

ORIGINAL ARTICLE

Timothy grass pollen extract-induced gene expression and signalling pathways in airway epithelial cells

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Summary

Background Grass pollen allergy is one of the most common allergies worldwide and airborne allergens are the major cause of allergic rhinitis. Airway epithelial cells (AECs) are the first to encounter and respond to aeroallergens and are therefore interesting targets for the development of new therapeutics. Our understanding of the epithelial contribution to immune responses is limited as most studies focus on only a few individual genes or proteins.

Objective To describe in detail the Timothy grass pollen extract (GPE)-induced gene expression in AECs.

Methods NCI-H292 cells were exposed to GPE for 24 h, and isolated RNA and cell culture supernatants were used for microarray analysis and multiplex ELISA, respectively.

Results Eleven thousand and seven hundred fifty-eight transcripts were affected after exposure to GPE, with 141 genes up-regulated and 121 genes down-regulated by more than threefold. The gene ontology group *cell communication* was among the most prominent categories. Network analysis revealed that a substantial part of regulated genes are related to the cytokines IL-6, IL-8, IL-1A, and the transcription factor FOS. After analysing significantly regulated signalling pathways, we found, among others, epidermal growth factor receptor 1, IL-1, Notch-, and Wnt-related signalling members. Unexpectedly, we found Jagged to be down-regulated and an increased release of IL-12, in line with a more Th1-biased response induced by GPE.

Conclusion and Clinical Relevance Our data show that the stimulation of AECs with GPE results in the induction of a broad response on RNA and protein level by which they are able to affect the initiation and regulation of local immune responses. Detailed understanding of GPE-induced genes and signalling pathways will allow us to better define the pathogenesis of the allergic response and to identify new targets for treatment.

Keywords airway epithelial cells, allergen, cytokines, grass pollen, microarray

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Introduction

Grass pollen allergy is one of the most common allergies worldwide and airborne allergens are the major cause of allergic rhinitis, affecting approximately 20% of the population of western European countries [1]. The acute allergic reaction within type I hypersensitivity is mediated by allergen-induced cross-linking of specific IgE molecules bound to the surface of mast cells in sensitized individuals, resulting in mast cell degranulation and the release of inflammatory mediators, causing bronchoconstriction and inflammation. Mast cell-derived IL-4

induces a shift of the T cell response towards a Th2 phenotype and isotype class switch of B cells towards the production of IgE. Besides these well-known players in allergic reactions and antigen recognition, abundant evidence also points to a pro-inflammatory role for structural cells in airway inflammation, including endothelial cells [2], airway smooth muscle cells [3], fibroblasts [4], and epithelial cells [5].

Airway and especially nasal epithelial cells are the first to encounter aeroallergens and are therefore interesting targets for the development of new therapeutical

approaches to treat pollen allergy. They are no longer considered to be only passive structural cells affected in allergy and asthma, but also to be effector cells, actively contributing to airway inflammation by responding to environmental factors and producing mediators [6, 7]. Airway epithelial cells (AEC) generate, either constitutively or following activation, a wide variety of mediators, including growth factors and cytokines [5]. Cytokines and chemokines released by AEC upon allergen exposure may amplify ongoing inflammatory processes via the recruitment and activation of specific subsets of immune competent cells, as well as by creating a micro-environment in which these cells may function.

As most of the current studies on the interaction of aeroallergens and AEC are limited by only considering a few individual genes or proteins in relation to allergy [8, 9], we aimed to build a detailed description of grass pollen extract (GPE)-induced gene expression of AEC. For this purpose, NCI-H292 cells were stimulated with an extract from Timothy grass pollen (*Phleum pratense*) for 24 h and allergen-induced gene expression, signalling pathways, and mediator release were analysed using genomic and proteomic approaches.

Methods

Cell culture

NCI-H292 cells, a mucoepidermoid carcinoma cell line derived from human lungs (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 penicillin, 100 µg/mL streptomycin, and 10% (v/v) fetal bovine serum (HyClone, Logan, UT, USA). Cells were grown in fully humidified air containing 5% CO₂ at 37 °C and were subcultured weekly. NCI-H292 cells were cultured in six-well plates to 80% confluence. Before stimulation, the culture medium was removed and the cells were preincubated with Hanks' balanced salt solution (HBSS) for 24 h. Cells were stimulated with GPE (250 µg/mL) diluted in HBSS or with HBSS alone (control). Twenty-four hours after stimulation, supernatants were collected and used for further analysis. Cells were used for RNA isolation.

Preparation of grass pollen extract

Timothy GPE was purified from pollen (Artu Biologicals, Lelystad, the Netherlands) as described by Suck et al. [10]. Briefly, 10 g of timothy grass pollen was incubated for 30 min in Tris/HCl (20 mM, pH 8.0) at 37 °C and under an agitation of 225 rpm, centrifuged at 4 °C and 12 000 g, and filtrated (pore size 0.45 µm). The extract was lyophilized and stored at -20 °C.

Ribonucleic acid isolation

Total RNA from each sample was extracted using Trizol (Life Technologies Inc., Gaithersburg, MD, USA) according to the manufacturer's instruction, followed by purification by nucleospin RNA II (Machery-Nagel, Düren, Germany). The concentration of RNA was measured using nanodrop ND-1000 (NanoDrop Technologies Inc., Wilmington, DE, USA). RNA quality was determined using an Agilent 2100 bio-analyzer (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) was used to analyse GPE-induced gene expression. Technical handling of microarray experiments was performed at the MicroArray Department at the University of Amsterdam (Amsterdam, the Netherlands), a fully licensed microarray technology centre for Affymetrix Genechip[®] platforms and official Dutch Affymetrix Service Provider. Briefly, biotin-labelled cRNA samples were prepared as described in the Affymetrix expression analysis technical manual (Affymetrix Inc.) using 3 µg of purified total RNA as a template per reaction. For the reaction, the One-Cycle cDNA Synthesis Kit (Affymetrix Inc.) was used. The Array images were acquired using a GeneChip Scanner 3000 (Affymetrix Inc.) and analysed using Rosetta Resolver (Rosetta Biosoftware, Seattle, WA, USA).

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 [11]. The normalized data were analysed using R (version 2.7.0) and the Bioconductor Limma package [12]. Experiments were performed in triplicate and simultaneously. Statistical analysis for differential gene expression was performed using ANOVA (MAANOVA package, version 0.98.8). The permutation-based F_s-test was used for hypothesis testing [13], and all *P*-values were adjusted for false discovery rate correction [14]. For gene ontology (GO) and network interaction analysis, GeneSpring GX11 (Agilent Technologies, Amstelveen, the Netherlands) was used. Correlations between fold changes (FC) within the microarray data and the real-time PCR data were determined using Pearson's correlation.

