

Immunological Determinants in a Mouse Model of Chemical-Induced Asthma After Multiple Exposures

J. A. J. Vanoirbeek, M. Tarkowski, V. De Vooght, B. Nemery & P. H. M. Hoet

Katholieke Universiteit Leuven, Research Unit of Lung Toxicology, Leuven, Belgium

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Correspondence to: Peter Hoet, Katholieke Universiteit Leuven, Occupational, Environmental and Insurance Medicine, Research Unit of Lung Toxicology, Herestraat 49 bus 706, B-3000 Leuven, Belgium. E-mail: peter.hoet@med.kuleuven.be

Abstract

In a mouse model of chemical-induced asthma, we investigated the effects of multiple challenges, using toluene diisocyanate (TDI), a known cause of occupational asthma. On days 1 and 7, BALB/*c* mice received TDI or vehicle (acetone/olive oil). On days 10, 13 and 16 the mice received an intranasal instillation of TDI. Ventilatory function (Penh) was monitored by whole body plethysmography for 40 min after each challenge. Reactivity to methacholine was measured 22 h later. Pulmonary inflammation, TNF- α and MIP-2 levels were assessed 24 h after the last challenge by broncho-alveolar lavage (BAL). Other immunological parameters included total IgE, lymphocyte sub-populations in auricular and cervical lymph nodes, and IL-4, IFN- γ and IL-13 levels in supernatants of lymph node cells, cultured with or without concanavalin A. Early ventilatory function and airway reactivity increased in all groups that received a dermal application and one or multiple intranasal challenges of TDI. After multiple challenges, lung inflammation was characterized by neutrophils (~15%), and eosinophils (~4%), along with an increase in BAL MIP-2 and TNF- α levels. The auricular and cervical lymph node cells of all sensitized mice showed an increase in B cells, Th cells and an increased concentration of *in vitro* release of IL-4, IFN- γ and IL-13 after stimulation with concanavalin A. Total serum IgE was elevated in dermally TDI-sensitized mice. This protocol including multiple challenges results in a model that resembles human asthma, indicating that responses found in the model using a single challenge could be a good first indication for the development of asthma.

Introduction

In the industrial world, occupational asthma is the leading cause of work-related respiratory disease [1, 2]. Exposure to low-molecular-weight sensitizers such as diisocyanates, acid anhydrides, reactive dyes and many other chemicals may induce adult-onset asthma [3, 4]. Although the clinical presentation of occupational asthma does not depend much on the causative agent, it is common to distinguish occupational asthma caused by sensitization to high-molecular-weight agents from that caused by sensitization to low molecular weight chemicals. The former agents consist of (glyco)proteins of biological origin, which may cause IgE-mediated allergy and asthma in a similar fashion to common inhalant allergens that cause atopic asthma. This working mechanism has been well described. Sensitization to low-molecular-weight chemicals is considered to result from a response

of the immune system to haptens conjugated with endogenous proteins. However, the exact pathways and mechanisms of sensitization to such chemicals and the pathogenesis of the subsequent respiratory reactions are much less well understood, as they seem to differ from those of classic IgE-mediated asthma [5].

Previously, we described a mouse model of chemical-induced asthma using toluene diisocyanate (TDI), a known respiratory sensitizer [6–9]. In this model, the mice were sensitized by dermal application, and challenged via a single intranasal instillation with a non-irritant concentration of TDI. We were able to reproduce several important features of human occupational asthma, including immediate antigen-specific ventilatory response after challenge (consistent with bronchoconstriction) and non-specific hyper-responsiveness to methacholine, as well as neutrophilic airway inflammation 24 h later. We were not able to find these changes in severe combined immunodeficient

(SCID) mice, indicating that lymphocytes are important to yield these effects. Auricular lymphocytes from TDI-treated mice produced, both Th2 and Th1 cytokines '*in vitro*'. The cervical lymph nodes only showed a very limited response after a single intranasal challenge [8]. These data were validated using another known respiratory sensitizer, trimellitic anhydride (TMA) [10].

The aim of this project was to determine, whether a multiple airway exposure protocol, after dermal sensitization, would yield a phenotype more closely mimicking human occupational asthma, compared with a single airway exposure protocol. In this multiple intranasal instillations protocol, we found the same neutrophil inflammation as in our single intranasal instillation protocol, but in the multiple exposure protocol this was accompanied by a transient influx of eosinophils. Furthermore, not only the auricular lymph nodes, but also the cervical lymph nodes clearly showed changed lymphocyte sub-populations in TDI-treated mice, compared with the vehicle-treated mice.

Materials and methods

Reagents. Toluene-2,4-diisocyanate (TDI) (98%) (Fluka, CAS 584-84-9), acetyl-B-methylcholine (methacholine), acetone and diethyl ether were obtained from Sigma Aldrich (Bornem, Belgium). Pentobarbital (Nembutal) was obtained from Sanofi Santé animale (CEVA, Brussels, Belgium). The vehicle (AOO) used to dissolve TDI consisted of a mixture of two volumes of acetone (A) and three volumes of olive oil (OO) ('extra virgin', Carbonell, Spain). Concentrations of TDI are given as percent (v/v) in AOO.

