



Dermal exposure determines the outcome of repeated airway exposure in a long-term chemical-induced asthma-like mouse model

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ABSTRACT

Background: Exposure to diisocyanates is an important cause of occupational asthma (OA) in the industrialized world. Since OA occurs after long-term exposure to diisocyanates, we developed a chronic mouse model of chemical-induced asthma where toluene diisocyanate (TDI) was administered at two different exposure sites.

Objectives: Evaluating the effect of long-term respiratory isocyanate exposure - with or without prior dermal exposure- on sensitization, inflammatory responses and airway hyperreactivity (AHR).

Methods: On days 1 and 8, BALB/c mice were dermally treated (20 µl/ear) with 0.5% 2,4-toluene diisocyanate TDI or the vehicle acetone olive oil (AOO) (3:2). Starting from day 15, mice received intranasal instillations with 0.1% TDI of vehicle five times in a week, for five successive weeks. One day after the last instillation airway hyperreactivity (AHR) to methacholine was assessed, followed by an evaluation of pulmonary inflammation and structural lung changes. Immune-related parameters were assessed in the lungs (BAL and tissue), blood, cervical- and auricular lymph nodes.

Results: Mice repeatedly intranasally exposed to TDI showed systemic sensitization and a mixed Th1/Th2 type immune response, without the presence of AHR. However, when mice are first dermally sensitized with TDI, followed by repeated intranasal TDI challenges, this results in a pronounced Th2 response and AHR.

Conclusion: Dermal exposure to TDI determines airway hyperreactivity after repeated airway exposure to TDI.

1. Introduction

Exposure to diisocyanates is an important cause of occupational asthma (OA) (Maestrelli et al., 2009). Diisocyanates are low molecular weight (LMW) compounds, used in spray foam insulation, plastic packaging and paints. They may act as haptens and bind with self-proteins to form functional antigens recognized by the immune system (Hettick and Siegel, 2011; Wisniewski and Liu, 2013). To date, a growing number of animal and workplace observational studies suggest that skin exposure to isocyanates elicits immune sensitization. After sensitization, very low airway exposures, can induce asthma (Tarlo, 2003; Bello et al., 2007; Redlich and Herrick, 2008; Wisniewski et al.,

2011). Therefore, the American Conference of Governmental Industrial Hygienist lowered the threshold limit value time-weighted average (TLV-TWA) from 5 ppb to 1 ppb (ACGIH, 2016). However, Lynch et al (2018) criticized the basis for lowering the TLV (Lynch et al., 2018). The incidence of diisocyanate-induced-asthma varies from 6.1/100 person years (Jaakkola et al., 2013) to 9/100 person years (Collins et al., 2017). Yet, the incidence of allergic skin disease induced by diisocyanates is not well investigated.

Sub-chronic and chronic pulmonary exposure to diisocyanates has been studied before in rat and mouse to mimic human asthma (Scheerens et al., 1999; Matheson et al., 2005b; Ban et al., 2006; Lee et al., 2011). While adaptive immune responses have been investigated

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in diisocyanate asthma, little is known about the involvement of dendritic cells (DC) in this type of asthma (Matheson et al., 2005a). DC are the most important antigen presenting cells (APC) responsible for initiating an immune response. They are located near the epithelium, where they can sense and capture inhaled antigens present in the environment. After collecting, DC process the allergens into small peptides and migrate to the T cell zone of the local draining lymph nodes. There they present the processed antigens via the major histocompatibility complexes (MHC class I and II) to naive T cells, inducing polarized T-cells (Holgate, 2012; van Helden and Lambrecht, 2013). In total, three distinct DC subsets are defined in the lung each with their own surface markers and functional specialization: conventional DC (cDC), which can be further divided in CD11b⁺ and CD103⁺ cDC, plasmacytoid DC (pDC) and monocyte derived DC (moDC) (Plantinga et al., 2013). Each of those DC subsets are geared to induce a particular helper T cell response. These responses are context dependent: environmental signals (house dust mite, cigarette smoke, diesel exhaust particles etc...), dose, routes of exposures and cell-released molecular patterns influence the function of DC which emphasize the plasticity of DC (Willart and Lambrecht, 2009; Provoost et al., 2010; Plantinga et al., 2013; Kopf et al., 2015).

In this manuscript, we investigated the importance of exposure route (dermal/respiratory) on adaptive immunity, lung inflammatory responses and AHR in a chronic mouse model consisting of long-term nasal mucosa exposure, with or without a brief prior skin exposure.

2. Methods

2.1. Reagents

Toluene-2,4-diisocyanate (TDI) (98%, CAS584-84-9), acetyl- β -methylcholine (methacholine) and acetone were obtained from Sigma-Aldrich (Bornem, Belgium). Pentobarbital (Nembutal®) was obtained from the internal animal facility at the University of Leuven. The vehicle acetone olive oil (AOO) used to dissolve TDI consists of a mixture of 2 volumes acetone and 3 volumes of olive oil (highly refined, low acidity, CAS8001-25-0) obtained from Sigma Aldrich. Concentration of TDI is given as percentage (v/v) in AOO.

2.2. Mice

Male BALB/c mice (6–8 weeks old) were obtained from the internal stock of the animalium (University of Leuven, Belgium). All mice were housed in a conventional animal house with 12 h dark/light cycles, in filter top cages. They received lightly acidified water and pelleted food ad libitum. All experimental procedures were approved by the local Ethical Committee for animal experiments (P063/2015).

2.3. Experimental protocols of mouse experiments

On days 1 and 8, mice received dermal applications of 20 μ l of 0.5% TDI or the vehicle AOO. From day 15, mice received, under light isoflurane anesthesia, intranasal instillations (40 μ l) of 0.1% TDI or the vehicle AOO, 5 times a week for 5 consecutive weeks. All experimental groups are indicated with two abbreviations: the first abbreviation indicates the dermal treatment on days 1 and 8, whereas the second abbreviation indicates the intranasal instillations. The AOO/AOO group never received TDI, the AOO/TDI group only received intranasal TDI and the TDI/TDI group received TDI dermally and intranasally.

2.4. Analysis in different sets of experiments

In this study we have three different sets of experiments. In the first set we evaluated the AHR and histological changes in the lung, IgE and IgG in blood serum, IgE and inflammation in BAL and T and B cells with *in vitro* cytokine secretion of the auricular and cervical lymph nodes

(n = 6–7/group). To increase the number of the auricular and cervical lymph nodes, we included a second set where we evaluated the T and B-cells with *in vitro* cytokine secretion of the auricular and cervical lymph nodes (n = 4–6/group). To analyze the DC, we included a third set. In the latter set we analyzed IgE in blood serum (to confirm sensitization), inflammation in BAL and DC, T and B cells in lung cells (n = 8/group). In a subgroup of this set we also evaluated the T and B cells in the auricular and cervical lymph nodes (n = 4/group).