Real-time polymerase chain reaction

Quantitative real-time PCR was performed with the LightCycler 2.0 System (Roche Diagnostics, Mannheim, Germany) using LightCycler[®] Fast Start DNA Master^{PLUS} according to the manufacturer's instructions. The following primers were used: CCL20; sense: GCT-GCT-TTG-ATG-TCA-GTG-CT, antisense: TCA-AAG-TTG-CTT-GCT-GCT-TC, IL-8; sense: AGA-CAG-CAG-AGC-ACA-CAA-GC, antisense: ATG-GTT-CCT-TCC-GGT-GGT, PTX3; sense: GCG-GTG-CTA-GAG-GAG-CTG, antisense: GGA-ATA-AAA-TAG-CTG-TTT-CAC-AAC-CT, IL13RA2; sense: AGC-AGT-CAC-CCT-TGA-ATT-GT, antisense: AGA-GAA-AGT-AGA-TTC-GTG-GTT-GGT, SERPINB4; sense: CAA-AGG-CAA-AGA-TCT-AAG-CAT-GA, antisense: CAA-TTT-CTC-AGC-AGT-GAG-TTT-CTC, TNPAIP6; sense: GGC-CAT-CTC-GCA-ACT-TAC-A, antisense: GCA-GCA-CAG-ACA-TGA-AAT-CC, AKR1C3; sense: CAT-TGG-GGT-GTC-AAA-CTT-CA, antisense: CCG-GTT-GAA-ATA-CGG-ATG-AC, JAG2; sense: TGG-GAC-TGG-GAC-AAC-GAT-AC, antisense: ATG-CGA-CAC-TCG-CTC-GAT, COL8A1; sense: TCT-CAA-GAA-CAA-AAG-ACA-ACT-GAA-A, antisense: CTG-GTT-TCT-GTC-TCT-TCA-GCA-G, VTCN1; sense: CCT-GGG-GCA-GAT-CCT-CTT, antisense: AAA-GCC-AAT-GAT-GAG-TGC-AA, LRP8; sense: CTC-CCG-AAG-AAC-CCT-CTT-TC, antisense: GCT-TAA-TGC-CAC-TCG-CTT-G, PTGS2; sense: CTT-CAC-GCA-TCA-GTT-TTT-CAA-G, antisense: TCA-CCG-TAA-ATA-TGA-TTT-AAG-TCC-AC, CDH1; sense: CCC-GGG-ACA-ACG-TTT-ATT-AC, antisense: GCT-GGC-TCA-AGT-CAA-AGT-CC, CXCL2; sense: CAT-CGA-AAA-GAT-GCT-GAA-AAA-TG, antisense: TTC-AGG-AAC-AGC-CAC-CAA-TA, CXCR4; sense: AGG-ATA-TAA-TGA-AGT-CAC-TAT-GGG-AAA, antisense: AAG-GGC-ACA-AGA-GAA-TTA-ATG-TAG-A, TNFRSF21; sense: GCA-CAT-GGA-AAC-CCA-TGA-A, antisense: AGA-AGA-GTT-GGA-TTC-TGT-TGA-GTT-C. The relative cDNA amount was calculated compared with the expression of the house-keeping genes GAPDH and HPRT.

Protein multiplex Luminex Bio-Plex assay

Cell-free supernatants of GPE- and control-treated cells were stored at -20°C until analysis. Cytokine levels in the supernatants of the cells were determined using a Bio-Plex Human Cytokine 30-Plex Panel Kit in combination with a Bio-Plex workstation (Bio-Rad, Veenendaal, the Netherlands). All standards were diluted in HBSS. Concentrations were calculated from a dilution series of standards using the Luminex software. FC were calculated compared with the detected release in control-stimulated cells. If the measured mediator concentration in the control was below the detection level, the FC was calculated on the basis of the detection limit for the respective mediator.

Results

Grass pollen extract-induced changes in gene expression level in airway epithelial cells

NCI-H292 cells have been stimulated for 24 h with GPE diluted in HBSS or HBSS only as a control. Cell culture supernatants were collected to evaluate mediator release and cells were used for RNA isolation. The overall gene expression ratio between HBSS- and GPE-stimulated cells from a triplicate experiment was determined using Rosetta resolver software. Expression was considered to be significantly differentially expressed with $P \leq 0.01$ after one-way Bonferroni's multiple test correction.

A strong response was observed after the stimulation of NCI-H292 cells with GPE. In total, 11 758 probe sets showed a significant up- or down-regulation of gene expression, representing about 20% of all probe sets. When this response was limited to those that were up- or down-regulated by more than threefold, we identified 221 probe sets as up-regulated and 191 probe sets as down-regulated. As some genes are represented by more than one probe set, this corresponds to 141 up-regulated and 121 down-regulated genes.

Validation of microarray data

To validate the microarray data, we first determined the influence of GPE stimulation on some housekeeping genes. Analysis of the expression of these genes revealed that their expression is not affected by stimulation with GPE. For example, the average expression ratio between the control and the stimulated condition for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was 0.93 (± 0.00) and hypoxanthine-phosphoribosyl-transferase (HPRT) displayed a FC of 0.98 (± 0.04). To confirm the expression levels calculated on the basis of the microarray data, we randomly chose 16 genes from the 262 genes that showed an up- or down-regulation by more than threefold for confirmatory real-time PCR. Table 1 shows the ratios calculated from the microarray data and the real-time PCR-derived expression. The real-time PCR data strongly resemble the ratios calculated on the basis of the microarray data (Fig. 1). Statistical analysis revealed a high level of correspondence ($R = 0.82$, $P \leq 0.001$), pointing towards a correlation of 67% ($R^2 = 0.67$) between the microarray data and the real-time PCR. Only the GPE-induced expression of PTX3 and IL13RA2 could not be validated by real-time PCR as the expression level of these genes was below the detection limit of the PCR.

Gene ontology

After positive validation of the microarray data, we sorted the genes that were up- or down-regulated more than

threefold after GPE stimulation into functional classes. Using their ascribed GO, genes were assorted by their molecular function or the biological process they are involved in (Table 2). Of the genes that have been assigned to a GO cluster, we found 19 genes to be in the group *immunity and defense*, 14 genes in the group *receptor activity*, 6 genes in *enzyme inhibitor activity*, 17 genes in *transport*, 32 genes in *nucleic acid binding*, and 10 genes in *binding*. *Metabolism* and *cell communication* appeared

Table 1. Confirmatory real-time PCR of 16 selected genes

Gene	Microarray ratio	Real-time PCR (fold change)
CCL 20	16.4 (± 0.2)	75.5 (± 9.3)
IL-8	3.6 (± 0.1)	2.6 (± 0.7)
PTX3	13.3 (± 0.9)	ND
IL-13RA2	22.7 (± 1.9)	ND
SERPINF4	15.5 (± 0.4)	11.3 (± 2.9)
TNFAIP6	17.4 (± 0.9)	76 (± 5.7)
AKR1C3	9.2 (± 0.03)	15.2 (± 0.9)
JAG2	-3.1 (± 0.02)	-3.1 (± 0.3)
COL8A1	4.5 (± 0.5)	4.0 (± 0.3)
VTN1	-3.7 (± 0.2)	-14.3 (± 1.4)
LRP8	5.7 (± 0.1)	2.3 (± 1.4)
PTGS2	3.4 (± 0.1)	3.9 (± 0.4)
CDH1	-3.6 (± 0.02)	-5.1 (± 2.3)
CXCL2	3.0 (± 0.05)	3.7 (± 1.5)
CXCR4	7.1 (± 0.3)	8.8 (± 3.9)
TNFRSF21	7.4 (± 0.1)	9.5 (± 0.9)

All values are given as mean ($\pm$ SD). Real-time PCR values are stated as fold change between control and GPE-stimulated cells and normalized to GAPDH and HPRT.