Animals. Male BALB/c mice (approximately 20 g, 6 weeks old) were obtained from Harlan (the Netherlands). The mice were housed in a conventional animal house with 12-h dark/light cycles. They received lightly acidified water and pelleted food (Trouw Nutrition, Gent, Belgium) *ad libitum*. All experimental procedures were approved by the local Ethical Committee for Animal Experiments.

Groups of animals and treatment protocol. On days 1 and 7, the mice received dermal applications (20 μ l) of vehicle or 0.3% TDI on each ear. On day 10, 13 and 16 they received, under light anaesthesia using diethyl ether, an intranasal instillation of 10 μ l of vehicle or 10 μ l TDI (0.1%) in each nostril. In all experiments, treatment with TDI is indicated as 1, while treatment with vehicle (AOO) is indicated as 0. For the coding, we have used 2–4 digits: the first digit represents the dermal applications (days 1 and 7), the second, third and fourth digit represent the intranasal challenge on days 10, 13 and 16, respectively. Thus, the 1/1 group consists of mice that received dermal applications of TDI (days 1 and 7) and one intranasal challenge of TDI (day 10), while the 0/0

control group consists of mice that received the AOO vehicle on all occasions, other groups are 1/0 and 0/1. After two intranasal challenges the treatment groups include 1/1/1, 1/0/0, 0/1/1 and 0/0/0; after three intranasal challenges we have following groups: 1/1/1/1, 1/0/0/1, 0/1/1/1 and 0/0/0/0 group.

Early ventilatory response and airway hyperreactivity. On day 10, 13 and 16 just before the intranasal instillation, the ventilatory function of each mouse was recorded, in resting condition, for 5 min in a whole body plethysmograph (EMKA Technologies, Paris, France). Immediately after the intranasal instillation, mice were placed again in the whole body plethysmograph (zero time point) and their ventilatory parameters were measured for 40 min. Every 30 s, the enhanced pause (Penh), which is a composite index indicative of airways obstruction, was calculated. The area under the curve (AUC) of Penh against time between 0 and 40 min was calculated for each individual mouse and this figure was used for statistical analyses.

On days 11, 14 and 17, i.e. 24 h after intranasal instillation, reactivity to methacholine was assessed in the whole body plethysmograph. Briefly, Penh was calculated for each mouse under resting condition (baseline) and after inhalation, during 1 min, of nebulized methacholine (successively 0, 10, 25, 50 and 100 mg/ml). An average Penh over 30 s was calculated during 3 min (six measurements), and the mean of these six values was used for each concentration. For each mouse, Penh was plotted against methacholine concentration (from 0 to 100 mg/ml) and the AUC was calculated.

Broncho-alveolar lavage. On day 17, 24 h after the last challenge the mice were sacrificed by an overdose (90 mg/kg, ip) of pentobarbital, and a broncho-alveolar lavage (BAL) was performed. The lungs were lavaged *in situ* three times with 0.7 ml sterile saline (0.9% NaCl) at room temperature, and the recovered fluid was pooled. Cells were counted using a Bürker hemocytometer (total cells) and the BAL fluid was centrifuged (1500 g, 10 min). The supernatant was frozen (–80 °C) until further analyses. For differential cell counts, 250 μ l (100,000 cells/ml) of the resuspended cells were spun (1400 g, 6 min) (Cytospin 3, Shandon, TechGen, Zellik, Belgium) onto microscope slides, air-dried and stained (Diff-Quik® method). For each sample, 200 cells were counted for the number of macrophages, eosinophils, neutrophils and lymphocytes.

Levels of tumour necrosis factor-alpha (TNF- α) (Bio-source, Nivelles, Belgium), and macrophage inflammatory protein-2 (MIP-2) (R&D Systems, Abingdon, UK) were measured in undiluted BAL fluid by a sandwich enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's instructions. Lower limits of detection were 3.0 pg/ml and 1.5 pg/ml for TNF- α and MIP-2, respectively.

Lymph node cell analysis. Retro-auricular and superficial cervical lymph nodes were also obtained from the same mice 24 h after the last intranasal instillation. The lymph nodes from four to five mice were pooled and kept on ice in RPMI-1640 (Invitrogen, Merelbeke, Belgium). Cell suspensions were obtained by pressing the lymph nodes through a cell strainer (100 μ m) (BD Biosciences, Erembodegem, Belgium) and rinsing with 10-ml tissue culture medium (RPMI-1640). Cells were counted using a Bürker hemocytometer. Lymphocytes were washed three times and suspended in complete tissue culture medium (RPMI-1640 supplemented with 10% heat-inactivated foetal bovine serum, 10 mg/ml streptomycin, 100 IU/ml penicillin, 1mM sodium pyruvate, and non-essential amino acids) at concentrations of 10^7 cells/ml.

