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Original Article

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Involvement of Innate Lymphoid Cells and Dendritic Cells in a Mouse Model of Chemical-induced Asthma

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ABSTRACT

Purpose: Exposure to low concentrations of toluene diisocyanate (TDI) leads to immune-mediated chemical-induced asthma. The role of the adaptive immune system has already been thoroughly investigated; nevertheless, the involvement of innate immune cells in the pathophysiology of chemical-induced asthma is still unresolved. The aim of the study is to investigate the role of innate lymphoid cells (ILCs) and dendritic cells (DCs) in a mouse model for chemical-induced asthma.
Methods: On days 1 and 8, BALB/c mice were dermally treated (20 μL/ear) with 0.5% TDI or the vehicle acetone olive oil (AOO; 2:3). On days 15, 17, 19, 22 and 24, the mice received an oropharyngeal challenge with 0.01% TDI or AOO (1:4). One day after the last challenge, airway hyperreactivity (AHR) to methacholine was assessed, followed by an evaluation of pulmonary inflammation and immune-related parameters, including the cytokine pattern in bronchoalveolar lavage fluid, lymphocyte subpopulations of the lymph nodes and their *ex vivo* cytokine production profile, blood immunoglobulins and DC and ILC subpopulations in the lungs.
Results: Both DC and ILC2 were recruited to the lungs after multiple airway exposures to TDI, regardless of the prior dermal sensitization. However, prior dermal sensitization with TDI alone results in AHR and predominant eosinophilic airway inflammation, accompanied by a typical type 2 helper T (Th2) cytokine profile.
Conclusions: TDI-induced asthma is mediated by a predominant type 2 immune response, with the involvement of adaptive Th2 cells. However, from our study we suggest that the innate ILC2 cells are important additional players in the development of TDI-induced asthma.

Keywords: Toluene diisocyanate; mice; dendritic cells; innate lymphoid cells; immune system; asthma; bronchoalveolar lavage fluid; methacholine; T-lymphocyte

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There are no financial or other issues that might lead to conflict of interest.

INTRODUCTION

Mostly, asthma develops during childhood, depending on sensitization to common environmental allergens. Yet, asthma can also emerge later in life. This late-onset asthma in adults is often non-allergic and tends to be more severe compared to early-onset asthma.[1] Of adults with late-onset asthma, 5%–25% suffer from occupational asthma, which is a consequence of exposure to harmful chemical agents at the workplace, such as diisocyanates.[2]

Skin exposure to diisocyanates, such as toluene diisocyanate (TDI), induces systemic T cell-dependent sensitization.[3,4] After sensitization, exposure to low concentrations of diisocyanates can result in an asthmatic airway response.[5,6] Tarkowski *et al.*[7] and Devos *et al.*[8] have shown in severe combined immune-deficient mice, which lack T and B cells, that ventilatory and inflammatory airway responses in chemical-induced asthma are lymphocyte dependent. Interestingly, these responses were not only type 2 helper T (Th2) cell dependent but also showed the characteristics of a Th1 response, such as elevated interferon (IFN)-γ levels.[9] However, lymphocytes were not present in the bronchoalveolar lavage (BAL) fluid of mice with asthma-like symptoms.[7] On the other hand, some studies were able to observe lymphocytes in BAL fluid. These contradictory results question the exact role and importance of lymphocytes in chemical-induced asthma.[10-12] Until now, the known underlying mechanisms cannot completely explain respiratory responses in diisocyanate-induced asthma.

Animal models of allergic asthma have also observed that T and B cells alone cannot explain observed asthmatic responses. For example, studies in RAG-deficient mice (no T and B cells) showed persistent eosinophilic airway inflammation and Th2 cytokine production, after administration of house dust mite (HDM). This suggests the involvement of non-T or B cells, such as innate lymphoid cells (ILCs). In these models, ILC2 are proposed as the additional key player. ILC2 are the most common subgroup of ILC in HDM-challenged mice and have a Th2-like cytokine expression pattern (interleukin [IL]-5, IL-4 and IL-13) in response to IL-33, IL-25 and thymic stromal lymphopoietin (TSLP). They are proposed to be involved in the very early phase after allergen exposure.[13-16] Additionally, ILC1 and ILC3 are 2 other ILC subsets that have been described, respectively characterised by a Th1-and a Th17-like cytokine pattern. These subsets are associated with non-allergic late-onset asthma, caused by exposure to pollutants, infections, occupational agents and intensive exercise.[13,15-17] Yet, the role of the ILC subsets in chemical-induced asthma, specifically in diisocyanate-induced asthma, is not well studied.

In addition to ILCs, dendritic cells (DCs) are recognised as crucial cells during both the sensitization and effector phases of allergic asthma. DCs are important antigen presenting cells in the lungs, bridging innate and adaptive immunity.[18] Different DC subsets are identified in the lung based on the presence of surface markers, such as CD11c, CD103, CD11b, CD64 and Siglec-H. Using these markers, DCs are divided into conventional DC ($CD103^+CD11b^-$ conventional dendritic cell [cDC] or cDC1 and $CD103^-CD11b^+$ cDC or cDC2), monocyte-derived DC ($CD64^+$ moDC) and plasmacytoid DC ($CD11b^-$Siglec-H^+ pDC). Each of them seems to have specific functions at their specific anatomical location in the lungs.[18] A limited number of studies have investigated and suggested the involvement of DC in chemical-induced asthma.[19-21] For example, Ban *et al.*[19] have already shown the accumulation of DC in lung-associated lymph nodes after TDI exposure. The role of the different DC subsets is never been investigated in more detail in chemical-induced asthma.

In this study, we used an oropharyngeal multi-challenge mouse model for diisocyanate-induced asthma to investigate the contribution and role of the innate immune system in chemical-induced asthma, which is still unexplored.

MATERIALS AND METHODS

Reagents

Toluene-2,4-diisocyanate (98%, CAS584-84-9), acetyl-β-methylcholine (methacholine) olive oil and acetone were obtained from Sigma-Aldrich (Bornem, Belgium). Pentobarbital (Nembutal®) was obtained from the internal animal facility at the University of Leuven (Leuven, Belgium). The vehicle acetone olive oil (AOO) was used to dissolve TDI. For the sensitization phase, a mixture of 2 volumes acetone and 3 volumes of olive oil was used. For the challenge phase, a mixture of 1 volume of acetone and 4 volumes of olive oil was used. Concentration of TDI is given as percentage (v/v) in AOO.

Mice

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

Experimental protocol

On days 1 and 8, mice received dermal applications of 20 μL 0.5% TDI or the vehicle AOO (2:3) on the dorsum of both ears. On days 15, 17, 19, 22 and 24, mice received an oropharyngeal instillation with 20 μL 0.01% TDI or the vehicle AOO (1:4) (**Fig. 1**). All experimental groups are indicated with 2 abbreviations: the first abbreviation indicates the dermal treatment on days 1 and 8, whereas the second abbreviation indicated the oropharyngeal instillations.

In this study, we had 3 different sets of experiments. In the first set, we evaluated airway hyperreactivity (AHR), airway inflammation, lung histology and immunoglobulins (Igs) in blood serum. We measured the cytokine secretion of T and B cells in the auricular lymph nodes (ALNs) (n = 7–9/group). In a second set of mice, we studied DC, T and B cells in the ALN and lung tissue. BAL was collected to confirm lung inflammation and total serum IgE was analyzed to confirm sensitization (n = 8/group). The third set of mice was used to study ILCs in lung tissue and cytokine levels in BAL fluid. In this set of mice, BAL airway inflammation was also evaluated to confirm previous findings. Depending on the endpoint, each group consisted of 6 to 17 mice.

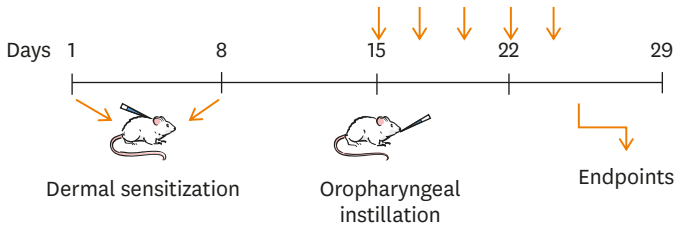

Fig. 1. Sub-chronic exposure protocol.

