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RESEARCH ARTICLE



Comparison of airway response in naïve and ovalbumin-sensitized mice during short-term inhalation exposure to chlorine

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ABSTRACT

Objective: It has been suggested that asthmatics are more susceptible than healthy individuals to air-borne irritating chemicals in general. However, there is limited human data available to support this hypothesis due to ethical and practical difficulties. We explored a murine model of ovalbumin (OVA)-induced airway inflammation to study susceptibility during acute exposure to chemicals with chlorine as a model substance.

Methods: Naïve and OVA sensitized female BALB/c mice were exposed to chlorine at four different concentrations (0, 5, 30 and 80 ppm) for 15 minutes with online recording of the respiratory function by plethysmography. The specific effects on respiratory mechanics, inflammatory cells and inflammatory mediators (cytokines and chemokines) of the airways were measured 24 hours after the chlorine exposure as well as histopathological examination of the lungs.

Results: Similar concentration-dependent reductions in respiratory frequency were seen in the two groups, with a 50% reduction (RD₅₀) slightly above 5 ppm. Decreased body weight 24 hours after exposure to 80 ppm was also observed in both groups. Naïve, but not OVA-sensitized, mice showed increased bronchial reactivity and higher number of neutrophils in bronchoalveolar lavage fluid at 80 ppm.

Conclusions: The results do not support an increased susceptibility to chlorine among OVA-sensitized mice. This animal model, which represents a phenotype of eosinophilic airway inflammation, seems unsuitable to study susceptibility to inhalation of irritants in relation to asthma.

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susceptibility

Introduction

Asthma is one of the most common chronic immunological diseases in humans and affects individuals of all ages (Fahy, 2015). The World Health Organization (WHO, 2015) and the Global asthma network (2014) report that the incidence of asthma is approximately 235 or 334 million worldwide, depending on the inclusion criteria that are being used in the surveys. Asthma is a heterogeneous disease, usually characterized by chronic airway inflammation. It is generally defined by the history of respiratory symptoms such as wheeze, shortness of breath, chest tightness and cough that vary over time and in intensity, together with variable expiratory airflow limitation (GINA, 2016). The underlying cellular and biochemical mechanisms for these symptoms are not well understood and very hard to investigate in humans, due to ethical reasons. One way to approach this is to use animal models. Although the results from animal models cannot easily be translated to humans, they may gain knowledge about gene involvement, the pathogenesis of asthma and possible therapeutic approaches (Shin et al., 2009).

We have previously shown that there is a general lack of data on asthmatics and that the few human studies that are available are frequently omitted in health risk assessment of airborne irritant chemicals (Johansson et al., 2012).

Nevertheless, the available experimental data on humans show that persons with asthma are often more sensitive to airborne irritants and that the mechanistic understanding of individual susceptibility is to a large extent absent (Johansson et al., 2016). Animal asthma models might therefore be useful to address the issue of individual susceptibility in risk assessment.

An animal model that reflects susceptible individuals would enable the use of higher concentrations and more invasive methods to detect effects from exposure to airborne irritants. The mouse is one species, among others, that can be used in experimental models of allergic airway disease (Patel & Chorawala, 2011). The mouse has several advantages, e.g. a well characterized genome and immune system, availability of inbred and transgenic strains, short breeding periods and relatively low maintenance costs (Glaab et al., 2007). Although mice do not become asthmatic as humans, although it is possible to induce a systemic CD4 (T_H2) immune response and eosinophilic airway inflammation experimentally in mice of the BALB/c strain. This strain has increased sensitivity to ovalbumin (OVA) which initiates an immune activation by repeated intra-peritoneal (i.p.) injections and an eosinophilic airway inflammation following repeated allergen aerosol exposures.

In this study, we chose chlorine as a model compound for airborne irritants as it is well-known for its effects manifested as changes in e.g. respiratory frequency, respiratory mechanics and inflammatory markers. Chlorine is a highly irritating, high-volume chemical used in various industrial processes and applications of chemical products. Accidents during transportation and use of chlorine are frequently occurring events (Van Sickle et al., 2009). Chlorine exposure has also been observed as a course of deliberate actions during terrorist attacks or war (Russell et al., 2006). Chlorine is a reactive oxidant gas which is highly soluble in water (Yadav et al., 2010). When inhaled, chlorine dissolves in the aqueous mucus layer that covers the epithelial cells in the trachea and bronchi-alveolar region. The chlorine is hydrolyzed to hypochlorous acid and hydrochloric acid ($\text{Cl}_2 + \text{H}_2\text{O} \leftrightarrow \text{HCl} + \text{HClO}$, $2\text{HClO} \leftrightarrow 2\text{HCl} + \text{O}_2$) ($\text{pK}_a = 7.5$), which both react with biomolecules and tissues in the airways to form reactive oxygen species (White & Martin, 2010; Yadav et al., 2010). Given its high water solubility, chlorine is anticipated to hydrolyze and react primarily in the upper respiratory tract, only at high concentrations the inhaled chlorine penetrates to distal lung regions (Yadav et al., 2010).

Sensory nerves are extensively innervated in both human and rodent airways (Nielsen, 1991; Nielsen et al., 2007). A stimulation of nasal trigeminal sensory nerves initiates a sensory irritation response in rodents. This is characterized by decreased respiratory frequency due to a prolonged pause at the beginning of the expiration (Nielsen, 1991; Vijayaraghavan et al., 1993). In animal models, a decrease in respiratory frequency, so-called respiratory depression, following chemical exposure is considered to be a sign of sensory irritation in the airways. The concentration of a chemical irritant that induces 50% depression in respiratory frequency, the RD_{50} , has been used for categorization of irritant chemicals and as a tool in the derivation of guideline values and occupational exposure limits (Bessac & Jordt, 2008).

One human study has previously been performed to investigate the individual susceptibility following exposure to airborne irritant chemicals, like chlorine. D'Alessandro et al. (1996) used a human exposure chamber to study chlorine exposure in healthy and asthmatic subjects for 60 minutes using three different concentrations of chlorine (0, 0.4 and 1 ppm). Symptoms and significant changes in specific airway resistance were seen following 0.4 ppm chlorine in asthmatic but not in healthy subjects, suggesting a higher sensitivity among asthmatics. Significant changes in respiratory effects were seen in both groups following exposure to 1 ppm chlorine. This study was previously identified as a key study for risk assessment of chlorine (Johansson et al., 2016).