Five hundred thousand cells were stained with anti-CD3 (PE), anti-CD4 (FITC), anti-CD25 (PerCP), or anti-CD19 (PE) labelled antibodies (BD Biosciences), according to standard procedures, and with control samples being labelled with isotype match control

antibodies (BD Biosciences). Flow cytometry (CellQuest, BD Biosciences) was performed using at least 10^4 cells.

Cells were also seeded into 48-well culture plates at a density of 2×10^6 /ml and incubated for 18 h without or with concanavalin A (Con A) (Sigma) (2.5 μ g/ml). Cells were then centrifuged and supernatants were stored at -80°C . Concentrations of interleukin-4 (IL-4), IL-13 and interferon-gamma (IFN- γ) were measured in undiluted supernatants by standard ELISA technique, according to the manufacturer's instructions (Biosource, Nivelles, Belgium). Lower limits of detection were 5, 2 and 1 pg/ml for IL-4, IL-13 and IFN- γ , respectively.

Total serum IgE. On day 17, i.e. 24 h after the last intranasal instillation, blood was drawn (prior to BAL) from the retro-orbital plexus. Serum was obtained by centrifugation of blood at room temperature (14 000g, 10 min). Serum samples were stored at -80°C for further analyses. The OptEIATM Mouse IgE set from Pharmingen (BD Biosciences) was used to measure total serum

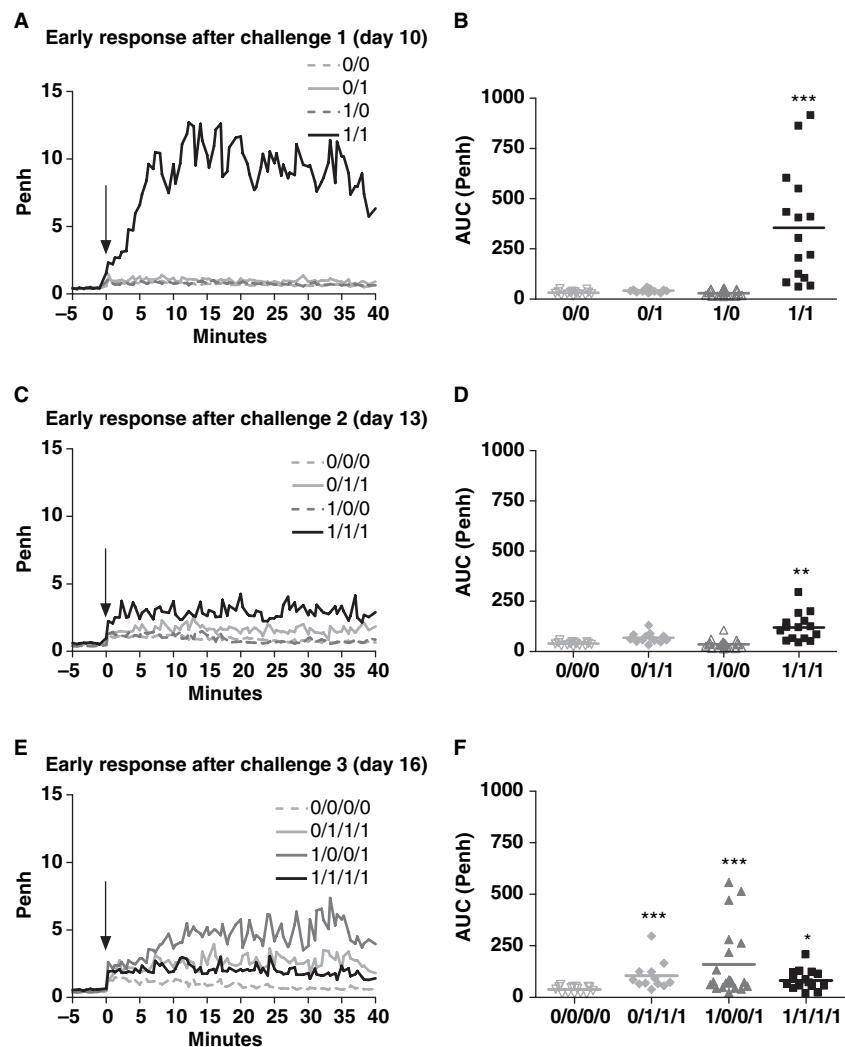


Figure 1 Early ventilatory response. Ventilation, expressed as Penh (enhanced pause), was measured by whole body plethysmography before and after each intranasal challenge (indicated with arrow) of vehicle or 0.1% TDI. (A+B) Early response after the first intranasal challenge on day 10. (C+D) Early response after the second intranasal challenge on day 13. (E+F) Early response after the third intranasal challenge on day 16. Panels A, C and E show mean values of Penh measured during 5 min before and 40 min after challenge; panels B, D and F show individual values of the Area Under the Curve (AUC) of Penh against time between 0 and 40 min. Experimental groups are identified by a 0 and 1, representing administration of vehicle (acetone:olive oil) and TDI, respectively. The first number identifies dermal application (days 1 and 7). The second, third and fourth numbers identify the agent instilled intranasally on day 10, 13 and 16. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to the completely vehicle-treated control group.