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Airway and tissue hyperreactivity measurements

Twenty-four hours after the last challenge, AHR and tissue hyperreactivity to methacholine were measured using a forced oscillation technique (FlexiVent 5; SCIREQ, Montreal, Canada). Mice were anesthetized with pentobarbital (70 mg/kg body weight, Nembutal®). Using the 'quick prime 3' (QP3) perturbation, airway resistance (Rn), tissue damping (G) and tissue elasticity (H) to increasing concentrations of methacholine (0, 1.25, 2.5, 5, 10 and 20 mg/mL) were measured. After each concentration, the QP3 perturbation was performed 5 times spread over 2 minutes. If the 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, G and H were plotted against the methacholine concentration and the area under the curve was calculated to perform statistical analysis.

Serum analysis

Serum sampling

After measuring AHR, mice were sacrificed. Blood was sampled from the retro-orbital plexus, centrifuged (14,000 g, 4°C, 10 minutes) and serum samples were stored at −80°C until analysis.

Enzyme-linked immunosorbent assay

The OptEIA Mouse IgE set (BD Pharmingen™, Erembodegem, Belgium) was used to measure total serum IgE (diluted 1/70). Measurements were performed according to the manufacturer's instructions. For the measurement of total serum IgG1 (diluted 1/8,000 or 1/20,000) and IgG2a (diluted 1/1,000), plates were coated using purified rat anti-mouse IgG1 (Cat: 553445; BD Pharmingen™) and rat anti-mouse IgG2a (Cat: 553446; BD Pharmingen™). A standard was created using purified mouse IgG1 (Cat: 557273; BD Pharmingen™) and mouse IgG2a (Cat: 553454; BD Pharmingen™). Further measurements were performed according to the manufacturer's instructions with the use of biotinylated anti-mouse avidin horseradish peroxidase (HRP) conjugate (HRP rat anti-mouse IgG1 [Cat: 559626], biotin rat anti-mouse IgG2a [Cat: 553388] and Streptavidin HRP [Cat: 554066]; BD Pharmingen™).

Lymph node analysis

Single cell suspension: lymph node cells

Retro-ALNs 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 μm) (BD Biosciences, Erembodegem, Belgium) and rinsing with 10 mL of tissue culture medium (RPMI-1640 (1X) + GlutaMAX™-I). After centrifugation (1,000 g, 10 minutes), cells were counted using a Bürker hemocytometer and resuspended (10^7 cells/mL) in complete tissue culture medium (RPMI-1640 (1X) + GlutaMAX™-I supplemented with 10% heat-inactivated forward scatter (FSC) and 10 mg/mL streptomycin/penicillin).

Lymphocyte subpopulations

Five-hundred thousand cells from the ALNs and lung were stained with anti-CD3 (APC), anti-CD4 (APC-Cy7), anti-CD8 (PerCP-Cy5.5) and anti-CD25 (PE), or received single staining with anti-CD19 (PE) labeled antibodies, according to standard procedures (BD Biosciences). Percentages of labeled cells were determined by performing flow cytometry (FACSArray; BD Biosciences) on at least 10^5 cells.

Cytokine analysis

Cells of the ALNs were seeded onto 48-well culture plates at a density of 10^6 cells/mL and incubated in complete RPMI-1640 (1X) + GlutaMAX™-I medium for 42 hours with 2.5 μg/

mL concanavalin A (ConA) (Sigma-Aldrich). Cell suspension was centrifuged (1,000 g, 10 minutes) and supernatant was stored at −80°C. Concentrations of IL-4, IL-10, IL-13 and IFN-γ were measured via the LSR Fortessa (BD Biosciences). Their 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.

Lung analysis

BAL

The lungs were lavaged, *in situ*, 3 times with 0.7 mL of 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 (1,000 g, 10 minutes). For differential cell count, 250 μL of the resuspended cells (100,000 cells/mL) were spun (300 g, 6 minutes) (Cytospin 3; Shandon, TechGen, Zellik, Belgium) onto microscope slides, air-dried and stained (Diff-Quik® method; Medical Diagnostics, Düdingen, Germany). For each sample, 200 cells were counted for the number of macrophages, eosinophils, neutrophils and lymphocytes. In experiment 1, the left lung was snap frozen in liquid nitrogen to be further analyzed, while the right lung was instilled with 4% formaldehyde until fill inflation of all lobes, as juged visually.

Cytokine analysis

The snap frozen lungs were homogenized in a 5% BSA solution, using an Ultra-Turrax T25 (Ika Works, Staufen, Germany). Afterwards, homogenates were centrifuged at 4°C (1,200 g, 10 minutes) and the supernatant was stored at −80°C. Cytokine and chemokine concentrations of IFN-γ, IL-10, IL-13, IL-17A, IL-17F, IL-33, IL-4, IL-5, monocyte chemoattractant protein 1 (MCP-1) and macrophage inflammatory protein 3 alpha (MIP-3α) were measured in BAL fluid and serum using a MSD U-plex Assay (Meso Scale Diagnostics, Rockville, MD, USA). Measurements were taken according manufacturer's instructions. Their detection limits were 0.16, 3.8, 2.7, 0.30, 2.4, 2.2, 0.56 and 0.63 pg/mL, respectively.

Histology

Lungs from experiment 1 were instilled with 4% formaldehyde until full inflation of all lobes. An experienced pathologist evaluated lung injury on slides stained with hematoxylin and eosin, 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 a 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).

Single-cell suspension: lung cells

The pulmonary circulation was rinsed with saline/ethylenediaminetetraacetic acid to remove the intravascular pool of cells. The cell suspensions of the lungs were obtained using digestion medium (RPMI-1640 supplemented with 5% FSC, 2 mM L-glutamine, 0.05 mM 2-mercaptoethanol [Gibco, Invitrogen, Paisley, UK], 100 U/mL penicillin, 100 mg/mL streptomycin [Invitrogen], 1 mg/mL collagenase type 2 [Worthington Biochemical, Lakewood, NY, USA], and 0.02 mg/mL DNase I [grade II from bovine pancreas; Boehringer Ingelheim, Ingelheim, Germany]) for 45 minutes at 37°C and at 5% CO_2. Red blood cells were lyzed by using ammonium chloride buffer. Cells were counted using a Bürker hemocytometer and resuspended (10^7 cells/mL) in phosphate buffered saline.

DC subpopulations

To minimize nonspecific bindings, 2 million cells of lung and ALNs were pre-incubated with anti-CD16/CD32 antibody (clone 2.4G2; BD Biosciences). 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 (clone N418), MHCII (clone M5/114.15.2), CD11b (clone M1/70), CD103 (clone 2E7), CD64 (clone X54-5/7.1) and Siglec-H (clone 551) (all from Biolegend, San Diego, CA, USA). Data acquisition was performed on a LSR Fortessa flow cytometer running DIVA software (BD Biosciences). FlowJo software (BD Biosciences) was used for data analysis.

ILC subpopulations

Two million cells were stained with Fixable Viability Dye FluorTM 780 (eBioscience, Invitrogen) to perform a live/dead staining and preïncubated with CD16/CD32 (clone 2.4G2; BD Biosciences) to block non-antigen-specific binding of Igs. Combinations of anti-mouse fluorochrome-conjugated mAbs against CD45 (clone 30-F11), CD127 (clone SB/199), CD117 (clone 2B8), CD90.2 (clone 53-2.1), KLGR-1 (clone 2F1), NK1.1 (clone PK136), CD11b (clone M1/70), CD19 (clone 1D3), CD3e (clone G4.18), CD45RB (clone 16A), CD5 (clone 53-7.3), CD94 (clone 20d5), Gr-1 (clone RB6-8C5), TCRγδ (clone GL3) and Ter-119 (clone TER-119) (all from BD Biosciences) were used to label the cells (see gating strategy in **Supplementary Figs. S1** and **S2**). Data acquisition was performed on a LSR Fortessa flow cytometer running DIVA software (BD Biosciences). Data analysis was performed by using FlowJo software.

Statistical analysis

The data are presented as mean with standard deviation or median with interquartile range, depending on the distribution of the data. Normality was assessed by a Kolmogorov-Smirnov test, followed by an one-way parametric analysis of variance (ANOVA) combined with a Bonferroni multiple comparison *post hoc* test or a non-parametric Kruskal-Wallis test. Dose-response data of the AHR was analyzed via a 2-way ANOVA (Graph Pad Prism 8.02; Graphpad Software Inc, San Diego, CA, USA). A P value of < 0.05 (2-tailed) was considered significant.