2.5. Airway and lung tissue hyperreactivity measurements

Twenty-four hours after the last instillation, airway and lung tissue reactivity to methacholine was measured using a forced oscillation technique (FlexiVent 7.1, SCIREQ, Montreal, Canada). Mice were anesthetized with pentobarbital (70 mg/kg body weight, Nembutal®). Applying the 'quick prime 3'(QP3) perturbation, airway resistance (Rn), and tissue elasticity (H) to increasing concentrations of methacholine (0, 1.25, 2.5, 5, 10, 20 mg/ml) was measured. After each concentration, the QP3 perturbation was performed 5 times spread over 2 min. If the COD (Coefficient of Determination) of a QP3 perturbation was lower than 0.90, the measurement was excluded and not used to calculate the average. For each mouse Rn and H were plotted against methacholine concentration and the area under the curve (AUC) was calculated to perform statistical analysis.

2.6. Lung, lymph node and serum analysis

2.6.1. Serum sampling

After measuring airway hyperreactivity to methacholine, mice were sacrificed. Blood was sampled from the retro-orbital plexus, centrifuged (14,000 g, 4 °C, 10 min) and serum samples were stored at –80 °C until analysis.

2.6.2. Broncho-alveolar lavage (BAL)

The lungs were lavaged, *in situ*, three times with 0.7 ml sterile saline (0.9% NaCl), and the recovered fluid was pooled. Cells were counted using a Bürker hemocytometer (total cell count) and the BAL fluid was centrifuged (1000 g, 10 min). For differential cell counts, 250 μ l of the resuspended cells (100,000 cells/ml) were spun (300 g, 6 min) (Cytospin 3, Shandon, TechGen, Zellik, Belgium) onto microscope slides, air-dried and stained (Diff-Quik® method, Medical Diagnostics, Düringen, Germany). For each sample, 200 cells were counted for the number of macrophages, eosinophils, neutrophils.

2.6.3. Single cell suspension

The pulmonary circulation was rinsed with saline/EDTA to remove the intravascular pool of cells. A cell suspension of the lungs was obtained using digestion medium (RPMI 1640 supplemented with 5% FCS, 2 mM L-glutamine, 0.05 mM 2-mercaptoethanol [Gibco; Invitrogen, Paisley, United Kingdom], 100 U/mL penicillin–100 mg/ml streptomycin [Invitrogen], 1 mg/ml collagenase type 2 [Worthington Biochemical, Lakewood, NY], and 0.02 mg/ml DNase I [grade II from bovine pancreas; Boehringer Ingelheim, Ingelheim, Germany]) for 45 min at 37 °C and 5% CO₂. Red blood cells were lysed by using ammonium chloride buffer. Cells were counted using a Bürker hemocytometer and resuspended (10⁷ cells/ml) in PBS.

The left and right retro-auricular as well as the two cervical lymph nodes from the same mice were pooled and kept on ice in RPMI-1640 (1X) + GlutaMAX™-I (Invitrogen, Merelbeke, Belgium). Cell suspensions were obtained by pressing the lymph nodes through a cell strainer (100 μ m) (BD Bioscience, Erembodegem, Belgium) and rinsing with 10 ml tissue culture medium (RPMI-1640 (1X) + GlutaMAX™-I). After centrifugation (1000 g, 10 min), cells were counted using a Bürker hemocytometer and resuspended (10⁷ cells/ml) in complete tissue culture medium (RPMI-1640 (1X) + GlutaMAX™-I supplemented with 10% heat-inactivated fetal bovine serum, 10 mg/ml streptomycin/penicillin).

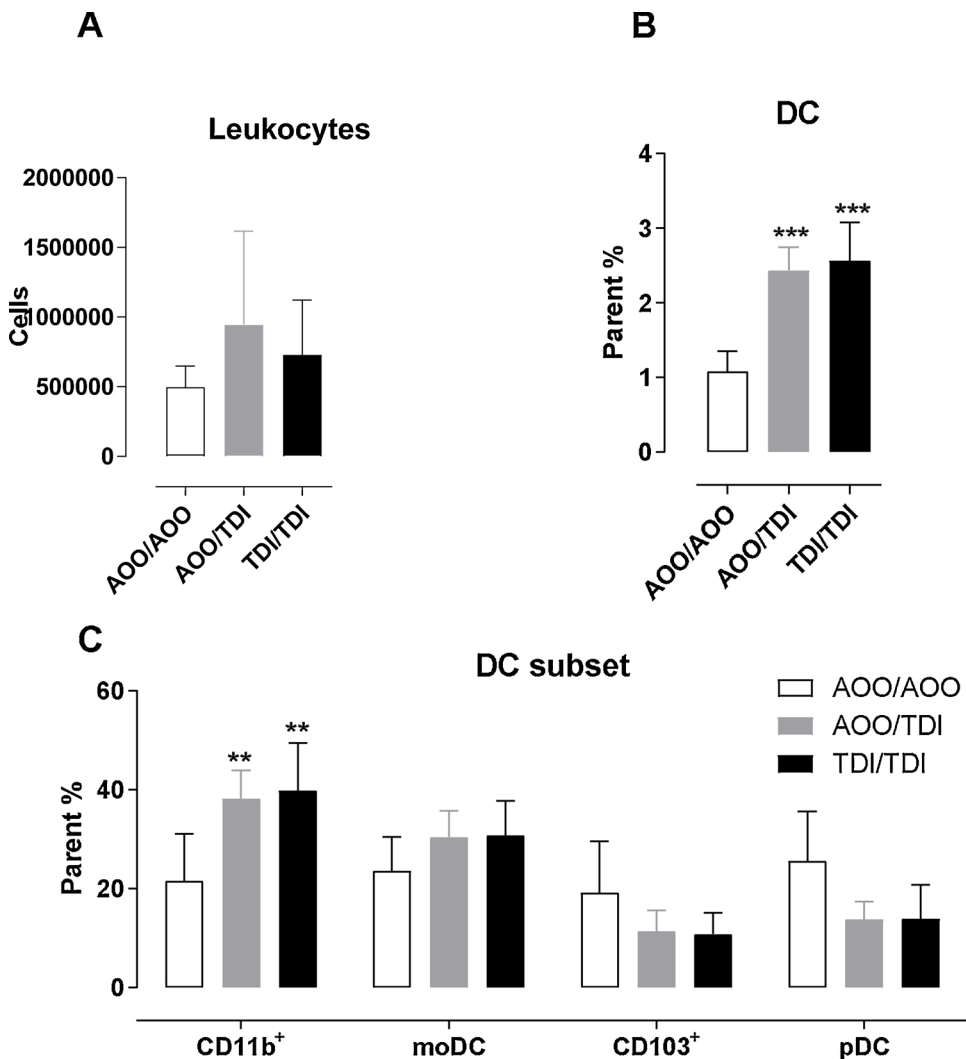


Fig. 1. Leukocytes and dendritic cells ALN. Cells were analyzed, using a flow cytometer. Leukocytes were assessed as CD45⁺ cells (a). DC were detected as low auto fluorescent MHCII⁺CD11c⁺ DC (b). Fig. 1c demonstrates the parent % of CD45⁺ low auto fluorescent MHCII⁺CD11c⁺CD11b⁺CD103⁺ cDC (CD103⁺ cDC), CD45⁺ low auto fluorescent MHCII⁺CD11c⁺CD11b⁺CD103[−] conventional DC (CD11b⁺ cDC), CD45⁺ low auto fluorescent MHCII⁺CD11c⁺CD11b⁺CD64⁺ monocyte derived DC (moDC), and CD45⁺ low auto fluorescent MHCII⁺CD11c⁺ SiglecH⁺ plasmacytoid DC (pDC). Experimental groups are identified by two abbreviations (AOO = acetone olive oil; TDI = toluene-2,4-diisocyanate); the first abbreviation indicates the dermal treatment on days 1 and 8, whereas the second abbreviation indicates the intranasal instillations. n = 8 per group. Bars shows the mean with SD. **p < 0.01 and ***p < 0.001 compared with the AOO/AOO control group.

