EUROPEAN RESPIRATORY journal

FLAGSHIP SCIENTIFIC JOURNAL OF ERS

Early View

Original article

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Please cite this article as: Blomme EE, Provoost S, Bazzan E, *et al*. Innate lymphoid cells in isocyanate-induced asthma: role of microRNA-155. *Eur Respir J* 2020; in press (https://doi.org/10.1183/13993003.01289-2019).

INNATE LYMPHOID CELLS IN ISOCYANATE-INDUCED ASTHMA: ROLE OF MICRORNA-155

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Total word count: 4241

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The research described here was supported by the Fund for Scientific Research in Flanders (FWO Vlaanderen, G053516N); FWO-EOS project G0G2318N and a Ghent University grant (BOF19-GOA-008). MB received an ERS short-term research training fellowship 2015-2016 which supported his internship in the Laboratory for Translational Research in Obstructive Pulmonary Diseases, Ghent.

ABSTRACT

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

Objective: Determine whether TDI exposure modifies the number of ILC 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 sensitized and challenged with TDI or vehicle. Intracellular cytokine expression in ILC and T cells was evaluated in bronchoalveolar lavage fluid (BAL) by flow cytometry. Peribronchial eosinophilia and goblet cells were evaluated on lung tissue and airway hyperresponsiveness was measured with the forced oscillation technique. Putative ILC2 cells 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 ILC, with a predominant type 2 response and tended to have airway hyperresponsiveness. In TDI-exposed microRNA-155 knockout mice, inflammation and airway hyperresponsiveness was 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 ILC. The proinflammatory microRNA-155 is crucial in a murine model of TDI asthma, suggesting its involvement in the pathogenesis of occupational asthma due to LMW agents.

KEY MESSAGES

- **Innate lymphoid cells are increased in a mouse model of TDI-induced asthma and $GATA3^{+}CD3^{-}$ cells are present in bronchial biopsies of subjects with TDI-induced occupational asthma.**
- **TDI exposure can induce expression of the alarmin IL-33 in human bronchial epithelial cells *in vitro* and in murine lungs *in vivo*, which was microRNA-155 dependent in mice.**
- **MicroRNA-155 plays a key proinflammatory role in an experimental model of TDI-induced asthma, which is characterized by type 2 immunity-driven eosinophilic airway inflammation**

Capsule summary: Our translational research data show that innate lymphoid cells are involved in isocyanate-induced occupational asthma. MicroRNA-155 has a proinflammatory role in a preclinical mouse model, suggesting that it could be a promising therapeutic target.

Key words: asthma, eosinophils, isocyanate, miR-155, innate lymphoid cells, ILC, occupational, TDI

ABBREVIATIONS

AHR airway hyperresponsiveness

ALI air-liquid interface

AOO acetone olive oil

BAL bronchoalveolar lavage fluid

concA concanavalin A

EGDME ethylene glycol dimethyl ether

HBEC human bronchial epithelial cells

HMW high molecular weight

ILC innate lymphoid cells

ILC2 innate lymphoid cell(s) type 2

KO knockout

LMW low molecular weight

LN lymph node

miR-155 microRNA-155

miRNAs microRNAs

PAS periodic acid-Schiff

R resistance

TDI toluene 2,4-diisocyanate

TSLP thymic stromal lymphopoietin

WT wild-type

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 sensitizers or inhaled irritants. Occupational asthma is a growing public health burden in industrialized countries. Respiratory sensitizers encompass high molecular weight (HMW) agents (e.g. wheat flour, natural rubber latex, animal proteins) and low molecular weight (LMW) 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 long-term exposed 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 non-specific airway hyperresponsiveness (AHR) (6-9). Both type 1 and type 2 immune responses can be induced (6). The response to TDI exposure in sensitized 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 signaling (15) and affects innate lymphoid cell type 2 (ILC2) expression (15-17). While Johansson and Zhu showed that miR-155 expression regulates ILC2 expansion and function (15, 16), Knolle *et al.* showed that ILC2-intrinsic miR-155 expression is required to prevent apoptosis of ILC2 (17). Zhu and colleagues 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 did not only upregulate ILC2 numbers, it also promoted allergic symptoms and type 2 cytokine production in their allergic rhinitis mouse model (16). ILC, the innate counterpart of T helper cells, are present in mucosal tissues and produce cytokines upon activation by alarmins (e.g. IL-33 and TSLP (thymic stromal lymphopoietin)) 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 (LN) (20). However, whether miR-155 and ILCs contribute to airway responses towards isocyanates remains to be elucidated.

In this paper we used a mouse model of TDI-induced asthma to investigate changes in ILC 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, 5-8 weeks old) were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and bred at Charles River (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 (Ghent University (ECD 16/34)) and was carried out in accordance with institutional guidelines for animal care.

Exposure protocol:

On day 1 and 8, mice were dermally sensitized 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 day 15, 22 and 29, isoflurane-anaesthetized mice were challenged (oropharyngeal administration) with 20 µl 0.01% TDI or vehicle (AOO ratio 1:4). On day 31, the animals were euthanized (exposure protocol adapted from De Vooght *et al.* (21)) (Fig. 1A). The AOO/AOO control group (also called vehicle group) was neither sensitized nor challenged with TDI, while the TDI/TDI group received TDI both dermally and oropharyngeally. Also an AOO/TDI group receiving no sensitization but only TDI oropharygeally was included in the experiments. Toluene 2,4-diisocyanate, acetone and olive oil were purchased from Sigma-Aldrich.

Bronchoalveolar lavage and tissue harvest:

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

Flow cytometry:

All staining procedures were performed in PBS without Ca^{2+} and Mg^{2+} containing 5 mM EDTA and 1% BSA. 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 TCR-β (H57-597). For cytoplasmatic 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 hours. Cell subsets were analyzed using an LSR Fortessa cytometer and FlowJo software. $CD45^+$ and $CD45^-$ lung cells were sorted (>95% purity, data not shown) using an OctoMACS separator and CD45 microbeads (Miltenyi Biotec).

Protein measurements:

Blood was collected from the retro-orbital plexus and centrifuged (10 min, 2500 rpm) 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 by ELISA (R&D systems) in supernatants of LN cultures, in total lung homogenate and BAL supernatants respectively (*see Online repository material for details on sample preparation*).

qRT-PCR:

RNA was extracted using the miRNeasy mini kit (Qiagen) 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) and normalized based on the expression of SNORD68 and SNORD95. The expression of mouse IL-33 and HPRT was analysed with TaqMan Gene Expression assays.

Histology:

To quantify eosinophils and goblet cells, lung sections were stained with Congo Red or periodic acid-Schiff (PAS) staining respectively. Quantitative measurements were performed on an Axio Imager running AxioVision software. Hematoxylin/eosin staining was executed according to standard protocols (22, 23).

