

Primary Nasal Epithelium Exposed to House Dust Mite Extract Shows Activated Expression in Allergic Individuals

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Nasal epithelial cells form the outermost protective layer against environmental factors. However, this defense is not just physical; it has been shown that epithelial cells respond by the production of inflammatory mediators that may affect local immune responses. In this research we set out to characterize potential differences between the responses of nasal epithelium from healthy and allergic individuals to house dust mite (HDM) allergen. These differences will help us to define local mechanisms that could contribute to allergic disease expression. Epithelial cells were cultured from nasal biopsies taken from five healthy and five allergic individuals. These cultures were exposed for 24 hours to culture medium containing HDM allergen, or to culture medium alone. Isolated RNA was used for microarray analysis. Gene-ontology of the response in healthy epithelium revealed mainly up-regulation of chemokines, growth factors, and structural proteins. Moreover, we saw increased expression of two transcription factors (NF- κ B and AP-1) and their regulatory members. The expression pattern of epithelium from allergic individuals in the absence of the HDM stimulus suggests that it is already in an activated state. Most striking is that, while the already activated NF- κ B regulatory pathway remained unchanged in allergic epithelium, the AP-1 pathway is down-regulated upon exposure to HDM allergen; this is contrary to what we see in healthy epithelium. Clear differences in the expression pattern exist between epithelial cells isolated from healthy and allergic individuals at baseline and between their responses to allergen exposure; these differences may contribute to the inflammatory response.

Keywords: allergy; epithelial response; innate immunity; local tissue response

The mucosal layer in the nose is constantly exposed to viruses, bacteria, parasites, and harmless allergens. It is crucial that a correct immune response is initiated to all these environmental factors. When harmless allergens are mistaken for dangerous pathogens the immune system will mount an unwanted inflammatory response to the allergens, resulting in allergic inflammation. An important player in the initiation of immune responses is the antigen-presenting cell that resides in the mucosal tissue; the dendritic cell (DC). In recent years it has become increasingly clear that the peripheral DC initiates the immune response within an active local tissue environment, and that epithelial cells can play a role in this initiation process. Epithelial cells are more than a physical barrier and are themselves able to detect and respond

CLINICAL RELEVANCE

Response of epithelial cells to allergen has not before been studied on a genome expression level. Characterization of the responses will give new insight to the pathophysiology, and open roads to new treatments.

to environmental signals. Epithelial cells can produce mediators that affect recruitment of immunocompetent cells to the local tissue and help create a micro-environment in which these cells function (1, 2).

In relation to the initiation of the immune response, it is very interesting that epithelial cells produce mediators that can influence DCs. An example of such a mediator is CCL20/MIP3 α , the chemokine for CCR6-positive Langerhans cells (LCs), which is produced by bronchial epithelial cells after a variety of stimuli (3, 4). Not only recruitment is affected by epithelial mediators, but epithelial expressed granulocyte macrophage-colony-stimulating factor and transforming growth factor- β guide the differentiation of myeloid DCs and LCs, respectively, from their precursors (5, 6). Activation of tissue resident LCs is partly dependent on locally produced TNF- α and IL-1 (7, 8), and recently thymic stromal lymphopoietin produced by epithelial cells was shown to be important in the activation of DC-mediated allergic inflammation (9–11). Currently, there are just a few players for which the effect on DC function has been documented. Even less is known about potential differences in mediators produced by epithelial cells from healthy or allergic individuals, or whether any of these differences contribute to the expression or development of allergic disease.

In previous experiments we have investigated the epithelial response of a bronchial epithelial cell line, H292, to house dust mite (HDM) allergen. There we could show that epithelial cells display a broad and diverse expression of genes in response to exposure to allergen, and that a substantial number of these regulated genes have a function in cell communication based on their gene ontology classification. Moreover, we identified a potential regulatory network centered around TNF- α and NF- κ B (12).

In this research we wanted to expand on these observations and investigate the response induced by HDM allergen in primary epithelium from healthy individuals and from individuals allergic to HDM. To this end, cultures of primary epithelial cells obtained from nasal biopsies were exposed to HDM extract diluted in saline, or to saline solution alone. RNA from this experiment was used for microarray analysis, and the resulting expression profiles were subjected to bioinformatical, biostatistical, and interaction network analysis. Characterization of potential differences in the expression pattern at baseline or in response to allergen exposure will provide valuable insight into the role of the epithelium in the allergic response. Identification of the mechanism by which nasal epithelium influences the allergic response can lead to development of new therapies that target the epithelial cells.

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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 GSE9150.

