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Barrier responses of human bronchial epithelial cells to grass pollen exposure

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METHODS

Subjects and primary cell culture

Subjects with severe asthma were classified according to the Global Initiative for Asthma guidelines and the control group were subjects without asthma (Table E1). Primary bronchial epithelial cells (PBECs) were cultured in bronchial epithelial growth medium (Lonza, Basel, Switzerland) and differentiation was induced at passage 2 as previously described [8]. Briefly, PBECs were plated on Transwell permeable supports (diameter 6.5mm, polyester membrane with 0.4μm pores, Corning Life Sciences, Amsterdam, The Netherlands) and differentiated at an air-liquid interface (ALI) for 21 days. Transepithelial electrical resistance (TER) was monitored weekly using a EVOM voltohmmeter (World Precision Instruments, Aston, UK) and cells with a TER <1000Ω on day 21 were used for experiments.

Grass pollen extract

Timothy grass (*Phleum pratense*) pollen was purchased from Allergon (Ängelholm, Sweden). Extracts were obtained by incubating 30mg/ml pollen in bronchial epithelial basal medium (BEBM, Lonza) for 30min at 37°C. Extracts were centrifuged, and supernatants were sterile filtered using a low protein binding filter, according to previously published protocols [E1-5]. LPS contamination was removed using Detoxi-Gel Endotoxin Removing Gel (Thermo Scientific, Bonn, Germany) according to the manufacturer's instructions and aliquots were stored at -80°C until use. Stated concentrations in experiments always refer to the initial amount of pollen used for extraction. Protease activity of the extract was determined by using a Protease Fluorescent Detection Kit (Sigma, Poole, UK) according to the manufacturer's instructions.

Stimulation of ALI Cultures

Fully differentiated epithelial cultures (21 day post ALI) were starved for 24h before stimulation with BEBM supplemented with 1x ITS Liquid Media Supplement (Sigma), 50U/ml penicillin, 50µg/ml streptomycin (Invitrogen, Paisley, UK) and 1.5µg/ml BSA (Sigma). After measuring the TER, cells were stimulated apically with 67µl of Timothy grass (*Phleum pratense*) pollen extract diluted to reflect an initial concentration of 0.1-2mg pollen grains. BEBM was used as a control. TER was monitored over time without removal of the apical supernatants. After 24 hours, apical supernatants were collected, replaced with 100µl pre-warmed Hanks Buffered Saline Solution (HBSS; Invitrogen) and after 15min incubation at 37°C and 5% CO_2 in a humidified atmosphere TER was measured again. Apical secretions and basolateral supernatants were taken and the apical HBSS supernatants were combined with the previously harvested apical secretions. Cell debris in the secretions was removed by centrifugation and supernatants were stored at -80°C until further analysis. Cells were either re-stimulated with pollen extract, fixed with 4% paraformaldehyde for immunofluorescence staining or lysed for Western Blot or RNA expression analysis. Involvement of mitogen-activated protein kinase (MAPK) signalling pathways in the regulation of barrier functions was analysed using specific pharmacological inhibitors U0126, SB203580 and SP600125 (Sigma, Poole, UK). Cells were apically pre-treated for 30min with 25µM of the inhibitors [36] and subsequently exposed to pollen extract in the presence of the inhibitors. DMSO was diluted for use as vehicle control.

Characterization of ALI cultures.

Differentiation of the ALI cultures was monitored on day 21 by immunostaining and counting cytospins for the presence of β-tubulin and MUC5AC positive cells. Briefly, the apical and basolateral compartments of the cultures were washed in HBSS then 500µl trypsin was added into the basal compartment and 200µl of trypsin into the apical compartment.

After 5 minutes, the trypsin was removed and detached cells resuspended in10% FBS/DMEM. Trypsinization was repeated and the cell suspensions combined for preparation of cytospins. After air drying, fixing in acetone and blocking endogenous peroxidase, the cells were immunostained for β-tubulin (Sigma, Catalogue number T5293) or MUC5AC (Neomarkers, catalogue number MS-145-PABX) using the immunoperoxidase technique and counterstained with Mayer's haematoxylin. The number of β-tubulin or MUC5AC positive cells in 5 fields were counted and expressed as a percentage of the total cells in the fields.