Airway Hyperresponsiveness:

48 hours after the last instillation, airway hyperresponsiveness was measured as previously described (19). A “snapshot perturbation” maneuver was imposed to measure the resistance (R) of the whole respiratory system.

Analysis on human samples:

Study population:

Patient characteristics can be found in Table I. Bronchial biopsies from 9 nonatopic subjects, non-smokers, with TDI-induced occupational asthma exposed to TDI at work were studied. Biopsies from 7 subjects were taken at time of diagnosis, 2-22 days after their last TDI exposure. Biopsies from 2 subjects were taken six months after cessation of exposure to TDI. From 1 patient, biopsies were obtained both at time of diagnosis and at six months after the last TDI exposure. The study conformed to the declaration of Helsinki, and informed written consent was obtained from each subject.

Human sample processing and immunohistochemistry:

Biopsies were processed as previously described (24, 25). ILC2 were detected with specific antibodies anti-CD3 and anti-GATA3 (*see Online repository material*). Sections were counterstained with hematoxylin. 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 (HBEC) were purchased from Lonza and primary HBEC were in-house isolated (see *Online repository material)* from lung resection specimens from 3 different never-smoking donors obtained at the Department of Respiratory Medicine, Ghent University Hospital. Cells were grown in air-liquid interface (ALI) culture and on day 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 HBSS) on the apical side of the culture. Cells were harvested for RNA extraction and supernatant was collected 6h post exposure.

Protein measurements and qRT-PCR:

Please see *Online repository material* for protein measurements. Human IL-33 and TSLP mRNA levels and reference genes HPRT1 and RPL13A were measured in HBEC with Bio-Rad PrimePCR SYBR Green Assays. miR-155/RNU48 cDNA was obtained with the TaqMan MicroRNA Reverse Transcription Kit (Thermofisher Scientific). LC480 Probes Master (Roche) and Taqman microRNA assay primers were used for the PCR reactions.

Data analysis:

Statistical analysis was performed with SPSS, version 25.0. Non-parametric tests (Kruskal-Wallis and Mann-Whitney-U) 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 sensitized, followed by oropharyngeal challenges on days 15, 22 and 29 (Fig. 1A). A mixture of acetone and olive oil (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 (Fig. 1B,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 (Fig. 1D,E). To distinguish the T-helper 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 PMA/ionomycin to measure intracellular cytokine production (gating strategy see Fig. 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^+ ILC2s (Fig. 1G,H). The increase in ILC2 and ILC1/3 was confirmed in an independent experiment via alternative gating with CD127 and KLRG1 (Suppl. Fig. S1A-C). Both %ILC2 as well as %Th2 cells were significantly positively correlated with the % eosinophils in BAL (Suppl. Fig. 1D,E). Also %ILC1 and %Th1 cells 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 (Fig. 1I-L). ILC numbers in lung single cell suspensions did not significantly change upon TDI exposure (Suppl. Fig. S1F).

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

Since the presence of ILC2s 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 ILC2s 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 ILC2s- were detected in the airway submucosa in 5 out of 7 patients (Fig. 2A,B). High numbers of $CD3^{+}$ cells were present in biopsies (Fig. 2C). We found a weakly significant, positive correlation between eosinophils and the number of $CD3^{+}$ cells (Rs:0.6727, P:0.039) (Suppl. Fig. S1G), but not with the number of $GATA3^{+}CD3^{-}$ cells (putative ILC2, data not shown). This however 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 at least 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 human bronchial epithelial cells and in the mouse model of TDI asthma

Given that IL-33 and TSLP activate ILC2s 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 human bronchial epithelial cells (HBEC) to TDI or vehicle. TDI exposure in commercially available HBEC (Lonza) led to an induction of IL-33 mRNA levels compared to vehicle control (Fig. 3A). Of note, in in-house isolated primary HBEC, the impact of TDI on IL-33 expression varied between donors, as a similar trend was only observed for 1 out of 3 donors (Suppl. Fig. 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 (Fig. 3B). Murine IL-33 mRNA was predominantly expressed in the non-hematopoietic ($CD45^{-}$) compartment and was highest in the AOO/TDI group (Fig. 3C).

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

Since proliferation of lymphoid cells –including ILC2s- is regulated by miR-155, a microRNA associated with allergic airway inflammation (15), we evaluated the expression of miR-155 in HBEC and in the mouse model of TDI asthma. TDI exposure in commercially available HBEC (Lonza) led to an induction of miR-155 expression compared to vehicle control (Fig. 3D), yet this was less apparent using the in-house generated HBEC (Suppl. Fig. S2B). In mice, the expression of miR-155 was detectable in total lung tissue and significantly higher in the hematopoietic ($CD45^+$) cell compartment compared to the non-hematopoietic ($CD45^-$) cell compartment (Fig. 3E,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 (Fig. 3E and Suppl. Fig. 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 (Fig. 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 (Fig. 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 (Suppl. Fig. 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 (Fig. 4F,G). The TDI-induced inflammation in lung tissue in WT mice, visualized by Haematoxylin & Eosin-staining, was absent in miR-155 KO mice (Fig. 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 cytokine IL-33, 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 sensitization and airway challenge (TDI/TDI), but not after sole airway challenge (AOO/TDI) (Fig. 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 (Fig. 5B).
TDI-exposure also resulted in a strong increase of CCL5 expression compared to the vehicle group, which was however similar in WT and miR-155 KO mice (Fig. 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 (Fig. 5D). TDI exposure led to elevated total IgE levels in serum in WT mice, which was absent in the miR-155 KO mice (Fig. 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 (Fig. 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 ILC2s in a mouse TDI asthma model, that is dependent on miR-155. We also 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 LMW 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 characterized by an increase in $CD4^{+}$ T cells and all $CD4^{+}$ T helper 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. We moreover confirmed sensitization 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 sensitization led to a rise in ILC subsets: IFN-γ^{+} ILC1, IL-13^{+} ILC2, and IL-17^{+} ILC3. Especially 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 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 ILC2s, in bronchial biopsies from patients suffering from diisocyanate-induced asthma. In the 3 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 HBEC to a non-cytotoxic TDI concentration and observed an increased IL-33 mRNA expression in commercial HBEC compared with the control group, which corresponds with an earlier report (42). The response was however donor-dependent in in-house generated HBEC. 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 also the type 2 promoting cytokine TSLP is increased upon TDI exposure and that blocking TSLP can reduce TDI-inflammation in murine models (47, 48). In our HBEC experiments, the TDI-induced changes in TSLP expression were however 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 hematopoietic cells, consistent with previous reports (17, 51). We did not observe TDI-induced expression of miR-155 in lungs or LN of WT mice, which is in contrast to earlier findings of increased miR-155 expression in LN of TDI exposed mice (20), possibly due to the timing or experimental setup. Also, the vehicle itself, a mixture of acetone and olive oil, may have already modulated miR-155 expression compared to e.g. untreated control mice. Notably miR-155 was detectable in non-hematopoietic cells in mice (highest in AOO/TDI group) and increased in TDI-exposed human bronchial epithelial cells (Fig. 3D), suggesting it could play a role in epithelial responses to TDI.