Inhalation studies with chlorine have been performed previously using naïve (Jonasson et al., 2013; McGovern et al., 2010) and OVA-sensitized BALB/c mice (Hansen et al., 2016; Hox et al., 2011). The BALB/c represents a genome-wide characterized strain and the functional OVA-sensitization is verified as a part of the present study. In addition, several experiments with chlorine have been performed using naïve mice from other strains; male A/J

(Tuck et al., 2008), female C57Bl/6J (Morris et al., 2005), male Swiss Webster (Barrow et al., 1977), male OF1 (Gagnaire et al., 1994) and FVB/N (gender not stated) (Chang et al., 2012; Hoyle et al., 2010). To our knowledge, there are no animal studies that combine an OVA-induced airway inflammation model with acute exposure to chlorine. Additionally, no experiments have been reported with comparative analyses or with the combination of head-out plethysmography and methacholine provocation using both naïve and OVA-sensitized mice. The aim of this study was to explore the use of OVA-sensitized mice as a model to study exposure to irritant chemicals in relation to asthma.

Materials and methods

Animals

Fifty-six female BALB/c OlaHsd mice (age 9–10 weeks) (Envigo, Horst, Netherlands) were housed in plastic cages with bedding and maintained in a 12-hour daylight cycle. Animals were fed with food (LAB FOR, R36, Lantmännen, Stockholm, Sweden) and water *ad libitum*. Ethical approval was received from the regional ethics committee in Umeå, Sweden.

OVA-induced airway inflammation

Mice were divided into four naïve groups and four OVA-sensitized groups. The sensitization was performed by two i.p. injections of 10 µg OVA (Sigma-Aldrich, St. Louis, MO) emulsified in aluminum hydroxide ($\text{Al}(\text{OH})_3$), (Alu-Gel-S suspension 1.3%, research grade sterile, cat no.: 12261, SERVA electrophoresis Thermo Fisher Scientific Inc., Waltham, MA) on day 0 and 7. Control naïve groups were treated with $\text{Al}(\text{OH})_3$ alone. Mice were challenged with 1% OVA-aerosol diluted in water (aerosol size 0.1–0.3 µm; detailed information in Koch et al., 1999) on days 13–15 in nose-only containers via nebulizer (DeVilbiss UltraNeb, Sunrise Medical Ltd., Brierley Hill, UK) for 30 minutes. Naïve groups were likewise exposed to water aerosol. The experimental design is presented in Figure 1.

Chlorine exposures

Naïve and OVA-sensitized mice were exposed to chlorine (Air Liquide, Frankfurt am Main, Germany; compressed gas in gas cylinders: 1 mol% chlorine, 99 mol% nitrogen) in nose-only tubes (plethysmographic compartments) at either of four concentrations; 0, 5, 30 and 80 ppm, for 15 minutes at day 16. The naïve and OVA-sensitized mice exposed to

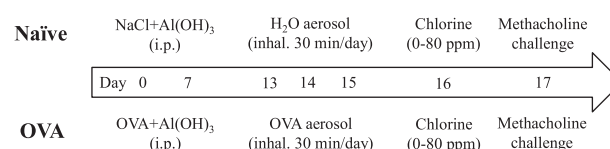


Figure 1. Experimental design for naïve and OVA-sensitized mice. Respiratory effects were measured during chlorine exposure (day 16). Lung mechanics were measured and biological samples collected at day 17.

air (0 ppm) are used as control groups for naïve and OVA-sensitized mice, respectively. Seven animals per group were used in the experiment, except for the group with OVA-sensitized mice receiving 0 ppm that only had six animals. The head of the mouse was guided gently into the nose-only tube through a latex rubber collar. All mice were weighed prior to any treatment (i.e. day 0, 7 and 13–17). The gain of bodyweight was calculated based on the body weights at the day of chlorine exposure (day 16) and at the day of terminal of the experiment (day 17).

Analysis of respiratory function

Several respiratory function parameters were measured in both naïve and OVA-sensitized mice at the time of chlorine exposure. The respiratory function was assessed with a single head-out plethysmographic compartments connected to pneumotachographs measuring the pressure (Lfx System, version 7.0, Mumed Systems, Pr 800 MUMED Physiological recorder, Ltd, London, UK) as previously described in Koch et al. (1999). The mice were allowed to acclimatize in the animal tubes (10 min) before the recordings of the baseline exposure (10 min), chlorine exposure (15 min) and the recovery period (5 min).

When recording the respiratory function, we experienced noise in the data which yielded abnormally high values. This noise was filtered out by the use of cutoff values (lower = 1 breaths/min, upper = 400 breaths/min). A more detailed description of the method used is presented in the supplemental material (Appendix A). The change in respiratory frequency during chlorine exposure (15 minute exposure) compared to pre-exposure baseline (10 minute of clean air) was calculated for each animal.

Respiratory mechanics

The mice were anesthetized by an i.p. injection of 90 mg/kg pentobarbital sodium, tracheostomized with an 18 gauge cannula and mechanically ventilated (flexiVent, Scireq, Montreal, PQ, Canada) at a frequency of 3 Hz and a tidal volume (V_T) of 12 ml/kg body weight (0.2 ml $\times$ 180 breaths). Paralyzation was performed by an i.p. injection of pancuronium (0.1 mg/kg, i.p.). The positive end-expiratory pressure (PEEP) was set to 3 cm H_2O . The animal was kept at 37°C with a warming pad. To establish a stable baseline and ensure similar volume history before the experiments, four sigh maneuvers at $3 \times V_T$ were performed. Before the experiment started, the mice were allowed to rest for 2 min. Airway hyperresponsiveness was measured by inhalation of methacholine (MCh, acetyl- β -methylcholine chloride, Sigma-Aldrich, St. Louis, MO) at increasing doses of 0, 25, 50 and 100 mg/ml given with 5 min intervals. The MCh was diluted in saline to a volume of 20 μ l and given as an aerosol (Aeroneb, Scireq, Montreal, Canada) during 10 seconds. Respiratory resistance was allowed to return to baseline level before the next MCh dose was administered and all respiratory parameters were measured continuously using a standardized script. The maximum response to a given concentration of MCh is reported. Respiratory resistance

(R_{RS}), respiratory compliance (C_{RS}), Newtonian resistance (R_n), peripheral tissue resistance (G) and peripheral tissue elastance (H) were obtained. The experiment ended at day 17, i.e. 40–46 hours after the final OVA exposure or 24–30 hours after the chlorine exposure. A detailed description of the performance and script for measurement of respiratory mechanics are given in Wigenstam et al. (2012).

OVA-specific IgE-analysis in serum

In order to confirm the allergic immune sensitization in the mice, OVA-specific IgE-antibodies were measured in serum. All mice that had been immunized and challenged to OVA had increased levels of OVA-specific IgE-antibodies in serum, compared to the naïve mice (for details, see supplemental material Appendix B). Blood was sampled by orbital sinus venipuncture on anesthetized mice. Blood samples were centrifuged and serum samples were stored in aliquots at -80°C until analysis. The OVA-specific immunoglobulin antibody (IgE) was analyzed in serum by using enzyme-linked immunosorbent assay (ELISA). Briefly, the measurements were done by using polyclonal anti-mouse IgE (rat-anti-mouse IgE, PharMingen, clone R35-72, BD Biosciences, Stockholm, Sweden) at a concentration of 16 μ g/ml in coating buffer. For optimal concentration, the serum samples were diluted 1:2 in blocking buffer. A detailed methodological description is outlined in Gustafsson et al. (2015).