RESULTS

Markers of sensitization

The lymphocyte subpopulations in ALNs were determined using flow cytometry. **Fig. 2** shows that TDI-sensitized mice (TDI/TDI) have a significant increase of all T cell subpopulations ($CD4^+$ helper T cells, $CD4^+CD25^+$ activated/regulatory T cells (Tregs) and $CD8^+$ cytotoxic T cells) and B cells ($CD19^+$) compared to the AOO/AOO and AOO/TDI- treated groups.

Lymphocytes of the ALNs were *ex vivo* stimulated with ConA. **Fig. 2** shows a significant higher secretion of IL-4 (**Fig. 2F**), IL-13 (**Fig. 2G**), IL-10 (**Fig. 2H**) and IFN-γ (**Fig. 2I**) from the auricular lymphocytes of TDI/TDI-treated mice compared to both AOO/AOO and AOO/TDI-treated groups.

As DCs are important during the sensitization phase, we determined in a second set of mice the number of DC in the ALNs. **Fig. 2B-E** show the total number of different subpopulations of DC in the ALN. The total number of DC (**Fig. 2B**) and the $CD11b^+$ cDC2 subpopulation (**Fig. 2C**) are significantly increased in the TDI/TDI-treated group compared to the control group (AOO/AOO) and AOO/TDI-treated groups. For the cDC1 and pDC (**Fig. 2D and E**), there is only a significant increase in the TDI/TDI-group compared to the AOO/AOO-treated group.

The serum analysis shows a significant increase in total serum IgE (**Fig. 3A**) and IgG1 (**Fig. 3B**) in TDI-sensitized and challenged mice (TDI/TDI), compared to the AOO/AOO and AOO/TDI-

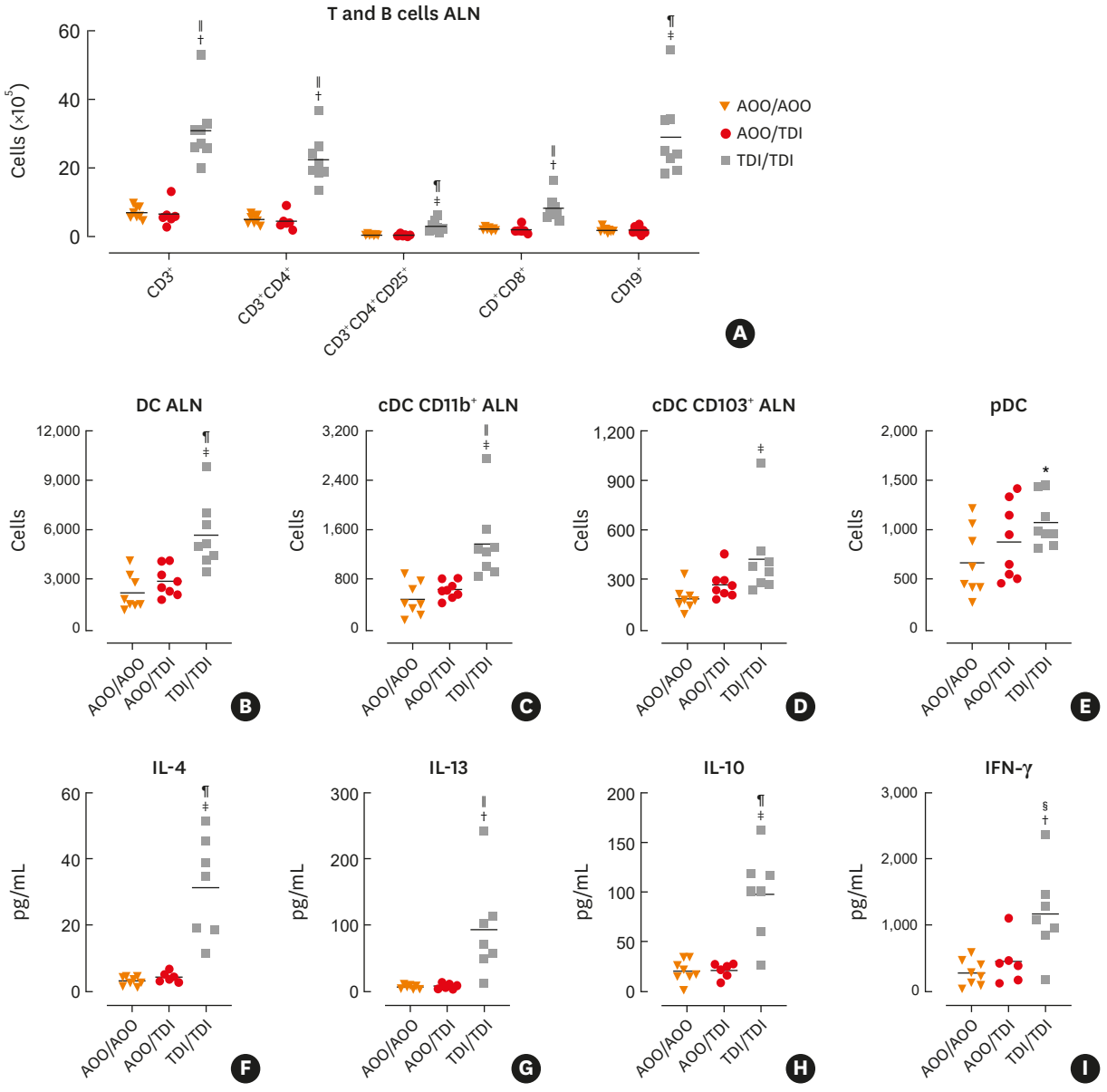

Fig. 2. ALN analysis. (A) Lymphocyte subpopulations in ALNs were measured using flow cytometry. ALN cells were stained with anti-CD3$^+$ (T lymphocytes), anti-CD3$^+$CD4$^+$ (Th-lymphocytes), anti-CD3$^+$CD4$^+$CD25$^+$ (activated/regulatory Th-lymphocytes) and anti-CD3$^+$CD8$^+$ (Tc-lymphocytes) or stained with a single anti-CD19$^+$ (B-lymphocytes). (B-E) Dendritic cell populations in ALNs were measured using flow cytometry. ALN cells were analyzed as CD45$^+$ low auto fluorescent MHCII$^+$CD11c$^+$ DC (DC) (B), CD45$^+$ low auto fluorescent MHCII$^+$CD11c$^+$CD11b$^+$CD103$^-$ cDC (CD11b$^+$ cDC or cDC2) (C), CD45$^+$ low auto fluorescent MHCII$^+$CD11c$^+$CD11b$^-$CD103$^+$ conventional DC (CD103$^+$ cDC or cDC1) (D), and CD45$^+$ low auto fluorescent MHCII$^+$ CD11c$^+$Siglec-H$^+$ pDC (**Fig. 1E**). (F-I) *Ex vivo* ConA-induced cytokine production of auricular lymphocytes was evaluated by measuring cytokine levels of IL-4 (F), IL-13 (G), IL-10 (H) and IFN-γ (I) in supernatant of cultured auricular lymphocytes, using Cytometric Bead Array. The experimental groups are identified by 2 abbreviations (AOO, TDI); the first abbreviation indicates the dermal treatment on days 1 and 8, whereas the second one indicates the oropharyngeal instillations (n = 6–8 per group, depending on the experimental endpoint). Data shown as individual data points and group means.
ALN, auricular lymph node; Th, helper T; Tc, cytotoxic T; AOO, acetone olive oil; TDI, toluene diisocyanate; DC, dendritic cell; cDC, conventional dendritic cell; pDC, plasmacytoid dendritic cell; ConA, concanavalin A; IL, interleukin; IFN, interferon.
$^*P < 0.05$, $^\dagger P < 0.01$, $^\ddagger P < 0.001$ compared with the AOO/AOO control group; $^\S P < 0.05$, $^\parallel P < 0.01$, $^\P P < 0.001$ compared to the AOO/TDI-treated group (1-way analysis of variance).

treated groups. There was no difference in total serum IgG2a between the different groups (**Fig. 3C**).

