

Original article

Allergen induced gene expression of airway epithelial cells shows a possible role for TNF- α

Background: Epithelium is more than a physical barrier for pathogens and allergens, as it is also capable of producing mediators in response to these environmental factors. Some of these mediators have an immuno-modulatory function, suggesting that epithelium is an active component of the immune response. Here, we fully characterize the expression profile of airway epithelial cells in response to house dust mite (HDM) allergen.

Methods: H292 cells were exposed to HDM extract for 24 h, RNA and supernatant was used for microarray analysis and multiplex enzyme-linked immunosorbent assay (ELISA) respectively.

Results: A total of 38 500 genes, 813 were differentially expressed by more than twofold and 116 even more than fivefold. Interestingly, among the most up-regulated genes, a large number are involved in cell-to-cell communication. These include chemokines (CCL-8 and -20, CXCL-1, -2 and -3), cytokines (IL-1 α , -6 and -11), anti-inflammatory factors [PTX-3, interleukin (IL)-13R α , tumour necrosis factor (TNF)- α IP3], and factors that are involved in repair of the mucosal tissue (LOXL-2, NID-2, HBEGF, MUC-5AC and MUC-5B). Pathway analysis showed that a number of these genes are transcriptionally regulated by TNF- α , which we could detect by quantitative polymerase chain reaction at earlier time points after HDM exposure. In addition, we could detect increased protein levels for TNF- α , IL-6, IL8, granulocyte-macrophage colony-stimulating factor, granulocyte colony stimulating factor and interferon (IFN)- γ using ELISA.

Conclusion: Our data show that a broad range of mediators produced upon allergen exposure by these mediators' epithelial cells can participate in the immune response via recruitment and activation of cells of the immune system.

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Allergic disease affects a large part of the western population, with a prevalence of more than 20% in the USA (1). Patients can have symptoms like runny nose, itching of the nose and/or eyes, sneezing, skin rashes, shortness of breath, nausea, fatigue, and can even die of anaphylactic shock. These symptoms can significantly affect the quality of life and performance on the job (2). The impact on society is enormous; it has been estimated that absenteeism and low productivity because of allergies cost USA companies more than \$250 million in 1998 (3, 4). Although these indirect costs are high, they are just a fraction compared with the estimated overall health care expenditures attributable to sinusitis, which in 1996 were more than \$6 billion (5).

Effective treatment will improve quality of life and will also reduce the indirect and direct costs associated with the disease. Although the two predominant treatments for nasal allergies, corticosteroids and antihistamines are effective in a large group of patients, not all patients benefit from it (4). Corticosteroids, because of their broad

action may cause unwanted side-effects, whereas antihistamines only work at the end of the immunological cascade, which may be responsible for their limited efficacy in asthma (6). By developing new medication that specifically targets cells in the beginning of the allergic response instead of effector cells, the efficacy and specificity of treatment might be increased.

An interesting target for the development of new drugs are airway epithelial cells, being the first cells an allergen encounters (7). Recent research has shown that they are more than just a physical barrier. They are capable of responding to environmental changes, such as invading pathogens, allergen exposure or wounding (8–10). One of the methods that has been used to investigate this is in a model where cultured epithelial cells (e.g. squamous cell carcinoma cell line NCI-H292) are subjected to various stimuli. These reports have shown that they can produce inflammatory mediators, such as interleukin (IL)-6, IL-8 and prostaglandin-2 upon activation by allergens (11, 12). Epithelial cells are activated via the protease activated

receptors (PARs), and in particular PAR2 has been described for activation by allergens (13). Protease activated receptors are innate receptors that have originally been described on endothelial cells as part of the blood clotting system, but that can also be found on many other cell types. It has been shown that not only thrombin but also the proteases that can be found in allergenic mixtures can activate PAR2. The epithelial response to the allergenic mixture is not preceded by earlier sensitization to the allergen, and many proteolytic allergens are capable of activating these epithelial cells (14, 15). In our own research, we have investigated the mediator release by epithelial cells in response to disruption, with or without concomitant PAR activation. We found a variety of produced mediators much more diverse than the previously described production of IL-6 and IL-8. For instance, we found activation of increased production of IL-1 β , interferon (IFN)- γ , tumour necrosis factor (TNF)- α , IL-4, granulocyte colony stimulating factor (G-CSF), growth related oncogene, epidermal growth factor, insulin-like growth factor binding protein 3, leukemia inhibitory factor (LIF), induced protein-10 (10 kDa interferon – gamma-IP), tumour growth factor (TGF)- β and vascular endothelial growth factor (submitted).

The aim of the experiments described in this manuscript was to map the response of airway epithelial cells to house dust mite allergen (HDM). By better knowledge of the affected genes, we can get closer to understanding the role of the epithelium in the mucosal response to allergens. This understanding will help to develop new medication that could inhibit the contribution of the epithelium to the initiation and maintenance of the allergic response. To characterize the expression profile of airway epithelial cells upon HDM stimulation, we used a transcriptomics and proteomics approach. The complete dataset from the time course is accessible through the NCBI Gene Expression Omnibus repository (GEO; <http://www.ncbi.nlm.nih.gov/geo/>), series accession number GSE9151.

Materials and methods

Cell culture

NCI-H292 human airway epithelial cells (American Type Culture Collection, Manassas, VA, USA) were cultured in RPMI 1640 medium (Invitrogen, Breda, The Netherlands) supplemented with 1.25 mM of L-glutamine, 100 U/ml of penicillin, 100 μ g/ml of streptomycin and 10% (v/v) of fetal bovine serum (HyClone, Logan, UT, USA). Cells were grown in fully humidified air containing 5% of CO₂ at 37°C and were subcultured weekly.