Okoye and colleagues reported that miR-155 is required for Th cell recruitment (52). Correspondingly, we observed that a deficiency in miR-155 attenuated the TDI-induced increase in Th cells. We also 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 and Knolle *et al.*, who demonstrated that miR-155 deficient mice exhibit reduced ILC2 levels as well as IL-33 (15, 17). The IL-33 expression seems not only a main driver of Th2 and ILC2 responses 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 8 (TRPM8) activation are potential mechanisms leading to IL-33 release following TDI exposure (42, 55, 56). Additionally, several damage-associated molecular patterns (DAMPs) and pro-inflammatory cytokines that could be associated with IL-33 release have been found in models of TDI exposure, e.g. HMGB1, IL-6, TNFα, IL-1α and IL-1β (28, 48, 57-59). We investigated the potential implication of IL-1R 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 AHR 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 ILC2s 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 ILC2s from miR-155 KO mice did not show a lower IL-13 production (17). It remains unclear from our experiments whether lung ILC2s 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 AHR. 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 Idzko *et al.*, who showed that microRNA-155 has a proinflammatory role in models of OVA and HDM-induced allergic airway inflammation and AHR (50).

A limitation of our study is that we used constitutive miR-155 KO mice, which may have some developmental differences impacting our data. In the future, an oropharyngeally delivered antagomir against miR-155 could be tested. Yet, in an OVA-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 innate lymphoid cells 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 LMW agents such as TDI.

ACKNOWLEDGEMENTS

The authors would like to thank Ann Neesen, Indra De Borle, Katleen De Saedeleer, Anouck Goethals, and Greet Barbier from the laboratory for Translational Research in Obstructive Pulmonary Diseases, Department of Respiratory Medicine (Ghent University Hospital, Ghent, Belgium) for their excellent technical assistance.

AUTHOR CONTRIBUTIONS

TM, SP, JV, LP, EB GGB and EEB conceived the project and designed the experiments; FV,SP, HVE, MR, AB, LV, EB, MB, SP and EEB conducted the experiments; MS and MC were responsible for supervision and data collection from subjects with diisocyanate-induced asthma; TM, GGB, GFJ and EEB contributed to the data analysis and interpretation; EEB wrote the manuscript and all authors contributed to writing and editing of the manuscript. All authors have approved the final version of the paper.

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Table I

Characteristics of study population (n = 9)

	Median (IQR)
Number	9
Gender ratio (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)

FIGURE LEGENDS

FIGURE 1: Exposure to TDI induces airway inflammation. WT mice were exposed to AOO/AOO (white bars), AOO/TDI (light gray bars), or TDI/TDI (dark gray bars). Exposure protocol in days **(A)**, total BAL cells **(B)** and eosinophils **(C)**, **B** and **C** were determined by cytospin. BAL cells were stimulated for 4 hours with phorbol 12-myristate 13-acetate/ionomycin + protein transport inhibitors, intracellular labeled and analyzed using flow cytometry **(D-H)** $CD4^+$ T cells ($CD45^+$, $CD11c^-$, $CD11b^-$, $CD45R^-$, $CD5^+$, $TCR\beta^+$, $CD4^+$) **(D),** ILCs ($CD45^+$, Lin^- ($CD11c^-$, $CD11b^-$, $CD45R^-$, $CD5^-$, $TCR\beta^-$), $CD90^+$) **(E),** gating strategy **(F),** IFN-γ^+ (IL-17^-) $CD4^+$ T cells, $IL13^+$ $CD4^+$ T cells, IL-17^+ (IFN-γ^-) $CD4^+$ T cells **(G)**, IFN-γ expressing ILC, IL-13^+ ILC and IL-17^+ ILC **(H)** are shown. Data are representative of three independent experiments. Representative photomicrographs and quantification of congo-red stained peribronchial eosinophils **(I-J)** and periodic acid–Schiff–stained mucus-producing goblet cells **(K-L**). Airways with a perimeter of the basement membrane larger than 800 µm and smaller than 2000 µm are included. Data are expressed as mean ± SD. N =8. * P <0.05.

FIGURE 2: Immunohistochemical detection of $GATA3^+CD3^-$ cells and T cells in human lung tissue. GATA3 in pink, CD3 in brown. Upper panel: representative examples of immunohistochemistry for $GATA3^+CD3^-$ 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 TDI. Original magnification 63X. Lower panel: $GATA3^+CD3^+$ cell (blue arrow) and $GATA3^+CD3^-$ cell (black arrow), $CD3^+$ cells in brown. Original magnification 100X. **(A)**. Quantification of $GATA3^+CD3^-$ cells **(B)** and $CD3^+$ cells **(C)** at time of diagnosis and 6 months post exposure. Data are expressed as mean ± SD. N =3-6.

FIGURE 3: IL-33 and miR-155 expression following TDI exposure. IL-33 mRNA levels were determined *in vitro* in HBEC (N=3 technical replicates) and normalized to HPRT and RPL13A. Vehicle=1/50 EGDME in HBSS **(A)** Murine IL-33 protein levels in lung homogenate **(B)** and IL-33 mRNA expression in murine sorted $CD45^+$ and $CD45^-$ lung cells normalized to HPRT **(C)**. miR-155/RNU48 mRNA expression in HBEC **(D)**, miR-155/snorD95 mRNA expression in murine whole lung tissue **(E)** miR-155 mRNA expression in murine sorted $CD45^+$ and $CD45^-$ lung cells normalized to

SNORD95 and SNORD68 **(F)**. Murine data are expressed as mean ± SD. N =6-8. * P <0.05.