ND, not detectable; GPE, grass pollen extract; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HPRT, hypoxanthine-phosphoribosyl-transferase.

to be the most prominent ontology groups, with 47 and 33 genes, respectively. The group *cell communication*, representing cytokines, chemokines, and growth factors, became even more prominent when the cut-off was increased to fivefold up- or down-regulated, with 13 identified genes (30.2%) out of 43 regulated genes.

Network analysis

To identify the interactions between genes we found to be regulated, we performed a network analysis. After analysing genes with a statistically significant change in the expression level by more than threefold, 80 genes were identified to interact directly. Interestingly, a number of hubs emerged, showing that a substantial part of the responding genes are related to the cytokines IL-6, IL-8, IL-1A, and the transcription factor FOS (Fig. 2).

There is growing awareness of the role of allergen-induced signalling pathways and the induction of auto-crine loops contributing to the initiation and maintenance of allergic responses. We first investigated significantly induced signalling pathways within genes that changed more than threefold. Corresponding to the IL-1 hub observed in the computational model of direct gene-gene interactions, the IL-1-signalling cascade was among the significantly regulated pathways ($P = 0.03$). In addition, the Wnt signalling cascade ($P = 0.03$) was identified. Next, we screened all significantly GPE-regulated transcripts independent of their FC for the presence of specific signalling pathways. As shown in Table 3, the following additional pathways were significantly regulated: epidermal growth factor receptor 1 (EGFR1), IL-3, IL-6, IL-7, Notch, Wnt, TGF- β -receptor (TGFB β), TNF- α , and nuclear factor κ -light-chain-enhancer of activated B cells (NF κ B).

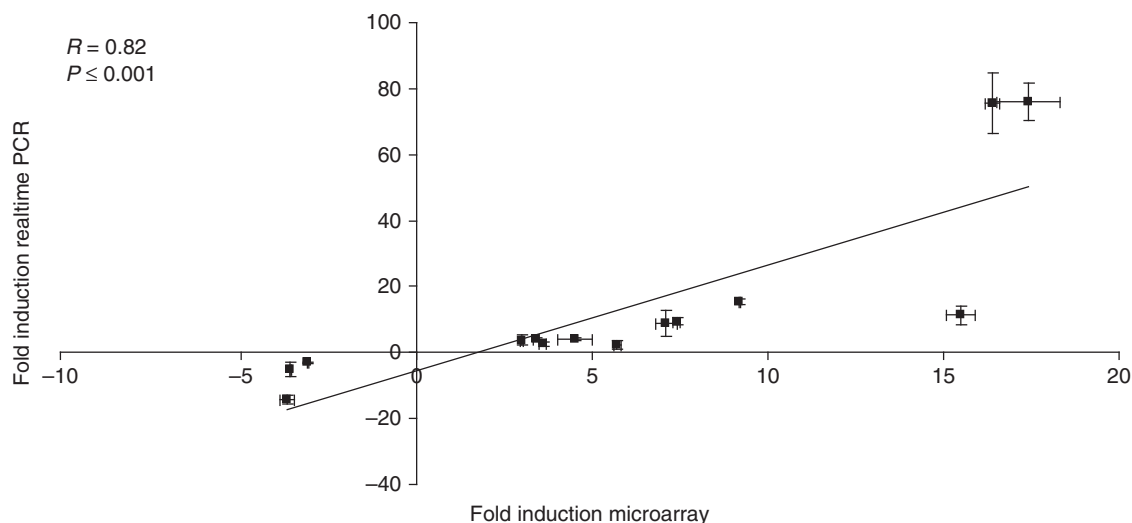


Fig. 1. Correlation plot of real-time PCR data and microarray results. Correlations between observed fold changes within the microarray data and the real-time PCR data were determined using Pearson's correlation.

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

Gene alias	Fold change	Gene name/description	P-value	Gene ID
<i>Cell communication</i>				
HMOX1	24.2	Haeme oxygenase (decycling) 1	5.25E-09	203665_at
CCL20	16.4	Chemokine (C-C motif) ligand 20	3.59E-10	205476_at
EREG	11.5	Epiregulin	1.00E-08	205767_at
FZD10	9.8	Frizzled homologue 10 (<i>Drosophila</i>)	6.34E-08	219764_at
IL6	8.7	IL-6	2.53E-08	205207_at
IL1A	8.3	IL-1. α	5.04E-09	210118_s_at
SHC4	8.2	SHC (Src homology 2 domain containing) family. member 4	6.96E-08	235238_at
CXCL3	4.9	Chemokine (C-X-C motif) ligand 3	2.03E-08	207850_at
BCL2A1	4.8	BCL2-related protein A1	2.02E-07	205681_at
COL8A1	4.5	Collagen type VIII α 1	9.89E-06	226237_at
IL24	4.5	IL-24	7.71E-07	206569_at
AREG	4.4	Amphiregulin (Schwannoma-derived growth factor)	3.14E-08	205239_at
RASGRP1	4.2	RAS guanyl releasing protein 1	7.30E-07	205590_at
MUCL1	4.2	Mucin-like 1	9.35E-07	1553602_at
ARTN	4.1	artemin	3.51E-08	210237_at
FLRT3	3.6	Fibronectin leucine-rich transmembrane protein 3	3.75E-06	222853_at
IL1RL1	3.6	IL-1 receptor-like 1	4.24E-06	242809_at
IL8	3.6	IL-8	1.34E-07	202859_x_at
NAMPT	3.4	nicotinamide phosphoribosyltransferase	2.07E-07	243296_at
CXCL2	3.0	Chemokine (C-X-C motif) ligand 2	2.70E-07	209774_x_at
TNFSF9	-3.0	TNF (ligand) superfamily. member 9	1.18E-07	206907_at
JAG2	-3.1	Jagged 2	3.38E-08	32137_at
ANXA9	-3.6	Annexin A9	5.01E-08	211712_s_at
ASB9	-3.5	Ankyrin repeat and SOCS box-containing 9	4.20E-08	205673_s_at
ARHGDI1	-4.1	Rho GDP dissociation inhibitor (GDI) β	1.86E-07	201288_at
FOS	-5.0	v-fos FBJ murine osteosarcoma viral Oncogene homologue	1.99E-08	209189_at
PTH1L	-5.7	Parathyroid hormone-like hormone	1.14E-07	210355_at
WISP2	-6.1	WNT1 inducible signalling pathway protein 2	3.45E-09	205792_at
<i>Immunity and defense</i>				
TNFAIP6	17.4	TNF- α -induced protein 6	2.02E-08	206026_s_at
PTX3	13.3	Pentraxin-related gene. rapidly induced by IL-1 β	2.95E-08	206157_at
GAL	5.6	Galanin prepropeptide	1.88E-08	214240_at
IRAK2	4.0	IL-1 receptor-associated kinase 2	6.73E-07	231779_at
TFPI	3.9	Tissue factor pathway inhibitor	1.81E-07	210665_at
F12	3.5	Coagulation factor XII (Hageman factor)	5.42E-08	205774_at
TNFAIP3	3.3	TNF- α -induced protein 3	2.59E-08	202643_s_at
OAS1	3.1	2',5'-oligoadenylate synthetase 1. 40/46kDa	1.82E-06	205552_s_at
PXDN	-3.1	Peroxidasin homologue (<i>Drosophila</i>)	2.71E-07	212012_at
HSPB1	-3.2	Heat shock 27kDa protein 1	2.96E-08	201841_s_at
VTG1	-3.7	V-set domain containing T cell activation inhibitor 1	1.39E-06	219768_at
HSPB8	-4.2	Heat shock 22kDa protein 8	2.04E-08	221667_s_at
RNASE7	-5.5	Ribonuclease RNase A family 7	1.63E-07	234700_s_at
<i>Receptor activity</i>				
IL13RA2	22.7	IL-13 receptor. α 2	2.02E-08	206172_at
TNFRSF21	7.4	TNF receptor superfamily. member 21	1.46E-08	218856_at
CXCR4	7.1	Chemokine (C-X-C motif) receptor 4	8.40E-09	211919_s_at
KISS1R	3.7	KISS1 receptor	4.59E-07	242517_at
BDKRB2	-3.3	Bradykinin receptor B2	2.33E-08	205870_at
OLFML2A	-5.1	Olfactomedin-like 2A	1.18E-08	213075_at
<i>Enzyme inhibitor activity</i>				
SERPINF4	15.5	Serpin peptidase inhibitor. clade B (ovalbumin). member 4	4.18E-10	211906_s_at
SERPINF3	5.5	Serpin peptidase inhibitor. clade B (ovalbumin). member 3	9.73E-09	209719_x_at
SERPINF2	3.3	Serpin peptidase inhibitor. clade E (nexin. plasminogen activator inhibitor type 1). member 2	1.10E-07	212190_at
SERPINF1	-3.8	Serpin peptidase inhibitor. clade F	2.64E-08	202283_at

