Online Data supplement

The composition of house dust mite is critical for mucosal barrier dysfunction and allergic sensitization

S. Post[*,1], M.C. Nawijn[*,1], T.L. Hackett[†], M. Baranowska[*], R. Gras[*], A.J.M. van Oosterhout[*,2] and I.H. Heijink[*,2]

AFFILIATIONS

[*] Laboratory of Allergology and Pulmonary Diseases, Dept of Pathology and Medical Biology, University Medical Center Groningen, GRIAC Research Institute, University of Groningen, Groningen, the Netherlands,

and,

[†] James Hogg Research Centre, Department of Anaesthesiology, Pharmacology and Therapeutics University of British Columbia, St Paul's Hospital, Vancouver, Canada.

[1] These authors contributed equally to the manuscript.

[2] These authors share senior authorship.

CORRESPONDENCE

A. J. M. van Oosterhout, University Medical Center Groningen (UMCG), Department of Pathology and Medical Biology, Laboratory of Allergology and Pulmonary Diseases, Hanzeplein 1, NL-9713GZ, Groningen, The Netherlands.

E-mail: a.j.m.van.oosterhout@umcg.nl

METHODS

Total Protease activity assay

Total protease activity of all four HDM extracts was determined by using the IRDye 800RS Casein Protease Substrate Kit (LI-COR Biosciences, Lincoln, Nebraska, USA) according to the manufacturer's guidelines. All HDM extracts were analyzed for their protease content at a concentration of 2.5 mg/ml dry weight.

Specific serine and cysteine protease assay

The specific serine and cysteine protease activity was determined by adding 25 µl HDM extract (2.5 mg/ml) in a 96-well plate. Protease was measured by adding 50 µl 10 nM Boc-Gln-Ala-Arg-7-amido-4-methylcoumarin (Sigma, St Louis, MO, USA) for serine determination or 50 µl Boc-Gln-Gly-Arg-MCA (Peptide Institute Inc., Osaka, Japan) for cysteine determination. The plate was measured by a spectrophotometer**.**

Chitinase assay

The presence of chitinase activity in all four HDM extracts was determined using the Chitinase Assay Kit (Sigma, St Louis, MO, USA). The kit provides three different substrates; 4-Nitrophenyl N,N'-diacetyl-ß-chitobioside, 4-Nitrophenyl N-acetyl-ß-D-glucosaminide, 4-Nitrophenyl ß-D-N,N', N''-triacetylchitotriose, for the detection of exo- and endochitinase activity. The assay was used according to the manufacturer's guidelines. All HDM extracts were analyzed for their chitinase content at a concentration of 2.5 mg/ml.

Endotoxin levels

Endotoxin levels were measured at the University Medical Center Pharmacy (Groningen, the Netherlands) by the Limulus Amebocyte Lysate (LAL) endotoxin test. The assay was used according to the pharmacy's guidelines (SOP nr. 02APA00017). All HDM extracts were analyzed for their endotoxin content at a concentration of 1 mg/ml, with the exception of HDM-A, which was analyzed at a concentration of 100 μg/ml.

Cell culture

16HBE cells were cultured in EMEM/10%FCS and seeded in 24-well plates at $5x10^4$ cells/well (Immunoblotting), ECIS arrays at $8x10^4$ cells/well (transepithelial resistance) or in LabTek arrays at $6x10^4$ cells/well (immunofluorescence) and grown to 95-98% confluence, serum deprived overnight and exposed for 0.25-24 hours to the HDM extracts (for concentrations see Table 1) or a protease cocktail (containing serine-type proteases, zinc endopeptidases, zinc leucine aminopeptidases and zinc carboxypeptidases), at a concentration of 2.5 μg/ml (Sigma, St Louis, MO, USA).