FIGURE 4: Absence of miR-155 attenuates TDI-induced inflammation. WT and miR-155 KO mice were exposed to AOO/AOO (white bars), AOO/TDI (light gray bars) or TDI/TDI (dark gray bars). Total eosinophils **(A)** were determined on cytospin. BAL cells were stimulated for 4 hours with phorbol 12-myristate 13-acetate/ionomycin + protein transport inhibitors, intracellular labeled and analyzed using flow cytometry **(B-E)**. $CD4^+$ T cells ($CD45^+$, $CD11c^-$, $CD11b^-$, $CD45R^-$, $CD5^+$, $TCR\beta^+$, $CD4^+$) **(B),** $IL13^+$ $CD4^+$ T cells **(C),** ILCs ($CD45^+$, Lin^- ($CD11c^-$, $CD11b^-$, $CD45R^-$, $CD5^-$, $TCR\beta^-$), $CD3^-$, $CD4^-$, $CD90^+$) **(D),** IL-13^+ ILC **(E).** Data are representative of at least two independent experiments and expressed as mean ± SD. N =8. * P <0.05. Quantification of congo-red stained peribronchial eosinophils **(F)** periodic acid–Schiff–stained mucus-producing goblet cells **(G)** and photomicrographs of Haematoxylin & Eosin-stained lung tissue **(H)**. Data are combined of two independent experiments and represented as mean ± SD. N =13-18. * P <0.05

FIGURE 5: Role of miR-155 in TDI-induced type 2 responses and airway hyperresponsiveness. Murine IL-33 protein levels in whole lung homogenate **(A)**, IL-1α protein levels in whole lung homogenate **(B),** CCL5 protein levels in BAL **(C)**. IL-13 protein levels in the supernatants of concanavaline A stimulated auricular LNs **(D)** and IgE titers in serum **(E)** were determined by ELISA. Airway resistance (R) of WT mice (black & blue lines) or miR-155 KO mice (red lines) exposed to TDI or vehicle (AOO/AOO) was measured in response to increasing doses of carbachol **(F).** No R-measurement in AOO/TDI miR-155 KO group due to limited availability of miR-155 KO mice. Data of **A-D** are expressed as mean ± SD, data of **(E)** are expressed as mean ± SEM, N =8-10. * P <0.05

Figure 1

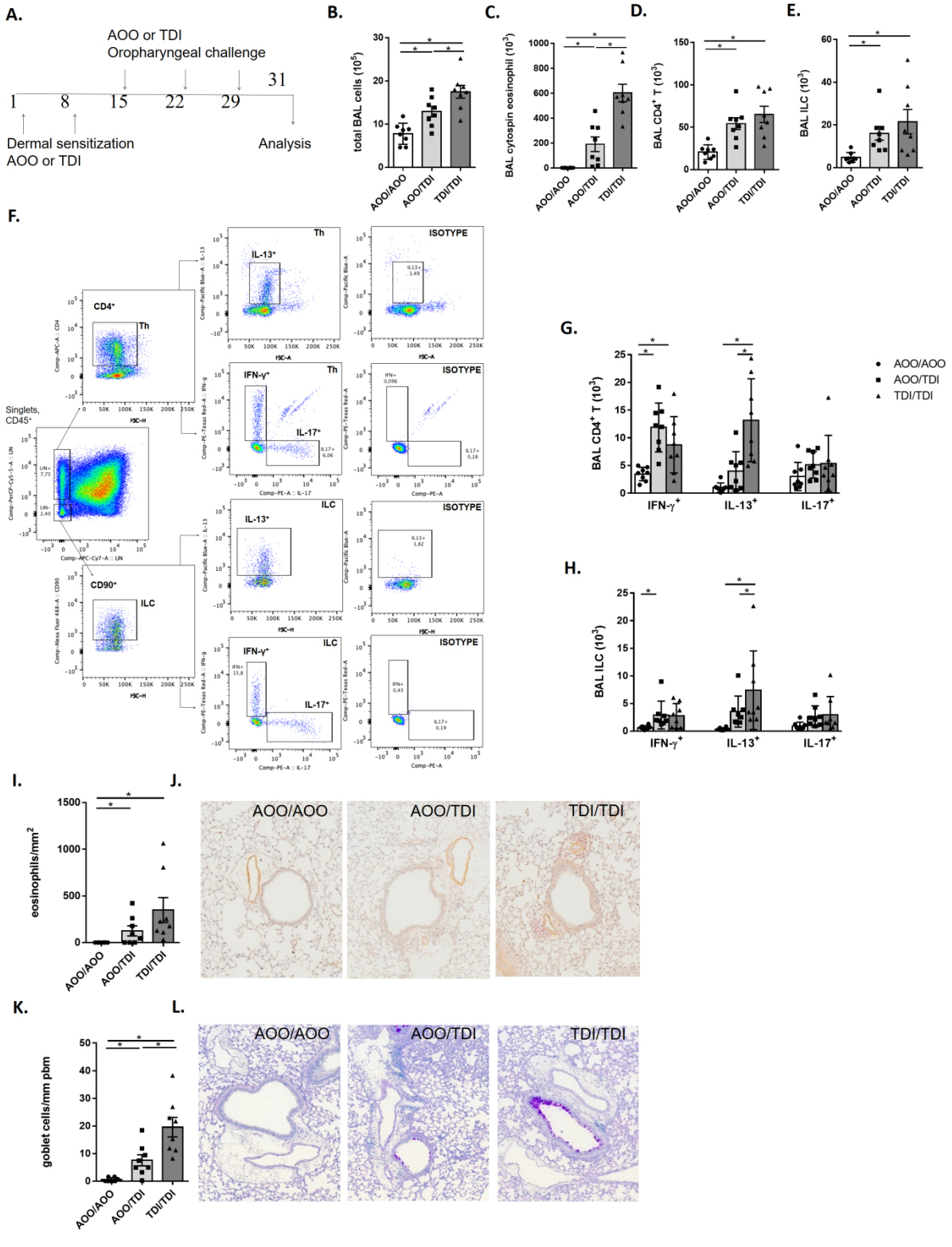
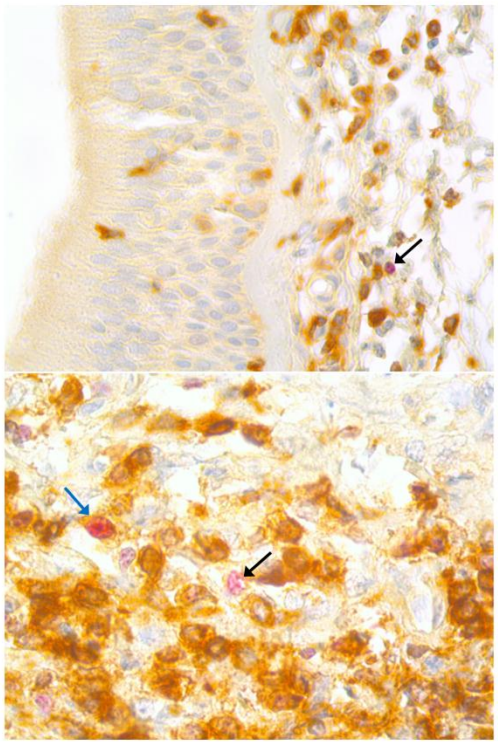
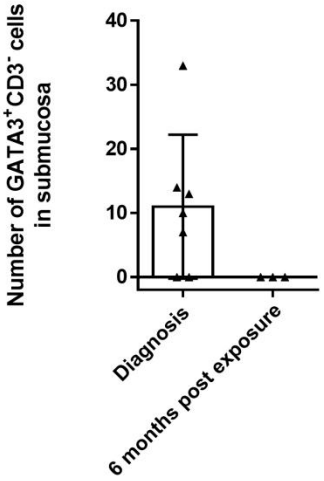