2.6.4. Flow cytometry

2.6.4.1. T- and B cells. Five-hundred thousand cells from the auricular/cervical lymph nodes and lung were stained with anti-CD3⁺ (APC), anti-CD4⁺ (APC-Cy7), anti-CD8⁺ (PerCP-Cy5.5) and anti-CD25⁺ (PE), or received a single staining with anti-CD19⁺ (PE) labeled antibodies, according to standard procedures (BD Biosciences, Erembodegem, Belgium). Percentages of labeled cells were determined by performing flow cytometry (FACSArray, BD Biosciences, Erembodegem, Belgium) on at least 10⁵ cells.

2.6.4.2. Dendritic cells. To minimize nonspecific bindings, two million cells of lung and auricular lymph nodes, were preincubated with anti-CD16/CD32 (Clone 2.4G2) (from BD Biosciences, San Jose, Calif). After live/dead staining using the Zombie Aqua™ Fixable Viability Kit, cells were labeled with combinations of anti-mouse fluorochrome-conjugated mAbs against CD45 (clone 30-F11), CD11c (N418), MHCII (M5/114.15.2), CD11b (M1/70), CD103 (2E7), CD64 (X54-5/7.1), Siglec-H (551) (all from Biolegend, San Diego, Calif). Data acquisition was performed on a LSR Fortessa flow cytometer running DIVA software (BD Biosciences, San Jose, Calif). FlowJo software (TreeStar, Inc, Ashland, Ore) was used for data analysis. We composed our DC panel based on those of Provoost et al. and Plantinga et al. (Provoost et al., 2010, 2012; Plantinga et al., 2013)

2.6.4.3. Cytometric bead array. Cells of the auricular and cervical lymph nodes were seeded into 48-well culture plates at a density of

10⁶ cells/ml and incubated in complete RPMI-1640 (1X) + GlutaMAX[™]-I medium for 42 h with 2.5 µg/ml of concanavalin A (ConA) (Sigma–Aldrich, Bornem, Belgium). Cell suspension were centrifuged (1000 g, 10 min) and supernatant was stored at −80 °C. Concentrations of interleukin-4 (IL-4), IL-10, IL-13 and interferon gamma (IFN-γ) were measured via the LSR Fortessa (BD Biosciences, Erembodegem, Belgium). Detection limits were 0.2 pg/ml, 0.3 pg/ml, 9.6 pg/ml, 2.4 pg/ml and 0.5 pg/ml, respectively.

2.6.5. Enzyme-linked immuno sorbent assay (ELISA)

The OptEIA Mouse IgE set from pharmingen (BD Biosciences) was used to measure total IgE in serum (diluted 1/70 for AOO/AOO and 1/1000 for the AOO/TDI and TDI/TDI treated group) and BAL (undiluted). Measurements were performed according to the manufacturer's instructions. To measure total serum IgG₁ (diluted 1/8000 or 1/10⁶) an IgG_{2a} (diluted 1/4000) plates were coated using purified rat anti-mouse IgG₁ (Cat: 553445, BD Pharmingen™) and purified rat anti-mouse IgG_{2a} (Cat: 553446, BD Pharmingen™). A standard was created using purified mouse IgG₁ (Cat: 557273, BD Pharmingen™) and purified Mouse IgG_{2a} (Cat: 553454, BD Pharmingen™). The further measurements were performed according to the manufacturer's (BD Pharmingen) instructions with the use of biotinylated anti-mouse avidin horseradish peroxidase conjugate (HRP rat anti-mouse IgG₁, Cat: 559626, biotin rat anti-mouse IgG_{2a}, Cat: 553388, and streptavidin HRP, Cat: 554066, BD Pharmingen™).

The Quantikine mouse IL-13 ELISA from R&D systems (Abingdon, UK) was used to measure IL-13 levels in serum. The ELISA was

performed according to the manufacturer's instructions, using undiluted serum samples. The detection limit was 1.5 pg/mL.

2.6.6. Histology

Lungs were instilled with 4% formaldehyde until full inflation of all lobes. Evaluation of lung injury on slides stained by hematoxylin and eosin (H&E) was performed by an experienced pathologist in a blinded manner. Inflammation (i.e., infiltration of macrophages/monocytes or leukocytes), epithelial damage (i.e., disruption of the epithelial barrier in the airways) and alveolar widening (airspace enlargements) were scored on semi-quantitative scale of 0–4 (0 is no changes; 1 is minor changes; 2 is moderate changes; 3 is substantial changes; and 4 is severe changes).

2.7. Data analyses

The data are presented as means with standard deviation (SD), or as individual mice and group mean. Normality of distribution was assessed by the Kolmogorov-Smirnov test, followed by a one-way parametric ANOVA combined with a Bonferroni multiple comparison post hoc test. Dose-response curves (AHR) were analysed using two-way parametric ANOVA, followed by a Bonferroni multiple comparison post hoc test (Graph Pad Prism 5.01. Graphpad Software Inc, San Diego, USA). A level of $p < 0.05$ (two tailed) was considered significant.

3. Results

3.1. Markers of sensitization

3.1.1. Dendritic cells in auricular lymph nodes

Fig. 1b and c shows a significant increase of DC, CD11b⁺ cDC in both TDI-treated groups TDI (AOO/TDI and TDI/TDI) compared to the control group (AOO/AOO). There seems to be an increasing trend of moDC and a decreasing trend of CD103⁺ cDC and pDC in both TDI-treated groups, yet significance is not reached (Fig. 1c). Total number of leukocytes were not significantly increased (Fig. 1a).

3.1.2. T- and B cells in auricular and cervical lymph nodes

Fig. 2a shows that mice, treated with TDI (AOO/TDI and TDI/TDI), have significantly more T-(CD3⁺) and B-(CD19⁺) cells in the auricular lymph nodes than the AOO/AOO control group. More specifically, all T-cell subpopulations (CD4⁺ T-helper, CD4⁺CD25⁺ activated/regulatory T-cells and CD8⁺ cytotoxic T-cells) were significantly increased in both TDI-treated groups. In the cervical lymph nodes (Fig. 2b), CD19⁺ B-cells were increased in TDI/TDI treated group while CD8⁺ cytotoxic T-cells were only increased in the AOO/TDI treated group (Fig. 2b).