IgE (diluted 1/70). Measurements were performed according to the manufacturer's instructions.

Data analyses. All data were analysed using the non-parametric Kruskal–Wallis test (GraphPad Prism 4.01; Graphpad Software Inc., San Diego, CA, USA). A level of $P < 0.05$ was considered significant. A level of $P < 0.05$ (two-tailed) was considered significant.

Results

Early ventilatory response and airway hyperreactivity

Fig. 1A shows significantly increased ventilatory responses in TDI-challenged mice that had previously received dermal applications of TDI (1/1). The AUC showed an 11-fold increases, compared with the 0/0 group. Both the non-sensitized, but TDI-challenged group (0/1) and the sensitized, but vehicle-challenged group (1/0) did not show this ventilatory response. Fig. 1B shows the early response after the second intranasal challenge. Only the 1/1/1 group show a significant twofold increased Penh,

compared with the 0/0/0 control group. Fig. 1C shows the early response after the third intranasal challenge. All TDI-challenged groups show a significant increase in AUC (two- to fourfold), compared with the 0/0/0/0 control group.

Fig. 2A shows the airway responsiveness to methacholine (on day 11) as measured by whole body plethysmography. BALB/*c* mice that received TDI on the skin and were challenged with TDI (1/1) showed an increased methacholine responsiveness compared to the 0/0 control group. The mean AUC of the 1/1 was increased threefold, compared to the 0/0 group. The 0/1 and 1/0 group of mice showed no increased airway responsiveness to methacholine compared to the control group. Fig. 2B shows the methacholine reactivity (on day 14) after two intranasal challenges. The 1/1/1 group is the only responding group, with a significant twofold increase in AUC compared to the 0/0/0 control group. On day 17, after three intranasal challenges, all TDI-challenged groups, showed an increased methacholine reactivity compared with the 0/0/0/0 control group (Fig. 2C). The

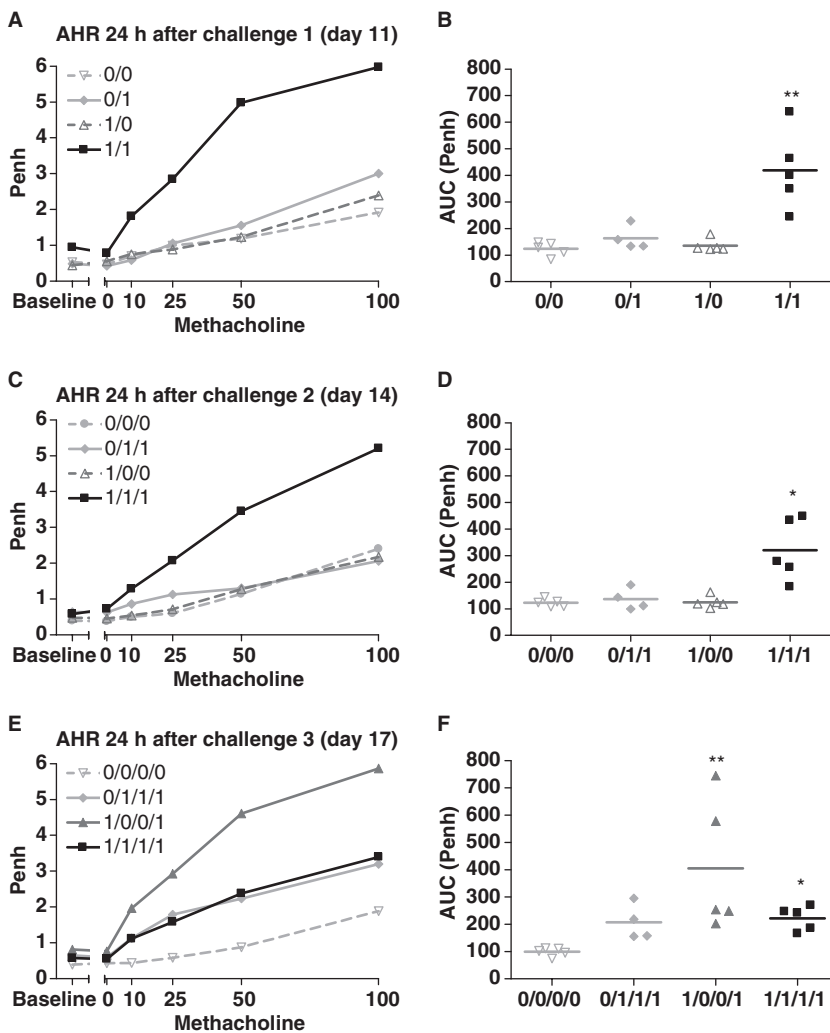


Figure 2 Non-specific methacholine responsiveness. Airway responsiveness (Penh) to methacholine was measured 24 h after each intranasal challenge. Panels A, C and E show mean values of Penh; panels B, D and F show individual values of the area under the curve (AUC) of Penh against concentration of methacholine between 0 and 100 mg/ml. Experimental group legends are as in Fig. 1. A+B. Methacholine responsiveness on day 11, i.e. 24 h after the first intranasal challenge. C+D. Methacholine responsiveness on day 14, i.e. 24 h after the second intranasal challenge. E+F. Methacholine responsiveness on day 17, i.e. 24 h after the third intranasal challenge. * $P < 0.05$, ** $P < 0.01$ compared to the completely vehicle-treated control group.