A.
AOO or TDI
Oropharyngeal challenge
1
8
15
22
29
31
Dermal sensitization
AOO or TDI
Analysis
B.
total BAL cells (10^5)
C.
BAL cytospin eosinophil (10^3)
D.
BAL CD4+ T (10^3)
E.
BAL ILC (10^3)
AOO/AOO
AOO/TDI
TDI/TDI
F.
Singlets, CD45+
CD4+
Th
IL-13+
IFN-γ+
IL-17+
ISOTYPE
CD90+
ILC
G.
BAL CD4+ T (10^3)
IFN-γ+
IL-13+
IL-17+
H.
BAL ILC (10^3)
I.
eosinophils/mm2
J.
AOO/AOO
AOO/TDI
TDI/TDI
K.
goblet cells/mm pbm
L.

Figure 2

A.



B.


Number of GATA3+CD3- cells in submucosa
40
30
20
10
0
Diagnosis
6 months post exposure

C.

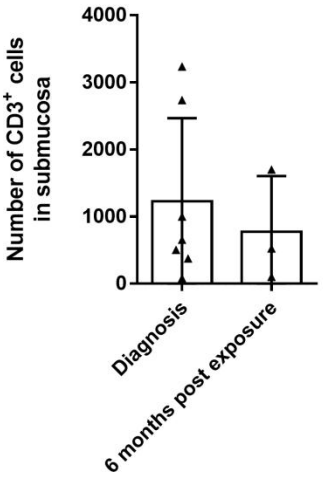

Number of CD3+ cells in submucosa
4000
3000
2000
1000
0
Diagnosis
6 months post exposure

Figure 3

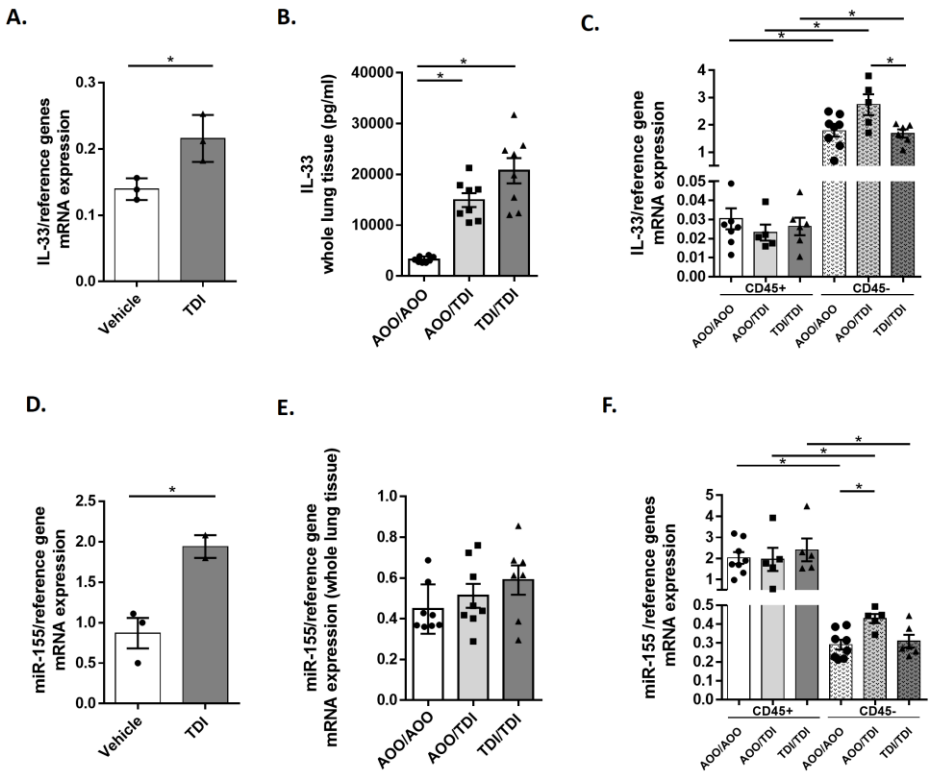

A.
IL-33/reference genes mRNA expression
Vehicle
TDI
B.
IL-33 whole lung tissue (pg/ml)
AOO/AOO
AOO/TDI
TDI/TDI
C.
IL-33/reference gene mRNA expression
CD45+
CD45-
AOO/AOO
AOO/TDI
TDI/TDI
D.
miR-155/reference gene mRNA expression
Vehicle
TDI
E.
miR-155/reference gene mRNA expression (whole lung tissue)
AOO/AOO
AOO/TDI
TDI/TDI
F.
miR-155 /reference genes mRNA expression
CD45+
CD45-
AOO/AOO
AOO/TDI
TDI/TDI