Induction experiment

Cells were cultured to 80% confluence in a six wells plate. Before stimulation experiment, the cells were preincubated with Hanks' balanced salt solution (HBSS) for 24 h. Culture medium was removed and the cells were then stimulated with HDM extract

diluted in HBSS (2 μ g/ml) or with HBSS alone (control condition) for 24 h. Supernatants were removed and stored for further analysis; cells were used for RNA extraction.

HDM extract

House dust mite extract was kindly provided by Prof. Dr M. L. Kapsenberg (AMC, The Netherlands) as a lyophilized powder. It was dissolved in phosphate buffer saline (PBS), and then dialysed against PBS and diluted to a final concentration of 8 μ g/ml.

RNA extraction

Total RNA from each sample was extracted using Trizol (Life Technologies, Inc., Gaithersburg, MD, USA) according to manufacturer's protocol, followed by purification by nucleospin RNA II (Machery-Nagel, Düren, Germany). The RNA concentration was measured on the nanodrop ND-1000 (NanoDrop Technologies Inc., Wilmington, DE, USA). RNA quality was checked by using Agilent 2100 bio-analyser (Agilent Technologies, Palo Alto, CA, USA).

Microarray Affymetrix U133 Plus 2

Human Genome U133 Plus 2.0 Genechip Array (Affymetrix Inc., Santa Clara, CA, USA) representing 47 000 transcripts, including 38 500 well-characterized genes was used in the analysis of HDM-induced genes. Technical handling of microarray experiments was performed at the MicroArray Department of the University of Amsterdam (Amsterdam, The Netherlands), a fully licensed microarray technology centre for Affymetrix Genechip® platforms and official Dutch Affymetrix Service Provider. In short, biotin-labelled cRNA samples were prepared as described in the Affymetrix expression analysis technical manual (Affymetrix) using 7.5 μ g of purified total RNA as template for the reaction. For this, the One-Cycle cDNA Synthesis Kit (Affymetrix) was used. The Array images were acquired using a GeneChip Scanner 3000 (Affymetrix) and analysed with Rosetta Resolver (Rosetta Biosoftware, Seattle, WA, USA).

Microarray data analysis

Genechip images were loaded into Rosetta Resolver where we used a factorial design to compare the three chips that were hybridized with RNA of control treated cells with the three chips that were hybridized with RNA of the HDM treated cells. This experimental design for the analysis will only yield genes of which the average expression in one condition statistically differs from the average expression in the other condition. To select for all statistically significant differentially expressed genes we used post-test statistics, which are described in statistical analysis section.

Real-time RT-PCR

Quantitative polymerase chain reaction (PCR) was used to validate the differential expression of selected genes. RNA isolated from control treated and HDM treated cells using the nucleospin RNA II, subsequently cDNA was synthesized using the MBI Fermentas first strand cDNA synthesis kit (Fermentas GmbH, St Leon-Rot, Germany). Polymerase chain reaction was performed on Bio-Rad iCycler (Bio-Rad, Veenendaal, the Netherlands). TaqMan® primer (Roche Molecular Systems, Pleasanton, CA, USA) and probe sequences for IL-6, IL-8, GAPDH (glyceraldehyde-3-phosphate

dehydrogenase) and β -actin (16–19) were obtained from Sigma-Aldrich (Haverhill, UK). For the other genes we ordered TaqMan® gene expression assays from Applied Biosystems (Nieuwerkerk a/d IJssel, the Netherlands). The sequences for PCR reactions are: IL6; sense: 5'-TGA-CAA-ACA-AAT-TCG-GTA-CAT-CCT-3', probe: 5'-FAM-TTA-CTC-TTG-TTA-CAT-GTC-TCC-TTT-CTC-AGG-GCT-G-TAMRA-3', antisense: 5'-AGT-GCC-TCT-TTG-CTG-CTT-TCA-C-3', IL8; sense: 5'-CCA-CAC-TGC-GCC-AAC-ACA-GAA-ATT-ATT-G-3', probe: 5'-FAM-AAG-CTT-TCT-GAT-GGA-AGA-GAG-CTC-TGT-C-TAMRA-3', antisense: 5'-GCC-CTC-TTC-AAA-AAC-TTC-TCC-ACA-ACC-C-3', β -actin; sense: 5'-TGA-GCG-CGG-CTA-CAG-CTT-3', probe: 5'-Texas red-ACC-ACC-ACG-GCC-GAG-CGG-BHQ2-3', antisense: 5'-TCC-TTA-ATG-TCA-CGC-ACG-ATT-T-3', GAPDH; sense: 5'-GAA-GGT-GAA-GGT-CGG-AGT-C-3, probe: 5'-Texas red-CAA-GCT-TCC-CGT-TCT-CAG-CC-BHQ2-3', antisense: 5'-GAA-GAT-GGT-GAT-GGG-ATT-TC-3'. The ordered assays had the following assay IDs: CCL20; Hs00171125_m1, IL11; Hs00174148_m1, IRAK2; Hs00176394_m1, COL5A1; Hs00609088_m1, PTX3; Hs00173615_m1, SCARA3; Hs00212206_m1, SERPINE2; Hs00299953_m1, SERPINF1; Hs00171467_m1, TNFAIP3; Hs00234712_m1, ZNF488; Hs00399237_m1, KRT4; Hs00361611_m1, NID2; Hs00201233_m1, IL13RA2; Hs00152924_m1, and MCTP1; Hs00226801_m1. We performed all PCR assays three times, on all three experimental replicates. Expression changes are presented as $\delta\delta_{ct}$, indicating the difference in threshold cycle between the control condition and the HDM treatment, after correcting a sample for the expression of the housekeeping gene.