Table 2. continued

Gene alias	Fold change	Gene name/description	P-value	Gene ID
<i>Metabolism</i>				
AKR1C2	35.6	Aldo-keto reductase family 1. member C2	4.18E-10	217626_at
CYP24A1	27.1	Cytochrome P450. family 24. subfamily A. polypeptide 1	4.18E-10	206504_at
DHRS2	24.6	Dehydrogenase/reductase (SDR family) member 2	1.74E-09	214079_at
AKR1C1	18.0	Aldo-keto reductase family 1. member C1	9.06E-11	216594_x_at
AKR1C3	9.2	Aldo-keto reductase family 1. member C3	1.08E-09	209160_at
UGT1A3	7.4	UDP glucuronosyltransferase 1 family. polypeptide A3	4.82E-08	208596_s_at
ALDH3	7.1	Aldehyde dehydrogenase 3 family. member A1	1.52E-07	205623_at
CYP1B1	6.4	Cytochrome P450. family 1. subfamily B. polypeptide 1	3.16E-08	202436_s_at
LRP8	5.7	Low-density lipoprotein receptor-related protein 8. apolipoprotein e receptor	1.00E-08	205282_at
UGDH	5.5	UDP-glucose dehydrogenase	1.07E-07	203343_at
CYP4F3	4.8	Cytochrome P450. family 4. subfamily F. polypeptide 3	1.57E-07	206515_at
GCLM	4.3	Glutamate-cysteine ligase. modifier subunit	2.33E-07	236140_at
NQO1	3.9	NAD(P)H dehydrogenase. quinone 1	5.24E-08	201467_s_at
PLA2G4A	3.8	Phospholipase A2. group IVA (cytosolic. calcium-dependent)	1.20E-06	210145_at
AKR1B1	3.7	Aldo-keto reductase family 1. member B1 (aldose reductase)	2.97E-08	201272_at
PMAIP1	3.6	Phorbol-12-myristate-13-acetate-induced protein 1	2.26E-08	204286_s_at
G0S2	3.5	G0/G1switch 2	1.88E-05	213524_s_at
PTGS2	3.4	Prostaglandin-endoperoxide synthase 2	2.70E-06	1554997_a_at
GCLC	3.0	Glutamate-cysteine ligase. catalytic subunit	6.44E-08	202922_at
PAK6	-3.2	p21(CDKN1A)-activated kinase 6	5.52E-08	219461_at
CDH1	-3.6	Cadherin 1. type 1. E-cadherin (epithelial)	1.14E-08	201131_s_at
BACE2	-3.6	β -site APP-cleaving enzyme 2	1.36E-08	222446_s_at
SLC2A3	-3.7	Solute carrier family 2 (facilitated glucose transporter). member 3	2.80E-08	222088_s_at
EGLN3	-7.5	EGL nine homologue 3 (<i>Caenorhabditis elegans</i>)	6.33E-08	219232_s_at
KRT13	-6.9	Keratin 13	1.89E-08	207935_s_at
KRT15	-7.3	Keratin 15	2.19E-07	204734_at
KRT4	-8.1	Keratin 4	1.36E-08	213240_s_at
GPNUMB	-10.8	Glycoprotein (transmembrane)	1.07E-07	201141_at
CA9	-17.3	Carbonic anhydrase IX	2.61E-10	205199_at
<i>Transport</i>				
SLC7A11	5.4	Solute carrier family 7. (cationic amino acid transporter. y+ system) member 11	1.40E-07	217678_at
STEAP1	4.2	Six transmembrane epithelial antigen of the prostate 1	8.43E-08	205542_at
SCG5	4.2	Secretogranin V (7B2 protein)	2.48E-06	203889_at
SLC47A1	3.8	Solute carrier family 47. member 1	1.21E-06	219525_at
ATP6V0A4	-4.3	ATPase. H ⁺ transporting. lysosomal V0 subunit a4	4.02E-07	220197_at
CHP2	-4.5	Calcineurin B homologous protein 2	1.49E-07	206149_at

Table indicates gene aliases, fold change, gene name, P-value, and gene ID.

As the induction of Notch and Wnt signalling was rather unexpected, we investigated the GPE-induced gene expression within these pathways. As shown in supporting information Table S1, stimulation with GPE-induced altered gene expression levels in 38 genes out of 74 genes that are involved in Notch signalling. For Wnt signalling, we identified 46 genes out of 105 to be regulated after GPE stimulation (supporting information, Table S2).

Grass pollen extract-induced protein expression

As the category *cell communication* was found to be one of the most dominant groups of GPE-regulated genes, we continued to investigate the induction of some of these genes on the translational level and further explore GPE-induced

mediator release. Measurement of the release of several cytokines and chemokines revealed a strong GPE-induced release of IL1-receptor antagonist (RA), IL-6, and IL-8 (Table 4), which was also reflected in the up-regulation of the respective genes. IL-12 also showed a clear enhanced release upon stimulation, with an increase of detectable IL-12p40/p70 by more than threefold. Interestingly, this was not reflected in the GPE-induced expression level of the IL-12 gene, with IL12A (p35) being slightly up-regulated (1.3-fold) and IL12B (p40) remaining unaltered. For the macrophage inflammatory protein-1 β (MIP-1 β), we observed a moderate increase in release (2.2-fold), which corresponds to moderate up-regulation of the corresponding gene (1.4-fold). Although the monocyte chemotactic protein-1 (MCP-1) showed an increased release of 4.4-fold, the corresponding gene showed