MATERIALS AND METHODS

Patient Characteristics

This study was reviewed and approved by the medical ethical committee of the Amsterdam Medical Center, and all participants read and signed an informed consent. Five allergic volunteers (aged 19–55 years) and five healthy nonsmoking volunteers (aged 21–32 years) were selected based on skin prick test for HDM and other common allergens, and on a nasal allergen provocation to assess their response. Only monotypically HDM-allergic and nonallergic volunteers were included. Allergic individuals had refrained from using any medication for their allergy in the 4 weeks before the visit when biopsies were taken. Biopsies were taken from the lower edge of the inferior turbinate, 1 and 2 cm from the anterior end, using Fokkens' forceps with a cup diameter of 2.5 mm. Local anesthesia was achieved by application of adrenalin and cocaine under the inferior turbinate without touching the biopsy site, for a period of 10 minutes.

Primary Epithelial Cell Culture

Primary cells were obtained by digesting nasal biopsies of volunteers with 0.5 mg/ml collagenase 4 (Worthington Biochemical Corp., Lakewood, NJ) for 1 hour in Hanks' balanced salt solution (HBSS; Sigma-Aldrich, Zwijndrecht, The Netherlands). Subsequently cells were washed with HBSS and resuspended in bovine epithelial growth medium (Lonza Clonetics, Breda, The Netherlands) and seeded in two wells of a 6-well plate. Culture medium was replaced every other day. Cells were grown in fully humidified air containing 5% CO₂ at 37°C.

HDM Extract and Exposure Experiment

HDM extract containing biologically nonrelevant trace amounts of LPS was kindly provided by Prof. Dr. M. L. Kapsenberg (AMC, The Netherlands) as a lyophilized powder. It was dissolved in PBS, then dialyzed against PBS and diluted to a stock concentration of 8 µg/µl. Cells were cultured for 2 weeks to 80% confluence and were subsequently pre-incubated with HBSS for 8 hours before exposure to HDM. Pre-incubation medium was removed and cells were then exposed to HBSS containing a previously (12) determined optimal concentration of house dust mite extract (2 µg/ml) or with HBSS alone (control condition) for 24 hours. Supernatants were removed and stored for further analysis; cells were used for RNA extraction.

RNA Extraction

Total RNA from each sample was extracted using Trizol (Life Technologies, Inc., Gaithersburg, MD) 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) and RNA quality was checked on the Agilent 2100 bio-analyzer (Agilent Technologies, Palo Alto, CA).

Microarray Affymetrix u133 Plus 2

Human Genome U133 Plus 2.0 Genechip Array (Affymetrix inc., Santa Clara, CA) 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 (MAD) of the University of Amsterdam (Amsterdam, The Netherlands), a fully licensed microarray technology center for Affymetrix Genechip platforms and official Dutch Affymetrix Service Provider. In short, biotin-labeled cRNA samples were prepared as described in the Affymetrix expression analysis technical manual (Affymetrix) using 3 µg 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 3,000 (Affymetrix) and analyzed with Affymetrix GeneChip Operating Software (Affymetrix).

Microarray Data Analysis and Statistics

The quality of the images was checked by visual inspection, and all raw data passed the quality criteria for average background, scale factors, percentage present calls, 3'/5' ratios GAPDH, 3'/5' ratios β-actin, hybridization spike-in controls, and poly-A spike-in controls. The data also passed a set of quality control checks provided by the affy, affyPLM, and affyQCReport packages from Bioconductor (<http://www.bioconductor.org/>). Expression values were calculated using the robust multi-array average (RMA) algorithm (13), and statistically analyzed for differential gene expression using ANOVA (MAANOVA package, version 0.98.8 [14]). The permutation based Fs test was used for hypothesis testing (15), and all *P* values were adjusted for false discovery rate correction (16). To quantify the effect of HDM extract on gene expression, pairwise statistical tests were performed to analyze: (1) the effect of HDM on epithelial cells from healthy individuals separately, (2) the effect of HDM on epithelial cells from allergic individuals separately, and (3) the difference in response to HDM in allergic compared with healthy individuals.

Ontology, Cluster, and Network Analysis

Gene ontology was done using the online gene ontology program Gostat (<http://gostat.wehi.edu.au/>), with which we used curated datasets and subsets to investigate the overrepresentation of geneontology groups (17). Cluster analysis on all significantly affected genes was done by transforming the means of the expression values for a gene in the four groups (healthy or allergic after control exposure or after HDM exposure) to Z-scores and using unsupervised K-means clustering based on correlation; for this we used Spotfire DecisionSite Functional Genomics. Network analysis was performed using Pathway Architect software (Stratagene, La Jolla, CA); here we used the curated dataset to build a regulation interaction network. We overlaid the colors used in our K-means cluster analysis to clarify the relative expression of the genes in our network.