Protein multiplex Luminex Bio-Plex assay

Supernatants of HDM and control treated cells were stored at -20°C until analysis. Cytokine levels in supernatant of cells were determined using the xMAP technology (Luminex Corporation, Austin, TX, USA). A Bio-Plex Human Cytokine 17-Plex Panel kit (Bio-Rad, Veenendaal, The Netherlands) was used and analysed on the Bio-Plex workstation (Bio-Rad). All standards were diluted in the same serum free culture medium where the cells were put in during treatment. Concentrations were calculated from a dilution series of standards using the Luminex software. Lower detection limits are indicated per cytokine.

Statistical analysis

Experiments were performed in triplicate and simultaneously. Genechip images and data sets were uploaded into the server of the Netherlands Bioinformatics Centre using the Rosetta Resolver Bioinformatics package, which was also used for statistical analyses. Triplicates were compared in a factorial design using an error-weighted one-way analysis of variance (ANOVA). To control for multiple comparisons, we applied a Bonferroni multiple test correction. We reported as significant genes only those that reached significance at level $P \leq 0.01$. Testing 54 675 probes at this level, we expected the average number of false positive results to be 550 or less. We found that 3491 reached the significance level of 0.01, exceeding the average number of false positives.

Results

House dust mite protease specific induction of epithelial cells

To see whether our model functions as described in literature and to investigate the properties of our stimuli

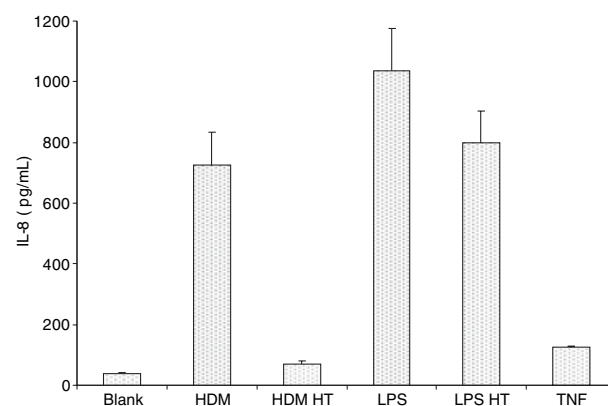


Figure 1. IL-8 production by H292 cells after different stimuli. Values are average and standard deviation over triplicate samples. Blank = culture medium exposed control; HDM = house dust mite exposed (2 $\mu\text{g}/\text{ml}$); HDM HT = heat treated HDM (2 $\mu\text{g}/\text{ml}$); LPS = lipopolysaccharide (1 $\mu\text{g}/\text{ml}$); LPS HT = heat treated LPS (1 $\mu\text{g}/\text{ml}$); TNF = tumour necrosis factor alpha (50 pg/ml).

we exposed epithelial cells to (i) HDM extract 2 $\mu\text{g}/\text{ml}$; (ii) heat treated HDM extract 2 $\mu\text{g}/\text{ml}$ (30 min at 95°C); (iii) LPS 1 $\mu\text{g}/\text{ml}$ and (iv) heat treated LPS 1 $\mu\text{g}/\text{ml}$ and to TNF- α 50 pg/ml . When cells were exposed to HDM, their IL-8 production increased 19 times, this increase could almost fully be abrogated by heat treatment of the HDM extract, inactivating the protease activity. When cells are exposed to LPS, they also show an increased production of IL-8, this increase could not be abrogated by heat treatment (see Fig. 1).

Gene expression ratio in airway epithelial cells between HDM stimulated and controls

Global expression values were measured in three experiments where cells were either stimulated with HDM diluted in HBSS or with HBSS alone as the control condition. Using the Rosetta Resolver software, we calculated the ratio of the expression between the two conditions. Genes were considered significantly differentially expressed when $P \leq 0.01$ after one-way ANOVA with a Bonferroni multiple test correction. The 54 675 sequences that are present on the Affymetrix U133 plus 2.0 represent approximately 38 500 genes. In our experiments, we found 614 sequences significantly up-regulated and 474 sequences down-regulated by more than twofold. These represent 458 up-regulated and 353 down-regulated genes.

Validation of the microarray data

Sixty-two out of 56 675 sequences on the chip were marked as controls by Affymetrix, based on their constitutive expression levels. Among these were sequences for the stably expressed genes 18S, 28S rRNA, β -actin and GAPDH, which are commonly used for normalization in

gene expression profiling experiments. These sequences were used as an internal validation. The average expression ratio between control and HDM stimulated conditions of these 62 assigned sequences in our experiment was on average $1.05 (\pm 0.31)$ showing that these control genes were indeed not affected by HDM stimulation.

Additionally, we selected 11 up-regulated genes [CCL-20 (Macrophage inflammatory protein 3 alpha)], PTX (pentraxin)-3, IL-8, IL-6, IL-11, IL-13RA, SERPIN (serine proteinase inhibitor)-E2, TNF-AIP3 (TNF- α inducible protein 3), NID (nidogen)-2, MCTP (multiple C2-domains with two transmembrane regions)-1, IRAK (interleukin-1 receptor-associated kinase)-2 and 5 down-regulated genes [COL (collagen)-5A, SERPIN-F1, KRT (cytokeratin)-4, SCARA (scavenger receptor class A)-3, and ZNF (zinc finger protein)-488] for independent confirmatory PCR. Expression levels in all PCR samples were normalized against GAPDH and β -actin. These housekeeping genes were selected as their expression was not affected by HDM stimulation, with their fold-change in our microarray experiment being -1.15 and -1.01 respectively. We could not calculate an expression ratio for PTX-3 as expression of PTX-3 in the control treated cells was below detection level of the PCR. Table 1 shows the ratios derived from the microarray experiment and the expression ratios calculated from the PCR. The correlation between the PCR results and the microarray results is high (uncorrected $R = 0.73$ with $R^2 = 0.54$) and even higher when corrected for the outlier CCL-20 (corrected $R = 0.92$ with $R^2 = 0.85$), indicating an 85% correlation between the PCR results and the microarray results.