3.1.3. Ex vivo cytokine secretion

Ex vivo stimulation of auricular lymphocytes with concanavalin A had in significantly increased concentrations of IL-4, IL-10, IL-13 and IFN- γ in the TDI/TDI treated group, compared to the control group (AOO/AOO) (Fig. 3a–d). IL-4, IL-10 and IL-13 concentrations in the cultured auricular lymphocytes of the TDI/TDI group were also significantly higher compared to the non-sensitized, and only TDI challenged group (AOO/TDI) (Fig. 3a–c). The AOO/TDI treated group showed a significant increase in IL-13 and IFN- γ compared to the control group (AOO/AOO) (Fig. 3b and d). The ex vivo cytokine production of the cervical lymphocytes was substantially lower than in the auricular lymph nodes (Fig. 3e–h). In the TDI/TDI treated group increased concentrations of IL-4, IL-13 and IL-10 were found, while in the AOO/TDI treated group only IL-13 was significantly increased compared to the control group (AOO/AOO) (Fig. 3e–g). The IFN- γ levels were not significantly changed (Fig. 3h).

3.1.4. Immunoglobulins and serum IL-13

Fig. 4a shows that mice, which received weekly intranasal instillations with TDI (AOO/TDI), resulted in a significant increase of total serum IgE

compared to the control group (AOO/AOO). However, TDI skin exposure followed by TDI-intranasal instillations (TDI/TDI) resulted in a significantly higher concentration of IgE in serum compared to the TDI challenged and control groups (AOO/TDI and AOO/AOO) (Fig. 4a). Besides the serum, IgE was also significantly increased in the BAL fluid of the TDI/TDI group (Fig. 4d). Serum IgG₁ was significantly increased and IgG_{2a} was significantly decreased in the TDI/TDI group, compared to the control group (AOO/AOO) (Fig. 4b and c). IL-13 in serum showed a significant increase in both TDI treated groups (AOO/TDI and TDI/TDI) compared to the control group (AOO/AOO). Moreover, the IL-13 level in the TDI/TDI group is significantly higher compared to AOO/TDI group (Fig. 4e).

3.2. Airway response

3.2.1. Airway hyperreactivity

Fig. 5a and c, shows the dose-response increase of methacholine-induced airway resistance (Rn) and tissue elasticity (H), while Fig. 5b and d show the area under the curve of the individual mice and the group average. Airway hyperreactivity (AHR) in the TDI/TDI group is significantly increased compared to both other groups (AOO/TDI and AOO/AOO).

3.2.2. Broncho-alveolar lavage and lung histology

The AHR in TDI/TDI treated group was not accompanied by increases in inflammatory cells (neutrophils, eosinophils or lymphocytes) in BAL fluid, where only macrophages were present (mean $\pm$ SD: AOO/AOO 7.5 $\pm$ 3.9 ($\times 10^4$); AOO/TDI 5.3 $\pm$ 4.2 ($\times 10^4$); TDI/TDI 5.9 $\pm$ 2.4 ($\times 10^4$)). Histological analysis revealed that long-term intranasal exposure to TDI resulted in limited increased inflammation round the blood vessels, characterized by macrophages/monocytes and leucocytes, epithelial shedding, with limited disruption of the epithelial barrier in the airways of mice challenged with TDI and alveolar widening, characterized by airspace enlargements. Yet, none of the semi-quantitatively judged parameters are significantly different (Fig. 6a–c).

3.2.3. T- and B cells in the lung

Table 1 shows a significant increase in CD3⁺CD8⁺ cytotoxic T cells in the TDI/TDI treated group compared to the AOO/AOO group. No statistical differences were found in CD3⁺ T helper cells of the TDI-treated groups (AOO/TDI and TDI/TDI), compared to the control group (AOO/AOO).

3.2.4. Dendritic cells in the lung

Only the moDC subpopulation in the lung from the TDI/TDI treated group are significantly higher than the control group (AOO/AOO) (Table 2). There were no significant differences in total number of leukocytes, dendritic cells, nor in the DC subpopulations: 103⁺cDC, CD11b⁺cDC, pDC and macrophages between the different treatment groups.

4. Discussion

Many efforts have been done to elucidate the mechanisms of chemical-induced asthma. Most of the work is performed in acute mouse models of chemical-induced asthma, with limited numbers of airway exposures (Vanoirbeek et al., 2004, 2008; Tarkowski et al., 2007; Lee et al., 2011; De Vooght et al., 2013; Pauluhn, 2014; Devos et al., 2015, 2016; Cruz et al., 2016; Pollaris et al., 2016). However, it takes weeks to years before initial onset of work-related asthma symptoms occurs. Here, we show that TDI skin exposure in combination with repeated five-week TDI airway exposure results in a pronounced Th2 hypersensitivity and AHR, while mice only exposed via the nasal mucosa to TDI, without prior dermal contact, showed a more Th1/Th2 systemic response without airway hyperreactivity.

TDI is able to migrate from the skin to the T-cell rich area of the cortex in the dermal lymph nodes (Nayak et al., 2014). This suggests