Normal human bronchial epithelium (NHBE) cells were cultured in hormonally supplemented bronchial epithelium growth medium (Lonza) on collagen/fibronectin-coated flasks. Cells were seeded in 24-well plates at $5x10^4$ cells/well (Immunoblotting) and in LabTek arrays at $7.5x10^4$ cells/well (immunofluorescence) and grown to 95-98% confluence and exposed for 0.25-24 hours to the HDM extracts

Immunoblot

Total cell lysates were obtained by resuspension of the cells in 1x Laemmli sample buffer containing 10% glycerol, 2% SDS, 60 mM Tris-HCL pH 6.8., 2 % ß-mercaptoethanol and 1% bromophenol blue, followed by boiling for 5 minutes. The immunodetection was performed as previously described (E1) for occludin (Life span Biosciences, Seattle, WA, USA) and ß-actin (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) by standard procedures, according to the manufacturer's guidelines (ECL, Amersham, Buckinghamshire, UK).

Immunofluorescent staining

Cells grown on LabTeks were washed with PBS/$CaCl_2$ and fixed in ice-cold acetone (90%) for 30 min and blocked in PBS/5% BSA for 60 min. Cells were stained with anti-E-cadherin (1:50; BD Biosciences, Erembodegem, Belgium), anti-occludin (1:50; Life span Biosciences, Seattle, WA, USA), or anti-ZO-1 (1:200; Invitrogen, Carlsbad, CA, USA), and detected by incubation with Alexa green 488-labeled anti-rabbit IgG conjugate or Rhodamin red-labelled anti-mouse IgG conjugate (1:200; Southern Biotech, Birmingham, AL, USA. Nuclei were stained using DAPI (Sigma, St Louis, MO, USA) in Citifluor (Agar Scientific). Fluorescence was analyzed by fluorescence microscopy (Leica, Solms, Germany).

Measurement of ear swelling after HDM injection

Prior to the last sensitization, HDM-A and HDM-D were injected subcutaneously (at a concentration of 25 µg/ml) in the right ear of HDM-A and HDM-D treated mice, while PBS was injected in the left ear as control. Ear thickness was measured after 2 hours by a

digimatic force-limited micrometer at 0.5N, with measure accuracy at ±0.15N (Mitutoyo, Japan).

Measurement of airway responsiveness in mice

The measurement of airway responsiveness was performed as previously described (E2). Briefly, mice were anesthetized by intraperitoneal injection of ketamine (100 mg/kg; Pfizer, New York, NY) and dormitor (1 mg/kg; Pfizer), tracheotomised using a 20-gauge intravenous cannula (Becton Dickinson, Alphen a/d Rijn, The Netherlands), and intravenously cannulised through the jugular vein. Then, mice were attached to a computer-controlled small-animal ventilator (Flexivent; SCIREQ, Montreal, Quebec, Canada). Ventilation was maintained at a breeding frequency of 300 breaths/min and a tidal volume of 10 mL/kg. Tidal volume was pressure limited at 300 mm H2O. Resistance in response to intravenous administration of increasing doses of methacholine (acetyl-b-methylcholine chloride, Sigma-Aldrich) was calculated from the pressure response to a 2-second pseudorandom pressure wave.

Collection and measurement of the bronchial lavage fluid

Briefly, lavage fluid was made of 10 ml PBS, containing 5% BSA and a protease inhibitor cocktail tablet (Roche, Mannheim, Germany). The trachea was cannulated and lungs were lavaged once with the lavage fluid. Cells were pelleted, and supernatants were stored at -80° C for cytokine measurements by ELISA. Subsequently, lungs were lavaged with 4 ml PBS containing 1% BSA, and BAL cells were pooled and counted using a coulter counter. Cytospin preparations were stained with Diff-Quick (Merz & Dade,

Dudingen, Switzerland) and evaluated in a blinded fashion. Cells were distinguished into mononuclear cells, lymphocytes, neutrophils, and eosinophils by standardmorphology. Per cytospin preparation, 200 cells were counted.