Bronchoalveolar lavage

Bronchoalveolar lavage fluid (BALF) was obtained by lavage, four times, via a tracheal tube with $\text{Ca}^{2+}/\text{Mg}^{2+}$ free Hanks' balanced salt solution (HBSS, Sigma-Aldrich, Steinheim, Germany) to a total volume of 4 ml (1 ml $+ 3 \times 1$ ml). The BALF was centrifuged (10 min, 4°C , 1500 rpm). After removing the supernatant, the cell pellet was resuspended and diluted in 1 ml phosphate buffered saline (PBS). The first 1 ml of supernatant was saved for further cytokine and chemokine analyses. The total number of leukocytes was counted manually using trypan blue exclusion in a Bürker chamber and 30 000 cells were loaded onto slides using a Cytospin centrifuge (Shandon cytospin 3 cyto-centrifuge, cell preparation system). Centrifuged cells were stained with May-Grünwald-Giemsa reagents and differential cell counts of pulmonary cells (macrophages, neutrophils, lymphocytes and eosinophils) were made in duplicates using standard morphological criteria and counting 300 cells per cytospin preparation in a blinded manner.

Inflammatory cytokines and chemokines in BALF

Nine cytokines and chemokines were analyzed with Luminex Bio-Plex 200 (Milliplex MAP Kit, Merck Chemicals and Life Science AB, Stockholm, Sweden); CCL11 (Eotaxin), CXCL1 (KC), CXCL9 (MIG), CXCL10 (IP-10), CCL3 (MIP-1 α), IL-1b, IL-6, IL-10 and TNF- α . TGF- β was analyzed with ELISA according to the manufacturer's instructions (R&D systems, Abingdon, UK).

Histopathology

The right lung lobe from lavaged mice was stored in formaldehyde 4% prior to embedding in paraffin. Lung sections were stained with hematoxylin and eosin for histopathological examination.

Statistical analyses

The results are presented as the mean $\pm$ standard error of means (SEM). Statistical significant differences were assessed by one-way ANOVA with Dunnett's multiple comparison test, two-way ANOVA, Bonferroni's multiple comparisons and Student's unpaired *t*-test. A statistical result with $p < 0.05$ was considered as significant. The statistical analyses were made with GraphPad Prism (version 5.0, GraphPad Software Inc., San Diego, CA).

Results

Respiratory function during chlorine exposures

Both naïve and OVA-sensitized mice showed concentration-dependent decreases in respiratory frequency. However, the changes were not significantly different between the two groups (Figure 2). The exposure to 5 ppm chlorine resulted

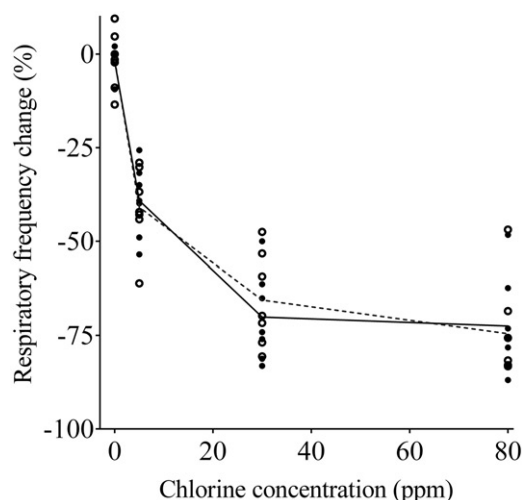


Figure 2. Percent change in respiratory frequency for naïve (--- ○ ---) and OVA-sensitized (— ● —) mice following exposure to chlorine for 15 minutes. Lines indicate arithmetic mean.

in mean depressions in respiratory frequency of 41% and 39%, in naïve and OVA-sensitized mice, respectively.

Body weight change

Minor differences in body weight change were observed in both naïve and OVA-sensitized mice 24 hours after exposure to 80 ppm, whereas no or little effect was seen at lower concentration (Figure 3). The reduction in body weight was significantly higher in naïve as compared to OVA-sensitized mice.

Respiratory mechanics

The respiratory mechanics were measured during exposure to increased concentrations of MCh, in both naïve and OVA-sensitized mice 24 hours after exposure to chlorine (Figure 4). Naïve mice, exposed to 80 ppm of chlorine, showed a marked increase in respiratory resistance (R_s), Newtonian resistance (R_n), peripheral tissue resistance (G) and peripheral tissue elastance (H), as well as a decrease in respiratory compliance (C_{RS}) (Figure 5). No such exposure-related effects on airway hyper responsiveness were observed in OVA-sensitized mice. However, the OVA-sensitized mice had a higher baseline respiratory resistance (R_{RS}), peripheral tissue resistance (G) and peripheral tissue elastance (H), as well as a slightly lower respiratory compliance (C_{RS}), confirming an OVA-related airway inflammation.

Inflammatory cells in BAL-fluid

The total number of inflammatory cells and number of eosinophils were 10 times higher in OVA-sensitized compared to naïve mice (Figure 6) confirming an OVA induced eosinophilic inflammation. The number of neutrophils was significantly increased in naïve mice exposed to 80 ppm of chlorine. In addition, few lung epithelial cells were noted in naïve mice exposed to 80 ppm of chlorine but not in other dose groups.

Cytokines and chemokines in bronchoalveolar lavage fluid

OVA-sensitized mice showed higher concentrations of several cytokines and chemokines in BALF (Figure 7).

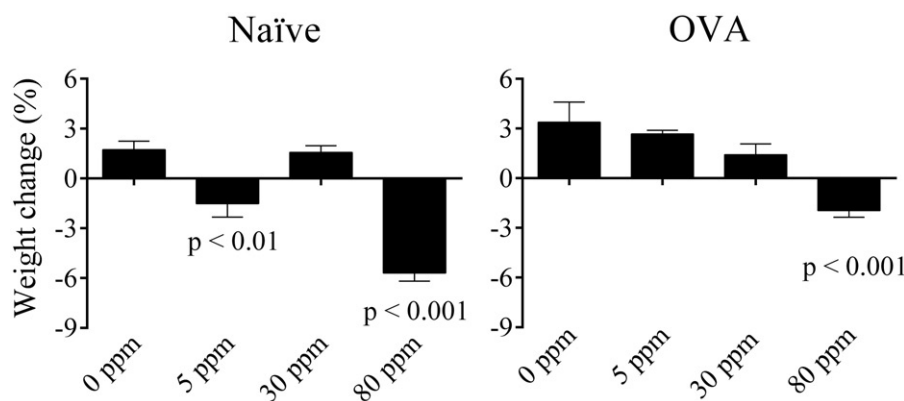


Figure 3. Body weight change (mean $\pm$ SEM) from day 16 to day 17 in naïve and OVA-sensitized mice exposed to clean air or chlorine.