Immunofluorescent staining of TJs

TJs were immunostained using mouse monoclonal antibodies directed to ZO-1 (clone ZO1-1A12) and occludin (clone OC-3F10 labelled with Alexa Fluor 488) (both from Invitrogen). The ZO-1 antibody was labelled with Alexa Fluor 647 using Alexa Fluor 647 Monoclonal Antibody Labeling Kit (Invitrogen). Actin filaments were stained with Acti-Stain 555 phalloidin (Cytoskeleton, Denver, Colorado, US). After fixation with 4% paraformaldehyde cells were permeabilised with 0.1% Triton X-100, blocked with 1% BSA in PBS and stained over night at 4°C. Subsequently, cells were washed extensively and mounted on slides using ProLong Gold antifade reagent with DAPI (Invitrogen). Z-stacks were taken using LSM 6000 microscope (Leica Microsystems, Wetzlar, Germany). After deconvolution using Leica Application Suite software orthogonal views were performed using ImageJ software.

Western Blot analysis

After apical stimulation with pollen extract, cells were lysed in Lysis Buffer (62.5mM Tris-HCl, 10% glycerol, 2% SDS, pH6.8 supplemented with Complete Protease and PhosSTOP Phosphatase Inhibitor Cocktails (Roche, Burgess Hill, United Kingdom) and sonicated. Protein concentration was determined by measuring the absorbance at 280nm. Samples were

diluted to a protein concentration of 0.5mg/ml and 7.5µg protein of each total cell lysate was separated by SDS-PAGE and transferred to a PVDF membrane. After blocking with 5% BSA in TBS+0.1% Tween-20, membranes were incubation over night with phospho-p38 MAPK (Thr180/Tyr182) specific polyclonal rabbit antibody and developed using anti-rabbit IgG HRP-linked antibody and ECL reagent. Bound antibodies were stripped using 62.5mM Tris-HCl, 2% SDS, 100mM β-mercaptoethanol and reprobed with p38 MAPK specific polyclonal rabbit antibody and developed as described above. Band intensity was determined by densitometry and the ratio of phospho-p38/p-38 was normalised to the unstimulated control. Antibodies were obtained from Cell Signaling, Danvers, MA.

CXCL8/IL-8 mRNA expression analysis

After treatment, cells were lysed with TRIzol (life technologies) and RNA was extracted using chloroform. After DNA removal using Ambion DNA-free DNase Treatment & Removal kit (life technologies), 1ug RNA was transcribed into cDNA using Reverse Transcription kit (Primer Design, Southampton, United Kingdom). Quantitative real-time PCR was performed using PerfectProbe CXCL8/IL-8 and UBC/GAPDH primers and Precision qPCR MasterMix (all reagents from Primer Design). Expression levels were calculated using the ΔΔCT method.

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Table E1. Characteristics of PBEC donors used for characterisation of ALI cultures.

non-asthmatic						
donor	**age**	**sex**	**skin prick test**	**FEV1%**	**medication**	**smoking**
donor A	23	M	Non-atopic	149	None	no
donor B	20	F	Non-atopic	96	None	no
donor C	22	F	Non-atopic	119	None	no
donor D	33	M	Atopic	105	None	no
donor E	24	M	Atopic	115	None	no
donor F	22	F	Atopic	108	None	no
donor G	22	F	Atopic	127	None	no
donor H	20	M	Non-atopic	98	None	no
donor I	18	M	Non-atopic	90	None	no
donor J	21	F	Non-atopic	90	None	no
donor K	22	F	Atopic	93	None	no