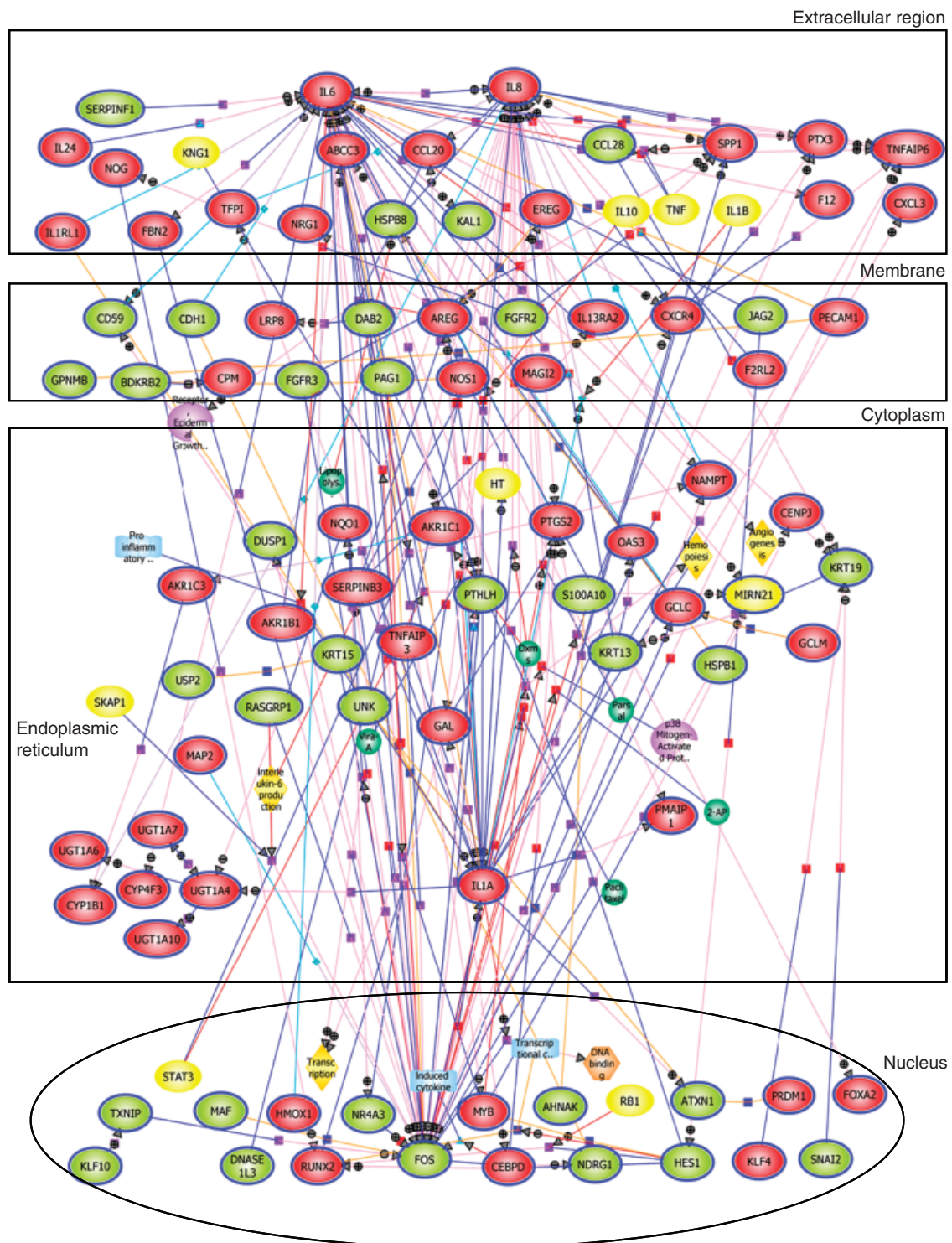


Fig. 2. Direct interaction network after the stimulation of NCI-H292 cells with grass pollen extract (GPE). Input has been all genes with significant up- or down-regulation by more than threefold ($P \leq 0.01$, fold change ≥ 3.0). Genes are sorted by their cellular location and are coloured by their expression, with green for down-regulation, red for up-regulation, and yellow for not regulated within our data set.

no altered expression upon stimulation. Albeit at lower levels, the growth factors vascular endothelial growth factor (VEGF) and EGF, as well as IP-10 (CXCL10) displayed an increase in released mediator concentration. The correspond-

ing genes exhibited no GPE-induced altered expression, however, with VEGF even being slightly down-regulated. Granulocyte-macrophage colony-stimulating factor (GM-CSF), IFN- α , and IL-2-receptor (IL-2R) showed a moderate

increase in mediator levels, with gene expression levels remaining unaltered upon stimulation. Monokine induced by IFN- γ (MIG) displayed a low basal expression, which remained unaltered after GPE stimulation. IL-1 β and IL-7 could not be detected in the supernatant of GPE-stimulated NCI-H292 cells, although the gene expression levels showed a clear up-regulation with a FC of 1.8 and 2.4. A similar picture emerged for TNF- α and IL-17, which were also

undetectable within the supernatant of GPE-stimulated AEC, albeit the gene expression levels were only slightly up-regulated. The following mediators were undetectable within the supernatant of stimulated cells and showed, although expressed, no altered gene expression level: IL-2, IL-4, IL-5, IL-10, IL-13, IL-15, IFN- γ , eotaxin, FGF- β , and HGF.

Discussion

Different grass extracts have been shown to induce the release of IL-6, IL-8, and MCP-1 from AEC [15]. Our data show that stimulation with Timothy GPE results in a broad response by airway epithelium, which extends far beyond the commonly reported.

In total, we identified 262 genes that were regulated by more than threefold up or down after GPE stimulation. After analysing these genes for their link to common GO clusters, the category *cell communication* was identified to be among the most prominent functional classes. CCL20, IL-1RA, IL-1A, IL-6, and IL-8 were among the most highly up-regulated genes, which, for IL-1RA, IL-6, and IL-8, also resulted in markedly enhanced protein release. CCL20 is the unique ligand for CCR6 expressed on immature dendritic cells (DCs), suggesting a possible mechanism by which GPE-stimulated AEC may facilitate the recruitment

Table 3. Pathways significantly regulated by grass pollen extract (GPE)

Pathway	Involved genes*	Genes regulated by GPE	P-value
EGFR1	178	97	2.33E-06
IL-1	24	16	1.38E-03
IL-3	71	33	2.32E-02
IL-6	52	32	1.14E-04
IL-7	16	10	2.11E-02
Notch	74	38	1.82E-03
TGFBR	137	72	3.25E-06
TNF- α /NF- κ B	193	94	4.77E-06
Wnt	105	46	5.00E-03

*Genes matched with Agilent Technologies.

EGFR1, epidermal growth factor receptor 1; TGFBR, TGF- β -receptor; NF- κ B, nuclear factor κ -light-chain-enhancer of activated B cells.