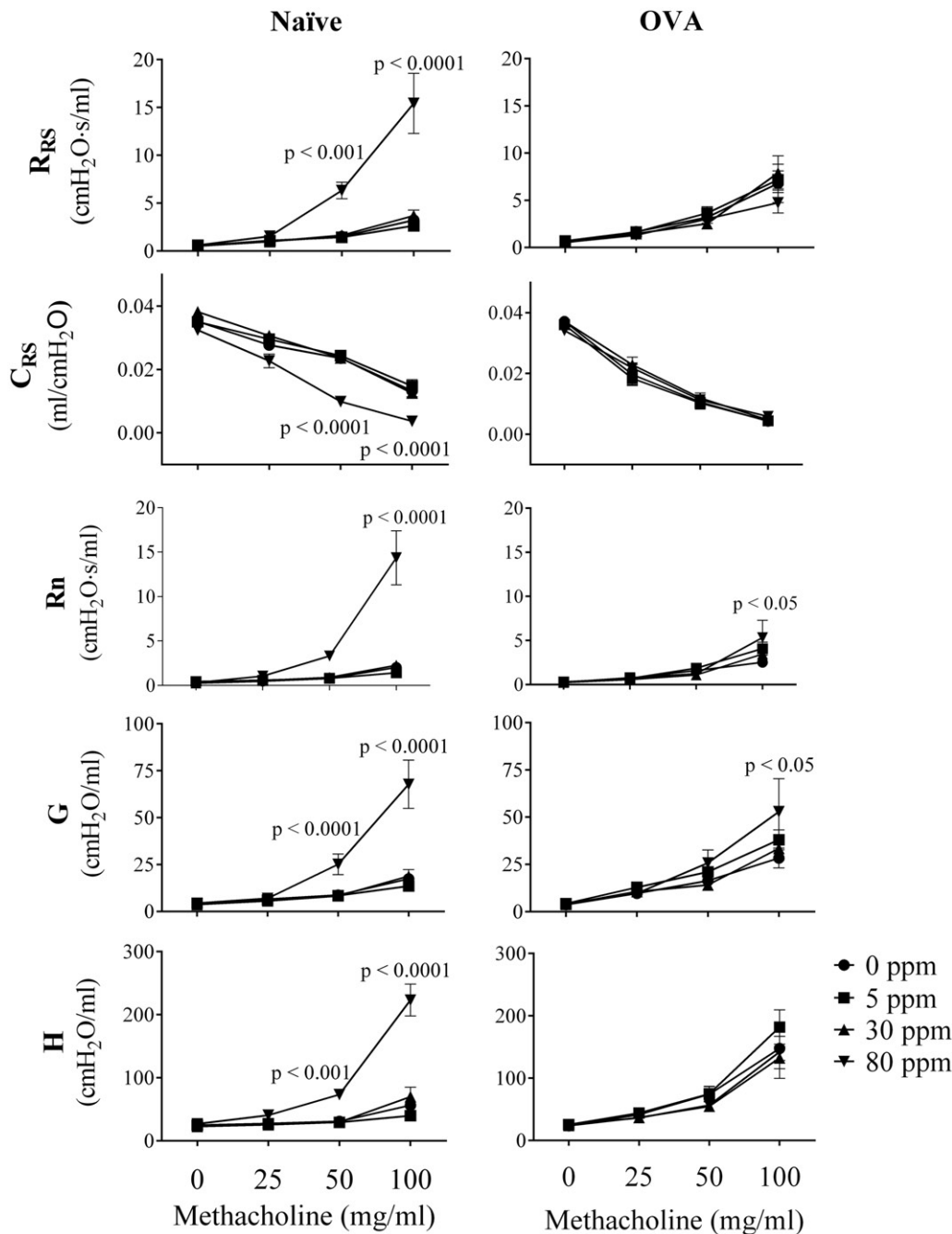


Figure 4. Methacholine induced changes in respiratory mechanics (mean $\pm$ SEM) in naïve and OVA-sensitized mice 24 h after exposure to clean air or chlorine. R_{RS} : respiratory resistance; C_{RS} : respiratory compliance; R_N : Newtonian resistance; G : peripheral tissue resistance; H : peripheral tissue elastance.

A sensitization increased the levels of CCL11 whereas CXCL1 were slightly decreased after exposure to 80 ppm chlorine. No exposure-related effects were observed in naïve mice. In total, 10 cytokines and chemokines in BALF were analyzed. The levels of IL-1 β and CCL3 (MIP-1 α) were out of range (3.2–10 000 pg/ml) and the measured levels of IL-6, TNF- α and IL-10 were close to or below 3.2 pg/ml (data not shown).

Histopathological analysis of lungs

No chlorine exposure-related histopathological changes in the lungs were observed in this study. Moderate to severe

signs of lung injury were evident in all groups of OVA-sensitized mice (Figure 8), including increased cellularity in alveolar walls, perivascular inflammatory cell infiltration and peribronchiolar inflammatory cells (Figure 9).

Discussion

We explored a murine model of asthma as a means to study susceptibility during acute exposure to irritant chemicals, using chlorine as a model substance. The most sensitive outcome was a marked decrease in respiratory frequency which was very similar in the naïve and OVA-sensitized mice. This indicates that there is no difference of the sensory irritation

