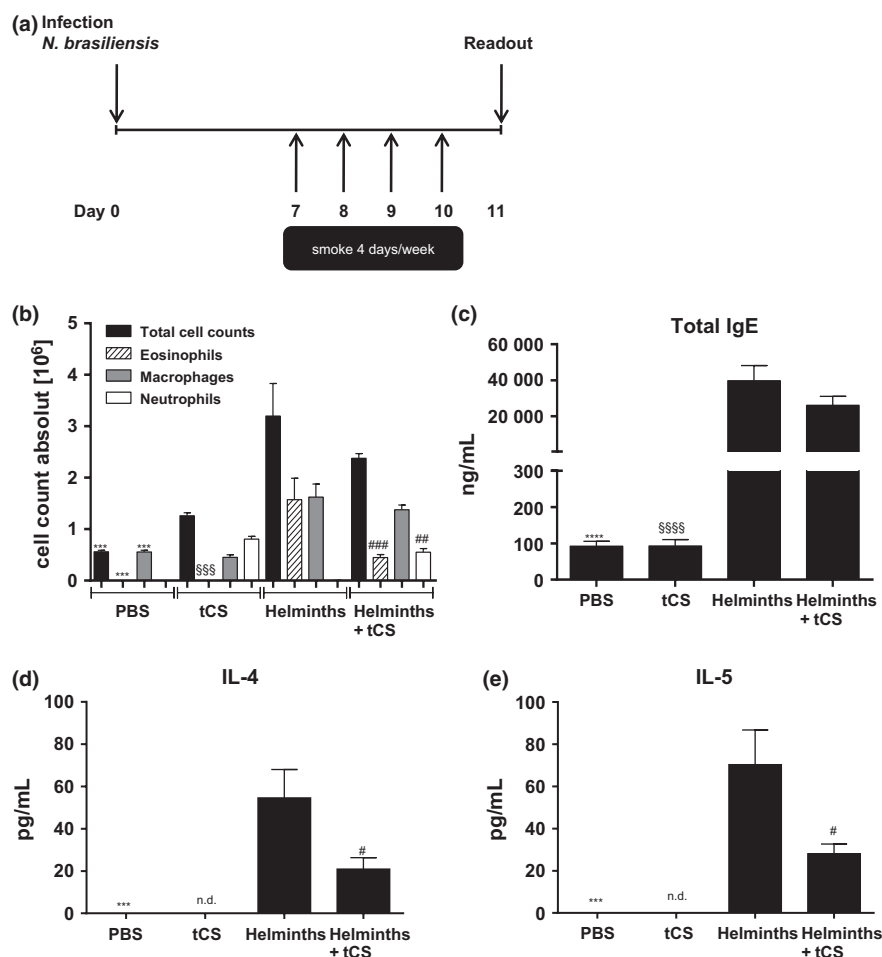


Fig. 8. Exposure to tobacco cigarette smoke reduces helminth infection-induced Th2 response in the lung. (a) C57BL/6 mice were either treated with PBS, infected intraperitoneally on day 0 with 750 *N. brasiliensis* larvae (helminths), exposed to tCS on days 7 to 10 or infected with *N. brasiliensis* and exposed to tCS (helminths + tCS). Mice were sacrificed and analysed on day 11. (b) Total cell, eosinophil, macrophage and neutrophil numbers in the BAL fluid of the different groups of mice. (c) Levels of total IgE in the serum and IL-4 (d) and IL-5 (e) levels in the BAL fluid in the different groups of mice. Results are presented as mean $\pm$ SEM ($n = 8$ per group). Significant changes between PBS group and helminth-infected group: *** $P < 0.001$, **** $P < 0.0001$; between the tCS group and the helminth-infected group: \$\$\$ $P < 0.001$, \$\$\$\$ $P < 0.0001$; between helminth + tCS group and the helminth-infected group: # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$.

