Innate lymphoid cells in isocyanate-induced asthma: role of microRNA-155

Evy E. Blomme [1], Sharen Provoost[1], Erica Bazzan[2],
Hannelore P. Van Eeckhoutte[1], Mirjam P. Roffel[1,3], Lore Pollaris[4],
Annelies Bontinck[1], Matteo Bonato[2], Louise Vandenbroucke[1], Fien Verhamme[1],
Guy F. Joos [1], Manuel G. Cosio[2,5], Jeroen A. J. Vanoirbeek [4],
Guy G. Brusselle [1], Marina Saetta [2] and Tania Maes [1]

Affiliations: [1]Dept of Respiratory Medicine, Laboratory for Translational Research in Obstructive Pulmonary Diseases, Ghent University Hospital, Ghent, Belgium. [2]Dept of Cardiac, Thoracic, Vascular Sciences and Public Health, University of Padova, Padova, Italy. [3]University of Groningen, University Medical Center Groningen, GRIAC (Groningen Research Institute for Asthma and COPD), Groningen, The Netherlands. [4]Centre for Environment and Health, KU Leuven, Leuven, Belgium. [5]Meakins Christie Laboratories, Respiratory Division, McGill University, Montreal, QC, Canada.

Correspondence: Tania Maes, Dept of Respiratory Medicine, Ghent University Hospital, Medical Research Building (MRB) II, 2nd floor, Corneel Heymanslaan 10, 9000 Ghent, Belgium. E-mail: tania.maes@UGent.be

ABSTRACT

Background: Occupational asthma, induced by workplace exposures to low molecular weight agents such as toluene 2,4-diisocyanate (TDI), causes a significant burden to patients and society. Little is known about innate lymphoid cells (ILCs) in TDI-induced asthma. A critical regulator of ILC function is microRNA-155, a microRNA associated with asthma.

Objective: To determine whether TDI exposure modifies the number of ILCs in the lung and whether microRNA-155 contributes to TDI-induced airway inflammation and hyperresponsiveness.

Methods: C57BL/6 wild-type and microRNA-155 knockout mice were sensitised and challenged with TDI or vehicle. Intracellular cytokine expression in ILCs and T-cells was evaluated in bronchoalveolar lavage (BAL) fluid using flow cytometry. Peribronchial eosinophilia and goblet cells were evaluated on lung tissue, and airway hyperresponsiveness was measured using the forced oscillation technique. Putative type 2 ILCs (ILC2) were identified in bronchial biopsies of subjects with TDI-induced occupational asthma using immunohistochemistry. Human bronchial epithelial cells were exposed to TDI or vehicle.

Results: TDI-exposed mice had higher numbers of airway goblet cells, BAL eosinophils, $CD4^+$ T-cells and ILCs, with a predominant type 2 response, and tended to have airway hyperresponsiveness. In TDI-exposed microRNA-155 knockout mice, inflammation and airway hyperresponsiveness were attenuated. TDI exposure induced IL-33 expression in human bronchial epithelial cells and in murine lungs, which was microRNA-155 dependent in mice. $GATA3^+CD3^-$ cells, presumably ILC2, were present in bronchial biopsies.

Conclusion: TDI exposure is associated with increased numbers of ILCs. The proinflammatory microRNA-155 is crucial in a murine model of TDI asthma, suggesting its involvement in the pathogenesis of occupational asthma due to low molecular weight agents.

Introduction

Asthma is a heterogeneous airway disease with many phenotypes. Whereas the mechanisms of early-onset allergic asthma are well understood, the mechanisms leading to late onset asthma are less known. One important example of late onset asthma is occupational asthma caused by workplace exposures to respiratory sensitisers or inhaled irritants. Occupational asthma is a growing public health burden in industrialised countries. Respiratory sensitisers encompass high molecular weight agents (*e.g.* wheat flour, natural rubber latex, animal proteins) and low molecular weight agents (*e.g.* acrylates and diisocyanates). Toluene 2,4-diisocyanate (TDI) is an industrial intermediate which is processed into polyurethanes, used for the manufacture of foams, adhesives, paints and varnishes [1–3]. Approximately 5–20% of workers exposed long-term to TDI develop asthma symptoms and have an accelerated decline in lung function [4, 5].

TDI-induced occupational asthma generally develops within 2–3 years since the first exposure and may be the outcome of both an inflammatory reaction and nonspecific airway hyperresponsiveness [6–9]. Both type 1 and type 2 immune responses can be induced [6]. The response to TDI exposure in sensitised asthmatics can be very severe and removal from the workplace is often the only effective approach to prevent asthma attacks [3, 7, 9, 10]. Elucidation of the pathogenesis of TDI-induced asthma is necessary for prevention and treatment.

MicroRNAs (miRNAs) are short single-stranded RNAs that negatively regulate gene expression by inhibiting mRNA translation or by degrading mRNA targets. Altered miRNA levels have a regulatory role in biological processes including stress response and inflammation. Several miRNAs have already been identified in the disease pathogenesis of asthma [11–13]. One particular miRNA of interest is miR-155, yet contradictory findings regarding its expression in airway samples and role in inflammatory response in mouse models [13, 14] have been reported. Recently it was demonstrated that miR-155 is a critical regulator for IL-33 signalling [15] and affects innate lymphoid cell type 2 (ILC2) expression [15–17]. While JOHANSSON *et al.* [15] and ZHU *et al.* [16] showed that miR-155 expression regulates ILC2 expansion and function, KNOLLE *et al.* [17] showed that ILC2-intrinsic miR-155 expression is required to prevent apoptosis of ILC2. ZHU *et al.* [16] found that ILC2 frequencies in peripheral blood positively correlated with miR-155 expression in nasal mucosa of patients with allergic rhinitis. miR-155 agomir administration not only upregulated ILC2 numbers, it also promoted allergic symptoms and type 2 cytokine production in their allergic rhinitis mouse model. ILCs, the innate counterpart of T-helper (Th) cells, are present in mucosal tissues and produce cytokines upon activation by alarmins (*e.g.* interleukin (IL)-33 and thymic stromal lymphopoietin (TSLP)) released by damaged epithelium. The importance of ILC2 has been investigated in several human studies and mouse models of atopic asthma [18, 19], but little is known about this cell population in isocyanate-induced asthma. Previously it was shown that dermal exposure to TDI increases miR-155 expression in mouse parotid draining lymph nodes [20]. However, whether miR-155 and ILCs contribute to airway responses towards isocyanates remains to be elucidated.

In this study we used a mouse model of TDI-induced asthma to investigate changes in ILCs and T-cell subsets upon isocyanate exposure. The presence of ILC2 was examined in bronchial biopsies of TDI-induced asthmatic patients. In addition, we exposed miR-155 knockout (KO) and wild-type (WT) mice to TDI to investigate whether miR-155 contributes to isocyanate-induced airway inflammation and hyperresponsiveness.

Methods

Murine experiments

Mice

Male B6.Cg-*Mir155*$^{tm1.1Rsky}$/J mice (n=6–10 per group, age 5–8 weeks) were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and bred at Charles River (Lyon, France). Male C57BL/6J wild-type mice were obtained from Charles River. The experimental protocol was approved by the animal ethical committee of the Faculty of Medicine and Health Sciences (ECD 16/34; Ghent University, Ghent, Belgium) and was carried out in accordance with institutional guidelines for animal care.