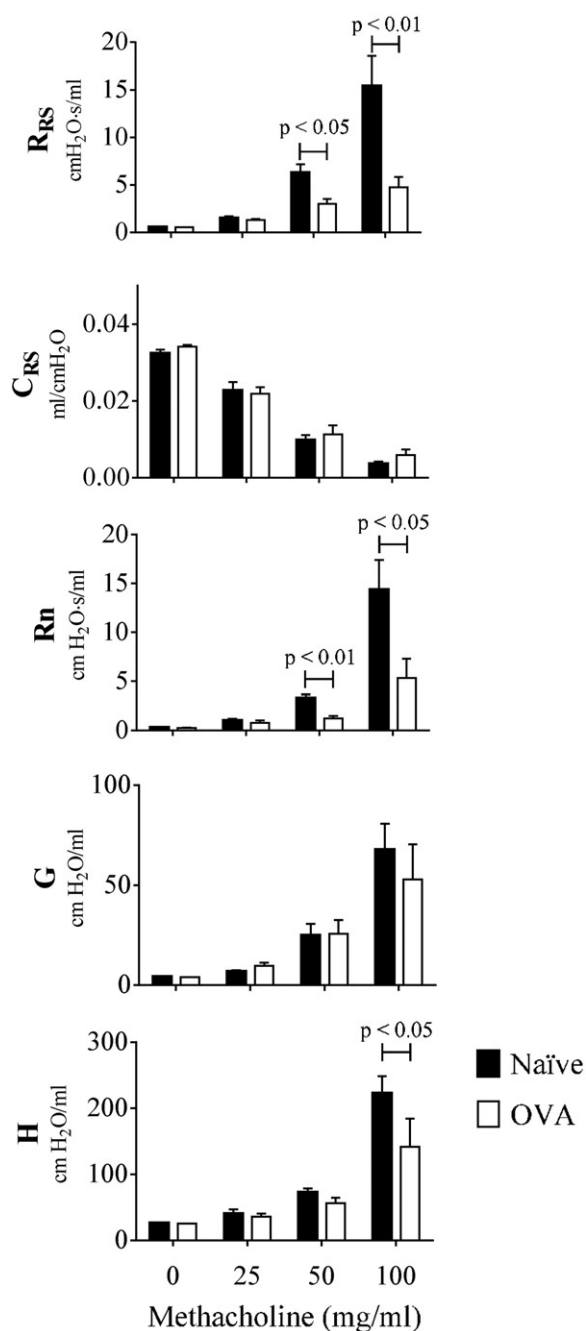


Figure 5. Methacholine induced changes in respiratory mechanics (mean $\pm$ SEM) in naïve and OVA-sensitized mice 24 h after exposure to 80 ppm chlorine. R_{RS} : respiratory resistance; C_{RS} : respiratory compliance; R_n : Newtonian resistance; G : peripheral tissue resistance; H : peripheral tissue elastance.

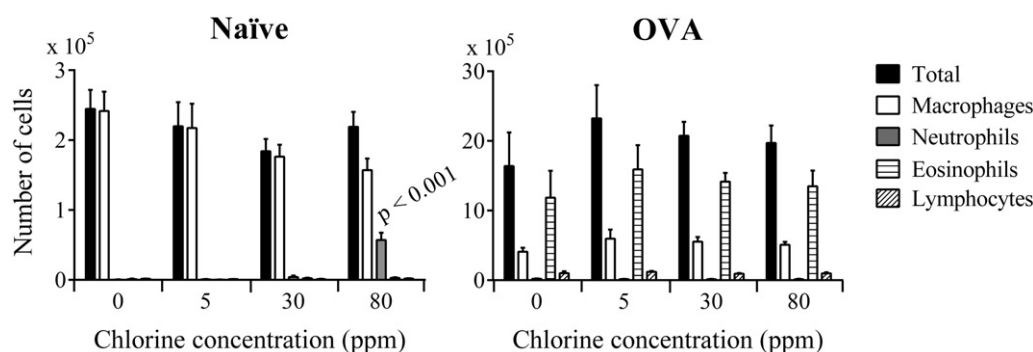


Figure 6. Number of leukocytes (mean $\pm$ SEM) in bronchoalveolar lavage fluid from naïve and OVA-sensitized mice following exposure to clean air or chlorine.

which mirror the upper airways between naïve and OVA-sensitized mice. The concentration–effect curve of respiratory depression suggests an RD_{50} -value slightly above 5 ppm. To our knowledge, the RD_{50} has not been determined in BALB/c mice previously. However, this value agrees with previously reported RD_{50} -values for chlorine from other mouse strains, indicating a low inter-strain variability for irritation; 9.3 ppm (10 min) in male Swiss Webster mice (Barrow et al., 1977), 3.5 ppm (60 min) in male OF1 mice (Gagnaire et al., 1994) and 2.3 ppm (15 min) in female C57Bl/6J mice (Morris et al., 2005). For mice it has been empirically observed that, on average, 0.03 times the RD_{50} in mice corresponds to the eight-hour occupational exposure limits for substances with sensory irritation properties (Alarie, 1981). It may be of interest to note that 0.03 times the approximate RD_{50} value of 5 ppm in our study, i.e. 0.15 ppm, is in line with the no-observed adverse effect concentration (NOAEC) in asthmatic human subjects of 0.4 ppm (D'Alessandro et al., 1996) as well as the AEGL-1 value of 0.5 ppm (NRC, 2004).

A slight decrease in body weight 24 hours after exposure to chlorine was observed in both naïve and OVA-sensitized mice after exposure to 80 ppm. Interestingly, a clear increase in airway hyper responsiveness was seen among the naïve, but not among the OVA-sensitized mice, after exposure to 80 ppm chlorine. Similarly, Jonasson et al. (2013) found increased airway hyper responsiveness after exposure to 50 and 200 ppm chlorine, using a similar experimental set-up with naïve BALB/c mice.

The present study includes several markers of inflammation, including cell counts as well as analysis of selected cytokines and chemokines. Chlorine (80 ppm) resulted in a cellular infiltration of neutrophils in naïve mice, but not in OVA-sensitized mice. This finding is consistent with those by Jonasson et al. (2013) (50 and 200 ppm chlorine) and McGovern et al. (2015) (100 ppm chlorine for 5 min). After depletion of neutrophils with antibodies directed against granulocyte receptor-1 or Ly6G, there was no recruitment of neutrophils to the airways and the hyper responsiveness was decreased during MCh challenge. Both types of antibodies caused decreases in R_{RS} and R_n but not in G and H (McGovern et al., 2015). This could be the explanation to our findings of increased R_{RS} and R_n and the possession of a neutrophil inflammation in naïve mice compared to the OVA-sensitized mice groups which have low levels of

