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

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SUPPLEMENTARY INFORMATION

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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% CO₂. 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 E₁, E₂). 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% CO₂) 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 (E₃, E₄). 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 mm² of examined area.

E₁. 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.

E₂. 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.

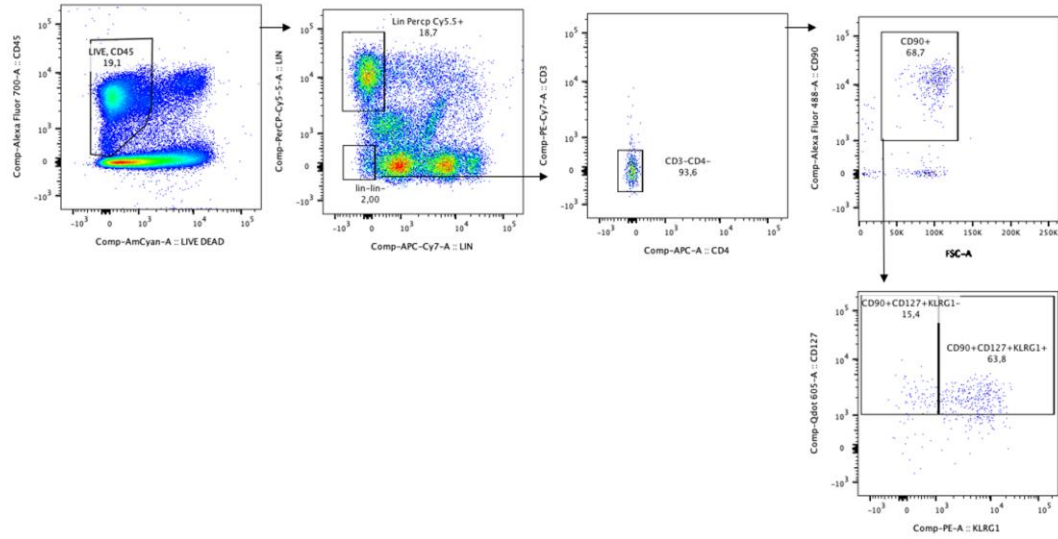
E₃. Saetta M, Di Stefano A, Maestrelli P, Turato G, Mapp CE, Pieno M, et al. Airway eosinophilia and expression of interleukin-5 protein in asthma and in exacerbations of chronic bronchitis. *Clinical & Experimental Allergy*. 1996;26(7):766-74.

E₄. 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.

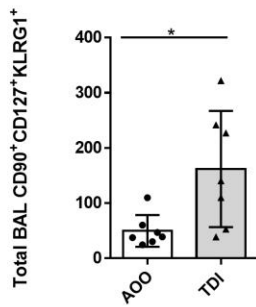
FIGURE S1: Gating strategy of live, CD45⁺,CD5⁻NK1.1⁻TCRb⁻GR-1⁻FcεRI⁻CD11b⁻CD11c⁻CD45R⁻,CD3⁻CD4⁻, CD90⁺, CD127⁺, KLRG1⁺ lung ILC2 **(A)**. C57BL6 mice were intratracheally exposed to TDI or AOO on day 1, 8 and 15. Endpoints were performed on day 17. CD127⁺KLRG1⁺ ILC2 in BAL **(B)** CD127⁺KLRG1⁻ ILC **(C)**. 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)**.