Table 4. Mediator release from NCI-H292 cells after stimulation with grass pollen extract (GPE) for 24 h

Mediator	HBSS (ctrl)	GPE	FC ELISA	FC microarray
IL1-RA (14.2)	129.7 ($\pm$ 24.)	1271 ($\pm$ 91)	9.8 ($\pm$ 3.3)	2.8 [†]
IL1- β (5.8)	ND	ND	–	1.8 [†]
IL-2R (10.7)	27.7 ($\pm$ 3.1)	45 ($\pm$ 8.2)	1.6 ($\pm$ 0.7)	1.0 [#]
IL-6 (3.2)	39 ($\pm$ 10)	2117 ($\pm$ 425)	54.4 ($\pm$ 14.3)	8.7 [†]
IL-7 (17.9)	ND	ND	–	2.4 [†]
IL-8 (2.3)	180 ($\pm$ 81)	6415 ($\pm$ 1171)	35.6 ($\pm$ 6.8)	3.6 [†]
IL-12 (4.8)	16.2 ($\pm$ 2.4)	52 ($\pm$ 5.7)	3.2 ($\pm$ 1.2)	1.3 [†]
IL-17 (11)	ND	ND	–	1.5 [†]
TNF- α (2.6)	ND	ND	–	1.3 [†]
IFN- α (10.6)	11.2 ($\pm$ 3.6)	22.1 ($\pm$ 5.4)	2 ($\pm$ 0.2)	1.0 [#]
MCP-1 (3.9)	204 ($\pm$ 57)	906 ($\pm$ 59)	4.4 ($\pm$ 1.1)	1.0 [#]
MIP-1 α (3.9)	13 ($\pm$ 5.11)	12.8 ($\pm$ 2.9)	1 ($\pm$ 0.4)	1.2 [#]
MIP-1 β (5.4)	ND	11.9 ($\pm$ 0.3)	2.2 ($\pm$ 0.1)	1.4 [†]
RANTES (3.8)	ND	12.2 ($\pm$ 1.5)	3.2 ($\pm$ 0.4)	1.0 [#]
GM-CSF (9.4)	18.8 ($\pm$ 0)	33.6 ($\pm$ 4.1)	1.8 ($\pm$ 0.2)	1.0 [#]
G-CSF (6.2)	22.2 ($\pm$ 9.4)	33 ($\pm$ 7.9)	1.5 ($\pm$ 1)	1.1 [#]
VEGF (4.6)	ND	19.9 ($\pm$ 1.5)	4.3 ($\pm$ 0.3)	–1.3 [†]
EGF (5.4)	ND	14.3 ($\pm$ 1.3)	2.7 ($\pm$ 0.2)	1.0 [#]
IP-10 (1.1)	ND	3.9 ($\pm$ 0.3)	3.6 ($\pm$ 0.3)	1.1 [#]
MIG (1.3)	8.0 ($\pm$ 0.3)	8.8 ($\pm$ 1.4)	1.1 ($\pm$ 0.2)	1.1 [#]

After stimulation, cell-free supernatants were collected and analysed for the presence of chemokines and cytokines. Stated values represent the average ($\pm$ SD) of a triplicate experiment and are given in pg/mL. Fold change (FC) was calculated in relation to the level of control-stimulated cells.

[†] $P \leq 0.05$.

[#]not significant.

MCP, monocyte chemoattractant protein-1; MIP, macrophage inflammatory protein; RANTES, regulated on activation normal T cell expressed and secreted; GM-CSF, granulocyte-macrophage colony-stimulating factor; VEGF, vascular endothelial growth factor; EGF, epidermal growth factor; MIG, monokine induced by IFN- γ ; ND = not detectable (below detection limit).

of DCs to the airways [16]. IL-1RA binds to the IL-1 receptor and blocks signalling [17]. Interestingly, high levels of IL-1RA expression are associated with a reduction of the inflammatory response, while the moderate release of IL-1RA has been shown to promote inflammation [18]. Furthermore, a polymorphism within the IL-1RA gene is associated with asthma and single nucleotide polymorphisms within the IL-1A gene are associated with atopy and the development of nasal polyps [19, 20], confirming the important role of these mediators and their signalling pathway within allergy. In our model, we used enhanced gene expression and release of IL-6 and IL-8 as internal controls for a successful stimulation of AEC with GPE, but these mediators also contribute to the immune response of AEC upon allergen stimulation. IL-8 and IL-6 are produced by the airway epithelium in response to various stimuli, such as bacteria or allergens [21, 22]. Vroiling et al. [23, 24] were able to show that NCI-H292 cells, as well as primary nasal epithelial cells from healthy individuals displayed an increased IL-8 release after stimulation with HDM extract when compared with HDM-allergic donors, indicating the response of NCI-H292 cells to allergen stimulation resembling more the response of healthy AEC. Interestingly, also birch pollen allergens induce a defence response in healthy subjects [25]. Therefore, our data extend the observation that AEC show an increased immune response upon allergen encounter for GPE as an aeroallergen source. The expression of these genes and the resulting increase in mediator release might contribute to the inflammatory response or to the maintenance of a balance between inflammatory and anti-inflammatory mediators by creating a local microenvironment allowing immune competent cells to function.

To depict interactions specific for GPE exposure, we performed a network analysis to identify direct interactions between GPE-induced genes. IL-6, IL-8, and IL-1A were shown to hold central positions within this model, which is likely due to the enormous amount of data available on these genes. Interestingly, all of them are under the transcriptional control of the transcription factor FOS, which is down-regulated after GPE stimulation and also holds a central position within the model. Fos is part of the transcription factor activator protein-1 (AP-1), which is composed of members of the Fos and Jun family of DNA-binding proteins. Besides the well-documented roles of Fos and Jun in oncogenesis, AP-1 proteins also function as regulators of cytokine expression and modulators in inflammatory diseases such as arthritis or psoriasis. Interestingly, after stimulation with HDM extract, NCI-H292 cells, as well as primary nasal epithelial cells from healthy donors showed an increased FOS gene expression [23], indicating different mechanisms of regulation and induction of signalling pathways in comparison with GPE stimulation.

In contrast to HDM-stimulated AECs, the Th1-cytokine IL-12 showed a significant increase in release (3.2-fold) after stimulation with GPE. IL-12p70 is predominantly produced by professional antigen-presenting cells (APC) and shifts the T cell response towards a Th1-phenotype. In addition to the Th1-polarizing capacity of IL-12, it has also been shown to exacerbate Helminth-mediated corneal pathology in mice by augmenting the recruitment of inflammatory cells [26]. IL-12p35 mRNA has been shown to be expressed in human nasal epithelial cells [27] and IL-12p40 mRNA was identified to be up-regulated after the application of HDM allergens in the epidermis of atopic dermatitis patients [28], but to our knowledge, the release of functional IL-12p70 from AEC has not been reported so far. Furthermore, none of the classical Th2-cytokines, such as IL-4, IL-5, or IL-13, showed an altered gene expression profile or increased release upon GPE stimulation, indicating a potential Th1-inducing capacity of GPE.