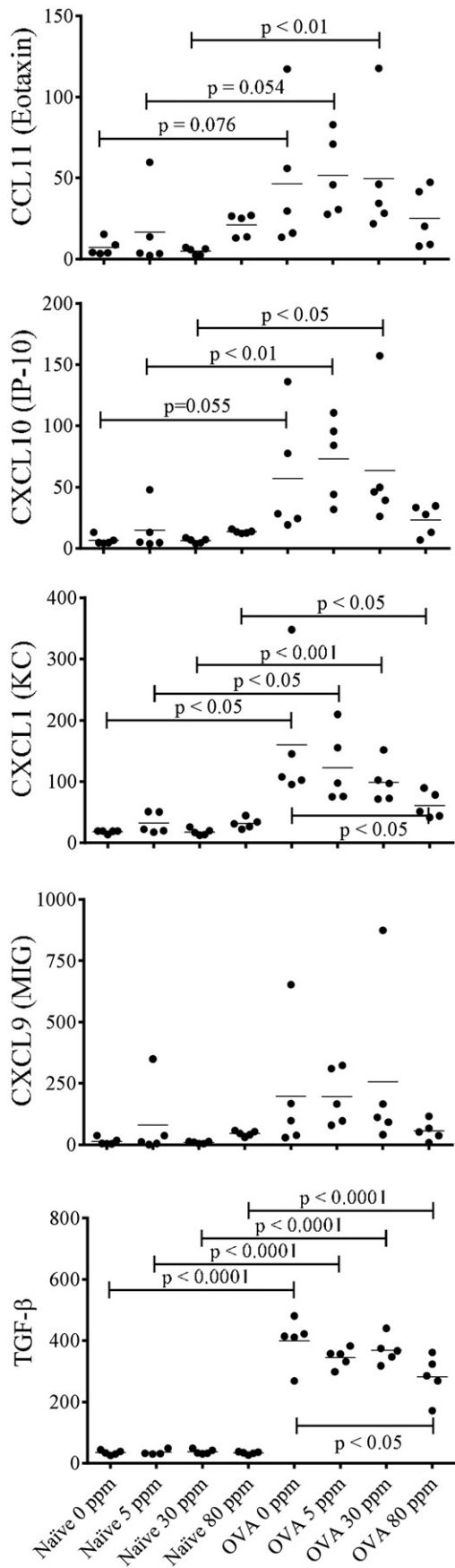


Figure 7. Concentrations of cytokines and chemokines in bronchoalveolar lavage fluid in naïve and OVA-sensitized mice exposed to clean air or chlorine. Arithmetic mean indicated.

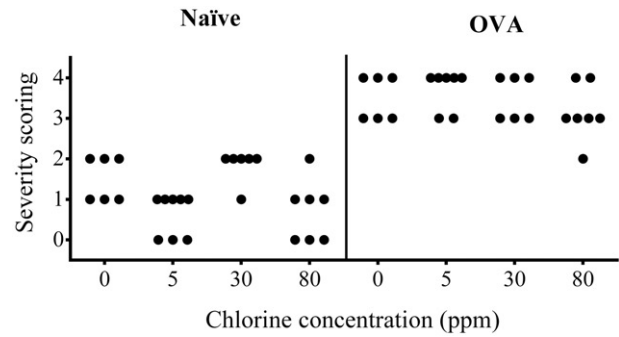


Figure 8. Lung histopathology. Severity scoring of effects on lung tissue in naïve and OVA-sensitized mice exposed to clean air or chlorine for 15 minutes. 0 = nothing abnormal detected; 1 = minimal; 2 = slight; 3 = moderate; 4 = severe. Scoring criteria are fully described in supplementary materials.

neutrophils in the airways. The underlying causes of the observed changes in respiratory mechanics (Figure 5) in naïve mice exposed to a high concentration of chlorine (80 ppm) might not be directly comparable to the observations in OVA-sensitized mice. The higher baseline resistance and slightly lower compliance confirm the strong airway inflammation among the OVA-sensitized mice. In pathologies such as acute lung injury or asthma, the parenchymal tissue becomes heterogeneous which results in alteration of the mechanical properties (Bates & Suki, 2008). During a challenge to a bronchial agonist it is therefore hard to distinguish whether this heterogeneity occurs due to bronchoconstriction in the central airways (R_n) or due to airway narrowing or increased closure of small peripheral airways (G and H).

McGovern et al. (2015) characterize chlorine and its hydrolyzed metabolites as strong oxidants, but they also conclude that the presence of neutrophils is a major player for the oxidative burden in the airways (McGovern et al., 2015). Additional studies are needed to confirm this hypothesis. Interestingly, and similar to the results in our study, Stenfors et al. (2004) observed an increase of neutrophils in the airways of healthy non-atopic, but not mild asthmatic, volunteer 6 h after chamber exposure to diesel exhaust ($108 \mu\text{g PM}_{10}/\text{m}^3$ for 2 h with intermittent exercise).

No chlorine-related increases in cytokine and chemokine levels from BAL-fluid were observed in samples from neither naïve nor OVA-sensitized mice. Sensitized mice generally show increased levels of cytokines and chemokines related to respiratory inflammation (i.e. CCL11 (Eotaxin), CXCL1 (KC), CXCL9 (MIG), CXCL10 (IP-10) and TGF- β). The neutrophil activating protein (CXCL1) is higher in the OVA-sensitized mice, but so is also the background number of neutrophils. The only exception is the group of naïve mice exposed to 80 ppm chlorine that has an increased number of without a corresponding induction of CXCL1. Jonasson et al. (2013) showed increased levels of IL-6 and CXCL1 (KC) from 2 h to 12 h, peaking at 6 h, following chlorine inhalation (50 and 200 ppm) in naïve mice. This indicates that our time point of measurement (i.e. 24 h) was too late. At 200 ppm chlorine, they also found an early increase (after 2 h) of IL-1 β followed by increases in TNF- α ,

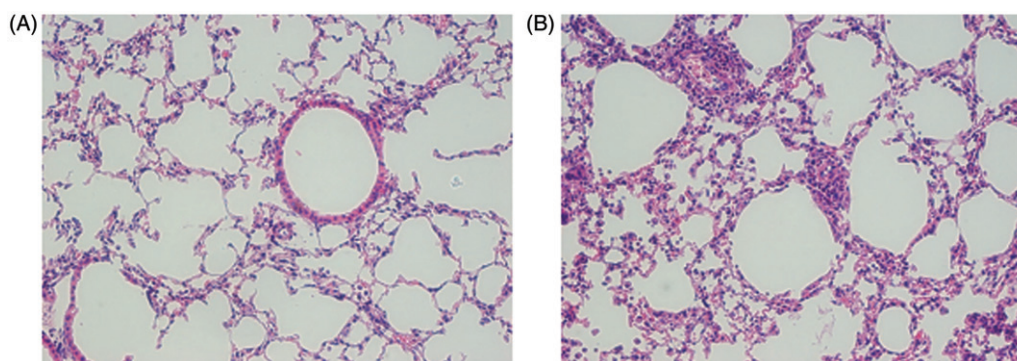


Figure 9. Histopathological examination of the lung. Sections stained with hematoxylin and eosin. Pictures from light microscope (20 $\times$). (A) Severity score 0 – naïve mouse exposed to 80 ppm chlorine. (B) Severity score 4 – OVA-sensitized mouse exposed to clean air. Scoring of histopathology data refer to effects in the lower section with including cellularity (mononuclear inflammatory cells, neutrophilic granulocytes, multinucleated macrophages) in alveolar walls; perivascular inflammatory cell infiltration (neutrophilic granulocytes, mononuclear inflammatory cells, multinucleated macrophages); and peribronchiolar inflammatory cells. Details in supplementary materials.

and CCL11 (Eotaxin) after 6 h. The chosen time point (24 h after exposure) in our study might have been too late to detect transient changes.