Exposure protocol

On days 1 and 8, mice were dermally sensitised on the dorsum of both ears to 20 µL of 2% TDI (v/v) dissolved in vehicle (a mixture of acetone and olive oil (AOO), ratio 2:3) or sole vehicle. On days 15, 22 and 29, isoflurane-anaesthetised mice were challenged (oropharyngeal administration) with 20 µL 0.01% TDI or vehicle (AOO ratio 1:4). On day 31, the animals were euthanised (exposure protocol adapted from DE VOOGHT *et al.* [21]) (figure 1a). The AOO/AOO control group (also called vehicle group) was neither sensitised nor challenged with TDI, while the TDI/TDI group received TDI both dermally and oropharyngeally. In addition, an AOO/TDI group receiving no sensitisation but only TDI oropharyngeally
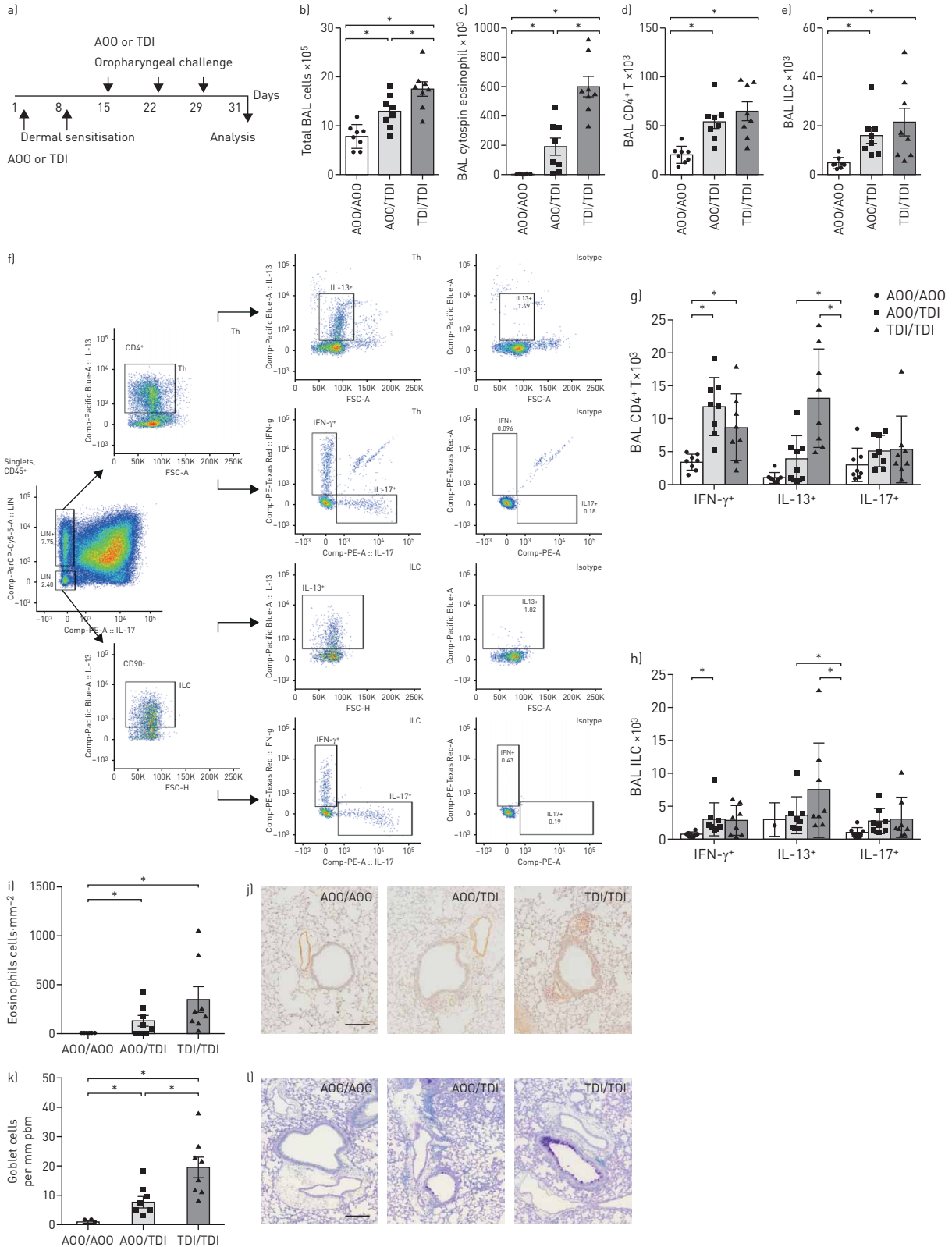
FIGURE 1 Exposure to toluene 2,4-diisocyanate (TDI) induces airway inflammation. Wild-type (WT) mice were exposed to acetone and olive oil (AOO)/AOO, AOO/TDI or TDI/TDI. a) Exposure protocol, b) total bronchoalveolar lavage (BAL) cells and c) eosinophils; b and c were determined by cytospin. BAL cells were stimulated for 4 h with phorbol 12-myristate 13-acetate/ionomycin+protein transport inhibitors, intracellular labelled and analysed using flow cytometry d) CD4$^+$ T-cells (CD45$^+$, CD11c$^-$, CD11b$^-$, CD45R$^-$, CD5$^+$, TCRβ$^+$, CD4$^+$), e) innate lymphoid cells (ILCs) (CD45$^+$, Lin$^-$ (CD11c$^-$, CD11b$^-$, CD45R$^-$, CD5$^-$, TCRβ$^-$), CD90$^+$), f) gating strategy, g) interferon (IFN)-γ$^+$ (interleukin (IL)-17$^-$) CD4$^+$ T-cells, IL13$^+$ CD4$^+$ T-cells,

IL-17$^+$ (IFN-γ$^-$) CD4$^+$ T-cells and h) IFN-γ expressing ILCs, IL-13$^+$ ILCs and IL-17$^+$ ILCs are shown. Data are representative of three independent experiments. Representative photomicrographs and quantification of i and j) Congo-red stained peribronchial eosinophils and k and l) periodic acid–Schiff-stained mucus-producing goblet cells. Airways with a perimeter of the basement membrane >800 µm and <2000 µm are included. Data are presented as mean±SD. n=8. Scale bars=100 µm. *: p<0.05.

was included in the experiments. TDI, acetone and olive oil were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Bronchoalveolar lavage and tissue harvest

Bronchoalveolar lavage was performed and cell counts were measured as described previously. Lungs and auricular lymph nodes were harvested and single cell suspensions were prepared [19].

Flow cytometry

All staining procedures were performed in PBS without calcium and magnesium containing 5 mM EDTA and 1% bovine serum albumin. Bronchoalveolar lavage (BAL) cells were preincubated with FcR-blocking antibody (anti-CD16/CD32, clone 2.4G2). Cells were stained with a combination of anti-mouse fluorochrome-conjugated monoclonal antibodies against CD3 (145–2C11), CD5 (53-7.3), CD4 (GK1.5), CD45 (30-F11), CD11b (M1/70), CD11c (N418), CD45R (RA3-6B2), CD90.2 (30-H12), Ly6G (1A8), Siglec-F (E50-2440), NK1.1(PK136), KLRG1(2F1/KLRG1), Ly-6G/Ly-6C(Gr-1)(RB6-8C5), FcεRIα (MAR-1), CD127(A7R34) and T-cell receptor-β (H57-597). For cytoplasmatic interferon (IFN)-γ (XMG1.2), IL-13 (eBio13A), IL-17 (17B7) or matched isotype staining, cells were stimulated with ionomycin and phorbol 12-myristate 13-acetate, supplemented with brefeldin A and monensin at 37°C for 4 h. Cell subsets were analysed using an LSR Fortessa cytometer (BD Biosciences, San Jose, CA, USA) and FlowJo software (Ashland, OR, USA). CD45$^+$ and CD45$^-$ lung cells were sorted (>95% purity, data not shown) using an OctoMACS separator and CD45 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany).

Protein measurements

Blood was collected from the retro-orbital plexus and centrifuged (10 min, 1210×g) for the isolation of serum. Total IgE measurement (BD OptEIA set mouse IgE) on serum was performed. IL-13, IL-33, IL-1α and CCL5 levels were determined using ELISA (R&D Systems, Abingdon, UK) in supernatants of LN cultures, in total lung homogenate and BAL supernatants (details on sample preparation are given in the supplementary material).

Quantitative reverse transcriptase PCR

RNA was extracted using the miRNeasy mini kit (Qiagen, Hilden, Germany) and cDNA was prepared with the miScript II RT kit. miR-155 expression was determined with the miScript System (Qiagen) on a LightCycler 96 detection system (Roche, Mannheim, Germany) and normalised based on the expression of SNORD68 and SNORD95. The expression of mouse IL-33 and HPRT was analysed using TaqMan Gene Expression assays.

Histology

To quantify eosinophils and goblet cells, lung sections were stained with Congo Red or periodic acid–Schiff stain, respectively. Quantitative measurements were performed on an Axio Imager running AxioVision software (Zeiss, Oberkochen, Germany). Haematoxylin and eosin (H&E) staining was executed according to standard protocols [22, 23].

Airway hyperresponsiveness

48 h after the last instillation, airway hyperresponsiveness was measured as described previously [19]. A "snapshot perturbation" manoeuvre was imposed to measure the resistance of the whole respiratory system.

