

**Shifted T-cell polarization after agricultural dust exposure in mice and men.**

P. Robbe<sup>1, 2</sup>, E.A.J. Spierenburg<sup>3</sup>, C. Draijer<sup>2,4</sup>, C.A. Brandsma<sup>1,2</sup>, E. Telenga<sup>2,5</sup>, A.J.M van Oosterhout<sup>2,6</sup>, M. van den Berge<sup>2,5</sup>, M. Luinge<sup>1,2</sup>, B.N. Melgert<sup>2,4</sup>, D. Heederik<sup>3</sup>, W. Timens<sup>1,2</sup>, I.M. Wouters<sup>3</sup>, M.N. Hylkema<sup>1,2</sup>

<sup>1</sup> University of Groningen, University Medical Center Groningen, Department of Pathology,  
Groningen, The Netherlands

<sup>2</sup> University of Groningen, University Medical Center Groningen, GRIAC- Groningen Research  
Institute for Asthma and COPD

<sup>3</sup> Institute for Risk Assessment Sciences (IRAS), division of Environmental Epidemiology,  
University of Utrecht, Utrecht, The Netherlands

<sup>4</sup> University of Groningen, Department of Pharmacokinetics, toxicology and targeting, Groningen,  
The Netherlands

<sup>5</sup> University of Groningen, University Medical Center Groningen Department of Pulmonology,  
University of Groningen, Groningen, The Netherlands

<sup>6</sup> University of Groningen, University Medical Center Groningen, Department of Medical Biology,  
University of Groningen, Groningen, The Netherlands

Corresponding author: Machteld N. Hylkema

Postal address: Hanzeplein 1

9713 GZ Groningen

Telephone: +31503619850

Telefax: +31503619107

Email: m.n.hylkema@umcg.nl

## ONLINE SUPPLEMENT

### METHODS

#### Animal study design

8 week old specific pathogen free female BALB/c mice (Harlan, Horst, The Netherlands) were housed in 10 groups of 8 and had access to standard food and water *ad libitum*. Female mice were chosen because, according to our previous study, female mice of this strain have a more prominent response to the HDM model of asthma. More specifically, female mice have more HDM specific IgE, more eosinophils in the lungs and are more responsive to methacholine than male mice.[1] Mice were anesthetized with isoflurane and intranasally exposed to each different dust extract (DE; details below; DE exposure: 1mg/ml in PBS, 50  $\mu$ l/day), house dust mite (HDM exposure: 40  $\mu$ g/day in 50  $\mu$ l PBS) or PBS (50  $\mu$ l/day) as a control, four times per week, during five consecutive weeks. To investigate the protective effect of dust exposure, mice were exposed to the combination of HDM and each type of DE. Mice had access to standard food and water *ad libitum*. All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Groningen (Permit Number: 4788E). A volume of 50  $\mu$ l was chosen as studies on distribution of intranasal instillations in mice have shown that a maximum distribution to the lungs is achieved with this volume.[2]

#### Dust extracts

Settled dust was collected from 30 cattle and 7 pig farms, and from 2 flower bulb and 3 onions agricultural industries in the Netherlands and were pooled by type of agricultural setting. Pooled dust was extracted applying the protocol from Peters *et al* with some adaptations [3]. Briefly, aliquots of approximately 0.5 gram of settled dust samples collected at farm or in agricultural industries were pooled. Mixed dust samples for each location (cattle, pig, flower bulb and onion)

ranged between 2.5 and 7.5 gram of settled dust and were mixed with glass beads in a 1:5 ratio (dust:beads) in a 50 ml Greiner tube, ~2.5 gram dust/tube. Braun sterile water was added to a volume of 50ml. Samples were then mixed for 5min on end-over-end roller at room temperature and subsequently crushed for 5 minutes in ultrasonic bath with crushed ice and transferred to a glass Erlenmeyer. Then sterile water was added to a final concentration of 10 mg dust per ml. Sodium chloride solution was added to obtain a final concentration of 0.9% followed by shaking for 6 hours on horizontal shaker at room temperature. Then samples were centrifuged at 20.000g/4°C/45min and after centrifugation the dust extract was dialyzed (MWCO 3500) against di-distilled water, sterilized through filtration through a 0.22 um filter and lyophilized. Hereafter, the extracted dust from both farms and agricultural industries is referred to as 'DE'. Dose responses experiments have been performed by the group of Peters *et al.* [3] using similar extraction, in which optimal dose was determined to be 50µg/ day (personal communication).

### **Lung homogenates**

Snap frozen lung tissue was mechanically homogenized (50% w/v) in 50 mM Tris-HCl buffer, containing 150 mM NaCl, 0.002% Tween-20 (pH 7.5) and a protease inhibitor (Sigma Aldrich). Homogenates were centrifuged at 12000xg for 10 minutes to remove any insoluble material. Supernatants were subsequently stored at -80°C until further analysis.

### **Semi quantitative analysis of inflammatory infiltrates**

Sections (3 µm) of zinc-fixed and paraffin-embedded lung tissue were stained for Hematoxylin-eosin. Semi quantitative scores for degree of inflammation from 0 to 3 (the greater the score, the greater the inflammatory infiltration in the lung) were determined by a blinded reviewer.

Evaluation of the whole lung tissue present in each slide included the bronchiolar compartment as well as the parenchyma. For representative pictures of each inflammatory score, see figure S2.