Gene ontology

After checking our microarray data by PCR and checking the expression of assigned housekeeping genes, we con-

Table 1. Validatory PCR of significantly different genes

	PCR expression $\delta\delta_{ct}$	Microarray ratio's
CCL20	7.8 (± 0.4)	100.0 (± 1.5)
IL8	9.2 (± 1.3)	39.3 (± 1.3)
IL13RA	5.4 (± 0.4)	29.8 (± 1.5)
IL6	7.5 (± 1.1)	26.8 (± 1.3)
IL11	1.8 (± 0.3)	23.2 (± 1.3)
SERPINE2	4.7 (± 0.4)	18.9 (± 1.3)
TNFAIP3	3.7 (± 0.4)	12.3 (± 1.1)
NID2	4.8 (± 0.5)	10.4 (± 1.4)
MCTP1	2.9 (± 0.4)	8.8 (± 1.1)
IRAK2	3.4 (± 0.4)	7.4 (± 1.3)
COL5A	-1.7 (± 0.4)	-5.6 (± 1.5)
SERPINF1	-2.1 (± 0.4)	-8.3 (± 1.2)
KRT4	-3.0 (± 0.3)	-10.0 (± 1.6)
SCARA	-1.3 (± 0.3)	-10.3 (± 1.4)
ZNF488	-1.6 (± 0.4)	-11.2 (± 1.5)
PTX3	n.d.	47.7 (± 1.5)

16 genes were selected for confirmatory PCR. All values are given as mean ($\pm$ SD). PCR expression is given as $\delta\delta_{ct}$ normalized against GAPDH and β -actin
n.d., below detection level.

cluded that our microarray data were indeed valid. For further detailed analysis, we focused initially on the genes that were up- or down-regulated more than fivefold, limiting the number to 46 down-regulated and 70 up-regulated genes. Using their ascribed gene ontology, we sorted the genes by their biological process or molecular function. We found 38 genes known to be associated with cell communication; in this group there were cytokines, chemokines, growth factors and receptors. Other groups we found are immunity and defense (5), receptor activity (4), enzyme inhibitor activity (5), nucleic acid binding (9), metabolism (14), transport (4), calcium ion binding (8). A number of genes (6) were single members of an ontology group, and are here pooled as 'miscellaneous'. No ontology was known for 24 genes. The ontology groups, the genes in these groups, with their gene symbol and name, fold change, P -value, chromosome location and accession number are shown in Table 2.

The ontology analysis revealed that from the group of genes that was up-regulated the strongest (more than 15-fold), a substantial number (10/16), belong to the cell communication group, and that a partly overlapping number had been reported to be under the transcriptional control of TNF- α signalling. Using network analysis we built a computational model from interactions between genes described in literature. In this case, we used our geneset containing genes whose expression was statically significantly changed by more than twofold. Figure 2 shows the scheme containing genes within our data set that have a known interaction with TNF- α . The found expression pattern corresponds to the expected expression pattern based on the effects of TNF- α on the genes described in literature. To explore this observation further, we looked for expression of the TNF receptor (CD120a) and TNF- α in our microarray experiments. mRNA for the receptor (CD120a) was present in both control and HDM-stimulated samples, with no significant difference in expression levels between these two conditions. However, TNF- α mRNA was undetectable in the microarray. When we investigated the TNF- α expression using a TaqMan[®] gene expression assay, we could detect TNF- α mRNA; however, the expression levels were quite low, on average 4000 times lower than β -2 microglobulin (ranged: 1000–17 000), a highly abundant constitutively expressed gene in epithelial cells. When we look at the expression profile of TNF- α at different time-points after HDM stimulation, and compare that to medium control, we see that TNF- α is the highest after 2.5 h, but is still higher at 24 h (see Fig. 3).

Protein expression of chemokines and cytokines under stimulating and control conditions

As indicated above, our initial analysis showed that a substantial number of highly up-regulated genes belong to the cell communication group (Table 2). Next, we

Table 2. Highly differentially expressed genes sorted by ontology in functional groups