Quantitative PCR

Quantitative PCR was used to validate the differential expression of selected genes. Isolated RNA from control-treated and HDM-treated cells was used to synthesize cDNA using the MBI Fermentas first-strand cDNA synthesis kit (Fermentas GmbH, St. Leon-Rot, Germany). PCR was performed on Bio-Rad iCycler (Bio-Rad, Veenendaal, The Netherlands). mRNA-specific TaqMan gene expression assays for ACTB, ATF3, CCL20, CNFN, EGR1, FLG, GAPDH, GATA3, GCSF, GROA, IL1RA, IL12B, IL13-Ra2, IL8, KRT4, MCP1, MIP1B, PLAUR, SPINK7, TNFA, TNFR1, and TNF-IP3 were ordered from Applied Biosystems (Nieuwerkerk a/d IJssel, The Netherlands). We performed all PCR assays three times, on all samples. Fold changes were calculated using the $\delta\delta Ct$ method.

RESULTS

Validation of the Microarray Results

After incubation with HDM extract, we identified 555 probe sets that were statistically differentially expressed in primary epithelium from healthy individuals and 301 probe sets that were statistically differentially expressed in primary epithelium from allergic patients. This collection of probe sets was first curated by, when possible, discarding the less specific x_at probe sets and the splice variant specific s_at probe sets. For analysis we further selected only those genes for which the expression level of their probe sets change by more than 1.5-fold (*see* Table E1 in the online supplement). In healthy epithelium, the original 555 probe sets correspond to 209 uniquely annotated genes and 19 unannotated and/or hypothetical sequences in our curated dataset. Of these genes, 206 were up-regulated and 22 were down-regulated. In allergic epithelium the original 301 probe sets correspond to 87 uniquely annotated genes and 12 unannotated and/or hypothetical sequences in our curated dataset. Here 62 genes showed increased and 37 decreased expression.

The results of this microarray experiment were validated by independent real-time PCR on the same starting material used for the microarray analysis. A selection of 20 genes that had revealed an increased, decreased, or unchanged expression level in either the healthy or allergic curated gene set was used for this validation experiment (see MATERIALS AND METHODS). After normalization for three household genes (GAPDH, β -actin, and β_2 -microglobulin), the relative change in expression of these genes measured by PCR was directly comparable to the relative change obtained from our microarray experiment, both for the healthy epithelium ($r = 0.621$, $P = 0.006$) and for the allergic epithelium ($r = 0.735$, $P < 0.001$) (data are shown in Table E2).

Global Analysis Reveals an Activated State in Primary Allergic Epithelium

Within our curated dataset, the expression of only a few genes (9/311) shows identical statistically significant changes upon HDM exposure in healthy and allergic epithelium. The expression of the other genes either change in healthy or in allergic epithelium, and in many cases shows a differential response in both groups (Figure 1). The absence of an overlap in the response could be due to a differential change in the expression profile by the HDM extract, to intrinsic differences between healthy and allergic epithelium at baseline, or a combination of both. To investigate the relative contribution of these factors, we first used principal components analysis (PCA) on the whole data collection of microarray chips. Most of the variation observed for the chips (57%, Figure 2) lies in the difference between healthy and allergic epithelium (PCA1). What was unexpected was that the next largest contribution (12%) comes from the variability of the individuals within the healthy epithelium group (PCA2), with only a very limited variation in the allergic epithelium group. Exposure to HDM extract only has the third largest contribution (7%) to the observed variation between all the genes in the healthy and allergic groups.

The PCA showed that not only differences in response to allergen, but also differences at baseline between healthy and allergic epithelium contribute to variation; therefore, we used K-means clustering on the expression levels in the four groups. This allows us to compare groups of genes that share specific expression patterns. The 311 genes that change their expression in healthy and/or allergic epithelium can be effectively separated into 12 clusters (Figure 3). Baseline expression of a given gene in 5 out of 12 clusters is substantially higher in allergic epithelium than in healthy epithelium. Together these clusters (numbers 7, 9, 10, 11, and 12) represent 74% of all regulated genes. The K-mean cluster analysis shows that for some of these