Figure 4

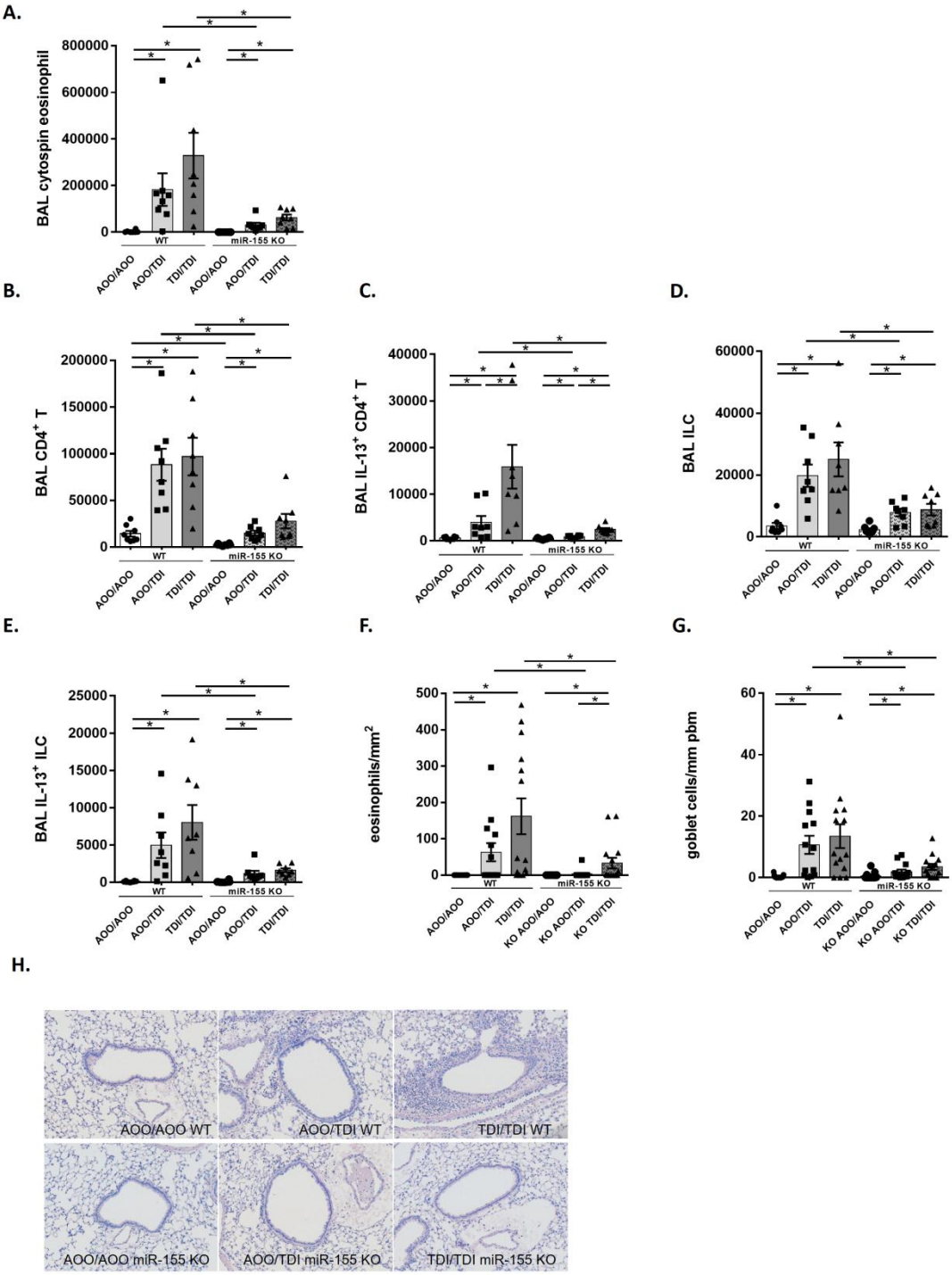
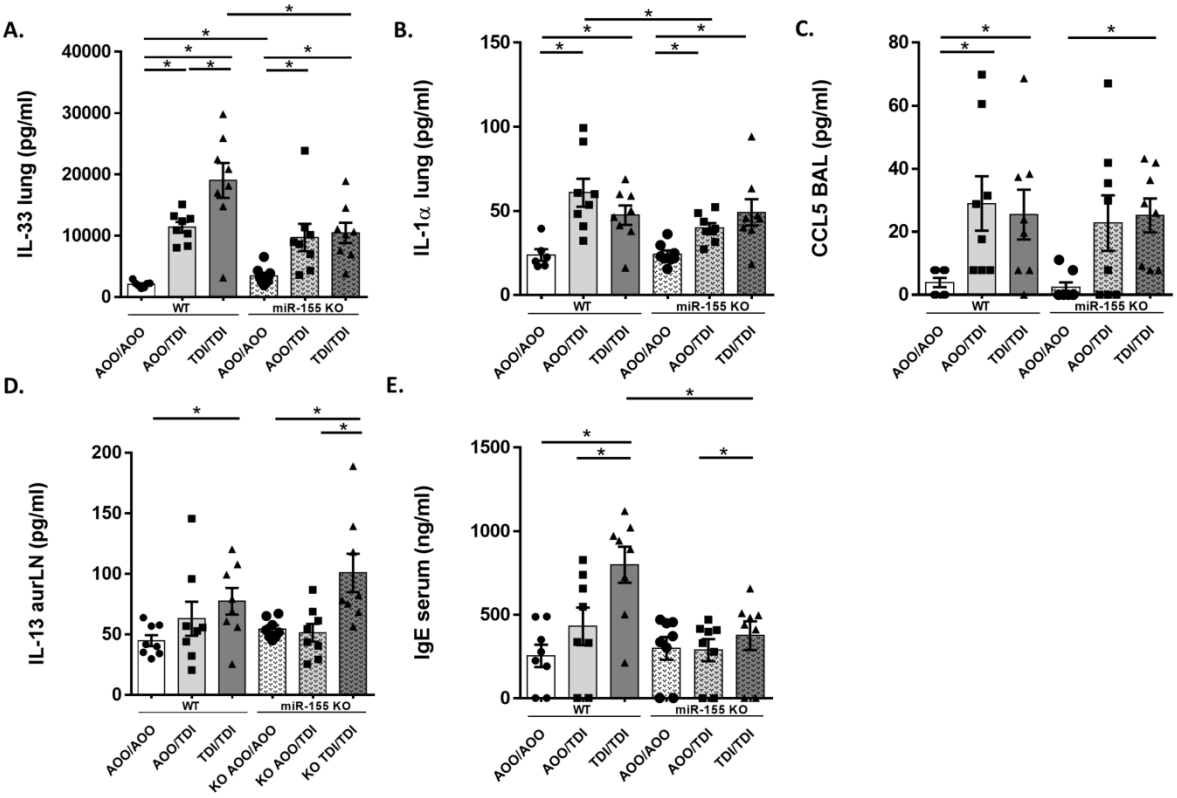
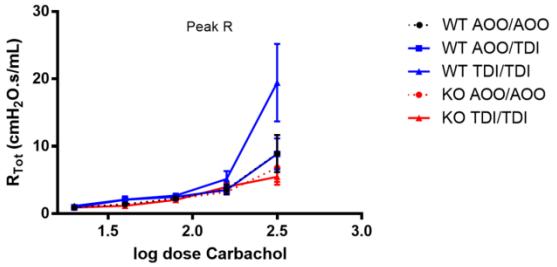

A.
BAL cytospin eosinophil
800000
600000
400000
200000
0
WT
miR-155 KO
AOO/AOO
AOO/TDI
TDI/TDI
AOO/AOO
AOO/TDI
TDI/TDI
B.
BAL CD4+ T
200000
150000
100000
50000
0
C.
BAL IL-13+ CD4+ T
40000
30000
20000
10000
0
D.
BAL ILC
60000
40000
20000
0
E.
BAL IL-13+ ILC
25000
20000
15000
10000
5000
0
F.
eosinophils/mm2
500
400
300
200
100
0
KO AOO/AOO
KO AOO/TDI
KO TDI/TDI
G.
goblet cells/mm pbm
60
40
20
0
H.
AOO/AOO WT
AOO/TDI WT
TDI/TDI WT
AOO/AOO miR-155 KO
AOO/TDI miR-155 KO
TDI/TDI miR-155 KO

Figure 5


A.
IL-33 lung (pg/ml)
40000
30000
20000
10000
0
WT
miR-155 KO
AOO/AOO
AOO/TDI
TDI/TDI
AOO/AOO
AOO/TDI
TDI/TDI
B.
IL-1α lung (pg/ml)
150
100
50
0
C.
CCL5 BAL (pg/ml)
80
60
40
20
0
D.
IL-13 aurLN (pg/ml)
200
150
100
50
0
KO AOO/AOO
KO AOO/TDI
KO TDI/TDI
E.
IgE serum (ng/ml)
1500
1000
500
0

F.