Gene alias	Fold change	Gene name/description	P-value	Chromosome location	Accession no.
Cell communication					
CCL20	100.0	MIP-3 α	0	2q33–q37	NM_004591
IL1A	41.0	Interleukin 1 α	0	2q14	NM_000575
IL8	39.3	Interleukin 8	0	4q13–q21	NM_000584
CXCL3	37.9	GRO- γ	0	4q21	NM_002090
CXCL2	31.5	GRObeta	0	4q21	NM_002089
IL6	26.8	Interleukin 6	0	7p21	NM_000600
STC1	25.3	Stanniocalcin 1	0	8p21–p11.2	NM_003155
IL11	23.2	Interleukin 11	0	19q13.3/4	NM_000641
EREG	18.7	Epiregulin	3.64E-43	4q13.3	NM_001432
FST	17.7	Follistatin	0	5q11.2	NM_013409
LOXL2	12.6	Lysyl oxidase-like 2	0	8p21.3–p21.2	NM_002318
MUC5AC	12.1	Mucin 5, subtypes A and C	2.06E-43	11p15.5	AW192795
CXCL1	11.9	GRO- α	0	4q21	NM_001511
IL1RL1	11.3	Interleukin 1 receptor-like 1	1.23E-16	2q12	NM_003856
DUSP6	11.3	Dual specificity phosphatase 6	0	12q22–q23	NM_001946
AREG	10.3	Amphiregulin	0	4q13–q21	NM_001657
HBEGF	10.1	Heparin-binding EGF-like growth factor	0	5q23	NM_001945
S100A9	8.3	Calgranulin B, S100 calcium binding protein A9	8.94E-17	1q21	NM_002965
SLIT2	8.3	Slit homolog 2 (Drosophila)	2.58E-42	4p15.2	NM_004787
EDNRA	8.2	Endothelin receptor type A	8.23E-20	4q31.22/23	NM_001957
IRAK2	7.4	Interleukin-1 receptor-associated kinase 2	3.24E-24	3p25.3	NM_001570
COL8A1	7.1	Collagen VIII alpha 1	0	3q12.3	NM_001850
MUC5B	6.6	Mucin 5, subtype B	1.55E-26	11p15	AI697108
PARK7	6.4	Parkinson disease 7	0	1p36.33/12	NM_007262
RASD1	5.5	RAS, dexamethasone-induced 1	4.74E-17	17p11.2	NM_002341
LTB	5.5	Lymphotoxin beta	4.74E-17	6p21.3	NM_002341
ITGB8	5.4	Integrin beta 8	5.89E-16	7p21.1	NM_002214
BIRC3	5.3	Baculoviral IAP repeat-containing 3	9.08E-20	11q22	NM_001165
IL1B	5.2	Interleukin 1 beta	1.44E-17	2q14	NM_000576
SPRY4	5.2	Sprouty homolog 4	3.11E-09	5q31.3	NM_030964
CHRNA1	5.1	Cholinergic receptor, nicotinic, beta polypeptide 1	8.18E-13	17p13.1	NM_000747
ASB9	–5.1	Ankyrin repeat and SOCS box-containing 9	8.66E-18	xp22.2	NM_024087
CRABP2	–5.2	Cellular retinoic acid binding protein 2	1.59E-16	1q21.3	NM_001878
EPHA4	–5.3	Ephrin receptor A4	2.43E-09	2q36.1	NM_004438
MASS1	–5.3	Monogenic, audiogenic seizure susceptibility 1 homolog	5.98E-08	5q13	NM_032119
COL5A1	–5.6	Collagen V alpha 1	2.76E-09	9q34.2–q34.3	NM_000093
VIPR1	–5.8	Vasoactive intestinal peptide receptor 1	2.85E-15	3p22	NM_004624
PPP2R2B	–7.3	Protein phosphatase 2, regulatory subunit B, beta isoform	0	5q31–5q32	NM_004576
Immunity and defense					
PTX3	47.7	Pentraxin 3	0	3q25	NM_002852
PBEF1	5.8	Pre-B-cell colony enhancing factor 1	0	7q22.3	AA873350
C1QTNF1	5.4	C1q and tumour necrosis factor related protein 1	2.50E-22	17q25.3	NM_030968
SELENBP1	–5.2	Selenium binding protein 1	8.08E-12	1q21–q22	NM_003944
DEFB1	–5.6	Defensin, beta 1	1.12E-17	8p23.2–p23.1	NM_005218
Receptor activity					
IL13RA2	29.8	Interleukin 13 receptor, alpha 2	0	Xq13.1–q28	NM_000640
F3	5.3	Coagulation factor III	2.52E-41	1p22–p21	NM_001993
PTPRB	5.4	Protein tyrosine phosphatase, receptor type, B	6.40E-29	12q15–q21	NM_002837
SCARA3	–10.3	Scavenger receptor class A, member 3	4.46E-43	8p21	NM_016240
Enzyme inhibitor activity					
SERPINE2	18.9	Serine proteinase inhibitor, clade E, member 2	0	2q33–q35	NM_006216
SERPINB4	8.0	Serine proteinase inhibitor, clade B, member 4	0	18q21.3	NM_002974
SERPINB8	5.9	Serine proteinase inhibitor, clade B, member 8	5.56E-14	18q21.3	NM_002640
CDKN1C	–8.1	Cyclin-dependent kinase inhibitor 1C	1.52E-12	11p15.5	NM_000076
SERPINF1	–8.3	Serine proteinase inhibitor, clade F, member 1	0	17p13.1	NM_002615
Nucleic acid binding					
TNFAIP3	12.3	Tumour necrosis factor, alpha-induced protein 3	0	6q23	NM_006290
C10orf48	10.2	Chromosome 10 open reading frame 48	1.46E-28	10p12	NM_173576
TNIP2	6.8	TNFAIP3 interacting protein 2	7.57E-23	4p16.3	NM_024309
ETV5	6.2	ETS translocation variant 5	3.64E-19	3q28	NM_004454

Table 2. (Continued)