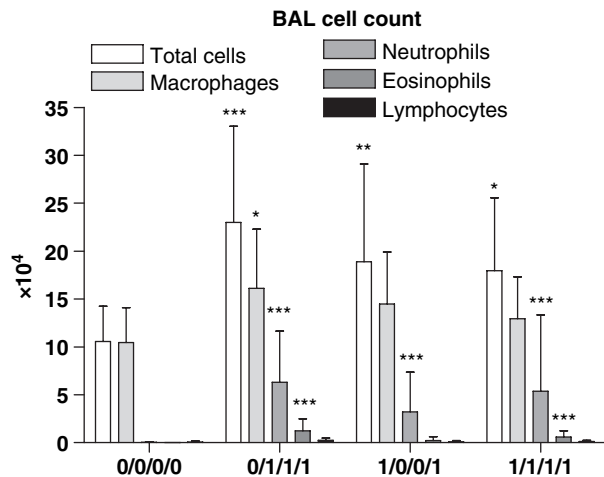


Figure 3 Total and differential cell counts. On day 17, i.e. 24 h after the last intranasal challenge, the lungs were lavaged and total and differential cell counts were assessed. Experimental group legends are as in Fig. 1. Bars represent mean $\pm$ SD. * P < 0.05, ** P < 0.01, *** P < 0.001 compared to 0/0/0/0 control group.

0/1/1/1, 1/0/0/1 and 1/1/1/1) showed a 3.5-, 4- and 3.5-fold significant increase, respectively, in AUC compared to the 0/0/0/0 control group.

BAL analysis

Fig. 3 shows the BAL total and differential cell counts on day 17, 24 h after the last intranasal challenge. All TDI-challenged groups show an increase in total cell counts. The 0/1/1/1, 1/0/0/1 and the 1/1/1/1 groups show a significant twofold increase compared with the control group. In the completely vehicle-treated control mice almost only macrophages were found. In all TDI-treated mice an increase of macrophages was found, but this was only statistically different in the vehicle-sensitized and TDI-challenged mice (0/1/1/1), compared with the control mice. The amount of neutrophils was significantly increased in all TDI-challenged groups compared with the control groups. On the other hand, eosinophils were only increased in the groups that received three intranasal instillations with TDI (0/1/1/1 and 1/1/1/1). No differences were found in the amount of BAL lymphocytes in the different groups, compared with the control group.

Fig. 4A, B shows MIP-2 and TNF- α concentrations in BAL fluid respectively. All TDI-sensitized and TDI-challenged mice showed a clear statistical increase in both MIP-2 and TNF- α levels in BAL, compared with the completely vehicle-treated control mice.

Lymph node cell analysis

Fig. 5A, B shows results of flow cytometric analyses of lymphocyte sub-populations in auricular and cervical

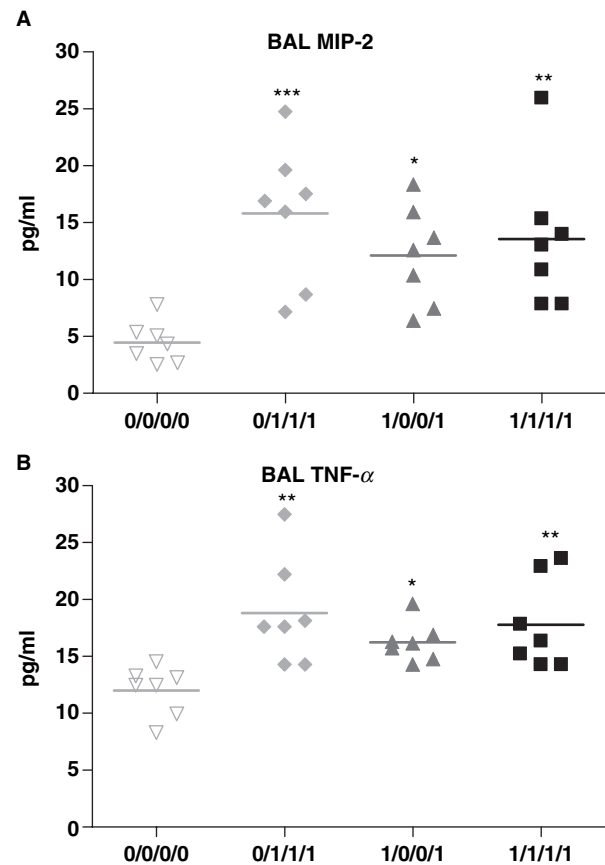


Figure 4 MIP-2 and TNF- α levels in BAL. On day 17, i.e. 24 h after the last intranasal challenge, the lungs of BALB/c mice were lavaged and cytokine levels of MIP-2 (A) and TNF- α (B) were measured in supernatants. Experimental group legends are as in Fig. 1. Bars represent mean $\pm$ SD. * P < 0.05, ** P < 0.01, *** P < 0.001 compared to 0/0/0/0 control group.