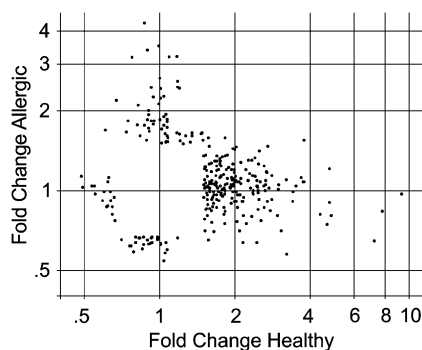


Figure 1. Gene expressions in different groups. This scatter plot shows the fold changes for the genes in our curated dataset as calculated in the healthy control group (x axis), and in the allergic group (y axis).

clusters expression after HDM exposure is high and remains largely unchanged in the allergic group, and is up-regulated to a certain degree in the healthy group. For cluster 9 (8 genes) the expression level in healthy epithelium after HDM exposure is even higher than in the allergic group, in cluster 11 (46 genes) the expression level is similar, while in cluster 12 (129 genes) the expression level in the healthy group does not quite reach the expression level of the allergic group. Evidently, a substantial group of genes in the epithelium from allergic individuals already displays an activated state at baseline. Interestingly, some clusters (numbers 3, 4, 7, and 10) show changes in the allergic group, whereas the expression in the healthy group remains unchanged, while two genes (cluster 5) even show an opposite response to allergen exposure.

The Activated State in Allergic Epithelium Is Reflected by Only a Few Ontology Classes

With only a limited number of expression profiles describing the effect of HDM exposure in healthy and allergic epithelium, we wondered whether these patterns could be linked to specific functions of these genes.

Ontology analysis for genes that change their expression due to exposure to HDM extract in the primary epithelium from healthy individuals shows that these genes are principally involved in cell-to-cell contact. *Cell communication* (GO:7154) is by far the biggest group (66 genes) that is significantly over-represented in the curated healthy data set ($P = 2.4 \times 10^{-5}$). Related ontology groups are also overrepresented: *signal transduction* (GO:7165, $P = 4.6 \times 10^{-4}$), *receptor binding* (GO:5102, $P = 2.8 \times 10^{-5}$), *extra-cellular space* (GO:5615, $P = 8.9 \times 10^{-5}$), and *transcription factor activity* (GO:3700, $P = 2.9 \times 10^{-6}$). These changes seem to be a consequence of a general response of healthy epithelium to environmental factors as the ontology group *response to external stimulus* (GO:9605, $P = 4.4 \times 10^{-7}$) and the related groups *response to wounding* (GO:9611, $P = 5.8 \times 10^{-6}$), *response to abiotic factors* (GO:9628, $P = 1 \times 10^{-4}$), and *response to stress* (GO:6950, $P = 2.4 \times 10^{-5}$) are all significantly over-represented in healthy gene set. Other processes that are affected are *cell proliferation* (GO:8283, $P = 3.3 \times 10^{-9}$), (*negative regulation*) of *cell death* (GO:43069 $P = 3.5 \times 10^{-4}$ and GO:8219 $P = 1.9 \times 10^{-4}$), and *intracellular junctions* (GO:5911, $P = 3 \times 10^{-4}$). A similar picture emerges when we combine the K-mean cluster analysis with the gene ontology class analysis. As clusters 9, 11, and 12 represent 58% of all genes that behave differently between healthy and allergic epithelium with expression in healthy epithelium going up by HDM exposure and expression remaining high in allergic epithelium, the activated status of allergic epithelium is best described by genes involved in *cell communication* and *cell proliferation*.

Although HDM extract also affects the expression pattern in the primary epithelium of allergic individuals, these genes do not seem to represent a specific gene ontology class. The only class that is significantly overrepresented is *protease inhibitor activity* (GO:0030414, $P = 8.9 \times 10^{-3}$), but of this group only a few genes are statistically altered in allergic epithelium (SPINK5, SPINK6, SPINK7, SERPINB3, C3, and WFDC5).

Involvement of the NF- κ B and AP-1 Transcription Factor Complexes in the Differential Gene Expression of Healthy and Allergic Epithelium

Now we found *cell communication* and *cell proliferation* activated in epithelial cells of allergic individuals, we wanted to find regulators that could be responsible for this activated state. To find these regulators, we used *regulation network analysis*. In the network (Figure 4) we saw the expected players involved in cell