Peak R
R_{Tot} ($cmH_2O.s/mL$)
30
20
10
0
1.5
2.0
2.5
3.0
log dose Carbachol
WT AOO/AOO
WT AOO/TDI
WT TDI/TDI
KO AOO/AOO
KO TDI/TDI

Online Repository

INNATE LYMPHOID CELLS IN ISOCYANATE-INDUCED ASTHMA: ROLE OF MICRORNA-155

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SUPPLEMENTARY INFORMATION

Online Repository text

Auricular LN cell culture:

LN single cells were cultured in culture medium (RPMI 1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine, 0.05 mM 2-mercaptoethanol, 100 U/ml penicillin, 100 mg/ml streptomycin) supplemented with concanavaline A (2,5 µg/ml) (concA) or

dehydrated culture medium (DCM) in round bottom, 96-well plates and incubated in a humidified 37°C incubator with 5% CO_2. After 46h, supernatants were harvested for protein measurements.

Preparation for protein measurements:

The superior lobe of the right lung was snap frozen in liquid nitrogen and stored at -80°C until analysis. Lobes were transferred into T-PER extraction buffer (Protease inhibitor cocktail kit added) and homogenized on ice using TissueRuptor. Homogenates were centrifuged (5 min, 10 000 g at 4°C) and the middle layer was transferred to micro centrifuge tubes. Total protein concentration was measured using the Pierce BCA protein Assay kit and samples were diluted with T-PER buffer to obtain the same concentration. Lung homogenate samples were stored at -80°C until further analysis. All reagents were purchased from Thermo Scientific. Blood was collected from the retro-orbital plexus and centrifuged (10 min, 2500 rpm) for the isolation of serum. Serum samples were stored at -20°C.

In vitro human bronchial epithelial cell culture:

HBEC were isolated from bronchial ring tissue resected at maximum distance of the tumor lesion and cultured at the air-liquid (ALI) interface (protocol based on E_1, E_2). Briefly, bronchi were cleared from excess connective tissue and washed three times with cold medium (MEM (Sigma), supplemented with L-glutamine (2 mM, Gibco) and 1% Pen/Strep (10000 U/ml, Gibco)), followed by digestion with 1mg/ml pronase E (Sigma, P5147) in medium. After 20h digestion on a rotor at 4°C, pronase E was inactivated with 10% FCS. Epithelial cells were scraped from the inner surface of the bronchi with a scalpel. After resuspension with a 19G needle, cells were centrifuged for 5min at 20°C, 220 g and collected in warm BEGM (Lonza, CC-3170), supplemented with 1% Pen/Strep. Fibroblasts were removed by plating the cell suspension in a dish without collagen coating for 3h and collecting the supernatant. Next, HBEC were seeded in a collagen I coated dish, incubated at 37°C (5% CO_2) and the medium was changed every second day until a confluency of 80%. P1 cells were seeded in Nunclon Delta coated flasks for further expansion until final seeding in inserts of 12-well plates for ALI cell culture.

Written informed consents were obtained from all donors, according to the protocol approved by the medical ethical committee of Ghent University Hospital (2016/0132).

IL-33 and TSLP levels were determined by ELISA (R&D systems) in apical supernatants of the ALI cultures from HBEC.

Human sample processing and immunohistochemistry:

Biopsies were processed as previously described (E_3, E_4). For the detection of ILC2, sections were processed for IHC analysis (Leica BOND Max) with specific antibodies anti-CD3 and anti-GATA3. Briefly, the first immunohistochemical staining was performed by quenching endogenous peroxidase using an enzyme-blocking reagent (BOND Polymer Refine Detection Kit) for 10 minutes. Next, sections were treated with the primary antibody (anti-CD3) for 60 minutes. Sections were then incubated with a dextran polymer reagent containing secondary antibodies, peroxidase molecules and non-permeable chromogen DAB (BOND Polymer Refine Detection Kit). Consecutively, in the second immunohistochemical staining sections were treated with the primary antibody (anti-GATA3) for 60 minutes. Sections were then incubated a biotin-free, polymeric alkaline phosphatase (AP)-linker antibody conjugate system (Bond Polymer Refine Red Detection). Finally, sections were counterstained with hematoxylin. Digital images from the stained sections were obtained with a light microscope (Leica DM 2000) connected to a video recorder and a computerized image analysis system (Leica LAS w3.8, Leica Application Suite). 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.

E_1. Smirnova NF, Schamberger AC, Nayakanti S, Hatz R, Behr J, Eickelberg O. Detection and quantification of epithelial progenitor cell populations in human healthy and IPF lungs. Respiratory research. 2016;17(1):83.

E_2. Zarcone MC, van Schadewijk A, Duistermaat E, Hiemstra PS, Kooter IM. Diesel exhaust alters the response of cultured primary bronchial epithelial cells from patients with chronic obstructive pulmonary disease (COPD) to non-typeable Haemophilus influenzae. Respiratory research. 2017;18(1):27.

E_3. Saetta M, Di Stefano A, Maestrelli P, Turato G, Mapp CE, Pieno M, et al. Airway eosinophilia and expression of interleukin-5 protein in asthma and in exacerbations of chronic bronchitis. Clinical & Experimental Allergy. 1996;26(7):766-74.