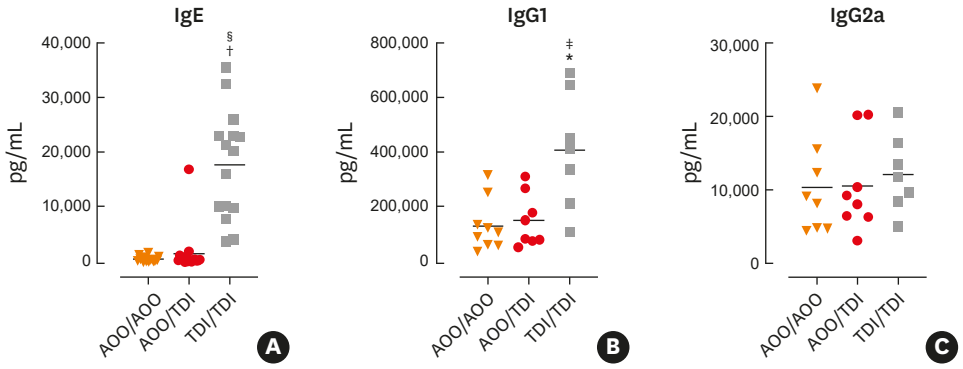

Fig. 3. Total serum Igs. Total serum IgE (A), IgG1 (B) and IgG2 (C) were measured using enzyme-linked immunosorbent assay. Blood was collected 24 hours after the last oropharyngeal challenge. The experimental groups are identified by 2 abbreviations (AOO, TDI); the first abbreviation indicates the dermal treatment on days 1 and 8, whereas the second one indicates the oropharyngeal instillations (n = 15–17 for IgE and n = 7–9 for IgG1 and IgG2 per group). Data shown as individual data points and group medians or means.
Ig, immunoglobulin; AOO, acetone olive oil; TDI, toluene diisocyanate.
$^{*}P < 0.01$ and $^{\dagger}P < 0.001$ compared to the AOO/AOO control group; $^{\ddagger}P < 0.01$, $^{\S}P < 0.001$ compared to the AOO/TDI-treated group (1-way analysis of variance for IgG1 and IgG2a and Kruskal-Wallis test for IgE).

Airway and tissue hyperreactivity

Fig. 4 shows the dose-response of methacholine-induced Rn (**Fig. 4A**), G (**Fig. 4B**) and H (**Fig. 4C**). Mice sensitized and challenged with TDI (TDI/TDI-treated group) showed significant increases in Rn, G and H after methacholine challenge compared to both AOO/AOO and AOO/TDI- treated groups.

Lung immune inflammatory response

Fig. 5A shows the differential cell count of the BAL fluid. Mice not sensitized and only exposed via the airways to TDI (AOO/TDI-treated group) showed a slight increase in neutrophils compared to the AOO/AOO-treated group. Mice both sensitized and challenged with TDI (TDI/TDI) showed a highly significant influx of eosinophils in the BAL fluid compared to both AOO/AOO and AOO/TDI-treated groups. Lung histological analysis, shown in **Supplementary Fig. S3** show an increase of alveolar widening (**Supplementary Fig. S3A**), inflammation

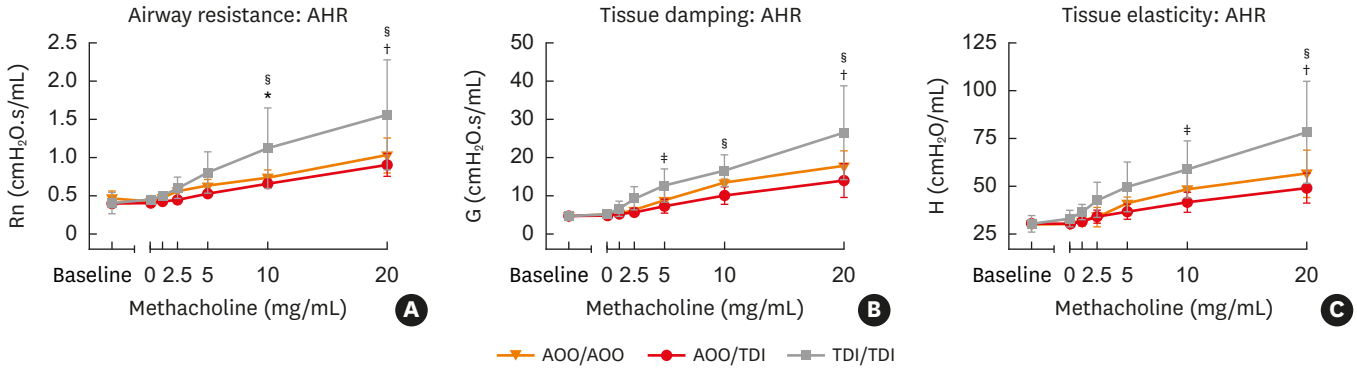

Fig. 4. Airway and tissue hyperreactivity. Methacholine responsiveness was assessed 24 hours after the last oropharyngeal challenge. The Rn, G and H to methacholine were measured using a forced oscillation technique (QP3). Fig. 4 shows the mean values of Rn, G and H against methacholine concentrations between 0 and 20 mg/mL. The experimental groups are identified by 2 abbreviations (AOO, TDI); the first abbreviation indicates the dermal treatment on days 1 and 8, whereas the second one indicates the oropharyngeal instillations (n = 8–9 per group). Data are shown as means with standard deviations.
Rn, airway resistance; G, tissue damping; H, tissue elasticity; QP3, quick prime 3; AOO, acetone olive oil; TDI, toluene diisocyanate; AHR, airway hyperreactivity.
$^{*}P < 0.01$, $^{\dagger}P < 0.001$ compared to the AOO/AOO control group; $^{\ddagger}P < 0.01$, $^{\S}P < 0.001$ compared to the AOO/TDI group (2-way analysis of variance).

(**Supplementary Fig. S3B**) and epithelial shedding (**Supplementary Fig. S3C**), in the TDI/TDI group, compared with both control groups (AOO/AOO and AOO/TDI).

Fig. 5B-H show the total number of leukocytes (**Fig. 5B**), macrophages (**Fig. 5C**), DC (**Fig. 5D**) and DC subpopulations (**Fig. 5E-H**) in the lungs. Leucocytes are significantly increased