lymph nodes removed 24 h after the last intranasal challenge of TDI or vehicle. A significant increase in total number of CD3⁺CD4⁺ lymphocytes (Th cells); CD3⁺CD4⁺CD25⁺ (activated Th cells) and CD19⁺ lymphocytes (B cells) were apparent in both auricular (Fig. 5A) and cervical (Fig. 5B) lymph nodes of all TDI-challenged groups (0/1/1/1, 1/0/0/1 and 1/1/1/1) compared to the 0/0/0/0 group.

Fig. 6A, B and C shows the levels of IL-4, IL-13 and IFN- γ , respectively, in supernatants of auricular (left panel) and cervical (right panel) lymph node cells cultured for 18 h with ConA stimulation. The spontaneous release, without ConA stimulation, of IL-4, IFN- γ and IL-13 was low and not statistically different between the groups (data not shown). In ConA-stimulated auricular lymphocytes (Fig. 6A, C and E), levels of all three cytokines were significantly increased in the supernatants from the TDI-dermally sensitized mice (1/0/0/1 and 1/1/1/1) in comparison with those from the 0/0/0/0 control group. IFN- γ and IL-13 concentration in

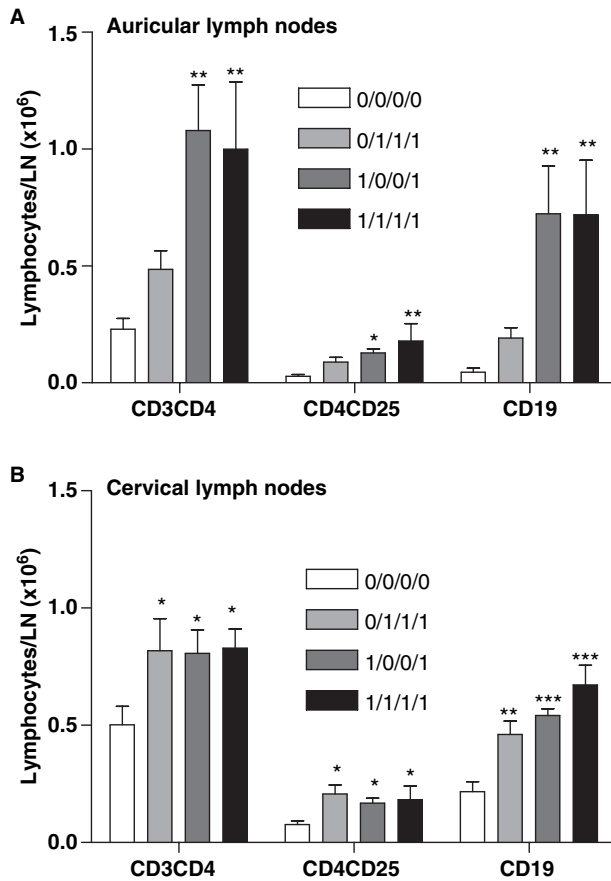


Figure 5 Auricular and cervical lymph node subpopulation analysis. Auricular (A) and cervical (B) lymph nodes were obtained on day 17, i.e. 24 h after the last intranasal challenge. The lymph nodes of four to five mice were pooled. Experimental group legends are as in Fig. 1. $n = 3-4$ experiments, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to the 0/0/0/0 control group.

supernatant of the non-sensitized and only TDI-challenged group (0/1/1/1) was also significantly increased compared with the control group. In the supernatant of cervical lymphocytes (Fig. 6B, D and E), levels of IL-4 were significantly increased in the 0/1/1/1 and 1/1/1/1 group of mice after ConA stimulation, compared with the control mice. IFN- γ concentrations, after ConA stimulation of cervical lymphocytes, were only significantly increased in the 0/1/1/1 group, whereas IL-13 concentration was increased in all TDI-challenged groups, but only reached significance in the 0/1/1/1 and 1/1/1/1 group, compared with the control group.

Total serum IgE

Fig. 7 shows total serum IgE levels on day 17, 1 day after the last intranasal challenge. Total serum IgE levels were significantly higher in the mice that had been dermally sensitized (1/0/0/1 and 1/1/1/1), compared with

the 0/0/0/0 control group. There were no increase in total serum IgE levels of the 0/1/1/1.