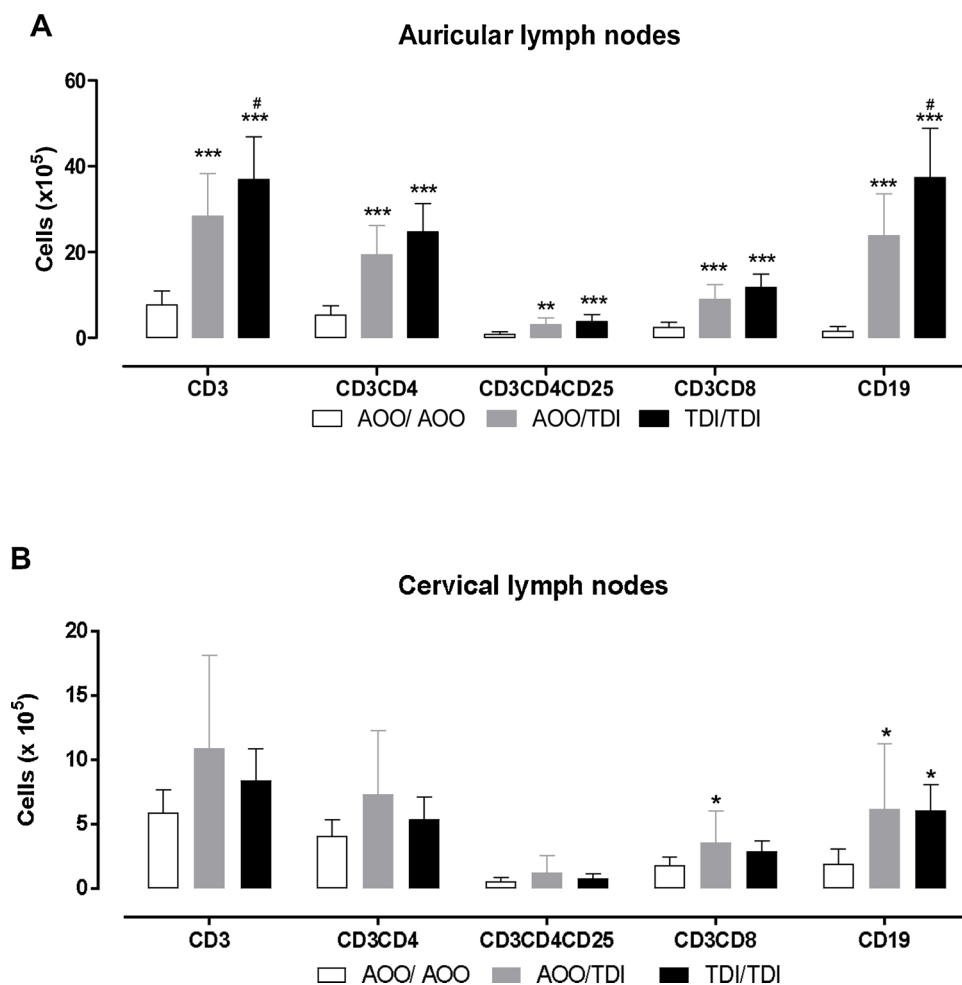


Fig. 2. Lymphocyte subpopulations in auricular and cervical lymph nodes. Lymphocyte subpopulations in ALN (a) and CLN (b) were measured using flow cytometry. Lymphocytes were stained with anti-CD3⁺ (T lymphocytes), anti-CD3⁺CD4⁺ (Th-lymphocytes), anti-CD3⁺CD4⁺CD25⁺ (activated/regulatory Th-lymphocytes) and anti-CD3⁺CD8⁺ (Tc-lymphocytes) or stained with a single anti-CD19⁺ (B-lymphocytes). Experimental groups are identified by two abbreviations (AOO = acetone olive oil; TDI = toluene-2,4-diisocyanate); the first abbreviation indicates the dermal treatment on days 1 and 8, whereas the second abbreviation indicates the intranasal instillations. *n* = 14 per group. Bars show the mean with SD. ***p* < 0.01 and ****p* < 0.001 compared with the AOO/AOO control group. #*p* < 0.05 compared to the AOO/TDI treated group.

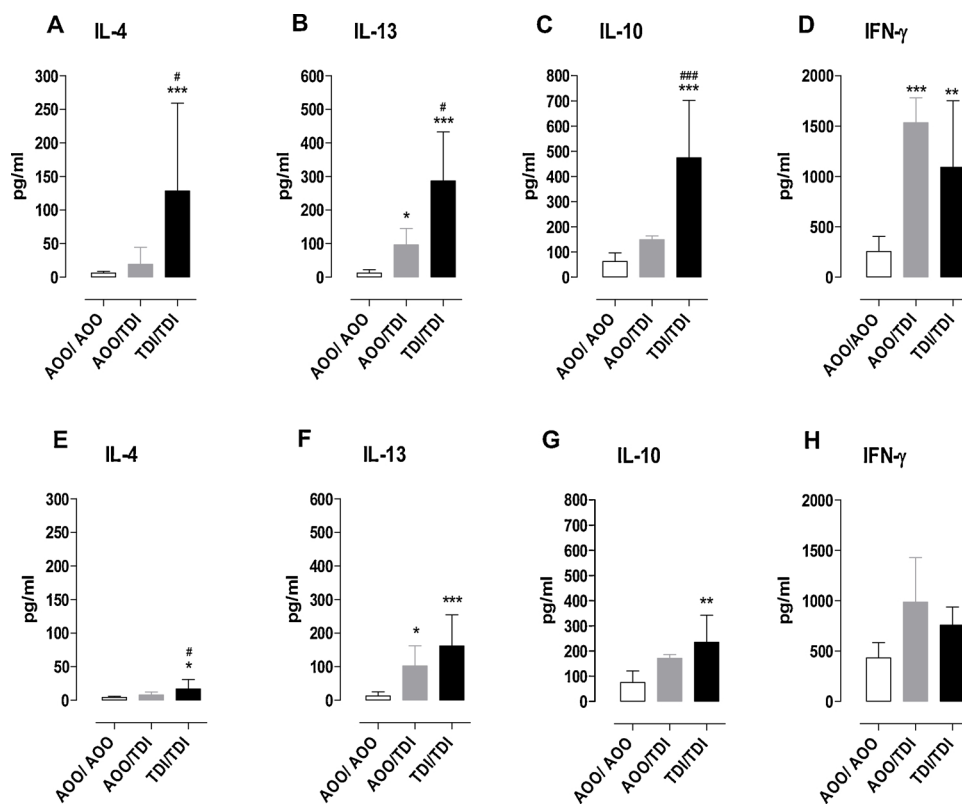


Fig. 3. Ex vivo cytokine production by auricular (a–d) and cervical (e–h) lymphocytes. Levels of IL-4 (a, e), IL-13 (b, f), IL-10 (c, g) and IFN-γ (d, h) were measured by Cytometric Bead Array in supernatant of auricular and cervical lymphocytes. Experimental groups are identified by two abbreviations (AOO = acetone olive oil; TDI = toluene-2,4-diisocyanate); the first abbreviation indicates the dermal treatment on days 1 and 8, whereas the second abbreviation indicates the intranasal instillations. *n* = 10–11 per group for the ALN, and 6–10 for the CLN. Bars show the mean and SD. **p* < 0.05, ***p* < 0.01 and ****p* < 0.001 compared with the AOO/AOO control group. #*p* < 0.05 compared to AOO/TDI.