Gene alias	Fold change	Gene name/description	P-value	Chromosome location	Accession no.
KLF4	6.1	Kruppel-like factor 4	8.39E-13	9q31	NM_004235
MAF	-6.1	Transcription factor Maf	1.05E-25	16q22-q23	NM_005360
SEMA6D	-6.8	Semaphorin 6D	2.30E-28	15q21.1	AL036088
KIAA1305	-8.5	Unknown	4.27E-13	14q11.2	XM_370756
ZNF488	-11.2	Zinc finger protein 488	1.75E-13	10q11.22	NM_153034
Metabolism					
ADAM8	14.8	A disintegrin and metalloproteinase domain 8	0	10q26.3	NM_001109
SUV420H2	8.4	Suppressor of variegation 4-20 homolog 2 (Drosophila)	5.25E-12	19q13.42	NM_032701
RAB3B	7.7	RAS related protein 3B	2.29E-10	1p32-p31	AU156710
ABCC3	6.9	ATP-binding cassette, sub-family C, member 3	1.38E-34	17q22	NM_020037
CYP26B1	5.6	Cytochrome P450, family 26, subfamily B, polypeptide 1	1.20E-09	2p13.2	NM_019885
KIAA0703	5.5	Probable calcium-transporting ATPase KIAA0703	0	16q24.1	NM_014861
MICAL2	5.0	Microtubule associated monooxygenase, calponin and LIM domain cont. 2	3.87E-23	11p15.3	NM_014632
HPGD	-5.3	Hydroxyprostaglandin dehydrogenase 15-(NAD)	1.53E-12	4q34-q35	NM_000860
AKR1C3	-5.4	Aldo-keto reductase family 1, member C3	0	10p15-p14	NM_003739
PAPSS2	-6.3	3'-phosphoadenosine 5'-phosphosulfate synthase 2	0	10q23-q24	NM_004670
ALPP	-7.2	Alkaline phosphatase, placental type precursor	1.06E-22	2q37	NM_001632
C5orf4	-7.4	Chromosome 5 open reading frame 4	4.55E-10	5q31-q32	NM_016348
KRT5	-8.6	Cytokeratin 5	1.59E-18	12q12-q13	NM_000424
KRT4	-10.0	Cytokeratin 4	3.07E-13	12q12-q13	NM_002272
Transport					
CHAC1	9.6	Cation transport regulator-like 1	3.78E-42	15q15.1	NM_024111
SLC04A1	9.1	Solute carrier organic anion transporter family, member 4A1	0	20q13.33	NM_016354
SLC7A11	6.9	Solute carrier family 7, member 11	0	4q28-q32	NM_014331
DMBT1	5.6	Deleted in malignant brain tumours 1	1.10E-30	10q25.3/26.1	NM_004406
Calcium ion binding					
NID2	10.4	Nidogen 2	0	14q21-q22	NM_007361
CAPN8	5.4	Calpain 8	8.50E-39	13q26	AW242997
CACNG4	-5.9	Calcium channel, voltage-dependent, gamma subunit 4	3.80E-06	17q24	NM_014405
MYL9	-6.0	Myosin, light polypeptide 9	1.43E-09	20q11.23	NM_006097
MATN2	-6.4	Matrilin 2	0	8q22	NM_002380
LMCD1	-8.1	LIM and cysteine-rich domains 1	6.78E-19	3p26-p24	NM_014583
SLC40A1	-8.5	Solute carrier family 40, member 1	0	2q32	NM_014585
LOC63928	-17.0	Hepatocellular carcinoma antigen gene 520	2.21E-38	16p12.1	NM_022097
Miscellaneous					
TMEM46	35.9	Transmembrane protein 46	0	13q12.13	AW664964
IER3	7.1	Immediate early response 3	0	6p21.3	NM_003897
SYNGR3	-5.3	Synaptogyrin 3	1.55E-25	16p13	NM_004209
FLJ32115	-5.7	Chromosome 12 open reading frame 46	3.15E-32	12p12.3	NM_152321
SNCA	-5.7	Synuclein, alpha	2.43E-19	5q35	BG260394
DKFZP586A0522	-15.5	Methyltransferase like 7A	0	12q13.12	NM_014033
Unclassified					
IGFL1	25.4	Insulin growth factor-like family member 1	0	19q13.32	NM_198541
230778_at	21.2	Unknown	3.43E-19	11p15.3	AA010315
TRBV5-4	13.5	T cell receptor beta variable 5-4	6.09E-25	7q34	AF043179
ARRDC3	12.1	Arrestin domain containing 3	0	5q14.3	NM_020801
MCTP1	8.8	Multiple C2-domains with two transmembrane regions 1	0	5q15	NM_024717
DKFZP564D166	7.8	Tetratricopeptide repeat, ankyrin repeat	6.37E-12	17q23.3	XM_371074
APBB1	6.6	Amyloid beta (A4) precursor protein-binding, B1	3.33E-09	11p15	U62325
LRRC49	6.4	Leucine rich repeat containing 49	1.40E-45	15q23	NM_017691
LOC149194	5.3	Unknown	6.91E-09	1p36.13	AI937119
SCEL	5.2	Sciellin	1.04E-27	13q22	NM_003843
LOC339260	-5.2	Unknown	1.02E-17	17p11.2	BC043529
MGC24665	-5.2	Unknown	2.73E-11	16p13.13	NM_152308
238463_at	-5.3	Unknown	7.20E-11	9q21.33	AA448328
KARCA1	-7.1	Kelch/ankyrin repeat containing cyclin A1 interacting protein	6.53E-07	1q23.3	NM_001007255.1
C1orf21	-7.5	Chromosome 1 open reading frame 21	1.54E-12	1q25	NM_030806
242396_at	-7.7	Unknown	1.68E-11	15q26.2	AA195408
228653_at	-8.1	SAM domain containing 1	2.62E-07	6q24.3	NM_001030060
LBH	-9.0	Likely ortholog of mouse limb-bud and heart gene	5.02E-12	2p23.1	NM_030915
239638_at	-9.0	Unknown	7.71E-16	15q14	AI608696

Table 2. (Continued)