Discussion

This study describes the results of a multiple challenge protocol using TDI as a relevant sensitizer for inducing occupational (chemical-induced) asthma. Several phenotypical characteristics of occupational asthma became more obvious in this multi-exposure protocol, in comparison with the previously published single challenge protocol [7, 8]. Immediate airway obstruction after TDI-exposure, non-specific bronchial hyperreactivity to methacholine, inflammation characterized by neutrophils and eosinophils, along with BAL cytokine increases indicative of lung inflammation were found. Furthermore, clear changes in immunological parameters were found, e.g. increased number of Th-, Treg- and B cells in draining lymph nodes, *in vitro*, IL-4, IFN- γ , IL-13 production and an increased total serum IgE level in TDI-sensitized and TDI-challenged mice.

We are aware of the fact that we did not actually measure bronchoconstriction, when assessing changes in ventilatory function by unrestrained whole body plethysmography. The use of whole body plethysmography has been heavily criticized as being inadequate for assessing airway mechanics [11, 12]. While an increase in Penh does not necessarily reflect bronchial obstruction correctly, it remains that whole body plethysmography does assess changes in ventilatory patterns. Consequently, regardless of the physiological basis for the observed ventilatory responses in our experiments, there is no doubt (because our design included all necessary control groups) that early ventilatory responses and increases in methacholine responsiveness occurred in TDI-sensitized and TDI-challenged mice. We infer that these changes are of an obstructive character. Furthermore, data published by our group have shown similar changes between Penh and correctly measured lung resistance using the FlexiVent system, thereby suggesting that our Penh findings are valid [8, 9].

In the three responsive groups (TDI-challenged groups), an increase in BAL neutrophils was shown 24 h after the last intranasal instillation. Increased levels of MIP-2, a known chemo-attractant for neutrophils in mice [13], were also found in BAL. In our single intranasal instillation protocol, we found only macrophages and neutrophils in the lungs of the responding mice [8]. We thought that increasing the number of exposures to the airways would also lead to an influx of eosinophils and lymphocytes in the BAL, as normally found in ovalbumin-models of asthma [14, 15]. This was only the case for mice receiving three intranasal instillations, and the increase was limited. The absence of lymphocytes in the BAL remains puzzling. We know that lymphocytes are

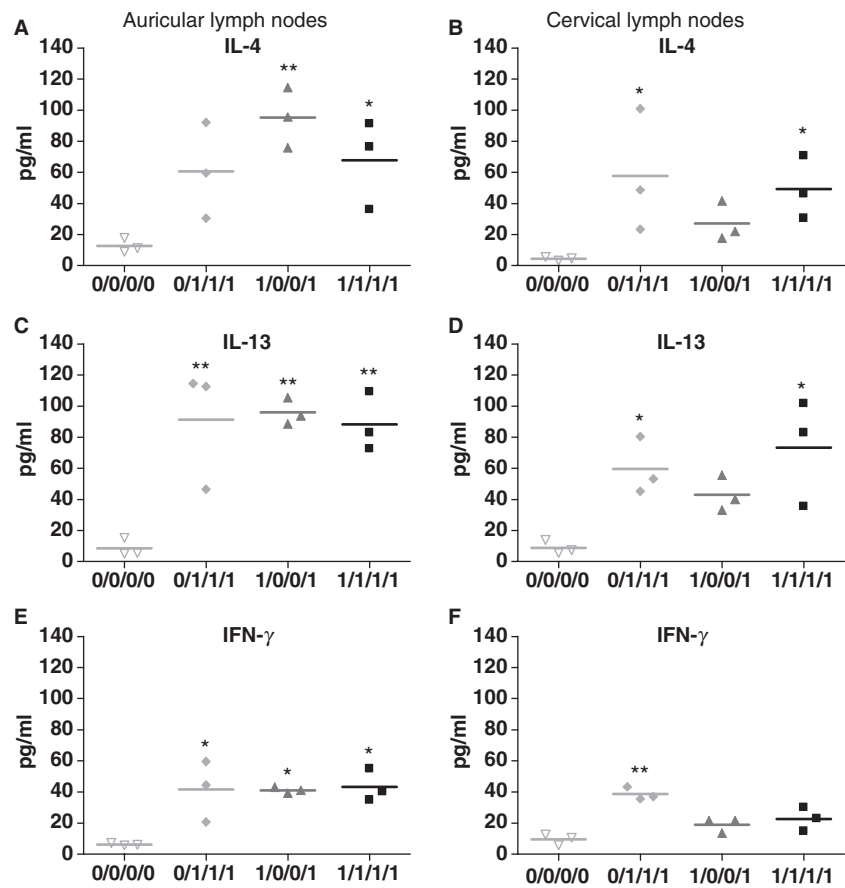


Figure 6 Cytokine concentrations in supernatants of *in vitro* cultured auricular and cervical lymphocytes. Auricular (A, C and E) and cervical (B, D and F) lymph nodes were obtained on day 17, i.e. 24 h after the last intranasal challenge. The lymph nodes of four to five mice were pooled, and cultured with concanavalin A. Levels of IL-4, IL-13 and IFN- γ , were measured in undiluted supernatants. Experimental group legends are as in Fig. 1. Individual data of three to four experiments are presented. A+B IL-4 cytokine levels C+D IL-13 cytokine levels E+F IFN- γ cytokine levels. * P < 0.05, ** P < 0.01 compared to the 0/0/0/0 control group.