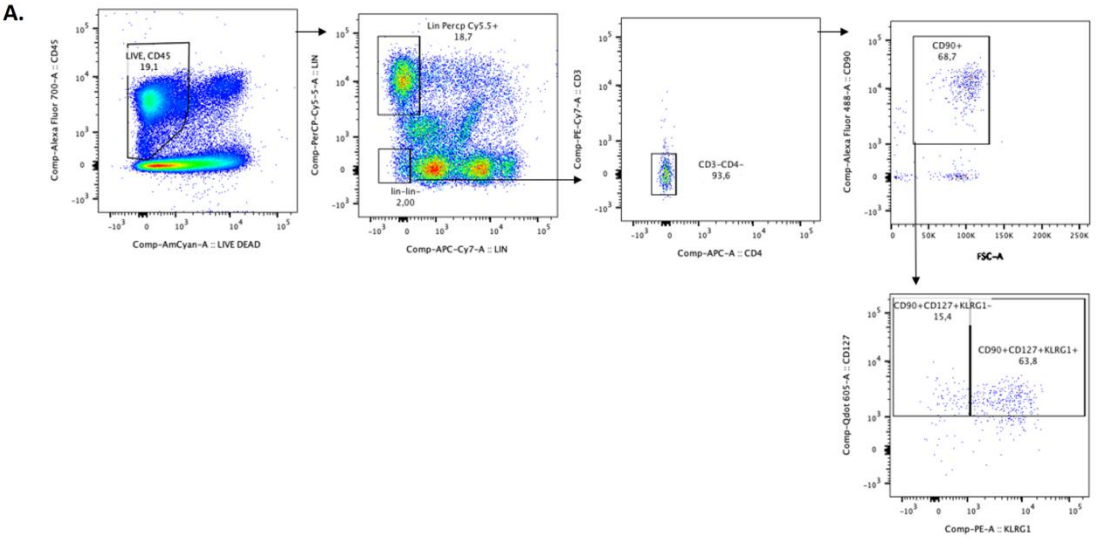
E_4. Di Stefano A, Turato G, Maestrelli P, Mapp CE, Ruggieri MP, Roggeri A, et al. Airflow limitation in chronic bronchitis is associated with T-lymphocyte and macrophage infiltration of the bronchial mucosa. American journal of respiratory and critical care medicine. 1996;153(2):629-32.

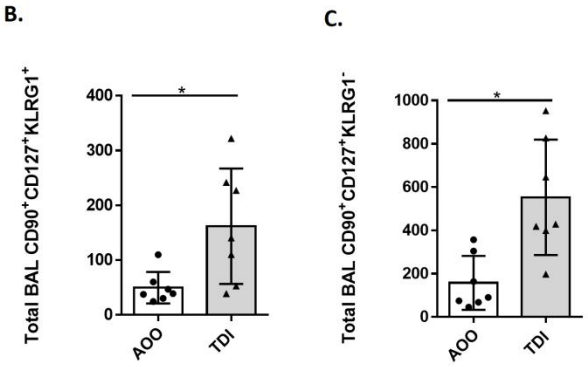
FIGURE LEGENDS

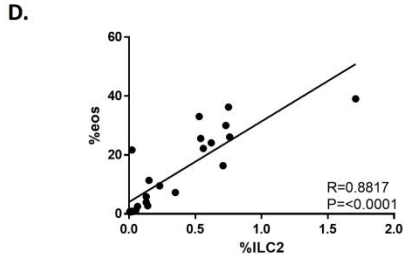
FIGURE S1: Gating strategy of live, $CD45^{+}$,$CD5^{-}NK1.1^{-}TCRb^{-}GR\text{-}1^{-}Fc\varepsilon RI^{-}CD11b^{-}$ $CD11c^{-}CD45R^{-}$,$CD3^{-}CD4^{-}$, $CD90^{+}$, $CD127^{+}$, $KLRG1^{+}$ lung ILC2 **(A)**. C57BL6 mice were intratracheally exposed to TDI or AOO on day 1, 8 and 15. Endpoints were performed on day 17. $CD127^{+}KLRG1^{+}$ ILC2 in BAL **(B)** $CD127^{+}KLRG1^{-}$ ILC **(C).** Murine data are expressed as mean ± SD. N =6-8. * P <0.05. Spearman correlation between inflammatory cells in the mouse model: %eosinophils and %ILC2 **(D),** %eosinophils and %Th2 **(E).** C57BL6 mice were dermally exposed to TDI or AOO on day 1, 8 and intratracheally on day 15, 22 and 29. Endpoints were performed on day 31. After 4h stimulation, lung ILCs were gated as live, $CD45^{+}CD5^{-}TCRb^{-}CD11b^{-}$ $CD11c^{-}CD45R^{-}CD90^{+}$ and discriminated based on IL-13, IL-17 and IFN-γ **(F)**. Results are expressed as mean ± SD. n = 7-8 mice per group. * P < 0.05. Correlation between eosinophils and $CD3^{+}$ cells in submucosa from human bronchial biopsies **(G)**.

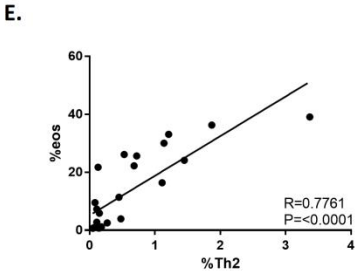
FIGURE S2: A-B mRNA expression in HBEC from never-smoking donors (n=3), ALI cultures from HBEC were exposed to 0.5mM TDI or vehicle for 25min. The dots represent the average of 2-4 technical replicates. IL-33/HPRT+RPL13A mRNA expression **(A)**, miR-155/RNU48 expression **(B)**. miR-155/snorD95 mRNA levels in murine auricular LN **(C)**. C57BL6 mice were dermally exposed to TDI or AOO on day 1, 8 and intratracheally on day 15, 22, 29. Endpoints were performed on day 31. IFN^{+} γ $CD4^{+}$ T cells **(D)** IFN-γ expressing ILC **(E)** $IL\text{-}17^{+}$ $CD4^{+}$ T cells **(F)** and $IL\text{-}17^{+}$ ILC **(G)** in BAL. Data are expressed as mean ± SD. N =8. * P <0.05.

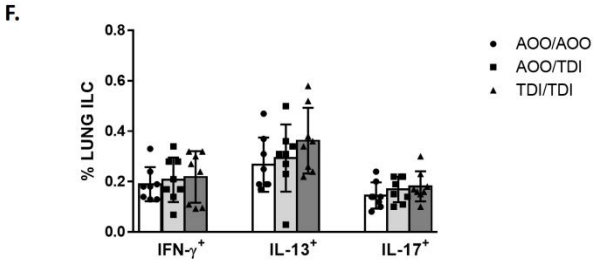
ONLINE SUPPLEMENT
Figure S1

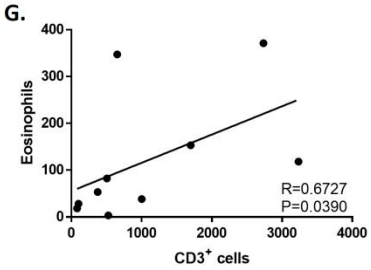












ONLINE SUPPLEMENT
Figure S2