Analysis on human samples

Study population

Patient characteristics are described in table 1. Bronchial biopsies from nine nonatopic subjects, all nonsmokers, with TDI-induced occupational asthma were studied. Biopsies from seven subjects were taken at time of diagnosis, 2–22 days after their last TDI exposure. Biopsies from two subjects were taken 6 months after cessation of exposure to TDI. From one patient, biopsies were obtained both at time of diagnosis and at 6 months after the last TDI exposure. The study conformed to the declaration of Helsinki, and informed written consent was obtained from each subject.

TABLE 1 Characteristics of study population	
Subjects	9
Male/female	6/3
Age years	39 (26.5–47)
Atopy yes/no	0/9
Onset of asthma years	2 (0.5–4.5)
Exposure to TDI years	2.5 (2.25–25.75)
Interval between last exposure and biopsy days	21 (10.5–26)

Data are presented as n or median (interquartile range). TDI: toluene 2,4-diisocyanate.

Human sample processing and immunohistochemistry

Biopsies were processed as described previously [24, 25]. ILC2 were detected with specific antibodies anti-CD3 and anti-GATA3 (supplementary material). Sections were counterstained with haematoxylin. The number of positive cells was counted in the submucosal area up to 100 μm below the basement membrane. Positively stained cells were expressed as the number of cells per mm^2 of examined area.

In vitro human bronchial epithelial cell culture

Primary normal human bronchial epithelial cells (HBECs) were purchased from Lonza (Basel, Switzerland) and primary HBECs were isolated in-house (supplementary material) from lung resection specimens from three different never-smoking donors obtained from the Department of Respiratory Medicine, Ghent University Hospital. Cells were grown in air–liquid interface (ALI) culture and on days 26 (donors) and 29 (Lonza), cells were exposed for 25 min to TDI (predissolved in ethylene glycol dimethyl ether (EGDME)) or to vehicle (1/50 EGDME in Hanks' buffered salt solution) on the apical side of the culture. Cells were harvested for RNA extraction and supernatant was collected 6 h post-exposure.

Protein measurements and quantitative reverse transcriptase PCR

Protein measurements are provided in the supplementary material. Human IL-33 and TSLP mRNA levels and reference genes HPRT1 and RPL13A were measured in HBECs with Bio-Rad PrimePCR SYBR Green Assays (Hercules, CA, USA). miR-155/RNU48 cDNA was obtained with the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Thermofisher Scientific, Foster City, CA, USA). LC480 Probes Master (Roche) and TaqMan microRNA assay primers were used for the PCR reactions.

Data analysis

Statistical analysis was performed using SPSS (version 25.0; IBM, Armonk, NY, USA). Nonparametric tests (Kruskal–Wallis test and Mann–Whitney U-test) were used to compare different groups, according to the standard statistical criteria. Values were reported as mean±SD. p-values <0.05 were considered as statistically significant.

Results

Isocyanate exposure in mice induces a predominant type 2 airway inflammation

To investigate the inflammatory responses towards TDI, mice were twice dermally sensitised, followed by oropharyngeal challenges on days 15, 22 and 29 (figure 1a). AOO was used as a vehicle to dissolve TDI, leading to three groups: AOO/AOO, AOO/TDI (only TDI airway challenge) and TDI/TDI (dermal and airway TDI challenge). Mice exposed to TDI/TDI had higher numbers of total cells and eosinophils in BAL compared to mice exposed to vehicle (figure 1b and c), which was also confirmed by flow cytometry (data not shown). Mice exposed to TDI/TDI also displayed higher number of CD4^{+} T-cells and ILCs (figure 1d and e). To distinguish the Th-cell subsets as well as the three ILC subsets (IFN-γ-producing ILC1, IL-13-producing ILC2 and IL-17-producing ILC3 [26]), BAL cells were stimulated with phorbol myristate acetate/ionomycin to measure intracellular cytokine production (gating strategy, see figure 1f). All examined T-cell and ILC subpopulations were elevated after TDI/TDI exposure, with a predominant increase in IL-13^{+} CD4^{+} Th2 cells and IL-13^{+} ILC2 (figure 1g and h). The increase in ILC2 and ILC1/3 was confirmed in an independent experiment via alternative gating with CD127 and KLRG1 (supplementary figure S1a–c). Both ILC2 and Th2-cell percentages were significantly positively correlated with the percentage eosinophils in BAL (supplementary figure S1d and e). In addition, ILC1 and Th1-cell percentages were positively correlated with eosinophils, but less pronounced (data not shown). Regarding neutrophils, no significant correlations with Th subsets or ILC subsets could be demonstrated (data not shown). In the AOO/TDI group, most inflammatory markers tended to be intermediate between the fully challenged group (TDI/TDI) and the vehicle-exposed group.

In lung tissue, TDI exposure increased the numbers of peribronchial eosinophils and airway goblet cells (figure 1i–l). ILC numbers in lung single-cell suspensions did not significantly change upon TDI exposure (supplementary figure S1f).

GATA3+CD3− cells are present in bronchial biopsies of isocyanate-induced asthma patients

Since the presence of ILC2 had not yet been reported within the context of isocyanate-induced asthma, we wanted to evaluate whether ILC2 are also present in airway samples of TDI-exposed human subjects. Therefore, we performed an immunohistochemical double staining for GATA3 (expressed both in ILC2 and Th2 cells) and CD3 (specific for T-cells) to identify putative ILC2 in precious bronchial biopsies from patients diagnosed with isocyanate-induced asthma (n=9). In biopsies taken at the time of diagnosis of TDI-induced asthma (within 2–22 days after the last TDI exposure), GATA3+CD3− cells (compatible with ILC2) were detected in the airway submucosa in five out of seven patients (figure 2a and b). High numbers of CD3+ cells were present in biopsies (figure 2c). We found a weakly significant positive correlation between eosinophils and the number of CD3+ cells (R=0.6727, p=0.039) (supplementary figure S1g), but not with the number of GATA3+CD3− cells (putative ILC2, data not shown). However, this does not exclude an association between ILC2 and eosinophils in the biopsies, since both the number of identified cells as well as the number of samples was too low to have sufficient power. Remarkably, GATA3+CD3− cells could not be demonstrated in biopsies from patients (n=3) after cessation of TDI exposure for 6 months. In the patient from whom specimens were available at both time points, GATA3+ CD3− cells, presumably ILC2, were detectable at diagnosis, but disappeared after ⩾6 months' cessation of TDI exposure, while the number of eosinophils was reduced (data not shown).

Isocyanate exposure can induce IL-33 expression in HBECs and in the mouse model of TDI asthma

Given that IL-33 and TSLP activate ILC2 and promote the induction of type 2 responses, we next assessed whether TDI can activate the epithelial-driven IL-33 or TSLP pathway by exposing HBECs to TDI or vehicle. TDI exposure in commercially available HBECs (Lonza) led to an induction of IL-33 mRNA levels
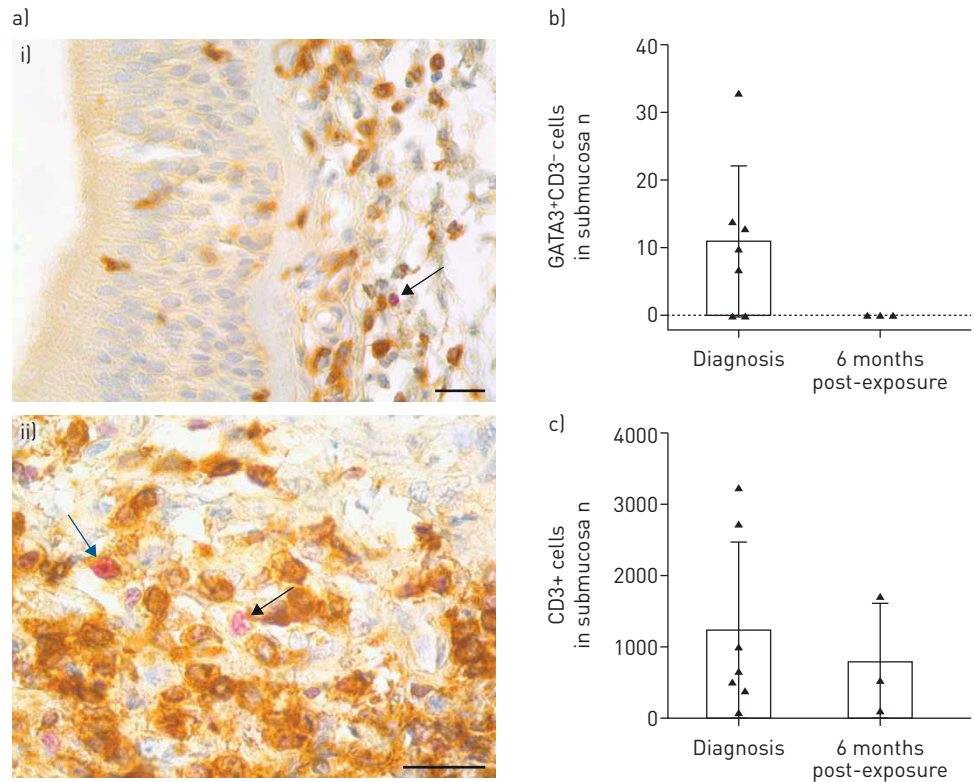