Histology and immunohistochemistry

Lungs were inflated with TissueTek O.C.T. Compound (Sakura Finetek Europe B.V, Zouterwoude, The Netherlands), and fixed in 10% Formalin for 24-hours, embedded in paraffin and cut in 3 μm-thick sections. Lung sections were stained with haemotoxylin/eosin (HE) and Periodic acid-Schiff (PAS). For immunohistochemistry, lung sections were deparaffinised in xylene, dehydrated in ethanol and washed in PBS. Antigen retrieval was performed by heating lung sections to the boiling point in 10 mM Tris/1 mM EDTA at pH 9.0. Sections were washed with PBS and blocked with PBS containing 30% H_2O_2 for 30 min. Lung sections were immunostained with mouse-anti-E-cadherin (1/800; BD Biosciences, Erembodegem, Belgium). The secondary Ab (Rabbit-anti-mouse-PO; DAKO, Glostrup, Denmark) was applied at a concentration of 1/100, and the tertiary Ab (Rabbit-anti-Goat-PO; DAKO, Glostrup, Denmark) was applied at a concentration 1/100. The immunostains were developed by using 3-amino-9-ethylcarbazole (AEC) substrate. Slides were examined and images were acquired by a microscope (Olympus) attached to a Color digital camera (Zeiss) using the Axiovision System (Zeiss). The percent area of membrane E-cadherin staining (mean ± SEM; n = 5 airways per mouse) was measured by the Image-Pro Plus software (Media Cybernetics, Inc., USA).

Measurement of Total and HDM-specific IgE levels in serum

Total IgE levels were measured in serum. Briefly, a 96-well plate was coated with anti-mouse IgE (BD Pharmingen, San Diego, CA, USA) overnight. The plate was washed with wash buffer and blocked for 1 hour with ELISA buffer. Samples and standard (purified mouse IgEκ control; BD Pharmingen, San Diego, CA, USA) were incubated at room temperature for 2 hours. After the plate was washed, the samples were first labelled with biotin-anti-mouse IgE (BD Pharmingen, San Diego, CA, USA) by incubation for 2 hours and then labelled with horseradish-peroxidase by incubation for 1 hour. After the last wash, the plate was incubated with OPD (Sigma, St Louis, MO, USA) for about 20 min, where after the reaction was stopped with 4M H_2SO_4. The plate was read at 490 nm.

HDM-specific IgE was also measured in serum. Briefly, a 96-well plate was coated with anti-mouse IgE (BD Pharmingen, San Diego, CA, USA) overnight. The plate was washed with wash buffer and blocked for 1 hour with ELISA buffer. Samples were incubated at room temperature for 2 hours. After the plate was washed, the samples were first labelled with biotinylated-HDM by incubation for 1 hour and then labelled with horseradish-peroxidase by incubation for 30 minutes. After the last wash, the plate was incubated with OPD (Sigma, St Louis, MO, USA) for about 20 min, where after the reaction was stopped with 4M H_2SO_4. The plate was read at 490 nm.

References

(E1) Heijink IH, Kies PM, Kauffman HF, Postma DS, van Oosterhout AJ, Vellenga E. Down-regulation of E-cadherin in human bronchial epithelial cells leads to epidermal growth factor receptor-dependent Th2 cell-promoting activity. J Immunol 2007;**178**(12):7678-7685.

(E2) Taher YA, Piavaux BJ, Gras R, van Esch BC, Hofman GA, Bloksma N, et al. Indoleamine 2,3-dioxygenase-dependent tryptophan metabolites contribute to tolerance induction during allergen immunotherapy in a mouse model. J Allergy Clin Immunol 2008; **121**(4):983-91.e2.