No histopathological findings were observed in lungs from naïve mice following exposure to chlorine. In contrast, all examined lungs from OVA-sensitized mice had clear histopathological findings, but there was no correlation to chlorine exposure. Jonasson et al. (2013) showed that injuries were restricted to larger airways following inhalation of chlorine (200 ppm, for 15 minutes). Hoyle et al. (2010) found consistent damage in the large airways in FVB/N mice exposed to 100 ppm chlorine gas for 60 minutes but detached epithelial cells were not visible in deeper parts of the lung. McGovern et al. (2015) show that neutrophils are visualized in the airway lumen from male BALB/c mice 6 h and 24 h after 100 ppm chlorine gas exposure for five minutes. The lack of histopathological findings in naïve mice in our study might be due to the lower exposure level (80 ppm) compared to the above mentioned studies. Chlorine hydrolyzes upon contact with the mucus in the upper respiratory tract (nose) and in the tracheobronchiolar region, indicating that small amounts of chlorine reached the lower regions where histological examination were performed. Higher concentrations of chlorine have a lower retained proportion of the dose downstream due to respiratory depression. In addition, dissolved chlorine may also change the mucus ability to interact with chlorine in a dose-time-dependent manner. Meanwhile, the OVA-induced inflammation with increased cellular recruitment to both the alveolar walls and the perivascular region were observed in all OVA-sensitized groups independent on the concentration of chlorine exposure. Although 1% OVA is suitable for inducing eosinophilic airway inflammation and has been widely used to evaluate antiallergic drugs, the concentration may be too high to evaluate the deterioration of inflammation.

In humans, asthma is a heterogeneous disease with many phenotypes, mainly characterized by eosinophilic, neutrophilic or mixed infiltration in the airways. While neutrophilic asthma is considered as the most severe there is a wide spectrum of severity within phenotypes.

Eosinophilic asthma is mostly associated with an immune response mediated by T_H2 related cytokines, whereas neutrophilic asthma is related to a $TH17$ immune response (Pelaia et al., 2015). The sensitized BALB/c mice model is a widely used model to study eosinophilic asthma. Recently, the BALB/c model was also used to study the potency of ozone to attenuate allergic lung inflammation (Hansen et al., 2016). The results showed that allergic mice were protected from pulmonary irritation caused by ozone. The authors ascribe this to a scavenging effect of the mucus in the airways. Our study is in line with the findings by Hansen et al. and confirm the changes of the airways by clear histopathological findings in lungs from OVA-challenged mice only. The model has some but not all characteristics of the human disease, so caution should be used in extrapolating data obtained in the model to humans. In human individuals possessing an eosinophilic asthma, eosinophils constitute about 1–3% of cells in BALF, whereas the percentage of eosinophils in BALF collected from OVA-sensitized mice is as high as 40–80% (Fulkersson et al., 2005). Comparing the lack of exacerbation of inflammation by chlorine in OVA-induced mice with the sensitivity among asthmatics observed in humans (D'Alessandro et al., 1996) one need to recall the large difference between mild asthmatic humans and the strong ongoing inflammation in OVA-induced mice. The animal model is already showing such a strong inflammation that a further increase might be unlikely.

In summary, no major differences in susceptibility were observed in naïve and OVA-sensitized mice. Similar concentration-dependent reductions in respiratory frequency, a marker of sensory irritation, were seen in the two groups, with a RD_{50} slightly above 5 ppm. Decreased body weight 24 hours after exposure to 80 ppm (but not at 5 or 30 ppm) was also observed in both groups. Naïve, but not OVA-sensitized, mice showed increased bronchial reactivity and higher number of neutrophils in BALF at 80 ppm. In conclusion, the results do not support an increased susceptibility to chlorine among OVA-sensitized mice. This animal model, which represents a phenotype of eosinophilic airway inflammation, seems unsuitable to study susceptibility to inhalation of irritants in relation to asthma.

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Disclosure statement

The authors report no conflicts of interest.

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Appendix A

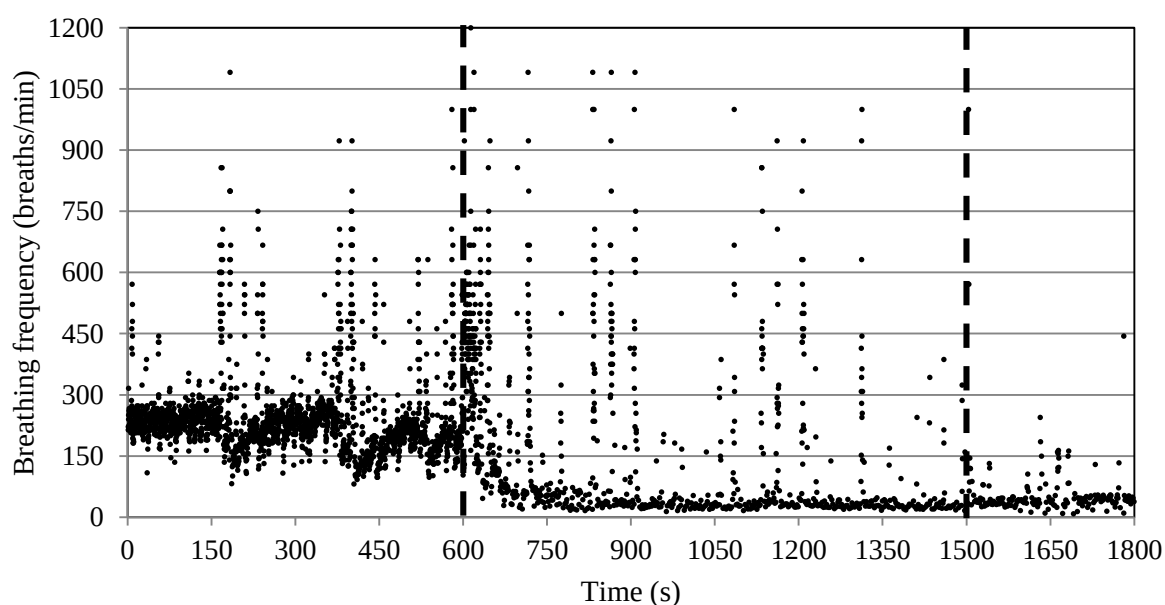
Method for analyzing plethysmography data.

As presented by Barrow and colleagues (1977) a short period of increased respiratory frequency and some body movements are expected at the start of the chlorine exposure (each lasting 10 to 20 seconds) and additional peaks can be seen during the exposure due to transient body movement.

In our study, high transient peaks were present during exposure to air and chlorine (Figure 1). A recorded value was considered abnormal if it was outside of the chosen cut-off values (lower=1 breaths/min, upper=400 breaths/min).

For values within the cut-off-range, we calculated the median breathing frequency during air exposure (0 to 10 minutes) and during chlorine exposure (11 to 25 minutes), separately. The first minute of chlorine exposure (minute 10-11) was excluded due to body movements and the delay in response to chlorine. The percent change following chlorine exposure compared to air exposure was calculated: $(\text{median}_{\text{Cl}_2} / \text{median}_{\text{air}} - 1) * 100$.

Figure 1. Example: A recording of breathing frequency in a naïve mouse. Exposures consisted of baseline air (0-600 s), 30 ppm chlorine (600-1500 s) and air (1500-1800 s). We applied cut-off values of 1-400 breaths/min.