### **Immunohistochemistry**

To determine the presence of T cells, B cells, neutrophils and macrophages, 4 µm-thick cryosections of lung tissue were stained with hamster anti-mouse CD3 (BD Bioscience), rat anti-mouse CD19 (BD bioscience), rat anti-mouse GR1 (BD Bioscience) and rat anti-mouse CD68 (AbD serotec) antibodies respectively. To determine the presence of Th17 and Tc17 cells, lung cryosections were stained with a monoclonal rat anti-mouse IL-17 antibody (*R&D systems*) in combination with a monoclonal hamster anti-mouse CD3 antibody (*BD Biosciences*), a monoclonal rat anti-mouse CD4 antibody (*BD Pharmingen*), a monoclonal rat anti-mouse CD8 antibody (*BD Pharmingen*), or a monoclonal hamster anti-mouse  $\gamma\delta$  antibody (*BD Pharmingen*).

### **Sample preparation and *in vitro* stimulation**

Peripheral Blood Mononuclear cells (PBMCs) were isolated by standard protocols using Lymphoprep for AW and Ficoll-Paque density gradient centrifugation for healthy controls. Agricultural workers' PBMC were stored in aliquots of  $3 - 7 \times 10^6$  cells in liquid nitrogen in a solution of 10% DMSO in Iscove's Modified Dulbecco's Medium (IMDM, Lonza, Basel, Switzerland) supplemented with 50U/ml Penicillin/Streptomycin, 10% FCS and 0.1%  $\beta$ -Mercapto-ethanol (Merk, Darmstadt, Germany) until use.  $5-10 \times 10^6$  cells from healthy controls were stored in liquid nitrogen in a solution of 10% Dimethylsulphoxide (DMSO, Sigma-Aldrich, Steinheim, Germany), 50% FCS (Invitrogen, New York, USA) and 40% Hank's Balanced Salt Solution (HBSS, Lonza, Basel, Switzerland). Both isolation and freezing methods were compared and showed difference in PBMC yield, but no influence on T-cell populations (data not shown). Cells were quickly thawed in a water bath and slowly diluted in warm RPMI1640 with 10% FCS to dilute the DMSO avoiding osmotic stress. After washing, cells were resuspended in warm RPMI1640 with 10% FCS and  $8-10 \times 10^5$  per well were stimulated overnight with 25ng/ml phorbol myristate acetate (PMA, Sigma-Aldrich, Steinheim, Germany) and 1 µg/ml calcium-ionophore (Ca-Io, Sigma-Aldrich, Steinheim, Germany) at 37 degrees Celcius, 5%CO<sub>2</sub>. Monensin (2 µg/ml, Sigma-Aldrich, Steinheim, Germany) was added to each sample to inhibit cytokine secretion. As a control, one sample of each subject was cultured overnight in complete RPMI at 37°C, 5%CO<sub>2</sub> without stimulation.

### **Immunofluorescent staining**

After stimulation the cells were washed in 2% BSA/PBS and counted.  $10^6$  cells were used for the flowcytometry staining and incubated with 0.5% human serum (Lonza, Breda, The Netherlands) to block nonspecific bindingsites. Then, cells were washed and incubated with an antibody cocktail containing FITC-conjugated anti-CD3 (BD Biosciences, Breda, the Netherlands), Krome orange- conjugated anti-CD8 (Beckman Coulter, Woerden, the Netherlands), PECy7-conjugated anti-CD25 (eBioscience, Vienna, Austria) and APCCy7-conjugated anti-CD69 (Biolegend, San Diego, USA) for 30 min in the dark on ice.

Following membrane staining, cells were fixed and permeabilized (Fix/Perm buffer, eBioscience, Vienna, Austria) and washed with cold permeabilization buffer. To block nonspecific bindingsites, cells were incubated for 15 min. with 2% human serum, followed by an antibody cocktail containing Pacific Blue-conjugated anti-Foxp3 (Biolegend, San Diego, USA), PE-conjugated anti-IL-4 (eBioscience Vienna, Austria), Alexa Fluor 647-conjugated anti-IL-17 (eBioscience, Vienna, Austria) and Alexa Fluor 700-conjugated anti-IFN $\gamma$  (eBioscience, Vienna, Austria). After incubation for 30 min in the dark on ice, cell suspensions were washed twice with cold permeabilization buffer and resuspended in fluorescence-activated cell sorting (FACS) lysing solution (BD Biosciences, Breda, the Netherlands). Multi-color fluorescent staining was immediately measured on a LSR-II flow cytometer (BD Biosciences, Breda, the Netherlands) and analyzed using FACS Diva software (BD Biosciences, Breda, the Netherlands).

### **Flow cytometric analysis**

Flowcytometry data were analyzed using FlowJo software 7.6.5 (Tree Star Inc., Ashland, USA). Because stimulation reduces surface expression of CD4 on T cells, CD4<sup>+</sup> T cells were identified indirectly by gating on CD3<sup>+</sup> and CD8<sup>-</sup> lymphocytes. Positively and negatively stained populations were calculated by dot plot analysis, as determined by unstained samples. The appropriate isotype controls for the cytokine (IL-4, IL-17 and IFN $\gamma$ ) staining (mouse IgG1 labeled with PE, AF647, and AF700 respectively) are represented in figure S6.

## REFERENCES

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