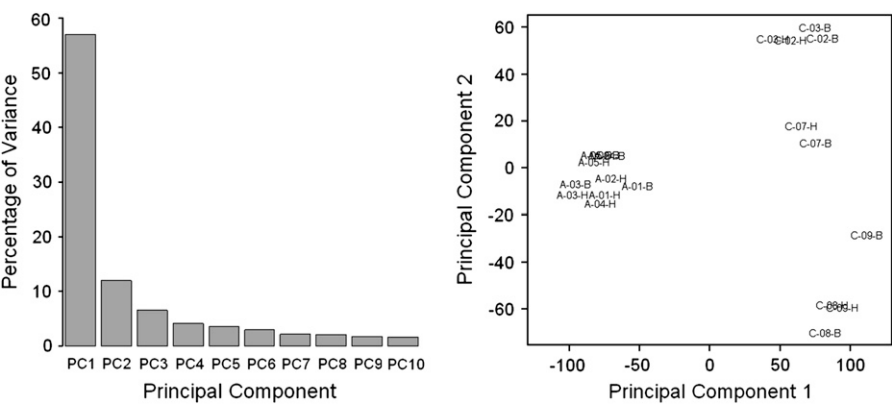


Figure 2. Principal components analysis. (Left panel) The relative contribution to variance for the first 10 principal components. (Right panel) A scatter plot of all 20 arrays and their individual relative contribution to principal component 1 (PC1) and principal component 2 (PC2). Array IDs are indicated in the graph.

communication: inflammatory markers (IL-1 α , IL-1 β , and TNF- α), chemokines (CXCL8/IL-8, CXCL1/GRO- α , CXCL2/GRO- β , and CXCL10/IP-10), growth factors (EREG, AREG, and HBEGF), and receptors (TLR3, PLAUR, PTGER4, and IL-7R). More interestingly, this regulation seems to be mediated by proteins from the transcription factor complexes NF- κ B (NFKB1, NFKB2, NFKBIA, and NFKBIZ), and AP-1 (FOS, JUN, JUNB, FOSL1, and ATF3). When we use a color code to identify the different gene clusters from Figure 3 in the *regulation network analysis* we see that these transcription factor complexes also share a similar expression pattern. The activated state we identified above is also reflected at the transcription factor level, with the NF- κ B and the noncanonical AP-1 transcription factors (JUNB and FOSL1) mapping to cluster 11. Interestingly, the canonical AP-1 transcription factors (FOS and JUN) behave differently. FOS and ATF3 are expressed at similar levels at baseline in healthy and allergic epithelium (cluster 8), and expression goes up in healthy epithelium, but goes down in allergic epithelium (cluster 9), but

again expression in allergic epithelium is down-regulated and is up-regulated in healthy epithelium upon allergen exposure.

DISCUSSION

The first important observation coming from our data is that for a selective group of genes the allergic epithelium already is in an activated state. Baseline expression levels for these genes are higher in allergic individuals than corresponding levels in healthy individuals. Moreover, for these genes, allergen exposure in healthy individuals leads to an increase in their expression levels, while the levels in allergic individuals remain largely unchanged. In our analysis of which processes are affected by the allergic status, we find the genes belonging to the ontology class *cell communication* and *proliferation*. In *cell communication* we find chemokines (IL-8, GRO- α , GRO- β , and IP-10) and cytokines (IL1- α , IL1 β , IL1 ϵ , and TNF- α) which have a known and well-documented functions in the recruitment and activation of cells of the immune system. In *cell communication* we also find genes for