severe asthmatic										
patient	**age**	**sex**	**skin prick test**	**FEV1%**	**medication**					**smoking**
					ICS (µg/d)	**OCS (mg/d)**	**LABA**	**LTRA**	**other**	
donor L	22	F	Atopic	87	200	none	none	none	Salbutamol PRN	no
donor M	23	M	Atopic	118	100	none	none	none	Salbutamol PRN	no
donor N	44	F	Atopic	82	400	none	none	none	Salbutamol PRN	no
donor O	22	M	Atopic	105	200	none	none	none	Salbutamol PRN	no
donor P	24	F	Atopic	58	400	none	yes	none	Salbutamol PRN	no
donor Q	24	F	Atopic	69	800	none	yes	none	Salbutamol PRN	no
donor R	33	M	Atopic	101	200	none	none	none	Salbutamol PRN	no
donor S	64	M	Atopic	41	500	none	yes	none	Salbutamol PRN	no
donor T	23	M	Atopic	90	400	none	none	none	Salbutamol PRN	no

ICS, inhaled corticosteroids equalised to Beclamethasone dipropionate; OCS, oral corticosteroids; LABA, long-acting β_2 agonists; LTRA, leukotriene receptor antagonist

Table E2. Characteristics of PBEC donors used for analysing pollen induced mediator release by multiplex immunoassay.

non-asthmatic											
donor	**age**	**sex**	**skin prick test**	**FEV 1%**	**medication**						**smoking**
donor 1	20	F	negative	101	none					---	no
donor 2	20	F	negative	93	none					---	no
donor 3	25	M	negative	101	none					---	no
donor 4	19	F	negative	77	none					---	no
donor 5	20	F	negative	97	none					---	no
donor 6	20	F	negative	109	none					---	no
donor 7	22	F	negative	102	none					---	no
donor 8	44	F	negative	88	none					---	ex(>1yr)
donor 9	24	F	negative	110	none					---	no
donor 10	19	M	negative	104	none					---	no
severe asthmatic											
donor	**age**	**sex**	**skin prick test**	**FEV 1%**	**medication**					**exacerbation last 12 month**	**smoking**
					ICS (µg/d)	**OCS (mg/d)**	**LABA**	**LTRA**	**other**		
donor 11	51	F	grass, feather	76	2400	none	yes	none		4	ex(>10yr)
donor 12	51	F	cat	55	1000	none	yes	yes		10	no
donor 13	45	M	Asp.fum, birch	31	1000	none	yes	none		6	no
donor 14	34	M	grass, birch, feather, alternaria, D.pter, cat, dog	80	2000	none	yes	yes		8	no
donor 15	45	F	grass, birch, rape, D.pter, D.far, cat, dog	94	3000	none	yes	yes		5	no
donor 16	35	F	rape	41	2000	none	yes	yes	Omali zumab	12	no

ICS, inhaled corticosteroids equalised to Beclamethasone dipropionate; OCS, oral corticosteroids; LABA, long-acting β_2 agonists; LTRA, leukotriene receptor antagonist

FIGURE LEGENDS

Figure E1. Protease activity in grass pollen extract. Detection of protease activity in pollen extract (PE) using a fluorescence protease assay (n=3). Data are corrected for the reagent control; the lower limit of detection for the assay was 0.1μg/ml trypsin. The activity for the pollen extract was significantly higher than that found with 0.1μg/ml trypsin (*: $p \leq 0.05$).

Figure E2. Baseline basolateral release of CCL20/MIP-3α (a), CCL2/MCP-1 (b) and TNF-α (c) by ALI from non-asthmatic (n=10) and severe asthmatic (n=6) subjects. (*: $p \leq 0.05$ non-asthmatic vs. severe asthmatic).

Figure E3. Apical release of CCL20/MIP-3α (a), CCL22/MDC (b) and TNF-α of ALI by cultures from non-asthmatic (n=10) and severe asthmatic (n=6) subjects after exposure to 1mg of grass pollen for 24h. (*: $p \leq 0.05$ compared to untreated control).

Figure E4. Apical release of CXCL10/IP-10 (a), CCL11/eotaxin (b) and CCL17/TARC by ALI cultures from non-asthmatic (n=10) and severe asthmatic (n=6) subjects after exposure to 1mg of grass pollen for 24h. (*: $p \leq 0.05$ compared to untreated control).