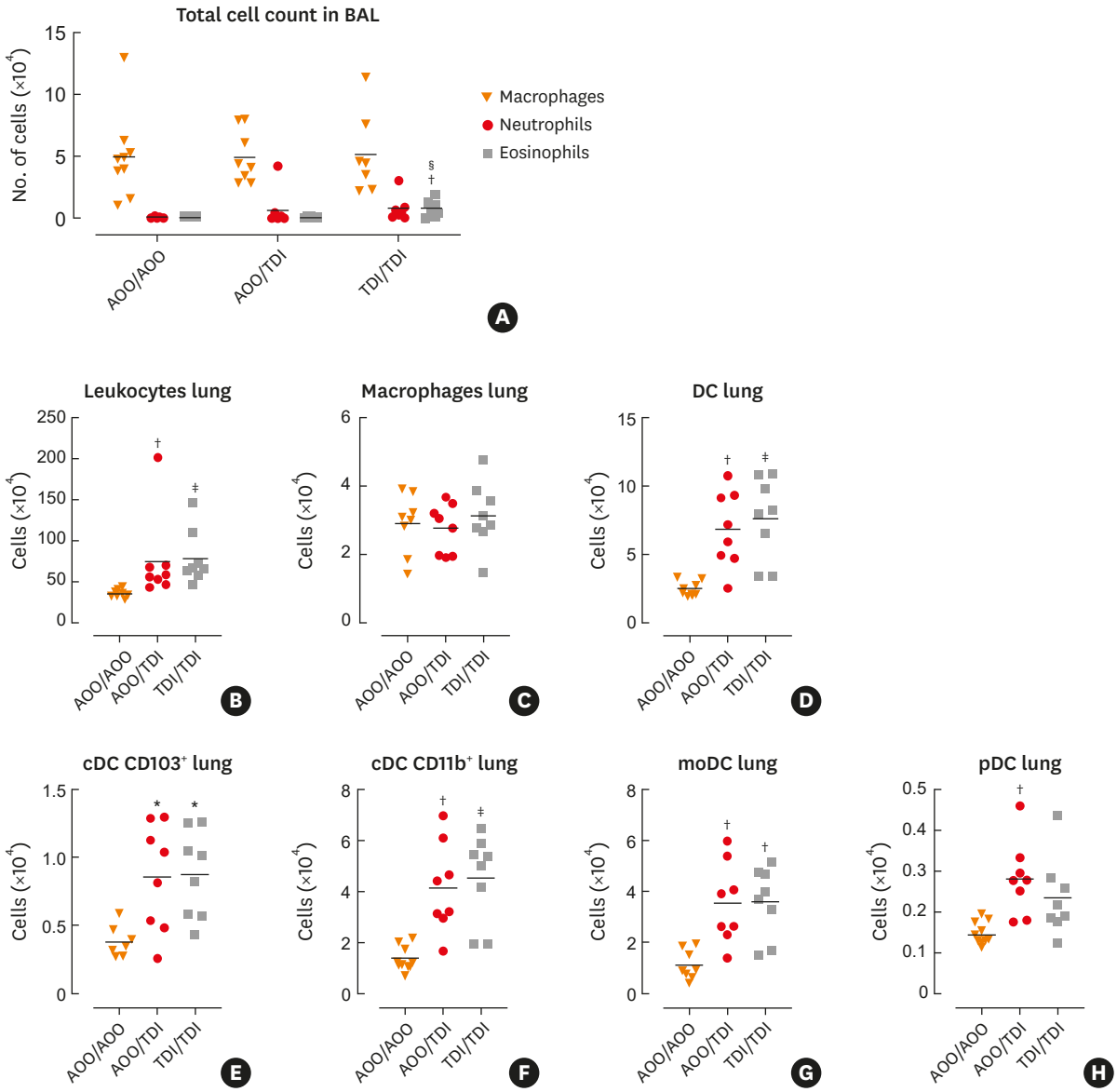

Fig. 5. Inflammatory and antigen presenting cells in the lung. (A) Total amount of macrophages, neutrophils and eosinophils was evaluated in bronchoalveolar lavage fluid. (B-H) Leukocytes and antigen presenting cells in lung tissue were measured using flow cytometry. Cells were analyzed as CD45⁺ leukocytes (B), auto fluorescent macrophages (C) CD45⁺ low auto fluorescent MHCII⁺CD11c⁺ DC (DC) (D), CD45⁺ low auto fluorescent MHCII⁺CD11c⁺CD11b⁻CD103⁺ cDC (CD103⁺ cDC or cDC1) (E), CD45⁺ low auto fluorescent MHCII⁺CD11c⁺CD11b⁺CD103⁻ conventional DC (CD11b⁺ cDC or cDC2) (F), CD45⁺ low auto fluorescent MHCII⁺CD11c⁺CD11b⁺CD64⁺ moDC (G) and CD45⁺ low auto fluorescent MHCII⁺CD11c⁺Siglec-H⁺ pDC (H). The experimental groups are identified by 2 abbreviations (AOO, TDI); the first abbreviation indicates the dermal treatment on days 1 and 8, whereas the second one indicates the oropharyngeal instillations (n = 7–8 per group, depending on the experimental endpoint). Data are shown as individual data points with means or medians.
AOO, acetone olive oil; TDI, toluene diisocyanate; DC, dendritic cell; cDC, conventional dendritic cell; moDC, monocyte derived dendritic cell; pDC, plasmacytoid dendritic cell; BAL, bronchoalveolar lavage.
*$P < 0.05$, †$P < 0.01$, ‡$P < 0.001$ compared to the AOO/AOO control group; §$P < 0.01$ compared to the AOO/TDI group (1-way analysis of variance for macrophages in BAL and lung, DC, CD103⁺ cDC, moDC and pDC and the Kruskal-Wallis test for neutrophils and eosinophils in BAL, leucocytes and CD11b⁺ cDC).

in both TDI-treated groups (AOO/TDI and TDI/TDI) compared to the AOO/AOO control group (**Fig. 5B**). The total number of DC (**Fig. 5D**), cDC1 (**Fig. 5E**), cDC2 (**Fig. 5F**) and moDC (**Fig. 5G**) are also significantly increased in both TDI-treated groups, compared to the AOO/AOO control group. Furthermore, pDC were increased in both TDI-treated groups, but were only significantly higher in the AOO/TDI-treated group (**Fig. 5H**). Macrophages did not show significant differences (**Fig. 5C**).

In the third set of mice, we investigated the presence of the ILC subpopulations after TDI-exposure. The total number of ILC, shown in **Fig. 6**, shows no significant differences between the 3 different treatment groups (**Fig. 6A**). However, the percentage ILC2 is significantly higher in the AOO/TDI and TDI/TDI treated groups compared to the AOO/AOO control group (**Fig. 6C**). On the other hand, the percentage of ILC3 is significantly decreased in both TDI-treated groups compared to the control group (**Fig. 6D**).

Cytokine concentrations were evaluated in BAL fluid of the third set of mice and are shown in **Table**. IFN-γ, IL-10 and IL-17A were significantly higher in both TDI-challenged groups (TDI/TDI and AOO/TDI) compared to the AOO/AOO control group. Other cytokines, such as IL-13, IL-17F, IL-4 and IL-5, are only significantly elevated in the TDI/TDI-treated group compared

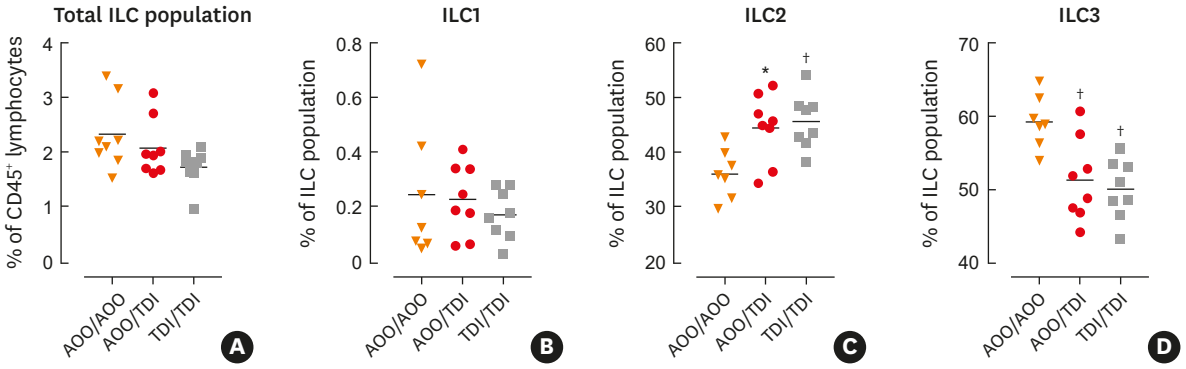

Fig. 6. ILCs in the lung. (A) The total amount of ILCs in lung tissue (CD45⁺, Lin⁻, CD90.2⁺ and CD127⁺) were measured using flow cytometry. ILC subpopulations were analysed as KLRG-1⁻ and NK1.1⁺ ILC1 (B), KLRG-1⁺ ILC2 (C) and KLRG-1⁻ and NK1.1⁺ ILC3 (D). The experimental groups are identified by 2 abbreviations (AOO, TDI); the first abbreviation indicates the dermal treatment on days 1 and 8, whereas the second one indicates the oropharyngeal instillations (n = 7–8 per group). Cell populations are shown as mean or median.
AOO, acetone olive oil; TDI, toluene diisocyanate; ILC, innate lymphoid cell.
*$P < 0.05$, †$P < 0.01$ compared to AOO/AOO (1-way analysis of variance for ILC subpopulations and the Kruskal-Wallis test for total ILC).