challenge period compared to alternating between the allergens (data not shown). We expected to detect a more severe phenotype in mice treated with allergen and exposed to tCS compared to the mice only treated with allergen according to the human situation. Surprisingly, we found that tCS strongly reduced all measured Th2 response parameters in the lung, AHR and collagen I deposition measured 24 h after the last allergen challenge. Interestingly, allergen-specific IgE levels were significantly reduced in the airways but also in the serum of the allergen + tCS-treated mice compared to the allergen-only treated group. In addition, spleen cells from mice of the allergen + tCS group produced significantly lower amounts of IL-5 and lower amounts of IL-4 and IL-13 in comparison with cultures obtained from the allergen group after allergen re-stimulation.

Together, this suggests that tCS reduces Th2 responses not only in the lung but also systemically.

In the protocol, we used we alternated the allergens finishing with a HDM challenge. To rule out that the observed suppressive effects of tCS were only limited to HDM induced inflammation, we repeated the experiment where the last two challenges were performed with a mixture of all three allergens instead of HDM only. We found similar suppressive effects of tCS on the asthmatic phenotype, indicating that tCS has suppressive effects also on inflammation induced by the challenge with all three allergens (Figure S2). In addition, we also found that tCS also strongly reduced all measured Th2 response parameters, AHR and collagen I deposition when the mice were analysed 1 week instead of 1 day after the last HDM challenge on day 70 and

were exposed to conventional tCS upto day 69 (Figure S1).

Our findings are in direct contrast to numerous publications showing enhanced Th2 inflammatory responses when allergen exposure and tCS are combined [15–19, 41]. It is possible that this is due to the different protocols, cigarettes (Roth-Händle without filter vs. Kentucky reference cigarette 2R4F) or smoke exposure chambers used. However, other publications (also using 2R4F reference cigarettes) support our results showing anti Th2 response effects of tCS [20–24, 33, 34].

To shed some light on the mechanism(s) by which tCS mediates these anti-Th2 response effects, we performed additional experiments. Our first hypothesis was that the anti-Th2 inflammatory effects of tCS might be mediated by LPS (through TLR4 mediated signalling) since we and others have previously shown that LPS suppresses allergic inflammation in mice [42–45], and LPS is a component of tobacco smoke [46]. To address this question, we repeated the experiments combining tCS and allergen challenges in TLR4-deficient mice and their wild-type control mice (C3H/HeOuJ). We found that the suppressive effects of tCS on allergen-induced Th2 responses was similar to the effects seen in Balb/c mice and C3H/HeOuJ mice, indicating that these anti-Th2 effects are not mediated by LPS contained in the tCS. The only effect observed in Balb/c mice and C3H/HeOuJ but not in TLR4-deficient mice was a reduction in collagen I levels, suggesting that this effect is dependent on TLR-4.

A further component of tCS known to have anti-inflammatory properties is nicotine [33, 35, 47–51]. In order to investigate the contribution of nicotine to the tCS-induced reduction in Th2 responses, we treated allergen challenged mice with nicotine or exposed them to hCS not containing nicotine. Nicotine significantly lowered cell influx of eosinophils, IL-4 and IL-5 levels in the BAL and smooth muscle mass in the airways. However, no effects of nicotine were observed on AHR, IgE levels or collagen I levels. Comparing the effects of hCS with tCS, we found that neutrophil numbers were much lower in the allergen + hCS-treated groups compared to the allergen + tCS-treated groups, indicating that the smoke of the tobacco cigarettes has a stronger inflammatory effect than the smoke of the herbal cigarettes we used. Nicotine in the tCS may be responsible for the increase in neutrophil numbers since it was shown that nicotine delays cell death of neutrophils [52] and increases their chemotactic activity [53]. Nevertheless, nicotine-free hCS exposure also reduced all measured Th2 parameters (except IgE in serum) and AHR in allergen-treated mice. The only remodelling parameter reduced by tCS but not hCS was collagen I deposition in the

airways of allergen + smoke-treated mice. Taken together, the data suggests that tCS-mediated suppression of Th2 responses is mediated partly by nicotine but also additional substances/particles released by burning tobacco contribute to the anti-Th2 effect. Tobacco smoke contains a mixture of more than 4000 components, making it difficult to assess which ones are contributing to the anti-Th2 effects [37, 54, 55]. One component which may play a role is carbon monoxide (CO). CO has anti-inflammatory properties including reducing allergen-induced AHR [24, 56–59] and is released after burning tobacco [60].