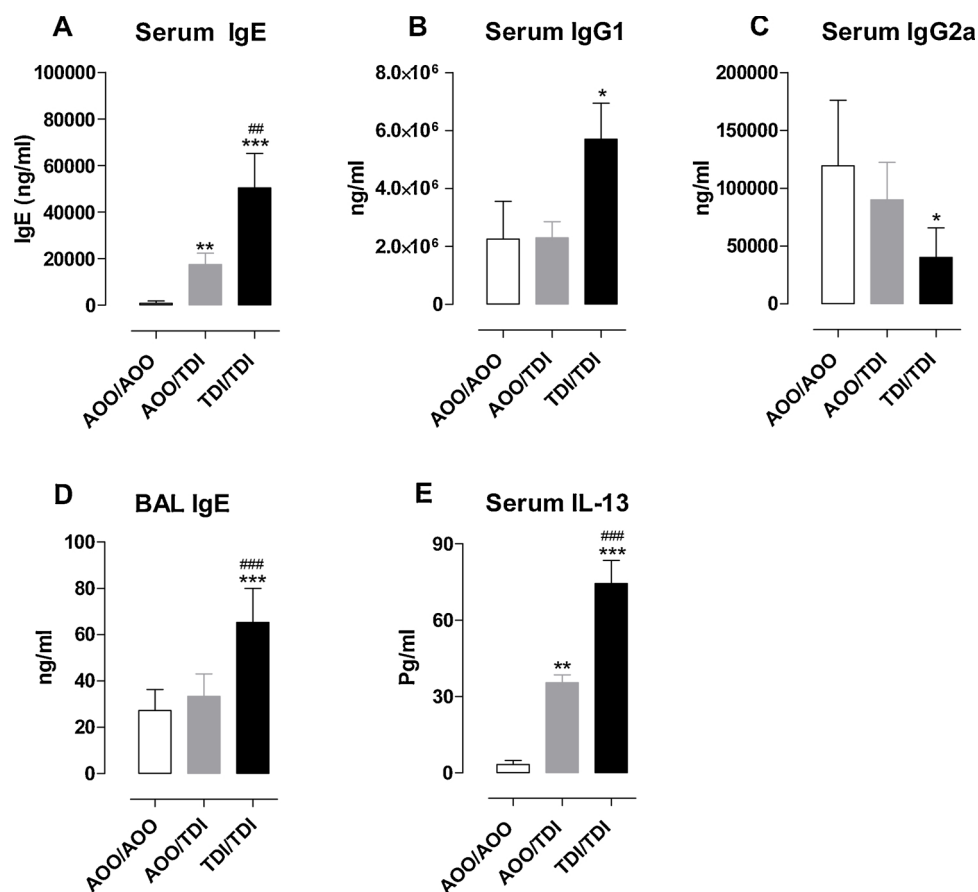


Fig. 4. Total levels of immunoglobulin (Ig) E, IgG₁ and IgG_{2a} and IL-13. Serum IgE (a), BAL IgE (d), serum IgG₁ (b), serum IgG_{2a} (c), and serum IL-13 (e) were measured using ELISA. Blood and BAL were collected 24 h after the last intranasal challenge. Experimental groups are identified by two abbreviations (AOO = acetone olive oil; TDI = toluene-2,4-diisocyanate); the first abbreviation indicates the dermal treatment on days 1 and 8, whereas the second abbreviation indicates the intranasal instillations. n = 6–7 per group for serum IgG_{2a}, IgG₁, IL-13 and BAL IgE, and n = 14–15 for serum IgE. Bars show the mean and SD. *p < 0.05 and ***p < 0.001 compared with the AOO/AOO control group. ###p < 0.001 compared to the AOO/TDI treated group. ##p < 0.01 and ###p < 0.001 compared to AOO/TDI.

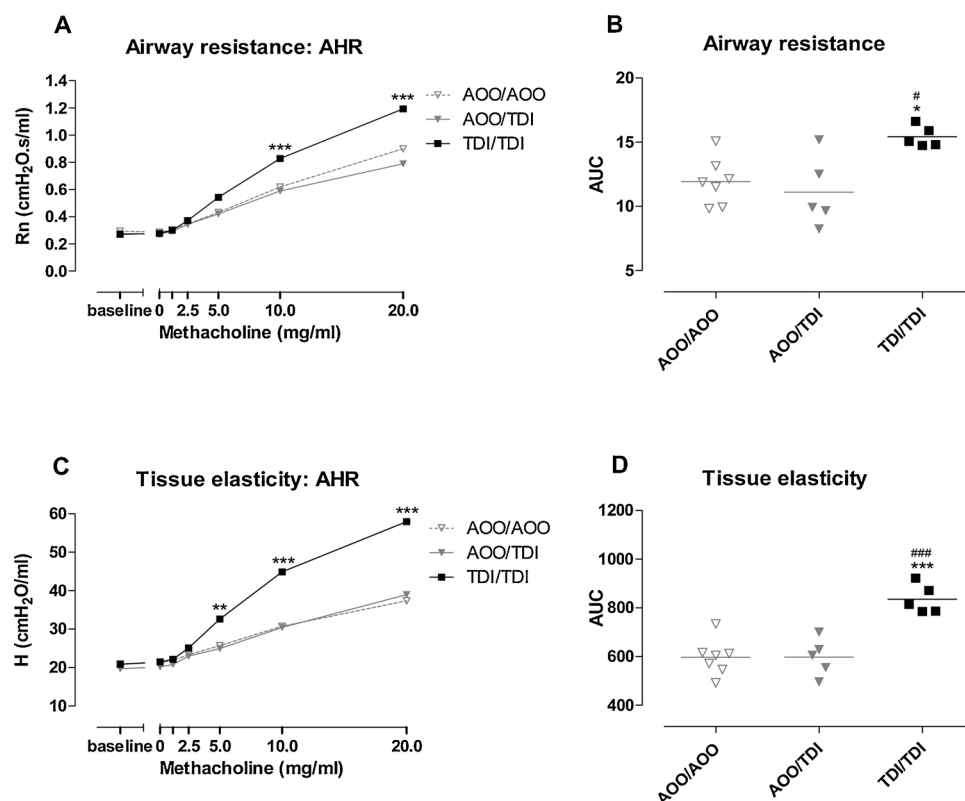


Fig. 5. Airway and tissue hyperreactivity. Methacholine responsiveness was assessed 24 h after the last intranasal challenge. The airway reactivity to methacholine was measured using a forced oscillation technique. Fig a and c show the mean values of Rn and H, respectively. Fig b and d, respectively, show individual values and the groups mean of the area under the curve (AUC) of Rn and H against methacholine concentrations between 0 and 20 mg/ml. Experimental groups are identified by two abbreviations (AOO = acetone olive oil; TDI = toluene-2,4-diisocyanate); the first abbreviation indicates the dermal treatment on days 1 and 8, whereas the second abbreviation indicates the intranasal instillations. n = 5–7 per group. Graphs show the mean. *p < 0.05, ***p < 0.001 compared with the AOO/AOO control group. #p < 0.05 and ###p < 0.001 compared to AOO/TDI treated group.