FIGURE 2 Immunohistochemical detection of GATA3+CD3− cells and T-cells in human lung tissue. a) Representative examples of immunohistochemistry for i) GATA3+CD3− cells (in pink, black arrow) and CD3+ cells (in brown) at time of diagnosis in the submucosa of an asthmatic subject who developed asthma symptoms after a period of exposure to toluene 2,4-diisocyanate (TDI). Original magnification ×63; ii) GATA3+CD3+ cell (blue arrow) and GATA3+CD3− cell (black arrow), CD3+ cells in brown. Original magnification ×100. Quantification of b) GATA3+CD3− cells and c) CD3+ cells at time of diagnosis and 6 months post-exposure. Data are expressed as mean±SD. n=3–6. Scale bars=20 μm.
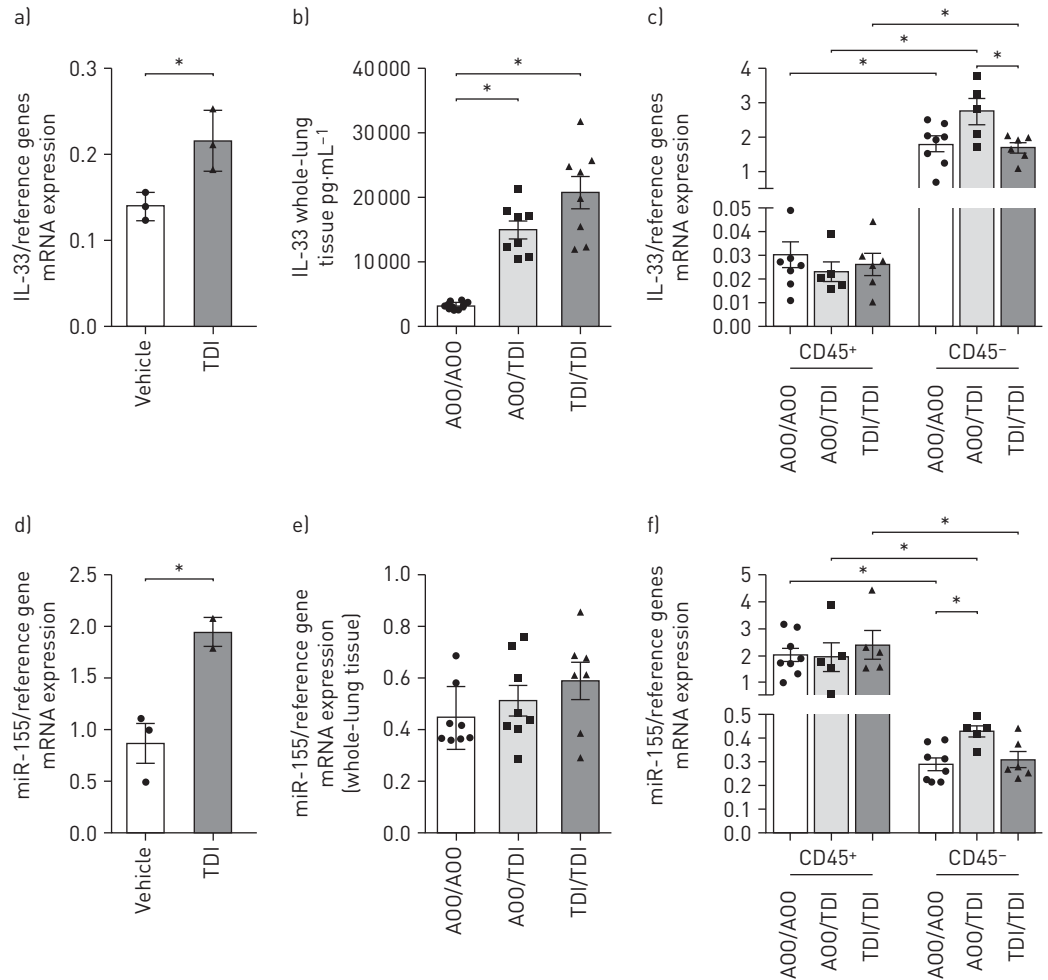
FIGURE 3 Interleukin (IL)-33 and microRNA (miR)-155 expression following toluene 2,4-diisocyanate (TDI) exposure. a) IL-33 mRNA levels were determined *in vitro* in human bronchial epithelial cells (HBECs) (n=3 technical replicates) and normalised to HPRT and RPL13A. Vehicle=1/50 ethylene glycol dimethyl ether in Hanks' buffered salt solution; b) murine IL-33 protein levels in lung homogenate and c) IL-33 mRNA expression in murine sorted CD45$^+$ and CD45$^-$ lung cells normalised to HPRT. d) miR-155/RNU48 mRNA expression in HBEC, e) miR-155/snorD95 mRNA expression in murine whole-lung tissue and f) miR-155 mRNA expression in murine sorted CD45$^+$ and CD45$^-$ lung cells normalised to SNORD95 and SNORD68. AOO: acetone and olive oil. Murine data are expressed as mean±SD. n=6–8. *: p<0.05.

compared to vehicle control (figure 3a). Of note, in in-house isolated primary HBECs, the impact of TDI on IL-33 expression varied between donors, as a similar trend was only observed for one out of three donors (supplementary figure S2a). TSLP mRNA expression either tended to increase or decrease upon TDI exposure, depending on the donor (data not shown).

In murine lung homogenates, IL-33 protein levels were significantly increased in response to TDI (figure 3b). Murine IL-33 mRNA was predominantly expressed in the nonhaematopoietic (CD45$^-$) compartment and was highest in the AOO/TDI group (figure 3c).

Isocyanate-induced airway inflammation is miR-155 dependent in mice

Since proliferation of lymphoid cells (including ILC2) is regulated by miR-155, a miRNA associated with allergic airway inflammation [15], we evaluated the expression of miR-155 in HBECs and in the mouse model of TDI asthma. TDI exposure in commercially available HBECs (Lonza) led to an induction of miR-155 expression compared to vehicle control (figure 3d), yet this was less apparent using the in-house generated HBECs (supplementary figure S2b). In mice, the expression of miR-155 was detectable in total lung tissue and significantly higher in the haematopoietic (CD45$^+$) cell compartment compared to the nonhaematopoietic (CD45$^-$) cell compartment (figure 3e and f). Interestingly, in the CD45$^-$ cell compartment, miR-155 expression was upregulated in the AOO/TDI group. However, the miR-155

expression in total lung tissue or LN was not significantly modulated by TDI exposure (figure 3e and supplementary figure S2c). Considering the controversial role of miR-155 in asthma, we subsequently evaluated the effect of miR-155 deficiency on isocyanate-induced type 2 airway inflammation by exposing miR-155 KO and WT mice to vehicle or TDI. The number of TDI-induced eosinophils was strongly reduced in miR-155 KO mice (figure 4a). Moreover, the TDI-induced increase in total and IL-13 producing CD4$^+$ T-cells and ILCs was significantly attenuated in TDI-exposed miR-155 KO mice compared to WT mice (figure 4b–e). An attenuated increase in the number of IFN-γ- or IL-17-producing T-cells and IL-17-producing ILCs was also observed (supplementary figure S2d–g).

In lung tissue, peribronchovascular eosinophilic inflammation and airway goblet cell metaplasia upon TDI exposure were significantly reduced in miR-155 KO mice compared to the corresponding WT mice (figure 4f and g). The TDI-induced inflammation in lung tissue in WT mice, visualised by H&E-staining, was absent in miR-155 KO mice (figure 4h).