Increasing attention is being paid to the role of signalling pathways in association with allergic responses. Among the pathways that were likely to be induced after the stimulation of AEC with GPE like IL-6 signalling, the induction of Notch and Wnt signalling was rather unexpected. We found 38 of 74 genes that are involved in Notch signalling to be significantly regulated by GPE (supporting information, Table S1). Beyond its developmental role, Notch signalling also seems to be crucial in providing mechanisms by which APCs can influence T cell proliferation. Studies *in vitro* have suggested that both Delta and Jagged ligand families may be associated with T cell differentiation, but that Delta ligands promote Th1, whereas Jagged ligands promote Th2 polarization [29]. We found JAG2, encoding for the Notch ligand Jagged2, to be down-regulated by more than threefold after stimulation with GPE. It is tempting to speculate that AECs, which have been shown to be able to function as unprofessional APC [30], might directly interact locally with T cells and in the case of an encounter with GPE shift the polarization of mucosal T lymphocytes towards a Th1 response. Wang et al. [31] were able to show that lung epithelial cells inhibit T cell activation in healthy airway mucosa and induce T-regulatory cells. However, further investigations are required to determine the capacity of AECs to directly influence the T cell response after exposure to GPE. Surprisingly, Wnt signalling was also identified to be significantly regulated by GPE (supporting information, Table S2). The Wnt signalling pathway is essential to organ development in general and lung morphogenesis in particular. Originally identified as a developmentally active signalling pathway, the Wnt pathway has recently been linked to the pathogenesis of lung diseases like lung cancer and pulmonary fibrosis [32]. The role of Wnt in lung inflammation is largely unexplored. Recent evidence suggests a role of Wnt proteins in

rheumatoid arthritis [33] and in a mouse model Wnt signalling is supposed to be associated with impaired lung function in asthma [34]. Interestingly, we found WNT5A, which is specific for the non-canonical Wnt pathway, to be up-regulated upon exposure to GPE. The corresponding protein Wnt5a has already been shown to be up-regulated in psoriasis [35] and Wnt2b was identified to be significantly higher expressed within nasal fluids of AR patients, suggesting a general role for this pathway within allergy and inflammation.

Recent evidence suggests that airway epithelial surface signalling through the EGFR is a convergent pathway producing innate immune responses to a variety of infectious and non-infectious stimuli. The EGFR signalling pathway leads to airway mucin production, neutrophil recruitment via the production of IL-8, and airway epithelial repair. Furthermore, EGFR expression is increased in bronchial epithelium of asthmatic individuals and the activation of this pathway by its ligand EGF is related to an increase in mucin MUC5AC expression contributing to mucus hypersecretion as a characteristic feature of the asthmatic phenotype [36, 37]. We found EGFR signalling to be significantly regulated upon stimulation of AEC with GPE with 97 regulated genes from 178 involved genes. The release of EGF showed a 2.7-fold increase, although the measured concentrations appeared to be rather low, which seems to be in line with MUC5AC expression remaining unaltered upon stimulation with GPE. Given the fact that the EGFR gene is associated with susceptibility to and severity of asthma [38, 39] GPE-induced EGFR-signalling might contribute to the allergic immune response and the EGFR cascade might therefore be a pivotal mechanism with regard to its potential as a pharmacological target.

Although mitogen-activated protein kinases (MAPKs) pathways were not directly identified to be among GPE-regulated signalling events, we found the dual-specificity phosphatase 1 (DUSP1) to be down-regulated upon GPE stimulation by more than fourfold. For several allergens, it has been shown that their activating capacity of AEC is associated with the phosphorylation of MAPKs [40, 41]. The MAPKs JNK and p38 were recently found to be negatively regulated via their dephosphorylation by the dual-specificity phosphatase 1 (DUSP1), with the expression pattern of DUSP1 dictating the kinetics of activation [42]. Therefore, DUSP1 can be regarded as a central regulator of innate immunity and its expression can profoundly affect the outcome of inflammatory challenges. DUSP1 might therefore be an interesting target for the development of new therapeutic strategies to block allergen-induced inflammatory cascades.

Taken together, we were able to identify the induction of a broad variety of genes and mediators after the stimulation of AEC with GPE. Although this study has been confined to a bronchial epithelial cell line, the

reported outcome far exceeds that previously described and might at least partially reflect the response of primary AECs to GPE-exposure [24]. Related to their size (30 µm), pollen grains are only able to reach the upper airways; however, upon encounter with the mucus of AECs, the grains release their content and allergens associated with starch granules can reach the lower airways [43, 44]. Currently, we are extending the study with primary nasal epithelial cells from patients mono-sensitized to grass pollen as well as healthy controls to define a core response of AECs to GPE in an approach similar to our studies on the influence of HDM allergens [23]. Furthermore, the extent of the response would be influenced by our use of a whole GPE instead of using a single allergic component. The use of isolated or recombinant allergens instead of complex extracts is becoming increasingly popular as it offers some important advantages for quantitative measures in diagnosis and research and it would be interesting to investigate the contribution of isolated components to the GPE-induced response of AECs. However, the response to purified allergens would not resemble the natural exposure situation *in vivo*. In general, an estimation of the bioactive components responsible within extract preparations is challenging as pollen do not only release allergens but also bioactive lipids, phytoprostanes, and lipid mediators [45–47]. Especially in allergens derived from plants, complex carbohydrates, in particular glucans, have been shown to promote Th2 responses [48]. The ability of binding lipids directly links allergic sensitization to Toll-like receptor (TLR) signalling and several major allergens have been shown to display a lipid-binding activity [49]. Furthermore, self-collected as well as commercially available pollen contain a broad range of microbial components (own unpublished data) and even standardized allergen vaccines contain endotoxin, complicating the identification of biological active components responsible for the induction of immune responses.

The analysis of GPE-induced gene expression revealed genes belonging to the GO clusters *cell communication*, *metabolism*, and *nucleic acid binding* to be the most prominent. Network analysis displayed a central role not only for IL-6 and IL-8 but also for IL-1A and the transcription factor FOS. Besides the commonly reported allergen-induced released mediators, we found an increase in IL-1RA and IL-12 concentrations, with the release of IL-12 not been reported by AEC so far. Furthermore, the Notch signalling pathway was identified to be significantly regulated upon GPE stimulation of AEC, together with the observed down-regulation of JAG2 linking GPE-induced activation of AEC to a possible interaction of epithelium with mucosal T lymphocytes. A very first comparison between HDM- and GPE-induced gene expression revealed that although

addressing the same signal transduction pathways, different players seem to be differentially regulated. These results will be helpful for a better understanding of the allergic mucosal response in general and therefore may contribute to the development of new therapeutically strategies for the treatment of grass pollen-allergic individuals.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Genes involved in Notch signalling regulated by GPE.

Table S2. Genes involved in Wnt signalling regulated by GPE.

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Supplementary table 1: Genes involved in Notch signalling regulated by GPE