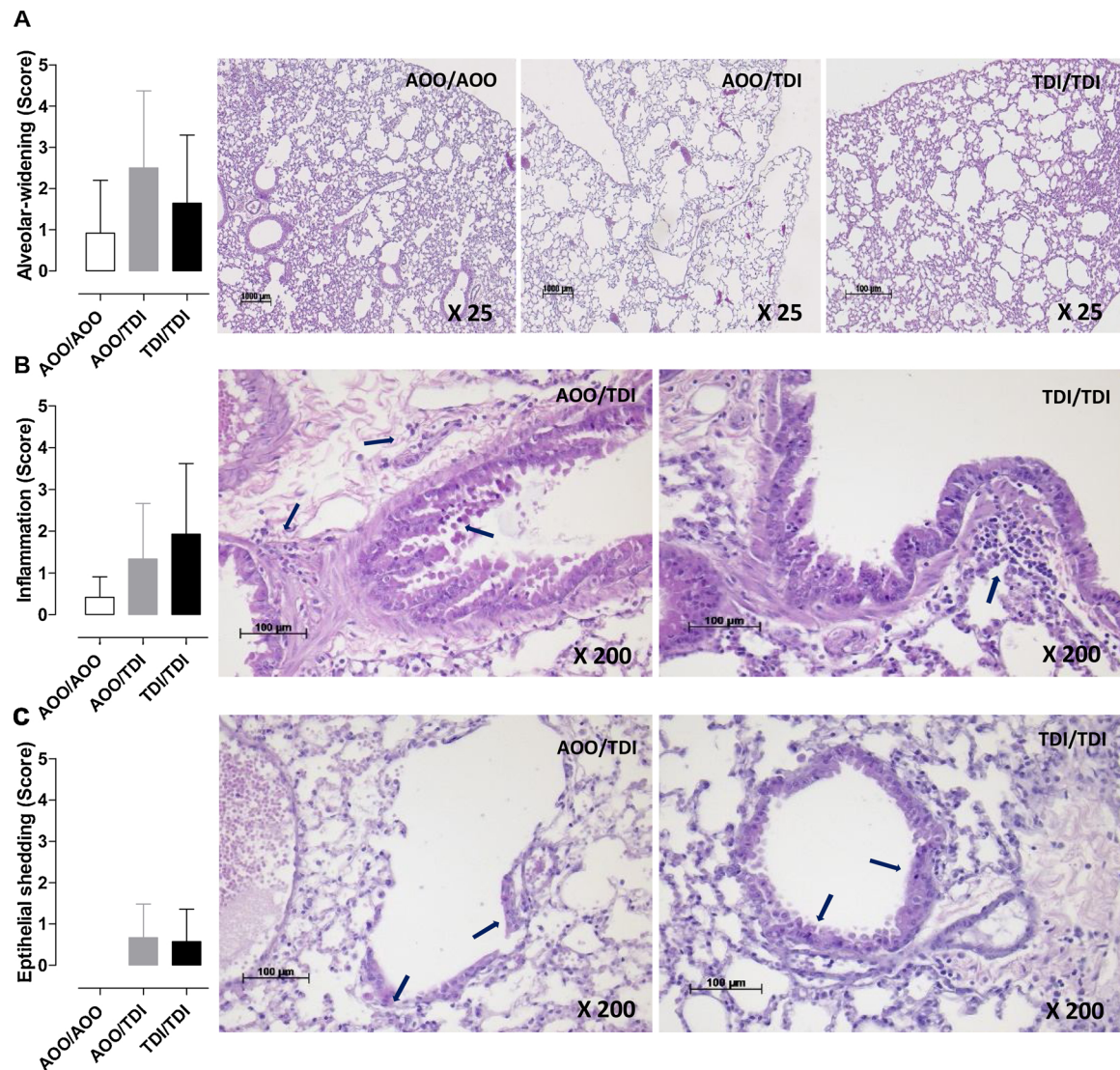


Fig. 6. Lung histology. H&E staining was performed on lung slices 24 after the last intranasal instillation. Alveolar widening (fig. 6a, 25x amplification), inflammation (fig. 6b, 200x amplification) and epithelial shedding (fig. 6c, 200x amplification) were scored on a semi-quantitative scale from 0 to 4, in a blinded manner. The score for the epithelial shedding of the AOO/AOO group is zero. Experimental groups are identified by two abbreviations (AOO = acetone olive oil; TDI = toluene-2,4-diisocyanate); the first abbreviation indicates the dermal treatment on days 1 and 8, whereas the second abbreviation indicates the intranasal instillations. $n = 6-7$ per group. Bars show the mean with SD.

Table 1
Lymphocyte subpopulations in the lung.

T cells lung (%)	AOO/AOO	AOO/TDI	TDI/TDI
CD3 ⁺	9,09 ± 1,97	10,38 ± 1,76	10,77 ± 2,21
CD3CD4 ⁺	5,9 ± 1,38	6,76 ± 1,30	6,83 ± 0,98
CD3CD4CD25 ⁺	0,82 ± 0,37	1,12 ± 0,53	0,96 ± 0,22
CD3CD8 ⁺	2,63 ± 0,66	3,12 ± 0,62	3,50 ± 0,96*
CD19 ⁺	12,16 ± 3,59	9,69 ± 2,38	10,64 ± 3,42

Lung cells were stained with anti-CD3⁺ (T-lymphocytes), anti-CD3⁺CD4⁺ (Th-lymphocytes), anti-CD3⁺CD4⁺CD25⁺ (activated/regulatory Th-lymphocytes) and anti-CD3⁺CD8⁺ (Tc-lymphocytes) or stained with a single anti-CD19⁺ (B-lymphocytes). Experimental groups are as described in Fig. 1. $n = 12-14$ per group. The data is expressed as the mean with SD. * $p < 0.05$ compared with the AOO/AOO control group.

that after TDI uptake in the skin, the APC migrate to the local lymph nodes and present TDI to the local T cells. Interestingly, the AOO/TDI group also showed partly similar signs of systemic sensitization suggesting that, next to the skin, the auricular lymph nodes also drain the

nasal mucosa. This was strengthened by the fact that DC, T- and B cells were significantly increased in the ALN of both TDI treated groups. When we investigated the different subpopulations in detail, a similar significant increase of cytotoxic T cells, regulatory T cells, helper T cells, B cells, CD11c⁺ and CD11b⁺ cDC, and an increasing trend of moDC was found in the ALN of both TDI-treated groups, independently of initial dermal TDI contact. As CD103⁺ and pDC both can induce T regulatory cells that damp allergic immune responses, the decreasing trend of both cells confirms the sensitization to TDI (van Helden and Lambrecht, 2013). As we focused on the panel composition of DC in the lung, we are aware that we might have missed some dermal DC subpopulations in the TDI/TDI group (Malissen et al., 2014).

Sensitization is also shown by a significant increase of serum IgE in both groups, confirming previous research (Matheson et al., 2005b; Ban et al., 2006). Yet, in the TDI-dermally sensitized mice, the IgE serum concentration was three times higher than the AOO/TDI treated group. This suggests that contact of a sensitizer with two different exposure sites, skin and airway mucosa, results in a much stronger immune response compared to only TDI airway exposure.

An important immunological difference between AOO/TDI and

Table 2
Leukocytes, macrophages and DC subpopulations in the lung.

Lung	AOO/AOO	AOO/TDI	TDI/TDI
Leukocytes (# cells)			
Leukocytes	294486 ± 80507	345315 ± 93944	374588 ± 88273
Antigen presenting cells (%)			
DC	5,04 ± 1,19	5,28 ± 1,34	5,52 ± 1,69
103 ⁺ cDC	10,74 ± 2,12	12,31 ± 2,14	11,06 ± 1,44
CD11b ⁺ cDC	45,09 ± 3,59	44,49 ± 5,39	48,54 ± 5,01
moDC	43,96 ± 3,25	44,96 ± 5,46	49,48 ± 2,78*
pDC	8,65 ± 1,71	8,58 ± 2,67	6,62 ± 2,87
Macrophages	4,89 ± 0,81	4,83 ± 1,36	5,11 ± 0,59

Cells were analyzed using flow cytometry as CD45⁺ leukocytes, CD45⁺ low auto fluorescent MHCII⁺CD11c⁺ DC (DC), CD45⁺ low auto fluorescent MHCII⁺CD11c⁺CD11b⁺CD103⁺ cDC (CD103⁺ cDC), CD45⁺ low auto fluorescent MHCII⁺CD11c⁺CD11b⁺CD103⁺ conventional DC (CD11b⁺ cDC), CD45⁺ low auto fluorescent MHCII⁺CD11c⁺CD11b⁺CD64⁺ monocyte derived DC (moDC), and CD45⁺ low auto fluorescent MHCII⁺CD11c⁺ SigleCH⁺ plasmacytoid DC (pDC). Macrophages were analyzed as auto fluorescent cells. Experimental groups are as described in Fig. 1. n = 8 per group. The data is expressed as the mean with SD. *p < 0.05 compared with the AOO/AOO control group.