TDI-induced pulmonary expression of IL-33 and IL-1α, but not CCL5, is miR-155 dependent

To unravel the mechanisms underlying the attenuated inflammatory responses in the TDI-exposed miR-155 KO mice, we investigated the expression of the type 2-promoting cytokines IL-33 and IL-1α as well as the T-cell and eosinophil-attracting chemokine CCL5 [27, 28]. The TDI-induced IL-33 protein levels in lung homogenate of miR-155 KO mice were significantly decreased compared to the corresponding TDI-exposed WT mice after combined dermal sensitisation and airway challenge (TDI/TDI), but not after sole airway challenge (AOO/TDI) (figure 5a). IL-1α protein levels also increased upon TDI exposure compared to vehicle. Notably, IL-1α expression was miR-155 dependent after AOO/TDI exposure, but not after TDI/TDI exposure (figure 5b).

In addition, TDI exposure resulted in a strong increase of CCL5 expression compared to the vehicle group, which was similar in WT and miR-155 KO mice (figure 5c).

Role of miR-155 in TDI-induced type 2 responses in lymph nodes and in serum IgE

To investigate type 2 responses in the lymph nodes, auricular lymph nodes of WT and miR-155 KO mice were isolated and stimulated with concanavalin A; next, IL-13 protein levels were measured in the cell culture supernatant. The auricular lymph nodes from TDI/TDI-exposed mice showed a significant increase in IL-13, with no difference between the genotypes (figure 5d). TDI exposure led to elevated total IgE levels in serum in WT mice, which was absent in the miR-155 KO mice (figure 5e).

Effect of miR-155 deficiency on isocyanate-induced airway hyperresponsiveness

To investigate the involvement of miR-155 in TDI-induced airway hyperresponsiveness, the peak resistance in response to increasing concentrations of carbachol was measured in WT and miR-155 KO mice. In the TDI/TDI group, there was a nominal increase in the peak resistance in WT mice, compared to the vehicle-exposed control group and the AOO/TDI group. TDI exposure in the miR-155 KO mice did not increase airway responsiveness (figure 5f).

Discussion

Diisocyanates such as TDI are highly reactive chemicals that can induce asthma upon workplace exposures. In this study, we show a predominant type 2 inflammatory response with higher numbers of Th2 cells and ILC2 in a mouse TDI asthma model, that is dependent on miR-155. In addition, we demonstrated the presence of GATA3$^+$CD3$^-$ cells, presumably ILC2, in human bronchial biopsies of subjects with diisocyanate-induced asthma. Together, these findings suggest a role for ILC2 and miR-155 in the pathogenesis of occupational asthma due to low molecular weight agents such as TDI.

Our TDI asthma model shows increased eosinophils in BAL, which is in line with observations in sputum [29] and in bronchial biopsies [30] of human subjects. The inflammatory response was further characterised by an increase in CD4$^+$ T-cells and all CD4$^+$ Th-cell subpopulations (IFN-γ^+, IL-13$^+$ and IL-17$^+$), as previously described in other TDI mouse models [7, 21, 31–36]. The prominent increase in IL-13-producing T-cells, together with peribronchial eosinophilia and goblet cells, point to a predominant Th2 type response. Moreover, we confirmed sensitisation by elevated total IgE in serum after TDI exposure [6, 7, 21, 32, 33, 37]

The novelty of our research data is that we demonstrated that TDI challenge following dermal sensitisation led to a rise in ILC subsets: IFN-γ^+ ILC1, IL-13$^+$ ILC2 and IL-17$^+$ ILC3. In particular the ILC2 subset, which is known to induce inflammation in mouse airways upon allergen exposure [26], was expanded. In atopic asthmatics, increased numbers of ILC2 have been detected in sputum, BAL and blood [18, 38]. The altered ILC2 numbers in BAL of our murine TDI asthma model suggest that this cell population might also be involved in the pathogenesis of chemical-induced asthma. To address this hypothesis, we used very
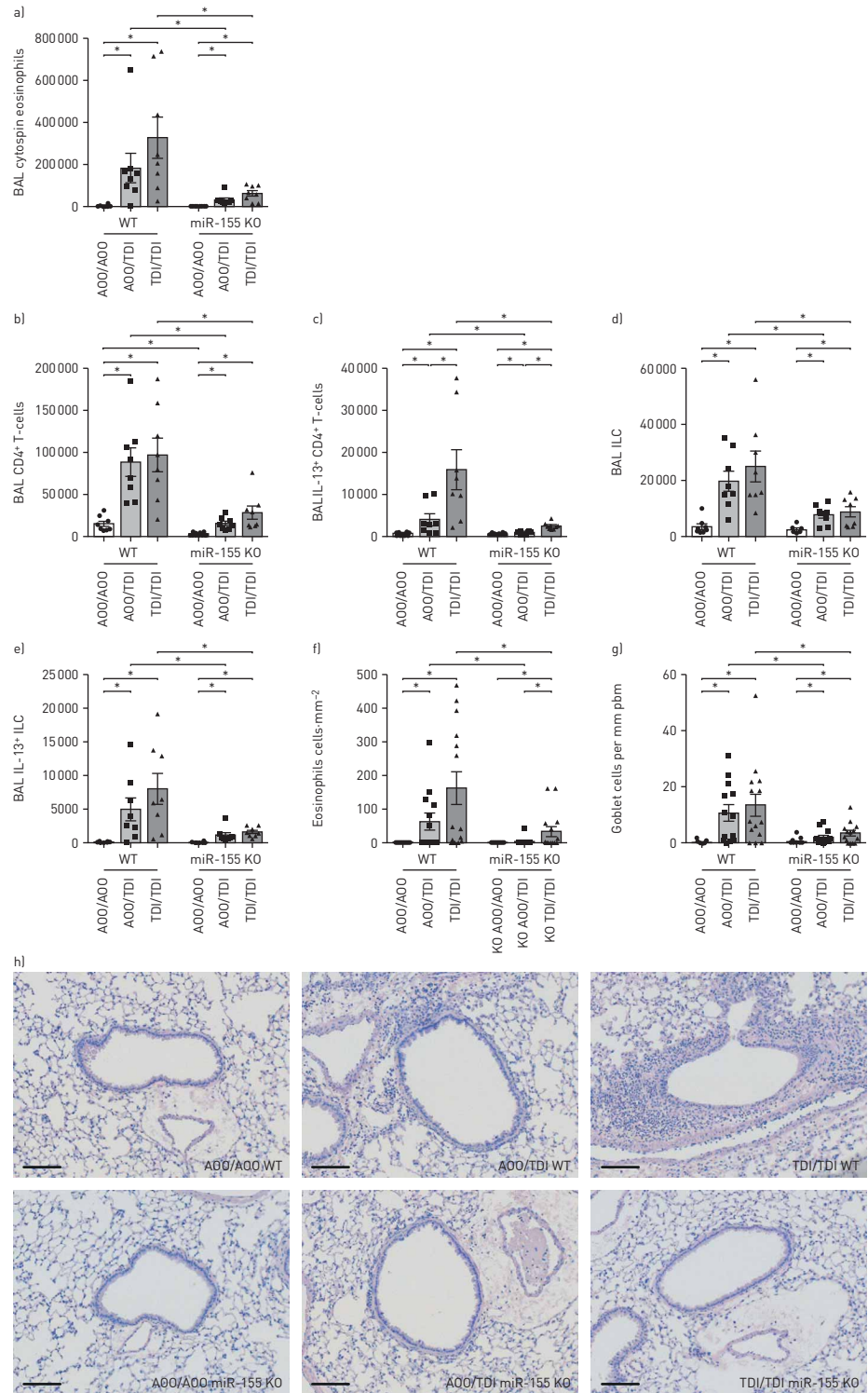
FIGURE 4 Absence of microRNA (miR)-155 attenuates toluene 2,4-diisocyanate (TDI)-induced inflammation. Wild-type (WT) and miR-155 knockout (KO) mice were exposed to acetone and olive oil (AOO)/AOO, AOO/TDI or TDI/TDI. a) Total eosinophils were determined on cytospin. Bronchoalveolar lavage (BAL) cells were stimulated for 4 h with phorbol 12-myristate 13-acetate/ionomycin+protein transport inhibitors, intracellular-labelled and analysed using flow cytometry: b) CD4+ T-cells (CD45+, CD11c−, CD11b−, CD45R−, CD5+, TCRβ+, CD4+), c) interleukin (IL)-13+ CD4+ T-cells, d) innate lymphoid cells (ILCs) (CD45+, Lin− (CD11c−, CD11b−, CD45R−, CD5−, TCRβ−), CD3−, CD4−, CD90+) and e) IL-13+ ILC. Data are representative of at least two independent experiments and expressed as mean±SD. n=8. *: p<0.05. Quantification of f) Congo-red stained peribronchial eosinophils, g) periodic acid–Schiff-stained mucus-producing goblet cells and h) photomicrographs of haematoxylin and eosin-stained lung tissue. Data are combined from two independent experiments and represented as mean±SD. n=13–18. Scale bar=100 µm. *: p<0.05.
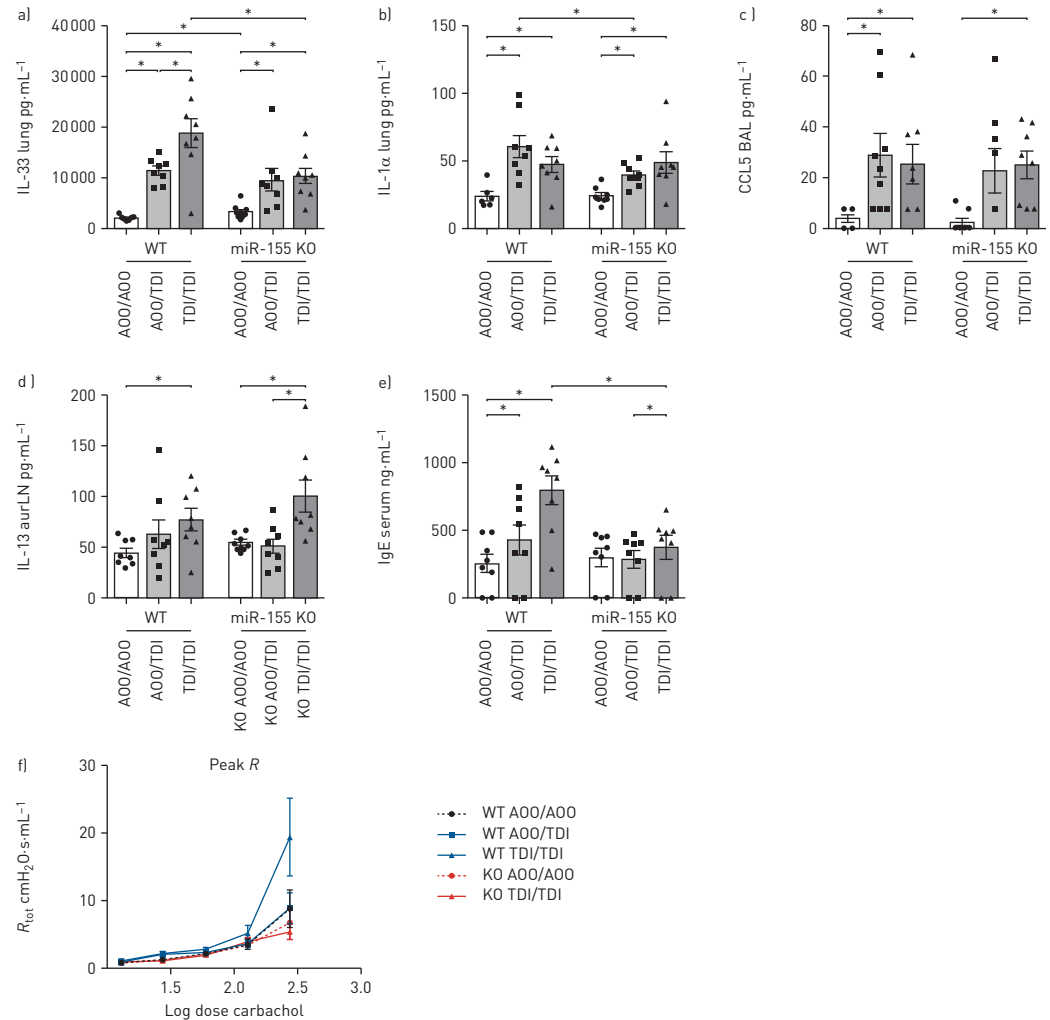
FIGURE 5 Role of microRNA (miR)-155 in toluene 2,4-diisocyanate (TDI)-induced type 2 responses and airway hyperresponsiveness. a) Murine interleukin (IL)-33 protein levels in whole-lung homogenate; b) IL-1α protein levels in whole-lung homogenate; and c) CCL5 protein levels in bronchoalveolar lavage (BAL). d) IL-13 protein levels in the supernatants of concanavaline A-stimulated auricular lymph nodes (aurLN) and e) immunoglobulin (Ig)E titres in serum were determined using ELISA. f) Airway resistance (R) of wild-type (WT) mice or miR-155 knockout (KO) mice exposed to TDI or vehicle (acetone and olive oil (AOO)/AOO) was measured in response to increasing doses of carbachol. There is no R-measurement in AOO/TDI miR-155 KO group due to limited availability of miR-155 KO mice. a–e) Data are expressed as mean±SD; e) data are expressed as mean±SEM. n=8–10. *: p<0.05.