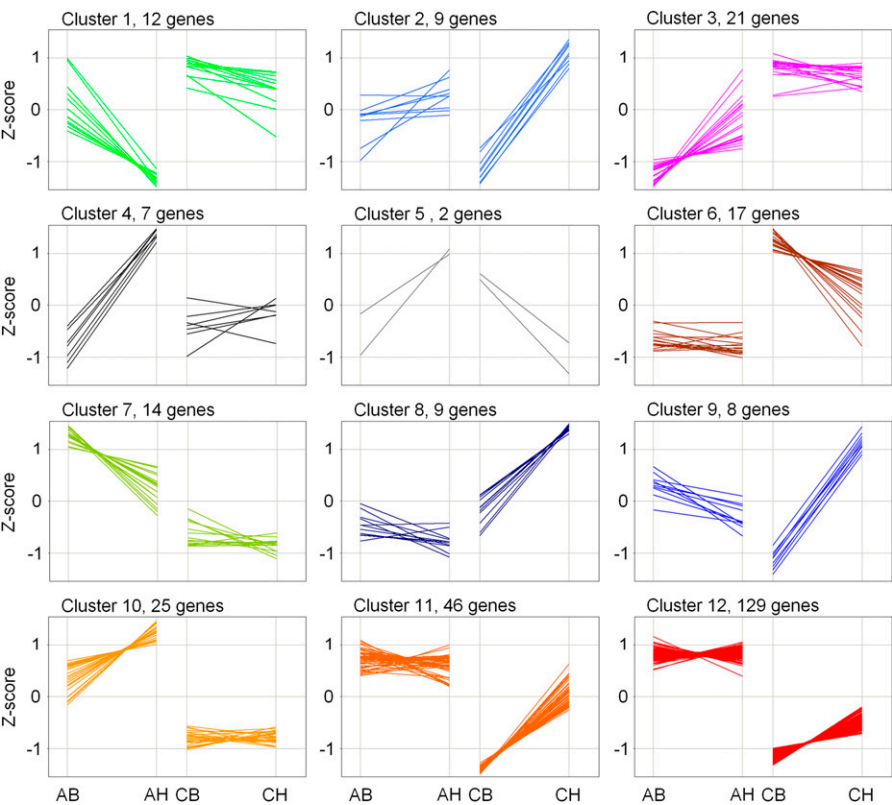


Figure 3. K-means clusters. Here all genes from curated dataset (see RESULTS) were transformed to Z-scores, and were organized in 12 clusters using K-means clustering. Table E1 gives all 311 genes and their respective cluster number. AB, allergic at baseline; AH, allergic after HDM exposure; CB, healthy control at baseline; CH, healthy control after HDM exposure.

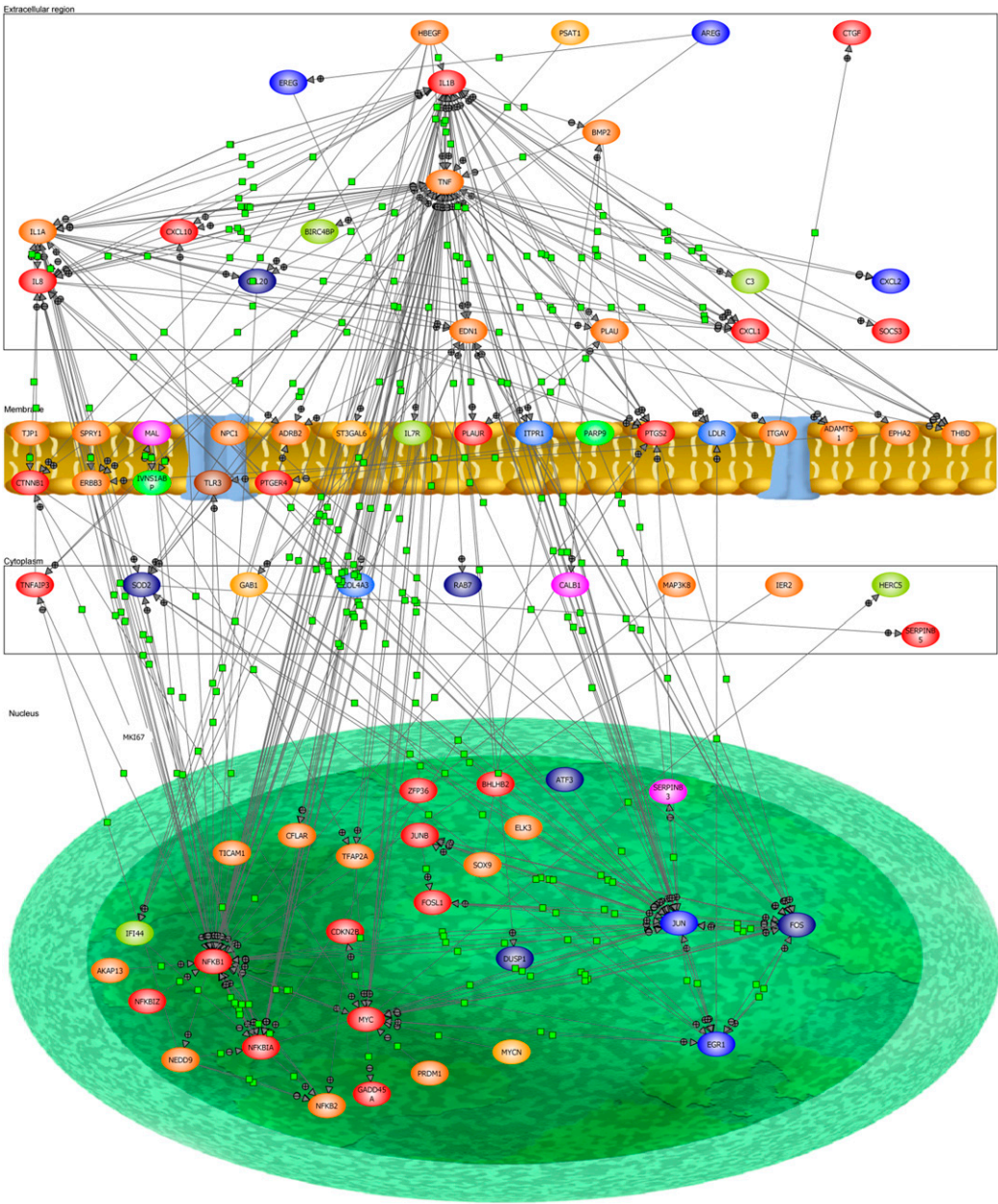


Figure 4. Regulation interaction network. Here genes within our dataset that are known to have a regulatory effect on each other are presented in their cellular location, with *arrows* indicating the interactions. Genes are colored by the K-means cluster to which they were assigned.