Appendix B

Analysis of ovalbumin-specific IgE-antibodies in serum from naïve and OVA-sensitized mice exposed to chlorine.

Figure 1. OVA-specific IgE-antibodies in serum.

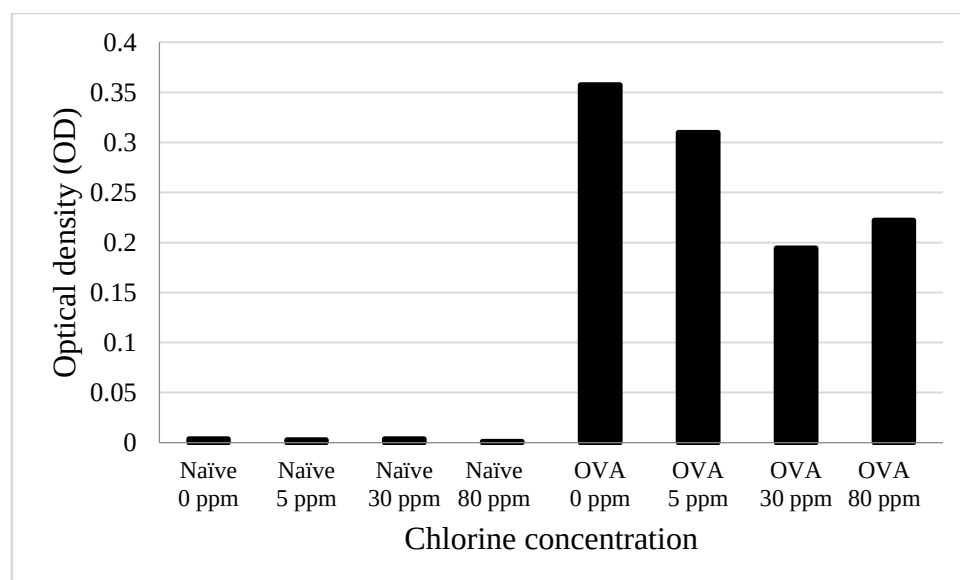


Table 1. OVA-specific IgE-antibodies in serum presented for each animal and as arithmetic mean.

	Naïve 0 ppm	Naïve 5 ppm	Naïve 30 ppm	Naïve 80 ppm	OVA 0 ppm	OVA 5 ppm	OVA 30 ppm	OVA 80 ppm
	0	0,00095	0,01185	5E-05	0,14555	0,18435	0,28185	0,23755
	0,01465	0	0,00795	0,00255	0,14395	0,19715	0,12425	0,21865
	0,00135	0	0	0	0,98605	0,12805	0,20765	0,29495
	0,00225	0,00245	0,00055	0,00025	0,16525	0,40825	0,12645	0,25565
	0,00515	0,00155	0,00085	0	0,22675	0,59485	0,22895	0,18245
	0,00105	0	0,00125	0,00225	0,47625	0,25775	0,21245	0,08265
	0,00135	0,01395	0,00195	0,00295		0,39675	0,17915	0,28365
Arithmetic mean	0,003686	0,0027	0,003486	0,00115	0,3573	0,309593	0,194393	0,222221

Appendix C

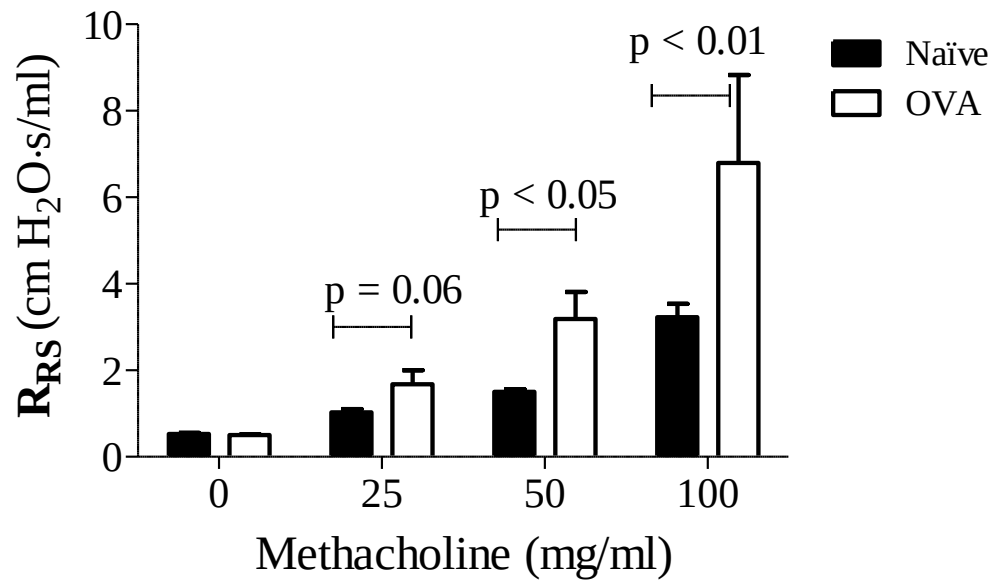


Figure 1. Increase (mean $\pm$ SEM) in respiratory resistance (R_{RS}) in response to methacholine in naïve and ovalbumin (OVA) sensitized mice not exposed to clean air.

Appendix D

The scoring used for lung histopathology was based on the following criteria:

0 = NAD (Nothing Abnormal Detected)

1 = Increased cellularity (mononuclear inflammatory cells, neutrophilic granulocytes) in alveolar walls, **minimal**.

Perivascular oedema, **slight**.

Peribronchiolar inflammatory cells, **minimal**.

2 = Increased cellularity (mononuclear inflammatory cells, neutrophilic granulocytes) in alveolar walls, **slight**.

Perivascular oedema, **slight**.

Perivascular inflammatory cell infiltration, **slight**.

Peribronchiolar inflammatory cells, **slight**.

3 = Increased cellularity (mononuclear inflammatory cells, neutrophilic granulocytes) in alveolar walls, **moderate**.

Perivascular inflammatory cell infiltration (neutrophilic granulocytes, mononuclear inflammatory cells, multinucleated macrophages), **moderate**.

Peribronchiolar inflammatory cells, **moderate**.

Increased alveolar macrophages, **slight**.

Subpleural inflammatory cell infiltrates, **minimal to slight**.

4 = Increased cellularity (mononuclear inflammatory cells, neutrophilic granulocytes, multinucleated macrophages) in alveolar walls, **moderate**.

Perivascular inflammatory cell infiltration (neutrophilic granulocytes, mononuclear inflammatory cells, multinucleated macrophages), **moderate to severe**.

Peribronchiolar inflammatory cells, **moderate**.

Increased alveolar macrophages, **moderate**.

Subpleural inflammatory cell infiltrates, **moderate**.