ONLINE SUPPLEMENT Figure S1

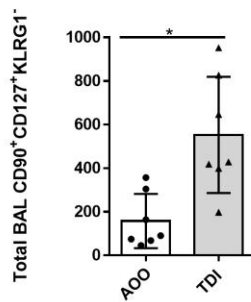
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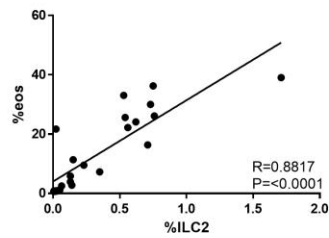
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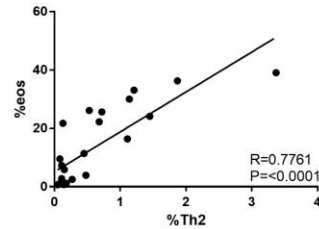
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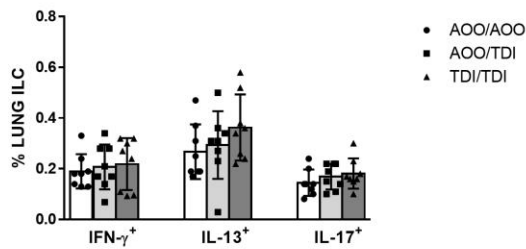
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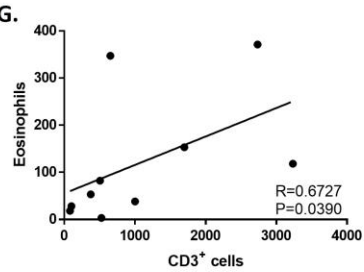
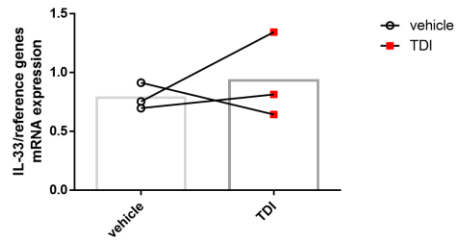


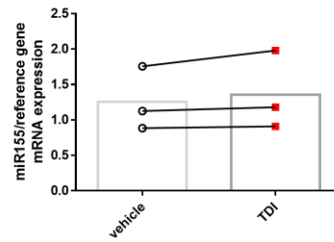
FIGURE S2: A-B mRNA expression in HBEC from never-smoking donors (n=3), ALI cultures from HBEC were exposed to 0.5mM TDI or vehicle for 25min. The dots represent the average of 2-4 technical replicates. IL-33/HPRT+RPL13A mRNA expression **(A)**, miR-155/RNU48 expression **(B)**. miR-155/snorD95 mRNA 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-17 $^{+}$ CD4 $^{+}$ T cells **(F)** and IL-17 $^{+}$ ILC **(G)** in BAL. Data are expressed as mean \pm SD. N =8. * P <0.05.

ONLINE SUPPLEMENT Figure S2

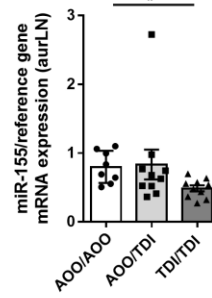
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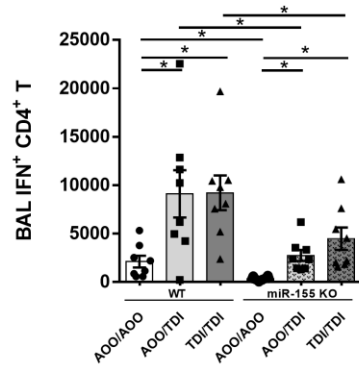
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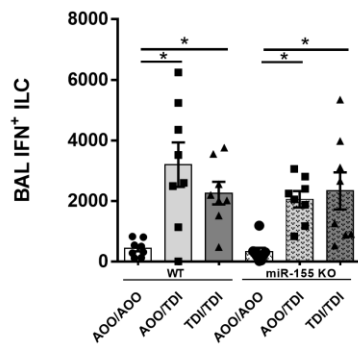
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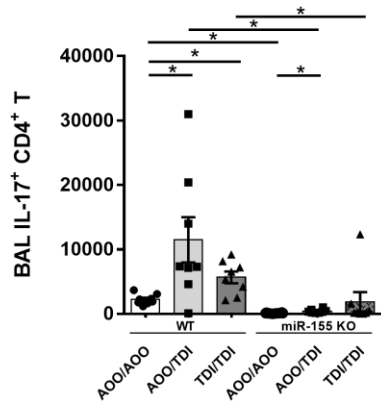
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