growth factors (CTGF, HBEGF, AREG, EREG, and FGF5) and intercellular junction (TJP-1, -2, CLDN-1, -4, and OCLN). The latter groups are known to be involved in the repair of damaged epithelium that, in this case, could be the consequence of cleavage of intracellular junction proteins by the protease activity contained within the HDM allergen extract. Where a substantial number of the regulated genes in epithelium from healthy individuals can be categorized in well-defined gene ontology classes, this does not hold true for the regulated genes in allergic epithelium. Only a single class of *protease inhibitors* is significantly enriched. Most likely this reflects a protective mechanism in allergic epithelium to counteract the activity of the protease activities contained within the allergen extract or those released by tissue resident mast cells (tryptase, chymase). Although relatively little attention has been given to the role of proteases in health and disease, it is important to note that mutations in one of the protease inhibitors SPINK5, as well as the protease ADAM33, have been associated to asthma (18, 19). Other proteases like the

matrix metalloproteinases (MMP) have a crucial role in the local tissue remodeling associated with asthma (20, 21). In this research we set out to characterize the response of epithelial cells; to do so we chose to culture cells taken from a biopsy for 2 weeks, after which the cell cultures contain only epithelial cells, thus eliminating any contamination of gene expression by other cell types. One of our concerns was that culturing the cells would alter the baseline expression, or the ability to respond, in a way that would prevent us from detecting differences between healthy and allergic individuals. Given that we detect so many well-established mediators that have been confirmed both *in vitro* and *in vivo* gives us confidence that the differences in the expression profiles are likely to reflect to situation *in situ*. Another deliberate choice in our model was to stimulate the cells for 24 hours and then investigate the mRNA profile. We wanted to mimic the continuous exposure to which the nasal epithelium is subject *in vivo*. As a consequence we were able to determine the relevant expression patterns of the NF- κ B and AP-1

transcription factor families. However, our data do not reveal how this state is achieved after allergen exposure. For this purpose, earlier time points after induction should be investigated, and this could well have consequence for the overall outcome of our experiment, with other genes and/or regulatory pathways playing a more prominent role.

There is little overlap in the response to HDM extract in primary epithelium from healthy and allergic individuals. As described above, a substantial group of genes that are up-regulated in healthy individuals are already expressed at high levels in allergic epithelium even without exposure to allergen. To expand on this observation it would seem that the induced expression of these “activated” genes is part of the normal response in healthy primary epithelium. How is this activated status in allergic epithelium induced or maintained? Both healthy and allergic individuals are constantly exposed to HDM allergen in every day life. Epithelial cells of healthy individuals do express PAR2 (protease activated receptor 2), though at lower levels than in allergic individuals, so *in vivo* activation of epithelial cells by the proteases contained within HDM extract before isolation could occur in both (22). However, *in vitro* experiments with the epithelial cell line H292 have shown that the effect of PAR2 activation normally is transient, with expression levels of the responding genes returning to baseline levels 72 hours after the initial exposure to HDM extract. Therefore it does not seem likely that the activated state we see at baseline can be directly maintained during the 2 weeks of culturing that precedes our HDM induction experiment (not without a positive feedback loop).

In previous work we have suggested that TNF- α , induced by HDM exposure, could be a central player both in mediating the HDM effect and in restimulating epithelial cells. No matter whether we assume a direct effect of HDM or an indirect effect through an HDM-induced feedback loop, the outcome is different for healthy or allergic epithelium. This would lead us to conclude that the allergic status could be aggravated in allergic individuals because of an inability to shut this response down. This would explain the high level of correspondence between genes activated in healthy epithelium and those already activated in allergic epithelium. Alternatively, the “activated” status could be not related to the *in vivo* action of HDM on epithelium directly, but to the indirect effect that allergen-induced mediators from other tissue resident cells like mast cells have on the epithelium. However, it is not entirely clear how this would explain the correspondence between the HDM-induced genes in our *in vitro* experiment and the “activated” state in allergic epithelium.