Gene symbol	Gene name	fold change	P-value	Gene ID
ADAM10	ADAM metallopeptidase domain 10	-1.5	1.91E-04	202604_x_at
ARHGEF7	Rho guanine nucleotide exchange factor (GEF) 7	-1.3	7.00E-04	202548_s_at
CIR	CBF1 interacting corepressor	-1.3	1.13E-04	209571_at
DLL1	delta-like 1 (Drosophila)	-2.4	2.01E-07	224215_s_at
EP300	E1A binding protein p300	-1.4	6.83E-05	213579_s_at
FHL1	four and a half LIM domains 1	1.9	3.30E-05	210299_s_at
GSK3B	glycogen synthase kinase 3 beta	-1.1	1.98E-01	209945_s_at
HDAC1	histone deacetylase 1	-1.2	5.10E-03	201209_at
HES1	hairy and enhancer of split 1, (Drosophila)	-3.3	2.02E-08	203394_s_at
HES6	hairy and enhancer of split 6 (Drosophila)	1.3	1.12E-03	226446_at
HEY1	hairy/enhancer-of-split related with YRPW motif 1	2.2	8.44E-06	44783_s_at
ITCH	itchy E3 ubiquitin protein ligase homolog (mouse)	-1.5	2.41E-04	239101_at
JAG1	jagged 1 (Alagille syndrome)	-1.3	8.85E-03	232406_at
JAG2	jagged 2	-3	6.21E-08	209784_s_at
LEF1	lymphoid enhancer-binding factor 1	1.6	1.36E-03	221558_s_at
MAML1	mastermind-like 1 (Drosophila)	-1.2	4.39E-03	202360_at
MAML3	mastermind-like 3 (Drosophila)	1.7	2.03E-03	235841_at
MAPK3	mitogen-activated protein kinase 3	-1.2	6.41E-03	212046_x_at
MFNG	MFNG O-fucosylpeptide 3-beta-N-acetylglucosaminyltransferase	-1.3	7.62E-03	204153_s_at
NCOR1	nuclear receptor co-repressor 1	1.3	1.86E-04	200854_at
NOTCH1	Notch homolog 1, translocation-associated (Drosophila)	-2	1.28E-05	218902_at
NOTCH2	Notch homolog 2 (Drosophila)	-1.5	5.09E-05	212377_s_at
NOTCH3	Notch homolog 3 (Drosophila)	-2.5	5.04E-08	203238_s_at
NUMB	numb homolog (Drosophila)	-1.4	4.76E-04	209073_s_at
PCAF	p300/CBP-associated factor	-2.4	1.11E-04	203845_at
PSEN2	presenilin 2 (Alzheimer disease 4)	-1.6	1.04E-05	211373_s_at
PSENEN	presenilin enhancer 2 homolog (C. elegans)	-1.2	3.50E-03	218302_at
SIN3A	SIN3 homolog A, transcription regulator (yeast)	1	9.84E-01	238189_at
SKP1	S-phase kinase-associated protein 1	-1.6	8.22E-05	200719_at
SKP2	S-phase kinase-associated protein 2 (p45)	1.7	9.02E-06	210567_s_at
SMAD1	SMAD family member 1	1.2	3.71E-03	210993_s_at
SMAD3	SMAD family member 3	-1.4	4.03E-05	218284_at
SMAD4	SMAD family member 4	-1.6	1.49E-03	202526_at
STAT3	signal transducer and activator of transcription 3 (acute-phase response factor)	-1.2	2.15E-03	208992_s_at
TLE1	transducin-like enhancer of split 1 (E(sp1) homolog, Drosophila)	1.3	5.63E-04	203221_at
WDR12	WD repeat domain 12	-1.5	4.25E-05	218512_at
YY1	YY1 transcription factor	-1.3	1.90E-04	224711_at
ZFPM1	zinc finger protein, multitype 1	1.3	8.17E-04	242282_at

Table indicates gene aliases, fold change, gene name, *P*-value and gene ID

Supplementary table 2: Genes involved in Wnt signalling regulated by GPE

Gene alias	Gene name	Fold change	P-value	Gene ID
ANKRD6	ankyrin repeat domain 6	1.6	3.03E-04	204672_s_at
CDC2	cell division cycle 2, G1 to S and G2 to M	1.9	1.26E-05	203213_at
CDH1	cadherin 1, type 1, E-cadherin (epithelial)	-3.6	1.14E-08	201131_s_at
CSNK1A1	casein kinase 1, alpha 1	1.3	1.02E-04	208865_at
CSNK1E	casein kinase 1, epsilon	-1.6	2.33E-04	226858_at
CTBP2	C-terminal binding protein 2	-1.3	3.29E-04	201220_x_at
CTNNB1	catenin (cadherin-associated protein), beta 1, 88kDa	-1.6	1.07E-04	223679_at
DAAM1	dishevelled associated activator of morphogenesis 1	-1.9	8.88E-06	216060_s_at
DAB2	disabled homolog 2 (Drosophila)	-3	1.10E-07	201280_s_at
DKK1	dickkopf homolog 1 (Xenopus laevis)	-1.2	1.62E-03	204602_at
DLG1	discs, large homolog 1 (Drosophila)	-1.5	8.12E-06	202514_at
DVL3	dishevelled, dsh homolog 3 (Drosophila)	-1.3	4.02E-03	201908_at
EP300	E1A binding protein p300	-1.4	6.83E-05	213579_s_at
FZD1	frizzled homolog 1 (Drosophila)	-1.2	2.24E-03	204451_at
FZD2	frizzled homolog 2 (Drosophila)	-1.6	9.81E-06	210220_at
FZD4	frizzled homolog 4 (Drosophila)	1.6	2.62E-05	218665_at
FZD5	frizzled homolog 5 (Drosophila)	1.4	3.84E-05	221245_s_at
FZD7	frizzled homolog 7 (Drosophila)	2.2	4.01E-07	203706_s_at
FZD8	frizzled homolog 8 (Drosophila)	1.9	8.18E-06	224325_at
GLMN	glomulin, FKBP associated protein	1.2	3.11E-03	207153_s_at
GSK3B	glycogen synthase kinase 3 beta	-1.1	1.98E-01	209945_s_at
JUP	junction plakoglobin	-1.8	2.66E-06	201015_s_at
LEF1	lymphoid enhancer-binding factor 1	1.6	1.36E-03	221558_s_at
LRP5	low density lipoprotein receptor-related protein 5	-1.7	4.27E-05	209468_at
LRP6	low density lipoprotein receptor-related protein 6	-1.3	8.76E-05	225745_at
MAP1B	microtubule-associated protein 1B	1.4	3.69E-03	212233_at
MAP3K7	mitogen-activated protein kinase kinase kinase 7	1.4	4.79E-04	206853_s_at
MAPK8	mitogen-activated protein kinase 8	-1.3	2.67E-02	243280_at
MPP2	membrane protein, palmitoylated 2 (MAGUK p55 subfamily member 2)	-2.6	9.40E-07	207984_s_at
MYB	v-myb myeloblastosis viral oncogene homolog (avian)	3.3	5.77E-07	204798_at
NLK	nemo-like kinase	1.4	5.74E-04	222589_at
PPP2R5B	protein phosphatase 2, regulatory subunit B	-1.2	5.10E-03	635_s_at
PRKCA	protein kinase C, alpha	-1.2	2.59E-03	213093_at
RAC1	ras-related C3 botulinum toxin substrate 1	-1.2	4.66E-03	208641_s_at
RUNX2	runt-related transcription factor 2	6.8	1.57E-08	232231_at
RUVBL1	RuvB-like 1 (E. coli)	1.2	8.94E-04	201614_s_at
SF1	splicing factor 1	-1.4	3.77E-05	208313_s_at
SFRP1	secreted frizzled-related protein 1	-1.5	6.43E-05	202036_s_at
SKP1	S-phase kinase-associated protein 1	-1.6	8.22E-05	200719_at
TAX1BP3	Tax1 (human T-cell leukemia virus type I) binding protein 3	-1.7	2.15E-06	215464_s_at
TCF7L2	transcription factor 7-like 2 (T-cell specific, HMG-box)	-2.2	1.69E-07	216035_x_at
TFAP2A	transcription factor AP-2 alpha	-1.3	3.62E-04	204653_at
VANGL2	vang-like 2 (van gogh, Drosophila)	-1.5	1.09E-04	226029_at
WISP2	WNT1 inducible signalling pathway protein 2	-6.3	3.45E-09	205792_at
WNT3	wingless-type MMTV integration site family, member 3	1.4	6.11E-04	229103_at
WNT4	wingless-type MMTV integration site family, member 4	-1.9	2.50E-05	208606_s_at
WNT5A	wingless-type MMTV integration site family, member 5A	1.9	4.60E-06	205990_s_at

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