Gene alias	Fold change	Gene name/description	P-value	Chromosome location	Accession no.
235892_at	-10.2	Unknown	2.55E-27	11q23.1	AI620881
APCDD1	-12.4	Adenomatosis polyposis coli down-regulated 1	0	18p11.22	NM_153000
ARD38	-16.0	Ankyrin repeat domain 38	0	1p31.3	NM_181712
RCSD1	-21.8	RCSD domain containing 1	0	1 h2.3	AI659418

This table indicates gene aliases, fold change (decreasing per group) gene name/description, P-value, chromosomal location and genbank accession number.

investigated if we could confirm expression of some of these genes, and especially TNF- α , on a protein level. Using the Luminex platform, we measured 17 known chemokines and cytokines in the supernatant of the HDM stimulated or control condition cells (Table 3). In the samples that were taken after 24 h exposure to HBSS, we could detect secreted protein for IL-6, IL-7, IL-8, IL-13, granulocyte-macrophage colony-stimulating factor (GM-CSF), and IFN- γ . For all these proteins, we could also see a significant mRNA levels in our microarrays. Moreover, the protein concentrations of IL-6, IL-8, GM-CSF, IFN- γ , and IL-13 were statistically significantly higher after HDM stimulation than after culture medium condition. In the supernatants taken after 24 h of HDM stimulation, we could detect significant expression of TNF- α and the other chemokines and cytokines, but not for IL-12.

Discussion

Our results show that airway epithelial cells are able to express a wide variety of genes when exposed to HDM. Furthermore, the expression profile upon HDM provocation cells shows genes that indicate that these cells can interact with cells of the immune system and in this way contribute to the immune response. In our experiments, we identified 813 genes out of 38 500 that were either up- or down-regulated by more than twofold; 116 of these genes even more than fivefold (70 up- and 46 down-regulated). Among these are genes known to be produced by epithelial cells upon allergen stimulation (IL-6, IL-8) (11), and genes known to be produced by epithelial cells in stress or damage situations [IL-1 β , intercellular adhesion molecule (ICAM)-1, TGF- β] (20–22). Additionally, we found genes not previously associated with the response to allergens [folliculin FST, PTX-3, LOXL (lysyl oxidase-like)-2] (23–25), and genes not previously described to be expressed by epithelial cells (IL-13RA2) (26). The major value of this study lies in the extent of the investigated response, which we show here in our experiments to extend far beyond the responses described in similar models, where expression of only a limited number of proteins or genes was possible.

Interestingly, of the genes up-regulated more than 20-fold, almost half (8/17) are involved in cell communication, and 'cell communication' is also the largest

group of the ontology groups we found. When we look at cytokine and chemokine expression profiles in our microarray data there are differences. Of the 42 chemokines that are known, nine are expressed in our microarray experiment, three of which are unaltered [CCL-16 (HCC-4), XCL-1 (lymphotactin) and XCL-2 (SCM-1 β)]. The other expressed chemokines are regulated by the HDM extract [CXCL-1 (+11.9), CXCL-2 (+31.5), CXCL-3 (+37.9), CXCL-8 (+39.3), CCL-20 (+100), CCL-28 (-4.6)]. In contrast to the chemokines, a substantial number of the 53 known cytokine genes are present on the array and reveal a constitutive expression (IL-7, IL-13, IL-15, IL-20, IL-23, IL-1RA, LIF, CD40 L, FasL, 4-1BBL, APRIL, BlyS, MIF). An additional eight show differential regulation [IL6 (+26.8), IL11 (+23.2), LTB (+5.5), TRAIL (-2.9), TGF- β 1 (-2.3), TGF- β 2 (+2.4), IL1- α (+41.4), IL1- β (+5.2)]. The expression of these genes may play a role in the maintenance of the milieu in the mucosa in which the cells of the innate and adaptive immune system function. This indicates that expression of chemokines may be more regulated than continuous process, suggesting that the epithelium may not contribute strongly to constant turnover of inflammatory cells by chemotaxis under normal conditions but contribute to recruitment under inflammatory conditions. The epithelium does constitutively express cytokines, thereby constantly influencing the milieu in which the cells of the immune system function.

When we looked for common regulators of the 813 differentially expressed genes, we found that 49 (6%) of all the differentially expressed genes had been reported to be influenced by TNF- α . In the group of fivefold up- or down-regulated genes this is even more; 16% (18 genes out of 116). In the microarray experiment, we could not detect any significant mRNA expression of TNF- α in culture medium controls, or at 24 h after stimulation with allergen. However, using a more sensitive PCR we could show that TNF- α mRNA was present, and its expression was significantly higher in HDM stimulated cells than controls, at earlier time points, showing the transient nature of this expression. Moreover, we could detect TNF- α protein in the supernatant after HDM stimulation, which corresponds with the transient expression of the TNF- α mRNA.

In the group of proteins linked to TNF- α , we found genes that had been investigated in connection with allergic disease (ICAM-1, ICAM-2, IL-6, IL-8, IL-11,

Figure 2. Interaction network of TNF- α regulated genes. Computational model of interactions as described in literature. The input for our model was a list of differentially expressed genes ($P \leq 0.01$, abs. fold change > 2) with known transcriptional regulation by TNF- α . In this scheme genes are depicted at their cellular location, and are coloured by fold change in expression between saline and HDM exposure (green = down-regulated; red is up-regulated).