When we assume a differential regulation of the HDM response in epithelium of healthy and allergic individuals, this seems also reflected in the response of two important transcription factor complexes. The transcription factor NF- κ B is a family of (hetero)dimeric proteins that has been linked to inflammation in many cell types, and is known to regulate (and to be regulated by) many different mediators (23, 24). That we find genes belonging to the NF- κ B family up-regulated in response to allergen in epithelium from healthy individuals is perhaps not surprising, but given the complex interactions between the family members it is hard to firmly establish NF- κ B as the responsible factor for the observed induction of genes (25). The NF- κ B proteins NFKB1/p105 and NFKB2/p100 are the cytoplasmic precursors of, respectively, the nuclear factors p50 and p52 (26, 27). Whereas homodimers of p50 or p52 are linked to transcriptional repression, the formation of heterodimers with the NF- κ B Rel-family members is linked to transcriptional activation (28, 29). In our microarray experiments RELB is expressed at a considerably higher level in allergic compared with healthy

epithelium and the expression levels are unaffected by allergen exposure. Interestingly, it has been described that knockout mice for NFKB1 show a reduced stress response with lower IL-6 and COX-2/PTGS2 levels, suggesting that at least in the overall outcome NFKB1 is required for a correct response to environmental signals (30). Given that the expression of these genes remains high when allergic epithelial cells are cultured for 2 weeks in the absence of HDM suggests that NF- κ B in allergic individuals could be (partly) responsible for maintaining the “activated” state. Although these observations could well explain the “activated” state in allergic epithelium or the induction of genes in healthy epithelium, it fails to account for how this “activated” state is maintained or why this does not occur in epithelium from healthy individuals. Further experiments should investigate whether epigenetic modification of key regulators differs between healthy and allergic epithelium, or the presence of an activating auto-feedback loop in allergic epithelium could be responsible for the maintenance of the allergic phenotype in tissue culture. Also, one might wonder whether the observed effect can be observed for other allergens and whether allergen-specific PAMP receptors could be involved in the response of epithelial cells to allergens.

What is interesting is that epithelial cells also show differential expression for some of the AP-1 family members. In these family members it is strange that the noncanonical members FOSL1 and JUND show the same pattern as NF- κ B, whereas the FOS, and JUN, the genes for c-FOS and c-JUN protein, do show an increase in healthy individuals, but are actually down-regulated in allergic individuals. If this expression could be linked to a protective mechanism against the effects of NF- κ B activation, then this could explain some of the differences we see between healthy and allergic individuals. It has been described that in *Drosophila* AP-1 is required to down-regulate NF- κ B target genes by interfering with promoter binding (31). If this mechanism also applies in humans, then the up-regulation of FOS/JUN in healthy epithelium may contribute to the down-regulation of NF- κ B-regulated genes, and the absence of this response in allergic epithelium may contribute to the maintenance of the activated state seen in allergic epithelium. Interestingly, the FOS-related AP-1 family member ATF3 displays an expression profile similar to that of the FOS gene itself. Identified as a stress factor, this bZIP protein in its homodimeric form acts as transcriptional repressor, but when it heterodimerizes with members of the JUN family it acts as a transcriptional activator (32).

Airway epithelium is becoming more widely accepted as an active player in the response to allergens; however, it was unknown if the response to allergen exposure would differ between allergic and healthy individuals. We have shown that not only the response differs, but also that the expression at baseline is different between healthy and allergic individuals. Linking our transcriptional observations to functional consequences is hard. Foremost, it is not clear how differences in the transcription level translate into effects on the protein level. This is further compounded by the formation of different homo- and hetero-dimers that each can have their distinct effects, by the functional interaction of the NF- κ B and AP-1 transcription factors at promoter sites, and even by the formation of hetero-dimers between NF- κ B and AP-1 family members. Despite all these considerations, the allergic epithelium seems an important target in the treatment, and possibly prevention, of allergic disease. Reducing the “activated” state in allergic epithelium may have direct consequence for the expression of allergic complaints, for instance by reducing the influx of effector cells, and understanding how and why the response in allergic epithelium differs from that in healthy epithelium may help in preventing the initiation of the allergic response.

Conflict of Interest Statement: A.B.V. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. M.J.J. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. S.L. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. T.M.B. is head of an academic microarray core facility. He also acts as service provider for for-profit parties, and is involved in two government-funded projects that have about 25% matching funding from various companies such as Organon, Eli Lilly, GSM, Biodetection Systems, and Notox. W.J.F. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. C.M.v.D. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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