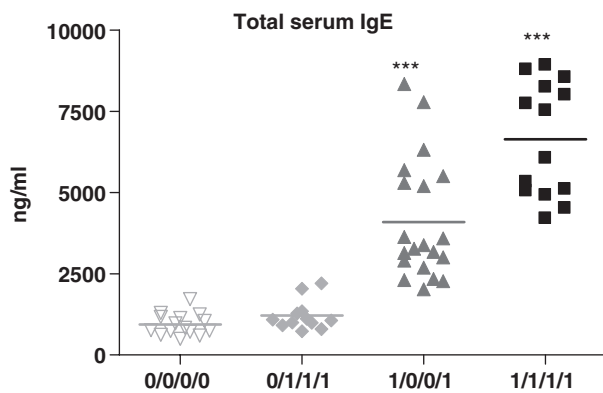


Figure 7 Total serum IgE level. On day 17, i.e. 24h after the last intranasal challenge, total serum immunoglobulin E levels were measured. Experimental group legends are as in Fig. 1. *** P < 0.001 compared to the 0/0/0/0 control group.

crucial for the elicitation of the ventilatory and inflammatory changes in TDI-treated mice, as we cannot find a response in SCID mice [8], but it remains intriguing that no lymphocytes are found in BAL. Further research is necessary to reveal this working mechanism.

In our single intranasal challenge protocol, concentrations of TNF- α were not changed in TDI-sensitized and

TDI-challenged mice [8]. On the other hand, using TMA in the same model we did find significant increases in TMA-sensitized and TMA-challenged mice [10]. These contradictory results made us doubt about the influence of TNF- α in this mouse model, because in other established models of isocyanates, it had been demonstrated that TNF- α was a key player [16]. Our current results now show a clear increase of TNF- α in BAL after multiple intranasal instillations of TDI, even without prior dermal sensitization (0/1/1/1). This probably reflects the irritant properties of TDI, as TNF- α can be released upon cell injury caused by irritants [17].

Although the involvement of Th2 cytokines and IgE is well accepted in atopic asthma, examples are known where asthma exists in the absence of detectable levels of IgE [18, 19]. Haptens do not necessarily need intracellular processing and protein binding to induce antigenic response: some chemicals can cross-link MHC-II molecules of antigen-presenting cells and T cell receptor [20, 21]. We found an increase in Th-lymphocytes, and B-lymphocytes in the draining lymph nodes of both the ears and the nose of TDI-challenged mice. We presume that the diminishing airway responses in the completely TDI-treated mice (1/1/1/1) could be linked to the increase in CD25⁺ Th cells, as regulatory T cells (T-reg,

CD25⁺FoxP3⁺) are a subpopulation of the activated Th cells (CD25⁺) and it has been shown in both human and mouse asthma studies, that T-reg down-regulate, via IL-10, the asthmatic phenotype [22–24].

Significant increases in total serum IgE were only found in dermally sensitized mice (1/0/0/1 and 1/1/1/1), supporting the notion that Th2 stimulation occurred. However, the absence of an increase in total serum IgE in the vehicle 'sensitized' and TDI-challenged mice (0/1/1/1), in which the respiratory and inflammatory responses were equally pronounced suggests that the increase in total serum IgE is not critical for this model.

When the lymphocytes from the auricular and cervical lymph nodes were cultured in the presence of conA, we not only found clear increase in Th2 cytokines IL-4 and IL-13, but also in Th1 cytokine IFN- γ . Our experiments do not provide definitive answers to the question of the nature of the lymphocyte activation in TDI-induced asthma, but they do suggest that both Th1 and Th2 lymphocytes are involved, not only in a single provocation model [8, 10], but also in a multiple provocation model, at least at the time of the analysis. Although this increase in both cytokines could indicate a lack of polarization of the response of TDI toward Th2 cytokines, we do observe that the relative increases in IL-4 and IL-13 are higher than the relative increase of IFN- γ . This mixed Th2–Th1 cytokine profile has also been observed in other mouse models [25–27]. In contrast to our single challenge protocol, we did find the cytokine profiles of the auricular lymphocytes also in the cervical lymphocytes when the mice were given multiple intranasal instillations. For future experiments, we should also investigate the mediastinal lymph nodes draining the site of the lungs. These lymph nodes could give us further information on what is happening in the lung itself.

In conclusion, to improve our mouse model of chemical-induced asthma and yield a more pronounced asthmatic response, we challenged our mice three times instead of once. Although, we found significantly more lung inflammation (both eosinophils and neutrophils) compared with the single challenge model, physiological airway responses diminished with increasing numbers of intranasal challenges in dermally sensitized mice, suggesting the induction of tolerance as shown previously [28]. Better insights in these mechanisms can lead to new therapeutic interventions for allergic asthma induced by either high or low molecular weight agents.

Acknowledgment

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