Further mechanism of how tCS could suppress allergic responses in the airways is that tCS may directly act as a barrier for allergen contact in the airways, as suggested by Hjern et al. [3]. Another possible mechanism for the anti-Th2 response properties of tCS is the induction of Prostaglandin E₂ (PGE₂). CS has been shown to enhance PGE₂ levels in airway macrophages [61] and to directly inhibit bronchoconstriction as well as early and late asthmatic responses [61, 62]. *In vitro* studies show that PGE₂ reduces mast cell mediator release and eosinophil activation [61, 63].

A recent publication by Gaschler et al., demonstrated that tCS exposure increased the levels of nontypeable Haemophilus influenza IgA titers in the lung [64]. We also investigated whether tCS increased allergen-specific IgA levels in our model, because IgA binding to the allergen may directly reduce mast cell degranulation via IgE possibly, thereby explaining the reduced Th2 responses. However, we found no increase in allergen-specific IgA in the allergen + smoke-exposed mice compared to the allergen-only exposed mice in lung homogenate (Table S2).

Taken together, our results demonstrate that tCS reduces the allergen-induced Th2 response in the lung and the development of AHR in a severe model of asthma in mice, which is TLR4 independent. Nicotine and hCS also both reduced Th2 responses, hCS stronger than nicotine and albeit both lower compared to tCS. This suggests that tCS reduces the Th2 responses through nicotine and other products released by burning tobacco. The anti-Th2 effects of tCS were also observed when they were induced by an infection with *N. brasiliensis*. However, we believe that this is a phenomenon observed in mice under these experimental conditions and most likely does not reflect the much more complex human situation. Furthermore, it is absolutely clear that generally asthmatics who smoke dramatically increase their risk of developing more severe asthma symptoms and to develop other serious health conditions including stroke, heart failure and different cancers compared to asthmatics who do not smoke.

Conflict of interest

The authors have the following interests. Cornelia Tilp, Hannes Bucher, Matthias J. Duechs, Hannah Haas, Eva Wex and Klaus J. Erb are employees of Boehringer Ingelheim Pharma GmbH & Co. KG which is a research-based pharmaceutical company that discovers, develops, manufactures and markets prescription

medicines for humans and animals. Boehringer Ingelheim Pharma GmbH & Co. KG provided funding for the experiments. There are no patents, products in development or marketed products to declare. This does not alter the authors' adherence to all the Clinical and Experimental Allergy policies on sharing data and materials, as detailed online in the guide for authors.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Tobacco cigarette smoke exposure reduces allergen-induced Th2 responses, airway hyperreactivity and airway remodelling in Balb/c mice 7 days after the allergen challenge. Mice were treated as described in material and methods.

Figure S2. Anti-inflammatory effects of tobacco cigarette smoke on allergic inflammation in mice challenged with a combination of OVA, CRA and HDM.

Figure S3. Tobacco cigarette exposure reduces

allergen-induced Th2 responses, airway hyperreactivity and airway remodelling in C3H/HeO_uJ mice.

Figure S4. Tobacco cigarette smoke does not reduce allergen-induced collagen I increase in the lung of TLR4 deficient mice.

Table S1. Cytokine levels in the lung of TLR4 deficient mice treated with allergen, tCS, and allergen + tCS. Different groups of mice were treated as described in Fig. 1.

Table S2. Allergen-specific IgA levels in lung homogenate of Balb/c mice treated with allergen, tCS, and allergen + tCS. Different groups of mice were treated as described in Fig. 1.