unique and scarce biopsy specimens from patients with TDI-induced asthma [30] to investigate the presence of ILC2. While increased numbers of activated T-cells and Th2 cytokines have already been found in diisocyanate asthmatic individuals [39–41], we are the first to detect GATA3^{+}CD3^{−} cells, possibly ILC2, in bronchial biopsies from patients suffering from diisocyanate-induced asthma. In the three biopsies of subjects who stopped exposure to TDI for 6 months, these GATA3^{+}CD3^{−} cells could not be detected. Although no firm conclusions can be drawn from this observative finding, it is noteworthy that cessation of TDI exposure also decreased eosinophil numbers [30].

To investigate if IL-33, a known activator of Th2 and ILC2 [15], is an important factor upstream of TDI-induced asthma, we exposed HBECs to a noncytotoxic TDI concentration and observed an increased IL-33 mRNA expression in commercial HBECs compared with the control group, which corresponds with an earlier report [42]. The response was donor-dependent in in-house generated HBECs. Interestingly, IL-33 protein levels were detectable in our TDI asthma model and increased upon TDI exposure. However, it is challenging to draw firm conclusions, since IL-33 is processed into mature bioactive forms and because IL-33 also acts as a chromatin-associated nuclear factor, besides its proinflammatory cytokine function [43, 44]. Thus, high levels of nuclear IL-33 do not necessarily mean that there is high secretion of

bioactive IL-33. It remains to be investigated whether the interaction between epithelial cells and inflammatory cells in our model could further perpetuate epithelial IL-33 release. It has been demonstrated before that exposing airway epithelial cells (A549) to TDI or a co-culture of A549 with blood neutrophils led to enhanced expression and release of epithelial folliculin, which is important in airway epithelial integrity and survival [45]. A similar mechanism exists for aspirin-exacerbated respiratory disease, where persistent airway inflammation is associated with eosinophil–epithelium interactions [46].

Previous studies have shown that the type 2 promoting cytokine TSLP is increased upon TDI exposure and that blocking TSLP can reduce TDI-inflammation in murine models [47, 48]. However, in our HBEC experiments, the TDI-induced changes in TSLP expression were donor-dependent.

miR-155 is a microRNA involved in regulating ILC2 function and allergic upper and lower airway inflammation [15, 16, 49, 50]. In WT mice, miR-155 was mostly expressed in haematopoietic cells, consistent with previous reports [17, 51]. We did not observe TDI-induced expression of miR-155 in lungs or lymph nodes of WT mice, which contrasts with earlier findings of increased miR-155 expression in lymph nodes of TDI-exposed mice [20], possibly due to the timing or experimental setup. Furthermore, the vehicle itself, a mixture of acetone and olive oil, may have already modulated miR-155 expression compared to untreated control mice, for example. Notably, miR-155 was detectable in nonhaematopoietic cells in mice (highest in the AOO/TDI group) and increased in TDI-exposed human bronchial epithelial cells (figure 3d), suggesting that it could play a role in epithelial responses to TDI.

OKOYE et al. [52] reported that miR-155 is required for Th-cell recruitment. Correspondingly, we observed that a deficiency in miR-155 attenuated the TDI-induced increase in Th-cells. In addition, we observed that TDI exposure induces CCL5, an important T-cell chemoattractant, in a miR-155 independent way, which corresponds with data from MATHESON et al. [53] and MALMHÄLL et al. [54].

Our data correspond with the work from JOHANSSON et al. [15] and KNOLLE et al. [17], who demonstrated that miR-155 deficient mice exhibit reduced ILC2 levels as well as IL-33. The IL-33 expression seems to be a main driver of Th2 and ILC2 responses not only upon allergen exposure, but also upon isocyanate exposure. How miR-155 actually affects IL-33 expression remains to be further investigated, but is likely due to an indirect effect, since miRNAs downregulate the expression of specific targets.