Table 3. Cytokine and Chemokine production in BALB/c mice after HDM exposure

	Experiment I			***Experiment II***		
	PBS *n = 6 & 8*	***ALK*** *n = 6*	***Greer*** *n = 6 & 8*	***PBS*** *n = 6*	***Citeq*** *n = 6*	***LPS*** *n = 6*
IL-5 (ng/ml)	16.50 [1.4 – 50]	19.61 [1.9 – 62.2]	76.98 * [23.3 – 174.4]	3.022 [0.6 – 4.1]	11.21 [1.1 – 23.7]	3.215 [0.8 – 16.3]
IL-13 (ng/ml)	158.6 [67.2 – 233.7]	155.9 [78.1 – 254.3]	229.9 [149.7 – 374]	14.87 [5.8 – 18.8]	49.04 ** [17.7 – 83.5]	15.89 [7.7 – 63.4]
CCL20 (pg/ml)	4.043 [1.6 – 19.2]	9.279 [2.7 – 19.6]	7.897 * [1.4 – 20.1]	5.065 [5.1 – 13.2]	8.249 [6.5 – 11.1]	5.1 [5.1 – 13.63]
CCL17 (pg/ml)	12.69 [6.2– 15.5]	11.14 [0.1 – 15.4]	30.23 * [12 – 260.1]	203.2 [1229 – 307.5	222.1 [123.6 – 616.4]	239.2 [197.8 – 945.9]
TSLP (pg/ml)	20.12 [13.1 – 31]	14.82 [8.9 – 28.9]	19.40 [13.3 – 27.8]	21.96 [12.7 – 42]	30.71 [15.1 – 50.5]	28.50 [22.1 – 149.6]
GM-CSF (pg/ml)	4.54 [2.7 – 11.2]	2.423 [1.1 – 5.7]	3.067 [1.4 – 4.0]	9.064 [7.8 – 19.3]	11.71 [7.8 – 21.6]	19.09 [7.8 – 31.04]
IgE (ng/ml)	2046 [865.9 – 3486]	1376 [1133 – 2482]	2876 [1514 – 6429]	608.9 [580.6 – 980.5]	2040 [810.9 – 3090]	ND
KC (pg/ml)	55.7 [44.2 - 92.5]	ND	99.29 * [44.73– 191.8]	54.54 [40.2 - 123.7]	98.64 [49.8 – 157.8]	ND
CCL11 (pg/ml)	67.6 [49.5– 86.7]	ND	97.59 ** [46.7 – 270.3]	71.1 [47.1 -163.3]	143.9 * [60.3 – 176.2]	ND
IL-17 (pg/ml)	218.3 [133.7 – 325.1]	ND	313.8 [146.1 – 560.2]	192.8 [166.3 – 417.5]	382.3 ** [228.3 – 543.1]	ND

The original absolute values (median [range]) obtained from two separate experiments by ELISA.. Abbreviations: ND; not determined

Figure E1 The effect of heat-inactivated HDM extracts on tight junction (TJ) and adhesion junction (AJ) expression. 16HBE cells were grown for 3-5 days in LabTeks, serum deprived overnight and stimulated without or with heat-inactivated (HI) HDM extracts (A) E-cadherin, zonula occludens (ZO)-1 and occludin were detected 15 minutes after HDM stimulation by immunofluorescent staining. Representative pictures from three independent experiments are shown.

Figure E2 The effects of the HDM-A extract on epithelial barrier function in epithelial cells. 16HBE cells were seeded in duplicate in Electric Cell-Substrate Impedance Sensing (ECIS) arrays or 24-well plates, serum deprived overnight and stimulated with HDM-A. (A) Normalized resistance measured after HDM-A exposure (n=3). (B) 16HBE cells after control and 10 ug/ml HDM-A exposure for 2 h. Representative pictures from three independent experiments are shown.

Figure E3 Effect of the HDM extracts in a mouse model of asthma. Balb/c mice (n = 6-14 per group) were exposed to 10 µl of Citeq and Greer extracts (2.5 mg/ml) or PBS twice a week for five weeks. ELISA measurements of (A) Eotaxin-1 (B) KC and (C) IL-17 in homogenized lung tissue, 24 h after the final intranasal challenge. Values were normalized to total protein content and expressed as percentages of control values. Relative levels and medians are shown. * = $p<0.05$ and ** = $p<0.01$ between HDM-treated and PBS-treated mice.