Table. Measurements of Cytokines (IL-4, IL-5, IL-13, IL-10, MCP-1, IL-33, MIP-3α, IFN-γ, IL17-A and IL17-F pg/mL) in bronchoalveolar lavage fluid using a U-PLEX Assay Platform

Cytokines (pg/mL)	AOO/AOO	AOO/TDI	TDI/TDI
IL-4	0.58 (0.44–1.02)	0.92 (0.52–2.40)	14.42†,§ (4.55–36.85)
IL-5	1.12 (0.28–1.66)	19.36 (5.10–27.17)	64.97†,‡ (28.35–217.00)
IL-13	0 (0–28.37)	46.79 (11.29–61.93)	66.49† (40.78–111.10)
IL-10	0 (0–2.83)	5.06* (2.08–6.44)	4.02* (1.49–12.62)
MCP-1	82.45 (55.32–108.80)	405.30* (151.10–732.00)	221.90 (92.65–1,093.00)
IL-33	12.18 (10.18–19.80)	11.75 (8.14–20.61)	16.89 (12.40–20.53)
MIP-3α	185.90 (130.50–242.40)	177.20 (132.30–271.30)	108.00 (82.94–158.30)
IFN-γ	0.29 (0.08–0.73)	2.05*** (1.57–3.40)	0.99* (0.79–2.87)
IL-17A	0.47 (0.35–0.77)	2.60† (1.04–4.05)	1.601* (0.95–1.93)
IL-17F	3.61 (0–9.88)	8.76 (3.39–17.54)	19.34* (8.43–46.66)

Data is shown as median with interquartile range (25th and 75th percentile). The experimental groups are identified by 2 abbreviations (AOO, TDI); the first abbreviation indicates the dermal treatment on days 1 and 8, whereas the second one indicates the oropharyngeal instillations (n = 7–8 per group).
AOO, acetone olive oil; TDI, toluene diisocyanate; IL, interleukin; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; IFN, interferon.
*$P < 0.05$ and †$P < 0.01$ compared to AOO/AOO; ‡$P < 0.05$ and §$P < 0.01$ compared to AOO/TDI.

to the AOO/AOO control, from which IL-4 and IL-5 were also significantly higher compared to the AOO/TDI-treated group. Significant higher concentrations of MCP-1 were only detected in AOO/TDI-treated mice compared to the control group. IL-33 and MIP-3α showed no significant differences between the treatment groups.

To confirm the phenotype of our model in the second and third sets of mice, airway inflammation was evaluated in BAL fluid. **Supplementary Fig. S4** shows that both mouse sets 2 and 3 have similar inflammatory patterns as in set 1 (**Fig. 5**), confirming our TDI-induced asthma model (data shown in supplement).

DISCUSSION

In this study, we showed that multiple TDI airway challenges resulted in the recruitment of conventional and monocyte-derived DCs, along with ILC2 in the lungs. Yet, only if the TDI airway challenges were preceded by TDI dermal sensitizations, a full Th2 mediated eosinophilic asthmatic response, with AHR, the presence of associated cytokines, such as IL-13, IL-17F, IL-4 and IL-5, and increased total serum IgE and IgG1, was initiated. In the non-dermally sensitized mice, some neutrophilic inflammation, along with IL-17A, MCP-1 and IFN-γ was present in the lungs, but there was no AHR.

We have previously described that dermal sensitization with TDI results in significantly proliferated ALNs, where both T and B cells are stimulated.[7,8,22] Additionally, we showed a significant increase in several DC subpopulations in the ALNs of TDI-sensitized mice. Previous studies have already shown that diisocyanates are transported by DC from the skin to the local lymph nodes.[23] Depending on the exposure context, each DC subset is geared to induce particular T cell responses.[24] Both $CD103^{-}CD11b^{+}$ cDC (cDC2) and $CD103^{+}CD11b^{-}$ cDC (cDC1) have shown to be able to induce Th2 and Th1 immunization, respectively.[23] Their significant increase can therefore be directly linked to the increased number of $CD4^{+}$ T cells in the ALN. Also, cDC1 are also described to cross present antigens to $CD8^{+}$ T cells, which is in line with the significant increased numbers of $CD8^{+}$ T cells in the ALN.[23] Increased numbers of cDC1 and pDC, are associated with a significant increase in $CD25^{+}$ Tregs, which are known to down-regulate immune responses, and induce immune tolerance.[25,26] Both humoral and *ex vivo* cell mediated cytokine secretion of the ALNs demonstrated a mixed Th2 (IgE, IgG1, IL-4, IL-10 and IL-13) *vs.* Th1 (IFN-γ) cytokine profile. The production of Th2 cytokines by the ALN is probably initiated by moDC and cDC2, as they are known Th2-immunity inducers, while cDC1 are responsible for the Th1 response in the ALN as they have been shown to induce a Th1 response *ex vivo*.[25] For the first time, we identified key initiators of immune-sensitization by contact of TDI to the skin, explaining the lymphocyte subpopulations and the cytokine secretion profile we have described before.[9,27-29] Admittedly, since we have focused on the panel composition for the lung DC, we are aware that we missed some other DC subpopulations in the skin.[30]

Next, DC also play a crucial role during the effector phase of asthma. In ovalbumin (OVA) models, it is shown that depletion of cDC2 during OVA airway exposures results in abrogation of all the characteristic features of asthma, which were restored after adoptive transfer of $CD11c^{+}$ cells.[31,32] Thus, the essential role of DC in allergic Th2-mediated asthma is well studies, but almost nothing is known concerning the involvement of DC during the effector phase of chemical-induced asthma. Our results show that DC are recruited to the

lungs after TDI challenges, independently of initial dermal TDI sensitization. In fact, there is no difference in the DC subpopulations between both TDI-exposed groups (AOO/TDI and TDI/TDI). Also, other studies confirmed recruitment of DC in the lungs after airway exposure to diesel exhaust particles and HDM without prior sensitization.[23,33-35] Several subsets of DC are increased in the lungs, but most substantial increases after TDI challenges can be found in the cDC2 and the moDC, which are known to stimulate the Th2 response. Moreover, the cDC1 are also significantly increased, but to a lesser extent, and are initiators of Th1 response and $CD8^+$-cell activation.[23] The pDC, known for their regulatory function, are only significantly increased in the AOO/TDI group, but show the same increasing trend in the TDI/TDI group. This trend towards type 2 response is also observed for ILCs in the lungs. Increased numbers of ILC2 and decreased numbers of ILC3 are shown in both TDI-treated groups, indicating that these ILCs act independently of prior sensitization. Halim *et al.*[36] have shown a link between ILC2, $CD11b^+$ cDC (or cDC2) and the Th2 response in the lung tissue of an allergic model. In their study, ILC2 seem to be the most important source of IL-13, which will activate cDC2. These cDC2 will produce on their turn CCL17, resulting in Th2 recruitment. This theory is in line with our findings in both TDI-challenged groups, which show increased levels of ILC2, IL-13 and cDC2. However, an extensive Th2 cytokine pattern is only present in the TDI-treated group, indicating that the complete type 2 response in this chemical-induced asthmatic model needs prior sensitization.

Differences in the cytokine profile of BAL fluid between all 3 groups (AOO/AOO, AOO/TDI and TDI/TDI) are illustrated in **Fig. 7**, and furthermore confirms the involvement of the initial TDI sensitization and the activation of the adaptive immune system after TDI challenge. As mentioned above, a type 2 cytokine signature with increased levels of IL-4, IL-5 and IL-13 was only identified in the TDI/TDI-treated mice. Increased levels of IL-5, exclusively in the TDI/TDI-treated mice, explain the presence of eosinophils in the lungs, which survival and maturation is IL-5 dependent.[37] AHR, only seen in the TDI treated mice, can be explained by the presence of IL-13, a well-known driver of AHR.[38] Additionally to the classic-Th2 cytokines, IL-17F also

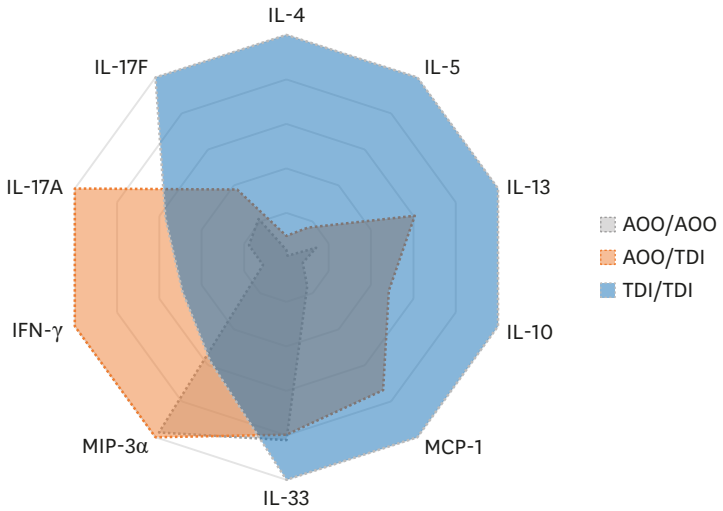

Fig. 7. Cytokine patterns in BAL. Cytokines in BAL fluid were measured using a U-PLEX Assay Platform. Each cytokine is normalized compared to the group with the highest concentration. The experimental groups are identified by 2 abbreviations (AOO, TDI); the first abbreviation indicates the dermal treatment on days 1 and 8, whereas the second one indicates the oropharyngeal instillations (n = 7–8 per group).
BAL, bronchoalveolar lavage; AOO, acetone olive oil; TDI, toluene diisocyanate; IL, interleukin; IFN, interferon; MIP, macrophage inflammatory protein; MCP, monocyte chemoattractant protein.