Oxidative stress and transient receptor potential melastatin (TRPM)8 activation are potential mechanisms leading to IL-33 release following TDI exposure [42, 55, 56]. Additionally, several damage-associated molecular patterns and proinflammatory cytokines that could be associated with IL-33 release have been found in models of TDI exposure, $e.g.$ High mobility group box (HMGB)1, IL-6, tumour necrosis factor-α, IL-1α and IL-1β [28, 48, 57–59]. We investigated the potential implication of IL-1 receptor signalling, since this pathway (especially through IL-1α) is upstream of IL-33 in a model of allergic airway inflammation [60]. Interestingly, prevention of IL-1 signalling can attenuate airway hyperresponsiveness and inflammation in a TDI model [28]. We demonstrated (similar to IL-33) an increase in IL-1α protein levels upon TDI exposure. Notably, whereas TDI-induced IL-33 expression was decreased in miR-155-deficient mice significantly for TDI/TDI (trend for AOO/TDI), IL-1α expression was only miR-155 dependent after AOO/TDI exposure, suggesting a transient miR-155 dependency.

Whereas miR-155 deficiency clearly affected the number of IL-13-producing T-cells and ILC2 in the alveolar compartment of TDI-exposed mice, lymph node cells derived from TDI-exposed WT and miR-155 KO mice produced similar amounts of IL-13 protein. This is in agreement with a previous report, where ILC2 from miR-155 KO mice did not show a lower IL-13 production [17]. It remains unclear from our experiments whether lung ILC2 in miR-155 KO mice are intrinsically affected, or (partly) because of defects in local Th2-cell activation. Total IgE levels were reduced in miR-155 KO mice, which is probably due to an intrinsic defect of miR-155-deficient B-cells in class switching [61] or due to the lower number of ILC2 present. Indeed, ILC2 influence B-cell proliferation and antibody production [62].

TDI/TDI exposures tended to induce airway hyperresponsiveness. This response was completely abolished in the miR-155 KO mice, which corresponds with the attenuated inflammation, including reduced numbers of IL-13$^+$ T-cells and ILC2. This observation is in agreement with ZECH et al. [50], who showed that microRNA-155 has a proinflammatory role in models of ovalbumin and house dust mite-induced allergic airway inflammation and airway hyperresponsiveness.

A limitation of our study is that we used constitutive miR-155 KO mice, which may have some developmental differences impacting our data. In future, an oropharyngeally delivered antagomir against miR-155 could be tested. Yet, in an ovalbumin-induced model of asthma, uptake of this antagomir was efficient in myeloid cells, but not in lymphocytes, and therefore failed to reduce airway inflammation [51]. Another limitation is that we had no access to fresh samples of isocyanate-asthmatic patients to perform flow cytometric analysis for ILC subsets.

In conclusion, this study demonstrates for the first time that ILCs are involved in TDI-induced occupational asthma. We could identify GATA3$^+$CD3$^-$ cells, presumably ILC2, in bronchial tissue of occupational asthma patients. Finally, we showed that miR-155 has a type 2 proinflammatory role in a mouse model of TDI asthma. Together, these data suggest that ILC2 and miR-155 could be promising therapeutic targets for subjects with occupational asthma due to low molecular weight agents such as TDI.

References

1   Vandenplas O. Occupational asthma: etiologies and risk factors. *Allergy Asthma Immunol Res* 2011; 3: 157–167.
2   Daniels RD. Occupational asthma risk from exposures to toluene diisocyanate: a review and risk assessment. *Am J Ind Med* 2018; 61: 282–292.
3   Dykewicz MS. Occupational asthma: current concepts in pathogenesis, diagnosis, and management. *J Allergy Clin Immunol* 2009; 123: 519–528.
4   Bernstein JA. Overview of diisocyanate occupational asthma. *Toxicology* 1996; 111: 181–189.
5   Ott MG, Diller WF, Jolly AT. Respiratory effects of toluene diisocyanate in the workplace: a discussion of exposure-response relationships. *Crit Rev Toxicol* 2003; 33: 1–59.
6   Ban M, Morel G, Langonné I, *et al.* TDI can induce respiratory allergy with Th2-dominated response in mice. *Toxicology* 2006; 218: 39–47.
7   De Vooght V, Smulders S, Haenen S, *et al.* Neutrophil and eosinophil granulocytes as key players in a mouse model of chemical-induced asthma. *Toxicol Sci* 2013; 131: 406–418.
8   Devos FC, Boonen B, Alpizar YA, *et al.* Neuro-immune interactions in chemical-induced airway hyperreactivity. *Eur Respir J* 2016; 48: 380–392.
9   Lau A, Tarlo SM. Update on the management of occupational asthma and work-exacerbated asthma. *Allergy Asthma Immunol Res* 2019; 11: 188–200.
10 Meca O, Cruz MJ, Sánchez-Ortiz M, *et al.* Do low molecular weight agents cause more severe asthma than high molecular weight agents? *PLoS One* 2016; 11: e0156141.
11 Maes T, Cobos FA, Schleich F, *et al.* Asthma inflammatory phenotypes show differential microRNA expression in sputum. *J Allergy Clin Immunol* 2016; 137: 1433–1446.
12 Perry MM, Baker JE, Gibeon DS, *et al.* Airway smooth muscle hyperproliferation is regulated by microRNA-221 in severe asthma. *Am J Respir Cell Mol Biol* 2014; 50: 7–17.
13 Malmhall C, Johansson K, Winkler C, *et al.* Altered miR-155 expression in allergic asthmatic airways. *Scand J Immunol* 2017; 85: 300–307. 10.1111/sji.12535.
14 Rodriguez A, Vigorito E, Clare S, *et al.* Requirement of bic/microRNA-155 for normal immune function. *Science* 2007; 316: 608–611.
15 Johansson K, Malmhäll C, Ramos-Ramírez P, *et al.* MicroRNA-155 is a critical regulator of type 2 innate lymphoid cells and IL-33 signaling in experimental models of allergic airway inflammation. *J Allergy Clin Immunol* 2017; 139: 1007–1016.
16 Zhu YQ, Liao B, Liu YH, *et al.* MicroRNA-155 plays critical effects on Th2 factors expression and allergic inflammatory response in type-2 innate lymphoid cells in allergic rhinitis. *Eur Rev Med Pharmacol Sci* 2019; 23: 4097–4109.
17 Knolle MD, Chin SB, Rana BMJ, *et al.* MicroRNA-155 protects group 2 innate lymphoid cells from apoptosis to promote type-2 immunity. *Front Immunol* 2018; 9: 2232.

18   Smith SG, Chen R, Kjarsgaard M, et al. Increased numbers of activated group 2 innate lymphoid cells in the airways of patients with severe asthma and persistent airway eosinophilia. J Allergy Clin Immunol 2016; 137: 75–86.

19   De Grove KC, Provoost S, Hendriks RW, et al. Dysregulation of type 2 innate lymphoid cells and T_H2 cells impairs pollutant-induced allergic airway responses. J Allergy Clin Immunol 2017; 139: 246–257.

20   Anderson SE, Beezhold K, Lukomska E, et al. Expression kinetics of miRNA involved in dermal toluene 2,4-diisocyanate sensitization. J Immunotoxicol 2014; 11: 250–259.

21   De Vooght V, Vanoirbeek JA, Haenen S, et al. Oropharyngeal aspiration: an alternative route for challenging in a mouse model of chemical-induced asthma. Toxicology 2009; 259: 84–89.

22   De Grove KC, Provoost S, Braun H, et al. IL-33 signalling contributes to pollutant-induced allergic airway inflammation. Clin Exp Allergy 2018; 48: 1665–1675.

23   Van Hove CL, Maes T, Cataldo DD, et al. Comparison of acute inflammatory and chronic structural asthma-like responses between C57BL/6 and BALB/c mice. Int Arch Allergy Immunol 2009; 149: 195–207.

24   Saetta M, Di Stefano A, Maestrelli P, et al. Airway eosinophilia and expression of interleukin-5 protein in asthma and in exacerbations of chronic bronchitis. Clin Exp Allergy 1996; 26: 766–774.

25   Di Stefano A, Turato G, Maestrelli P, et al. Airflow limitation in chronic bronchitis is associated with T-lymphocyte and macrophage infiltration of the bronchial mucosa. Am J Respir Crit Care Med 1996; 153: 629–632.