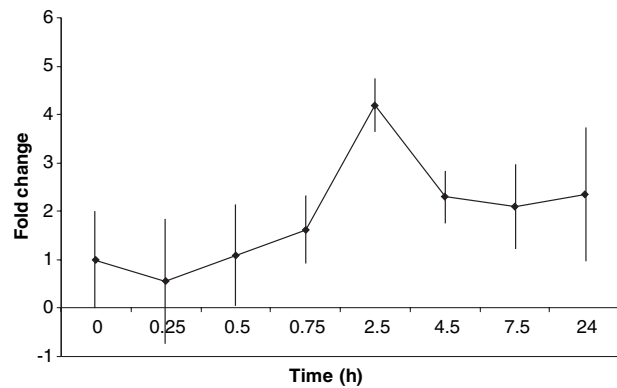


Figure 3. TNF α mRNA expression following HDM exposure. Fold changes calculated compared with control stimulated cells, after normalization for β -2-microglobulin.

Table 3. Protein production of H292 cells in 24 h in which they are exposed to HDM or control condition

mediator	control	HDM
IL2 (8)	n.d.	36.7 ($\pm$ 10.1)
IL4 (2)	n.d.	42.8 ($\pm$ 2.7)
IL6 (8)	302.7 ($\pm$ 16.4)	$\geq$ 1500
IL8 (2)	496.1 ($\pm$ 32.2)	$\geq$ 1500
IL10 (2)	n.d.	5.3 ($\pm$ 1.9)
GM-CSF (2)	8.2 ($\pm$ 14.3)	76.5 ($\pm$ 15.4)
IFN- γ (8)	10.6 ($\pm$ 2.1)	97.9 ($\pm$ 5.0)
TNF- α (2)	n.d.	26.7 ($\pm$ 2.1)
IL1- β (2)	n.d.	6.4 ($\pm$ 0.5)
IL5 (8)	n.d.	8.5 ($\pm$ 1.9)
IL7 (8)	55.5 ($\pm$ 5.2)	39.7 ($\pm$ 4.5)
IL12 (8)	n.d.	n.d.
IL13 (2)	11.7 ($\pm$ 0.0)	19.1 ($\pm$ 6.4)
IL17 (2)	n.d.	57.6 ($\pm$ 2.0)
G-CSF (30)	n.d.	161.4 ($\pm$ 6.2)
MCP-1 (30)	n.d.	37.1 ($\pm$ 2.6)
MIP-1 β (8)	n.d.	22.3 ($\pm$ 2.1)

Cytokine concentrations are presented as average ($\pm$ SD) of triplicate experiment in pg/ml. Lower detection limits are indicated behind respective mediator (pg/ml). n.d., below detection level.

MUC) or with severe asthma-associated aspirin intolerant nasal polyposis (COX-2/PTGS-2). The TNF-superfamily is also represented by TRAIL (TNFAIP3), a protein regulating apoptosis. TNF-superfamily is also indirectly represented by tissue inhibitor of metalloproteinase (TIMP)-3, an inhibitor of the protease responsible for the release of soluble TNF- α from the membrane-bound precursor. The prominent presence of TNF-induced genes in our expression screen suggests this ‘TNF-cluster’ may play a role in the allergen-induced response of epithelium. Indeed, some of the proteins have been reported to be induced by TNF- α , and we have shown that even concentrations that epithelial cells are capable of secreting can induce IL-8 production (see Fig. 1). However, these results cannot exclude that another factor may also be involved in signalling of cells to neighbouring cells or to themselves.

Tumour necrosis factor is normally associated with T_H1 diseases, such as rheumatoid arthritis, and the gene expression that is influenced by TNF may contribute to the pathophysiology of these diseases (27). Our data show that TNF and TNF-induced genes may also be involved in a T_H2-mediated response. Given that there is growing interest in adopting anti-TNF- α in T_H2-mediated diseases, it might be well worth the effort to try and discriminate which of the genes regulated by TNF- α are responsible for the efficacy of the anti-TNF- α treatment (28, 29).

In our experimental setup, we chose to look at the mRNA profile after 24 h of continuous exposure, rather than immediately after initial exposure, because epithelium in patients was exposed to allergens for a prolonged time. A consequence of this approach is that genes that are transiently up-regulated upon PAR activation might not be detected, but it does allow us to detect mediators that are induced by previously secreted mediators (as in the case of TNF- α). Even though our data are derived from a model that uses a squamous cell carcinoma cell line in an *in vitro* experiment, we were able to find some similarities to the data that Lilly et al. (30) presented in their study where they compared the expression profile of cells obtained from bronchial brushes from asthmatics before and after allergen challenge. As in their study, we also find genes involved in the immune function and cell growth, and we find a number of genes previously associated with allergy, but not before being linked to epithelial cells.

The epithelial response is not specific for a single type of allergen or epitope, as was the case with allergen-mediated cross-linking of specific IgE antibodies on the surface of mast cells causing degranulation and histamine release. In our model, the proteolytic activity of the allergen is the cause of response and other allergenic mixtures could potentially induce a similar response. In both *in vitro* and *in vivo* experiments, involvement of airway epithelial cells in the response to allergens has been established. As a result of the methods used, the magnitude and the variety of signalling molecules that the airway epithelium can express has previously not been shown to be so vast. The role of these mediators can lie in maintaining the physical integrity of the mucosal tissue, in attracting cells that lead to an increased immune response, or to alter the activity of those cells. It may also lie in deviating the response away from immune activation, for instance by attracting phagocytosing cells. More likely, epithelial cells are capable of influencing all these processes, some to a greater extent than others, and further research is needed to find out how the epithelium modifies the allergic response. Our research provided us with a large number of leads through which the epithelium may alter or influence the tissue response to allergens. In this respect, it will be of importance to see how, and if this mapped response to allergen may differ between allergic or healthy individuals.

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