seems to be involved in the development of the asthmatic airway response in the TDI/TDI-treated mice, as also shown by Suzuki *et al.*[39] in an OVA-induced allergic mouse model. In contrast to IL-17F, IL-17A was significantly higher in both TDI-challenged groups, suggesting that this is a marker of lung or epithelial irritation, rather than an allergic response. This also confirms the findings of Yao *et al.*[40] which states that IL-17F and not IL-17A is playing a role in development of allergic airway inflammation in chemical-induced asthma. Both IL-17 cytokines are mainly produced by Th17 cells. Moreover, IL-17A can also be secreted by ILC3, which were also evaluated in our model. However, the increased levels of IL-17A in both TDI-challenged groups are in contrast with the significantly decreased amount of ILC3 in lung tissue. These findings suggest that Th17 cells, rather than ILC3, play a role in the immunological response to irritant exposure. Nevertheless, in our model, Th17 cells in the lungs or ALN were not evaluated and need further research. Additionally to IL-17A, both IL-10 and IFN-γ are also significantly elevated in TDI-challenged mice, with and without prior sensitization. Probably, we can attribute these increases to the recruitment and activation of cDC1 and pDC. This suggests the involvement of mixed Th2/Th1 response after TDI airway challenge.

Surprisingly, IL-33 which is a known damage-associated pattern used to evaluate epithelial damage was not changed after dermal and/or airway challenge with TDI. We expected increased levels of IL-33 caused by irritant exposure to TDI on the airway epithelium, which was already proven *in vitro* by Kim *et al.*[41] It is also known that IL-33 initiates the production of Th2 cytokines, such as IL-4, IL-5 and IL-13, which were increased in the TDI/TDI- treated group.[41] It needs to be mentioned that IL-33 is known to be released from the epithelium immediately after exposure and can already be cleared from the body at the time of collection, which is 24 hours after the last exposure. Additionally, these findings also indicate that other damage-associated patterns (IL-25 or TSLP) are possibly responsible for the induction of cytokine production and play a more important role in our model for chemical-induced asthma.[42] Further investigations are warranted.

Our data indicate that dermal sensitization influences the activation of inflammation in the lungs. Mice both dermally sensitized and oropharyngeal instilled with TDI (TDI/TDI) illustrated predominant eosinophilic inflammation, reflecting Th2 mediated allergic immune response.[23] While mice that were only oropharyngeal challenged with TDI only showed minor neutrophilic airway inflammation. In addition, AHR was also only present in the fully treated TDI/TDI group. Again, this confirms that the skin exposure to TDI is a potent activator of the adaptive immune system, where after low concentrations of TDI exposure to the lungs lead to the development of airway inflammation and AHR. We and other groups already emphasized the importance of previous skin exposure in inducing a pronounced airway response.[43-46]

In conclusion, multiple airway exposure to TDI, with or without prior dermal sensitization, results in recruitment of DC and ILC2, which indicates a mixed response of the adaptive and innate immunity. Prior skin exposure and consequent systemic immune sensitization result in predominant eosinophilic airway inflammation accompanied by AHR and a classic Th2-cytokine signature, with the obvious cytokines IL-4, IL-5 and IL-13 playing a role for IL-17F. While airway exposure without prior skin contact leads to a more irritant response characterized by minor neutrophilic airway inflammation without AHR and the presence of cytokines, such as IL17A and MCP-1.

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SUPPLEMENTARY MATERIALS

Supplementary Fig. S1

Representative flow cytometry data and gating strategy for DC populations in lung. DC were marked as low-autofluorescent, CD11c^{high} and MHC^{+}. CD11c^{high} and high-autofluorescent cells were marked as macrophages. pDC were gated as CD11^{-} and Siglec-H^{+}. cDC were subdivided into CD11b^{+} and CD103^{-} cells or CD11b^{-} and CD103^{+} cells. moDC were identified as CD11b^{+}, CD103^{-} and CD64^{+}. Data shown of TDI/TDI challenged mice.

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Supplementary Fig. S2

Representative flow cytometry data and gating strategy for ILC populations in the lungs. ILC were marked as Lin^{-} (CD11b, CD19, CD3e, CD45RB, CD5, CD94, Gr-1, TCRγδ and Ter-119), CD90.2^{+} and CD127^{+} cells. ILC2 were marked as KLRG-1^{+}, ILC1 were marked as KLRG-1^{neg} and NK1.1^{+} and ILC3 were marked as KRLG-1^{neg} and NK1.1^{-}.

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Supplementary Fig. S3

Histology of lung tissue. Hematoxylin and eosin staining was performed on lung slices 24 hours after the last oropharyngeal instillation. Alveolar widening (A, 50 × amplification), inflammation (B, 200 × amplification) and epithelial shedding (C, 200 × amplification) were validated. The experimental groups are identified by 2 abbreviations (AOO, TDI); the first abbreviation indicates the dermal treatment on days 1 and 8, whereas the second one indicates toropharyngeal instillations (n = 7–9 per group). Bars show the mean with standard deviation.

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Supplementary Fig. S4

BAL cell count of experiment 2 (DC) and 3 (ILC). A second and third set of mice were used to evaluate DC in auricular lymph node and lungs and ILC in lungs. Percentages of macrophages, neutrophils, eosinophils and lymphocytes were assessed in BAL 24 hours after the last oropharyngeal instillation (A, C) This was performed in both sets to confirm the phenotype as found in experiment 1 (**Fig. 3**). The total amount of macrophages, neutrophils and eosinophils are shown in (B, D). The experimental groups are identified by 2 abbreviations (AOO, TDI); the first abbreviation indicates the dermal treatment on days 1 and 8, whereas the second one indicates the oropharyngeal instillations (n = 7–8 per group). Individual data points are shown with means.

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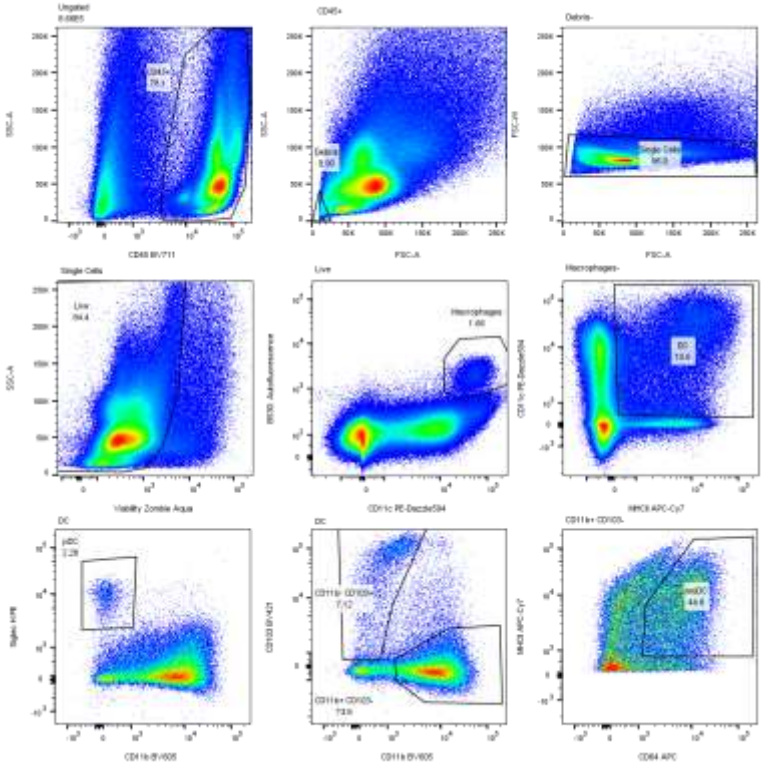

Supplementary Fig. S1. Representative flow cytometry data and gating strategy for DC populations in lung. DC were marked as low-autofluorescent, CD11c^{high} and MHC^{+}. CD11c^{high} and high-autofluorescent cells were marked as macrophages. pDC were gated as CD11^{-} and Siglec-H^{+}. cDC were subdivided into CD11b^{+} and CD103^{-} cells or CD11b^{-} and CD103^{+} cells. moDC were identified as CD11b^{+}, CD103^{-} and CD64^{+}. Data shown of TDI/TDI challenged mice.