26   Spits H, Artis D, Colonna M, et al. Innate lymphoid cells – a proposal for uniform nomenclature. Nat Rev Immunol 2013; 13: 145–149.

27   Teran LM, Noso N, Carroll M, et al. Eosinophil recruitment following allergen challenge is associated with the release of the chemokine RANTES into asthmatic airways. J Immunol 1996; 157: 1806–1812.

28   Johnson VJ, Yucesoy B, Luster MI. Prevention of IL-1 signaling attenuates airway hyperresponsiveness and inflammation in a murine model of toluene diisocyanate-induced asthma. J Allergy Clin Immunol 2005; 116: 851–858.

29   Lemière C, Romeo P, Chaboillez S, et al. Airway inflammation and functional changes after exposure to different concentrations of isocyanates. J Allergy Clin Immunol 2002; 110: 641–646.

30   Saetta M, Maestrelli P, Di Stefano A, et al. Effect of cessation of exposure to toluene diisocyanate (TDI) on bronchial mucosa of subjects with TDI-induced asthma. Am Rev Respir Dis 1992; 145: 169–174.

31   Pollaris L, Devos F, De Vooght V, et al. Toluene diisocyanate and methylene diphenyl diisocyanate: asthmatic response and cross-reactivity in a mouse model. Arch Toxicol 2016; 90: 1709–1717.

32   Matheson JM, Johnson VJ, Vallyathan V, et al. Exposure and immunological determinants in a murine model for toluene diisocyanate (TDI) asthma. Toxicol Sci 2005; 84: 88–98.

33   Yao L, Chen S, Tang H, et al. Transient receptor potential ion channels mediate adherens junctions dysfunction in a toluene diisocyanate-induced murine asthma model. Toxicol Sci 2019; 168: 160–170.

34   Tarkowski M, Vanoirbeek JA, Vanhooren HM, et al. Immunological determinants of ventilatory changes induced in mice by dermal sensitization and respiratory challenge with toluene diisocyanate. Am J Physiol Lung Cell Mol Physiol 2007; 292: L207–L214.

35   Zhao W, Lin Y, Xiong J, et al. RAGE mediates β-catenin stabilization via activation of the Src/p-Cav-1 axis in a chemical-induced asthma model. Toxicol Lett 2018; 299: 149–158.

36   Pollaris L, Van Den Broucke S, Decaesteker T, et al. Dermal exposure determines the outcome of repeated airway exposure in a long-term chemical-induced asthma-like mouse model. Toxicology 2019; 421: 84–92.

37   Wisnewski AV, Xu L, Robinson E, et al. Immune sensitization to methylene diphenyl diisocyanate (MDI) resulting from skin exposure: albumin as a carrier protein connecting skin exposure to subsequent respiratory responses. J Occup Med Toxicol 2011; 6: 6.

38   Christianson CA, Goplen NP, Zafar I, et al. Persistence of asthma requires multiple feedback circuits involving type 2 innate lymphoid cells and IL-33. J Allergy Clin Immunol 2015; 136: 59–68.

39   Maestrelli P, Occari P, Turato G, et al. Expression of interleukin (IL)-4 and IL-5 proteins in asthma induced by toluene diisocyanate (TDI). Clin Exp Allergy 1997; 27: 1292–1298.

40   Bentley AM, Maestrelli P, Saetta M, et al. Activated T-lymphocytes and eosinophils in the bronchial mucosa in isocyanate-induced asthma. J Allergy Clin Immunol 1992; 89: 821–829.

41   Redlich CA, Karol MH, Graham C, et al. Airway isocyanate-adducts in asthma induced by exposure to hexamethylene diisocyanate. Scand J Work Environ Health 1997; 23: 227–231.

42   Kim JH, Jang YS, Jang SH, et al. Toluene diisocyanate exposure induces airway inflammation of bronchial epithelial cells via the activation of transient receptor potential melastatin 8. Exp Mol Med 2017; 49: e299.

43   Carriere V, Roussel L, Ortega N, et al. IL-33, the IL-1-like cytokine ligand for ST2 receptor, is a chromatin-associated nuclear factor in vivo. Proc Natl Acad Sci USA 2007; 104: 282–287.

44   Lefrançais E, Roga S, Gautier V, et al. IL-33 is processed into mature bioactive forms by neutrophil elastase and cathepsin G. Proc Natl Acad Sci USA 2012; 109: 1673–1678.

45   Pham DL, Trinh TH, Ban GY, et al. Epithelial folliculin is involved in airway inflammation in workers exposed to toluene diisocyanate. Exp Mol Med 2017; 49: e395.

46   Choi Y, Lee Y, Park HS. Which factors associated with activated eosinophils contribute to the pathogenesis of aspirin-exacerbated respiratory disease? Allergy Asthma Immunol Res 2019; 11: 320–329.

47   Yu G, Zhang Y, Wang X, et al. Thymic stromal lymphopoietin (TSLP) and toluene-diisocyanate-induced airway inflammation: alleviation by TSLP neutralizing antibody. Toxicol Lett 2019; 317: 59–67.

48   Wang Y, Le Y, Zhao W, et al. Short thymic stromal lymphopoietin attenuates toluene diisocyanate-induced airway inflammation and inhibits high mobility group box 1-receptor for advanced glycation end products and long thymic stromal lymphopoietin expression. Toxicol Sci 2017; 157: 276–290.

49   Lu TX, Rothenberg ME. Diagnostic, functional, and therapeutic roles of microRNA in allergic diseases. J Allergy Clin Immunol 2013; 132: 3–13.

50   Zech A, Ayata CK, Pankratz F, et al. MicroRNA-155 modulates P2R signaling and Th2 priming of dendritic cells during allergic airway inflammation in mice. Allergy 2015; 70: 1121–1129.

51   Plank MW, Maltby S, Tay HL, et al. MicroRNA expression is altered in an ovalbumin-induced asthma model and targeting miR-155 with antagomirs reveals cellular specificity. PLoS One 2015; 10: e0144810.

52   Okoye IS, Czieso S, Ktistaki E, *et al.* Transcriptomics identified a critical role for Th2 cell-intrinsic miR-155 in mediating allergy and antihelminth immunity. *Proc Natl Acad Sci USA* 2014; 111: E3081–E3090.

53   Matheson JM, Lange RW, Lemus R, *et al.* Importance of inflammatory and immune components in a mouse model of airway reactivity to toluene diisocyanate (TDI). *Clin Exp Allergy* 2001; 31: 1067–1076.

54   Malmhäll C, Alawieh S, Lu Y, *et al.* MicroRNA-155 is essential for T_H2-mediated allergen-induced eosinophilic inflammation in the lung. *J Allergy Clin Immunol* 2014; 133: 1429–1438.

55   Lantz RC, Lemus R, Lange RW, *et al.* Rapid reduction of intracellular glutathione in human bronchial epithelial cells exposed to occupational levels of toluene diisocyanate. *Toxicol Sci* 2001; 60: 348–355.

56   Miller AM. Role of IL-33 in inflammation and disease. *J Inflamm* 2011; 8: 22.

57   Oczypok EA, Milutinovic PS, Alcorn JF, *et al.* Pulmonary receptor for advanced glycation end-products promotes asthma pathogenesis through IL-33 and accumulation of group 2 innate lymphoid cells. *J Allergy Clin Immunol* 2015; 136: 747–756.

58   Mattoli S, Colotta F, Fincato G, *et al.* Time course of IL1 and IL6 synthesis and release in human bronchial epithelial cell cultures exposed to toluene diisocyanate. *J Cell Physiol* 1991; 149: 260–268.

59   Maestrelli P, di Stefano A, Occari P, *et al.* Cytokines in the airway mucosa of subjects with asthma induced by toluene diisocyanate. *Am J Respir Crit Care Med* 1995; 151: 607–612.

60   Willart MA, Deswarte K, Pouliot P, *et al.* Interleukin-1α controls allergic sensitization to inhaled house dust mite *via* the epithelial release of GM-CSF and IL-33. *J Exp Med* 2012; 209: 1505–1517.

61   Dorsett Y, McBride KM, Jankovic M, *et al.* MicroRNA-155 suppresses activation-induced cytidine deaminase-mediated Myc-Igh translocation. *Immunity* 2008; 28: 630–638.

62   Stier MT, Peebles RS Jr. Innate lymphoid cells and allergic disease. *Ann Allergy Asthma Immunol* 2017; 119: 480–488.