TDI/TDI groups is illustrated by the cytokines production profiles of the auricular and cervical lymph nodes. The AOO/TDI treated group showed a mixed Th1/Th2 response; indicated by an increase of *ex vivo* IFN- γ secretion and IL-13 concentration both in serum and *ex vivo* secretion. Whereas, the TDI/TDI treated group had a similar Th1 response, but a much more pronounced Th2 phenotype response, as shown by a high *ex vivo* Th2 cytokine secretion (IL-4, IL-10 and IL-13), along with high IL-13 serum levels. The pronounced Th2 response in the fully TDI-treated group is also demonstrated by an increase of serum IgG₁, along with IgE and decrease of IgG_{2a} in the serum. Again, this indicates that skin sensitization in combination with airway exposure leads to a pronounced Th2 response. Previously, Ban et al., 2006 showed similar results when topical sensitization was followed by tracheal instillation, leading to a Th2 type lung inflammatory response, whereas sensitization via the airways was not effective (Ban et al., 2006).

Yet, although both TDI treated groups (AOO/TDI and TDI/TDI) showed pronounced sensitization, only the TDI/TDI treated group showed AHR. The AHR was not accompanied by a neutrophilic, nor an eosinophilic airway inflammation in BAL, and only very limited histological alteration in the blood vessels was found. An explanation could be that the delivered volume/dose of TDI into the lungs was too limited. To estimate the volume of the intranasal instillation, we based on findings of Southam et al., who demonstrated that the instillation volume and anesthesia affect the distribution of substances in the lungs (Southam et al., 2002). Therefore, we performed the instillation under isoflurane anesthesia and increased the instillation volume to 40 μ l. At this volume, 40–60% of the substance is distributed to the lungs (Southam et al., 2002). Yet, these distribution ranges are only demonstrated for substances dissolved in aqueous solutions. As TDI is not dissolvable in an aqueous solution we used the viscous vehicle acetone/olive oil which will reduce substance distribution and volume that reaches the lower airways (Ebino et al., 1999).

Our results suggest that the Th2 immune response, induced by the combined skin and (upper) airway exposure, is necessary to induce AHR. Probably, as we showed previously in an acute mouse model of chemical-induced asthma, a complex interplay between neurogenic mechanisms (TRPA1 and NK1 receptor), innate immune mechanisms (mast cells) and adaptive immune mechanisms are involved in the induction of airway hyperreactivity (Devos et al., 2016). Notably, the serum IgE levels of the TDI/TDI treated group were three times higher compared to the AOO/TDI treated group, even five weeks after the initial dermal sensitization phase. Moreover, the levels of total serum

IgE are 20 times higher than in our acute mouse model of chemical-induced asthma (Devos et al., 2016; Pollaris et al., 2016). The literature already showed a significant correlation between total serum IgE and the presence of asthma and AHR. It is suggested that high levels of IgE can induce ongoing mast cell activation, a cell shown to be crucial in our chemical-induced asthma mouse model (Sears et al., 1991; Sunyer et al., 1995; Devos et al., 2016; Virk et al., 2016). To further investigate the presence of IgE in the lung, we measured IgE directly in the BAL fluid. IgE was only significantly increased in TDI/TDI treated group, which can explain the presence of AHR only in the TDI/TDI treated group. Unfortunately, we could not prove that these IgE's were TDI specific as this is technically challenging (Wisniewski et al., 2011).

IL-13 has already been shown to play a key role for inducing AHR (Hacha et al., 2012). Also, we found very high levels of the Th2 marker IL-13 both in serum and *ex vivo* T cell secretion in the TDI/TDI treated group, which might further explain the presence of AHR in this group. Also, in the AOO/TDI group IL-13 was increased but to a significant lesser extent. As the choice of mouse strain is known to have an impact on the phenotypical outcome in asthma mouse models (De Vooght et al., 2010), we used the Th2-prone BALB/c mouse strain. This mouse strain is known to be more susceptible to reproduce asthmatic hallmarks. Yet, despite this Th2 predisposition, we were unable to induce lung inflammation, while the Th2 marker, IL-4, IL-13 and IgE were highly upregulated.

All these experiments further confirm the importance of initial topical exposure to chemicals on the airway response as also demonstrated by our and other groups (Herrick et al., 2003; Vanoirbeek et al., 2004, 2008; Ban et al., 2006; Wisniewski et al., 2011; Pauluhn and Poole, 2011; De Vooght et al., 2013; Devos et al., 2016; Pollaris et al., 2016).

An important novelty in our research is that we investigated whether DC, the most 'professional' APCs in the lung and responsible for initiating an immune response and the induction of an asthmatic response, still play a role in the effector phase 46 days after the initial TDI contact (Provoost et al., 2010, 2012; Plantinga et al., 2013). As this is still unstudied in isocyanate-induced asthma, we analyzed the different DC and T cell subsets in the lung (Lambrecht and Hammad, 2003; Kopf et al., 2015). No significant increase of DC was found in the lung after long-term exposures to TDI. This could be because DC, after allergen capture, migrate to the T cell area of the draining mediastinal lymph nodes (MLN) within twelve hours via a CCR7 dependent manner (Lambrecht and Hammad, 2003). Although there was no significant increase of DC, there was a shift between the DC subpopulations, illustrated by a significant increase of moDC in the TDI/TDI treated group. As they do not express CCR7, moDC do not migrate well to the draining nodes leading to an accumulation into the lung (Lambrecht and Hammad, 2014). As moDC have the capacity to initiate and maintain a Th2 immune response it can explain the pronounced Th2 type sensitization in the TDI/TDI treated group (Plantinga et al., 2013). The absence of the increase of DC in the lung after a long-term exposure protocol is however in contrast with other findings (Johnson et al., 2004; Provoost et al., 2010, 2012; Plantinga et al., 2013). These differences in DC response can be due to the different allergen exposure (house dust mite and diesel exhaust particles) and different airway exposure. Often an intratracheal aspiration technique is used, which leads to a higher distribution to the lungs (80%) compared to the intranasal instillation method (50–60%) (Foster et al., 2001; Southam et al., 2002; Provoost et al., 2010; Plantinga et al., 2013). Along with the instillation technique, the vehicle (aqueous vehicle), versus our much more viscous vehicle (olive oil) influences the distribution in the lungs (Ebino et al., 1999).

Our findings prove that repeated airway exposure to TDI causes immunological alterations indicative of sensitization, but are not accompanied by physiological changes indicative of asthma (i.e. AHR). However, the same type of airway exposure does lead to AHR if there has been previous dermal contact with TDI. These results implicate that

long-term airway exposure to low TDI concentrations have a higher risk of resulting in asthma if dermal exposure occurred earlier, again suggesting that dermal protection is important to protect TDI-induced OA.

Conflict of interest

The authors declare no conflict of interest.

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