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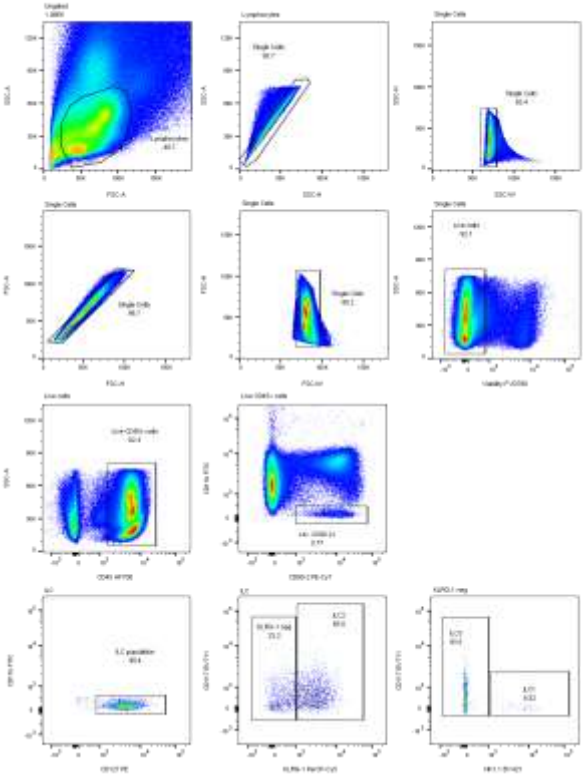

Supplementary Fig. S2. Representative flow cytometry data and gating strategy for ILC populations in the lungs. ILC were marked as Lin^{-} (CD11b, CD19, CD3e, CD45RB, CD5, CD94, Gr-1, TCRγδ and Ter-119), CD90.2^{+} and CD127^{+} cells. ILC2 were marked as KLRG-1^{+}, ILC1 were marked as KLRG-1^{neg} and NK1.1^{+} and ILC3 were marked as KRLG-1^{neg} and NK1.1^{-}.

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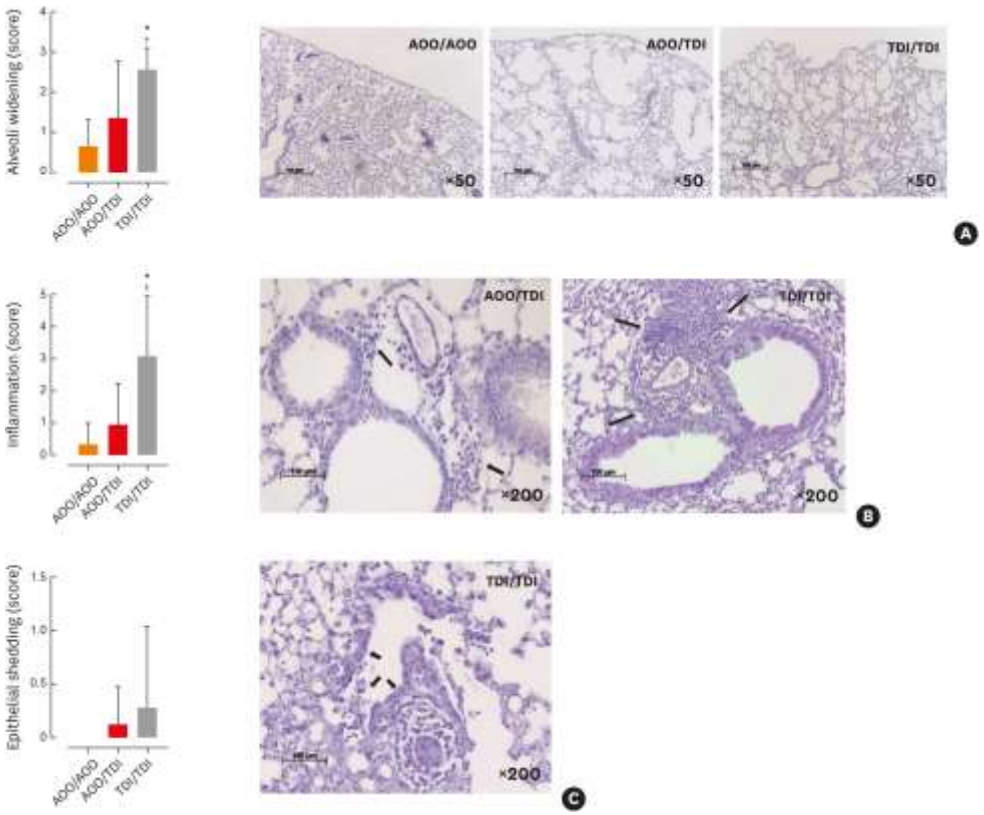

Supplementary Fig. S3. Histology of lung tissue. Hematoxylin and eosin staining was performed on lung slices 24 hours after the last oropharyngeal instillation. Alveolar widening (A, 50 × amplification), inflammation (B, 200 × amplification) and epithelial shedding (C, 200 × amplification) were validated. The experimental groups are identified by 2 abbreviations (AOO, TDI); the first abbreviation indicates the dermal treatment on days 1 and 8, whereas the second one indicates toropharyngeal instillations . . .

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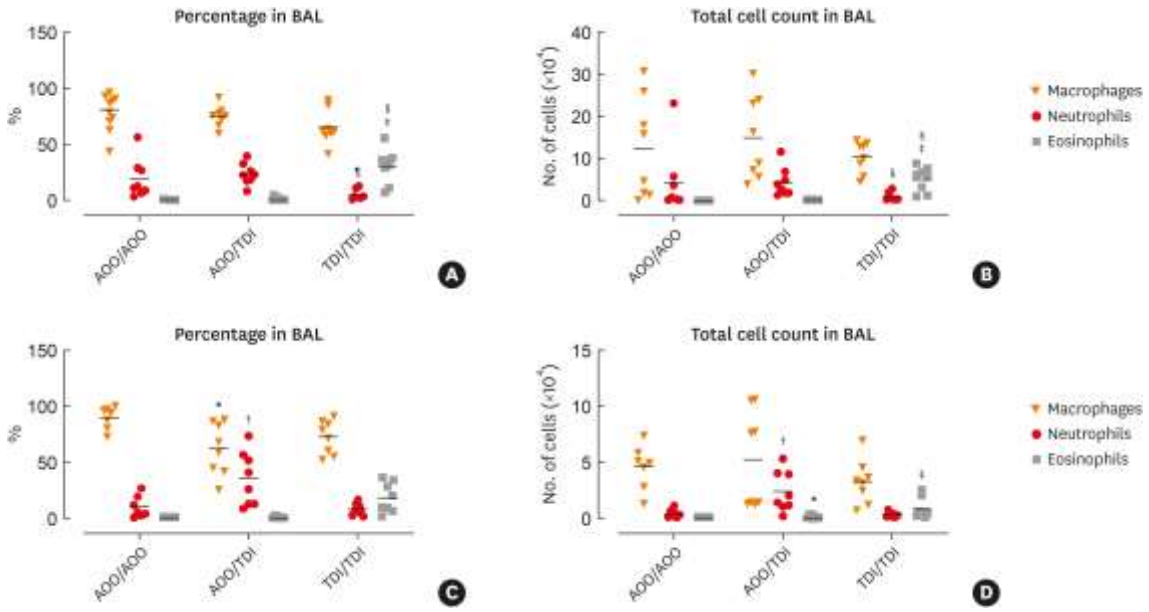

Supplementary Fig. S4. BAL cell count of experiment 2 (DC) and 3 (ILC). A second and third set of mice were used to evaluate DC in auricular lymph node and lungs and ILC in lungs. Percentages of macrophages, neutrophils, eosinophils and lymphocytes were assessed in BAL 24 hours after the last oropharyngeal instillation (A, C) This was performed in both sets to confirm the . . .

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